112. *An Antibacterial Substance from* Aspergillus clavatus.

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An antibacterial substance from *Aspergillus clavatus* is identical with patulin from *Penicillium patulum*. Additional evidence for the chemical structure is presented which confirms the formulæ advanced by Raistrick *et al.* (*Lancet*, 1943, **245**, 625).

The isolation of β-*n*-propyl-γ-butyrolactone following reductive degradation and the isolation of γ-keto-β-methyl-*n*-hexoic acid, and the intermediates preceding its formation, *viz.*, αε-di-iodo-γ-keto-β-methyl-*n*-hexoic acid, 3-methyltetrahydro-γ-pyrone-2-carboxylic acid, and 3-chloromethylenetetrahydro-γ-pyrone-2-carboxylic acid, confirm the presence of a condensed system of two heterocyclic rings. Further experimental work helps to characterise the position and properties of the double bond and the keto-group. The results of oxidative and other degradations strongly suggest the existence of predominant tautomeric forms such as anhydro-4-hydroxy-5-hydroxymethyl-1 : 2-pyran-6-carboxylic acid and anhydro-4-hydroxy-5-hydroxymethyl-ene-5 : 6-dihydro-1 : 2-pyran-6-carboxylic acid.

An antibacterial substance isolated from *Aspergillus clavatus* metabolism solution (cf. Bergel *et al.*, *Nature*, 1943, **152**, 750; Hooper *et al.*, *Science*, 1944, **99**, 16) has been shown by us to be identical with claviformin from *Penicillium claviforme* (Chain, Florey, and Jennings, *Brit. J. Exp. Path.*, 1942, **23**, 202) and very probably with patulin isolated from *Penicillium patulum*, Bainier, by Raistrick *et al.* (*Lancet*, 1943, **245**, 625). Through the courtesy of Professor Raistrick in furnishing a sample of patulin we were able to carry out mixed m. p.'s and thus conclusively established the identity of these three mould metabolites. Chain *et al.* (*Lancet*, 1944, **246**, 112) have shown independently the identity of patulin with claviformin by *X*-ray crystallographic examination. Many of our results on the degradation of this antibacterial substance are identical with those published for patulin; and in the following communication we wish to record only hitherto unpublished observations on the constitution of this chemically most interesting substance. In the absence of an accepted common name for this metabolite we have used the name clavatin as a convenient designation.

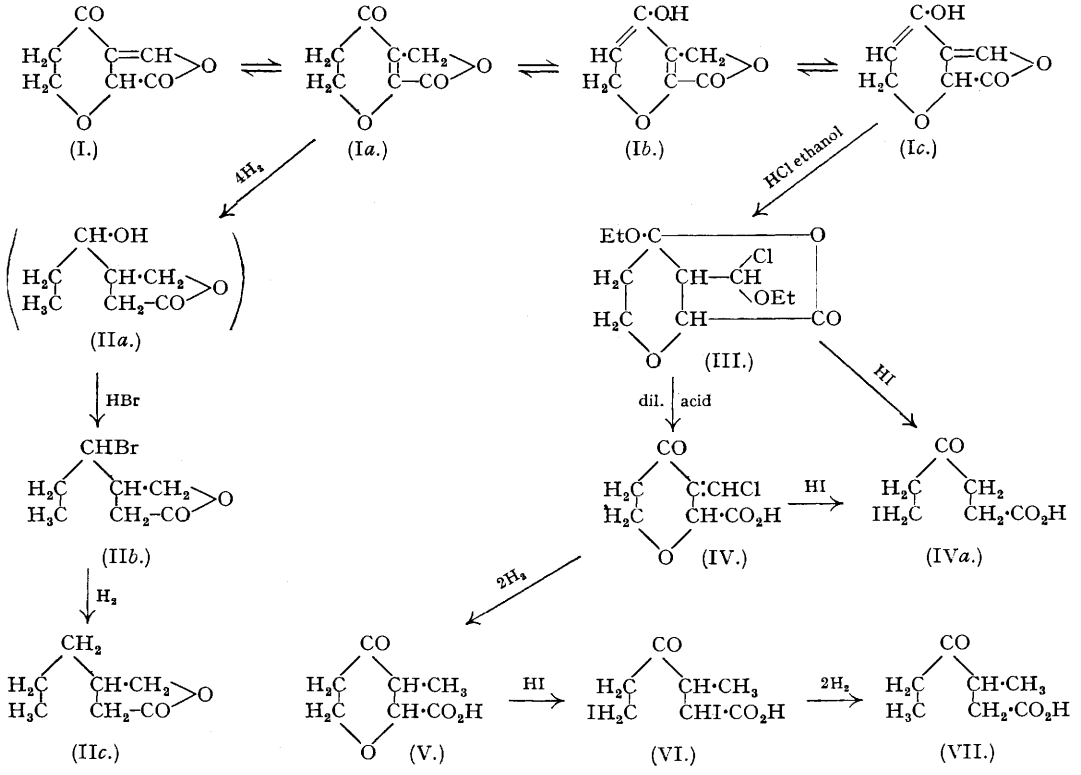
Raistrick *et al.* have based their proposed structure for patulin as anhydro-3-hydroxymethylenetetrahydro-γ-pyrone-2-carboxylic acid (I) mainly on the isolation, following acid hydrolysis, of formic acid and in 10% yield of tetrahydro-γ-pyrone-2-carboxylic acid; on the isolation of β-methylhexoic acid and β-methyl-γ-hexolactone, following hydrogenation and further reduction with hydriodic acid and red phosphorus; and on the formation in 60% yield of ε-iodo-γ-keto-*n*-hexoic acid by direct hydriodic acid treatment of patulin. In addition it was deduced from all the properties of patulin that the substituents in positions 2 and 3 of the pyrone ring are combined to an unsaturated lactone.

Similar evidence from alkaline titration and degradation of clavatin or derivatives led us early in our work to suspect the existence of an unsaturated lactone group. The identification of this group as an unsaturated butyrolactone and its relation to the remaining three carbon atoms of the molecule were established by the isolation of β-*n*-propylbutyrolactone (II*c*) from perhydrogenated clavatin after treatment with hydrobromic acid and reduction of the monobromide so obtained. The mechanism of the hydrogenation involved will be discussed later.

Our evidence for the structure of the remainder of the clavatin molecule, derived from ozonolysis, in which the main products were formic acid and glyoxal with traces of oxalic acid, did not enable us to envisage a simple oxygen ring system. The structure of a reduced γ-pyrone put forward by Raistrick *et al.* (*loc. cit.*) was based mainly on their isolation of tetrahydro-γ-pyrone-2-carboxylic acid. The low yield in which this substance was obtained, however, left room for further confirmatory evidence, which we now present. This is based on work in hand at the time of their publication.

Clavatin in ethanol solution on treatment with hydrogen chloride gave an oil, which was hydrolysed with dilute acid to a crystalline ketochloro-acid, $C_7H_7O_4Cl$. Hydrogenation of this substance gave as the main product a chlorine-free keto-acid, $C_7H_{10}O_4$, which on treatment with hydriodic acid was transformed into a

di-iodo-acid, $C_7H_{10}O_3I_2$. Replacement of the iodine atoms in this compound by hydrogen produced an acid, $C_7H_{12}O_3$, identified as γ-keto-β-methyl-*n*-hexoic acid (VII). The isolation of this compound and β-*n*-propylbutyrolactone (*IIc*), as mentioned before, both containing all seven carbon atoms of clavatin, fixes definitely the relative positions of the keto-group and the original lactone group. The double bond in clavatin must therefore be in the αβ-position to the keto-group, a position suggested by the relationship between the ultraviolet absorption spectra of clavatin phenylhydrazone and dihydroclavatin phenylhydrazone.



Consideration of the structure and reactions of the intermediates preceding the formation of γ-keto-β-methyl-*n*-hexoic acid (VII) determined the nature and position of the fourth oxygen atom. The fact that the iodine of the di-iodo acid, $C_7H_{10}O_3I_2$, was easily removed with aqueous alkali to give a doubly unsaturated acid suggested that the iodine atoms were located in β- and β′-positions to the keto-group. The isolation of β-*n*-propylbutyrolactone being taken into consideration, this acid can only be formulated as *αε-di-iodo-γ-keto-β-methyl*-n-*hexoic acid* (VI). It follows that the parent substance, the monoketo-acid, $C_7H_{10}O_4$, must contain the fourth oxygen in cyclic ether linkage and is therefore *3-methyltetrahydro-γ-pyrone-2-carboxylic acid* (V).

An empirical formula $C_7H_7O_4Cl$ for the preceding chloro-acid was established by analysis and equivalent weight titrations. The presence of a keto-group was proved by hydroxylamine titration and the isolation of a dinitrophenylhydrazone. Addition of one mole of bromine and the uptake of two moles of hydrogen with elimination of chlorine established the presence of a double bond. The pattern of the alkaline titration, the preparation in high yield of ε-iodo-γ-keto-*n*-hexoic acid (IV*a*) by heating with hydriodic acid, and the simultaneous formation of formic acid and hydrogen chloride by alkaline degradation leave little doubt as to the existence of a chloromethylene group in the molecule. The substance is therefore *3-chloromethylenetetrahydro-γ-pyrone-2-carboxylic acid* (IV). It is noteworthy that, whereas the saturated pyronecarboxylic acid (V) gave on fission with hydriodic acid a di-iodo-compound (VI), the unsaturated acid (IV), as Raistrick *et al.* had found with patulin itself, underwent reduction at the 2-position during identical treatment and thus yielded a monoiodo-compound (IV*a*).

The precise constitution of the initial product obtained from clavatin by treatment with hydrogen chloride–ethanol is not so certain; but the following indications have been found. Analytical data indicated that it contained two ethoxy-groups and agreed approximately with the formula $C_{11}H_{17}O_5Cl$, and reactions showed that the substance was neutral and saturated. The isolation of two distinct 2 : 4-dinitrophenylhydrazones when the substance was treated with 2 : 4-dinitrophenylhydrazine in ethanol–sulphuric acid and in methanol–sulphuric acid respectively is explicable by assuming the acidolysis and alcoholysis of an etherified hydroxy-lactone, with accompanying loss of hydrogen chloride. These facts, together with the production of (IV*a*) by hydriodic acid degradation and the evidence as to position of the chlorine atom afforded by acid hydrolysis to (IV), can be reconciled in the formula (III).

With the existence of a *condensed system of two heterocyclic rings* firmly established, there remains to be discussed the position of the double bond and with it the peculiar character of the keto-group.

The acid and the alkaline degradations and the hydriodic acid treatment of clavatin and patulin (Raistrick *et al.*, *loc. cit.*) favour formula (I). However, the results of our hydrogenation experiments, together with the lack of optical activity and the behaviour of clavatin in the formation of its functional derivatives and in certain types of degradation, can only be explained by the coexistence of the tautomeric forms (I*a*, *b*, and *c*), to which Raistrick *et al.* have already alluded.

In contrast to the findings of Raistrick *et al.*, who report the addition of only 1 or 2 moles of hydrogen to patulin, the hydrogenation of clavatin under our conditions proceeded with the uptake of 3—4 moles of hydrogen. The complex nature of our product indicated that the reaction was not uni-directional and also that a considerable degree of polymerisation had taken place. The distillable material could be separated into neutral and acid fractions. The latter was not further investigated. The neutral fraction behaved as a lactone on titration. Treatment of a crude undistilled hydrogenation product with hydrobromic acid yielded a small amount of a lactone monobromide, $C_7H_{11}O_2Br$, which gave a crystalline *piperidino hydriodide*, $C_{12}H_{22}O_2NI$. The monobromide was hydrogenated to a bromine-free lactone, which yielded a phenylhydrazide identical with that prepared from β-*n*-propylbutyrolactone (II*c*). The bromo-lactone was not identical with α-bromo-β-*n*-propylbutyrolactone and, as it contained a $C{\cdot}CH_3$ group, the only remaining structure possible is that of β-(α′-*bromo*-n-*propyl*)*butyrolactone* (II*b*). The formation of the parent hydroxy-compound from clavatin may be explained by hydrogenolysis of the tautomeric form (I*a*) to give β-(γ′-hydroxy-α′-keto-*n*-propyl)butyrolactone, which was further hydrogenated to β-(α′-hydroxy-*n*-propyl)butyrolactone (II*a*), neither of which was isolated. We believe that the acidic fraction is formed by hydrogenolysis of the tautomeric form (I) (cf. Jacobs and Scott, *J. Biol. Chem.*, 1931, **93**, 139).

It was surprising to find that the keto-group in clavatin is acylated and etherified under unusually mild conditions, forming, *e.g.*, a monoacetate, *monobenzoate*, and *monomethyl ether*. That these derivatives are hydrolysed with equal ease (cf. Raistrick *et al.*, *loc. cit.*) was shown by the close similarity of the hydroxylamine titration of clavatin and its acetate and by the formation of the same *oxime* from both these substances. Finally, clavatin could be regenerated from clavatin acetate by mild acid hydrolysis. Although it did not give any coloration with ferric chloride, we believe that under normal conditions the clavatin molecule exists predominantly in the enol forms (I*b*) and (I*c*). This was strongly supported by the almost identical ultra-violet absorption spectra of clavatin and its acetate, our inability to obtain a benzylidene derivative, and by the smooth formation of a considerable amount of glyoxal (at least 0·5 mol.) in the ozonolysis and permanganate oxidation of the mould metabolite. This involved the oxidative fission of the cyclic ether linkage (cf. Fischer, *Annalen*, 1929, **476**, 233). The small amount of oxalic acid and the large amount of formic acid (0·73 mol.) formed in these oxidations favour formula (I*c*) as the predominant tautomer.

Taking into consideration the smallness of the clavatin molecule, some of its reactions have been found unexpectedly complicated. This characteristic, as well as the very powerful effect which it exerts on biological systems, must be accepted as the functions of this unique chemical structure.

Experimental.

Isolation and Purification of Clavatin.—The crude concentrate of clavatin (Wiesner, *Nature*, 1942, **149**, 357) was further purified by treatment with a small amount of warm water, which dissolved all the clavatin and left a residue of phenolic by-product. Concentration of the aqueous solution gave an oil in early experiments; later it was always obtained crystalline. From the non-crystalline concentrate, crystalline derivatives such as the acetate (m. p. 118—120° *ex* methanol) and phenylhydrazone (m. p. 149—150°) were readily obtained. Pure clavatin was obtained from the crystalline concentrate by pressing it on porous plate and crystallising the oil-free material several times from chloroform (charcoal); m. p. 109·5—110·5° (Found: C, 54·2; H, 3·8. Calc. for $C_7H_6O_4$: C, 54·5; H, 3·9%).

Molecular-weight determinations were carried out on clavatin and its acetate by the cryoscopic method. The value indicated for clavatin in acetic acid solution was 172 ± 5, and in nitrobenzene solution, 150 ± 4. The value found for clavatin acetate in acetic acid solution was 198 ± 5.

The phenolic by-product, crystallised from methanol–water, had m. p. 184—186° and empirical formula $C_{10}H_{12}O_3$. It did not possess any antibacterial activity. Work on its constitution will be reported later.

Some Properties of Clavatin and Clavatin Acetate.—The ultra-violet absorption spectrum of clavatin consists of a single band having $\lambda_{max.}$ at 2765 A., $\varepsilon_{max.}$ 16,600. That of clavatin acetate is very similar, the single band having a maximum at 2770 A., $\varepsilon_{max.}$ 17,600.

Treatment of clavatin or the acetate with bromine in glacial acetic acid resulted in the slow absorption of one molecular equivalent. The products were viscous liquids which could not be induced to crystallise. In chloroform solution clavatin and the acetate did not react with bromine or with perbenzoic acid.

An alkaline hydrolysis of clavatin, performed in a closed system, gave a distillate which yielded a trace of crystalline 2 : 4-dinitrophenylhydrazone, m. p. 120—122° after recrystallisation. A mixed m. p. with acetone-2 : 4-dinitrophenylhydrazone was not depressed; but a large depression resulted from admixture with the 2 : 4-dinitrophenylhydrazone of methyl ethyl ketone.

A hydroxylamine titration was performed in the following manner: Aqueous solutions were prepared: (A) of clavatin (0·33 mM in 5 ml.), (B) of hydroxylamine hydrochloride (2 mM in 5 ml.), (C) of sodium hydroxide (1 mM in 5 ml.). A number of mixtures containing 5 ml. each of (A), (B) and (C) were prepared, kept at room temperature for definite periods, and then titrated to the end-point of bromophenol-blue with N/10-hydrochloric acid. Typical runs were as follows: after ½ hour, 6·63 ml.; after 1 hour, 5·24 ml.; and after 3½ hours, 3·2 ml. Titration of a mixture containing (B) (5 ml.) and (C) (5 ml.) required 10·0 ml. of acid. The results indicate absorption of 1 molar equivalent of hydroxylamine in ½—1 hour and of 1·9 molar equivalents within 3½ hours. A yellow colour developed in the solutions soon after mixing and gradually increased in depth, so that it was impossible to observe the indicator colour change in runs of more than 3½ hours' duration.

An experiment carried out in the same manner with clavatin acetate gave very similar results as regards amount and rapidity of hydroxylamine uptake. There was also a development of yellow colour and appreciable buffering at the end-point due to liberated acetic acid.

Clavatin acetate (0·5 g.), shaken with 0·2N-hydrochloric acid (5 ml.), gradually passed into solution. After neutralisation this solution was evaporated to dryness to give an oily residue. A chloroform extract deposited crystals which were identified as clavatin.

Other Derivatives of Clavatin.—*Clavatin oxime* (as I or I*a*). From a solution of clavatin (0·5 g.) and hydroxylamine hydrochloride (0·55 g.) in 0·2N-hydrochloric acid (5 ml.) at 20°, *clavatin oxime* slowly crystallised during about 3 days. Recrystallised from water, it had m. p. 152—153° (decomp.) (Found: C, 49·6; H, 4·3; N, 8·3. $C_7H_7O_4N$ requires C, 49·6; H, 4·2; N, 8·3%). This oxime was also formed when clavatin acetate was shaken with a solution of hydroxylamine hydrochloride in 0·2N-hydrochloric acid.

Clavatin oxime monoacetate. Acetylation of clavatin oxime with acetic anhydride and pyridine in the usual manner yielded a *monoacetate* which, recrystallised from dilute acetic acid, had m. p. 82—84° (Found: C, 51·2; H, 4·7; N, 5·8. $C_9H_9O_5N$ requires C, 51·2; H, 4·3; N, 6·6%).

Dihydroclavatin phenylhydrazone. The red solution of clavatin phenylhydrazone (2·4 g.) in ethanol (250 ml.) was shaken with hydrogen in presence of a neutral catalyst prepared from charcoal (1 g.) and 5% palladous chloride solution (6 ml.) until approximately one molar equivalent of hydrogen had been absorbed. The alcoholic solution, which was now yellow, was filtered from catalyst and concentrated to dryness at 12 mm. The crystalline product, recrystallised from methanol, had m. p. 180—183° and was shown to be identical with the phenylhydrazone prepared from crude dihydroclavatin (Raistrick *et al.*, *loc. cit.*) by the action of phenylhydrazine hydrochloride (Found: C, 63·2; H, 5·5; N, 11·7. Calc. for $C_{13}H_{14}O_3N_2$: C, 63·4; H, 5·7; N, 11·4%).

The ultra-violet absorption spectrum of this compound has maxima at 3800 A. and at 2490 A.; $\varepsilon_{max.}$ respectively 35,700 and 15,400. This shows a general shift towards shorter wave-lengths as compared with the ultra-violet absorption spectrum of clavatin phenylhydrazone, which has $\lambda_{max.}$ 4130 A. and 2690 A.; $\varepsilon_{max.}$ respectively 17,400 and 13,300.

Clavatin methyl ether (as I*b* or I*c*). Clavatin (1·0 g.), dissolved in anhydrous ether (10 ml.) and methyl iodide (4 ml.), was refluxed with dry silver oxide (2·3 g.) for 40 hours. The liquid was filtered, and the residue washed with ether. Evaporation of solvent left a pale yellow oil which crystallised completely. The compound was unstable and could not be recrystallised from solvent, but it sublimed readily at 45—50°/0·1 mm. in colourless stars, m. p. 69—71° (Found: C, 57·2; H, 5·1; OMe, 17·2. $C_8H_8O_4$ requires C, 57·1; H, 4·8; OMe, 18·4%). On standing at room temperature even in a sealed tube, a fall in m. p. was apparent within 12 hours, and the whole material was rapidly transformed into an unsublimable, non-melting polymer.

Clavatin benzoate (as I*b* or I*c*). Clavatin (0·31 g.) was treated with benzoic anhydride in pyridine, and the product worked up in the usual manner. The crystalline substance (0·36 g.) obtained, recrystallised from methanol, formed colourless needles, m. p. 143·5—144·5° (Found: C, 64·8; H, 4·0. $C_{14}H_{10}O_5$ requires C, 65·1; H, 3·9%).

Hydrogenation of Clavatin.—Hydrogenation in 90% ethanol–water or acetic acid at normal pressure with a palladium–charcoal catalyst resulted in hydrogen uptakes varying from 2·9 to 4·0 molar equivalents and produced oils containing *ca.* 90% of lactone and 10% of acid. The crude products reacted with phenylhydrazine or hydrazine hydrate in ethanol; but crystalline derivatives did not result. The hydrogenation products resinified on heating and only small heterogeneous distillates could be obtained. One such distillate (1·0 g.), b. p. 143—149°/15 mm., gave with phenylhydrazine a crystalline substance, which was recrystallised from ethyl acetate and finally from ethanol–water, forming nearly colourless plates, m. p. 164—166° (Found: C, 67·1; H, 7·0; N, 16·5; *C*-Me, 0·6. $C_{19}H_{24}O_2N_4$ requires C, 67·0; H, 7·0; N, 16·5%). Negative Knorr tests were obtained on this substance and on the product of treatment with sodium in ethanol.

In a pressure hydrogenation, clavatin (3·0 g.), Adams's catalyst (0·3 g.), and glacial acetic acid (170 ml.) were stirred at room temperature for 45 minutes under a hydrogen pressure of 110 atms. A second portion of catalyst (0·2 g.) was added, and the mixture stirred for a further 3 hours. The catalyst was filtered off, and the solvent evaporated under 15 mm. The product, dissolved in 49% hydrobromic acid (50 ml.), was heated in a bomb tube at 110—120° for 4 hours. The cooled reaction mixture, containing some charred material, was diluted with water (50 ml.) and extracted four times with ether (50 ml. portions). The combined ethereal extracts were washed twice with water, twice with ice-cold N-sodium carbonate, again with water, and finally dried over sodium sulphate. Evaporation of the ether left a greyish-yellow oil (1·68 g.). On distillation this material (1·4 g.) yielded a pale yellow oil (0·75 g.), b. p. 175—180°/15 mm. [Found: C, 40·5; H, 5·3; Br, 41·8. $C_7H_{11}O_2Br$ (II*b*) requires C, 40·6; H, 5·3; Br, 38·8%].

Hydrogenation of the *monobromide* (0·7 g.), dissolved in ethanol (70 ml.) and water (10 ml.), with a palladium–charcoal catalyst (1·0 g.) yielded an oil (0·39 g.), for which alkaline titration indicated a lactone : acid ratio of 7 : 1 and an equivalent weight of 145. This crude hydrogenation product (0·37 g.) was dissolved in ethanol (5 ml.) containing phenylhydrazine (0·32 g.), and the mixture refluxed for 3 hours. The solvent was evaporated, and the residual oil leached with dry ether. A small amount of ether-insoluble solid was found to be phenylhydrazine hydrobromide. The ethereal solution yielded on evaporation an oil which crystallised almost completely. Treatment with benzene separated this material into colourless crystals and a red oil, from which more crystalline material was obtained after a further reaction with phenylhydrazine (0·1 g.). Combined crystals (0·12 g.) from these procedures were recrystallised from benzene, separating in small lustrous plates, m. p. 112·5—113·5° [Found: C, 66·0; H, 8·7; N, 12·1. Calc. for $C_{13}H_{20}O_2N_2$ (cf. II*c*): C, 66·1; H, 8·5; N, 11·9%]. A mixed m. p. with the phenylhydrazide from β-propylbutyrolactone prepared according to Clutterbuck *et al.* (*Biochem. J.*, 1937, **31**, 987) was not depressed.

Perhydroclavatin monobromide (0·25 g.) obtained in a second experiment was dissolved in benzene (3 ml.), and freshly distilled piperidine (0·23 ml.) added. On 3 days' standing at room temperature, long colourless needles separated and were identified as piperidine hydrobromide (0·15 g.). Benzene was evaporated from the filtrate, and the residue leached with dry ether. A small amount of gummy insoluble material was removed, and the ethereal solution extracted twice with 2N-hydrochloric acid (10 ml.). The acid aqueous phase was cooled to 0° and made alkaline by slow addition of ice-cold 2N-sodium hydroxide (22 ml.). Two extractions with ether then removed from the alkaline mixture a viscous, almost colourless oil (0·17 g.). This substance was dissolved in freshly distilled acetone (25 ml.), and N-alcoholic hydrogen chloride (0·8 ml.) added. After a few minutes, sodium iodide (0·2 g.) in acetone (10 ml.) was added, and the mixture kept for 30 minutes with occasional agitation. Precipitated sodium chloride was filtered off, and the hydriodide recovered as an oil by evaporation of the acetone. It became crystalline on addition of ethyl acetate and was recrystallised from ethanol (2·5 ml.) and ethyl acetate (6 ml.); m. p. 170—171° (Found: C, 42·3; H, 6·4; N, 4·6; I, 35·3; *C*-CH_3, 3·6. $C_{12}H_{22}O_2NI$ requires C, 42·5; H, 6·5; N, 4·1; I, 37·5; *C*-CH_3, 7·1%).

Hydrogenation of Clavatin Acetate.—Clavatin acetate (0·5 g.) in ethanol (50 ml.) was added to a neutral washed catalyst prepared from Merck charcoal (1·0 g.) and palladous chloride (0·15 g.) suspended in water (50 ml.); 3·65 molar equivalents of hydrogen were absorbed in 1¼ hours. Catalyst was filtered off and washed with water. The combined filtrate and washings were evaporated until distillation at 15 mm. ceased with a bath temperature of 50°. The distillate contained acid equivalent to 26·1 ml. of N/10-alkali, which was isolated as a *S*-benzylthiuronium salt, m. p. 143—144°. A mixed m. p. with *S*-benzylthiuronium acetate was undepressed.

In a repetition of the above experiment with nitrogen replacing hydrogen, the liquid was filtered and distilled after 2½ hours' shaking with the catalyst. The distillate contained 91% of the theoretical quantity of acetic acid. The distilland residue did not crystallise on seeding with clavatin and had physical properties suggestive of polymerisation.

Ozonolysis of Clavatin.—A solution of clavatin (1·0 g.) in water (50 ml.), cooled to 0—5°, was treated with ozonised oxygen for 3½ hours. The ozonised solution was kept for 20 hours at room temperature and then evaporated in a vacuum until distillation ceased with a bath temperature maximum of 40°. Distillate—Fr. 1; distilland—Fr. 2.

Fr. 1 gave a positive test with Schiff's reagent and required the addition of 47·6 ml. of 0·1N-sodium hydroxide (0·73 molar equivalent) for neutralisation to pH 6·5. The neutralised solution was evaporated, and the distillate (Fr. 3) collected. The residue of sodium salt was converted into a *S*-benzylthiuronium salt, and the latter recrystallised from methyl ethyl ketone. It formed laminæ, m. p. 150—151° (Found: C, 50·1; H, 5·6; N, 12·9; S, 14·9. Calc. for $C_9H_{12}O_2N_2S$: C, 50·9; H, 5·7; N, 13·2; S, 15·1%), identified by mixed m. p. as *S*-benzylthiuronium formate.

Fr. 3 with Brady's reagent gave an orange-yellow, apparently amorphous powder (0·27 g.), m. p. 322—324° (frothing). Two crystallisations from nitrobenzene gave dark red needles, m. p. 334—335° (frothing) (Found: C, 40·4; H, 2·5; N, 26·5. Calc. for $C_{14}H_{10}O_8N_8$: C, 40·2; H, 2·4; N, 26·8%). A mixed m. p. with the bis-2 : 4-dinitrophenylhydrazone of glyoxal was undepressed.

Fr. 2, a medium yellow, viscous oil (1·09 g.), was taken up in water (50 ml.), the solution acidified to Congo-red and refluxed for 30 minutes. It was then treated with a slight excess of 2 : 4-dinitrophenylhydrazine in ethanol and heated. The solid was filtered off (Filtrate—Fr. 4), washed, and dried (1·1 g.), m. p. 315—320° (frothing). A portion was triturated with cold 15% sodium carbonate solution (10 ml.); but very little colour appeared in solution and only a trace of amorphous solid was deposited when the filtrate was acidified. The fraction insoluble in sodium carbonate crystallised readily from nitrobenzene and the purified material was identified as the bis-2 : 4-dinitrophenylhydrazone of glyoxal (total amount isolated, 0·5 molar equivalent).

Fr. 4 was evaporated, the residue treated with water (100 ml.), and the extract decolourised with charcoal. Evaporation of the solution gave a crystalline solid (0·27 g.), which in aqueous solution required 38·6 ml. of 0·1N-sodium hydroxide for neutralisation to pH 6·5. The resultant sodium salt was extracted with hot ethanol, a small amount of oil, which was not further examined, thereby being removed. The ethanol-insoluble fraction, treated with *S*-benzylthiuronium chloride, yielded a gummy solid (0·28 g.) which slowly crystallised. It was extracted first with boiling methyl ethyl ketone and then with boiling ethanol. The insoluble residue crystallised from water in small plates, m. p. 199—200° (frothing). A mixed m. p. with *S*-benzylthiuronium oxalate was undepressed. The ethanol extract contained a solid (0·15 g.) which crystallised from ethanol (12 ml.) in long colourless needles, m. p. 154·5—155·5° (frothing) (Found: C, 50·4; H, 5·4; N, 12·5; S, 13·5. $C_{20}H_{24}O_5N_4S_2$ requires C, 51·7; H, 5·2; N, 12·1; S, 13·8. $C_{19}H_{22}O_4N_4S_2$ requires C, 50·7; H, 4·9; N, 12·5; S, 14·2%). A mixed m. p. with *S*-benzylthiuronium *dl*-malate [m. p. 152—153·5° (slow frothing)] was depressed. *S*-Benzylthiuronium ketosuccinate (from hydroxyfumaric and hydroxymaleic acids), m. p. 125—126° (frothing), and *S*-benzylthiuronium ethylene oxide dicarboxylate, m. p. 184—186° (frothing), were prepared in unsuccessful attempts to identify this compound.

A second ozonolysis of clavatin in chloroform solution gave qualitatively identical results.

Oxidation of Clavatin by Permanganate.—To a stirred solution of clavatin (0·5 g.) in water (20 ml.), cooled in ice, was added dropwise a solution of potassium permanganate (1·37 g.) in water (150 ml.) during 5 hours, decolorisation of the entering salt being instantaneous during the whole period. The filtrate from manganese dioxide was acidified and worked up substantially as described for the ozonolysis. 0·92 Molar equivalent of distillable acid was identified as formic acid and a small amount of glyoxal obtained in the form of its bis-2 : 4-dinitrophenylhydrazone. Addition of Brady's reagent to the distilland produced an orange-red flocculent precipitate (0·5 g.), having an indefinite decomposition m. p. *ca* 160°. It was not found possible to crystallise this substance and it was not further examined.

Treatment of Clavatin with Hydrogen Chloride in Ethanol.—Dry hydrogen chloride was passed into a solution of clavatin (4·4 g.) in ethanol (70 ml.) cooled to −10°. Saturation of the solution was complete in 6 hours. Benzene (50 ml.) was added to the reaction product, and the mixture evaporated under a water pump vacuum to a small volume. After a second addition of benzene and subsequent evaporation, the residual solution was diluted with benzene (150 ml.) and transferred to a separating funnel. This solution was then washed once with water (20 ml.), twice with 1% sodium bicarbonate solution (30 ml. portions), twice with water (20 ml.), and finally dried over sodium sulphate. Removal of solvent left a pale yellow-brown oil (5·9 g.). A portion kept at room temperature showed progressive development of amorphous ethanol-insoluble substance and underwent gradual resinification. The *substance* distilled, with a very small forerun, at 114—116°/0·15 mm. as a colourless oil (4·4 g.) (Found: C, 50·5; H, 5·6; Cl, 13·6; OEt, 26·8. $C_{11}H_{17}O_5Cl$ requires C, 49·9; H, 6·4; Cl, 13·4; OEt, 34·0%). Gradual decomposition took place in the distilland, producing a dark brown resin.

Reactions and Derivatives of Substance $C_{11}H_{17}O_5Cl$ (III).—Quantitative studies with freshly distilled material showed that (*a*) bromine was not absorbed from acetic acid or chloroform solution; (*b*) no reaction occurred with monoperphthalic acid in ethereal solution.

2 : 4-*Dinitrophenylhydrazones.* Freshly prepared crude material (0·53 g.), dissolved in ethanol (5 ml.), was added to a solution of 2 : 4-dinitrophenylhydrazine (0·2 g.) in sulphuric acid (0·4 ml.) and ethanol (3 ml.). A red colour rapidly developed and orange-coloured crystals separated. Recrystallised three times from benzene (3 ml.) and light petroleum (b. p. 40—60°) (6 ml.), the substance had m. p. 168—170° (frothing) (Found: C, 49·4; H, 4·9; N, 13·8; Cl, 1·8; OEt, 20·2. $C_{17}H_{20}O_8N_4$ requires C, 50·0; H, 5·1; N, 13·7; OEt, 22·2. 23% $C_{17}H_{21}O_8N_4Cl$ + 77% $C_{17}H_{20}O_8N_4$ require C, 49·0; H, 4·8; N, 13·5; Cl, 1·8; OEt, 21·6%).

The experiment was repeated, methanol being used in place of ethanol. Crystals, similarly recrystallised, had m. p. 164—166° (frothing), depressed on admixture with the substance, m. p. 168—170°, from ethanol solution (Found: C, 48·0; H, 4·5; N, 13·7; Cl, 1·5. $C_{16}H_{18}O_8N_4$ requires C, 48·6; H, 4·6; N, 14·1. 18·2% $C_{16}H_{19}O_8N_4Cl$ + 81·8% $C_{16}H_{18}O_8N_4$ require C, 47·9; H, 4·5; N, 14·0; Cl, 1·5%).

Degradation by hydriodic acid. Freshly prepared crude material (0·4 g.), dissolved in hydriodic acid (*d* 1·7) (13 ml.), was heated in an atmosphere of carbon dioxide for 2 hours, the bath temperature being raised gradually from 70° to 125° during 1 hour and then maintained at 130—135° for 1 hour. The reaction mixture was cooled, diluted with water (30 ml.) and extracted with ether (200 ml.) in four portions. The combined ethereal extracts were washed with water (20 ml.), in two portions, with small volumes of N-sodium thiosulphate until colourless, finally with water (40 ml.) in three portions, and dried over sodium sulphate. Removal of solvent yielded an oil (0·35 g.) which crystallised immediately. Recrystallised from benzene, it melted at 92—94°. A mixed m. p. with ε-iodo-γ-ketohexoic acid (IV*a*) was not depressed.

Acid hydrolysis. Freshly prepared crude reaction product (5·9 g.) obtained in a second experiment was dissolved in ethanol (50 ml.) and 2N-sulphuric acid (70 ml.), and the solution boiled in a nitrogen atmosphere so that slow distillation took place. Boiling was continued in this manner for 2 hours, additions of water being made when the volume reached one-third of the original. At the end of the period, the solution was evaporated under 15 mm. pressure until distillation ceased. The distilland residue crystallised during this procedure. The distillate required 31·8 ml. of N/10-sodium hydroxide for neutralisation to pH 6·5.

Analysis showed that 49% of the distillate acid was hydrochloric, and 51% formic acid.

The crystalline distilland residue was filtered off, washed with ice-cold 2N-sulphuric acid (15 ml.) in four portions, with ice-cold water (20 ml.) in five portions, and finally dried in a vacuum over phosphoric oxide. The product was a light brown, crystalline solid (2·75 g.), m. p. 128·5—129·5°. A further quantity (0·3 g.) was obtained by working up the mother-liquors. The total yield was dissolved in chloroform (100 ml.), the boiling solution treated with charcoal and evaporated to *ca.* 30 ml. On cooling, colourless needles separated (2·58 g.), m. p. 129—130° [Found : C, 43·8; H, 3·8; Cl, 18·4. $C_7H_7O_4Cl$ (IV) requires C, 44·1; H, 3·7; Cl, 18·6%].

Reactions and Derivatives of the Chloro-acid (IV).—*Titrations.* An aqueous solution was acid to Congo-red but gave no coloration with ferric chloride. Titration of the acid group with N/50-sodium hydroxide to pH 4·9 indicated an equivalent weight of 197 and titration to pH 6·5, one of 185. Slow further absorption of alkali beyond the first transient indicator colour occurred at room temperature to a total of 3 molar equivalents (assuming *M*, 190·5). A typical experiment is described in detail.

To the chloro-acid (IV) (0·0828 g.) in water (5 ml.), N/10-sodium hydroxide was added, phenolphthalein being used as an indicator. The first indicator colour, lasting 5 secs., was obtained with 4·58 ml. A further 9·16 ml. of alkali were added dropwise during 24 hours, the mixture being stored at −15° overnight. Speed of indicator colour discharge slowed progressively during entry of the third molar equivalent. The final indicator colour was stable for 30 minutes. The solution contained as ionised chlorine 82% of the chlorine content of the starting material.

A strongly alkaline solution of the chloro-acid was boiled for 15 minutes. The mixture was acidified and distilled. In the distillate formic and hydrochloric acids were identified by methods previously described. Oxalic acid could not be detected in the distilland.

Degradation by hydriodic acid. The chloro-acid (0·198 g.) was dissolved in freshly distilled hydriodic acid (*d* 1·7) (10 ml.), and the solution treated and finally worked up as described previously. Removal of solvent left an oil (0·24 g.) which crystallised rapidly and completely. Crystallisation from benzene gave a substance, m. p. 92—94°. A mixed m. p. with ε-iodo-γ-ketohexoic acid (IV*a*) was undepressed.

Hydroxylamine titration. This was carried out substantially as described for clavatin, chloro-acid (0·1 mM), hydroxylamine hydrochloride (0·56 mM), and sodium hydroxide (0·38 mM) being used for each determination. The solutions were titrated to pH 4·8, a lower pH being impossible owing to buffering caused by the carboxyl group of the chloro-acid. At pH 4·8 only about 82% of the free hydroxylamine was titrated. Titrations indicated that in 1½ hours 0·80, in 3 hours 0·82, and in 18 hours 0·84 molar equivalent of hydroxylamine was absorbed.

2 : 4-*Dinitrophenylhydrazone.* The chloro-acid (IV) (0·2 g.) in ethanol (2 ml.) was added to a solution containing 2 : 4-dinitrophenylhydrazine (0·15 g.) in sulphuric acid (0·3 ml.) and ethanol (2·2 ml.). An orange-coloured solid was deposited and was filtered off after 2 days (0·10 g.). In two crystallisations from benzene it separated in rosettes of red needles, m. p. 189—190° (Found : C, 45·9; H, 3·8; N, 14·6; Cl, 9·0; OEt, 10·4. $C_{15}H_{15}O_7N_4Cl$ requires C, 45·2; H, 3·8; N, 14·1; Cl, 8·9; OEt, 11·3%). The substance did not dissolve in sodium carbonate solution and the Knorr pyrazoline test was negative. Esterification of the carboxyl group had apparently taken place.

Bromine addition. The chloro-acid (IV) (0·0934 g.) in glacial acetic acid (5 ml.) was treated with bromine (0·16 g.), and the residual bromine estimated by the usual method. The bromination required 11·25 ml. of 0·1N-sodium thiosulphate as compared with 18·8 ml. for the control. Further iodine was rapidly liberated on standing after the first end-point had been reached.

In a second experiment, the chloro-acid (IV) (0·0927 g.) in chloroform (5 ml.) was mixed with bromine (0·16 g.) in chloroform (1 ml.). There was a rapid reduction in colour as compared with a control solution. After standing overnight, the solvent was evaporated under 0·1 mm. The solvent-free residue was a viscous oil (0·17 g.). This substance was dissolved in ethanol (10 ml.) and titrated with N/20-sodium hydroxide : 11·4 ml. were required to bring the pH to 6·5 and on standing further acid rapidly appeared. In 36 hours a further total of 11·06 ml. of N/20-sodium hydroxide were required for neutralisation. The final end-point was stable.

Hydrogenation. Neutral washed catalyst prepared from Merck charcoal (0·15 g.) and 10% palladous chloride solution (0·25 ml.) was suspended in water (20 ml.) under hydrogen. The chloro-acid (IV) (1·48 g.), dissolved in water (30 ml.), was added. During 1 hour a total of 2·4 molar equivalents of hydrogen were absorbed. Catalyst was filtered off, and the colourless filtrate evaporated under 15 mm. to a viscous oil (1·23 g.). A portion of this material (0·014 g.), dissolved in water and titrated with N/20-sodium hydroxide, required 3·95 ml. for neutralisation to pH 6·5. On boiling, neutralisation of a further 0·4 ml. occurred slowly. The remaining material was dissolved in water, and the cold solution brought to pH 6·5 by cautious addition of sodium hydroxide and extracted continuously with ether for 7 hours. The extracted solution was evaporated to dryness, the residue dissolved in water (3 ml.) and ethanol (17 ml.), and this solution divided into four equal parts.

From three parts the following derivatives were prepared by methods in general use : S-*Benzylthiuronium* salt, crystallised from methyl ethyl ketone, m. p. 149—150° [Found : C, 56·0; H, 6·4; N, 8·6; S, 9·6. $C_{15}H_{20}O_4N_2S$ (cf. V) requires C, 55·6; H, 6·2; N, 8·6; S, 9·9%]; p-*phenylphenacyl* ester, crystallised four times from benzene–light petroleum (b. p. 60—80°) and twice from ethanol, burrs of colourless needles, m. p. 125—127° [Found : C, 71·2; H, 5·9; *C*-CH_3, 1·6. $C_{21}H_{20}O_5$ (cf. V) requires C, 71·6; H, 5·7; *C*-CH_3, 4·3%]; 2 : 4-*dinitrophenylhydrazone*, crystallised twice from 80% ethanol–water and twice from toluene, m. p. 197—199° [Found : C, 46·3; H, 4·1; N, 16·2. $C_{13}H_{14}O_7N_4$ (cf. V) requires C, 46·2; H, 4·1; N, 16·6%].

Isolation of γ-Keto-β-methylhexoic acid (VII). The fourth portion was evaporated to dryness, and the residue dissolved in freshly distilled hydriodic acid (*d* 1·7) (12 ml.). This solution was treated and worked up as previously described for hydriodic acid degradations. In this way there was obtained a pale yellow oil which crystallised completely (0·45 g.). A portion for analysis was crystallised twice from light petroleum (b. p. 100—120°) after treatment with charcoal, separating in feathery aggregates of needles, m. p. 103—105° [Found : C, 21·4; H, 2·6; I, 62·3. $C_7H_{10}O_3I_2$ (VI) requires C, 21·2; H, 2·5; I, 64·1%].

The substance (0·0252 g.), dissolved in ethanol (3 ml.) and water (2 ml.) and titrated with N/50-sodium hydroxide, required 3·7 ml. to the first phenolphthalein colour in the cold and at boiling temperature absorbed a further 5·88 ml. The titrated solution contained ionised iodine. Indicated equivalent weight, 131. $C_7H_{10}O_3I_2$ (VI) with loss of both iodine atoms requires 131·6.

The crude iodo-acid (VI) (0·17 g.) in ethanol (20 ml.) was titrated at boiling temperature with N/10-sodium hydroxide. Decolourisation of the indicator ceased when 12 ml. had been absorbed. The titrated solution was added to a suspension of palladium–charcoal catalyst and shaken under hydrogen until uptake ceased. The hydrogenated solution was evaporated under 15 mm. to one-third volume, acidified to Congo-red with sulphuric acid, and extracted with ether (100 ml. in four portions). The extract was thoroughly dried over sodium sulphate, and the solvent distilled off to leave a small residue of oil requiring 3·8 ml. of N/10-sodium hydroxide for neutralisation to pH 6·5. The resultant sodium salt was converted into a S-*benzylthiuronium* salt. Crystallised and recrystallised from freshly distilled methyl ethyl ketone, it separated in colourless laminæ, m. p. 144·5—145·5° [Found : C, 58·5; H, 7·0; N, 9·2; S, 10·7. $C_{15}H_{22}O_3N_2S$ (cf. VII) requires C, 58·1; H, 7·1; N, 9·0; S, 10·3%].

The *S*-benzylthiuronium salt of γ-keto-β-methylhexoic acid was prepared synthetically for comparison. Ethyl 2-keto-1-methyl-*n*-butylmalonate, b. p. 91—93°/0·1 mm., was obtained in 60% yield by slightly modifying the method of Raistrick *et al.* (*loc. cit.*). The ester (7·0 g.) was hydrolysed with aqueous alkali, and the malonic acid decarboxylated by heating at 135°/12 mm. Two distillations of the product gave γ-keto-β-methylhexoic acid (3·0 g.), b. p. 142—143°/12 mm. The *S*-benzylthiuronium salt prepared from this acid had m. p. 145·5—146·5°. A mixture with the substance isolated from clavatin melted at 145—146·5°.

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