Appl Microbiol Biotechnol (1999) 52: 553–557

ORIGINAL PAPER

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Biotransformation of *N*-acetylphenothiazine by fungi

Received: 15 January 1999 / Received revision: 7 May 1999 / Accepted: 21 May 1999

Abstract Cultures of the fungi *Aspergillus niger*, *Cunninghamella verticillata*, and *Penicillium simplicissimum*, grown in a sucrose/peptone medium, transformed *N*-acetylphenothiazine to *N*-acetylphenothiazine sulfoxide (from 13% to 28% of the total) and phenothiazine sulfoxide (from 5% to 27%). Phenothiazin-3-one (4%) and phenothiazine *N*-glucoside (4%) were also produced by *C. verticillata*. The probable intermediate, phenothiazine, was detected only in cultures of *P. simplicissimum* (6%).

Introduction

Phenothiazine and its derivatives, many of which are valuable as psychiatric and cardiovascular drugs, are metabolized by several different pathways in mammalian systems (Vikhlyaev and Zherdev 1975; Mitchell and Waring 1979; Richards et al. 1997). Microbial models have been used in studies of the biotransformation of these and other drugs; for instance, *Cunninghamella elegans* metabolizes the *N*-alkylated phenothiazines, chlorpromazine and methdilazine, to several different products (Zhang et al. 1996). Using *N*-acetylphenothiazine as a model compound, we have continued to study the influence of *N*-substituents on the fungal biotransformation of phenothiazines.

Phenothiazines with *N*-carbonyl substituents, including moricizine, chloracizine, fluacizine, and ethacizine, have been used as antiarrhythmic drugs, coronary vasodilators, and antidepressants (Vikhlyaev et al. 1974; Buryi et al. 1986; Clyne et al. 1992; Kaverina and Sokolov 1992). We investigated *N*-acetylphenothiazine because it "can be considered as a model compound for the phenothiazines having a carbonyl function next to the nitrogen atom" (Roseboom and Förch 1979). Three strains of fungi transformed this compound to five metabolites; these fungi may be useful in studies of the bioconversion processes of drugs in mammalian systems.

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Materials and methods

Aspergillus niger VKM F-1119 was from the All-Russian Microorganism Collection, *Cunninghamella verticillata* VKPM F-430 from the All-Russian Collection of Industrial Microorganisms, and *Penicillium simplicissimum* KM-16 from the Biological Faculty of Moscow State University. Stock cultures were maintained on glucose/malt-extract/peptone/agar slants.

For inoculum, cultures on agar slants were incubated for 10–14 days at 28 °C. The spores were suspended in 5 ml sterile water and used to inoculate 500-ml flasks, each with 100 ml liquid medium containing (per liter): 30 g sucrose, 5.0 g peptone, 3.0 g NaCl, 3.0 g NaH_2PO_4, 3.0 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.1 g $FeSO_4$, 1 mg $MnSO_4$, and 1000 ml deionized water. The pH was adjusted to 5.0. The fungi were grown at 28 °C with rotary shaking at 200 rpm. After 24 h, 20 mg *N*-acetylphenothiazine for each culture was dissolved in 1 ml of *N*,*N*-dimethylformamide and filter-sterilized. The cultures were dosed and then incubated for an additional 72 h at 28 °C with shaking at 200 rpm.

The mycelia were collected by paper filtration. The filtrates were brought to pH 7.0 and extracted with three equal volumes of chloroform, which then was evaporated in vacuo.

The extracts were analyzed by thin-layer chromatography (TLC), using silica gel plates (E. Merck; 0.25 mm) developed in hexane/ethyl acetate/methanol (10:10:2) (Modyanova et al. 1999). Metabolites were separated by reversed-phase high-performance liquid chromatography (HPLC), using a Shimadzu (Kyoto, Japan) LC-600 liquid chromatograph with a PD-6AV photodiode array detector and a Phenomenex (Torrance, Calif.) Prodigy 5 μm ODS-3 column (4.6 × 250 mm). They were eluted with a 20-min gradient of 50%–90% methanol in buffer (50 mM ammonium acetate, pH 5.5) followed by 90% methanol for 10 min. The flow rate was 1 ml/min.

After purification by HPLC, the metabolites were concentrated in vacuo to dryness. Metabolites were identified by the retention times and the UV/visible, mass, and ^{1}H-NMR spectra. They were quantified by the HPLC peak areas at 231 nm, which is a λ_{max} for *N*-acetylphenothiazine.

Direct-exposure-probe/electron-ionization (DEP/EI) and gas-chromatography/electron-ionization (GC/EI) analyses were performed on a Finnigan (San Jose, Calif.) TSQ 700 mass spectrometer in the single quadrupole mode. The DEP was heated with a linear current ramp of 10 mA/s to 800 mA. The quadrupole (Q1) was scanned from *m/z* 50–500 with a 0.5-s cycle time. The ion source temperature and electron voltage for GC/EI mass spectrometry were 175 °C and 70 V respectively.

^{1}H nuclear magnetic resonance (NMR) spectral analyses were performed at 500.13 MHz on a Bruker (Billerica, Mass.) AM500 NMR spectrometer at 28 °C. Metabolites were dissolved in [$^2H_6$]acetone or [$^2H_2$]dichloromethane; chemical shifts are reported on the ppm scale by assigning the residual solvent signals as 2.04 and 5.32 respectively. First-order coupling constants are reported in hertz; non-first-order coupling constants and those of overlapping resonances are not reported. Proton assignments are based on chemical shifts, integration, homonuclear decoupling measurements, and nuclear Overhauser enhancement (NOE) experiments.

N-Acetylphenothiazine (Fig. 1, **I**) was synthesized by heating phenothiazine (Sigma Chemical Co., St. Louis, Mo.) in the presence of excess acetic anhydride and extracting the product with dichloromethane (Blau 1993). The product eluted from the HPLC column at 18.7 min. The GC/EI mass spectrum (Table 1) had ions at *m/z* 241 [$M^{+}\cdot$], 200, 199 [$M^{+}\cdot-42$], 198 [$M^{+}\cdot-43$], 167, and 154. The fragment ion at *m/z* 199 resulted from the loss of ketene ($CH_2{=}C{=}O$) from the molecular ion (*m/z* 241), which is a typical loss for compounds with an acetyl moiety (Hallberg et al. 1984). The ^{1}H NMR spectrum (Table 2) consisted of four aromatic resonances, shifted downfield from those of phenothiazine, and a three-proton singlet in the aliphatic region. The absence of proton 10 and its chemical shift indicated that the singlet represented an *N*-acetyl group. Selective saturation of the singlet resulted in an NOE to the resonance at 7.62 ppm (H1 and H9) and allowed the assignment of the other aromatic resonances by homonuclear decoupling experiments. The purity of *N*-acetylphenothiazine (**I**) was 98% as shown by HPLC, and 95% as shown by mass spectrometry. Since the residual phenothiazine content was negligible, the synthetic product was used without further purification.

Results

HPLC analysis of the extracts from cultures dosed with *N*-acetylphenothiazine (Fig. 1) showed that *A. niger*, *C. verticillata*, and *P. simplicissimum* transformed *N*-acetylphenothiazine (**I**) to several metabolites (Table 3). The apparent metabolites were collected from multiple HPLC injections for further analysis.

Metabolite **II**, which was produced from *N*-acetylphenothiazine (**I**) by all three of the fungi, eluted from the HPLC column at 7.7 min. The GC/EI mass spectrum (Table 1) had ions at *m/z* 257 [$M^{+}\cdot$], 241 [$M^{+}\cdot-16$], 215 [$M^{+}\cdot-42$], 199 [$M^{+}\cdot-16-42$], 198 [$M^{+}\cdot-16-43$], 186, and 167, corresponding to *N*-acetylphenothiazine sulfoxide. The fragment ions at *m/z* 241 and 215 resulted from the losses of oxygen and ketene, respectively, from the molecular ion, and the fragment ion at *m/z* 199 resulted from the combined losses of oxygen and ketene. The fragment ion at *m/z* 198 resulted from the combined losses of oxygen and the acetyl ($CH_3{-}C{=}O$) radical. These losses are consistent with the presence of acetyl and sulfoxide moieties. The numbers and types of resonances in the ^{1}H NMR spectrum of metabolite **II** (Table 2) were the same as those of **I**, except that they were shifted downfield, which is also consistent with the identification of *N*-acetylphenothiazine sulfoxide.

Metabolite **III**, produced by all three fungi dosed with *N*-acetylphenothiazine, eluted from the HPLC column at 11.1 min. The GC/EI mass spectrum (Table 1), which had ions at *m/z* 215 [$M^{+}\cdot$], 199 [$M^{+}\cdot-16$], 198, 186, 167 [$M^{+}\cdot-48$], 166, and 154, was similar to that of phenothiazine sulfoxide (Mitchell and Waring 1979). The fragment ion at *m/z* 199 resulted from the loss of oxygen from the molecular ion (*m/z* 215); the fragment ion at *m/z* 167 may have resulted from the loss of SO from the molecular ion. Both losses are common for sulfoxide compounds. In the ^{1}H-NMR spectrum (Table 2), differences between the chemical shifts of the aromatic protons of metabolite **III** and those of metabolite **II** were evident. Most important were the absence of an aliphatic singlet and the presence of a broad singlet at 10.00 ppm. Selective saturation of the broad singlet resulted in an NOE to H1 and H9 (7.38 ppm). Since the aromatic resonances were still downfield from those of phenothiazine and the proton

Table 1 Analytical data for *N*-acetylphenothiazine and metabolites produced by fungi. R_F values were obtained by thin-layer chromatography on silica gel plates developed in hexane/ethyl-acetate/methanol (10:10:2). All mass spectra were obtained by GC/EI-MS, except for metabolite **IV**, which was analyzed by DEP/EI-MS

Compound	R_F	UV λ_{max} (nm)	Electron ionization mass spectrum, *m/z* (relative intensity, %)
N-Acetylphenothiazine (**I**)	0.91	231, 263	241 [$M^{+}\cdot$] (23), 200 (15), 199 (100), 198 (68), 171(5), 167 (17), 166 (8), 154 (10), 140 (5), 127 (5)
N-Acetylphenothiazine sulfoxide (**II**)	0.76	217, 248, 278, 307	257 [$M^{+}\cdot$] (1), 241 (2), 215 (26), 199 (24), 198 (100), 186 (10), 167 (11), 154 (6)
Phenothiazine sulfoxide (**III**)	0.51	225, 273, 307, 344	215 [$M^{+}\cdot$] (40), 199 (100), 198 (13), 186 (41), 167 (79), 166 (19), 154 (10)
Phenothiazine *N*-glucoside (**IV**)	0.29	244, 302	361 [$M^{+}\cdot$] (3), 241 (3), 211 (2), 201 (5), 199 (100), 198 (13), 127 (14), 113 (11), 111 (12), 97 (23), 84 (12), 83 (23), 73 (30), 71 (10), 69 (23), 57 (22), 56 (18), 55 (51)
Phenothiazin-3-one (**V**)	0.92	236, 272, 368, 504	214 (11), 213 [$M^{+}\cdot$] (100), 185 (74), 154 (42), 70 (11)
Phenothiazine (**VI**)	0.96	255, 321	200 (13), 199 [$M^{+}\cdot$] (100), 198 (11), 167 (53), 154 (7), 100 (11)

Table 2 ^{1}H-NMR spectral parameters of *N*-acetylphenothiazine and metabolites produced by fungi. All spectra were recorded in [$^2H_2$]acetone except where otherwise noted. The numbered column headings refer to positions assigned to the atoms

Compound	Chemical shift (ppm)									
	1	2	3	4	6	7	8	9	10	CH_3
N-Acetylphenothiazine[a] (**I**)	7.62	7.38	7.29	7.51	7.51	7.29	7.38	7.62		2.14
N-Acetylphenothiazine sulfoxide[b] (**II**)	7.85	7.62	7.56	7.82	7.82	7.56	7.62	7.85		2.32
Phenothiazine sulfoxide[c] (**III**)	7.38	7.56	7.21	7.89	7.89	7.21	7.56	7.38	10.00	
Phenothiazine *N*-glucoside[d] (**IV**)	7.24	7.06	7.22	7.34	7.34	7.22	7.06	7.24		
Phenothiazin-3-one[e] (**V**)	7.63	6.86		6.76	7.89[f]	7.58	7.58	7.66[f]		
Phenothiazine[g] (**VI**)	6.70	6.97	6.77	6.92	6.92	6.77	6.97	6.70	7.77	

[a] Coupling constants: $J_{1,2} = 8.0$, $J_{1,3} = 1.5$, $J_{2,3} = 7.5$, $J_{2,4} = 1.5$, $J_{3,4} = 7.5$
[b] Coupling constants: $J_{1,2} = 8.0$, $J_{1,3} = 1.3$, $J_{2,3} = 7.7$, $J_{2,4} = 1.7$, $J_{3,4} = 7.5$
[c] Coupling constants: $J_{1,2} = 8.2$, $J_{1,3} = 1.1$, $J_{2,3} = 7.7$, $J_{2,4} = 1.5$, $J_{3,4} = 7.5$
[d] Spectrum was recorded in [$^2H_2$]dichloromethane. Chemical shifts (ppm) of the glucose moiety: H1′ = 4.88, H2′ = 3.98, H3′ = 3.65, H4′ = 3.71, H5′ = 3.65, H6a′ = 3.95, H6b′ = 4.05. Coupling constants: $J_{1,3} = 1.3$, $J_{2,3} = 7.5$, $J_{2,4} = 1.1$, $J_{3,4} = 7.7$
[e] Coupling constants: $J_{1,2} = 9.9$, $J_{2,4} = 2.2$
[f] Assignments may be reversed
[g] Coupling constants: $J_{1,2} = 8.0$, $J_{1,3} = 1.3$, $J_{2,3} = 7.7$, $J_{2,4} = 1.3$, $J_{3,4} = 7.5$

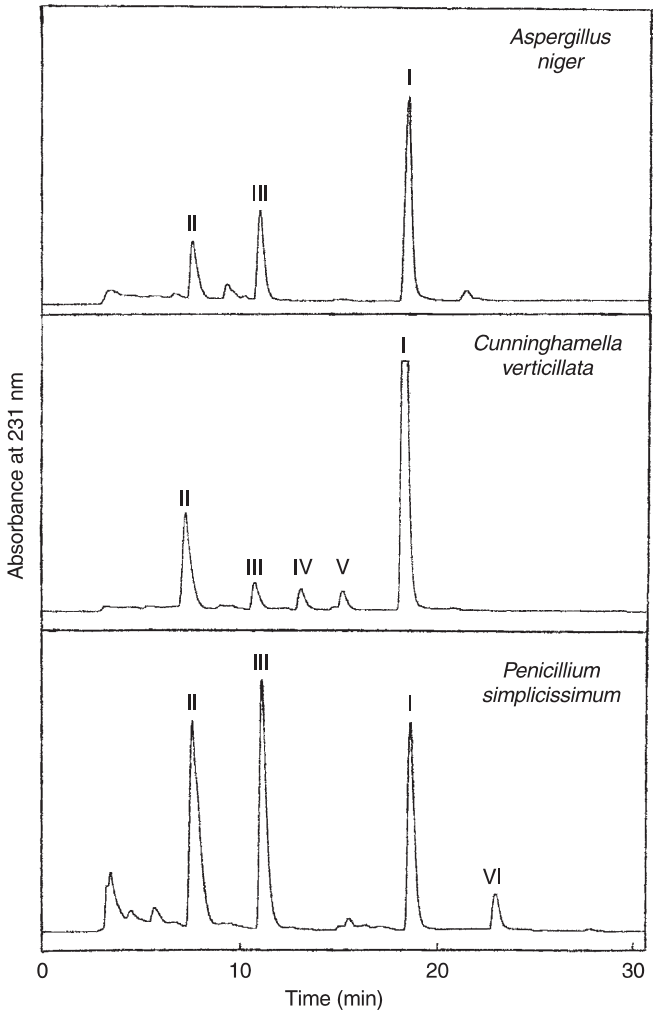

Fig. 1A–C HPLC chromatograms obtained at 231 nm showing the biotransformation of *N*-acetylphenothiazine by (**A**) *Aspergillus niger*, (**B**) *Cunninghamella verticillata*, and (**C**) *Penicillium simplicissimum*. (***I*** *N*-acetylphenothiazine, ***II*** *N*-acetylphenothiazine sulfoxide, ***III*** phenothiazine sulfoxide, ***IV*** phenothiazine *N*-glucoside, ***V*** phenothiazin-3-one, ***VI*** phenothiazine)

was present on the nitrogen, consistent with the mass spectrum, this metabolite was identified as phenothiazine sulfoxide.

Metabolite **IV**, produced only by *C. verticillata*, eluted from the HPLC column at 12.9 min. The DEP/EI mass spectrum (Table 1) had ions at m/z 361 [$M^{+\cdot}$], 199, 198, etc. The fragment ion at m/z 199 resulted from the loss of a sugar moiety from the molecular ion (m/z 361) by either a thermal or an ionization process. The ^{1}H-NMR spectrum of metabolite **IV** (Table 2) showed seven protons between 3.65 ppm and 4.06 ppm with coupling patterns similar to those of a glucosyl moiety. All of the aromatic protons were present except for proton 10, indicating that it had been replaced by the glucosyl moiety. Metabolite **IV** thus was identified as a conjugate, phenothiazine *N*-glucoside.

Metabolite **V**, produced only by *C. verticillata*, eluted from the HPLC column at 15.8 min. The GC/EI mass spectrum (Table 1) had ions at m/z 214, 213 [$M^{+\cdot}$], 185 [$M^{+\cdot}-28$], 154, and 70. The fragment ion at m/z 185 resulted from the loss of carbon monoxide from the molecular ion (m/z 213), which would be expected for an aromatic carbonyl moiety. The ^{1}H-NMR spectrum of metabolite **V** (Table 2) consisted of seven aromatic protons, indicating ring substitution. The upfield shifts of two protons (6.76 ppm and 6.86 ppm) and their coupling patterns (*meta*-coupled doublet and doublet of doublets respectively) indicated that the substitution was either at H2 or H3 and contained an oxygen atom. Oxidation of C3 to a keto group, with rearrangement of the double bonds to retain conjugation, accounts for the absence of proton 10. Metabolite **V** thus was identified as phenothiazin-3-one.

Metabolite **VI**, which was detected only in cultures of *P. simplicissimum*, eluted from the HPLC column at 23.0 min. The GC/EI mass spectrum (Table 1), which had ions at m/z 200, 199 [$M^{+\cdot}$], 198, 167 [$M^{+\cdot}-32$], 154, and 100, was identical to that of phenothiazine. The fragment ion at m/z 167 resulted from the loss of sulfur

Table 3 Percentages of different metabolites produced from *N*-acetylphenothiazine by fungi. The peak area for residual *N*-acetylphenothiazine (**I**) was 63% of the total for *Aspergillus niger*, 70% for *Cunninghamella verticillata*, and 39% for *Penicillium simplicissimum*

Fungus	Relative peak area of metabolite (231 nm)				
	N-Acetylphenothiazine sulfoxide (**II**)	Phenothiazine sulfoxide (**III**)	Phenothiazine *N*-glucoside (**IV**)	Phenothiazin-3-one (**V**)	Phenothiazine (**VI**)
A. niger	13	24	0	0	0
C. verticillata	17	5	4	4	0
P. simplicissimum	28	27	0	0	6

from the molecular ion (*m*/*z* 199). The ^{1}H-NMR spectrum of metabolite **VI** (Table 2) also was identical to that of authentic phenothiazine.

Discussion

When dosed with *N*-acetylphenothiazine (**I**), *A. niger*, *C. verticillata*, and *P. simplicissimum* all oxidized the sulfur atom to produce *N*-acetylphenothiazine sulfoxide (**II**) and phenothiazine sulfoxide (**III**). Formation of any of the metabolites other than **II** would require hydrolysis of the acetyl group to form phenothiazine. *C. verticillata* transformed this postulated intermediate further to phenothiazine *N*-glucoside (**IV**) and phenothiazin-3-one (**V**). Phenothiazine (**VI**), the expected intermediate, was detected in cultures of *P. simplicissimum* but not in the other cultures or noninoculated controls.

The structure of *N*-acetylphenothiazine is more stable than that of phenothiazine (Roseboom and Förch 1979); however, the acetyl group of *N*-acetylphenothiazine was removed by *P. simplicissimum* and presumably also by the other two fungi.

N-Acetylphenothiazine sulfoxide was found in cultures of all three fungi, showing their ability to oxidize the sulfur atom even in the presence of the *N*-acetyl group. In humans, fluacizine and moricizine are also converted directly to the sulfoxides without hydrolysis (Vikhlyaev and Zherdev 1975; Richards et al. 1997). *C. elegans* oxidizes the sulfur atoms of chlorpromazine and methdilazine without removal of the side chains (Zhang et al. 1996).

In cultures of *C. verticillata*, the appearance of phenothiazin-3-one and phenothiazine *N*-glucoside suggested that the acetyl group was hydrolyzed and the resulting phenothiazine was either oxidized to the ketone or conjugated to the *N*-glucoside. Guinea pigs dosed with phenothiazine produce small amounts of phenothiazine sulfoxide and phenothiazin-3-one, but the major metabolites are phenothiazine *N*-glucuronide and phenothiazine 3-sulfate (Mitchell and Waring 1979). It is likely that *C. verticillata* produced a phenothiazine

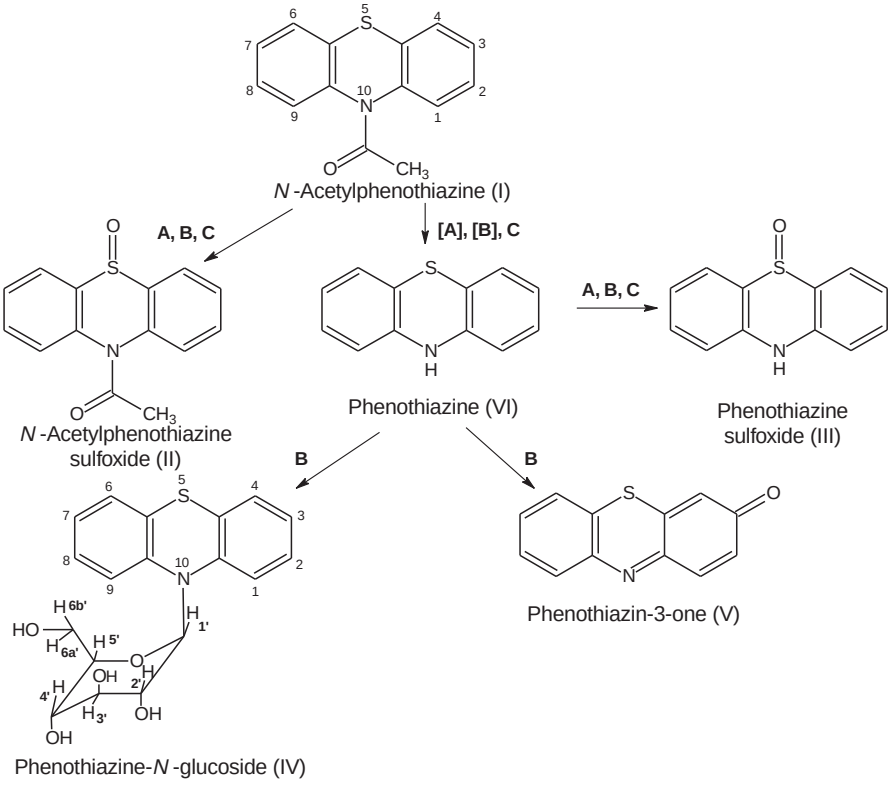

Fig. 2 Proposed pathways for the metabolism of *N*-acetylphenothiazine (***I***) by (*A*) *A. niger*, (*B*) *C. verticillata*, and (*C*) *P. simplicissimum*. Although phenothiazine (***VI***) was detected only in cultures of *P. simplicissimum*, it is postulated as an intermediate in cultures of the other two fungi, [*A*] and [*B*]

intermediate and oxidized it to 3-hydroxyphenothiazine, which was subsequently autooxidized to form phenothiazin-3-one (Mitchell and Waring 1979). Ring hydroxylation also occurs with substituted phenothiazine drugs (Zhang et al. 1996).

In the fungal transformation of chlorpromazine and methdilazine, Zhang et al. (1996) observed hydrolysis of a substituent, *S*-oxidation, *N*-oxidation, hydroxylation of aromatic rings, and *N*-demethylation. In the transformation of *N*-acetylphenothiazine, we observed some similar pathways, including hydrolysis, *S*-oxidation, and ring oxidation, as well as glucose conjugation. Our proposed scheme for the biotransformation of *N*-acetylphenothiazine by the three fungi is shown in Fig. 2. We conclude that these fungi can be used to transform *N*-acetylphenothiazine, and perhaps also other *N*-carbonyl-substituted phenothiazine drugs, by hydrolytic, oxidative, and conjugative processes.

Acknowledgements We thank C.E. Cerniglia and T.M. Heinze for helpful discussions and for valuable comments on this manuscript. This work was supported in part by an appointment to the Postgraduate Research Program at the National Center for Toxicological Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration.

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