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SYNTHESIS OF THE PROTECTED GLYCOTETRAPEPTIDE CORRESPONDING TO THE N-TERMINAL AMINO ACID SEQUENCE OF [Thr^3-O-α-D-GALACTOPYRANOSYL]-VESPULAKININ 1

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Abstract - The synthesis of the protected glycopeptide Z-Thr(tBu)-Ala-Thr[α-D-Gal(Bzl)$_4$]-Thr(tBu)-NHNH-Boc was performed in solution by a stepwise coupling strategy. Fmoc-Thr[(α+β)-D-Gal(Bzl)$_4$]-OSu, obtained from the reducing sugar and the protected amino acid by the trifluoromethanesulfonic anhydride procedure, was reacted with H-Thr(tBu)-NHNH-Boc. The two diastereoisomers of the resulting glycosylated dipeptide derivative were purified and separated by column chromatography. The anomeric configuration of the O-glycosidic linkage was assigned by ^{1}H-NMR-spectroscopy. The α-anomeric glycodipeptide, obtained in a larger amount, was selectively deblocked at the amino function and acylated with Z-Thr(tBu)-Ala-OH in the presence of either IIDQ or EEDQ, to afford the desired protected glycotetrapeptide.

INTRODUCTION

Vespulakinin 1 (VSK1) and Vespulakinin 2 (VSK2) are the first reported naturally occuring glycopeptide derivatives of bradykinin (1). The structure deduced for VSK1 is shown in fig. 1.

carbohydrate carbohydrate bradykinin

H-Thr(1)-Ala-Thr-Thr-Arg-Arg-Arg-Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg(17)-OH

FIG. 1 - Structure of vespulakinin 1.

Most unique is the presence of carbohydrate O-glycosides attached to the hydroxyl function of Thr-3 and Thr-4 (a). The precise composition of the carbohydrates is not known but some evidence suggests that they are tri- or tetra-saccharides containing N-acetyl-galactosamine and galactose.

The pentadecapeptide VSK2 lacks the amino terminal dipeptide Thr-Ala and has the same carbohydrate composition of VSK1.

(a) **Abbreviations** - The amino acid residues are of L-configuration. Standard abbreviations for amino acid derivatives and peptides are used according to the suggestion of the IUPAC-IUB Commission of Biochemical Nomenclature, European J. Biochem. 1984, 138, 9-37. Other abbreviations are: Boc, tert-butyloxycarbonyl; Fmoc, fluorenylmetoxycarbonyl; Z, benzyloxycarbonyl; tBu, tert-butyl; Su, succinimidyl; Gal(Bzl)$_4$, 2,3,4,6-tetra-O-benzyl-D-galactopyranosyl; DMF, dimethyl-formamide, HOBt, 1-hydroxybenzotriazole; IIDQ, 1-isopropyloxycarbonyl-2-isopropyloxy-1,2-dihydro-quinoline; EEDQ, 1-ethyloxycarbonyl-2-ethyloxy-1,2-dihydroquinoline; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; t.l.c., thin layer chromatography.

These strucuturally similar glycopeptides, which were isolated from extracts of venom sacs of the yellow jacket Vespula Maculifrons, possess high activity in lowering rat blood pressure (1).

In order to investigate the extent to which the carbohydrate moieties contribute to the properties of biologically active glycopeptides, the synthesis was undertaken of analogues of VSK1 and VSK2, either devoid of carbohydrate or differing from the native molecules by the number and the composition of the carbohydrate side chain.

The carbohydrate-free VSK1 has been synthesized recently in our laboratory, both by the continuous flow-solid phase procedure and by a combination of solid phase and solution procedures (2). The synthesis of a glycopeptide analogue of VSK1 in which a galactose residue is O-glycosidically linked to one of two naturally glycosylated threonines, is now in progress.

In this communication we report the solution synthesis of the protected glycotetrapeptide Z-Thr(tBu)-Ala-Thr[α-D-Gal(Bzl)$_4$]-Thr(tBu)-N_2H_2-Boc corresponding to the N-terminal amino acid sequence of VSK1.

RESULTS AND DISCUSSION

The stepwise strategy of O-glycopeptide synthesis (3,4) was used for preparing the glycotetrapeptide fragment of VSK1, starting from an appropriately protected and O-glycosylated threonine residue. Elongation was carried out at both C- and N-terminal sides.

In our synthetic scheme for preparing the VSK 1 glycosylated analogue, the last coupling step will involve the reaction, via the azide procedure, of Z-Thr-Ala-Thr[α-D-Gal(Bzl)$_4$]-Thr-N_2H_3 with the α-NH_2 free C-terminal tridecapeptide sequence of VSK 1, whose N-terminal sequence is Arg-Arg-Arg. Difficulties could be expected in coupling two peptide segments through a reaction involving the troublesome arginine residue and a sterically hindered O-glycosyl-threonine residue. Threonine 3 was thus preferred to threonine 4 for glycosylation. The glycosylated threonine derivative Fmoc-Thr[(α+β)-D-Gal(Bzl)$_4$]-OSu was synthetized according to the literature (3).

An unexpected discrepancy with the literature data was found during the characterization of the intermediate Fmoc-Thr(tBu)-OSu. The value of the optical rotation we measured was almost identical to that previously reported with respect to the absolute value but opposite in sign. The synthesis of a Fmoc-Thr(tBu)-OSu derivative was thus carried out. Comparison with literature data demonstrated that the optical rotation we measured was the correct one (see Experimental for details). The synthetic route used for the preparation of the protected glycotetrapeptide corresponding to the N-terminal fragment of VSK1 analogue is illustrated in fig. 2.

2,3,4,6-Tetra-O-benzyl-D-galactopyranose (5) was reacted with Fmoc-Thr-OSu by the trifluoromethanesulfonic anhydride procedure (3). The resulting diastereomeric mixture of the (α+β)-O-galactopyranosyl amino acid derivative was directly reacted with H-Thr(tBu)-N_2H_2-Boc (6) in the presence of 1-hydroxybenzotriazole to minimize the risk of Fmoc-deblocking (7). Chromatography on silica gel of the resulting protected glycopeptide allowed the separation of α and β anomers of Fmoc-Thr[D-Gal(Bzl)$_4$]-Thr(tBu)-N_2H_2-Boc [1α and 1β].

The ^{1}H-NMR spectrum of compound [1α] is shown in fig. 3. The α or β configuration of the O-glycosidic linkage was assigned on the basis of decoupling and NOE (8) ^{1}H-NMR experiments, described in the following. The same analysis allowed the attribution of most resonances.

The low field broad resonances at 8.62, 7.93, 6.17 and 6.05 ppm can be attributed to amidic protons. The resonances at 8.62 and 6.17 ppm are reciprocally perturbed through decoupling experiments and are therefore assigned to hydrazidic protons.

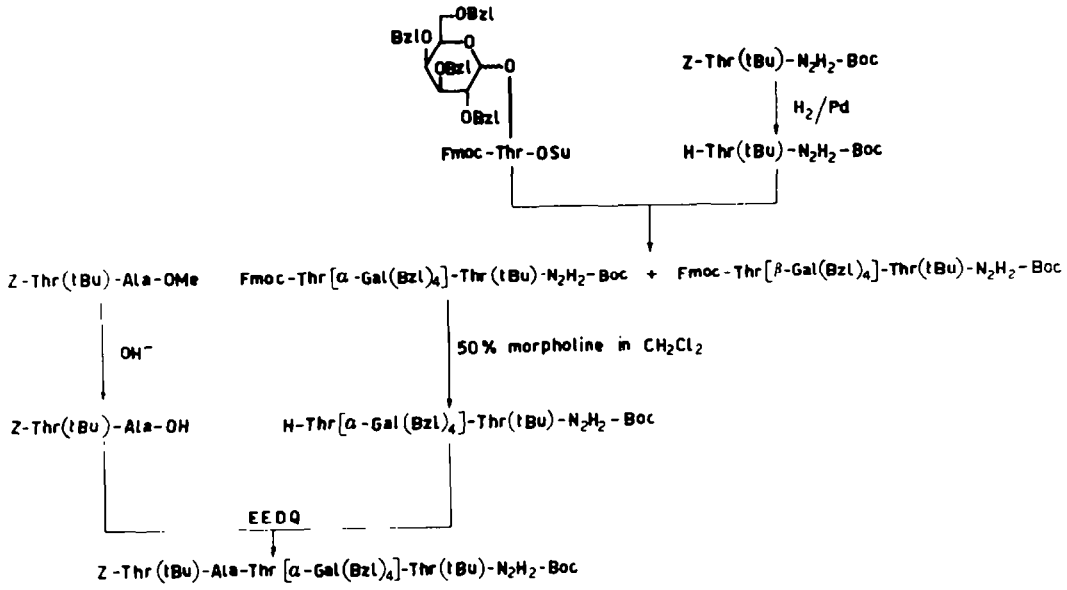

FIG. 2 - Synthesis of Fmoc-Thr(tBu)-Ala-Thr[α-D-Gal(Bzl)$_4$]-Thr(tBu)-N$_2$H$_2$-Boc (Procedure b).

The sugar anomeric H-1 proton can be localized in the doublet at 5.20 ppm. This resonance frequency and the $^3J_{1,2}$ coupling constant (3.8 Hz) are diagnostic for the α configuration of the O-glycosidic bond. Decoupling of this doublet reveals the resonance of the proton on the C-2 carbon of the galactopyranosidic ring (H-2) at 4.05 ppm. NOE perturbation of H-1 confirms this

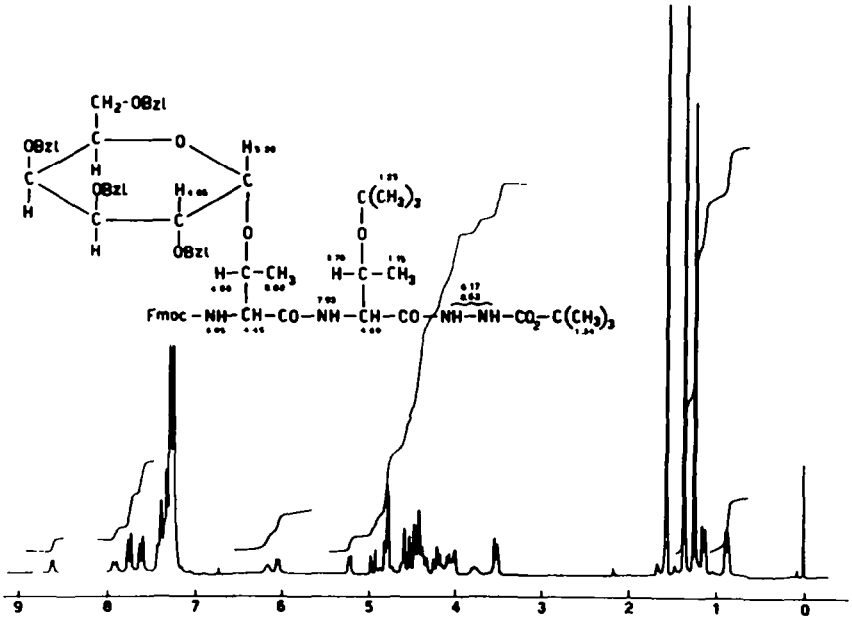

FIG. 3 - 200 MHz ^{1}H-NMR spectrum of Fmoc-Thr[α-D-Gal(Bzl)$_4$]-Thr(tBu)-N$_2$H$_2$-Boc in CDCl$_3$.

assignement, allowing at the same time the identification of Hβ-Thr at 4.08 ppm. This last resonance is NOE enhanced when the high field Hγ-Thr methyl doublet at 0.88 ppm is saturated. The signals at 4.08 and 0.88 ppm are to be assigned to the glycosylated threonine residue.

On the other hand, decoupling of the amidic resonance at 7.93 ppm modifies a multiplet at 4.60 ppm. This same system is NOE enhanced from a saturation of a relatively isolated multiplet at 3.78 ppm, which in turn is enhanced following the saturation of the high field Hγ-Thr methyl

doublet at 1.15 ppm. All these signals belong to non-glycosylated threonine residue.

Finally, from decoupling of the last amidic resonance at 6.05 ppm, the Hα-Thr resonance at 4.45 ppm can be localized. By exclusion, these signals are to be assigned to the glycosylated threonine residue. Fmoc-Thr[α-D-Gal(Bzl)$_4$]-Thr(tBu)-N$_2$H$_2$-Boc [1α], obtained in a larger amount, was selectively deblocked at NH$_2$-terminus and acylated with Z-Thr(tBu)-Ala-OH, which was prepared by saponification of the corresponding methyl ester (9). Removal of the Fmoc group from glycodipeptide 1α was achieved either with 10% piperidine in CH_2Cl_2 or 50% morpholine in CH_2Cl_2. No β-elimination of the carbohydrate moiety was detected.

Fragment condensation was performed in the presence of either IIDQ (10) or EEDQ (11). These coupling reagents do not require a tertiary base in the mixed anhydride forming reaction and are thus particularly useful for the synthesis of alkaly-labile O-glycopeptides (12,13).

It has been reported (14) that the use of IIDQ gives rise to a lower formation of the second acylation product by attack of the amine component at the carbonic acid carbonyl. In a first experiment we decided to use IIDQ in the acylation of H-Thr[α-D-Gal(Bzl)$_4$]-Thr(tBu)-N$_2$H$_2$-Boc with Z-Thr(tBu)-Ala-OH. Two-fold molar excess of the coupling reagent was used to increase the reaction rate (14). Nevertheless, t.l.c. monitoring indicated that even after 4 days the reaction was incomplete. The IIDQ excess made the chromatographic purification of the crude material difficult and the desired glycotetrapeptide Z-Thr(tBu)-Ala-Thr[α-D-Gal(Bzl)$_4$]-Thr(tBu)-N$_2$H$_2$-Boc [2α] was obtained in only 60% yield.

In a second experiment carried out in the presence of an equimolar amount of EEDQ, after some hours the amino component was undetectable in the reaction mixture by t.l.c. analysis. The chromatographic separation was easier and the glycotetrapeptide 2α was obtained in 85% yield.

EXPERIMENTAL

All chemicals were reagent grade or the best commercially available grade. Solvents were freshly distilled and evaporations were carried out under reduced pressure, at 25-35° using a rotary evaporator. Sodium sulfate was used for drying purposes. Hydrogenations were carried out at room temperature and atmospheric pressure, in the presence of 10% Pd/C. Yields are based on the weight of vacuum-dried product. Melting points were taken on a Buchi 150 melting point apparatus in open capillary tubes and are not corrected. Optical rotations were determined with a Perkin Elmer 241 polarimeter. Amino acid analyses were performed with a Carlo Erba 3A 28 amino acid analyzer equipped with a Perkin Elmer Sigma 10 Chromatography Data Station, following hydrolysis for 22 h at 110° in sealed, evacuated vials in constant boiling hydrochloric acid. The figures in parentheses are average recoveries of amino acid based on formula weights.

Ascending t.l.c. was routinely performed on precoated Silica Gel 60 F_{254} plates, Merck A.G., in the following solvent systems: I - chloroform-methanol 95:5 (v/v); II - n-hexane-ethyl acetate 60:40 (v/v); III - n-hexane-ethyl acetate 50:50 (v/v); IV - chloroform-acetic acid-cyclohexane (85:10:5 by vol.); V - chloroform-acetic acid-methanol (90:2:8 by vol); VI - chloroform-acetic acid-methanol (10:1:10 by vol); VII - n-butanol-acetic acid-water (3:1:1 by vol.).

Amino acid derivatives and peptides were located by spraying the chromatograms with ninhydrin (16), the hydrazide reagent (17) for C-terminal hydrazides and a modified chlorine reagent (18) for all peptide derivatives. Amino acid, peptide and sugar derivatives containing benzyl groups were also visualized by UV light. Sugar derivatives, glycosylated amino acid and

peptides were located by spraying the plates with 10% sulfuric acid in ethanol followed by heating for 18 min at 100°. Preparative liquid column chromatography was performed on a Jobin-Yvon Chromatospac-Prep 100 Instrument (column 4x50 cm), loaded with approximately 120 g of silica gel 60, 15-40 μm (Merck A.G.), under nitrogen pressure (approximately 1.5 atm). Column eluates were monitored with an Uvicord 2138 (LKB) detector connected to a 2210 Potentiometric Recorder (LKB).

^{1}H-NMR spectra at 200 MHz were recorded on a Bruker WP 200 SY, at 20°. Chemical shifts (in pmm) are relative to tetramethylsilane (TMS) as internal standard. In assignements, H-1 denotes the anomeric proton of galactose and Thr* denotes the glycosylated threonine residue. For NOE experiments, the sample in $CDCl_3$ was freed from oxygen by sonication under a nitrogen flow. The usual gated perturbative procedure was modified (19), and the selected resonance region was saturated by a 8 sec cyclic perturbation of all lines in the region with a 43 dB attenuation of a nominal 0.2 W decoupling power. The enhancements were detected in the differential mode (20).

Fmoc-Thr[(α+β)-D-Gal(Bzl)$_4$]-Thr(tBu)-N_2H_2-Boc [1α and 1β]. Z-Thr(tBu)-N_2H_2-Boc (6) (0.29 g, 0.70 mmol) was hydrogenated for 30 min in methanol (40 ml). The catalyst was removed by filtration and the filtrate was evaporated to dryness at room temperature. The residue (0.18 g, 90%) was homogeneus according to the t.l.c. analysis (Rf_I 0.23).

The diastereomeric mixture of Fmoc-Thr[(α+β)-D-Gal(Bzl)$_4$]-OSu (3) (0.46 g, 0.47 mmol) was dissolved in CH_2Cl_2 (25 ml) followed by addition of HOBt (0.07 g, 0.47 mmol) in DMF (2.5 ml). The solution was cooled at 0° and H-Thr(tBu)-N_2H_2-Boc (0.12 g, 0.43 mmol) in CH_2Cl_2 (30 ml) was added in portions in about 30 min. The mixture was kept at room temperature for 28 h and the reaction progress was monitored by t.l.c. in solvent system II. The solvent was removed in vacuo and the residue was taken up in CH_2Cl_2, washed with ice-cold 0.1 N $KHSO_4$ (50 ml), water (50 ml), 5% $NaHCO_3$ (50 ml), water (50 ml) and dried. Evaporation of the solvent gave a residue which was applied to the silica gel column of the Chromatospac-Prep 100 apparatus (hexane-ethyl acetate 65:35, v/v; flow rate 38 ml/min). Separation of pure diastereoisomers of the title compound (1α and 1β) was achieved.

1α: oil, 0.24 g (50%); Rf_{II} 0.72. Anal. calcd. for $C_{66}H_{78}N_4O_{13}$ (1135.37): C 69.82, H 6.92, N 4.93. Found: C 69.09, H 6.91, N 4.56; $[\alpha]_D^{21°}$ +44.05° (c 1.04, chloroform). ^{1}H-NMR ($CDCl_3$): 0.88 (d, 3H, Hγ-Thr*); 1.15 (d, 3H, Hγ-Thr); 1.25 (s, 9H, tBu); 1.34 (s, 9H, Boc); 3.78 (1H, Hβ-Thr); 4.08 (1H, Hβ-Thr*); 4.45 (1H, Hα-Thr*); 4.60 (m, 1H, Hα-Thr); 5.20 (d, 1H, H-1, J=3.8 Hz); 6.05 (d, 1H, NH-Thr*); 6.17 (1H, NH-NH); 7.93 (d, 1H, NH-Thr); 8.62 (1H, NH-NH).

1β: oil, 0.07 g (14%); Rf_{II} 0.41. Anal. calcd. for $C_{66}H_{78}N_4O_{13}$ (1135.37): C 69.82, H 6.92, N 4.93. Found: C 69.02, H 7.14, N 4.59; $[\alpha]_D^{21°}$ +16.1° (c 0.50, chloroform). ^{1}H-NMR ($CDCl_3$): 1.05 (d, 3H, Hγ-Thr*); 1.23 (d, 3H, Hγ-Thr); 1.17 (s, 9H, tBu); 1.40 (s, 9H, Boc); 4.18 (m, Hβ-Thr* + Hβ-Thr).

Z-Thr(tBu)-Ala-Thr[α-D-Gal(Bzl)$_4$]-Thr(tBu)-N_2H_2-Boc [2α] - 1 M NaOH was added in portions to a solution of Z-Thr(tBu)-Ala-OMe (9) (1.0 g, 2.43 mmol) in 90% dioxane-water (8 ml) in the presence of thymolphthalein. After 150 min stirring at room temeprature, the mixture was neutralized with 1 M HCl, concentrated to small volume in vacuo and taken up in ethyl acetate (30 ml). The organic phase was extracted with saturated aqueous $NaHCO_3$ (2x15 ml), the combined aqueous layers were acidified to pH 5 with 2 M HCl and extracted with ethyl acetate (3x25 ml). The organic extracts were combined, washed with water, dried and evaporated to dryness. Z-Thr(tBu)-Ala-OH was obtained as an oil (0.87 g, 90%, Rf_{IV} 0.66), which was directly used in next coupling step.

Procedure a - Compound **1α** (0.18 g, 0.15 mmol) was treated with 10% piperidine in CH_2Cl_2 (15 ml) and the mixture was stirred at room temperature for 80 min (t.l.c. monitoring in solvent system V) and evaporated to dryness. The oily residue was dissolved in CH_2Cl_2 (100 ml), washed with ice-cold 0.1 N $KHSO_4$ (3x50 ml), water (50 ml), 5% $NaHCO_3$ (2x50 ml), and dried. The solvent was removed in vacuo and the oily residue (0.13 g, Rf_V 0.78) was immediately dissolved in the minimum amount of CH_2Cl_2 and cooled at 0°. A solution of Z-Thr(tBu)-Ala-OH (0.059 g, 0.14 mmol) and IIDQ (0.087 g, 0.28 mmol) in CH_2Cl_2 (2.5 ml) was added and the reaction mixture was stirred at room temperature (t.l.c. monitoring in solvent system III). After 4 days CH_2Cl_2 (60 ml) was added and the solution was washed with ice-cold 0.1 N $KHSO_4$ (3x30 ml) and water (2x30 ml), dried and evaporated to dryness. Purification of the crude residue (0.19 g) by silica gel column chromatography (n-hexane-ethyl acetate 50:50, v/v, flow rate 32 ml/min) afforded the title compound as an oil (0.11 g, 60%).

2α - Rf_{III} 0.35; $[\alpha]_D^{23°}$ +42.2° (c 1.08; ethyl acetate). Anal. calcd. for $C_{70}H_{94}N_6O_{16}$ (1275.56): C 65.91, H 7.43, N 6.56. Found: C 65.98, H 7.76, N 6.54. Amino acid ratios in acid hydrolysate: Thr 2.99, Ala 1.00 (92%)

Procedure b - Morpholine (1 ml) was added to a stirred solution of compound **1α** (0.13 g, 0.12 mmol) in CH_2Cl_2 (1 ml). After 4 h reaction at room temperature (t.l.c. monitoring in solvent system III) the mixture was concentrated in vacuo and the residue dissolved in CH_2Cl_2 and worked up as previously described. The resulting oil (0.12 g) was dissolved in CH_2Cl_2 (2 ml), cooled to -15°, and Z-Thr(tBu)-Ala-OH (0.048 g, 0.12 mmol) and EEDQ (0.045 g, 0.12 mmol) in CH_2Cl_2 (1 ml) were added. After 12 h at room temperature, the reaction mixture was worked up as described in **Procedure a** and the crude residue (0.18 g) was purified by column chromatography (n-hexane-ethyl acetate 55:45, v/v, flow rate 60 ml/min) yielding the pure oily glycopeptide **2α** (0.13 g, 85%).

2α - Rf_{III} 0.35; $[\alpha]_D^{20°}$ + 42.6° (c 1.17, ethyl acetate). Anal. calcd. for $C_{70}H_{94}N_6O_{16}$ (1275.56): C 65.91, H 7.43, N 6.56, Found: C 65.15, H 7.48, N 6.39. Amino acid ratios in acid hydrolysate: Thr 3.01, Ala 1.00 (98%).

Assignement of the optical rotation of Fmoc-Thr(tBu)-OSu

Fmoc-Thr(tBu)-OSu - This compound was prepared from commercial Fmoc-Thr(tBu)-OH (Fluka) according to the previously described procedure (3, 14): m.p. 149-150°; $[\alpha]_D^{25°}$ -14.22° (c 0.97, ethyl acetate); Anal. calcd. for $C_{27}H_{30}N_2O_7$ (494.6): C 65.57, H 6.11, N 5.66. Found: C 65.54, H 6.19, N 5.77 [lit. (14) m.p. 148.5-153°, $[\alpha]_D^{20°}$ +14.58° (c 1, ethyl acetate); lit. (3) m.p. 144-145°, $[\alpha]_D^{20°}$ +16° (c 1, ethyl acetate).

The correctness of the negative sign of the optical rotation of Fmoc-Thr(tBu)-OSu has been established through the preparation of H-Thr-Ala-OH and the comparison of its optical rotation value with the literature data (15).

Fmoc-Thr-Ala-OH - Fmoc-Thr(tBu)-OSu (1 g, 2.0 mmol) was dissolved in ice-cold dioxane (4 ml) followed by dropwise addition of a solution of alanine (0.18 g, 2.0 mmol) in 10% aqueous Na_2CO_3 (4 ml). After 24 h at room temperature HOBt (0.15 g, 1 mmol) and additional 10% aqueous Na_2CO_3 (3.5 ml) were added and the mixture was kept at room temperature for 8 h. The reaction mixture was diluted with cold water (50 ml) and acidified to pH 2 by adding 2 N HCl. The resulting white precipitate was collected by filtration, dissolved in ethyl acetate (30 ml), washed with 0.01 N HCl (3x30 ml) and water (2x30 ml) and dried. The white foam (0.9 g, Rf_V 0.5) obtained after

evaporation of the solvent was dissolved in 50% trifluoroacetic acid in CH_2Cl_2 (2 ml). After 3 h at room temperature most of the solvent was removed and the residue taken up with ethyl acetate (100 ml) and washed with 0.5 M citric acid (2x50 ml). The aqueous washings were combined and re-extracted with ethyl acetate (3x50 ml). Finally, the combined organic extracts were washed with water (2x50 ml) and dried. Evaporation of the solvent afforded a residue which was crystallyzed from ethyl acetate. 0.42 g (90%); Rf_{VII} 0.77; m.p. 201-202°; $[\alpha]_D^{22°}$ -20.6° (c 1.05, methanol). Anal. calcd. for $C_{22}H_{24}N_2O_6$ (412.4): C 64.06, H 5.86, N 6.79. Found: C 63.96, H 5.89, N 6.75.

H-Thr-Ala-OH - A solution of Fmoc-Thr-Ala-OH (0.38 g, 0.92 mmol) in piperidine (4 ml) was kept 30 min at room temperature, concentrated in vacuo and diluted with cold water. The precipitated dibenzofulvene-piperidine adduct was separated by centrifugation, the aqueous supernatant evaporated to dryness and the residue taken up with water and re-evaporated to dryness (three times). The oily residue dissolved in 1 M acetic acid (2 ml) and precipitated by addition of ethanol, yielding the desired product: 0.154 g (88%); Rf_{VI} 0.12; m.p. 246° dec.; $[\alpha]_{578}^{20°}$ -10.8° (c 1.13, methanol- 1 N hydrochloric acid 9:1, v/v). Anal. calcd. for $C_{17}H_{14}N_2O_4$ (190.20): C 44.20, H 7.42, N 14.73. Found: C 43.93, H 7.59, N 14.44 [lit.(15) m.p. 224-228°; $[\alpha]_{578}^{20°}$ -11.5° (c 1, methanol- 1 N hydrochloric acid 9:1, v/v)].

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