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Microbial Metabolism Studies on the Major Microbial and Mammalian Metabolite of Primaquine

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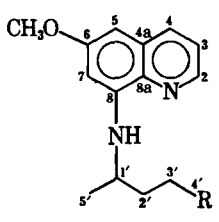
Abstract □ The microbial metabolism of 8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline (II), a known microbial and mammalian metabolite of the antimalarial drug primaquine (I), was investigated using selected organisms. *Streptomyces rimosus* produced a single metabolite that was identified as an amide derivative of II (V) by spectroscopic methods. The amide (V) was synthesized by ammonia treatment of the methyl ester (IV). The lactam derivative (VI) was also prepared by treatment of II with *N*-ethoxycarbonyl-2-ethoxy-1,3-dihydroquinoline.

Keyphrases □ Primaquine—microbial studies on the major microbial and mammalian metabolite, high-performance liquid chromatography □ High-performance liquid chromatography—microbial metabolism studies on the major microbial and mammalian metabolite of primaquine

The utilization of microorganisms as models for studying mammalian metabolism is a concept that has been advocated as an aid for future metabolic studies (1). Recent studies with phencyclidine (2) and imipramine (3) have shown that microorganisms do produce the same metabolites as mammalian systems. Primaquine (I), an antimalarial drug, has also been subjected to a microbial metabolic study since little or no information has been reported regarding its mammalian metabolism (4). The two major microbial metabolites identified were the carboxylic acid derivative (II) and the *N*-acetyl derivative of the primary amine (III). In parallel studies, the mammalian metabolism of I using rats has been studied, and II has been identified as the major mammalian metabolite as well (5). The tissue distribution of I has been studied, and even though substantial evidence indicated that I was rapidly metabolized by the rat, no mammalian metabolites were identified (6). Having microbial metabolites as reference standards for conducting mammalian metabolic studies can be useful. The purpose of this investigation was to study further the biotransformations of the major mammalian metabolite (II) of primaquine using microbial systems.



I: R = $-CH_2NH_2$
II: R = $-CO_2H$
III: R = $-CH_2NHCOCH_3$
IV: R = $-CO_2CH_3$
V: R = $-CONH_2$

RESULTS AND DISCUSSION

A total of 60 microorganisms typical of those used previously (3, 7) were used in the screening of 8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline (II) for microbial metabolites. Of these, *Streptomyces rimosus*[1] was chosen for preparative scale fermentations, since TLC indicated complete conversion in 3 days to one major metabolite. Other microorganisms showing this same metabolite as determined by TLC were *Streptomyces flocculus*[2], *Polyporus sanguineus*[3], and *Fusarium oxysporum* f. sp. *cepae*[4] although large amounts of II were also present even after 7–10 days.

Incubation of 300 mg of II with submerged cultures of *S. rimosus* for 4 days and extraction of the entire culture with ethyl acetate resulted in 700 mg of ethyl acetate solubles. Chromatography of a 400-mg sample of this residue using preparative layer silica gel and alumina plates resulted in the isolation of 18 mg of the pure metabolite by TLC and HPLC (overall yield, 12%).

The ^{1}H-NMR spectrum of the metabolite showed the aromatic protons to have nearly the same δ positions and J values as reported for II (4). The spectrum also confirmed that the methoxyl group and the secondary methyl were present. This suggested that the biotransformation had occurred in the side chain. The mass spectrum was consistent for $C_{15}H_{19}O_2N_3$ (M^+ 273, 10%) and showed significant fragments at m/z 215 (51%) and 201 (100%), further confirming that the aromatic nucleus had not been transformed (4). The IR spectrum indicated absorption at 3400, 3510, and 1760 cm^{-1}. The ^{13}C-NMR spectral data (Table I) was nearly identical to that of the methyl ester of II (IV). The collective spectroscopic data suggested the microbial metabolite was an amide derivative represented by V. This was confirmed by treating IV with methanol saturated with ammonia in a sealed tube at 140°. The product of this reaction was identical in all respects to the metabolite (V). No evidence of the lactam derivative (VI) was noted in this reaction, although VI was noted as a product of heating IV in the gas chromatograph (5). Attempts to prepare

[1] American Type Culture Collection (ATCC), 23955, Rockville, Md.
[2] ATCC 25453.
[3] ATCC 14622.
[4] ATCC 11711.

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Table I—^{13}C-NMR Data for IV, V, and VI [a]

Carbon No.	IV	V	VI [b]
2	144.4d	144.4d	147.7d
3	121.9d	121.9d	121.8d
4	134.8d	134.9d	134.0d
4a	130.1s	130.0s	130.3s
5	92.3d	92.3d	105.6d
6	159.7s	159.6s	157.4s
7	97.1d	97.2d	123.0d
8	145.2s	145.2s	140.8s[1]
8a	135.6s	135.5s	136.4s[1]
1′	47.7d	47.7d	57.1d
2′	31.0t[1]	32.4t	31.3t[2]
3′	32.0t[1]	32.4t	28.0t[2]
4′	174.0s	175.3s	175.8s
5′	20.6q	20.7q	20.6q
OCH_3	55.3q	55.2q	55.7q
CO_2CH_3	51.5q	—	—

[a] Data were obtained in deuterochloroform. The data and assignments for IV have been reported (4) and are listed here for comparison with V and VI. Signals bearing the same numerical superscript may be reversed. [b] The assignments listed for VI are based on comparison with IV and V and SFORD.

V from II by using such reagents as thionyl chloride and ethyl chloroformate followed by ammonia treatment were unsuccessful. The major product appeared to be VI (TLC). The lactam (VI) could be efficiently prepared by treatment of II with *N*-ethoxycarbonyl-2-ethoxy-1,3-dihydroquinoline (VII).

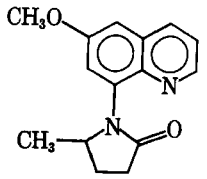
Similar biotransformations of carboxylic acids to primary amides have been reported for biotin and desthiobiotin (8, 9), but they do not appear to be common microbial reactions. The conversion of mycophenolic acid to amide derivatives of glycine and alanine have also been reported (10). Whether these biotransformations play a role in the mammalian metabolism of II remains to be established, but conjugation of glycine with similar compounds is common (11).

EXPERIMENTAL[5]

Fermentation Procedures—Initial screening studies to identify microorganisms capable of metabolizing 8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline (II) were accomplished using the usual two-stage fermentation procedure (2–4, 7). Cultures were grown in 25 ml of dextrose–yeast extract–peptone medium (4) held in 125-ml Erlenmeyer flasks equipped with stainless steel tops. The substrate (II) was added to 24-hr-old second-stage cultures (0.2 mg of substrate/milliliter of culture medium) as a 5% solution in dimethylformamide. Cultures were incubated on a rotary shaker[6] at 250 rpm and room temperature.

Culture controls consisted of fermentation blanks, in which organisms were grown under identical conditions but without the substrate. Substrate controls consisted of sterilized 24-hr-old second-stage cultures containing the substrate and incubated at 250 rpm and room temperature.

The cultures were sampled at periodic intervals by homogenizing 5 ml of the culture, adjusting to pH 7–8, and extracting with 5 ml of ethyl acetate. The ethyl acetate layer was evaporated to dryness, the residue redissolved in 100 μl of ethyl acetate, and 20 μl of the solution was spotted on silica gel G TLC plates. The plates were developed in either acetone (solvent A), ether–acetone (1:1, solvent B), or 10% methanol in chloroform (solvent C). In solvent A the R_f values were 0.6 (II) and 0.56 (V); in solvent B, R_f = 0.58 (II), and 0.49 (V); and in solvent C, R_f = 0.45 (II) and 0.39 (V). Visualization was accomplished with UV light and by spraying with diazotized *p*-nitroaniline followed by spraying with concentrated hydrochloric acid. All spots were dull yellow after spraying. HPLC analyses were carried out using a C_{18}-reversed-phase column[7] and a mobile phase consisting of 6.6 g of K_2HPO_4, 8.4 g or KH_2PO_4, 4.0 g of *N,N*-dimethyl-*n*-octylamine, 2.4 liters of water, and 1.6 liters of methanol at a flow rate of 1.0 ml/min. An HPLC pump[8] and a microsyringe-loaded loop injector[9] were used with a dual wavelength UV detector[10] (254 and 280 nm). Extracts were dissolved in 1.0 ml of methanol and 40 μl was injected for analysis. In this system, the metabolite (V) has a relative retention time (relative to II) of 0.73 and an A_{254}/A_{280} value equal to 4.73.



VI

Microbiological Preparation of 8-(3-Carboxamido-1-methylpropylamino)-6-methoxyquinoline (V)—*Streptomyces rimosus*[1] was grown in 1.5 liters of medium held in fifteen 500-ml Erlenmeyer flasks. A total of 300 mg of II was distributed evenly among the cultures. The cultures were incubated at room temperature and 250 rpm for 4 days after substrate addition. The cultures were pooled and extracted with ethyl acetate (2 × 700 ml, 1 × 1000 ml). The combined ethyl acetate layers were dried (sodium sulfate) and evaporated *in vacuo* to leave an oily residue (702 mg). Preparative layer chromatography of 400 mg of the residue was carried out using precoated silica gel G plates[11] (2.0 mm thick, 20 × 20 cm) developed in ether–acetone (1:1). The major band, corresponding to metabolite V, was located by UV light, scraped off, and the silica gel was extracted with ethyl acetate. Evaporation of the solvent afforded 66 mg of a crystalline residue. Crystallization from ether yielded a crystalline material, which was shown by ^{1}H-NMR to be unrelated to the primaquine nucleus. It appeared to be a simple organic constituent that was not further characterized. This substance could be separated from the metabolite on TLC alumina plates[11] using benzene–ethyl acetate–ammonium hydroxide (50:45:5). It could be visualized under UV light but it did not react with the spray reagent.

The mother liquor residue was subjected to another preparative layer chromatography over alumina plates (1.0 mm thick, 20 × 20 cm) using benzene–ethyl acetate–ammonium hydroxide (50:45:5). The metabolite band was extracted with ethyl acetate and after evaporation yielded 18 mg of the metabolite V pure by TLC and HPLC as an oily residue; IR ($CHCl_3$) ν_{max} 3510, 3490, 3400, 1670, 1612, 1595, 1575, and 1520 cm^{-1}, ^{1}H-NMR ($CDCl_3$) δ 8.53 (1H, dd, *J* = 1.5, 4.5 Hz, C-2 H), 7.93 (1H, dd, *J* = 1.5, 9.0 Hz, C-4 H), 7.30 (1H, dd, *J* = 4.5, 9.0 Hz, C-3 H), 6.34 (2H, s, C-5 H, and H-7), 6.0 (1H, br, NH), 5.6 (2H, br, NH_2), 3.87 (3H, s, OCH_3), 3.7 (1H, m, H-1′), 2.4 (2H, m, H-3′), 2.1 (2H, m, H-2′), and 1.30 (3H, d, *J* = 6.0 Hz, CH_3); CD (0.005% methanol) $[\theta]_{350-210}$ = 0; mass spectrum: M^+ at *m/z* 273 (10%), 257 (1%), 215 (51%), and 201 (100%). The metabolite sample was identical to that prepared from IV (TLC, cobalt-TLC, HPLC, IR, ^{1}H- and ^{13}C-NMR).

Synthesis of 8-(3-Carboxamido-1-methylpropylamino)-6-methoxyquinoline (V) from IV—8-(3-Carbomethoxy-1-methylpropylamino)-6-methoxyquinoline (IV, 80 mg) (4) was dissolved in a few drops of methanol and placed in a glass tube (2-mm thick, 3-mm i.d. × 30 cm) and treated with 0.5 ml of methanol saturated with anhydrous ammonia gas. Additional ammonia was bubbled into the tube while cooling in an ice bath. The tube was then sealed and heated in an oven at 140° for 4 days. The reaction mixture was evaporated *in vacuo* to leave a brown oily residue (98 mg). TLC analysis of the mixture showed the presence of two major components corresponding to starting material (IV) and the amide product (V). There was no evidence (TLC) of the presence of the lactam derivative (VI) [solvent B, R_f = 0.69 (IV), 0.22 (VI)[12], 0.49 (V)]. The reaction mixture was purified by preparative layer chromatography on alumina plates[11] (20 × 20 cm, 1.0-mm thick) using benzene–ethyl acetate–ammonium hydroxide (50:45:5) as the developing solvent. The band corresponding to the product (R_f = 0.30) was located under UV light, scraped off, and extracted with 25% methanol–ether. Filtration and evaporation of the solvent afforded 17 mg of an oily residue that was pure by TLC, HPLC, and ^{1}H-NMR. For ^{13}C-NMR data see Table I; mass spectrum: M^+ at *m/z* 273 (14%), 257 (2%), 215 (48%), 201 (100%).

Synthesis of VI From II—A 50-mg sample of II (4) was dissolved in 1 ml of methylene chloride containing 0.03 ml (1.1 eq.) of triethylamine to which was added 75 mg of VII (1.5 eq.). The clear solution was allowed to stand at room temperature for 3 days, after which time the solution was extracted with 1 ml of 1 *N* sodium hydroxide to remove traces of II. The methylene chloride layer was filtered through cotton, evaporated,

[5] IR spectra were run in chloroform using a Perkin-Elmer 281b spectrophotometer. The ^{1}H-NMR spectra (90 MHz) were recorded in deuterochloroform on a Varian EM 390 spectrometer using tetramethylsilane as an internal standard. The ^{13}C-NMR spectra (15.03 MHz) were recorded on a JEOL-FX60 FT spectrometer using tetramethylsilane as internal standard.

[6] New Brunswick Model G10-21.

[7] μBondapak, Waters Associates, Milford, Mass.

[8] Model M-6000, Waters Associates, Milford, Mass.

[9] Model U6-K, Waters Associates, Milford, Mass.

[10] Model 440, Waters Associates, Milford, Mass.

[11] Brinkmann Instruments, Westbury, N.Y.

[12] The lactam VI does not react with the spray reagent but can be visualized under UV light.

and the residue (142 mg) was purified using flash chromatography (12) (silica gel, ether, then ether–acetone, 1:1). A total of 24 mg of VI was obtained, mp 109–110° (*iso*-propyl ether); IR (KBr) ν_{max} 1690, 1615, 1590, and 1490 cm^{-1}; ^1H-NMR ($CDCl_3$) δ 8.75 (1H, dd, J = 1.5, 4.5 Hz, C-2 H), 8.06 (1H, dd, J = 1.5, 8.5 Hz, C-4 H), 7.35 (1H, dd, J = 4.2, 8.5 Hz, C-3 H), 7.29 (1H, d, J = 2.7 Hz, C-7 H), 7.07 (1H, d, J = 2.7 Hz, C-5 H), 4.72 (1H, ddq, J = 6.0, 6.0, 6.0 Hz, C-1′ H), 3.90 (3H, s OCH_3), 2.6, and 1.8 (3H and 1H, m, C-3′ H, C-2′ H), 1.03 (3H, d, J = 6.0 Hz, CH_3); mass spectrum M^+ at m/z 256 (18%), 228 (18%), 213 (21%), 200 (81%), 187 (52%), 186 (51%), 159 (100%); ^{13}C-NMR data (Table I); TLC, solvent B, R_f 0.22[12].

Anal.—Calc. for $C_{15}H_{16}N_2O_2$: C, 70.29; H, 6.29; N, 10.93. Found: C, 70.32; H, 6.40; N, 10.86.

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Synthesis and Anticonvulsant Activity of Some 2-Methyl-3-phenylcarbamoyl-2,3-diazabicyclo-[2.2.1]heptanes

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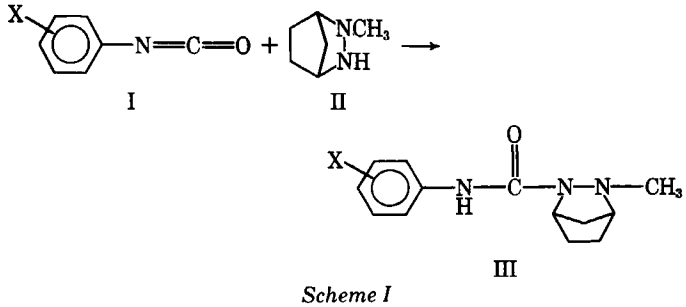
Abstract □ A series of 2-methyl-3-phenylcarbamoyl-2,3-diazabicyclo[2.2.1]heptanes were obtained by treating aryl isocyanates with 2-methyl-2,3-diazabicyclo[2.2.1]heptane. The compounds showed only minimal anticonvulsant activity.

Keyphrases □ 2-Methyl-3-phenylcarbamoyl-2,3-diazabicyclo[2.2.1]-heptanes—synthesis and anticonvulsant activity □ Anticonvulsants—synthesis of some 2-methyl-3-phenylcarbamoyl-2,3-diazabicyclo[2.2.1]-heptanes

A previous report (1) describes the synthesis and anticonvulsant activity of a series of 1-methyl-2-phenylcarbamoylpiperidazines. Four members of this series showed significant activity. The present report describes the synthesis and anticonvulsant activity of a series of 2-methyl-3-phenylcarbamoyl-2,3-diazabicyclo[2.2.1]heptanes (III). The series of III, with their cage structure, would be expected to provide different steric and basic properties in comparison with the monocyclic compounds (1).

BACKGROUND

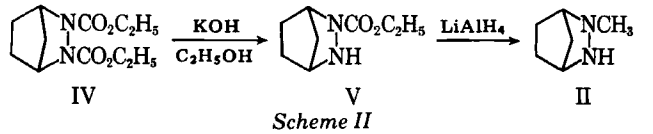
The synthesis of 2-methyl-2,3-diazabicyclo[2.2.1]heptane (II) was accomplished in two steps from 2,3-dicarboethoxy-2,3-diazabicyclo[2.2.1]heptane (IV) (2). Partial saponification of IV gave 2-carboethoxy-2,3-diazabicyclo[2.2.1]heptane (V), which underwent reduction with lithium aluminum hydride to afford the base, II (Scheme I). Compound II reacted with aryl isocyanates (I) to produce III in good yields (Scheme II; Table I).



Scheme I

Compounds III*a–j* were tested in the maximal electroshock seizure and subcutaneous pentylenetetrazol seizure threshold tests for anticonvulsant activity and in the rotorod test for neurotoxicity in male mice[1] by reported procedures (3). None of the compounds showed activity in either test at 100 mg/kg.

In the maximal electroshock seizure test, compounds III*a*, *d*, *g*, *i*, and *j* exhibited activity at 300 mg/kg at 30 min. Compounds III*a* and *i* showed no toxicity at this dose level, whereas III*d*, *g*, and *j* displayed some toxicity. Two compounds were active in the subcutaneous pentylenetetrazol seizure test at 300 mg/kg. Compound III*j* was active at 30 min; compound III*b* showed activity at 4 hr with no toxicity. Apparently, the introduction



Scheme II

[1] Carworth Farms No. 1 mice.

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