Synthesis and Some Pharmacological Properties of [3-β-(2-Thienyl)-L-alanine]-8-lysine-vasopressin†

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[3-β-(2-Thienyl)-L-alanine]-8-lysine-vasopressin was synthesized by solution techniques. The partially protected heptapeptide Boc-Cys(Ec)-Tyr-Thi-Gln-Asn-Cys(Ec)-Pro (1) was synthesized in a stepwise manner using the active ester method or the dicyclohexylcarbodiimide (DCC) coupling technique mediated by 1-hydroxybenzotriazole (HBt). The protected nonapeptide amide Boc-Cys(Ec)-Tyr-Thi-Gln-Asn-Cys(Ec)-Pro-Lys(Boc)-Gly-NH_2 (2) was prepared by coupling 1 with Lys(Boc)-Gly-NH_2 using DCC–HBt. From 2, [3-thienylalanine]-8-lysine-vasopressin was obtained by removing the Boc-protecting groups with trifluoroacetic acid and ethylcarbamoyl (Ec) protecting groups in refluxing liquid NH_3 followed by oxidative cyclization in H_2O–MeOH using ICH_2CH_2I. Purification was effected by partition chromatography followed by gel filtration. The highly purified product possesses activities in the oxytocic, avian vasodepressor, rat pressor, and antidiuretic assays of 19.0 ± 0.5, 87 ± 4, 243 ± 5, and 332 ± 32 units/mg, respectively. Thus [3-thienylalanine]-8-lysine-vasopressin has higher oxytocic, avian vasodepressor, and antidiuretic potencies than does 8-lysine-vasopressin, whereas its pressor potency is about the same as or slightly lower than that of 8-lysine-vasopressin.

The proof by synthesis of the structures of the mammalian neurohypophyseal hormones—oxytocin,[2] lysine vasopressin[3] (LVP), and arginine vasopressin[4] (AVP)—established unequivocally the fact that their primary structures differ only in the amino acid residues at positions 3 and 8 (Figure 1). Oxytocin has an isoleucine residue at position 3 and a leucine residue at position 8, whereas the vasopressins have a phenylalanine residue at position 3 and a lysine or arginine residue at position 8. Thus the striking difference at position 3 is the presence of an aliphatic side chain in oxytocin and an aromatic side chain in the vasopressins. Many 3-position analogs of oxytocin with aliphatic side chains different from that of isoleucine have been prepared. This work has demonstrated a necessity for a lipophilic interaction between the side chain of the residue at position 3 and the receptor for biological activity[5] and further that uterotonic activity is quite sensitive to structural modifications at that position.

The formal replacement of the phenylalanine residue by an isoleucine residue in LVP and AVP yields, respectively, lysine-vasotocin[6-8] (LVT) and arginine-vasotocin[8,9] (AVT). The latter is also a naturally occurring compound.[10] These two compounds, which may also be regarded as analogs of oxytocin with changes at position 8, have potencies intermediate between those of oxytocin and lysine- or arginine-vasopressin in each of the characteristic pharmacological assays for oxytocin and the vasopressins. Some indication that steric size plays a significant role in the manifestation of vasopressin-like activity is shown by the pressor potency of [3-cyclopentylglycine]oxytocin, which is higher than that of oxytocin.[11]

Very little additional work has been done to investigate the importance of the aromatic nature of the side chain of the phenylalanine residue to the characteristic pharmacological activities of the vasopressins. Only three other analogs of the vasopressins with single substitutions at position 3 appear in the literature. They are [3-tyrosine]-LVP,[12] [3-serine]-LVP,[13] and [3-tryptophan]-LVP.[13] All have side-chain substituents, whether aromatic or aliphatic, that are hydrophilic in nature, and all have little or no activity. Thus it would appear as in the case of oxytocin that a lipophilic side chain at position 3 is necessary for the expression of biological activities.

An interesting candidate for the replacement of the phenylalanine residue in LVP is β-(2-thienyl)-L-alanine.[14] The thiophene ring is isosteric with the benzene ring but has less aromatic character, having a resonance energy of 25 kcal/mol as compared to 36 kcal/mol for benzene. The formal substitution of a β-(2-thienyl)-L-alanine residue for either one or both of the phenylalanine residues in bradykinin resulted in analogs which are more potent than bradykinin itself in the rat uterus assay.[15] Replacement of the phenylalanine residue with a β-(2-thienyl)-L-alanine residue in angiotensin II results in an analog which has 27% of the pressor potency of the parent hormone and which functions as a noncompetitive inhibitor of the myotropic response to angiotensin II.[16] The synthesis of [3-thienylalanine]-8-lysine-vasopressin ([3-Thi]-LVP) was undertaken to investigate the effect of isosteric substitution and decreased aromaticity at the 3 position on the pharmacological activities characteristic of LVP.

The presence of the reactive thiophene ring in the β-(2-thienyl)-L-alanine residue imposed several restrictions on the choice of protecting groups for the synthesis. Model reactions showed that the thiophene ring is reduced by Na in liquid NH_3. It presence is thus incompatible with the use of the benzyl group for protection of the sulfhydryl moiety of cysteine. Benzyloxycarbonyl protection of the amino components was avoided, as the reagent required for its removal (HBr–AcOH) often contains small amounts of Br_2 capable of substitution reactions on the thiophene ring.

The sulfhydryl moiety of cysteine was protected by the ethylcarbamoyl (Ec) group which is easily removed by mild base.[17] *tert*-Butyloxycarbonyl (Boc) protection was employed for the α-amino nitrogen. A facile, one-vessel synthesis of Boc-Cys(Ec) was developed starting from $(Boc\text{-}Cys)_2$, which was obtained routinely in yields greater than 95% from cystine by the method of Ferraro.[18] Reduction of $(Boc\text{-}Cys)_2$ was accomplished by treatment with 1,4-butanedithiol and followed by introduction of the Ec group with ethyl isocyanate. In order that the easily removable Boc group could also be employed on the ε-amino nitrogen of the lysine residue, a synthetic scheme similar to the one developed by Jones et al.[19] for a synthesis of AVP

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Table I. Pharmacological Potencies of Analogs of Lysine-vasopressin

Compound	Oxytocic[a]	AVD[a]	Antidiuretic[a]	Pressor[a]
8-Lysine-vasopressin	4.8 ± 0.3[b]	48 ± 2[b]	203 ± 7[b]	243 ± 3[b,c]
[3-Thienylalanine]-8-lysine-vasopressin	19.0 ± 0.5	87 ± 4	332 ± 32	243 ± 5
[3-Isoleucine-8-lysine-vasopressin (lysine-vasotocin)[d]	78 ± 10	210 ± 3	24 ± 3	130 ± 13

[a] Activities are in units/mg ± SE. [b] Kimbrough et al.[34] [c] Higher values ranging up to 308 units/mg[35] have also been reported, and most fall within the range of 250–290 units/mg originally reported by Bartlett et al.[36] Uncontrolled differences in H_2O and HOAc content of the lyophilized samples used for assay may account for some of the variability. [d] Huguenin and Boissonnas.[8]

was used. The partially protected heptapeptide Boc-Cys(Ec)-Tyr-Thi-Gln-Asn-Cys(Ec)-Pro (1) was prepared in a stepwise manner from the C-terminal end using Boc-protected amino acid active esters except in the synthesis of Boc-Cys(Ec)-Pro. This dipeptide was prepared by treating Boc-Cys(Ec) with dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole (HBt)[20] in dimethylformamide (DMF) and adding this preactivation mixture to a suspension of proline in DMF. In all cases the Boc group was removed either by treatment with trifluoroacetic acid (TFA) or by HCl in EtOAc. The latter reagent was especially useful for removing the Boc group from Boc-Gln-Asn-Cys(Ec)-Pro, as it effectively prevented the formation of <Glu-Asn-Cys(Ec)-Pro. The isolation of the protected tri- through heptapeptides was complicated by the fact that these compounds exhibited a marked solubility in water. The protected nonapeptide Boc-Cys(Ec)-Tyr-Thi-Gln-Asn-Cys(Ec)-Pro-Lys(Boc)-GlyNH_2 (2) was prepared by coupling 1 with Lys(Boc)-Gly-NH_2[21] using DCC–HBt.[20] The Boc-protecting groups of 2 were removed by treatment with TFA, and the Ec groups were removed by refluxing in liquid NH_3.[17] Cyclization was accomplished by oxidation with ICH_2CH_2I[22] in H_2O–MeOH. The crude product was purified by partition chromatography[23] followed by gel filtration on Sephadex G-25.[24]

The highly purified product was tested for oxytocic, avian vasodepressor (AVD), antidiuretic, and pressor activities against the U.S.P. posterior pituitary reference standard.[‡] The results are shown in Table I. [3-Thi]-LVP possesses substantially higher oxytocic, AVD, and antidiuretic potencies than does LVP. The pressor potency of [3-Thi]-LVP is perhaps slightly lower than, but not substantially different from, that of LVP.

The substitution of the less aromatic side chain of thienylalanine for that of phenylalanine at position 3 e enhances the oxytocin-like (oxytocic and AVD) activities of LVP but not nearly to the same extent as does substitution of the lipophilic aliphatic side chain of isoleucine. However, unlike the case of [3-Ile]-LVP, the vasopressin-like (pressor and antidiuretic) activities of [3-Thi]-LVP remain high. Since the thiophene ring is isosteric with the benzene ring, the findings here support earlier studies with oxytocin analogs[11] which suggested that steric size in the 3 position plays a significant role in the manifestation of vasopressin-like activities.

‡ Oxytocic assays were performed on isolated uteri from virgin rats in natural estrus according to the method of Holton,[25] as modified by Munsick[26] with the use of Mg-free van Dyke–Hastings solution as bathing fluid. AVD assays were performed on conscious chickens by the method of Coon,[27] as described in the U.S. Pharmacopeia,[28] as modified by Munsick, Sawyer, and van Dyke.[29] Pressor assays were carried out on anesthetized male rats as described in the U.S Pharmacopeia.[30] Antidiuretic assays were performed on anesthetized male rats according to the method of Jeffers et al.[31] as modified by Sawyer.[32] The four-point assay design of Schild was used.[33]

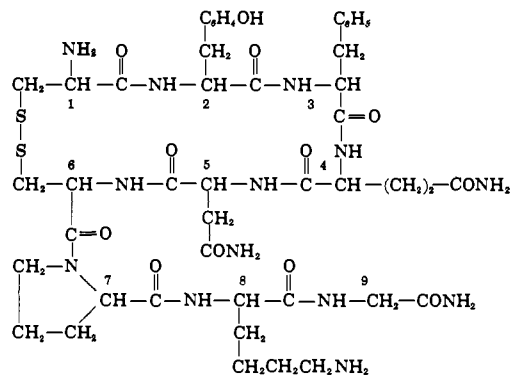

Figure 1. Structure of lysine-vasopressin with numbers indicating the positions of the individual amino acid residues. Oxytocin has an isoleucine residue at position 3 and a leucine residue at position 8. Arginine-vasopressin has an arginine residue at position 8.

Experimental Section[§]

Boc-Cys(Ec). A solution of $(Boc\text{-}Cys)_2$[19] (4.4 g, 10 mmol), diisopropylethylamine (*i*-Pr_2NEt) (3.5 ml, 20 mmol), and 1,4-butanedithiol (6.0 ml, 50 mmol) was prepared in DMF (50 ml) and allowed to stand at room temperature for 12 hr. The solution was cooled to 0° and 4.5 ml of EtOAc saturated with anhydrous HCl at 0° (4.5–5 *M*) was added dropwise with stirring. The solvents and excess 1,4-butanedithiol were removed by rotary evaporation at 0.5 Torr and 40°. The residue was redissolved in DMF (50 ml), cooled to 0°, and freshly distilled ethyl isocyanate (1.62 ml, 20.5 mmol) was added. After 12 hr the DMF was removed by rotary evaporation. The residue, dissolved in EtOAc (100 ml), was extracted with three portions of H_2O (50, 25, 25 ml). The combined aqueous extracts with added brine (100 ml) were reextracted with two 50-ml portions of EtOAc. The combined EtOAc extracts were dried (Na_2SO_4) and evaporated to dryness. The solid residue was dissolved in boiling EtOAc (25 ml). To the cooled solution was added dicylohexylam-

§ All melting points were determined in open capillary tubes and are corrected. Thin-layer chromatography was performed on precoated glass plates of silica gel GF_{254} (0.25 mm, E. Merck) in the following solvent systems: (A) acetone–AcOH 9:1; (B) $CHCl_3$–MeOH–AcOH 9:1:1; (C) acetone–AcOH–H_2O 8:1:1; (D) $CHCl_3$–MeOH 9:1; (E) acetone–AcOH–H_2O 7:2:1; (F) acetone–AcOH–H_2O 16:2:1; (G) BuOH–pyridine–AcOH–H_2O 15:10:3:12; (H) PrOH–AcOH–H_2O 3:1:1. The load size was 10–30 μg, and chromatogram lengths were 100–150 mm. Detection was made by uv, ninhydrin, and chlorination followed by NaI–starch treatment. In all cases, unless otherwise noted, single symmetrical spots were observed for purified material. Amino acid analysis was performed on a Beckman Model 116 amino acid analyzer on a single column (0.9 × 58 cm) of AA-15 resin employing 0.066 *M* sodium citrate buffers at pH 3.28, 4.30, and 6.40 (Na^+ concentrations 0.2, 0.2, and 1.0 *N*, respectively) at 55°. At a flow rate of 72.7 ml/hr with buffer changes at 85 and 140 min, β-(2-thienyl)-L-alanine emerged as a completely resolved peak with a retention time of 156 min preceded by leucine at 146 min and followed by tyrosine at 167 min. Optical rotations were determined on a Perkin-Elmer Model 141 polarimeter. Where analyses are indicated only by the symbols of the elements, analytical results obtained for the elements were within ±0.4% of the theoretical values. Where compounds are formulated as containing solvent this does not necessarily imply that they are defined solvates; particularly in the case of amorphous products, solvent retention may be due to mild drying conditions (24 hr at 22°/0.005 Torr over P_2O_5).

ine (DCHA) (4.0 ml, 20 mmol) and Et_2O (50 ml). The product crystallized slowly as the solution was cooled to −17°. The crystals were collected, washed with Et_2O, and dried in vacuo: 7.85 g (83%); mp 148–149.5°. The product gave two spots on TLC in system A corresponding to Boc-Cys(Ec) at R_f 0.78 and DCHA at R_f 0.35. The DCHA salt was converted quantitatively to free Boc-Cys(Ec) by the following procedure. Boc-Cys(Ec)·DCHA (0.979 g, 2.07 mmol) was dissolved in EtOAc (50 ml) and EtOH (2.5 ml). The DCHA·HCl, which precipitated upon the addition of 0.5 ml of HCl–EtOAc and cooling to 0°, was removed by filtration. The product crystallized as the filtrate was rotary evaporated to dryness and was dried in vacuo: 0.605 g (100%); mp 137.5–138.5°; $[\alpha]^{22}D$ −24.0° (c 0.9, EtOH) [lit.[37] mp 139; $[\alpha]^{24}D$ −22.2° (c 1.0, EtOH); lit.[38] mp 139–140°; $[\alpha]^{25}D$ −21.8° (c 1.0, EtOH)]; TLC (A) 0.78, (B) 0.70.

Boc-Cys(Ec)-Pro (3). A solution of Boc-Cys(Ec) (2.93 g, 10.0 mmol) and HBt[20] (2.0 g, 15 mmol) was prepared in DMF (10 ml) and cooled to 0°. DCC (2.06 g, 10.0 mmol) was added and the solution was stirred at room temperature for 60 min. Precipitated dicyclohexylurea (DCU) was removed by filtration, and the filtrate was added (with two 5-ml rinses of DMF) to a suspension of proline (1.21 g, 10.5 mmol) in DMF (5 ml). The reaction mixture was allowed to stand overnight, and the DMF was removed by rotary evaporation (oil pump). The residue was dissolved in 1 *N* HCl (50 ml) and $CHCl_3$ (50 ml) and shaken vigorously. The two-phase system was filtered to remove the precipitated HBt. The phases were separated and the organic phase was washed with 1 *N* HCl (50 ml), three 50-ml portions of H_2O, and two 50-ml portions of brine. After being dried with Na_2SO_4 the $CHCl_3$ was removed by rotary evaporation. The residue was dissolved in EtOAc (50 ml), DCHA (1.96 ml, 10 mmol) was added, and the salt precipitated immediately as a gel-like solid. Et_2O (50 ml) and hexane (100 ml) were added to obtain a workable slurry. The product was collected by filtration, washed with Et_2O, and dried in vacuo: 4.2 g; mp 141–144°. This material was dissolved in boiling EtOAc (250 ml), and hexane (250 ml) was added. The precipitate that formed as the solution was cooled to −17° was collected, washed with hexane, and dried in vacuo: 3.41 g (60%); mp 147–148.5°; $[\alpha]^{21}D$ −35.7° (c 0.95, DMF). This material gave two spots by TLC in system A: R_f 0.66, corresponding to Boc-Cys(Ec)-Pro, and R_f 0.35, corresponding to DCHA. For analytical purposes a small portion of Boc-Cys(Ec)-Pro was obtained in 80% recovery by partitioning the DCHA salt between EtOAc and aqueous citric acid, followed by precipitation from Et_2O with hexane: mp 84–87°; $[\alpha]^{21}D$ −62.3° (c 1.0, EtOH); TLC (A) 0.67. Anal. ($C_{16}H_{27}N_3O_6S$) C, H, N.

Boc-Asn-Cys(Ec)-Pro (4). Boc-Cys(Ec)-Pro·DCHA (4.57 g, 8.0 mmol) was partitioned between EtOAc and aqueous citric acid. The resulting dipeptide, obtained as an oil, was used without further purification. After removal of the Boc group by treatment with HCl–EtOAc (40 ml) for 30 min and rotary evaporation to dryness, the resulting salt was dissolved in DMF (40 ml) and adjusted to pH 7.5 (Gramercy indicator solution) with *N*-methylmorpholine (1.6 ml, 14.4 mmol). Boc-Asn-ONp (3.5 g, 9.6 mmol) was added, and the progress of the reaction was followed by the quantitative ninhydrin test.[39] The reaction was complete after 19 hr. *N*-Methylpiperazine (0.35 ml, 3.2 mmol) was added to destroy the excess Boc-Asn-ONp and allowed to react for 1.5 hr. The solvents were removed by rotary evaporation and the residue dissolved in H_2O (50 ml). Tetrahydrofuran (THF) (150 ml) was added, the solution was cooled to 0°, and the pH adjusted to 1.5 with concentrated HCl. The solution was saturated with NaCl and the resulting two phases were separated. The aqueous phase was extracted with three 50-ml portions of THF, and the combined THF solutions were reextracted with three 50-ml portions of brine. The THF solution was partially dried over Na_2SO_4 and evaporated to dryness. The residue was redissolved in THF (25 ml) and the product precipitated by the addition of Et_2O (200 ml). The precipitate was collected, washed with Et_2O, and dried in vacuo: 3.71 g. The product was crystallized from *i*-PrOH: 1.99 g (49.4%); mp 174.5–175.5°; $[\alpha]^{22}D$ −60.3° (c 1.2, DMF). Anal. ($C_{20}H_{33}N_5O_8S$) C, H, N. A second crop was obtained by adding Et_2O to the filtrate: 1.27 g (31.5%); mp 172–174°. Both crops gave identical single spots on TLC [(A) 0.35, (B) 0.72] and were used interchangeably for further steps.

Boc-Gln-Asn-Cys(Ec)-Pro (5). The protected tripeptide 4 (2.1 g, 4.0 mmol) was treated with TFA (20 ml) for 20 min and the TFA was removed by rotary evaporation. The residue was triturated with Et_2O, collected, washed with Et_2O, and dried in vacuo. The peptide salt was dissolved in DMF (13 ml) and the pH of the solution adjusted to 7.5 with *i*-Pr_2NEt (1.0 ml, 6.0 mmol). Boc-Gln-ONp (1.7 g, 4.8 mmol) was added and the reaction followed by the ninhydrin test. After 20 hr the pH was adjusted back up to 6.5 with *i*-Pr_2NEt (0.5 ml, 3 mmol) and the reaction continued for 2 hr. The solution was cooled to 0° and TFA (0.7 ml, 9.5 mmol) was added. The reaction mixture was added slowly to EtOAc (150 ml) with rapid stirring. The product precipitated during the addition and the resulting mixture was stored for 2 hr at 8°. The precipitate was collected, washed with EtOAc and Et_2O, and dried in vacuo: 2.58 g; mp 119–124°. This material was triturated in boiling EtOAc (50 ml), the mixture cooled to 8°, and the precipitate collected, washed with EtOAc and Et_2O, and dried in vacuo: 2.37 g (94%); mp 133–135°; TLC (C) 0.52. For elemental analysis a 29.4-mg portion was dissolved in *i*-PrOH and precipitated with Et_2O in 82% recovery: mp 122–124°; $[\alpha]^{22}D$ −51.2° (c 0.88, DMF); TLC (C) 0.52. Anal. [$C_{25}H_{41}N_7O_{10}S·H_2O·0.5(CH_3)_2CHOH$] C, H, N.

Boc-Thi-ONp. Boc-L-Thi[15] (1.00 g, 3.7 mmol) and *p*-nitrophenol (0.61 g, 4.4 mmol) were dissolved in EtOAc (5 ml) and cooled to 0°. DCC (0.80 g, 3.9 mmol) in EtOAc (1 ml) was added and the solution was kept at 4° overnight. Precipitated DCU was collected on a frit and washed with EtOAc. The filtrate was rotary evaporated to dryness and the crystalline residue recrystallized from EtOAc–hexane (1:3): 1.02 g (70%); mp 145.5–146.0°; $[\alpha]^{22}D$ −28.2° (c 0.51, MeOH); $[\alpha]^{22}D$ −28.3° (c 1.08, EtOAc); TLC (D) 0.74. Anal. ($C_{18}H_{20}N_2O_6S$) C, H, N. S.

Boc-Thi-Gln-Asn-Cys(Ec)-Pro (6). A solution of 5 (631 mg, 1.00 mmol) was prepared in 15 ml of HCl–EtOAc. Precipitation of the peptide hydrochloride salt occurred immediately. After 30 min the solvents were removed by rotary evaporation. The salt was dried in vacuo, dissolved in DMF (4 ml), and neutralized to pH 7.5 with *i*-Pr_2NEt (0.35 ml, 2.0 mmol). The neutral peptide or its *i*-Pr_2NEt salt precipitated from solution. The addition of 10 ml of DMF and gentle warming did not completely redissolve the precipitate. Boc-Thi-ONp (451 mg, 1.15 mmol) was added to the suspension after it had cooled to room temperature. After 5 hr the pH had dropped to ~5, but undissolved precipitate remained. The volume of the solution was reduced to approximately 7 ml by rotary evaporation (oil pump) and dimethyl sulfoxide (DMSO) (6 ml) was added. The precipitate dissolved and after 8 hr the ninhydrin test indicated complete reaction. TFA (80 μl, 1.1 mmol) was added and the solvents were removed by rotary evaporation (oil pump). The residue was dissolved in 95% EtOH (5 ml), and the product was precipitated by adding the EtOH solution to 100 ml of rapidly stirred EtOAc. The precipitate was collected, washed with EtOAc and Et_2O, and dried in vacuo: 726 mg (92.6%); mp 155–160°. TLC revealed a major component at R_f 0.80 (E) and two small trailing impurities. The product was reprecipitated from 90 ml of EtOH–EtOAc–Et_2O (1:1:1): 665 mg (91.7% recovery); mp 189.5–191.5°; $[\alpha]^{21}D$ −46.4° (c 1.03, DMF); TLC (E) 0.80. Anal. ($C_{32}H_{48}N_8O_{11}S_2·H_2O$) C, H, N, S.

Boc-Tyr(Boc)-Thi-Gln-Asn-Cys(Ec)-Pro (7). The Boc group of 6 (784 mg, 1.00 mmol) was removed in TFA (5 ml) as described for the preparation of 5. The peptide salt was dissolved in DMF (13 ml); the pH was adjusted to 7.5 with *i*-Pr_2NEt (0.26 ml, 1.5 mmol), and Boc-Tyr(Boc)-ONp[40] (603 mg, 1.2 mmol) was added. After 2 hr the pH had dropped to 5.5 and was readjusted to 7 with *i*-Pr_2NEt (0.08 ml, 0.5 mmol). The reaction went to completion overnight. TFA (0.15 ml, 2.0 mmol) was added and the product was isolated as described for 6: 954 mg (91%); mp 160–162° dec. TLC (F) showed a major component at R_f 0.52 and a small impurity at R_f 0.18. The product was used for the next step without further purification. For analysis a 32-mg portion was reprecipitated from DMF with EtOAc with 80% recovery: mp 158.5–161° dec; $[\alpha]^{21}D$ −40.4° (c 0.67, DMF). TLC showed one trace impurity still present at R_f 0.18 (F) and R_f 0.51 (G), with the product spot at R_f 0.52 (F) and R_f 0.58 (G). Anal. ($C_{46}H_{65}N_9O_{15}S_2·H_2O$) C, H, N.

Boc-Cys(Ec)-Tyr-Thi-Gln-Asn-Cys(Ec)-Pro (1). The Boc groups of 7 (837 mg, 0.80 mmol) were removed by TFA (5 ml) as described for 5. The peptide salt was dissolved in DMF (8 ml), the pH was adjusted to 7.5 with *i*-Pr_2NEt (0.20 ml, 1.2 mmol), and Boc-Cys(Ec)-OTCP[38] (452 mg, 0.96 mmol) was added. After 40 min the pH was readjusted from 5.5 to 7 with *i*-Pr_2NEt (0.07 ml, 0.4 mmol). The ninhydrin test indicated complete reaction overnight. TFA (0.12 ml, 1.6 mmol) was added, and the solvents were removed by rotary evaporation. The residue was dissolved in hot 95% EtOH (10 ml). The product precipitated as the warm solution was added to 75 ml of stirred THF–EtOAc (1:2) and was isolated as described for 6: 857 mg (95%); mp 164–166° dec. TLC (C) revealed two impurities in addition to the product at R_f 0.59. The product was reprecipitated from 95% EtOH–EtOAc (5:1) (30 ml) by the addition of Et_2O (20 ml): 804 mg (82% recovery); mp 163–

165° dec; $[\alpha]^{20}D$ −53.3° (c 1.04, DMF); TLC (C) 0.60, (G) 0.58. Anal. ($C_{47}H_{67}N_{11}O_{15}S_3 \cdot H_2O \cdot CH_3CH_2OH$) C, H, N.

Boc-Cys(Ec)-Tyr-Thi-Gln-Asn-Cys(Ec)-Pro-Lys(Boc)-Gly-NH_2 (2). A solution of 1 (224 mg, 0.20 mmol), HBt[20] (54 mg, 0.4 mmol), and Lys(Boc)-Gly-NH_2[21] (84.5 mg, 0.28 mmol) in DMF (2.5 ml) was prepared and cooled to 0°, and DCC (43.6 mg, 0.21 mmol) was added. The solution was stirred for 1 hr at 0° and then allowed to warm to room temperature. The reaction was judged complete after 53 hr. The solvent was removed by rotary evaporation (oil pump) and the residue was precipitated from 95% EtOH (6 ml) solution by cooling to −17°. The precipitate was collected, washed with cold 95% EtOH and Et_2O, and dried in vacuo: 220 mg (78.3%); mp 199.5–202.5° dec; $[\alpha]^{22}D$ −48.1° (c 0.92, DMF); TLC (F) 0.82, (G) 0.72. Anal. ($C_{60}H_{91}N_{15}O_{18}S_3 \cdot 2H_2O$) C, H, N.

[3-Thienylalanine]-8-lysine-vasopressin. A solution of **2** (141 mg, 0.10 mmol) in TFA (2 ml) was prepared. After 30 min the TFA was removed by rotary evaporation and the peptide salt was isolated by lyophilization from redistilled AcOH. The lyophilizate was dissolved in anhydrous liquid NH_3 (100 ml), freshly distilled from Na, and refluxed for 4 hr. The NH_3 was removed by evaporation and lyophilization. The reaction vessel was opened under an atmosphere of N_2, and the residue was dissolved in 90 ml of H_2O and 80 ml of MeOH which had been flushed with N_2. The Ellman test[41] revealed 94% of the theoretical amount of sulfhydryl. A solution of ICH_2CH_2I (28.2 mg, 0.10 mmol) in 10 ml of MeOH was added. The oxidation reaction was followed by the Ellman test and was judged complete after 20 min. After the addition of AcOH (5 ml), the solvents were removed by rotary evaporation. The residue was dissolved in 3 ml of the upper phase and 0.5 ml of the lower phase of the solvent system BuOH–EtOH–pyridine–AcOH–H_2O (4:1:1:0.4:6.4) for purification by partition chromatography.[23] The solution was applied to a column of Sephadex G-25 (100–200 mesh, 2.15 × 111 cm) which had been equilibrated with both phases of the solvent system. The column was eluted with upper phase at a flow rate of 15 ml/hr, and the eluate was collected in 7.0-ml fractions. The peptide material was detected by the Folin–Lowry method.[42] The product emerged as a sharp symmetrical peak with a maximum at R_f 0.13, well resolved from a small unsymmetrical peak at R_f 0.34. Fractions comprising the major peak (R_f 0.15–0.11) were pooled and the solvents removed by rotary evaporation and lyophilization: 73.3 mg (69%); TLC (H) 0.15. This material was further purified by gel filtration[24] on a column of Sephadex G-25 (200–270 mesh, 2.82 × 68 cm) in 0.2 *N* AcOH at a flow rate of 26 ml/hr. Fractions of 4.0 ml were read at 280 nm. The product emerged as a single sharp symmetrical peak with a maximum at 79% of the column volume. Fractions 77–90, comprising the peak area, were pooled and the solvent was removed by lyophilization: 69.7 mg (65%); $[\alpha]^{22}D$ −30.6° (c 0.49, 1 *N* AcOH); TLC (G) 0.31, (H) 0.15. Amino acid analysis[43] following 22 hr of hydrolysis in deaerated 6 *N* HCl at 110° gave the following ratios: Asp, 1.03; Glu, 1.05; Pro, 1.04; Gly, 0.97; 1/2 Cys, 1.92; Thi, 1.05; Try, 1.03; Lys 1.01; NH_3, 2.88. Anal. ($C_{44}H_{63}N_{13}O_{12}S_3 \cdot 2H_2O \cdot 2CH_3CO_2H$) C, H, N.

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References and Notes

(1) *J. Biol. Chem.*, **247**, 977 (1972).
(2) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954).
(3) V. du Vigneaud, M. F. Bartlett, and A. Jöhl, *J. Am. Chem. Soc.*, **79**, 5572 (1957).
(4) V. du Vigneaud, D. T. Gish, P. G. Katsoyannis, and G. P. Hess, *J. Am. Chem. Soc.*, **80**, 3355 (1958).
(5) H. Nesvadba, J. Honzl, and J. Rudinger, *Collect. Czech. Chem. Commun.*, **28**, 1691 (1963); A. Chimiak and J. Rudinger, *ibid.*, **30**, 2592 (1965); J. Rudinger and I. Krejčí, *Experientia*, **18**, 585 (1962).
(6) R. A. Boissonnas and R. L. Huguenin, *Helv. Chim. Acta*, **43**, 182 (1960).
(7) R. D. Kimbrough, Jr., and V. du Vigneaud, *J. Biol. Chem.*, **236**, 778 (1961).
(8) R. L. Huguenin and R. A. Boissonnas, *Helv. Chim. Acta*, **45**, 1629 (1962).
(9) P. G. Katsoyannis and V. du Vigneaud, *J. Biol. Chem.*, **233**, 1352 (1958).
(10) R. Acher, J. Chauvet, M. T. Lenci, F. Morel, and J. Maetz, *Biochim. Biophys. Acta*, **42**, 379 (1960); W. H. Sawyer, R. A. Munsick, and H. B. van Dyke, *Nature (London)*, **184**, 1464 (1959).
(11) K. Eisler, J. Rudinger, and F. Šorm, *Collect. Czech. Chem. Commun.*, **31**, 4563 (1966).
(12) R. A. Boissonnas and St. Guttmann, *Helv. Chim. Acta*, **43**, 190 (1960).
(13) St. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **43**, 200 (1960).
(14) M. F. Ferger and V. du Vigneaud, *J. Biol. Chem.*, **174**, 241 (1948).
(15) F. W. Dunn and J. M. Stewart, *J. Med. Chem.*, **14**, 779 (1971).
(16) M. C. Khosla, M. M. Hall, R. R. Smeby, and F. M. Bumpus, *J. Med. Chem.*, **17**, 1156 (1974).
(17) St. Guttmann, *Helv. Chim. Acta*, **49**, 83 (1966).
(18) J. J. Ferraro, *Biochem. Prep.*, **13**, 39 (1971).
(19) D. A. Jones, Jr., R. A. Mikulec, and R. H. Mazur, *J. Org. Chem.*, **38**, 2865 (1973).
(20) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
(21) R. L. Huguenin and St. Guttmann, *Helv. Chim. Acta*, **48**, 1885 (1965).
(22) F. Weygand and G. Zumach, *Z. Naturforsch. B*, **17**, 807 (1962).
(23) D. Yamashiro, *Nature (London)*, **201**, 76 (1964); D. Yamashiro, D. Gillessen, and V. du Vigneaud, *J. Am. Chem. Soc.*, **88**, 1310 (1966).
(24) J. Porath and P. Flodin, *Nature (London)*, **183**, 1657 (1959).
(25) P. Holton, *Br. J. Pharmacol. Chemother.*, **3**, 328 (1948).
(26) R. A. Munsick, *Endocrinology*, **66**, 451 (1960).
(27) J. M. Coon, *Arch. Int. Pharmacodyn. Ther.*, **62**, 79 (1939).
(28) "The Pharmacopeia of the United States of America", 18th revision, Mack Publishing Co., Easton, Pa., 1970, p 469.
(29) R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, **66**, 860 (1960).
(30) See ref 28, p 771.
(31) W. A. Jeffers, M. M. Livezey, and J. H. Austin, *Proc. Soc. Exp. Biol. Med.*, **50**, 184 (1942).
(32) W. H. Sawyer, *Endocrinology*, **63**, 694 (1958).
(33) H. O. Schild, *J. Physiol. (London)*, **101**, 115 (1942).
(34) R. D. Kimbrough, Jr., W. D. Cash, L. A. Branda, W. Y. Chan, and V. du Vigneaud, *J. Biol. Chem.*, **238**, 1411 (1963).
(35) J. Meienhofer and Y. Sano, *J. Am. Chem. Soc.*, **90**, 2996 (1968).
(36) M. F. Bartlett, A. Jöhl, R. Roeske, R. J. Stedman, F. H. C. Stewart, D. N. Ward, and V. du Vigneaud, *J. Am. Chem. Soc.* **78**, 2905 (1956).
(37) H. Zahn and K. Hammerström, *Chem. Ber.*, **102**, 1048 (1969).
(38) H. T. Storey, J. Beacham, S. F. Cernosek, F. M. Finn, C. Yanaihara, and K. Hofmann, *J. Am. Chem. Soc.*, **94**, 6170 (1972).
(39) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970); D. F. Dyckes, J. J. Nestor, Jr., M. F. Ferger, and V. du Vigneaud, *J. Med. Chem.*, **17**, 250 (1974).
(40) E. Schnabel, H. Herzog, P. Hoffmann, E. Klauke, and I. Ugi, *Justus Liebigs Ann. Chem.*, **716**, 175 (1968).
(41) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
(42) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
(43) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).