MICROBIAL *N*-ACETYLATION OF PRIMAQUINE BY TWO *STREPTOMYCES* SPECIES: TIME COURSE STUDIES AND HPLC ANALYSES

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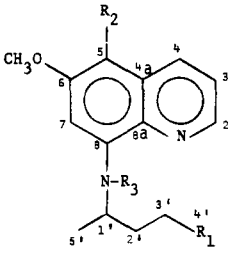
ABSTRACT.—The time course of *N*-acetylation of primaquine (**1**) by *Streptomyces roseochromogenus* (ATCC 13400) and *Streptomyces rimosus* (ATCC 23955) was determined by quantitative high pressure liquid chromatographic analyses of the culture broths. The *N,5-bis*-trifluoroacetyl derivative of primaquine (**4**) was used as an internal standard in the hplc analysis for the quantitation of primaquine-*N*-acetate (**2**) in microbial culture broths. It was shown that *S. roseochromogenus* forms the highest level of primaquine-*N*-acetate (**2**) at 24–36 hours after substrate addition, while *S. rimosus* is slower in its acetylation, peaking at three days after substrate addition. An assay procedure utilizing hplc analysis for the quantitation of primaquine-*N*-acetate (**2**) in microbial culture broth is described. The formation of a novel dimeric compound (**6**) from the reaction of primaquine (**1**) with 8-(4-phthalimido-1-methylbutylamino)-6-methoxyquinoline (**5**) is also reported.

It is well documented that microorganisms are capable of performing a wide variety of metabolic transformations, many of which parallel mammalian metabolic reactions. Rosazza and Smith (1) have suggested that mammalian metabolism may be studied using microorganisms as model systems. A study of the microbial metabolism of primaquine has been undertaken in an attempt to isolate and identify possible mammalian metabolites (2).

The two major microbial metabolites of primaquine result from *N*-acetylation (**2**) and oxidative deamination (**3**) of the terminal primary amine (2). The scope and substrate specificity of mammalian *N*-acetyltransferase enzymes are well known (3,4) and *N*-acetylation does not represent a major metabolic route for primary aliphatic amines in mammals (3). However, *N*-acetylation appears to be the major route of primaquine metabolism by *Streptomycetes*. Although *N*-acetylation appears to be a common metabolic route of primary aliphatic amines by bacteria, detailed studies of the scope and substrate specificity of

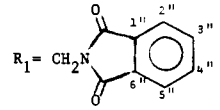


1 R_1= CH_2NH_2; R_2= R_3= H

2 R_1= $CH_2NHCOCH_3$; R_2= R_3= H

3 R_1= CO_2H; R_2= R_3= H

4 R_1= $CH_2NHCOCF_3$; R_2= $COCF_3$; R_3= H



5 R_1= CH_2N ; R_2= R_3= H

9 R_1= $CH_2NHCOCF_3$; R_2= H; R_3= $COCF_3$

microbial *N*-acetyltransferase systems have not been reported. Since the *N*-acetate is the most common and major metabolite of primaquine produced by this class of microorganisms, it was speculated that it may itself serve as a substrate for further transformation. For these reasons we were interested in determining the time course of the *N*-acetylation of primaquine by microorganisms, and the results of this study are described herein.

RESULTS AND DISCUSSION

Primaquine-*N*-acetate (**2**) is the major metabolite of primaquine in a number of microorganisms, particularly Streptomycetes (2). Although almost all the Streptomycetes screened are capable of *N*-acetylation of primaquine, the rate and degree of *N*-acetylation varies from one organism to another. This was established by determining the time course of *N*-acetate production by two Streptomycetes: *S. roseochromogenus* (ATCC 13400) and *S. rimosus* (ATCC 23955).

The time course of *N*-acetate production by each organism was determined by hplc analysis of culture extracts harvested at various times. Second-stage cultures were harvested in triplicate at designated times after substrate addition and were adjusted to pH 9.0, and 4 ml were withdrawn from each culture. The *N*,5-*bis*-trifluoroacetyl derivative of primaquine (**4**) was added as an internal

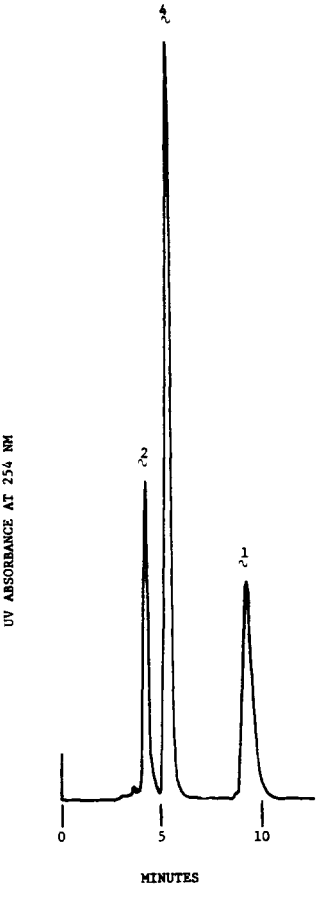


FIG. 1. HPLC of standard samples of primaquine (**1**), primaquine-*N*-acetate (**2**), and *N*,5-*bis*-trifluoroacetylprimaquine, (**4**).

standard and the samples were extracted with ether. The extracts were analyzed by hplc with a reverse phase column and a methanol-phosphate buffer mobile phase. In this system, primaquine (1) had a relative retention time (t_R) of 1.76 and primaquine-*N*-acetate (2) had a t_R of 0.79 (fig. 1). Analysis of extracts of culture controls (cultures without substrate) showed no interfering peaks in the hplc chromatograms.

The levels of primaquine-*N*-acetate (2) were determined by comparison of the peak height ratio (PHR) of the *N*-acetate to the internal standard (4) with the PHR's of standard solutions of 2 and 4. Recovery of the *N*-acetate (2) under these assay conditions was 89%. The glucose levels and pH were also determined at the time of harvest to establish if either had an effect on the rate and/or degree of *N*-acetylation.

With *S. roseochromogenus*, the production of *N*-acetate (2) was monitored over a 48-hour period after substrate addition. It was found (fig. 2) that the *N*-acetate production increased steadily over a period of 24 hours, at which time the maximum yield was 59%. The level of *N*-acetate remained at 59% for an additional 24-hour period then began to decrease. The glucose levels drop dramatically within the first 24 hours, but this does not appear to be related to the level of *N*-acetate (2), which exhibits a steady increase over the same time period (fig. 2). There also does not appear to be any relationship between *N*-acetylation and pH since the average pH varied only between 6.68 at 12 hours and 6.82 at 48 hours.

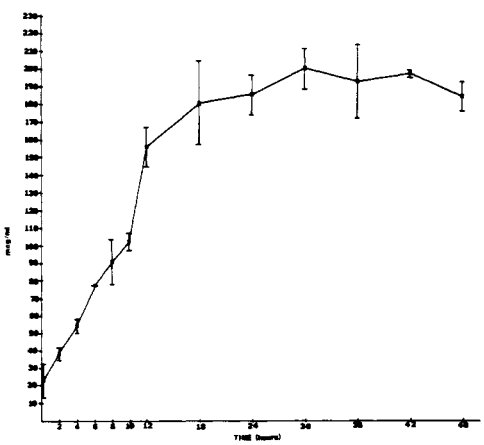


FIG.2. Time course of production of primaquine-*N*-acetate (2) by *Streptomyces roseochromogenus* ATCC 13400.

With *S. rimosus*, the production of *N*-acetate was monitored over a 4-day period after substrate addition. It was found (fig. 3) that the *N*-acetate production increased steadily beginning within 15 minutes after substrate addition and leveling off at three days, after which the *N*-acetate level decreases. The pH levels varied from 6.26 at 15 minutes to 6.88 at 4 days. Unlike *S. roseochromogenus*, glucose consumption in *S. rimosus* cultures was not complete even after four days incubation.

The acetylation of primary amines by microorganisms appears to be a widespread capability possessed by both fungi and bacteria (2,5–9). Mammals also possess *N*-acetylation capability. However, aromatic primary amines are normally more susceptible to metabolism by mammals via *N*-acetylation than are aliphatic primary amines (3,4).

In order to quantitate the *N*-acetylation of primaquine by microorganisms, it was necessary to develop an analytical system for the quantitative assay of

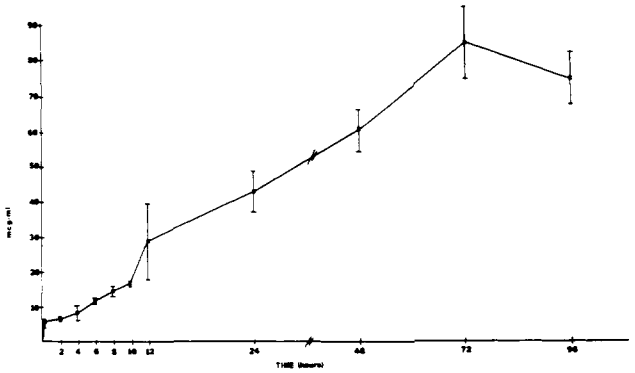
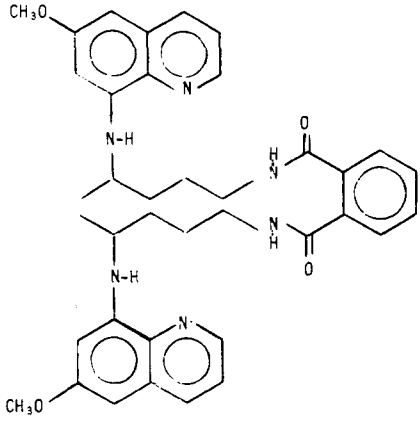


FIG.3. Time course of production of primaquine-*N*-acetate (**2**) by *Streptomyces rimosus* ATCC 23955.

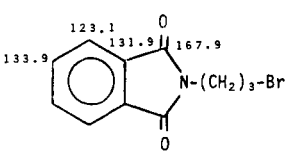
the microbial extracts. Our first choice for an internal standard for hplc analysis was 8-(4-phthalimido-1-methylbutylamino)-6-methoxyquinoline (**5**) since it was expected to exhibit extraction properties similar to **2**. In addition, this product was nicely crystalline and had a retention time between the *N*-acetate (**2**) ($t_R = 0.64$) and primaquine (**1**) ($t_R = 1.49$). However, when **5** was used as an internal standard, an additional peak was evident in the hplc chromatograms ($t_R = 1.28$) which did not correspond to **1**, **2** or **5**. Control studies were conducted in which sterile medium containing **1**, **5**, and both **1** and **5** were analyzed. In these studies the product was found only in the extracts of the medium containing both **1** and **5**, suggesting that it was formed as a result of a reaction between **1** and **5**. This was substantiated by demonstrating that the same product was formed when **1** and **5** were allowed to stand in ether at room temperature. The reaction of **1** and **5** is very facile and occurs to a significant extent in the ether layer after extraction of **1** and **5** from the aqueous culture broth. Although **5** was abandoned as an internal standard, it was of interest to determine the nature of this product. Structure **6** is proposed for the reaction product based on the following data.



6

The ir spectrum of **6** did not show absorption bands at ν max 1770 and 1705 cm^{-1} which are characteristic of the phthalimidyl moiety, but instead showed a band at 1625 cm^{-1} for a secondary amide. In addition, the mass spectrum of **6** showed a molecular ion at m/z 648 ($C_{38}H_{44}N_6O_4$). The ^1H nmr spectrum of **6** was similar to that of **5** except for the presence of additional exchangeable signals at δ 6.97,

consistent with the presence of secondary amide protons. The ^{13}C nmr spectral data for **6** is very similar to that of the phthalimidyl derivative **5** except for the positions of the signals for C–2" and C–3" as would be expected for loss of the imide ring. The assignments for C–2" and C–3" in **5** and **6** were made by comparison with *N*(3-bromopropyl)-phthalimide (**7**) and *bis*(2-methoxyethyl)-phthalate (**8**) (10).



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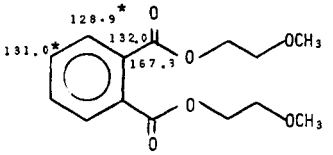

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Attempts to use the monotrifluoroacetate derivative of primaquine as an internal standard were also abandoned. Treatment of primaquine (**1**) with trifluoroacetic anhydride in pyridine (room temp.) resulted in a mixture of the mono- and ditrifluoroacetyl derivatives. Separation of the mixture gave the monotrifluoroacetate which could not be induced to crystallize. Treatment of **1** with trifluoroacetic anhydride in refluxing pyridine did not yield the expected *N*,*N*-ditrifluoroacetate (**9**) but, instead, gave the *N*,5-*bis*-trifluoroacetyl derivative **4**. This compound (**4**) did prove to be an acceptable internal standard and its structure was established by detailed analysis of its spectroscopic data.

The mass spectrum of the product showed a parent ion at *m/z* 451, indicating that the product contained two trifluoroacetyl units. However, the ^1H nmr spectrum of the product showed signals for two D_2O exchangeable protons (one proton exchanged slowly), suggesting that only the primary amine had been acetylated. The ^1H nmr spectrum of the product also showed that the side chain protons were essentially the same as in primaquine-*N*-acetate (**2**) (2), but there were significant changes in the aromatic region of the spectrum. The signal at δ 6.22 integrated for only one proton, indicating that either C–5 or C–7 was substituted (2). In addition, the signal for H–4 underwent a downfield shift to δ 8.70 from δ 7.82 in primaquine (**1**) (2). The ^{13}C nmr spectrum (pnd) of the product (table 1) showed a quartet of 157.7 ppm ($J_{CCF}=37.1$ Hz) consistent with a trifluoroacetamide carbonyl (11), a second carbonyl quartet at 182.4 ppm ($J_{CCF}=35.1$ Hz), supporting the presence of a trifluoromethyl ketone group (12),[2] and one singlet at 102.1 ppm and one doublet at 89.2 ppm for C–5 and/or C–7. The location of the trifluoromethyl ketone was suggested by the ^1H nmr spectrum to be C–5 (downfield shift of H–4). This was confirmed by analysis of the proton-coupled ^{13}C nmr spectrum. The signal at 102.1 ppm appears as a broadened triplet due to equal three-bond coupling to H–4 and H–7 ($^3J_{C-H}=3.9$ Hz). The signal for C–7 (89.2 ppm) occurs as a double doublet due to one-bond coupling with H–7 and three-bond coupling with the amine proton ($^1J_{C7-H7}=158.2$; $^3J_{C7-NH}=5.9$ Hz). This assignment was confirmed by adding D_2O to the sample and rerunning the spectrum. Upon addition of D_2O to the sample, the signal at 89.2 ppm collapsed to a doublet ($J_{C7-H7}=158.2$ Hz) due to loss of coupling of C–7 with the amine proton. The loss of the long range coupling of C–7 to the amine proton upon addition of D_2O was also observed with primaquine (2). The signal at 102.1 ppm for C–5 remained unchanged.

[2]The ^{13}C nmr spectrum ($CDCl_3$) of a commercial sample of trifluoroacetophenone showed the carbonyl as a quartet at 180.9 ppm ($J_{CCF}=35.2$ Hz) and the CF_3 carbon as a quartet at 117.3 ppm ($J_{CF}=291.0$ Hz).

TABLE 1. ^{13}C nmr assignments of primaquine derivatives

Carbon No.	Assignment (multiplicity)[a]			
	2	**4**	**5**	**6**
2	144.3(d)	145.3(d)	144.3(d)	144.3(d)
3	121.8(d)	124.6(d)	121.8(d)	121.8(d)
4	134.8(d)	132.6(d)	134.6(d)	134.7(d)
4a	130.0(s)	129.1(s)	130.0(s)	130.0(s)
5	91.9(d)	102.1(s)	92.1(d)	92.0(d)
6	159.6(s)	164.8(s)	159.7(s)	159.7(s)
7	96.9(d)	89.2(d)	96.9(d)	96.9(d)
8	145.1(s)	150.7(s)	145.2(s)	145.2(s)
8a	135.5(s)	134.1(s)	135.6(s)	135.5(s)
1'	48.0(d)	48.0(d)	48.0(d)	48.0(d)
2'	34.1(t)	33.7(t)	34.1(t)	34.1(t)
3'	26.3(t)	25.6(t)	25.4(t)	26.2(t)
4'	39.6(t)	39.7(t)	38.1(t)	40.1(t)
5'	20.6(q)	20.3(q)	20.6(q)	20.5(q)
Ar-OCH_3	55.2(q)	56.1(q)	55.1(q)	55.1(q)
-NHCOCH_3	23.1(q)	—	—	—
-NRCO-	170.2(s)	157.7(q)[b]	168.3(s)	169.3(s)
NHCOCF_3	—	116.2(q)[c1]	—	—
Ar$COCF_3$	—	182.4(q)[d]	—	—
Ar$CO$$CF_3$	—	117.1(q)[e1]	—	—
1", 6"	—	—	132.3(s)	134.7(s)
2", 5"	—	—	123.1(d)	129.8(d)[1]
3", 4"	—	—	133.7(d)	128.5(d)[1]

[a]The ^{13}C nmr assignments for **2** were reported previously (2) and are shown here for comparison purposes. Assignments bearing the same numerical superscript in any one column may be reversed.

[b]J_{CCF}=37.1 Hz.

[c]J_{CF}=288.0 Hz.

[d]J_{CCF}=35.1 Hz.

[e]J_{CF}=291.0 Hz.

EXPERIMENTAL[3]

TIME COURSE OF *N*-ACETYLATION OF PRIMAQUINE (**1**) BY *Streptomyces roseochromogenus* (ATCC 13400).—The production of primaquine-*N*-acetate (**2**) was accomplished by the usual two-stage fermentation procedure (2) in a medium consisting of (per liter of H_2O): dextrose, 20 g; NaCl, 5 g; K_2HPO_4, 5 g; yeast extract, 5 g; peptone, 5 g. Two ml of the first-stage cultures were used to initiate triplicate second-stage cultures in 23 ml of medium held in 125 ml erlenmeyer flasks to give a total volume of 25 ml for second-stage cultures. The cultures were then incubated at 250 rpm and room temperature for 24 hr, at which time 150 μl of a solution of primaquine diphosphate (50 mg/ml dimethylformamide) were added to each culture. Cultures were harvested, in triplicate, at the following time periods (after substrate addition): 15 min, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 12 hr, 18 hr, 24 hr, 30 hr, 36 hr, 42 hr, and 48 hr. The pH of each culture was measured and a semi-quantitative determination of glucose content was done with Tes-Tape.[4] The cultures were adjusted to pH 9 with 10% NH_4OH and a 4-ml sample was taken from each culture. The internal standard (**4**, 1.0 mg) was added and each sample was extracted with 5 ml of ether. After centrifugation, 4 ml of the ether layer were withdrawn and the samples were extracted with an additional 4 ml of ether. After centrifugation, 4 ml of the ether layer were withdrawn and the combined ether layers were dried and evaporated *in vacuo* (40°). The extracts were then dissolved in 500 μl of methanol and 2.5 μl were taken for hplc analyses.

TIME COURSE OF *N*-ACETYLATION OF PRIMAQUINE (**1**) BY *Streptomyces rimosus* (ATCC 23955).—Time course studies done with *S. rimosus* were conducted as described for *S. roseochromogenus* except that the cultures were harvested at the following time periods: 15 min., 2 hr, 6 hr, 8 hr, 10 hr, 12 hr, 24 hr, 48 hr, 72 hr, 96 hr.

[3]Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Ir spectra were run in potassium bromide using a Perkin-Elmer 281b spectrophotometer. The ^{13}C nmr spectra (15.03 MHz) were recorded in deuterochloroform on a JEOL-FX60 FT NMR spectrometer with tetramethylsilane as internal standard. Mass spectra were obtained on a Finnigan model 3200 spectrometer with the INCOS data system. Primaquine diphosphate was obtained from Aldrich Chemical Co. and was used as received after checking its purity (2). Primaquine-*N*-acetate (2) was prepared as previously described (2). All solvents for hplc analyses were of spectral grade quality.

[4]Eli Lilly and Co., Indianapolis IN.

HPLC ANALYSES.—Culture extracts were analyzed by use of a hplc system consisting of a 3.9 mm x 30 cm octadecyl reversed-phase column[5] (10 μm particle size). The mobile phase was prepared with 2.2 g KH_2PO_4, 3.3 g K_2HPO_4, 1.2 liters distilled water, and 2.8 liters methanol and was used at a flow rate of 1 ml/min. An hplc pump[6], a microsyringe-loaded loop injector[7] and a uv detector (254 nm) were also used. Culture extracts were dissolved in 500 μl methanol and 2.5 μl of the solutions were used for analysis.

PREPARATION OF 8-(4-TRIFLUOROACETAMIDO-1-METHYLBUTYLAMINO)-6-METHOXY-5-TRIFLUOROACETYLQUINOLINE (4).—To a solution of 998 mg of primaquine (free base) in 10 ml dry pyridine was added 2 ml of trifluoroacetic anhydride. The reaction mixture was refluxed for 4 hr then cooled and diluted with 100 ml of ice water. The mixture was extracted with ether (6 x 50 ml); the combined ether layers, when dried (Na_2SO_4) and evaporated *in vacuo* (40°), gave an oily residue (1.79 g). Purification of the reaction product by chromatography over alumina (neutral, grade I) with $CHCl_3$ as eluent afforded 816 mg of a crystalline residue which, when crystallized from methanol, gave 499 mg of **4**, mp 124–126°; ir νmax (KBr) 3365, 3305, 1701, 1665, 1605, 1565, 1530, 1387 cm^{-1}; ^1H nmr ($CDCl_3$) δ 1.33 (3H, d, J=6 Hz, 1'-CH_3), 1.57–1.97 (4H, H-2', H-3'), 3.2–4.0 (3H, H-1', H-4'), 3.97 (3H, s, $ArOCH_3$), 6.21 (1H, s, H-7), 7.1 (2H, exchange D_2O; -N*H*-; -N*H*-$COCF_3$) 7.40 (1H, dd, J=4, 8.8 Hz, H-3), 8.52 (1H, dd, J=1.5, 4 Hz, H-2), 8.70 (1H, dd, J=1.5, 8.8 Hz, H-4); ms m/z (% rel. abund.) 451 (M^+, 44%), 382 (M^+-CF_3, 100%), 297 (M^+-$(CH_2)_3NHCOCF_3$, 82%), 201 (28%); ^{13}C nmr see table 1; Anal.: Calcd. for $C_{19}H_{19}N_3O_3F_6$: C, 50.56, H, 4.24, N, 9.31; Found: C, 50.56, H, 4.31, N, 9.25.

PREPARATION OF 8-(4-PHTHALIMIDO-1-METHYLBUTYLAMINO)-6-METHOXYQUINOLINE (5).—To a solution of primaquine free base (424 mg, 0.0016 M) in 50 ml of toluene and 0.5 ml TEA was added 242 mg (0.0016 M) of phthalic anhydride. The reaction mixture was refluxed for 2 hr and water was collected in a Dean-Stark trap. After refluxing, the reaction mixture was evaporated *in vacuo* and the residue was taken up in dilute HCl and extracted with ether. The ether was evaporated to give a residue (453 mg) which was purified by chromatography over silica gel 60 (50 g, 70–230 mesh, 1.5 cm (i.d.) x 58 cm) with 1% methanol-chloroform as eluent. Fractions 1 and 2 were combined and crystallized from methanol to give 215 mg of **4**, mp 87–89° [lit (13): mp 89–90.5°]; ir νmax (KBr) 3400, 1770, 1705, 1615, 1515, 1400, 1061, 825 cm^{-1}; ^1H nmr ($CDCl_3$) δ 1.30 (3H, d, J=6 Hz, 1'-CH_3), 1.63–2.00 (4H, H-2', H-3'), 3.53–4.00 (3H, H-1', H-4'), 3.87 (3H, s, Ar-OCH_3), 6.0 (1H, br s, exchanges D_2O, -NH-), 6.30 (2H, s, H-5, H-7), 7.27 (1H, dd, J=4, 9 Hz, H-3), 7.57–8.0 (5H, m, H-4, H-2", 3", 4", 5"), 8.51 (1H, dd, J=2, 4 Hz, H-2); ms m/z (% rel. abund.) 389 (M^+, 12%), 201 (100%), 186 (33%); ^{13}C nmr see table 1; Anal.: Calcd. for $C_{23}H_{23}N_3O_3$: C, 70.93, H, 5.95, N, 10.80; Found: C, 70.77, H, 6.01, N, 10.73.

PREPARATION OF 6.—Primaquine base (227 mg) was dissolved in 25 ml of ether and 200 mg of **5** was added to the solution. The mixture was allowed to stand at room temperature for 1 hour. The reaction mixture was concentrated *in vacuo* (40°) and the reaction product crystallized. Filtration of the mixture afforded 184 mg (55%) of crude **6**. Crystallization twice from methanol afforded 66 mg of **6**, mp 165–167°; ir νmax (KBr) 3385, 3266, 1625 (NHCO) 1557, 1520, 1390, 1160, 825, 795; ^1H nmr ($CDCl_3$) δ 1.25 (6H, d, J=6 Hz, 1'-CH_3), 1.47–2.0 (8H, H-2' and H-3'), 3.13–3.97 (6H, H-1' and H-4'), 3.87 (6H, s, $ArOCH_3$), 5.99 (2H, br d, J=8 Hz, exchanges D_2O, -$CH(CH_3)N$*H*-), 6.30 (4H, s, H-5 and H-7), 6.97 (2H, br t, J=6 Hz, exchanges D_2O overnight, -CH_2N*H*CO-), 7.12–7.57 (6H, m, H-3' and H-2"–5"), 7.89 (2H, dd, J=2,9 Hz, H-4), 8.49 (2H, dd, J=2,4 Hz, H-2); ms m/z (% rel. abund.) 648 (M^+, 0.02%), 389 (M^+-259, 3.6%), 259 (M^+-389, 2.2%), 241 (2.9%), 201 (100%); ^{13}C nmr see table 1; Anal.: Calcd. for $C_{38}H_{44}N_6O_4$: C, 70.35, H, 6.84, N, 12.95; Found: C, 70.02, H, 6.89, N, 12.80.

ACKNOWLEDGMENTS

This investigation received the financial support of the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases. SLE acknowledges the National Science Foundation for a grant for Undergraduate Research Participation, SPI-7926577. The authors would also like to acknowledge Dr. John K. Baker, Department of Medicinal Chemistry, School of Pharmacy, The University of Mississippi, for many helpful suggestions and advice on analytical methodology.

Received 14 December 1981

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