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RESEARCH ARTICLE

Protection of the Amide Side-Chain of Asparagine with the 1-Tetralinyl Group in the Solid-Phase Peptide Synthesis of Lysine-Vasopressin

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Abstract

Lysine-vasopressin, a nonapeptide, was synthesised on a benzhydryl-resin using solid-phase peptide synthesis via the Boc-strategy. The benzyl group was used in the protection of the side-chains of tyrosine and cysteine, while 1-tetralinyl, benzhydryl and benzyloxycarbonyl groups were used in the protection of the side-chains of asparagine, glutamine and lysine, respectively. Thioanisole–1,2-ethanedithiol–trifluoroacetic acid–trifluoromethanesulphonic acid (2:1:20:2 v/v) was used to cleave the peptide-resin under different temperature conditions. The cleavage at 40 °C for two hours gave lysine-vasopressin in a one-pot reaction; the yield after reversed-phase HPLC purification was 62%.

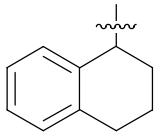
Keywords solid-phase peptide synthesis; cyclic peptide; electrospray mass spectrum; benzhydryl-resin; 1-tetralinyl, amide protection.

1. Introduction

Lysine-vasopressin is a hormone of the mammalian neurohypophysis.[1] This hormone is responsible for pressor and diuretic effects. It exerts a marked effect on the kidneys, accelerating the rate of water reabsorption. This leads to the excretion of

urine, which contains increased concentrations of sodium and chloride ions, total nitrogen and phosphates. Its secretion is augmented in circumstances of dehydration and increased salt intake. This nonapeptide hormone was first synthesised in solution using the classical methods of peptide chemistry.[2] Its synthesis has been made more efficient with the development of solid-phase peptide synthetic methods (SPPS).[3,4]

Side-chain amide protection of asparagine (Asn) or glutamine (Gln) has been considered optional.[5] These amide side-chains are liable to undergo dehydration during the coupling steps.[6–8] This side reaction does not occur when active esters are used.[5,6,8–10] Several carboxamide-protecting groups such as 2,4,6-trimethoxybenzyl,[11,12] 4,4'-dimethoxybenzhydryl,[13] triphenylmethyl,[14] 4-methyltrityl,[15] 2,4-dimethoxybenzyl[11,12,16] and 1-tetralinyl[17,18] have been developed in order to reduce or eliminate the aforementioned side reaction during peptide synthesis. In this project, we were encouraged to examine whether the 1-tetralinyl group (see insert) is viable as an asparagine amide-protecting group in the solid-phase peptide synthesis of lysine-vasopressin.



The most popular cleavage reagent for *tert*-butoxycarbonyl (Boc)-based peptide resins is anhydrous hydrogen fluoride. Whereas it is least harmful to a variety of peptides, its major drawback is its high toxicity and reactivity. The use of trifluoromethanesulphonic acid (TFMSA)[19–21] as an alternative to hydrogen fluoride cleavage has been encountered in the literature. This cleavage reagent is capable of removing most protecting groups at room temperature. Other stable groups can be removed at a slightly elevated temperature (40 °C).

Disulphide bonds in peptides are important for the maintenance of biological activity and conformational stability. These bonds in peptides are usually formed after the linear peptide has been synthesised, the side-chain-protecting groups removed, and the peptide detached from the resin.[22] This procedure is quite time-consuming. A one-pot synthesis has been developed for disulphide-containing bonds, which simultaneously cleaves, deprotects and oxidizes the cysteines.[23] Thus, cystine-containing peptides have been obtained after treatment of the protected peptidyl resin with trifluoroacetic acid (TFA) in the presence of iodine.[23] Other oxidants include air, potassium ferricyanide[24] and dimethyl sulphoxide.[25,26]

2. Experimental

General

Protected amino acid derivatives and benzhydrylamine hydrochloride salt resin (loading: 0.9 mmol g^{-1}) were obtained from Sigma (St. Louis, USA). TFA, TFMSA, thioanisole and 1,2-ethanedithiol (EDT) were obtained from Fluka (Buchs, Switzerland). All solvents were of analytical grade or of equivalent purity, and were used without further purification. Dichloromethane, methanol, ethanol and *N,N*-dimethylformamide 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 and tyrosine; benzyloxycarbonyl for lysine; benzhydryl for glutamine and 1-tetralinyl for asparagine. Boc removal was achieved with TFA–CH_2Cl_2–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 thioanisole–EDT–TFA–TFMSA (2:1:20:2 v/v). Peptide couplings were monitored using ninhydrin.

IR (cm^{-1}) spectra were recorded on a Perkin Elmer 1600 series (FTIR). Ion Electrospray Mass Spectra were determined on a Sciex API III TAGA 6000 (Toronto, Canada). ES–MS samples were prepared by dissolving 1 mg of peptide in 1 mL of 5% acetic acid (AcOH), MeCN or MeOH–H_2O (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 × 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 samples (1 mg mL^{-1} of MeOH) were chromatographed at 0.3 mL min^{-1} using a linear gradient of 0.1% aqueous TFA and 0.1% TFA in MeCN (10:90 to 0:100 over 45 min), detection at 214 and 280 nm. Semi-preparative HPLC was performed using a Grom semi-preparative nucleosil C-18 reversed-phase column (7 μm, 250 × 8 mm) on a Waters 600 Multi Solvent Delivery System (Milford, Massachusetts, USA) using manual injection (0.5 mL, 5 mg of peptide per run) and elution at 3.5 mL min^{-1} using 0.1% aqueous TFA and 0.1% TFA in MeCN (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). Peptide (1–2 nmol) was dissolved in 10 μL of MeOH or MeCN-H_2O (1:1 v/v). Hydrolysis was done using aq. HCl (6 mol L^{-1}) at 170 °C for 3 h. Sequencing of amino acids was performed using Applied Biosystems Model 476A and 477A (Foster City, California, USA). Samples were prepared by dissolving peptide (*ca.* 1 pmol) in MeOH (15 μL).

Synthesis of Glycinamide Resin

In a 60 mL solid-phase reaction vessel, the benzhydrylamine hydrochloride salt resin (5 g) was washed three times each with aq. Na_2CO_3 solution (10%), H_2O–dioxane (3:1 v/v), MeOH and CH_2Cl_2. The resin was then suspended in CH_2Cl_2 (35 mL) and shaken for 10 min. After removal of the solvent, the resin was treated with Boc-glycine (1.82 g, 10.4 mmol) in CH_2Cl_2 (8 mL) and *N,N*-dicyclohexylcarbodiimide (DCC) (2.14 g, 10.4 mmol) in the same solvent (18 mL) for 20 min at room temperature. After several washes with CH_2Cl_2 and EtOH, the coupling procedure was repeated twice more for 60 min each with half the quantities of Boc-glycine and DCC in the same volume of CH_2Cl_2. The resin was then washed sequentially with CH_2Cl_2, EtOH and CH_2Cl_2 (3 × 35 mL each), and the unreacted amino groups were blocked by treatment with *N*-acetylimidazole (8 g, 72 mmol) in CH_2Cl_2 (60 mL) for 2 h at 23 °C. The ninhydrin test for the presence of free amino groups indicated that there were no exposed amino groups. The weight of the resin was 5.408 g. The weight increase was 0.408 g, giving a glycine substitution of 0.48 mmol g^{-1} of the resin.

Boc-Cys(Bn)-Tyr(Bn)-Phe-Gln(Bzh)-Asn(Tet)-Cys(Bn)-Pro-Lys(Cbz)-Glycinamide Resin (Lysine-vasopressin resin, R3)

The glycinamide resin (2 g) 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) washing with CH_2Cl_2 (3 × 18 mL); (2) cleavage of the Boc group by treating with TFA–CH_2Cl_2–anisole (50:48:2 v/v; 18 mL) for 25 min at room temperature; (3) washing with CH_2Cl_2 (5 × 18 mL); (4) washing with $CHCl_3$ (4 × 18 mL); (5) neutralization with triethylamine in $CHCl_3$ (7:93 v/v; 2 × 15 mL) for 6 min at room temperature; (6) washing with $CHCl_3$ (3 × 18 mL); (7) washing with CH_2Cl_2 (4 × 18 mL); (8) addition of appropriate Boc-amino acid (2 mmol) in CH_2Cl_2 (9 mL) and 5 min of mixing; (9) addition of DCC

(0.41 g, 2 mmol) in CH_2Cl_2 (9 mL) followed by a reaction period of 90 min at room temperature; (10) washing with CH_2Cl_2 (3 × 18 mL); (11) washing with EtOH (3 × 18 mL); (12) washing with CH_2Cl_2 (3 × 18 mL); (13) repetition of steps 8 and 9 but using Boc-amino acid (1 mmol) and DCC (1 mmol), respectively; (14) washing with CH_2Cl_2 (3 × 18 mL); (15) washing with EtOH (3 × 18 mL). 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 (89%, based on the glycinamide substitution on the resin); ν_{max}/cm^{-1} (KBr) 3297 (N–H), 1678 (broad, C=O).

Lysine-vasopressin Resin Cleavage at Room Temperature for Two Hours (P3)

To the dried resin (250 mg) was added thioanisole–EDT (2:1 v/v; 750 μL). TFA (5 mL) was then added and the mixture was stirred at room temperature for about 10 min. TFMSA (500 μL) was then added slowly to the chilled mixture with vigorous stirring to dissipate the heat generated. Stirring was continued at room temperature for 2 h. The mixture was then filtered using a sintered glass funnel and the peptide was precipitated by adding Et_2O (60 mL). The solvent was decanted and the procedure was repeated twice. TFA (5 mL) was then added to dissolve the peptide, followed by Et_2O (60 mL) for re-precipitation, and decanting of the supernatant solvent. This was repeated five more times for the complete removal of the scavengers. The product was then dried under vacuum to give crude peptide P3 (42 mg, 60%); ν_{max}/cm^{-1} (KBr) 3296 (N–H), 3086.1 (Ar–H), 1667.9 (C=O);
ES-MS *m/z* 1239 (21.3%) (calcd for $C_{60}H_{80}N_{13}O_{12}S_2$, M^+, 1239.5), 1369 (17%) (calcd for $C_{70}H_{90}N_{13}O_{12}S_2$, M^+, 1369.7), 1406 (40.4%) (calcd for $C_{73}H_{90}N_{13}O_{12}S_2$, M^+, 1405.7), 1536 (25.5%) (calcd for $C_{83}H_{100}N_{13}O_{12}S_2$, M^+, 1535.9).

Lysine-vasopressin Cleavage at 40 °C for Half an Hour (PP3)

The resin was cleaved as done in the preparation of P3, but the cleavage was done at 40 °C for 30 min to give crude peptide PP3 (63.12 mg, 90%); ν_{max}/cm^{-1} (KBr) 3318 (N–H), 3096.6 (Ar–H), 1662.6 (C=O); ES-MS *m/z* 1239 (10%) (calcd for $C_{60}H_{80}N_{13}O_{12}S_2$, M^+, 1239.5), 1406 (18.5%), (calcd for $C_{73}H_{90}N_{13}O_{12}S_2$, M^+, 1405.7).

Lysine-vasopressin Cleavage at 40 °C for Two Hours (PPP3)

The resin was cleaved as done in the preparation of P3, but the cleavage was done at 40 °C for 2 h to give crude peptide PPP3 (66.31 mg, 94.6%); ν_{max}/cm^{-1} (KBr) 3360.2 (N–H), 3096.6 (Ar–H), 1683.7 (C=O); ES-MS *m/z* 1056.5 (100%) (calcd for $C_{46}H_{66}N_{13}O_{12}S_2$, M^+, 1057.2), 1148.5 (44.6%) (calcd for $C_{53}H_{73}N_{13}O_{12}S_2$, M^+, 1148.4), 1239 (17.7%) (calcd for $C_{60}H_{80}N_{13}O_{12}S_2$, M^+, 1239.5). Amino acid analysis: Asp 1.17 (1); Glu 0.88 (1); Gly 1.12 (1); Pro 1.00 (1); Tyr 0.39 (1); Cys 1.82 (2); Phe 0.82 (1); Lys 0.85 (1); Sequence analysis: order of amino acid residues was Tyr, Phe, Gln, Asn, Pro, Lys and Gly (residues 1 and 6 were not determined owing to formation of cystine derivative); Analytical HPLC gave major peaks with retention times (min) of 12.30 (*m/z* 1056.5), 16.24 (*m/z* 1148) and 27.91. Detection was at 214 nm. Crude peptide (10 mg) was used in the separation of the pure peptide by semi-preparative HPLC. Fraction 1 with the correct peptide was pooled and lyophilized to give a white powder (6.2 mg, 5.8 μmol, 62% isolated yield).

3. Results and Discussion

3.1. Lysine-vasopressin Resin (R3)

The nonapeptide was synthesised by SPPS via the Boc-strategy from 2 g of Boc-glycinamide resin. Cys and Tyr side-chains were protected with benzyl (Bn) groups. Side-chains of Gln, Asn and Lys were protected with benzhydryl (Bzh), 1-tetralinyl (Tet) and benzyloxycarbonyl (Cbz) groups respectively. The weight of the resin after the synthesis was 3.22 g (89%, based on the first amino acid anchored on the resin). 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 (Table 1). No peak due to the cyano functional group was seen in the IR spectrum.

Table 1 Solid-phase peptide synthesis of lysine-vasopressin resin (R3).

AA Residue	MM Boc-AA	MM AA residue	Weight increase (g)	Resin weight (g)	Substitution mmol/g
Gly	175.17	157.15	0.1509	2.0000	0.480
Lys	380.42	262.42	0.2519	2.2519	0.426
Pro	215.23	97.23	0.0933	2.3452	0.409
Cys	311.36	193.36	0.1856	2.5308	0.379
Asn	362.43	244.43	0.2347	2.7655	0.347
Gln	412.49	294.49	0.2827	3.0482	0.315
Phe	265.29	147.29	0.1414	3.1896	0.301
Tyr	371.39	253.39	0.2433	3.4329	0.280
Cys	311.36	193.36	0.1856	3.6185	0.265

3.2. Lysine-vasopressin Resin Cleavage at Room Temperature for Two Hours (P3)

Under the cleavage conditions with thioanisole–EDT–TFA–TFMSA as described in the experimental section, the benzyl group used in the protection of cysteine was not removed, while 1-tetralinyl (for asparagine protection) and benzhydryl (for glutamine protection), were partially cleaved. No cyclic lysine-vasopressin was formed.

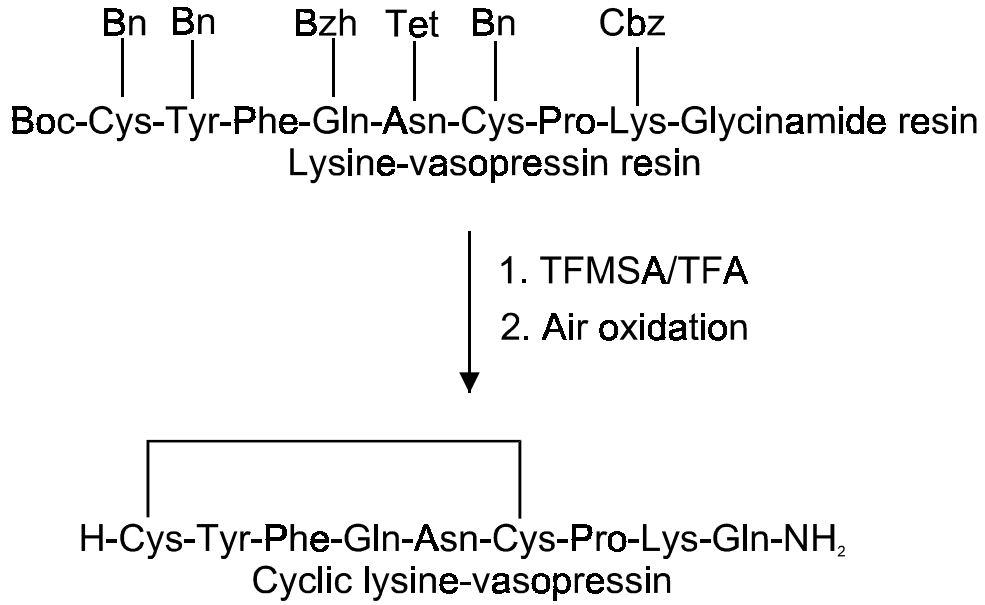
3.3. Lysine-vasopressin Cleavage at 40 °C for Half an Hour (PP3)

Under this cleavage condition, benzyl (cysteine protection) was not removed at all, while benzhydryl (glutamine protection) was partially removed. The 1-tetralinyl (asparagine protection) group, on the other hand, was completely removed. No cyclic lysine-vasopressin was formed.

3.4. Lysine-vasopressin Resin Cleavage at 40 °C for Two Hours (PPP3) (Scheme 1)

Under this cleavage condition, benzyl (for cysteine protection), benzhydryl (for glutamine protection) and 1-tetralinyl (for asparagine protection) groups were completely removed. A large quantity of cyclic lysine-vasopressin was formed. The peak at *m/z* 1056.5 is cyclic lysine-vasopressin, with all protecting groups removed and the free mercapto groups oxidised. 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.

In cases where benzyl and benzhydryl are resistant to cleavage, incorporating electron-donating group(s) on the aromatic ring(s) will make their removal more facile.[17] Electron-donating group(s) on the aromatic ring of 1-tetralinyl group will have the same effect. This is more enhanced if the electron-donating group is on position 6 or 8 of the 1-tetralinyl group.



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

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

4. Conclusion

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

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