A Solid-Phase Synthesis of [8-Arginine]-vasopressin through a Crystalline Protected Nonapeptide Intermediate and Biological Properties of the Hormone[1-3]

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Abstract: The neurohypophyseal hormone [8-arginine]-vasopressin has been synthesized by the solid-phase method. The protected nonapeptide intermediate, *S*-benzyl-*N*-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-N^G-tosyl-L-arginylglycinamide, has been obtained in crystalline form. Removal of the protecting groups from the crystalline protected nonapeptide followed by oxidative cyclization provided highly active hormone preparations in excellent yields. Further purification by ion exchange chromatography afforded [8-arginine]-vasopressin possessing 503 ± 53 units/per mg of rat antidiuretic activity, 487 ± 15 units/mg of rat pressor activity, 12.0 ± 0.2 units/mg of rat oxytocic activity, 30–120 units/mg of rabbit milk-ejecting activity, and 100 ± 5 units/mg of avian vasodepressor activity.

The solid-phase method of peptide synthesis[5] has been successfully used for the preparation of several naturally occurring neurohypophyseal peptides such as oxytocin,[6] [8-lysine]-vasopressin,[7] and glumitocin.[8] This paper describes the application of this method to the synthesis of the mammalian antidiuretic principle, [8-arginine]-vasopressin,[9] Figure 1. Chiefly through the crystallization of the protected nonapeptide (*S*-benzyl-*N*-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-N^G-tosyl-L-arginylglycinamide, I, Figure 2) the hormone was obtained in excellent yield and with high biological potency.

Earlier syntheses of [8-arginine]-vasopressin gave preparations exhibiting considerable variation in the degree of biological activities; rat pressor activities ranging from 200 to 500 units/mg have been obtained.[11-15] One reason for this variability could be that the protected nonapeptide intermediates were invariably obtained as amorphous powders which were not freed from by-products. The final hormone preparations were probably still contaminated to various degrees with by-products in spite of extensive purification by such modern techniques as gel filtration, partition and ion exchange chromatography, countercurrent distribution, or high-voltage electrophoresis. The power of crystallization of a protected nonapeptide from organic solvent for removal of impurities was previously demonstrated in syntheses of [8-lysine]-vasopressin;[7,16] highly active hormone preparations were obtained in excellent yields from crystalline II (Figure 2).[17]

When designing our solid-phase synthesis of [8-arginine]-vasopressin, we decided to explore whether crystalline II could serve as seed to induce crystallization of I. The protecting groups for I were therefore selected accordingly, *i.e.*, the *p*-toluenesulfonyl group for both the guanidino function of the arginine residue and for the amino group of the *N*-terminal cysteine residue, the benzyl group for the sulfhydryl groups of the cysteine residues. The general procedure de-

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(2) Abbreviations follow the rules of the IUPAC–IUB Commission on Biochemical Nomenclature, in *Biochemistry*, **5**, 2485, 1445 (1966); **6**, 362 (1967); *J. Biol. Chem.*, **241**, 2491 (1966).

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(4) (a) Children's Cancer Research Foundation; (b) Mount Sinai Medical and Graduate Schools.

(5) R. B. Merrifield, *J. Amer. Chem. Soc.* **85**, 2149 (1963); *Advan. Enzymol.*, **32**, 221 (1969).

(6) M. Manning, *J. Amer. Chem. Soc.*, **90**, 1348 (1968); H. C. Beyerman, C. A. M. Boers-Boonekamp, and H. Maassen van den Brink–Zimmermannová, *Recl. Trav. Chim. Pays-Bas*, **87**, 257 (1968); E. Bayer and H. Hagenmaier, *Tetrahedron Lett.*, 2037 (1968); N. Inukai, K. Nakano, and M. Murakami, *Bull. Chem. Soc. Jap.*, **41**, 182 (1968).

(7) J. Meienhofer and Y. Sano, *J. Amer. Chem. Soc.*, **90**, 2996 (1968).

(8) M. Manning, T.-C. Wuu, J. W. M. Baxter, and W. H. Sawyer, *Experientia*, **24**, 659 (1968).

(9) K. Lübke reported a solid-phase synthesis of [8-arginine]-vasopressin using different protecting groups; see ref 1.

(10) V. du Vigneaud, H. C. Lawler, and E. A. Popenoe, *J. Amer. Chem. Soc.*, **75**, 4880 (1953); R. Acher and J. Chauvet, *Biochim. Biophys. Acta*, **12**, 487 (1953).

(11) V. du Vigneaud, D. T. Gish, and P. G. Katsoyannis, *J. Amer. Chem. Soc.*, **76**, 4751 (1954); P. G. Katsoyannis, D. T Gish, and V. du Vigneaud, *ibid.*, **79**, 4516 (1957); V. du Vigneaud, D. T. Gish, P. G. Katsoyannis, and G. P. Hess, *ibid.*, **80**, 3355 (1958).

(12) R. O. Studer and V. du Vigneaud, *ibid.*, **82**, 1499 (1960).

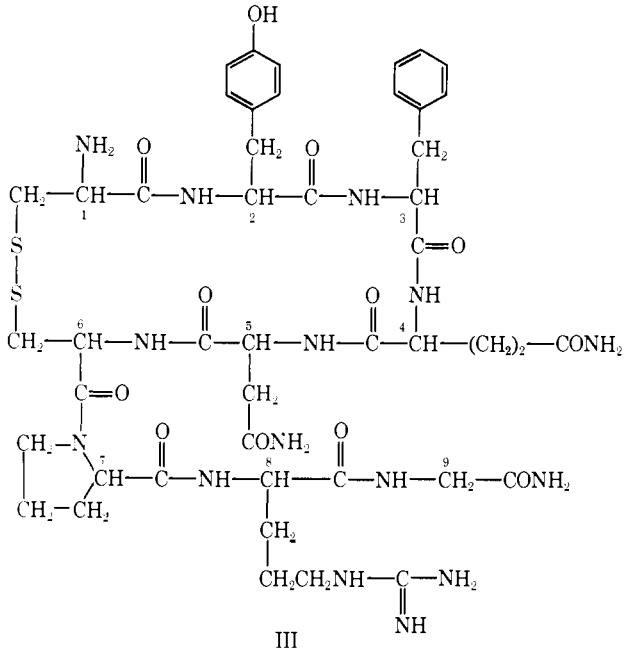
(13) R. L. Huguenin and R. A. Boissonnas, *Helv. Chim. Acta*, **45**, 1629 (1962).

(14) R. O. Studer, *Chimia*, **17**, 22 (1963); *Helv. Chim. Acta*, **46**, 421 (1963).

(15) M. Bodanszky, M. A. Ondetti, B. Rubin, J. J. Piala, J. Fried, J. T. Sheehan, and C. A. Birkhimer, *Nature (London)*, **194**, 485 (1962); M. Bodanszky, M. A. Ondetti, C. A. Birkhimer, and P. L. Thomas, *J. Amer. Chem. Soc.*, **86**, 4452 (1964).

(16) J. Meienhofer and V. du Vigneaud, *ibid.*, **82**, 2279 (1960).

(17) Deprotection of recrystallized II by sodium in liquid ammonia, oxidative cyclization (disulfide bond formation), desalting, and lyophilization gave 95–100% yields of [8-lysine]-vasopressin with approximately 250 units/mg of rat pressor activity. In contrast, amorphous II after the same operations gave crude materials with approximately 60–120 units/mg of rat pressor activity.

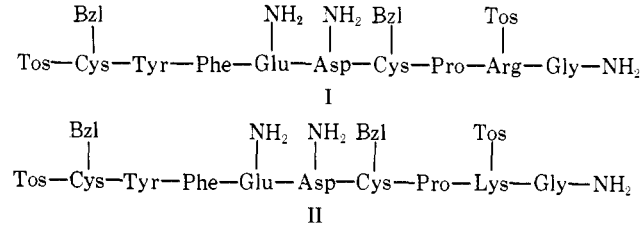


III

Figure 1. The structure of [8-arginine]-vasopressin.[10]

scribed by Marshall and Merrifield[18] for solid-phase peptide synthesis was modified in various ways: reaction times were generally longer, the amount of free amino groups after each deprotection step was determined by titration,[19] and the N-protecting groups of the asparagine and glutamine moieties were removed by treatment with anhydrous trifluoroacetic acid.[20] Dicyclohexylcarbodiimide was used as the coupling reagent for N^{α}-*tert*-butyloxycarbonyl-N^{G}-tosyl-L-arginine,[21,22] *tert*-butyloxycarbonyl-L-proline,[22,23] *tert*-butyloxycarbonyl-L-phenylalanine,[22,23] *tert*-butyloxycarbonyl-L-tyrosine,[22,23] and *S*-benzyl-*N*-tosyl-L-cysteine.[24] The remaining optically active amino acid residues were introduced into the growing peptide chain *via* active esters, *i.e.*, *S*-benzyl-*N*-*tert*-butyloxycarbonyl-L-cysteine *p*-nitrophenyl ester,[25] *tert*-butyloxycarbonyl-L-asparagine *p*-nitrophenyl ester[26] or *o*-nitrophenylsulfenyl-L-asparagine *N*-hydroxysuccinimide ester,[27] and *tert*-butyloxycarbonyl-L-glutamine *p*-nitrophenyl ester[28] or *o*-nitrophenylsulfenyl-L-glutamine *N*-hydroxysuccinimide ester.[27]

Removal of the completed, protected peptide chain from the polymer was effected by ammonolysis as



I

II

Figure 2. [8-Arginine]-vasopressin protected nonapeptide,[13] I, and [8-lysine]-vasopressin protected nonapeptide,[7,16] II.

described for [8-lysine]-vasopressin.[7] Resulting crude oils were converted into solid powders by precipitation with ether or absolute ethanol from dimethylformamide solution. Crystalline II did not induce crystallization of I but the latter crystallized spontaneously from dimethylformamide–ethanol and subsequently also from glacial acetic acid–ethanol. Each preparation was recrystallized to constant melting point and optical rotation. Yields of crystalline I ranged from 14 to 16% based on the glycine content of the starting *tert*-butyloxycarbonylglycyl-resins. Approximately the same yield of crystalline I was obtained when the protected peptide chain had been removed from the solid support by *N*-methylpiperidine catalyzed transesterification[29] followed by conversion of the isolated protected nonapeptide methyl ester to the amide; hence this route offered no advantage over the direct peptide-resin ammonolysis, which has been shown to proceed, in part, *via* intermediate transesterification.[29]

Conversion of I to [8-arginine]-vasopressin was performed as described[7,16] for [8-lysine]-vasopressin: treatment with sodium in liquid ammonia to remove all protecting groups, oxidative cyclization, desalting on an Amberlite IRC-50 column,[30,31] and lyophilization. A colorless powder was obtained which had a rat pressor activity of 350 units/mg prior to further purification. In several experiments the yield of highly active hormone from I was between 85 and 95%. These favorable results depended largely on the degree of purity of the protected nonapeptide (I); crystallization of this intermediate proved to be of great value in the synthesis of [8-arginine]-vasopressin.

The hormone was further purified by ion exchange chromatography on IRC-50 in 0.5 *M* ammonium acetate buffer at pH 6.4.[32] The center fractions of the main peak gave a 32% yield of highly purified hormone; material obtained from both sides (31%) had lower rat pressor potency. The total recovery of activity from such a chromatographic purification[33] in several experiments was about 80%. The homogeneity of the purified [8-arginine]-vasopressin from the center fractions was checked by electrophoresis on paper and

(18) G. R. Marshall and R. B. Merrifield, *Biochemistry*, **4**, 2394 (1965).
(19) E. Bayer and H. Hagenmaier, *Tetrahedron Lett.*, 2037 (1968).
(20) H. Takashima, V. du Vigneaud, and R. B. Merrifield, *J. Amer. Chem. Soc.*, **90**, 1323 (1968).
(21) J. Ramachandran and C. H. Li, *J. Org. Chem.*, 27, 4006 (1962).
(22) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 188 (1967).
(23) G. W. Anderson and A. C. McGregor, *J. Amer. Chem. Soc.*, **79**, 6180 (1957).
(24) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, 20, 1190 (1955); V. du Vigneaud, M. F. Bartlett, and A. Jöhl. *J. Amer. Chem. Soc.*, **79**, 5572 (1957); J. A. MacLaren, W. E. Savige, and J. M. Swan, *Aust. J. Chem.*, 11, 345 (1958).
(25) S. Hörnle, *Hoppe-Seyler's Z. Physiol. Chem.*, **348**, 1355 (1967); H. C. Beyerman, C. A. M. Boers-Boonekamp, and H. Maassen van den Brink-Zimmermannová, *Recl. Trav. Chim. Pays-Bas*, **87**, 257 (1968).
(26) E. Sandrin and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1637 (1963); E. Schröder and E. Klieger, *Justus Liebigs Ann. Chem.*, 673, 208 (1964).
(27) C. Meyers, R. T. Havran, I. L. Schwartz, and R. Walter, *Chem. Ind. (London)*, 136 (1969).
(28) H. Zahn, W. Danho, and B. Gutte, *Z. Naturforsch. B*, **21**, 763 (1966).
(29) H. C. Beyerman, H. Hindriks, and E. W. B. De Leer, *Chem. Commun.*, 1668 (1968).
(30) H. B. F. Dixon and M. P. Stack-Dunne, *Biochem. J.*, **61**, 483 (1955).
(31) Desalting by dialysis using a Diaflo ultrafilter (UM-5, retaining solutes of molecular weights above 500) and 0.2 *N* acetic acid at 4° gave repeatedly slightly colored powders in about 90% yields which had lower rat pressor activities than preparations desalted on IRC-50.
(32) A. Light, R. Acher, and V. du Vigneaud, *J. Biol. Chem.*, **228**, 633 (1957).
(33) Gel filtration on Sephadex G-15 in 0.2 *N* acetic acid has been used for the purification of neurohypophyseal hormones [M. Manning, T.-C. Wuu, and J. W. M. Baxter, *J. Chromatogr.*, **38**, 396 (1968)]. [8-Arginine]-vasopressin purified by this technique had lower rat pressor activity than preparations purified on IRC-50 and contained an impurity as revealed by paper electrophoresis.

on cellulose polyacetate. Amino acid and elemental analyses gave the expected values.

This preparation of the mammalian antidiuretic hormone was assayed for biological activities characteristic of the posterior pituitary hormones. In the rat antidiuretic assay the hormonal preparation exhibited an activity of 503 ± 53 units/mg which is an increase over the highest value reported earlier.[34] The rat pressor activity of the hormone amounted to 487 ± 15 units/mg, a value comparable to that previously reported for this peptide by Bodanszky, *et al.*[15] With respect to the "oxytocin-like" activities, the [8-arginine]-vasopressin exhibited an activity of 12.0 ± 0.2 units/mg in the *in vitro* rat uterotonic assay (without magnesium added to the ambient fluid); this value was also found by Heller[35] and van Dyke, *et al.*[34] The milk-ejecting potency ranged from 30 to 120 units/mg. Autoinhibition and, particularly, the inhibition of responses to the reference standard given subsquent to [8-arginine]-vasopressin were responsible for the considerable variation of potency observed. The inhibition evoked by the hormone was reduced by repeated injections (up to eight times) of reference standard. In the avian vasodepressor assay an activity of 100 ± 5 units/mg was found; other investigators reported values ranging from 42 units/mg[36] to 57 ± 6 units/mg.[13]

Experimental Section

Materials and Methods. *tert*-Butyloxycarbonyl amino acids were prepared from *tert*-butyloxycarbonyl azide[37] (Aldrich Chemical Co., Inc., Milwaukee, Wis.) with pH-stat controlled addition of NaOH according to Schnabel.[22] HCl (2 *N*) was used for final acidification instead of citric or acetic acid. All protected amino acid derivatives were repeatedly recrystallized until the criteria of purity compared well with reported data.[38] The Merrifield resin (chloromethylated copolystyrene–2% divinylbenzene, containing 1.04 mequiv of Cl/gram) was obtained from Cyclo Chemical Corp., Los Angeles, Calif. The glass vessel and the mechanical shaker[5] used for synthesis were custom built. Analytical grade solvents were obtained and were distilled prior to use: glacial acetic acid, trifluoroacetic acid, triethylamine from ninhydrin and subsequently from sodium under nitrogen, dimethylformamide *in vacuo* under nitrogen (stored over Linde 4A Molecular Sieve), and methylene chloride from P_2O_5 (stored over Linde type 4A Molecular Sieve). Dicyclohexylcarbodiimide was distilled *in vacuo* under nitrogen. IRC-50 (XE-64 or IRP-64) was obtained from Serva Entwicklungslabor, Heidelberg, Germany. Ammonium acetate buffer (0.5 *N*) was prepared by introducing ammonia gas (17 g; Matheson Gases, East Rutherford, N. J.) into acetic acid (1 *N*, 1000 ml) and diluting to 2000 ml. Ultrapure water was used, provided by a tank filter system (Hydro Service and Supplies, Inc., Hingham, Mass.). Commercial analytical grade ammonium acetate contains 0.08% impurities which remain in a lyophilisate (1 mg from 300 ml of 0.5 *N* buffer).

Melting points were determined on a Fisher-Johns apparatus and are corrected. Optical rotations were done with a Rudolph Model 200 manual spectropolarimeter. High-voltage paper electrophoresis (Whatman 3MM) was performed in a Gilson Electrophorator, Model D. Electrophoresis on cellulose polyacetate strips (Gelman, Sepraphore III, 2.5 × 15 cm) was done in a Gelman Chamber No. 51101 using a Beckman Duostat Model RD power supply. Detection was achieved by ninhydrin spray. Elemental analyses were performed by Werby Laboratories, Inc., Boston, Mass. Amino acid analyses[39] were done with a Phoenix analyzer, Model M-6800. Chromatographic separations were monitored by optical density at 280 mμ. Solutions for lyophilization were shell-frozen at controlled mild temperatures, not lower than −30°. Lower freezing temperatures were found to have denaturing effects on peptides and proteins.

***S*-Benzyl-*N*-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-N^G-tosyl-L-arginylglycyl-resin.** *tert*-Butyloxycarbonylglycyl-resin, containing 0.5 mmol of *tert*-butyloxycarbonylglycine/g, was prepared from chloromethylated copolystyrene–2% divinylbenzene following the standard procedure[5] and using ethyl acetate as a solvent. A cycle for the incorporation of each amino acid into the growing peptide chain involved the following washing and reaction steps (8 ml of solvent/g of starting resin was used throughout): (1) three washings with glacial acetic acid; (2) 1.25 *N* HCl in glacial acetic acid for 30 min; (3) three washings with glacial acetic acid; (4) three washings with ethanol; (5) three washings with dimethylformamide; (6) 10% triethylamine in dimethylformamide for 10 min; (7) three washings with dimethylformamide; (7a) the combined collected washings from (6) and (7) were evaporated to dryness and the chloride content of the residue was titrated according to Volhard;[19] (8) three washings with methylene chloride; (9) addition of 4–8 equiv of *tert*-butyloxycarbonyl amino acid in methylene chloride and mixing for 10 min; (10) 4–8 equiv of dicyclohexylcarbodiimide in methylene chloride, reaction for 12–16 hr; (11) three washings with methylene chloride; (12) three washings with ethanol. For the active ester reactions [Boc-Cys(Bzl)-ONp, Boc-Asn-ONp, Nps-Asn-OSu, Boc-Gln-ONp, Nps-Gln-OSu] step 8 involved addition of 4–8 equiv of Boc-amino acid active ester in dimethylformamide and reaction for 15–22 hr, followed by (9) three washings with dimethylformamide and (10) three washings with ethanol. The removal of the *tert*-butyloxycarbonyl protecting group from the asparagine and glutamine residues was performed with anhydrous trifluoroacetic acid (step 2, 15 min).[20] After the completion of the protected nonapeptide, the peptide-resin was removed from the reaction vessel, thoroughly washed with ethanol and ether, and dried *in vacuo* (P_2O_5 and KOH).

***S*-Benzyl-*N*-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-N^G-tosyl-L-arginylglycinamide. I. A. By Ammonolysis of the Peptide-Resin.** The protected nonapeptide-resin was suspended in a 1:1 mixture of dimethylformamide–ethanol (40 ml/g of peptide-resin). The mixture was saturated with dry ammonia at −5°. The flask was sealed and the mixture stirred for 72 hr at room temperature. The resin was removed by filtration and washed several times with dimethylformamide. The combined filtrate was evaporated *in vacuo* to give an oil which was precipitated from dimethylformamide by ether or ethanol at −70°. The gelatinous precipitate was filtered, thoroughly washed with ethanol and ether, and dried *in vacuo* (P_2O_5 and KOH) to give a cream-colored powder (412 mg from 3 g of peptide-resin, 28%, based on 0.5 mmol of glycine/g of starting resin), mp 210–225°. Crystallization was effected from acetic acid–absolute ethanol. After a second recrystallization, colorless crystals were obtained (225 mg, 15.3%): mp 230–231°, $[\alpha]^{22}D$ −21.9° (*c* 1, dimethylformamide); lit.[13] mp 198–199° dec, $[\alpha]^{22}D$ −18.5 ± 1° (*c* 1, dimethylformamide). *Anal.* Calcd for $C_{74}H_{91}N_{15}O_{16}S_4$ (1574.8): C, 56.4; H, 5.82; N, 13.3; S, 8.14. Found: C, 56.3; H, 6.13; N, 13.2; S, 8.39.

Amino acid analysis[35] (6 *N* HCl, 110°, 24 hr) gave the following molar ratios, Asp being taken as 1.0: Arg, 1.08; Asp, 1.0; Glu, 1.03; Pro, 1.05; Gly, 0.94; Tyr, 0.94; Phe, 0.97.

B. *Via* **Alcoholysis of the Peptide-Resin.** The protected nonapeptide-resin (1 g) was suspended in a 1:5 mixture of dimethylformamide–2 *N* methanolic *N*-methylpiperidine[29] (30 ml). After stirring the mixture for 18 hr at room temperature, the resin was removed by filtration and washed several times with dimethylformamide. The combined filtrate was evaporated *in vacuo* to give an oil (0.6 g), which was purified by column chromatography on Sephadex LH-20 (1.2 × 96 cm) in dimethylformamide. An oil (418 mg) was obtained by evaporating *in vacuo* the fractions from the main peak. The oil was taken up in dimethylformamide (5 ml), absolute ethanol (50 ml) was added, and the mixture was saturated with dry ammonia at −5° and kept in a sealed flask for 54 hr at room temperature. Working up as described in (A) gave colorless crystals (60 mg, 12%), mp 229–230°, $[\alpha]^{20}D$ −21.4° (*c* 0.6, dimethylformamide).

(34) H. B. van Dyke, W. H. Sawyer, and N. I. A. Overweg, *Endocrinology*, 73, 637 (1963).
(35) H. Heller, *Symp. Zool. Soc. (London)*, 9, 93 (1963).
(36) R. A. Munsick, W. H. Sawyer, and H. B. van Dyke, *Endocrinology*, 66, 860 (1960).
(37) L. A. Carpino, *J. Amer. Chem. Soc.*, 79, 4427 (1957).
(38) E. Bayer, G. Jung, and H. Hagenmaier, *Tetrahedron*, 24, 4853 (1968).
(39) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, 30, 1190 (1958).

[8-Arginine]-vasopressin, L-Half-cystinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-half-cystinyl-L-prolyl-L-arginyl-glycinamide 1,6-Disulfide, III. Recrystallized protected nonapeptide I (73.2 mg, 46 μmol) was dissolved in liquid ammonia (150 ml) which had been distilled from sodium. Sodium was added in small quantities to the boiling ammonia solution over a period of 15–20 min until a permanent blue color remained for 30 sec, approximately 10 mg of sodium being necessary. Glacial acetic acid (2 drops) was added, the ammonia was evaporated to a small volume, and the remainder removed from the frozen state (at approximately −77°) at water pump vacuum. The lyophilisate was dissolved in deoxygenated 0.2 *N* acetic acid (200 ml) under nitrogen. The pH was adjusted to 6.8 with 0.5 *N* ammonia and air was passed through the solution for 2 hr after which the pH was adjusted to 4.8 with acetic acid. A total of 18,000 units of rat pressor activity was obtained. The solution was applied to an IRC-50 column (2 × 7 cm) in the H^+ form for desalting.[30] The column was washed with 0.25% acetic acid (250 ml) until the pH of the effluent reached approximately 3.5. The hormone was eluted with a pyridine–acetate solution (30 ml of pyridine and 4 ml of acetic acid diluted with water to 100 ml). The eluate was lyophilized to give a colorless powder (56.5 mg, 98% based on I) with a rat pressor activity of 270 units/mg. Following another deprotection of I (40 mg) a 90% yield of III (28.5 mg) with an activity of 350 rat pressor units/mg was obtained.

Purification of [8-Arginine]-vasopressin by Ion Exchange Chromatography. A preparation of III with 270 rat pressor units/mg (59 mg) was dissolved in 0.5 *M* ammonium acetate buffer at pH 6.4 (2 ml) and applied to a column (1.1 × 55 cm) of IRC-50 which had been equilibrated with the buffer at 4°. The column was eluted with the same buffer at 4° with a flow rate of 5 ml/hr; the main peak was preceeded by two small peaks. Lyophilization of the center portion of the main peak gave [8-arginine]-vasopressin (19 mg, 32%) possessing approximately 500 rat pressor units/mg. Lyophilized material from both sides of the main peak (18 mg, 31%) exhibited lower rat pressor activity; the total recovery of activity was 80%.

Purified III exhibited $[\alpha]^{22}D$ −22° (*c* 0.22, 1 *N* acetic acid); high voltage paper electrophoresis (3450 V, 65 V cm^{-1}, 40 min, room temperature) gave single spots: mobility, 11.5 cm at pH 2.1 ($HCOOH–CH_3COOH–H_2O$, 25:87:888 v/v), and 8.6 cm at pH 6.5 (pyridine–$CH_3COOH–H_2O$, 100:4:896 v/v); electrophoresis on cellulose polyacetate (200 V, 14.3 V cm^{-1}, 20 min, room temperature, 0.02 *M* sodium phosphate buffer pH 6.5) gave a single spot, mobility 0.8 cm. A sample for analysis was dried at 105° for 16 hr *in vacuo* (P_2O_5 and KOH).

Anal. Calcd for $C_{46}H_{65}O_{12}N_{15}S_2$–$2CH_3COOH$–$2H_2O$ (1240.4): C, 48.4; H, 6.26; N, 16.9; H_2O, 2.90. Found: C, 48.3; H, 6.16; N, 16.5; H_2O, 2.80 (Karl Fischer).

Amino acid analysis (6 *N* HCl, 105°, 24 hr) gave the following molar ratios, glycine being taken as 1.0: Arg, 1.02; Asp, 1.02; Glu, 1.08; Pro, 1.01; Gly, 1.0; Cys, 1.9; Tyr, 0.98; Phe, 1.03; NH_3, 2.9.

Bioassay Method. Assays for antidiuretic activity were performed on anesthetized, hydrated Sprague-Dawley male rats according to the method of Jeffers, Livezey, and Austin[40] as modified by Sawyer;[41] maximal depression of urine flow, in contrast to average duration of the response, was used to measure the antidiuretic activity. Assays were carried out on 12 rats; not more than six hormone injections[42] were given to each animal. Rat pressor assays were carried out on nine atropinized, urethane-anesthetized male rats as described in the United States Pharmacopeia.[43] Oxytocic assays were performed on six isolated uterine horns from three rats in natural estrus according to the method of Holton,[44] modified by Munsick[45] with the use of magnesium-free van Dyke–Hastings solution as the bathing fluid. The milk-ejecting activity was determined in three anesthetized, lactating rabbits following the procedure of Chan.[46] Avian vasodepressor assays were performed on four conscious chickens according to the procedure employed by Munsick, Sawyer, and van Dyke.[36] The biological activities were measured against the USP posterior pituitary reference standard; in all of these bioassays the four-point design was used and standard errors were calculated according to the method of Bliss.[47]

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(40) W. A. Jeffers, M. M. Livezey, and J. H. Austin, *Proc. Soc. Exp. Biol. Med.*, **50**, 184 (1942).
(41) W. H. Sawyer, *Endocrinology*, **63**, 694 (1958).
(42) P. S. Tata and O. H. Gauer, *Pfluegers Arch.*, **290**, 279 (1966).
(43) "The Pharmacopeia of the United States," 17th Revision, Mack, Easton, Pa., 1965, p 750.
(44) P. Holton, *Brit. J. Pharmacol.*, **3**, 328 (1948).
(45) R. A. Munsick, *Endocrinology*, **66**, 451 (1960).
(46) W. Y. Chan, *J. Pharmacol. Exp. Ther.*, **147**, 48 (1965).
(47) C. I. Bliss, "The Statistics of Bioassay," Academic Press, New York, N. Y., 1952.

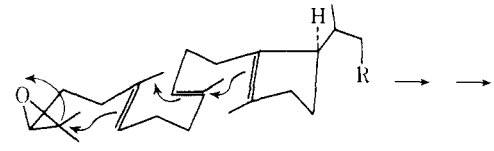
Communications to the Editor

Biogenetic-Type Synthesis of the Isoeuphenol System

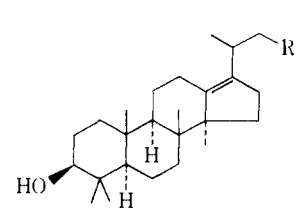
Sir:

As a further development in our continuing bioorganic studies of terpenoid terminal epoxides,[1] we wish to report the total synthesis of the isoeuphenol system (**3**), featuring the stereoselective generation of five asymmetric centers during cyclization of epoxide **1** (Mechanism A).[2] This step represents the closest nonenzymic approach thus far to a basic biosynthetic (all-chair) cyclization scheme[3]—modified (as in Mechanism B) to include the established intermediate,

Mechanism A



1, R = H
2, R = $CH_2CH{=}C(CH_3)_2$



3, R = H
4, R = $CH_2CH{=}C(CH_3)_2$

(1) E. E. van Tamelen, *Accounts Chem. Res.*, **1**, 111 (1968).
(2) For preceding examples of stereoselective, biogenetic-type polycyclizations of terpene terminal epoxides, see *e.g.*, E. E. van Tamelen, A. Storni, E. J. Hessler, and M. Schwartz, *J. Amer. Chem. Soc.*, **85**, 3295 (1963); E. E. van Tamelen and R. G. Nadeau, *ibid.*, **89**, 176 (1967).
(3) (a) G. Stork and A. W. Burgstahler, *ibid.*, 77, 5068 (1955); (b) A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chim. Acta*, **38**, 1890 (1955).