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Solid Phase Synthesis of Partially Protected Tocinoic Acid: Optimization with Respect to Resin and Protecting Groups

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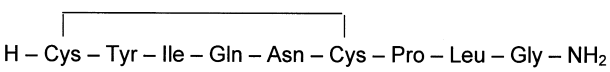
Abstract: A few solid phase and solution approaches of good repute were applied in parallel with the aim to provide optimized routes to Boc- and Fmoc-tocinoic acid (**3a** and **3c**) and the corresponding Tyr(Bu^t) derivatives (**3b** and **3d**). Boc-tocinoic acid is known to couple with tripeptide amides to give substituted oxytocin precursors in high yields, requiring only Boc-cleavage to furnish the corresponding hormone analogs with minimal loss of material. For comparison, two protected linear hexapeptides (**2a** and **2b**) were prepared on three polystyrene supports, two with acid-labile handles and one a conventional chloromethylated resin, in yields of 62–82 and 58–76%, respectively. The intermediate **2a** could be converted to **3a** with physical data in agreement with those earlier reported. Similarly, the intermediate **2b** was converted to **3b**. The highest yields for both **2a** and **2b** were obtained with a 2-chlorotrityl chloride resin, which in addition provided advantages with respect to overall speed and convenience. Additional syntheses of **3c** and **3d** on this and of **3c** on SASRIN resin, in conjunction with trityl instead of benzyl for side-chain protection of cysteine, were also elaborated. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: optimized synthesis; oxytocin segment; solid phase synthesis; synthetic methodology; tocinoic acid

INTRODUCTION

With respect to methodology, the landmark synthesis of oxytocin **1** by du Vigneaud and coworkers can be described as a $2 + (3 + 4)$ segment condensation [1]. Later, the protected *N*-terminal hexapeptide was identified as a useful segment for the synthesis of analogues modified in the *C*-terminal tripeptide amide part, i.e. by a $6 + 3$ approach. When the analogue contains expensive components like non-coded or labelled amino acid residues, such a segment condensation appears to be the method of choice [2–10]. Both the linear hexapeptide with various N^{α}- and side-chain protecting groups, and the corresponding cyclic species containing the preformed 1-6 disulphide bridge have been exploited in this context. In several cases, the latter has been coupled to tripeptide amides in high yields, from which the desired analogues were obtained after a simple additional deprotection step.



1, oxytocin

In connection with a project aiming at isotope-labelling of the backbone of the *C*-terminal tripeptide amide part of oxytocin, we have performed several syntheses of N^{α}-protected tocinoic acid by the solid phase method [11] in order to develop an optimized procedure. The synthetic strategies investigated included alternative protection (Boc, Fmoc, Bu^t, Bzl, Trt) of this linear hexapeptide and also involved an

Abbreviations: The nomenclature and symbols of amino acids follow published recommendations of the IUPAC/IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984; **138**: 9–37). Amino acids used in this work are of the L configuration; AAA, amino acid analysis; $\frac{1}{2}Cys_2$, half cystine; DIE, 1,2-diiodoethane; MeCN, acetonitrile; PFP, 2,3,4,5,6-pentafluorophenol; TDM, 4,4′-bis(dimethylamino)diphenylmethane.

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examination of different resins (Merrifield, SASRIN, 2-chlorotrityl chloride) as solid supports. After cleavage of the protected hexapeptides from the resin and *S*-deprotection of its cysteine residues when required, the disulphide bond was closed by oxidation under suitable conditions with formation of the required N^{α}-Boc or N^{α}-Fmoc-protected tocinoic acid. Although all the individual protecting groups, resins and other reagents employed are in common use, to our knowledge no such comparison has been performed in a similar context previously.

RESULTS

As a first step towards an optimized synthetic procedure for N^{α}-protected tocinoic acid, in parallel experiments the protected hexapeptides **2a** and **2b** were assembled on Merrifield, SASRIN [12,13] and 2-chlorotrityl chloride [14–16] resins. For both peptides Fmoc/Bzl were applied as protecting groups up to the penultimate step except for Tyr in **2b**, which was incorporated as Tyr(Bu^t). Boc-Cys(Bzl)-OH was applied in the final step. Further synthetic experiments were conducted with SASRIN (**2c**) and 2-chlorotrityl chloride (**2d**) resins in which cases exclusively Fmoc-amino acids were used and Tyr was incorporated as in **2b** and Cys as *S*-Trt derivative. All couplings on the Merrifield and SASRIN resins were mediated by DIC and HOBt in DMF and those on the 2-chlorotrityl resin by TBTU and HOBt in the presence of DIEA in the same solvent. The Fmoc groups were cleaved after each coupling step by a 20% piperidine–5%TFE mixture in DMF:DCM 9:1.

R^1-Cys(R^2)-Tyr(R^3)-Ile-Gln-Asn-Cys(R^2)-OR^4

2a, R^1 = Boc, R^2 = Bzl, R^3 = Bzl, R^4 = H,

2b, R^1 = Boc, R^2 = Bzl, R^3 = Bu^t, R^4 = H,

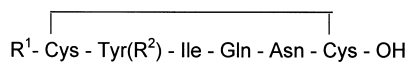
2c, R^1 = Fmoc, R^2 = Trt, R^3 = Bu^t,

R^4 = SASRIN resin,

2d, R^1 = Fmoc, R^2 = Trt, R^3 = Bu^t,

R^4 = 2-chlorotrityl resin

The cleavage of **2a** and **2b** from the Merrifield resin was carried out according to the method of Buis *et al.* [17], involving mild alkaline hydrolysis in the presence of methanol. This step was, however, accompanied by formation of methyl ester as a side-product. In the case of the SASRIN resin, the corresponding step was accomplished by 2% TFA in DCM [13] and of the 2-chlorotrityl resin by an AcOH-TFE-DCM mixture [14–16]. This was followed by deprotection of the Cys(Bzl) residues with Na in liquid ammonia. From the resin **2c**, a simultaneous detachment and side-chain deprotection of the hexapeptide was performed with a TFA–anisole–DCM mixture, whereas the peptide was cleaved with intact Tyr(Bu^t) side-chain protection from **2d** with AcOH–TFE–DCM as above. Oxidation was mediated by DIE in all cases, except the last one, for which it was performed with I_2 in MeOH, to afford the corresponding partially protected tocinoic acids **3a–3d**. During the synthesis of **3c** from **2d**, the derivative **3d** was also isolated and characterized. The derivatives **3b** and **3d** should also be useful for peptide segment condensation.



3a, R^1 = Boc, R^2 = H; **3b**, R^1 = Boc, R^2 = Bu^t

3c, R^1 = Fmoc, R^2 = H; **3d**, R^1 = Fmoc, R^2 = Bu^t

In general, the three mentioned resins were useful and afforded good partially protected tocinoic acid. However, some experimental details spoke in favour of the 2-chlorotrityl chloride resin, such as its quick and facile loading by the first amino acid and mild detachment of the protected hexapeptide after completed synthesis. Combined with application of Cys(Trt) the *S*-protecting group could be removed and the sulphhydryl groups oxidized '*in situ*' with formation of the required disulphide bond. As a result, the overall highest yields were obtained with this resin.

Whereas in the case of the Merrifield resin the standard method of Gisin [18], utilizing a cysteine cesium salt, could be applied for esterification, some problems arose with the SASRIN resin. We neither succeeded in acylating this by a DCC/DMAP/(HOBt) mixture [12,13] nor with a cesium salt, using its chloromethylated modification [19]; the substitution attained was in both cases below 0.1 mmol/g. Therefore, we also attempted a few other reagents like TBTU, HBTU and BOP used for *in situ* generation of active esters, but without success. Finally, we chose the PFP/DCC complex [20] with HOBt and a catalytic amount of DMAP for cysteine acylation and a satisfactory substitution was obtained (Table 1). After Fmoc deprotection, standard acidic hydrolysis and suitable sample treatment, the product $[\alpha]_D$ values obtained agreed

Table 1 Determination of Resin Loading for N^{α},*S*-protected Cysteine by Three Different Methods (in mmol/g)

Resin/amino acid derivative	Weight increase[a]	UV absorption[b]	AAA[c]	Loading time[d]
Merrifield[e]				
Boc-Cys(Bzl)-OH	0.56	0.54	0.52	20
SASRIN[f]				
Fmoc-Cys(Bzl)-OH	0.58	0.55	0.52	96
Fmoc-Cys(Trt)-OH	0.52	0.41	0.30	96
2-Chlorotrityl chloride[g]				
Fmoc-Cys(Bzl)-OH	0.74	0.72	0.71	1
Fmoc-Cys(Trt)-OH	0.68	0.71	0.67	1

[a] Calculated from resin weight increase after loading of N^{α},*S*-protected cysteine.
[b] Calculated from absorption of dibenzofulvene–piperidine complex at 300 nm, after treatment of Fmoc-Cys(X)-resin with 5% piperidine in DCM-DMF 1:1 for 10 min, and 20% piperidine in DMF for 15 min.
[c] Determined as H-Cys(Bzl)-OH after hydrolysis of H-Cys(Bzl)-resin or Boc-Cys(Bzl)-resin with 6 M HCl at 110°C for 20 h or as H-Cys(SO_3)-OH after similar hydrolysis of H-Cys(Trt)-resin and performic acid oxidation of H-Cys(SH)-OH.
[d] Loading time in hours.
[e] Resin substitution 0.96 mmol of Cl/g.
[f] Resin substitution 0.88 mmol of OH/g.
[g] Resin substitution 1.28 mmol of Cl/g.

with literature data [21,22]. As to the 2-chlorotrityl chloride resin [14–16], esterification is believed to take place by the attack of the trityl carbonium ion, which should guarantee racemization-free reaction. This is especially advantageous for the synthesis of peptides with Cys at the *C*-terminus. The extreme steric hindrance of the trityl group is believed to effectively suppress formation of dioxopiperazine at the dipeptide stage and premature cleavage of peptide from the resin.

Although excess of soluble reagent is always required in solid phase synthesis to drive reactions to completion, it is important to have an accurate value for the amount of amino acid on the resin, not least to determine the yield. In this context, we have compared three different methods to determine the amount of protected Cys originally present on the resins. These data are presented in Table 1. In general they are in good agreement except in one case for the SASRIN resin (Table 1).

DISCUSSION

Up to now, two syntheses of Boc-tocinoic acid **3a** have been described in the literature, and both were performed in solution [2–4]. On coupling with Pro-Leu-Gly-NH_2 it afforded Boc-oxytocin, from which fully active oxytocin was isolated in high yield after Boc-cleavage and chromatography [2]. Subsequently, it was applied to make a few oxytocin analogues [3,4,8]. Free tocinoic acid was first prepared by du Vigneaud *et al.* [23]. The synthesis of the related substance deamino-tocinoic acid was reported from three laboratories [2,10,23], one of which applied solid phase synthesis to make the linear hexapeptide [10]. Deamino-tocinoic acid has been used to make deamino-oxytocin [2,10] and two derivatives [10].

In the present work, using only reliable and convenient procedures, we have prepared the two linear hexapeptides **2a** and **2b** successfully by solid phase synthesis, with and without resin handles. After cleavage from the supports, the peptides were converted to the corresponding tocinoic acids **3a** and **3b**. The physical data for **3a** prepared in this way agreed with those earlier reported. In parallel, the corresponding Fmoc-derivatives, **3c** and **3d**, have been prepared similarly.

The detachment of the protected hexapeptide **2a** from the Merrifield resin, using mild alkaline hydrolysis [17] was exploited in the synthesis of some oxytocin analogues containing non-coded amino acid residues in the *C*-terminal tripeptide amide [8,9]. Later, we noticed that this hydrolysis is indeed accompanied by formation of methyl ester as pointed out by Tesser *et al.* [17]. This side-product could be removed by repeated precipitation from

DMF-water, although at the cost of lower yields of the protected hexapeptides. As a consequence, we, therefore, decided to carry out parallel experiments on resins with acid-labile handles. Several such resins are available nowadays [24], and we chose to investigate commercial SASRIN and 2-chlorotrityl chloride resins. Their application allowed utilization of 2%TFA and AcOH, respectively, for detachment of **2a** and **2b** from the supports without formation of any side-products. Their Cys(Bzl) deprotection required reduction with sodium in liquid ammonia, before the disulphide bond could be closed by oxidation. To avoid this step, in our final experiments, we, therefore, decided to use Cys(Trt) protection, which was applied by Jones *et al.* [4] in their solution synthesis of the tocinoic acid, in combination with the SASRIN and 2-chlorotrityl resins. This protection was found to be compatible [14–16] with the mild acidic deprotecting reagents just mentioned. As the 2-chlorotrityl resin was shown to be the most convenient also with respect to the loading of the first *N*-protected amino acid (Cys) to the resin (Table 1), the combination of this support with Cys(Trt) protection led us to the synthesis of **2d**, which was found to be the best precursor for N^{α}-protected tocinoic acid of the ones examined in this paper. For example, while the loading of the corresponding Cs-salt or pentafluorophenyl ester of protected Cys residues to the Merrifield or SASRIN resins required 24 h and 96 h, respectively, the coupling to the 2-chlorotrityl resin required only 1 h, moreover, without any activation and risk of racemization.

In conclusion, the application of the SASRIN and, especially, the 2-chlorotrityl resin allowed utilization of milder and more convenient conditions for loading and detachment of the protected hexapeptide from the supports. Besides, the preparation of N^{α}-Fmoc-tocinoic acids **3c** and **3d** from the protected precursors **2c** and **2d**, involving Cys(Trt) protection, proceeded without any apparent formation of side-product and resulted in more pure products in higher overall yields. Moreover, after detritylation both the linear hexapeptides could be directly oxidized to close the disulphide bridge, and complete the synthesis of the partially protected tocinoic acids.

MATERIALS AND METHODS

General

Merrifield (200–400 mesh, 1% DVB, 0.78 mmol Cl/g) and 2-chlorotrityl chloride (200–400 mesh, 1% DVB, 1.3 mmol/g) resins were purchased from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland) and SASRIN resin (200–400 mesh, 1% DVB, 0.9 mmol/g), TBTU and orthogonally protected amino acids from Bachem AG (Bubendorf, Switzerland), whereas N^{α}-protected ones were prepared in our laboratory following general protocols [21]. In the preparation of Fmoc- and Boc-amino acids, the prescribed pH was maintained using a pH meter with an automatic titrator (Radiometer, Copenhagen, Denmark). Protected amino acids were checked for their purity by TLC, HPLC and FAB MS. TLC was performed on precoated silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) in the following systems: 2-butanol:98% formic acid:water 75:13.5:11.5, S1; 2-butanol:25% aqueous ammonia:water 85:7.5:7.5, S2 and 1-butanol:acetic acid:water 4:1:1, S3. Spots were visualized directly under UV light or by ninhydrin and chlorine–TDM [25]. For HPLC, a Spectra Physics instrument with an SP 8800 pump, an SP 4290 integrator and a Thermo Separation Products Spectra 100 UV detector was used. Melting points were determined on a Kofler block and are uncorrected. Optical rotations were measured on a Perkin-Elmer 141 MCA polarimeter at 22°C. Solvents were evaporated *in vacuo* on a rotary evaporator (bath temperature 30°C); DMF was evaporated at 30°C and 150 Pa. Loadings of Fmoc-amino acids on resins are average values determined by (a) weight increase, (b) measurement of the dibenzofulvene-piperidine complex absorption after cleavage by 5% piperidine in DCM:DMF 1:1 for 10 min and 20% piperidine in DMF for 15 min, and (c) AAA, whereas for Boc-amino acids, they are based only on (a) and (c). Progress in peptide synthesis was monitored by the Kaiser [26] and bromophenol blue [27] tests. Samples for AAA were hydrolysed in 6 M HCl containing 3% of phenol for 20 h at 110°C, and analysed on a Biochrom 20 instrument (Pharmacia, Sweden). Peptide molecular weights were determined by FAB MS technique (Micromass, Manchester, UK). Peptides were purified by preparative HPLC on a 25×2.5 cm, 10 μm Vydac RP-18 column (The Separations Group, Hesperia CA, USA), flow rate 6 ml/min using a gradient 0–100% of MeCN in 0.05% aqueous TFA (60 min) with detection at 280 nm. Analytical HPLC was carried out on a 25×0.4 cm, 5 μm LiChrospher WP-300 RP-18 column (Merck, Darmstadt, Germany), flow rate 1 ml/min using the same gradient, but with detection at 220 nm, unless otherwise stated.

Solid Phase Synthesis of Boc-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-OH (2a) and Boc-Cys(Bzl)-Tyr(Bu^t)-Ile-Gln-Asn-Cys(Bzl)-OH (2b)

Experiment with Merrifield resin. Boc-Cys(Bzl)-O^-Cs^+ was prepared by treatment of Boc-Cys(Bzl)-OH (3.2 mmol; 1 g) in EtOH (32 ml)-water (12 ml) solution with 2 M Cs_2CO_3 at pH 7 followed by evaporation of the solvents and threefold evaporation with DMF. The Cs-salt was stirred in DMF (50 ml) with Merrifield resin (2 g) and dibenzo-18-crown-6 (2,3,11,12-dibenzo-1,4,7,10,13,16-hexaoxacyclooctadeca-2,11-diene; 2 mmol; 0.62 g) at 50°C for 20 h. The resin, loaded with the first amino acid, was filtered off, washed with DMF and EtOH (3 × 20 ml each) and dried over P_2O_5. It contained 0.54 mmol/g. Boc deprotection was carried out with a TFA:anisole:DCM 48:2:50 mixture for 15 and 30 min and neutralization before each coupling step with 10% DIEA in DCM. A threefold amount (3.2 mmol) of the following amino acid derivatives were employed: Fmoc-Asn-OH (1.15 g), Fmoc-Gln-OH (1.19 g) and Fmoc-Ile-OH (1.18 g). They were coupled stepwise to the H-Cys(Bzl)-O-Bzl-resin using DIC (0.57 ml; 3.66 mmol) and HOBt hemihydrate (0.53 g) with DIEA (0.3 equiv., 0.19 ml). Fmoc deprotection was performed in two steps with 20% piperidine–5%TFE in DMF:DCM 9:1 (20 ml) for 15 min, and with 20% piperidine in DMF (20 ml) for 30 min. After three coupling steps, the protected peptide–resin was washed with DMF and DCM and dried, whereupon the material (2.7 g) was divided into two parts. Each part was subjected to Fmoc deprotection and stepwise coupling either with Fmoc-Tyr(Bzl)-OH (0.79 g) and Boc-Cys(Bzl)-OH (0.5 g) (**2a**) or with Fmoc-Tyr(Bu^t)-OH (0.74 g) and Boc-Cys(Bzl)-OH (0.5 g) (**2b**) under conditions described above. The protected hexapeptide resins were washed with DMF, 2-propanol and EtOH, and were dried over P_2O_5 to give 1.46 g peptide polymer ester of **2a** and 1.43 g such of **2b**. Each of them was treated with a dioxane (60 ml)–MeOH (20 ml)–4 M NaOH (0.2 ml) mixture at room temperature for 2 × 3 min, whereupon AcOH (1 ml) was added, the resin washed consecutively with 2 × 30 ml portions of MeOH, DCM, DMF and again MeOH. The solvents were evaporated, toluene added and evaporated twice, and the residue triturated with water before the solid was filtered off, washed with diethyl ether and dried over solid KOH. To remove an impurity, later identified as the corresponding methyl ester by FAB MS, the material was crystallized twice from DMF-water to give 0.38 g (0.34 mmol, 62%) of **2a** [HPLC retention time 39.24 min; m.p. 220–222°C; TLC, R_F 0.63, S1; 0.42, S2; 0.62, S3; ([5], m.p. 219–222°C; TLC, R_F 0.62, S1; 0.42, S2; 0.63, S3)]; AAA: Asp 0.92, Glu 1.02, Ile 1.0, Tyr 1.04, Cys(Bzl) 1.78; for $C_{56}H_{72}N_8O_{12}S_2$ (1113.4) found FAB MS, m/z: 1114.1 ($M^+ + 1$). Similarly was obtained 0.34 g (0.32 mmol, 58%) of **2b** [HPLC retention time 39.74 min; AAA: Asp 0.94, Glu 1.01, Ile 1.0, Tyr 1.07, Cys(Bzl) 1.86; for $C_{53}H_{74}N_8O_{12}S_2$ (1079.4) found FAB MS, m/z: 1079.9 ($M^+ + 1$)].

Experiment with SASRIN resin. A mixture of Fmoc-Cys(Bzl)-OH (5.4 mmol; 2.34 g), HOBt (11 mmol; 1.47 g) and the complex PFP-DCC (10.2 mmol; 7.4 g) in DMF (15 ml) was stirred at 0°C for 30 min, whereupon the active ester formed was added to SASRIN resin (2 g) and DMAP (0.54 mmol; 0.06 g) in DMF (50 ml). The temperature was allowed to increase to room temperature and the pH of the solution was adjusted to 7 with DIEA. After 4 days stirring, the amino acid–resin was filtered and washed with DMF, EtOH, 2-propanol and MeOH (5 × 10 ml each). Remaining hydroxyl groups on the resin were blocked by acetic anhydride (1.3 ml)–DIEA (2 ml) in DCM (8 ml) for 1 h. Finally, the Fmoc–Cys(Bzl)–resin was filtered, washed with DCM, 2-propanol and DMF. The main part of the Fmoc-Cys(Bzl)-resin[1] was placed in a reaction vessel of a synthesizer. It contained 0.55 mmol/g. The removal of the N^{α}-Fmoc protecting groups and addition of the individual amino acid residues to the growing peptide chain, including dividing the resin in two parts after the Ile coupling step, followed the scheme used on Merrifield resin. Finally, each of the protected hexapeptide SASRIN-resins was washed with DMF and MeOH, dried over solid KOH to give 1.52 g of peptide polymer ester of **2a** and 1.49 g such of **2b**. After cleavage of the peptides from the resins with 2% TFA in DCM (2 × 50 ml) and filtration, the solutions were evaporated. Each of the peptides was dissolved under heating in DMF (5 ml), and precipitated with water. The precipitates were collected by filtration, washed on the filter with diethyl ether and dried. HPLC revealed that these crude products contained 56% and 59% of **2a** and **2b**, respectively, as a result of which, they were again purified from DMF-water in overall yields of 0.40 g (0.36 mmol, 65%) for **2a** and 0.36 g (0.33 mmol, 60%) for **2b**. Their analytical parameters were in accordance with those obtained for peptides **2a** and **2b** synthesized on Merrifield resin.

Experiment with 2-chlorotrityl chloride resin. Fmoc-Cys(Bzl)-OH (1.8 mmol; 0.78 g), 2-chlorotrityl chloride resin (2 g) dried over KOH overnight and DIEA (4.5 mmol; 0.78 ml) in DCM (20 ml)-DMF (4 ml)

mixture were stirred for 1 h at room temperature in the reaction vessel of a solid phase synthesizer. Unreacted chlorotrityl handle was quenched by addition of excess of MeOH (2 ml) and stirring for 10 min. The Fmoc-Cys(Bzl)-*O*-2-chlorotrityl-resin was washed consecutively with 3 × 10-ml portions of DCM, DMF, 2-propanol, DMF, MeOH and diethyl ether and then dried over KOH in desiccator overnight. The first Fmoc deprotection was performed with 5% piperidine in DCM:DMF 1:1 (10 ml) for 10 min and 20% piperidine in DMF (10 min) for 15 min. The loading was 0.72 mmol/g and N^{α}-Fmoc-amino acids were applied in 2.5-fold excess. With respect to the synthesis, the Fmoc deprotection and stepwise coupling of the individual amino acid residues followed those on Merrifield resin, except that TBTU and HOBt (3.6 mmol) with a twofold excess of DIEA were used instead of DIC. Both the protected hexapeptide resins were washed with DMF, 2-propanol and MeOH and then dried over solid KOH to give 1.71 g of peptide polymer ester of **2a** and 1.69 g such of **2b**. Each was treated with an AcOH:TFE:DCM 2:2:6 mixture (3 × 30 ml) at room temperature for 1 h, separated from the resin by filtration and washed with the same mixture (3 × 10 ml). The solutions of the protected hexapeptides **2a** and **2b** were concentrated to remove DCM and TFE and water was added. The precipitated solids were collected by filtration, washed with 2-propanol and MeOH and dried. HPLC revealed that the crude products contained 68% and 70% of **2a** and **2b**, respectively. They were further purified by crystallization from DMF–water to give 0.65 g (0.58 mmol, 81%) of **2a** and 0.59 g (0.55 mmol, 76%) of **2b**, the analytical data of which were comparable with those obtained for these peptides, synthesized on Merrifield and SASRIN resins.

Solid Phase Synthesis of Fmoc-Cys(Trt)-Tyr(Bu^t)-Ile-Gln-Asn-Cys(Trt)-*O*-SASRIN Resin (2c)

Fmoc-Cys(Trt)-SASRIN resin was prepared according to the procedure described above for **2a** and **2b** on SASRIN resin from Fmoc-Cys(Trt)-OH (2.7 mmol; 1.6 g), HOBt (5.4 mmol; 0.72 g), PFP-DCC complex (5.4 mmol; 3.9 g) and SASRIN resin (1 g) in DMF (40 ml) in the presence of DMAP (0.03 g; 0.27 mmol). Remaining hydroxyl groups were capped with an acetic anhydride (0.7 ml)–DIEA (1 ml)–DCM (6 ml) mixture, after which analysis indicated a substitution degree 0.41 mmol/g of resin.[2] A 3-fold excess of N^{α}-Fmoc-amino acids was used. They were coupled and N^{α}-deprotected, using the reagents and conditions described in the synthesis of **2a** and **2b** on Merrifield resin. The protected hexapeptide resin **2c** was washed with DMF, 2-propanol and MeOH and finally dried over KOH (1.32 g).

Solid Phase Synthesis of Fmoc-Cys(Trt)-Tyr(Bu^t)-Ile-Gln-Asn-Cys(Trt)-*O*-2-Chlorotrityl Resin (2d)

Fmoc-Cys(Trt)-*O*-2-chlorotrityl resin was prepared according to the procedure described above for **2a** and **2b** on 2-chlorotrityl chloride resin from Fmoc-Cys(Trt)-OH (0.53 g; 0.9 mmol), resin (1 g) and DIEA (0.39 ml) in DCM (10 ml)–DMF (2 ml). Quenching with MeOH (1 ml), washing and drying provided a resin with a substitution of 0.71 mmol/g. After Fmoc deprotection with 5% piperidine in DCM:DMF 1:1 (10 ml) for 10 min and 20% piperidine in DMF (10 ml) for 15 min, the corresponding N^{α}-Fmoc amino acids were coupled stepwise, in 2.5-fold excess, according to the procedure used in the synthesis of **2a** and **2b** on Merrifield resin, except that TBTU and HOBt (1.8 mmol) with a twofold excess of DIEA were used as coupling reagents. The protected hexapeptide–resin **2d** was washed with DMF and DCM and then dried over KOH (2.05 g).

Synthesis of Boc-tocinoic acid (3a) and Boc-(Tyr(Bu^t)^2)-tocinoic acid (3b)

Each of the protected hexapeptides **2a** (0.3 mmol; 0.33 g) and **2b** (0.3 mmol; 0.32 g) was treated with sodium in liquid ammonia (300 ml) until the dark blue colour persisted for 1 min, when AcOH was added dropwise to discharge the colour and the ammonia was evaporated at reduced pressure and a bath temperature of 5–10°C. The solid residues were dissolved in 2 M AcOH (30 ml) and pH under stirring in argon atmosphere adjusted to 8.5 with 10% NH_4OH. The solutions were diluted with 20% aqueous MeOH (200 ml) and DIE (0.1g) in MeOH (60 ml) was added dropwise during 30 min. The reaction mixtures were stirred for 2 h, when their volumes were reduced to about 60 ml, and pH was adjusted to 4.5 with AcOH. These solutions on freeze-drying furnished crude Boc-tocinoic acids **3a** and **3b** containing some sodium salts. They were purified by preparative HPLC.

The crude Boc-tocinoic acid **3a** was dissolved in MeCN (3 ml) and the insoluble material was removed by centrifugation (2500 rpm, 10 min). The supernatant was injected in several portions onto the HPLC VYDAC column 25 × 2.5 cm. Elution was carried out with a 5–95% gradient of MeCN in 0.05% aqueous TFA, 40 min with flow rate

6 ml/min, affording 67 mg of pure **3a**. Analytical HPLC was performed using the same gradient, flow 1 ml/min, HPLC elution time was 18.01 min; m.p. 196–198°C; $[\alpha]_D$ −66°(c 0.5, EtOH) [[2], m.p. 195–198°C; $[\alpha]_D$ −67.3° (c 0.5, EtOH)]. AAA: Asp 1.00, Glu 0.98, Ile 0.96, Tyr 0.97, 1/2 Cys_2 2.02; for $C_{35}H_{52}N_8O_{12}S_2$ (841.0) found FAB MS, m/z: 841.3 ($M^+ + 1$).

The crude Boc-[Tyr(Bu^t)2]-tocinoic acid **3b** was purified by preparative HPLC under the same conditions as described for **3a** to yield 70 mg. Analytical HPLC using the same gradient and flow revealed a peak at 23.61 min. AAA: Asp 1.00, Glu 1.02, Ile 0.98, Tyr 0.95, 1/2 Cys_2 1.97; for $C_{39}H_{60}N_8O_{12}S_2$ (897.1), FAB MS, m/z: 897.4 ($M^+ + 1$).

Synthesis of Fmoc-tocinoic acid (3c)

The protected hexapeptide–SASRIN resin **2c** (0.66 g) was treated with a TFA (15 ml)–anisole(1.5 ml)–DCM(13 ml) mixture for 3 × 30 min. The spent resin was washed with DCM (3 × 15 ml), DMF (3 × 15 ml) and DCM (3 × 5 ml). The combined solutions were evaporated to dryness and the residue was triturated with diethyl ether and dried. The simultaneous side-chain deprotection and peptide cleavage off the resin afforded the Fmoc-hexapeptide acid (0.12 g, 0.12 mmol) that was directly subjected to oxidation. The solid was dissolved in 20% aqueous MeOH (300 ml), pH adjusted to 8 with 10% NH_4OH and a solution of DIE (0.1 g) in MeOH (60 ml) was added dropwise over a period of 2 h. The reaction mixture was stirred for another 2 h at this pH, reduced to a volume of 80 ml and pH adjusted to 4.5 with AcOH. This solution was freeze-dried and the lyofilizate was purified by preparative HPLC under the same conditions as described for **3a**, affording 73 mg of the pure **3c**. The product was analysed by HPLC using conditions described in the general section of Materials and Methods; elution time 31.79 min; AAA after Fmoc deprotection of the sample: Asp 1.0, Glu 1.1, Ile 1.0,Tyr 0.92, 1/2 Cys_2 1.72. For $C_{45}H_{54}N_8O_{12}S_2$ (963.1) found FAB MS, m/z: 963 ($M^+ + 1$).

The protected hexapeptide-*O*-2-chlorotrityl resin **2d** (1.1 g) was treated with an AcOH:TFE:DCM 2:2:6 mixture (20 ml) at room temperature for 1 h. The spent resin was washed with the above mixture (3 × 10 ml). To pooled solutions of the protected hexapeptide, I_2 (0.2 g) in MeOH (20 ml) was added dropwise within 30 min, whereupon the solution was first concentrated to remove DCM with TFE and then 10% ascorbic acid in water (10 ml) was added to discharge the excess of I_2. The resulting solution was washed with ethyl acetate (3 × 50 ml) and the aqueous phase was freeze-dried. The residue was dissolved in MeCN (3 ml) and then applied, in several portions, to the preparative Vydac column 25 × 2.5 cm using the HPLC conditions described above for **3a** to furnish 86 mg of pure **3d**. Analytical HPLC retention time 37.65 min; AAA: Asp 1.1, Glu 1.1, Ile 1.0, Tyr 0.98, 1/2 Cys_2 1.78; for $C_{49}H_{62}N_8O_{12}S_2$ (1019.2), FAB MS, m/z: 1019.4 ($M^+ + 1$). On treatment of **3d** with a TFA (5 ml)–anisole (0.5 ml) mixture for 2 h, evaporation and trituration with diethyl ether, crude **3c** was obtained. Purification by HPLC under standard conditions afforded pure **3c** (70 mg) with analytical data identical to those described above.

CONCLUSION

Boc-tocinoic acid, previously prepared by stepwise synthesis in solution, can more conveniently be made by the solid phase technique. Various polystyrene supports, including the chloromethylated and such with acid-labile handles, are applicable, but although, after completed synthesis, cleavage under basic conditions can be used, acidic detachment is preferable. The yields for the two linear Boc-hexapeptides, with and without a protecting group in the tyrosine side-chains, were 58–82%, in both cases, highest for those based on 2-chlorotrityl chloride resin. Boc-tocinoic acid made from linear intermediate exhibited physical data, in agreement with those earlier reported. The side-chain of cysteine can be protected by benzyl or trityl, but as the former requires reduction by sodium in liquid ammonia, the latter is more convenient. Finally, Fmoc-tocinoic acid and its Tyr(But) derivative were prepared under these optimized solid phase conditions.

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NOTES

1. A sample (0.1 g) was checked for the optical purity of the cysteine residue: after *N*-deprotection, as described

in the preceding paragraph and cleavage with 2% TFA in DCM, the acidic solution was filtered off and evaporated. The remaining material was triturated with 20% NH_4OH, filtered, washed with water, and dried over P_2O_5 to yield H-Cys(Bzl)-OH with $[\alpha]_D$ +24.1° (c 0.2; 1 M NaOH); [[21], $[\alpha]_D$ +24.5° (c 1; 1 M NaOH)].

2. A sample (0.1 g) was checked for the optical purity of the cysteine residue, as described above, except that the material after evaporation of the acidic solution was triturated with EtOH, filtered and washed with EtOH. After drying over P_2O_5, it afforded H-Cys-OH with $[\alpha]_D$ +5.8°(c 0.2; 5 M HCl); [[22], $[\alpha]_D$ +6.0° (c 1; 5 M HCl)].

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