Pergamon

Tetrahedron, Vol. 53, No. 21, pp. 7231-7236, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00408-0

Protein Mimetics (TASP) by Sequential Condensation of Peptide Loops to an Immobilised Topological Template

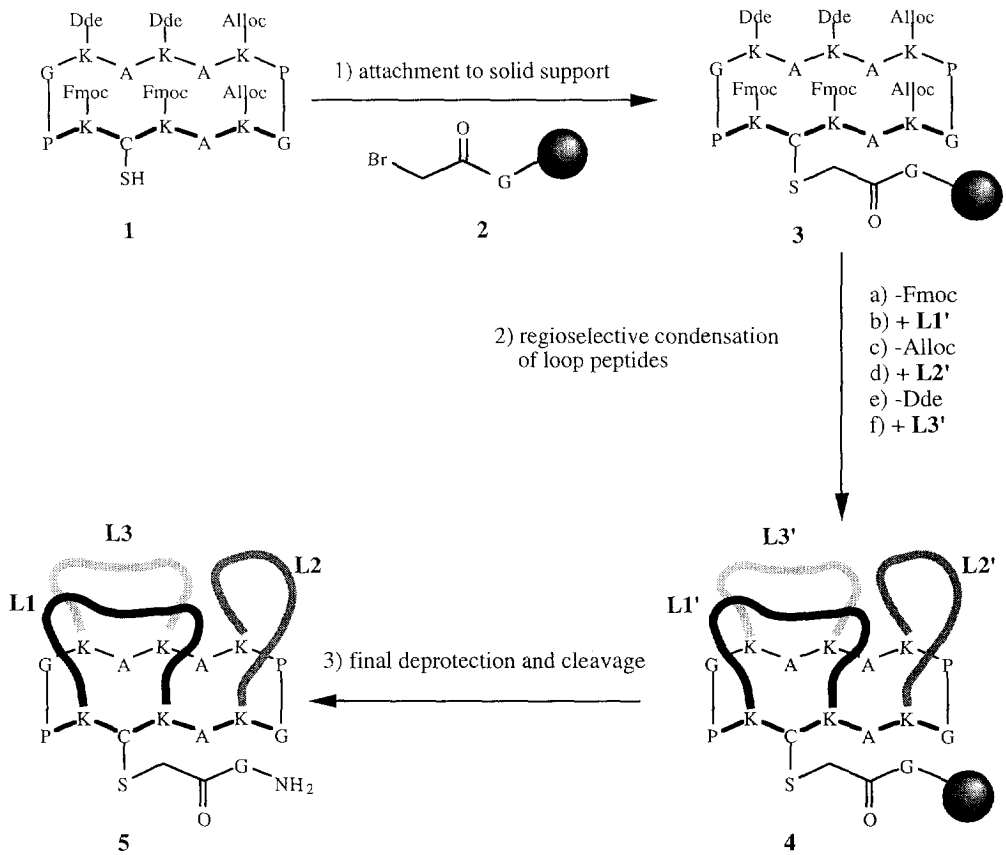
Stéphane Peluso, Pascal Dumy, Ian M. Eggleston, Patrick Garrouste and Manfred Mutter*

Institute of Organic Chemistry, University of Lausanne, BCH-Dorigny, CH-1015 Lausanne, Switzerland

Abstract : The sequential condensation of peptide loops onto selectively addressable templates immobilised on solid supports allows the rapid and efficient synthesis of protein mimetics applying the TASP (Template Assembled Synthetic Protein) concept. As an example of the versatility of this methodology, we describe here the solid phase assembly of a representative 3-loop receptor mimetic.
© 1997 Elsevier Science Ltd.

Recent progress in the methodology of peptide synthesis allows one to access peptide and protein mimetics of high structural complexity. In particular, the introduction of chemoselective ligation procedures[1-3] and orthogonal protection techniques have strongly stimulated the concept of non-native chain architectures to bypass the folding problem in protein design and mimicry[4-6]. In applying these methodological innovations, we have recently proposed the replacement of the structural part of receptors by topological templates, which serve as scaffolds for the regioselective attachment of binding loops, mimicking the functional part of the native molecule[7]. Due to the branched chain topology, the synthesis of this novel generation of Template Assembled Synthetic Proteins (TASP)[5,6] largely relies on the availability of Regioselectively Addressable Functionalised Templates (RAFT) developed in our laboratories[8]. So far, solution methodologies have been applied for the assembly of these constructs[6,7,9]. On the other hand, various studies have demonstrated the feasibility of solid phase peptide synthesis[10] (SPPS) for the efficient synthesis of cyclic[11] and branched peptides[12]. Here, we elaborate a new strategy for the regiospecific condensation of peptide loops to topological templates in the framework of SPPS.

The methodology is illustrated by the solid phase assembly[13] of a three-loop TASP molecule (**5** in Scheme 1) intended to mimic the complementary-determining region of the monoclonal antibody McPC603[7,14]. Critical steps of our strategy are (1) attachment of the cyclic template **1** bearing three pairs of orthogonally protected attachment sites to the solid support, (2) condensation of the bifunctional side-chain protected peptide loops **L1'** - **L3'** onto the polymer-bound template **3** upon sequential removal of protecting groups, (3) final deprotection and cleavage to release the target molecule **5**.

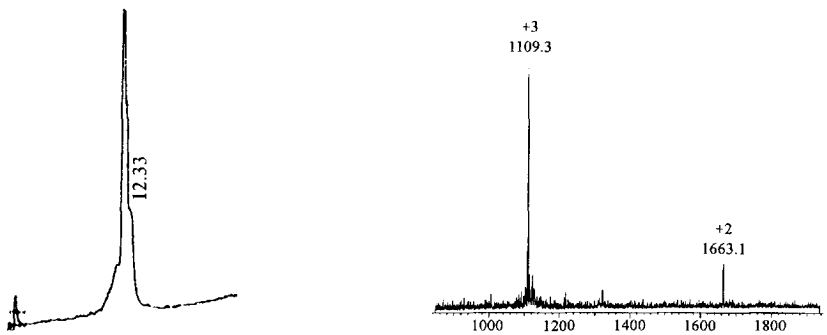


Scheme 1. Solid phase assembly of a three-loop TASP[15]. 1) 3eq. of **1** in DCM/DMF 1/1, 16h. 2a) piperidine/DMF 1/4. 2b) **L1'**: HO-Suc-F-G-L-Y(tBu)-G-OH, 5eq.; PyBOP, DIPEA, 2h. 2c) $Pd(PPh_3)_4$ / DMSO/THF/0.5M HCl/NMM 2/2/1/0.1. 2d) **L2'**: HO-Suc-E(OtBu)-L-G-R(Pmc)-G-OH, 5eq.; PyBOP, DIPEA, 2h. 2e) 2% hydrazine in DMF. 2f) **L3'**: HO-Suc-K(Boc)-G-Y(tBu)-N(Trt)-G-OH, 5eq.; PyBOP, DIPEA, 8h. 3) TFA:H_2O:TIS 95:2.5:2.5.

As depicted in Scheme 1, the attachment of the template **1** was performed via thioether formation with bromoacetylated resin **2**. This reaction was adopted since it proceeds quickly under mild basic conditions, without need for any activating reagent, allowing an easy recovery of unreacted template. Furthermore, in combining amino- and sulfhydryl protecting groups, we increase the number of regioselectively addressable attachment sites on the template. The immobilisation reaction was investigated in different solvents such as DMF, TFE, DMSO, DCM/DMF and mixtures thereof. As determined by UV-measurements of Fmoc release from the attached template, best results were obtained using 1:1 mixtures of DCM/DMF. For example, 3-fold excess of template resulted in 25% substitution of the bromoacetyl groups of the resin.

The peptide loops **L1'** - **L3'** were condensed to the polymer-bound template as side chain protected peptide segments bearing C- and N- terminal carboxylic groups. The preactivated peptides were condensed in five fold excess to the regioselectively deprotected attachment sites of the template. It is quite remarkable that the

fixation of **L1'** and **L2'** proceeded to completion within less than two hours; quantitative condensation of **L3'** required an extended reaction time as observed for the solution synthesis of **5**[7]. Most notably, no side reactions, e.g. attachment of two linear peptides to both ε-amino groups on the template or intermolecular reactions of the noncyclised loops instead of intramolecular cyclisation were detected. The observed high cyclisation rates may be attributed to the preferential template conformation featuring the loop attachment sites on the same face of the β-sheet forming template plane[16] in agreement with macrocyclisation theory of peptides[17]. Simultaneous cleavage from the resin and side chain deprotection afforded the desired TASP molecule **5** in good yield and purity as confirmed by HPLC, ESI-MS (Fig. 1) and amino acid analysis[18]. It should be noted that the simultaneous condensation of the N- and C-terminal chain ends of each loop peptide onto the template (featuring two attachment sites) results in two orientational isomers in each step, amounting to eight structural isomers of identical molecular mass (Fig. 1).



a) b)

Figure 1 : Analysis of TASP **5** (8 structural isomers) a) HPLC of final product (Vydac, C_4, buffer A : H_2O 0.09% TFA; buffer B : MeCN/H_2O 0.09% TFA; gradient : 15% B to 55% B in 20 min). b) ESI-MS obtained (calculated) : $[(M+2)/2]^+$=1663.1(1662.8), $[(M+3)/3]^+$=1109.3(1108.8).

In summary, the sequential condensation of loop peptides to regioselectively addressable templates immobilised on a hydrophilic support represents a versatile alternative to solution methodologies for accessing TASP molecules. In particular, the synthetic scheme outlined here opens the way for the development of TASP libraries according to the principles of combinatorial chemistry[19], allowing for rapid functional screening of these protein mimetics.

EXPERIMENTAL SECTION

All protected amino acids were purchased from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland), Neosystem Laboratoire (Strasbourg, France) and BACHEM Feinchemikalien AG (Bubendorf, Switzerland); SASRIN™ resins were obtained from BACHEM, Fmoc-PAL-PEG-PS resin from PerSeptive Biosystems GmbH (Hamburg, Germany); reagents and solvents were purchased from Fluka (Buchs, Switzerland). HPLC were performed on Waters equipment using columns packed with Vydac Nucleosil 300A 5µm C_{18} (preparative) and C_4 (analytical) or columns packed with Merck Lichrospher® 100 RP-18 5µm (analytical). The analytical column C_4

(250x4.6mm) was operated at 1ml/min, the analytical column C_{18} (250x4mm) at 0.7ml/min and the preparative column (250x21mm) at 18ml/min, with UV monitoring at 214 nm. Solvent A consisted of 0.09% TFA and solvent B of 0.09% TFA in 90% MeCN. Flash chromatography was performed on Merck silica gel 60 (40-63 mesh). Solid phase extractions were carried out using Sep-Pak® Vac cartridge tC_{18} 35cc from Waters (Milford, Massachusetts). Mass spectra were obtained by electron spray ionization (ESI-MS) on a Finnigan LC710.

*General procedure for the solid phase synthesis of peptide loops **L1'-L3'** (Scheme 1).*

Peptide loops were synthesised manually in a glass reaction vessel fitted with a sintered glass frit, on Fmoc-G-SASRIN™, according to Fmoc/tBu strategy. All the reaction vessels were silylated using Me_2SiCl_2:toluene 1:4 for 16 hours and then washed with toluene, MeOH and DCM. The resin (1g, 0.6meq/g) was washed with DCM (3x10ml x1min) and then swollen in DCM (20ml x30min) and DMF (20ml x30min). Coupling reactions were performed using, relative to the resin loading, 1.5eq. of N-α-Fmoc protected amino acid activated *in situ* with 1.5eq. of PyBOP® and 1.5eq. of DIPEA in 10ml DMF for 30min. The N-terminal succinic moiety was obtained by reacting the resin with 1.5eq. of succinic anhydride and 1.5eq. DIPEA in 10ml DMF for 30min. The completeness of each coupling was confirmed by the Kaiser test[20]. N-α-Fmoc protecting groups were removed by treatment with piperidine:DMF 1:4 (10ml x 5+10+10min), the completeness of each deprotection being verified by the U.V. absorption of the piperidine washings at 301nm[21]. The protected peptides were cleaved from the resin by repeated treatment with TFA:DCM 1:99 until the resin beads became dark purple (15x10ml x3min). Each washing solution was neutralised with pyridine:DCM 1:99 (15x12ml). The combined washings were concentrated under reduced pressure and the protected peptide was obtained by precipitation from DCM: Et_2O. The peptide loops were further purified by solid phase extraction or preparative HPLC.
L1' (HO-Suc-F-G-L-Y(tBu)-G-OH); $C_{36}H_{49}N_5O_{10}$ (711.81); t_R= 21.90min (0-100% B, 30min, C_{18}); ESI-MS : 712.4 $[M+1]^+$.
L2' (HO-Suc-E(OtBu)-L-G-R(Pmc)-G-OH); $C_{43}H_{68}N_8O_{14}S$ (953.12); t_R= 24.28min (0-100% B, 30min, C_{18}); ESI-MS : 953.4 $[M+1]^+$.
L3' (HO-Suc-K(Boc)-G-Y(tBu)-N(Trt)-G-OH); $C_{55}H_{69}N_7O_{13}$ (1036.19); t_R= 26.56min (0-100% B, 30 min, C_{18}); ESI-MS : 1036.9 $[M+1]^+$.

*Synthesis of template **1** (Scheme 1).*

c[A-K(Alloc)-G-P-K(Alloc)-A-K(Dde)-A-K(Dde)-G-P-K(Boc)-C(Acm)-K(Boc)]$^{\alpha}$, I.
The linear peptide sequence was assembled on Fmoc-A-SASRIN™ resin (2g, 0.5meq/g) according to the above procedure and was further reacted without additional purification. $C_{103}H_{167}N_{21}O_{28}S$ (2179.65); t_R= 22.49 min (0-100% B, 30 min, C_{18}); ESI-MS : 1090.9 $[(M+2)/2]^+$. The peptide (2.17g, 1mmol) was dissolved in DMF (1l) and the pH adjusted to 8-9 by addition of DIPEA. PyBOP® (545mg, 1.05eq) was added and the solution stirred at rt for 3h. Solvent was removed in vacuo and the residue treated with Et_2O to obtain a white powder which was desalted by solid phase extraction using a discontinuous gradient 30-70% B and lyophilised to afford 2.01g (93.0%) of cyclic peptide **I**. $C_{103}H_{165}N_{21}O_{27}S$ (2161.63); t_R= 21.30min (10-100% B, 27min, C_{18}); ESI-MS : 1081.9 $[(M+2)/2]^+$.

c[A-K(Alloc)-G-P-K(Alloc)-A-K(Dde)-A-K(Dde)-G-P-K(NH_2)-C(Acm)-K(NH_2)], II.
Compound **I** (1.20g, 555μmol) was dissolved in TFA (30ml) and allowed to stand for 30min at rt. The solution was then concentrated under reduced pressure and the residue triturated with Et_2O to yield 2.10g (99.5%) of the TFA salt of **II** as a white solid. $C_{103}H_{149}N_{21}O_{23}S$ (1961.40); t_R= 19.82 min (0-100% B, 30 min, C_{18}). ESI-MS : 1962.8 $[M+1]^+$.

c[A-K(Alloc)-G-P-K(Alloc)-A-K(Dde)-A-K(Dde)-G-P-K(Fmoc)-C(Acm)-K(Fmoc)][22], III.
Compound **II** (1.16g, 531μmol) was dissolved in DMF (40ml) and the pH of the solution was adjusted to 8-9 with DIPEA. FmocOSu (0.36g, 1.05eq.) was added and the reaction stirred at room temperature for 90min. Solvent was removed in vacuo and the residue triturated with Et_2O to afford 1.22g of crude **III** which was used without further purification. $C_{123}H_{169}N_{21}O_{27}S$ (2405.88); t_R= 30.06 min (0-100%B 30min and 100% B 5min, C_{18}); ESI-MS : 1204.0 $[(M+2)/2]^+$.

c[A-K(Alloc)-G-P-K(Alloc)-A-K(Dde)-A-K(Dde)-G-P-K(Fmoc)-C(SH)-K(Fmoc)][23], 1 (Scheme 1).
Compound **III** (1.20g, 500μmol) and anisole (550μl, 10eq.) were dissolved in TFA (120ml) at 0°C. $AgBF_4$ (1.95g, 20eq.) was added and the reaction stirred at 0°C under N_2 for 3h before adding Et_2O (200ml). The resulting white precipitate was separated by centrifugation and dissolved in AcOH:H_2O 2:1 (90ml) to give a yellowish solution. A precipitate formed upon the addition of HCl 30% (2ml, 40eq.). The reaction mixture was stirred under N_2 for 5 hours and centrifuged, then the supernatant was filtered and concentrated to dryness in vacuo to afford 1.05g of a slightly grey powder. 312mg of the crude product was purified by flash chromatography (eluent DCM:MeOH:AcOH 90:10:5) to obtain 117mg of pure **1**. $C_{120}H_{164}N_{20}O_{26}S$ (2405.88); t_R= 31.02 min (0-100%B 30min and 100% B 5min, C_{18}); ESI-MS : 1168.3 $[(M+2)/2]^+$.

*Preparation of resin **2** (Scheme 1).*

Resin **2** was prepared from Fmoc-PAL-PEG-PS resin (2.0g, 0.14meq/g) according to the above procedure. The bromoacetyl moiety was fixed to the N-terminus by reacting bromoacetic acid (0.39g, 10eq.) and DIC (450ml, 10eq.) with the resin in DMF (20ml) for 1 hour.

Substitution of resin **2** was determined according to the following procedure : dry resin (59.5mg) was swollen in DCM (2x5ml x5min) and in DMF (2x5ml x5min and then 1x5ml x30min). It was then reacted with Fmoc-Cys-OH (29.5mg, 10eq.) and DIPEA (pH 8) in DMF for 4 hours and finally washed with DMF (3x5ml x1min). N-α Fmoc protecting groups were removed from the resin by treatment with piperidine:DMF 1:4 (5ml x 5+10+10min) followed by washing with DMF (3x5ml x1min). Absorbance of the combined washings at 301nm indicated a level of substitution of 136μmol/g for resin **2**.

*Attachment of template **1** on solid support.*

Resin **2** (108mg) was washed with DCM:DMF 1:1 (3x5ml x1min) and swollen in this solvent (5ml x30min). The resin was then reacted with template **1** (98.7mg, 42.3μmol) and DIPEA (50μl) in DCM:DMF 1:1 (3.5ml). After 16h, the resin was washed with DCM:DMF 1:1 (3x5mlx5min). Unreacted template was recovered by concentrating the recombined washings in vacuo and treating the residue with Et_2O. Treatment of the resin by piperidine:DMF 1:4 (5ml x 5+10+10min) allowed the capping of unreacted bromoacetyl moieties and the removal of N-ε-Fmoc protecting groups from the immobilised template. The level of substitution of the resin (25%) was determined by measurement of UV absorption of the piperidine washings at 301nm.

General procedure for loop condensation onto the immobilised template

Peptide loops were condensed onto the immobilised template upon sequential removal of Fmoc, Alloc[11] and Dde[24] protecting groups according to published procedures.
Typically, **L1'** (12.1mg, 17μmol) was preactivated with PyBOP® (17.7mg, 34μmol) and DIPEA (17μl, 99μmol) in DCM:DMF 1:1 (2.5ml) for 15min and then allowed to react with the above described immobilised template for 2 hours. The resin was washed with DMF (2x5ml x1min) and DCM (2x5ml x1min). Completeness of the reaction was confirmed by the Kaiser test. An aliquot of the resin (5-10mg) was treated with TFA:H_2O 95:5 (0.5ml) for 2 hours. The cleavage solution was concentrated to dryness under reduced pressure and the residue dissolved in MeCN (0.5ml) and analysed by HPLC and ESI-MS. $C_{126}H_{187}N_{27}O_{32}S$ (2624.10). t_R= 19.36 min (0-100%B, 30min, C_{18}). ESI-MS : 1312.8 $[(M+2)/2]^+$.

Final cleavage and deprotection

The dry resin was treated with TFA:H_2O:TIS 95:2.5:2.5 (5ml) for 2 hours. The cleavage solution was concentrated under reduced pressure and the residue treated with Et_2O to obtain a white solid which was isolated by centrifugation and purified by preparative HPLC (isocratic 32%B). $C_{150}H_{228}N_{42}O_{42}S$ (3323.79). t_R= 12.33min (15-55%B, 20min, C_4). ESI-MS : 1663.1 $[(M+2)/2]^+$.

ACKNOWLEDGMENTS

This work was supported by the Swiss National Science Foundation.

REFERENCES AND NOTES

Dedicated to Ernst Bayer on the occasion of his 70th birthday.

1. Dawson, P.E.; Kent, S.B.H. *J. Am. Chem. Soc.* **1993**, 115, 7263-7266.
2. Rose, K. *J. Am. Chem. Soc.* **1994**, 116, 30-33.
3. Liu, C.F.; Tam, J.P. *Proc. Natl. Acad. Sci. USA* **1994**, 91, 6584-6588.
4. DeGrado, W.F.; Wassermann, Z.R.; Lear, J.D.; *Science* **1989**, 243, 622-628; Bryson, J.W.; Betz, S.F.; Lu, H.S.; Suich, D.J.; Zhou, H.X.; O'Neil, K.T.; DeGrado, W.F. *Science* **1995**, 270, 935-941.
5. Mutter, M.; Vuilleumier, S. *Angew. Chem. Int. Ed. Engl.* **1989**, 28, 535-554.
6. Tuchscherer, G.; Mutter, M. Highlights in Bioorganic Chemistry. In *Pure & Appl. Chem.* **1996**, 68, 2153-2162.
7. Mutter, M.; Dumy, P.; Garrouste, P.; Lehmann, C.; Mathieu, M.; Peggion, C.; Peluso, S.; Razaname, A.; Tuchscherer, G. *Angew. Chem. Int. Ed. Engl.* **1996**, 35, 1481-1485.
8. Dumy, P.; Eggleston, I.M.; Cervigni, S.; Sila, U.; Sun, X.; Mutter, M. *Tetrahedron Lett.* **1995**, 36, 1255-1258.
9. Tuchscherer, G. *Tetrahedron Lett.* **1993**, 34, 8419-8422.
10. Stewart, J.M.; Young, J.D. *Solid Phase Peptide Synthesis*; 2nd ed., Pierce Chemical : Rockford, IL, 1984.
11. Kates, S.A.; Solé, N.A.; Johnson, C.R.; Hudson, D.; Barany, G.; Albericio, F. *Tetrahedron Lett.* **1993**, 34, 1549-1552.

12. Blomberg, G.B.; Askin, D.; Gargaro, A.R.; Tanner, M.J.A. *Tetrahedron Lett.* **1993**, 34, 4709-4712; Emery, F.; Bisang, C.; Favre, M.; Jiang, L.; Robinson, J.A. *J. Chem. Soc. Chem. Comm.* **1996**, 2155-2156.
13. For a recent review see : Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron* **1993**, 49, 11065-11133.
14. Satow, Y.; Cohen, G.H.; Padlan, E.A.; Davies, D.R. *J. Mol. Biol.* **1986**, 190, 593-604.
15. Abbreviations : A, alanine; Acm, acetamide; Alloc, allyloxycarbonyl; Boc, *ter.*-butyloxycarbonyl; C, cysteine; D, aspartic acid; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; DIC, diisopropyl carbodiimide; DIPEA, N,N-diisopropylethylamine; E, glutamic acid; F, phenylalanine; Fmoc, 9-fluorenylmethyloxycarbonyl; G, glycine; K, lysine; L, leucine; N, asparagine; NMM, N-methyl morpholine; P, proline; PAL, 5-(4-(9-fluorenylmethyloxycarbonyl)-aminomethyl-3,5-dimethoxy-phenoxy) valeric acid; PEG-PS, polyethylene glycol-polystyrene graft polymer; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulphonyl; PyBOP®, benzotriazole-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; Q, glutamine; R, arginine; Suc, succinic acid; TIS, triisopropylsilane; Trt, trityl; Y, tyrosine.
16. Dumy, P.; Eggleston, I.M.; Esposito, G.; Nicula, S.; Mutter, M. *Biopolymers* **1996**, 39, 297-308.
17. Mutter, M. *J. Am. Chem. Soc.* **1977**, 99, 8307-8314.
18. Amino acid analysis obtained (calculated) of molecule **5** : D+N 1.2(1), E+Q 1.3(1), G 9.2(9), A 2.9(3), R 1.2(1), P 1.9(2), L 1.7(2), F 0.9(1), C 0.6(1), K 6.6(7), Y 2.2(2).
19. Gallop, M.A.; Barret, R.W.; Dower, W.J.; Fodor, S.P.A.; Gordon, E.M. *J.Med. Chem.* **1994**, 37, 1233-1251.
20. Kaiser, E.; Colescott, R.L.; Bossinger, C.D.; Cook, P.I. *Anal. Biochem.* **1970**, 34, 595-598.
21. Meienhofer, J.; Waki, M.; Heimer, E.P.; Lambros, T.J.; Makofske, R.C.; Chang, C.D. *Int. J. Peptide Protein Res.* **1979**, 13, 35-42.
22. Paquet, A. *Can. J. Chem.* **1982**, 60, 976-980.
23. Yoshida, M.; Akaji, K.; Tatsumi, T.; Iinuma, S.; Fujiwara, Y.; Kimura, T.; Kiso, Y. *Chem. Pharm. Bull.* **1990**, 38(1), 273-275.
24. Bycroft, B.W.; Chan, W.C.; Chhabra, S.R.; Hone, N.D. *J. Chem. Soc. Chem. Comm.* **1993**, 778-779.

(*Received in Germany* 3 *April* 1997; *accepted* 8 *April* 1997)