Novel Sulfur-Containing Microbial Metabolite of Primaquine

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Microbial metabolism studies of the antimalarial drug primaquine, using Streptomyces roseochromogenus (ATCC 13400) have produced an N-acetylated metabolite and a methylene-linked dimeric product, both of which have been previously reported, and a novel sulfur-containing microbial metabolite. The structure of the metabolite as a sulfur-linked dimer was proposed on the basis of spectral and chemical data. The molecular formula C38H44N6O4S was established from field-desorption mass spectroscopy and analytical data. The 1H- and 13C-nuclear magnetic resonance spectral data firmly established that the novel metabolite was a symmetrically substituted dimer of primaquine N-acetate with a sulfur atom linking the two units at C-5. The metabolite has been shown to be a mixture of stereoisomers which can equilibrate in solution. This observation was confirmed by microbial synthesis of the metabolite from optically active primaquine.

MATERIALS AND METHODS

General. Infrared spectra were run in chloroform by using a Perkin-Elmer 281B spectrophotometer. 1H-nuclear magnetic resonance (NMR) spectra were obtained on a Varian EM-390 90-MHz NMR spectrometer (Varian Associates, Inc., Palo Alto, Calif.) and the 13C-NMR spectra (15.03 MHz) were recorded on a JEOL-FX60 FT NMR spectrometer (JEOL U.S.A., Inc., Peabody, Mass.). Field-desorption mass spectra were obtained from the Department of Medicinal Chemistry, University of Utah, Salt Lake City. Primaquine diphosphate was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.) and was used as received after checking its purity (6).

Cultures and fermentation screening procedures. Stock cultures were maintained on Sabouraud-maltose agar or Mycophil agar slants (BBL Microbiology Systems, Cockeysville, Md.) and stored at 4°C. Initial screening was carried out in 125-ml Erlenmeyer flasks containing 25 ml of medium consisting of (per liter of distilled water): glucose, 20 g; NaCl, 5 g; K2HPO4, 5 g; yeast extract, 5 g; peptone (Difco Laboratories, Detroit, Mich.), 5 g. A two-stage fermentation procedure was utilized as previously described (6). Primaquine diphosphate was added to 24-h-old stage II cultures (0.5 mg/ml) as a 25% suspension in dimethylformamide.

Culture controls consisted of fermentation blanks in which organisms were grown under identical conditions, but without substrate. Substrate controls consisted of sterile media containing the same amount of substrate and were incubated under the same conditions.

Fermentation sampling and thin-layer chromatographic analyses. The fermentations were sampled by withdrawing 5 ml of culture, adjusting it to pH 8, and then extracting it with 5 ml of diethyl ether. The ether layer was evaporated, and the residue was redissolved in ether and spotted on precoated silica gel G plates (0.25 mm; Sil G-25 UV254; Brinkmann Instruments, Inc., Westbury, N.Y.). The plates were developed in 8% methanol in benzene-ethyl acetate-diethylamine (5:4:1) and were visualized by spraying with diazotized p-nitroaniline followed by spraying with concentrated hydrochloric acid. The Rf values and colors are as follows: primaquine (I), 0.47 (dull yellow); primaquine N-acetate (II), 0.50 (bright yellow); metabolite IV, 0.38 (bright orange); metabolite V, 0.38 (bright orange).

HPLC. A 5-μm particle size, C-18 reverse-phase column (Whatman PXS 5/25 ODS, Whatman, Inc., Clifton, N.J.) was used for high-performance liquid chromatography (HPLC) of the mixtures of V (peaks A and B) and IV (peaks A and B). It was also found that a 10-μm-particle size column (Waters μ-Bondapak C-18; Waters Associates, Inc., Milford, Mass.) gave base-line separation of V-A and V-B or IV-A and IV-B, but the column did not resolve V-A from IV-A or V-B from IV-B. The mobile phase (1.0 ml/min) was prepared by using 8.4 g of KH2PO4, 6.6 g of K2HPO4, 4.0 g of N,N-dimethylacetamide, 2.8 liters of methanol, and 1.2 liters of water. The chromatographic peaks were detected by using a dual-wavelength UV detector (model 440; Waters Associates, Inc., Milford, Mass.) at A254 and at A380 in conjunction with a dual-pen recorder. Identification of the components in the mixtures was based on a comparison of the retention time with the A254/A380 ratio of each component. Retention volumes for each of the compounds are as follows: V-A, 26.8 ml; V-B, 32.0 ml; IV-A, 28.8 ml; IV-B, 34.0 ml.

Preparative scale production of dimer V from primaquine by Streptomyces roseochromogenus (ATCC 13400). Second-stage cultures of S. roseochromogenus (ATCC 13400) were initiated in 24 1-liter Erlenmeyer flasks containing 200 ml of medium per flask and in 15 2-liter Erlenmeyer flasks containing 400 ml of medium per flask. After 24 h of incubation (250 rpm), (+)-primaquine diphosphate was administered to each of the cultures as a suspension in dimethylformamide to give
TABLE 1. Comparative $^1$H-NMR and $^{13}$C-NMR data for metabolites II and V

<table>
<thead>
<tr>
<th>Positions</th>
<th>$\delta$ H(J)</th>
<th>$\delta$ C</th>
<th>$\delta$ H(J)</th>
<th>$\delta$ C</th>
</tr>
</thead>
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<tr>
<td>2</td>
<td>8.48, dd (2, 4)</td>
<td>144.3, d</td>
<td>8.50, dd (1.5, 4)</td>
<td>144.2, d</td>
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<tr>
<td>3</td>
<td>7.26, dd (4, 9)</td>
<td>121.8, s</td>
<td>7.41, dd (4, 8)</td>
<td>121.8, d</td>
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<tr>
<td>4</td>
<td>7.88, dd (2, 9)</td>
<td>134.8, d</td>
<td>9.27, dd (1.5, 8)</td>
<td>134.7, d</td>
</tr>
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<td>4a</td>
<td>130.0, s</td>
<td>132.1, s</td>
<td>102.6, s</td>
<td>160.5, s</td>
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<tr>
<td>5</td>
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<td>91.9, d</td>
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<td>96.9, d</td>
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<tr>
<td>8</td>
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<td>135.5, s</td>
<td>135.3, s</td>
<td>136.0, s</td>
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<tr>
<td>1’</td>
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<td>48.0, d</td>
<td>3.0-3.5, m</td>
<td>47.8, d</td>
</tr>
<tr>
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<td>34.1, t</td>
<td>1.5-1.8, m</td>
<td>34.2, t</td>
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<tr>
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<td>1.5-1.8, m</td>
<td>26.3, t</td>
<td>1.5-1.8, m</td>
<td>26.3, t</td>
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<tr>
<td>4’</td>
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<td>39.6, t</td>
<td>3.0-3.5, m</td>
<td>39.5, t</td>
</tr>
<tr>
<td>5’ (Me)</td>
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<td>20.6, q</td>
<td>1.29, d (6)</td>
<td>20.6, q</td>
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<tr>
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<td>55.2, q</td>
<td>3.76, s</td>
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<td>NH</td>
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<td>170.2, s</td>
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<td>NHCO</td>
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<td>23.1, q</td>
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* Data were obtained in CDCl$_3$ at 90 MHz (H) and at 15 MHz (C), using tetramethylsilane as internal standard. Data and assignments for II have been reported previously (6, 11) and are listed here for comparison with V.

a final concentration of 0.5 mg/ml of culture medium. The stage II cultures were incubated at room temperature in a rotary shaker at 250 rpm for 19 days. The whole cultures were pooled, adjusted to pH 8 with ammonium hydroxide, and extracted with an equal volume of ethyl acetate (250 ml portions). The combined ethyl acetate layers were dried (Na$_2$SO$_4$) and evaporated in vacuo (40°C) to leave a dark brown oily residue (2.443 g). The residue was adsorbed onto alumina (Woelm TSC, neutral, for dry-column chromatography; activity, II/30 mm) and applied as a plug to a column (2.1 by 40 cm, 150 g) of the same adsorbent packed in ethyl acetate-hexane (1:2). Elution of the column with ethyl acetate-hexane (3:1) afforded a fraction (444 mg), shown as one spot on a thin-layer chromatography (TLC) plate but was shown by HPLC to be a mixture of predominantly sulfur-linked dimer V with a small amount of methylene-linked dimer IV. The dimers III, IV, and V all give the same $R_f$ value on all the TLC systems used; however, they can be distinguished by HPLC (5-µm column). The HPLC analyses were critical in determining a culture that produced the sulfur-linked dimer V as the major metabolite in determining the optimum time of harvest. The fraction (444 mg) was adsorbed onto 2 g of alumina (as above) and applied as a plug to a column of the same adsorbent (1.5 by 160 cm, 300 g) packed in ethyl acetate-hexane (1:1). The column was eluted with ethyl acetate-hexane (3:1), and 5-ml fractions were collected when the compounds began to elute, which could be seen by the bright yellow color of both IV and V. A small amount of the methylene-linked dimer IV eluted first and was identified by HPLC comparison with an authentic sample (5). Continued elution of the column ultimately afforded a fraction containing pure sulfur-linked dimer V (104 mg), as determined by HPLC. Crystalization from 10% H$_3$PO$_4$ in ethanol-OH-H$_2$O produced metabolite V as its tetraphosphate salt (51 mg), mp 169 to 171°C (Calculated for C$_8$H$_8$N$_2$O$_8$S·4H$_2$PO$_4$: C, 39.85; H, 5.50; N, 8.20; S, 3.13. Found: C, 38.32; H, 5.68; N, 8.11; S, 3.09). Spectral data were obtained on the free base: $\nu$ max (CHCl$_3$) 3440, 3000, 2920, 1659, 1600, 1526 cm$^{-1}$; $\lambda$ max (methanol) 389 (ε 8437), 263 (46,875), 213 (25,000); $\lambda$ max (methanol plus HCl), 293 (sh, 15,313), 268 (29,688), 230 (sh, 15,938), 213 (18,438); CD (0.002% methanol), $\theta$$_{s0-210} = 0$. $^1$H- and $^{13}$C-NMR data (Table 1); field-desorption mass spectrum mlz 632 (M* 100%), 634 (15.38%).

HPLC separation of V-A and V-B and determination of half-life. A mixture of V-A and V-B (1:1) was injected onto a 10-µm, C-18 reverse-phase column (Waters; µ-Bondapak), using the same mobile phase described above, but at a 1.5-ml/min flow rate. The chromatograms showed base-line separation of V-A and V-B. The two pure fractions were stored at room temperature in the mobile phase. A second C-18 reverse-phase HPLC system then was used to follow the kinetics of the conversion of V-A to the 1:1 equilibrium mixture of V-A and V-B. A least-squares regression analysis of the data showed a half-life of 28.8 h for the interconversion at room temperature in the methanol-phosphate buffer system.

Microbial synthesis of (+)-V and (-)-V by S. roseochromogenus. Two stage II cultures of S. roseochromogenus were initiated in 125-ml deLong culture flasks containing 25 ml of medium. After 24 h of incubation at room temperature and 250 rpm, 10 mg of (+)-primaquine diphasate in 0.15 ml of dimethylformamide was administered to one culture. (-)-Primaquine diphasate (10 mg/0.15 ml of dimethylformamide) was administered to the other culture. After a 13-day incubation period (room temperature, 250 rpm), each culture (pH 8) was extracted three times with 20 ml of ethyl acetate. The ethyl acetate layers were dried (Na$_2$SO$_4$) and evaporated in vacuo (40°C) to give bright yellow-orange residues. Each extract was purified by flash chromatography (to remove N-acetate, II) on a column of alumina (TSC, Woelm, 1 g) packed in a Pasteur pipette. Elution of each column with 10 ml of ethyl acetate-hexane (2:1) afforded the N-acetate (II). Elution of each column with 5 ml of ethyl acetate-methanol (9:1) yielded the optically active dimers, (+)-V and (-)-V. HPLC of (+)-V showed only one peak which had the same retention time and the A$_{254}$/A$_{380}$ ratio as the peak for V-B. HPLC of (-)-V showed only one peak and it was also identical to V-B. When stored separately for 1 week in methanol at room temperature, both (+)-V and (-)-V showed only the one peak. However, when (+)-V and (-)-V were mixed together and allowed to stand at room temperature in methanol for 1 week, an equilibrium mixture of V-A and V-B was detected by HPLC.

Hydrogenolysis of metabolite V to N-acetate II. A 45-mg amount of PO$_4$ was added to a solution of metabolite V (43 mg, 0.068 mM) in ethanol (5 ml). The suspension was shaken with hydrogen in a Parr hydrogenator at 48 lb/in$^2$ for 23 h. The catalyst was removed by filtration through diatomaceous earth, and the filtrate was evaporated in vacuo (40°C). The oily residue (25 mg) was chromatographed on alumina (TSC, Woelm, 1 g) packed in a Pasteur pipette. Elution of the column with ethyl acetate-hexane (1:1) afforded a fraction (29 mg) which contained predominantly the N-acetate II (TLC). Further purification of the hydrogenolysis product by a second column chromatography on alumina afforded pure N-acetate II (6 mg), which was identified by TLC and by $^1$H-NMR spectral comparisons with an authentic sample of the N-acetate II (6).

RESULTS AND DISCUSSION

Preparative scale fermentation of primaquine (I) with submerged cultures of S. roseochromogenus (ATCC 13400) afforded, in addition to the N-acetate (II,6) and a small amount of the methylene-linked dimer IV (5), the novel
metabolite V after column chromatography of the fermentation extract (Fig. 1). The $^{13}$C-NMR spectral data for metabolite V (Table 1) were very similar to those for the N-acetate II (6). The major differences were in the upfield aromatic region, where two signals were observed at δ 92.7 (doublet) and δ 102.6 (singlet) (II) has two doublets at δ 91.9 and at δ 96.9) (6). This suggested that substitution at C-5 or C-7 occurred in the primaquine nucleus but without new carbon signals present in the $^{13}$C-NMR spectrum.

The 1H-NMR spectral data for the metabolite V (Table 1) were also very similar to those for the N-acetate II, with the only major difference the downfield position of H-4 (δ 9.27) in V relative to H-4 in II. Since an anisotropic effect on H-4 is much more likely by substitution at C-5 than C-7, a substitution at C-5 seemed more plausible.

The molecular formula C$_{34}$H$_{42}$N$_{2}$O$_{3}$S was established from elemental analysis and from the field-desorption mass spectrum, which not only showed the parent ion at m/z 632, but showed the characteristic isotope peak for one sulfur atom (M$^+$ + 2).

The field-desorption mass spectra and 1H-NMR and 13C-NMR data were consistent only with a symmetrical dimer of the N-acetate (II) linked through sulfur at either C-5 or C-7.

Confirmation of substitution at C-5 was obtained by analysis of the proton-coupled $^{13}$C-NMR spectrum of the metabolite. The signal at δ 102.6 (s) was observed as a triplet (J$_{C-H}$ = 5Hz; equal three-bond couplings to H-4 and H-7), while the signal at δ 92.7 (d) appeared as a sharp doublet (J$_{C-H}$ = 156.2 Hz; H-7). The three-bond coupling patterns of the aromatic carbons have been very useful in assigning C-5 and C-7 (6, 11). The data recorded here were obtained after exchange with D$_2$O. It is known that C-7 is coupled to the NH proton (6). The signal at δ 92.7 sharpened after D$_2$O exchange, whereas the signal at δ 102.6 remained unchanged. These patterns are consistent only with substitution at C-5. If the sulfur atom was substituted at C-7, the signal at δ 102.6 would appear as a doublet (J$_{C-H}$), while the signal at δ 92.7 would appear as a double doublet (J$_{C,H5}$ and J$_{C,H4}$). Therefore, the collective data lead to formulation of structure V for the metabolite. Catalytic hydrogenation of metabolite V led to the formation of the N-acetate II as the only isolable product.

Dimeric metabolites III and IV are composed of mixtures of stereoisomers (9). Metabolite V exhibited behavior similar to that reported for the methylene-linked dimer IV. HPLC of V showed two peaks (designated A and B) which were
separated by preparative HPLC, but each equilibrated back to the original mixture (1:1) when left in solution at room temperature. The half-life for this equilibration is 28.8 h at room temperature (cf. 10.6 h for IV). Thus, one of the peaks represents an enantiomeric mixture of R-sulfur-R and S-sulfur-S and the other peak represents R-sulfur-S. Since the R-sulfur-S dimer can form in two ways, the ratio of 1:1 resulted. The equilibration of the separate components back to the original mixture can be explained if one assumes that an equilibrium between the two diastereomeric pairs is mediated by interaction of the dimer V and the N-acetate II and intermediate VI. Since both + and - forms of II and VI were present [because (±)-primaquine was used as substrate], reformation of the dimer V accomplished interconversion between the two HPLC peaks (9).

Microbial synthesis of the dimer V separately from (+)-primaquine and from (−)-primaquine (2) (the absolute stereochemistry has not been established) yielded only the HPLC peak with the longer retention time, even after standing in solution for a week. Thus, the peak with the longer retention time in the chromatogram of the dimer V represents the enantiomeric mixture (R-sulfur-R and S-sulfur-S). When V prepared from (+)-primaquine was mixed (in methanol) with V prepared from (−)-primaquine, the appearance of the peak with the shorter retention time was detected by HPLC (complete in 1 week). These experiments confirm that the two peaks in the chromatogram for the sulfur-linked dimer V represent stereoisomers which can be interconverted via the N-acetate II and the intermediate VI.

The isolation of the novel sulfur-containing microbial metabolite, V, has interesting stereochemical features and may also have some important metabolic implications as well. It is becoming increasingly apparent that many drugs conjugate with glutathione and that such conjugation may have toxicological implications (8, 12, 13). Primaquine is an important antimalarial drug which has significant toxicities (3) and perhaps glutathione conjugation may play a role. While glutathione may be considered a likely source of sulfur for this metabolite, other organic or inorganic sources cannot be ruled out at this time.

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LITERATURE CITED