CLVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLV. THE METABOLIC PRODUCTS OF *PENICILLIUM CHARLESII* G. SMITH. IV. *l*- γ -METHYLTETRONIC ACID, WITH OBSERVATIONS ON THE FORMATION AND STRUCTURE OF RAMIGENIC AND VERTICILIC ACIDS.

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It was recently shown [Clutterbuck *et al.*, 1934] that *Penicillium Charlesii* G. Smith, when grown on nutrient solutions containing glucose, produces two new polysaccharides, mannocarlose [Haworth *et al.*, 1935] and galactocarlose, together with a number of new organic acids. The polysaccharides were isolated by precipitation from the evaporated metabolism solution with acetone, and the acids by solvent extraction of the filtrate from the polysaccharides, after removal of the acetone. Of these acids, carolic acid $C_8H_{10}O_4$ (I), carolinic acid $C_9H_{10}O_6$ (II), carlic acid $C_{10}H_{10}O_6$ (III), and carlosic acid $C_{10}H_{12}O_6$ (IV) have been shown [Clutterbuck *et al.*, 1935, 1, 2] to have the following molecular constitutions:

In addition to these four acids, two further acids, ramigenic acid $C_{16}H_{20}O_6$ and verticilic acid $C_{26}H_{32}O_{12}$ ($C_{26}H_{30}O_{11} \cdot H_2O$), were also isolated in small amounts from the ether and chloroform extracts, using both Czapek-Dox and Raulin-Thom media.

During our investigation of the constitutions of carolic and carolinic acids, it was found that both acids on bromination gave *d*- α -bromo- γ -methyltetronic acid $C_5H_5O_3Br$, which on catalytic reduction gave *l*- γ -methyltetronic acid

$C_5H_6O_3$ (V). We have found that one of the most characteristic properties of *l*- γ -methyltetronic acid is the ease of its condensation with acetone. Thus on keeping a mixture of *l*- γ -methyltetronic acid and acetone in dilute aqueous solution at the ordinary temperature for 2–3 hours, especially in presence of HCl, or on boiling the mixture for a few minutes in absolute alcoholic solution, a practically quantitative yield of isopropylidene-bis- γ -methyltetronic acid (VI) is obtained.

If either of the above experiments is allowed to proceed for a longer time, a second molecule of acetone undergoes condensation and a beautifully crystalline acid, acetonylisopropylidene-bis- γ -methyltetronic acid (VII) is formed. This acid has now been shown to be identical with ramigenic acid.

Both of the above condensation reactions are strictly analogous to those obtained by Wolff and Schimpff [1901] with tetronic acid and acetone.

Further, we have found that verticillic acid (1 mol.) on standing overnight in dilute HCl, or more rapidly in acetic acid solution at room temperature is hydrolysed and gives 1 mol. of ramigenic acid and 2 mols. of *l*- γ -methyltetronic acid:

From its general properties, the structure of anhydrous verticillic acid is therefore almost certainly given by formula VIII.

In view of the fact that in the early work the polysaccharide fraction had been separated by precipitation with acetone (alcohol had been avoided because

slight esterification of the metabolic acids occurred) it appeared a reasonable supposition that verticillic and ramigenic acids were artefacts arising from *l*- γ -methyltetronic acid by reaction with acetone during the separation of the polysaccharide fraction. That this view is correct has now been proved by repeating the metabolism experiments exactly as in our first experiments but replacing acetone with alcohol as a means of precipitation of the polysaccharide fraction. The combined ether extracts of the alcohol- and polysaccharide-free metabolism fluid then gave an oil from which *l*- γ -methyltetronic acid was isolated in a yield of the same order as would be expected from the previously obtained yields of ramigenic and verticillic acids, assuming that these acids were formed by reaction of 2 and 4 mols. respectively of *l*- γ -methyltetronic acid with acetone. Further, ramigenic and verticillic acids were entirely absent, while, on the other hand, carolic, carolinic, carlic and carlosic acids were isolated from the ether and chloroform extracts of the metabolism solution by methods similar to those previously employed, and in comparable yields.

Carolic, carolinic, carlic, carlosic and *l*- γ -methyltetronic acids should therefore be regarded as true metabolism products, whilst the ramigenic and verticillic acids of our earlier experiments must now be regarded as artefacts arising from *l*- γ -methyltetronic acid by condensation with the acetone used for precipitation of the polysaccharide fraction.

During this work a small amount of synthetic γ -methyltetronic acid was required for comparison with our natural product. In our earlier paper, the method of Benary [1911] was used for its synthesis. This method, although repeated several times under varying conditions, always gave only a very small yield of γ -methyltetronic acid and a new synthesis was therefore elaborated. Ethyl propionylacetate was first synthesised by a Grignard condensation of ethyl iodide and ethyl cyanoacetate with subsequent hydrolysis and was then brominated under conditions which gave the α - γ -dibromo-derivative of this ester. This compound on heating *in vacuo* readily lost ethyl bromide with ring closure and formation of α -bromo- γ -methyltetronic acid, $C_5H_5O_3Br$, which on catalytic reduction was converted into *dl*- γ -methyltetronic acid, $C_5H_6O_3$:

The acid obtained was in every way identical with the acid synthesised by Benary's method. Like the latter it did, however, give a slight depression of M.P. with the *l*-variety of this acid, M.P. 115° obtained by degradation of carolic and carolinic acids.

EXPERIMENTAL.

Earlier experiments.

In our previously described experiments, the polysaccharide fraction was separated from the evaporated metabolism solution by precipitation with acetone, the mother-liquors from which were concentrated to 600 ml., acidified with concentrated HCl and extracted several times first with ether and subsequently with $CHCl_3$.

The material extracted with ether, on grinding with a small volume of $CHCl_3$, filtering and washing with $CHCl_3$, gave a crystalline powder consisting in the

Czapek-Dox experiments of carlosic and carolinic acids, and in the Raulin-Thom experiments of carolinic and verticillic acids. The CHCl_3 filtrates from these acids, after removal of solvent, gave syrups which in both cases were ground with water. The fraction soluble in water was extracted with CHCl_3 and gave a yellow oil which slowly crystallised and consisted chiefly of carolic acid, whilst the material insoluble in water gave, on standing, crystals of ramigenic acid, and the residual syrups on neutralising with NaOH and adding HgCl_2 gave a precipitate of the mercuric salt of verticillic acid.

The material extracted with CHCl_3 was in the first extract almost pure carolic acid and in the remaining extracts carlic acid.

Present experiments.

In the present experiments the initial procedure was the same as described above except that absolute alcohol replaced acetone as the polysaccharide precipitant. After filtering off the polysaccharide fraction and concentrating *in vacuo* at 40–45° to remove alcohol, the mother-liquors were extracted as before with ether and chloroform.

The yellow syrups obtained by ether extraction of the concentrated metabolism solutions using Raulin-Thom and Czapek-Dox medium respectively should (according to our earlier work) contain carolinic acid, some carolic acid ramigenic and verticillic acids, and from Czapek-Dox medium carlosic acid in addition. This fraction also should contain most of the *l*- γ -methyltetronic acid if this acid were present. Of all these acids only *l*- γ -methyltetronic, ramigenic and verticillic acids give insoluble mercuric salts. In the present series of experiments therefore the ether-extracted material was divided in the case of each medium into two equal portions. One portion was neutralised with NaOH and treated with a large excess of saturated HgCl_2 . The precipitate consisted almost entirely of the mercuric salt of *l*- γ -methyltetronic acid, ramigenic and verticillic acids being entirely absent. The second portion of the ether-extracted material was examined exactly as in our earlier experiments for the presence of carolinic, carlosic and carolic acids.

The material extracted with CHCl_3 was separated, as in our earlier experiments, into carolic and carlic acids.

Isolation of the products of metabolism of Penicillium Charlesii G. Smith using ethyl alcohol as carbohydrate precipitant.

One hundred 1-litre conical flasks containing 350 ml. Czapek-Dox solution and a similar number of flasks of Raulin-Thom solution were sterilised and sown as in our earlier paper [Clutterbuck *et al.*, 1934] with a spore suspension of *P. Charlesii* and incubated at 24° until the glucose had just disappeared. This occupied about 7 weeks with Czapek-Dox and about 4 weeks with Raulin-Thom medium. The metabolism solution was filtered from the mycelium and the latter well pressed and washed with water. The filtrate and washings were evaporated *in vacuo* at 45–50° to about 800 ml. and kept for 1 day at 0°. A small amount of amorphous material was filtered off and to the filtrate were added 4 volumes of absolute alcohol, the mixture being well stirred and allowed to stand until the precipitated polysaccharide mixture had completely settled. The clear liquid was then decanted and evaporated *in vacuo* to about 600 ml. to remove the alcohol. To the concentrate 125 ml. concentrated HCl were added, the fluid was extracted 10 times with an equal volume of ether, the combined ethereal extracts evaporated and the extracted material dried *in vacuo*. In this way were obtained,

using Czapek-Dox medium, 153.2 g. and with Raulin-Thom medium 87.2 g. of a yellow oil.

The ether-extracted solution was then re-extracted with chloroform for the separation of carolic and carlic acids. The number of extracts was increased to 25 and this, together with the fact that a larger amount of HCl had been employed for acidification, led to the isolation of almost twice as much carlic acid as had been previously recorded. It was noticed in our earlier experiments that even after protracted extraction with ether and chloroform the mother-liquors still retained a small negative rotation, and this is now shown undoubtedly to be due to the incomplete extraction of carlic acid. The weights, after drying, of the first CHCl_3 extract were, on Czapek-Dox medium, 29.8 g. and on Raulin-Thom medium 24.9 g., whilst the remaining 24 extracts of the former medium weighed 17.5 g. and of the latter 20.0 g.

The ether extract was next divided into two equal portions, half being examined by the mercury salt method (a) for the presence of *l*- γ -methyltetronic, verticillic and ramigenic acids, and the remaining half being worked up (b) for carolic, carolinic and carlosic acids as in our earlier experiments.

(a) *Isolation of l*- γ -methyltetronic acid as a product of metabolism of *P. Charlesii*. The half-portion of ether-extracted material from experiments on each medium were first neutralised with NaOH using litmus paper as an external indicator, treated with a large excess of aqueous HgCl_2 and kept overnight. The resultant precipitate was separated by centrifuging, washed in the centrifuge pots, suspended in water and saturated with H_2S . The HgS was filtered off, H_2S removed from the filtrate in a stream of air and the free acids extracted with ether. On removing the ether a product was obtained which immediately crystallised but contained a little yellow oil. The total weight of this crude product with Czapek-Dox medium was 6.9 g. and with Raulin-Thom medium 12.6 g. The products were rubbed up with a little ether, transferred to a filter and washed with ether. The yellow oil was thus completely removed and dry crystalline powders, weights 4.5 and 9.2 g. respectively, remained. The material in both cases melted at 114° and consisted of almost pure *l*- γ -methyltetronic acid. No ramigenic or verticillic acid was present. After sublimation the *l*- γ -methyltetronic acid melted at 115° . (Found (Schoeller): C, 52.63, 52.61; H, 5.27, 5.28 %. $\text{C}_5\text{H}_6\text{O}_3$ requires C, 52.61; H, 5.30 %.)

0.066 g. required 5.80 ml. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 114 ($\text{C}_5\text{H}_6\text{O}_3$ titrating as a monobasic acid requires 114). The free acid in water ($c=0.526$) had $[\alpha]_{5461} - 21^\circ$. The rotation was therefore identical with that of the acid obtained from carolic and carolinic acids by bromination followed by catalytic reduction of the resultant *d*- α -bromo- γ -methyltetronic acid.

The acid obtained both from Czapek-Dox and Raulin-Thom media did not depress the m.p. of a sample of *l*- γ -methyltetronic acid obtained from carolic acid, and gave red FeCl_3 and violet NaNO_2 colours and a mono-2:4-dinitrophenylhydrazone, m.p. 235° , not depressed by mixing with the corresponding dinitrophenylhydrazone of synthetic *dl*- γ -methyltetronic acid. A neutral solution of the acid gave with FeCl_3 a very intense brownish red colour similar to that noted in our earlier paper with a neutral solution of verticillic acid.

It is certain therefore that when alcohol is used to precipitate the polysaccharide fraction, *l*- γ -methyltetronic acid can be isolated as a true metabolism product, whilst the ramigenic and verticillic acids of our earlier experiments are entirely absent and have probably arisen as artefacts by condensation of *l*- γ -methyltetronic acid with the acetone then used as polysaccharide precipitant.

(b) *Isolation of carolic, carolinic and carlosic acids from the ether extract.* This material, which after standing was for the most part crystalline, was ground in a glass mortar with a small amount (20 ml.) of chloroform, filtered and washed with chloroform. In this way the ether extract from each medium was separated into a quantity of light brown crystalline material and a chloroform mother-liquor which, after removal of solvent, was dried down to a syrup.

Using Czapek-Dox medium, the crystalline fraction consisted of a mixture of carlosic and carolinic acids. These acids were separated by boiling with a large volume of benzene and filtering, when carlosic acid immediately separated on cooling, whilst carolinic acid remained for the most part in solution and was again obtained by concentrating the benzene solution, adding a little light petroleum and setting aside to crystallise. Carlosic acid after several recrystallisations from boiling benzene had m.p. 181° , not depressed by admixture with an authentic specimen. The carolinic acid fraction was recrystallised once from a small volume of water and then several times from acetone-light petroleum and melted at 123° , not depressed by admixture with an authentic specimen.

Using Raulin-Thom medium, the crystalline fraction consisted almost entirely of carolinic acid, which after one recrystallisation from a small volume of water and then from acetone-light petroleum had m.p. 123° , not depressed by admixture with an authentic specimen. It will be noted that in our earlier experiments verticillic acid accompanied carolinic acid in this fraction but was now absent.

The syrups remaining after drying down the chloroform washings from the crystalline fractions referred to above contained in addition to a reddish brown oil both carolic and *l*- γ -methyltetronic acids. Thus on grinding half of this syrup with water, extracting the aqueous phase once with chloroform and removing the chloroform, a pale yellow oil remained which on standing slowly crystallised. This material was rubbed up with a small volume of alcohol, filtered, washed with a small volume of alcohol and finally recrystallised from this solvent. It had m.p. 132° , not depressed on admixture with an authentic specimen of carolic acid. The presence of *l*- γ -methyltetronic acid in the remaining portion of the syrup was shown by the mercury salt method used above in its isolation.

(c) *Isolation of carolic and carlic acids from the chloroform extract.* The isolation of carolic and carlic acids from the material extracted by CHCl_3 from the ether-extracted, carbohydrate-free metabolism solution was carried out exactly as in our earlier paper. Thus the semi-crystalline extracts were ground with a small amount (20 ml.) of absolute ethyl alcohol, filtered and washed with a little alcohol. In this way the first CHCl_3 extract using both Czapek-Dox and Raulin-Thom media gave almost pure carolic acid which after recrystallisation from alcohol had m.p. 132° , not depressed by admixture with an authentic specimen. The material from the remaining 24 CHCl_3 extracts on the other hand gave almost pure carlic acid, which after recrystallisation from a small volume of ethyl alcohol had m.p. 176° , not depressed by admixture with an authentic specimen.

The total yields in g. of the almost pure crystalline acids obtained from 100 flasks are given in the following table. The yields of *l*- γ -methyltetronic,

Medium	Carolic acid $\text{C}_9\text{H}_{10}\text{O}_4$	Carolinic acid $\text{C}_9\text{H}_{10}\text{O}_6$	Carlic acid $\text{C}_{10}\text{H}_{10}\text{O}_6$	Carlosic acid $\text{C}_{10}\text{H}_{12}\text{O}_6$	<i>l</i> - γ -Methyltetronic acid $\text{C}_5\text{H}_8\text{O}_3$
Czapek-Dox	27.2	6.5	19.3	6.3	4.5
Raulin-Thom	17.2	14.7	5.1	None	9.2

carlosic, carolinic and carolic acids actually isolated from half of the ether extracts have been doubled so as to refer to the total ether extract.

These figures are of the same order as in our earlier experiments except that the yield of carlic acid on Czapek-Dox medium is higher, owing to more thorough extraction, whilst the yields of verticillic and ramigenic acids obtained in the earlier experiments are replaced by a yield of *l*- γ -methyltetronic acid roughly corresponding to the combined weights of these two acids.

Hydrolysis of verticillic acid to give ramigenic and l- γ -methyltetronic acids.

This hydrolysis occurs only slowly in dilute HCl solution at room temperature because of the extremely small solubility of verticillic acid. The reaction was readily effected in the following way. Verticillic acid hydrate (VIII) (2 g.) was dissolved in 64 ml. 8 % aqueous sodium acetate solution, diluted to 100 ml. and 2*N* HCl added to faint turbidity (14 ml.). The solution was kept overnight. Large colourless prisms (1.12 g., m.p. 166–7°) separated, were filtered off and shown to be the hydrate of ramigenic acid (VII). Thus after recrystallisation from acetone-light petroleum the acid had m.p. 171°, not depressed on admixture with an authentic specimen of ramigenic acid, but depressed 10–12° on admixture with verticillic acid (m.p. 171°). It gave the characteristic rotation values of ramigenic acid, the anhydrous free acid in alcohol having $[\alpha]_{5461} + 28^\circ$ and the sodium salt $[\alpha]_{5461} + 85^\circ$. (Found (Schoeller) on anhydrous material: C, 62.34, 62.27; H, 6.47, 6.59 %. $C_{16}H_{20}O_6$ requires C, 62.30; H, 6.54 %.) The acid also gave on methylation the characteristic monomethyl derivative of ramigenic acid. Thus on suspending the finely powdered acid in ether and adding an ethereal solution of diazomethane there was an immediate and vigorous reaction and a methyl derivative separated, m.p. 192°. On dissolving in a larger volume of boiling ether, filtering and adding an equal volume of boiling light petroleum, the methyl derivative separated in long colourless needles, m.p. 199°. (Found: C, 63.41, 63.47; H, 6.91, 6.93; OMe, 9.60, 9.78 %; Mol. wt. (cryoscopic in camphor) 332, 340. $C_{16}H_{19}O_5OCH_3$ requires: C, 63.31; H, 6.88; OMe, 9.62 %. Mol. wt. 322.)

The mother-liquors from which ramigenic acid had been separated were continuously extracted with ether, and the extracted material, which contained some acetic acid, was rapidly dried over sticks of KOH at very low pressure. A solid remained (0.90 g.) which contained a trace of yellow oil but consisted essentially of colourless crystalline material. This sublimed with great ease and after two sublimations had m.p. 115°, not depressed by admixture with a sample of *l*- γ -methyltetronic acid from carolic acid. (Found: C, 52.82, 52.80; H, 5.37, 5.37 %. $C_5H_6O_3$ requires C, 52.61; H, 5.30 %.) 0.052 g. required 4.60 ml. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 113. ($C_5H_6O_3$ titrating as a monobasic acid requires 114.) This acid gave red $FeCl_3$ and violet $NaNO_2$ colours and a mono-dinitrophenylhydrazone, m.p. 235°, not depressed by mixing with the corresponding dinitrophenylhydrazone of synthetic *dl*- γ -methyltetronic acid. (Found: C, 45.03, 45.18; H, 3.47, 3.57; N, 19.04, 19.01 %. $C_{11}H_{10}O_6N_4$ requires C, 44.89; H, 3.43; N, 19.04 %.)

The theoretical yield from 2 g. of verticillic acid hydrate is 1.22 g. of ramigenic acid $C_{16}H_{20}O_6 \cdot H_2O$ (assumed 1 mol.), and of *l*- γ -methyltetronic acid (assumed 2 mols.) is 0.85 g. It appears therefore that verticillic acid (1 mol) on mild hydrolysis gives rise to 1 mol. of ramigenic acid and 2 mols. of *l*- γ -methyltetronic acid. This fact fixes the empirical formula of verticillic acid hydrate as $C_{26}H_{32}O_{12}$, and confirms the formula previously assigned to this substance.

Synthesis of ramigenic acid and isopropylidene-bis- γ -methyltetronic acid.

For this purpose the natural *l*- γ -methyltetronic acid obtained as a metabolic product of *P. Charlesii* and also a sample of the same acid prepared from carolic acid by bromination and reduction of the so-formed α -bromo- γ -methyltetronic acid were used.

l- γ -Methyltetronic acid on standing in dilute aqueous solution with acetone for 2–3 hours readily deposits crystals of isopropylidene-bis- γ -methyltetronic acid (VI). After filtering off this compound, the mother-liquors on keeping for 48 hours deposit crystals of ramigenic acid (VII). Both of these reactions proceed somewhat more quickly in presence of HCl. The reactions can be best carried out by boiling a solution of *l*- γ -methyltetronic acid (0.25 g.) in absolute alcohol (5 ml.) containing acetone (5 ml.) on the water-bath under reflux. If the reaction is stopped after 30 minutes, the acetone distilled off and water added, isopropylidene-bis- γ -methyltetronic acid crystallises out. If boiling is continued for 4–5 hours, the product is pure ramigenic acid. An exact analogy for both of these reactions is given by Wolff and Schimpff [1901] in their reactions of tetronic acid and acetone which give isopropylidene-bis-tetronic acid and acetonyl-isopropylidene-bis-tetronic acid respectively.

Isopropylidene-bis- γ -methyltetronic acid (VI) crystallises from water with $2\text{H}_2\text{O}$, which is readily lost on heating at 63° *in vacuo*. The anhydrous acid melts at 172° . (Found (Schoeller): C, 58.09, 58.18; H, 6.09, 6.00 %. $\text{C}_{13}\text{H}_{16}\text{O}_6$ requires C, 58.19; H, 6.02 %.) The acid in alcohol ($c=0.513$) had $[\alpha]_{5461} -39^\circ$.

0.0298 g. required 2.19 ml. *N*/10 NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 136 ($\text{C}_{13}\text{H}_{16}\text{O}_6$, titrating as a dibasic acid requires 134). The acid gives a red FeCl_3 colour in aqueous alcohol but does not give an immediate colour with NaNO_2 .

Acetonylisopropylidene-bis- γ -methyltetronic acid (ramigenic acid, VII) synthesised as described above showed exactly the same properties as those of ramigenic acid as described in our earlier paper. Both acids crystallised in the same characteristically well-formed prisms, M.P. 171° , the mixed M.P. showing no depression. Both acids can be crystallised both in the anhydrous form $\text{C}_{16}\text{H}_{20}\text{O}_6$ and as the monohydrate. Both acids show the same abnormality in rotation. Thus in each case the free acid in absolute alcohol ($c=0.22$) had $[\alpha]_{5461} +28^\circ$ whereas the sodium salt (calculated as monosodium salt) in water ($c=0.27$) had $[\alpha]_{5461} +85^\circ$. Both acids when suspended in ether in finely powdered condition reacted immediately with an ethereal solution of diazomethane and gave the same monomethyl derivative, which after recrystallising from ether-light petroleum melted at 199° , the mixed M.P. showing no depression. The synthetic acid showed the same solubilities and behaviour on titration and gave the same reactions with salt solutions as were described in our earlier paper for ramigenic acid. It is certain therefore that the synthetic acetonylisopropylidene-bis- γ -methyltetronic acid (VII) is identical with the ramigenic acid isolated in earlier experiments from the metabolism fluid and the ease of its formation in dilute aqueous solutions containing *l*- γ -methyltetronic acid and acetone makes it certain that the ramigenic acid of our earlier experiments was an artefact arising from these substances during the separation of the polysaccharide fraction with acetone.

A new synthesis of dl- γ -methyltetronic acid.

In our earlier paper [Clutterbuck *et al.*, 1935, 1] this acid was synthesised by the method of Benary [1911] by condensation of bromopropionylbromide with ethyl sodiomalonate with the usual ring closure by loss of ethyl bromide on

heating and subsequent hydrolytic decarboxylation. With this method however the yield we obtained was always very small and a better method was desirable.

In our new method, ethyl propionylacetate was first synthesised by the Grignard reaction of Blaise [1901] as modified by Willstätter and Clarke [1914]. This ester was then brominated under conditions, which by analogy with the work of Wolff [1896] should result in the formation of ethyl $\alpha\gamma$ -dibromopropionylacetate. Ring closure was then effected by heating as usual giving α -bromo- γ -methyltetronic acid,

which on catalytic reduction with palladium-charcoal-hydrogen readily gave *dl*- γ -methyltetronic acid.

Ethyl propionylacetate (11 g.) was dissolved in ether (100 ml.) and 7.8 ml. bromine ($\equiv 4$ atoms Br) were slowly added, the flask being cooled in running water. The reaction was immediate. The ether layer was washed with water until free from HBr, dried over MgSO_4 and the ether removed *in vacuo*. The resulting dibromoester was highly lachrymatory and had a very disagreeable odour. It was not therefore isolated in the pure state but was immediately heated at reduced pressure (about 30 mm.) for 3 hours in an oil-bath, temperature 130 – 140° . In this way ring closure with loss of ethyl bromide was effected. On cooling the product set to a semi-crystalline mass and was kept overnight in the cold room. On filtering and washing with benzene 3.35 g. of crystalline α -bromo- γ -methyltetronic acid remained. The filtrate, which on adding benzene deposited a small amount of tar, was filtered and evaporated and the residue heated for a further period as above. On standing in the cold room overnight, filtering and washing with benzene, a second crop of the bromide (0.62 g.) was obtained. The total yield of α -bromo- γ -methyltetronic acid was thus 3.97 g. or 26.8 % of theory. The product melted at 173° and gave red FeCl_3 and violet NaNO_2 colour reactions. It was reduced catalytically with palladium-charcoal-hydrogen in the same way as were the bromides from carolic and carolinic acids in our earlier work, and gave quantitatively *dl*- γ -methyltetronic acid $\text{C}_5\text{H}_6\text{O}_3$, which crystallised from benzene-light petroleum in curved feathery needles, m.p. 117° . It readily sublimed *in vacuo* and the sublimate melted at 117° . (Found (Weiler): C, 52.94; H, 5.69 %. $\text{C}_5\text{H}_6\text{O}_3$ requires C, 52.61; H, 5.30 %.) 0.0745 g. required 6.60 ml. $N/10$ NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 113 ($\text{C}_5\text{H}_6\text{O}_3$ titrating as a monobasic acid requires 114).

With Brady's reagent it readily gave a crystalline mono-dinitrophenylhydrazone, m.p. 235° , unchanged by recrystallisation from nitrobenzene-toluene and not depressed by admixture with the dinitrophenylhydrazone from the acid prepared by earlier methods. The acid on boiling with $2N$ H_2SO_4 was readily hydrolysed giving CO_2 , and the hydrolysate slowly, after the manner of acetoin, gave the bis-dinitrophenylhydrazone of diacetyl, m.p. 318° , not depressed by admixture with an authentic specimen.

The acid $\text{C}_5\text{H}_6\text{O}_3$ is therefore undoubtedly the *dl*-variety of γ -methyltetronic acid.

SUMMARY.

It is shown that *l*- γ -methyltetronic acid $C_5H_6O_3$ is formed as a metabolic product of *Penicillium Charlesii* G. Smith when this mould is grown on synthetic solutions containing glucose. A new synthesis of the *dl*-form of this acid is described.

Ramigenic acid, $C_{16}H_{20}O_6$, and verticillic acid, $C_{26}H_{32}O_{12}$, previously reported as metabolic products of *P. Charlesii*, are now shown to be artefacts arising by condensation of *l*- γ -methyltetronic acid with the acetone previously used for precipitation of the polysaccharide fraction from the evaporated metabolism solution. Ramigenic acid has been synthesised from *l*- γ -methyltetronic acid and acetone and shown to be acetonylisopropylidene-bis- γ -methyltetronic acid. Verticillic acid has been shown to be the bis- γ -methyltetronic acid derivative of ramigenic acid.

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