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.

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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9 T.F. Spande, unpublished results.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11 D.J. Hart and Y.-M. Tsai, unpublished results.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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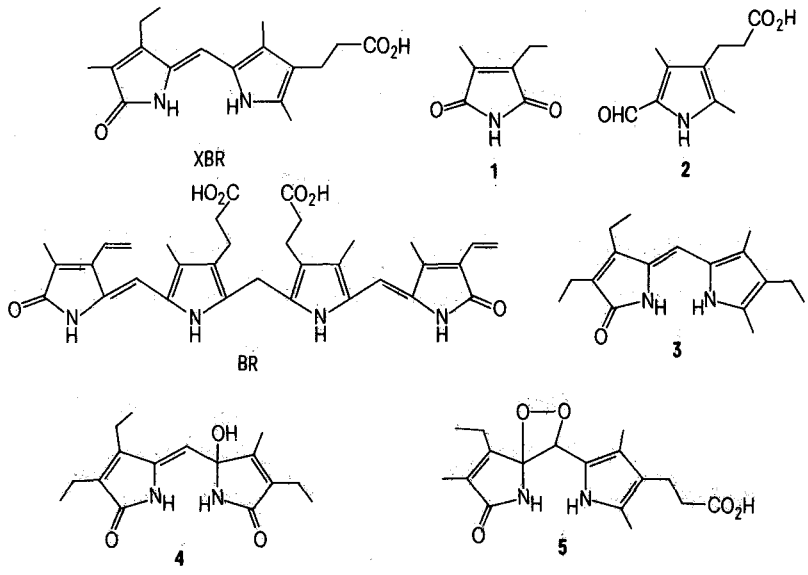
*Summary.* Xanthobilirubic acid (XBR) undergoes enzymatic oxidation in the presence of horseradish peroxidase and tert-butyl hydroperoxide to give cleanly methylethylmaleimide (**1**) and 5-formyl-2,4-dimethyl-1*H*-pyrrole-3-propanoic acid (**2**).

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.




ammonium hydroxide and diluted with 10 ml of water to give a 0.01 M solution of pH 7-8, was added 0.1 ml of a solution of 1 mg of HRP in 1 ml of water. Tert-butylhydroperoxide (0.2 ml) was added (immediate bleaching observed), and the solution was stirred at 25 °C for 12 h. After lyophilization, the resulting tan powder was washed with chloroform (10 ml) then dissolved in methanol (10 ml). The chloroform and methanol fractions (containing 20 and 10 mg of BR derivatives, respectively) were analyzed by analytical HPLC using a Perkin-Elmer Series 3 liquid chromatograph equipped with a Reodyne 7105 injector and an LC-55 detector. The column used was a DuPont Zorbax-SIL (25 cm × 4.6 mm inside diameter). Separations were achieved at 25 °C. The variable wavelength detector was set at 275 nm, the elution solvent was chloroform (Fisher, HPLC grade containing 0.75% ethanol stabilizer) - 1% acetic acid, and the flow rate was 0.2 ml/min. The components were separated preparatively by LC on a 20 cm × 1.5 cm inner diameter glass column packed with Woelm silica gel (32-63 µm particle size) using chloroform (Fisher, with 0.75% ethanol stabilizer) - 1% acetic acid. Clean separation was achieved using a flow rate of 2 ml/min (Milton-Roy mini-pump) as detected by an Altex model 150 detector equipped with preparative flow cell and 254 nm filter.

*Results and discussion.* Our HPLC analyses of either the analytical or preparative scale HRP-tert-butylhydroperoxide oxidation of XBR showed only 1 faster moving major and 1 slower moving minor product in the chloroform washings. These same products appeared in an HPLC analysis of the methanol solution, but with more nearly equal relative intensities, in addition to a 3rd, more minor product, which had the longest retention time. No other products could be detected. The products were separated by preparative LC. The major product (**1**) [UV ($CH_3OH$): $\varepsilon_{221}=12{,}660$; IR, $\nu$ ($CCl_4$): 2950, 1720, 1620 $cm^{-1}$; $^1$H-NMR, $\delta(CCl_4)$: 1.1 (3H, t, J=7Hz), 1.9 (3H, s), 3.0 (2H, q, J=7Hz), 6.1-6.5 (1H, br.s., NH)ppm] was identical in all respects with a standard sample[8] of ethylmethylmaleimide[9]. The other principal component (**2**) was characterized as its ethyl ester [UV ($CH_3OH$): $\varepsilon_{279}=14{,}600$, $\varepsilon_{313}$ shoulder; IR, $\nu(CCl_4)$: 3250, 3000, 1710, 1660, 1250 $cm^{-1}$; $^1$H-NMR, ($CDCl_3$) $\delta$: 1.3 (3H, t, J=7Hz), 2.2 (6H, s), 2.3-2.7 (4H, m), 4.3 (2H, q, J=7), 9.5 (1H, s, CHO) ppm] which was identical with synthetic material[10] prepared from methyl 2,5-dimethyl-1*H*-pyrrole-3-propanoic acid[7] using the Vilsmeier reaction[11,12]. The most minor product is methylethylmaleic acid, produced by hydrolysis of methylethylmaleimide during the reaction[13]. Treatment of methylethylmaleimide under the reaction conditions leads to the same hydrolysis product. It should be noted that reaction of XBR with either HRP or tert-butylhydroperoxide alone leads to essentially no rection, XBR being recovered under our conditions; only the hydroperoxide in the presence of the enzyme results in rapid degradation of XBR. Use of hydrogen peroxide in place of tert-butylhydroperoxide gives qualitatively similar results.

The course of the enzymatic oxidation of XBR to give **1** and **2** is of special interest in relation to the self-sensitized or dye-sensitized photooxidation, reported by Grunewald et al.[14] to give the same principal products, **1** and **2**. It is thought that the photooxidation might involve production and reaction of singlet oxygen [$^1O_2$] with the ene-amide C=C of XBR, or possibly a radical mechanism involving ground state oxygen[11,14]. On the other hand, in a reaction which must surely involve a radical mechanism, the structurally similar 5′-oxo-3′,4′-triethyl-3,5-dimethyl-1′,5′-dihydro (2.2′)-dipyrromethene (**3**) autoxidizes on a silica surface and gives neither **1** nor **2** but **4** and the corresponding mesobiliverdin[15]. Curiously, products like **4** are found in the photooxidation of BR but not in the photooxidation of **3**[11] or XBR[14]. Although it is a striking fact that the products from the enzymatic oxidation and the photooxidation of XBR are the same, those observations per se do not necessarily suggest the intermediacy of $^1O_2$ in the enzymatic oxidation or radical oxidations in the photooxidation. However, because other evidence has been reported supporting radical processes in the self-sensitized photooxidations of XBR[14,17] and related oxopyrromethenes[9,16-18], and possibly even BR[17,18] it would appear likely that both the photooxidation and the enzymatic oxidation of XBR arise via radical mechanisms - possibly involving the same oxygen radical species. We assume that the dioxetane (**5**) of XBR must be formed at some stage in the reaction[9,15,16,19]. Although peroxidase oxidations have been investigated[20,21] the exact mechanism with XBR as substrate is as yet unclear. Work is in progress on this point as are explorations with other peroxidases.

As expected from the results with XBR, in the HRP-tert-butylhydroperoxide reaction with BR we have obtained evidence that methylvinylmaleimide is a product. Studies are underway to determine the structures of the other products.

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## Spermatozoid-attracting substance in hermaphrodite brown algae, *Pelvetia wrightii* and *Fucus evanescens*

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*Summary.* A spermatozoid-attracting substance of the hermaphrodite brown algae, *Pelvetia wrightii* and *Fucus evanescens*, was identified as 1, trans-3, cis-5-octatriene, respectively, by $^1$H-NMR and $^{13}$C-NMR data and biological activities.

Müller and Jaenicke[3,4], in 1973, isolated the volatile attractant of spermatozoids; 1, trans-3, cis-5-octatriene (fucoserratene, **1**) from the eggs and oogonia of a dioecious brown alga, *F. serratus*. However, spermatozoid-attracting substances of hermaphrodite brown algae have not been explored so far.

Receptacles of the hermaphrodite brown algae, *P. wrightii* and *F. evanescens* were collected in the tidal flat along the Charatsunai coast of Muroran, Hokkaido, in October and June respectively. The mature receptacles were detached from the plants, wiped with gauze and rinsed in filtered sea water to remove diatoms and microorganisms[5]. The cleaned receptacles were kept at 18 °C in a growth chamber illuminated with white fluorescent lamps at about 2500 lx for 8 h and then transferred to Yamaguchi University, in a container at 5 °C with dry ice. The receptacles (22.4 kg), treated in the cold, were soaked in MeOH saturated with pentane for 3 days and the pentane-soluble portion was chromatographed on an alumina gel and florisil gel with pentane. Early fractions were further separated by $AgNO_3$-silica gel column chromatography (pentane and increasing amounts of diethyl ether) followed by preparative GC (Varian Model 920 gas chromatograph; 5% PEG 20M 1 m × 5 mm, column temp. 70 °C, flow rate 35 ml/min) to give a spermatozoid-attracting substance (6.3 mg). The structure of the attractant was fully substantiated by comparison of UV ($\lambda_{max}^{hexane}$ 252, 261 and 272 nm), MS [m/z 108 (39%, $M^+$); m/z 79 (100%, M-29)][3,4], $^1$H-NMR and $^{13}$C-NMR data (table 1)[3,6] with those of authentic **1** which was prepared by a Wittig reaction between 1, trans-3-pentadienal and propyltriphenyl phosphonium bromide in THF in solid-liquid 2 phase using 18-crown ether - *t*-BuOK[7]. The natural **1** from *P. wrightii* was shown to contain a small amount (~5%) of 1, trans-3, trans-5-octatriene[7-9] (**2**) by $^{13}$C-NMR[6] (table 1). From a hermaphrodite alga, *F. evanescens*, the male-gamete attractant, **1** ($4 \times 10^{-5}$% based on the weight of fresh receptacles), was separated according to the procedure described above. The separated attractant was found to be contaminated with a trace of 2-methyl-1,3,5-heptatriene (**3**) (tentatively identified) by gas chromatography (Shimadzu GC-6A, flame ionization detector, 15% TCEP Chromosorb WAW, 6 m × 3 mm, column temp. 70 °C, $N_2$ flow rate 80 ml/min) and mass spectral analysis; **1** [15.5 min. 98% (based on peak area)] and **3** (18.2 min. 2%); m/z 108 (40%, $M^+$), 93 (base peak, $M^+$-15), 77 (51%), 65 (11%), 39 (17%), 27 (11%).

This procedure, including fluorescent illumination and cold treatment is an effective method of attractant extraction. We have demonstrated that synthetic **1** attracts spermatozoids of *F. evanescens*[7-10]. When we compared directly the biological activities of **1** and **3** for the males of *F. evanescens* and *P. wrightii* according to the method of Müller[11,12], the preference of the males for **1** was quite unambigurous (table 2). The spermatozoid-attracting activity of **2** for

Table 1. $^{13}$C-NMR and $^1$H-NMR data of fucoserratene from *P. wrightii*

| Carbon No. | $^{13}$C-NMR* $\delta$ (ppm) | $^1$H-NMR* $\delta$ (ppm), Multiplicity, J (Hz) |
|---|---|---|
| 1 | 116.8 | A 5.08 dd $J_{1A,2}=10$, $J_{1A,B}=1.5$<br>B 5.19 dd $J_{1B,2}=17$, $J_{1A,B}=1.5$ |
| 2 | 137.2 | 6.15 m |
| 3 | 135.0** | 5.72 m |
| 4 | 132.9** | 6.44 m |
| 5 | 127.7** | 5.98 t $J_{4,5}=J_{5,6}=10$ |
| 6 | 128.5 | 5.42 dt $J_{5,6}=10$, $J_{6,7}=6$ |
| 7 | 21.2<br>25.9*** | 2.21 quin. $J_{6,7}=J_{7,8}=6$ |
| 8 | 14.2 | 1.01 t $J_{7,8}=6$ |

* $^{13}$C-NMR and $^1$H-NMR spectra in $CDCl_3$ were recorded on a JEOL EX-60 and FX-100 NMR-spectrometer. TMS served as an internal reference ($\delta=0$). ** Assignments for values marked may be interchanged. *** The $^{13}$C-NMR chemical shift of C-7 in **2** characteristically appears downfield compared with C-7 in **1**[6].