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Microbial Transformations of Antitumor Compounds. 1. Conversion of Acronycine to 9-Hydroxyacronycine by *Cunninghamella echinulata*

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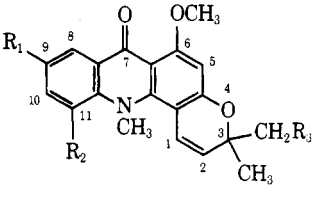
Microbial transformations have been employed as a method for producing quantities of a potentially active metabolite of the antitumor alkaloid acronycine. More than 40 microorganisms were screened for their abilities to convert acronycine to metabolites in small-scale fermentations. Ten cultures were found to accumulate one or more acronycine derivatives in culture media. In larger scale fermentations, *Cunninghamella echinulata* (NRRL 3655) converted acronycine to the phenolic metabolite, 9-hydroxyacronycine, in 30% yield. The extremely insoluble metabolite was acetylated and its structure established by spectral methods. The potential of microbial transformations as a tool for producing synthetically difficult derivatives of antitumor agents is discussed.

The ability of microorganisms to accomplish structural modifications of many types of organic compounds has been well documented.[1-4] With structurally complex compounds such as the steroids and alkaloids, selected microorganisms have been advantageously used to perform single, specific chemical transformations. To date, this technique has found widespread use, especially in the preparation of therapeutically important steroid derivatives. We have initiated a series of studies to establish microbial transformations as a convenient general method for obtaining novel and difficult-to-synthesize analogs of antitumor compounds.

This report is concerned with the antitumor alkaloid, acronycine (**1a**). Acronycine was chosen for study because it has exhibited broad antitumor activity in several experimental tumor systems,[5] and it is currently being studied in the clinic.[6] In addition, the alkaloid is readily available in large amounts from the bark of *Acronychia Baueri* Schott (Rutaceae),[5] and it has been synthesized.[7,8]



	R_1	R_2	R_3
acronycine **1a**	H	H	H
1b	OH	H	H
1c	H	OH	H
1d	OH	OH	H
1e	H	OH	OH
1f	$OCOCH_3$	H	H

Microbial Transformations. Preliminary small-scale fermentation experiments were conducted in order to obtain microorganisms capable of metabolizing acronycine. Organisms were selected on the basis of prior experi-

Table I. Acronycine Metabolizing Microorganisms

Culture name	Culture collection no.[a]
Cunninghamella blakesleeana	ATCC 9245
Cunninghamella bainieri	ATCC 9244
Cunninghamella echinulata	NRRL 3655
Cunninghamella blakesleeana	NRRL 1369
Cunninghamella echinulata	SP-WISC 1387
Cunninghamella echinulata	SP-WISC 1386
Cunninghamella blakesleeana	ATCC 8688a
Streptomyces rimosus	ATCC 23955
Aspergillus niger	ATCC 9142
Zygorhynchus japonicus	UI-1234

[a] 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.; UI, University of Iowa, College of Pharmacy Culture Collection, Iowa City, Iowa.

ence[9-11] and on literature precedence which indicated their potential for metabolizing the alkaloid.[12] In addition, a number of steroid transforming fungi were used for initial experiments. Cultures chosen for screening experiments represented a selection from a broad taxonomic range of microorganisms including species of *Penicillium, Cunninghamella, Rhodotorula, Rhizopus, Zygorrhynchus, Aspergillus, Botrytus, Syncephalastrum, Kicksella, Trichothecium, Gliocladium, Streptomyces, Mucor, Curvularia, Stemphylium, Nadsonia, Helicostylum, Pseudomonas, Ophiobolus, and Chromobacterium.* Of 47 cultures initially screened, ten actively metabolized acronycine to several more polar metabolites (Table I). Interestingly, seven of the eight *Cunninghamella* species examined in the screening experiment could produce metabolites of acronycine.

Cunninghamella echinulata (NRRL 3655) was chosen for larger scale production of the acronycine metabolites. When this organism was grown in a stirred fermentor, a single metabolite was produced. The metabolite was isolated by liquid–liquid extraction of the acidified culture and was obtained in 14% overall yield. In a second preparative scale fermentation, 4 g of acronycine was metabolized by *C. echinulata* grown in numerous shaken-flask cultures to yield 1.26 g (30% yield) of the purified metabolite.

Discussion

For our experiments, acronycine was synthesized by the method of Hlubucek, *et al.*,[7] but it was obtained in low overall yield. It was much more convenient to obtain the compound from *A. Baueri* bark† by a modification of literature extraction procedures whereby the alkaloid was isolated in amounts representing 0.5% of the bark.

The high-resolution mass spectrum indicated that the metabolite possessed a molecular weight of 337 ($C_{20}H_{19}NO_4$), consistent with a monohydroxylated acronycine derivative. The metabolite was very insoluble in all common solvents, except for aqueous NaOH. Therefore, nmr spectral data were obtained on the acetate derivative. A portion of the nmr spectrum of the acetylated metabolite is shown in Figure 1.

In acronycine, the signal for 8 H exists as a quartet at δ 8.43 while the remaining aromatic protons give complex overlapping signals centered at approximately δ 7.40. The signal for 8 H of the acetylated metabolite **1f** occurs as a doublet at δ 7.93. The magnitude of the coupling constant

†*Achronychia Baueri* Schott stem bark (50 lb) was purchased from S. B. Pennick and Co., New York, N. Y.

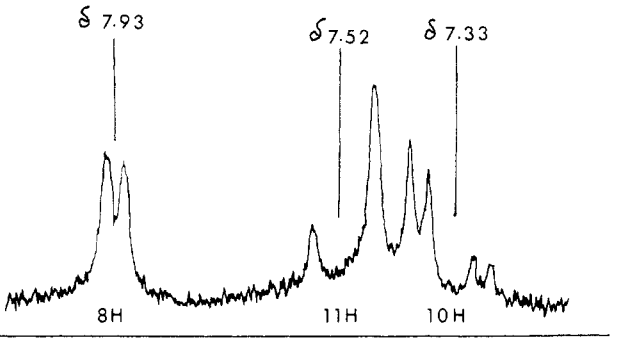
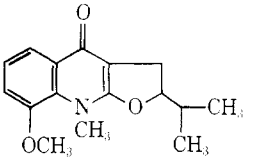


Figure 1. Aromatic portion of the 100-MHz nmr spectrum of 9-acetoxyacronycine (**1f**) (acetone-d_6).

(J = 2 Hz) is consistent with the location of the acetoxy substituent on position 9. Signals for 10 H and 11 H appear as an ABC quartet exhibiting ortho coupling between 10 H and 11 H (J = 7.5 Hz) and meta coupling between 8 H and 10 H (J = 2 Hz). All of the remaining proton signals in the nmr spectrum of the metabolite are unchanged relative to acronycine. It is therefore possible to unambiguously assign the phenolic hydroxyl group of the metabolite to position 9 of the acronycine ring system. Comparison of the nmr spectrum of the acetylated metabolite with that of lunacrine, a furoquinoline alkaloid (**2**), rules out the possibility that the hydroxyl group appears on position 11.[13]



Microbial hydroxylation of acronycine yielded 9-hydroxyacronycine (**1b**) as the principal metabolite. In addition to this compound, *C. echinulata* (NRRL 3655), *C. blakesleeana* (ATCC 9245), and *C. bainieri* (ATCC 9244) produced traces of other acronycine metabolites. On the basis of tlc data[14] obtained from mammalian metabolism studies, these other metabolites were tentatively identified as 11-hydroxyacronycine (**1c**), 9,11-dihydroxyacronycine (**1d**), and 3-hydroxymethyl-11-hydroxyacronycine (**1e**). All of these compounds are produced by mammals, and 9-hydroxyacronycine is also the major mammalian metabolite of acronycine.[14]

Presumably the mammalian metabolites are produced as a result of the action of monooxygenase enzyme systems. Both the microbial and mammalian systems produced 9-hydroxyacronycine as the principal metabolite. The hydroxylated position occurs para to the nitrogen and meta to the carbonyl of the acridone ring system. Because 9-hydroxyacronycine and 9,11-dihydroxyacronycine were isolated in the mammalian metabolism study, it appears that the nitrogen atom exerts a directing influence for hydroxylation of the aromatic ring. Hydroxylations of aromatic rings which are mediated by monooxygenases usually behave according to the rules of electrophilic aromatic substitution.[15,16]

Smith and Rosazza have recently shown that microorganisms may be effectively utilized as models of mammalian metabolism.[9] This potential has been alluded to by others.[17] It has recently been shown that *Cunninghamella bainieri* (ATCC 9244) possesses monooxygenase enzyme activity similar to that displayed by liver microsomes in the oxidation of a variety of organic compounds.[18] It is of interest that we have independently found *C. bainieri*

(ATCC 9244) and other *Cunninghamella* species to be capable of actively metabolizing acronycine and other aromatic compounds as well.[9]

The value of microbial transformations as a tool for providing gram quantities of difficult-to-synthesize mammalian metabolites of acronycine and the ability of microorganisms to mimic the mammalian metabolism of acronycine have been demonstrated.

Experimental Section

Melting points were determined in open-ended capillaries in a Thomas-Hoover capillary melting point apparatus and are corrected. Nmr spectra were obtained on all compounds with a Varian Associates Model T-60 spectrometer using TMS as an internal standard. Ir spectra were measured on a Beckman Ir-10 instrument. Low-resolution mass spectra were obtained on a RMU-6 Hitachi Perkin-Elmer mass spectrometer, while high-resolution spectra were obtained through the chemistry department of the Massachusetts Institute of Technology. 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.

Fermentation Methods. Cultures used in this work are maintained on Sabouraud-maltose agar slants and are stored in a refrigerator at 4° prior to use. All organisms mentioned in this work are maintained in the culture collection at the College of Pharmacy of the University of Iowa.

Cultures were grown in a soybean meal-glucose 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, 1000 ml; pH 7.0 adjusted 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°, usually in erlenmeyer flasks holding one-fifth of their volumes of medium.

To initiate fermentations, the surface growth from slants of microorganism was suspended in 5 ml of sterile medium. The resulting suspension was used to initiate stage I cultures which were incubated for 48 hr. The thick 48-hr growth started from slants was used as inoculum for stage II cultures. The inoculum volume was 10% of the volume of stage II fermentation medium in all cases. Acronycine was added to 24-hr-old stage II cultures as a solution in DMF (125 mg/ml) in order to facilitate dispersion of the compound in aqueous fermentation media.

Small-scale screening experiments were conducted in 25 ml of medium contained in 125-ml cotton-plugged erlenmeyer flasks. Preparative scale fermentations were conducted either in a Microferm (New Brunswick Scientific Co.) stirred fermentor in 7.5 l. of medium or in numerous 500- and/or 1000-ml erlenmeyer flasks containing 100 and 200 ml of medium, respectively. For preparative scale fermentations, stage I cultures were conducted in 500-ml erlenmeyer flasks with 100 ml of medium.

Sampling Procedure. A routine procedure was used in preparing fermentation samples for tlc analysis. Samples of 4 ml were withdrawn from fermentations at various time intervals and were adjusted to pH 2 with 2 drops of 5 *N* HCl. The acidified samples were extracted with 1 ml of ethyl acetate, and approximately 50 μl of the extracts was spotted on tlc plates. Plates were developed in a number of solvent systems including (A) $CHCl_3$-EtOH (90:6); (B) EtOAc-MeOH-$(C_2H_5)_2NH$ (85:10:5); (C) C_6H_6-MeOH (20:4); (D) C_6H_6-EtOAc (2:1); (E) C_6H_6-EtOAc-HOAc (65:30:5). Visualization was accomplished by quenching of uv fluorescence and by spraying developed plates with *p*-anisaldehyde-60% $HClO_4$-Me_2CO-H_2O (0.5:5:10:40) reagent and by warming sprayed plates with a heat gun.

Screening of Microorganisms. Small-scale fermentations were used to screen 47 microorganisms for their abilities to metabolize acronycine. Cultures were grown according to the general fermentation procedure in 125-ml erlenmeyer flasks. A total of 12.5 mg of acronycine in 0.1 ml of DMF was added to each 24-hr-old stage II culture. Substrate-containing flasks were incubated under the usual conditions, and 5-ml samples were taken at 24, 48, and 72 hr after substrate addition. Fermentations were monitored by tlc using solvent system A. The 24-hr sample indicated that little metabolism of the acronycine had occurred. At 48 hr, several cultures accumulated at least one metabolite, while at 72 hr, ten microorganisms (Table I) had produced one or more metabolites in relatively large amounts.

The experiment was repeated with controls using only the ten active cultures. Control flasks consisting of substrate added to sterile medium without organism were examined. Acronycine was the only substance detectable in these controls after they had been incubated under the usual conditions for 48 hr. In addition, each of the ten microorganisms was grown in duplicate flasks, one containing acronycine, the other only DMF as a control. Fermentations were monitored by tlc using solvent system B. The three most active cultures were *Cunninghamella bainieri* (ATCC 9244), *Cunninghamella echinulata* (NRRL 3655), and *Cunninghamella blakesleeana* (ATCC 9245). After 5 days of incubation, each of these cultures used approximately 80% of the acronycine substrate to produce four metabolites at R_f 0.55, 0.38, 0.30, and 0.10, respectively. The metabolite at R_f 0.38 was the most abundant one.

Preparation of the Metabolite in a Stirred Fermentor. *Cunninghamella echinulata* (NRRL 3655) was grown according to the usual fermentation procedure. Stage II cultures were grown in 7.5 l. of medium in a Microferm fermentor at a constant temperature of 26° and an aeration rate of 300 ml/l./min. The fermentor was agitated at 300 rpm initially, and after 47 hr when the culture became extremely thick the agitation rate was increased to 500 rpm. Foaming was controlled with 0.4 ml of 2-octanol during the first 24 hr and with antifoam A (Dow) thereafter. A total of 3.5 g of acronycine in 22 ml of DMF was added to the fermentor at 24 hr. Tlc analysis of the fermentation at 24, 46, and 70 hr after substrate addition with solvent systems A and B revealed a single, yellow metabolite with R_f values 0.27 and 0.40, respectively. The fermentation was terminated after 70 hr, the pH of the medium being 6.85.

Isolation of 9-Hydroxyacronycine (1b). The fermentation was adjusted to pH 2 with HCl and was defatted with hexane in a liquid-liquid extractor. The hexane was replaced with ethyl acetate, and extraction was continued until the extracts yielded no more acronycine or the metabolite. The ethyl acetate was removed under vacuum, and the resulting nondrying oil was dissolved in methanol and refrigerated at 4° to yield 574 mg of a mixture of acronycine-metabolite in a ratio of approximately 1:4. The yellow mixture was dissolved in 200 ml of boiling ethyl acetate to ultimately yield 480 mg of the crystalline metabolite. The analytical sample was prepared by further recrystallization of the metabolite from boiling methanol. Purity of the analytical sample was determined by tlc using solvent systems A, B, C, and E before analytical data were recorded: mp 343–344° dec; λ_{max}^{EtOH} 281 nm (log ε 4.41); mass spectrum, $M\cdot^+$ 337.12979 ($C_{20}H_{19}NO_4$), requires $M\cdot^+$ 337.13139; other mass spectral signals, *m/e* (rel intensity) 337 (72), 323 (24), 322 (100), 308 (30), 278 (29).

Preparation of 9-Hydroxyacronycine (1b) by Shaken Flask Culture. *C. echinulata* was grown as usual. Stage II fermentations were conducted in 7.5 l. of medium held in 500-ml and 1-l. erlenmeyer flasks. A total of 4.0 g of acronycine in 30 ml of DMF was distributed among the stage II cultures. After 9 days, the fermentation was terminated. Cultures were centrifuged at 7000 rpm for 1 min to seprate the solids and cells from the fermentation beer. The resulting supernatant was acidified to pH 2, extracted with ethyl acetate, and examined by tlc (solvent system D) to reveal only minute amounts of metabolite and acronycine.

The sedimented solids were stirred in 1.5 l. of cold 95% ethanol and filtered. The cold ethanol extract contained a wide variety of substances including acronycine but contained little of the metabolite. The remaining solid residue was stirred in hot ethanol five times and filtered, and the combined filtrates gave 1.26 g of crystalline metabolite (30% yield).

9-Acetoxyacronycine (1f). The acetoxy derivative was prepared by refluxing 100 mg of 9-hydroxyacronycine with 89 mg of NaOAc in 27 ml of Ac_2O for 2 hr. The reaction was quenched by pouring onto crushed ice, and the cold mixture was allowed to stand overnight. The acetoxy derivative was extracted from the brown mixture with four 200-ml portions of ethyl acetate. Combined EtOAc layers were washed with two 200-ml portions of water and dried over anhydrous Na_2SO_4. After removing the solvent under vacuum, the residue was dissolved in 10 ml of boiling methanol and after cooling the solution was refrigerated at −12° to yield 37 mg of pure **1f**: mp 185.5–188°; λ_{max}^{EtOH} 280 nm (log ε 4.50); mass spectrum *m/e* (rel intensity) 379 (96), 364 (100), 338 (21), 322 (70), 308 (31), 306 (32), 278 (29); nmr (acetone-d_6) (see Figure 1) δ 1.50 [s, 6, $-C(CH_3)_2$], 2.30 (s, 3, $-COCH_3$), 3.88 (s, 6, $-OCH_3$, NCH_3), 5.60 (d, 1, H_2), 6.30 (s, 1, H_5), 6.68 (d, 1, H_1), 7.33 (q, 1, H_{10}), 7.52 (d, 1, H_{11}), 7.93 (d, 1, H_8). *Anal.* Calcd for $C_{22}H_{21}NO_5$: C, 69.65; H, 5.58; N, 3.69. Found: C, 69.77; H, 5.61; N, 3.73.

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Adamantanealkanamines as Potential Antidepressant and Anti-Parkinson Agents†

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Various adamantanealkanamines were prepared and their activity in antagonizing reserpine-induced hypothermia was compared with nortriptyline. A structure–activity relationship in respect of the substitution on the adamantane ring and the nature of the alkanamino side chain is discussed. Norepinephrine responses *in vivo* as well as *in vitro* are potentiated at low doses with only one compound, *N*,α-dimethyl-2-phenyl-1-adamantaneethanamine (**52**). However, unlike the tricyclic antidepressants, **52** fails to block the pressor response to tyramine in the cat. The anti-Parkinson activity of some of these potent amines in reversing the reserpine-induced catalepsy in rats was evaluated and compared with amantadine.

While screening adamantane derivatives for biological activity, it was found that certain adamantanealkanamines antagonized reserpine hypothermia in mice, indicating possible antidepressant activity. The present paper describes the synthesis and evaluation of antidepressant properties, in terms of reserpine antagonism, of these amines. The anti-Parkinson properties of some of the amines, as determined by reversal of reserpine-induced catalepsy in rats, are also described. The compounds concerned are adamantanes substituted on the 1 position with an alkyl (2–4 carbon atom, straight/branched) amino group. In some of the compounds, the other nuclear positions of adamantane are also substituted with methyl, phenyl, halo, or hydroxyl groups. Adamantane derivatives substituted on the 2 position with an aminopropylidene group, analogous to amitriptyline or nortriptyline, and their corresponding reduction products are also described. It is interesting to note that these amines bear structural resemblance to the tricyclic antidepressants in respect of their nonplanar fused ring structure being replaced by the adamantane ring system. It is also possible that the pronounced lipophilic nature of the adamantane group may facilitate access of these amines into the brain and cerebrospinal fluid.[2]

Chemistry. 1-Adamantanealkanamines II and VI were obtained by procedures as represented in Scheme I.

(i) Reductive Amination of the Appropriate Ketones I (Method A). The preparation of these ketones was effected either by reacting an acid chloride with diethylethoxymagnesium malonate and subsequent hydrolysis and decarboxylation as described in our earlier paper[3] or by the reaction of an acid with MeLi. The latter procedure is preferred as it reduces the number of steps and also gives excellent yields.

(ii) Reduction of an Amide with $LiAlH_4$ (Method C). The amides were obtained by reacting an acid chloride III with the appropriate amine or, where a lengthening of the alkyl chain was intended, through an Arndt–Eistert synthesis, as in the case of V.

(iii) Reaction of an Alkyl Halide with an Appropriate Amine (Method D). The starting 1-(2-bromoethyl)adamantane VII was obtained according to the procedure described by Stetter, *et al.*[4] When the magnesium Grignard reagent of this bromide was allowed to react with formaldehyde, acetaldehyde, or ethylene oxide, the corresponding higher alkanols VIII were obtained. These alkanols on reaction with either HBr or $SOCl_2$ produced the corresponding alkyl halides IX.

N-Methyl-1-adamantanepropanamine was also prepared by an alternative route as follows. 1-Adamantanepropanonitrile was obtained in almost quantitative yield by reacting VII with NaCN in DMSO. This nitrile on reaction with dimethoxycarbonium fluoroborate[5] yielded the corresponding nitrilium salt, which on reduction with $NaBH_4$ produced the desired amine.

1,2-Disubstituted adamantane derivatives were obtained by using intramolecular free-radical reactions.[3,6] We described[3] the reaction of 1-adamantaneethanol with $Pb(OAc)_4$ to give adamantano[2,1-*b*]tetrahydrofuran (octahydro-4*H*-3a,7:5,9-dimethanocycloocta[*b*]furan). This

† Part VII of the series, Chemistry of Adamantane. For part VI, see ref 1.