

Microbiological Metabolism of Naphthyridines¹

P. B. HAMILTON,² D. ROSI, G. P. PERUZZOTTI,³ AND E. D. NIELSON⁴

Sterling-Winthrop Research Institute, Rensselaer, New York 12144

Received for publication 14 November 1968

Penicillium adametzi and seven other species convert nalidixic acid, 1,4-dihydro-1-ethyl-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid, to 1,4-dihydro-1-ethyl-7-hydroxymethyl-4-oxo-1,8-naphthyridine-3-carboxylic acid. Forty-seven other species from six orders of fungi seem to achieve the same conversion as judged by chromatographic and spectral evidence. Under special conditions, *P. adametzi* also produces a second metabolite which was identified as the corresponding 7-carboxylic acid. The metabolic attack on the ring substituent is identical with the pathway previously established with humans. No evidence was obtained for metabolic attack on the naphthyridine nucleus itself.

Nalidixic acid, 1,4-dihydro-1-ethyl-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid, is a synthetic antibacterial compound which is a representative of a new class of chemotherapeutic agents (9). It has found clinical use in the treatment of urinary tract infections (2, 14). It is more active against gram-negative than gram-positive organisms (3), and the mechanism of its action is the specific inhibition of deoxyribonucleic acid synthesis in the susceptible microorganism (4). Nalidixic acid is metabolized in humans and experimental animals to the 7-hydroxymethyl and the 7-carboxylic acid analogs (10, 11).

The 7-hydroxymethyl derivative shows the same order of activity *in vitro* as the parent drug (6). Its occurrence as a metabolite suggested that a suitable route to the preparation of the pharmaceutically interesting 7-hydroxymethyl derivative might be transformation of the parent compound by fungi analogous to the well-known transformation of steroids. This approach might be expected also to provide information about the microbiological metabolism of naphthyridines. To our knowledge, there have been no previous reports on the effects of microorganisms on naphthyridines. The present report details the widespread occurrence among fungi of the ability to metabolize nalidixic acid.

MATERIALS AND METHODS

The microorganisms were maintained on slants of Sabouraud Maltose Agar (Difco) or Potato Dextrose

Agar (Difco). Inoculated slants were incubated at 25 C for 1 week before a spore suspension in distilled water was made. The suspension was transferred to 100 ml of soy-dextrose medium (7) in a 500-ml Erlenmeyer flask which was incubated at 25 C for 24 hr on a shaker rotating at 210 rev/min with a 1-inch (2.54 cm) radius of rotation. After this period, 10 ml of the submerged growth was added to 500-ml Erlenmeyer flasks containing 100 ml of soy-dextrose medium, Edamine-Dextrose medium (5), and dextrin-cornsteep medium (dextrin, 10.0 g; cornsteep liquor, 80.0 g; KH_2PO_4 , 1.0 g; NaCl, 5.0 g; tap water, 1.0 liter; pH adjusted to 4.0).

The inoculated flasks were incubated 48 hr, at which time 10.0 mg of nalidixic acid dissolved in 0.25 ml of warm *N,N*-dimethylformamide was added. The flasks then were agitated another 24 hr before being acidified with 2.0 ml of 10 N HCl. The acidified whole cultures were extracted once with an equal volume of dichloromethane and once with half that volume. The extracts were combined in beakers and evaporated to dryness in a water bath at 60 C. Each residue was dissolved in 10 ml of dichloromethane for application to paper for chromatographic analysis. The solvent system was toluene-dioxane (9:1) saturated with propylene glycol (mobile phase; 17); dry paper (Whatman no. 1) dipped into 35% aqueous propylene glycol solution and blotted between two sheets of paper towels to remove the excess solvents was the stationary phase. The papers were photographed by using ultraviolet (UV) light by a modification of the method of Haines and Drake (Federation Proc., p. 180, 1950). The phosphor plate was omitted and G3530 photographic paper (Transcopy Inc., Boston, Mass.) was used as a negative; paper G451 was the positive and G40 was the processing fluid.

Large quantities of culture for the isolation of the primary transformation product were obtained by inoculating 1.0 liter of 48-hr cultures in soy-dextrose medium into a 14-liter stirred jar fermentor. The fermentor contained 10.0 liters of soy-dextrose me-

¹ Portions of this report are taken from U.S. Patent 3,317,401.

² Present address: Department of Poultry Science, North Carolina State University, Raleigh, N.C. 27607.

³ Present address: School of Pharmacy, University of Wisconsin, Madison, Wis. 53706.

⁴ Present address: R. J. Reynolds Tobacco Co., Winston-Salem, N.C. 27101.

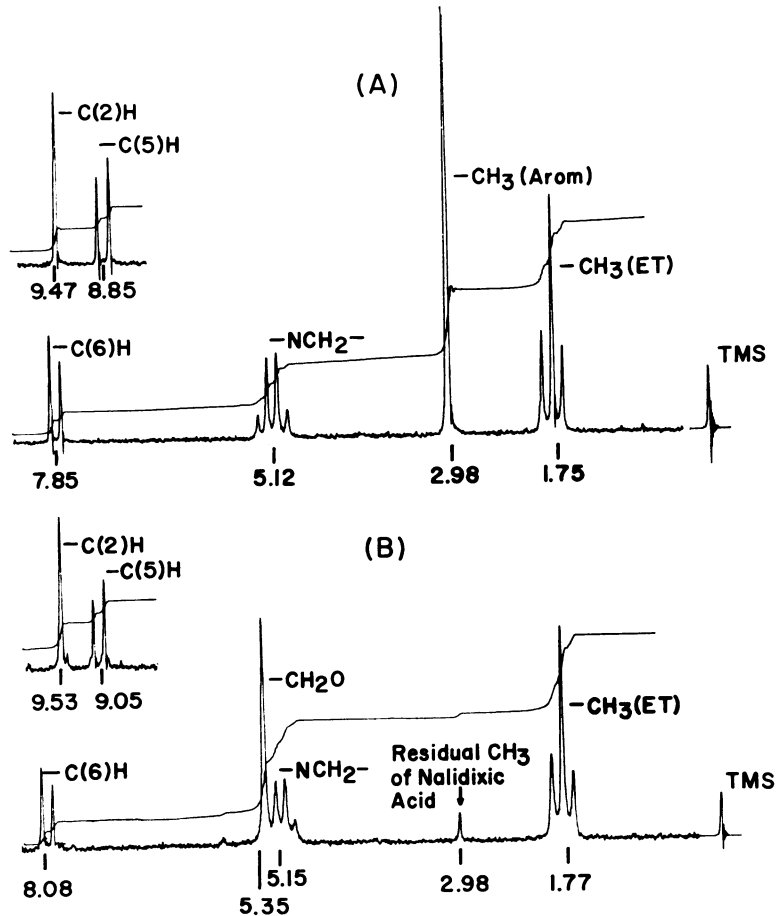


FIG. 1. NMR spectra of nalidixic acid (A) and its 7-hydroxymethyl derivative (B). Concentrations were 20% in trifluoroacetic acid with tetramethylsilane (TMS) as internal standard. Signals are given in parts per million.

dium autoclaved 45 min at 121 C. The inoculated medium was incubated at 30 C with a filtered airflow of 2 liters per min and with agitation of 450 rev/min with a double impellor. After 48 hr, 1.0 g of nalidixic acid in 50 ml of dimethylformamide was added, and the fermentation was continued for 24 hr. The course of the fermentation was followed by chromatography of samples as described above. The fermentation was stopped by adding 200 ml of 10 N HCl.

The 3,7-dicarboxylic acid derivative was isolated from flask fermentations. The medium was triple-strength soy-dextrose (25 ml per 500-ml Erlenmeyer flask). The flasks were inoculated, incubated, and exposed to substrate as described above. Ten flasks which had received a total of 100 mg of nalidixic acid were used for isolation of the product after being acidified with 1 ml of 10 N HCl.

RESULTS

Penicillium adamezti NRRL 737, one of the first microorganisms to be tested, was observed to convert all the added nalidixic acid to a com-

pound with an R_F value of about one-tenth that of nalidixic acid in the chromatographic system. The product of this conversion was isolated from a stirred fermentation. The acidified fermentation beer was extracted three times with 4 liters of dichloromethane. The extracts were combined and concentrated to about 100 ml. The crystalline product that separated was collected by filtration. This product was recrystallized three times from ethyl acetate and once from acetone to yield 0.6 g of chromatographically pure material melting at 256 to 257 C. Analysis: $C_{12}H_{12}N_2O_4$; calculated: C, 58.06; H, 4.87; N, 11.29. Found: C, 57.71; H, 4.56; N, 11.33.

The product differed in its composition from the parent nalidixic acid only in having one more oxygen atom. The infrared (IR) spectra of the parent and product compounds were very similar except that the product had a hydroxyl peak at 2.99 μ m. The near identity of the UV spectrum

TABLE 1. *Microorganisms capable of oxidizing nalidixic acid to its 7-hydroxymethyl derivative*

Species	Order	ATCC ^a
	Hypocreales	
<i>Melanospora parasitica</i>		11103
	Melanconiales	
<i>Septomyxa affinis</i>		6737
<i>S. affinis</i>		13414
	Moniliales	
<i>Alternaria porri</i> (Ell.) Sacc		CBS
<i>Aspergillus alliaceus</i>		10060
<i>A. clavatus</i>		9192
<i>A. conicus</i>		11911
<i>A. fischerii</i>		1020
<i>A. flavipes</i>		1030
<i>A. flavus</i>		9170
<i>A. itaconicus</i>		10021
<i>A. malignus</i>	CMI	16061
<i>A. nidulans</i>		11267
<i>A. niger</i>		11394
<i>A. ochraceus</i>		12337
<i>A. ustus</i>		10032
<i>A. wentii</i>		1023
<i>Beauveria densa</i>		9452
<i>Botrytis cinerea</i>		12481
<i>Cephalosporium gregatum</i>		11073
<i>Curvularia falcata</i> (Tehon)		CBS
<i>C. lunata</i> (Wakker) Boedijn		CBS
<i>Gliocladium catenulatum</i>		10523
<i>G. roseum</i>		10521
<i>G. vermoeseni</i>		10522
<i>Penicillium aculeatum</i>		10409
<i>P. adametzi</i>	NRRL	737
<i>P. aurantio-violaceum</i>		10412
<i>P. bervi-compactum</i>		9056
<i>P. canescens</i>		10419
<i>P. charlesii</i>		8730
<i>P. chermesinum</i>		10424
<i>P. chrysogenum</i>		10106
<i>P. expansum</i>		7861
<i>P. frequentans</i>		10444
<i>P. frequentans</i>		10494
<i>P. lilacinum</i>		10114
<i>P. nicricans</i>		10115
<i>P. novae-zeelandiae</i>		10473
<i>P. patulum</i>		9260
<i>P. tardum</i>		10503
<i>P. thomii</i>		10506
<i>P. thomii</i>		10596
<i>P. sp.</i>		12556
<i>Pleospora herbarum</i> (Pers.)		CBS
<i>Spondylocladium atrovierens</i>		10846
<i>Tricothecium roseum</i>		8685
<i>T. roseum</i>		12519
<i>T. roseum</i>		12543
	Mucorales	
<i>Absidia blakesleeana</i>		10148a (+)
<i>Cunninghamella elegans</i>		9245
<i>C. elegans</i>		9246
<i>C. elegans</i>		6795b (-)

TABLE 1—Continued

Species	Order	ATCC ^a
<i>C. verticillata</i>		8983
<i>Helicostylum piriforme</i>		8992
<i>Mortierella alpina</i>		8979
<i>Mucor mucedo</i>		7941
<i>Rhizopus oryzae</i>		8477
<i>Syncephalis nodosa</i>		7943
	Sphaeriales	
<i>Botryosphaeria ribis</i>		11232
<i>Hypomyces rosellus</i> (alb. et Schw. Tal.)		CBS
	Spirogyrales	
<i>Syncephalolastrum racemosum</i>		1332b (-)

^a American Type Culture Collection number.

^b Northern Regional Research Laboratory number.

^c Commonwealth Mycological Institute number.

^d CBS indicates the organism was obtained from the Central Bureau voor Schimmelcultures, Netherlands.

of the product which had absorption maxima at 258 nm ($\epsilon = 26,800$) and 320 nm ($\epsilon = 11,700$) with the UV spectrum of the parent nalidixic acid suggested that the hydroxylation did not occur in the ring position but rather on one of the two alkyl groups. This supposition was confirmed by the nuclear magnetic resonance (NMR) spectra shown in Fig. 1. It clearly shows that the methyl signal of the parent nalidixic acid at 2.98 ppm (s, 3) was replaced in the fermentation product by a signal at 5.35 ppm (s, 2) for a methylene group adjacent to an oxygen.

Further evidence on the nature of the product was obtained by oxidizing it to 1,4-dihydro-1-ethyl-4-oxo-1,8-naphthyridine-3,7-dicarboxylic acid (Lesher et al., *submitted for publication*). The oxidation was done by stirring for 2 hr at room temperature a 1% aqueous solution containing 2% KMnO_4 and 3% KOH . The excess KMnO_4 was discharged by using an aqueous solution of NaHSO_3 . The resulting MnO_2 was filtered and washed with water. The filtrate and washings were combined and acidified with 10 N HCl . The resulting white precipitate was collected by filtration and recrystallized from ethyl alcohol. The oxidation product and the authentic dicarboxylic acid prepared by oxidation of 1-ethyl-4-oxo-7-styryl-1,8-naphthyridine-3-carboxylic acid had identical UV and IR spectra, and a mixture of the two samples melted without depression at 282 to 283 C (decomp). The identity of the fermentation product with one of the human metabolites was established by their UV, IR, and NMR spectra and by the un-

depressed melting point of a mixed sample (253.5 to 254 C).

The recovery of only 60% of the added nalidixic acid as the 7-hydroxymethyl derivative suggested there might be other transformation products similar to human metabolism. Chromatographic examination of extracts from flasks and the fermentor failed to reveal any other UV-absorbing spots. However, when nalidixic acid was fermented in 500-ml flasks containing only 25 ml of triple-strength medium, a new spot more polar than the 7-hydroxymethyl compound was observed. The spot was eluted from the chromatogram with methanol and the UV spectrum was determined. The material exhibited peaks at 258 and 330 nm with a 258:330 ratio of 2.5 in common with the 3,7-dicarboxylic acid derivative. The extracts from 10 flasks were combined and 43 mg of pure material was obtained by crystallization from ethyl alcohol. This material was identical with authentic 1,4-dihydro-1-ethyl-4-oxo-1,8-naphthyridine-3,7-dicarboxylic acid as judged by UV and IR spectra and melting point of a mixed sample.

Screening of a collection of fungi for their ability to metabolize nalidixic acid revealed that the representatives of six orders (Table 1) yielded a more polar metabolite. The product from *Absidia blakesleeana* ATCC 10148a(+), *Aspergillus wentii* ATCC 1023, *Botryosphaeria ribis* ATCC 11232, *Penicillium sp.* ATCC 12556, *P. frequentans* ATCC 10444, *P. lilacinum* ATCC 10114, and *P. thomii* ATCC 10506, was isolated in the pure state and identified as the 7-hydroxymethyl derivative by IR spectra and melting point of a mixed sample. The other cultures were assumed to produce this derivative because their product also had a chromatographic mobility of

about one-tenth that of the starting nalidixic acid and a similar UV spectrum.

DISCUSSION

The conversion of nalidixic acid to its 7-hydroxymethyl derivative by fungi is a finding with considerable potential importance. The hydroxylated product has been reported to be of the same order of activity as the parent drug against meningococci resistant to sulfonamide (6). Undoubtedly some of the chemotherapeutic activity of nalidixic acid is attributable to its conversion to this active metabolite (10). Although this metabolite can be synthesized chemically, the process is difficult and the yield is low. A microbiological transformation might be of considerable utility.

Fungi appear to metabolize nalidixic acid by first oxidizing the 7-methyl group to the alcohol and then to the carboxylic acid as shown in Fig. 2. The latter step probably proceeds through the aldehyde similar to the oxidation of lucanone by fungi (13). The initial metabolic attack on many aromatic systems is an oxidation of methyl groups (12, 15), and the fungal attack on nalidixic acid is no exception. This metabolic route is identical with the pathway established in animals and humans for the metabolism of nalidixic acid (10).

Although naturally occurring naphthyridines have been reported (8), their occurrence is rare and this suggested that there might be a molecular recalcitrance similar to that discovered with synthetic detergents and herbicides (1). The present findings and the survey of fungi, in particular, demonstrate no such refractoriness with nalidixic acid. However, it is worth noting that all of the metabolites isolated had their basic ring structure

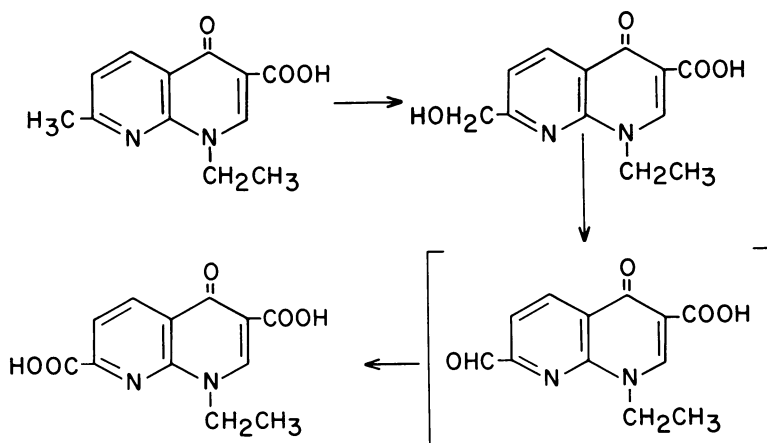


FIG. 2. Presumptive pathway of fungal oxidation of nalidixic acid.

intact. The screening results gave similar indications, since the assay was dependent on the UV absorption of the naphthyridine nucleus and not a single microorganism tested was able to destroy this UV-absorbing ability. Hence, the widespread ability of fungi to attack nalidixic acid might be a reflection of the lability of methyl groups rather than the lability of naphthyridines. Further experiments on nalidixic acid and other naphthyridines will be necessary before this can be settled.

ACKNOWLEDGMENTS

We are indebted to F. C. Nachod, R. K. Kullnig, and Catherine M. Martini for spectral determinations and to K. D. Fleischer and staff for elemental analyses.

LITERATURE CITED

- Alexander, M. 1965. Biodegradation. Problems of molecular recalcitrance and microbial fallibility. *Advan. Appl. Microbiol.* 7:35-76.
- Buchbinder, M., J. C. Webb, L. V. Anderson, and W. R. McCabe. 1963. Laboratory studies and clinical pharmacology of nalidixic acid (Win 18,320). *Antimicrobial Agents and Chemotherapy*—1962, p. 308-317.
- Deitz, W. H., J. H. Bailey, and E. J. Froelich. 1964. In vitro antibacterial properties of nalidixic acid, a new drug active against gram-negative organisms. *Antimicrobial Agents and Chemotherapy*—1963, p. 583-587.
- Goss, W. A., W. H. Deitz, and T. M. Cook. 1965. Mechanism of action of nalidixic acid on *Escherichia coli*. II. Inhibition of deoxyribonucleic acid synthesis. *J. Bacteriol.* 89:1068-1074.
- Dulaney, E. L., W. J. McAleer, M. Koslowski, E. O. Stapley, and J. Jaglom. 1955. Hydroxylation of progesterone and 11-deoxy-17-corticosterone by *Aspergillus* and *Penicillium*. *Appl. Microbiol.* 3:336-340.
- Feldman, H. A., and C. Melynk. 1965. In vitro susceptibility of sulfonamide-resistant meningococci to nalidixic and hydroxynalidixic acids. *Antimicrobial Agents and Chemotherapy*—1964, p. 440-443.
- Hanson, F. R., K. M. Mann, E. D. Nielson, H. V. Anderson, M. P. Brunner, J. N. Kornermatt, D. R. Colingsworth, and W. J. Haines. 1953. Microbiological transformation of steroids. VIII. Preparation of 17 α -hydroxycorticosterone. *J. Am. Chem. Soc.* 75:5369-5370.
- Jewell, D. M., and G. K. Hartung. 1964. Identification of nitrogen bases in heavy gas oil. *J. Chem. Eng. Data* 9:297-304.
- Leshner, G. Y., E. J. Froelich, M. D. Gruett, and J. H. Bailey. 1962. 1,8-Naphthyridine derivatives. A new class of chemotherapeutic agents. *J. Med. Pharm. Chem.* 5:1063-1065.
- McChesney, E. W., E. J. Froelich, G. Y. Leshner, A. V. R. Crain, and D. Rosi. 1964. Absorption, excretion and metabolism of a new antibacterial agent, nalidixic acid. *Toxicol. Appl. Pharmacol.* 6:292-309.
- Portmann, G. A., E. W. McChesney, H. Stander, and W. E. Moore. 1966. Pharmacokinetic model for nalidixic acid in man. I. Kinetic pathways for hydroxynalidixic acid. *J. Pharm. Sci.* 55:59-62.
- Rosi, D., T. R. Lewis, R. Lorenz, H. Freele, D. A. Berberian, and S. Archer. 1967. Preparation and schistosomicidal activity of some 4-hydroxymethyl-3-chloroanilines. *J. Med. Chem.* 10:877-880.
- Rosi, D., G. Peruzzotti, E. W. Dennis, D. A. Berberian, H. Freele, B. F. Tullar, and S. Archer. 1967. Hycanthonone, a new active metabolite of lucanthonone. *J. Med. Chem.* 10:867-876.
- Sinclair, H. 1964. Current therapy of infections of the renal excretory system. *J. Am. Geriat. Soc.* 12:1100-1127.
- Williams, R. T. 1959. *Detoxification mechanisms*. John Wiley and Sons, New York.
- Zaffaroni, A., R. Burton, and E. H. Kentmann. 1950. Adrenal cortical hormones. Analysis by paper partition chromatography and occurrence in the urine of normal persons. *Science* 111:6-8.