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Incorporation of 5-Hydroxytryptophan in Oligopeptides

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Abstract: In the discovery of new inhibitors of enzymes, peptide libraries are actually representing a major point of interest. For this purpose, protecting group strategies are developed for the incorporation of modified amino acids in oligopeptides. Here we describe the incorporation of 5-hydroxytryptophan in oligopeptides using 5-*O*-benzyl-N^{α}-Fmoc-N^{in}-Boc-5-hydroxytryptophan as reagent. To evaluate the usefulness of this amino acid in solid phase peptide synthesis, protected 5-hydroxytryptophan was incorporated in a pentagastrine analogue. After deprotection, cleavage from the solid support, HPLC purification was performed and the identity of the obtained products was established by mass spectrometry. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Research on the incorporation of unnatural amino acids has been of considerable interest due to the increased enzymatic stability of the resulting peptide and their potential higher biological activity. Recently, chemical diversity methods[1-5] for the preparation of large libraries of molecules to screen for biological activity have further increased the interest in modified amino acids. Combinatorial libraries using the "one bead one peptide" concept are actually intensively investigated. This approach requires the availibility of protected amino acids of different origin. Moreover, the primary ligands, obtained by this method, possess in many cases low inhibitory and/or stability features and structural analogues need to be synthesized. Replacement of individual amino acids of an oligopeptide might also emphasize the importance of certain structural elements in an oligopeptide. Here we report on the incorporation of 5-hydroxytryptophan in oligopeptides. This amino acid can be considered as an isoster of tryptophan with increased hydrogen bonding capacity due to the presence of the 5-hydroxyl group. Tryptophan is known to be essential for the activity of various endogenous peptides: galanine[6], gastrine[7], endotheline[8] and cholecystokinine. Recently, several L-tryptophan derivatives with potent and selective antagonist activity at the NK_1 receptor were identified[9].

During peptide synthesis, an orthogonal protection strategy is used. During the last years the Fmoc-group[10] [9-(Fluorenylmethyloxycarbonyl)] has been prevailing the Boc-group (tert-butyloxycarbonyl) as α-amino protecting group, as less side reactions are observed[11]. The Fmoc group is easily removed by β-elimination using secondary amines in polar solvents[12]. This invokes that the orthogonal side chain protecting groups should be base stable. Therefore, the acid labile Boc and benzyl-based protecting group are the most attractive and useful.

Tryptophan derivatives, and particularly 5-hydroxytryptophan, are known to be prone to two main side reactions. The first is oxidation of tryptophan during coupling and the second is due to alkylation of the indole ring by carbenium ions generated during final deprotection and cleavage of the peptide from the resin. White *et al.*[13] proved these problems could be overcome by the introduction of a Boc group on the indole nitrogen. The 5-hydroxyl may be protected by a benzyl group in order to avoid peptide elongation. It should be easily removed using a thioanisol trifluoroacetic acid cocktail[14]. The aim of our work was to synthesize a fully orthogonal protected 5-hydroxytryptophan, ready to be incorporated in peptides using the Fmoc-strategy. To evaluate the usefulness and the limitations of 5-hydroxytryptophan, the obtained synthon was incorporated in pentagastrine (H_2N-βAla-Trp-Met-Asp-PheCOOH) containing the active center of natural gastrine. After deprotection and cleavage of the pentapeptide from the solid support, it was purified and the main products formed were determined.

RESULTS

In order to be able to efficiently protect the indole nitrogen and the 5-hydroxyl group, a temporary protection of the α-amino group and the carboxylic acid function is necessary. For the protection of the α-amino group, different protecting groups were used (Fmoc, Troc, Teoc). Best results were obtained with the trimethylsilylethyloxycarbonyl group (Teoc). For the temporary protection of the carboxylic acid function, the *p*-nitrobenzyl group seems to be most appropriate. The reaction scheme leading to **7a** is depicted in scheme 1.

2-(Trimethylsilyl)ethyl-4-nitrophenylcarbonate was prepared according to the procedure of Rosowsky[15]. Using this reactive ester, the α-aminogroup of 5-hydroxytryptophan (**1a**) was blocked in the presence of triethylamine in 91 % yield giving **2a**. Next, the carboxylic acid was converted into its cesium salt by titration to neutrality with aqueous Cs_2CO_3 solution. After evaporation to dryness the neutral salt was treated with *p*-nitrobenzylbromide in DMF to form the corresponding ester **3a** in 75 % yield[16].

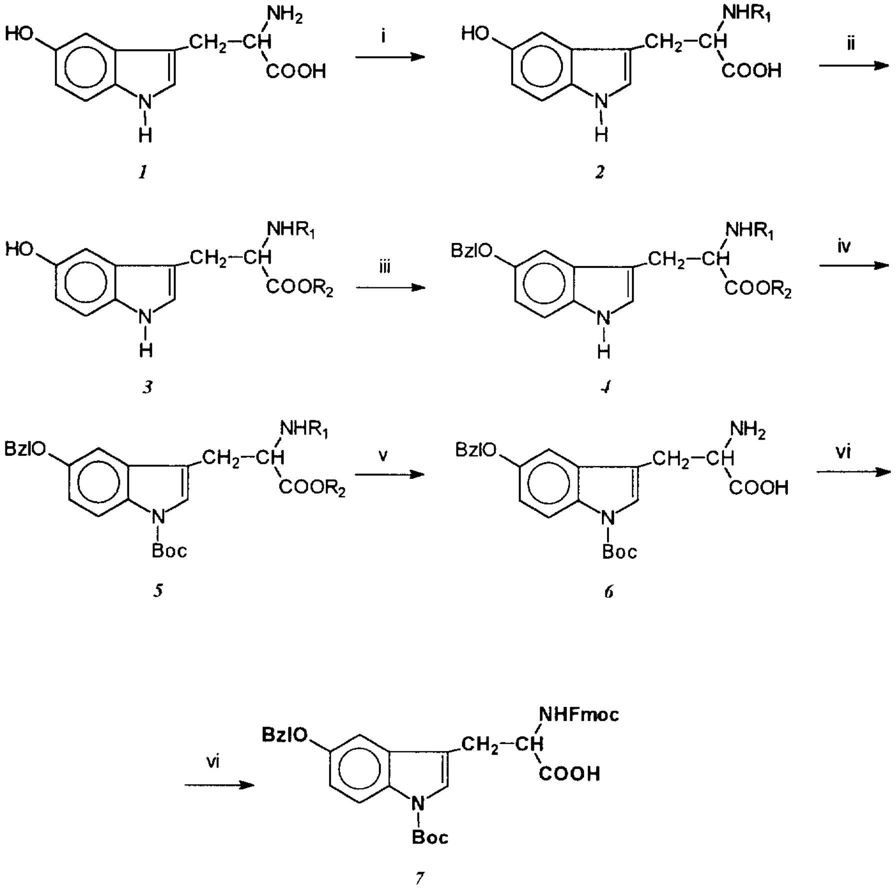
Benzyl protection of the 5-hydroxylgroup was carried out with benzylalcohol using Mitsunobu conditions[17]. Best results (68 %) for the synthesis of **4a** were obtained in anhydrous CH_2Cl_2. Finally the Boc group[13] on the indole nitrogen was introduced using 1 equivalent di-*tert*-butyldipyrocarbonate in the presence of a catalytic amount of DMAP in CH_3CN. Compound **5a** was obtained as the only product in 91 % yield.

A solution of 6 equivalents of Et_4NF dihydrate in DMF was used as well for the removal of the nitrobenzylgroup[18] as for the removal the Teoc-group[19]. Compound **6a** was chromatographically purified on silica gel. By addition of 1.1 eq FmocCl to a solution of **6a** in 1,4-dioxane/Na_2CO_3 10 % in H_2O/MeOH (1:1:1) the desired product **7a** was readily prepared under Schotten-Baumann conditions (yield = 64 %).

To evaluate the usefulness of 5-hydroxytryptophan in peptide synthesis, **7a** was used to incorporate 5-hydroxytryptophan instead of natural tryptophan in a pentagastrine analogue[7]. Therefore, the solid phase was first modified with a 4-hydroxybutyric acid spacer. A TFA-cleavable resin has not been tried.

The 4-hydroxybutyric acid linker was bound to Tentagel-S resin (ϕ = 130 μm, 290 μmol/g) in 95 % yield using the 4-*O*-monomethoxytrityl protected form. After removal of this acid labile trityl group, the first amino acid (phenylalanine) was introduced using DCC/HOBt in the presence of DMAP as catalyst. The remaining part of the hexapeptide (H_2N-βAla-Trp-Met-Asp-PheCOOH) was synthesized by a standard active ester method using Fmoc-AA/DCC/HOBt/DIEA in a 1:1:1:1 ratio.

The protected resin-bound peptide was deprotected using a mixture of 4 mL TFA, 0.75 mL EDT, 0.25 mL *m*-cresol, 0.25 mL thioanisol containing 110 mg of 5-hydroxyindole over a period of 1 x 90 minutes and 2 x 90 minutes respectively. After removal of the Fmoc-group at the amino terminus of the peptide, the gastrine analogue was hydrolized from the solid support using 0.1 N NaOH in the presence of 0.25 % $NaHSO_3$. The filtrate was freeze-dried and the resulting light brown solid was purified on a PLRP-S®column. The different products were identified using LSIMS and yields were determined.



a) R_1 = Teoc; R_2 = *p*-nitrobenzyl
b) R_1 = Fmoc; R_2 = *p*-nitrobenzyl
c) R_1 = Troc; R_2 = trichloroethyl

i. 2-(trimethylsilyl)ethyl-4-nitrophenyl carbonate 1.2 eq in MeOH/TEA; ii. a. Cs_2CO_3 pH = 7, b. 1.35 eq *p*-nitrobenzyl bromide; iii. Ph_3P 1.5 eq, DEAD 1.5 eq, BzlOH 1.2 eq in CH_2Cl_2; iv. $(Boc)_2O$ 1 eq, DMAP 0.2 eq. in CH_3CN v. $Et_4NF.2H_2O$ 6 eq in DMF; vi. FmocCl 1.2 eq in 10 % Na_2CO_3 in H_2O/MeOH/1,4-dioxane.

Scheme 1: Protection of 5-hydroxytryptophan

DISCUSSION

For the synthesis of protected hydroxytryptophan we first considered the synthesis of **7b** and **7c**. For the synthesis of compound **7b**, a direct protection of the α-aminofunction of compound **1** by a Fmoc-group represents the most straightforward strategy (Scheme 1, Method b). Unfortunately, the need of even a catalytic amount of DMAP during the Boc protection of the indole ring was sufficient to remove the Fmoc group. Therefore, temporary protection of the amino acid functionalities was necessary using Teoc and *p*-nitrobenzyl (approach a).

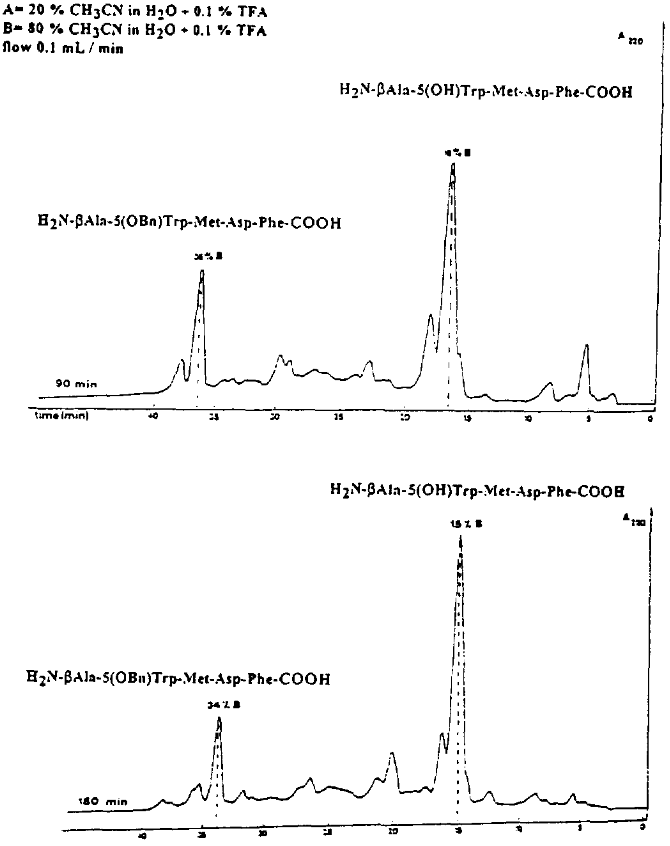


Fig. 1. HPLC profiles after 90 minutes (A) and 180 minutes (B) treatment with the deprotection cocktail

An alternative for the Teoc-group is the Troc-group (trichloroethyloxycarbonyl; Scheme 1, Method c). Again, protection of the indole nitrogen did not result in compound 5c. ^1H NMR as well as ^{13}C NMR indicated the presence of a second Boc-group with a different shift. Both the indole ring and the α-amino group were protected with a Boc group. Due to the strong electronwithdrawing capacity of the trichloroethyl group the remaining hydrogen on the urethane nitrogen was acidic enough to be abstracted by DMAP. As a consequence, selective protection of the indole moiety was not possible using less than 1 equivalent di-*tert*-butyl-dipyrocarbonate. Substitution of the trichloroethyl (Troc group) into trimethylsilyl group (Teoc group) induced a selective indole protection under the same reaction conditions.

A lot of literature[20-25] has been published concerning the minimization of side reactions during peptide deprotection. In the case of the pentagastrine analogue, two amino acids require some precautions: 5-hydroxytryptophan and methionine. Based on actual data, a cocktail of scavengers was formulated: EDT has shown to be highly efficient as a *tert*-butyltrifluoroacetate scavenger; thioanisol was suppressing the oxidation of methionine and 5-hydroxyindole was added as a competitive inhibitor for the alkylation. After hydrolysis under basic conditions during 1 hour, the freeze dried filtrate was purified on a PLRP-S column monitored at 220 nm. The HPLC profiles for the different deprotection times are represented in figure 1.

After 90 minutes of treatment with the cocktail, two main compounds were present, isolated and determined using mass spectrometry. The most polar compound proved to be the fully deprotected pentapeptide, while the second product isolated was its benzylated congener. The ratio was 2:1. Increasing the deprotection time to 3 h increased the ratio to 4:1. After 16 hours of treatment, the benzylated hexapeptide was almost completely converted in the unprotected pentagastrine analogue (HOOC-Phe-Met-Asp-5(OH)Trp-βAla-NH_2). The isolated yield of the deprotected pentapeptide was 45 % after deprotection for 3 h.

EXPERIMENTAL SECTION

The amino acid derivatives Fmoc-L-Phe-COOH, Fmoc-L-Met-COOH, Fmoc-L-Asp(*t*Bu)-COOH and Fmoc-β-Ala-COOH were purchased from Advanced Chem. and used without any further purification. Melting points were determined in capillary tubes with a Buchi-Tottoli apparatus and are uncorrected. Ultraviolet spectra were recorded with a Philips PU 8740 UV/Vis spectrofotometer. The ^{13}C NMR spectra were recorded with Varian Gemini 200. Exact mass measurements by liquid secondary ion mass spectrometry were obtained using a Kratos Concept 1H mass spectrometer. Precoated Machery-Nagel Alugram^R Sil G/UV_{254} plates were used for TLC and the spots were examined with UV light, sulfuric acid-anisaldehyde spray or ninhydrine spray. Column chromatography was performed on Janssen Chimica Silica gel (0.06 - 200 nm). Anhydrous solvents were obtained as follows: THF was refluxed on $LiAlH_4$ overnight and was distilled; dichloromethane was stored on calcium hydride, refluxed and distilled. Pyridine, triethylamine and *N,N*-diisopropylethylamine were refluxed overnight on potassium hydroxide and distilled. The CH_3CN for HPLC was purchased from Rathburn and water for HPLC purification was doubly distilled.

N^{α}-2-(trimethylsilyl)ethyloxycarbonyl-5-hydroxytryptophan (2a)

To a solution of 10.0 g (45 mmol) of 5-hydroxytryptophan in 80 mL of MeOH, 7.5 mL of TEA (54 mmol, 1.2 eq) and 15.4 g 2-(trimethylsilyl)ethyl-4-nitrophenylcarbonate (54 mmol, 1.2 eq) were added. The reaction mixture was stirred at room temperature overnight. The solvent was evaporated under vacuum, and a saturated solution of sodium bicarbonate was added. This mixture was extracted three times with diethyl ether. The aqueous layer was acidified with a 2 N HCl and extracted three times with ethyl acetate. The combined organic layer was dried over anhydrous magnesium sulphate. After filtration and evaporation, the yellow oil was purified on silica gel affording 15.07 g (41 mmol, 91 %) of compound **2a**. ^{13}C NMR ($CDCl_3$): δ -1.6 (CH_3, Teoc), 17.6 (CH_2-Si), 27.7 (β-CH_2), 54.1 (α-CH), 64.0 (CH_2-O), 103.2, 108.7, 112.0, 112.1, 124.4, 128.0, 131.5 and 149.4 (indole), 156.9 (HNCOO; Teoc), 175.9 (COOH). Exact mass (thioglycerol-NaOAc) calculated for $C_{17}H_{25}N_2O_5Si$ [M+H]+ 365.1533, found 365.1534.

N^{α}-2-(trimethylsilyl)ethyloxycarbonyl-5-hydroxytryptophan-p-nitrobenzylester (3a)

Compound **2a** (15.07, 41.4 mmol) was dissolved in 200 mL of ethanol and 20 mL of water was added. The solution was titrated to pH 7 with a 20 % aqueous solution of Cs_2CO_3 (6.73 g, 20.7 mmol). The mixture was evaporated to dryness and the residual water was removed by coevaporation with ethanol. The cesium salt of compound **2a** was dissolved in 200 mL of anhydrous DMF and 11.78 g (54 mmol, 1.35 eq.) of *p*-nitrobenzyl bromide was added. The reaction was stirred for 6 hours. After removal of the solvent, the oily residue was taken up in a saturated aqeous solution of sodium bicarbonate and was extracted three times with ethyl acetate. The combined organic layers were dried on anhydrous magnesium sulphate. After filtration, the solvent was evaporated and the obtained yellow oil was adsorbed on silica gel and purified by column chromatography yielding 14.9 g (30 mmol, 75 %) of compound **3a**. ^{13}C NMR ($CDCl_3$): δ -1.6 (CH_3, Teoc), 17.6 (CH_2-Si), 28.4 (β-CH_2), 54.4 (α-CH), 63.7 (CH_2-O, Teoc), 65.5 (CH_2-O, *p*-nitroBzl), 102.9 (indole), 108.9, 111.9 and 112.3 (indole), 123.5 (p-nitroBzl), 128.2, and 131.3 (indole), 142.2 and 147.5 (p-nitroBzl), 149.9 (indole), 156.3 (NHCOO, Teoc), 172.1 (COOR). Exact mass (thioglycerol) calculated for $C_{24}H_{24}N_2O_7Si$ $[M+H]^+$ 522.1673, found 522.1666

5-O-benzyloxy-N^{α}-2-(trimethylsilyl)ethyloxycarbonyl-tryptophan-p-nitrobenzylester (4a)

To a solution of 5.69 g (11.38 mmol) of compound **3a** in 200 mL dry CH_2Cl_2, 4.47 g Ph_3P (17 mmol, 1.5 eq) and 2.68 mL anhydrous benzyl alcohol (22.6 mmol, 2 eq) were added. A solution 2.68 mL of DEAD (17.07 mmol, 1.5 eq) in 75 mL anhydrous CH_2Cl_2 was added dropwise over a period of 1 hour. The reaction mixture was stirred at room temperature for 2 hours. The organic phase was washed twice with a saturated solution of sodium bicarbonate. The organic layer was dried on anhydrous sodium sulphate and adsorbed on silica gel. Chromatography eluting with CH_2Cl_2 gave 9 mmol (68 %) of **4a** as a yellow foam. ^{13}C NMR ($CDCl_3$): δ -1.6 (CH_3, Teoc), 17.6 (CH_2-Si), 28.2 (β-CH_2), 54.4 (α-CH), 63.5 (CH_2-O, Teoc), 65.4 (CH_2O, *p*-nitroBzl), 70.8 (CH_2O, Bzl), 102.1, 109.4, 111.9 and 113.0 (indole), 123.5 (indole + Bzl), 127.3 (*p*-nitroBzl), 127.6 (Bzl), 127.8 (Bzl), 128.2 (indole), 128.5 (Bzl), 131.5 (indole), 137.4 (Bzl), 142.3 and 147.5 (*p*-nitroBzl), 153.4 (indole), 156.1 (NHCOO, Teoc), 171.9 (COOR). Exact mass (thioglycerol-NaOAc) calculated for $C_{29}H_{36}N_3O_9SiNa$ $[M+Na]^+$ 612.2142, found 612.2141.

5-O-benzyloxy-N^{in}-tert-butyloxycarbonyl-N^{α}-2(trimethylsilyl)ethyloxycarbonyl-tryptophan-p-nitrobenzylester (5a)

To a solution of 5.29 g of compound **4a** (8.9 mmol) in 60 mL CH_3CN, 2.12 g di-*tert*-butylpyrocarbonate (1 eq) and 274 mg DMAP (2.2 mmol, 0.2 eq) were added. The reaction mixture was stirred overnight at room temperature. The solvent was evaporated and the light yellow oil was adsorbed on silica gel. Compound **5a** was purified chromatographically on silica gel to give 5.58 g (6.89 mmol, 91 %) of a foam. ^{13}C NMR ($CDCl_3$): δ -1.6 (CH_3,Teoc), 17.6 (CH_2-Si, Teoc), 28.1 (CH_3, Boc; β-CH_2), 53.8 (α-CH), 63.6 (CH_2O, Teoc), 65,6 (CH_2O, *p*-nitroBzl), 70.5 (CH_2O, Bzl), 83.8 (C-O, Boc), 103.2, 113.7, 114.3 and 116.0 (indole), 123.6 (*p*-nitroBzl), 124.8 (indole), 127.5 (*p*-nitroBzl), 127.9, 128.3 and 128.5 (Bzl), 130.1 and 131.0 (indole), 136.9 (Bzl), 141.9 and 147.6 (*p*-nitroBzl), 149.3 (indole), 155.0 (NHCOO, Boc), 155.9 (NHCOO, Teoc), 171.5 (COOR). Exact mass (LSIMS, thiogycerol) for $C_{36}H_{44}N_3O_9Si$ $[M]^{+\cdot}$calculated 689.2768, found 689.2760. El. Anal: $C_{36}H_{43}O_9N_3Si$ calculated C 62.68, H 6.28, N 6.09; found C 62.42, H 6.27, N 6.11.

N^{α}-fluorenylmethyloxycarbonyl-5-O-benzyl-N^{in}-tert-butyloxycarbonyl-5-hydroxytryptophan (7a)

To a solution of 1 g (1.45 mmol) of compound **5a** in 40 mL anhydrous DMF, (10 mmol, 6 eq) Et_4NF dihydrate was added. The solution was stirred overnight and solvent was removed under reduced pressure. The obtained brown oily residue was dissolved in ethyl acetate and was extracted twice with water. The organic layer was dried on anhydrous magnesium sulphate and evaporated under reduced pressure. After chromatographic purification on silica gel 470 mg (1.14 mmol, 79 %) of compound **6a** was obtained. Compound **6a** (440 mg, 1 mmol) was dissolved in a mixture of 20 mL MeOH, 15 mL 1,4-dioxane and 15 mL

10 % Na_2CO_3 in H_2O. The mixture was cooled in an ice bath and 1.2 eq FmocCl (300 mg , 1.16 mmol) was added. After stirring for 2 hours, the reaction mixture was poured in 100 mL of water, acidified with 10 % citric acid in H_2O and extracted twice with EtOAc. The organic layer was dried on magnesium sulphate, filtred and evaporated. The remaining oil was purified chromatographically on silica gel eluting with CH_2Cl_2 affording 470 mg (0.73 mmol, 65 %) compound **7a** which could be crystallized from $EtOH/Et_2O$. Data for compound **6a**: ^{13}C NMR (CD_3OD): δ 27.3 (β-CH_2), 27.5 (CH_3, Boc), 54.5 (α-CH), 70.6 (CH_2O, Bzl), 83.9 (C-O, Boc), 103.4, 114.1, 115.1, 116.1 and 125.7 (indole), 127.8 and 128.5 (Bzl), 130.9 and 131.2 (indole), 137.8 (Bzl), 149.9 (indole), 155.6 (NHCOO, Boc), 175.5 (COOH). Data compound **7a**: ^{13}C NMR ($CDCl_3$): δ 27.4 (β-CH_2), 28.1 (CH_3, Boc), 47.0 (Fmoc), 53.7 (α-CH), 67.3 (CH_2O, Fmoc), 70.5 (CH_2O, Bzl), 83.8 (C-O, Boc), 109.1, 113.8, 114.6 and 116.1 (indole), 120.0 (Fmoc), 125.1 (Fmoc + indole), 127.0 (Fmoc), 127.5 (Bzl), 127.7 (Fmoc), 128.5 (Bzl), 130.1 and 131.3 (indole), 137.3 (Bzl), 141.2 and 143.6 (Fmoc), 155.0 (NHCOO, Boc), 156.0 (NHCOO, Fmoc), 175.4 (COOH). Exact mass (NBA-NaOAc) for $C_{38}H_{35}N_2O_7Na_2$ $[M-H+2Na]^+$ calculated 677.2240, found 677.2239.

Synthesis of the pentagastrine analogue

A. Introduction of the linker. To a solution of 4-*O*-monomethoxytrityl-4-hydroxybutyric acid (2 mmol, 0.784 g) in 12 mL anhydrous pyridine, 192 µl TEA, 0.42 g DEC and 250 mg DMAP were added. After 20 minutes preactivation, 1.2 g Tentagel-S-NH_2 (130 µm, 290 mmol NH_2/g) was added and the suspension was shaken at room temperature for 24 hours. The beads were filtered from the solution, washed with anhydrous pyridine and CH_2Cl_2 and dried in vacuo. By measuring the absorption of a solution of 6 mg beads in 50.0 mL of a mixture of $HClO_4$:EtOH (3:2) at 475 nm, the loading was calculated and was found to be complete (95 %).

B. Attachment of the first amino acid. The beads were treated four times with a solution of 3 % CCl_3COOH in CH_2Cl_2 during 5 minutes and washed extensively with CH_2Cl_2. The solid phase was moistened with a solution of 110 mg DMAP in 1 mL anhydrous THF. After 20 minutes preactivation, a solution of 1.25 g (10 eq) of Fmoc-Phe-COOH, 870 mg (20 eq) of HOBt and 512 µL (10 eq) of DIC in 3 mL dry THF was added. Beads were shaken during 6 hours and washed with DMF (4 x 25 mL) and CH_2Cl_2 (4 x 25 mL). Coupling yield was 75 % which could be determined measuring the absorption of the fulvene piperidine adduct in a solution of 10 mg beads in 25.0 mL of a solution of 20 % piperidine in DMF.

C. Peptide synthesis. The synthesis protocol was as follows: 1. deprotection: 1x 20 min 20 % piperidine in DMF; 2. Washing 4 x 25 mL DMF, 4 x 25 mL CH_2Cl_2; 3. Coupling: 4 eq. Fmoc-AA-COOH, 4 eq. HOBt, 4 eq. DIC, 4 eq. DIEA in CH_2Cl_2/DMF (2:1) during 12 hours; 4. Washing: 4 x 25 mL DMF, 4 x 25 mL CH_2Cl_2; 5. Capping: pyridine/*N*-methylimidazole/acetic anhydride (4:1:1) 10 minutes; 6. Washing: 4 x 25 mL CH_2Cl_2. After each coupling, approximately 10 mg beads were isolated and treated with 25.0 mL of a solution of 20 % piperidine in DMF and the absorbance was measured at 301 nm in order to determine the amount of *N*-(9-fluor-enyl)methylpiperidine liberated.

D. Solid phase deprotection and cleavage of the pentagastrine analogue from the solid support. The adduct-protected peptide resin (300 mg) was treated twice with a mixture of 4 mL TFA, 0.75 mL of EDT, 0.25 mL of thioanisol, 0.25 mL of *m*-cresol and 110 mg of 5-hydroxyindole for 90 minutes. The deprotection cocktail was removed and beads were washed with CH_2Cl_2 (4 x 25 mL) and DMF (4 x 25 mL). Finally, the Fmoc group was removed using 20 % piperidine in DMF. The pentagastrine analogue was cleaved from the solid support using 10 mL of a mixture of 0.2 N NaOH/CH_3CN containing 55 mg $NaHSO_3$. The aqueous phase was pooled and acidified with 1 N HCl to pH = 2 and then freeze-dried. The light brown powder was purified on a PLRP-S® column using a gradual increase of CH_3CN. Two main products were identified at 220 nm. These compounds were isolated and identified by high resolution mass spectrometry. Main product: Exact mass (LSIMS, thioglycerol) for $C_{32}H_{41}O_9N_6S_1$ $[M+H]^+$ calculated 685.2655, found 685.2670.

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