Biochemistry of the Wood-rotting Fungi

7. METABOLIC PRODUCTS OF *POLYPORUS BENZOINUS* (WAHL.) FR.

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The fungus *Polyporus bovinus* (Wahl.) Fr., although comparatively rare in Britain, is fairly common on the Continent, particularly in Sweden, where it attacks coniferous logs, especially *Abies* species. It produces in culture a marked aromatic odour and for this reason came under examination in our investigation of the odoriferous products formed by some of the wood-rotting fungi (Birkinshaw & Findlay, 1940; Birkinshaw, Bracken & Findlay, 1944; Birkinshaw & Morgan, 1950). The present communication gives a complete account of this work so far as it has proceeded. A summary of our findings has already appeared (Morgan, 1951).

In preliminary investigations the fungus was grown on aqueous synthetic medium, containing glucose, nutrient salts and Marmite. Ether extraction of the wet minced mycelium gave a crude mixture of metabolic products with a pronounced aromatic odour. Treatment of the ether residue with fresh ether gave a white powder, acid to litmus, of m.p. 285–290° (decomp.). The ether filtrate after removal of this sparingly soluble acid was extracted with aqueous sodium bicarbonate. Acidification of the separated bicarbonate layer afforded a white precipitate of a complex aliphatic acid, of m.p. 53–54.5°. The bicarbonate-extracted ether layer, after removal of solvent, left a fatty residue which still retained the aromatic odour. Steam distillation of this material gave a colourless aromatic oil which was identified as anisaldehyde. This substance is probably the main contributor to the odour of the fungus in culture. Anisaldehyde has already been reported as one of the metabolic products of *Trametes suaveolens* (Birkinshaw *et al.* 1944).

The odorous constituent having been identified, further attention was given to the solid substances extracted from the wet mycelium by ether. The medium so far employed gave poor growth and mycelial development, and towards the end of the long incubation period there were signs of incipient autolysis. After continued cultivation of the fungus on this medium the yields of the two acidic products fell almost to zero. A search for a more suitable medium led to the use of an aqueous Vimaltol-Marmite medium which permitted vigorous growth and gave much enhanced yields of the acidic products, in addition to the odorous constituent.

The extraction procedure with the new medium was similar to that previously adopted, but it was found that when the ether filtrate was treated with aqueous sodium bicarbonate the separated bicarbonate layers gave on standing a crystalline sodium salt of the aliphatic acid. The salt was only slightly soluble in cold water and was obtained in almost pure condition, much purer than the acid which had previously been obtained by acidification of the total bicarbonate layer.

In the later stages of the work the mycelium, on removal from the culture flasks, was submitted to the action of a high-speed disintegrator. This reduced it to a viscous paste and considerably shortened the time necessary for its complete extraction by ether. In addition, it led to a further increase in the amount of the aliphatic acid recovered as the sodium salt. The acid of m.p. 285–290° (decomp.) was found by analysis to have a composition corresponding with $C_{30}H_{46}O_4$, or possibly $C_{30}H_{48}O_4$. It gave characteristic colour reactions with the Liebermann reagent and with the Salkowski reagent. Etheral diazomethane converted it into a neutral monomethyl ester $C_{31}H_{48}O_4$ (or $C_{31}H_{50}O_4$), m.p. 192–193°. Acetylation, preferably at room temperature, gave a crystalline diacetate, m.p. 212–214° (decomp.), titrating in the cold as a monobasic acid. When subjected to ozonolysis in chloroform solution it afforded 53% of 1 mol. of formaldehyde.

These properties indicate that the substance is in all probability a dihydroxymonocarboxylic acid of formula $C_{30}H_{46}O_4$, and possessing a terminal methylene group. It would seem to fall into the group of triterpenes, a number of which occur naturally and have already been described. Amongst these the triterpene alcohols, baeoeol, lupeol ($C_{30}H_{50}O$; Heilbron, Moffat & Spring, 1934) and betulin ($C_{30}H_{50}O_2$; Vesterberg, 1923) are each characterized by the presence of an exocyclic methylene group. A comparison of the acid from *Polyporus bovinus* with certain triterpenoid acids isolated from *P. betulinus* by Cross, Eliot, Heilbron & Jones (1940) shows striking points of resemblance. These authors minced the fresh fungus, as found in nature, and extracted it with cold ethanol and subsequently under reflux with a mixture of acetone and

ether. From the ethanolic extract after saponification they isolated a mixture of sterols and a crystalline acid $C_{30}H_{48}O_4$ or $C_{30}H_{50}O_4$, m.p. 194° , which they named polyporenic acid *A*. Hydrolysis of the acetone-ether extract yielded two further acids, polyporenic acid *B*, m.p. $300\text{--}310^\circ$ (decomp.), of similar composition to *A*, and, in small amount, polyporenic acid *C* m.p. $270\text{--}275^\circ$. The last named acid was analysed only as its methyl ester, $C_{31}H_{48(50)}O_4$, m.p. $192\text{--}193^\circ$. The melting point of the free polyporenic acid *C* and of its methyl ester and the analysis of the methyl ester were so close to those of our triterpenoid acid that the possibility arose that the two acids might be identical. This possibility was further strengthened by the observation that the colour reactions of our acid with the Liebermann and Salkowski reagents were similar to those described for polyporenic acid *C*. Through the courtesy of Prof. E. R. H. Jones, F.R.S., and Dr T. G. Halsall of Manchester University, a small sample of the methyl ester of polyporenic acid *C* was made available to us. The sample showed no depression in melting point when mixed with our methyl ester, indicating that our acid was in fact identical with polyporenic acid *C*.

The suggestion of Cross *et al.* (1940) that polyporenic acid *C* may be identical with gypsogenin would appear to be discounted if the identity of polyporenic acid *C* with our triterpenoid acid is accepted, since ozonolysis of the latter has afforded evidence of a terminal methylene group which is not present in gypsogenin. Further, gypsogenin contains an aldehyde group, which cannot be present in our triterpenoid acid since all the oxygen atoms are otherwise accounted for, two being present in a carboxyl group and one in each of two hydroxyl groups.

Cross *et al.* (1940) put forward an interesting speculation as to the possible interrelationship between the acids of the parasitic fungus, *P. betulinus*, and the dihydric triterpene alcohol betulin, $C_{30}H_{50}O_2$, which forms a major constituent of the bark of the host tree (*Betula alba*). This hypothetical relationship involves the interchange of a methyl and carboxyl group ($CO_2H \rightleftharpoons CH_3$), and would apparently imply that the triterpenoid acids found in the fungus are derived with some modification from the host tree and are not elaborated by the parasite. The present work shows that a fungal species of the same genus, *Polyporus benzoinus*, is capable of synthesizing one of these triterpenoid acids when grown on a synthetic medium containing only glucose (and a little Marmite) as source of carbon. Hence it appears likely that *P. betulinus* has the same power of synthesis of the triterpenoid nucleus and is not dependent on the host plant for its supply of this complex molecule.

It is interesting to note that the polyporenic acids

A, *B* and *C* were isolated after saponification of the extracts of the naturally occurring sporophore of *P. betulinus*. Extracts from mycelium of *P. benzoinus* cultured by us in the laboratory yielded the acid *C* directly without previous saponification, indicating that, in this fungus, it occurs in the free state.

Further examples of triterpenoid acids derived from wood-rotting fungi have recently been described. Eburicoic acid, $C_{30}H_{48}O_3$, m.p. 293° , was first isolated by Kariyone & Kuruno (1940) from *Fomes officinalis* Fr. and was further characterized by Gascoigne, Holker, Ralph & Robertson (1950, 1951) and by Lahey & Strasser (1951) who obtained the acid from *Polyporus anthracophilus* Cooke. It was also isolated from the mycelium of *P. eucalyptorum* Fr., *P. sulphureus* (Bull.) Fr. and *Lentinus dactyloides* Cleland. The acid contains in addition to carboxyl a secondary hydroxyl and an exocyclic methylene group.

From *Trametes odorata* (Wulf.) Fr., Gruber & Proske (1950) isolated trametenolic acid, $C_{30}H_{48}O_3$, m.p. $259\text{--}261^\circ$, which also contains a secondary hydroxyl group.

The crystalline sodium salt obtained from the bicarbonate extraction of the ether extract of the mycelium is converted on acidification into one or other of two compounds depending on the procedure employed. The more stable form, m.p. $53\text{--}54.5^\circ$, is prepared by acidification of an aqueous solution of the sodium salt. Analysis indicates the formula $C_{22}H_{36}O_5$ or $C_{22}H_{35}O_5$. The equivalent determined by titration in the cold is intermediate between the values required for a dibasic and a tribasic acid; after refluxing with excess of standard alkali the acid becomes fully tribasic. Acidification of the hydrolysate leads to recovery of the original acid of m.p. $53\text{--}54.5^\circ$. If the argument which follows as to the function of the five oxygen atoms is accepted, namely that three are present in an acid anhydride grouping and two in a lactone group, then the formula $C_{22}H_{35}O_5$ is untenable, since the formula $C_{22}H_{36}O_5$ would represent a fully saturated compound with no carbocyclic ring.

The less stable form of the acid is prepared by shaking a suspension of the sodium salt in ether with dilute hydrochloric acid (2*N*). It has m.p. 78° and is less soluble in organic solvents than the lower melting form, particularly in light petroleum. Analysis of this acid indicates that it is a hydrated form of the acid of m.p. $53\text{--}54.5^\circ$, agreeing with a formula of $C_{22}H_{38}O_6$ with a little additional water. This acid is readily dehydrated to the acid of lower melting point; the melting point falls from 78 to $55\text{--}65^\circ$ on keeping for 6 weeks at room temperature in a stoppered tube. The simplest interpretation of this behaviour is to regard this dehydration as due to the formation of an acid anhydride. We therefore

propose for the higher melting acid the name of ungulinic acid derived from *Ungulina fuliginosa* (Scop.) Pat, a synonym of *Polyporus bozoinus* (Wahl.) Fr. The acid of lower melting point then becomes ungulinic acid anhydride.

Ungulinic acid is apparently optically inactive. Ethereal diazomethane converts it into a dimethyl ester $C_{24}H_{42}O_6$, a pale-yellow oil. This ester is convertible into a trihydrazide, m.p. 190–195° (decomp.), which could not be recrystallized. Both ungulinic acid and its anhydride give colour reactions which are identical with those described for agaricic acid, which is a hexadecylcitric acid.

The neutral sodium salt of ungulinic acid which crystallizes from the bicarbonate layer during the extraction process has a sodium content in agreement with that of the disodium salt $C_{22}H_{36}O_6Na_2$. The equivalent of this salt, determined by titration after refluxing with excess standard alkali, agrees with the formula $C_{22}H_{36}O_6Na_2$ reacting as a monobasic acid. If a stream of carbon dioxide is passed through the neutralized solution after titration, a crystalline precipitate of a monosodium salt is obtained and when this is decomposed with ether-dilute hydrochloric acid, ungulinic acid is recovered.

Fusion of the disodium salt with potassium hydroxide afforded a mixture of stearic, acetic and oxalic acids. A consideration of the experimental results indicates that ungulinic acid probably bears a general structural relationship to certain polybasic aliphatic acids with long carbon chains which are metabolic products of other fungi and of lichens. Amongst these are spiculisporic, minioluteic and agaricic acids.

Like these acids ungulinic acid is potentially tribasic. If we assume that the three acidic groups are carboxyls, then the stable form of the acid $C_{22}H_{36}O_6$ must be accounted for by anhydride formation. This indicates a constitution of a substituted succinic or glutaric acid type. Tetraethylsuccinic acid (Walker & Walker, 1905) shows a similar ready convertibility to the anhydride, since this change occurs on melting the acid or on keeping it in aqueous solution for 2 days. The third carboxyl group is assumed to be present in lactonic form since the disodium salt is neutral, yet one acid equivalent is revealed on alkaline hydrolysis. This again emphasizes the relationship with spiculisporic and minioluteic acids. Since the lactone ring appears to be stable and readily reformed after being opened by alkaline hydrolysis, ungulinic acid is probably a γ -lactone. The formation by ungulinic acid of a dimethyl ester with diazomethane, giving rise to a trihydrazide, is again evidence for the lactone structure.

The precipitation of the monosodium salt after complete hydrolysis of ungulinic acid with excess alkali and passage of carbon dioxide is in line with the observation of Hesse (1898) on roccellic acid which is an α -methyl- α' -decylsuccinic acid. Hesse found that passage of carbon dioxide through a solution of the acid in potassium carbonate gives rise to the deposition of a crystalline precipitate of the monopotassium salt.

The production of stearic acid on fusion with potassium hydroxide indicates the presence of a long aliphatic side chain and again stresses the resemblance to the other fungal and lichen acids cited. In view of these similarities it is permissible to favour a substituted tricarballic acid configuration for ungulinic acid, which might then be represented by one of the three formulae:

One point which arises in connexion with this formulation calls for further comment. A natural product of the above configuration would be expected to be optically active since II and III contain two and I contains three asymmetric carbon atoms. It is, of course, possible that a racemic compound is produced as in the case of fumarylalanine, a metabolic product of the mould *Penicillium reticulosum* (Birkinshaw, Raistrick & Smith, 1942). Alternatively, the rotation may be so small as to escape observation. In the case of the mould product palitantin, the substance was reported as optically inactive (Birkinshaw & Raistrick, 1936) but an examination of some of its crystalline derivatives

and breakdown products revealed optical activity (unpublished observation). It is not possible on the evidence so far obtained to advance a definite structural formula for ungulinic acid. The substituted tricarballic acid formulae are to be regarded as purely tentative and are advanced merely as a basis for further work on the constitution of ungulinic acid which is to be undertaken.

EXPERIMENTAL

Organism

The culture, F.P.R.L., No. 137 A, obtained from the Forest Products Research Laboratory, Princes Risborough, had originally been prepared from the tissue of a sporophore of *Polyporus benzoinus* (Wahl.) Fr. found on a fallen trunk of silver fir, *Abies alba*, in Norfolk.

Preparation of the inoculum

The culture was maintained in the laboratory on malt agar slopes. The culture used for inoculation was developed on a moist Norway spruce sawdust medium containing 10% of the accelerator exactly as used in the investigation of species of *Endoconidiophora* (Birkinshaw & Morgan, 1950). The fungus grew slowly throughout the sawdust medium, and progress of the infection could be followed visibly by the spread of the white hyphae of the developing mycelium.

Media

The following media were employed:

(I) A synthetic medium containing glucose, 30 g.; KH_2PO_4 , 2.5 g.; NH_4Cl , 2.5 g.; MgSO_4 , 1.0 g.; Marmite, 1.0 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.005 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.005 g.; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.005 g.; tap water to 1 l.

(II) An aqueous solution of Vimaltol (4% w/v) and Marmite (0.1% w/v). Vimaltol is a proprietary preparation prepared from malt extract, calcium glycerophosphate, orange juice, iron and ammonium citrate, halibut oil, vitamin D, and vitamin A concentrate. Marmite, also a proprietary preparation, is a concentrated extract obtained from autolysed yeast. It contains vitamins of the B complex.

Method of culture

The medium was distributed (350 ml./flask) in 1 l. conical flasks which were plugged with cotton wool and sterilized by autoclaving at 110° for 30 min. The flasks were inoculated by dropping in a little of the infected sawdust and were then incubated in the dark at 24°. In preliminary work the best time of harvesting was decided by examining single flasks at weekly intervals after the mycelium completely covered the surface of the culture fluid. The weight of ether-extractable material in the wet minced mycelium was determined in each case. After 10 weeks the weight of ether extract did not increase appreciably. The residual sugar in the culture filtrate estimated polarimetrically as glucose (medium I) had fallen to about 1.5% and the pH was approximately 4.0. The period of about 10 weeks was therefore adopted as the standard time of incubation.

Isolation of metabolic products

On the synthetic medium (I). The total wet mycelium from forty-five culture flasks was roughly minced and mechanically stirred under ether for several hours. The solvent layer was removed and the operation repeated until the extract, after removal of ether, weighed less than 0.1 g. The separate ether layers were combined, dried over Na_2SO_4 and evaporated. A viscous fawn-coloured paste (7.25 g.) remained, which was acid to litmus and possessed an aromatic odour.

This ether residue when treated with fresh ether (200 ml.) did not all redissolve. The white insoluble portion when collected and washed with ether formed a white powder (0.11 g., m.p. 285–290°, decomp.) with acid reaction to litmus.

The red-brown ether filtrate obtained after removal of the product of m.p. 285–290° was extracted with aqueous NaHCO_3 (2% w/v, 3 × 50 ml.). Acidification to congo red of the combined aqueous layers with dilute HCl gave a semi-solid light brown precipitate (1.96 g.). This was collected and recrystallized from light petroleum (b.p. 60–80°) at low temperature, giving ungulinic acid anhydride as a colourless crystalline product (0.33 g., m.p. 53–54° after previous slight shrinkage), reacting acid to litmus. The bicarbonate-extracted ether layer was washed with water, dried over Na_2SO_4 and evaporated to leave a yellow-brown waxy solid which still retained the aromatic odour. Steam distillation of this residue gave a colourless distillate with a colourless oil floating on the surface. This oil was collected by ether; and after removal of solvent weighed 0.61 g. The oil (0.15 g.) in ethanol (3 ml.), when treated with Brady's reagent (100 ml.), gave a characteristic 2,4-dinitrophenylhydrazone (0.30 g.). Recrystallization from ethyl acetate or xylene afforded glistening bright-red needles, m.p. 248–250°. The melting point was not depressed on admixture with the corresponding derivative of authentic anisaldehyde, m.p. 248–250°. Brady (1931) recorded a melting point of 250° for this compound. (Found: C, 53.0; H, 3.9; N, 17.3. Calc. for $\text{C}_{14}\text{H}_{12}\text{O}_6\text{N}_4$: C, 53.2; H, 3.8; N, 17.7%.)

On the aqueous Vimaltol-Marmite medium. Mycelial development on this medium was much healthier in appearance and more vigorous than when the fungus was grown on the synthetic medium. The incubation period was the same, viz. 10 weeks, and the method of isolation of the acidic metabolic products was similar, but the yields of the two acids previously encountered were greatly improved.

The triterpenoid acid of m.p. 285–290° (decomp.), was obtained in the same manner as described above. Owing to its slight solubility in ether and to the increased amount present, it began to precipitate during the early stages of evaporation of the ether extract.

After removal of the triterpenoid product by filtration, the filtrate was shaken as before with aqueous NaHCO_3 . Owing to the increased amount of ether-soluble acid present, there was greater tendency to emulsification during the extraction and separation, particularly with the first extraction, was very slow. After combining the aqueous layers the last traces of ether were removed *in vacuo*, when the aliphatic acid began to precipitate as a sodium salt in the form of microcrystalline glistening leaflets. As it was only sparingly soluble in cold water it was well washed until free from NaHCO_3 . When dry it formed a light, white powder with an odour of a higher fatty acid, e.g. palmitic acid.

Table 1. *Yields of products obtained from Polyporus bovinus*

(Medium II; 10 weeks' incubation.)

No. of flasks	Weight of crude extract (g.)	pH of culture filtrate	Yield of acid (m.p. 285–290°, decomp.) (g.)	Yield of aliphatic acid as Na salt (g.)
75	13.78	3.9	4.26	2.08
96	18.08	3.9	1.65	2.37
100	19.20	3.9	3.02	2.32
100	23.83	4.2	3.39	2.26
99	20.77	4.0	4.27	2.73
99	27.18	3.8	4.14	5.19
97	22.40	3.9	2.75	4.12
98*	26.40	3.9	3.22	9.46

* Using high-speed disintegrator.

Acidification of the filtrate after removal of the sodium salt gave only a slight amount of oily material from which none of the acid of m.p. 53–54° could be obtained on treatment with light petroleum.

The culture filtrate exhibited no antibacterial activity against *Staphylococcus aureus*.

On a later batch the effect was tried of submitting the mycelium to the action of a high-speed disintegrator. This reduced it to a viscous paste and considerably shortened the time required for complete extraction with ether. It also markedly increased the recovery of the low melting-point acid (as the sodium salt).

The yields of acids obtained on medium II by the procedure outlined are given in Table 1.

Investigation of the triterpenoid acid

Properties. The acid is fairly soluble in pyridine, moderately so in ethanol in the cold. It is moderately soluble in hot glacial acetic acid, dioxan, CHCl_3 and nitrobenzene, but only sparingly in acetone and ethyl acetate. When crystallized from acetic acid it separates in microcrystalline aggregates.

The behaviour of the acid on heating in the melting-point tube is as follows: at 250° it begins to discolour, at 275–280° it begins to soften and the colour deepens from yellow to dark brown. It melts at 285–290° with liberation of gas. The recrystallized acid was analysed after drying in high vacuum at 100–110°. (Found: C, 76.1, 76.4; H, 10.1, 10.0. $\text{C}_{30}\text{H}_{46}\text{O}_4$ requires C, 76.7; H, 9.9; $\text{C}_{30}\text{H}_{48}\text{O}_4$ requires C, 76.3, H, 10.3%.) $[\alpha]_{\text{D}}^{20} + 8.19^\circ$ (c, 5.1) in pyridine.

Equivalent. The acid (24.0 mg.) dissolved in ethanol (10 ml.) required 0.50 ml. of 0.1 N-NaOH for neutralization to phenolphthalein. This indicates an equivalent of 480, or if the acid is monobasic a mol.wt. of 480 ($\text{C}_{30}\text{H}_{46}\text{O}_4$ requires 471; $\text{C}_{30}\text{H}_{48}\text{O}_4$ requires 473).

Colour reactions. With CHCl_3 and H_2SO_4 it forms an orange lower layer. With acetic anhydride and H_2SO_4 it affords a blue-violet colour. These colour reactions are identical with those described for polyporenic acid C by Cross *et al.* (1940).

Methyl ester. The acid (0.50 g.) suspended in acetone (20 ml.), when treated with excess ethereal diazomethane, slowly passed into solution. After 3 hr. at room temperature the solvent was removed and the residue dried *in vacuo* over P_2O_5 . Recrystallization of the product (0.49 g.; m.p. 160–170°) from methanol afforded homogeneous needles, m.p. 192–193°, after slight softening from 190°. $[\alpha]_{\text{D}}^{21}$ in CHCl_3 + 19.5° (c, 6.7); $[\alpha]_{\text{D}}^{21}$ in CHCl_3 + 22.3° (c, 6.7). (Found:

C, 76.9; H, 9.7; OMe, 6.3; $\text{C}_{31}\text{H}_{48}\text{O}_4$ requires C, 76.8; H, 10.0; OMe, 6.4; $\text{C}_{31}\text{H}_{50}\text{O}_4$ requires C, 76.5; H, 10.4; OMe 6.4%.) The methyl ester of polyporenic acid C is stated by Cross *et al.* (1940) to have m.p. 192–193°, after softening from 189°. The melting points of the methyl ester of polyporenic acid C obtained from Dr T. G. Halsall and of the methyl ester of the *Polyporus bovinus* acid were compared under the same conditions of heating with the following results. Methyl ester of polyporenic acid C, m.p. 190–191°; methyl ester of *P. bovinus* acid, m.p. 191–192°; mixed m.p. 191–192°.

The ester does not titrate with standard NaOH in the cold.

Diacetate

Hot acetylation. The acid (1.0 g.) was refluxed with acetic anhydride (7 ml.), acetic acid (5 ml.) and anhydrous sodium acetate (2 g.) for 4 hr. When the reaction mixture was cooled and decomposed with ice water a gummy product was precipitated. Recrystallization of this material (1.20 g.) from glacial acetic acid gave only a small yield (0.1 g.) of the diacetate, m.p. 212–214° (decomp.).

Cold acetylation. The acid (2.0 g.) was dissolved in pyridine (12 ml.) and acetic anhydride (3.0 ml.) was added. The reaction mixture was kept at room temperature overnight and then poured into iced water. The white flocculent precipitate was collected, dried and recrystallized from hot glacial acetic acid to which just sufficient water was added to induce turbidity. The product (0.5 g.), rectangular plates, had m.p. 212–214° (decomp.) with preliminary softening at 205° which could not be eliminated by repeated recrystallization. (Found: C, 73.8, 73.8; H, 9.7, 9.2; CH_3CO , 15.5. $\text{C}_{34}\text{H}_{50}\text{O}_6$ (i.e. the diacetate of $\text{C}_{30}\text{H}_{46}\text{O}_4$) requires C, 73.6; H, 9.1; CH_3CO , 15.5; mol.wt. 554.8; $\text{C}_{34}\text{H}_{52}\text{O}_6$ requires C, 73.3; H, 9.4; CH_3CO , 15.5%, mol.wt. 556.8.)

The diacetate (28.05 mg.) in ethanol required 0.500 ml. of 0.1 N-NaOH for neutralization to phenolphthalein in the cold, indicating an equivalent of 561. $[\alpha]_{\text{D}}^{21}$ in CHCl_3 + 3.2° (c, 8.6). $[\alpha]_{\text{D}}^{23}$ + 4.2° in CHCl_3 (c, 6.0).

Both the monomethyl ester and the diacetate give immediate red-brown colorations with tetranitromethane indicating the presence of an olefinic double bond.

Ozonolysis of the diacetate. The diacetate (0.8 g.) in CHCl_3 (25 ml.) was treated with ozonized oxygen at room temperature for 3 hr. The ozonide was decomposed by warming the CHCl_3 solution with water at 60° whilst a current of nitrogen was passed through the liquid into a bubbler containing Brady's reagent. (Brady & Elmslie, 1926). No volatile

carbonyl compound was detected in the issuing gas. The layers were separated and the CHCl_3 was washed with water, the washings being added to the water layer. Treatment of the latter with Brady's reagent (250 ml.) gave immediate yellow turbidity and flocculation. The precipitate was collected and dried. The crude product (160 mg., m.p. 155–160°) was crystallized from light petroleum and then from methanol giving yellow-orange needles of m.p. 164°, not depressed on admixture with authentic formaldehyde 2:4-dinitrophenylhydrazone of m.p. 164°. (Found C, 40.2; H, 3.2; N, 26.0. Calc. for $\text{C}_7\text{H}_8\text{N}_4\text{O}_4$: C, 40.0; H, 2.9; N, 26.7 %.) The weight of crude material recovered represents a yield of 53 % of theoretical for one methylene group. The CHCl_3 layer was not further examined.

Hydrogenation of triterpenoid acid

The acid (0.5000 g.), m.p. 285–290°, dissolved in ethanol (100 ml.), was hydrogenated at room temperature and ordinary pressure in the presence of Adams's PtO_2 catalyst (0.1000 g.). The corrected uptake of hydrogen was 24.6 ml. at N.T.P. in 1 hr. (uptake of 2H requires 23.8 ml.). The mixture was then filtered and the precipitated product washed out of the catalyst by warm ethanol. On evaporation a crude product (0.495 g., m.p. 295–297° (decomp.)) was obtained, which on recrystallization from acetic acid gave microcrystals (70 mg.), elongated plates or flattened needles which began to darken at ~275°, shrank at ~280°, softened at ~295° and melted with effervescence at 295–297° (decomp.) $[\alpha]_D^{20}$ in pyridine + 13.1° (c, 6.1). (Found: C, 75.8, 75.8; H, 10.2, 10.1. $\text{C}_{30}\text{H}_{48}\text{O}_4$ requires C, 76.3; H, 10.2; $\text{C}_{30}\text{H}_{50}\text{O}_4$ requires C, 75.9; H, 10.6%.)

Ungulinic acid

Anhydrous disodium salt. The crystalline salt obtained from the bicarbonate extract was dried in a vacuum desiccator (water-pump vacuum) over H_2SO_4 to constant weight and then exposed to a high vacuum at room temperature. No further loss in weight occurred. When the drying was continued at 55–60° in high vacuum, 98.89 mg. of the salt lost 4.01 mg., i.e. 4.05 %, before attaining constant weight. $\text{C}_{22}\text{H}_{36}\text{O}_5\text{Na}_2 \cdot \text{H}_2\text{O}$ requires 3.91 % water. The anhydrous salt is hygroscopic.

Equivalent of the anhydrous salt

By sulphated ash. The disodium salt (94.29 mg.) when ashed with H_2SO_4 gave 29.33 mg. of Na_2SO_4 . The Na content of 10.1 % indicates a mol. wt. 456 for an anhydrous disodium salt. $\text{C}_{22}\text{H}_{36}\text{O}_5\text{Na}_2$ requires Na 10.4 %, mol. wt. 442.5.

By back titration. The anhydrous disodium salt (90.61 mg.), after refluxing for 1 hr. with 0.1N-NaOH (5.02 ml.) with exclusion of CO_2 , required 2.99 ml. of 0.1N-HCl for neutralization to phenolphthalein. This corresponds with an apparent equivalent of 446, or a mol. wt. of 446 for a monoacid salt. Passage of CO_2 through the neutralized hydrolysate resulted in the almost immediate precipitation of a white crystalline salt. The reaction mixture was kept overnight in the refrigerator, and the precipitate was collected, washed and dried finally, in high vacuum at 55–60°; wt. 80 mg.

Monosodium salt

A sulphated ash determination showed that this was a monosodium salt; 79.06 mg. of the anhydrous salt afforded 14.05 mg. of Na_2SO_4 ; Na content 5.79 %.

$\text{C}_{22}\text{H}_{37}\text{O}_5\text{Na}$ requires 5.47 % Na. Decomposition of this salt with dil. HCl in the presence of ether gave the parent acid, m.p. 78–80° with slight previous softening. This acid has been named *ungulinic acid*.

Preparation and properties of the derived acids

Ungulinic acid anhydride, m.p. 53–54.5°

The hydrated disodium salt (0.50 g.) was dissolved in water by warming to about 50°. The solution frothed easily in the manner of a soap solution. After cooling to room temperature the solution was acidified with 2N-HCl, dropwise, until acid to congo red. A white flocculent precipitate was formed. The mixture was chilled overnight and the precipitate was collected and dried over H_2SO_4 to yield 0.36 g. of a crude acid of fatty appearance. On recrystallization from light petroleum (b.p. 60–80°) it formed colourless, thin, elongated plates, m.p. 53–54.5°. This substance, which we regard as the acid anhydride, is more stable than ungulinic acid. It is very soluble in all the common organic solvents. (Found: C, 69.3; H, 9.4; OCH_3 , nil. $\text{C}_{22}\text{H}_{36}\text{O}_5$ requires C, 69.4; H, 9.5; mol. wt. 380.5; $\text{C}_{22}\text{H}_{38}\text{O}_5$ requires C, 69.1; H, 10.0%; mol. wt. 382.5.)

Equivalent. The acid anhydride (79.97 mg.), dissolved in ethanol (5 ml.), required 4.61 ml. of 0.1N-NaOH for neutralization to phenolphthalein, corresponding to an equivalent of 173.4. $\text{C}_{22}\text{H}_{36}\text{O}_5$ as dibasic acid requires 190.3, as tribasic acid, 126.8. Further NaOH (5.00 ml. of 0.1N) was then added to the neutralized solution and the mixture was refluxed for 1.75 hr. at 100°. The solution now required 3.25 ml. of 0.1N-HCl for neutralization, indicating a further uptake of 1.75 ml. of alkali. The total alkali required is thus 6.36 ml. of 0.1N, indicating an equivalent of 126. Ungulinic acid anhydride thus behaves as a tribasic acid when treated with excess of hot alkali, but in the cold the equivalent is intermediate between that of a di- and a tri-basic acid.

After removal of the ethanol *in vacuo* the acid anhydride was precipitated by HCl. It had m.p. 51–52°, which was raised to 53–54° by recrystallization from light petroleum (wt. 60 mg.). It did not depress the melting point of a sample of the acid anhydride, m.p. 53–54.5°, which was the original material.

Molecular weight. The mol. wt. of the acid anhydride as determined by the cryoscopic method in dioxan was 358. $\text{C}_{22}\text{H}_{36}\text{O}_5$ requires 380.5.

Ungulinic acid, m.p. 78–80°

The hydrated salt (0.30 g.) was suspended in ether (100 ml.) and shaken with 2N-HCl (10 ml.), when the liberated acid passed into the ether layer. Evaporation of the ether left a white solid (0.25 g., m.p. 74–76°), which when recrystallized from ether-light petroleum (b.p. 40–60°) gave microcrystalline needles, m.p. 78–80°, after previous slight softening at 76°. The melt reset on cooling and remelted at 39–45°. The acid was less soluble in organic solvents than the anhydride, particularly in ether and light petroleum. The melting point of the acid, kept in a corked tube for 6 weeks, had fallen to 55–65°. (Found on the fresh sample: C, 65.3, 65.7, 65.1; H, 9.3, 9.3, 9.4. $\text{C}_{22}\text{H}_{38}\text{O}_5$ requires C, 66.3; H, 9.6. $\text{C}_{22}\text{H}_{36}\text{O}_5 \cdot \frac{1}{2}\text{H}_2\text{O}$ requires C, 65.6; H, 9.6%.)

Optical activity. The acid, m.p. 78–80° (1.150 g.) in ethanol (30.0 ml.) was examined in a 4 dm. tube. The observed optical rotation was zero. Taking the maximum reading error as 0.01° the value for $[\alpha]_{4861}^{21}$ is 0° within $\pm 0.07^\circ$.

Dimethyl ester. The crystalline acid (0.1 g.) on treatment with an excess of diazomethane in ether quickly dissolved, with effervescence. The solution was left overnight and then the ether and excess reagent were removed *in vacuo* leaving a very pale-yellow viscous oil (0.12 g.). This was soluble in all the commoner organic solvents but could not be obtained crystalline. It was analysed after drying for 12 hr. in high vacuum at 40°. (Found: C, 67.7; H, 9.7; OMe, 14.8. $C_{22}H_{42}O_6$ (the dimethyl ester of $C_{22}H_{38}O_4$) requires C, 67.6; H, 9.9; OMe, 14.3 %.) The dimethyl ester does not give the acetic anhydride-acetone colour reaction described for the parent acid.

Trihydrazide. The dimethyl ester obtained from 0.20 g. of the crystalline acid as above was dissolved in ethanol (3 ml.), treated with aqueous hydrazine hydrate (50 % w/v; 1 ml.) and allowed to stand at room temperature. After a few hours a flocculent precipitate began to separate. After 24 hr. the precipitate was collected by filtration. The crude hydrazide (0.11 g.) was a white powder, m.p. 170–175°, which resisted all attempts at recrystallization. After refluxing several times with ethanol and drying over P_2O_5 , it had m.p. 190–195° (decomp.). (Found: C, 59.8; H, 10.3; N, 17.8, 19.9. $C_{22}H_{44}O_3N_6$, the trihydrazide of $C_{22}H_{38}O_4$, requires C, 60.0; H, 10.1; N, 19.1 %.)

Colour reactions of unguinic acid and anhydride

Both acid and anhydride give the following colour reactions:

When warmed with acetic anhydride at 100° for a few minutes a pink colour develops, which on addition of excess acetone gives rise to a pronounced green fluorescence.

When dissolved in a few drops of pyridine and treated with a few drops of acetic anhydride in the cold a deep-red colour is produced with a pronounced green fluorescence. On slight warming the colour changes to a dark brown-violet, retaining the green fluorescence. These reactions are also given by agaricic acid.

Interconversion of unguinic acid and its anhydride

Conversion of unguinic acid, m.p. 78–80°, to the anhydride, m.p. 53–54.5°. The acid was quickly dried to constant weight in a vacuum desiccator. When further dried in a high vacuum at 48–52°, 102.27 mg. lost 4.47 mg. before the weight again became constant. The original material gradually passed to a pale-brown, effervescent mass, which eventually ceased to effervesce; this weighed 97.8 mg. If this loss corresponds to 1 mol. of water the mol.wt. of the acid would be 412. $C_{22}H_{38}O_4$ requires mol.wt. 398.5. The melting point of the dried material was 42–46°, but recrystallization from light petroleum (b.p. 40–60°) raised it to 53–54°, undepressed on admixture with the anhydride of m.p. 53–54.5°.

Conversion of the anhydride to unguinic acid. The anhydride (0.15 g.) was dissolved in $N-Na_2CO_3$ (15 ml.) and filtered to remove a slight turbidity. CO_2 was passed through the clear filtrate giving a crystalline Na salt (0.12 g.).

Regeneration of the acid by shaking the salt with ether in presence of 2N-HCl gave 0.10 g. of product of m.p. 74–76°, raised by recrystallization from light petroleum to 78–80° (with previous slight softening). The melting point was undepressed on admixture with a sample of unguinic acid of the same melting point, prepared independently.

Potassium hydroxide fusion of the disodium salt

The disodium salt (0.55 g.) of unguinic acid was heated with KOH (2.5 g.) and water (1.5 ml.). As the temperature rose effervescence occurred at 190–200° and again at about 290°. The temperature was maintained at 310° for 1 hr. The reaction mixture after cooling was treated with water. The product did not dissolve readily, but was replaced by a flocculent precipitate which passed slowly into solution on warming. The solution was clarified by filtration through a sintered-glass filter and the filtrate was acidified, dropwise, with H_2SO_4 (30 % v/v) to give an almost white flocculent precipitate (0.31 g.) of m.p. 55–60°. Several recrystallizations from light petroleum (b.p. 40–60°) raised the melting point to 65–67°, and gave a product of colourless shining leaflets. (Found: C, 76.1; H, 12.4, equivalent by micro-titration 274. $C_{18}H_{36}O_2$ requires C, 76.0; H, 12.8, equivalent (monobasic) 284.)

This product did not depress the melting point of an authentic sample of stearic acid, m.p. 66–68°; when mixed with palmitic acid, the sample produced a definite depression.

The acidified filtrate was evaporated repeatedly *in vacuo* to remove any volatile acids. The aqueous distillate required 9.45 ml. of 0.1N-NaOH for neutralization. The solution of Na salt was evaporated to small volume (4 ml.) and refluxed with *p*-bromophenacyl bromide (0.28 g.) in ethanol (6 ml.) for 2 hr. The reaction mixture deposited crystals (80 mg., 70–75°) on cooling which when recrystallized from light petroleum had m.p. 83–85° and did not depress the m.p. (84–85°) of an authentic sample of *p*-bromophenacyl acetate.

The acidified concentrated aqueous solution containing the non-volatile material was continuously extracted with ether (14 hr.) to give a further quantity of crystalline material (0.10 g.). This was dissolved in water, neutralized and the oxalic acid precipitated with aqueous $CaCl_2$ as calcium oxalate (0.13 g.). No other acid was detectable after removal of the oxalic acid.

Potassium hydroxide fusion of unguinic acid (disodium salt) thus gives rise to stearic, acetic and oxalic acids.

All melting points in this paper are uncorrected.

SUMMARY

1. The metabolic products of the wood-rotting fungus *Polyporus bovinus* (Wahl.) Fr., when cultivated on laboratory media, have been examined.

2. Anisaldehyde, isolated as the 2:4-dinitrophenylhydrazone, is probably the main contributor to the aromatic odour.

3. A triterpenoid acid, $C_{30}H_{46}O_4$ (or $C_{30}H_{48}O_4$), m.p. 285–290° (decomp.), $[\alpha]_{D}^{20} + 8.19^\circ$ in pyridine, was isolated. This is a dihydroxy-monobasic acid with a terminal methylene group. It appears to be identical with polyporenic acid C, obtained by Cross *et al.* (1940) from the natural sporophore of *P. betulinus*.

4. A second acid, unguinic acid, probably $C_{22}H_{38}O_4$, m.p. 78°, was obtained from the metabolic products. This acid gives a more stable anhydride

$C_{22}H_{36}O_5$, m.p. 53–54.5°. The acid is potentially tribasic; it appears to have two carboxyl groups and one lactonic grouping. It affords stearic, oxalic and acetic acids on potassium hydroxide fusion. Its structure is not determined, but a hydroxytri-

carballylic structure with a normal C_{16} alkyl chain as substituent is tentatively suggested.

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APPENDIX

Antibacterial activity of the triterpenoid acid (polyporenic acid C) and of ungulinic acid, metabolic products of *Polyporus benzoinus* (Wahl.) Fr.

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METHODS

For the determination of antibacterial activity the sodium salts of the respective acids were used for preparing a series of dilutions in broth which were inoculated with the test organisms, incubated at 37° and observed after a suitable interval for growth, and in the case of the Mycobacteria, for pellicle formation. The dilutions were made as follows:

Triterpenoid acid. The purified acid of m.p. 285–290° (decomp.) (10 mg.) was dissolved in ethanol (5 ml.) with slight warming. The equivalent amount of 0.1 N-NaOH (0.255 ml.) was then added and the solution of the sodium salt was diluted to 10 ml. to give a 1:1000 solution and from this the desired twofold dilutions in Lab-Lemco-peptone broth + 0.2% glucose, pH 7.5, were prepared, starting at 1:10,000.

Ungulinic acid. The hydrated disodium salt of the acid was used. A 1:1000 solution was obtained by dissolving the salt (30.0 mg.) in glucose broth (30 ml.) with careful warming to 40–50°. From this solution twofold dilutions in broth were prepared, starting at 1:2000.

The serial dilutions (4.5 ml. quantities in $6 \times \frac{5}{8}$ in. test tubes) of the two substances were inoculated with one loopful of an undiluted culture of the micro-organisms used, viz. *Staphylococcus aureus*, Oxford H; *Bacillus coli*, N.C.T.C. 86; *Mycobacterium phlei*, N.C.T.C. 525; and *M. smegmatis*, N.C.T.C. 523.

RESULTS

The triterpenoid acid (Table 1) was found to have a relatively low antibacterial activity against *Staph. aureus* and *B. coli* (24 hr.) whereas the activity against the Mycobacteria was high, no growth being obtained with *M. phlei* after 72 hr. with a dilution of 1:1,280,000 of the antibiotic. In this case the activity disappeared on further incubation up to 7 days. The ungulinic acid (Table 2), on the other hand, showed a moderate activity against *Staph. aureus* (1:128,000), none against *B. coli* (24 hr.) and only low activity against *M. phlei* (1:8000) after 77 hr., which, however, was only slightly reduced in 7 days.

An attempt was made to determine the antibacterial action of the two metabolic products