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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. **06.020.917**
Solicitud de patente a nombre de **NOVARTIS AG**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, actuando como apoderada sustituta de acuerdo con la sustitución adjunta, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **NOVARTIS AG.**, domiciliada en Basilea Suiza, solicitante de la patente de la referencia, en respuesta al oficio No. 9379 notificado por fijación en lista el 14 de agosto de 2009, relacionado con el concepto técnico de fondo No. 02157, me permito informar lo siguiente:

Acompañamos copia informal del artículo de Blackburn C y Steven A. Kates "Solid-phase Synthesis of Cyclic Homodetic Peptides" publicado en Methods in Enzymology, vol 289. , páginas 175-198. 1997.

Con este artículo se proporciona cabal respuesta al requerimiento exigido por el señor Examinador con base en lo manifestado en el artículo 46 de la Decisión 486.

Señor Superintendente,

DILIA MARIA RODRIGUEZ D'ALEMAN
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TP 20824 CSJ
NEA

[9] Solid-Phase Synthesis of Cyclic Homodetic Peptides

By CHRISTOPHER BLACKBURN and STEVEN A. KATES

Introduction

There are two general classes of cyclic peptides: homodetic and heterodetic. Ring formation may occur to form the usual amide linkage between an amino and carboxylic acid function to give homodetic cyclic peptides, whereas any other linkages such as lactone, ether, thioether, and, most commonly, the disulfide bridge are referred to as heterodetic. The interest in cyclic homodetic peptides began in the late 1940s when the antibiotic gramicidin S was found to be a cyclic decapeptide.[1] Commercially available drugs such as the immunosuppressant cyclosporin indicate that research in this field continues. Many antibiotics and toxins have been shown to contain cyclic amino acid sequences that result in metabolic stability, increased potency, receptor selectivity, and bioavailability, and they are synthetically challenging. The constrained geometry of a cyclic peptide allows for conformational investigations such as determining the connection between primary and secondary structures of proteins and sequences related to protein folding and as selective metal ion chelating agents (ionophores). Initial work in this area focused on preparing peptides with a small ring size. Cyclic hexapeptides containing a proline to further restrict conformational possibilities were prepared and extensively studied. With the development of improved synthetic methods, larger ring sizes and more intriguing targets have been successfully attempted.

In the past, cyclic peptides have been prepared totally in solution[2] or by assembling the linear peptide in the solid phase,[3] cleaving the peptide from the resin, and cyclizing in solution. Unfortunately, there are still limitations to the cyclization of peptides via classic solution phase synthesis despite efforts to develop practical and convenient methods.[4] For instance, a "doubling reaction" has been observed during ring closure.[3] As a result, literature methods rely on suitably protected linear precursors, which are selectively activated and cyclized in solution under highly dilute conditions.

[1] R. J. Consden, A. H. Gordon, A. J. P. Martin, and R. D. M. Synge, *Biochem. J.* **41**, 596 (1947).
[2] K. D. Kopple, *J. Pharm. Sci.* **61**, 1345 (1972).
[3] A. E. Tonelli, *in* "Cyclic Polymers" (J. A. Semlyen, ed.), p. 261. Elsevier Applied Science, London, 1986.
[4] D. S. Perlow, J. M. Erb, N. P. Gould, R. D. Tung, R. M. Freidinger, P. D. Williams, and D. F. Veber, *J. Org. Chem.* **57**, 4394 (1992).

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Cyclodimerizations and cyclooligomerizations may also occur even under high dilution usually as unwanted side reactions but occasionally to benefit for certain symmetrical targets, as temperature, solvent, and the concentration and structure of the linear peptide are other important factors.[2,4,5]

There are advantages to performing peptide cyclizations on solid phase rather than in solution. A pseudodilution phenomenon is achieved by having the linear peptide attached to a polymeric support. Intramolecular cyclization is favored over intermolecular because the solid support provides a large distance between chains.[3] Furthermore, reactions can be driven to completion by the use of excess soluble reagents that can be removed by simple filtration and washing, and in an automated process.

There are three classic modes of medium- and long-range lactam cyclization of peptides. A ring may be formed via cyclization of the amino terminus to the carboxyl terminus. This mode of ring closure is called head to tail or end to end. Alternatively, the functional groups of side chains, usually the amino group of lysine or ornithine, can provide a method for lactam formation with the carboxyclic acid of aspartic acid or glutamic acid. This type of cyclization is referred to as side chain-to-side chain or backbone-to-backbone cyclization. Lastly, cyclization can occur through a side chain such as the amino side-chain function of Lys/Orn to the carboxyl terminus. Alternatively, the carboxylic acid function of Asp/Glu can cyclize onto the amino terminus. Solid-phase syntheses of these classes of peptides have been attempted through different strategies outlined in Schemes 1, 2, and 3.

This article reviews the synthesis of cyclic homodetic peptides by solid-phase methods with an emphasis on solid supports, protecting group strategy, reagents, side reactions, and the emergence of cyclic peptide combinatorial libraries.[6]

Solid Supports

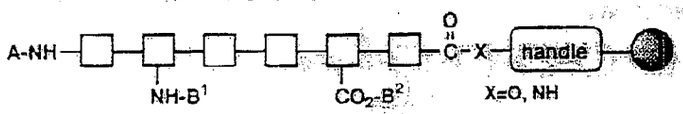
A wide range of polymeric supports has been successfully used to prepare cyclic peptides such as polystyrene (PS) resins and cellulose membranes.[7] With the development of milder orthogonal protection schemes

[5] M. Rothe, A. Sander, W. Fischer, W. Mastle, and B. Nelson, *in* "Peptides: Proceedings of the Fifth American Peptide Symposium" (M. Goodman and J. Meienhofer, eds.) p. 506. Wiley, New York, 1977.

[6] For a recent review on solid-phase synthesis of cyclic peptides, see S. A. Kates, N. A. Solé, F. Albericio, and G. Barany, *in* "Peptides: Design, Synthesis and Biological Activity" (C. Basava and G. M. Anantharamaiah, eds.), p. 39. Birkhaeuser, Boston, 1994.

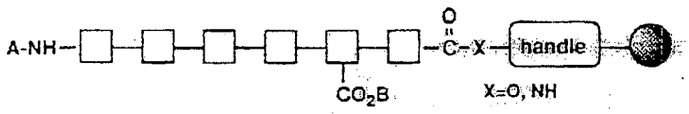
[7] D. Winkler, A. Schuster, B. Hoffmann, and J. Schneider-Mergener, *in* "Peptides 1994: Proceedings of the Twenty-third European Peptide Symposium" (H. L. S. Maia, ed.), p. 485. Escom, Leiden, The Netherlands, 1995.

a. side chain to side chain



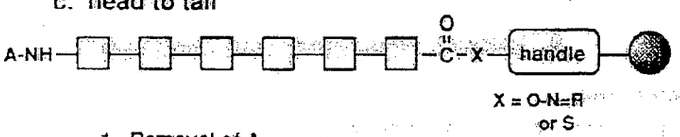
1. Removal of B^1 and B^2
2. Cyclization
3. Final deprotection and cleavage

b. *N* terminal to side chain



1. Removal of A and B
2. Cyclization
3. Final deprotection and cleavage

c. head to tail



1. Removal of A
2. Cyclization and concomitant release of protected peptide from the resin
3. Removal of protecting groups in solution

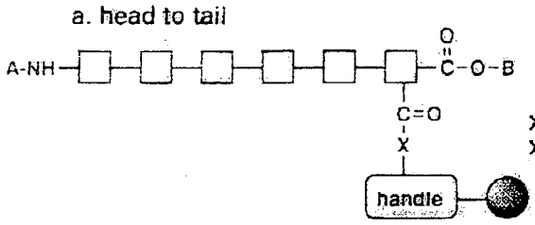
SCHEME 1. C-terminal anchoring strategy for different classes of cyclic peptides.

and more efficient reagents for cyclization, polyethylene glycol–polystyrene graft supports (PEG–PS) have been shown to be excellent resins.[8] The PEG spacers allow complete solvation of the reactive sites, which increases coupling efficiency for both linear assembly and cyclization. Moreover, PEG–PS supports do exist that are compatible with both 9-fluorenylmethyloxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu) and *tert*-butyloxycarbonyl (Boc)/ben-

[8] G. Barany, F. Albericio, N. A. Solé, G. W. Griffin, S. A. Kates, and D. Hudson, *in* "Peptides 1992: Proceedings of the Twenty-second European Peptide Symposium" (C. H. Schneider and A. N. Eberle, eds.), p. 267. Escom, Leiden, The Netherlands, 1993.

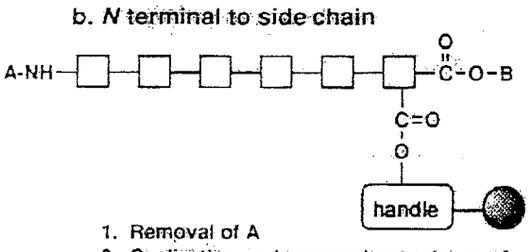
I. Through carboxylic acid

a. head to tail



1. Removal of A and B (usually two steps)
2. Cyclization
3. Final deprotection and cleavage

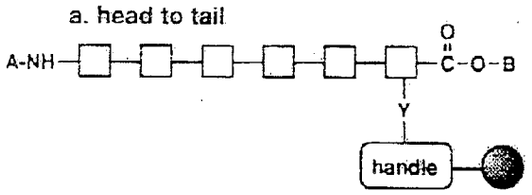
b. *N* terminal to side chain



1. Removal of A
2. Cyclization and concomitant release of peptide from the resin
3. Removal of protecting groups in solution

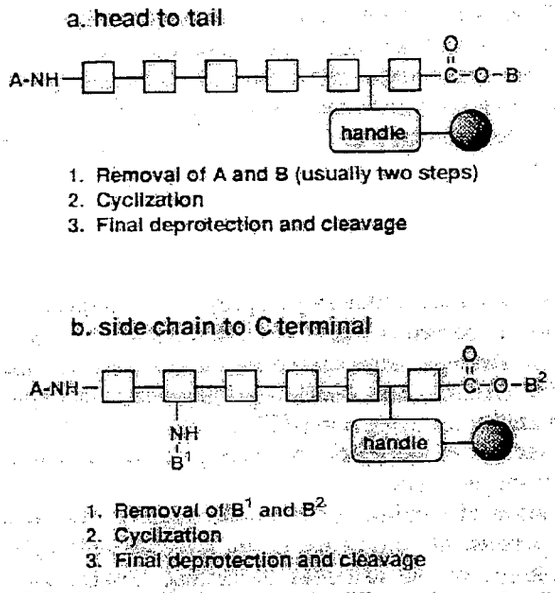
II. Through other amino acid side chain

a. head to tail



1. Removal of A and B (usually two steps)
2. Cyclization
3. Final deprotection and cleavage

SCHEME 2. Side-chain anchoring strategy for different classes of cyclic peptides.



SCHEME 3. Backbone anchoring strategy for different classes of cyclic peptides.

zyl (Bzl) methods.[9] Finally, PepsynK (polyamide–kieselguhr), a resin developed for continuous-flow instrumentation, has also been successfully used.[10]

Resin Loading

The amount of dimerization and oligomerization is dependent on the substitution level of the polymeric support. Plaué has prepared 4-methylbenzhydrylamine (MBHA) resins with an initial loading of 0.1–0.6 mmol/g and examined the cyclization of a model compound.[11] Analysis of the high-performance liquid chromatography (HPLC) data clearly indicate that as the resin substitution increases from 0.1 to 0.6 mmol/g, the percentage of cyclodimer increases from 8 to 19%. PEG–PS supports with 0.40 and 0.20 mmol/g substitutions have been used in conjunction with an Fmoc/

[9] F. Albericio, J. Bacardit, G. Barany, J. M. Coull, M. Egholm, E. Giralt, G. W. Griffin, S. A. Kates, E. Nicolás, and N. A. Solé, *in* "Peptides 1994: Proceedings of the Twenty-third European Peptide Symposium" (H. L. S. Maia, ed.), p. 271. Escom, Leiden, The Netherlands, 1995.

[10] C. Mendre, R. Pascal, and B. Calas, *Tetrahedron Lett.* 35, 5429 (1994).

[11] S. Plaué, *Int. J. Pept. Protein Res.* 35, 510 (1990).

*t*Bu/allyl strategy for the preparation of a head-to-tail cyclic peptide.[12] The product purity from the on-resin cyclization is greater with the lower loaded PEG–PS support. In contrast, when the repeating unit of gramicidin S is constructed on an oxime resin with 0.54 and 0.17 mmol/g loadings, the resin of lower loading gives more dimer due to interchain reactions.[13] Cyclic monomeric peptide is formed at a faster rate and higher yield from the higher loaded resin.

Handles

Anchors that have been commonly employed for on-resin lactamization of cyclic peptides are shown in Fig. 1. The selection of a handle is governed by the protection strategy incorporated into a synthetic scheme. Initially, benzhydrylamine (BHA)– and MBHA–polystyrene resins were used because of their stability to strong acid (HF), which was commonly employed for final deprotection of side-chain protecting groups and release of the cyclic peptide from the resin.

The preparation of head-to-tail or a side chain-to-tail cyclic peptides requires "semipermanent" protection of the terminal carboxylic acid function (for definitions of the different protecting groups, see Protection Schemes). In standard linear assembly, the α-carboxylic acid is used for attachment to a solid support. Therefore, construction of these two classes of cyclic peptides demands side-chain anchoring of a trifunctional amino acid to a polymeric support. Side-chain anchoring on route to head-to-tail cyclic peptides has been accomplished by attachment of the ω-carboxyl groups of Asp or Glu to hydroxymethyl or benzhydrylamine resins.[14] The linear peptide was assembled on a phenylacetamido (PAM) resin starting with attachment of Boc-Asp(OH)-OFm (where Fm is 9-fluorenylmethyl) followed by peptide chain elongation with Boc/Bzl amino acids. Tromelin *et al.* reported the synthesis of a head-to-tail cyclic peptide on MBHA resin via side-chain anchoring of Boc-Asp(OH)-OFm through an amide link.[15] After acetylation of residual MBHA sites, the peptide chain was assembled using Boc/Bzl amino acids. Following removal of the N-terminal Boc group

[12] B. F. McGuinness, S. A. Kates, G. W. Grifin, L. W. Herman, N. A. Solé, J. Vágner, F. Albericio, and G. Barany, *in* "Peptides: Chemistry and Biology, Proceedings of the Fourteenth American Peptide Symposium" (P. T. P. Kaumaya and R. S. Hodges, eds.), p. 125. Mayflower Worldwide, Birmingham, UK, 1996.

[13] M. Bouvier and J. W. Taylor, *in* "Peptide: Chemistry and Biology, Proceedings of the Twelfth American Peptide Symposium" (J. A. Smith and J. E. Rivier, eds.), p. 535. Escom, Leiden, The Netherlands, 1992.

[14] P. Rovero, L. Quartara, and G. Fabbri, *Tetrahedron Lett.* **32**, 2639 (1991).

[15] A. Tromelin, M. H. Fulachier, G. Mourier, and A. Ménez, *Tetrahedron Lett.* **33**, 5197 (1992).

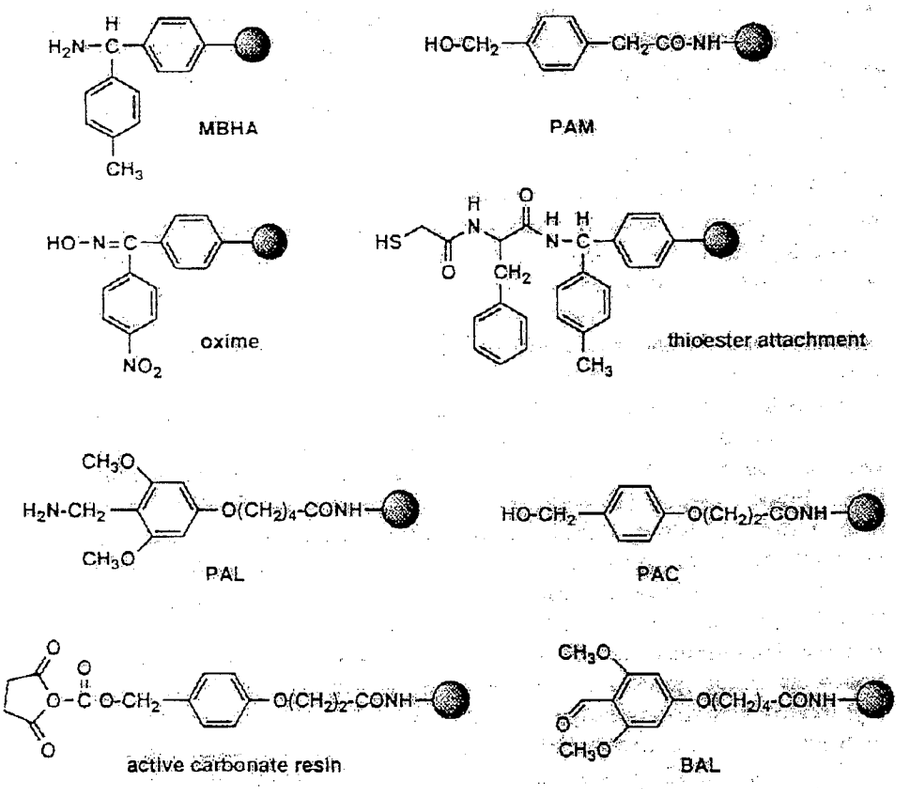


FIG. 1. Handles used for the preparation of cyclic peptides.

and the C-terminal Fmoc group and cyclization, HF cleavage afforded a cyclic peptide in which the Asn residue was derived from the Asp group initially attached to the resin.

The side-chain anchoring strategy for head-to-tail cyclic peptides in Fmoc/*t*Bu-based chemistry was first conducted by McMurray[16] by attaching Fmoc-Glu(OH)-ODmb (2,4-dimethoxybenzyl) to a 4-hydroxymethylphenoxyacetylaminomethyl–resin for the preparation of cyclo(Ala-Ala-Arg-DPhe-Pro-Glu-Asp-Asn-Tyr-Glu). Albericio and co-workers applied an Fmoc/*t*Bu/allyl strategy to the preparation of the same sequence using four different starting resins: (a) Fmoc-Glu(OPAC–PEG–PS)-OAl (where PAC is *p*-alkoxybenzyl alcohol and Al is allyl) at positions Glu-6 and Glu-10; (b) Fmoc-Asp(OPAC–PEG–PS)-OAl at position Asp-7, and (c) Fmoc-

[16] J. S. McMurray, *Tetrahedron Lett.* **32,** 7679 (1991).

Asp(OPAL–PEG–PS)-OAl at position Asn-8. In strategy (c), Asn-8 was derived from an aspartic acid with its β-carboxylic acid bound to the resin via the 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid (PAL) handle. All four strategies gave the desired peptide; the best yield and purity (71%) were obtained with the Asn-8 strategy, and the Glu-10 strategy gave the highest level of by-products.[17]

Side-chain anchoring an amino acid to a solid support has been accomplished most frequently through a carboxylic acid function, which restricts linear sequences to those containing Asx or Glx. As an extension to this approach, Alsina *et al.*[18] prepared an active carbonate resin to which the side chain of Lys was attached. The novel solid support was prepared in quantitative yield by treatment of PAC resin with *N,N'*-disuccinimidyl carbonate and 4-dimethylaminopyridine (DMAP) followed by coupling with Fmoc-Lys-OAl in the presence of *N,N*-diisopropylethylamine (DIEA).

Procedure. PAC–PS resin is suspended in *N,N*-dimethylformamide (DMF), and disuccinimidyl carbonate (10 equivalent) and DMAP (1 equivalent) are added under Ar for 2 hr. The resin is filtered and washed with DMF. Fmoc-Lys-OAl (10 equivalent) is dissolved in DMF, added to the resin in the presence of DIEA (20 equivalent), and allowed to react for 4 hr at 25°. The Fmoc group is removed with piperidine–DMF followed by peptide chain elaboration. Allyl removal is carried out using 1.9 *M* $Pd(PPh_3)_4$ in dimethyl sulfoxide (DMSO)–tetrahydrofuran (THF)–0.5 *N* aqueous HCl–morpholine (2:2:1:0.1, by volume), for 150 min at 25°. Following Fmoc group removal, the resin is treated with benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP)/1-hydroxy-7-azabenzotriazole (HOAt)/DIEA (5:5:10 equivalents) in DMF for 2 hr at 25° to effect cyclization. The product is cleaved from the resin with concomitant removal of protecting group side chains with reagent R (TFA–thioanisole–1,2-ethanedithiol–anisole, 90:5:3:2, by volume) for 2 hr at 25°.

The above methods rely on the concept of side-chain anchoring an amino acid to a resin via a carboxylic acid or amine function, which restricts the sequences that can be cyclized on a solid support. The BAL handle (backbone amide linker) has been developed for Fmoc/*t*Bu methods to attach a peptide chain via a backbone amide nitrogen.[19] An example of

[17] S. A. Kates, N. A. Solé, C. R. Johnson, D. Hudson, G. Barany, and F. Albericio, *Tetrahedron Lett.* **34**, 1549 (1993).

[18] J. Alsina, F. Rabanal, E. Giralt, and F. Albericio, *Tetrahedron Lett.* **35**, 9633 (1994).

[19] K. J. Jensen, M. F. Songster, J. Vágner, J. Alsina, F. Albericio, and G. Barany, *in* "Peptides: Chemistry and Biology, Proceedings of the Fourteenth American Peptide Symposium" (P. T. P. Kaumaya and R. S. Hodges, eds.), p. 30. Mayflower Worldwide, Birmingham, UK, 1996.

the use of this handle involves the attachment of Fmoc-(BAL-OH)Ala-OAl to MBHA resin. Using the BAL anchor, head-to-tail cyclic peptides devoid of a trifunctional amino acid residue can be readily constructed.

Anchoring the first amino acid to a PAM-type handle for the synthesis of head-to-tail cyclic peptides via a Boc/Bzl/OFm strategy has been investigated.[20] Coupling Boc-Asp(OH)-OFm to hydroxymethyl PAM resins with *N,N'*-diisopropylcarbodiimide (DIPCDI)/DMAP using various conditions gives low yields and high levels of racemization. Nucleophilic substitution of bromomethyl-Pam handles with the cesium and zinc salts of Boc-Asp(OH)-OFm substantially improves the anchoring yield and lowers the amount of racemization.

Protection Schemes

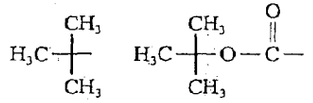
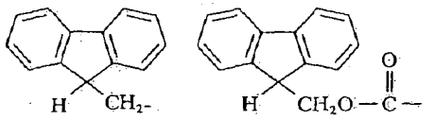
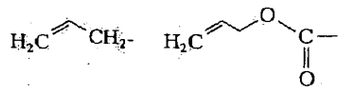
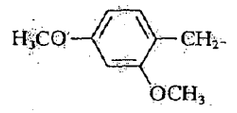
The success of solid-phase synthesis of cyclic peptides is based on the proper combination of temporary, semipermanent, and permanent protecting groups (Table I) and the corresponding methods for their removal (Fig. 2). Temporary groups are for N^{α}-protection; semipermanent groups are for Lys/Orn-Asp/Glu side-chain protection, and permanent groups are for side-chain protection and resin–peptide anchorage. Two or more classes of groups that are removed by differing chemical mechanisms in any order and in the presence of other classes is called an orthogonal protection scheme. This discussion is organized according to the following format: semipermanent/temporary/permanent/handle (anchoring strategy and class of cyclic peptide, based on Schemes 1, 2, and 3) accompanied with a brief experimental section.

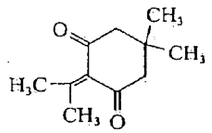
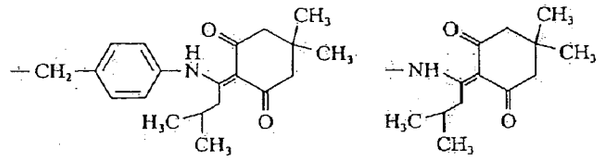
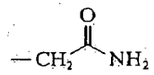
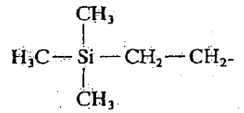
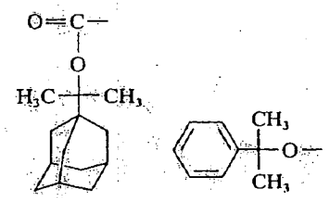
Oxime/Boc/Bzl/oxime Handle (Scheme 1c)

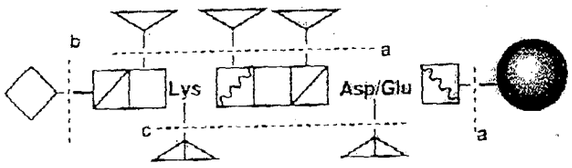
A polystyrene-based resin frequently used is the *p*-nitrobenzophenone oxime–resin[21,22] which acts as both a semipermanent and permanent protecting group for the C terminus. This resin provides a method for preparation of head-to-tail cyclic peptides with concomitant cleavage from the resin via intramolecular nucleophilic displacement of the anchoring linkage by the amine terminus of a peptide.[23,24] This strategy requires N^{α}-Boc amino acids for linear chain elongation because oxime resins are not compatible with secondary amines used for N^{α}-Fmoc removal. To avoid premature cleavage of the peptide from the resin, coupling of amino acids is accom-

[20] M.-L. Valero, E. Giralt, and D. Andreu, *Tetrahedron Lett.* **37,** 4229 (1996).
[21] W. F. DeGrado and E. T. Kaiser, *J. Org. Chem.* **45,** 1295 (1980).
[22] W. F. DeGrado and E. T. Kaiser, *J. Org. Chem.* **47,** 3258 (1982).
[23] G. Ösapay, M. Bouvier, and J. W. Taylor, *Tetrahedron Lett.* **31,** 6121 (1990).
[24] G. Ösapay and J. W. Taylor, *J. Am. Chem. Soc.* **112,** 6046 (1990).

TABLE I
PROTECTING GROUPS FOR SOLID-PHASE CYCLIC PEPTIDES

Semipermanent	Structure	Removal	Temporary	Permanent	Final cleavage
*t*Bu, Boc		TFA	Fmoc	Bzl	HF
Fm-Fmoc		Piperidine	Boc	Bzl	HF
Allyl-Aloc		Pd(0)	Fmoc/Boc	*t*Bu/Bzl	TFA/HF
Dmb		1% TFA	Fmoc	*t*Bu	TFA

Dde		Hydrazine	Fmoc	*t*Bu	TFA
Dmab-*N*-ivDde		Hydrazine	Fmoc	*t*Bu	TFA
CAM		NaOH	Fmoc	*t*Bu	TFA
TMSE		Tetrabutylammonium fluoride (TBAF)	Fmoc	*t*Bu	TFA
Adpoc-OPp		1% TFA	Fmoc	*t*Bu	TFA



a. Side-chain protecting groups and resin–peptide anchorange ("permanent")
b. N^{α}-Protecting group ("temporary")
c. Lys-Asp/Glu side-chain protecting groups ("semipermanent")

FIG. 2. Protection scheme for synthesis of cyclic peptides.

plished with BOP in conjunction with *in situ* neutralization using DIEA. Although final products have been obtained in high purity, a limitation is that the lability of the oxime residue can result in peptide lost at each step, resulting in low yields.

Procedure. The linear peptide chain is assembled on Boc-Leu-oxime resin (0.11 mmol/g). The Boc protecting group is removed by treatment of the resin with trifluoroacetic acid (TFA)–CH_2Cl_2 (3:3, v/v) for 30 min. After washing, the appropriate Boc-amino acid (5 equivalent), BOP (5 equivalent), and DIEA (5 equivalent) in DMF are added to the resin and allowed to couple for 2 hr. Couplings of Boc-Asn-OH and Boc-Gln-OH are repeated using 2.5 equivalents of reactants. After the final coupling, the Boc group is removed from the N terminus as described above and the resin treated with DIEA (1.5 equivalent) in CH_2Cl_2 for 24 hr. The protected cyclic peptide is isolated from the solution phase and purified by chromatography.

The selection of the amino acid to be anchored to an oxime resin influences the yield and purity of the desired target. Cyclo[Arg(Tos)-Gly-Asp(OcHex)-Phg] (where Tos is 4-toluenesulfonyl, cHex is cyclohexyl, and Phg is phenylglycine) has been synthesized and the monomer to dimer ratio studied as a function of the order of attachment of the amino acids to the resin.[25] The highest yield of monomer is achieved when Phg is the C terminus residue, whereas Arg(Tos) gives the lowest yield.

Oxime resins in conjunction with phenacyl (Pac) and allyl (Al) protecting groups have been used for the preparation of protected side chain-to-side chain cyclic peptides.[26] The linear peptide is anchored to the oxime

[25] N. Nishino, M. Xu, H. Mihara, T. Fujimoto, Y. Ueno, and H. Kumagai, *Tetrahedron Lett.* 33, 1479 (1992).
[26] A. Kapimiolu and J. W. Taylor, *Tetrahedron Lett.* 34, 7031 (1993).

resin via the β-carboxyl group of Boc-Asp(OH)-OX, where X is phenacyl or allyl ester. Fmoc-Lys(Boc)-OH is incorporated as the final amino acid following peptide elongation. Deprotection of the side chain with TFA–CH_2Cl_2 (1:3, v/v) followed by neutralization with DIEA–CH_2Cl_2 (1:19, v/v), releases the peptide from the resin and gives the cyclized, fully protected peptide.

As an alternative approach, Richter *et al.* have used the reactivity of thioesters toward nucleophilic attack for the preparation of head-to-tail cyclic peptides without Asx or Glx residues.[27] *S*-Tritylthioglycolic acid has been attached to Phe-MBHA resin and deprotected to give a handle terminating in a thiol group. Coupling of the first amino acid to the free thiol is achieved using DIPCDI and DIEA in CH_2Cl_2 with minimal epimerization. A series of linear penta-, hexa-, and heptapeptides is assembled from the thiol function using Boc chemistry and cyclized in the presence of DIEA (3 equivalent) and DMAP (0.1 equivalent) in *N,N*-dimethylacetamide (DMA) for 2–7 days.

Fmoc-Fm/Boc/Bzl/BHA Handle (Scheme 1b)

Felix *et al.* have described the Boc/Bzl/Fmoc approach for the construction of side chain-to-side chain cyclic peptides.[28] Linear peptides are prepared on BHA–resin by Boc/Bzl chemistry using Boc-Asp(OFm)-OH and Boc-Lys(Fmoc)-OH as precursors to the lactam residue. The Fm-based side-chain protecting groups have been shown to be stable to DIEA–CH_2Cl_2 used in neutralization steps. Selective removal of these groups using piperidine–DMF (1:4, v/v), cyclization, and HF cleavage gives the desired product.

Procedure. Boc-[Asp^8(OFm),Lys^{12}[Asp^8,Ala^{15}]GRF(1–29)]-BHA–resin (0.4 mmol) is deprotected at the Asp side chain using piperidine–DMF (1:4, v/v) (once for 1 min and once for 20 min) and then washed successively with DMF, methanol, CH_2Cl_2, and methanol (1 min each). The peptide is cyclized by addition of BOP (2.5 equivalent) in DMF containing triethylamine (2.8 equivalent) three times for 4 hr until the resin gives a negative ninhydrin test. The cyclic peptide is cleaved from the resin by treatment with HF–1,2-ethanedithiol at 0° for 2 hr. The product is isolated by evaporation of HF, washing with ethyl acetate, extraction with TFA, evaporation, and trituration with ether.

[27] L. S. Richter, J. Y. K. Tom, and J. P. Burnier, *Tetrahedron Lett.* **35,** 5547 (1994).

[28] A. M. Felix, E. P. Heimer, C. T. Wang, T. J. Lambros, A. Fournier, T. F. Mowles, S. Maines, R. M. Campbell, B. B. Wegrzynski, V. Toome, D. Fry, and V. S. Madison, *Int. J. Pept. Protein Res.* **32,** 441 (1988).

Boc-tBu/Fmoc/Bzl/BHA Handle (Scheme 1c)

Schiller *et al.* have developed the first method for the preparation of side chain-to-side chain cyclic lactam peptides on BHA–resin incorporating N^{α}-Fmoc-amino acids with Bzl side-chain protecting groups for linear assembly and with Boc and *t*Bu protection for amino- and carboxylate-containing side chains for cyclization.[29] Removal of the Boc and *t*Bu side-chain protecting groups with TFA–CH_2Cl_2, neutralization using DIEA–CH_2Cl_2, cyclization, and subsequent HF treatment remove benzyl side-chain protecting groups and cleave the peptide from the solid support.

Later, Schiller *et al.* reported a procedure enabling the preparation of demanding and larger cyclic peptides.[30] The linear sequences are synthesized on MBHA–resin using N^{α}-Boc-amino acids and changing to N^{α}-Fmoc-amino acids with Boc and *t*Bu side-chain protection for Orn and Asp, respectively, for the lactam region. Removal of the side-chain protecting groups with TFA is followed by neutralization and cyclization. Removal of the N^{α}-Fmoc group allows for the completion of the chain elongation using Boc/Bzl chemistry. The cyclic peptide is obtained from the resin on HF treatment.

Procedure. The linear peptide is constructed on MBHA–resin with the terminal N^{α}-Fmoc of Orn retained. Treatment with TFA–CH_2Cl_2 (1:1, v/v) to remove the side chains of Orn and Asp residues is followed by neutralization using DIEA–CH_2Cl_2 (1:9, v/v). After washing with DMF, cyclization is conducted by addition of *N,N'*-dicyclohexylcarbodiimide (DCC) (5 equivalent) and 1-hydroxybenzotriazole (HOBt) (5 equivalent) in DMF over a period of several days adding fresh DCC and HOBt every 48 hr. The N-terminal Fmoc group is removed with piperidine–CH_2Cl_2 (1:1, v/v) and the final residue coupled in the usual manner. The peptide is released from the solid support with removal of the Bzl side-chain protecting groups with HF.

Dmb/Fmoc/tBu/PAC Handle (Scheme 2a)

The utility of 2,4-dimethoxybenzyl (Dmb) ester protection for the α-carboxylic acid of Glu in conjunction with side-chain anchoring has been reported.[16] Following linear peptide chain elaboration, removal of the Dmb group is accomplished with TFA–CH_2Cl_2 (1:99, v/v), conditions which are compatible with *t*Bu and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) side-chain protecting groups but not with Trt (trityl) protection for Cys

[29] P. W. Schiller, T. M. Nguyen, and J. Miller, *Int. J. Pept. Protein Res.* **25**, 171 (1985).
[30] P. W. Schiller, T. M.-D. Nguyen, L. A. Maziak, B. C. Wilkes, and C. Lemieux, *J. Med. Chem.* **30**, 2094 (1987).

and His residues. N^{α}-Fmoc removal, cyclization, release of the peptide from the resin and removal of *t*Bu side chains with prolonged treatment with TFA–phenol, and gel filtration to remove oligomers give a monomeric cyclic peptide.

Procedure. Fmoc-Glu(OH)-ODmb is coupled to PAC–PS resin (0.45 mmol/g) using a DMAP-catalyzed symmetric anhydride procedure. After assembly of the desired linear sequence, the Dmb group is removed by treatment with TFA–CH_2Cl_2 (1:99, v/v, six times for 5 min). The Fmoc group is removed (piperidine–DMF, 1:4, v/v) and the peptide cyclized by addition of BOP/HOBt (3 equivalent) and *N*-methylmorpholine (NMM) (6 equivalent) for 5 hr. After DMF washing, the resin is cleaved by treatment with TFA–phenol (95:5, v/v), to give the cyclic peptide.

Alloc-Al/Fmoc/tBu/PAC-PAL Handle (Scheme 2a)

The synthesis of cyclic head-to-tail peptides by a three-dimensional orthogonal protecting strategy was accomplished independently by Trzeciak and Bannwarth[31] and by Albericio and co-workers[17] with an Fmoc/*t*Bu/allyl scheme. Allyl-based protecting groups for peptide synthesis have been introduced independently by Lyttle and Hudson[32] and by Loffet and Zhang[33] and are removed smoothly and quantitatively with mild palladium-catalyzed transfer without the undesired back-alkylation side reactions that result when carbocations are generated. Furthermore, owing to their lower steric hindrance and less hydrophobic character, allyl derivatives couple more efficiently than do *t*Bu-based derivatives.

The Fmoc/*t*Bu/allyl strategy has become an attractive approach and is incorporated in a variety of sequences. The antibiotic bacitracin has been synthesized by initially attaching the side chain of L-Fmoc-Asp(OH)-OAl to PAL–PS.[34] Linear assembly, allyl removal with $Pd(PPh_3)_4$, acetic acid and triethylamine, cyclization, addition of the N-terminal thiazoline dipeptide, and cleavage afford the cyclic peptide.

The preparation of cyclic peptides on solid phase with Fmoc/*t*Bu/allyl-based methods has been automated.[35] Standard allyl removal uses a sus-

[31] A. Trzeciak and W. Bannwarth, *Tetrahedron Lett.* **33**, 4557 (1992).

[32] M. H. Lyttle and D. Hudson, *in* "Peptides: Chemistry and Biology, Proceedings of the Twelfth American Peptide Symposium" (J. A. Smith and J. E. Rivier, eds.), p. 583. Escom, Leiden, The Netherlands, 1992.

[33] A. Loffet and H. X. Zhang, *in* "Innovation and Perspectives in Solid Phase Synthesis: Peptides, Polypeptides and Oligonucleotides" (R. Epton, ed.), p. 77. Intercept, Andover, UK, 1992.

[34] J. Lee, J. H. Griffin, and T. Nicas, *J. Org. Chem.* **61**, 3983 (1996).

[35] S. A. Kates, S. B. Daniels, and F. Albericio, *Anal. Biochem.* **212**, 303 (1993).

pended palladium catalyst, which is not feasible on a batch and continuous-flow peptide synthesizer. Solvent conditions have been examined and optimized to solubilize the catalyst, prevent undesired Fmoc deblocking, and be compatible with sensitive amino acids (Trp and Met) and with glyco- and sulfopeptides. $CHCl_3$–acetic acid–NMM (37:2:1, by volume), has proved to be most effective for the use in automated instruments, and the results give comparable purity to that obtained by performing the cyclization manually.

Procedure. Automated side-chain deblocking of the allyl group is performed by placing a vial containing $Pd(PPh_3)_4$ (125 mg) under Ar in the amino acid module following the last amino acid in the sequence. The catalyst is dissolved to a final concentration of 0.14 *M* in $CHCl_3$–acetic acid–NMM (37:2:1, by volume), delivered to a 0.3 mmol/g substituted peptidyl–resin (500 mg), and recycled through the column for 2 hr. The column is washed with a solution of 0.5% DIEA and 0.5% sodium diethyldithiocarbamate in DMF (10 min, 6 ml/min) and DMF (10 min, 6 ml/min). The N^{α}-Fmoc group of the final amino acid is removed, and benzotriazol-1-yl-*N*-oxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP)/HOBt (4 equivalent) is dissolved to a final concentration of 0.3 *M* in a solution of 0.6 *M* DIEA in DMF and delivered to the solid support. The cyclization is allowed to occur until the resin gives a negative ninhydrin test, and the resin is washed with DMF (15 sec, 3 ml/min) and CH_2Cl_2 (6 min, 3 ml/min). The cyclic peptide is cleaved from the resin using TFA–anisole–2-mercaptoethanol (95:3:2, by volume), for 2 hr and the filtrate collected. The resin is washed with TFA and the combined filtrates treated with ether and cooled to −70°. After removal of the supernatant, the precipitated cyclic peptide is washed with ether, dissolved in acetic acid, and lyophilized.

After assembly of a linear peptide on MBHA–resin (0.4 mmol/g) the N^{α}-allyloxycarbonyl (Alloc) protecting group is removed by addition of $PdCl_2(PPh_3)_2$ (0.04 equivalent) and acetic acid (3.5 equivalent) in CH_2Cl_2 (3 ml/g of resin).[36] Tributyltin hydride (3 equivalent) is then added and allowed to react for 5 min before washing with CH_2Cl_2, triethylamine–CH_2Cl_2 (1:9, v/v), CH_2Cl_2, methanol, and CH_2Cl_2.

Dde/allyl/Fmoc/tBu/PAL Handle (Scheme 1a)

A branched, cyclic peptide has been prepared by Bloomberg *et al.*[37] with an Fmoc/*t*Bu/allyl/1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidine)ethyl (Dde)

[36] O. Dangles, F. Guibé, G. Balavoine, S. Lavielle, and A. Marquet, *J. Org. Chem.* **52**, 4984 (1987).

[37] G. B. Bloomberg, D. Askin, A. R. Gargaro, and M. J. A. Tanner, *Tetrahedron Lett.* **34**, 4709 (1993).

protection scheme. The first branch is assembled by sequential coupling of Fmoc-Lys(Dde)-OH, Fmoc-Ala-OH, Fmoc-Glu(OAl)-OH, and Fmoc-Cys(Trt)-OH to PAL–PEG–PS followed by acetylation. The Dde function is removed with hydrazine, and the second branch is added. Allyl removal of the carboxyl side chain of Glu, N^{α}-Fmoc removal, cyclization, and cleavage from the resin give the crude peptide, which is purified by HPLC.

Procedure. Following assembly of the appropriate linear peptide the Dde group is removed by flowing a 1.5% solution of hydrazine in DMF through the resin for 9 min. Elongation from the side chain is carried out with Fmoc-amino acids. Allyl removal is achieved by suspending the resin in DMSO–THF–0.5 *M* HCl–NMM (20:10:10:1, by volume) (5 ml/100 mg of resin) and $Pd(PPh_3)_4$ (0.15 to 1.0 equivalent based on resin substitution) for 3 hr in an inert atmosphere. The Fmoc group is removed and cyclization is carried out by treatment of the resin with 1.5 equivalents of *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU)/HOBt/NMM (1:1:2, by volume) in DMF (2 ml/100 mg resin) for 2 hr. The cyclic peptide is cleaved from the resin using TFA–1,2-ethanedithiol–phenol–water–triisobutylsilane (92:3:2:2:1, by volume) and the product isolated by precipitation with ether.

N-ivDde-Dmab/Fmoc/tBu/PAC–PAL Handle (Scheme 1a,b)

To complement the Dde protecting group, 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidine)-3-methylbutyl]amino}benzyl (ODmab) ester protection for the carboxylic acid function of Asp and Glu residues and the more sterically hindered 1-(4,4-dimethyl-2,6-dioxocyclohexylidine)-3-methylbutyl (*N*-ivDde) group for amine protection have been reported.[38] Both protecting groups are orthogonal to Fmoc/*t*Bu methods, and cleavage is achieved within minutes on treatment with hydrazine–DMF (1:49, v/v).

Procedure. Fmoc-Asp(ODmab)-OH is coupled to 2-chlorotrityl resin and the linear sequence assembled using Fmoc/*t*Bu method. The Dmab group is removed by reaction with hydrazine–DMF (1:49, v/v) for 9 min. Cyclization is carried out by addition of DIPCDI/HOAt in DMF and the product cleaved from the resin using TFA–CH_2Cl_2 (1:99, v/v).

CAM/Fmoc/tBu/PAC Handle (Scheme 1b)

Starting with Fmoc-Trp–PepsynK resin, Mendre *et al.* have assembled a head-to-side chain cyclic peptide.[10] To avoid aspartimide formation, dimer

[38] D. J. Evans, B. W. Bycroft, W. C. Chan, and P. D. White, *in* "Peptides 1996: Proceedings of the Twenty-fourth European Peptide Symposium" (R. Ramage, ed.), in press. Mayflower Worldwide, Birmingham, UK, 1996.

Fmoc-Asp([Z]-Dpr-CAM)-OH (CAM, carboxyamidomethyl) is prepared in solution and incorporated into the linear sequence. Hydrolysis of the CAM group is achieved with NaOH; cyclization and cleavage from the resin with concomitant removal of *t*Bu-based side-chain protecting groups give the desired target.

Procedure. The CAM side-chain protecting group is removed by treatment with NaOH (3 equivalent) in 2-propanol–water (7:3, v/v) for 1 hr at 25° followed by neutralization with 1.75 *N* acetic acid (5 equivalent) and washed with acetic acid in 2-propanol–water (7:3, v/v), 2-propanol–water (7:3, v/v), 2-propanol and diethyl ether. The linear peptide is cyclized by three successive treatments with *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate-*N*-oxide (TBTU)/HOBt (3 equivalent) and DIEA (6 equivalent) for 2 hr. After washing, side-chain protecting groups are removed and the product cleaved from the resin by treatment with reagent K (TFA–water–phenol–thioanisole-1,2-ethanedithiol, 34:2:2:1:1, by volume) for 1.5 hr at 25°.

TMSE/Fmoc/tBu/PAL Handle (Scheme 1b)

The trimethylsilylethyl ester (TMSE) group has been used for side-chain carboxyl protection in a three-dimensional orthogonal scheme for preparing cyclic peptides on Rink amine resin.[39] Fmoc-Asp(OTMSE)-OH is first coupled to the resin following standard Fmoc/*t*Bu elongation to elaborate the linear sequence. Treatment of the resin-bound peptide with 1 *M* tetrabutylammonium fluoride (TBAF) in DMF removes both the TMSE side chain and the N^{α}-Fmoc group; cyclization and cleavage give the desired target.

Procedure. Fmoc-Asp(OTMSE)-OH is coupled to Rink amide resin using DCC/HOBt, and residual amino groups are capped by treatment with acetic anhydride. After construction of the linear sequence, treatment with 1 *M* TBAF in DMF for 20 min removes both the TMSE and N^{α}-terminal Fmoc protecting groups. Cyclization is carried out by treatment with BOP (10 equivalent) and DIEA (5 equivalent) in DMF for 4 hr. Treatment with reagent K removes the side-chain remaining protecting groups and releases the cyclic peptide from the resin.

Adpoc-OPp/Fmoc/tBu/PAC-PAL Handle (Scheme 1a,b)

2-Phenyl-2-propyl (OPp) ester and 2-(1′-adamantyl)-2-propoxycarbonyl (Adpoc) carbamate protection for carboxylic acids and amines, respec-

[39] C. K. Marlowe, *Bioorg. Med. Chem. Lett.* **3**, 437 (1993).

tively, have been introduced as a third level of orthogonality in Fmoc/*t*Bu methods.[40] Linear sequences are assembled on *p*-alkoxybenzyl alcohol and 5-amino-10,11-dihydro-5*H*-dibenzo[*a,d*]cycloheptenyl-2-oxyacetyl-Nle-methylbenzhydrylamine resins for C-terminal acids and amides, respectively, and the OPp and Adpoc protecting groups are removed with TFA–CH_2Cl_2 (1:99, v/v). Neutralization, on-resin cyclization, and final cleavage yield the desired targets in high purity.

Procedure. Adpoc and OPp deprotections are conducted by mixing the resin with TFA–CH_2Cl_2 (1:99, v/v) six times for 10 min. The resin is washed with CH_2Cl_2 (twice, 1 min), DIEA–CH_2Cl_2 (1:49, v:v, twice, 1 min), and DMF (three times, 1 min). The peptide is cyclized with TBTU (1.2 equivalent) and DIEA (1.8 equivalent) in DMF three times for 1.5 hr. Treatment of the resin with TFA–water–*p*-cresol–1,2-ethanedithiol–2-methylindole (75:5:5:12.5:2.5, by volume) gives the crude peptide.

Reagents for Cyclization

Once a protection scheme has been chosen and successfully incorporated into a synthetic strategy, the penultimate step for on-resin cyclization requires an efficient coupling reagent (Fig. 3). Initially, carbodiimides were used for ring closure. In the first report for the assembly of side chain-to-side chain cyclic peptides, representative opioid analogs were treated with DCC (5 equivalent) and HOBt (5 equivalent) in DMF over a period of several days, adding fresh DCC and HOBt every 48 hr, to give 6–22% yields.[29]

A comparison of different activators for lactamization was studied by Felix *et al.*[41] Cyclization of a linear peptidyl–resin with 6 equivalents of DCC/HOBt in DMF required 6 days for a negative ninhydrin test, but the cleaved product was contaminated with side products. Alternatively, treatment with 6 equivalent of BOP in DMF containing excess DIEA gave a purer product in 4 hr. Bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl)- and diphenylphosphoryl azide (DPPA)-mediated cyclizations gave even less efficient cyclization in comparison to DCC as measured by quantitative ninhydrin. In a similar study, Hruby *et al.* investigated the cyclization

[40] F. Dick, U. Fritschi, G. Haas, O. Hässler, R. Nyfeler, and E. Rapp, *in* "Peptides 1996: Proceedings of the Twenty-fourth European Peptide Symposium" (R. Ramage, ed.), in press. Mayflower Worldwide, Birmingham, UK, 1996.

[41] A. M. Felix, C. T. Wang, E. P. Heimer, and A. Fournier, *Int. J. Pept. Protein Res.* **31**, 231 (1988).

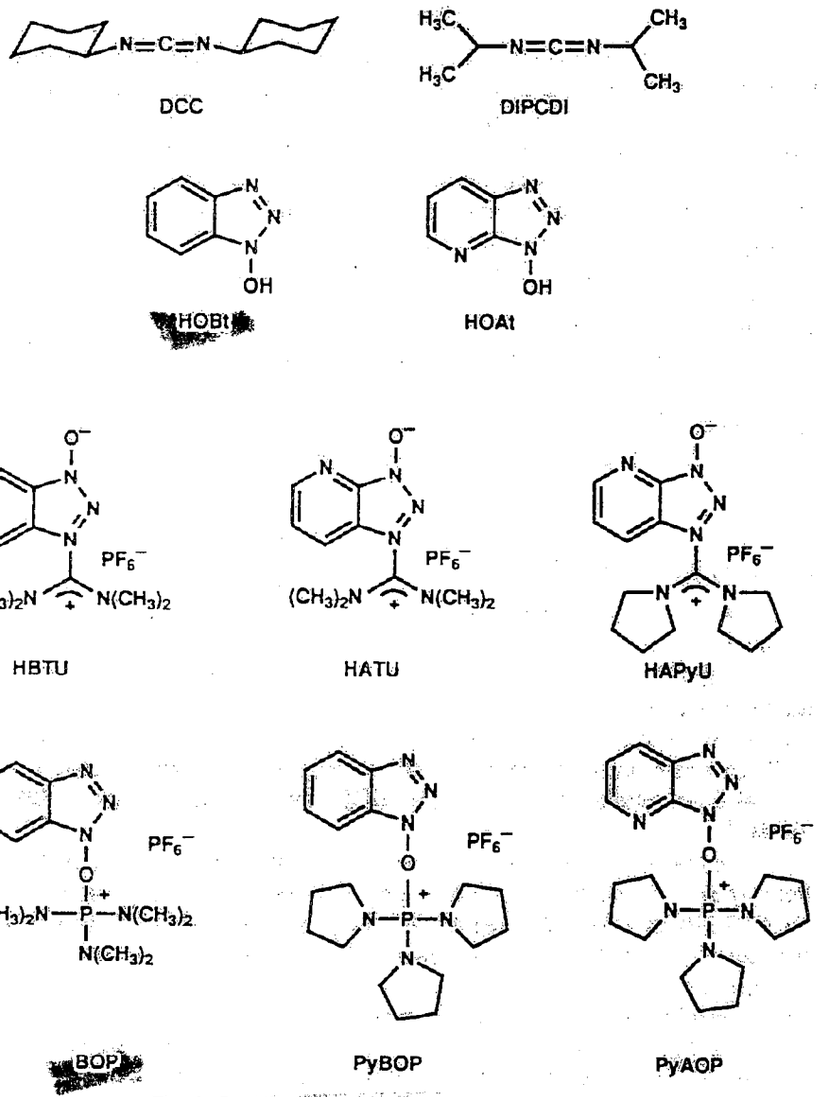


FIG. 3. Coupling reagents used for on-resin cyclization.

of model peptides.[42] To facilitate ring closure and minimize cyclodimerizations, D-amino acids were incorporated in the design, and linear peptides were assembled on chloromethyl and MBHA resins at loadings of 0.2 to 0.45 mmol/g. For on-resin cyclizations, BOP was found to be superior to DIPCDI/HOBt and DPPA, and to solution methods in terms of product yield, purity, and reaction rate.

Plaué found that BOP/HOBt/DIEA-mediated cyclization of a model peptide occurred in 2 hr, whereas with DIPCDI/HOBt lactamization required 3 days for completion.[11] The same ratio of cyclic monomer to dimer was obtained, but the amount of polymeric material formed was greater when using the BOP-containing mixture. For the formation of large loop peptides, cyclodimerization occurs at high resin loading if the cyclization rate is slow (DIPCDI). Polymerization occurs at high loading when cyclization is fast (BOP).

McMurray *et al.* studied the side reactions of head-to-tail cyclizations of resin-bound peptides.[43] BOP/HOBt/DIEA-mediated cyclizations gave more racemization and oligomerization than DIPCDI/HOBt. In addition, oligomerization and racemization were resin dependent but solvent independent with DIPCIDI (BOP in CH_2Cl_2 gave greater amounts of oligomer). Story and Aldrich found that HBTU-mediated on-resin cyclizations gave poor results due to formation of a tetramethylguanidinium (Tmg) Schiff base derivative of the ω-amino group of α,β-diaminopropionic acid (Dap).[44] BOP- and DIPCDI/HOBt-activated cyclizations gave higher yields, but the reaction proceeded more slowly.

Applications of 1-hydroxy-7-azabenzotriazole (HOAt) and its uronium {*N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU)} and phosphonium [7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP)] derivatives have been thoroughly examined for both solution and solid-phase peptide synthesis.[45] These reagents enhance coupling rates and yields, reduce racemization, and are suitable for the synthesis of difficult peptides such as those which incorporate hindered amino acids and hydrophobic peptides or involve the coupling of protected segments. To study the utility of azabenzotriazole derivatives, the linear

[42] V. J. Hruby, F. Al-Obeidi, D. G. Sanderson, and D. D. Smith, *in* "Innovation and Perspectives in Solid Phase Synthesis: Peptides, Polypeptides and Oligonucleotides" (R. Epton, ed.), p. 197. SPCC, Birmingham, U.K., 1990.

[43] J. S. McMurray, C. A. Lewis, and N. U. Obeyesekere, *Pept. Res.* **7**, 195 (1994).

[44] S. C. Story and J. V. Aldrich, *Int. J. Pept. Protein Res.* **43**, 292 (1994).

[45] S. A. Kates, L. A. Carpino, and F. Albericio, *in* "Peptides: Chemistry and Biology, Proceedings of the Fourteenth American Peptide Symposium" (P. T. P. Kaumaya and R. S. Hodges, eds.), p. 893. Mayflower Worldwide, Birmingham, UK, 1996.

TABLE II
COMPARISON OF COUPLING REAGENTS FOR THE LACTAMIZATION STEP OF CYCLO(Asp-Tyr-DTrp-Val-DTrp-DTrp-Arg)

Coupling method	Linear (%)	Cyclic (%)	Purity (%)
PyAOP/HOAt	5	74	61
PyBOP/HOBt	5	69	51
HATU/HOAt	5	70	50
HBTU/HOBt	5	63	44

heptapeptide cyclo(Asp-Tyr-DTrp-Val-DTrp-DTrp-Arg) was cyclized. For this model, azabenzotriazole-based reagents performed slightly better than the benzotriazole analogs, with PyAOP producing the peptide in highest yield (74%) and purity (61%) (Table II).[46]

Aldrich and co-workers surveyed several activating reagents for their efficiency of resin-bound lactam formation in the cyclic dynorphin A model.[47] Uronium-based reagents HBTU, HATU, and 1-(1-pyrrolidinyl-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene) pyrrolidinium hexafluorophosphate *N*-oxide (HAPyU) gave low yields of desired product and a high formation of the linear alkyl guanidinium by-products even when a low concentration of reagent was used. HATU and HAPyU gave better results than HBTU, but the dipyrrolidinylguanidinium (Dpg) by-product from HAPyU was more difficult to remove during purification. On the contrary, the phosphonium-based reagents BOP, PyBOP, and PyAOP gave satisfactory yields of cyclic peptide, but the reactions progressed more slowly (3 days). PyXOP cyclizations were faster than those with BOP, but all phosphonium reagents gave similar yields of cyclic peptide, undesired linear starting peptide, linear peptide imide, and cyclodimer.

Zhang and Taylor examined solvent conditions for solid-phase cyclizations.[48] Solvent mixtures were chosen that combine high polarity with excellent resin swelling properties. For BOP/DIEA lactamization on a *p*-chloromethylbenzyl–polystyrene resin, optimal monomer–dimer ratios were obtained with DMSO–*N*-methylpyrrolidone (NMP) (1:4, v/v) > THF–NMP (35:65, v/v), DMF, and DMF–CH_2Cl_2 (1:1, v/v) > CH_2Cl_2–DMF (96:4, v/v).

[46] S. A. Kates, C. A. Minor, H. Shroff, R. C. Haaseth, S. Triolo, A. El-Faham, L. A. Carpino, and F. Albericio, *in* "Peptides 1994: Proceedings of the Twenty-third European Peptide Symposium" (H. L. S. Maia, ed.), p. 248. Escon, Leiden, The Netherlands, 1995.
[47] S. Arttamangkul, B. Arbogast, D. Barofsky, and J. V. Aldrich, *Lett. Pept. Sci.* **3**, 357 (1996).
[48] W. Zhang and J. W. Taylor, *Tetrahedron Lett.* **37**, 2173 (1996).

Cyclic Peptide Libraries

Combinatorial libraries serve as a source of diverse potential therapeutic candidates and have become an important tool in the drug discovery field. Pioneering work involving the creation and screening of linear peptide libraries has expanded to the preparation of cyclic peptide libraries.[49] Spatola *et al.* coupled the side chain of Boc-Asp-OFm to a benzyl alcohol resin (Merrifield) using Mitsunobu conditions and then constructed combinatorial libraries of cyclic pentapeptides.[50] The linear chain was assembled using Boc-amino acids with the exception of the last amino acid, which was added as the Fmoc derivative. To avoid partial racemization of the C-terminal Asp residue during chain elongation, 1 equivalent of DIEA in the couplings was used. Removal of Fmoc and Fm groups was accomplished using piperidine–DMF (1 : 4, v/v). Initially cyclizations were accomplished using BOP/HOBt, but the final lactam bond was formed faster using BOP/HOAt activation. Cleavage of peptide mixtures from the resin was carried out using HF.

Mihara *et al.* prepared cyclic peptide mixtures using oxime–resin. The cyclic peptides contained ε-aminocaproic acid (Aca) as a flexible residue to enhance the cyclization step.[51] The synthesis of cell adhesion inhibitors cyclo[Arg(Tos)-Gly-Asp(OcHex)-Ser(Bzl)-Aca] was accomplished by BOP/HOBt-induced coupling of Boc-amino acids to Aca-oxime resin. After removal of the N-terminal Boc group, cyclization was effected by treatment with acetic acid–DIEA. A combinatorial library based on this structure in which Ser was replaced by Lys, Gln, Ala, Glu, Pro, Val, Tyr, Phe, and Trp was constructed via both the "mix and split" and mixture methods. For the mix and split technique, the cyclic peptides were formed in almost equal amount throughout the coupling and cyclization reactions. The mixed protocol (0.3 equivalent of each amino acid) gave similar results except lower yields for Pro and Val.

Side Reactions

Although fluoride-labile protecting groups and linkers have been developed as an extra level of orthogonality, a side reaction was observed when peptidyl–resins were treated with tetrabutylammonium fluoride. For Glu(O*t*Bu)- and Asp(O*t*Bu)-containing peptides, fluoride ions were found

[49] K.-H. Wiesmüller, S. Feiertag, B. Fleckenstein, S. Kienle, D. Stoll, M. Herrmann, and G. Jung, *in* "Peptides and Nonpeptide Libraries—A Handbook" (G. Jung, ed.), p. 203. VCH, Germany, 1996.

[50] A. F. Spatola, K. Darlak, and P. Romanovskis, *Tetrahedron Lett.* **37,** 591 (1996).

[51] H. Mihara, S. Yamabe, T. Niidome, and H. Aoyagi, *Tetrahedron Lett.* **36,** 4837 (1995).

to convert α-peptides to their corresponding γ- or β-rearranged sequence.[52] Although HPLC, matrix-assisted laser desorption/time-of-flight (MALDI-TOF) mass spectrometry, and amino acid analysis are powerful techniques for peptide identification, the use of capillary zone electrophoresis (CZE) was required to detect this phenomenon.

In the synthesis of small loop peptides using Fmoc/Bzl amino acids by Plaué,[11] HF cleavage afforded the desired cyclic peptide and a second product that was identified to be the result of imide formation between Asp-146 and Phe-147. To avoid the succinimide side reaction, cyclohexyl side-chain protection of Asp-146 was incorporated into the linear sequence.

With Fmoc/Fm protecting groups, following standard treatment with piperidine–DMF (1:4, v/v), a wash with 0.4% concentrated HCl in DMF,[17] HOBt in DMF,[43] or a tertiary amine[50] was recommended prior to cyclization. This step ensured complete removal of piperidine and avoided the formation of C-terminal-piperidylamides as a by-product in the cyclization step. If DBU–piperidine–DMF (1:1:48, by volume) was used, the acid wash was not required.

Epimerization during linear assembly was identified to be problematic for strategies that include side-chain anchoring of Asp residues. Coupling with preactivated pentafluorophenyl esters or reducing the amount of base to 1 equivalent for BOP/HOBt significantly reduced the level of racemization.[50] The degree of racemization during on-resin cyclization is affected by the amino acid sequence.[43]

[52] S. A. Kates and F. Albericio, *Lett. Pept. Sci.* **1,** 213 (1994).

[10] Disulfide Bond Formation in Peptides

By IOANA ANNIS, BALAZS HARGITTAI, and GEORGE BARANY

Introduction

Disulfide bridges represent important evolutionarily conserved structural motifs in many biologically important peptides and proteins, including hormones, enzymes, growth factors, toxins, and immunoglobulins.[1–5] Intra-

[1] J. M. Thornton, *J. Mol. Biol.* **151,** 261 (1981).
[2] T. E. Creighton, *BioEssays* **8,** 57 (1988).
[3] N. Srinivasan, R. Sowdhamini, C. Ramakrishnan, and P. Balaram, *Int. J. Pept. Protein Res.* **36,** 147 (1990).

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Clarke, Modet & Cº

COLOMBIA

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SEÑORES
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

REF: **EXPEDIENTE No. 06.020.917**
SOLICITUD DE PATENTE A NOMBRE DE NOVARTIS AG

Yo, **JIMENA ESCOBAR URIBE,** identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53 Piso 6, atentamente digo a usted que sustituyo el poder con que obro a la doctora **DILIA RODRIGUEZ D´ALEMAN**, domiciliada en Bogotá, D.C., portadora de la tarjeta profesional No. 20.824, con las mismas facultades con que me fueron conferidas para este caso.

JIMENA ESCOBAR URIBE
C.C. 39.779.452 de Usaquén
T.P. 68.228

Acepto sustitución

DILIA RODRIGUEZ D´ALEMAN
C.C. 41.654.882 de Bogotá
T.P. 20.824

PRESENTACION PERSONAL

El anterior escrito fue presentado ante el

NOTARIO ONCE DEL CIRCULO DE BOGOTA, D.C.

personalmente por

quien exhibió la C.C.

y Tarjeta Profesional No.

Fecha 21 OCT 2009

REPUBLICA DE COLOMBIA

Guillermo Chávez Cristancho

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Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/10192 A2

(54) Title: SOMATOSTATIN ANALOGUES

(57) Abstract: The invention provides cyclo[{4-(NH_2-C_2H_4-NH-CO-O-)Pro}-Phg-DTrp-Lys-Tyr(4-Benzyl)-Phe], optionally in protected form, or a pharmaceutically acceptable salt or complex thereof, which has interesting pharmaceutical properties.

D3

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United States Patent [19]

Veber

[11] **4,190,648**

[45] **Feb. 26, 1980**

[54] PEPTIDES HAVING SOMATOSTATIN ACTIVITY

[75] Inventor: **Daniel F. Veber, Ambler, Pa.**

[73] Assignee: **Merck & Co., Inc., Rahway, N.J.**

[21] Appl. No.: **20,148**

[22] Filed: **Mar. 13, 1979**

[51] **Int. Cl.**[2] **A61K 37/100; C07C 103/52**

[52] **U.S. Cl.** ..... **424/177; 260/112.5 S**

[58] **Field of Search** ..... 260/112.5 S, 112.5 R; 424/177

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,146,612 3/1979 Veber ..... 260/112.5 S

OTHER PUBLICATIONS

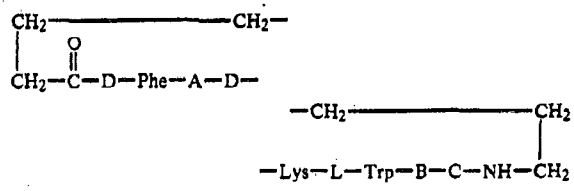
Immer, H., Proceeding of Fourteenth European Peptide Symposium, 471-476 (1976).

Shemyakin, M., et al., Angew Chem., Int. Ed. Engl., 8, 492-499 (1969).

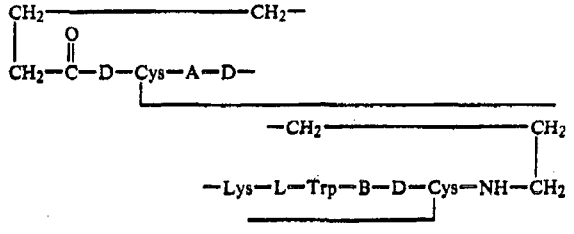
Primary Examiner—Delbert R. Phillips
Assistant Examiner—Blondel Hazel
Attorney, Agent, or Firm—David L. Rose; Harry E. Westlake

[57] **ABSTRACT**

Peptides having the structural formula:



and



wherein

A is D-Thr, D-Val;

B is D-Phe, D-Tyr;

C is D-Phe, D-Tyr, O-Me-D-Tyr;

wherein the ring formed by the peptide backbone contains 26 atoms and pharmaceutically acceptable nontoxic acid addition salts thereof are prepared by the solid phase method. These peptides have the property of inhibiting release of insulin, inhibiting growth hormone release and inhibiting glucagon release in humans and animals without materially affecting gastric secretion. They have a longer duration of action than somatostatin.

3 Claims, No Drawings

and the pharmaceutically acceptable non-toxic acid addition salts thereof.

Illustrative of acid addition salts are hydrochloride, hydrobromide, sulfate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate and the like. The acid addition salts can be conveniently prepared by dissolving the above novel compounds in water, adding two equivalents of appropriate acid and lyophilizing.

The abbreviated designations, which are used herein for the amino acid components, certain preferred protecting groups, amino acid activating groups, condensing agents, reagents and solvents employed in the process of this invention are as follows:

TABLE I

Abbreviated Designation	Amino Acid
Lys	L-lysine
Phe	L-phenylalanine
Trp	L-tryptophan
D-Trp	D-tryptophan
Thr	L-threonine
Aha	7-aminoheptanoic acid
Tyr	L-tyrosine
O-Me-Tyr	O-Me-L-tyrosine
Val	L-valine
D-Lys	D-lysine
D-Phe	D-phenylalanine
D-Thr	D-threonine
D-Tyr	D-tyrosine
O-Me-D-Tyr	O-Me-D-tyrosine
D-Val	D-valine
Cys	L-cysteine
D-Cys	D-cysteine
Acm-D-Cys	acetamidomethyl-D-cysteine

Abbreviated Designation	Protecting Groups
BOC	tert-butyloxycarbonyl
Bzl	benzyl
2-Cl-CBZ	2-chlorobenzyloxycarbonyl
$NHNH_2$	hydrazide

Abbreviated Designation	Activating Groups
HBT	1-hydroxybenzotriazole

Abbreviated Designation	Condensing Agents
DCCI	dicyclohexylcarbodiimide

Abbreviated Designation	Reagents
TFA	trifluoroacetic acid
TEA	triethylamine
DIPEA	diisopropylethylamine

Abbreviated Designation	Solvents
EPAW	ethyl acetate-pyridine-acetic acid-water
BAW	butanol-acetic acid-water
CMW	chloroform-methanol-water
DMF	dimethylformamide
THF	tetrahydrofuran
CMA	chloroform-methanol-concentrated NH_4OH

In accordance with the present invention, the novel somatostatin analogs are prepared by cyclizing corresponding linear peptides. The linear peptides are prepared by using the solid phase sequential synthesis technique. Accordingly, the process for preparing the somatostatin analogs of the present invention comprises (a) preparing a corresponding blocked linear peptide attached to a solid phase resin; (b) selectively deblocking the N-terminal amine group; (c) removing the linear peptide from the resin; (d) treating the linear peptide with a cyclizing agent to obtain the cyclic peptide; and (e) removing the remaining blocking groups.

When the linear peptide is prepared on the resin, it is not critical which amino acid is selected to be at the C-terminal position provided only that the sequence of amino acids in the linear peptide corresponds to that in the desired somatostatin analog. Once a linear peptide has been cyclized one can no longer determine which amino acid was at the C-terminus of the linear peptide. As an example to illustrate this, either of the two following linear peptides, when cyclized, will give the identical somatostatin analog:

(O—Bzl)—D—Thr—(ε-2-Cl—CBZ)—D—Lys—L—Trp—D—Phe—D—Phe—Aha—D—Phe—N_3

or

D—Phe—Aha—D—Phe—(O—Bzl)—D—Thr—(ε-2-Cl—CBZ)—D—Lys—L—Trp—D—Phe—N_3

↓ cyclization

cyclo[(O—Bzl)—D—Thr—(ε-2-Cl—CBZ)—D—Lys—L—Trp—D—Phe—D—Phe—Aha—D—Phe]

It is evident that since the linear peptide is going to be cyclized, it does not matter which amino acid is used to start the chain. Starting with D-Phe at the carboxyl end, as illustrated in the first of the two examples above, has an advantage over the second example. In the first example, Trp, which reacts with t-butyl carbonium ions formed when BOC groups are removed, is in position closer to the N-terminal amino acid and thus will be subjected to a lesser number of exposures to t-butyl carbonium ion.

The synthesis of the linear peptides by the solid phase technique is conducted in a stepwise manner on chloromethylated resin. The resin is composed of fine beads (20–70 microns in diameter) of a synthetic resin prepared by copolymerization of styrene with 1 to 2 percent divinylbenzene. The benzene rings in the resin are chloromethylated in a Friedel-Crafts reaction with chloromethyl methyl ether and stannic chloride. The Friedel-Crafts reaction is continued until the resin contains 0.5 to 5 mmoles of chloride per gram of resin.

The amino acid selected to be the C-terminal amino acid of the linear peptide is converted to its amino protected derivative. The carboxyl group of the selected C-terminal amino acid is bound covalently to the insoluble polymeric resin support, as for example, as the carboxylic ester of the resin-bonded benzyl chloride present in chloromethyl-substituted polystyrene-divinylben-

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2020
Bogotá, D.C.,

Abogado
DILIA MARIA RODRÍGUEZ D'ALEMAN
Apoderado

ASUNTO		
	Radicación	06-020917
	Trámite	002
	Evento	001
	Actuación	430
	Oficio	No 06459
	Folios	07

Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (fase nacional) ☒ Cap.I ☒ Cap. II ☐

No. Sol. Internacional PCT/EP2004/008850
Fecha de Presentación Internal. 06 de Agosto de 2004

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados

Descripción:	Folios: 6 a 39 como se radicó inicialmente
Reivindicaciones:	Folios 40 a 44 como se radicó inicialmente Folios 132 a 135 modificadas
Prioridad:	Folios: 1 08 de Agosto de 2003, UK, 0318682.2
Respuesta del solicitante:	Folios 127 a 131

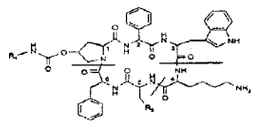
2. Análisis de la Prioridad

La fecha de prioridad del 08 de Agosto de 2003 y su correspondiente documento, son aceptados para el estudio de patentabilidad.

3. Objeto de la invención

Título de la solicitud: preparación de péptidos de somatostatina

El objeto de la presente solicitud de patente de invención consiste en el procedimiento para producir el compuesto de fórmula:



El problema planteado en esta solicitud es proveer un procedimiento de preparación de un análogo somatostatina cíclica y sus intermediarios mediante ciclización de un péptido lineal.

Para solucionar este problema técnico la solicitud en estudio proporciona un procedimiento de preparación de un análogo somatostatina cíclica y sus intermediarios mediante ciclización de un péptido lineal en forma protegida.

IPC: C07 K 7/00

4. De la solicitud de Patente

Reivindicaciones (artículo 30 D 486)

La reivindicación 1 referente al grupo protector amino y donde se realiza la remoción del grupo protector, el cual no se encuentra definido; encontrándose de forma general ya amplia dentro del campo de los grupos protectores .Se sugiere al solicitante restringirlos
a una razonable generalización de los que se encuentran ejemplificados
La reivindicación 6 se refiere a la reacción común de formación de péptidos a través del enlace amida, se sugiere al solicitante restringir a una razonable generalización de acuerdo con lo que presenta ejemplificado.

5. Determinación del Estado de la Técnica

La fecha que se ha tenido en cuenta para determinar el Estado de la Técnica es:

- Fecha de presentación de la solicitud prioritaria: 08 de Agosto de 2003

Considerando que la fecha de presentación de la primera solicitud de patente de la cual se reivindica prioridad es: 08/08/2003 se encontraron los siguientes documentos del estado de la técnica relacionados, que pueden afectar la patentabilidad del objeto de la presente solicitud:

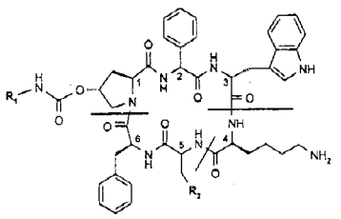
Nº	Documento	Título	Fecha de publicación
D1	WO 02/10192	Somatostatin analogues	07/02/2002
D3	US 4,190,648	Peptides having somatostatine activity	26/02/1980

6. Análisis de patentabilidad (artículo 14 D486)

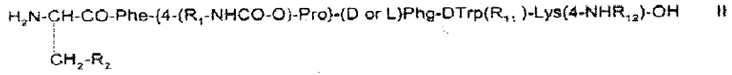
Nivel inventivo (artículo18 D486)

La reivindicación 1 consiste en:

Un procedimiento para producir el compuesto de la fórmula:

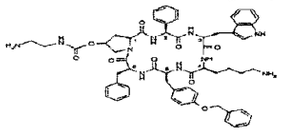


caracterizado porque comprende la ciclización de un análogo de somatostatina lineal, de la fórmula II, en donde la remoción requerida del grupo (s) protector, y la recuperación del compuesto de la fórmula I de esta manera, se obtiene en forma libre o en la forma de la sal.



II

El Estado de la Técnica más cercano es **D1**, que da a conocer el proceso de preparación de análogos de somatostatina de fórmula I y, enseña el compuesto A (Ver resumen, página 1, líneas 3-11), el cual puede ser producido por ciclización de un péptido linear protegido de fórmula Phe-{4-(NHR$_1$-C$_2$H$_4$-NH-CO-O-)Pro}-Phg-DTrp(R$_2$)-Lys(ε-NH$_3$)-Tyr(4-Bzl)-OH, unido a polímero o en forma no protegida de tal forma que el compuesto A es obtenido cuando opcionalmente se remueve los grupos protectores (Ver página 3, líneas 18-22)



cyclo[{4-(NH$_2$-C$_2$H$_4$-NH-CO-O-)Pro}-Phg-DTrp-Lys-Tyr(4-Bzl)-Phe],

Compuesto A –**D1**

D1 enseña que generalmente no es crítico cuál aminoácido es seleccionado en la posición C-terminal para iniciar la cadena de péptido a ser ciclizada, pues sólo se proveen los aminoácidos que hacen parte de la cadena lineal de péptidos que corresponden al compuesto A y que la construcción de la cadena de péptido puede ser efectuada en la forma convencional, por ejemplo usando unidades de aminoácido donde el grupo amino terminal es Fmoc-protegida y los grupos de aminoácidos de cadena lateral, pueden ser protegidos con un grupo amino protector diferente. El péptido lineal es ciclizado de tal forma que se produce una unión entre Tyr(4-Bzl)-OH y Phe por ejemplo Phe-{4-(NHR$_1$-C$_2$H$_4$-NH-CO-O-)Pro}-Phg-DTrp(R$_2$)-Lys(ε-NH$_3$)-Tyr(4-Bzl)-OH o un derivado funcional.

La ciclización puede llevarse a cabo por los métodos conocidos como por ejemplo por vía azida, un éster activo, un anhídrido mezclado o carbodiimida.

Los grupos protectores pueden ser removidos por trifluoroacético o por hidrogenación. (Ver página 4, todo el texto-**D1**)

El proceso objeto de esta solicitud se diferencia del procedimiento de **D1**, en que la conexión del péptido se realiza entre los aminoácidos 4 y 5.

El hecho de realizar la conexión de los aminoácidos 4 y 5, causa un procedimiento de preparación alterno de análogos de somatostatina cíclica, sin ser tóxico.

El Problema Técnico Objetivo era cómo proveer un procedimiento de preparación alternativo de un análogo somatostatina cíclica, realizando la conexión de los aminoácidos 4 y 5, sin ser tóxico.

D3 describe una manera de solucionar el problema técnico, pues enseña la preparación de análogos de somatostatina por ciclización de péptidos lineares. (Ver columna 4, líneas 9-12) y donde no importa cuál aminoácido va a ser utilizado en el comienzo de la cadena del péptido linear a ser ciclizado (Ver columna 4, líneas 42-45) y donde las sales de adición obtenidas de los compuestos, no son tóxicas (Ver resumen)

La reivindicación es obvia, porque el Experto en la Materia enfrentado al problema de "proveer un procedimiento de preparación de un análogo somatostatina cíclica y sus intermediarios mediante ciclización de un péptido lineal conectando los aminoácidos 4 y 5, sin ser tóxico", incluiría "la conexión de otros aminoácidos" cómo se revela en **D3**, en el procedimiento de preparación que se enseña en **D1**, logrando de esta forma el proceso de preparación objeto de esta solicitud. Por tanto, la reivindicación 1 no tiene Nivel Inventivo. Las reivindicaciones dependientes 2-7, no mencionan alguna característica que haga inventivo el procedimiento de preparación de la reivindicación 1, por lo cual se considera que no tienen nivel inventivo.

7. Conclusión:

Los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

N°	Documento N°	Reivindicaciones afectadas	Requisito que afecta
D1	WO 02/10192	1-7	Nivel Inventivo
D3	US 4,190,648	1-7	Nivel Inventivo

8. Respuesta a los argumentos del solicitante:

El solicitante afirma en el Folio 128 que la conexión de los aminoácidos para la formación del péptido, conecta los aminoácidos 4 y 5; sin embargo en **D1** se afirma que no es crítico el aminoácido en la posición C-terminal empleado; sugiriendo que el experto en la materia puede iniciar la ciclización a partir de cualquier aminoácido en la cadena lineal.

El solicitante en el Folio 128 además se refiere a que el arte previo falla al sustituir la azida, sin embargo ya se reportaba en el documento **D3** el empleo de agentes de acoplamiento de péptidos como los empleados en la solicitud, sin usar azidas durante el proceso de producción.

El solicitante se refiere al problema técnico y su solución en los Folios 129 y 130, donde se refiere a los reactivos empleados como HOBT y HBTU, sin embargo el solicitante no los refiere en el capítulo reivindicatorio.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de 60 días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.

ALIX CARMENZA CESPEDES DE VERGEL
Dirección de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 14 MAYO 2010

CONCEPTO TÉCNICO No. 0292	**EXAMINADOR TÉCNICO** Código No. 202070