Improved Method for Purification of Epidithiodioxopiperazines from Fungal Extracts: Purification of Sporidesmin A

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A simple and convenient method for purification of the epidithiodioxopiperazine mycotoxin sporidesmin A from crude extracts of *Pithomyces chartarum* has been developed. The method involves reduction of sporidesmin to the water-soluble dithiol using $NaBH_4$, followed by removal of lipophilic contaminants by liquid–liquid partition. The dithiol is then oxidized back to the lipophilic sporidesmin A, which is then extracted, purified by flash chromatography, and crystallized from $CHCl_3$ to give the pure material as the chloroform solvate in overall yields of 44–63%. The method may be applicable to purification of other dithiodioxopiperazines by judicious choice of reducing and oxidizing agents. The published ^{13}C NMR assignments of some of the quaternary resonances of sporidesmin A are revised as a result of a series of one- and two-dimensional NMR experiments which allowed unambiguous assignment of the ^1H and ^{13}C resonances.

INTRODUCTION

In New Zealand, farm animals are susceptible to a seasonal hepatogenous photosensitization disease known as facial eczema, caused by ingestion of spores of the fungus *Pithomyces chartarum*. The principal hepatotoxin, the epidithiodioxopiperazine mycotoxin sporidesmin A (1) (Figure 1), was first isolated in 1959 and fully characterized in the early 1960s [for two excellent reviews of the history of facial eczema and the chemistry and toxicity of the sporidesmins, see Mortimer and Ronaldson (1983) and White et al. (1977)].

Current immunological studies involving 1 (Gallagher et al., 1992) and studies of the metabolic fate of 1 in sheep require gram quantities of the pure mycotoxin. The standard methods for purification of 1 (Mortimer and Ronaldson, 1983) are tedious, hazardous, low-yielding, and inappropriate for large-scale operation. Furthermore, separation of sporidesmin A from other nonpolar compounds, especially sporidesmins D (2) and E (3), pithomycolide, and the sporidesmolides, has proved to be difficult. Even after cleanup by preparative HPLC followed by precipitation from benzene with hexane (Ronaldson, 1982), the material analyzed by HPLC contained only 90–95% 1 and 2–5% each of 2 and 3.

Here we report a rapid (≤1 day) and convenient procedure for purifying hundreds of milligrams of 1 from crude extracts of *P. chartarum*. The procedure utilizes the chemical reactivity of the disulfide bond of 1 and affords a 44–63% recovery of crystalline 1 (as the chloroform solvate) containing no detectable 2 and only traces of 3 and sporidesmin B (4), which are readily removed by recrystallization.

MATERIALS AND METHODS

General. Flash chromatography was performed according to the method of Still et al. (1978) using dichloromethane–methanol (99:1) as eluent. HPLC analyses were performed using a 4.6 mm × 25 cm, 5 μm Zorbax ODS column with acetonitrile–water–methanol (9:9:2) as eluent (1.5 mL min^{-1}) and detection by absorbance at 254 nm. The HPLC system was fitted with an LC Autocontrol, an LC-85B spectrophotometric detector, and an ASV-1 automatic switching valve (all from Perkin-Elmer), allowing stopped-flow UV scans (200–500 nm) of eluting peaks to be performed. Quantitative analyses were performed with the aid of freshly prepared standard solutions of solvate-free sporidesmins available from earlier work in this laboratory [see Mortimer and Ronaldson (1983)]. The retention times of 1, 2, 3, and 4 were 6.0, 4.7, 7.3, and 7.8 min, respectively. Laboratory grade sodium perborate and AR grade iodine were obtained from BDH Ltd., and sodium borohydride was from Sigma. All solvents were of analytical or HPLC grade. Sporidesmin extracts were available from material routinely prepared (Mortimer and Ronaldson, 1983) in this laboratory by extraction of cultures with methanol–water (17:3), concentration in vacuo to an aqueous suspension, extraction with diethyl ether, and evaporation of the ethereal extract in vacuo. TLC was performed on silica gel plates (E. Merck art. 5554) using chloroform–methanol (24:1) as eluent (R_f of 1, 0.51). Elemental analysis was performed by the University of Otago Microanalytical Laboratory. ^1H and ^{13}C NMR spectra were obtained from a deuterioacetone solution using a Bruker AC-300 instrument operating at 300 and 75 MHz, respectively. Chemical shifts are reported relative to internal TMS. Two-dimensional ^{13}C–^1H correlated spectra, optimized for ^1J or for long-range ($^2J/^3J$) couplings were acquired in absolute value mode using the XHCORR sequence with D_3 = 3.8 ms and D_4 = 1.9 ms (for ^1J couplings) or with D_3 = 35 ms and D_4 = 25 ms (long-range couplings). Electron impact mass spectra (EIMS) from direct probe sample insertion were obtained under low- and high-resolution conditions using a Kratos MS80 RFA instrument.

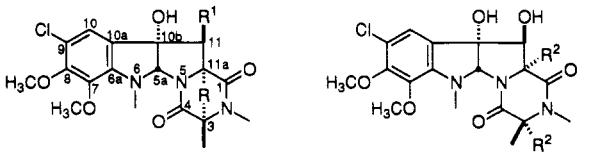

Figure 1. Structures of sporidesmin A (1) and related compounds.

Purification of 1. A. Using Sodium Perborate Oxidation. [Caution: Studies with animals indicate that 1, 3, and 4 are highly toxic by ingestion and cause severe irritation upon contact with skin and eyes (Mortimer and Ronaldson, 1983).] Sporidesmin extract (viscous brown oil) (4.428 g) containing 1 (580 mg), 2 (66 mg), 3 (13 mg), and 4 (9 mg) was dissolved in absolute ethanol (20 mL). To the stirred solution was added $NaBH_4$ (ca. 1 g)

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(Caution: Exothermic reaction necessitates the use of a water bath to keep the reaction at room temperature). After the vigorous effervescence [Caution: Because hydrogen gas (flammable) and volatile sulfides (stench) are produced, it is necessary to perform this reaction in a fume hood.] had ceased, a further portion of $NaBH_4$ (ca. 1 g) was added. After a total reaction time of about 1 h, TLC indicated that very little 1 remained, although 2 was still present. The reaction mixture was diluted with water (150 mL) and extracted with diethyl ether (50 mL) to remove remaining lipophilic materials (e.g., 2, sporidesmolides, pithomycolide). Acetone (10 mL) was cautiously added to the aqueous fraction to consume excess hydride, followed by sodium perborate (2 g) as a slurry in water (5 mL). The mixture was stirred for 2 h, saturated with NaCl, and extracted with dichloromethane (2 × 50 mL). This extract was dried ($MgSO_4$), concentrated in vacuo, and purified by flash chromatography to a pale green oil (541 mg). Two crystallizations, performed by slow addition of petroleum spirit (40–60 °C) to a solution of this material in chloroform, yielded 1·$CHCl_3$ (439 mg, 63%) as pale yellow crystals: mp 110–115 °C. Analysis (HPLC): 1, 82.8%; 2, not detected; 3, 0.5%; 4, 0.6%. A portion of this material was recrystallized by vapor diffusion of petroleum spirit (40–60 °C) into a chloroform solution, to give 1·$CHCl_3$ as pale yellow rods and prisms: mp 112–115 °C. Analysis (HPLC): 1, 83.5%; 2–4 not detected. Analysis (elemental) found: C, 38.6; H, 3.73; N, 7.23; Cl, 24.5. $C_{18}H_{20}N_3O_6ClS_2$·$CHCl_3$ requires: C, 38.5; H, 3.56; N, 7.08; Cl, 23.9. δ ^{1}H 2.09 (3 H, s, 3-CH_3), 3.09 (3 H, s, 2-CH_3), 3.27 (3 H, s, 6-CH_3), 3.78 (3 H, s, 7-OCH_3), 3.84 (3 H, s, 8-OCH_3), 4.61 (1 H, d, J = 1.7 Hz, H-11), 5.05 (1 H, d, J = 1.7 Hz, 11-OH), 5.29 (1 H, s, H-5a), 5.65 (1 H, s, 10b-OH), 7.08 (1 H, s, H-10), 8.00 (1 H, s, $CHCl_3$); δ ^{13}C 18.3 (3-CH_3), 27.1 (2-CH_3), 39.4 (6-CH_3), 61.1 (2 × OCH_3), 74.7 (C-3), 77.9 (C-11a), 79.2 ($CHCl_3$), 82.9 (C-11), 90.7 (C-10b), 95.5 (C-5a), 119.0 (C-9), 121.3 (C-10), 127.2 (C-10a), 141.1 (C-7), 145.7 (C-6a), 152.0 (C-8), 165.2 (C-4), 167.1 (C-1); EIMS m/z 475 (1%, ^{37}Cl M^+), 473.0475 (2, M^+, $C_{18}H_{20}N_3O_6{}^{35}ClS_2$ requires 473.0482), 441 (1), 411 (2), 409 (8), 407 (4), 391 (4), 374 (3), 358 (2), 279 (1), 273 (2), 271 (6), 252 (5), 244 (25), 243 (74), 242 (59), 241 (100), 240 (37), 228 (65), 227 (46), 226 (90), 213 (50), 212 (49), 211 (66), 210 (25).

B. Using Iodine Oxidation. Sporidesmin extract (2.51 g) containing 1 (606 mg), 2 (87 mg), 3 (10 mg), and 4 (9 mg) was treated as above, except that the oxidation was performed by addition of a saturated solution of iodine in acetone, until the color of iodine persisted, to give crystalline 1·$CHCl_3$: mp 104–110 °C, 329 mg (44%). Analysis (HPLC): 1 (80.3%); 2 and 4 not detected; 3 trace amount.

The ethereal wash of the reduction reaction was dried ($MgSO_4$) and the solvent removed in vacuo to leave a pale brown oil (1.41 g) which contained (HPLC) 1 (5.1 mg), 2 (39.3 mg), 3 (5.0 mg), and 4 (0.4 mg).

The mother liquors from the crystallizations were combined and concentrated in vacuo to leave a pale brown oil (275 mg). HPLC analysis indicated the presence of 1 (73.5 mg), 2 (0.6 mg), 3 (45.8 mg), and 4 (present but peak not fully resolved from that of 3).

DISCUSSION

One of the limiting factors in mycotoxin research is the availability of the required toxins in a pure form and in sufficient quantity. Because of the unsatisfactory performance of standard procedures for preparing 1, we required an alternative method. The presence in the crude sporidesmin extracts of contaminants with chromatographic mobilities similar to that of 1 suggested that purification by HPLC might be difficult. We therefore devised a purification route based on the chemical properties of 1. In particular, 1 is readily interconvertible with its reduced form (5) in vivo, a feature that appears to be responsible for its toxicity (Munday, 1989). While 1 is lipophilic, with a solubility of ca. 30 mg L^{-1} in water (Mortimer and Ronaldson, 1983), 5 should be highly soluble in water, especially at high pH. We therefore anticipated that reaction of crude 1 with a mild reducing agent (to give 5) followed by solvent extraction of lipophilic materials and then reoxidation of 5 with a selective oxidant would provide substantially pure 1. In particular, it was anticipated that impurities such as 2, pithomycolide, and sporidesmolides would be readily extracted from the aqueous solution of 5.

Sodium borohydride is the reagent most commonly used for reduction of 1 (and 3) during conversion to 2 with methylating agents (White et al., 1977). The high pH of aqueous solutions of $NaBH_4$ (ca. 10) ensures that 5 will remain in aqueous solution until its oxidation to 1; indeed, Ronaldson (1978) reports having to acidify a solution of 5 to extract sporidesmin dithiol (6) into chloroform.

A number of reagents are available for oxidation of dithiols to disulfides, including H_2O_2 and I_2 (Capozzi and Modena, 1974), sodium perborate (McKillop et al., 1990), and DMSO (Tam et al., 1991). Of these, $NaBO_3$, H_2O_2, and I_2 were tried on small-scale reactions, and all oxidized 5 to 1, although H_2O_2 tended to cause overoxidation (unpublished observations). Preparative-scale reactions were therefore attempted using $NaBO_3$ or I_2 as the oxidant.

The reduction step proceeded smoothly, as shown by TLC monitoring. In preparation B, the subsequent ether extraction removed 1.41 g of material (56% by weight of the crude sporidesmin extract), which contained 98.5 and 1% of the total 2 and 1, respectively, that was recovered in all of the various fractions isolated during the course of the purification.

After oxidation of 5 to 1, extraction and flash chromatography provided 1 of >90% purity. Crystallization by addition of petroleum spirit to a chloroform solution of the semipure sporidesmin provided 1 as its chloroform solvate, substantially free from impurities. Pure 1·$CHCl_3$ was then readily obtained through crystallization by vapor diffusion [recrystallization from hot solutions is inadvisable due to the well-known thermal instability of 1 (Mortimer and Ronaldson, 1983)]. The purified 1·$CHCl_3$ coeluted with authentic 1 during TLC and HPLC analyses, and its UV spectrum (stopped-flow HPLC scan) was identical to that of 1. 1·$CHCl_3$ is calculated to contain 79.9% by weight of 1, whereas HPLC analysis showed 83.5% of 1 in a sample of 1·$CHCl_3$ purified by vapor diffusion. Since this sample gave a satisfactory elemental analysis for 1·$CHCl_3$, we suspect that the reason for this anomaly is that the 1 used for the HPLC standard contains small amounts of impurities.

The ^{13}C NMR spectrum of 1·$CHCl_3$ (20 mg) in $(CD_3)_2CO$ was identical to that reported by Ronaldson (1976) for 1, except for the presence of an extra resonance at 79.2 ppm attributable to $CHCl_3$. However a long-range ^{13}C–^{1}H correlated spectrum, optimized for $^2J/^3J$ couplings, indicated that the assignments given by Ronaldson (1976) for C-6a and C-7 should be reversed (see Table I). The ^{1}H NMR spectrum contained a resonance at 8.00 ppm attributable to $CHCl_3$ from the solvent of crystallization. Upon addition of $CHCl_3$ (5 μL) to the NMR sample, the intensities of the ^{13}C and ^{1}H resonances assigned to $CHCl_3$ approximately doubled relative to those of 1. Relative to the 7- and 8-OCH_3, the integrated peak areas of the H-11, H-5a, H-10, and $CHCl_3$ resonances of 1·$CHCl_3$ were 0.98, 0.98, 0.97, and 0.94 protons, respectively, consistent with a 1:1 solvate.

During purification B, analysis of the mother liquor from the crystallization step revealed the presence of 45.8 mg of 3, whereas only 10 mg of 3 was present in the original sporidesmin extract. We attribute this to the interconversion of 1 and 3 during the purification procedure. Using HPLC, we have observed similar interconversions of

Table I. ^{13}C–^{1}H Correlations Observed for 1·$CHCl_3$

^{1}H resonance	correlated ^{13}C resonance	
	^{1}J	$^{2}J/^{3}J$
2.09 (3-CH_3)	18.3 (3-CH_3)	74.7 (C-3), 165.2 (C-4)
3.09 (2-CH_3)	27.1 (2-CH_3)	74.7 (C-3), 167.1 (C-1)
3.27 (6-CH_3)	39.4 (6-CH_3)	95.5 (C-5a), 145.7 (C-6a)
3.78 (7-OCH_3)	61.1 (OCH_3)	141.1 (C-7)
3.84 (8-OCH_3)	61.1 (OCH_3)	152.0 (C-8)
4.61 (H-11)	82.9 (C-11)	90.7 (C-10b)
5.05 (11-OH)		90.7 (C-10b)
5.29 (H-5a)	95.5 (C-5a)	
5.65 (10b-OH)		82.9 (C-11)
7.08 (H-10)	121.3 (C-10)	119.0 (C-9), 145.7 (C-6a), 152.0 (C-8)
8.00 ($CHCl_3$)	79.2 ($CHCl_3$)[a]	

[a] Observed in the long-range experiment.

initially pure solutions of 1 or 3 in aqueous alcohol solutions at room temperature (unpublished observations). Furthermore, Ronaldson (1978) reports that 1 can be converted to 3 simply by treatment with sulfur in pyridine; it would therefore not be surprising if sulfur-containing compounds in the crude sporidesmin extract converted some of the 1 that is present into 3 during the course of the purification procedure.

In our laboratory, the new procedure (with perborate oxidation) described above has superseded earlier methods (Mortimer and Ronaldson, 1983) for the purification of sporidesmin A because of its efficiency and convenience. Epidithiodioxopiperazines are a common class of natural products (Nagarajan, 1984), and our method of purification should be applicable to other compounds of this type, so long as reducing and oxidizing agents are chosen which do not affect other functional groups that are present in the molecule.

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