Table 1. *Limiting dilutions of mycelianamide for complete inhibition of growth of various bacteria*

1 : 20,000 inhibits	1 : 33,000 inhibits	1 : 50,000 inhibits
Staph. aureus strains* 3093, 3095, 3750 and 3761	*Staph. albus* 3256*	*B. anthracis* 5444*
Strep. viridans 3166*	*Staph. aureus* 4163*	
Strep. pyogenes 2432* (on plain broth)		

* Of the National Collection of Type Cultures.

Table 1, from which it will be seen that *Staphylococcus aureus* and other Gram positive bacteria are completely inhibited by mycelianamide at concentrations of 0·002–0·005%. The compound has no marked action on Gram negative bacteria.

Mycelianamide was equally effective against Gram positive bacteria on plain heart broth containing no added glucose, and almost equally effective if an undiluted culture was used for sowing the tubes. Through the courtesy of Dr A. J. Ewins, F.R.S., we were able to compare the bacteriostatic action of mycelianamide with that of certain aromatic diamidines of comparable molecular complexity, and have found that 4:4′-diamidino-$\alpha\gamma$-diphenoxypropane ($C_{17}H_{20}O_2N_4$; mol. wt. = 312) is almost identically potent when tested in dilute aqueous solution, in that 1 part in 40,000 would inhibit the growth of *Staph. aureus* No. 3095, and 1 part in 20,000 would inhibit the growth of *Staph. aureus* No. 3750 for 1 day.

SUMMARY

1. A hitherto undescribed, colourless, crystalline, metabolic product, *mycelianamide*, $C_{22}H_{28}O_5N_2$, m.p. 170–172° decomp., has been isolated from the mycelium of *Penicillium griseo-fulvum* Dierckx.

2. Acid hydrolysis of mycelianamide under different conditions gives a new hydrocarbon, *mycelene*, $C_{10}H_{16}$, ω-amino-*p*-hydroxyacetophenone, acetaldehyde, ammonia and carbon dioxide.

3. Alkaline hydrolysis gives ammonia and a monobasic acid, $C_{17}H_{22}O_3$, which on acid hydrolysis affords mycelene and *p*-hydroxybenzoic acid.

4. Consideration of these and other reactions leads to the tentative conclusion that mycelianamide may be the *amide* of O-*mycelyl*-N-*pyruvyl*-β-*keto-tyrosine*.

5. Mycelianamide completely inhibits the growth of a number of Gram positive bacteria *in vitro* at concentrations of 1 : 20,000 to 1 : 50,000, but has no marked action on Gram negative bacteria.

We desire to thank Prof. J. L. Simonsen, F.R.S., for his examination of the hydrocarbon, mycelene; Dr A. J. Ewins, F.R.S., for a gift of aromatic diamidines; and Dr T. A. Henry for a gift of ω-amino-*p*-hydroxyacetophenone.

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Biochemistry of the Wood-rotting Fungi

5. THE PRODUCTION OF D-THREITOL (*l*-ERYTHRITOL) BY *ARMILLARIA MELLEA* (VAHL) QUÉLET

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The tetrahydric alcohol until recently known as erythritol is capable of existing in three stereoisomeric forms (if we exclude external compensation), the optical relationships being the same as for tartaric acid. The optically inactive internally compensated form, known as *meso*erythritol or *i*-erythritol has frequently been observed in nature. It is found in the algae *Protococcus vulgaris* (Lamy, 1852) and *Trentepohlia Jolithus* (Bamberger & Landsiedl, 1900). It also occurs in lichens of the genus *Roccella* (Stenhouse, 1848; Hesse, 1904) usually in the form of erythrin from which it can be obtained in the free state by hydrolysis. It has also been isolated from various fungi. Zellner (1910) obtained it from spores of *Ustilago maydis*, Oxford & Raistrick (1935) from the mycelium of *Penicillium brevi-*

compactum Dierckx and of *P. cyclopium* Westling, and Stodola (1946) from *Aspergillus terreus* Thom.

There is, however, no record in the literature of the natural occurrence of the optically active forms of erythritol, although they have been prepared by synthetic methods by Maquenne (1900) and others. This gap has now been partially filled by the isolation from a natural source of the form of erythritol which is dextrorotatory in water and laevorotatory in ethanol and which in accordance with the modern nomenclature is described as D-threitol. It was obtained in a yield of about 13 % on dry weight from the mycelial tissue of a freshly isolated laboratory culture of *Armillaria mellea* (Vahl) Quélet, one of the higher fungi. When subcultured in the laboratory under our conditions, the fungus rapidly lost its power of producing D-threitol. The species is widely distributed and has been reported as a cause of damage to a variety of plants ranging from forest trees to shrubs and herbaceous plants. It is very common on tree stumps, but may become parasitic, attacking the base of living trees.

The new product is apparently identical with the substance synthesized by Maquenne from natural xylose and described under the name of '*l*-erythritol'. The name was later changed to '*d*-erythritol' in accordance with the Rosanoff convention for sugars, but recently it has been thought advisable to change the name to D-threitol to relate it more closely to the tetrose threose.

EXPERIMENTAL

Culture. The culture was obtained by transplantation on to malt agar of a fragment of tissue from the fruiting body of *Armillaria mellea* found growing in a large clump at the foot of a horse-chestnut tree at Pinner, Middlesex. The inoculum was taken under sterile conditions from a newly exposed surface obtained by breaking the pileus.

Medium and conditions of culture. Two culture solutions were prepared from a basal medium of the following composition: glucose 30 g.; KH_2PO_4 2·5 g.; $MgSO_4.7H_2O$ 1·0 g.; $FeSO_4.7H_2O$ 0·01 g.; $ZnSO_4.7H_2O$ 0·01 g.; $MnSO_4.4H_2O$ 0·005 g.; $CuSO_4.5H_2O$ 0·005 g.; $Na_2B_4O_7.10H_2O$ 0·005 g.; NH_4Cl 2·5 g.; water 1000 ml. For culture medium *A* an addition of 10 g. of corn-steep powder, and for medium *B* an addition of 0·1 g. of Marmite was made.

The medium was distributed in conical flasks of 1 l. capacity, 350 ml. to each flask. It was sterilized by autoclaving at 110° and inoculated with fragments of tissue from slopes on malt agar containing an addition of 1 % of corn-steep solids. In culture on liquid media of depth about 1 in. the fungus forms a mass of tangled rhizomorphs, the lower portion of the mass eventually coming to rest on the bottom of the vessel and the upper portion appearing above the surface of the liquid.

Growth and harvesting of fungus. The rate of growth and utilization of sugar is much more rapid on medium *A* (corn steep) than on medium *B* (Marmite). The contents of single flasks were harvested after varying intervals of time. The culture solution was filtered from the total mycelium obtained from the six single flasks (Table 1). The mycelium was dried *in vacuo* (dry weight 20·00 g.), powdered and subjected to continuous extraction. After a preliminary extraction with light petroleum to remove lipid material the solid was subjected to exhaustive extraction with dry ether (16 working days). Even after this length of time, matter was still being extracted, but the daily yield had fallen to less than 0·1 g. of extractive. During the extraction a crystalline substance separated on the walls of the extraction flask and was scraped off daily before renewing the extraction. Evaporation of the ether gave a little more of this crystalline material (0·12 g.) and 0·25 g. of a non-crystalline oil. The extraction was continued with ethyl acetate, but only a further 0·05 g. of the crystalline solid was obtained; the total weight of crystals extracted was 2·62 g. or 13 % of the dry weight of mycelium.

Table 1. *Rate of growth and utilization of glucose by* Armillaria mellea *on synthetic media with added corn-steep solids* (*A*) or *Marmite* (*B*)

	Dry mycelium (g./flask)		Residual glucose by polarimeter (%)	
Medium	*A*	*B*	*A*	*B*
Period of growth (days)				
11	3·02	—	2·05	—
24	4·82	1·32	0·09	2·78
35	6·82	1·79	0·00	2·28
45	—	2·23	—	2·11

Identification of the crystalline solid as D-*threitol.* The crystalline solid was recrystallized from absolute ethanol and was thus obtained in the form of long colourless silky needles with a sweet taste. The m.p. was 90–91° (corr.), not changed by further recrystallization. The product dissolved readily in water to give a neutral solution, and gave a strong reaction for primary or secondary hydroxyl groups in the test of Fearon & Mitchell (1932). (Found: C, 39·4, 39·5; H, 8·3, 8·3. Calc. for $C_4H_{10}O_4$: C, 39·4; H, 8·3 %.) Tests for N, S, and halogen were negative. The substance was found to be optically active; the following rotations were observed. In 90 % ethanol, $[\alpha]_{5780}^{20°} = -13·3°$, $[\alpha]_{5461}^{20°} = -14·5°$ ($c = 5$ %). In water, $[\alpha]_{5780}^{20°} = +4·7°$, $[\alpha]_{5461}^{20°} = +5·5°$ ($c = 5$ %).

Dibenzylidene derivative. When the substance (0·1 g.) was treated with conc. HCl (2 ml.) and benzaldehyde (0·2 ml.) a colourless crystalline solid, insoluble in water, was obtained. This was collected, washed, dried and recrystallized from ethanol, then from benzene, and was thus obtained in the form of needles of m.p. 215–222° (corr.). (Found: C, 72·2; H, 6·2. Calc. for $C_{18}H_{18}O_4$: C, 72·5; H, 6·1 %.)

The properties of the fungal product are thus in general agreement with those described by Maquenne (1900) for '*l*-erythritol' now known as D-threitol. Complete identity of the product with D-threitol was established by the synthesis of the latter substance.

Preparation of D-*threitol.* The method chosen was that of Hockett (1935) who prepared dibenzylidene-D-threitol via D-threose from D-xylose. The dibenzylidene compound was hydrolyzed in aqueous H_2SO_4 to yield D-threitol.

Tetra-acetyl-D-xylononitrile. Early experiments gave very low yields even when the reactants were vigorously dried. The following reacetylation procedure increased the yield from about 20 to 52% of theoretical (Hockett (1935) claims 48%).

After the acetic anhydride had all been added the mixture was held at 70–75° for 15 min. and evaporated at this temperature under reduced pressure. When most of the solvent was removed a volume of acetic anhydride equal to half that originally employed was added. After 15 min. at 70–75° the solvent was evaporated and the procedure repeated with fresh acetic anhydride. After the final evaporation water was added and the mixture was boiled for 0·5 min. to decompose acetic anhydride. On cooling, the product solidified. It was collected, washed, dried and recrystallized from ethanol. It had m.p. 82–83° and was coloured slightly brown, but was sufficiently pure for the next stage.

D-Threose diacetamide. As the purification method of Hockett gave only a low yield it was decided to dispense with purification at this stage. The crude product, after evaporation of NH_3, partly crystallized on standing.

Dibenzylidene-D-threitol. The crude D-threose diacetamide was hydrolyzed in 0·1 N-H_2SO_4 at 100° for 6 hr. the solution being maintained acid to congo red by addition of 2 N-H_2SO_4 as required. The deep red solution was reduced with Na amalgam. Complete reduction (as indicated by Fehling's test) could not be effected, but when 80–90% reduction was attained the solution was adjusted to pH 6 and evaporated to small bulk. Ethanol was added and the mixture was cooled and filtered. The filtrate was evaporated to dryness, and the residue was taken up in conc. HCl (10 vol. based on the theoretical content of D-threitol) and shaken with benzaldehyde (2 vol. on same basis); the dibenzylidene derivative separated at once. The mixture was poured into ice and ether, warmed to 0° and the product collected, washed and dried. Yields were very variable and never greater than 23% of theoretical, based on nitrile, the best yields being obtained with small scale preparations using up to 12 g. of the nitrile. Recrystallized from benzene the product had m.p. 217–222° (corr.).

D-Threitol. The dibenzylidene compound (3·5 g.) was added to 2 N-H_2SO_4 (100 ml.), the mixture boiled until solution was complete and the benzaldehyde distilled off in steam. A slight excess of $CaCO_3$ was added, and the mixture was filtered. The filtrate was adjusted to pH 6 with H_2SO_4 and evaporated to dryness. The D-threitol was extracted from the solid residue by boiling with ethanol. The ethanol was removed and the residue crystallized from *n*-butanol. Yield 0·94 g. (66%), m.p. 90–91° (corr.). A further 10% was obtained from the mother liquors. The melting points and specific rotations of the natural and synthetic D-threitol and of their benzylidene derivatives are shown in Table 2.

Table 2. *Comparison of natural and synthetic* D-*threitol*

	D-Threitol							Dibenzylidene-D-threitol			
		Optical rotation							Optical rotation in chloroform ($c=1$%)		
		In water ($c=5$%)			In 90% ethanol ($c=5$%)						
	Melting point	$[\alpha]_{5461}^{20°}$	$[\alpha]_{5780}^{20°}$	$[\alpha]_{D}^{20°}$	$[\alpha]_{5461}^{20°}$	$[\alpha]_{5780}^{20°}$	$[\alpha]_{D}^{20°}$	Melting point	$[\alpha]_{5461}^{20°}$	$[\alpha]_{5780}^{20°}$	$[\alpha]_{D}^{20°}$
Natural	90–91° (corr.)	+5·5	+4·7	—	−14·5	−13·3	—	215–222° (corr.)	−97	−84	—
Synthetic (our observation)	90–91° (corr.)	+5·5	+4·5	—	−14·3	−13·0	—	217–222° (corr.)	−96	−87	—
Mixture of above	90–91° (corr.)	—	—	—	—	—	—	217–220° (corr.)	—	—	—
Synthetic (Maquenne, 1900)	88°	—	—	+4·33 ($c=6$%)	—	—	−10·50	231°	—	—	—
Synthetic (Hockett, 1935)	—	—	—	—	—	—	—	218–222°	—	—	−77·9
	—	—	—	—	—	—	—	220–222° (corr.)	—	—	−78·2

Rotation figures liable to an error of ±5%.

The figures for the melting point of the dibenzylidene compound of threitol quoted in the literature show a considerable range: 231° (Maquenne, 1900), 204–205° (Ruff, 1901), 218–222°, 220–222° (Hockett, 1935). Since the preparation of the compound introduces two new asymmetric carbon atoms into the molecule it is probable that the substances as obtained after recrystallization are mixtures of different stereoisomers. Melting points of dibenzylidene derivatives of other polyhydric alcohols quoted in the literature also show wide variations. The only reference found to this possibility, however, is in a paper by Radulescu & Tanasescu (1924) where two forms of *bis-o*-nitrobenzylidene-erythritol and two of *bis-m*-nitrobenzylidene-erythritol are described.

SUMMARY

A freshly isolated culture of the wood-rotting fungus *Armillaria mellea* (Vahl) Quélet, grown on a liquid culture medium, gave rise to D-threitol in the mycelium, the yield being about 13% of dry weight.

We have to thank Sir Norman Haworth, F.R.S., for valuable advice on the nomenclature of the polyhydric alcohols. One of us (C.E.S.) is indebted to the Therapeutic Research Corporation of Great Britain Ltd. for a grant which has enabled him to take part in this work.

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An Alkali-producing Mechanism in Macerated Leaves

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While investigating the enzymic demethylation of pectin in macerated leaves, a phenomenon not previously described was observed in some members of the Cucurbitaceae (Holden, 1945). When the fibre residue, obtained by squeezing out the sap from minced leaves, was washed with distilled water, it was found that successive washes had higher pH values until a maximum of pH 9·4–9·6 was reached, and this value was maintained on continued washing. The present paper concerns further investigations of this spontaneous rise of pH. The phenomenon would appear to be of importance in the extraction of normal and virus proteins from green leaves since the infectivity of a number of plant viruses is decreased by pH values greater than 9.

MATERIAL AND METHODS

The plants examined were obtained from various sources. All the Cucurbitaceae (except marrow, cucumber and bryony) were from the University Botanic Garden, Cambridge. *Nicotiana tabacum* and *N. glutinosa* were glasshouse grown and the rest of the plants were from gardens or were found growing wild in the neighbourhood of Rothamsted.

The leaves were minced with a domestic meat mincer and the sap squeezed out by hand through madapollam. The fibre was washed by suspending in distilled water (about 5 ml./g. fibre wet weight) and again squeezing out. Measurements of pH were made with a glass electrode.

Calcium was determined on ashed material. Ashing was done in porcelain crucibles over a Bunsen burner at *c.* 500° on weighed portions of fibre or measured volumes of sap that had been dried at 100°. The dried material was moistened with a few drops of HNO_3 to assist ashing. After weighing, the ash was dissolved in N-HCl, filtered, and calcium precipitated as oxalate; the latter was dissolved in H_2SO_4 and titrated with $KMnO_4$ (0·05 N or 0·01 N depending on the amount of oxalate).

Carbonate determinations were made by the method of Hutchinson & MacLennan as described by Piper (1942) except that $Ba(OH)_2$ was used instead of NaOH to absorb the CO_2 liberated.

Phosphorus was determined colorimetrically by a modification of the method of Kuttner & Lichtenstein (1932). Values for inorganic phosphorus were obtained by developing the colour in samples without previous incineration.

EXPERIMENTAL AND RESULTS

Plants showing alkaline drift

Plants of different families, including ten genera of the Cucurbitaceae, were examined and are listed in Tables 1 and 2. As the pH of the expressed sap from the leaves in which the pH rise was first observed was neutral or slightly alkaline, in contrast to the somewhat acid saps found in most plants, other plants known to have more alkaline sap were examined. Haas (1920) had recorded a pH as high as 8·5 for the expressed sap of sweet clover leaves. These were not available, but the sap of common yellow melilot (*Melilotus altissima*), which is closely related, had a pH of 7·8. On washing the fibre, however, the pH fell to 6·3. The pH of sunflower-leaf sap was given by Gustafson (1924) as between 6·3 and 6·9. This was confirmed and both sunflower (*Helianthus annuus*) and Jerusalem artichoke (*H. tuberosus*) were found to show the pH rise, though other members of the Compositae that were examined did not. All other members of the Cucurbitaceae as well as comfrey (*Symphytum officinale*) of the Boraginaceae and stinging nettle (*Urtica dioica*) of the Urticaceae showed the phenomenon. Although the pH tended to be higher in sap from leaves which showed the drift than from those which did not, this was not always so as can be seen from Tables 1 and 2. Young leaves either did not show the pH rise at all or only to a much less marked extent.

Fig leaves (*Ficus carica*) were anomalous in that the pH rose to 8 but no higher; these cannot therefore be included in either group.