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1-Tetralinyl as Carboxamide-Protecting Group for Asparagine and Application to N-α- t-Butyloxycarbonyl (Boc) Solid-phase Peptide Synthesis of Oxytocin

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Abstract

Oxytocin, a nonapeptide amide, was synthesized on a benzhydryl-resin using the Boc strategy. Benzyl group was used in the protection of sulfhydryl group of cysteine and tyrosine side-chain. Benzhydryl and tetralinyl groups were used in the protection of glutamine and asparagine side-chains respectively. TFMSA-TFA-thioanisole-1,2-ethanedithiol (2:20:2:1 v/v) was used on the peptide-resin under different cleavage conditions to obtain oxytocin in a one-pot reaction. The cleavage at 40°C for two hours gave oxytocin quantitatively. Oxytocin could be isolated in 56% yield.

Keywords: Boc-strategy, Solid-phase peptide Synthesis, nonapeptide, resin, protecting group

1. Introduction

Oxytocin is a nonapeptide human neurohyphysial hormone which is secreted by the posterior pituitary gland (Bodansky *et al.* 1953). Its role is in sexual reproduction during and after birth of a child. It causes contraction and expansion of the uterus during baby delivery and thereafter causes milk production. It is also thought to be involved in sexual arousal (Blaicher *et al.* 1999; Kosfeld *et al.* 2005).

A good carboxamide-protecting group should be stable in reagents like trifluoroacetic acid used for deprotecting α-amino-protecting groups, but readily removed by strong deprotecting reagents that are used for complete removal of most of the protecting groups. These include hydrogen fluoride (Stewart & Young 1969; Hruby *et al.* 1972), boron tristrifluoroacetate (Pless & Bauer 1973; Gitu 1974) and trifluoromethane sulphonic acid (*Yajima et al.* 1974). Some of the carboxamide-protecting groups that have been studied includes xanthenyl (Han *et al.* 1996), 2,4,6-trimethoxybenzyl (Pietta & Marshall 1970; Weygand *et al.* 1968), 4,4'-dimethoxybenzhydryl (Koenig & Geiger 1970).

Asparagine and glutamine derivatives can be incorporated without side-chain protection. However, they are known to undergo several side reactions of which the most important is nitrile formation via dehydration of the carboxamide side-chain during acylation (Katsoyanis *et al.* 1958). This side reaction has been reported to occur with a variety of activating agents, including carbodiimides (Katsoyanis *et al.* 1958; Kashelikar & Ressler 1964; Ressler & Ratzkin 1961) and o-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU). This side reaction can be minimised by the addition of 1-hydroxybenzotriazole (HOBT) to the coupling reaction or acylation with active esters (Mojsov *et al.* 1980; Bodansky & du Vigneaud 1959).

We recently studied other protecting groups which includes 1,2,3,4-tetrahydro-1-naphthyl, 7-methoxy-1,2,3,4-tetrahydro-1-naphthyl and 5,7-dimethyl-1,2,3,4-tetrahydro-1-naphthyl (Yusuf 1989).

In this research, we were encouraged to examine whether 1-tetralinyl group is viable as a protecting group in the solid phase peptide synthesis (Merrifield 1963, 1965). A one pot reaction has been developed for disulfide-containing bonds, which simultaneously cleave, deprotect and oxidize the cysteine (Spetzler & Meldal 1966; Hope *et al.* 1962; Otaka *et al.* 1991; Tam *et al.* 1991). Biological and conformational stability in peptides is achieved by the formation of disulfide bonds. In order to do this, cystine-containing peptides have also been obtained after treatment of the protected peptidyl resin with TFA in the presence of iodine. Other oxidants include air, potassium ferricyanide (Hope *et al.* 1962), iodine and dimethyl sulfoxide (Spetzler & Meldal 1966; Hope *et al.* 1962; Otaka *et al.* 1991; Tam *et al.* 1991).

Leuckart reaction (Crossley & Moore 1944) was used in the synthesis of benzhydryl and tetralinyl amines, which were then used as precursors in the synthesis of carboxamide protected derivatives.

2. Materials and Methods

2.1 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). 1-Tetralone and benzophenone were purchased from Aldrich (Milwaukee, USA). All solvents were of analytical grade or of equivalent purity, and used 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; carbobenzoxy (Cbz) for lysine; tosyl (Tos) for arginine; benzhydryl (Bzh) for glutamine and tetralinyl (Tet) for asparagine. Boc removal was achieved with TFA/dichloromethane/anisole (50:48:2 v/v) for 25 minutes 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). Thin-layer chromatography (TLC) was performed on Macherey-Nagel (Duren, Germany) silica gel precoated plates with fluorescent indicator (0.25mm, 40 × 80mm), developed with chloroform-ethyl acetate (3:1 v/v) (CE). The compounds were observed by both iodine and ultraviolet (UV) lamp.

^{1}H-NMR (δ) spectra were recorded at 250 MHz on a Bruker A250 and ^{13}C-NMR (δ) spectra were recorded at 69 MHz on the same instrument. IR (cm^{-1}) spectra were recorded on a Perkin Elmer 1600 series (FTIR). Mass spectra were recorded on a TSQ 70 and MAT 711A (FINNIGAN MAT). 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 1mL 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, 250X2mm) 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 (1mg/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 minutes), detection at 214 and 280 nm. Semipreparative HPLC was performed using a Grom semi-preparative nucleosil C-18 reversed-phase column (7 µm, 250X8mm) 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 minutes), 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 6N 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.

2.2 N-1,2,3,4-Tetrahydro-1-naphthylformamide (1)

To a three-necked flask equipped with a dropping funnel, thermometer and down-directed condenser, was added with care 6.99 g (410.4 mmol) of 25% ammonia and 18.9 g (410.4 mmol) of 90% formic acid. The temperature of the solution was raised to 160°C by distilling out water and 12 g (82.1 mmol) of 1-tetralone was added at once. The temperature was maintained at 170-180°C (oil-bath temperature was 180°C) for three hours and any ketone which distilled was returned to the flask at intervals. On cooling, a brown mass was formed. Yield: 9.3 g (64.67%); mp 80-81°C; R_f (CE) 0.45; IR (KBr) 3275 (NH), 3062 (Ar-H), 1656 (CO),1538 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ1.75-2.12 (m, 4H, methylene protons of C-2,C-3), 2.68-2.88 (m, 2H, C-4), 5.20-5.28 (q, 1H, C-1 methine), 5.82-6.05 (broad peak,1H, NH), 7.06-7.27 (m, 4H, Ar-H), 8.14-8.17 (s, 1H, CHO); ^{13}C-NMR ($CDCl_3$) δ20.07 (C-3), 29.1 (C-4), 30.18 (C-2), 46.26 (C-1), 126.34-129.39 (Ar carbons), 136.11-137.62 (bridging Cs), 160.47 (CO); MS (m/z) 176.5 (MH^+, 2.3), 129.7 (M-$HNHCHO^+$, 100), 90.7 ($PhCH^+$, 10), 77 (Ph^+, 8.85).

2.3 1,2,3,4-Tetrahydro-1-naphthylamine (2)

2 g (11.41 mmol) of 1,2,3,4-Tetrahydro-1-naphthylformamide was hydrolysed with 25 ml of 10% sodium hydroxide solution under reflux at 140°C for three hours. After cooling to room temperature, the reaction mixture was extracted

with several portions of dichloromethane. The combined dichloromethane extract was shaken with concentrated hydrochloric acid solution. Unreacted ketone may be recovered from dichloromethane layer. The amine was regenerated from the acid layer by the addition of 45% sodium hydroxide solution and then extracted with dichloromethane. The dichloromethane solution of the amine was dried over sodium sulphate and filtered. Dichloromethane was removed by distillation under reduced pressure to give the product. Yield: 1.26 g (75%) ; R_f (CE) 0.11; b.p. 244-247°C/714 mm; IR (Neat) 3381,3308 (d, NH_2), 3054 (Ar-H), 2938-2875 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ1.64-2.14 (m, 6H, CH_2), 2.7-2.92 (m, 2H, NH_2), 3.98-4.03 (t, 1H, methine), 7.09-7.21 (m, 4H, Ar-H); ^{13}C-NMR ($CDCl_3$) δ19.17 (C-3), 29.13 (C-4), 32.71 (C-2), 48.96 (C-1), 125.66-128.63 (Ar-C), 136.36, 139.99 (bridging Cs); MS (m/z) 146 (M-H^+, 31.34), 131.2 (M-2H-N^+, 23.88), 129.6 (M-3H-N^+, 100), 118.3 ($PhCH_2CH_2CH_2^+$, 67.16), 103.9 ($PhCH_2CH_2^+$, 8.96), 92.2 ($PhCH_3^+$, 14.93).

2.4 α-Benzyl t-butyloxycarbonyl-N^{CA}-diphenylmethylglutaminate

A mixture of 5.06 g (15 mmol) of α-benzyl t-butyloxycarbonylglutamate and 2.6 g (22.5 mmol) of N-hydroxysuccinimide in 30 ml of methylene chloride was cooled to -5°C with stirring. A solution of 3.58 g (17.4 mmol) of DCC in 14 ml of methylene chloride was added, and the mixture was stirred at -5° C for 50 minutes. A solution of 3.02 g (16.5 mmol) of benzhydrylamine was added and the mixture stirred at -5 C for an additional 50 minutes and at room temperature for 24 hours. Acetic acid (1 ml) was added, the mixture was stirred for 15 minutes and the DCHU was filtered off and washed with three 30-ml portions of methylene chloride. The solvents were removed in vacuo on a rotary evaporator and the residue was dissolved in 20 ml of methylene chloride. The insoluble material was filtered off and washed with two 5-ml portions of methylene chloride. 60 ml of chloroform was added to the filtrate and the solution was washed with three 90-ml portions of 5% citric acid, two 120-ml portions of 5% aqueous sodium bicarbonate and three 180-ml portions of deionised water. The organic layer was dried over anhydrous sodium sulphate, and the solvents were removed in vacuo on a rotary evaporator. The semi-solid was dissolved in 60 ml of hot ethyl acetate, cooled to room temperature and filtered off. To the filtrate, a solution of 240 ml of petroleum spirit (40-60°C) was added dropwise, and the mixture kept at 0-5°C overnight. The precipitate was filtered, washed with three 45-ml portions of petroleum spirit (40-60°C)-ethyl acetate (4:1 v/v) and dried in vacuo to give the product. Yield: 5.07 g (67.24%); mp 135-136° C; R_f (CE) 0.80; IR (KBr) 3350 (NH), 3063 (Ar-H), 1756 (COO), 1688, 1644 (CONH), 1525 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ1.41 (s, 9H, CH_3), 1.82-1.96 (m, 2H, Gln methylene), 2.19-2.31 (m, 2H, Gln methylene), 4.31 (m, 1H, Gln methine), 5.07-5.19 (q, 2H, $PhCH_2$), 5.34-5.37 (broad d, 1H, NH next to Boc), 6.19-6.22 (d, 1H, Ph_2CH), 6.82-6.85 (broad d, 1H, $NHCHPh_2$), 7.21-7.34 (m, 15H, Ar-H); ^{13}C-NMR ($CDCl_3$) δ28.33 ($(CH_3)_3C$), 29.07 (CCH_2C), 32.51 (CCH_2CO), 53.13 (Gln methine), 57.15 (Ph_2CH), 67.23 ($PhCH_2$), 80.24 ($(CH_3)_3C$), 127.43-128.65 (Ar-C), 135.35 (bridging C of Bzl), 141.61 (aromatic bridging C of Bzh), 155.89 (OCONH), 172.16 ($CCOOCH_2$), 170.91 (CCONH); MS (m/z) 402.3 (M-Boc+H^+, 10.45), 182.2 (Ph_2CHNH^+, 100), 167.1 (Ph_2CH^+, 64.55), 90.9 ($PhCH_2^+$, 29.10), 77.1 (Ph^+, 11.94), 56.9 (Boc^+, 72.01).

2.5 α-Benzyl t-butyloxycarbonyl-N^{CA}-1,2,3,4-tetrahydro-1-naphthylasparaginate This compound was prepared from 4.85 g (15 mmol) of α-benzyl t-butyl-oxycarbonylaspartate and 2.4 g (16.5 mmol) of 1,2,3,4-tetrahydro-1-naphthylamine in the same manner as described for the preparation of 5:Yield: 4.43 g (65.24%); m.p. 121-122°C; R_f (CE) 0.83; IR (KBr) 3325 (NH), 3050 (Ar-H), 1750 (COO), 1694, 1644 (CON), 1538 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ1.41 (s, 9H, CH_3), 1.66-2.01 (m, 4H, C-2 and C-3 tetralinyl methylenes), 2.66-2.92 (m, 4H, methylenes of Asn and C-4 of tetralinyl), 4.94-4.57 (m, 1H, Asn methine), 5.12-5.24 (m, 3H, OCH_2Ph and tetralinyl methine), 5.85-5.94 (broad peak, 2H, amide protons), 7.02-7.22 (m, 4H, Ar-H of tetralinyl), 7.30-7.36 (s, 5H, CH_2Ph); ^{13}C-NMR ($CDCl_3$) δ28.33 (CH_3), 47.61, 30.12, 19.96, 29.20 (C-1 to C-4 of tetralinyl respectively), 38.13 (CCH_2CO), 50.82 (NCHCO), 67.36 (OCH_2Ph), 79.99 ($(CH_3)_3C$)), 126.32-129.19 (Ar-C), 137.57-136.31 (C-9, C-10 of tetralinyl), 135.52 (Ph bridging Cs), 155.66 (OCONH), 171.37 (CCO_2C), 168.99 (CCON); MS (m/z) 396 (M-Bu^{t+}, 19.62), 352.1 (M-Boc+H^+, 25), 146.1 (tetralinylamine-H^+, 100), 130.4 (tetralinylamine-NH_2^+, 80.77), 91.1 ($PhCH_2^+$, 67.69), 57 (Bu^{t+}, 59.23)

2.6 N-tert-Butyloxycarbonyl-N^{CA}-diphenylmethyl-L-glutamine

α -Benzyl t-butyloxycarbonyl-N^{CA}-benzhydrylglutaminate (1 g, 1.99 mmol) in methylene chloride (20 ml) was hydrogenated at room temperature and pressure over 5% palladium on charcoal (0.2 g) for 8 hours. The reaction was followed by TLC using solvent system CE. After the end of reaction, the catalyst was removed by filtration and the filtrate evaporated on a rotary evaporator in vacuo. The residue was dissolved in ethyl acetate. Petroleum spirit (40-60°C) was then added and crystallization ensued. The contents were left in the freezer overnight. Precipitate was filtered and dried, yielding the product. Yield: 0.63 g (76.8%); m.p. 136-137°C; R_f (CE) 0.06; IR (KBr) 3363 (NH),

3050 (Ar-H), 1700 (COOH), 1631 (CONH), 3725-2375 (COOH), 1531 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ1.41 (s, 9H, CH_3), 2.03-2.36 (m, 4H, Gln methylenes), 4.13 (broad peak, 1H, Gln methine), 5.55 (broad peak, 1H, amide H next to Boc), 6.18 (broad peak, 1H, Bzh methine), 7.23 (broad s, 11H, Ar-H and amide H next to Bzh), 9.06 (broad peak, 1H, COOH); ^{13}C-NMR ($CDCl_3$) δ28.33 (CH_3), 29.14 (CCH_2C), 32.5 (CCH_2CO), 52.92 (NCHCO), 57.34 (Ph_2CH), 80.38 (($CH_3)_3C$)), 127.46-128.67 (Ar-C), 141.3 (Bzh Ar bridging C), 156.18 (OCON), 172.52 (CCON), 174.16 (COOH); MS (m/z) 412.4 (M^+, 5.38), 311.6 (M-Boc$^+$, 50.77), 182.3 (Ph_2CHNH^+, 77.69), 167.4 (Ph_2CH^+, 29.23), 77 (Ph^+, 76.15).

2.7 N-tert-Butyloxycarbonyl-N^{CA}-1,2,3,4-tetrahydro-1-naphthyl-L-asparagine

α -Benzyl t-butyloxycarbonyl-N^{CA}-1,2,3,4-tetrahydro-1-naphthylasparaginate (0.9 g, 1.99 mmol) in methylene chloride was hydrogenated at room temperature and pressure over 5% palladium on charcoal (0.2 g) for 8 hours. After removal of the catalyst, solution was evaporated and the residue recrystallized from ethyl acetate-petroleum spirit (40-60°C). The precipitate was filtered and dried, yielding the product: Yield: 0.45 g(62.5%); m.p. 146-147°C; R_f (CE) 0.10; IR (KBr) 3325 (NH), 3025 (Ar-H), 3700-2338 (OH), 1713 (COOH), 1638 (CONH), 1538 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ1.38 (s, 9H, CH_3), 1.82-2.03 (m, 4H, tetralinyl methylenes of C-2, C-3), 2.62-2.87 (m, 4H, methylenes of Asn and C-4 of tetralinyl), 4.07-4.23 (broad peak, 1H, Asn methine), 5.09-5.14 (broad t, 1H, tetralinyl methine), 5.85-5.87 (broad t, 1H, amide H next to tetralinyl), 6.92-6.95 (broad d, 1H, amide H next to Boc), 7.06-7.22 (m, 4H, Ar-H), 9.80 (broad peak, 1H, COOH); ^{13}C-NMR ($CDCl_3$) δ28.31 (CH_3), 48.3, 29.8, 19.96, 29.01 (C-1 to C-4 of tetralinyl), 37.86 (CCCO), 50.18 (Asn methine), 80.44 (($CH_3)_3C$)), 126.32-129.27 (Ar-C), 135.5, 137.56 (tetralinyl C-10, C-9), 155.6 (OCONH), 170.88 (CCONH), 172.56 (COOH); MS (m/z) 362.4 (M^+, 6.92), 305.5 (M-Bu^{t+}, 46.14), 262.5 (M-Boc+H^+, 20.77), 130.2 (tetralinyl-H^+, 100), 90.9 ($PhCH_2^+$, 62.31).

2.8 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 minutes. After removal of the solvent, the resin was treated with 1.82 g (10.4 mmol) of t-butyloxycarbonylglycine in 8 ml of methylene chloride and 2.14 g (10.4 mmol) of DCC in 18 ml of the same solvent for 20 minutes at room temperature. After several washes with methylene chloride and ethanol, the coupling procedure was repeated twice more for 60 minutes 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-acetyl-imidazole 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.

2.9 Boc-Cys(Bzl)-Tyr(Bzl)-Ile-Gln(Bzh)-Asn(Tet)-Cys(Bzl)-Pro-Leu-Glycinamide resin (Oxytocin resin, R1)

2 g of the glycinamide resin was utilized for the preparation of this compound. The following cycles of deprotection, neutralization 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 minutes at room temperature; (3) five washings with 18-ml portions of methylene chloride; (4) four washings with 18-ml portions of chloroform; (5) neutralization with two 15-ml portions of triethylamine in chloroform (7:93 v/v) for 6 minutes 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-Leu-OH in 9 ml of methylene chloride and 5 minutes 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 minutes 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-Leu-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 minutes. The ninhydrin test was run to monitor the coupling steps (sample was run just before step 2). Yield: 1.47 g (42.7%, based on the glycinamide substitution on the resin); IR (KBr) 3308 (NH), 1678 cm^{-1} (broad, CO).

2.9.1 Oxytocin resin cleavage at room temperature for two hours (P1)

To 250 mg of the dried resin was added 750 µL of thioanisole-1,2-ethanedithiol (2:1 v/v). 5 mL of TFA was then added and the mixture stirred at room temperature for about 10 minutes. 500 µL of trifluoromethanesulfonic acid (TFMSA) was then added slowly to the chilled mixture 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 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 59 mg (84.09%) of the crude peptide P1.

2.9.2 Oxytocin resin cleavage at 40°C for half an hour (PP1)

This resin was cleaved as already done in the preparation of P1 but the cleavage was done at 40°C for half an hour to give 61.42 mg (87.54%) of crude peptide PP1.

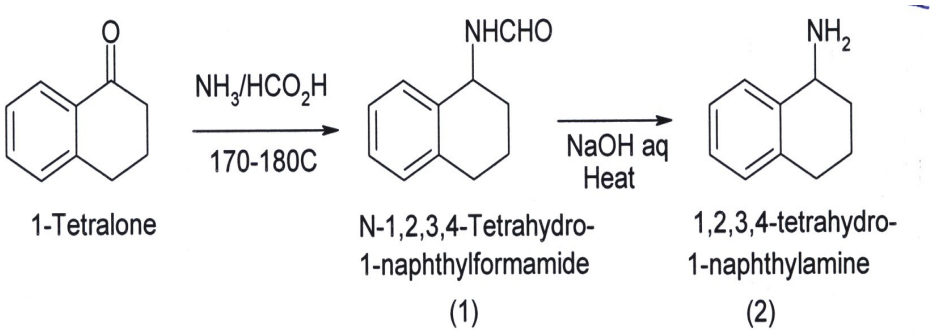
2.9.3 Oxytocin resin cleavage at 40°C for two hours (PPP1)

The resin was cleaved as outlined in the preparation of P1 but the cleavage was done at 40°C for two hours to give 67.80 mg (96.64%) of crude peptide PPP1.

3. Results and Discussion

*3.1 N-1,2,3,4-Tetrahydro-1-naphthylformamide (**1**)*

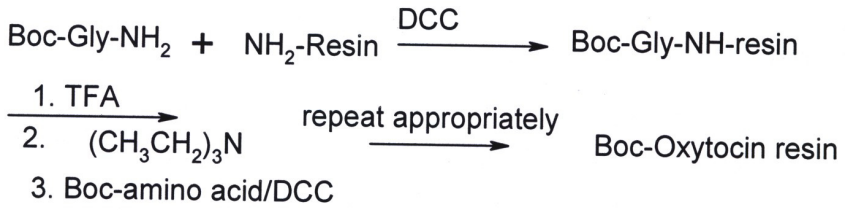
This was prepared by reacting 1-tetralone with ammonia and formic acid at elevated temperature. This was then refluxed with base to form the amine **2**, 1,2,3,4-tetrahydro-1-naphthylamine. This was then used as a precursor in the protection of asparagine side-chain. The synthetic route is given in Scheme 1.



Scheme 1: Synthesis of 1,2,3,4-tetrahydro-1-naphthylamine

3.2 Oxytocin-resin (R1)

The nonapeptide was synthesized by SPPS via the Boc strategy from 2 g of Boc-glycinamide resin. The side-chains of cysteine and tyrosine were protected with benzyl while those of asparagine and glutamine were protected with tetralinyl and benzhydryl groups respectively. Oxytocin-resin weight was 1.47 g (43%, based on first amino acid on the resin). The synthesis of oxytocin resin is shown in scheme 2.



Scheme 2: Synthesis of protected Boc-oxytocin resin

Table 1 shows the masses of amino acids, weight increases of amino acids and peptide resin and the substitution on the resin.

Table 1: SPPS of oxytocin resin (R1)

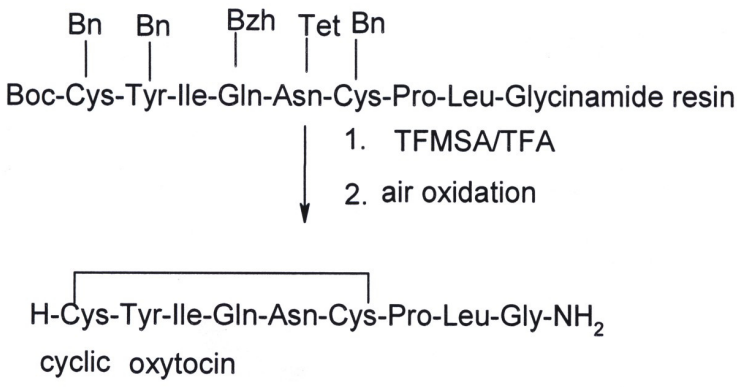
AA-Residue	MM-Boc-AA	MM-AA residue	Wt increase(g)	Peptide resin wt(g)	Subs mmol/g
Gly	175.17	157.15	0.1509	2	0.48
Leu	231.28	113.28	0.1087	2.1087	0.455
Pro	215.23	97.23	0.0933	2.202	0.436
Cys	311.36	193.36	0.1856	2.3876	0.402
Asn	362.43	244.43	0.2347	2.6223	0.366
Gln	412.49	294.49	0.2827	2.905	0.330
Ile	231.28	113.28	0.1087	3.0137	0.318
Tyr	371.39	253.39	0.2433	3.257	0.295
Cys	311.36	193.36	0.1856	3.4426	0.279

KEY: Subs= substitution; AA= amino acid; MM= molecular mass; SPPS=solid phase peptide synthesis

During the incorporation of the first amino acid on the resin, it loses water to give a residue whose molecular mass (MM) is less by 18. In the subsequent coupling of the other amino acids, there will be loss of Boc (MM 101) and OH (MM 17) to give a residue that has molecular mass that is less by 118. IR (cm^{-1}) gave peaks at 3297 (N-H), and a broad peak centred at 1652 (-CO-). There was no peak at about 2200 which showed the absence of cyano functional group. This was a clear indication that tetralinyl group used in the protection of asparagine side-chain was effective in preventing dehydration at this centre by DCC.

3.3 Deprotection and cleavage of peptide from the resin

The fully protected peptides were deprotected and cleaved from the resin by a cleavage cocktail of TFMSA/TFA/thioanisole/EDT (2:20:2:1 v/v). These were done at room temperature for two hours, 40°C for half an hour and 40°C for two hours (Scheme 3).



Bz= Benzyl; Bzh= Benzhydryl; Tet= Tetralinyl; TFMSA= Trifluoromethanesuolfonic acid; TFA= Trifluoroacetic acid

Scheme 3: Synthetic scheme for the one-pot cleavage, deprotection and disulfide bond formation of oxytocin

3.3.1 Oxytocin resin cleavage at room temperature for two hours

The data is shown in Table 2.

Table 2: ESMS analysis of crude oxytocin (P1)

Peptide derivative	Calc m/z MH^+	Expe m/z MH^+	% inten
C(Bz)-Y-I-Q-N-C(Bz)-P-L-G	1189.61	1190	21.3
C(Bz)-Y-I-Q-N(Tet)-C(Bz)-P-L-G	1319.7	1320	21.3
C(Bz)-Y-I-Q(Bzh)-N-C(Bz)-P-L-G	1355.7	1356	100
C(Bz)-Y-I-Q(Bzh)-N(Tet)-C(Bz)-P-L-G	1485.79	1486	12.8

KEY: Calc= calculated; Expe= experimental; Inten= intensity

Oxytocin resin was cleaved at room temperature for two hours to give 59 mg (84.09%) of crude oxytocin (P1). Weight of the peptide was 59 mg (84.09%). ESMS revealed the presence of four peptides. The first had all protecting groups removed except benzyl (Cys) (m/z 1190). The second had all groups removed except benzyl (Cys) and tetralinyl (Asn) (m/z 1320). The third had all protecting groups removed except benzyl (Cys) and benzhydryl (Gln) (m/z 1356). The last peptide had all protecting groups deprotected except benzyl (Cys), tetralinyl (Asn) and benzhydryl (Gln) (m/z 1486). No cyclic oxytocin was produced. Benzyl (Cys) was not removed while tetralinyl(Asn) and benzhydryl (Gln) were partially deprotected. Under the given condition, benzyl (Cys) was stable, while tetralinyl (Asn) and benzhydryl (Gln) groups were partially stable. G is a glycinamide residue. Peak corresponding to MH^{2+} appeared at m/z 678, 56.52% (MH^+ 1356). IR (cm^{-1}) gave peaks at 3296.9 (N-H), 3086.1 (Ar-H), 1662.6 (-CO-).

3.3.2 Oxytocin resin cleavage at 40°C for half an hour

Oxytocin resin was cleaved at 40°C for half an hour to give 61.42 mg (87.54%) of crude oxytocin (PP1). This is shown in Table 3.

Table 3: ESMS analysis of crude oxytocin (PP1)

Peptide derivative	Calc. m/z MH^+	Experimental m/z MH^+	% Intensity
C-Y-I-Q-N-C-P-L-G Cyclization at C-C	1007.41	1007.5	11.5
C-Y-I-Q-N-C(Bz)-P-L-G	1099.52	1099.5	67.7
C(Bz)-Y-I-Q-N-C(Bz)-P-L-G	1189.61	1190	100
C-Y-I-Q(Bzh)-N-C(Bz)-P-L-G	1264.61	1266.0	24.6
C(Bz)-Y-I-Q(Bzh)-N-C(Bz)-P-L-G	1355.7	1356.5	27.7

ESMS revealed the presence of five peptides. The first had all protecting groups removed and the free mercapto groups oxidised to cyclic oxytocin between the first and the sixth amino acids (m/z 1007.5). The second had all groups removed except one of the benzyls on Cys (m/z 1099.5). The third peptide had all protecting groups removed except benzyl (Cys) (m/z 1190). The fourth peptide had all protecting groups removed except one of the benzyls on Cys and benzhydryl (Gln) (m/z 1266.0). The last peptide had all protecting groups deprotected except benzyl (Cys) and benzhydryl (Gln) (m/z 1356.5). Benzyl (Cys) and benzhydryl (Gln) groups were partially cleaved while tetralinyl (Asn) group was completely removed. Under the given condition, tetralinyl group was unstable, while benzyl and benzhydryl groups were partially stable. G is a glycinamide residue. IR (cm^{-1}) gave peaks at 3318 (N-H), 3086.1 (Ar-H), 1667.9 (-CO-).

3.3.3 Oxytocin resin cleavage at 40°C for two hours

Oxytocin resin was cleaved at 40°C for two hours to give 67.80 mg (96.64%) of crude oxytocin (PPP1) as given in Table 4.

Table 4: ESMS analysis of crude oxytocin (PPP1)

Peptide derivative	Calculated m/z MH^+	Experimental m/z MH^+	% Intensity
C-Y-I-Q-N-C-P-L-G Cyclization at C-C	1007.41	1007.5	100
C-Y-I-Q-N-C(Bz)-P-L-G	1099.52	1099.5	73.8

Two peptides were revealed by ESMS. The first had all protecting groups removed and the free mercapto groups oxidized to cyclic oxytocin between the first and the sixth amino acids (m/z 1007.5). The other had all protecting groups removed except one of the benzyls on Cys (m/z 1099.5). Under these conditions, tetralinyl (Asn) and benzhydryl (Gln) were completely deprotected. Benzyl (Cys) was partially cleaved. At this temperature, benzyl group was partially stable, while tetralinyl and benzhydryl groups were found to be unstable. G is a glycinamide residue. IR (cm^{-1}) gave peaks at 3381.3 (N-H), 3075.5 (Ar-H), 1678.4 (-CO-).

Analytical HPLC of crude peptide gave two major peaks with retention times (min) of 14.25, 21.72 and 29.78 at 214 nm. 10 mg of the crude peptide was used in the separation of the pure peptide by semi-preparative HPLC (Table 5). Detection was also done at 214 nm.

Table 5: Semipreparative HPLC of crude oxytocin (PPP1)

Fraction	ESMS (m/z) MH^+	Retention Time (min)
4	1007.5	14.25
11	1099.0	18.93
14	1189.5	21.72

Fraction 4 with the correct peptide was pooled and lyophilized to provide a white powder (5.8 mg, 5.7 µmol, 58% isolated yield). Hydrolysis and amino acid analysis of the peptide showed the expected ratios: Asp 1.17 (1); Glu 1.15 (1); Gly 1.08 (1); Pro 1.00 (1); Tyr 0.42 (1); Cys 2.42 (2); Ile 1.01 (1); Leu 0.77 (1). Sequence analysis gave the following results as given in Table 6.

Table 6: Sequence analysis of oxytocin (Edman method)

Residue no.	Abbreviation	Amino acid
1	-	-
2	Y	Tyr
3	I	Ile
4	Q	Gln
5	N	Asn
6	-	-
7	P	Pro
8	L	Leu
9	G	Gly

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

4. Conclusion

When cleavage was done at room temperature for two hours, benzyl group (Cys) was not removed at all in oxytocin. This protecting group was therefore stable as a protecting group for the side chain of cysteine. Tetralinyl and benzhydryl groups used for the protection of amide side-chains of asparagine and glutamine respectively were partially removed.

At 40°C for half an hour, benzyl protecting group (Cys) and benzhydryl group (Gln) were partially removed in. Tetralinyl group used in the protection of asparagine side chain was completely removed and cyclic derivative of oxytocin was also formed.

At 40°C for two hours, benzyl group (Cys) was partially removed. Benzhydryl (Gln) and tetralinyl (Asn) groups were completely removed. A large quantity of cyclic oxytocin was formed.

Electron-donating group(s) on the aromatic ring of tetralinyl group will make its removal more facile. This is more enhanced if the electron-donating group is on position 6 or 8 of the 1, 2, 3,4-Tetrahydro-1-naphthyl group.

Comparing the three reaction conditions, the one done at 40°C for two hours gave the best results with the target peptide (cyclic oxytocin) formed in good yield.

These studies have demonstrated the usefulness of 1-tetralinyl group in the side chain protection of asparagine and its use in the Boc solid-phase peptide synthesis of oxytocin. Oxytocin synthesized on a solid support, can be successfully deprotected and cleaved from the resin at 40°C for two hours with consecutive disulfide bond formation in a one-pot reaction using TFMSA-thioanisole-1,2-ethanedithiol-TFA (2:2:1:20 v/v).

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References

Blaiche, W., Gruber D., Biegelmayer C., Blaicher, A.M., Knogler, W. & Huber, J.C. (1999), "The role of oxytocin in relation to female sexual arousal", *Gynecologic and Obstetric Investigatio* **47**(2), 125-126.

Bodanszky, M., Meienhofer, J. & du Vigneaud, V. (1953), "The sequence of amino acids in oxytocin, with a proposal for its structure", *J. Biol. Chem.* **205**, 949-957.

Bodanszky, M. & du Vigneaud, V. (1959). "A method of synthesis of long peptide chains using a synthesis of oxytocin as an example", *J. Am. Chem. Soc.* **81,** 5688-5691.

Crossley, F.S. & Moore, M.L. (1944), "Studies on the Leuckart reaction", *J. Org. Chem.* **9**, 529-536.

Gitu, P.M. (1974)," Benzyl and naphthyl derivatives as potential protecting groups of the carboxamide side chain of asparagine and glutamine in peptide synthesis", *Ph.D. Dissertation*, The University of Arizona, Tucson.

Han, Y., Sole, N.A., Tejbrant, J. & Barany, G. (1996), "Novel N-xanthenyl-protecting groups for asparagine and glutamine, and applications to N-9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis", *Pept. Res*. **9**, 166-173.

Hope, D.B., Murti, V.V.S. & du Vigneaud, V. (1962), "A highly potent analogue of oxytocin, deamino-oxytocin", *J. Biol. Chem*. **237**, 1563-1566.

Hruby, V.J., Muscio, F.O., Brown, W. & Gitu, P.M. (1972), *Chemistry and Biology of Peptide*, J. Meienhofer (Ed.), Ann Arbor Science Publishers, Inc., Ann Arbor, p. 331-333.

Kashelikar, D.V. & Ressler, C. (1964), "An O-18 study of the dehydration of asparagine amide with DCC and p-toluenesulfonyl chloride", *J. Am. Chem. Soc.* **86**, 2467-2473.

Katsoyannis, P.G., Gish, D.T., Hess, G.P, & du Vigneaud, V. (1958)," Synthesis of two protected hexapeptides containing the N-terminal and C-terminal sequence of arginine-vasopressin", *J. Am. Chem. Soc*. **80**, 2558-2562.

Koenig, W. & Geiger, R. (1970), " Eine neue amid-schtzgruppe", *Chem. Ber*. **103**, 2041-2051.

Kosfeld M., Heinrichs M., Zak P.J. Fischbacher U. & Fehr, E. (2005), "Oxytocin increases trust in humans", *Nature* **435** (7042), 673-676

Merrifield, R.B. (1963), "The synthesis of a tetrapeptide", *J. Am. Chem. Soc.* **85**, 2149-2154.

Merrifield, R.B. (1965), "Solid-phase peptide synthesis, a simple and rapid synthetic method, has now been automated", *Science* **150**, 178-185.

Mojsov, S., Mitchell, A.R. & Merrifield, R.B. (1980), "A quantitative evaluation of methods for coupling asparagines", *J. Org. Chem*. **45**, 555-560.

Otaka, A., Koide, T., Shide, A. & Fujii, N. (1991), "Application of dimethyl sulfoxide (DMSO)/trifluoroacetic acid (TFA) oxidation to the synthesis of cystine-containing peptide", *Tett. Lett*. **32**, 1223-1226.

Pietta, P.G. & Marshall, G.R. (1970), "Amide protection and amide supports in solid-phase peptide synthesis", *J. Chem. Soc. Chem. Comm*. 650-651.

Pietta, P.G., Cavallo, P. & Marshall, G.R. (1971), "2,4-Dimethoxybenzyl as a protecting group for glutamine and asparagine in peptide synthesis", *J. Org. Chem.* **36**, 3966-3970.

Pless, J. & Bauer, W.(1973), "Boron tris(trifluoroacetate) zur abspaltung von schutzgruppen in der peptid chemie", *Angew. Chem.* **85**, 142.

Ressler, C. & Ratzkin, H. (1961), "Synthesis of ß-cyano-L-alanine and γ-cyano-L-aminobutyric acid, dehydration products of asparagine and glutamine, a new synthesis of amino acid nitriles". *J. Org. Chem.* **26**, 3356-3360.

Spetzler, J.C. & Meldal, M. (1996), "Evaluation of strategies for "one-pot" deprotection, cleavage and disulfide bond formation in the preparation of cystine-containing peptide", *Lett. in Pept. Sci*. **3**, 327-332.

Stewart, J.M. & Young, J.D. (1969), *Solid-phase peptide synthesis*, Freeman, San Francisco.

Tam, J.P., Wu, C-R., Liu, W. & Zhang, J.W. (1991), "Disulfide bond formation in peptide by dimethyl sulfoxide : Scope and applications", *J. Am. Chem. Soc*. **113**, 6657-6662.

Weygand, F., Steglich, W. & Bjarnason, J. (1968), "Derivate des asparagins und glutamins mit 2,4-dimethoxybenzyl und 2,4,6-trimethoxybenzyl-geschuetzten amidgruppen", *Chem. Ber*. **101**, 3642-3648.

Yajima, H., Fujii, N., Ogawa, H. & Kawatani, H. (1974), "Trifluoromethanesulfonic acid, as a deprotecting reagent in peptide chemistry", *J. Chem. Soc. Chem. Comm*. 107-108.

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