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Microbial Models of Mammalian Metabolism. O-Dealkylation of 10,11-Dimethoxyaporphine

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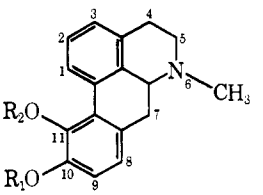
Microbial transformations of 10,11-dimethoxyaporphine were studied to determine the potential of microorganisms to produce monomethoxyaporphines. Ten microorganisms were identified as being capable of yielding apocodeine and/or isoapocodeine as the major metabolite of this compound. A *Streptomyces* species (SP-WISC 1158) gave a mixture of apocodeine and isoapocodeine in 24 and 20% yield, respectively. *Cunninghamella blakesleeana* (ATCC 9245) converted 10,11-dimethoxyaporphine quantitatively into isoapocodeine. O-Dealkylation of this aporphine system is a facile microbial transformation, and the 10-methoxyl group is more susceptible to metabolic cleavage than the sterically hindered 11-methoxyl group. Selectivity in O-dealkylation may be accomplished with different microorganisms. This is the first report dealing with the microbial transformation of an aporphine system.

Smith and Rosazza have commenced a series of studies designed to develop microbial transformation systems as useful adjuncts in drug metabolism studies.[1-3] Recent developments in comparative biochemistry have made it possible to link diverse metabolic systems through similarities in the pathways by which they alter xenobiotics. It is suggested that microbial metabolic systems consisting of a selected series of microorganisms may be used to produce metabolites normally obtained in very low amounts in mammalian systems.[1] In practice, microbial and mammalian biotransformation studies may be conducted simultaneously. Metabolites produced in common by both metabolic systems could be readily obtained by routine fermentation scale-up procedures. Other advantages of this application of microbial transformation systems center about the mild conditions and the selectivity with which such biotransformations are accomplished, especially with polyfunctional substrates. Initial studies using microbial models of mammalian metabolism have been successfully conducted on aromatic hydroxylation as a reaction type.[1] Microbial patterns of phenolic metabolites from a broad array of aromatic substrates were similar to those obtained with cytochrome P-450 monooxygenases of hepatic microsomes and/or in vivo mammalian systems. This report is the first one dealing with O-dealkylation as a reaction type and is concerned with the metabolism of 10,11-dimethoxyaporphine by microorganisms.

Considerable interest has been demonstrated in apomorphine (1) due to its application in the treatment of Parkinsonism[4] and because of suggested relationships of this compound to dopamine.[5-7] The metabolic fate of apomorphine in mammalian systems has been studied by several groups, and glucuronidation[8-11] and methylation[12,13] appear to be important pathways in the biodisposition of this compound.[14,15] Both metabolic reactions occur predominantly at the 10-phenolic position of apomorphine (1). A COMT preparation from rat liver yielded a mixture of apocodeine (2) and isoapocodeine (3) in a ratio of 81:1.[12] To facilitate studies on the COMT reaction with apomorphine (1), three possible O-methylation products, 10,11-dimethoxyaporphine (4), 10-methoxy-11-hydroxyaporphine (apocodeine,



1, $R_1 = R_2 = H$ (apomorphine)
2, $R_1 = CH_3$, $R_2 = H$ (apocodeine)
3, $R_1 = H$; $R_2 = CH_3$ (isoapocodeine)
4, $R_1 = R_2 = CH_3$ (10,11-dimethoxyaporphine)

2), and 10-hydroxy-11-methoxyaporphine (isoapocodeine, 3), were prepared.[12] Isoapocodeine (3) could be prepared in only 5% yield, while 2 and 4 were more readily obtained. Smith and Cook have shown that apocodeine (2) may be O- and N-dealkylated in vivo by rats to 1 and norapomorphine.[16]

Results and Discussion

Initial screening experiments were conducted to obtain microorganisms which metabolized 10,11-dimethoxyaporphine (4). For this purpose, 65 microorganisms were selected from our culture collection, based on previous work by which O-dealkylation had been observed[1,3] and on literature reports describing cultures capable of accomplishing O-dealkylation.[17-20] Some steroid metabolizing cultures were also chosen for early screening experiments. The ten cultures which actively metabolized 4 included representatives of seven genera (Table I). It is interesting that *Cun-*

Table I. Various Cultures Capable of Yielding Apocodeine (2), Isoapocodeine (3), or Other Metabolites of 10,11-Dimethoxyaporphine (4)

Culture name (collection no.)[b]	Compounds identified in extracts by TLC[a]			
	Dimethoxyaporphine (4)	Apocodeine (2)	Isoapocodeine (3)	Other
Cunninghamella bainieri (ATCC 3065)	−	−	++	+
Cunninghamella blakesleeana (ATCC 9245)	−	−	+−++	+
Cunninghamella echimulata (NRRL 3655)	++	Trace	++	+
Helicostylum piriforme (QM 6945)	+++	+	+	−
Microsporum gypseum (ATCC 11395)	+++	−	+	+
Mucor mucedo (SP-WISC 4605)	+++	−	++	+
Penicillium duclauxi (NRRL 2020)	+++	−	+	+
Sepedonium chryospermum (ATCC 13378)	++	−	−	++
Streptomyces species (SP-WISC 1158)	+	+++	++	+
Streptomyces rimosus (ATCC 23955)	++	++	Trace	−

[a] Estimates of relative amounts of metabolites and substrate present in fermentation extracts were made on 48-hr Stage II culture samples using the silica gel GF_{254}, cyclohexane–chloroform–piperidine (8:1:1) solvent system. [b] ATCC = American Type Culture Collection, Rockville, Md; NRRL = Northern Regional Research Laboratories, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill.; SP-WISC = School of Pharmacy, University of Wisconsin, Madison, Wis.

ninghamella species, as noted earlier,[1,21-23] demonstrated high metabolic activity with an unusual substrate.

Among the ten cultures which converted 4 into its O-dealkylated metabolites, yields were high, and cultures could selectively cleave either the 10-methoxy or 11-methoxy ether groups. The least hindered methoxyl function (on position 10) was removed by most cultures to yield isoapocodeine (3) as the most common metabolite. *Cunninghamella blakesleeana* and *C. bainieri* selectively cleaved the 10-methyl ether from 4 to give 3 almost exclusively, while *Streptomyces rimosus* cleaved the 11-methyl ether giving 2 as the sole O-dealkylation product. On the basis of preliminary screening experiments, *C. blakesleeana* (ATCC 9245) and *Streptomyces* species (SP-WISC 1158) were selected for further study.

It was anticipated that analytical difficulties might be encountered in the isolation and quantitation of substrates and metabolites from the complex fermentation medium. Therefore, incubations using resting cell suspensions of *C. blakesleeana* and *Streptomyces* species in pH 7.0 phosphate buffer were conducted to determine whether the biotransformation of 4 could be conducted using a simpler incubation medium. Only traces of metabolites 2 and 3 could be detected in these incubation mixtures over a 72-hr incubation period.

Apocodeine and isoapocodeine were obtained in quantities sufficient for structure elucidation by incubating 1 g of 4 with Stage II cultures of *Streptomyces* species (SP-WISC 1158). The yields of 2 and 3 were 24 and 19.5%, respectively. Although the yield of isoapocodeine obtained in this incubation was superior to that obtained synthetically,[12] difficulties were encountered in the separation of the isomeric monomethoxyaporphines and in the final purification of 3. These difficulties could be easily by-passed by using *C. blakesleeana* or *C. bainieri* which gave 3 as the only monomethoxyaporphine metabolite. In an analytical experiment, yields of isoapocodeine by *C. blakesleeana* were essentially 100% (Figure 1) with maximum metabolite production occurring 48 hr after substrate addition to Stage II cultures. After 48 hr, isopocodeine levels decreased, probably as a result of further metabolism of 3 to unidentified metabolites. As noted in earlier screening experiments, other more polar metabolites were found in fermentations of *C. blakesleeana* and *C. bainieri*. These unidentified metabolites gave the same color reactions on TLC plates as the monomethoxyaporphines.

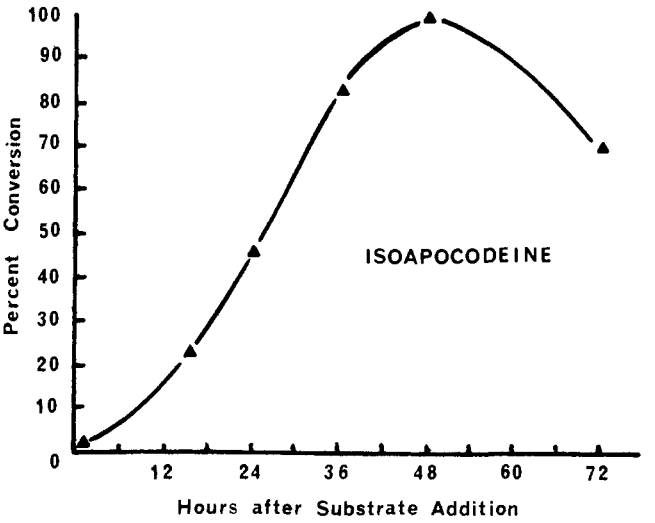


Figure 1. Conversion of 10,11-dimethoxyaporphine (4) to isoapocodeine (3) by *Cunninghamella blakesleeana* (ATCC 9245).

O-Dealkylation studies on 4 in mammalian systems have not yet been reported. However, Smith and Cook[16] have shown that Sprague–Dawley rats are capable of O-dealkylating apocodeine to yield apomorphine and norapomorphine. It is interesting that the COMT methylation of apomorphine by in vitro rat liver preparations leads to the formation of much larger amounts of 2 than 3.[12] The enzyme system apparently displays a preference for introducing methyl groups on the least sterically hindered phenolic functions of 1. It appears that when microorganisms accomplish the reverse reaction, O-demethylation, the least sterically hindered methoxyl group (position 10) of 4 is also preferentially removed. It is of interest that the enzyme systems of *Streptomyces* species appear to display a preference for removal of the more sterically hindered 11-methoxyl group of 4.

Microbial O-dealkylation reactions are generally considered to be monooxygenase mediated enzyme processes. An enzyme system capable of conducting these reactions on a

limited variety of substrates (methoxybenzoates) has been purified from *Pseudomonas putida* cells.[24] This system consists of an iron-flavoprotein and an iron–sulfur protein requiring NADH and oxygen to function. Demethylation of more complicated aryl ether substrates has also been accomplished by microorganisms, and these include aconitine,[19] thebaine,[17,18] and griseofulvin.[20] Griseofulvin possesses three methoxyl groups, each of which is labile to microbial O-dealkylation enzymes. Boothroyd *et al.*[20] characterized microorganisms which could singly and selectively remove the methoxyl groups of this antibiotic. Our results with *C. bainieri*, *C. blakesleeana*, and *S. rimosus* support the findings of these workers that selective O-dealkylation may be accomplished with different microorganisms.

Experimental Section

NMR spectra were obtained on compounds with a Varian T-60 spectrometer using TMS as an internal standard. Low-resolution mass spectra were obtained on a Finnigan Model 3200 mass spectrometer. Gas chromatographic analyses were performed on a Hewlett-Packard 5710A gas chromatograph equipped with dual flame ionization detectors.

Analytical Methods. Gas chromatographic analyses were performed on glass columns containing 3% OV-17 on 100–120 mesh Gas Chrom Q (Applied Science) as described by Smith and Stocklinski.[25]

Thin-layer chromatography (TLC) was performed on 0.25-mm thick layers of silica gel GF_{254} prepared on glass plates with a Quickfit Industries spreader. Plates were developed in cyclohexane–chloroform–piperidine (8:1:1).[12] Alternatively, 0.25-mm thick Woelm, neutral alumina plates (Analtech) were developed in chloroform–benzene (9:1). Woelm neutral alumina preparative TLC plates (1 mm thick) were prepared with the Quickfit industries spreader and were used in the final purification of apocodeine and isoapocodeine. Compounds were visualized on developed chromatograms by spraying with Dragendorff's reagent; by allowing developed plates to stand in air for 24 hr when characteristic colors would develop (dimethoxyaporphine, yellow; apocodeine and isoapocodeine, green); or by fluorescence detection under 254- or 366-nm ultraviolet irradiation.

Substrate and Metabolite Synthetic Standards. The following compounds were prepared according to literature procedures: 10,11-dimethoxyaporphine;[12] 10-methoxy-11-hydroxyaporphine (apocodeine, **2**);[26] and 10-hydroxy-11-methoxyaporphine (isoapocodeine, **3**).[12] Each compound melted in accordance to reported melting point values and was evaluated for purity chromatographically.

Fermentation Procedures. Cultures used in this study are maintained in the University of Iowa, College of Pharmacy culture collection, and are stored at 4° in a refrigerator. Organisms were grown according to two-stage fermentation procedure in a medium of the following composition: soybean meal, 5 g; glucose, 20 g; yeast extract, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; distilled water to 1000 ml; pH adjusted to 7.0 with 5 *N* HCl. Media were sterilized in an autoclave at 121° for 15 min.

Fermentations were conducted on rotary shakers (Model G-25, New Brunswick Scientific Co.) operating at 250 rpm (1-in. stroke) at 27° in erlenmeyer flasks holding one-fifth of their volumes of medium. The surface growth from slants was suspended in 5 ml of sterile medium and used to inoculate Stage I cultures which were incubated as described for 72 hr. The thick 72-hr Stage I growth was used as inoculum for Stage II cultures, the inoculum volume being 10% of the volume of Stage II fermentation medium in all cases. 10,11-Dimethoxyaporphine (**4**) was added to 24-hr-old Stage II cultures in 1% HCl solutions to achieve a concentration of substrate of 0.5 mg/ml of Stage II culture medium. Substrate containing Stage II cultures were sampled at various time intervals for TLC and gas chromatographic analyses.

Fermentation Sampling. Samples of 5 ml were withdrawn from fermentations at various time intervals and were adjusted to pH 8.6 with 2.5 *N* NaOH. The adjusted samples were extracted with four, 5-ml volumes of *n*-heptane–isoamyl alcohol (99:1), and 5 μl of the combined extracts were spotted on TLC plates.

Screening of Microorganisms. Small-scale fermentations were conducted in 125-ml erlenmeyer flasks to screen 65 selected microorganisms for their abilities to metabolize 10,11-dimethoxyaporphine (**4**). Cultures were grown as described, and a total of 12.5 mg of 10,11-dimethoxyaporphine was added to each 24-hr Stage II culture. Substrate-containing flasks were incubated with shaking, and 5-ml samples were withdrawn at 24 and 72 hr after substrate addition. Of the 65 organisms screened, 10 were found to utilize substrate to produce apocodeine (**2**), isoapocodeine (**3**), and/or other unidentified metabolites (Table I).

The experiment was repeated with controls using only the active cultures. Controls consisted of substrate added to sterile medium and of fermentation blanks containing no substrate. The most active metabolizing cultures were *Streptomyces* species (SP-WISC 1158) and *Cunninghamella blakesleeana* (ATCC 9245). Both of these microorganisms utilized nearly all of the substrate after 72 hr of incubation. These microorganisms were selected for further study.

Preparative Scale Production of Apocodeine (2) and Isoapocodeine (3) by *Streptomyces sp* (SP-WISC 1158). *Streptomyces* species (1158) was grown according to the usual fermentation procedure. Stage II fermentations were conducted in 2.0 l. of medium held in 500-ml erlenmeyer flasks. A total of 1.0 g of **4** in 20 ml of 1% HCl was distributed evenly among 20 Stage II culture flasks, and the substrate-containing cultures were incubated for 72 hr. At this time, the contents of each flask were pooled, adjusted to pH 8.6, and exhaustively extracted with *n*-heptane–isoamyl alcohol (99:1). The extracts were dried over anhydrous Na_2SO_4 and concentrated. After adjusting the concentrate to exactly 1.0 l., a 1-ml aliquot was taken and used to determine quantitatively the amount of metabolites produced. The remaining organic extract was concentrated to dryness and applied to a neutral alumina column (Bio-Rad, AG-7 alumina; column dimensions 70 × 2.5 cm). The column was eluted with chloroform–benzene (9:1). Fractions containing **2** or **3** were combined, evaporated to dryness, and purified further by preparative TLC. Alumina zones corresponding to **2** and **3** were scraped from developed preparative TLC plates and exhaustively extracted with methanol. The metabolites isolated in this way were characterized by comparison with authentic standards using TLC,[9] gas chromatography,[17] NMR, and mass spectral analyses.

The microbial conversion of 10,11-dimethoxyaporphine afforded 225 mg of apocodeine (24% yield): NMR ($CDCl_3$) δ 2.55 (s, NCH_3), 3.90 (s, 10-OCH_3), 6.79 (s, 8 H, 9 H), 8.31 (q, 1 H); mass spectrum, *m/e* (% relative abundance) 281 (88), 280 (100), 265 (18), 238 (34), 236 (12), 223 (21), 221 (11), 205 (18), 178 (13), 165 (27).

The *Streptomyces* species afforded 185 mg (19.5% yield) of isoapocodeine (**3**): NMR ($CDCl_3$) δ 2.54, (s, N-CH_3), 3.62 (s, 11-OCH_3), 5.90 (s, 10 H), 6.88 (s, 8 H, 9 H), 8.18 (q, 1 H); D_2O exchange causes the broad singlet at 5.90 to disappear; mass spectrum, *m/e* (% relative abundance) 281 (99), 280 (100), 265 (33), 238 (26), 236 (16), 223 (26), 221 (12), 205 (12), 178 (23), 165 (38).

Production of Isoapocodeine by *Cunninghamella blakesleeana* (ATCC 9245). *Cunninghamella blakesleeana* (ATCC 9245) was grown in 20-ml portions of medium contained in 125-ml erlenmeyer flasks. The substrate **4** was added to a final concentration of 0.5 mg/ml in each 24-hr-old Stage II flask. These experiments were performed with controls consisting of substrate added to sterile medium without organism and blank samples consisting of normal fermentations without substrate. All experiments were conducted in duplicate.

Samples of Stage II, substrate-containing flasks, controls, and blanks were harvested at 1, 16, 24, 36, 48, and 72 hr after substrate addition. The total contents of each flask was adjusted to 25 ml in volumetric flasks. Aliquots of 5 ml were used to analyze for isoapocodeine as previously described.[25] The only metabolite detected in incubation mixtures was isoapocodeine, and the results of this experiment are shown in Figure 1.

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Antiprotozoal Thiazoles. 2-(5-Nitro-2-thienyl)thiazoles

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A series of 2-(5-nitro-2-thienyl)thiazoles and their vinylogs having substituted methylamine side chains has been prepared by halogen displacement on the corresponding 4-chloromethylthiazole. Of these, 4-morpholinomethyl-2-(5-nitro-2-thienyl)thiazole showed moderate activity against *Trypanosoma cruzi* and *Trypanosoma rhodesiense* in mice. This compound formed the lead for a series of analogous thiazole-4-carboxaldehyde hydrazones. Some of the latter were found active in curing murine *Tryp. cruzi* and *Tryp. rhodesiense* infections and to have low acute toxicity. A comparison with known active compounds is given and some structural features necessary for activity are discussed.

As part of a program for the discovery of new antiprotozoal agents, we have investigated the synthesis and in vivo antitrypanosomal activity of some thiazole compounds bearing a nitro-substituted five-membered heterocyclic ring in the thiazole 2 position. In this paper, we report the synthesis of such compounds carrying a 5-nitro-2-thienyl substituent and their activity against *Trypanosoma cruzi* and *Trypanosoma rhodesiense*.

Chemistry. A search of the literature has revealed several reports of the synthesis and biological activity of 2-(5-nitro-2-furyl)thiazoles[1-3] and 2-[2-(5-nitro-2-thienyl)vinyl]thiazoles.[4] However, no description of the title compounds was found, which may be a consequence of the fact that a key intermediate, 5-nitro-2-thiophenethiocarboxamide (**1a**), was unknown. Sherman and Von Esch[3] have described the synthesis of the furan analog of **1a** in moderate yield, using the method of Taylor and Zoltewicz.[5] By a modification of this method, we obtained **1a** in 90% yield and the vinylog **1b** in 72% yield.

The treatment of compounds **1a,b** with 1,3-dichloroacetone, by a typical Hantzch[6] procedure, provided the 4-chloromethylthiazoles **2a,b** in good yield. These were formed as the free thiazoles with the evolution of gaseous HCl, the thiazole ring having a low basicity when bearing a 5-nitro-2-thienyl group.

Compounds **2a,b** were used as intermediates for the introduction of biologically active side chains. Thus, displacement of the chlorine atom in **2a,b** with a secondary amine yielded the substituted thiazolemethylamines **3a-g**. These were relatively unstable (with the exception of **3a**) and were biologically screened as their stable hydrochloride salts. See Table I.

The aldehydes **6a,b** were obtained by the treatment of **2a,b** by the method of Kröhnke[7] and, more simply, by that of Sommelet.[8] See Scheme I.

The aldehydes **6a,b** were used to broaden the scope of

Scheme I