SOLID PHASE SYNTHESIS OF A SOMATOSTATIN AMIDE ANALOGUE USING ACID LABILE t-BUMEOC PROTECTION AND AN ACID LABILE ANCHOR GROUP

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Dedicated to the memory of Professor Iphigenia Photaki.

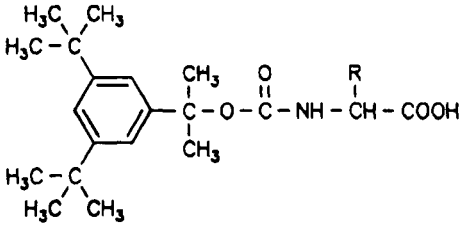
The solid phase synthesis of an amidated somatostatin analogue based on the principle of differentiated acidolysis is described. The acid labile and smoothly cleavable t-Bumeoc moiety (1% TFA/DCM) is used for temporary N^{α}-protection of D- and L-amino acids and [4-[[[9*H*-fluoren-9-yl-methoxycarbonyl]amino](4-methoxyphenyl)methyl]-2-methylphenoxy]acetic acid, attached to an aminomethylated polystyrene resin is used as acid sensitive linker of the solid carrier which releases the peptide in its amide form by treatment with TFA. Its preparation is described in detail.

Though the t-Bumeoc* (1-(3,5-di-tert-butylphenyl)-1-methylethoxycarbonyl) residue as an amino protecting group was reported a couple of years ago[1] no detailed experimental report exists so far in the literature about its application for the synthesis of peptides containing trifunctional, oxydation and acid sensitive amino acids. This lack of information should be eliminated by the present communication on the synthesis of a

* Abbreviations are used in accordance with the rules of IUPAC–IUB Commission on Biochemical Nomenclature, J. Biol. Chem. *247*, 977 (1972), Biochem. J. *219*, 345 (1984). Boc tert-butoxycarbonyl; BOP benzotriazole-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; Bu^t tert-butyl; DCC N,N'-dicyclohexylcarbodiimide; DCM dichloromethane; DIC N,N'-diisopropylcarbodiimide; DMF N,N'-dimethylformamide; Fmoc 9-fluorenylmethoxycarbonyl; Fmoc-NH_2 9-fluorenylmethyl carbamate; HOBt 1-hydroxybenzotriazole; HOSu N-hydroxysuccinimide; OPA/MPA *o*-phthalaldehyde/3-mercaptopropionic acid; PE petroleum ether; SBu^t S-tert-butylthio; SPPS solid phase peptide synthesis; TFA trifluoroacetic acid.

somatostatin amide analogue using t-Bumeoc N^{α}-protected amino acids and [4-[[[9*H*-fluoren-9-yl-methoxycarbonyl]amino](4-methoxyphenyl)methyl]-2-methylphenoxy]acetic acid linked to an aminomethylated polystyrene resin as anchor[2].

Because of the two tert-butyl residues in meta position of the benzene ring and the nature of the quarternary carbon atom neighbouring the oxygen atom of the carbamate system, the t-Bumeoc residue provides a suitable molecular structure for an amino protecting group. It is cleaved similar to known urethane-like groups[3 - 7] under mild acidolytic conditions according to pseudofirst order kinetics. Due to their lipophilicity, t-Bumeoc amino acids (see below), their basic salts as well as the protected peptides are easily solubilized in organic solvents. This property is advantageous with reactions in hydrophobic media resulting in short coupling and cleavage times.



The synthesis of the t-Bumeoc residue is started from di-tert-butylated toluene which is converted to the alcohol 2-(3,5-di-tert-butylphenyl)-2-propanol (t-Bumeoc-OH) via oxidation, esterification and Grignard reaction. The desired reagent for the introduction of the t-Bumeoc group ([1-(3,5-di-tert-butylphenyl)-1-methylethyl]fluoroformate, t-Bumeoc-F) is obtained by the reaction of t-Bumeoc-OH with carbonyl chloride fluoride. The resulting compound acylates amino acids (and derivatives) in high yields in alkaline/aqueous or basic/organic media (pH 8, pH-stat)[1].

The physicochemical data of the t-Bumeoc amino acid derivatives applied for the described synthesis are listed in Table I.

t-Bumeoc-protected amino acids can be coupled to free amino functions with conventional methods (DCC/HOBt, DCC/HOSu, BOP); besides, the crystalline N-hydroxysuccinimide esters are reactive synthons[8].

The acidolytic cleavage of the t-Bumeoc residue with 1% TFA in organic solvents yields the hydrophobic, oily olefin 2-(3,5-di-tert-butylphenyl)-2-methylethene and the free amino acid (derivative) within 1 – 3 min. Though the tryptophan-containing peptide is not destroyed by the low acid concentration, repetitive treatments with 1% TFA/CH_2Cl_2 or 1% TFA/$CHCl_3$ are carried out in the presence of 2-methylindole, once the acid-labile amino acid is incorporated.

While solid phase peptide synthesis has been developed to a well established method, numerous efforts are undertaken to develop linker groups cleaving the peptide

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TABLE I

Physicochemical data of t-Bumeoc-protected amino acids and derivatives used for SPPS of the somatostatin analogue

t-Bumeoc derivative	Yield %	M. p., °C (solvent)	$[\alpha]_D^{25}$ (*c* 1, MeOH)	pH[a] R_F[b]	Formula (M. w.)	Calculated/Found		
						% C	% H	% N
L-Cys(SBu^t)-OH•DCHA	85	159	−41.20	10.2	$C_{37}H_{64}N_2O_4S_2$	66.82	9.68	4.21[c]
		(hexane)		0.72	(665.1)	66.93	9.76	4.17[c]
N$^\varepsilon$-Boc-L-Lys-OH•DCHA	87	71 – 74	+8.6	10.0	$C_{41}H_{71}N_3O_6$	70.14	10.19	5.95
		(PE)		0.53	(702.0)	70.61	10.50	5.85
L-Phe-OH•DCHA	86	80 – 83	+32.2	10.2	$C_{39}H_{60}N_2O_4$	75.37	9.66	4.50
		(hexane)		0.58	(620.9)	74.90	9.86	4.36
D-Phe-OH•DCHA	65	79 – 83	−32.1	10.2	$C_{39}H_{60}N_2O_4$	75.37	9.66	4.50
		(hexane)		0.58	(620.9)	75.02	9.83	4.40
L-Thr(Bu^t)-OH•CHA	93	188	+1.3	10.0	$C_{32}H_{56}N_2O_5$	70.03	10.28	5.10
		(hexane)		0.70	(548.8)	70.51	10.57	4.77
D-Thr(Bu^t)-OH•CHA	99	187	−1.3	10.0	$C_{32}H_{56}N_2O_5$	70.03	10.28	5.10
		(hexane)		0.70	(548.8)	70.98	10.61	4.61
D-Trp-OH•DCHA	92	197	−19.6	9.5	$C_{41}H_{61}N_3O_3$	74.63	9.32	6.37
		(hexane)		0.58	(660.0)	74.69	9.45	6.08

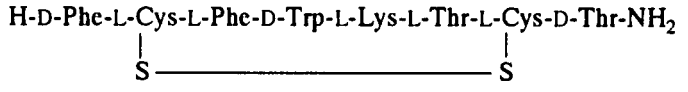
[a] pH-stat - method. [b] $CHCl_3/MeOH/C_6H_6/H_2O$ (40 : 40 : 40 : 5). [c] Calculated 9.64% S; found 9.31% S.

in its amide form. Conventional methods using anhydrous liquid hydrogen fluoride, trifluoromethanesulfonic acid or ammonolysis should be avoided for reasons of side reactions. Consequently, most anchor groups published in the last years[9 - 15] eliminate the peptide amide after exposure to media of varying acidity.

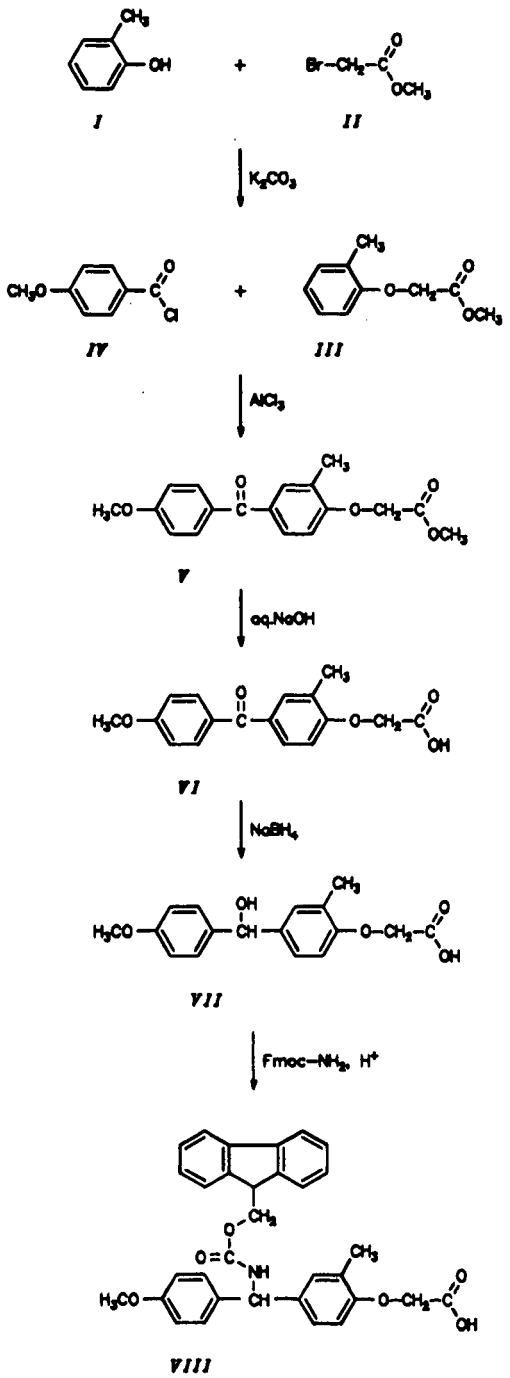
Based on the acidolytic cleavage of the Mbh amide protecting group[16] by trifluoroacetic acid at 20 °C the [4-[[hydroxy](4-methoxyphenyl)methyl]-2-methylphenoxy]-acetic acid moiety (*VII*) (Scheme 1) is reacted with Fmoc-amide to [4-[[[9*H*-fluoren-9-yl-methoxycarbonyl]amino](4-methoxyphenyl)methyl]-2-methylphenoxy]-acetic acid (*VIII*) (ref.[2]). The attachment to the commercially available aminomethylated polystyrene resin yields the corresponding polymeric support, which releases the synthesized somatostatin analogue in acidic medium (90% TFA/10% scavenger).

The synthesis of compound *VIII* (Scheme 1) is performed starting from commercially available 2-methylphenol (*I*) which is reacted with methyl bromoacetate (*II*) to give methyl 2-methylphenoxyacetate (*III*). This oily liquid is coupled with 4-methoxybenzoyl chloride (*IV*) via a Friedel–Crafts acylation and the resulting methyl 4-(4'-methoxybenzoyl)-2-methylphenoxyacetate (*V*) is saponified to 4-(4'-methoxybenzoyl)-2-methylphenoxyacetic acid (*VI*). Reduction of the ketone moiety by sodium borohydride yields compound *VII*, which is assumed to form oligomers[2,15] in acidic solution. The carbinol acid is therefore reacted immediately with Fmoc-amide in acetic acid by addition of a catalytic amount of sulfuric acid to the desired linker moiety *VIII*. In the last step of the synthesis the Fmoc-amide anchor group is coupled to the aminomethylated polystyrene resin using N,N'-diisopropylcarbodiimide in the presence of 1-hydroxybenzotriazole and 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine as additives in N,N-dimethylformamide. The loading is calculated after the cleavage of Fmoc-amide with 90% (95% TFA/H_2O)/DCM and its quantitative HPLC determination[17] using an authentic sample as standard (0.6 mmol/g resin).

To prove the utility of the acid labile t-Bumeoc-protecting group for solid phase synthesis the preparation of the cyclic somatostatin analogue[18] is performed using the



Fmoc-MMBHA resin. After removal of the Fmoc group with 20% piperidine/DMF the t-Bumeoc-protected amino acid derivatives are reacted with three-fold excess (related to the Fmoc loading) in methylene chloride by addition of DCC/HOBt/DMF. Complete coupling is observed using bromophenol blue as an indicator. A 1% solution in N,N'-dimethylacetamide of this acid-base indicator is added to the solution of the acylating agent during the coupling reactions[19] . The t-Bumeoc residue is cleaved with 1%



SCHEME 1

TFA/methylene chloride and the peptide resin neutralized with 10% N,N'-diisopropylethylamine/methylene chloride. The solid phase protocol for the synthesis of the somatostatin analogue using the N^{α}-t-Bumeoc and tert-butyl side-chain protected amino acid derivatives is presented in the experimental part of this communication.

After completion of the synthesis the peptide resin is treated with TFA/anisole/2-methylindole at room temperature for 2 h in a stream of nitrogen to yield the S-tert-butylthio-protected peptide amide. Disappointed by the low yield of cleavage (15% crude peptide) a more rigorous TFA-treatment at 50 °C for 30 min was risked. Surprisingly the somatostatin analogue survived this procedure without damage. After removal of the scavengers using Sephadex LH-20 column chromatography and methanol as eluent the S-tert-butylthio-protected peptide amide was isolated in 45% yield. At this stage of our work we ensured the identity of the S-tert-butylthio-substituted somatostatin amide by comparison with a reference sample synthesized by conventional solution method[20] (Fig. 1).

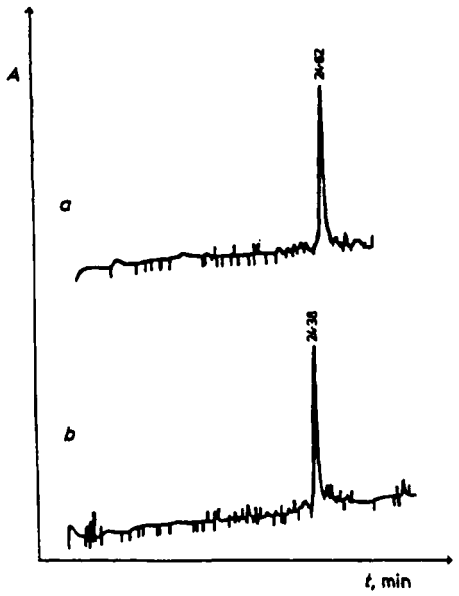


FIG. 1

Comparison of HPLC profiles of H-D-Phe-L-Cys(SBu^t)-L-Phe-D-Trp-L-Lys-L-Thr-L-Cys(SBu^t)-D-Thr-NH_2 synthesized by the solid phase using the t-Bumeoc strategy (*a*) resp. solution method (*b*), (ref.[8]). Column: 4 × 250 mm, Li-Chrosorb RP-18, 5 μm. Eluents: A, 0.05% TFA in H_2O; B, CH_3CN (80%)/0.05% TFA in H_2O (20%); linear gradient from 30 to 100% B in 30 min. Detection: 220 nm. Injection: 10 μl

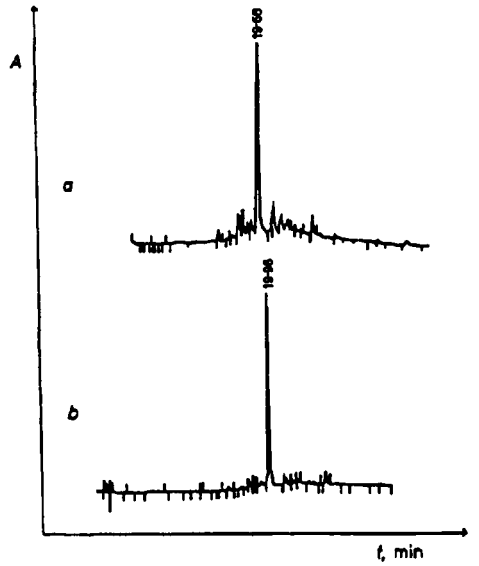


FIG. 2

Comparison of HPLC profiles of the somatostatin analogue synthesized by the solid phase method using the t-Bumeoc strategy (*a*) and reference sample (*b*). Column: 4 × 250 mm, Li-Chrosorb RP-18, 5 μm. Eluents: A, 0.01M-$N(CH_3)_4OH$ in H_2O, H_3PO_4, pH 1.9; B, A/CH_3CN (1 : 1); linear gradient from 10 to 100% B in 30 min. Detection: 220 nm. Injection: 10 μl

Removal of the cysteine protecting groups is carried out by tributylphosphine in trifluoroethanol (1 : 40, 2 h). After precipitation with ether the linear octapeptide amide is cyclized by air oxidation in a mixture of dioxane/ammonium acetate (pH 6.8) (negative Ellman test). After evaporation to a small volume and lyophilisation the sample is dissolved in acetic acid (5%)/H_2O and separated on a Sephadex G-25 column using acetic acid (5%)/H_2O as eluent. In Fig. 2 the HPLC profile of the fraction containing the desired product is compared with a reference sample (kindly provided by Dr W. Bauer, Sandoz AG, Basel, Switzerland).

EXPERIMENTAL

Chemicals and Methods

All reagents and solvents used for the synthesis and chromatography are of p.a. quality and purchased from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) and Fluka (Neu-Ulm, Germany). According to the general procedures described in the literature[21] O-tert-butyl-L-(and D-)threonine and N^{ε}-tert-butyloxycarbonyl-L-lysine are synthesized. Chromatographic separations are performed on columns packed with silica gel 60 (100 × 2.0 cm, 70 – 230 mesh ASCM, Merck and Sephadex LH-20 (80 × 2.6 cm, Pharmacia, Freiburg, Germany) in combination with a fraction collector Multirac 211 and an Uvicord I photometer (E_{254} nm, both LKB, Stockholm, Sweden). Thin-layer chromatography is performed on precoated silica gel plates 60 F_{254} (Merck) in the solvent systems: S1: benzene–chloroform–methanol–water (40 : 40 : 40 : 5), S2: chloroform–methanol–acetic acid–water (70 : 30 : 5 : 5), S3: 1-butanol–acetic acid–water–pyridine (60 : 6 : 24 : 20). The spots are visualized by UV light at 254 nm, ninhydrin, chlorine-*o*-tolidine and Ehrlich's reagent. The melting points are determined on a Tottoli capillary melting point apparatus (Büchi, Flavil, Switzerland) and are not corrected. Elemental analyses are carried out on an Elemental Analyzer, type 240/B (Perkin–Elmer, Konstanz, Germany). Optical rotations are measured in a jacketed 1-dm cell using a LEP A2 polarimeter (Zeiss, Oberkochen, Germany) at 546 nm. The $[\alpha]_D$ values are calculated by multiplication with the factor 0.85103. ^1H NMR spectra are recorded with a WP 60 and AM 270 spectrometer (Bruker–Physik, Karlsruhe, Germany) with tetramethylsilane as internal standard. Mass spectra are taken with Kratos M9 (Kratos, Berlin, Germany) and MAT 711A (Varian, Bremen, Germany) spectrometers coupled with a data System SS 200 at a temperature of the ion source of 30 °C. The HPLC system used for peptide separations consists of a sample injector Rheodyne 7125 (Rheodyne, Berkeley, U.S.A.), a low-pressure gradient former M 2500 (Gynkothek, Munich, Germany), a constant-flow pump M 600/200 (Gynkothek) and a variable UV wavelength detector BT 3030 (Biotronik, Maintal, Germany). The acid hydrolyses are performed in 6M-HCl at 110 °C for 24 h in the presence of 2.5% thioglycolic acid. Determination of amino acid compositions are carried out by classical ion exchange chromatography using the HPLC Liquimat III (Kontron, Eching, Germany), a column (5 μm, 180 × 4 mm) packed with Ck 10F (Mitsubishi, Japan) and a Pico buffer system 2 (Pierce, Rockford, U.S.A.) as eluent at 52, 57, 77 °C. Amino acid analyses using precolumn derivatization with *o*-phthalaldehyde/3-mercaptopropionic acid[22] are performed using a HPLC system BT 8100 (Biotronik, Maintal, Germany), equipped with a LiChrospher 100 RP-18e (250 × 4 mm, 5 μm) column (Merck) and a fluorometer RF-535 (Shimadzu, Düsseldorf, Germany). The amount of unprotected SH groups as well as the oxidative process of cyclization are determined by the Ellman test[23] and performed as published[24] using a Beckmann 24 photometer (Beckmann Instruments, Munich, Germany). The solid phase synthesis is carried out in an ordinary shaker reactor.

Synthesis of Fmoc-Amide Anchor Resin

Methyl 2-Methylphenoxyacetate (*III*)

2-Methylphenol (108 g, 1 mol) is dissolved in dry acetone (500 ml) and powdered potassium carbonate (165.8 g, 1.2 mol) is added. Then methyl bromoacetate (113 ml, 1.2 mol) is added to the stirred suspension and the mixture is left at room temperature with exclusion of moisture. After the reaction is complete, salts are filtered off by suction, washed with acetone and the filtrate is concentrated. The residue is taken up in ethyl acetate, the organic phase is extracted with water, dried over magnesium sulfate and concentrated to give an oily liquid which is used directly for the next reaction. Yield 180 g (100%).

Methyl 4-(4'-Methoxybenzoyl)-2-methylphenoxyacetate (*V*)

Aluminium chloride (146.6 g, 1.1 mol) is suspended in dry 1,2-dichloroethane (500 ml). To the stirred solution 4-methoxybenzoyl chloride (187 g, 1.2 mol) and methyl 2-methylphenoxyacetate (180 g, 1 mol) are successively added dropwise at 0 °C. The mixture is heated at 50 °C to complete the reaction and then poured onto crushed ice (2 kg). The pH is adjusted to 2 with 2M-HCl and the precipitated product filtered off by suction and washed with water and a little amount of ether. The precipitate is dissolved in hot ethyl acetate and a small amount of charcoal is added. Then the solution is filtered and cooled to −10 °C. The crystalline product is filtered, washed with ether and dried at high vacuum. Yield 172.6 g (55%); m.p. 92 – 95 °C; ^{1}H NMR spectrum ($CDCl_3$): 2.30 s, 3 H; 3.85 s, 3 H; 3.92 s, 3 H; 4.85 s, 2 H; 6.6 – 7.9 m, 7 H.

4-(4'-Methoxybenzoyl)-2-methylphenoxyacetic Acid (*VI*)

Methyl 4-(4'-methoxybenzoyl)-2-methylphenoxyacetate (35.8 g, 114 mmol) is stirred with a mixture of dioxane (240 ml) and 0.5M-NaOH (240 ml) at room temperature. After the saponification is complete, the organic solvent is removed in vacuo and the aqueous phase is adjusted to pH 3 with 2M-HCl and extracted with ethyl acetate. The organic layer is washed with water, dried over magnesium sulfate and evaporated to dryness. Pale yellow crystals remain. Yield 30.2 g (83%); m.p. 149 – 151 °C.

[4-[[Hydroxy](4-methoxyphenyl)methyl]-2-methylphenoxy]acetic Acid (*VII*)

4-(4'-Methoxybenzoyl)-2-methylphenoxyacetic acid (22.5 g, 75 mmol) is dissolved in a mixture of dioxane (100 ml) and water (200 ml), adding 1M-NaOH to pH 9. Sodium borohydride (2.8 g, 75 mmol) is added in portions to the stirred solution, and the mixture is left to react overnight. Then the dioxane is removed in vacuo, the aqueous phase is adjusted to pH 3 with 2M-HCl and extracted with ethyl acetate. The organic layer is dried over magnesium sulfate and concentrated. The remaining colourless foam is triturated with hexane to give an amorphous powder which is filtered off by suction. As the product oligomerizes, it is used without further treatment and characterization for the next reaction. Yield 19.2 g (84%).

[4-[[[9*H*-Fluoren-9-yl-methoxycarbonyl]amino](4-methoxyphenyl)-methyl]-2-methylphenoxy]acetic Acid (*VIII*)

Compound *VII* (3.6 g, 12 mmol) is dissolved together with 9-fluorenylmethyl carbamate (2.8 g, 6 mmol) in glacial acetic acid (100 ml). Under stirring 10 drops of concentrated sulfuric acid are added. The solution turns light red and after 10 min the product starts to precipitate. After 90 min the reaction is complete. The slurry is poured into ice-water (400 ml) and the product is filtered off by suction and washed with water. After drying the product in a desiccator it is recrystallized from an ethyl acetate–methanol–petrol ether mixture. Yield 4.34 g (69%); m.p. 160 – 163 °C. For $C_{32}H_{29}NO_6$ (523.7) calculated: 73.26% C, 5.67% H, 2.68% N; found: 73.74% C, 5.58% H, 2.67% N. FAB MS (*m/z*): 524 (M^+). ^{1}H NMR spectrum (hexa-

deuteriodimethyl sulfoxide): 2.25 s, 3 H; 3.78 s, 3 H; 4.20 m, 1 H; 4.45 m, 2 H; 4.68 s, 2 H; 5.30 bs, 1 H; 6.6 – 7.8 m, 15 H.

Fmoc-Amide Anchor Resin

Phenoxyacetic acid *VIII* (3.67 g, 7 mmol) is dissolved with 1-hydroxybenzotriazole (0.95 g, 7 mmol) and 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (0.16 g, 1 mmol) in dry N,N-dimethylformamide (70 ml). The mixture is added to aminomethylated polystyrene resin (5 g, 1.07 mmol NH_2/g), diisopropylcarbodiimide (1.2 g, 10 mmol) is added and the slurry mixed by rotating overnight. After completion of the reaction (negative ninhydrin test) the resin is filtered off by suction, washed with DMF, 2-propanol, dichloromethane and methyl tert-butyl ether and dried in high vacuum. Yield 7.4 g (96%).

Determination of Fmoc-Amide Loading

The Fmoc loading of the resin is determined by treatment of the polymer (5 mg) with 90% (95% TFA/H_2O)/DCM yielding Fmoc-amide, whose quantity is determined by HPLC using an authentic sample for calibration[17]. From the experimental data a loading of 0.6 mmol/g resin is calculated.

Solid Phase Synthesis

Based on the analytical data reagents are used in the following amounts for the synthesis: 1.8 mmol t-Bumeoc-protected amino acid, 2.7 mmol 1-hydroxybenzotriazole/DMF, 1.8 mmol N,N'-dicyclohexylcarbodiimide/CH_2Cl_2, 12 ml 1% bromophenol blue/N,N-dimethylacetamide.

Fmoc Cleavage from the Resin

In a shaker reactor 0.5 g Fmoc-amide resin are swollen in dichloromethane for 10 min and treated as shown in Table II.

Coupling of t-Bumeoc-D-Thr(Bu^t)-OH and Determination of the Coupling Ratio

The amino resin is suspended in dichloromethane (10 ml) and then t-Bumeoc-D-Thr(Bu^t)-OH (1.8 mmol) and HOBt (2.7 mmol), dissolved in DMF (5 ml), are added. After addition of bromophenol blue in N,N-dimethylacetamide (12 μl) the dark blue solution is equilibrated for 2 min and N,N'-dicyclohexylcarbodiimide (1.8 mmol) is added. The mixture is shaken for 90 min while the colour changes from blue to yellow, indicating the termination of the coupling reaction. The resin is collected by suction and washed as described in the solid phase protocol. Uncoupled amino groups are capped by treatment of the polymer with acetic anhydride (0.57 ml), pyridine (0.48 ml) and dichloromethane (10 ml) for 30 min at room temperature. After the usual washing procedures the resin is dried in vacuo and the coupling ratio of threonine is determined after hydrolysis of the resin with 6M-HCl by OPA/MPA derivatization to yield 0.2 mmole Thr/g resin.

Solid Phase Synthesis Protocol

After the attachment of t-Bumeoc-D-Thr(Bu^t)-OH the t-Bumeoc-protected amino acids H-L-Cys(SBu^t)-OH, H-L-Thr(Bu^t)-OH, H-L-Lys(Boc)-OH, H-D-Trp-OH, H-L-Phe-OH, H-L-Cys(SBu^t)-OH, H-D-Phe-OH are coupled to the amino acid (peptide) resin in the listed order using the protocol given in Table III.

After completion of the synthesis of the pentapeptide resin 2-methylindole (100 mg) is added during each deprotection step (see protocol, steps 2 and 3). The octapeptide resin, subjected to 6M-HCl hydrolysis, exhibits the following amino acid ratios: Phe 2.00 (2), Lys 1.11 (1), Thr 2.00 (2), Trp 0.71 (1), (OPA/MPA method);

Phe 2.00 (2), Lys 0.83 (1), Thr 1.99 (2), Trp 0.41 (1), (ion exchange chromatography method, the Cys ratio is not determined and a loading of 0.2 mmol/g resin is calculated).

D-Phenylalanyl-S-tert-butylthio-L-cysteinyl-L-phenylalanyl-D-tryptophyl-L-lysyl-L-threonyl-S-tert-butylthio-L-cysteinyl-D-threoninamide (H-D-Phe-L-Cys(SBu^t)-L-Phe-D-Trp-L-Lys-L-Thr-L-Cys(SBu^t)-D-Thr-NH_2)

The fully protected peptide resin (500 mg) is suspended in anisole (2 ml) in the presence of 2-methylindole (400 mg). Nitrogen gas is bubbled through the reaction mixture and trifluoroacetic acid (18 ml) is added. After stirring for 2 h at room temperature and another 30 min at 50 °C the resin is filtered off by suction

TABLE II
Protocol of resin pretreatment

Step	Time min	Volume ml	Reagent
1. Wash	3 × 10	10	CH_2Cl_2
2. Wash	2 × 10	10	DMF
3. Fmoc cleveage	1 × 10	10	20% piperidine/DMF
4. Fmoc cleveage	1 × 25	10	20% piperidine/DMF
5. Wash	2 × 10	10	DMF
6. Wash	2 × 10	10	dioxane/water (2 : 1)
7. Wash	3 × 10	10	DMF

TABLE III
Solid phase synthesis protocol

Step	Time min	Volume ml	Reagent
1. Wash	3 × 5	10	CH_2Cl_2
2. t-Bumeoc cleveage	1 × 5	10	1% TFA/CH_2Cl_2
3. t-Bumeoc cleveage	1 × 10	10	1% TFA/CH_2Cl_2
4. Wash	3 × 1	10	CH_2Cl_2
5. Neutralization	1 × 5	10	N,N-diisopropylethylamine
6. Neutralization	1 × 10	10	N,N-diisopropylethylamine
7. Wash	6 × 1	10	CH_2Cl_2
8. Equilibration	1 × 2	10	1.8 meq t-Bumeoc amino acid, 2.7 meq HOBt, 12 µl bromophenol blue
9. Coupling	45 – 120	10	1.8 meq DCC/CH_2Cl_2
10. Wash	3 × 5	10	CH_2Cl_2
11. Wash	3 × 1	10	N,N-dimethylacetamide
12. Wash	3 × 3	10	2-propanol

and the filtrate is evaporated in vacuo. The remaining red oil is dissolved in methanol and slowly poured in precooled ether. The solid product is filtered off, dissolved in a few drops of methanol, chromatographed on a Sephadex LH-20 column using methanol as eluent (detection at λ 254 nm). The peptide-containing fractions are collected, evaporated to a small volume and precipitated with ether. After filtration and washing with ether the peptide is dried in a desiccator at high vacuum over potassium hydroxide. Yield: 64 mg (43%); m.p. 204 – 208 °C; R_F: 0.43 (S1), 0.89 (S2). $[\alpha]_D^{24}$ -74.9° (*c* 1, MeOH). Amino acid ratios in a 6M-HCl hydrolysate: Phe 2.00 (2), Lys 1.00 (1), Thr 2.00 (2), Trp 0.53 (1) (OPA/MPA method); Phe 2.00 (2), Lys 1.12 (1), Thr 1.97 (2), Trp 0.42 (1) (ion exchange chromatography method).

Dihydro-D-phenylalanyl-L-cysteinyl-L-phenylalanyl-D-tryptophyl--L-lysyl-L-threonyl-L-cysteinyl-D-threoninamide (H-D-Phe-L-Cys-L-Phe-D-Trp-L-Lys-L-Thr-L-Cys-D-Thr-NH_2)

Nitrogen is bubbled through a solution of tributylphosphine (233 μl, 0.94 mmol) in trifluoroethanol (20 ml) and the partly protected peptide amide (57 mg, 38 μmol) is added. The mixture is stirred for 2 h at room temperature and the solvents are evaporated in vacuo to a small volume. After adding dry ether, the crude product precipitates. It is recrystallized using methanol and ether, dried under reduced pressure in a desiccator, dissolved in 1% acetic acid (5 ml) and lyophilized. Yield: 37 mg (77%); m.p. 183 – 189 °C. R_F: 0.72 (S2), 0.34 (S3). $[\alpha]_D^{24}$ -36.38° (*c* 1, 1M acetic acid). FAB MS (*m/z*): 1 034 (M^+). Ellman test: 1.28 mmol SH/compound (87%).

D-Phenylalanyl-L-cysteinyl$^{2-7}$-L-phenylalanyl-D-tryptophyl-L-lysyl-L-threonyl-L-cysteinyl$^{7-2}$-D-threoninamide H-D-Phe-L-Cys-L-Phe-D-Trp-L-Lys-L-Thr-L-Cys-D-Thr-NH_2

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The unprotected octapeptide amide (37 mg, 27.2 μmol) is dissolved in 1,4-dioxane (5 ml) and diluted with 0.1M ammonium acetate (5 ml, pH 6.8). The reaction mixture is added dropwise to 0.1M ammonium acetate solution (380 ml, pH 6.8) at room temperature. The Ellman test performed after 5 min indicates 25 μmol (91.9%) of starting material. After stirring for five days, evaporation to a small volume, adding acetic acid (5 ml, 5%) and lyophilization, the product is chromatographed on a Sephadex G-25 column (eluent: 5% acetic acid, detection at 278 nm). The peptide-containing fraction is rechromatographed (same conditions, detection at λ 254 nm) and lyophilized. The final product is compared with a reference sample by HPLC and thin-layer chromatography. Yield: 15 mg (53%), m.p. 155 °C (decomposition). R_F 0.45 (S2), 0.45 (S3). $[\alpha]_D^{25}$ -51.1° (*c* 1, 5 acetic acid).

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