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STRUCTURE OF MALHAMENSILIPIN A, AN INHIBITOR OF PROTEIN TYROSINE KINASE, FROM THE CULTURED CHRYSOPHYTE *POTERIOOCHROMONAS MALHAMENSIS*

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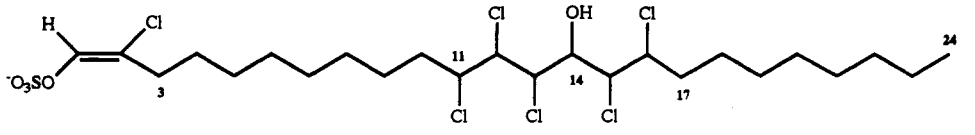
ABSTRACT.—A new chlorosulfolipid, malhamensilipin A [**1**] was isolated from the cultured chrysophyte *Poterioochromonas malhamensis*. Malhamensilipin A was demonstrated to be a modest inhibitor of $pp60^{v\text{-}src}$ protein tyrosine kinase. The structure was determined by detailed spectral analysis to be a novel C_{24} hexachloro lipid containing a vinyl sulfate ester (2,11,12,13,15,16-hexachloro-14-hydroxy-*n*-tetracos-1*E*-enol-1-sulfate).

The protein tyrosine kinase (PTK) signaling pathway mediates the effects of growth factors and oncogenes on cell proliferation and has been identified as a new molecular target for anticancer drug development (1). A number of natural products have been identified as inhibitors of this kinase, most recently the marine sponge metabolite, halenaquinone (2,3). While evaluating microalgae for new pharmaceutical agents, we found extracts of the cultured chrysophyte, *Poterioochromonas malhamensis*, to inhibit $pp60^{v\text{-}src}$ PTK and to exhibit antiviral and antimicrobial activity.

A number of unusual chlorine-substituted docosane and tetracosane disulfates have been previously reported in the algae *Ochromonas danica* and *P. malhamensis* (Chrysophyta, Chrysophyceae) (4,5). The chlorosulfolipids of *P. malhamensis* were found to be predominantly of the tetracosane-1,14-diol disulfate variety (6) and displayed broad-spectrum antimicrobial activity (7). We report here the isolation and structure determination of a new chlorosulfolipid, malhamensilipin A [**1**], which is responsible for the major antimicrobial activity in the extract of *P. malhamensis* and has moderate inhibitory activity against $pp60^{v\text{-}src}$ PTK (IC_{50} of 35 μM).

Bioassays were used to direct the fractionation of the antimicrobial extract of cultured *P. malhamensis* cells (see Experimental), giving pure **1** as a colorless oil [$C_{24}H_{42}Cl_6O_5S$ by negative-ion hrfabms]. The positive- and negative-ion ci mass spectra were consistent with **1** being a hexachloro monosulfate. For example, the negative-ion ci mass spectrum of **1** showed a fragment ion cluster at *m/z* 580/578/576/574/572 for $[M-SO_3]^-$, ion clusters corresponding to subsequent loss of Cl, Cl_2, Cl_3, Cl_4, and Cl_5, as well as intense fragment ions at *m/z* 97 $[HSO_4]^-$ and 80 $[SO_3]^-$.

Detailed analysis of the ^1H- and ^{13}C-nmr spectra, aided by two-dimensional ^1H-^1H COSY, ^1H-^1H LRCOSY (8), HMQC (9), and HMBC (10) experiments, enabled assignment of the ^1H and ^{13}C signals and partial structures **a–c** (Figure 1). A broad singlet proton signal at δ 6.73 and carbon signals at δ 136.26 (d, C-1) and 125.46 (s, C-2) indicated the



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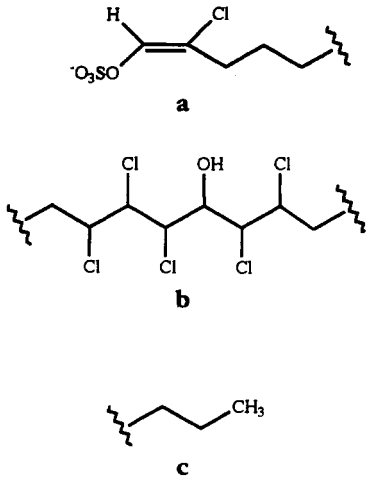

FIGURE 1. Partial Structures **a–c** for Malhamensilipin A [**1**].

presence of a trisubstituted olefin. The chemical shifts of these olefinic carbon atoms suggested that a sulfate ester and chlorine atom were located at C-1 and C-2, respectively (11,12). The olefinic proton at C-1 showed a small long-range coupling to protons at δ 2.41 (2H, bt) with an associated carbon at δ 31.86 (t, C-3). Long-range ^{1}H-^{13}C couplings indicated that the C-3 protons were correlated to two methylene carbons at δ 30.06 and 27.29 (C-4, C-5) (partial structure **a**). The remaining five chlorines were attached to methine carbons resonating at δ 62.09 (C-16), 63.11 (C-11), 65.98 (C-15), 67.49 (C-13), and 69.03 (C-12), whereas an oxygen atom was present on a methine carbon absorbing at δ 77.50 (C-14). The ^{1}H-^{1}H COSY, HMQC, and HMBC spectra of **1** established an overall linear structure for these chlorinated and oxygenated carbons (-CHCl-CHCl-CHCl-CHOH-CHCl-CHCl-). An isotope shift of $\Delta\delta = 0.12$ for the carbon at δ 77.50 in a CH_3OH/CD_3OD solution of **1** was consistent with the presence of a hydroxyl group at this position in the spin system (C-14). The methine protons at the termini of this unit were coupled to methylene protons at δ 1.92 (2H, m) and 1.81 (2H, m), with associated carbons at δ 37.75 and 38.42 (partial structure **b**). The remainder of **1** was composed of 10 methylenes (20H at δ 1.32–1.41 with associated carbons at δ 23.73–33.01) and one methyl group (3H at δ 0.89 (t); associated carbon at δ 14.43, C-24). Long-range ^{1}H-^{13}C couplings located H_3-24 in the proximity of carbons at δ 23.73 (2-bond, C-23) and 33.01 (3-bond, C-22) (partial structure **c**).

Definitive placement of the series of sequentially chlorinated or oxidized carbon atoms in the chain was not possible on the basis of nmr analysis alone. Connectivity between these three partial structures and the remaining methylene groups was determined by mass spectrometry of the TMS ether derivative of malhamensilipin A [**1**]. Two major fragment ion clusters at *m/z* 315/313/311 and 441/439/437/435 were observed for $[C_{14}H_{29}Cl_2OSi]^+$ and $[C_{17}H_{31}Cl_4O_2Si]^+$. These fragment ions are consistent with cleavage α to a TMS ether at C-14. Placement of the hydroxyl group at C-14 indicates that the six sequentially functionalized centers span C-11 to C-16, thereby defining the gross structure of malhamensilipin A [**1**].

The geometry of the C-1,2 olefin is proposed to be *E* based on the absence of nOe for H-1↔H-3. The lack of adequate model compounds for the *E* and *Z* isomers of this α-chloro vinyl sulfate precluded confirmation of the olefin geometry through nmr shift data. Assignment of relative stereochemistry at C-11 to C-16 was not possible from nmr data alone owing to conformational flexibility of the system.

Malhamensilipin A [**1**] represents a new addition to the chlorosulfolipid structure class. The chlorovinyl sulfate present in **1** is an unprecedented functionality that expands our appreciation of the range of functional groups produced in nature. Although we hypothesize a biogenetic relationship between malhamensilipin A and other chlorosulfolipids known from

this and related genera, it is conceivable that the free alcohol at C-14 and the C-1 olefin in malhamensilipin A are formed artifactually. However, we feel this to be unlikely given the presence of the more labile sulfate ester at C-1 in metabolite **1** and the mildness of conditions used to process the extract. As a class, chlorosulfolipids have been found to be responsible for the antibiotic activity associated with the extracts of several chrysophyte species. This may be relevant to the ecological role of these amphipathic lipids.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were recorded on a Bruker AC 300 spectrometer. ^{1}H-Nmr chemical shifts are referenced in MeOH-d_4 to the CHD_2OD residual signal (3.30 ppm). ^{13}C-Nmr chemical shifts are referenced in MeOH-d_4 to the solvent signal (49.0 ppm). Mass spectra were recorded on Kratos MS 50 TC and Finnigan 4023 mass spectrometers. Uv spectra were recorded on a Hewlett-Packard 8452A uv-vis spectrophotometer and ir spectra on a Nicolet 510 spectrophotometer. Merck aluminum-backed thin-layer chromatography sheets were used for tlc, and all solvents were distilled from glass prior to use.

CULTURE AND EXTRACTION.—The alga *P. malhamensis* (Pringsheim) Peterfi. (UTEX L1297) was cultured in a 50 liter Nalgene carboy containing 30 liters of *Ochromonas* medium (13) with continuous stirring at 28°. The light regime was 16 h light/8 h dark/day. The cells were harvested after 22 days (pH=7.7) by using a De Laval (Laval) cream separator and then filtered through 30 g of washed Celite. Cells were lyophilized and kept frozen prior to extraction.

Dried cell material (5.56 g) was extracted with $CHCl_3$ (3×50 ml) and then left overnight in CH_3OH-H_2O (3:1) solution (120 ml) at room temperature. The solution was filtered using a Millipore GF/F filter and then the cells were extracted a second time overnight with CH_3OH/H_2O (120 ml). The combined CH_3OH/H_2O filtrates were reduced *in vacuo*, mixed with ca. 80 g of Amerlite® XAD-7 resin (Sigma) in a small volume of H_2O and allowed to sit overnight. The XAD-7 resin was sequentially rinsed with 3 liters of H_2O and then an excess of Me_2CO (150 ml) and CH_3OH (200 ml). The CH_3OH-soluble material (598 mg) from the combined organic eluents was tested for activity to PTK and in standard antimicrobial sensitivity assays at 2 mg/disc [*Bacillus subtilis* (ATCC 6081, 14 mm), and *Staphylococcus aureus* (ATCC 12600, 19 mm), *Escherichia coli* (ATCC 11775, 8.5 mm), *Pseudomonas aeruginosa* (ATCC 9721, 13 mm), and *Candida albicans* (ATCC 14053, 15 mm)]. Inhibitory activity to $pp60^{v\text{-}src}$ PTK was determined following previously described methodology (2,3).

FRACTIONATION AND BIOAUTOGRAPHY.—A portion of the crude bioactive fraction (560 mg) was chromatographed by gel filtration [Amberlite® XAD-7 column with Me_2CO-H_2O (1:1) and then CH_3OH]. The CH_3OH eluent (230 mg) showed pronounced antimicrobial activity. Bioautographic analysis of this fraction revealed that the active component had an R_f of 0.42–0.47 [Si gel tlc plate, 33% $CH_3OH/CHCl_3$, Muller-Hinton agar, *Bacillus subtilis* (ATCC 6081), 11–13 mm/200 μg].

Isolation of malhamensilipin A [**1**].—Repetitive chromatography of the above gel filtration fraction (230 mg) over Si gel with 12% $CH_3OH/CHCl_3$ gave **1** as a colorless oil (13.8 mg, ca. 0.25% yield relative to dry wt of cells). The remainder of this crude fraction was composed largely of other chlorosulfolipids of undetermined structure. Tlc analysis of malhamensilipin A [**1**] showed it to be an acid-charring compound of R_f 0.46 (33% $CH_3OH/CHCl_3$) with the following spectral characteristics: $[\alpha]_D$ +28° (c=0.5, CH_3OH); uv λ max (CH_3OH) 206 nm (ε 13200); ir ν max (neat) 3450, 2930, 2860, 1640, 1460, 1260, 1230, 1050, 940, 815, 720 cm^{-1}; negative-ion hrfabms, glycerol/H_2O matrix, $[M-H]^-$ cluster at m/z 659.0666 (7), 657.0690 (19), 655.0741 (38), 653.0716 (46), 651.0738 (27, 0.2 mmu dev. from $C_{24}H_{41}{}^{35}Cl_6O_5S$); negative-ion cims, m/z $[M-SO_3]^-$ ion cluster 580 (12)/578 (45)/576 (86)/574 (100)/572 (48), $[M-SO_3-Cl]^-$ ion cluster 545 (3)/543 (14)/541 (41)/539 (62)/537 (45), $[M-SO_3-2Cl]^-$ ion cluster 508 (10)/506 (21)/504 (33)/502 (27), $[M-SO_3-3Cl]^-$ ion cluster 471 (4)/469 (12)/467 (13), $[M-SO_3-4Cl]^-$ ion cluster 436 (1)/434 (4)/432 (7), $[M-SO_3-5Cl]^-$ ion cluster 395 (1)/397 (4), $[HSO_4]^-$ 97 (69), $[SO_3]^-$ 80 (38); ^{1}H nmr (CD_3OD, 300 MHz) δ 6.73 (1H, br s, H-1), 5.02 (1H, dd, J=9.0 and 1.0 Hz, H-14), 4.93 (1H, m, H-11), 4.82 (1H, dd, J=10 and 1.0 Hz, H-13), 4.76 (1H, ddd, J=6.2, 3.8, and 1.0 Hz, H-16), 4.62 (1H, dd, J=10 and 1.6 Hz, H-12), 4.27 (1H, dd, J=9.0 and 1.0 Hz, H-15), 2.41 (2H, br t, H_2-3), 1.92 (2H, m, H-10a+H-17a), 1.81 (2H, m, H-10b+H-17b), 1.52 (4H, m), 1.32–1.41 (20H, m), 0.89 (3H, t, J=6.7 Hz); ^{13}C nmr (CD_3OD, 75 MHz) δ 136.26 (d, C-1), 125.46 (s, C-2), 77.50 (d, C-14), 69.03 (d, C-12), 67.49 (d, C-13), 65.98 (d, C-15), 63.11 (d, C-11), 62.09 (d, C-16), 38.42 (t, C-10 or C-17), 37.75 (t, C-17 or C-10), 33.01 (t, C-22), 31.86 (t, C-3), 30.56 (t), 30.50 (t), 30.39 (t), 30.31 (t), 30.17 (t), 30.06 (t, C-4 or C-5), 29.75 (t), 27.75 (t), 27.68 (t), 27.29 (t, C-5 or C-4), 23.73 (t, C-23), 14.43 (q, C-24).

Malhamensilipin A-TMS ether.—Malhamensilipin A [**1**] (0.1 mg) was treated with 50 μl of pyridine-TMS-imidazole (1:1) at 60° for 30 min. The resulting mixture was subjected to gc-eims (SE54 column, 30 m×0.25 mm, 50 to 300° at 20°/min, R_t=15.23 min) and probe cims analyses. Both mass spectra showed two major fragment ion clusters for $[M-C_{10}H_{19}Cl_2-SO_3]^+$ and $[C_{14}H_{29}Cl_2OSi]^+$ at *m/z* 441 (4)/439 (20)/437 (41)/435 (30) and 315 (12)/313 (65)/311 (95), respectively. Other fragment ions were observed at *m/z* 149 (76), 147 (96), 95 (97), 93 (97), 83 (96), 81 (100), 79 (62), 75 (95).

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