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A NATURALLY OCCURRING ERYTHRO DIASTEREOMER OF PUMILIOTOXIN B.

by

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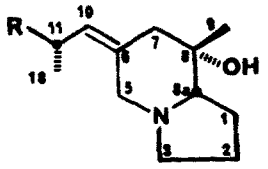
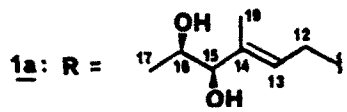
Summary: The 15R,16S erythro diastereomer of pumiliotoxin B has been discovered in the skin of an Australian myobatrachid frog, Pseudophryne coriacea.

INTRODUCTION

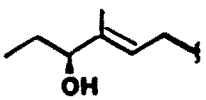
Pumiliotoxin B (1a) (PTX-B) is a major alkaloid in skin extracts of the Panamanian poison frog, Dendrobates pumilio[1]. The gross structure of PTX-B was first proposed based upon NMR analysis and analogies to the simpler alkaloid, pumiliotoxin 251D (5)[2]. The threo configuration of the side chain diol was demonstrated by ^{1}H NMR model studies[3,4], the E-configuration of the C(13)-C(14) double bond was shown by NOE experiments[4] and the 15R,16R diol chirality by degradation to a derivative of (-) tartaric acid[5]. The resulting structure was confirmed by enantioselective synthesis[6].



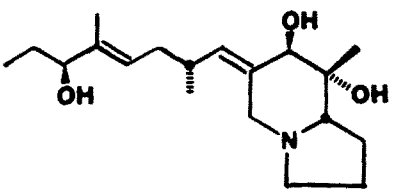


1a: R = (PTX-B)

5: R = *n*-Bu (251D)



6: R = (PTX-A)



7 (323B)

PTX-B, pumiliotoxin A (6) and related pumiliotoxins and allopumiliotoxins (7-hydroxy-PTXs) occur in a large number of species of dendrobatid frogs from Central and South America as determined either by isolation or gas chromatography-mass spectrometry (GC-MS)[7]. PTX-B and related alkaloids have also been detected in certain non-dendrobatid frogs from the myobatrachid, ranid (subfamily mantellinae) and bufonid families[8].

Recently, a PTX-B-like alkaloid was detected by a characteristic profile of biological activity[9,10] in skin extracts of the myobatrachid frog Pseudophryne coriacea collected in Australia in 1973. Purification by alumina column chromatography yielded a fraction (II) containing relatively pure material[9] that appeared identical in chemical properties to PTX-B (see Experimental). Further studies on an earlier alkaloid fraction (I) from this frog led to the isolation and structural elucidation of a group of cyclized N-methyltryptamines with isoprene-derived side chains and to the isolation of a small amount of material that also appeared to be similar to PTX-B in chemical properties[11].

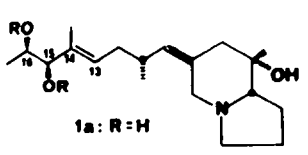
RESULTS AND DISCUSSION

The PTX-B-like material from fraction I cochromatographed with PTX-B (1a) on GC with packed or capillary columns (OV-1, OV-17) and had a mass spectrum virtually identical to that of 1a. However, slight differences were noted in the ^{13}C and ^{1}H NMR spectra of this substance when compared with spectra from 1a. In particular, the carbon signals for C-17 and C-15 were displaced downfield from those of 1a by 1.3 and 2.1 ppm, respectively, and the proton signals for H-16 and H-15 in the PTX-B-like material appeared at δ3.83(m) and δ3.91(d, J=4.9 Hz), respectively, shifted slightly from 1a where they occurred at δ3.74(m) and δ3.65(d, J=7 Hz)[12]. It was also observed that this PTX-B-like alkaloid had low biological activity compared to that of PTX-B itself or to a PTX-B-like alkaloid present in the later Al_2O_3 chromatographic fraction (II) from P. coriacea[13]. In this paper we present evidence that the earlier Al_2O_3 fraction (I) contained the 15R,16S erythro diastereomer (2a) of PTX-B, while the later Al_2O_3 fraction (II) contained PTX-B (1a).

Acetylation (Ac_2O/Py, 2 h,20°) of a portion of each fraction and examination by capillary GC (see Table I) revealed a small difference, confirmed by coinjection, in the retention times (t_r) of the two resulting 15,16-diacetates. The diacetate from fraction I (t_r 18.03 min) proved identical in t_r to the diacetate (2b) of the synthetic[14] 15R,16S erythro diastereomer of PTX-B, while the diacetate from fraction II had the same t_r (18.34 min) as the diacetate (1b) of either natural or synthetic[6] PTX-B. Both diacetates (1b, 2b) had virtually identical E.I. mass spectrometric fragmentation patterns (see Experimental). The NMR of the alkaloid from fraction I differed from that of 1a principally in the chemical shifts of carbons 17 and 15 and in protons 16 and 15. The GC evidence and the close correspondence between the H-16 and H-15 signals of the PTX-B-like material and equivalent protons of a synthetic model namely erythro (E)-4-methylhept-4-en-2,3-diol, (H-2, δ3.85(m); H-3, δ3.91(d, J=5.2 Hz)),[4] suggested that the new alkaloid isolated from fraction I was the 15R,16S erythro diastereomer (2a) of PTX-B, not previously known to exist in nature. This diastereomer is known from studies on synthetic 2a to have low biological activity[14]. Although GC data on the diacetate from fraction II suggested it to be 1a, insufficient alkaloid was present in this fraction for an NMR confirmation of its identity. However, a similar Al_2O_3 chromatographic fraction of a P. coriacea extract obtained in 1987 and having high biological activity afforded an alkaloid with ^{13}C and ^{1}H NMR spectra identical to those reported[12] for 1a. This diacetate

TABLE I

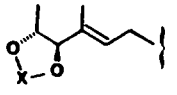
GC RETENTION TIMES (t_r, min) OF PTX-B DERIVATIVES



1a: R=H

1b: R=Ac

15R,16R threo

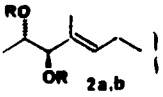


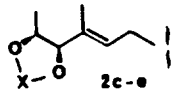
1c: X=C(CH$_3$)$_2$

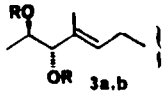
1d: X=B-Ph

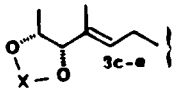
1e: X=B-n-Bu

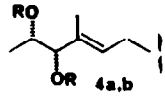
	1b-4b	1c-4c	1d-4d	1e-4e
15R,16R threo	18.34	13.21	26.70	18.27
15R,16S erythro	18.03	14.26	28.26	19.67
15S,16R erythro	18.03	14.29	28.44	19.81
15S,16S threo	18.44	13.21	26.85	18.42

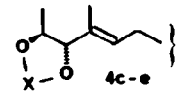












Conditions: A 30 m x 0.32 mm i.d. fused silica OV-1 column (Alltech) was used with He carrier, injector temp = 250°, detector (FID) temp = 275° and a 1:40 split. Derivatives b, c and e used a program from 200 to 250° at 2.5°/min, while d used a program from 200 to 275° at 2.5°/min.

had a t_r (18.34 min) identical to that of the diacetate of 1a. Thus, the active alkaloid[9,10] present in fraction II appears likely to be 1a. Certainly, the biologically active alkaloid isolated in the same manner from extracts of P. coriacea in 1987 is, in all the chemical properties examined, identical with PTX-B.

There still remained the possibility that the PTX-B-like material for which an erythro configuration was supported by ^{1}H NMR was instead the 15S,16R isomer (3a)[15]. This isomer has not been synthesized per se, but is a by-product (6%) in the synthesis of 1a where it was originally detected by ^{1}H NMR[6]. We found that the diacetate of the synthetic mixture of 1a and 3a exhibited a small GC peak (3b, 6%) at exactly the same t_r (18.03 min) as the major peak (2b) from the acetylation of the synthetic 15R,16S diastereomer (2a)[16]. Thus, these conditions cannot separate the two erythro isomers (2b, 3b).

The acetonide derivative of synthetic 1a ((CH$_3$)$_2$CO, p-TsOH, 2 h, 20°) likewise showed two GC peaks. The minor peak (3c, 5%), as in the case of the diacetate derivative, cochromatographed with the major peak (2c) in the mixture of acetonides formed from synthetic 2a. Furthermore the acetonides of the two threo samples (1c and 4c) had identical retention times (Table I).

We were successful, however, in separating all four diastereomers (1a-4a) using either their phenyl- or butyl-boronide derivatives (PhB(OH)$_2$ or n-BuB(OH)$_2$, $CHCl_3$ (EtOH-free), 1 h, 20°). Using these derivatives, the PTX-B-like alkaloid in fraction I from P. coriacea was unambiguously identified as the 15R,16S erythro (2a) structure by GC retention times in comparison with derivatives (2d, 2e) prepared from synthetic 2a, and had clearly different retention times from the analogous derivatives (3d, 3e) of the 15S,16R erythro isomer[17]. Phenyl- and butyl-boronides of fraction II from P. coriacea had identical retention times with

derivatives (1d, 1e) prepared from natural PTX-B.

We have examined by GC and GC-MS, acetylated samples from six different populations of P. coriacea collected in Australia in 1987[18,19]. Three observations are particularly noteworthy:

1. The level of 1a in skin varied greatly among these populations, being in some populations 50 to 80 fold higher than others.

2. One population contained no 1a whatsoever, but instead gave the allopumiliotoxin 323B (7).

3. The erythro isomer 2a was detected in only one of the six populations where it was present with 1a in a ratio of 2a/1a, 0.22.

We are presently examining extracts from other Pseudophryne species and a number of species of dendrobatid frogs as well, for the presence of 1a and 2a[20].

In summary, it now appears that during alumina column chromatography of the 1973 P. coriacea extract, a fortuitous separation of erythro and threo diastereomers must have occurred[21]. One column fraction (I) (95% ethanol) which contained mainly indole alkaloids, contained a small amount of nearly pure 2a (GC analysis 2b/1b, 93/7), while a subsequent 95% ethanol fraction (II) contained nearly pure 1a in a very small amount (0.1 mg/mL) (1b/2b,94/6). A sample of the original crude extract before chromatography was found by acetylation to contain 2a and 1a in a 1:5 ratio.

ACKNOWLEDGEMENT. The generous support of fieldwork (J.W.D. and C.S.) in Australia in January 1987 by the Fidia Research Laboratory, Alba Terme, Italy is gratefully acknowledged.

EXPERIMENTAL:

Gas chromatography employed a Hewlett-Packard instrument, model 5890 with a 3390A recorder-integrator (see Table I for conditions). GC-MS used a Finnigan model 4500 mass spectrometer, scanning from mass 50 to 450 (one scan per second) attached to an INCOS data system and a 25 m X 0.25 mm i.d. OV-17 column (Alltech) programmed from 150° to 300° at 3°/min (condition A) or a Finnigan MAT model 700 ion trap detector with an ITDS data system and a 25 m X 0.25 mm i.d. OV-1 column (Alltech) programmed from 150° to 280° at 15°/min (condition B). NMR spectra were obtained in $CDCl_3$ with a Varian Assoc. XL-300 instrument with TMS internal standard. The ^{13}C NMR spectrum was obtained at 75.429 MHz.

Isolation:

2a: Approximately 3 mg of 2a were obtained by LH-20 chromatography with methanol of an alumina chromatographic fraction from P. coriacea[11]. Another sample was isolated by HPLC (Altex model 420 liquid chromatograph equipped with a Zorbax ODS column, 6.2 mm X 25 cm) using 4:1, CH_3OH/H_2O at a flow rate of 1.5 mL/min and further purified using a small column of neutral alumina.

Characterization:

2a: ^{13}C NMR: 23.1[1], 21.0[2], 54.3[3], 52.8[5], 129.5[6], 48.5[7], 68.5[8], 71.7[8a], 24.6[9], 134.6[10], 32.6[11], 35.4[12], 126.8[13], 134.9[14], 80.7[15], 68.7[16], 17.7[17], 21.3[18], 12.9[19]. Signals for all carbons except 15 and 17 are within ±0.8 ppm of 1a[6,12].

2a: ^{1}H NMR: δ5.42(t, J=7.2)[H-13], 5.08(d, J=9.8)[H-10], 3.91(d, J=4.9)[H-15], 3.83(m, J=6.0)[H-16], 3.78(d, J=10.7)[H-5eq]. All other signals are virtually identical to PTX-B[6,12] (see also ref. 14 for 250 MHz data on synthetic 2a).

M.S. (E.I.):

2b (contains 18% 3b): 407(16), 348(57), 320(8), 288(20), 206(19), 194(55), 193(44), 176(21), 166(80), 84(24), 70(100), (condition A).

2c: 363(8), 288(8), 206(36), 194(13), 193(28), 176(17), 166(100), 134(18), 84(13), 70(77), (condition A).

2d: 409(2), 206(20), 166(100), 105(44), 84(12), 70(62), (condition A).

1b: 407(10), 348(35), 320(15), 288(8), 206(10), 194(40), 193(27), 176(16), 166(50), 84(22), 70(100), (condition A).

1c: 363(32), 348(18), 288(12), 262(5), 248(8), 220(5), 206(30), 194(30), 193(30), 176(12), 166(100), 84(10), (condition B, not scanned below m/z 80.)

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15. Diastereomer 3a was of particular interest in the light of the facile acid-catalyzed epimerization of the 15-hydroxy group, which we have observed with PTX-A (6) and allopumiliotoxin 323B (7). We find, however, that natural 1a or synthetic 2a when exposed to these conditions (0.1 N HCl, 18 h, 20°), gives after phenylboronation, no evidence of any epimerization. Furthermore 1a (natural) on exposure to 0.1 N HCl in $H_2{}^{18}O$ gives after 1 week, no incorporation of heavy isotope. Although these studies are unfinished (results will appear elsewhere), a significant effect (probably inductive) evidently exists that must prevent the formation of a carbocation at C-15 necessary for the epimerization. Thus, an artifactural erythro isomer seems unlikely for PTX-B. The occurrence of both the erythro isomer (2a) and the threo isomer (1a) in nature, suggests either a lack of absolute stereo control in the generation of a hydroxy group at C-16 or the presence of alternative metabolic pathways.

16. Two barely resolved minor peaks in this sample were identified as the 15R,16R (1a, 3%) and 15S,16S (4a, 13%) threo diastereomers (see footnote 17).

17. Phenylboronation of synthetic 2a shows the mixture to be composed of 66% 2a, 18% 3a, 13% 4a and 3% 1a.

18. We have found unacetylated PTX-B (1a) to behave erratically on gas chromatography, particularly on capillary columns. Detector response to concentration of 1a is non-linear. Where acetylation indicated some erythro isomer, we have confirmed this by phenylboronation.

19. A more complete account of this survey, including both pumiliotoxins and the tricyclic pyrroloindole alkaloids, is in preparation.

20. The results of this survey on 1a and 2a in dendrobatid, mantellid, myobatrachid and bufonid frogs will appear elsewhere.

21. While conditions are not comparable, it should be noted that a separation of 2a from 1a does result on alumina thin layer plates where R_f values of 0.41 and 0.30, respectively, result. Merck (5 cm x 20 cm x 0.25 mm) plates, developed with 5% CH_3OH-$CHCl_3$ and exposed to iodine vapor or 20% H_2SO_4 spray and heat, were used. A very weak spot can also be detected at R_f 0.41 in a heavily loaded sample of synthetic 1a, suggesting that the erythro contaminant, 3a, has the same R_f as 2a on alumina. No difference between 2a and 1a on silica gel thin layer chromatography could be detected.