Studies in the Biochemistry of Micro-organisms

101. THE COLOURING MATTERS OF SPECIES IN THE *ASPERGILLUS NIDULANS* GROUP. PART 2. FURTHER OBSERVATIONS ON THE STRUCTURE OF ASPERTHECIN*

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Asperthecin, $C_{15}H_{10}O_8$, is a chestnut-brown crystalline colouring matter which was isolated by Howard & Raistrick (1955) from laboratory cultures of two strains of *Aspergillus quadrilineatus* Thom & Raper. It is of particular interest since it is characteristic of the purple-red perithecia and ascospores of a number of other species in the *Aspergillus nidulans* group in addition to *A. quadrilineatus*.

Howard & Raistrick (1955) established the partial structure (I) for asperthecin, but were unable to determine with certainty the position of the two remaining hydroxyl groups. They tentatively advanced two possible structural formulae (II and III), structure (II) illustrating best the available chemical evidence, while structure (III) was more in accord with the ultraviolet and infrared absorption spectra of asperthecin.

Attempts to degrade asperthecin by oxidation to smaller recognizable fragments were frustrated by the difficulty of obtaining satisfactory yields of its pentamethyl ether, in which the five nuclear hydroxyl groups, but not the side-chain $-CH_2 \cdot OH$ group, are methylated. Thus while methylation with dimethyl sulphate and potassium carbonate in acetone did indeed lead to the desired pentamethyl ether, the yields obtained were very small, probably because of the extreme instability of asperthecin towards alkali. Quick methylation of asperthecin with ethereal diazomethane in acetone solution gave only a dimethyl ether. It has been known for some time that certain *o*-hydroxy compounds, including hydroxylated anthraquinones, e.g. alizarin 2-methyl ether (Herzig & Klimosch, 1909), 1-hydroxy-, 1:5-dihydroxy- and 1:4-dihydroxy-anthraquinone (Schönberg & Mustafa, 1946), are not fully methylated by an ethereal solution of diazomethane, probably because of the formation of a chelated ring system in these compounds. Schönberg & Mustafa showed, however, that alizarin 2-methyl ether and 1:5-dihydroxyanthraquinone are readily methylated by ethereal diazomethane if methanol is added to the solution. In confirmation of their claims we have found that by

slow methylation under these conditions purpurin (1:2:4-trihydroxyanthraquinone) readily gives a trimethyl ether, and asperthecin gives a mixture of about 50 % each of a tetramethyl and a pentamethyl ether.

Oxidation of either the tetra- or penta-methyl ether of asperthecin with potassium permanganate leads to the formation of a substance existing as colourless needles, m.p. 157–158°, having the molecular formula $C_{11}H_8O_7$, and containing two methoxyl groups. Examination of the substance

* Part 100: Neill & Raistrick (1957).

indicated that it was probably a carboxydimethoxyphthalic anhydride.

Assuming that the carboxyl group arises by oxidation of the $-\text{CH}_2\cdot\text{OH}$ group in asperthecin and that the anhydride group arises from the quinone grouping, it follows that, if asperthecin has structure (III), oxidation of its pentamethyl ether would lead to (IV), i.e. 4-carboxy-3:6-dimethoxyphthalic anhydride. This substance, previously undescribed, was synthesized by oxidation of 3:6-dimethoxy-4-methylphthalic anhydride (Anslow & Raistrick, 1940). It was obtained as bright-yellow crystals, m.p. 220–221°, which are obviously not identical with the oxidation product of asperthecin pentamethyl ether. Hence (III) must be abandoned as a possible structure for asperthecin.

If the same assumptions as have just been made in considering structure (III) for asperthecin are now applied to structure (II), it follows that the corresponding oxidation product would have structure (V), i.e. 5-carboxy-3:4-dimethoxyphthalic anhydride. This substance, also previously undescribed, was synthesized by oxidation with hot potassium permanganate of 5-methylmeconin (VI), which was prepared substantially as described by Blair & Newbold (1954). The resulting anhydride (V) formed colourless needles, m.p. 157–158°, not depressed on admixture with the oxidation product of asperthecin pentamethyl ether of the same melting point.

It follows from this evidence, coupled with the fact that Howard & Raistrick (1955) obtained emodin (4:5:7-trihydroxy-2-methylanthraquinone) by the degradation of asperthecin, that the partial structure (I) for asperthecin may be expanded to structure (VII), so that asperthecin must then be either 3:4:5:6:7-pentahydroxy-2-hydroxymethylanthraquinone of structure (II) or 3:4:5:7:8-pentahydroxy-2-hydroxymethylanthraquinone of structure (VIII).

In the synthesis of 5-carboxy-3:4-dimethoxyphthalic anhydride (V) just described, 5-methylmeconin (VI) was oxidized with hot potassium permanganate. We found that when the oxidation

was carried out at room temperature a very good yield was obtained of 3:4-dimethoxy-5-methylphthalic acid, from which 3:4-dimethoxy-5-methylphthalic anhydride was readily prepared. It is suggested that this substance might well prove a useful starting point for syntheses of structures (II) and (VIII), and so determine with certainty the structure of asperthecin.

EXPERIMENTAL

Methylations with diazomethane in methanol

Purpurin trimethyl ether. An ethereal solution (400 ml.) of diazomethane ($\equiv 20$ g. of nitrosomethylurea) was added to a suspension of purpurin (3 g.) in methanol (150 ml.). The mixture was kept at room temp. for 5 days and was then filtered from a dark-purple precipitate (0.51 g.). The filtrate was evaporated to 50 ml., diluted with ether (200 ml.) and extracted with aqueous N-NaOH (2×50 ml.). The ethereal solution was concentrated, giving almost pure purpurin trimethyl ether (1.2 g.) which, after crystallization from ethanol, gave yellow needles, m.p. 165–166°. [Briggs & Nicholls (1951) give m.p. 169–171°; Wiles (1952) gives m.p. 168°] (Found: C, 68.45; H, 4.60; OMe, 30.9. Calc. for $\text{C}_{17}\text{H}_{14}\text{O}_6$: C, 68.4; H, 4.73; 3OMe, 31.2%). The ether is not soluble in aqueous NaOH . Its solution in cold conc. H_2SO_4 is deep cherry-red in colour with a bluish shade.

Asperthecin tetramethyl ether. An ethereal solution (300 ml.) of diazomethane ($\equiv 15$ g. of nitrosomethylurea) was added to a suspension of asperthecin (1 g.) in methanol (50 ml.). The mixture was held at room temp. for 1 day when a small amount of dark-red amorphous material was separated by filtration. The reddish-orange filtrate was evaporated to 15 ml. and the rosettes of orange needles which separated on cooling were collected by filtration (0.5 g.) (for treatment of the filtrate see next paragraph) and recrystallized from methanol, giving *asperthecin tetramethyl ether* as orange needles, m.p. 179–180° (Found: C, 60.9, 60.95; H, 4.76, 4.88; OMe, 33.4, 33.2. $\text{C}_{19}\text{H}_{16}\text{O}_8$ requires C, 60.95; H, 4.85; 4OMe, 33.2%). The tetramethyl ether is insoluble in aqueous Na_2CO_3 , but dissolves in aqueous NaOH to a strong pinkish-red solution. Its purple solution in cold conc. H_2SO_4 is not fluorescent. It gives a ruby-red ferric colour in ethanol solution.

Asperthecin pentamethyl ether. The methanolic filtrate from the tetramethyl ether was diluted with ether (100 ml.) and shaken with aqueous 2N-NaOH (20 ml.) to remove incompletely methylated asperthecin, and then with water. The washed ether solution, on standing, deposited yellow needles (0.45 g.), m.p. 145°, which were purified by crystallization from ethanol-light petroleum, b.p. 40–60°, giving *asperthecin pentamethyl ether* as yellow needles, m.p. 149–150° [Howard & Raistrick (1955) give m.p. 134–135°], which are insoluble in aqueous NaOH , do not give a ferric colour in ethanol and give a strong violet solution in cold conc. H_2SO_4 (Found: C, 62.0, 61.95; H, 5.35, 5.34; OMe, 40.0, 39.8. Calc. for $\text{C}_{20}\text{H}_{20}\text{O}_8$: C, 61.85; H, 5.19; 5OMe, 40.0%).

Oxidation experiments on asperthecin methyl ethers

Asperthecin tetramethyl ether. A solution of this methyl ether (0.15 g.) and KMnO_4 (1 g.) in acetone (50 ml.) was refluxed for 4 hr., when all the KMnO_4 had been used. The

mixture was then cooled and filtered, and the acetone filtrate was discarded since it contained only traces of an amorphous product. The MnO_2 precipitate was suspended in $2\text{N-H}_2\text{SO}_4$ (20 ml.) and SO_2 was passed until the MnO_2 dissolved. There remained in suspension an orange substance (m.p. $240-260^\circ$; soluble in NaHCO_3 ; strong purple colour in conc. H_2SO_4) which was oxidized further by boiling its suspension in $2\text{N-H}_2\text{SO}_4$ with excess of KMnO_4 for 10 min. KMnO_4 and MnO_2 were destroyed with SO_2 and the clear colourless solution was extracted with ether. The ether extract was evaporated to dryness and the residue was heated a little above its m.p. and then sublimed in high vacuum at $130-140^\circ$, giving a colourless crystalline sublimate (*A*) (30 mg.). The small unsublimed residue, which has not been identified, gave a slightly pink sublimate in high vacuum at $180-190^\circ$. This sublimate melts at $190-200^\circ$ and gives a purple ferric colour in ethanol, resembling that given by salicylic acid.

The crystalline sublimate *A* formed colourless prismatic needles, m.p. $145-155^\circ$ without obvious decomposition. It was purified for analysis as follows. Its solution in ether was shaken with aqueous NaHCO_3 . The bicarbonate extract was acidified with cold 2N-HCl and extracted with ether. The extract was evaporated to dryness and the residue, contained in a sublimation tube, was heated above its m.p. for 10 min. It was then cooled and sublimed in high vacuum at 120° . The sublimate was crystallized from dry isopropyl ether, giving the oxidation product as colourless needles which dissolve in NaHCO_3 with evolution of gas and give no colour with FeCl_3 . The product melts at $157-158^\circ$, not depressed on admixture with an authentic synthetic specimen of 5-carboxy-3:4-dimethoxyphthalic anhydride (structure V) of the same m.p. (see below) (Found: C, 52.6; H, 3.29; OMe, 24.5%; titration equiv., 85.5. $\text{C}_{11}\text{H}_8\text{O}_7$ requires C, 52.4; H, 3.20; 2 OMe, 24.6%; equiv. titrating as a tribasic acid, 84).

Asperthecin pentamethyl ether. KMnO_4 (about 3 g.) was added in portions over a period of 4 hr. to a suspension of asperthecin pentamethyl ether (0.4 g.; m.p. $148-150^\circ$) in aqueous 0.5N-NaOH (100 ml.) heated at 100° . The mixture was acidified with $2\text{N-H}_2\text{SO}_4$ and SO_2 was passed until clear and colourless. The solution was extracted with ether and, on removal of the solvent from the extract, the residue was sublimed in high vacuum at $120-140^\circ$, giving a slightly yellow sublimate, wt. 75 mg., m.p. $135-165^\circ$. This was purified as described for sublimate *A* above. After final crystallization from dry isopropyl ether the oxidation product was obtained as colourless needles, m.p. $157-158^\circ$ alone or in admixture with synthetic 5-carboxy-3:4-dimethoxyphthalic anhydride (*V*).

Syntheses

4-Carboxy-3:6-dimethoxyphthalic anhydride (IV). A solution of 3:6-dimethoxy-4-methylphthalic anhydride (0.3 g.; Anslow & Raistrick, 1940) in aqueous 0.5N-NaOH (100 ml.) and KMnO_4 (1 g.) was refluxed for 1 hr. The mixture was filtered while still hot and the precipitate was well washed with hot water. The clear alkaline filtrate and washings were acidified with conc. HCl , giving a yellowish precipitate which was extracted with ether. The dried (Na_2SO_4) ether extract was evaporated to dryness and the residue was purified by repeated sublimation in high vacuum. *4-Carboxy-3:6-dimethoxyphthalic anhydride* (0.25 g.) was thus obtained as a bright-yellow crystalline sublimate, m.p.

$220-221^\circ$, which is obviously not identical with the colourless KMnO_4 oxidation product (m.p. $157-158^\circ$) of asperthecin tetra- and penta-methyl ethers (Found: C, 52.2; H, 3.20. $\text{C}_{11}\text{H}_8\text{O}_7$ requires C, 52.4; H, 3.20%).

5-Methylmeconin. The starting point of this synthesis was *p*-toluic acid. This was sulphonated to give 1-carboxy-4-methylbenzene-3-sulphonic acid (Meldrum & Perkin, 1908), which on fusion with KOH gave 3-hydroxy-4-methylbenzoic acid (Meldrum & Perkin, 1908). The latter was methylated to give 3-methoxy-4-methylbenzoic acid, which was converted into 6-methoxy-5-methylphthalide, m.p. $143-144^\circ$, by treatment with aqueous formaldehyde and conc. HCl [Charlesworth, Rennie, Sinder & Yan (1945) who give m.p. 144°]. The remaining steps in the synthesis, (a) 6-methoxy-5-methylphthalide $\rightarrow$ (b) 6-methoxy-5-methyl-7-nitrophthalide $\rightarrow$ (c) 7-amino-6-methoxy-5-methylphthalide $\rightarrow$ (d) 7-iodo-6-methoxy-5-methylphthalide $\rightarrow$ (e) 5-methylmeconin, were those described by Blair & Newbold (1954), with the exception of step (b) $\rightarrow$ (c), which they carried out by catalytic hydrogenation with Raney nickel in ethyl acetate solution. Our method was as follows: 6-Methoxy-5-methyl-7-nitrophthalide (5 g.) was dissolved in hot aqueous KOH (5 g. in 50 ml. of H_2O). Sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$; 20 g.) was added to the cooled, stirred solution in the course of 20 min., the temperature being kept below 40° . The solution was acidified to Congo red with HCl and kept at 0° . The reduction product, which separated as needles, was collected and crystallized from ethanol as colourless needles (3.2 g.), m.p. $106-107^\circ$. Blair & Newbold (1954) give m.p. $109-110^\circ$.

Our preparation of 5-methylmeconin crystallized from light petroleum, b.p. $40-60^\circ$, or aqueous methanol in colourless needles, m.p. $81-81.5^\circ$. Blair & Newbold (1954) give m.p. 82.5° .

5-Carboxy-3:4-dimethoxyphthalic acid and its anhydride (V). A solution of KMnO_4 (2.53 g. $\equiv 5$ atoms of O) in water (100 ml.) was added in portions over a period of 3 hr. to a continuously refluxed solution of 5-methylmeconin (*VI*; 1 g.) in aqueous N-NaOH (5 ml.) and water (45 ml.). The mixture was cooled and acidified to Congo red with $2\text{N-H}_2\text{SO}_4$, and SO_2 was bubbled through to dissolve MnO_2 . The resulting colourless solution was evaporated *in vacuo* to 50 ml. and extracted with ether. The dried (Na_2SO_4) ether extract was evaporated to dryness, giving a colourless solid (1.1 g.; m.p. $198-200^\circ$, with effervescence); this was crystallized from ethyl acetate-light petroleum, b.p. $60-80^\circ$, giving 5-carboxy-3:4-dimethoxyphthalic acid as small colourless prisms, m.p. $204-206^\circ$ (with effervescence) (Found: C, 49.2; H, 3.81; OMe, 23.1%; equiv. 90.7. $\text{C}_{11}\text{H}_{10}\text{O}_8$ requires C, 48.9; H, 3.73; 2 OMe, 23.0%; equiv. as a tribasic acid, 90.1).

The above acid (0.3627 g.) was converted into its anhydride by heating above its m.p. for 15 min. Water (0.0255 g.; calc. 0.0242 g.) was evolved. The crude anhydride was purified by partition between ether and aqueous NaHCO_3 , followed by heating above its m.p. and sublimation in high vacuum at 120° and crystallization from dry isopropyl ether substantially as described for sublimate *A* (see above). *5-Carboxy-3:4-dimethoxyphthalic anhydride* was thus obtained as colourless needles, m.p. $157-158^\circ$, not depressed on admixture with the KMnO_4 oxidation products of asperthecin tetra- and penta-methyl ethers (Found: C, 52.4; H, 3.22; OMe, 24.7. $\text{C}_{11}\text{H}_8\text{O}_7$ requires C, 52.4; H, 3.20; 2 OMe, 24.6%).

3:4-Dimethoxy-5-methylphthalic acid and its anhydride. 5-Methylmeconin (2.08 g.; 10 m-moles) was dissolved by

heating in aqueous N -NaOH (12.0 ml.). The solution, cooled to room temp., was mixed with a cold solution of $KMnO_4$ (2.107 g. $\equiv$ 3 atoms of O) in water (100 ml.). The mixture was kept at room temp. overnight and acidified with $2N$ - H_2SO_4 , and SO_2 was passed to give a clear colourless solution, which was evaporated to about 50 ml. The resulting colourless crystalline solid (1.38 g.; m.p. 180–181° with effervescence) was collected, and ether extraction of the filtrate gave 0.94 g. of less pure material, m.p. 140–150°. The first crop was purified by crystallization from water, giving 3:4-dimethoxy-5-methylphthalic acid as colourless prisms, m.p. 184–185° (with effervescence) (Found: C, 54.6; H, 4.99; OMe, 26.2%; titration equiv., 123.6. $C_{11}H_{10}O_6$ requires C, 55.0; H, 5.03; 2 OMe, 25.8%; equiv. titrating as a dibasic acid, 120.1).

The above acid (114 mg.) was heated at 185–190° for 10 min., cooled and sublimed in high vacuum at 85–90°. The sublimate (96 mg., m.p. 114°) was crystallized from dry isopropyl ether, giving 3:4-dimethoxy-5-methylphthalic anhydride (61 mg.) as colourless needles, m.p. 118° (Found: C, 59.5; H, 4.49; OMe, 27.6. $C_{11}H_{10}O_5$ requires C, 59.4; H, 4.53; 2 OMe, 27.9%).

SUMMARY

1. Asperthecin has been shown to be either 3:4:5:6:7-pentahydroxy-2-hydroxymethylanthraquinone or 3:4:5:7:8-pentahydroxy-2-hydroxymethylanthraquinone.

2. It has been confirmed that full methylation of polyhydroxyanthraquinones with ethereal diazo-

methane is greatly facilitated by the addition of methanol to the methylation mixture.

3. The syntheses of 4-carboxy-3:6-dimethoxyphthalic anhydride, 5-carboxy-3:4-dimethoxyphthalic acid and its anhydride and 3:4-dimethoxy-5-methylphthalic acid and its anhydride are described.

4. 5-Carboxy-3:4-dimethoxyphthalic anhydride has been identified as an oxidation product of asperthecin tetra- and penta-methyl ethers.

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The Action of Diisopropyl Phosphorofluoridate and Other Anticholinesterases on Amino Acids

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The work described in this paper, which was carried out in 1950–52 and which has been the subject of a preliminary communication (Ashbolt & Rydon, 1952), was undertaken in an attempt to find some chemical parallel for the marked biochemical and toxicological difference between diisopropyl phosphorofluoridate (DFP) and its chlorine-containing analogue diisopropyl phosphorochloridate (DCIP); as is well known, DFP is very toxic and a potent inhibitor of several esterases (notably cholinesterase, chymotrypsin and trypsin), whereas DCIP is not markedly toxic and is not a powerful enzyme inhibitor. The discrepancy between the chemical and biochemical activities of the two compounds is very striking, since in general DCIP is chemically the

more reactive of the two (cf. Saunders & Stacey, 1948; Wagner-Jauregg, O'Neill & Summerson, 1951).

It is well established that the inhibition of chymotrypsin by DFP involves the diisopropylphosphorylation of a single amino acid side chain (Jansen, Nutting, Jang & Balls, 1949, 1950; Jansen, Nutting & Balls, 1949) and the same mechanism is highly probable for the inhibition of other enzymes by DFP. We decided accordingly to study the action of DFP and DCIP on a number of amino acids under 'physiological' conditions (aqueous solution, pH about 7, 37°) in the hope of finding some difference between the two phosphorus compounds in this respect; a number of other anticholinesterases were included for the sake of completeness.

Mazur (1946) found that the rate of liberation of acid, as measured by carbon dioxide evolution, from

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