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SOLID-PHASE PEPTIDE SYNTHESIS OF ISOTOCIN WITH AMIDE OF ASPARAGINE PROTECTED WITH 1-TETRALINYL. TRIFLUOROMETHANESULPHONIC ACID (TFMSA) DEPROTECTION, CLEAVAGE AND AIR OXIDATION OF MERCAPTO GROUPS TO DISULPHIDE

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ABSTRACT. Isotocin, a nonapeptide amide, was synthesised on a benzhydryl-resin using the Boc-strategy. Benzyl group was used in the protection of the side-chains of tyrosine, serine and cysteine. Tetralinyl group was used to protect asparagine side-chain. TFMSA-TFA-thioanisole-1,2-ethanedithiol (2:20:2:1 v/v) was used on the peptide-resin under different cleavage conditions to obtain isotocin in a one-pot reaction. The cleavage at 40 °C for two hours gave isotocin quantitatively. Isotocin could be isolated in 61% yield.

KEY WORDS: Isotocin, Nonapeptide amide, Tyrosine, Serine, Cysteine, Asparagine, Tetralinyl group

INTRODUCTION

During solid-phase peptide synthesis (SPPS), side chain amide protection of asparagine (Asn) or glutamine (Gln) has been considered optional [1]. These amide side chains are liable to undergo dehydration during the coupling steps [2-4]. This side reaction does not occur when active esters are used [1, 2, 4-6]. Several carboxamide protecting groups, like 2,4,6-trimethoxybenzyl [7, 8], 4,4'-dimethoxybenzhydryl [9], triphenylmethyl [10], 4-methyltrityl [11], 2,4-dimethoxybenzyl [7, 8, 12] and tetralinyl [13, 14] have been developed with the goal to reduce or eliminate the aforementioned side reaction during peptide synthesis. In this project, we were encouraged to examine whether tetralinyl group is viable as an amide-protecting group (asparagine) in the solid-phase peptide synthesis of isotocin [15, 16]. Disulphide bonds are important for peptides in the maintenance of biological activity and conformational stability. Disulphide bonds in peptides are usually formed after the linear peptide is synthesised, side chain protecting group removed, and the peptide detached from the resin [17]. This procedure is quite time-consuming. A one-pot synthesis has been developed for disulphide-containing bonds, which simultaneously cleave, deprotect and oxidise the cysteines [18]. Thus, cystine-containing peptides have been obtained after treatment of the protected peptidyl resin with TFA in the presence of iodine [18]. Other oxidants include air, potassium ferricyanide [19] and dimethyl sulphoxide [20, 21].

EXPERIMENTAL

General. Protected amino acid derivatives, benzhydrylamine hydrochloride salt (loading: 0.9 mmol/g) were obtained from Sigma (St. Louis, USA). Trifluoroacetic acid (TFA), trifluoromethanesulphonic acid (TFMSA), thioanisole, 1,2-ethanedithiol (EDT) were obtained from Fluka (Buchs, Switzerland). All solvents were of analytical grade or of equivalent purity, and used

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without further purification. Dichloromethane, methanol, ethanol, N,N-dimethylformamide (DMF) were purchased from BDH Chemicals Ltd (Poole, England).

Peptide chain assemblies by Boc chemistry were carried out manually. Side chain protection was provided by benzyl for cysteine, tyrosine and serine; tetralinyl (Tet) for asparagine. Boc removal was achieved with TFA/dichloromethane/anisole (50:48:2 v/v) for 25 min at room temperature. Final release of peptide from the support and concomitant cleavage of side chain protecting groups was achieved with TFMSA-TFA-thioanisole-EDT (2:20:2:1 v/v).

IR spectra were recorded on a Perkin Elmer 1600 series (FTIR). Ion Electrospray mass spectra (ESMS) were determined on a Sciex API III TAGA 6000 (Toronto, Canada). ESMS samples were prepared by dissolving 1mg of peptide in 1 mL of 5% acetic acid (AcOH), acetonitrile or methanol-water (80:20 v/v). Analytical high performance liquid chromatography (HPLC) of crude peptide was performed using a Grom analytical nucleosil C-18 reversed-phase column (5 µm, 250 mm x 2 mm) on a Beckman system, configured with a Programmable Solvent Module 126 with Auto Sampler 507 and a variable wavelength Diode Array Detector Module 168. This was controlled from a computer with Beckman System Gold Software. Peptide (1 mg/mL of methanol) samples were chromatographed at 0.3 mL/min using a linear gradient of 0.1% aqueous TFA and 0.1% TFA in acetonitrile (10:90 to 0:100 over 45 min), detection at 214 and 280 nm. Semipreparative HPLC was performed using a Grom semi-preparative nucleosil C-18 reversed-phase column (7 µm, 250 mm x 8 mm) on a Waters 600 (Milford, Massachusetts, USA) Multi Solvent Delivery System using manual injection (0.5 mL, 5 mg of peptide per run) and elution at 3.5 mL/min using 0.1% aqueous TFA and 0.1% TFA in acetonitrile (90:10 to 30:70 over 45 min), detection at 214 nm.

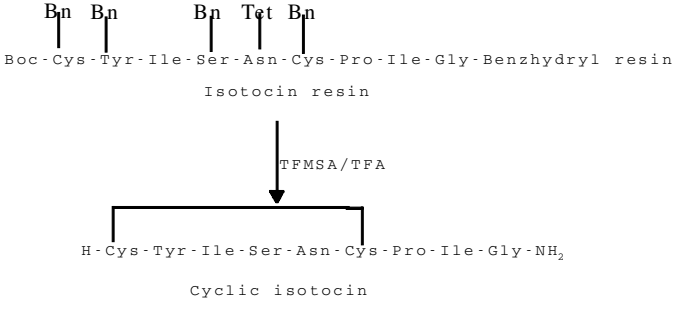
Amino acid analysis was done using Applied Biosystems Model 420A Derivatizer 8 coupled to an Applied Biosystems Model 130A Micro Separation System (Foster City, California, USA). 1-2 nmol of peptide was dissolved in 10 µL of methanol or acetonitrile-water (1:1 v/v). Hydrolysis was done using 6 N HCl at 170 °C for three hours. Sequencing of amino acids was performed using Applied Biosystems Model 476A and 477A (Foster City, California, USA). Samples were prepared by dissolving about 1 pmol of peptide in 15 µL of methanol. Single-letter notation is used for amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

Synthesis of glycinamide resin. In a 60-mL solid-phase reaction vessel, 5 g of the benzhydrylamine hydrochloride salt resin was washed three times each with 10% aqueous sodium carbonate, water-dioxane (3:1 v/v), methanol and methylene chloride. The resin was then suspended in 35 mL of methylene chloride and shaken for 10 min. After removal of the solvent, the resin was treated with 1.82 g (10.4 mmol) of *t*-butyl-oxycarbonylglycine in 8 mL of methylene chloride and 2.14 g (10.4 mmol) of DCC in 18 mL of the same solvent for 20 min at room temperature. After several washes with methylene chloride and ethanol, the coupling procedure was repeated twice more for 60 min each with half the quantities of *t*-butyloxycarbonylglycine and DCC in the same volume of methylene chloride. Then the resin was washed with three 35-mL portions each of methylene chloride, ethanol and methylene chloride, and the unreacted amino groups were blocked by treatment with 8 g (72 mmol) of N-acetylimidazole in 60 mL of methylene chloride for 2 hours at 23 °C. The ninhydrin test for the presence of free amino groups indicated that there were no exposed amino groups. Weight of the resin was 5.408 g. The weight increase was 0.408 g giving a glycine substitution of 0.48 mmol/g of the resin.

Boc-Cys(Bn)-Tyr(Bn)-Ile-Ser(Bn)-Asn(Tet)-Cys(Bn)-Pro-Ile-Glycinamide resin (isotocin resin, R2). 2 g of the glycinamide resin was utilised for the preparation of this compound (Scheme 1). The

following cycles of deprotection, neutralisation and coupling were carried out for the introduction of each new residue in the peptide: (1) three washings with 18-mL portions of methylene chloride; (2) cleavage of the Boc group by treating with 18 mL of trifluoroacetic acid-methylene chloride-anisole (50:48:2 v/v) for 25 min at room temperature; (3) five washings with 18-mL portions of methylene chloride; (4) four washings with 18-mL portions of chloroform; (5) neutralisation with two 15-mL portions of triethylamine in chloroform (7:93 v/v) for 6 min at room temperature; (6) three washings with 18-mL portions of chloroform; (7) four washings with 18-mL portions of methylene chloride; (8) addition of 0.46 g (2 mmol) of Boc-Ile-OH in 9 mL of methylene chloride and 5 min of mixing; (9) addition of 0.41 g (2 mmol) of DCC in 9 mL of methylene chloride followed by a reaction period of 90 min at room temperature; (10) three washings with 18-mL portions of methylene chloride; (11) three washings with 18-mL portions of ethanol; (12) three washings with 18-mL portions of methylene chloride; (13) repetition of steps 8 and 9 but using 1 mmol of Boc-Ile-OH and 1 mmol of DCC, respectively; (14) three washings with 18-mL portions of methylene chloride; (15) three washings with 18-mL portions of ethanol. Unless otherwise specified, each washing and mixing step lasted for 2 min. The ninhydrin test was run to monitor the coupling steps (sample was run just before step 2). Yield: 3.22 g (96.69%, based on the glycinamide substitution on the resin); IR (KBr, cm^{-1}) 3308 (NH), 1678 (broad, CO).

Isotocin resin cleavage at room temperature for two hours (P2). To 250 mg of the dried resin was added 750 µL of thioanisole-1,2-ethanedithiol (2:1 v/v). 5 mL of trifluoroacetic acid (TFA) was then added and the mixture stirred at room temperature for 10 min. This mixture was then cooled in ice-cold water and 500 µL of trifluoromethanesulphonic acid (TFMSA) was added slowly with vigorous stirring to dissipate the heat generated. Stirring was then done at room temperature for two hours. The mixture was then filtered using a sintered glass funnel. The peptide was then precipitated by adding 60 mL of diethyl ether and ether removed by decantation. The procedure was then repeated twice. TFA (5 mL) was then added to dissolve the peptide. Diethyl ether (60 mL) was added to precipitate the peptide and decanted. This was repeated five more times for the complete removal of the scavengers. The peptide was then dried under vacuum to give 54.71 mg (78.53%) of crude peptide P2.



Bn = benzyl; Tet = tetralinyl; TFMSA = trifluoromethanesulphonic acid; TFA = trifluoroacetic acid

Scheme 1. Synthetic scheme for the one-pot cleavage, deprotection and disulphide bond formation of isotocin.

Isotocin resin cleavage at 40°C for half an hour (PP2). The resin was cleaved as already done for the preparation of P2, but the cleavage was done at 40 °C for half an hour to give 59.86 mg (85.92%) of crude peptide PP2.

Isotocin resin cleavage at 40°C for two hours (PPP2). The resin was cleaved as done in the preparation of P2, but the cleavage was done at 40 °C for two hours to give 66.52 mg (95.48%) of crude peptide PPP2.

RESULTS AND DISCUSSION

Isotocin-resin (R2). The nonapeptide was synthesised by SPPS via the Boc-strategy from 2 g of Boc-glycinamide resin. The side chains of cysteine, tyrosine and serine were protected with benzyl while that of asparagine was protected with tetralinyl group. The weight of resin was 3.22 g (97%, based on the first amino acid anchored on the resin) (Table 1). IR (cm^{-1}) gave peaks at 3308 (N-H) and a broad peak centred at 1678 (-CO-). No peaks due to cyano by-product were found, indicating effective side-chain protection of asparagine.

Table 1. SPPS of isotocin resin (R2).

AA-residue	MM-Boc-AA	MM-AA residue	Wt increase (g)	Peptide resin wt (g)	Substitution mmol/g
Gly	175.17	157.15	0.1509	2	0.48
Ile	231.28	113.28	0.1087	2.1087	0.455
Pro	215.23	97.23	0.0933	2.202	0.436
Cys	311.36	193.36	0.1856	2.3876	0.402
Asn	362.43	244.43	0.2347	2.6223	0.366
Ser	295.29	177.29	0.1702	2.7925	0.344
Ile	231.28	113.28	0.1087	2.9012	0.331
Tyr	371.39	253.39	0.2433	3.1445	0.305
Cys	311.36	193.36	0.1856	3.3301	0.288

Key: AA= amino acid; MM= molecular mass.

Isotocin resin cleavage at room temperature for two hours. Isotocin resin was cleaved at room temperature for two hours to give 54.71 mg (78.53%) of crude isotocin (P2). Two peptides were revealed by ESMS. One had all protecting groups removed except one of the benzyls on Cys (*m/z* 1059). The other had all groups removed except benzyl (Cys) (*m/z* 1149) (Table 2). No cyclic isotocin was formed. This showed that all protecting groups were deprotected except benzyl (Cys), which was partially removed. Benzyl group was partially stable, while tetralinyl group was unstable under the given condition. G is a glycinamide residue. IR (cm^{-1}) gave peaks at 3296.9 (N-H), 3086.1 (Ar-H), 1667.9 (-CO-).

Table 2. ESMS analysis of crude isotocin (P2).

Peptide derivative	Calculated *m/z* (MH^+)	Experimental *m/z* (MH^+)	% Intensity
C(Bn)-Y-I-S-N-C-P-I-G or C-Y-I-S-N-C(Bn)-P-I-G	1058.52	1059	44.7
C(Bn)-Y-I-S-N-C(Bn)-P-I-G	1148.61	1149	100

Isotocin resin cleavage at 40°C for half an hour. This was cleaved at 40 °C for half an hour and gave 59.86 mg (85.92%) of crude isotocin (PP2). Two peptides were revealed by ESMS. One had all protecting groups removed except one of the benzyls on Cys (*m/z* 1059). The other had all groups removed except benzyl (*m/z* 1149.5) (Table 3). Tetralinyl (Asn) was completely deprotected, while benzyl (Cys) was partially removed. Under this condition, benzyl was partially stable while tetralinyl group was unstable. No cyclic isotocin was formed. G is a glycinamide residue. IR (cm^{-1}) of the product gave peaks at 3328.6 (N-H), 3096.6 (Ar-H), and 1667.9 (-CO-).

Table 3. ESMS analysis of crude isotocin (PP2).

Peptide derivative	Calculated *m/z* (MH^+)	Experimental *m/z* (MH^+)	% Intensity
C-Y-I-S-N-C(Bn)-P-I-G or C(Bn)-Y-I-S-N-C-P-I-G	1058.52	1059.0	100
C(Bn)-Y-I-S-N-C(Bn)-P-I-G	1148.61	1149.5	100

Isotocin resin cleavage at 40°C for two hours. This was done at 40 °C for two hours to give 66.52 mg (95.48%) of crude isotocin (PPP2). ESMS showed the presence of three peptides. The first had all protecting groups removed and free mercapto groups oxidised to cyclic isotocin between the first and the sixth amino acids (*m/z* 966.5). The second had all protecting groups removed except one of the benzyls on Cys (*m/z* 1058.5). The last had all protecting groups removed except benzyl (Cys) (*m/z* 1148.5) (Table 4). At the given reaction conditions, all protecting groups were removed except benzyl (Cys), which cleaved partially. This showed that benzyl was partially stable while tetralinyl (Asn) group was unstable. G is a glycinamide residue. IR (cm^{-1}) gave peaks at 3391.8 (N-H), 3096.6 (Ar-H), 1673.1 (-CO-).

Table 4. ESMS analysis of crude isotocin (PPP2).

Peptide derivative	Calculated *m/z* (MH^+)	Experimental *m/z* (MH^+)	% Intensity
C-Y-I-S-N-C-P-I-G	966.41	966.5	100
C-Y-I-S-N-C(Bn)-P-I-G or C(Bn)-Y-I-S-N-C-P-I-G	1058.52	1058.5	53
C(Bn)-Y-I-S-N-C(Bn)-P-I-G	1148.61	1148.5	10

In cases where benzyl group protection on cysteine is resistant to cleavage, incorporating electron-donating group(s) on the aromatic ring will make the removal more facile. Electron-donating group(s) on the aromatic ring of tetralinyl group will have the same effect. This is more enhanced if the electron-donating group is on position 6 or 8 of the tetralinyl group. Comparing the three reaction conditions, the one done at 40 °C for two hours gave the best results with the target peptide formed in good yield.

Amino acid analysis of the peptide had a substantial amount of tyrosine residue destroyed during hydrolysis. This could have been due to the elevated temperature employed during automated hydrolysis. This was done at 160-170 °C for three hours. This destruction of tyrosine could have been reduced if the duration of hydrolysis was reduced.

HPLC analysis of crude isotocin (PPP2). Analytical HPLC gave major peaks with retention times (min) of 13.75, 18.07 and 27.95 (Table 5). 10 mg of the crude peptide was used in the separation of the pure peptide by semipreparative HPLC. Detection was done at 214 nm.

Table 5. Semipreparative HPLC analysis of crude isotocin (PPP2).

Fraction 11	ESMS *m/z* (MH^+)	Retention time (min)
11	966.5	13.75
20	1058.0	18.07
26	1148.5	21.62

Fraction 11 with the correct peptide was pooled and lyophilised to provide a white powder (6.1 mg, 6.3 µmol, 61% isolated yield). Hydrolysis and amino acid analysis of the peptide showed the expected ratios: Asp 0.89 (1); Ser 0.88 (1); Gly 1.04 (1); Pro 1.00 (1); Tyr 0.34 (1); Cys 2.19 (2); Ile 1.95 (2). The results of sequence analysis as given in Table 6 showed the expected results.

Table 6. Sequence analysis of isotocin (Edman method).

Residue	Abbreviation	Amino acid
1	-	-
2	Y	Tyr
3	I	Ile
4	S	Ser
5	N	Asn
6	-	-
7	P	Pro
8	I	Ile
9	G	Gly

Sequence analysis of residues 1 and 6 were not determined due to formation of cystine derivative.

CONCLUSION

This study has demonstrated the usefulness of 1-tetralinyl group in protecting the amide side-chain of asparagine in Boc solid-phase peptide synthesis. With the 1-tetralinyl group, the results obtained show that isotocin synthesised on a solid support, can be successfully deprotected and cleaved from the resin at 40 °C for two hours with consecutive disulphide bond formation in a one-pot reaction using TFMSA-thioanisole-1,2-ethanedithiol-TFA (2:2:1:20 v/v).

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