Microbial Transformation of Artificial Estrogens of the Allenolic Group

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When 2,2-dimethyl 3-(2′-naphthyl 6′-hydroxy) pentanoic acid, an artificial estrogen of the allenolic acid group, was added to an exponential-phase growth culture of Neurospora crassa (in Horowitz medium), it was transformed into its hydroxylated derivative, 2,2-dimethyl 3-(2′-naphthyl 4′,6′-dihydroxy)pentanoic acid. To study this transformation, radioactive 2-methyl-[2-14C]methyl 3-(2′-naphthyl 6′-hydroxy) pentanoic acid has been prepared. The rate of metabolism of allenolic acids varies in the same way as their estrogenic activity.

In the course of studies regarding the biotransformation of estrogenic hormones by the fungus Neurospora crassa, we previously reported that the main product of the transformation of 17β estradiol is 3,11β,17β estriol. This compound is also formed when estrone is the substrate (we also reported the reduction of estrone into estriadiol and, vice-versa, the oxidation of estriadiol into estrone) (20). In the same way, equilenin [3-hydroxy 17-oxo 1,3,5 (10), 6,8-estrapentaene] is converted into its hydroxylated derivative 3,12β,17β-trihydroxy 1,3,5 (10), 6,8-estrapentaene (19). The different behavior of these two metabolites in hydroxylation reactions can be explained by the structure of the substrates. The presence of the B aromatic ring in equilenin modifies the spacial structure of the C ring, and it can be verified that the hydrogen atom bonded to carbon number 12 is in the same region of space as hydrogen bonded to carbon 11 in estradiol.

We now describe the biotransformation of an artificial estrogenic hormone, 2,2-dimethyl 3-(2’-naphthyl 6’-hydroxy) pentanoic acid. This acid, synthesized by Courrier, Horreau, and Jacques (5, 7, 12), possesses an estrogenic activity as great as that of the natural hormone estradiol.

This activity was explained by the similarity of the geometry of this compound with that of natural hormone (6, 13, 15). The acid which does not possess the cycles C and D cannot be hydroxylated at the positions observed in estradiol and equilenin. Therefore, it was interesting to look for a hydroxylation reaction with this substrate. For this purpose we prepared radioactive 2-methyl-[2-14C]methyl 3-(2′-naphthyl 6′-hydroxy) pentanoic acid in order to detect its metabolites.

MATERIALS AND METHODS

Materials. (i) Synthesis of allenolic acids. Acid I, 2,2-dimethyl 3-(2′-naphthyl 6′-hydroxy) pentanoic acid, was prepared by the method described by Courrier et al. (5, 7, 12). Acids II, 2-methyl 3-(2′-naphthyl 6′-hydroxy) pentanoic acids, were prepared by the method described by Jacques and Horreau (16). These acids possess two adjacent asymmetric carbon atoms. The mixture of diastereoisomers was separated as methyl ethers by chromatography on a silica gel column (3/4 silicic acid and 1/4 celite in anhydrous benzene). Elution was carried out with mixtures of anhydrous benzene and diethyl ether. The two acids are characterized by their melting points, 78 to 82 and 178 to 182 C.

(ii) Synthesis of radioactive 2-methyl-[2-14C]methyl 3-(2′-naphthyl 6′-hydroxy) pentanoic acid. Acid I, with a radioactive C methyl group bonded at position 2, was prepared by the reaction of alkylolation described in Fig. 1. This reaction was used to synthesize alkylated allenolic acids (10). The reaction performed by the authors was not quantitative and produced some by-products, which were formed from the alkyl halide and triphenyl-methyl sodium. Therefore, we investigated conditions which permit greater efficiency when working with small quantities of radioactive compounds. The following experimental method was used. Radioactive methyl iodide (0.15 mmol; specific activity, 13 μCi/mmol) was introduced in a vacuum-sealed glass vessel containing a magnetic hammer. A solution (0.087 mmol) of the methyl ester of 2-methyl 3-(2′-naphthyl 6′-methoxy) pentanoic acid (isomer mp, 110 C) in benzene was introduced in the upper compartment with 0.14 ml of a 0.15 N solution in diethyl ether or triphenyl methyl sodium (Fig. 2). After 40 min at room temperature, the container was connected to a...
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pentanoic acid. We thus obtained 26.7 mg of 2,2-dimethyl 3-(2'-naphthyl 6'-methoxy) pentanoic acid III with a 4°C atom bonded at position 2. Its specific activity is 50 μCi/mmol.

The radioactive phenol acid was obtained by heating the methyl ether, previously prepared with five times its weight of pyridine hydrochloride, at 200°C for 5 h. After cooling, the reaction mixture was diluted with water and the acid was extracted with diethyl ether. The solution was washed with diluted hydrogen chloride and water and dried over anhydrous sodium sulfate. After dilution with 2,2-dimethyl 3-(2'-naphthyl 6'-hydroxy) pentanoic acid and several recrystallizations from benzene, we obtained 2-methyl-[2-14C]methyl 3-(2'-naphthyl 6'-hydroxy) pentanoic acid I (specific activity, 11 μCi/mmol).

Growth of the microorganism. N. crassa Me- (American Type Culture Collection no. 10780) was stored at 4°C on slants made from Horowitz medium (14). Submerged cultures (80 ml of Horowitz medium supplemented with 40 μg of DL-methionine per liter) were inoculated with a conidial suspension (3 × 10^9 to 6 × 10^9 conidia) and incubated at 25°C on a rotary shaker. Under such conditions, after 48 h, the fungus was in the exponential phase of growth. The biotransformation of compounds was studied by adding them to the growth medium.

Purification and identification of the products of biotransformation. (i) Thin-layer chromatography. The products of biotransformation were analyzed by thin-layer chromatography of silicic acid (Kieselgel G, Merck AG). Elution was performed with mixtures of the solvents benzene, dioxane, and acetic acid (90:10:2 or 85:15:5, vol/vol/vol). The purification of the biotransformation products was accomplished, after esterification by a solution of diazomethane, in diethyl ether, with preparative thin-layer chromatography of silicic acid (Kieselgel H, Merck AG) as adsorbent and the same eluent.

Phenolic compounds were visualized by first spraying a mixture of parasulfanilic acid (0.3%) in 3% HCl and an equal volume of 0.5% sodium nitrite solution and then in 10% Na2CO3 solution.

Radioactive compounds were detected with a Berthold scanner.

(ii) Liquid-phase chromatography. Columns were packed with a suspension of 75% silicic acid and 25% celite (wt/wt) in anhydrous benzene. Elution was carried out with mixtures of benzene and ethyl acetate.

Infrared, nuclear magnetic resonance, mass spectroscopy spectra. Infrared spectra were performed with a Perkin-Elmer 237 apparatus, nuclear magnetic resonance spectra were done with a Varian
apparatus operating at 100 MHz, and mass spectra were performed with a Thomson-Houston 208 spectrometer.

RESULTS

Biotransformation of acid I [2,2-dimethyl 3-(2'-naphthyl 6'-hydroxy)pentanoic acid]. (i) Observation of a metabolite and studies of growth conditions of the fungus to obtain a metabolite accumulation. Preliminary experiments showed that a phenolic product appears in the culture medium of N. crassa several hours after addition of acid I. This product, colored red by sulfanilic acid reagent, has a slower migration rate than acid I in chromatographic analysis (Rf, 0.4 and 0.2 under the conditions described in Materials and Methods).

To prove that this compound proceeds from acid I, radioactive acid I was added to culture. Chromatographic analysis of the product extracted from the medium several hours after acid I was added shows the formation of a radioactive product which appears while acid I disappears (Fig. 3). Before determination of the structure of the metabolite, we studied the growth conditions which provide the best transformation of acid I. Only chromatographic analysis was used because this technique allows the observation of other possible biotransformation compounds.

Thus we determined that a metabolite of acid I appears while the acid I disappears. The metabolite disappears rapidly from the culture medium when acid I has been completely metabolized. The best transformation rate was obtained when acid I was added to 3-day-old cultures in the exponential phase of growth. A 80-ml inoculum containing 3 × 10⁹ to 6 × 10⁹ conidia was used. The dry mycelium weight was then 150 to 200 mg. The pH of the culture medium, which was slightly acid before inoculation (pH 5.7), became more acidic (pH 3 to 4) during biotransformation of acid I. If the substrate was added at the end of the exponential phase of growth, its metabolism was more rapid and several compounds were formed. If basic pH was maintained (pH 8), other compounds besides the observed metabolite were formed.

The optimal quantity of acid I, which was 10 mg for 80 ml of culture medium (125 µg/ml), caused little growth inhibition. In this latter case, acid I was slowly metabolized while its metabolite disappeared from the medium as soon as it was formed. The aeration of cultures produced by rotary shaking provided the best conditions. By bubbling sterile air in the culture medium, acid I was rapidly and thoroughly metabolized.

(ii) Structure of the metabolite of acid I. To determine the structure of the metabolite of acid I, a great number of cultures were extracted as described. After purification by preparative thin-layer chromatography, followed by liquid-column chromatography and esterification with diazomethane, we obtained crystals with 178 to 182 C mp. The infrared spectrum obtained from a potassium bromide disk was similar to that of acid I methyl ester in all regions except near 3,300 and 1,100 cm⁻¹.

The mass spectrum indicates a mass 302 corresponding to the possible formula C₁₆H₂₄O₄, which corresponds to the addition of one oxygen atom to acid I. Peaks at m/e 201 (M⁺ – 101) and m/e 173 (M⁺ – 129) are attributed to the cleavage of the side chain of the ester of acid I. Thus it is proved that biotransformation does not affect the side chain but rather the naphthyl moiety of the molecule.

The proton magnetic resonance spectrum shown in Fig. 4 was obtained from a solution in deuterated dimethyl sulfoxide (Me₂SO) with tetramethyl silane as internal reference. In this spectrum, the two lines at δ = 0.98 and 1.1 ppm

![Fig. 3. Thin-layer chromatograms of the extracts of culture medium of N. crassa 0, 12, 24, and 48 h after radioactive 2,2-dimethyl 3-(2'-naphthyl 6'-hydroxy) pentanoic acid was added to the culture. D is the radioactive substrate; C is its metabolite; and C and D, respectively, are red and orange colored by spraying parasulfanilic acid reagent.](http://jb.asm.org/)

FIG. 3. Thin-layer chromatograms of the extracts of culture medium of N. crassa 0, 12, 24, and 48 h after radioactive 2,2-dimethyl 3-(2'-naphthyl 6'-hydroxy) pentanoic acid was added to the culture. D is the radioactive substrate; C is its metabolite; and C and D, respectively, are red and orange colored by spraying parasulfanilic acid reagent.
come from the two methyls on carbon 2. The triplet at δ = 0.64 ppm is due to the protons on carbon 5 and are coupled to methylene protons at C-4 which appear as a broad line at δ = 1.6 ppm. The proton at C-3 is located at 2.7 ppm. The signal of the methyl ester group is at δ = 3.60 ppm. Lines at δ = 2.52 and δ = 3.27 ppm come from Me₂SO-D₄ and the remaining water, respectively. This last peak is shifted to δ = 3.62 ppm when D₂O is added to the solution. The two phenolic protons are located at δ = 9.46 and δ = 9.6 ppm. These lines disappear when deuterium oxide is added (Fig. 4). The chemical shifts of the different protons of the side chain are equal to those of the side chain of the substrate except for the proton on carbon 3, the chemical shift of which is 2.92 ppm. In the molecule studied, the position of one hydroxyl group was unknown and the analysis of the spectrum corresponding to the aromatic region was used to determine it.

The analysis was as follows: (i) two doublets not well resolved at δ = 6.57 and δ = 6.94 ppm with a coupling constant on the order of 1.5 Hz which correspond to two meta-coupled protons with no other neighboring protons; (ii) an A-B-C system with a doublet at δ = 7.55 ppm with J = 9 Hz which corresponds to an ortho coupling (1) between protons located on carbons 7', 8', or equivalent ones; (iii) a doublet at δ = 7.28 ppm with J = 2.3 Hz which corresponds to a meta coupling; and (iv) a quadruplet at δ = 6.94 ppm with J = 9 and J = 2.3 Hz, which indicates that this proton is coupled to the two preceding ones.

Then we have two meta-coupled protons on one cycle of the naphthalene skeleton and two ortho-coupled protons, one of them meta coupled to a third proton on the same cycle. From this analysis, since there is the side chain on carbon C-2' and an hydroxy group on carbon C-6', two possible positions remain for the second hydroxyl group: C-4' or C-8'.

The two observed meta couplings are quite different: 1.5 and 2.3 Hz. From the study of a number of naphthalene derivatives (3; Granger and Maugras [submitted for publication]), it appears that when a substituent on carbon 2', or the equivalent, is a proton or a carbon the meta-coupling constant J₁,₂ is lower than 1.8 Hz. On the contrary, when this carbon is bonded to an oxygen atom, as for hydroxy, methoxy, acetoxy groups, this coupling constant is greater than 2.2 Hz. In our case the meta coupling corresponding to the A-B-C system was the greater one (2.3 Hz) and corresponded to an hydroxy group between two protons. The only possibility was to bond the second hydroxyl group to the C-4' carbon. This assignment may be confirmed by the fact that the resonance of the proton on carbon 3 is slightly shifted when compared to the substrate (δ = 2.9 ppm), the other protons of the side chain being unshifted.

Thus, the compound of the biotransformation of acid I by N. crassa is the 2,2-dimethyl 3-(2'-naphthyl 4',6'-dihydroxy) pentanoic acid IV (Fig. 5).

(iii) Optical activity. Acid I has an asymmetric center. The product added to the culture media is the racemic form. It is of some interest
Fig. 5. Biotransformations of acids of the allenolic group. (A) Biotransformation of 2,2-dimethyl 3-(2'-naphthyl 6'-hydroxy) pentanoic acid I; (B) supposed biotransformation of 2-methyl 3-(2'-naphthyl 6'-hydroxy) pentanoic acid II; (C) possible routes of biotransformation of the methyl ether of acid I.

to know whether both optical isomers are metabolized at the same rate. For this study we stopped the cultures before acid I was completely metabolized and purified the nontransformed acid from cultures to which 100 mg of allenolic acid I had been added. The optical activity of the acid was: \([\alpha]_D = +4.25^\circ\). This is a low value compared with \([\alpha]_D = +25.5^\circ\) for the resolved acid (17), but the fact that nontransformed allenolic acid I possesses an optical activity shows that the levorotatory isomer, which is the most active, is metabolized more rapidly than the dextrorotatory one.

**Biotransformation of 2-methyl 3-(2'-naphthyl 6'-hydroxy) pentanoic acids II.** The biotransformations were only examined by thin-
layer chromatographic analysis. Chromatographic analysis of extracts of the culture medium at different intervals of time reveals that these acids were metabolized at different rates, and two metabolites appear, one more rapidly than the other. Their migration rates in chromatographic analysis were similar to that of the metabolite of acid I.

We compared the rates of the metabolism of the diastereoisomers. With the growth culture conditions used, 24 h after the addition of the isomer with mp 78 to 82 °C, it was completely metabolized, whereas the isomer with mp 178 to 182 °C was still present. This was also true when two isomers were added separately, under the same conditions and in similar cultures. We emphasize that the isomer with mp 78 to 82 °C is the one which had the strongest estrogenic activity (8).

**Biotransformation of 2,2-dimethyl 3-(2'-naphthyl 6'-methoxy) pentanoic acid III.** For this study we used radioactive acid III with the conditions described above. Qualitative analysis indicated two metabolites. The first metabolite that appeared had the same migration rate as acid I and the second one had the same chromatographic characteristics as the metabolite of acid I (Fig. 6). We did not study this any further due to the small quantities of product formed. We only tried to identify the first metabolite. A great number of cultures were made, and this metabolite, assumed to be acid I, was purified by thin-layer chromatography. Its infrared spectrum was similar, but not identical, to the one of acid I.

**DISCUSSION**

We have shown that acid I 2,2-dimethyl 3-(2'-naphthyl 6'-hydroxy) pentanoic acid is transformed into its hydroxylated derivative 2,2-dimethyl 3-(2'-naphthyl 4',6'-dihydroxy) pentanoic acid IV. This metabolite is the main product of the biotransformation of acid I. Culture conditions have permitted its accumulation in the culture medium. Since this product disappears when the cultures are not stopped at the proper time, we suppose that it is a product of the metabolic pathway of the degradation of acid I. This result agrees with the literature. Indeed, nine types of fungi have been shown to hydroxylate anisole (2) and a great number of compounds are hydroxylated by fungi. For example, Grabbe, Laskin, and Casas-Campillo (4, 11, 18) described hydroxylation of estradiol and estrone with Glomerella fusaroides, Glomerella glycines, Fusarium moniliforme, Gibberella fujikuroi, Mortiella alpina, and Aspergillus carneus. N. crassa has been shown to hydroxylate deoxycorticosterone (21), estradiol, and equilenin (19, 20). All the results obtained with one fungus do not permit prediction of the positions of hydroxylation at 11β, 12β, and 8 or 9 for estradiol, equilenin, and deoxycorticosterone, respectively. The use of acid I after estradiol and equilenin provides evidence for a hydroxylation of the aromatic B ring under the same conditions of hydroxylation for the C ring of estradiol and equilenin. We could explain the different positions of hydroxylation: 11β and 12β for estradiol and equilenin by the role played by the aromatic B ring, which imposes different orientation of the hydrogen atoms bonded to carbons of the C ring (19). In the case of acid I we could predict a hydroxylation in the ortho position of the phenolic function to give an orthodiphenol, a preliminary form of degradation of aromatic rings. The use of other aromatic substrates will perhaps explain the position of the hydroxylation of acid I. Studies in this direction are in progress.

![Fig. 6. Thin-layer chromatograms of the extracts of culture medium of N. crassa 0, 12, 24, and 48 h after radioactive 2,2-dimethyl 3-(2'-naphthyl 6'-methoxy) pentanoic acid was added in the culture. B is the radioactive substrate; D and C are its metabolites; D and C, respectively, are orange and red colored after spraying parasulfanilic acid reagent; and B is brown colored after spraying sulfochromic reagent.](http://jb.asm.org/)
Studies with 2-methyl 3-(2'-naphthyl 6'-hydroxy) pentanoic acid II do not permit the determination of the structures of the metabolites of the two diastereoisomeric acids. From the chromatographic analysis of the metabolites it is inferred that these acids are hydroxylated at the same position as allenolic acid I.

Results obtained with methyl ether III can be explained by the scheme given in Fig. 5. It is assumed that hydroxylation is possible on the ether but not necessarily after an O-demethylation reaction.

These results should provide information on the biotransformation of allenolic acids in mammals because of the analogy between hydroxylating enzymatic systems of fungi and of mammals (9).

In another way, when cultures were stopped before complete biotransformation of racemic acid I was achieved, the optical activity of the remaining nontransformed acid I is \([\alpha]_{D}^o = +4.25^\circ\). This value is weak in regard to that of the resolved acid I, \([\alpha]_{D}^o = +25.5^\circ\) (7). This result agrees with two different rates of metabolism for the two isomers. The levorotatory isomer is metabolized more rapidly than the dextrorotary one. We must note that the levo-rotatory acid has an estrogenic activity greater than that of the dextrorotary one; so the isomer having the greatest biological activity appears to be metabolized more rapidly. In the same way, 2-methyl 3-(2'-naphthyl 6'-hydroxy) pentanoic acid II, diastereoisomer with mp 78 to 82 C, is metabolized more rapidly than the other isomer with mp 178 to 182 C, which possesses the weaker biological activity. This observation still holds if we compare the rate of metabolism of the methyl ether of acid I and that of the corresponding phenolic acid, which possesses a more important estrogenic activity.

All these results indicate a correlation between the estrogenic activity and the rate of metabolism. One explanation is that the fungus has been in contact with a long time with the natural hormone, which possesses the common stereo-chemical structure of steroids. Thus, enzymatic systems for their degradation or elimination have been induced. Since the biological activity of an allenolic acid is the greatest when its structure is the most similar to the structure of the natural hormone, it is not surprising that the rate of metabolism shows the same variations.

In conclusion, we have shown that 2,2-dimethyl 3-(2'-naphthyl 6'-hydroxy) pentanoic acid I is hydroxylated in vivo by the fungus N. crassa to form 2,2-dimethyl 3-(2'-naphthyl 4', 6'-dihydroxy) pentanoic acid IV. Comparison with other acids of the allenolic group establishes that the rate of metabolism of allenolic acids varies in the same way as their estrogenic activity.

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LITERATURE CITED


