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(54) Title: PEPTIDE PRODRUGS CONTAINING AN ALPHA-HYDROXYACID LINKER

(57) Abstract

Peptide prodrugs of the general formula (I): X-L-Z, wherein X designates a pharmaceutically active peptide sequence, e.g. Leu-enkephalin; Z designates a peptide pre-sequence of 2 to 20 amino acid units, preferably comprising lysine and glutamic acid; and L is a linking group comprising from 3 to 9 backbone atoms, wherein the bond between the C-terminal carbonyl of X and L is different from a C-N amide bond. Preferably, the bond between X and L is an ester bond. It has been found that it is possible to obtain a remarkable increase in the resistance towards degradation by proteolytic enzymes such as carboxypeptidase A, pepsin A, leucine aminopeptidase, α-chymotrypsin when masking a pharmaceutically active peptide as a prodrug of the formula (I). The prodrugs of formula (I) are cleaved by the blood plasma enzyme butyryl cholinesterase indicating a readily bioreversibility. It is believed that the stability towards enzymatic cleavage is due to an induced helix-like structure.

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PEPTIDE PRODRUGS CONTAINING AN ALPHA-HYDROXYACID LINKER

FIELD OF THE INVENTION

The present invention relates to prodrugs of pharmaceutically active peptides having reduced tendency towards hydrolysis.

BACKGROUND OF THE INVENTION

There exist a large number of pharmaceutically active peptides, e.g. naturally occurring in man or in animals, or synthetic analogues of such peptides. An illustrative example of such a peptide is the analgetically active peptide enkephalin which has given rise to a vast number of synthetic analogues. However, due to precisely their peptide nature, the routes of administration thereof have been rather limited. Thus, peptides are rapidly and very effectively degraded by enzymes, generally with half-lives in the range of minutes. Proteases and other proteolytic enzymes are ubiquitous, particularly in the gastro-intestinal tract, therefore peptides are usually susceptible to degradation in multiple sites upon oral administration, and to some extent in the blood, the liver, the kidney, and the vascular endothelia. Furthermore, a given peptide is usually susceptible to degradation at more than one linkage within the backbone; each locus of hydrolysis is mediated by a certain protease.

There have been a number of attempts at protecting peptides against premature degradation, such as by modification of the peptide structure, co-administration of protease inhibitors, or special formulation strategies, but they have only been met with limited success.

SUMMARY OF THE INVENTION

It has now surprisingly been found that by equipping a pharmaceutically active peptide, at its C-terminal, with a suitable bioreversible amino acid pre-sequence, it is possible to render the peptide significantly less susceptible to degradation by proteases. Without being bound to any specific model for this effect, it is believed that the presence of the pre-sequence induces a degree of structuring of a helix-like nature of the pharmaceutically active peptide, whereby the peptide is less susceptible to proteases in contrast to peptides in the random-coil conformation. As a result of the structuring, the peptide is much more difficult for a protease to degrade. The bioreversible property of the pre-sequence is obtained by linking the peptide and the pre-sequence by means of a linking group bound to the peptide in a manner different from a normal peptide bond.

Thus, the invention concerns prodrugs of pharmaceutically active peptides (X-OH), peptide amides ($X-NH_2$), or peptide esters (X-OR), wherein the prodrug has the general formula I

X - L - Z I

wherein X is bound to L at the C-terminal carbonyl function of X;

L is a linking group, comprising from 3 to 9 backbone atoms, wherein the bond between the C-terminal carbonyl of X and L is different from a C-N amide bond; and

Z is a peptide sequence of 2-20 amino acid units and bound to L at the N-terminal nitrogen atom of Z, each amino acid unit being independently selected from Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, Met, Orn, and amino acid units of the formula II

$$-NH-C(R^3)(R^4)-C(=O)- \qquad II$$

wherein R^3 and R^4 independently are selected from C_{1-6}-alkyl, phenyl, and phenyl-methyl, wherein C_{1-6}-alkyl is optionally substituted with from one to three substituents selected from halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, and phenyl and phenyl-methyl is optionally substituted with from one to three substituents selected from C_{1-6}-alkyl, C_{2-6}-alkenyl, halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, or R^3 and R^4 together with the carbon atom to which they are bound form a cyclopentyl, cyclohexyl, or cycloheptyl ring:

or a salt thereof.

The present invention also relates to the use of a prodrug of the general formula I in therapy, and the use of a prodrug of the general formula I in the preparation of a composition for use in therapy, and a pharmaceutical composition comprising a prodrug of the general formula I and a pharmaceutically acceptable carrier.

Another aspect of the present invention relates to an immobilised linker-peptide sequence L-Z-SSM, wherein L and Z are as defined above, and SSM designates a solid support material, the use of an immobilised linker-peptide sequence L-Z-SSM for the preparation of a prodrug of the general formula I, and methods for preparation of prodrugs of the general formula I comprising the use of an immobilised linker-peptide sequence L-Z-SSM.

As the prodrugs of the general formula I are novel in themselves, a further aspect of the present invention relates to compounds of the general formula I.

DETAILED DESCRIPTION OF THE INVENTION

Peptides are utilised in a number of processes, e.g., cell-to-cell communication, some being present in the autonomic and central nervous system. Some of the latter peptides, and a number of other peptides, exert important effects on vascular and other smooth muscles. These peptides include, e.g., the vasoconstrictors angiotensin II, vasopressin, endothelin, neuropeptide Y, vasoactive intestinal peptide, substance P, neurotensin, and calcitonin, calcitonin gene-related peptide, and calcitonin gene-related peptide II. Among other pharmaceutically interesting peptides may be mentioned analgetic, antidiabetic, antibiotic, and anaesthetic peptides, etc. and, thus, the peptide may be or be reminiscent of endorphins, enkephalins, insulin, gramicidin, paracelsin, delta-sleep inducing peptide, ANF, vasotocin, bradykinin, dynorphin, endothelin, growth hormone release factor, growth hormone release peptide, oxytocin, tachykinin, ACTH, brain natriuretic polypeptide, cholecystokinin, corticotropin releasing factor, diazepam binding inhibitor fragment, FMRF-amide, galanin, gastric releasing polypeptide, gastrin, gastrin releasing peptide, glucagon, glucagon-like peptide-1, glucagon-like peptide-2, LHRH, melanin concentrating hormone, alpha-MSH, morphine modulating peptides, motilin, neurokinins, neuromedins, neuropeptide K, neuropeptide Y, PACAP, pancreatic polypeptide, peptide YY, PHM, secretin, somatostatin, substance K, substance P, TRH, vasoactive intestinal polypeptide, and such biologically active peptides as described in H.L. Lee, "Peptide and Protein Drug Delivery", Marcel Dekker Inc. 1991, Chapter 9, and references therein, Phoenix Pharmaceuticals, Inc. "The Peptide Elite", 1997-1998 Catalogue, and Bachem, "Feinchemikalien AG", Catalog S15-1995.

It should be understood that the above-mentioned peptides as well as the pharmaceutically active peptide sequence of these

peptides can be incorporated in the prodrugs (and the compounds) of the invention.

In the present context, the term "pharmaceutically active peptide sequence" as applied to X is intended to mean any peptide or peptide-containing structure, either naturally occurring or synthetic, having two or more amino acid units (preferably three or more amino acid units) and exerting a pharmaceutical effect in mammals such as humans. In the present context, the term "amino acid unit" as used in connection with X means any naturally occurring or synthetic α, β, and γ-amino acid, as well as side-chain modified amino acids such as modified tyrosines wherein the aromatic ring is further substituted with e.g. one or more halogens, sulfono groups, nitro groups etc., and/or the phenol group is converted into an ester group, etc, including side-chain protected amino acids, wherein the amino acid side-chains are protected in accordance with methods known to the person skilled in peptide chemistry, such as described in, e.g., M. Bodanszky and A. Bodanszky, "The Practice of Peptide Synthesis", 2. Ed, Springer-Verlag, 1994, and J. Jones, "The Chemical Synthesis of Peptides", Clarendon Press, 1991, whether in the L-form or the corresponding D-form.

The pharmaceutically active peptide sequence X preferably consists of 2-200 amino acid units, more preferably 2-100 amino acid units (e.g. 3-100), even more preferably 2-50 amino acid units (e.g. 3-50 or 4-30), in particular 2-20 amino acid units (e.g. 3-20 or 4-20), especially 2-10 amino acid units (e.g. 3-10 or 4-10), such as 2-8 amino acid units (e.g. 3-8 or 4-8).

In the present context, a pharmaceutically active peptide sequence X which in the native form is present as the C-terminal free carboxylic acid, such as Leu-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH), is denoted X-OH. In a similar way, a pharmaceutically active peptide sequence X with a C-terminal amide group, such as oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-

Gly-NH_2), is denoted X-NH_2, and a pharmaceutically active peptide sequence X with a C-terminal ester groups, is denoted X-OR, wherein OR, e.g. is the alkoxy moiety of the alcohol which, together with the pharmaceutically active peptide sequence X, constitutes the ester. R may designate C_{1-6}-alkyl, aryl such as phenyl, aryl-C_{1-6}-alkyl such as benzyl, etc.

Thus, even though the pharmaceutically active peptide sequence X is bound to the linker via the C-terminal carbonyl, it should be understood that any peptide sequences corresponding to pharmaceutically active peptides having a free C-terminal carboxy group (i.e. X-OH) as well as peptides corresponding to pharmaceutically active peptides having a C-terminal amide (i.e. X-NH_2) or ester group (i.e. X-OR) may be used in the compounds and prodrugs of the invention.

It is well known that many biologically active peptides also exert their desired biological effect when present in a modified or truncated form. In the case of for example insulin, porcine insulin differ from human insulin by only one amino acid unit, the B30 amino acid in porcine insulin being Ala and the B30 amino acid in human insulin being Thr. Despite this difference porcine insulin has been used as an effective diabetes drug for many years. In a similar way it has been found that the essential features for activity in the heptadecanepeptide Porcine gastrin I are all contained in the C-terminal tetrapeptide and that essentially all biological effects of neurotensin are associated with the C-terminal hexapeptide. Furthermore, pharmaceutically active peptides, wherein one or more amide bonds have been modified, e.g. reduced, often exhibit a similar or even enhanced biological activity; for example the $Cys^2\psi[CH_2NH]Tyr^3$ analogue of somatostatin was found to be an even more potent growth hormone releasing agent than somatostatin itself, and also the transition state analogue $Leu^{10}\psi[CH(OH)CH_2]Val^{11}$ of angiotensin

has been found to show strong inhibitory effect against the aspartic acid protease Renin. Thus, the term "modified or truncated analogue thereof" is intended to mean such peptides that is modified by changing and/or deleting one or more amino acid units in the sequence of the native peptide, including modification of the side-chain, stereochemistry, and backbone in the individual amino acid units, such as changing one or more carboxamide bonds (-C(=O)-N-) into e.g. reduced forms such as (-CH(OH)-N-), ($-CH_2$-N-), and other peptide bond mimetics such as (-C(=O)-O), (-C(=O)-CH_2-), (-CH=CH-), (-PO_2-NH-), (SO-CH_2-), (SO_2-N-), etc.

This being said, it should be understood that the peptide sequence in question should preferably comprise at least one amide bond (preferably two amide bonds (this naturally does not apply for a dipeptide)) susceptible to enzymatic degradation in order to fully take advantage of the present invention.

The most interesting prospect of the present invention is that it is possible to prepare "peptide prodrugs" for the treatment of mammals, such as humans, which are stabilised towards degradation by proteases and which subsequently are able to be released in an environment in which the peptide or the pharmaceutically active peptide sequence (X-OH) will exhibit a pharmaceutical action or will be transported to the desired location. Although the pharmaceutically active peptide sequence X preferably are released as a free acid (due to splitting of e.g. an ester bond between X and L) it is envisaged that the free acid may also posses a pharmaceutical relevant effect in cases where the native paharmaceutically active peptide sequence X is an amide (X-NH_2) or ester (X-OR).

Thus, in an interesting embodiment, the bond between the C-terminal carbonyl function of X and L is capable of being cleaved by blood plasma enzymes such as e.g. butyryl cholinesterase, acetyl cholinesterase, etc. In particular, the

bond between the C-terminal carbonyl function of X and L is a thiolester bond or an ester bond, preferably an ester bond.

In order to release the pharmaceutically active peptide sequence X-OH, the bond between X and L in the peptide prodrugs of the invention must be capable of being cleaved *in vivo*. It will be understood from the examples provided herein that the bond between X and L (which is preferably an ester bond) is capable of being cleaved by the enzyme butyryl cholinesterase. Thus, it is envisaged that the peptide prodrugs of the invention is capable of being cleaved by e.g. esterases present in the blood plasma and thereby releasing the desired pharmaceutically active peptide X-OH at a desired location.

The rate of enzymatic cleavage of the peptide may be adjusted in order for a medicament comprising the prodrug I to have a prolonged or retarded effect. Adjustment of the cleavage rate may, e.g., be carried out by increasing or decreasing the bulkiness and/or the electron-donating effect of substituents on L.

In the present context, the term "C_{1-6}-alkyl" used alone or as part of another group designates a straight, branched or cyclic saturated hydrocarbon group having from one to six carbon atoms, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec.butyl, tert.butyl, n-pentyl, n-hexyl, cyclohexyl, etc. Similarly, the term "C_{1-5}-alkyl" covers a straight, branched or cyclic saturated hydrocarbon group having from one to five carbon atoms, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec.butyl, tert.butyl, n-pentyl, isopentyl, cyclopentyl, etc. The term "C_{1-4}-alkyl" used alone or as part of another group designates a straight or branched saturated hydrocarbon group having from one to four carbon atoms, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec.butyl, tert.butyl, etc., and similarly, the term "C_{1-3}-alkyl" covers a straight or branched saturated hydrocarbon

group having from one to three carbon atoms, such as methyl, ethyl, n-propyl, and isopropyl.

In the present context, the term "C_{2-6}-alkenyl" designates a hydrocarbon group having from two to six carbon atoms, which may be straight, branched, or cyclic and may contain one or more double bonds, such as vinyl, allyl, 1-butenyl, 2-butenyl, isobutenyl, 1-pentenyl, 2-pentenyl, 4-pentenyl, 3-methyl-1-butenyl, 2-hexenyl, 5-hexenyl, cyclohexenyl, 2,3-dimethyl-2-butenyl etc., which may have *cis* and/or *trans* configuration. Similarly, the term "C_{2-5}-alkenyl" designates a hydrocarbon group having from two to five carbon atoms, which may be straight, branched, or cyclic and may contain one or more double bonds, such as vinyl, allyl, 1-butenyl, 2-butenyl, isobutenyl, 1-pentenyl, 2-pentenyl, 4-pentenyl, 3-methyl-1-butenyl, cyclopentenyl, etc., which may have *cis* and/or *trans* configuration, and the term "C_{2-4}-alkenyl" designates a hydrocarbon group having from two to four carbon atoms, which may be straight or branched and may contain one or more double bonds, such as vinyl, allyl, 1-butenyl, 2-butenyl, isobutenyl, etc., which may have *cis* and/or *trans* configuration.

The term "alkoxy" means alkyl-oxy.

The term "aryl" is intended to mean an aromatic, carbocyclic group such as phenyl or naphthyl.

The term "halogen" includes fluorine, chlorine, bromine, and iodine.

The term "heteroaryl" includes 5- or 6-membered aromatic monocyclic heterocyclic groups containing 1-4 heteroatoms selected from nitrogen, oxygen and sulfur, such as pyrrolyl, furyl, pyrazolyl, imidazolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, oxadiazolyl, thiadiazolyl, triazolyl, pyridyl, and aromatic bicyclic heterocyclic groups containing 1-6

heteroatoms selected from nitrogen, oxygen and sulfur, such as quinolinyl.

The peptide sequence Z is the part of the compound I responsible for introduction and/or stabilisation of a certain secondary structure into the molecule which will render the compound more stable towards degradation by proteases, thus, it is believed that Z needs to include at least 2 amino acid units (preferably at least 3 amino acid units) in order to introduce such a structural element either alone or in combination with the linker L. On the other hand it is also believed that a sequence of more than around 20 amino acid units will not improved the stability further. Thus, Z is a peptide sequence of 2-20 amino acid units (preferably 3-20), preferably in the range of 3-15, more preferably 3-9 (such as 4-9), in particular 3-6 amino acid units, such as 4-6 amino acid units.

Each of the amino acid units in the peptide sequence Z are independently selected from Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, Met, Orn, and amino acids of the formula II as defined herein. Preferably, the amino acid units are selected from Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, and Met, more preferably from Glu, Lys, and Met, especially Glu and Lys. The above-mentioned amino acids may have either D- or L-configuration, but preferably the above-mentioned amino acids have L-configuration. As the pharmaceutically active peptide sequence X usually consists exclusively of L-amino acids, it must be expected, in order to preserve the helix structure of the entire prodrug, that a peptide sequence Z consisting only or principally of L-amino acids will be advantageous compared to a peptide sequence Z consisting only or principally of D-amino acids. Furthermore, it is envisaged that a peptide sequence Z consisting only or principally of D-amino acids may exert toxicological effects due to the resistance of D-peptides and D-amino acids towards biodegradation.

The amino acid units of Z may of course all be different or all be identical. However, in interesting embodiments of the present invention, the amino acid units in Z are selected from three different amino acids or from two different amino acids, or are identical amino acids, preferably the amino acid units in Z are identical such as $(Lys)_n$ or $(Glu)_n$, wherein n is an integer in the range from 4 to 6, or a combination of two amino acid units such as $(LysGlu)_2$, $(LysGlu)_3$, $(GluLys)_2$, or $(GluLys)_3$, or a combination of three amino acid units, e.g. Xaa-$(Lys)_x$-$(Glu)_y$, Xaa-$(Glu)_x$-$(Lys)_y$, $(Lys)_x$-$(Glu)_y$-Xaa, $(Glu)_x$-$(Lys)_y$-Xaa, $(Lys)_x$-Xaa-$(Glu)_y$, $(Glu)_x$-Xaa-$(Lys)_y$, Xaa-Lys-Glu-Lys, Xaa-Lys-Glu-Lys-Glu, Xaa-Lys-Glu-Lys-Glu-Lys, Xaa-Glu-Lys-Glu, Xaa-Glu-Lys-Glu-Lys, Xaa-Glu-Lys-Glu-Lys-Glu, Lys-Glu-Lys-Xaa, Lys-Glu-Lys-Glu-Xaa, Lys-Glu-Lys-Glu-Lys-Xaa, Glu-Lys-Glu-Xaa, Glu-Lys-Glu-Lys-Xaa, Glu-Lys-Glu-Lys-Glu-Xaa, etc., wherein x and y are integers in the range from 1 to 4 with the proviso that x+y is at the most 5, and Xaa denotes Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Arg, His, Met, Orn, and amino acids of the formula II as defined herein.

With respect to the peptide sequence Z, it is envisaged that the specific amino acid units mentioned as constituents of the peptide sequence Z, i.e. Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, Met, Orn, and amino acid units of the formula II, are amino acid units which, due to their sterical arrangement around the α-carbon atom, and probably also due to a specific electronic configuration, have certain preferences for participating in, or even stabilising or initiating, helix-like structures. The Chou-Fasman approach (Chou, P.Y. & Fasman, G.D. *Ann. Rev. Biochem.* **47**, 251-276 (1978)) is one attempt to quantize (empirically) the likelihood for a specific amino acid unit to be involved in an α-helix structure (expressed as the "Conformational parameter P_α"). Chou and Fasman's studies and related studies have, however, shown that amino acid units

which have a low parameter P_α, may be found in α-helices, but of course not as often as amino acid units having a higher P_α. Thus, in the peptide sequence Z, it is considered possible to include a small proportion of amino acid units which are not among the amino acid units selected above as constituents of Z, and still obtain the desired effect from the peptide sequence Z, in that the selected amino acid units are believed to compensate for any negative or neutral effect of such an alternative amino acid unit.

Thus, in embodiments which are within the scope of the present invention, it may be realistic to include up to 25% of amino acid units which are not among the amino acids preferred as constituents of Z. (By "25% percent" is referred to the number of amino acid units, i.e. no alternative amino acid units are allowed in di- and tripeptides, up to one alternative amino acid unit is allowed in tetra-, penta-, hexa-, and hepta-peptides, up to two alternative amino acid units are allowed in octapeptides, etc.) Such alternative amino acid units may be selected from Val, Ile, Pro, Phe, Gly, Trp, as well as N-methyl amino acid units, however, preferably not Pro, Gly and N-methyl amino acid units.

Illustrative examples of the peptide sequences Z are:
Lys-Lys-Lys-Lys, Glu-Lys-Lys-Lys, Lys-Glu-Lys-Lys, Lys-Lys-Glu-Lys, Lys-Lys-Lys-Glu, Glu-Glu-Lys-Lys, Glu-Lys-Gly-Lys, Glu-Lys-Lys-Glu, Lys-Glu-Glu-Lys, Lys-Glu-Lys-Glu, Lys-Lys-Glu-Glu, Glu-Glu-Glu-Lys, Glu-Glu-Lys-Glu, Glu-Lys-Glu-Glu, Lys-Glu-Glu-Glu, Glu-Glu-Glu-Glu, Lys-Lys-Lys-Lys-Lys, Glu-Lys-Lys-Lys-Lys, Lys-Glu-Lys-Lys-Lys, Lys-Lys-Glu-Lys-Lys, Lys-Lys-Lys-Glu-Lys, Lys-Lys-Lys-Lys-Glu, Glu-Glu-Lys-Lys-Lys, Glu-Lys-Glu-Lys-Lys, Glu-Lys-Lys-Glu-Lys, Glu-Lys-Lys-Lys-Glu, Lys-Glu-Glu-Lys-Lys, Lys-Glu-Lys-Glu-Lys, Lys-Glu-Lys-Lys-Glu, Lys-Lys-Glu-Glu-Lys, Lys-Lys-Glu-Lys-Glu, Lys-Lys-Lys-Glu-Glu, Lys-Lys-Glu-Glu-Glu, Lys-Glu-Lys-Glu-Glu, Lys-Glu-Glu-Lys-Glu, Lys-Glu-Glu-Glu-Lys,

Glu-Lys-Lys-Glu-Glu, Glu-Lys-Glu-Glu-Lys, Glu-Glu-Lys-Lys-Glu, Glu-Glu-Lys-Glu-Lys, Glu-Glu-Glu-Lys-Lys, Lys-Glu-Glu-Glu-Glu, Glu-Lys-Glu-Glu-Glu, Glu-Glu-Lys-Glu-Glu, Glu-Glu-Glu-Lys-Glu, Glu-Glu-Glu-Glu-Lys, Glu-Glu-Glu-Glu-Glu, Lys-Lys-Lys-Lys-Lys-Lys, Glu-Lys-Lys-Lys-Lys-Lys, Lys-Glu-Lys-Lys-Lys-Lys, Lys-Lys-Glu-Lys-Lys-Lys, Lys-Lys-Lys-Glu-Lys-Lys, Lys-Lys-Lys-Lys-Glu-Lys, Lys-Lys-Lys-Lys-Lys-Glu, Glu-Glu-Lys-Lys-Lys-Lys, Glu-Lys-Glu-Lys-Lys-Lys, Glu-Lys-Lys-Glu-Lys-Lys, Glu-Lys-Lys-Lys-Glu-Lys, Glu-Lys-Lys-Lys-Lys-Glu, Lys-Glu-Glu-Lys-Lys-Lys, Lys-Glu-Lys-Glu-Lys-Lys, Lys-Glu-Lys-Lys-Glu-Lys, Lys-Glu-Lys-Lys-Lys-Glu, Lys-Lys-Glu-Glu-Lys-Lys, Lys-Lys-Glu-Lys-Glu-Lys, Lys-Lys-Glu-Lys-Lys-Glu, Lys-Lys-Lys-Glu-Glu-Lys, Lys-Lys-Lys-Glu-Lys-Glu, Lys-Lys-Lys-Lys-Glu-Glu, Glu-Glu-Glu-Lys-Lys-Lys, Glu-Glu-Lys-Glu-Lys-Lys, Glu-Glu-Lys-Lys-Glu-Lys, Glu-Glu-Lys-Lys-Lys-Glu, Glu-Lys-Glu-Glu-Lys-Lys, Glu-Lys-Glu-Lys-Glu-Lys, Glu-Lys-Glu-Lys-Lys-Glu, Glu-Lys-Lys-Glu-Glu-Lys, Glu-Lys-Lys-Glu-Lys-Glu, Glu-Lys-Lys-Lys-Glu-Glu, Lys-Lys-Lys-Glu-Glu-Glu, Lys-Lys-Glu-Lys-Glu-Glu, Lys-Lys-Glu-Glu-Lys-Glu, Lys-Lys-Glu-Glu-Glu-Lys, Lys-Glu-Lys-Lys-Glu-Glu, Lys-Glu-Lys-Glu-Lys-Glu, Lys-Glu-Lys-Glu-Glu-Lys, Lys-Glu-Glu-Lys-Lys-Glu, Lys-Glu-Glu-Lys-Glu-Lys, Lys-Glu-Glu-Glu-Lys-Lys, Lys-Lys-Glu-Glu-Glu-Glu, Lys-Glu-Lys-Glu-Glu-Glu, Lys-Glu-Glu-Lys-Glu-Glu, Lys-Glu-Glu-Glu-Lys-Glu, Lys-Glu-Glu-Glu-Glu-Lys, Glu-Lys-Lys-Glu-Glu-Glu, Glu-Lys-Glu-Lys-Glu-Glu, Glu-Lys-Glu-Glu-Lys-Glu, Glu-Lys-Glu-Glu-Glu-Lys, Glu-Glu-Lys-Lys-Glu-Glu, Glu-Glu-Lys-Glu-Lys-Glu, Glu-Glu-Lys-Glu-Glu-Lys, Glu-Glu-Glu-Lys-Lys-Glu, Glu-Glu-Glu-Lys-Glu-Lys, Glu-Glu-Glu-Glu-Lys-Lys, Lys-Glu-Glu-Glu-Glu-Glu, Glu-Lys-Glu-Glu-Glu-Glu, Glu-Glu-Lys-Glu-Glu-Glu, Glu-Glu-Glu-Lys-Glu-Glu, Glu-Glu-Glu-Glu-Lys-Glu, Glu-Glu-Glu-Glu-Glu-Lys, Glu-Glu-Glu-Glu-Glu-Glu.

It should be understood that the C-terminal of Z may be presented in the form of the free acid, the amide, or the ester, e.g. depending on the type of solid support material and cleavage conditions used in connection with the syntheses as will be clear to the person skilled in the art.

It should also be understood that L is bound at the N-terminal nitrogen atom of Z, i.e. the possible bond types between L and Z are those which involve a nitrogen atom, e.g. a carboxamide bond (-C(=O)-N-), a sulfonamide bond ($-SO_2-N-$), or an alkylamine bond (-C-N-), a carbamate bond (-O-C(=O)-N-), a thiocarbamate bond (-S-C(=O)-N-), an urea bond (-N-C(=O)-N-), a thiourea bond (-N-C(=S)-N-), a thioamide bond (-C(=S)-N-), a cyanomethyleneamino bond (-C(CN)-N-), or an N-methylamide bond ($-C(=O)-N(CH_3)-$). (In these examples the nitrogen atom (on the right-hand side) arises from Z and the remaining part of the "bond" arises from L.) Preferred bonds are -C(=O)-N-, $-SO_2-N-$, -C-N-, -C(=S)-N-, -C(CN)-N-, and $-C(=O)-N(CH_3)-$, among which -C(=O)-NH- and -C(=S)-NH- are preferred as they have the geometry of an ordinary peptide bond.

The linker L should preferably be able to participate in a helix-like structure initiated or stabilised by Z. Apart from the fact that the bond between X and L is not an amide bond, the geometry of L should preferably correspond to the geometry of an amino acid (or two or more amino acids), i.e. the linker L preferably comprises 3 backbone atoms or a multiple thereof such as 6 or 9 backbone atoms. In the present context, the term "backbone atoms" when used in connection with the linker L, therefore refers to the atoms in the linker L directly linking the pharmaceutically active peptide sequence X and the pre-sequence Z.

Thus, L is preferably derived from a hydroxy-carboxylic acid, in particular an α-hydroxy-carboxylic acid. More specifically, L is derived from an α-hydroxy-carboxylic acid of the general formula $HO-C(R^1)(R^2)-COOH$ wherein R^1 and R^2 independently is selected from H, C_{1-6}-alkyl, C_{2-6}-alkenyl, aryl, aryl-C_{1-4}-alkyl, heteroaryl, heteroaryl-C_{1-4}-alkyl, or R^1 and R^2 together with the carbon atom to which they are bound form a cyclopentyl,

cyclohexyl, or cycloheptyl ring, where an alkyl or alkenyl group may be substituted with from one to three substituents selected from amino, cyano, halogen, isocyano, isothiocyano, thiocyano, sulfamyl, C_{1-4}-alkylthio, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-alkoxy, aryl, heteroaryl, aryloxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, aminocarbonyl, mono- or di-C_{1-4}-alkyl-aminocarbonyl, mono- or di-C_{1-4}-alkyl-amino, mono- or di-C_{1-4}-alkyl-amino-C_{1-4}-alkyl, C_{1-4}-alkylcarbonylamino, sulfono, and sulfino, and where an aryl or a heteroaryl group may be substituted with from one to three substituents selected from C_{1-4}-alkyl, C_{2-4}-alkenyl, nitro, amino, cyano, halogen, isocyano, isothiocyano, thiocyano, sulfamyl, C_{1-4}-alkylthio, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-alkoxy, aryloxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, aminocarbonyl, mono- or di-C_{1-4}-alkyl-aminocarbonyl, mono- or di-C_{1-4}-alkyl-amino, mono- or di-C_{1-4}-alkyl-amino-C_{1-4}-alkyl, C_{1-4}-alkylcarbonylamino, sulfono, and sulfino.

From structural analysis of the prodrugs of the invention, it is envisaged that further stabilization of the helix-like structure of the prodrugs may be achieved when the linker L is derived from an α-hydroxy-carboxylic acid which also bears a methylene ($-CH_2-$) group in the α-position. Thus, in an interesting embodiment, the linker L is derived from an α-hydroxy-carboxylic acid with the general formula HO-C(CH_2-R^5)(R^2)-COOH, wherein R^5 is selected from H, C_{1-5}-alkyl, C_{2-5}-alkenyl, aryl, aryl-C_{1-3}-alkyl, heteroaryl, heteroaryl-C_{1-3}-alkyl, where an alkyl or alkenyl group may be substituted with from one to three substituents selected from amino, halogen, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-alkoxy, aryl, heteroaryl, aryloxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, and aminocarbonyl and where an aryl or heteroaryl may be substituted with from one to three substituents selected from C_{1-4}-alkyl, C_{2-4}-alkenyl, nitro, amino, halogen, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-

alkoxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, and aminocarbonyl; and R^2 is as defined above, preferably H, C_{1-6}-alkyl, C_{2-6}-alkenyl, aryl, aryl-C_{1-4}-alkyl, heteroaryl, heteroaryl-C_{1-4}-alkyl, where an alkyl or alkenyl group may be substituted with from one to three substituents selected from amino, halogen, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-alkoxy, aryl, heteroaryl, aryloxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, and aminocarbonyl, and where an aryl or heteroaryl may be substituted with from one to three substituents selected from C_{1-4}-alkyl, C_{2-4}-alkenyl, nitro, amino, halogen, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-alkoxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, and aminocarbonyl.

The above mentioned adjustment of the cleavage rate by increasing or decreasing the bulkiness and/or the electron-donating effect of substituents on L may e.g. be carried out by increasing or decreasing the bulkiness and/or the electron-donating effect of R^1 and/or R^2 (or R^5).

In especially interesting embodiments, L is derived from hydroxyacetic acid, (S)-(+)-mandelic acid, L-lactic acid ((S)-(+)-2-hydroxypropanoic acid), L-α-hydroxy-butyric acid ((S)-2-hydroxybutanoic acid), and α-hydroxy-isobutyric acid.

It should be understood that the prodrugs of the invention may also be in the form of a salt thereof. Salts include pharmaceutically acceptable salts, such as acid addition salts and basic salts. Examples of acid addition salts are hydrochloride slats, sodium salts, calcium salts, potassium salts, etc.. Examples of basic salts are salts where the cation is selected from alkali metals, such as sodium and potassium, alkaline earth metals, such as calcium, and ammonium ions $^+N(R^6)_3(R^7)$, where R^6 and R^7 independently designates optionally substituted C_{1-6}-alkyl, C_{2-6}-alkenyl, optionally substituted

aryl, or optionally substituted heteroaryl. Other examples of pharmaceutically acceptable salts are, e.g., those described in "Remington's Pharmaceutical Sciences" 17. Ed. Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, U.S.A., 1985 and more recent editions and in Encyclopedia of Pharmaceutical Technology.

As mentioned above the routes of administration of pharmaceutically active peptides have thus far been rather limited due to the fast biodegradation by proteases such as chymotrypsin, trypsin, carboxypeptidase A, pepsin, leucine aminopeptidase, etc. As it will be understood from the examples provided herein, the tendency of prodrugs of the general formula I (X-L-Z) to resist protease-catalysed hydrolysis can be measured directly by the *in vitro* enzyme assays shown in the examples. The tendency of X-L-Z to resist degradation can for example be expressed as a pseudo-first-order rate constant and/or as the half-life of said prodrugs, which may be compared to the corresponding values of X-OH, X-NH_2, and/or X-OR. Furthermore, the ability of the prodrugs of the invention to exert the desired biological effect was tested in various *in vitro* and *in vivo* assay procedures. A detailed description of the above-mentioned tests are given in the examples.

It has been found that it is possible to obtain a remarkable increase in the half-life ($t_{½}$) of a peptide or pharmaceutically active peptide sequence by protecting the peptide in question as a prodrug according to the invention.

Thus in a preferred embodiment of the invention, the ratio between the half-life of the prodrug in question in the "Hydrolysis in enzyme solution test", as defined herein, and the half-life of the corresponding peptide (X-OH), in the "Hydrolysis in enzyme solution test", is at least 2, preferably at least 5, and even more preferably at least 10, especially at

least 20, when using one of the enzymes carboxypeptidase A and leucine aminopeptidase.

The invention also concerns a pharmaceutical composition comprising a prodrug of the general formula I as defined above in combination with a pharmaceutically acceptable carrier.

Such compositions may be in a form adapted to oral, parenteral (intravenous, intraperitoneal), rectal, intranasal, dermal, vaginal, buccal, ocularly, or pulmonary administration, and such compositions may be prepared in a manner well-known to the person skilled in the art, e.g. as generally described in "Remington's Pharmaceutical Sciences", 17. Ed. Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, U.S.A., 1985 and more recent editions and in the monographs in the "Drugs and the Pharmaceutical Sciences" series, Marcel Dekker.

The invention also concerns use of a prodrug of the general formula I as defined above or a salt thereof in the preparation of a composition for use in therapy, e.g. in the treatment of disorders in the central nerveous system, in vaccine therapy, and in the treatment of HIV, cancer, diabetes, incontinence, hypertension, and as analgesics and contraceptives, and such indications known to be treated by therapy comprising administration of pharmaceutically active peptides.

The prodrugs of the invention may be prepared by methods known per se in the art. Thus, the peptide sequences X and Z may be prepared by standard peptide-preparation techniques such as solution synthesis or Merrifield-type solid phase synthesis. It is believed that the Boc (tert.butyloxycarbonyl) as well as the Fmoc (9-fluorenylmethyloxycarbonyl) strategies are applicable.

In one possible synthesis strategy, the prodrugs of the invention may be prepared by solid phase synthesis by first constructing the peptide sequence Z using well-known standard

protection, coupling and deprotection procedures, subsequently coupling the linking group L, thereafter sequentially coupling the pharmaceutically active sequence X on the linking group L in a manner similar to the construction of Z, and finally cleaving off the entire prodrug X-L-Z from the carrier.

Another possible strategy is to prepare one or both of the two sequences X and Z separately by solution synthesis, solid phase synthesis, recombinant techniques, or enzymatic synthesis, followed by coupling of the two sequences and the linking group L by well-known segment condensation procedures, either in solution or using solid phase techniques or a combination thereof.

Furthermore, it is envisaged that a combination of the above-mentioned strategies may be especially applicable where a modified peptide sequence, e.g. from a biologically active peptide comprising reduced peptide bonds, is to be coupled to a peptide sequence Z via a linker L. In this case it may be advantageous to prepare the immobilised fragment L-Z by successive coupling of amino acids (and the linker) first and then couple a complete biologically active peptide sequence X (prepared in solution or fully or partially using solid phase techniques) to the fragment L-Z.

Thus, the present invention also relates to an immobilized linker-peptide sequence Prot-L-Z-SSM, where L designates a linker of the general formula $-O-C(R^1)(R^2)C(=O)$, wherein R^1 and R^2 are as defined above, and Prot designates H or a hydroxy protecting group, where the hydroxy protecting group is selected from dimethoxytrityl, monomethoxytrityl, trityl, 9-(9-phenyl)xanthenyl (pixyl), tetrahydropyranol, methoxytetrahydropyranol, trimethylsilyl, triisopropylsilyl, tert.butyldimethylsilyl, triethylsilyl, phenyldimethylsilyl, benzyloxycarbonyl, substituted benzyloxycarbonyl ethers, such as 2-bromo benzyloxycarbonyl, tert.butylethers, methyl ethers, acetyl,

halogen substituted acetyls, such as chloroacetyl and fluoroacetyl, isobutyryl, pivaloyl, benzoyl and substituted benzoyls, methoxymethyl, benzyl ethers and benzyl ethers, such as 2,6-dichlorobenzyl, etc; SSM designates a solid support material selected from e.g. functionalised resins such as polystyrene, polyacrylamide, polydimethylacrylamide, polyethyleneglycol, cellulose, polyethylene, polyethyleneglycol grafted on polystyrene, latex, dynabeads, etc.; and Z is as defined above.

It should be understood that it may be necessary or desirable that the C-terminal amino acid of the pre-sequence Z is attached to the solid support material by means of a common linker such as 2,4-dimethoxy-4'-hydroxy-benzophenone, 4-(4-hydroxy-methyl-3-methoxyphenoxy)-butyric acid, 4-hydroxy-methylbenzoic acid, 4-hydroxymethyl-phenoxyacetic acid, 3-(4-hydroxymethylphenoxy)propionic acid, and p-[(R,S)-a[1-(9H-fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl]-phenoxy-acetic acid.

Consequently, the present invention also relates to the use of an immobilised linker-peptide sequence Prot-L-Z-SSM for the preparation of a prodrug according to the invention, and to a method for the preparation of a prodrug of a peptide (X-OH), a peptide amide ($X-NH_2$), or a peptide ester (X-OR) comprising coupling the corresponding peptide in a C-terminal activated form (X-Act) to an immobilised linker-peptide sequence H-L-Z-SSM.

The present invention also relates to a further method for the preparation of a prodrug of a peptide (X-OH), a peptide amide ($X-NH_2$), or a peptide ester (X-OR) comprising the steps of:

a) coupling an N-α-protected amino acid in the carboxyl activated form, or an N-α-protected dipeptide in the C-

terminal activated form to an immobilised linker peptide sequence H-L-Z-SSM, thereby forming an immobilised N-α-protected peptide fragment,

b) removing the N-α-protecting group, thereby forming an immobilised peptide fragment having an unprotected N-terminal end,

c) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the unprotected N-terminal end of the immobilised peptide fragment, and

repeating the removal/coupling procedure in step b) and c) until the desired peptide sequence X is obtained, and then

d) cleaving off the prodrug X-L-Z from the solid support material to obtain the free prodrug in the form of a C-terminal carboxylic acid, amide, or ester.

The coupling, removal and cleavage step is performed by methods known to the person skilled in the art taking into consideration the protection strategy and the selected solid phase material.

With respect to establishing the bonds on the one hand between X and L, and on the other hand between L and Z, of the types indicated above, such bonds may be established by methods known *per se* for establishing thiol ester, ester, carboxamide, sulfonamide, alkylamine, carbamate, thiocarbamate, urea, thiourea, thioamide, cyanomethyleneamino, or N-methylamide bonds or groupings, see e.g. J. March, "Advanced Organic Chemistry", 3rd edition, John Wiley & Sons, 1985 as well as references cited therein. Thus, e.g. an ester may be formed from an activated derivative (acid halide, acid anhydride,

activated ester e.g. HObt-ester etc.) of the appropriate carboxylic acid by reaction with the relevant hydroxy compound. Likewise, a carboxamide may be formed by reacting an activated derivative (acid halide, acid anhydride, activated ester e.g. HObt-ester etc.) of the appropriate carboxylic acid with the relevant amino compound as known to a person skilled in peptide chemistry; a sulfonamide may be formed by reacting a sulfonyl chloride with the appropriate amino compound; an alkyl amine bond or grouping may be formed by reacting the appropriate compound carrying a leaving group such as tosyl, halogen, and mesityl on the carbon atom in question with the relevant amino compound in a nucleophilic substitution reaction; a carbamate bond or grouping may be formed by treating the appropriate alcohol with phosgene to afford the corresponding chlorocarbonate which is then reacted with the relevant amino compound; a thiocarbamate bond or grouping may be formed by treating the appropriate alcohol with thiophosgene to afford the corresponding chlorothiocarbonate which is then reacted with the relevant amino compound; a urea bond or grouping may be formed by reacting the appropriate compound carrying a isocyanate group on the carbon atom in question with the relevant amino compound; a thiourea bond or grouping may be formed by reacting the appropriate compound carrying a isothiocyanate group on the carbon atom in question with the relevant amino compound; a thioamide bond or grouping may be formed by reacting the thiono ester of the appropriate carboxylic acid with the relevant amino compound, the thiono ester being formed e.g. from the corresponding piperidide.

Furthermore, it may be necessary or desirable to include side-chain protection groups when using amino acid units carrying functional groups which are reactive under the prevailing conditions. The necessary protection scheme will be known to the person skilled in the art (see e.g. M. Bodanszky and A. Bodanszky, "The Practice of Peptide Synthesis", 2. Ed,

Springer-Verlag, 1994, and J. Jones, "The Chemical Synthesis of Peptides", Clarendon Press, 1991).

Thus, the peptide prodrug of the invention may be cleaved from the solid support material by means of an acid such as trifluoracetic acid, trifluoromethanesulfonic acid, hydrogenbromide, hydrogenchloride, hydrogenfluoride, etc. or a base such as ammonia, hydrazine, an alkoxide, such as sodium ethoxide, an hydroxide, such as sodium hydroxide, etc.

As the prodrugs of the invention represent a novel class of compounds, a still further aspect of the present invention relates to compounds of the general formula I

$$X - L - Z \qquad I$$

wherein X is a peptide sequence which is bound to L at the C-terminal carbonyl function of X;

L is a linking group, comprising from 3 to 9 backbone atoms, wherein the bond between the C-terminal carbonyl of X and L is different from an C-N amide bond; and

Z is a peptide sequence of 2-20 amino acid units and bound to L at the N-terminal nitrogen atom of Z, each amino acid unit being independently selected from Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, Met, Orn, and amino acid units of the formula II

$$-NH-C(R^3)(R^4)-C(=O)- \qquad II$$

wherein R^3 and R^4 independently are selected from C_{1-6}-alkyl, phenyl, and phenyl-methyl, wherein C_{1-6}-alkyl is optionally substituted with from one to three substituents selected from halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, and phenyl and phenyl-methyl is

optionally substituted with from one to three substituents selected from C_{1-6}-alkyl, C_{2-6}-alkenyl, halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, or R^3 and R^4 together with the carbon atom to which they are bound form a cyclopentyl, cyclohexyl, or cycloheptyl ring;

or a salt thereof.

The invention is further illustrated by the following examples.

EXPERIMENTAL

Peptide synthesis

General procedures

Abbreviations used:

tBu = tert.butyl
DAMGO = Tyr-(D-Ala)-Gly-ψ[-C(=O)-N(CH_3)-]Phe-NH-CH_2-CH_2OH
DCC = dicyclohexylcarbodiimide
DCM = dichloromethane
DIC = diisopropylcarbodiimide
DIEA = N,N-diisopropylethylamine
DMAP = 4-(N,N-dimethylamino)-pyridine
Dhbt-OH = 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
DMF = N,N-dimethylformamide
DSIP = Delta-Sleep Inducing Peptide,
H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-OH
EDT = ethanedithiol
ES-MS = electrospray mass spectrometry
Fmoc = 9-fluorenylmethyloxycarbonyl
cHex = cyclohexyl
HAA = hydroxyacetic acid
HMPA = 4-hydroxymethylphenoxyacetic acid

HObt = 1-hydroxybenzotriazole

HPLC = high performance liquid chromatography

Ma = mandelic acid

NHS = N-hydroxy-succinic acid imido ester

PEG-PS = polyethyleneglycol grafted on polystyrene

Pfp = pentaflourophenyl

SEM = Standard Error of Mean

TFA = trifluoroacetic acid

Z = benzyloxycarbonyl

Apparatus and synthetic strategy

Peptides were synthesized batchwise in a polyethylene vessel equipped with a polypropylene filter for filtration using 9-fluorenylmethyloxycarbonyl (Fmoc) as N-α-amino protecting group and suitable common protection groups for side-chain functionalities (Dryland, A. and Sheppard, R.C. (1986) J. Chem. Soc., Perkin Trans. 1, 125-137).

Solvents

Solvent DMF (*N,N*-dimethylformamide, Riedel de-Häen, Germany) was purified by passing through a column packed with a strong cation exchange resin (Lewatit S 100 MB/H strong acid, Bayer AG Leverkusen, Germany) and analyzed for free amines prior to use by addition of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) giving rise to a yellow colour (Dhbt-O-anion) if free amines are present. Solvent DCM (dichloromethane, analytical grade, Riedel de-Häen, Germany) was used directly without purification.

Amino acids and dipeptides

Fmoc-protected amino acids were purchased from MilliGen (UK) in suitable side-chain protected forms. Otherwise protected amino acids (H-Glu(OtBu)-OtBu; H-Glu(cHex)-OH; Z-Glu(OtBu)-OH) and the dipeptides Fmoc-Phe-Gly-OH and H-Phe-Leu-OH were purchased from Bachem (Switzerland).

Coupling reagents

Coupling reagent diisopropylcarbodiimide (DIC) was purchased from (Riedel de-Häen, Germany) and distilled prior to use, dicyclohexylcarbodiimide (DCC) was purchased from Merck-Schuchardt, München, Germany, and purified by distillation.

Linkers

Linkers (4-hydroxymethylphenoxy)acetic acid (HMPA), Novabiochem, Switzerland; hydroxyacetic acid, (S)-(+)-mandelic acid 99% pure, Aldrich, Germany, and (R)-(-)-mandelic acid 98.5% pure, M&R, England, were coupled to the resin or to the N-terminal of the pre-sequence Z as a preformed 1-hydroxybenzotriazole (HObt) ester generated by means of DIC.

Solid supports

Peptides synthesised according to the Fmoc-strategy were synthesised on two different types of solid support using 0.05 M or higher concentrations of Fmoc-protected activated amino acid in DMF: 1) PEG-PS (polyethyleneglycol grafted on polystyrene; NovaSyn TG resin, 0.29 mmol/g, Novabiochem, Switzerland); 2) NovaSyn K 125 (Kieselguhr supported polydimethylacrylamide resin functionalised with sarcosine methyl ester 0.11 mmol/g; Novabiochem, Switzerland).

Catalysts and other reagents

Diisopropylethylamine (DIEA) was purchased from Aldrich, Germany, and ethylenediamine from Fluka, piperidine and pyridine from Riedel-de Häen, Frankfurt, Germany. 4-(N,N-dimethylamino)pyridine (DMAP) was purchased from Fluka, Switzerland and used as a catalyst in coupling reactions involving symmetrical anhydrides. Ethanedithiol was purchased from Riedel-de Häen, Frankfurt, Germany. 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) and 1-hydroxybenzotriazole (HObt) were obtained from Fluka, Switzerland. FmocNHS was purchased from Aldrich, Germany.

Enzymes

Carboxypeptidase A (EC 3.4.17.1) type I from Bovine Pancreas, leucine aminopeptidase (EC 3.4.11.1) type III-CP from Porcine Kidney, butyryl cholinesterase (EC 3.1.1.8) from Horse Serum, α-chymotrypsin (EC 4.4.21.1) from Bovine Pancreas, and pepsin A (EC 3.4.23.1) from Porcine Stomarch Mucosa Bovine Pancreas were obtained from Sigma, UK.

Coupling procedures

The first amino acid was coupled as a symmetrical anhydride in DMF generated from the appropriate N-α-protected amino acid and DIC or DCC. The following amino acids were coupled as preformed HObt esters made from appropriate N-α-protected amino acids and HObt by means of DIC in DMF. Acylations were checked by the ninhydrin test performed at 80°C in order to prevent Fmoc deprotection during the test (Larsen, B. D. and Holm, A., Int. J. Peptide Protein Res. **43**, 1994, 1-9).

Deprotection of the N-α-amino protecting group

Deprotection of the Fmoc group was performed by treatment with 20% piperidine in DMF (1x3 and 1x7 min.), followed by wash with DMF until no yellow colour (Dhbt-O-) could be detected after addition of Dhbt-OH to the drained DMF.

Cleavage of peptide from resin with acid

Peptides were cleaved from the resins by treatment with 95% trifluoroacetic acid (TFA, Riedel-de Häen, Frankfurt, Germany)-water v/v or with 95% TFA and 5% ethanedithiol v/v at r.t. for 2 h. The filtered resins were washed with 95% TFA-water and filtrates and washings evaporated under reduced pressure. The residue was washed with ether and freeze dried from acetic acid-water. The crude freeze dried product was analysed by high-performance liquid chromatography (HPLC) and identified by electrospray ionisation mass spectrometry (ESMS).

Preformed HObt-ester

3 eq. N-α-amino protected amino acid or hydroxyacetic acid or (S)-(+)-mandelic acid was dissolved in DMF together with 3 eq. HObt and 3 eq DIC. The solution was left at r.t. for 10 minutes and then added to the resin, which had been washed with a solution of 0.2% Dhbt-OH in DMF prior to the addition of the preactivated amino acid.

Preformed symmetrical anhydride

6 eq. N-α-amino protected amino acid was dissolved in DCM and cooled to 0°C. DCC (3 eq.) was added and the reaction continued for 10 min. The solvent was removed *in vacuo* and the remanence dissolved in DMF. The solution was filtered and immediately added to the resin followed by 0.1 eq. of DMAP.

Estimation of the coupling yield of the first N-α-amino protected amino acid

3-5 mg dry Fmoc-protected peptide-resin was treated with 5 ml 20% piperidine in DMF for 10 min at r.t. and the UV absorption for the dibenzofulvene-piperidine adduct was estimated at 301 nm. The yield was determined using a calculated extension coefficient ε_{301} based on a Fmoc-Ala-OH standard.

Peptide synthesis on PepSyn K resin

Dry PepSyn K (ca 500 mg), was covered by ethylenediamine and left at r.t. over night. The resin was drained and washed with DMF 10 x 15 ml, 5 min each. After draining the resin was washed with 10% DIEA in DMF v/v (2 x 15 ml, 5 min each) and finally washed with DMF until no yellow colour could be detected by addition of Dhbt-OH to the drained DMF. 3 eq. HMPA 3 eq. HObt and 3 eq. DIC was dissolved in 10 ml DMF and left for activation for 10 min, after which the mixture was added to the resin and the coupling continued for 24 h. The resin was drained and washed with DMF (10 x 15 ml, 5 min each), and the acylation was checked by the ninhydrin test. The first amino

acid was coupled as the preformed symmetrical anhydride (see above), and the coupling yields estimated as described above. It was in all cases better than 70%. The synthesis was then continued as "*batchwise* ".

Continued batchwise peptide synthesis on PepSyn K

The resin (ca. 500 mg) with the first amino acid attached was placed in a polyethylene vessel equipped with a polypropylene filter for filtration, and the Fmoc-group deprotected as described above. The remaining amino acids according to the sequence were coupled as preformed Fmoc-protected, if necessary side-chain protected, HObt esters (3 eq.) in DMF (5 ml) prepared as described above. The couplings were continued for 2 h unless otherwise specified. Excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min) All acylations were checked by the ninhydrin test performed at 80°C. After completed synthesis the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (5x5 ml, 1 min each) and finally diethyl ether (5x5 ml, 1 min each) and dried *in vacuo*.

Batchwise peptide synthesis on PEG-PS

NovaSyn TG resin (250 mg, 0.27-0.29 mmol/g) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration. The resin was swelled in DMF (5 ml), and treated with 20% piperidine in DMF to secure the presence of non-protonated amino groups on the resin. The resin was drained and washed with DMF until no yellow colour could be detected after addition of Dhbt-OH to the drained DMF. HMPA (3 eq.) was coupled as a preformed HObt-ester as described above and the coupling was continued for 24 h. The resin was drained and washed with DMF (5 x 5 ml, 5 min each) and the acylation checked by the ninhydrin test. The first amino acid was coupled as a preformed symmetrical anhydride as described above. The coupling yields of the first Fmoc-protected amino acids were estimated as described above. It was in all cases better than 60%. The following amino acids according to the sequence were

coupled as preformed Fmoc-protected, if necessary side-chain protected, HObt esters (3 eq.) as described above. The couplings were continued for 2 h, unless otherwise specified. The resin was drained and washed with DMF (5 x 5 ml, 5 min each) in order to remove excess reagent. All acylations were checked by the ninhydrin test performed at 80°C. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 5 min each), DCM (3x5 ml, 1 min each) and finally diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

HPLC conditions

Isocratic HPLC analysis was preformed on a Shimadzu system consisting of an LC-6A pump, an MERCK HITACHI L-4000 UV detector operated at 215 nm and a Rheodyne 7125 injection valve with a 2, 20, or 100 µl loop. The column used for isocratic analysis was a Spherisorb ODS-2 (100 x 3 mm; 5-µm particles). HPLC analysis using gradients was performed on a MERCK-HITACHI L-6200 Intelligent pump, an MERCK HITACHI L-4000 UV detector operated at 215 nm and a Rheodyne 7125 injection valve with a 20 µl loop. The column used was a Rescorce™ RPC 1 ml.

Buffer A was 0.1 vol % TFA in water and buffer B 90 vol% acetonitrile, 9.9 vol% water and 0.1 vol% TFA. The Buffers were pumped through the column at a flow rate of 1.3-1.5 ml/min using the following gradient for peptide analysis 1. Linear gradient from 0% - 100% B (30 min), for enzymatic studies 2. Linear gradient from 40 - 100% B (15 min), 3. Linear gradient from 10 - 40% B (15 min), or 4. Linear gradient from 0 - 50% B (15 min). The mobile phase used for isocratic analysis will be mentioned under the description of the individual experiments.

Mass spectroscopy

Mass spectra were obtained on a Finnigan Mat LCQ instrument equipped with an electrospray (ESI) probe (ES-MS).

Peptide synthesis of individual peptides

1. Peptide synthesis of H-Tyr-Gly-Gly-Phe-Leu-Glu_6-OH on NovaSyn TentaGel

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the pre-sequence Glu_6. The following amino acids forming the Leu-enkephalin sequence were coupled as preformed Fmoc-protected, if necessary side-chain protected, HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before each of the last five couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceeded. When the yellow colour was no longer visible the couplings were interrupted by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 90% and the identity of the peptide was confirmed by ES-MS. Yield 76%.

2. Synthesis of H-Tyr-Gly-Gly-Phe-Leu-HAA-Glu_6-OH on a PepSyn K resin

Dry PepSyn K (ca 500 mg, 0.1 mmol/g) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated with ethylenediamine as earlier described. The first 6 glutamic acid units forming the pre-sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with

the addition of Dhbt-OH (1 eq.). The acylations were checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was deprotected as described above. After finishing the pre-sequence the deprotected peptide-resin was reacted with 6 eq. hydroxyacetic acid as a preactivated HObt-ester as described above and the coupling was continued for 24 h. Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min). The acylation was checked by the ninhydrin test. The next amino acid according to the sequence (leucine) was coupled as preformed symmetrical anhydride as described above and the reaction was continued for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above, and found to be 90%. The synthesis was then continued by cleavage of the Fmoc group as described above.

The next coupling according to the sequence was in order to prevent diketopiperazine formation performed as a dipeptide coupling. Thus Fmoc-Gly-Phe-OH was coupled as a preformed HObt ester (3 eq.) in DMF (5 ml) prepared as described above for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min) and the acylation was checked by the ninhydrin test performed 80°C as described above. The Fmoc group was then removed by treatment with 20% piperidine in DMF as described above. The remaining amino acids according to the sequence were coupled as preformed Fmoc-protected, if necessary side-chain protected, HObt esters (3 eq.) with the addition of 1 eq Dhbt-OH in DMF (2 ml) for 2 h. The acylation was checked by the ninhydrin test performed as described above. The remaining amino acids according to the sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml). Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min) and acylations were checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was deprotected as described above. After completed

synthesis the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from ammonium hydrogencarbonate (0.1 M). The crude freeze dried product was analysed by HPLC and found to be homogeneous, the purity was found to be better than 80%, the identity of the peptide was confirmed by ESMS, and the yield 58%.

3. Synthesis of H-Tyr-Gly-Gly-Phe-Leu-((S)-(+)-Ma)-Glu_6-OH on a PepSyn K resin.

Dry PepSyn K (ca 500 mg, 0.1 mmol/g) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated with ethylenediamine as earlier described. The first 6 glutamic acids forming the pre-sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The acylations were checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was deprotected as described above. After finishing the pre-sequence the deprotected peptide-resin was reacted with 6 eq. (S)-(+)-mandelic acid as a preactivated HObt-ester as described above and the coupling was continued for 24 h. Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min). The acylation was checked by the ninhydrin test. The next amino acid according to the sequence (leucine) was coupled as preformed symmetrical anhydride as described above and the reaction was continued for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above, and found to be 85%. The synthesis was then continued by cleavage of the Fmoc group as described above.

The next coupling according to the sequence was in order to prevent diketopiperazine formation performed as a dipeptide coupling. Thus Fmoc-Gly-Phe-OH was coupled as a preformed HObt ester (3 eq.) in DMF (5 ml) prepared as described above for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min) and the acylation was checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was then removed by treatment with 20% piperidine in DMF as described above. The remaining amino acids according to the sequence were coupled preformed Fmoc-protected HObt esters (3 eq.) with the addition of 1 eq Dhbt-OH in DMF (2 ml) for 2 h. The acylation was checked by the ninhydrin test performed as described above. The remaining amino acids according to the sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml). Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min) and acylations were checked by the ninhydrin test performed 80°C as described above. The Fmoc group was deprotected as described above. After completed synthesis the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from ammonium hydrogencarbonate (0.1 M). The crude freeze dried product was analysed by HPLC and found to be homogeneous, the purity was found to be better than 80%, the identity of the peptide was confirmed by ESMS, and the yield 57%.

The prodrug H-Tyr-Gly-Gly-Phe-Leu-((R)-(-)-Ma)-Glu_6-OH was prepared as described above for 3.

<u>4. Synthesis of H-Tyr-Gly-Gly-Phe-Leu-((S)-(+)-Ma)-Lys_6-OH on a PepSyn K resin.</u>

Dry PepSyn K (ca 500 mg, 0.1 mmol/g) was placed in a polyethylene vessel equipped with a polypropylene filter for

filtration and treated with ethylenediamine as earlier described. The first 6 lysines forming the pre-sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The acylations were checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was deprotected as described above. After finishing the pre-sequence the deprotected peptide-resin was reacted with 6 eq. (S)-(+)-mandelic acid as a preactivated HObt-ester as described above and the coupling was continued for 24 h. Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min). The acylation was checked by the ninhydrin test. The next amino acid according to the sequence (leucine) was coupled as preformed symmetrical anhydride as described above and the reaction was continued for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above, and found to be 85%. The synthesis was then continued by cleavage of the Fmoc group as described above.

The next coupling according to the sequence was in order to prevent diketopiperazine formation performed as a dipeptide coupling. Thus Fmoc-Gly-Phe-OH was coupled as a preformed HObt ester (3 eq.) in DMF (5 ml) prepared as described above for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min) and the acylation was checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was then removed by treatment with 20% piperidine in DMF as described above. The remaining amino acids according to the sequence were coupled preformed Fmoc-protected HObt esters (3 eq.) with the addition of 1 eq Dhbt-OH in DMF (2 ml) for 2 h. The acylation was checked by the ninhydrin test performed as described above. The remaining amino acids according to the sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml). Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min) and

acylations were checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was deprotected as described above. After completed synthesis the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from ammonium hydrogencarbonate (0.1 M). The crude freeze dried product was analysed by HPLC and found to be homogeneous, the purity was found to be better than 80%, the identity of the peptide was confirmed by ESMS, and the yield 57%.

The prodrug H-Tyr-Gly-Gly-Phe-Leu-((R)-(-)-Ma)-Lys_6-OH was prepared as described above for 4.

5. Synthesis of H-Tyr-Gly-Gly-Phe-Leu-((S)-(+)-Ma)-$(LysGlu)_3$-OH on a PepSyn K resin.

Dry PepSyn K (ca 500 mg, 0.1 mmol/g) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated with ethylenediamine as earlier described. The first 6 amino acids forming the pre-sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The acylations were checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was deprotected as described above. After finishing the pre-sequence the deprotected peptide-resin was reacted with 6 eq. (S)-(+)-mandelic acid as a preactivated HObt-ester as described above and the coupling was continued for 24 h. Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min). The acylation was checked by the ninhydrin test. The next amino acid according to the sequence (leucine) was coupled as preformed symmetrical anhydride as described above and the reaction was continued for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which

was estimated as described above, and found to be 85%. The synthesis was then continued by cleavage of the Fmoc group as described above.

The next coupling according to the sequence was in order to prevent diketopiperazine formation performed as a dipeptide coupling. Thus Fmoc-Gly-Phe-OH was coupled as a preformed HObt ester (3 eq.) in DMF (5 ml) prepared as described above for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min) and the acylation was checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was then removed by treatment with 20% piperidine in DMF as described above. The remaining amino acids according to the sequence were coupled preformed Fmoc-protected HObt esters (3 eq.) with the addition of 1 eq Dhbt-OH in DMF (2 ml) for 2 h. The acylation was checked by the ninhydrin test performed as described above. The remaining amino acids according to the sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml). Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min) and acylations were checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was deprotected as described above. After completed synthesis the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from ammonium hydrogencarbonate (0.1 M). The crude freeze dried product was analysed by HPLC and found to be homogeneous, the purity was found to be better than 80%, the identity of the peptide was confirmed by ESMS, and the yield 63%.

The prodrug H-Tyr-Gly-Gly-Phe-Leu-((R)-(-)-Ma)-$(LysGlu)_3$-OH was prepared as described above for 5.

6. Peptide synthesis of Fmoc-Phe-Leu-HAA-Glu_6-OH on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the pre-sequence Glu_6. The peptide-resin was then reacted with 6 eq. hydroxyacetic acid as a preactivated HObt-ester as described above and the coupling was continued for 24 h. Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min). The acylation was checked by the ninhydrin test. The next amino acid according to the sequence (leucine) was coupled as preformed symmetrical anhydride as described above and the reaction was continued for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above, and found to be ~100%. The following amino acid according to the sequence was coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before the last two couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceeded. When the yellow colour was no longer visible the couplings were interrupted by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above freeze dried from ammonium hydrogencarbonate (0.1 M). The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 90% and the identity of the peptide was confirmed by ES-MS. Yield 83%.

7. Peptide synthesis of Fmoc-Phe-Leu-((S)-(+)-Ma)-Glu_6-OH on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the pre-sequence Glu_6. The peptide-resin was then reacted with 6 eq. (S)-(+)-mandelic acid as a preactivated HObt-ester as described above and the coupling was continued for 24 h. Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min). The acylation was checked by the ninhydrin test. The next amino acid according to the sequence (leucine) was coupled as preformed symmetrical anhydride as described above and the reaction was continued for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above, and found to be 90%. The following amino acid according to the sequence was coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before the last two couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceeded. When the yellow colour was no longer visible the couplings were interrupted by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above freeze dried from ammonium hydrogencarbonate (0.1 M). The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 90% and the identity of the peptide was confirmed by ES-MS. Yield 71%.

8. Peptide synthesis of H-Tyr-Gly-Gly-Phe-Leu-Lys_6-OH on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the pre-sequence Lys_6. The following amino acids forming the Leu-enkephalin sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before each of the last five couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceed. When the yellow colour was no longer visible the couplings were interrupted by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80oC as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide was confirmed by ES-MS. Yield 84%.

9. Peptide synthesis of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-Glu_6-OH on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the pre-sequence Glu_6. The following amino acids forming the DSIP sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml)

generated by means of DIC. Before each of the last nine couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceeds. When the yellow colour was no longer visible the couplings were interrupt by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each), and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide was confirmed by ES-MS. Yield 80%.

10. Peptide synthesis of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(LysGlu)$_3$-OH on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the pre-sequence (LysGlu)$_3$. The following amino acids forming the DSIP sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before each of the last nine couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceeds. When the yellow colour was no longer visible the couplings were interrupt by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed

synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each), and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide was confirmed by ES-MS. Yield 91%.

11. Peptide synthesis of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-OH (DSIP) on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" The first amino acid was coupled as a preformed symmetrical anhydride as described above. The coupling yields of the first Fmoc-protected amino acids were estimated as described above. It was in all cases better than 60%. The following amino acids forming the DSIP sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before each of the last eight couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceeds. When the yellow colour was no longer visible the couplings were interrupt by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each), and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from acetic acid. The crude freeze dried product

was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide was confirmed by ES-MS. Yield 78%.

General synthesis

12. Synthesis of Fmoc-Phe-Leu-OH:

1.0 g (3.6 mmol) H-Phe-Leu-OH was dissolved in 25 ml 10% sodium carbonate w/v and 25 ml dioxane was added. To this mixture 1.24 g (3.68 mmol) FmocNHS dissolved in 10 ml dioxane was added drop wise. The resulting mixture was stirred over night at room temperature. The dioxane was removed by evaporation *in vacuo* and the resulting basic aqueous solution was extracted 3 times with ether (5 ml each). The pH was adjusted to 2 by adding HCl (1 M) and the aqueous phase was extracted with ethyl acetate 3 times (20 ml each). The combined ethyl acetate phase was washed with water 3 times (10 ml each), and evaporated to dryness. The remanence was crystallised by adding petroleum ether. Yield 1.45 g (86%). Mp. 153-158; MS calculated 500.59 found MH+: 501.3.

13. Synthesis of Z-Glu(OtBu)-Glu(OtBu)-OtBu:

1.14 g (3.38 mmol) Z-Glu(OtBu)-OH, 0.457 g HOBt (3.38 mmol) and 520 µl DIC (3.38 mmol) was dissolved in 10 ml THF and preactivated for 10 min, and added to a solution of 1.0 g (3.38 mmol) H-Glu(OtBu)-OtBu, HCl in 10 ml THF. The mixture was stirred over night at room temperature. The solvent was removed by evaporation *in vacuo*, and the residue was dissolved in DCM (10 ml) and extracted with 10% acetic acid water v/v 3 times (10 ml each) and 10% sodium hydrogencarbonate water w/v 3 times (10 ml each) and finally with water 2 times (10 ml each). The organic phase was dried over sodium sulfate filtered and evaporated. The residue a colourless oil was used directly without further purification.

14. Synthesis of Z-Glu-Glu-OH:

The oil from 13 was dissolved in 20 ml 50% TFA-DCM v/v and stirred for 2 h at room temperature. The solution was evaporated to dryness and the remanence was crystallised from petroleum ether. Yield 1.2 g (86.5%). Mp. 174-176°C; MS: calculated 410.4, found MH+ 411.0.

Hydrolysis in enzyme solution test

The decomposition of the peptide prodrug (X-L-Z) and the corresponding peptide (X-OH) is studied at 37°C in a 0.05 M phosphate buffer solution. The buffer solutions contains leucine aminopeptidase (25 u/ml) at pH 7.4, or carboxypeptidase A (25 u/ml) at pH 7.4. The decomposition is initiated by addition of an aliquot (~10^{-7}-10^{-8} mol) from a stock solution of the peptide or peptide prodrug, respectively, to the test solution giving a total volume of ~5 ml reaction mixture which is kept in a water-bath at 37°C. At appropriate time intervals samples of 50 µl are withdrawn and analysed by reversed phase HPLC as described above without previous precipitation of proteins. Pseudo-first-order rate constants for the degradation of the peptide prodrog (X-L-Z) and the corresponding peptide (X-OH) are determined from the slopes (i.e. k_{obs}) of the linear plots of the logarithm to the concentration of the residual derivative (HPLC peak heights) against time using the formula $t_{½}=(\ln 2)/(k_{obs})$. The ratio between the half-life of the prodrug and the corresponding peptide is calculated according to the formula: ratio=$(t_{½}(\text{prodrug}))/(t_{½}(\text{X-OH}))$.

Kinetic Measurements

Hydrolysis in buffer solution.

The decomposition of some of the peptide prodrugs were studied in aqueous phosphate or carbonate buffer solutions with a total

buffer concentration of 0.1 - 0.05 M. In order to maintain a constant ionic strength (μ) of 0.5 a calculated amount of potassium chloride was added to the buffer solutions unless otherwise stated. The temperature was kept at 37°C during the degradation studies and pH was adjusted by adding hydrochloric acid (4M) or sodium hydroxide (2M). Hydrolysis experiments were carried out at pH 2, 7.4, and 11.

The rates of decomposition were determined by using reversed phase HPLC. The mobile phase systems used for isocratic separation were 20% acetonitrile 79.9% water 0.1% trifluoroacetic acid or 10% acetonitrile 89.9% water 0.1% trifluoroacetic acid. When using a linear gradient (40 - 100% B in 30 min) buffer A was 0.1% TFA in water v/v and buffer B was 90% acetonitrile 9.9% water 0.1% TFA v/v.

Hydrolysis in enzyme solution.

The decomposition of the peptides and the peptide prodrugs were studied at 37°C in a 0.05 M phosphate buffer solution containing leucine aminopeptidase (25 u/ml) at pH 7.4, carboxypeptidase A (25 u/ml) at pH 7.4, α-chymotrypsin (25 u/ml) at pH 7.4, pepsin A (25 u/ml) at pH 2.0, or butyryl cholinesterase (at two concentrations: 25 and 50 u/ml) at pH 7.4. The decomposition was initiated by adding an aliquot ($\sim 10^{-7}$-10^{-8} mol) from a stock solution of the peptide or peptide prodrug to the test solution giving a total volume of ~5 ml reaction mixture which was kept in a water-bath at 37°C and at appropriate intervals samples of ~50 µl were withdrawn and analysed by reversed phase HPLC as described above without previous precipitation of proteins. Pseudo-first-order rate constants for the degradations were determined from the slopes (i.e. k_{obs}) of the linear plots of the logarithm to the concentration of the residual derivative (HPLC peak heights) against time using the formula $t_{½}=(\ln 2)/(k_{obs})$. The individual assay conditions are given in the below examples.

Hydrolysis in plasma solution

The decomposition of the peptides and the peptide prodrugs were studied at 37°C in 80% human plasma. The decomposition was initiated by adding an aliquot (~10^{-7}-10^{-8} mol) from a stock solution of the peptide to the test solution giving a total volume of ~5 ml reaction mixture which was kept in a water-bath at 37°C and at appropriate intervals samples of ~50 µl were withdrawn and the samples were treated with 50 µl of 2% (w/v) solution of zinc sulphate in methanol-water (1:1 v/v) to deproteinize the samples and stop the reactions. After immediate centrifugation for 3 min. at 13000 rpm., 20 µl of the clear supernatant was analysed by reverse phase HPLC as described previously. Pseudo-first-order rate constants for the degradations were determined from the slopes (i.e. k_{obs}) of the linear plots of the logarithm to the concentration of the residual derivative (HPLC peak heights) against time using the formula $t_{½}=(\ln 2)/(k_{obs})$. The individual assay conditions are given in the below examples.

H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH:

Hydrolysis in buffer solution

The degradation of H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH (~5 x 10^{-6} M) was studied in different aqueous buffers (0.1 M) as described above. The decomposition was followed at pH = 2, pH = 7.4 and pH = 11. The peptide was found stable at the above mentioned pH values, thus only ~5% of the peptide was degraded over a period of 24 h.

Hydrolysis in leucine aminopeptidase

The degradation of H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing leucine aminopeptidase (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as earlier

described and was found to be 5.2×10^{-3} min^{-1}. The half-life was calculated to 133 min.

Hydrolysis in carboxypeptidase A

The degradation of H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH ($\sim 10^{-5}$ M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing carboxypeptidase A (25 u/ml) was studied as described above. The peptide was characterised as stable. Approximately 15% of the peptide was degraded over a period of 24 h.

H-Tyr-Gly-Gly-Phe-Leu-$(Lys)_6$-OH

Hydrolysis in leucine aminopetidase

The degradation of H-Tyr-Gly-Gly-Phe-Leu-$(Lys)_6$-OH ($\sim 10^{-5}$ M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing leucine aminopeptidase (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as earlier described and was found to be 3.6×10^{-3} min^{-1}. The half-life was calculated to 191 min.

Hydrolysis in pepsin A

The degradation of H-Tyr-Gly-Gly-Phe-Leu-$(Lys)_6$-OH ($\sim 10^{-5}$ M) in 0.05 M phosphate buffer solutions (pH = 2.0) containing pepsin A (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as earlier described and was found to be 1.2×10^{-3} min^{-1}. The half-life was calculated to 580 min.

Fmoc-Phe-Leu-((S)-(+)-mandelic acid)-$(Glu)_6$-OH:

Hydrolysis in butyryl cholinesterase

The hydrolysis of the ester linkage generated via mandelic acid in the peptide prodrug Fmoc-Phe-Leu-((S)-(+)-mandelic acid)-$(Glu)_6$-OH ($\sim 10^{-5}$ M) was studied in 0.05 M phosphate buffer solutions (pH = 7.4) containing butyryl cholinesterase (50 u/ml) as described above. The half-life was estimated to $t_{1/2}$ =

212 min as described under general procedures. Based on the recovery of Fmoc-Phe-Leu-OH the half-life was estimated to $t_{1/2}$ = 164 min. The difference in the values is probably due to further degradation of Fmoc-Phe-Leu-OH at urethane the bond in the Fmoc protecting group.

Fmoc-Phe-Leu-(hydroxyacetic acid)-$(Glu)_6$-OH:

Hydrolysis in butyryl cholinesterase

The hydrolysis of the ester linkage generated via hydroxyacetic acid in the peptide prodrug Fmoc-Phe-Leu-(hydroxyacetic acid)-$(Glu)_6$-OH (~10^{-5} M) was studied in 0.05 M phosphate buffer solutions (pH = 7.4) containing butyryl cholinesterase (50 u/ml) as described above. Based on the recovery of Fmoc-Phe-Leu-OH the half-life was estimated to $t_{1/2}$ = 144 min.

Fmoc-Phe-Leu-OH:

Hydrolysis in Butyryl cholinesterase

The degradation of Fmoc-Phe-Leu-OH (~5 x 10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing butyryl cholinesterase (50 u/ml) was studied as described above. The half-life was estimated to $t_{1/2}$ = 25 h.

H-Tyr-Gly-Gly-Phe-Leu-((S)-(+)-mandelic acid)-$(Glu)_6$-OH:

Hydrolysis in buffer solution

The degradation of H-Tyr-Gly-Gly-Phe-Leu-((S)-(+)-mandelic acid)-$(Glu)_6$-OH (1 x 10^{-5} M) was studied in different aqueous buffers (0.1 M) as described above. The decomposition was followed at pH = 2, pH = 7.4 and pH = 11. The peptide was characterised as stable at the above mentioned pH values, thus less than 5 % of the peptide was degraded over a period of 24 h.

Hydrolysis in leucine aminopeptidase

The degradation of H-Tyr-Gly-Gly-Phe-Leu-((S)-(+)-mandelic acid)-$(Glu)_6$-OH (1×10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing leucine aminopeptidase (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as earlier described and the half-life was calculated to 93 min.

Hydrolysis in carboxypeptidase A

The degradation of H-Tyr-Gly-Gly-Phe-Leu-((S)-(+)-mandelic acid)-$(Glu)_6$-OH (1×10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing carboxypeptidase A (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as earlier described and the half-life was calculated to 10.3 min.

Hydrolysis in butyryl cholinesterase

The hydrolysis of the ester linkage generated via mandelic acid in the peptide prodrug H-Tyr-Gly-Gly-Phe-Leu-((S)-(+)-mandelic acid)-$(Glu)_6$-OH (1.75×10^{-5} M) was studied in 0.05 M phosphate buffer solutions (pH = 7.4) containing butyryl cholinesterase (50 u/ml) as described above. The half-life was estimated to $t_{1/2}$ = 50.2 min.

H-Tyr-Gly-Gly-Phe-Leu-(Hydroxyacetic acid)-$(Glu)_6$-OH:

Hydrolysis in buffer solution

The decomposition of H-Tyr-Gly-Gly-Phe-Leu-(hydroxyacetic acid)-$(Glu)_6$-OH (1×10^{-5} M) was studied in different aqueous buffers (0.1 M) as described above. The decomposition was followed at pH = 2, pH = 7.4 and pH = 11. The peptide was characterised as stable at the above mentioned pH values, thus less than 5% of the peptide was degraded over a period of 24 h.

Hydrolysis in leucine aminopeptidase

The degradation of H-Tyr-Gly-Gly-Phe-Leu-(Hydroxyacetic acid)-$(Glu)_6$-OH (1×10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing leucine aminopeptidase (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as earlier described and the half-life was calculated to 16.1 min.

Hydrolysis in carboxypeptidase A

The degradation of H-Tyr-Gly-Gly-Phe-Leu-(Hydroxyacetic acid)-$(Glu)_6$-OH (1×10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing carboxypeptidase A 25 (u/ml) was studied as described above. The peptide was characterised as stable, thus less than 13% of the peptide was degraded over a period of 24 h.

Hydrolysis in butyryl cholinesterase

The hydrolysis of the ester linkage generated via hydroxyacetic acid in the peptide prodrug H-Tyr-Gly-Gly-Phe-Leu-(Hydroxyacetic acid)-$(Glu)_6$-OH (1.75×10^{-5} M) was studied in 0.05 M phosphate buffer solutions (pH = 7.4) containing butyryl cholinesterase (25 u/ml or 50 u/ml) as described above. The half-life was estimated using 25 u butyryl cholinesterase/ml to $t_{1/2}$ = 119.3 min and $t_{1/2}$ = 86.6 min when 50 u/ml was used.

Hydrolysis in human plasma

The decomposition of the peptide prodrug H-Tyr-Gly-Gly-Phe-Leu-(Hydroxyacetic acid)-$(Glu)_6$-OH was studied by adding 1 ml 0.05 M phosphate buffer solutions (pH = 7.4) of the peptide (7×10^{-5} M) at 37°C to 4 ml human plasma and treated as described above. The pseudo-first-order rate constant was determined as earlier described and the half-life was calculated to $t_{1/2}$ =19.2 min.

Z-Glu-Glu-OH:

Hydrolysis in carboxypeptidase A

The degradation of Z-Glu-Glu-OH (1×10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing carboxypeptidase A (25 u/ml) was studied as described above. The peptide was characterised as stable over a period of 24 h.

H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Lys-Glu)$_3$-OH

Hydrolysis in carboxypeptidase A

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Lys-Glu)$_3$-OH ($\sim 10^{-5}$ M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing carboxypeptidase A 25 (u/ml) was studied as described above. The pseudo-first-order rate constant was determined as earlier described and the half-life was calculated to 396 min.

Hydrolysis in leucine aminopeptidase

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Lys-Glu)$_3$-OH ($\sim 10^{-5}$ M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing leucine aminopeptidase (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as earlier described and the half-life was calculated to 145 min.

Hydrolysis in α-chymotrypsin

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Lys-Glu)$_3$-OH ($\sim 10^{-5}$ M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing α-chymotrypsin (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as earlier described and the half-life was calculated to 613 min.

Hydrolysis in pepsin A

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Lys-Glu)$_3$-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 2.0) containing pepsin A (25 u/ml) was studied as described above. The peptide was characterised as stable.

H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Glu)$_6$-OH

Hydrolysis in α-chymotrypsin

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Glu)$_6$-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing α-chymotrypsin (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as earlier described and the half-life was calculated to 523 min.

H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-OH

Hydrolysis in leucine aminopeptidase

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing leucine aminopeptidase (25 u/ml) was studied as described above. The half-life was calculated to be less than 20 min.

***In Vitro* Studies**

μ-opioid receptor activity - 1

The affinity of the prodrugs of the invention for the μ-opioid receptor in calf brain was determined as described by Kristensen et al. (1994) [K. Kristensen, C.B. Christensen, L.L. Christrup, and L.C. Nielsen (1994). The mu_1, mu_2, delta, kappa opioid receptor binding profiles of methadone stereoisomers and morphine. *Life Sci.* **56**, PL45-PL50.]. The activity of the

prodrugs was determined in freshly made solutions and in solutions stored for 20 h at room temperature.
The experimental data are summarised in Table 1.

Table 1. Inhibition of 3H-DAMGO (2 nM)

Compound	IC_{50} ± SEM (nM)	
	2 hours	20 hours
Leu-enkephalin-((R)-(-)-Ma)-$(Lys)_6$-OH	100 ± 11	200 ± 61
Leu-enkephalin-((S)-(+)-Ma)-$(Lys)_6$-OH	150 ± 73	110 ± 9
Leu-enkephalin-((R)-(-)-Ma)-$(Glu)_6$-OH	3500 ± 3200	220 ± 42
Leu-enkephalin-((S)-(+)-Ma)-$(Glu)_6$-OH	1200 ± 910	190 ± 55
Leu-enkephalin-(R(-)-Ma)-$(LysGlu)_3$-OH	1200 ± 920	170 ± 63
Leu-enkephalin-(S(+)-Ma)-$(LysGlu)_3$-OH	450 ± 210	230 ± 73
Leu-enkephalin-OH	97 ± 9	56 ± 14
Met-enkephalin-OH	30	30
Naloxone	9 ± 1	4 ± 2
DAMGO	7 ± 1	8 ± 3

μ-opioid receptor activity - 2

The affinity of the prodrugs of the invention as μ-opioid receptor agonists was determined using the isolated mouse vas deferens *in vitro* model described by Kramer et al. (1997) [T.H. Kramer, H. Bartosz-Bechowski, P. Davis, V.J. Hruby, and F. Porreca (1997). Extraordinary potency of a novel delta opioid receptor agonist is due in part to increased efficacy. *Life Sci.* **61:2**, 129-135.]. The activity of the prodrugs was determined in freshly made solutions and in solutions stored for 48 h at room temperature. The experimental data are summarised in Table 2 and 3.

Table 2. Vas Deferens activity

Peptide/prodrug	Fresh	48 h
Leu-enkephalin-((R)-(-)-Ma)-$(Lys)_6$-OH	a	aa
Leu-enkephalin-((S)-(+)-Ma)-$(Lys)_6$-OH	aa	aa
Leu-enkephalin-((R)-(-)-Ma)-$(Glu)_6$-OH	aaa	aaa
Leu-enkephalin-((S)-(+)-Ma)-$(Glu)_6$-OH	aaa	aaa
Leu-enkephalin-((R)-(-)-Ma)-$(LysGlu)_3$-OH	aa	aa
Leu-enkephalin-((S)-(+)-Ma)-$(LysGlu)_3$-OH	aa	aa
Leu-enkephalin-OH	aaa	aaa

% reduction at 100 nM: a: <25%; aa: <50%; aaa: <75%

Table 3. Vas Deferens activity

Peptide/prodrug	IC_{50} ± SEM (nM)
Leu-enkephalin-((R)-(-)-Ma)-$(Lys)_6$-OH	590 ± 200
Leu-enkephalin-((S)-(+)-Ma)-$(Lys)_6$-OH	240 ± 130
Leu-enkephalin-((R)-(-)-Ma)-$(Glu)_6$-OH	110 ± 23
Leu-enkephalin-((S)-(+)-Ma)-$(Glu)_6$-OH	58 ± 11
Leu-enkephalin-((R)-(-)-Ma)-$(LysGlu)_3$-OH	140 ± 11
Leu-enkephalin-((S)-(+)-Ma)-$(LysGlu)_3$-OH	200 ± 26
Leu-enkephalin-OH	41 ± 13

***In Vivo* Studies**

Analgesic activity

Attempts to determine the *in vivo* activity in mouse were carried out using the grid-shock model described by Swedberg (1994) [M.D. Swedberg (1994). The mouse grid-shock analgesia test: pharmacological characterization of latency to vocalization threshold as an index of antinociception. *J. Pharmacol. Exp. Ther.* **269:3**, 1021-1028.]. The experimental results are summerised in Table 4.

Table 4. Mouse analgesic activity

Prodrug	i.p.	i.v.
Leu-enkephalin-((R)-(-)-Ma)-$(Lys)_6$-OH	NA	NT
Leu-enkephalin-((S)-(+)-Ma)-$(Lys)_6$-OH	WA	WA
Leu-enkephalin-((R)-(-)-Ma)-$(Glu)_6$-OH	NT	NT
Leu-enkephalin-((S)(+)-Ma)-$(Glu)_6$-OH	NT	NT
Leu-enkephalin-((R)-(-)-Ma)-$(LysGlu)_3$-OH	NA	NT
Leu-enkephalin-((S)-(+)-Ma)-$(LysGlu)_3$-OH	NA	NT

NT: Not tested. NA: Not active. WA: Weakly active at 20 mg/kg.

Conclusion

Since native enkephalin degrades with a half-life of 6.0 minutes in 80% human plasma, with a half-life of 10.0 minutes in aminopeptidase (20 u/ml), and with a half-life of 2.0 minutes in carboxypeptidase (1 u/ml) (see G.J. Rasmussen and H.Bundgaard, *Int. J. Pharm.*, **79**, pp 113-122 (1991)), it is concluded that the invention provides a significant protection of a peptide sequence compared with the native peptide sequence. This is further corroborated by the results obtained for DSIP; native DSIP degrades with a half-life of less than 20 minutes in leucine aminopeptidase (25 u/ml), whereas DSIP-$(LysGlu)_3$-OH degrades with a half-life of 145 minutes under identical conditions. In general, the half-lives of pre-sequence-containing DSIP molecules in solutions containing α-chymotrypsin or carboxypeptidase A (25 u/ml) were in the order of several hours. Although native DSIP has not been tested under these conditions it must be expected that the corresponding half-lives are significantly lower than the values obtained for the pre-sequence-containing DSIP molecules as it is well established that native DSIP is rapidly degraded in both plasma and tissue extracts (see H.L. Lee, "Peptide and Protein Drug Delivery", Marcel Dekker Inc. 1991, Chapter 9)

Furthermore, the peptide prodrugs tested were all cleaved by butyryl cholinesterase indicating a readily bioreversibility.

From the *in vitro* tests performed it can be concluded that the pre-sequence influences the biological activity significantly. The results presented in Tables 1, 2 and 3 clearly indicate that the prodrugs of the invention have a reduced affinity towards the μ-opoid receptor compared to native Leu-enkephalin. Thus, in order to exert the desired activity, in this case binding to the μ-opoid receptor, the prodrug must be hydrolysed by e.g. blood plasma enzymes such as butyryl cholinesterase in order to release the native pharmaceutically active peptide.

The difference between the results obtained from freshly prepared solutions and solutions kept at room temperature for 20 or 48 h, may be due to two different factors: The solutions contaning the prodrugs of the invention may be hydrolysed to some extent when stored for 20 or 48 h thereby releasing the native Leu-enkaphalin. However, as shown by the kinetic measurements, the prodrugs of the invention are stable over the entire pH range. The most pronounced effect on standing is observed when applying the pre-sequence $(Glu)_6$. Thus, a more plausible explanation is the rather low water-solubility of the $(Glu)_6$-containing prodrugs: It is very likely that due to slow solution kinetics only a fraction of the prodrug is dissolved in the freshly prepared solutions. However, when left for 20 or 48 h in solution the compounds will slowly dissolve and thereby increasing the available amount of active substance in the assays.

From the *in vivo* analgesia activity studies it can be concluded that the pre-sequence as well as the linker is of importance. Apparently, the positively charged pre-sequence $(Lys)_6$ in combination with the (S) enantiomer of mandelic acid exhibited the desired effect whereas enkephalin containing the pre-

sequence $(Lys)_6$ in combination with the (R) enantiomer of mandelic acid did not show any activity. Furthermore, enkephalin prodrugs with the electroneutral pre-sequence $(LysGlu)_3$ whether in combination with (R) or (S) mandelic acid did not show the desired effect. In conclusion, the combination of linker and pre-sequence is of importance in e.g. the ability of the prodrugs of the invention to cross biological barriers such as the blood-brain-barrier, and the present invention opens up the prospect of transporting prodrugs to the desired region by selecting an appropriate combination of linker and pre-sequence.

CLAIMS

1. A prodrug of a pharmaceutically active peptide (X-OH), peptide amide ($X-NH_2$), or peptide ester (X-OR), wherein the prodrug has the general formula I

$$X - L - Z \qquad I$$

wherein X is bound to L at the C-terminal carbonyl function of X;

L is a linking group, comprising from 3 to 9 backbone atoms, wherein the bond between the C-terminal carbonyl of X and L is different from an C-N amide bond; and

Z is a peptide sequence of 2-20 amino acid units and bound to L at the N-terminal nitrogen atom of Z, each amino acid unit being independently selected from Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, Met, Orn, and amino acid units of the formula II

$$-NH-C(R^3)(R^4)-C(=O)- \qquad II$$

wherein R^3 and R^4 independently are selected from C_{1-6}-alkyl, phenyl, and phenyl-methyl, wherein C_{1-6}-alkyl is optionally substituted with from one to three substituents selected from halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, and phenyl and phenyl-methyl is optionally substituted with from one to three substituents selected from C_{1-6}-alkyl, C_{2-6}-alkenyl, halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, or R^3 and R^4 together with the carbon atom to which they are bound form a cyclopentyl, cyclohexyl, or cycloheptyl ring;

or a salt thereof.

2. A prodrug as claimed in claim 1 wherein the amino acid units in Z are selected from three or from two different amino acids, or are identical amino acid.

3. A prodrug as claimed in claim 1 or 2 wherein the amino acid units in Z independently are selected from Glu, Met, and Lys, in particular from Glu and Lys.

4. A prodrug as claimed in any of the preceding claims wherein the pharmaceutically active peptide (X-OH), peptide amide ($X-NH_2$), or peptide ester (X-R) consists of 2-200 amino acid units.

5. A prodrug as claimed in any of the preceding claims wherein Z consists of 3-15 amino acid units.

6. A prodrug as claimed in any of the preceding claims wherein the bond between the C-terminal carbonyl function of X and L is capable of being cleaved by blood plasma enzymes.

7. A prodrug as claimed in any of the preceding claims wherein the bond between the C-terminal carbonyl function of X and L is a thiolester bond or an ester bond.

8. A prodrug as claimed in any of the preceding claims wherein the bond between L and the N-terminal nitrogen atom in Z is a carboxamide bond (-C(=O)-N-), a sulfonamide bond ($-SO_2-N-$), an alkylamine bond (-C-N-), a carbamate bond (-O-C(=O)-N-), a thiocarbamate bond (-S-C(=O)-N-), an urea bond (-N-C(=O)-N-), a thioamide bond (-C(=S)-N-), a cyanomethyleneamino bond (-C(CN)-N-), or an N-methylamide bond ($-C(=O)-N(CH_3)-$).

9. A prodrug as claimed in any of the preceding claims wherein L is derived from a hydroxy-carboxylic acid.

10. A prodrug according to any of the preceding claims wherein L is derived from an α-hydroxy carboxylic acid.

11. A prodrug as claimed in claim 10 wherein L is derived from an α-hydroxy carboxylic acid of the general formula HO-C(R^1)(R^2)-COOH wherein R^1 and R^2 independently is H, C_{1-6}-alkyl, C_{2-6}-alkenyl, aryl, aryl-C_{1-4}-alkyl, heteroaryl or heteroaryl-C_{1-4}-alkyl, or R^1 and R^2 together with the carbon atom to which they are bound form a cyclopentyl, cyclohexyl, or cycloheptyl ring, where an alkyl or alkenyl group may be substituted with from one to three substituents selected from amino, cyano, halogen, isocyano, isothiocyano, thiocyano, sulfamyl, C_{1-4}-alkylthio, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-alkoxy, aryl, heteroaryl, aryloxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, aminocarbonyl, mono- or di-C_{1-4}-alkyl-aminocarbonyl, mono- or di-C_{1-4}-alkyl-amino, mono- or di-C_{1-4}-alkyl-amino-C_{1-4}-alkyl, C_{1-4}-alkylcarbonylamino, sulfono, and sulfino, and where an aryl or a heteroaryl group may be substituted with from one to three substituents selected from C_{1-4}-alkyl, C_{2-4}-alkenyl, nitro, amino, cyano, halogen, isocyano, isothiocyano, thiocyano, sulfamyl, C_{1-4}-alkylthio, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-alkoxy, aryloxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, aminocarbonyl, mono- or di-C_{1-4}-alkyl-aminocarbonyl, mono- or di-C_{1-4}-alkyl-amino, mono- or di-C_{1-4}-alkyl-amino-C_{1-4}-alkyl, C_{1-4}-alkylcarbonylamino, sulfono, and sulfino.

12. A prodrug as claimed in any of the preceding claims wherein L is derived from hydroxyacetic acid, (S)-(+)-mandelic acid, L-lactic acid ((S)-(+)-2-hydroxypropanoic acid), L-α-hydroxy-butyric acid ((S)-2-hydroxybutanoic acid), and α-hydroxy-isobutyric acid.

13. A prodrug as claimed in claim 11 wherein L is derived from an α-hydroxy carboxylic acid of the general formula HO-C(CH_2-R^5)(R^2)-COOH, wherein R^5 is selected from H, C_{1-5}-alkyl, C_{2-5}-alkenyl, aryl, aryl-C_{1-3}-alkyl, heteroaryl, heteroaryl-C_{1-3}-alkyl, where an alkyl or alkenyl group may be substituted with from one to three substituents selected from amino, halogen, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-alkoxy, aryl, heteroaryl, aryloxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, and aminocarbonyl, and where an aryl or heteroaryl may be substituted with from one to three substituents selected from C_{1-4}-alkyl, C_{2-4}-alkenyl, nitro, amino, halogen, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-alkoxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, and aminocarbonyl; and R^2 is as defined in claim 11.

14. A prodrug as claimed in any of the preceding claims wherein X is the peptide sequence of an enkephalin, angiotensin II, vasopressin, endothelin, neuropeptide Y, vasoactive intestinal peptide, substance P, neurotensin, endorphins, insulin, gramicidin, paracelsin, delta-sleep inducing peptide, ANF, vasotocin, bradykinin, dynorphin, growth hormone release factor, growth hormone release peptide, oxytocin, calcitonin, calcitonin gene-related peptide, calcitonin gene-related peptide II, growth hormone release peptide, tachykinin, ACTH, brain natriuretic polypeptide, cholecystokinin, corticotropin releasing factor, diazepam binding inhibitor fragment, FMRF-amide, galanin, gastric releasing polypeptide, gastrin, gastrin releasing peptide, glucagon, glucagon-like peptide-1, glucagon-like peptide-2, LHRH, melanin concentrating hormone, alpha-MSH, morphine modulating peptides, motilin, neurokinins, neuromedins, neuropeptide K, neuropeptide Y, PACAP, pancreatic polypeptide, peptide YY, PHM, secretin, somatostatin, substance K, substance P, TRH, vasoactive intestinal polypeptide, or any modified or truncated analogue thereof.

15. A prodrug according to any of the proceeding claims, wherein the ratio between the half-life of the prodrug in question in the "Hydrolysis in enzyme solution test", as defined herein, and the half-life of the corresponding peptide (X-OH), in the "Hydrolysis in enzyme solution test", is at least 2, when using the enzyme carboxypeptidase A.

16. A prodrug according to claim 15, wherein the ratio is at least 5.

17. A prodrug according to claim 16, wherein the ratio is at least 10.

18. A prodrug according to any of the proceeding claims, wherein the ratio between the half-life of the prodrug in question in the "Hydrolysis in enzyme solution test", as defined herein, and the half-life of the corresponding peptide (X-OH), in the "Hydrolysis in enzyme solution" test, is at least 2, when using the enzyme leucine aminopeptidase.

19. A prodrug according to claim 18, wherein the ratio is at least 5.

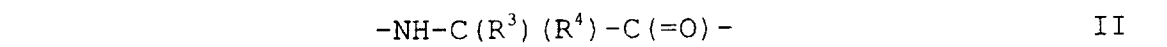
20. A prodrug according to claim 19, wherein the ratio is at least 10.

21. A pharmaceutical composition comprising a prodrug as defined in any of the claims 1-20, and a pharmaceutically acceptable carrier.

22. A prodrug as defined in any of the claims 1-20 for use in therapy.

23. Use of a prodrug as defined in any of the claims 1-20 for the preparation of a pharmaceutically composition for use in therapy.

24. An immobilised linker-peptide sequence Prot-L-Z-SSM, where L designates a linker of the general formula -O-C(R^1)(R^2)-C(=O)- wherein R^1 and R^2 independently is H, C_{1-6}-alkyl, C_{2-6}-alkenyl, aryl, aryl-C_{1-4}-alkyl, heteroaryl or heteroaryl-C_{1-4}-alkyl, or R^1 and R^2 together with the carbon atom to which they are bound form a cyclopentyl, cyclohexyl, or cycloheptyl ring, where an alkyl or alkenyl group may be substituted with from one to three substituents selected from amino, cyano, halogen, isocyano, isothiocyano, thiocyano, sulfamyl, C_{1-4}-alkylthio, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-alkoxy, aryl, heteroaryl, aryloxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, aminocarbonyl, mono- or di-C_{1-4}-alkyl-aminocarbonyl, mono- or di-C_{1-4}-alkyl-amino, mono- or di-C_{1-4}-alkyl-amino-C_{1-4}-alkyl, C_{1-4}-alkylcarbonylamino, sulfono, and sulfino, and where an aryl or a heteroaryl group may be substituted with from one to three substituents selected from C_{1-4}-alkyl, C_{2-4}-alkenyl, nitro, amino, cyano, halogen, isocyano, isothiocyano, thiocyano, sulfamyl, C_{1-4}-alkylthio, mono- or di-C_{1-4}-alkyl-amino, hydroxy, C_{1-4}-alkoxy, aryloxy, carboxy, C_{1-4}-alkoxycarbonyl, C_{1-4}-alkylcarbonyloxy, aminocarbonyl, mono- or di-C_{1-4}-alkyl-aminocarbonyl, mono- or di-C_{1-4}-alkyl-amino, mono- or di-C_{1-4}-alkyl-amino-C_{1-4}-alkyl, C_{1-4}-alkylcarbonylamino, sulfono, and sulfino; Z designates a peptide sequence comprising 2-20 amino acid units, each amino acid unit being independently selected from Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, Met, Orn, and amino acid units of the formula II



$$-NH-C(R^3)(R^4)-C(=O)- \qquad II$$

wherein R^3 and R^4 independently are selected from C_{1-6}-alkyl, phenyl, and phenyl-methyl, wherein C_{1-6}-alkyl is optionally substituted with from one to three substituents selected from halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, and phenyl and phenyl-methyl is optionally substituted with

from one to three substituents selected from C_{1-6}-akyl, C_{2-6}-alkenyl, halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, or R^3 and R^4 together with the carbon atom to which they are bound form a cyclopentyl, cyclohexyl, or cycloheptyl ring; SSM designates a solid support material; and Prot designates H or a hydroxy protecting group.

25. An immobilised linker-peptide sequence Prot-L-Z-SSM according to claim 24, wherein the solid support material (SSM) is selected from polystyrene, polyacrylamide, polydimethylacrylamide, polyethyleneglycol, cellulose, polyethylene, polyethyleneglycol grafted on polystyrene, latex, and dynabeads

26. The use of an immobilised linker-peptide sequence Prot-L-Z-SSM for the preparation of a prodrug of a peptide, a peptide amide, or a peptide ester.

27. A method for the preparation of a prodrug of a peptide (X-OH), a peptide amide ($X-NH_2$), or a peptide ester (X-OR) comprising coupling the corresponding peptide in a C-terminal activated form (X-Act) to an immobilised linker-peptide sequence H-L-Z-SSM.

28. A method for the preparation of a prodrug of a peptide (X-OH), a peptide amide ($X-NH_2$), or a peptide ester (X-OR) comprising the steps of:

a) coupling an N-α-protected amino acid in the carboxyl activated form, or an N-α-protected dipeptide in the C-terminal activated form to an immobilised linker peptide sequence H-L-Z-SSM, thereby forming an immobilised N-α-protected peptide fragment,

b) removing the N-α-protecting group, thereby forming an immobilised peptide fragment having an unprotected N-terminal end,

c) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the unprotected N-terminal end of the immobilised peptide fragment, and

repeating the removal/coupling procedure in step b) and c) until the desired peptide sequence X is obtained, and then

d) cleaving off the prodrug X-L-Z from the solid support material to obtain the free prodrug in the form of a C-terminal carboxylic acid, amide, or ester.

29. A method according to claim 28, wherein the N-α-protecting group is selected from tert.butyloxycarbonyl and 9-fluorenylmethyloxycarbonyl.

30. A compound of the general formula I

X - L - Z I

wherein X is a peptide sequence which is bound to L at the C-terminal carbonyl function of X;

L is a linking group, comprising from 3 to 9 backbone atoms, wherein the bond between the C-terminal carbonyl of X and L is different from an C-N amide bond; and

Z is a peptide sequence of 2-20 amino acid units and bound to L at the N-terminal nitrogen atom of Z, each amino acid unit being independently selected from Ala, Leu, Ser, Thr, Tyr, Asn,

Gln, Asp, Glu, Lys, Arg, His, Met, Orn, and amino acid units of the formula II

$$-NH-C(R^3)(R^4)-C(=O)- \qquad II$$

wherein R^3 and R^4 independently are selected from C_{1-6}-alkyl, phenyl, and phenyl-methyl, wherein C_{1-6}-alkyl is optionally substituted with from one to three substituents selected from halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, and phenyl and phenyl-methyl is optionally substituted with from one to three substituents selected from C_{1-6}-alkyl, C_{2-6}-alkenyl, halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, or R^3 and R^4 together with the carbon atom to which they are bound form a cyclopentyl, cyclohexyl, or cycloheptyl ring;

or a salt thereof.

INTERNATIONAL SEARCH REPORT

Interi nal Application No
PCT/DK 97/00376

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K1/107 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 00846 A (J F TAM) 5 January 1995 see the whole document ---	1-30
A	E J F FRANSSEN ET AL.: "Low molecular weight proteins as carriers for renal drug targeting. Preparation of drug-protein conjugates and drug-spacer derivatives and their catabolism in renal cortex homogenates and lysosomal lysates " JOURNAL OF MEDICINAL CHEMISTRY., vol. 35, no. 7, July 1992, WASHINGTON US, pages 1246-1259, XP002050524 see the whole document --- -/--	1-30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1997	21.01.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/DK 97/00376

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	M MUTTER ET AL.: "Template-assembled syntetic proteins (TASPS) containing two folding domains" PEPTIDES 1988, PROCEEDINGS OF THE 20TH EUROPEAN PEPTIDE SYMPOSIUM, SEPTEMBER 4-9, 1988, UNIVERSITY OF TÜBINGEN, TÜBINGEN, GERMANY, 1989, W DE GRUYTER, BERLIN/NEW YORK, pages 193-195, XP002050525 see the whole document -----	1-30

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00376

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-30
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

The subject matter of claims 1-30 is too broad and vaguely defined. This does not permit a complete search for economical reasons. A limited search has been performed on the claimed subject matter only insofar as further defined by the claims 3 and 12 taken together.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr nal Application No
PCT/DK 97/00376

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9500846 A	05-01-95	US 5589356 A	31-12-96
		AU 7317894 A	17-01-95