Gladiolic Acid, a Metabolic Product of *Penicillium gladioli*

1. STRUCTURE

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Wilkins & Harris (1943, 1944) found that *Penicillium gladioli* Machacek ($\equiv P. gladioli$ McCull. & Thom) produced an antibacterial substance when grown on liquid nutrient media, and Brian *et al.* (1946) showed that culture filtrates of *P. gladioli* were toxic to fungi, the fungistatic effect being more pronounced than the antibacterial effect. The production and isolation of the active material named gladiolic acid, $C_{11}H_{10}O_5$, m.p. 160° , have already been described (Brian *et al.* 1946) (Brian, Curtis & Hemming, 1948), and chemical work designed to elucidate the structural formula of this compound has now been completed. Early in the investigation it was realized that in solution under the influence of chemical reagents, gladiolic acid and certain of its derivatives, showed keto-lactol tautomerism (Grove & Willis, 1951) and could not be represented by a single structural formula of the conventional type. The present paper is concerned mainly with the chemistry of gladiolic acid and with the chemical evidence in support of the tautomeric structural formulae; the determination, by spectrographic methods, of the precise structures of gladiolic acid and some of its derivatives in the solid state, will be the subject of a separate communication.

Three compounds without antifungal properties have also been isolated, though in very low yield, from the crude solid obtained by charcoal extraction of the culture filtrates of *P. gladioli* and elution of the charcoal with ether, and their respective structural relationships to gladiolic acid have been determined.

The crude gladiolic acid, m.p. $140-155^\circ$, obtained by the charcoal extraction procedure is purified by solution in ether and extraction into sodium bicarbonate. Acidification of the bicarbonate extract and repeated crystallization of the product from water yields pure gladiolic acid, m.p. 160° . The bicarbonate-insoluble fraction normally consists of a phenolic substance, $C_{10}H_{10}O_3$, m.p. 158° , giving a deep-blue colour with ferric chloride, and is present to the extent of about 0.3% in the crude gladiolic acid. As is described below, Clemmensen reduction of gladiolic acid followed by demethylation of the reduction product gives a phenolic substance, $C_{10}H_{10}O_3$, identical with the above compound. One

particular batch of crude gladiolic acid was found to have an abnormally low melting point ($120-140^\circ$) and a much reduced solubility in ether. Crystallization of the ether-insoluble fraction and fractional crystallization of the portion soluble in ether and bicarbonate, led to the isolation of a second acid, $C_{11}H_{12}O_5$, m.p. $132-135^\circ$ (decomp.). This acid, on treatment with hot dilute mineral acids, readily forms a neutral lactone, $C_{11}H_{10}O_4$, m.p. 173° , identical with deoxygladiolic acid, $C_{11}H_{10}O_4$, obtained by the reduction of gladiolic acid with iron powder and acetic acid. The acid, m.p. $132-135^\circ$ (decomp.), may therefore be regarded as a dihydrogladiolic acid. Rather surprisingly, the bicarbonate-insoluble fraction of the same batch of crude gladiolic acid did not contain any of the phenol, $C_{10}H_{10}O_3$; in its place only deoxygladiolic acid, $C_{11}H_{10}O_4$, was obtained. In view of the ease with which dihydrogladiolic acid undergoes lactonization, deoxygladiolic acid may not be a true metabolic product of *P. gladioli*, but an artifact arising from the method of extraction and purification of the crude gladiolic acid.

Subsequently, dihydrogladiolic acid was shown to be identical with the dihydrogladiolic acid of Raistrick & Ross (1952); deoxygladiolic acid was identical with the dihydrogladiolide of the same workers.

Gladiolic acid, $C_{11}H_{10}O_5$, forms long colourless needles, m.p. 160° . It is optically inactive. The ultraviolet absorption curves (Fig. 1) show maxima at 3070 Å. $\log \epsilon$ 3.65 and 2690 Å. $\log \epsilon$ 4.04 in water, and 3430 Å. $\log \epsilon$ 2.82 and 2750 Å. $\log \epsilon$ 3.53 in 0.1N-sodium hydroxide. Gladiolic acid contains one methoxyl group and titrates as a monobasic acid. It gives no colour with ferric chloride and the hydroxamic acid test for the carboxylic acid group (Feigl, 1947) is positive. The pK determined in the usual way from the potentiometric titration curve (Fig. 2) is 4.45 at 18° . Gladiolic acid is stable to hydrolysis by dilute mineral acids, but 1 mol. of carbon dioxide is liberated on heating with copper chromite in quinoline. On heating under reflux with methanol in the presence of dry HCl gas a methyl ester, $C_{12}H_{12}O_5$, is formed; esterification with ethanol and concentrated sulphuric acid gives an

ethyl ester, $C_{13}H_{14}O_5$. Gladiolic acid reacts with diazomethane, but the course of the reaction is not straightforward and nitrogen-containing material can be separated from the crude product. Gladiolic acid neither reduces Fehling's solution nor restores the colour to Schiff's reagent, but ammoniacal silver nitrate is reduced immediately in the cold. Neutral permanganate in acetone is rapidly decolorized on standing at room temperature, but bromine water is

(Brian *et al.* 1946), has considerable value as a diagnostic test. Gladiolic acid also gives an interesting colour reaction with technical benzene and concentrated sulphuric acid, a deep-red ring being formed at the benzene/acid interface; this colour reaction is also given by many aromatic aldehydes. Gladiolic acid does not give the iodoform reaction, nor does it give a red colour with sodium nitroprusside and alkali. It is only very slowly oxidized by periodic acid, 42% being recovered unchanged after 3 weeks at 30°. Gladiolic acid does not give the nitrochromic acid test for primary and secondary alcoholic —OH groups.

Three compounds have been isolated following the acetylation of gladiolic acid under different conditions. Acetylgladiolic acid, $C_{13}H_{12}O_6$, formed on heating gladiolic acid with acetic anhydride and glacial acetic acid for 1 hr. at 100° is no longer acidic, but is a neutral compound insoluble in cold sodium hydroxide and containing one methoxyl group. It no longer gives the colour reaction with ammonia solution given by gladiolic acid, but an intense red colour is produced with benzene and concentrated sulphuric acid. It reduces ammoniacal silver nitrate immediately in the cold, and contains a reactive carbonyl group, forming a dinitrophenylhydrazone, $C_{19}H_{16}O_8N_4$, and a semicarbazone, $C_{14}H_{15}O_6N_2$. Acetylgladiolic acid regenerates gladiolic acid on hydrolysis with hot dilute mineral acid. When gladiolic acid is heated with the acetic anhydride/acetic acid acetylating mixture at 100° for some hours a second neutral product, $C_{17}H_{18}O_8$, is obtained. It contains three acetyl groups and may be regarded as the triacetate of a hypothetical gladiolic acid hydrate, $C_{11}H_{12}O_6$. The triacetyl compound does not reduce ammoniacal silver nitrate and shows no carbonyl reactivity, although on prolonged heating in aqueous solution with semicarbazide acetate, acetylgladiolic acid semicarbazone is formed in low yield. The triacetyl compound is also formed when acetylgladiolic acid is heated at 100° with acetic anhydride in the presence of concentrated sulphuric acid. Under more drastic conditions of acetylation, namely, heating under reflux with acetic anhydride and fused sodium acetate, gladiolic acid is converted into an unsaturated monobasic acid, $C_{15}H_{14}O_7$, which contains one methoxyl group and one acetyl group which is liberated as acetic acid on acid hydrolysis. The acid, $C_{15}H_{14}O_7$, is non-ketonic and does not reduce ammoniacal silver nitrate.

Gladiolic acid is sensitive to alkali and, on boiling with 3N-sodium hydroxide for some minutes or on standing with 2N-sodium hydroxide for 24 hr. at room temperature, is transformed quantitatively into *isogladiolic acid*, $C_{11}H_{10}O_5$, m.p. 234°. However, gladiolic acid is unaffected by solution in 0.1N-sodium hydroxide for 30 min. at 25° and on acidi-

Fig. 1. Ultraviolet absorption spectra of gladiolic acid and *isogladiolic acid*. —, gladiolic acid in water; ----, gladiolic acid on 0.1N-NaOH; ····, *isogladiolic acid* in ethanol.

Fig. 2. Potentiometric titration of gladiolic acid at 18° with 0.1067N-NaOH. Gladiolic acid approx. 5×10^{-3} M.

only decolorized very slowly on warming. On treatment with Brady's reagent gladiolic acid forms a dinitrophenylhydrazone, $C_{17}H_{14}O_8N_4$. With other carbonyl reagents such as phenylhydrazine and semicarbazide and with primary amines and diamines, the reactions are complex in nature, and will be the subject of a further communication, but in general, 1 mol. of gladiolic acid reacts with 2 mol. of the reagent or amine. The formation of an intense green colour with ammonia solution, sp.gr. 0.880

fication is recovered unchanged. *iso*Gladiolic acid contains one methoxyl group and gives no colour with ferric chloride. It titrates as a monobasic acid, but lactone titration discloses the presence of a second acidic grouping. Although the lactone ring is readily opened, for example on standing with excess 0.1N alkali at 20°, *isog*ladiolic acid is recovered unchanged on acidification even when this is carried out carefully with ice-cold solutions. On heating *isog*ladiolic acid with copper chromite in quinoline at 220°, 1 mol. of carbon dioxide is evolved and a phenolic compound, $C_9H_8O_3$, is obtained in poor yield. This phenol, $C_9H_8O_3$, gives no colour with ferric chloride; it contains a lactone ring and no methoxyl group, and *isog*ladiolic acid appears to have undergone demethylation in addition to the loss of the carboxyl group.

*iso*Gladiolic acid does not give a coloration with ammonia solution, sp.gr. 0.880, or with benzene and concentrated sulphuric acid. In marked contrast to gladiolic acid, *isog*ladiolic acid does not form a precipitate with Brady's reagent and is recovered unchanged even after heating under reflux for 6 hr. with acetic anhydride and fused sodium acetate. On esterification under Fischer-Speier conditions, *isog*ladiolic acid forms a neutral methyl ester, $C_{12}H_{12}O_5$, which like the parent acid shows no carbonyl reactivity. The ultraviolet absorption curve, λ_{max} . 2980 Å., $\log \epsilon$ 3.72 in ethanol (Fig. 1) supports the conclusion that a molecular rearrangement is involved in the formation of *isog*ladiolic acid.

As might be expected, *isog*ladiolic acid is also formed by alkaline hydrolysis of acetylgladiolic acid, triacetylgladiolic acid hydrate, methyl gladiolate and ethyl gladiolate.

Demethylation of *isog*ladiolic acid with hydriodic acid or better with hydrobromic acid gives *noriso*gladiolic acid, $C_{10}H_8O_5$, a phenolic compound giving a deep reddish-purple colour with ferric chloride. *noriso*Gladiolic acid couples immediately with diazotized *p*-nitraniline but only very slowly with diazotized aniline. The Millon reaction is negative. In the test with 2:6-dibromoquinone chloroimide (Gibbs reaction: Gibbs, 1927), a faint blue colour develops slowly. *Noriso*gladiolic acid is also formed, together with much tarry product, when gladiolic acid is heated under reflux with hydrobromic acid.

Like acetylgladiolic acid, methyl gladiolate does not give a green coloration with ammonia solution, but gives a red colour with benzene and concentrated sulphuric acid and reduces ammoniacal silver nitrate. With Brady's reagent, methyl gladiolate gives an immediate precipitate of the dinitrophenylhydrazones, $C_{18}H_{16}O_8N_4$. On reaction with phenylhydrazine hydrochloride in aqueous solution the phenylhydrazone, $C_{18}H_{18}O_4N_2$, is formed, and on treatment with acetic anhydride and concentrated

sulphuric acid at 0°, the diacetate, $C_{16}H_{18}O_8$, of a hydrate of methyl gladiolate is produced. The diacetyl compound does not immediately form a precipitate with Brady's reagent, but on standing methyl gladiolate dinitrophenylhydrazones separates. It reduces ammoniacal silver nitrate on warming but more slowly than methyl gladiolate.

Ethyl gladiolate forms a similar series of derivatives including a semicarbazone, $C_{14}H_{17}O_5N_3$, and a diacetate, $C_{17}H_{20}O_8$, corresponding to the diacetate, $C_{16}H_{18}O_8$, of methyl gladiolate. Ethyl gladiolate slowly restores the colour to Schiff's reagent.

On reduction of gladiolic acid with iron powder and acetic acid the main product is deoxygladiolic acid, $C_{11}H_{10}O_4$, m.p. 173°, which is no longer an acidic substance. An acidic compound is also produced but in an amount too small for adequate characterization. Deoxygladiolic acid contains a lactone ring and is identical, by mixed melting points and comparison of infrared absorption spectra, both with the lactone, $C_{11}H_{10}O_4$, obtained from dihydrogladiolic acid isolated from the crude gladiolic acid, and also with the dihydrogladiolide of Raistrick & Ross (1952). Deoxygladiolic acid contains one methoxyl group, reduces ammoniacal silver nitrate and shows carbonyl reactivity forming an immediate precipitate of the dinitrophenylhydrazones, $C_{17}H_{14}O_4N_4$, with Brady's reagent. Cautious demethylation with hydrobromic acid yields *nor*-deoxygladiolic acid, $C_{10}H_8O_4$, which gives a deep reddish-violet colour with ferric chloride. *iso*-Gladiolic acid, $C_{11}H_{10}O_5$, is formed on oxidation of deoxygladiolic acid with acid permanganate.

The main product of the Clemmensen reduction of gladiolic acid is a neutral lactone, $C_{11}H_{12}O_3$, which contains one methoxyl group and is non-ketonic. Demethylation of this substance with hydrobromic acid yields a phenolic lactone, $C_{10}H_{10}O_3$, m.p. 158°, identical with the phenolic substance, $C_{10}H_{10}O_3$, present in crude gladiolic acid. It gives an intense blue colour with ferric chloride. It does not couple with diazotized *p*-nitraniline and the Millon and Gibbs reactions are negative. A second neutral substance having the probable molecular formula, $C_{22}H_{20}O_6$, resulting from the condensation of two molecules of gladiolic acid, is present in the tarry by-products of the Clemmensen reduction and can be isolated therefrom by trituration with ethanol, in which it is sparingly soluble, followed by vacuum sublimation.

Oxidation of gladiolic acid, $C_{11}H_{10}O_5$, with permanganate gives rise to a series of acidic products according to the conditions employed. Substance A, obtained in good yield by the action of 1% potassium permanganate solution equivalent to one oxygen atom per molecule of gladiolic acid in sodium carbonate at room temperature, is a dibasic acid, $C_{11}H_{10}O_6$. It contains one methoxyl group and

gives no coloration with ferric chloride. The fluorescein and phenolphthalein colour tests for an *o*-phthalic acid are negative. It does not reduce ammoniacal silver nitrate, but nevertheless shows carbonyl reactivity forming a dinitrophenylhydrazone, $C_{17}H_{14}O_8N_4$, a semicarbazone, $C_{12}H_{13}O_6N_3$, which titrates as a dibasic acid, and a monobasic anhydrophenylhydrazone, $C_{17}H_{14}O_4N_2$. In contrast to gladiolic acid, substance *A* is unaffected by boiling 3*N*-sodium hydroxide. It is recovered unchanged after standing 12 hr. with 40% aqueous potassium hydroxide at room temperature. On mild acetylation with acetic anhydride and glacial acetic acid, substance *A* forms an acetate, $C_{13}H_{12}O_7$, which titrates as a monobasic acid and shows no ketonic properties. This acetate is dimorphous, forming needles with m.p. 199° from dilute acetic acid and prisms, m.p. 213°, from aqueous ethanol, and is also formed in the oxidation of acetylgladiolic acid by permanganate in acetone solution.

Oxidation of gladiolic acid with excess permanganate in sodium carbonate at room temperature or further oxidation of substance *A* under the same conditions gives substance *B*, $C_{11}H_{10}O_7$, m.p. 162°, a tribasic acid, which contains one methoxyl group, forms a trimethyl ester, $C_{14}H_{16}O_7$, gives no colour with ferric chloride and is non-ketonic. Substance *B* gives strongly positive results in the usual tests for an *o*-phthalic acid.

Two products have been obtained when gladiolic acid is oxidized in alkaline solution with excess boiling potassium permanganate, a tribasic acid substance *C*, $C_{10}H_8O_7$, m.p. 160–165° (decomp.), and a tetrabasic acid substance *D*, $C_{11}H_8O_9$. Crystallized from ethyl acetate substance *D* has m.p. 175–180° (decomp.) resetting about 220° and remelting at 259° (decomp.); crystallized from dilute hydrochloric acid the initial melting point is somewhat higher (190° decomp.), but the melt then resets and remelts at 259° (decomp.) as already described. Substance *C* was obtained, although in poor yield, in one experiment only, in which 5% potassium permanganate was added to gladiolic acid in boiling sodium carbonate, and attempts to repeat this preparation have only given traces of material. Unfortunately, it has not been possible to accumulate sufficient material for the preparation of a derivative. When the oxidation is carried out initially at room temperature, and then continued, when the rate of uptake of permanganate has slowed, by heating under reflux, the main product is substance *D* and only traces of substance *C* are obtained. Substance *D* is the sole product both of the oxidation of *isog*ladiolic acid by permanganate in boiling potassium hydroxide and of gladiolic acid in the same medium. Undoubtedly under these conditions gladiolic acid is first converted into *isog*ladiolic

acid. The melting point of substance *B* is not depressed by admixture with substance *C* or with substance *D*, but the three acids may be recognized by their infrared absorption spectra which show characteristic bands.

Whereas substance *C* is readily soluble in ether and can be sublimed at 150°/10⁻⁴ mm., substance *D* is only slightly soluble in ether and does not sublime at 160°/10⁻⁴ mm.; hence the separation of the two acids when they occur together is a relatively simple matter. In the original oxidation experiment in which permanganate was added to gladiolic acid in boiling sodium carbonate, the resulting aqueous solution was evaporated to dryness with concentrated hydrochloric acid to avoid the possible isolation of mono-sodium or mono-potassium salts and the dry solid residue extracted with ether. It is not surprising, therefore, that only substance *C* and no substance *D* was obtained. Both substances *C* and *D* give positive reactions in the tests for *o*-phthalic acids, and it is reasonable to regard substance *C* as decarboxy substance *D*. Substance *D* is not decarboxylated by heating at 150° *in vacuo* and it follows that the decarboxylation must take place either during the early stages of the oxidation or directly as a result of the method adopted for working up the reaction mixture. For decarboxylation to occur as a result of either of these possibilities is unusual; however, cases have been reported in the literature where aromatic polycarboxylic acids undergo decarboxylation on heating with concentrated mineral acids (Baeyer, 1873), and this provides a possible explanation for the formation of substance *C*.

Substance *D* gives no colour with ferric chloride. It contains one methoxyl group and forms a liquid tetramethyl ester, $C_{15}H_{16}O_9$. It is clearly a methoxybenzenetetra-carboxylic acid and on demethylation with hydrobromic acid gives the corresponding hydroxybenzenetetra-carboxylic acid, $C_{10}H_6O_9$, m.p. 302°. The latter compound gives a red colour with ferric chloride, and on methylation with ethereal diazomethane in methanol the tetramethyl ester of substance *D* is produced. The hydroxybenzenetetra-carboxylic acid couples very slowly with diazotized amines, but both the Millon and Gibbs reactions are negative. Although, prior to this investigation, none of the three possible hydroxybenzenetetra-carboxylic acids or their methyl ethers had actually been synthesized, one hydroxybenzenetetra-carboxylic acid and one methoxybenzenetetra-carboxylic acid are reported in the literature as having been obtained by the degradation of certain natural products, the structures of which are known with such certainty as to leave little doubt as to the configuration of the degradation products. Thus Dimroth & Goldschmidt (1913) obtained 5-hydroxybenzene-1:2:3:4-tetra-carboxylic

acid, m.p. 212°, with anhydride formation by the degradation of laccaic acid, and Nikuni (1941) isolated 3-methoxybenzene-1:2:4:5-tetracarboxylic acid, m.p. 250–251° (decomp.), by oxidation of dimethyl- α -sorijenin. It seemed probable, therefore, that substance *D* was in fact 4-methoxybenzene-1:2:3:5-tetracarboxylic acid, and this conclusion was supported by the negative Millon and Gibbs reactions given by the demethylated acid.

Accordingly, 4-methoxybenzene-1:2:3:5-tetracarboxylic acid has been synthesized as follows: the Friedel-Crafts reaction between mesityl methyl

and 3-acetoxy-2:4:6-trimethylacetophenone. Very similar results were obtained using 3 moles of aluminium chloride and acetic anhydride, in place of acetyl chloride. The crude neutral product was separated by distillation into two arbitrary fractions, b.p. 200–240°, and 250–275° respectively, and each fraction separately hydrolysed with boiling 3*N*-sodium hydroxide to remove the acetates. 3-Methoxy-2:4:6-trimethylacetophenone, b.p. 268–270°, was obtained from the high-boiling fraction; acidification of the alkaline mother liquors yielded 3-hydroxy-2:4:6-trimethylacetophenone. Oxidation

ether and acetyl chloride in the presence of 1.5 moles of anhydrous aluminium chloride was carried out according to Auwers & Mauss (1928), and the crude product separated into phenolic and neutral fractions by extraction with sodium hydroxide. Auwers & Mauss obtained a 20% yield of 3-hydroxy-2:4:6-trimethylacetophenone, but in their hands the neutral fraction consisted of only a little yellow oil in addition to unchanged mesityl methyl ether. In the present investigation the phenolic fraction consisted of only a trace of 3-hydroxy-2:4:6-trimethylacetophenone, whereas the neutral fraction, a yellow oil, b.p. 200–275°, contained, in addition to unreacted mesityl methyl ether, mesityl acetate, 3-methoxy-2:4:6-trimethylacetophenone

of 3-methoxy-2:4:6-trimethylacetophenone, first with boiling permanganate in alkaline solution and then with acid permanganate to bring about decarboxylation of the resultant phthalonic acid (Posternak, 1940) gives in good yield, 4-methoxybenzene-1:2:3:5-tetracarboxylic acid, identical by mixed melting-point determinations and comparison of the infrared absorption spectra with substance *D*, the methoxybenzenetetracarboxylic acid from gladiolic acid. 4-Hydroxybenzene-1:2:3:5-tetracarboxylic acid, obtained on demethylation of the synthetic material with hydrobromic acid, is identical with the hydroxybenzenetetracarboxylic acid, m.p. 302°, obtained by demethylation of substance *D*.

DISCUSSION

Isomeric structural formulae for gladiolic acid are readily deduced from the chemical work described above. Many of the compounds obtained from gladiolic acid, in particular the products of oxidation and the demethylated reduction products, are clearly aromatic in character. The presence of a benzene nucleus in gladiolic acid itself is indicated by the stability of the compound towards bromine, and is supported by the ultraviolet absorption spectrum which is typical of a substituted aromatic compound in which benzenoid and carbonyl chromophores are conjugated. Gladiolic acid, $C_{11}H_{10}O_5$, is a monobasic carboxylic acid. It contains a carbonyl group as shown by the formation of a dinitrophenylhydrazone, and this must be a formyl group, since on mild oxidation with permanganate, the dibasic acid substance *A*, $C_{11}H_{10}O_6$, is produced and the reducing properties of gladiolic acid are eliminated. The presence of a tautomeric system is most easily seen from an examination of the chemistry of substance *A*. Of the six oxygen atoms in this compound, four occur in the two $-\text{COOH}$ groups, one of which was originally present in the gladiolic acid molecule and the second formed during the oxidation. The fifth oxygen atom is contained in a $-\text{OCH}_3$ group and the sixth is present as a $>\text{C}=\text{O}$ group as shown by

tautomeric system I, since the neutral acetylgladiolic acid, $C_{13}H_{12}O_6$, which is derived from the lactol form retains the carbonyl reactivity and reducing properties of gladiolic acid.

*iso*Gladiolic acid, $C_{11}H_{10}O_5$, on the other hand, shows no reducing properties or carbonyl reactivity and the presence of a hydroxyl group cannot be detected; the five oxygen atoms are in fact contained in one $-\text{OCH}_3$ group, one carboxyl group and one lactone ring. It may be concluded, therefore, that the molecular rearrangement concerned in the formation of *isog*ladiolic acid involves both the formyl group and the carbonyl group of the keto-lactol system of gladiolic acid.

All the oxygen and carbon atoms in gladiolic acid have been accounted for in the various groupings detected and it only remains to determine how these groupings are oriented with respect to each other. Thus the additional nuclear substituents in the partial structure I may be $-\text{CH}_3$, $-\text{CHO}$, or $-\text{CH}_2$ and $-\text{CHO}$, depending on whether R is $-\text{CHO}$, $-\text{CH}_3$, or H respectively.

the formation of the semicarbazone which titrates as a dibasic acid. Nevertheless, the presence of a hydroxyl group in substance *A* is indicated by the formation on acetylation under mild conditions of a monobasic monoacetate, $C_{13}H_{12}O_7$, and this can be explained if substance *A* contains a γ - or δ -keto acid structure capable of showing keto-lactol tautomerism. Kühn-Roth oxidations on gladiolic acid and its derivatives suggest the presence of $1\text{C}-\text{CH}_3$ group and if this is admitted, substance *A* must be a γ -keto acid and contains the partial structure (I). Of the five oxygen atoms in gladiolic acid, four are present in the methoxyl substituted keto-acid structure I; the fifth is contained in a formyl group which cannot be the carbonyl group involved in the

Gladiolic acid gives neither the iodoform reaction, nor the nitroprusside test for the $\text{CH}_3\cdot\text{CO}-$ group and it may be concluded that this grouping is absent. Following the original oxidation experiment which led to the isolation of the methoxybenzenetricarboxylic acid, substance *C*, it was concluded that gladiolic acid contained a benzene ring with a methoxyl substituent and three carbon side chains, and in a preliminary communication (Brian *et al.* 1946) a 2-carboxyphenylglyoxal structure II was suggested as being consistent with the chemistry of gladiolic acid as then known. On this structure the formation of *isog*ladiolic acid from gladiolic acid in alkali was visualized as involving the normal rearrangement, under such conditions, of the phenyl

glyoxal portion of the molecule to the corresponding mandelic acid followed by lactonization to the phthalide-3-carboxylic acid (III). Subsequently, it was discovered that gladiolic acid did not undergo

fission with periodate with the formation of a methylmethoxyphthalic acid as might have been expected on structure II; moreover, when the oxidation with permanganate was re-examined the

isolation of a tetracarboxylic acid, shown by synthesis to be 4-methoxybenzene-1:2:3:5-tetracarboxylic acid (IV), from both gladiolic acid and *iso*-gladiolic acid clearly excluded structures II and III

for these compounds. The isolation of the tetracarboxylic acid in fact admits of no other arrangement but V for gladiolic acid, comprising (in the open form) a benzene nucleus with methyl, methoxyl, carboxyl and two formyl substituents, and VI for *isog*ladiolic acid, namely a phthalide ring with nuclear methyl, methoxyl and carboxyl substituents.

The well known rearrangement of *o*-phthalaldehyde under alkaline conditions to give hydroxymethylbenzoic acid usually isolated as its lactone, phthalide, provides a ready explanation of the formation of *isog*ladiolic acid. VII and VIII are therefore seen to be the more probable structures for gladiolic acid. (For simplicity, only the open, keto, structures will be considered in the arguments which follow.)

Nevertheless structures IX and X, in which the formyl substituents are 1:4, and XI and XII, in which they are 1:3, cannot at this stage be completely disregarded as *isog*ladiolic acid can theoretically be derived from them by a type of crossed Cannizzaro reaction followed by lactonization. For example, by this reaction sequence, X can give rise to XIII, and IX and XI to XIX and XX respectively. A second product, namely the substituted phthalic acid XIV, might also be expected from X, and similar considerations apply to crossed Cannizzaro reactions with IX and XI. The isolation of a phthalic acid from gladiolic acid under the influence of alkali has not been realized experimentally: however, the absence of a second product cannot be used as an argument against the *iso*- and *tere*-phthalaldehyde structures VII and VIII are each capable of rearrangement to three products, the phthalide carboxylic acids

XV-XVII, and XVI-XVIII respectively. Under the experimental conditions employed, a mixture of phthalide carboxylic acids is not obtained: *iso*-gladiolic acid is a pure substance and the only product of the reaction. A possible explanation for this observation is advanced in the closing paragraphs of the discussion.

Returning to a consideration of the possible structures for gladiolic acid, XII can be disregarded, as the dicarboxylic acid substance A is not an *o*-phthalic acid. Some support for the *o*-phthalaldehyde structures VII and VIII comes from the colour reaction of gladiolic acid with ammonia; *o*-phthalaldehyde, but not *iso*- or *tere*-phthalaldehyde, gives a bluish-green colour on acidification of an ammoniacal solution with dilute acetic acid. The final decision between the isomeric structures VII–XI comes from a consideration of the structures of *isog*ladiolic acid and also of the reduction products of gladiolic acid, deoxygladiolic acid, $C_{11}H_{10}O_4$, obtained by the action of iron powder and acetic acid, and the lactone, $C_{11}H_{12}O_3$, obtained by Clemmensen's method.

Deoxygladiolic acid retains the carbonyl reactivity and reducing properties and hence the reactive formyl group of gladiolic acid. Apparently only the phthalaldehydic acid portion of the molecule is affected by the reduction; either reduction of the formyl group in the tautomeric system (I, R = H) occurs preferentially and is followed by lactonization or more probably, gladiolic acid is reduced in the lactol form directly to the corresponding phthalide. Deoxygladiolic acid then has the partial structure XXI.

Reduction of the 3-hydroxyphthalide system to phthalide occurs in the Clemmensen reduction, but in addition the formyl group is reduced to $-CH_3$, and the compound $C_{11}H_{12}O_3$ has the partial structure XXII.

Oxidation of deoxygladiolic acid in dilute mineral acid gives *isog*ladiolic acid and if we assume, as seems reasonable, that the phthalide ring remains intact under these conditions, then the reactive formyl group of gladiolic acid must correspond to the free carboxyl group in *isog*ladiolic acid. Consequently, structures XV and XVI for *isog*ladiolic acid can only come from VIII, and XVII and XVIII can only come from VII. (As will be seen below, evidence obtained at one stage of the investigation threw some doubt on the validity of the above assumption and on the deductions made.)

All three substances, *isog*ladiolic acid, deoxygladiolic acid and the Clemmensen reduction product $C_{11}H_{12}O_3$ can be demethylated to phenolic substances, but little information on the orientation of these compounds can be obtained from the Millon and Gibbs tests or from coupling reactions which are negative, except in the case of *norisog*ladiolic acid. The positive coupling with diazotized *p*-

nitraniline shown by *norisog*ladiolic acid no doubt involves displacement of the carboxyl group. *Norisog*ladiolic acid gives an intense reddish-purple colour with ferric chloride showing that the hydroxyl substituent must be ortho to a carbonyl group present either in the carboxylic acid residue or in the phthalide ring and eliminating structure XVIII for *isog*ladiolic acid. Further information on the orientation of the substituents in *norisog*ladiolic acid can be obtained from the infrared spectrum.

Recent work on the correlation of absorption frequency with structure for the $>C=O$ stretching vibration has been summarized by Grove & Willis (1951). Chelation in *o*-hydroxycarbonyl compounds results in very large displacements of the characteristic absorption frequencies of both the $>C=O$ and $-OH$ groups (Gordy, 1940; Martin, 1950; Hunsberger, 1950). It follows that these displacements may be used to detect chelation in compounds of unknown orientation which contain a carbonyl and a hydroxyl group, and the method is more reliable if the corresponding methyl ether is also available for purposes of comparison. If two $>C=O$ groups of different types each having its own characteristic absorption band are present in the same molecule, the method may be extended to detecting which of the two $>C=O$ groups is chelated. *iso*Gladiolic acid shows two bands in the double bond-stretching region at 1758 and 1688 cm^{-1} due to phthalide ring $>C=O$ and aromatic carboxylic acid $>C=O$ respectively (Grove & Willis, 1951). *Norisog*ladiolic acid shows only a single broad absorption band with its peak at 1695 cm^{-1} and a definite shoulder at 1727 cm^{-1} . The $-OH$ absorption frequency of *norisog*ladiolic acid is so low that it is masked by the $C-H$ absorption of the 'Nujol' medium, and it may be deduced that the compound contains a chelate ring and that chelation involves the $>C=O$ group of the phthalide ring and not the $>C=O$ group of the carboxyl group. *Norisog*ladiolic acid must therefore contain the partial structure XXIII

(R = H), and XIII, XV and XX for *isog*ladiolic acid are thereby eliminated as they cannot, under the experimental conditions employed in the demethylation, give a *norisog*ladiolic acid with structure XXIII (R = H).

The elimination of XIII and XX for *isogradiolic* acids disposes of X and XI respectively as possible structures for gladiolic acid. Now the phenol, $C_9H_8O_3$, obtained by decarboxylation of *isogradiolic* acid with copper chromite in quinoline gives no colour with ferric chloride. The phenol derived from structure XIX for *isogradiolic* acid by decarboxylation and demethylation should give an intense ferric reaction, and structures XIX for *isogradiolic* acid and IX for gladiolic acid may therefore be excluded. Of the two remaining structures for *isogradiolic* acid, XVI and XVII, the absence of a ferric reaction in the phenol, $C_9H_8O_3$, clearly favours the salicylic acid structure XVI; on the other hand, the partial structure XXIII deduced above for *norisogradiolic* acid is derived from XVII. It must be borne in mind that during the demethylation of *isogradiolic* acid under acid conditions with hydrobromic acid, a rearrangement of type XXIV, although unlikely, is not impossible. A rearrangement of this type is of course possible in the decarboxylation experiment and indeed is more likely than under the acid conditions of the demethylation. Therefore, if we assume

that there is no rearrangement of type XXIV involved in the formation of *norisogradiolic* acid, *isogradiolic* acid must be 7-methoxy-6-methyl-phthalide 4-carboxylic acid XVII. Although this line of reasoning appears to exclude VIII and establishes gladiolic acid as VII, 4-methoxy-5-methyl-o-phthalaldehyde 3-carboxylic acid (McGowan, 1950), the assumption involved, namely that there is no rearrangement of type XXIV during the oxidation of deoxygladiolic acid to *isogradiolic* acid, is precisely that used to exclude XVI as a possible structure for *isogradiolic* acid following the establishment of *norisogradiolic* acid as containing XXIII ($R = H$). It is relevant to point out here that the open structure XXV ($R = CH_3$) must be present as the sodium salt in alkaline solution following the rearrangement of gladiolic acid, and that on acidification this closes preferentially to give only one product.

In view of the ambiguity involved, further evidence was sought by the examination of the phenol, $C_{10}H_{10}O_3$, obtained by demethylation of the Clemmensen reduction product, $C_{11}H_{12}O_3$. This phenol gives a blue colour with ferric chloride in contrast to *norisogradiolic* acid which gives a reddish purple. In the infrared spectrum, the $-OH$ and $>C=O$ groups absorb at 3378 cm^{-1} ($2.96\text{ }\mu$.) and 1742 cm^{-1} respectively, the normal positions for non-chelated phenolic hydroxyl and phthalide ring $>C=O$ respectively. In the methyl ether, the phthalide ring $>C=O$ band has its maximum at 1745 cm^{-1} . It was concluded, therefore, that the phenol had in fact the structure XXVII ($R = H$) and not XXVI ($R = H$) corresponding to VII. On this basis it was concluded that gladiolic acid was

VIII, 3-methoxy-4-methyl-o-phthalaldehyde 6-carboxylic acid and that a rearrangement of type XXIV did take place either during the oxidation of deoxygladiolic acid to *isogradiolic* acid or during the demethylation of *isogradiolic* acid. Moreover, the absence of a ferric reaction in the phenol $C_9H_8O_3$ supported the conclusions that *isogradiolic* acid had the structure XVI and that a rearrangement occurred during the demethylation of *isogradiolic* acid.

At this point in the investigation the information that the phenol $C_{10}H_{10}O_3$, which can also be obtained by Clemmensen reduction of dihydrogladiolic acid and demethylation of the reduction product (Raistrick & Ross, 1952), gave *ψ*-cumenol (2:4:5-trimethylphenol) XXVIII on treatment with hydriodic acid and red phosphorus, was communicated to the present author by Prof. H. Raistrick, F.R.S. In this case there is no possibility of a rearrangement of type XXIV and this evidence, subject to the proviso that there is no migration of methyl groups round the benzene ring, establishes the structure of the phenol $C_{10}H_{10}O_3$ as 7-hydroxy-4:6-dimethylphthalide XXVI ($R = H$). It follows that gladiolic acid has structure VII, 4-methoxy-5-

methyl-*o*-phthaldehyde 3-carboxylic acid, a structure which is certainly favoured by the bulk of the chemical evidence and is now believed to be correct.

Additional evidence in support of this structure comes from an examination of the infrared spectrum of nordeoxygladiolic acid. Nordeoxygladiolic acid gives an intense reddish-purple colour with ferric chloride which cannot be used to distinguish between XXIX ($R=H$) (derived from VII) and XXX

(XXIX)

(XXX)

(XXXI)

(XXXII)

($R=H$) (derived from VIII), both salicylaldehyde and salicylic acid giving very similar colours with ferric chloride. However, the infrared spectrum shows two bands due to $>C=O$ groups at 1733 and 1688 cm^{-1} compared with 1758 and 1695 cm^{-1} in deoxygladiolic acid. As in the case of norisogradiolic acid, the shift of 25 cm^{-1} shows that the $>C=O$ group in the phthalide ring is chelated and XXIX ($R=H$) is therefore preferred to XXX ($R=H$). In this series there is no possibility of molecular rearrangement and deoxygladiolic acid must be XXIX ($R=CH_3$). It follows that gladiolic acid has the structure VII.

The failure of the infrared method in the case of the phenol $C_{10}H_{10}O_3$ is surprising, and is at present under investigation in these laboratories. There is no reason for doubting the validity of the method in those cases where displacements of the bands are actually observed.

The structures of the majority of the derivatives and degradation products of gladiolic acid follow immediately the orientation of the substituents has been established. Deoxygladiolic acid and dihydrogladiolic acid are 4-formyl-7-methoxy-6-methylphthalide XXIX ($R=CH_3$) and 3-formyl-2-hydroxymethyl-6-methoxy-5-methylbenzoic acid XXXI respectively, as conclusively and independently established by Raistrick & Ross. It seems more reasonable to suppose that the partial structure XXIII ($R=CH_3$ or H) persists throughout the sequence deoxygladiolic acid $\rightarrow$ isogradiolic acid $\rightarrow$ norisogradiolic acid and that isogradiolic acid is 7-methoxy-6-methylphthalide 4-carboxylic acid

XVII. Moreover, the methylisogradiolate must be derived from XVII since ortho disubstituted benzoic acids do not readily form normal esters under Fischer conditions. However, the alternative structure XVI, 5-methoxy-6-methylphthalide 4-carboxylic acid cannot be completely excluded in view of the results obtained in the decarboxylation experiment.

(XXXIII)

(XXXIV)

(XXXV)

Of the acetylation products of gladiolic acid, acetylgladiolic acid has the structure XXXIII ($R=Ac$) and the triacetyl compound, XXXII ($R=Ac$), the diacetate of the hydrated form of acetylgladiolic acid. Compounds of similar structure are formed by many reactive aldehydes and are described in the literature (e.g. *o*-phthalaldehyde tetra-acetate and phenylglyoxal diacetate). The unsaturated monobasic acid, $C_{15}H_{14}O_7$, XXIV, which contains one acetyl group is formed by Perkin condensation between the reactive formyl group of acetylgladiolic acid and acetic anhydride. The formation by methyl and ethyl gladiolate of diacetyl compounds, XXXII, which show no carbonyl reactions, suggests that these esters are pseudo esters of structure XXXIII ($R=CH_3$ and C_2H_5 respectively), rather than normal esters (XXXV) which might be expected to form either tetra-acetates or aldehydic diacetates. Conclusive spectroscopic evidence for the structures of these esters will be reported elsewhere. It may be noted that although ortho disubstituted benzoic acids do not form normal esters under Fischer conditions, their ability to form pseudo esters, where this is possible, is not greatly inhibited (Newman & McCleary, 1941; Newman & Lord, 1944).

Of the oxidation products, substance B is 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid, XXXVIII. Substance A has the tautomeric structure XXXVI, the monobasic monoacetate, $C_{13}H_{12}O_7$, XXXVII, being derived from the lactol form XXXVIb since it is also formed on oxidation of acetylgladiolic acid XXXIII ($R=Ac$). A derivative of the lactol form XXXVIc has not been obtained.

The precise structure of the monobasic anhydro-phenylhydrazone which is now seen to be a methoxymethylphthalazone carboxylic acid (XXXIX or

absorption spectra (Fig. 1) of the compound in water and in 0.1N-sodium hydroxide in which gladiolic acid is stable.

XL), derived from the keto form XXXVIa of substance A, is uncertain. The failure of substance A to undergo the Cannizzaro reaction is consistent with XXXVIa; it is well known that ortho disubstituted benzaldehydes do not readily undergo this reaction owing to steric hindrance.

Keto-lactol tautomerism in gladiolic acid (VII) is shown by the rearrangement of the keto form VIIa to isogladiolic acid and by the formation of the neutral acetylgladiolic acid and the ψ esters derived

One further topic of gladiolic acid chemistry which remains to be discussed is the formation of a single product, isogladiolic acid, on treatment of gladiolic acid with alkali. If, in the rearrangement of o-phthalaldehyde, we assume a mechanism involving an intramolecular transfer of H^- similar to that recently advanced (Alexander, 1947; Doering *et al.* 1948) for the rearrangement of phenylglyoxal to mandelic acid, then the formyl group which undergoes the initial attack by the hydroxyl ion must become a carboxyl group in the resulting hydroxymethylbenzoic acid.

In the gladiolic acid molecule this initial attack is most likely to take place on the more reactive of the two formyl groups, namely that in position 1, and hence only one substituted hydroxymethylbenzoic acid (XLI) is formed. The preferential lactonization of this intermediate to isogladiolic acid (XVII) is no

from the lactol form VIIb, 4-formyl-3-hydroxy-7-methoxy-6-methylphthalide. It is most clearly demonstrated by the differences in the ultraviolet

doubt due to steric considerations arising from the presence of the methoxy substituent ortho to one of the carboxyl groups.

The somewhat involved series of relationships in gladiolic acid chemistry is illustrated in the diagram (p. 652). Gladiolic acid, dihydrogladiolic acid, deoxygladiolic acid and the phenol $C_{10}H_{10}O_3$ make up an interesting series of closely related metabolic products of *P. gladioli*. The obvious structural relationship of the phenol $C_{10}H_{10}O_3$ to mycophenolic acid, and

Phenol $C_{10}H_{10}O_3$

Mycophenolic acid

hence to the series of derivatives of 3:5-dihydroxyphthalic acid discussed by Raistrick (1950), gives the gladiolic acid series of compounds a definite place in the general picture of the metabolic products of the fungi.

EXPERIMENTAL

Micro-analyses are by Weiler and Strauss, Oxford, and by Mr W. Brown. Melting points are corrected. The infrared spectra of solid compounds were obtained using 'Nujol' suspensions in the apparatus described by Grove & Willis (1951).

(1) Isolation and purification of gladiolic acid

Penicillium gladioli McCull. & Thom was grown on a simplified Raulin-Thom medium (7.5% glucose) of initial pH 5.0, and the culture filtrate treated with activated charcoal (British Drug Houses Ltd.) followed by elution of the charcoal with ether according to the procedure described by Brian *et al.* (1946) and Brian *et al.* (1948). On removal of the ether by distillation, the crude yellow-brown solid product obtained had, in general, a melting point of 140–155° and further purification was carried out as follows: in a typical experiment, crude gladiolic acid (5 g.) was heated under reflux with ether (500 ml.), a small quantity of amorphous insoluble material was filtered off and discarded and the cooled ethereal solution was extracted with a saturated solution of $NaHCO_3$. Cautious acidification of the yellow bicarbonate extract with dilute HCl brought down a white precipitate which was filtered off and crystallized twice from water (charcoal). The gladiolic acid thus obtained was in the form of colourless needles, m.p. 158–160° (2.5 g.), and was sufficiently pure for the preparation of derivatives and for degradative work.

Evaporation of the ether left a small bicarbonate-insoluble fraction in the form of a brown microcrystalline powder, m.p. 155°. Sublimation of this material at 100°/10⁻⁴ mm. followed by crystallization from ethanol yielded a phenolic compound, long colourless needles (12 mg.), m.p. 156–158°, soluble in NaOH solution and giving an intense $FeCl_3$ colour which was blue in both ethanol and aqueous solution. (Found: C, 67.8; H, 6.0%; mol.wt. (Rast), 169. $C_{10}H_{10}O_3$ requires C, 67.4; H, 5.7%; mol. wt., 178.)

(2) Isolation of a second acidic substance from the culture filtrate (with Mrs J. Zealley)

One particular batch of crude product (6 g.) from ether elution of the charcoal extract of the culture filtrate was found to have an abnormally low melting point (120–140°) and was only partly soluble in the normal volume of ether.

The ether-insoluble material (fraction A, 395 mg.) could not be purified by sublimation *in vacuo*, but two crystallizations from water gave a colourless crystalline acid (47 mg.), m.p. 125–130° (decomp.). The ether-soluble portion yielded gladiolic acid (2.1 g.) by extraction with $NaHCO_3$ in the usual way, but the bulked aqueous mother liquors from the recrystallization of this material gave, on concentration, colourless needles (23 mg.), m.p. 128–128° (decomp.), of an acid, identical by mixed melting-point determination with the substance obtained from fraction A. It formed colourless needles, m.p. 128–130° (decomp.), from water (found: C, 58.7; H, 5.5%; equiv., 236) and colourless prisms, m.p. 132–135° (decomp.), from ethyl acetate/benzene. (Found: C, 59.0; H, 5.3%; mol.wt. (Rast), 188 (some decomp.); equiv., 230. $C_{11}H_{12}O_5$ requires C, 58.9; H, 5.4%; mol.wt., 224; equiv., 224, monobasic acid.) This acid $C_{11}H_{12}O_5$ gave no coloration with $FeCl_3$ or with ammonia solution, sp.gr. 0.880 (cf. gladiolic acid). It was soluble in ethanol but sparingly soluble in ether. It did not sublime *in vacuo* at 125°. On solution in NaOH, acidification with concentrated HCl and warming, it readily formed a neutral lactone, m.p. 170–171°, insoluble in $NaHCO_3$. (Found: C, 64.1; H, 5.0. $C_{11}H_{10}O_4$ requires C, 64.1; H, 4.9%.) This lactone was subsequently shown to be identical by mixed melting-point determinations and comparison of the infrared absorption spectra both with deoxygladiolic acid, $C_{11}H_{10}O_4$, a reduction product of gladiolic acid (see § 8), and with the dihydrogladiolide of Raistrick & Ross (1952). On evaporation of the ethereal solution, the bicarbonate-insoluble fraction proved to be insoluble in NaOH, and gave no coloration with $FeCl_3$. On sublimation at 100°/10⁻⁴ mm. a colourless sublimate was obtained which yielded, on crystallization from water, two neutral substances the more soluble of which was identical with the lactone $C_{11}H_{10}O_4$ described above. The less soluble substance crystallized from ethanol in needles, m.p. 135–137° (8 mg.), but was not investigated further.

Subsequently, the acid $C_{11}H_{12}O_5$ was shown to be identical with the dihydrogladiolic acid of the London School of Hygiene workers by mixed melting-point determinations and by comparison of the infrared absorption spectra.

(3) Analysis and properties of gladiolic acid

For analysis the purified material, m.p. 158–160°, was recrystallized three times from water and formed long colourless needles, m.p. 160°. The analytical specimens

contained no halogen, sulphur or nitrogen and were optically inactive. (Found: C, 59.7, 59.7, 59.4, 59.6; H, 4.6, 4.7, 4.6, 4.4; OCH₃, 13.2%; mol.wt. (Rast), 212; equiv., 228, 207. C₁₁H₁₀O₅ requires C, 59.45; H, 4.5; 1OCH₃, 14.0%; mol.wt., 222.) A Kühn-Roth oxidation gave 4.1% C-Me; C₁₁H₁₀O₅ requires 6.8% for 1C-Me. Gladiolic acid was readily soluble in hot water but only sparingly in the cold. It was very soluble in acetone, dioxan, ethanol and in glacial acetic acid, sparingly soluble in CHCl₃ and ether, but only very slightly soluble in carbon tetrachloride, benzene and in light petroleum. It dissolved without charring in cold conc. H₂SO₄; however, it was observed that when conc. H₂SO₄ was added cautiously to a dilute solution of gladiolic acid in technical benzene, an intense red coloration was produced at the benzene/acid interface. Gladiolic acid was soluble in hot dilute mineral acids. It was recovered unchanged in 70% yield after 4 hr. heating under reflux in 2N-H₂SO₄ in an atmosphere of N₂, and no CO₂ or volatile acids were produced. Gladiolic acid dissolved in Na₂CO₃ and in NaHCO₃ with liberation of CO₂ and the formation of a faintly yellow solution. The same yellow coloration was also produced on solution in NaOH. The colour changes that occur when gladiolic acid is dissolved in ammonia, sp.gr. 0.880, and the solution is allowed to stand, have already been described (Brian *et al.* 1946). Solid gladiolic acid suspended in air on a spatula over 0.880 ammonia solution is rapidly attacked with the formation of a brown pasty mass; this gives a deep greenish-brown colour in ammonia and acidification of the solution with dilute HCl brings down a heavy blue-green precipitate which may be filtered off and dried. The chemical composition of this material has not been investigated.

Gladiolic acid gave no colour with FeCl₃. It did not give iodoform with KI and NaOCl and did not give a red colour with sodium nitroprusside and alkali. The hydroxamic acid test for the carboxyl group (Feigl, 1947) was positive. It did not reduce Fehling's solution or restore the colour to Schiff's reagent; it appeared to reduce ammoniacal silver nitrate in the cold, but observation of this reaction was made difficult by the deep greenish-brown colour produced when gladiolic acid is treated with ammonia. Gladiolic acid reduced KMnO₄ on standing in acetone solution at room temperature, but bromine water was not rapidly decolorized even on warming at 100°. Similarly, gladiolic acid did not appear to react with bromine at room temperature either in solution in acetic acid or in suspension in carbon tetrachloride. The nitrochromic acid test for primary and secondary alcoholic —OH groups (Fearon & Mitchell, 1932) was negative. Nevertheless, gladiolic acid contained an —OH group which was readily acetylated (§ 5). It reacted readily with phenylhydrazine and with primary aromatic amines and diamines, but the reactions were complex and simple derivatives were not formed. The *dinitrophenylhydrazones* formed orange needles from glacial acetic acid, m.p. 232° (decomp.), readily soluble in NaOH. In ethanol it gave a red colour with a drop of NaOH. (Found: C, 50.9; H, 3.3; N, 13.8. C₁₇H₁₄O₈N₄ requires C, 50.7; H, 3.5; N, 14.0%.)

Formal identification of the alkoxy substituent. Gladiolic acid (0.1 g.) and hydriodic acid, sp.gr. 1.7 (15 ml.), were maintained at 150° for 1.5 hr. in the conventional type of apparatus and the alkyl iodide produced swept out by a stream of CO₂ and absorbed in redistilled dimethylaniline. A white precipitate formed and was identified as dimethylaniline methiodide, m.p. 204° in a sealed tube, by comparison with a synthetic specimen.

(4) Reaction with alkali: formation of isogladiolic acid

(A) *With boiling 3N-NaOH.* Gladiolic acid (0.2 g.) was heated under reflux for 10 min. with 3N-NaOH (10 ml.), the yellow solution cooled and acidified with dilute HCl, and the solid which separated, filtered off and recrystallized from aqueous ethanol. Long colourless needles of *isogladiolic acid* (0.14 g.), m.p. 234°. (Found: C, 59.3, 59.1; H, 4.4, 4.8; OCH₃, 13.3%; mol.wt. (Rast), 217, 227; equiv., 218, 225. C₁₁H₁₀O₅ requires C, 59.45; H, 4.5; OCH₃, 14.0%; mol.wt., 222.) A Kühn-Roth estimation gave 3.4% C-Me; C₁₁H₁₀O₅ requires 6.8% for 1C-Me. The infrared spectrum showed

two bands in the >C=O stretching region at 1758 and 1688 cm.⁻¹. *iso*Gladiolic acid was insoluble in benzene and light petroleum and only slightly soluble in ethanol and in CHCl₃; it was only slightly soluble in hot water, i.e. much less soluble than gladiolic acid. It dissolved in NaHCO₃ solution with liberation of CO₂ and the formation of a colourless solution. *iso*Gladiolic acid (0.104 g.) suspended in water (20 ml.) neutralized 4.96 ml. 0.0928N-NaOH (equiv. 225). After the addition of a further 9.90 ml. NaOH the solution was allowed to stand at room temperature for 18 hr. and then back titrated to the phenolphthalein end point with 0.0826N-H₂SO₄. 5.46 ml. were used, corresponding to an apparent equiv. of 112. *iso*Gladiolic acid did not give the colour reactions with concentrated ammonia solution or with concentrated H₂SO₄ and benzene given by gladiolic acid. It was insoluble in boiling 2N-H₂SO₄. It had no reducing properties, and did not give derivatives either with Brady's reagent or with other reagents for the carbonyl group. It did not contain an acetyltable —OH group and was recovered unchanged (melting point and mixed melting point with starting material) after 4 hr. heating with acetic acid/acetic anhydride, 1.5 hr. heating under reflux with acetic anhydride in pyridine and 6 hr. heating under reflux with acetic anhydride and fused sodium acetate.

The *methyl ester* obtained by heating under reflux with 10% methanolic HCl for 2 hr. formed colourless needles, m.p. 142°. (Found: C, 60.6; H, 5.2. C₁₂H₁₂O₅ requires C, 61.0; H, 5.1%.) A mixed melting point with the gladiolic acid methyl ester of m.p. 140° showed a 15° depression. Like *isogladiolic acid*, methyl *isogladiolate* did not form a derivative on treatment with Brady's reagent.

(B) *With 2N-NaOH at room temperature.* Gladiolic acid (0.1 g.) in 2N-NaOH (5 ml.) was allowed to stand 24 hr. On acidification with conc. HCl, *isogladiolic acid* (90 mg.), m.p. 232°, was precipitated.

(C) *With 0.1N-NaOH.* Gladiolic acid (0.1 g.) in 0.1N-NaOH (20 ml.) was allowed to stand 30 min. at 25°. On acidification, gladiolic acid, m.p. 156–158°, was recovered unchanged.

(5) Acetylation of gladiolic acid

(A) *With acetic anhydride/acetic acid.* (i) Gladiolic acid (0.1 g.) was heated on the water bath with acetic anhydride (1 ml.) and glacial acetic acid (1 ml.) for 1 hr. The reaction was incomplete after only 30 min. heating, but periods in excess of 1 hr. gave a mixture of products (see below) and should not be employed. After cooling and pouring into ice water, the precipitated *neutral monoacetylgladiolic acid* was collected. It crystallized in colourless plates, m.p. 144°, from dilute acetic acid; colourless needles, m.p. 145°, from ethanol. (Found: C, 59.1, 58.8; H, 4.75, 4.6; OCH₃, 12.4;

CH_3CO , 18.8%; mol.wt. (Rast), 256. $\text{C}_{15}\text{H}_{18}\text{O}_6$ requires C, 59.1; H, 4.6; 10CH_3 , 11.7; $1\text{CH}_3\text{CO}$, 16.3%; mol.wt., 264.) Acetylgladiolic acid was insoluble in cold NaOH and an aqueous suspension was neutral to the British Drug Houses Ltd. Universal Indicator. It reduced ammoniacal silver solution immediately in the cold, but did not reduce Fehling's solution or restore the colour to Schiff's reagent. It gave no coloration with FeCl_3 or with 0.880 ammonia solution. It gave the deep-red colour with concentrated H_2SO_4 and benzene given by gladiolic acid, and contained a very reactive carbonyl group. The *dinitrophenylhydrazones* formed orange needles from glacial acetic acid, m.p. 245–250° (decomp.). (Found: C, 51.3; H, 4.0; N, 12.2. $\text{C}_{19}\text{H}_{18}\text{O}_8\text{N}_4$ requires C, 51.3; H, 3.6; N, 12.6%.) The *semicarbazone* crystallized from ethanol in colourless prisms, m.p. 240° (decomp.). (Found: C, 52.5, 52.5; H, 5.0, 5.2; N, 12.4, 12.5. $\text{C}_{14}\text{H}_{16}\text{O}_5\text{N}_2$ requires C, 52.3; H, 4.7; N, 13.1%.) Acetylgladiolic acid was converted into *isogladiolic acid* on hydrolysis with boiling 3N-NaOH. On hydrolysis with boiling 3N- H_2SO_4 acid, gladiolic acid was regenerated and identified by a mixed melting-point determination and by the colour reaction with ammonia.

(ii) When the heating period was extended to 4 hr. using the quantities of gladiolic acid and the acetic anhydride/acetic acid mixture given above, the main product was found to be the *triacetate of gladiolic acid hydrate*, colourless prisms from ethanol, m.p. 131°. (Found: C, 55.4, 55.9; H, 5.1, 4.9; OCH_3 , 7.6; CH_3CO , 36.5; *C-Me*, 14.8%; mol.wt. (Rast), 323. $\text{C}_{17}\text{H}_{18}\text{O}_9$ requires C, 55.7; H, 4.95; 10CH_3 , 8.5; $3\text{CH}_3\text{CO}$, 35.2; *4C-Me*, 14.8%; mol.wt., 366.) The acetyl determinations were carried out by alkaline hydrolysis followed by acidification, steam distillation and titration of the volatile acid produced. The required *C-Me* content was calculated on the basis of 100% for each CH_3CO group and 60% for the *C-Me* group already detected in gladiolic acid (see § 3) and which gives low values under the standard conditions of the estimation. The low result in the Rast determination of molecular weight is no doubt due to the instability of the compound at the melting point of camphor. The melting point of the triacetyl compound was depressed by acetylgladiolic acid.

The triacetyl compound like acetylgladiolic acid gave no colour with 0.880 ammonia solution and an intense red colour with benzene and concentrated H_2SO_4 . It was insoluble in cold NaOH. Unlike acetylgladiolic acid it did not react immediately with carbonyl reagents; but on heating at 100° for 1 hr. with excess semicarbazide acetate, *acetylgladiolic acid semicarbazone*, m.p. 240° (decomp.), was formed in low yield. It did not reduce ammoniacal silver nitrate. On hydrolysis with boiling 3N-NaOH the triacetyl compound was converted into *isogladiolic acid*.

(iii) The triacetyl compound, m.p. 131°, was also formed though in poor yield (10 mg.) when acetylgladiolic acid (0.2 g.) was heated for 6 hr. on the water bath with the acetic anhydride/acetic acid acetylating mixture (2 ml.).

(B) *With acetic anhydride and concentrated H_2SO_4 .* Gladiolic acid (0.1 g.) was suspended in acetic anhydride (1.5 ml.) and a trace of concentrated H_2SO_4 added. The gladiolic acid dissolved at once and after warming for a few minutes the solution was poured on to crushed ice and after working up in the usual way the product was crystallized from ethanol. Colourless prisms, m.p. 131°, identical with the *triacetyl compound* (§ 5A, ii, above) by a mixed melting-point determination.

(C) *With acetic anhydride and fused sodium acetate.*

Gladiolic acid (0.2 g.), acetic anhydride (2 ml.) and fused sodium acetate (0.6 g.) were heated under reflux for 4 hr., and after pouring on to crushed ice, the *acid* product obtained was crystallized from acetone in colourless needles, m.p. 280° (decomp.). (Found: C, 58.6; H, 4.55; OCH_3 , 10.2; CH_3CO , 20.7%; equiv., 282. $\text{C}_{15}\text{H}_{14}\text{O}_7$ requires C, 58.8; H, 4.6; 10CH_3 , 10.1; $1\text{CH}_3\text{CO}$, 14.1%; equiv. (monobasic acid), 306.) The molecular weight could not be determined in camphor owing to the low solubility of the material. It rapidly reduced KMnO_4 in acetone at room temperature and decolorized bromine water on warming. It did not give a derivative with Brady's reagent and ammoniacal silver nitrate was not reduced.

(6) Esterification of gladiolic acid

(A) *With methanolic HCl.* Gladiolic acid (0.1 g.) and methanol containing 10% HCl (2 ml.) were heated under reflux for 2 hr. The *methyl ester* separated during the course of the reaction and on cooling was filtered off, washed with water and recrystallized twice from methanol. It formed colourless needles, m.p. 140° (55 mg.). (Found: C, 60.7, 61.1; H, 5.3, 5.1; OCH_3 , 23.8; *C-Me* (Kühn-Roth), 7.5%; mol.wt. (Rast), 214. $\text{C}_{12}\text{H}_{12}\text{O}_6$ requires C, 61.0; H, 5.1; 2OCH_3 , 26.2; *C-Me*, 6.4%; mol.wt., 236.) The methyl ester was insoluble in water, cold NaOH and aqueous Na_2CO_3 . It gave no coloration with 0.880 ammonia solution or with conc. H_2SO_4 . With conc. H_2SO_4 and benzene an intense purple colour was produced at the interface. It did not reduce Fehling's solution, but reduced ammoniacal silver nitrate solution on warming. It contained a reactive carbonyl group. The *dinitrophenylhydrazones*, formed immediately on treatment of an ethanolic solution with Brady's reagent, was a yellow microcrystalline powder, m.p. 245–250° (decomp.). (Found: N, 12.6. $\text{C}_{18}\text{H}_{16}\text{O}_8\text{N}_4$ requires N, 13.4%.) The *phenylhydrazones* obtained on warming for a few minutes with phenylhydrazine hydrochloride in aqueous ethanol formed yellowish prisms, m.p. 160°. (Found: N, 8.5. $\text{C}_{18}\text{H}_{18}\text{O}_4\text{N}_2$ requires N, 8.6%.)

The *methyl diacetylgladiolate hydrate* was prepared as follows: The ester (0.1 g.) was suspended in acetic anhydride (2 ml.), cooled to 0° and a drop of conc. H_2SO_4 added. The ester rapidly dissolved and the clear solution was allowed to stand for 30 min. at room temperature and then poured into ice water. The product after crystallization from ethanol melted at 106°. (Found: C, 56.9; H, 5.0; OCH_3 , 16.2; CH_3CO , 28.2%; mol.wt. (Rast), 312. $\text{C}_{16}\text{H}_{16}\text{O}_8$ requires C, 56.8; H, 5.4; 2OCH_3 , 18.3; $2\text{CH}_3\text{CO}$, 25.5%; mol.wt., 338.)

The methyl ester was hydrolysed quantitatively by boiling 2N-NaOH to *isogladiolic acid*, m.p. 232°.

(B) *With ethanolic H_2SO_4 .* Gladiolic acid (0.1 g.), absolute ethanol (1 ml.) and a drop of conc. H_2SO_4 were heated under reflux for 30 min., the yellow solution poured into water and the solid product collected. By crystallization from ethanol, colourless needles, m.p. 105°, of the *ethyl ester* were obtained. (Found: C, 61.6; H, 5.7%; mol.wt. (Rast), 244, 262. $\text{C}_{15}\text{H}_{14}\text{O}_6$ requires C, 62.4; H, 5.6%; mol.wt., 250.) The ethyl ester was insoluble in Na_2CO_3 , in NaOH and in 0.880 ammonia solutions. It gave no colour with FeCl_3 . It restored the colour to Schiff's reagent, reduced ammoniacal silver solution in the cold and readily formed a precipitate with Brady's reagent. The *semicarbazone* formed colourless prisms, m.p. 190° (decomp.). (Found: C, 54.9; H, 5.7; N, 13.5. $\text{C}_{14}\text{H}_{17}\text{O}_5\text{N}_2$ requires C, 54.7; H, 5.6; N, 13.7%.)

The ethyl ester could be acetylated in the same way as the methyl ester. The ethyl ester (0.1 g.) was suspended in acetic anhydride (1 ml.) and a drop of concentrated H_2SO_4 added. After standing some hours at room temperature the solution was poured on to crushed ice and the *ethyl diacetylgladiolate hydrate* recrystallized twice from ethanol. It formed colourless prisms, m.p. 103°. A mixed melting-point determination with the starting material showed a 20° depression. (Found: C, 57.6; H, 5.4. $\text{C}_{17}\text{H}_{20}\text{O}_8$ requires C, 57.9; H, 5.6%.) *iso*Gladiolic acid was formed when ethyl gladiolate was hydrolysed with hot 3N-NaOH.

(C) *With diazomethane.* Gladiolic acid (1.0 g.) suspended in ether was treated with an excess of an ethereal solution of diazomethane and allowed to stand 2 days. After removal of the ether and diazomethane the residual gum was distilled at 5×10^{-2} mm. pressure. The distillate which passed over between 180 and 210° partly solidified and the solid portion crystallized from methanol in colourless leaflets, m.p. 105°, but the quantity obtained was too small for further work. The oil was subjected to chromatography on activated alumina using the technique of fractional elution (Shoppee & Reichstein, 1941) and shown to be a mixture of substances; the main fraction contained a small amount of nitrogen (2.7%) and was not further investigated.

(7) Oxidation of gladiolic acid, *isogladiolic acid and related compounds*

(A) *Gladiolic acid.* (i) *With potassium permanganate* $\equiv$ 1 O atom per molecule gladiolic acid in alkaline solution at room temperature. 1% KMnO_4 solution (10.5 ml.: 1.3 oxygen atoms) was added dropwise from a burette to a well stirred solution of gladiolic acid (0.14 g.) in Na_2CO_3 (15 ml.) at 20°. After filtering off the MnO_2 formed, the solution was acidified with concentrated HCl and evaporated to small bulk. On cooling a white solid crystallized, was collected, and recrystallized from hot water (substance A). Colourless needles (0.1 g.), m.p. 219°. (Found: C, 54.8, 55.6; H, 4.2, 4.5; OCH_3 , 13.7%; mol.wt. (Rast), 244; equiv., 116. $\text{C}_{11}\text{H}_{10}\text{O}_8$ requires C, 55.4; H, 4.2; IOCH_3 , 13.0%; mol.wt., 238; equiv. (dibasic acid), 119.) Substance A dissolved in NaHCO_3 with liberation of CO_2 and the formation of a colourless solution. It did not give the colour reactions with ammonia, and with benzene and H_2SO_4 given by gladiolic acid. No red coloration was produced on fusing with a crystal of phenol and a trace of conc. H_2SO_4 , diluting with water and adding NaOH. The fluorescein test was also negative. It was readily acylated under the conditions used for the acetylation of gladiolic acid (§ 5).

Substance A (50 mg.) heated 2 hr. on the water bath with acetic anhydride (1 ml.) and acetic acid (1 ml.) yielded after working up in the usual way, the *monoacetate*, colourless needles from dilute acetic acid, m.p. 199°. (Found: C, 55.8; H, 4.4%; equiv., 259. $\text{C}_{13}\text{H}_{12}\text{O}_7$ requires C, 55.7; H, 4.3%; mol.wt., 280.)

Substance A did not give an immediate precipitate on warming with Brady's reagent, but carbonyl derivatives were formed under somewhat more drastic conditions. Substance A (50 mg.) dissolved in boiling 2N-HCl was added to 2,4-dinitrophenylhydrazine (50 mg.) in 2N-HCl and the whole heated under reflux for 5 min. and then at 100° for 30 min. The *dinitrophenylhydrazone* was obtained as a yellow microcrystalline powder, m.p. 245–250° decomp., giving a deep-red solution in NaOH. (Found: C, 48.4; H, 3.1; N, 13.7. $\text{C}_{17}\text{H}_{14}\text{O}_8\text{N}_4$ requires C, 48.8; H, 3.4; N, 13.4%.)

Substance A (20 mg.) and phenylhydrazine hydrochloride (30 mg.) were heated in aqueous solution at 100° for 2 hr. On cooling, the *anhydraphenylhydrazone* separated, m.p. 190° decomp., soluble in Na_2CO_3 . (Found: C, 65.0; H, 4.7; N, 9.5%; equiv., 282. $\text{C}_{17}\text{H}_{14}\text{O}_4\text{N}_2$ requires C, 65.8; H, 4.55; N, 9.0%; equiv., 310, monobasic acid.)

The *semicarbazone* formed colourless prisms, m.p. 192° decomp. (Found: C, 48.5; H, 4.6; N, 13.2%; equiv., 154. $\text{C}_{12}\text{H}_{13}\text{O}_6\text{N}_3$ requires C, 48.8; H, 4.4; N, 14.2%; equiv., 147.5, dibasic acid.)

Substance A was recovered unchanged after boiling for 10 min. with 3N-NaOH followed by cooling and acidification. It was similarly unaffected by standing overnight with 40% aqueous KOH.

(ii) *Excess alkaline potassium permanganate at room temperature.* Gladiolic acid (1.0 g.), Na_2CO_3 (20 ml.) and 5% KMnO_4 (40 ml.) were stirred together for 12 hr.; after removal of the MnO_2 , the filtrate was acidified, the excess permanganate removed with SO_2 , and the solution concentrated to small bulk. In one experiment a small amount (70 mg.) of a colourless acid, m.p. 218°, separated and was found to be substance A, but in general inorganic material was the first to separate and the solution was evaporated to dryness and the solid product extracted with ether in a Soxhlet apparatus. Removal of the ether left a gummy solid which crystallized from ethyl acetate in colourless prisms, substance B (0.12 g.), m.p. 162°. A much better yield of substance B (0.85 g. from 1.0 g. gladiolic acid) was obtained when the solution, after acidification and destruction of the excess permanganate, was continuously extracted with ethyl acetate. (Found: C, 52.1; H, 3.9; OCH_3 , 12.5%; mol.wt. (Rast), 222; equiv., 85. $\text{C}_{11}\text{H}_{10}\text{O}_7$ requires C, 52.0; H, 4.0; IOCH_3 , 12.2%; mol.wt., 254; equiv., 85, tribasic acid.) Substance B was very soluble in water. It gave no colour with FeCl_3 , but gave strongly positive colour reactions for an *o*-phthalic acid (fluorescein and phenolphthalein tests). The melting point of 162° recorded above was unaffected by several cycles of resetting and remelting and was unchanged even after the melt had been maintained at 200° for 10 min. The infrared spectrum showed a strong band at 927 cm^{-1} absent in the spectra of substances C and D (see below). Substance B (0.12 g.) in methanol, treated with an excess of ethereal diazomethane and allowed to stand 24 hr., formed the *trimethyl ester* (90 mg.), colourless prisms from methanol m.p. 98°. (Found: C, 56.9; H, 5.85; OCH_3 , 38.3. $\text{C}_{14}\text{H}_{16}\text{O}_7$ requires C, 56.75; H, 5.45; 4OCH_3 , 41.9%.)

(iii) *Excess boiling potassium permanganate in sodium carbonate.* (a) Gladiolic acid (0.2 g.) was dissolved in Na_2CO_3 (10 ml.) and 5% KMnO_4 added dropwise to the boiling solution. At the end of 30 min. when 10.7 ml. had been added a permanent pink colour remained 5 min. after the addition of four drops of permanganate solution. After removal of the MnO_2 and acidification with conc. HCl, the solution was evaporated cautiously to dryness and the residue extracted with ether. The colourless solid obtained, substance C, m.p. 160–165° decomp., was very soluble in water and in ether. (Found: C, 49.7, 50.0; H, 3.7, 4.0; OCH_3 , 14.6%; mol.wt., 232; equiv., 82. $\text{C}_{10}\text{H}_8\text{O}_7$ requires C, 50.0; H, 3.4; OCH_3 , 12.9%; mol.wt., 240; equiv., 80, tribasic acid.) Substance C gave positive results in the usual tests for an *o*-phthalic acid. The melting point was not depressed by admixture with substance B, but the infrared spectrum showed bands at 1123 and 1143 cm^{-1} which were absent in the spectra both of substances B and D (see below).

(b) Gladiolic acid (1.0 g.) was dissolved in Na_2CO_3 (20 ml.) and oxidized with 5% KMnO_4 . The reaction was started at room temperature and when uptake of permanganate ceased, was continued by heating under reflux for 4 hr. In all, 53.0 ml. of permanganate solution were used, corresponding to approx. 5 O atoms per molecule of gladiolic acid. After removal of the MnO_2 and acidification, the solution was evaporated to small bulk and extracted with 5×10 ml. portions of ethyl acetate. Evaporation of the solvent left a colourless solid (0.2 g.), m.p. $171-181^\circ$ decomp., with gas evolution (fraction 1). The aqueous solution on standing deposited a crop of colourless crystals (0.45 g., fraction 2), insoluble in ethyl acetate, but readily soluble in water. These on heating lost water at 120° and then remelted with decomposition at 280° . The crystals did not sublime at 210° and 10^{-4} mm. Hg pressure, and a white residue remained after ignition on a porcelain tile.

Fraction 1 was sublimed at $130^\circ/10^{-4}$ mm. The sublimate (30 mg.), m.p. $152-164^\circ$ decomp. after recrystallization from ethyl acetate, was identical with substance *C* by comparison of the infrared absorption spectra. The residue was crystallized from ethyl acetate and obtained as a micro-crystalline powder, substance *D*, m.p. $175-180^\circ$ decomp., with gas evolution, resetting at 220° and remelting at 259° with further decomp. (Found on a specimen dried *in vacuo* over P_2O_5 at room temperature: C, 43.8; H, 3.7%; equiv., 78.1. $\text{C}_{11}\text{H}_{18}\text{O}_9 \cdot \text{H}_2\text{O}$ requires C, 43.5; H, 3.3%; equiv., 75.5 (tetrabasic acid). Found on a specimen dried at 100° *in vacuo* over P_2O_5 : C, 46.3, 47.3; H, 2.8, 2.95; OCH_3 , 9.9, 11.4%; mol.wt. (Rast), 304; equiv., 74.5. $\text{C}_{11}\text{H}_{18}\text{O}_9$ requires C, 46.5; H, 2.8; OCH_3 , 10.9%; mol.wt., 284; equiv., 71, tetrabasic acid.)

Substance *D* crystallized from a small volume of dilute HCl in colourless needles, m.p. 190° , with gas evolution, resetting at 210° and remelting at 259° decomp. (Found on a specimen dried 3 hr. *in vacuo* over P_2O_5 at room temperature: C, 43.5; H, 3.3. $\text{C}_{11}\text{H}_{18}\text{O}_9 \cdot \text{H}_2\text{O}$ requires C, 43.5; H, 3.3%. Found on a specimen dried 6 hr. *in vacuo* over P_2O_5 at 100° : C, 46.7; H, 2.95. $\text{C}_{11}\text{H}_{18}\text{O}_9$ requires C, 46.5; H, 2.8%.)

Substance *D* did not depress the melting point of substance *B* or substance *C*. The infrared spectrum showed a strong band at 1165 cm^{-1} and weak bands at 865 and 940 cm^{-1} , which were absent in the spectra of substances *B* and *C*. It was very soluble in water but only slightly soluble in ether. It showed positive reactions in the usual tests for an *o*-phthalic acid. It gave no coloration with FeCl_3 .

Fraction 2 was boiled for a few minutes with conc. HCl (5 ml.), adding a little water to dissolve the KCl formed. On cooling, colourless needles of substance *D* (0.15 g.), m.p. 180° , remelting at 256° (decomp.) were obtained. Extraction of the acid solution with ethyl acetate yielded a further small quantity (20 mg.) of the same product.

Substance *D* (0.5 g.) in methanol treated with an excess of ethereal diazomethane and allowed to stand 1 week formed the *tetramethyl ester*, a colourless oil (0.4 g.), b.p. $180-182^\circ/10^{-2}$ mm. (Found: C, 53.1; H, 4.8; OCH_3 , 41.5. $\text{C}_{15}\text{H}_{26}\text{O}_9$ requires C, 52.9; H, 4.7; 5OCH_3 , 45.5%.)

(iv) *With excess boiling potassium permanganate in potassium hydroxide.* Gladiolic acid (2.0 g.) in boiling *N*-KOH (25 ml.) consumed 135 ml. 5% KMnO_4 , and 0.95 g. crude substance *D*, m.p. $175-180^\circ$ decomp., was obtained by ethyl acetate extraction of the concentrated aqueous filtrate. Sublimation of this crude material at $150^\circ/10^{-3}$ mm. did not yield any substance *C*.

(v) *Attempted oxidation with periodic acid.* The method adopted was essentially that of Clutterbuck & Reuter (1935). In a preliminary experiment gladiolic acid (0.1322 g.) was dissolved in water (100 ml.), a standard approx. 0.1 *N* solution of HIO_4 (100 ml.) added and the mixture allowed to stand for 48 hr. at room temperature. At the end of this time the solution was titrated, after the addition of acid and excess KI, against standard $\text{Na}_2\text{S}_2\text{O}_3$ solution. The difference between this titre and a blank titration gave the amount of periodate consumed. In this experiment it corresponded to only 32% of the theoretical for the oxidation of 1 mol. of gladiolic acid by 1 mol. of periodic acid.

In a second experiment using 0.1277 g. gladiolic acid, the solution in 0.1 *N*- HIO_4 (100 ml.) and 3 *N*-HCl (10 ml.) was made up to 250 ml. and allowed to stand with occasional shaking at 30° . 10 ml. Portions were withdrawn at intervals and titrated in the usual way:

Time (days) ...	...	...	1	3	6	9	12	21
Percentage 1 mol. periodic acid consumed per mol. gladiolic acid			17	26	34	55	55	68

At the end of 3 weeks, concentration of the residual solution yielded 0.055 g. (42%) pure gladiolic acid, colourless needles, m.p. 156° .

Repetition of this experiment on a somewhat larger scale (0.5 g. gladiolic acid) gave very similar results.

(B) *isoGladiolic acid.* *Excess potassium permanganate in alkali* at 100° . *isoGladiolic acid* (1.1 g.) was dissolved in *N*-KOH (20 ml.), the solution heated to boiling with vigorous mechanical stirring and oxidized in the usual way with 5% KMnO_4 solution added dropwise from a burette. After the addition of 50 ml. the rate of consumption of the permanganate was reduced considerably; a further 20 ml. were then added and the boiling continued 2 hr. more. At the end of this period the excess permanganate was destroyed by the addition of methanol and the mixture cooled and the MnO_2 filtered off and washed with *N*-KOH solution. After acidification of the bulked filtrate and washings, the solution was concentrated and continuously extracted with ethyl acetate for some hours. Concentration of the ethyl acetate gave 0.57 g. of an acid, m.p. $175-180^\circ$ decomp., with gas evolution, resetting at 220° and remelting at 256° (decomp.). This behaviour on heating was unaltered by admixture with substance *D* from gladiolic acid.

(C) *Acetylgladiolic acid.* Acetylgladiolic acid (0.1 g.) was dissolved in acetone (2 ml.) and solid KMnO_4 (0.09 g.) added. After standing 1 hr. at room temperature, water (2 ml.) and saturated Na_2CO_3 (1 ml.) were added and the MnO_2 removed by filtration. The filtrate was acidified with concentrated HCl and after standing overnight at 0° the colourless crystals were filtered off and recrystallized from 50% aqueous ethanol. Colourless prisms, m.p. 213° (20 mg.), soluble in cold Na_2CO_3 solution with liberation of CO_2 . (Found: C, 55.8, 55.5; H, 4.5, 4.2%; mol.wt. (Rast), 272; equiv., 263. $\text{C}_{13}\text{H}_{22}\text{O}_7$ requires C, 55.7; H, 4.3%; mol.wt., 280.) On the addition of a single crystal of this acid to a melt maintained at 205° of the needles of the acetate of substance *A*, m.p. 199° , the whole solidified immediately and remelted at 213° . In a mixed melting-point determination the mixture melted at the higher temperature (213°).

(D) *Substance A.* (i) *With acidified potassium permanganate* at 100° . Substance *A* (0.1 g.) was dissolved by warming in 5% H_2SO_4 (5 ml.) and 1% KMnO_4 added to the boiling

solution. On cooling, unchanged substance *A* separated, identified by melting point and mixed melting point.

(ii) *With potassium permanganate in alkaline medium at room temperature.* Substance *A* (0.1 g.), Na_2CO_3 (2 ml.) and 1% KMnO_4 solution (5 ml.) were stirred at 20°. After 12 hr. all the permanganate had been used up and a further 5 ml. were added and stirring continued 12 hr. more. After removal of the MnO_2 , acidification of the filtrate and extraction with ethyl acetate, a colourless solid (70 mg.), m.p. 158–162°, was obtained, which gave no depression in a mixed melting-point determination with substance *B*. (Found: C, 52.1; H, 4.1%; equiv., 92. $\text{C}_{11}\text{H}_{10}\text{O}_7$ requires C, 52.0; H, 4.0%; equiv., 85, tribasic acid.)

(E) *Deoxygladiolic acid.* Deoxygladiolic acid (15 mg.) was dissolved in $\text{N-H}_2\text{SO}_4$ (30 ml.) at 70° and 1% KMnO_4 solution (2 ml.) added. After 10 min. the excess permanganate was removed with SO_2 and on cooling overnight at 0° the solution deposited colourless needles, m.p. 230°, identical with isogladiolic acid by mixed melting point and comparison of the infrared spectra.

(8) Reduction of gladiolic acid

(A) *With iron powder and acetic acid.* Gladiolic acid (0.5 g.), activated iron powder (1.0 g.) and 50% acetic acid (10 ml.) were heated on the steam bath for 2 hr. The dark-coloured solution was diluted with an equal volume of water and extracted with ether. Removal of the ether and residual acetic acid left a yellow pasty material (0.15 g.) which was taken up in ether and extracted with NaHCO_3 . The neutral fraction crystallized from ethanol in stout colourless needles of deoxygladiolic acid, m.p. 173° (60 mg.). The melt partly resolidified on maintaining at 180° for a few minutes. (Found: C, 63.8; H, 5.05; OCH_3 , 14.2%; mol.wt. (Rast), 215. $\text{C}_{11}\text{H}_{10}\text{O}_4$ requires C, 64.1; H, 4.9; OCH_3 , 15.0%; mol.wt., 206.) The infrared spectrum showed bands at 1758 and 1695 cm^{-1} due to >C=O groups. Deoxygladiolic acid was insoluble in cold NaOH , but readily dissolved on warming. 12.0 mg. deoxygladiolic acid were dissolved in 5.06 ml. 0.128 N-NaOH by warming on the water bath. Back titration with 0.128 N-HCl to the phenolphthalein end point required 4.62 ml., whence the apparent equivalent weight is 213. It gave a dinitrophenylhydrazone decomp. above 320° on treatment with Brady's reagent. (Found: N, 14.8. $\text{C}_{17}\text{H}_{14}\text{O}_7\text{N}_4$ requires N, 14.5%.) Deoxygladiolic acid was identical by mixed melting-point determination and comparison of the infrared spectra both with the lactone obtained from crude gladiolic acid and from dihydrogladiolic acid and with the dihydrogladiolide of the London School of Hygiene workers (§ 2). The bicarbonate-soluble fraction on acidification and extraction with ether yielded only a trace of acidic material, m.p. 125–135°.

(B) *By Clemmensen's method.* Granulated zinc (10 g.), HgCl_2 (1 g.), concentrated HCl (0.5 ml.) and water (10 ml.) were shaken together for 5 min. after which the aqueous solution was decanted and the zinc covered with water (7.5 ml.) and conc. HCl (10 ml.). Gladiolic acid (1.0 g.) was added and the mixture heated under reflux for 5 hr. The hot solution was decanted from tarry by-products and on cooling at 0° deposited a neutral lactone, a colourless, crystalline solid which was recrystallized from water (0.25 g.), m.p. 113°. (Found: C, 68.4, 68.9; H, 6.5, 6.5%; mol.wt. (Rast), 212; equiv. (by lactone titration), 188. $\text{C}_{11}\text{H}_{12}\text{O}_3$ requires

C, 68.7; H, 6.3%; mol. wt., 192.) The lactone >C=O group absorbed at 1745 cm^{-1} in the infrared. This reduction product was insoluble in cold NaOH , but was readily soluble on warming and was precipitated unchanged therefrom by dilute HCl . It showed no carbonyl reactivity. The tarry by-products were treated with ethanol and the insoluble material crystallized from acetone in colourless needles, m.p. 282–286° decomp., which could be sublimed at 250°/10⁻⁴ mm. without change of melting point (10 mg.). (Found: C, 69.1; H, 5.5. $\text{C}_{22}\text{H}_{20}\text{O}_8$ requires C, 69.6; H, 5.3%.) This substance was also neutral and non-ketonic.

(9) Demethylation of degradation products

(A) *Substance D.* Substance *D* (0.1 g.) and HBr , sp.gr. 1.49 (4 ml.), were heated under reflux for 30 min. The hydroxybenzenetetra-carboxylic acid separating on cooling was dried *in vacuo* over H_2SO_4 and crystallized from a small volume of water in colourless prisms, m.p. 302° decomp. It gave a red coloration with FeCl_3 . (Found on a specimen dried 6 hr. *in vacuo* at 100° over P_2O_5 : C, 41.7, 41.7; H, 2.9, 2.9%; equiv., 75, 73. $\text{C}_{10}\text{H}_8\text{O}_{10}$ ($\text{C}_{10}\text{H}_8\text{O}_9 \cdot \text{H}_2\text{O}$) requires C, 41.7; H, 2.8%; equiv., 72, tetrabasic acid.) It coupled very slowly with diazotized amines, but both the Millon and Gibbs tests were negative. On methylation with diazomethane in methanol the tetramethyl ester of substance *D* was formed and identified from its infrared spectrum.

(B) *isoGladiolic acid.* (a) *isoGladiolic acid* (0.2 g.) and HBr (7 ml.) were heated under reflux for 40 min. On cooling norisogladiolic acid separated and crystallized from aqueous ethanol in prisms, m.p. 254–256° decomp. (Found: C, 57.75; H, 3.9%; equiv., 196. $\text{C}_{10}\text{H}_8\text{O}_8$ requires C, 57.7; H, 3.9%; mol.wt., 208.) In the >C=O region the infrared spectrum showed a broad band with maximum at 1695 cm^{-1} and a shoulder at 1727 cm^{-1} . Norisogladiolic acid gave a beautiful reddish-purple colour with ferric chloride. It coupled immediately with diazotized *p*-nitraniline, but only very slowly with diazotized aniline. It gave a negative Millon reaction, but with the Gibbs reagent a faint blue colour developed slowly. (b) *isoGladiolic acid* (0.15 g.) and HI , sp.gr. 1.7 (6 ml.), were heated under reflux for 3 hr. in an atmosphere of N_2 , cooled, diluted with water and extracted with ether. The ethereal extract was washed successively with water, $\text{Na}_2\text{S}_2\text{O}_3$ and water, dried, and the ether removed. Norisogladiolic acid, m.p. 250–255° decomp., was obtained from the tarry residue by crystallization from ethanol (charcoal).

(C) *Gladiolic acid.* When gladiolic acid (0.1 g.) was heated under reflux with HBr (2 ml.) as described under isogladiolic acid above, the tarry product, after purification by sublimation *in vacuo*, was identified as norisogladiolic acid by a mixed melting-point determination. (Found: C, 57.6; H, 4.1. $\text{C}_{10}\text{H}_8\text{O}_8$ requires C, 57.7; H, 3.9%.)

(D) *Clemmensen reduction product* $\text{C}_{11}\text{H}_{12}\text{O}_3$. The compound (60 mg.) was heated under reflux with HBr (2 ml.) for 20 min. when a solid product began to separate. After filtration and drying over KOH , it crystallized from ethanol in needles, m.p. 158°. (Found: C, 67.2; H, 5.7%; equiv. (by lactone titration), 175. $\text{C}_{10}\text{H}_{10}\text{O}_3$ requires C, 67.4; H, 5.7%; mol.wt., 178.) The infrared spectrum showed bands at 3378 and 1745 cm^{-1} due to —OH and >C=O groups respectively. It gave an intense blue colour with FeCl_3 in

ethanol and in aqueous solution. It did not couple with diazotized *p*-nitraniline and showed negative Millon and Gibbs reactions. It was identical by mixed melting point and by comparison of the infrared absorption spectra with the phenol isolated from crude gladiolic acid (§ 1).

(E) *Deoxygladiolic acid*. Deoxygladiolic acid (30 mg.) was cautiously dissolved in HBr (2 ml.) by warming on the steam bath and then heated under reflux for a few minutes. Heating was stopped as soon as red amorphous material began to appear and the solution cooled. The amorphous precipitate was filtered off, the filtrate diluted with water (6 ml.) and extracted with ether, and the ethereal layer extracted with cold 0.1 N-NaOH. The yellow alkaline layer was acidified with concentrated HCl and extracted with ether. Removal of the ether left a solid substance purified by sublimation *in vacuo* at 150°/10⁻³ mm. followed by crystallization from ethanol affording *nordeoxygladiolic acid* as colourless plates, m.p. 205–208° decomp. (Found: C, 62.3; H, 4.7. C₁₀H₈O₄ requires C, 62.5; H, 4.2%). It readily sublimed at 200°, gave a reddish-violet FeCl₃ colour and formed a precipitate with Brady's reagent. In the infrared spectrum the >C=O groups absorbed at 1733 and 1688 cm.⁻¹.

(10) *Decarboxylation of isogladiolic acid and gladiolic acid*

(A) *isoGladiolic acid*. *isoGladiolic acid* (0.5 g.) in redistilled quinoline (7 g.) was heated in a stream of CO₂-free N₂ with a copper chromite catalyst (0.2 g.). CO₂ was rapidly evolved at a bath temperature of 220° and was swept out and absorbed in a series of wash bottles containing standard baryta. The CO₂ evolved after 2.5 hr. corresponded to 87% of 1 mol. CO₂/mol. of *isogladiolic acid* and the reaction mixture was cooled, the catalyst filtered off and the filtrate poured into excess 2 N-HCl (25 ml.). The solution was extracted with ether and the tarry ethereal extract sublimed at 200°/10⁻³ mm. The orange-coloured sublimate crystallized from ethanol in colourless needles, m.p. 244° (27 mg.). (Found: C, 65.7, 66.2; H, 4.7, 5.1; OCH₃, nil; mol.wt. (Rast), 186. C₉H₈O₃ requires C, 65.8; H, 4.9%; mol.wt., 164.) It was soluble in cold NaOH, but insoluble in NaHCO₃ and an aqueous suspension was neutral to the Universal indicator. It gave no colour with FeCl₃. The Millon reaction was negative. The infrared spectrum showed a band at 1738 cm.⁻¹ attributed to a phthalide ring >C=O group.

(B) *Gladiolic acid*. Under precisely the same conditions 95% of 1 mol. of CO₂ was evolved from gladiolic acid in 30 min. at 220°, but the tarry product obtained by ether extraction proved intractable.

(11) *Synthesis of*

4-methoxybenzene-1:2:3:5-tetracarboxylic acid (with Mrs J. Zealley)

Mesityl methyl ether. Nitromesitylene was converted in 86% yield into mesidine by hydrogenation at 25° in the presence of a Raney nickel catalyst. Diazotization of mesidine and replacement of —NH₂ by —OH (Porter & Thurber, 1921) gave mesitol which was methylated with dimethyl sulphate and caustic soda in the usual way. The mesityl methyl ether had b.p. 205–208°.

Acylation of mesityl methyl ether. (a) Powdered anhydrous AlCl₃ (13.5 g.) was added with stirring to CS₂ (20 ml.) in a

flask fitted with a mechanical stirrer, dropping funnel and reflux condenser and immersed in an ice bath, and a mixture of mesityl methyl ether (10 g.) and acetyl chloride (7.5 g.) added at such a rate that the temperature did not exceed 5°. The solution was then stirred for 2 hr. at 20° and decomposed with ice and HCl in the usual way. The oily layer was extracted with ether and after removal of the ether and CS₂ the residue was taken up again in ether and extracted with NaOH. Acidification of the aqueous alkaline layer followed by extraction with ether yielded only a trace of phenolic material. The neutral oil from the ether layer was distilled and fractions, b.p. 220–240° (2.0 g.) and 250–275° (6.4 g.), collected. The former, b.p. > 220°, consisted of unchanged mesityl methyl ether. The yield of oil, b.p. 250–275°, was not improved by carrying out the reaction at 45° after the addition of mesityl methyl ether and acetyl chloride to the AlCl₃ at 5°.

(b) Mesityl methyl ether (4.2 g.) and powdered anhydrous AlCl₃ (12 g.) in CS₂ (20 ml.) were stirred together and acetic anhydride (3.0 g.) added at room temperature. The temperature of the reaction mixture rose to 35°, and after stirring for 2 hr. the product was worked up as before. Fractions, b.p. 200–240° (0.82 g.) and 255–275° (2.65 g.), were collected.

The infrared absorption spectra of the high boiling fractions from both experiments showed two bands in the >C=O stretching region at 1760 and 1700 cm.⁻¹ respectively. The former frequency is characteristic of phenyl ester >C=O and the latter of aryl alkyl ketone >C=O (Grove & Willis, 1951).

The oil, b.p. 250–275° (4.4 g.) and 3 N-NaOH (40 ml.), were heated under reflux for 1 hr. and the oily layer remaining extracted with ether. Removal of the ether and distillation of the residual oil gave 3-methoxy-2:4:6-trimethylacetophenone, b.p. 268–270° (3.0 g.). (Found: C, 74.8; H, 8.5; OCH₃, 14.9. C₁₃H₁₆O₂ requires C, 75.0; H, 8.4; OCH₃, 16.2%.)

Acidification of the aqueous alkaline layer and repeated recrystallization from petroleum ether, b.p. 40–60°, of the solid obtained gave 3-hydroxy-2:4:6-trimethylacetophenone, m.p. 81–82° (0.4 g.). (Found: C, 74.2; H, 8.1. Calc. for C₁₁H₁₄O₃: C, 74.1; H, 7.9%.) Auwers & Mauss (1928) give m.p. 81° for 3-hydroxy-2:4:6-trimethylacetophenone. Alkaline hydrolysis of the fraction, b.p. 200–240°, in a similar manner gave mesityl methyl ether, and mesitol, melting point and mixed melting point with an authentic sample, 71°.

4-Methoxybenzene-1:2:3:5-tetracarboxylic acid. 3-Methoxy-2:4:6-trimethylacetophenone was dispersed by stirring in saturated Na₂CO₃ (20 ml.) and 5% KMnO₄ added slowly first to the warm and then to the boiling solution. The reaction was continued by heating under reflux for 4 hr. by which time 300 ml. permanganate solution had been added. The excess of KMnO₄ was destroyed by the addition of methanol, the MnO₂ filtered off and washed with Na₂CO₃ and the bulked filtrate and washings acidified to congo red with concentrated HCl and evaporated to 100 ml. Continuous extraction of this solution with ether furnished a fluffy amorphous solid (1.9 g.) which was markedly hygroscopic. It was dissolved in 2 N-H₂SO₄ (20 ml.) and 5% KMnO₄ added to the warm solution. CO₂ was evolved and when uptake of permanganate ceased, the solution was cooled, made strongly acid with concentrated HCl and continuously extracted with ethyl acetate. Removal of the solvent left

a solid product (1.12 g.), m.p. 175–180° decomp., resetting at 220° and remelting at 256° (decomp.), and identical by mixed melting point and comparison of the infrared spectrum with substance *D*, the methoxybenzenetetracarboxylic acid from gladiolic acid. For analysis, a portion of the material was crystallized from ethyl acetate and dried *in vacuo* over P_2O_5 at 100° for 12 hr. (Found: C, 46.8; H, 3.1%; equiv., 72. $C_{11}H_8O_8$ requires C, 46.5; H, 2.8%; equiv., 71, tetrabasic acid.)

4-Hydroxybenzene-1:2:3:5-tetracarboxylic acid. 4-Methoxybenzene-1:2:3:5-tetracarboxylic acid (100 mg.) was heated under reflux with hydrobromic acid for 30 min. On cooling 4-hydroxybenzene-1:2:3:5-tetracarboxylic acid separated and formed colourless prisms, m.p. 302°, decomp. from 2N-HCl. (Found: C, 42.0; H, 3.0; equiv., 72. $C_{10}H_6O_8 \cdot H_2O$ requires C, 41.7; H, 2.8%; equiv., 72, tetrabasic acid.) It was identical with the hydroxybenzenetetracarboxylic acid from gladiolic acid by mixed melting-point determinations and comparison of the infrared spectra.

SUMMARY

1. Chemical work is described showing that gladiolic acid $C_{11}H_{10}O_5$, a weakly antibacterial but strongly antifungal metabolic product of *Penicillium gladioli*, may be represented by the tautomeric structural formulae 4-methoxy-5-methyl-o-phthal-

aldehyde-3-carboxylic acid (in the keto form) and 4-formyl-3-hydroxy-7-methoxy-6-methylphthalide (in the lactol form).

2. The structures of a number of derivatives and degradation products of gladiolic acid have also been established and the synthesis of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid is reported.

3. Three impurities, an acid $C_{11}H_{12}O_5$, a lactone $C_{11}H_{10}O_4$ and a phenolic compound $C_{10}H_{10}O_3$, have been isolated from the crude gladiolic acid and their respective structural relationships to gladiolic acid established.

4. The structural relationship of the phenol $C_{10}H_{10}O_3$ to mycophenolic acid gives the gladiolic acid series of compounds a definite place in the general picture of the metabolic products of the fungi.

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