Studies in the Biochemistry of Micro-organisms

113. PENCOLIDE, A NITROGEN-CONTAINING METABOLITE OF *PENICILLIUM MULTICOLOR* GRIGORIEVA-MANILOVA AND PORADIELOVA*

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Several strains of the mould *Penicillium multicolor* Grigorieva-Manilova and Poradielova have been shown to produce, in the mycelium, sclerotiorin (Birkinshaw, 1952), a chlorine-containing pigment, the complete structure of which was finally determined by Dean, Staunton & Whalley (1959). Of the five strains tested, four gave sclerotiorin.

In the present investigation three strains of *P. multicolor* were examined for production of new metabolites that might be present in the culture fluid. These strains were: (1) N.R.R.L. 2058, which had produced no sclerotiorin, (2) N.R.R.L. 2324, which produced sclerotiorin and (originally) new products of probable empirical formulae $C_{13}H_{16}O_6$ and $C_{13}H_{18}O_7$ in the culture solution, and (3) N.R.R.L. 4036, not previously examined by us. Only the last-named strain afforded the new nitrogen-containing product now reported, which has been named pencolide.

Pencolide was obtained on Raulin–Thom medium in best yield after 8 days' incubation. It was isolated by ether extraction from the culture fluid with subsequent sublimation and finally crystallization from ether–light petroleum. It was then obtained as colourless rosettes, m.p. 135·5°. Analysis and determination of mol.wt. established the empirical formula as $C_9H_9NO_4$. On titration it showed an immediate (unstable) end point corresponding with one acidic group and a final end point, reached after several hours, corresponding with neutralization of a second acidic group. Pencolide could be recovered on acidification of the solution after titration with either one or two equivalents of alkali. This behaviour indicates the presence of a free acidic group, probably carboxyl, and a masked acidic group such as occurs in a lactone. Pencolide did not afford an acetyl derivative, indicating the probable absence of a hydroxyl group, but with diazomethane a product, $C_{11}H_{13}N_3O_4$, with one methoxyl group was obtained, representing addition of diazomethane in addition to methylation (probably esterification). Additional evidence of a free carboxyl group was the production of a *p*-nitrobenzyl derivative. On catalytic reduction (platinum dioxide) one molecule of hydrogen was taken up, and the dihydropencolide was isolated as the *p*-nitrobenzyl ester, indicating the presence of at least one double bond.

Pencolide was cleaved by either acid or alkaline hydrolysis. In the former case, the product identified was α-oxobutyric acid, isolated as the 2,4-dinitrophenylhydrazone. A second volatile acid was obtained, but not identified. The products of alkaline hydrolysis were ammonia, α-oxobutyric acid (III) and itaconic acid (IV). An unidentified volatile acid was again produced. A crystalline dibasic acid, $C_9H_{11}NO_5$, was also obtained in small amount. In another experiment, mesaconic acid (V) was present among the non-volatile products. On alkaline hydrolysis of dihydropencolide, the non-volatile fraction of the hydrolysate was found to contain methylsuccinic acid. Since mesaconic acid, itaconic acid and citraconic acid form an equilibrium mixture in alkaline solution (Linstead & Mann, 1931), and only one of these, namely citraconic acid (II), is volatile in steam, it seemed likely that the unidentified volatile acid was citraconic acid, and that the initial hydrolysis product could be any one of these three acids. The double bond of this moiety had evidently been saturated in the production of dihydropencolide.

Oxidation of pencolide with permanganate gave pyruvamide and oxamic acid, indicating the

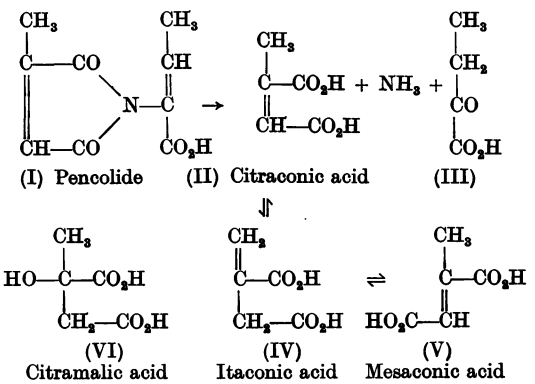


Fig. 1. Hydrolysis of pencolide.

* Part 112: Agosti, Birkinshaw & Chaplen (1962).

presence of an amide or imide group. Oxalic acid and acetic acid were also obtained in this reaction. In another experiment, pyruvic acid was detected amongst the oxidation products.

Ozonolysis afforded oxalic acid, acetic acid, acetaldehyde and ammonium oxalate.

From these facts it appears that the molecule of pencolide is composed of the molecules of α-oxobutyric acid, ammonia and one of the above three C_5 acids, with the elimination of three molecules of water. A Kuhn–Roth determination indicated the presence of two *C*-methyl groups (1·36 mol.prop. of acetic acid), which argues against an itaconic acid residue, and in favour of a citraconic or mesaconic residue. The isolation of pyruvic acid and pyruvamide by oxidation is also more readily explained on this basis, and suggests a citraconic or mesaconic amide. Further, when pencolide was subjected to mild alkaline hydrolysis, there was obtained, in addition to α-oxobutyric acid, a small amount of a crystalline product, $C_5H_9NO_4$, m.p. 270°, containing a *C*-methyl group; it was probably a monoamide of citramalic acid (VI). An itaconic amide derivative would be expected to add water to produce a hydroxymethyl rather than a terminal methyl group.

The citraconic or mesaconic amide must be linked to α-oxobutyric acid with the loss of two molecules of water; in the process, one acid function and the carbonyl function must be eliminated. Since acetaldehyde is obtained by ozonolysis, it is probable that the α-oxobutyric acid residue is in the enolic form, which would dispose of the carbonyl function.

Decisive evidence of the arrangement of the hydrogen atoms was obtained by examination of the nuclear-magnetic-resonance spectrum of pencolide (see Appendix), and was fully in accord with the above arguments.

Examination of the infrared-absorption spectrum of pencolide and its derivatives indicates the absence of imino or hydroxyl groups: pencolide absorbs in the 2500–3100 cm.$^{-1}$ region, indicative of the free carboxyl group, but only very weak absorption is shown at higher frequencies, and the *p*-nitrobenzyl ester and the diazomethane product show only weak absorption in the 3000–3500 cm.$^{-1}$ region.

The simplest method of joining the two fragments, in view of the above considerations, is formula (I), and we believe this adequately explains the experimental results. Thus it contains a free carboxyl group, neutralization of which would correspond with the first rapid stage of the titration. The second (slow) stage would then be due to the opening of the imide ring to give a substituted amide and a second free carboxyl group. This ring-opening must be reversible, since pencolide is formed again on acidification. The product, $C_9H_{11}NO_5$, obtained on alkaline hydrolysis of pencolide, is probably also a substituted amide produced by opening of the imide ring. It differs from the gummy product recovered from titration to a permanent end point, since it does not revert to pencolide. This could be due to a rearrangement of the citraconic moiety to the mesaconic or itaconic form. The path of hydrolysis is outlined in Fig. 1. Pencolide gives a red colour with ethanolic potassium hydroxide, a reaction shown by Piutti (1906) to be typical of unsaturated, *N*-substituted imides.

The products of oxidation are accounted for by assuming fission of the double bonds and hydrolysis of C–N links, which would give acetaldehyde and acetic acid, pyruvic acid and its amide, and oxalic acid and its monoamide (Fig. 2).

The ultraviolet-absorption spectrum of pencolide shows a single peak, $\lambda_{max.}$ 219 mμ (log ε 4·30). The spectrum of cyclohexylcitraconimide is similar in shape, $\lambda_{max.}$ 222 mμ (log ε 4·13), whereas crotonic acid absorbs at 214 mμ (log ε 3·89). A simple addition spectrum gives a single peak at about 217 mμ (log ε 4·28) of similar shape to that of pencolide. The nitrogen attached to the doubly linked carbon atom might be expected to have a bathochromic effect, but according to Braude (1945) nitrogen attached to C=O has a hypsochromic effect. An analogy could be drawn with the absorption maxima of *N*-(1-naphthyl)maleimide, $\lambda_{max.}$ 223, 270 mμ (Tsou, Barrnett & Seligman, 1955), which differ little from those of 1-methylnaphthalene, $\lambda_{max.}$ 224, 280 mμ (Friedel & Orchin, 1951).

The infrared-absorption maxima of pencolide and its derivatives, and of several model compounds, in the double-bond-stretching region are shown in Table 1. According to Cross (1960), the imide group gives rise to two strong bands in the 1790–1720 and 1710–1670 cm.$^{-1}$ regions. It appears from the Table that in *N*-substituted maleimides the higher-frequency peak is always weak and occasionally absent, and the strong lower-frequency peak is shifted to somewhat higher frequencies. The spectra of pencolide and its derivatives are consistent with this; the strong lower band may also include carboxyl or ester carbonyl absorption.

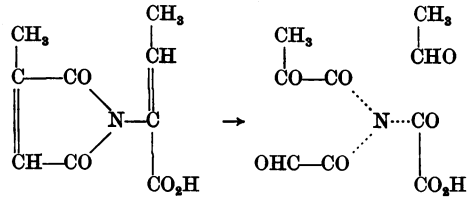
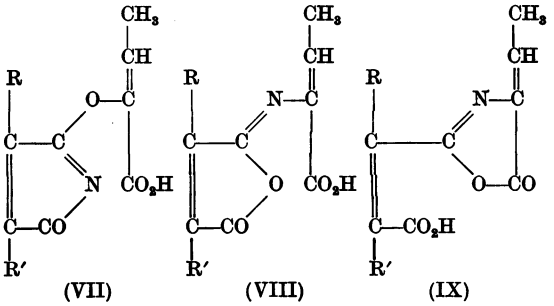


Fig. 2. Oxidation of pencolide.

There are two possible geometrical isomers of formula (I), depending on whether the methyl and carboxyl groups of the crotonic acid moiety are *cis* or *trans* to each other. We have no evidence on this point.

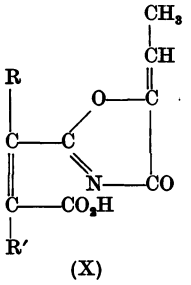
Structures (VII), (VIII), (IX) and (X) (R = CH_3, R′ = H; or R = H, R′ = CH_3), are also consistent with the nuclear-magnetic-resonance spectrum and some chemical properties. Little is known of such isoimide and imino ester groups, but we would not expect them to have the stability of pencolide. In addition, (VIII) and (IX) have extended conjugated double-bond systems which would be expected to give a higher-wavelength absorption in the ultraviolet.



(VII)

(VIII)

(IX)



(X)

The imide structure of pencolide is most unusual in natural products; so far as we are aware, it is unique. Nitrogenous metabolites of fungi were reviewed by Birkinshaw & Stickings (1962) and pencolide bears little structural resemblance to any of the compounds cited. No evidence is yet available about its mode of biosynthesis, but it could well be formed from citraconic acid and threonine or some related amino acid. Citraconic acid has not been recorded from natural sources, but its two isomers are both known: mesaconic acid was isolated from cabbage leaves by Buston & Schryver (1923), and itaconic acid is a metabolite of *Aspergillus itaconicus* (Kinoshita, 1931), *A. terreus* (Calam, Oxford & Raistrick, 1939) and other fungi. Itaconic acid has been shown to arise from Krebs-cycle intermediates (Bentley & Thiessen, 1957), and citraconic acid could be derived in a similar way.

EXPERIMENTAL

The C, H and N determinations were by Dr A. Schoeller; the *C*-Me analyses by Weiler and Strauss; other analyses were by M. G. K.; m.p. are uncorrected. Absorption spectra were measured on a Hilger Uvispek instrument and a Perkin–Elmer Infracord instrument.

Light petroleum refers to the fraction b.p. 60–80°.

Cultures. The following strains of *Penicillium multicolor* Grigorieva-Manilova and Poradielova were examined for the production of pencolide. (1) N.R.R.L. 4036. Received in November 1954 from the Northern Regional Research Laboratory, Bureau of Agriculture, Peoria, Ill., U.S.A. L.S.H.T.M. catalogue no. G.A. 217. (2) N.R.R.L. 2324. Obtained from Dr K. B. Raper of the Northern Regional Research Laboratory in 1949. (3) N.R.R.L. 2058. Obtained from Dr Raper in 1948 (Raper & Thom, 1949).

Cultural conditions and characteristics. Since pencolide was obtained only from the first of these strains, N.R.R.L. 4036, this strain was employed in all except a preliminary experiment. The strains were grown on Raulin–Thom

Table 1. *Infrared-absorption maxima of pencolide and derivatives, and of some model compounds, in the* 1800–1600 *cm.*$^{-1}$ *region*

vw, Very weak; w, weak; m, medium; s, strong; vs, very strong; sh, shoulder.

		Absorption maxima (cm.$^{-1}$)		
	Medium	1780–1745	1725–1705	1685–1595
Pencolide	Nujol	1775 w	1710 s	1685 s 1650 m
	$CHCl_3$	1780 w	1717 vs	1660 s
Diazomethane product from pencolide	Nujol	1780 m	1720 s	1670 s
p-Nitrobenzyl ester of pencolide	Nujol	—	1720 s 1710 s	— —
N-Cyclohexylcitraconimide	Nujol	1750 w	1705 vs	—
N-Ethylmaleimide*	$CHCl_3$	—	1712 s	1595 w
N-(1-Naphthyl)maleimide*	$CHCl_3$	1745 sh	1724 s	1603 m
N-(4-Acetoxy-1-naphthyl)maleimide*	$CHCl_3$	1770 vw	1724 s	1603 m
N-(4-Hydroxy-1-naphthyl)maleimide*	Dioxan	1754 vw	1706 s	1632 vw
N-(5-Hydroxy-1-naphthyl)maleimide*	Dioxan	1761 w, sh	1706 s	1600 s

* Figures calculated from those recorded in μ by Tsou *et al.* (1955).

medium, of the composition: glucose monohydrate, 54·5 g.; tartaric acid, 2·67 g.; ammonium tartrate, 2·67 g.; $(NH_4)_2HPO_4$, 0·4 g.; $MgCO_3$ (heavy), 0·27 g.; $ZnSO_4,7H_2O$, 0·047 g.; $FeSO_4,7H_2O$, 0·047 g.; $(NH_4)_2SO_4$, 0·17 g.; K_2CO_3, 0·4 g.; water, 1000 ml. This medium was distributed in 1 l. conical flasks (350 ml./flask). The flasks were plugged with cotton wool, sterilized by steaming and inoculated with spore suspensions derived from malt–agar slopes. They were incubated in the dark at 24°. When harvested, the culture medium was pale orange in colour and the mycelium showed a mixture of green, red, orange and white patches, the green being more predominant in the earlier stages; the reverse was orange.

Harvesting and isolation of product. The culture fluid was separated from the mycelium by filtration. It gave no colour with $FeCl_3$, but decolorized bromine water and $KMnO_4$. The filtrate was extracted with ether in a counter-current liquid extractor and the ether extracts were dried over Na_2SO_4 and concentrated. Evaporation of the culture filtrate under reduced pressure in a climbing-film evaporator before extraction was detrimental; the concentrate gave only traces of crystalline material on extraction. The residue from the ether extract was a gum containing crystals. Most of the gum could be removed by washing with ether, but much crystalline matter was also dissolved. Further crystalline material was obtained from the washings by addition of light petroleum. The crystals were then recrystallized from ether–light petroleum, giving colourless rosettes of m.p. 135·5°.

This method involved much loss. It was found that sublimation of the gummy ether extract in high vacuum at 110° gave, in much better yield, a pale-yellow sublimate of m.p. 130–132°, which was then recrystallized from ether–light petroleum. This method, though more laborious, was therefore employed in all subsequent isolations of pencolide.

Time of incubation for optimum yield of product. One hundred flasks inoculated with *P. multicolor* were incubated at 24° and harvested in batches of ten after varying periods. The pH and residual glucose (by polarimeter) were determined in each batch, which was then extracted three times with ether. The crude residues from the ether were weighed and subjected to sublimation. The results, recorded in Table 2, show that, although the yield of the crude ether extracts was highest at 14 days, the best yield of pencolide was obtained after 8 days' incubation.

Table 2. *Production of pencolide by* Penicillium multicolor *N.R.R.L.* 4036

Period of incubation (days)	pH	Residual glucose (%)	Crude extract from 10 flasks (mg.)	Sublimed pencolide (mg.)
5	3·3	2·98	370	132
6	3·2	2·53	405	179
7	3·0	2·10	470	192
8	2·7	1·76	504	283
9	3·0	1·22	538	264
10	3·1	1·10	566	214
12	3·3	0·92	643	177
14	3·5	0·68	791	130
16	3·8	0·38	593	90
20	4·3	0·24	586	37

Properties of pencolide

Pencolide crystallizes from ether–light petroleum as colourless rosettes, m.p. 135·5°. It sets again on cooling and melts again at the same temperature. It is soluble in water and most organic solvents except light petroleum. It contains nitrogen but no halogens or sulphur [Found: C, 55·5, 55·55; H, 4·5, 4·7; N, 7·3; OMe, nil; *C*-Me, 10·5%; mol.wt. (Rast) 196. $C_9H_9NO_4$ requires C, 55·4; H, 4·65; N, 7·2; *C*-Me, (1) 7·7, (2) 15·4%; mol.wt. 195]. An ethanolic solution treated with ethanolic KOH gives a red colour which fades after a few minutes.

Light-absorption values: $\lambda_{max.}$ 219 mμ, log ε 4·30; $\lambda_{max.}$ 216 mμ, log ε 4·27, in ethanol and water respectively. A 1% solution in $CHCl_3$ showed no optical rotation.

Titration with dilute alkali. Pencolide (0·040 g.) was dissolved in water (5 ml.) with slight warming and, after cooling to room temperature, was titrated with 0·1 N-NaOH to phenolphthalein. The first (rapid) end point, obtained after the addition of 2·13 ml. (apparent equivalent 188) was unstable and the solution gradually took up more alkali. At the final end point of the titration, which lasted several hours, the alkali used was 4·25 ml. (equivalent 94). Material recovered by acidification with HCl and extraction with ether from the titration to the first unstable end point was found to be unchanged pencolide.

The material from the titration to the permanent end point was a gum. However, on trituration with light petroleum, and storage at 0–5° for several days, a colourless crystalline substance was obtained, m.p. 135° undepressed when mixed with pencolide.

When pencolide (0·0245 g.) was dissolved in 0·1 N-NaOH (5 ml.) and back-titrated the value for the equivalent was 94.

Functional derivatives of pencolide

All attempts to prepare an acetyl derivative resulted in recovery of unchanged pencolide. Of the usual methylating agents only diazomethane formed a derivative with pencolide.

Pencolide (0·5 g.) suspended in ether was treated with an ethereal solution of diazomethane, added slowly until effervescence ceased. After evaporation of the ether and trituration of the residue with light petroleum, colourless crystals (0·42 g.) were obtained. These, on recrystallization from ether, gave colourless rosettes, m.p. 119–120°, of a *product* insol. in $NaHCO_3$ [Found: C, 52·95; H, 5·1; N, 16·8; OMe, 12·6. $C_{11}H_{13}N_3O_4$ requires C, 52·6; H, 5·2; N, 16·7; OMe (1), 12·35%]. This represents addition of diazomethane, presumably to a double bond, in addition to methylation.

p-*Nitrobenzyl ester.* Pencolide (0·3 g.), dissolved in water (10 ml.) with slight warming, was neutralized with 0·1 N-NaOH and treated with *p*-nitrobenzyl bromide (0·47 g.) in ethanol (15 ml.). The mixture was refluxed for 1 hr., diluted with water and chilled. The colourless solid (0·41 g.) which separated, when recrystallized from light petroleum, afforded glistening plates, m.p. 105°, of *pencolide* p-*nitrobenzyl ester* (Found: C, 58·4; H, 4·45; N, 8·5. $C_{16}H_{14}N_2O_6$ requires C, 58·2; H, 4·3; N, 8·5%).

Catalytic reduction of pencolide

Pencolide (0·52 g.) in ethanol (15 ml.) was hydrogenated with freshly reduced Adams catalyst (hydrated PtO_2, 0·1 g. suspended in ethanol). A rapid uptake of H_2

(1 mol.prop.) occurred. After filtration of the solution and removal of solvent, an oily product (0·54 g.) was obtained, soluble in aq. $NaHCO_3$.

p-*Nitrobenzyl ester of dihydropencolide.* The reduced product (0·115 g.) was converted into the *p*-nitrobenzyl ester by the method used for pencolide. The product (0·07 g.), which separated on chilling the diluted reaction mixture, gave, on recrystallization from light petroleum, colourless plates, m.p. 97·5–98°, of *dihydropencolide* p-*nitrobenzyl ester* (Found: C, 57·9; H, 5·0; N, 8·2. $C_{16}H_{16}N_2O_6$ requires C, 57·8; H, 4·85; N, 8·4%).

Degradation of pencolide

Acid hydrolysis. Pencolide (0·30 g.) in N-H_2SO_4 (10 ml.) was refluxed for 5 hr. in a stream of N_2. The effluent gases were bubbled in succession through aq. solutions of (*a*) 2,4-dinitrophenylhydrazine hydrochloride and (*b*) $Ba(OH)_2$. No precipitate was obtained in either solution. The hydrolysate was extracted with ether in a continuous extractor for 12 hr. The oily residue from the ether gave an immediate cryst. ppt. with Brady's reagent. The ether residue was dissolved in water and subjected to distillation under reduced pressure, more water being added at intervals to replace the loss. The undistilled portion left a gummy residue giving no reaction with Brady's reagent. No solid could be obtained from this portion.

Half of the distillate was treated with Brady's reagent. The yellow ppt. was separated and recrystallized from aq. ethanol. It thus afforded orange–yellow needles, m.p. 200°, of α-oxobutyric acid 2,4-dinitrophenylhydrazone (Found: C, 42·7; H, 3·7; N, 19·4. Calc. for $C_{10}H_{10}N_4O_6$: C, 42·6; H, 3·6; N, 19·9%). The product did not depress the m.p. of an authentic sample of the 2,4-dinitrophenylhydrazone of α-oxobutyric acid. The two products also showed the same R_F on a paper chromatogram. Owen (1945) gives m.p. 197° for this derivative.

The remainder of the distillate was titrated to phenolphthalein with 0·1N-NaOH. The volume of alkali required, 18·3 ml., considerably exceeded the theoretical amount (7·7 ml.) required for 1 mol.prop. of α-oxobutyric acid from 1 mol.prop. of pencolide. It therefore seemed that the distillate contained a second volatile acid. An attempt to identify this by conversion into *p*-bromophenacyl ester was not successful; only the unchanged reagent *p*-bromophenacyl bromide was isolated from the reaction mixture.

Alkaline hydrolysis of pencolide. (*a*) Isolation of α-oxobutyric acid and itaconic acid. Pencolide (1·0 g.) was refluxed for 2 hr. with N-NaOH (100 ml.). The hydrolysate smelt of ammonia. It was acidified with 2N-H_2SO_4 and subjected to distillation in a stream of N_2. A baryta trap showed no evidence of evolution of CO_2. The distillation was continued under reduced pressure to dryness. Half of the distillate was treated with Brady's reagent. It gave an immediate yellow ppt. (0·17 g.), which was recrystallized and identified as the 2,4-dinitrophenylhydrazone of α-oxobutyric acid. The remaining distillate required 31·4 ml. of 0·1N-NaOH for neutralization whereas only 6·0 ml. would be required for the α-oxobutyric acid present, based on the weight of dinitrophenylhydrazone isolated.

The residue (0·49 g.) from the hydrolysis was resolved into two fractions by extraction with ether. The more soluble portion was repeatedly crystallized from ethyl acetate and sublimed in high vacuum at 90°, the m.p. being thus raised to 165°. It was found to be identical with itaconic acid, and showed no depression in m.p. when mixed with an authentic sample [Found: C, 46·1; H, 4·6; equivalent 65·5 by titration. Calc. for $C_5H_6O_4$: C, 46·2; H, 4·6%; equivalent (dibasic) 65]. A portion was converted into the *p*-bromophenacyl ester, plates, m.p. 117°, not depressed on admixture with an authentic specimen of itaconic acid *p*-bromophenacyl ester.

The less-soluble portion of the residue from alkaline hydrolysis was recrystallized from ethyl acetate, giving colourless rosettes, m.p. 229–230° (decomp.). The *substance* turned red from 215° onwards and melted with strong effervescence to a deep-red solution [Found: C, 50·8, 50·85; H, 5·5, 5·5; N, 6·5%; equivalent by titration, 111. $C_9H_{11}NO_5$ requires C, 50·7; H, 5·7; N, 6·6%; equivalent (dibasic) 107]. The amount of this product obtained was too small for identification.

(*b*) Isolation of ammonia and mesaconic acid. Pencolide (1·0 g.) was refluxed with N-NaOH (100 ml.) for 2 hr. The hydrolysate was steam-distilled and the distillate collected in 20 ml. of a solution of the composition: boric acid, 40 g.; 0·2% methyl red solution, 6·66 ml.; 0·2% methylene blue solution, 3·33 ml.; water to 1l. The original bluish-purple colour of the indicator changed to green on absorption of ammonia. It was titrated against 0·1N-H_2SO_4 until the original colour was restored. About 70% of the equivalent of 1 mol.prop. of NH_3/mol.prop. of pencolide was present in the distillate. With Nessler's reagent the distillate gave a positive test for NH_3, which was also detected by the test of Cheronis & Entrikin (1957). In this test, NH_3 gives a blue colour, whereas methylamine gives a pink colour.

After distillation of the NH_3, the hydrolysate was acidified with 2N-H_2SO_4 and evaporated to dryness. α-Oxobutyric acid was again present in the distillate. The dry residue, containing Na_2SO_4, was refluxed with ether. The ether extracts on evaporation afforded a colourless residue (0·31 g.), containing two fractions, one (A) more readily soluble in ether than the other (B). Fraction A, m.p. 194–196°, was sublimed at 80° in high vacuum to separate it completely from fraction B and recrystallized from ether–light petroleum. It thus afforded mesaconic acid, m.p. 204°, undepressed on admixture with an authentic specimen [Found: C, 46·2; H, 4·6; equivalent by titration 66. Calc. for $C_5H_6O_4$: C, 46·2; H, 4·6; equivalent (dibasic) 65]. Fraction B, obtained in very small amount, had m.p. 220–223° and was identical with the product, $C_9H_{11}NO_5$, previously obtained by alkaline hydrolysis.

Alkaline hydrolysis of dihydropencolide. The oily product (0·3 g.) obtained by catalytic hydrogenation of pencolide was refluxed with N-NaOH (25 ml.) for 2 hr. The hydrolysate was acidified with H_2SO_4 and evaporated under reduced pressure. The distillate afforded α-oxobutyric acid, isolated as the 2,4-dinitrophenylhydrazone. The dry residue was extracted with ether. The ether was evaporated, giving an oil which crystallized after a few hours. The product was purified by recrystallization from ether–light petroleum, affording needles, m.p. 107–108°, of DL-methylsuccinic acid. No depression in m.p. was observed on admixture with an authentic sample. The identity was confirmed by conversion into the bis-*p*-bromophenacyl ester, plates, m.p. 121°, unchanged on admixture with an authentic specimen (Found: C, 48·2; H, 3·8; Br, 30·5. Calc. for $C_{21}H_{18}Br_2O_6$: C, 47·9; H, 3·4; Br, 30·4%). Wiley *et al.* (1957) give m.p. 120–125°.

Mild alkaline hydrolysis of pencolide. Pencolide (1·0 g.), dissolved in warm water (20 ml.) and then cooled, was treated with a saturated solution of $Ba(OH)_2$ (250 ml.; approx. 0·2 N). The mixture was kept at room temp. for 20 hr. and neutralized with 2N-H_2SO_4 to pH 7·0. The $BaSO_4$ was removed by centrifuging and adjustments were made until neither Ba^{2+} nor SO_4^{2-} ions were present. The hydrolysate was then evaporated to dryness under reduced pressure. A gummy residue (0·2 g.) was obtained from which rosettes slowly crystallized. The product was extracted with boiling ether. The soluble portion (0·07 g.) consisted of unchanged pencolide. The insoluble fraction was crystallized several times from ethanol–light petroleum and eventually afforded glistening plates, m.p. 169–170° (10 mg.) [Found: C, 41·05; H, 6·1; N, 9·2; *C*-Me (Kuhn–Roth), 8·2. $C_5H_9NO_4$ requires C, 40·8; H, 6·2; N, 9·5; (one) *C*-Me, 10·2%]. This was probably a monoamide of citramalic acid.

The distillate from the hydrolysate, when treated with Brady's reagent, gave a yellow ppt. (0·45 g.) which was identified as the 2,4-dinitrophenylhydrazone of α-oxobutyric acid.

Oxidations of pencolide

Oxidation with potassium permanganate. 2N-Potassium permanganate was added to an aq. solution of pencolide (1·0 g.) until no further decolorization occurred. The slight excess of reagent was destroyed with Na_2SO_3. The mixture was filtered and the filtrate was extracted with ether continuously for 20 hr. The ether extract was dried with Na_2SO_4 and evaporated, affording a cryst. product (0·1 g.), m.p. 123°. The crude product was difficult to recrystallize. As it was found to react with Brady's reagent, it was converted into the 2,4-dinitrophenylhydrazone. Recrystallization of the product from ethanol afforded pyruvamide 2,4-dinitrophenylhydrazone, yellow needles, m.p. 228–229°, undepressed on mixing with an authentic specimen (Found: C, 40·6; H, 3·1; N, 26·2. Calc. for $C_9H_9N_5O_5$: C, 40·4; H, 3·4; N, 26·2%). Strauser & Dyer (1956) give m.p. 217·5–219° for this derivative.

After all the pyruvamide had been removed by ether extraction, the oxidation mixture was acidified with 2N-H_2SO_4 and the extraction was continued for 24 hr. On evaporation of the ether solution, a crystalline product separated and was collected. This material (0·29 g.) was recrystallized from ethanol and then afforded an acidic product, m.p. 208° (decomp.), which did not depress the m.p. of authentic oxamic acid on mixing (Found: C, 27·3; H, 3·7; N, 15·7. Calc. for $C_2H_3NO_3$: C, 27·0; H, 3·4; N, 15·7%).

The ether residue was an oily liquid which was dissolved in water and evaporated to dryness several times under reduced pressure, the water being restored after each distillation. The dry residue (0·15 g.) was recrystallized from ether–light petroleum and obtained as rods, m.p. 99–100°, resetting and finally melting at 189° with sublimation. This was shown to be oxalic acid by conversion into the methyl ester with diazomethane. The product had m.p. 54°, undepressed on admixture with authentic methyl oxalate.

The volatile acid obtained by distillation of the acidified oxidation mixture was converted in the usual way into the *p*-bromophenacyl ester, obtained as needles, m.p. 83°, unchanged on admixture with authentic *p*-bromophenacyl acetate.

In another oxidation, with the same conditions, the amount of pyruvamide was much smaller. From the ether extract of the acidified oxidation mixture, oxamic acid was obtained as before. The oily residue from the ether mother liquor, on treatment with Brady's reagent, gave a ppt. which was recrystallized from ethanol, affording yellow needles, m.p. 215°, unchanged on admixture with the 2,4-dinitrophenylhydrazone of pyruvic acid (Found: C, 40·9; H, 3·2; N, 20·6. Calc. for $C_9H_8N_4O_6$: C, 40·3; H, 3·0; N, 20·9%).

Ozonolysis. Ozonized O_2 was passed for 5 hr. through pencolide (1·1 g.) dissolved in $CHCl_3$ (50 ml.) in an ice-bath. A cryst. product (0·07 g.) separated during the ozonolysis and was collected. This fraction was identified as oxalic acid by m.p. 99–100°, resetting and remelting at 188–189°; and by conversion into the methyl ester, m.p. 54°, undepressed on mixing with methyl oxalate.

The $CHCl_3$ solution was shaken with water and the two layers were separated. From the $CHCl_3$ layer unchanged pencolide (0·12 g.) was obtained. The aqueous layer was distilled several times under reduced pressure to remove volatile acid. One-half of the distillate was neutralized, requiring 10·8 ml. of N-NaOH, reduced in volume and refluxed with *p*-bromophenacyl bromide in ethanol. Colourless needles (0·21 g.), m.p. 83°, undepressed by admixture with *p*-bromophenacyl acetate, were obtained. The rest of the distillate was treated with Brady's reagent. The yellow ppt., collected and recrystallized from ethanol, afforded pale-orange needles, m.p. 147°, undepressed on admixture with authentic acetaldehyde 2,4-dinitrophenylhydrazone. On a paper chromatogram, the derivative of the ozonolysis product and the authentic material had the same R_F.

The non-volatile residue of the aqueous extract was washed with ether and then had m.p. 229–230° and sublimed unchanged at 120° in high vacuum. It was found to consist of ammonium oxalate.

Synthetic material

N-Cyclohexylcitraconimide, m.p. 92–96°, was synthesized from citraconic anhydride and cyclohexylamine by the method of Sheremeteva & Larina (1959).

SUMMARY

1. A new nitrogen-containing product, pencolide, has been obtained from the culture fluid of a strain of *Penicillium multicolor* Grigorieva-Manilova and Poradielova, grown on Raulin–Thom medium.

2. Pencolide, $C_9H_9NO_4$, m.p. 135·5, titrates to a fugitive end point as monobasic acid and to a permanent end point as dibasic acid. It yields no acetyl derivative, but with diazomethane affords a product, $C_{11}H_{13}N_3O_4$, with one methoxyl group, also a *p*-nitrobenzyl derivative.

3. The products of alkaline hydrolysis are ammonia, α-oxobutyric acid, itaconic acid, mesaconic acid and probably citraconic acid.

4. Oxidation with permanganate gave pyruvamide and oxamic acid. Ozonolysis afforded oxalic acid and acetic acid, acetaldehyde and ammonium oxalate.

5. Based on these facts, and on ultraviolet, infrared and nuclear-magnetic-resonance spectral data, the most probable structure for pencolide is 2-citraconimidobut-2-enoic-acid.

We thank Mr A. Manchanda for carrying out the preparation of *N*-cyclohexylcitraconimide and for repeating several other preparations. The Uvispek spectrophotometer employed in this work was purchased by means of a grant from the Central Research Fund of London University. We also thank Dr J. K. Sutherland, Imperial College, for many helpful suggestions.

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APPENDIX

The Proton-Magnetic-Resonance Spectrum of Pencolide

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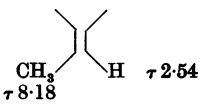
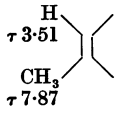
The spectrum of pencolide is simple, consisting of two doublets, each representing three protons, and two quartets, each accounting for one proton. The positions (Jackman, 1959) of the two doublets, τ 8·18 and 7·87 p.p.m., with coupling constants of 7·5 and 2 cyc./sec. respectively, are consistent with there being present two methyl groups each on a double bond. The other two protons, τ 3·51 (*J* 2 cyc./sec.) and 2·54 p.p.m. (*J* 7·5 cyc./sec.) must be vinylic; further, from the splitting pattern and the identity of the coupling constants, the methyl group, τ 8·18 p.p.m., must be on the same double bond as the vinyl proton, τ 2·54 p.p.m., and from the magnitude of the coupling constant (Jackman, 1959) they must be on the same carbon atom, i.e. an ethylidene group (I). The smaller coupling constant in the other case best agrees with



(I)



(II)

allylic coupling across the double bond (II). This accounts for eight of the nine protons present; the ninth, present as an hydroxyl group, was not observed in the spectrum down to τ 1·7 p.p.m.

We thank Mr R. G. Foster, B.Sc., for determining the spectrum on a Varian A-60 Spectrometer with $CDCl_3$ as solvent and tetramethylsilane as internal standard.

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