THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 236, No. 2, February 1961
Printed in U.S.A.

Biosynthesis of Aspergillic Acid*

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(Received for publication, September 28, 1960)

Aspergillic acid is an antibiotic which was first isolated by White and Hill (1) and Jones *et al.* (2) from cultures of certain strains of *Aspergillus flavus* grown on a tryptone-saline medium. The structure of aspergillic acid was established by Dutcher (3), Dunn *et al.* (4, 5), and Newbold *et al.* (6) to be that shown in Fig. 1. Apart from some unsuccessful attempts to obtain production

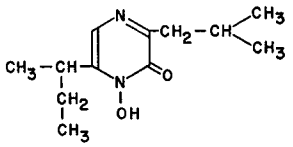

FIG. 1. Aspergillic acid

of aspergillic acid from isoleucine in replacement cultures (3), there has been no work reported previously on the biogenesis of this antibiotic.

In the present work, *A. flavus* was grown on a medium containing radioactive leucine or isoleucine, and these compounds were found to be incorporated into the aspergillic acid formed. Aspergillic acid was degraded to determine what part of the molecule contained the isotope and to determine the optical configuration of the *sec*-butyl side chain.

EXPERIMENTAL PROCEDURES

A sufficient number of potato dextrose agar (Difco) slants of *A. flavus* PRL 932 were made at one time to provide spore inoculum for all the experiments reported here. A loopful of dry spores was used to inoculate each 250-ml flask containing 50 ml of sterile medium (2% tryptone, 0.05% NaCl). Isotope solutions were autoclaved separately and 2 ml (containing 10 mg of DL-leucine or 5 mg of D-leucine, L-leucine, or L-isoleucine) were added to each of five flasks. The growth conditions were those described by Woodward (7).

Crude aspergillic acid was isolated from the culture filtrate as described by Dutcher (3). The crude acid (54 to 160 mg from five flasks) was next dissolved in 10 ml of $CHCl_3$ and extracted three times with 5 ml of 0.1 M $NaHCO_3$ to remove an impurity with a higher melting point than that of aspergillic acid. The $CHCl_3$ layer was then extracted three times with 10 ml of 0.1 M Na_2CO_3, the aqueous layer made slightly acid, and the aspergillic acid extracted into ether. After evaporation of the ether, the aspergillic acid was further purified by sublimation at 80° and 5 mm of pressure, and the major fraction resublimed at 60° and 0.03 mm with, in both cases, an apparatus similar in principle to that described by Thomas *et al.* (8).

* Issued as National Research Council No. 6088.

Radioactive aspergillic acid plus carrier aspergillic acid was degraded by methods described by Dutcher (3) and by Dunn *et al.* (5) to yield leucine, alloisoleucine, and isoleucine. The degradation was done, without the isolation of intermediate products, in the following manner. The reaction mixture from the bromination of 85 mg of aspergillic acid was extracted several times with ether. The residue left after evaporating the ether was reduced with zinc and acetic acid. This reaction mixture was then adjusted to pH 4.5 with sodium hydroxide and extracted several times with chloroform. The residue left after evaporating the chloroform was hydrolyzed with HBr.

Mycelium from five flasks was pooled, washed twice with boiling water, and then hydrolyzed and chromatographed on an Amberlite CG-120 column (9). The column was eluted first with 2 liters of 0.2 M citric acid buffer pH 3.25, and then with pH 3.9 buffer. The latter buffer eluted and separated leucine and isoleucine. The appropriate eluate fractions were desalted and the amino acids purified by sublimation (9). The amino acids from the degradation of aspergillic acid were separated and purified the same way. This procedure did not separate alloisoleucine and isoleucine. In the one experiment in which these were separated, citric acid buffer of pH 3.7 was used as the eluant. The amount of leucine and isoleucine in a tryptone hydrolysate was determined by adding a known amount of radioactive leucine and isoleucine to the hydrolysate, separating and purifying the isoleucine and leucine as outlined above, and determining the isotope dilution.

Leucine was decarboxylated with ninhydrin and the products collected as $BaCO_3$ and the 2,4-dinitrophenylhydrazone derivative as described elsewhere (9).

DL-Leucine-1-C^{14} was obtained from Atomic Energy of Canada, Commercial Products Division, and uniformly labeled L-leucine-C^{14} and L-isoleucine-C^{14} from Merck and Company, Montreal. Unlabeled L-isoleucine and D-alloisoleucine were obtained from the California Corporation for Biochemical Research, Los Angeles. D-Leucine-1-C^{14} was prepared from DL-leucine-1-C^{14} by essentially the same method used to prepare D-valine-1-C^{14} from DL-valine-1-C^{14} (9). Radioactivity in samples was determined as described elsewhere (9).

RESULTS

In preliminary experiments, it was found that the yield of aspergillic acid from *A. flavus* PRL 932 decreased on repeated subculture. Such an observation has previously been made for other strains of *A. flavus* by White and Hill (1). To minimize this effect, about a dozen slants were made of a selected culture which produced satisfactory yields of aspergillic acid; these slants were stored at 10° for use as inoculum.

The crude aspergillic acid produced by the organism melted between 80 and 90°, uncorrected (Fischer-Johns melting point apparatus), whereas the purified acid (yield 44 to 60 mg from five flasks) melted between 90 and 95°. The infrared spectrum and x-ray diffraction pattern of the purified material were the same as those obtained from an authentic sample of aspergillic acid (m.p. 93.5–94.5°), kindly supplied by Dr. G. T. Newbold, The Royal College of Science and Technology, Glasgow. Degradation of the aspergillic acid gave a yield of leucine from 20 to 58% of theory. The amount of combined isoleucine plus alloisoleucine was 50 to 60% of that of the leucine. In one experiment, 459 mg of aspergillic acid were degraded, and isoleucine was separated from alloisoleucine. The optical rotations in 6 M HCl were $[\alpha]_D^{25°}$ +38.9° (c = 3.85) for isoleucine and $[\alpha]_D^{28°}$ −38.2° (c = 3.02) for alloisoleucine. These samples had the same infrared spectrum and same optical rotation (within 0.4°) as authentic samples of L-isoleucine and D-alloisoleucine, respectively. The leucine sample from this experiment had the same infrared spectrum as authentic DL-leucine and was optically inactive. Because L-isoleucine and D-alloisoleucine have the same optical configuration at carbon 3 (10), it follows that the *sec*-butyl side chain of aspergillic acid also has this configuration. The asymmetry at carbon 2 of leucine and isoleucine is introduced by the degradation procedure and consequently, the isomers identified above are the ones expected.

The results of experiments with isotopic leucine and isoleucine (Table I) show that these compounds are incorporated to a somewhat greater extent into aspergillic acid than into mycelial leucine and isoleucine. DL-Leucine-1-C^{14} was used for the biosynthesis of aspergillic acid, which was subsequently chemically degraded to leucine and alloisoleucine plus isoleucine. The activity was all present in the leucine so obtained (Table I), and on further degradation, 87% of the activity in leucine was found in the carboxyl group and less than 2% in the rest of the molecule. These results indicate that there is little or no conversion of leucine to isoleucine, and that the carbon skeleton of leucine is incorporated intact, without randomization of activity, in the biosynthesis of aspergillic acid. The incorporation of both D-leucine-1-C^{14} and uniformly labeled L-leucine into aspergillic acid and mycelial leucine was high (Table I); therefore, the question of which isomer is the more immediate precursor of the label in aspergillic acid cannot be decided.

L-Isoleucine was used for the biosynthesis of mycelial isoleucine and the part of aspergillic acid which yields isoleucine plus alloisoleucine on degradation (Table I). In this case, there was a relatively small but significant amount of activity in mycelial leucine and leucine from the degradation of aspergillic acid. Some of the uniformly labeled L-isoleucine-C^{14} purchased for the experiment was diluted with unlabeled L-isoleucine and L-leucine, and these were separated by chromatography. Approximately 85% of the activity was present in the isoleucine and 15% in the leucine. Therefore, it is likely that the small activity in the leucine fractions arose from the presence of labeled leucine in the isotope preparation used.

The amount of leucine and isoleucine in the tryptone was determined so that some indication of the amount of dilution of the radioisotope could be obtained. It was not possible to obtain a separation of free leucine and isoleucine from other ninhydrin-reactive material in tryptone on chromatography, probably because of interference by peptides. However, the amount of leucine and isoleucine in an acid hydrolysate of tryptone was determined, and the results showed that 1 g of tryptone contained 100 mg of leucine and 52 mg of isoleucine. If one assumes that these amino acids in the tryptone are used by the organism at the same rate as the isotopic amino acids added, and that no endogenous synthesis of these amino acids takes place, the expected specific activity of leucine in Experiments 1, 2, 3, and 4 would be 1.04, 0.39, 1.21, and 0.18 μc per mmole, respectively, and that of isoleucine in Experiment 4, 1.77 μc per mmole. The assumptions made for these calculations may not be strictly true, for the calculated specific activities are only of the same order of magnitude as, and not necessarily identical to, the specific activities of leucine or isoleucine from the mycelium of *A. flavus* or from the degradation of aspergillic acid (Table I). These calculations indicate, however, that exogenous leucine and isoleucine are incorporated to a significant extent into the mycelial leucine and isoleucine and into aspergillic acid.

TABLE I

Incorporation of labeled leucine and isoleucine into mycelium and aspergillic acid by A. flavus

Experiment No.	Labeled compound and activity	S.A.* in mycelium hydrolysate of		S.A. aspergillic acid	S.A. of degradation products of aspergillic acid	
		Leucine	Isoleucine		Leucine	Isoleucine isomers
1	DL-Leucine-1-C^{14}, 4.36 μc	1.04	<0.01	1.19	1.21	<0.02
2	D-Leucine-1-C^{14}, 1.57 μc	0.53	<0.01	0.62		
3	Uniformly labeled L-Leucine-C^{14}, 4.84 μc	0.84	0.02	1.26		
4	Uniformly labeled L-Isoleucine-C^{14}, 4.58 μc	0.19	1.68	2.29	0.22	2.32

* S.A. = specific activity, μc per mmole.

DISCUSSION

The results of the present work support the hypothesis that the molecule of aspergillic acid is synthesized by *A. flavus* from one molecule of leucine plus one molecule of isoleucine. There are other reports in the literature which indicate that structures similar in some respects to that of aspergillic acid are derived from the appropriate amino acids. Winstead and Suhadolnik (11) have provided evidence to show that gliotoxin (Fig. 2) is derived in part from *m*-tyrosine and serine. Birch *et al.* (12) have mentioned that the heterocyclic ring in mycelianamide (Fig. 3) is derived in part from tyrosine, with alanine presumably

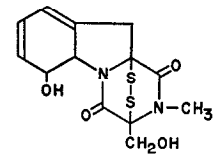

FIG. 2. Gliotoxin

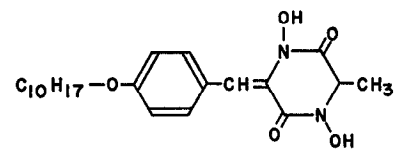

FIG. 3. Mycelianamide

being used for the synthesis of the rest of the ring. A possible route for the biosynthesis of such compounds by the oxidation of appropriate peptides has been outlined by Birch and Smith (12).

The degradation of one molecule of aspergillic acid might be expected to yield one molecule of leucine and one of isoleucine (or alloisoleucine). The actual yield of leucine obtained was about double that of the isoleucine plus alloisoleucine, but less than the theoretical maximum. It seems unlikely that the aspergillic acid was heavily contaminated with a similar compound (as yet unknown) derived from two molecules of leucine. The properties of the compound used here were the same as those of an authentic sample of aspergillic acid. In addition, the specific activity of the leucine or isoleucine obtained in Experiments 1 or 4, respectively, from the degradation of aspergillic acid agreed well with the activity in the whole molecule. It is more likely that the degradation procedure produced compounds which gave a higher yield of leucine than isoleucine isomers on hydrolysis.

The specific activities of aspergillic acid or the amino acids derived from it on degradation were significantly higher than the specific activity of the corresponding amino acids in the mycelium. It may be that mycelial leucine and isoleucine are derived to a greater extent from endogenously synthesized leucine or isoleucine than is the antibiotic. *A. flavus* can undoubtedly synthesize these amino acids, for it grows well on media composed of sucrose, nitrate, and salts. However, it is not known whether the organism will synthesize these amino acids if they are supplied in the medium. Further work will have to be done before the differences in specific activity mentioned above can be explained.

SUMMARY

1. When *Aspergillus flavus* was grown on media containing DL-leucine-1-C^{14}, D-leucine-1-C^{14}, uniformly labeled L-leucine-C^{14} or L-isoleucine-C^{14}, significant amounts of radioactivity were found in mycelial leucine or isoleucine, and in aspergillic acid.

2 When the aspergillic acid isolated from medium containing DL-leucine-1-C^{14} was degraded to leucine and isoleucine isomers, the radioactivity was found in carbon atom 1 of leucine and none in the isoleucine isomers.

3. When the aspergillic acid isolated from medium containing uniformly labeled L-isoleucine-C^{14} and a small amount of leucine-C^{14} was degraded, most of the radioactivity was found in isoleucine isomers and a small amount in leucine.

4. The *sec*-butyl side chain of aspergillic acid has the same optical configuration as that of carbon atom 3 of L-isoleucine.

5. The results support the hypothesis that aspergillic acid is synthesized by *A. flavus* from leucine and isoleucine.

Acknowledgments—The author is greatly indebted to Mr. D. M. Tenaschuk for technical assistance, to Mr. J. Dyck and Mr. M. Mazurek for radioisotope analyses, and to Miss I. M. Gaffney for infrared and x-ray diffraction analyses.

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