Comparative Study of Supports for Solid-Phase Coupling of Protected-Peptide Segments[1,2]

Fernando Albericio, Miquel Pons, Enrique Pedroso, and Ernest Giralt*

Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain

Received March 1, 1988

Five different polymers [polystyrene-1%-divinylbenzene (I), polystyrene-macroporous (II), Kel-F-g-styrene (III), polyacrylamide (IV), and controlled pore glass (V)] were tested in order to evaluate the influence of the chemical and physical constitution of the support on the coupling efficiency of protected peptides. The Fmoc-(60-67)-uteroglobin-OH protected segment was coupled onto H-(68-70)-uteroglobin previously assembled on each one of the polymeric supports. For this model, I gave the best result, III and IV also allowed coupling of the protected peptide but at a lower rate, while II and V proved to be unsuitable. ^{19}F NMR and ^{13}C NMR studies made on Boc-fluorotryptophan bound to supports I-IV showed differences in the chemical environment and dynamic behavior that correlated with the different reactivities observed for peptide synthesis.

Peptide synthesis by a convergent solid-phase approach is a promising alternative to stepwise methodology[3,4] for the preparation of large peptides with high purity.[5-12] Convergent solid-phase peptide synthesis involves (i) solid-phase synthesis of protected peptides and subsequent purification in solution, (ii) assembly of several purified protected peptides on a new solid support, and (iii) cleavage of the full unprotected peptide and final purification. Previous papers from this laboratory[13-16] described syntheses of various protected peptides using two different strategies and some improvements in the purification of these protected peptides. In the present work, we sought to determine the polymer support of choice for the coupling of these protected peptides.

One of the most serious problems in convergent solid-phase methodology can be low yields for coupling protected peptides on the solid support. Reaction of the bulky protected peptide may be hindered by the polymer matrix. Nevertheless, the situation can be alleviated if both the growing peptide chain and the solid support show a high degree of mobility. The physical and chemical nature of the polymer, and other factors such as temperature and solvent, are likely to influence the coupling yields. Therefore we have studied five different polymers in an attempt to determine optimal conditions for coupling of peptide segments and to understand the physical basis of the found differences. Thus, in addition to the commonly used polystyrene-1%-divinylbenzene resin (I), we have examined a more porous support (polystyrene-macroporous) (II), a non-cross-linked one (Kel-F-g-styrene) (III), a more polar one (polyacrylamide resin) (IV), and a rather rigid matrix (controlled pore glass, CPG) (V).

Supports I and IV have a microporous morphology, with sites uniformly distributed throughout the polymeric matrix.[17] Support I has been employed with considerable success to obtain many biologically active peptides and analogues.[4,18] Support IV has also been used for the synthesis of unprotected peptides using both Fmoc/tBu[19,20] and Boc/Bzl[21,22] methodologies. It has been claimed that IV has advantages over I, on the basis of the idea that solvation properties of IV are similar to those of a peptide chain.[23] The morphology of II is of the macroporous type, involving a rigid structure with substantially increased surface area. These kinds of supports are used in ion-exchange chromatography, and satisfactory results have been reported as well for its use in the synthesis of small peptides.[24,25] Support V is currently being used for oligonucleotides synthesis[26,27] and has also been applied for

(1) A preliminary report of a portion of this work has been presented: 19th European Peptide Symposium, Porto Carras, Greece, August 31-September 5, 1986. See: Albericio, F.; Porta, A.; Pedroso, E.; Giralt, E. In *Peptides 1986*; Theodoropoulos, D., Ed.; Walter de Gruyter: Berlin, 1987; pp 167-170.

(2) Abbreviations used: BFT, Boc-5-fluorotryptophan; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; CPG, controlled pore glass; DCC, *N,N'*-dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; Fmoc, (9-fluorenylmethoxy)carbonyl; HPLC, high-performance liquid chromatography; HOBt, 1-hydroxybenzotriazole; tBu, *tert*-butyl; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Z, benzyloxycarbonyl. Amino acid symbols denote the L configuration where applicable.

(3) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149.

(4) Barany, G.; Merrifield, R. B. In *The Peptides: Analysis, Synthesis, Biology*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 2, pp 1-284.

(5) Protein Synthesis Group, Shanghai Institute of Biochemistry, *Sci. Sin* **1975**, *18*, 745.

(6) Camble, R.; Petter, N. N. In *Peptides 1976: Proceedings of the Fourteenth European Peptide Symposium*; Loffet, A., Ed.; Editions de l'Université de Bruxelles: Bruxelles, 1976; pp 299-307.

(7) Pedroso, E.; Grandas, A.; Saralegui, M. A.; Giralt, E.; Granier, C.; van Rietschoten, J. *Tetrahedron* **1982**, *38*, 1183.

(8) Giralt, E.; Albericio, F.; Pedroso, E.; Granier, C.; van Rietschoten, J. *Tetrahedron* **1982**, *38*, 1193.

(9) Voss, C.; DiMarchi, R.; Whitney, D. B.; Tjoeng, F. S.; Merrifield, R. B.; Tam, J. P. *Int. J. Pept. Protein Res.* **1983**, *22*, 204.

(10) Ljungqvist, A.; Folkers, K. *Acta Chem. Scand.* **1984**, *B38*, 375.

(11) Nakagawa, S. H.; Lau, H. S. H.; Kézdy, F. J.; Kaiser, E. T. *J. Am. Chem. Soc.* **1985**, *107*, 7087.

(12) Lyle, T. A.; Brady, S. F.; Ciccarone, T. M.; Colton, C. D.; Paleveda, W. J.; Veber, D. F.; Nutt, R. F. *J. Org. Chem.* **1987**, *52*, 3752.

(13) Giralt, E.; Eritja, R.; Pedroso, E.; Granier, C.; van Rietschoten, J. *Tetrahedron* **1986**, *42*, 691.

(14) Grandas, A.; Pedroso, E.; Giralt, E.; Granier, C.; van Rietschoten, J. *Tetrahedron* **1986**, *42*, 6703.

(15) Albericio, F.; Nicolás, E.; Josa, J.; Grandas, A.; Pedroso, E.; Giralt, E.; Granier, C.; van Rietschoten, J. *Tetrahedron* **1987**, *43*, 5961.

(16) Sabatier, J. M.; Tessier-Rochat, M.; Granier, C.; van Rietschoten, J.; Pedroso, E.; Grandas, A.; Albericio, F.; Giralt, E. *Tetrahedron* **1987**, *43*, 5973.

(17) Altgelt, K. H.; Segal, L., Eds. In *Gel Permeation Chromatography*; Dekker: New York, 1971.

(18) Barany, G.; Cordonier-Kneib, N.; Mullen, D. G. *Int. J. Pept. Protein Res.* **1987**, *30*, 705.

(19) Atherton, E.; Gait, M. J.; Sheppard, R. C.; Williams, B. J. *Bioorg. Chem.* **1979**, *8*, 351.

(20) Atherton, E.; Sheppard, R. C.; Ward, P. *J. Chem. Soc., Perkin Trans. I* **1985**, 2065-2073.

(21) Baleux, F.; Daunis, J.; Jacquier, R.; Calas, B. *Tetrahedron Lett.* **1984**, *25*, 5893.

(22) Calas, B.; Mery, J.; Parelló, J.; Cavé, A. *Tetrahedron* **1985**, *41*, 5331.

(23) Atherton, E.; Clive, D. L. J.; Sheppard, R. C. *J. Am. Chem. Soc.* **1975**, *97*, 6584.

(24) Sano, S.; Tokunaga, R.; Kun, K. A. *Biochim. Biophys. Acta* **1971**, *244*, 201.

(25) Losse, A. *Tetrahedron* **1973**, *29*, 1203.

0022-3263/89/1954-0360$01.50/0

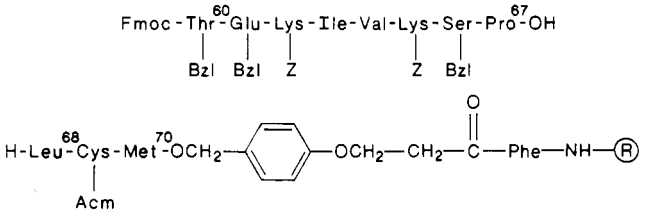
Table I. Syntheses of LAGV on Five Different Supports[a]

support	loading, mmol/g	cleavage yield,[b] %	LAGV	AGV	GV	LAV	LGV	LA[c]	LV
I	0.67	99	99.07	0.08	0.12	0.32	0.10	0.31	
II	0.67	98	90.54	0.17	0.09	4.23	1.22	1.13	2.48
III	0.13	97	97.98	0.03	0.22	0.71	0.17	0.85	0.04
IV	0.50	85	98.94	0.14	0.13	0.49	0.08	0.22	
V	0.04	95	99.07	0.02	0.15	0.18	0.25	0.31	0.02

[a] Syntheses were carried out by using the Fmoc group for α-amino protection and TFA-labile p-alkoxybenzyl anchoring linkage. [b] Yields were determined by internal reference amino acid techniques.[34,35] [c] Due to diketopiperazine formation during deprotection step of second residue.[36]

some syntheses of small peptides.[28] Finally, III has a pellicular morphology, with an impermeable core surrounded by a mobile layer of linear polystyrene chains where the peptide syntheses takes place. This support, which was designed to avoid problems derived from steric hindrance of the matrix, has been successfully used for synthesis of unprotected peptides.[29,30]

In these studies, we have determined the kinetics of coupling of the Fmoc-(60–67)-uteroglobin-OH protected segment onto the H-(68–70)-uteroglobin tripeptide previously assembled on each of the polymeric supports.



Furthermore, ^{19}F NMR and ^{13}C NMR have been used to assess the physical characteristics of 5-fluorotryptophan linked to polymeric supports I–IV in different solvents. The results (chemical shifts, line widths, relaxation times) correlate with the reactivity observed in segment condensation experiments and may be used to understand the origin of the differences.

Results and Discussion

Preparation of Supports. Styrene-type supports (I, II, III) were functionalized as aminomethyl-resins by the method described by Mitchell et al.[31] In order to get similar degrees of substitution, 1 mmol, 10 mmol, and 100 mmol of *N*-(hydroxymethyl)phthalimide per gram of styrene were used respectively for supports I, II, and III. The reaction with a 1:1 ratio on the macroporous resin led to a substitution degree 10 times lower (0.067 mmol/g) than that in the other cases. Polyacrylamide resin (IV) and controlled pore glass (V) were obtained from commercial sources and used without any chemical modification.

Syntheses of Leu-Ala-Gly-Val. Initially, Merrifield's model tetrapeptide[3] was synthesized on each support, using a combination of the base-labile Fmoc group for α-amino protection and a TFA-labile *p*-alkoxybenzyl anchoring linkage. The first protected amino acid was incorporated onto each amino-functionalized support via a preformed handle,[32] a method that provides maximal control. The Fmoc group was removed by piperidine–CH_2Cl_2 (1:1) (3 × 1 min); these conditions ensure complete deprotection but avoid long exposure of the peptide resin to the strong nucleophile and base piperidine. The remaining amino acids (2.5-fold excess) were attached by a single DCC-mediated coupling in CH_2Cl_2. Final cleavage was achieved by TFA–CH_2Cl_2 (1:1) for 1 h at 25 °C and was >85% in all cases (Table I). The purity of the tetrapeptides was judged by ion-exchange chromatography[33] with ninhydrin detection on an amino acid analyzer and shown to be 97–99%, except for the synthesis carried out on the macroporous resin II (Table I).

The results of Table I clearly indicate that supports I, III, IV, and V are compatible with solid-phase peptide synthesis, but no distinction can be made among them. On the other hand, the heterogeneity of the peptide synthesized on support II suggests problems of the macroporous resin with solid-phase peptide synthesis. Interestingly, on this support, the extent of reaction seemed to increase progressively as the peptide chain grew. Thus, the coupling yield for glycine was only 95.7%, compared to 98.7% for alanine and 99.8% for leucine (Table I). Consequently, a further synthesis was carried out by using five glycine residues as a spacer[37] between the handle and the polymeric matrix II, but in this new case the distribution of products obtained (LAGV, 83.69; AGV, 0.03; GV, 0.11; LAV, 2.62; LGV, 2.96; LA, 9.43; LV, 1.16) was similar to that without a spacer. A tetrapeptide with homogeneity comparable to the ones obtained with the other supports (LAGV, 98.83; AGV, 0.09; GV, 0.13; LAV, 0.54; LGV, 0.15; LA, 0.27) could be obtained with polymer II only by use of substantially larger excesses (12.5-fold) of protected amino acids in each coupling cycle.

Synthesis and Purification of Fmoc-(60–67)-uteroglobin-Protected Segment. The title peptide was synthesized on a photolabile resin (Nbb-resin),[7] by using HF-labile side-chain protecting groups and the Boc group for temporary α-amino protection. The amino terminal Thr residue was blocked with the Fmoc group, and the presence of this chromophore on the protected peptide allowed monitoring of the purification by UV absorbance (280–300 nm). The anchoring of Boc-Pro-OH onto the resin was carried out by the cesium salt method.[38] In order to avoid diketopiperazine formation, the third amino acid was incorporated by using the method described by Suzuki et al.[8,39] No decrease in the substitution level was found by picric acid titration.[40] The remaining amino acids were

(26) Köster, H.; Stumpe, A.; Wolter, A. *Tetrahedron Lett.* **1983**, *24*, 747.
(27) Adams, S. P.; Kauka, K. S.; Wykes, E. J.; Holder, S. B.; Galluppi, G. R. *J. Am. Chem. Soc.* **1983**, *105*, 661.
(28) Parr, W.; Grohmann, K.; Hagele, K. *Justus Liebigs Ann. Chem.* **1974**, 655.
(29) Tregear, G. W. In *Chemistry and Biology of Peptides*; Meienhofer, J., Ed.; Ann Arbor Sci. Plub.: Michigan, 1972; p 175.
(30) Kent, S. B. H.; Merrifield, R. B. *Isr. J. Chem.* **1978**, *17*, 243.
(31) Mitchell, A. R.; Kent, S. B. H.; Engelhard, M.; Merrifield, R. B. *J. Org. Chem.* **1978**, *43*, 2845.
(32) Albericio, F.; Barany, G. *Int. J. Pept. Protein Res.* **1985**, *26*, 92.
(33) Merrifield, R. B.; Mitchell, A. R.; Clark, J. E. *J. Org. Chem.* **1974**, *39*, 660.
(34) Arshady, R.; Atherton, E.; Clive, D. L. J.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. I* **1981**, 529–537.
(35) Matsueda, G. R.; Haber, E. *Anal. Biochem.* **1980**, *104*, 215.
(36) Pedroso, E.; Grandas, A.; de las Heras, X.; Eritja, R.; Giralt, E. *Tetrahedron Lett.* **1986**, *27*, 743.
(37) Sparrow, J. T. *J. Org. Chem.* **1976**, *41*, 1350.
(38) Gisin, B. F. *Helv. Chim. Acta* **1973**, *56*, 1476.
(39) Suzuki, K.; Nitta, K.; Endo, N. *Chem. Pharm. Bull.* **1975**, *23*, 222.
(40) Gisin, B. F. *Anal. Chim. Acta* **1972**, *58*, 248.

Table II. Yields of Coupling of Fmoc-(60–67)uteroglobin-OH onto H-(68–70)-uteroglobin Resins

support	equiv of peptide	yield, % 2 h	7 h	24 h	48 h	72 h
I	2.5	31	100			
I	1.25	8	24	56	–	80
II	2.5	6	12	22	34	37
II	1.25	2	4	–	8	17
III	2.5	24	49	92	100	
III	1.25	3	9	15	23	35
IV	2.5	24	87	100		
IV	1.25	4	13	22	32	44
V	2.5	18	35	41	46	66
V	1.25	7	21	31	53	64

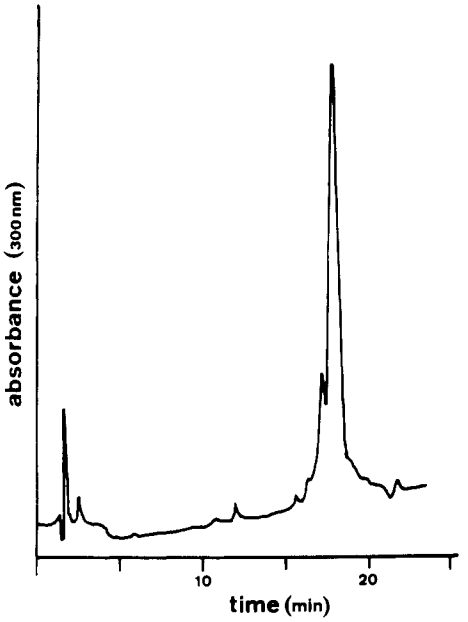


Figure 1. Analytical HPLC on reversed phase C_{18} of crude Fmoc-(60–67)-uteroglobin-OH. Linear gradient starting from CH_3CN–0.1% TFA in H_2O (1:9) taken over 20 min to pure CH_3CN; flow rate 1.1 mL/min.

assembled by standard solid-phase procedures (details in Experimental Section), and the completeness of coupling was assessed by the ninhydrin test.[41] Single couplings were adequate, and acetylations were not necessary. The protected peptide was obtained by photolysis (65% yield) in TFE–CH_2Cl_2 (3:7)[42] and showed a homogeneity of 85% by HPLC (Figure 1). The crude product was purified on a Merck Lobar RP-8 column (Figure 2a) eluted with a convex gradient (DMF/AcCN/H_2O/propionic acid mixtures) to provide the pure protected peptide (Figure 2b) with 60% recovery and the correct amino acid composition and 200-MHz ^1H NMR spectra. Use of reversed phase semipreparative columns made possible purification of protected peptides on medium scales (0.1 mmol, 170 mg), thereby overcoming one of the main problems associated with convergent solid-phase peptide synthesis.

Syntheses of H-(68–70)-uteroglobin Resins. The different peptide resins were synthesized by the same techniques already described for the preparation of Merrifield's model tetrapeptide. Samples of peptide resins were subjected to TFA–CH_2Cl_2 (1:1) treatments and the

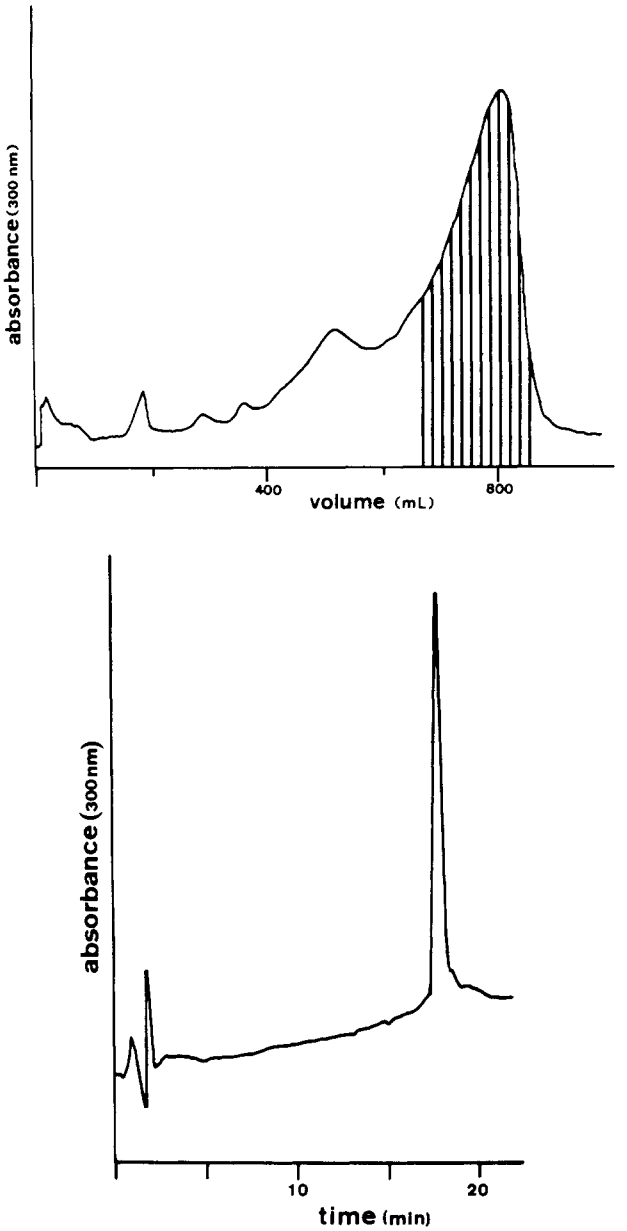


Figure 2. (a) MPLC profile on reversed phase C_8 of Fmoc-(60–67)-uteroglobin-OH. Convex gradient starting from DMF–CH_3CN–H_2O–propionic acid (50:10:40:0.5) (400 mL) to DMF–CH_3CN–propionic acid (50:50:0.5) (400 mL); flow rate 3 mL/min. (b) Analytical HPLC of purified Fmoc-(60–67)-uteroglobin-OH (same conditions as Figure 1).

purities of protected tripeptides were >95% as judged by HPLC.

Coupling Experiments. Fmoc-(60–67)-uteroglobin-OH (1.25/2.5 equiv) was coupled onto H-(68–70)-uteroglobin

(41) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595.

(42) In an attempt to increase the yield, a photolysis experiment was performed in 10% TFA–CH_2Cl_2, because besides the nature of peptide, the photolysis yield has been attributed to some experimental conditions such as solvent/resin ratio (ref 13), and it is well known that mixtures of TFA–CH_2Cl_2 are the best solvents to swell resins. The yield obtained (18%) did not confirm the expectations.

resins by use of DCC and HOBt[7,8,43] in DMF (solutions 1.25 × 10^{-2} M). The extent of coupling was checked by acid hydrolysis and amino acid analysis on samples of peptide resins removed at different times (Table II).

Coupling of the Fmoc-(60–67)-uteroglobin-OH-protected segment onto H-(68–70)-uteroglobin resins was always a rather slow reaction. However, in spite of the hydrophobicity and high density of protecting groups, quantitative coupling yields could be obtained when a sufficient excess, between 1.25 and 2.5 equiv, was used. Conventional microporous polystyrene–1%-divinylbenzene (I) was the best choice for this model. Both polystyrene grafted on Kel-F (III) and polyacrylamide (IV) allow the assemblage of (60–70)-uteroglobin with a quantitative yield, but at a lower rate. In our hands, controlled pore glass (V) and the strongly cross-linked macroporous polystyrene (II) were not suitable polymers for the coupling of protected peptide segments.[44]

NMR Experiments. All of the polymer supports used in this study allow the synthesis of the model tetrapeptide Leu-Ala-Gly-Val by stepwise *amino acid coupling*. Yields were better than 98% in all cases with the exception of macroporous polystyrene (II), where the yield was 90%. This result contrasts with the behavior of the same supports in the *segment coupling* experiments, showing clearly that coupling of a segment is a much more demanding process. Reasons for the low reactivity include inter- and intramolecular interactions between peptide segments. The former can give precipitation and is a common drawback in peptide synthesis by solution methods. Interaction with the polymer strongly influences the reactivity in solid-phase segment coupling. A beneficial effect can be the reduction of the intermolecular interactions between polymer-bound peptides (site isolation), which is equivalent to a solubilization of the peptide by the polymer. On the other hand, undesired effects can be unaccesibility of the reactive points to the incoming peptide and restricted mobility of the bound peptide. Unaccesibility is probably related to site heterogeneity and to local peptide–polymer interactions. Restricted mobility can be understood mainly in terms of reduced rotational mobility, which has to be compensated by increased translational motions that imply movement of the complete solvent shell. Gel-phase NMR[45–49] is sensitive to the same effects and could provide an easy test for potential new supports. ^{1}H NMR is not very suitable for these studies due to the large line widths arising from nonaveraged dipole–dipole interactions and the small chemical shift range.[46] On the other hand, ^{13}C NMR gives relatively sharp lines and a large chemical shift spread but the low receptivity of ^{13}C and the low effective concentration of peptide on the resin poses a sensitivity problem. ^{19}F NMR has both a high receptivity (4730 times that of carbon-13) and a very large chemical shift range and was therefore chosen for this NMR study. The use of gel-phase ^{19}F NMR has already been reported as an aid to solid-phase peptide synthesis with specially designed protecting groups.[50]

Table III. Line Widths at Half-Height (Hz)[a]

nucleus solvent	^{19}F ($CDCl_3$)	^{19}F (DMF)	^{13}C[b] ($CDCl_3$)
BFT-NH-CH_2-I	81	120	6
BFT-NH-CH_2-II	347	275	37
BFT-NH-CH_2-III	299	370	40
BFT-IV	158	101	15

[a] ^{19}F NMR spectra at 188.2 MHz. ^{13}C NMR spectra at 50.5 MHz. [b] Measured at the signal from methyl carbons of the Boc group.

Table IV. Field Dependence of the Line Widths (Hz)

sample	line width at 188.2 MHz	line width at 75.4 MHz	ratio (188.2/75.4 = 2.5)
BFT-NH-CH_2-I	81	32	2.5
BFT-NH-CH_2-II	347	230	1.5

In order to compare our results with other ^{13}C NMR data on polymer-bound peptides, a Boc-protected fluorinated amino acid was used, since the signal from the Boc group can be detected even under low sensitivity conditions.[51]

Chemical Shifts. Fluorine chemical shifts can show a considerable solvent dependence. In the case of Boc-5-fluorotryptophan (BFT) bound to resins I, II, and III a 2 ppm downfield shift was observed when the solvent was changed from DMF to $CDCl_3$ (Figure 3). On the other hand, the corresponding signal from BFT–IV moved only 0.9 ppm downfield when changing from DMF to $CDCl_3$. This result is consistent with the polar nature of polymer IV and indicates that even in less polar solvents this polymer can provide an environment to the peptide that mimics the solvent DMF.

Line Widths. Line widths (Table III) of the fluorine signal of resin-bound BFT are sensitive both to peptide–support interactions and to restricted mobility.[52] In the first case, line broadening arises from a spread of chemical shifts from fluorine atoms in different chemical environments. Restricted mobility causes broadening because of the enhanced transverse relaxation. An indication of the relative importance of the two contributions can be obtained from the field dependence of the line widths.

Supports I and IV give considerably sharper lines both in DMF and $CDCl_3$ than supports II and III. In terms of solvent effect, the lines for I and III are sharper in $CDCl_3$,

(43) Albericio, F.; Granier, C.; Labbé-Juillé, C.; Seagar, M.; Couraud, F.; van Rietschoten, J. *Tetrahedron* **1984**, *40*, 4313.

(44) For the support II, an additional coupling experiment was carried out by the same procedure (2.5-fold excess) at 50 °C. The coupling yields were 2 h, 14%; 7 h, 38%; 24 h, 65%; and 72 h, 78%.

(45) Mazure, M.; Calas, B.; Cavé, A.; Parellõ, J. *C.R. Acad. Sci. Paris* **1986**, *303*, 553.

(46) Live, D.; Kent, S. B. H. In *Elastomers and Rubber Elasticity*, ACS Symposium Series 193; Mark, J., Lol, J., Eds.; American Chemical Society: Washington, DC, 1982; pp 501–515.

(47) Giralt, E.; Rizo, J.; Pedroso, E. *Tetrahedron* **1984**, *40*, 4141.

(48) Live, D. H.; Kent, S. B. H. In *Peptides: Structure and Function*; Hruby, V., Rich, D., Eds.; Pierce Chemical Co.: Rockford, IL, 1983; pp 65–68.

(49) Ludwick, A.; Jelinski, L. W.; Live, D.; Kintanar, A.; Dumais, J. J. *J. Am. Chem. Soc.* **1986**, *108*, 6493.

(50) Manatt, S. L.; Amsden, C. F.; Bettison, C. A.; Frazer, W. T.; Gudman, J. T.; Lenk, B. E.; Lubetich, J. F.; McNelly, E. A.; Smith, S. C.; Templeton, D. J.; Pinnell, R. P. *Tetrahedron Lett.* **1980**, *21*, 1397.

(51) The following NMR results are limited to supports I, II, III, and IV, as molecules bound to support V, because of its rigidity, give signals too broad to be observed.

(52) Other contributions to line widths such as magnetic field inhomogeneity caused by the heterogeneous system or residual (nonaverage) chemical shift anisotropy can be neglected as the former should also broaden the ^{13}C NMR lines to the same extent (corrected by the different gyromagnetic ratios of the two nuclei) and the latter would not be consistent with the correlation times of 10^{-8} s derived from ^{13}C NMR relaxation data (ref 53) and the static ^{19}F shielding anisotropies of fluorinated aromatic compounds (ref 54).

(53) Ford, W. T.; Balakrishnan, T. *Macromolecules* **1981**, *14*, 284.

(54) Mehring, M. "High Resolution NMR Spectroscopy in Solids" in *NMR Basic Principles and Progress*; Springer-Verlag: Berlin, 1976; Vol. 11.

(55) The other possible field-dependent contribution to line width, namely, the chemical shift anisotropy contribution to relaxation, would require a quadratic dependence. Such field-dependent line widths have been previously reported in the proton and ^{13}C NMR gel-phase spectrum of polystyrene (ref 46).

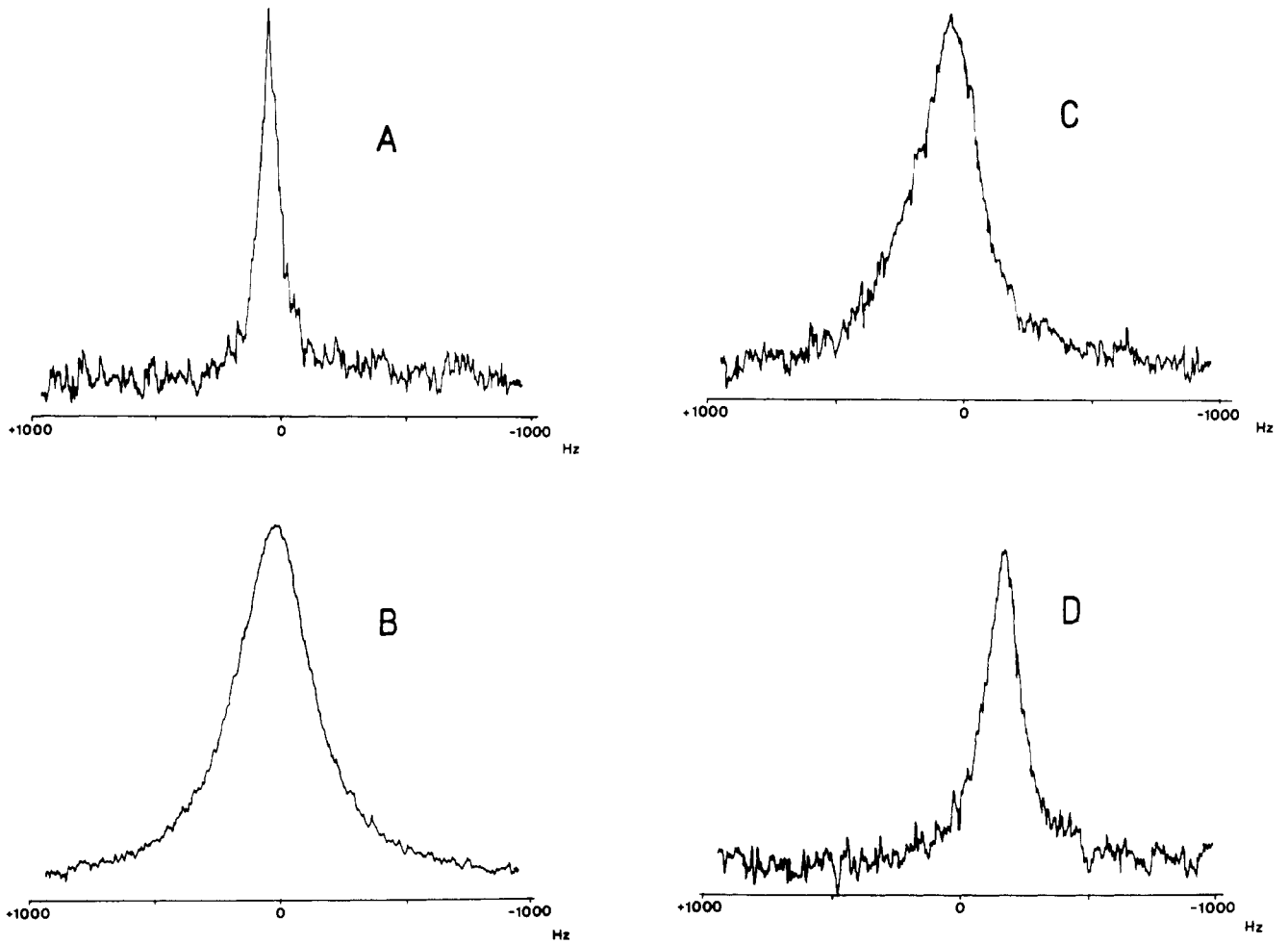


Figure 3. ^{19}F NMR signal of Boc-5-fluorotryptophan (BFT) bound to resins I (A), II (B), III (C), and IV (D) swollen in $CDCl_3$. Spectra were run at 188 MHz, with a 30-kHz spectral width using 90° pulses and an 8-s recycling time.

while those of II and IV have smaller widths in DMF. It is surprising that solvent dependences of the line widths are reversed between 1% cross-linked (I) and macroporous (II) polystyrene despite their similar chemical nature.

Spectra of BFT-NH-CH_2-I and BFT-NH-CH_2-II suspended in $CDCl_3$ were measured at a lower field strength (Table IV). The line width is directly proportional to the field strength for BFT-NH-CH_2-I while the decrease in line width for BFT-NH-CH_2-II at low field was smaller. The proportionality between observed width and field strength points to an important contribution arising from a spread in chemical shift in the case of BFT-NH-CH_2-I. On the other hand the lower sensitivity of the line width to the field strength suggest that, at low field, the line width of BFT-NH-CH_2-II is dominated by relaxation.

The chemical shift contribution to the observed line widths in polymer-bound molecules is not unexpected as the interactions with the polymer matrix can cause shifts even in noncovalently bound molecules: the ^{19}F NMR of BFT dissolved in $CDCl_3$ in the presence of 1% cross-linked polystyrene also shows two partially resolved peaks. Similar effects have been reported in the ^{19}F NMR and ^{13}C NMR spectra measured in solutions containing insoluble polymers.[50,56] Finally, it should be noted that line widths offer dynamic information even in the cases where they are not dominated by relaxation as they reflect the averaging of conformationally distinct sites. The sensitivity nevertheless is shifted to a much slower time scale and also reflects the heterogeneity, i.e., the maximum difference, among different sites.

(56) Ford, W. T.; Periyasamy, M.; Olin Spivey, H.; Chandler, J. P. *J. Magn. Reson.* **1985**, *63*, 298.

Table V. Relaxation Times (s)

	$CDCl_3$	DMF
BFT-NH-CH_2-I	0.95 ± 0.08	0.71 ● 0.04
BFT-NH-CH_2-II	0.61 ± 0.01	0.49 ± 0.01
BFT-NH-CH_2-III	(0.7)[a]	(0.5)[a]
BFT-IV	0.61 ± 0.01	0.50 ± 0.03

[a] Multiexponential decay due to the underlying broad resonance from the polymer. Estimated values for the fast component.

Relaxation Times. Mobility differences among the four supports can be independently estimated through T_1 measurements. T_1 values for the ^{19}F signal of BFT bound to I, II, and IV are reported in Table V. No precise T_1 values could be measured in the case of BFT bound to a Kel-F support (III) due to highly nonexponential behavior arising from the relaxation of the underlying very broad signal from the polymer. Estimated values are 0.7 s in $CDCl_3$ and 0.5 s in DMF, similar to those observed for BFT-NH-CH_2-II and BFT-IV. This solvent dependence has not been found in ^{13}C NMR data on polystyrene polymers and probably simply reflects the efficiency of the intermolecular relaxation pathway involving the solvent.

Two main conclusions can be extracted from these data. First BFT bound to polystyrene–1%-*co*-divinylbenzene clearly has higher mobility by comparison to the remaining supports tested that display similar lower relaxation times. Second, our findings of constant T_1 for three of the four supports studied are in agreement with previous reports[53] on the low sensitivity to the degree of cross-linking of T_1

values from ^{13}C NMR spectra of polystyrene polymers, in contrast to line-width measurements.[57]

Conclusions

Polyacrylamide (IV) and microporous polystyrene (I) appear to be the most convenient supports for segment coupling. This correlates very well with the smaller line widths at half-height observed for supports I and IV in gel-phase ^{19}F NMR spectra in $CDCl_3$ or DMF of Boc-5-fluorotryptophane (BFT) covalently bound to resins, and in ^{13}C NMR spectra of the same materials observed in $CDCl_3$. The faster coupling rates observed in our system using microporous polystyrene (I) as compared to polyacrylamide (IV) are in agreement with the T_1 measurements that show a clear difference between resin I and the remaining resins. It is interesting that in spite of the larger line width and poorer swelling properties of BFT–I in DMF, it still has a T_1 value 40% larger than BFT–IV.

The unsuitability of IV for segment coupling contrasts with the reliability of this support for the assembly of a short peptide via amino acid coupling (Table I). Kel-F–styrene, although by design not cross-linked, facilitates quantitative segment coupling but with clearly slow reaction rates. NMR experiments suggest that for this support, the increased flexibility caused by the lengthening of the polystyrene chain could be balanced by the lack of mobility due to grafting to a Kel-F core. The qualitative correlation between NMR data and reactivity gives support to the idea that the same basic principles are involved in the two kinds of experiments. Nevertheless, the very different physical and chemical characteristics of the polymers studied, the superposition of effects, and the small number of examples prevent a quantitative fit.

Experimental Section

Boc-5-fluorotryptophan was synthesized from 5-fluorotryptophan (Sigma, St. Louis, MO) according to the method of Moroder et al.[58] Other Boc-amino acids were from Protein Research Foundation (Japan). Fmoc amino acids were synthesized according to previously described procedures.[59,60] 2,4,5-Trichlorophenyl 3′-[4″-[[[(Fmoc-amino)acyl]oxy]methyl]phenoxy]-propionates were prepared according to the method described by one of us.[32] Bromomethyl-Nbb–resin was prepared starting from polystyrene–*co*-1%-divinylbenzene, 200–400 mesh, from Bio-Rad Laboratories (Richmond, CA), as previously described.[8] Macroporous-polystyrene, Kel-F–styrene, polyacrylamide resin, and controlled pore glass were obtained respectively from Rohm & Hass (Italy), ICI (Australia), Expansia (France), and Pierce (Rockford, IL). All these supports were thoroughly washed to remove any impurities: 2 × toluene; 3 × MeOH; 3 × MeOH–H_2O (1:1); 3 × H_2O; 1 N NaOH, 1 h with mechanical stirring; 3 × H_2O; 1 N HCl, 1 h with mechanical stirring; 3 × H_2O; 3 × H_2O–MeOH (1:1); 3 × MeOH; DMF, 30 min with mechanical stirring at 80 °C; 3 × DMF; 3 × MeOH, 1 h. Resins were dried in vacuo over P_2O_5, KOH pellets, and paraffin. Hydrazine was used freshly after distillation over NaOH. *N*-(Hydroxymethyl)phthalimide was prepared according to the procedure of Winstead and Heine.[61] Boc-Phe-resins and Boc-BFT-resins were prepared following the synthetic program I (see next section).

Peptides and peptide-resins were hydrolyzed at 130 °C and 150 °C with 6 N HCl and 12 N HCl/AcOH for 3 h and 1 h, respectively, under vacuum degassed tubes. Amino acid analyses were done on a Biotronik Model LC 7000. NMR spectra were recorded on a Varian XL-200 spectrometer. High performance liquid chromatography was carried out in a Waters Associates apparatus with two-solvent delivery systems and a variable wavelength UV monitor; a Spherisorb ODS-2 C_{18} column (0.39 × 30 cm, 10 μm) was used. Preparative medium pressure liquid chromatography was achieved on a Merck Lobar size A RP-8 column, using a Milton Roy pump to achieve a flow rate of 3 mL/min and a LKB 2158 Uvicord SD single path monitor connected to a Servoscribe 1S recorder for detection of peaks.

General Procedure for Solid-Phase Assembly of Peptides. Peptide syntheses were performed manually in silanized screw-cap tube reaction vessels[4] each with a Teflon-lined cap, sintered glass frit, and stopcock. Two synthetic programs were used, one for Boc-amino acids (I) and the other for Fmoc-amino acids (II).

Program I (except for the third amino acid on the Nbb-resin): (1) CH_2Cl_2, 4 × 5 mL, 1 min; (2) TFA–CH_2Cl_2 (3:7), 1 × 5 mL, 1 min + 1 × 5 mL, 30 min; (3) CH_2Cl_2, 5 × 5 mL, 1 min; (4) DIEA–CH_2Cl_2 (1:19), 3 × 5 mL, 1 min; (5) CH_2Cl_2, 5 × 5 mL, 1 min; (6) Boc-amino acid in CH_2Cl_2 (2 mL), after 1 min add equivalent amount of DCC in CH_2Cl_2 (2 mL), shake 60 min; (7) CH_2Cl_2, 5 × 5 mL, 1 min; (8) DMF, 2 × 5 mL, 1 min.

The third protected amino acid on the Nbb-resin was incorporated by following the method of Suzuki:[39] (1) CH_2Cl_2, 3 × 5 mL, 1 min; (2) dioxane, 2 × 5 mL, 1 min; dioxane/HCl 4 N, 1 × 5 mL, 1 min + 1 × 5 mL, 30 min; (4) dioxane, 2 × 5 mL, 1 min; (5) CH_2Cl_2, 4 × 5 mL, 1 min; (6) DCC in CH_2Cl_2 (2 mL), after 1 min add the equivalent amount of Boc-amino acid *N*-methylmorpholinium salt, shake 120 min; (7) CH_2Cl_2, 5 × 5 mL, 1 min; (8) DMF, 2 × 5 mL, 1 min.

Program II: (1) CH_2Cl_2, 4 × 5 mL, 1 min; (2) piperidine–CH_2Cl_2 (1:1), 3 × 5 mL, 1 min; (3) CH_2Cl_2, 5 × 5 mL, 1 min; (4) Fmoc-amino acid in CH_2Cl_2 (2 mL), after 1 min add the equivalent amount of DCC in CH_2Cl_2 (2 mL), shake 60 min; (5) CH_2Cl_2, 5 × 5 mL, 1 min; (6) 2-propanol, 3 × 5 mL, 1 min.

General Procedure for Photolytic Cleavage of *o*-Nitrobenzyl Ester Anchoring Linkages of Peptide-Nbb-resins. Peptide-Nbb-resin was placed in a silanized three-necked cylindrical reaction vessel and suspended in TFE–CH_2Cl_2 (3:7) (100 mL). Oxygen was removed at 0 °C by alternated connection to vacuum and nitrogen lines for about 10 min. The photochemical reaction was carried out for 9–14 h with three H 125 BL eye lamps on an improved apparatus similar to the one previously described.[8] The cleaved peptide-resin was filtered and washed with TFE–CH_2Cl_2 (1:9), CH_2Cl_2, and MeOH, and the combined filtrates and washings were evaporated to dryness at 25 °C.

Aminomethyl-resins. Resins (I, 25 g; II, 3.5 g; III, 5 g) were suspended in TFA–CH_2Cl_2 (1:1) (250 mL, 50 mL, 65 mL); then *N*-(hydroxymethyl)phthalimide (5.3 g, 30 mmol; 7.1 g, 40 mmol; 5.3 g, 30 mmol) and trifluoromethanesulfonic acid (8.1 mL, 90 mmol; 1.8 mL, 20 mmol; 1.8 mL, 20 mmol) were added. The suspensions were mechanically stirred at 25 °C. Reactions were stopped when IR spectra showed that absorption at 1720 cm^{-1} corresponding to the phthalimide carbonyl was constant (ca. 5 h). Resins were filtered and washed with TFA–CH_2Cl_2 (1:1), CH_2Cl_2, and MeOH and dried in vacuo over P_2O_5.

Phthalimidomethyl-resins (29 g, 3.2 g, 5.2 g) were refluxed overnight in EtOH (500 mL, 50 mL, 50 mL) containing 5% hydrazine. Resins were filtered and washed with hot EtOH, CH_2Cl_2, and EtOH and dried in vacuo over P_2O_5. Picric acid titration[40] showed 0.67 mmol of NH_2/g resin of support I, 0.67 mmol of NH_2/g of support II, and 0.13 mmol of NH_2/g of support III.

Syntheses of Leu-Ala-Gly-Val. After deprotection of Boc-Phe-NH-CH_2-resins and neutralization (steps 1–5 of the synthetic program I), a solution of 2,4,5-(trichlorophenyl) 3′-[4″-[[[(Fmoc-valyl)oxy]methyl]phenoxy]propionate (2 equiv) and HOBt (2 equiv) in DMF was added. After overnight reaction, the ninhydrin test was negative for supports I, III, IV, and V. For macroporous resin a second coupling (3 equiv) with HOBt (3 equiv) in the presence of DMAP (0.3 equiv) was done. After this extra coupling, the ninhydrin test was slightly positive and an acetylation (20 μL of AcOH, 66 mg of DCC) was performed.

Fmoc-Gly-OH, Fmoc-Val-OH, and Fmoc-Leu-OH were incorporated by following the synthetic program II. A portion of the peptide–resin (ca. 100 mg) was deprotected with piperidine–CH_2Cl_2 (1:1) (3 × 1 min), washed with CH_2Cl_2 (5 × 1 min), and cleaved with TFA–CH_2Cl_2 (7:3) at 25 °C. After 1 h, the filtrate

(57) Mohanraj, S.; Ford, W. T. *Macromolecules* **1985**, *18*, 351.
(58) Moroder, L.; Hallett, A.; Wünsch, E.; Keller, O.; Wersin, G. *Hoppe-Seyler's Z. Physiol. Chem.* **1976**, *357*, 1651.
(59) Tessier, M.; Albericio, F.; Pedroso, E.; Grandas, A.; Eritja, R.; Giralt, E.; Granier, C.; van Rietschoten, J. *Int. J. Pept. Protein Res.* **1983**, *22*, 125.
(60) Albericio, F.; Grandas, A.; Porta, A.; Pedroso, E.; Giralt, E. *Synthesis* **1987**, 271.
(61) Winstead, M. B.; Heine, H. W. *J. Am. Chem. Soc.* **1955**, *77*, 1913.

was collected, combined with TFA-CH_2Cl_2 (1:1) washes of the resin, and evaporated to dryness. The ratio upon acid hydrolysis of amino acids remaining on the cleaved resin to the Phe "internal reference"[34,35] indicated the yield of the cleavage (Table I). The peptide mixture from the cleavage was dissolved in 0.2 N sodium citrate buffer, pH 2.2, applied to the amino acid analyzer and eluted at pH 3.25 and 75 °C (Table I).

Synthesis and Purification of Fmoc-(60-67)-uteroglobin-Protected Segment. Cesium Boc-prolinate[38] (0.69 g, 2.0 mmol) was added to Nbb-resin (3.0 g, 1.8 mmol of Br) in DMF (30 mL), and the suspension was stirred overnight at 50 °C. The reaction was then filtered and carefully washed with DMF (3 × 25 mL), DMF-H_2O (9:1) (3 × 25 mL), DMF (3 × 25 mL), CH_2Cl_2 (3 × 25 mL), and MeOH (3 × 25 mL). The Boc was removed and picric acid titration[40] revealed a loading of 0.58 mmol of Pro/g resin (quantitative incorporation).

Boc-Ser(Bzl)-OH, Boc-Val-OH, Boc-Ile-OH, Boc-Lys(Z)-OH, and Fmoc-Thr(Bzl)-OH were incorporated by using the synthetic program I; the first Lys residue was incorporated according to the method of Suzuki.[39] The ninhydrin test was negative after each coupling. Once the protected peptide was assembled on the resin, a portion was hydrolyzed and subjected to amino acid analysis: $Thr_{0.82}$, $Ser_{1.06}$, $Glu_{1.03}$, $Pro_{0.99}$, $Val_{0.91}$, $Ile_{0.94}$, $Lys_{2.07}$, with a substitution level of 0.19 mmol/g.

Boc-peptide-OCH_2-Nbb-resin (3 g, 0.57 mmol) was photolyzed in different batches (300-500 mg) to provide 0.35 mmol (62% yield) of crude protected peptide, which was about 85% pure by analytical HPLC (Figure 1). This material was purified by MPLC (Figure 2a) (ca. 90 μmol was injected at each run, convex gradient formed from 400 mL each of 5:1:4 and 5:5:0 of DMF, CH_3CN, and H_2O containing 0.5% of propionic acid) to provide 0.21 mmol (60%) of pure protected peptide (Figure 2b). Amino acid analysis: $Thr_{0.92}$, $Ser_{0.95}$, $Glu_{0.98}$, $Pro_{1.02}$, $Val_{1.03}$, $Ile_{1.00}$, $Lys_{2.06}$. ^1H NMR (CD_3SOCD_3): 7.2-8.3 (m, Fmoc), 7.1-7.3 (m), 5.00 (s, CH_2 Glu-Bzl), 4.97 (s, CH_2 Lys-Z), 4.1-4.5 (m, CH_2 Ser-Bzl, CH_2 Thr-Bzl), 2.8-3.0 (m, CH_2 Lys), 1.14 (d, CH_3 Thr), 0.7-0.9 (m, CH_3).

Syntheses of H-(68-70)-uteroglobin-handle-Phe-NH-CH_2-resins. Boc-Phe-NH-CH_2-resins (200-300 mg) were subjected to steps 1-5 of synthetic program I and then shaken overnight with a solution of 2,4,5-trichlorophenyl 3′-[4″-[[[(Fmoc-methionyl)oxy]methyl]phenoxy]propionate (1.5 equiv) and HOBt (1.5 equiv) in DMF. After the usual washings, resins I, III, IV, and V were ninhydrin negative. Deprotection of an aliquot of Fmoc-handle-Phe-resin II gave an incorporation yield of 80%, so an acetylation as described above was carried out. Fmoc-Cys(Acm)-OH and Fmoc-Leu-OH were added by using synthetic program II. Samples of the resultant Fmoc-Leu-Cys(Acm)-Met-resins were cleaved for 1 h with TFA-CH_2Cl_2 (1:1) containing 1% of β-mercaptoethanol to give crude peptides, which were about 85-97% pure by analytical HPLC.

Coupling Experiments. Fmoc-Leu-Cys(Acm)-Met-resins (4-6 μmol, 20-30 mg) were deprotected and then Fmoc-(60-67)-uteroglobin (1.25 equiv/2.5 equiv) in 300 μL of DMF and HOBt (1.25 equiv/2.5 equiv) in 50 μL of DMF were added at 0 °C. After 2 min of mechanical stirring, DCC (1.25 equiv/2.5 equiv) in 50 μL of DMF was added and the mixture (total volume 400 μL) was stirred for 2 h at 0 °C and then at room temperature. Aliquots of the resin were removed at different times in order to determine the extent of coupling (Table II).

NMR Experiments. Samples of 41 mg of BFT-NH-CH_2-I, 29 mg of BFT-NH-CH_2-II, 100 mg of BFT-NH-CH_2-III, and 44 mg of BFT-IV where used in all the experiments. Initially they were suspended in $CDCl_3$ and allowed to swell completely inside a 5-mm NMR tube. Argon was carefully bubbled through the gels to degass them. At the time of the measurements the gels were confined to the coil region by bottom and anti-vortex Teflon plugs. For the measurements in DMF the $CDCl_3$ was evaporated, and the resins were washed with DMF and finally suspended in this solvent. The spectra in DMF were recorded without lock.

Spectra were run at 188 MHz for ^{19}F NMR and 50 MHz for ^{13}C NMR on a Varian XL-200 instrument. ^{19}F NMR spectra were recorded with a 30 kHz spectral width using 90° pulses and an 8-s recycling time. Under these conditions all samples gave signal-to-noise ratios on excess of 10:1 after 20 transients. ^{19}F NMR spectra at 75 MHz were measured on a Brucker WP-80 operating at a probe temperature of 304 K. Chemical shifts were measured in separate experiments by using a capillary containing trifluoroethanol and $CDCl_3$.

T_1 measurements were made by the inversion-recovery method with an 8-s relaxation delay and a minimum of 10 increments ranging from less than 0.06 s to more than 4 s. Intensities were fitted to a three-parameter exponential curve. All measurements were made at the probe room temperature (294-297 K unless otherwise stated).

Acknowledgment. We thank Dr. G. Barany for critically reading the manuscript. The excellent assistance of Anna Porta in the developing of macroporous resin and Francisco Cárdenas in running the amino acid analyzer is gratefully acknowledged. This work was supported by funds from CIRIT, CAICYT (grant n BT-18/86), and U.S.-Spain Joint Committee for Scientific and Technological Cooperation. We thank ICI (Australia), Expansia (France), and Rohm & Haas (Italy) for the gift of Kel-F-g-styrene, polyacrylamide, and polystyrene-macroporous, respectively.

Registry No. I, 9003-70-7; II, 9003-53-6; III, 110651-21-3; IV, 9003-05-8; H-Leu-Ala-Gly-Val-OH, 17195-26-5; Fmoc-Val-OC_6H_4-p-OCH_2CH_2COO-2,4,5-$Cl_3C_6H_2$, 117872-74-9; Fmoc-Gly-OH, 29022-11-5; Fmoc-Leu-OH, 35661-60-0; fmoc-Thr(Bzl)-Glu-(OBzl)-Lys(Z)-Ile-Val-Lys(Z)-Ser(Bzl)-Pro-OH, 117860-20-5; BOC-Pro-OH-Cs, 42538-66-9; BOC-Ser(Bzl)-OH, 23680-31-1; BOC-Val-OH, 13734-41-3; BOC-Ile-OH, 13139-16-7; BOC-Lys-(Z)-OH, 2389-45-9; Fmoc-Thr(Bzl)-OH, 117872-75-0; Fmoc-Met-OC_6H_4-p-OCH_2CH_2COO-2,4,5-$Cl_3C_6H_2$, 117860-21-6; Fmoc-Cys(Acm)-OH, 86060-81-3.

Two Epimeric Aliphatic Amino Alcohols from a Sponge, *Xestospongia* sp.[1]

Nanda K. Gulavita and Paul J. Scheuer*

Department of Chemistry, University of Hawaii at Manoa, Honolulu, Hawaii 96822

Received June 29, 1988

Two epimeric amino alcohols, 2(*S*)-aminotetradeca-5,7-dien-3(*S*)- and -3(*R*)ol, were isolated from a Papua-New Guinea sponge, *Xestospongia* sp. Their structures were determined spectrally, relative stereochemistry by derivatization, and absolute stereochemistry by degradation to L-alanine. In contrast to the widely distributed sphinganines, which are derived from fatty acids and serine, these compounds are derivatives of alanine. Both compounds inhibit the growth of *Candida albicans*.

Sphinganines are long-chain aliphatic 2-amino-1,3-diols, exemplified by sphingosine (1). Their *N*-acylamides (ceramides), usually occurring as C-1 phosphates or C-1 glycosides, are widely distributed in nature.[2] A few

0022-3263/89/1954-0366$01.50/0