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Biochemistry of the Wood-rotting Fungi

8. VOLATILE METABOLIC PRODUCTS OF *DAEDALEA JUNIPERINA* MURR.*

BY J. H. BIRKINSHAW AND P. CHAPLEN

Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

(Received 20 November 1954)

A sample of East African pencil cedar, *Juniperus procera* Hochst., which showed a crumbling brown rot in the heartwood, was received in 1937 at the Forest Products Research Laboratory and a fungus was isolated but could not at first be identified. However, some years afterwards a culture of *Daedalea juniperina* Murr. isolated by Dr R. W. Davidson of the U.S. Department of Agriculture in Washington was obtained and this proved to be identical with the fungus isolated from the decayed pencil cedar. An unusual rather sickly sweet aromatic smell was produced in these cultures. Through the courtesy of Dr W. P. K. Findlay, who supplied the cultures, we were able to investigate the metabolic products of the fungus in continuation of previous work on the odorous constituents of wood-rotting fungi (cf. Birkinshaw, Morgan & Findlay, 1952).

* Paper 7 of this series: Birkinshaw, Morgan & Findlay (1952).

Daedalea juniperina is widely distributed but not common in the eastern and southern parts of the U.S.A. on living trees, worked timber, and stumps of *Juniperus virginiana* and has been reported on *J. excelsa* from Russia. The sporophore, which is a thick greyish corky bracket with a daedaloid pore surface, is generally similar in appearance to *Daedalea quercina*, which is so common on oak.

The fungus was grown mainly on a synthetic medium containing glucose and inorganic salts together with a little Marmite to supply vitamin B₁. At harvesting, the whole contents of the culture flasks was subjected to steam distillation, and the volatile products thus separated were examined and form the subject of the present communication. Other metabolic products present in the culture fluid and in the mycelium are still under investigation. The steam distillates on extraction with ether afforded a small amount of oil from which, by fractionation with light petroleum, colourless

crystals of m.p. 80° were eventually obtained. This substance, which for convenience has been given the trivial name of junipal, has the sweetish aromatic odour of the cultures from which it is derived. It is neutral in reaction, and rather unexpectedly was found to contain sulphur, the empirical formula being C_8H_8OS . Since it was found to give typical aldehyde reactions, afforded a 2:4-dinitrophenylhydrazone and was readily oxidized by means of the Doeuvre (1927) reagent (mercuric iodide and potassium iodide in alkaline solution) to a monobasic acid, $C_8H_8O_2S$, it was evident that junipal contained an aldehyde group and could be written $C_7H_7S(CHO)$, the oxidation product being $C_7H_7S(COOH)$ (junipic acid). On oxidation with permanganate, methyl junipate, $C_7H_7S(COOCH_3)$, gave the monomethyl ester, $C_4H_2S \begin{Bmatrix} COOH \\ COOCH_3 \end{Bmatrix}$, of a dibasic acid, the carbomethoxy group remaining intact. This formula and the general properties of the monoacid mono ester suggested that the permanganate oxidation product was a thiophen derivative. It was converted into the dimethyl ester, which was found to be identical with the dimethyl ester of 2:5-thiophendicarboxylic acid, synthesized for comparison.

The new carboxyl group obtained by permanganate oxidation must have arisen from the oxidation of a C_3H_3 residue attached to the thiophen ring. The possibility that this could be attached either as two separate substituents or in ring form to the thiophen nucleus appeared remote, since in this case oxidation should leave two new carboxyl groups as substituents of the thiophen ring, one of which must have been subsequently lost. As the permanganate oxidation was carried out in the cold, loss of a carboxyl was very unlikely.

Assuming then that besides the original aldehyde group in the 2-position there was only one other substituent, namely C_3H_3 in the 5-position, this could have one of three forms, namely $CH:C.CH_2$, $CH_3.C:C$ or $CH_2.C:CH$. Only one of these forms, namely $CH_3.C:C$, contains a carbon-methyl group. Since on examination such a group was detected in junipal, the constitution of junipal must be 5-(α -propynyl)-2-formylthiophen. The oxidation and degradation reactions are illustrated below.

Although the experimental work was restricted by the small yields of junipal obtained, a total of less than 1 g. of pure material being available, the chemical evidence of structure outlined above is supported by the information obtained from the ultraviolet and (particularly) from the infrared absorption spectra.

The ultraviolet curve of junipal determined in ethanol (Fig. 1) was of the general form associated with the simpler thiophen derivatives, e.g. 2-vinylthiophen (Hartough, 1952) but showed a pronounced bathochromic shift. The main band showed a maximum at $\lambda_{320} m\mu$. ($\log \epsilon 4.29$) as compared with a maximum for vinylthiophen at $\lambda_{275} m\mu$. ($\log \epsilon 3.96$). There was a subsidiary maximum (little more than an inflexion) in the junipal curve at 286.5 ($\log \epsilon 3.95$) and a definite band at 216.5 ($\log \epsilon 3.73$) which has no doubt appeared because of the bathochromic shift; it would be below the range of observation for the simpler thiophen derivatives. The $C=C$ or $C=O$ substituents in the 2-position of the thiophen nucleus produce a bathochromic shift for λ_{max} of about $+40 m\mu$. In the present case the shift in comparison with thiophen ($\lambda_{max} \sim 235 m\mu$) is about $85 m\mu$, which could well be produced by two groups capable of conjugation with the thiophen nucleus, namely $.CHO$ and $CH_3.C:CH$.

The infrared spectrum was kindly determined by Dr Almin of the Swedish Forest Products Research Laboratory. He found strong evidence for (a) the absence of hydroxyl groups (no band at $3300 cm^{-1}$), (b) the presence of a triple bond (band at $2232 cm^{-1}$), and (c) the probable presence of an aldehyde group (with conjugation) (band at $1666 cm^{-1}$). Although it was not possible to state with certainty that a thiophen group was present, the infrared evidence was not inconsistent with this assumption.

Indication of another S-containing product present in the steam distillates was obtained. The uncrystallizable mother liquors from the light petroleum extract afforded in very low yield a 2:4-dinitrophenylhydrazone of m.p. 198° . Elemental analyses were unsatisfactory and could not be repeated for lack of material. A possible formula would be $C_{16}H_{12(14)}O_4N_4S$, which would be derived from a parent carbonyl compound $C_{10}H_8(10)OS$,

differing from junipal by $C_2H_{2(4)}$. The ultraviolet absorption spectrum of this dinitrophenylhydrazone resembled that of the corresponding junipal derivative, but there was a bathochromic displacement of the principal band from 395 to 405 $m\mu$, a difference of 10 $m\mu$. This product, presumably related to junipal, may thus possess a further unsaturated group in conjugation with the chromophoric system of junipal.

The light-petroleum-insoluble tarry material from the steam distillates was subjected to a second steam distillation. Most of it was non-volatile and probably consisted of condensation or polymerization products. By treatment of the distillate with Brady's reagent, however, a micro-crystalline precipitate was obtained from which was isolated the 2:4-dinitrophenylhydrazone of anisaldehyde. Anisaldehyde therefore represents a further metabolic product of *Daedalea juniperina*. It has previously been reported as a metabolic product of wood-rotting fungi. It was detected in small amount amongst the volatile products of *Trametes suaveolens* accompanying methyl anisate, the major product (Birkinshaw, Bracken & Findlay, 1944), and is produced in larger amount by *Polyporus benzoinus* (Birkinshaw *et al.* 1952).

The isolation of junipal as a fungal metabolic product is of considerable interest since this substance contains both the thiophen nucleus and a triple bond. This is believed to be the first instance recorded of the occurrence of the thiophen nucleus amongst the products of fungi and micro-organisms, although β -biotin, a product of yeast, contains a urea residue condensed with a tetrahydrothiophen ring carrying a $-(CH_2)_4 \cdot COOH$ side-chain. Biocytin (ϵ -*N*-biotyllysine) has also been isolated from yeast; like biotin itself it promotes the growth of certain micro-organisms.

Acetylenic derivatives have already been shown to occur as metabolic products of fungi. Several highly unstable antibiotic substances, isolated by Anchel, Polatnick & Kavanagh (1950) from Basidiomycetes were shown on the basis of their ultraviolet absorption spectra to be acetylenic compounds (Anchel, 1952). A crystalline antibiotic, mycomycin, obtained from an Actinomycete by Johnson & Burdon (1947) was shown to have the constitution 3:5:7:8-tridecatetraene-10:12-diyneic acid (Celmer & Solomons, 1952). Similar systems of conjugate double and triple bonds had already been found to occur in the products of higher plants, particularly in the genera *Matricaria*, *Artemisia* and *Erigeron*, all of the family Compositae. These were investigated by Sørensen and co-workers (cf. Birkinshaw, 1953).

Zechmeister & Sease (1947) isolated α -terthienyl from the flowers of the Indian marigold, *Tagetes erecta* (Compositae). This is the only recorded

instance of the occurrence of a true thiophen derivative in plants. Challenger & Holmes (1953) have pointed out that it may be more than a coincidence that this solitary example should be found in the family Compositae, of which so many members produce polyacetylenes. They surmise that the α -terthienyl may arise by interaction of hydrogen sulphide (possibly derived from cystine) with a straight-chain compound containing an acetylenic-olefinic system. This hypothesis is considerably strengthened by the isolation of junipal as a product of fungal metabolism, since both the acetylenic linkage and the thiophen nucleus are present in the same molecule.

The two volatile products identified, namely junipal, C_8H_6OS , and anisaldehyde, $C_8H_8O_2$, show certain points of similarity. Thus both are aldehydes, both contain methyl groups, one *C*-methyl and one *O*-methyl and both contain the same number of carbon atoms. The possibility is suggested that these two aldehydes are derived from a common precursor, a straight chain C_7 aldehyde, say $C_7H_{14}O$, with unsaturated linkages. Carbon-to-carbon ring closure (C_2 to C_7 to form the benzene ring) with addition of water would give $C_7H_6O_2$ in the one case, whereas ring formation by incorporation of an external sulphur atom (attached to C_2 and C_6 to form the thiophen ring) would give C_7H_4OS . Subsequent *O*-methylation in the one case and *C*-methylation in the other would then afford $C_8H_8O_2$ and C_8H_6OS respectively. Some such set of operations, not necessarily in the chronological order outlined above, appears biogenetically possible. Although purely hypothetical, the scheme may suggest a method of attack on the problem of the mechanism involved in the biosynthesis.

EXPERIMENTAL

All m.p.'s are uncorrected. Analyses are by Weiler and Strauss.

Organism

Two strains of *Daedalea juniperina* Murr. were available. These were obtained from the Forest Products Research Laboratory, Princes Risborough, by courtesy of Dr W. P. K. Findlay, and bore their catalogue numbers as follows: (1) Catalogue no. 136. Originally isolated by Davidson in U.S.A. (2) Catalogue no. 136A. Isolated by Findlay from decayed pencil cedar wood from Kenya, and identified by comparison with Davidson's culture. From a preliminary investigation it appeared that the volatile products from the two strains were generally similar. Strain 136A was employed for most of the work here recorded.

Preparation of the inoculum. The cultures were maintained in the laboratory on malt-agar slopes. For inoculation the culture was developed on a moist spruce sawdust medium as employed in the investigation of species of *Endoconidiophora* (Birkinshaw & Morgan, 1950).

Media. The following media were employed: (1) Synthetic medium (suggested by Dr Findlay) containing glucose,

20 g.; KH_2PO_4 , 1.5 g.; NH_4NO_3 , 0.6 g.; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.005 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g.; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.005 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.005 g.; Marmite, 1 g.; water, 1 l. This medium had a pH of 4.8–5.0 after sterilization by steaming for 1 hr. on 3 successive days. (2) The aqueous Vimaltol–Marmite medium as used by Birkinshaw *et al.* (1952) for growth of *Polyporus benzoinus*. The pH was 5.4 after sterilization by autoclaving at 110° for 30 min. This medium did not increase the yield of steam-volatile products and was used for only two batches of flasks.

Method of culture. The medium was distributed (350 ml./flask) in 1 l. conical flasks plugged with cotton wool, sterilized and inoculated by dropping into each flask a little of the infected sawdust. The flasks were then incubated in the dark at 24°.

Culture characteristics

Strain 136 A. Good growth was observed on the synthetic medium only after 3–4 weeks' incubation, and thence onward little change occurred in growth or appearance. The mycelium was very thin, smooth, creamy-white in colour, with some salmon-pink mottled patches and with a uniform yellowish buff reverse. The culture solution was a deep straw colour. The culture had a pleasant, characteristic odour. After several subcultures had been made the pink mottling was gradually lost and the mycelium presented a uniform creamy-white appearance. On the malt medium the mycelium was thicker and had a deeper brown reverse. Good growth was obtained after 2 weeks.

Strain 136 (cultured on synthetic medium only). In general the appearance was similar to that of strain 136 A, but initially the pink mottling was absent and only appeared after several subcultures. Growth was rather slower than that of strain 136 A. The culture solution had the same colour and characteristic odour as strain 136 A.

136 A (synthetic medium) gave no reaction with aqueous ferric chloride, conc. HCl or bromine water. Cold aqueous KMnO_4 was slowly decolorized and with 2:4-dinitrophenylhydrazine in dilute HCl (Brady's reagent) it gave an orange-red turbidity after 20–30 min. with deposition of a heavy amorphous orange-red ppt. after 3 hr. With aqueous CaCl_2 a heavy granular ppt. formed immediately. In the case of the malt medium no ppt. was obtained with CaCl_2 . The test with Brady's reagent was not performed as the original medium gave a strong positive reaction.

Isolation of volatile products

The whole contents of the culture flasks was subjected to steam distillation. The steam distillates were extracted with ether ($4 \times \frac{1}{4}$ vol.), the combined extracts were dried (Na_2SO_4) and reduced to small vol. on the water bath. After spontaneous evaporation of the residual ether, the golden-brown oily residue partly crystallized on long cooling. By treatment with light petroleum (b.p. 60–80°) this material was separated into an insoluble dark brown tar, which showed no further crystallization even on long keeping at 0°, and a soluble fraction, which yielded crystals on evaporation and chilling. It was sometimes necessary to employ solid CO_2 as refrigerant to induce crystallization. The separated crystalline material was collected, washed with CO_2 -cooled light petroleum and dried. Further crops were obtained from the mother liquors. The crude crystalline material usually had m.p. 76–78° which after sublimation and recrystallization from aqueous ethanol (1:1) was raised to 80°. For this new fungal metabolic product we propose the trivial name junipal. The yields of junipal were small, only 1.24 g. of crude crystalline material being obtained by harvesting a total of 1061 flasks (see Table 1). Further purification reduced the final yield to about 70% of this amount. The light petroleum mother liquors eventually yielded a golden-brown oil from which no further crystals could be obtained.

Table 1. *Cultural details and yields of volatile products of Daedalea juniperina*

Strain	Medium	No. of flasks	Period of incubation at 24° (weeks)	Residual glucose (%)	Final pH	Wt. of ether extract of distillate (g.)	Wt. of crude cryst. solid (g.)
136	Synthetic	48	16	0.19	2.0	0.31	0.010
136 A	Malt	74	12	—	3.0	0.61	0.012
136 A	Synthetic	20	12	0.24	1.7	0.16	0.012
136 A	Synthetic	27	15	0.19	1.7	0.19	0.015
136 A	Synthetic	279	12	0.18	1.8	3.8	0.22
136 A	Synthetic	166	10	0.20	1.8	1.6	0.25
136 A	Synthetic	124	10	0.20	1.8	1.3	0.17
136 A	Synthetic	141	10	0.18	1.8	1.5	0.21
136 A	Synthetic	182	10	0.20	1.8	3.2	0.35

Harvesting and preliminary investigation

The best harvesting time (with reference to the steam-volatile products) was determined by examination of single flasks at weekly intervals. The contents of the flask was subjected to steam distillation and the weight of the material extracted by ether from the distillate determined. The maximum yield was obtained 10–12 weeks after inoculation. In the synthetic medium the glucose content had then fallen to about 0.2% and the pH to 1.8. On the malt medium the pH was 3.0 after 12 weeks. At this stage the culture fluid of

Properties of junipal

Junipal is obtained from light petroleum as thick colourless needles, m.p. 80°, and has the characteristic odour of growing cultures of *D. juniperina*. It slowly darkens on keeping in the light but remains colourless in the dark. It is readily soluble in most organic solvents, very slightly in cold water, but fairly soluble in hot water from which it separates as an oily turbidity, which crystallizes on keeping. It is neutral in reaction, gives no colour with FeCl_3 , conc. H_2SO_4 , NaOH, or Na_2CO_3 and is obtained unchanged from

hot alkaline solution on cooling. It decolorizes aqueous KMnO_4 rapidly, slowly gives a positive Schiff reaction and gives in ethanolic solution an immediate orange-red crystalline ppt. with Brady's reagent. It reduces Fehling's solution and gives a silver mirror with ammoniacal AgNO_3 . It is optically inactive. (Found: C, 64.5, 64.3, 63.6; H, 3.9, 4.35, 3.6; S, 21.3, $\text{CH}_3(\text{C})$ 9.5%, OCH_3 nil. Mol.wt. (cryoscopic) in camphor 120, 152, 182. $\text{C}_8\text{H}_8\text{OS}$ requires C, 64.0; H, 4.0; S, 21.3; $1\text{CH}_3(\text{C})$ 10.0%; mol.wt. 150.)

Ultraviolet absorption. λ_{max} 216.5, 286.5, 320 $\text{m}\mu$; $\log \epsilon$ 3.73, 3.95, 4.29, in ethanol (see Fig. 1).

Fig. 1. Ultraviolet absorption of junipal in ethanol.

Fig. 2. Infrared absorption of junipal. The two curves represent results of two experiments done with different amounts of material.

Infrared absorption. The infrared absorption spectrum (Fig. 2) was determined by Dr K. E. Almin using a Perkin Elmer spectrograph, model 112 with NaCl prism. The KBr preparation technique was used. Dr Almin gives the following interpretation: 'It is impossible to state with certainty that a thiophen group is present in the compound. However, infrared evidence is by no means inconsistent with this assumption. The following can be said with certainty. (1) The lack of a strong band at about 3300 cm^{-1} speaks against the presence of hydroxyl groups. (2) The 2232 cm^{-1} band strongly indicates the presence of a triple bond. (3) The 1666 cm^{-1} band very probably derives from an aldehyde group. Aldehyde bands normally occur in the region of $1725\text{--}1690\text{ cm}^{-1}$ but their frequency is lower when conjugated double bonds are present. The low frequency found for your substance thus indicates conjugation.' Junipal did not inhibit the growth of *Escherichia coli* (N.C.T.C. 86) or of *Staphylococcus aureus* (Oxford H strain, N.C.T.C. 6571) at a dilution of 1:5000 in heart broth-2% glucose medium.

Derivative and oxidation products of junipal

2:4-Dinitrophenylhydrazone. Junipal (25 mg.) in ethanol (10 ml.) was treated with Brady's reagent (40 ml.) and left for 5 hr. The orange-red microcrystalline deposit (30 mg.) was collected, m.p. $152\text{--}154^\circ$, raised to 165° (decomp.) by three recrystallizations from ethanol. The product in ethanol gave a cherry-red colour with a drop of dil. NaOH. (Found: C, 51.05; H, 3.4; N, 16.7; S, 9.9. $\text{C}_{14}\text{H}_{10}\text{O}_4\text{N}_4\text{S}$ (i.e. the monodinitrophenylhydrazone of $\text{C}_8\text{H}_8\text{OS}$) requires C, 50.9; H, 3.05; N, 17.0; S, 9.7%.) Ultraviolet absorption: λ_{max} 250 (inflection), 320, 395; $\log \epsilon$ 4.15, 3.99, 4.45, in ethanol.

Junipic acid (by Doeuvre (1927) oxidation of junipal). Junipal (45 mg.) was suspended in water (20 ml.) and HgI_2 (0.25 g.) and KI (2 g.) in water (5 ml.) were added, followed by 0.1N-NaOH (30 ml.). The mixture was shaken frequently and left overnight. As some of the junipal remained undissolved, N-NaOH (5 ml.) was added and the mixture left for 24 hr. with frequent shaking. All was now in solution except for a ppt. of Hg, which was removed by filtration through kieselguhr and the filtrate was titrated to phenolphthalein with N-HCl. The total acid produced in the reaction, 0.91 ml. N, corresponds with the formation of carboxyl equivalent to 0.30 ml. N, which agrees with the theoretical value, 0.30 ml. N, calc. for the reaction $\text{C}_8\text{H}_8\text{OS} \rightarrow \text{C}_8\text{H}_6\text{O}_2\text{S}$, in which $\text{R} \cdot \text{CHO} \rightarrow \text{R} \cdot \text{COOH}$.

On addition of 0.35 ml. N-HCl to the neutralized solution, straw-coloured needles of *junipic acid* slowly separated (43 mg. m.p. $169\text{--}171^\circ$). These were soluble in cold NaHCO_3 and did not react with Brady's reagent. Recrystallization from light petroleum, then from aqueous ethanol (1:1) gave very pale straw coloured needles of constant m.p. 180° . (Found (on a specimen sublimed in high vacuum at $140\text{--}145^\circ$): C, 58.15; H, 3.9; S, 19.4%, equivalent by titration 166. $\text{C}_8\text{H}_6\text{O}_2\text{S}$ requires C, 57.8; H, 3.6; S, 19.3%, equivalent (monobasic) 166.) Ultraviolet absorption: λ_{max} 293 $\text{m}\mu$; $\log \epsilon$ 4.07, in ethanol.

Methyl junipate. Junipic acid (47 mg.) was treated with excess diazomethane in ether. A rapid evolution of nitrogen occurred and the reaction appeared complete in a few seconds. On removal of the ether, small hexagonal plates slowly appeared in the residual oil (52 mg. m.p. $60\text{--}62^\circ$). Recrystallization from aqueous methanol afforded *methyl junipate* (38 mg.) of m.p. 62° unchanged on sublimation at $50\text{--}55^\circ/16\text{ mm}$. (Found: C, 60.0; H, 4.6; S, 18.05; OMe, 17.0%; mol.wt. (cryoscopic in camphor) 177, 169. $\text{C}_8\text{H}_8\text{O}_2\text{S}$ requires C, 60.0; H, 4.5; S, 17.8; OMe, 17.2%; mol.wt. 180.)

Oxidation by KMnO_4 of methyl junipate to methyl hydrogen thiophen-2:5-dicarboxylate. Methyl junipate (24 mg.) was dissolved in purified acetone (15 ml.) and powdered KMnO_4 (0.13 g.) was added portionwise until the colour persisted for 3-4 hr. The solution was filtered and the deposited MnO_2 washed with acetone. The acetone contained a negligible amount of residual solid. The MnO_2 was extracted with warm water ($2 \times 20\text{ ml.}$) and the aqueous extracts were acidified with HCl. A small amount of needles separated (12 mg. m.p. $190\text{--}191^\circ$) a further amount (11 mg. m.p. $189\text{--}190^\circ$) was obtained by ether extraction of the filtrate. After recrystallization from light petroleum, methyl hydrogen thiophen-2:5-dicarboxylate had m.p. 192° , unchanged on sublimation in high vacuum. (Found: C, 45.0; H, 2.9; S, 15.6; OMe, 16.5. $\text{C}_7\text{H}_6\text{O}_4\text{S}$ requires C, 45.2; H, 3.25; S, 17.2; OMe, 16.7%.) (The sulphur determination could not be

repeated as material was not available.) Ultraviolet absorption: $\lambda_{\max}$ 274 μ , $\log \epsilon$ 4.47, in ethanol.

Dimethyl thiophen-2:5-dicarboxylate from the mono-ester. The mono-ester (13 mg.) treated with diazomethane in ether afforded crystalline needles (13 mg.) of the dimethyl ester, m.p. 142–144°. The m.p. was raised to a constant value of 146° by sublimation in high vacuum. The mixed m.p. with the authentic sample (m.p. 146–147°) synthesized for comparison, showed no depression. (Found: C, 48.05; H, 3.9; OMe, 30.8. Calc. for $C_8H_8O_4S$: C, 48.0; H, 4.0, 2OMe, 31.0%.)

Oxidation by $KMnO_4$ of junipic acid to thiophen-2:5-dicarboxylic acid. Junipic acid (32 mg.) was dissolved in purified acetone (10 ml.) and powdered $KMnO_4$ was slowly added until there was no further utilization. The MnO_2 residue was extracted with warm water (2×20 ml.) and the extracts were acidified with HCl. No solid separated, but ether extraction gave pale-straw coloured non-crystalline material (29 mg.). This did not melt in an open tube but slowly sublimed between 220 and 330°. In a sealed tube in metal block it darkened and shrank slightly at 330–335° but did not melt below 360°. From a survey of the literature, this behaviour appears to be characteristic of thiophen-2:5-dicarboxylic acid, although Hartough & Kosak (1947) state for this acid, 'Since no m.p. was listed in the literature due to sublimation, a m.p. was run in a sealed tube on a melting point block, m.p. 358.5–359.5°.' Confirmation of the identity of this product was obtained by conversion into the dimethyl ester.

Dimethyl thiophen-2:5-dicarboxylate from the free acid. This was prepared by the action of diazomethane (in ether) on the free acid (28 mg.). On removal of ether the residue crystallized in needles (25 mg.) of m.p. 139–142° raised by recrystallization from aqueous methanol (1:1) to 145–146°, and by sublimation in high vacuum to a constant value of 146°. (Found: C, 48.5, 48.2; H, 4.1, 3.9; S, 15.8. $C_8H_8O_4S$ requires C, 48.0; H, 4.0; S, 16.0%.) Ultraviolet absorption: $\lambda_{\max}$ 275 μ , $\log \epsilon$ 4.21, in ethanol.

The m.p. of this product was not depressed when mixed with an authentic specimen of dimethyl thiophen-2:5-dicarboxylate synthesized for comparison. Further the u.v. spectra of the synthetic and junipal-derived dimethyl esters were identical.

Proof that junipal is not an artifact

The culture fluid from five incubated flasks was filtered from the mycelium and extracted with ether (6×400 ml.). After evaporation to small volume the ether solution was shaken with aqueous $NaHCO_3$ (freshly prepared) to remove any acidic products. After washing with water and drying (Na_2SO_4) the ether was removed. The residue, a thin oily film, having the characteristic odour of junipal, partly crystallized on long cooling. A little solid material was obtained from this mixture by sublimation, which when recrystallized from aqueous ethanol (1:1) had m.p. 72–73° (~ 5 mg.), raised by two further sublimations to 79–80°. This substance did not depress the m.p. of junipal on admixture. Hence junipal is present as such in the metabolic products of *D. juniperina*.

Residual oil from the light petroleum extract

The uncrystallizable material from the light petroleum extract consisted of a thick oil which could not be purified by chromatography on alumina, since it was eluted from

the column apparently unchanged. The ultraviolet absorption spectrum showed only the main band characteristic of junipal. With Brady's reagent the oil gave a small amount of deep red ppt., which after 6 hr. had hardened to a microcrystalline mass. It was collected, washed and dried, m.p. 196–198° (decomp.). After 2 recrystallizations from ethanol the m.p. was constant at 198°, and the substance dissolved in ethanol gave with dil. NaOH a wine-red colour, indicating that it was not an α -dicarbonyl compound. The combustion figures were unsatisfactory. (Found: C, 53.6, 51.4; H, 4.1, 3.2; N, 14.4, 17.3; S, 10.5, 8.1%.) The most reasonable fit for these values is perhaps $C_{16}H_{14}O_4N_4S$ which requires C, 53.6; H, 3.9; N, 15.6; S, 8.9 or $C_{16}H_{12}O_4N_4S$ which requires C, 53.9; H, 3.4; N, 15.7; S, 9.0%. Ultraviolet absorption: $\lambda_{\max}$ 250 (inflection), 320, 405 μ , $\log \epsilon$ 4.30, 4.12, 4.60, in ethanol.

Light-petroleum-insoluble tarry material

The combined material (10 g.) from the steam distillates which was insoluble in light petroleum was exhaustively distilled in steam. Most of the material remained in the distillation flask in the form of a semi-solid tar. The distillate (3 l.) was extracted with ether (4×750 ml.) and the ether was removed leaving a residue (0.4 g.) consisting of a golden-yellow mobile oil with a pleasant aromatic odour. The oil was dissolved in ethanol and poured into a large excess of Brady's reagent. An immediate orange-yellow turbidity was followed by rapid deposition of a microcrystalline ppt., which was collected after keeping overnight, 0.35 g., m.p. 210–213° (decomp.). It gave a red colour in ethanol with dil. NaOH. Recrystallization successively from acetic acid, benzene and ethyl acetate afforded orange-red needles (0.12 g.) of m.p. 247–8°. It gave no depression in m.p. on admixture with an authentic specimen of anisaldehyde 2:4-dinitrophenylhydrazone of m.p. 249–250°. (Found: C, 53.4, 53.3; H, 4.0, 3.6; N, 16.4, 17.3; OMe, 9.8, 9.7. Calc. for $C_{14}H_{12}O_2N_4$: C, 53.2; H, 3.8; N, 17.7; OMe, 9.6%.)

Syntheses

2:5-Thiophendicarboxylic acid. 2:5-Bis(chloromethyl)-thiophen was prepared from 5.5 g. of thiophen according to Griffing & Salisbury (1948). The crude reaction product was directly oxidized by dissolution in acetone and addition of powdered $KMnO_4$ until no further decolorization occurred. The MnO_2 was filtered off, dried and extracted with warm water (4×150 ml.). The extract was acidified and the solid material separating combined with further material extracted by ether. The slightly sticky solid (1.27 g.) obtained was refluxed with benzene to remove any monocarboxylic acid. The residual light brown powder (0.93 g.) did not melt but sublimed slowly in an open tube. In a sealed tube in metal block it softened and partly decomposed at 335–340°.

Dimethyl ester. The acid (100 mg.) was converted without further purification into the dimethyl ester by means of diazomethane in ether. On removal of the ether a yellow cryst. residue (m.p. 143–144°) was obtained. Two recrystallizations from aqueous methanol (1:1) gave thick flattened needles, m.p. 146–147° (70 mg.) of the dimethyl ester. Sublimation at 80–95° in high vacuum afforded small plates, m.p. 146–147°. (Found: C, 48.3; H, 3.9; S, 16.3; OMe, 30.7. Calc. for $C_8H_8O_4S$: C, 48.0; H, 4.0; S, 16.0, 2OMe, 31.0%.) Ultraviolet absorption: $\lambda_{\max}$ 275 μ , $\log \epsilon$ 4.27, in ethanol.

SUMMARY

1. The steam-volatile metabolic products of *Daedalea juniperina* Murr. have been investigated.

2. A new fungal metabolic product 5-(α -propynyl)-2-formylthiophen, m.p. 80°, has been isolated and characterized. It has been given the trivial name of junipal.

3. On oxidation with the Doeuvre reagent junipal affords junipic acid, $C_8H_6O_2S$, m.p. 180°.

4. Oxidation of junipic acid with $KMnO_4$ gives 2:5-thiophendicarboxylic acid, identified as the dimethyl ester, m.p. 145–6°.

5. The ultraviolet and infrared absorption spectra of junipal have been determined and are consistent with the structure advanced.

6. Indications of a second metabolic product closely related to junipal have been obtained.

7. A third metabolic product has been identified as anisaldehyde.

The authors wish to thank Dr W. P. K. Findlay of the Forest Products Research Laboratory, Princes Risborough, for supplying the cultures used, and Dr K. E. Almin of the Swedish Forest Products Research Laboratory, Stockholm, for the determination of the infrared absorption spectrum of junipal. The ultraviolet absorption spectra were determined by means of a Hilger Uvispek spectrophotometer. For the

grant for the purchase of this instrument the authors are indebted to the Central Research Fund Committee of London University.

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Micro-determination of Bromide in Body Fluids

By G. HUNTER

Stoke Mandeville Hospital, Aylesbury, Bucks

(Received 11 December 1954)

A micro method for the estimation of bromide in presence of chloride and in the absence of organic matter has been described by Hunter & Goldspink (1954). It is based on the quantitative conversion of bromide into bromate by hypochlorite: $Br^- + 3 OCl^- \rightarrow BrO_3^- + 3 Cl^-$, but instead of determining by iodometric titration as already described (Hunter, 1953), bromine is freed under suitable conditions according to: $BrO_3^- + 5 Br^- + 6 H^+ \rightarrow 3 Br_2 + 3 H_2O$ with quantitative conversion into tetrabromorosaniline, which is measured colorimetrically. The method has now been applied to the estimation of bromide in blood and other body fluids and it would appear to be applicable to other tissues. The essential problem here is to destroy the organic matter without loss of bromine. A procedure will be described for the treatment of blood and body fluids which permits precise determination of bromide in many samples in a short time. The material for analysis should not contain more than 100 and preferably less than 25 μg . Br; 1 μg . Br suffices.

EXPERIMENTAL

Reagents. These are described and discussed by Hunter & Goldspink (1954) and are as follows: sodium hypochlorite, N in 0.1N-NaOH, and bromine-free; sodium formate, 50% (w/v); buffer solution (pH 6.35) made by mixing 10 vol. 40% (w/v) NaH_2PO_4 , 2H₂O, 7 vol. 2N-KOH and 5 vol. water; rosaniline solution, 6 mg. in 100 ml. 2N-H₂SO₄; bromide-molybdate mixture, i.e. 0.15 g. KBr and 3 g. ammonium molybdate made to 100 ml. with water; sulphuric acid, 14N; *tert.*-butanol, with 5% (v/v) ethanol; potassium hydroxide, 2N.

Methods

Preparation of the test solution. Place in a nickel crucible (26 mm. diam., 10 ml. cap.) 1 ml. or less of serum or cerebrospinal fluid, add 2 drops 2N-KOH and dry in an oven at 110°. Remove to a muffle furnace at 600° and ignite for 30 min. Remove crucible, cool and add exactly 4 ml. water. Stir thoroughly with a small glass rod and transfer the solution to a test tube (16 × 125 mm.). Centrifuge. Remove 3.5 ml. of the supernatant fluid with a pro-pipette into another test tube (16 × 125 mm.) and add 1 ml. buffer, pH 6.35. Add 0.25 ml. sodium hypochlorite solution, mix