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FUNGAL DETOXICATION—IX.

METABOLISM OF 1-NAPHTHALENEACETIC ACID BY *ASPERGILLUS NIGER* VAN TIEGH

D. R. CLIFFORD and D. WOODCOCK

Long Ashton Research Station, University of Bristol

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Abstract—A study of the detoxication products of 1-naphthalene acetic acid by *Aspergillus niger* using a replacement culture technique indicated 5-hydroxy NAA to be the major metabolite together with 4- and 6-hydroxy NAA's as very minor products. This represents yet another example of the similarity of the hydroxylation systems in this fungus and in higher plants.

INTRODUCTION

1-NAPHTHALENE acetic acid (NAA) is a growth regulator employed in fruit-growing practice for reducing the pre-harvest drop of apples and pears and as a fruit-thinning agent for apples.

The absorption, translocation and metabolism of NAA in a number of plant species[1–6] have yielded some interesting results, although unequivocal identification of metabolites was not always possible. Zenk,[2] working with pea epicotyls and with eighteen arbitrarily chosen plants, concluded that formation of NAA-aspartate and NAA-β-d-glucoside represented the two main detoxication mechanisms, although he also recognised the formation of a hydroxylated NAA (partially bound to glucose) as a further detoxication product. Klämbt,[3–5] working with wheat coleoptiles, showed the presence of the asparagine, glucose, gentiobiose and malonic acid derivatives together with the glucoside of a ring-hydroxylated NAA. Veen,[6] used explants of *Coleus rhenaltianus* and reported that six compounds represented more than 90 per cent of the total metabolic products. He also acknowledged the presence of a ring-hydroxylated NAA glucoside.

The fact that the same hydroxylated metabolic products obtained by the detoxication of 2,4-D[7,8] using *Aspergillus niger* were obtained from 2,4-D in the runner bean (*Phaseolus vulgaris*)[9] prompted this investigation.

RESULTS AND DISCUSSION

Concentration and extraction of the substrate from the mycelial mats gave an ethereal solution of the metabolites. Sodium bicarbonate extraction of this solution enabled partition

[1] L. C. LUCKWILL and C. P. LLOYD-JONES, *J. Hort. Sci.* **37**, 190–206 (1962).
[2] M. H. ZENK, *Planta* **58**, 75–84 (1962).
[3] H-D. KLÄMBT, *Planta* **57**, 339–353 (1961).
[4] H-D. KLÄMBT, *Planta* **57**, 391–407 (1961).
[5] H-D. KLÄMBT, *Planta* **60**, 453–460 (1964).
[6] H. VEEN, *Acta Botan. Neerland.* **15**, 419–433 (1966).
[7] J. K. FAULKNER and D. WOODCOCK, *Nature* **203**, 865 (1964).
[8] J. K. FAULKNER and D. WOODCOCK, *J. Chem. Soc.* 1187–91 (1965).
[9] E. W. THOMAS, B. C. LOUGHMAN and R. G. POWELL, *Nature* **204**, 884 (1964).

of the metabolites into "acidic" and "non-acidic" components, the former representing by far the major fraction although fungal debris was present in both.

TLC investigation of the material insoluble in $NaHCO_3$ solution showed that at least eight compounds are present, four of which are phenolic or naphtholic in nature. These compounds have not been positively identified, but no evidence was found for the presence of phenol, 1- or 2-naphthol, or the lactones of 2- and 8-hydroxy NAA's. Of the high R_f components, a spot originally thought to be 1-methyl naphthalene from decarboxylation of NAA, proved otherwise by comparison with an authentic specimen using GLC and TLC. Attempts to isolate individual metabolites by column, thin-layer and gas–liquid chromatography were unsuccessful.

Zenk[2] observed that decarboxylation of NAA in plants amounts to less than 0·1 per cent of the material taken up during 12 hr. Luckwill and Lloyd-Jones[1] showed that NAA is extensively decarboxylated when irradiated with u.v. light and work is in progress in this laboratory on the isolation and characterization of these breakdown products.[10]

The $NaHCO_3$ soluble fraction of the ethereal extract represented 95 per cent of the total metabolic products. This material always contained unchanged NAA which could be removed by esterification of the mixture and isolation of the hydroxy esters from the ethereal extract of the reaction mixture by partition into dilute sodium hydroxide solution. This process also enabled removal of fungal debris from the mixture thus achieving a useful "clean up" prior to chromatography. The use of acetone dimethyl acetal[11] as an esterifying agent gave far superior results to ethanol–gaseous HCl, whilst ethanol–sulphuric acid gave only tars. The lactones of 2- or 8-hydroxy NAA's could not be detected in the residual ether from the NaOH washes. TLC evaluation of the saponified hydroxy ester mixture indicated the presence of six or more "diazo positive" compounds, some of which were present in only trace amounts. It was clear that one component predominated and that it was always associated on the thin-layer plate with two other compounds, irrespective of solvent system or support used. This also proved to be the case with all GLC systems tried but the major metabolite was isolated by fractional crystallization of a mixture from a preparative run using XE 60 as the stationary phase. It was identified as 5-hydroxy NAA from i.r. spectra and TLC data, and by mixed m.p. with an authentic specimen. Legler *et al.*[12] showed that 5-hydroxy NAA glucoside was the major hydroxylated component produced by wheat coleoptiles, but we found no evidence for the presence of NAA glucosides in the fungal substrate.

All attempts (column chromatography, TLC, GLC, paper chromatography, ion-exchange chromatography) to separate and isolate the two other hydroxy acids proved abortive. However, TLC chromatograms incorporating the metabolite mixture, authentic specimens of individual hydroxy NAA's and mixtures of authentic NAA's gave a strong indication that these two components were the 4- and 6-hydroxy isomers. The former appears as a cherry-red centre to the orange 5-hydroxy NAA spot and the latter as a pink cusp beneath it when the plate is sprayed with *p*-nitrobenzene diazonium fluoroborate solution.

The metabolism of 1-naphthalene acetic acid does not parallel that of phenyl acetic acid in that *ortho*-hydroxylation is here unimportant.[13] Earlier work on the metabolism of 2-naphthyloxy acetic acid[14] and on nerolin[15] supports the inference that a different enzyme

[10] D. A. M. WATKINS and D. WOODCOCK, unpublished work.
[11] N. B. LORETTE and J. H. BROWN, JR., *J. Org. Chem.* **24**, 261–262 (1959).
[12] G. LEGLER, H-D. KLÄMBT and J. P. GAREL, *Z. Naturforsch.* **20b**, 643–645 (1965).
[13] S. M. BOCKS, *Phytochem.* **6**, 785–789 (1967).
[14] R. J. W. BYRDE, J. F. HARRIS and D. WOODCOCK, *Biochem. J.* **64**, 154–160 (1956).
[15] R. J. W. BYRDE, D. F. DOWNING and D. WOODCOCK, *Biochem. J.* **72**, 344–348 (1959).

system is operative in the breakdown of naphthalene compounds as opposed to that of single ring aromatic compounds.

The conversion of NAA to its 5-hydroxy analogue by *Aspergillus niger* is yet another example of the similarity of the hydroxylation systems in this fungus and in higher plants.

EXPERIMENTAL

Cultures of *Aspergillus niger* van Tiegh. (Mulder strain) obtained from C.B.S. (Baarn) were grown in penicillin flasks in a glucose–mineral salts medium for 3 days. After pouring off the nutrient liquor, the undersurface of the mycelial mats was washed with water before the introduction of a 5×10^{-4} M solution of NAA in 10^{-2} M aqueous disodium hydrogen phosphate buffer, and then further incubated at 25° for varying periods, the optimum being 3 days. The fungal substrate was poured off, concentrated in a cyclone evaporator at 30–40°, acidified and continuously extracted with ether for 16 hr. The ethereal solution was extracted with sodium bicarbonate solution and the crude materials stored as $NaHCO_3$-soluble and $NaHCO_3$-insoluble fractions.

Thin-layer chromatography was carried out on silica gel "G" using twelve different solvent systems, the most successful being ether–light petroleum–formic acid (60:40:2). Plates were sprayed either with *p*-nitrobenzene diazonium fluoroborate in acetone to indicate phenolic or naphtholic compounds or with ceric sulphate–H_2SO_4 followed by baking at 140°.

TABLE 1.

Compound	Retention time of TMS deriv. relative to NAA (min)
NAA	1
2-Hydroxy NAA	6·2
4-Hydroxy NAA	6·65
5-Hydroxy NAA	6·6
6-Hydroxy NAA	7·25

Gas–liquid chromatography. A Varian Aerograph A90 P3 instrument with linear temperature programming and flame ionization detector was used. Blackley[16] successfully separated the isomeric hydroxyphenylacetic acids as their trimethylsilyl ethers–esters using 10 per cent SE 52 column packing on 60/80 mesh silanized Gas Chrom Z, but whilst this system satisfactorily separated NAA from the metabolic mixture it did not separate the individual hydroxy acids. Other column packings tried with little success were: LAC-2R-446 (2.5 per cent) on Chromosorb G; S.E.30 on Chromosorb G (acid washed, silanized); 5 per cent O.V.17 plus Bentone 34 on 70/80 mesh Aeropack.

The most favourable results were obtained by the following method: authentic hydroxy NAA's (5 mg) or metabolite mixture (20 mg) were dissolved in N,O-bis(trimethylsilyl) acetamide (0·1 ml) and 2–4 μl of this solution were injected into a 10 ft × $\frac{1}{8}$ in. stainless-steel column packed with stabilized diethylene glycol adipate (Analabs C5)–phosphoric acid (2·5 per cent:0·2 per cent) on Chromosorb G (acid washed; silanized). Operating conditions were as follows: Flow rate 64 ml/min nitrogen; injector temperature 280°; detector temperature 290°; initial column temperature 180° programmed to 260° at 10°/min. Relative retention times with this system using NAA as standard are shown in Table 1, the retention time for NAA being 4·6 min. Authentic mixtures of the 4- and 6-hydroxy isomers were separable, but no useful separation of an authentic mixture of the 4-, 5- and 6-isomers or of the crude acidic mixture was possible, despite variation of gas flow rates, column temperatures and programming rates. It would appear that the lack of separation is due to the lack of volatility of the trimethylsilyl ethers–esters coupled with the extreme similarity in physical properties of these compounds.

Infra-red spectra were determined as KCl discs on a Perkin–Elmer Infracord 237 spectrophotometer.

2-Hydroxy naphthaleneacetic acid was prepared from nerolin by Griehl's method[17] followed by demethylation of the methoxy compound.

[16] E. R. BLACKLEY, *Anal. Biochem.* **15** (2), 350 (1966).
[17] W. GRIEHL, *Chem. Ber.* **80**, 410–412 (1947).

4-*Hydroxy naphthaleneacetic acid.* In our hands the method of Inamdar *et al.*[18] for the preparation of 4-methoxy naphthalene acetic acid gave best results both in respect of ease of handling the product and yield. Demethylation of this material gave the hydroxy compound, m.p. 197–198°, from water.[12]

5-*Hydroxy naphthaleneacetic acid.* The methoxy acid was prepared from 5-methoxy tetralone by a Reformatsky reaction.[19] Demethylation with pyridine hydrochloride yielded the hydroxy acid, m.p. 200–200·5°, after crystallization[12] from water.

6-*Hydroxy naphthaleneacetic acid.* 6-Methoxy tetralone subjected to a Reformatsky reaction with ethyl bromoacetate followed by dehydration, hydrolysis and dehydrogenation yielded 6-methoxy naphthalene acetic acid.[19] Demethylation yielded the hydroxy acid, m.p. 203–204°. (Süss *et al.*[20] give m.p. 199°.)

3- *and* 7-*hydroxy naphthaleneacetic acids* are new compounds and details of their preparation will be published elsewhere.

8-*Methoxynaphthaleneacetic acid* was kindly donated by Dr. H-D. Klämbt. Demethylation of this compound yielded the lactone.[12]

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[18] A. R. INAMDAR, S. M. KULKARNI and K. S. NARGUND, *J. Indian Chem. Soc.* **44**, 398–399 (1967).
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[20] O. SÜSS, K. MÖLLER, R. DIETRICH, H. EBERHARDT, M. GLOS, M. GRUNDKÖTTER, H. HOFFMANN and H. SCHÄFER, *Annalen.* **593**, 91–126 (1955).