protons, and the positive A_{ZZ} with protons in the methionine group.

Conclusion

The spin distribution obtained from SCCEH calculations for ferricytochrome *c* have provided a satisfactory explanation of observed ^{14}N and ^{1}H ENDOR data. The unpaired electron spin is found to be a π state involving admixture of d_{XZ} and d_{YZ}-like states and the sulfur of methionine is found to carry a small positive charge, in keeping with the proposition made in the literature in a qualitative description[10] of the mechanism of electron transfer to and from cytochrome. It is felt that the calculated electronic structure in this paper will be a useful starting point for further quantitative investigations of electron transfer between cytochrome *c* and donor or acceptor systems.

Acknowledgment. We are grateful to Professor C. P. Scholes and to Dr. R. de Beer from Technische Hogeschool Delft, The Netherlands for valuable discussions.

Registry No. Cytochrome *c*, 9007-43-6.

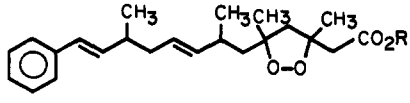
Antifungal Peroxide-Containing Acids from Two Caribbean Sponges†

Douglas W. Phillipson and Kenneth L. Rinehart, Jr.*

Contribution from the Roger Adams Laboratory, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801. *Received March 23, 1983*

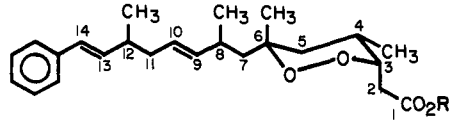
Abstract: Plakinic acids A and B, two new antifungal peroxy acids isolated from a Caribbean sponge, were assigned structures by spectroscopic techniques and by chemical degradation of their methyl esters, which were more easily purified. One of the acids contains the peroxide function as part of a five-membered ring. Plakortic acid, a carboxylic acid corresponding to plakortin, a previously described methyl ester, was isolated from another sponge. While the acids are potent antimicrobial compounds, the methyl esters, including plakortin, are essentially inactive.

A Caribbean sponge that grows like a shelf fungus in fairly deep water (at least down to 60 m) on rock or coral and has a smooth pink frosting-like outer layer over a fibrous charcoal-gray inner layer gave extracts among those most active from the Alpha Helix Caribbean Expedition[1] against *Saccharomyces cerevisiae* (a yeast) and *Penicillium atrovenetum* (a filamentous fungus) in a disk assay. The extracts also inhibited L1210 leukemia cells (ID_{50} 0.14 μg/mL). From samples of that sponge, collected by SCUBA, stored frozen or in isopropyl alcohol, and most recently identified as belonging to an apparently previously undescribed and still unnamed genus of the family Plakinidae,[2] we have isolated two peroxy acids that are responsible for the antifungal activity—plakinic acids A (**1**) and B (**2**), whose structure assignments we report here.



1 R = H

3 R = CH_3



2: R = H

4: R = CH_3

The toluene phase from sponge samples extracted in the usual manner with 3:1 methanol–toluene[1] was applied to a reversed-phase medium-performance liquid chromatography (LC) column (Waters Associates C_{18} column packing from cracked Prep 500 cartridges, in Altex glass columns), gradient eluted in 10% steps from 70% methanol water through 100% methanol. Reversed-phase high-performance (HP)LC of bioactive fractions using 72.5% methanol–27.5% 0.01 N sodium acetate buffer (pH 4.6) yielded plakinic acids A and B (**1** and **2**), whose molecular formulas were established as $C_{23}H_{32}O_4$ and $C_{24}H_{34}O_4$, respectively, by high-resolution fast atom bombardment mass spectrometry (HRFABMS) employing xenon and glycerol[3] (417.2005, Δ 1.3 mmu, $M_A - H + Na_2$, $C_{23}H_{31}Na_2O_4$ requires 417.2018; 431.2138, Δ 3.7 mmu, $M_B - H + Na_2$, $C_{24}H_{33}Na_2O_4$ requires 431.2175).

The compounds were established as carboxylic acids by their IR spectra (**1**: broad band 3600–2600 cm^{-1}, carbonyl 1716 cm^{-1}) and by their conversion with diazomethane to the corresponding methyl esters **3** [$[\alpha]^{21}_D$ −57.8° (*c* 1.15)] and **4** [$[\alpha]^{21}_D$ −186.0° (*c* 5.00)] (carbonyl 1735 cm^{-1}), which could be easily separated by flash chromatography[4] on silica gel using 7% ethyl acetate in hexane (estimate of acids **1** and **2**: 0.01% and 0.1% in sponge). Crude methyl plakinate A (**3**) eluted first in the flash chromatography and was purified by HPLC on a semipreparative silica gel column with 2% ethyl acetate in hexane as the mobile phase.

The major skeletal fragment of **3** was assigned as unit a, $C_6H_5CH{=}CHCH(CH_3)CH_2CH{=}CHCH(CH_3)CHH-$, from extensive decoupling of its ^{1}H NMR spectrum [360 MHz; cf. supplementary material (see paragraph at end of paper regarding supplementary material)], with signals at δ 7.35–7.16 (m, 5 H), 6.33 (d, J = 16 Hz), 6.13 (dd, 16, 7.5 Hz), 2.36 (m), 1.07 (d, 3 H, 7 Hz), 2.1 (m, 2 H), 5.35 (m), 5.35 (m), 2.27 (m), 1.00 (d, 3 H, 7 Hz), 1.54 (dd, 14, 6 Hz), and 1.66 (dd, 14, 8 Hz) for the respective hydrogens and from its UV spectrum [$\lambda^{95\%EtOH}_{max}$ 246 nm

† This work was presented in part at the 186th National Meeting of the American Chemical Society, Washington, DC, Aug 28–Sept 2, 1983.

(1) Rinehart, K. L., Jr.; Shaw, P. D.; Shield, L. S.; Gloer, J. B.; Harbour, G. C.; Koker, M. E. S.; Samain, D.; Schwartz, R. E.; Tymiak, A. A.; Weller, D. L.; Carter, G. T.; Munro, M. H. G.; Hughes, R. G., Jr.; Renis, H. E.; Swynenberg, E. B.; Stringfellow, D. A.; Vavra, J. J.; Coats, J. H.; Zurenko, G. E.; Kuentzel, S. L.; Li, L. H.; Bakus, G. J.; Brusca, R. C.; Craft, L. L.; Young, D. N.; Connor, J. L. *Pure Appl. Chem.* **1981**, *53*, 795–817.

(2) Identification by Dr. G. J. Bakus, Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089

(3) Rinehart, K. L., Jr. *Science (Washington, D.C.)* **1982**, *218*, 254–260.

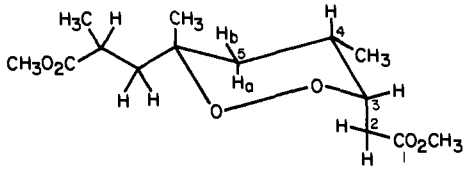
(4) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923–2925.

0002-7863/83/1505-7735$01.50/0

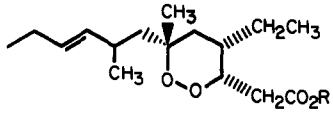
(ε 20000), shoulders at 282 and 291 nm], characteristic of a styrene unit.[5] The geometry of the unconjugated olefin was shown to be trans by addition of a shift reagent [Eu(fod)$_3$], which separated the resonances at 5.35 ppm and allowed measurement of the observed 15-Hz coupling constant.

Five other isolated spin systems [two CH_3–C–O groups, δ 1.41 (s, 3 H), 1.30 (s, 3 H); two isolated $-CH_2-$ groups, 2.74 (d, J = 14 Hz) and 2.61 (d, 14 Hz), 2.57 (d, 12 Hz) and 2.07 (d, 12 Hz); one $COOCH_3$ group, 3.66 (s, 3 H)] account for the remaining atoms of methyl plakinate A and must be assembled as shown in **3** in view of the groups' lack of coupling to unit a, thereby giving structures **3** for methyl plakinate A and **1** for plakinic acid A. The ^{13}C NMR spectrum of **3** contains the expected number of quaternary, methine, methylene, and methyl signals (cf. supplementary material).

Similar decoupling experiments identified unit a (with slightly different chemical shifts) as a part of methyl plakinate B (**4**, a



5



6 R = H

7 R = CH_3

homologue of **3**) and also identified, by decoupling, a unit containing C-1 through C-5 as $-CH_2-CH(CH_3)-C(-O-)H-CHH-CO_2CH_3$ [δ 1.4 (m, 2 H), 2.25 (m), 0.79 (d, 3 H, J = 7 Hz), 4.34 (m), 2.88 (dd, 16, 9 Hz), 2.38 (dd, 16, 4 Hz), and 3.68 (s, 3 H), respectively]. As for **3**, the remaining methyl-substituted quaternary carbon, C-6 [CH_3–C–O, δ 1.28 (s, 3 H)], must be attached to a, leading directly to structures **2** and **4** for plakinic acid B and methyl plakinate B. Again, the ^{13}C NMR spectrum of **4** was consistent with the proposed structure.

Reductive ozonolysis (ozone at −78 °C, followed by dimethyl sulfide) of **4**, followed by oxidation of the resulting aldehydes with Jones reagent and esterification of the carboxylic acids with diazomethane, gave methyl benzoate and dimethyl α-methylsuccinate (identified by GC coelution with reference materials on Carbowax 20M, SP 2100, or Tabsorb and GC/MS analysis using Carbowax 20M column) and **5** $[[\alpha]^{23}_D$ −218° (*c* 2.13, $MeCl_2$); $C_{14}H_{24}O_6$, HREIMS 288.1576, Δ −0.3 mmu, isolated by flash chromatography on silica gel using 50% ether in pentane].

The 360-MHz ^1H NMR spectrum (C_6D_6) of **5** showed every proton resonance and extensive decoupling of every geminally and vicinally coupled proton in the C-2 through C-5 spin system of **5** was carried out. Assuming a chair conformation for the six-membered ring, the coupling constant between H-5a and H-4 (11.9 Hz) indicates a trans-diaxial relationship between those hydrogens, and the methyl group at C-4 must be equatorial. The coupling constant between H-4 and H-3, 5 Hz, indicates a cis relationship between those hydrogens, and the CH_2COOCH_3 group must be axial, requiring the large group on C-6 to be equatorial and the methyl group on C-6 to be axial. Thus, the relative stereochemistry of the six-membered ring is assigned as shown in **2** and **5**.

We believe this is the first observation of a five-membered ring peroxide among marine natural products, although the peroxide functionality occurs in marine-derived compounds such as plakortin (**7**).[6] From a Caribbean sponge most recently identified as *Plakortis zyggompha* (de Laubenfels, 1934),[2] whose extracts were active against *S. cerevisiae* and *P. atrovenetum*,[1] we have isolated for the first time[7] the active compound **6** and have named it plakortic acid since its treatment with diazomethane gives a compound identical to the methyl ester **7** (MS, GC retention time on OV-17 and Tabsorb columns).[6,8] Plakortin itself (**7**, ^1H and ^{13}C NMR chemical shift data in agreement with literature values) was the major, but essentially bioinactive, product from the same sponge [no activity vs. *Escherichia coli*; *Bacillus subtilis* (Gram-positive) inhibition only at concentrations of 100 mg/disk, in contrast to its previously reported activity], accompanied by its 3-epimer, both in 0.2% yields. The much more difficultly isolable plakortic acid is a potent antifungal as well as antibacterial agent, giving 40-, 34-, and 23-mm zones of inhibition at 100 μg/disk vs. *S. cerevisiae*, *P. atrovenetum*, and *B. subtilis*, respectively.

Similarly, the esters **3** and **4** are essentially inactive in our disk assay while plakinic acids A and B (**1** and **2**) are quite active antifungal agents, giving zones of 24 and 20 mm, respectively, vs. *S. cerevisiae* and 25 and 18 mm vs. *P. atrovenetum* at 100 μg/disk.

Note Added in Proof. We have recently isolated a third antifungal acid from the family Plakinidae sponge with the same structure as **1** except for an additional $-CHCH_3CH_2-$ group between the five-membered ring and unit a, i.e., $C_6H_5CH{=}CHCH(CH_3)CH_2CH{=}CHCH(CH_3)CH_2CH(CH_3)CH_2C(CH_3)CH_2C(CH_3)CH_2COOH$.

Acknowledgment. This work was supported by grants from the National Institute of Allergy and Infectious Diseases (AI 04769) and the National Science Foundation (PCM 77-12584). Mass spectra and NMR spectra were obtained on instruments supported respectively in part by grants from the National Institute of General Medical Sciences (GM 27029) and the National Science Foundation (CHE 79-16100). We thank J. C. Cook, who performed the high-resolution FAB analyses, and Dr. M. Geckle, Bruker Instruments, who obtained the ^{13}C NMR spectrum of methyl plakinate A. We also thank the government of Belize for permission to carry out scientific studies in its territorial waters and L. S. Shield for assistance with preparation of the manuscript.

Supplementary Material Available: Description of the experimental procedures (7 pages). Ordering information is given on any current masthead page.

(5) Overberger, C. G.; Tanner, D. *J. Am. Chem. Soc.* **1955**, *77*, 369–373.

(6) Higgs, M. D.; Faulkner, D. J. *J. Org. Chem.* **1978**, *43*, 3454–3457.

(7) This portion of the present report was described in brief at the IUPAC 4th International Symposium on Marine Natural Products, Tenerife, Spain, July 26–30, 1982.

(8) It may be of some biosynthetic interest that the stereochemical assignments we have made for **2** and **4** yield the same relative stereochemistry for the ring as found for plakortin by Higgs and Faulkner[6] using different reasoning.