Studies in the Biochemistry of Micro-organisms

87. DIHYDROGLADIOLIC ACID, A METABOLIC PRODUCT OF *PENICILLIUM GLADIOLI* MACHACEK

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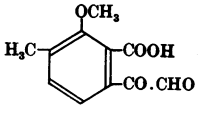
(*Received* 31 *July* 1951)

The *Penicillium gladioli* series in the subsection Fasciculata of the Asymmetrica section of species of *Penicillium* is at present represented by a single well defined species, *P. gladioli* Machacek (Raper & Thom, 1949, p. 474). Wakefield & Moore (1936), however, prefer to describe the species as *P. gladioli* McCulloch & Thom. *P. gladioli* is a common cause of the *Penicillium* rot of gladiolus corms and is occasionally responsible for serious destruction of the corms in storage.

Brian, Curtis, Grove, Hemming & McGowan (1946) showed that strains of *P. gladioli* produce a strongly antifungal and weakly antibacterial substance, gladiolic acid, $C_{11}H_{10}O_5$, the chemical properties of which 'are consistent with those that might be expected for a methoxy methyl-2-carboxyphenyl glyoxal'. Brian, Curtis & Hemming (1948) reported a detailed study of the conditions of production of gladiolic acid and of its biological properties. They showed that the acid is highly fungistatic at low pH (3·5), but that it is almost inactive at pH 7·0. Grove (1947) described a number of derivatives and breakdown products of gladiolic acid, the formation of which could best be explained by assigning to gladiolic acid the provisional structure (I).



(I)

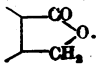
We have now found that when a strain of *P. gladioli*, known to be descended from one of the strains used by Brian *et al.* (1948) for the production of gladiolic acid, is grown on a medium, the constituents of which are qualitatively the same as, but are present in quantitatively different amounts from, those used by Brian *et al.* (1948), there is formed in place of gladiolic acid, and in good yield, a hitherto undescribed mould metabolic product. This substance, which has the empirical formula $C_{11}H_{12}O_5$ and, as will be shown later, is readily convertible into gladiolic acid *in vitro*, we propose to name dihydrogladiolic acid. We have further found that when the same strain of *P. gladioli* is grown for the same length of time (14 days) and at the same temperature (24°) on the Raulin-Thom medium containing 7·5% glucose and with initial pH 5·0, such as was used for the production of gladiolic acid by Brian *et al.* (1948), both dihydrogladiolic acid and gladiolic acid are formed in the ratio of about 3·5 parts of the former to 1 part of the latter.

Dihydrogladiolic acid, $C_{11}H_{12}O_5$, forms colourless lustrous plates, m.p. 135–136° (decomp.), or when finely ground, m.p. 132–133° (decomp.). It has no optical activity. It contains one methoxyl group and one methyl group attached to carbon. It is a strong acid dissolving at once with effervescence in aqueous sodium bicarbonate solution and in aqueous sodium hydroxide to a colourless solution. It titrates sharply to phenolphthalein as a monobasic acid. It dissolves readily in water, ethyl acetate, ethanol and acetone, less readily in ether and is almost insoluble in benzene and in light petroleum (b.p. 60–80°). An aqueous solution of dihydrogladiolic acid gives no colour with ferric chloride but readily forms a precipitate of orange needles with Brady's reagent (0·32% 2:4-dinitrophenylhydrazine in 2N-hydrochloric acid). It gives a negative Schiff's reaction and does not reduce Fehling's solution or ammoniacal silver nitrate solution on heating for a few minutes. It gives no colour when dissolved in concentrated ammonia solution (*d*, 0·88), whereas under the same conditions gladiolic acid gives a very deep-green colour changing after some time to red and finally to orange (Brian *et al.* 1946).

The following functional derivatives have been prepared. Dihydrogladiolic acid mono-2:4-dinitrophenylhydrazone, $C_{11}H_{12}O_4{:}N.NH.C_6H_3(NO_2)_2.1H_2O$, orange microrods which do not melt up to 360°; dihydrogladiolic acid mono-semicarbazone, $C_{11}H_{12}O_4{:}N.NH.CO.NH_2.1H_2O$, colourless lustrous tablets, m.p. 130° (decomp.); dihydrogladiolic acid mono-anil, $C_{11}H_{12}O_4{:}N.C_6H_5$, colourless tetragonal plates, m.p. 161·5–162·5°, these three derivatives being soluble in cold aqueous sodium bicarbonate; crude methyl dihydrogladiolate, $C_{12}H_{14}O_5$, a thick colourless gum which could not be purified and

giving in ethanolic solution an orange-yellow precipitate with Brady's reagent. When dihydrogladiolic acid is acetylated with acetic anhydride and pyridine there is formed a diacetate, $C_{15}H_{16}O_7$, m.p. 70–70·5°, as colourless needles, which, on hydrolysis with dilute sulphuric acid, give a good yield of dihydrogladiolide, $C_{11}H_{10}O_4$, m.p. 172·5–173°, as colourless needles. Dihydrogladiolide is also formed, though in much smaller yield, by sublimation of dihydrogladiolic acid in a high vacuum. Neither dihydrogladiolide nor the above dihydrogladiolide diacetate, m.p. 70–70·5°, is soluble in cold aqueous sodium hydroxide. Further, the diacetate gives no precipitate in ethanolic solution with Brady's reagent although, under the same conditions, dihydrogladiolide forms a mono-2:4-dinitrophenylhydrazone, orange tetragonal prisms from ethyl acetate which do not melt up to 320°.

The experimental evidence presented so far justifies the working hypothesis that the following groups are present in the molecule of dihydrogladiolic acid: one OCH_3 group actually estimated; one *C*-CH_3 group actually estimated; one reactive carbonyl group, probably a CHO group explaining the formation of its mono-2:4-dinitrophenylhydrazone, mono-semicarbazone and mono-anil; one COOH group accounting for the fact that dihydrogladiolic acid is a strong acid which liberates carbon dioxide from aqueous sodium bicarbonate, and which is esterified in methyl dihydrogladiolate; one CH_2OH group, vicinal to the COOH group, to account for the formation of dihydrogladiolide with the loss of one molecule of water, dihydrogladiolide thus containing the grouping

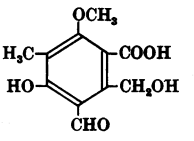


The formation of a diacetate from dihydrogladiolic acid would then involve the acetylation of the CHO group, believed to be present in dihydrogladiolide, into $CH(O.CO.CH_3)_2$. On this hypothesis the formula for dihydrogladiolic acid may be written

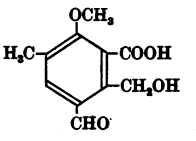
$$C_6.(H).(OCH_3).(CH_3).(CHO).(COOH).(CH_2OH) = C_{11}H_{12}O_5.$$

Dihydrogladiolic acid would then be a pentasubstituted benzene derivative, four of the substituents having C—C linkages and one of them being the OCH_3 linkage.

At this stage of the investigation of the molecular structure of dihydrogladiolic acid it became clear that there is a close similarity in the general reactions of this acid and its derivatives to those of cyclopolic acid and its corresponding derivatives. Cyclopolic acid, $C_{11}H_{12}O_6$, is a metabolic product of *P. cyclopium* Westling and a full account of the determination of its molecular structure (II) is given on p. 610 (Birkinshaw, Raistrick, Ross & Stickings, 1952*b*). The empirical formula for cyclopolic acid, $C_{11}H_{12}O_6$, differs from that of dihydrogladiolic acid, $C_{11}H_{12}O_5$, in containing one extra atom of oxygen, which may well be that contained in the phenolic group present in cyclopolic acid and accounting, on the one hand, for its intense stable purple colour with ferric chloride and, on the other hand, for the complete absence of any ferric colour with dihydrogladiolic acid. On this basis dihydrogladiolic acid might have structure (III). To test the validity of this hypothesis, which in fact proved to be correct, a number of degradation experiments was carried out.

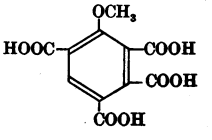


(II) Cyclopolic acid

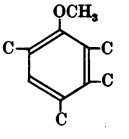


(III) Dihydrogladiolic acid

(*a*) Dihydrogladiolic acid was oxidized with hot alkaline potassium permanganate. The resulting oxidation acid, $C_{11}H_8O_9$, 4-methoxybenzene-1:2:3:5-tetracarboxylic acid of structure (IV), was obtained as colourless tetragonal prisms which on heating melted at 176–181° (decomp.), reset at about 220° and remelted at 256° (decomp.). The acid titrates sharply as a tetrabasic acid and its aqueous solution gives no colour with ferric chloride. It forms a liquid tetramethyl ester, $C_6H(OCH_3)(COOCH_3)_4$, and on demethylation gives 4-hydroxybenzene-1:2:3:5-tetracarboxylic acid, m.p. 298–299° (decomp.), an aqueous solution of which gives a stable deep orange-red colour with ferric chloride. We are greatly indebted to Mr J. F. Grove of the Imperial Chemical Industries Ltd., Butterwick Research Laboratories, Welwyn, who compared our oxidation acid with a synthetic specimen of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid prepared by himself (Grove, 1952) and with his permanganate oxidation product from gladiolic acid. He found that the three specimens were identical. It follows, therefore, that dihydrogladiolic acid contains the skeleton structure (V).



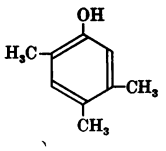
(IV)



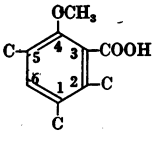
(V)

(*b*) Dihydrogladiolic acid was reduced by the Clemmensen method. The resulting reduction product, $C_{11}H_{12}O_3$, forms colourless needles, m.p. 116–116·5°, which, unlike the parent compound, are insoluble in cold aqueous sodium hydroxide—although they are soluble in the hot reagent—and

do not give in ethanolic solution any precipitate with Brady's reagent. This reduction product was heated for 5 hr. with hydriodic acid (*d*, 1·7) and a little red phosphorus. Demethylation occurred, one molecule of carbon dioxide was evolved and was estimated, and pseudocumenol, 5-hydroxy-1:2:4-trimethylbenzene of structure (VI), was formed. It was isolated as colourless needles, m.p. 71–71·5° not depressed on admixture with authentic synthetic pseudocumenol of the same melting point (for synthesis see p. 646). Its identity was confirmed by conversion into its 3:5-dinitrobenzoate, colourless needles, m.p. 179·5–180°, also not depressed on admixture with an authentic synthetic specimen. Thus, since it has been proved that dihydrogladiolic acid has the skeleton structure (V), it follows that the carboxyl group present in dihydrogladiolic acid, and evolved as carbon dioxide on heating the Clemmensen reduction product, $C_{11}H_{12}O_3$, with hydriodic acid and red phosphorus, must be in position 3 in structure (VII), since if it were in position 6, then 4-methoxybenzene-1:2:3:5-tetracarboxylic acid (IV) could not arise by the oxidation of dihydrogladiolic acid with potassium permanganate. The skeleton structure of dihydrogladiolic acid may therefore be expanded to (VII). This conclusion is confirmed by the following fact. Previous work on cyclopolic acid (Birkinshaw *et al.* 1952*b*) and on mycophenolic acid (Birkinshaw, Raistrick & Ross, 1952*a*) has shown that a carboxyl group *ortho* to a phenolic OH group or a methoxyl group is readily eliminated as carbon dioxide on heating with hydriodic acid and a little red phosphorus. A carboxyl group meta to the OH group is not, however, eliminated by this means.

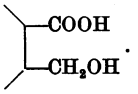


(VI)
Pseudocumenol

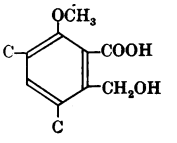


(VII)

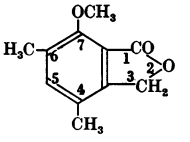
It has already been mentioned that, in order to explain the formation of dihydrogladiolide from dihydrogladiolic acid with the loss of one molecule of water, and also to explain the properties of dihydrogladiolide, it is necessary to postulate that dihydrogladiolide is the phthalide of dihydrogladiolic acid, in which case dihydrogladiolic acid must contain the grouping



If this postulate is accepted the skeleton structure of dihydrogladiolic acid may be expanded to (VIII) and structure (IX) may be deduced for its Clemmensen reduction product since it alone explains all the known properties of this substance.



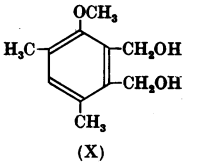
(VIII)



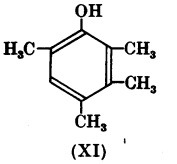
(IX)

Thus, its empirical formula is $C_{11}H_{12}O_3$, it is insoluble in cold aqueous 2N-sodium hydroxide, but dissolves on heating. It contains two methyl groups attached to carbon. It gives no precipitate with Brady's reagent. On heating with hydriodic acid and red phosphorus it would be demethylated, the phthalide ring would be slowly opened and the resulting COOH group liberated as carbon dioxide, the resulting CH_2OH group would be reduced to CH_3 and the final product would be pseudocumenol (VI). Structure (IX) for the Clemmensen reduction product, $C_{11}H_{12}O_3$, is confirmed by the fact that, although this substance gives no colour with ferric chloride, when it is demethylated but not otherwise changed the resultant product, $C_{10}H_{10}O_3$, colourless needles, m.p. 159·5–160·5°, gives a stable deep-blue colour with ferric chloride. A carboxyl group or the carbonyl group of a lactone ring ortho to a phenolic hydroxyl group would be expected to give rise to an intense colour with ferric chloride. Thus Scheussner & Voswinckel (1921) have shown that 7-hydroxy-5-methylphthalide, and Perkin & Trikojus (1926) have shown that 3-carboxy-7-hydroxy-6-methoxyphthalide, give deep-blue colours with ferric chloride. Hence, the Clemmensen reduction product $C_{11}H_{12}O_3$, is formulated as 7-methoxy-4:6-dimethylphthalide and its demethylation product, $C_{10}H_{10}O_3$, as 7-hydroxy-4:6-dimethylphthalide.

The Clemmensen reduction phthalide (IX) was further reduced with lithium aluminium hydride in ether, a process which is known to reduce lactones to diols (Adams, 1951). The resulting reduction product, a di-alcohol, $C_{11}H_{16}O_3$, was obtained in good yield as colourless needles, m.p. 97–97·5°,



(X)

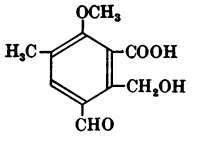


(XI)
*iso*Durenol

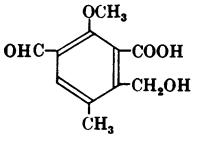
which on heating with *p*-nitrobenzoyl chloride in pyridine solution readily gave the di-*p*-nitrobenzoate, $C_{25}H_{22}O_9N_2$, as colourless needles, m.p. 157–158°. We believe the di-alcohol to be 2:3-di(hydroxymethyl)-4-methoxy-1:5-dimethylbenzene of structure (X).

The di-alcohol (X) was exhaustively reduced in glacial acetic acid solution with hydrogen and a palladium oxide catalyst. After removal of the catalyst by filtration, the reduction product still in acetic acid solution was demethylated by the addition of concentrated aqueous hydrobromic acid and boiling for 1·5 hr. The demethylated reduction product was isolated and purified and was obtained as colourless needles, $C_{10}H_{14}O$, m.p. 78–79°, not depressed on admixture with an authentic synthetic specimen of *iso*durenol, 4-hydroxy-1:2:3:5-tetramethylbenzene of structure (XI) (for synthesis see p. 647). Its identity was confirmed by conversion into its 3:5-dinitrobenzoate, colourless needles, m.p. 173·5–174·5° also not depressed on admixture with an authentic synthetic specimen. The isolation of *iso*durenol (XI) confirms the skeleton structure (V) of dihydrogladiolic acid inferred from the isolation of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid (IV) by hot permanganate oxidation of dihydrogladiolic acid (see p. 636).

It is clear that one of the two CH_3 groups in the Clemmensen reduction phthalide (IX) must have arisen by reduction of the CHO group in dihydrogladiolic acid to account for the empirical formula for (IX), i.e. $C_{11}H_{12}O_3$, and its lack of carbonyl properties. Hence the structure of dihydrogladiolic acid must be either (XII) or (XIII).



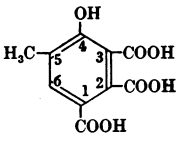
(XII)



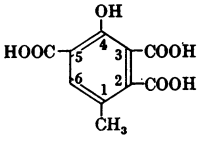
(XIII)

(*c*) A choice between (XII) and (XIII) for the structure of dihydrogladiolic acid was made as follows. Dihydrogladiolic acid was oxidized with cold alkaline potassium permanganate. The resulting oxidation product, $C_{11}H_{10}O_7$, isolated in good yield, forms colourless tetragonal microprisms, m.p. 153·5–154·5°. It contains one methyl group attached to carbon, titrates sharply as a tribasic acid, readily gives an anhydride but gives no colour with ferric chloride and no precipitate with Brady's reagent. The same tribasic acid was obtained by methylation of the oxidation acid obtained when dihydrogladiolic acid was heated at 300° in air with potassium hydroxide. This oxidation acid, $C_{10}H_8O_7$, colourless plates, m.p. 167·5–168°, which gives an intense magenta colour with ferric chloride and forms an anhydride, $C_{10}H_6O_6$, must clearly have either structure (XIV), derived from (XII), or structure (XV), derived from (XIII), the cold permanganate oxidation product of dihydrogladiolic acid being its monomethyl ether.

When the acid $C_{10}H_8O_7$ (XIV or XV) was heated with quinoline at 210–215° one molecule of carbon dioxide was evolved and the resulting decarboxylation product, $C_9H_8O_5.H_2O$, colourless tetragonal plates, m.p. 193·5–194·5° (decomp.), had the

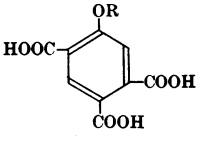


(XIV)

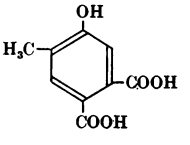


(XV)

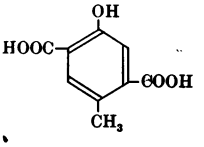
following important properties. (i) Unlike its parent substance, it gives only a pale yellow-orange colour with ferric chloride. (ii) It readily forms an anhydride, m.p. 175–175·5°. (iii) Oxidation of its methyl ether with boiling alkaline potassium permanganate gave 5-methoxybenzene-1:2:4-tricarboxylic acid (XVI, R = CH_3) identical with a synthetic specimen made by oxidizing pseudocumenol monomethyl ether (5-methoxy-1:2:4-trimethylbenzene) with boiling alkaline potassium permanganate.



(XVI)



(XVII)



(XVIII)

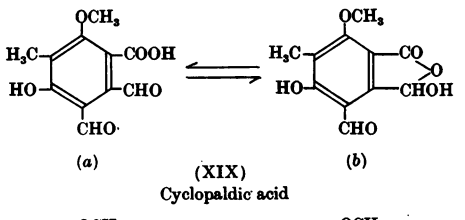
In order to obtain (XVI, R = H) from (XIV) it is necessary to remove the carboxyl group in position 3. This would lead to the decarboxylation product having structure (XVII). Similarly, to satisfy the same condition with (XV), its COOH group in position 3 must be removed, and this would lead to structure (XVIII) for its decarboxylation product. It is clear that (XVIII), being a substituted salicylic acid, would be expected to give an intense ferric colour and could not form an anhydride. On the other hand, (XVII) would not be expected to give an intense ferric colour and would readily form an anhydride. Hence, we may with confidence assign structure (XIV), i.e. 4-hydroxy-5-methylbenzene-1:2:3-tricarboxylic acid to the potassium hydroxide fusion oxidation product of dihydrogladiolic acid, and 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid to the cold potassium permanganate oxidation product. It therefore follows that dihydrogladiolic acid itself must have

structure (XII) and is in fact 5-formyl-6-hydroxymethyl-2-methoxy-3-methylbenzoic acid.

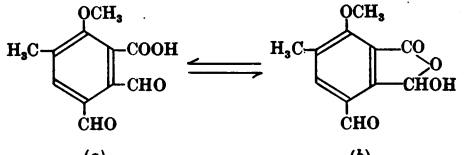
(*d*) When the neutral sodium salt of dihydrogladiolic acid is oxidized with hydrogen peroxide at 100° for 10 hr. an acid, $C_{11}H_{10}O_5$, is formed. The same acid is also obtained by treating dihydrogladiolic acid with alkaline iodine for 2 hr. at room temperature. The acid forms colourless hexagonal prisms, m.p. 216–216·5° without decomposition. *iso*-Gladiolic acid, $C_{11}H_{10}O_5$ (see p. 646), which is produced when a solution of gladiolic acid in aqueous 2N-sodium hydroxide is boiled for 10 min., forms colourless long needles, m.p. 235·5–236·5° without decomposition. A mixture of the two acids melts at 195–205°, so that they are clearly different substances. Both acids titrate sharply in the cold as monobasic acids and, on heating, as dibasic acids (mono-acid mono-lactone) and neither of them gives a precipitate with Brady's reagent. We believe that the acid formed by oxidizing dihydrogladiolic acid either with hydrogen peroxide or with alkaline iodine is either 4-carboxy-5 (or 7)-methoxy-6-methylphthalide or 7-carboxy-6-methoxy-5-methylphthalide, but we have no experimental evidence to enable us to decide which of these formulations is correct. The structure of *iso*gladiolic acid is discussed by Grove (1952).

GLADIOLIC ACID

Workers in this laboratory (Birkinshaw *et al.* 1952*b*) found that when cyclopolic acid (II) is heated with potassium periodate in dilute sulphuric acid solution cyclopaldic acid (XIX, *a* and *b*) is formed. We



(XIX)
Cyclopaldic acid

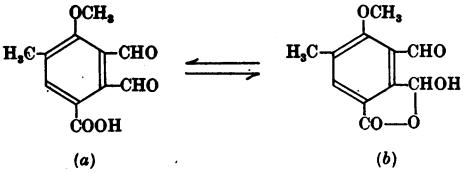


(XX)
Gladiolic acid

found that when dihydrogladiolic acid is submitted to the same reaction gladiolic acid is formed in good yield. By analogy, therefore, we arrived at structure (XX, *a* and *b*) for gladiolic acid, since this structure offers the only really feasible explanation for the transformation of dihydrogladiolic acid into gladiolic acid by the loss of two atoms of hydrogen. Our gladiolic acid was identified by direct comparison with an authentic specimen obtained from *P. gladioli* and kindly given to us by Mr J. F. Grove and also by direct comparison of specimens of *iso*-gladiolic acid obtained by heating a solution of gladiolic acid in aqueous 2N-sodium hydroxide for 10 min. We also showed that our specimen of gladiolic acid gave, on Clemmensen reduction, the same 7-methoxy-4:6-dimethylphthalide (IX) as was obtained by Clemmensen reduction of dihydrogladiolic acid.

It is thus clear that dihydrogladiolic acid bears the same structural relationship to gladiolic acid as does cyclopolic acid to cyclopaldic acid, and that cyclopolic acid is 4-hydroxydihydrogladiolic acid and cyclopaldic acid is 4-hydroxygladiolic acid. This conclusion is supported by the fact that, as shown by Birkinshaw *et al.* (1952*b*), although cyclopolic acid and dihydrogladiolic acid have little if any antifungal activity, both cyclopaldic acid and gladiolic acid have marked antifungal activity, cyclopaldic acid being about four times as potent as gladiolic acid. The fact that cyclopaldic acid is more active than gladiolic acid is doubtless due to the presence in cyclopaldic acid of a nuclear hydroxyl group ortho to an aldehyde group and the absence of this nuclear hydroxyl group in gladiolic acid.

Our work on the structure of dihydrogladiolic and gladiolic acids had reached the above stage when one of us (H.R.) was informed by Mr J. F. Grove, in a letter dated 18 January 1951, that, having obtained a methoxybenzenetetracarboxylic acid by oxidation of gladiolic acid, he had abandoned structure (I) for gladiolic acid 2 years ago and now favoured structure (XXI, *a* and *b*). Following receipt of this information we and the Imperial Chemical Industries workers on gladiolic acid interchanged our experimental results and agreed on simultaneous publication of our findings (see Grove, 1952).



(XXI)

EXPERIMENTAL

All melting points are uncorrected. Methoxyl and equivalent determinations were carried out by one of us (D.J.R.), all other micro-analyses by Weiler and Strauss, Oxford.

History of culture

The culture of *P. gladioli* Machacek used throughout this work was obtained from the Commonwealth Mycological Institute on 5 April 1950, bearing their catalogue number C.M.I. 38567, and was given our number P 251. This strain is

descended from a culture labelled *Penicillium B* which was pathogenic on, and isolated from, Gladiolus corms by Prof. F. T. Brooks, School of Botany, University of Cambridge, and was given by him to one of us (H.R.) on 22 January 1925. It was then assigned the Ardeer catalogue number Ad. 65 and was identified by Dr C. Thom of the U.S. Department of Agriculture, who wrote 'Ad. 65 is *P. gladioli* Machacek. The same name was published independently by McCulloch and Thom but later in date'. A subculture of this strain was deposited by one of us (H.R.) in 1931 with the British National Collection of Type Cultures who gave it their catalogue number N.C.T.C. 3994, and transferred it some 3 years ago, together with the rest of their collection of fungi, to the Commonwealth Mycological Institute. The same strain (N.C.T.C. 3994) was used by Brian *et al.* (1948) in their original work on gladiolic acid, although they preferred to give it the name of *P. gladioli* McCull. and Thom. Strain C.M.I. 38567 is no longer really typical of *P. gladioli* since it is floccose rather than fasciculate and does not produce sclerotia although it freely produces conidia. On the other hand, strain Ad. 65, from which it is descended and which has been kept in laboratory culture first at Ardeer and, since 1929, in this Department, produces sclerotia freely with little formation of conidia.

Cultural conditions

The following solutions were made up. (*A*) Glucose, 2625 g. in 15 l. distilled water. This solution was sterilized separately. (*B*) Tartaric acid, 53·4 g.; ammonium tartrate, 53·4 g.; $(NH_4)_2HPO_4$, 8·0 g.; $(NH_4)_2SO_4$, 8·0 g.; K_2CO_3, 3·4 g.; $MgCO_3$, 5·4 g.; $FeSO_4.7H_2O$, 0·94 g.; $ZnSO_4.7H_2O$, 0·94 g.; distilled water, 20 l. This solution was adjusted to pH 5·0 by the addition of 2 N-NaOH, and 200 ml. quantities were distributed in each of one hundred 1 l. conical flasks, plugged with cotton wool and sterilized. After sterilization, 150 ml. of glucose solution (*A*) were added to each of the 100 flasks under aseptic conditions. Each flask was then inoculated with *P. gladioli* (C.M.I. no. 38567) grown at 24° on malt agar slopes for approximately 3 weeks, a spore suspension from each slope, prepared with sterile distilled water, being used for inoculating three flasks. The hundred flasks were then incubated in the dark at 24°.

After 4 days, growth had extended over the whole surface of the culture medium which, after 9 days, was pale yellow in colour while the upper surface of the mycelial felt was white with a few dull brown patches where the mycelial reverse was exposed to the air. After 14 days, the upper surface of the mycelium was pale cream and the lower surface dull greenish yellow in colour. The culture medium was still pale yellow.

Isolation of dihydrogladiolic acid

After 14 days' incubation in the dark at 24° the culture medium from each batch of one hundred flasks was filtered through a coarse filter paper. The pH of a representative sample of the culture filtrate was determined colorimetrically and the residual glucose was determined polarimetrically. The pH of the culture filtrate was then adjusted to pH 4·0 by the addition of 2 N-NaOH (12–15 ml./l.). Activated charcoal (British Drug Houses; 5 g./l.) was added and the suspension was stirred vigorously for 3 hr. at room temperature. After standing at 0° overnight, the bulk of the supernatant liquid was decanted from the charcoal and discarded. The thick sludge remaining was drained by filtration and the damp charcoal + adsorbate was dried *in vacuo* over conc. H_2SO_4 and then over P_2O_5 for several days. The dried charcoal was added cautiously to ether (1 l.), the sludge was placed in a percolator and the charcoal was exhaustively extracted with ether under reflux. The rate of percolation was slow and several days were required for complete extraction of ether-soluble material.

The charcoal + adsorbate from batch no. 1 was fractionally extracted with ether in an attempt to separate dihydrogladiolic acid from any other metabolic products present. In the first extraction of 12 hr. *dihydrogladiolic acid* crystallized from the ethereal extract (1500 ml.) as colourless needles (4·7 g.), m.p. 128–129·5° (decomp.), which were removed by filtration and washed with a little cold ether (20 ml.). The filtrate from this extract and the ethereal solutions from subsequent fractional extractions of the charcoal were separately evaporated. The resulting gummy crystals in each case were triturated with cold ether, filtered and washed with a little ether.

In the extraction of charcoal + adsorbate from batch no. 2 only two fractional extractions with ether were carried out. In each case dihydrogladiolic acid crystallized from the ethereal extracts as colourless needles which were removed by filtration, washed with cold ether (30 ml.) and dried. The yields obtained from batches 1 and 2 and melting points are given in Table 1, column 5.

Finally, the ether mother liquors from all fractions of batches 1 and 2 were combined. On removal of the solvent there remained a yellow gum (21·5 g. from 200 flasks) containing some needles from which, however, no other pure crystalline product could be isolated. A portion of this gum, when dissolved in conc. NH_3, gave a dark-green solution indicative of the presence of gladiolic acid.

Table 1. *Details of extraction of dihydrogladiolic acid from charcoal adsorbates of culture filtrates of* P. gladioli *C.M.I.* 38567

Batch no.	Glucose by polarimeter (% w/v)	pH	Extraction time (hr.)	Dihydrogladiolic acid (g.)	Dihydrogladiolic acid M.p. (decomp.) (°)	Total extraction time (hr.)	Total dihydrogladiolic acid (g.)
1	2·20	3·2	12	4·7	128–129·5		
				1·7	120–124		
			20	4·2	125–127		
			20	3·1	122·5–125	96	16·3
			36	2·4	122–124		
			8	0·2	122–125		
2	1·18	3·2	20	6·8	129–131	95	17·7
			75	10·9	128–130		

Isolation of dihydrogladiolic and gladiolic acids from P. gladioli *grown on Raulin-Thom medium (7·5 % glucose, initial* pH *5·0)*

Fifty 1 l. conical flasks, each containing 350 ml. of Raulin-Thom medium (7·5 % glucose, initial pH 5·0), were sterilized, inoculated with *P. gladioli* C.M.I. 38567 and incubated at 24° in the dark for 14 days. The culture filtrate from these flasks was then worked up in the same way as is described on p. 640. The following results were obtained. Residual glucose by polarimeter, 0·44 %; pH 4·5; crops of crystalline material obtained after a total extraction time of 47·5 hr., 5·95 g.; m.p. 128·5–129·5°; 4·9 g., m.p. 123–127°; 1·8 g., m.p. 120–123°; 0·4 g., m.p. 123–125°; total weight of isolated solid, 13·05 g.; residual gum, 9·7 g.

Proportionate amounts of all crops were combined. The resulting crude solid (1·64 g. equivalent to one-eighth of the total solids) was crystallized from ethyl acetate (66 ml.) + benzene (264 ml.). Pure dihydrogladiolic acid (0·74 g.), m.p. 132–133° (decomp.), was thus obtained as colourless lustrous plates. The mother liquors were evaporated to dryness at 50° *in vacuo* giving a slightly gummy solid which was triturated with cold benzene (5 ml.), filtered and washed with benzene (5 ml.). The benzene filtrate and washings were shown to contain no gladiolic acid. The colourless solid (0·50 g.), m.p. 144–146°, remaining on the filter was shown as follows to consist of crude gladiolic acid. A portion of it (100 mg. equivalent to one-fifth) was fractionally sublimed in a high vacuum. The fraction subliming between 125 and 130° (50 mg.), m.p. 156·5–158°, was collected and resublimed at the same temperature giving gladiolic acid (41 mg.), m.p. 157·5–158·5°, which on admixture with an authentic specimen (ex *P. gladioli* from I.C.I.), m.p. 159–159·5°, melted at 158–159°. The mixed melt reset and remelted at 157–158°. The sublimed sample also gave the characteristic intense colour reaction with conc. NH_3. Thus, calculating on the total solids isolated from fifty flasks, the yields obtained were as follows. (*a*) Dihydrogladiolic acid, m.p. 132–133° (decomp.), 5·9 g. equiv. to 340 mg./l. of culture solution. (*b*) Gladiolic acid, m.p. 157·5–158·5°, 1·64 g. equiv. to 94 mg./l. of culture solution.

Purification of dihydrogladiolic acid

Most of the fractions of dihydrogladiolic acid separating from ether and given in Table 1 were already of a good degree of purity and were readily completely purified by one or two crystallizations from ethyl acetate (50 ml./g.) + benzene (200 ml./g.). For example, the first fraction (4·7 g.), m.p. 128–129·5° (decomp.), from batch 1, on crystallization from these solvents, gave pure dihydrogladiolic acid as colourless lustrous plates (3·8 g.), m.p. 135–136° (decomp.), or when finely ground m.p. 132–133° (decomp.).

An attempt was made to isolate other metabolic products, including gladiolic acid, from the combined ethyl acetate-benzene mother liquors from all fractions of crude dihydrogladiolic acid from batch 1. These liquors were evaporated to 150 ml. at 45° *in vacuo*. Crude dihydrogladiolic acid (2·0 g.), m.p. 128–129° (decomp.), separated and was collected by filtration. The filtrate was evaporated at 50° *in vacuo* giving a gummy solid, trituration of which with cold benzene (10 ml.) gave further crude dihydrogladiolic acid (2·6 g.), m.p. 116–120° (decomp.), from which, or from the evaporated benzene mother liquors, no other pure compound could be isolated by fractional crystallization from water. Finally, sublimation of the crude dihydrogladiolic acid, m.p. 116–120° (decomp.) in a high vacuum at 140°, at which temperature gladiolic acid readily sublimes, gave a sublimate, m.p. 150–155° depressed to 134–138° on admixture with gladiolic acid, m.p. 159–159·5°. The sublimate probably consisted of crude dihydrogladiolide since its melting point was not depressed on admixture with pure dihydrogladiolide, m.p. 172–172·5° (see p. 642).

General properties of dihydrogladiolic acid

Dihydrogladiolic acid forms colourless lustrous plates which melt at 135–136° or, when finely ground, at 132–133°. It melts with effervescence and loss of water to a clear straw-coloured liquid which does not reset on cooling. (Found: C, 58·9, 58·9; H, 5·3, 5·1; OCH_3, 13·9; *C*-CH_3, 5·5; equiv. by titration, 223. $C_{11}H_{12}O_5$ requires C, 58·9; H, 5·4; $1OCH_3$, 13·9; $1C$-CH_3, 6·7 %; equiv. titrating as a monobasic acid, 224.) A 1·5 % solution in methanol shows no optical activity when viewed through a 2 dm. tube. The acid dissolves readily in water, ethyl acetate, ethanol and acetone, is slightly soluble in ether, but is almost insoluble in benzene and light petroleum (b.p. 60–80°). It dissolves at once with effervescence in cold aqueous $NaHCO_3$ and in aqueous 2 N-NaOH to a colourless solution. Its aqueous solution gives the following reactions: (*a*) no colour with $FeCl_3$; (*b*) a flocculent precipitate of orange needles with Brady's reagent (0·32 % 2:4-dinitrophenylhydrazine in aqueous 2 N-HCl); (*c*) a negative reaction with Schiff's reagent; (*d*) does not reduce Fehling's solution or ammoniacal $AgNO_3$ even on boiling for a few minutes; (*e*) with bromine water slowly gives a turbidity insoluble in excess of the reagent even on standing for several hours at room temperature. The solid acid gives no colour on solution in conc. aqueous NH_3 (*d*, 0·880) (cf. gladiolic acid), but dissolves in cold conc. H_2SO_4 to a yellow solution which becomes a deeper yellow on warming.

Functional derivatives

Dihydrogladiolic acid mono-2:4-dinitrophenylhydrazone. Brady's reagent (40 ml.) was added to a solution of dihydrogladiolic acid (63 mg.) in hot water (10 ml.). The flocculent precipitate of orange microneedles which rapidly formed was removed by filtration, washed with water and dried, wt. 119 mg. Crystallization from ethanol gave *dihydrogladiolic acid mono-2:4-dinitrophenylhydrazone* as orange microrods which on heating commenced to shrink at 198° and decomposed as the temperature was raised, but did not melt up to 360°. (Found: C, 48·2; H, 4·3; N, 13·2. $C_{17}H_{16}O_8N_4.1H_2O$ requires C, 48·3; H, 4·3; N, 13·3 %.) The water of crystallization could not be removed except by heating at a high temperature when obvious decomposition occurred. The compound dissolves slowly in cold aqueous $NaHCO_3$ giving a yellow solution. It gives a red Neuberg reaction (solution in cold ethanolic NaOH) confirming a mono-derivative.

Dihydrogladiolic acid mono-anil. A solution of dihydrogladiolic acid (100 mg.) in aqueous acetone (80 %; 2·5 ml.) was warmed, and aniline (0·1 ml.) was added. The colourless tetragonal prisms, formed after standing overnight at room temperature, were removed by filtration, washed with aqueous acetone and dried, wt. 93 mg., m.p. 161·5–162·5°. Crystallization from aqueous methanol gave *dihydrogladiolic acid mono-anil* as glistening colourless tetragonal plates of the same melting point. (Found: C, 67·9; H, 5·4; N, 4·2;

OCH_3, 10·3. $C_{17}H_{17}O_4N$ requires C, 68·2; H, 5·7; N, 4·7; $1OCH_3$, 10·4%.) The compound is readily soluble in methanol, but only slightly soluble in water. It dissolves slowly in cold aqueous $NaHCO_3$ and rapidly in cold 2N-NaOH.

Dihydrogladiolic acid mono-semicarbazone. A solution of dihydrogladiolic acid (45 mg.) in hot water (10 ml.) was mixed with a solution of semicarbazide hydrochloride (26 mg.) and anhydrous sodium acetate (23 mg.) in water (0·5 ml.), and heated at 100° for 5 min. After standing overnight at room temperature the clear solution deposited lustrous colourless tablets of *dihydrogladiolic acid mono-semicarbazone* which were collected, washed with water and dried, wt. 36 mg., m.p. 130° (decomp.) with shrinking and softening from 120°. Crystallization from water, in which the compound is readily soluble, effected no change of melting point. (Found: C, 48·2; H, 5·7; N, 13·8. $C_{12}H_{15}O_5N_3$. $1H_2O$ requires C, 48·2; H, 5·7; N, 14·0%.) The compound is soluble in aqueous $NaHCO_3$. An aqueous solution of this compound and a methanolic solution of the mono-anil both give precipitates of orange microneedles with Brady's reagent, though much more slowly than dihydrogladiolic acid itself under the same conditions. This is probably due to acid hydrolysis of the two compounds to their constituents.

Methyl dihydrogladiolate. Excess ethereal diazomethane was added to dihydrogladiolic acid (250 mg.). The acid dissolved slowly with gentle effervescence. After standing overnight, the solution was filtered. On removal of the solvent there remained a thick colourless gum (265 mg.) which did not solidify on standing. It could not be crystallized and did not sublime or distil on heating in a high vacuum at 150°. Crude *methyl dihydrogladiolate* is insoluble in N-NaOH. (Found, on sample dried in a high vacuum at room temperature: OCH_3, 24·5. $C_{12}H_{14}O_5$ requires $2OCH_3$, 26·1%.) An ethanolic solution of crude methyl dihydrogladiolate gives an orange-yellow precipitate with Brady's reagent.

Acetylation of dihydrogladiolic acid. Formation of dihydrogladiolide diacetate. A solution of dihydrogladiolic acid (30 mg.) in pyridine (0·1 ml.) and acetic anhydride (0·1 ml.) was held for 18 hr. at room temperature. Addition of water precipitated a colourless oil which slowly solidified, wt. 35 mg., m.p. 65·5–67·5°. Crystallization from aqueous methanol gave *dihydrogladiolide diacetate* as colourless needles, m.p. 70–70·5°. (Found: C, 58·5; H, 5·2; OCH_3, 10·2. $C_{12}H_{16}O_7$ requires C, 58·4; H, 5·2; $1OCH_3$, 10·1%.) The diacetate is insoluble in 2N-NaOH. Its ethanolic solution gives no precipitate with Brady's reagent on standing for 0·5 hr. Under the same conditions both dihydrogladiolic acid and dihydrogladiolide give orange precipitates with Brady's reagent.

Dihydrogladiolide. (*a*) *From dihydrogladiolide diacetate.* The diacetate (360 mg.) was heated under reflux for 4 hr. with 2N-H_2SO_4 (7 ml.). On cooling, the separated light-brown solid was collected, wt. 230 mg., m.p. 157–160°. Two crystallizations from aqueous methanol gave *dihydrogladiolide* as colourless needles, m.p. 172·5–173°, which reset and remelted at the same temperature. The trivial name dihydrogladiolide is given to this substance despite the fact that it is not strictly accurate nomenclature. (Found: C, 64·3; H, 5·1; OCH_3, 15·3. $C_{11}H_{10}O_4$ requires C, 64·1; H, 4·9; $1OCH_3$, 15·1%.) It is insoluble in N-NaOH. Its ethanolic solution gives an immediate gelatinous orange precipitate with Brady's reagent.

(*b*) Dihydrogladiolic acid (70 mg.) was heated in a high vacuum at 140° for 15 min. and gave a sublimate of colourless tetragonal microprisms (9 mg.), m.p. 171–171·5° not depressed on admixture with dihydrogladiolide, m.p. 172·5–173° prepared by method (*a*).

Dihydrogladiolide mono-2:4-dinitrophenylhydrazone. Brady's reagent (40 ml.) was added to a solution of dihydrogladiolide (30 mg.) in ethanol (200 ml.). The rapidly formed gelatinous orange precipitate was collected (45 mg.) and crystallized from ethyl acetate, giving *dihydrogladiolide mono-2:4-dinitrophenylhydrazone* as small orange tetragonal prisms which decomposed, but did not melt up to 320°. (Found: C, 52·7; H, 3·6; N, 14·1. $C_{17}H_{14}O_7N_4$ requires C, 52·8; H, 3·7; N, 14·5%.) The compound is slightly soluble in acetic acid, ethanol and ethyl acetate, and moderately soluble in pyridine. Its ethanolic solution gives a red colour in the Neuberg reaction confirming a mono-derivative.

Oxidation products of dihydrogladiolic acid

(*a*) *Oxidation of dihydrogladiolic acid with cold alkaline* $KMnO_4$. *Isolation of 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid, methyl ether of* (*XIV*)

An aqueous solution of $KMnO_4$ (2% (w/v); 18 ml. equiv. to 3·8 atoms O) was added to a solution of dihydrogladiolic acid (200 mg.) in N-KOH (4 ml.). The mixture was held at room temperature overnight. The residual $KMnO_4$ was destroyed by the addition of $NaHSO_3$. MnO_2 was removed by filtration and washed with water. The filtrate and washings were acidified with HCl, saturated with NaCl and extracted with ethyl acetate (4 × 20 ml.). The water-washed extract was dried over Na_2SO_4 and evaporated *in vacuo* at 45–50° giving a pale-buff coloured solid (202 mg.), m.p. 154–155·5° (decomp.). Crystallization from ethyl acetate-benzene-light petroleum (b.p. 60–80°) gave *4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid* as colourless tetragonal microprisms, m.p. 153·5–154·5° (decomp.) (see also p. 644). (Found, on sample dried at 100° *in vacuo*: C, 51·9; H, 4·2; OCH_3, 12·0; *C*-CH_3, 7·5; equiv. by titration, 84. $C_{11}H_{10}O_7$ requires C, 52·0; H, 4·0; $1OCH_3$, 12·2; 1*C*-CH_3, 5·9%; equiv. titrating as a tribasic acid, 85.) An aqueous solution of the acid gives no colour with $FeCl_3$ and no precipitate with Brady's reagent.

The acid (30 mg.) was heated at 120–125° in a high vacuum giving a sublimate of colourless needles (22 mg.), m.p. 163–165°, which on crystallization from benzene-light petroleum (b.p. 60–80°) gave the *anhydride of 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid* as colourless needles, m.p. 165–166·5°. (Found: C, 56·1; H, 3·7; OCH_3, 13·1; equiv. by titration, 79. $C_{11}H_8O_6$ requires C, 55·9; H, 3·4; $1OCH_3$, 13·1%; equiv. titrating as a tribasic acid (mono-acid mono-anhydride), 79.)

(*b*) *Oxidation of dihydrogladiolic acid with hot alkaline* $KMnO_4$. *Isolation of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid* (*IV*)

A solution of dihydrogladiolic acid (0·45 g.) in 0·1 N-NaOH (25 ml.) was heated under reflux, and powdered $KMnO_4$ (1·48 g., equiv. to 7·5 atoms O) was added gradually over 1·5 hr. The heating was continued for a further 20 min. Excess $KMnO_4$ was destroyed with $NaHSO_3$ and MnO_2 was removed by filtration. The filtrate and water washings were adjusted to pH 2 with HCl, and $CaCl_2$ (0·2 g.) was added. The small precipitate of calcium oxalate which

formed overnight was removed by filtration and discarded. The filtrate was acidified with conc. HCl (2 ml.), saturated with NaCl, and extracted with ethyl acetate (5 × 15 ml.). Removal of the solvent *in vacuo* from the dried (Na_2SO_4) extract gave a colourless solid (0·45 g.), m.p. 182–185° (decomp.), which, on crystallization from ethyl acetate-benzene-light petroleum (b.p. 60–80°), gave *4-methoxybenzene-1:2:3:5-tetracarboxylic acid* as colourless tetragonal microprisms (0·36 g.), which on heating shrink at 177°, soften at 180° and melt with slight effervescence at 182·5–184·5° (decomp.). (Found, on sample dried at 100° in a high vacuum: C, 46·7; H, 3·0; OCH_3, 11·1; equiv. by titration, 72. $C_{11}H_8O_9$ requires C, 46·5; H, 2·9; $1OCH_3$, 10·9%; equiv. titrating as a tetrabasic acid, 71.) The acid is very soluble in water, methanol and acetone, soluble in ethyl acetate and ether and almost insoluble in benzene. Its aqueous solution gives no colour with $FeCl_3$. All attempts to form a homogeneous anhydride failed.

Mr J. F. Grove of the Imperial Chemical Industries Ltd., Butterwick Research Laboratories, found the above specimen to melt in his apparatus at 176–181° (decomp.), resetting and remelting at 256° (decomp.), and to be identical with a synthetic specimen of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid and with the same acid prepared by the permanganate oxidation of gladiolic acid (see Grove, 1952).

The acid (60 mg.) was methylated by treatment with an excess of ethereal diazomethane, when an immediate vigorous effervescence took place. Excess diazomethane was removed by distillation and the residual ethereal solution was washed with aqueous $NaHCO_3$ and water and dried over Na_2SO_4. Removal of the solvent gave a very pale-yellow gum (72 mg.) which could not be solidified or crystallized. It was purified by fractional distillation in a high vacuum in a 'cold-finger' apparatus. The small amount of oil distilling below 100° was rejected and the *tetramethyl ester of 4-methoxybenzene-1:2:3:5-tetracarboxylic acid* was obtained as a colourless viscous oil (50 mg.). (Found: C, 53·3; H, 5·0; OCH_3, 45·8. $C_{15}H_{16}O_9$ requires C, 52·9; H, 4·7; $5OCH_3$, 45·6%.) The ester is very soluble in ether and methanol and sparingly soluble in benzene.

The acid (80 mg.) was heated under reflux for 0·5 hr. with HI (*d*, 1·7; 4 ml.) and a little red P which was then removed by filtration from the hot solution. The filtrate, on cooling to 0°, deposited clusters of small blades (53 mg.) which were collected by filtration on a sintered-glass filter and washed with a little ice-cold conc. HCl. Crystallization of this product from acetone-benzene gave *4-hydroxybenzene-1:2:3:5-tetracarboxylic acid* as colourless tetragonal microprisms, which on heating shrink at 290°, soften at 296°, and melt with vigorous effervescence at 298–299°. (Found, on sample dried at 110° in a high vacuum: C, 44·3; H, 2·5. $C_{10}H_6O_9$ requires C, 44·4; H, 2·2%.) An aqueous solution of the acid gives a stable deep orange-red colour with $FeCl_3$. The acid proved to be identical with a synthetic specimen supplied by Mr J. F. Grove, i.e. same ferric reaction, no depression of melting point on mixing.

(*c*) *Oxidation of dihydrogladiolic acid with* (*i*) H_2O_2 *and* (*ii*) *alkaline* I_2. *Isolation of a carboxymethoxymethylphthalide*

(i) *With* H_2O_2. Dihydrogladiolic acid (150 mg.) was converted into its sodium salt by the addition of 0·1 N-NaOH (6·7 ml.). H_2O_2 ('100 vol.'; 0·4 ml.) was added and the solution was maintained at 100° for 10 hr. The solution was cooled, acidified with conc. HCl and held at room temperature overnight. The resulting colourless prisms were collected, wt. 85 mg., m.p. 209–212°. Sublimation at 155–175° in a high vacuum and two subsequent crystallizations from water gave *a carboxymethoxymethylphthalide* as large colourless hexagonal prisms, m.p. 216–216·5°, which reset and remelted at the same temperature. (Found: C, 59·4; H, 4·5; OCH_3, 14·0; titration equiv. cold, 217, hot, 110. $C_{11}H_{10}O_5$ requires C, 59·4; H, 4·5; $1OCH_3$, 14·0%; equiv. titrating as a monobasic acid, 222, as a dibasic acid (mono-acid mono-lactone), 111.) The acid dissolves with effervescence in aqueous $NaHCO_3$. Its aqueous solution gives no precipitate with Brady's reagent.

(ii) *With alkaline* I_2. 0·1 N-I_2 (25 ml.) and 0·1 N-NaOH (50 ml.) were added to a solution of dihydrogladiolic acid (112 mg.) in water (20 ml.). After 2 hr. at room temperature titration with aqueous $Na_2S_2O_3$ of an acidified portion (10 ml.) showed the absorption of 2·10 equiv. of $I_2 = 1·05$ atoms O. The remainder of the reaction solution (85 ml.) was acidified with 2 N-H_2SO_4 (5 ml.), and the liberated I_2 was removed by the addition of a little $NaHSO_3$. The colourless solution was saturated with NaCl and extracted with ethyl acetate (3 × 30 ml.). Removal of the solvent from the dried (Na_2SO_4) extract at 40° *in vacuo* gave slightly yellow microprisms (85 mg.), m.p. 206–208°. The crystals were purified by sublimation in a high vacuum at 150°, followed by one crystallization from water giving a carboxymethoxymethylphthalide as large colourless prisms, m.p. 216–216·5° not depressed on admixture with the H_2O_2 oxidation product (see § (i) above). The mixed melt reset and remelted at the same temperature.

The above phthalide (25 mg.) was methylated in the usual way with dimethyl sulphate (0·04 ml.), acetone (5 ml.) and K_2CO_3 (50 mg.). The methylated product (20 mg.), m.p. 132·5–134°, on crystallization from aqueous methanol gave *a carboxymethoxymethoxymethylphthalide* as thick colourless needles, m.p. 135–135·5°. (Found: C, 60·7; H, 5·0; OCH_3, 26·3. $C_{12}H_{12}O_5$ requires C, 61·0; H, 5·1; $2OCH_3$, 26·3%.) This ester is insoluble in cold aqueous 2 N-NaOH.

Degradation products of dihydrogladiolic acid

(*a*) *Clemmensen reduction of dihydrogladiolic acid. Isolation of 7-methoxy-4:6-dimethylphthalide* (*IX*). Granulated zinc (20 g.) was immersed in an aqueous solution of $HgCl_2$ (5%; 40 ml.) for 1 hr. A suspension of the washed amalgam (20 g.) was heated under reflux with conc. HCl (20 ml.), and a solution of dihydrogladiolic acid (0·65 g.) in warm water (20 ml.) was added gradually over 0·75 hr. Heating was continued for a further 0·5 hr. when the solution no longer gave a precipitate with Brady's reagent. On cooling, the slightly gummy product was mechanically separated from the amalgamated zinc, filtered, washed with water and dried, wt. 0·225 g., m.p. 109–111°. Sublimation in a high vacuum at 90–95° and subsequent crystallization from light petroleum (b.p. 60–80°), in which it is readily soluble, gave the *phthalide* as colourless needles, m.p. 116–116·5°, which reset and remelted at the same temperature. (Found: C, 68·5; H, 6·2; OCH_3, 16·3; *C*-CH_3, 11·9 equiv. 1·52 *C*-CH_3 groups. $C_{11}H_{12}O_3$ requires C, 68·7; H, 6·3; $1OCH_3$, 16·1; 2 *C*-CH_3, 15·6%.) The phthalide is insoluble in cold 2 N-NaOH, but dissolves in hot 2 N-NaOH. Its ethanolic solution gives no colour with $FeCl_3$ and no precipitate with Brady's reagent.

The above phthalide (44 mg.) was demethylated by heating under reflux for 20 min. with HI (*d*, 1·7; 3 ml.) and a little

red P. Hot water (10 ml.) was added and P was removed by filtration. On cooling, slightly brown needles (24 mg.), m.p. 155–156°, were deposited and were collected. They were sublimed in a high vacuum at 120° and subsequently crystallized from aqueous methanol giving the demethylation product, *7-hydroxy-4:6-dimethylphthalide*, as colourless needles, m.p. 159·5–160·5°, which reset and remelted at the same temperature. (Found: C, 67·4; H, 5·7. $C_{10}H_{10}O_3$ requires C, 67·4; H, 5·7%.) Its ethanolic solution gives a stable, deep blue colour with ethanolic $FeCl_3$. Addition of water causes deepening, but no alteration in the shade, of the blue colour.

(*b*) *Prolonged action of* HI *and red* P *on the Clemmensen reduction phthalide* $C_{11}H_{12}O_3$. *Isolation of pseudocumenol* (*5-hydroxy-*1:2:4-*trimethylbenzene*) (*VI*). The above Clemmensen reduction phthalide, $C_{11}H_{12}O_3$ (90 mg.) was refluxed in a stream of CO_2-free N_2 with HI (*d*, 1·7; 4 ml.) and red P (0·3 g.). CO_2, estimated by absorption in 0·2N-$Ba(OH)_2$, was evolved as follows. After 0·75 hr., 0·33 mol. equiv.; after 5·0 hr., 0·94 mol. equiv. During the reaction, oily globules collected in the lower part of the condenser system and on cooling readily solidified into needles. Long needles also separated from the cooled reaction liquid. The whole was extracted with ether (3 × 15 ml.) after dilution with water, and the ethereal extract was washed successively with water, aqueous $Na_2S_2O_3$, aqueous $NaHCO_3$ and again with water. On removal of the solvent from the ethereal extract there remained long needles, wt. 60 mg., m.p. 65·5–66·5°. This product was purified by sublimation *in vacuo* in a 'cold-finger' apparatus and two subsequent crystallizations from water giving pseudocumenol (5-hydroxy-1:2:4-trimethylbenzene) (VI) as fine colourless glistening needles, m.p. 71–71·5° not depressed on admixture with authentic synthetic pseudocumenol of the same melting point (for synthesis see p. 646). (Found, on sample dried over P_2O_5: C, 79·0; H, 9·2. Calc. for $C_9H_{12}O$: C, 79·4; H, 8·9%.) An aqueous solution of the above pseudocumenol gives with $FeCl_3$ a transient pale-blue colour fading to colourless within a few minutes with the rapid formation of a turbidity and, finally, with the formation of a colourless precipitate.

A solution of the above pseudocumenol (10 mg.) and 3:5-dinitrobenzoyl chloride (17·5 mg.) in pyridine (0·25 ml.) was refluxed for 0·5 hr. After cooling, N-H_2SO_4 (3 ml.) was added, and the resulting pale-yellow needles were separated by filtration, washed with water and triturated with 0·5N-NaOH (3 ml.). Subsequent filtration and washing with water gave needles, wt. 14 mg., m.p. 176·5–177·5°, which, on sublimation in a high vacuum at 140–145°, gave *pseudocumenol* 3:5-*dinitrobenzoate* as colourless needles, m.p. 179·5–180° not depressed on admixture with an authentic synthetic specimen of the same melting point (for synthesis see p. 646). The mixed melt reset and remelted at the same temperature. (Found: C, 58·3; H, 4·4; N, 8·3. $C_{16}H_{14}O_6N_2$ requires C, 58·2; H, 4·3; N, 8·5%.)

(*c*) *Further reduction of the Clemmensen reduction phthalide* $C_{11}H_{12}O_3$. *Isolation of* 2:3-*di*(*hydroxymethyl*)-4-*methoxy*-1:5-*dimethylbenzene* (*X*). Powdered lithium aluminium hydride (0·35 g.) was refluxed for 2 hr. with ether (30 ml.), dried over a Na-K liquid alloy. Refluxing was continued while a solution of the Clemmensen reduction phthalide (0·245 g.) in dry ether (25 ml.) was added over 20 min. with constant stirring. The suspension was refluxed for a further 20 min. and was then cooled to 0°. The stirring was continued and water (5 ml.), followed by 2N-H_2SO_4 (25 ml.) was cautiously added. The ethereal layer was separated and the aqueous layer was re-extracted with ether (3 × 25 ml.). Removal of the solvent from the combined dried (Na_2SO_4) extracts gave long colourless needles (0·23 g.), m.p. 88–88·5°, resetting and remelting at 95·5–96°. Crystallization from light petroleum (b.p. 60–80°), in which the compound is readily soluble, gave 2:3-*di*(*hydroxymethyl*)-4-*methoxy*-1:5-*dimethylbenzene* as fine glistening colourless needles, m.p. 97–97·5°, which rapidly reset and remelted at 96·5–97°. (Found: C, 67·0; H, 8·3; OCH_3, 16·0. $C_{11}H_{16}O_3$ requires C, 67·3; H, 8·2; 1OCH_3, 15·8%.)

The *di*-p-*nitrobenzoate* of the above substance was prepared by heating a solution of it (10 mg.) in pyridine (0·15 ml.) with *p*-nitrobenzoyl chloride (20 mg.) at 100° for 0·5 hr. The product was recovered in the usual way and on crystallization from methanol gave the di-*p*-nitrobenzoate as small colourless needles, m.p. 157–158°. (Found: C, 60·4; H, 4·4; N, 5·5. $C_{25}H_{22}O_9N_2$ requires C, 60·7; H, 4·5; N, 5·7%.)

(*d*) *Catalytic reduction of the di-alcohol* (*X*). *Isolation of* iso*durenol* (*XI*) (*4-hydroxy-*1:2:3:5-*tetramethylbenzene*). A solution of the above 2:3-di(hydroxymethyl)-4-methoxy-1:5-dimethylbenzene (X) (0·3 g.) in glacial acetic acid (20 ml.) was shaken with H_2 in the presence of palladium oxide catalyst (0·2 g.) supplied by Johnson, Matthey & Co. Ltd. Two further additions, each of 0·1 g., of the catalyst were made during the reduction before the uptake of H_2 finally ceased after 3·25 hr. 65 ml. H_2 (corrected for catalyst absorption and at N.T.P.) were absorbed; theoretical for absorption of 2 mol. H_2, 69 ml. The catalyst was removed by filtration and washed with a little glacial acetic acid. HBr (constant b.p.; 10 ml.) was added to the acetic acid filtrate and washings and the whole was refluxed for 1·5 hr. to demethylate the reduction product. The clear reddish reaction liquors were then adjusted to about pH 6 by the addition of aqueous 10N-NaOH and the oily solid which thus separated was extracted with ether (2 × 40 ml.). The ether extract was washed with water and dried (Na_2SO_4). Removal of the solvent gave a gum which rapidly solidified to long slightly brown needles (0·21 g.), m.p. 72–74·5°, smelling faintly of acetic acid. This crude product was distilled in steam and gave colourless solidified globules (0·14 g.), m.p. 72–74·5°, which were collected and crystallized twice from aqueous methanol. *iso*Durenol (0·06 g.) was thus obtained as long glistening colourless needles, m.p. 78–79°, resetting on cooling and remelting at the same temperature. Admixture with authentic synthetic *iso*durenol (see p. 647), m.p. 77·5–78·5°, gave no depression of melting point, the mixture melting at 78–78·5°, resetting and remelting at 77·5–78·5°. (Found, on sample dried over P_2O_5: C, 79·7; H, 9·3. Calc. for $C_{10}H_{14}O$: C, 80·0; H, 9·4%.)

The reduction *iso*durenol (20 mg.) was heated at 110–120° with 3:5-dinitrobenzoyl chloride (31 mg.) in pyridine (0·3 ml.). The product was recovered in the usual way and crystallized from methanol giving iso*durenol* 3:5-*dinitrobenzoate* as colourless needles (18 mg.), m.p. 173·5–174·5°, which reset and remelted at 171–173°. Admixture with an authentic synthetic specimen, m.p. 173·5–174·5° (see p. 647) gave no depression of melting point, the mixed melt resetting and remelting at 171–172°. (Found: C, 59·4; H, 4·6; N, 8·2. $C_{17}H_{16}O_6N_2$ requires C, 59·3; H, 4·7; N, 8·1%.)

Potash fusion of dihydrogladiolic acid

Isolation and identification of 4-*hydroxy*-5-*methylbenzene*-1:2:3-*tricarboxylic acid* (*XIV*). A mixture of dihydrogladiolic acid (1·0 g.), KOH (5·0 g.) and water (1 ml.) was heated

in air at 160° in a Wood's metal bath. The melt was raised during 15 min. to 300° and maintained at this temperature for a further 10 min. The cooled, light brown, melt was dissolved in water (25 ml.), filtered, and the filtrate was acidified with conc. HCl. The resulting cream solid precipitate (0·86 g.) was collected. It consisted essentially of a crude potassium salt which is only slightly soluble in water, but dissolves immediately in 2N-Na_2CO_3. It was dissolved in hot water (50 ml.), adjusted to pH 5 with 2N-NaOH, acidified with excess HCl and extracted with ethyl acetate (4 × 25 ml.). Removal of the solvent from the dried (Na_2SO_4) extract by evaporation *in vacuo* at 50° gave a light-brown solid (0·41 g.). This substance, on crystallization from ethyl acetate-benzene, gave *4-hydroxy-5-methylbenzene*-1:2:3-*tricarboxylic acid* as colourless lustrous plates, m.p. 167·5–168° (decomp.). (Found, on sample dried at 100° in a high vacuum: C, 50·2; H, 3·8. $C_{10}H_8O_7$ requires C, 50·0; H, 3·4%.) The acid is very soluble in water, methanol and ethyl acetate, and is almost insoluble in benzene. It dissolves immediately with effervescence in aqueous $NaHCO_3$. Its aqueous solution gives no precipitate with Brady's reagent, but gives a stable intense magenta colour with $FeCl_3$. On methylation it gives 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid identical with the acid, m.p. 153·5–154·5° (decomp.), obtained by cold $KMnO_4$ oxidation of dihydrogladiolic acid (see p. 642).

The KOH fusion acid (30 mg.) was heated at 155° in a high vacuum giving a sublimate of small tetragonal plates (17 mg.), m.p. 146·5–148°, which on crystallization from benzene, gave the *anhydride of 4-hydroxy-5-methylbenzene*-1:2:3-*tricarboxylic acid* as small colourless hexagonal prisms, m.p. 146·5–147°, which reset and remelted at 145–146·5°. (Found: C, 54·2; H, 2·6. $C_{10}H_6O_6$ requires C, 54·1; H, 2·7%.) The anhydride dissolves slowly in cold water. It dissolves rapidly with effervescence in aqueous $NaHCO_3$ to a deep-yellow solution which fades to a colourless solution within a few minutes. Its ethanolic solution gives a deep-magenta colour with ethanolic $FeCl_3$, the colour becoming more intense on the addition of water.

In a quantitative experiment, the KOH fusion acid, 4-hydroxy-5-methylbenzene-1:2:3-tricarboxylic acid, (205 mg.) was heated with quinoline (0·6 ml.) at 210–215° in a stream of CO_2-free N_2. CO_2, absorbed in standard baryta, was evolved as follows. After 5 min., 0·42 mol. equiv.; 20 min., 0·84 mol. equiv.; 30 min., 0·92 mol. equiv.; 45 min., 0·96 mol. equiv. In a preparative experiment, the acid (644 mg.) was heated with quinoline (3 ml.) at 210° for 25 min. 2N-HCl (30 ml.) was added, the solution was saturated with NaCl and extracted with ethyl acetate (4 × 25 ml.). Removal of the solvent from the washed and dried (Na_2SO_4) extract gave yellow gummy needles (440 mg.) which were purified by two sublimations at 125–150° in a high vacuum, followed by crystallization from ethyl acetate-benzene-light petroleum (b.p. 60–80°) giving *4-hydroxy-5-methylphthalic anhydride* as fine colourless needles (190 mg.), m.p. 175–175·5°, which reset and remelted at practically the same temperature. (Found: C, 60·4; H, 3·5. $C_9H_6O_4$ requires C, 60·7; H, 3·4%.) The anhydride is of low solubility in cold water, but dissolves readily on warming. It is readily soluble in ethyl acetate and methanol and almost insoluble in benzene and light petroleum (b.p. 60–80°). It dissolves readily in saturated aqueous $NaHCO_3$ to a yellow solution which quickly fades to colourless. Its aqueous solution gives a pale yellow-orange colour with $FeCl_3$.

The above anhydride (25 mg.) was boiled for 2 min. with water (0·5 ml.). The colourless solution was evaporated to dryness *in vacuo* over P_2O_5 and the residue was crystallized from ethyl acetate-benzene giving *4-hydroxy-5-methylphthalic acid* (XVII) as colourless lustrous tetragonal plates (19 mg.), m.p. 193·5–194·5° (decomp.). The compound softened slightly at 122°, partially melted at 142°, reset by 150° and then melted sharply at 193·5–194·5° with effervescence. When the acid was dried at 100° *in vacuo* no initial softening occurred. (Found, on sample dried at 100° in a high vacuum: C, 50·7; H, 4·9. $C_9H_8O_5$, $1H_2O$ requires C, 50·5; H, 4·7%.) Its aqueous solution gives a pale yellow-orange colour with $FeCl_3$.

A mixture of 4-hydroxy-5-methylphthalic anhydride (75 mg.), dimethyl sulphate (0·4 ml.), acetone (10 ml.) and K_2CO_3 (100 mg.) was refluxed for 1·5 hr. The solvent was removed and the residue was saponified with methanolic N-NaOH (10 ml.) for 0·5 hr. Methanol was removed, the residue was dissolved in water (10 ml.) and acidified with HCl. The resulting pale-yellow needles were collected (69 mg.) and crystallized twice from water giving 4-methoxy-5-methylphthalic acid as pale-yellow needles, m.p. 180–180·5° (decomp.). (Found: C, 57·2; H, 4·7; OCH_3, 14·8. Calc. for $C_{10}H_{10}O_5$: C, 57·1; H, 4·8; $1OCH_3$, 14·8%.)

The above acid (30 mg.) was refluxed for 0·5 hr. at 140° with acetic anhydride (0·9 ml.). The solvent was removed *in vacuo* and the residue was sublimed at 90–95° in a high vacuum giving *4-methoxy-5-methylphthalic anhydride* as colourless plates (23 mg.), m.p. 158·5–159° unchanged on crystallization from light petroleum (b.p. 60–80°), and resetting and remelting at the same temperature. (Found: C, 62·4; H, 4·4; OCH_3, 16·3. $C_{10}H_8O_4$ requires C, 62·5; H, 4·2; $1OCH_3$, 16·2%.)

A boiling solution of 4-methoxy-5-methylphthalic acid (83 mg.) in 0·1N-NaOH (10 ml.) was oxidized with $KMnO_4$ (0·14 g., equiv. to 3·4 atoms O) added gradually over 1·25 hr. The oxidation product was isolated as described on p. 642 and was recovered as a colourless solid (66 mg.), m.p. 210–212·5° (decomp.). It was crystallized from acetone-benzene giving 5-methoxybenzene-1:2:4-tricarboxylic acid (XVI, R = CH_3) as colourless tetragonal microprisms, m.p. 214–216·5° (decomp.) with shrinking from 204° and softening at 210°. The melting point was undepressed on admixture with an authentic synthetic specimen of the same melting point (for synthesis see p. 646). (Found: C, 50·3; H, 3·5; OCH_3, 12·9. Calc. for $C_{10}H_8O_7$: C, 50·0; H, 3·4; $1OCH_3$, 12·9%.) The acid is readily soluble in water, methanol and acetone and is almost insoluble in benzene.

Meldrum & Kapadia (1932) synthesized an acid which they claimed to be 4-hydroxy-5-methylphthalic acid, m.p. 244–245°. Their evidence for its structure is ambiguous. No ferric reaction was described and the methyl ether and anhydride were not prepared. Charlesworth, Rennie, Sinder & Yan (1945), who accepted Meldrum & Kapadia's orientation, synthesized what they claimed to be 4-methoxy-5-methylphthalic acid and described it as colourless crystals. m.p. 166·5–167° (decomp.). No derivatives of this acid were reported.

Gladiolic acid

Action of potassium periodate on dihydrogladiolic acid. Isolation of gladiolic acid

In a quantitative experiment a solution of dihydrogladiolic acid (200 mg.) in water (15 ml.), aqueous KIO_4 (approx. 0·12N; 50 ml.) and 2N-H_2SO_4 (5 ml.) was held at room temperature for 24 hr. KI was then added to a

measured portion and the liberated I_2 was titrated with 0·1 N-$Na_2S_2O_3$. No KIO_4 had, however, been utilized and dihydrogladiolic acid (190 mg.) was recovered after extraction with ethyl acetate. The recovered acid gave no colour with conc. aqueous NH_3 indicating the absence of gladiolic acid.

A vigorous reaction occurred, however, on boiling. A solution of pure dihydrogladiolic acid (0·5 g.), KIO_4 (0·525 g.) and N-H_2SO_4 (25 ml.) was boiled for 15 min. The solution turned brown and crystals of I_2 were evolved. On cooling, fine colourless needles (0·30 g.), m.p. 140–143°, separated and were collected. Crystallization from water (10 ml.) gave gladiolic acid as fine colourless needles (0·24 g.), m.p. 157–157·5° raised to 159–159·5° by sublimation in a high vacuum at 130° and not depressed on admixture with authentic gladiolic acid of the same melting point. The mixed melt reset and remelted at 157·5–158°. (Found: C, 59·5; H, 4·8; OCH_3, 14·2; equiv. by titration, 221. Calc. for $C_{11}H_{10}O_5$: C, 59·4; H, 4·5; 1OCH_3 14·0%; equiv. titrating as a monobasic acid, 222.) We are indebted to Mr J. F. Grove for the authentic specimen of gladiolic acid from *P. gladioli* and also for a specimen of *iso*gladiolic acid (see next section). The specimen of gladiolic acid prepared from dihydrogladiolic acid gave the following reactions, previously recorded by the Imperial Chemical Industries Ltd. workers as typical of gladiolic acid. An aqueous solution of the acid gives a gelatinous orange precipitate with Brady's reagent and reduces ammoniacal $AgNO_3$ on heating. When the acid is dissolved in conc. aqueous NH_3 (*d*, 0·88) a green-brown colour is initially formed and rapidly darkens to a very deep brown-green colour. After 1 hr. the colour changes to deep brown, gradually changing to orange-red over 18 hr. and to orange over 3 days. Under the same conditions cyclopaldic acid gives initially a brown colour rapidly changing to deep purple and slowly fading to orange with a yellow fluorescence (Birkinshaw *et al.* 1952*b*).

Action of alkali on gladiolic acid. Isolation of iso*gladiolic acid*

A solution of gladiolic acid (44 mg., from dihydrogladiolic acid) in aqueous 2 N-NaOH (10 ml.) was refluxed for 10 min. when a test portion of the solution no longer gave a precipitate with excess of Brady's reagent. The solution was then acidified with HCl giving rapid separation of colourless needles which were collected (wt. 38 mg.), m.p. 235·5–236·5°. Crystallization from hot water (25 ml.), in which the compound is sparingly soluble, gave *iso*gladiolic acid as fine long colourless needles (32 mg.) of the same melting point. Admixture with authentic *iso*gladiolic acid, m.p. 234–235°, gave no depression of melting point, the mixed melt resetting and remelting at 232·5–234°. (Found: C, 59·5; H, 4·4; OCH_3, 14·1; equiv. by titration in ethanol, cold 219, hot 114. Calc. for $C_{11}H_{10}O_5$: C, 59·4; H, 4·5; 1OCH_3, 14·0%; equiv. titrating as a monobasic acid, 222, as a dibasic acid, i.e. mono-acid mono-lactone, 111.) Unlike gladiolic acid, *iso*gladiolic acid dissolves in conc. aqueous NH_3 to a colourless solution and its aqueous solution gives no precipitate with Brady's reagent.

Clemmensen reduction of gladiolic acid. Isolation of 7-methoxy-4:6-dimethylphthalide (*IX*)

A solution of gladiolic acid (200 mg., from dihydrogladiolic acid) in hot water (12 ml.) was added during 0·5 hr. to mercury-amalgamated zinc (10 g.) heated under reflux with conc. hydrochloric acid (10 ml.). The heating was continued for a further 0·5 hr. when a test portion no longer gave a precipitate with Brady's reagent. The aqueous liquors were cooled to 30° and decanted from the amalgamated zinc and from some oily material which had separated. The decanted liquid was cooled to 0° and the colourless solid which separated was collected (wt. 45 mg.), m.p. 106–109°. Sublimation at 75–80° in a high vacuum gave 7-methoxy-4:6-dimethylphthalide as thick colourless needles (35 mg.), m.p. 115·5–116° not depressed on admixture with the phthalide, m.p. 116–116·5°, obtained by Clemmensen reduction of dihydrogladiolic acid (see p. 643).

Syntheses

(*a*) *Pseudocumenol* (*VI*). Pseudocumenol was synthesized from pseudocumenesulphonic acid (1:2:4-trimethylbenzene-5-sulphonic acid) according to the method of Reuter (1878), who employed the method of Jacobsen (1878) for the preparation of xylenols from xylenesulphonic acids. A mixture of pseudocumene sulphonic acid (1:2:4-trimethylbenzene-5-sulphonic acid) (15 g.), m.p. 112°, supplied by British Chemicals and Biologicals Ltd., Genatosan Division (Fine Chemicals), KOH (32 g.) and water (5 ml.) was heated with constant stirring in a nickel crucible over a naked flame. The contents rapidly set to a lumpy mass. At approximately 300°, the mass melted to a brown liquid and the melt was maintained at this temperature for 10 min. After cooling, water (500 ml.) and conc. HCl (75 ml.) were added, and the whole was extracted with ether (2 × 150 ml.). The brown ethereal extract was washed successively with water, aqueous $NaHCO_3$ and water. On removal of the solvent from the dried (Na_2SO_4) ethereal extract there remained long needles (3·0 g.) which were purified first by distillation in steam giving colourless needles (2·1 g.), m.p. 62·5–65°, followed by two crystallizations from water. Pseudocumenol was thus obtained as long glistening colourless needles, m.p. 71–71·5°, which gave the same $FeCl_3$ reaction as did the pseudocumenol obtained by degradation of the Clemmensen reduction phthalide (see p. 644). (Found, on sample dried over P_2O_5: C, 79·5; H, 9·1. Calc. for $C_9H_{12}O$: C, 79·4; H, 8·9%.) There are three possible monohydroxy derivatives of pseudocumene (1:2:4-trimethylbenzene). (*a*) Pseudocumenol (5-hydroxypseudocumene), the melting point of which, given by a number of authors, ranges from 70 to 72°; (*b*) 6-hydroxypseudocumene, m.p. 95° (Jacobsen, 1886); (*c*) 3-hydroxypseudocumene, m.p. 62° (Morgan & Pettet, 1934).

A portion of the above synthetic pseudocumenol was converted into its 3:5-dinitrobenzoate by the same method as is described on p. 644. Crystallization from methanol gave *pseudocumenol 3:5-dinitrobenzoate* as colourless needles, m.p. 179·5–180°, which reset and remelted at the same temperature. (Found: C, 58·3; H, 4·3; N, 8·2. $C_{16}H_{14}O_6N_2$ requires C, 58·2; H, 4·3; N, 8·5%.)

(*b*) *5-Methoxybenzene-1:2:4-tricarboxylic acid* (*XVI*, R = CH_3). A mixture of pseudocumenol (0·5 g.), dimethyl sulphate (1 ml.), acetone (15 ml.) and K_2CO_3 (0·6 g.) was refluxed for 1·5 hr. The solvent was removed and the residue was shaken vigorously for 15 min. with aqueous 0·5 N-KOH (30 ml.). The insoluble gum was extracted with ether (2 × 25 ml.). Removal of the solvent from the dried (Na_2SO_4) ethereal extracts gave crude pseudocumenol methyl ether as a colourless gum (0·44 g.) which was not further purified. A suspension of this gum in aqueous

0·1 N-NaOH (50 ml.) was refluxed, and powdered $KMnO_4$ (3·8 g.) was added in portions over 6 hr. Excess $KMnO_4$ was destroyed with $NaHSO_3$, and MnO_2 was removed by filtration and washed with water. The filtrate and washings were adjusted to pH 2 with HCl, and $CaCl_2$ (0·5 g.) was added. The resulting precipitate of calcium oxalate (0·19 g.) was filtered after 18 hr. and discarded. The filtrate was acidified with conc. HCl (5 ml.), saturated with NaCl and extracted with ethyl acetate (4 × 30 ml.). Removal of the solvent from the extract *in vacuo* at 45° gave an almost colourless solid (0·17 g.) which was crystallized twice from acetone-benzene-light petroleum (b.p. 60–80°) giving 5-methoxybenzene-1:2:4-tricarboxylic acid as colourless tetragonal microprisms, m.p. 214–216·5° (decomp.). (Found: C, 49·8; H, 3·4; OCH_3, 12·7; equiv. by titration, 81. Calc. for $C_{10}H_8O_7$: C, 50·0; H, 3·4; $1OCH_3$, 12·9%; equiv. titrating as a tribasic acid, 80.) Buehler, Spees & Sanguinetti (1949) give the melting point of 5-methoxybenzene-1:2:4-tricarboxylic acid as 224–225° with effervescence.

(*c*) iso*Durenol*, 4-*hydroxy*-1:2:3:5-*tetramethylbenzene* (*XI*). *iso*Durenesulphonic acid (1:2:3:5-tetramethylbenzene-4-sulphonic acid) was prepared essentially as described by Smith & Cass (1932). *iso*Durene (15 g.), supplied by British Chemicals and Biologicals Ltd., Genatosan Division (Fine Chemicals), was shaken for 10 min. at room temperature with conc. H_2SO_4 (30 ml.). The brownish solution was then poured, with stirring, into crushed ice (30 g.) surrounded by an ice bath. The precipitated solid was removed by filtration on a sintered-glass filter, pressed and dried on a porous plate, wt. 28 g., m.p. 72–76°. A solution of the crude product (14 g.) in water (35 ml.), cooled in ice, was saturated with gaseous HCl. The precipitated solid was collected and air-dried giving *iso*durenesulphonic acid (11 g.) as a nearly colourless solid, m.p. 75–79°. (Smith & Cass give m.p. 79° for pure *iso*durenesulphonic acid.)

*iso*Durenol was prepared by fusion with KOH of the above *iso*durenesulphonic acid by the same method as was used for the preparation of pseudocumenol (see syntheses, § *a*). The crude *iso*durenol obtained by steam distillation was purified by repeated crystallization from aqueous methanol giving pure *iso*durenol as long glistening colourless needles, m.p. 77·5–78·5°, which reset on cooling and remelted at the same temperature. (The melting point of *iso*durenol given by a number of authors ranges from 79 to 81°.) (Found, on sample dried over P_2O_5: C, 79·9; H, 9·5. Calc. for $C_{10}H_{14}O$: C, 80·0; H, 9·4%.)

A portion of the above *iso*durenol (20 mg.) was heated at 110–120° for 0·5 hr. with 3:5-dinitrobenzoyl chloride (30 mg.) in pyridine (0·3 ml.). The product was recovered in the usual way and was crystallized from methanol giving iso*durenol* 3:5-*dinitrobenzoate* as colourless needles (26 mg.), m.p. 173·5–174·5°, resetting and remelting at 171·5–172°. (Found: C, 59·1; H, 4·7; N, 8·2. $C_{17}H_{16}O_6N_2$ requires C, 59·3; H, 4·7; N, 8·1%.)

SUMMARY

1. A hitherto undescribed mould metabolic product, dihydrogladiolic acid, has been isolated from culture filtrates of *Penicillium gladioli* Machacek grown on a modified Raulin-Thom solution.

2. Dihydrogladiolic acid, $C_{11}H_{12}O_5$, forms colourless lustrous plates, m.p. 135–136° (decomp.). It is believed to be 5-formyl-6-hydroxymethyl-2-methoxy-3-methylbenzoic acid. A number of its functional derivatives and degradation products have been described.

3. Dihydrogladiolic acid is readily oxidized *in vitro* to gladiolic acid by heating with potassium periodate in dilute sulphuric acid solution. Gladiolic acid is believed to be 5:6-diformyl-2-methoxy-3-methylbenzoic acid, or its tautomer.

4. Cyclopolic and cyclopaldic acids from *P. cyclopium* Westling (Birkinshaw *et al.* 1952*b*) are thus 4-hydroxydihydrogladiolic acid and 4-hydroxy gladiolic acid respectively.

One of us (D. J. R.) wishes to thank the New Zealand Defence Science Corps for financial support. We also wish to thank the workers in the Imperial Chemical Industries Ltd., Butterwick Research Laboratories, The Frythe, Welwyn, Herts, for their helpful co-operation.

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