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Fungal Detoxication

THE METABOLISM OF ω-(2-NAPHTHYLOXY)-*n*-ALKYLCARBOXYLIC ACIDS BY *ASPERGILLUS NIGER*

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(*Received* 26 *January* 1956)

Detoxication studies in higher animals have been extensively reviewed by Williams (1947), who has used the term 'detoxication' to cover all drug metabolism, whether or not associated with a decrease in toxicity. Its importance in other biological systems is now becoming increasingly apparent, e.g. in the development of drug resistance in bacteria (Albert, 1951, p. 168), in selective weed control (Wain, 1954) and in insecticidal action (Casida, 1955). The metabolism of toxic materials by fungi, however, has received little attention except in a number of specific instances (Rich & Horsfall, 1954; Byrde & Fielding, 1955). In view of its fundamental importance in fungicidal action, we have now commenced a systematic study of fungal detoxication.

The fungicidal activity of certain ω-aryloxyalkylcarboxylic acids (Crowdy, 1948) and the knowledge that they are capable of translocation in plants led to investigations of their possible value as systemic fungicides against plant disease (Crowdy & Wain, 1951; Byrde, Crowdy & Woodcock, 1953). Such studies were confined to assessments of their fungitoxicity both *in vivo* and *in vitro*, no attempt being made to investigate the biochemistry of the host–parasite–fungicide relationships.

From other sources, however, there is considerable evidence, both direct and indirect, that ω-aryloxyalkylcarboxylic acids undergo β-oxidation in plants, forming the corresponding aryloxyacetic acid or phenol according as the side chain possesses an even or an odd number of carbon atoms (Synerholm & Zimmerman, 1947; Fawcett, Ingram & Wain, 1954; Wain & Wightman, 1954; Luckwill & Woodcock, 1955). It has also been suggested (Holley, Boyle & Hand, 1950) that nuclear hydroxylation (in an unspecified position) may occur during the metabolism of 2:4-dichlorophenoxyacetic acid in plants.

Little is known of the metabolism of such acids by micro-organisms, however, though ω-phenyl-substituted fatty acids have been studied (Rittenberg & Ivler, 1952; Webley, Duff & Farmer, 1955). It was demonstrated by Webley *et al.* (1955), who used *Nocardia opaca* Waksman & Henrici, that, although acids with an odd number of carbon atoms in the side chain underwent β-oxidation to benzoic acid, *o*-hydroxyphenylacetic acid was the only product identified from the even-numbered acids. They considered that this acid was formed by a side reaction from phenylacetic acid, the remainder of which was metabolized further. Evans & Smith (1954) isolated a small motile bacterium from soil, which converted 4-chlorophenoxyacetic acid into 2-hydroxy-4-chlorophenoxyacetic acid and 4-chlorocatechol. A small Gram-negative motile rod was also isolated, which converted 2:4-dichlorophenoxyacetic acid into a phenolic acid, presumed, on chromatographic evidence, to be 6-hydroxy-2:4-dichlorophenoxyacetic acid. Brown & McCall (1955), however, have reported that this metabolite is not 2:4-dichloro-6-, 4-chloro-2- or 2-chloro-4-hydroxyphenoxy acetic acid.

The investigations described in this and subsequent papers were undertaken to explore the metabolism of ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids by fungi. Some of the results formed the basis of a preliminary communication (Woodcock & Byrde, 1955).

EXPERIMENTAL

Materials and reference compounds

ω-(2-Naphthyloxy)-n-alkylcarboxylic acids. These were prepared as described by Pope & Woodcock (1954).

(6-Nitro-2-naphthyloxy)acetic acid. 6-Nitro-2-naphthol (4 g.) and ethyl bromoacetate (4 ml.) were refluxed overnight in a solution of sodium (0·52 g.) in ethanol (50 ml.). After the addition of 10% NaOH (10 ml.) the solution was refluxed for a further 1 hr., cooled, acidified and extracted with ether. Re-acidification of the bicarbonate-soluble material gave the required *acid*, which crystallized from aqueous ethanol in cream-coloured monoclinic prisms, m.p. 211–212°. (Found: C, 58·2; H, 3·8; N, 5·6. $C_{12}H_9O_5N$ requires C, 58·3; H, 3·6; N, 5·7%.) This compound was obtained by Shibata & Okuyama (1936), who gave m.p. 157–185°, but no analytical data. The *ethyl ester* crystallized from benzene in pale-yellow needles, m.p. 111–112°. (Found: C, 61·3; H, 4·7; N, 4·9. $C_{14}H_{13}O_5N$ requires C, 61·1; H, 4·7; N, 5·1%.)

Ethyl (6-amino-2-naphthyloxy)acetate. A solution of the ethyl ester (1·7 g.) in tetrahydrofuran (10 ml.) was shaken in hydrogen with Raney nickel until no further gas was absorbed. Removal of the solvent from the filtered solution and crystallization of the residual solid from ethanol gave the *amine* (1·3 g.) as nacreous plates, m.p. 80–81°. (Found: C, 68·1; H, 6·0; N, 5·7. $C_{14}H_{15}O_3N$ requires C, 68·6; H, 6·1; N, 5·7%.)

(6-Hydroxy-2-naphthyloxy)acetic acid. Ethyl (6-amino-2-naphthyloxy)acetate (1·3 g.) was diazotized at 0° in HCl solution with $NaNO_2$ (0·4 g.), and the solution poured into boiling 20% H_2SO_4 (100 ml.). The reddish solid product was extracted with ether and crystallized from aqueous methanol. Recrystallization of the *acid* from benzene gave prisms, m.p. 165–166°. (Found: C, 65·9; H, 4·8. $C_{12}H_{10}O_4$ requires C, 66·0; H, 4·6%.) The *methyl ester*, prepared in ethereal solution by the use of diazomethane, crystallized from benzene in prisms, m.p. 131–132°. (Found: C, 67·5; H, 5·3. $C_{13}H_{12}O_4$ requires C, 67·2; H, 5·2%.)

β-(6-Nitro-2-naphthyloxy)propionic acid. β-Propiolactone (2·5 g. = 1·5 equivalents) was added dropwise during 10 min. to a boiling stirred solution of 6-nitro-2-naphthol (4·7 g.) and NaOH (1 g.) in water (50 ml.). After the boiling solution had been stirred for a further 15 min. the solution was cooled, acidified and extracted with ether. The *acid*, isolated from the ethereal solution by shaking with $NaHCO_3$ solution and acidifying, was re-extracted with ether, the extract dried and the solvent removed. The residue crystallized from 50% aqueous methanol in long brown rectangular prisms, m.p. 163–164°. (Found: C, 59·5; H, 4·4; N, 5·3. $C_{13}H_{11}O_5N$ requires C, 59·8; H, 4·2; N, 5·4%.) The *ethyl ester*, prepared in the usual way, crystallized from ethanol in pale-yellow prisms, m.p. 110–111°. (Found: C, 62·6; H, 5·2; N, 4·9. $C_{15}H_{15}O_5N$ requires C, 62·3; H, 5·2; N, 4·8%.)

Ethyl β-(6-amino-2-naphthyloxy)propionate. This *ester* was prepared from the appropriate nitro ester as described above for the corresponding acetate. It crystallized from ethanol in colourless plates, m.p. 86–87°. (Found: C, 69·7; H, 6·7; N, 5·6. $C_{15}H_{17}O_3N$ requires C, 69·5; H, 6·6; N, 5·4%.)

β-(6-Hydroxy-2-naphthyloxy)propionic acid. A solution of the aminopropionic ester (2·3 g.) in a mixture of conc. HCl (5 ml.) and water (20 ml.) was diazotized at 0–5° by the gradual addition of $NaNO_2$ (0·7 g.) dissolved in a little water. After being stirred for 1 hr. the mixture was poured into boiling 20% H_2SO_4 (200 ml.) and cooled, and the mauve-coloured product collected, washed with water and dried at 100° (1·7 g., m.p. 155–180°). Repeated crystallizations of the *acid* from aqueous methanol (charcoal) finally gave prismatic plates, m.p. 194–195°. (Found: C, 67·3; H, 5·3. $C_{13}H_{12}O_4$ requires C, 67·2; H, 5·2%.) The *methyl ester* prepared in ethereal solution by the use of diazomethane, crystallized from benzene in large prismatic plates, m.p. 152–153°. (Found: C, 68·0; H, 6·0. $C_{14}H_{14}O_4$ requires C, 68·3; H, 5·7%.) The *p-nitrobenzyl ester*, prepared by refluxing in ethanolic solution, crystallized from acetone in prisms, m.p. 175–176°. (Found: C, 65·5; H, 4·3. $C_{20}H_{17}O_6N$ requires C, 65·4; H, 4·6%.)

γ-(6-Nitro-2-naphthyloxy)butyronitrile. 6-Nitro-2-naphthol (7·7 g.) was refluxed for 5 hr. with γ-bromobutyronitrile (8·2 ml.) in a solution of sodium (0·95 g.) in ethanol (50 ml.). After most of the ethanol had been distilled off the mixture was cooled, diluted with water and the *nitrile* extracted with ether. The ethereal solution was washed with dilute NaOH solution and then water, dried and the solvent removed. The residual solid crystallized from methanol in large orange-red plates, m.p. 99–99·5°. (Found: C, 65·5; H, 4·7; N, 10·8. $C_{14}H_{12}O_3N_2$ requires C, 65·6; H, 4·7; N, 10·9%.)

γ-(6-Nitro-2-naphthyloxy)-n-butyric acid. The nitrile (6·7 g.) was refluxed with water (50 ml.), conc. H_2SO_4 (30 ml.) and ethanol (100 ml.) for 18 hr. After the excess of ethanol had been distilled off, the solution was diluted with water and extracted with ether. The *acid* (0·45 g.) isolated with $NaHCO_3$ from the ethereal solution crystallized from aqueous ethanol in minute brown prisms, m.p. 184–185°. (Found: C, 61·0; H, 4·9; N, 5·2. $C_{14}H_{13}O_5N$ requires C, 61·1; H, 4·7; N, 5·1%.) Evaporation of the residual ether solution gave the *ethyl ester* (6·7 g.) m.p. 53–58° (67–68° after recrystallization from aqueous methanol). (Found: C, 63·5; H, 5·6; N, 4·7. $C_{16}H_{17}O_5N$ requires C, 63·4; H, 5·6; N, 4·6%.)

Ethyl γ-(6-amino-2-naphthyloxy)-n-butyrate. A solution of the nitro ester (2·3 g.) in tetrahydrofuran was shaken with Raney nickel in an atmosphere of hydrogen until no further uptake of gas was observed. Removal of the solvent gave the *amine*, which crystallized from aqueous ethanol in large nacreous plates, m.p. 86–87°. (Found: C, 70·6; H, 6·9; N, 5·2. $C_{16}H_{19}O_3N$ requires C, 70·3; H, 6·95; N, 5·1%.)

γ-(6-Hydroxy-2-naphthyloxy)-n-butyric acid. A solution of the amino-ester (1·9 g.) in HCl (5 ml.) and water (20 ml.) was diazotized at 0–5° by the dropwise addition of $NaNO_2$ (0·55 g.) dissolved in water (2 ml.). After stirring for 1 hr., the material was hydrolysed by adding the solution to boiling 20% H_2SO_4 (200 ml.). The product, collected after cooling, consisted of a mixture of the required acid and its ethyl ester; hydrolysis was completed by heating for a further 2 hr. with 10% NaOH (10 ml.). After cooling and acidifying, the *acid* was isolated with ether and washed with water, the ethereal solution dried and the solvent removed. The acid crystallized from aqueous methanol in aggregates of rectangular prisms, m.p. 157–158°. (Found: C, 68·0; H, 5·7. $C_{14}H_{14}O_4$ requires C, 68·3; H, 5·7%.)

Methods

Replacement culture technique and isolation of metabolites. Cultures of *Aspergillus niger* van Tiegh. (Mulder strain) were grown on the following medium, based on that described by Nicholas & Fielding (1951): glucose, 50 g.; KNO_3, 5 g.; KH_2PO_4, 2·5 g.; $MgSO_4,7H_2O$, 1 g.; Na_2SO_4, 1 g.; water, 1 l. The medium was sterilized in bulk (5 lb./sq.in. for

25 min.), and after addition of trace elements $FeCl_3,6H_2O$ (20 mg.), $ZnSO_4,7H_2O$ (10 mg.), $MnSO_4,4H_2O$ (3 mg.), $Na_2MoO_4,2H_2O$ (1·5 mg.) and $CuSO_4,5H_2O$ (1 mg.) was inoculated with a heavy spore suspension of *A. niger* and dispensed in sterile 20 oz. medicinal 'flats' (100 ml. each) or in penicillin culture flasks (500 ml. each).

After incubation at 25° for 3 days, the medium was poured off from the fungal mat, which was then washed with distilled water. It was replaced by a solution (or suspension, for acids of low solubility) of the test acid (0·001 M) in 0·01 M-Na_2HPO_4, the pH of which was approx. 8·1. After a further 7 days' incubation at 25°, the substrate was removed, strained through muslin and continuously extracted with ether for 18 hr. The ethereal solution was then shaken successively with saturated $NaHCO_3$, dilute NaOH and then water. Acidic products were isolated with ether after acidifying the $NaHCO_3$ solution and examined chromatographically as described below.

Sporulation was usually beginning when the new substrate was introduced, and marked pigmentation was apparent by the following day. In one experiment when fungal growth was poor and no pigmentation occurred, the usual metabolites could not be isolated.

Chromatographic examination of ether extracts. The ether extracts were evaporated to dryness, and the residue was dissolved in ether–methanol (4:1) (2·5 ml./l. of initial aqueous substrate). Samples (9·5 µl.), together with known compounds as required, were applied to Whatman no. 1 paper by means of a capillary pipette, and the chromatogram was developed overnight at laboratory temp. by downward solvent flow. The solvents used were *n*-butanol–ethanol–3 N ammonia (4:1:5) and *n*-butanol–acetic acid–water (4:1:5). The former gave best resolution, and was used for routine examinations.

After being dried at laboratory temperature, chromatograms were exposed under ultraviolet light (wavelength of maximum emission, 2539 Å) and fluorescent spots marked. They were subsequently sprayed with one of the following reagents: (1) *p*-nitrobenzenediazonium fluoroborate, as a 1% (w/v) solution in 20% (w/v) aqueous sodium acetate, for the detection of phenolic compounds as coloured spots (Freeman, 1952). If the papers were subsequently exposed to ammonia vapour while still moist, unhydroxylated acids showed as pale spots on a pink background. (2) Bromocresol green as a 0·04% solution in 95% (v/v) aqueous ethanol, adjusted to pH 5·5 (Lugg & Overell, 1948), for the detection of acids. This reagent was not suitable for papers developed in the ammoniacal solvent.

Where chromatographic evidence indicated the presence of only one metabolite, it was often possible to isolate it directly from the extracts by conventional methods. Where the presence of two major metabolites was indicated, however, and difficulty was encountered in isolating them, large-scale chromatographic separation on Whatman no. 3 MM paper was carried out. Bands fluorescing under ultraviolet light were marked and a marker strip from the edge of each sheet was sprayed with reagent (1). The appropriate bands on the main sheets were then cut out and eluted overnight with methanol. After evaporation of the solvents the residue was acidified with a few drops of conc. HCl and the acid extracted with ether.

In vitro fungitoxicity. For comparisons of fungitoxicity the medium already described was modified by increasing the KH_2PO_4 concn. to 3·4 g./l. (0·025 M) to ensure adequate buffering. The pH was adjusted to approx. 2·2 by the addition of H_3PO_4, the final phosphate concentration being 0·05 M.

The medium was sterilized in bulk, inoculated with a heavy spore suspension of *A. niger*, and dispensed in 50 ml. lots in 100 ml. conical flasks. To each was added 0·5 ml. of an ethanolic solution of the acid being tested; after thorough agitation, three replicates (16 ml. each) were dispensed into 4 oz. medicinal 'flats'. A control treatment, to which 0·5 ml. of ethanol alone was added, was included.

After incubation of the bottles on their sides for 6 days at 25°, the fungal mats were harvested, washed with distilled water, dried between sheets of coarse filter paper and then at 80° for 2–3 days. Mycelial dry weights were determined after cooling in a desiccator.

For statistical analysis the data (y) were transformed to x values, where $x = \log(y+1)$, since it was found that there was an approx. linear relationship between $\sqrt{[\text{variance}\ (y)]}$ and $\bar{y}$ (Anscombe, 1948).

RESULTS

Identification of metabolites

The metabolism of ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids by *A. niger* gave hydroxy acids which were detected by their characteristic deep-pink colour when papers were sprayed with reagent (1). The identification of the principal metabolites is summarized in Table 1. Chromatographic examination of $NaHCO_3$-insoluble extracts yielded negative results in all cases.

Metabolism of 2-naphthyloxyacetic acid. The acidic material extracted from the fungal substrate after incubation with 2-naphthyloxyacetic acid was a light-brown amorphous powder, m.p. 148–150°, which, after several crystallizations from water (with charcoal), gave prismatic plates, m.p. 163–164°, not depressed by admixture with (6-hydroxy-2-naphthyloxy)acetic acid. (Found: C, 65·8; H, 4·3%.) The *methyl ester*, prepared as described above, crystallized from benzene in rosettes of prisms, m.p. 132–133°, not depressed by admixture with the authentic methyl ester. (Found: C, 67·5; H, 5·1%.)

Metabolism of β-(2-naphthyloxy)propionic acid. The acidic material extracted from the fungal substrate after incubation with β-(2-naphthyloxy)-propionic acid had m.p. 175–185°. Several recrystallizations from aqueous ethanol (with charcoal) gave plates, m.p. 193–195°, not depressed by admixture with β-(6-hydroxy-2-naphthyloxy)propionic acid. (Found: C, 67·4; H, 5·4%.) The *methyl ester*, prepared as described above, crystallized from benzene in plates, m.p. 147–150°, not depressed by admixture with the authentic methyl ester, m.p. 152–153°.

Metabolism of γ-(2-naphthyloxy)-n-butyric acid. Although 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids usually gave only single phenolic products, the presence of more than one phenolic spot was occasionally apparent with the

Table 1. *Identification of principal metabolites of* ω-(2-*naphthyloxy*)-n-*alkylcarboxylic acids incubated with* A. niger

Substrate [ω-(2-naphthyloxy) derivative of the acid shown]	Principal metabolite	R_F value*	Identification
Acetic acid	(6-Hydroxy-2-naphthyloxy)acetic acid (I)	0·30	Mixed m.p. and mixed m.p. of methyl ester with authentic specimens
Propionic acid	β-(6-Hydroxy-2-naphthyloxy)propionic acid (II)	0·46	Mixed m.p. and mixed m.p. of methyl ester with authentic specimens
n-Butyric acid	(I)	0·31	Mixed m.p. with authentic specimen
n-Valeric acid	(II)	0·46	Mixed m.p. with authentic specimen
n-Hexanoic acid	(I)	0·31	Mixed m.p. with authentic specimen
n-Heptanoic acid	(II)	0·45	Mixed m.p. with authentic specimen
n-Octanoic acid	(I)	0·30	R_F value
n-Nonanoic acid	(II)	0·45	Mixed m.p. with authentic specimen
n-Decanoic acid	(I)	0·30	R_F value
n-Dodecanoic acid	(I)	0·32	R_F value

* In *n*-butanol–ethanol–3N ammonia (4:1:5).

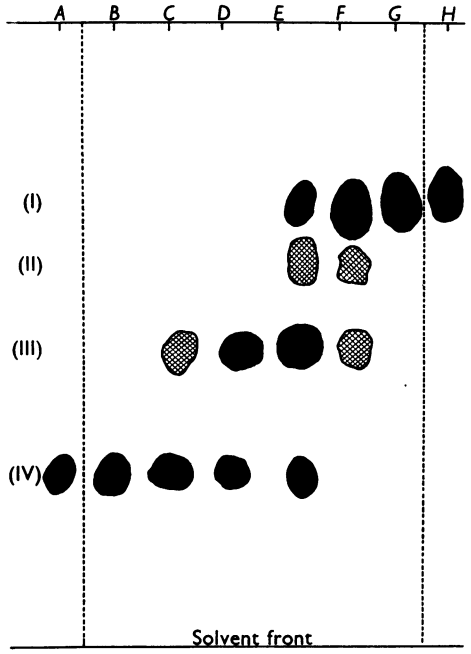


Fig. 1. Tracing of chromatogram of extracts from samples taken at intervals from γ-(2-naphthyloxy)-*n*-butyric acid incubated with *A. niger*. *A*: authentic γ-(2-naphthyloxy)-*n*-butyric acid. *B–G*: samples taken after 1, 2, 4, 8, 24 and 72 hr. respectively. *H*: authentic (6-hydroxy-2-naphthyloxy)acetic acid. All spots showed purple fluorescence under ultraviolet light (2539 Å). Spots (I), (II) and (III) gave deep-pink colour with *p*-nitrobenzenediazonium fluoroborate. Faint spots shaded.

higher members of this series. γ-(2-Naphthyloxy)-*n*-butyric acid was therefore selected for a more detailed study. Samples (25 ml.) from each of two flasks, initially containing 500 ml. of substrate, were taken after intervals of 1, 2, 4, 8, 24 and 72 hr. After the usual extraction procedure the acidic material was examined chromatographically together with the appropriate known compounds. The results are illustrated diagrammatically in Fig. 1. The gradual conversion of the butyric acid (IV) into (6-hydroxy-2-naphthyloxy)acetic acid (I) is obvious. Large-scale paper-chromatographic separation of acidic extracts obtained after 6 hr. enabled compound (III) (R_F 0·52) to be isolated. It crystallized from water and had m.p. 145–150°, not depressed by admixture with γ-(6-hydroxy-2-naphthyloxy)-*n*-butyric acid, m.p. 157–158°. The diagram also indicates the presence of a second, transient and less clearly marked intermediate (II) (R_F 0·39) between γ-(6-hydroxy-2-naphthyloxy)-*n*-butyric acid and (6-hydroxy-2-naphthyloxy)acetic acid, but no attempt was made to isolate this compound.

The conversion of the butyric acid (IV) into (6-hydroxy-2-naphthyloxy)acetic acid (I) was apparently unaffected by the presence of β-(2-naphthyloxy)propionic acid or 2:6-dihydroxynaphthalene.

Hydroxylation with a model system

Hydroxylation was further investigated with the ascorbic acid system developed by Udenfriend, Clark, Axelrod & Brodie (1954), which under physiological conditions of temperature and pH hydroxylates aromatic substances in a manner shown to be closely analogous to that occurring *in vivo*. A solution of 2-naphthyloxyacetic acid (0·12 g.), ascorbic acid (0·25 g.), $FeSO_4,7H_2O$ (0·04 g.) and sodium ethylenediaminetetraacetate (0·23 g.) in 0·2M phosphate buffer pH 6·8 (30 ml.) and acetone (10 ml.) was maintained at 37° for 2 hr. during the passage of a stream of air. The acidified solution was then extracted with ether and acidic substances were separated from other organic material with $NaHCO_3$ solution. Chromatographic examination of the bicarbonate-soluble fraction

showed the presence of unchanged 2-naphthyloxyacetic acid (R_F 0·63), (6-hydroxy-2-naphthyloxy)acetic acid (R_F 0·34) and an unknown substance giving a dark-brown spot with diazotized *p*-nitraniline (R_F 0·44). The bicarbonate-insoluble fraction gave only one spot (bright orange, R_F 0·97), which appears to be β-naphthol.

In vitro *fungitoxicity of unsubstituted acids*

Table 2 summarizes the results of toxicity tests with the unsubstituted acids against *A. niger* at pH 2·2. The pK_a value of 2-naphthyloxyacetic acid, determined experimentally, was 3·7, and a reliable comparison of inherent toxicity is therefore obtained at pH 2·2 where the acids are predominantly undissociated and thus more active (Simon & Blackman, 1949).

Table 2. *Fungistatic activity against* A. niger *of acids of the general formula* $C_{10}H_7.O.[CH_2]_n.CO_2H$ *at a concn. of* 10^{-4} M

n	Mean mycelial wt. (mg.)	Mean of transformed data*
1	335·4	2·53
2	5·9	0·66
3	5·3	0·76
4	28·3	1·33
5†	225·4	2·35
Control	295·2	2·47

Least significant difference between means of transformed data: ($P=0·05$), 0·39.

* After applying the transformation $x=\log(y+1)$ (see text).

† No significant inhibition of growth for values of *n* from 6 to 8.

Relative toxicity of hydroxylated and unsubstituted acids

In preliminary experiments, provisional dosage–response lines for the unsubstituted and the corresponding hydroxylated acids were not parallel, and it was therefore impossible to obtain an absolute figure for relative potency (Finney, 1952, p. 65). The data of Table 3, however, show that the overall growth of the fungus in the presence of three fungistatic concentrations of 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids was significantly less than that in the presence of the corresponding hydroxylated acids at concentrations eight times higher.

DISCUSSION

The most significant feature of the present work on the metabolism of the ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids is their nuclear hydroxylation, together with β-oxidation of the side chain. Though this substitution of a hydroxyl group in position 6 in preference to position 1 is chemically unusual, hydroxylation in both positions with 2-naphthylamine has been reported with rat-liver slices (Booth, Boyland & Manson, 1955), and in the animal body (Boyland, Manson, Sims & Williams, 1955). The same substitution was shown by the former workers to be possible with the ascorbic acid–$FeSO_4$

Table 3. *Effect of 6-hydroxylation on the fungistatic activity of 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids against* A. niger

2-Naphthyloxyacetic acid			(6-Hydroxy-2-naphthyloxy)acetic acid		
Concn. ($\times 10^{-4}$ M)	Mean mycelial wt. (mg.)	Mean of transformed data*	Concn. ($\times 10^{-4}$ M)	Mean mycelial wt. (mg.)	Mean of transformed data*
2·50	0·0	0·000	20·0	73·5	1·853
1·75	2·7	0·367	14·0	140·1	2·140
1·25	84·0	1·470	10·0	148·6	2·167
Overall mean	28·9	**0·612**	Overall mean	120·7	**2·053**
β-(2-Naphthyloxy)propionic acid			β-(6-Hydroxy-2-naphthyloxy)propionic acid		
Concn. ($\times 10^{-4}$ M)	Mean mycelial wt. (mg.)	Mean of transformed data*	Concn. ($\times 10^{-4}$ M)	Mean mycelial wt. (mg.)	Mean of transformed data*
1·25	0·0	0·000	10·0	2·1	0·477
0·88	0·0	0·000	7·0	5·0	0·753
0·63	2·3	0·410	5·0	45·4	1·663
Overall mean	0·8	**0·137**	Overall mean	17·5	**0·964**

Least significant difference between individual means of transformed data: ($P=0·05$), 0·562; ($P=0·01$), 0·761; ($P=0·001$), 1·019.

Least significant difference between overall means of transformed data: ($P=0·05$), 0·324; ($P=0·01$), 0·439; ($P=0·000$), 0·588.

Comparable mean wt. of controls = 234·4 mg. (mean of transformed data = 2·370).

* After applying the transformation $x=\log(y+1)$ (see text).

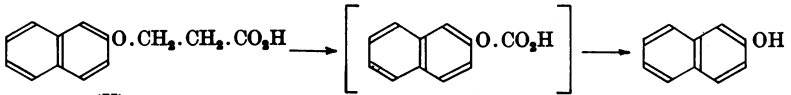
reagent of Udenfriend *et al.* (1954). This system has also been shown by Dalgliesh (1955) to hydroxylate phenylacetic acid in the *ortho-* and *para*-positions, and we have found that 2-naphthyloxyacetic acid may under these conditions give another hydroxy acid in addition to (6-hydroxy-2-naphthyloxy)-acetic acid. Identification of the unknown acid has not been possible, however, owing to the inaccessibility of (1-hydroxy-2-naphthyloxy)acetic acid. Synthetic routes to acids of this type are being currently explored.

Although those acids having an even number of carbon atoms in the side chain were successively β-oxidized to (6-hydroxy-2-naphthyloxy)acetic acid, as might be expected from earlier work with these compounds in certain plants, the corresponding acids with an odd number of carbon atoms in the side chain provided an anomaly. It has previously been postulated that, in plants, β-(2-naphthyloxy)-propionic acid (V) would be oxidized via the hypothetical carbonate (VI) to β-naphthol (VII). The latter compound has in fact been detected chromatographically after incubation of *Sclerotinia laxa* Aderh. & Ruhl. with β-(2-naphthyloxy)-propionic acid (Byrde & Woodcock, unpublished work). It was to be expected, therefore, that the corresponding end-product with *A. niger* would be 2:6-dihydroxynaphthalene, but no diazo-positive material was observed when the alkali-soluble (as distinct from the bicarbonate-soluble) fraction was chromatographed, nor was there any evidence of 2:6-naphthaquinone formation. An unambiguous synthesis has confirmed that the product isolated was β-(6-hydroxy-2-naphthyloxy)propionic acid.

The reason for the failure of β-oxidation at this stage is not yet clear. Though the possibility that β-oxidation does initially occur, forming successively 2:6-dihydroxynaphthalene and then 2:6-naphthaquinone, is an attractive one, we have been unable to obtain any evidence that the former compound can interfere with this process. Moreover, incubation of γ-(2-naphthyloxy)-*n*-butyric acid with *A. niger* in the presence of β-(2-naphthyloxy)propionic acid has shown that the γ-compound is converted into (6-hydroxy-2-naphthyloxy)-acetic acid. Inhibition of the β-oxidation process by any metabolic product of β-(2-naphthyloxy)-propionic acid thus seems most unlikely. Ring closure of the propionic acid to form a substituted γ-pyrone would presumably prevent β-oxidation, but again there is no evidence to support this suggestion.

Chromatographic examination of the metabolism of γ-(2-naphthyloxy)-*n*-butyric acid (IV) (Fig. 1) indicated that nuclear hydroxylation occurred rapidly and took precedence over β-oxidation. (6-Hydroxy-2-naphthyloxy)acetic acid was also formed by incubation of γ-(6-hydroxy-2-naphthyloxy)butyric acid (III) with *A. niger*. The possibility of limited metabolism by the alternative pathway (β-oxidation followed by hydroxylation) cannot be excluded. Compound (II) is presumably an intermediate in the β-oxidation of (III).

In the homologous series $C_{10}H_7.O.[CH_2]_n.CO_2H$, where $n = 1$–9, there are therefore two principal phenolic end-products, and the fungitoxicity of each has been shown to be less than that of the corresponding unsubstituted acid. The hydroxylation of 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids thus appears to be a true detoxication mechanism (Williams, 1947, p. 241). Although this may be due to the effect of the hydroxyl group in lowering the 'lipophilic–hydrophilic ratio' (Veldstra & Booij, 1949) the difference in slope of the dosage–response lines observed in preliminary experiments implies a different mode of fungistatic action (Finney, 1952, p. 124).



(V) (VI) (VII)

The toxicity of the unsubstituted acids followed the normal pattern found in homologous series (Ferguson, 1939; Albert, 1951, p. 32). Following maximal values at $n = 2$–3, toxicity fell off markedly as the series was further ascended. It is thus possible that, at a certain length of side chain, the toxicity of the acids may in fact be lower than that of the hydroxylated end-product. Above $n = 3$ there was also some slight indication of an alternation in toxicity between odd and even values of n, suggesting that β-oxidation may be occurring.

The mechanism of hydroxylation in biological systems is obscure. Brodie *et al.* (1955) have suggested that it is brought about by non-specific peroxidase-type enzymes, the hydrogen peroxide arising from the enzymic breakdown of reduced triphosphopyridine nucleotide (TPN). Dalgliesh (1955), however, suggested that a free-radical electron acceptor of the dehydroascorbic acid type (which is probably concerned in TPN breakdown) may itself be the active oxidizing agent.

In studies of steroid metabolism by fungi it has been shown by Dulaney, Stapley & Hlavac (1955) that zinc is a factor necessary for the 6β-hydroxylation of progesterone by *A. ochraceus* Wilhelm. In the present work with *A. niger*, it is significant that in a preliminary experiment the addition of sodium

diethyldithiocarbamate (0·001 M) markedly inhibited the hydroxylation of 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids. There is also some evidence that hydroxylation is associated with sporulation and the production of melanin by the fungus. Thus, in an experiment where the fungus was grown in submerged culture, no sporulation or pigmentation occurred and no hydroxy acids could be detected. Similar results were obtained when growth of *A. niger* in surface cultures was poor, and also with cultures of *S. laxa* which were not pigmented.

An extension of this investigation to other fungi will be reported later.

SUMMARY

1. By means of a replacement-culture technique 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids were hydroxylated in the 6-position by *Aspergillus niger*.

2. γ-(2-Naphthyloxy)-*n*-butyric acid was similarly converted into γ-(6-hydroxy-2-naphthyloxy)-*n*-butyric acid, which was subsequently β-oxidized to (6-hydroxy-2-naphthyloxy)acetic acid.

3. Higher members of the ω-(2-naphthyloxy)-*n*-alkylcarboxylic acid series ($C_{10}H_7.O.[CH_2]_n.CO_2H$, where $n = 4$–9), likewise underwent hydroxylation and β-oxidation.

4. There was no evidence to suggest why β-(6-hydroxy-2-naphthyloxy)propionic acid did not undergo β-oxidation.

5. Hydroxylation of the acetic and propionic acids led to a decrease in toxicity to the fungus.

The authors wish to thank Mr G. M. Clarke for statistical advice, Miss M. Waller and Miss N. Waugh for valuable technical assistance, Mr D. R. Clifford for nitrogen analyses, and the Goodrich Chemical Co. for a gift of β-propiolactone.

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