620. *Gliotoxin. Part II. Degradative and Synthetic Studies on Dethiogliotoxin.*

By J. A. Elvidge and F. S. Spring.

It is shown that dethiogliotoxin, like gliotoxin, contains three hydroxyl groups and, with the object of locating these, attempts have been made to obtain monodehydration products from dethiogliotoxin. Although the latter is stable to thermal treatment, it is sensitive to dry hydrogen chloride, yielding a series of reaction products. Of these, the isomeric *compounds* A and F, $C_{13}H_{14}O_3N_2$, derived from dethiogliotoxin by the loss of the elements of one molecule of water, have received more particular attention. Each of the two isomers yields *N*-(indole-2-carboxy)-*N*-methylalanine (VI) on alkaline hydrolysis, and we suggest that they are stereoisomeric forms of the structure (IV) (or IV*a*). The alternative structure (III) for these isomers is shown to be improbable by experiments designed to synthesise this diol. Oxidation of the tetrahydropyrazinoindole (VII) with osmium tetroxide in the presence of pyridine gave in high yield the crystalline osmic ester–pyridine *complex* (VIII) which under relatively mild conditions of hydrolysis was degraded to indole-2-carboxymethylamide (X). The intermediate *cis*-diol (III) was not isolated, apparently because it decomposes spontaneously to yield the amide (X); that this instability is also shared by the *trans*-isomer appears probable from an attempt to synthesise the latter. Treatment of the tetrahydropyrazinoindole (VII) with peracetic acid gives a crystalline *diol monoacetate* to which we ascribe the structure (XI). Attempts to hydrolyse this ester with mineral acid failed to yield the diol (III) and the action of dilute methanolic ammonia afforded the amide (X). Treatment of the diol monoacetate with 2 : 4-dinitrophenylhydrazine in hydrochloric acid gave the bisdinitrophenylhydrazone of methylglyoxal, a result which again indicates profound instability of the diol (III).

A preliminary account is given of attempts to synthesise a dihydroindole derivative of the type (IV). Satisfactory surface-culture production of gliotoxin is described.

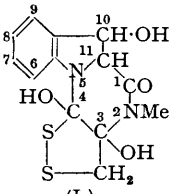
In Part I (Elvidge and Spring, this vol., p. S135) we discussed the points of attachment of the disulphide group to the nucleus in gliotoxin. We now report an examination of some of the structural features of dethiogliotoxin, the product obtained from gliotoxin by reduction with aluminium amalgam. Dethiogliotoxin (II) is represented by Dutcher, Johnson, and Bruce (*J. Amer. Chem. Soc.*, 1945, **67**, 1736) as a derivative of gliotoxin (I) in which the two sulphur atoms of the latter are replaced by two hydrogens. Support for this view was found in the close similarity in the ultra-violet absorption spectra of gliotoxin and dethiogliotoxin. Evidence in favour of this simple relationship was obtained by Elvidge and Spring, who showed that dethiogliotoxin, like gliotoxin, yields a diacyl derivative, and further support is now provided by determinations of active hydrogen in gliotoxin and dethiogliotoxin derivatives. By the Zerewitinoff method, gliotoxin and its dibenzoate give values corresponding to the presence of three and one active hydrogen atoms, respectively. Although dethiogliotoxin gives rather low values, its diacetate contains one active hydrogen, and we conclude that dethiogliotoxin, like the parent gliotoxin, contains three hydroxyl groups. These results render untenable the tentative suggestion (Dutcher, Johnson, and Bruce, *loc. cit.*) that gliotoxin (and consequently dethiogliotoxin) may be hydrated.

Active-hydrogen determinations.

	Found, %.	Calc., %, for :
Gliotoxin	0·95	3H 0·92
Gliotoxin dibenzoate	0·24	1H 0·19
Dethiogliotoxin	0·93, 0·78	2H 0·76; 3H 1·14
Dethiogliotoxin diacetate	0·39	1H 0·29

The conversion of dethiogliotoxin into DL-*N*-(indole-2-carboxy)-*N*-methylalanine (VI) by treatment with alkali (*idem, loc. cit.*) requires that the three hydroxyl groups in the pyrazinoindole nucleus are at 3 and 4 and either 10 or 11, from which it follows that dethioglio-

toxin is to be represented as either (II) or (II*a*) and that gliotoxin is either (I) or (I*a*). To test these formulations, efforts have been made to prepare a mono-dehydration product from dethiogliotoxin. Dethiogliotoxin and its acetate sublime unchanged at 230° and 150°, respectively, in a high vacuum. Such stability to thermal treatment is remarkable in a compound of structure (II) or (II*a*).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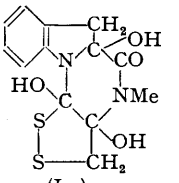
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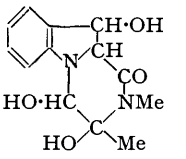
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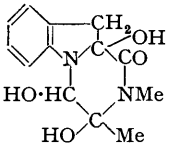
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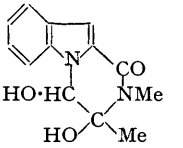
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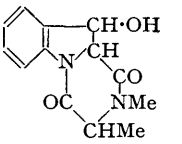
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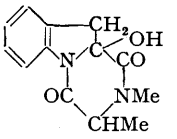
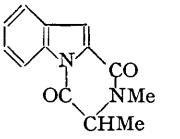

(IV*a*.)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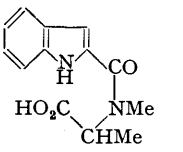

(VI.)

Treatment of a dioxan solution of dethiogliotoxin with hydrogen chloride gave a neutral product, *compound A*, of molecular formula $C_{13}H_{14}O_3N_2$. This compound is derived from dethiogliotoxin by the loss of the elements of one molecule of water. Similar treatment of dethiogliotoxin in dry methanol gave a mixture from which compound A and a second neutral product, *compound B*, were separated. Analysis of compound B, which does not contain methoxyl, established the molecular formula $C_{13}H_{18}O_5N_2$; it is thus derived from dethiogliotoxin by the addition of the elements of one molecule of water. It is to be noted that dethiogliotoxin does not form a hydrate when crystallised from an aqueous solvent. Compound B exhibits a double melting point and appears to be a hydrate since on being heated in a vacuum it loses water. Less vigorous heating in a high vacuum, followed by crystallisation under anhydrous conditions, unexpectedly yielded an isomeric *compound B'* which has a simple melting point and a different ultra-violet absorption spectrum from that of compound B.

With ethanol as solvent, treatment of dethiogliotoxin with dry hydrogen chloride gave a more complex mixture of products. From the mixture a hydrochloride, compound C, and a neutral compound D have been isolated in extremely low yields, insufficient for their satisfactory characterisation. In addition two neutral products of this reaction have been characterised; these are *compound E*, which has the molecular formula $C_{13}H_{16}O_4N_2$ and is therefore isomeric with dethiogliotoxin, and *compound F* which has the molecular formula $C_{13}H_{14}O_3N_2$ and is thus derived from dethiogliotoxin by the loss of the elements of one molecule of water, and is isomeric with compound A. A method for the isolation of compound F in relatively high yield was developed in which dethiogliotoxin is added to methanol previously saturated with hydrogen chloride; this observation is but one example of the great variation in the course of the reaction following slight changes in the experimental technique. In this case, compound F is accompanied by *compound G*, $C_{13}H_{18}O_5N_2$, isomeric with compound B. Like compound B, compound G shows a double melting point but this is lower than that of compound B. The ultra-violet absorption spectra of the two compounds G and B are significantly different. When heated in a high vacuum, compound G loses the elements of two molecules of water and gives compound F, a change which appears to be a simple dehydration of a hydrate since the ultra-violet absorption spectra of the two compounds G and F are almost identical. The spectra referred to are shown below.

Of these compounds, A and F are of more immediate interest since they both appear to

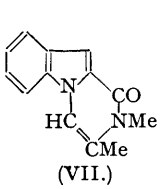
have been obtained from dethiogliotoxin by the loss of one molecule of water. They both dissolve in water to yield neutral solutions, and neither reacts with a mineral acid solution of 2 : 4-dinitrophenylhydrazine or gives a coloration with aqueous ferric chloride. When hydrolysed with alcoholic potassium hydroxide both compounds A and F give DL-*N*-(indole-2-carbonyl)-*N*-methylalanine (VI), together with small amounts of a high-melting by-product. The compounds A and F thus show some striking similarities to dethiogliotoxin itself. In terms of the formulation (II) or (II*a*) for dethiogliotoxin, compounds A and F can be represented either as (III), in which the hydroxyl group associated with the dihydroindole nucleus in dethiogliotoxin has been removed by a dehydration mechanism, or as (IV) or (IV*a*) in which the dehydration has involved the diol system in the pyrazine nucleus of dethiogliotoxin. Each of the dehydration compounds A and F contains one active hydrogen (Zerewitinoff) which leads us to the view that they are stereoisomers of the structure (IV (or IV*a*). We are confirmed in this view by a consideration of the ultra-violet absorption characteristics of the two compounds. Each shows a principal absorption maximum at approximately 2500 A.; in comparison, indole-2-carboxymethylamide exhibits an intense absorption maximum at 2930 A., as also does the compound (XI) (see below).

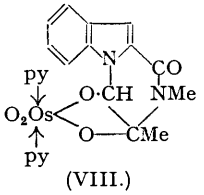
	Formula.	M. p.	$[\alpha]_D$.*	λ max., A.	ε.
Dethiogliotoxin	$C_{13}H_{16}O_4N_2$	248—249°	−130° (*c*, 0·2) †	2670	3,960
Compound A	$C_{13}H_{14}O_3N_2$	190	±0° (*c*, 0·43)	2540	10,000
				2790	2,455
Compound F	$C_{13}H_{14}O_3N_2$	156	−100°±5° (*c*, 0·48)	2530	10,370
				2750	5,980
Compound E	$C_{13}H_{16}O_4N_2$	168	—	2530	15,700
Compound B	$C_{13}H_{18}O_5N_2$	74 and 156	−87°±5° (*c*, 0·41)	2560	4,200
Compound B′	$C_{13}H_{18}O_5N_2$	156	—	2680	2,930
Compound G	$C_{13}H_{18}O_5N_2$	68 and 149	—	2520	9,800
				2760	4,700

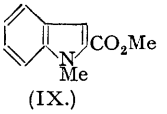
* Measured in a 1-dm. tube in ethanol. † Dutcher, Johnson, and Bruce (*loc. cit.*).

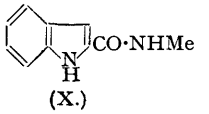
Compound F is remarkably stable, and when heated in a vacuum it sublimes unchanged. Attempts to convert it ($[\alpha]_D$ −100°) into the optically inactive compound A were unsuccessful, compound F being recovered unchanged after relatively vigorous treatment with methanolic hydrogen chloride. It is of some importance that the diketopiperazine (V), which can be considered as derived from compound A or F by the loss of one molecule of water, or as derived from dethiogliotoxin by the loss of two molecules of water, is also recovered unchanged after treatment with methanolic hydrogen chloride. With alkali the diketopiperazine is hydrolysed to DL-*N*-(indole-2-carboxy)-*N*-methylalanine (VI), but does not give the high-melting by-product obtained under these conditions from compounds A and F and from dethiogliotoxin.

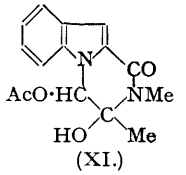
Concomitantly with the examination of the dehydration products A and F, attempts have been made to synthesise compounds of the structures (III) and (IV). A synthesis of (III) was attempted by direct hydroxylation of 3′-keto-4′ : 5′-dimethyl-1′ : 2′ : 3′ : 4′-tetrahydropyrazino-(1′ : 2′-1 : 2)indole (VII), with results which have a bearing on the structure of the compounds A and F. With *tert.*-butyl hydroperoxide in *tert.*-butanol containing a trace of osmium tetroxide (Milas and Sussman, *J. Amer. Chem. Soc.*, 1936, **58**, 1302) (VII) underwent no change. Treatment of (VII) in benzene with osmium tetroxide and pyridine on the other hand gave a crystalline osmic ester *complex* (VIII) in high yield. Evidence that the double bond between


(VII.)


(VIII.)

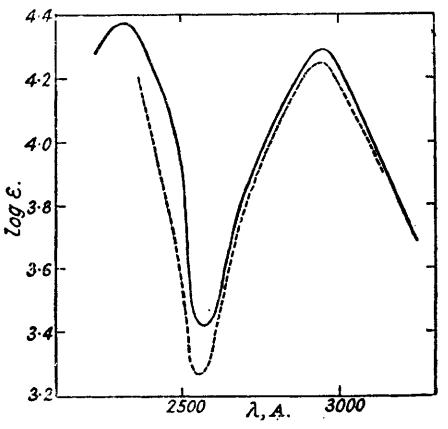

(IX.)


(X.)


(XI.)

positions 2 and 3 was not involved in this reaction was provided by the observation that methyl 1-methylindole-2-carboxylate (IX) did not give a similar osmic ester complex even after a considerably longer reaction period than that employed in the case of (VII). Treatment of the osmic ester complex (VIII) with the calculated quantity of potassium hydroxide in the presence of mannitol (cf. Criegee, Marchand, and Wannowius, *Annalen*, 1942, **550**, 99) gave indole-2-carboxymethylamide (X). This reaction represents a degradation of the *cis*-diol (III), the product expected from the decomposition of the osmic ester complex. Decomposition

of the complex with sodium sulphite (cf. Wieland and Benend, *Ber.*, 1942, **75**, 1708) also gave indole-2-carboxymethylamide in excellent yield. Although this instability appeared to eliminate (III) as a possible structure for compounds A and F, it was conceivable that the instability was a property of the *cis*-diol as distinct from the *trans*-isomer. Efforts were therefore made to synthesise the latter. Oxidation of 3′-keto-4′ : 5′-dimethyl-1′ : 2′ : 3′ : 4′-tetrahydropyrazino(1′ : 2′-1 : 2)indole (VII) with performic acid rapidly gave a deep green, high-melting, amorphous compound. On using peracetic acid, however, a crystalline compound $C_{15}H_{16}O_4N_2$ was obtained which we consider is the *monoacetate* (XI) of the required *trans*-diol. An attempt to convert this acetate into the diol (III) by storage in cold dilute methanolic ammonia, as in the successful hydrolysis of dethiogliotoxin diacetate (Part I, this vol., p. S135) resulted in the formation of indole-2-carboxymethylamide in good yield. When treated with 2 : 4-dinitrophenylhydrazine in hydrochloric acid, the acetate slowly gave the bis-2 : 4-dinitrophenylhydrazone of methylglyoxal. These reactions indicate a marked instability of the *trans*-diol (III); in the latter case the larger fragment of the degradation (indole-2-carboxylic acid or its *N*-methylamide) eluded isolation. Hydrolysis of the *trans*-diol monoacetate with mineral acid unexpectedly gave a product closely resembling that obtained by direct oxidation of (VII) with performic acid.

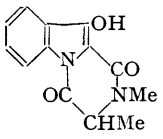


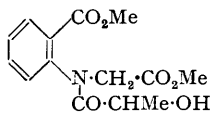
2 : 5-*Diketo*-3′-*acetoxy*-6-*methylindolo*(1′ : 2′-3 : 4)*morpholine* (XV; R = Ac). ——— *Methyl* 3-*acetoxy*-1-*lactylindole*-2-*carboxylate* (XIV; R = Ac). – – – – –

It is clear that both the *cis*- and the *trans*-isomer of the diol (III) are extremely sensitive. In our opinion, the observations described above point to an alternative structure for the compounds A and F, and stereoisomeric forms of (IV) or (IV*a*) appear to be satisfactory. It is also evident that in dethiogliotoxin the hydroxylated hydropyrazine ring, if present as shown in (II) or (II*a*), must be considerably stabilised. Curiously, the relative stability seems to be achieved through the proximity of a hydroxydihydroindole system, a system itself normally regarded as unstable.

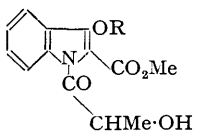
The synthesis of the dihydroindole derivative (IV), for comparison with compounds A and F, may be expected to offer some difficulty. 2 : 3-Dihydroindoxyl and its *N*-acetyl derivative have been described (B.P. 326,523; *Zentr.*, 1930, II, 811; D.R.-PP. 516,675, 518,515; *Zentr.*, 1931, I, 1832; 1931, II, 2388). They are apparently stable crystalline compounds, although they revert to indole and *N*-acetylindole, respectively, when heated in neutral, acid, or alkaline aqueous suspension. Our first objective was 3-hydroxy-3′ : 6′-diketo-4′ : 5′-dimethylpiperazino-

(XII.)





(XIII.)



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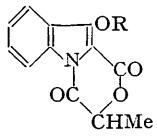

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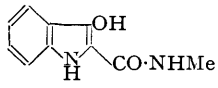

(XVI.)

(1′ : 2′-1 : 2)indole (XII), the reduction of which to (IV) appears feasible. Treatment of *o*-carbomethoxyphenylglycine methyl ester with acetyl-lactyl chloride gave N-*lactyl*-N-*o*-*carbomethoxyphenylglycine methyl ester* (XIII), the condensation being accompanied by deacetylation. The *N*-lactyl derivative (XIII) was treated with sodium methoxide with the object of obtaining methyl *N*-lactylindoxylate (XIV; R = H). Instead, there was obtained a compound $C_{12}H_9O_4N$ which has pseudo-acidic properties and is clearly 3′-*hydroxy*-2 : 5-*diketo*-6-*methylindolo*(1′ : 2′-3 : 4)*morpholine* (XV; R = H). This compound gives a neutral *acetate* (XV; R = Ac). With the object of converting (XV; R = H) into (XII) the pseudo-acid

was treated with methylamine. Reaction at 70° gave rise to *indoxyl-2-carboxymethylamide* (XVI), presumably by opening of the lactone ring in (XV; R = H) followed by removal of the 1-lactyl grouping. The product of reaction in the cold, however, was a methylamine *salt* which was also obtained by treatment of the monoacetate (XV; R = Ac) with methylamine. Treatment of the salt with mineral acid regenerated the compound (XV; R = H). The structures ascribed to the pseudo-acid and its acetate receive confirmation from a consideration of the ultra-violet absorption characteristics (see figure). The spectrum of the acetate (XV; R = Ac) is very similar to that of *methyl 3-acetoxy-1-lactylindole-2-carboxylate* (XIV; R = Ac) which was obtained by treatment of methyl 3-acetoxyindole-2-carboxylate with *O*-acetyllactyl chloride: again the acylation is accompanied by deacetylation. Appropriate routes are now being sought for the conversion of (XIV; R = Ac) and (XV) into (XII).

Experimental.

The culture conditions most suited to the production of gliotoxin by means of *Gliocladium fimbriatum* have been studied by Weindling (for bibliography, see Elvidge and Spring, this vol., p. S135). It appeared clear from this work that a shake-culture technique was most suited for the production of gliotoxin in high yield. Since a shake-culture has marked limitations, alternative methods were examined by Johnson, Bruce, and Dutcher. None of these methods gave a yield of gliotoxin approaching that obtained by using a shake-culture, and consequently Johnson *et al.* developed a shaking machine with a capacity of 60 l. In our earlier work using *Trichoderma viride* (strain 211) we avoided the mechanical difficulties inherent in a shake-culture by employing Brian's drip-culture technique which in our hands gave yields of approximately 80 mg./l. or a total of 8 g. over a period of 40 days. We now find that a surface culture of *Trichoderma viride* (strain 211) on Weindling's medium (pH 3·5) gives considerably higher yields of gliotoxin (up to 140 mg./l. in a few days) if the optimum temperature of 28° is maintained. Under these conditions, production of gliotoxin is rapid and the metabolite is not contaminated with pigment. Using the drip-culture technique we had observed that, provided aeration was efficient, pigment formation became apparent to a slight degree only after the culture had grown for several weeks. However, if aeration was stopped, pigment was formed in appreciable quantity. Irrespective of the method of culture of *Trichoderma viride* (strain 211) pigment formation tends to increase with the age of the culture but it can be inhibited by ample aeration. Consequently, with a surface culture technique (no aeration) the gliotoxin is purer the shorter the culture time, and this implies culturing at an optimum temperature. Purification of pigment-contaminated gliotoxin can be achieved by repeated crystallisation from ethyl acetate or by chromatography in benzene on acid-washed alumina (pH 3—4).

Sublimation of Dethiogliotoxin and its Acetate.—Dethiogliotoxin (*ca.* 50 mg.), m. p. 248—249° (decomp.), was kept at 230°/3 × 10^{-5} mm. Sublimation occurred slowly to yield a dense white solid (30 mg.) during 8—9 hours. The sublimate had m. p. 240—248° (decomp.), undepressed in admixture with dethiogliotoxin.

Dethiogliotoxin diacetate (12 mg., m. p. 175°) sublimed completely at 150°/10^{-4} mm. during 1·5 hours. The sublimate had m. p. 170—174° and a mixture with the starting material had m. p. 171—175°.

Action of Hydrogen Chloride on Dethiogliotoxin.—(*a*) A solution of dethiogliotoxin (50 mg.) in dry dioxan (15 c.c.) was saturated with anhydrous hydrogen chloride and after 16 hours at room temperature the solution was evaporated under reduced pressure. Treatment of the gummy residue with acetone induced crystallisation. The solid (small prisms) was washed with *n*-propanol and then had m. p. 181—185°, not depressed when mixed with compound A described below. No other solid product was isolated.

(*b*) A solution of dethiogliotoxin (600 mg.) in dry methanol (80 c.c.) was cooled in ice and saturated with anhydrous hydrogen chloride. After 14 hours at room temperature, the solution was evaporated under reduced pressure, and the residual gum dissolved in methanol (2 c.c.). The solution was filtered from a trace of insoluble matter, ether was added almost to turbidity, and the solution allowed to evaporate spontaneously. During 3 days a crop of prismatic crystals (96 mg.; m. p. 184—188°) separated; they gave a negative Beilstein test. Sublimation at 160—165°/10^{-5} mm. followed by recrystallisation from dioxan–light petroleum (b. p. 60—80°) yielded *compound A* as triangular tablets, m. p. 190° (Found: C, 63·6; H, 5·9; N, 11·2; *C*-Me, 3·4, 3·8; active H, 0·46. $C_{13}H_{14}O_3N_2$ requires C, 63·4; H, 5·7; N, 11·4; 1 *C*-Me, 6·1; 1 active H, 0·41%). Compound A dissolves in hot water to give a neutral solution and does not react with 2 : 4-dinitrophenylhydrazine in 5N-hydrochloric acid.

The ethereal methanolic mother-liquors from compound A were evaporated under reduced pressure, and the residue dissolved in a little warm water. Overnight, a mass of needle-shaped crystals (97 mg.; m. p. 69—72° with gradual resolidification and then m. p. 156°) separated. Concentration of the filtrate *in vacuo* over calcium chloride gave a second crop (89 mg.) of the same compound. From hot water (the solution is neutral) *compound B* crystallised as needles, m. p. *ca.* 74° (depending on rate of heating) with resolidification and then m. p. 156° (Found, on an air-dried specimen: C, 55·5; H, 6·4; N, 10·4; OMe, 0·0. $C_{13}H_{18}O_5N_2$ requires C, 55·3; H, 6·4; N, 9·9%).

(*c*) Dry hydrogen chloride was passed into a cooled suspension of dethiogliotoxin (807 mg.) in ethanol (80 c.c.) and the solution, nearly saturated with hydrogen chloride, was kept at room temperature for 16 hours. The solvent was removed by distillation under reduced pressure, and the gum dissolved in ethanol and treated with ether. A trace of brown flocculent solid was removed by filtration, and the clear pale-brown solution allowed to evaporate spontaneously; a crop of prismatic crystals [56 mg.; m. p. 172—174° (decomp.)] separated. Recrystallisation from ethanol gave compound C as small rectangular prisms, m. p. 196° (decomp.) (Found: C, 49·65; H, 5·9; N, 7·7%). It gives a positive

Beilstein reaction, and a curdy white precipitate immediately with aqueous ethanolic silver nitrate and dilute nitric acid. Light absorption in ethanol: rising end-absorption only.

The ethanolic mother-liquors from compound C were evaporated under reduced pressure, the viscous oil was redissolved in absolute ethanol, and the solution diluted with ether. Spontaneous evaporation was accompanied by crystallisation to yield prisms (151 mg.), m. p. 150—153°. Recrystallisation of the prisms from ethanol–ether gave a mixture of (i) small stout prisms (26 mg.), m. p. 178—180°, and (ii) clusters of laths (13·5 mg.), m. p. 150—152°, which were separated mechanically. Recrystallisation of fraction (i) from dioxan–light petroleum and then from ethanol–water gave compound D as prismatic needles, m. p. 204° (Found: C, 74·25; H, 8·0; N, 13·3%). Light absorption in ethanol: maximum at 2560 A., $E^{1\%}_{1\,cm.} = 604$.

The mother-liquors from fractions (i) and (ii) were evaporated under reduced pressure, and the residue fractionally crystallised from dioxan–light petroleum (b. p. 60—80°) to yield (iii) prisms (52 mg.), m. p. 150—165°, and (iv) needles (8·5 mg.), m. p. 168°. Fraction (iii) was chromatographed in ethanol (4 c.c.) on a column (15 × 1 cm.) of alumina (Light's, activated at 200° for 14 hours), and by continued elution with ethanol, collection of fractions and evaporation of solvent, the following fractions were obtained: (v) prismatic needles (23 mg.), m. p. 153°, (vi) prisms (11 mg.), m. p. 161—164°, and (vii) prisms (2 mg.), m. p. 162—165°. Fractions (ii) and (v) were combined and recrystallised from dioxan–light petroleum (b. p. 60—80°) to give prismatic needles, m. p. 155—156°, identified (mixed m. p.) with compound F described below (Found: N, 11·7%). Light absorption in ethanol: Max. at 2540 A., $\varepsilon = 9930$, and 2820 A., $\varepsilon = 3800$.

Fractions (iv), (vi), and (vii) were combined and recrystallised from dioxan–light petroleum (b. p. 60—80°) to give *compound E* as needles, m. p. 168° with slight softening at *ca.* 70° (Found: C, 59·4; H, 6·5; N, 10·4. $C_{13}H_{16}O_4N_2$ requires C, 59·1; H, 6·1; N, 10·6%).

(*d*) Dry methanol (80 c.c.) was saturated at 0° with anhydrous hydrogen chloride. Dethiogliotoxin (600 mg.) was added, and the solution kept at room temperature for 24 hours. Evaporation under reduced pressure yielded a gummy residue which was redissolved in methanol (2 c.c.). Ether was added in amount nearly sufficient to cause turbidity, and the solution allowed to evaporate spontaneously; prismatic crystals (159 mg.; m. p. 154—156°) (filtrate *A*) separated slowly. Recrystallisation from dioxan–light petroleum (b. p. 60—80°) gave *compound F* as sheaves of laths, m. p. 156° (Found: C, 63·5, 63·4; H, 5·7, 5·8; N, 11·7; active H, 0·30. $C_{13}H_{14}O_3N_2$ requires C, 63·4; H, 5·7; N, 11·4; I active H, 0·41%). Compound F gave a neutral solution in hot water. When heated at 140°/2 × 10^{-4} mm., it slowly gave a hard glassy sublimate which gradually crystallised and then had m. p. 154—155° undepressed in admixture with the starting material.

A solution of compound F (10 mg.) in methanol (10 c.c.) which had been saturated with dry hydrogen chloride was kept at 40—50° for 35 minutes. Evaporation of the solvent under reduced pressure left a residue which crystallised completely. Recrystallisation from dry dioxan–light petroleum gave prismatic needles (9 mg.), m. p. 156° not depressed in admixture with the starting material. Light absorption in ethanol: Max. at 2530 A., $\varepsilon = 11{,}000$, and inflection at 2850 A., $\varepsilon = 6600$.

The filtrate A was evaporated under reduced pressure, and the residual gum dissolved in a little warm water. On cooling, a crop of needles (165 mg.; m. p. *ca.* 130°) rapidly separated. Recrystallisation from hot water (the solution is neutral) gave *compound G* as laths which softened at *ca.* 68° (depending on rate of heating) and then resolidified with m. p. 149°; the higher m. p. was not raised on repeated crystallisation from water (Found: C, 55·4; H, 6·4; N, 9·9. $C_{13}H_{18}O_5N_2$ requires C, 55·3; H, 6·4; N, 9·9%).

Action of Heat on Compound B.—(*a*) A recrystallised specimen of compound B was dried at 78° *in vacuo* for 2 hours. The *product* sintered and then had m. p. 150—156° (Found: C, 60·2; H, 6·0; N, 11·1. ($C_{13}H_{18}O_5N_2 - 1\frac{1}{2}H_2O$) requires C, 61·2; H, 5·9; N, 11·0%). Light absorption in ethanol: Max. at 2520 A., $\varepsilon = 12{,}000$

(*b*) Compound B (8 mg.) was heated at 120°/10^{-2} mm. for 6·5 hours during which time sublimation was slight. The solid had m. p. *ca.* 153° (Found: N, 12·1%). Light absorption in ethanol: maxima at 2540 A., $E^{1\%}_{1\,cm.} = 371$, and 2580 A., $E^{1\%}_{1\,cm.} = 62$.

(*c*) Compound B was dried at 56° *in vacuo* for 1·5 hours and recrystallised from dry dioxan–ether to yield *compound B′* as sheaves of laths, m. p. 156° (Found: C, 55·5; H, 6·6; N, 10·0; active H, 1·36. $C_{13}H_{18}O_5N_2$ requires C, 55·3; H, 6·4; N, 9·9; 4 active H, 1·42%).

Conversion of Compound G into Compound F.—Compound G (10 mg.) was heated at 100°/5 × 10^{-3} mm. for 1·5 hours. Practically no sublimation occurred, but the substance sintered to a hard cake, m. p. 153—154°, which on crystallisation from dioxan–light petroleum (b. p. 60—80°) yielded prismatic laths, m. p. 154—156°, identified by mixed m. p. as compound F.

Alkaline Hydrolysis of Compounds F and A.—Compound F (35 mg.) was heated under reflux with methanolic 2N-potassium hydroxide (1·5 c.c.). A solid separated almost at once. After 10 minutes the solid (9·6 mg.; m. p. 365—370° with charring) was collected and washed with methanol. This solid left no residue when ignited on platinum foil. The filtrate was acidified with concentrated hydrochloric acid and evaporated to dryness under reduced pressure. The residue was extracted with boiling acetone (4 × 5 c.c.), the filtered extract evaporated, and the residue crystallised from chloroform–light petroleum (b. p. 60—80°) to yield *N*-(indole-2-carboxy)-*N*-methylalanine (17·5 mg., m. p. 179—181°, identified by its solubility (with effervescence) in sodium hydrogen carbonate solution and by mixed m. p. with an authentic specimen (Found: N, 11·6. Calc. for $C_{13}H_{14}O_3N_2$: N, 11·4%).

Similar hydrolysis of compound A (10 mg.) with methanolic potassium hydroxide (1 c.c.; 2N) gave an insoluble fraction (2 mg., m. p. >350°) and *N*-(indole-2-carboxy)-*N*-methylalanine (3·5 mg.), m. p. 170—173°, undepressed (m. p. 173—178°) when mixed with an authentic specimen (m. p. 183—185°).

Treatment of the Diketopiperazine (V) *with Methanolic Hydrogen Chloride.*—A solution of the diketopiperazine (20 mg.) (Johnson, Andreen, and Holley, *J. Amer. Chem. Soc.*, 1947, **69**, 2370) in methanol (10 c.c.) which had been saturated with dry hydrogen chloride was kept at room temperature for 24 hours, then evaporated under reduced pressure. The residue was taken up in methanol (a few drops)

and ether was added. On storage, prismatic crystals (19 mg.) separated, m. p. 121—122°, not depressed in admixture with the starting material.

Hydroxylation of 3′-Keto-4′ : 5′-dimethyl-1′ : 2′ : 3′ : 4′-tetrahydropyrazino(1′ : 2′-1 : 2)indole (VII).—(*a*) A solution of the pyrazinoindole (424 mg.) in anhydrous benzene (100 c.c.) containing pyridine (0·32 c.c.) was treated with osmium tetroxide (510 mg.). A deep reddish-brown coloration rapidly developed and within 2 hours dark brown prismatic crystals began to separate. After 3 days the osmic ester–pyridine *complex* (which contained benzene of crystallisation) was collected (1·064 g.; decomp. >200°) (Found: C, 50·2; H, 4·35; N, 8·0. $C_{13}H_{12}O_5N_2Os,2C_5H_5N,C_6H_6$ requires C, 49·6; H, 4·0; N, 8·0%).

The osmium complex (1·014 g.) was shaken for 1 hour with ethanol (5·9 c.c.) and 0·553N-potassium hydroxide (5·9 c.c.) in the presence of mannitol (400 mg.), and the solution evaporated under reduced pressure at room temperature. The residue was dissolved in a little water, and the solution extracted with ethyl acetate (4 × 100 c.c.). The extract was washed with water, evaporated under reduced pressure, and the crystalline residue recrystallised from benzene containing a little ethyl acetate to yield indole-2-carboxymethylamide as needles (41 mg.), m. p. 220° (Found: N, 16·4. Calc. for $C_{10}H_{10}ON_2$: N, 16·1%). Light absorption in ethanol: Max. at 2930 A., $\varepsilon = 18{,}700$. The m. p. was not depressed when the substance was mixed with an authentic specimen.

The osmic ester complex (500 mg.) in ethanol (30 c.c.) was heated under reflux with sodium sulphite (5 g.) in water (20 c.c.). After 60 minutes, ethanol (150 c.c.) was added, and when the precipitate had settled, the supernatant liquor was decanted. The solid was further extracted with boiling ethanol (2 × 25 c.c.) and the combined ethanolic extracts evaporated under reduced pressure to yield a crystalline residue. The material separated in needles (95 mg.; 77%) from benzene and had m. p. 220° undepressed in admixture with indole-2-carboxymethylamide.

(*b*) The pyrazinoindole (400 mg.) in acetic acid (30 c.c.) was treated with hydrogen peroxide (0·5 c.c.; 100-vol.) and after 4 days the yellowish-green solution was evaporated under reduced pressure. The dark green residue was dissolved in ethyl acetate, and the solution washed with aqueous sodium hydrogen carbonate and water, and dried (Na_2SO_4). The solution was concentrated under reduced pressure to small bulk, ether was added, and on spontaneous evaporation, small prisms (200 mg.) slowly separated. From a very small volume of ethyl acetate, on addition of ethylene dichloride, the *acetate* (XI) crystallised as minute prisms, m. p. 146° (frothing) (Found: C, 62·3; H, 6·2; N, 9·4. $C_{15}H_{16}O_4N_2$ requires C, 62·5; H, 5·55; N, 9·7%). Light absorption in ethanol: Max. at 2970 A., $\varepsilon = 22{,}000$. The acetate is insoluble in warm water and aqueous sodium hydrogen carbonate and does not give a coloration with ferric chloride in aqueous methanol, or with Schiff's reagent: Tollens's reagent was not reduced.

A solution of the monoacetate (18 mg.) in ethanol (2 c.c.) was treated at room temperature with 2 : 4-dinitrophenylhydrazine in 2N-hydrochloric acid (30 c.c.). A turbidity appeared within 30 minutes. After 48 hours, the orange-red precipitate was collected, washed with ethanol, and dried (yield, 20 mg.; m. p. *ca.* 285—295°). With methanolic potassium hydroxide, a trace of the precipitate gave an intense purplish-blue coloration, a reaction characteristic of a dinitrophenylosazone (Strain, *J. Amer. Chem. Soc.*, 1935, **57**, 758). A solution of the solid in nitrobenzene was treated with a little alcohol, and small orange-red prismatic needles separated, m. p. 300—301°, undepressed in admixture with an authentic specimen, m. p. 300—301°, of the bis-2 : 4-dinitrophenylhydrazone of methylglyoxal.

The filtrate from the bis-2 : 4-dinitrophenylhydrazone was treated with acetone (0·5 c.c.) to remove excess of dinitrophenylhydrazine and, after filtration, the solution was evaporated to dryness under reduced pressure. The residual solid was shaken with aqueous sodium carbonate (2 c.c.), and the extract acidified with hydrochloric acid and evaporated to dryness. The salt was extracted with hot acetone (4 × 5 c.c.), and the extract evaporated. To the trace of residue, alcoholic *p*-dimethylaminobenzaldehyde (3 drops) was added followed by concentrated hydrochloric acid (2 drops). There was no coloration. A portion of the yellow-brown solid which had been shaken with sodium carbonate solution was similarly treated with the Ehrlich reagent. A fine stable green coloration resulted; indole-2-carboxylic acid similarly treated gave a pink-violet colour.

The monoacetate (14 mg.) in methanolic hydrogen chloride was kept at room temperature for 15 hours. Evaporation of the solution under reduced pressure afforded a greenish amorphous solid which had a very high m. p.

A solution of the monoacetate (30 mg.) in methanol (2 c.c.) was treated with ammonia (3 drops; *d* 0·880) overnight and then evaporated at room temperature under reduced pressure. The crystalline residue (14 mg.; 78%) had m. p. 220—221°, alone or when mixed with a specimen of indole-2-carboxymethylamide of m. p. 220—221°.

N-*Lactyl*-N-*o*-*carbomethoxyphenylglycine Methyl Ester* (XIII).—A mixture of *N*-*o*-carbomethoxyphenylglycine methyl ester (4·3 g.) (Vorländer and von Schilling, *Annalen*, 1898, **301**, 349), dry pyridine (40 c.c.), and *O*-acetyl-lactyl chloride (2·8 g.) was heated on the steam-bath for 3 hours. Next day, the excess of pyridine was distilled off under reduced pressure, the residue taken up in chloroform, and the solution washed with dilute hydrochloric acid, sodium hydrogen carbonate solution, and water, and finally evaporated; the residue slowly crystallised. Recrystallisation from methanol–water gave the original ester as needles, m. p. 93—94°, and, after dilution of the mother-liquor with water, a second crop (1·5 g.) as prisms, m. p. 70—80°, which on recrystallisation from methanol–water, gave N-*lactyl*-N-*o*-*carbomethoxyphenylglycine methyl ester* as prisms, m. p. 80° (Found: C, 56·9; H, 5·6; N, 4·9, 5·0. $C_{14}H_{17}O_6N$ requires C, 56·9; H, 5·8; N, 4·75%). With ferric chloride in aqueous methanol it gave a yellow coloration.

3′-*Hydroxy*-2 : 5-*diketo*-6-*methylindolo*(1′ : 2′-3 : 4)*morpholine* (XV; R = H).—The preceding lactyl ester (19·1 g.) was heated under reflux in benzene (100 c.c.) with powdered sodium (2 g.). As no reaction appeared to have taken place during 3 hours, methanol (3·5 c.c.) was added and refluxing continued for 9 hours. During this time a white solid separated. The benzene was evaporated off under reduced pressure, and the solid residue treated with ice and water. The filtered aqueous solution was acidified with acetic acid, and the precipitate (7·9 g.) crystallised from methanol to give 3′-*hydroxy*-2 : 5-*diketo*-6-

methylindolo(1′ : 2′-3 : 4)*morpholine* as silky needles, m. p. 204° (Found: C, 61·9, 62·3; H, 3·9, 4·1; N, 5·8. $C_{12}H_9O_4N$ requires C, 62·3; H, 3·9; N, 6·1%). This is soluble in dilute aqueous alkalis and gives a deep greenish-blue coloration with ferric chloride in aqueous ethanol. It was recovered unchanged after 3 hours' shaking in ethyl acetate solution with hydrogen at atmospheric pressure in the presence of Adams's catalyst.

Acetyl derivative (XV; R = Ac). A solution of (XV; R = H) (0·5 g.) in acetic anhydride (10 c.c.) was heated under reflux for one hour and then evaporated under reduced pressure. Crystallisation of the solid residue (0·5 g.) from ethanol gave the *acetyl* derivative as laths, m. p. 141°; it does not give a coloration with the ferric chloride reagent (Found: C, 61·5; H, 4·2; N, 5·4. $C_{14}H_{11}O_5N$ requires C, 61·5; H, 4·0; N, 5·1%).

Treatment of the acetyl derivative in ethanol with ethanolic methylamine rapidly precipitated a substance as needles, m. p. 215—219° (decomp.), identified with the salt described below.

Action of Methylamine on 3′-Hydroxy-2 : 5-diketo-6-methylindolo(1′ : 2′-3 : 4)*morpholine.*—(*a*) A solution of the hydroxyindolomorpholine (1 g.) in hot ethanol was treated with excess of methylamine. The voluminous precipitate was collected after 30 minutes, recrystallised from hot water, and washed with ethanol and ether. The *methylamine* salt forms felted needles, m. p. 221—222° (decomp.), and gives a greenish-blue coloration with aqueous ferric chloride (Found: C, 59·3; H, 5·2; N, 10·6. $C_{13}H_{14}O_4N_2$ requires C, 59·5; H, 5·3; N, 10·7%).

Acidification of an aqueous solution of the salt with dilute hydrochloric acid gave 3′-hydroxy-2 : 5-diketo-6-methylindolo(1′ : 2′-3 : 4)morpholine, m. p. 204°.

(*b*) The hydroxyindolomorpholine (0·5 g.) was heated with excess of liquid methylamine (10 c.c.) in a sealed tube at 70° for 14 hours. Evaporation of the methylamine left a solid residue (0·3 g.) which was washed with dilute hydrochloric acid. Recrystallisation from ethanol–water gave prismatic needles, m. p. 240—242° (decomp.), of *indoxyl-2-carboxymethylamide* (Found: C, 62·6; H, 5·1; N, 14·8. $C_{10}H_{10}O_2N_2$ requires C, 63·15; H, 5·3; N, 14·7%).

Methyl 3-Acetoxy-1-lactylindole-2-carboxylate.—A solution of methyl 3-acetoxyindole-2-carboxylate (2·8 g.) in dry pyridine (20 c.c.) was treated with *O*-acetyl-lactyl chloride (1·9 g.) and heated for 8 hours at 100°. After being kept at room temperature for 18 days, the mixture was evaporated under reduced pressure, the residue dissolved in chloroform, and the solution washed with dilute hydrochloric acid, aqueous sodium hydrogen carbonate, and water. Evaporation of the chloroform gave a brown gum which was dissolved in methanol (15 c.c.). After standing over-night, solid separated (0·5 g.; m. p. >200°). The filtrate was evaporated to yield a crystalline residue (2·1 g.; m. p. 90—110°). Recrystallisation from methanol–water gave *methyl 3-acetoxy-1-lactylindole-2-carboxylate* as plates, m. p. 128° (Found: C, 59·0; H, 5·2; N, 4·6, 4·7. $C_{15}H_{15}O_6N$ requires C, 59·1; H, 4·9; N, 4·6%).

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