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<b>(21) International Application Number:</b> PCT/GB94/02483 <b>(22) International Filing Date:</b> 11 November 1994 (11.11.94) <b>(30) Priority Data:</b> 9323429.2 12 November 1993 (12.11.93) GB <b>(71) Applicant (for all designated States except US):</b> THE WELL-COME FOUNDATION LIMITED [GB/GB]; Unicorn House, 160 Euston Road, London NW1 2BP (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SMITH, Gary, Keith [US/US]; 521 Mills Street, Raleigh, NC 27608 (US). BLUMENKOPF, Todd, Andrew [US/US]; 201 Hunter Hill Road, Chapel Hill, NC 27516 (US). CORY, Michael [US/US]; 55 Cedar Street, Chapel Hill, NC 27514 (US). <b>(74) Agent:</b> STOTT, Michael, John; The Wellcome Foundation Limited, Langley Court, Beckenham, Kent BR3 3BS (GB).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> THERAPY  <b>(57) Abstract</b>  The present invention relates to improvements in targetted enzyme prodrug therapy including antibody-directed enzyme prodrug therapy (ADEPT), it particularly relates to novel enzymes and prodrugs for use in ADEPT.		

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## THERAPY

The present invention relates to improvements in targetted enzyme prodrug therapy including antibody-directed enzyme prodrug therapy (ADEPT), it particularly relates to novel enzymes and prodrugs for use in ADEPT.

In the therapy of certain conditions it is preferable that a drug be delivered only to a particular cellular subpopulation. For example the use of drugs in the treatment of cancer is limited by the inability of the cytotoxic drug to differentiate between cells exhibiting normal cell division and those exhibiting neoplastic division. Hence the therapy is not targeted to a clinically acceptable extent and healthy cells are exposed to cytotoxin. Conjugation of a drug to an antibody, preferably a monoclonal antibody (mAb), allows the targeting of the drug to a particular cellular subpopulation expressing the antigenic determinant to which the targeting antibody binds. However factors, such as the inability of the conjugate to penetrate the relevant tissue, poor release of the drug from the antigen bound conjugate and the limitation placed on the amount of drug which can be delivered by the number of available antibody-binding sites, have limited the effectiveness of this approach.

Avoidance of such limitations led to the concept of targeting conjugates of antibodies and enzymes capable of converting relatively non-cytotoxic 'prodrugs' into low molecular weight cytotoxins at the antibody-binding site. This general concept was disclosed by Rose in European Patent application 84302218.7. Bagshawe and collaborators have referred to the concept as ADEPT, Antibody Directed Enzyme Prodrug Therapy. (Bagshawe K.D. et.al., Br. J. Cancer [1987] 56, 531-532, Bagshawe K.D. *et al.*, Br. J. Cancer [1988] 50, 700-703 and WO 90/10140). In this way one conjugate could generate a proportionately larger amount of drug at the target site by repeated rounds of enzymatic catalysis of prodrug activation.

EP 382 411 describes ADEPT wherein a prodrug may be converted to a cytotoxic agent by enzymes including beta-lactamases isolated from various micro-organisms, L-pyroglutamate aminopeptidase, beta-galactosidase, D-amino acid peptidase, isoenzymes of alkaline phosphatase and various carboxypeptidases.

WO 91/11201 describes ADEPT wherein a prodrug may be enzymatically cleaved to generate cyanide by  $\beta$ -glycosidases or  $\beta$ -glucosidases, generally of plant origin.

EP 302 473 describes ADEPT wherein the enzyme alkaline phosphatase may be used to cleave novel prodrugs of mitomycin, penicillin V amidase may be used to cleave novel prodrugs of adriamycin, or cytosine deaminase may be used with the prodrug 5-fluorocytosine.

EP 484 870 describes ADEPT wherein  $\beta$ -lactamase may be used to activate a cephalosporin prodrug to yield a cytotoxic nitrogen mustard.

WO 88/07378 describes ADEPT wherein benzoic acid nitrogen mustard glutamides may be converted to the nitrogen mustard under the action of carboxypeptidases.

Vitols, K. S., *et al*, Pteridines 3, [1992], 125-126, discloses a number of MTX-amino acid prodrugs which may be activated by carboxypeptidase-mAb conjugates as part of ADEPT.

WO 91/09134 discloses bispecific hybrid mAbs for use in ADEPT wherein the mAb has specificities against both human cancer cell antigens and a prodrug-activating enzyme.

All previously disclosed methods of ADEPT may be divided into two categories: those which employ human enzymes and those which employ non-human (eg. bacterial) enzymes to activate the relevant prodrug. Both strategies retain inherent problems which limit their potential to provide effective therapy. Use of a human enzyme results in instability of the associated prodrug *in vivo*, as it may be activated at sites distant to the target site where endogenous human enzymatic activity may occur naturally. Clearly this will also have the highly undesirable effect of generating potentially cytotoxic compounds in non-targetted areas of the body with possibly lethal consequences. The use of a non-human enzyme permits the associated prodrug, which is only activated by the non-human enzymatic activity, to avoid activation by endogenous enzymes and so remain stable *in vivo* until converted to drug at the target site. However, such a non-human enzyme may elicit an immune response when introduced *in vivo* and antibodies generated to the enzyme will limit or destroy its ability to activate the prodrug.



WO 90/07929 identifies the desideratum of a non-endogenous catalytic activity being provided by a non-immunogenic enzyme but teaches only that this may be achieved by the use of "genetically conserved" enzymes or those from a "genetically similar species". However it is not taught how this may be accomplished.

It has now been found that the apparent conflict arising from the need to provide a prodrug which is stable *in vivo* and yet activated by a non-immunogenic enzyme may be resolved by generation of a mutant mammalian enzyme which retains catalytic activity but possesses a novel substrate specificity. The associated prodrug may be activated by the catalytic activity of the mutant enzyme but since the substrate specificity of the mutant enzyme is not a naturally occurring one, the prodrug remains stable *in vivo* until converted to drug at the target site. The ability to use a non-immunogenic enzyme according to the present invention provides the further advantage that repeated rounds of therapy may be administered. This is in contrast to known processes for ADEPT, in which the initial introduction of the enzyme to the system elicits an immune response which effectively precludes further treatment with the same enzyme as this will be removed from the body by an immune reaction 'primed' during the first round of therapy.

One aspect of the present invention provides a method of targetting a chemotherapeutic agent to a specific cell-type comprising the administration to a mammal of (i) an effective amount of a conjugate of a cell-type specific targetting molecule with a mutant mammalian enzyme capable of catalysing a functionally inactive precursor of a chemotherapeutic agent to its active form and (ii) an effective amount of the functionally inactive precursor of the chemotherapeutic agent which is refractory to endogenous catalysis to the chemotherapeutic agent.

Endogenous catalysis implies conversion of functionally inactive precursor of a chemotherapeutic agent to that chemotherapeutic agent by enzymes naturally present *in vivo*. Clearly, conversion occurring at the target site, catalysed by the targetted mutant mammalian enzyme, is not considered to be endogenous catalysis.

A further aspect of the present invention provides a method of treatment of a mammal requiring therapy for any of the conditions hereinafter described comprising the administration to the mammal of an effective amount of a cell-type specific targetting molecule conjugated with a mutant mammalian enzyme which is capable of catalysing a

functionally inactive precursor of a drug to its active form in combination with an effective amount of a functionally inactive precursor of the drug which is refractory to endogenous catalysis.

A mutant human enzyme as used herein shall be taken to be any human enzyme with a sequence differing by at least one amino acid from the amino acid sequence or sequences of that enzyme in the patient to which the therapy is applied.

As used herein the term "cell-type" means any localised or dispersed population of cells possessing a common determinant essentially selective for a pathological state, for example, cancer cells expressing carcinoembryonic antigen, Tag-72, mucin, or the antigens recognised by the antibodies Ing-1, 17-1A, 323/A3, NR-LU-10, c174, PR.1A3, MOV18, G250, U36, E48, NR-CO-02 or any of the other antigens shown in Table 3.

The cell-type specific targetting molecule may be any molecule capable of being noncovalently or covalently linked or conjugated with the mutant enzyme and which can demonstrate a selectivity in its binding affinity for cell-surface markers. Such molecules include polyclonal and monoclonal antibodies, including bispecific antibodies in which one antibody arm noncovalently binds the mutant enzyme and the other performs the cell-type specific targetting (U. Sahin, et al, Cancer Research 50, 6944, 1990).

Alternative targetting molecules which may be linked to mutant mammalian enzymes to form conjugates include hormones, ligands, cytokines, antigens, oligonucleotides and peptidomimetics.

The choice of targetting molecule will depend upon the nature of the cells to be targetted but will most probably be an antibody and preferably a monoclonal antibody as mAbs produce the greatest selectivity.

A "chemotherapeutic agent" which may also be referred to herein as a "drug" includes any molecule which has activity in human therapy. Such chemotherapeutic agents include but are not limited to cytostatic or cytotoxic compounds used in the therapy of cancers or viral infections. A "functionally inactive precursor" which may also be referred to herein as a "prodrug" includes any compound which may be converted into a

chemotherapeutic agent under the action of an enzyme. Such functionally inactive precursors may typically be converted to a chemotherapeutic agent by the enzymatic cleavage of the functionally inactive precursor to yield a chemotherapeutic agent and a "prodrug moiety". Such conversion from functionally inactive precursor to chemotherapeutic agent may also occur by enzymatically mediated isomerisation.

A functionally inactive precursor will generally not exhibit clinically significant levels of the therapeutic activity possessed by the chemotherapeutic agent into which it may be enzymatically converted having been chemically derivitized to decrease its normal pharmacological activity. The functionally inactive precursor of a cytotoxic chemotherapeutic agent will not itself exhibit clinically significant cytotoxicity and will be sufficiently stable *in vivo* such that during therapy, clinically significant level of cytotoxicity are largely only generated at the site of conversion of functionally inactive precursor to chemotherapeutic agent i.e. at the site to which the mutant enzyme has been targetted.

The mutant mammalian enzyme is preferably a wild-type mammalian enzyme with one or more mutations which generate novel substrate specificities without engendering a significant immunological response when administered during human therapy. A significant immunological response may be regarded as one which would preclude the clinical use of such an enzyme in human therapy.

The essential characteristic of a mutant mammalian enzyme of the present invention is the presence of a mutant substrate-binding site irrespective of the amino acid sequences flanking this mutant substrate-binding site. Hence a chimaeric enzyme comprising a mutant substrate-binding site and flanking sequences derived from one or more different enzymes is included with the definitions of a mutant mammalian enzyme of the present invention. Such a chimaeric enzyme may be generated by recombinant DNA technology and/or protein engineering.

Enzymes suitable for directed mutagenesis include any enzymes possessing a catalytic activity capable of converting a prodrug to a drug. Such catalytic activities include transferase, hydrolase, oxido-reductase, isomerase, lyase, or ligase. The directed mutagenesis will generate a novel substrate specificity but preserve the class of catalytic activity involved. For example a mutant isomerase of the present invention will possess a

novel isomerase activity sufficiently different from the isomerase from which it was derived to ensure that prodrugs susceptible to activation by the mutant isomerase remain substantially stable in the presence of the isomerase from which the mutant enzyme was derived. As will be shown this dramatic shift in activity may be achieved by the alteration of as little as one residue at the catalytic site. The alteration of the minimum number of residues necessary to obtain the required shift in activity ensures continuity of enzyme structure and hence avoids negative immunological effects when the mutant enzyme is introduced to the body.

A preferred mutant enzyme of the present invention is mutant mammalian carboxypeptidase A (CPA). This enzyme has the general activity of cleaving carboxy-terminal aromatic and aliphatic amino acid residues and has been characterised in a diversity of species and tissue-specific variants. Particularly preferred mutant carboxypeptidases include mutants derived from human pancreatic carboxypeptidase A1 (Catasus L. *et al.*, Biochem. J. 287, 299-303, 1992), human mast cell carboxypeptidase A (Reynolds D.S. *et al.* J. Clin. Invest. 89 273-282, 1992) and human pancreatic carboxypeptidase A2 (Fig 9 hereinafter). It will be appreciated that the common characteristic of these carboxypeptidases is the presence of a CPA-like substrate-binding pocket and associated enzymatic activity irrespective of overall sequence or structure of the enzyme and that any enzyme possessing this CPA-like substrate-binding pocket is amenable to mutation to a mutant carboxypeptidase of the present invention.

In a particularly preferred embodiment of the present invention the mutant enzyme is mutant human pancreatic carboxypeptidase A1 or A2 (CPA1 or CPA2) and yet more preferably, CPA1 or CPA2 wherein amino acid substitutions are generated at one or more of residues 203, 210, 242, 244, 250, 253, 255, 267, 268, 269 and 305, of the amino acid sequences shown in Table 4 which represent the wild-type (w.t.) sequences of CPA1 and CPA2 respectively. Particularly preferred combinations of residue substitution include Gly at residues 250 and 268 when 253 and 255 are w.t.; Gly at 253 and 268 when 250 and 255 are w.t.; Gly at 250 and His at 268 when 253 and 255 are w.t.; Gly at 250 when 253, 255 and 268 are w.t.; Ala at 255 and His at 268 when 250 and 253 are w.t. and His at 268 when 250, 253 and 255 are w.t. The most preferred mutants are carboxypeptidase A1 or A2 mutants comprising a single substitution; Gly at 268.

The most preferred conjugate of the present invention comprises all or part of either of mAbs CAMPATH-1H<sup>®</sup>, 323/A3 or ING-1 and at least the substrate-binding domain of either of mutant mammalian enzymes CPA1 or CPA2 having glycine present at amino acid position 268 as described in Table 5.

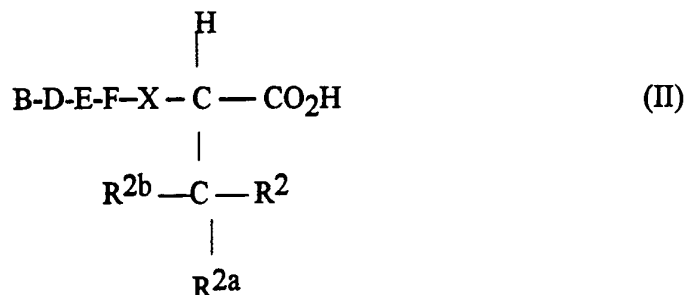
Human pancreatic carboxypeptidase A is expressed as a preproenzyme (Fig. 2) which is processed to a proenzyme and subsequently to the mature enzyme. Mutant carboxypeptidases of the present invention may be mutants of the preproenzyme, the proenzyme or of the mature enzyme but are preferably mutants of the mature enzyme. These mutants may be derived from either preproenzyme, the proenzyme or the mature enzyme but are preferably derived from the proenzyme. The mutated proenzyme (or preproenzyme) may then be converted to the corresponding mutant mature enzyme by standard methods such as trypsinisation.

A mutant enzyme of the present invention may be generated from the DNA or RNA source of any enzyme possessing the previously discussed activities by methods well known in the art of molecular biology and more particularly by the methods described hereinafter in the Examples.

The selection of the enzymatic activity and the primary sequence of a mutant enzyme of the present invention will clearly depend upon the nature of the relevant prodrug and, if a prodrug of the type which is activated by the removal of a prodrug moiety, on the precise structure of that moiety.

The substrate-binding or active site of the mutant enzyme must, unlike the corresponding non-mutant enzyme, be capable of interacting with the prodrug in such a way that enzymatic catalysis is facilitated. The ability of the mutant enzyme to perform this activity will depend upon subtle alterations in the 3-dimensional structure of the active site, which is in turn dependent upon the primary amino acid sequence of this region of the protein. Alterations of the primary sequence of an enzyme by standard techniques of protein engineering will allow the generation of an appropriate mutant enzyme for use according to the invention with a corresponding prodrug.

Novel prodrugs comprise those of formula (II)



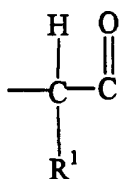
wherein,

X represents NH or O and

$\text{R}^2$  represents  $\text{CO}_2\text{H}$ ,  $\text{SO}_3\text{H}$ ,  $\text{SO}_2\text{H}$ ,  $\text{OSO}_3\text{H}$ ,  $\text{PO}_3\text{H}_2$ ,  $\text{OPO}_3\text{H}_2$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{CO}_2\text{H}$ ,  $\text{C}_{1-6}$  esterified  $\text{PO}_3\text{H}_2$ ,  $\text{C}_{4-12}$  alkyl,  $\text{C}_{4-12}$  alkenyl,  $\text{C}_{4-12}$  alkynyl,  $\text{C}_{4-12}$  branched alkyl,  $\text{C}_{1-8}$  alkyl or  $\text{C}_{3-8}$  branched alkyl; substituted with  $\text{CO}_2\text{H}$ ,  $\text{SO}_3\text{H}$ ,  $\text{SO}_2\text{H}$ ,  $\text{OSO}_3\text{H}$ ,  $\text{PO}_3\text{H}_2$ ,  $\text{OPO}_3\text{H}_2$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{CO}_2\text{H}$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{PO}_3\text{H}_2$ , hydroxyl, alkoxy, halo, haloalkyl, cyano or carboxamide;  $\text{C}_{3-8}$  cycloalkyl, aryl or heteroaryl optionally substituted with  $\text{C}_{1-8}$  alkyl,  $\text{C}_{3-8}$  branched alkyl,  $\text{C}_{3-8}$  cycloalkyl,  $\text{C}_{3-6}$  alkenyl,  $\text{C}_{2-6}$  alkynyl,  $\text{C}_{4-6}$  cycloalkenyl, trisubstituted-silyl i.e.  $(\text{R}^{13})(\text{R}^{14})(\text{R}^{15})\text{Si}$ , where  $\text{R}^{13}$ ,  $\text{R}^{14}$ , and  $\text{R}^{15}$  are  $\text{C}_{1-6}$  alkyl,  $\text{C}_{3-6}$  branched alkyl,  $\text{C}_{3-6}$  cycloalkyl,  $\text{C}_{3-6}$  alkenyl,  $\text{C}_{2-6}$  alkynyl,  $\text{C}_{4-6}$  cycloalkenyl, aryl, or heteroaryl, and where  $\text{R}^{13}$ ,  $\text{R}^{14}$ , and  $\text{R}^{15}$  are each the same group or different groups (e.g. trimethylsilyl, tert-butyldimethylsilyl, cyclohexyldimethylsilyl, or phenyldimethylsilyl), aryl,  $\text{CO}_2\text{H}$ ,  $\text{SO}_3\text{H}$ ,  $\text{SO}_2\text{H}$ ,  $\text{OSO}_3\text{H}$ ,  $\text{PO}_3\text{H}_2$ ,  $\text{OPO}_3\text{H}_2$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{CO}_2\text{H}$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{PO}_3\text{H}_2$ , carboxamide, hydroxyl,  $\text{C}_{1-6}$  alkoxy,  $\text{C}_{1-6}$  alkylthio, mercapto, halo, haloalkyl, nitro or cyano,  $\text{R}^{2a}$  and  $\text{R}^{2b}$  represent H, hydroxy, mercapto, alkoxy, alkylthio, halo, cyano,  $\text{CO}_2\text{H}$ ,  $\text{SO}_3\text{H}$ ,  $\text{SO}_2\text{H}$ ,  $\text{OSO}_3\text{H}$ ,  $\text{PO}_3\text{H}_2$ ,  $\text{OPO}_3\text{H}_2$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{CO}_2\text{H}$ , carboxamide,  $\text{C}_{1-6}$  alkyl esterified  $\text{PO}_3\text{H}_2$ , cyclic  $\text{C}_{2-6}$  [i.e.,  $\text{R}^{2a}$  and  $\text{R}^{2b}$  together represent  $(\text{CH}_2)_{2-6}$ ], trisubstituted-silyl i.e.  $(\text{R}^{13})(\text{R}^{14})(\text{R}^{15})\text{Si}$ , where  $\text{R}^{13}$ ,  $\text{R}^{14}$ , and  $\text{R}^{15}$  are  $\text{C}_{1-6}$  alkyl,  $\text{C}_{3-6}$  branched alkyl,  $\text{C}_{3-6}$  cycloalkyl,  $\text{C}_{3-6}$  alkenyl,  $\text{C}_{2-6}$  alkynyl,  $\text{C}_{4-6}$  cycloalkenyl, aryl, or heteroaryl and where  $\text{R}^{13}$ ,  $\text{R}^{14}$ , and  $\text{R}^{15}$  could be the same group or different groups, e.g. trimethylsilyl, tert-butyldimethylsilyl, cyclohexyldimethylsilyl, or phenyldimethylsilyl; or  $\text{C}_{1-6}$  alkyl or  $\text{C}_{1-6}$  branched alkyl or  $\text{C}_{1-6}$  cycloalkyl or aryl or heteroaryl optionally substituted with hydroxy, mercapto,  $\text{C}_{1-6}$

6 alkoxy, C<sub>1-6</sub> alkylthio, halo, cyano, CO<sub>2</sub>H, SO<sub>3</sub>H, SO<sub>2</sub>H, OSO<sub>3</sub>H, nitro, PO<sub>3</sub>H<sub>2</sub>, OPO<sub>3</sub>H<sub>2</sub>, C<sub>1-6</sub> alkyl esterified CO<sub>2</sub>H, carboxamide, C<sub>1-6</sub> alkyl esterified PO<sub>3</sub>H<sub>2</sub>, C<sub>1-6</sub> alkyl, C<sub>1-6</sub> branched alkyl, C<sub>1-6</sub> cycloalkyl, trisubstituted-silyl i.e.(R<sup>13</sup>)(R<sup>14</sup>)(R<sup>15</sup>)Si, where R<sup>13</sup>, R<sup>14</sup>, and R<sup>15</sup> are C<sub>1-6</sub> alkyl, C<sub>3-6</sub> branched alkyl, C<sub>3-6</sub> cycloalkyl, aryl, or heteroaryl, and where R<sup>13</sup>, R<sup>14</sup>, and R<sup>15</sup> could be the same group or different groups, e.g. trimethylsilyl, tertbutyldimethylsilyl or phenyldimethylsilyl; with the proviso that R<sup>2a</sup> and R<sup>2b</sup> are not both hydroxy or mercapto.

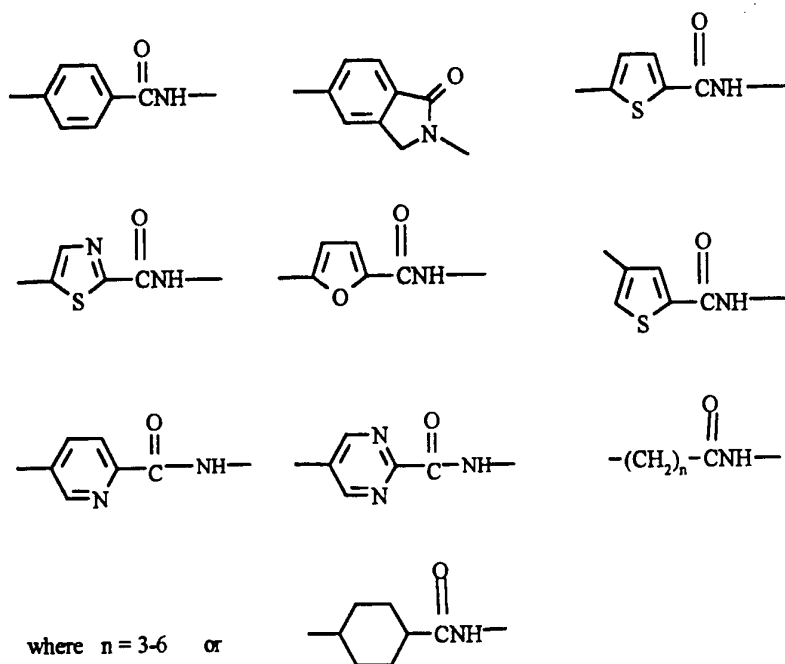
F comprises a moiety of formula (IV)



(IV)

wherein R<sup>1</sup> represents a group corresponding to the side chain of any α- amino acid for example, H, C<sub>1-6</sub> alkyl, C<sub>1-6</sub> alkenyl, C<sub>1-6</sub> alkynyl optionally substituted with CO<sub>2</sub>H, C<sub>1-6</sub> alkyl esterified CO<sub>2</sub>H, OPO<sub>3</sub>, PO<sub>3</sub>H<sub>2</sub>, C<sub>1-6</sub> alkyl esterified PO<sub>3</sub>H<sub>2</sub>, halo, hydroxy, carboxamide, amino optionally substituted with C<sub>1-6</sub> alkyl, cyano or nitro,

E represents

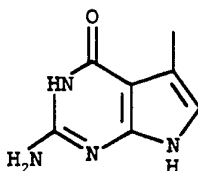
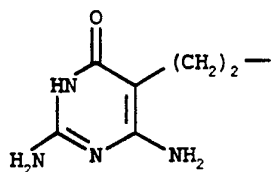
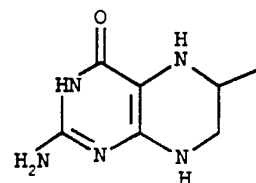
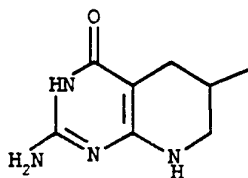
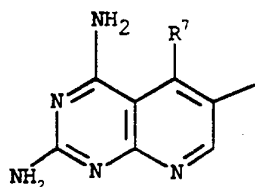
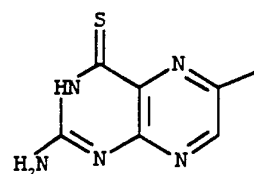
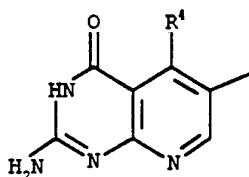
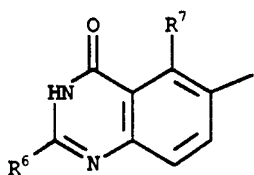
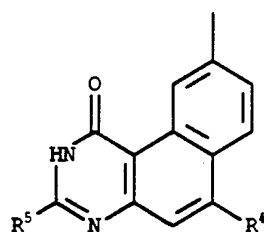
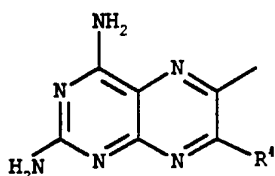


each of which may be optionally substituted with one or more of hydroxy, one or more of alkoxy, halo, or  $\text{C}_{1-6}$  alkyl optionally substituted with one or more of hydroxy,  $\text{C}_{1-6}$  alkoxy or halo.

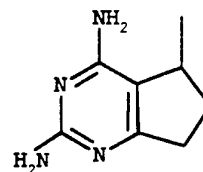
D represents  $\text{—CH}_2\text{—CH(R}^3\text{)—}$ ,  $\text{—CH}_2\text{—NR}^3\text{—}$ ,  $\text{—NR}^3\text{—CH}_2\text{—}$ ,  $\text{—CH}_2\text{S—}$  or  $\text{—CH}_2\text{O—}$  where  $\text{R}^3$  is H,  $\text{C}_{1-6}$ alkyl, allyl or propargyl, optionally substituted with one or more of  $\text{C}_{1-6}$  alkoxy halo, hydroxy or cyano; and

B represents





or



wherein,

$R^4$  represents H,  $C_{3-6}$  branched alkyl,  $C_{3-6}$  cycloalkyl, or  $C_{1-6}$  alkyl;

$R^5$  represents  $C_{1-6}$  alkyl,  $C_{3-6}$  branched alkyl,  $C_{3-6}$  cycloalkyl, amino (optionally substituted with  $C_{1-6}$  alkyl,  $C_{1-6}$  alkanoyl or benzyl);

$R^6$  represents H,  $C_{1-6}$  alkyl,  $C_{3-6}$  branched alkyl,  $C_{3-6}$  cycloalkyl, amino (optionally substituted with  $C_{1-6}$  alkyl,  $C_{1-6}$  alkanoyl or benzyl),  $C_{1-6}$  alkoxy or  $C_{1-6}$  alkylthio; and

$R^7$  represents H,  $C_{1-6}$  alkyl,  $C_{3-6}$  branched alkyl,  $C_{3-6}$  cycloalkyl, halo  $C_{1-6}$  alkyl or halo

and salts N-oxides, solvates and physiologically functional derivatives thereof,

with the proviso that the following compounds are not included within the definition of novel prodrugs although the use of these compounds in therapy does form one embodiment of the present invention:

N-(N-(4-(((2-amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl)(2-propynyl)amino)benzoyl)-L-glutam-1-yl)-L-glutamic acid,

N-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl)-L-glutamic acid,

N-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl)-L-aspartic acid,

N-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl)-L-phenylalanine,

4-bis(2-chloroethyl)amino)-phenylalanylphenylalanine and

4-bis(2-chloroethyl)amino)-phenylalanyl-3,5-dimethyl-4-methoxyphenylalanine

In a preferred embodiment of prodrugs according to the present invention,

X is NH;

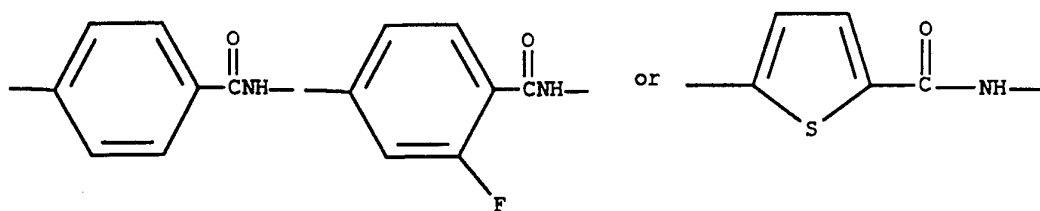
R<sup>2</sup> is C<sub>3-8</sub> cycloalkyl or aryl substituted with C<sub>1-8</sub> alkyl, C<sub>3-8</sub> cycloalkyl, C<sub>3-9</sub> branched alkyl or trisubstituted-silyl i.e. (R<sup>13</sup>)(R<sup>14</sup>)(R<sup>15</sup>)Si, where R<sup>13</sup>, R<sup>14</sup>, and R<sup>15</sup> are C<sub>1-6</sub> alkyl, C<sub>3-6</sub> branched alkyl, C<sub>3-6</sub> cycloalkyl, aryl, or heteroaryl, and where R<sup>13</sup>, R<sup>14</sup>, and R<sup>15</sup> are each the same group or different groups (e.g. trimethylsilyl, tert-butyldimethylsilyl, cyclohexyldimethylsilyl, or phenyldimethylsilyl);

R<sup>2a</sup> and R<sup>2b</sup> are both H;

R<sup>1</sup> is H, C<sub>1-6</sub> alkyl, CH<sub>2</sub>CO<sub>2</sub>H, CH<sub>2</sub>CH<sub>2</sub>CO<sub>2</sub>H or CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>,

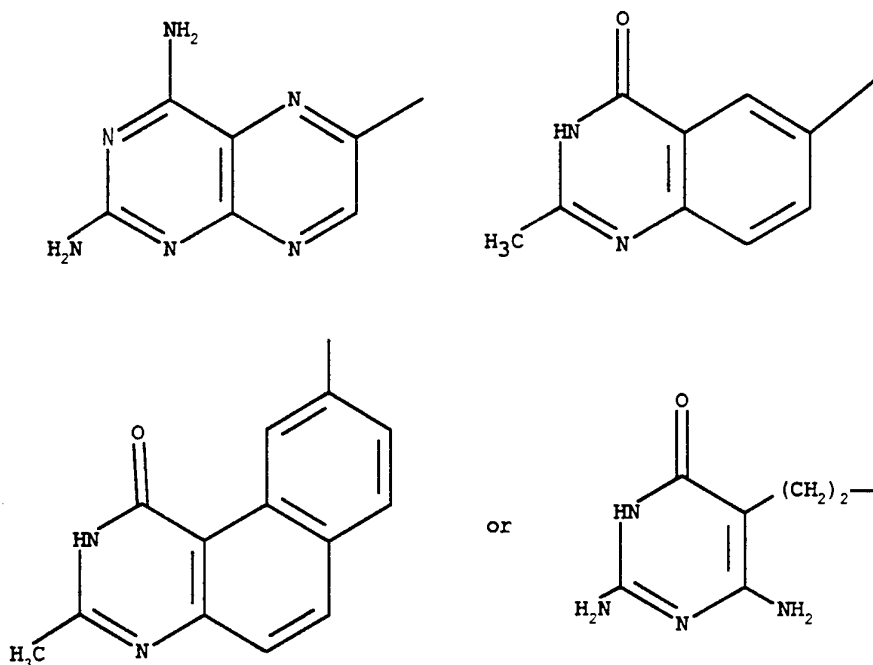
E is

13



D is  $-\text{CH}_2\text{NH}-$ ,  $-\text{CH}_2\text{N}(\text{CH}_3)-$ ,  $-\text{CH}_2\text{N}(\text{CH}_2\text{C}\equiv\text{CH})-$ ,  $-\text{CH}_2\text{CH}_2-$  or  $-\text{CH}_2-\text{CH}(\text{C}_2\text{H}_5)-$

and B is



and salts, N-oxides, solvates and physiologically functional derivatives thereof.

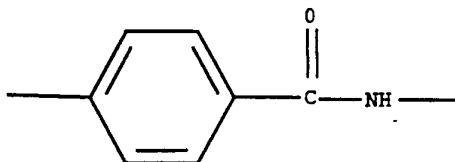
In the most preferred embodiment of prodrugs according to the present invention,

X is NH;

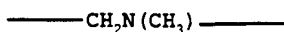
$\text{R}^2$  is phenyl or para-hydroxyphenyl substituted with cyclopentyl, cyclobutyl or tert-butyl. The cyclopentyl, cyclobutyl and tert-butyl groups may both be either ortho- or meta- but are preferably meta-,  $\text{R}^{2a}$  and  $\text{R}^{2b}$  are H,

$\text{R}^1$  is  $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$

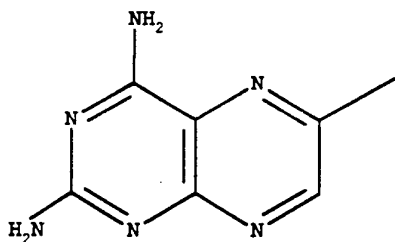
E is



D is



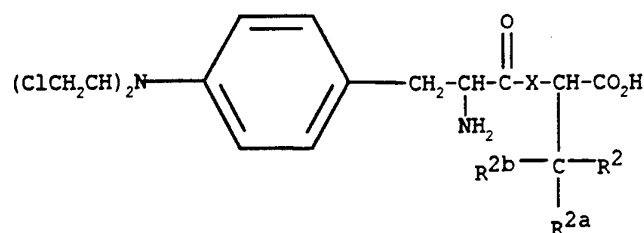
and B is



and salts, N-oxides, solvates and physiologically functional derivatives thereof.

Where chiral centres of prodrugs as defined above are present they may, independently, each be either in the S or R configuration or may be a mixture of the S and R configuration. Preferably they are in the S-configuration for 'F' and the chiral carbon of formula (II) adjacent to X.

A preferable prodrug for use according to the present invention is a prodrug of the cytotoxic agent melphalan (UK 750, 155) which is commercially available under the name Alkeran (T. M. The Wellcome Foundation Limited) and has the chemical name 4-[bis(2-chloroethyl)amino]-1-phenylalanine. Prodrugs of melphalan according to the present invention have the general formula



wherein  $R^2$ ,  $R^{2a}$ ,  $R^{2b}$  and  $X$  are as herein defined.

The following are particularly preferred novel prodrugs according to the present invention:

N-((S)-4-carboxy-2-(5-(((1,2-dihydro-3-methyl-1-oxobenzo(F)quinazolin-9-yl)methyl)amino)-1-oxo-2-isindoliny)butanoyl)-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclobutyl-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-trimethylsilyl-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-tert-butyl-L-phenylalanine,

N-(4-((3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine,

N-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(F)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-tyrosine,

N-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-L-glutam-1-yl-3,5-diiodo-L-tyrosine,

N-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-tyrosine,

(S)-3-(3-cyclopentyl-4-hydroxyphenyl)-2-((N-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-L-glutam-1-yl)oxy)propionic acid,

N-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-tert-butyl-L-tyrosine

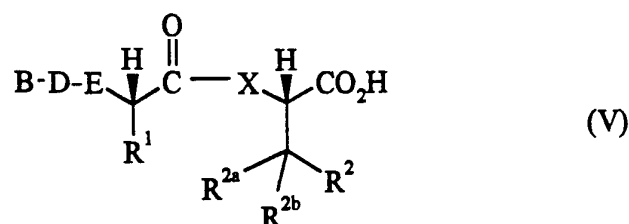
and salts, N-oxides, solvates and physiologically functional derivatives thereof.

The most preferred prodrug according to the present invention is:

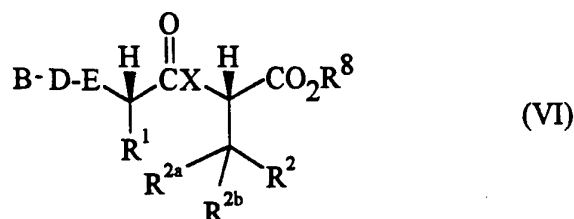
N-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-tyrosine.

Any chiral centers of any of the chemical structures on compounds described herein can be in the R and S configuration. Although these chiral centers of the chemical structures drawn hereinafter which are depicted in the preferred S-configuration, it is understood that the corresponding chemical structures having the chiral centers in the R-configuration as well as enantiomeric and diastereomeric mixtures of these compounds are also considered as being party of the invention.

A prodrug of formula (V), where B, D and E are as defined in formula (III), and X, R<sup>1</sup> and R<sup>2</sup> are as defined in formulas (IV) and (II),



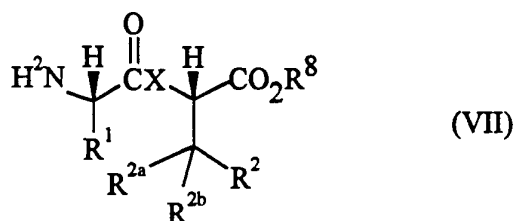
can be prepared by hydrolysis of an ester of formula (VI); where  $\text{R}^8$  is alkyl, branched alkyl, or cycloalkyl group such as methyl, ethyl, tert-butyl or cyclohexyl; or an aryl group such as phenyl, 2,6-dimethylphenyl; benzyl (optionally substituted with halo, alkoxy, or alkyl groups such as methyl or tert-butyl); or 9-fluorenylmethyl (Fm); normally in the presence of a base, such as NaOH, LiOH; or, in the case where  $\text{R}^8$  is Fm, diethylamine or piperidine; or in the presence of an acid such as HCl or trifluoroacetic acid; in a solvent or solvent mixture such as THF, nitromethane or THF:H<sub>2</sub>O; at a temperature, for example 0°C to reflux temperature, conveniently at room temperature; or in the presence of an acid, such as HCl or trifluoroacetic acid;



in a solvent such as THF or nitromethane; at a temperature, for example 0°C to reflux temperature, conveniently at room temperature; or by hydrogenolysis where  $\text{R}^8$  is 9-fluorenylmethyl (Fm) or benzyl optionally substituted with alkyl groups such as methyl or tert-butyl using hydrogen at ambient or elevated pressure, for example between 1.75 kgcm<sup>-2</sup> and 7.0 kgcm<sup>-2</sup>, conveniently at 3.5 kgcm<sup>-2</sup>, in the presence of a catalyst such as palladium-on-carbon, in a polar solvent, for example methanol or ethanol, or a mixture of methanol and dichloromethane, at ambient temperature, or by methods which are known to anyone skilled in the art such as those methods described in Rylander, P. N., Catalytic Hydrogenation in Organic Syntheses, Academic Press: New York, 1979, or in Rylander, P.N., Hydrogenation Methods, Academic Press: London, 1985, or in Bodanszky, M., *et.al.*, The Practice of Peptide Synthesis, Springer-Verlag: Berlin, 1984, which are incorporated herein by reference; or by oxidation where  $\text{R}^8$  is phenyl or benzyl substituted with alkoxy (preferably in the 2-, 4-, or 6- position of the phenyl ring) optionally substituted with suitable alkyl groups such as methyl or tert-butyl, in the presence of a suitable oxidizing agent such as ceric ammonium nitrate or dichlorodicyanoquinone (DDQ) in a suitable solvent such as methanol, water,

dichloromethane, or combinations thereof, by methods which are known to anyone skilled in the art such as those methods described in Heathcock, C.H., *et.al.*, Tetrahedron, 1981, 37, 4087; or those methods described in Greene, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference; or where R<sup>8</sup> is 2-(trimethylsilyl)ethyl by reaction with a source of a suitable nucleophile such as potassium fluoride or tetrabutylammonium fluoride for which methods are known to anyone skilled in the art such as those methods described in Greene, T. W., *et al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, or in Bodanszky, M., *et.al.*, the Practice of Peptide Synthesis, Springer-Verlag: Berlin, 1984, or in Jones, J., The Chemical Synthesis of Peptides, Clarendon Press: Oxford, England, 1991, which are incorporated herein by reference; or where R<sup>8</sup> is a photoremovable group such as ortho-nitrophenyl by photolytic methods which are known to anyone skilled in the art such as those described in Cama, L.D., *et.al.*, J. Am. Chem. Soc., 1978, 100, 8006, or in Pillai, V.N.R., Synthesis, 1980, 1, or in Pillai, V.N.R., Org. Photochem., 1987, 9, 225 which are incorporated herein by reference. Esters present on sidechains R<sup>1</sup> or R<sup>2</sup> may or may not be hydrolyzed in the same reaction mixture utilised to hydrolyze R<sup>8</sup>, depending on the choice of ester group and the reaction conditions.

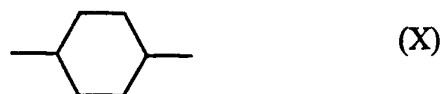
Ester (VI) can be prepared by reaction of an amine of formula (VII) where R<sup>1</sup> is defined as in formula (IV), R<sup>2</sup> and X are defined as in formula (II), and R<sup>8</sup> is defined as in formula (VI); a carboxylic acid of formula (VIII),



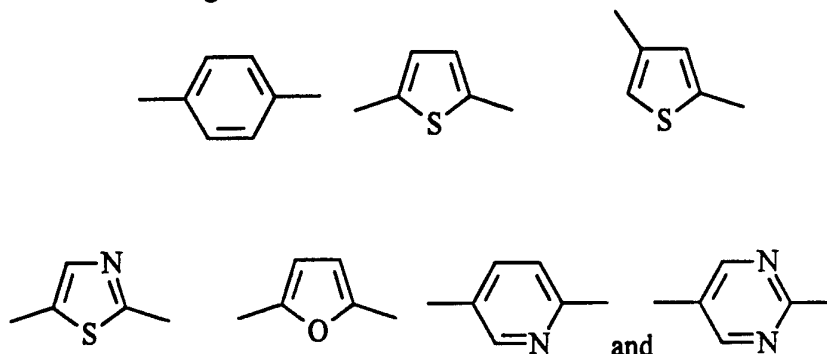
where B and D are as defined in hereinbefore and G represents a group (IX),



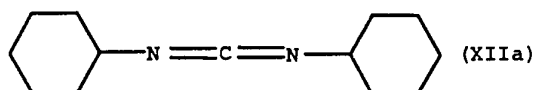
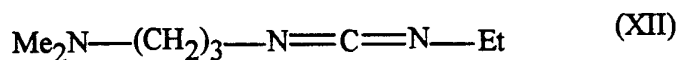
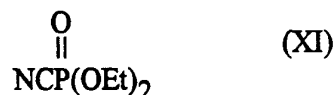




where  $n = 3-7$ , or 1,4-cyclohexyl (X) where the 1- and 4-substituents may be either in the cis- or trans- configuration or mixtures of the two configurations, or an aromatic group chosen from the following:

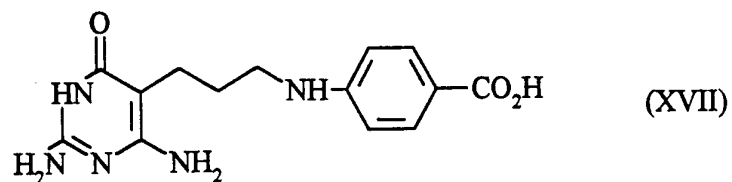
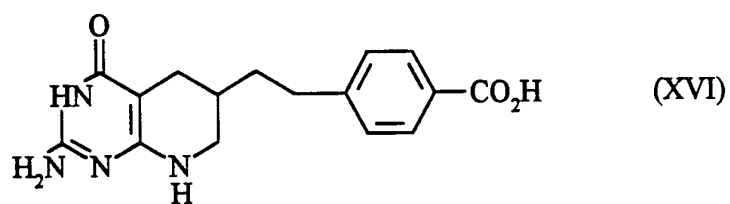
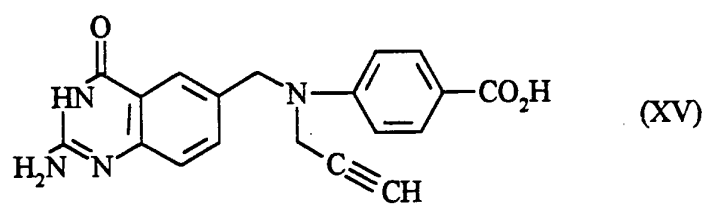
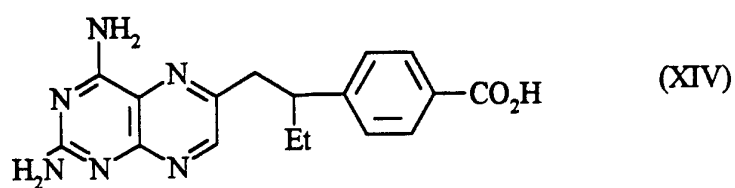
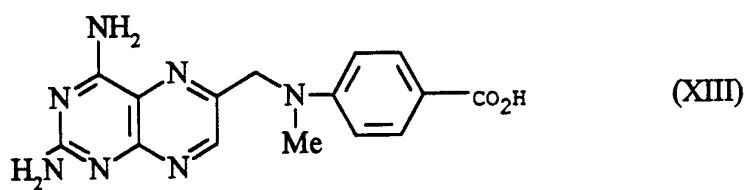


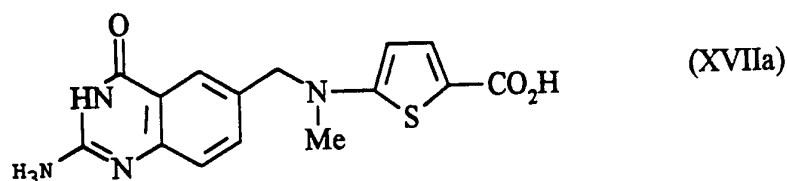
The reaction of amines of formula (VII) with carboxylic acids of formula (VIII) are carried out in the presence of a coupling agent; including but not limited to diethyl cyanophosphonate (XI), 1-ethyl-3-(dimethylaminopropyl)carbodiimide (XII), or dicyclohexylcarbodiimide (XIIa), optionally in the presence of an activating group such as 1-hydroxybenzotriazole (HOBT), in a polar, aprotic solvent, conveniently N,N-dimethylformamide (DMF) or N,N-dimethylpropyleneurea (DMPU) at a temperature, for example 0°C to 80°C,



conveniently at room temperature. Alternatively, an ester of formula (VI) can be prepared by treating a mixture of carboxylic acid (VIII) and a tertiary amine base; such as triethylamine, diisopropylethylamine, or N-methylmorpholine; in an aprotic solvent, such as DMF or DMPU; at a temperature from 0°C to 25°C; with an acylating agent, such as isobutyl chloroformate or ethyl chloroformate; followed by addition of amine (VII).

Several of the preferred carboxylic acids (VIII) can be purchased from commercial suppliers, eg. (XIII) can be purchased from Aldrich Chemical Co; or can be prepared via literature methods, eg. (XIV): De Graw, J I, *et al*, J. Med Chem, 1982, 25, 1227; Ibid, 1986, 29, 1056. (XV): Jones, T R, *et al*, J. Med Chem, 1986, 29, 1114; Nair, M G, *et al*, Ibid, 1986, 29, 1754; Ghazola, M, *et al*, Ibid, 1986, 29, 1263; Acharya S.P. *et.al*, J. Heterocyclic Chem, 1975, 12, 1283. (XVI): Barnett, C J, *et al*, Tetrahedron Lett, 1989, 30, 6291. (XVII): Styles, V L, *et al*, J. Med Chem, 1990, 33, 561 Taylor, E.c., *et al*. J. Med.Chem. 1992, 32 1517: (XV11a) and Bisset, G.M.F. *et al* J. Med. Chem. 1992 35 859;





Carboxylic acids of formula (VIII) can be prepared by hydrolysis of esters of formula (XVIII), wherein B and D are as defined in formula (III), G is as defined in formula (VIII) and  $R^9$  is a suitable group such as methyl, ethyl, or tert-butyl; normally in the presence of a suitable base, such as NaOH or LiOH; or a suitable acid, such as HCl or trifluoroacetic acid; in a suitable solvent or solvent mixture, such as THF, nitromethane, or THF:H<sub>2</sub>O; at a temperature, for example from 0°C to reflux temperature, conveniently at room temperature.



For prodrugs where D in formula (III) or formula (XVIII) represents  $-CH_2-NR^3-$ , esters of formula (XVIII) can be prepared by:

- (a) reaction of a compound of formula (XIX), where G is as defined in formula (VIII),  $R^9$  is as defined in formula (XVIII), and  $R^{10}$  represents

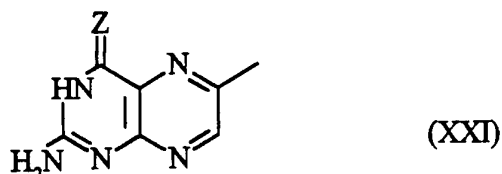


$NHR^3$  wherein  $R^3$  is as defined in formula (III); and a compound of formula (XX)



wherein  $R^{11}$  is an aldehyde group or cyano group and K is a heterocyclic group such as those defined as B in formula (III) or a group such as (XXI) where  $Z=O$ , or a group such as defined as B in formula (III) or a group such as (XXI) where  $Z=O$  substituted with a suitable protecting group to provide a more suitable reactant, suitable groups including but not limited to N-pivaloyl, N-benzoyl, N-carbobenzyloxy (N-cbz), or N-tert-butoxycarbonyl (N-t-BOC). Such protecting groups can be removed at a later stage in the synthesis, where appropriate, using

standard methods known to anyone skilled in the art such as those methods described in Green, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference.



Esters of formula (XVIII) are prepared by reaction of (XVI) and (XX) in a solvent such as THF, DMPU, DMF, ethanol, or acetic acid at a temperature between 25°C and 100°C, optionally with removal of water during the course of the reaction, followed by reduction with sodium cyanoborohydride or hydrogen gas in the presence of a suitable catalyst, such as Raney nickel, using standard reaction conditions known to anyone skilled in the art.

- (b) reaction of a compound of formula (XIX), as defined in (a), with a compound of formula (XX), where K is as defined in (a) but R<sup>11</sup> is defined as CH<sub>2</sub>Y, where Y is defined as a leaving group such as chloro, bromo, iodo, mesyl, or tosyl, in a suitable solvent such as THF, in the presence of a base such as NaHCO<sub>3</sub>, Na<sub>2</sub>CO<sub>3</sub>, K<sub>2</sub>CO<sub>3</sub>, triethylamine, or diisopropylethylamine, at an elevated temperature, for example 50°C to 150°C and conveniently 80°C to 120°C.

For prodrugs where D in formula (III) and formula (XVIII) represents -NR<sup>3</sup>-CH<sub>2</sub>-, esters of formula (XVIII) can be prepared by reaction of a compound of formula (XIX) where R<sup>10</sup> represents an aldehyde group and G is as defined in formula (VIII), and a compound of formula (XX), where K is as defined in formula (XX) and R<sup>11</sup> is defined as HNR<sup>3</sup> where R<sup>3</sup> is as defined in formula (III), in a solvent such as THF, DMF, DMPU, or acetic acid at a temperature between 25°C and 100°C, optionally with removal of water during the course of the reaction, followed by reduction with sodium cyanoborohydride or hydrogen gas in the presence of a suitable catalyst, such as Raney nickel.

For prodrugs where D in formula (III) and formula (XVIII) represents -CH<sub>2</sub>O-or-CH<sub>2</sub>S- esters of formula (XVIII) are prepared by reaction of a compound of formula (XIX),

where  $R^9$  is as defined in formula (XVIII), G is as defined in formula (VIII), and  $R^{10}$  is OH or SH; with a compound of formula (XX), where K is as defined in formula (XX) and  $R^{11}$  is  $CH_2Y$ , where Y is defined as a leaving group such as chloro, bromo, iodo, mesyl, or tosyl; in a suitable solvent such as DMF, DMPU, or acetone; in the presence of a base such as triethylamine, diisopropylethylamine, cesium carbonate, cesium bicarbonate, sodium carbonate, or sodium bicarbonate, at a temperature from 25°C to 80°C.

For prodrugs where D in formula (III) and formula (XVIII) represents  $-CH_2CH_2-$ , esters of formula (XVIII) are prepared by:

- (a) reaction of a compound of formula (XX) where K is defined as in formula (XX) and where  $R^{11}$  is  $CH_2Br$ , with triphenylphosphine in a polar solvent, for example a  $C_{1-4}$  alkanol or glycol, conveniently methanol or ethanol, normally in the presence of a base, for example a metal alkoxide conveniently formed from the metal and the solvent, ie. sodium methoxide or sodium ethoxide at a temperature of 0°C to reflux temperature; followed by addition of a compound of formula (XIX), where  $R^9$  is as defined in formula (XVIII), G is as defined in formula (VIII), and  $R^{10}$  is an aldehyde group; followed by reduction with hydrogen at elevated pressure, for example between 1.75 kgcm<sup>-2</sup> and 7.0 kgcm<sup>-2</sup>, conveniently at 3.5 kgcm<sup>-2</sup> in the presence of a suitable catalyst such as palladium-on-carbon, in a polar solvent, for example methanol or ethanol or a mixture of methanol and dichloromethane, at ambient temperature.
- (b) reaction of a compound of formula (XIX) where  $R^9$  is defined as in formula (XVIII) and G is defined as in formula (VIII), and  $R^{10}$  is  $CH_2Br$ , with triphenylphosphine in a polar solvent, for example a  $C_{1-4}$  alkanol or glycol, conveniently methanol or ethanol, normally in the presence of a base, for example a metal alkoxide conveniently formed from the metal and the solvent, ie. sodium methoxide or sodium ethoxide at a temperature of 0°C to reflux temperature; followed by addition of a compound of formula (XX), where K is as defined in formula (XX), and  $R^{11}$  is an aldehyde group; followed by reduction with hydrogen at elevated pressure, for example between 1.75 kgcm<sup>-2</sup> and 7.0 kgcm<sup>-2</sup>, conveniently at 3.5 kgcm<sup>-2</sup>, in the presence of a suitable catalyst such as

palladium-on-carbon, in a polar solvent, for example methanol or ethanol or a mixture of methanol and dichloromethane, at ambient temperature.

- (c) reaction of a compound of formula (XX), where K is defined as in formula (XX) and R<sup>11</sup> is chloro, bromo, or iodo; with a compound of formula (XIX), where R<sup>9</sup> is defined as in formula (XVIII), G is as defined in formula (VIII), and R<sup>10</sup> is a acetylene group C≡CH.

The reaction is carried out in the presence of a Pd(O) catalyst, conveniently tetrakis-(triphenylphosphine)palladium; and a base such as triethylamine or diisopropylethylamine; in a polar aprotic solvent such as DMF or DMPU; at a temperature of 25°C to 50°C; followed by reduction with hydrogen at elevated pressure, for example between 1.75kgcm<sup>-2</sup> and 7.0kgcm<sup>-2</sup>, conveniently at 3.5kgcm<sup>-2</sup>, in the presence of a suitable catalyst such as palladium-on-carbon, in a polar solvent, for example methanol or ethanol or a mixture of methanol and dichloromethane, at ambient temperature.

For prodrugs where D in formula (III) and formula (XVIII) represents -CH<sub>2</sub>-CH(R<sup>3</sup>)-, R<sup>3</sup> is as defined in formula (III), esters of formula (XVIII) are prepared by reaction of a compound of formula (XX), where K is defined as in formula (XX) and where R<sup>11</sup> is CH<sub>2</sub>Br, with tributylphosphine or triphenylphosphine in a polar solvent, for example a C<sub>1-4</sub> alkanol or glycol, conveniently methanol or ethanol, normally in the presence of a base, for example a metal alkoxide conveniently formed from the metal and the solvent, ie. sodium methoxide or sodium ethoxide, or in a solution of dimethyl sulfoxide and a base such as sodium hydride at a temperature from 25°C to 80°C; followed by addition of a compound of formula (XIX), where R<sup>9</sup> is defined as in formula (XVIII), G is defined as in formula (VIII), and R<sup>10</sup> is a keto group -(CO)-R<sup>3</sup> where R<sup>3</sup> is defined as in formula (III); subsequently stirring the reaction mixture at a temperature from 25°C to reflux temperature for an extended period from 4 to 96 hours, conveniently 24 to 48 hours; followed by reduction with hydrogen at elevated pressure, for example between 1.75kgcm<sup>-2</sup> and 7.0kgcm<sup>-2</sup>, conveniently at 3.5kgcm<sup>-2</sup>, in the presence of a suitable catalyst such as palladium-on-carbon or platinum oxide, in a polar solvent, for example methanol ethanol or acetic acid.

Compounds of formula (XX) where K is as defined above and R<sup>11</sup> is CH<sub>2</sub>Br can be prepared using methodology available to those skilled in the art. (Piper, J R *et al*, J Org

Chem., 1977, 42, 208; Piper, J R *et al*, J Med Chem, 1986, 29, 1080; Oakes, V, *et al*, J Chem Soc, 1956, 4433; Acharya, S P, *et al*, J Heterocyclic Chem, 1975, 12, 1283; Bird, O D, *et al* in Pteridine Chemistry, Pfleiderer W & Taylor E C, Eds, Pergamon Press, Oxford, 1964, p. 417; Piper, J R, *et al*, J Heterocyclic Chem, 1974, 279.)

Compounds of formula (XX) where K is as defined above and R<sup>11</sup> is an aldehyde group can be prepared using methodology available to those skilled in the art. (Taylor, E C, *et al*, J Org Chem, 1983, 48, 4852; Arnold, A, *et al*, Collect Czech Chem Commun, 1960, 25, 1318; Beardsley, G P, *et al*, Proc Am Assoc Cancer Res, 1986, 1027.)

Compounds of formula (XX) where K is as defined above and R<sup>11</sup> is a cyano group can be prepared using methodology available to those skilled in the art. (Elsager, E F, *et al*, Lect Heterocyclic Chem, 1974, 2, S-97; Davoll, J, *et al*, J Chem Soc (C), 1970, 997; Jones, T.R., Eur. J. Cancer, 1981, 17, 11; Kondo, T., *et al.*, Chem. Lett., 1980, 559.

Compounds of formula (XX) where K is as defined above and R<sup>11</sup> is an amino group can be prepared using methodology available to those skilled in the art. (Hynes, J B, *et al*, J Med Chem, 1975, 18, 632, 1191; Davoll, J, *et al*, J Med Chem, 1972, 15, 837; Bernetti, R *et al*, J Org Chem, 1962, 27, 2863.)

Compounds of formula (XX) where K is as defined above and R<sup>11</sup> is a bromo, chloro, or iodo group can be prepared using methodology available to those skilled in the art. Taylor, E C, *et al*, J Med Chem, 1992, 35, 4450; Taylor, E C, *et al*, J Org Chem, 1989, 54, 3618; Jones, J H, *et al*, J Med Chem, 1968, 11, 322.)

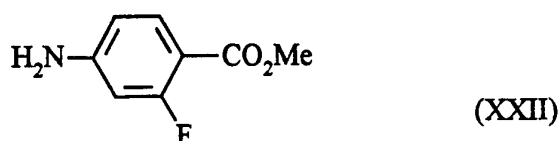
Carboxylic acids of formula (VIII) where A is a group of formula (XXI) where Z=S, can be prepared from carboxylic acids of formula (VIII) where C is a group of formula (XXI) wherein Z=O, by reaction with a sulfurating agent such as P<sub>2</sub>S<sub>5</sub> in a solvent such as pyridine at a temperature between 25°C and reflux temperature.

Compounds of formula (XIX) where G is defined as in formula (VIII), R<sup>9</sup> is as defined in formula (XVIII), and R<sup>10</sup> is NHR<sup>3</sup> where R<sup>3</sup> is as defined in formula (III) can be prepared from compounds of formula (XIX) where G and R<sup>9</sup> are as defined above and R<sup>10</sup> is NH<sub>2</sub> by reaction with a compound R<sup>3</sup>-Y where Y is defined as a leaving group such as chloro, bromo, iodo, mesyl, or tosyl; in the presence of a suitable base such as

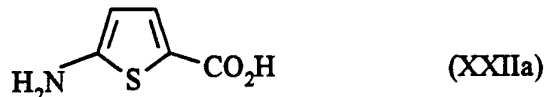


2,6-lutidine, in a polar aprotic solvent such as DMF or dimethylacetamide at an elevated temperature between 50°C and 100°C.

Compounds of formula (XIX) where G is a phenyl or furyl group, R<sup>10</sup> is NH<sub>2</sub>, and R<sup>9</sup> is defined as in formula (XVIII) can be obtained from commercial suppliers, or can be readily prepared from such compounds that are available from commercial suppliers using methods known to anyone skilled in the art. One such preferred compound of formula (XXII)



can be prepared by methods described by N Soundararajan and M S Platz. (Soundararajan N *et al*, J Org Chem, 1990, 55, 2034.) One such preferred compound is of formula (XXIIa) and can be prepared by methods described in Mackay, D., Can J. Chem, 1966, 44, 2881; or in Paul, H., *et.al.*, Arch. Pharm, 1978, 52 538.



Compounds of formula (XIX) where G is a cyclohexyl group, R<sup>10</sup> is NH<sub>2</sub>, and R<sup>9</sup> is as defined in formula (XVIII) can be obtained from commercial suppliers, or can be readily prepared using standard methods available to anyone skilled in the art. (Banfi, A, *et al*, Synth Commun, 1989, 19, 1787; Krieg, R, *et al*, J Prakt Chem, 1987, 329, 1123; Johnston, T P, *et al*, J Med Chem, 1977, 20, 279.)

Compounds of formula (XIX) where G is a thienyl group, R<sup>10</sup> is NH<sub>2</sub>, and R<sup>9</sup> is as defined in formula (XVIII) can be prepared by methodology available to anyone skilled in the art. (Goddard, C J, J Heterocyclic Chem, 1991, 28, 17; Decroix, B, *et al*, J Chem Res (S), 1978, 134; Paul, H, *et al*, Arch Pharm, 1978, 311, 679; Mackay, D, Can J Chem, 1966, 44, 2881.)

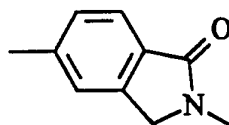
Compounds of formula (XIX) where G is a thiazole group, R<sup>10</sup> is NH<sub>2</sub>, and R<sup>9</sup> is as defined in formula (XVIII) can be prepared from 5-nitrothiazole-2-carboxylic acid (Strehlke, P, Chem Ber, 1973, 106, 721) using esterification and reduction methods known to anyone skilled in the art.

Compounds of formula (XIX) where G is a pyridyl group,  $R^{10}$  is  $NH_2$ , and  $R^9$  is as defined in formula (XVIII) can be prepared using methodology available to anyone skilled in the art. (Dawson, M I, *et al*, J Med Chem, 1983, 26, 1282; Finch, N, *et al*, J Med Chem, 1978, 21, 1269; Cooper, G H, *et al*, J Chem Soc(C), 1971, 3257; Deady, L W, *et al*, Aust J Chem, 1971, 24, 385.)

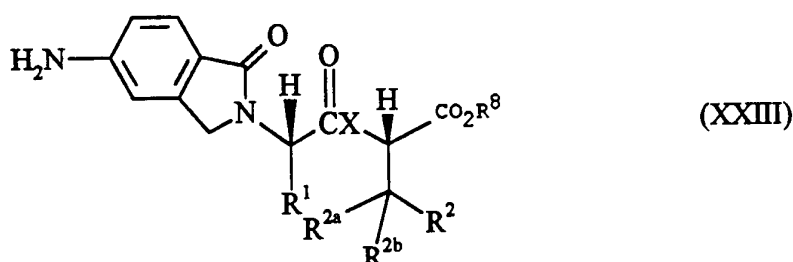
Compounds of formula (XIX) where G is a pyrimidyl group,  $R^{10}$  is  $NH_2$ , and  $R^9$  is as defined in formula (XVIII) can be prepared using methods analogous to those described by P R Marsham, *et al*. (Marsham, P R, *et al*, J Med Chem, 1991, 34, 1594.)

General methods for synthesis of compounds of formula (VIII) are described in two recent reviews. (Palmer, D C, *et al*, Prog Med Chem, 1988, 25, 85; Rosowsky, A, Prog Med Chem, 1989, 26, 1.)

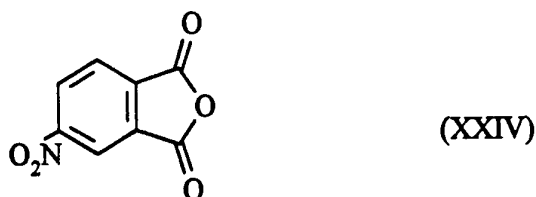
Esters of formula (VI) where E is defined as



can be prepared by reaction of a compound of formula (XX) where  $R^{11}$  is  $CH_2Br$ ; and a compound of formula (XXIII) where  $R^1$  is defined



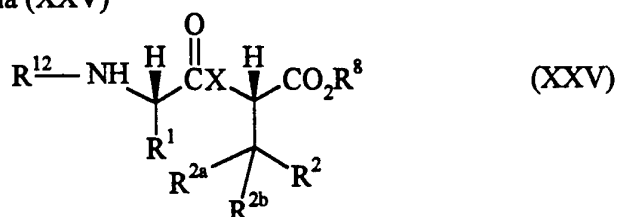
as in formula (IV),  $R^2$  and X are defined as in formula (II), and  $R^8$  is defined as in formula (VI); in a polar solvent, such as DMF or DMPU; in the presence of a base, such as sodium bicarbonate, cesium bicarbonate, sodium carbonate or cesium carbonate; at an elevated temperature, for example from  $50^\circ C$  to  $100^\circ C$ , conveniently at  $100^\circ C$ . Compounds of formula (XXIII) can be prepared from compounds of formula (VII) and a compound of formula (XXIV) by methods analogous to those described by



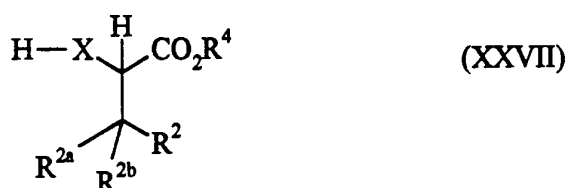
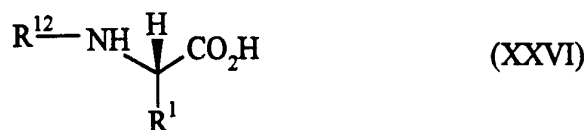
W Pendergast, *et al.* (Pendergast, W, *et al.*, J Med Chem, [1994], 37 p 838) or in patent application WO 01/19700 which is incorporated in its entirety by reference herein.

Compounds of formula (VII) can be prepared from compounds of formula (XXV), where  $R^1$  is as defined in formula (IV),  $R^2$  and X are as defined in formula (II),  $R^8$  is as defined in formula (VI), and  $R^{12}$  represents a suitable protecting group such as N-tert-butoxycarbonyl(N-t-Boc), or N-carbobenzyloxy (N-Cbz), for which methods are known to anyone skilled in the art such as those methods described in Greene, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference.

Compounds of formula (XXV)

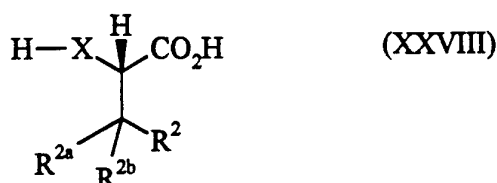


can be prepared by a reaction of carboxylic acid of formula (XXVI) and a compound of formula (XXVII), where X is defined as in formula (II); for compounds where X is NH, compounds of formula (XXV) can be prepared by reaction of a carboxylic acid of formula (XXVI) and an amine of formula (XXVII), where X is NH, in the presence of a suitable coupling agent such as 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide or dicyclohexylcarbodiimide, in the presence of a suitable base such as N-methylmorpholine, in an aprotic solvent such as dichloromethane or DMF at 0°C to 50°C or by methods which are known to anyone skilled in the art such as those methods described in Bodanszky, M., *et.al.*, The Practice of Peptide Synthesis, Springer-Verlag: Berlin, 1984, or in Jones, J., The Chemical Synthesis of Peptides, Clarendon Press: Oxford, England, 1991, which are incorporated herein by reference.



dichloromethane or DMF at 0°C to 50°C for which methods are known to anyone skilled in the art such as those methods described in Greene, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference.

For compounds of formula (XXV) where X is O, compounds of formula (XXV) can be prepared by reaction of a carboxylic acid of formula (XXVI) with a suitable activating agent such as para-toluenesulfonyl chloride or benzenesulfonyl chloride, in a suitable solvent such as pyridine or lutidine, at a temperature between 0°C and 25°C; followed by addition of an alcohol of formula (XXVII) where X is O.

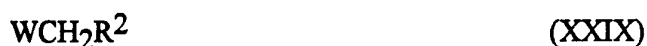


Esters of formula (XXVII), where X is defined as in formula (II), can be prepared from carboxylic acids of formula (XXVIII) using standard methods known to anyone skilled in the art for which methods are known to anyone skilled in the art such as those methods described in Greene, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference.

Carboxylic acids of formula (XXVIII), where X is O, can be made from carboxylic acids of formula (XXVIII) where X is NH by methods analogous to those described by C

Morin, *et al.* (Morin, C, *et al.*, Synthesis, 1987, 479) and K Mori, *et al.* (Mori, K, *et al.*, Tetrahedron, 1982, 38, 3705).

Carboxylic acids of formula (XXVIII) where X is NH can be prepared from compounds of formula (XXIX); where W is a leaving group such as chloro, bromo, iodo, mesyl, or tosyl and R<sup>2</sup> is defined as in formula (II); by methods analogous to



described by R M Williams (Williams, R M, Aldrichimica Acta, 1992, 25, 11 and references cited therein), or by M J O'Donnell, *et al.* (O'Donnell, M J, *et al.*, J Am Chem Soc, 1989, 111, 2353.)

Compounds of formula (XXIX), where W is a leaving group such as chloro, bromo, iodo, mesyl, or tosyl can be prepared from compounds of formula (XXIX) where W is a hydroxyl group for which methods are known to anyone skilled in the art such as those methods described in March, J., Advanced Organic Chemistry, fourth edition, John Wiley & Sons: New York, 1992, which are incorporated herein by reference.

Some of the preferred compounds of formula (XXIX) where R<sup>2</sup> is a phenyl group substituted with C<sub>1-8</sub> alkyl, C<sub>3-8</sub> branched alkyl, or C<sub>3-8</sub> cycloalkyl can be prepared by reaction of a compound of (XXIX) where W is a hydroxyl group, or a hydroxyl group with a suitable protecting group which can be attached and later removed where appropriate in the synthesis, by standard methods known to anyone skilled in the art, and R<sup>2</sup> is a phenyl group substituted with an iodo group or a bromo group; the reaction can proceed by addition of a suitable olefinic compound in the presence of a Pd catalyst by methods analogous to those described by H A Dieck, *et al.* (Dieck, H A, *et al.*, J Am Chem Soc, 1974, 96, 1133), followed by reduction with hydrogen gas in the presence of a suitable catalyst using standard conditions known to anyone skilled in the art for which methods are known to anyone skilled in the art such as those methods described in Rylander, P.N., Catalytic Hydrogenation in Organic Syntheses, Academic Press: New York, 1979, or in Rylander, P.N., Hydrogenation Methods, Academic Press: London, 1985, which are incorporated herein by reference; or the reaction can proceed by reaction with a suitable alkyl lithium reagent such as n-butyllithium reagent, in an ethereal solvent such as THF, at a reduced temperature, for example -100°C to -20°C,

conveniently at  $-78^{\circ}\text{C}$ , followed by addition of the resulting benzylic alcohol group by methods analogous to those described by C T West, *et al*, (West, C T, *et al*, J Org Chem, 1973, 38, 2675).

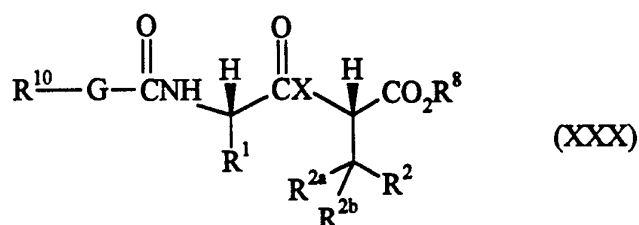
Some of the preferred compounds of formula (XXIX) where  $\text{R}^2$  is a phenyl group substituted with a carboxyl or esterified carboxyl group can be prepared by reaction of a compound of formula (XXIX) where W is a hydroxyl group and  $\text{R}^2$  is a phenyl group substituted with a bromo or iodo group, with carbon monoxide in a suitable alkanol solvent by methods analogous to those described by A Schoenber, *et al*, (Schoenber, A, *et al*, J Org Chem, 1974, 39, 3318), followed by hydrolysis of the resulting ester, if desired, for which methods are known to anyone skilled in the art such as those methods described in Greene, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference.

Some of the preferred carboxylic acids of formula (XXVIII) where X is NH and  $\text{R}^2$  is a para-hydroxyphenyl group substituted with  $\text{C}_{1-8}$  alkyl,  $\text{C}_{3-8}$  branched alkyl, or  $\text{C}_{3-8}$  cycloalkyl can be prepared by reaction of tyrosine substituted on the phenyl ring with a bromo or an iodo group; where the amino and/or carboxyl groups can be optionally substituted with a suitable protecting group which can be attached and later removed where appropriate in the synthesis, for which methods are known to anyone skilled in the art such as those methods described in Greene, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference with a suitable olefinic compound in the presence of a Pd catalyst by methods analogous to those described by H A Dieck, *et al*, (Dieck, H A, *et al*, J Am Chem Soc, 1974, 96, 1133), followed by reduction with hydrogen gas in the presence of a suitable catalyst using standard conditions known to anyone skilled in the art.

Some of the preferred carboxylic acids of formula (XXVIII) where X is NH and  $\text{R}^2$  is a para-hydroxyphenyl group substituted with carboxyl or esterified carboxyl can be prepared by reaction of tyrosine substituted on the phenyl ring with a bromo or an iodo group; where the amino and/or carboxyl groups can be optionally substituted with a suitable protecting group which can be attached and later removed where appropriate in the synthesis, for which methods are known to anyone skilled in the art such as those

methods described in Greene, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference; with carbon monoxide in a suitable alkanol solvent by methods analagous to those described by (Schoenberg, A, *et al*, J Org Chem, 1974, 39, 3318), followed by hydrolysis of the resulting ester, if desired, by using standard methods known to anyone skilled in the art.

An alternate method for the synthesis of compounds of formula (VI) involves reaction of a compound of formula (XX), where K is as defined in formula (XX) and R<sup>11</sup> is an aldehyde group, a cyano group, NHR<sup>3</sup> where R<sup>3</sup> is as defined in formula (III), or a group CH<sub>2</sub>Y where Y is defined as a leaving group such as chloro, bromo, iodo, mesyl, or tosyl; with a compound of formula (XXX), where R<sup>1</sup> is as defined in formula (IV), R<sup>2</sup> and X are as defined in formula (II), R<sup>8</sup> is as defined in formula (VI), G is as defined in formula (VIII), and R<sup>10</sup> is defined as OH, SH, NHR<sup>3</sup> where R<sup>3</sup> is as defined in formula (III), an aldehyde group, a keto group -(CO)-R<sup>3</sup> where R<sup>3</sup> is as defined in formula (III), or a group CH<sub>2</sub>Y where Y is a leaving group such as chloro, bromo, iodo, mesyl, or tosyl; by methods analogous to



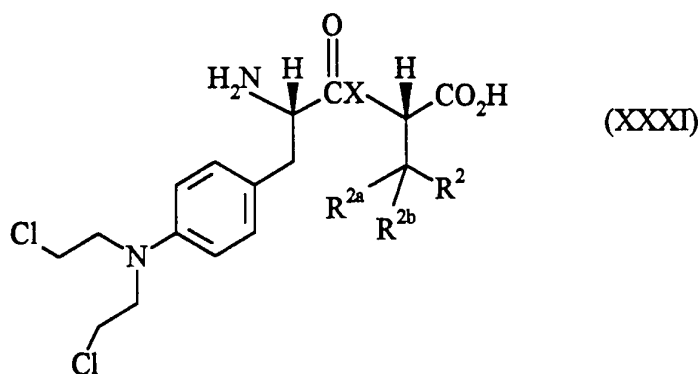
those described for the preparation of compounds of formula (XVIII) by reaction of compounds of formula (XX) with compounds of formula (XIX).

Compounds of formula (XXX) where R<sup>1</sup> is as defined in formula (IV), R<sup>2</sup> and X are as defined in formula (II), R<sup>8</sup> is as defined in formula (VI), G is defined as in formula (VIII), and R<sup>10</sup> is CH<sub>2</sub>Y where Y is a leaving group such as chloro, bromo, iodo, mesyl, or tosyl can be prepared from compounds of formula (XXX) where R<sup>1</sup>, R<sup>2</sup>, R<sup>8</sup>, X and G are as defined above, and where R<sup>10</sup> is CH<sub>2</sub>OH for which methods are known to anyone skilled in the art such as those methods described in March, J., Advanced Organic Chemistry, fourth edition, John Wiley & sons: New York, 1992, which are incorporated herein by reference.

Compounds of formula (XXX), where  $R^1$  is as defined in formula (IV),  $R^2$  and X are as defined in formula (II),  $R^8$  is as defined in formula (VI), G is as defined in formula (VIII), and  $R^{10}$  is defined as OH, SH,  $CH_2OH$ ,  $NHR^3$  where  $R^3$  is as defined in formula (III), an aldehyde group, or a keto group  $-(CO)-R^3$  can be prepared from compounds of formula (VII), where  $R^1$ ,  $R^2$ ,  $R^8$ , and X are as defined above, with a compound of formula (XIX), where  $R^9$  is hydrogen and  $R^{10}$  is as defined above, by methods analogous to those described for the preparation of compounds of formula (VI) by reaction of compounds of formula (VII) with compounds of formula (VIII). In cases where  $R^{10}$  is OH, SH,  $CH_2OH$ ,  $NHR^3$ , it may be preferable to add a protecting group to  $R^{10}$  prior to reaction with a compound of formula (VII). Such protecting groups can be added and can be removed at a later stage in the synthesis, for which methods are known to anyone skilled in the art such as those methods described in Greene, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference.

Compounds of formula (XIX), where  $R^9$  is hydrogen and  $R^{10}$  is defined as OH, SH,  $CH_2OH$ ,  $NHR^3$  where  $R^3$  is as defined in formula (III), an aldehyde group, or a keto group  $-(CO)-R^3$  above, can be prepared from compounds of formula (XIX), where  $R^9$  is as defined in formula (XVIII) and  $R^{10}$  is as defined above, for which methods are known to anyone skilled in the art such as those methods described in Greene, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference.

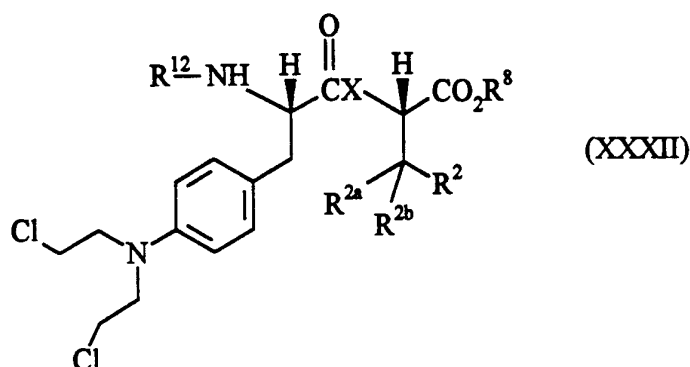
Prodrugs of general structure (XXXI) where X and  $R^2$  are as defined in formula (II) can be prepared from compounds of formula (XXXII)



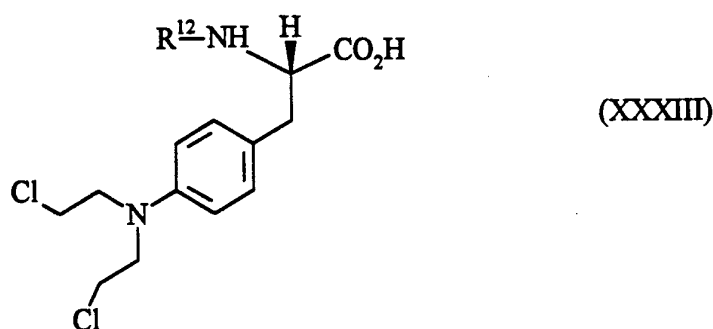
wherein  $R^{12}$  is as defined in formula (XXV),  $R^2$  is as defined in formula (II), and  $R^8$  is as defined in



formula (VI), for which methods are known to anyone skilled in the art such as those methods described in Greene, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference.



Compounds of formula (XXXII) can be prepared by reaction of compounds of formula (XXXIII) where  $R^{12}$  is as defined in formula (XXV);



with compounds of formula (XXVII) where X is NH or O,  $R^2$  is as defined in formula (II), and  $R^8$  is as defined in formula (VI); using methods analogous to those described for the preparation of compounds of formula (XXV) from compounds of formulas (XXVI) and (XXVII).

Compounds of formula (XXXIII), where  $R^{12}$  is a suitable protecting group as defined in formula (XXV), can be prepared for which methods are known to anyone skilled in the art such as those methods described in Greene, T.W., *et.al.*, Protective Groups in Organic Synthesis, second edition, John Wiley & Sons: New York, 1991, which are incorporated herein by reference; from melphalan (UK 750, 155) which is commercially available under the name Alkeran (T.M. The Wellcome Foundation Limited) and has the chemical name of 4-[bis(2-chloroethyl)amino]-1-phenylalanine.

The term protein engineering has come to mean the structurally directed modification of a protein to obtain a specific, desired new set of protein properties or, in this context, a precise enzymatic activity. Two approaches to modification of protein specificity and reactivity can be used. The differences in these approaches depend upon the chemical and structural information available for a specific enzyme. If no chemical structural information is known about a protein, random mutations can be introduced singly or in groups to determine the importance of specific amino acid residues using techniques well known in the art of molecular biology. However, a more directed approach using site directed mutagenesis, can be performed, if there is detailed chemical and structural information available on a protein. This involves only specific changes to amino acid residues that the chemical and structural data suggest are the residues critical to determination of the desired enzymatic activity. If 3-dimensional structural data are available, the methodology can be carried further to provide molecular models of proposed mutations. These models can provide estimates of the relative stability or reactivity of a specific site directed mutation.

Typically, in the computer-assisted protein engineering approach, computer models based upon x-ray crystallographic structures or models built, by homology model building, from x-ray structures of homologous proteins are used. In many cases the starting crystallographic structures can be obtained from the publicly available Brookhaven Protein Database.

Extensive studies of protein structure and the function of individual amino acids have allowed the classification of residues in respect of their importance in structure, stability, reactivity or catalysis. Many active site amino acid residues in proteins show unusual chemical reactivity, these chemical reactivities characterise the catalytic properties of proteins. This chemical reactivity aids in the identification of the active site and the non-reactive associated residues in the active site. Crystallographic studies in concert with chemical reactivity information can indicate distinctly the active site locations. Additionally, some proteins will crystallise with substrates or inhibitors in the active site. All of these pieces of structural information can be combined to enhance the focus of design efforts towards specific residues as candidates for site directed mutagenesis.

The specific desired change in protein reactivity can be obtained by specific changes in the amino acid at a given site of interest. Amino acid residues can be inserted, thus

separating functional groups in space, or existing residues can be deleted, bringing functional groups closer together.

Additionally, changes in protein structure can be effected by changing the side chain or main chain torsion angles and the functional groups on the amino acid residues. These torsion angles can be changed by exchange, deletion or insertion of amino acid residues. If structural data are available, all these changes can be modelled using computer-assisted molecular modelling techniques. Forces interacting on the protein structures can be modelled in the computer using molecular mechanics or molecular dynamics force fields.

The space available for ligand binding within a protein can be modified by changes in the bulk of amino acid side chains. Mutating bulky residues, such as isoleucine or leucine, to alanine or glycine, can provide pockets within a ligand binding site without appreciably changing the hydrophobicity of the site. The opposite mutation can exclude bulky substrates from binding to a specific protein.

Mutations that involve changes in the functional groups of the amino acids that make up an active site can provide more drastic changes than bulk tolerance or steric changes. Examples of many of these types of changes are available in the literature (see, eg: Recktenwald, A, *et al*, J. Biotech, 1993, 28, 1-23 and references cited therein; and Lesk, A, *et al*, "Antibody Engineering: A Practical Guide", Ed. C. A K. Borrebaeck *et al*, 1991, 1-75). In systems with high quality structural data, specific hydrogen bonding groups can be inserted to improve the interaction of polar substrates. Replacing uncharged residues with arginine, lysine, or histidine inserts groups that could interact with negatively charged substrates. Replacing residues with glutamic acid or aspartic acid can provide interactions with positively charged substrates and thus change the specificity of an enzyme substrate interaction.

In a further aspect of the present invention there is provided a recombinant conjugate comprising a targetting antibody as hereinbefore described and a mutant enzyme of the present invention; such recombinant conjugate having been expressed from a single DNA construct or translated from a single RNA transcript. Also included within the scope of the present invention are the corresponding DNA and RNA molecules encoding such a conjugate. Preferred embodiments of such a conjugate reflect a combination of the preferred embodiments for its two component activities as hereinbefore expressed.

Such recombinant conjugates may be produced by expression of fusion constructs, generated by joining, *in vitro*, fragments of DNA that separately encode the enzyme and all or a part of a mAb that confers antigen specificity (Pastan, I. *et al*, (1986) Cell, 47, 641-648). Such fusions may be constructed by standard techniques of recombinant molecular biology in ways that place the substrate-binding or active site of the polypeptide at the amino terminus or the carboxy terminus and preferably at the amino terminus. The mAb portion of the construct may be designed so that a single chain species of the antibody is connected to the enzyme portion either with the variable light or the variable heavy domain at the 5'- end. In either case, the variable light and variable heavy gene regions will be connected with a synthetic DNA fragment encoding a peptide linker that achieves the correct spatial arrangement of the antibody variable regions. Such a synthetic DNA region connecting the antibody portion of the fusion construct to the enzyme portion may also be desirable. In other forms, the gene encoding the enzyme may be fused to the 5'-end of the variable region of the heavy or light chain gene that extends to form a Fab or F(ab')<sub>2</sub> species. Other variations of the heavy chain gene may be used to form the enzyme/antibody recombinant conjugate. Such variations may include natural heavy chain isotypes, modified full-length heavy chain genes of all antibody isotypes, and heavy chains with various deletions, specifically, deletion of the CH<sub>2</sub> domain may confer desirable pharmacokinetic properties. Alternatively, the fusion may produce a covalent bispecific antibody:enzyme conjugate where one arm of the antibody is replaced by the enzyme in a fashion similar to but not limited to that described in De Sutter, K and Fiers, W. Mol Immunol 31 261 (1994). The term 'conjugate' as used herein includes such recombinant conjugates.

Antibodies designed to have particular cellular specificities may be generated in any way well known in the art. Fragments of antibodies generated by recombinant DNA technology may also be used as targeting molecules.

Although the preferred antibodies for use will be those expressed from natural or recombinant human derived cell-lines, it is also possible to exploit antibodies from other antibody producing mammalian cell-lines providing such antibodies are not immunogenic to an extent liable to interfere significantly with therapy or that the antibodies have been 'humanised' such that they no longer elicit an immune reaction *in vivo*. Such a humanised antibody may be a chimaeric antibody (Morrison *et al* P.N.A.S. (1984), 81, 6851-6855;

Boulianne *et al*, Nature, 1985, 314, 268-270 and Neuberger *et al*, Nature, 1985, 314, 268-70), or a CDR-grafted antibody (James *et al*, Nature, 1986, 321, 522-525; Riechmann *et al*, Nature, 1988, 332, 323-327).

An antibody for use according to the present invention preferably is a monoclonal antibody or a fragment thereof. The antibody may therefore comprise a complete antibody, a (Fab')<sub>2</sub> fragment, a Fab' fragment, a Fab fragment, a light chain dimer or a heavy chain dimer or single chain species comprising the variable regions from heavy and light chains. The antibody may be an IgG such as IgG1, IgG2, IgG3, or IgG4; or IgM, IgA, IgE or IgD. The constant domain of the antibody heavy chain may be selected accordingly. The light chain constant domain may be a kappa or lambda constant domain.

The antibody may be a chimaeric antibody of the type described in WO 86/01533. A chimaeric antibody according to WO 86/01533 comprises an antigen binding region and a non-immunoglobulin region. The antigen binding region is an antibody light chain variable domain and/or heavy chain variable domain. Typically the chimaeric antibody comprises both light and heavy chain variable domains. The non-immunoglobulin region is fused to the C-terminus of the antigen binding region. The non-immunoglobulin region is typically a non-immunoglobulin protein and is preferably an enzyme region. The two regions of the chimaeric antibody may be connected via a cleavable linker sequence.

Antibodies of any of the classes referred to above may be raised against any antigen characteristic of and essentially limited to, a particular target. Such targets include all types of human pathogens, cells expressing antigens as a consequence of transformation or viral infection, cells expressing particular histocompatibility antigens, cells involved in inflammatory responses, blood clots which may be targetted by antibodies against fibrin, etc.

Preferred antibodies for use according to the invention, the antigens which they target and the associated indication are detailed in fig 8.

Particularly preferred antibodies for use according to the invention include CAMPATH 1H<sup>®</sup>, ING-1, c174, PR1.A3, 323/A3, G250, MOV18, 17-1A, NR-LU-10, U36, NR-CO-O2 as well as antibodies targetting mucins or carcinoembryonic antigen.

For the treatment of humans when the targetting moiety is an antibody, it is preferred to use an antibody which does not carry with it the risk of the development of an immune reaction against the antibody itself. Accordingly whilst it is possible to use mouse or rat mono-clonal antibodies it is preferred to use antibodies which have been produced by recombinant DNA technology and which have been engineered to reduce the risk of causing an immune reaction. Thus it is possible to use a chimaeric antibody in which the constant domains of a mouse or rat antibody have been replaced by the constant domains of a human antibody. However, it is preferred to use a humanised or CDR-grafted antibody for the treatment of humans, i.e. an antibody in which the complementarity determining regions from a mouse or rat antibody are combined with framework regions and constant domains from one or more human antibodies.

In one preferred embodiment the method according to the invention is carried out using the CDR-grafted antibody CAMPATH-1H (see Riechmann *et al* Nature, 322, 323-327 (1988)). As noted above, the antibody CAMPATH-1H can be produced in rat myeloma cells as originally described or it can be produced in any other expression system, particularly an expression system suitable for the production of a correctly folded, glycosylated, mammalian protein. High yields of CAMPATH-1H have been obtained by expression in a genetically manipulated CHO cell line (Page and Sydenham, Biotechnology, 9, 64-68 (1991)).

The conjugation of the targetting molecule to the enzyme may be carried out in any appropriate way which will neither interfere with the binding specificity and capability of the targetting molecule nor inhibit the catalytic activity of the enzyme. In the case of proteinic targetting molecules the conjugation may be carried out either by direct covalent linkage following the generation of reactive groups by treatment with protein modifying agents or by the use of homo or heterobifunctional cross-linking molecules.

Commercially available linkers with amino or hydrazide groups may be reacted with aldehydes generated by oxidation of the sugar moieties of glycoproteins and carbohydrates to form a Schiff's base (Sela M. *et al*, Conjugates of Antibodies with Cytotoxic Drugs. Immunoconjugates. Antibody Conjugates in Radioimaging and Therapy of Cancer (C.-W. Vogel, ed.) 189-216, (1987)). Alternatively linkers may be reacted with amino, carboxyl or sulfhydryl groups of the antibody or enzyme. (Wawrzynczak P.E. *et al*, Methods for Preparing Immunotoxins: Effect of the Linkage

on Activity & Stability In : Immunoconjugates : Antibody Conjugates in Radioimaging and Therapy of Cancer (C. - W. Vogel, ed.) 78-85, (1987)).

Covalent linkage may be obtained by the generation of sulfhydryl groups and, particularly in the case of antibodies, by reduction of disulfide bonds. Such free sulfhydryl groups may be reacted with haloalkyl groups, p-mercuribenzoate groups and groups capable of Michael-type addition reactions, such as maleimides or groups as per (Mitra *et al*, J Amer Chem Soc, 101, 3097-3110, 1979).

Another method for linking enzyme to antibody comprises utilising the epsilon amino groups of lysine. Reactive succinimide esters, cyclic thio esters or anhydrides may be used to introduce carbonyl functions which may react with the lysine amino group or to introduce a thiol reactive group such as those discussed herein. Alternatively, carbonyl functions may be activated with carbodimides to form carboxamide bonds or the lysine amino group may be reacted with methyl amidate esters to form an amidinium bond.

One particular utility of the present invention is its application to in vitro or in vivo diagnostics. For example, in vitro detection of a particular antigen may be achieved by exposing a diagnostic sample to a conjugate of the present invention comprising a targetting moiety such as an antibody capable of binding to the antigen and subsequent exposure to a prodrug such as a methotrexate prodrug of the present invention, which may be catalysed to methotrexate by the enzyme of the conjugate if antigen is present and conjugate is bound to the antigen. Prodrug should be added after unbound conjugate has been removed. Catalysed prodrug such as MTX may be detected by standard HPLC analysis, or by the coupled spectrophotometric assay described herein.

In vivo diagnostic applications of the present invention include its ability to be used in tumour imaging. Particularly preferred conjugates for use in this application include those comprising an antibodies to a tumour associated antigen and a mutant enzyme capable of catalysing to methotrexate, an MTX-prodrug labelled in the benzene ring with  $^{131}\text{I}$ . Such a prodrug should be one which is otherwise refractory to endogenous catalysis. Conjugate and prodrug may be administered in a manner analogous to the therapeutic application of the invention and detection of the location of catalysed prodrug i.e.  $^{131}\text{I}$  MTX achieved using standard equipment for detection of radioisotopic decay.

Salts of prodrugs of the present invention include pharmaceutically acceptable base salts, derived from an appropriate base, such as alkali metal (e.g. sodium), alkaline earth metal (e.g. magnesium) salts, ammonium and  $NX_4$  (wherein X is  $C_{1-4}$  alkyl) salts. Suitable pharmaceutically acceptable salts also include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, salicylic, p-toluene-sulfonic, tartaric, citric, acetic, trifluoroacetic methanesulfonic, formic, succinic, naphthalene-2-sulfonic, isethionic, lactobionic and benzenesulfonic. The pharmaceutically acceptable prodrug salts may be prepared in conventional manner for example by treatment with the appropriate base or acid.

In a method of treatment according to the present invention the means of administration of conjugate and of prodrug will depend upon the disease and its severity and will be at the discretion of the practising physician. Both the conjugate and prodrug may be administered by the conventional methods well known to those skilled in the art including intravenously, intraperitoneally, intralymphatically, subcutaneously, intradermally, intramuscularly, orally or in the case of a tumour by direct injection into the tumour. Both the prodrug and the conjugate will preferably be administered intravenously as a bolus or by continuous infusion. Preferably the dose of conjugate administered is in the range 0.1 to 100 mg per kg of bodyweight per day and most preferably between 1.0 and 40 mg per kg of bodyweight per day. The preferred dose of the prodrug administered is in the range 0.01 to 100 mg per kg of bodyweight per day and preferably between 1.0 and 50 mg per kg of bodyweight per day.

It is preferred that the conjugate of targetting molecule and mutant enzyme be administered to the patient prior to administration of the prodrug. The period between administration of conjugate and prodrug must be sufficient to permit targetting of the conjugate to the targetted cells and effective removal from the system of excess conjugate which has not bound to the targetted cells. Unbound conjugate must be effectively removed to avoid activation of the subsequently administered prodrug by unbound conjugate present at sites distant from the target cells. The period between administration of conjugate and prodrug is preferably between 0.5 days and 7 days but may be reduced by use of any known technique for the accelerated clearance of such conjugates from the system; particularly by delivery of an effective amount of anti-enzyme antibody, which binds to the mutant enzyme and in turn accelerates elimination,



or by extra corporeal circulation (Nilsson, I M, *et al*, Plasma Ther. Transfus Technol., 1984, 5, 127; Wallmark, A, *et al*, Artificial Organs, 1984, 8, 72) involving removal of the unbound conjugate by methods such as passage through a column of an appropriate affinity matrix. It may also be desirable to couple galactosyl residues to the conjugate to facilitate unbound conjugate clearance via hepatocyte lectins.

Conditions susceptible to therapy according to the present invention will clearly include those for which there exists a known chemotherapeutic agent and a corresponding prodrug thereof as hereinbefore defined and which presents a pathology specific feature permitting binding of a conjugate of the present invention. Such conditions include neoplastic and non-neoplastic cellular transformation, autoimmune disease, inflammatory disease and viral, bacterial, fungal, mycoplasmal or parasitic infection.

A method of treatment according to the present invention may further comprise the co-administration of a number of agents capable of increasing the efficiency of the therapy. For example, interferons are known to increase the level of expression of certain potential ADEPT target antigens and therefore administration of an appropriate interferon before administration of conjugate may increase level of conjugate binding to target cells.

It is further preferred that a combination of a prodrug of a purine synthesis inhibitor such as an inhibitor of glycineamide ribonucleotide transformylase (GAR TFase) or a prodrug of a pyrimidine synthesis inhibitor such as an inhibitor of thymidine synthase (TS) be administered in combination with a prodrug of a dihydrofolate (DHFR) inhibitor as part of such therapy (Galivan, J. *et al*, J. Biol. Chem. 264, 10685, 1989, Ferguson, K. *et al* Cancer Chemother. Pharmacol. 23 173, 1989). It is further preferred that a combination of a prodrug of purine synthesis inhibitor such as an inhibitor of GAR TFase or a prodrug of a pyrimidine synthesis inhibitor such as an inhibitor of TS be administered in combination with DHFR inhibitor, or that a combination of a purine synthesis inhibitor such as an inhibitor of GAR TFase or a pyrimidine synthesis inhibitor such as an inhibitor of TS be administered in combination with a prodrug of a DHFR inhibitor as part of such therapy.

A further aspect of the present invention comprises a method for the potentiation of any antiviral compound, the efficacy of which may be augmented by elevation of intracellular kinase activity.

According to a preferred embodiment of this further aspect of the present invention, a conjugate of the present invention comprising a targeting molecule capable of directing conjugate to a virally-infected cell and a prodrug of a folate antagonist which may be one or more of a thymidylate synthase (TS) inhibitor, dihydrofolate reductase (DFHR) inhibitor or GAR transformylase inhibitor is used to deliver high doses of folate antagonist directly to the virally-infected cells of a patient who is undergoing therapy. As viral infection is associated with increased levels of DNA synthesis, it is proposed that the delivery of an inhibitor of DNA synthesis in combination with such an antiviral may prove therapeutic as the inhibition of DNA synthesis will increase the level of intracellular kinases available for phosphorylating the antiviral compound.

In a preferred embodiment of this further aspect of the invention, the anti-viral is an anti-herpes compound selected from acyclovir (GB 1523865), valacyclovir (EP308065), famciclovir EP182024), penciclovir (EP141927), ganciclovir (EP0072027), foscarnet (GB 1585615), iododeoxyuridine, 5-bromovinyl-2'-deoxyuridine (GB1601020), arabinofuranosyl-5-bromovinyl-2'-deoxyuridine (EP0031128), benzimidazole nucleosides, 1-( $\beta$ -D-arabinofuranosyl)-5-(1-propynyl)uracil (EP272065) and 2'-deoxy-5-ethyl- $\beta$ -4'-thiouridine (EP409575), or is an anti-HIV compound selected from 2',3'-dideoxyinosine (EP206497), 2',3'-dideoxycytidine (J. Org. Chem. 32(3) 817-818 (1967)), (2R,5S)-5-fluoro-1-(2-(hydroxymethyl)-1,3-oxathiolan-5-yl)cytosine (US5210085), (2R,5S)-1-(2-(hydroxymethyl)-1,3-oxathiolan-5-yl)cytosine (EP0382526), 5-chloro-2',3'-dideoxy-3'-fluorouridine (EP0305117) and (1S,4R)-4-(2-amino-6-(cyclopropylamino)-9H-purin-9-yl)-2-cyclopentene-1-methanol (EP0434450) or is the anti-influenza compound 9-(2-Deoxy-2-fluoro- $\beta$ -D-ribofuranosyl) guanine) (EP0417999).

In the most preferred embodiment of this further aspect of the invention, the antiviral compound is an anti-HIV compound such as zidovudine (EP196185), (Retrovir: registered trade mark of The Wellcome Foundation Limited), the conjugate comprises an antibody against an HIV-associated antigen such as GP120 and the prodrug is a prodrug of a TS inhibitor.

The present invention provides for the use of a novel prodrug of the present invention as hereinbefore defined, or a pharmaceutically acceptable salt, solvate, N-oxide or physiologically functional derivative thereof, in therapy or in the preparation of a medicament for use in the treatment of any of the conditions hereinbefore described.

Also provided is the use of a mutant enzyme of the present invention, or a conjugate thereof as hereinbefore described, in therapy or in the preparation of a medicament for use in the treatment of any of the conditions hereinbefore described.

Pharmaceutical formulations for mutant enzyme, antibody and prodrug may each include but are not limited to solutions, suspensions, tablets, powders, and other formulations known to those skilled in the art. The formulations may contain pharmaceutically acceptable stabilisers and carriers including human serum albumin or other proteins, pH buffering agents, and physiological or other nontoxic salts as well as non toxic solubilisers known to those skilled in the art. Preferably a pharmaceutical formulation of mutant enzyme and antibody will comprise conjugate in a sterile solution. A pharmaceutical formulation of prodrug will preferably comprise a sterile solution or a solid.

A yet further aspect of the present invention comprises a kit comprising a pharmaceutical formulation of conjugate and a pharmaceutical formulation of prodrug which may be converted to drug by that conjugate.

A yet further aspect of the present invention comprises the combination of a prodrug and a conjugate of the present invention as hereinbefore defined.

A yet further aspect of the present invention comprises the use of a novel prodrug as hereinbefore defined but excluding the previously stated proviso, for use in the preparation of a medicament for use in the treatment of any of the conditions hereinbefore described.

A yet further aspect of the present invention comprises the use of a novel prodrug as hereinbefore defined but excluding the previously stated proviso, for use in the treatment of any of the conditions hereinbefore described.

A yet further aspect of the present invention comprises the use of a prodrug in the preparation of a medicament for use in the treatment of a patient who has previously been administered with a conjugate of the present invention as hereinbefore defined.

A further aspect of the invention comprises the cloned version of human carboxypeptidase A2 described herein as well as the DNA encoding carboxypeptidase A2.

A further aspect of the invention comprises any DNA sequence encoding a mutant enzyme for use according to the present invention and plasmids, expression vectors, and cell lines containing such DNA. Preferably such DNA encodes mutant carboxypeptidase A1 or A2 mutants as herein described and most preferable mutant carboxypeptidase A1 or A2 wherein position 268 is glycine.

A further aspect of the invention comprises DNA sequence encoding a conjugate according to the present invention and plasmids, expression vectors, and cell lines containing such DNA.

A yet further agreed aspect of the invention is a method of converting a prodrug to a drug which is cytotoxic to cells.

All references to alternatives herein, such as alternative patterns of chemical substitutions, shall be construed as inclusive and not as exclusive.

As used herein the term "halo" means fluoro, chloro, bromo or iodo.

The terms "aryl" means phenyl, naphthyl or anthracenyl optionally substituted by one or more halo, hydroxy, nitro, cyano, trifluoromethyl, C<sub>1-6</sub> alkyl, C<sub>1-6</sub> alkoxy or amino optionally substituted with C<sub>1-6</sub> alkyl.

The term "heteroaryl" means an aromatic monocyclic or bicyclic fused ring system comprising 5 to 10 carbon atoms wherein one or more ring atoms are independently selected from nitrogen, oxygen or sulfur.

"Carboxamide" groups can be unsubstituted or substituted C<sub>1-6</sub> alkyl.

A mutant mammalian enzyme as used herein shall be taken to be any enzyme with a sequence differing by at least one amino acid from the amino acid sequence or sequences of that enzyme in the mammal to which the therapy is applied. Preferably the mammal to which the therapy is applied is a human patient and preferably the amino acid sequence of the mutant mammalian enzyme does not differ by more than 15% from the amino acid sequence or sequences of that enzyme in the mammal to which the therapy is applied.

All references hereinbefore and in the Claims hereinafter to residue numbers of amino acids relate to the residue number indicated in the Tables contained herein and not to the residue numbers given in the Sequence Listing herein which follows a different numbering convention.

## Experimental Procedures

### General Comments

All solvents were reagent grade and were used without further purification. Anhydrous solvents were dispensed from Aldrich Sure Seal bottles using a dry syringe. Chemicals are reagent grade and were used without purification. The full name and address of the suppliers of the reagents is given when first cited. Thereafter, an abbreviated name is used.

$^1\text{H}$ -NMR spectra were obtained using Varian XL-200 or XL-300 spectrometers at 200 and 300MHz respectively. Chemical shifts are expressed in ppm downfield from tetramethylsilane. Spectral data are tabulated in order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), number of hydrogens, coupling constant(s) in Hertz, descriptor. Chemical ionization (CI) mass spectra were performed by Oneida Research Services, Inc., Whitesboro, NY 13492. Atmospheric pressure chemical ionization (APCI) mass spectra were obtained using a Fisons platform mass spectrometer. Fast atom bombardment (FAB) mass spectra were obtained using a VG Analytical 70SQ spectrometer. Ion spray mass spectra were obtained using a Sciex API III spectrometer. Elemental analyses were performed by Atlantic Microlabs, Inc., Atlanta, GA 30366. Reverse phase analytical HPLC's were run on C18 analytical columns using a Waters HPLC system consisting of a 600E System Controller, a 715 UltraWisp sample processor, and a 991 Photodiode Array Detector. Semi-preparative HPLC's were run on a Regis column (C18, Prep-100-60-ODS-FEC, 10mm packing, 25cm x 21.1mm i.d.) using a similar system equipped with a manual injector.

Abbreviations used are: grams (g), milliliters (mL), liters (L), hours (h), minutes (min), room temperature (RT), calculated (calcd.), decomposition (dec.), molecular weight (mw), tetrahydrofuran (THF), dimethyl formamide (DMF), dimethyl sulphoxide (DMSO), 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (DMPU), 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC), dicyclohexylcarbodiimide (DCC), diethyl cyanophosphonate (DECP), (benzyloxy)carbonyl (Cbz), and trifluoroacetic acid (TFA).

Some examples prepared were very tenacious of water and/or other hydroxylic solvents (e.g. DMF), and/or were obtained as whole or partial salts of organic or inorganic acids (e.g. trifluoroacetic acid, hydrochloric acid). Such addends are indicated in the analytical data for appropriate examples; although these addends may not be indicated as part of the compound name, their presence in the isolated products is noted by the inclusion of salt and hydration data in the molecular formulas and analytical data in appropriate examples.

Example 1: 2-Fluoro-4-nitrobenzoic acid

A 3L 3-necked flask equipped with a magnetic stirrer was charged with 25.00 g of 2-fluoro-4-nitrotoluene (Aldrich Chemical Co., Milwaukee, WI, 53233), 1.65 L of 1N NaOH, and 25.00 g of KMnO<sub>4</sub>. The resulting mixture was heated to 95°C with stirring. Additional 25.00 g portions of KMnO<sub>4</sub> were added after 1h and 2h. After stirring at 95°C for an additional hour the reaction mixture was cooled to RT and filtered through celite to remove MnO<sub>2</sub>. The filtrate was concentrated *in vacuo* to 500 mL and acidified to pH 2 by addition of concentrated H<sub>2</sub>SO<sub>4</sub>. A yellow-orange precipitate formed which was collected by vacuum filtration. The crude solid was dissolved in

300 mL of 1N aqueous NaOH, acidified to pH 2 and the mixture extracted with CH<sub>2</sub>Cl<sub>2</sub> (4 x 150 mL). The combined CH<sub>2</sub>Cl<sub>2</sub> extracts were washed with 0.1 N aqueous HCl (2 x 100 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo* to afford 12.44 g (42%) of 2-fluoro-4-nitrobenzoic acid as a yellow crystalline solid, m.p. 165-167°C.

Example 2: Ethyl 2-fluoro-4-nitrobenzoate

To a dry 100 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet were added 3.00 g of 2-fluoro-4-nitrobenzoic acid, 3.00 g of NaHCO<sub>3</sub>, and 20 mL of anhydrous dimethylacetamide. The suspension was treated with 3.50 mL of ethyl iodide and stirred at RT under N<sub>2</sub> for 18h. The reaction mixture was filtered to remove solids and the filtrate concentrated *in vacuo* to give an orange residue which was dissolved in 100 mL of CH<sub>2</sub>Cl<sub>2</sub>. The solution was washed with saturated aqueous NaHCO<sub>3</sub> (3 x 50 mL), water (1 x 60 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Removal of drying agent by filtration and passage through a silica gel plug (3 x 5 cm) gave a light yellow solution which was concentrated *in vacuo* to afford 3.10 g (90%) of ethyl 2-fluoro-4-nitrobenzoate as a light yellow crystalline solid, m.p. 70-73°C.

Example 3: Ethyl 4-amino-2-fluorobenzoate

To a 250 mL round bottomed flask equipped with a magnetic stirrer were added 3.00 g of ethyl 2-fluoro-4-nitro-benzoate and 100 mL of MeOH. The solution was deoxygenated by bubbling nitrogen through for 10 min and then treated with 2.00 g of Raney nickel (Raney 3200, Davison Chemical, Chattanooga, TN 37406) which had been washed with water and EtOH. The solution was subjected to hydrogenation at 1 atm with stirring for 40 min. The flask was purged with nitrogen and the catalyst was



removed by filtration. The filtrate was concentrated *in vacuo* to afford 2.40 g (93%) of ethyl-4-amino-2-fluorobenzoate as a tan crystalline solid, m.p. 110-112°C.

**Example 4: 9-Bromomethyl-3-methylbenzo(f)quinazolin-1(2H)-one**

To a 3L 3-necked flask equipped with a magnetic stirrer, a nitrogen inlet, and a reflux condenser were added 1.4 L of 1,2-dichloroethane. The solvent was heated to reflux and 10.00 g of 3,9-dimethylbenzo(f)-quinazolin-1(2H)-one (prepared according to the method described in International Patent Application WO 91/19700) were slowly added. This was followed by addition of 8.73 g of *N*-bromosuccinimide (Aldrich) and 1.00 g of benzoyl peroxide (Aldrich). After heating at reflux for 2h the reaction mixture was cooled to room temperature and concentrated *in vacuo*. The residue was slurried with 60 mL of EtOH and the solid was collected by vacuum filtration. The product was washed with 50 mL of EtOH and dried *in vacuo* to afford 12.39 g of a light tan solid, m.p. >200°C. Analysis by <sup>1</sup>H-NMR indicated a mixture consisting of 80% of 9-bromomethyl-3-methylbenzo(f)quinazolin-1(2H)-one and 20% of starting material. The material was carried on to the next step without further purification.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 9.86 (s, 1H, aromatic CH), 8.28 (d, 1H, J=9.0, aromatic CH), 8.06 (d, 1H, J=8.4, aromatic CH), 7.72 (d, 1H, J=8.2, aromatic CH), 7.65 (d, 1H, J=8.8, aromatic CH), 4.94 (s, 2H, ArCH<sub>2</sub>Br), 2.49 (s, 3H, CH<sub>3</sub>).

**Example 5: Ethyl 4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoate**

To a 500 mL 3-neck flask equipped with a magnetic stirrer, a nitrogen inlet, and a reflux condenser were added 5.00 g of 9-bromomethyl-3-methylbenzo(f)quinazolin-1(2H)-one, 3.02 g of ethyl 4-amino-2-fluorobenzoate, 4.17 g of NaHCO<sub>3</sub>, and 42 mL of anhydrous DMF. The reaction mixture was heated to 100°C with stirring under nitrogen for 1.5h.

After cooling to RT the mixture was filtered to remove solids and the crude product was deposited on 30 g of silicon gel by concentrating the filtrate *in vacuo* in the presence of silica gel. The product/silica gel mixture was applied to a column (360 g SiO<sub>2</sub>) and purified by flash chromatography using MeOH-EtOAc gradient elution (EtOAc → 2% MeOH-EtOAc). This afforded 3.50 g (65%) of ethyl 4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoate as a white powder, m.p. >200°C.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 12.54 (s, 1H, NH), 9.85 (s, 1H, aromatic CH), 8.23 (d, 1H, J=8.7, aromatic CH), 8.01 (d, 1H, J=8.2, aromatic CH), 7.69-7.50 (m, 3H, aromatic CH), 6.52 (dd; 1H; J=1.8, 8.9; aromatic CH), 6.40 (dd; 1H; J=1.7, 14.7; aromatic CH), 4.58 (d, 2H, J=5.6, ArCH<sub>2</sub>N), 4.19 (q, 2H, J=7.0, ethyl CH<sub>2</sub>), 2.44 (s, 3H, CH<sub>3</sub>), 1.24 (t, 3H, J=7.0, ethyl CH<sub>3</sub>).

Example 6: 4-(((1,2-Dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoic acid

A 100 mL 3-necked flask equipped with a magnetic stirrer was charged with 0.200 g of ethyl 4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoate, 18 mL of 1N NaOH, and 20 mL of EtOH. After stirring overnight at RT, the solution was concentrated to 20 mL *in vacuo* and acidified to pH 2.5 by addition of concentrated HCl. The resulting precipitate was collected by vacuum filtration, washed with 15 mL of water, and dried *in vacuo* to afford 0.15 g (77%) of 4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoic acid as a white powder, m.p. >250°C.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 13.20-12.30 (br s, 1H, COOH), 9.83 (s, 1H, aromatic CH), 8.24 (d, 1H, J=8.6, aromatic CH), 8.06 (d, 1H, J=8.4, aromatic CH),

7.80-7.40 (m, 4H, aromatic CH), 6.51 (d, 1H, J=7.9, aromatic CH), 6.37 (d, 1H, J=14.2, aromatic CH), 4.60 (s, 2H, ArCH<sub>2</sub>N), 2.48 (s, 3H, CH<sub>3</sub>).

Example 7: *N*-((Benzyloxy)carbonyl)-5-*O*-ethyl-L-glutamic acid

A 2L 3-necked flask equipped with a magnetic stirrer and a thermometer was charged with 25.0 g of glutamic acid  $\gamma$ -ethyl ester (Sigma Chemical Co., St. Louis, MO, 63178) and 1L of distilled water. The solution was heated to 60°C with stirring and treated with 30 g of NaHCO<sub>3</sub> followed by 25 mL of benzyl chloroformate (Aldrich). The flask was allowed to cool to RT and stirring was continued for 2h. The mixture was extracted with ethyl ether (5 x 100 mL) and the ether extracts were discarded. The aqueous phase was acidified to a congo red end point by addition of concentrated aqueous HCl and the resulting emulsion was extracted with CH<sub>2</sub>Cl<sub>2</sub> (5 x 100 mL). The combined CH<sub>2</sub>Cl<sub>2</sub> extracts were washed with 0.05 N aqueous HCl (2 x 100 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo* to a volume of 50 mL. The solution was chilled in an ice water bath and triturated with addition of 200 mL of pentane. The resulting solid was collected by vacuum filtration and dried *in vacuo* to afford 37.0 g (83%) of *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutamic acid as a white powder, m.p. 82-84°C.

Example 8: Ethyl 3-(1-naphthyl)-L-alaninate

To a 250 mL 3-necked flask equipped with a magnetic stirrer and a reflux condenser were added 3.00 g of 3-(1'-naphthyl)-L-alanine (Schweizerhall, Inc., Piscataway, NJ, 08854) and 75 mL of absolute EtOH. The suspension was acidified by bubbling HCl gas through until a clear solution was obtained and the solution was heated at reflux with stirring for 5h. The solution was cooled to RT and concentrated *in vacuo* to give a white solid, which was suspended in 100 mL of 5% aqueous NaHCO<sub>3</sub> and extracted with EtOAc (3 x 60 mL). The combined EtOAc extracts were washed with 5% aqueous

NaHCO<sub>3</sub> (3 x 50 mL), water (1 x 50 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The drying agent was removed by filtration and the filtrate concentrated *in vacuo* to give an oil which was dissolved in 40 mL of ethyl ether. The ether solution was treated with 13.9 mL of 1N ethereal HCl and the resulting precipitate was collected by vacuum filtration and dried *in vacuo* to afford 3.70 g (95%) of ethyl 3-(1-naphthyl)-L-alaninate·HCl as a white powder, m.p. 182-184°C.

Example 9: Ethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-3-(1-naphthyl)-L-alaninate

A 250 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 3.70 g of ethyl 3-(1-naphthyl)-L-alaninate·HCl, 4.09 g of *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutamic acid, 60 mL of CH<sub>2</sub>Cl<sub>2</sub>, and 1.45 mL of *N*-methylmorpholine. The mixture was chilled to 0°C in an ice bath, treated with 2.66 g of EDC (Aldrich) and stirred under nitrogen. After 1h at 0°C the solution was allowed to warm to RT and stirring was continued for an additional 3h. The solution was concentrated *in vacuo* and the residue was dissolved in 100 mL of EtOAc. The EtOAc solution was washed with 10% aqueous citric acid (3 x 50 mL) followed by 5% aqueous NaHCO<sub>3</sub> (3 x 50 mL). The solution was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, concentrated *in vacuo* to 20 mL, and triturated with addition of 50 mL of pentane. The resulting solid was collected by vacuum filtration and dried *in vacuo* to afford 5.80 g (82%) of ethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-3-(1-naphthyl)-L-alaninate as a white powder, m.p. 124-126°C.

Example 10: Ethyl 5-*O*-ethyl-L-glutam-1-yl-3-(1-naphthyl)-L-alaninate

A 500 mL Parr bottle was charged with 0.19 g of 10% Pd(C) and 60 mL of absolute EtOH under nitrogen. The catalyst-solvent mixture was deoxygenated by bubbling

nitrogen through for 5 minutes and then treated with 0.14 mL of acetyl chloride followed by 1.02 g of ethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-3-(1-naphthyl)-L-alaninate. The mixture was hydrogenated at 45 psi for 18h. The bottle was purged with nitrogen and the catalyst was removed by filtration through celite. The filtrate was concentrated *in vacuo* to afford 0.81 g (97%) of ethyl 5-*O*-ethyl-L-glutam-1-yl-3-(1-naphthyl)-L-alaninate·HCl as a light yellow foam, m.p. 85-90°C (dec.).

Example 11: Ethyl *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-ethyl-L-glutam-1-yl)-3-(1-naphthyl)-L-alaninate

A 100 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer was charged with 0.50 g of 4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoic acid and 10 mL of DMPU (Aldrich). The mixture was alternately heated and sonicated to dissolve the solid and the resulting solution was stirred under nitrogen in the presence of 0.50 g of 3Å molecular sieves for 18h. The mixture was cooled to 0°C in an ice bath and treated with 0.15 mL of *N*-methylmorpholine followed by 0.17 mL of isobutyl chloroformate. After stirring at 0°C for 15 min the reaction mixture was treated with a solution of 0.69 g of ethyl 5-*O*-ethyl-L-glutam-1-yl-3-(1-naphthyl)-L-alaninate·HCl and 0.17 mL of *N*-methylmorpholine in 4 mL of anhydrous DMF. The reaction was maintained at 0°C for 1h and was then allowed to warm to RT and stirred for an additional 4h. The mixture was filtered to remove solids and the filtrate was mixed with 150 mL of water. The resulting crude solid was collected by filtration, dried *in vacuo*, and subjected to flash chromatography on 200 g of silica gel (1% MeOH/EtOAc → 5% MeOH/EtOAc) to afford 0.40 g (40%) of ethyl *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-ethyl-L-glutam-1-yl)-3-(1-naphthyl)-L-alaninate as a white powder, m.p. 225°C (dec.).

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 12.55 (s, 1H, benzaquinazoline NH), 9.87 (s, 1H, aromatic CH), 8.63 (d, 1H, J=7.5, amide NH), 8.24 (d, 1H, J=8.3, aromatic CH), 8.09 (d, 1H, J=7.7, aromatic CH), 8.03 (d, 1H, J=8.0, aromatic CH), 7.92 (d, 1H, J=7.1, aromatic CH), 7.79 (t, 1H, J=5.6, 4-aminobenzoyl NH), 7.68-7.30 (m, 9H, aromatic CH, amide NH), 6.56 (d, 1H, J=7.9, aromatic CH), 6.42 (d, 1H, J=14.4, aromatic CH), 4.68-4.43 (m, 4H, benzoquinazoline-9-CH<sub>2</sub>, glu methine, naphthylala methine), 4.10-3.92 (m, 4H, ethyl CH<sub>2</sub>), 3.63-3.30 (m, 2H, naphthyl-CH<sub>2</sub>), 2.45 (benzoquinazoline CH<sub>3</sub>), 2.29 (m, 2H, glu 4-CH<sub>2</sub>), 2.07-1.74 (m, 2H, glu 3-CH<sub>2</sub>), 1.14(t, 3H, J=7.1, ethyl CH<sub>3</sub>), 1.03 (t, 3H, J=7.1, ethyl CH<sub>3</sub>).

Example 12: *N*-(*N*-(4-(((1,2-Dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl)-3-(1-naphthyl)-L-alanine

To a 50 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet were added 0.15 g of ethyl *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-ethyl-L-glutam-1-yl)-3-(1-naphthyl)-L-alaninate, 8 mL of 1 : 1 THF : H<sub>2</sub>O, and 20 mg of LiOH·H<sub>2</sub>O. The mixture was stirred at RT under nitrogen. After 1h analysis of the solution by tlc (SiO<sub>2</sub>, 3% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) showed no remaining starting material and a new component at R<sub>f</sub>=0.0. The solution was acidified to pH 5 by addition of 1N aqueous HCl and the resulting white suspension was concentrated *in vacuo* to dryness. Analysis of the residue by reverse phase HPLC (C18, 65 : 35 : 0.1 H<sub>2</sub>O : MeCN : TFA) indicated a major component (95%) at k'=3.17 and a minor component (5%) at k'=4.90. The crude product was purified by semi-preparative reverse phase HPLC using gradient elution (C18, 70 : 30 : 0.1 Æ 65 : 35 : 0.1 H<sub>2</sub>O : MeCN : TFA over 10 min). Pure fractions (as determined by analytical HPLC) were combined, concentrated to 20 mL *in vacuo* and lyophilized to afford 76 mg (50%) of *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)-quinazolin-9-

yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl)-3-(1-naphthyl)-L-alanine as an off white powder, m.p. 180°C (dec.).

HPLC: one peak on C18,  $k'=3.53$ , 65 : 35 : 0.1 H<sub>2</sub>O:MeCN:TFA, flow rate = 1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  13.30-12.20 (br m, COOH), 9.82 (s, 1H, aromatic CH), 8.44 (d, 1H,  $J=8.1$ , amide NH), 8.26 (d, 1H,  $J=8.9$ , aromatic CH), 8.11 (d, 1H,  $J=8.3$ , aromatic CH), 8.03 (d, 1H,  $J=8.3$ , aromatic CH), 7.84 (d, 1H,  $J=8.2$ , aromatic CH), 7.73 (d, 1H,  $J=7.8$ , aromatic CH), 7.67-7.27 (m, 9H, aromatic CH, amide NH), 6.53 (d, 1H,  $J=8.5$ , aromatic CH), 6.38 (d, 1H,  $J=14.7$ , aromatic CH), 4.63-4.38 (m, 4H, benzoquinazoline-9-CH<sub>2</sub>, glu methine, naphthylala methine), 3.55 (m, 1H, naphthyl-CH<sub>2</sub>), 3.27 (m, 1H, naphthyl-CH<sub>2</sub>), 2.43 (s, 3H, benzoquinazoline CH<sub>3</sub>), 2.18 (m, 2H, glu 4-CH<sub>2</sub>), 2.00-1.70 (m, 2H, glu 3-CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>39</sub>H<sub>34</sub>N<sub>5</sub>O<sub>7</sub>F·1.5 H<sub>2</sub>O·0.5 TFA (MW 787.76): C, 60.99; H, 4.80; N, 8.89. Found: C, 60.96; H, 4.82; N, 8.98.

Mass Spectrum: (FAB) 704 (M + H)<sup>+</sup>, 613, 489, 371, 309.

UV Spectrum: (pH 7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 223.4 (82600), 266.4 (53100), 348.6 (5500).  $\lambda_{\min}$  ( $\epsilon$ ) 243.2 (23500), 340.2 (3430).

Example 13: Ethyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-ethyl-L-glutam-1-yl-3-(1-naphthyl)-L-alaninate

To a solution of 0.24 mL of DECP (Aldrich) and 0.22 mL of Et<sub>3</sub>N in 20 mL of anhydrous DMF in a 100 mL 3-necked flask were added 0.20 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich Chemical Co., Milwaukee, WI, 53233) and 0.04 mL of Et<sub>3</sub>N dissolved in 3 mL of DMPU. The solution was stirred at RT under nitrogen for 3h and treated with a solution of 0.23 g of ethyl 5-*O*-ethyl-L-glutam-1-yl-3-(1-naphthyl)-L-alaninate·HCl and 0.15 mL

of Et<sub>3</sub>N in 2 mL of anhydrous DMF. After stirring for 70h the reaction mixture was concentrated *in vacuo* to 3 mL and treated with 60 mL of ethyl ether to precipitate the crude product as a tacky orange solid. The ether was decanted and the product dissolved in 60 mL of CHCl<sub>3</sub>. The solution was extracted with 1% aqueous NH<sub>4</sub>OH, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo* to dryness. The yellow-orange residue was purified by flash chromatography on 150 g of silica gel (7 : 7 : 1 CH<sub>2</sub>Cl<sub>2</sub> : acetone : MeOH) to afford 0.26 g (70%) of ethyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-ethyl-L-glutam-1-yl-3-(1-naphthyl)-L-alaninate as a yellow powder, m.p. 135°C (dec).

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 8.59 (s, 1H, pteridiny-7-CH), 8.45 (d, 1H, J=7.2, amide NH), 8.10 (d, 1H, J=8.0, aromatic CH), 8.04 (d, 1H, J=8.2, aromatic CH), 7.92 (d, 1H, J=6.8, aromatic CH), 7.83-7.45 (m, 6H, aromatic CH, NH<sub>2</sub>), 7.41-7.23 (m, 2H, aromatic CH), 6.84 (d, 2H, J=8.8, aromatic CH), 6.65 (br s, 2H, NH<sub>2</sub>), 4.81 (s, 2H, pteridiny-CH<sub>2</sub>), 4.63-4.39 (m, 2H, glu methine, naphthylala methine), 4.13-3.91 (m, 4H, ethyl CH<sub>2</sub>), 3.63-3.32 (m, 2H, naphthyl-CH<sub>2</sub>), 3.24 (s, 3H, N-CH<sub>3</sub>), 2.34 (t, 2H, J=7.6, glu-4-CH<sub>2</sub>), 2.10-1.80 (m, 2H, glu-3-CH<sub>2</sub>), 1.17 (t, 3H, J=7.1, ethyl-CH<sub>3</sub>), 1.03 (t, 3H, J=7.1, ethyl-CH<sub>3</sub>).

Example 14: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-(1-naphthyl)-L-alanine

To a 50 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet were added 0.13 g of ethyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-ethyl-L-glutam-1-yl-3-(1-naphthyl)-L-alaninate, 10 mL of EtOH and 15 mL of 0.2 N NaOH. The resulting solution was stirred at RT under N<sub>2</sub> for 2.5h, acidified to pH 5 by addition of 1N aqueous HCl, and concentrated *in vacuo* to dryness. Analysis of the yellow solid by reverse phase HPLC (C18, 70 : 30 : 0.1 H<sub>2</sub>O : MeCN : TFA)



indicated a major component (93%) at  $k'=4.3$  and four minor components at  $k'=6.8$ , 3.5, 2.9 and 1.9. A 70 mg portion of the crude product was purified by semi-preparative reverse phase HPLC (C18, 70 : 30 : 0.1 H<sub>2</sub>O : MeCN : TFA). Pure fractions (as determined by analytical HPLC) were combined, concentrated *in vacuo* to 20 mL and lyophilized to afford 36 mg (53%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-(1-naphthyl)-L-alanine as a yellow powder, m.p. 175°C (dec).

HPLC: one peak on C18,  $k'=3.23$ , 70 : 30 : 0.1 H<sub>2</sub>O : MeCN : TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  13.00-11.95 (br m, 2H, COOH), 8.65 (s, 1H, pteridiny-7-CH), 8.45 (br, 1H, NH<sub>2</sub>), 8.36-8.18 (br, 1H, NH<sub>2</sub>), 8.25 (d, 1H,  $J=7.5$ , amide NH), 8.10 (d, 1H,  $J=8.6$ , aromatic CH), 7.96 (d, 1H,  $J=7.8$ , aromatic CH), 7.90 (d, 1H,  $J=7.7$ , aromatic CH), 7.70 (m, 3H, aromatic CH), 7.60-7.45 (m, 3H, aromatic CH, NH<sub>2</sub>), 7.39-7.15 (m, 3H, aromatic CH, amide NH, NH<sub>2</sub>), 6.81 (d, 2H,  $J=8.6$ , aromatic CH), 4.84 (s, 2H, pteridiny-CH<sub>2</sub>), 4.51 (m, 1H, naphthylala methine), 4.42 (m, 1H, glu methine), 3.57 (m, 1H, naphthylala-CH<sub>2</sub>), 3.28 (m, overlapping H<sub>2</sub>O peak, naphthylala CH<sub>2</sub>), 3.25 (s, 3H, N-CH<sub>3</sub>), 2.23 (t, 2H,  $J=7.5$ , glu-4-CH<sub>2</sub>), 2.02-1.72 (m, 2H, glu-3-CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>33</sub>H<sub>33</sub>N<sub>9</sub>O<sub>6</sub>·1.5 H<sub>2</sub>O·0.5 TFA (MW 735.72): C, 55.51; H, 5.00; N, 17.13. Found: C, 55.65; H, 5.09; N, 16.92.

Mass Spectrum: (FAB) 652 (M + H)<sup>+</sup>, 581, 461, 371, 309.

UV Spectrum: (pH 7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 224.3 (74700), 260.3 (24900), 295.9 (24100), 372.5 (7150).  $\lambda_{\min}$  ( $\epsilon$ ) 242.3 (15200), 271.0 (20500), 345.2 (5550).

Example 15: *N*-((Benzyloxy)carbonyl)-5-*O*-methyl-L-glutamic acid

To a 1L 3-necked flask equipped with a thermometer and a magnetic stirrer were added 4.90 g of L-glutamic acid  $\gamma$ -methyl ester (Sigma) and 200 mL of water. The solution was heated to 60°C, treated with 6.40 g of NaHCO<sub>3</sub> followed by 5.20 mL of benzyl chloroformate (Aldrich) and allowed to cool to RT with vigorous stirring. After 4h the reaction mixture was extracted with ethyl ether (4 x 100 mL) and the ether extracts were discarded. The aqueous solution was acidified to a congo red endpoint by addition of concentrated HCl. An oily emulsion resulted which was extracted with CH<sub>2</sub>Cl<sub>2</sub> (4 x 60 mL). The combined organic extracts were washed with 0.05 N aqueous HCl (2 x 60 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo* to afford 8.23 g (92%) of *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutamic acid as a white crystalline solid, m.p. 61-63°C.

Example 16: Glycine *tert*-butyl ester-benzophenone imine

A 500 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 10.00 g of glycine-*tert*-butyl ester-HCl (Sigma) and 200 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub>. The solution was treated with 10.80 g of benzophenone imine (Aldrich) and stirred at RT under nitrogen for 24h. A fine white precipitate of NH<sub>4</sub>Cl formed during the reaction. The mixture was concentrated *in vacuo* to dryness to give a white solid which was suspended in 250 mL of ethyl ether and washed with water (3 x 200 mL) to remove NH<sub>4</sub>Cl. The ether solution was dried over anhydrous MgSO<sub>4</sub> and concentrated *in vacuo* to afford a white solid. The material was dissolved in 30 mL of CH<sub>2</sub>Cl<sub>2</sub> and triturated with addition of 150 mL of pertane. A white crystalline solid resulted which was collected by filtration, dried *in vacuo*, and identified as glycine *tert*-butyl ester-benzophenone imine by <sup>1</sup>H-NMR. yield=15.0 g (85%), m.p. 110 -112°C.

Example 17: 2-Iodobenzyl bromide

To a dry 100 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer were added 5.00 g of 2-iodobenzyl alcohol (Aldrich) and 20 mL of anhydrous  $\text{CH}_2\text{Cl}_2$ . The resulting suspension was cooled to  $0^\circ\text{C}$  and treated with 0.71 mL of  $\text{PBr}_3$  (Aldrich) by dropwise addition over a 2 minute period. The solid slowly dissolved giving a light yellow solution which was stirred under nitrogen at  $0^\circ\text{C}$  for 2h and then allowed to warm to RT with continued stirring for 18h. The solution was concentrated *in vacuo* to dryness and the residue was dissolved in 60 mL of ethyl ether. The ether solution was washed with ice cold 5% aqueous  $\text{NaHCO}_3$  (3 x 50 mL), dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and concentrated *in vacuo* to afford 5.65 g (89%) of 2-iodobenzyl bromide as a white crystalline solid, m.p.  $52-54^\circ\text{C}$ .

Example 18: 2-Iodo-L-phenylalanine *tert*-butylester-benzophenone imine

A 500 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer was charged with 20.00 g of glycine *tert*-butyl ester-benzophenone imine, 24.13 g of 2-iodobenzyl bromide, 2.85 g of *N*-benzylcinchonidinium chloride (Fluka Chemical Corp., Ronkonkoma, NY, 11779), and 105 mL of  $\text{CH}_2\text{Cl}_2$ . The resulting solution was treated with 110 mL of 50% (W/W) aqueous  $\text{NaOH}$  and the mixture was stirred vigorously at RT under nitrogen. After 24h, tlc (silica gel, 7 : 1 hexane : EtOAc) indicated a major new component at  $R_f=0.65$ , unreacted starting material at  $R_f=0.85$  and three minor components at  $R_f=0.20-0.45$ . The  $\text{CH}_2\text{Cl}_2$  phase was separated, washed with water (3 x 70 mL), dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated *in vacuo* to afford a thick pink oil. The crude product was subjected to flash chromatography on 550 g of silica gel eluting with 7 : 1 hexane : EtOAc (column was pretreated by passing through two column volumes of 0.5%  $\text{Et}_3\text{N}$  in 7 : 1 hexane : EtOAc) to give 26.4 g (76%) of the desired enantiomer mixture as identified by  $^1\text{H-NMR}$ . The product was determined to

be a mixture of two enantiomers by chiral HPLC (Pirkle phenylglycine, 99 : 1 hexane : isopropanol, flow rate=1 mL/min):  $k'_1=3.66$  (76%),  $k'_2=3.28$  (24%). The mixture was dissolved in 160 mL of hexane and allowed to crystallize at 4°C for 2 days. The solid was separated by filtration and dried *in vacuo* to afford 10.72 g (31%) of racemic material. The filtrate was concentrated *in vacuo* to give 15.72 g (45%) of enantioenriched material (2-iodo-L-phenylalanine *tert*-butyl ester-benzophenone imine, ee=86%) as a transparent, viscous oil. The product was used in subsequent steps without further purification.

**<sup>1</sup>H-NMR:** (200 MHz, CDCl<sub>3</sub>) δ 7.72 (d, 1H, J=7.8, aromatic CH), 7.60 (m, 2H, aromatic CH), 7.41-7.21 (m, 6H, aromatic CH), 7.19 (m, 2H, aromatic CH), 6.86 (m, 1H, aromatic CH), 6.55 (d, 2H, J=6.6, aromatic CH), 4.34 (dd, 1H; J=3.9, 9.6; methine), 3.48-3.21 (m, 2H, Ar-CH<sub>2</sub>), 1.47 (s, 9H, t-Bu).

**Mass Spectrum:** (CI, CH<sub>4</sub>) 512 (M + H)<sup>+</sup>, 484, 456, 410, 384, 217.

#### Example 19: 2-Iodo-L-phenylalanine

A 500 mL round bottomed flask equipped with a reflux condenser and a magnetic stirrer was charged with 15.72 g of 2-iodo-L-phenylalanine *tert*-butyl ester-benzophenone imine (86% ee) and 165 mL of 6N aqueous HCl. The mixture was heated at reflux with stirring for 4h and cooled to RT to afford a mixture of white crystalline solid and an immiscible organic phase (benzophenone). The mixture was partitioned between water (100 mL) and ethyl ether (70 mL). The aqueous phase was separated, washed with four additional 70 mL portions of ether and concentrated *in vacuo* to dryness to give 9.5 g (94%) of 2-iodo-L-phenylalanine·HCl as a fluffy white solid, m.p. 240°C (dec).

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.90-8.23 (br s, 3H, -NH<sub>3</sub><sup>+</sup>), 7.88 (d, 1H, J=8.1, aromatic CH), 7.39 (m, 2H, aromatic CH), 7.04 (m, 1H, aromatic CH), 4.06 (t, 1H, J=7.4, methine), 3.23 (m, 2H, Ar-CH<sub>2</sub>).

Example 20: Methyl 2-iodo-L-phenylalaninate

To a 500 mL 3-necked flask equipped with a magnetic stirrer and a reflux condenser were added 5.00 g of 2-iodo-L-phenylalanine·HCl and 100 mL of anhydrous MeOH. The mixture was stirred and acidified by bubbling HCl gas through for 3 min. A clear solution resulted which was heated at reflux for 5h. The solution was cooled to RT and concentrated *in vacuo* to dryness to give a white solid. The material was suspended in 120 mL of CH<sub>2</sub>Cl<sub>2</sub> and washed with 5% aqueous NaHCO<sub>3</sub> (4 x 80 mL). The CH<sub>2</sub>Cl<sub>2</sub> solution was concentrated to a volume of 20 mL, diluted to 60 mL with ethyl ether and treated with 16 mL of 1M ethereal HCl. A white solid precipitated which was collected by vacuum filtration and dried *in vacuo* to afford 4.90 g (94%) of methyl 2-iodo-L-phenylalaninate·HCl, m.p. 194-196°C.

Example 21: Methyl N-((benzyloxy)carbonyl)-5-O-methyl-L-glutam-1-yl-2-iodo-L-phenylalaninate

A dry 500 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer was charged with 3.00 g of methyl 2-iodo-L-phenylalaninate·HCl, 2.59 g of N-((benzyloxy)carbonyl)-5-O-methyl-L-glutamic acid and 80 mL of CH<sub>2</sub>Cl<sub>2</sub>. The mixture was cooled to 0°C in an ice water bath and was treated with 0.97 mL of N-methylmorpholine followed by 1.77 g of EDC (Aldrich). The reaction mixture was stirred at 0°C for 1h and allowed to warm to RT with continued stirring for an additional 3h. The solution was diluted with 100 mL of CH<sub>2</sub>Cl<sub>2</sub>, washed with 5% aqueous citric acid (3 x 70 mL), 5% aqueous NaHCO<sub>3</sub> (3 x 70 mL), and dried over anhydrous

Na<sub>2</sub>SO<sub>4</sub>. Removal of the drying agent by filtration followed by *in vacuo* concentration of the filtrate afforded 4.30 g (84%) of an off-white crystalline solid, m.p. 110-112°C. Examination by <sup>1</sup>H-NMR indicated methyl *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutam-1-yl-2-iodo-L-phenylalaninate contaminated with approximately 4% of the (*S,R*)-diastereomer. The crude product was recrystallized twice from 1:1 EtOH-H<sub>2</sub>O to give 3.47 g (68%) of pure (*S,S*)-diastereomer as a white crystalline solid, m.p. 114-116°C.

Example 22: Methyl *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutam-1-yl-2-(methoxycarbonyl)-L-phenylalaninate

To a dry 100 mL 3-necked flask equipped with a thermometer, a magnetic stirrer, and a reflux condenser were added 1.50 g of methyl *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutam-1-yl-2-iodo-L-phenylalaninate, 0.149 g of Pd(Ph<sub>3</sub>P)<sub>4</sub> (Aldrich), and 35 mL of 1:1 MeOH:THF under a nitrogen flush. The solution was deoxygenated by bubbling nitrogen through for 10 min and was then saturated with CO by bubbling CO through for 10 min. The solution changed color from yellow to bright orange during this period. The reaction vessel was put under CO balloon pressure by attaching a CO filled balloon to the condenser via a gas inlet adapter. The reaction mixture was heated to 60°C with stirring. After 3h, analysis by tlc (SiO<sub>2</sub>, 1:1 hexane:EtOAc) indicated no remaining starting material, a new component at R<sub>f</sub>=0.40, and two minor components at R<sub>f</sub>=0.90-0.95. The solution was cooled to RT and concentrated *in vacuo* to give a yellow solid. This material was purified by flash chromatography on 85 g of silica gel (1:1 hexane:EtOAc) to afford 1.23 g (93%) of methyl *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutam-1-yl-2-(methoxycarbonyl)-L-phenylalaninate as a white crystalline solid, m.p. 128-130°C.

Example 23: Methyl 5-*O*-methyl-L-glutam-1-yl-2-(methoxycarbonyl)-L-phenylalaninate

According to example 10, 1.10 g of methyl *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutam-1-yl-2-(methoxycarbonyl)-L-phenylalaninate was hydrogenolyzed in the presence of 0.214 g of 10% Pd(C) and 0.18 mL of acetyl chloride to afford 0.86 g (97%) of methyl 5-*O*-methyl-L-glutam-1-yl-2-(methoxycarbonyl)-L-phenylalaninate-HCl as a light yellow foam, m.p. 77-80°C.

Example 24: Methyl

*N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxo-benzo(f)quinazolin-9-yl)methyl)-amino)-2-fluorobenzoyl)-5-*O*-methyl-L-glutam-1-yl)-2-(methoxycarbonyl)-L-phenylalaninate

According to example 11, 0.50 g of 4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoic acid was coupled with 0.55 g of methyl 5-*O*-methyl-L-glutam-1-yl-2-(methoxycarbonyl)-L-phenylalaninate-HCl using 0.17 mL of isobutyl chloroformate and 0.14 mL (2x) of *N*-methylmorpholine. The crude product was purified by flash chromatography on 250 g of silica gel (97:3→95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.29 g (30%) of methyl *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxo-benzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-methyl-L-glutam-1-yl)-2-(methoxycarbonyl)-L-phenylalaninate as a white powder, m.p. 180°C (dec).

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 12.52 (s, 1H, benzoquinazoline NH), 9.86 (s, 1H, aromatic CH), 8.52 (d, 1H, J=7.6, amide NH), 8.23 (d, 1H, J=8.8, aromatic CH), 8.03 (d, 1H, J=8.4, aromatic CH), 7.80 (d, 1H, J=7.2, aromatic CH), 7.59 (m, 2H, aromatic CH, 4-amino-benzoyl NH), 7.50 (t, 1H, J=7.6, aromatic CH), 7.44-7.21 (m, 5H, aromatic CH, amide NH), 6.53 (d, 1H, J=8.0, aromatic CH), 6.40 (d, 1H, J=14.5, aromatic CH), 4.58 (m, 3H, benzoquinazoline 9-CH<sub>2</sub>, methine), 4.45 (m, 1H, methine), 3.82 (s, 3H, ArCO<sub>2</sub>CH<sub>3</sub>), 3.57 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.55 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.48 (m, 1H,

phe Ar-CH<sub>2</sub>), 3.10 (m, 1H, phe Ar-CH<sub>2</sub>), 2.45 (s, 3H, CH<sub>3</sub>), 2.27 (t, 2H, J=7.5, glu 4-CH<sub>2</sub>), 2.01-1.73 (m, 2H, glu 3-CH<sub>2</sub>).

Example 25: *N*-(*N*-(4-(((1,2-Dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl)-2-carboxy-L-phenylalanine

A 50 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer was charged with 0.11 g of methyl

*N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxo-benzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-methyl-L-glutam-1-yl)-2-(methoxycarbonyl)-L-phenylalaninate and 15 mL of 1:1 THF:H<sub>2</sub>O. The solution was treated with 62 mg of LiOH (Fisher Scientific Co., Fair Lawn, NJ) and stirred at RT under nitrogen. After 18h, analysis by HPLC (C18, 70:30:0.1 H<sub>2</sub>O:MeCN:TFA) showed no remaining starting material (*k'*=4.89), a new component at *k'*=2.29 (92%), and three minor components between *k'*=0.72 and 1.13. The solution was concentrated *in vacuo* to 7 mL and subjected to semipreparative HPLC (C18, 80:20:0.1 ÷ 70:30:0.1 H<sub>2</sub>O:MeCN:TFA over 25 min). Pure fractions (as determined by analytical HPLC) were combined and concentrated *in vacuo* to dryness. The residue was dissolved in 20 mL of 0.17 M aqueous LiOH, filtered, and acidified to pH=3.5 by addition of 1N aqueous HCl. A white solid resulted which was separated by centrifugation and washed with four cycles of aqueous suspension-centrifugation-decantation. The product was suspended in 10 mL of water, frozen, and lyophilized to afford 60 mg (56%) of *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl)-2-carboxy-L-phenylalanine as a fluffy white solid, m.p. 245°C (dec).

HPLC: one peak on C18, *k'*=1.86, 70:30:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>) δ 13.00-11.70 (br s, COOH), 12.54 (s, 1H, benzoquinazoline NH), 9.84 (s, 1H, aromatic CH), 8.35 (d, 1H, J=7.9, amide NH), 8.22



(d, 1H, J=9.1, aromatic CH), 8.01 (d, 1H, J=8.2, aromatic CH), 7.78 (d, 1H, J=7.8, aromatic CH), 7.60 (m, 2H, aromatic CH, 4-aminobenzoyl NH), 7.50 (t, 1H, J=7.8, aromatic CH), 7.45-7.18 (m, 5H, aromatic CH, amide NH), 6.53 (d, 1H, J=8.5, aromatic CH), 6.39 (d, 1H, J=14.7, aromatic CH), 4.67-4.46 (m, 3H, benzoquinazoline 9-CH<sub>2</sub>, methine), 4.41 (m, 1H, methine), 3.55 (dd; 1H; J=4.0, 12.3; phe Ar-CH<sub>2</sub>), 3.06 (dd; 1H, J=10.2, 12.9; phe Ar-CH<sub>2</sub>), 2.42 (s, 3H, CH<sub>3</sub>), 2.16 (t, 2H, J=7.3, glu 4-CH<sub>2</sub>), 1.98-1.64 (m, 2H, glu 3-CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>36</sub>H<sub>32</sub>N<sub>5</sub>O<sub>9</sub>F·H<sub>2</sub>O (MW 715.69): C, 60.42; H, 4.79; N, 9.79. Found: C, 60.49; H, 4.83; N, 9.76.

Mass Spectrum: (Ion Spray) 698 (M+H)<sup>+</sup>, 489, 361.

UV Spectrum: (pH7 Buffer) λ<sub>max</sub> (ε) 265.2 (43700), 298.4 (25600), 347.9 (5320).  
λ<sub>min</sub>(ε) 241.5 (20300), 284.9 (24600), 340.0 (3140).

Example 26: Methyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-methyl-L-glutam-1-yl-2-(methoxycarbonyl)-L-phenylalaninate

According to example 13, 0.29 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was reacted with 0.32 g of methyl 5-*O*-methyl-L-glutam-1-yl-2-(methoxycarbonyl)-L-phenylalaninate-HCl using 0.35 mL of DECP (Aldrich) and 0.58 mL of Et<sub>3</sub>N. The crude product was purified by flash chromatography on 100 g of silica gel (7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH) to afford 0.27 (51%) of methyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-methyl-L-glutam-1-yl-2-(methoxycarbonyl)-L-phenylalaninate as a yellow powder, m.p. 125-130°C.

Example 27: *N*-(4-(((2,4-Diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-carboxy-L-phenylalanine

To a 50 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet were added 0.12 g of methyl *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-5-*O*-methyl-L-glutam-1-yl-2-(methoxycarbonyl)-L-phenylalaninate and 10 mL of 1:1 H<sub>2</sub>O:THF. The mixture was treated with 29 mg of LiOH·H<sub>2</sub>O (J.T. Baker Chemical Co., Phillipsburg, NJ), and was stirred at RT under nitrogen. After 48h, analysis by HPLC (C18, 75:25:0.1 H<sub>2</sub>O:MeCN:TFA) indicated a major new component at *k'*=1.70 (ca. 90%) and three minor components at *k'*=0.60, 1.03, and 2.58. The solution was neutralized by dropwise addition of 1N aqueous HCl and concentrated *in vacuo* to dryness. The residue was dissolved in 10 mL of MeOH, filtered, and diluted with 20 mL of water to precipitate the product. The MeOH was removed by rotary evaporation and the remaining suspension was frozen and lyophilized to afford 65 mg (45%) of *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-carboxy-L-phenylalanine as a yellow powder, m.p. 160°C (dec).

HPLC: two peaks on C18; *k'*=2.49 (98%), *k'*=5.65 (2%); 78:22:0.1 H<sub>2</sub>O:MeCN:TFA; flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>) δ 13.10-11.90 (br s, COOH), 8.64 (s, 1H, pteridiny 7-CH), 8.62 (br s, 1H, NH<sub>2</sub>), 8.18 (d, 1H, *J*=8.0, amide NH), 7.92 (d, 1H, *J*=8.0, amide NH), 7.80-7.20 (br m, 2H, NH<sub>2</sub>), 7.77 (d, 1H, *J*=7.3, aromatic CH), 7.70 (d, 2H, *J*=8.9, aromatic CH), 7.25 (m, 3H, aromatic CH), 6.81 (d, 2H, *J*=9.0, aromatic CH), 4.84 (s, 2H, pteridiny-CH<sub>2</sub>), 4.49 (m, 1H, methine), 4.39 (m, 1H, methine), 3.51 (m, 1H, phe Ar-CH<sub>2</sub>), 3.24 (s, 3H, *N*-CH<sub>3</sub>), 3.08 (m, 1H, phe Ar-CH<sub>2</sub>), 2.19 (t, 2H, *J*=7.6, glu 4-CH<sub>2</sub>), 1.97-1.72 (m, 2H, glu 3-CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>30</sub>H<sub>31</sub>N<sub>9</sub>O<sub>8</sub>·2.7H<sub>2</sub>O·1.2TFA (MW 831.10): C, 46.82; H, 4.56; N, 15.17. Found: C, 46.76; H, 4.48; N, 15.15.

**Mass Spectrum:** (Ion Spray) 646 (M+H)<sup>+</sup>, 410, 369, 185.

**UV Spectrum:** (pH7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 258.8 (23400), 307.0 (24200), 372.8 (7540).

$\lambda_{\min}$ ( $\epsilon$ ) 242.0 (16000), 273.2 (16200), 344.3 (5900).

**Example 28: Dimethyl *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutam-1-yl-L-aspartate**

According to example 9, 2.01 g of L-aspartic acid dimethylester-HCl (Sigma) was coupled with 3.00 g of *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutamic acid using 2.05 g of EDC (Aldrich) and 1.12 mL of *N*-methyldmorpholine. This gave 3.93 g (88%) of dimethyl *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutam-1-yl-L-aspartate as a crystalline white solid, m.p. 86-88°C.

**Example 29: Dimethyl 5-*O*-methyl-L-glutam-1-yl-L-aspartate**

According to example 10, 2.00 g of dimethyl *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutam-1-yl-L-aspartate was hydrogenolyzed in the presence of 0.45 g of 10% Pd(C) and 0.39 mL of acetyl chloride to afford 1.56 g (100%) of dimethyl 5-*O*-methyl-L-glutam-1-yl-L-aspartate-HCl as a light yellow foam.

**<sup>1</sup>H-NMR:** (200 MHz, DMSO-d<sub>6</sub>)  $\delta$  9.14 (d, 1H, J=7.4, NH), 8.40 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 4.72 (m, 1H, glu methine), 3.89 (m, 1H, asp methine), 3.66 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.65 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.64 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 2.85 (d, 2H, J=6.2, asp CH<sub>2</sub>), 2.49 (m, 2H, glu 4-CH<sub>2</sub>), 2.04 (m, 2H, glu 3-CH<sub>2</sub>).

**Example 30: Dimethyl *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxo-benzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-methyl-L-glutam-1-yl)-L-aspartate**

According to example 11, 0.50 g of 4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoic acid was coupled with 0.45 g of dimethyl 5-*O*-methyl-L-glutam-1-yl-L-aspartate-HCl using 0.17 mL of isobutyl

chloroformate and 0.14 mL (2x) of *N*-methyl morpholine. The crude product was purified by flash chromatography on 250 g of silica gel (95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.33 g (38%) of dimethyl

*N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxo-benzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-O-methyl-L-glutam-1-yl)-L-aspartate as a white powder, m.p. 198-200°C.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 12.54 (s, 1H, benzoquinazoline NH), 9.85 (s, 1H, aromatic CH), 8.55 (d, 1H, J=7.7, amide NH), 8.23 (d, 1H, J=8.8, aromatic CH), 8.02 (d, 1H, J=8.4, aromatic CH), 7.70-7.45 (m, 4H, aromatic CH, amide NH), 7.33 (m, 1H, 4-amino-benzoyl NH), 6.55 (dd; 1H; J=8.8, 1.9; aromatic CH), 6.42 (dd; 1H; J=15.3, 1.7; aromatic CH), 4.72-4.41 (m, 4H, benzoquinazoline 9-CH<sub>2</sub>, glu and asp methines), 3.62 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.60 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.55 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 2.91-2.66 (m, 2H, asp CH<sub>2</sub>), 2.42 (s, 3H, CH<sub>3</sub>), 2.34 (t, 2H, J=7.7, glu 4-CH<sub>2</sub>), 2.13-1.80 (m, 2H, glu 3-CH<sub>2</sub>).

Example 31: *N*-(*N*-(4-((1,2-Dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl-L-aspartic acid

According to example 12, 0.12 g of dimethyl *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl) methyl)amino)-2-fluorobenzoyl)-5-O-methyl-L-glutam-1-yl)-L-aspartate was saponified in 8 mL of 1:1 THF-H<sub>2</sub>O using 45 mg of LiOH·H<sub>2</sub>O (Baker). Analysis of the crude product by reverse phase HPLC (C18, 80:20:0.1 H<sub>2</sub>O:MeCN:TFA) indicated a major component at k'=1.73 (90%) and a minor component at k'=1.28. The material was purified by semi-preparative HPLC using gradient elution (C18, 80:20:0.1 → 75:25:0.1 H<sub>2</sub>O:MeCN:TFA over 20 min ). Pure fractions (as determined by analytical HPLC) were combined and concentrated *in vacuo*. The residue was mixed with 20 mL of water and treated with enough LiOH·H<sub>2</sub>O to

dissolve the solid. The solution was filtered and acidified to pH=3.0 by addition of 1N HCl. A white solid precipitated which was separated by centrifugation, washed with four cycles of aqueous suspension-centrifugation-decantation, and lyophilized to afford 57 mg (48%) of *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl)-L-aspartic acid as an off-white solid, m.p. 185°C (dec). HPLC: one peak on C18,  $k'=1.42$ , 75:25:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  12.85-12.00 (br s, COOH), 12.53 (s, 1H, benzoquinazoline NH), 9.84 (s, 1H, aromatic CH), 8.36 (d, 1H,  $J=7.9$ , amide NH), 8.21 (d, 1H,  $J=8.8$ , aromatic CH), 8.01 (d, 1H,  $J=8.3$ , aromatic CH), 7.67-7.46 (m, 4H, aromatic CH, amide NH), 7.32 (m, 1H, 4-aminobenzoyl NH), 6.53 (d, 1H,  $J=8.5$ , aromatic CH), 6.40 (d, 1H,  $J=14.8$ , aromatic CH), 4.64-4.44 (m, 4H, benzoquinazoline 9-CH<sub>2</sub>, glu methine, asp methine), 2.75-2.53 (m, 2H, asp CH<sub>2</sub>), 2.42 (s, 3H, CH<sub>3</sub>), 2.25 (t, 2H,  $J=7.3$ , glu 4-CH<sub>2</sub>), 1.98 (m, 1H, glu 3-CH<sub>2</sub>), 1.83 (m, 1H, glu 3-CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>30</sub>H<sub>28</sub>N<sub>5</sub>O<sub>9</sub>F·1.8H<sub>2</sub>O (MW 654.01): C, 55.10; H, 4.87; N, 10.71. Found: C, 55.04; H, 4.82; N, 10.61.

Mass Spectrum: (Ion Spray) 622 (M+H)<sup>+</sup>, 316, 213, 156.

UV Spectrum: (pH7 Buffer)  $\lambda_{\max}$  (ε) 264.8 (46200), 298.4 (28500), 347.8 (6180).

$\lambda_{\min}$ (ε) 237.9 (19500), 285.7

(27900), 340.0 (3820).

#### Example 32: Dimethyl

*N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-5-*O*-methyl-L-glutam-1-yl)-L-aspartate

According to example 13, 0.200 g of 4-(*N*-(2,4-diamino-6-pteridinylmethyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was coupled with 0.18 g of dimethyl 5-*O*-methyl-L-glutam-1-yl-L-aspartate·HCl using 0.24 mL of DECP

(Aldrich) and 0.41 mL of Et<sub>3</sub>N. The crude product was purified by flash chromatography on 100 g of silica gel (7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH) to afford 0.17 g (53%) of dimethyl *N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-5-*O*-methyl-L-glutam-1-yl)-L-aspartate as a yellow powder, m.p. 130-135°C.

Example 33: *N*-(4-(((2,4-Diamino-6-pteridinyl)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-aspartic acid (Procedure A)

For Procedure B to prepare this compound, see Example 125.

A 50 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 0.16 g of dimethyl *N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-5-*O*-methyl-L-glutam-1-yl)-L-aspartate and 10 mL of 1:1 THF:H<sub>2</sub>O. The solution was treated with 44 mg of LiOH·H<sub>2</sub>O (Baker) and stirred at RT under N<sub>2</sub>. After 3h, tlc (SiO<sub>2</sub>, 7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH) indicated no remaining starting material at R<sub>f</sub>=0.30 and a single spot at R<sub>f</sub>=0.0. Analysis by reverse phase HPLC (C18, 85:15:0.1 H<sub>2</sub>O:MeCN:TFA) indicated a major component at k'=2.65 (90%) and a minor component at k'=1.94. The solution was neutralized by addition of 1N HCl and concentrated *in vacuo* to dryness. The crude product was purified by semi-preparative HPLC (C18, 87:13:0.1 H<sub>2</sub>O:MeCN:TFA). Pure fractions (as determined by analytical HPLC) were combined and concentrated *in vacuo* to dryness. The residue was dissolved in 20 mL of H<sub>2</sub>O and lyophilized to afford 65 mg (32%) of *N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-aspartic acid as a yellow-orange powder, m.p. 165-180°C (dec).

HPLC: one peak on C18, k'=2.45, 85:15:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 13.00-11.85 (br s, COOH), 9.17 (br s, 1H, NH<sub>2</sub>), 8.96 (br s, 1H, NH<sub>2</sub>), 8.71 (s, 1H, pteridinyl 7-CH), 8.60-8.30 (br s, 1H, NH<sub>2</sub>), 8.21 (d, 1H, J=8.0, amide NH), 8.06 (d, 1H, J=7.8, amide NH), 7.85-7.43 (br s, 1H, NH<sub>2</sub>), 7.75

(d, 2H,  $J=8.7$ , aromatic CH), 6.82 (d, 2H,  $J=8.8$ , aromatic CH), 4.87 (s, 2H, pteridinyl-CH<sub>2</sub>), 4.59-4.38 (m, 2H, 2 methines), 3.25 (s, 3H, N-CH<sub>3</sub>), 2.75-2.53 (m, 2H, asp CH<sub>2</sub>), 2.28 (t, 2H,  $J=7.5$ , glu 4-CH<sub>2</sub>), 2.01 (m, 1H, glu 3-CH<sub>2</sub>), 1.88 (m, 1H, glu 3-CH<sub>2</sub>).

**Elemental Analysis:** Calcd. for C<sub>24</sub>H<sub>27</sub>N<sub>9</sub>O<sub>8</sub>·1.9H<sub>2</sub>O·1.6TFA (MW 786.20): C, 41.55; H, 4.15; N, 16.03. Found: C, 41.53; H, 4.19; N, 16.00.

**Mass Spectrum:** (Ion Spray) 570 (M+H)<sup>+</sup>, 223.

**UV Spectrum:** (pH7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 258.7 (24400), 307.3 (26800), 372.9 (8080).  
 $\lambda_{\min}$ ( $\epsilon$ ) 240.0 (14800), 272.9 (17000), 345.0 (6420).

**Example 34: Diethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-L-glutamate**

According to example 9, 3.17 g of L-glutamic acid diethyl ester·HCl (Sigma) was coupled with 4.09 g of *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutamic acid using 2.66 g of EDC (Aldrich) and 1.45 mL of *N*-methylmorpholine. This gave 4.80 g (74%) of diethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-L-glutamate as a white crystalline solid, m.p. 104°C.

**Example 35: Diethyl 5-*O*-ethyl-L-glutam-1-yl-L-glutamate**

To a 500 mL Parr bottle were added 0.75 of diethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-L-glutamate, 0.14 g of 10% Pd(C) and 60 mL of EtOH under a N<sub>2</sub> flush. The mixture was deoxygenated by bubbling N<sub>2</sub> through for 7 min, treated with 1.52 mL of 1M ethereal HCl, and hydrogenated at 45 psi for 18h. The bottle was purged with N<sub>2</sub>, catalyst was removed by filtration through celite, and the filtrate was concentrated *in vacuo* to dryness to afford 0.60 (99%) of diethyl 5-*O*-ethyl-L-glutam-1-yl-L-glutamate·HCl as a light yellow foam.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 9.11 (d, 1H, J=7.5, NH), 8.41 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 4.32 (m, 1H, methine), 4.06 (m, 6H, ethyl CH<sub>2</sub>), 3.92 (m, 1H, methine), 2.47 (m, 4H, glu 4-CH<sub>2</sub>), 2.12-1.79 (m, 4H, glu 3-CH<sub>2</sub>), 1.19 (t, 9H, J=7.1, ethyl CH<sub>3</sub>).

**Example 36: Diethyl *N*-(*N*-(4-**

**(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-ethyl-L-glutam-1-yl)-L-glutamate**

According to example 11, 0.57 g of 4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoic acid was coupled with 0.60 g of diethyl 5-*O*-ethyl-L-glutam-1-yl-L-glutamate·HCl using 0.20 mL of isobutyl chloroformate and 0.17 mL (2x) of *N*-methylmorpholine. The crude product was purified by flash chromatography on 350 g of silica gel (EtOAc→98:2 EtOAc:MeOH) to afford 0.27 g (25%) of diethyl *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-ethyl-L-glutam-1-yl)-L-glutamate as a white powder, m.p. 185-187°C.

**Example 37: *N*-(*N*-(4-(((1,2-Dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl)-L-glutamic acid**

A 250 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 0.217 g of diethyl *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-ethyl-L-glutam-1-yl)-L-glutamate, 40 mL of 0.2 N aqueous NaOH, and 15 mL of EtOH. The mixture was stirred at RT under N<sub>2</sub>. The solid starting material slowly dissolved to give a clear solution. After 2h, tlc (SiO<sub>2</sub>, EtOAc) indicated no remaining starting material at R<sub>f</sub>=0.15 and a new spot at R<sub>f</sub>=0.0. The solution was acidified to pH=3.00 by addition of 1N HCl. A white solid precipitated which was separated by centrifugation, washed with



four cycles of aqueous suspension-centrifugation-decantation, and lyophilized to afford 0.17 g (84%) of *N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl)-L-glutamic acid as a white powder, m.p. 180°C (dec). The product was determined to be pure by HPLC.

HPLC: one peak on C18,  $k'$ =1.66, 75:25:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  12.65-11.90 (br s, COOH), 12.53 (s, 1H, benzoquinazoline NH), 9.84 (s, 1H, aromatic CH), 8.29 (d, 1H,  $J$ =7.5, amide NH), 8.22 (d, 1H,  $J$ =8.9, aromatic CH), 8.01 (d, 1H,  $J$ =8.0, aromatic CH), 7.60 (m, 3H, aromatic CH, amide NH), 7.51 (t, 1H,  $J$ =9.0, aromatic CH), 7.33 (m, 1H, 4-aminobenzoyl NH), 6.54 (dd; 1H;  $J$ =8.5, 1.9; aromatic CH), 6.41 (dd; 1H;  $J$ =13.9, 1.4; aromatic CH), 4.57 (d, 2H,  $J$ =5.2, benzoquinazoline 9-CH<sub>2</sub>), 4.49 (m, 1H, methine), 4.22 (m, 1H, methine), 2.43 (s, 3H, CH<sub>3</sub>), 2.28 (m, 4H, glu 4-CH<sub>2</sub>), 2.10-1.70 (m, 4H, glu 3-CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>31</sub>H<sub>30</sub>N<sub>5</sub>O<sub>9</sub>F·2H<sub>2</sub>O (MW 671.64): C, 55.44; H, 5.10; N, 10.43. Found: C, 55.38; H, 5.12; N, 10.26.

Mass Spectrum: (FAB) 636 (M+H)<sup>+</sup>, 613, 581, 549.

UV Spectrum: (pH7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 264.9 (45100), 296.1 (28000), 331.4 (7280), 348.1 (5990).  $\lambda_{\min}$ ( $\epsilon$ ) 285.6 (27200), 329.3 (7040), 339.8 (3700).  $\lambda_{\text{sh}}$ ( $\epsilon$ ) 271.5 (41900).

#### Example 38: Diethyl

*N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-5-*O*-ethyl-

L-glutam-1-yl-L-glutamate

According to example 13, 0.142 g of 4-(*N*-(2,4-diamino-6-pteridinylmethyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was coupled with 0.15 g of diethyl 5-*O*-ethyl-L-glutam-1-yl-L-glutamate·HCl using 0.18 mL of DECP (Aldrich) and 0.31 mL of Et<sub>3</sub>N. The crude product was purified by flash chromatography on 120 g of silica gel (7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH) to afford 0.14 g

(56%) of diethyl *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-5-*O*-ethyl-L-glutam-1-yl-L-glutamate as a yellow powder, m.p. 105-109°C.

Example 39: *N*-(4-(((2,4-Diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-glutamic acid (Procedure A)

For Procedure B to prepare this compound, see Example 129.

According to example 33, 0.125 g of diethyl *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-5-*O*-ethyl-L-glutam-1-yl-L-glutamate was saponified in 10 mL of 1:1 THF:H<sub>2</sub>O using 23 mg of LiOH (Fisher). After stirring at RT under N<sub>2</sub> for 18 h, the solution was acidified to pH=2.0 by addition of 1N HCl and was then concentrated *in vacuo* to dryness. Analysis of the residue by HPLC (C18, 85:15:0.1 H<sub>2</sub>O:MeCN:TFA) indicated a major component at *k'*=2.89 (90%) and two minor components at *k'*=2.46 and 1.17. The crude product was purified by semi-preparative HPLC (C18, 87:13:0.5 H<sub>2</sub>O:MeCN:TFA) and lyophilized to afford 37 mg (24%) of *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-glutamic acid as a yellow powder, m.p. 178°C.

HPLC: one peak on C18, *k'*=2.90, 85:15:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>) δ 13.20-12.00 (br s, COOH), 9.30 (br s, 1H, NH<sub>2</sub>), 9.10 (br s, 1H, NH<sub>2</sub>), 8.74 (s, 1H, pteridiny 7-CH), 8.72-8.42 (br s, 1H, NH<sub>2</sub>), 8.20 (d, 1H, *J*=7.5, amide NH), 8.07 (d, 1H, *J*=7.5, amide NH), 7.76 (d, 2H, *J*=8.8, aromatic CH), 7.61-7.40 (br s, 1H, NH<sub>2</sub>), 6.83 (d, 2H, *J*=8.8, aromatic CH), 4.90 (s, 2H, pteridiny-CH<sub>2</sub>), 4.43 (m, 1H, methine), 4.20 (m, 1H, methine), 3.27 (s, 3H, N-CH<sub>3</sub>), 2.30 (m, 4H, glu 4-CH<sub>2</sub>), 2.08-1.77 (m, 4H, glu 3-CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>25</sub>H<sub>29</sub>N<sub>9</sub>O<sub>8</sub>·2.3H<sub>2</sub>O·1.7TFA (MW 818.84): C, 41.66; H, 4.35; N, 15.40. Found: C, 41.61; H, 4.25; N, 15.44.

Mass Spectrum: (FAB) 584 (M+H)<sup>+</sup>, 309, 275, 176, 155, 119.

UV Spectrum: (pH7 Buffer)  $\lambda_{\text{max}}$  ( $\epsilon$ ) 220.9 (20500), 258.5 (21500), 306.7 (23200), 372.5 (7180).  $\lambda_{\text{min}}$ ( $\epsilon$ ) 240.1 (13400), 272.5 (15100), 344.8 (5730).

Example 40: 3-Cyano-L-tyrosine

A solution of 5.70 g of 3-amino-L-tyrosine (Aldrich) in 35 mL of 2.15 N aqueous HCl was cooled to 0°C and treated with a solution of 1.41 g of NaNO<sub>2</sub> (Aldrich) in 5 mL of water followed by 2.97 g of Na<sub>2</sub>CO<sub>3</sub>. After stirring at 0°C for 7 min, the solution was slowly added (via an addition funnel) to a stirred mixture of 3.58 g of CuCN (Aldrich), 5.88 g of NaCN (Aldrich) and 5.72 g of Na<sub>2</sub>CO<sub>3</sub> in 40 mL of water maintained at 75°C in a 250 mL 3-necked flask equipped with a condenser. During the addition, vigorous evolution of nitrogen occurred and the reaction mixture changed color to dark red-orange. The reaction mixture was stirred at 75°C for 2h, heated to 95°C, and stirred for an additional 1h. After cooling to RT, the reaction mixture was acidified to pH=2.0 by addition of concentrated HCl via the addition funnel. The evolved gas was scrubbed through a 20% aqueous NaOH trap followed by a 5% NaOCl (Chlorox) trap. The mixture was filtered and the pH of the filtrate was adjusted to 6.0 with concentrated NH<sub>4</sub>OH. The brown solution was mixed with 300 mL of water and stirred with 300 g of DOWEX 50WX-8 ion exchange resin (acid form). The resin was filtered, washed with 1L of water, mixed with 500 mL of water, and the mixture was basified to pH 11 by addition of concentrated NH<sub>4</sub>OH. The mixture was filtered and the filtrate concentrated *in vacuo* to dryness. The dark brown residue was suspended in 20 mL of water and treated with enough 1N aqueous NaOH to dissolve the solid. The pH was then adjusted to 6.0 by addition of 1N aqueous HCl. A precipitate formed which was collected by filtration and dried *in vacuo* to afford 1.45 g (35%) of 3-cyano-L-tyrosine as a chocolate brown solid, m.p. >250°C.

**<sup>1</sup>H-NMR:** (200 MHz, DMSO-d<sub>6</sub>) δ 9.60-7.20 (br s, NH<sub>2</sub>), 7.39 (s, 1H, aromatic CH), 7.30 (m, 1H, aromatic CH), 6.98 (m, 1H, aromatic CH), 3.52 (m, 1H, methine), 2.92 (m, 2H, ArCH<sub>2</sub>).

**Example 41: Methyl 3-(methoxycarbonyl)-L-tyrosinate**

A 250 mL 3-necked flask equipped with a reflux condenser and a magnetic stirrer was charged with 1.45 g of 3-cyano-L-tyrosine, 50 mL of concentrated HCl, and 50 mL of water. The solution was heated to reflux with stirring. After 2 days, analysis of the reaction mixture by HPLC (C18, 90:10:0.1 H<sub>2</sub>O:MeCN:TFA) indicated a major component at k'=2.23 (75%) and a minor component at k'=1.59 (25%). After 4 days HPLC indicated only the k'=2.23 material. The reaction mixture was cooled to RT, filtered, and the filtrate was concentrated *in vacuo* to dryness to give 1.2 g of 3-carboxy-L-tyrosine as a dark brown solid. IR analysis (nujol) indicated loss of the nitrile stretch of 3-cyano-L-tyrosine, at 2223 cm<sup>-1</sup>. The crude solid was added to 60 mL of anhydrous MeOH in a 250 mL 3-necked flask equipped with a condenser and a magnetic stirrer. The mixture was acidified by bubbling HCl gas through for 5 min and heated to reflux with stirring. After 6h, the reaction mixture was cooled to RT and neutralized with solid NaHCO<sub>3</sub>. The mixture was concentrated *in vacuo* to dryness, mixed with 100 mL of water, and extracted with CH<sub>2</sub>Cl<sub>2</sub> (4x50 mL). The combined extracts were washed with 5% aqueous NaHCO<sub>3</sub> (2 x 50 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo* to 10 mL. The solution was diluted with 35 mL of ether and treated with 5.5 mL of 1M ethereal HCl. A solid precipitated which was collected by filtration, washed with ether, and dried *in vacuo* to afford 0.90 (50%) of methyl 3-(methoxycarbonyl)-L-tyrosinate-HCl as an off-white powder, m.p. 211-213°C.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>) δ 10.45 (s, 1H, OH), 8.59 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.66 (s, 1H, aromatic CH), 7.38 (dd; 1H; J=7.9, 1.1, aromatic CH), 6.97 (d, 1H, J=7.9, aromatic

CH), 4.26 (m, 1H, methine), 3.89 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.68 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.09 (m, 2H, ArCH<sub>2</sub>).

Example 42: Methyl *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutam-1-yl-3-(methoxycarbonyl)-L-tyrosinate

According to example 9, 0.80 g of methyl 3-(methoxycarbonyl)-L-tyrosinate-HCl was coupled with 0.82 g of *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutamic acid using 0.56 g of EDC (Aldrich) and 0.30 mL of *N*-methylmorpholine. This gave 1.24 g (85%) of methyl *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutam-1-yl-3-(methoxycarbonyl)-L-tyrosinate as a white solid, m.p. 142-144°C.

Example 43: Methyl 5-*O*-methyl-L-glutam-1-yl-3-(methoxycarbonyl)-L-tyrosinate

According to example 10, 0.99 g of methyl *N*-((benzyloxy)carbonyl)-5-*O*-methyl-L-glutam-1-yl-3-(methoxycarbonyl)-L-tyrosinate was hydrogenolyzed using 0.19 g of 10% Pd(C) and 0.15 mL of acetyl chloride in 90 mL of 2:1 EtOH:EtOAc to afford 0.75 g (93%) of methyl 5-*O*-methyl-L-glutam-1-yl-3-(methoxycarbonyl)-L-tyrosinate-HCl as a light yellow foam, m.p. 95-97°C (dec).

Example 44: Methyl *N*-(*N*-(4(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-methyl-L-glutam-1-yl)-3-(methoxycarbonyl)-L-tyrosinate

According to example 11, 0.65 g of 4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoic acid was coupled with 0.75 g of methyl 5-*O*-methyl-L-glutam-1-yl-3-(methoxycarbonyl)-L-tyrosinate-HCl using 0.22 mL of isobutyl chloroformate and 0.19 mL (2x) of *N*-methylmorpholine. The crude product was purified by flash chromatography on 300 g of silica gel (99:1 → 94:6 EtOAc:MeOH) to

afford 0.53 g (40%) of methyl *N*-(*N*-(4((((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-methyl-L-glutam-1-yl)-3-(methoxycarbonyl)-L-tyrosinate as a white powder, m.p. 195-203°C (dec).

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 12.52 (benzoquinazoline NH), 10.38 (s, 1H, OH), 9.83 (s, 1H, aromatic CH), 8.45 (d, 1H, J=7.5, amide NH), 8.21 (d, 1H, J=8.9, aromatic CH), 8.00 (d, 1H, J=8.3, aromatic CH), 7.60 (m, 3H, aromatic CH, 4-aminobenzoyl NH), 7.48 (m, 2H, aromatic CH), 7.36 (m, 2H, aromatic CH, amide NH), 6.86 (d, 1H, J=8.6, aromatic CH), 6.52 (dd; 1H; J=8.5, 1.9; aromatic CH), 6.39 (dd; 1H; J=13.9, 1.5; aromatic CH), 4.57 (d 2H, J=5.6, benzoquinazoline 9-CH<sub>2</sub>), 4.45 (m, 2H, methines), 3.84 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.58 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.52 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.05-2.80 (m, 2H, ArCH<sub>2</sub>), 2.42 (s, 3H, CH<sub>3</sub>), 2.25 (t, 2H, J=7.8, glu 4-CH<sub>2</sub>), 2.00-1.77 (m, 2H, glu 3-CH<sub>2</sub>).

Example 45: 3-Carboxy-*N*-(*N*-(4((((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl)-L-tyrosine

A solution of 0.15 g of methyl *N*-(*N*-(4((((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-methyl-L-glutam-1-yl)-3-(methoxycarbonyl)-L-tyrosinate in 20 mL of 0.2 N aqueous NaOH was stirred at RT under N<sub>2</sub> for 18h.

Analysis of the solution by HPLC (C18, 70:30:0.1 H<sub>2</sub>O:MeCN:TFA) indicated a major component at k'=1.9 (90%) and two minor components at k'=1.0 and 2.6. The solution was acidified to pH 2.5 by addition of 1N HCl. A white precipitate resulted which was separated by centrifugation and purified by semi-preparative HPLC (C18, 75:25:0.1 H<sub>2</sub>O:MeCN:TFA). Pure fractions (as determined by analytical HPLC) were combined and concentrated *in vacuo* to dryness. The residue was dissolved in 20 mL of 0.15 N aqueous NaOH and the solution was acidified to pH=3.0 by addition of 1N HCl. The resulting white precipitate was separated by centrifugation, washed with four cycles of

aqueous suspension-centrifugation-decantation, and lyophilized to afford 39 mg (26%) of 3-carboxy-*N*-(*N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl)-L-tyrosine as a white powder, m.p. 198-205°C (dec).

**HPLC:** one peak on C18,  $k'=1.86$ , 70:30:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

**<sup>1</sup>H-NMR:** (200 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  13.00-11.00 (br m, COOH, benzoquinazoline NH), 9.86 (s, 1H, aromatic CH), 8.28 (d, 1H,  $J=7.5$ , amide NH), 8.24 (d, 1H,  $J=8.9$ , aromatic CH), 8.03 (d, 1H,  $J=8.4$ , aromatic CH), 7.70-7.42 (m, 5H, aromatic CH, 4-aminobenzoyl NH), 7.40-7.24 (m, 2H, aromatic CH, amide NH), 6.78 (d, 1H,  $J=8.6$ , aromatic CH), 6.55 (dd; 1H;  $J=8.5$ , 1.9; aromatic CH), 6.42 (dd; 1H;  $J=13.9$ , 1.5; aromatic CH), 4.58 (d, 2H,  $J=5.8$ , benzoquinazoline 9-CH<sub>2</sub>), 4.55-4.31 (m, 2H, methines), 3.06-2.75 (m, 2H, ArCH<sub>2</sub>), 2.24 (s, 3H, CH<sub>3</sub>), 2.21 (t, 2H,  $J=7.6$ , glu 4-CH<sub>2</sub>), 2.08-1.70 (m, 2H, glu 3-CH<sub>2</sub>).

**Elemental Analysis:** Calcd. for C<sub>36</sub>H<sub>32</sub>N<sub>5</sub>O<sub>10</sub>F·2.2 H<sub>2</sub>O (MW 753.31): C, 57.40; H, 4.87; N, 9.30. Found: C, 57.37; H, 4.97; N, 9.28.

**Mass Spectrum:** (FAB) 714 (M+H)<sup>+</sup>, 613, 549, 461, 360, 309.

**UV Spectrum:** (pH7 Buffer)  $\lambda_{\max}(e)$  265.9 (44700), 300.2 (28200), 348.3 (5270).  $\lambda_{\min}(e)$  246.3 (23400), 285.2 (25500), 340.8 (3630).

**Example 46: *tert*-Butyl 3-(*tert*-butoxycarbonyl)-L-tyrosinate**

A 250 mL 3-necked flask equipped with a magnetic stirrer and a condenser was charged with 4.1 g of 3-cyano-L-tyrosine and 100 mL of 6N aqueous HCl. The mixture was heated to reflux with stirring. After 36h, HPLC (C18, 90:10:0.1 H<sub>2</sub>O:MeCN:TFA) indicated no remaining starting material at  $k'=1.33$  and a major new component at  $k'=1.69$ . The reaction mixture was cooled to RT, filtered to remove solids, and concentrated *in vacuo* to dryness. The residue was dissolved in 100 mL of 8:2

H<sub>2</sub>O:MeOH and the solution was passed through two C18 plugs (3.5 x 4.5 cm, EM separations LiChroprep RP-18) washing with 8:2 H<sub>2</sub>O:MeOH. The filtrate was concentrated *in vacuo* to dryness to afford 4.5 g of 3-carboxytyrosine as a brown solid. A portion of this material (1.30 g) was dissolved in 40 mL of 1,4-dioxane with 2 mL of concentrated H<sub>2</sub>SO<sub>4</sub> and the solution was added to 30 mL of isobutylene (condensed at -78°C) in a 300 mL pressure bottle equipped with a magnetic stirrer. The vessel was stoppered, warmed to RT and stirred. After 48h, the bottle was cooled to 0°C in an ice water bath, opened, and the mixture was treated with 20 g of NaHCO<sub>3</sub>. After allowing the isobutylene to evaporate, the mixture was suspended in 300 mL of water and extracted with CH<sub>2</sub>Cl<sub>2</sub> (4 x 100 mL). The combined CH<sub>2</sub>Cl<sub>2</sub> extracts were washed with 5% aqueous NaHCO<sub>3</sub> (2 x 50 mL), dried over anhydrous MgSO<sub>4</sub>, and concentrated *in vacuo* to give a yellow-brown oil. Analysis by tlc (SiO<sub>2</sub>, 97:3 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) indicated a major component at R<sub>f</sub>=0.41 and three minor components at R<sub>f</sub>=0.30, 0.25 and 0.0. The crude material was purified by flash chromatography on 75 g of silica gel (94:6 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to give 1.2 g of a light yellow oil. The oil was dissolved in 40 mL of ether and treated with 3.9 mL of 1M ethereal HCl. A precipitate formed which was collected by filtration and dried *in vacuo* to afford 1.14 g (53%) of *tert*-butyl 3-(*tert*-butoxycarbonyl)-L-tyrosinate-HCl as a fluffy white solid. An analytical sample (26 mg) was prepared by recrystallization from MeOH-ether, m.p. 205°C (dec). <sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 10.69 (s, 1H, OH), 8.39 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.57 (d, 1H, J=2.2, aromatic CH), 7.43 (dd; 1H; J=8.6, 2.2; aromatic CH), 6.96 (d, 1H, J=8.4, aromatic CH), 4.12 (m, 1H, methine), 3.11 (dd; 1H; J=14.3, 5.3; ArCH<sub>2</sub>), 2.97 (dd; 1H, J=14.1, 8.1; ArCH<sub>2</sub>), 1.57 (s, 9H, t-Bu), 1.36 (s, 9H, t-Bu).



Example 47: *tert*-Butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(*tert*-butoxycarbonyl)-L-tyrosinate

According to example 9, 0.323 g of *tert*-butyl 3-(*tert*-butoxycarbonyl)-L-tyrosinate·HCl was coupled with 0.292 g of *N*-benzyloxycarbonyl-L-glutamic acid  $\gamma$ -*t*-butyl ester (Sigma) using 0.174 g of EDC (Aldrich) and 0.095 mL of *N*-methyldimorpholine. The crude product was purified by flash chromatography on 85 g of silica gel (75:25 hexane:EtOAc) to afford 0.49 g (89%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(*tert*-butoxycarbonyl)-L-tyrosinate as a tacky white foam.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  10.67 (s, 1H, OH), 8.21 (d, 1H, J=7.4, NH), 7.56 (s, 1H, aromatic CH), 7.46-7.26 (m, 7H, aromatic CH, NH), 6.86 (d, 1H, J=8.5, aromatic CH), 5.00 (m, 2H, PhCH<sub>2</sub>O), 4.31 (m, 1H, methine), 4.06 (m, 1H, methine), 2.89 (d, 2H, J=6.9, ArCH<sub>2</sub>), 2.21 (t, 2H, J=7.7, glu 4-CH<sub>2</sub>), 1.92-1.64 (m, 2H, glu 3-CH<sub>2</sub>), 1.57 (s, 9H, *t*-Bu), 1.38 (s, 9H, *t*-Bu), 1.33 (s, 9H, *t*-Bu).

Example 48: *tert*-Butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-(*tert*-butoxycarbonyl)-L-tyrosinate

To a 300 mL Parr bottle were added 0.076 g of 10% Pd(C) and 20 mL of MeOH under a nitrogen flush. To this was added a solution of 0.49 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(*tert*-butoxycarbonyl)-L-tyrosinate in 50 mL of MeOH. The mixture was deoxygenated by bubbling nitrogen through for 7 min and was hydrogenated at 50 psi for 3h. The vessel was purged with nitrogen, catalyst removed by filtration through celite, and the filtrate concentrated *in vacuo* to give a thick oil.

This material was purified by flash chromatography on 70 g of silica gel (98:2 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.31 g (82%) of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-

(*tert*-butoxycarbonyl)-L-tyrosinate as a thick transparent oil. A small sample of the corresponding HCl salt for  $^1\text{H-NMR}$  analysis was prepared by treating 5 mg of the product with excess 1N ethereal HCl and concentrating to dryness.

$^1\text{H-NMR}$ : (200 MHz, DMSO- $d_6$ )  $\delta$  10.68 (s, 1H, OH), 8.99 (d, 1H,  $J=7.1$ , NH), 8.33 (br s, 3H,  $\text{NH}_3^+$ ), 7.60 (d, 1H;  $J=2.0$ , aromatic CH), 7.47 (dd; 1H;  $J=8.6$ , 2.2; aromatic CH), 6.92 (d, 1H,  $J=8.4$ , aromatic CH), 4.38 (m, 1H, methine), 3.87 (m, 1H, methine), 2.92 (d, 2H,  $J=7.1$ ,  $\text{ArCH}_2$ ), 2.34 (m, 2H, glu 4- $\text{CH}_2$ ), 2.00 (m, 2H, glu 3- $\text{CH}_2$ ), 1.59 (5, 9H, t-Bu), 1.41 (s, 9H, t-Bu), 1.35 (s, 9H, t-Bu).

Example 49: *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-5-*O-tert*-butyl-L-glutam-1-yl-3-(*tert*-butoxycarbonyl)-L-tyrosinate

A dry 100 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer was charged with 25 mL of anhydrous DMF, 0.26 mL of DECP (Aldrich) and 0.28 mL of  $\text{Et}_3\text{N}$ . To this solution was added 0.217 g of 4-(*N*-(2,4-diamino-6-pteridinylmethyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich). The solid starting material slowly dissolved to give a light yellow solution. After stirring for 3h under  $\text{N}_2$ , the solution was treated with 0.29 g of *tert*-butyl 5-*O-tert*-butyl-L-glutam-1-yl-3-(*tert*-butoxycarbonyl)-L-tyrosinate in 5 mL of DMF. The solution was stirred for 18h at RT and was then concentrated *in vacuo* to dryness. The residue was dissolved in 85 mL of  $\text{CHCl}_3$ . The resulting solution was washed with 1% aqueous  $\text{NH}_4\text{OH}$  (3x60 mL), dried over anhydrous  $\text{MgSO}_4$ , and concentrated to give a thick, yellow-orange oil. This material was purified by flash chromatography on 150 g of silica gel (7:7:1  $\text{CH}_2\text{Cl}_2$ :acetone: MeOH) to afford 0.245 g (52%) of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-5-*O-tert*-butyl-L-glutam-1-yl-3-(*tert*-butoxycarbonyl)-L-tyrosinate as a light yellow powder, m.p. 140-143°C.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>) δ 10.64 (s, 1H, OH), 8.57 (s, 1H, pteridinyl 7-CH), 8.15 (d, 1H, J=7.4, amide NH), 7.99 (d, 1H, J=7.9, amide NH), 7.71 (d, 2H, J=8.8, aromatic CH), 7.67 (br s, 1H, NH<sub>2</sub>), 7.53 (d, 1H, J=2.0, aromatic CH), 7.43 (br s, 1H, NH<sub>2</sub>), 7.37 (dd; 1H; J=8.6, 2.1; aromatic CH), 6.81 (m, 3H, aromatic CH), 6.59 (br s, 2H, NH<sub>2</sub>), 4.79 (s, 2H, pteridinyl-CH<sub>2</sub>), 4.43 (m, 1H, methine), 4.32 (m, 1H, methine), 3.21 (s, 3H, N-CH<sub>3</sub>), 2.90 (d, 2H, J=7.1, ArCH<sub>2</sub>), 2.24 (t, 2H, J=7.9, glu 4-CH<sub>2</sub>), 2.03-1.78 (m, 2H, glu 3-CH<sub>2</sub>), 1.54 (s, 9H, t-Bu), 1.36 (s, 9H, t-Bu), 1.30 (s, 9H, t-Bu).

**Example 50: *N*-(4-(((2,4-Diamino-6-pteridinyl)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-carboxy-L-tyrosine**

A 100 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 0.20 g of *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(*tert*-butoxycarbonyl)-L-tyrosinate and 20 mL of CH<sub>3</sub>NO<sub>2</sub>. The yellow solution was stirred at RT and acidified by bubbling gaseous HCl through for 10 min. Upon HCl treatment, the color rapidly changed from yellow to orange to lighter yellow and a solid began to precipitate. The mixture was stirred at RT under N<sub>2</sub> for 1.5 h and was then concentrated *in vacuo* to dryness. The resulting yellow solid was suspended in 15 mL of water and treated with sufficient 1N aqueous NaOH to give complete solution. The solution was filtered and neutralized by addition of 1N aqueous HCl. A yellow precipitate resulted which was separated by centrifugation, washed with four cycles of aqueous suspension-centrifugation-decantation, and lyophilized to afford 140 mg (88%) of *N*-(4-(((2,4-Diamino-6-pteridinyl)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-carboxy-L-tyrosine as a yellow-orange powder, m.p. 195°C (dec).

**HPLC:** two peaks on C18; k'=3.16 (98%), k'=6.07 (2%); 80:20:0.1

H<sub>2</sub>O:MeCN:TFA:flow rate=1 mL/min.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  13.15-11.80 (br s, COOH), 8.62 (s, 1H, pteridinyl 7-CH), 8.19 (br s, 1H, NH<sub>2</sub>), 8.00 (m, 3H, amide NH (2), NH<sub>2</sub>), 7.72 (d, 2H, J=8.7, aromatic CH), 7.61 (d, 1H, J=2.1, aromatic CH), 7.22 (m, 3H, NH<sub>2</sub>(2), aromatic CH), 6.81 (d, 2H, J=8.7, aromatic CH), 6.66 (d, 1H, J=7.8, aromatic CH), 4.81(s, 2H, pteridinyl-CH<sub>2</sub>), 3.39 (m, 2H, methines), 3.21 (s, 3H, *N*-CH<sub>3</sub>), 2.94 (m, 1H, ArCH<sub>2</sub>), 2.86 (m, 1H, ArCH<sub>2</sub>), 2.27 (t, 2H, J=7.9, glu 4-CH<sub>2</sub>), 2.05-1.72 (m, 2H, glu 3-CH<sub>2</sub>).

**Elemental Analysis:** Calcd. for C<sub>30</sub>H<sub>31</sub>N<sub>9</sub>O<sub>9</sub>·1.8H<sub>2</sub>O (MW 694.06): C, 51.92; H, 5.02; N, 18.16. Found: C, 51.84; H, 5.02; N, 18.19.

**Mass Spectrum:** (Ion Spray) 662 (M+H)<sup>+</sup>, 385, 306, 235, 157.

**UV Spectrum:** (pH7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 258.6 (19900), 306.3 (23500), 372.6 (5490).  $\lambda_{\min}$ ( $\epsilon$ ) 245.4 (14800), 272.8 (12600), 347.0 (4040).

**Example 51: (2*R*, 3*S*, 5*S*)-Benzyl 3-(3-iodobenzyl)-2-oxo-2,3-diphenyl-4-morpholinecarboxylate**

A dry 500 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 3.0 g of benzyl (2*R*, 3*S*)-6-oxo-2,3-diphenyl-4-morpholinecarboxylate (Aldrich) and 180 mL of anhydrous THF. After gently warming the mixture to dissolve the solid starting material, the solution was cooled to -78°C and was treated with 8.13 mL of 1M sodium bis(trimethylsilyl)amide in THF (Aldrich) by slow addition via syringe through a rubber septum. The solution was stirred at -78°C under N<sub>2</sub> for 40 min and was then treated with a solution of 2.41 g of 3-iodobenzyl bromide (Lancaster, Windham, NH 03087) in 5 mL of anhydrous THF. The reaction mixture was stirred at -78°C for 2.5h, mixed with 100 mL of water and the resulting mixture was concentrated *in vacuo* to dryness. The residue was dissolved in 100 mL of EtOAc, washed with water (3x100 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo* to dryness to

give a tan solid. Analysis by tlc (SiO<sub>2</sub>, 8:2 hexane:EtOAc) indicated no remaining starting material at R<sub>f</sub>=0.35, a major new component at R<sub>f</sub>=0.50 and some unreacted 3-iodobenzyl bromide at R<sub>f</sub>=0.80. The crude product was purified by flash chromatography on 150 g of silica gel (8:2 hexane:EtOAc) to afford 2.8 g (60%) of (2*R*, 3*S*, 5*S*)-benzyl 3-(3-iodobenzyl)-2-oxo-2,3-diphenyl-4-morpholinecarboxylate as a white crystalline solid, m.p. 167-168°C.

Example 52: (2*R*, 3*S*, 5*S*)-Benzyl 3-(3-methoxycarbonyl)-2-oxo-2,3-diphenyl-4-morpholinecarboxylate

A dry 100 mL 3-necked flask equipped with a magnetic stirrer, a reflux condenser, and a thermometer was charged with 2.56 g of (2*R*, 3*S*, 5*S*)-benzyl 3-(3-iodobenzyl)-2-oxo-5,6-diphenyl-4-morpholinecarboxylate and 50 mL of 1:1 THF:MeOH. The mixture was deoxygenated by bubbling N<sub>2</sub> through for 10 min and was treated with 1.0 mL of Et<sub>3</sub>N followed by 0.24 g of Pd(Ph<sub>3</sub>P)<sub>4</sub> (Aldrich). The mixture was saturated with CO and was heated to reflux (65°C) with stirring. A slight positive pressure of CO was maintained by attaching a CO gas inlet adapter equipped with a mineral oil bubbler to the condenser. After 4h at reflux, tlc (SiO<sub>2</sub>, 8:2 hexane:EtOAc) indicated no remaining starting material at R<sub>f</sub>=0.50 and a new component at R<sub>f</sub>=0.30. The reaction mixture was cooled to RT at which point a voluminous white solid precipitated. The suspension was mixed with 100 mL of CH<sub>2</sub>Cl<sub>2</sub> and the resulting solution was filtered through a silica gel plug (2.5x3 cm) and concentrated *in vacuo* to give a light yellow solid. This material was purified by flash chromatography on 150 g of silica gel (8:2 hexane:EtOAc) to afford 2.0 g (88%) of (2*R*, 3*S*, 5*S*)-benzyl 3-(3-methoxycarbonyl)-2-oxo-5,6-diphenyl-4-morpholinecarboxylate as an off-white crystalline solid, m.p. 180-182°C.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 8.05-7.80 (m, 2H, aromatic CH), 7.72-6.97 (m, 14H, aromatic CH), 6.73 (m, 1H, aromatic CH), 6.58 (m, 2H, aromatic CH), 6.25, 6.16

(d, J=2.2; d, J=2.6; 1H total; 2 resonances for morpholine 2-H due to carbamate conformers), 5.36, 5.31 (d, J=2.6; d, J=2.6; 1H total; 2 resonances for morpholine 3-H due to carbamate conformers), 5.16-4.88 (m, 3H, morpholine 5-H, PhCH<sub>2</sub>O), 3.88, 3.85 (5, 5, 3H total, CO<sub>2</sub>CH<sub>3</sub>, conformers), 3.71-3.40 (m, 2H, ArCH<sub>2</sub>).

**Example 53: 3-Methoxycarbonyl-L-phenylalanine**

To a 500 mL Parr bottle were added 2.0 g of (2*R*, 3*S*, 5*S*)-benzyl 3-(3-methoxycarbonyl)-2-oxo-5,6-diphenyl-4-morpholinecarboxylate, 0.46 g of PdCl<sub>2</sub> (Aldrich) and 60 mL of 1:1 THF:MeOH. The mixture was deoxygenated by bubbling N<sub>2</sub> through for 10 min and was then hydrogenolyzed at 45 psi for 18 h. The bottle was purged with N<sub>2</sub> and the mixture filtered to remove catalyst. The filtrate was concentrated *in vacuo* to 5 mL and triturated with addition of 50 mL of ether. A white solid precipitated which was collected by vacuum filtration, washed with 30 mL of ether, and dried *in vacuo* to afford 0.88 g (91%) of 3-methoxycarbonyl-L-phenylalanine-HCl, m.p. >200°C.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 8.50 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.89 (m, 2H, aromatic CH), 7.67-7.43 (m, 2H, aromatic CH), 4.21 (m, 1H, methine), 3.87 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.22 (d, 2H, J=6.5, ArCH<sub>2</sub>).

**Example 54: *tert*-Butyl 3-methoxycarbonyl-L-phenylalaninate**

A solution of 0.88 g of 3-methoxycarbonyl-L-phenylalanine-HCl, and 1.5 mL of conc. H<sub>2</sub>SO<sub>4</sub> in 20 mL of 1,4-dioxane was added to 25 mL of liquid isobutylene (condensed at -78°C) in a 300 mL pressure bottle equipped with a magnetic stirrer. The bottle was sealed and the mixture allowed to warm to RT with stirring. The initially heterogeneous mixture afforded a slightly cloudy solution after stirring for 18h at RT. After 48h the vessel was chilled in an ice water bath, opened, and the contents treated with 13 g of

solid NaHCO<sub>3</sub>. The isobutylene was allowed to evaporate and the remaining mixture was concentrated *in vacuo* to dryness. The residue was suspended in 100 mL of water and extracted with EtOAc (4x40 mL). The combined EtOAc extracts were washed with 5% aqueous NaHCO<sub>3</sub> (3x50 mL), dried over anhydrous MgSO<sub>4</sub>, and concentrated *in vacuo* to give an oil. This material was dissolved in 30 mL of ether and the solution was treated with 3.4 mL of 1M ethereal HCl. A white solid precipitated which was collected by filtration and dried *in vacuo* to afford 0.79 g (74%) of *tert*-butyl 3-methoxycarbonyl-L-phenylalaninate·HCl, m.p. 165°C (dec).

Example 55: *tert*-Butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-L-glutam-1-yl-3-methoxycarbonyl-L-phenylalaninate

According to example 9, 0.79 g of *tert*-butyl 3-methoxycarbonyl-L-phenylalaninate·HCl was coupled with 0.84 g of *N*-benzyloxycarbonyl-L-glutamic acid  $\gamma$ -*tert*-butyl ester (Sigma) using 0.50 g of EDC (Aldrich) and 0.27 mL of *N*-methymorpholine. The crude product was purified by flash chromatography on 85 g of silica gel (70:30 hexane:EtOAc) to afford 1.38 g (93%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-L-glutam-1-yl-3-methoxycarbonyl-L-phenylalaninate as a transparent glass.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.26 (d, 1H, J=7.5, NH), 7.80 (m, 2H, NH, aromatic CH), 7.43 (d, 1H, J=7.7, aromatic CH), 7.44-7.23 (m, 7H, aromatic CH), 4.99 (m, 2H, PhCH<sub>2</sub>O), 4.38 (m, 1H, methine), 4.01 (m, 1H, methine), 3.83 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.02 (m, 2H, ArCH<sub>2</sub>), 2.19 (t, 2H, J=7.7, glu 4-CH<sub>2</sub>), 1.90-1.61 (m, 2H, glu 3-CH<sub>2</sub>), 1.38 (s, 9H, t-Bu), 1.32 (s, 9H, t-Bu).

Example 56: *tert*-Butyl 5-*O*-*tert*-L-glutam-1-yl-3-methoxycarbonyl-L-phenylalaninate

According to example 48, 1.38 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-L-glutam-1-yl-3-methoxycarbonyl-L-phenylalaninate was hydrogenolyzed using 0.23 g of

10% Pd(C) in 60 mL of MeOH. This afforded 1.0 g (94%) of *tert*-butyl 5-*O*-*tert*-L-glutam-1-yl-3-methoxycarbonyl-L-phenylalaninate as a thick, transparent oil. The product was carried on to the next step without purification. A sample of the corresponding HCl salt for  $^1\text{H}$ -NMR analysis was prepared by treating 7 mg of the product with excess 1M ethereal HCl and concentrating to dryness.

$^1\text{H}$ -NMR: (300 MHz, DMSO- $d_6$ )  $\delta$  9.12 (d, 1H,  $J=7.6$ , NH), 8.39 (br s, 3H,  $\text{NH}_3^+$ ), 7.82 (m, 2H, aromatic CH), 7.60 (d, 1H,  $J=7.5$ , aromatic CH), 7.46 (t, 1H,  $J=6.9$ , aromatic CH), 4.40 (m, 1H, methine), 3.85 (m, 1H, methine), 3.83 (s, 3H,  $\text{CO}_2\text{CH}_3$ ), 3.05 (d, 2H,  $J=8.4$ ,  $\text{ArCH}_2$ ), 2.33 (t, 2H,  $J=7.7$ , glu 4- $\text{CH}_2$ ), 1.97 (m, 2H, glu 3- $\text{CH}_2$ ), 1.37 (s, 9H, *t*-Bu), 1.31 (s, 9H, *t*-Bu).

Example 57: *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(methoxycarbonyl)-L-phenylalaninate

According to example 49, 0.200 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was coupled with 0.245 g of *tert*-butyl 5-*O*-*tert*-L-glutam-1-yl-3-methoxycarbonyl-L-phenylalaninate using 0.24 mL of DECP (Aldrich) and 0.26 mL of  $\text{Et}_3\text{N}$  in 20 mL of DMF. The crude product was purified by flash chromatography on 130 g of silica gel (12:12:1  $\text{CH}_2\text{Cl}_2$ :acetone:MeOH) to afford 0.297 g (73%) of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(methoxycarbonyl)-L-phenylalaninate as a yellow powder, m.p. 130°C (dec).

Example 58: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-carboxy-L-phenylalanine

According to example 50, 0.15 g of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-



(methoxycarbonyl)-L-phenylalaninate was treated with gaseous HCl in 20 mL of CH<sub>3</sub>NO<sub>2</sub>. After stirring the mixture at RT for 1h, the mixture was concentrated *in vacuo* to dryness to give a yellow-orange solid. Analysis of this material by <sup>1</sup>H-NMR indicated complete loss of the *tert*-butyl groups of the starting material. HPLC (C18, 75:25:0.1 H<sub>2</sub>O:MeCN:TFA) indicated a single component at k'=3.50. The intermediate methyl ester was dissolved in 25 mL of 1:1 THF:H<sub>2</sub>O, treated with 49 mg of LiOH·H<sub>2</sub>O (Baker), and the solution was stirred at RT under N<sub>2</sub> for 18h. Analysis by HPLC indicated no remaining k'=3.50 material and a single new component at k'=1.58. The solution was neutralized by addition of 1N aqueous HCl. A yellow slurry resulted which was concentrated *in vacuo* to 2 mL and diluted with 20 mL of water. The precipitate was separated by centrifugation, washed with four cycles of aqueous suspension-centrifugation-decantation, and lyophilized to afford 0.109 g (84%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-carboxy-L-phenylalanine as a yellow-orange powder, m.p. 195°C (dec).

HPLC: one peak on C18, k'=1.93, 78:22:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 13.00-12.10 (br s, COOH), 8.58 (s, 1H, pteridiny 7-CH), 8.08 (d, 1H, J=7.8, amide NH), 7.98 (d, 1H, J=8.0, amide NH), 7.80 (s, 1H, aromatic CH), 7.79-7.60 (m, 4H, NH<sub>2</sub>(1), aromatic CH), 7.49 (br s, 1H, NH<sub>2</sub>), 7.44 (d, 1H, J=7.7, aromatic CH), 7.30 (t, 1H, J=7.6, aromatic CH), 6.81 (d, 2H, J=9.0, aromatic CH), 6.63 (br s, 2H, NH<sub>2</sub>), 4.79 (s, 2H, pteridiny-CH<sub>2</sub>), 4.41 (m, 2H, methines), 3.20 (s, 3H, N-CH<sub>3</sub>), 3.10 (m, 1H, ArCH<sub>2</sub>), 2.97 (m, 1H, ArCH<sub>2</sub>), 2.23 (t, 2H, J=7.8, glu 4-CH<sub>2</sub>), 2.00-1.73 (m, 2H, glu 3-CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>30</sub>H<sub>31</sub>N<sub>9</sub>O<sub>8</sub>·1.5H<sub>2</sub>O (MW 672.65) C, 53.57; H, 5.09; N, 18.74. Found: C, 53.58; H, 5.11; N, 18.72.

Mass Spectrum: (Ion Spray) 646 (M+H)<sup>+</sup>, 437, 340, 308, 175.

UV Spectrum: (pH7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 258.7 (24300), 307.5 (25000), 372.7 (7880).  
 $\lambda_{\min}$  ( $\epsilon$ ) 244.0 (17900), 272.7 (16900), 346.2 (6440).

Example 59: *tert*-Butyl *N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)-quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(methoxycarbonyl)-L-phenylalaninate

A 100 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 0.150 g of 4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)-quinazolin-9-yl)methyl)amino)-2-fluorobenzoic acid, 0.221 g of *tert*-butyl 5-*O*-*tert*-L-glutam-1-yl-3-methoxycarbonyl-L-phenylalaninate, 65 mg of 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (Aldrich), and 24 mL of anhydrous DMF. The resulting light yellow solution was treated with 84 mg of EDC (Aldrich) and was stirred at RT under N<sub>2</sub> for 48h. The DMF was removed *in vacuo* and the residue purified by flash chromatography on 150 g of silica gel (98:2→96:4 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.255 g (78%) of *tert*-butyl *N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)-quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(methoxycarbonyl)-L-phenylalaninate as a white powder, m.p. 155-157°C.

Example 60: *N*-(4-(((1,2-Dihydro-3-methyl-1-oxobenzo(f)-quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl-3-carboxy-L-phenylalanine

According to example 50, 0.219 g of *tert*-butyl *N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)-quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(methoxycarbonyl)-L-phenylalaninate was treated with gaseous HCl in 20 mL of CH<sub>3</sub>NO<sub>2</sub>. After removal of the CH<sub>3</sub>NO<sub>2</sub> *in vacuo*, the residue was saponified with 66 mg of LiOH·H<sub>2</sub>O (Baker) in 20 mL of 1:1 THF:H<sub>2</sub>O for 18h. The solution was acidified to pH=2.5 by addition of 1N HCl and the resulting precipitate was isolated and

lyophilized in the usual manner to afford 0.162 g (84%) of *N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl-3-carboxy-L-phenylalanine as a white powder, m.p. 187°C (dec).

HPLC: one peak on C18,  $k' = 4.29$ , 75:25:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate = 1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  13.00-12.25 (br m, COOH, benzoquinazoline NH), 9.83 (s, 1H, aromatic CH), 8.32 (d, 1H,  $J = 7.4$ , amide NH), 8.22 (d, 1H,  $J = 9.0$ , aromatic CH), 8.00 (d, 1H,  $J = 8.5$ , aromatic CH), 7.81 (s, 1H, aromatic CH), 7.75 (d, 1H,  $J = 8.0$ , aromatic CH), 7.60 (m, 2H, aromatic CH), 7.49 (m, 3H, aromatic CH, 4-aminobenzoyl NH), 7.35 (m, 2H aromatic CH, amide NH), 6.52 (d, 1H,  $J = 8.8$ , aromatic CH), 6.39 (d, 1H,  $J = 15.2$ , aromatic CH), 4.57 (br s, 2H, benzoquinazoline 9-CH<sub>2</sub>), 4.45 (m, 2H, methines), 3.12 (m, 1H, ArCH<sub>2</sub>), 2.96 (m, 1H, ArCH<sub>2</sub>), 2.42 (s, 3H, CH<sub>3</sub>), 2.19 (m, 2H, glu 4-CH<sub>2</sub>), 2.01-1.70 (m, 2H, glu 3-CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>36</sub>H<sub>32</sub>N<sub>5</sub>O<sub>9</sub>F·1.7H<sub>2</sub>O (MW 728.30): C, 59.37; H, 4.90; N, 9.62. Found: C, 59.45; H, 4.93; N, 9.42.

Mass Spectrum: (Ion Spray) 698 (M+H)<sup>+</sup>.

UV Spectrum: (pH7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 265.3 (45500), 296.5 (26100), 348.0 (5530).

$\lambda_{\min}$  ( $\epsilon$ ) 244.4 (22600), 287.0 (25500), 340.1 (3540).

#### Example 61: 2-Cyclopentylbenzyl alcohol

A 300 mL Parr bomb was charged with 10.0 g of 2-iodobenzyl alcohol (Aldrich), 80 mL of toluene, 30 mL of cyclopentene (Aldrich), 2.60 g of tri-ortho-tolylphosphine (Aldrich), and 6.6 mL of Et<sub>3</sub>N. The mixture was deoxygenated by bubbling N<sub>2</sub> through for 10 min and was then treated with 0.96 g of Pd(OAc)<sub>2</sub> (Aldrich). The bomb was flushed with N<sub>2</sub>, sealed, and heated to 100°C for 18h. The vessel was cooled to RT, purged with N<sub>2</sub> and opened. The dark red reaction mixture was concentrated *in vacuo* to give a red-brown syrup. This material was dissolved in 200 mL of CH<sub>2</sub>Cl<sub>2</sub>, washed

with water (4x100 mL), dried over anhydrous  $\text{MgSO}_4$ , and concentrated *in vacuo* to afford a brown oil. Analysis by tlc ( $\text{SiO}_2$ , 8:2 hexane:EtOAc) indicated two new major components at  $R_f=0.45$  and 0.50, tri-*o*-tolylphosphine at  $R_f=0.90$ , and 4 minor components at  $R_f=0.60$ , 0.70, 0.85 and 0.0. The crude product was purified by flash chromatography (twice) on 200 g of silica gel (9:1 hexane:EtOAc) to afford 6.1 g (82%) of a mixture of the  $R_f=0.45$  and 0.50 materials. This material was determined to be a mixture of double bond isomers by  $^1\text{H-NMR}$ . The product (6.0 g) was then hydrogenated at 40 psi in 40 mL of MeOH in the presence of 0.60 g of 5% Pt(C) (Aldrich) for 4h. The catalyst was removed by filtration through celite and the filtrate was concentrated *in vacuo* to afford 5.47 g (74% from 2-iodobenzyl alcohol) of 2-cyclopentylbenzyl alcohol as a light yellow-brown liquid.

$^1\text{H-NMR}$ : (200 MHz,  $\text{DMSO-d}_6$ )  $\delta$  7.40-7.07 (m, 4H, aromatic CH), 5.06 (t, 1H,  $J=5.4$ , OH), 4.58 (d, 2H,  $J=5.4$ ,  $\text{ArCH}_2\text{O}$ ), 3.24 (m, 1H, cyclopentyl methine), 1.97 (m, 2H, cyclopentyl  $\text{CH}_2$ ), 1.89-1.42 (m, 6H, cyclopentyl  $\text{CH}_2$ ).

#### Example 62: 2-Cyclopentylbenzyl bromide

According to example 17, 5.47 g of 2-cyclopentylbenzyl alcohol was treated with 1.03 mL of  $\text{PBr}_3$  in 25 mL of anhydrous  $\text{CH}_2\text{Cl}_2$  to afford 6.9 g (93%) of 2-cyclopentylbenzyl bromide as a clear liquid.

$^1\text{H-NMR}$ : (300 MHz,  $\text{DMSO-d}_6$ )  $\delta$  7.40-7.24 (m, 3H, aromatic CH), 7.14 (m, 1H, aromatic CH), 4.78 (s, 2H,  $\text{ArCH}_2\text{Br}$ ), 3.31 (m, 1H, cyclopentyl methine), 2.05 (m, 2H, cyclopentyl  $\text{CH}_2$ ), 1.88-1.47 (m, 6H, cyclopentyl  $\text{CH}_2$ ).

**Example 63: (2R, 3S, 5S)-Benzyl 3-(3-cyclopentylbenzyl)-2-oxo-2,3-diphenyl-4-morpholinecarboxylate**

According to example 51, 1.47 g of benzyl (2R, 3S)-6-oxo-2,3-diphenyl-4-morpholinecarboxylate (Aldrich) was alkylated with 1.00 g of 2-cyclopentylbenzyl bromide using 3.99 mL of 1M sodium bis(trimethylsilyl)amide in THF (Aldrich). The crude product was purified by flash chromatography on 125 g of silica gel (8:2 hexane:EtOAc) to afford 1.07 g (52%) of (2R, 3S, 5S)-benzyl 3-(3-cyclopentylbenzyl)-2-oxo-2,3-diphenyl-4-morpholinecarboxylate as a white solid, m.p. 161-163°C.

**Example 64: 2-Cyclopentyl-L-phenylalanine**

According to example 53, 1.0 g of (2R, 3S, 5S)-benzyl 3-(3-cyclopentylbenzyl)-2-oxo-5,6-diphenyl-4-morpholinecarboxylate was hydrogenolyzed in 40 mL of 1:1 THF:MeOH using 0.23 g of PdCl<sub>2</sub> to afford 0.373 g (76%) of 2-cyclopentyl-L-phenylalanine·HCl as an off-white powder, m.p. 175°C (dec).

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 8.49 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.38-7.07 (m, 4H, aromatic CH), 3.92 (m, 1H, methine), 3.18 (m, 1H, cyclopentyl methine), 1.98 (m, 2H, cyclopentyl CH<sub>2</sub>), 1.90-1.41 (m, 6H, cyclopentyl CH<sub>2</sub>).

**Example 65: *tert*-Butyl 2-cyclopentyl-L-phenylalaninate**

According to example 54, 0.23 g of 2-cyclopentyl-L-phenylalanine·HCl was treated with 25 mL of isobutylene in 20 mL of 1,4-dioxane in the presence of 0.13 mL of conc. H<sub>2</sub>SO<sub>4</sub>. Work-up afforded a clear oil which was dissolved in 20 mL of ether. The solution was treated with 1 mL of 1 M ethereal HCl and concentrated *in vacuo* to dryness to afford 0.28 g (100%) of *tert*-butyl 2-cyclopentyl-L-phenylalaninate·HCl as a light yellow glass.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>) δ 8.59 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.28 (m, 2H, aromatic CH), 7.10 (m, 2H, aromatic CH), 3.91 (m, 1H, methine), 3.30 (m, 1H, ArCH<sub>2</sub>), 3.18 (m, 1H, cyclopentyl methine), 2.99 (dd; 1H; J=10.1, 12.4; ArCH<sub>2</sub>), 1.98 (m, 2H, cyclopentyl CH<sub>2</sub>), 1.89-1.46 (m, 6H, cyclopentyl CH<sub>2</sub>), 1.19 (s, 9H, t-Bu).

**Example 66: *tert*-Butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclopentyl-L-phenylalaninate**

According to example 9, 0.25 g of *tert*-butyl 2-cyclopentyl-L-phenylalaninate-HCl was coupled with 0.26 g of *N*-benzyloxycarbonyl-L-glutamic acid γ-*tert*-butyl ester (Sigma) using 0.16 g of EDC (Aldrich) and 0.084 mL of *N*-methyldmorpholine. The crude product was purified by flash chromatography on 85 g of silica gel (8:2 hexane:EtOAc) to afford 0.355 g (76%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclopentyl-L-phenylalaninate as a white solid, m.p. 95-96°C.

**Example 67: *tert*-Butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclopentyl-L-phenylalaninate**

According to example 48, 0.355 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclopentyl-L-phenylalaninate was hydrogenolyzed using 60 mg of 10% Pd(C) in 45 mL of MeOH. This afforded 0.236 g (85%) of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclopentyl-L-phenylalaninate as a thick, transparent oil.

**<sup>1</sup>H-NMR:** (200 MHz, DMSO-d<sub>6</sub>) δ 8.23 (m, 1H, NH), 7.34-7.00 (m, 4H, aromatic CH), 4.37 (m, 1H, methine), 3.36-2.85 (m, 5H, ArCH<sub>2</sub>, cyclopentyl methine, NH<sub>2</sub>), 2.27-1.92 (m, 4H, glu 4-CH<sub>2</sub>, glu 3-CH<sub>2</sub>), 1.90-1.46 (m, 8H, cyclopentyl CH<sub>2</sub>), 1.39 (s, 9H, t-Bu), 1.32 (s, 9H, t-Bu).

Example 68: *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclopentyl-L-phenylalaninate

According to example 49, 0.189 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-(methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was coupled with 0.236 g of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclopentyl-L-phenylalaninate using 0.23 mL of DECP (Aldrich) and 0.24 mL of Et<sub>3</sub>N in 20 mL of DMF. The crude product was purified by flash chromatography on 85 g of silica gel (12:12:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH) to afford 0.195 g (50%) of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclopentyl-L-phenylalaninate as a yellow powder, m.p. 130-140°C.

Example 69: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-phenylalanine

According to example 50, 0.167 g of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclopentyl-L-phenylalaninate was treated with gaseous HCl in 15 mL of CH<sub>3</sub>NO<sub>2</sub> for 1h. The reaction mixture was concentrated *in vacuo* to dryness to give a yellow solid. This material was suspended in ether, collected by vacuum filtration, and dried *in vacuo* to afford 0.15 g (91%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-phenylalanine as a light yellow powder, m.p. 155°C (dec).

HPLC: one peak on C18, *k'*=3.25, 65:35:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>) δ 13.25-12.95 (br s, COOH), 9.31 (s, 1H, NH<sub>2</sub>), 9.09 (s, 1H, NH<sub>2</sub>), 8.80-8.58 (br s, 1N, NH<sub>2</sub>), 8.72 (s, 1H, pteridiny 7-CH), 8.24 (d, 1H, *J*=8.0, amide NH), 8.01 (d, 1H, *J*=8.0, amide NH), 7.74 (m, 3H, NH<sub>2</sub>(1), aromatic CH), 7.22 (d, 1H, *J*=8.1, aromatic CH), 7.12 (m, 2H, aromatic CH), 6.93 (t, 1H, *J*=7.4,

aromatic CH), 6.82 (d, 2H,  $J=9.0$ , aromatic CH), 4.88 (s, 2H, pteridinyl-CH<sub>2</sub>), 4.45 (m, 1H, methine), 4.34 (m, 1H, methine), 3.26-3.10 (m, 2H, ArCH<sub>2</sub>, cyclopentyl methine), 2.88 (m, 1H, ArCH<sub>2</sub>), 2.23 (t, 2H,  $J=7.6$ , glu 4-CH<sub>2</sub>), 2.06-1.40 (m, 10H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>).

**Elemental Analysis:** Calcd. for C<sub>34</sub>H<sub>39</sub>N<sub>9</sub>O<sub>6</sub>·1.7 HCl·2.2 H<sub>2</sub>O (MW 771.36): C, 52.94; H, 5.89; N, 16.34; Cl, 7.81. Found: C, 52.57; H, 5.74; N, 16.13; Cl, 7.52.

**Mass Spectrum:** (Ion Spray) 670 (M+H)<sup>+</sup>, 613, 459, 391, 309, 275.

**UV Spectrum:** (pH7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 259.5 (24000), 308.5 (24000), 373.0 (7450).  
 $\lambda_{\min}$  ( $\epsilon$ ) 241.0 (14400), 274.2 (16200), 346.5 (5840).

#### Example 70: 3-Cyclopentylbenzyl alcohol

According to example 61, 10.0 g of 3-iodobenzyl alcohol (Aldrich) was treated with 30 mL of cyclopentene using 0.96 g of Pd(OAc)<sub>2</sub>, 2.60 g of tri-ortho-tolylphosphine, and 6.6 mL of Et<sub>3</sub>N. The resulting olefin mixture was hydrogenated for 2.5h using 0.62 g of 5% Pt(C) in MeOH to afford 5.77 g (76%) of 3-cyclopentylbenzyl alcohol as a light yellow liquid.

**<sup>1</sup>H-NMR:** (200 MHz, DMSO-d<sub>6</sub>)  $\delta$  7.32-7.17 (m, 2H, aromatic CH), 7.15 (m, 2H, aromatic CH), 5.14 (t, 1H,  $J=5.5$ , OH), 4.48 (d, 2H,  $J=5.6$ , ArCH<sub>2</sub>O), 2.96 (m, 1H, cyclopentyl methine), 2.01 (m, 2H, cyclopentyl CH<sub>2</sub>), 1.88-1.42 (m, 6H, cyclopentyl CH<sub>2</sub>).

#### Example 71: 3-Cyclopentylbenzyl bromide

According to example 17, 5.77 g of 3-cyclopentylbenzyl alcohol was treated with 1.09 mL of PBr<sub>3</sub> in 25 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub> to afford 7.85 g (100%) of 3-cyclopentylbenzyl bromide as a light yellow liquid.



<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 7.37-7.14 (m, 4H, aromatic CH), 4.68 (s, 2H, ArCH<sub>2</sub>Br), 2.96 (m, 1H, cyclopentyl methine), 2.00 (m, 2H, cyclopentyl CH<sub>2</sub>), 1.84-1.42 (m, 6H, cyclopentyl CH<sub>2</sub>).

Example 72: (2R, 3S, 5S)-Benzyl 3-(3-cyclopentylbenzyl)-2-oxo-2,3-diphenyl-4-morpholinecarboxylate

A dry 500 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer was charged with 1.47 g of benzyl (2R, 3S)-6-oxo-2,3-diphenyl-4-morpholinecarboxylate (Aldrich), 1.00 g of 3-cyclopentylbenzyl bromide, and 80 mL of anhydrous THF. The mixture was gently heated with stirring to dissolve the solid starting material and the resulting solution was cooled to -78°C. The solution was treated with 4.0 mL of 1M sodium bis(trimethylsilyl)amide in THF (Aldrich) by slow addition via syringe through a rubber septum. After stirring at -78°C for 4h, the solution was mixed with 80 mL of water and worked-up according to example 51. The crude product was purified by flash chromatography on 85 g of silica gel (85:15 hexane:EtOAc) to afford 1.44 g (70%) of (2R, 3S, 5S)-Benzyl 3-(3-cyclopentylbenzyl)-2-oxo-2,3-diphenyl-4-morpholinecarboxylate as a white powder, m.p. 82-84 °C.

Example 73: 3-Cyclopentyl-L-phenylalanine

According to example 53, 1.37 g of (2R, 3S, 5S)-benzyl 3-(3-cyclopentylbenzyl)-2-oxo-5,6-diphenyl-4-morpholinecarboxylate was hydrogenolyzed in 80 mL of 1:1 THF:MeOH using 0.31 g of PdCl<sub>2</sub> to afford 0.63 g (93%) of 3-cyclopentyl-L-phenylalanine·HCl as a white powder, m.p. >200°C.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 8.39 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.31-7.12 (m, 3H, aromatic CH), 7.08 (d, 1H, J=7.3, aromatic CH), 4.17 (m, 1H, methine), 3.11 (d, 2H, J=6.1,

ArCH<sub>2</sub>), 2.96 (m, 1H, cyclopentyl methine), 1.99 (m, 2H, cyclopentyl CH<sub>2</sub>), 1.88-1.45 (m, 6H, cyclopentyl CH<sub>2</sub>).

Example 74: *tert*-Butyl 3-cyclopentyl-L-phenylalaninate

According to example 54, 0.50 g of 3-cyclopentyl-L-phenylalanine-HCl was treated with 25 mL of isobutylene in 20 mL of 1,4-dioxane in the presence of 0.3 mL of conc. H<sub>2</sub>SO<sub>4</sub>. Work-up followed by acidification of the free amine with ethereal HCl afforded 0.49 g (82%) of *tert*-butyl 3-cyclopentyl-L-phenylalaninate-HCl as a white powder, m.p. 154-156°C.

Example 75: *tert*-Butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate

According to example 9, 0.49 g of *tert*-butyl 3-cyclopentyl-L-phenylalaninate-HCl was coupled with 0.51 g of *N*-Cbz-L-glutamic acid  $\gamma$ -*tert*-butyl ester (Sigma) using 0.30 g of EDC (Aldrich) and 0.16 mL of *N*-methylmorpholine. The crude product was purified by flash chromatography on 80 g of silica gel (8:2 hexane:EtOAc) to afford 0.75 g (82%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate as a thick transparent oil.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.22 (d, 1H, J=7.3, NH), 7.33 (m, 6H, aromatic CH), 7.20-6.97 (m, 3H, aromatic CH, NH), 5.01 (m, 2H, PhCH<sub>2</sub>O), 4.33 (m, 1H, methine), 4.05 (m, 1H, methine), 2.91 (m, 3H, ArCH<sub>2</sub>, cyclopentyl methine), 2.20 (t, 2H, J=7.7, glu 4-CH<sub>2</sub>), 1.97 (m, 2H, glu 3-CH<sub>2</sub>), 1.88-1.43 (m, 8H, cyclopentyl CH<sub>2</sub>), 1.38 (s, 9H, t-Bu), 1.31 (s, 9H, t-Bu).

**Example 76: *tert*-Butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate**

According to example 48, 0.75 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate was hydrogenolyzed using 0.12 g of 10% Pd(C) in 60 mL of MeOH. This afforded 0.58 g (99%) of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate as a transparent oil. A sample of the corresponding HCl salt for <sup>1</sup>H-NMR analysis was prepared by treating 10 mg of the product with excess 1N ethereal HCl and concentrating to dryness.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.99 (d, 1H, J=7.0, NH). 8.32 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.24-7.01 (m, 4H, aromatic CH), 4.38 (m, 1H, methine), 3.85 (m, 1H, methine), 2.93 (m, 3H, ArCH<sub>2</sub>, cyclopentyl methine), 2.34 (t, 2H, J=7.7, glu 4-CH<sub>2</sub>), 1.96 (m, 4H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.82-1.44 (m, 6H, cyclopentyl CH<sub>2</sub>), 1.40 (s, 9H, t-Bu), 1.31 (s, 9H, t-Bu).

**Example 77: *tert*-Butyl 5-*O*-*tert*-butyl-*N*-(4-(((2,4-diamino-6-**

pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate

According to example 49, 0.200 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was coupled with 0.25 g of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate using 0.24 mL of DECP (Aldrich) and 0.26 mL of Et<sub>3</sub>N in 20 mL of DMF. The crude product was purified by flash chromatography on 150 g of silica gel (7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH) to afford 0.195 g (47%) of *tert*-butyl 5-*O*-*tert*-butyl-*N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate as a yellow powder, m.p. 115-120°C.

Example 78: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine

According to example 50, 0.18 g of *tert*-butyl 5-*O-tert*-butyl-*N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate was treated with gaseous HCl in 20 mL of CH<sub>3</sub>NO<sub>2</sub> for 1.25h. Work-up and isolation in the usual manner afforded 0.135 g (84%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine as a yellow-orange powder, m.p. 170°C (dec).

HPLC: one peak on C18, *k'*=2.86, 65:35:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>) δ 12.90-11.85 (br m, COOH), 8.58 (s, 1H, pteridiny 7-CH), 8.04 (d, 1H, *J*=7.7, amide NH), 8.00 (d, 1H, *J*=8.1, amide NH), 7.72 (br s, 1H, NH<sub>2</sub>), 7.71 (d, 2H, *J*=8.8, aromatic CH), 7.53 (br s, 1H, NH<sub>2</sub>), 7.08-6.93 (m, 4H, aromatic CH), 6.81 (d, 2H, *J*=8.9, aromatic CH), 6.69 (br s, 2N, NH<sub>2</sub>), 4.79 (s, 2H, pteridiny-CH<sub>2</sub>), 4.40 (m, 2H, methines), 3.22 (s, 3H, N-CH<sub>3</sub>), 3.01 (m, 1H, ArCH<sub>2</sub>), 2.94-2.75 (m, 2H, ArCH<sub>2</sub>, cyclopentyl methine), 2.24 (t, 2H, *J*=7.7, glu 4-CH<sub>2</sub>), 2.03-1.76 (m, 4H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.73-1.35 (m, 6H, cyclopentyl CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>34</sub>H<sub>39</sub>N<sub>9</sub>O<sub>6</sub>·1.6 H<sub>2</sub>O (MW 698.56): C, 58.46; H, 6.09; N, 18.05. Found: C, 58.50; H, 6.08; N, 18.10.

Mass Spectrum: (FAB) 670 (M+H)<sup>+</sup>, 613, 437, 309, 275.

UV Spectrum: (pH7 Buffer) λ<sub>max</sub> (ε) 259.7 (23300), 308.3 (23500), 371.7 (7030). λ<sub>min</sub> (ε) 241.0 (13500), 274.8 (15300), 346.1 (5260).

Example 79: 2-Cyclohexylbenzyl alcohol

According to example 61, 10.0 g of 2-iodobenzyl alcohol was treated with 25 mL of cyclohexene (Aldrich) using 0.96 g of Pd(OAc)<sub>2</sub>, 2.60 g of tri-*ortho*-tolylphosphine, and 6.6 mL of Et<sub>3</sub>N at 110°C for 48h. The resulting olefin mixture was hydrogenated for

6.5h using 0.36 g of 5% Pt(C) in MeOH to afford 3.37 g (42%) of 2-cyclohexyl benzyl alcohol as a light yellow liquid.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>) δ 7.33 (d, 1H, J=7.4, aromatic CH), 7.27-7.08 (m, 3H, aromatic CH), 5.03 (t, 1H, J=5.4, OH), 4.34 (d, 2H, J=5.4, ArCH<sub>2</sub>O), 2.76 (m, 1H, cyclohexyl methine), 1.85-1.61 (m, 5H, cyclohexyl CH<sub>2</sub>), 1.48-1.22 (m, 5H, cyclohexyl CH<sub>2</sub>).

**Example 80: 2-Cyclohexylbenzyl bromide**

According to example 17, 3.36 g of 2-cyclohexylbenzyl alcohol was treated with 0.59 mL of PBr<sub>3</sub> in 25 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub> to afford 3.90 g of crude product (90% pure by <sup>1</sup>H-NMR). This material was vacuum distilled via short path (1.0 mmHg, 117-120°C) to afford 3.54 g (79%) of 2-cyclohexylbenzyl bromide as a clear liquid.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>) δ 7.39 (d, 1H, J=5.7, aromatic CH), 7.29 (m, 2H, aromatic CH), 7.13 (m, 1H, aromatic CH), 4.76 (s, 2H, ArCH<sub>2</sub>Br), 2.86 (m, 1H, cyclohexyl methine), 1.83-1.63 (m, 5H, cyclohexyl CH<sub>2</sub>), 1.53-1.20 (m, 5H, cyclohexyl CH<sub>2</sub>).

**Example 81: (2R, 3S, 5S)-tert-Butyl 3-(2-cyclohexylbenzyl)-2-oxo-2,3-diphenyl-4-morpholinecarboxylate**

To a dry 250 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was added a solution of 1.47 g of *tert*-butyl (2R, 3S)-(-)-6-oxo-2,3-diphenyl-4-morpholinecarboxylate (Aldrich) in 30 mL of THF and a solution of 1.00 g of 2-cyclohexylbenzyl bromide in 8 mL of THF. Both solutions had been dried over 3 Å molecular sieves for 24h. Anhydrous THF was used for rinse (30 mL) to give a total solution volume of 68 mL. The resulting solution was cooled to -78°C and was treated with 4.15 mL of 1M sodium bis-(trimethylsilyl) amide in THF (Aldrich) by slow addition

via syringe through a rubber septum. After stirring at  $-78^{\circ}\text{C}$  for 3.5h, the reaction mixture was mixed with 80 mL of water and subjected to rotary evaporation to remove THF. The resulting mixture was extracted with EtOAc (4x50 mL). The combined EtOAc extracts were washed with saturated aqueous NaCl (2x70 mL), dried over anhydrous  $\text{MgSO}_4$  and concentrated *in vacuo* to afford a white solid. Purification of this material by flash chromatography on 200 g of silica gel (8:2 hexane:EtOAc) afforded 1.2 g (58%) of (2*R*, 3*S*, 5*S*)-*tert*-butyl 3-(2-cyclohexylbenzyl)-2-oxo-2,3-diphenyl-4-morpholinecarboxylate as a white, crystalline solid, m.p.  $195\text{--}198^{\circ}\text{C}$ .

Example 82: (2*R*, 3*S*, 5*S*)-6-Oxo-2,3-diphenyl-5-(2-cyclohexylbenzyl)morpholine

A dry 50 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer was charged with 2.10 g of (2*R*, 3*S*, 5*S*)-*tert*-butyl 3-(2-cyclohexylbenzyl)-2-oxo-5,6-diphenyl-4-morpholinecarboxylate and 33 mL of anhydrous  $\text{CH}_2\text{Cl}_2$ . The solution was stirred at RT and treated with 3.3 mL of TFA. After 3.5h, tlc ( $\text{SiO}_2$ , 85:15 hexane:EtOAc) indicated no remaining starting material, a major new component at  $R_f=0.45$ , and two minor components at  $R_f=0.68$  and 0.70. The solution was neutralized by addition of 9 mL of  $\text{Et}_3\text{N}$  and was concentrated *in vacuo* to give a thick oil. This material was dissolved in 80 mL of  $\text{CH}_2\text{Cl}_2$ , washed with water (3x60 mL) followed by 5% aqueous  $\text{NaHCO}_3$  (3x60 mL) and dried over anhydrous  $\text{MgSO}_4$ . Drying agent was removed by filtration and the filtrate concentrated *in vacuo* to afford a white solid. Purification of the crude product by flash chromatography on 150 g of silica gel (8:2 hexane:EtOAc) followed by recrystallization from EtOH- $\text{H}_2\text{O}$  afforded 1.27 g (75%) of (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(2-cyclohexylbenzyl)morpholine as a white crystalline solid, m.p.  $159\text{--}160^{\circ}\text{C}$ .

**Example 83: 2-Cyclohexyl-L-phenylalanine**

According to example 53, 1.17 g of (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(2-cyclohexylbenzyl)morpholine was hydrogenolyzed in 60 mL of 1:1 THF:EtOH using 0.34 g of PdCl<sub>2</sub> to afford 0.83 g of 2-cyclohexyl-L-phenylalanine·HCl as a fluffy white solid, m.p. 140°C (dec).

**Example 84: *tert*-Butyl 2-cyclohexyl-L-phenylalaninate**

According to example 54, 0.73 g of 2-cyclohexyl-L-phenylalanine·HCl was treated with 25 mL of isobutylene in 25 mL of 1,4-dioxane in the presence of 0.4 mL of conc. H<sub>2</sub>SO<sub>4</sub>. Work-up followed by acidification of the free amine with ethereal HCl afforded 0.70 g (80%) of *tert*-butyl 2-cyclohexyl-L-phenylalaninate·HCl as a yellow foam.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.53 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.27 (m, 2H, aromatic CH), 7.10 (m, 2H, aromatic CH), 3.86 (m, 1H, methine), 3.24 (m, 1H, ArCH<sub>2</sub>), 2.97 (m, 1H, ArCH<sub>2</sub>), 2.72 (m, 1H, cyclohexyl methine), 1.86-1.60 (m, 5H, cyclohexyl CH<sub>2</sub>), 1.56-1.30 (m, 5H, cyclohexyl CH<sub>2</sub>), 1.19 (s, 9H, *t*-Bu).

**Example 85: *tert*-Butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclohexyl-L-phenylalaninate**

According to example 9, 0.70 g of *tert*-butyl 2-cyclohexyl-L-phenylalaninate·HCl was coupled with 0.70 g of *N*-Cbz-L-glutamic acid-γ-*tert*-butyl ester (Sigma) using 0.42 g of EDC (Aldrich) and 0.23 mL of *N*-methylmorpholine. The crude product was purified by flash chromatography on 150 g of silica gel (8:2 hexane:EtOAc) to afford 0.97 g (76%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclohexyl-L-phenylalaninate as a white foam.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.32 (d, 1H, J=7.6, NH), 7.40-6.97 (m, 10H, aromatic CH, NH), 5.00 (m, 2H, PhCH<sub>2</sub>O), 4.26 (m, 1H, methine), 4.04 (m, 1H,

methine), 3.07 (m, 1H, ArCH<sub>2</sub>), 2.93-2.70 (m, 2H, ArCH<sub>2</sub>, cyclohexyl methine), 2.19 (t, 2H, J=7.7, glu 4-CH<sub>2</sub>), 1.90-1.62 (m, 7H, glu 3-CH<sub>2</sub>, cyclohexyl CH<sub>2</sub>), 1.56-1.33 (m, 5H, cyclohexyl CH<sub>2</sub>), 1.38 (s, 9H, t-Bu), 1.32 (s, 9H, t-Bu).

Mass Spectrum: (CI, CH<sub>4</sub>) 623 (M+H)<sup>+</sup>, 567, 539, 511, 489, 433, 193.

Example 86: *tert*-Butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclohexyl-L-phenylalaninate

According to example 48, 0.97 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclohexyl-L-phenylalaninate was hydrogenolyzed using 0.16 g of 10% Pd(C) in 50 mL of MeOH. This afforded 0.76 g (100%) of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclohexyl-L-phenylalaninate as a thick transparent oil. A sample of the corresponding HCl salt for <sup>1</sup>H-NMR analysis was prepared by treating 10 mg of the product with excess 1M ethereal HCl and concentrating to dryness.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 9.05 (d, 1H, J=7.5, NH), 8.24 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.20 (m, 3H, aromatic CH), 7.07 (t, 1H, J=7.1, aromatic CH), 4.28 (m, 1H, methine), 3.83 (m, 1H, methine), 3.08 (m, 1H, ArCH<sub>2</sub>), 2.91 (m, 1H, ArCH<sub>2</sub>), 1.78 (m, 1H, cyclohexyl methine), 2.34 (m, 2H, glu 4-CH<sub>2</sub>), 2.00 (m, 2H, glu 3-CH<sub>2</sub>), 1.89-1.60 (m, 5H, cyclohexyl CH<sub>2</sub>), 1.57-1.20 (m, 5H, cyclohexyl CH<sub>2</sub>), 1.40 (s, 9H, t-Bu), 1.33 (s, 9H, t-Bu).

Example 87: *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclohexyl-L-phenylalaninate

According to example 49, 0.247 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was coupled with 0.35 g of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-2-cyclohexyl-L-phenylalaninate using 0.30 mL of DECP (Aldrich) and 0.32 mL of Et<sub>3</sub>N in 25 mL of DMF. The crude product was subjected to flash chromatography twice (150 g SiO<sub>2</sub>, 7:7:1



CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH; 150 g SiO<sub>2</sub>, 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.297 g (57%) of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-5-*O-tert*-butyl-L-glutam-1-yl-2-cyclohexyl-L-phenylalaninate as a yellow powder, m.p. 135-140°C.

Example 88: *N*-(4-(((2,4-Diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-cyclohexyl-L-phenylalanine

According to example 50, 0.271 g of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-5-*O-tert*-butyl-L-glutam-1-yl-2-cyclohexyl-L-phenylalaninate was treated with gaseous HCl in 20 mL of CH<sub>3</sub>NO<sub>2</sub> for 1.5 h. Work-up and isolation in the usual manner afforded 0.191 g (79%) of *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-cyclohexyl-L-phenylalanine as a yellow-orange powder, m.p. 175°C (dec).

HPLC: one peak on C18, *k'*=4.05, 65:35:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 12.75-11.90 (br s, COOH), 8.58 (s, 1H pteridiny 7-CH), 8.20 (d, 1H, *J*=7.9, amide NH), 7.98 (d, 1H, *J*=8.1, amide NH), 7.79 (br s, 1H, NH<sub>2</sub>), 7.72 (d, 2H, *J*=8.9, aromatic CH), 7.59 (br s, 1H, NH<sub>2</sub>), 7.19 (m, 1H, aromatic CH), 7.08 (m, 2H, aromatic CH), 6.92 (m, 1H, aromatic CH), 6.83 (d, 2H, *J*=8.9, aromatic CH), 6.75 (br s, 2H, NH<sub>2</sub>), 4.80 (s, 2H, pteridiny-CH<sub>2</sub>), 4.43 (m, 1H, methine), 4.31 (m, 1H, methine), 3.21 (s, 3H, N-CH<sub>3</sub>), 3.12 (m, 1H, ArCH<sub>2</sub>), 3.92-3.70 (m, 2H, ArCH<sub>2</sub>, cyclohexyl methine), 2.23 (t, 2H, *J*=7.7, glu 4-CH<sub>2</sub>), 2.05-1.60 (m, 7H, glu 3-CH<sub>2</sub>, cyclohexyl CH<sub>2</sub>), 1.50-1.16 (m, 5H, cyclohexyl CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>35</sub>H<sub>41</sub>N<sub>9</sub>O<sub>6</sub>·1.5 H<sub>2</sub>O (MW 710.79): C, 59.14; H, 6.24; N, 17.74. Found: C, 59.11; H, 6.20; N, 17.73.

Mass Spectrum: (FAB) 684 (M+H)<sup>+</sup>, 613, 510, 385, 309.

UV Spectrum: (pH7 Buffer) λ<sub>max</sub> (ε) 259.7 (22300), 308.8 (22700), 374.0 (6850). λ<sub>min</sub> (ε) 241.7 (12800), 275.2 (14700), 346.3 (5170).

**Example 89: 3-*tert*-Butylbenzyl bromide**

A dry 100 mL 3-necked flask equipped with a nitrogen inlet, a condenser, a thermometer, and a magnetic stirrer was charged with 2.0 g of 3-*tert*-butyltoluene (Wiley Organics, Coshocton, OH 43812), 2.40 g of *N*-bromosuccinimide (Aldrich), and 30 mL of CCl<sub>4</sub>. The mixture was treated with 0.16 g of benzoyl peroxide (Aldrich) and heated at 60°C under N<sub>2</sub>. Careful temperature control is important to minimize by-product formation. After 1 h at 60°C the reaction mixture was cooled to 0°C, filtered to remove succinimide, and the filtrate was concentrated *in vacuo* to afford a light yellow liquid.

Analysis of this material by tlc (SiO<sub>2</sub>, hexane) indicated a major new component at R<sub>f</sub>=0.50, a minor component at R<sub>f</sub>=0.55, and a trace of 3-*tert*-butyltoluene at R<sub>f</sub>=0.75.

The crude product was purified by flash chromatography on 100 g of silica gel (hexane) to afford 1.06 g (35%) of 3-*tert*-butylbenzyl bromide as a clear liquid.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 7.47 (s, 1H, aromatic CH), 7.39-7.21 (m, 3H, aromatic CH), 4.69 (s, 2H, ArCH<sub>2</sub>Br), 1.27 (s, 9H, t-Bu).

**Example 90: (2*R*, 3*S*, 5*S*)-*tert*-Butyl 3-(3-*tert*-butylbenzyl)-2-oxo-2,3-diphenyl-4-morpholinecarboxylate**

According to example 81, 1.63 g of *tert*-butyl (2*R*, 3*S*)-(-)-6-oxo-2,3-diphenyl-4-morpholinecarboxylate (Aldrich) was alkylated with 1.0 g of 3-*tert*-butylbenzyl bromide in 70 mL of anhydrous THF using 4.62 mL of 1M sodium bis(trimethylsilyl)amide in THF (Aldrich). The crude product was purified by flash chromatography on 150 g of silica gel (9:1 hexane: EtOAc) to afford 1.84 g (80%) of (2*R*, 3*S*, 5*S*)-*tert*-butyl 3-(3-*tert*-butylbenzyl)-2-oxo-2,3-diphenyl-4-morpholinecarboxylate as a white foam, m.p 65-90°C.

Example 91: (2*R*, 3*S*, 5*S*)-6-Oxo-2,3-diphenyl-5-(3-*tert*-butylbenzyl)morpholine

According to example 82, 1.79 g of (2*R*, 3*S*, 5*S*)-*tert*-butyl 3-(3-*tert*-butylbenzyl)-2-oxo-5,6-diphenyl-4-morpholinecarboxylate was treated with 3 mL of TFA in 30 mL of CH<sub>2</sub>Cl<sub>2</sub> for 3.4h. Neutralization with 7.7 mL of Et<sub>3</sub>N and work-up in the usual manner gave a thick oil. Purification of the crude material by flash chromatography on 125 g of silica gel (75:25 hexane:EtOAc) afforded 0.90 g (63%) of (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-*tert*-butylbenzyl)morpholine as a white crystalline solid, m.p. 136-137°C.

Example 92: 3-*tert*-Butyl-L-phenylalanine

According to example 53, 0.89 g of (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-*tert*-butylbenzyl)morpholine was hydrogenolyzed in 40 mL of 1:1 THF:EtOH using 0.28 g of PdCl<sub>2</sub> to afford 0.57 g (99%) of 3-*tert*-butyl-L-phenylalanine·HCl as a white powder, m.p. >250°C.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.42 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.26 (m, 3H, aromatic CH), 7.08 (dd; 1H; J=7.0, 1.3; aromatic CH), 4.12 (m, 1H, methine), 3.12 (d, 2H, J=6.1, ArCH<sub>2</sub>), 1.27 (s, 9H, t-Bu).

Example 93: *tert*-Butyl 3-*tert*-butyl-L-phenylalaninate

According to example 54, 0.50 g of 3-*tert*-butyl-L-phenylalanine·HCl was treated with 30 mL of isobutylene in 25 mL of 1,4-dioxane in the presence of 0.4 mL of conc. H<sub>2</sub>SO<sub>4</sub>. Work-up in the usual manner followed by acidification of the free amine with ethereal HCl afforded 0.53 g (87%) of *tert*-butyl 3-*tert*-butyl-L-phenylalaninate·HCl as a light yellow foam.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.48 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.36-7.20 (m, 3H, aromatic CH), 7.08 (d, 1H, J=7.1, aromatic CH), 4.16 (m, 1H, methine), 3.19 (dd; 1H; J=13.9, 5.7; ArCH<sub>2</sub>), 2.95 (dd; 1H; J=13.9, 8.6; ArCH<sub>2</sub>), 1.28 (s, 9H, t-Bu), 1.27 (s, 9H, t-Bu).

Example 94: *tert*-Butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-phenylalaninate

According to example 9, 0.52 g of *tert*-butyl 3-*tert*-butyl-L-phenylalaninate·HCl was coupled with 0.56 g of *N*-Cbz-L-glutamic acid γ-*tert*-butyl ester (Sigma) using 0.33 g of EDC (Aldrich) and 0.18 mL of *N*-methymorpholine. The crude product was purified by flash chromatography on 110 g of silica gel (75:25 hexane:EtOAc) to afford 0.77 g (78%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-phenylalaninate as a thick transparent oil.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 8.28 (d, 1H, J=7.3, NH), 7.42-7.13 (m, 9H, aromatic CH, NH), 7.04 (d, 1H, J=6.6, aromatic CH), 5.02 (s, 2H, PhCH<sub>2</sub>O), 4.37 (m, 1H, methine), 4.07 (m, 1H, methine), 2.96 (d, 2H, J=7.6, ArCH<sub>2</sub>), 2.21 (t, 2N, J=7.7, glu 4-CH<sub>2</sub>), 1.97-1.60 (m, 2H, glu 3-CH<sub>2</sub>), 1.39 (s, 9H, t-Bu), 1.31 (s, 9H, t-Bu), 1.27 (s, 9H, t-Bu).

Example 95: *tert*-Butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-phenylalaninate

According to example 48, 0.76 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-phenylalaninate was hydrogenolyzed using 0.13 g of 10% Pd(C) in 60 mL of MeOH. This afforded 0.58 g (99%) of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-phenylalaninate as a thick transparent oil. A sample of the corresponding HCl salt for <sup>1</sup>H-NMR analysis was prepared by treating 10 mg of the product with excess 1N ethereal HCl and concentrating to dryness.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.97 (d, 1H, J=7.0, NH), 8.27 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.31-7.15 (m, 3H, aromatic CH), 7.06 (d, 1H, J=6.8, aromatic CH), 4.38 (m, 1H, methine), 3.82 (m, 1H, methine), 2.96 (d, 2H, J=7.2, ArCH<sub>2</sub>), 2.33 (m, 2H, glu 4-CH<sub>2</sub>), 1.98 (m, 2H, glu 3-CH<sub>2</sub>), 1.38 (s, 9H, t-Bu), 1.29 (s, 9H, t-Bu), 1.26 (s, 9H, t-Bu).

Example 96: *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-phenylalaninate

According to example 49, 0.223 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was coupled with 0.30 g of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-phenylalaninate using 0.27 mL of DECP (Aldrich) and 0.29 mL of Et<sub>3</sub>N in 25 mL of DMF. The crude product was subjected to flash chromatography twice (180 g SiO<sub>2</sub>, 7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH; 185 g SiO<sub>2</sub>, 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.323 g (71%) of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-phenylalaninate as a yellow powder, m.p. 128-135°C.

Example 97: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-*tert*-butyl-L-phenylalanine (Procedure A)

For Procedure B to prepare this compound, see Example 130.

According to example 50, 0.303 g of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-phenylalaninate was treated with gaseous HCl in 40 mL of CH<sub>3</sub>NO<sub>2</sub> for 1.5 h. Work-up and isolation in the usual manner afforded 0.215 g (79%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-*tert*-butyl-L-phenylalanine as a yellow powder, m.p. 183°C.

HPLC: one peak on C18, k'<sup>1</sup>=2.54, 70:30:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 12.90-11.80 (br s, COOH), 8.59 (s, 1H, pteridinyl 7-CH), 8.08 (d, 1H, J=7.6, amide NH), 7.98 (d, 1H, J=8.0, amide NH), 7.89 (br s, 1H, NH<sub>2</sub>), 7.70 (d, 2H, J=8.9, aromatic CH), 7.69 (br s, 1H, NH<sub>2</sub>), 7.21 (s, 1H, aromatic CH), 7.16 (d, 1H, J=7.7, aromatic CH), 7.08 (t, 1H, J=7.6, aromatic CH), 6.99 (d, 1H, J=7.4, aromatic CH), 6.81 (m, 4H, aromatic CH, NH<sub>2</sub>), 4.79 (s, 2H, pteridinyl CH<sub>2</sub>), 4.43 (m, 2H, methines), 3.21 (s, 3H, N-CH<sub>3</sub>), 3.03 (m, 1H, ArCH<sub>2</sub>), 2.91 (m, 1H, ArCH<sub>2</sub>), 2.24 (t, 2H, J=7.8, glu 4-CH<sub>2</sub>), 2.04-1.74 (m, 2H, glu 3-CH<sub>2</sub>), 1.20 (s, 9H, t-Bu).

Elemental Analysis: Calcd. for C<sub>33</sub>H<sub>39</sub>N<sub>9</sub>O<sub>6</sub>·1.8 H<sub>2</sub>O (MW 690.16): C, 57.43; H, 6.22; N, 18.27. Found: C, 57.34; H, 6.07; N, 18.26.

Mass Spectrum: (FAB) 658 (M+H)<sup>+</sup>, 459, 307.

UV Spectrum: (pH7 Buffer) λ<sub>max</sub> (ε) 259.4 (22800), 308.1 (23000), 372.5 (7040). λ<sub>min</sub> (ε) 241.0 (13400), 274.6 (15200), 345.2 (5380).

Example 98: *N*-((*tert*-Butoxy)carbonyl)-4-(bis(2-chloroethyl)amino)-L-phenylalanine

A dry 50 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 0.10 g of 4'-(bis(2-chloroethyl)amino)-L-phenylalanine·H<sub>2</sub>O (Sigma), 14 mL of 1:1 DMF:CH<sub>3</sub>CN, and 0.19 mL of Et<sub>3</sub>N. The mixture was treated with 91 mg of 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON, Fluka) and stirred at RT under N<sub>2</sub>. After 18h, the solid starting material had dissolved giving a light yellow solution which was concentrated *in vacuo* to dryness. Analysis of the residue by tlc (SiO<sub>2</sub>, 9:1 CHCl<sub>3</sub>:MeOH) indicated a major new component at R<sub>f</sub>=0.65, BOC-ON derived components at R<sub>f</sub>=1.0 and 0.71 and two trace components at R<sub>f</sub>=0.60 and 0.35. The crude product was purified by flash chromatography on 60 g of silica gel (9:1 CHCl<sub>3</sub>:MeOH) to afford 0.10 g (80%) of *N*-((*tert*-butoxy)carbonyl)-4-(bis(2-chloroethyl)amino)-L-phenylalanine as light yellow glass.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 7.04 (d, 2H, J=8.6, aromatic CH), 6.62 (d, 2H, J=8.5, aromatic CH), 6.46 (br s, 1H, NH), 3.87 (m, 1H, methine), 3.67 (s, 8H, N (CH<sub>2</sub>CH<sub>2</sub>Cl)<sub>2</sub>), 2.90 (m, 1H, ArCH<sub>2</sub>), 2.73 (m, 1H, ArCH<sub>2</sub>), 1.33 (s, 9H, t-Bu).

Example 99: *tert*-Butyl *N*-((*tert*-butoxy)carbonyl)-4-(bis(2-chloroethyl)amino)-L-phenylalanyl-(3-cyclopentyl)-L-phenylalaninate

A 50 mL round bottomed flask equipped with a magnetic stirrer was charged with 96 mg of *N*-((*tert*-butoxy)carbonyl)-4-(bis(2-chloroethyl)amino)-L-phenylalanine 77 mg of *tert*-butyl 3-cyclopentyl-L-phenylalaninate-HCl, and 10 mL of CH<sub>2</sub>Cl<sub>2</sub>. The solution was cooled to 0°C and treated with 48 mg of EDC (Aldrich) followed by 0.026 mL of *N*-methylmorpholine. The reaction mixture was stirred at 0°C for 20 min, allowed to warm to RT, and stirred for an additional 2h. Analysis of the solution by tlc (SiO<sub>2</sub>, 7:3 hexane:EtOAc) indicated a major new component at R<sub>f</sub>=0.54 and four minor components at R<sub>f</sub>=0.4, 0.37, 0.16 and 0.0. The solution was concentrated *in vacuo* to dryness and the residue was subjected to flash chromatography on 50 g of silica gel to afford 73 mg (46%) of *tert*-butyl *N*-((*tert*-butoxy)carbonyl)-4-(bis(2-chloroethyl)amino)-L-phenylalanyl-(3-cyclopentyl)-L-phenylalaninate as a thick semi-solid.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.18 (d, 1H, J=7.8, NH), 7.19 (m, 1H, aromatic CH), 7.13-7.00 (m, 5H, aromatic CH, NH), 6.77 (d, 1H, J=8.8, aromatic CH), 6.63 (d, 2H, J=8.9, aromatic CH), 4.35 (m, 1H, methine), 4.05 (m, 1H, methine), 3.68 (s, 8H, N (CH<sub>2</sub>CH<sub>2</sub>Cl)<sub>2</sub>), 2.93 (m, 3H, ArCH<sub>2</sub>, cyclopentyl methine), 2.79 (m, 1H, ArCH<sub>2</sub>), 2.58 (m, 1H, ArCH<sub>2</sub>), 1.80-1.43 (m, 8H, cyclopentyl CH<sub>2</sub>), 1.29 (s, 18H, t-Bu).

Example 100: 4-(Bis(2-chloroethyl)amino)-L-phenylalanyl-3-cyclopentyl-L-phenylalanine

A 25 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer was charged with 65 mg of *tert*-butyl *N*-((*tert*-butoxy)carbonyl)-4-(bis(2-chloroethyl)amino)-L-phenylalanyl-(3-cyclopentyl)-L-phenylalaninate and 8 mL of CH<sub>3</sub>NO<sub>2</sub>. The solution was acidified by bubbling HCl gas through for 10 min. After stirring at RT under N<sub>2</sub> for 30 min, the solution was concentrated *in vacuo* to dryness. Analysis of the resulting solid by HPLC (C18, 50:50:0.1 H<sub>2</sub>O:MeCN:TFA) indicated a major component at *k'*=1.14 (96%) and a minor component at *k'*=1.60 (4%). The crude product was purified by semi-preparative HPLC (C18, 55:45:0.1→50:50:0.1 H<sub>2</sub>O:MeCN:TFA over 20 min, flow rate=15 mL/min). Fractions containing pure product (*k'*=1.14 by analytical HPLC) were combined and concentrated *in vacuo* to 20 mL. The resulting suspension was frozen and lyophilized to afford 35 mg (55%) of 4-(bis(2-chloroethyl)amino)-L-phenylalanyl-3-cyclopentyl-L-phenylalanine as a white powder, m.p. 80-90°C.

HPLC: one peak on C18, *k'*=1.05, 50:50:0.1 MeCN:H<sub>2</sub>O:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>) δ 8.85 (d, 1H, *J*=7.8, NH), 8.00 br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.25-7.00 (m, 6H, aromatic CH), 6.70 (d, 2H, *J*=8.5, aromatic CH), 4.50 (m, 1H, methine), 3.89 (m, 1H, methine), 3.69 (s, 8H, N (CH<sub>2</sub>CH<sub>2</sub>Cl)<sub>2</sub>), 3.12-2.83 (m, 4H, ArCH<sub>2</sub>, cyclopentyl methine), 2.77 (m, 1H, ArCH<sub>2</sub>), 1.97 (m, 2H, cyclopentyl CH<sub>2</sub>), 1.84-1.43 (m, 6H, cyclopentyl CH<sub>2</sub>).

<sup>19</sup>F-NMR: (282 MHz, DMSO-*d*<sub>6</sub>): d 1.26 (relative to TFA external standard).

Elemental Analysis: Calcd. for C<sub>27</sub>H<sub>35</sub>N<sub>3</sub>O<sub>3</sub>Cl<sub>2</sub>·TFA·1.6 H<sub>2</sub>O (MW 663.35): C, 52.51; H, 5.96; N, 6.33. Found: C, 52.16; H, 5.69; N, 6.69.

Mass Spectrum: (FAB) 520 (M+H)<sup>+</sup>, 273.

UV Spectrum: (2.5×10<sup>-3</sup>M NaOH) λ<sub>max</sub> (ε) 261.5 (18700), 303.9 (2090), λ<sub>min</sub> (ε) 232.3 (5270), 288.2 (1760).



**Example 101: 3-Iodo-O-(tert-butyldimethylsilyl)benzyl alcohol**

A 100 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 5.0 g of 3-iodobenzyl alcohol (Aldrich), 3.86 g of *tert*-butyldimethylsilyl chloride (Aldrich), 3.20 g of imidazole (Aldrich), and 30 mL of anhydrous DMF. The resulting solution was stirred under nitrogen for 4 h and concentrated *in vacuo* to give a clear viscous oil. This material was dissolved in 150 mL of EtOAc and the solution was washed with 5% aqueous NaHCO<sub>3</sub> (3x70 mL), dried over anhydrous MgSO<sub>4</sub> and concentrated *in vacuo* to give a clear liquid. The crude product was purified by flash chromatography on 200 g of silica gel (hexane) to afford 7.01 g (94%) of 3-iodo-O-(*tert*-butyldimethylsilyl)benzyl alcohol as a clear liquid.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>) δ 7.67 (s, 1H, aromatic CH), 7.56 (d, 1H, J=7.3, aromatic CH), 7.27 (d, 1H, J=9.2, aromatic CH), 7.06 (t, 1H, J=7.6, aromatic CH), 4.68 (s, 2H, ArCH<sub>2</sub>O), 0.94 (s, 9H, t-Bu), 0.10 (s, 6H, SiMe<sub>2</sub>).

**Example 102: 3-(1-Hydroxycyclobutyl)-O-(tert-butyltrimethylsilyl)benzyl alcohol**

To a dry 500 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer were added 7.0 g of 3-iodo-O-(*tert*-butyldimethylsilyl)benzyl alcohol and 100 mL of anhydrous THF. The solution was cooled to -78°C in a dry ice-isopropanol bath and treated with 9.7 mL of 2.5 M n-butyllithium/hexane (Aldrich) by dropwise addition via syringe through a rubber septum. After stirring at -78°C for 20 min, the solution was treated with 1.80 mL of cyclobutanone (Aldrich) by dropwise addition. The reaction mixture was stirred at -78°C for 20 min and then allowed to warm to RT. After 30 min at RT the solution was mixed with 100mL of water and subjected to rotary evaporation to remove THF. The resulting emulsion was extracted with EtOAc (4x60 mL). The combined EtOAc extracts were washed with saturated brine, dried over anhydrous

MgSO<sub>4</sub>, and concentrated *in vacuo* to give a light yellow oil. This material was purified by flash chromatography on 160 g of silica gel (9:1 hexane:EtOAc) to afford 3.98 g (68%) of 3-(1-hydroxycyclobutyl)-*O*-(*tert*-butyltrimethylsilyl)benzyl alcohol as a clear liquid.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 7.44 (s, 1H, aromatic CH), 7.38-7.23 (m, 2H, aromatic CH), 7.15 (d, 1H, J=7.4, aromatic CH), 5.45 (s, 1H, OH), 4.72 (s, 2H, ArCH<sub>2</sub>O), 2.41-2.18 (m, 4H, cyclobutyl CH<sub>2</sub>), 1.88 (m, 1H, cyclobutyl CH<sub>2</sub>), 1.61 (m, 1H, cyclobutyl CH<sub>2</sub>), 0.90 (s, 9H, *t*-Bu), 0.08 (s, 6H, SiMe<sub>2</sub>).

#### Example 103: 3-Cyclobutylbenzyl alcohol

A dry 500 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer was charged with 3.98 g of 3-(1-hydroxycyclobutyl)-*O*-(*tert*-butyltrimethylsilyl)benzyl alcohol and 85 mL of CH<sub>2</sub>Cl<sub>2</sub>. The solution was cooled to 0°C under nitrogen and treated with 15.7 mL of TFA followed by 4.78 mL of triethylsilane. After stirring at 0°C for 1 h, the solution was allowed to warm to RT. Analysis by tlc (SiO<sub>2</sub>, 8:2 hexane:EtOAc) after 3.5 h at RT indicated unreacted starting material at R<sub>f</sub>=0.15 and a new component at R<sub>f</sub>=0.65. The solution was treated with an additional 3 mL of triethylsilane and stirred at RT for 18 h. The solution was cooled to 0°C and neutralized by slow addition of 30 mL of Et<sub>3</sub>N. The mixture was concentrated *in vacuo* to give a light yellow liquid which was dissolved in 100 mL of EtOAc. The resulting solution was washed with saturated brine (3x80 mL), dried over anhydrous MgSO<sub>4</sub>, and concentrated to give a clear liquid. This material was dissolved in 80 mL of anhydrous THF and treated with 60 mL of 1 M (n-Bu)<sub>4</sub>NF (Aldrich) in a 500 mL 3-necked flask. After stirring at RT for 18 h, the solution was concentrated *in vacuo* and the residue dissolved in 100 mL of EtOAc. The solution was washed with saturated brine (4x80 mL), dried over anhydrous MgSO<sub>4</sub>, and concentrated to give a clear oil. The crude product was

purified by flash chromatography on 120 g of silica gel (8:2 hexane:EtOAc) to afford 1.29 g (59%) of 3-cyclobutylbenzyl alcohol as a clear liquid.

**<sup>1</sup>H-NMR:** (300MHz, DMSO-d<sub>6</sub>) δ 7.29-7.05 (m, 4H, aromatic CH), 5.14 (t, J=5.7, OH), 4.48 (d, J=5.8, ArCH<sub>2</sub>O), 3.51 (m, 1H, cyclobutyl methine), 2.30 (m, 2H, cyclobutyl CH<sub>2</sub>), 2.17-1.88 (m, 3H, cyclobutyl CH<sub>2</sub>), 1.82 (m, 1H, cyclobutyl CH<sub>2</sub>).

**Example 104: 3-Cyclobutylbenzyl bromide**

According to example 17, 1.29 g of 3-cyclobutylbenzyl alcohol was treated with 0.26 mL of PBr<sub>3</sub> in 15 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub> to afford 1.69 g of crude product (92% pure by <sup>1</sup>H-NMR). This material was purified by flash chromatography on 65 g of silica gel (hexane) to afford 1.59 g (89%) of 3-cyclobutyl benzyl bromide as a clear liquid.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>) δ 7.35-7.13 (m, 4H, aromatic CH), 4.70 (s, 2H, ArCH<sub>2</sub>Br), 3.53 (m, 1H, cyclobutyl methine), 2.39-1.75 (m, 6H, cyclobutyl CH<sub>2</sub>).

**Example 105: *tert*-Butyl (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-cyclobutylbenzyl)-4-morpholinecarboxylate**

According to example 81, 2.50 g of *tert*-butyl (2*R*, 3*S*)-(-)-6-oxo-2,3-diphenyl-4-morpholinecarboxylate (Aldrich) was alkylated with 1.59 g of 3-cyclobutylbenzyl bromide in 80 mL of anhydrous THF using 7.41 mL of 1 M sodium bis(trimethylsilyl)amide in THF (Aldrich). The crude product was subjected to flash chromatography on 120 g of silica gel (8:2 hexane:EtOAc) and then recrystallization from EtOH-water to afford 1.90 g (54%) of *tert*-butyl (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-cyclobutylbenzyl)-4-morpholinecarboxylate as a white powder, m.p. 145-147°C.

Example 106: (2R, 3S, 5S)-6-Oxo-2,3-diphenyl-5-(3-cyclobutylbenzyl)morpholine

According to example 82, 1.87 g of *tert*-butyl (2R, 3S, 5S)-6-oxo-2,3-diphenyl-5-(3-cyclobutylbenzyl)-4-morpholinecarboxylate was treated with 3.1 mL of TFA in 30 mL of CH<sub>2</sub>Cl<sub>2</sub> for 4 h. Neutralization with 8 mL of Et<sub>3</sub>N and work-up in the usual manner gave a light yellow residue. Purification of the crude material by flash chromatography on 85 g of silica gel (8:2 hexane:EtOAc) afforded 1.35 g (91%) of (2R, 3S, 5S)-6-oxo-2,3-diphenyl-5-(3-cyclobutylbenzyl)morpholine as a thick, transparent semi-solid.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 7.24-7.02 (m, 10H, aromatic CH), 6.92 (m, 4H, aromatic CH), 5.71 (s, 1H, morpholine 2-H), 4.64 (m, 1H, morpholine 3-H), 4.24 (m, 1H, morpholine 5-H), 3.42 (m, 1H, cyclobutyl methine), 3.26 (m, 1H, ArCH<sub>2</sub>), 3.10 (m, 1H, ArCH<sub>2</sub>), 2.76 (t, 1H, J=5.3, NH), 2.15 (m, 2H, cyclobutyl CH<sub>2</sub>), 2.02-2.81 (m, 3H, cyclobutyl CH<sub>2</sub>), 1.71 (m, 1H, cyclobutyl CH<sub>2</sub>).

Example 107: 3-Cyclobutyl-L-phenylalanine

According to example 53, 1.35 g of (2R, 3S, 5S)-6-oxo-2,3-diphenyl-5-(3-cyclobutylbenzyl)morpholine was hydrogenolyzed in 60 mL of 1:1 THF:EtOH using 0.42 g of PdCl<sub>2</sub> to afford 0.79 g (91%) of 3-cyclobutyl-L-phenylalanine-HCl as a white powder, m.p. >200°C.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.40(br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.26 (m, 1H, aromatic CH), 7.13 (m, 2H, aromatic CH), 7.08 (d, 1H, J=7.4, aromatic CH), 4.13 (m, 1H, methine), 3.50 (m, 1H, cyclobutyl methine), 3.11 (d, 2H, J=7.4, ArCH<sub>2</sub>), 2.29 (m, 2H, cyclobutyl CH<sub>2</sub>), 2.18-1.88 (m, 3H, cyclobutyl CH<sub>2</sub>), 1.83 (m, 1H, cyclobutyl CH<sub>2</sub>).

Example 108: *tert*-Butyl 3-cyclobutyl-L-phenylalaninate

According to example 54, 0.65 g of 3-cyclobutyl-L-phenylalanine-HCl was treated with 25 mL of isobutylene in 25 mL of 1,4-dioxane in the presence of 0.5 mL of conc.

H<sub>2</sub>SO<sub>4</sub>. Work-up in the usual manner followed by acidification of the free amine with ethereal HCl afforded 0.64 g (81%) of *tert*-butyl 3-cyclobutyl-L-phenylalaninate-HCl as a white powder, m.p. 173-174°C.

Example 109: *tert*-Butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclobutyl-L-phenylalaninate

According to example 9, 0.64 g of *tert*-butyl 3-cyclobutyl-L-phenylalaninate-HCl was coupled with 0.69 g of *N*-Cbz-L-glutamic acid  $\gamma$ -*tert*-butyl ester (Sigma) using 0.41 g of EDC (Aldrich) and 0.23 mL of *N*-methylmorpholine. The crude product was purified by flash chromatography on 85 g of silica gel (7:3 hexane:EtOAc) to afford 0.98 g (80%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclobutyl-L-phenylalaninate as a thick transparent oil.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.21 (d, 1H, J=7.3, NH), 7.33 (m, 6H, aromatic CH, NH), 7.18 (m, 1H, aromatic CH), 7.02 (m, 3H, aromatic CH), 5.00 (m, 2H, PhCH<sub>2</sub>O), 4.32 (m, 1H, methine), 4.05 (m, 1H, methine), 3.47 (m, 1H, cyclobutyl methine), 2.92 (d, 2H, J=7.6, ArCH<sub>2</sub>), 2.31-2.12 (m, 4H, glu 4-CH<sub>2</sub>, cyclobutyl CH<sub>2</sub>), 2.11-1.62 (m, 6H, glu 3-CH<sub>2</sub>, cyclobutyl CH<sub>2</sub>), 1.37 (s, 9H, t-Bu), 1.29 (s, 9H, t-Bu).

Example 110: *tert*-Butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclobutyl-L-phenylalaninate

According to example 48, 0.98 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclobutyl-L-phenylalaninate was hydrogenolyzed using 0.17 g of 10% Pd(C) in 55 mL of MeOH. This afforded 0.76 g (100%) of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclobutyl-L-phenylalaninate as a thick transparent oil. A sample of the corresponding HCl salt for <sup>1</sup>H-NMR analysis was prepared by treating 10 mg of the product with excess 1N ethereal HCl and concentrating to dryness.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.92 (d, 1H, J=7.0, NH), 8.25 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.20 (m, 1H, aromatic CH), 7.08 (m, 3H, aromatic CH), 4.38 (m, 1H, methine), 3.83 (m, 1H, methine), 3.48 (m, 1H, cyclobutyl methine), 2.94 (d, 2H, J=7.8, ArCH<sub>2</sub>), 2.16 (m, 4H, glu 4-CH<sub>2</sub>, cyclobutyl CH<sub>2</sub>), 2.15-1.86 (m, 5H, glu 3-CH<sub>2</sub>, cyclobutyl CH<sub>2</sub>), 1.79 (m, 1H, cyclobutyl CH<sub>2</sub>), 1.38 (s, 9H, t-Bu), 1.31 (s, 9H, t-Bu).

Example 111: *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclobutyl-L-phenylalaninate

According to example 49, 0.285 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was coupled with 0.38 g of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclobutyl-L-phenylalaninate using 0.34 mL of DECP (Aldrich) and 0.37 mL of Et<sub>3</sub>N in 25 mL of DMF. The crude product was subjected to flash chromatography on 100 g of silica gel (7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH) to afford 0.45 g (78%) of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclobutyl-L-phenylalaninate as a yellow powder, m.p. 120-125°C.

Example 112: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclobutyl-L-phenylalanine

According to example 50, 0.40 g of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclobutyl-L-phenylalaninate was treated with gaseous HCl in 60 mL of CH<sub>3</sub>NO<sub>2</sub> for 1 h. Work-up and isolation in the usual manner afforded 0.281 g (79%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclobutyl-L-phenylalanine as a yellow powder, m.p. 175°C (dec).

HPLC: one peak on C18,  $k'=2.50$ , 65:35:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  12.85-11.75 (br m, COOH), 8.58 (s, 1H, pteridinyl 7-CH), 8.04 (d, 1H,  $J=8.1$ , amide NH), 8.00 (d, 1H,  $J=8.7$ , amide NH), 7.75 (br s, 1H, NH<sub>2</sub>), 7.71 (d, 2H,  $J=8.9$ , aromatic CH), 7.55 (br s, 1H, NH<sub>2</sub>), 7.10-6.92 (m, 4H, aromatic CH), 6.82 (d, 2H,  $J=8.9$ , aromatic CH), 6.70 (br s, 2H, NH<sub>2</sub>), 4.79 (s, 2H, pteridinyl-CH<sub>2</sub>), 4.39 (m, 2H, methines), 3.40 (m, 1H, cyclobutyl methine), 3.21 (s, 3H, N-CH<sub>3</sub>), 3.00 (m, 1H, ArCH<sub>2</sub>), 2.88 (m, 1H, ArCH<sub>2</sub>), 2.29-2.08 (m, 4H, glu 4-CH<sub>2</sub>, cyclobutyl CH<sub>2</sub>), 2.07-1.65 (m, 6H, glu 3-CH<sub>2</sub>, cyclobutyl CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>33</sub>H<sub>37</sub>N<sub>9</sub>O<sub>6</sub>·1.6 H<sub>2</sub>O (MW 684.54): C, 57.90; H, 5.92; N, 18.42. Found: C, 57.88; H, 5.90; N, 18.52.

Mass Spectrum: (Ion Spray) 656 (M+H)<sup>+</sup>.

UV Spectrum: (pH 7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 259.5(22100), 308.0 (22300), 373.7 (6740).  $\lambda_{\min}$  ( $\epsilon$ ) 241.2 (12900), 274.5 (14600), 345.6 (5130).

Example 113: 6-Bromomethyl-3,4-dihydro-2-methyl-4-oxoquinazoline

According to example 4, 1.00 g of 3,4-dihydro-2,6-dimethyl-4-oxoquinazoline (Sen, A. B.; Gupta, J. K. *J. Indian Chem. Soc.*, 1962, 31, 369) was subjected to bromination using 1.05 g of *N*-bromosuccinimide (Aldrich) and 0.17 g of benzoyl peroxide in 50 mL of 1,2-dichloro-ethane for 30 min to afford 0.95 g (64%) of 6-bromomethyl-3,4-dihydro-2-methyl-4-oxoquinazoline as a tan solid.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.17 (s, 1H, aromatic CH), 7.85 (d, 1H,  $J=8.5$ , aromatic CH), 7.58 (d, 1H,  $J=8.5$ , aromatic CH), 4.88 (s, 2H, ArCH<sub>2</sub>Br), 2.39 (s, 3H, CH<sub>3</sub>).

Example 114: Ethyl 5-*N*-methylamino-2-thiophenecarboxylate

A mixture of 2.0 g of ethyl 5-amino-2-thiophenecarboxylate (Mackay, D. *Can. J. Chem.*, 1966, 44, 2881-2891; Paul, H.; Migulla, H. *Arch. Pharm.*, 1978, 311, 679-691.), 2.05 g of methyl iodide (Aldrich), and 2.58 g of 2,6-lutidine (Aldrich) in 80 mL of DMF was stirred at 70°C under nitrogen for 24h. Analysis of the reaction mixture by tlc (SiO<sub>2</sub>, 3:2 hexane:EtOAc) indicated starting material at R<sub>f</sub>=0.51 and a new component at R<sub>f</sub>=0.60. The mixture was treated with an additional 1.71 g of methyl iodide and was stirred at 70°C for 8h, RT for 72h and 70°C for an additional 8h. The reaction mixture was mixed with 75 mL of water and extracted with EtOAc (3x150 mL). The combined EtOAc extracts were washed with saturated aqueous brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo* to dryness. The crude residue was subjected to flash chromatography on silica gel (75:25 hexane:EtOAc) to afford 0.67 g (38%) of ethyl 5-*N*-methylamino-2-thiophenecarboxylate as a brown solid, m.p. 41-43°C.

Example 115: Ethyl 5-(((3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)methylamino)-2-thiophene-carboxylate

A 100 mL round bottomed flask equipped with a condenser and a magnetic stirrer was charged with 0.66 g of 6-bromomethyl-3,4-dihydro-2-methyl-4-oxoquinazoline, 0.44 g of ethyl 5-*N*-methylamino-2-thiophenecarboxylate, 0.25 g of 2,6-lutidine, and 30 mL of anhydrous DMF. The mixture was stirred at 80°C for 18h. Analysis of the reaction mixture by tlc (SiO<sub>2</sub>, 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) indicated starting materials and a new component at R<sub>f</sub>=0.41. The mixture was concentrated *in vacuo* to dryness and the residue was subjected to flash chromatography on 110 g of silica gel (95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH). Fractions containing the R<sub>f</sub>=0.41 component were combined and concentrated to afford 0.42 g (49%) of ethyl 5-(((3,4-dihydro-2-methyl-4-oxo-6-



quinazolinyl)methyl)methylamino)-2-thiophenecarboxylate as a light tan powder, m.p. >200°C.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 12.22 (br s, 1H, NH), 7.92 (s, 1H, aromatic CH), 7.65 (d, 1H, J=8.4, aromatic CH), 7.56 (d, 1H, J=8.5, aromatic CH), 7.48 (d, 1H, J=4.4, thienyl CH), 6.07 (d, 1H, J=4.3, thienyl CH), 4.70 (s, 2H, quinazolinyl-CH<sub>2</sub>), 4.16 (q, 2H, J=7.2, ethyl CH<sub>2</sub>), 3.09 (s, 3H, N-CH<sub>3</sub>), 2.33 (s, 3H, quinazolinyl 2-CH<sub>3</sub>), 1.22 (t, 3H, J=7.2, ethyl CH<sub>3</sub>).

Example 116: 5-(((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)methylamino)-2-thiophene-carboxylic acid

A 25 mL 3-necked flask equipped with a condenser, a thermometer, and a magnetic stirrer was charged with 0.20 g of ethyl 5-(((3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)methylamino)-2-thiophene-carboxylate, 6 mL of EtOH, and 6 mL of 1N aqueous NaOH. The resulting solution was stirred under nitrogen at RT for 25 min and then at 55°C for 2h. Analysis by tlc (SiO<sub>2</sub>, 95:5 CH<sub>2</sub>Cl<sub>2</sub>) indicated no remaining starting material and a new component at R<sub>f</sub>=0.06. The solution was cooled to RT and subjected to rotary evaporation to remove EtOH. The remaining solution was diluted with 10 mL of water and acidified to pH=3.5 by addition of 1N aqueous HCl. A tan precipitate formed which was collected by centrifugation, washed with four cycles of aqueous suspension-centrifugation-decantation, and lyophilized to afford 0.116 g (63%) of 5-(((3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)methylamino)-2-thiophene-carboxylic acid as a white powder, m.p. 195°C (dec).

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 12.40-11.90 (br m, COOH, NH), 7.95 (s, 1H, aromatic CH), 7.69 (m, 1H, aromatic CH), 7.58 (d, 1H, J=8.2, aromatic CH), 7.42 (d, 1H, J=4.2, thienyl CH), 6.04 (d, 1H, J=4.2, thienyl CH), 4.70 (s, 2H, quinazolinyl-CH<sub>2</sub>), 3.09 (s, 3H, N-CH<sub>3</sub>), 2.35 (s, 3H, quinazolinyl 2-CH<sub>3</sub>).

Example 117: *tert*-Butyl *N*-(((5-(((3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)methylamino)-2-thienyl)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate

A dry 50 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer was charged with 0.115 g of 5-(((3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)methylamino)-2-thiophene-carboxylic acid, 0.165 g of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate 0.06 mL of Et<sub>3</sub>N, and 7 mL of anhydrous DMF. The yellow solution was treated with 0.06 mL of DECP and was stirred at RT under nitrogen. After 2h, tlc (SiO<sub>2</sub>, 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) indicated starting material at R<sub>f</sub>=0.02, a major new component at R<sub>f</sub>=0.37, and minor components at R<sub>f</sub>=0.31 and 0.40. The reaction mixture was treated with an additional 0.03 mL of DECP and 0.03 mL of Et<sub>3</sub>N and was stirred for another 1h at RT. TLC indicated little additional conversion. The solution was concentrated *in vacuo* to dryness and the residue dissolved in 60 mL of CHCl<sub>3</sub>. The solution was washed with 5% aqueous NaHCO<sub>3</sub> (3x50 mL), dried over anhydrous MgSO<sub>4</sub>, and concentrated to give a light yellow oil. The crude product was subjected to flash chromatography twice (SiO<sub>2</sub>, 95:5 EtOAc:MeOH; SiO<sub>2</sub>, 97:3 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.119 g (46%) of *tert*-butyl *N*-(((5-(((3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)methylamino)-2-thienyl)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate as a tan foam.

<sup>1</sup>H-NMR: (400 MHz, DMSO-d<sub>6</sub>) δ 12.19 (s, 1H, quinazoline NH), 8.18 (d, 1H, J=7.1, amide NH), 7.92 (m, 2H, amide NH, aromatic CH), 7.63 (dd; 1H; J=7.9, 1.8; aromatic CH), 7.57 (d, 1H, J=4.1, thienyl CH), 7.54 (d, 1H, J=8.0, aromatic CH), 7.12-6.94 (m, 4H, aromatic CH), 5.97 (d, 1H, J=4.1, thienyl CH), 4.63 (s, 2H, quinazolinyl-CH<sub>2</sub>), 4.40-4.27 (m, 2H, methines), 3.02 (s, 3H, N-CH<sub>3</sub>), 2.87 (m, 3H, ArCH<sub>2</sub>, cyclopentyl methine), 2.31 (s, 3H, quinazolinyl 2-CH<sub>3</sub>), 2.20 (t, 2H, J=7.7, glu 4-CH<sub>2</sub>), 1.91 (m, 3H,

glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.83-1.40 (m, 7H, cyclopentyl CH<sub>2</sub>), 1.35 (s, 9H, t-Bu), 1.27 (s, 9H, t-Bu).

Example 118: *N*-((5-(((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)methylamino)-2-thienyl)carbonyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine

According to example 50, 0.117 g of *tert*-butyl *N*-((5-(((3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)methylamino)-2-thienyl)carbonyl)-5-*O-tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate was treated with gaseous HCl in 25 mL of CH<sub>3</sub>NO<sub>2</sub> for 45 min. Removal of the CH<sub>3</sub>NO<sub>2</sub> *in vacuo* gave a tan residue which was purified by semi-preparative reverse phase HPLC (C18, 70 : 30 : 0.1 → 60 : 40 : 0.1 H<sub>2</sub>O : MeCN : TFA over 25min ). Fractions containing the major component (*k'*=1.61 on analytical HPLC, C18, 60:40:0.1 H<sub>2</sub>O:MeCN:TFA) were combined, concentrated to 25 mL and lyophilized to afford 0.052 g (42%) of *N*-((5-(((3,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)methylamino)-2-thienyl)carbonyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine as a yellow powder, m.p. 125°C (dec).

HPLC: one peak on C18, *k'*=1.62, 60:40:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.09 (d, 1H, *J*=7.5, amide NH), 7.93 (m, 2H, amide NH, aromatic CH), 7.69 (d, 1H, *J*=8.0, aromatic CH), 7.56 (m, 2H, aromatic CH, thienyl CH), 7.10-6.92 (m, 4H, aromatic CH), 5.97 (d, 1H, *J*=4.3, thienyl CH), 4.66 (s, 2H, quinazolinyl-CH<sub>2</sub>), 4.33 (m, 2H, methines), 3.06 (s, 3H, N-CH<sub>3</sub>), 2.99 (m, 1H, ArCH<sub>2</sub>), 2.85 (m, 1H, ArCH<sub>2</sub>), 2.38 (s, 3H, quinazolinyl 2-CH<sub>3</sub>), 2.22 (t, 2H, *J*=7.5, glu 4-CH<sub>2</sub>), 2.02-1.37 (m, 10H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>).

<sup>19</sup>F-NMR: (376 MHz, DMSO-d<sub>6</sub>) δ 0.57 (relative to TFA external standard).

Elemental Analysis: Calcd. for C<sub>35</sub>H<sub>39</sub>N<sub>5</sub>O<sub>7</sub>S·1.2 TFA·1.3 H<sub>2</sub>O (MW 834.03): C, 53.86; H, 5.17; N, 8.40; S, 3.84. Found: C, 53.85; H, 5.11; N, 8.36; S, 3.92.

Mass Spectrum: (Ion Spray) 691 ( $M+NH_4^+$ ).

UV Spectrum: (pH 7 Buffer)  $\lambda_{max}$  ( $\epsilon$ ) 225.2 (36600), 265.6 (10300), 353.1 (22200).  
 $\lambda_{min}$  ( $\epsilon$ ) 214.3 (35200), 252.1 (9230), 288.4 (4120).

Example 119: *tert*-Butyl 5-*O-tert*-butyl-*N*-(4-((3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)-trifluoroacetamido)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate

A 25 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 0.258 g of 4-(*N*-(3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)trifluoroacetamido)benzoic acid (Styles, V. L., *et al*, J. Heterocyclic Chem., 1990, 27, 1809), 0.250 g of *tert*-butyl 5-*O-tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate, 0.077 g of 1-hydroxybenzotriazole hydrate (Aldrich), 5 mL of anhydrous DMF, and 0.07 mL of Et<sub>3</sub>N. The solution was treated with 0.104 g of DCC (Fluka) and stirred at RT under nitrogen for 24h. The mixture was filtered and the filtrate was concentrated *in vacuo* to dryness. The residue was dissolved in 70 mL of CH<sub>2</sub>Cl<sub>2</sub>, washed with 5% aqueous NaHCO<sub>3</sub> (3x50 mL), dried over anhydrous MgSO<sub>4</sub> and concentrated to give a tan oil. This material was purified by flash chromatography on silica gel (95:5  $\pm$  90:10 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.304 g (70%) of *tert*-butyl 5-*O-tert*-butyl-*N*-(4-((3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)trifluoroacetamido)-benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate as a tan glass, m.p. 124-130°C.

Example 120: *tert*-Butyl 5-*O-tert*-butyl-*N*-(4-((3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate

To a 100 mL round bottomed flask equipped with a nitrogen inlet and a magnetic stirrer were added 0.268 g of *tert*-butyl 5-*O-tert*-butyl-*N*-(4-((3-(2,4-diamino-1,6-dihydro-6-

oxo-5-pyrimidinyl)propyl)trifluoroacetamido)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate, and 7 mL of MeOH. The solution was treated with 0.18 mL of 40% (w/w) dimethylamine/water (Aldrich) followed by 0.45 mL of water and was stirred at RT under nitrogen for 24h. The solution was concentrated *in vacuo* to dryness and the residue was subjected to flash chromatography on 60 g of silica gel (92:8 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.185 g (78%) of *tert*-butyl 5-*O-tert*-butyl-*N*-(4-((3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate as a thick transparent semi-solid.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 9.80 (br s, 1H, pyrimidinyl NH), 8.20 (d, 1H, J=7.2, amide NH), 7.90 (d, 1H, J=8.0, amide NH), 7.67 (d, 2H, J=8.6, aromatic CH), 7.20-6.98 (m, 4H, aromatic CH), 6.55 (d, 2H, J=8.6, aromatic CH), 6.25 (m, 1H, NH), 5.94 (br s, 2H, NH<sub>2</sub>), 5.77 (br s, 2H, NH<sub>2</sub>), 4.54-4.29 (m, 2H, methines), 3.12-2.80 (m, 5H, cyclopentyl methine, ArCH<sub>2</sub>, pyrimidinyl-CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N), 2.27 (m, 4H, glu 4-CH<sub>2</sub>, pyrimidinyl-CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N), 2.10-1.82 (4H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.80-1.45 (m, 8H, cyclopentyl CH<sub>2</sub>, pyrimidinyl-CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>N), 1.39 (s, 9H, t-Bu), 1.32 (s, 9H, t-Bu).

Example 121: *N*-(4-((3-(2,4-Diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine

A 100 mL round bottomed flask equipped with a magnetic stirrer was charged with 0.180 g of *tert*-butyl 5-*O-tert*-butyl-*N*-(4-((3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate and 25 mL of CH<sub>3</sub>NO<sub>2</sub>. The mixture was acidified by bubbling HCl gas through for 5 min, however, the solid starting material did not dissolve. Analysis of the reaction mixture by tlc (SiO<sub>2</sub>, 92:8 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) indicated minimal conversion of the starting material. The mixture was concentrated *in vacuo* to dryness. A yellow residue resulted which was

suspended in 20 mL of  $\text{CH}_2\text{Cl}_2$  and treated with 1 mL of anisole followed by 15 mL of TFA. The solid quickly dissolved giving a yellow solution which was stirred at RT under nitrogen. After 1h, tlc indicated no remaining starting material and a new spot at  $R_f=0.0$ . The solution was concentrated to dryness and the residue was purified by semi-preparative reverse phase HPLC (C18, 70:30:0.1  $\text{H}_2\text{O}$ : MeCN:TFA). Fractions containing pure material were combined, concentrated to 30 mL and lyophilized to afford 0.098 g (47%) of *N*-(4-((3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine as a fluffy white solid, m.p. 125°C (dec).

HPLC: one peak on C18,  $k'=1.64$ , 70:30:0.1  $\text{H}_2\text{O}$ :MeCN:TFA, flow rate=1mL/min.

$^1\text{H}$ -NMR: (300 MHz, DMSO- $d_6$ )  $\delta$  12.91-11.10 (br s, COOH, pyrimidinyl NH), 8.07 (d, 1H,  $J=7.6$ , amide NH), 7.88 (d, 1H,  $J=7.9$ , amide NH), 7.70 (br s, 1H,  $\text{NH}_2$ ), 7.62 (m, 3H,  $\text{NH}_2$ , aromatic CH), 7.13-6.70 (m, 6H,  $\text{NH}_2$ , aromatic CH), 6.53 (d, 2H,  $J=8.6$ , aromatic CH), 4.40 (m, 2H, methines), 3.01 (m, 3H, cyclopentyl methine, pyrimidinyl- $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$ ), 2.86 (m, 2H,  $\text{ArCH}_2$ ), 2.25 (m, 4H, glu 4- $\text{CH}_2$ , pyrimidinyl- $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$ ), 2.01-1.78 (m, 4H, glu 3- $\text{CH}_2$ , cyclopentyl  $\text{CH}_2$ ), 1.76-1.37 (m, 8H, cyclopentyl  $\text{CH}_2$ , pyrimidinyl- $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$ ).

$^{19}\text{F}$ -NMR: (282 MHz, DMSO- $d_6$ )  $\delta$  3.92 (relative to TFA external standard).

Elemental Analysis: Calcd. for  $\text{C}_{33}\text{H}_{41}\text{N}_7\text{O}_7 \cdot 1.8 \text{ TFA} \cdot 1.6 \text{ H}_2\text{O}$  (MW 881.80): C, 49.85; H, 5.26; N, 11.12. Found: C, 49.81; H, 5.24; N, 11.11.

Mass Spectrum: (Ion Spray) 648 ( $\text{M}+\text{H}$ ) $^+$ .

UV Spectrum: (pH 7 Buffer)  $\lambda_{\text{max}}$  ( $\epsilon$ ) 280.1 (29700).  $\lambda_{\text{min}}$  ( $\epsilon$ ) 247.4 (7590). sh ( $\epsilon$ ) 302.9 (20400).

**Example 122: Di-*tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O-tert*-butyl-L-glutam-1-yl-L-aspartate**

According to example 9, 1.00 g of L-aspartic acid di-*t*-butyl ester hydrochloride (Sigma) was coupled with 1.20 g of *N*-Cbz-L-glutamic acid  $\gamma$ -*t*-butyl ester (Sigma) using 0.72 g of EDC (Aldrich) and 0.39 mL of *N*-methylmorpholine. The crude product was purified by flash chromatography on 80 g of silica gel (75:25 hexane:EtOAc) to afford 1.10 g (55%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O-tert*-butyl-L-glutam-1-yl-4-*O-tert*-butyl-L-aspartate as a white solid, m.p. 119-120°C.

**Example 123: Di-*tert*-butyl 5-*O-tert*-butyl-L-glutam-1-yl-L-aspartate**

According to example 48, 0.389 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O-tert*-butyl-L-glutam-1-yl-4-*O-tert*-butyl-L-aspartate was hydrogenolyzed using 0.07 g of 10% Pd(C) in 40 mL of MeOH. This afforded 0.29 g (98%) of di-*tert*-butyl 5-*O-tert*-butyl-L-glutam-1-yl-L-aspartate as a thick transparent oil. A sample of the corresponding HCl salt for <sup>1</sup>H-NMR analysis was prepared by treating 10 mg of the product with excess 1N ethereal HCl and concentrating to dryness.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.91 (d, 1H, J=7.8, NH), 8.31 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 4.52 (m, 1H, methine), 3.83 (m, 1H, methine), 2.63 (d, 2H, J=6.1, asp CH<sub>2</sub>), 2.36 (m, 2H, glu 4-CH<sub>2</sub>), 1.94 (m, 2H, glu 3-CH<sub>2</sub>), 1.38 (s, 27H, t-Bu).

**Example 124: Di-*tert*-butyl 5-*O-tert*-butyl-*N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-aspartate**

According to example 49, 0.30 g of 4-(*N*-(2,4-diamino-6-pteridinylmethyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was coupled with 0.34 g of di-*tert*-butyl 5-*O-tert*-butyl-L-glutam-1-yl-L-aspartate using 0.36 mL of DECP (Aldrich) and 0.39 mL of Et<sub>3</sub>N in 25 mL of DMF. The crude product was subjected to

flash chromatography twice (SiO<sub>2</sub>, 7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH; SiO<sub>2</sub>, 96:4 → 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.213 g (37%) of di-*tert*-butyl 5-*O-tert*-butyl-*N*-(4-(((2,4-diamino-6-pteridiny))methyl)methylamino)benzoyl)-L-glutam-1-yl-L-aspartate as a yellow powder, m.p. 130-135°C.

Example 125: *N*-(4-(((2,4-Diamino-6-pteridiny))methyl)methylamino)benzoyl)-L-glutam-1-yl-L-aspartic acid (Procedure B)

For Procedure A to prepare this compound, see Example 33.

According to example 50, 0.307 g of di-*tert*-butyl 5-*O-tert*-butyl-*N*-(4-(((2,4-diamino-6-pteridiny))methyl)methylamino)benzoyl)-L-glutam-1-yl-L-aspartate was treated with gaseous HCl in 40 mL of CH<sub>3</sub>NO<sub>2</sub> for 1.5h. The reaction mixture was concentrated to 5 mL and mixed with 50 mL of ether. The resulting solid was collected by filtration and dried *in vacuo*. The product was dissolved in 25 mL of water, filtered and lyophilized to afford 0.246 g (89%) of *N*-(4-(((2,4-diamino-6-pteridiny))methyl)methylamino)benzoyl)-L-glutam-1-yl-L-aspartic acid as a yellow-orange powder, m.p. 160°C (dec).

HPLC: one peak on C18, *k'*=0.70, 85:15:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>) δ 13.12 (br s, COOH), 9.31 (s, 1H, NH<sub>2</sub>), 9.08 (s, 1H, NH<sub>2</sub>), 8.72 (s, 1H, pteridiny 7-CH), 8.68 (br s, 1H, NH<sub>2</sub>), 8.22 (d, 1H, *J*=8.0, amide NH), 8.09 (d, 1H, *J*=7.9, amide NH), 7.79 (br s, 1H, NH<sub>2</sub>), 7.75 (d, 2H, *J*=8.9, aromatic CH), 6.82 (d, 2H, *J*=8.9, aromatic CH), 4.87 (s, 2H, pteridiny-CH<sub>2</sub>), 4.59-4.40 (m, 2H, methines), 3.24 (s, 3H, N-CH<sub>3</sub>), 2.75-2.52 (m, 2H, asp CH<sub>2</sub>), 2.27 (t, 2H, *J*=7.8, glu 4-CH<sub>2</sub>), 2.10-1.78 (m, 2H, glu 3-CH<sub>2</sub>) .

Elemental Analysis: Calcd. for C<sub>24</sub>H<sub>27</sub>N<sub>9</sub>O<sub>8</sub>·2 HCl·1.3 H<sub>2</sub>O (MW 665.87): C, 43.29; H, 4.78; N, 18.93; Cl, 10.65. Found: C, 43.42; H, 4.90; N, 18.83; Cl, 10.46.

Mass Spectrum: (FAB) 570 (M+H)<sup>+</sup>.

UV Spectrum: (pH 7 Buffer) λ<sub>max</sub> (ε) 258.7 (21900), 306.4 (23800), 372.2 (7250).



$\lambda_{\min}$  ( $\epsilon$ ) 240.1 (13400), 272.4 (15200), 345.0 (5740).

**Example 126: Di-*tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O-tert*-butyl-L-glutam-1-yl-L-glutamate**

According to example 9, 0.88 g of L-glutamic acid di-*t*-butyl ester hydrochloride (Sigma) was coupled with 1.00 g of *N*-Cbz-L-glutamic acid  $\gamma$ -*t*-butyl ester (Sigma) using 0.60 g of EDC (Aldrich) and 0.33 mL of *N*-methyilmorpholine. The crude product was purified by flash chromatography on silica gel (75:25 hexane:EtOAc) to afford 1.49 g (87%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O-tert*-butyl-L-glutam-1-yl-5-*O-tert*-butyl-L-glutamate as a thick transparent oil.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO- $d_6$ )  $\delta$  8.21 (d, 1H,  $J=7.6$ , NH), 7.42 (d, 1H,  $J=8.2$ , NH), 7.33 (m, 5H, aromatic CH), 5.01 (m, 2H, PhCH<sub>2</sub>O), 4.17-3.96 (m, 2H, methines), 2.24 (m, 4H, glu 4-CH<sub>2</sub>), 1.96-1.64 (m, 4H, glu 3-CH<sub>2</sub>), 1.37 (s, 27H, *t*-Bu).

**Example 127: Di-*tert*-butyl 5-*O-tert*-butyl-L-glutam-1-yl-L-glutamate**

According to example 48, 0.60 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O-tert*-butyl-L-glutam-1-yl-5-*O-tert*-butyl-L-glutamate was hydrogenolyzed using 0.10 g of 10% Pd(C) in 45 mL of MeOH. This afforded 0.45 g (98%) of di-*tert*-butyl 5-*O-tert*-butyl-L-glutam-1-yl-L-glutamate as a thick transparent oil. A sample of the corresponding HCl salt for <sup>1</sup>H-NMR analysis was prepared by treating 10 mg of the product with excess 1N ethereal HCl and concentrating to dryness.

**<sup>1</sup>H-NMR:** (200 MHz, DMSO- $d_6$ )  $\delta$  8.86 (d, 1H,  $J=7.3$ , NH), 8.31 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 4.21 (m, 1H, methine), 3.87 (m, 1H, methine), 2.35 (m, 4H, glu 4-CH<sub>2</sub>), 2.07-1.66 (m, 4H, glu 3-CH<sub>2</sub>), 1.39 (s, 27H, *t*-Bu).

Example 128: Di-*tert*-butyl 5-*O-tert*-butyl-*N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-glutamate

According to example 49, 0.30 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was coupled with 0.386 g of di-*tert*-butyl 5-*O-tert*-butyl-L-glutam-1-yl-L-glutamate using 0.36 mL of DECP (Aldrich) and 0.39 mL of Et<sub>3</sub>N in 25 mL of DMF. The crude product was subjected to flash chromatography on 150 g of silica gel (7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH) to afford 0.432 g (73%) of di-*tert*-butyl 5-*O-tert*-butyl-*N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-glutamate as a yellow powder, m.p. 130-145°C.

Example 129: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-glutamic acid (Procedure B)

For Procedure A to prepare this compound, see Example 39.

According to example 50, 0.382 g of di-*tert*-butyl 5-*O-tert*-butyl-*N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-glutamate was treated with gaseous HCl in 50 mL of CH<sub>3</sub>NO<sub>2</sub> for 1h. The reaction mixture was concentrated to 10 mL and mixed with 50 mL of ether. The resulting solid was collected by filtration and dried *in vacuo*. The product was dissolved in 25 mL of water, filtered and lyophilized to afford 0.265 g (76%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-glutamic acid as a yellow-orange powder, m.p. 155°C (dec).

HPLC: one peak on C18, *k'*=0.88, 85:15:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>) δ 13.18 (br s, COOH), 9.29 (s, 1H, NH<sub>2</sub>), 9.07 (s, 1H, NH<sub>2</sub>), 8.72 (s, 1H, pteridiny 7-CH), 8.69 (br s, 1H, NH<sub>2</sub>), 8.19 (d, 1H, *J*=7.7, amide NH), 8.09 (d, 1H, *J*=7.9, amide NH), 7.87 (br s, 1H, NH<sub>2</sub>), 7.74 (d, 2H, *J*=8.9, aromatic CH), 6.81 (d, 2H, *J*=8.9, aromatic CH), 4.87 (s, 2H, pteridiny-CH<sub>2</sub>), 4.42 (m,

1H, methine), 4.19 (m, 1H, methine), 3.24 (s, 3H, N-CH<sub>3</sub>), 2.28 (m, 4H, glu 4-CH<sub>2</sub>), 2.07-1.71 (m, 4H, glu 3-CH<sub>2</sub>).

**Elemental Analysis:** Calcd. for C<sub>25</sub>H<sub>29</sub>N<sub>9</sub>O<sub>8</sub>·1.9 HCl·2 H<sub>2</sub>O (MW 688.87): C, 43.59; H, 5.11; N, 18.30; Cl, 9.78. Found: C, 43.72; H, 5.07; N, 18.16; Cl, 9.97.

**Mass Spectrum:** (FAB) 584 (M+H)<sup>+</sup>.

**UV Spectrum:** (pH 7 Buffer) λ<sub>max</sub> (ε) 258.8(23700), 306.2 (25600), 371.9 (7830). λ<sub>min</sub> (ε) 240.4 (14300), 272.9 (16600), 344.6 (6150).

**Example 130: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-*tert*-butyl-L-phenylalanine (Procedure B)**

For Procedure A to prepare this compound, see Example 97.

According to example 50, 3.51 g of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-phenylalaninate was treated with gaseous HCl in 150 mL of CH<sub>3</sub>NO<sub>2</sub> for 1h. The reaction mixture was concentrated *in vacuo* to a slurry and mixed with 100 mL of ether. The solid was collected by vacuum filtration and dissolved in 150 mL of 6:4 MeCN:H<sub>2</sub>O. The solution was subjected to rotary evaporation to remove MeCN and the resulting suspension was frozen and lyophilized to afford 3.167 g (93%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-*tert*-butyl-L-phenylalanine as a yellow-orange powder, m.p. 165°C (dec).

**HPLC:** one peak on C18, k'=2.30, 70:30:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>) δ 13.35-12.95 (br s, COOH), 9.30 (s, 1H, NH<sub>2</sub>), 9.07 (s, 1H, NH<sub>2</sub>), 8.71 (s, 1H, pteridiny 7-CH), 8.68 (br s, 1H, NH<sub>2</sub>), 8.14 (d, 1H, J=7.6, amide NH), 8.02 (d, 1H, J=7.8, amide NH), 7.89 (br s, 1H, NH<sub>2</sub>), 7.71 (d, 2H, J=8.9, aromatic CH), 7.21 (s, 1H, aromatic CH), 7.18-7.05 (m, 2H, aromatic CH), 6.98 (d, 1H, J=7.4, aromatic CH), 6.79 (d, 2H, J=8.9, aromatic CH), 4.85 (s, 2H, pteridiny-

CH<sub>2</sub>), 4.39 (m, 2H, methines), 3.23 (s, 3H, N-CH<sub>3</sub>), 3.03 (m, 1H, ArCH<sub>2</sub>), 2.90 (m, 1H, ArCH<sub>2</sub>), 2.20 (t, 2H, J=7.6, glu 4-CH<sub>2</sub>), 2.01-1.76 (m, 2H, glu 3-CH<sub>2</sub>), 1.19 (s, 9H, t-Bu).

**Elemental Analysis:** Calcd. for C<sub>33</sub>H<sub>39</sub>N<sub>9</sub>O<sub>6</sub>·1.5HCl·1.8 H<sub>2</sub>O (MW 744.85): C, 53.21; H, 5.97; N, 16.92; Cl, 7.14. Found: C, 53.20; H, 5.97; N, 16.96; Cl, 7.20.

**Mass Spectrum:** (Ion Spray) 658 (M+H)<sup>+</sup>.

**UV Spectrum:** (pH 7 Buffer) λ<sub>max</sub> (ε) 259.5(24400), 308.0 (24400), 371.5 (7410).  
λ<sub>min</sub> (ε) 241.4 (14600), 274.2 (16200), 345.1 (5680).

**Example 131: tert-Butyl 5-O-tert-butyl-N-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate**

A dry 50 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 0.150 g of 4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoic acid, 0.198 g of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate, and 7 mL of anhydrous DMF. The mixture was cooled to 0°C and treated with 0.058 mL of Et<sub>3</sub>N followed by 0.063 mL of DECP. The solid starting material rapidly dissolved to give a clear solution. The solution was allowed to warm to RT and was stirred at RT under nitrogen. After 3h, tlc (SiO<sub>2</sub>, 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) indicated a major new component at R<sub>f</sub>=0.41 and minor components at R<sub>f</sub>=0.38 and 0.06. The solution was concentrated *in vacuo* to dryness and the residue was dissolved in 70 mL of CHCl<sub>3</sub>. The CHCl<sub>3</sub> solution was washed with 5% aqueous NaHCO<sub>3</sub> (3x60 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated to give a yellow glass. The crude product was purified by flash chromatography on silica gel (95:5 → 93:7 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.258 g (80%) of *tert*-butyl 5-*O*-*tert*-butyl-N-(4-(((1,2-

dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate as a transparent glass.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 12.54 (s, 1H, benzoquinazoline NH), 9.86 (s, 1H, aromatic CH), 8.39 (d, 1H, J=7.6, amide NH), 8.23 (d, 1H, J=9.0, aromatic CH), 8.02 (d, 1H, J=8.4, aromatic CH), 7.68-7.41 (m, 4H, aromatic CH, amide NH), 7.35 (m, 1H, 4-aminobenzoyl NH), 7.21-6.97 (m, 4H, aromatic CH), 6.56 (d, 1H, J=8.8, aromatic CH), 6.41 (d, 1H, J=15.0, aromatic CH), 4.67-4.30 (m, 4H, benzoquinazoline 9-CH<sub>2</sub>, methines), 3.06-2.77 (m, 3H, ArCH<sub>2</sub>, cyclopentyl methine), 2.43 (s, 3H, CH<sub>3</sub>), 2.19 (m, 2H, glu 4-CH<sub>2</sub>), 2.00-1.38 (m, 10H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.35 (s, 9H, t-Bu), 1.32 (s, 9H, t-Bu).

Example 132: N-(4-(((1,2-Dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine

According to example 50, 0.25 g of *tert*-butyl 5-*O-tert*-butyl-N-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate was treated with gaseous HCl in 40 mL of CH<sub>3</sub>NO<sub>2</sub> for 1h. The reaction mixture was concentrated *in vacuo* to give a yellow residue. Analysis of this material by HPLC (C18, 65:35:0.1 H<sub>2</sub>O:MeCN:TFA) indicated a major component at k'=2.82 and minor components at k'=3.33, 1.53, and 0.83. The crude product was subjected to semi-preparative reverse phase HPLC (C18, 65:35:0.1 H<sub>2</sub>O:MeCN:TFA). Fractions containing pure product (as determined by analytical HPLC) were combined and concentrated to dryness. The residue was mixed with 20 mL of water and treated with sufficient 1N aqueous NaOH to give complete solution. The solution was filtered and acidified to pH 3.0 by addition of 1N aqueous HCl. A white precipitate resulted which was separated by centrifugation, washed with 4 cycles of aqueous suspension-centrifugation-decantation, and lyophilized to afford 0.141 g (62%)

of *N*-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine as a fluffy white powder, m.p. >200°C.

**HPLC:** one peak on C18,  $k'=2.77$ , 65:55:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  12.90-11.80 (br m, COOH, benzoquinazoline NH), 9.82 (s, 1H, aromatic CH), 8.27 (d, 1H,  $J=7.9$ , amide NH), 8.19 (d, 1H,  $J=8.7$ , aromatic CH), 7.99 (d, 1H,  $J=8.4$ , aromatic CH), 7.58 (m, 2H, aromatic CH), 7.45 (m, 2H, aromatic CH, amide NH), 7.31 (m, 1H, 4-aminobenzoyl NH), 7.05 (m, 2H, aromatic CH), 6.97 (d, 2H,  $J=7.9$ , aromatic CH), 6.51 (d, 1H,  $J=8.7$ , aromatic CH), 6.37 (d, 1H,  $J=15.2$ , aromatic CH), 4.55 (d, 2H,  $J=5.2$ , benzoquinazoline 9-CH<sub>2</sub>), 4.43 (m, 2H, methines), 3.02 (m, 1H, ArCH<sub>2</sub>), 2.82 (m, 2H, ArCH<sub>2</sub>, cyclopentyl methine), 2.41 (s, 3H, CH<sub>3</sub>), 2.16 (t, 2H,  $J=7.7$ , glu 4-CH<sub>2</sub>), 1.98-1.71 (m, 4H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.69-1.31 (m, 6H, cyclopentyl CH<sub>2</sub>).

**Elemental Analysis:** Calcd. for C<sub>40</sub>H<sub>40</sub>N<sub>5</sub>O<sub>7</sub>F·0.5H<sub>2</sub>O (MW 730.79): C, 65.74; H, 5.65; N, 9.58. Found: C, 65.76; H, 5.68; N, 9.57.

**Mass Spectrum:** (FAB) 722 (M+H)<sup>+</sup>.

**UV Spectrum:** (pH 7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 265.9 (47900), 299.6 (27300), 348.4 (5710).  $\lambda_{\min}$  ( $\epsilon$ ) 240.6 (19900), 284.5 (24900), 340.0 (3450). sh ( $\epsilon$ ) 332.3 (6780).

**Example 133:** *tert*-Butyl *N*-(4-(((2-amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl)(2-propynyl)amino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate

A 50 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 0.097 g of 4-(((2-amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl)(2-propynyl)amino)benzoic acid (Jones, T.R., *et al*, *J. Med. Chem.*, 1986, 29, 1114; Nair, M.G., *et al*, *J. Med. Chem.*, 1986, 29, 1754; Ghazala, M., *et al*, *J. Med. Chem.*, 1986, 29, 1263; Acharya, S.P., *et al*, *J. Heterocyclic Chem.*, 1975, 12, 1283), 0.142 g of *tert*-

butyl 5-*O-tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate, 0.044 g of 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (Aldrich), and 7 mL of anhydrous DMF. The mixture was treated with 0.057 g of EDC and was stirred at RT under nitrogen. The solid starting material slowly dissolved to give a clear solution. After 3 days the solution was concentrated *in vacuo* to dryness and the residue was subjected to flash chromatography on silica gel (98:2 → 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.142 g (65%) of *tert*-butyl *N*-(4-(((2-amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl)(2-propynyl)amino)benzoyl)-5-*O-tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate as a white solid, m.p. 115°C (dec).

Example 134: *N*-(4-(((2-Amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl)(2-propynyl)amino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine

According to example 50, 0.114 g of *tert*-butyl *N*-(4-(((2-amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl)(2-propynyl)amino)benzoyl)-5-*O-tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-phenylalaninate was treated with gaseous HCl in CH<sub>3</sub>NO<sub>2</sub> for 2h. The reaction mixture was concentrated to dryness and the residue was suspended in 50 mL of ether. The resulting solid was collected by filtration and dried *in vacuo*. The crude product was purified by semi-preparative reverse phase HPLC (C18, 68:32:0.1 → 60:40:0.1 H<sub>2</sub>O:MeCN:TFA). Fractions containing pure product (as determined by analytical HPLC) were combined and concentrated to dryness. The residue was suspended in water and lyophilized to afford 0.053 g (45%) of *N*-(4-(((2-amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl)(2-propynyl)amino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine as a fluffy white solid, m.p. 158°C (dec).

HPLC: two peaks on C18; *k'*=1.27 (98.8%), *k'*=0.50 (1.2%); 62:38:0.1

H<sub>2</sub>O:MeCN:TFA; flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 12.90-11.80 (br m, COOH), 8.07 (d, 1H, J=7.9, amide NH), 8.03 (d, 1H, J=7.9, amide NH), 7.87 (s, 1H, aromatic CH), 7.71 (d, 2H, J=8.7, aromatic CH), 7.62 (d, 1H, J=8.6, aromatic CH), 7.57-7.24 (br s, 2H, NH<sub>2</sub>), 7.31 (d, 1H, J=8.6, aromatic CH), 7.13-6.95 (m, 4H, aromatic CH), 6.82 (d, 2H, J=8.8, aromatic CH), 4.71 (s, 2H, quinazolinyl-CH<sub>2</sub>), 4.41 (m, 2H, methines), 4.30 (s, 2H, propargyl CH<sub>2</sub>), 3.19 (s, 1H, propargyl CH), 3.10-2.77 (m, 3H, ArCH<sub>2</sub>, cyclopentyl methine), 2.23 (t, 2H, J=7.6, glu 4-CH<sub>2</sub>), 2.02-1.76 (m, 4H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.75-1.38 (m, 6H, cyclopentyl CH<sub>2</sub>).

<sup>19</sup>F-NMR: (282 MHz, DMSO-d<sub>6</sub>) δ 1.53 (relative to TFA external standard).

Elemental Analysis: Calcd. for C<sub>38</sub>H<sub>40</sub>N<sub>6</sub>O<sub>7</sub>·TFA·2.2H<sub>2</sub>O (MW 846.43): C, 56.76; H, 5.41; N, 9.93. Found: C, 56.79; H, 5.42; N, 9.95.

Mass Spectrum: (FAB) 693 (M+H)<sup>+</sup>.

UV Spectrum: (pH 7 Buffer) λ<sub>max</sub> (ε) 227.2 (48400), 306.4 (24500). λ<sub>min</sub> (ε) 213.5 (40200), 252.2 (11500).

Example 135: Ethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-L-phenylalaninate

A 500 mL round bottomed flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 10.0 g of *N*-benzyloxycarbonyl-L-glutamic acid γ-ethyl ester, 7.43 g of L-phenylalanine ethyl ester hydrochloride (Aldrich), 100 mL of CH<sub>2</sub>Cl<sub>2</sub>, and 4.51 mL of Et<sub>3</sub>N. The solution was cooled to 0°C and treated with a solution of 6.67 g of DCC (Fluka) in 25 mL of CH<sub>2</sub>Cl<sub>2</sub>. The solution was stirred at 0°C for 1h and then allowed to warm to RT. After an additional 3h, the mixture was filtered to remove dicyclohexylurea. The filtrate was washed with 10% aqueous citric acid (3x50mL), saturated aqueous NaHCO<sub>3</sub> (3x50 mL), followed by one 60 mL portion of water. The solution was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, concentrated to 30 mL, and triturated with



addition of 50 mL of ether followed by 150 mL of pentane. The resulting precipitate was collected by filtration and dried *in vacuo* to afford 12.9 g (82%) of ethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-L-phenylalaninate as a white crystalline solid, m.p. 85-87°C.

**Example 136: Ethyl 5-*O*-ethyl-L-glutam-1-yl-L-phenylalaninate**

According to example 10, 13.08 g of ethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-L-phenylalaninate was hydrogenolyzed in 300 mL of EtOH using 2.43 g of 10% Pd(C) and 2 mL of acetyl chloride to afford 9.84 g (94%) of ethyl 5-*O*-ethyl-L-glutam-1-yl-L-phenylalaninate·HCl as a white foam.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 9.08 (d, 1H, J=7.0, NH), 7.90-7.40 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.29 (m, 5H, aromatic CH), 4.50 (m, 1H, methine), 4.07 (m, 4H, ethyl CH<sub>2</sub>), 3.80 (m, 1H, methine), 3.04 (m, 2H, ArCH<sub>2</sub>), 2.46 (m, 2H, glu 4-CH<sub>2</sub>), 1.99 (m, 2H, glu 3-CH<sub>2</sub>), 1.20 (t, 3H, J=7.1, ethyl CH<sub>3</sub>), 1.13 (t, 3H, J=7.1, ethyl CH<sub>3</sub>).

**Example 137: Ethyl *N*-((S)-4-(ethoxycarbonyl)-2-(4-nitrophthalimido)butanoyl)-L-phenylalaninate**

A 1L round bottomed flask equipped with a magnetic stirrer, a condenser, a nitrogen inlet, and a Dean Stark trap was charged with 7.28 g of ethyl 5-*O*-ethyl-L-glutam-1-yl-L-phenylalaninate·HCl, 3.68 g of 4-nitrophthalic anhydride (American Tokyo Kasei, Portland, OR 97203), 300 mL of toluene, and 2.43 g of diisopropylethylamine (Aldrich). The reaction mixture was heated at reflux for 2h, allowed to cool to RT, and stirred at RT overnight under nitrogen. The toluene was removed by rotary evaporation and the residue was dissolved in 400 mL of CH<sub>2</sub>Cl<sub>2</sub>. The solution was washed with water (2x200 mL), 5% aqueous NaHCO<sub>3</sub> (2x250 mL), and dried over anhydrous MgSO<sub>4</sub>. The drying agent was removed by filtration and the filtrate was concentrated to 100 mL.

The concentrate was mixed with excess ether to induce precipitation. The precipitate was collected by filtration and dried *in vacuo* to afford 6.72 g (67%) of ethyl *N*-((*S*)-4-(ethoxycarbonyl)-2-(4-nitrophthalimido)butanoyl)-*L*-phenylalaninate as a white solid.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>) δ 8.65 (m, 2H, aromatic CH, NH), 8.49 (s, 1H, aromatic CH), 8.13 (d, 1H, J=8.6, aromatic CH), 7.27-7.06 (m, 5H, aromatic CH), 4.70 (m, 1H, methine), 4.39 (m, 1H, methine), 4.04 (q, 2H, J=7.1, ethyl CH<sub>2</sub>), 3.93 (q, 2H, J=7.1, ethyl CH<sub>2</sub>), 2.94 (dd; 1H; J=13.7, 5.8; ArCH<sub>2</sub>), 2.82 (dd; 1H; J=13.6, 9.4; ArCH<sub>2</sub>), 2.34 (m, 3H, glu 4-CH<sub>2</sub>, glu 3-CH<sub>2</sub>), 2.18 (m, 1H, glu 3-CH<sub>2</sub>), 1.11 (t, 6H, J=7.1, ethyl CH<sub>3</sub>).

**Example 138: Ethyl *N*-((*S*)-2-(4-aminophthalimido)-4-(ethoxycarbonyl)butanoyl)-*L*-phenylalaninate**

To a 300 mL Parr bottle were added 1.17 g of 10% Pd(C) and 20 mL of EtOAc under a nitrogen flush. To this mixture was added a solution of 6.72 g of ethyl *N*-((*S*)-4-(ethoxycarbonyl)-2-(4-nitrophthalimido)butanoyl)-*L*-phenylalaninate in 200 mL of EtOAc. The mixture was deoxygenated by bubbling nitrogen through and then hydrogenated at 45 psi for 1h. The bottle was purged with nitrogen and the catalyst was removed by filtration through celite. The filtrate was concentrated *in vacuo* to give a residue which was purified by flash chromatography on silica gel (1:1 → 3:7 hexane:EtOAc). This afforded 5.66 g (88%) of ethyl *N*-((*S*)-2-(4-aminophthalimido)-4-(ethoxycarbonyl)butanoyl)-*L*-phenylalaninate as a bright yellow solid.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>) δ 8.50 (d, 1H, J=7.4, NH), 7.48 (d, 1H, J=8.2, aromatic CH), 7.26-7.08 (m, 5H, aromatic CH), 6.92 (s, 1H, aromatic CH), 6.82 (dd; 1H; J=8.3, 1.6; aromatic CH), 6.47 (s, 2H, NH<sub>2</sub>), 4.54 (m, 1H, methine), 4.35 (q, 1H, J=7.4, methine), 4.07-3.86 (m, 4H, ethyl CH<sub>2</sub>), 2.92 (m, 2H, ArCH<sub>2</sub>), 2.40-2.13 (m, 4H, glu 4-CH<sub>2</sub>, glu 3-CH<sub>2</sub>), 1.09 (m, 6H, ethyl CH<sub>3</sub>).

Example 139: Ethyl N-((S)-2-(5-amino-1-oxo-2-isoindoliny)-4-(ethoxycarbonyl)butanoyl)-L-phenylalaninate

A zinc-mercury amalgam was prepared by stirring 19.8 g of zinc dust (Mallinckrodt, Inc., Paris, KY, 40361) in 500 mL of 10% aqueous HCl for 10 min. The HCl solution was decanted and the zinc was washed with water until neutral pH was achieved. A warm solution of 0.10 g of HgCl<sub>2</sub> in water was added to the zinc and the mixture was stirred for 10 min. The amalgam was collected by filtration, washed consecutively with water, EtOH, and ether and air dried. The dry amalgam was added to a solution of 3.0 g of ethyl N-((S)-2-(4-aminophthalimido)-4-(ethoxycarbonyl)butanoyl)-L-phenylalaninate and 60 mL of 1N ethereal HCl in 250 mL of EtOH which was maintained at 0°C in an ice bath. After stirring at 0°C for 25 min, tlc (SiO<sub>2</sub>, 3:7 hexane:EtOAc) indicated only a trace of starting material at R<sub>f</sub>=0.58 and two new components at R<sub>f</sub>=0.52 and 0.63. The reaction mixture was filtered to remove the amalgam and the filtrate was concentrated *in vacuo* to 200 mL. The solution was hydrogenated at 50 psi in the presence of 0.54 g of 10% Pd(C) for 18h. The catalyst was removed by filtration through celite and the filtrate was neutralized by addition of excess NaHCO<sub>3</sub> and then concentrated to dryness. The residue was partitioned between water and CH<sub>2</sub>Cl<sub>2</sub> and the layers were separated. The CH<sub>2</sub>Cl<sub>2</sub> solution was washed with water (2x200 mL) and the water layer was extracted with EtOAc (200 mL). The CH<sub>2</sub>Cl<sub>2</sub> and EtOAc solutions were combined, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated to give 2.77 g of a pale yellow foam. Analysis of this material by tlc (SiO<sub>2</sub>, 9:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone) indicated four major components at R<sub>f</sub>=0.50, 0.41, 0.29, and 0.20. These materials were separated by flash chromatography on silica gel (9:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone). Comparative <sup>1</sup>H-NMR analysis indicated the R<sub>f</sub>=0.29 material to be

ethyl *N*-((*S*)-2-(5-amino-1-oxo-2-isoindoliny)-4-(ethoxycarbonyl)butanoyl)-*L*-phenylalaninate. The product was obtained as a pale yellow foam, 0.646 g (22%).  
<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 8.48 (d, 1H, J=7.7, NH), 7.32 (d, 1H, J=8.2, aromatic CH), 7.14 (s, 5H, aromatic CH), 6.59 (m, 2H, aromatic CH), 5.78 (s, 2H, NH<sub>2</sub>), 4.73 (m, 1H, methine), 4.43 (m, 1H, methine), 4.13-3.90 (m, 6H, ethyl CH<sub>2</sub>, isoindoliny methylene), 3.07-2.85 (m, 2H, ArCH<sub>2</sub>), 2.26-2.01 (m, 3H, glu 4-CH<sub>2</sub>, glu 3-CH<sub>2</sub>), 1.88 (m, 1H, glu 3-CH<sub>2</sub>), 1.12 (m, 6H, ethyl CH<sub>3</sub>).

Example 140: Ethyl *N*-((*S*)-2-(5-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-1-oxo-2-isoindoliny)-4-(ethoxycarbonyl)butanoyl)-*L*-phenylalaninate

According to example 5, 1.04 g of 9-bromomethyl-3-methylbenzo(f)quinazolin-1(2H)-one was reacted with 1.65 g of ethyl *N*-((*S*)-2-(5-amino-1-oxo-2-isoindoliny)-4-(ethoxycarbonyl)butanoyl)-*L*-phenylalaninate using 0.84 g of NaHCO<sub>3</sub>. The reaction mixture was stirred at 100°C for 5h, cooled to RT, and stirred at RT for 18h. Analysis by tlc (SiO<sub>2</sub>, 4:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone) indicated incomplete reaction. An additional 0.3 g of 9-bromomethyl-3-methylbenzo(f)quinazolin-1(2H)-one was added and the reaction mixture was stirred at 100°C for 4h. The mixture was cooled to RT and filtered to remove solids. The crude product was adsorbed onto 25 g of silica gel by adding silica gel to the filtrate, concentrating to dryness, and storing *in vacuo*. The product/silica gel mixture was loaded onto a column (1000 g SiO<sub>2</sub>) and subjected to flash chromatography (CH<sub>2</sub>Cl<sub>2</sub> → 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to give 0.30 g of pure product. Impure chromatography fractions were combined, concentrated, and subjected to flash chromatography a second time (98:2 → 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to give 0.26 g of pure product. This afforded a total yield of 0.56 g (23%) of ethyl *N*-((*S*)-2-(5-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-1-oxo-2-isoindoliny)-4-(ethoxycarbonyl)butanoyl)-*L*-phenylalaninate as a white solid, m.p. 140°C (dec).

Example 141: *N*-((*S*)-4-Carboxy-2-(5-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-1-oxo-2-isoindoliny)butanoyl)-L-phenylalanine

To a 100 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet were added 0.90 g of ethyl *N*-((*S*)-2-(5-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-1-oxo-2-isoindoliny)-4-(ethoxycarbonyl)butanoyl)-L-phenylalaninate, 3 mL of EtOH, and 10 mL of 0.2N aqueous NaOH. The solution was stirred at RT under nitrogen for 2h and was acidified to pH 3.0 by addition of 1N HCl. A white precipitate resulted which was separated by centrifugation. Analysis of this material by analytical HPLC (C18, 70:30:0.10 H<sub>2</sub>O:MeCN:TFA) indicated a major component at  $k'=1.93$  (90%) and a minor component at  $k'=2.41$ . The crude product was purified by semi-preparative reverse phase HPLC (C18, 75:25:0.1 H<sub>2</sub>O:MeCN:TFA). Fractions containing pure product ( $k'=1.93$  by analytical HPLC using above conditions) were combined and concentrated *in vacuo* to dryness. The residue was dissolved in 15 mL of 0.1N aqueous NaOH and the solution was adjusted to pH 3.0 by addition of 1N HCl. The resulting precipitate was collected by centrifugation, washed with four cycles of aqueous suspension-centrifugation-decantation, and lyophilized to afford 0.035 g (40%) of *N*-((*S*)-4-carboxy-2-(5-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)methyl)amino)-1-oxo-2-isoindoliny)butanoyl)-L-phenylalanine as a white powder, m.p. 180°C.

HPLC: one peak on C18,  $k'=2.3$ , 70:30:0.10 H<sub>2</sub>O:MeCN:TFA, flow rate=1mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  12.83-12.56 (br s, 1H, COOH), 12.53 (s, 1H, benzoquinazoline NH), 12.20-11.90 (br s, 1H, COOH), 9.88 (s, 1H, aromatic CH), 8.19 (m, 2H, amide NH, aromatic CH), 8.00 (d, 1H,  $J=8.4$ , aromatic CH), 7.66 (d, 1H,  $J=7.4$ , aromatic CH), 7.57 (d, 1H,  $J=8.7$ , aromatic CH), 7.33 (d, 1H,  $J=8.6$ , aromatic CH), 7.17

(m, 1H, isoindoliny 5-NH), 7.03 (d, 2H, J=7.2, aromatic CH), 6.90 (m, 3H, aromatic CH), 6.72 (d, 1H, J=7.8, aromatic CH), 6.59 (s, 1H, aromatic CH), 4.67 (m, 1H, methine), 4.57 (d, 2H, J=5.9, benzoquinazoline 9-CH<sub>2</sub>), 4.38 (m, 1H, methine), 4.02 (d, 1H, J=16.8, isoindoliny methylene), 3.76 (d, 1H, J=16.9, isoindoliny methylene), 3.00 (m, 1H, ArCH<sub>2</sub>), 2.80 (m, 1H, ArCH<sub>2</sub>), 2.41 (s, 3H, CH<sub>3</sub>), 2.02 (m, 3H, glu 4-CH<sub>2</sub>, glu 3-CH<sub>2</sub>), 1.76 (m, 1H, glu 3-CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>36</sub>H<sub>33</sub>N<sub>5</sub>O<sub>7</sub>·2.3H<sub>2</sub>O (MW 689.12): C, 62.75; H, 5.50; N, 10.16. Found: C, 62.82; H, 5.50; N, 10.18.

Mass Spectrum: (FAB) 648 (M+H)<sup>+</sup>.

UV Spectrum: (pH 7 Buffer) λ<sub>max</sub> (ε) 265.6 (38900), 301.7 (21100), 348.4 (4870). λ<sub>min</sub> (ε) 245.1 (19700), 284.1 (18300), 341.8 (3420). sh (ε) 331.7 (7570).

Example 142: *tert*-Butyl (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-(trimethylsilyl)benzyl)-4-morpholinecarboxylate

A dry 250 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 2.08 g of *t*-butyl (2*R*, 3*S*)-(-)-6-oxo-2,3-diphenyl-4-morpholinecarboxylate (Aldrich), 1.43 g of 3-(trimethylsilyl)benzyl bromide (Yamakawa, T., *et al*, J. Med. Chem., 1990, **33**, 1430), and 45 mL of anhydrous THF. The solution was cooled to -78°C and was treated with 6.17 mL of 1 M sodium bis(trimethylsilyl)amide/THF (Aldrich) via syringe through a rubber septum. The solution was stirred at -78°C for 3.5h and was then poured into 150 mL of water. The resulting mixture was extracted with EtOAc (4x50 mL). The combined EtOAc extracts were washed with saturated brine (3x100 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo* to give a light yellow oil. The crude material was purified by flash chromatography on silica gel (9:1 hexane:EtOAc) to afford 2.6 g (88%) of *tert*-butyl (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-(trimethylsilyl)benzyl)-4-morpholinecarboxylate as a white powder, m.p. 88-90°C.

Example 143: (2R, 3S, 5S)-6-Oxo-2,3-diphenyl-5-(3-(trimethylsilyl)benzyl)morpholine

According to example 82, 2.47 g of *tert*-butyl (2R, 3S, 5S)-6-oxo-2,3-diphenyl-5-(3-(trimethylsilyl)benzyl)-4-morpholinecarboxylate was treated with 4.1 mL of TFA in 40 mL of CH<sub>2</sub>Cl<sub>2</sub> for 3 h. Neutralization with 8 mL of Et<sub>3</sub>N and work-up in the usual manner gave a thick oil. Purification of the crude material by flash chromatography on 80 g of silica gel (85:15 hexane:EtOAc) afforded 1.70 g (83%) of (2R, 3S, 5S)-6-oxo-2,3-diphenyl-5-(3-(trimethylsilyl)benzyl)morpholine as a white crystalline solid, m.p. 94-95°C.

Example 144: *tert*-Butyl 3-(trimethylsilyl)-L-phenylalaninate

According to example 53, 1.58 g of (2R, 3S, 5S)-6-oxo-2,3-diphenyl-5-(3-(trimethylsilyl)benzyl)morpholine was hydrogenolyzed in 70 mL of 1:1 THF:EtOH using 0.70 g of PdCl<sub>2</sub>. Analysis of the product by <sup>1</sup>H-NMR indicated partial desilylation of the phenyl ring. The mixture was subjected to esterification according to example 54 using 25 mL of isobutylene and 0.5 mL of conc. H<sub>2</sub>SO<sub>4</sub> in 25 mL of 1,4-dioxane. Work-up in the usual manner afforded a light yellow oil which by tlc (SiO<sub>2</sub>, EtOAc) consisted of two components at R<sub>f</sub>=0.53 and 0.47. The R<sub>f</sub>=0.53 material was isolated by flash chromatography twice on silica gel (EtOAc then 1:1 hexane:EtOAc). This gave 0.161 g (15%) of *tert*-butyl 3-(trimethylsilyl)-L-phenylalaninate as a clear oil. A sample of the corresponding HCl salt was prepared for <sup>1</sup>H-NMR by treating 5mg of product with excess 1N ethereal HCl and concentrating to dryness.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 8.53 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.50-7.20 (m, 4H, aromatic CH), 4.14 (m, 1H, methine), 3.21 (m, 1H, ArCH<sub>2</sub>), 2.98 (m, 1H, ArCH<sub>2</sub>), 1.26 (s, 9H, t-Bu), 0.23 (s, 9H, trimethylsilyl).

Example 145: *tert*-Butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(trimethylsilyl)-L-phenylalaninate

To a 50 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet were added 0.161 g of *tert*-butyl 3-(trimethylsilyl)-L-phenylalaninate, 0.194 g of *N*-Cbz-L-glutamic acid  $\gamma$ -*tert*-butyl ester (Sigma), and 10 mL of CH<sub>2</sub>Cl<sub>2</sub>. The solution was cooled in to 0°C and was treated with 0.110 g of EDC. The reaction mixture was stirred at 0°C for 1.5h and was then allowed to warm to RT. After an additional 16h of stirring at RT, the solution was concentrated *in vacuo* to dryness in the presence of 5 g of silica gel. The resulting mixture was applied to a column and the product isolated by flash chromatography (SiO<sub>2</sub>, 75:25 hexane:EtOAc) to afford 0.320 g (95%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(trimethylsilyl)-L-phenylalaninate as a thick, transparent glass.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>)  $\delta$  8.29 (d, 1H, J=7.0, NH), 7.50-7.18 (m, 10H, aromatic CH, NH), 5.02 (s, 2H, PhCH<sub>2</sub>O), 4.38 (m, 1H, methine), 4.07 (m, 1H, methine), 2.97 (d, 2H, J=7.3, ArCH<sub>2</sub>), 2.23 (t, 2H, J=7.6, glu 4-CH<sub>2</sub>), 1.96-1.60 (m, 2H, glu 3-CH<sub>2</sub>), 1.40 (s, 9H, t-Bu), 1.31 (s, 9H, t-Bu), 0.24 (s, 9H, trimethylsilyl).

Example 146: *tert*-Butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-(trimethylsilyl)-L-phenylalaninate

According to example 48, 0.315 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(trimethylsilyl)-L-phenylalaninate was hydrogenolyzed using 0.051 g of 10% Pd(C) in 35 mL of MeOH. This afforded 0.24 g (98%) of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-(trimethylsilyl)-L-phenylalaninate as a thick transparent oil. A sample of the corresponding HCl salt for <sup>1</sup>H-NMR analysis was prepared by treating 5 mg of the product with excess 1N ethereal HCl and concentrating to dryness.



<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 8.99 (d, 1H, J=7.2, NH), 8.28 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.47-7.24 (m, 4H, aromatic CH), 4.42 (m, 1H, methine), 3.86 (m, 1H, methine), 3.01 (d, 2H, J=7.2, ArCH<sub>2</sub>), 2.37 (m, 2H, glu 4-CH<sub>2</sub>), 2.01 (m, 2H, glu 3-CH<sub>2</sub>), 1.42 (s, 9H, t-Bu), 1.32 (s, 9H, t-Bu), 0.26 (s, 9H, trimethylsilyl).

Example 147: *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(trimethylsilyl)-L-phenylalaninate

According to example 49, 0.095 g of 4-(*N*-(2,4-diamino-6-pteridinylmethyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich) was coupled with 0.12 g of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-(trimethylsilyl)-L-phenylalaninate using 0.11 mL of DECP (Aldrich) and 0.12 mL of Et<sub>3</sub>N in 11 mL of DMF. The crude product was subjected to flash chromatography on silica gel (7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH) to afford 0.141 g (72%) of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(trimethylsilyl)-L-phenylalaninate as a yellow powder, m.p. 118-125°C.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 8.58 (s, 1H, pteridinyl 7-CH), 8.23 (d, 1H, J=7.5, amide NH), 8.00 (d, 1H, J=7.8, amide NH), 7.74 (d, 2H, J=8.7, aromatic CH), 7.70 (br s, 1H, NH<sub>2</sub>), 7.48 (br s, 1H, NH<sub>2</sub>), 7.33 (m, 2H, aromatic CH), 7.22 (m, 2H, aromatic CH), 6.83 (d, 2H, J=8.8, aromatic CH), 6.63 (br s, 2H, NH<sub>2</sub>), 4.79 (s, 2H, pteridinyl-CH<sub>2</sub>), 4.53-4.29 (m, 2H, methines), 3.23 (s, 3H, N-CH<sub>3</sub>), 2.96 (d, 2H, J=7.0, ArCH<sub>2</sub>), 2.23 (m, 2H, glu 4-CH<sub>2</sub>), 2.04-1.77 (m, 2H, glu 3-CH<sub>2</sub>), 1.38 (s, 9H, t-Bu), 1.28 (s, 9H, t-Bu), 0.21 (s, 9H, trimethylsilyl).

Example 148: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-(trimethylsilyl)-L-phenylalanine

A 100 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 0.111 g of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(trimethylsilyl)-L-phenylalaninate and 25 mL of CH<sub>3</sub>NO<sub>2</sub>. The resulting solution was treated with 5 mL of TFA and stirred at RT. After 1h, the solution was concentrated *in vacuo* to dryness. The residue was dissolved in 3 mL of CH<sub>3</sub>NO<sub>2</sub> and the solution was treated with 20 mL of ethyl ether. A yellow solid precipitated which was collected by filtration and dried *in vacuo*. The product was dissolved in 3 mL of 1:1 H<sub>2</sub>O:CH<sub>3</sub>CN and the solution mixed with 50 mL of water. A suspension resulted which was frozen and lyophilized to afford 0.90 g (82%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-(trimethylsilyl)-L-phenylalanine as a yellow powder, m.p. 155°C (dec).

HPLC: one peak on C18, *k'*=1.55, 65:35:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 13.00-11.75 (br s, COOH), 8.64 (s, 1H, pteridiny 7-CH), 8.60 (br s, 1H, NH<sub>2</sub>), 8.39 (br s, 1H, NH<sub>2</sub>), 8.11 (d, 1H, *J*=7.7, amide NH), 7.97 (d, 1H, *J*=8.1, amide NH), 7.75-7.10 (br m, 2H, NH<sub>2</sub>), 7.69 (d, 2H, *J*=8.9, aromatic CH), 7.28 (m, 2H, aromatic CH), 7.14 (m, 2H, aromatic CH), 6.79 (d, 2H, *J*=8.9, aromatic CH), 4.82 (s, 2H, pteridiny-CH<sub>2</sub>), 4.39 (m, 2H, methines), 3.21 (s, 3H, N-CH<sub>3</sub>), 3.02 (m, 1H, ArCH<sub>2</sub>), 2.88 (m, 1H, ArCH<sub>2</sub>), 2.20 (t, 2H, *J*=7.8, glu 4-CH<sub>2</sub>), 2.00-1.72 (m, 2H, glu 3-CH<sub>2</sub>), 0.17 (s, 9H, trimethylsilyl).

Elemental Analysis: Calcd. for C<sub>32</sub>H<sub>39</sub>N<sub>9</sub>O<sub>6</sub>Si·0.8 TFA·1.2 H<sub>2</sub>O (MW 786.64): C, 51.30; H, 5.41; N, 16.03. Found: C, 51.24; H, 5.39; N, 16.07.

Mass Spectrum: (FAB) 674 (M+H)<sup>+</sup>.

UV Spectrum: (pH 7 Buffer) λ<sub>max</sub> (ε) 259.5 (23500), 308.0 (24300), 373.2 (7210). λ<sub>min</sub> (ε) 240.7 (12700), 273.9 (15500), 345.0 (5210).

Example 149: *tert*-Butyl 3-iodo-L-tyrosinate

A mixture of 5.0 g of 3-iodo-L-tyrosine (Aldrich) and 2.5 mL of conc. H<sub>2</sub>SO<sub>4</sub> in 85 mL of 1,4-dioxane was added to 30 mL of liquid isobutylene (condensed at -78°C) in a 300 mL pressure bottle. The vessel was stoppered and the contents stirred at RT. After 3 days the bottle was cooled in an ice water bath, opened and the solution poured into a mixture of 10 g of NaHCO<sub>3</sub> in 150 mL of water. The resulting mixture was subjected to rotary evaporation to remove isobutylene and dioxane. A white solid resulted which was collected by filtration and dried *in vacuo*. Analysis by tlc (SiO<sub>2</sub>, 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) indicated major and minor components at R<sub>f</sub>=0.52 and 0.60 respectively. The crude product was purified by flash chromatography on silica gel (95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 3.4 g (58%) of *tert*-butyl 3-iodo-L-tyrosinate as a white crystalline solid, m.p. 158-159°C.

Example 150: *tert*-Butyl *N*-((benzyloxy)carbonyl)-4-*O*-((benzyloxy)carbonyl)-3-iodo-L-tyrosinate

A 500 mL round bottomed flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 5.15 g of *tert*-butyl 3-iodo-L-tyrosinate, 250 mL of 1,4-dioxane, and 4.55 mL of Et<sub>3</sub>N. The resulting solution was treated with 4.66 mL of benzyl chloroformate by slow addition. After 30 min, tlc (SiO<sub>2</sub>, 8:2 hexane:EtOAc) indicated no remaining starting material and a single new component at R<sub>f</sub>=0.50. The solution was concentrated *in vacuo* and the residue mixed with 200 mL of water. The mixture was extracted with EtOAc (4x50mL). The combined EtOAc extracts were washed with 5% aqueous NaHCO<sub>3</sub>, dried over anhydrous MgSO<sub>4</sub>, and concentrated *in vacuo*. The crude product was subjected to flash chromatography on silica gel (8:2 hexane:EtOAc) to

afford 7.49 g (84%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-4-*O*-((benzyloxy)carbonyl)-3-iodo-L-tyrosinate as a transparent foam.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 7.77 (m, 2H, NH, aromatic CH), 7.53-7.21 (m, 12H, aromatic CH), 5.32 (s, 2H, PhCH<sub>2</sub>O), 5.20 (s, 2H, PhCH<sub>2</sub>O), 4.12 (m, 1H, methine), 2.92 (m, 2H, ArCH<sub>2</sub>), 1.35 (s, 9H, t-Bu).

Example 151: *tert*-Butyl 3-cyclopentyl-L-tyrosinate

A 300 mL Parr bomb was charged with 5.13 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-4-*O*-((benzyloxy)carbonyl)-3-iodo-L-tyrosinate, 120 mL of toluene, 30 mL of cyclopentene and 0.49 g of tri-ortho-tolylphosphine. The solution was deoxygenated by bubbling nitrogen through for 10 min and was treated with 0.182 g of Pd(OAc)<sub>2</sub> and 1.24 mL of Et<sub>3</sub>N. The bomb was flushed with nitrogen, sealed, and heated to 110°C for 48h. The vessel was cooled to RT, purged with nitrogen and opened. The reaction mixture was filtered to remove solids and the filtrate was concentrated *in vacuo* to dryness. The residue was dissolved in 200 mL of EtOAc and the solution was washed with water (4x100 mL), dried over anhydrous NaSO<sub>4</sub> and concentrated to give a thick brown oil. Analysis by tlc (SiO<sub>2</sub>, 8:2 hexane:EtOAc) indicated two major components at R<sub>f</sub>=0.50 and 0.40. The crude mixture was subjected to flash chromatography on silica gel (8:2 hexane:EtOAc). This afforded 2.0 g (43%) of the R<sub>f</sub>=0.50 material identified as the bis(benzyloxycarbonyl) olefin mixture by <sup>1</sup>H-NMR. In addition, 0.90 g (25%) of the R<sub>f</sub>=0.40 material was obtained and was identified as the *N*-benzyloxycarbonyl olefin mixture resulting from loss of the *O*-benzyloxycarbonyl group. The bis(benzyloxycarbonyl) olefin mixture (2.00 g) was subjected to catalytic hydrogenation (45 psi) in the presence of 0.35 g of 10% Pd(C) in 70 mL of MeOH for 18h. The crude product was purified by flash chromatography (SiO<sub>2</sub>, EtOAc) to give 0.99 g (93%) of *tert*-butyl 3-cyclopentyl-L-tyrosinate as a transparent glass. The *N*-benzyloxycarbonyl

olefin mixture (0.90 g) was similarly hydrogenated and purified to afford 0.52 g (83%) of *tert*-butyl 3-cyclopentyl-L-tyrosinate.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 9.05 (s, 1H, OH), 6.92 (d, 1H, J=1.9, aromatic CH), 6.80 (dd; 1H; J=8.0, 2.0; aromatic CH), 6.66 (d, 1H, J=8.0, aromatic CH), 3.16 (m, 1H, cyclopentyl methine), 2.67 (d, 2H, J=6.6, ArCH<sub>2</sub>), 2.00-1.40 (m, 10H, cyclopentyl CH<sub>2</sub>, NH<sub>2</sub>), 1.33 (s, 9H, t-Bu).

Example 152: *tert*-Butyl N-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-tyrosinate

According to example 145, 0.99 g of *tert*-butyl 3-cyclopentyl-L-tyrosinate was coupled with 1.09 g of *N*-Cbz-L-glutamic acid γ-*tert*-butyl ester (Sigma) using 0.65 g of EDC in 20 mL of CH<sub>2</sub>Cl<sub>2</sub> for 4h. The crude product was purified by flash chromatography on silica gel (7:3 hexane:EtOAc) to afford 1.8 g (89%) of *tert*-butyl N-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-tyrosinate as a colorless foam.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 9.08 (s, 1H, OH), 8.17 (d, 1H, J=7.5, NH), 7.36 (m, 6H, aromatic CH, NH), 6.96 (s, 1H, aromatic CH), 6.81 (d, 1H, J=8.1, aromatic CH), 6.67 (d, 1H, J=8.1, aromatic CH), 5.01 (m, 2H, PhCH<sub>2</sub>O), 4.28 (m, 1H, methine), 4.08 (m, 1H, methine), 3.17 (m, 1H, cyclopentyl methine), 2.82 (d, 2H, J=6.9, ArCH<sub>2</sub>), 2.21 (t, 2H, J=7.7, glu 4-CH<sub>2</sub>), 1.99-1.46 (m, 10H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.40 (s, 9H, t-Bu), 1.33 (s, 9H, t-Bu).

Example 153: *tert*-Butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-tyrosinate

According to example 48, 1.75 g of *tert*-butyl N-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-tyrosinate was hydrogenolyzed using 0.28 g of 10% Pd(C) in 70 mL of MeOH for 3h. The crude product was subjected to flash chromatography

on silica gel (95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 1.26 g (92%) of *tert*-butyl 5-*O-tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-tyrosinate as a white crystalline solid, m.p. 108-110°C.

Example 154: *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O-tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-tyrosinate

To a dry 100 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet were added 35 mL of anhydrous DMF, 1.14 mL of Et<sub>3</sub>N, and 0.99 mL of DECP. To the resulting solution were added 1.24 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate (Aldrich). The solid starting material slowly dissolved to give a yellow solution. After 45 min, a solution of 1.60 g of *tert*-butyl 5-*O-tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-tyrosinate in 8 mL of DMF was added. The solution was stirred at RT for 24h and was then concentrated *in vacuo* to dryness. The residue was dissolved in 150 mL of CHCl<sub>3</sub>. The solution was washed with 5% aqueous NH<sub>4</sub>OH (3x70 mL), dried over anhydrous MgSO<sub>4</sub>, and concentrated *in vacuo*. The crude product was subjected to flash chromatography on silica gel twice (7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH, 93:7 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 2.08 g (80%) of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O-tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-tyrosinate as a yellow powder, m.p. 145-148°C.

Example 155: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-tyrosine

According to example 50, 2.04 g of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O-tert*-butyl-L-glutam-1-yl-3-cyclopentyl-L-tyrosinate was treated with gaseous HCl in 65 mL of CH<sub>3</sub>NO<sub>2</sub> for 1h. The yellow suspension was concentrated to a slurry and mixed with 80 mL of ethyl ether. The

resulting precipitate was collected by filtration and dried *in vacuo*. The product was dissolved in 40 mL of 8:2 H<sub>2</sub>O:MeCN and the solution was filtered and concentrated to 30 mL. A yellow suspension was obtained which was diluted with 150 mL of water, frozen, and lyophilized to give 1.71 g (86%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-tyrosine as a yellow-orange powder, m.p. 175°C (dec).

HPLC: one peak on C18,  $k' = 2.98$ , 75:25:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  13.12 (br m, COOH), 9.30 (s, 1H, NH<sub>2</sub>), 9.09 (s, 1H, NH<sub>2</sub>), 8.71 (s, 1H, pteridiny 7-CH), 8.67 (br s, 1H, NH<sub>2</sub>), 8.02 (m, 2H, amide NH's), 7.78 (br s, 1H, NH<sub>2</sub>), 7.71 (d, 2H,  $J = 8.7$ , aromatic CH), 6.91 (s, 1H, aromatic CH), 6.79 (m, 3H, aromatic CH), 6.61 (d, 1H,  $J = 8.1$ , aromatic CH), 4.86 (s, 2H, pteridiny-CH<sub>2</sub>), 4.42 (m, 1H, methine), 4.29 (m, 1H, methine), 3.24 (s, 3H, N-CH<sub>3</sub>), 3.09 (m, 1H, cyclopentyl methine), 2.93-2.71 (m, 2H, ArCH<sub>2</sub>), 2.23 (t, 2H,  $J = 7.8$ , glu 4-CH<sub>2</sub>), 2.03-1.74 (m, 4H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.73-1.37 (m, 6H, cyclopentyl CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>34</sub>H<sub>39</sub>N<sub>9</sub>O<sub>7</sub>·1.6 HCl·1.7 H<sub>2</sub>O (MW 774.70): C, 52.71; H, 5.72; N, 16.27; Cl, 7.32. Found: C, 52.79; H, 5.71; N, 16.16; Cl, 7.35.

Mass Spectrum: (Ion Spray) 686 (M+H)<sup>+</sup>.

UV Spectrum: (pH 7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 259.3 (24700), 307.8 (24800), 372.5 (7780).  $\lambda_{\min}$  ( $\epsilon$ ) 242.3 (15500), 272.9 (17800), 344.6 (6080). sh ( $\epsilon$ ) 221.3 (28900), 286.4 (21100).

Example 156: 3-(3-hydroxy-3-pentyl)-*O*-(*tert*-butyltrimethylsilyl)benzyl alcohol

To a dry 250 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet were added 5.0 g of 3-iodo-*O*-(*tert*-butyldimethylsilyl)benzyl alcohol and 40 mL of anhydrous ethyl ether. The solution was cooled to -78°C under nitrogen and was treated

with 23.2 mL of 1.3 M *sec*-butyllithium/cyclohexane (Aldrich) via syringe. After 20 min, the solution was treated with 3.1 mL of 3-pentanone (Aldrich). The reaction mixture was stirred at -78°C for 1.5h and was then allowed to warm to RT. After 3h at RT the solution was quenched by addition of 5 mL of water, stirred for several minutes and then mixed with 80 mL of water. The two phases were separated and the aqueous solution was extracted with an additional 50 mL portion of ether. The ether extract was combined with the original ether solution. This solution was washed with saturated aqueous brine (3x50 mL), dried over anhydrous MgSO<sub>4</sub>, and concentrated to afford a clear liquid. Analysis by tlc (SiO<sub>2</sub>, 9:1 hexane:EtOAc) indicated a major new component at R<sub>f</sub>=0.47. The crude product was purified by flash chromatography on silica gel (9:1 hexane:EtOAc) to afford 3.9 g (88%) of 3-(3-hydroxy-3-pentyl)-*O*-(*tert*-butyltrimethylsilyl)benzyl alcohol as a clear liquid.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 7.33 (s, 1H, aromatic CH), 7.26 (m, 2H, aromatic CH), 7.12 (m, 1H, aromatic CH), 4.72 (s, 2H, ArCH<sub>2</sub>O), 4.48 (s, 1H, OH), 1.72 (m, 4H, pentyl CH<sub>2</sub>), 0.90 (s, 9H, *t*-Bu), 0.64 (t, 6H, J=7.5, pentyl CH<sub>3</sub>), 0.06 (s, 6H, SiMe<sub>2</sub>).

Example 157: 3-(3-Pentyl)benzyl alcohol

A 250 mL round bottomed flask equipped with a nitrogen inlet and a magnetic stirrer was charged with 3.9 g of 3-(3-hydroxy-3-pentyl)-*O*-(*tert*-butyltrimethylsilyl)benzyl alcohol and 25 mL of CH<sub>2</sub>Cl<sub>2</sub>. The solution was cooled to 0°C in an ice bath and was treated with 15 mL of TFA followed by 5 mL of Et<sub>3</sub>SiH (Aldrich). After 30 min at 0°C, the ice bath was removed and the solution was stirred at RT for 18h. The solution was concentrated to a viscous liquid. This material was dissolved in ether (60 mL), washed with 5% aqueous NaHCO<sub>3</sub> (3x50 mL), dried over anhydrous MgSO<sub>4</sub>, and concentrated *in vacuo*. The resulting liquid was dissolved in 10 mL of THF and treated with 63 mL of 1M Bu<sub>4</sub>NF/THF (Aldrich). After stirring at RT for 18h, the solution was concentrated



and the resulting syrup mixed with 80 mL of water. The mixture was extracted with ether (4x30 mL). The combined extracts were washed with 5% aqueous NaHCO<sub>3</sub> (3x50 mL), dried over anhydrous MgSO<sub>4</sub>, and concentrated to afford a light yellow liquid. Analysis by tlc (SiO<sub>2</sub>, 9:1 hexane:EtOAc) indicated a major new component at R<sub>f</sub>=0.30. The crude product was purified by flash chromatography on silica gel (85:15 hexane:EtOAc) to give 1.84 g (82%) of 3-(3-pentyl)benzyl alcohol as a clear liquid.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 7.21 (t, 1H, J=7.5, aromatic CH), 7.10 (m, 2H, aromatic CH), 6.99 (d, 1H, J=7.4, aromatic CH), 5.12 (t, 1H, J=5.8, OH), 4.46 (d, 2H, J=5.8, ArCH<sub>2</sub>O), 2.26 (m, 1H, pentyl methine), 1.62 (m, 2H, pentyl CH<sub>2</sub>), 1.49 (m, 2H, pentyl CH<sub>2</sub>), 0.69 (t, 6H, J=7.4, pentyl CH<sub>3</sub>).

Example 158: 3-(3-Pentyl)benzyl bromide

According to example 17, 1.84 g of 3-(3-pentyl)benzyl alcohol was treated with 0.34 mL of PBr<sub>3</sub> in 25 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub> to afford 2.23 g (90%) of 3-(3-Pentyl)benzyl bromide as a clear liquid.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 7.26 (m, 3H, aromatic CH), 7.12 (m, 1H, aromatic CH), 4.70 (s, 2H, ArCH<sub>2</sub>), 2.33 (m, 1H, pentyl methine), 1.78-1.40 (m, 4H, pentyl CH<sub>2</sub>), 0.73 (t, 6H, J=7.3, pentyl CH<sub>3</sub>).

Example 159: *tert*-Butyl (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-(3-pentyl)benzyl)-4-morpholinecarboxylate

According to example 142, 3.27 g of *tert*-butyl (2*R*, 3*S*)-(-)-6-oxo-2,3-diphenyl-4-morpholinecarboxylate was alkylated with 2.23 g of 3-(3-pentyl)benzyl bromide in 70 mL of anhydrous THF using 9.71 mL of 1 M sodium bis(trimethylsilyl)amide in THF. The crude product was subjected to flash chromatography on silica gel (9:1 hexane:EtOAc) and then recrystallization from EtOH-water to afford 2.26 g (48%) of

*tert*-butyl (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-(3-pentyl)benzyl)-4-morpholinecarboxylate as a white crystalline solid, m.p. 143-144°C.

Example 160: (2*R*, 3*S*, 5*S*)-6-Oxo-2,3-diphenyl-5-(3-(3-pentyl)benzyl)morpholine

To a 250 mL round bottomed flask equipped with a magnetic stirrer and a nitrogen inlet were added 2.19 g of *tert*-butyl (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-(3-pentyl)benzyl)-4-morpholinecarboxylate and 45 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub>. The solution was cooled to 0°C and was treated with 3.5 mL of TFA. The solution was allowed to warm to RT and was stirred under nitrogen. After 5h, tlc (SiO<sub>2</sub>, 9:1 hexane:EtOAc) indicated a trace of starting material and a major new component at R<sub>f</sub>=0.42. The reaction mixture was poured into 150 mL of 6% aqueous NaHCO<sub>3</sub> and stirred for several minutes. The phases were separated and the aqueous portion was extracted with 3 additional 40 mL portions of CH<sub>2</sub>Cl<sub>2</sub>. The extracts were combined with the original CH<sub>2</sub>Cl<sub>2</sub> solution and washed with 5% aqueous NaHCO<sub>3</sub> (3x60mL). The solution was dried over anhydrous MgSO<sub>4</sub> and concentrated to give a light tan solid. This material was purified by flash chromatography on silica gel (85:15 hexane:EtOAc) to afford 1.47 g (84%) of (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-(3-pentyl)benzyl)morpholine as a white crystalline solid. An analytical sample was prepared by recrystallization from EtOH-H<sub>2</sub>O. Melting point, 96-97°C.

Example 161: 3-(3-Pentyl)-L-phenylalanine

To a 300 mL Parr bottle were added 0.41 g of PdCl<sub>2</sub>, 1.36 g of (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-(3-pentyl)benzyl)morpholine, 50 mL of EtOH, and 30 mL of THF. The mixture was deoxygenated by bubbling nitrogen through for 10min and was hydrogenated at 45 psi for 18h. The vessel was purged with nitrogen, catalyst removed by filtration through celite, and the filtrate concentrated *in vacuo* to a volume of 15 mL.

The solution was chilled in an ice bath and triturated with addition of 50 mL of hexane. A fine precipitate slowly formed. After storing at 5°C overnight, the solid was collected by filtration and dried *in vacuo* to afford 0.65 g (72%) of 3-(3-pentyl)-L-phenylalanine-HCl as a white powder, m.p. 170°C (dec).

Example 162: *tert*-Butyl 3-(3-pentyl)-L-phenylalaninate

According to example 54, 0.60 g of 3-(3-pentyl)-L-phenylalanine-HCl was treated with 30 mL of isobutylene in 35 mL of 1,4-dioxane in the presence of 0.5 mL of conc. H<sub>2</sub>SO<sub>4</sub>. Work-up in the usual manner followed by acidification with 2 mL of 1N ethereal HCl afforded 0.53 g (74%) of *tert*-butyl 3-(3-pentyl)-L-phenylalaninate-HCl as a light yellow glass.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 8.63 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.28 (t, 1H, J=7.45, aromatic CH), 7.10 (m, 3H, aromatic CH), 4.12 (m, 1H, methine), 3.23 (dd, 1H; J=13.9, 5.1; ArCH<sub>2</sub>), 2.96 (dd, 1H; J=13.9, 9.1; ArCH<sub>2</sub>), 2.32 (m, 1H, pentyl methine), 1.78-1.40 (m, 4H, pentyl CH<sub>2</sub>), 1.28 (s, 9H, t-Bu), 0.74 (t, 6H, J=7.3, pentyl CH<sub>3</sub>).

Example 163: *tert*-Butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(3-pentyl)-L-phenylalaninate

According to example 145, 0.53 g of *tert*-butyl 3-(3-pentyl)-L-phenylalaninate-HCl was coupled with 0.55 g of *N*-Cbz-L-glutamic acid γ-*tert*-butyl ester using 0.33 g of EDC and 0.18 mL of *N*-methylmorpholine in 15 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub>. The crude product was purified by flash chromatography on silica gel (8:2 hexane: EtOAc) to afford 0.80 g (81%) of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(3-pentyl)-L-phenylalaninate as a white crystalline solid, m.p. 50-53°C.

Example 164: *tert*-Butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-(3-pentyl)-L-phenylalaninate

According to example 48, 0.72 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(3-pentyl)-L-phenylalaninate was hydrogenolyzed using 0.12 g of 10% Pd(C) in 70 mL of MeOH. This afforded 0.51 g (91%) of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-(3-pentyl)-L-phenylalaninate as a thick transparent oil. A sample of the corresponding HCl salt for <sup>1</sup>H-NMR analysis was prepared by treating 5 mg of the product with excess 1N ethereal HCl and concentrating to dryness.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 9.03 (d, 1H, J=6.9, NH), 8.38 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.23 (t, 1H, J=7.2, aromatic CH), 7.06 (m, 3H, aromatic CH), 4.39 (m, 1H, methine), 3.87 (m, 1H, methine), 3.01 (d, 2H, J=7.4, ArCH<sub>2</sub>), 2.35 (m, 3H, pentyl methine, glu 4-CH<sub>2</sub>), 2.01 (m, 2H, glu 3-CH<sub>2</sub>), 1.75-1.48 (m, 4H, pentyl CH<sub>2</sub>), 1.41 (s, 9H, t-Bu), 1.32 (s, 9H, t-Bu), 0.73 (t, 6H, J=7.3, pentyl CH<sub>3</sub>).

Example 165: *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(3-pentyl)-L-phenylalaninate

According to example 49, 0.199 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate was coupled with 0.25 g of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-(3-pentyl)-L-phenylalaninate using 0.24 mL of DECP and 0.26 mL of Et<sub>3</sub>N in 25 mL of DMF. The crude product was subjected to flash chromatography twice on silica gel (7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH; 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH ) to afford 0.215 g (52%) of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(3-pentyl)-L-phenylalaninate as a yellow powder, m.p. 175°C (dec).

Example 166: *N*-(4-(((2,4-Diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-(3-pentyl)-L-phenylalanine

According to example 50, 0.184 g of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-(3-pentyl)-L-phenylalaninate was treated with gaseous HCl in 25 mL of CH<sub>3</sub>NO<sub>2</sub> for 45 min. The yellow suspension was concentrated *in vacuo* to dryness. Analysis of the product by HPLC (C18, 65:35:0.1 H<sub>2</sub>O:MeCN:TFA) indicated a major component at *k'*=1.61 and a minor component at *k'*=0.25. The crude product was purified by semi-preparative HPLC (C18, 65:35:0.1 H<sub>2</sub>O:MeCN:TFA). Fractions containing pure product (analytical HPLC) were combined and subjected to rotary evaporation to remove CH<sub>3</sub>CN. A yellow suspension resulted which was frozen and lyophilized to afford 0.12 g (58%) of *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-(3-pentyl)-L-phenylalanine as a yellow powder, m.p. 120°C (dec).

HPLC: one peak on C18, *k'*=1.61, 65:35:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>) δ 12.87-11.90 (br m, 2H, COOH), 9.20 (br s, 1H, NH<sub>2</sub>), 9.00 (br s, 1H, NH<sub>2</sub>), 8.70 (s, 1H, pteridiny 7-CH), 8.60-8.23 (br m, 2H, NH<sub>2</sub>), 8.11 (d, 1H, *J*=7.6, amide NH), 7.96 (d, 1H, *J*=7.8, amide NH), 7.70 (d, 2H, *J*=8.7, aromatic CH), 7.07 (m, 1H, aromatic CH), 6.98 (d, 2H, *J*=7.9, aromatic CH), 6.90 (d, 1H, *J*=7.6, aromatic CH), 6.80 (d, 2H, *J*=8.6, aromatic CH), 4.86 (s, 2H, pteridiny-CH<sub>2</sub>), 4.41 (m, 2H, methines), 3.23 (s, 3H, N-CH<sub>3</sub>), 2.92 (m, 2H, ArCH<sub>2</sub>), 2.21 (m, 3H, pentyl methine, glu 4-CH<sub>2</sub>), 2.03-1.77 (m, 2H, glu 3-CH<sub>2</sub>), 1.68-1.37 (m, 4H, pentyl CH<sub>2</sub>), 0.62 (m, 6H, pentyl CH<sub>3</sub>).

Elemental Analysis: Calcd. for C<sub>34</sub>H<sub>41</sub>N<sub>9</sub>O<sub>6</sub>·1.5TFA·1.8 H<sub>2</sub>O (MW 875.22): C, 50.78; H, 5.31; N, 14.40. Found: C, 50.76; H, 5.40; N, 14.34.

Mass Spectrum: (Ion Spray) 672 (M+H)<sup>+</sup>.

UV Spectrum: (pH 7 Buffer) λ<sub>max</sub> (ε) 259.8 (20000), 308.7 (19800), 373.7 (5890).

$\lambda_{\text{min}}$  ( $\epsilon$ ) 241.5 (11600), 275.0 (13200), 345.8 (4420).

Example 167: 2-Iodo-4-nitro-L-phenylalanine

A 250 mL round bottomed flask equipped with a magnetic stirrer was charged with 60 mL of fuming sulfuric acid (Aldrich) and 12.1 g of iodine (Aldrich). To the resulting dark red solution was added 10.0 g of 4-nitro-L-phenylalanine by slow addition. The flask was fitted with a CaCl<sub>2</sub> drying tube and the reaction mixture was stirred at RT for 18h. The mixture was then poured onto 300 g of crushed ice. A yellow-brown emulsion resulted which was treated with 7.4 g of sodium thiosulfate and stirred. The resulting light brown suspension was basified to pH=9 by addition of solid NaCO<sub>3</sub>. This was followed by acidification to pH=5.5 by addition of conc. H<sub>2</sub>SO<sub>4</sub>. A brown solid precipitated which was collected by filtration and dried *in vacuo*. This afforded 6.54 g (41%) of 2-iodo-4-nitro-L-phenylalanine as a brown powder, m.p.>200°C.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>/TFA)  $\delta$  8.62 (s, 1H, aromatic CH), 8.46 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 8.26 (d, 1H, J=8.5, aromatic CH), 7.65 (d, 1H, J=8.5, aromatic CH), 4.22 (m, 1H, methine), 3.34 (m, 2H, ArCH<sub>2</sub>).

Example 168: Ethyl 2-iodo-4-nitro-L-phenylalaninate

According to example 8, 1.40 g of 2-iodo-4-nitro-L-phenylalanine was esterified by refluxing in 50 mL of ethanolic HCl for 18h. Work-up in the usual manner followed by flash chromatography on silica gel (95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) afforded 1.19 g (78%) of ethyl 2-iodo-4-nitro-L-phenylalaninate as a yellow crystalline solid, m.p.33-34°C.

Example 169: Ethyl N-((benzyloxy)carbonyl)-2-iodo-4-nitro-L-phenylalaninate

To a 250 mL round bottomed flask equipped with a magnetic stirrer and a nitrogen inlet were added 4.7 g of ethyl 2-iodo-4-nitro-L-phenylalaninate, 125 mL of 1,4-dioxane, and

2.2 mL of Et<sub>3</sub>N. The solution was cooled in an ice water bath and treated with 2.2 mL of benzyl chloroformate by slow addition. The ice bath was removed and the reaction mixture was stirred at RT for 18h. The mixture was filtered and the filtrate was concentrated to dryness. The brown residue was dissolved in EtOAc, washed with 5% aqueous NaHCO<sub>3</sub> (4x75 mL), dried over anhydrous MgSO<sub>4</sub> and concentrated *in vacuo*. Analysis of the crude product by tlc (SiO<sub>2</sub>, 75:25 hexane:EtOAc) indicated a major new component at R<sub>f</sub>=0.35 and minor components at R<sub>f</sub>=0.40 and 0.25. The R<sub>f</sub>=0.35 product was isolated by flash chromatography on silica gel (75:25 hexane:EtOAc). This afforded 2.08 g (32%) of ethyl *N*-((benzyloxy)carbonyl)-2-iodo-4-nitro-L-phenylalaninate as a white powder, m.p. 85-87°C.

Example 170: Ethyl 4-amino-2-cyclopentyl-L-phenylalaninate

According to example 151, 1.65 g of ethyl *N*-((benzyloxy)carbonyl)-2-iodo-4-nitro-L-phenylalaninate was subjected to Heck coupling with 20 mL of cyclopentene using 60 mL of toluene, 0.074 g of Pd(OAc)<sub>2</sub>, 0.20 g of tri-ortho-tolylphosphine, and 0.51 mL of Et<sub>3</sub>N. The reaction was carried out at 110°C for 24h. Analysis of the crude product by tlc (SiO<sub>2</sub>, 8:2 hexane:EtOAc) indicated a major product at R<sub>f</sub>=0.40 and minor components at R<sub>f</sub>=0.38, 0.60, and 0.95. The R<sub>f</sub>=0.40 material was isolated by flash chromatography on silica gel (8:2 hexane:EtOAc). This afforded 1.03 g (71%) of the desired olefin mixture as a clear liquid. This material was subjected to catalytic hydrogenation at 45 psi for 18h using 55 mL of MeOH and 0.25 g of 10% Pd(C). The crude product was purified by flash chromatography (SiO<sub>2</sub>, 99:1 EtOAc:MeOH) to give 0.56 g (61% for both steps) of ethyl 4-amino-2-cyclopentyl-L-phenylalaninate as a thick transparent oil.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 6.72 (d, 1H, J=8.2, aromatic CH), 6.50 (d, 1H, J=2.3, aromatic CH), 6.29 (dd, 1H, J=8.1, 2.3; aromatic CH), 4.80 (br s, 2H, aromatic

NH<sub>2</sub>), 4.00 (q, 2H, J=7.1, ethyl CH<sub>2</sub>), 3.12 (m, 1H, cyclopentyl methine), 2.72 (m, 2H, ArCH<sub>2</sub>), 2.03-1.37 (m, 10H, cyclopentyl CH<sub>2</sub>, NH<sub>2</sub>), 1.11 (t, 3H, J=7.1, ethyl CH<sub>3</sub>).

Example 171: Ethyl 2-cyclopentyl-L-tyrosinate

To a 500 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer were added a solution of 0.56 g of ethyl 4-amino-2-cyclopentyl-L-phenylalaninate in 60 mL of 0.1 M aqueous H<sub>2</sub>SO<sub>4</sub>. After cooling to 3°C, the solution was treated with a solution of 0.148 g of sodium nitrite (Aldrich) in 10 mL of water. The solution was stirred at 3°C for 10 min and was then treated with 95 mL of 1.5 M aqueous CuSO<sub>4</sub> followed by 0.25 g of Cu<sub>2</sub>O (Aldrich). The mixture was heated to 45°C for 35 min with stirring. Gas evolution was evident during this period. The mixture was cooled to RT and filtered. The blue filtrate was treated with 1N aqueous NaOH to pH=5.5. The resulting blue-green suspension was transferred to a separatory funnel and extracted with CHCl<sub>3</sub> (5x100 mL). The combined CHCl<sub>3</sub> extracts were dried over MgSO<sub>4</sub> and concentrated to give 0.12 g of a green oil. The blue-green suspension was then treated with solid NaHCO<sub>3</sub> until gas evolution ceased and was extracted with CHCl<sub>3</sub> a second time (4x50 mL). The combined extracts were washed with water, dried (MgSO<sub>4</sub>), and concentrated to give 0.076 g of an oil. This material was combined with the original 0.12 g of product and purified by flash chromatography (SiO<sub>2</sub>, 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.168 g (30%) of ethyl 2-cyclopentyl-L-tyrosinate as a thick, transparent oil.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 9.07 (s, 1H, OH), 6.87 (d, 1H, J=8.2, aromatic CH), 6.66 (d, 1H, J=2.4, aromatic CH), 6.48 (dd; 1H; J=8.1, 2.4; aromatic CH), 4.00 (q, 2H, J=7.1, ethyl CH<sub>2</sub>), 3.16 (m, 1H, cyclopentyl methine), 2.78 (m, 2H, ArCH<sub>2</sub>), 2.05-1.32 (m, 10H, cyclopentyl CH<sub>2</sub>, NH<sub>2</sub>), 1.10 (t, 3H, J=7.1, ethyl CH<sub>3</sub>).



Example 172: Ethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-2-cyclopentyl-L-tyrosinate

According to example 145, 0.153 g of ethyl 2-cyclopentyl-L-tyrosinate was coupled with 0.171 g of *N*-Cbz-L-glutamic acid  $\gamma$ -ethyl ester using 0.111 g of EDC in 8 mL of CH<sub>2</sub>Cl<sub>2</sub> for 4.5h. The crude product was purified by flash chromatography twice on silica gel (1:1 hexane:EtOAc; 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.245 g (78%) of ethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-2-cyclopentyl-L-tyrosinate as a colorless foam.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  9.09 (s, 1H, OH), 8.37 (d, 1H, J=7.3 NH), 7.33 (m, 6H, aromatic CH, NH), 6.88 (d, 1H, J=8.4, aromatic CH), 6.63 (s, 1H, aromatic CH), 6.43 (d, 1H, J=8.3, aromatic CH), 4.99 (m, 2H, PhCH<sub>2</sub>O), 4.28 (m, 1H, methine), 4.00 (m, 5H, ethyl CH<sub>2</sub>, methine), 3.09 (m, 1H, cyclopentyl methine), 2.96 (m, 1H, ArCH<sub>2</sub>), 2.83 (m, 1H, ArCH<sub>2</sub>), 2.29 (t, 2H, J=7.9, glu 4-CH<sub>2</sub>), 2.02-1.53 (m, 8H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.46 (m, 2H, cyclopentyl CH<sub>2</sub>), 1.15 (t, 3H, J=7.1, ethyl CH<sub>3</sub>), 1.04 (t, 3H, J=7.1, ethyl CH<sub>3</sub>).

Example 173: Ethyl 5-*O*-ethyl-L-glutam-1-yl-2-cyclopentyl-L-tyrosinate

According to example 48, 0.24 g of ethyl *N*-((benzyloxy)carbonyl)-5-*O*-ethyl-L-glutam-1-yl-2-cyclopentyl-L-tyrosinate was hydrogenolyzed using 0.042 g of 10% Pd(C) in 85 mL of MeOH. The crude product was purified by flash chromatography (SiO<sub>2</sub>, 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.154 g (84%) of ethyl 5-*O*-ethyl-L-glutam-1-yl-2-cyclopentyl-L-tyrosinate as a thick transparent oil.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  9.08 (s, 1H, OH), 8.23 (d, 1H, J=7.9, NH), 6.87 (d, 1H, J=8.1, aromatic CH), 6.63 (d, 1H, J=2.1, aromatic CH), 6.44 (dd; 1H; J=8.1, 2.1; aromatic CH), 4.31 (m, 1H, methine), 4.00 (m, 4H, ethyl CH<sub>2</sub>), 3.12 (m, 2H, glu methine, cyclopentyl methine), 2.98 (m, 1H, ArCH<sub>2</sub>), 2.82 (m, 1H, ArCH<sub>2</sub>), 2.27 (t, 2H,

J=7.9, glu 4-CH<sub>2</sub>), 2.00-1.35 (m, 12H, glu 3-CH<sub>2</sub>, NH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.07 (t, 3H, J=7.1, ethyl CH<sub>3</sub>), 1.16 (t, 3H, J=7.1, ethyl CH<sub>3</sub>).

Example 174: Ethyl *N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-5-*O*-ethyl-L-glutam-1-yl-2-cyclopentyl-L-tyrosinate

According to example 49, 0.131 g of 4-(*N*-(2,4-diamino-6-pteridinylmethyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate was coupled with 0.15 g of ethyl 5-*O*-ethyl-L-glutam-1-yl-2-cyclopentyl-L-tyrosinate using 0.08 mL of DECP and 0.10 mL of Et<sub>3</sub>N in 12 mL of DMF. The crude product was subjected to flash chromatography twice on silica gel (93:7 CH<sub>2</sub>Cl<sub>2</sub>:MeOH; 92:8 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.134 g (52%) of ethyl *N*-(4-(((2,4-diamino-6-pteridinyl)methyl)methylamino)benzoyl)-5-*O*-ethyl-L-glutam-1-yl-2-cyclopentyl-L-tyrosinate as a yellow powder, m.p. 135-150°C.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 9.06 (s, 1H, OH), 8.54 (s, 1H, pteridinyl 7-CH), 8.34 (d, 1H, J=7.4, amide NH), 7.96 (d, 1H, J=8.2, amide NH), 7.70 (d, 2H, J=8.6, aromatic CH), 7.63 (br s, 1H, NH<sub>2</sub>), 7.43 (br s, 1H, NH<sub>2</sub>), 6.86 (d, 1H, J=8.4, aromatic CH), 6.80 (d, 2H, J=8.9, aromatic CH), 6.60 (m, 3H, aromatic CH, NH<sub>2</sub>), 6.40 (dd; 1H, J=8.2, 1.9; aromatic CH), 4.77 (s, 2H, pteridinyl-CH<sub>2</sub>), 4.42 (m, 1H, methine), 4.25 (m, 1H, methine), 4.07-3.89 (m, 4H, ethyl CH<sub>2</sub>), 3.19 (s, 3H, N-CH<sub>3</sub>), 3.08 (m, 1H, cyclopentyl methine), 2.94 (m, 1H, ArCH<sub>2</sub>), 2.82 (m, 1H, ArCH<sub>2</sub>), 2.31 (t, 2H, J=7.9, glu 4-CH<sub>2</sub>), 2.04-1.32 (m, 10H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.13 (t, 3H, J=7.1, ethyl CH<sub>3</sub>), 1.02 (t, 3H, J=7.1, ethyl CH<sub>3</sub>).

Example 175: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-tyrosine

A solution of 0.130 g of ethyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-ethyl-L-glutam-1-yl-2-cyclopentyl-L-tyrosinate in 10 mL of 1:1 THF:H<sub>2</sub>O was treated with 17 mg of LiOH and the solution was stirred at RT under nitrogen. The hydrolysis was monitored by HPLC (C18, 70:30:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min). An additional 10 mg portion of LiOH was added after 3h. Analysis by HPLC (above conditions) 2h after the second LiOH addition indicated a single component at *k'*=0.62. The solution was neutralized by addition of 1N HCl. A yellow precipitate resulted which was collected by centrifugation, washed with four cycles of aqueous suspension-centrifugation-decantation, frozen, and lyophilized to afford 0.106 g (83%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-tyrosine as a yellow powder, m.p. 185°C (dec).

HPLC: one peak on C18, *k'*=2.10, 75:25:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>) δ 12.75-11.85 (br m, COOH), 9.03 (br s, 1H, OH), 8.56 (s, 1H, pteridiny 7-CH), 8.13 (d, 1H, *J*=7.9, amide NH), 7.96 (d, 1H, *J*=7.8, amide NH), 7.72 (br s, 1H, NH<sub>2</sub>), 7.70 (d, 2H, *J*=8.7, aromatic CH), 7.53 (br s, 1H, NH<sub>2</sub>), 6.89 (d, 1H, *J*=8.4, aromatic CH), 6.80 (m, 3H, aromatic CH, NH<sub>2</sub>), 6.69 (br s, 1H, NH<sub>2</sub>), 6.60 (s, 1H, aromatic CH), 6.38 (dd; 1H; *J*=8.3, 2.2; aromatic CH), 4.78 (s, 2H, pteridiny-CH<sub>2</sub>), 4.41 (m, 1H, methine), 4.22 (m, 1H, methine), 3.21 (s, 3H, N-CH<sub>3</sub>), 3.12 (m, 1H, cyclopentyl methine), 3.00 (dd; 1H; *J*=14.3, 5.3; ArCH<sub>2</sub>), 2.76 (dd; 1H; *J*=14.3, 8.8; ArCH<sub>2</sub>), 2.23 (t, 2H, *J*=7.9, glu 4-CH<sub>2</sub>), 2.05-1.30 (m, 10H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>).

Elemental Analysis: Calcd. for C<sub>34</sub>H<sub>39</sub>N<sub>9</sub>O<sub>7</sub>·2.5 H<sub>2</sub>O (MW 730.78): C, 55.88; H, 6.07; N, 17.25. Found: C, 55.91; H, 5.94; N, 17.10.

Mass Spectrum: (Ion Spray) 686 (M+H)<sup>+</sup>.

UV Spectrum: (pH 7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 259.0 (25000), 308.2 (25500), 371.5 (7960).

$\lambda_{\min}$  ( $\epsilon$ ) 242.3 (16000), 273.0 (17700), 346.8 (6350). sh ( $\epsilon$ ) 220.9 (30100), 288.1 (21000).

Example 176: *tert*-Butyl 3,5-diiodo-L-tyrosinate

A solution of 5.0 g of 3,5-diiodo-L-tyrosine and 0.95 mL of 70% aqueous HClO<sub>4</sub> in 155 mL of *tert*-butyl acetate was stirred at RT under nitrogen for 4 days. The solution was neutralized by addition of 150 mL of 5% aqueous NaHCO<sub>3</sub> and the mixture was subjected to rotary evaporation to remove *tert*-butyl acetate. An off-white precipitate resulted which was collected by filtration and dried *in vacuo*. The crude product was subjected to flash chromatography on silica gel (97:3 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 4.2 g (75%) of *tert*-butyl 3,5-diiodo-L-tyrosinate as a white powder, m.p. 165-168°C.

Example 177: *tert*-Butyl *N*-(9-fluorenylmethyloxycarbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3,5-diiodo-L-tyrosinate

According to example 145, 1.50 g of *tert*-butyl 3,5-diiodo-L-tyrosinate was coupled with 1.31 g of *N*-Fmoc-L-glutamic acid  $\gamma$ -*tert*-butyl ester using 0.62 g of EDC in 45 mL of CH<sub>2</sub>Cl<sub>2</sub> for 3h. The crude product was purified by flash chromatography on silica gel (7:3 hexane:EtOAc) to afford 1.78 g (65%) of *tert*-butyl *N*-(9-fluorenylmethyloxycarbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3,5-diiodo-L-tyrosinate as a light brown foam.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>)  $\delta$  9.41 (s, 1H, OH), 8.27 (d, 1H, J=7.0, NH), 7.91 (d, 2H, J=7.2, aromatic CH), 7.75 (m, 2H, aromatic CH), 7.61 (s, 2H, aromatic CH), 7.57-7.28 (m, 5H, aromatic CH, NH), 4.26 (m, 4H, fluorenyl-CH<sub>2</sub>O, fluorenyl methine,

$\alpha$ -methine), 4.08 (m, 1H,  $\alpha$ -methine), 2.83 (d, 2H,  $J=7.4$ , ArCH<sub>2</sub>), 2.25 (t, 2H,  $J=7.9$ , glu 4-CH<sub>2</sub>), 1.83 (br m, 2H, glu 3-CH<sub>2</sub>), 1.41 (s, 9H, t-Bu), 1.33 (s, 9H, t-Bu).

**Example 178: *tert*-Butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3,5-diiodo-L-tyrosinate**

To a solution of 1.66 g of *tert*-butyl *N*-(9-fluorenylmethyloxycarbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3,5-diiodo-L-tyrosinate in 7 mL of anhydrous DMF were added 1.71 mL of piperidine (Sigma). After stirring at RT under nitrogen for 1h, the solution was mixed with 75 mL of water and was extracted with CH<sub>2</sub>Cl<sub>2</sub>. The combined CH<sub>2</sub>Cl<sub>2</sub> solutions were washed with aqueous NaCl (4x30 mL), dried over MgSO<sub>4</sub>, and concentrated *in vacuo* to give a white solid. Analysis of the crude product by tlc (SiO<sub>2</sub>, 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) indicated major new components at  $R_f=0.4$  and 0.5. The  $R_f=0.4$  material was isolated by flash chromatography on silica gel (95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 1.1 g (87%) of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3,5-diiodo-L-tyrosinate as a white foam, m.p. 45-57°C. A sample of the corresponding HCl salt was prepared for NMR by treating 10 mg of product with 1N ethereal HCl and concentrating to dryness. <sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>)  $\delta$  9.42 (br s, 1H, OH), 8.98 (d, 1H,  $J=7.0$ , NH), 8.33 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.67 (s, 2H, aromatic CH), 4.36 (m, 1H, methine), 3.88 (m, 1H, methine), 2.86 (d, 2H,  $J=7.0$ , ArCH<sub>2</sub>), 2.38 (m, 2H, glu 4-CH<sub>2</sub>), 2.01 (m, 2H, glu 3-CH<sub>2</sub>), 1.41 (s, 9H, t-Bu), 1.35 (s, 9H, t-Bu).

**Example 179: *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3,5-diiodo-L-tyrosinate**

According to example 154, 0.25 g of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3,5-diiodo-L-tyrosinate was coupled with 0.132 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate using 0.06 mL of DECP, 0.07

mL of Et<sub>3</sub>N, and 10 mL of DMF. The crude product was purified by flash chromatography on silica gel (95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.18 g (50%) of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3,5-diiodo-L-tyrosinate as a yellow powder, m.p. 172°C (dec).

Example 180: *N*-(4-(((2,4-Diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-3,5-diiodo-L-tyrosine

According to example 50, 0.119 g of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3,5-diiodo-L-tyrosinate was treated with gaseous HCl in 15 mL of CH<sub>3</sub>NO<sub>2</sub> for 30 min. The yellow suspension was concentrated *in vacuo* to dryness. Analysis of the product by <sup>1</sup>H-NMR and reverse phase HPLC indicated the desired product contaminated with minor impurities. The mixture was purified by semi-preparative HPLC (C18, 77:23:0.1 H<sub>2</sub>O:MeCN:TFA). Fractions containing pure product were combined and subjected to rotary evaporation to remove MeCN. The resulting suspension was frozen and lyophilized to afford 0.024 g (18%) of *N*-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-3,5-diiodo-L-tyrosine as a yellow powder, m.p. 205°C (dec).

HPLC: one peak on C18, k'=2.74, 77:23:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 13.20-11.90 (br m, COOH), 9.35 (s, 1H, OH), 9.21 (br s, 1H, NH<sub>2</sub>), 9.02 (br s, 1H, NH<sub>2</sub>), 8.70 (s, 1H, pteridiny 7-CH), 8.52 (br s, 1H, NH<sub>2</sub>), 8.13 (d, 1H, J=7.6, amide NH), 7.98 (d, 1H, J=7.9, amide NH), 7.72 (d, 2H, J=9.0, aromatic CH), 7.57 (s, 2H, aromatic CH), 7.51 (br s, 1H, NH<sub>2</sub>), 6.80 (d, 2H, J=8.9, aromatic CH), 4.86 (s, 2H, pteridiny-CH<sub>2</sub>), 4.41 (m, 1H, methine), 4.30 (m, 1H, methine), 3.23 (s, 3H, N-CH<sub>3</sub>), 2.81 (m, 2H, ArCH<sub>2</sub>), 2.25 (t, 2H, J=7.6, glu 4-CH<sub>2</sub>), 1.90 (m, 2H, glu 3-CH<sub>2</sub>).

**Elemental Analysis:** Calcd. for  $C_{29}H_{29}N_9O_7I_2 \cdot 1.7 \text{ TFA} \cdot 2.3 \text{ H}_2\text{O}$  (MW 1104.69): C, 35.23; H, 3.22; N, 11.41. Found: C, 35.25; H, 3.24; N, 11.44.

**Mass Spectrum:** (Ion Spray) 870 (M+H)<sup>+</sup>.

**UV Spectrum:** (pH 7 Buffer)  $\lambda_{\text{max}}$  ( $\epsilon$ ) 219.7 (48900), 258.0 (26500), 306.0 (26200), 373.3 (7780).  $\lambda_{\text{min}}$  ( $\epsilon$ ) 214.9 (48300), 246.0 (23700), 273.7 (18400), 347.6 (6200).

**Example 181: Methyl 4-hydroxy-3-iodobenzoate**

To a 500 mL 3-necked flask equipped with a magnetic stirrer, a thermometer, and a condenser were added 10.0 g of methyl 4-hydroxybenzoate (Aldrich) and 30 mL of glacial acetic acid. To this suspension was added a solution of 11.74 g of iodine monochloride in 30 mL of glacial acetic acid via addition funnel. The solution was heated at 80°C for 1h, 45°C for 18h, and 90°C for an additional 4h. Cooling to RT afforded a thick red-orange suspension which was transferred to a 1L separatory funnel and dissolved in 500 mL of  $\text{CHCl}_3$  by shaking vigorously. The dark purple solution was washed with water (2x100 mL), saturated aqueous sodium thiosulfate (2x100 mL), and 5% aqueous  $\text{NaHCO}_3$  (2x100 mL). A light yellow solution resulted which was dried over  $\text{MgSO}_4$ , and concentrated *in vacuo* to give a white solid. This material was recrystallized from water-MeOH to afford 14.92 g (82%) of methyl 4-hydroxy-3-iodobenzoate as a white crystalline solid, m.p. 145-146°C.

**Example 182: Methyl 4-hydroxy-3-cyclopentylbenzoate**

According to example 151, 5.0 g of methyl 4-hydroxy-3-iodobenzoate was subjected to Heck coupling with 25 mL of cyclopentene using 80 mL of toluene, 0.40 g of  $\text{Pd}(\text{OAc})_2$ , 1.10 g of tri-ortho-tolylphosphine, and 2.76 mL of  $\text{Et}_3\text{N}$ . The reaction was carried out at 110°C for 24h. Analysis of the crude product by tlc ( $\text{SiO}_2$ , 75:25 hexane:EtOAc) indicated a major new component at  $R_f=0.50$ . This material was isolated by flash

chromatography on silica gel (75:25 hexane:EtOAc). This gave 2.97 g (76%) of the desired olefin mixture ( $^1\text{H-NMR}$ ) as a brown solid. This material was subjected to catalytic hydrogenation at 45 psi for 18h using 80 mL of MeOH and 1.0 g of 10% Pd(C). This afforded 2.59 g (65% for both steps) of methyl 4-hydroxy-3-cyclopentylbenzoate as a white solid, m.p. 114-116°C.

Example 183: Methyl 4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylbenzoate

A 100 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet was charged with 1.79 g of methyl 4-hydroxy-3-cyclopentylbenzoate, 1.22 g of imidazole (Aldrich), 30 mL of anhydrous DMF, and 1.47 g of *tert*-butyldimethylsilyl chloride (Aldrich). The solution was stirred at RT under nitrogen for 24h and then concentrated *in vacuo* to give a thick oil. This material was subjected to flash chromatography on silica gel (95:5 hexane:EtOAc) to afford 2.43 g (89%) of methyl 4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylbenzoate as a clear liquid.

$^1\text{H-NMR}$ : (200 MHz, DMSO- $d_6$ )  $\delta$  7.81 (d, 1H,  $J=2.0$ , aromatic CH), 7.72 (dd, 1H;  $J=8.4$ , 2.0; aromatic CH), 6.93 (d, 1H,  $J=8.4$ , aromatic CH), 3.80 (s, 3H, CH<sub>3</sub>), 3.30 (m, 1H, cyclopentyl methine), 2.06-1.37 (m, 8H, cyclopentyl CH<sub>2</sub>), 0.98 (s, 9H, *t*-Bu), 0.27 (s, 6H, SiMe<sub>2</sub>).

Example 184: 4-((*tert*-Butyldimethylsilyl)oxy)-3-cyclopentylbenzyl alcohol

To a 100 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet were added 2.58 g of methyl 4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylbenzoate and 15 mL of anhydrous toluene. The solution was cooled in an ice water bath and was treated with 19.3 mL of 1M diisobutylaluminum hydride in toluene (Aldrich). The solution was stirred at 0°C for 30 min and was allowed to warm to RT. After 2h at RT the solution was poured into 130 mL of saturated aqueous potassium sodium tartrate and the



resulting mixture was stirred for 20 min. The mixture was then extracted with ethyl ether (3x70 mL). The combined ether extracts were washed with water (3x70 mL), dried over anhydrous MgSO<sub>4</sub>, and concentrated to give a cloudy oil. The crude product was purified by flash chromatography on silica gel (8:2 hexane:EtOAc) to afford 1.94 g (82%) of 4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylbenzyl alcohol as a clear liquid. <sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 7.18 (d, 1H, J=2.0, aromatic CH), 7.02 (dd, 1H; J=8.2, 2.2; aromatic CH), 6.76 (d, 1H, J=8.0, aromatic CH), 5.02 (t, 1H, J=5.8, OH), 4.40 (d, 2H, J=5.6, ArCH<sub>2</sub>O), 3.29 (m, 1H, cyclopentyl methine), 2.04-1.38 (m, 8H, cyclopentyl CH<sub>2</sub>), 1.03 (s, 9H, *t*-Bu), 0.22 (s, 6H, SiMe<sub>2</sub>).

Example 185: 4-((*tert*-Butyldimethylsilyl)oxy)-3-cyclopentylbenzaldehyde

A solution of 1.94 g of 4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylbenzyl alcohol in 70 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub> was treated with 2.05 g of pyridinium chlorochromate (Aldrich) and was stirred at RT under nitrogen. After 30 min, tlc (SiO<sub>2</sub>, 85:15 hexane:EtOAc) indicated no remaining starting material and a major new component at R<sub>f</sub>=0.80. The mixture was filtered to remove solids and the filtrate was washed with 5% aqueous citric acid (4x50 mL) followed by 5% aqueous NaHCO<sub>3</sub> (4x50 mL). The CH<sub>2</sub>Cl<sub>2</sub> solution was dried over anhydrous MgSO<sub>4</sub> and concentrated *in vacuo* to give a brown oil. This material was purified by flash chromatography on silica gel (9:1 hexane:EtOAc) to afford 1.52 g (79%) of 4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylbenzaldehyde as a clear oil.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 9.88 (s, 1H, aldehyde CH), 7.80 (d, 1H, J=2.0, aromatic CH), 7.69 (dd, 1H; J=8.2, 2.0; aromatic CH), 7.03 (d, 1H, J=8.3, aromatic CH), 3.29 (m, 1H, cyclopentyl methine), 2.08-1.43 (m, 8H, cyclopentyl CH<sub>2</sub>), 1.01 (s, 9H, *t*-Bu), 0.30 (s, 6H, SiMe<sub>2</sub>).

Example 186: *tert*-Butyl *trans*-4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylcinnamate

To a 100 mL 3-necked flask equipped with a magnetic stirrer, a nitrogen inlet and a reflux condenser were added 1.57 g of *tert*-butyl diethylphosphonoacetate (Aldrich) and 20 mL of anhydrous ethyl ether. This was followed by addition of 0.40 g of 60% NaH mineral oil dispersion. The resulting cloudy solution was heated at reflux for 25 min, cooled to 0°C and treated with a solution of 1.52 g of 4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylbenzaldehyde in 10 mL of ether. A thick yellow gel resulted which was broken up with a spatula. The mixture was allowed to warm to RT and stirring was continued for an additional 1h. Analysis by tlc (SiO<sub>2</sub>, 95:5 hexane:EtOAc) indicated a major new component at R<sub>f</sub>=0.48 and minor components at R<sub>f</sub>=0.06, 0.41 and 0.98. The mixture was diluted with 70 mL of ether, washed with saturated brine, and dried over anhydrous MgSO<sub>4</sub>. The drying agent was removed by filtration and the filtrate concentrated to give a yellow oil. This material was purified by flash chromatography on silica gel (97:3 hexane:EtOAc) to afford 1.53 g (77%) of *tert*-butyl *trans*-4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylcinnamate as a clear oil.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 7.59-7.36 (m, 3H, aromatic CH, olefinic CH), 6.83 (d, 1H, J=8.4, aromatic CH), 6.38 (d, 1H, J=15.8, olefinic CH), 3.27 (m, 1H, cyclopentyl methine), 2.02-1.52 (m, 8H, cyclopentyl CH<sub>2</sub>), 1.49 (s, 9H, t-Bu), 1.00 (s, 9H, Si-t-Bu), 0.25 (s, 6H, SiMe<sub>2</sub>).

Example 187: (2*R*, 3*S*)-*tert*-Butyl 3-(4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylphenyl)-2,3-dihydroxypropionate

To a 250 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet were added 20 mL of *tert*-butyl alcohol, 20 mL of water, 5.32 g of AD-mix-α (Sharpless, K.B.; et al. *J. Org. Chem.*, 1992, 57, 2768-2771; reagent purchased from Aldrich) and 0.36 g of methanesulfonamide (Aldrich). This was followed by addition of 1.53 g of

*tert*-butyl *trans*-4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylcinnamate. The reaction mixture was stirred at RT under nitrogen. After 18h, 5.7 g of sodium sulfite were added and the mixture was stirred for 30 min. The mixture was concentrated *in vacuo* to 15 mL, diluted with 100 mL of water and extracted with EtOAc (4x50 mL). The combined EtOAc extracts were washed with water (3x50 mL), dried over anhydrous MgSO<sub>4</sub> and concentrated to give a cloudy oil. This material was purified by flash chromatography on silica gel (75:25 hexane:EtOAc) to afford 1.44 g (87%) of *tert*-butyl (2*R*, 3*S*)-3-(4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylphenyl)-2,3-dihydroxypropionate as a thick clear oil.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 7.16 (s, 1H, aromatic CH), 7.01 (d, 1H, J=8.2, aromatic CH), 6.73 (d, 1H, J=8.2, aromatic CH), 5.28 (d, 1H, J=5.4, OH), 5.25 (d, 1H, J=7.1, OH), 4.55 (t, 1H, J=5.6, propionate 2-methine), 3.93 (t, 1H, J=6.8, propionate 3-methine), 3.27 (m, 1H, cyclopentyl methine), 2.04-1.37 (m, 8H, cyclopentyl CH<sub>2</sub>), 1.24 (s, 9H, *t*-Bu), 0.99 (s, 9H, Si-*t*-Bu), 0.21 (s, 6H, SiMe<sub>2</sub>).

Example 188: (*R*)-*tert*-Butyl 3-(4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylphenyl)-2-hydroxypropionate

To a 300 mL Parr bottle were added 0.35 g of 10% Pd(C) and 20 mL of *tert*-butyl alcohol under a nitrogen flush. To this was added a solution of 1.42 g of *tert*-butyl (2*R*, 3*S*)-3-(4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylphenyl)-2,3-dihydroxypropionate in 40 mL of *tert*-butyl alcohol. The mixture was deoxygenated by bubbling nitrogen through for 5 min while gently warming the bottle to avoid freezing of *tert*-butyl alcohol. The mixture was treated with 4 drops of concentrated HClO<sub>4</sub> and hydrogenated at 45 psi and 40°C for 72h. The vessel was purged with nitrogen, catalyst removed by filtration through celite, and the filtrate concentrated *in vacuo* to 20 mL. The solution was mixed with 60 mL of 5% aqueous NaHCO<sub>3</sub> and the mixture extracted with EtOAc (4x40 mL).

The combined EtOAc extracts were washed with 5% aqueous NaHCO<sub>3</sub> (3x50 mL), dried over anhydrous MgSO<sub>4</sub> and concentrated to give a cloudy oil. This material was purified by flash chromatography on silica gel (9:1 hexane:EtOAc) to afford 1.17 g (86%) of *tert*-butyl (*R*)-3-(4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylphenyl)-2-hydroxypropionate as a thick transparent oil.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 7.05 (d, 1H, J=2.1, aromatic CH), 6.92 (dd, 1H, J=8.4, 2.1; aromatic CH), 6.69 (d, 1H, J=8.2, aromatic CH), 5.33 (d, 1H, J=5.7, OH), 4.08 (m, 1H, methine), 3.23 (m, 1H, cyclopentyl methine), 2.78 (d, 2H, J=6.1, ArCH<sub>2</sub>), 2.00-1.38 (m, 8H, cyclopentyl CH<sub>2</sub>), 1.32 (s, 9H, *t*-Bu), 0.99 (s, 9H, Si-*t*-Bu), 0.20 (s, 6H, SiMe<sub>2</sub>).

Example 189: (*S*)-*tert*-Butyl 2-((*N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl)oxy)-3-(4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylphenyl)propionate

To a 100 mL 3-necked flask equipped with a magnetic stirrer and a nitrogen inlet were added 1.14 g of *tert*-butyl (*R*)-3-(4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylphenyl)-2-hydroxypropionate, 20 mL of anhydrous THF, 1.01 g of *N*-Cbz-L-glutamic acid  $\gamma$ -*tert*-butyl ester (Sigma), and 0.78 g of triphenylphosphine (Aldrich). The solution was cooled to 0°C and was treated with 0.47 mL of diethyl azodicarboxylate (Aldrich) by dropwise addition. The solution was allowed to warm to RT and stirring under nitrogen was continued. After 3.5h, tlc (SiO<sub>2</sub>, 85:15 hexane:EtOAc) indicated a major new component at R<sub>f</sub>=0.46 and some remaining alcohol starting material at R<sub>f</sub>=0.51. The solution was again cooled to 0°C and treated with an additional 0.18 g of *N*-Cbz-L-glutamic acid  $\gamma$ -*tert*-butyl ester, 0.14 g of triphenylphosphine, and 0.085 mL of diethyl azodicarboxylate. After warming to RT and stirring for 18h tlc indicated only a trace of starting material. The solution was concentrated *in vacuo* to dryness and the crude product purified by flash chromatography (SiO<sub>2</sub>, 85:15 hexane:EtOAc). This afforded

1.86 g (84%) of (S)-*tert*-butyl 2-((N-((benzyloxy)carbonyl)-5-*O-tert*-butyl-L-glutam-1-yl)oxy)-3-(4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylphenyl)propionate as a thick transparent oil.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 7.76 (d, 1H, J=8.0, NH), 7.34 (m, 5H, aromatic CH), 7.11 (s, 1H, aromatic CH), 6.96 (d, 1H, J=8.1, aromatic CH), 6.68 (d, 1H, J=8.1, aromatic CH), 5.01 (m, 3H, PhCH<sub>2</sub>O, propionate methine), 4.19 (m, 1H, glu methine), 3.21 (m, 1H, cyclopentyl methine), 3.01 (d, 2H, J=5.1, ArCH<sub>2</sub>), 2.32 (t, 2H, J=7.8, glu 4-CH<sub>2</sub>), 2.12-1.43 (m, 10H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.39 (s, 9H, t-Bu), 1.26 (s, 9H, t-Bu), 0.98 (s, 9H, Si-t-Bu), 0.19 (s, 9H, SiMe<sub>2</sub>).

Example 190: (S)-*tert*-Butyl 2-((N-((benzyloxy)carbonyl)-5-*O-tert*-butyl-L-glutam-1-yl)oxy)-3-(3-cyclopentyl-4-hydroxyphenyl)propionate

A solution of 1.85 g of (S)-*tert*-butyl 2-((N-((benzyloxy)carbonyl)-5-*O-tert*-butyl-L-glutam-1-yl)oxy)-3-(4-((*tert*-butyldimethylsilyl)oxy)-3-cyclopentylphenyl)propionate in 20 mL of anhydrous THF was treated with 2.63 mL of 1M tetrabutylammonium fluoride/THF (Aldrich) and was stirred at 0°C under nitrogen. After 15 min, tlc (SiO<sub>2</sub>, 75:25 hexane:EtOAc) indicated no remaining starting material and a single new component at R<sub>f</sub>=0.41. The solution was mixed with 80 mL of water and was extracted with EtOAc (4x40 mL). The combined EtOAc extracts were washed with saturated brine (3x60 mL), dried over anhydrous MgSO<sub>4</sub> and concentrated *in vacuo* to give a clear oil. This material was subjected to flash chromatography on silica gel (75:25 hexane:EtOAc) to afford 1.5 g (96%) of (S)-*tert*-butyl 2-((N-((benzyloxy)carbonyl)-5-*O-tert*-butyl-L-glutam-1-yl)oxy)-3-(3-cyclopentyl-4-hydroxyphenyl)propionate as a white foam.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 9.11 (s, 1H, OH), 7.77 (d, 1H, J=8.2, NH), 7.33 (m, 5H, aromatic CH), 7.01 (d, 1H, J=1.6, aromatic CH), 8.85 (dd, 1H; J=8.2, 1.7;

aromatic CH), 6.68 (d, 1H, J=8.2, aromatic CH), 5.04 (s, 2H, PhCH<sub>2</sub>O), 4.97 (m, 1H, propionate methine), 4.18 (m, 1H, glu methine), 3.17 (m, 1H, cyclopentyl methine), 2.96 (d, 2H, J=6.0, ArCH<sub>2</sub>), 2.34 (t, 2H, J=7.4, glu 4-CH<sub>2</sub>), 2.13-1.43 (m, 10H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.40 (s, 9H, t-Bu), 1.29 (s, 9H, t-Bu).

Example 191: (S)-tert-Butyl 2-((5-O-tert-butyl-L-glutam-1-yl)oxy)-3-(3-cyclopentyl-4-hydroxyphenyl)propionate

According to example 48, 1.49 g of (S)-tert-butyl 2-((N-((benzyloxy)carbonyl)-5-O-tert-butyl-L-glutam-1-yl)oxy)-3-(3-cyclopentyl-4-hydroxyphenyl)propionate was hydrogenolyzed using 0.24 g of 10% Pd(C) in 70 mL of MeOH. Analysis of the crude product by tlc (SiO<sub>2</sub>, 4:6 hexane:EtOAc) indicated a major component at R<sub>f</sub> 0.47 and minor components at R<sub>f</sub> 0.60 and 0.15. The crude product was purified by flash chromatography on silica gel (4:6 hexane:EtOAc) to afford 0.76 g (65%) of (S)-tert-butyl 2-((5-O-tert-butyl-L-glutam-1-yl)oxy)-3-(3-cyclopentyl-4-hydroxyphenyl)propionate as a white foam.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 9.12 (s, 1H, OH), 7.00 (s, 1H, aromatic CH), 6.86 (d, 1H, J=8.2, aromatic CH), 6.68 (d, 1H, J=8.2, aromatic CH), 4.92 (t, 1H, J=6.2, propionate methine), 4.04 (q, 1H, J=7.0, glu methine), 3.19 (m, 1H, cyclopentyl methine), 2.95 (d, 2H, J=6.4, ArCH<sub>2</sub>), 2.33 (t, 2H, J=7.4, glu 4-CH<sub>2</sub>), 2.00-1.48 (m, 12H, glu 3-CH<sub>2</sub>, NH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.39 (s, 9H, t-Bu), 1.31 (s, 9H, t-Bu).

Example 192: (S)-tert-Butyl 3-(3-cyclopentyl-4-hydroxyphenyl)-2-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl-L-glutam-1-yl)oxy)propionate

According to example 154, 0.414 g of (S)-tert-butyl 2-((5-O-tert-butyl-L-glutam-1-yl)oxy)-3-(3-cyclopentyl-4-hydroxyphenyl)propionate was coupled with 0.32 g of 4-(N-(2,4-diamino-6-pteridiny)methyl)-N-methylamino)benzoic acid hemihydrochloride

dihydrate using 0.19 mL of DECP, 0.23 mL of Et<sub>3</sub>N, and 25 mL of DMF. The crude product was subjected to flash chromatography twice on silica gel (14:14:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH; 95:5:91:9 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.46 g (69%) of (*S*)-*tert*-butyl 3-(3-cyclopentyl-4-hydroxyphenyl)-2-((5-*O*-*tert*-butyl-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl)oxy)propionate as a yellow powder, m.p. 135-137°C.

<sup>1</sup>H-NMR: (300 MHz, DMSO-d<sub>6</sub>) δ 9.07 (s, 1H, OH), 8.55 (s, 1H, pteridiny 7-CH), 8.30 (d, 1H, J=7.6, amide NH), 7.71 (d, 2H, J=8.6, aromatic CH), 7.64 (br s, 1H, NH<sub>2</sub>), 7.42 (br s, 1H, NH<sub>2</sub>), 6.97 (d, 1H, J=1.6, aromatic CH), 6.80 (m, 3H, aromatic CH), 6.59 (m, 3H, aromatic CH, NH<sub>2</sub>), 4.92 (t, 1H, J=6.1, propionate methine), 4.77 (s, 2H, pteridiny-CH<sub>2</sub>), 4.49 (m, 1H, glu methine), 3.19 (s, 3H, N-CH<sub>3</sub>), 3.12 (m, 1H, cyclopentyl methine), 2.93 (m, 2H, ArCH<sub>2</sub>), 2.33 (t, 2H, J=7.2, glu 4-CH<sub>2</sub>), 2.08 (m, 1H, glu 3-CH<sub>2</sub>), 1.87 (m, 3H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.77-1.40 (m, 6H, cyclopentyl CH<sub>2</sub>), 1.36 (s, 9H, t-Bu), 1.25 (s, 9H, t-Bu).

Example 193: (*S*)-3-(3-Cyclopentyl-4-hydroxyphenyl)-2-((*N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl)oxy)propionic acid

According to example 50, 0.43 g of (*S*)-*tert*-butyl 3-(3-cyclopentyl-4-hydroxyphenyl)-2-((5-*O*-*tert*-butyl-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl)oxy)propionate was treated with gaseous HCl in 50 mL of CH<sub>3</sub>NO<sub>2</sub> for 45 min.

The yellow suspension was concentrated *in vacuo* to dryness. The product was suspended in 25 mL of water and was treated with sufficient 1N aqueous NaOH to give complete solution. The solution was filtered and the pH adjusted to 5.5 by addition of 1N HCl. A yellow precipitate resulted which was separated by centrifugation, washed with four cycles of aqueous suspension-centrifugation-decantation, frozen and lyophilized to afford 0.345 g (88%) of (*S*)-3-(3-cyclopentyl-4-hydroxyphenyl)-2-((*N*-(4-

(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl)oxy)propionic acid as a yellow-orange powder, m.p. 165°C (dec).

**HPLC:** one major peak on C18,  $k'=2.02$ ; 3.6% of methotrexate was detected at  $k'=0.09$ ; 70:30:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

**<sup>1</sup>H-NMR:** (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  12.40-11.90 (br s, COOH), 9.03 (br s, 1H, OH), 8.57 (s, 1H, pteridiny 7-CH), 8.27 (d, 1H,  $J=7.6$ , amide NH), 7.86 (br s, 1H, NH<sub>2</sub>), 7.70 (d, 2H,  $J=8.9$ , aromatic CH), 7.65 (br s, 1H, NH<sub>2</sub>), 6.97 (d, 1H,  $J=1.6$ , aromatic CH), 6.80 (m, 5H, NH<sub>2</sub>, aromatic CH), 6.61 (d, 1H,  $J=8.2$ , aromatic CH), 4.96 (t, 1H,  $J=5.9$ , methine), 4.78 (s, 2H, pteridiny-CH<sub>2</sub>), 4.49 (m, 1H, methine), 3.19 (s, 3H, N-CH<sub>3</sub>), 3.16 (m, 1H, cyclopentyl methine), 2.94 (d, 2H,  $J=5.8$ , ArCH<sub>2</sub>), 2.34 (t, 2H,  $J=7.5$ , glu 4-CH<sub>2</sub>), 2.10 (m, 1H, glu 3-CH<sub>2</sub>), 1.87 (m, 3H, glu 3-CH<sub>2</sub>, cyclopentyl CH<sub>2</sub>), 1.78-1.40 (m, 6H, cyclopentyl CH<sub>2</sub>).

**Elemental Analysis:** Calcd. for C<sub>34</sub>H<sub>38</sub>N<sub>8</sub>O<sub>8</sub>·2.1 H<sub>2</sub>O (MW 724.56): C, 56.36; H, 5.87; N, 15.47. Found: C, 56.13; H, 5.59; N, 15.46.

**Mass Spectrum:** (Ion Spray) 687 (M+H)<sup>+</sup>.

**UV Spectrum:** (pH 7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 259.2 (23100), 307.3 (23300), 372.4 (7190).  $\lambda_{\min}$  ( $\epsilon$ ) 242.0 (14300), 272.9 (16900), 343.6 (5460). sh ( $\epsilon$ ) 219.8 (26400), 280.7 (19900).

#### Example 194: Methyl 3-*tert*-butyl-4-hydroxybenzoate

To a 500 mL 3-necked flask equipped with a magnetic stirrer, a nitrogen inlet, and a condenser were added 10.0 g of 3-*tert*-butyl-4-hydroxybenzoic acid (ICN Biomedicals Inc., Aurora, Ohio 44202), and 200 mL of MeOH. The solution was acidified by bubbling HCl gas through for 5 min. and was then heated at reflux for 5h. The solution was cooled to RT and concentrated *in vacuo* to dryness. The resulting solid was dissolved in EtOAc. The solution was washed three times with 5% aqueous NaHCO<sub>3</sub>,



dried over anhydrous  $\text{MgSO}_4$  and concentrated to dryness to afford 9.65 g (90%) of methyl 3-*tert*-butyl-4-hydroxybenzoate as a white crystalline solid, m.p. 144-145°C.

Example 195: Methyl 3-*tert*-butyl-4-((*tert*-butyldimethylsilyl)oxy)benzoate

According to example 183, 5.00 g of methyl 3-*tert*-butyl-4-hydroxybenzoate was silylated using 4.34 g of *tert*-butyldimethylsilyl chloride, 3.59 g of imidazole, and 70 mL of anhydrous DMF. The crude product was purified by flash chromatography on silica gel (9:1 hexane:EtOAc) to afford 6.1 g (79%) of methyl 3-*tert*-butyl-4-((*tert*-butyldimethylsilyl)oxy)benzoate as a clear liquid.

$^1\text{H-NMR}$ : (200 MHz,  $\text{DMSO-d}_6$ )  $\delta$  7.89 (d, 1H,  $J=2.3$ , aromatic CH), 7.76 (dd, 1H,  $J=8.5, 2.3$ ; aromatic CH), 6.98 (d, 1H,  $J=8.4$ , aromatic CH), 3.82 (s, 3H,  $\text{CO}_2\text{CH}_3$ ), 1.37 (s, 9H, *t*-Bu), 1.03 (s, 9H, Si-*t*-Bu), 0.37 (s, 6H,  $\text{SiMe}_2$ ).

Example 196: 3-*tert*-Butyl-4-((*tert*-butyldimethylsilyl)oxy)benzyl alcohol

According to example 184, 5.00 g of methyl 3-*tert*-butyl-4-((*tert*-butyldimethylsilyl)oxy)benzoate was reduced using 31.8 mL of 1M diisobutylaluminum hydride/toluene and 50 mL of anhydrous toluene. Analysis of the reaction mixture by tlc after 30 min indicated some remaining starting material. To ensure complete reduction, an additional 5 mL of 1M diisobutylaluminum hydride/toluene was added and the reaction was allowed to proceed for an additional 5 min. Work-up in the usual manner afforded 4.11 g (90%) of 3-*tert*-butyl-4-((*tert*-butyldimethylsilyl)oxy)benzyl alcohol as a cloudy oil. The product was used without further purification.

$^1\text{H-NMR}$ : (200 MHz,  $\text{DMSO-d}_6$ )  $\delta$  7.20 (d, 1H,  $J=2.0$ , aromatic CH), 7.04 (dd, 1H,  $J=8.2, 2.1$ ; aromatic CH), 6.80 (d, 1H,  $J=8.0$ , aromatic CH), 5.01 (t, 1H,  $J=5.7$ , OH), 4.39 (d, 2H,  $\text{ArCH}_2\text{O}$ ), 1.36 (s, 9H, *t*-Bu), 1.02 (s, 9H, Si-*t*-Bu), 0.32 (s, 6H,  $\text{SiMe}_2$ ).

Example 197: 3-*tert*-Butyl-4-((*tert*-butyldimethylsilyl)oxy)benzyl bromide

A 500 mL round-bottomed flask equipped with a magnetic stirrer was charged with 3.6 g of 3-*tert*-butyl-4-((*tert*-butyldimethylsilyl)oxy)benzyl alcohol and 25 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub>. The solution was cooled in an ice water bath and was treated with 5.68 g of dibromotriphenylphosphorane (Aldrich). The reaction mixture was warmed to RT, stirred for 2.5h and then diluted with 100 mL of CH<sub>2</sub>Cl<sub>2</sub>. The resulting solution was washed with 5% aqueous NaHCO<sub>3</sub> (4x60 mL), dried over anhydrous MgSO<sub>4</sub> and concentrated to give a white solid. The crude product was purified by flash chromatography on silica gel (95:5 hexane:EtOAc) to afford 3.86 g (88%) of 3-*tert*-butyl-4-((*tert*-butyldimethylsilyl)oxy)benzyl bromide as a white crystalline solid, m.p. 54-55°C.

Example 198: *tert*-Butyl (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-*tert*-butyl-4-((*tert*-butyldimethylsilyl)oxy)benzyl)-4-morpholinecarboxylate

According to example 142, 3.82 g of *tert*-butyl (2*R*, 3*S*)-(-)-6-oxo-2,3-diphenyl-4-morpholinecarboxylate was alkylated with 3.86 g of 3-*tert*-butyl-4-((*tert*-butyldimethylsilyl)oxy)benzyl bromide in 80 mL of anhydrous THF using 11.34 mL of 1 M sodium bis(trimethylsilyl)amide in THF. The crude product was subjected to flash chromatography on silica gel (85:15 hexane:EtOAc) to afford 4.85 g (71%) of *tert*-butyl (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-*tert*-butyl-4-((*tert*-butyldimethylsilyl)oxy)benzyl)-4-morpholinecarboxylate as a white foam, m.p. 75-83°C.

Example 199: (2*R*, 3*S*, 5*S*)-6-Oxo-2,3-diphenyl-5-(3-*tert*-butyl-4-((*tert*-hydroxybenzyl)morpholine

To a 250 mL 3-necked flask equipped with a nitrogen inlet and a magnetic stirrer were added 4.51 g of *tert*-butyl (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-*tert*-butyl-4-((*tert*-

butyldimethylsilyl)oxy)benzyl)-4-morpholinecarboxylate and 50 mL of anhydrous THF. The solution was cooled in an ice water bath, treated with 7.51 mL of 1M nBu<sub>4</sub>NF/THF (Aldrich), and then allowed to warm to RT with stirring under nitrogen. After 1.5h, tlc (SiO<sub>2</sub>, 85:15 hexane:EtOAc) indicated no remaining starting material and a single new component at R<sub>f</sub>=0.52. The solution was diluted with 125 mL of EtOAc, washed with water (4x100 mL), dried over MgSO<sub>4</sub>, and concentrated to give a thick, clear oil. Mass spectral analysis (APCI) indicated the desired intermediate with m/e=538 (M+H+Na)<sup>+</sup>. The oil was dissolved in 50 mL of CH<sub>2</sub>Cl<sub>2</sub> and the solution treated with 15 mL of TFA. After stirring for 40 min, tlc (SiO<sub>2</sub>, 65:35 hexane:EtOAc) indicated a single new component at R<sub>f</sub>=0.36. The solution was neutralized by addition of saturated aqueous NaHCO<sub>3</sub>. The mixture was transferred to a separatory funnel and the layers separated. The CH<sub>2</sub>Cl<sub>2</sub> solution was washed with additional saturated NaHCO<sub>3</sub> (3x50 mL), dried over MgSO<sub>4</sub> and concentrated *in vacuo* to give a clear foam. The crude product was purified by flash chromatography (SiO<sub>2</sub>, 7:3 hexane:EtOAc) to afford 2.66 g (89%) of (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-*tert*-butyl-4-hydroxybenzyl)morpholine as a white foam, m.p. 76-80°C.

Example 200: 3-*tert*-Butyl-L-tyrosine

To a 300 mL Parr bottle were added 0.73 g of PdCl<sub>2</sub> and 20 mL of 1:1 THF:EtOH. To this was added a solution of 2.43 g of (2*R*, 3*S*, 5*S*)-6-oxo-2,3-diphenyl-5-(3-*tert*-butyl-4-hydroxybenzyl)morpholine in 80 mL of 1:1 THF:EtOH. The mixture was deoxygenated by bubbling nitrogen through for 10 min and was hydrogenated at 45 psi for 18h. The vessel was purged with nitrogen, catalyst removed by filtration, and the filtrate concentrated *in vacuo*. The residue was partitioned between water and hexane. The aqueous solution was washed with four additional 50 mL portions of hexane, subjected

to brief rotary evaporation to remove residual hexane, frozen and lyophilized to afford 1.47 g (92%) of 3-*tert*-butyl-L-tyrosine-HCl as a tan hygroscopic solid.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 9.40 (br s, 1H, OH), 8.36 (br s, 3H, NH<sub>3</sub><sup>+</sup>), 7.04 (s, 1H, aromatic CH), 6.91 (d, 1H, J=8.2, aromatic CH), 6.78 (d, 1H, J=8.2, aromatic CH), 4.05 (m, 1H, methine), 3.02 (d, 2H, J=5.6, ArCH<sub>2</sub>), 1.34 (s, 9H, t-Bu).

Example 201: *tert*-Butyl 3-*tert*-butyl-L-tyrosinate

To a 250 mL round bottomed flask equipped with a magnetic stirrer were added 30 mL of isobutylene (condensed at -78°C), 1.30 g of 3-*tert*-butyl-L-tyrosine-HCl, 60 mL of 1,4-dioxane, and 0.5 mL of concentrated H<sub>2</sub>SO<sub>4</sub>. The flask was sealed with a rubber septum and wired shut. After stirring at RT for 18h, the flask was vented and the contents poured into a solution of 10 g of NaHCO<sub>3</sub> in 150 mL of water. The mixture was subjected to rotary evaporation to remove isobutylene. An emulsion resulted which was extracted with EtOAc (4x40 mL). The combined extracts were washed with 5% aqueous NaHCO<sub>3</sub> (3x50 mL), dried over anhydrous MgSO<sub>4</sub>, and concentrated to give a light yellow foam. Analysis of the product by tlc (SiO<sub>2</sub>, 95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) indicated major and minor components at R<sub>f</sub>=0.45 and 0.49 respectively. The R<sub>f</sub>=0.45 product was isolated by flash chromatography on silica gel (95:5 CH<sub>2</sub>Cl<sub>2</sub>:MeOH) to afford 0.61 g (44%) of *tert*-butyl 3-*tert*-butyl-L-tyrosinate as a white foam, m.p. 43-52°C.

Example 202: *tert*-Butyl N-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-tyrosinate

According to example 145, 0.57 g of *tert*-butyl 3-*tert*-butyl-L-tyrosinate was coupled with 0.66 g of *N*-Cbz-L-glutamic acid γ-*tert*-butyl ester (Sigma) using 0.39 g of EDC in 20 mL of CH<sub>2</sub>Cl<sub>2</sub> for 4h. The crude product was purified by flash chromatography on silica gel (65:35 hexane:EtOAc) to afford 0.99 g (83%) of *tert*-butyl *N*-

((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-tyrosinate as a white foam, m.p. 62-66°C.

Example 203: *tert*-Butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-tyrosinate

According to example 48, 0.93 g of *tert*-butyl *N*-((benzyloxy)carbonyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-tyrosinate was hydrogenolyzed using 0.15 g of 10% Pd(C) in 70 mL of MeOH for 3.5h. The crude product was subjected to flash chromatography on silica gel (EtOAc) to afford 0.63 g (87%) of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-tyrosinate as a thick transparent oil.

<sup>1</sup>H-NMR: (200 MHz, DMSO-d<sub>6</sub>) δ 9.17 (s, 1H, OH), 8.11 (d, 1H, J=7.8, NH), 6.96 (d, 1H, J=1.7, aromatic CH), 6.84 (dd; 1H; J=8.1, 1.9; aromatic CH), 6.68 (d, 1H, J=8.0, aromatic CH), 4.33 (m, 1H, methine), 3.14 (m, 1H, methine), 2.84 (d, 2H, J=7.0, ArCH<sub>2</sub>), 2.23 (t, 2H, J=7.9, glu 4-CH<sub>2</sub>), 1.88-1.46 (m, 4H, glu 3-CH<sub>2</sub>, NH<sub>2</sub>), 1.40 (s, 9H, t-Bu), 1.33 (s, 18H, t-Bu's).

Example 204: *tert*-Butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-tyrosinate

According to example 154, 0.320 g of *tert*-butyl 5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-tyrosinate was coupled with 0.254 g of 4-(*N*-(2,4-diamino-6-pteridiny)methyl)-*N*-methylamino)benzoic acid hemihydrochloride dihydrate using 0.15 mL of DECP, 0.19 mL of Et<sub>3</sub>N, and 20 mL of DMF. The crude product was purified by flash chromatography on silica gel (7:7:1 CH<sub>2</sub>Cl<sub>2</sub>:acetone:MeOH) to afford 0.32 g (61%) of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-tyrosinate as a yellow powder, m.p. 125°C (dec).

Example 205: *N*-(4-(((2,4-Diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-*tert*-butyl-L-tyrosine

According to example 50, 0.287 g of *tert*-butyl *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-5-*O*-*tert*-butyl-L-glutam-1-yl-3-*tert*-butyl-L-tyrosinate was treated with gaseous HCl in 55 mL of CH<sub>3</sub>NO<sub>2</sub> for 1h. The suspension was concentrated *in vacuo* to give a yellow powder. This material was suspended in 30 mL of water and treated with sufficient 1N aqueous NaOH to give complete solution. The yellow solution was filtered and acidified to pH=5.5 by addition of 1N aqueous HCl. The resulting yellow precipitate was separated by centrifugation, washed with four cycles of aqueous suspension-centrifugation-decantation, frozen and lyophilized to afford 0.178 g (68%) of *N*-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-*tert*-butyl-L-tyrosine as a yellow powder, m.p. 185°C (dec).

HPLC: one peak on C18,  $k'=3.14$ , 75:25:0.1 H<sub>2</sub>O:MeCN:TFA, flow rate=1 mL/min.

<sup>1</sup>H-NMR: (300 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  12.80-11.80 (br m), 9.09 (s, 1H, OH), 8.55 (s, 1H, pteridiny 7-CH), 8.01 (d, 1H,  $J=7.9$ , amide NH), 7.97 (d, 1H,  $J=8.1$ , amide NH), 7.78 (br s, 1H, NH<sub>2</sub>), 7.68 (d, 2H,  $J=8.9$ , aromatic CH), 7.57 (br s, 1H, NH<sub>2</sub>), 6.93 (d, 1H,  $J=1.6$ , aromatic CH), 6.85-6.64 (m, 5H, aromatic CH, NH<sub>2</sub>), 6.60 (d, 1H,  $J=8.2$ , aromatic CH), 4.77 (s, 2H, pteridiny-CH<sub>2</sub>), 4.42 (m, 1H, methine), 4.29 (m, 1H, methine), 3.19 (s, 3H, N-CH<sub>3</sub>), 2.83 (m, 2H, ArCH<sub>2</sub>), 2.23 (t, 2H,  $J=7.8$ , glu 4-CH<sub>2</sub>), 1.90 (m, 2H, glu 3-CH<sub>2</sub>), 1.25 (s, 9H, *t*-Bu).

Elemental Analysis: Calcd. for C<sub>33</sub>H<sub>39</sub>N<sub>9</sub>O<sub>7</sub>·2.3 H<sub>2</sub>O (MW 715.16): C, 55.42; H, 6.14; N, 17.63. Found: C, 55.58; H, 5.97; N, 17.47.

Mass Spectrum: (Ion Spray) 674 (M+H)<sup>+</sup>.

UV Spectrum: (pH 7 Buffer)  $\lambda_{\max}$  ( $\epsilon$ ) 259.4 (23700), 307.4 (23500), 372.5 (7330).  $\lambda_{\min}$  ( $\epsilon$ ) 242.8 (14900), 273.0 (17000), 344.7 (5660). sh ( $\epsilon$ ) 222.1 (26400), 287.2 (19600).

**Example 206****Sequencing of Human Carboxypeptidase A1 cDNA:**

A Hind III - Sal I cDNA fragment encoding the rat preprocarboxypeptidase A1 (CPA1) (Gardell *et al.*, Nature, 317:551-555, 1985) was isolated from pMP36 provided by Dr. M. Phillips (UCSF). The 1.2 kb Hind III - Sal I rat CPA1 cDNA was radiolabeled with [<sup>32</sup>P]dCTP and Prime-it kit (Stratagene) and then used to screen a lambda gt11 human pancreas cDNA library (Clontech) according to the method of Grunstein and Hogness (Proc. Nat. Acad. Sci 72: 5016-5020, 1975). Purified isolated plaques that hybridized to the rat CPA1 cDNA probe were obtained after three rounds of screening.

Lambda DNA from purified plaques was prepared from overnight growth liquid cultures using Qiagen columns and according to the manufacturer's protocol (Qiagen). Human CPA1 cDNA inserts were liberated from lambda DNA by digestion with EcoRI and purification of the cDNA inserts by low-melting agarose gel electrophoresis. The EcoRI cDNA inserts were cloned into the EcoRI site of pGEM 7z(+) (Promega) and denoted as pHCPA plasmids. Following overnight growth in liquid culture, plasmid pHCPA DNA was prepared using Qiagen columns.

DNA sequencing of isolated HCPA1 lambda plaques and of plasmids containing HCPA1 cDNA was performed with [<sup>35</sup>S]dATP and the fmol sequencing system (Promega). Oligonucleotide primers that flanked the cloning site of lambda gt11 or the multiple cloning sites in pGEM 7(+) and the TA vector (Invitrogen) were used initially to determine cDNA sequence. Oligonucleotide primers that corresponded to regions of the determined sequence were synthesized which permitted the entire cDNA sequence of both strands of HCPA1 to be determined. (Seq ID 1)

### Example 207

#### Cloning of Human Carboxypeptidase A2

Human carboxypeptidase A2 (CPA2) was cloned from a human pancreas cDNA library constructed in lambda gt11 (Clontech). The probe used was an 1198 base fragment of rat CPA2. This probe was amplified from QUICK-Clone rat pancreas cDNA (Clontech) using PCR. The upstream primer corresponded to the sequence between positions 12-33 in the rat cDNA coding sequence (5'-CCTGTTATTGGCTGCCCTACTT-3'), while the downstream primer was the complement to the sequence between positions 1888-1209 (5'-AAGCCAGGTCTCTTCTGCTGTC-3')(Gardell, S.J. *J.Biol. Chem.* **263** 17828 (1988)). The standard PCR conditions were a 94°C preincubation followed by 30 cycles of one minute at 94°C, two mins at 56°C, and three mins at 72°C. The 1198 bp PCR product was extracted with 100 ul chloroform and the upper aqueous phase containing the DNA was passed through a CHROMA SPIN-100 column (Clontech) to remove the primers. The sequence of the rat CPA2 PCR product was confirmed with DNA sequencing using the PCR primers as sequencing primers. Sequencing of the DNA was done with [<sup>32</sup>P] dATP using the fmol sequencing kit (Promega).

The rat CPA2 DNA was radiolabelled with [<sup>32</sup>P] dCTP and the Prime-it II Random Primer Labelling kit (Stratagene) and then used to screen the human pancreas cDNA library. The library was plated using E. coli strain Y1090r<sup>-</sup> on 150 mm plates (approximately 10,000 plaques/plate) and duplicate nitrocellulose lifts made. The filters were blocked in hybridization solution (50% formamide, 5X SSPE, 50X Denhardt's solution, 0.1% SDS, and 100 ug/ml sonicated salmon sperm DNA) for 3 hours at 42°C. Labelled rat CPA2 PCR product was added and hybridized to the filters overnight at 45 °C. The filters were washed for 1 hour at 60°C in 0.1X SSC, 0.1% SDS and exposed to autoradiography film while moist. One duplicate plaque from each plate was picked and subjected to another round of screening as described above. Positive plaques after the second round of screening were picked and the size of the lambda gt11 insert DNA was determined using PCR. Lambda gt11 forward and reverse sequencing primers (Clontech) that flanked the EcoRI cloning site of lambda gt11 were used as the upstream and downstream PCR primers, respectively. Well-isolated plaques were picked, with a portion suspended in 10 ul water, and heated at 95°C for 5 min, and the remainder being used to make a phage stock for future screening. The 10 ul containing the plaque DNA was used directly as the template in PCR using the conditions described above.



Amplified insert DNA was visualized on agarose gels and the two plaques giving the largest insert DNA were screened a third time. Lambda DNA from the two plaques was isolated and sequenced in both directions using the fmol kit (Promega) and the lambda gt11 forward and reverse primers (Clontech). One clone contained regions highly homologous to the 5' and 3' regions of rat CPA2 and was chosen for further study.

The insert cDNA was liberated from the lambda DNA by digestion with EcoRI, gel purified, and cloned in the EcoRI site of the pGEM-7Zf(+) vector (Promega). Sequencing was carried out using [<sup>33</sup>P] dATP and the fmol kit (Promega) as described above. Oligonucleotide primers that corresponded to regions of the determined sequence were synthesized which permitted the entire cDNA sequence of hCPA2 to be determined. The cDNA was found to contain an open reading frame coding for a protein of 417 amino acids. This protein shares 87% identity with rat CPA2 at the amino acid level and 85% identity at the cDNA level. This protein shares 63% identity with human CPA1 at both the amino acid and nucleotide levels. Thus the protein was classified as human carboxypeptidase A2. Table 4 shows a comparison between the amino acid sequences of human CPA1 and CPA2. The amino acid numbering scheme used throughout this document is that of CPA1. All mutations described for CPA 1 and CPA2 refer to this numbering scheme.

#### Example 208

##### Expression of HCPA1 in Yeast:

The size of cDNA inserts within isolated HCPA1 lambda plaques was determined using oligonucleotide primers that flanked the EcoRI cloning site of lambda gt11 and polymerase chain reaction (PCR). Single isolated, plaques were picked, suspended in 200µl water and heated at 95°C for 5 minutes. Ten µl of each suspension was used for PCR amplification according to manufacturer's instructions (Perkin Elmer/Cetus). The standard PCR reaction conditions consisted of 1 minute at 95°C, .5 minute at 50°C, and 3 minutes at 72°C. The reaction products were visualized following agarose gel electrophoresis.

CPA-11 5'-gCT gAA gCT TCg gAg gAC TTT-3'

CPA-12 5'-TCT TgA CCg CCT ggA TgC Tg-3'

The expression of HCPA1 in *S. cerevisiae* was based on the strategy employed by Gardell *et al* (Nature, 317; 551-555, 1985). Two oligonucleotide primers, CPA 11 and 12, were used to amplify a 200 bp fragment from pHCPA by PCR as described above. The amplified fragment contained a new 5' Hind III site created by CPA 11 that would allow ligation in frame with the alpha factor leader of pMP36 as described by Phillips *et al* (J. Biol. Chem. 265; 20692-20698, 1990). The PCR fragment was ligated into the TA vector (Invitrogen) and the entire DNA sequence of the fragment determined as described earlier. A colony that contained the desired 5' alteration was selected for further use, TAHCPAexp.

Plasmid pHCPA was restricted with Aat II and EcoR1 to liberate a 1.1 kb HCPA1 fragment. This fragment was purified following low melting point agarose gel electrophoresis and ligated into TAHCPAexp that had been restricted with AatII and EcoR1 to yield the plasmid TAHCPA. TAHCPA was digested with EcoR1, treated with T4 DNA polymerase, and Sal I linkers were added and ligated to the 3' end of HCPA1 as described by Maniatis (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, 1989). TAHCPA that now contained 5' Hind III and 3' Sal I sites was restricted with Hind III and Sal I to liberate a 1.2 kb HCPA1 cDNA fragment. This fragment was ligated together with the large Hind III fragment and the small Hind III-SalI fragment of pMP36 to produce pMP36 HCPA1.

Yeast expression of the cloned human enzyme is based on the method of Phillips, M. *et al*, J. Biol. Chem., 265, 20692 (1990). Plasmid pMP36 containing HCPA1 was restricted sequentially with BAMH1, SAL1 and SSP1 with intervening purifications by either phenol extractions or use of Promega Magic Mini Columns with manufacturer supplied procedures. Alternatively, the One-Phor-All buffer system (Pharmacia) can be used in a concurrent restriction by all three enzymes.

Following SSP1 restriction, the mixture was separated on a 1% low melting gel giving 5 distinct bands. The band of interest at about 2.8 kb (the second from top) was excised from the gel and purified using a Schleicher and Schuell Elutip-D column with the supplied procedures.

The BAM/SAL/SSP fragment was then ligated into pBS24.1 shuttle vector (produced from pBS24.1-AGC3 kindly provided by Dr M Phillips, UCSF with BAMH1/SAL1

restriction) overnight at 16°C using the T4 ligase system (Gibco BRL). The ligated vector was precipitated with ammonium acetate and ethanol at -80°C for 4 hrs and resuspended in 10µl TE.

4µl of the vector was electroporated into 40µl of DH5alpha E. coli using a Bio Rad Gene Pulser at 2000 volts with a time constant around 4. One ml of Miller Hinton broth was added and the sample incubated at 37°C for one hour. Three 100µl samples of each was plated out onto ampicillin containing LB plates.

The resultant colonies were replated on fresh plates. 100ml volumes of Mueller-Hinton/ampicillin broth were inoculated with colonies and incubated overnight at 37°C. The plasmid was extracted using the Qiagen Maxi extraction procedure.

Approximately 500 to 2000ng of the PBS24.1 plasmid DNA extracted above was electroporated into 40 µl of competent DLM101 alpha yeast cells. One ml of sorbitol was added immediately after electroporation to rescue the cells. 100 µl samples were plated out on dishes of YNB (yeast nitrogen broth) lacking uracil which were incubated at 30°C. Positive clones were picked. Transformants were grown in minimal synthetic medium (Wickersham, L.J., U.S. Dept. of Agric. Tech. Bull. No. 1029 (1951), lacking leucine, for 48 hrs. This culture was then used to seed a 350 litre fermenter.

#### Example 209

##### Purification of HCPA1 or its mutants from yeast:

A 350 l culture of proCPA-producing *S. cerevisiae* (T268G/p2619) was grown in a 500 l New Brunswick fermentor for 65 h at 30°C with 175 lpm air flow, 5 psig head pressure and 150 rpm agitation. The medium contained 1% yeast extract (Difco), 2% tryptone (Difco), 1% glucose, 20 mM KPO<sub>4</sub>, pH 7.5, 50µg/ml ampicillin and 0.007% (v/v) Mazu DF 60P antifoaming agent (Mazei' Chemicals, Inc.). The final OD<sub>600</sub> was 14. After cooling to 15-20°C the cells were removed from the medium by cross-flow filtration on a Sartorius Sartocon II system equipped with 7 0.6m<sup>2</sup> polyolefin filter units. The protein in the medium was concentrated with 10K MWCO cross-flow filters and the fluid exchanged by diafiltration with 20mM Tris-Cl. pH 8, 0.1 mM Zn-acetate (buffer A). All subsequent buffers contained 0.1 mM Zn-acetate. Ammonium sulfate (AS) was added to 0.5M to the 10K concentrate and it was loaded onto a 250 ml radial

flow Phenyl-sepharose column equilibrated in buffer A with 0.5 M AS. The proCPA was eluted with a 1.5 l 0.5-0 M AS gradient followed by a buffer A wash. The Phenyl-Sepharose procedure was repeated with the breakthrough material to recover unbound proCPA from the first run. Fractions containing proCPA were pooled and concentrated and dialyzed on a Sartorius 10K Easy Flow filter (0.2m<sup>2</sup>) into buffer A. This was loaded onto a Hi Load 26/10 Q-Sepharose High Performance (Pharmacia) column (53ml) and eluted with a 1 litre 0-0.5M NaCl gradient in buffer A. The proCPA eluted between 120-220 mM NaCl with the peak tube at ~150 mM. Fractions containing proCPA were pooled and the proCPA was cleaved to mature CPA with trypsin (2µg/ml) at 37° C for 1 h. PMSF was added (0.5 mM) and the pool was dialyzed in buffer A. The Q-Sepharose HP step was repeated and the activated CPA eluted between 80-140 mM with the peak tube at 120 mM. Previous IEF experiments have shown that proCPA has a pI=4.75 while the mature CPA has a pI=6.2. The CPA fractions were pooled and concentrated with 10K Centripreps (Amicon) to 15 ml and this was passed through a HiLoad 26/60 Superdex 75 (Pharmacia) column in 3 runs (5 ml each). The elution buffer was buffer A with 150mM NaCl. The CPA fractions were pooled, concentrated with 10K Centripreps and exchanged with Sigma PBS containing 0.1 mM Zn-acetate. The final volume (15ml) was passed through a 0.2µ syringe filter and stored at 4°C. The protein was 24.5 mg/ml by the Lowry assay using BSA standards. The specific activity for a typical preparation of the mutant with Gly at 268 was 163 u/mg. Total protein = 368 mg.

### **Example 210**

#### **Expression of HCPA2 in Yeast**

The expression of HCPA2 in *S. cerevisiae* was employed the same strategy described for HCPA1 (Example 208) with the following exceptions: 1) the PCR primers used to introduce the new 5' Hind III site were :

YEAST 1: 5' - gCA gAA gCT TCA gAA ACg TTT gTg ggA gAT CAA gTTCTT  
gAg ATT gTA CC - 3'

YEAST 2: 5' - CTC TTT gTC CAA CAg gAC CTg - 3'

2) a Hinc II site rather than Aat II was used to subclone the mutagenic PCR fragment to the 5' end of the HCPA2 cDNA, and 3) the cloned PCR product was cut from the TA

vector and subcloned into the pGEM-7Zf(+) vector containing the HCPA2 cDNA. PCR was carried out with the YEAST 1 and 2 primers as described for HCPA1. The mutagenic PCR product was cloned into the TA vector and the sequence confirmed. This fragment was cut from the TA vector using Hind III and Hinc II and cloned into the corresponding sites of pHCPA2 and the resulting construct designated pHCPA2-Y. The unique 3' Eco RI site of pHCPA2-Y was changed to a Sal I site using linkers as described above with the resulting construct designated pHCPA2-YSal I. The 1.2 kb Hind III/Sal I HCPA2 fragment derived from pHCPA2-YSal I was excised from the vector and ligated together with the large Hind III/Sal I fragment of pMP36 (Phillips, M. *et al*, J. Biol. Chem., 265, 20692-20698 (1990)) to produce pMP36HCPA2.

Yeast expression of clone HCPA2 was as described above for HCPA1 (Example 144). Human CPA2 has two internal Bam HI sites not present in human CPA1 so partial digestion was needed for the final subcloning step. pMP36HCPA2 was digested to completion with Sal I and partially digested with Bam HI (as described above) to yield the 2.7 kb fragment containing the alcohol dehydrogenase/GADPH promotor and regulatory region (A/G promotor) attached to human CPA2 fused in frame to the yeast  $\alpha$  factor leader. This fragment was ligated into the Sal I/Hind III site of pBS24.1 forming pBSHCPA2. The construct was amplified in DH5 $\alpha$  and used to electroporate yeast as described above (Example 208).

#### **Example 211**

##### **Purification of HCPA2 or its Mutants from Yeast**

An overnight culture (grown in Leu<sup>-</sup> medium) of *S. cerevisiae* expressing proCPA2 was used to start 2 l of culture in 1% yeast extract (Difco), 2% tryptone (Difco), 1% glucose, 20 mM KPO<sub>4</sub>, pH 7.5, and 50  $\mu$ g/ml ampicillin. The culture was grown for 48 hrs at 30°C and the supernatant cleared by centrifugation at 6000 X g for 10 min at 4°C. The protein in the supernatant was concentrated on an Amicon PM-10 Ultrafiltration membrane to 114 ml. The concentrated supernatant was made to 20 mM Tris-HCl (pH 8.0), 0.1 mM Zn-acetate and 0.5 M ammonium sulfate and loaded onto a 1.8 X 20 cm Phenyl-Sepharose column (Pharmacia) equilibrated in buffer A with 0.5 M ammonium sulfate. The protein was eluted with a 120 ml linear gradient (1 ml/min) from buffer B (20 mM Tris-HCl pH 7.5, 0.1mM Zn-acetate) with 0.5 M ammonium sulfate to buffer B with 20 % (v/v) ethanol. Fractions containing carboxypeptidase activity were pooled

and dialyzed against buffer B. The dialyzed pool was loaded onto an HR 5/5 (1 ml) Mono Q column (Pharmacia) and eluted with a 30 ml linear gradient (1ml/min) from 0-0.5 M NaCl in buffer B. Fractions containing carboxypeptidase activity were pooled and dialyzed against buffer B. The pooled proCPA2 was activated with trypsin (10 µg/ml) at 37°C for 1 hr and the trypsin inactivated with PMSF added to 0.5 mM. The mature CPA2 was dialyzed against buffer B and again loaded onto the 1 ml Mono Q column (Pharmacia) and eluted as above. Fractions containing carboxypeptidase activity were pooled, dialyzed against buffer B, and concentrated 10 fold in an Amicon Ultrafiltration cell using a PM-10 membrane. The concentrated mature CPA2 was loaded onto an HR 10/30 Superose 12 column (25 ml) and eluted isocratically (0.4 ml/min.) in phosphate buffered saline (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.15 M NaCl, pH 7.2). For wild type HCPA2 the protein was 0.65 mg/ml (4.13 mg total) and for the 268 Gly mutation the concentration was 0.3 mg/ml (4.47 mg total) as determined with the Micro BCA Assay (Pierce Chemical Co.) using BSA standards.

#### Example 212

##### Expression of Mutant HCPA2

The A250G and T268G mutations of HCPA2 were constructed as described above (Example 149) for mutant forms of HCPA1 using the T7-Gen *In Vitro* Mutagenesis Kit (United States Biochemical, No. 74500). The mutagenic oligonucleotide primers (Oligos Etc.) to mutate the A250 (gCC) and T268 (ACC) to Gly (ggC) are shown below:

A250G 5' - TCC TCC ACT gCC TTg gTA gAT - 3' Gly = ggC

T268G 5' - CAg TTC AAA gCC AAA TgA gTA - 3' Gly = ggC

Mutagenic codons are underlined.

Using these oligonucleotide primers the following mutant forms of HCPA2 were produced:

1. A250G
2. T268G

Each of the above mutagenized HCPA2 cassettes was sequenced to verify that only the desired DNA mutations were produced. The mutagenized cassettes were subcloned into pMP36HCPA2 that could further be processed for expression in *S. cerevisiae* using methods described in Example 152.

### **Example 213**

#### **Synthesis of a Chemical conjugate between Campath-1-H or ING-1 and Human carboxypeptidase A mutants**

2mg of mutant carboxypeptidase A was combined with 0.15 mg of Sulfo-SMCC (Pierce Chemical Co) in 475  $\mu$ l of Dulbecco's Phosphate Buffered Saline (PBS). The resulting solution was stirred for 45 minutes at 25°C. The modified enzyme was purified through a 1 x 13 cm G-25 medium column equilibrated with PBS. Purified product can be stored at 4°C for 24 hrs.

Maleimide content was determined by combining 0.5 ml of 6.3  $\mu$ M modified enzyme with 6  $\mu$ l of 1mM mercaptoethanolamine. After 30 minutes, 20  $\mu$ l of 4 mg/ml Ellman's reagent was added, and after another 20 minutes absorbance at 412 nm was determined. Based upon a molar absorptivity of 13.6 mM<sup>-1</sup>, the purified product was found to contain 1 maleimide per enzyme molecule. The modification had no effect upon enzyme activity.

The antibody was modified with 2-iminothiolane (Pierce Chemical Co). 0.35 mg of antibody solution was combined with 0.055 mg of 2-iminothiolane in 0.1 M triethanolamine-HCl, 2mM EDTA, pH 8.0 under anaerobic conditions, reacted with stirring for 1 hr and 45 mins. The modified antibody was purified through a 1 x 13 cm G-25 medium column equilibrated with 0.02 M sodium acetate, 0.1 M NaCl, pH 5.8, bubbled with and maintained under a He atmosphere. The purified modified antibody solution was collected directly into the solution of modified carboxypeptidase A. The resulting solution was adjusted to pH 7.4 with NaOH, made anaerobic, and allowed to react, with stirring, at 4°C for 18 hours. (Optionally free maleimide groups may be removed by reacting the solution with 0.3 mM mercaptoethanolamine at room temperature for one hour). The solution concentrated to 1 ml. The resulting concentrated conjugate was purified from aggregates, unreacted enzyme and small molecules by chromatography on Superose 12 HR 10/30. The enzyme specific activity

of the conjugate was found to be appropriate for a one to one conjugate with antibody. Antibody (Campath-IH® or ING-1) % immunnoreactivity and antigen binding affinity of the conjugate were both found to be high.

#### **Example 214**

The enzyme was modified by the protocol described for Example 4 above, while the antibody was modified with dithiothreitol. 0.7 mg of antibody solution was combined with 0.035  $\mu$ mol of dithiothreitol in 350  $\mu$ l of 0.1 M triethanolamine-HCl, 2 mM EDTA, pH 8.0 under anaerobic conditions and reacted with stirring for 1 hr. The modified antibody was purified through a 1 x 13 cm G-25 medium column equilibrated with 0.02 M sodium acetate, 0.1 M NaCl, pH 5.8, bubbled with and maintained under a He atmosphere. The purified antibody solution was collected directly into the solution of modified carboxypeptidase A. The resulting solution was adjusted to pH 7.4 with NaOH, made anaerobic, and allowed to react, with stirring, at 24°C for 30 min and at 4°C for 18 hrs. Then the solution was concentrated to 1ml. The resulting concentrated conjugate was purified from aggregates, unreacted enzyme and small molecules by chromatography on Superose 12 HR 10/30. The enzyme specific activity of the conjugate was found to be appropriate for a one to one conjugate with antibody. Antibody % immunoreactivity and antigen binding affinity were both found to be high.

#### **Example 215**

The enzyme was modified by the protocol described for Example 4 above, while the antibody was modified with SATA (Pierce Chemical Co). 0.225 mg of antibody solution was combined with 0.003 mg of SATA in a total volume of 150  $\mu$ l of Dulbecco's Phosphate Buffered Saline (PBS) and allowed to react with stirring for 45 mins. The modified antibody was purified through a 1 x 13 cm G-25 medium column equilibrated with PBS. The purified modified antibody solution was mixed with the modified enzyme and made anaerobic, 300  $\mu$ l of anaerobic 0.5 M  $\text{NH}_2\text{OH}$  was added and the solution allowed to react, with stirring, at 25°C for 2 hrs followed by 4°C for 18 hrs. Free maleimide groups were removed by reacting the solution with 0.3 mM mercaptoethanolamine at room temperature for one hour, and the solution was then concentrated to 1 ml. The resulting concentrated conjugate was purified from aggregates, unreacted enzyme and small molecules by chromatography on Superose 12



IIR 10/30. The enzyme specific activity of the conjugate was found to be appropriate for a one to one conjugate with antibody. Antibody antigen binding affinity was found to be high.

### **Example 216**

#### **Expression of Mutant HCPA1**

pMP36 containing wild type (WT) proHCPA1 cDNA (as a fusion with yeast alpha-factor leader described above) was restricted with Nco I and Sal I to liberate a 481 bp cDNA fragment. This fragment begins at nucleotide 893, proceeds to the very 3' end of HCPA1 cDNA and encodes amino acids 186-309 of mature HCPA1. The HCPA1 Nco I-Sal I fragment was ligated into the Nco I and Sal I cloning sites of pGEM5zf(-) (Promega) to generate pHCPAINS (Figure 3). pHCPAINS was then restricted with Sph I and Sal I to liberate the HCPA1 Nco I-Sal I fragment and additional (9 bp) pGEM5zf(-) sequence.. This Sph I-Sal I fragment was cloned into M13mp19 (BRL) using its Sph I and Sal I cloning sites to generate M13mp19HCPA1 (Figure 4).

Single stranded M13mp19HCPA1 DNA was used as template for oligonucleotide-directed mutagenesis using the T7-GEN *In Vitro* Mutagenesis Kit (United States Biochemical, No. 74500). Mutagenic oligonucleotide primers (Oligos Etc) listed below were used to mutate residues I255 (ATT) and T268 (ACC) either separately or in tandem.

1. I255A 5'- ggT CCA gTC AgC AgT gCT TCC -3' Ala = gCT
2. T268A 5'- gAg CTC gAA ggC gAA ggA gTA -3' Ala = gCC
3. T268G 5'- gAg CTC gAA gCC gAA ggA gTA -3' Gly = ggC

Mutagenic codons are underlined.

Using these oligonucleotide primers the following mutant forms of HCPA1 were produced:

1. I255A
2. T268A
3. T268G
4. I255A/T268A

Each of the above mutagenized HCPA1 cassettes was sequenced to verify that only the desired DNA mutations were produced.

pMP36 HCPA1 w.t. was restricted with NcoI to liberate a 1.2 kb fragment that was cloned into the Nco I site of M13mp19HCPA1 mutants. After the correct orientation of the Nco I fragment within M13mp19 proHCPA1 mutants (Figure 5) was verified the DNAs were restricted with Hind III and Sal I which liberated a 1.2 kb cDNA fragment encoding the entire proHCPA1 mutant enzyme. This fragment was then ligated into the Hind III and Sal I sites of pMP36 yielding pMPHCPA1 mutants (Figure 6) that could then be further processed for expression in *S. cerevisiae* using the methods described in Examples 2 and 3.

#### **Example 217**

##### **Expression of Mutant HCPA1**

Alternatively, the mutations have been conveniently generated in hCPA in the pMP36 plasmid (pMP36 HCPA1) by the unique site elimination (USE) procedure of Clontech Laboratories, Inc. using the manufacturers' reagents and protocols.

In the protocol, elimination of a unique EcoRI in the pMP36 HCPA1 plasmid was used for selection with the oligonucleotide XS5 (TCC CCC GGG CTG CAG GAT ATC GAT AGC TGG GCT GTG T). The oligonucleotides used to generate three specific mutations in hCPA by this protocol follow:

T 268 H (ACC codon to CAC) with oligonucleotide XT2  
(ATC AAG TAC TCC TCC CAG CTC CGG GAC)

S 253 G (AGC codon to GGT) with oligonucleotide XT3  
(TTT ATC AAG CCA GTG GAG GTA TTG ACT GGA CC)

A 250 G (GCC codon to GGC) with oligonucleotide XT4  
(AAG GCA ATT TAT CAA GGC AGT GGA AGC ACT ATT)

The mutations were isolated using the oligonucleotides listed above and XS5 (TCC CCC GGG CTG CAG GAT ATC GAT AGC TGG GCT GTG T) which eliminates a unique EcoRI site. Plasmids containing the desired mutations were selected by digesting the wild type pMP36(hCPA) with EcoRI (uncut plasmids transform 10 to 100x more efficiently than linearized plasmid). In addition, the XS5 selection oligonucleotide introduces an EcoRV site which can then be used to screen for mutant plasmids. In the mutant plasmids the EcoRV is unique and can therefore be used as a selection site to construct double mutants with an oligonucleotide that converts the EcoRV site back to an EcoRI site.

The mutant pMP36 HCPA so generated could then be further processed for expression in S. cerevisiae using the methods described in Examples 2 and 3.

#### Example 218

##### Stability and biodistribution of prodrugs *in vivo*:

Mice were dosed i.p. or i.v. bolus with 50 mg/kg of prodrug by administration of a 5 mg/ml solution in PBS, pH 6-8. Animals were sacrificed after 30 min and 2 h and the plasma, and tissues were collected. Plasma was frozen at -70°C and tissues were snap frozen in liquid nitrogen and stored at -70°C. Duplicate animals were sacrificed for each time point. Plasma was prepared for extraction by 4-fold dilution with ice cold 0.1 N HCl. Tissues were prepared for extraction by homogenization in 5 volumes of ice cold 0.1 N HCl with a Polytron equipped with a PTA 7 generator (Brinkman Instruments, Westbury, NY). Drugs were subsequently extracted by adding 500 µl of -20°C acetonitrile to 200 µl of the 0.1N HCl homogenate. After a 5 min incubation on ice, samples were centrifuged at 12,400 x g for 15 min. Supernatants were collected and diluted 3.57 fold with PBS to reduce the final acetonitrile concentration to 20%. Diluted supernatants were then analyzed by HPLC as described below. Drug recovery was estimated by spiking control tissue homogenates with either drug or prodrug prior to the acetonitrile extraction step. After correction for recovery of these standards, the levels of drug and prodrug in tissues and the stability of the prodrug in these tissues were calculated using HPLC.

Samples were analyzed on a 4 µm C18 Nova-Pak 3.9 x 300 mm steel HPLC column equipped with a C18 guard column with UV detection at a flow rate of 1 ml/min.

Samples were injected onto the HPLC column equilibrated with 2% acetic acid, pH 3.0, containing either 5% or 20% acetonitrile depending upon the compound being analyzed. Upon sample injection a 25 or 35 min linear gradient was initiated raising the acetonitrile concentration to 50% acetonitrile. Three compounds (ASP-MTX, o-cyclopentyl-PHE-MTX and m-cyclopentyl-PHE-MTX) were analyzed on a 10  $\mu$ m C18 uBondapak column (3.9 x 300 mm) equilibrated with 16% acetonitrile in 5 mM tetrabutylammonium hydrogen sulfate, 10 mM  $\text{NH}_4\text{H}_2\text{PO}_4$ . The column was then eluted with a 30 min linear gradient of up to 50% acetonitrile.

Results indicated in Table 1. The results show that a compound that is a good substrate for wild type CPA, such as compound # 10, is not stable *in vivo*. This type of compound is not useful for ADEPT. However, compounds which are good substrates for a mutant enzyme but not for the wild type enzyme, such as compounds 8, 9, 11, 12, 13 and 14 and others, are stable *in vivo* and are as such useful in ADEPT.

The data shown in Table 1 indicate the concentration of prodrug detected over time in minutes as either  $\mu$ M in the case of plasma or nmol/g in the case of tissues. The figures in brackets indicate the stability of the prodrug expressed as the percentage of material (sum of drug and prodrug) observed as intact prodrug.

Except as indicated all experiments were carried out in CD-1 nude mice.

**Table 1**

Prodrug	Time	PLASMA		LIVER		KIDNEY		SPLEEN	
1 259W91	30	41.5	(99)	770.3	(98)	175.8	(98)	23.4	(100)
	120	4.2	(100)	579.8	(97)	70.3	(97)	1.8	(100)
2 519W91	30	33.0	(100)	42.0	(98)	9.0	(98)	ND	
	90	5.5	(98)	9.1	(100)	1.3	(98)	ND	
	180	3.5	(97)	0.8	(100)	0.2	(100)	ND	
2 519W91	30	22.1	(100)						
	120	0.3	(93)						
3 552W92	30	96.3	(93)	128.8	(79)	41.7	(82)	76.6	(95)
	120	5.1	(87)	267.9	(74)	9.2	(74)	12.1	(89)
3 552W92	30	32.4	(93)	135.3	(87)	29.2	(89)	30.9	(100)
	120	2.8	(48)	371.2	(90)	4.0	(74)	1.5	(100)
4 2484W92	30	71.4	(100)	343.4	(99)	94.5	(100)	30.9	(99)
	120	1.4	(99)	33.3	(98)	14.5	(99)	ND	
5 2749W92	30	114.6	(98)	118.2	(81)	42.5	(93)	34.3	(85)
	120	9.4	(95)	7.5	(41)	3.0	(96)	3.2	(81)
6 3347W92	30	46.7	(100)	287.8	(97)	93.4	(98)	3.4	(70)
	120	2.0	(100)	161.6	(93)	12.6	(98)	0.0	(0)
7 3855W92	30	51.4	(100)	171.9	(100)	55.4	(100)	12.4	(100)
	120	0.2	(100)	7.5	(89)	3.8	(100)	1.0	(100)
8 250W93	30	28.5	(95)	113.5	(90)	5.6	(84)	1.4	(98)
	120	1.1	(90)	43.9	(76)	3.2	(81)	1.1	(100)
9 637W93 (BALB/C)	30	1.1	(100)	54.3	(83)	6.8	(100)	8.4	(100)
	120	ND		126.2	(94)	1.3	(100)	ND	
	30	4.9	(100)						
10 1311W92	30	17.8	(30.2)	335.1	(94.425)	26.9	(100)	19.9	(80.4)
	120	1.1	(2.1)	0.6	(7.55)	0.0		ND	
11 3352W93	30	22.2	(97.075)	53.8	(81.35)	7.3	(83.475)	2.7	(96.45)
	120	ND		47.8	(73.325)	3.0	(82.3)	ND	
12 1834W93	30	2.6	(100)	58.6	(91.325)	2.2	(53.433)	ND	
	120	0.7	(100)	39.5	(89.25)	0.2	(21.55)	ND	
13 5755U93	30	3.3	(100)	47.7	(100)	3.7	(100)	0.6	(100)
	120	0.6	(100)	129.0	(100)	0.5	(100)	0.2	(100)
14 4204W93	30	1.1	(100)	22.5	(100)	4.6	(100)	2.4	(100)
	120	ND		42.1	(100)	ND		ND	

**Table 1 (Continued)**

Prodrug	Time	LARGE INTESTINE		CECUM		SMALL INTESTINE		FECES		TUMOR	
1 259W91	30 120	12.2 1.0	(93) (100)	6.0 1.2	(96) (76)	48.2 55.6	(83) (68)			6.0 1.2	(96) (76)
2 519W91	30 90 180	5.9 0.7 1.3	(93) (60) (52)	2.7 ND ND	(98)					2.7 ND ND	(98)
2 519W91	30 120					170.2 17.6	(91) (47)				
3 552W92	30 120	23.4 1.7	(87) (89)								
3 552W92	30 120	14.9 1.1	(94) (87)			27.7 1.8	(10) (1)				
4 2484W92	30 120	25.3 45.4	(100) (100)	6.7 1.3	(100) (100)	188.1 69.1	(99) (98)			6.7 1.3	(100) (100)
5 2749W92	30 120	28.4 1.6	(93) (8)	4.7 2.9	(95) (88)	32.1 1.6	(40) (2)			4.7 2.9	(95) (88)
6 3347W92	30 120	5.7 19.4	(100) (65)	7.5 0.8	(82) (59)	21.3 14.2	(86) (54)	4.2 207	(21) (52)	7.5 0.8	(82) (59)
7 3855W92	30 120	12.1 91.8	(100) (100)	6.9 1.5	(100) (100)	127.4 24.1	(100) (97)			6.9 1.5	(100) (100)
8 250W93	30 120	5.2 27.3	(93) (57)	6.8 235.1	(75) (56)	364.5 31.7	(77) (35)	150.2 646.2	(71) (55)		
9 637W93 (BALB/C)	30 120 30	2.6 6.2	(100) (100)	4.1 108.3	(100) (99)	377.9 211.0 420.3	(97) (93) (98)	1104.1 1505.7 1604.27	(97) (94) (98)		
10 1311W92	30 120	19.3 1.1	(65.35) (21.6)	3.0 ND	(65)	17.3 ND	(16) (0)			3.0 ND	(65)
11 3352W93	30 120	2.7 4.4	(74.45) (84.95)	4.5 0.8	(100) (100)	301.3 140.0	(86.725) (67.7)	426.8 389.8	(92.275) (67.925)	4.8 2.4	(100) (88.1)
12 1834W93	30 120	ND 14.7	(66.925)	16.3 402.3	(100) (80.7)	550.8 44.9	(91) (67)	1205 1941	(93) (82)	1.6 ND	(92.8)
13 5755U93	30 120	7.9 12.5	(100) (100)	14.4 4.8	(100) (100)	373.7 322.1	(100) (100)	665.8 738.4	(100) (100)		
14 4204W93	30 120	9.4 34.6	(100) (76.775)	4.1 65.3	(100) (48.75)	218.3 127.8	(100) (100)	535.0 932.8	(90.675) (88.05)		

Prodrugs 1 to 14 shown in Table 1 are as follows:

1. N-(N-(4((((1,2-dihydro-3-methyl-1-oxobenzo(F)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl)-L-glutamic acid
2. N-((S)-4-carboxy-2-(5-((((1,2-dihydro-3-methyl-1-oxobenzo(F)quinazolin-9-yl)methyl)amino)-1-oxo-2-isoindoliny)butanoyl)-L-phenylalanine
3. N-(N-(2-fluoro-4-((((1,2-dihydro-3-methyl-1-oxobenzo(F)quinazolin-9-yl)methyl)amino)benzoyl)-L-glutam-1-yl)-3-(1-naphthyl)-L-alanine
4. N-(N-(4((((1,2-dihydro-3-methyl-1-oxobenzo(F)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl)-2-carboxy-L-phenylalanine
5. N-(N-(4((((1,2-dihydro-3-methyl-1-oxobenzo(F)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl-2-iodo-L-phenylalanine
6. N-(4-((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-aspartic acid
7. N-(4-((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-carboxy-L-phenylalanine
8. N-(4-((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-phenylalanine
9. N-(4-((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine
10. N-(4-((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-phenylalanine.
11. N-(4-((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclobutyl-L-phenylalanine.

12. N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-tert-butyl-L-phenylalanine.
13. N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-tyrosine.
14. N-(4-((3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine.



### **Example 219**

#### **Molecular modelling of human CPA1:**

The Brookhaven Protein Database (Bernstein F.C. *et al.*, J. Mol. Biol. 112, 535-542 [1977]) was searched and bovine CPA selected as that likely to have the greatest structural similarity to human CPA (HCPA) based on base sequence homology. The COMPOSER algorithm which was used to develop the HCPA model is part of the commercially available SYBYL molecular modelling package sold by TRIPOS Associates (SYBYL Molecular Modeling Software, Version 5.3, November 1989, TRIPOS Associates Inc., St Louis, MO 63144). The COMPOSER algorithm was used to fit the amino acid residues of the human CPA sequence into the 3D coordinates of the crystal structure of bovine CPA to determine a best fit and consequently a projected 3D structure for HCPA.

### **Example 220**

#### **Cell Culture Targetting and Therapy**

Wein-133 B-cell lymphoma cells were incubated with a conjugate of wild-type HCPA1 and Campath for 30 min. Cells were then washed to remove unbound conjugate and returned to cell culture at 500,000 cells ml<sup>-1</sup>. Cells were cultured in the presence of varying concentrations of the prodrug MTX-Phe and levels of inhibition of growth analysed after 3 days. Results are illustrated in Fig 1. Similar results were obtained when a conjugate of mutant 268 Gly with Campath was substituted for the wild type conjugate.

### **Example 221**

#### **Prodrug toxicity**

The stable prodrug of the present invention should in addition to being stable *in vivo* also be less toxic in the host than the parent drug in order to permit larger dosing levels of prodrug and in turn generation of local high concentrations of active agent. We have found that the maximum tolerated dose for methotrexate in the mouse (female, strain CD-1 nu/nu) is 4 mg/kg IV once daily for 5 days. Doses of N-(4-(((2,4-diamino-6-pteridiny)l) methyl) methylamino) benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-phenylalanine up to 20 mg/kg IV once daily for 5 days were tolerated, and doses of up to 40mg/kg of

N-(4-(((2,4-diamino-6-pteridiny) methyl) methylamino) benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine or N-(4-(((2,4-diamino-6- pteridiny) methyl) methylamino) benzoyl)-L-glutam-1-yl-3-tert-butyl-L-phenylalanine IV once daily for 5 days produced no toxicity; therefore the maximally tolerated doses of the latter two compounds are greater than 40mg/kg. In Swiss nu/nu mice, the maximum tolerated dose of methotrexate is 2.5 mg/kg IV daily for 5 days. In these mice, N-(4-(((2,4-diamino-6- pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-tyrosine was tolerated at doses to 50 mg/kg IV daily for 5 days while N-(4-(((2,4-diamino-6- pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclobutyl-L-phenylalanine showed some toxicity at doses of 30 mg/kg IV daily for 5 days. Thus, in Swiss nu/nu mice, with this daily x 5 schedule, the MTD for N-(4-(((2,4-diamino-6- pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-tyrosine and N-(4-(((2,4-diamino-6- pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclobutyl-L-phenylalanine would be > 50 mg/kg and < 30 mg/kg, respectively. Thus the prodrugs of the present invention are indeed much better tolerated than the corresponding active agent.

### **Example 222**

#### **Activity of a mutant human carboxypeptidase A1 with a stable prodrug**

As indicated herein, the *in vivo* stable prodrug of the present invention should be a better substrate for a mutant enzyme than the corresponding wild type enzyme in order for the stable prodrug to be activated selectively at the tumour. Table 2 shows the enzyme kinetics for several prodrugs of methotrexate with wild type human carboxypeptidase A1 and mutants with thr 268 mutated to either Ala or Gly. The most relevant kinetic constant in the table is the  $k_{cat}/K_m$ , which provides an estimate of the second order rate constant for the enzyme catalyzed conversion of prodrug to methotrexate. Thus, the larger the  $k_{cat}/K_m$ , the more efficient the enzyme with a particular substrate. It can be seen that the  $k_{cat}/K_m$  for human CPA1 with one of the best substrates for this wild type enzyme N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-phenylalanine the prodrug of MTX, is 0.44/uM/sec. The  $k_{cat}/K_m$  values for N-(4-(((2,4-diamino-6-pteridiny) methyl) methylamino) benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-phenylalanine, N-(4-(((2,4-diamino-6-pteridiny) methyl) methylamino) benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine, N-(4-(((2,4-diamino-6-pteridiny) methyl) methylamino) benzoyl)-L-glutam-1-yl-3-tertbutyl-L-phenylalanine, N-(4-(((2,4-

diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclobutyl-L-phenylalanine, and N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-tyrosine for the human CPA1 are much lower; indeed the human CPA catalyzed reactions with the latter four prodrugs were not measureable. However, the  $k_{cat}/K_m$  for these five stable prodrugs with 268Gly are 0.157, 0.092, 0.38, 1.8 and 0.18/uM/sec, respectively. Thus, the single mutation at 268 from thr to gly converted very poor substrates in the wild type enzyme to excellent substrates in the mutant enzyme. Indeed in the case of N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-tertbutyl-L-phenylalanine the activity of 268Gly with the prodrug is not different than the human CPA1 with N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-phenylalanine, one of its best substrates. Further, in the case of N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclobutyl-L-phenylalanine the activity of 268Gly is 4 times better than CPA1 with N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-phenylalanine. Thus, the compounds are much better substrates for the mutant enzyme than the wild type enzyme which permits very specific tumor targeted activation of the nontoxic, stable prodrug to the active agent.

**Table 2**

<b>Prodrug</b>	<b>kinetic constant</b>	<b>Human CPA1</b>	<b>268ala</b>	<b>268gly</b>
N-(4-(((2,4-diamino-6-pteridiny)methylmethylamino)benzoyl)-L-glutam-1-yl-L-phenylalanine	Km (uM)	4.3	0.9	0.3
	kcat (1/sec)	1.9	2.0	2.2
	kcat/Km	0.44	2.25	7.35
N-(4-(((2,4-diamino-6-pteridiny)methylmethylamino)benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-phenylalanine	Km (uM)	61	56	15
	kcat (1/sec)	0.012	0.088	2.35
	kcat/Km	0.0002	0.0016	0.157
N-(4-(((2,4-diamino-6-pteridiny)methylmethylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine	Km (uM)	ND	31	15
	kcat (1/sec)	ND	0.001	1.4
	kcat/Km	ND	0.00003	0.092
N-(4-(((2,4-diamino-6-pteridiny)methylmethylamino)benzoyl)-L-glutam-1-yl-3-tertbutyl-L-phenylalanine	Km (uM)	ND	ND	2.1
	kcat (1/sec)	ND	ND	0.81
	kcat/Km	ND	ND	0.38
N-(4-(((2,4-diamino-6-pteridiny)methylmethylamino)benzoyl)-L-glutam-1-yl-3-cyclobutyl-L-phenylalanine	Km (uM)	ND	160	1.8
	kcat (1/sec)	ND	0.12	3.0
	kcat/Km	ND	0.00075	1.8
N-(4-(((2,4-diamino-6-pteridiny)methylmethylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-tyrosine	Km (uM)	ND	50	18
	kcat (1/sec)	ND	0.0006	3.0
	kcat/Km	ND	0.00001	0.18

ND = not determined; rate too slow to assay. kcat  $\leq$  0.0005/sec.

Kinetics were determined by a modification of the coupled assay of Kuefner, et al (1989) *Biochemistry* 28 2288-2297 in which the product MTX is converted by excess carboxypeptidase G to the pterate of MTX. In this assay the reaction is followed spectrophotometrically at 315 nm; at this wavelength the change in molar absorptivity for the coupled reaction was determined to be  $9.57 \text{ mM}^{-1}\text{cm}^{-1}$ . Assays were performed at 25°C in 25 mM tris-HCl, 100 mM NaCl, pH 7.4. Prodrugs were preincubated at 25 °C with buffer and 17 milliunits of Sigma #C-9658 carboxypeptidase G in 1 ml total volume. Assay was initiated with carboxypeptidase A or the appropriate mutant. Initial velocities

were obtained, and standard Michaelis-Menten calculations were used to determine kinetic constants (in Biochemistry, Lehninger, A.L. 1970, Worth Publishers, Inc, NY).

TABLE 3

**Antibodies and Antigens**

<b><u>Antigen</u></b>	<b><u>Antibody</u></b>	<b><u>Current uses</u></b>
<b><u>Tumor associated Antigens</u></b>		
Pan Adenocarcinoma	ING-1 (Wellcome)	Imaging and therapy of various carcinomas.
Cytokeratins	c174 (Biomira)	Imaging and therapy of Squamous cell carcinomas.
Cytokeratins	16.88 (Organon Teknika)	Imaging and therapy of Squamous cell carcinomas.
Carcinoembryonic Antigen	NR-CO-02 (NeoRex)	Imaging and therapy of colon/gastrointestinal tumors
Carcinoembryonic Antigen	PR.1A3 (ICRF)	Imaging and therapy of colon/gastrointestinal tumors
Carcinoembryonic Antigen	Immu-14 (Immunomedics)	Imaging and therapy of colon/gastrointestinal tumors
Carcinoembryonic Antigen	Col-1 (Dow)	Imaging and therapy of colon/gastrointestinal tumors
Carcinoembryonic Antigen	C110 (Abbott)	Imaging and therapy of colon/gastrointestinal tumors
Carcinoembryonic Antigen	A5B7 (Brit. J. Cancer, 1986, <u>54</u> , 75)	Imaging and therapy of colon/gastrointestinal tumors
Carcinoembryonic Antigen	BW431/26 (Br J. Cancer 1992, <u>65</u> , 234) (Cancer Immunol Immunother 1992, <u>34</u> , 343)	Imaging and therapy of colon/gastrointestinal tumors
Carcinoembryonic Antigen	C46.3 (Cytogen)	Imaging and therapy of colon/gastrointestinal tumors

Pan Adenocarcinoma	MAB 12.8 (Biochem Pharmacol 1991, <u>42</u> , 2062)	Imaging and therapy of various carcinomas.
Pan Adenocarcinoma (TAG-72)	CC49 and B72.3 (Dow)	Imaging and therapy of various carcinomas.
Histones	TNT-1 and TNT-2 (Techniclone)	All tumors with necrosis
Folate binding Protein	MOV18 (Centocor)	Ovarian Carcinoma
Folate binding Protein	MOV19 (Int. J. Cancer <u>39</u> , 297, 1987)	Ovarian Carcinoma
Pan Adenocarcinoma	323/A3 (Centocor)	Imaging and therapy of various carcinomas.
Pan Adenocarcinoma	17.1A (Centocor)	Imaging and therapy of various carcinomas.
Renal cell	G250 (Centocor)	Imaging and therapy of Renal Cell carcinomas.
200Kd squamous cell surface antigen	U36 (Centocor)	Imaging and therapy of Squamous cell carcinomas.
22Kd squamous cell surface antigen	E48 (Centocor)	Imaging and therapy of Squamous cell carcinomas.
125Kd colon adenocarcinoma cell surface glycoprotein	SF-25 (Centocor)	Imaging and therapy of various carcinomas.
High molecular weight mucins	Various mucin targeting antibodies (Trends Biochemical Science, 1992, <u>17</u> , 359)	Imaging and therapy of various carcinomas.
High molecular weight Mucin	HMFG1 (Cancer Research, 1992, <u>52</u> , 904)	Imaging and therapy of various carcinomas.
High molecular weight Mucin	BrE-3 (Hybridoma, 1993, <u>12</u> , 15)	Imaging and therapy of various carcinomas.
Lung squamous cell carcinoma antigen	RS7-3G11 (Antibody, Immunoconjugates and Radiopharmaceuticals, 1991 <u>4</u> 703)	Imaging and therapy of lung squamous cell carcinomas.
LEY related tumor antigen	BR64 and BR96 (Bristol-Meyers Squibb)	Imaging and therapy of various carcinomas.

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HER2 protooncogene product	P185HER2 (NeoRx)	Imaging and therapy of various carcinomas, especially breast.
human carcinoma antigen	15A82a (Cytogen)	Imaging and therapy of various carcinomas.
human Chorionic gonadotropin	W14A and SB10 (Int J. Cancer, 1984, <u>33</u> , 429. Br. J. Cancer, 1990, <u>61</u> , 659)	Imaging and therapy of various carcinomas.



**TABLE 4**  
**Amino acid coding sequences for human CPA1 and CPA2.\***

HCPA1	-110	-100	-90	-80	-70
HCPA2	MRGLLVLSVL	LGAVFGKEDF	VGHQVLRISV	ADEAQVQKVK	ELEDLEHLQL
	::LI:FPGA:	F:HIYCL:T:	::D::E:VP	SN:E:IKNLL	Q::AQ::::
HCPA1	-60	-50	-40	-30	-20
HCPA2	DFWRGPAHPG	SPIDVRMPFP	SIQAVKIFLE	SHGISYETMI	EDVQSLLEDE
	:::KS:TTS:	ETAH::V::V	NV::::V::	:Q::A:SI::	:::V:::K:
HCPA1	-10	1	11	21	31
HCPA2	QEQMFAFRSR	ARSTDTFNVA	TYHTLEEIYD	FLDLLVAENP	HLVSKIQIGN
	N:E:LFN:R:	E:-SGN::FG	A:::::SQ	EM:N::::H:	G::::VN::S
HCPA1	41	51	61	71	81
HCPA2	TYEGRPIYVL	KFSTGGSKRP	AIWIDTGIHS	REWVTQASGV	WFAKKITQDY
	SF:N::MN::	:::-DK:	:::L:A::A	::::::TAL	:T:N::VS::
HCPA1	91	101	111	121	131
HCPA2	GQDAFTAIL	DTLDIFLEIV	TNPDGFAFTH	STNRMWRKTR	SHTAGSLCIG
	:K:PSI:S::	:A::::LP:	:::YV:SQ	TK:::::	:KVS::::V:
HCPA1	141	151	161	171	181
HCPA2	VDPNRNWDAG	FGLSGASSNP	CSETYHGKFA	NSEVEVKSIV	DFVKDHGNIK
	:::~::~:	::GP:A:::	::DS::PS:	:::~::~:	::I:S::KV:
HCPA1	191	201	211	221	231
HCPA2	AFISIHYSQ	LLMPYGYKT	EPVPDQDELD	QLSKAAVTAL	ASLYGTFKNY
	:::TL::::	:::F::::C	TKLD:F::S	EVAQK:AQS:	R::H::YKV
HCPA1	241	251	261	271	281
HCPA2	GSIIKAIYQA	SGSTIDWTYS	QGIKYSFTFE	LRDTGRYGFL	LPASQIIPTA
	:P:CSV:::	::GS::S:D	Y::::A::	:::~::~:	:::R::L::
HCPA1	291	301			
HCPA2	KETWLALLTI	MEHTLNHPY			
	E::::G:KA:	:::VRD::			

\* Numbering scheme for CPA1 is used. Dashes at positions 3 and 57 indicate a gap in the A2 sequence required for optimal alignment with A1.

Colons indicate identity between A1 and A2.

Prepro-CPA begins at amino acid -110 and runs to +309.  
 Pro-CPA begins at amino acid -94 and runs to +309.  
 mature CPA begins at amino acid +1 and runs to +309.

TABLE 5

Amino acid sequences for the 268Gly mutations of human CPA1 and CPA2.\*

(mut.)	-110	-100	-90	-80	-70
HCPA1	MRGLLVLSVL	LGAVFGKEDF	VGHQVLRISV	ADEAQVQKVK	ELEDLEHLQL
HCPA2	::LI:FFGA:	F:HIYCL:T:	::D:::E:VP	SN:E:IKNLL	Q::AQ::::
(mut.)	-60	-50	-40	-30	-20
HCPA1	DFWRGPAHPG	SPIDVRMPFP	SIQAVKIFLE	SHGISYETMI	EDVQSLLDEE
HCPA2	:::KS:TTS:	ETAH::V::V	NV:::V:::	:Q::A:SI::	:::V:::K:
(mut.)	-10	1	11	21	31
HCPA1	QEOMFAFRSR	ARSTDTFNYA	TYHTLEEIYD	FLDLLVAENP	HLVSKIQIGN
HCPA2	N:E:LFN:R:	E:-SGN::FG	A::::::SQ	EM:N:::H:	G:::VN::S
(mut.)	41	51	61	71	81
HCPA1	TYEGRPIYVL	KFSTGGSKRP	AIWIDTGIHS	REWVTQASGV	WFAKKITQDY
HCPA2	SF:N::MN::	:::--DK:	:::L:A:::A	:::--TAL	:T:N::VS::
(mut.)	91	101	111	121	131
HCPA1	GQDAAFTAIL	DTLDIFLEIV	TNPDGFAFTH	STNRMWRKTR	SHTAGSLCIG
HCPA2	:K:PSI:S::	:A:::LP:	:::YV:SQ	TK:::--	:KVS:::V:
(mut.)	141	151	161	171	181
HCPA1	VDPNRNWDAG	FGLSGASSNP	CSETYHGKFA	NSEVEVKSIV	DFVKDHGNIK
HCPA2	:::--	::GP:A:::	::DS::PS:	:::--	::I:S::KV:
(mut.)	191	201	211	221	231
HCPA1	AFISIHYSYSQ	LLMYPYGYKT	EPVPDQDELD	QLSKAAVTAL	ASLYGTFKFN
HCPA2	:::TL:::--	:::F:::C	TKLD:F::S	EVAQK:AQS:	R::H::YKV
(mut.)	241	251	261	271	281
HCPA1	GSIKAIYQA	SGSTIDWTYS	QGIKYSFGFE	LRDTGRYGFL	LPASQIIPTA
HCPA2	:P:CSV:::	::GS::S:D	Y:::G::	:::--	:::R::L::
(mut.)	291	301			
HCPA1	KETWLALLTI	MEHTLNHPY			
HCPA2	E:::G:KA:	:::VRD:::			

\* Numbering scheme for CPA1 is used. Dashes at positions 3 and 57 indicate a gap in the A2 sequence required for optimal alignment with A1.

Colons indicate identity between A1 and A2.

## SEQUENCE LISTING

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(C) CITY: Chapel Hill  
(D) STATE: North Carolina  
(E) COUNTRY: United States of America  
(F) POSTAL CODE (ZIP): 27514

## (ii) TITLE OF INVENTION: THERAPY

## (iii) NUMBER OF SEQUENCES: 22

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1257 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA to mRNA

214

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..1257

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATG CGC GGG TTG CTG GTG TTG AGT GTC CTG TTG GGG GCT GTC TTT GGC	48
Met Arg Gly Leu Leu Val Leu Ser Val Leu Leu Gly Ala Val Phe Gly	
1 5 10 15	
AAG GAG GAC TTT GTG GGG CAT CAG GTG CTC CGA ATC TCT GTA GCC GAT	96
Lys Glu Asp Phe Val Gly His Gln Val Leu Arg Ile Ser Val Ala Asp	
20 25 30	
GAG GCC CAG GTA CAG AAG GTG AAG GAG CTG GAG GAC CTG GAG CAC CTG	144
Glu Ala Gln Val Gln Lys Val Lys Glu Leu Glu Asp Leu Glu His Leu	
35 40 45	
CAG CTG GAC TTC TGG CGG GGC CCT GCC CAC CCT GGC TCC CCC ATC GAC	192
Gln Leu Asp Phe Trp Arg Gly Pro Ala His Pro Gly Ser Pro Ile Asp	
50 55 60	
GTC CGA ATG CCC TTC CCC AGC ATC CAG GCG GTC AAG ATC TTT CTG GAG	240
Val Arg Met Pro Phe Pro Ser Ile Gln Ala Val Lys Ile Phe Leu Glu	
65 70 75 80	
TCC CAC GGC ATC AGC TAT GAG ACC ATG ATC GAG GAC GTG CAG TCG CTG	288
Ser His Gly Ile Ser Tyr Glu Thr Met Ile Glu Asp Val Gln Ser Leu	
85 90 95	
CTG GAC GAG GAG CAG GAG CAG ATG TTC GCC TTC CGG TCC CGG GCG CGC	336
Leu Asp Glu Glu Gln Glu Gln Met Phe Ala Phe Arg Ser Arg Ala Arg	
100 105 110	
TCC ACC GAC ACT TTT AAC TAC GCC ACC TAC CAC ACC CTG GAG GAG ATC	384
Ser Thr Asp Thr Phe Asn Tyr Ala Thr Tyr His Thr Leu Glu Glu Ile	
115 120 125	
TAT GAC TTC CTG GAC CTG CTG GTG GCG GAG AAC CCG CAC CTT GTC AGC	432
Tyr Asp Phe Leu Asp Leu Leu Val Ala Glu Asn Pro His Leu Val Ser	
130 135 140	
AAG ATC CAG ATT GGC AAC ACC TAT GAA GGG CGT CCC ATT TAC GTG CTG	480
Lys Ile Gln Ile Gly Asn Thr Tyr Glu Gly Arg Pro Ile Tyr Val Leu	
145 150 155 160	
AAG TTC AGC ACG GGG GGC AGT AAG CGT CCA GCC ATC TGG ATC GAC ACG	528
Lys Phe Ser Thr Gly Gly Ser Lys Arg Pro Ala Ile Trp Ile Asp Thr	
165 170 175	
GGC ATC CAT TCC CGG GAG TGG GTC ACC CAG GCC AGT GGG GTC TGG TTT	576
Gly Ile His Ser Arg Glu Trp Val Thr Gln Ala Ser Gly Val Trp Phe	
180 185 190	
GCA AAG AAG ATC ACT CAA GAC TAT GGG CAG GAT GCA GCT TTC ACC GCC	624
Ala Lys Lys Ile Thr Gln Asp Tyr Gly Gln Asp Ala Ala Phe Thr Ala	
195 200 205	

215

ATT CTC GAC ACC TTG GAC ATC TTC CTG GAG ATC GTC ACC AAC CCT GAT Ile Leu Asp Thr Leu Asp Ile Phe Leu Glu Ile Val Thr Asn Pro Asp 210 215 220	672
GGC TTT GCC TTC ACG CAC AGC ACG AAT CGC ATG TGG CGC AAG ACT CGG Gly Phe Ala Phe Thr His Ser Thr Asn Arg Met Trp Arg Lys Thr Arg 225 230 235 240	720
TCC CAC ACA GCA GGC TCC CTC TGT ATT GGC GTG GAC CCC AAC AGG AAC Ser His Thr Ala Gly Ser Leu Cys Ile Gly Val Asp Pro Asn Arg Asn 245 250 255	768
TGG GAC GCT GGC TTT GGG TTG TCC GGA GCC AGC AGT AAC CCC TGC TCG Trp Asp Ala Gly Phe Gly Leu Ser Gly Ala Ser Ser Asn Pro Cys Ser 260 265 270	816
GAG ACT TAC CAC GGC AAG TTT GCC AAT TCC GAA GTG GAG GTC AAG TCC Glu Thr Tyr His Gly Lys Phe Ala Asn Ser Glu Val Glu Val Lys Ser 275 280 285	864
ATT GTA GAC TTT GTG AAG GAC CAT GGG AAC ATC AAG GCC TTC ATC TCC Ile Val Asp Phe Val Lys Asp His Gly Asn Ile Lys Ala Phe Ile Ser 290 295 300	912
ATC CAC AGC TAC TCC CAG CTC CTC ATG TAT CCC TAT GGC TAC AAA ACA Ile His Ser Tyr Ser Gln Leu Leu Met Tyr Pro Tyr Gly Tyr Lys Thr 305 310 315 320	960
GAA CCA GTC CCT GAC CAG GAT GAG CTG GAT CAG CTT TCC AAG GCT GCT Glu Pro Val Pro Asp Gln Asp Glu Leu Asp Gln Leu Ser Lys Ala Ala 325 330 335	1008
GTG ACA GCC CTG GCC TCT CTC TAC GGG ACC AAG TTC AAC TAT GGC AGC Val Thr Ala Leu Ala Ser Leu Tyr Gly Thr Lys Phe Asn Tyr Gly Ser 340 345 350	1056
ATC ATC AAG GCA ATT TAT CAA GCC AGT GGA AGC ACT ATT GAC TGG ACC Ile Ile Lys Ala Ile Tyr Gln Ala Ser Gly Ser Thr Ile Asp Trp Thr 355 360 365	1104
TAC AGC CAG GGC ATC AAG TAC TCC TTC ACC TTC GAG CTC CGG GAC ACT Tyr Ser Gln Gly Ile Lys Tyr Ser Phe Thr Phe Glu Leu Arg Asp Thr 370 375 380	1152
GGG CGC TAT GGC TTC CTG CTG CCA GCC TCC CAG ATC ATC CCC ACA GCC Gly Arg Tyr Gly Phe Leu Leu Pro Ala Ser Gln Ile Ile Pro Thr Ala 385 390 395 400	1200
AAG GAG ACG TGG CTG GCG CTT CTG ACC ATC ATG GAG CAC ACC CTG AAT Lys Glu Thr Trp Leu Ala Leu Leu Thr Ile Met Glu His Thr Leu Asn 405 410 415	1248
CAC CCC TAC His Pro Tyr	1257

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 419 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

Met Arg Gly Leu Leu Val Leu Ser Val Leu Leu Gly Ala Val Phe Gly
 1           5           10           15
Lys Glu Asp Phe Val Gly His Gln Val Leu Arg Ile Ser Val Ala Asp
          20           25           30
Glu Ala Gln Val Gln Lys Val Lys Glu Leu Glu Asp Leu Glu His Leu
          35           40           45
Gln Leu Asp Phe Trp Arg Gly Pro Ala His Pro Gly Ser Pro Ile Asp
          50           55           60
Val Arg Met Pro Phe Pro Ser Ile Gln Ala Val Lys Ile Phe Leu Glu
          65           70           75           80
Ser His Gly Ile Ser Tyr Glu Thr Met Ile Glu Asp Val Gln Ser Leu
          85           90           95
Leu Asp Glu Glu Gln Glu Gln Met Phe Ala Phe Arg Ser Arg Ala Arg
          100          105          110
Ser Thr Asp Thr Phe Asn Tyr Ala Thr Tyr His Thr Leu Glu Glu Ile
          115          120          125
Tyr Asp Phe Leu Asp Leu Leu Val Ala Glu Asn Pro His Leu Val Ser
          130          135          140
Lys Ile Gln Ile Gly Asn Thr Tyr Glu Gly Arg Pro Ile Tyr Val Leu
          145          150          155          160
Lys Phe Ser Thr Gly Gly Ser Lys Arg Pro Ala Ile Trp Ile Asp Thr
          165          170          175
Gly Ile His Ser Arg Glu Trp Val Thr Gln Ala Ser Gly Val Trp Phe
          180          185          190
Ala Lys Lys Ile Thr Gln Asp Tyr Gly Gln Asp Ala Ala Phe Thr Ala
          195          200          205
Ile Leu Asp Thr Leu Asp Ile Phe Leu Glu Ile Val Thr Asn Pro Asp
          210          215          220
Gly Phe Ala Phe Thr His Ser Thr Asn Arg Met Trp Arg Lys Thr Arg
          225          230          235          240
Ser His Thr Ala Gly Ser Leu Cys Ile Gly Val Asp Pro Asn Arg Asn
          245          250          255

```

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1251 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 1..1251

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATG	AGG	TTG	ATC	CTG	TTT	TTT	GGT	GCC	CTT	TTT	GGG	CAT	ATC	TAC	TGT	48
Met	Arg	Leu	Ile	Leu	Phe	Phe	Gly	Ala	Leu	Phe	Gly	His	Ile	Tyr	Cys	
1				5					10					15		
CTA	GAA	ACA	TTT	GTG	GGA	GAC	CAA	GTT	CTT	GAG	ATT	GTA	CCA	AGC	AAT	96
Leu	Glu	Thr	Phe	Val	Gly	Asp	Gln	Val	Leu	Glu	Ile	Val	Pro	Ser	Asn	
			20					25					30			

218																
GAA	GAA	CAA	ATT	AAA	AAT	CTG	CTA	CAA	TTG	GAG	GCT	CAA	GAA	CAT	CTC	144
Glu	Glu	Gln	Ile	Lys	Asn	Leu	Leu	Gln	Leu	Glu	Ala	Gln	Glu	His	Leu	
		35					40					45				
CAG	CTT	GAT	TTT	TGG	AAA	TCA	CCC	ACC	ACC	TCA	GGG	GAG	ACA	GCC	CAC	192
Gln	Leu	Asp	Phe	Trp	Lys	Ser	Pro	Thr	Thr	Ser	Gly	Glu	Thr	Ala	His	
	50					55					60					
GTC	CGA	GTT	CCC	TTC	GTC	AAC	GTC	CAG	GCA	GTC	AAA	GTG	TTC	TTG	GAG	240
Val	Arg	Val	Pro	Phe	Val	Asn	Val	Gln	Ala	Val	Lys	Val	Phe	Leu	Glu	
	65				70				75					80		
TCC	CAG	GGA	ATT	GCC	TAT	TCC	ATC	ATG	ATT	GAA	GAC	GTG	CAG	GTC	CTG	288
Ser	Gln	Gly	Ile	Ala	Tyr	Ser	Ile	Met	Ile	Glu	Asp	Val	Gln	Val	Leu	
				85				90						95		
TTG	GAC	AAA	GAG	AAT	GAA	GAA	ATG	CTT	TTT	AAT	AGG	AGA	AGA	GAA	CGG	336
Leu	Asp	Lys	Glu	Asn	Glu	Glu	Met	Leu	Phe	Asn	Arg	Arg	Arg	Glu	Arg	
			100					105					110			
AGT	GGT	AAC	TTC	AAT	TTT	GGG	GCC	TAC	CAT	ACC	CTG	GAA	GAG	ATT	TCC	384
Ser	Gly	Asn	Phe	Asn	Phe	Gly	Ala	Tyr	His	Thr	Leu	Glu	Glu	Ile	Ser	
		115					120					125				
CAA	GAA	ATG	GAT	AAC	CTC	GTG	GCT	GAG	CAC	CCT	GGT	CTA	GTG	AGC	AAA	432
Gln	Glu	Met	Asp	Asn	Leu	Val	Ala	Glu	His	Pro	Gly	Leu	Val	Ser	Lys	
		130				135					140					
GTG	AAT	ATT	GGC	TCT	TCT	TTT	GAG	AAC	CGG	CCT	ATG	AAC	GTG	CTC	AAG	480
Val	Asn	Ile	Gly	Ser	Ser	Phe	Glu	Asn	Arg	Pro	Met	Asn	Val	Leu	Lys	
	145				150				155					160		
TTC	AGC	ACC	GGA	GGA	GAC	AAG	CCA	GCT	ATC	TGG	CTG	GAT	GCG	GGG	ATC	528
Phe	Ser	Thr	Gly	Gly	Asp	Lys	Pro	Ala	Ile	Trp	Leu	Asp	Ala	Gly	Ile	
				165					170					175		
CAT	GCT	CGA	GAG	TGG	GTT	ACA	CAA	GCT	ACG	GCA	CTT	TGG	ACA	GCA	AAT	576
His	Ala	Arg	Glu	Trp	Val	Thr	Gln	Ala	Thr	Ala	Leu	Trp	Thr	Ala	Asn	
			180					185					190			
AAG	ATT	GTT	TCT	GAT	TAT	GGA	AAG	GAC	CCA	TCC	ATC	ACT	TCC	ATT	CTG	624
Lys	Ile	Val	Ser	Asp	Tyr	Gly	Lys	Asp	Pro	Ser	Ile	Thr	Ser	Ile	Leu	
		195					200					205				
GAC	GCC	CTG	GAT	ATC	TTC	CTC	CTG	CCA	GTC	ACA	AAC	CCT	GAT	GGA	TAC	672
Asp	Ala	Leu	Asp	Ile	Phe	Leu	Leu	Pro	Val	Thr	Asn	Pro	Asp	Gly	Tyr	
		210				215						220				
GTG	TTC	TCT	CAA	ACC	AAA	AAT	CGT	ATG	TGG	CGG	AAG	ACC	CGG	TCC	AAG	720
Val	Phe	Ser	Gln	Thr	Lys	Asn	Arg	Met	Trp	Arg	Lys	Thr	Arg	Ser	Lys	
	225				230					235				240		
GTA	TCT	GGA	AGC	CTC	TGT	GTT	GGT	GTG	GAT	CCT	AAC	CGG	AAC	TGG	GAT	768
Val	Ser	Gly	Ser	Leu	Cys	Val	Gly	Val	Asp	Pro	Asn	Arg	Asn	Trp	Asp	
				245				250						255		



219

GCA GGT TTT GGA GGA CCT GGA GCC AGC AGC AAC CCT TGC TCT GAT TCA Ala Gly Phe Gly Gly Pro Gly Ala Ser Ser Asn Pro Cys Ser Asp Ser 260 265 270	816
TAC CAC GGA CCC AGT GCC AAC TCT GAA GTT GAA GTG AAA TCC ATA GTG Tyr His Gly Pro Ser Ala Asn Ser Glu Val Glu Val Lys Ser Ile Val 275 280 285	864
GAC TTC ATC AAG AGT CAT GGA AAA GTC AAG GCC TTC ATT ACC CTC CAC Asp Phe Ile Lys Ser His Gly Lys Val Lys Ala Phe Ile Thr Leu His 290 295 300	912
AGC TAT TCC CAG CTG CTG ATG TTC CCC TAT GGG TAC AAA TGT ACC AAG Ser Tyr Ser Gln Leu Leu Met Phe Pro Tyr Gly Tyr Lys Cys Thr Lys 305 310 315 320	960
TTA GAT GAC TTT GAT GAG CTG AGT GAA GTG GCC CAA AAG GCT GCC CAA Leu Asp Asp Phe Asp Glu Leu Ser Glu Val Ala Gln Lys Ala Ala Gln 325 330 335	1008
TCT CTG AGA AGC CTG CAT GGC ACC AAG TAC AAA GTG GGA CCA ATC TGC Ser Leu Arg Ser Leu His Gly Thr Lys Tyr Lys Val Gly Pro Ile Cys 340 345 350	1056
TCT GTC ATC TAC CAA GCC AGT GGA GGA AGC ATT GAC TGG TCC TAT GAT Ser Val Ile Tyr Gln Ala Ser Gly Gly Ser Ile Asp Trp Ser Tyr Asp 355 360 365	1104
TAT GGC ATC AAG TAC TCA TTT GCC TTT GAA CTG AGA GAC ACA GGG CGC Tyr Gly Ile Lys Tyr Ser Phe Ala Phe Glu Leu Arg Asp Thr Gly Arg 370 375 380	1152
TAC GGC TTC CTC TTG CCA GCC CGT CAG ATC CTG CCC ACA GCC GAG GAG Tyr Gly Phe Leu Leu Pro Ala Arg Gln Ile Leu Pro Thr Ala Glu Glu 385 390 395 400	1200
ACC TGG CTT GGC TTG AAG GCA ATC ATG GAG CAT GTG CGA GAC CAC CCC Thr Trp Leu Gly Leu Lys Ala Ile Met Glu His Val Arg Asp His Pro 405 410 415	1248
TAT Tyr	1251

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 417 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

220

Met Arg Leu Ile Leu Phe Phe Gly Ala Leu Phe Gly His Ile Tyr Cys  
 1 5 10 15  
 Leu Glu Thr Phe Val Gly Asp Gln Val Leu Glu Ile Val Pro Ser Asn  
 20 25 30  
 Glu Glu Gln Ile Lys Asn Leu Leu Gln Leu Glu Ala Gln Glu His Leu  
 35 40 45  
 Gln Leu Asp Phe Trp Lys Ser Pro Thr Thr Ser Gly Glu Thr Ala His  
 50 55 60  
 Val Arg Val Pro Phe Val Asn Val Gln Ala Val Lys Val Phe Leu Glu  
 65 70 75 80  
 Ser Gln Gly Ile Ala Tyr Ser Ile Met Ile Glu Asp Val Gln Val Leu  
 85 90 95  
 Leu Asp Lys Glu Asn Glu Glu Met Leu Phe Asn Arg Arg Arg Glu Arg  
 100 105 110  
 Ser Gly Asn Phe Asn Phe Gly Ala Tyr His Thr Leu Glu Glu Ile Ser  
 115 120 125  
 Gln Glu Met Asp Asn Leu Val Ala Glu His Pro Gly Leu Val Ser Lys  
 130 135 140  
 Val Asn Ile Gly Ser Ser Phe Glu Asn Arg Pro Met Asn Val Leu Lys  
 145 150 155 160  
 Phe Ser Thr Gly Gly Asp Lys Pro Ala Ile Trp Leu Asp Ala Gly Ile  
 165 170 175  
 His Ala Arg Glu Trp Val Thr Gln Ala Thr Ala Leu Trp Thr Ala Asn  
 180 185 190  
 Lys Ile Val Ser Asp Tyr Gly Lys Asp Pro Ser Ile Thr Ser Ile Leu  
 195 200 205  
 Asp Ala Leu Asp Ile Phe Leu Leu Pro Val Thr Asn Pro Asp Gly Tyr  
 210 215 220  
 Val Phe Ser Gln Thr Lys Asn Arg Met Trp Arg Lys Thr Arg Ser Lys  
 225 230 235 240  
 Val Ser Gly Ser Leu Cys Val Gly Val Asp Pro Asn Arg Asn Trp Asp  
 245 250 255  
 Ala Gly Phe Gly Gly Pro Gly Ala Ser Ser Asn Pro Cys Ser Asp Ser  
 260 265 270  
 Tyr His Gly Pro Ser Ala Asn Ser Glu Val Glu Val Lys Ser Ile Val  
 275 280 285  
 Asp Phe Ile Lys Ser His Gly Lys Val Lys Ala Phe Ile Thr Leu His  
 290 295 300

221

Ser Tyr Ser Gln Leu Leu Met Phe Pro Tyr Gly Tyr Lys Cys Thr Lys  
 305 310 315 320

Leu Asp Asp Phe Asp Glu Leu Ser Glu Val Ala Gln Lys Ala Ala Gln  
 325 330 335

Ser Leu Arg Ser Leu His Gly Thr Lys Tyr Lys Val Gly Pro Ile Cys  
 340 345 350

Ser Val Ile Tyr Gln Ala Ser Gly Gly Ser Ile Asp Trp Ser Tyr Asp  
 355 360 365

Tyr Gly Ile Lys Tyr Ser Phe Ala Phe Glu Leu Arg Asp Thr Gly Arg  
 370 375 380

Tyr Gly Phe Leu Leu Pro Ala Arg Gln Ile Leu Pro Thr Ala Glu Glu  
 385 390 395 400

Thr Trp Leu Gly Leu Lys Ala Ile Met Glu His Val Arg Asp His Pro  
 405 410 415

Tyr

## (2) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCTGTTATTG GCTGCCCTAC TT

22

## (2) INFORMATION FOR SEQ ID NO: 6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AAGCCAGGTC TCTTCTGCTG TC

22

(2) INFORMATION FOR SEQ ID NO: <sup>222</sup>7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGTCCAGTCA GCAGTGCTTC C

21

## (2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAGCTCGAAG GCGAAGGAGT A

21

## (2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GAGCTCGAAG CCGAAGGAGT A

21

## (2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 37 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear <sup>223</sup>

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TCCCCCGGGC TGCAGGATAT CGATAGCTGG GCTGTGT

37

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATCAAGTACT CCTCCCAGCT CCGGGAC

27

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TTTATCAAGC CAGTGGAGGT ATTGACTGGA CC

32

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

224

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AAGGCAATTT ATCAAGGCAG TGGAAGCACT ATT

33

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TCCCCCGGGC TGCAGGATAT CGATAGCTGG GCTGTGT

37

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GCTGAAGCTT CGGAGGACTT T

21

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TCTTGACCGC CTGGATGCTG

20

(2) INFORMATION FOR SEQ ID NO: 17:<sup>225</sup>

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 419 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```

Met Arg Gly Leu Leu Val Leu Ser Val Leu Leu Gly Ala Val Phe Gly
 1           5           10           15
Lys Glu Asp Phe Val Gly His Gln Val Leu Arg Ile Ser Val Ala Asp
 20           25           30
Glu Ala Gln Val Gln Lys Val Lys Glu Leu Glu Asp Leu Glu His Leu
 35           40           45
Gln Leu Asp Phe Trp Arg Gly Pro Ala His Pro Gly Ser Pro Ile Asp
 50           55           60
Val Arg Met Pro Phe Pro Ser Ile Gln Ala Val Lys Ile Phe Leu Glu
 65           70           75           80
Ser His Gly Ile Ser Tyr Glu Thr Met Ile Glu Asp Val Gln Ser Leu
 85           90           95
Leu Asp Glu Glu Gln Glu Gln Met Phe Ala Phe Arg Ser Arg Ala Arg
100           105           110
Ser Thr Asp Thr Phe Asn Tyr Ala Thr Tyr His Thr Leu Glu Glu Ile
115           120           125
Tyr Asp Phe Leu Asp Leu Leu Val Ala Glu Asn Pro His Leu Val Ser
130           135           140
Lys Ile Gln Ile Gly Asn Thr Tyr Glu Gly Arg Pro Ile Tyr Val Leu
145           150           155           160
Lys Phe Ser Thr Gly Gly Ser Lys Arg Pro Ala Ile Trp Ile Asp Thr
165           170           175
Gly Ile His Ser Arg Glu Trp Val Thr Gln Ala Ser Gly Val Trp Phe
180           185           190
Ala Lys Lys Ile Thr Gln Asp Tyr Gly Gln Asp Ala Ala Phe Thr Ala
195           200           205
Ile Leu Asp Thr Leu Asp Ile Phe Leu Glu Ile Val Thr Asn Pro Asp
210           215           220
Gly Phe Ala Phe Thr His Ser Thr Asn Arg Met Trp Arg Lys Thr Arg
225           230           235           240
Ser His Thr Ala Gly Ser Leu Cys Ile Gly Val Asp Pro Asn Arg Asn
245           250           255

```

(2) INFORMATION FOR SEQ ID NO: 18:

SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 417 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Met Arg Leu Ile Leu Phe Phe Gly Ala Leu Phe Gly His Ile Tyr Cys  
1 5 10 15  
Leu Glu Thr Phe Val Gly Asp Gln Val Leu Glu Ile Val Pro Ser Asn  
20 25 30  
Glu Glu Gln Ile Lys Asn Leu Leu Gln Leu Glu Ala Gln Glu His Leu  
35 40 45  
Gln Leu Asp Phe Trp Lys Ser Pro Thr Thr Ser Gly Glu Thr Ala His  
50 55 60



227

Val Arg Val Pro Phe Val Asn Val Gln Ala Val Lys Val Phe Leu Glu  
 65 70 75 80  
 Ser Gln Gly Ile Ala Tyr Ser Ile Met Ile Glu Asp Val Gln Val Leu  
 85 90 95  
 Leu Asp Lys Glu Asn Glu Glu Met Leu Phe Asn Arg Arg Arg Glu Arg  
 100 105 110  
 Ser Gly Asn Phe Asn Phe Gly Ala Tyr His Thr Leu Glu Glu Ile Ser  
 115 120 125  
 Gln Glu Met Asp Asn Leu Val Ala Glu His Pro Gly Leu Val Ser Lys  
 130 135 140  
 Val Asn Ile Gly Ser Ser Phe Glu Asn Arg Pro Met Asn Val Leu Lys  
 145 150 155 160  
 Phe Ser Thr Gly Gly Asp Lys Pro Ala Ile Trp Leu Asp Ala Gly Ile  
 165 170 175  
 His Ala Arg Glu Trp Val Thr Gln Ala Thr Ala Leu Trp Thr Ala Asn  
 180 185 190  
 Lys Ile Val Ser Asp Tyr Gly Lys Asp Pro Ser Ile Thr Ser Ile Leu  
 195 200 205  
 Asp Ala Leu Asp Ile Phe Leu Leu Pro Val Thr Asn Pro Asp Gly Tyr  
 210 215 220  
 Val Phe Ser Gln Thr Lys Asn Arg Met Trp Arg Lys Thr Arg Ser Lys  
 225 230 235 240  
 Val Ser Gly Ser Leu Cys Val Gly Val Asp Pro Asn Arg Asn Trp Asp  
 245 250 255  
 Ala Gly Phe Gly Gly Pro Gly Ala Ser Ser Asn Pro Cys Ser Asp Ser  
 260 265 270  
 Tyr His Gly Pro Ser Ala Asn Ser Glu Val Glu Val Lys Ser Ile Val  
 275 280 285  
 Asp Phe Ile Lys Ser His Gly Lys Val Lys Ala Phe Ile Thr Leu His  
 290 295 300  
 Ser Tyr Ser Gln Leu Leu Met Phe Pro Tyr Gly Tyr Lys Cys Thr Lys  
 305 310 315 320  
 Leu Asp Asp Phe Asp Glu Leu Ser Glu Val Ala Gln Lys Ala Ala Gln  
 325 330 335  
 Ser Leu Arg Ser Leu His Gly Thr Lys Tyr Lys Val Gly Pro Ile Cys  
 340 345 350  
 Ser Val Ile Tyr Gln Ala Ser Gly Gly Ser Ile Asp Trp Ser Tyr Asp  
 355 360 365

228

Tyr Gly Ile Lys Tyr Ser Phe Gly Phe Glu Leu Arg Asp Thr Gly Arg  
 370 375 380

Tyr Gly Phe Leu Leu Pro Ala Arg Gln Ile Leu Pro Thr Ala Glu Glu  
 385 390 395 400

Thr Trp Leu Gly Leu Lys Ala Ile Met Glu His Val Arg Asp His Pro  
 405 410 415

Tyr

## (2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 50 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GCAGAAGCTT CAGAAACGTT TGTGGGAGAT CAAGTTCTTG AGATTGTACC

50

## (2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CTCTTTGTCC AACAGGACCT G

21

## (2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

229

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

TCCTCCACTG CCTTGGTAGA T

21

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CAGTTCAAAG CCAAATGAGT A

21

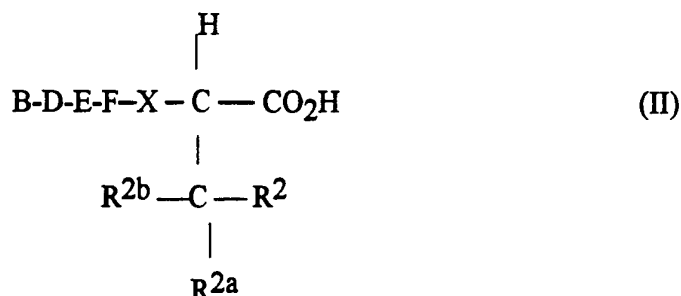
**CLAIMS**

1. A conjugate comprising a targetting molecule capable of delivering an enzyme to a particular mammalian cell and a mammalian enzyme conjugated to the targetting molecule and capable of catalysing a functionally inactive precursor of a chemotherapeutic agent to its active form wherein the enzyme is a mutant and the precursor is refractory to endogenous catalysis by the wild-type form of the enzyme.
2. A conjugate according to Claim 1, wherein the enzyme is one of a hydrolase, a transferase, an oxido-reductase, an isomerase, a lyase and a ligase.
3. A conjugate according to Claim 2, wherein the hydrolase is a peptidase.
4. A conjugate according to Claim 2, wherein the hydrolase is an esterase.
5. A conjugate according to Claim 3, wherein the peptidase is an exopeptidase.
6. A conjugate according either Claim 3 or 4, wherein the peptidase of Claim 3 or the esterase of Claim 4 is a carboxypeptidase.
7. A conjugate according to Claim 6 wherein the carboxypeptidase is a human carboxypeptidase A.
8. A conjugate according to Claim 7 wherein the human carboxypeptidase A is HCPA 1 or HCPA 2 possessing amino acid substitutions at one or more of residues 203, 210, 242, 244, 250, 253, 255, 267, 268, 269 and 305.
9. A conjugate according to Claim 8 wherein the amino acid substitutions are selected from the following:  
  
Gly at 250 and 268,  
Gly at 253 and 268,  
Gly at 250 and His at 268,  
Gly at 250,

Ala at 255 and His 268,  
His at 268, and  
Gly at 268.

10. A conjugate according to Claim 9 wherein the substitution is Gly at 268.
11. A conjugate according to any of Claims 8 to 10 wherein all other residues are wild-type.
12. A conjugate according to any of Claims 1 to 11 wherein the targetting molecule is one of an antibody, a hormone, a ligand, a cytokine, an antigen, an oligonucleotide and a peptidomimetic.
13. A conjugate according to Claim 12 wherein the antibody is a polyclonal antibody, a monoclonal antibody or a fragment thereof.
14. A conjugate according to Claim 13 wherein the antibody or fragment thereof is humanised.
15. A conjugate according to any of Claims 1 to 14, wherein the targetting molecule is joined to the enzyme by a linker molecule.
16. A conjugate according to any of Claims 1 to 14 which is translated from a single RNA transcript or multiple RNA transcripts.
17. A DNA or RNA molecule encoding a conjugate as claimed in Claim 16.
18. A vector containing the DNA or RNA claimed in Claim 17.
19. A cell line containing the DNA or RNA claimed in Claim 17 or a vector as claimed in Claim 18.

20. A compound of general formula II



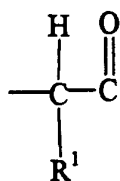
wherein,

X represents NH or O and

$\text{R}^2$  represents  $\text{CO}_2\text{H}$ ,  $\text{SO}_3\text{H}$ ,  $\text{SO}_2\text{H}$ ,  $\text{OSO}_3\text{H}$ ,  $\text{PO}_3\text{H}_2$ ,  $\text{OPO}_3\text{H}_2$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{CO}_2\text{H}$ ,  $\text{C}_{1-6}$  esterified  $\text{PO}_3\text{H}_2$ ,  $\text{C}_{4-12}$  alkyl,  $\text{C}_{4-12}$  alkenyl,  $\text{C}_{4-12}$  alkynyl,  $\text{C}_{4-12}$  branched alkyl;  $\text{C}_{1-8}$  alkyl or  $\text{C}_{3-8}$  branched alkyl; substituted with  $\text{CO}_2\text{H}$ ,  $\text{SO}_3\text{H}$ ,  $\text{SO}_2\text{H}$ ,  $\text{OSO}_3\text{H}$ ,  $\text{PO}_3\text{H}_2$ ,  $\text{OPO}_3\text{H}_2$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{CO}_2\text{H}$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{PO}_3\text{H}_2$ , hydroxyl, alkoxy, halo, haloalkyl, cyano or carboxamide;  $\text{C}_{3-8}$  cycloalkyl, aryl or heteroaryl optionally substituted with  $\text{C}_{1-8}$  alkyl,  $\text{C}_{3-8}$  branched alkyl,  $\text{C}_{3-8}$  cycloalkyl,  $\text{C}_{3-6}$  alkenyl,  $\text{C}_{2-6}$  alkynyl,  $\text{C}_{4-6}$  cycloalkenyl, trisubstituted-silyl i.e.  $(\text{R}^{13})(\text{R}^{14})(\text{R}^{15})\text{Si}$ , where  $\text{R}^{13}$ ,  $\text{R}^{14}$ , and  $\text{R}^{15}$  are  $\text{C}_{1-6}$  alkyl,  $\text{C}_{3-6}$  branched alkyl,  $\text{C}_{3-6}$  cycloalkyl,  $\text{C}_{3-6}$  alkenyl,  $\text{C}_{2-6}$  alkynyl,  $\text{C}_{4-6}$  cycloalkenyl, aryl, or heteroaryl, and where  $\text{R}^{13}$ ,  $\text{R}^{14}$ , and  $\text{R}^{15}$  are each the same group or different groups (e.g. trimethylsilyl, tert-butyl dimethylsilyl, cyclohexyldimethylsilyl, or phenyldimethylsilyl), aryl,  $\text{CO}_2\text{H}$ ,  $\text{SO}_3\text{H}$ ,  $\text{SO}_2\text{H}$ ,  $\text{OSO}_3\text{H}$ ,  $\text{PO}_3\text{H}_2$ ,  $\text{OPO}_3\text{H}_2$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{CO}_2\text{H}$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{PO}_3\text{H}_2$ , carboxamide, hydroxyl,  $\text{C}_{1-6}$  alkoxy,  $\text{C}_{1-6}$  alkylthio, mercapto, halo, haloalkyl, nitro or cyano,  $\text{R}^{2a}$  and  $\text{R}^{2b}$  represent H, hydroxy, mercapto, alkoxy, alkylthio, halo, cyano,  $\text{CO}_2\text{H}$ ,  $\text{SO}_3\text{H}$ ,  $\text{SO}_2\text{H}$ ,  $\text{OSO}_3\text{H}$ ,  $\text{PO}_3\text{H}_2$ ,  $\text{OPO}_3\text{H}_2$ ,  $\text{C}_{1-6}$  alkyl esterified  $\text{CO}_2\text{H}$ , carboxamide,  $\text{C}_{1-6}$  alkyl esterified  $\text{PO}_3\text{H}_2$ , cyclic  $\text{C}_{2-6}$  [i.e.,  $\text{R}^{2a}$  and  $\text{R}^{2b}$  together represent  $(\text{CH}_2)_{2-6}$ ], trisubstituted-silyl i.e.  $(\text{R}^{13})(\text{R}^{14})(\text{R}^{15})\text{Si}$ , where  $\text{R}^{13}$ ,  $\text{R}^{14}$ , and  $\text{R}^{15}$  are  $\text{C}_{1-6}$  alkyl,  $\text{C}_{3-6}$  branched alkyl,  $\text{C}_{3-6}$  cycloalkyl,  $\text{C}_{3-6}$  alkenyl;  $\text{C}_{2-6}$  alkynyl,  $\text{C}_{4-6}$  cycloalkenyl, aryl, or heteroaryl and where  $\text{R}^{13}$ ,  $\text{R}^{14}$ , and  $\text{R}^{15}$  could be the same group or different groups, e.g. trimethylsilyl, tert-butyl dimethylsilyl,

cyclohexyldimethylsilyl, or phenyldimethylsilyl; or C<sub>1-6</sub> alkyl or C<sub>1-6</sub> branched alkyl or C<sub>1-6</sub> cycloalkyl or aryl or heteroaryl optionally substituted with hydroxy, mercapto, C<sub>1-6</sub> alkoxy, C<sub>1-6</sub> alkylthio, halo, cyano, CO<sub>2</sub>H, SO<sub>3</sub>H, SO<sub>2</sub>H, OSO<sub>3</sub>H, nitro, PO<sub>3</sub>H<sub>2</sub>, OPO<sub>3</sub>H<sub>2</sub>, C<sub>1-6</sub> alkyl esterified CO<sub>2</sub>H, carboxamide, C<sub>1-6</sub> alkyl esterified PO<sub>3</sub>H<sub>2</sub>, C<sub>1-6</sub> alkyl, C<sub>1-6</sub> branched alkyl, C<sub>1-6</sub> cycloalkyl, trisubstituted-silyl i.e. (R<sup>13</sup>)(R<sup>14</sup>)(R<sup>15</sup>)Si, where R<sup>13</sup>, R<sup>14</sup>, and R<sup>15</sup> are C<sub>1-6</sub> alkyl, C<sub>3-6</sub> branched alkyl, C<sub>3-6</sub> cycloalkyl, aryl, or heteroaryl, and where R<sup>13</sup>, R<sup>14</sup>, and R<sup>15</sup> could be the same group or different groups, e.g. trimethylsilyl, tertbutyldimethylsilyl or phenyldimethylsilyl; with the proviso that R<sup>2a</sup> and R<sup>2b</sup> are not both hydroxy or mercapto.

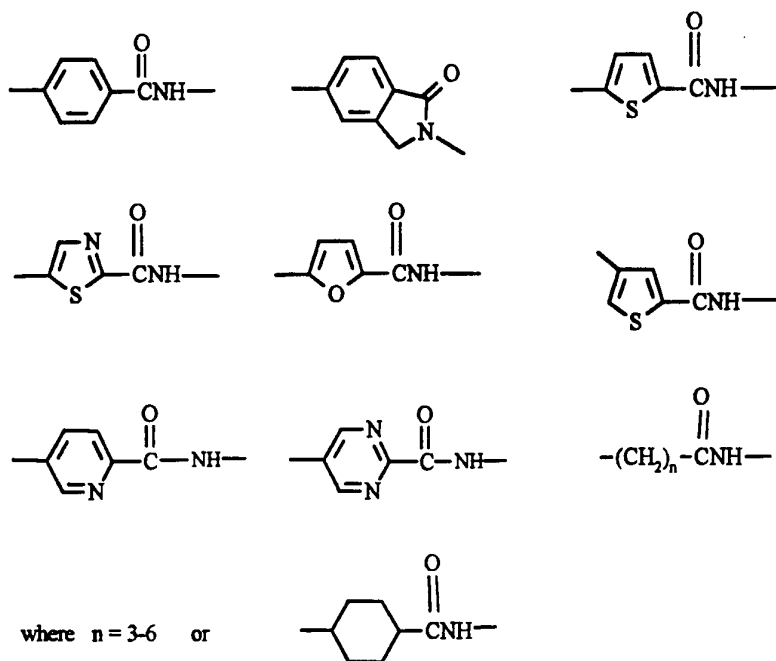
F comprises a moiety of formula (IV)



(IV)

wherein R<sup>1</sup> represents a group corresponding to the side chain of any α- amino acid for example, H, C<sub>1-6</sub> alkyl, C<sub>1-6</sub> alkenyl, C<sub>1-6</sub> alkynyl optionally substituted with CO<sub>2</sub>H, C<sub>1-6</sub> alkyl esterified CO<sub>2</sub>H, OPO<sub>3</sub>, PO<sub>3</sub>H<sub>2</sub>, C<sub>1-6</sub> alkyl esterified PO<sub>3</sub>H<sub>2</sub>, halo, hydroxy, carboxamide, amino optionally substituted with C<sub>1-6</sub> alkyl, cyano or nitro,

E represents

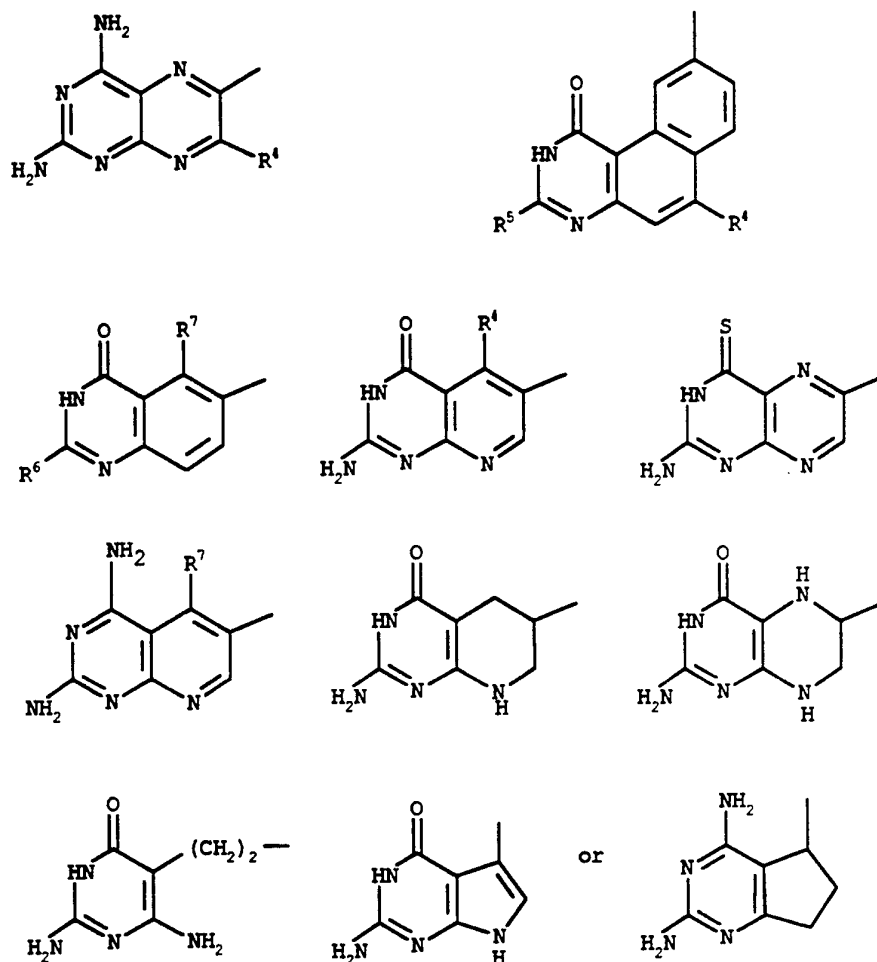


each of which may be optionally substituted with one or more of hydroxy, one or more of alkoxy, halo, or  $C_{1-6}$  alkyl optionally substituted with one or more of hydroxy,  $C_{1-6}$  alkoxy or halo.

D represents  $-CH_2-CH(R^3)-$ ,  $-CH_2-NR^3-$ ,  $-NR^3-CH_2-$ ,  $-CH_2S-$  or  $-CH_2O-$  where  $R^3$  is H,  $C_{1-6}$  alkyl, allyl or propargyl, optionally substituted with one or more of  $C_{1-6}$  alkoxy halo, hydroxy or cyano; and

B represents





wherein,

$\text{R}^4$  represents H,  $\text{C}_{3-6}$  branched alkyl,  $\text{C}_{3-6}$  cycloalkyl, or  $\text{C}_{1-6}$  alkyl;

$\text{R}^5$  represents  $\text{C}_{1-6}$  alkyl,  $\text{C}_{3-6}$  branched alkyl,  $\text{C}_{3-6}$  cycloalkyl, amino (optionally substituted with  $\text{C}_{1-6}$  alkyl,  $\text{C}_{1-6}$  alkanoyl or benzyl);

$\text{R}^6$  represents H,  $\text{C}_{1-6}$  alkyl,  $\text{C}_{3-6}$  branched alkyl,  $\text{C}_{3-6}$  cycloalkyl, amino (optionally substituted with  $\text{C}_{1-6}$  alkyl,  $\text{C}_{1-6}$  alkanoyl or benzyl),  $\text{C}_{1-6}$  alkoxy or  $\text{C}_{1-6}$  alkylthio; and

$\text{R}^7$  represents H,  $\text{C}_{1-6}$  alkyl,  $\text{C}_{3-6}$  branched alkyl,  $\text{C}_{3-6}$  cycloalkyl, halo  $\text{C}_{1-6}$  alkyl or halo

and salts N-oxides, solvates and physiologically functional derivatives thereof, with the proviso that the following compounds are not included within the formula.

N-(N-(4-(((2-amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl)(2-propynyl)amino)benzoyl)-L-glutam-1-yl)-L-glutamic acid,

N-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-glutamic acid,

N-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-aspartic acid,

N-(4-(((2,4-diamino-6-pteridiny)l)methyl)methylamino)benzoyl)-L-glutam-1-yl-L-phenylalanine,

4-bis(2-chloroethyl)amino)-phenylalanylphenylalanine and

4-bis(2-chloroethyl)amino)-phenylalanyl-3,5-dimethyl-4-methoxyphenylalanine

21. A compound according to Claim 20 and salts, N-oxides, solvates and physiologically functional derivatives thereof wherein

X is NH;

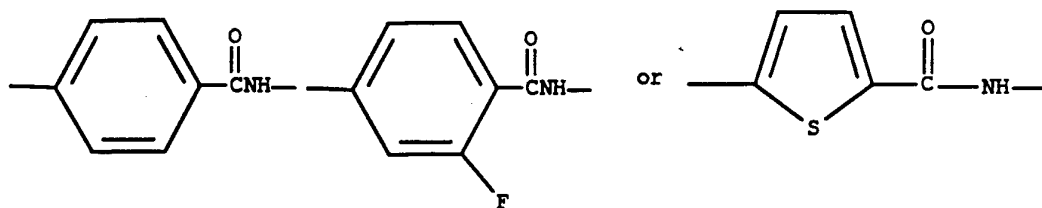
R<sup>2</sup> is C<sub>3-8</sub> cycloalkyl or aryl substituted with C<sub>1-8</sub>alkyl, C<sub>3-8</sub>cycloalkyl, C<sub>3-9</sub> branched alkyl or trisubstituted-silyl i.e. (R<sup>13</sup>)(R<sup>14</sup>)(R<sup>15</sup>)Si, where R<sup>13</sup>, R<sup>14</sup>, and R<sup>15</sup> are C<sub>1-6</sub> alkyl, C<sub>3-6</sub> branched alkyl, C<sub>3-6</sub> cycloalkyl, aryl, or heteroaryl, and where R<sup>13</sup>, R<sup>14</sup>, and R<sup>15</sup> are each the same group or different groups (e.g. trimethylsilyl, tert-butyldimethylsilyl, cyclohexyldimethylsilyl, or phenyldimethylsilyl);

R<sup>2a</sup> and R<sup>2b</sup> are both H;

R<sup>1</sup> is H, C<sub>1-6</sub> alkyl, CH<sub>2</sub>CO<sub>2</sub>H, CH<sub>2</sub>CH<sub>2</sub>CO<sub>2</sub>H or CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>,

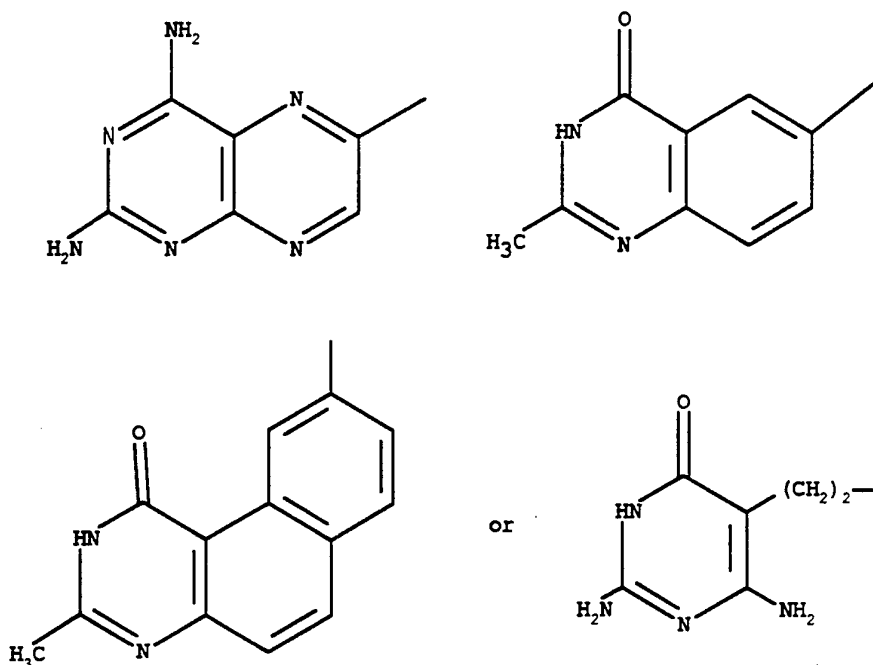
E is

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D is  $-\text{CH}_2\text{NH}-$ ,  $-\text{CH}_2\text{N}(\text{CH}_3)-$ ,  $-\text{CH}_2\text{N}(\text{CH}_2\text{C}\equiv\text{CH})-$ ,  $-\text{CH}_2\text{CH}_2-$  or  $-\text{CH}_2-\text{CH}(\text{C}_2\text{H}_5)-$

and B is



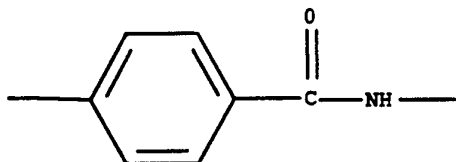
22. A compound according to Claim 20 and salts, N-oxides, solvates and physiologically functional derivatives thereof wherein

X is  $\text{NH}$ ;

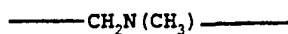
$\text{R}^2$  is phenyl or para-hydroxyphenyl substituted with cyclopentyl, cyclobutyl or tert-butyl. The cyclopentyl, cyclobutyl and tert-butyl groups may both be either ortho- or meta- but are preferably meta-,  $\text{R}^{2a}$  and  $\text{R}^{2b}$  are H,

R<sup>1</sup> is CH<sub>2</sub>CH<sub>2</sub>CO<sub>2</sub>H

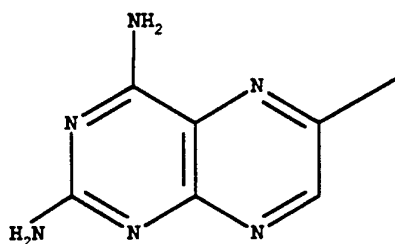
E is



D is



and B is



23. The following compounds and salts, N-oxides, solvates and physiologically functional derivatives thereof:

N-((S)-4-carboxy-2-(5-(((1,2-dihydro-3-methyl-1-oxobenzo(F)quinazolin-9-yl)methyl)amino)-1-oxo-2-isoindolyl)butanoyl)-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclobutyl-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-trimethylsilyl-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-tert-butyl-L-phenylalanine,

N-(4-((3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl)amino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine,

N-(4-(((1,2-dihydro-3-methyl-1-oxobenzo(F)quinazolin-9-yl)methyl)amino)-2-fluorobenzoyl)-L-glutam-1-yl-3-cyclopentyl-L-phenylalanine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-tyrosine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3,5-diiodo-L-tyrosine,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-2-cyclopentyl-L-tyrosine,

(S)-3-(3-cyclopentyl-4-hydroxyphenyl)-2-((N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl)oxy)propionic acid,

N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-tert-butyl-L-tyrosine.

24. N-(4-(((2,4-diamino-6-pteridiny)methyl)methylamino)benzoyl)-L-glutam-1-yl-3-cyclopentyl-L-tyrosine and salts, N-oxides, solvates and physiologically functional derivatives thereof.
25. The use of a compound according to any of Claims 20 to 24 or of a conjugate according to any of Claims 1 to 16 in medical therapy.

26. The use of a compound according to any of Claims 20 to 24 or of a conjugate according to any of Claims 1 to 16 or of a cell line according to Claim 19 or of a vector according to Claim 18 or of DNA or RNA according to Claim 17 in the preparation of a medicament for use in medical therapy.
27. A method of targetted chemotherapy consisting of the administration of a conjugate comprising a mutant mammalian enzyme and a cell-type specific targetting molecule wherein the enzyme is capable of catalysing a functionally inactive precursor of a chemotherapeutic agent to its active form which said precursor is refractory to endogenous catalysis.
28. The use of a precursor of a chemotherapeutic agent in the preparation of a medicament for use in the treatment of a patient requiring therapy to a particular cell population who has a conjugate as claimed in any of Claims 1 to 16 bound to that particular cell-population.
29. A mutant mammalian enzyme capable of catalysing a functionally inactive precursor of a chemotherapeutic agent to its active form wherein the precursor is refractory to endogenous catalysis.
30. An enzyme according to Claim 29 wherein the enzyme possesses carboxypeptidase activity.
31. An enzyme according to Claim 30 wherein the enzyme is a human carboxypeptidase A.
32. An enzyme according to Claim 31 wherein the enzyme is HCPA 1 or HCPA 2 possessing amino acid substitutions at one or more of residues 203, 210, 242, 244, 250, 253, 255, 267, 268, 269 and 305.
33. An enzyme according to Claim 32 wherein the amino acid substitutions are selected from the following:

Gly at 250 and 268,

Gly at 253 and 268,

Gly at 250 and His at 268,  
Gly at 250,  
Ala at 255 and His 268,  
His at 268, and  
Gly at 268.

34. An enzyme according to Claim 33 wherein the substitution is Gly at 268.
35. An enzyme according to any of Claims 32 to 34 wherein all other residues are wild-type.
36. A DNA or RNA molecule encoding an enzyme claimed in any of Claims 29 to 35.
37. A vector containing a DNA or RNA molecule claimed in Claim 36.
38. A cell line combining a DNA or RNA molecule as claimed in Claim 36 or a vector as claimed in Claim 37.

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## INHIBITION OF WEIN-133 GROWTH BY MTX-PHE IN THE PRESENCE OF CPA-CAMPATH-1H

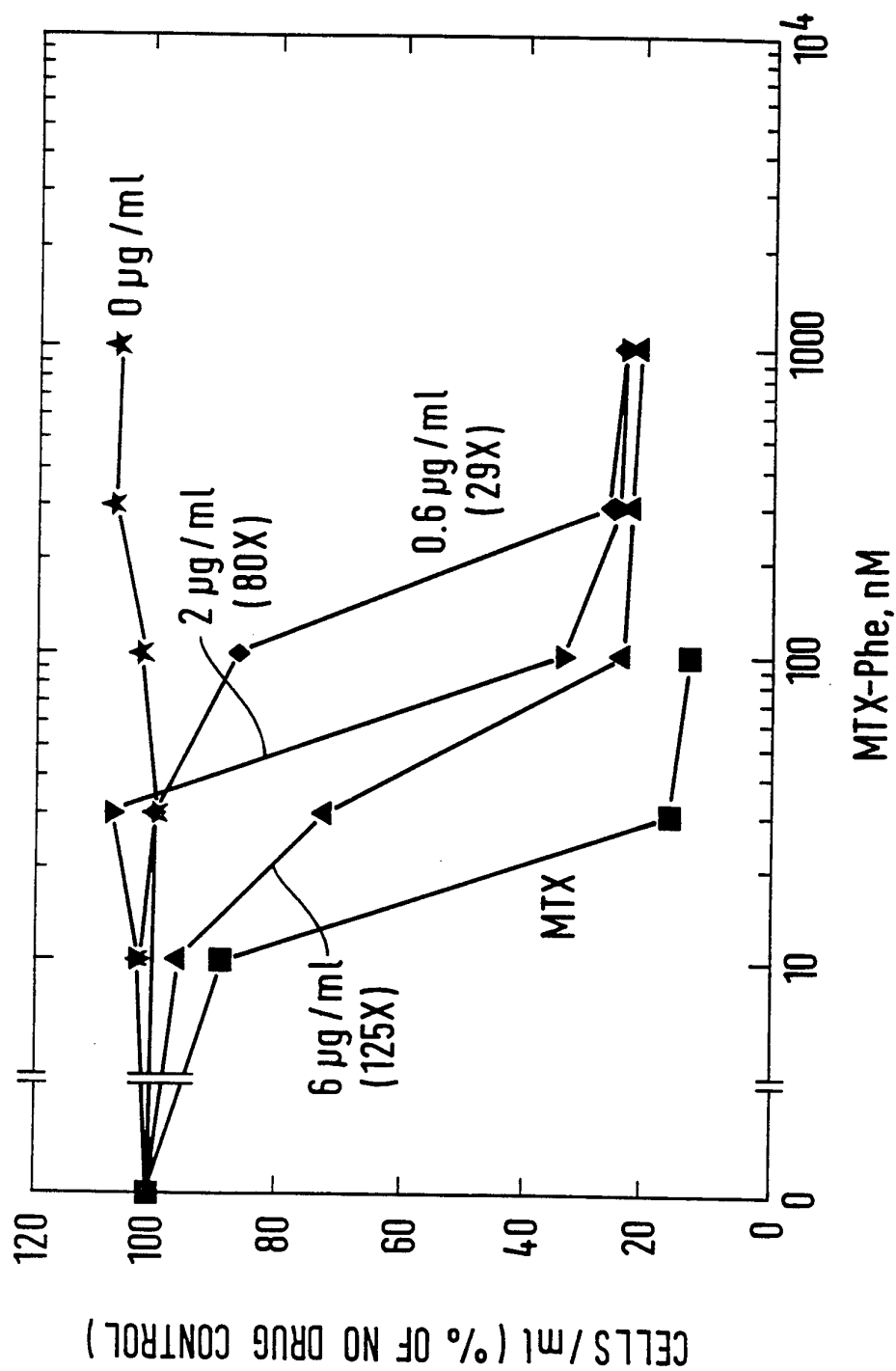
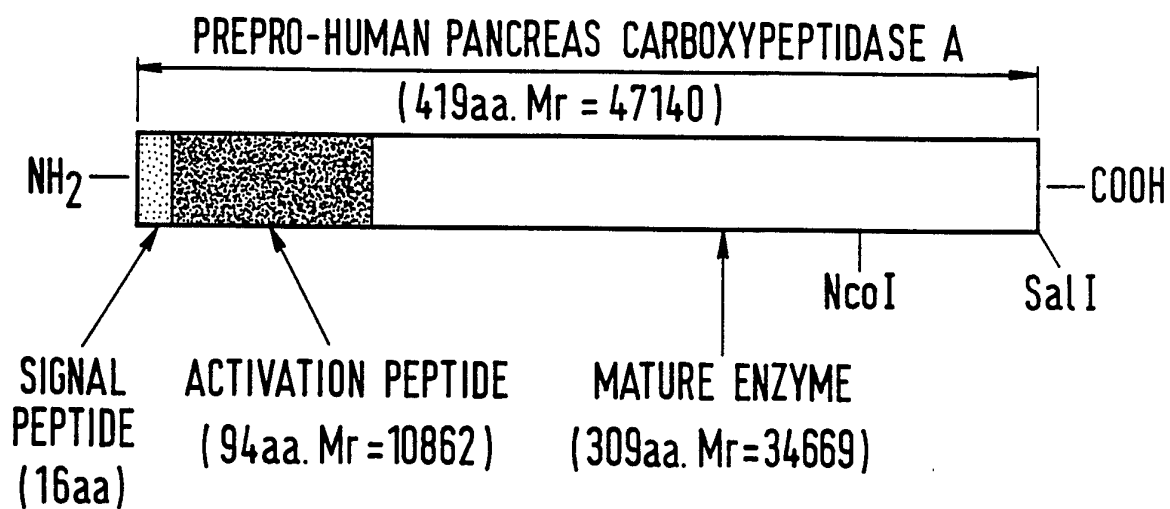
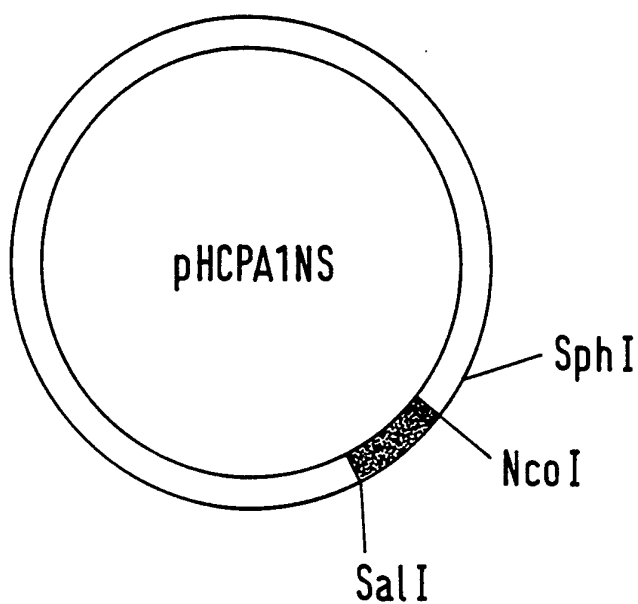


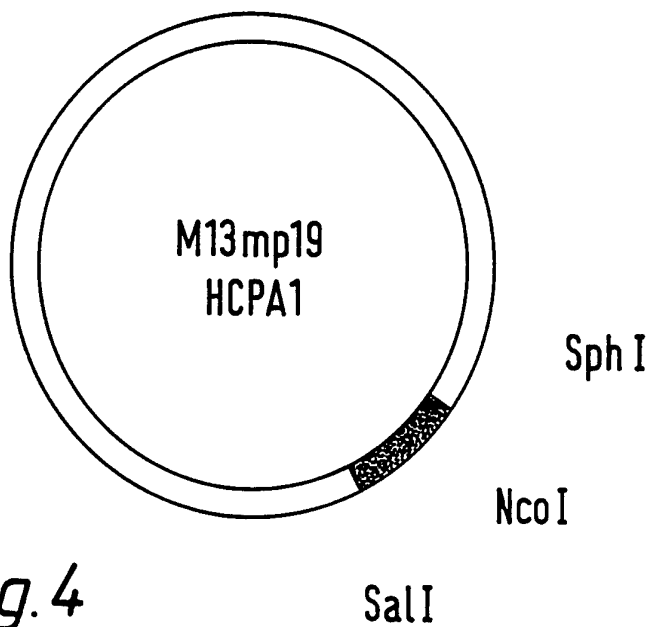
Fig. 1



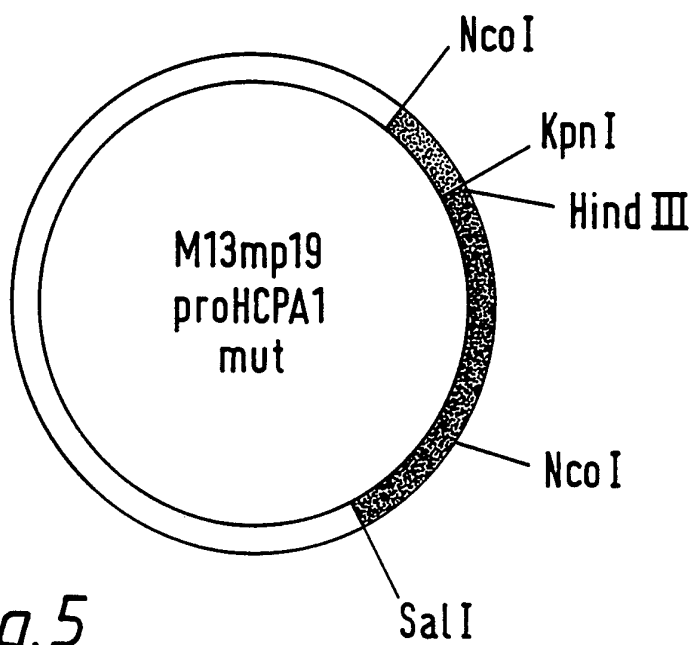
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*Fig. 2**Fig. 3*

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*Fig. 4*



*Fig. 5*

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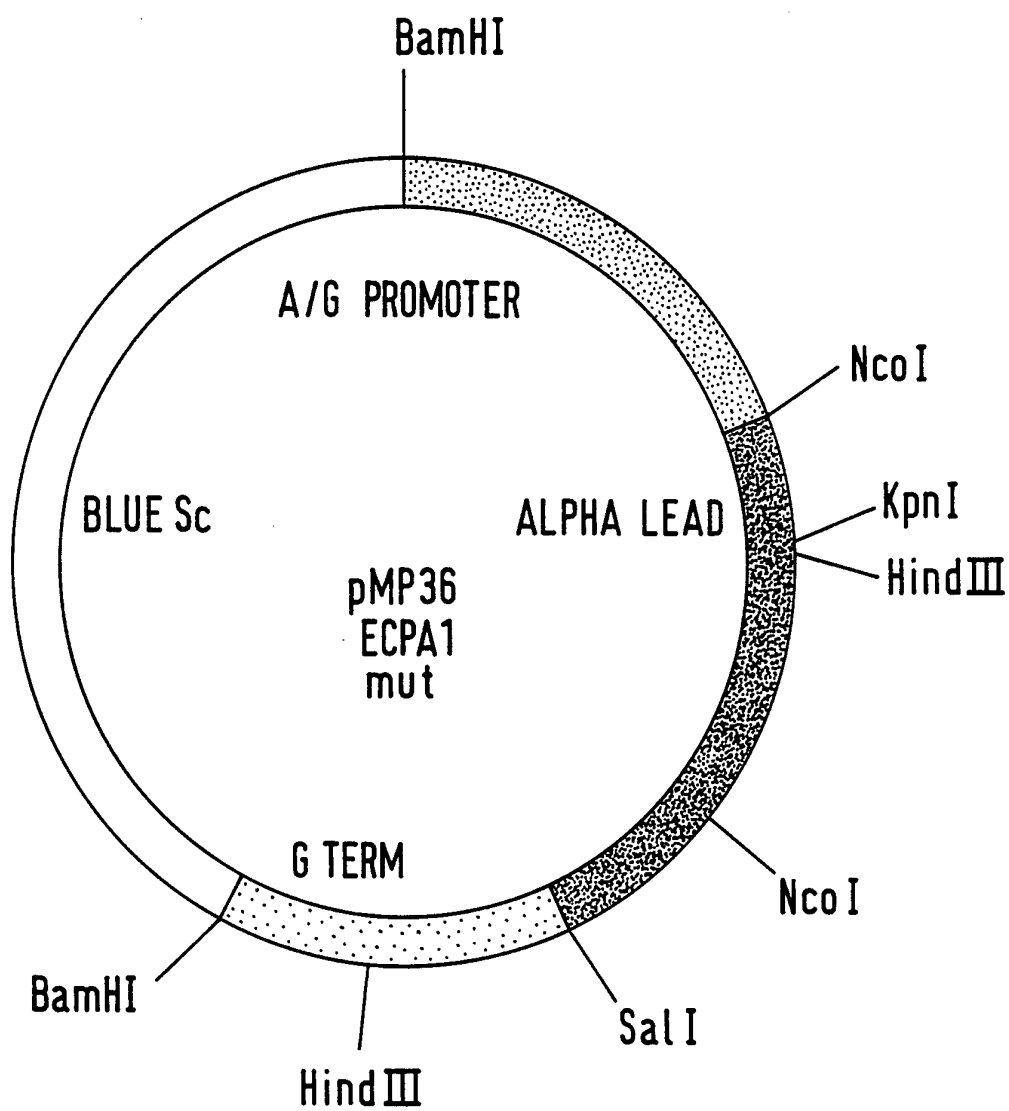
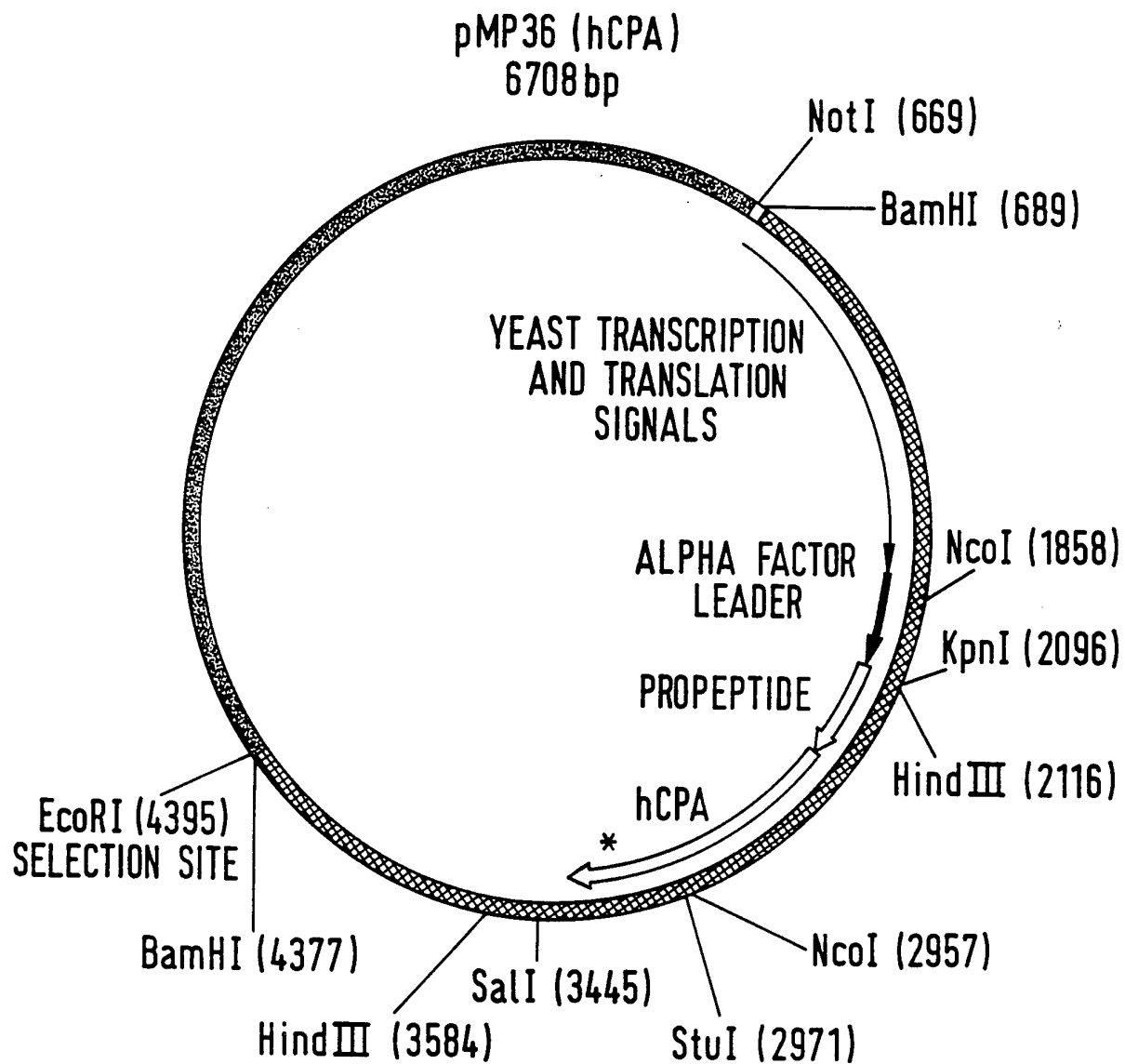


Fig. 6

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\* SITE OF hCPA MUTATIONS

*Fig. 7*