

Biosynthesis of Neoaspergillic and Neohydroxyaspergillic Acids*

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Neohydroxyaspergillic acid is an antibiotic that was first isolated by Weiss, Strelitz, Flon, and Asheshov (1) from cultures of *Aspergillus sclerotiorum*. The most probable structure (2) for this compound is shown in Fig. 1. Under suitable conditions, *A. sclerotiorum* synthesizes another antibiotic at the same time, the structure of which (2) is shown in Fig. 2, and which we have named neoaspergillic acid (3). Neoaspergillic acid can be reduced chemically (2) to flavacol (Fig. 3). The latter has been isolated by Dunn, Newbold, and Spring (4) from cultures of *Aspergillus flavus*.

Aspergillic acid and hydroxyaspergillic acid have been shown to be synthesized by *A. flavus* from leucine plus isoleucine (5, 6), and a comparison of their structures with those of neoaspergillic acid and neohydroxyaspergillic acid suggested that the latter compounds would be synthesized by *A. sclerotiorum* entirely from leucine. The results of the present work support this postulate and indicate a biosynthetic relationship between flavacol, neoaspergillic acid, and neohydroxyaspergillic acid.

EXPERIMENTAL PROCEDURE

A. sclerotiorum PRL 1930 was grown as outlined elsewhere (2). In Experiments 1 and 2 (Table I), L-leucine-1-¹⁴C was added to 2 liters of 2% yeast extract medium prior to autoclaving. In Experiments 3 to 6 (Table I), the radioactive compounds were dissolved in chloroform, and portions of the solution containing approximately 10 mg of the compound were added to four (Experiment 3) or five sterile flasks. The chloroform was evaporated off under vacuum, 50 ml of sterile 2% yeast extract solution were added to each flask, and the medium was inoculated with spores.

In Experiments 1, 3, 5, and 6 (Table I), the neoaspergillic and neohydroxyaspergillic acids were isolated from the culture filtrate and purified essentially as described elsewhere (2), with appropriate decreases in the volume of extracting solvents for smaller volumes of culture filtrate. However, a better separation of these compounds, and higher initial purity, was obtained in other experiments by the following procedure. The culture filtrate from five flasks was acidified and then extracted by shaking with chloroform (6 × 12 ml), and the chloroform was extracted with 0.1 M sodium carbonate (6 × 3 ml) which was then acidified to pH 2 to 3 with hydrochloric acid. The acidified solution was extracted with chloroform (4 × 10 ml), the chloroform evaporated off in a vacuum and the residue

weighed. Where more than five flasks were used, the volume of extracting solvents was increased proportionately. The residue was dissolved in chloroform and subjected to a five-tube counter-current distribution, with chloroform (0.5 ml per mg of residue) as the mobile phase and an equal volume of 0.1 M sodium phosphate buffer, pH 7.0, as the stationary phase. The contents of the first two tubes were combined, the aqueous layer was acidified to pH 2 to 3, and the aqueous layer was extracted several times with chloroform. After evaporation of the chloroform, the residue was recrystallized from *n*-heptane to give neohydroxyaspergillic acid. The contents of the last two tubes were treated similarly, except that the residue was sublimed at 95° and 0.02 mm of Hg to give neoaspergillic acid.

In Experiment 5 (Table I), the chloroform extract of the culture filtrate was extracted with sodium bicarbonate and sodium carbonate solutions to remove neohydroxyaspergillic and neoaspergillic acids (2). Then the chloroform was evaporated off in a vacuum to leave a residue which contained flavacol. The residue was sublimed at 120° and 0.02 mm of Hg to give flavacol, m.p. 149–150°.

Mycelium from Experiments 1 and 3 to 6 (Table I) was extracted for over 24 hours in a Soxhlet apparatus with organic solvents or water to remove traces of isotopic compound used in the experiment. The mycelium was dried, and radioactivity was determined (7). If radioactivity was present, the mycelium was hydrolyzed and leucine was isolated (5).

Radioactive neoaspergillic and neohydroxyaspergillic acids for Experiments 3 and 4 were prepared biosynthetically in Experiment 1 or a similar experiment. Flavacol-¹⁴C was prepared either by the reduction of neoaspergillic acid-¹⁴C (2) or from leucine-1-¹⁴C by the method of Dunn *et al.* (4). The cyclic dipeptide of L-leucine, cyclo-L-leucyl-L-leucyl, was prepared in the following manner. L-Leucine-1-¹⁴C (500 mg) was converted to the methyl ester hydrochloride (8), which was dried in a vacuum over soda lime. To the hydrochloride were added 1.5 ml of an aqueous solution containing 0.5 g of sodium carbonate, and the methyl ester was extracted with methylene chloride, centrifuging to break emulsions. Most of the methylene chloride was evaporated off with a stream of nitrogen, and 0.01 ml of glacial acetic acid was added to the liquid residue (460 mg). This solution was then heated at 50° in a small stoppered flask for 40 hours, and a solid product was obtained. This solid was purified by fractional sublimation. The first fraction, obtained by subliming at 87° and 20 mm of Hg, was discarded, and the second fraction, obtained by subliming at 170° and 0.02 mm of Hg, was crystallized from ethanol-water to

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give 106 mg of cyclo-L-leucyl-L-leucyl, which was identified by its infrared spectrum in comparison to authentic cycloleucylleucyl (racemic) and by the optical rotation of a nonradioactive sample prepared in exactly the same way, $[\alpha]_D^{25} -42^\circ$ (c, 4.2 in glacial acetic acid). Aberhalden and Funk reported $[\alpha]_D^{25} -42.5^\circ$ for cyclo-L-leucyl-L-leucyl in glacial acetic acid (9).

For experiments involving replacement cultures, *A. sclerotiorum* was grown on yeast extract medium (2), the medium was poured off, and the mycelial mat was washed several times with sterile distilled water. The mat from one flask was transferred to a 250-ml Erlenmeyer flask containing either 50 ml of sterile water or 50 ml of sterile water plus either 10 mg of flavacol- ^{14}C or 100 mg of L-leucine. Flasks prepared in this manner were incubated in the dark for 2 days on a rotary shaker at 25° . The possible presence of neoaspergillic or neohydroxyaspergillic acids was tested spectrophotometrically (2), and if they were present in amounts sufficient for isolation, the mycelium was filtered off and the filtrate was made 0.1 M with respect to sodium carbonate and extracted with chloroform to remove any unused flavacol. The residue after evaporation of the chloroform was sublimed at 150° and 0.02 mm of Hg to attempt to recover unused flavacol (flavacol sublimes under these conditions). The aqueous layer was acidified and neoaspergillic and neohydroxyaspergillic acids isolated and purified as described above. In a separate experiment done in essentially the same way, deoxyaspergillic acid (10 mg per flask) was used instead of flavacol, and an attempt was made to isolate unused deoxyaspergillic acid in the manner just mentioned for flavacol, prior to the isolation of aspergillic acid as described elsewhere (6).

Neoaspergillic acid- ^{14}C was degraded in the following manner to determine the distribution of radioactivity. The labeled compound was diluted with carrier, if necessary, and 80 mg were dissolved in 8 ml of ethanol plus 12 ml of 5 M hydrochloric acid. Saturated bromine-water was added dropwise with stirring until a slight excess was present. The brominated product was extracted and reduced with zinc-acetic acid, and the product was extracted into chloroform by the same methods that were used for the degradation of aspergillic acid (5). After evaporation of the chloroform, the residue was purified by sublimation at 150° and 0.02 mm of Hg, and the sublimate, cycloleucylleucyl, was recrystallized from ethanol-water and identified by its infrared spectrum. The cyclic dipeptide was hydrolyzed with hydrobromic acid, leucine was isolated (10), and its specific activity was determined and expressed as that which would have been obtained if no carrier neoaspergillic acid had been added. The yield of leucine from neoaspergillic acid was approximately 42% of theory. The leucine was decarboxylated with ninhydrin, and the products isolated as barium carbonate and the 2,4-dinitrophenylhydrazones of isovaleraldehyde. Radioactivity was determined as described previously (7).

RESULTS AND DISCUSSION

Derivation of Neoaspergillic and Neohydroxyaspergillic Acids from L-Leucine—The results of Experiments 1 and 2 (Table I) show that leucine- ^{14}C was used in the biosynthesis of these compounds by *A. sclerotiorum*. The leucine from mycelium (Experiment 1) was degraded and 92% of the radioactivity was found in the carboxyl group and less than 1% in the rest of the molecule. Neoaspergillic acid, specific activity $1.66 \mu\text{c}$ per mmole, from Experiment 2 was chemically degraded to leucine (theory, 2 molecules of leucine from 1 molecule of neoaspergillic

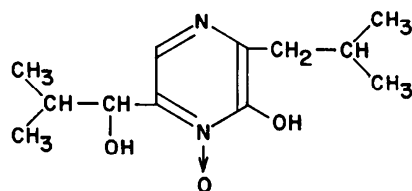


FIG. 1. Neohydroxyaspergillic acid

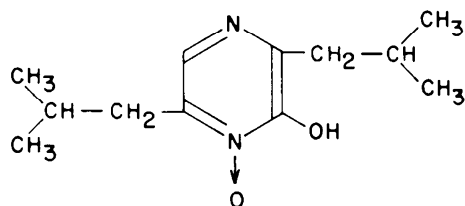


FIG. 2. Neoaspergillic acid

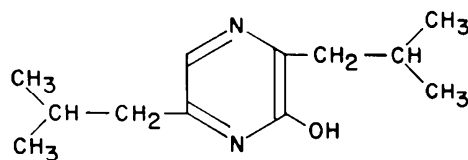


FIG. 3. Flavacol

acid) of specific activity $0.85 \mu\text{c}$ per mmole, in which 93% of the radioactivity was found in the carboxyl group and less than 3% in the rest of the molecule. The above results indicate that the carbon skeleton of leucine was incorporated intact either into mycelial leucine or into neoaspergillic acid, without significant randomization of activity.

The figures for the specific activity of the L-leucine- ^{14}C present in the medium before growth of the organism (Table I, Experiments 1 and 2) were determined as described previously (6), and were based on the free leucine in the yeast extract. If one calculates the specific activity on the basis of total leucine in the yeast extract, as determined by acid hydrolysis and isotope dilution analysis (6), the specific activity of leucine added to the medium in Experiment 1 would be $0.90 \mu\text{c}$ per mmole, and in Experiment 2 would be $0.97 \mu\text{c}$ per mmole. The specific activity ($0.62 \mu\text{c}$ per mmole) of the leucine from the mycelium in Experiment 1 of Table I, was lower than that of the leucine added to the medium (either 1.01 or $0.90 \mu\text{c}$ per mmole, based on either free or total leucine content) which indicates that some endogenous synthesis of leucine from unlabeled precursors took place. The neoaspergillic and neohydroxyaspergillic acids had a specific activity of approximately 1.5 times that of the leucine originally present in the medium (Experiments 1 and 2), or 2.4 times that of the leucine isolated from mycelium hydrolysate (Experiment 1). These findings, together with the finding that no significant randomization of the labeled carbon of leucine- ^{14}C occurred in the incorporation of leucine into neoaspergillic acid from Experiment 2 (see data in preceding paragraph), indicate that 2 molecules of leucine were used to synthesize 1 molecule of neoaspergillic acid (2-hydroxy-3,6-diisobutylpyrazine-1-oxide) which was labeled in positions 2 and 5. Since neoaspergillic and neohydroxyaspergillic acids have closely related structures and had similar specific activities, and since neohydroxyaspergillic acid can be derived from neoaspergillic acid (see below), it would seem

TABLE I
Incorporation of labeled compounds into various products by *A. sclerotiorum* grown on yeast extract medium

Experiment No.	Labeled compound added	Amount per flask	Specific activity	Compounds isolated from the culture	Amount per flask	Specific activity
		mg	$\mu\text{c/mmole}$		mg	$\mu\text{c/mmole}$
1	L-Leucine-1- ¹⁴ C	35.2	1.01	Mycelial leucine		0.62
				Neospergillie acid	8.25	1.48
2	L-Leucine-1- ¹⁴ C	35.2	1.09	Neohydroxyaspergillie acid	5.13	1.42
				Neospergillie acid	8.67	1.66
3	Neospergillie- ¹⁴ C	10.2	1.48	Neohydroxyaspergillie acid	2.85	1.56
				Neospergillie acid	11.0	0.44
4	Neohydroxyaspergillie- ¹⁴ C	10.0	1.84	Neohydroxyaspergillie acid	8.1	0.33
				Neospergillie acid	12.4	<0.002
5	Flavacol- ¹⁴ C	10.0	1.43	Neohydroxyaspergillie acid	9.9	1.22
				Flavacol	1.3	1.09
6	Cyclo-L-leucyl-L-leucyl- ¹⁴ C	9.8	5.26	Neospergillie acid	10.6	0.29
				Neohydroxyaspergillie acid	3.6	0.18
				Mycelial leucine		0.64
				Neospergillie acid	10.0	0.30
				Neohydroxyaspergillie acid	4.3	0.40

probable that neohydroxyaspergillie acid was also synthesized from 2 molecules of leucine in Experiments 1 and 2.

Conversion of Neospergillie Acid to Neohydroxyaspergillie Acid—Radioactivity from neospergillie acid-¹⁴C (2-hydroxy-3,6-diisobutylpyrazine-1-oxide-2,5-¹⁴C) was incorporated by growing cultures of *A. sclerotiorum* into neohydroxyaspergillie acid (Experiment 3, Table I), while radioactivity from neohydroxyaspergillie acid-¹⁴C did not enter neospergillie acid (Experiment 4). There was synthesis of neospergillie acid from unlabeled precursors in both these experiments, as was shown by the lowering of the specific activity of this compound in Experiment 3 and the isolation of unlabeled neospergillie acid in Experiment 4. Although such endogenous synthesis of unlabeled neospergillie acid complicates the results in Experiment 3, the specific activity of neohydroxyaspergillie acid was high enough compared to that of the neospergillie acid reisolated from the medium to indicate that neohydroxyaspergillie acid was formed from neospergillie acid. Since mycelium from Experiments 3 and 4 contained no radioactivity, there was apparently no extensive breakdown of these compounds into metabolites that could be used for synthesis of mycelium, and the conversion of neospergillie to neohydroxyaspergillie acid was presumably direct. The postulate that neospergillie acid was the precursor of neohydroxyaspergillie acid is in accord with studies on the biosynthesis of hydroxyaspergillie acid (6), in which it was shown that aspergillie acid was the precursor of hydroxyaspergillie acid.

In a preliminary experiment, results were obtained which indicated that there might be a small amount of conversion of neohydroxyaspergillie acid-¹⁴C to neospergillie acid by *A. sclerotiorum*. However, when extra precautions were taken to remove any trace of neospergillie acid-¹⁴C from the neohydroxyaspergillie acid-¹⁴C added to the medium, and to remove any trace of neohydroxyaspergillie acid-¹⁴C from neospergillie acid isolated from the medium, the results in Experiment 4 were obtained. These precautions consisted of the addition of unlabeled neospergillie acid to the neohydroxyaspergillie acid-¹⁴C, and reisolation of the latter before adding it to the medium, the use of an improved procedure for the isolation of the two acids from the medium (see "Experimental Procedure"), and the addition of unlabeled neohydroxyaspergillie acid to the

neospergillie acid isolated from the medium, followed by reisolation of the latter for determination of radioactivity.

Conversion of Flavacol to Neospergillie Acid—The results of Experiment 5 (Table I) show that radioactivity from flavacol-¹⁴C was incorporated into neospergillie and neohydroxyaspergillie acids by growing cultures of *A. sclerotiorum*. No radioactivity was found in the mycelium so there was apparently little degradation of flavacol to a compound such as leucine which could be used for the synthesis of mycelium. The specific activity of the flavacol recovered was less than that of the flavacol added, which suggests that this compound may be produced by the organism, although it did not accumulate in sufficient amounts to be isolated in cultures of *A. sclerotiorum* grown on yeast extract medium alone. Flavacol was poorly soluble in the medium, and crystalline flavacol was visible in the flasks during the early stages of growth. At later stages of growth one could not see, because of heavy growth of the mycelium, whether crystals of flavacol were present or not. The reisolated flavacol might be expected to have a high specific activity if, because of its poor solubility, it could not equilibrate with endogenously synthesized flavacol. Since the specific activity of neospergillie and neohydroxyaspergillie acids was lower than that of reisolated flavacol-¹⁴C, there was endogenous synthesis of these acids from unlabeled precursors (probably mostly from leucine) in the medium, as well as from flavacol-¹⁴C.

Preliminary experiments had shown that washed mycelium of *A. sclerotiorum* did not synthesize appreciable amounts of neospergillie or neohydroxyaspergillie acid when incubated either with water or leucine solutions. In such preliminary experiments, these acids were synthesized, and were isolated, when mycelium was incubated with flavacol suspensions, while the flavacol crystals dissolved during the incubation and no flavacol could be reisolated at the end of the incubation. There was no conversion of flavacol to neospergillie or neohydroxyaspergillie acid in a control flask incubated in the absence of mycelium. This was shown by the presence of undissolved flavacol in the flask and by the ultraviolet spectrum of an appropriate dilution of a portion of the solution in the flask with 0.1 M Na₂CO₃. This spectrum was identical with that of solutions of flavacol in 0.1 M Na₂CO₃ (absorption maximum at 322 m μ), and different from that of solutions of neospergillie or

neohydroxyaspergilliac acid in the same solvent (absorption maximum at 337 $m\mu$). These findings made it appear possible that replacement cultures could be used to investigate the conversion of flavacol- ^{14}C to neospergilliac or neohydroxyaspergilliac acids, without the complication of excessive endogenous synthesis of these compounds from other precursors. This possibility was confirmed by the following experiment. Six flasks containing a total of 60 mg of flavacol- ^{14}C , specific activity 1.03 μc per mmole, were incubated with washed mycelium. After 48 hours, 50.1 mg of neospergilliac acid, specific activity of 0.94 μc per mmole, was isolated along with 5.9 mg of neohydroxyaspergilliac acid, specific activity 0.82 μc per mmole. No flavacol could be reisolated. The neospergilliac acid was degraded to leucine with a specific activity of 0.49 μc per mmole (2 molecules of leucine are theoretically obtained from 1 of neospergilliac acid in the degradation), in which 90% of the radioactivity was found in the carboxyl group and no detectable amount in the rest of the molecule.

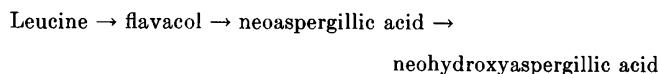
The flavacol- ^{14}C used in the experiment with washed mycelium had been synthesized chemically from leucine- ^{14}C . The neospergilliac acid was derived from the flavacol- ^{14}C to the extent of 0.94:1.03, or 91%, without detectable randomization of the labeled carbon. Since externally added leucine was not converted to neospergilliac acid by washed mycelium, it is not likely that flavacol was degraded to leucine prior to its incorporation into neospergilliac acid.

Conversion of Deoxyaspergilliac Acid to Aspergilliac Acid—Aspergilliac acid was not produced in detectable amounts by *A. sclerotiorum* grown on yeast extract medium. However, it was thought that since washed mycelium of this organism could convert flavacol to neospergilliac acid, it might also convert deoxyaspergilliac acid (3-isobutyl-5-*sec*-butyl-2-hydroxypyrazine) to aspergilliac acid (3-isobutyl-5-*sec*-butyl-2-hydroxypyrazine-1-oxide). Nineteen flasks containing a total of 190 mg of deoxyaspergilliac acid in water were incubated with washed mycelium. After 48 hours, 6.5 mg of unused deoxyaspergilliac acid was isolated along with 133 mg of aspergilliac acid (identified by its infrared spectrum, solid in KBr). Since *A. sclerotiorum* can convert deoxyaspergilliac to aspergilliac acid, which is not ordinarily a fermentation product of this organism, it would appear that the organism had the ability to oxidize certain hydroxypyrazines to their *N*-1-oxides, and this is supporting evidence that the conversion of flavacol to neospergilliac acid was relatively direct.

Incorporation of Cyclo-L-leucyl-L-leucyl into Neospergilliac and Neohydroxyaspergilliac Acids—One of the early steps in the biosynthesis of neospergilliac acid could be the formation of cyclo-L-leucyl-L-leucyl. However, the results obtained with this radioactive compound in Table I, Experiment 6, cannot be used to support this hypothesis, for the specific activity of neospergilliac and neohydroxyaspergilliac acids was even less than that found in mycelial leucine. A similar result also had been found when cyclo-L-leucyl- ^{14}C -L-isoleucyl was used in investigations on the biosynthesis of aspergilliac acid.¹ It would seem possible

that such cyclic dipeptides were hydrolyzed by the organism and that the free amino acids were used for the synthesis of cell constituents and metabolic products. Any involvement of cyclic dipeptides in the biosynthesis of compounds similar to neospergilliac acid, although possible, remains to be proven.

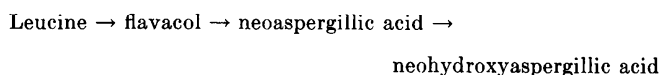
The results of the present work can be most simply explained by the postulate that intermediates in the biosynthesis of neohydroxyaspergilliac acid occurred in the order



SUMMARY

When *Aspergillus sclerotiorum* was grown on yeast extract medium containing L-leucine- ^{14}C , the incorporation of radioactivity indicated that each molecule of neospergilliac acid or neohydroxyaspergilliac acid was derived from 2 molecules of leucine, without significant randomization of the labeled carbon in leucine. When the medium contained neospergilliac acid- ^{14}C , the organism incorporated appreciable radioactivity into neohydroxyaspergilliac acid. Neohydroxyaspergilliac acid- ^{14}C was not used by the organism for the biosynthesis of neospergilliac acid. Flavacol- ^{14}C was used for the biosynthesis of neospergilliac and neohydroxyaspergilliac acids by either growing or replacement cultures of *A. sclerotiorum*. Replacement cultures could also oxidize deoxyaspergilliac acid to aspergilliac acid. Radioactivity from cyclo-L-leucyl-L-leucyl- ^{14}C was incorporated to a greater extent into mycelial leucine than into neospergilliac and neohydroxyaspergilliac acids.

The results were interpreted by the postulate that the intermediates in the biosynthesis of neohydroxyaspergilliac acid occurred in the order



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¹ J. C. MacDonald and L. C. Vining, unpublished results.