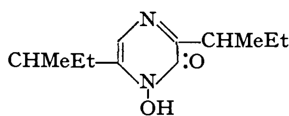
S 27. *Aspergillic Acid. Part I.*

By George Dunn, J. J. Gallagher, G. T. Newbold, and F. S. Spring.

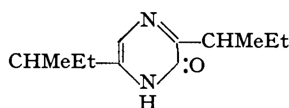
From culture filtrates of the mould *Aspergillus flavus* grown on a medium containing sodium chloride and a casein hydrolysate, aspergillic acid and an alkyl-substituted *hydroxypyrazine*, $C_{12}H_{20}ON_2$, isomeric with deoxyaspergillic acid, have been isolated.

Deoxyaspergillic acid is racemised on vigorous treatment with alkali, and the *racemate* is different from racemic 3-hydroxy-2 : 5-di-*sec*.-butylpyrazine, which itself is unaffected on similar treatment with alkali. It is concluded that deoxyaspergillic acid is not an optically active 3-hydroxy-2 : 5-di-*sec*.-butylpyrazine. The diketopiperazine DL-*norleucyl*-DL-iso*leucine anhydride* (IX) has been synthesised, and, whilst it is very similar to DL-*iso*leucine anhydride (III), it differs from an isomeric compound obtained from aspergillic acid. DL-*Leucyl*-DL-iso*leucine anhydride* (VIII) closely resembles DL-*iso*leucine anhydride and the compound $C_{12}H_{22}O_2N_2$ from aspergillic acid. These facts suggest that deoxyaspergillic acid may be either 3-hydroxy-2-*iso*butyl-5-*sec*.-butylpyrazine (XIV) or 3-hydroxy-5-*iso*butyl-2-*sec*.-butylpyrazine (XIII), and that aspergillic acid may be either (IV) or (V).

Dutcher and Wintersteiner (*J. Biol. Chem.*, 1944, **155**, 359; Dutcher, *ibid.*, 1947, **171**, 321, 341) have ascribed the structure (I) to the antibacterial compound aspergillic acid, from which it follows that its simple reduction product, deoxyaspergillic acid, is to be represented as 3-hydroxy-2 : 5-di-*sec.*-butylpyrazine (II). Newbold and Spring (*J.*, 1947, 373) synthesised 3-hydroxy-2 : 5-di-*sec.*-butylpyrazine (II), but were unable to resolve the racemate; the same racemate has subsequently been synthesised by two different routes (Baxter and Spring, *J.*, 1947, 1179; Newbold and Spring, *ibid.*, p. 1183). A comparison of the ultra-violet absorption



(I.)



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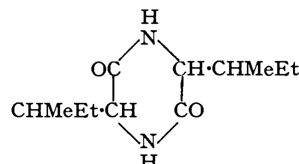


(III.)

spectrum of deoxyaspergillic acid with the spectra of racemic 3-hydroxy 2 : 5-di-*sec.*-butylpyrazine and other hydroxypyrazines together with the general similarity in the properties of these compounds led Newbold and Spring to the view that deoxyaspergillic acid is a hydroxypyrazine derivative. The differences in the intensities of absorption in the ultra-violet region did not allow of the decision that deoxyaspergillic acid and 3-hydroxy-2 : 5-di-*sec.*-butylpyrazine are structurally identical. We have, therefore, undertaken a re-examination of aspergillic acid and deoxyaspergillic acid with the object of obtaining further information concerning the nature of the alkyl side chains.

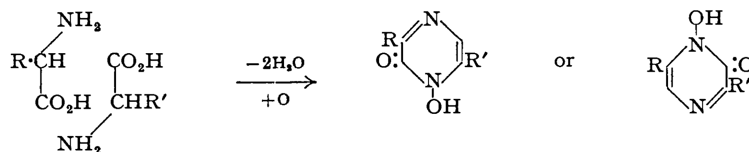
The strain of *Aspergillus flavus* employed was a variant of White's original strain, described by Jones, Rake, and Hamre (*J. Bact.*, 1943, **45**, 461), and the culture medium was an aqueous solution of the casein hydrolysate " Pronutrin " and sodium chloride. Under the conditions described in the experimental section, the yield of aspergillic acid averaged 250 mg. per l. of culture filtrate; the acid, after sublimation, had m. p. 97—99° and $[\alpha]_D^{18}$ $+13{\cdot}3°$; White and Hill (*J. Bact.*, 1943, **45**, 433) give m. p. 96° and Dutcher (*loc. cit.*) gives m. p. 93°, $[\alpha]_D$ $+12°$ for aspergillic acid.

From the culture filtrate, we have isolated, in low yield, a compound of molecular formula $C_{12}H_{20}ON_2$. This compound is soluble in 3N-sodium hydroxide and in 3N-hydrochloric acid, but unlike aspergillic acid it is insoluble in sodium hydrogen carbonate solution; this property was used in separating it from aspergillic acid. Again, unlike aspergillic acid, it does not give a colouration with ferric chloride. The general properties of this compound, particularly its weak acidic and basic properties and the location and intensity of its ultra-violet absorption maxima, suggest that it is a hydroxypyrazine derivative. It is isomeric with deoxyaspergillic acid and with 3-hydroxy-2 : 5-di-*sec.*-butylpyrazine.

Aspergillic acid is reduced by hydrazine in good yield to deoxyaspergillic acid (Dutcher, *loc. cit.*). Deoxyaspergillic acid couples with benzenediazonium chloride to give a *phenylazo*-derivative. This reaction establishes the presence of an unsubstituted nuclear position in the pyrazine ring and agrees with the formulation of deoxyaspergillic acid as a 3-hydroxy-2 : 5-dialkylsubstituted pyrazine. 3-Hydroxy-2 : 5-di-*sec.*-butylpyrazine also yields a 6-*phenylazo*-derivative. When heated with alkali, deoxyaspergillic acid gives a *racemate*, m. p. 103—104°, which is undepressed in melting point when mixed with dextrorotatory deoxyaspergillic acid, but is depressed in melting point when mixed with racemic 3-hydroxy-2 : 5-di-*sec.*-butylpyrazine. Racemic 3-hydroxy-2 : 5-di-*sec.*-butylpyrazine is unaffected when treated with alkali using the same conditions; if deoxyaspergillic acid and the synthetic isomer simply differed in stereochemical orientation, this treatment would have been expected to produce the same racemic mixture. This observation leads to the conclusion that the alkyl side chains in deoxyaspergillic acid and in aspergillic acid are not both *sec.*-butyl groups.

Summarising the evidence, it is established that deoxyaspergillic acid contains a pyrazine ring, a nuclear hydroxyl group in the 3-position, and at least one unsubstituted nuclear position, most probably the 6-position. The remaining C_8H_{18} fragment is to be distributed either as a single alkyl side chain, which must contain at least one asymmetric centre, located at the 2- or the 5-position, or as two alkyl groups, at least one of which must contain an asymmetric centre, located at the 2- and 5-positions. The production of aspergillic acid by *Aspergillus flavus* requires the presence of an amino-acid source in the culture medium (White and Hill, *J. Bact.*, 1943, **45**, 433). This, taken in conjunction with the fact that the side chains are not

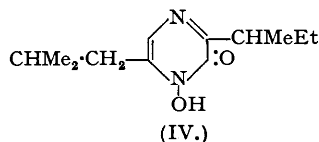
both *sec.*-butyl groups, suggests that aspergillic acid is derived from two different amino-acids thus : *



If leucine and *iso*leucine are assumed to be the parent amino-acids, the derived cyclic hydroxamic acid would be (IV) or (V); similarly, norleucine with *iso*leucine would give rise to the cyclic hydroxamic acids (VI) or (VII).

One of the major reasons which led Dutcher and Wintersteiner to assign the specific structure (I) to aspergillic acid was the fact that the acid can be converted into a compound $C_{12}H_{22}O_2N_2$ which, apart from optical differences, appeared to be identical with DL-*iso*leucine anhydride (III). If aspergillic acid is (IV) or (V), the derived diketopiperazine will be leucyl*iso*leucine anhydride (VIII), and if the acid is (VI) or (VII) the diketopiperazine will be norleucyl*iso*leucine anhydride (IX). For purposes of comparison, we have prepared the three diketopiperazines (III), (VIII), and (IX), and the compound $C_{12}H_{22}O_2N_2$ from aspergillic acid. DL-*iso*Leucine anhydride was prepared by a method described by Baxter and Spring (*loc. cit.*) and also by a method described by Dutcher (*loc. cit.*).

A leucyl*iso*leucine anhydride, m. p. 273—277°, $[\alpha]_D$ −15·9°, has been isolated from hog bristles (Abderhalden and Komm, *Z. physiol. Chem.*, 1924, **134**, 113), and a leucyl*iso*leucine anhydride, m. p. 291°, $[\alpha]_D$ −35·8°, has been obtained from L-leucyl-D-*iso*leucine methyl ester (Abderhalden and Hirsch, *Ber.*, 1910, **43**, 2435). We have prepared DL-*leucyl*-DL-iso*leucine anhydride* (2 : 5-*diketo*-3-iso*butyl*-6-sec.-*butylpiperazine*) (VIII) by two different methods, in the first of which the dipeptide DL-leucyl-DL-*iso*leucine (X) (Abderhalden, Hirsch, and Schuler, *Ber.*, 1909, **42**, 3394) is dehydrated to the diketopiperazine by heating with β-naphthol (cf.



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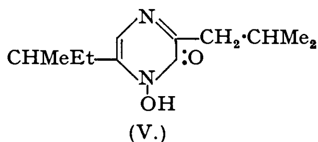

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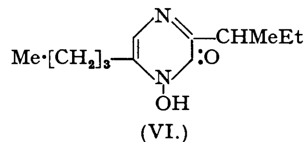

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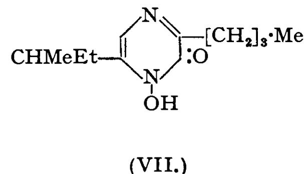

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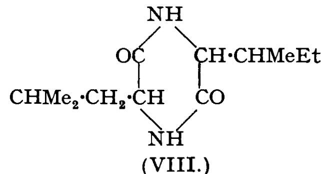
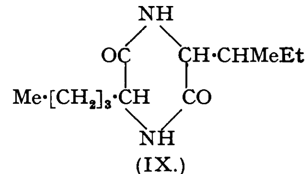


(VIII.)



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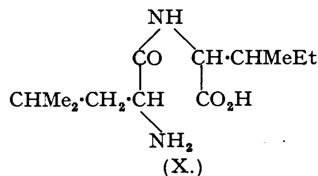

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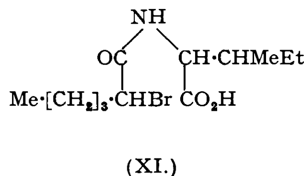

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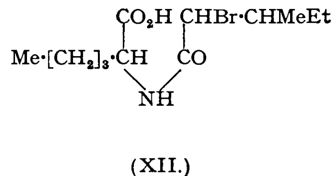

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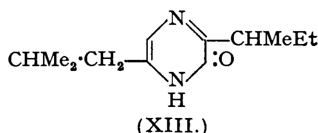

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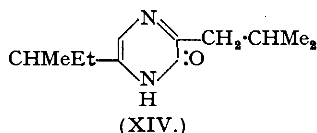

(XIV.)

* Biogenetic considerations led Dutcher to postulate that the side chains are both *sec.*-butyl groups, and the possibility of *iso*leucine being the biochemical precursor of aspergillic acid was discussed since two moles of *iso*leucine supply the necessary carbon and nitrogen skeleton for the structure (I). This author reported that experiment failed to disclose the direct utilisation of this amino-acid for the formation of aspergillic acid. We have found that if an enzymic hydrolysate of casein is replaced by an acid hydrolysate of the same protein, *A. flavus* does not produce aspergillic acid.

Lichtenstein, *J. Amer. Chem. Soc.*, 1938, **60**, 560). The second method was by esterification of α-bromo*iso*hexoyl-DL-*iso*leucine followed by treatment of the product with ammonia under pressure. DL-*Norleucyl*-DL-iso*leucine anhydride* (2 : 5-*diketo*-3-n-*butyl*-6-sec.-*butylpiperazine*) (IX) was obtained by the condensation of α-bromo-*n*-hexoyl bromide with DL-*iso*leucine to give α-*bromo*-n-*hexoyl*-DL-iso*leucine* (XI). Esterification of (XI) followed by treatment of the ester with alcoholic ammonia yielded the diketopiperazine (IX). A second method was by condensation of α-bromo-β-methylvaleryl chloride with DL-norleucine to give α-*bromo*-β-*methyl-valeryl*-DL-*norleucine* (XII) which was esterified and the ester treated with ammonia. The solubility properties of *iso*leucine anhydride, DL-leucyl-DL-*iso*leucine anhydride, DL-norleucyl-DL-*iso*leucine, and the compound $C_{12}H_{22}O_2N_2$ from aspergillic acid are similar, and they each melt within the range 255—282°. The melting point of these compounds is not a satisfactory criterion; it is complicated by a tendency to sublimation, and varies according to the rate of heating and the initial temperature of the bath.

We confirmed the observation of Dutcher that the melting point of the compound $C_{12}H_{22}O_2N_2$ from aspergillic acid is not depressed when mixed with DL-*iso*leucine anhydride. A mixture of the former with DL-norleucyl-DL-*iso*leucine anhydride on the other hand shows a slight but consistent depression. The compound $C_{12}H_{22}O_2N_2$ from aspergillic acid is not depressed in melting point when mixed with DL-leucyl-DL-*iso*leucine anhydride. This latter observation nullifies the most important evidence adduced in favour of the specific structure (I) for aspergillic acid. Furthermore, it leads to the conclusion that (XIII) and (XIV) are possible structures for deoxyaspergillic acid, and that aspergillic acid may be either (IV) or (V).

EXPERIMENTAL.

Aspergillic Acid.—The medium was prepared by dissolving the casein hydrolysate " Pronutrin " (20 g.) and sodium chloride (5 g.) in distilled water (1 l.). The culture solution was distributed in flasks each containing, approximately, 380 c.c. of solution (1·5 cm. depth) which were then steam-autoclaved for 15 minutes at 15 lbs. After this treatment, the medium contained a slight sediment and had pH 5·4—5·6. The inoculum consisted of the spores from two ten-day beer wort–agar slopes of *A. flavus* incubated at 25°, suspended in distilled water (100 c.c.); 5 c.c. of this inoculum were added to each flask. The flasks were incubated at 23° ± 1° for 14 days; growth was scanty, and there was some sporulation on about the twelfth day. The mycelium was removed, and the filtrate from 100 flasks (33 l.; pH 7·8) adjusted to pH 4·0 by addition of 3N-hydrochloric acid. The mixture was filtered, and the filtrate stirred for 3 hours with charcoal (700 g.). The charcoal was dried in air and extracted (Soxhlet) with ether. The red ethereal solution was concentrated to 250 c.c., extracted with 2N-sodium hydroxide (2 × 150 c.c.), the extract acidified with 33% acetic acid, and the mixture kept at 0° overnight. The separated solid was collected and shaken for 6 hours with 2% sodium hydrogen carbonate solution. The insoluble fraction (430 mg.; solid *A*) was removed, and the filtrate was acidified by the dropwise addition of 33% acetic acid with stirring during 3 hours. The solid was collected, dried (8 g.; m. p. 80—85°), and purified by again dissolving it in sodium hydrogen carbonate solution followed by precipitation with 33% acetic acid as described above. Crystallisation from methanol gave aspergillic acid as radial clusters of yellow needles. After sublimation at 80°/10^{-3} mm., it has m. p. 97—99°, $[\alpha]_D^{18°}$ + 13·3° ± 2° (*c*, 3·9 in ethanol), pK_a = 5·95. In ethanol solution it exhibits absorption maxima at 3280 A., ε = 8,300, and 2340 A., ε = 6,500 (Found: C, 64·2, 64·5; H, 8·9, 8·8; N, 12·3. Calc. for $C_{12}H_{20}O_2N_2$: C, 64·3; H, 8·9; N, 12·5%).

Compound, $C_{12}H_{20}ON_2$.—The solid *A* was shaken with 2N-sodium hydroxide for 1 hour, the mixture filtered from resinous matter, and the filtrate acidified by addition of 33% acetic acid. The solid was collected, and after repeated crystallisation from aqueous methanol the *compound* separated as needles, m. p. 143—145°. It is insoluble in water and in sodium hydrogen carbonate solution, but soluble in 3N-hydrochloric acid and in 3N-sodium hydroxide. It does not give a coloration with aqueous ferric chloride solution, and it sublimes unchanged at 115°/10^{-3} mm. [Found (two different preparations): C, 68·9, 68·9, 69·0; H, 9·35, 9·55, 9·3; N, 13·7. $C_{12}H_{20}ON_2$ requires C, 69·2; H, 9·6; N, 13·5%].

Deoxyaspergillic Acid.—A solution of aspergillic acid (2 g.) in ethanol (60 c.c.) was treated with 99% hydrazine hydrate (2 c.c.) and heated in an autoclave at 170° for 9 hours. The mixture, which then gave a negative test with ferric chloride, was evaporated under reduced pressure, the residue dissolved in ether, and the solution extracted with 3N-hydrochloric acid (5 × 10 c.c.). The extract was neutralised (litmus) by the addition of sodium hydroxide solution, the solid collected and dissolved in ether, and the solution extracted with 3N-sodium hydroxide. The alkaline extract was neutralised by addition of hydrochloric acid and purified by sublimation and crystallisation from aqueous methanol, from which deoxyaspergillic acid separates as needles, m. p. 98—100°, $[\alpha]_D^{18°}$ +21·3° ± 1° (*c*, 2·55 in ethanol) (yield, 65%) (Found: C, 69·3, 69·35; H, 9·3, 9·6; N, 13·4. Calc. for $C_{12}H_{20}ON_2$: C, 69·2; H, 9·6; N, 13·5%).

Racemisation of Deoxyaspergillic Acid.—A solution of deoxyaspergillic acid (260 mg.) in N-potassium hydroxide (12·5 c.c.) was heated in an autoclave at 170° for 24 hours. The solution was neutralised by addition of dilute hydrochloric acid, and the precipitated solid collected and crystallised from aqueous methanol, from which the *racemate* separates as needles, m. p. 103—104°, $[\alpha]_D^{17°}$ 0° ± 1° (*c*, 3·5 in ethanol). A mixture of deoxyaspergillic acid (m. p. 98—100°) and racemic deoxyaspergillic acid had m. p. 100—102°. A mixture of racemic deoxyaspergillic acid and 3-hydroxy-2 : 5-di-*sec.*-butylpyrazine (m. p. 122°) melted at 80—88°. Light absorption in alcohol: Maxima at 2280 A., ε = 7000, and 3250 A., ε = 8400

(Found: C, 69·0; H, 9·6. $C_{12}H_{20}ON_2$ requires C, 69·2; H, 9·6%). 3-Hydroxy-2 : 5-di-*sec*.-butylpyrazine was recovered unchanged (m. p. and mixed m. p.) after similar treatment with alkali.

Phenylazodeoxyaspergillic Acid.—A solution of deoxyaspergillic acid (0·7 g.) in 3N-sodium hydroxide (35 c.c.) was treated at 0° with an ice-cold solution of benzenediazonium chloride prepared from aniline (1·2 c.c.), hydrochloric acid (*d* 1·16; 10·5 c.c.), water (10·5 c.c.), and sodium nitrite solution. The yellow solid was collected and washed with a little water. It was suspended in warm water, and the mixture acidified to Congo-red with dilute hydrochloric acid. The solid (0·87 g.) was filtered and crystallised from aqueous alcohol (charcoal) from which *phenylazodeoxyaspergillic acid* separates as red needles, m. p. 188—190° (Found: C, 69·4; H, 7·4; N, 18·1, 17·8. $C_{18}H_{24}ON_4$ requires C, 69·3; H, 7·7; N, 17·95%).

The phenylazo-derivative of racemic deoxyaspergillic acid was obtained using the same procedure (yield 75%). It separates from aqueous alcohol as red needles, m. p. 188—190° either alone or with phenylazodeoxyaspergillic acid (Found: N, 18·1%).

6-*Phenylazo*-3-*hydroxy*-2 : 5-*di*-sec.-*butylpyrazine*, prepared by the method described above, separates from aqueous ethanol as light red needles, m. p. 200° (yield, 94%). A mixture of this and the phenylazo-derivative of deoxyaspergillic acid had m. p. 188—190°, and a mixture with the phenylazo-derivative of racemic deoxyaspergillic acid had m. p. 188—190·5° (Found: C, 69·2; H, 7·5; N, 17·6. $C_{18}H_{24}ON_4$ requires C, 69·3; H, 7·7; N, 17·95%).

DL-*Leucyl*-DL-isoleucine *Anhydride* (2 : 5-*Diketo*-3-iso*butyl*-6-sec.-*butylpiperazine*).—(*a*) α-Bromo*iso*hexoyl-DL-*iso*leucine was prepared by the method described by Abderhalden, Hirsch, and Schuler (*loc. cit.*) who give m. p. 146—149° (corr.). According to our repeated observations, this derivative has a considerably higher m. p. The reaction solution obtained, using the conditions described by Abderhalden *et al.*, was filtered and acidified with 5N-hydrochloric acid; the product separated as an oil which quickly solidified, and then had m. p. 142—144°. After 6 recrystallisations from aqueous alcohol, α-bromo*iso*hexoyl-DL-*iso*leucine separated as plates, m. p. 178·5° (uncorr.) (Found: C, 46·8; H, 7·1; N, 4·4. Calc. for $C_{12}H_{22}O_3NBr$: C, 46·75; H, 7·1; N, 4·5%). A solution of α-bromo*iso*hexoyl-DL-*iso*leucine (65 g.) in ammonia (*d* 0·88; 370 c.c.) was kept in a tightly stoppered flask at room temperature for 4 days. The ammonia was removed, and the solution concentrated to 100 c.c. DL-Leucyl-DL-*iso*leucine separated on standing as rhombic prisms, m. p. 261—262°; it was recrystallised from water containing a little alcohol from which it separates as rhombic prisms, m. p. 272° (266—267° in a sealed tube) [Abderhalden, Hirsch, and Schuler give m. p. 262—263° (corr.) (yield, 33%)]. The dipeptide gives a positive ninhydrin reaction which is slow in developing. The colour (grey-blue) is weak compared with the colours produced using either leucine or *iso*leucine (Found: C, 59·1; H, 10·25; N, 11·5. Calc. for $C_{12}H_{24}O_3N_2$: C, 59·0; H, 9·8; N, 11·5%).

The dipeptide (15 g.) was heated for 3 hours with β-naphthol (65 g.) at 135—150°. The cooled mixture was ground to a fine powder and extracted with ether. The insoluble solid, m. p. 272—273° (11·4 g., 84%), was crystallised from alcohol, from which DL-*leucyl*-DL-iso*leucine anhydride* separated as long felted needles, m. p. 275—276° (sealed tube), with some sublimation. A mixture of this diketopiperazine with DL-*iso*leucine anhydride, m. p. 280—281° (sealed tube), prepared by the ethylene glycol method described by Baxter and Spring (*loc. cit.*), had m. p. 270—272°. A mixture of DL-leucyl-DL-*iso*leucine anhydride with the compound $C_{12}H_{22}O_2N_2$ (m. p. 260—262°) from aspergillic acid had m. p. 263—265° (Found: C, 63·8; H, 9·8; N, 12·7. $C_{12}H_{22}O_2N_2$ requires C, 63·7; H, 9·7; N, 12·4%).

(*b*) A solution of α-bromo*iso*hexoyl-DL-*iso*leucine (19·5 g.), prepared as described above, in dry ethanol (200 c.c.) was saturated with dry hydrogen chloride and heated under reflux for 2 hours. The solution was evaporated under reduced pressure, and a mixture of the gum, alcohol (200 c.c.), and liquid ammonia (*ca.* 75 c.c.) heated for 4 hours at 140° in an autoclave. The solid separating on cooling was recrystallised from ethanol, from which DL-leucyl-DL-*iso*leucine anhydride (5 g.) separated as needles, m. p. 261—262°. It was recrystallised 4 times from ethyl acetate, from which it separates as long needles, m. p. 266—267°, undepressed with the specimen described above. A mixture with DL-*iso*leucine anhydride (m. p. 280—282°, sealed tube), prepared either by the method of Baxter and Spring (*loc. cit.*) or by that described by Dutcher (*loc. cit.*), had m. p. 266—269°. A mixture with the compound $C_{12}H_{22}O_2N_2$ from aspergillic acid (m. p. 260—262°) had m. p. 263—265° (Found: C, 63·4; H, 9·7; N, 12·4%).

α-*Bromo*-n-*hexoyl*-DL-iso*leucine.*—α-Bromo-*n*-hexoyl bromide (b. p. 54—58°/2 mm.) (48 g.) and N-sodium hydroxide (270 c.c.) were added simultaneously with vigorous stirring at 0° to an ice-cooled solution of DL-*iso*leucine (20 g.) in N-sodium hydroxide (150 c.c.) during 30 minutes. The mixture was acidified by addition of 5N-hydrochloric acid (54 c.c.), and extracted with ether. The extract was dried (Na_2SO_4) and the solvent removed. The crystalline solid (8·5 g.), m. p. 165—167°, was recrystallised repeatedly from aqueous ethanol, from which α-*bromo*-n-*hexoyl*-DL-iso*leucine* separated in plates, m. p. 168—170° (Found: C, 47·1; H, 6·9; N, 4·5. $C_{12}H_{22}O_3NBr$ requires C, 46·75; H, 7·1; N, 4·5%).

α-*Bromo*-β-*methylvaleryl*-DL-*norleucine.*—α-Bromo-β-methylvaleryl chloride was condensed with DL-norleucine by the above method. The product was crystallised from ether–light petroleum (b. p. 60—80°), from which α-*bromo*-β-*methylvaleryl*-DL-*norleucine* separated in needles, m. p. 115—116° (yield 65%) (Found: C, 47·0; H, 7·3; N, 4·8. $C_{12}H_{22}O_3NBr$ requires C, 46·75; H, 7·1; N, 4·5%).

DL-*Norleucyl*-DL-iso*leucine Anhydride* (2 : 5-*Diketo*-3-n-*butyl*-6-sec.-*butylpiperazine*).—(*a*) A solution of α-bromo-*n*-hexoyl-DL-*iso*leucine (1 g.) in dry ethanol (20 c.c.) was saturated with dry hydrogen chloride and heated under reflux for 2 hours. The alcohol and hydrogen chloride were removed under reduced pressure, and the residual gum heated at 140° with alcohol (20 c.c.) and excess liquid ammonia in an autoclave for 4 hours. The mixture was concentrated and cooled, and the crystalline solid separating (240 mg.), m. p. 253—255°, collected. Recrystallisation from ethyl acetate gave DL-*norleucyl*-DL-iso*leucine anhydride* as felted needles, m. p. 258—260°; it is readily soluble in alcohol and acetone, moderately soluble in ether and ethyl acetate, and insoluble in water. This diketopiperazine is undepressed in m. p. when mixed with DL-leucyl-DL-*iso*leucine anhydride, m. p. 266—267°, and also when mixed with *iso*leucine anhydride, m. p. 280—282°. A mixture of DL-norleucyl-DL-*iso*leucine anhydride and the compound $C_{12}H_{22}O_2N_2$ from aspergillic acid (m. p. 259—260°) had m. p. 257—259°;

similar slight depressions in m. p. were observed on mixing several different preparations of the synthetic diketopiperazine with the compound $C_{12}H_{22}O_2N_2$ from aspergillic acid (Found: 64·0; H, 9·7; N, 12·2. $C_{12}H_{22}O_2N_2$ requires C, 63·7; H, 9·7; N, 12·4%).

(*b*) A solution of α-bromo-β-methylvaleryl-DL-norleucine (25 g.) in dry ethanol (250 c.c.) was saturated with dry hydrogen chloride and heated under reflux for 4 hours. The mixture was evaporated under reduced pressure, and the product heated at 120° in an autoclave with alcohol (250 c.c.) and excess of liquid ammonia (*ca.* 75 c.c.). The solid which separated on cooling was recrystallised from ethyl acetate and then from aqueous ethanol, from which DL-norleucyl-DL-*iso*leucine anhydride (3·5 g.) separated as felted needles, m. p. 259—260°, undepressed when mixed with the specimen described under (*a*) (Found: C, 63·6; H, 9·6; N, 12·2%).

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