Metabolism of (±)-N-(n-Propyl)Amphetamine by
Cunninghamella echinulata

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(±)-N-(n-propyl)amphetamine (I), a secondary amine, was readily metabolized by Cunninghamella echinulata. The products included known C- and N-oxygenated mammalian metabolites as well as N-acetylamphetamine and were identified by gas chromatography and mass spectrometry.

In recent reviews on microbial transformations (1, 5, 15–18), numerous microorganisms have been identified which metabolize various drugs and xenobiotics to products that are also formed by animal species. Most of the metabolites formed by microorganisms are the products of C-oxidation of the substrate, but examples of microbial N-oxidation have also been reported. Some primary aromatic amines are known to be metabolized to nitro compounds via hydroxylamine intermediates (18) and tertiary amines are readily converted to their N-oxides (18), but to our knowledge no reports have been made of fungal microbial oxidation of secondary aliphatic amines. It was of interest to us, therefore, to determine whether a microorganism could be found which would metabolize the secondary amine (±)-N-(n-propyl)amphetamine (NPA; I) (Fig. 1) in a manner comparable to mammalian metabolism.

Mammalian metabolism of N-alkylamphetamines is species dependent, and products formed in in vivo studies differ from those formed in vitro (8–11). The supernatant fraction of rat liver homogenate converted NPA to six products (9), which were the results of C-oxidation (IIa, IIb, and IIIa) and N-oxidation (IVA, IVb, and V), but no p-hydroxylated products (Ib) were formed. In contrast, in vivo metabolism of NPA in rats (10) resulted in C-oxidation (Ib, IIb, and IIIa) but not N-oxidation.

The objective of this study was to determine whether microbial metabolism of NPA could mimic in vivo or in vitro mammalian metabolism.

MATERIALS AND METHODS

Fermentation procedure. Cultures were maintained on Sabouraud dextrose agar slants at 4°C. Spores from the slants were used to inoculate the medium, which had the following composition: trypticase soy broth (BBL), 30 g; glucose, 15 g; KH₂PO₄, 7 g; yeast extract, 5 g; distilled water, to 1,000 ml; adjusted to pH 7.4 with solid KOH. The medium was sterilized at 121°C for 15 min before use.

Fermentations were conducted on a gyrotary shaker (model G-2, New Brunswick Scientific Co.) operating at 250 rpm at 27°C in 125-ml Erlenmeyer flasks containing 25 ml of medium. Thick 3-day culture was homogenized and added to fresh medium as a 10% inoculum. NPA-HCl was added after 24 h of growth to give a final concentration of 0.24 mg/ml. Controls consisted of cultures grown without NPA and sterile medium with NPA.

Sampling procedure. Samples of the fermentation broths were withdrawn at various time intervals for analysis and treated in one of two manners depending on the stability of the metabolites. Broth sampled for N-oxygenated metabolites was adjusted to pH 7.5 and extracted three times with 5 volumes of a mixture of freshly distilled diethyl ether-methylene chloride (55:45, vol/vol), and the extracts were combined. The remaining metabolites were extracted from alkaline (solid potassium carbonate) broth with the same solvent mixture. Each combined solvent extract was concentrated to about 50 µl under nitrogen at 45°C before gas-liquid chromatography (GLC) analysis.

Instrumentation. GLC analysis was performed on a Hewlett-Packard model 5700A gas chromatograph equipped with a flame ionization detector. GLC/mass spectrometry (MS) analysis was carried out in a Hewlett-Packard model 5710A gas chromatograph coupled to a Hewlett-Packard model 5981A mass spectrometer.

Gas chromatography. Two different columns were used for analysis: (i) column A—5% OV-101 on Chromosorb 750 (80/100 mesh) in a glass column, 4 mm in ID and 150 cm in length; column B—1% Carbowax 20M on Chromosorb 750 (80/100 mesh) in a glass column, 4 mm in ID and 120 cm in length. Constant operating conditions were: carrier gas (He), 60 ml/min; flow, 40 ml/min; air, 240 ml/min; injector port and detector temperatures, 250°C.

Retention times are given in minutes, and oven temperatures are listed in Table 1.
Combined GLC/MS. The columns used for GLC were used for combined GLC/MS, with He, 60 ml/min, as the carrier gas. The injection port and transfer line temperature was 250°C. The operating conditions for the MS were: electron energy, 70 eV; emission ion current, 35 mA; source temperature, 180°C; separator temperature, same as oven temperature.

Substrates and reference compounds. NPA-HCl, mp 155 to 157°C, was prepared and purified by a reported method (9). Syntheses of C- and N-oxygenated metabolites have been reported previously (2, 3, 10). N-acetylamphetamine was formed by the addition of 1.2 M acetic anhydride to amphetamine base in ether at 4°C and recrystallized from ether-n-pentane, mp 93 to 95°C. Phenylethylamine was purchased from Aldrich Chemical Co., Inc., and 1-phenyl-2-propanol was obtained from Fluka AG.

RESULTS AND DISCUSSION

Small-scale screening experiments were conducted with Aspergillus niger ATCC 9142, A. ochraceus ATCC 1008, Cunninghamella echinulata ATCC 3655 and 9244, and Streptomyces platensis ATCC 13865. Metabolism of NPA by C. echinulata ATCC 3655 and 9244 produced 10 metabolites which have been identified. With one exception (IIc), all are known mammalian metabolites of NPA (8, 10). This observation is in agreement with that of Ferris and colleagues (12), who concluded from their studies that no other fungal or bacterial system exhibited such a metabolic similarity to the liver microsomal metabolizing system as C. echinulata ATCC 9244. This organism was chosen for further studies. The GLC trace (Fig. 2) of an extract of a 6-day incubation contained several peaks not seen in the traces of the controls. The MS of each peak was recorded. Comparison of GLC retention times and the MS traces of metabolites (A through I, Table 1) with those of authentic standards enabled us to identify these compounds.

Compounds F, G, H, and J were the N-oxygenated products N-hydroxyamphetamine (IVA), N-(n-propyl)hydroxyamphetamine (IVb), 1-phenyl-2-propanone oxime (IIb), and N-(1-benzylethyl)-a-ethyl nitrotr (V), respectively. These metabolites have been observed in vitro mammalian studies (9). Hydroxylamines are extremely labile under alkaline conditions (4). To minimize possible decomposition, the fermentation broth was buffered to pH 7.4 and metabolites were extracted for analysis at that pH value. GLC conditions were also selected to minimize on-column degradation of hydroxylamines (IVA and IVb) to the oxime (IIb) or the nitrotr (V) (4). In a control experiment using identical GLC conditions, hydroxylamine (IVb) degraded to a small extent (5%) to the nitrotr (V), and 35% of hydroxylamine (IVA) degraded to the oxime (IIb). The relative amounts of IIb and IVa

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**TABLE 1. Metabolites of NPA—GLC retention times (Rₜ) and MS data**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Column A Rₜ</th>
<th>Column B Rₜ</th>
<th>MS data [m/e (% relative abundance)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.5¹</td>
<td>108(41), 91(18), 79(100), 77(62), 51(21)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.99</td>
<td>0.66</td>
<td>91(8), 65(5), 44(100)</td>
</tr>
<tr>
<td>C¹</td>
<td>2.11</td>
<td>0.77</td>
<td>91(13), 86(100), 65(2), 44(10)</td>
</tr>
<tr>
<td>D</td>
<td>1.40⁵</td>
<td>134(47), 92(43), 91(100), 65(30), 43(33)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>2.03⁵</td>
<td>136(2), 92(100), 91(82), 65(25), 45(45)</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>4.94</td>
<td>149(3), 91(76), 65(20), 60(100)</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>4.33</td>
<td>5.66</td>
<td>118(15), 102(100), 91(53), 86(37), 60(50)</td>
</tr>
<tr>
<td>H</td>
<td>5.96</td>
<td>7.18</td>
<td>149(47), 116(47), 91(100), 65(37)</td>
</tr>
<tr>
<td>J</td>
<td>5.96</td>
<td>9.73</td>
<td>118(93), 117(44), 100(9), 91(100), 84(28)</td>
</tr>
<tr>
<td>K</td>
<td>4.33</td>
<td>15.06</td>
<td>177(1), 118(65), 91(35), 86(100), 44(18)</td>
</tr>
<tr>
<td>L</td>
<td>7.13</td>
<td>107(11), 86(100), 77(10), 44(34)</td>
<td></td>
</tr>
</tbody>
</table>

¹ GLC columns are described in the text; column A was operated at 155°C and column B was operated at 145°C except where otherwise noted.

⁵ 125°C.

¹ NPA.
Conclusion is consistent with the finding that metabolic p-hydroxylation predominates in fungal systems (12).

Cytochrome P-450 monooxygenases mediate N-dealkylation and aromatic hydroxylations in mammalian metabolism (18), and since cytochrome P-450 has been isolated from C. echinulata ATCC 9244 (13), it is probably involved in formation of amphetamine and 4-hydroxy-N-(n-propylamphetamine in the present study.

Three minor metabolites of NPA were identified on the basis of their GLC and MS properties. Metabolite A was benzyl alcohol (VI), metabolite D was the deamination product 1-phenyl-2-propanone (IIIa), and metabolic E was the corresponding alcohol 1-phenyl-2-propanol (IIb).

Compounds A, B, D, E, F, G, H, J, and L are known mammalian metabolites of NPA, but metabolite K is not (8–10). Diagnostic ions in the spectrum of K were located at m/e 177 (molecular ion), m/e 118 (Ph—CH==CHCH₃)²⁺, m/e 91 (C₄H₆)⁺, and m/e 86 (CH₃CH==NHCOCH₃⁺) (Fig. 3). The production of these ions is consistent with N-acetylamphetamine (11c). Authentic N-acetylamphetamine was synthesized and had GLC and MS properties identical to those of metabolite K. Microbial N-acetylation of tryptamine (14), aniline (17), and other compounds (5) has been documented.

This report represents another example of a

![GLC trace](image-url)

**Fig. 2.** GLC trace (system B; oven, 145°C) of metabolites of NPA by C. echinulata ATCC 9244. *Unidentified metabolite.

(Fig. 2, peaks H and F) and of V and IVb (Fig. 2, peaks J and G) detected in the present study suggest that some of the oxime and nitrone detected was formed metabolically.

Compound B was the major metabolite, based on its GLC peak area, and was readily identified as the N-dealkylated compound amphetamine (IIa), which is also a major metabolite in rat liver homogenates (9). Microbial N-dealkylation of secondary amines is well documented (6, 7, 16, 18). Interpretation of the MS of metabolite L indicated that it was a monoxygenated derivative of the NPA substrate. The site of the oxygenation was deduced to be the benzylic group of the NPA molecule since the base peak in the spectrum was at m/e 86 (CH₃CH==NHCH₂CH₂CH₃)⁺; this ion is also present in the MS of NPA. The ion at m/e 91 (C₄H₆)⁺ in the NPA spectrum, however, was absent from the spectrum of metabolite L, having been replaced by a fragment at m/e 107 (C₃H₇O)⁺. Metabolite L was identified as 4-hydroxy-N-(n-propyl)amphetamine (Ib), which is also a major in vivo metabolite in rats (10).
similarity between mammalian and microbial metabolism. It is believed that it describes the first example of fungal N-oxidation of a secondary amine.

ACKNOWLEDGMENT

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LITERATURE CITED


