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(54) Title
A PROCESS FOR ENZYMATIC PRODUCTION OF DIPEPTIDES

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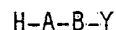
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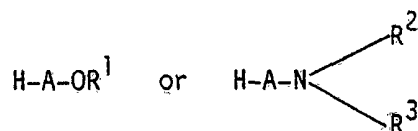
(56) Prior Art Documents
AU 72365/87 C12P 21/02

(57) Claim

1. A process for producing dipeptides having the general formula



wherein A represents an optionally side-chain protected L- or D- α -amino acid residue or ω -amino acid residue and B represents an optionally side-chain protected L- or D- α -aminocarboxylic acid residue which may be the same as or different from A, an L- or D-aminophosphonic acid residue or L- or D-aminosulfonic acid residue or the corresponding ω -amino acids or salts and hydrates thereof, and Y is OH or a C-terminal blocking group, characterized by reacting a substrate component, which is an amino acid derivative having the formula



wherein A is as defined above, R^1 represents hydrogen, alkyl, aryl or aralkyl optionally substituted with inert substituents or an α -des-amino fragment of an amino acid, and R^2 and R^3 are the same or different and each represents hydrogen, alkyl, aryl or aralkyl optionally substituted with inert substituents,

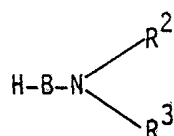
with a nucleophile component which when A = B may be formed in situ and is selected from

(a) amino acids having the formula



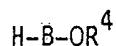
wherein B is an aminocarboxylic acid as defined above

(b) amino acid amides having the formula



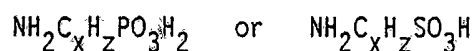
wherein B is an aminocarboxylic acid as defined above, and R^2 and R^3 have the above meaning, except that when R^2 represents hydrogen, R^3 may also represent hydroxy or amino

(c) amino acid esters having the formula



wherein B is an aminocarboxylic acid as defined above, and R^4 represents alkyl, aryl or aralkyl, and

(d) straight chain or branched aminophosphonic acids or aminosulfonic acids having the formula



wherein x is 1-6 and z is 2-12

in the presence of an optionally immobilized serine or thiol carboxypeptidase from yeast or of animal, vegetable or other microbial origin, in an aqueous solution or suspension having a pH value between 5 and 10.5 optionally containing an organic solvent and/or a salt, and then, if desired, cleaving a present side-chain protecting group or protective group Y and/or, if desired, converting the resulting dipeptide derivative to a salt or hydrate.

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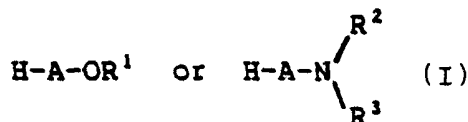
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(21) International Application Number: PCT/DK88/00022 (22) International Filing Date: 15 February 1988 (15.02.88) (31) Priority Application Number: 725/87 (32) Priority Date: 13 February 1987 (13.02.87) (33) Priority Country: DK (71) Applicant (for all designated States except US): CARLSBERG BIOTECHNOLOGY LTD. A/S [DK/DK]; Tagensvej 16, DK-2200 Copenhagen N (DK). (72) Inventors; and (75) Inventors/Applicants (for US only) : THORBEK, Pia [DK/DK]; Sdr. Jagtvej 39, DK-2970 Hørsholm (DK). WIDMER, Fred [CH/CH]; Brønsholmdalsvej 53, DK-2980 Kokkedal (DK). AASMUL-OLSEN, Stig [DK/DK]; Skodsborgvej 410, st.tv., DK-2942 Skodsborg (DK).		(74) Agent: HOFMAN-BANG & BOUTARD A/S; Adelgade 15, DK-1304 Copenhagen K (DK). (81) Designated States: AU, DK, FI, HU, JP, US. Published <i>With international search report.</i> <i>With amended claims and statement.</i> A.O.J.P. 13 OCT 1988 AUSTRALIAN 14 SEP '88 PATENT OFFICE

(54) Title: A PROCESS FOR ENZYMATIC PRODUCTION OF DIPEPTIDES**(57) Abstract**

Dipeptides having the general formula H-A-B-Y, wherein A represents an optionally side-chain protected L- or D- α -amino acid or ω -amino acid and B represents an optionally side-chain protected L- or D- α -amino-carboxylic acid which may be the same as or different from A, and L- or D-aminophosphonic acid or L- or D-aminosulfonic acid or the corresponding ω -amino acids or salts and hydrates thereof, and Y is OH or a C-terminal blocking group, are prepared by reacting a substrate component, which is an amino acid derivative having formula (I), wherein A is as defined above, R^1 represents hydrogen, alkyl, aryl or aralkyl optionally substituted with inert substituents or an α -des-amino fragment of an amino acid, and R^2 and R^3 are the same or different and each represents hydrogen, alkyl, aryl or aralkyl optionally substituted with inert substituents, with a nucleophile component which when A = B may be formed in situ and is selected from (a) L-amino acids having the formula H-B-OH, (b) L-amino acid amides having formula (II), wherein B is an L-amino acid, and R^2 and R^3 have the above meaning, except that when R^2 represents hydrogen, R^3 may also represent hydroxy or amino, (c) L-amino acid esters having the formula H-B-OR⁴, wherein B is an L-amino acid, and R^4 represents alkyl, aryl or aralkyl, and (d) optionally acid group protected straight chain or branched amino phosphonic acids or amino sulfonic acids having the formula $\text{NH}_2\text{C}_x\text{H}_2\text{PO}_3\text{H}_2$ or $\text{NH}_2\text{C}_x\text{H}_2\text{SO}_3\text{H}$, wherein x is 1-6 and z is 2-12 in the presence of a serine or thiol carboxypeptidase from yeast or of animal, vegetable or other microbial origin, preferably CPD-Y from yeast in an aqueous solution or suspension having a pH value between 5 and 10.5 optionally containing an organic solvent and/or a salt, and then, if desired cleaving a present side-chain protecting group or protective group Y and/or, if desired, converting the resulting dipeptide derivative to a salt or hydrate. The process allows for production of L,L-, LD-, DL- and DD-dipeptides without risk of racemization in a simple and economic manner.

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A process for enzymatic production of dipeptides

The present invention concerns a process for enzymatic production of dipeptides and derivatives of dipeptides and of the type stated in the introductory portion of claim 1.

In recent years there has been an increasing interest in dipeptides and dipeptide derivatives optionally containing an amino acid residue of D-configuration, with a view to their potential pharmacological effects, such as e.g. antibiotics. Likewise, there has been a great interest in dipeptides within fields such as artificial nutrition - human as well as veterinary, sweeteners and within agrochemistry, such as e.g. herbicides.

Such dipeptides H-A-B-Y can be produced by means of known chemical coupling reactions, but all these methods share the feature that, generally, it is necessary to protect the amino acids involved - A and B - on the amino group and the carboxylic acid group, respectively, and frequently also on the side chains if these carry functional groups. Further, there is an inherent risk of side reactions during the chemical coupling step because of the reagents and conditions employed, a major side reaction being racemization, particularly of the A-component. By replacing the chemical coupling step with an enzymatic coupling step proceeding under mild conditions, such side reactions and racemization can be avoided, yielding a stereochemically pure product.

The presence of amino- and carboxyl protective groups is mandatory in chemical coupling as well as in enzymatic coupling using endoproteases, and according to prior knowledge also on the amino function of the substrate in enzymatic exoprotease catalyzed formation of dipeptides.

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This adds several undesired features to these processes seriously afflicting their process economy on an industrial scale, particularly apparent in dipeptide synthesis.

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The disadvantages are concerned with the introduction of these groups, as well as their removal and presence during process operation, increasing overall process cost and time and affecting overall yield.

10

Typical examples of amino protective groups commonly used are those of the carbobenzoxy (Z-) and tert-butoxycarbonyl (Boc-) type, which are of a molecular weight comparable to those of the amino acid residues.

15

Firstly, the protective groups will have to be introduced in the starting materials by means of appropriate costly agents in a separate reaction step followed by an isolation step. While present, these hydrophobic groups often have a ^{drastic} ~~drastical~~ effect upon the solubility of the intermediates and reaction products, and may afflict both the nature and the amount of solvents required in their processing as well as ease of purification and of deprotection. The deprotection will also take place in a separate step with a following purification step.

25

For this purpose a series of reactions are available, but with the exception of ^{catalytic} ~~catalytical~~ hydrogenation, posing industrial problems of its own, these methods are occurring under violent often strongly acidic or basic conditions, frequently giving rise to a series of side reactions, resulting in an impure product or demanding laborious purification.

30

The last steps in this relatively long series of synthesis steps may thus be a rather comprehensive deprotection to obtain the desired ^{dipeptides} ~~peptides~~, and, owing

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to the almost inevitable secondary reactions, rather laborious purification procedures are frequently required to provide a product with the desired high purity.

5

Attempts to avoid amino terminal protection in the production of dipeptides have led to microbial fermentation approaches, like the fermentation process under formation of aspartame described in EP-A1-074095 and EP-A2-102529. This technique is fundamentally different from synthetic approaches and relies on specific organisms for each peptide, and is thus not generally applicable. In addition, yields are often low and recovery from the fermentation broth laborious.

15

Thus, it is an obvious advantage in terms of overall process economy to be able to avoid protective groups; also on the amino and carboxy terminus. It is the object of the present invention to make this possible in the synthesis of dipeptides mediated by serine- and thiol carboxypeptidase catalysis. In some cases, it may be of interest to be able to produce a dipeptide which may carry side-chain protection, but no terminal protection, and it will be shown that it is possible in the process according to this invention, starting from side-chain protected, but amino respectively carboxy unprotected starting materials. In this case, the same advantages of mild reaction conditions and overall process economy may be obtained. If desired, the side-chain protective group may be cleaved by chemical or enzymatic means.

20

The enzyme catalyzed coupling reactions enabling the use of side-chain unprotected amino acid derivatives and an optionally C-terminal unprotected B-component (nucleophile) are known. See e.g. the DK Patent Specification No. ¹⁵⁵⁶¹³ ~~(DK Application No. 5202/80)~~ as well as the corresponding EP Patent Specification No. 17 485

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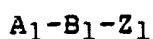
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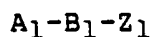
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(EP-B1-17485).

5 EP-B1-17485 describes a process for producing peptides
of the general formula

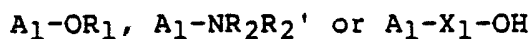


10 wherein A_1 represents an N-terminal protected ^{L-}amino acid
residue or an optionally N-terminal protected peptide
residue having a C-terminal L-amino acid residue, and B_1
represents an L-amino acid residue, and Z_1 is OH or a C-
15 terminal protective group, by reaction of a substrate
component with an amine component in the presence of an
enzyme, and, if desired, cleavage of optional terminal
protective groups to provide a peptide of the formula



20 which is characterized by reacting a substrate component
selected from

25 amino acid esters, peptide esters, depsipeptides, or
optionally N-substituted amino acid amides or
peptide amides, or optionally N-terminal protected
peptides of the formulae



30 wherein A_1 is as defined above,
 R_1 is alkyl, aryl, heteroaryl, aralkyl or an α -des-
amino fragment of an amino acid residue,
 R_2 and R_2' are each hydrogen, alkyl, aryl, heteroaryl
or aralkyl, and
35 X_1 is an L-amino acid residue,

with an amine component (nucleophile) selected from



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optionally N-substituted L-amino acid amides, L-amino acids or L-amino acid esters, of the formula

5
$$\text{H-B}_1\text{-NR}_3\text{R}_3' \text{ or } \text{H-B}_1\text{-OR}_4$$

wherein B_1 is as defined above,

R_3 and R_3' are each hydrogen, hydroxy, amino, alkyl, aryl, heteroaryl or aralkyl, and

10 R_4 is hydrogen, alkyl, aryl, heteroaryl or aralkyl,

in the presence of an L-specific serine or thiol carboxypeptidase enzyme originating from yeast or of animal, vegetable or other microbial origin, in an aqueous solution or dispersion at pH-5-10.5, preferably at a temperature of 20-50°C. The preferred enzyme is carboxypeptidase Y from yeast, called CPD-Y in the following. (It is noted that in order to avoid confusion with the present symbol meaning the above-mentioned symbol meanings are not identical with those used in EP-B-17485).

Thus, if a dipeptide is to be produced by the process of EP-B1-17485, the substrate component is ^{necessarily} obligatory, an N-terminal protected amino acid derivative, and the constituent amino acid is ^{necessarily} obligatory, an L-amino acid.

More generally, it is said that the need for N-terminal amino group protection of the substrate component decreases with increasing chain length of the peptide residue and is substantially absent when the peptide residue consists of 3 amino acids, depending, however, upon their type and sequence.

35 This is illustrated by Breddam et al, "Influence of the substrate structure on carboxypeptidase Y catalyzed peptide bond formation", Carlsberg Res. Commun., vol.



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45, p. 361-67, 30th December 1980, in which Ac-Ala-Ala-Ala-OMe and H-Ala-Ala-Ala-OMe were coupled with H-Leu-NH₂ in the presence of CPD-Y to form Ac-Ala-Ala-Ala-LeuNH₂ and H-Ala-Ala-Ala-LeuNH₂ in yields of 90 and 80%, respectively.

Breddam et al also examined the importance of the amino acid configuration for the coupling yield in CPD-Y catalyzed peptide synthesis, cf. the following table, where Ala represents L-alanine and ala represents D-alanine.

Substrate	Product	Yield (%)
H-Ala-Ala-Ala-OMe	H-Ala-Ala-Ala-Leu-NH ₂	80
H-Ala-ala-Ala-OMe	H-Ala-ala-Ala-Leu-NH ₂	40
H-Ala-Ala-ala-OMe	H-Ala-Ala-ala-Leu-NH ₂	0

Conditions: 25 mM substrate, 0.1 M KCl, 1 mM EDTA, pH 9.5, CPD-Y = 12 μ m, 0.2 M Leu-NH-2.
The reaction was stopped after 20 min.

It appears from the table that if the C-terminal is in D-form, like in H-Ala-Ala-ala-OMe, no peptide synthesis takes place because the ester is not reacted. With the D-amino acid juxtaposed^{to} the C-terminal like in H-Ala-ala-Ala-OMe, reaction takes place, but the coupling yield is reduced to 40% compared with the 80% in the pure L-configuration H-Ala-Ala-Ala-OMe.

Further, it has long been known that some endoproteases can catalyze oligomerization of certain N-unprotected amino acid esters with L-configuration, but it has never been attempted to use this for production of dipeptides which are not simple dimers. Generally, the results of such observations have been a mixture of a series of oligomers, sometimes long, and only in case of product



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precipitation has it been possible to isolate a single product.

5 For this reason the use of endoproteases for peptide synthesis has been limited to the use of amino and carboxy terminal protected starting materials, as exemplified by US-A-4.086.136.

10 The above-mentioned amino and carboxy terminal protected ~~these types of~~ starting materials are also mandatory if aspartate endoproteases are used as exemplified by US-A-3.972.773, and if metallo endoproteases are used, as exemplified by the synthesis of Z-AspPheOMe.PheOMe-salt in EP-A1-009585.

15 Finally, the synthesis of the diastereomeric dipeptides of DL, LD and DD-configuration as well as peptides containing beta-amino acid residues from amino- unprotected starting compounds has so far not been possible with carboxypeptidases nor in general with any
20 proteolytical enzymes (Class EC 3.4). Some efforts have been made with a different class of enzymes, aminoacyl-t-RNA-synthetase (Class EC 6.1) as exemplified by EP-A1-086053. In this case a specific enzyme must be used for each type of amino acid residue, and furthermore,
25 expensive Co-factors like ATP are required. At the same time, yields are very poor, so even though some product was isolated and identified, typically a ten fold excess of Co-factor and a hundred fold excess of nucleophile and up to a thousand fold excess of enzyme by weight was
30 required.

35 It has now surprisingly been found that the serine and thiol carboxypeptidases used in EP-B1-17485 are capable of utilizing N-unprotected amino acid esters as a substrate component in controlled reactions for synthesis of dipeptides and dipeptide derivatives, and that it is possible to suppress a possible oligomerization of the



substrate.

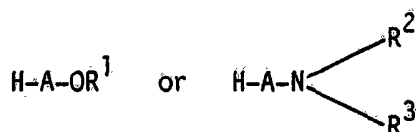
It has moreover surprisingly been found that also N-unprotected amino acid derivatives of D-configuration can be used as substrates in these reactions, so that, in addition to LL-dipeptides, it is also possible to synthesize DL-dipeptides. The reaction rate for D-substrates, however, is generally somewhat lower than for L-substrates under uniform conditions, but, as illustrated below, the difference in rate is much smaller than for the corresponding N-protected amino acid esters, the D-substrate being reacted at a rate which is much smaller than the rate for the L-substrate. The yields are often just as high or higher with the unprotected D-substrates in relation to the unprotected L-substrates as shown by the following examples.

Furthermore, it has even more surprisingly been found that with substrates of D-configuration, nucleophiles of D-configuration can be coupled by means of these enzymes, otherwise known to be L-specific on the amino side of the synthesis point. An amino acid ester incorporated in this manner is not hydrolyzed further.

According to a broad format, this invention provides a process for producing dipeptides having the general formula

20
$$\text{H-A-B-Y}$$

wherein A represents an optionally side-chain protected L- or D- α -amino acid residue or ω -amino acid residue and B represents an optionally side-chain protected L- or D- α -aminocarboxylic acid residue which may be the same as or different from A, an L- or D-aminophosphonic acid residue or L- or D-aminosulfonic acid residue or the corresponding ω -amino acids or salts and hydrates thereof, and Y is OH or a C-terminal blocking group, characterized by reacting a substrate component, which is an amino acid derivative having the formula

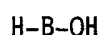


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wherein A is as defined above, R^1 represents hydrogen, alkyl, aryl or aralkyl optionally substituted with inert substituents or an α -des-amino fragment of an amino acid, and R^2 and R^3 are the same or different and each represents hydrogen, alkyl, aryl or aralkyl optionally substituted with inert substituents,

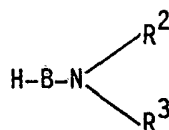
with a nucleophile component which when A = B may be formed in situ and is selected from

(a) amino acids having the formula



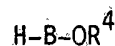
10 wherein B is an aminocarboxylic acid as defined above

(b) amino acid amides having the formula



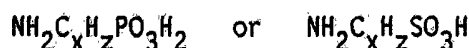
wherein B is an aminocarboxylic acid as defined above, and R^2 and R^3 have the above meaning, except that when R^2 represents hydrogen, R^3 may also represent hydroxy or amino

(c) amino acid esters having the formula



wherein B is an aminocarboxylic acid as defined above, and R^4 represents alkyl, aryl or aralkyl, and

20 (d) straight chain or branched aminophosphonic acids or aminosulfonic acids having the formula

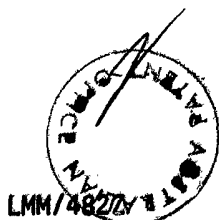


wherein x is 1-6 and z is 2-12



in the presence of an optionally immobilized serine or thiol
carboxypeptidase from yeast or of animal, vegetable or other microbial
origin, in an aqueous solution or suspension having a pH value between 5
and 10.5 optionally containing an organic solvent and/or a salt, and
5 then, if desired, cleaving a present side-chain protecting group or
protective group Y and/or, if desired, converting the resulting dipeptide
derivative to a salt or hydrate.

Examples of useful amino acids include aliphatic amino acids, such as
monoaminomonocarboxylic acids, e.g. glycine (Gly), alanine (Ala), valine
10 (Val), norvaline (Nval), leucine (Leu), isoleucine (iso-Leu) and
norleucine (Nleu), hydroxy amino acids, such as serine (Ser), threonine
(Thr) and homoserine (homo-Ser), sulfur-containing amino acids, such as
methionine (Met) or cystine (CysS) and cysteine (CysH), monoaminodi-
carboxylic acids, such as aspartic acid (Asp),



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glutamic acid (Glu) and amides thereof, such as asparagine (Asn) and glutamine (Gln), diaminomono-carboxylic acids, such as ornithine (Orn) and lysine (Lys), arginine (Arg), aromatic amino acids, such as phenylalanine (Phe) and tyrosine (Tyr), as well as heterocyclic amino acids, such as histidine (His), proline (Pro) and tryptophan (Trp). As examples of useful amino acids of a more unusual structure may be mentioned penicillamine (Pen) aminophosphonic acids, such as alanine-phosphonic acid (AlaP) aminosulfonic acids, such as taurine (Tau), or omega amino acids, such as beta alanine (BAla). As mentioned, they may be included in D-form in the substrate component and they may also be present in D-form in the nucleophile component.

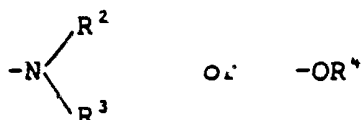
The advantages of the process of the invention over the mentioned known methods are minimum or no side chain protection, no N-protection of the substrate component which may have both D- and L-configuration, no risk of racemization, few synthesis steps and an expected relatively pure end product, which in combination provides an extremely simple and economic method of production.

Preferred substrate components are esters in which R^1 is a straight or branched alkyl having 1 to 6 carbon atoms, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert-butyl, amyl and hexyl, or the aralkyl group benzyl. Particularly expedient nucleophile components are free L-amino acids or amino acid amides, in which R^2 is H, and R^3 is H or C_1 - C_6 alkyl, or amino acid esters in which R^4 is a straight or branched alkyl having 1 to 6 carbon atoms such as the above-mentioned ones. As mentioned, R^1 may be alkyl, aryl or aralkyl optionally substituted with inert substituents, e.g. hydroxy or nitro.

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The invention also comprises the processes involving intermediate formation of a peptide containing the group

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following which this group is cleaved to form a carboxylic acid group. This cleavage may be catalyzed by another or the same enzyme as was used to form the peptide.

15

Enzymes may also be used to cleave side-chain protective groups, applicable enzymes being proteolytic enzymes, lipases and esterases, according to the nature of the protective group, see "The peptides, Analysis, Synthesis, Biology" Vol 9, Special Methods in peptide Synthesis Part C. J.A. Glaas, Enzymatic manipulation of Protecting Groups in Peptide Synthesis, Academic Press 1987.

20

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The process of the invention may be performed with CPD-Y, which is the presently preferred enzyme, and which is characterized more fully in EP-B1-17485, and also with other serine or thiol carboxypeptidases, such as those mentioned in the survey below, since these have a common mechanism of activity via acyl enzyme intermediates.

30

35

Enzyme

Origin

Fungi

Penicillocarboxypeptidase S-1

Penicillium janthinellum

- S-2

- -

5 Carboxypeptidase(s) from

Aspergillus saitoi

-

Aspergillus oryzae

Plants

Carboxypeptidase(s) C

Orange leaves

Orange peels

10 Carboxypeptidase C_N

Citrus natsudaikai Hayata

Phaseoline

Bean leaves

Carboxypeptidase(s) from

Sprouted barley or malt

eg. carboxypeptidase MII

Carboxypeptidase(s) from

Sprouted cotton plants

15

Tomatoes

Watermelons

Bromelain (pineapple) powder

Carboxypeptidase W

Wheat

The close relation between a plurality of the abovementioned carboxypeptidases has been discussed by Kubota et al, Carboxypeptidase C. J. Biochem., Vol. 74 (1973), p. 757-770. Carboxypeptidase from malt, wheat and other sources have been described by Breddam, Carlsberg Res. Comm. Vol. 51, p. 83-128, 1986.

The carboxypeptidase used may also be chemically modified or be a biosynthetic mutant of a natural form.

As illustrated more fully below, the process of the invention is rather simple; however, it is important to maintain a rather constant pH value in the reaction mixture. This pH value is between 5 and 10.5, however, preferably between 7 and 9.5 and also depends upon the



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concrete starting materials, the peptide formed and the enzyme.

5 The reaction is performed in an aqueous reaction medium, if desired containing up to 70% of an organic solvent which is miscible or immiscible with water, and compatible with the enzyme under the conditions specified. Preferred solvents are lower alcohols, dimethyl formamide, dimethyl sulfoxide, dimethoxy
10 ethane, ethylene glycol and ethyl acetate.

The reaction temperature is preferably room temperature and above, 20 to 50°C, but temperatures in the range 0 to 60°C may be used, if advantage is under the
15 conditions otherwise given.

The concentration of the two reaction components may vary within wide limits, but the nucleophile component is frequently in excess, and to avoid oligomerization of
20 the substrate component, said component is often added in minor portions at intervals during the entire reaction sequence. If the ester used is ethyl or higher, surprisingly no oligomerization is observed, and very high substrate concentrations may be used without side
25 reactions. In this case, a homo-dipeptide in which A = B may be formed without oligomerization from a single starting compound, the nucleophile being generated in situ by hydrolysis of this ester. In some cases this is a further technical advantage, cf. Fig. 2.

30 Thus, the starting concentration of the substrate component may typically be 0.005 to 2 molar and for the nucleophile component in cases, where it is added separately, 0.005 to 3 molar. In most situations it is
35 possible to recover excess of the nucleophile component and the hydrolysis product from the substrate component for optional reesterification and reuse. Recycling of

- 13 -

the components is particularly easy because of their simple structure and the absence of side reactions and deprotective losses.

5 The enzyme concentration may likewise vary, but is frequently somewhat higher (5-50 μ m) than the concentrations appropriate in the use of N-protected amino acid ester substrates, but as illustrated by the following examples, the amount required for synthetic
10 purposes may be reduced more than tenfold by using a stable immobilized enzyme preparation, thereby enabling the enzyme to be used in a continuous process.

The reaction medium may also contain salts, such as
15 NaCl, which influences the binding of the enzyme to the substrate, as well as a complex binding agent for present metal ions, such as EDTA, which stabilizes the enzyme.

20 The reaction rate differences between D- and L-substrates mentioned initially are illustrated in fig. 1, which shows the reaction sequence for the CPD-Y hydrolysis of a protected and unprotected amino acid ester (Tyr-OEt) D- and L-form.

25 It will be seen from the figure that while L-AcTyrOEt is hydrolyzed almost instantaneously, only insignificant hydrolysis (below 5%) of D-AcTyrOEt will have taken place after 2 hours.

30 In contrast, there are only small differences in the hydrolysis sequence for the unprotected esters in L- and D-form.

35 This surprising hydrolysis sequence is reflected in the following examples, which illustrate the production of various dipeptides in the process of the invention using

- 14 -

the enzymes CPD-Y and malt carboxypeptidase II, (CPD-MII) and wheat carboxypeptidase (CPA-W).

General method for examples 1-15

5

The reactions, performed on an analytical scale with a reaction volume of 1 ml, were carried out in a pH-stat, and the selected pH value was kept constant by automatic addition of 1 N NaOH. Reaction temperature was room
10 temperature, unless otherwise stated. The table also includes reaction concentrations, content of organic solvent, product and yield. Reaction times are typically between 0.5 and 5 hours at the enzyme concentrations are typically 10-20 μ m. unless otherwise stated.

15

Product identification and determination of product yield were performed by means of reverse phase HPLC (Waters 6000 A pumps, 660 gradient blender, UK 6 injector) on a C₁₈NOVA PAK column (Waters, RCM) using
20 suitable gradients of elution systems containing 50 mM triethylammonium phosphate, pH 3.0 from 0% to 80% acetonitrile with a flow of 2 ml/min. Elution was monitored by means of a UV detector (Waters 480) at 230 nm, 254 nm, 278 nm or 290 nm.

25

The products were identified by amino acid analysis of fractions from the HPLC analysis, which corresponded to the assumed product peak and/or by HPLC comparison with a chemically synthesized reference product. These were
30 produced according to known principles, usually via reaction between BOC-A-OSu - tertiary butyloxy carbonyl - the succine imide ester derivative of the substrate amino acid - and the used nucleophile component followed by deblocking of the N-terminal amino acid residue. In
35 all cases, it was possible to separate LL-and DD-dipeptides from the diastereomeric DL-and LD-dipeptide products.

- 15 -

For the products which can only be detected at 230 nm, the product yields were determined by means of the absorption/concentration curve of the chemically synthesized reference compound. For the other products, the yields were determined on the basis of the ratio between the integrated areas below the peaks in the elution chromatogram, corresponding to product respectively the reactant which absorbs at the wavelength concerned.

The reaction conditions in the preparative examples 16-²⁵~~21~~ are described in the individual examples. The reactions were followed on analytical HPLC as described. The enzyme concentrations are generally lower and the reaction times longer than in the corresponding analytical examples, but no attempt to optimize the reaction conditions has been made.

35



Example 1

Carboxypeptidase Y ^{a)} catalyzed synthesis of L-L-dipeptides with L-Tyrosine ethylester (50 mM) as substrate component and free amino acids as nucleophiles

Nucleophile (conc.)		Solvent	pH	Product	Yield %
Alanine	(1.9 M)	Water	9.5	TyrAlaOH	10
Arginine	(0.8 M)	Water	9.5	TyrArgOH	31
Cysteine	(1 M)	Water	8.0	TyrCysOH	30
LD-Cysteine	(2 M)	Water	8.0	TyrCysOH(LL)	40
Leucine	(0.2 M)	Water	8.0	TyrLeuOH	1
Lysine	(2 M)	Water	9.5	TyrLysOH	18
Methionine	(0.3 M)	Water	8.0	TyrMetOH	8
Methionine	(0.3 M)	Water	9.0	TyrMetOH	9
Methionine	(0.3 M)	30% DMSO	9.0	TyrMetOH	15
Methionine	(0.3 M)	15% EtOH	9.0	TyrMetOH	7
Glutamine	(0.8 M)	Water	9.5	TyrGlnOH	8
Pennicil- amine	(0.5 M)	Water	8.0	TyrPenOH	7

^{a)} 10 μ M, 1 mM EDTA

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Example 2

Carboxypeptidase Y ^{a)} catalyzed synthesis of L-L-dipeptides with L-Methionine (0.3 M) as nucleophile component in water at pH 9.0

Substrate (50 mM)	Product	Yield %
Leucine methylester	LeuMetOH	30
Leucine isopropylester	LeuMetOH	36
Methionine ethylester ^{b)}	MetMetOH	25
Phenylalanine methylester	PheMetOH	16
Phenylalanine ethylester	PheMetOH	19
Phenylalanine isopropylester	PheMetOH	23
Serine isopropylester ^{c)}	SerMetOH	21
Tryptophane methylester	TrpMetOH	26
Tyrosine benzylester ^{d)}	TyrMetOH	19

a) 10 μ M, 1 mM EDTA

b) 5 mM

c) Reaction time > 20 hr

d) 30% DMSO

Example 3

Carboxypeptidase Y ^{a)} catalyzed synthesis of L-L-dipeptides with L-Tyrosine ethylester (50 mM) as substrate component and L-amino acid amides or esters as nucleophiles

Substrate	Nucleophile	(Conc.)	Solvent	pH	Product	Yield%
TyrOEt	Leucine amide	(0.2 M)	30% DMSO	9.5	TyrLeuNH ₂ ^{b)}	42
					TyrLeuOH	4
TyrOEt	Lysine amide	(0.3 M)	Water	9.5	TyrLysNH ₂	20
					TyrLysOH	22
TyrOEt	Arginine amide	(0.2 M)	Water	9.0	TyrArgNH ₂	50
TyrOEt	Valine amide	(0.3 M)	Water	9.0	TyrValNH ₂	77
TyrOEt	Leucine methylester	(0.2 M)	30% DMSO	9.0	TyrLeuOH	6

a) 20 μ M, 1 mM EDTA

b) Reaction time > 20 hr, 50% substrate converted

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Example 4

Carboxypeptidase Y ^{a)} catalyzed synthesis of L-L-homo-dipeptides from a single starting compound in water at pH 8.5

Substrate	(conc.)	Product	Yield %
Methionine methylester	(0.5 M) ^{b) c)}	MetMetOH	14
Methionine ethylester	(0.5 M)	MetMetOH	16
Methionine isopropylester	(0.5 M)	MetMetOH	14
Tyrosine methylester	(0.2 M)	TyrTyrOH ^{c)}	1
Tyrosine ethylester	(0.2 M)	TyrTyrOH ^{c)}	1
Phenylalanine ethylester	(0.2 M) ^{d) e)}	PhePheOH	2
Alanine amide	(0.2 M) ^{f) d)}	AlaAlaNH ₂	^{b)}

a) 10 μ M CPD-Y, 1 mM EDTA

b) Polymerization

c) Precipitation

d) pH 9.0

e) 30% DMSO

f) 50 μ M CPD-Y, 1 mM EDTA

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Example 5

Carboxypeptidase Y a) catalyzed synthesis of D,L-dipeptides with D-Tyrosine ethylester (50 mM) as substrate and free L-amino acids as nucleophiles in water

Nucleophile	(conc.)	pH	Product	Yield %
Arginine	(1 M)	9.0	tyrArgOH	75
Cysteine	(1 M)	8.0	tyrCysOH	86
LD-cysteine	(2 M)	8.0	tyrCysOH(DL)	45
Leucine	(0.2 M)	8.0	tyrLeuOH	22
Methionine	(1 M)	9.0	tyrMetOH	65
Pennicil- amine	(1 M) ^{b)}	8.0	tyrPenOH	27

a) 10 μ M, 1 mM EDTA

b) Reaction time > 20 hr

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Example 6

Carboxypeptidase Y a) catalyzed synthesis of D,L-dipeptides with L-Methionine (0.3 M) as nucleophile component in water at pH 9.0

D-substrate (50 mM)	Product	Yield %
leucine methylester	leuMetOH	50
leucine isopropylester	leuMetOH	71
methionine ethylester	metMetOH	68
phenylalanine ethylester	pheMetOH	71
phenylalanine isopropylester	pheMetOH	72
serine isopropylester	serMetOH b)	46
tryptophane ethylester	trpMetOH	72

a) 15 μ M, 1 mM EDTA

b) Reaction time 5 days

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Example 7

Carboxypeptidase Y ^{a)} catalyzed synthesis of D,L-dipeptide amides with D-Tyrosine or D-Phenylalanine ethylester (50 mM) as substrate component and L-amino acid amides as nucleophile components in water

Substrate	Nucleophile	(conc.)	pH	Product	Yield %
tyrOEt	Leucine amide	(0.2 M)	9.0	tyrLeuNH ₂	82
				tyrLeuOH	2
tyrOEt	Valine amide	(0.3 M)	9.0	tyrValNH ₂	94
tyrOEt	Arginine amide	(0.2 M)	9.0	tyrArgNH ₂	86
pheOEt	Alanine amide	(0.8 M)	9.0	pheAlaNH ₂ ^{b)}	50

a) 15 μ M, 1 mM EDTA

b) Some polymerization noted

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Example 8

Carboxypeptidase Y ^{a)} catalyzed synthesis of D,D-homodipeptide esters from D-substrate ester components in water at pH 9.0, also acting as nucleophile component

<u>D-substrate</u>	<u>(conc.)</u>	<u>Product</u>	<u>Yield %</u>
tyrosin ethylester	(0.05 M)	tyrtyrOEt	9
phenylalanine ethylester	(0.1 M)	phepheOEt	10
tyrosine ethylene glycolester	(0.05 M)	tyrtyrOEtOH	1
methionine methylester	(0.1 M)	metmetOMe	3

^{a)} 15 μ M, 1 mM EDTA

Example 9

Carboxypeptidase Y a) catalyzed synthesis of L-L-dipeptides with sidechain-protected carboxyterminal amino acids with L-TyrOEt (50 mM) as substrate component and sidechain-protected L-amino acids and amides as Nucleophiles

Nucleophile	(Conc.)	pH	Solvent	Product	Yield%
Acetamidomethyl cysteine	(1 M)	8.5	Water	TyrCys(-SAcn)OH	10
Acetamidomethyl cysteine amide	(0.4 M)	8.5	Water	TyrCys(-SAcn)NH ₂	12
				TyrCys(-SAcn)OH	1
Beta Benzyl Aspartic Acid	(0.1 M) ^b	9.0	30%DMSO	TyrAsp(OBzl)OH	13
Epsilon Triflouracetyl Lysine	(0.1 M) ^b	8.5	30%DMSO	TyrLys(Tfa)OH	12
Gamma Tertbutyl Glutamic Acid					
Amide	(0.1 M) ^b	8.0	30%DMSO	TyrGlu(OtBu)NH ₂	3
				TyrGlu(OtBu)OH	12
Gamma Methyl Glutamic Acid	(0.3 M)	8.5	Water	TyrGlu(OMe)OH	20
Gamma Ethyl Glutamic Acid	(0.3 M)	8.5	Water	TyrGlu(OEt)OH	18

a) 10 μ M, 1 mM EDTA

b) 25 mM Substrate, 20 μ m

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Example 10

Carboxypeptidase Y a) catalyzed synthesis of D-L-dipeptides with sidechain-protected carboxyterminal amino acids with D-TyrOEt (50 mM) as substrate component and sidechain-protected L-amino acids and amides as Nucleophiles

Nucleophile	(Conc.)	pH	Solvent	Product	Yield%
Acetamidomethyl cysteine	(1 M)	8.5	Water	tyrCys(-SAcn)OH	75
Acetamidomethyl cysteine amide	(0.4 M)	8.5	Water	tyrCys(-SAcn)NH ₂	71
				tyrCys(-SAcn)OH	6
Beta Benzyl Aspartic Acid	(0.1 M) ^b	9.0	30%DMSO	tyrAsp(OBzl)OH	54
Epsilon Triflouracetyl Lysine	(0.1 M) ^b	8.5	30%DMSO	tyrLys(Tfa)OH	51
Gamma Tertbutyl Glutamic Acid					
	Amide (0.1 M) ^b	8.0	30%DMSO	tyrGlu(OtBu)NH ₂	42
				tyrGlu(OtBu)OH	10
Gamma Methyl Glutamic Acid	(0.3 M)	8.5	Water	tyrGlu(OMe)OH	75
Gamma Ethyl Glutamic Acid	(0.3 M)	8.5	Water	tyrGlu(OEt)OH	71

a) 10 μ M, 1 mM EDTA

b) 25 mM Substrate, 20 μ m

Example 11

Carboxypeptidase Y ^{a)} catalyzed synthesis of L,L-dipeptides with sidechain-protected aminoterminal amino acid residues from sidechain-protected substrate components (25 mM) and L-Methionine (0.3 M) as nucleophile component in 30% DMSO at pH 9.0

Substrate	Product	Yield %
L-Aspartic Acid Dibenzylester ^{b)}	Asp(OBzl)MetOH	65
L-Glutamic Acid Dibenzylester ^{c)}	Glu(OBzl)MetOH	70

a) 20 μ M, 1 mM EDTA, Reaction time > 20 h

b) At 35 per cent conversion

c) At 60 per cent conversion

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Example 12

Carboxypeptidase Y ^{a)} catalyzed synthesis of dipeptide esters and amides containing omega amino acids from L- and D-amino acid ester substrates at 50 mM in water with beta alanine and beta alanine amide as nucleophiles

Substrate	Nucleophile	(conc.)	pH	Product	Yield %
tyrOEt	B-Alanine methyl ester	(0.2 M)	8.5	tyrBAlaOMe	51
tyrOEt	B-Alanine methyl ester	(0.5 M)	8.5	tyrBAlaOMe	72
pheOEt	B-Alanine methyl ester	(0.5 M)	9.0	pheBAlaOMe	83
PheOEt	B-Alanine methyl	(0.5 M)	9.0	PheBAlaOMe	15
pheOEt	B-Alanine amide	(0.5 M)	9.0	pheBAlaNH ₂	70
PheOEt	B-Alanine amide	(0.5 M)	9.0	PheBAlaNH ₂	3

^{a)} 20 μ M, 1 mM EDTA

Example 13

Carboxypeptidase γ ^{a)} catalyzed synthesis of L,L and D,L dipeptides with hydroxyalkylesters of L- and D-Tyrosine and L-Phenylalanine (50 mM) as substrate components and free L-Methionine (0.3 M) as nucleophile in water/ethyleneglycol mixtures at pH 9.0

Substrate	% glycol (vol/vol)	Product	Yield %
TyrOEtOH	0	TyrMetOH	10
TyrOEtOH	40	TyrMetOH	8
TyrOEtOH	60 ^{b)}	TyrMetOH	5
tyrOEtOH	0	tyrMetOH	56
PheOEtOH	0	PheMetOH	16

a) 5 μ M, 1 mM EDTA

b) 10 μ M, 1 mM EDTA

- 29 -

Example 14

Synthesis of L,L-dipeptides catalyzed by carboxypeptidases from barley and wheat at 50 mM initial L-substrate ester concentration at pH 8.0 in water and L-amino acid and amides as nucleophile components

Enzyme	Substrate	Nucleophile	(conc.)	Product	Yield%
CPD-MII ^{a)}	PheOEt	Methionine	(0.4 M)	PheMetOH	10
CPD-W ^{b)}	PheOEt	Arginine	(0.8 M)	PheArgOH	8

a) 20 μ M, 1 mM EDTA, 2 M NaCl

b) 10 μ M, 1 mM EDTA

- 30 -

Example 15

Synthesis of D,L-dipeptides catalyzed by carboxypeptidases from barley and wheat at 50 mM initial D-Phenylalanine ethylester concentration at pH 8.0 in water and free L-amino acids as nucleophile components

Enzyme	Substrate	Nucleophile	(conc.)	Product	Yield%
CPD-MII ^{a)}	pheOEt	Methionine	(0.4 M)	pheMetOH	15
CPD-W ^{b)}	pheOEt	Arginine	(0.8 M)	pheArgOH	13
CPD-W ^{b)}	pheOEt	Methionine	(0.4 M)	pheMetOH	40

a) 20 μ M, 1 mM EDTA, 2 M NaCl

b) 10 μ M, 1 mM EDTA, 2 M NaCl, Reaction time > 20 hr,
conversion less than 50 per cent

Example 16

=====

Preparative synthesis of L,L-tyrosylcysteine, TyrCysOH

5

Procedure

L-tyrosineethylester hydrochloride (1.5 g, 6 mmol) and L-cysteine (15.2 g, 125 mmol) were dissolved in 110 ml of-
10 0,1 M KCl solution containing 1 mM EDTA. pH was adjusted to 8.0 with triethylamine. The reaction was initiated by addition of 7.5 ml of carboxypeptidase Y-solution (16 mg/ml), and was kept at pH 8.0 for the duration of the reaction by the addition of triethylamine under continuous
15 stirring at room temperature. The remainder of the substrate (13.5 g, 54 mmol) was added in portions of 1,5 g during one hour. After 0,5 hour tyrosine precipitation started, and after 3.5 hours the reaction was stopped by heating to 45°C for 20 minutes.

20

The formed tyrosine was filtered off, and the filtrate was purified by R-preparative HPLC (Waters Prep. L C/system 500A) using two columns (5.7 x 30 cm) packed with 60 um C₁₈-particles and 50 mM acetic acid as eluent. Collected
25 fractions containing pure product were evaporated to dryness in vacuo under repeated additions of absolute ethanol. The remnant was stirred with diethyl-ether. Following this, 3.88 g of L,L-tyrosine cysteine (14 mmol, 22%) was isolated by filtration and drying.

30

Identification

Chloride and acetate was not detected, the product being present as a zwitter-ion.

- 32 -

Amino acid analysis following acid hydrolysis and derivation of Cys by acrylonitrile gave the result:

5 Tyr (1.00)
 Cys (1.08)

Purity

10 Following derivation of the cysteine sidechain with acrylonitrile, only one spot was detected on TLC on Kieselgel 60-F using ninhydrine detection (Rf 0.73, eluent: ethylacetate, butanol, acetic acid and water (1:1:1:1)).

15 HPLC-purity: 99.5% (nucleosile 7 C18, 0.1 M ammoniumphosphate, pH 3.0/acetonitrile, 220 nm).

UV-quantization: 98.5% (293 nm, tyrosine absorbance in 0,1 M NaOH).

Example 17

=====

Preparative synthesis of L,L-methionyl-methionine, MetMet-
5 OHProcedure

- 10 L-methionineethylester hydrochloride (24.6 g, 115 mmol) and L-methionine (20.6 g, 138 mmol) were dissolved in 190 ml of 0.1 M KCl solution containing 1 mM EDTA. pH was adjusted to 9.0 with sodium hydroxide solution, and the reaction was initiated by addition of 14.2 ml of carboxypeptidase Y solution (20 mg/ml). The reaction was stirred
- 15 overnight at room temperature, and pH was kept at 9.0 through the addition of sodium hydroxide solution by a pH-stat. pH was adjusted to 3.0 with HCl-solution at the end of reaction.
- 20 Precipitated methionine was filtered off, and the filtrate was purified by R-preparative HPLC as described in example 16. Collected fractions containing pure product were concentrated by evaporation and finally freeze-dried. This procedure gave 10.6 g (37.8 mmol, 33%) of L-methionyl-L-
- 25 methionine as a white amorphous powder.

Identification

- 30 No chloride was detected, and only 1.9% (w/w) of acetate was determined, the product being predominantly in zwitter-ionic form. Amino acid analysis showed methionine following acid hydrolysis and the absence of free methionine in unhydrolysed samples.

Purity

Purity by HPLC: 99.8% (nucleoside 7 C₁₈, 0.1 M ammonium-phosphate, pH 3.0/acetonitrile, 220 nm).

5

Quantization by reaction with trinitrobenzenesulfonic acid (TNBS) and UV-detection: 92.5%.

Water content by Karl Fisher: 1.5%.

Example 18

=====

Preparative synthesis of L,L-tyrosylvalineamide, TyrValNH₂

5

Procedure

L-tyrosineethyl-ester-hydrochloride (16.0 g, 65 mmol) and L-valineamide-hydrochloride (60 g, 390 mmol) were dissolved in 1150 ml of water and 65 ml of 20 mM EDTA was added. pH was adjusted to 9.0 with sodium hydroxide solution and the reaction was initiated by addition of 12 ml of a carboxypeptidase Y solution (20 mg/ml). The reaction was stirred at room temperature for four hours, and pH was maintained at 9.0 by the addition of sodium hydroxide solution. Following complete conversion the reaction was stopped by raising pH to 11.

The denatured enzyme was filtered off, and the reaction mixture was diluted and purified by successive anion exchange on a Dowex A61x4 column and cation exchange on a Dowex A650Wx4 column using sodium and ammonium acetate salt gradients, respectively, and was finally desalted. Product fractions were combined and evaporated to dryness in vacuo under repeated additions of absolute ethanol, giving 11.2 g L,L-tyrosylvalineamide (40 mmol, 62%) as a white powder.

Identification

30

1.8% (w/w) of acetate was determined, the product being predominantly in zwitter-ionic form.

- 36 -

Amino acid analysis following acid hydrolysis gave the following results:

5 Tyr (1.01)
 Val (0.99)

Purity

10 HPLC-purity: 99.5% (Novapak C₁₈, 0.1 M ammoniumphosphate
 containing alkylsulfonate, pH 4.5/acetonitrile, 220 nm).

UV-quantization: 97.8% (293 nm, tyrosine absorbance in 0.1
M NaOH).

Example 19

=====

5 Preparative synthesis of D,L-tyrosyl-arginine, D,L-tyrArg-
OHProcedure

10 105 g of L-arginine (105 g, 603 mmol) was dissolved in 400 ml of water, and pH was adjusted to 9.0 with HCl-solution. D-tyrosineethyl-ester-hydrochloride (6.1 g, 25 mmol) and 5 ml of 0.1 M EDTA were added, and pH readjusted to 9.0. The reaction was initiated by addition of 7 ml of a carboxypeptidase Y solution (20 mg/ml) and pH was kept at
15 9.0 by addition of NaOH-solution. After 4 hours the reaction was stopped by adjusting pH to 3 with HCl-solution.

20 The reaction mixture was diluted and purified by cation exchange on a Dowex A650Wx4 column using an ammonium acetate salt gradient, and finally desalted. Collected product fractions were concentrated by evaporation and finally freeze-dried, giving 6.45 g of D,L-tyrosyl-arginine (19 mmol, 77%) as a white powder.

25 Identification

Acetate and chloride could not be detected, the product being present as a zwitter-ion.

30 Amino acid analysis following acid hydrolysis gave the following result:

Arg (1.03)

Tyr (0.97)

- 38 -

Purity

HPLC-purity: 99.5% (Novapak C₁₈, 0.1 M ammoniumphosphate with alkylsulfonic acid, ph 4.5/acetonitrile, 220 nm).

5

Water content by Karl Fisher: 3.4%.

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Example 20

=====

Preparative synthesis of D,L-phenylalanylmethione, D,L-
5 pheMetOH by the use of immobilized carboxypeptidase Y

Immobilization procedure

Carboxypeptidase Y was immobilized on Eupergit C following
10 the recommended procedure of the manufacturer. The immobilization was carried out in a phosphatebuffer at pH 7.5, and residual active gel-groups were blocked with ethanolamine at pH 8.0 with subsequent washing.

15 93% of the enzyme was bound to the gel containing 2.5 mg protein/ml. The Eupergit C coupled enzyme was stored in 10 mM PIPES, 1 mM EDTA, 0.05% hydroxybenzoic acid ethyl ester, pH 7.0 at 4°C.

20 Synthetic procedure

D-phenylalanine ethylester hydrochloride (5.7 g, 25 mmol) and L-methionine (29.8, 200 mmol) were dissolved in 400 ml of H₂O, 5 ml of 0.1 N EDTA was added, and pH adjusted to
25 9.0 with sodium hydroxide solution, giving a reaction volume of 500 ml. The reaction mixture was continuously stirred and pH kept constant at 9.0 with sodium hydroxide solution, while the solution was being circulated over a column of immobilized CPD-Y at a flowrate of 3 ml/min. The
30 column contained immobilized CPD-Y on Eupergit C prepared as described above and was 2.5 cm x 5.5 cm with a volume of 27 ml, containing a total of 67 mg of CPD-Y. Circulation was continued for 10 hours. pH was then adjusted to 7 with HCl-solution and the product was purified on R-prepa-
35 rative HPLC as described in example 16.

- 40 -

Collected fractions containing pure product were combined, evaporated in vacuo and finally freeze-dried to give 3.5 g (12 mmol, 48%) of D,L-phenylalanylMethionine as a white, amorphous powder.

5

Stability of the enzyme preparation

The experiment could be repeated with the same enzyme gel preparation several times without noticable loss in conversion rate and comparable results. Thus, the enzyme is fairly stable at the reaction conditions, further improving process economy.

10

Identity of the product

15

The product was free of chloride, but contained 7.0% (w/w) of acetate and so was partially on acetate form.

Amino acid analysis showed the absence of free amino acids, and following acid hydrolysis a ratio of

20

Met (0.98)

Phe (1.03)

Specific optical rotation using sodium D-line at 25°C. and c=0.25 in water was -128.9°

25

Purity of product

HPLC: 99.6% (nucleosile 7 C₁₈, 0.1 M ammoniumphosphate, pH 3/acetonitrile, 220 nm).

30

Water content by Karl Fisher: 2.8%

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Example 21

=====

Preparative synthesis of D,D-phenylalanylphenylalanine
5 ethylester hydrochloride, D,D-phepheOEt.HClProcedure

10 D-phenylalanine ethylester hydrochloride (2.5 g, 11 mmol)
was dissolved in 45 ml of water, and 0.5 ml of 0.1 N EDTA
was added. pH was adjusted to 9.0 with sodium hydroxide
solution, the substrate being present as a partially oily
suspension at the beginning. The reaction was initiated by
addition of 3.4 ml of a carboxypeptidase Y solution (20
15 mg/ml) and was stirred for 2.5 hours at room temperature,
pH being kept at 9.0 with sodium hydroxide solution. The
reaction was stopped by adjusting pH to 3 with HCl-solu-
tion.

20 The reaction mixture was filtered and purified by R-prepa-
rative HPLC as described in example 16.

Collected product fractions were concentrated by evapora-
tion in vacuo and freeze-dried with added HCl-solution,
25 giving 0.49 g (1.3 mmol, 12%) as a white, amorphous pow-
der.

Identification

30 The product contained 9.2% (w/w) of chloride and no aceta-
te, the product being present as hydrochloride.

Amino acid analysis showed phenylalanine following acid
hydrolysis and no phenylalanine prior to acid hydrolysis.

- 42 -

The product could be further hydrolysed with base, yielding a product chromatographically different from D,L-Phe-PheOH, and the product itself coeluted with an L,L-PhePhe-OEt-standard in the used HPLC-system.

5

Finally, specific optical rotation using sodium D-line at 25°C and $c=0.5$ in acetic acid was found to be -42.7° . This compared with a pure standard of L,L-PhePheOEt, which gave $+52.1^\circ$ under similar conditions. In this case, the discrepancy is believed to be due to the lesser purity of the synthesized peptide.

10

Purity

15 HPLC-purity: 82.3% (nucleosile 7 C₁₈, 0.1 M ammonium phosphate, pH 3/acetonitrile, 220 nm).

The impurities detected were chromatographically different from substrate, substrate hydrolysis, product hydrolysis or diastereomers thereof.

20

Water content by Karl Fisher: 7.5%.

Example 22

Preparative synthesis of D-Tyrosyl- β -Alanine, D-Tyr- β -
5 Ala-OH via the ester D-Tyrosyl- β -Alanine methylester
through enzymatical deblocking

Procedure

10 D-Tyrosine-ethylester (1.07 g, 5.1 mmol) and β -Alanine
methylester (4.14 g, 40.1 mmol) were dissolved in 57 ml
H₂O containing 60 μ moles (22.3 mg) disodium EDTA and 3
mmoles (363.3 mg) TRIS. The reaction was initiated by
addition of 3.40 ml of a solution of Carboxypeptidase-Y
15 (353 μ M) and was kept at pH 8.5 for the duration of the
reaction.

After 2 hours the pH was adjusted to 3.0 by addition of
1 N hydrochloric acid. The reaction mixture was diluted
20 to 100 ml and purified by RP-preparative HPLC (Waters
Prep LC/System 500A) using one column (5.7 x 30 cm)
packed with 60 μ m C-18 particles and 50 mM acetic
acid/ethanol mixtures.

25 Collected fractions containing pure product were con-
centrated under reduced pressure and finally freeze-
dried.

The procedure gave 0.78 g of D-Tyrosyl- β -Alanine
30 methylester acetate (2.7 mmol, 58%).

Purity

HPLC purity: 98,0% (Nucleosite 7 C-18, 0.1 M ammonium
35 phosphate, pH 3.0/acetonitrile, 220 nm).



Identification

Amino acid analysis showed the absence of free amino acids but the following acid hydrolysis gave the
5 following results:

β -Ala (1.04)
D-Tyr (0.96)

10 Procedure

The dipeptide methylester (0.65 g, 2.3 mmol) was dissolved in 40 ml H₂O containing 4 ml 0.5 M phosphate buffer (pH 7.0). The reaction was initiated by addition of
15 2.8 ml of a solution of Pig Liver Esterase (PLE, 357 μ M) and was kept at pH 7.0 for the duration of the reaction.

After 4 hours the pH was adjusted to 3.0 by addition of 10 N hydrochloric acid. The reaction mixture was diluted
20 to 70 ml and purified by RP-preparative HPLC (Waters Prep LC/System 500A) using one column (5.7 x 30 cm) packed with 60 μ m C-18 particles and 50 mM acetic acid/ethanol mixtures.

25 Collected fractions containing pure product were concentrated under reduced pressure and finally freeze-dried.

This procedure gave 0.50 g of D-Tyrosyl- β -Alanine acetate (1.5 mmol, 65%).
30

Identification

Amino acid analysis showed the absence of free amino acids but the following acid hydrolysis gave the
35 following results:



β -Ala (1.04)

D-Tyr (0.96)

- 5 Acetate content by HPLC: 1.2% (w/w). No chloride was detected.

Specific optical rotation: -34.9° ($C = 0.01$ in 0.1 N HCl using the sodium D-line of 20°C).

10

Purity

HPLC purity: 98.7% (Nucleosite 7 C-18, $0.1\text{ M ammonium phosphate}$, $\text{pH } 3.0$ /acetonitrile, 220 nm).



Example 23

5 Synthesis of β -Alanyl-L-methionine catalysed by Carboxy-peptidase-Y^{a)} using β -Alanine-benzylester as substrate and free methionine acid as nucleophile at 2 different pH values in water.

Conc. of		Nucleophile	pH	Yield(%)
10	substrate			
	25 mM	0.4 M	8.0	5 ^{b)}
	25 mM	0.4 M	8.5	8

15

a) 50 μ M CPD-Y, 2 mM Na₂-EDTA.

b) Determined at less than 50% conversion.



Example 24

- 5 Synthesis of dipeptides catalysed by carboxypeptidases^{a)} using D-amino acid-ethylesters as substrates and amino methyl phosphonic acid (AMP) and 2-amino ethyl sulphonic acid (2-AES) as nucleophiles in water at different pH values.

10	Enzyme	Substrate	Nucleophile	pH	Yield (%)
	CPD-Y	D-Tyr-OEt (50 mM)	AMP (0.2 M)	9.3	2
	CPD-W	D-Tyr-OEt (50 mM)	AES (0.5 M)	8.5	4 ^{b)}

- 15 a) 20 μ M CPD-W, 2 mM Na₂ EDTA and 0.1 M NH₄Cl (pH adjusted)

b) Determined vs. hydrolysis via a standard at less than 100% conversion



Example 25

Synthesis of L,L-TyrValNH₂ catalysed by carboxypeptidase
5 Y in high organic solvent concentrations using L-Tyro-
sine ethylester as substrate and L-Valineamide as
nucleophile at pH 8.5.

Conc. of			
10	substrate	Nucleophile	Solvent ^{b)} Yield (%) ^{a)}
	25 mM	0.5 M	92% glycerol 53

15 a) Determined at 80% conversion, 100 μ M CPD-Y,
2 mM Na₂-EDTA.

b) Solvent concentration is calculated as the ratio
between water and organic solvent, excluding solids
20 in solution.

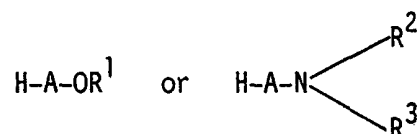


The claims defining the invention are as follows:

1. A process for producing dipeptides having the general formula



wherein A represents an optionally side-chain protected L- or D- α -amino
5 acid residue or ω -amino acid residue and B represents an optionally
side-chain protected L- or D- α -aminocarboxylic acid residue which may
be the same as or different from A, an L- or D-aminophosphonic acid
residue or L- or D-aminosulfonic acid residue or the corresponding
10 ω -amino acids or salts and hydrates thereof, and Y is OH or a
C-terminal blocking group, characterized by reacting a substrate
component, which is an amino acid derivative having the formula



wherein A is as defined above, R^1 represents hydrogen, alkyl, aryl or
aralkyl optionally substituted with inert substituents or an
15 α -des-amino fragment of an amino acid, and R^2 and R^3 are the same
or different and each represents hydrogen, alkyl, aryl or aralkyl
optionally substituted with inert substituents,

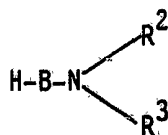
with a nucleophile component which when A = B may be formed in situ and
is selected from

20 (a) amino acids having the formula



wherein B is an aminocarboxylic acid as defined above

(b) amino acid amides having the formula



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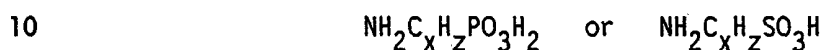
wherein B is an aminocarboxylic acid as defined above, and R^2 and R^3 have the above meaning, except that when R^2 represents hydrogen, R^3 may also represent hydroxy or amino

(c) amino acid esters having the formula



wherein B is an aminocarboxylic acid as defined above, and R^4 represents alkyl, aryl or aralkyl, and

(d) straight chain or branched aminophosphonic acids or aminosulfonic acids having the formula



wherein x is 1-6 and z is 2-12

in the presence of an optionally immobilized serine or thiol carboxypeptidase from yeast or of animal, vegetable or other microbial origin, in an aqueous solution or suspension having a pH value between 5 and 10.5 optionally containing an organic solvent and/or a salt, and then, if desired, cleaving a present side-chain protecting group or protective group Y and/or, if desired, converting the resulting dipeptide derivative to a salt or hydrate.

2. A process according to claim 1, characterized by using carboxypeptidase Y from yeast as the



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~~50~~

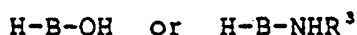
carboxypeptidase.

3. A process according to claim 2, c h a r a c t e r -
i z e d in that the carboxypeptidase used has been
5 purified by affinity chromatography over an affinity
resin consisting of a polymeric resin skeleton with a
plurality of coupled benzyl succinyl groups.
4. A process according to claim 1, c h a r a c t e r -
10 i z e d in that the carboxypeptidase used is
penicillocarboxypeptidase S-1 or S-2 from *Penicillium*
janthinellum, a carboxypeptidase from *Aspergillus saitoi*
or *Aspergillus oryzae*, a carboxypeptidase C from orange
leaves or orange peels, carboxypeptidase C_N from *Citrus*
15 *natsudaoidai* Hayata, phaseoline from bean leaves or a
carboxypeptidase from sprouted barley, malt, sprouted
cotton plants, tomatoes, watermelons or Bromelain
(pineapple) powder.
- 20 5. A process according to claims 1-4, c h a r a c -
t e r i z e d in that the carboxypeptidase used has
been chemically modified or is a biosynthetic mutant of
a natural form.
- 25 6. A process according to any of the preceding claims,
c h a r a c t e r i z e d in that the carboxypeptidase
enzyme used has been immobilized.
- 30 7. A process according to any of the preceding claims,
c h a r a c t e r i z e d by using an aqueous reaction
solution or reaction dispersion containing from 0 to 70%
of an organic solvent.
- 35 8. A process according to claim 7, c h a r a c t e r -
i z e d in that the organic solvent is selected from
alkanols, dimethyl sulfoxide, dimethyl formamide,
dimethoxy ethane, ethylene glycol or ethyl acetate.

52
~~51~~

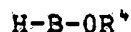
9. A process according to any of the preceding claims, characterized by using as substrate component a D- or L-amino acid ester selected from benzyl esters or straight or branched C₁-C₆ alkyl esters optionally substituted with inert substituents.

10. ~~—(Amended)—~~ A process according to any of the preceding claims, characterized by using as nucleophile component an amino acid or amino acid amide having the formulae



15 wherein R³ is hydrogen or C₁-C₃ alkyl and B is an L-amino acid residue.

11. A process according to claim 1, characterized by using as nucleophile component an ester having the formula



25 wherein B is an aminocarboxylic acid residue and R⁴ is C₁-C₃ alkyl.

12. A process according to any of the preceding claims, characterized in that the resulting dipeptide includes one or more C-terminal protective groups Y, and that the group or groups are cleaved enzymatically, preferably by means of the same carboxypeptidase enzyme as was used in the preceding reaction.

13. A process according to any of the preceding claims, characterized in that the resulting dipeptide includes one or more side-chain protective



groups and that the group or groups are cleaved enzymatically, preferably by means of an esterase or lipase or proteolytic enzyme.

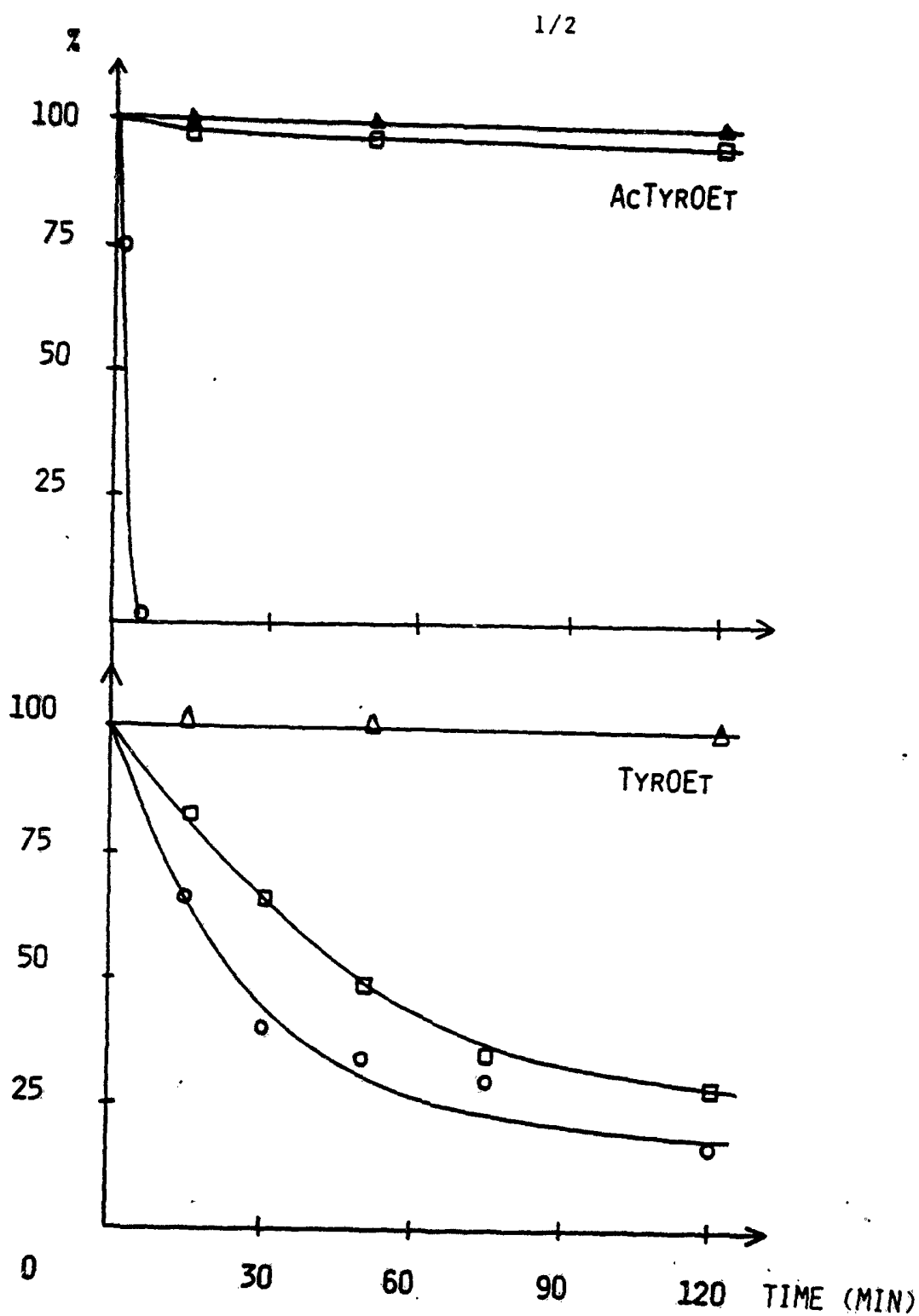
14. A process for producing dipeptides as defined in claim 1, which process is substantially as hereinbefore described with reference to any
5 one of Examples 1 to 25.

15. The product when produced by the process of any one of claims 1 to 14.

DATED this NINTH day of MAY 1991
Carlsberg Biotechnology Ltd. A/S

Patent Attorneys for the Applicant
SPRUSON & FERGUSON





○ L
□ D
△ CONTROL (SUBSTRATA WITHOUT ENZYME)

FIGURE 1
Carboxypeptidase Y (10 μ M) Catalyzed Hydrolysis
of L- and D-TyroEt and of L- and D-AcTyroEt (50 mM), 20%
DMSO, pH 9.0, 1 mM EDTA

2/2

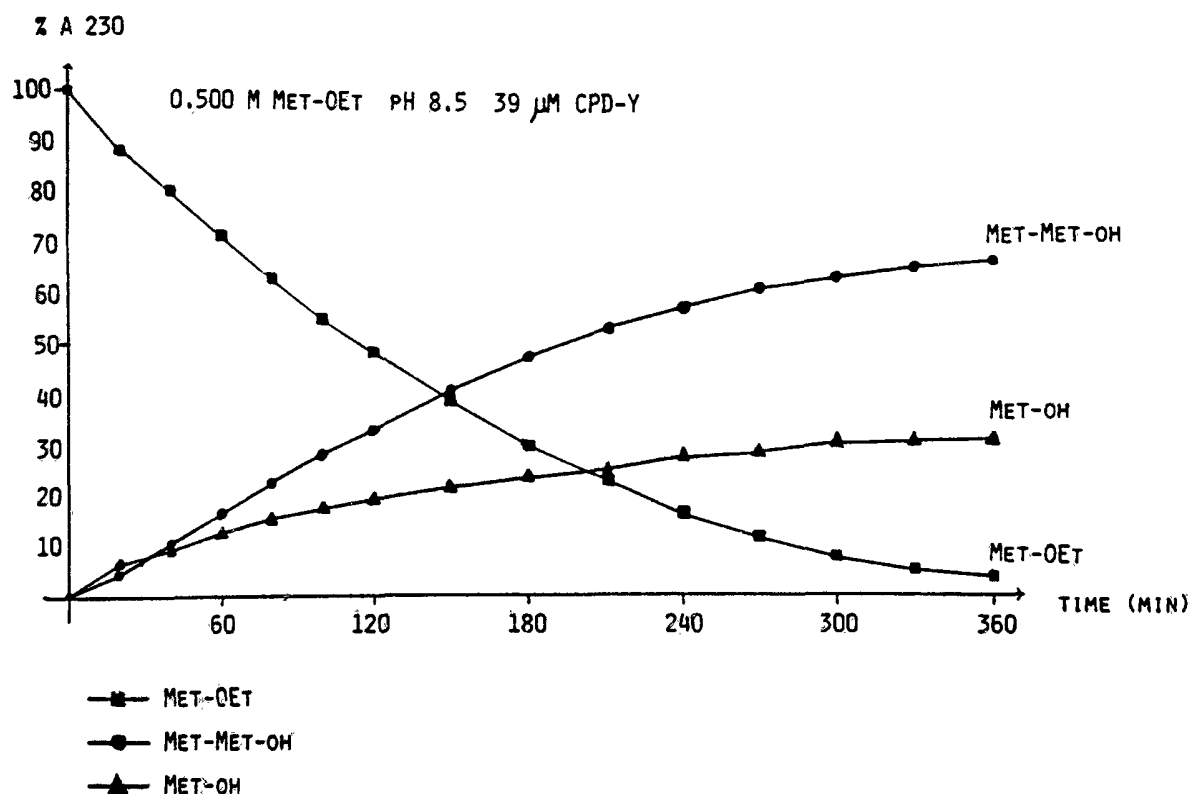
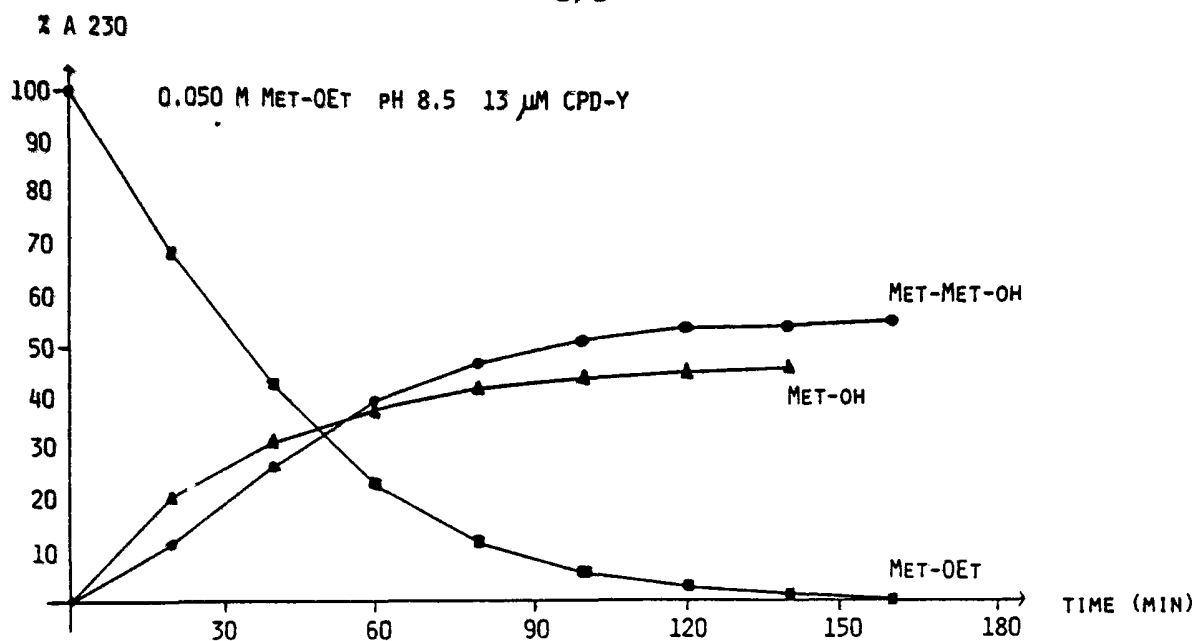


Figure 2

Carboxypeptidase Y catalyzed formation of the homodipeptide L,L-Methionyl-Methionine from the single starting compound L-Methionine ethylester at 0.2 M and 0.5 M initial concentration, the nucleophile methionine being generated in situ. No oligomerisation is observed.

%A230 are per cent arbitrary absorbance units at 230 nm with HPLC-detection.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK88/00022

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC 4		
C 12 P 21/02, C 07 K 1/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched 7		
Classification System	Classification Symbols	
IPC 4	C 12 P 21/02; C 07 K 1/00 - /14	
IPC 3	C 07 C 103/52; C 12 D 13/06 .../...	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8		
SE, NO, DK, FI classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT 9		
Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	EP, A1, 0 074 095 (TOYO SODA MANUFACTURING CO LTD) 16 March 1983 See page 17, lines 13-35	1-13
Y	EP, A1, 0 086 053 (UNIFIKA LTD) 17 August 1983 See the claims	1-12
Y	EP, A1, 0 099 585 (TOYO SODA MANUFACTURING CO LTD) 1 February 1984 See the claims	1-12
Y	EP, A1, 0 017 485 (DE FORENEDE BRYGGERIER A/S) 15 October 1980 See page 5, lines 1-5, page 6-7 and the claims	1-12
A	US, A, 3 972 773 (SAGAMI CHEMICAL RESEARCH CENTER) 3 August 1976 .../...	1-12
* Special categories of cited documents: 19 "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 "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		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
1988-04-15	1988-05-17	
International Searching Authority	Signature of Authorized Officer	
Swedish Patent Office	Yvonne Siösteen	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

II

Fields searched (cont)US C1 435:68-70; 195:4, 29, 30V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim numbers 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:
3. ☐ Claim numbers..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

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 of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ 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 claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest:

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US, A, 4 086 136 (ISOWA ET AL) 25 April 1978	1-12