MICROBIAL MODELS OF MAMMALIAN METABOLISM[1]

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INTRODUCTION

Drug discovery and development is a complex process involving several definitive phases. After discovery of a new chemical entity with interesting pharmacological activity, further investigations are pursued to determine the scope of activity in higher animals and man and to prepare suitable dosage forms that insure stability and the most effective drug administration to the host. The thrust of these efforts is to enhance desired therapeutic effects and diminish unwanted side-effects.

Few drugs are able to stimulate single or specific responses in complex living systems. Undesirable side-effects may be due to the intrinsic effects of a drug or may be caused by metabolites formed by the action of enzymes as the drug passes through actively metabolizing organs in the body such as the liver. Consequently, absorption, distribution, excretion and metabolic transformation studies of new drugs assume major importance. Through these studies, underlying reasons for drug action and/or toxicity may be gained, as well as a firm base for structure activity relationships.

MICROBIAL MODELS OF MAMMALIAN METABOLISM

Our first paper (1) on microbial models of mammalian metabolism was presented at the 1973 American Society of Pharmacognosy Meeting on Jekyll Island in Georgia. This work was subsequently published (2) and followed by a series of review articles (3–6). In this paper, we bring together concepts presented earlier, and we present additional examples of metabolic transformations which clearly underline the applicability of microbial transformations as a means for preparing mammalian drug metabolites.

The need for conducting metabolic studies of drugs and other xenobiotics is clear. Numerous problems, however, hinder rapid and satisfactory completion of this work. Included are deficiencies in animal models where species variation is observed. This phenomenon is often due to differences in pathways and degrees of metabolic transformations. Analytical chemical difficulties may occur especially concerning the complete establishment of structures of metabolites which may be available only in miniscule (*i.e.*, microgram) amounts. Additional problems are posed by the inability to detect nascent/highly reactive intermediates and in the production of sufficient amounts of metabolites for complete biological evaluation.

Solutions to some of the problems encountered in drug metabolism studies include the use of gc/mass spectral/computer systems and nmr and ir methods employing Fourier transform computer processes which enable structure elucidation work on small amounts of metabolites. Production of sufficient quantities of drug metabolites or potential metabolites for complete biological evaluation, however, remains as a continuing and major problem, especially with difficult-to-

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synthesize molecules. Attempts to circumvent the problem of metabolite availability have included the use of microsomal preparations or organ perfusion systems. These methods, however, have limitations in providing more than milligrams of metabolites—quantities insufficient for biological/toxicological evaluation.

Over 10 years ago we first suggested (1,2) that microbial systems could be defined which would mimic metabolic systems of mammals in the production of drug metabolites. These systems were labeled "Microbial Models of Mammalian Metabolism" (M^4). Ideally, microbial and mammalian xenobiotic metabolism studies would be conducted in parallel, and common microbial/mammalian metabolites would be produced by simple fermentation scale-up methods by use of the microbial systems (3,4). Microorganisms might also be employed in predictive fashion where microbial drug metabolites would presumably be similar or identical to those formed by mammalian systems. Microbially produced metabolites could then be used to establish analytical methods for use in the identification of mammalian metabolites.

Considerable recent work testifies to the amazing metabolic capabilities of single fungal, yeast or bacterial cultures which perform useful biotransformation reactions. We suggested earlier that it might be difficult to find individual microorganisms which could achieve many of the transformations achieved by mammalian liver (2). Many recent examples, however, affirm the versatility of single microorganisms.

CONDUCT OF M^4 STUDIES

Our strategy for conducting M^4 studies involves the following steps:

- Definition of type reactions desired
- Choice of microorganisms for screening studies
- Development of analytical methods for drug and expected or model metabolites
- Microbiological screening
- Optimization
- Preparative studies

Once the substrate and type reaction desired are defined, a literature search is conducted to define potentially useful microorganisms. Cultures for transformations may be obtained from natural environments such as sewage or soil. We recommend, however, that standardized culture collections (4) be used where possible for the sake of ready repetition of results in different laboratories. Often, listings of 20 or more microorganisms that are likely to affect given transformations can be compiled by the literature approach (6).

We use simple soybean-containing culture medium (2–4) in screening experiments; thus, analytical methodology necessary to detect and possibly quantitate substrate and expected metabolites is developed initially by use of media appropriately spiked. In the spiking step, the level of substrate is dictated by the incubation conditions but is generally around 250 μg/ml. Analytical procedures capable of detecting conversions of 1% or more are developed. By using approaches described for the determinations of drugs and metabolites in biological fluids (7), we have consistently been successful in developing necessary methodology. Tlc, gc or hplc procedures are most useful, and a number of these methods have been developed in our laboratories (8–10) or those of other investigators (11,12). These serve as examples of successful approaches.

Screening methods used by us have been described (4,5). Briefly, we find that stainless steel capped Delong culture flasks are simple to use and afford reproducibility in aeration of shaken-flask cultures. A two-stage fermentation protocol is routinely employed, and drug substrates are normally added to incubation media 24 hours after the inoculation of Stage 2 cultures. Samples of drugs

are typically added as concentrated solutions in water or in miscible solvents such as dimethyl-sulfoxide, acetone, alcohol, or dimethylformamide. Alternatively, solutions in methylene chloride or water in the presence of wetting agents like Tween 80 (0.001%) can be used (4). Samples are withdrawn from substrate containing incubation mixtures at appropriate time intervals (2 to 240 hours) and are assayed by gc, tlc or hplc. All of our screening procedures are conducted in 125 ml Delong flasks containing 25 ml of culture to which 5 to 10 mg of drug may be added. Preparative scale incubations are performed in multiple shaken flasks containing 25 ml of culture to which 5 to 10 mg of drug may be added or in stirred fermentors (4).

METABOLIC TRANSFORMATIONS COMMON TO MAMMALIAN AND MICROBIAL SYSTEMS

Practically all xenobiotics are metabolized enzymatically when administered to mammals. The metabolic process has been subdivided into Phase I and Phase II biotransformation reactions. Phase I transformations include oxidative, reductive, and hydrolytic reactions, while Phase II reactions consist of conjugation processes or synthetic reactions involving the drug or its metabolites and common biochemical intermediates found in the body. These biotransformation reactions often cause biological inactivation, yet, activation is possible and it is naive to refer to these processes as detoxification mechanisms.

It is noteworthy that many of the type-reactions found in mammals are described as occuring in microorganisms. These include aromatic hydroxylations, *O*- and *N*-dealkylations, *S*-oxidations, keto-reductions, hydrolyses (esters, amides), quinoid oxidations and dehydrogenations. A comparison of these transformations in mammals and microorganisms has been described (2–6). Also, the biochemical basis for similarities in oxidative transformations have been reviewed (4–6). In this paper, we briefly review the similarities between microbial and mammalian aromatic hydroxylations, *O*-dealkylations and *N*-dealkylations. Following a description of these fundamental studies, we provide selected examples of application of M^4 investigations with drugs.

Aromatic Hybroxylations

Aromatic compounds are commonly converted into phenols by mammalian Phase I transformation reactions, and phenols are also produced as major microbial metabolites. Electron-rich aromatic rings are readily hydroxylated, while electron deficient systems are hydroxylated sparingly or not at all. Hydroxylation reactions occur at positions predicted by the concepts of electrophilic substitution (*i.e.*, aniline and anisole (13–16) are hydroxylated at *ortho*- and para-positions, while deactivated ring systems like benzoic acid and benzamide (16) are hydroxylated in the *meta*-position). In di- and tri-substituted systems, hydroxylation is directed to positions predicted by a summation of substituent effects (15,17).

Aromatic hydroxylation can be viewed as proceeding by a number of different mechanisms, but most available information suggests the involvement of an oxenoid mechanism with *arene*-oxide intermediates which rearrange nonenzymatically to phenolic metabolites (18). This process is usually accompanied by the so-called "NIH-Shift" (13,14,19–21), a mechanism involving the migration of a substituent at the site of hydroxylation to an adjacent carbon atom. The same enzymes forming arene oxides from aromatic substrates appear to be capable of forming epoxides from isolated and conjugated olefins (18).

In many biological systems, cytochrome P–450 monooxygenases appear to be linked to epoxide hydrolases which convert arene-oxide intermediates of compounds like naphthalene (18,22) into their corresponding *trans*-dihydrodiols. In fact, the occurence of *trans*-dihydrodiols represents strong evidence for the involvement of arene-oxides derived from monooxygenase oxidation of aromatic compounds. The *trans*-dihydrodiols may be converted further into catechol products by the

action of soluble hepatic dehydrogenases. Such catechol metabolites and their conjugates are important metabolic products of aromatic substrates like chlorobenzene.

Daly, Jerina and their associates have provided a number of useful gauges to measure the possible involvement of monooxygenases in aromatic hydroxylations by diverse metabolic systems. Lack of a primary isotope effect in enzymatic phenol formation (*i.e.*, with mixtures of deuterated and non-deuterated aromatic substrates), demonstration of the NIH shift, and *trans*-dihydrodiol formation are considered strong evidence for the presence of oxenoid monooxygenase activity (13,14,23). These features of monooxygenases have been extremely valuable in differentiating monooxygenases from dioxygenases in microbial systems.

Much recent work implicates arene-oxide intermediates in the metabolism of aromatic substrates, particularly by eucaryotic fungal species in pathways directly analogous to those found in mammals (2,3). The presence of microbial cytochrome P–450 monooxygenase enzyme systems has been clearly established in numerous cases (5). The first clear evidence that monooxygenases existed in fungi came in 1971 through the work of Auret *et al.* (24,25). Several microorganisms including *Aspergillus niger*, *Sporotrichum sulfurescens*, *Cunninghamella elegans*, *Mucor parasiticus*, *Rhizopus arrhizus*, *Helicostylum piriforme*, *Rhizopus stolonifer*, and *Curvularia falcata* displayed the NIH shift during aromatic hydroxylation with 4D- and 2D-anisoles as substrates. The magnitude of the NIH shift observed was consistent with that reported for hydroxylations of anisole by hepatic microsomes. The results, therefore, implicated the involvement of arene oxide intermediates.

Ferris *et al.* (26) examined the monooxygenase enzyme system of *Cunninghamella bainieri* (ATCC 9244).[2] With resting cells of this fungus, monooxygenase activity nearly paralleled that observed with hepatic monooxygenase systems. Naphthalene was converted to a *trans*-dihydrodiol demonstrating the presence of epoxide hydrolase. A mixture of *alpha*- and *beta*-naphthols was obtained in the ratio 9:1, and this result closely parallels that obtained with *in vitro* or *in vivo* mammalian systems (19). Both 2- and 4-hydroxylations of anisole as well as *O*-dealkylation were observed with this microorganism, but the ratios of products were different from those obtained with mammalian systems (15). The close metabolic parrallelism of this fungal system to the drug metabolizing system of liver microsomes was noted by the authors. Later studies by Ferris and coworkers (27) demonstrated that the enzyme system(s) involved in these metabolic transformations was a cytochrome P–450 system again analogous to that found in mammals. Gibson, Cerniglia *et al.* (28) have demonstrated the monooxygenase capabilities of a *Cunninghamella elegans* originally isolated in studies on crude oil degradation by marine fungi (28–31). This culture was capable of oxidizing substrates, including naphthalene and biphenyl, to a variety of phenolic products, and evidence was provided to suggest the involvement of a cytochrome P–450 monooxygenase system in this organism. Numerous other fungi were also found capable of metabolizing naphthalene (28).

In our earliest work (2,32), we studied the abilities of selected fungi to achieve aromatic hydroxylation of a number of aromatic substrates. Cultures which we selected included *Cunninghamella blakesleeana* (ATCC 8688a), *Aspergillus niger* (ATCC 9142), *Penicillium chrysogenum* (ATCC 10002), *Aspergillus ochraceous* (ATCC 1008), *Gliocladium deliquescens* (1086), *Streptomyces* species (1158w), *Rhizopus stolonifer* (NRRL 1477), *Curvularia lunata* (NRRL 2178), *Streptomyces rimosus* (ATCC 23955), *Cunninghamella bainieri* (ATCC 9244)[2], and *Helicostylum piriforme* (QM 6945). These organisms were chosen as potential microbial models of mammalian aromatic hydroxylation with a number of substrates, including: acetanilide, aniline, anisole, benzene, benzoic acid, biphenyl, chlorobenzene,

[2]This organism is currently classified as *Cunninghamella echinulata* (ATCC 9244).

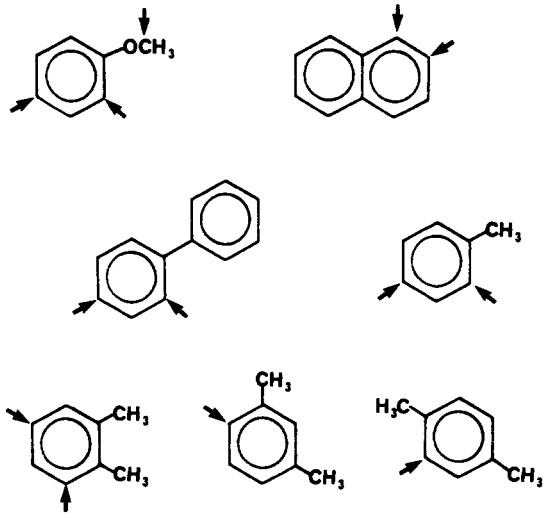


FIGURE 1. Mammalian and microbiological hydroxylations of aromatic substrates. Arrows indicate common sites of transformations.

coumarin, naphthalene, nitrobenzene, *trans*-stilbene, toluene and the three isomeric xylenes. The selection of substrates was based on the fact that each of the compounds had been studied in mammalian systems, and this permitted a comparison of mammalian and microbial aromatic hydroxylase activities. The aromatic substrates also represented the aromatic portions of numerous chemical entities of environmental importance and of drugs. Details of these experiments have been reviewed (2,3,32). In general, the degree of parallelism was high. Numerous phenolic metabolites were observed with nearly all substrates examined, and patterns of hydroxylation mimicked those seen in mammals (see figure 1).

The aromatic hydroxylations of biphenyl by fungi has been studied extensively by Smith *et al.* (33,34) and Gibson's group (31). Biphenyl is hydroxylated principally at the 4- and 4'-positions while small amounts of 2- and 3-hydroxy products are observed (31,33,34). One study (33) reported: exclusive 4-hydroxylation of biphenyl by *Cunninghamella echinulata* (ATCC 9244); complete conversion of biphenyl to 4,4'-dihydroxybiphenyl by *Aspergillus parasiticus* (ATCC 15517); exclusive 2-hydroxylation of biphenyl by *Helicostylum piriforme* (QM 6945). These results were obtained through isolation and complete structure elucidation and confirmed some earlier work which relied entirely on tlc for product confirmation (2).

The mechanism of 4-hydroxylation of biphenyl by *C. echinulata* (ATCC 9244) has been reported by Smith *et al.* (34). Gc-ms analyses indicated an absence of an isotope effect during hydroxylation of biphenyl-d_{10}. This prompted a study of a possible 1,2-hydride shift (NIH-shift) during hydroxylation which would implicate the intermediacy of an arene oxide. Therefore, the hydroxylation of biphenyl-2,2',3,3',4,4',5,5'-$^2H_8$(BP-d_8) was studied by gc-ms and ^{1}H-nmr. In one set of experiments, a 20.3% retention of ^{1}H was measured for the 4-hydroxy-product, whereas the 4,4'-dihydroxy-metabolite showed retentions of 37.6% (heptadeutero-product) and 2.8% (hexadeutero-product) as determined by gc-ms of heptafluorobutyrate derivatives of the phenols (9). It is noteworthy that the percent ^{1}H-retention in the 4,4-dehydroxy-product is close to calculated values based on sequential hydroxylations of the octadeutero-substrate (34). Overall, these data suggest that BP-d_8 undergoes an NIH shift during hydroxylation, thus implicating an intermediary arene oxide and monooxygenase activity in *C. echinulata* (ATCC 9244) (see figure 2). Since NIH shift studies performed with mammalian prepara-

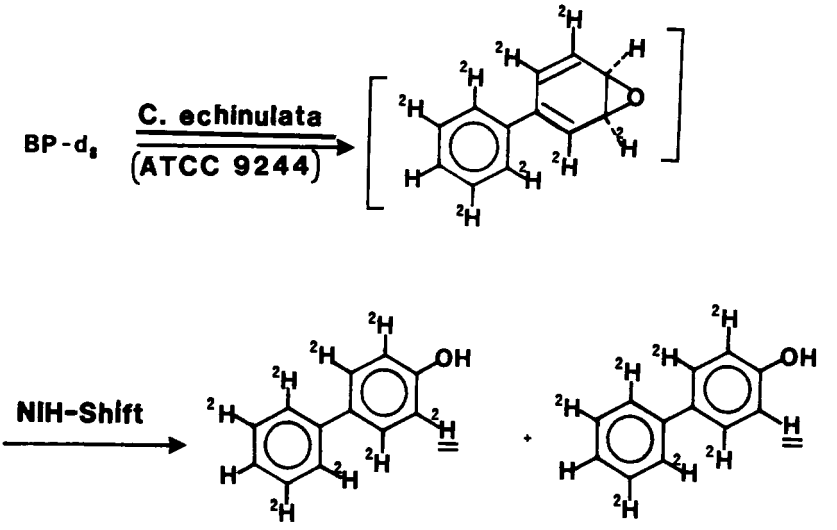


FIGURE 2. Hydroxylation of biphenyl-2,2',3,3',4,4',5,5'-$^2H_8$ (BP-d_8) by *C. echinulata* (ATCC 9244).

tions typically yield deuterium-retentions of 60 to 80% (14), the protium-retentions observed in our fungal studies are consistent with the arene oxide mechanism.

Final confirmation of an NIH shift was obtained after a preparative scale incubation of BP-d_8 with *C. echinulata* (ATCC 9244). A crystalline product was isolated that displayed mp, tlc, and gc behavior indicative of 4-hydroxybiphenyl (9). Mass-spectral analysis (solid probe) revealed a 23.3% rention of ^1H. ^1H-nmr analysis on a Fourier-transform 200 MHz instrument showed a rention of ^1H of 30.1% (34). These data confirmed the NIH shift and, to our knowledge, represents the first isolation of an NIH-product where isotope retention was determined by nmr.

O-Dealkylations

Alkyl-aryl ethers are often *O*-dealkylated in mammals. The reactions are commonly mediated by cytochrome P–450 monooxygenases, and the reactions proceed by attack of oxygen at the alkyl carbon *alpha* to the oxygen atom (35,36). As expected, the relative rates of *O*-dealkylation decrease as the size and/or complexity of the *O*-alkyl function increases (37,38). This relationship was first uncovered by an *in vitro* microsomal study with a large number of *p*-nitrophenyl ethers (38). In an analogous study with 4-alkoxybiphenyls, Davies and Creaven (39) found that the relative rates of *O*-dealkylation by rat liver microsomal preparations followed the order, methyl = ethyl > *n*-propyl > *n*-butyl. Interestingly, the same rank order of rates of *O*-dealkylation has also been reported with liver preparations from trout and frogs (40). Study of the *O*-dealkylation of alkyl-aryl ethers with alkyl functions larger than methyl can be complicated by hydroxylation at ω and ω-1 carbons producing aliphatic alcoholic metabolites.

Kirk and Lorenz (41) studied the metabolism of various 4-alkoxybenzoic acid derivatives by the wood fungus *Polyporus dichrous*. When incubated with 3-ethoxy-4-isopropoxybenzoic acid, dealkylation of the 4-isopropyl-group was observed along with aliphatic hydroxylation to form 2-[4-carboxy-2-ethoxyphenoxy]-propane-1-ol. Other wood-destroying fungi are known to O-dealkylate similar substrates. For example, *Polypous vesicolor* converts 4-hydroxy-3-methoxybenzoic acid to 3,4-dihydroxybenzoic acid (42,43) and *Fomes fomentarius* *O*-dealkylates 3,4-dimethoxybenzoic acid at the 4-position (44).

Recently, Smith *et al.* (45) completed a study of the *O*-dealkylation 4-alkoxy-

biphenyls by microorganisms. Forty-five fungi and actinomycetes were screened for their abilities to *O*-dealkylate 4-methoxybiphenyl. Of the 20 organisms with *O*-dealkylase activity, five were found to be most active, including: *Aspergillus flavus* (ATCC 24,741), *A. flavus* (ATCC 9170), *A. niger* (ATCC 10,548), *Penicillium brevi-compactum* (ATCC 10,418) and *P. claviforme* (UT/UI 376). These organisms were subsequently found to *O*-dealkylate a series of 4-alkoxybiphenyls to varying extents and the most active organism, *A. flavus* (ATCC 24,741), was chosen for rate studies. A prime consideration in this work was the limited aqueous solubilities of the proposed substrates: 4-methoxy-, 4-ethoxy-, 4-*n*-propoxy-, 4-isopropoxy-, and 4-*n*-butoxybiphenyl. Preliminary studies with a number of solubilizing agents revealed that 1% Triton X–100 affected a 100 μg/ml solubility of each substrate in water. Thus, solubility would not be a rate-limiting factor in *O*-dealkylation of the various substrates. Figure 3 presents data from a study of the relative rates and extents of *O*-dealkylation of 4-methoxy-, 4-ethoxy-, 4-*n*-propoxy-, 4-isopropoxy- and 4-*n*-butoxybiphenyl in 48 h Stage 2 cultures of *A. flavus* (ATCC 24,741). The values obtained for 4-methoxy- and 4-ethoxybiphenyl are of the same order of magnitude, while both of these substrates show significantly different conversions from those of the remaining more lipophilic substrates with bulkier alkyl groups. No attempt was made in this study to detect products of aliphatic hydroxylation of the higher alkyl ethers.

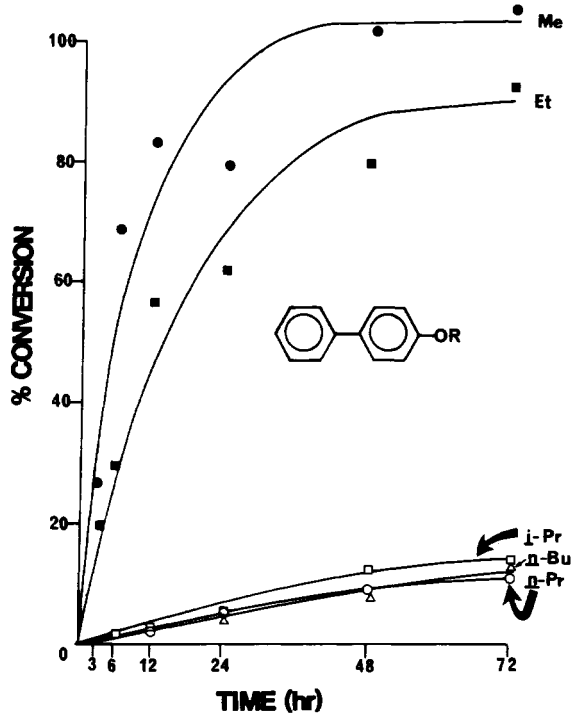


FIGURE 3. Time course of O-dealkylation of 4-alkoxybiphenyls by *A. flavus* (ATCC 24741).

The relative rate data derived from figure 3 is similar to that described in mammalian systems (38,39). Thus, the *O*-dealkylase(s) of *A. flavus* nicely parallels that observed in higher organisms.

N-Dealkylations

Various types of N-alkyl compounds are N-dealkylated in mammals. Most N-dealkylations are mediated by cytochrome P–450 monooxygenases, and the reactions proceed by attack of oxygen at a carbon *alpha* to the nitrogen atom

(46,47). Considerable evidence exists for a carbinolamine intermediate in the N-dealkylation process (48–50).

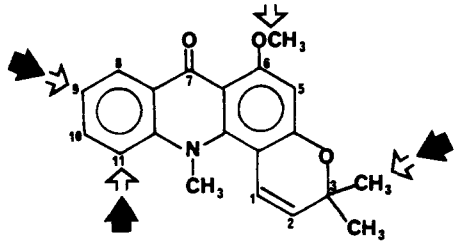
Rates of N-dealkylation are influenced by the basicity of the nitrogen and the size and/or complexity of alkyl function (3). For example, Abdel-Monem and Portoghese (51) found that the relative rates of rat liver microsomal N-dealkylations of a homologous series of N-alkylnormeperidines followed the order: methyl < ethyl < *n*-propyl > > *n*-butyl > *n*-pentyl≃n-hexyl through *n*-nonyl. Unfortunately, parallel types of studies have not been performed with microorganisms. There are, however, a number of isolated reports which suggest that N-dealkylation is a common transformation type in fungi. Included here are N-dealkylations observed with *Cunninghamella echinulata* (ATCC 9244) and substrates such as aminopyrine (26,27) and N-*n*-propylamphetamine (52).

APPLICATIONS

In the following sections, we provide selected examples of the M[4] approach in producing metabolites of drug substances. For each use, a brief comparison of the mammalian and microbial routes of metabolism is given in the text and in accompanying figures. In the figures noted, open arrows indicate positions where metabolism occurs in mammals; closed arrows denote sites of transformation in microorganisms.

Acronycine

Acronycine (**1**) is an alkaloid derived from the bark of *Achronychia Baueri* (see figure 4). Compound **1** is a yellow acridone derivative which possesses significant oral activity as an antitumor agent. Mammalian metabolic studies uncovered a number of hydroxylated metabolites including 9- and 11-hydroxy-



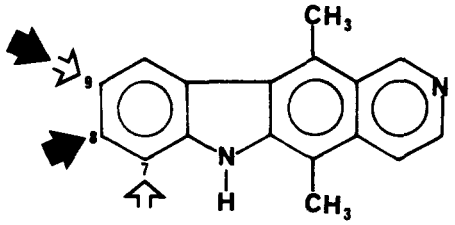
ACRONYCINE

FIGURE 4. Acronycine and its mammalian (⇨) and microbial (➡) transformation sites.

and 9,11-di- and 3,11-dihydroxyacronycines in several animal species. Microbial studies with **1** revealed microorganisms that gave high yields of the metabolites. *Cunninghamella echinulata* (NRRL 3655) provided a 30% yield of 9-hydroxyacronycine following incubation of **1** in a stirred fermentor (53). Brannon *et al.* (54) reported further microbial conversions of **1** including its hydroxylation at positions 3 and 11. All of the microbial metabolites were produced in sufficient quantities for biological evaluation, and none were as active as acronycine itself. The successful use of microbial transformations in both aliphatic and aromatic hydroxylation type reactions was cited (54).

Ellipticine

Ellipticine (**2**) is a brilliant yellow alkaloid found in many species of the plants *Ochrosia* and *Aspidosperma*. It possesses highly significant activity against several experimental tumors. In mammalian systems, 9-hydroxyellipticine is the major metabolite and 7-hydroxyellipticine is formed to a small extent (see figure 5).



ELLIPTICINE

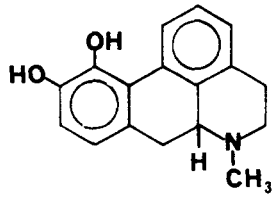
FIGURE 5. Ellipticine and its mammalian (⇨) and microbial (➡) transformation sites.

A study of the microbial transformation of **2** (55) led to *Aspergillus alliaceus* (NRRL 315) which provided the 9-hydroxy- and 8-hydroxy-metabolites of **3** in preparative yields of 40% and 10%, respectively. The 8-hydroxy-metabolite is yet to be found in mammals but has been proposed as a possible mammalian metabolite (6).

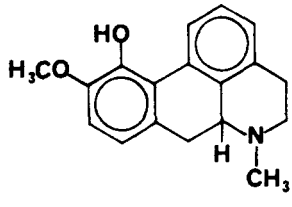
Both acronycine and ellipticine represent interesting examples of the usefulness of the M^4-approach in the preparation of products of aromatic hydroxylations.

Apocodeines

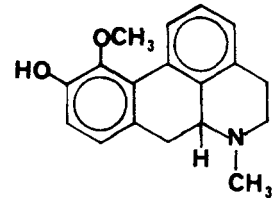
R-(−)-Apomorphine (**4**) (see figure 6) is used worldwide in neuropharmacological research as a prototypical dopaminergic agonist. Clinically, **4** has been proposed as a useful drug in the treatment of a variety of neurological disorders, including Parkinson's disease and schizophrenia (56–59).



APOMORPHINE



APOCODEINE



ISOAPOCODEINE

FIGURE 6. R-(−)-Apomorphine and its O-methyl metabolites, apocodeine and isoapocodeine.

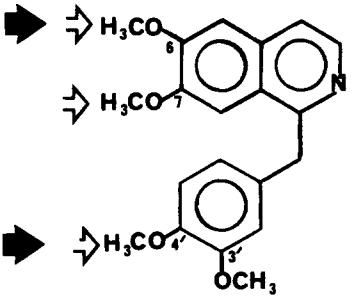
In mammalian systems, apomorphine undergoes methylation (60) and glucuronidation (61) primarily at the 10-position. A rat liver catechol *O*-methyltransferase preparation produced a mixture of the 10-methylated product apocodeine (**5**) and its 11-methylated isomer, isoapocodeine (**6**), in a ratio of 81:1 (60).

Apocodeine (**5**) is readily prepared by rearrangement of codeine (62) or by chemical *O*-demethylation of 10,11-dimethoxyaporphine (**7**) (63). In contrast, isoapocodeine (**6**) is much more difficult to prepare synthetically (60).

Rosazza *et al.* (64) studied the microbial *O*-demethylation of **7** by ten microorganisms. *Streptomyces griseus* (UI 1158) gave a mixture of **5** and **6** in 24% and 20% yields, respectively. Another organism, *Cunninghamella elegans* (ATCC 9245) appeared to regiospecifically convert **7** to **6**. Smith and Davis (65) studied the preparative utility of the latter transformation. Through studies of media composition, pH and incubation times, conditions were developed for the preparation of **6** in 59% yield (isolated product) from *C. elegans*. This work represents a novel microbial approach (*via* *O*-demethylation) to a mammalian metabolite resulting from the action of catechol O-methyltransferase on the catechol, **4**.

Papaverine

Papaverine (**8**) is used therapeutically in treating cerebral and peripheral ischemia. Mammalian metabolites of **8** include, 4'-desmethylpapaverine (**9**), 7-desmethylpapaverine (**10**), 6-desmethylpapaverine (**11**), and 4',6-didesmethylpapaverine (**12**) (see figure 7) (66–69). Interestingly, some of the *O*-demethylated metabolites are biologically active when tested at 3',5'-AMP phosphodiesterase inhibitors (70).



PAPAVERINE

FIGURE 7. Papaverine and its mammalian (⇨) and microbial (●) transformation sites.

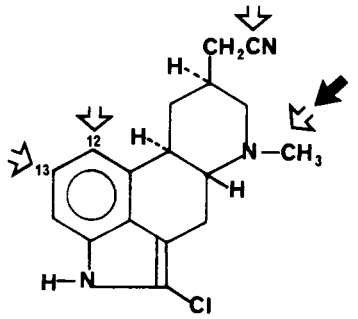
Rosazza *et al.* (71) studied the microbiological *O*-demethylations of papaverine. The M^4- approach was deemed potentially useful in preparing the desmethyl-metabolites since published synthetic schemes for compounds **9**, **10**, and **11** are lengthy or result in complicated mixtures of products (72,73).

In the microbial work (71), 60 organisms were screened for papaverine-*O*-demethylase activity and ten were found to actively metabolize the drug. Two *Aspergillus* species formed the 6-desmethyl-metabolite exclusively, while a number of *Cunninghamella* species appeared to primarily produce the 4'-desmethyl-compound (9). In a preparative-scale incubation, an *Aspergillus alliaceus* was used to produce gram quantities of **11**. Preparative amounts of **9** were produced with *Cunninghamella echinulata* (ATCC 9244) (71). Papaverine represents an interesting example of the versatility (*i.e.*, producing isomeric metabolites) and usefulness of the M^4-approach in the preparation of products of O-dealkylation.

Lergotrile

Clinical interest in lergotrile (**13**) stems from its putative dopaminergic activity and inhibition of prolactin secretion. Lergotrile was tested extensively in treatment of Parkinsonism, galactorrhea, acromegaly, and in reversing infertility in women (74–78); however, side-effects of orthostatic hypotension, mental change, and abnormal liver function were observed with its use. The hepatotoxicity was particularly disturbing and caused cancellation of clinical trials (78). This toxicity could be related to biotransformation(s) of **13** in man.

In guinea pigs, lergotrile is converted to desmethyl lergotrile (**14**). The drug is also hydroxylated at position 13 (see figure 8). The phenolic metabolite, 13-hydroxylergotrile, has also been found in humans, and the 12-hydroxy metabolite occurs in certain mammals (79).



LERGOTRILE

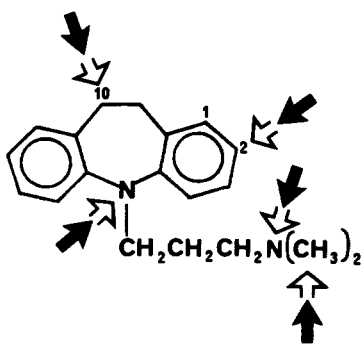
FIGURE 8. Lergotrile and its mammalian (⇨) and microbial (➡) transformation sites.

Davis *et al.* (80) screened over 30 organisms for their ability to produce metabolites of lergotrile. Five microorganisms were found to biotransform lergotrile, and *N*-desmethyl lergotrile was detected as the principal metabolite with most organisms. *Streptomyces platensis* (NRRL 2354) formed the metabolite in highest yield, and a preparative-scale conversion was accomplished with a recovered yield of 50% (80). All attempts to produce phenolic metabolites of **13** have thus far failed, perhaps due to the deactivation of the indole moiety by the 2-chloro-group.

Imipramine

The tricylic antidepressant, imipramine (**15**), is used widely in clinical medicine. In mammals, **15** is converted to a variety of metabolites including 2-hydroxyimipramine (**16**), 10-hydroxyimipramine (**17**), iminodibenzyl (**18**), imipramine-*N*-oxide (**19**) and desipramine (**20**) (see figure 9) (81–85).

Hufford *et al.* (86) studied the microbial metabolism of **15**. They described 14 organisms capable of metabolizing the drug, and four organisms were chosen for preparative experiments. *Cunninghamella blakesleeana* (ATCC 8688a) produced low yields of 2-hydroxy- and 10-hydroxyimipramine. *Fusorium oxysporum f. sp. cepae* (ATCC 11711) gave a 28% yield of iminodibenzyl in 14 days, while *Aspergillus*



IMIPRAMINE

FIGURE 9. Imipramine and its mammalian (⇨) and microbial (➡) transformation sites.

flavipes (ATCC 16795) affected a 13% conversion to imipramine-N-oxide after a 13-day incubation. *Mucor griseocyanus* (ATCC 1207a) was found to produce three metabolites after 20 days of incubation with **15**. These compounds were isolated by column chromatography and identified as 10-hydroxyimipramine, imipramine *N*-oxide, and desipramine, of which the latter was not produced in other preparative experiments.

The work with imipramine (86) supports the versatility of microbial models methodology in producing several different types of metabolites. Studies with lergotrile (80) and imipramine (86) represent interesting examples of the usefulness of M^4 approach in the preparation of products of *N*-dealkyation.

SUMMARY AND CONCLUSIONS

A solid basis for the M^4- approach has been developed over the past 10 years. Recent examples of the production of difficult-to-synthesize mammalian metabolites through microbial transformations attest to the utility of the methodology. There is, however, much more to be done. Model studies should be conducted to test parallels between microbial and mammalian *S*- and *N*-oxidations, *O*-glucuronidations, and ester and amide hydrolyses. Subsequently, even greater applications of M^4- work can be envisioned.

We have been pleased to see our colleagues in industry and academia adopt the M^4- approach to solve difficult pharmacological and toxicological problems. In large measure, this has been our greatest reward for efforts initially presented before the membership of the American Society of Pharmacognosy in 1973.

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