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(57) Abstract

A solid phase method for producing a compound comprising an unsubstituted or substituted diamine moiety, which method comprises: (i) reacting an N–protected amino aldehyde with an amino group attached to a solid resin to produce a resin–bound enamine product; and (ii) reducing the enamine product to produce a resin–bound N–protected diamine. The resin bound diamine may be further modified for example by further protection, reaction with an amino acid or by carrying out solid phase peptide synthesis to provide a peptide bonded at its C–terminus to a diamine moiety.

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SYNTHESIS OF DIAMINES

The present invention relates to a method of producing diamines in the solid phase. In particular it relates to a solid phase method for the synthesis of unsubstituted or substituted diamines including N-substituted diamines and N,N'-disubstituted diamines.

The key areas for generating chemical diversity within peptides are:

- modification of N-/C-termini;
- modification/replacement of the peptide bond of natural amino acids; and
- substitution of amino acids by their D-analogues or synthetic mimetics

One form of peptide modification is by reductive amination on a resin. This allows preparation of peptide analogues with a reduced peptide bond of general formula A-CONHCH(R)CH_2NHCH_2-B (*excluding C-terminal amide bond*) where A and B are peptide residues. These compounds display not only stability towards enzymatic degradation and bioavailability, but may also show improved potency and selectivity.

This simple amide bond replacement $\Psi[CH_2NH_2]$ in a peptide provides an N-acyl, N'-alkyl diamine. This substitution has been widely used to increase stability towards proteolytic enzymes of biologically active peptides, to achieve receptor selectivity and/or to obtain peptide antagonist. Potent ACE inhibitors, an antagonist against bombesin receptors, gastrin, substance P, secretin and peptide analogues which mimic the enzyme bond tetrahedral transition state of the scissile amide bond have all been synthesised which incorporate such a functional group.

The problems in the synthesis of monoprotected *gem*-diaminoalkylderivatives (H_2N-CH(R)-NH_2) and synthesis of retro-inverso peptides both in solution and on a solid-phase utilising classical Curtius or Hofmann-type rearrangements have been investigated and reviewed Chorev, M. and Goddamn, M., Acc. Chem. Res., 26, 266-273(1993). Most of the studied retro-inverso peptidomimetics displayed increased stability toward enzymatic degradation, bioavailability and also improved potency and selectivity.

Naturally occurring polyamines and derivatives showed varied biological activity: growth promoters, regulation of cell proliferation and differentiation, significant

tumor-inhibiting activity, diagnostic markers in a number of diseases (cystic fibrosis, malignancies, etc.), antihypertensive, hypoglycemic, fever-lowering, antistress-antiulcer, antagonists of glutamate receptor and in gene therapy programs as a non-viral vector for DNA-delivery (for review see Ganet, B, Acc. Chem. Res., 15, 190-198 (1982), Zang, E., Saller, P.J., Synthesis, 4, 410-411 (1996); Byk, G., Frederic, M., and Scherman, D., Tetr. Lett., 38 (Vol. 18) 3219-3222, 1997).

Syntheses of di-, polyamines are therefore based on difficult solution chemistry and are somewhat inflexible.

Summary of the Invention

The invention therefore seeks to provide a concise and versatile method of producing diamines, N-substituted diamines and N,N'-disubstituted diamines in high yields. In particular the invention provides a new solid phase method for producing amino acid and peptide substituted diamines in which the C-terminus of the amino acid or peptide is bound to the diamine moiety.

In one embodiment the invention provides a solid phase method for producing a compound comprising an unsubstituted or substituted diamine moiety, which method comprises:

(i) reacting an N-protected amino aldehyde with an amino group of a resin to produce a resin-bound enamine product; and

(ii) reducing the enamine product to produce a resin-bound N-protected diamine.

The resin-bound N-protected diamine can be modified prior to optionally removing the diamine from the resin so as to produce a variety of useful substituted diamine products. The resin-bound N-protected diamine can be modified in a number of ways including those set out below.

The resin-bound diamine may be modified by reacting the amine group bonded to the resin with an amine protecting group to produce a resin-bound diamine in which both amine groups are protected. If the protecting group used is the same as the protecting group

of the N-protected amino aldehyde used in step (i) then the resin-bound diprotected diamine will be symmetrically protected. If the protecting group used is different from the protecting group of the N-protected amino aldehyde used in step (i) then the resin-bound diprotected diamine will be asymmetrically protected.

Alternatively the resin-bound diamine may be modified by reaction with an amino acid. Thus the resin-bound diamine is acylated by reaction of the amine group bonded to the resin with an N-protected amino acid to produce a resin-bound amino acid-substituted diamine in which both terminal nitrogen atoms are protected. The amino acid may typically be a naturally occurring amino acid or the D-isomer thereof.

In a third alternative the resin-bound diamine may be modified by a solid phase peptide synthesis at either or both of the amine groups.

Thus the diamine may be modified by protecting the amine group bonded to the resin with an amine protecting group, selectively deprotecting the amine group derived from the N-protected aldehyde used in step (i), and carrying out the steps of a solid phase peptide synthesis to produce a peptide product comprising at its C-terminus a diamine, the terminal group of which is N-protected and is bonded to the resin.

Alternatively the diamine may be modified by carrying out the steps of a solid phase peptide synthesis without diprotecting the nitrogen derived from the N-protected aldehyde used in step (i) to produce a peptide product comprising at its C-terminus a diamine, which is bonded to the resin.

Peptide synthesis of both amines may be obtained by diprotecting the amine group derived from the N-protected aldehyde used in step (i), and carrying out the steps of a solid phase peptide synthesis so as to produce a homodimerised peptide product comprising two peptide moieties each of which is attached at the C-terminus to the diamine moiety.

Alternative peptide synthesis at both amines may be used to obtain a heterodimer. The resin-bound diamine may be modified by:

a) protecting the amine group bonded directly to the resin with an orthogonal protecting group or/and orthogonally protected amino acid, diprotecting the amine group derived from the N-protected amino aldehyde used in step (i) and carrying out the

steps of solid phase synthesis to produce a resin-bound peptide product comprising at its C-terminus a diamine; and

b) deprotecting the orthogonally-protected amine or amino acid group without removal of the N-terminus protecting group of the peptide formed in step (a) and carrying out further steps of solid phase peptide synthesis to produce a hetero dimerised peptide product comprising two different peptide moieties each of which is attached at the C-terminus to the diamine moiety.

Alternatively a peptide heterodimer may be obtained in a process in which the resin bound diamine is modified by:

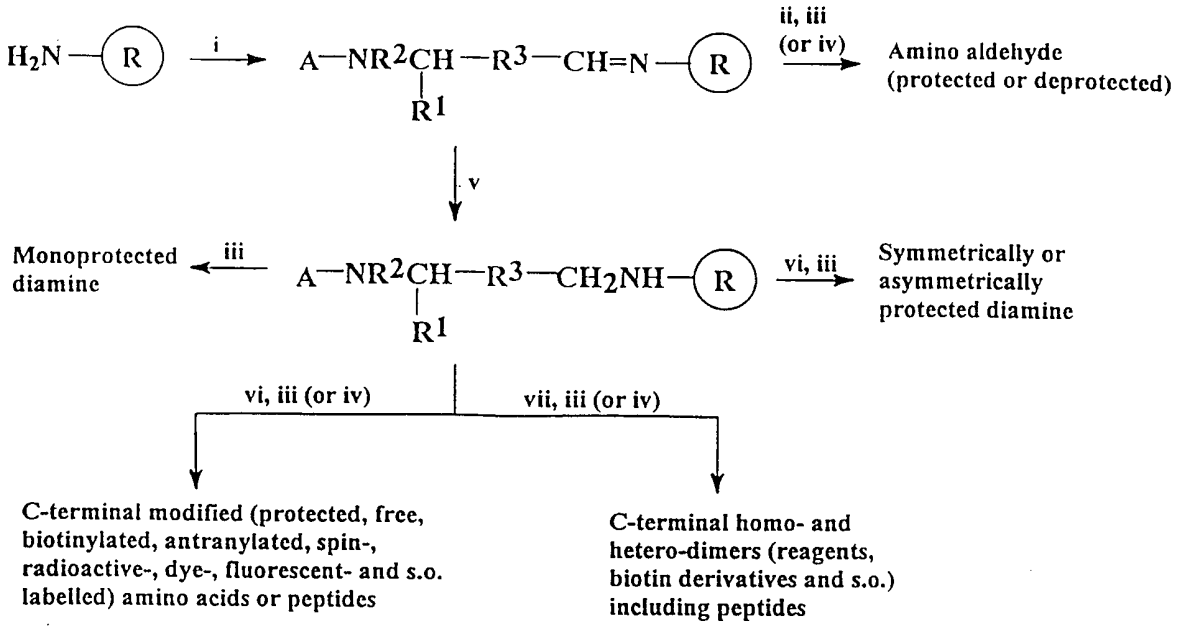
a) carrying out the steps of solid phase peptide synthesis using a reagent system comprising a protecting group orthogonal to the N-protecting group used in step (i); and

b) selectively deprotecting the amine protecting group derived from the N-protected amino aldehyde used in step (i) without removal of the orthogonal N-terminus protecting group of the peptide formed in step (a) and carrying out further steps of solid phase peptide synthesis so as to produce a hetero-dimerised peptide product comprising two different peptide moieties each of which is attached at the C-terminus to the diamine moiety.

In yet a further aspect of the invention a detectable label is attached to the resin-bound product (substituted or unsubstituted diamine moieties) produced by any embodiments of this invention.

It would be understood by one skilled in the art that the term bound or bonded refers to the presence of a chemical interaction so as to achieve attachment of two moieties. Preferably the attachment is a covalent attachment.

A schematic representation of selected aspects of this invention are presented as scheme 1.



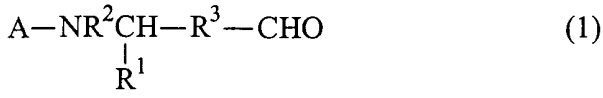
R =Benzhydrylamine-, 4-methylbenzhydrylamine, 4-(2', 4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy-, Fmoc-amino-xanthen-3-iloxy- type resins for solid phase organic synthesis

i N-protected amino aldehyde (2-10 eq.); 0.5-1.0% AcOH in DMF (NMP)/toluene (1/1); distillation of toluene at 35°C; 30-45 min.
ii -A
iii 1% TFA/DCM
iv 80-95% TFA/scavengers
v 4-10 eq. $NaBH_3CN$ in DMF/toluene/0.5-1% AcOH (30-60 min) or $NaBH_4$ in DMF/toluene/MeOH (3/3/2) (2-5 hrs)
vi B=Y-Xaa-COCl(F) or RCOCl/DIES or pyridine; B=(Y-Xaa-CO)$_2$ or $(RCO)_2O$; B=Y-Xaa-COOH (RCOOH)/HATU, BOP, PyBOP or PyBroP/DIEA (4/4/8); 4-8 eq., RT, 1-12 hrs

Scheme 1

Detailed description of the invention

Suitable N-protected amino aldehydes for use in the method of the invention include any substituted or unsubstituted amino aldehydes which have been protected with a suitable nitrogen protecting group. Preferred amino aldehydes include α- or ω-amino aldehydes. Preferred amino aldehydes are of general formula (1)



(1)

wherein: A is a N-protecting group (see below for details); R^1 is hydrogen, alkyl, a side chain of a natural amino acid or its stereoisomer, or a substituted derivative thereof and R^2 is hydrogen or alkyl or R^1 and R^2 with the atoms to which they are bonded form a heterocyclic ring; and R^3 is a C_{1-10}-alkylene group or a single bond.

In N-protected amino aldehydes where R^1 is a side chain of a natural amino acid, R^1 is preferably selected from -Me, $-(CH_2)_3NHC(=NH)NH_2$, $-CH_2CONH_2$, $-CH_2CO_2H$, $-CH_2SH$, $-(CH_2)_2CONH_2$, $-(CH_2)_2CO_2H$, $-CH_2$(4-imidazolyl), $-CH(CH_3)CH_2CH_3$, $-CH_2CH(CH_3)_2$, $-(CH_2)_4NH_2$, $-(CH_2)_2SCH_3$, $-CH_2Ph$, $-CH_2OH$, $-CH(CH_3)OH$, $-CH_2$(3-indolyl), $-CH_2$(4-hydroxylphenyl) and $-CH(CH_3)_2$. In N-protected amino aldehydes where R^1 is a substituted derivative of an alkyl group or a side chain of a natural amino acid or its stereoisomer the one or more substituents are preferably present on a carbon, oxygen, sulphur or nitrogen atom and may be halogen such as Cl, F, Br and I including radioactive I, oxygen, $-NO_2$, $-NH_2$, C_{1-4}-alkyl or PhCO-. Particularly preferred substituted R^1 groups are $-CH_2C_6H_4X$ wherein X is F, Cl, Br, I, $-NO_2$ or -COPh-4; $-(CH_2)_3NH_2$; $-(CH_2)_2S(O)CH_3$; $-CH_2$(2-halogen-4-hydroxy-phenyl).

Where R^1 or R^2 is an alkyl group it preferably contains from 1 to 6 carbons more preferably 1-4 carbons, for example, methyl, ethyl, n-propyl, i-propyl, n-butyl, s-butyl or t-butyl.

In N-protected amino aldehydes where R^1 and R^2 together with the atoms to which they are bonded from a heterocyclic ring, the ring preferably has from 5 to 7

members, more preferably it is substituted or unsubstituted pyrrolidinyl or piperidinyl ring.

R^3 may be a straight or branched alkylene group of 1 to 10 carbon atoms, typically 1 to 8 carbon atoms, preferably 1 to 6 atoms, more preferably 2 to 5 carbon atoms, for example $-(CH_2)_n-$ where n is 1 to 10, typically 1 to 8, preferably 1 to 6, and more preferably 2 to 5.

The most preferred N-protected amino aldehydes for use in the invention are the N-protected aldehyde derivatives of natural amino acids or stereoisomers thereof and N-protected amino aldehydes of formula (1) wherein R^1 and R^2 are both hydrogen and R^3 is a C_2, C_3, C_4 or C_5 alkylene group. Examples of amino aldehydes which when N-protected can be used include glycinal, L- and D-phenyl alaninal, prolinal and 1-amino-hexan-6-al.

Suitable nitrogen-protecting groups (A) for use in the method of the invention include any appropriate nitrogen-protecting groups known in the art. The skilled person can select the nitrogen-protecting group appropriately based on the subsequent reaction steps he or she wishes to perform. Examples of suitable protecting groups include acetyl (Ac), allyloxycarbonyl (Aloc), tert-amyloxycarbonyl (Aoc), tert-butoxycarbonyl (Boc), bromobenzyloxycarbonyl (BrZ), benzoyl (Bz), chlorobenzyloxycarbonyl (ClZ), 9-fluorenylmethoxycarbonyl (Fmoc), formyl (For), methoxybenzyloxycarbonyl (MeOZ), trityl (Trt), 4-methytrityl (Mtt) and benzyloxycarbonyl (Z). Other N protecting groups which can be used in the method are reviewed in Green, T.W. and Wuts P.G.M., Protective Groups in Organic Synthesis, (Second Edition, 1991) John Wiley and Sons Inc. New York, pp 309-405 and Kocieński, P.J., Protecting Groups, (1994) Georg Thieme Verlag, Stuttgart, New York, Chapter 6, pp 185-237. In preferred N-protected amino aldehydes for use in the invention the N-protecting group is Boc or Fmoc.

Resins suitable for use in the method of the invention include any resins suitable for solid phase organic synthesis which have a primary amine group which is capable of reacting with an aldehyde. Suitable resins may have a protected amine group which is deprotected prior to reaction. Preferred resins are aminobenzhydryl- or aminoxantyl-based resins. The resins may or may not posses a spacer arm. The most preferred resins are benzyhydrylamine-, 4-methylbenzhydrylamine-, 4-(2',4'-dimethoxyphenyl-Fmoc-

aminomethyl)phenoxy- and 9-Fmoc-amino-xanthen-3-iloxy-type resins.

Reaction Conditions

The reductive alkylation process is carried out using a two stage methodology. Stage 1 is the condensation reaction between the aldehyde functional group of the amino aldehyde and the primary amine group of the resin so as to form an enamine product (Schiff base). This reaction can be carried out using any suitable reaction conditions known in the art. Preferably, the reaction is carried out by treating the reactants with acetic acid (e.g. 1%) in a mixture of dimethyl formamide (DMF) and toluene (e.g. 1:1 mixture). More preferably the alkylation is carried out by treating the acetate salt of the primary amine of the resin with a 2 to 3 molar excess of N-protected amino aldehyde in a mixture of DMF/toluene (1:1) containing 0.5 to 1% acetic acid and removing water of reaction by distillation at 35-40°C for 10 to 15 minutes. If required this alkylation step can be repeated after filtration of the reaction mixture.

Stage 2 of the methodology is the reduction of the enamine carbon-nitrogen double bond to give a secondary amine group. This results in an N-protected diamine which is bound to the resin at its unprotected N-terminus. The reduction can be carried out using any appropriate reaction conditions known in the art. By appropriate selection of the N-protecting group (A) and the conditions used to reduce the enamine double bond, it is possible to reduce the enamine in the presence of protecting groups present in the substrate. Preferred reducing agents are $NaBH_3CN$, and $NaBH_4$ and BH_3.

Preferred reduction reactions using $NaBH_3CN$ comprise pre-swelling the enamine-resin (alkylated resin) in DMF or a mixture of DMF/toluene (1:1) containing 0.5-1% acetic acid and adding, over a period of 10 to 15 minutes, 4-10 molar equivalents (based on moles of bound enamine) of $NaBH_3CN$ dissolved in the minimum amount of DMF.

Preferred reduction conditions using $NaBH_4$ comprise preswelling the enamine resin in a mixture of DMF/methanol (3:2) or a mixture of DMF/toluene/methanol (3:3:2) and adding 10 to 15 molar equivalents of solid $NaBH_4$.

An alternative method for producing the resin bound diamine is to couple, using

standard methodology, a protected amino acid to an SA or MBHA type resin of the type known for use in the preparation of amides and then reduce the amide moiety using a suitable reducing agent preferably BH_3. Preferred reduction reactions using BH_3 comprise prewashing the amino acid resin in THF or THF/1 to 5% acetic acid, then adding 5-15 equivalents of BH_3.THF as approximately 1M solution in THF. This method is not convenient for the synthesis of diamines derived from Cys, Asp, Glu, Met, Gln, Arg, Trp and Asn.

Diamine Modifications

The resin-bound N-protected diamine may be modified by chemical steps carried out whilst the diamine moiety is attached to the resin, to produce a variety of products. Methods of producing these products from the resin-bound mono-protected diamine also form embodiments of the invention.

In one embodiment (iiia) the invention provides a method of producing free (mono) N-protected diamine by cleaving the N-protected diamine from the resin. Any reaction conditions known in the art to cleave the nitrogen to resin-component bond may be used. Preferably, the supported diamine is cleaved from the resin by treatment with trifluoro-acetic acid (TFA) (e.g. 1%) in dichloromethane (DCM), this is particularly useful for resins containing xanthyl linkers (SA- and TSGA-resins) and for the cleavage from the resin of moieties bearing acid sensitive groups including acid sensitive protecting groups.

A combined cleavage and deprotection step may be effected by treatment with 25 to 95% TFA in the presence of carbocation scavengers.

Alternative methodologies for cleaving the diamine or derivative from the resin with deprotection of acid sensitive groups can be effected by treatment with hydrogen fluoride in the presence of suitable scavengers or hydrogen bromide in TFA. These treatments are highly suited to use with benzhydryl type resins.

In another embodiment (iiib) the invention provides a method of producing N,N'-diprotected diamine, which method comprises reacting the amine group bonded directly to the resin with a suitable amino protecting group. The N,N'-diprotected diamine is then

cleaved from the resin using any one of the cleavage conditions mentioned above. Any protecting group known in the art which is capable of reacting with the secondary amine is suitable for use in this method (see above for examples of preferred N-protecting groups). If the protecting group is the same as the protecting group of the N-protected amino aldehyde used, then the diamine product is symmetrically protected. If the protecting group is different from the protecting group of the N-protected amino aldehyde then an asymmetrically protected diamine is produced.

In a modification of embodiment (iiib) the invention provides a method of producing a free N-protected-N'-amino acid substituted diamine, which method comprises acylating the amine group bonded directly to the resin with an N-protected amino acid or derivative thereof and then cleaving the amino acid substituted diamine from the resin. The N-protected amino acid or derivative used in this embodiment can be any N-protected amino acid or derivative known in the art. Preferably, the N-protected amino acid or derivative is the N-protected carboxylic acid or derivative corresponding to the preferred N-protected aldehydes mentioned hereinabove. For example Fmoc-alanine, Fmoc-glycine, Fmoc-leucine, Fmoc-tyrosine, Fmoc-valine and Fmoc-proline or derivatives thereof.

Acylation of the amine group is carried out using reaction conditions well known in the art, preferably conditions which involve activation of the carboxyl group of the amino acid, most preferably reaction conditions which use the carbonyl group activators benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), O-(7-azabenzotriazol-1-yl) -N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) or 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluroborate (TBTU) or use the corresponding N-protected amino acid chloride.

Preferred coupling reactions where TBTU, HBTU, BOP or PyBOP are used as the coupling agent comprise treating the resin bound diamine with a solution preferably in NMP or DMF and preferably of 0.3 to 0.45 molar concentration, comprising 4 to 8 equivalents of the coupling agent, equivalent of the N-protected amino acid, 1 equivalent of HOBt and 1.76-2.0 equivalents of NMM or DIEA. The reaction is preferably conducted for

1 to 1.5 hours at room temperature.

Preferred coupling reactions where HATU is used as the coupling agent comprise treating the resin bound diamine with a solution preferably in NMP or DMF and preferably of 0.3 to 0.43 molar concentration, comprising 4 equivalents of HATU, 4 equivalents of the N-protected amino acid and 7-8 equivalents of DIEA. The reaction is preferably conducted for 30-60 minutes at room temperature.

Peptide Synthesis

In another embodiment (iiic) the invention provides a method of producing a diamine having the C-terminus of a peptide moiety bonded to one nitrogen whilst having the other one protected by a suitable N-protecting group, which method comprises reacting the amine group bonded directly to the resin with an amine protecting group, selectively deprotecting the amine group of the N-protected aldehyde and carrying out the steps of solid phase peptide synthesis.

The diamine derivative may then be cleaved from the resin in the manner specified above. The amine protecting group which is reacted with the amine group bonded directly to the resin should be selected appropriately by the person skilled in the art so that it is orthogonal to the amine protecting group used in the N-protected aldehyde. The protecting group which is reacted with the amine group bonded directly to the resin should also be orthogonal to the protecting group used in the solid phase peptide synthesis steps.

The solid phase peptide synthesis technique used in this embodiment and other embodiments may be a conventional method of solid phase peptide synthesis known in the art. For examples see J. Jones, Amino Acid and Peptide Synthesis Oxford University Press 1992.

Preferred solid-phase peptide synthesis methods used in the invention use one or more of the following coupling systems: TBTU/HOBt/DIEA (1:1:2), HATU/DIEA (1:2), PyBroP/DIEA (1:2) and acid chloride/DIEA at neutral pH. SPPS can be conducted either manually or automatically using the following protocols:

A. Standard protocol for manual solid phase peptide synthesis see Girald and

Andreu, Peptides (Proceedings of the Twenty First European Peptide Symposium), ESCOM, The Netherlands, 1990, p174 *et seq*, for both Boc and modified Fmoc protocols.

B. Standard protocol for automated solid-phase peptide synthesis see Fields *et al* Peptide Res. 4(2), 95-101, (1991).

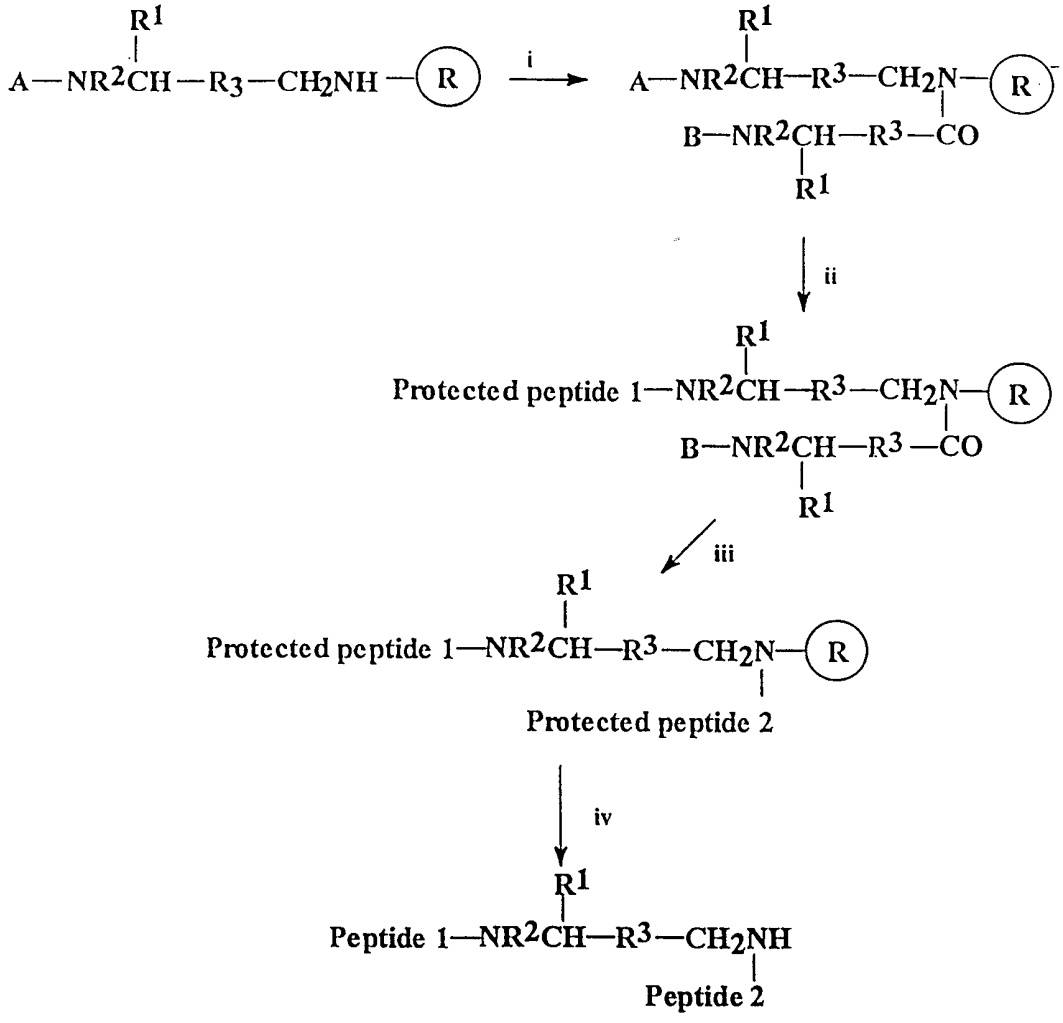
In another embodiment (iiid) the invention provides an alternative method of producing a diamine having a C-terminal peptide moiety bonded to one nitrogen whilst having the other one protected by a suitable N-protecting group, which method comprises carrying out the steps of solid phase peptide synthesis without deprotecting the nitrogen group of the N-protected aldehyde used in step (i).

The diamine derivative may then be cleaved from the resin in the manner specified above. The protocol used in solid phase peptide synthesis should be selected so that when solid phase peptide synthesis is effected it does not cause removal of the N-protecting group derived from the N-protected aldehyde used in step (i). The product produced will thus comprise a peptide moiety bonded at its C-terminal to the amino group bonded to the resin and an N-protected amino group derived from the N-protected amino aldehyde.

In another embodiment (iiie) the invention provides a method of producing a peptide which is C-homodimerised with diamine. This method comprises deprotecting the amine group derived from the N-protected aldehyde and then, without protection of the other amine group, carrying out steps of solid phase peptide synthesis. The peptide C-homodimerised with diamine can then be cleaved from the resin in the manner described above. The conditions used to deprotect the amine group will depend on which protecting group is used in the original N-protected aldehyde.

In another embodiment (iiif and ivf) the invention provides a method of producing a peptide heterodimer which is C-terminally dimerised with a diamine. This method may comprise acylation of the amine group bonded directly to the resin with a N-protected amino acid, selective deprotection of said N-protected amino acid, followed by steps of solid phase peptide synthesis, and then selective deprotection of the amine group derived from the N-protected amino aldehyde followed by further steps of solid phase peptide synthesis. (This is illustrated by scheme 2). In this method the N-protected amino aldehyde

must bear a protecting group orthogonal to the protecting group used in the N protected amino acid. Also, the protecting group used in the N-protected amino aldehyde must be removable in the presence of the N-protecting group used in the first implementation of solid phase peptide synthesis. Furthermore, the protecting group used in the first implementation of solid phase peptide synthesis must be orthogonal to the protecting group used in the second implementation of solid phase peptide synthesis.



A and B = orthogonal protecting groups

i acylation with first amino acid
ii selective A-deprotection, SPPS
iii selective B-deprotection, SPPS
iv cleavage or cleavage/deprotection

Scheme 2

Also in embodiment (iiif and ivf), the invention provides an alternative method of producing a peptide heterodimer which is C-terminally dimerised with a diamine The method comprises protection of the amine group bonded directly to the resin with an orthogonal protecting group or an orthogonally protected amino acid, followed by selective deprotection of the amine group derived from the N-protected aldehyde and followed by steps of solid phase peptide synthesis; and then deprotection of the orthogonally protected amine group bonded directly to the resin or the amine group of the orthogonally protected amino acid, followed by a further implementation of solid phase peptide synthesis. In this method the N-protecting group used to protect the amine group bonded directly to the resin or the amine group of the attached amino acid should be removable in the presence of the N-terminus protecting group of the first implementation of solid phase peptide synthesis. Also, the N-terminus protecting group used in the second implementation of solid phase peptide synthesis should be orthogonal to that used in the first.

Other Modifications and Cleavage

In a further embodiment, the invention provides a process for producing a substituted or unsubstituted diamine bearing a detectable label at least one of its N-termini. The process comprises attaching, for example by acylation, a detectable label to a deprotected primary or secondary amine whilst the diamine derivative is attached to the resin. The diamine may, at its other N-terminus, bear a protecting group, an amino acid or a peptide chain as a substituent. Preferably, the label is a biotinyl-, antranyl-, spin-, dye-, radioactive-, fluorescent- or spectrophotometric-labelling group, more preferably a biotinyl-group or a spectrophotometric-labelling group. In a most preferred embodiment the invention provides a method of producing a labelled diamine in which the non-labelled N-terminus bears a peptide chain as a substituent.

It should be appreciated that any diamine derivative product produced by synthesis whilst the diamine is attached to the resin can be cleaved from the resin to yield free product using the conditions detailed herein. Also it should be appreciated that any diamine derivative produced by a method according to this invention which bears one or

more protecting groups can be deprotected either individually or simultaneously, either whilst still attached to the resin or after having undergone cleavage from the resin. Methodology used for deprotection and cleavage would be within the knowledge of the skilled man and would be dependent on which protecting groups are present and are required to be removed.

The invention provides a method of a general application for producing a wide variety of diamine based compounds. Advantageously, all of the synthetic steps are carried out on a substrate bound to a solid support. The method provides an easy experimental regimen wherein any excess reagents and byproducts formed during reaction can be washed away. The products themselves are thus highly pure once they are cleaved from the resins. The method of the invention generates products in high yield and of high regiochemical and stereochemical accuracy. The use of solid phase synthesis in this method also allows the method to be automated if desired.

The invention provides a method of producing products such as C-terminally linked peptides or amino acids which can be either hetero- or homo-peptides or amino acids; C-terminally modified peptides and amino acids; C-terminally labelled peptides or amino acids; diamines; labelled diamines; and selectively protected (preferably orthogonally protected) versions of the above.

The C-terminally linked peptides or amino acids made by the method of the invention use the diamine moiety to link the amino acid or peptide C-terminals together. It is known from studies using retro-inverso peptides (peptidomimetics) wherein one link in a peptide chain is reversed by inclusion of a coupled product of a gem-diamine and gem-dicarboxylic acid, that reversal of the direction of the peptide bond within the peptide chain can increase stability towards enzymatic degradation and improve bioavailability, potency and selectivity when compared to the natural peptide (Chorev, M. and Goddamn, M., Acc. Chem. Res., 26, 266-273(1993). The C-terminally linked peptide or amino acid products resemble the retro-inverso compounds but have a normal peptide amide bond. For examples of such peptidomimetics produced by the method of the invention see Examples: 7.4 C-terminal modified analogues of human calcitonin, 10.1 symmetrical dimer of MSH-release inhibiting factor, 10.2 synthesis of Fmoc-Pro-$NHCH_2CH_2N$ (Fmoc-Pro-) and 10.3 Synthesis

of Fmoc-Leu-Ψ[CH_2NH(Fmoc-Leu)]-SA resin and Fmoc-Leu-Ψ[CH_2NH(Fmoc-Leu)]; Fmoc-Phe-Leu-Ψ[CH_2NH(Fmoc-Phe-Leu)].

The method of the invention also allows the production of C-terminally modified peptides or amino acids. This together with the abovementioned synthesis of retro-inverso peptides provides a method for generating chemical diversity within peptides. The method of the invention allows the production of a number of C-terminally modified peptides or amino acids the simplest being a Ψ[CH_2NH_2] replacement in the final amino acid C-terminal amide group. For Examples of C-terminally modified peptides made by the method of the invention see Example 7.1 C-terminal reduced amide bond Leucine enkephalin amide and Example 7.3 synthesis of C-terminal modified Leu-enkephalin analogues. Peptides and peptidomimetics which possess a Ψ[CH_2NH_2] group within their structure frequently display increased bioavailability, improved potency and improved selectivity.

C-terminally labelled amino acids or peptides made by the method of the invention use the diamine moiety to link the C-terminal of the amino acid of the peptide to the labelling group. Synthesis according to the method of the invention advantageously allows complete control over the position of the labelling group. The position of the labelling group may be important for optimal efficiency in detection. It is well known that side chain amino groups can bind a labelling group either in solution or on the resin. The present method attaches the labelling group specifically to the C-terminal. For an example of C-terminally labelled peptide see Example 7.2 C-terminal reduced amide bond, N'-terminal biotinylated leucine enkephalin amide.

The model peptides (peptidommetics) produced find application in technical fields such as ligand/receptor investigation, receptor mapping and affinity purification of receptors.

The method of the invention also advantageously allows N-protected amino aldehydes to be coupled to the resin and thus to form the C-terminal modification (last amino acid) or a reversed peptide linking group whilst retaining the stereochemical integrity.

The method of the invention allows the production of a wide variety of useful substituted and unsubstituted diamines and diamine derivatives some of which are known in

the art and others are new. The new substituted or unsubstituted diamines also form part of the invention.

The invention provides a diamine of formula

$$R^5R^2NCH(R^1)—R^3—CH_2NHR^4 \qquad (2)$$

wherein R^1, R^2 and R^3 are as defined previously for formula (1); R^4 and R^5 are the same or different and are selected from a hydrogen atom, a nitrogen protecting group, an amino acid or an amino acid derivative, a peptide chain comprising two or more amino acids or amino acid derivatives, and a detectable label; the diamine being capable of being produced by the method of the invention.

Of the diamine of formula (2) particularly prefered examples are those wherein R^1 is hydrogen or a side chain of a natural amino acid; R^3 is a single bond; and at least one of R^4 and R^5 is a peptide chain of at least 2 amino acids or amino acid derivatives.

Most prefered examples of diamines of formula 2 are those wherein R^1 is a side chain of the amino acid leucine; R^2 is hydrogen; R^3 is a single bond; R^4 is selected from a hydrogen atom, a biotinyl group and chemical groups of formulae Dde-Ahx-, H-Ahx-, Boc-Aoa-Ahx- and H-Aoa-Ahx-; and R^5 is a peptide chain of formula

A-Tyr(X)-Gly-Gly-Phe-

wherein (X) is hydrogen or a suitable tyrosine hydroxyl protecting group and A is a hydrogen atom, an Fmoc group or a Boc group, and those wherein R^1 and R^2 together with the atoms to which they are bonded form a pyrrolidine ring; R^3 is a single bond; R^4 is selected from a hydrogen atom, a biotinyl group, and chemical groups of formulae H-Ahx-, Boc-Aoa-Ahx-, Dde-Ahx- and H-Aoa-Ahx-; and R^5 is a peptide chain of formula

A-Gly-Val-Gly-Ala-

wherein A is a hydrogen atom, an Fmoc group or a Boc group.
Ahx is 2-aminohexanoic acid, Aoa is aminooxyacetic acid (O-(carboxymethyl) hydroxylamine) and Dde is 4,4-dimethyl-2,6-dioxocyclohexylidene methyl.

Examples

The following examples illustrate the invention.

1.0 Materials and methods

All Nα-Fmoc-amino acids, resins, PyBroP, TBTU, HOBt were obtained from NOVA Biochem AG (Läufelfingen, Switzerland). The DMF and NMP were from LAB-SCAN Limited (Dublin, Ireland). THF and DIPEA were from Fluka AG. HATU was from PerSeptiveBiosystem GmbH, Hamburg, Germany. All other reagents were the purest grade available.

HPLC was performed on a Waters instrument consisting of two Model 510 pumps, an automated gradient controller model 680 (Millipore Waters Chromatography Corp.) and a Spectra System 2000 UV/Vis detector (Thermo Separation Products Ltd.). The Gilson 712 HPLC System Controller Software Version 1.0 (Gilson Ltd.) was used for data collection. Peptides were eluted using 0.1% TFA in water (Buffer A) and 0.1% TFA in acetonitrile (Buffer B). The analytical HPLC was performed on a LiChrospher 100 5RP18 125x 4 mm i. d. or LiChrospher 100 5RP18 select B column (Merck GmbH) with either isocratic or linear gradient elution in a mobile phase of varying concentration of acetonitrile in aqueous TFA (0.10%); the preparative HPLC was performed on a Waters Radial Compressed Module (RCM) equipped with DeltaPak 300 15RP18 100X25 mm i.d. column cartridge (Millipore Waters Chromatography Corp.). The fractions were collected manually.

The IE HPLC was carried out on a Shodex SP-825 8x75 column (Waters Co.) using 10 mM sodium phosphate, pH 3.0 and linear gradient from 0 to 0.65 M KCl in 60 min Flow rates used were from 0.8 to 1.5 mL/min for analytical and ion exchange HPLC and 10 mL/min for preparative HPLC.

The amino acid composition of the peptide was determined using a Waters work-station and PicoTag HPLC System (Millipore Waters Chromatography Corp.). Samples were hydrolyzed with 6N HCl in the presence of crystalline phenol at 110°C for 18 hours and the amino acids were analysed after derivatization with PITC, as described previously (Bidlingmeyer, B.A., Cohen, S.A. & Tarvin, T.L. J. Chromatogr. 336, 93-104 (1984)).

Mass spectra were measured with an electrospray ionisation spectrometer (Perkin-Elmer Sciex API-1, Norwalk, CT, USA) either on samples directly eluted from the RP-HPLC column or freeze-dried, redissolved in 50% acetonitrile/2% formic acid. Mass scans were accumulated using a resolution of 0.1 mass unites, in the 300 - 1000 mass range using an orifice voltage of 5000 V and interface voltage 60-90 V.

The peptide concentration was determined on the basis of UV absorption at 278 nm using a standard curve of known peptide concentrations or on the base of amino acid composition analysis. Concentration of Fmoc-containing compounds was determined on the basis of UV absorbance at 301 nm upon treatment with DMF/20% piperidine for 10 min at room temperature and were compared with the standard curve, prepared for Fmoc-Gly-OH.

1.1 Resins

1. NovaSyn TG Rink amide (TGR) resin: 4-(2',4' - dimethoxyphenyl-Fmoc-aminomethyl)phenoxy-polyetylenglycol-polystyrene (0.20 mol/g (A15453) NovaBiochem AG)., Sieber amide (SA) resins: 9-Fmoc-amino-xanthen-3-iloxy - poly styrene(0.37 mmol/g(A15218) and 0.36 mmol/g (A16437) NovaBiochem AG,) and Nova Syn TG Sieber (TGSA) resin [9-Fmoc--amino-xanthen-3-iloxy -polyethylenglycol-polystyrene (0.15 mmol/g).

1.2 Fmoc/tBu based solid phase peptide synthesis protocols

A. Standard protocol for manual solid phase peptide synthesis (see Bidlingmeyer, B.A., Cohen, S.A. & Tarvin, T.L. J. Chromatogr. 336, 93-104 (1984)).

B. Standard protocol for automated solid phase peptide synthesis (see Fields, C.G., Lloyd, D.H., Macdonald, R.L., Otteson, K.M. and Noble, R.L., Peptide Res., 4(2), 95-101(1991)) was performed on 9050 PepSynthesizer (MilliGen), System software version 1.5.

C. Modified protocol for manual SPPS;

The resin, pre-swollen in NMP or DMF, was mixed with amino acid and activating reagent (HBTU, TBTU, BOP,) and HOBT , both as a 0.45 M solution in DMF or NMP and after 5 minutes, DIEA or NMM was added. The HATU activating reagent was used without HOBt. The excess and concentration of activated amino acid was from 3 to 8 eq. at 0.3 - 0.45 M in NMP or DMF and amino acid/coupling reagent/HOBt/DIEA (or NMM) were used in 1/1/1/2 ratio.

The first amino acid or reagent was introduced to the diamine-resin with the methods of symmetrical anhydrides, TBTU/HOBt, HATU, PyBroP or acid chlorides. Fmoc-amino acid chlorides were prepared according Carpino et al. (J. Org. Chem., 51, 3732-3234 (1986)). The conditions for the coupling of acid chlorides were similar to the conditions for stepwise Solid-Phase Synthesis by means of Fmoc-Amino Acid Fluorides: 8 molar excess of Fmoc-Amino acid chloride or reagent chloride as a 0.3-0.5 molar solution in DCM was neutralised with DIEA at -5°C and added to diamine-resin prewashed in DMF. For the

PyBOP activation was essentially used the protocol of Goodman et al. in the presence of 6 molar excess of activated carboxyl component (Spenser, J.R., Antonenko, V.V., Delaet, N,G.J., and Goodman, M., Int. J. Pept. Prot. Res., 40, 282-293, (1992)). The coupling was continued from 1 to 12 hr at room or elevate temperature. The coupling was monitored by RPHPLC. The peptide chain assembly was carried out manually or automatically on 0.02-0.2 mmol scale. The coupling steps were monitored with qualitative or quantitative ninhydrin test, 2,4.6-trinitrobenzenesulfonic acid, chloranyl test or spectrophotometric determination of Fmoc-piperidine adduct. According to these tests and the results of RPHPLC analysis if necessary, double coupling was performed.

The deprotection of the Nα-Fmoc protecting groups was carried out in flow at 3 mL/min and room temperature for 8 minutes (for manual deprotection, three times for 1, 2 and 5 minutes respectively) with piperidine/20% DMF. (For Asp, Asn, Glu, Gln -containing peptides, 0.1 M HOBt was added see Dšlling, R., et al. (1995) in Peptides 1994 (Maia, H. L.S. ed), ESCOM Leiden, The Netherlands, 244-245). At the end of the synthesis the resin was washed with DMF, PrOH(X2) and diethyl ether or DCM and dried overnight under vacuum over KOH.

1.3 Monitoring of N-protected aldehydes by RP HPLC

The reaction mixture containing 20-200 μg N-protected aminoaldehyde was allowed to react with 20-100 molar excess of 1M semicarbazide in 1.5 M NaOAc buffer pH 4.5. in an equal volume of MeOH. If necessary, pH was corrected to pH 4.5-5 and after 2 min a volume corresponding to 5-100 μg aldehyde was injected for monitoring the purity of crude or unreacted N-protected aldehydes by RPHPLC at $3.5 < pH < 7.0$.

1.4 Cleavage of protected diamine

a. cleavage of protected diamine containing acid sensitive protecting groups with low concentration of TFA

The dried resin-bound diamine (0.02 mmol) was preswollen for 20 min in

DCM, placed into 5 mL plastic tubes fitted with 20 μm Teflon filter and then cleaved by eluting with 1% TFA in DCM (flow 6 mL/min, 7-8 min elution) into a flask containing 15 ml 5% pyridine in MeOH. The mixture was evaporated to dryness in vacuo and purified by HPLC. Purified peptides were analysed by HPLC and MS.

b. cleavage and full deprotection of diamine containing acid sensitive groups.

For pilot deprotection studies, portions of the crude partially deprotected peptide were added to cleavage cocktails containing TFA and appropriate carbonation scavengers and shaken at room temperature at various intervals. Portions from the cleavage cocktail were diluted several times with MeCN, MeOH or H_2O, eventually extracted with diethylether and analysed by HPLC. The main component and/or the total mixture were collected and analysed by ESMS.

The aldehydes were synthesised from Fmoc-Xaa-N(Me)OMe according the racemization free method of Fehrentz and Castro Fehrentz, J.A. and Castro, B. Synthesis, 676-678 (1983), as modified by others Fisher, L.E. and Muchowski, J.M., Org. Prep. Proced. Int., 22, 399-404; Hocart, S.J., J. Med. Chem., 33, 1954-1958 (1990), with the exception that the activation of the carboxylic group was carried out using HBTU/HOBt instead of the BOP reagent. Corresponding Nα-protected N,O-dimethyl oxime was reduced with $LiAlH_4$ for 20 min at -5 to -10°C and was used without further purification Fehrentz, J.-A., Tetr. Lett., 36(43), 7871-7874 (1995). Alternatively, aldehydes were synthesised by oxidation of N-protected amino alcohols with sulfur trioxide/pyridine complex (see Pennigton, M.W.(1994) Solid-phase peptide synthesis of peptides containing the CH_2-NH reduced bond surrogate: in M.W Pennigton and B.M. Dunn (eds.) Methods in Molecular Biology, vol. 35: Peptide Synthesis Protocols. Humana Press, Totowa, N. J., pp 241-247 and references cited therein).

2.0 Methods for synthesis of N-protected resin bound diamine

2.1 General method A

0.1 mmol of resin (NH_2-groups 0.15 - 0.37 mmol/g) was washed with DMF/1% AcOH, DMF/toluene (1:1). The resin acetate form, was filtered and 2 mL DMF/1% AcOH (1/1, v/v) containing 0.5 - 1 mmol aminoaldehyde were added. The crude N-protected aldehyde (2-4 molar excess) was freshly prepared, dried by two time evaporation from toluene, 10 mL each one at 35-40°C and presented a purity of 75-95%. The reaction mixture was shaken for 5 min at room temperature; 5 mL toluene were added and then evaporated in vacuo at 30-40°C (residual pressure 30-40 Pa.). After 25 min new 5 mL toluene were added and re-evaporated at the same condition. At this stage the substitution (Fmoc-piperidine adduct spectrophotometric determination) of the washed resin was 85-99 % in respect to the initial substitution (after washing the resin with DMF and ethanol, 2,4,6-TNBS-test should be negative). The purity of the aldehyde after 8 min cleavage with 1% TFA in DCM was 87-97%. Several new hydrophobic impurities were detected in RPHPLC analysis. The resin was filtered, washed with DMF, PrOH(X2), DMF, 3% DIEA in DMF, toluene and DMF/toluene (1/1, v/v), containing 0.5%-1% AcOH. The resin was mixed by nitrogen bubbling and 4-10 equivalents $NaBH_3CN$ dissolved in minimal volume DMF were added over a period of 15 min. The average total time for reduction was 30-60 min and as exception no more than 90 min at room temperature. The progress of the reaction was monitored by analytical RPHPLC.

For analysis, in different intervals, small portions (2-3 mg) diamine/aldehyde-resin, were pre washed with DMF, MeOH and DCM, then the diamine was cleaved with 1-95% TFA for 7-30 min according to the starting resin. Collected solvents were evaporated under nitrogen, and the rest dissolved in 50-200 μl MeOH, then 50 μl 1 M semicarbazide in 1.5 M NaOAc, pH 5 were added. The reaction mixture was shaken for 2 min and 5 - 20 μl were injected for monitoring of the progress of the reaction by RPHPLC.

Finally, the resin was washed with DMF, PrOH(X2), H_2O, DMF, DCM/1-3% DIEA, DCM and dried in vacuo.

Kaiser ninhydrin test for 5 min at 90°C of the resin should be negative (red-brown).

2.2 One step enamine (Schiff's base) reduction procedure, convenient for sterically hindered N-protected aminoaldehydes (Leu, Pro, Val, Ile) Method B)

Acetate salt of the amino resin was prepared as above. The aldehyde (2-4 equivalents) in DMF/0.5% AcOH (10 mL/mmol amino resin) was added and the mixture bubbled with N_2. $NaBH_3CN$ (4-10 equivalents) pre-dissolved in a minimal volume of DMF, was added dropwise to the reaction mixture along 5-10 min and the reaction left to proceed for additional 15 to 30 min. The progress of the reaction was monitored by RPHPLC and Kaiser ninhydrine test as above.

2.3 $NaBH_4$ -assisted reduction of the Schiff's base (method C)

Acetate salt of the amino resin (100 mg) was prepared as above, then 5 equivalents aldehyde dissolved in 3 mL DMF/toluene/1% AcOH-49.5/49.9/1 (v/v/v) were added and treated as in method A. This procedure was repeated if necessary (according to TNBS-test) one more time. The resin was pre washed in DMF/MeOH (3/2, v/v) or DMF/toluene/ MeOH (1.5/1.5/2), 3 mL washing solvent was added and 10 equivalents solid $NaBH_4$ were added in small portions over 30 min. The progress of the reaction was monitored by RPHPLC as in method A and generally was complete after 3-6 hrs.

2.4 Borane assisted reduction of Fmoc-Xaa-SA-Resins (Method D , not convenient for Cys, Asp, Glu, Met, Gln, Arg, Trp and Asn)

N-uretan type protected amino acid (Boc, Fmoc, Z and others) was coupled quantitatively with standard procedures (PyBrop, TBTU, HATU or BOP) to SA or MBHA resins for preparation of amide (incl. peptide amide) mentioned and specified above. To 0.03 equivalents N-protected amino acid-resin, washed with THF, 1.5 mL THF, or THF/1-5% AcOH were added and then slowly, over a period of 15 - 45 min were added 5-15 equivalents BH_3.THF as an approximately 1 M solution in THF from RT to the temperature of boiling THF. During the reduction, aliquots (2-5 mg) from the resin were used, after washing and cleavage, for HPLC analysis. The reduction was not complete even after 4 hrs. The resin was filtered, washed with THF, PrOH(X2), DCM and diethyl ether and dried in

vacuo.

3.0 Acylation of secondary amino groups on the resin

3.1 Acid chloride method for coupling of first amino acid to diamine-resin

Fmoc-Xaa-Cl were prepared according Carpino L. et al., J. Org. Chem., 51, 3732-3234 (1986) (for review see Fields, G.B. and Noble, R.L., Int. J. Pept. Prot. Res., 35, 161-214 (1990), p182 and discussion therein.), dissolved in dry DCM (0.5 mmol solution), neutralised at -5°C with DIEA (1 M solution in DMF) and added (5-8 equivalents per amino group) to the pre washed diamine-resin. The reaction was monitored by RPHPLC.

3.2 PyBroP assisted method for coupling of first amino acid to diamine-resin

The resin was prewashed and filtered from DMF or NMP in a 5-20 mL syringe with teflon filter and piston. In a separate tube Fmoc-Xaa-OH and PyBroP (5-8 molar excess each one as a 0.3-0.5 solution in DMF or NMP, neutralised at -10 to 5°C with DIEA (2 molar excess as a 1 M solution in DMF) were dissolved and then immediately transferred to diamine resin. The progress of reaction was monitored by RPHPLC.

4.0 Synthesis of N-protected aldehydes

4.1 Synthesis of Fmoc-D-Phe-N(Me)OMe and Fmoc-D-Phe-H

1.00 g (2.58 mmol) Fmoc-D-Phe-OH (Bachem, B-1410, lot 123087), 0.38 g HCl.HN(Me)OMe (3.87 mmol, 1.5 mol excess), 0.91 g TBTU (2.84 mmol, 1.1 mol excess) 0.43 g HOBt (2.84 mmol, 1.1 mol excess) were suspended in 3.5 mL DMF and 1.61 mL (9.3 mmol) DIEA were added at 0°C while stirring. After 30 min pH was corrected with 0.2 mL DIEA. and the reaction mixture was stirred for 6 hrs at room temperature. The amide was isolated by standard procedure in 98% yield and 99.1% purity, after crystallisation from EtOAc/ petroleum ether (fraction 40-60°C). Fmoc-L-Phe-N(Me)OMe, Fmoc-N(Me)OMe,

Fmoc-Gly-N(Me)OMe, Fmoc-Ahx-N(Me)OMe, Fmoc-Leu-N(Me)OMe, Fmoc-Pro-N(Me)OMe were synthesized at the same conditions.

5. Synthesis of N-protected diamine resins

5.1 Synthesis of Fmoc-$NHCH_2CH_2NH_2$

Preparation of Fmoc-Gly-H: to 1.02 g (3 mmol) Fmoc-Gly-N(Me)OMe in 25 mL THF were added at – 40°C by vigorous stirring 114 mg $LiAlH_4$ (1.3 mL, as a 2.3 M solution in THF). The reaction mixture was stirred at -10 /-5°C for 20 min, and acidified under 5 mL EtOAc with 10% citric acid (8 mL). After 30 min the reaction mixture was diluted to 40 mL with saturated solution of NaCl and extracted five times with 25 mL EtOAc each one. Collected EtOAc extracts were washed with 5% citric acid, saturated solution of NaCl, saturated solution of $NaHCO_3$, again with saturated solution of NaCl, dried over Na_2SO_4, filtered from Na_2SO_4, then washed on the filter three times with 5 mL diethyl ether each one and the collected organic phase evaporated to dryness. Crystallised Fmoc-Gly-H was reevaporated two times with 20 mL toluene at 35°C, resuspended, filtered and washed with petroleum ether. Yield 98%, purity (HPLC) >92%, main impurity 3 % from the starting Fmoc-Gly-N(Me)OMe. The aldehyde was used without further purification.
All other aldehydes were prepared in a similar way.

5.1.1 Loading of the resin (Preparation of Fmoc-$NHCH_2CH_2NH$-SA resin), <u>scheme 1, b</u>

Fmoc-deprotected Sieber amide resin (SA) - 9-aminoxant-3-yloxy-Merrifield resin (0.833 g. 0.3 mmol, NH_2-groups substitution 0.36 mmol/g on the base of spectrophotometric determination at 301 nm of Fmoc-piperidine adduct ($\epsilon = 14700$) upon treatment with DMF/20% piperidine for 10 min at room temperature), was washed first with 1% AcOH in DMF/toluene (1/1, v/v), then with toluene and filtered.

276 mg (0.9 mmol, 3 equivalents) Fmoc-Gly-H (purity 92%) dissolved in 3 mL 1% AcOH in DMF/toluene (1/1, v/v) were added under nitrogen to the acetate salt of the resin. The resin was stirred under nitrogen for 15 min, 3 mL toluene were added and

evaporated. Resin was filtered from the reaction mixture, washed with DMF/toluene (1:1), containing 1% AcOH, new 3 equivalents Fmoc-Gly-H were added as above and the procedure was repeated one more time. The resin was washed with DMF, PrOH(X2), again with DMF and finally with toluene. Several milligrams resin were washed with DCM or DMF and analysed after cleavage.

1. The substitutions of several different batches of the resin, determined on the base of photometric determination of Fmoc-chromophore at 301 nm after deprotection, varied from 0.32 to 0.35 mmol/g or 88.9 -97.2% of the starting substitution.

1.1. 2.5 mg from the resin were cleaved for 10 min at room temperature with 200 µl TFA/DCM/water (47.5/47.5/5, v/v) in DCM (1/1/ - v/v). The resin was filtered and washed with 200 µl DCM. Collected solvents were evaporated in a stream of nitrogen at room temperature, dissolved in 500 µl HPLC buffer B and analysed by RPHPLC. The broad peak, with retention time centred at 19.9 min, corresponded to Fmoc-Gly-H.

5.1.2 Reduction of the Schiff's base. (see table 1 and table 2)

Method C: with 10 molar excess of $NaBH_4$ in DMF/MeOH = 3:2 (v/v) or 40 % MeOH in DMF/toluene=1/1 the reduction was complete (no more detection of Fmoc-Gly-H) after 5 -6 hours. Two main peaks corresponding to Fmoc-$NHCH_2CH_2NH_2$ and (Fmoc-$NHCH_2CH_2)_2NH$ (1:2, ratio) with several not identified side products monitored by RPHPLC.

Method B: Fmoc-deprotected Sieber amide resin acetate salt in the presence of 3 equivalents Fmoc-Gly-H was reduced *in situ* with 10 equivalents $NaBH_3CN$ in DMF, containing 0.5% AcOH for one hour at room temperature; after 15 min reduction, the area of the peak corresponding to Fmoc-$NHCH_2CH_2NH_2$ was 27%, and main peak (Fmoc-$NHCH_2CH_2)NH$ was 53%. This procedure was repeated two times. The main peak corresponding to (Fmoc-$NHCH_2CH_2)_2NH$ as determinated by ESMS was > 85% by HPLC (retention time 24.7 mins). The area of the peak corresponding to Fmoc-$NHCH_2CH_2NH_2$ was <7%.

Method A: portions of 4 - 8 equivalents of $NaBH_3CN$ in DMF, containing 0.5 - 1% AcOH for 30 - 45 min were added at room temperature. Main peak, corresponding to Fmoc-$NHCH_2CH_2NH_2$ was 79 - 85% (retention time 17.7 min). Both Fmoc-Gly-H and (Fmoc-$NHCH_2CH_2)_2NH$ were not detected.

5.2 Fmoc-NH-$(CH_2)_6$-NH-Sieber resin and Fmoc-NH $(CH_2)_6$-NH_2 (method A)

4 mmol (640 mg) $Py.SO_3$ in 2 ml DMSO were added along 10 min to 340 mg Fmoc-$NH(CH_2)_5CH_2OH$ (1 mmol) in 3 ml DMSO, containing 0.3 g NEt_3 at temperature 10-20 ^{o}C. The progress of the reaction was monitored by RPHPLC. The aldehyde was isolated by standard procedure and without purification was dissolved in 10 ml DMF. The solvent was evaporated in vacuo at 40 ^{o}C. The rest was dissolved in 15 ml toluene/DMF=1/1 containing 1 % AcOH and 5 mL from this mixture (0.2 mmol, purity 77%) were added to 100 mg (0.037 mmol) NH_2-Sieber resin, prewashed with the same solvent mixture. After 30 min the solvent was reduced to 1 mL in vacuo at 40^{o}C and evaporated to dryness after 1 h. The resin was washed with DMF and new 5 mL from aldehyde containing mixture were added and Schiff's base formation procedure was repeated one more time at the same condition. The resin was washed with DMF, PrOH(X2), DMF and DMF/MeOH (3/2 -v/v)., then was swollen in 2 mL DMF/MeOH (3/2) and Schiff base was reduced for 4 hrs at room temperature with 30 mg solid $NaBH_4$ added in three portions for a period of 30 min each one. After 60 min 87% , and after 3 h - 95% conversion of aldehyde to Fmoc-$NH(CH_2)_6NH_2$ was detected by RPHPLC and MS. The resin was washed with DMF/MeOH (1/1), H_2O, PrOH(X2), DCM and dried in vacuo.

RPHPLC analysis with a gradient from 10 to 70 % MeCN for 25 min at 1. 5 ml/min showed the following retention times: alcohol - 20.58 min; aldehyde - 22.7-22.9 min ; Fmoc- NH-$(CH_2)_6$-NH_2 - 16.48 min; resin loading was 0.32 mmol/g (89%) on the base of spectrophotometric determination of Fmoc/piperidine adduct at 301 nm in DMF; ES MS (H^+): 339.0 (calculated mass: 338.45-average), see Table 2.

This assay was repeated two times with 0.3 g (0.111 mmol) and 0.5 g (0.185

mmol) resin and determined substitutions were 0.34 mmol/g and 0.31 mmol/g resin, respectively.

5.3 Fmoc-Pro-Ψ[CH_2NH]-TGR resin (table 1, method B)

0.5 mmol (160 mg) Fmoc-Pro-H with purity 75% (3.75 equivalents) were added in 2 mL DMF/1% AcOH to AcOH salt of 0.1 mmol (500 mg, 0.2 mmol/g) TGR-resin pre swollen and filtered in toluene. Reaction mixture was stirred under nitrogen and solid 1 mmol $NaBH_3CN$ was added in portions over 30 min. After additional 30 min, the determined substitution of the resin was 0.198 mmol/g and the Kaiser test was negative (the resin had a red-brown colour); after cleavage of 5 mg resin with 95% TFA, 2.5% H_2O, 2.5% TIPS over 45 min at room temperature, the area of the main peak in RPHPLC was 83% with RT=21.0 min (0-100% B over 33.33 min), corresponding to Fmoc-Pro-Ψ[CH_2NH_2]. The level of by-product, corresponding to dialkylated resin was approximately 1.2%

Notes: Replacement of the Rink handle with the Sieber one (TGSA; 0.15 mmol/g) at the same reaction condition gave a mixture of products including Fmoc-Pro-Ψ[CH_2NH(Fmoc-Pro-Ψ[CH_2]) with RT = 24.4 min; the peaks area ratio with RT=21.0 (monoalkylated resin) in respect to RT= 24.4 min (dialkylated resin) was 2/1;

A similar result was observed with **Fmoc-Leu-H.**

6.0 Examples of acylation of secondary amino groups on the resin

6.1 HATU assisted synthesis of Fmoc-NH-$(CH_2)_6$-N(Biot)-SA resin and Fmoc-NH-$(CH_2)_6$-NH(Biot)

100 mg of the resin (example 3.1), pre swollen in DMF, was suspended in 0.5 mL NMP, containing 59 mg biotin and 91 mg HATU and sonified (Branson 2200 ultrasonic cleaner, Bransonic Co, Danbury, Connecticut, USA) for 2 min at room temperature. Then 84 µl DIEA were added and sonified for additional 10 min at 40-45°C. The progress of the reaction was monitored by RP HPLC (for the cleavage see above) and showed a conversion

of 89 % in 30 min and 95 % in 45 min. The area of the new peak monitored by HPLC (retention time 19.25 min) was 85.5% and the product had the correct molecular mass as calculated for the formula $C_{31}H_{39}N_4O_4S_2$ (563.74, average): $(M+H)^+$: 565.2; 582.4 = $+NH_3$; 587.2 = +Na; 603.2 = +K

6.2 TBTU/HOBt assisted synthesis of 4-BzBz-NH$(CH_2)_6$NH(Fmoc);N1-[(4-benzoyl)-benzyl)]-N6-(9 fluorenylmethoxycarbonyl)-hexyl-1,6-diamine. (see Table 2)

143 mg (0.031 mmol) of Fmoc-NH$(CH_2)_6$NH-SA resin (substitution 0.22 mmol/g) were swollen in DCM (20 min), filtered and washed with NMP. The reaction mixture containing 0.25 mmol (8 mol excess) 4-benzoylbenzoic acid (57 mg), 0.25 mmol (8 molar excess) HOBt (38 mg), 0.25 mmol (8 mol excess) TBTU (80 mg) as a 0.5 M solution in NMP was sonified for two minutes and then added to the resin. After two minutes of mixing by nitrogen bubbling, 0.5 mmol (16 molar excess) DIEA (87 μl) were added and the resin was stirred for 45 min at 45 ^{o}C and 6 hrs at room temperature. The resin was washed by standard procedure, 5 mg. were pre swollen in DCM and the reaction product was cleaved from the resin as described above. The analysis of this result shows 53% conversion to the desired compound, 33% unreacted Fmoc-NH$(CH_2)_6$ NH_2 and several unidentified very hydrophobic side products.

6.3 HATU assisted synthesis of 4-BzBz-NH$(CH_2)_6$ NH(Fmoc-); N1-[(4-benzoyl)-benzoyl)]-N6-(9-fluorenylmethoxycarbonyl)-hexyl-1,6-diamine

100 mg (0.022 mmol) of Fmoc-NH$(CH_2)_6$NH-Sieber resin were acylated with 4-benzoylbenzoic acid 22 mg (0.1 mmol), HATU (38 mg, 0.1 mmol) and DIEA (35 μL, 0.2 mmol) as a 0.45 mM solution in NMP for 1 h at room temperature. The resin was then treated as above. The HPLC-analysis showed 78.3 % conversion to the requested compound with 13% unreacted product and side reaction products.

6.4 Synthesis of 4-BzBz-NH$(CH_2)_6$NH(Fmoc-); N1-[(4-benzoyl)-benzoyl)]-N-(9-

fluorenylmethoxycarbonyl)-hexyl-1,6-diamine by the method of symmetrical anhydrides;

0.2 mmol (25.2mg) N,N'-diisopropylcarbodiimide were added to a suspension of 90 mg (4 mmol) of 4-benzoylbenzoic acid in 0.8 mL DCM. During the progress of the reaction, the material in suspension was completely dissolved and after 10 minutes the crystallised 4-benzoylbenzoic acid anhydride, without further purification, was added, together with 0.3 mmol (45 μl) DIEA to 100 mg (0.022 mmol) Fmoc-NH$(CH_2)_6$NH-SA resin. The resin was sonified for 5 min, mixed for one hour at room temperature and treated as above. The results from HPLC analysis and MS are presented in Table 2.

6.5 Synthesis of Fmoc-Phe-Ψ[CH_2NH(Boc)]

50 mg Fmoc-Phe-Ψ-[CH_2NH_2]-SA resin (0.33 mmol/g, 0.016 mmol) were pre swollen in DCM and allowed to react with 0.1 mmol (22 mg) Boc_2O in 0.2 mL DMF, containing 5 μL DIEA for 2 h at room temperature. According to RPHPLC analysis (RT 28.6 min; area under main peak 92.3%), Boc-capping was quantitative.

Under the same conditions were synthesised **Fmoc-Leu-Ψ[CH_2NH(Boc)]-SA** and **Fmoc-Leu-Ψ[CH_2NH(Boc)]-TGSA** resins.

7.0 Application of the method in the synthesis of peptides

7.1 C-terminal reduced amide bond Leucine enkephalin amide (H-Tyr-Gly-Gly-Phe-Leu-Ψ[CH_2NH_2])

100 mg resin Fmoc-Leu-Ψ[CH_2NH(SA)], prepared according to general **method B**, with substitution 0.33 mmol/g (0.03 mmol) were capped under sonification with 30 equivalents Boc_2O in DMF containing 0.2 equivalents DIEA for 0.5 hr at 40 ^{o}C (quantitative). The resin was then used for preparation of the compound described in the title by manual SPPS (protocol C) using 5.5 molar excess of amino acid/TBTU/HOBt and 11 molar excess of DIEA in NMP. The resin was washed by standard procedure, preswollen in

DCM and cleaved with 95% TFA/2.5%TIPS/2.5% H_2O (2.0 mL) for 45 min at room temperature, filtered and washed with DCM; finally the collected filtrates were evaporated in flow of N_2. Yield of the crude product was 92%. The rest was dissolved in 1 mL water and analysed by HPLC (the area of the main peak was 75-82%; retention time 14.75 min) and ESMS. 0.1 ml AcOH was added to the crude peptide solution, extracted 4 times with 2 ml diethyl ether each one and the water phases were purified by preparative RPHPLC (first run for 30% of crude product: gradient - 0-40 % B over 40 min; second, for the rest of the product, 0-40% B over 60 min, at flow rate 10 mL/min). The yield of purified H-Tyr-Gly-Gly-Phe-Leu-$\Psi[CH_2NH_2]$ was 42 %. (Purity > 97.8 % , RT=14.2 min, gradient 0-60% B over 30 min at flow rate 1mL/min). For other analytical data see table 2.

7.2 C-terminal reduced amide bond, N'-terminal biotinylated leucine enkephalin amide (H-Tyr-Gly-Gly-Phe-Leu-$\Psi[CH_2NH(biotinyl)]$)

144 mg Fmoc-Leu-$\Psi[CH_2NH(biot)]$-TGSA resin (substitution 0.14 mmol/g, 0.02 mmol, see table 2) was used for automated continuos-flow SPPS (protocol B) at room temperature and coupling solvent DMF. Fmoc- was removed by was piperidine/DMF (1/4, v/v) for 8 minutes. Fmoc-protected amino acid/TBTU/ HOBt/DIEA was 1/1/1/2 ratio at 5 molar excess. Following chain assembly, the protected peptide-resin was washed with DCM, propan-2-ol, diethyl ether and dried in vacuo. A portion of the resin (3 mg) was placed into a 1 mL syringe fitted with Teflon filter, pre swollen in DCM and filtered. The peptide was cleaved/ deprotected with 95%TFA, containing 2.5% TIPS and 2.5% water for 40 min at RT, the resin was washed with cleavage mixture and the collected filtrates were evaporated under nitrogen, dissolved in 1.5 mL water,/10% AcOH and analysed by RPHPLC and ESMS. The results are presented in table 2. The rest from the peptide-resin was cleaved/deprotected for 1 h at the same condition (20 mL cleavage mixture per gram resin) and the compound isolated by preparative RPHPLC (0-40% B over 60 min at 10 ml/min). Yield 55% with purity >98.2%.

7.3 Synthesis of C-terminal modified Leu-enkephalin analogues

(7, 8 and 38-51, table 2, scheme 2); (**A**-Tyr(X)-Gly-Gly-Phe-Leu-Ψ[CH_2NH(**B**-Ahx)] where **A**=Fmoc; Boc, H; **X**=But, H; **B**=Dde; H; Boc-Aoa-; H-Aoa;)

0.22 g Fmoc-Leu-Ψ[CH_2NH_2]-SA resin (0.34 mmol/g), prepared according to procedure C, was allowed to react in NMP with 4 equivalents Dde-Ahx-OH in the presence of 5 equivalents PyBroP and 10 equivalents DIEA for 10 min at -10 ^{o}C and 6 hrs at room temperature. The time for quantitative coupling of Dde-Ahx-OH to the resin was experimentally determined in a separate test experiments.

Leu-enkephalin analogues were synthesised manually on the resin according to protocol C with 4 molar excess of Fmoc-protected Phe, Gly, Gly and Tyr(But) in reaction solvent NMP and coupling reagent TBTU. Several intermediate products were also characterized.(see table 2). Final substitution of the resin was 0.32 mmol/g). After a final cycle of Fmoc-deprotection, the resin was capped with 10 equivalents Boc_2O and two equivalents DIEA in DCM for 1 h at room temperature. The Dde-group was deprotected with 2% hydrazine in NMP (4 times for 3 min each one) and washed with NMP. The progress of the reactions at all stages was monitored by Kaiser ninhydrin test (modified for Fmoc strategy, Byk, G. *et al.*, Tetr. Lett., 38(18) 3219-3222, 1997, RPHPLC and ESMS (table 2). This procedure was repeated one more time with 300 mg resin.

130 mg portions (0.03 mmol) from Boc-Y(But)GGFL-Ψ[CH_2NH(**H**-Dde)]-SA resin were allowed to react separately with 8 equivalents Boc-Aoa-OH (TBTU, protocol C), respectively Dns-Cl in the presence of two equivalents DIEA in DMF at 0.4 mmol solution in NMP for 1.5 hrs and A-Y(But)GGFL-Ψ[CH_2NH(**B**-Dde)], where A=Boc, H; B=Boc-Aoa, Dns. Protected amines were cleaved or cleaved/deprotected respectively with DCM/1% TFA (8 min) or 95% TFA (45 min), containing 2.5% TIPS and 2.5% water. Analytical data for these compounds are shown in table 2.

7.4 Preparation of C-terminal modified analogues of human calcitonin (31-35 A-Gly-Val-Gly-Ala-Pro-Ψ[CH_2NH(B-Ahx)] or A=H-; Boc-; B=H- or Dabs-(see Scheme 2, Table 2, Scheme 3))

Fmoc-Pro-Ψ[CH_2NH(Dde-Ahx)]-TGSA (420 mg, 0.14 mmol/g, 0.059 mmol)

was used for manual synthesis (modified for Fmoc-strategy protocol A) of peptide resin **Boc-Gly-Val-Gly-Ala-Pro-Ψ[CH_2NH(Dde-Ahx)]-TGSA**. At the last coupling cycle the peptide synthesis was carried out with Boc-Gly-OH and then washed with DMF, PrOH(X2) and DCM. During the synthesis aliquots from the peptide-resin were cleaved/deprotected and analysed with RPHPLC and ESMS. (see Table 2)

5 mg peptide resin were cleaved with DCM/1% TFA, containing 2.5% TIPS in flow for 8 min . The solvents were evaporated immediately in vacuo at room temperature. The peptide mixture was separated by RPHPLC. The main peak, corresponding to **Boc-Gly-Val-Gly-Ala-Pro-Ψ[CH_2NH(Dde-Ahx)]** (see Table 2) has correct mass determinated by ESMS.

The rest from the resin was washed with DMF and Dde-deprotected with NPM/2% hydrazine for 15 min at room temperature (with 5 mL portions each one for 1, 6 and 8 min respectively), washed with DMF, PrOH(X2) and DCM. A portion of 5 mg peptide-resin was cleaved with DCM/1% TFA, containing 2.5% TIPS in flow for 8 min. The solvents were evaporated immediately in vacuo at room temperature. The peptide mixture was separated by RPHPLC. The main peak, corresponding to **Boc-Gly-Val-Gly-Ala-Pro-Ψ[CH_2NH(H-Ahx)]** has correct mass determined by ESMS.

The rest from the resin was separated in two identical portions . First one was cleaved/deprotected for 45 min with 95% TFA, containing 2.5% TIPS and 2.5% water and the peptide **H-Gly-Val-Gly-Ala-Pro-Ψ[CH_2NH(H-Ahx)]** then elaborated with a standard ether extraction work-up procedure. The second, was capped with Dabsyl chloride (Dabs-Cl, 4 equivalents) in the presence of two equivalents DIEA and 5 equivalents pyridine for 1 hr at room temperature. The peptide-resins were washed with DMF, PrOH(X2) and DCM and peptides were cleaved/deprotected with 95% TFA, containing 2.5% water, 2.5 % TIPS for 45 min at room temperature. The peptide **H-Gly-Val-Gly-Ala-Pro-Ψ[CH_2NH(Dabs-Ahx)]** was isolated by standard procedure, purified by preparative RPHPLC and characterised by amino acid analysis and ESMS (see Table 2)

8.0 Preparation of samples for racemization test

8.1 Fmoc-D-Phe-Ψ[CH_2NH_2]-SA resin

556 mg SA resin (0.2 mmol, 0.36 mmol/g) was Fmoc-deprotected with 20% piperidine in DMF (v/v) - 3 times with 5 mL (for 1, 3, and 5 min respectively), washed with DMF, MeOH, DMF/1% AcOH, toluene and 149 mg (0.4 mmol, two equivalents) Fmoc-D-Phe-H in 2 ml 0.5% AcOH in DMF were added. After 5 min 3 mL toluene was added and then evaporated in vacuo (oil pump) at 30-35°C (5 min). After 20 min new 3 ml toluene were added and re-evaporated at the same conditions. The resin was filtered, washed with DMF, toluene, new 0.4 mmol (two equivalents) Fmoc-D-Phe-H in 2 ml 0.5% AcOH in DMF were added and the above described procedure was repeated one more time. (TNBS-test on this stage was negative). Resin was washed again with DMF, MeOH, toluene, DMF/1% AcOH (v/v), toluene and reduced in 3 mL DMF/1%AcOH with $NaBH_3CN$ (85% purity, 4-10 equivalents, dissolved in 0.3 ml DMF, added a portions by stirring for first 15 min under nitrogen). The resin was mixed under nitrogen for additional 30 min; total time for reduction was 45 min (only 1.0 - 1.5% aldehyde remains unreduced; area of the main peak >90.3%, conversion 98.3 %).

At the same conditions from 666 mg (0.1 mmmol, 0.15 mmol/g) and 333 mg (0.05 mmol) TGSA resin **Fmoc-D-Phe-Ψ[CH_2NH]-TGSA** resin (loading 0.143 mmol/g) and **Fmoc-D-Phe-Ψ[CH_2NH]-TGSA** (loading 0.140 mmol/g) were synthesized (see table 2)

8.2 Fmoc-D-Phe-Ψ[CH_2NH(Boc)]; Fmoc-Leu-D-Phe-Ψ[CH_2NH(Boc)]; and H-Leu-D-Phe-Ψ[CH_2NH_2]

120 mg Fmoc-D-Phe-Ψ[CH_2NH_2]-SA resin (0.35 mmol/g, 0.042 mmol) was swollen in DCM, filtered and then 0.42 mmol (92 mg, 10 molar excess) of di-tert-butyl dicarbonate (Boc_2O) in 250 μL DCM, containing 7.5 μl (1 equivalent) DIEA were added. After 2 h at room temperature, (Kaiser test was negative) resin was washed with DCM, DMF, PrOH(X2), again with DCM and 5 mg were HPLC analysed after 8 min deprotection in flow with DCM/1% TFA, containing 2.5 % TIPS. The main peak from RPHPLC (retention time 28.6 min, 94.3%, gradient 0-100% B over 33.33 min), corresponding to **Fmoc-D-Phe-Ψ[CH_2NH(Boc)]** was collected and analysed with ESMS. The rest from the

resin was washed with DMF and Fmoc-deprotected (3 times with DMF/20% piperidine 1.5 mL each one for 1, 3 and 5 min respectively), washed carefully with DMF and then coupled with 6 equivalent Fmoc-Leu-OH/TBTU/HOBt/DIEA (1/1/1/2), protocol C in NMP as solvent for coupling for 90 min at room temperature.

The resin was washed, again 5 mg resin was deprotected and isolated main peak from HPLC (retention time 29.4 min, 82.2%, gradient 0-100% B over 33.33 min), corresponding to **Fmoc-Leu-D-Phe-Ψ[CH_2NH(Boc)]** was analysed with ESMS. After standard Fmoc-deprotection , the rest from the resin was cleaved with 2 mL 2.5% TIPS in DCM/TFA (1/1, v/v) for 45 min at room temperature (**H-Leu-D-Phe-Ψ[CH_2NH(Boc)]**, RT=34.9 min, gradient 0-60% B over 60 min at 1.5 mL/min, 214 nm). The resin was filtered, washed with two portions of 0.5 mL DCM, collected filtrates were evaporated in stream of nitrogen and the rest redissolved in 1 mL DCM and reevaporated *in vacuo*. The crude peptide mixture [main peak from RPHPLC and MS analysis corresponding to **H-Leu-D-Phe-Ψ[CH_2NH_2)]** was used for racemization assay together with **H-Leu-Phe-Ψ[CH_2NH_2)]**, prepared from **Fmoc-Phe-Ψ[CH_2NH]-SA resin** (see next example).

8.3 Fmoc-Phe-Ψ[CH_2NH(Boc)]; Fmoc-Leu-Phe-Ψ[CH_2NH(Boc)]; and H-Leu-Phe-Ψ[CH_2NH_2]

Starting from 177 mg Fmoc-Phe-Ψ[CH_2NH]-SA resin (Fmoc loading 0.35 mmol/g. 0.062 mmol) and following the conditions described in above example, titled compounds were synthesised, isolated and analysed. (see table 2 Fmoc-Phe-Ψ[CH_2NH(Boc)] retention time 28.6 min; area under main peak 92.3%).

9.0 Synthesis of Fmoc-Pro-Ψ[CH_2NH(Dde-Ahx)]

Dde-Ahx-OH was synthesized according Bycroft (Bycroft B.W., Chan, W.C., Chhabra, S.R., Teesdale-Spittle, P.H and Hardy, P.M., J. Chem. Soc. Chem. Commin., 776-777(1993)), crystallised from ethyl acetate/petroleum ether (fraction 40 -60 ^{o}C), isolated with 86% yield and RPHPLC analysed (gradient 0-100 % B over 33.33 min, purity 99.4%,

RT= 17.3 min)

0.5 g (loading 0.14 mmol/g, 0.06 mmol) **Fmoc-Pro-Ψ[CH_2NH]-TGSA-resin** was allowed to react with 0.6 equivalents Dde-Ahx-OH/TBTU/HOBt = 1/1/1 (each one) and 1.2 equivalents DIEA as a 0.4 M solution in NMP for 45 min at 30 - 45 ^{o}C by sonification (at this time according to RPHPLC analysis the ratio of acylated to starting compound was 76/17) and then without sonification overnight at room temperature. The peak with retention time 21.7 min, corresponding to **Fmoc-Pro-Ψ[CH_2NH_2]** completely disappeared, titled compound was formed (retention time 27.2 min, 92.0%), the area of the peak that corresponded to Dde-Ahx-NH_2 was 0.2% , i.e. the conversion was >99.5%. The resin was used for preparation of analogues of human calcitonin (28-32).

RPHPLC analysis of the results of reaction between **Fmoc-Pro-Ψ[CH_2NH]-TGR-resin** (with loading 0.19 mmol/g) and Dde-Ahx-OH at the same reaction conditions shows only 1% reaction to titled compound and more then 90% from the total area corresponds to **Fmoc-Pro-Ψ[CH_2NH_2].** Similar result was registered when Trt-Ahx-OH instead of Dde-Ahx-OH was used.

10.0 Synthesis of symmetrically acylated diamines

10.1 Synthesis of symmetrical dimer of MSH-release inhibiting factor (H-Pro-Leu-Gly-NH-CH_2-)$_2$

Preparation of the starting resin (PyBroP-method):

Fmoc-Gly-Ψ[CH_2NH_2]-TGSA resin (0.200 g, 0.028 mmol) was Fmoc-deprotected and then allowed to react with 12 equivalents Fmoc-Gly-OH and 12 equivalents PyBroP in 0.9 mL NMP/DCM (1:1) in the presence of 24 equivalents DIEA for 5 min at -10 ^{o}C and 12 hrs at room temperature.

The progress of the reaction was monitored by RPHPLC (gradient 0-100% B over 33.33 min). After 15 min only 2% from starting Fmoc-$NHCH_2CH_2NH_2$, remains not reacted and new products, corresponding to Fmoc-Gly-$NHCH_2CH_2NH_2$ (RT=21.0 min) and side reaction product (RT=21.8 min) were formed. After 12 hrs the ratio of areas under the

peaks, corresponding to **Fmoc-Gly-NH_2**(RT=19.2)/ **Fmoc-Gly-$NHCH_2CH_2NH_2$**(RT=21.0)/**(Fmoc-Gly-$NHCH_2$-)$_2$** (RT=27.8 min) was 1:2:10. The coupling was repeated with 6 equivalents Fmoc-Gly-OH for 1 h by sonification at 45°C, when the level of the peak with RT = 21.0 min decrease to 2% from a total area. For manual SPPS was used (protocol C, 6 molar excess TBTU-assisted coupling), 160 mg (0.0416 mmol, loading 0.26 mmol/g) resin. After the last coupling 2 mg peptide-resin were cleaved and (Fmoc-PLG-$NHCH_2$-)$_2$ was isolated and characterised with ESMS and amino acid analysis. (see table 2.) The rest from peptide resin was Fmoc-deprotected, washed with DMF, PrOH(X2) and DCM and cleved with 95% TFA/2.5% TIPS/ 2.5% H_2O for 30 min at room temperature and (H-Pro-Leu-Gly-$NHCH_2$-)$_2$ was purified with semipreparative RPHPLC (gradient 0-30%B over 45 min)

10.2 Synthesis of Fmoc-Pro-$NHCH_2CH_2N$(Fmoc-Pro-)-SA

Two portions of **Fmoc-Gly-Ψ[CH_2NH_2]-SA (120 mg, 0.33 mmol/g)** were Fmoc-deprotected and then allowed to react separately first with 8 equivalents Fmoc-Pro-OH/8 equivalents PyBroP and 18 equivalents DIEA, later with 8 equivalents Fmoc-Pro-Cl in DCM/NMP, neutralised (after 80 min the pH was changed to acidic and was adjusted with 40 μl DIEA) with DIEA. (see the methods for coupling the first amino acid). The ratio Fmoc-Pro-$NHCH_2CH_2NH_2$(19.1 min, 0-100% B over 33.33 min)/Fmoc-Pro-$NHCH_2CH_2NH$(Fmoc-Pro-) after 10 min for first and after 36 hrs for second experiment was near to 1:1 and at this stage can be used for preparation of mixtures of C-terminal proline-aminoethylamide or C-terminal symmetrical peptide dimers with 1,2-diaminoethane.

10.3 Synthesis of Fmoc-Leu-Ψ[CH_2NH(Fmoc-Leu)]-SA resin and Fmoc-Leu-Ψ[CH_2NH(Fmoc-Leu)]; Fmoc-Phe-Leu-Ψ [CH_2NH(Fmoc-Phe-Leu)] and H-Phe-Leu-Ψ[CH_2NH(H-Phe-Leu)]

220 mg **Fmoc-Leu-Ψ[CH_2NH_2]-SA** resin (0.32 mmol/g, 0.07 mmol) was swollen in DCM, washed in DMF and allowed to react with Fmoc-Leu-OH (8 equivalents, 198 mg), PyBroP (8 equivalents, 262 mg) and 16 equivalents DIEA (98 μl) as a 0.3 M

solution in NMP for 36 hrs at 37°C,. The area ratio under the peaks after 3 h corresponding to **Fmoc-Leu-Ψ[CH_2NH_2]** and **Fmoc-Leu-Ψ[CH_2NH(Fmoc-Leu)]** was 32% /37%) and after 36 h 64.9 / 1.2 % (See table 2, gradient a, RT=32.2 min).

At the same conditions using **Fmoc-Leu-Ψ[CH_2NH_2]-TGR resin,** the area of the titled compound was below 2%.

Fmoc-Leu-Ψ[CH_2NH(Fmoc-Leu)]-SA resin was used for preparation of titled compound **Fmoc-Phe-Leu-Ψ[CH_2NH(Fmoc-Phe-Leu)] and H-Phe-Leu-Ψ[CH_2NH (H-Phe-Leu)].**

11.0 Reduction of Fmoc-Gly-SA resin with BH_3.THF

Example A

Sieber amide resin 108 mg (loading 0.36 mmol/g) was Fmoc-deprotected and manually coupled with 0.2 mmol Fmoc-Gly-OH/TBTU/HOBt each one and 0.4 mmol, 68 μl DIEA (protocol C) in NMP for 30 min, when Kaiser ninhydrin test was negative. After standard washing, the Fmoc-Gly-SA resin was transferred in a 10 mL syringe equipped with a sinter in bottom Teflon filter and stopped on the top with a septum with inlet needle for introducing reagents or nitrogen and another needle connected to a tube with KOH for protection of reaction mixture from CO_2. The resin was washed with THF and then 2 ml THF were added. The air was substituted with nitrogen and then to the resin vigorously stirred with a magnet bar, was added 0.5 mL BH_3.THF as 1M solution in THF over 15 min (gas liberation). After 30 min RPHPLC analysis (0-100% B over 33.33 min) shown Fmoc-$NH(CH_2)_2NH_2$(RT=17.8 min)/Fmoc-Gly-NH_2 (RT=18.8 min)=40/47 % from the total area, after 2 hr 37/39% and time increasing two peaks with RT 25.3 and 27.2 (4.8/1.7 after 0.5 h to 10.9/7.0 % from the total area). The peaks were identified by ESMS and coinjection with prepared in solution or on the resin Fmoc-$NH(CH_2)_2NH_2$ and Fmoc-Gly-NH_2.

Example B

At the same condition (Example R), solvent for reduction 3 mL THF/5% AcOH, after 1 h , the ratio Fmoc-Gly-NH_2/ Fmoc-$NHCH_2CH_2NH_2$ was 51/41 (25% of the total area), with low level of side reactions (the area of a main impurity (a side product) with

retention time 24.3 min was 2.9%).

Example C

At the same condition Fmoc-Leu-SA resin (120 mg) in 1 ml THF was reduced with 0.7 mL BH_3.THF and after 45 min were dropped THF/5% AcOH over 15 min until no more gas was released (1.8 mL). After 30 min RPHPLC analysis (0-100% B over 33.33 min) shown Fmoc-Leu-$\Psi[CH_2NH_2]$ (RT=18.7 min)/Fmoc-Leu-NH_2(RT=19.9 min) ratio 32/64 (as % of the total area). Increasing reduction time from 45 min to 12 h does not substantially change this ratio.

12.0 Racemization study

Optical purity of L-Phe/D-Phe was investigated, using RPHPLC separation of diastereomeric peptides L-Phe-L-Leu-NH_2/D-Phe-L-Leu-NH_2 (retention times 9.5 and 24.5 min respectively on Vydac 5RP18 (30X4.6 mm i. d.) in TEAP (pH 3.) buffer, gradient 0-16% B in 30 min at flow rate 1.5 mL/min). The pseudopeptide diasteromers L-Phe-$\Psi[CH_2NH]$-L-Leu-NH_2 and D-Phe-$\Psi[CH_2NH_2]$-L-Leu-NH_2 were eluted at 5.7 and 6.6 min respectively under 5-40% TEPA/acetonitrile gradient conditions.

We examined the optical purity of two model peptides Fmoc-L-Leu-L-Phe-$\Psi[CH_2NH_2]$ and Fmoc-L-Leu-D-Phe-$\Psi[CH_2NH_2]$ prepared on the resin using RPHPLC separation of diastereoisomeric peptide at following conditions:

Column: LiChrospher 60 Select B RP18 125x 4 mm i. d. (Hewlett Packard); gradient 0-30% B over 30 min at 1.5 mL/min, buffer A=H_2O/0.1% TFA; buffer B= MeCN/0.1% TFA; Peptide surrogate H-Leu-Phe-$\Psi[CH_2NH_2]$ and H-Leu-D-Phe-$\Psi[CH_2NH_2]$ were eluted at 14.6 and 17.3 min respectively as broad tailed peaks. Racemization was between 1% (method A) and 16.3 % (method C) by coupling with 4 equivalents Fmoc-Leu-OH/TBTU/HOBt/DIEA (1/1/1/2) in DMF for 12 h. Similar results were observed when H-Leu-Phe-$\Psi[CH_2NH(Boc)]$ and H-Leu-D-Phe-$\Psi[CH_2NH(Boc)]$ were used as a test compound. Column LiChrosper 100RP18 125X4 mm i.d, gradient 0-60% B over 60 min in the same buffer system (retention time 32.4 and 34.6 respectively, as good resolved broad peaks).

13.0 Results and Discussion

As a part of our investigation for synthesis of tail to tail peptide and protein dimers by SPPS or by methods of chemical ligation, our aim was to develope a method for the synthesis of selective protected diamine. This method should be technically simple, of a general application and all synthetic steps could be carried out on a diamine attached to the resin (for the advantages of Solid-Phase Organic Chemistry (SPOC) see Früchtel, J.S. and Jung, G, Angev. Chem. Int. Ed. Engl., 35, 17-42(1996)). The problem arose when we tried to find a general method for C-terminal modification of peptides. On the other hand, site-specific chemical modification procedures for peptide and small protein by SPPS are well established and reviewed (Pennigton, M.W.1994. Site-Specific Chemical Modification Procedures: in M. W Pennigton and B.M. Dunn (eds.) Methods in Molecular Biology, vol. 35: Peptide Synthesis Protocols. Humana Press, Totowa, NJ., pp 171-185). The presence of these groups at a specific location offers a number of interesting possibilities for bioassays, citochemisty/immunochemistry, fluorescent microscopy, indirect affinity chromatography, identification of important or essential residues in peptides and protein for understanding the mechanism of an enzyme action or the binding affinity of a peptide ligand to its receptors and many others. Peptide synthesis in solution by using orthogonal protecting groups is a possible alternative to this problem. A second one is the selective chemical modification of amino acid residues in purified peptides or proteins. Both methods are complicated with labour-intensive processes for isolation of desired modified peptide or protein. The second one additionally suffers from the low selectivity of the chemical modification reagents and as a consequence the difficult isolation of the modified peptide/protein from the heterogeneous mixture of close related products. Furthermore, a procedure for the determination of the number and place of the modified residues is very often necessary. For example, the position of the biotinylation (N-, C-terminal or side chain) may be important for optimal efficiency. Side chain thiol or amino groups biotinylation in solution or on the resin (solid-phase) are well established and reviewed. Possible ways for C-terminal biotinylation (e.g. C-terminal modification) of peptides and proteins are: 1) reversible proteolysis with amides of biotinylated diaminocarboxilic acids (biocitin, ω-biotinyl-L-lysinamide) in the presence of

carboxypeptidase Y (with limitation of C-terminal proline); 2) reaction in solution between selectively protected peptide or protein or C-terminal extended aminoalkylamide or hydrazide of peptides or protein with biotin derivatives, and 3) chemical ligation of biotin derivatives and C-terminally modified peptides or proteins with mutually reactive groups.

As a first step we investigated a well known procedure for reductive alkylation of amino acidyl-/aminopeptidyl-resins with N-protected aminoaldehyde, starting directly from amino resins. The resulting protected diamine resin was used for preparation of mono-, deprotected diamines, C-modified and tail to tail homo- and heterodimers of peptides. In our preliminary investigations we chose resins with Rink and Sieber linkers. The compounds from the latter can be cleaved from the resin by short time exposure to DCM/0.5-1%TFA. This permits synthesis of protected diamine, even including groups labile to high concentrated or strong acids and decreases the time for analysis. Several problems arose:

1) formation of dialkylated amino resin during first step of reductive alkylation;

2) racemization of the diamines during reductive alkylation complicated from generally unstable N-protected aminoaldehydes; and

3) elaboration of conditions and methods for acylation of very sterically hindered secondary amines on the resin. (see scheme 1 c,d,e)

N-protected amino aldehydes are relatively unstable both chemically and configurationally, particularly in solution and can racemize during purification with RPHPLC or flash chromatography on silica gel. In the present study we use freshly prepared crude aminoaldehydes by reduction of corresponding N, O-dimethylhydroxamate according to Castro's method. The problems associated with the synthesis of N-protected amino acid aldehydes have been discussed by Fehrentz and Castro (Synthesis, 676-678 (1983)), by Coy et al (Tetrahedron, 44(3), 835-841 (1988) and cited therein) and by Kaljuste and Unden (Int. J. Pept. Prot. Res., 43, 505-511(1994)). Only side chain arylsulfonyl protected arginine hydroxamate can not resist to condition for reduction with $LiAlH_4$ to the corresponding aldehyde. Orthogonally δ-protected ornithine, followed by the amidination of δ-amino group on the peptide-resin should be possible to overcome the problem. Traces of non-reduced hydroxamate or overeduced corresponding alcohol would not react with the amino groups of

the resin, and therefore do not require their removal prior to reductive alkylation. Such a way, Schiff's base formation between of crude aldehydes and the resin is also a method for their purification.(Scheme 1). For example, Fmoc-NH$(CH_2)_5$CHO (purity 78% determinated by RPHPLC), prepared from Fmoc-Ahx-N(Me)OMe by reduction with $LiAlH_4$, was cleaved from the resin with purity more then 95% and correct molecular mass, confirmed by ESMS : theoretical mass for $C_{21}H_{23}H_2NO_3$ (337.42, average; 337.17, monoisotopic) ESMS, molecular mass - in parenthesis are shown relative intensity of the peaks (M+H)+: (225.1(8.4); 276.2 (42.3); 320.1 (100); 338.1 (83.2); 355.1(18.70); 360(3.0); 376(2.3). Peak with molecular mass 320.1, corresponds to dehydrated aldehyde, with 355.1, to mass of aldehyde plus 17 mass units (+NH_3); with 360.0, to the molecular mass of aldehyde plus sodium (+Na); and 376, to the molecular mass of aldehyde plus potassium (+K). Additionally in the range of dimers the following peaks were detected; 607.1(2.3); 657.2(17.6) (dehydrated dimer, without 18 mass unites); 675.1(7.2), corresponding exactly to noncovalent dimer and finally 692.2(2.2) to noncovalent dimer plus 17 mass units (+NH, from the resin). Other Fmoc-protected aminoaldehydes, used as starting products for on resin enamine formation, after cleavage from the resin, have shown similar increasing of their purity in comparison with starting purity, showing correct molecular masses, determined by ESMS.

First we tried to adapt the procedure of Chan et al. (J. Chem. Soc. Chem. Commun. 1475-1477, (1995)) where Schiff's base formation has been achieved by exposure of amino group of the resin bound amino acid to different aldehydes in DMF/0.5-1%AcOH. In our case (Method C, see experimental part) a quantitative enamine formation between Fmoc-protected aldehyde was possible only after repeated procedure of enamine formation with high excess (> 10 equivalents) of Fmoc-amino protected aldehyde (example with Gly or Ahx).

During enamine formation the water liberated from the reaction was eliminated by molecular sieves or irreversible reacted with water reagents. We azeotroped the reaction water with toluene and found as optimal the following conditions: the amino group of the resin was transferred to acetate salt and preswollen in toluene; then 2-3 equivalent N-

protected amino aldehyde (calculated on the base of HPLC purity), dissolved in minimal volume DMF/0.5 - 1% AcOH were added and toluene was evaporated in vacuo at 35-40°C. A new volume of toluene was added and the procedure repeated one more time. Because of the inadequacy of the Kaiser (red-brown colour instead of blue) and chloranyl tests (brown instead of blue), the Schiff's base formation was monitored with quantitative Fmoc-determination or by undestructive dinitrobenzene sulfonic acid (DNBS) tests. Usually, after two times evaporation with toluene the enamine formation was quantitative or light positive according to DNBS-test. A positive test requires a recoupling step with new 2-3 equivalents of N-protected amino aldehyde.

Toluene plays a substantial role in one of the critical stages of the reaction, the Schiff's base formation:

1. polystyrene type resins swell better in toluene and DCM, then in DMF. Limiting factor in solid phase organic reactions is the diffusion of the reactant towards reactive groups on the resin and this factor depends directly from the swelling capacity of the reaction solvent. On the other hand, a correlation exist between the bad solubility of amino component (in this case amino resin) and formation of by-products (dialkylation) at the stage of reductive alkylation .

2. The water liberated from the reaction was azeotropically removed with toluene. This procedure accelerates substantially the reaction, moving the equilibrium, aldehyde/enamine to enamine.

3. It is possible that both effects of concentration and increasing of temperature during evaporation of toluene at 30-35°C play a role in increasing diffusion, solubility and reaction rate.

4. "Salting out" effect of toluene (Fmoc-Xaa-H usually are poorly soluble in toluene) probably also plays a role in accelerated formation of Schiff's base.

An important factor before reduction step of the resin is pre washing (neutralisation) with base in DMF or DCM. Several impurities could be eliminated and the quality of the N-protected diamine-resin could be improved.

A) Two points are critical for obtaining high loading and good quality of the

diamine resin when reducing the enamine resin:

- washing of the aldehyde excess by acidified solvent (DMF/0.5 - 1.0% AcOH or mixtures with toluene or DCM)

- usage of $NaBH_3CN$ for reduction in a second separate step in the above mentioned acidified solvent [Table 1, Scheme 1, reaction product (**3**)]

The reduction step proceeds quickly (30-45 minutes) with 2-5 equivalents $NaBH_3CN$ and was easily performed, without dimers formation, even with not-sterically hindered N-protected aldehydes of Gly or Ahx. Dialkylation could not be prevented if the reduction step was performed in the presence of a small amount of these aldehydes (stechiometric or even below stechiometric quantity). Additionally, the side products were minimal, when sterically hindered resins with Rink handle were used. If an excess of N-protected Gly or Ahx-aldehyde was added during the reduction step or to the already formed N-protected diamine-resin, [Fmoc-$NH(CH_2)_n$]NH, n=2 or 6 were isolated as main products in high yield. Three steps method for synthesis of symmetrical diamino acid have been described by Qasmi, D. et al., Tetr. Lett., 34(24), 3861-3862(1993). Another five step procedure for preparation of symmetrical branched handle (Bambino, F, Brownlee, R.T.C and Chiu, F.C.L., Tetr. Lett., 35(26), 4619-4622 (1994)) have been used for preparation by SPPS of asymmetrical or symmetrical dimers of peptides. Chloromethylated polymers have been modified to dinitriles in condition of phase-transfer catalysis, then reduced with excellent yield to corresponding diamine-resins and have been used extensively to prepare selective metal ions polymer complexing regents. Our method, one pot procedure for preparation of resin bound handles [Fmoc-$NHCH_2CH_2NH)_2$N-Resin] allows as additional opportunity the preparation of protected and deprotected symmetrical dimers, which could be, if desired, N-terminal modified in solution on the secondary amino group after cleavage of protected dimer (A-$NHCH_2CH_2NH)_2$N-M, where M= modifier, A=peptide (protected or deprotected) or acyl- /aryl residue.

Racemization (see below and experimental part) was below 1% judged from racemization test, diastereoisomer separation of H-Leu-Phe-Ψ[CH_2NHX] and H-Leu-D-Phe-Ψ[CH_2NHX], where X-Boc or H.

B) Direct *in situ* reductive alkylation of the resin with Rink handle was successfully achieved with 3-4 equivalents Fmoc-protected sterically hindered amino aldehyde (Leu and Pro, loading up to 98% from the starting resin loading) in acidified DMF with excess of $NaBH_3CN$. This method was not convenient for reductive alkylation of resins with Fmoc-protected Gly or Ahx, because a mixture of two products was isolated: Fmoc-$NH(CH_2)_nNH_2$ and [Fmoc-$NH(CH_2)_2]_2NH$; where n=2 or 6. This side reaction is more pronounced in case of SA and especially TGSA-resins. For example, RPHPLC analysis of a 20 min reductive alkylation of TGSA-resin with 4 molar excess Fmoc-Leu-H and $NaBH_3CN$ shows Fmoc-Leu-$\Psi[CH_2NH_2]$/Fmoc-Leu-$\Psi[CH_2NH$(Fmoc-Leu-$\Psi[CH_2$-)] in ratio 10:1. (area ratio 71.5:15.2); on the contrary no formation of a side product was observed when TGR-resin was used at the same conditions. A side reaction has been described by Coy et al in the case of reductive alkylation of amino acid or peptide-resins with N-protected glycine, a similar side reaction has also been noted in solution. In these cases, the formation of a second iminium moiety proceeds at least as fast as the first one. Three factors can influence and explain the formation of dimers on the resin :

1.) very low local concentration of amine component respect to aldehyde;

2.) sterical factors, resulting from sterically hindered amino aldehydes A-Xaa-H or used resin ;

3.) stabilisation of desired intermediate Fmoc-Xaa-$\Psi[CH_2NH$-Resin] in cases when hydrogen bond between Fmoc-carbonyl of amino acid and ammonium hydrogen - $CH_2NH_2^+$-Resin occurs.

This side reaction, reductive alkylation of reduced peptide bond analogues (secondary amines); has been successfully used for preparation of backbone-to-backbone cyclic peptides. Protection of the already formed secondary amine on the resin from acylation/reductive alkylation has been proposed; this protection can be achieved by reacting the imino group containing peptidyl resin with benzyl chloroformate (Carbobenzoxy-chloride, Z-Cl) or other convenient orthogonal protecting group Pennigton, M.W.(1994) Solid-phase peptide synthesis of peptides containing the CH_2-NH reduced bond surrogate: in M.W Pennigton and B.M. Dunn (eds.) Methods in Molecular Biology, vol. 35: Peptide Synthesis Protocols. Humana

Press, Totowa, N. J., pp 241-247 and references cited therein, Wen, J.J. and Spatola, A.F., Int. J. Pept. Res., 49(1), 3-14 (1997) and references cited therein.

C.) A method similar to method **A** but wherein the reduction of the Schiff's base on the resin is carried out in separate step after washing the excess of aldehyde with 10 molar excess of $NaBH_4$ in DMF/MeOH (3:2). In the present case the reduction proceeds smoothly (complete reduction of Fmoc-Phe-Ψ[CH-NH-Resin)] for 5 - 6 hrs; with formation of dialkylated byproducts when Gly and Ahx aldehydes were employed.

Other side reactions were observed, probably due to instability of Fmoc-protection in reduction conditions. Only when sterically hindered aldehydes like Fmoc-Leu-H or Fmoc-Pro-H were used for Schiff's base formation, acceptable level (75-92% from the starting resin substitution) without dimer formation was achieved. When Fmoc-Phe-H was used for enamine formation, racemization up to 15% was detected (see experimental part and racemization study). Using Fmoc-Leu-Ψ[CH_2NH]-resin, prepared according this procedure, for synthesis of C-terminal reduced peptide bond Leu-enkephalin amide analogues, additional peaks (7%) with the same molecular mass were isolated by RPHPLC. A lower concentration of acetic acid up to 0.5%, decreases but does not eliminate completely (3%) the problem of racemization of phenylalanine.

When low loaded polyethylenglycol type TGSA -resin was used, the reduction of enamine proceeded for 2-3 hrs, probably because the resin was better swollen in MeOH.

Finally, it was found that conditions using BH_3.THF assisted reduction of N-protected amino acid resins using small or large excess of BH_3.THF at room temperature or in boiling THF at neutral, basic or acidic conditions resulted in incomplete reduction of amide bond. Fmoc-NH-$CH_2CH_2NH_2$, together with unreduced Fmoc-Gly-NH_2 was isolated. Using all these conditions RPHPLC analysis detected side reactions, due probably to instability of Fmoc-protection during the reduction. Similar results were observed when Fmoc-Leu-SA resin was subjected to reduction. These resins can be used for preparation by SPOC of two different compound in the same time: C-terminal amides and C-terminal reduced peptide [CH_2NH_2] surrogate. The new imino group, resulting from reduction could be orthogonally protected.

Acylation

In a preliminary experiment, acylation of resulting secondary amino group on N-protected diamine-resin, which serves for C-modification, proved to be easy in the case of not-sterically hindered diamine-resin with not hindered acylation reagents, but it revealed to be very difficult for hindered ones. For example acylation by Fmoc-Leu-OH of Fmoc-Leu-Ψ[CH_2NH]-TGR resin and partially Fmoc-Leu-Ψ[CH_2NH]-SA did not, respectively react slowly and not quantitatively; more vigorous, double or prolonged couplings, changing of coupling methods or solvent and elevate temperature in order to have acceptable acylation rates lead to several side reactions. On the other hand, acylation of Fmoc-Leu-Ψ[CH_2NH]-TGSA or -SA resin with biotin was easy (87% yield for 5 min and quantitative after 45 min in the case of HATU activation. Acylation of Fmoc-NH$(CH_2)_6$NH-SA resin with 4-benzoyl-benzoic acid using the methods of symmetrical anhydrides, HATU, TBTU/HOBt even when elevate temperature, high excess and extended coupling time were applied, was not quantitative. Similar problems are frequently encountered with N-terminal N-methyl amino acid residues.

From investigated methods prolonged coupling with TBTU (up to 12 hr), short time (2 h) coupling with HATU or double coupling using the method of symmetrical anhydrides gave the best results and low level of racemization (1%) without a side reaction in the case of easy couplings. For sterically hindered amino acids (e.g. Pro) reduced peptide bond analogues, only a method with ByBroP assisted coupling was near to quantitative. The results from coupling of first amino acid to N-protected diamine-resins are presented in Table 3. Both the method of acid chloride and PyBroP in the presence of DIEA gave acceptable coupling rate for prolonged coupling time. The coupling of sterically hindered amino acid to sterically hindered N-protected or unprotected diamine-resin requires elevate temperature and or double coupling. For example Fmoc-Leu-OH was coupled in 45% yield for 3 hr and almost quantitatively after 48 hrs at 40°C to Fmoc-Leu-Ψ[CH_2NH]-SA resin using PyBroP/DIEA in NMP as a coupling reagent; anyway several side reactions occurred. The coupling of Fmoc-Pro-OH to $H_2NCH_2CH_2NH$-SA resin with the same method for 36 hrs at 40°C gave only 57% conversion to diacylated product. The result from RPHPLC analysis

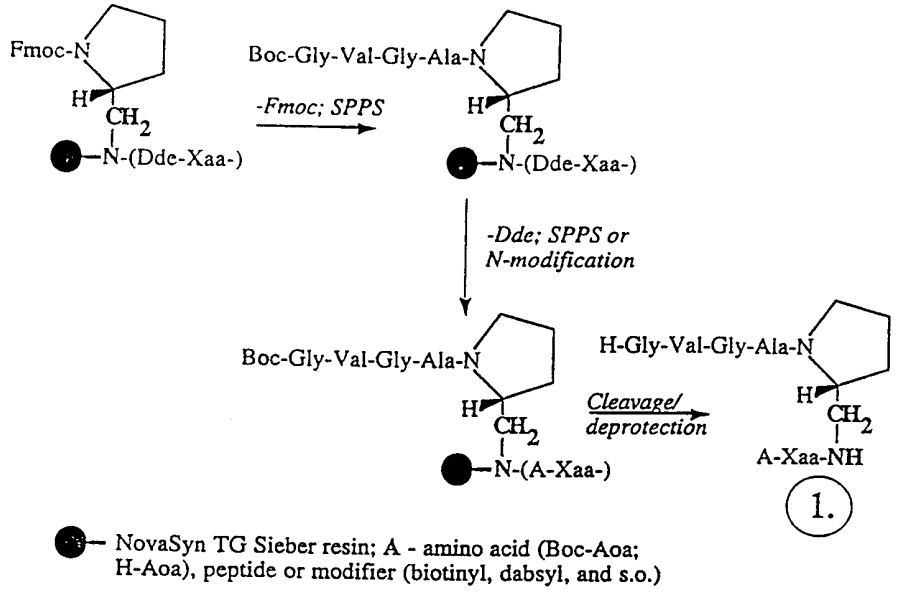
showed also in this case that several side reactions occurred. Acylation rates with Fmoc-Leu-Cl and Fmoc-Pro-Cl respectively, were similar but with a relative low level of side reactions. If the effect of the resin core is eliminated by a spacer arm, good levels of acylation of sterically hindered diamine-resin can be obtained only with non-sterically hindered reagents or N-protected amino acids using PyBroP activation. Both Fmoc-Pro-$\Psi[CH_2NH]$-TGSA or Fmoc-Leu-$\Psi[CH_2NH]$-TGSA were used for preparation of C-terminal modified analogues of human calcitonin (28-34) (scheme 3) and Leu-enkephalin amide respectively (scheme 1, scheme 4). These two peptides and the intermediate products, shown in table 2 and illustrated in Schemes 3 and 4 cover the following classes of diamine and peptides: mono-, asymmetrically protected diamines, C-modified amino acids and peptides: dye-, fluorescent-labelled amino acid and peptides, C-heterodimers of peptides and C-modified peptides with groups for chemical ligation. Aminooxyacetyl C-terminal modified analogues of enkephalin can be used (not shown) for ligation of labels (spin, -radioactive and s.o.) containing ketogroups.

Two different types C-terminal dimers of amino acid, respectively peptides were synthesised: symmetrical for example (Fmoc-Pro-$NHCH_2$-)$_2$, (Fmoc-Gly-$NHCH_2$-)$_2$; (H-Pro-Leu-Gly-$NHCH_2$-)$_2$ and asymmetrical (see table 2). Both Fmoc-Leu-$\Psi[CH_2NH$(Fmoc-Leu)] and Fmoc-Phe-Leu-$\Psi[CH_2NH$(Fmoc-Phe-Leu)] are also asymmetrical (pseudosymmetrical).

In conclusion carboxylic and N-protected amino acids can be coupled to sterically hindered diamine-resins using a method of symmetrical anhydride, acid chloride, PyBroP, HATU, TBTU/HOBt if the reaction is performed on relatively low sterically hindered diamine-resin. The diamine resins with Rink handle are convenient for synthesis of C-terminal reduced peptide bond peptide, but in general not useful for synthesis of N, N'-diprotected diamines, respectively C-terminal modified peptides (excluding sterically not hindered, "good couplers") and C-terminal peptide dimers. Low loaded, spacer arm containing and less sterically hindered diamine TGSA resin is, at least under above discussed conditions, the most suitable support for hindered sequences.

Scheme 3

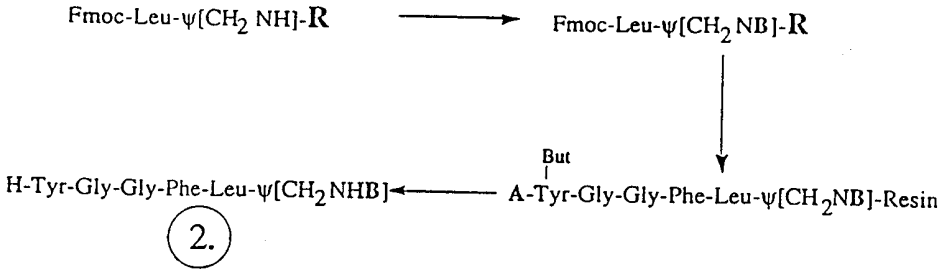
1. C-terminal modified analogues of Human calcitonin (28-32)



NovaSyn TG Sieber resin; A - amino acid (Boc-Aoa; H-Aoa), peptide or modifier (biotinyl, dabsyl, and s.o.)

Scheme 4

2. C-terminal modified analogues of Leu-enkephalin



A = Boc; H,
B = Biotinyl; Boc, H,
R = Sieber amide (SA) orTentaGel SA resin

Table 2. Analytical data of diamine and diamine derivatives

Compound		HPLC [area(%)/RT*(min)]	Cleavage yield #(%) crude/purified	Molecular formula (Molecular mass)	ESMS (+H)
1. Fmoc-NH(CH_2)$_6$$NH_2$	SA	89.3/11.2[c]	94/83	$C_{21}H_{26}N_2O_2$ (338.45)	339.0
2. Fmoc-NH(CH_2)$_6$NH(Biot)	TGR	85.4/19.2[b]	92/77	$C_{31}H_{39}N_4O_4S_1$(563.74)	565.2; 582.4(+NH3) 587.2(+Na);603.2(+K)
3. Fmoc-NH(CH_2)$_6$NH(Aloc)	SA	79.2/20.2[e]	90/69	$C_{25}H_{30}N_2O_4$ (422.53)	423.2; 440.4(+NH3) 587.2(+Na);603.2(+K); 865.6
4. Fmoc-Leu-ψ(CH_2NH_2)	SA	94-97/17.2[b]	98/91	$C_{21}H_{26}N_2O_2$ (338.45)	339.2
5. Fmoc-Leu-ψ[CH_2NH(Biot)]	TGSA	96.3/28.0[b]	94/92	$C_{31}H_{39}N_4O_4S_1$ (563.74)	565.0; 343= -Fmoc;
6. Fmoc-Leu-ψ[CH_2NH(Aloc)]	TGSA	84.0/30.9[a]	85/72	$C_{25}H_{30}N_2O_4$ (422.53)	422.9
7. H-Tyr-Gly-Gly-Phe-Leu-ψ(CH_2NH_2)	SA	61.3/14.8[k]	89/52	C28H40N6O5 (540.67)	541.5
8. Tyr-Gly-Gly-Phe--Leu-ψ[CH_2NH(Biot)]	TGSA	69.7/19.2[k]	91/55	$C_{38}H_{53}N_8O_7S_1$(765.96)	767.4;
9. Fmoc-Phe-ψ[CH_2NH_2]	SA	95.5/21.6[a]	96/89	$C_{24}H_{24}N_2O_4$ (372.47)	373.1
10. Fmoc-D-Phe-ψ[CH_2NH_2]	TGR	97.2/21.6[a]	90.0/ -	$C_{24}H_{24}N_2O_4$ (372.47)	373.4
11. Fmoc-Phe-ψ[CH_2NH(Biot)	SA	95.3/26.9[a]	87.3/ -	$C_{34}H_{37}N_4O_4$ (598.77)	599.1; 612.6(+NH_3)

Table 2, page 2

12. Fmoc-Phe-ψ[CH_2NH(Boc)]	SA	92.3/28.6[a]	92.0/-	$C_{29}H_{32}N_2O_4$ (472.59)	473.3
13. Fmoc-D-Phe-ψ[CH_2NH(Boc)]	SA	94.3/28.6[a]	n.d	$C_{29}H_{32}N_2O_4$ (472.59)	472.8; 373.2(-buten-2);
14. Fmoc-Leu-Phe-ψ[CH_2NH(Boc)]	SA	86.4/29.3[a]	n.d	$C_{35}H_{43}N_3O_5$ (472.59)	586.6; 530.4(buten-2); 486.3(-Boc)
15. Fmoc-Leu-Phe-ψ[CH_2NH(Boc)]	SA	87.9/29.4[a]	n.d	$C_{35}H_{43}N_3O_5$ (585.75)	586.4; 530.2(-buten-2);486.2(-Boc)
16. H-Leu-Phe-ψ[CH_2NH(Boc)]	SA	90.1/32.4[h]	81/73	$C_{20}H_{33}N_3O_3$ (472.59)	n.d.
18. H-Leu-D-Phe-ψ[CH_2NH(Boc)]	SA	91.3/34.6[h]	82.0/74.0	$C_{20}H_{33}N_3O_3$ (472.59)	n.d.
19. H-Leu-Phe-ψ[CH_2NH_2]	SA	78.1/14.6[h]	91/65	$C_{15}H_{25}N_3O_1$ (263.39)	264.0; 527.2(dimer)
20. H-Leu-D-Phe-ψ[CH_2NH_2]	SA	84.3/17.3[h]	90/69	$C_{15}H_{25}N_3O_1$ (263.39)	264.3 527.5(dimer)
21. Fmoc-R(Pbf)-R(Pbf)-$NH(CH_2)_6NH_2$	TGR	84.3-86.3/25.1[a]; 8.22[g]	n.d	$C_{68}H_{90}N_{11}O_{11}S_2$ (1 301.67)	1303.0; 1050; 861; 652;
22. Fmoc-NHCH2CH2NH2	TGSA	85.5/17.7[a]	95/71	$C_{17}H_{19}N_2O_2$ (282.35)	283.0; 219; 244; 316.4
23. Fmoc-(NHCH2CH2)2NH	TGSA	78.4/24.7[a]	96/73	$C_{34}H_{32}N_3O_4$ (548.67)	548.3; 326.2(-Fmoc);563;569
24. 4-BzBz-NH(CH2)6NH(Fmoc)	SA	55.2-71.3.2/17.7[f]	n.d	$C_{35}H_{34}N_2O_4$ (546.67)	547.7
25. Fmoc-Pro-ψ[CHNH(H-Ahx)]	TGSA	82.8/21.0[a]	- / 69	$C_{36}H_{45}N_3O_5$ (599.78)	601.0
26. Fmoc-Pro-ψ[CH_2NH_2]	TGSA	94.9/21.0[a]	91/90	$C_{20}H_{22}N_2O_2$ (322.41)	323.5

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27. Fmoc-D-Phe-ψ[CHNH(Fmoc-Leu)]	TGSA	67.0/31.9[a]	84/62	$C_{45}H_{46}N_3O_5$ (708.89)	709.0; 486.5(-Fmoc);
28. H-Leu-Phe-ψ[CH_2NH_2]	SA	92.0//16[h]	n.d	$C_{15}H_{25}N_3O_1$ (263.39)	264.2; 527.3(dimer)
29. H-Leu-D-Phe-ψ[CH_2NH_2]	SA	86//18.5[h]	n.d	$C_{15}H_{25}N_3O_1$ (263.39)	264; 527.2(dimer)
30. Fmoc-Pro-ψ[CH_2NH(Dde-Ahx)]	TGSA	92.0/27.1[a]	n.d	$C_{36}H_{45}N_3O_5$ (599.78)	600.2; 378.4(-Fmoc)
31. Fmoc-Val-Gly-Ala-Pro-ψ[CH_2NH(Dde-Ahx)]	TGSA	88.7/25.6[a]	94/90	$C_{46}H_{62}N_6O_8$ (827.04)	827.6
32. Boc-Gly-Val-Gly-Ala-Pro-ψ[CH_2NH(Dde-Ahx)]	TGSA	73.2/21.0[a]	n.d	$C_{38}H_{63}N_7O_9$ (761.97)	762.5; 662.5(-Boc);
33.H-Gly-Val-Gly-Ala-Pro-ψ[CH_2NH(Dde-Ahx)]	TGSA	95.1/18.6[a]	91/72	$C_{33}H_{55}N_7O_7$ (661.85)	662.4;
34. H-Gly-Val-Gly-Ala-Pro-ψ[CH_2NH(Dabsyl-Ahx)]	TGSA.77.2/16.7[a]; 15.2[b]24.5[i]		89/66	$C_{37}H_{56}N_{10}O_7S_1$ (784.99)	785.4; 501.2; 484.2; 400.2
35. H-Gly-Val-Gly-Ala-Pro-ψ[CH_2NH(H-Aoa-Ahx)]	TGSA	64.0/7.4[j]	83/45	$C_{25}H_{46}N_8O_7$ (570.69)	571.3
36. Fmoc-Leu ψ[CH_2NH(Dde-Ahx)]	SA	76.8/30.0[a]	92/87	$C_{37}H_{49}N_3O_5$ (615.82)	616.4; 420.4
37. H-Leu-ψ[CH_2NH(Dde-Ahx)]	SA	66.1/ 20.4[a]	n.d	$C_{22}H_{39}N_3O_3$ (393.57)	394.5; 395.3; 377.3; 376.5

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38. Fmoc-Phe-Leu-ψ[CH_2NH(Dde-Ahx)]	SA	75.5/30.8[a]	n.d	$C_{46}H_{58}N_4O_6$ (763.00)	763.5; 453.8
39. H-Phe-Leu-ψ[CH_2NH (Dde-Ahx)]	SA	71.8/22.3[a]	93/83	$C_{31}H_{48}N_4O_4$ (540.75)	541.5; 377.5
40. Fmoc-Gly-Phe-Leu-ψ[CH_2NH(Dde-Ahx)]	SA	82.1/29.8[a]16.2[d]	91/ -	$C_{48}H_{60}N_5O_7$ (818.95)	820.5; 763.6; 656.3; 616.4; 453.8
41. H-Gly-Phe-Leu-ψ[CH_2NH(Dde-Ahx)]	SA	63.3/21.8[a]	n.d	$C_{33}H_{51}N_5O_5$ (597.80)	n.d.
42. Fmoc-Gly-Gly-Phe-Leu-ψ[CH_2NH(Dde-Ahx)]	SA	90.9 - 91.2/28.5[a]15.1[d]	91/80	$C_{50}H_{64}N_6O_8$ (875.48)	877.6; 713.4
43. H-Gly-Gly-Phe-Leu-ψ[CH_2NH(Dde-Ahx)]	SA	79.1/21.7[a]	94/52	$C_{35}H_{54}N_6O_6$ (654.86)	n.d.
44. Fmoc-Tyr(But)-Gly-Gly-Phe-Leu-ψ[CH_2NH(Dde-Ahx)]	SA	91.7-95.3/31.4[a]; 17.3[d]	90/83	$C_{63}H_{81}N_7O_{10}$ (1096.39)	1096.8; 1040.5; 932.7; 818.5; 598.4
45. Boc-Tyr(But)-Gly-Gly-Phe-Leu-ψ[CH_2NH(Dde-Ahx)]	SA	90.7/29.4[a]	n.d.	$C_{53}H_{79}N_7O_{10}$ (974.26)	974.8; 918.8(-isobutene); 874.8 (-Boc)
46. Boc-Tyr(But)-Gly-Gly-Phe-Leu-ψ[CH_2NH(H-Ahx)]	SA	88.8-91.5/25.9[a]; 13.1[d]	n.d.	$C_{43}H_{67}N_7O_8$ (810.06)	810.6; 663.4; 576.4; 943.8
47. Boc-Tyr(But)-Gly-Gly-Phe-Leu-ψ[CH_2NH(Boc-Aoa-Ahx)]	SA	83.1- 89.6/28.5[a]; 15.2[d]	n.d.	$C_{50}H_{78}N_8O_{12}$ (983.23)	984.0; 928; 884 (-Boc); 828; 784(-2xBoc)

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48. Boc-Tyr(But)-Gly-Gly-Phe-Leu-ψ[CH_2NH(H-Aoa-Ahx)]	SA	78.3/L-21.2[a];D-21.5[a]	;8.1[d]	n.d.	$C_{40}H_{63}N_8O_8$ (784.40)	n.d.
49. H-Tyr-Gly-Gly-Phe-Leu-ψ[CH_2NH(H-Aoa-Ahx)]	SA	79.9/21.2[d]	84/62		$C_{36}H_{54}N_8O_8$ (726.88)	727.4; 800.4; 879.4
50. Boc-Tyr(But)-Gly-Gly-Phe-Leu-ψ[CH_2NH(Dns-Aoa)]	SA	68.9-69.1/26.0[a]; 16.2[d]	n.d.		$C_{55}H_{78}N_8O_{10}S_1$ (1043.35)	1043.6; 943.6(-Boc); 987.6; 887.6(-Boc;-
51.H-Tyr-Gly-Gly-Phe-Leu-ψ[CH_2NH(Dns-Aoa)]	SA	79.4/19.0[a]	- / 71		$C_{46}H_{62}N_8O_8S_1$ (887.12)	887.6; 463.4
52. Fmoc-Leu-ψ[CH_2NH(Fmoc-Leu)]	SA	64.9/32.2[a]	82/55		$C_{42}H_{47}N_3O_5$ (673.86)	674.5; 478.5; 452.5
53. Fmoc-Phe-Leu-ψ[CH_2NH(Fmoc-Phe-Leu)]	SA	62.5/20.1[c]	79/53		$C_{60}H_{65}N_5O_7$ (968.22)	969.0;
54. (Fmoc-Pro-$NHCH_2$-)2	TGSA	83.0/26.3[a]	93/66		$C_{42}H_{42}N_4O_6$(698.83)	699.5; 503.0; 477.5
55. Fmoc-Pro-$NHCH_2CH_2NH_2$	TGSA	52.1/19.0[a]	n.d.		$C_{22}H_{25}N_3O_3$(379.5)	380.4

*RPHPLC was performed on LiChrosper 100 5RP18 (125x4.6 mm i. d.) on a Waters liquid chromatographer as indicated in the text.
#-spectrophotometric determination of Fmoc-piperidine adduct at 301 nm liberaterd upon treatment of aliquotes from purified product using a standard curve for Fmoc-Gly-OH or amino acid analysis determination.

a Gradient B from 0 to100% over 33.33 min at flow rate 1 mL/min, λ= 256 nm

b- Gradient B from 10 to70% over 30.00 min at flow rate 1 mL/min, λ= 256 nm

c-Gradient B from 20 to100% over 20 min at flow rate 1 mL/min, λ= 256 nm

d-Gradient B from 20 to100% over 20 min at flow rate 1 mL/min, λ= 214 nm

e- Gradient B from 30 to 80% over 25 min at flow rate 1 mL/min, λ= 214 nm

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f-Gradient B from 20 to 95% over 25 min at flow rate 1 mL/min, λ= 256 nm (RT of Fmoc-NH-$(CH_2)_6$-NH_2 in separate run was 11.4 min)

g-Gradient B from 60 to100% over 20 min at flow rate 1 mL/min, λ= 256 nm

h-Gradient B from 0 to 60% over 60 min at flow rate 1.5 mL/min, λ= 256nm

i-Gradient B from 0 to 45% over 30 min at flow rate 1.0 mL/min, λ= 220 / 256 / 420 nm

j-Gradient B from 10 to 40% over 30 min at flow rate 1.5 mL/min, λ= 214 nm

k-Gradient B from 20 to 50% over 30 min at flow rate 1.0 mL/min, λ= 214 nm

n.d. - not determined

TABLE 3

Coupling first amino acid to Fmoc-NH$(CH_2)_6$NH-TGSA resin (1), Fmoc-NHCH[CH_2CH$(CH_3)_2$]CH_2NH-TGSA resin (2) and

Resin type	Conditions	Coupling yields (%)# Gly	Leu
1.	Fmoc-Xaa-Cl (4 eq), DIEA (4 eq), DMF:DCM(1:1),12 h	92	82
2.	Fmoc-Xaa-Cl (4 eq), DIEA (4 eq), DMF:DCM(1:1),12 h	85	79
2.	Fmoc-Xaa-Cl (4 eq), DIEA (4 eq), DMF:DCM(1:1), 2X12 h	93	88
1.	Fmoc-Xaa-OH (6 eq); PyBroP(6 eq), DIEA(12 eq),12 h	97	89
2.	Fmoc-Xaa-OH (6 eq); PyBroP(6 eq), DIEA(12 eq),12 h	91	82

#-spectrophotometric determination of Fmoc-piperidine adduct at 301 nm (ε=7800) after treatment in DMF/20% piperidine for 10 min. at room temperature. Yields (in %) were determined from the equation Y=[(S1-So)/So] X 100%, where S1 (in mmol/g) - was the substitution of the resin, mesured after the coupling with the methods, shown inTable 3. So - substitution of resin before the coupling (starting substitutuion). The presented results are in good correlation with these from RPHPLC and amino acid analysis.

Comparative study of methods to couple sterically hindered amino acids and peptides was investigated by Spenser, J.R., Antonenko, V.V., Delaet, N.G.J. and Goodman, M., *Int. J. Pept. Prot. Res.*, 40, 282-293(1992) and cited therein, Coste, J. and al., *J. Org. Chem.*, 59, 2437(1994) and cited therein and more recently by Caciagli, V., Cardinali, F., Righetti, G and Lombardi, P., *4-th Intern. Symp. Solid. Phase Synthesis*; Edinburgh, 12-16 September 1995, P17; Caciagli, V., Cardinali, F., Righetti, G and Lombardi, P., *24-th Symp. European Pept. Soc.*, 8-13 September 1996, Edinburgh, P.81. The methods with isolated (Carpino, L.A., Sadat-Aalaee, D., Chao, H.-G., deSelms, R.H., J. Am. Chem. Soc, 112, 9651(1990) or *in situ* generated (Carpino, L.A., El-Faham, A., J.Am. Chem. Soc., 117, 5401-5402(1995) Fmoc-amino acid fluorides (Carpino, L. et al., *Acc. Chem. Res.*, 29, 288 (1966) and references cited therein;) in the presence of silylating reagent (Wenschuh, H. et al., *Tetr. Lett.*, 37, 5483(1996) ; Wenschuh, H. et al., in *Peptides 1996*, Epton R.(Ed.) Mayflower Wordwide Ltd., Birmingham, 1977, in press) uretan type groups protected N-carboxyanhydrate (UNCAS) for coupling are also effective, and the results from these investigations will be published elsewhere.

For our comparative study of acylation of Fmoc-Leu-ψ[CH2NH-SA] resin with Dde-Ahx-OH in the precence of PyBroP or TBTU see experimental part.

Claims

1. A solid phase method for producing a compound comprising an unsubstituted or substituted diamine moiety, which method comprises:

(i) reacting a N-protected amino aldehyde with an amino group attached to a solid resin to produce a resin-bound enamine product; and

(ii) reducing the enamine product to produce a resin-bound N-protected diamine.

2. A method according to claim 1 which further comprises modifying the resin-bound N-protected diamine prior to optionally removing the diamine from the resin.

3. A method according to claim 2 in which the diamine is modified by reacting the amine group bonded to the resin with an amine protecting group to produce a resin-bound diamine in which both amine groups are protected.

4. A method according to claim 3 wherein the protecting group which is reacted with the amine group bonded to the resin is the same as the protecting group of the N-protected amino aldehyde used in step (i).

5. A method according to claim 3 wherein the protecting group which is reacted with the amine group bonded to the resin is different from the protecting group of the N-protected amino aldehyde used in step (i).

6. A method according to any one of the claims 3 to 5 wherein the protecting group that is reacted with the amine is an acyl group.

7. A method according to claim 2 in which the resin-bound diamine is modified by acylating the amine group bonded to the resin with an N-protected amino acid to

produce a resin-bound amino acid-substituted diamine in which both terminal nitrogen atoms are protected.

8. A method according to claim 2 in which the resin-bound diamine is modified by protecting the amine group bonded to the resin with an amine protecting group, selectively deprotecting the amine group derived from the N-protected aldehyde used in step (i), and carrying out the steps of a solid phase peptide synthesis to produce a peptide product comprising at its C-terminus a diamine, the terminal group of which is N-protected and is bonded to the resin.

9. A method according to claim 2 in which the resin-bound diamine is modified by carrying out the steps of a solid phase peptide synthesis without deprotecting the nitrogen group derived from the N-protected aldehyde used in step (i) to produce a peptide product comprising at its C-terminus a diamine, which is bonded to the resin.

10. A method according to claim 2 in which the resin-bound diamine is modified by deprotecting the amine group derived from the N-protected aldehyde used in step (i), and carrying out the steps of a solid phase peptide synthesis so as to produce a homodimerised peptide product comprising two peptide moieties each of which is attached at the C-terminus to the diamine moiety.

11. A method according to claim 2 in which the resin-bound diamine is modified by:

a) protecting the amine group bonded directly to the resin with an orthogonal protecting group or an orthogonally protected amino acid, deprotecting the amine group derived from the N-protected amino aldehyde used in step (i) and carrying out the steps of solid phase peptide synthesis to produce a resin-bound peptide product comprising at its C-terminus a diamine; and

b) deprotecting the orthogonally-protected amine or amino acid group

without removal of the N-terminus protecting group of the peptide formed in step (a) and carrying out further steps of solid phase peptide synthesis to produce a heterodimerised peptide product comprising two different peptide moieties each of which is attached at the C-terminus to the diamine moiety.

12. A method according to claim 2 in which the resin-bound diamine is modified by:

a) carrying out the steps of solid phase peptide synthesis using a reagent system comprising a protecting group orthogonal to the N-protecting group used in step (i); and

b) selectively deprotecting the amine protecting group derived from the N-protected amino aldehyde used in step (i) without removal of the orthogonal N-terminus protecting group of the peptide formed in step (a) and carrying out further steps of solid phase peptide synthesis so as to produce a heterodimerised peptide product comprising two different peptide moieties each of which is attached at the C-terminus to the diamine moiety.

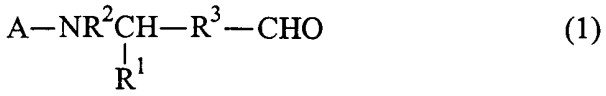
13. A method according to any one of the preceding claims which further comprises attaching a detectable label to the resin-bound product.

14. A method according to claim 13 which comprises attaching a detectable label to one of the amine groups of the diamine and carrying out steps of solid phase peptide synthesis from the other, so as to produce a resin bound label peptide.

15. A method according to claim 13 or 14 wherein the label is a biotin-label, anthranyl-label, spin-label, radioactive-label or spectrophotometric-label.

16. A method according to any one of the preceding claims which further comprises removing the modified or unmodified diamine product from the resin.

17. A method according to any one of the preceding claims wherein the N-protected amino aldehyde is of formula (1)



(1)

wherein: A is a N-protecting group; R^1 is hydrogen, alkyl, a side chain of a natural amino acid or its stereoisomer, or a substituted derivative thereof; R^2 is hydrogen or alkyl or R^1 and R^2 together with the atoms to which they are bonded form a heterocyclic ring; and R^3 is a C_{1-10}-alkylene group or a single bond.

18. A method according to claim 17 wherein A is selected from acetyl, allyloxycarbonyl, tert-amyloxycarbonyl, tert-butoxycarbonyl, bromobenzyloxycarbonyl, benzoyl, chlorobenzyloxycarbonyl, 9-fluorenylmethoxycarbonyl, formyl, methoxybenzyloxycarbonyl, trityl, 4-methyl trityl and benzyloxycarbonyl.

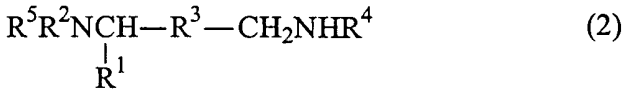
19. A method according to any one of the preceding claims wherein the N-protected amino acid is a N-protected α- or ω-amino aldehyde containing a moiety corresponding to the side chain of a natural α-amino acid or a stereoisomer thereof.

20. A method according to any one of the preceding claims wherein the resin is an aminobenzhydryl or aminoxantyl based resin, which has or does not have a spacer arm group.

21. A method according to any one of the preceding claims which further comprises removal of any remaining protecting group individually or simultaneously.

22. A free or resin-bound substituted or unsubstituted diamine obtained by the method of any one of the preceding claims.

23. A diamine of formula (2):



(2)

wherein R^1 is hydrogen, alkyl or a side chain of a natural amino acid or its stereoisomer, or a substituted derivative thereof; R^2 is hydrogen or alkyl or R^1 and R^2 together with the atoms to which they are bonded form a heterocyclic ring; R^3 is a C_{1-10} alkylene group or a single bond; R^4 and R^5 are on the same or different and are selected from a hydrogen atom, a nitrogen protecting group, an amino acid or an amino acid derivative, a peptide chain comprising two or more amino acids or amino acid derivatives, or a detectable label.

24. A diamine according to claim 23 wherein R^1 is hydrogen or a side chain of a natural amino acid; R^3 is a single bond; and at least one of R^4 and R^5 is a peptide chain of at least 2 amino acids or amino acid derivatives.

25. A diamine according to claim 23 wherein R^1 is a side chain of the amino

acid leucine; R^2 is hydrogen; R^3 is a single bond; R^4 is selected from a hydrogen atom, a biotinyl group and chemical groups of formulae Dde-Ahx-, H-Ahx-, Boc-Aoa-Ahx- and H-Aoa-Ahx-: R^5 is a peptide chain of formula

A-Tyr(X)-Gly-Gly-Phe-

wherein (X) is hydrogen or a suitable tyrosine hydroxyl protecting group and A is a hydrogen atom, an Fmoc group or a Boc group.

26. A diamine according to claim 23 wherein R^1 and R^2 together with the atoms to which they are bonded form a pyrrolidine ring; R^3 is a single bond; R^4 is selected from a hydrogen atom, a biotinyl group, and chemical groups of formulae H-Ahx-, Boc-Aoa-Ahx-, Dde-Ahx- and H-Aoa-Ahx; and R^5 is a peptide chain of formula

A-Gly-Val-Gly-Ala-

wherein A is a hydrogen atom, an Fmoc group or a Boc group.