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Solid-Phase Peptide Synthesis of Arginine-vasopressin with Amide Side-chain of Asparagine Protected with 1-Tetralinyl Group

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Abstract
Arginine-vasopressin, 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, tetralinyl and tosyl groups were used in the protection of glutamine, asparagine and arginine side-chains respectively. TFMSA-TFA-thioanisole-1,2-ethanedithiol (2:20:2:1 v/v) was used to cleave the peptide from the resin under different conditions to obtain arginine-vasopressin in a one-pot reaction. The cleavage at 40°C for two hours gave arginine-vasopressin quantitatively (77% yield)
Keywords: Solid-Phase Peptide Synthesis, resin, protecting group, cleavage, nonapeptide

1. Introduction

Arginine-vasopressin is a nonapeptide hormone of the mammalian neurohypophysis with cyclization at residues one and six (Fig 1) (Sivannandaiah & Gurusiddappa, 1981; Live et al. 1977). This hormone is responsible for pressor and diuretic effects (Barbar 2013). It exerts a marked effect on the kidneys, accelerating the rate of water re-absorption (Nielsen et al. 1995). Its secretion is augmented in circumstances of dehydration and increased salt intake. It is secreted by the posterior pituitary gland. It can also be synthesized by parvocellular neurosecretory neurons and then transported to anterior pituitary where it stimulates corticotrophin cells (Salata et al. 1988).

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

Fig 1: Structure of arginine-vasopressin

A good carboxamide-protecting group for asparagine should be stable in trifluoroacetic acid which is used for cleaving 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 studied includes xanthenyl (Han et al. 1996), 2,4,6-trimethoxybenzyl (Pietta & Marshall 1970; Weygand et al. 1968), and 4,4'-dimethoxybenzhydryl just to name a few (Koenig & Geiger 1970).

During acylation, asparagine and glutamine derivatives are known to undergo several side reactions of which the most important is nitrile formation via dehydration of the carboxamide side-chain (Katsoyanis et al. 1958). This side reaction has been reported to occur with carbodiimides (Katsoyanis et al. 1958; Kashelikar & Ressler, 1964; Ressler & Ratzkin 1961). 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).

In this research, we examined the possibility of using 1-tetralinyl group as asparagines side-chain protecting group in the solid phase peptide synthesis of arginine-vasopressin (Merrifield 1963, 1965). A one-pot reaction has been developed for disulfide-containing bonds, which simultaneously cleave, deprotect and oxidize the cysteine in preparation of arginine-vasopressin (Spetzler & Meldal 1966; Hope et al. 1962; Otaka et al. 1991; Tam et al. 1991).

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 and 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 were used without further purification. Dichloromethane, methanol, ethanol and 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 (Bn) for cysteine and tyrosine; tosyl (Tos) for arginine; benzhydryl (Bzh) for glutamine and 1-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, 40X80mm),

developed with chloroform-ethyl acetate (3:1 v/v) (CE). The compounds were observed by both iodine and ultraviolet (UV) lamp.

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 Electro spray 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-pressure 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: C, cysteine; F, phenylalanine; G, glycine; N, asparagine; P, proline; Q, glutamine; R, arginine; Y, tyrosine.

2.2 Synthesis of Boc-Gly-resin (t-Butyloxycarbonyl-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 ml each of 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 (20 ml X3) and ethanol (20 ml X3), 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.

2.3Boc-Cys(Bzl)-Tyr(Bzl)-Phe-Gln(Bzh)-Asn(Tet)-Cys(Bzl)-Pro-Arg(Tos)-Glycinamide resin (Arginine-vasopressin resin, R4)

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 2 mmol of Boc-amino acid 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-amino acid 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: 3.13 g (85.41%, based on the glycinamide substitution on the resin); IR (KBr) 3276 (NH), 1663 cm^{-1} (broad, CO).

2.4 Arginine-vasopressin resin cleavage at room temperature for two hours (P4)

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 P4.

2.5 Arginine-vasopressin resin cleavage at 40 C for half an hour (PP4)

The resin was cleaved as done in the preparation of P4, but the cleavage was done at 40°C for half an hour to give 66.16 mg (93.23%) of crude peptide PP4.

2.6 Arginine-vasopressin resin cleavage at 40 C for two hours (PPP4)

The resin was cleaved as done in the preparation of P4, but the cleavage was done at 40°C for two hours to give 68.3 mg (96.25%) of crude peptide PPP4.

3. Results and Discussion

3.1 Arginine-vasopressin resin (R4)

The nonapeptide-resin was synthesised by SPPS via the Boc strategy from 2 g of Boc-glycinamide resin. The side chains of cysteine and tyrosine were protected with benzyl. The protecting groups for other amino acid side-chains were tetralinyl for asparagine, benzhydryl for glutamine and tosyl for arginine. Peptide resin yield was 3.13 g (85%, based on first amino acid substitution on the resin). IR (cm^{-1}) gave peaks at 3276 (N-H) and a broad peak centred at 1663. No peaks due to cyano group were present.

Table 1 shows the various amino acid molecular masses, residue masses, peptide resin weights and resin substitution.

Table 1: SPPS of arginine-vasopressin resin (R4)

AA-Residue	MM-Boc-AA	MM-AA residue	Wt increase(g)	Peptide resin wt(g)	Substitutionn mmol/g
Gly	175.17	157.15	0.1509	2	0.48
Arg	428.48	310.48	0.2981	2.2981	0.418
Pro	215.23	97.23	0.0933	2.3914	0.401
Cys	311.36	193.36	0.1856	2.577	0.372
Asn	362.43	244.43	0.2347	2.8117	0.341
Gln	412.49	294.49	0.2827	3.0944	0.310
Phe	265.29	147.29	0.1414	3.2358	0.297
Tyr	371.39	253.39	0.2433	3.4791	0.276
Cys	311.36	193.36	0.1856	3.6647	0.262

KEY: AA= amino acid; MM=molecular mass

3.2 Arginine-vasopressin resin cleavage at room temperature for two hours

This resin was cleaved at room temperature for two hours to give 59 mg (84.09%) of crude arginine-vasopressin (P4). Peaks are shown in Table 2.

Table 2: Electro spray Mass Spectrometer analysis of crude arginine-vasopressin (P4)

Peptide derivative	Calc m/z MH^+	Expe m/z MH^+	% Inten
C(Bz)-Y-F-Q-N-C(Bz)-P-R-G	1266.61	1267	11.5
C(Bz)-Y-F-Q(Bzh)-N-C(Bz)-P-R-G	1432.7	1433.5	13

ESMS showed two peptides with one having all protecting groups removed except benzyl (Cys) (m/z 1267) while the other had all protecting groups removed except benzyl (Cys) and benzhydryl (Gln) (m/z 1433.5). This showed that benzyl on Cys was not removed at all, while benzhydryl was partially removed. 1-Tetralinyl (Asn) was completely removed. Under the given condition, benzyl was stable, while benzhydryl was partially stable. 1-Tetralinyl was unstable. No cyclic arginine-vasopressin was formed. G is a glycinamide residue. Peaks corresponding to MH^{2+} appeared at m/z 634.0, 19.23% (MH^+ 1267) and m/z 717.0, 100% (MH^+ 1433.5). IR (cm^{-1}) of the product gave peaks at 3307.5 (N-H), 3086.1 (Ar-H), 1667.9 (-CO-).

3.3 Arginine-vasopressin resin cleavage at 40 C for half an hour

This resin was cleaved at 40 C for half an hour to give 66.16 mg (99.23%) of the crude arginine-vasopressin (PP4). The ESMS peaks are shown in Table 3.

Table 3**:** *Electro spray Mass Spectrometer analysis of crude arginine-vasopressin (PP4)*

Peptide derivative	Calc m/z MH^+	Expe m/z MH^+	% Intensity
C(Bz)-Y-F-G-N-C(Bz)-P-R-G	1266.61	1267.0	16.9
C(Bz)-Y-F-Q(Bzh)-N-C(Bz)-P-R-G	1432.7	1433.5	13.8

ESMS showed the presence of two peptides. One had all protecting groups removed except benzyl (Cys) (m/z 1267.0), while the other had all the groups deprotected apart from benzyl (Cys) and benzhydryl (Gln) (m/z 1433.5). Benzyl was not cleaved hence stable under the given condition. Benzhydryl was partially removed while 1-tetralinyl (Asn) was completely removed. On stability, benzhydryl was partially stable while 1-tetralinyl was unstable. No cyclic arginine-vasopressin was formed. G is a glycinamide residue. Peaks corresponding to MH^{2+} appeared at m/z 634.0, 51.54% (MH^+ 1267.0) and m/z 717.0, 100% (MH^+ 1433.5). IR (cm^{-1}) gave peaks at 3328.6 (N-H), 3086.1 (Ar-H), 1667.9 (-CO-).

3.4 Arginine-vasopressin resin cleavage at 40 C for two hours

This resin was cleaved at 40 C for two hours to give 68.3 mg (96.25%) of the crude peptide (PPP4). Peaks are shown in Table 4.

Table 4: Electro spray Mass Spectrometer analysis of crude arginine-vasopressin (PPP4)

Peptide derivative	Calc m/z MH^+	Expe m/z MH^+	% Intensity
C-Y-F-Q-N-C-P-R-G	1084.41	1084.5	100
C-Y-F-Q-N-C(Bz)-P-R-G	1176.52	1176.5	29.2

Evidence of ESMS showed the presence of two peptides. The first had all protecting groups removed and the free mercapto groups oxidized to cyclic arginine-vasopressin between the first and the sixth amino acids (m/z 1084.5). The second had all protecting groups removed except benzyl (Cys) (m/z 1176.5). Benzyl (Cys) was cleaved partially. Benzhydryl (Gln) and 1-tetralinyl (Asn) were completely removed. Under the given condition, benzyl was partially stable, while benzhydryl and 1-tetralinyl were unstable. G is a glycinamide residue. Peaks corresponding to MH^{2+} appeared at m/z 542.5, 66.15% (MH^+ 1084.5) and m/z 588.5, 52.31% (MH^+ 1176.5). IR (cm^{-1}) of the product gave peaks at 3391.8 (N-H), 3075.5 (Ar-H), 1678.4 (-CO-). Analytical HPLC gave major peaks with retention times (min) of 12.59, 16.46 and 27.86. 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: Semi-preparative HPLC analysis of crude arginine-vasopressin (PPP4)

Fraction	ESMS m/z MH^+	RT (min)
2	1084.5	12.59
10	1176.0	16.46
14	1266.5	18.97

Fraction 2 with the correct peptide was pooled and lyophilized to give cyclic arginine-vasopressin which is a white powder (7.7 mg, 7.1 μmol, 77% isolated yield). (Table 5)

Amino acid analysis gave the expected ratios: Asp 1.15 (1); Glu 0.99 (1); Gly 1.07 (1); Arg 1.27 (1); Pro 1.00 (1); Tyr 0.47 (1); Cys 2.07 (2); Phe 0.84 (1). Sequence analysis of the pure peptide gave the expected results as given in Table 6.

Table 6: Sequence analysis of arginine-vasopressin (Edman method)

Residue	Abbreviation	Amino acid
1	-	-
2	Y	Tyr
3	F	Phe
4	Q	Gln
5	N	Asn
6	-	-
7	P	Pro
8	R	Arg
9	G	Gly

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

4. Conclusion

When cleavage was done at room temperature for two hours, benzyl group used in the protection of cysteine was not removed at all in arginine-vasopressin. Benzhydryl group was partially removed while 1,2,3,4-Tetrahydro-1-naphthyl group was completely removed. Arginine-vasopressin had all groups removed except benzyl (Cys) and benzhydryl (Gln).

When cleavage was done at 40°C for half an hour, benzyl-protecting group on cysteine was not removed at all. Benzhydryl group used in the protection of glutamine side chain was partially removed. 1,2,3,4-Tetrahydro-1-naphthyl group used in the protection of asparagine side chain was completely removed. Arginine-vasopressin had all groups removed except benzyl (Cys) and benzhydryl (Gln).

At 40°C for two hours, benzyl group on cysteine was partially removed. Benzhydryl and 1,2,3,4-tetrahydro-1-naphthyl groups were completely removed. Large quantities of cyclic arginine-vasopressin was formed. Benzyl was therefore partially stable while 1,2,3,4-tetrahydro-1-naphthyl and benzhydryl groups were unstable. Arginine-vasopressin had all protecting groups removed except benzyl (Cys). High yield of arginine-vasopressin was formed.

In cases where benzyl group protection on cysteine and benzhydryl on glutamine are resistant to cleavage, incorporating electron-donating group(s) on the aromatic ring(s) will make their removal more facile. Electron-donating group(s) on the aromatic ring of 1,2,3,4-tetrahydro-1-naphthyl group will have the same effect. 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. Compairing 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 peptides 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 shorter.
These studies have demonstrated the usefulness of 1,2,3,4-tetrahydro-1-naphthyl group in asparagine amide-protection and its use in Boc solid-phase peptide synthesis. With the 1-tetralinyl group, the results obtained show that arginine-vasopressin 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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