Studies in the Biochemistry of Micro-organisms

76. MYCELIANAMIDE, $C_{22}H_{28}O_5N_2$, A METABOLIC PRODUCT OF *PENICILLIUM GRISEO-FULVUM* DIERCKX. PART I. PREPARATION, PROPERTIES, AND BREAKDOWN PRODUCTS

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The occurrence of a crystalline, ether-soluble, nitrogenous compound in the mycelium of *Penicillium griseo-fulvum* Dierckx, grown on synthetic media, has previously been mentioned in several studies from this laboratory on the metabolic products of this mould. The compound was first encountered by Anslow & Raistrick (1931) in their work on the isolation of 6-methylsalicylic acid. It was mentioned again by Oxford, Raistrick & Simonart (1935) in their memoir on fulvic acid, and the same authors later (1939) devised a method for separating it from the accompanying griseofulvin. To extend the account of the synthetical achievements of this biochemically remarkable mould we now give a description of our researches, as far as they have gone, into the constitution of this nitrogenous product which we propose to name 'mycelianamide', meaning an amide found in mycelium.

Mycelianamide, m.p. 170–172° decomp., is a colourless, laevorotatory, crystalline substance of empirical formula $C_{22}H_{28}O_5N_2$. It is sparingly soluble in the usual organic solvents and is very readily split both by acids and alkalis. The following is a list of the relevant facts concerning its properties and breakdown products.

(1) The compound has no basic properties but is a very weak acid, soluble in sodium carbonate solution but not in sodium bicarbonate or baryta water. It is at least a monobasic acid, but does not titrate sharply and suffers some decomposition during the titration. It has no phenolic properties. It could not be induced to give any reaction for a $>CO$ group.

(2) It gives an intense reddish brown colour with ferric chloride in ethanolic solution.

(3) After hydrolysis by hot concentrated HCl two bases were isolated as hydrochlorides, viz. ammonia and the known compound ω -amino-*p*-hydroxyacetophenone (1).

(4) Hydrolysis by boiling dilute sulphuric acid yielded an inseparable mixture of non-volatile pro-

ducts. The volatile products included CO_2 (1 mol. if the hydrolysis was continued long enough), ammonia (1 mol.) and a sweet-smelling unsaturated hydrocarbon $\text{C}_{10}\text{H}_{16}$ (b.p. 177°) subsequently referred to as 'mycelene', which appears not to be identical with any known hydrocarbon of this empirical formula. Some observations concerning its constitution and relation to mycelianamide appear in a subsequent paragraph. A little acetaldehyde is also produced in this hydrolysis.

(5) Hydrolysis by boiling dilute sodium hydroxide or sodium carbonate (or even bicarbonate) yielded ammonia (approximately 1 mol.) and an optically inactive, monobasic acid $\text{C}_{17}\text{H}_{22}\text{O}_3$, m.p. 118–120°, which has no phenolic, alcoholic or ketonic properties. On further hydrolysis with dilute sulphuric acid it yielded mycelene and *p*-hydroxybenzoic acid ($\text{C}_7\text{H}_6\text{O}_3$). This decomposition can be represented very simply as follows:

and the acid $\text{C}_{17}\text{H}_{22}\text{O}_3$ is clearly *p*-myceloxybenzoic acid (2; $R = \text{OH}$),

the third oxygen atom possessing an ether function. It is reasonable to assume that mycelene arises from a hypothetical alcohol, mycelyl alcohol $\text{C}_{10}\text{H}_{17}\text{OH}$, by loss of water, and that the cause of the remarkable instability of the ether link in the acid $\text{C}_{17}\text{H}_{22}\text{O}_3$ must lie in the constitution of the mycelyl radical (see later).

(6) Hydrolysis by cold very dilute ammonia (0.03N), continued for several days at room temperature, yielded a neutral compound $\text{C}_{17}\text{H}_{23}\text{O}_2\text{N}$, m.p. 120°, which by further acid hydrolysis again yielded mycelene and *p*-hydroxybenzoic acid. This neutral compound is therefore *p*-myceloxybenzamide (2; $R = \text{NH}_2$). It is obviously of importance to find out if the N atom in this breakdown product arises from the ammonia used in the hydrolysis or whether it is an integral part of the molecule of mycelianamide. All attempts to obtain *p*-myceloxybenzamide from mycelianamide directly by the use of other

alkalis in dilute solution failed. These included NaOH (0.01N and 0.03N), methylamine, potassium carbonate, and various mixtures of potassium carbonate and sodium bicarbonate all acting in the cold at a concentration of 0.03N. Only with methylamine was an appreciable amount of an insoluble, neutral compound obtained, and this proved to be uncrystallizable. It must be concluded that there is no evidence at the moment against the view that the N atom of *p*-myceloxybenzamide is not an integral part of the mycelianamide molecule but originates in the ammonia used in the particular mode of hydrolysis which leads to its formation.

p-Myceloxylbenzamide, as obtained above, is optically inactive. Since it is almost inconceivable that racemization could occur under the particularly mild conditions of this hydrolysis the important consequence follows that the mycelyl radical does not contain an asymmetric C atom.

(7) It is an interesting fact, although not of immediate importance with respect to the structure of mycelianamide, that this mould product exerts a marked bacteriostatic action on *Staphylococcus aureus*, quantitatively almost identical with that of 4:4'-diamidino- α - γ -diphenoxypropane (Ashley, Barber, Ewins, Newberry & Self, 1942).

As the simplest partial structural formula which will explain the above facts we suggest (3), viz. the amide of O-mycelyl-N-pyruvyl- β -keto-tyrosine, a structure which is obviously plausible from a phytochemical point of view.

Accepting the view previously arrived at that the $\text{C}_{10}\text{H}_{17}$ radical does not contain an asymmetric C atom it will be seen that (3) contains one asymmetric C atom, marked with an asterisk. The fact that mycelianamide is optically active eliminates one structure which is otherwise as satisfactory as (3), viz. that in which the side chain is

An attempt will now be made to account for the known facts listed above, concerning mycelianamide on the basis of (3).

(1) (3) contains no basic N atom, nor a COOH group. It ought, however, to be weakly acidic by virtue of the grouping $\cdot \text{CO} \cdot \text{CH} \cdot \text{CO} \cdot$, which is known to confer acidity on benzoylacetate ester and related compounds.

(2) The presence of this same grouping will explain the reddish brown colour with ferric chloride.

(3 and 4) Acid hydrolysis may be supposed to remove the mycelyl radical leaving a phenolic OH, to convert the acid amide grouping to COOH, and also to split the NH.CO link. The resulting amino

derivative of hydroxybenzoylacetate acid would readily lose CO_2 on further acid hydrolysis to yield structure (1). The acetaldehyde, which is produced in very small yield, might arise from the acetyl group at the end of the side-chain in (3) by the following side-reaction:

(5 and 6) *p*-Myceloxylbenzoic acid is formed from (3) by the ordinary alkaline breakdown of a substituted benzoylacetate acid leading to the formation of a benzoic acid and an acetic acid. With ammonia the following reaction must be supposed to occur:

The nearest analogy in the literature is the production of benzamide from the addition compound of ammonia and benzophenone by the action of water (Houben, 1924). The ammonia produced by the action of caustic soda on mycelianamide arises from the acid amide grouping.

A word or two may now be said on the constitution of the mycelyl radical. Four atoms of hydrogen are readily taken up by the catalytic reduction of mycelianamide in presence of palladium. Hence the mycelyl radical probably contains two double bonds, and the hypothetical mycelyl alcohol ought to be an open-chain diolefinic alcohol, in fact a near relative of geraniol. When mycelianamide was oxidized by ozone the only recognizable product was methylglyoxal (isolated as the *bis*-2:4-dinitrophenylhydrazone), which may mean that the double bonds are conjugated, but may also be interpreted to mean that mycelianamide contains the grouping $\text{CH}_3 \cdot \text{C} \cdot \text{CH}_2 \cdot \text{O} \cdot$, which would yield acetol (pyruvyl

alcohol, $\text{CH}_3\text{CO} \cdot \text{CH}_2\text{OH}$) by the action of ozone. The latter alcohol would be oxidized to methylglyoxal by the action of dinitrophenylhydrazine. This last explanation implies that mycelianamide is a substituted allyl ether of phenol. The allyl group in such compounds is known to be labile (Claisen, 1912) and the ready formation of mycelene by dilute acid hydrolysis may thus be explained. Mycelene has been examined by Prof. J. L. Simonsen, F.R.S., to whom we desire to express our most sincere thanks, and his observations are included in the experimental section. In brief, mycelene appears to be a monocyclic hydrocarbon containing two double bonds which are not conjugated. One of these double bonds is probably terminal, since formaldehyde is formed in quantity by the action of ozone. It seems very unlikely that mycelene is an isoprene derivative. Owing to the somewhat drastic conditions employed in the production of mycelene from mycelianamide,

the constitution of the former, if known, would provide no certain clues to the constitution of the mycelyl radical in mycelianamide. It is only necessary to recall that the primary alcohol geraniol is converted into the tertiary alcohol linalol merely by heating with water, in order to render probable the supposition that mycelyl alcohol would be an extremely labile substance.

As regards the biochemical origin of mycelianamide, a possible explanation may be that *P. griseo-fulvum*, which, from our previous researches, is known to be an extraordinarily prolific producer of phenolic substances from glucose, also elaborates the phenolic amino-acid tyrosine far in excess of its requirements. By β -oxidation, amidation, alkylation of the OH group and acylation of the NH_2 group, the excess of tyrosine is converted into an insoluble substance, as long as the medium remains no more alkaline than sodium bicarbonate solution, and is stored in the mycelium. It is significant that mycelianamide is formed when the source of N in the medium is either NO_3^- or NH_4^+ , and that the mould produces phenolic substances on both media.

EXPERIMENTAL

History of the culture used

The culture of *Penicillium griseo-fulvum* Dierckx used throughout this work was received in December 1929 from Prof. Ph. Biourge of the University of Louvain, and was numbered B. 34. It bears the London School of Hygiene Catalogue No. P. 38.

Extraction of mycelianamide

The cultural conditions (as regards modified Czapek-Dox medium with NaNO_3 as source of N) and the method of extraction of both griseofulvin and mycelianamide have already been given by Oxford *et al.* (1939). Mycelianamide is best purified by repeated crystallization from ethyl acetate in which it is moderately soluble while hot. The total yield of mycelianamide was more than twice that recorded for griseofulvin in the above memoir, i.e. about 100 g. from 2970 g. of dry mycelium derived from the growth of the mould on about 259 l. of medium containing about 21 kg. of glucose and 650 g. NaNO_3 . Mycelianamide was also isolated from the mycelium of *P. griseo-fulvum* grown on a medium containing ammonium tartrate as source of nitrogen. The mycelium examined was obtained from our experiments in 1935 (Oxford *et al.* 1935), in which 150 flasks of the following medium were employed: glucose, 50 g.; ammonium tartrate, 5 g.; KH_2PO_4 , 1.0 g.; KCl, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1000 ml. The sterilized flasks, each containing 350 ml. of the above medium, were inoculated with a spore suspension of *P. griseo-fulvum* and incubated at 19–21° for 50 days. The total weight of dried mycelium from three such experiments was 1119 g., yielding 81 g. of crude solid by ether extraction. By crystallization from benzene 51.7 g. of fairly pure mycelianamide m.p. 165° was obtained, containing, however, about 7% of a neutral, amorphous impurity which

could not be removed by recrystallization from any solvent. About 20 g. of pure mycelianamide was eventually obtained by extraction with cold very dilute NaOH, immediate acidification of the filtrate, and repeated recrystallization of the dried precipitate. Because of the difficulty of removal of the amorphous impurity it is recommended that the mould be grown on the glucose- NaNO_3 medium if it is desired to prepare mycelianamide in quantity.

Analysis and general properties of mycelianamide

Mycelianamide crystallizes from ethyl acetate in colourless, shining leaflets, m.p. 170–172° with vigorous decomposition. (Found: C, 66.09, 66.12, 66.20, 66.15; H, 6.94, 7.12, 7.20, 7.07; N, 7.06, 6.69, 7.33, 7.46%; mol. wt. (cryoscopic in dioxan), 400.5. $\text{C}_{22}\text{H}_{28}\text{O}_5\text{N}_2$ requires C, 65.97; H, 7.05; N, 7.00%; mol. wt. 400.) It contains no S, Cl, OCH_3 or NCH_3 .

0.1729 g. dissolved in ethanol (10 ml.) and water (3 ml.) required 7.58 ml. 0.1 N-NaOH for neutralization to phenolphthalein; hence equivalent = 228, but the end point was very indefinite and was further complicated by the production of a yellow colour after the addition of the first equivalent of alkali. Some unchanged substance could be obtained by recrystallization of the product obtained by immediate acidification of the titrated liquid.

Optical rotation: $[\alpha]_{5461}^{19} = -217^\circ$; $[\alpha]_{5790}^{19} = -182^\circ$ ($c = 0.8688$ in chloroform).

It is sparingly soluble in the cold in ethanol, chloroform, ether, benzene, ethyl acetate and glacial acetic acid; readily soluble in acetone and dioxan.

Mycelianamide is unchanged by boiling water, but is very unstable to cold, dilute NaOH, the solution, which froths readily like a soap solution, soon darkening in colour and acquiring the fragrant lavender-like odour referred to earlier. It is also soluble in Na_2CO_3 solution but insoluble in dilute NaHCO_3 , $\text{Ba}(\text{OH})_2$ or HCl. It forms a series of insoluble salts and even the Na salt is partly precipitated by the addition of NaCl to its neutral aqueous solution. The insoluble ferric salt is reddish brown, and the insoluble Cu and Ni salts are green. The yellow insoluble Ag salt becomes black on standing.

Colour reactions. A deep reddish brown colour results when a few drops of very dilute FeCl_3 are added to a solution of mycelianamide in ethanol or glacial acetic acid. The colour is discharged by dilute HCl. With 70% H_2SO_4 the colour was first yellow, becoming orange and finally deep brownish red on standing. Mycelianamide does not give the Millon reaction, the murexide reaction, the biuret reaction, or the *p*-dimethylaminobenzaldehyde reaction for indole derivatives. It could not be induced to form a picrate or methiodide, or give any reaction for a >CO group.

Mycelianamide is highly unsaturated and absorbs Br_2 rapidly in chloroform solution. Nothing crystalline resulted from numerous oxidation experiments with KMnO_4 , H_2O_2 , HNO_3 in glacial acetic acid and alkaline I_2 . Iodoform was produced in the last instance. Attempted acetylation by acetic anhydride in pyridine solution soon yielded a very dark-coloured liquid from which only amorphous products could be obtained. Methylation by diazomethane yielded a yellow, viscid oil, with an odour of impure acetamide, which was insoluble in both acids and alkalis.

Catalytic reduction of mycelianamide

Mycelianamide (0.832 g.) dissolved in ethyl acetate (100 ml.) was reduced by shaking in an atmosphere of H_2 with a catalyst prepared from palladium chloride (0.1 g.) and norite charcoal (1 g.). Uptake of H_2 was at first rapid (60 ml. in 17 min.) and ceased when 100 ml. had been absorbed in 146 min. (theor. for saturation of two double bonds = 93 ml.). After filtration, the solvent was removed *in vacuo* below 40° to leave a thick brown syrup which became an amorphous solid on treatment with boiling light petroleum, from which nothing crystalline could be obtained. It would be expected that new asymmetric centres might be formed by reduction and the product would then be a complex mixture containing several stereoisomers.

Oxidation of mycelianamide by ozone

Ozonized O_2 was passed through a solution of mycelianamide (0.5 g.) in chloroform (50 ml.) for 6 hr., the temperature being maintained at -10° to 0° . The ozone was completely absorbed throughout and there appeared to be much unchanged substance at the end. The liquid was twice extracted with water and twice with $NaHCO_3$ solution, and the extracts, after acidification with dilute HCl, were added to excess of 2:4-dinitrophenylhydrazine in 2N-HCl. A precipitate slowly formed and was collected after several days. Fine, orange-red needles were obtained by crystallization from aqueous dioxan, m.p. 302° decomp., alone or mixed with the authentic *bis*-2:4-dinitrophenylhydrazone of methylglyoxal. The respective colour reactions with ethanolic KOH were also identical.

Action of hot conc. HCl on mycelianamide; formation of NH_4Cl and the hydrochloride of ω -amino-*p*-hydroxyacetophenone (1)

Mycelianamide (10 g.) and conc. HCl (1000 ml.; *d* 1.16) were heated together for 15 hr. at 100 – 105° with frequent shaking. The dark-coloured liquid was then filtered from tar, repeatedly extracted with ether until the extracts were almost colourless, freed from dissolved ether by the passage of a rapid stream of air for several hours, and finally taken to dryness *in vacuo* at 50 – 60° . The last traces of HCl were removed from the almost black solid residue by repeated shaking with ether. The residue was taken up in a little hot ethanol, and the undissolved part (mostly NH_4Cl) was benzoylated by the Schotten-Baumann method to yield benzamide, m.p. and mixed m.p. 126 – 128° . Fractional precipitation of the ethanolic extract with ether yielded a crystalline hydrochloride (prisms) and NH_4Cl . The latter, precipitated in a very finely divided condition, came through the filter on suction and was thus removed.

The crude hydrochloride remaining on the filter (1.2 g.) was purified by repeated crystallization from ethanol-ether. It formed almost colourless, compact prisms, m.p. 249 – 253° decomp., which were readily soluble in water to give a neutral solution which gave a purple colour with $FeCl_3$, an intense yellow colour with bleaching powder solution, a positive Millon reaction but a negative Denigé-Mörner reaction for tyrosine. The compound was the hydrochloride of a primary amine, since an effervescence was immediately

observed when very dilute HCl was added to its solution in very dilute $NaNO_2$. The NH_2 was not directly attached to a benzene nucleus since no dyestuff was produced by diazotization and coupling with β -naphthol. Furthermore, the phenolic OH appeared to have a free position *ortho* or *para* to it since an intense orange-red colour was given with diazotized sulphanilic acid. The amine contained no $-CHO$ group since it did not restore the colour to Schiff's reagent.

A *platinichloride* was prepared by addition of 5% platinum chloride solution (10 ml.) to a solution of the hydrochloride (0.15 g.) in water (2 ml.). The mixture was filtered and kept in the cold room when orange needles and prisms separated; m.p. 224° decomp. (Found, on material dried at 15° : C, 25.74; H, 3.31; N, 3.90, 4.02; Pt, 25.89%; loss at 100° *in vacuo*, 4.53%. $(C_8H_9O_2N)_2 \cdot H_2PtCl_6 \cdot 2H_2O$ requires C, 25.65; H, 3.23; N, 3.74; Pt, 26.08; $2H_2O$, 4.81%. Found, on material dried at 100° *in vacuo*: C, 26.94; H, 2.99; Pt, 26.66%. $(C_8H_9O_2N)_2 \cdot H_2PtCl_6$ requires C, 26.96; H, 2.83; Pt, 27.39%.)

The amine hydrochloride also yielded a picrate (needles, m.p. 188 – 190° decomp.) and a benzoate, by the Schotten-Baumann method, which formed long needles from ethanol, m.p. 177 – 178° . (Found: C, 73.49, 73.30; H, 4.83, 4.77; N, 4.33, 4.21%; mol. wt. (cryoscopic in dioxan), 303. Calc. for $C_{22}H_{17}O_4N$ (dibenzoate of $C_8H_9O_2N$): C, 73.52; H, 4.77; N, 3.90%; mol. wt. 359.)

The free amine is therefore $C_8H_9O_2N$, and the most likely structure which will fit the facts recorded above is $HO \cdot C_6H_4 \cdot CO \cdot CH_2 \cdot NH_2$.

ω -Amino-*p*-hydroxyacetophenone (1) was first made synthetically by Tutin, Caton & Hann (1909) who recorded the following constants: m.p. 190 – 193° decomp.; hydrochloride, prisms, m.p. 245 – 252° decomp.; picrate, needles, m.p. 192° decomp.; dibenzoate, needles, m.p. 173 – 174° . The melting points given for the three derivatives are all close to the values given above for the corresponding derivatives of the amine from mycelianamide. Through the great kindness of Dr T. A. Henry we have been able to make a direct comparison of our hydrochloride with that made by Tutin *et al.* in 1909 at the Wellcome Chemical Research Laboratories. The latter hydrochloride gave a purple colour with $FeCl_3$ and an intense yellow colour with bleaching powder solution; it melted at 246 – 253° alone or mixed with our hydrochloride, and readily yielded a benzoate of m.p. 176 – 178° , alone or mixed with the benzoate derived from our hydrochloride. There can thus be no doubt that the amine derived from mycelianamide is ω -amino-*p*-hydroxyacetophenone, but, in our opinion, the description of this compound given by Tutin *et al.* (1909) requires amendment in one or two particulars. We have prepared the free amine from their specimen of the hydrochloride, and also from our own, and find that it does not melt at 190 – 193° as stated, but rather begins to darken at 190° and progressively darkens and softens from 200 to 300° , with production of a sublimate of yellow needles which do not melt at 310° . The amine sublimes readily at 150 – 170° *in vacuo* to yield a product which behaves exactly as above. (Found on sublimed material from the product obtained by the action of Na_2CO_3 on our amine hydrochloride: C, 63.68; H, 6.07; N, 9.50%. $C_8H_9O_2N$ requires C, 63.54; H, 6.00; N, 9.27%.) Tutin *et al.* (1909) further stated that the *platinichloride* and *aureichloride* were both too soluble to admit of isolation. In our experience this statement applies only to the *aureichloride*.

Hydrolysis with boiling dilute H_2SO_4 ; production of NH_3 (1 mol.), CO_2 (1 mol.) and mycelene, $\text{C}_{10}\text{H}_{16}$

Mycelianamide (7.85 g.) suspended in a mixture of water (800 ml.) and $\text{N-H}_2\text{SO}_4$ (450 ml.) was refluxed for 9.5 hr., in a stream of N_2 , until the solid had completely disappeared and had been replaced by a dark-coloured oil floating on a pale-yellow liquid. CO_2 was evolved slowly but continuously throughout and was absorbed in baryta bubblers, the total amount being equivalent to 22.5 ml. of N-Ba(OH)_2 , i.e. 0.57 mol. of CO_2 /mol. of mycelianamide. In a separate experiment, in which the hydrolysis was continued until CO_2 ceased to be evolved, mycelianamide (1.0618 g.) was refluxed with $2\text{N-H}_2\text{SO}_4$ (60 ml.) in a stream of N_2 for 13 hr. CO_2 was evolved equivalent to 53.8 ml. 0.1N-Ba(OH)_2 . Theor. for 1 mol. CO_2 /mol. of mycelianamide, 53.1 ml.

The reaction liquid from the 7.85 g. of mycelianamide was distilled in steam for 5 hr. until no more oil came over.

The acid hydrolysis liquid was treated as follows. A sample was made alkaline and distilled to yield 55% of the N originally present in the mycelianamide, $\text{C}_{22}\text{H}_{28}\text{O}_5\text{N}_2$, as a volatile base which was identified as NH_3 by conversion into benzamide, m.p. and mixed m.p. $126\text{--}127^\circ$. Hence only one of the two N atoms present was accounted for. Part of the remaining N was present in an intractable tar (wt. 0.5 g.) which was formed during the hydrolysis. (Hydrolysis of mycelianamide with 30% (w/w) H_2SO_4 yielded much the same results, save that the amount of tar formed was much greater, and 52% of the N present in the mycelianamide was accounted for as NH_3 .) All attempts to isolate crystalline compounds in a state of purity from the hydrolysis liquid failed so that the fate of the second N atom present in mycelianamide remains undetermined. At least two crystalline compounds appeared to be present, viz. clusters of massive plates, m.p. about 150° , and small feathery crystals, m.p. about 100° , both soluble in ether and crystallizing from benzene. Neither substance could be obtained free from adhering oil in quantities sufficient for analysis and characterization. Neither of them was identical with *p*-myceloxybenzoic acid, $\text{C}_{17}\text{H}_{22}\text{O}_3$, m.p. $118\text{--}120^\circ$, formed in the alkaline hydrolysis (see p. 328).

The steam distillate was treated as follows. A sample was titrated but no volatile acid was detected. The oil was extracted with ether, and the extract dried over anhydrous Na_2SO_4 . After removal of the solvent the residual oil was distilled at atmospheric pressure to yield 1.56 g. of distillate, the middle fraction of which (0.68 g.) came over at $177\text{--}178^\circ$ and was almost colourless. By redistillation *in vacuo* it was obtained as a colourless limpid oil with a strong lavender-like odour. (Found: C, 84.26, 84.51; H, 11.21, 11.07; O (by difference), 4.53, 4.42%.) The atomic C : H ratio calculated from the above figures is 1 : 1.57, and since the boiling point is close to that of several terpenes, e.g. *d*-limonene, $\text{C}_{10}\text{H}_{16}$, boils at 176° , there can be little doubt that the product is essentially a hydrocarbon. The oxygen-containing impurity was not water since the oil submitted to analysis gave an absolutely clear and bright solution in light petroleum.

Several grams of the once-distilled hydrocarbon were prepared and were examined by Prof. J. L. Simonsen, F.R.S., at that time of the University College of North Wales, Bangor, who in a letter dated 24 April 1937, reported as follows.

'The hydrocarbon had b.p. $73\text{--}75^\circ/20\text{ mm.}$, d_{25}^{25} 0.8583, n_D^{20} 1.4780, $[\alpha]_{5461} = 0^\circ$. Molecular refractivity, 45.1; calc. $\text{C}_{10}\text{H}_{16}$ with two ethylenic linkages 44.6. (Found on sample distilled over sodium: C, 84.6; H, 11.4; after 3 days C, 83.5; H, 11.1; after 4 days C, 83.7; H, 11.0%.) The hydrocarbon is obviously very readily oxidized. It polymerizes to a gum on standing.

The hydrocarbon (3.5 g.) in ethyl acetate (30 ml.) was ozonized at 0° ; a considerable quantity of formaldehyde was present in the issuing gases (dimedone test). After removal of the ethyl acetate *in vacuo*, the ozonide was decomposed in water (10 ml.) by heating on the water bath for 1 hr. The oil was extracted with ether, the ethereal extract washed with Na_2CO_3 solution and NaOH , dried over MgSO_4 and the solvent removed. A pleasant smelling oil (2 g.) remained. This gave no colour with FeCl_3 , reduced Fehling's solution and gave a silver mirror (poor). Satisfactory products were not obtained with semicarbazide, phenylsemicarbazide, *p*-nitrophenylhydrazine. The 3,5-dinitrophenylhydrazine separated at once as an amorphous orange-yellow powder. It was very sparingly soluble in ethanol, but crystallized from ethyl acetate-ethanol in what appeared to be rosettes of terracotta prisms, m.p. $170\text{--}173^\circ$ raised to $180\text{--}182^\circ$ by recrystallization. (Found: C, 48.1; H, 4.4; N, 22.1. $\text{C}_{22}\text{H}_{22}\text{O}_8\text{N}_8$, i.e. *bis*-dinitrophenylhydrazine of $\text{C}_8\text{H}_{14}\text{O}_2$, requires C, 47.8; H, 4.3; N, 22.3%.)

The acid fractions weighed respectively 0.3 and 0.8 g. Both gave amorphous derivatives with carbonyl reagents, and bromoform with NaOBr . They were combined and oxidized in alkaline solution with this reagent. The resulting acid was a viscid oil. It did not give an anhydride with acetyl chloride; the *p*-bromophenacyl derivative was a viscid gum.

I would suggest that the following conclusions can be drawn tentatively:

(1) The hydrocarbon is cyclic and contains two ethylenic linkages; one of these is exocyclic (formaldehyde) and the other as the group Me.C:CH- . The formation of a keto-

aldehyde having the composition $\text{C}_8\text{H}_{14}\text{O}_2$ might result in accordance with the following scheme:

(2) This receives support from the fact that the acid fraction does contain the grouping MeCO- as shown by the NaOBr oxidation with the formation of bromoform. The non-formation of an anhydride suggests that the dibasic acid (if it be such) is an adipic acid.

(3) It seems very unlikely that the hydrocarbon is an isoprene derivative.'

*Hydrolysis with moderately strong H_2SO_4 ;
production of acetaldehyde*

A mixture of mycelianamide (2 g.) and 30% (w/w) H_2SO_4 (200 ml.) was boiled in a flask connected to a long condenser, the liquid being replaced by distilled water, from a dropping funnel, as fast as it distilled over. After 7 hr. the distillate (900 ml.) was filtered free from oil and redistilled, the first 100 ml. being collected in an ice-cooled receiver. To this was added conc. HCl (10 ml.) and a saturated solution of 2:4-dinitrophenylhydrazine in 2N- HCl (25 ml.), and the resulting mixture kept overnight at 0°. The precipitated dinitrophenylhydrazone, after two crystallizations from light petroleum, formed shining orange plates, m.p. 161–163°, alone or mixed with the authentic 2:4-dinitrophenylhydrazone of acetaldehyde. A mixed melting point with formaldehyde 2:4-dinitrophenylhydrazone showed a large depression. The yield of acetaldehyde in the above hydrolysis was very small.

*Hydrolysis with boiling dilute NaOH , Na_2CO_3 or NaHCO_3 ; formation of NH_3 and *p*-myceloxybenzoic acid (2; $R = \text{OH}$)*

Similar results were obtained with all three alkalis save that the liberation of NH_3 was very slow with NaHCO_3 as the hydrolyzing agent. The following is a typical experiment with Na_2CO_3 . A solution of mycelianamide (1 g.) in 0.5N- Na_2CO_3 (100 ml.) was very slowly distilled in a current of N_2 . After about 3 hr. the distillate no longer turned red litmus blue and the total volatile base which had distilled over was equivalent to 21.8 ml. of 0.1N- HCl , i.e. contained 44% of the N originally present in 1 g. of mycelianamide. The HCl -neutralized distillate, which contained a few drops of mycelene, was taken to dryness *in vacuo* to yield NH_4Cl (fern-like aggregates of needles, m.p. above 300°) which, when benzoylated by the Schotten-Baumann method, yielded benzamide, m.p. and mixed m.p. 126–128°. The brownish red liquid remaining in the distillation flask was acidified with dilute H_2SO_4 and exhaustively extracted with ether to yield a dark-brown syrup, a very small portion of which was soluble in hot light petroleum. Concentration of the petroleum extract yielded colourless slender needles (40 mg.), m.p. 118–120°, after purification by recrystallization from light petroleum. (Found: C, 74.20, 74.39; H, 8.10, 8.10%; N, nil.; mol. wt. (cryoscopic in camphor), 276, 280. $\text{C}_{17}\text{H}_{22}\text{O}_3$ requires C, 74.39; H, 8.09%; mol. wt. 274.)

The product (0.0548 g.) in a mixture of ethanol (6 ml.) and water (4 ml.) required 2.03 ml. of 0.1N- NaOH for neutralization to phenolphthalein; hence equivalent = 270 and the acid is monobasic. The titrated liquid was optically inactive and the unchanged acid was recovered from it by acidification.

The acid $\text{C}_{17}\text{H}_{22}\text{O}_3$ (*p*-myceloxybenzoic acid) is insoluble in water but readily soluble in all organic solvents with the exception of light petroleum. It forms a sparingly soluble Na salt, gives no colour with FeCl_3 and only a faint Millon reaction on warming. It could not be induced to form a semicarbazone or 2:4-dinitrophenylhydrazone, and was recovered unchanged after treatment with acetic anhydride in pyridine at room temperature for 2 days.

*Hydrolysis of the acid $\text{C}_{17}\text{H}_{22}\text{O}_3$ with production of mycelene and *p*-hydroxybenzoic acid*

The acid (0.6 g.) was treated for 3 hr. with boiling 0.5N- H_2SO_4 (240 ml.) under reflux condenser. One hundred ml. were then distilled off to yield discrete drops of oil in the first runnings, with the characteristic odour of mycelene. The liquid in the distillation flask was cooled, extracted four times with ether, and the extracts dried over anhydrous Na_2SO_4 . The yellow crystalline residue (0.3 g.) left after removal of the solvent was crystallized from benzene containing some chloroform to yield colourless flat prisms, m.p. 210–212°, alone or mixed with authentic *p*-hydroxybenzoic acid, m.p. 212°. (Found: C, 61.02, 60.87; H, 4.30, 4.38%. Calc. for $\text{C}_7\text{H}_6\text{O}_3$: C, 60.84; H, 4.38%.)

*Action of cold dilute ammonia on mycelianamide;
production of *p*-myceloxybenzamide (2; $R = \text{NH}_2$)*

Mycelianamide (50 mg.) was dissolved in N- NH_4OH (6.6 ml.) by gentle shaking, and the solution immediately diluted to 200 ml. with distilled water. A crystalline deposit began to appear after standing for 1 day at room temperature and was collected on the fourth day. The filtrate deposited very little more of the product after standing for a further 3 days; total yield, 15 mg. of m.p. about 120°. *p*-Myceloxylbenzamide formed colourless silky needles from benzene containing some light petroleum; m.p. 120°, considerably depressed on admixture with *p*-myceloxybenzoic acid, m.p. 118–120°. (Found: C, 74.65, 74.8; H, 8.43, 8.56; N, 5.0, 5.2%. $\text{C}_{17}\text{H}_{23}\text{O}_2\text{N}$ requires C, 74.66; H, 8.49; N, 5.1%.) A 1.1% solution in acetone gave absolutely no rotation in a 0.5 dm. tube.

Hydrolysis. The above compound, m.p. 120° (90 mg.), was treated with boiling 20% (w/w) H_2SO_4 (25 ml.) under reflux condenser for 3 hr. Drops of volatile oil were observed in the condenser after a very short time and the fragrant odour was in evidence (formation of mycelene). The reaction liquid was cooled, filtered, extracted once with a little light petroleum and then twice with ether. An almost colourless solid was obtained from the ether extract, which, after treatment with a little boiling benzene and boiling carbon tetrachloride, melted at 210–212°. Mixed with authentic *p*-hydroxybenzoic acid the m.p. was 212–214°. The colour reactions with Millon's reagent and with FeCl_3 were identical with those given by *p*-hydroxybenzoic acid.

Bacteriostatic action of mycelianamide

The method of testing used was that described by Oxford (1942) modified to meet the case of a compound insoluble in water but soluble in ammonia. A 1:4000 solution of mycelianamide in 0.03N- NH_4OH was made as described above, sterile N- NH_4OH and sterile distilled water being used, and employed immediately for the test. Volumes up to 1 ml. of this solution were added aseptically to 4 ml. quantities of sterile 2% glucose broth and the volumes in the tubes made up to 5 ml., if necessary, by the addition of sterile 0.03N- NH_4OH . The controls contained 4 ml. of sterile glucose broth plus 1 ml. of sterile 0.03N- NH_4OH . The inoculum given in all cases was 0.04 ml. of a 1-day culture in glucose broth which had been diluted 1000 times. The tubes were read after 1 day's incubation at 37°. All the controls grew normally. The results are summarized in

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
<i>Staph. aureus</i> strains* 3093, 3095, 3750 and 3761	<i>Staph. albus</i> 3256*	<i>B. anthracis</i> 5444*
<i>Strep. viridans</i> 3166*	<i>Staph. aureus</i> 4163*	
<i>Strep. pyogenes</i> 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 (*D*-ERYTHRITOL) BY *ARMILLARIA MELLEAE* (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 *mesoerythritol* 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 *Rocella* (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-*