Tetrahedron Letters No. 34, pp 3017 - 3020, 1975. Pergamon Press. Printed in Great Britain.

CARDENOBUFOTOXIN : NOVEL CONJUGATED CARDENOLIDE FROM JAPANESE TOAD[1]

Youichi Fujii, Kazutake Shimada, Yuriko Niizaki
and Toshio Nambara*
Pharmaceutical Institute, Tohoku University, Aobayama, Sendai, Japan

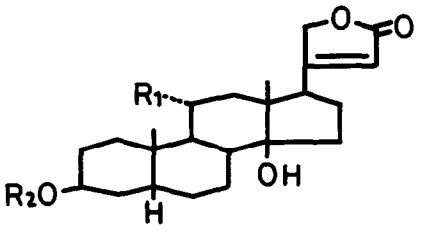
(Received in Japan 30 June 1975; received in UK for publication 14 July 1975)

In recent years three novel types of bufotoxins in which the succinoyl, adipoyl, and pimeloyl groups are displaced for the suberoyl residue of the so-called "bufotoxin", [2-4] have been isolated from the skin of Bufo vulgaris formosus Boulenger.[5-7] In addition the existence of bufalin-3-sulfate in the Japanese toad has been also demonstrated.[8] Separation of the cardenolide and its 3-hemisuberate from the Chinese preparation Ch'an Su by Meyer and his coworkers[9] strongly implied the possible occurrence of the analogous conjugates in the living animal. Now the isolation and characterization is reported of two novel conjugated cardenolides named cardenobufotoxin from the skin of Bufo vulgaris formosus Boulenger.

The ethanolic extract of the skin obtained from 1,800 toads was partitioned with ether-water and then with ethyl acetate-water systems. The organic layer was concentrated in vacuo and the residue was submitted to chromatography on silica gel. Subsequent purification by preparative thin-layer chromatography on silica gel HF_{254} provided digitoxigenin (I), mp 242-249°, as colorless prisms (from acetone-ether) and sarmentogenin (II), mp 256-263°, as colorless prisms (from MeOH-ether). These compounds were unequivocally characterized by direct comparison with the authentic samples.

The aqueous layer was chromatographed repeatedly in the manner as previously reported.[4,5] Further purification by high-speed liquid chromatography on a μ-Bondapak C_{18} column (Waters Associates Inc., Milford) using MeOH-H_2O (2:1) as

solvent afforded a new bufotoxin (V), mp 171-173°, $[\alpha]_D^{16}$ +14.2° (c=0.11 in MeOH), as colorless amorphous substance (from MeOH-ether). It showed a positive result with Sakaguchi's reagent and a negative test with ninhydrin, and exhibited the n.m.r. signals at δ : 0.89 (3H, s, 18-CH_3), 1.08 (3H, s, 19-CH_3), 2.90 (1H, m, 17α-H), 3.70 (1H, m, 11β-H), 4.26 (1H, m, Arg-CH), 5.06 (1H, m, 3α-H), 5.90 (1H, m, 22-H). Hydrolytic cleavage with 6N hydrochloric acid yielded arginine which was identified by thin-layer chromatography. Upon enzymatic hydrolysis with a hog pancreas lipase preparation (Sigma Chemical Co., St. Louis), followed by methylation with diazomethane compound V furnished the methyl ester of sarmentogenin 3-hemisuberate (III) as colorless leaflets (from ether), mp 115-116°, $[\alpha]_D^{16}$ +25.0° (c=0.08 in $CHCl_3$), n.m.r. δ : 3.62 (3H, s, $COOCH_3$), 3.75 (1H, m, $W^1/_2$=20 Hz, 11β-H), 4.82 and 4.90 (each 1H, d, J=18 Hz, 21-CH_2), 5.04 (1H, m, $W^1/_2$=10 Hz, 3α-H), 5.85 (1H, m, 22-H), ms m/e : 560 (M^+), 189, 171. Hydrolysis of compound III with 5% hydrochloric acid in aq. methanol under the mild conditions gave an aglycone, which was identified as sarmentogenin (II) by means of thin-layer chromatography and mass spectrometry. These evidences lent a support to assign the structure sarmentogenin 3-suberoylarginine ester (V) to this new type bufotoxin.



I : R_1=R_2=H

II : R_1=OH, R_2=H

III : R_1=OH, R_2=$CO(CH_2)_6COOCH_3$

IV : R_1=OH, R_2=$CO(CH_2)_5COOCH_3$

V : R_1=OH, R_2=$CO(CH_2)_6CONHCH(CH_2)_3NHCNH_2$ (COOH; NH)

VI : R_1=OH, R_2=$CO(CH_2)_5CONHCH(CH_2)_3NHCNH_2$ (COOH; NH)

Another cardenobufotoxin (VI), mp 184-187°, $[\alpha]_D^{17}$ +9.6° (c=0.15 in MeOH), n.m.r. δ : 0.90 (3H, s, 18-CH_3), 1.09 (3H, s, 19-CH_3), 2.88 (1H, m, 17α-H), 3.72

(1H, m, 11β-H), 4.26 (1H, m, Arg-CH), 5.92 (1H, m, 22-H), was similarly obtained as colorless amorphous substance (from MeOH-ether). The presence of a peptide bond involving the α-amino group of arginine was confirmed by the color tests and hydrolytic cleavage with hydrochloric acid. Enzymatic hydrolysis and subsequent methylation with diazomethane in the similar fashion yielded the methyl ester of sarmentogenin 3-hemipimelate (IV). Unfortunately compound IV could not be obtained crystalline, but the structure was unambiguously assignable on the basis of n.m.r. and mass spectral data, n.m.r. δ : 3.76 (1H, m, $W^1/_2$=20 Hz, 11β-H), 5.10 (1H, m, $W^1/_2$=10 Hz, 3α-H), ms m/e : 546 (M^+), 175, 157. Acid hydrolysis of IV in the manner as described above afforded sarmentogenin (II) as an aglycone. These results provided unequivocal support for the assignment of the structure sarmentogenin 3-pimeloylarginine ester (VI) to the second cardenobufotoxin.

To the best of our knowledges this is the first reported isolation of the conjugated cardenolide from an animal source. It should be emphasized that the cardenolide and its conjugate do occur in both animal and plant kingdoms. The physiological activity of these cardenobufotoxins will be the subject of a future communication.

Acknowledgement The authors express their deep gratitudes to Dr. Hiroshi Ishii, Shionogi & Co., Ltd., for the generous gift of sarmentogenin. They are indebted to all the staffs of analytical laboratories of this Institute and Teikoku Hormone Mfg. Co. for elemental analyses and spectral measurements. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Japan, which is gratefully acknowledged.

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