S 28. *Aspergillic Acid. Part II. The Conversion of Aspergillic Acid into Leucine and* iso*Leucine (or alloiso*Leucine).

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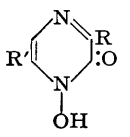
The compound $C_{12}H_{22}O_2N_2$, obtained from aspergillic acid by bromination followed by treatment with zinc and acetic acid, is shown to be 2 : 5-diketo-3-*iso*butyl-6-*sec.*-butylpiperazine (leucyl*iso*leucine anhydride) since, on hydrolysis, it yields a mixture of leucine and *iso*leucine (or *alloiso*leucine). It follows that aspergillic acid is either (I; R = Bu^s, R′ = Bu^i) or (I; R = Bu^i, R′ = Bu^s).

TREATMENT of aspergillic acid with bromine followed by reduction of the monobromo-derivative with zinc and acetic acid gives a compound, $C_{12}H_{22}O_2N_2$, considered by Dutcher (*J. Biol. Chem.*, 1947, **171**, 341) to be an active *iso*leucine anhydride. In Part I (this vol., p. S 126) it is shown that the side chains in aspergillic acid cannot both be *sec.*-butyl groups from which it follows that the compound $C_{12}H_{22}O_2N_2$ cannot be an *iso*leucine anhydride. Furthermore, we showed that the identification of the compound $C_{12}H_{22}O_2N_2$ as *iso*leucine anhydride by the absence of a mixed melting-point depression is not reliable since the compound $C_{12}H_{22}O_2N_2$ is also undepressed in melting point when mixed with leucyl*iso*leucine anhydride.

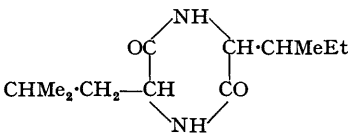
Assuming the general formula (I) for aspergillic acid, the only limitations upon the nature of the side chains are that R + R′ must equal C_8H_{18}, and that one of these side chains must contain an asymmetric centre. Furthermore, even the general formula (I) is a simplification which has not been rigorously established; although it is established that one nuclear position is unsubstituted, the decision that this is the 5-position (in a 2-hydroxypyrazine nucleus) is dependent upon the assumption that the compound $C_{12}H_{22}O_2N_2$ is a 2 : 5-diketopiperazine, a conclusion which has not been established. These considerations suggested that attempts to synthesise deoxyaspergillic acid should be preceded by attempts to obtain further information concerning the specific nature of the side chains.

The side chains of aspergillic acid have now been identified as a *sec.*-butyl and an *iso*butyl group by an examination of the compound $C_{12}H_{22}O_2N_2$. Complete hydrolysis of 2 : 5-diketopiperazines to the related amino-acids is in general a difficult procedure, and relatively few cases of such direct hydrolyses have been reported. Model experiments using *iso*leucine anhydride showed that this 2 : 5-diketopiperazine is converted into *iso*leucine in good yield by prolonged refluxing with 48% hydrobromic acid. This method when applied to the compound $C_{12}H_{22}O_2N_2$ gave, again in high yield, a product which had the general properties of an α-amino-acid. The absence of glycine, alanine, valine, and norvaline from this hydrolysis product was readily demonstrated by the paper strip partition chromatographic method of Consden, Gordon, and Martin (*Biochem. J.*, 1944, **38**, 224) with *n*-butanol saturated with water as the mobile phase. Furthermore, the behaviour of the hydrolysate on the strip was indistinguishable from that of leucine or that of *iso*leucine or from that of a mixture of these two amino-acids. The hydrolysate was converted into its copper salt which was separated into a relatively water-soluble fraction and a relatively water-insoluble fraction. Regeneration of the amino-acid from the latter gave DL-leucine (III) identified as its 3 : 5-dinitrobenzoyl derivative and by its conversion into *iso*valeraldehyde by reaction with ninhydrin. The more soluble copper salt was reconverted into the corresponding α-amino-acid, degradation of which with ninhydrin gave methylethyl-

acetaldehyde, thus proving that the α-amino-acid is *iso*leucine (or *alloiso*leucine) (IV). The compound $C_{12}H_{22}O_2N_2$ is, therefore leucyl*iso*leucine anhydride(2 : 5-diketo-6-*iso*butyl-3-*sec.*-butylpiperazine) (II) and deoxyaspergillic acid is either 3-hydroxy-5-*iso*butyl-2-*sec.*-butylpyrazine (V) or 3-hydroxy-2-*iso*butyl-5-*sec.*-butylpyrazine (VI) from which it follows that aspergillic acid is either (I; R = Bu^s, R′ = Bu^i), or (I; R = Bu^i, R′ = Bu^s).



(I.)



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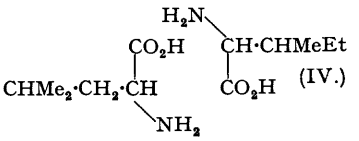

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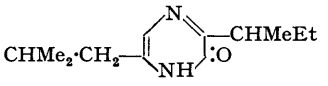
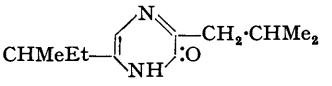


(V.)



(VI.)

Experimental.

3 : 5-*Dinitrobenzoyl*-DL-*norleucine* was prepared by using the method described by Saunders (*Biochem. J.*, 1934, **28**, 580). It separates from 50% ethanol as microcrystalline plates, m. p. 202—204° (Found : C, 47·6; H, 4·9; N, 12·9. $C_{13}H_{15}O_7N_3$ requires C, 48·0; H, 4·6; N, 12·9%).

3 : 5-*Dinitrobenzoyl*-DL-*leucine* separates as pale yellow plates from aqueous ethanol, m. p. 195—198° (with some sintering at 185°) (Found : C, 48·1; H, 4·6; N, 12·8%).

3 : 5-*Dinitrobenzoyl*-DL-iso*leucine* separates as plates from aqueous alcohol, m. p. 189—192° (sinters at 180°) (Found : C, 48·2; H, 4·6; N, 13·2%).

Diketopiperazine $C_{12}H_{22}O_2N_2$ *from Aspergillic Acid.*—The diketopiperazine was prepared from aspergillic acid in 27% yield by using the method described by Dutcher (*J. Biol. Chem.*, 1947, **171**, 321, 341). It separates from aqueous methanol as needles, m. p. 261—262° (sealed tube) with some sublimation; $[\alpha]_D^{18°}$ +10·7° (*l*, 1; *c*, 0·94 in methanol). Dutcher gives m. p. 249—250° with sublimation, $[\alpha]_D$ +13·8°, and does not record an analysis of this compound (Found : C, 63·7; H, 9·6; N, 12·5. $C_{12}H_{22}O_2N_2$ requires C, 63·7; H, 9·7; N, 12·4%).

Hydrolysis of iso*Leucine Anhydride.*—*iso*Leucine anhydride was prepared by two methods; a modification of Fischer's method (*Ber.*, 1901, **34**, 433; 1906, **39**, 2960) for the preparation of DL-leucine anhydride as described by Dutcher (*loc. cit.*) gave DL-*iso*leucine anhydride as needles from aqueous methanol, m. p. 280—283° (sealed tube), with sintering above 270° (Found : C, 63·4; H, 9·6. Calc. for $C_{12}H_{22}O_2N_2$: C, 63·7; H, 9·7%). By using the method described by Baxter and Spring (*J.*, 1947, 1179), *iso*leucine anhydride was obtained as needles from aqueous methanol, m. p. 277—280° (sinters 268°) (Found : C, 63·6; H, 10·1; N, 12·1. Calc. for $C_{12}H_{22}O_2N_2$: C, 63·7; H, 9·7; N, 12·4%).

*iso*Leucine anhydride (2 g.) was refluxed with 48% hydrobromic acid (20 c.c.), for 20 hours. The solution was evaporated to dryness under reduced pressure, the residue dissolved in a little water, and the solution treated with excess of silver hydroxide. The silver bromide–silver hydroxide mixture was removed by filtration, and the filtrate treated with hydrogen sulphide and filtered. The filtrate was evaporated to dryness, the residue dissolved in a little water, and the solution clarified by treatment with charcoal (50 mg.) and evaporated to dryness. The white powdery residue was crystallised from boiling water, from which *iso*leucine separated as small plates, m. p. 260—263° (sealed tube with sublimation) (yield, 60%); it gave a positive ninhydrin test (Found : C, 55·3; H, 10·1; N, 10·8. Calc. for $C_6H_{13}O_2N$: C, 55·0; H, 9·9; N, 10·7%).

The *N*-formyl derivative was obtained as plates, m. p. 117—119° undepressed when mixed with an authentic specimen.

Hydrolysis of Compound $C_{12}H_{22}O_2N_2$.—The compound $C_{12}H_{22}O_2N_2$ (500 mg.) was refluxed with 48% hydrobromic acid (10 c.c.) for 12 hours, and the product isolated by the procedure described above for *iso*leucine anhydride. The hydrolysis product (425 mg.) is a white powder, $[\alpha]_D = 0° \pm 1°$ (*c*, 0·95 in water), which gives a positive ninhydrin test.

A solution of the hydrolysate (420 mg.) in water (150 c.c.) was treated with a warm solution of cupric acetate (320 mg.) in water (50 c.c.). The copper salt was filtered off (filtrate *A*) and shaken with 150 c.c. of water and collected, the filtrate being rejected. The solid was shaken with methanol (75 c.c.), and again filtered. The copper salt was suspended in water (50 c.c.) and treated with hydrogen sulphide, and the mixture filtered. The filtrate was clarified by treatment with charcoal (50 mg.), concentrated to 3 c.c., and diluted with an equal volume of ethanol. On standing, DL-leucine separated; after 2 recrystallisations from aqueous ethanol, it was obtained as plates, m. p. 273—277° (65 mg.), which give a positive ninhydrin test (Found : C, 54·9; H, 9·5. Calc. for $C_6H_{13}O_2N$: C, 55·0; H, 9·9%).

The 3 : 5-dinitrobenzoyl derivative was prepared by the method described above. It separates from ethanol as plates, m. p. 194—196·5°. A mixture of this derivative with 3 : 5-dinitrobenzoyl-DL-leucine (m. p. 195·0—198°) had m. p. 195·5—197°, and a mixture with 3 : 5-dinitrobenzoyl-DL-*iso*leucine (m. p. 189—191°) had m. p. 180—183°.

A solution in water (25 c.c.) of DL-leucine (80 mg.) isolated from the hydrolysate was treated with ninhydrin (75 mg.), and distilled in a stream of carbon dioxide into an aqueous solution of 2 : 4-dinitrophenylhydrazine hydrochloride; 10 c.c. of distillate were collected in 30 minutes. The phenylhydrazone

was collected (60 mg.) and recrystallised from ethanol to yield *iso*valeraldehyde 2 : 4-dinitrophenylhydrazone as orange plates, m. p. 119—120°. undepressed when mixed with an authentic specimen. A mixture with methylethylacetaldehyde 2 : 4-dinitrophenylhydrazone (m. p. 129—130°) shows a considerable m. p. depression (Found : C, 49·6; H, 5·05; N, 21·3. Calc. for $C_{11}H_{14}O_4N_4$: C, 49·6; H, 5·3; N, 21·1%).

The filtrate *A* was evaporated to dryness, and the residue extracted with warm methanol (2 × 30 c.c.). The methanol extract was evaporated under reduced pressure, and the residue suspended in water (40 c.c.) and decomposed by treatment with hydrogen sulphide. After filtration, the solution was evaporated to dryness; the residue was dissolved in water (10 c.c.), and the solution clarified with charcoal and evaporated to dryness. The *iso*leucine (allo*iso*leucine) (92 mg.) separated from aqueous alcohol as small plates, m. p. 250—253°, $[\alpha]_D$ −0·35° ±0·7° (*l*, 2; *c*, 0·4 in water); it gives a positive ninhydrin reaction (Found : N, 10·1, 10·2. Calc. for $C_6H_{13}O_2N$: N, 9·9%).

A solution of the amino-acid (90 mg.) was treated with ninhydrin as described for DL-leucine to yield methylethylacetaldehyde 2 : 4-dinitrophenylhydrazone (80 mg.) which was twice recrystallised from ethanol from which it separated as orange plates, m. p. 127—129°, undepressed when mixed with a specimen prepared from authentic DL-isoleucine; a mixture with *iso*valeraldehyde 2 : 4-dinitrophenylhydrazone prepared from DL-leucine showed a depression in m. p. (Found : C, 49·4, 49·7; H, 5·0, 4·9; N, 20·9. Calc. for $C_{11}H_{14}O_4N_4$: C, 49·6; H, 5·3; N, 21·1%).

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