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ESPERAMICIN P, THE TETRASULFIDE ANALOG OF ESPERAMICIN A_1

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ABSTRACT.—A minor congener of esperamicin A_1 [**1**], designated esperamicin P (BMY-41339, **2**), was isolated from a fermentation broth of *Actinomadura verrucosospora* and determined to differ from esperamicin A_1 by having a methyl tetrasulfide moiety instead of a methyl trisulfide. It was active against xenografted tumors in mice and exhibited antimicrobial activity. Interconversions of **2** and **1** have been observed for DMSO solutions of both congeners at room temperature.

The esperamicins are an important group of enediyne antitumor antibiotics, discovered at Bristol-Myers in fermentation broths of the actinomycete *Actinomadura verrucosospora* (1). The lead compound in the series, esperamicin A_1 [**1**], was produced on a large scale by the Fermentation Production Facility at NCI-FCRDC for clinical trials in cancer.

During the isolation of esperamicin A_1, a minor congener was noted to occur in several fermentation runs, eluting just after esperamicin A_1 on C-18 analytical hplc. Inspection of its uv chromophore by diode array methods suggested that it was also an esperamicin, but different from the previously isolated minor esperamicins (A_{1b}, A_2) in hplc retention time. The preparative-scale isolation of **1** yielded sufficient amounts of the congener, esperamicin P [**2**], for structure elucidation. In the course of structure elucidation it was found that **2** could be converted into **1** at room temperature in DMSO. In addition, investigation of the oxidation of **1** was carried out to test the initial hypothesis that **2** was an oxidation product of **1**. Compound **1** was cleanly converted by reaction with H_2O_2 into two novel oxidation products, esperamicins O_1 and O_2 (**3** and **4**).

RESULTS AND DISCUSSION

Compound **2** was evident in chromatograms of crude broth extracts as a minor peak eluting just after **1**. It co-purified with **1** through the large-scale isolation protocol, which consisted of removal of antifoam, batch elution from Dicalite, and flash chromatography over Si gel. Final separation from **1** was made using prep. hplc on C-18 media with buffered CH_3CN/MeOH mixtures.

The ^{1}H-nmr spectrum of **2** was essentially identical to that of **1**, with the exception of a shift in the S_xMe signal from 2.54 ppm to 2.62 ppm, and a shift in the ^{13}C-nmr signal for C-15 from 39.5 to 40.1 ppm. Thermospray mass spectroscopy of the new metabolite gave a molecular ion at *m/z* 1357, indicating that it differed from **1** by the addition of 32 mass units. The additional substituent was clearly located on the "core" of the molecule, since the sugar fragments in the mass spectrum were identical to those of **1**. Because **2** was observed to be more abundant in samples that had been stored for several months, oxidation was considered to be a likely explanation for the increased mass.

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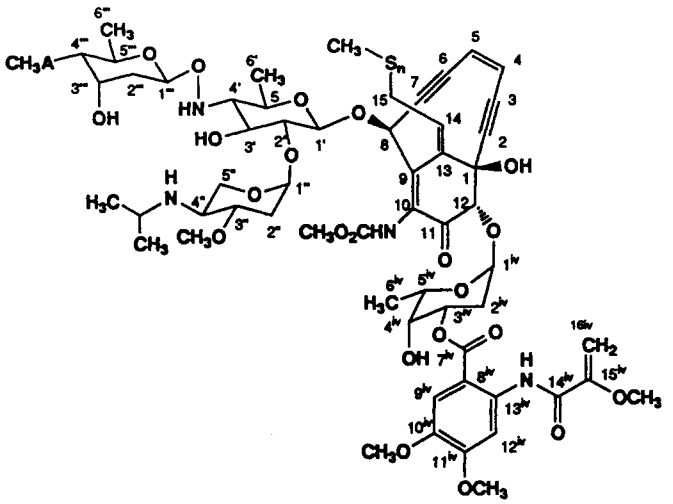

1 n=3, A=S
2 n=4, A=S
3 n=3, A=SO
4 n=3, A=SO
} Diastereoisomers

An attempt to mimic the proposed oxidation of **1** to **2** using H_2O_2 (2,3) gave a clean reaction yielding two products, **3** and **4**, which were clearly different (nmr, hplc retention times, reduced biological activity) from **2**. Instead of the expected oxidation of the trisulfide, these products appear to be the diastereomeric sulfoxides of the thiomethyl sugar. The principal evidence in support of this site of oxidation is the shift of the S-Me peak from 2.14 ppm to 2.80 ppm in the ^{1}H-nmr spectrum, as well as small shifts in the other thiomethyl sugar protons.

The preparative separation of **2** from **1** involved hplc injection of mixtures of esperamicins in DMSO solution. We noted an apparent interconversion of **2** to **1** as we attempted to purify it. This conversion was not observed when CH_3CN solutions were used to prepare material for injection. Controlled experiments using purified **2** validated this observation. Over a 4 h time-period (Figure 1) the content of **2** dropped, while the peak corresponding to **1** increased to ca. 50 percent of the total peak area. When the reaction was allowed to proceed overnight, however, degradation of both esperamicins to a complex mixture of products was observed. The reverse experiment, reaction of **1** in DMSO, gave minimal (ca. 4%) yields of **2** under the same conditions, suggesting that an equilibrium favoring **1** is involved. Reaction of **2** in MeOH at room temperature gave only complex degradation products, but no production of **1**.

The observation that **2** could be converted to **1** in DMSO solution at room temperature made it clear that oxidation of the trisulfide moiety of **1** could not possibly explain the higher molecular weight of **2**. Instead, the 32 mass unit difference had to be attributed to addition of S. Combustion analysis gave rather equivocal evidence for this proposition, with parallel combustions of **1** yielding 9.1 percent S, and **2** yielding 9.9 percent S. The similarity of nmr spectra demanded that the extra sulfur be placed in a position such that no proton would be substantially shielded or deshielded. The most reasonable placement is thus insertion into the trisulfide moiety.

A tetrasulfide functionality is consistent with the known proclivity of polysulfides to undergo exchange reactions, as with simple compounds isolated from garlic (4). Subsequent to this work, a report appeared on the reactions of the trisulfide moiety in

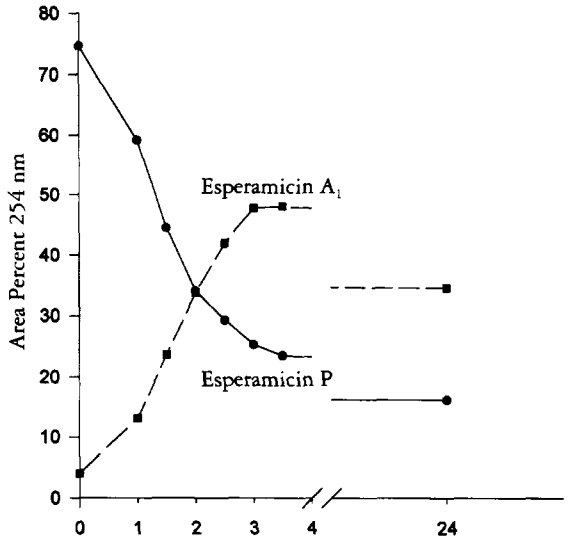

FIGURE 1. Conversion of Esperamicin P to Esperamicin A_1 in DMSO.

the related calicheamicins (5), in which dimeric trisulfide and disulfide compounds are produced with Ph_3P.

It is interesting to observe that the in vitro cytotoxicity and in vivo antitumor activity of **2** are very similar to that of **1**. While extensive biological characterization was not possible due to the limited amount of **2** at hand, **2** was active in a P-388 leukemia model (ip-ip at 1–3 μg/kg, maximum T/C 145%), similar in potency to **1**. Activity was not seen for **2** in an iv-iv P-388 model in which **1** was active, but toxic doses of **2** were not attained due to the limited supply. It would appear that **2** has somewhat weaker biological activity than **1**. It is possible that the reduced activity may be related to a difference in ability to form the anionic intermediate postulated as a reductive activation step that initiates the aromatization cascade of **1** (1,6).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All operations were carried out under yellow fluorescent lights (Sylvania Gold F40/60) due to the light-sensitive nature of the compounds. Flash chromatography was carried out essentially as described by Still *et al.* (7) with maximum loadings of 10 g on a 10-cm diameter column. Mass recoveries were typically greater than 90% of loaded mass. Si gel was Merck No. 9385. Compressed N_2 was used to elute the flash column. All fractions were evaporated to dryness at 35° and stored dry at −20°. Reversed-phase hplc was carried out using a Waters Delta-Prep 3000, with a Rainin Dynamax C-18 column (41.4×250 mm), with loadings of 250 mg per injection in DMSO or CH_3CN. All purification solvents were hplc grade, while recovery solvents were technical or ACS grade. Nmr was done on a Nicolet 300 MHz, a Bruker 400 MHz, or a Varian XL-200 system.

EXTRACTION AND ISOLATION.—The fermentation broth (680 liters) of *Actinomadura verrucosospora* strain ATCC 39638 was extracted with an equal volume of EtOAc and the mixture filtered with the addition of diatomaceous earth (Dicalite Speed-Plus). The phases were separated and the organic phase concentrated to 8 liters of an oil with a wiped-film evaporator. Antifoam was removed by suspension of the oil in ten volumes of hexane and filtration through a bag filter precoated with diatomite. The residual mass (236 g) was extracted with 19 liters of EtOAc and coated on diatomite by several cycles of slurrying with hexane and re-evaporation. The diatomite was packed as a hexane slurry into a chromatography column and eluted batchwise with hexane (47 liters), hexane/toluene (14 liters), CH_2Cl_2 (18 liters), $CHCl_3$ (18 liters), and MeOH (12 liters). The esperamicins eluted in the CH_2Cl_2 fraction, which was evaporated to dryness to give 33 g of solid.

Compound **2** copurified with **1** through four successive flash chromatography steps on Si gel: (a)

$CHCl_3$-MeOH (95:5); (b) hexane-Me_2CO (1:1); (c) $CHCl_3$-*t*-BuOH (100:6); and (d) $CHCl_3$-MeOH-HOAc (95:5:0.1). This left 5.5 g **1** with ca. 3% **2** as judged by hplc.

The two esperamicins were separated by reversed-phase hplc on C-18 using MeOH-CH_3CN-50 mM NH_4OAc pH 4.4 (32.5:32.5:35). Prompt dilution of the column effluent with equal volumes of $CHCl_3$ and H_2O was followed by extraction, phase separation, and re-extraction with a second volume of $CHCl_3$; the combined organic phases were evaporated to dryness. This yielded a 1:1 mixture of **1** and **2**. Rechromatography and back extraction under the same conditions gave 20 mg of pure **2**.

Esperamicin P (BMY-41339) [**2**].—$C_{59}H_{80}N_4O_{22}S_5$; ir (KBr disc) ν max 3534, 2955, 2920, 1720, 1670, 1590, 1575, 1425, 1405, 1395, 1350, 1290, 1230, 1190, 1135, 1095, 1050, 1000, 970, 730 cm^{-1}; uv (MeOH) λ max 318 (log ε=4.07), 277 (sh, 4.24), 252 (4.44) nm; $[\alpha]_D$ −272° (*c*=0.01 mg/ml, MeOH); ^1H nmr (CD_3OD, 300 MHz) δ 8.46 (s), 7.65 (s), 6.64 (dd, *J*=4.5 and 10.5 Hz), 6.22 (br d, *J*=1.1 Hz), 6.08 (d, *J*=9.5 Hz), 5.95 (dd, *J*=1.5 and 9.5 Hz), 5.56 (br s), 5.53 (br d, *J*=3.1 Hz), 5.47 (dd, *J*=2.8 and 5.1 Hz), 5.39 (d, *J*=2.5 Hz), 4.98 (dd, *J*=1.7 and 10.2 Hz), 4.68 (d, *J*=2.5 Hz), 4.64 (q, *J*=6.8 Hz), 4.57 (d, *J*=7.8 Hz), 4.26 (s), 4.23 (m), 4.19 (d, *J*=3.4 Hz), 3.94 (s), 3.91 (3H, s), 3.88 (m), 3.85 (3H, s), 3.80 (3H, s), 3.66 (br s), 3.5–3.65 (m), 2.98 (br m), 2.85 (br m), 2.62 (3H, s), 2.42 (dd, 2.5 and 4.1 Hz), 2.36 (dd, *J*=2.6 and 10.5 Hz), 2.32 (d, *J*=3.7 Hz), 2.25 (t, *J*=9.8 Hz), 2.13 (3H, s), 1.95 (m), 1.59 (2H, m), 1.38 (3H, d, *J*=6.2 Hz), 1.32 (3H, d, *J*=6.1 Hz), 1.26 (3H, d, *J*=6.5 Hz), 1.13 (3H, d, *J*=6.2 Hz), 1.07 (3H, d, *J*=6.3 Hz), 0.8–1.0 (m, impurity); ^{13}C nmr ($CDCl_3$, 100 MHz) δ 13.7, 16.5, 17.5, 19.8, 22.2, 23.4, 23.5, 29.0, 29.6, 34.0, 35.1, 40.1, 47.3, 52.6, 55.7, 56.0 (x2), 56.1 (x2), 62.4, 64.5, 66.7, 68.2, 68.9, 69.2, 69.6, 70.2, 71.9, 76.1, 77.1, 83.5, 86.5, 88.5, 90.6, 97.2, 98.2, 99.6 (x2), 103.8, 107.6, 112.6, 123.1, 124.9, 128.7, 129.8, 130.8, 136.7, 144.1, 154.0, 154.5, 160.7, 166.4, 174.9, 191.5; elemental analysis for S, 9.87%, single sample (calcd for S_5 11.8%), [**1** analyzed for S 9.12% in duplicate, calcd for S_4 9.66%]; hplc [C-18; CH_3CN-MeOH-50 mM NH_4OAc (32.5:32.5:35)], R_t 12.9 min (**1** R_t 8.8 min); fabms (+mode, magic bullet matrix) *m/z* 1403 (19), 1249 (3); thermospray ms-hplc *m/z* 1379 (0.9), [M+Na], 1357 (0.9), $[M]^+$, 511 (25), 509 (20), 493 (12), 333 (34), 282 (24), 264 (100), 232 (47), 172 (18), 154 (32), [direct inlet hplc, solvent system CH_3CN-MeOH-H_2O (33:33:34) v/v]; hplc system (a): CH_3CN-MeOH-0.05 N NH_4OAc (30:42:28 v/v), system (b): CH_3CN-MeOH-0.05 N NH_4OAc (10:40:50); both on Waters Nova-Pak C18, 10 cm×4.6 cm, 1 ml/min; detection by diode array uv. Compound **1** had a R_t of 8.8 min in system (a) and did not elute in system (b); compound **2** had a R_t of 12.9 min in system (a).

CONVERSION OF ESPERAMICIN P [**2**] TO ESPERAMICIN A_1 [**1**] IN DMSO.—A quantity (1.0 mg) of **2** was evaporated into a vial and dissolved in 1 ml DMSO at room temperature. Aliquots of the reaction mixture were injected directly into the analytical hplc system as noted above to monitor the course of reaction, illustrated in Figure 1. At 24 h polar degradation products begin to predominate. The same reaction with **1** produced minimal conversion to **2** (<4%); degradation to polar decomposition products occurred after 21 h.

REACTION OF ESPERAMICIN A_1 [**1**] WITH H_2O_2.—A quantity (109 mg) of **1** was dissolved in 10 ml MeOH, to which 10 ml of 10% H_2O_2 in MeOH was added. The mixture was allowed to stand at room temperature for 2 h, after which it was directly injected onto a 41.4×250 mm Dynamax C18 column and eluted with a gradient from MeOH-CH_3CN-NH_4OAc 50 mM (35:7:58) to (42:30:28). Flash chromatography on silica in $CHCl_3$-MeOH-HOAc (98:2:0.1 to 94:6:0.1, MeOH rinse) yielded two products, esperamicin O_1 (**3**, 21.6 mg) and O_2 (**4**, 26.6 mg).

ESPERAMICIN O_1 [**3**].—Uv λ max (MeOH) 320 (log ε=3.99), 282 (4.13), 252 (4.36) nm; mp >300° (dec); $[\alpha]_D$ −176° (MeOH, *c*=0.01 g/ml); thermospray ms-hplc *m/z* 1341 [M+H], 1363 [M+Na], solvent system MeOH/H_2O; ^1H nmr (CD_3OD, 300 MHz) same as **2** except the S-Me resonance shifted from 2.14 ppm to 2.80 ppm: δ 8.46 (s), 7.91 (s), 7.65 (s), 6.62 (dd, *J*=4.4 and 10.7 Hz), 6.25 (br s), 6.08 (d, *J*=9.5 Hz), 5.94 (dd, *J*=9.5 and 1.4 Hz), 5.54 (br), 5.51 (d, *J*=2.5 Hz), 5.48 (m), 5.39 (d, *J*=2.5 Hz), 5.09 (d, *J*=2.1 Hz), 5.05 (br s), 4.89 (obs.), 4.68 (d, *J*=2.4 Hz), 4.64 (q, *J*=7 Hz), 4.57 (d, *J*=7.8 Hz), 4.50 (dq, *J*=3.5 Hz), 4.35 (dd, *J*=9.6 and 6.3 Hz), 4.29 (s), 4.25 (s), 4.13 (dd, *J*=10.7 and 14.8 Hz), 3.94 (m), 3.91 (s), 3.85 (s), 3.80 (s), 3.63 (br s), 3.57 (dd), 3.40 (s), 2.80 (s), 2.66 (s), 2.53 (s), 2.3 (m), 2.15 (m), 1.94 (m), 1.5–1.7 (m), 1.45 (d, *J*=6.3 Hz), 1.34 (d, *J*=6.1 Hz), 1.25 (d, *J*=6.1 Hz), 1.25 (d, *J*=6.5 Hz), 1.07 (d, *J*=6.2 Hz), 1.01 (d, *J*=6.3 Hz); ^{13}C nmr (CD_3OD, 75 MHz) δ 168.1, 162.4, 155.9, 155.3, 145.9, 137.5, 136.5, 133.4, 131.9, 126.7, 123.9, 114.7, 109.6, 105 (br), 104.9, 101.5, 101.4, 99.8, 99.4, 91.2, 89.7, 84.9, 84.4, 80.4, 79.5, 78.1, 77.6, 72.7, 72.1, 71.8, 71.4, 69.8, 69.1, 68.0, 67.6, 66.0, 65.4, 57.0, 56.7, 56.5, 56.4, 53.2, [46–51 obscured by solvent], 41.0, 38.5, 36.4, 34.8, 29.9, 23.9, 22.8, 22.2, 20.1, 18.7, 17.1; tlc Si gel, $CHCl_3$-MeOH (95:5) R_f=0.20; hplc, system (a): 2.3 min, system (b): 4.0 min.

ESPERAMICIN O_2 [**4**].—Uv λ max (MeOH) 318 (log ε=4.03), 282 (4.18), 253 nm (4.41); mp >300° (dec); $[\alpha]_D$ −91° (MeOH, *c*=0.01 mg/ml); thermospray ms-hplc *m/z* 1341 [M+H], 1363 [M+Na], solvent

TABLE 1. Antibacterial Activity of Esperamicin P (**2**).

Organism	MIC (μg/ml)
E. faecalis A20688	0.002
E. faecalis A25707	0.002
E. faecalis A25708	0.002
S. aureus A9537	0.001
S. aureus A20698	0.001
S. aureus A 24407	0.001
E. coli A15119	0.25
E. coli A20697	0.25
E. coli A9751	0.016
K. pneumoniae A9664	1
K. pneumoniae A20468	1
P. vulgaris A21559	0.13
P. aeruginosa A9843	0.25
P. aeruginosa A20235	0.13

system MeOH/H_2O; ^1H nmr, ^{13}C nmr, identical to **3**; tlc Si gel, $CHCl_3$-MeOH (95:5) R_f=0.46; hplc, system (a): 4.5 min, system (b): did not elute.

BIOLOGICAL ACTIVITY.—Antimicrobial activity of **2** in a serial twofold dilution procedure versus a variety of organisms is listed in Table 1. In vitro cytotoxicity testing of **2** vs. cultured cell lines yielded IC_{50} values (μg/ml) as follows: A549 human lung, 0.01; A549 (VP-resistant), 0.01; B16-PRIM murine melanoma, <0.002; HCT-116 human colon, <0.002; HCT/VP35 (VP-resistant human colon), <0.002. The in vivo evaluation of **2** is reported in greater detail in Refs. (8) and (9).

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