Syntheses of Bufotoxin Analogs¹⁾

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In order to examine the physiological activity the arginine-linked cardiotonic steroids have been synthesized as bufotoxin analogs. Resibufogenin 3-suberoyl-*p*-nitrophenyl ester, derivable from the 3-suberate, was condensed with L-arginine hydrochloride to yield resibufogenin 3-suberoyl-L-arginine. In an analogous manner digitoxigenin 3-suberate was converted into 3-suberoyl-L-arginine, -L-arginyl-L-arginine and -L-arginyl-L-proline *via* the activated *p*-nitrophenyl ester. The structure of the product was verified by leading to the pyrimidine derivative, respectively.

The so-called "bufotoxin" which was isolated from the toad venom by Wieland, *et al.*,³⁾ has recently been characterized as bufogenin 3-suberoylarginine by the degradative means.⁴⁾ It has been suggested that the genuine steroid toad venom may be a peptide conjugate linked with L-arginyl-L-arginyl-L-proline.⁵⁾ The presence of physiologically active peptides in the skin of amphibian animals⁶⁾ also implies the possible occurrence of the steroidal peptide containing a segment of kinin as toad venom constituent. In addition the discovery of the cardenolide and its 3-suberate in the Chinese drug, Ch'an Su,⁷⁾ indicates that the analogous conjugated cardenolide may occur as the natural product. In these respects the physiological activity of the arginine-linked steroidal peptide appears to be of particular interest. The present paper deals with the syntheses of resibufogenin 3-suberoyl-L-arginine and digitoxigenin 3-suberoyl-L-arginine, -L-arginyl-L-arginine and -L-arginyl-L-proline.

In the previous work of this series we developed a synthetic route to digitoxigenin 3-suberoylamino acid from its 3-suberate *via* the activated *p*-nitrophenyl ester.⁸⁾ It is substantiated that condensation reaction of the *p*-nitrophenyl ester with arginine hydrogen halide takes place preferentially at the α -amino group rather than the guanidino residue.⁹⁾ The *p*-nitrophenyl ester method, therefore, appeared to be promising for preparation of the desired compounds. When digitoxigenin 3-suberoyl-*p*-nitrophenyl ester (Ib), derivable from the 3-suberate (Ia), was stirred with L-arginine hydrochloride in aqueous pyridine at room temperature, facile condensation was effected. Subsequent purification by column chromatography on silica gel provided digitoxigenin 3-suberoyl-L-arginine hydrochloride (Ic). This substance gave the positive result with the Sakaguchi's reagent and the negative test with

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derivatives (Ig, Ih) by treatment with acetylacetone and then with diazomethane verified the assigned structures of these peptide conjugates.

The availability of the arginine-linked steroidal peptides related to bufotoxin should serve as a useful reference compound for study of the toad venom constituents. The results of the pharmacological test will be reported elsewhere in the near future.

Experimental¹³⁾

Digitoxigenin 3-Suberoyl-L-arginine Hydrochloride (Ic)—To a solution of digitoxigenin 3-suberoyl-*p*-nitrophenyl ester (Ib)⁸⁾ (40 mg) in pyridine (2 ml) was added an aq. solution of L-arginine·HCl (15 mg in 1 ml) and allowed to stand at room temperature for 16 hr. The resulting solution was concentrated *in vacuo* and the residue obtained was chromatographed on silica gel (500 mg). Elution with AcOEt–MeOH (4:1) and recrystallization of the eluate from MeOH–acetone gave Ic (15 mg) as colorless amorphous substance. mp 109–111°. $[\alpha]_D^{25} +15.0^\circ$ ($c=0.10$, MeOH). Anal. Calcd. for $C_{37}H_{55}O_8N_4 \cdot HCl \cdot 2H_2O$: C, 58.52; H, 8.36; N, 7.38. Found: C, 58.42; H, 8.27; N, 7.19. IR ν_{max}^{KBr} cm⁻¹: 3280 (OH, NH), 1730 (C=O), 1665, 1625 (monosubstituted guanidine C=N).

Transformation of Ic into Pyrimidine Derivative (If)—A solution of Ic (50 mg) in acetylacetone–KHCO₃⁴⁾ (3 ml) was allowed to stand at 40° for 16 hr. The resulting solution was extracted with CHCl₃, and the organic layer was separated and concentrated *in vacuo*. The residue obtained was dissolved in MeOH (1 ml) and treated with an ethereal solution of CH₂N₂ in the usual manner. The crude product was submitted to the preparative thin-layer chromatography (TLC) using AcOEt as developing solvent. The adsorbent corresponding to the spot (*R*_f 0.70) was eluted with AcOEt. Unfortunately the eluate (15 mg) could not be crystallized, but it was substantially homogeneous according to TLC. UV λ_{max}^{EtOH} nm: 217, 239, 303. NMR (4% solution in CDCl₃) δ : 0.88 (3H, s, 18-CH₃), 0.97 (3H, s, 19-CH₃), 2.30 (6H, s, pyrimidine-4,6-CH₃), 3.73 (3H, s, -COOCH₃), 4.90 (2H, m, 21-CH₂), 5.10 (1H, m, 3 α -H), 5.50 (1H, m, -NH-pyrimidine), 5.90 (1H, m, 22-H), 6.31 (1H, s, pyrimidine-5-H), 6.45 (1H, m, -CONH-).

Resibufogenin 3-Suberoyl-*p*-nitrophenyl Ester (IIb)—To a solution of resibufogenin 3-suberate (IIa)¹²⁾ (50 mg) and *p*-nitrophenol (20 mg) in AcOEt was added N,N'-dicyclohexylcarbodiimide (10 mg) under ice-cooling and allowed to stand at room temperature for 16 hr. The precipitated dicyclohexylurea was filtered off and washed with AcOEt. The filtrate and washings were combined and concentrated *in vacuo*. The crude product was submitted to the preparative TLC using benzene–AcOEt (4:1) as developing solvent. The adsorbent corresponding to the spot (*R*_f 0.54) was eluted with AcOEt, and recrystallization of the eluate from acetone–MeOH gave IIb (30 mg) as colorless prisms. mp 97–98°. $[\alpha]_D^{25} +6.3^\circ$ ($c=0.16$, CHCl₃). Anal. Calcd. for $C_{38}H_{47}O_9N$: C, 68.96; H, 7.16; N, 2.12. Found: C, 68.63; H, 7.34; N, 2.18. NMR (4% solution in CDCl₃) δ : 0.78 (3H, s, 18-CH₃), 0.99 (3H, s, 19-CH₃), 3.57 (1H, s, 15 α -H), 5.10 (1H, m, 3 α -H), 6.23 (1H, d, *J*=10 Hz, 23-H), 7.25 (1H, d, *J*=2 Hz, 21-H), 7.30 (2H, d, *J*=10 Hz, , 7.80 (1H, q, *J*=10, 2 Hz, 22-H), 8.25 (2H, d, *J*=10 Hz,).

Resibufogenin 3-Suberoyl-L-arginine Hydrochloride (IIc)—A solution of IIb (50 mg) in pyridine (2 ml) was treated with an aq. solution of L-arginine·HCl (10 mg in 1 ml) in the manner as described in Ic. The crude product was chromatographed on silica gel (600 mg). Elution with AcOEt–MeOH (4:1) and recrystallization of the eluate from MeOH–AcOEt gave IIc (15 mg) as colorless amorphous substance. mp 123–125°. $[\alpha]_D^{25} +25.0^\circ$ ($c=0.08$, MeOH). Anal. Calcd. for $C_{38}H_{56}O_8N_4 \cdot HCl \cdot H_2O$: C, 60.74; H, 7.92; N, 7.46. Found: C, 60.44; H, 7.98; N, 7.36.

Digitoxigenin 3-Suberoyl-L-arginyl-L-arginine Diacetate (Id)—A solution of Ib (40 mg) in pyridine (2 ml) was treated with an aq. solution of L-arginyl-L-arginine·2AcOH¹⁴⁾ (40 mg in 0.5 ml) in the manner as described in Ic. The crude product obtained was chromatographed on silica gel (500 mg). Effluent with MeOH–AcOH–H₂O (3:1:1) was concentrated *in vacuo* below 40°, and the residue obtained was submitted to gel filtration on Sephadex LH-20 using MeOH as eluent. After evaporation of solvent trituration of the eluate with MeOH–ether gave Id (10 mg) as colorless amorphous substance. mp 167–174° (decomp.).

13) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Infrared (IR) and UV spectral measurements were run on JASCO Model IR-S and Hitachi EPS-3 spectrometers, respectively. NMR spectra were obtained on Hitachi Model R-20A spectrometer at 60 MHz; the chemical shifts are quoted as ppm downfield from tetramethylsilane used as an internal standard. Abbreviation used s=singlet, d=doublet, q=quartet, and m=multiplet. For preparative thin-layer chromatography (TLC) Silica gel HF (E. Merck AG, Darmstadt) was used as adsorbent.

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$[\alpha]_D^{25} + 10.2^\circ$ ($c=0.10$, MeOH). *Anal.* Calcd. for $C_{43}H_{70}O_9N_8 \cdot 2AcOH \cdot 2H_2O$: C, 56.49; H, 8.27; N, 11.22. Found: C, 56.35; H, 7.79; N, 11.70. NMR (5% solution in CD_3OD) δ : 0.85 (3H, s, 18- CH_3), 0.97 (3H, s, 19- CH_3), 5.00 (3H, m, 3 α -H, 21- CH_2), 5.90 (1H, m, 22-H).

Digitoxigenin 3-Suberoyl-L-arginyl-L-proline Acetate (Ie)—A solution of Ib (93 mg) in pyridine (10 ml) was treated with an aq. solution of L-arginyl-L-proline·AcOH¹⁵ (60 mg in 5 ml) in the manner as described in Ic. The crude product obtained was chromatographed on silica gel (500 mg). Eluate with AcOEt–MeOH (1:1) was further purified by gel filtration on Sephadex LH-20 using MeOH as eluent. After evaporation of solvent trituration of the eluate with hexane gave Ie (68 mg) as colorless amorphous substance. mp 184–190°. $[\alpha]_D^{25} - 27.3^\circ$ ($c=0.18$, MeOH). *Anal.* Calcd. for $C_{42}H_{65}O_9N_5 \cdot AcOH$: C, 62.61; H, 8.24; N, 8.30. Found: C, 62.27; H, 8.14; N, 8.72. NMR (12% solution in CD_3OD) δ : 0.88 (3H, s, 18- CH_3), 0.96 (3H, s, 19- CH_3), 3.70 (2H, m, Pro-5- CH_2), 4.38 (1H, m, Pro-2-H), 4.98 (2H, m, 21- CH_2), 5.10 (1H, m, 3 α -H), 5.90 (1H, m, 22-H).

Transformation of Id into Pyrimidine Derivative (Ig)—A solution of Id (60 mg) in acetylacetone– $KHCO_3$ (5.5 ml) was allowed to stand at 37° for 78 hr. The resulting solution was extracted with $CHCl_3$ and the extract was then methylated with CH_2N_2 in the manner as described in If. The crude product obtained was submitted to the preparative TLC using AcOEt–MeOH (20:1) as developing solvent. The adsorbent corresponding to the spot (R_f 0.10) was eluted with AcOEt–MeOH (10:1), and recrystallization of the eluate from MeOH–ether gave Ig (20 mg) as colorless prisms. mp 142–143°. $[\alpha]_D^{25} + 20.8^\circ$ ($c=0.12$, $CHCl_3$). *Anal.* Calcd. for $C_{54}H_{80}O_9N_8$: C, 65.83; H, 8.19; N, 11.37. Found: C, 65.31; H, 8.28; N, 10.98. UV λ_{max}^{MeOH} nm: 241, 304. NMR (4% solution in $CDCl_3$) δ : 0.85 (3H, s, 18- CH_3), 0.95 (3H, s, 19- CH_3), 2.28 (12H, s, pyrimidine-4,6,4',6'- CH_3), 3.70 (3H, s, - $COOCH_3$), 4.50 (1H, m, -NH-pyrimidine), 4.90 (2H, m, 21- CH_2), 5.05 (1H, m, 3 α -H), 5.50 (1H, m, -NH-pyrimidine), 5.85 (1H, m, 22-H), 6.28 (2H, s, pyrimidine-5,5'-H), 6.60 (1H, m, -CONH-), 7.10 (1H, m, -CONH-).

Transformation of Ie into Pyrimidine Derivative (Ih)—A solution of Ie (42 mg) in acetylacetone– $KHCO_3$ (2 ml) was allowed to stand at 37° for 72 hr. The resulting solution was extracted with $CHCl_3$ and the extract was then methylated with CH_2N_2 in the manner as described in If. The crude product obtained was submitted to the preparative TLC using AcOEt as developing solvent. The adsorbent corresponding to the spot (R_f 0.10) was eluted with AcOEt and the eluate was submitted to gel filtration on Sephadex LH-20 using MeOH as eluent. After evaporation of solvent trituration of the eluate with hexane gave Ih (17 mg) as colorless amorphous substance. mp 80–85°. $[\alpha]_D^{25} - 21.0^\circ$ ($c=0.10$, $CHCl_3$). *Anal.* Calcd. for $C_{48}H_{71}O_9N_5$: C, 66.87; H, 8.30; N, 8.12. Found: C, 66.40; H, 8.25; N, 8.02. UV λ_{max}^{MeOH} nm: 236.5, 304. NMR (8.5% solution in $CDCl_3$) δ : 0.88 (3H, s, 18- CH_3), 0.96 (3H, s, 19- CH_3), 2.30 (6H, s, pyrimidine-4,6- CH_3), 3.50 (2H, m, Pro-5- CH_2), 3.69 (3H, s, - $COOCH_3$), 4.50 (2H, m, Pro-2-H, -NH-pyrimidine), 4.90 (2H, m, 21- CH_2), 5.09 (1H, m, 3 α -H), 5.89 (1H, m, 22-H), 6.20 (1H, m, -CONH-), 6.30 (1H, s, pyrimidine-5-H).

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