SPECIALIA

The structure of gephyrotoxin (GTX) 223AB

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Summary. The structure 3-butyl-5-propylindolizidine (**2**), tentatively assigned to a minor alkaloid in skin extracts from a number of poison frogs of the Neotropical genus *Dendrobates,* has been confirmed and its stereochemistry determined as 5E, 9E (**2d**) by comparison on GC and GC-MS with the four synthetic diastereomers **2a–2d.**

A recent survey[2] by gas chromatography-mass spectrometry (GC-MS) of alkaloids found in the skins of brightly colored Central and South American frogs of the genus *Dendrobates* revealed a non-acetylatable amine, $C_{15}H_{29}N$, as a minor component in a number of species. On the basis of its electron-impact (EI) mass spectrum[2] and the likelihood of biosynthetic formation from a straight-chain, 2,6-disubstituted piperidine having one propyl substituent, the 3-butyl-5-propylindolizidine structure **2** was proposed for this alkaloid[2]. A number of more complex indolizidine alkaloids from *Dendrobates* species have recently been characterized[3,4] and (5Z, 9Z) 3-butyl-5-methylindolizidine **6a**[5a,b] has been identified as a component of the pharaoh ant trail marker by means of biological studies using synthetic (dl)-diastereomers[6]. Otherwise, naturally occurring indolizidines are comparatively rare. Because **2** appears to be the parent of a number of related side-chain hydroxylated materials[7], it seemed worthwhile to confirm the provisional structure and to determine its relative stereochemistry. A cooperative effort has led to the identification of GTX 223AB as (5E, 9E)-3-butyl-5-propylindolizidine (**2d**)[8]. Our evidence follows:

Catalytic reduction (H_2/Rh-Al_2O_3 (5%)/HOAc-EtOH (1:4)) of the pyrrole **1**[9] yielded an indolizidine mixture (eq'n 1) exhibiting four peaks (A-D) on GC analysis[10] whose relative areas in order of increasing retention times were 4.4(A), 3.8(B), 1.0(C) and 1.2(D) (column A). The major diastereomers, **2a** and **2b,** (peaks A and B, respectively) assumed to be cis-hydrogenation products (i.e. 9Z isomers, epimeric at C(5)) could be obtained pure by silica gel column chromatography (7–9% CH_3OH–$CHCl_3$). Their configuration at C(5) was secured by comparing them with the 2 diastereomers prepared[11] by a Speckamp-type cyclization of **4**. This cyclization was shown[11] by 360 MHz PMR examination of the product, lactam **7**, to proceed stereospecifically, giving a single lactam having a trans (E)-relationship between the protons at C(5) and C(9). This gave ultimately (eq'n 2) 2 indolizidines epimeric at C(3). One of these proved identical (^{13}C NMR, GC (peak B); columns A or B; silica gel TLC, 10% CH_3OH–$CHCl_3$) with one of the major hydrogenation products and must consequently possess the 5E,9Z configuration, **2b**[11]. From this it follows that the other major pyrrole hydrogenation product (peak A) and the other diastereomer resulting from **4** (peak C) must have the 5Z,9Z (**2a**) and 5Z,9E (**2c**) configurations, respectively (see table). Thus structures for **2a**, **2b** and **2c** can be assigned unambiguously, solely upon the basis of the observed C(5)-C(9)-E relationship in **7** and the reasonable (Oliver and Sonnet[6]) assumption that the 2 major hydrogenation products (**2a** and **2b**) represent products of cis-addition of hydrogen to the pyrrole-ring of **1.** Our ^{13}C NMR data[11,12,14] are consistent (table) with data reported[13] by Sonnet and coworkers for the related 3-butyl-5-methylindolizidines (**6a–6d**).

The remaining diastereomer – the 5E,9E isomer – had earlier been prepared using bis-alkylation of N-methoxycarbonyl-3-pyrroline to produce an intermediary 2,5-trans-dialkyl-3-pyrroline, **8,** and from this a 1:1 mixture of dehydroindolizidines, **9**, of the 9E configuration, epimeric at C(5) (eq'n 3). This mixture on hydrogenation gave solely[14] 1 indolizidine co-chromatographing with peak D. The assignment of the 5E,9E configuration (**2d**) for this product rests[14] upon the production in analogous fashion (from **10** and **11**) of a single 3-butyl-5-methylindolizidine ant trail marker diastereomer **6d** of the 5E,9E configura-

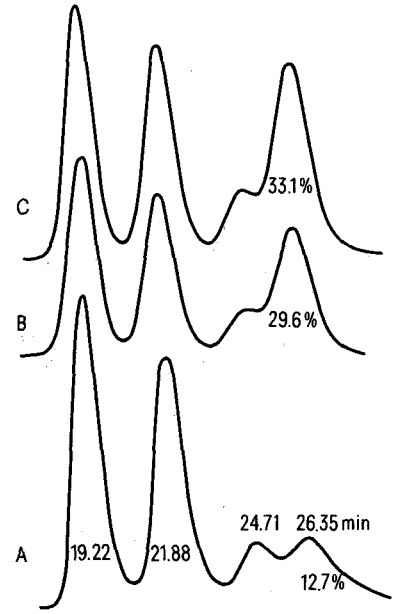
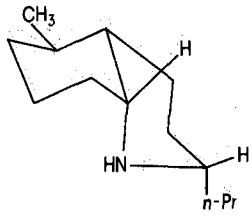


Figure 1. Chromatograms of: *A* hydrogenation mixture from **1**; *B* hydrogenation mixture plus *D. histrionicus* skin extract; *C* mixture B plus Macdonald's compound **2d**. GC column B was used at 95 °C. Percentages refer to relative amount of peak 4. See footnote 10 for other details.



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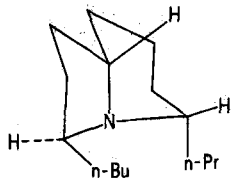
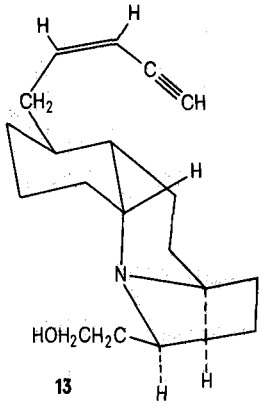


2d



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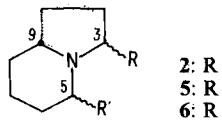
tion, unambiguously synthesized by Sonnet and Oliver[6]. This assignment is supported further by noting that the hydrogenation product from **9** differs from **2a, 2b** or **2c** in GC retention time and in its EI fragmentation pattern and that it cochromatographs with the pyrrole hydrogenation mixture at the peak position (D) expected for the remaining diastereomer.

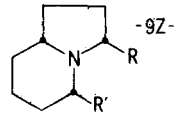
When the hydrogenation mixture was coinjected with the frog skin extract, only the peak corresponding to **2d** was enhanced. Simultaneous coinjection with synthetic **2d** led to further enhancement (fig. 1). GC-MS analysis[15] of the synthetic hydrogenation mixture gave the following EI spectra (fig. 2) for the four peaks. The EI spectrum of **2d** most nearly resembles that of the natural material (fig. 2).

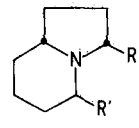
We have also prepared the isomeric 3-propyl-5-butylindolizidine diastereomeric mixture (**5a–5d**) by reduction of intermediate **3**. This led to 4 peaks (columns A or B, 90°) of relative areas 3.4:3.2:1.0:1.2 in order of increasing reten-

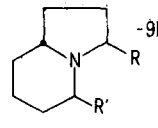
Indolizidine diastereomers, preparation and selected ^{13}C NMR assignments

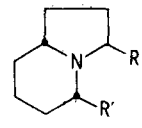


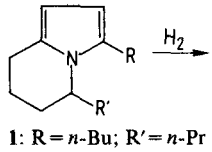
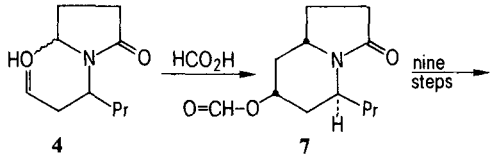
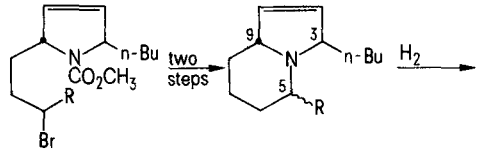
2: R = *n*-Bu; R′ = *n*-Pr
5: R = *n*-Br; R′ = *n*-Bu
6: R = *n*-Bu; R′ = CH_3









Synthetic route	(5Z,9Z) **2a,5a,6a**	(5E,9Z) **2b,5b,6b**	(5Z,9E) **2c,5c,6c**	(5E,9E) **2d,5d,6d**
(1)[9] 				
1: R = *n*-Bu; R′ = *n*-Pr **3**: R = *n*-Pr; R′ = *n*-Bu	42% **2a** 39% **5a**	37% **2b** 37% **5b**	10% **2c** 11% **5c**	12% **2d** 14% **5d**
(2)[11] 				
4 **7**		64% **2b**	36% **2c**	
(3)[14] 				
8: R = *n*-Pr **9**: R = *n*-Pr **10**: R = CH_3 **11**: R = CH_3				**2d** or **6d**
^{13}C NMR chemical shifts	**2a 6a**[b]	**2b**[a] **6b**[b]	**2c**[a] **6c**[b]	**2d**[c] **6d**[b]
C(3)	65.3 61.8	56.4 55.4	54.8 55.0	57.7 56.6
C(5)	62.3 59.7	52.7 47.2	52.3 48.4	55.9 51.8
C(9)	67.7 67.5	58.4 58.7	58.5 59.4	58.5 58.9

Percentages refer to composition of the isolated indolizidine product. Chemical shifts (δ_C) are in ppm from TMS in $CDCl_3$ unless indicated otherwise. a: ref. 11, C_6D_6, b: ref. 13, c: ref. 14. We have selected for comparison, δ_C for carbons 3, 5 and 9. Sonnet et al.[13] found for these the order C(9) < C(3) < C(5). We have assumed the same order. The additional downfield shift of 3–5 ppm for C(5) is consistent with the effect expected on replacement of a methyl group by a propyl group[17].

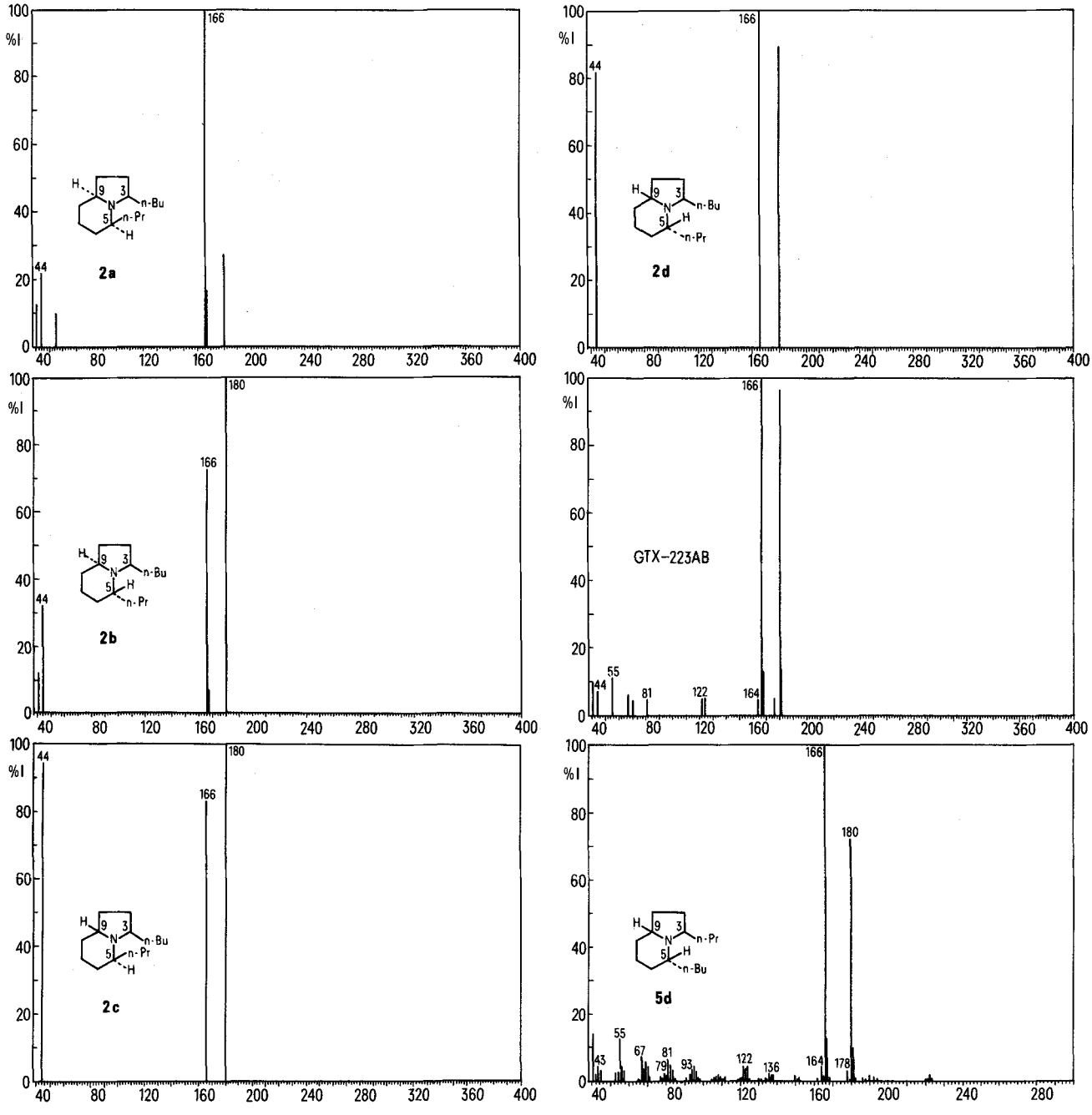


Figure 2. Representative electron impact mass spectra obtained on components **2a–2d** of hydrogenation mixture from **1**; the natural material GTX-223AB and the component, **5d**, possessing the greatest retention time from hydrogenation of **3**. GC-MS conditions are given in footnote 15. The peak at m/z 44 is an artefact. The labelling of minor peaks is computer-generated and is not meant to imply any significance. The parent ion at m/z 223 was in all cases less than 1% of the base peak.

tion times. This mixture co-chromatographed peak for peak with the mixture **2a–2d** (columns A or B). The EI spectrum[15] for the peak of the greatest retention time (fig. 2, **5d**) differs significantly enough from **2d** and the natural material to exclude the "iso" (5E,9E) 3-propyl-5-butylindolizidine structure (**5d**) for the natural material[16]. Thus we conclude GTX 223AB has the structure indicated as **2d** and is stereochemically related to pumiliotoxin-C, **12**, in the configuration of its piperidine-ring substituents rather than to gephyrotoxin, **13**, to which it appeared initially to be related.

1 Author for correspondence and reprints: J.W.D., Laboratory of Bioorganic Chemistry, NIAMDD, NIH, Bethesda, MD 20205.

2 J.W. Daly, G.B. Brown, M. Mensah-Dwumah and C.W. Myers, Toxicon *16*, 163 (1978). See discussion for GC peak 223AB. The EI spectrum (LKB 3000 spectrometer, 70eV) exhibited a parent ion at m/z 223 (intensity 1% of base peak) and only 2 major fragment ions, one at 180 (loss of C_3H_7, intensity 66% of base peak) and the other at 166 (loss of C_4H_9, base peak). This material has been referred to in ref. 14 as GTX-223, since it appeared to be related structurally to gephyrotoxin (GTX) **13**. Biosynthesis of most dendrobatid alkaloids

appears likely to derive from a precursor 2,6-disubstituted piperidine which has odd numbers of carbons in both substituents; cf. histrionicotoxins, pumiliotoxin-C, gephyrotoxin. Because of this, an alternative structure for GTX-223AB, viz. 3-propyl-5-butylindolizidine **5**, was considered quite unlikely since it would derive from a 2,6-disubstituted piperidine with 4 and 6 carbon substituents.

3 J.W. Daly, T. Tokuyama, R.J. Highet and I.L. Karle, J. Am. chem. Soc. *102,* 830 (1980).

4 J.W. Daly, B. Witkop, T. Tokuyama, T. Nishikawa and I.L. Karle, Helv. chim. Acta *60,* 1128 (1977).

5 a) F.J. Ritter, I.E.M. Rotgans, E. Talman, P.E.J. Verwiel and F. Stein, *Experientia 29,* 530 (1973). b) J.P. Edwards and D.B. Pinninger, Ann. appl. Biol. *89,* 395 (1978). We have adopted Sonnet's use[13] of the E,Z nomenclature to characterize indolizidine stereoisomers. The configuration of the hydrogen atom at C(5) and C(9) is referred to the hydrogen at C(3) and is either in a cis-(Z) or trans-(E) relationship.

6 a) J.E. Oliver and P.E. Sonnet, J. org. Chem. *39,* 2663 (1974). b) P.E. Sonnet and J.E. Oliver, J. het. Chem. *12,* 289 (1975).

7 Ref. 2. See e.g. alkaloids 239AB and 239CD.

8 Initially diastereomer **2d** was reported[14] as different from GTX-223AB since coinjection with the natural material gave rise to 2 peaks on a 3% OV-17 GC column. We have since been unable to repeat this result and we have no explanation for this discrepancy.

9 T.F. Spande, unpublished results.

10 "U"-Shaped 2 mm (i.d.) × 150 cm GC columns of 3% OV-17 (100–120 mesh) (Applied Science Laboratories, State College, PA) (column A) or 10% SP-1000 on Supelcoport (100–120 mesh) (Supelco, Inc., Bellefonte, PA) (column B). A Finnegan gas chromatograph, model 9500 (injection port 250°) equipped with a flame ionization detector (300°) was used with a Hewlett-Packard 3380A recorder-integrator. The carrier (N_2) flow rate was 18 cc/min. The following are typical retention times for **2a, 2b, 2c** and **2d,** respectively on column A (90 °C): 16.6, 19.1, 21.2, 22.7 min; column B (95 °C): 17.4, 19.9, 22.7, 24.2 min. Separation of all 4 diastereomers could not be obtained on the following columns: 1.5% OV-1, 3% SE-30, 3% OV-225, 3% Poly I-110, and 5% ECNSS. However, in all these cases, **2d** chromatographed with the natural compound.

11 D.J. Hart and Y.-M. Tsai, unpublished results. The 5E,9Z and 5Z,9E configurations for **2b** and **2c**, respectively, could also be assigned on the basis of conformational arguments. Compound **2b** from the hydrogenation of **1** eluted first from silica gel (7% CH_3OH–$CHCl_3$). ^{13}C NMR (C_6D_6) **2b**: 58.4, 56.4, 52.7, 33.5, 33.2, 30.4, 28.8, 28.5 (2), 23.6, 21.3, 20.0, 14.7, 14.4 ppm. **2c**: 58.5, 54.8, 52.3, 36.3, 35.5, 29.8, 28.9, 28.8, 27.7, 23.6 (2), 20.7, 20.1, 14.6, 14.5 ppm.

12 **2a**: ^{13}C NMR ($CDCl_3$): 67.7, 65.3, 62.3, 39.6, 38.0, 32.0, 31.0, 30.5, 29.8, 29.2 (2), 25.0, 22.9, 14.4, 14.0 ppm. M.S. (70 eV): m/z 166 (100%), 180 (52%), 222 (3%), 223 (1%). IR (neat): Bohlmann bands (see ref. 13) observed at 2550, 2630, 2700 and 2790 cm^{-1} in order of increasing intensity. Compound **2a** eluted last from silica gel (9% CH_3OH–$CHCl_3$).

13 P.E. Sonnet, D.A. Netzel and R. Mendoza, J. het. Chem. *16,* 1041 (1979).

14 T.L. Macdonald, J. org. Chem. *45,* 193 (1980). **2d**: ^{13}C NMR ($CDCl_3$): 58.5, 57.7, 55.9, 34.7, 30.9, 29.6, 28.7, 28.3, 25.4, 24.3, 23.6, 21.9, 17.9, 13.4, 13.0 ppm. The unusual hydrogenation result is discussed above.

15 GC column A (ref. 10) was used with a Perkin-Elmer Sigma 3 gas chromatograph attached to a V.G. Micromass Ltd., model 7070F mass spectrometer in the EI mode (70 eV). The carrier (He) flow rate was 25 cc/min. Slight differences in m/z 180/160 ratios are evident between EI spectra obtained directly and in the GC-MS mode (cf. **2a**, ref. 12, fig. 2). We thank Mr. Noel Whittaker (NIAMDD, NIH) for the EI spectra.

16 The EI spectra for the initial 3 'iso' diastereomers (**5a–5c**) in order of increasing retention times had the following m/z 166/180 ratios ± SEM for (n) scans through the GC peak: 0.42 ± 0.11 (8), 1.98 ± 0.38 (11) and 1.37 ± 0.36 (4). The G.C. peak corresponding to **5d** had 1.42 ± 0.15 (6) and 1.36 ± 0.37 (7) (2 runs). This corresponds to m/z 180/160 ratios of 0.30 ± 0.10 (12), 1.42 ± 0.35 (14), 1.04 ± 0.19 (6) and 0.89 ± 0.27 (11), for **2a, 2b, 2c** and **2d**, respectively. In both series, the ratios compared are for loss of the 5-substituent/loss of the 3-substituent; i.e., m/z 166/180 for the 'iso' compounds and m/z 180/166 for compounds **2a–d**. These data along with the relative peak areas support the same order of retention times for the 'iso' series as observed for **2a–2d**. The natural material had a 180/160 ratio of 0.93 ± 0.23 (8). The magnitude of these ratios depends very much on the source design of the spectrometer (cf. footnote 2, 15) but it is relatively invariant with the same instrument.

17 R.J. Abraham and P. Loftus, Proton and Carbon-13 NMR Spectroscopy, p. 25. Heyden, London 1979.

Enzymatic oxidation of xanthobilirubic acid

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Department of Chemistry, University of Nevada, Reno (Nevada 89557, USA), 31 March 1981

Summary. Xanthobilirubic acid (XBR) undergoes enzymatic oxidation in the presence of horseradish peroxidase and tert-butyl hydroperoxide to give cleanly methylethylmaleimide (**1**) and 5-formyl-2,4-dimethyl-1*H*-pyrrole-3-propanoic acid (**2**).

Bilirubin-IX*α* (BR) undergoes rapid bleaching with hydroperoxides, e.g., H_2O_2, C_2H_5OOH, in the presence of horseradish peroxidase (HRP) to give products of as yet unknown structures[2–4]. This enzymatic oxidation, which is very slow for albumin-bound BR and fast for unbound, has been used for the measurement of BR concentrations, down to nmolar, in water and for the determination of BR-to-albumin binding constants[4]. This and other enzymatic oxidations, including those with hemoglobin-H_2O_2, xanthine oxidase, cytochrome C, and oxidases from brain mitochondria, liver and heart, have been reported, again yielding products of uncertain structure[2,3]. One might infer from these observations biological mechanisms for controlling excessively high serum BR levels such as in the in vivo degradation of BR leading to its excretion as an 'alternate pathway' in congenitally jaundiced Gunn rats[5] of Crigler-Najjar babies[6]. We have begun a study of the HRP-peroxide oxidation or BR, with emphasis on product structure determination, first using a simple dipyrrole analog, xanthobilirubic acid (XBR) and report herein on the product structures.

Materials and methods. XBR was synthesized according to the method of Grunewald et al.[7]. BR was obtained from Sigma and purified by dissolving in chloroform, washing that solution 3 times with dilute aq. sodium bicarbonate, evaporation and crystallization from 1:1 chloroform-methanol. HRP was obtained from Sigma, and tert-butylhydroperoxide was obtained from MCB. Enzymatic oxidations were carried out using freshly prepared solutions as follows: to 30 mg of XBR, dissolved in 1 drop of conc.