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Structure of a Naturally Occurring Antagonist of Dihydrostreptomycin

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The isolation from culture filtrates of *Pseudomonas pyocyanea* of a crystalline product, which antagonizes under certain conditions the antibacterial action of dihydrostreptomycin, has been reported elsewhere (Lightbown, 1950, 1954). This paper records a chemical examination of the product, which has proved to be a mixture, and the synthesis of its principal constituents. The natural product is referred to hereafter as the 'antagonist'.

RESULTS AND EXPERIMENTAL

Use of partition chromatography for isolation of the antagonist. The yields of antagonist from the initial butanol extract of culture filtrates could be increased by evaporating most of the butanol in a rotary evaporator at approximately 30°, adding benzene until the mixture contained about 30% (v/v) benzene, and then running the solution on a kieselguhr column with a stationary phase of 0.1M phosphate buffer (pH 11.9) and butanol-benzene (7:3, v/v) as mobile phase. The column (3 ft. × 1½ in.) contained 200 g. of kieselguhr (Hyflo Super Cel) for a charge containing about 200 mg. of antagonist. The antagonist ran rapidly through the column and emerged free from most of the coloured impurities as well as from some contaminants absorbing in the ultraviolet. The product was further purified by crystallization from benzene-light petroleum.

Properties of the antagonist. The antagonist separated from ethanol or ethyl acetate in ill-defined plates. The melting point, which varied in different specimens, was in the range 136-148° and was little affected by repeated recrystallization. The antagonist was insoluble in water and dilute mineral acids, soluble in aqueous NaOH or Na₂CO₃,

to a yellow solution, and sparingly soluble in ether. It gave an orange-red colour with FeCl₃ in ethanol; it reacted instantly with Br₂-CHCl₃ with liberation of HBr; an aqueous alkaline solution reduced KMnO₄ rapidly in the cold. It did not reduce Fehling's solution; it liberated no iodine from NaI in isopropanol-acetic acid; it gave no azo-dye with diazotized sulphanilic acid. The ultraviolet absorption spectrum in aqueous solution at pH 9 showed a broad maximum at about 350 mμ. (*E*_{1%}^{1cm.} 250) and a sharper peak at 250 mμ. (*E*_{1%}^{1cm.} 700). Analysis indicated an approximate formula C₁₇H₂₃O₂N. (Found: C, 75.0, 74.8; H, 8.45, 8.2; N, 5.0; OMe, 0%.)

Isolation of the antagonist from an organism which has already been shown (Hays *et al.* 1945; Wells, Elliott, Thayer & Doisy, 1952; Wells, 1952) to produce 2-alkyl-4-hydroxyquinolines (I) suggested a chemical relationship, a suggestion further

supported by the general similarity of the ultraviolet absorption spectra. The only essential difference suggested by elementary analysis was the presence of an additional oxygen atom. One

possible formula, considered in view of the failure to couple with diazonium salts, was that of a 2-alkyl-3:4-dihydroxyquinoline (II) [2-alkyl-4-hydroxyquinolines do couple in the 3-position (Conrad & Limpach, 1888)]; but a substance of this type (II; R = Me), prepared by oxidation of 4-hydroxy-2-methylquinoline-3-aldehyde (III) with alkaline hydrogen peroxide, gave a pure-blue FeCl_3 reaction.

Preparation of 2-methyl-3:4-dihydroxyquinoline. The aldehyde (III) (187 mg.) (Conrad & Limpach, 1888) was dissolved in N-NaOH (1 ml.), and 3% H_2O_2 (1.4 ml.) was added. The solution became warm and the product crystallized in almost quantitative yield. Recrystallization from ethanol gave the *diol* (II; R = Me) in small leaflets, m.p. 304° (decomp.). (Found: C, 68.5; H, 5.1; N, 8.15. $\text{C}_{10}\text{H}_9\text{O}_2\text{N}$ requires C, 68.6; H, 5.1; N, 8.0%.)

Reduction of the antagonist. Decisive evidence of structure was obtained by reduction with zinc in acetic acid. The antagonist (100 mg.; m.p. $143\text{--}146^\circ$) in acetic acid (3 ml.) was warmed on a steam bath with zinc powder (0.5 g.) for 1 hr. with frequent shaking. Ether and water were added; the ether, after being freed from acetic acid by washing with water and NaHCO_3 , was evaporated. The residue was immediately dissolved in a little dry ether, from which the product (82 mg.) crystallized, m.p. $123\text{--}125^\circ$. A portion crystallized from ethyl acetate had m.p. 125° . (Found: C, 79.9; H, 9.06%.)

The analysis indicates that one atom of oxygen has been removed. The product resembled in composition and properties a 2-alkyl-4-hydroxyquinoline (I) with R approx. equal to C_8H_{17} ; in particular, the ultraviolet absorption

spectrum in ethanol was identical with that of 2-octyl-4-hydroxyquinoline, but the melting point was low. For comparison, the known 2-*n*-heptyl-4-hydroxyquinoline (I; R = $[\text{CH}_2]_6\text{CH}_3$) and 2-*n*-nonyl-4-hydroxyquinoline (I; R = $[\text{CH}_2]_8\text{CH}_3$) (Wells, 1952) were prepared, and by similar methods the new homologues 2-*n*-octyl-4-hydroxyquinoline (I; R = $[\text{CH}_2]_7\text{CH}_3$; colourless needles from ethyl acetate; m.p. $136\text{--}138^\circ$. Found: C, 79.0; H, 9.0; N, 5.5. $\text{C}_{17}\text{H}_{23}\text{ON}$ requires C, 79.4; H, 9.0; N, 5.45%) and 2-*n*-undecyl-4-hydroxyquinoline (I; R = $[\text{CH}_2]_{10}\text{CH}_3$; colourless aggregated needles from acetone; m.p. 135° . Found: C, 79.9; H, 9.3. $\text{C}_{20}\text{H}_{29}\text{ON}$ requires C, 80.2; H, 9.7%).

The resemblance between the reduced antagonist and the 'Pyo' compounds of Hays *et al.* (1945) was further tested by chromatography on paper. The paper was impregnated with ethylene glycol by immersion in a 5% (v/v) solution in acetone followed by removal of acetone. The substances were applied in ethanol. With a mobile phase of benzene the reduced antagonist was resolved into two spots, the slower of which corresponded in position with 2-*n*-heptyl-4-hydroxyquinoline (Pyo Ib) and the faster with 2-*n*-nonyl-4-hydroxyquinoline (Pyo Ic). Synthetic 2-*n*-octyl-4-hydroxyquinoline ran to an intermediate position. The spots were made visible by exposure to iodine vapour, when they appeared dark on a white ground.

The reduced antagonist was then run on a liquid-liquid column with ethylene glycol as stationary phase and benzene-*cyclohexane* (4:1, v/v) as mobile phase. By measuring the light absorption of the eluate at $328\text{ m}\mu$, at least five substances were shown to be present (Fig. 1, curve A, peaks 1-5). A mixture of synthetic 2-*n*-undecyl-, -*n*-nonyl-, -*n*-octyl- and -*n*-heptyl-4-hydroxyquinolines was run under the same conditions (Fig. 1, curve B). The

Fig. 1. Curve A. Separation of the components of the reduced antagonist. Load, 1 mg.; column length, 10 cm.; diameter, 1 cm.; stationary phase, ethylene glycol; mobile phase, benzene-20% *cyclohexane*.

Curve B. Separation of the components of a mixture of synthetic 2-alkyl-4-hydroxyquinolines, under the same conditions as in A. Peaks: (1) 2-*n*-undecyl-4-hydroxyquinoline; (3) 2-*n*-nonyl-4-hydroxyquinoline; (4) 2-*n*-octyl-4-hydroxyquinoline; (5) 2-*n*-heptyl-4-hydroxyquinoline. Conditions as for curve A.

n-undecyl compound corresponded in position with peak 1 in curve A, *n*-nonyl with peak 3, *n*-octyl with the trace component peak 4, and *n*-heptyl with the major component peak 5. These results suggested that the reduced antagonist was a mixture of 2-alkyl-4-hydroxyquinolines (I), the major components having as side chains heptyl and nonyl groups and the minor components octyl and undecyl groups. The minor component peak 2 might, from its position, have a decyl side chain.

Separation of 2-n-heptyl- and 2-n-nonyl-4-hydroxyquinolines from the reduced antagonist. The reduced antagonist (80 mg.) was run on a large (2 ft. $\times$ 1½ in.) liquid-liquid column with the ethylene glycol-benzene-cyclohexane system described above. The effluent corresponding to the main peak was washed with water and evaporated. The residue on crystallization from acetone gave white crystals (31 mg.), m.p. 142–144°. Another crystallization left 23 mg., m.p. 143–144.5°. A mixture with synthetic 2-*n*-heptyl-4-hydroxyquinoline (m.p. 143–144.5°) had the same m.p.

The first peak, representing the 2-*n*-nonyl component, showed insufficient resolution from faster-running components, two crystalline fractions (7 and 9 mg.) being obtained by crystallization, m.p. below 120° and 125–127° respectively. The total product divided into three equal parts was run on a smaller column (30 cm. $\times$ 1 cm.) with the same solvent system. The minor components were well separated from the 'nonyl' peak. The effluent corresponding to this peak after evaporation and crystallization from acetone gave 5 mg., m.p. 131–135°. This was further purified by leaving overnight with KMnO₄ (10 mg.) in acetone (1 ml.). A parallel oxidation was done with the synthetic nonyl compound. The recovered product after crystallization from acetone had m.p. 134–136°; a mixture with 2-*n*-nonyl-4-hydroxyquinoline (m.p. 136–137°) had m.p. 135–137°. X-ray powder photographs confirmed the identity of the two specimens. From the permanganate purification traces of fatty acids were obtained, and these were examined as the methyl esters by vapour-phase chromatography (see below for details). Methyl decanoate, nonanoate and octanoate were identified; the amounts of these esters obtained from the natural product were slightly greater than from the synthetic product.

Structure of the antagonist. The facile reduction of the antagonist to 2-alkyl-4-hydroxyquinoline limited the choice of structures. Only that of an *N*-oxide (IV) seemed worth considering, since the presence of a 4-hydroperoxide group seemed to be excluded by the failure to oxidize hydrogen iodide. Accordingly, syntheses of 2-alkyl-4-hydroxyquinoline *N*-oxides were begun.

2-Methyl-4-hydroxyquinoline *N*-oxide (IV; R=Me) was the only known example of this

chemical type; it was obtained by Gabriel & Gerhard (1921) and by McCluskey (1922) by reduction of *o*-nitrobenzoylacetone (V; R=Me). McCluskey also made it by decarboxylation of the 3-carboxylic acid (VI; R=Me), the ester of which had been prepared by reduction of ethyl *o*-nitrobenzoylacetate. We experimented first with a different method, the *N*-oxidation of a 2-alkyl-4-hydroxyquinoline. A direct oxidation failed, but when 2-*n*-heptyl-4-hydroxyquinoline was converted into the *O*-ethoxycarbonyl derivative (VII) and the product without isolation was oxidized with perbenzoic acid, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide could be obtained after alkaline hydrolysis. This substance resembled the antagonist in its properties, including the characteristic antagonism to dihydrostreptomycin. The yield was small, and for preparative purposes McCluskey's method was better. In this way 2-*n*-heptyl-, 2-*n*-nonyl-, and 2-*n*-undecyl-4-hydroxyquinoline *N*-oxides were prepared. In the heptyl series the route via the 3-carboxylic acid

was tried. This gave the same product but in poorer yield.

The requisite β -keto esters were synthesized according to Stållberg-Stenhagen (1945).

Synthesis of 2-n-heptyl-4-hydroxyquinoline N-oxide. (a) 2-*n*-Heptyl-4-hydroxyquinoline (1 g.) was converted into the sodium salt by dissolution in the calculated amount of ethanolic sodium ethoxide and evaporation at low pressure. The residue was suspended in benzene (20 ml.) and ethyl chloroformate (0.4 ml.) was added. The mixture was boiled under reflux until neutral and treated with perbenzoic acid in CHCl₃ (7.2 ml. of 7.9%, w/v). When the oxidant had been consumed the solution was washed with 0.5 *N*-Na₂CO₃ and water, and evaporated. The residue, a brown oil, was diluted with ethanol (10 ml.), and 2 *N*-KOH (5 ml.) was added. A precipitate formed slowly; next day this was removed and washed with *N*-KOH. The filtrate was acidified and the solid product was collected and triturated with ethyl acetate. The residue after two crystallizations from ethanol gave the *N*-oxide (IV; R = $\cdot[CH_2]_6 \cdot CH_3$) in colourless leaflets, m.p. 158–160°. (Found: C, 74.2; H, 8.3; N, 5.4. C₁₆H₂₁O₂N requires C, 74.1; H, 8.1; N, 5.4%). The ultraviolet absorption in 0.001 *N*-NaOH showed maxima at 346 m μ . (ϵ 9450) and 253 m μ . (ϵ 24000) (see Fig. 2). The substance easily formed a sparingly soluble Na salt with aqueous NaOH. On treatment with one equivalent of Br₂ in CHCl₃ a monobromo derivative (presumably 3-bromo-2-*n*-heptyl-4-hydroxyquinoline *N*-oxide) was formed; it was

isolated by evaporation of the CHCl_3 and treatment of the residue, a hydrobromide, with NaHCO_3 . Crystallization from ethanol gave leaflets, m.p. 178–179°. (Found: C, 56.5; H, 6.2. $\text{C}_{16}\text{H}_{20}\text{O}_2\text{NBr}$ requires C, 56.8; H, 5.9%.) A test for halogen was positive.

(b) Methyl 3-oxodecanoate (48 g.) was added dropwise to a stirred suspension of powdered sodium (5.52 g.) in toluene (100 ml.) and benzene (200 ml.) under N_2 . When the sodium was dissolved, *o*-nitrobenzoyl chloride (from 36.1 g. of *o*-nitrobenzoic acid, according to McCluskey, 1922) was added slowly with stirring and cooling. Next day dilute H_2SO_4 was added and the product (A; 84 g.) was isolated in the normal manner with the help of some more benzene. It was then boiled with dilute H_2SO_4 (500 ml. of 33%, w/w) and dioxan (68 ml.) for 7 hr. The cooled mixture was extracted with ether, and this, after washing with aqueous NaHCO_3 , was extracted with 0.5N-NaOH (1 l.) in seven portions which were acidified immediately after separation. The acidic product was recovered by means of ether; it was treated with cold light petroleum (b.p. 40–60°), and a residue (8 g.) of *o*-nitrobenzoic acid was removed. The light-petroleum-soluble product was a red oil (30 g.). A portion (5 g.) was added to a mixture of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (14 g.) and acetic acid (45 ml.), with sufficient dry HCl to effect dissolution. The mixture became warm; passage of hydrogen chloride was continued until it had cooled. After an hour the mixture was poured into water and extracted with chloroform. The chloroform was washed with water (emulsions were broken by filtration) and with aqueous NaHCO_3 and evaporated. The residue on trituration with ethyl acetate gave the crystalline *N*-oxide (1.5 g.), which was purified by recrystallization from ethanol: colourless leaflets, m.p. 158–160°, undepressed by material prepared by method (a).

(c) The crude diketone ester A (5 g.) was reduced with SnCl_2 as described above, and the product, which was semi-

crystalline, was hydrolysed by boiling for 1½ hr. with NaOH (20 ml. of 2N). The aqueous solution was extracted with ether and acidified. The precipitated solid was crystallized once from ethyl acetate and once from ethanol, 3-carboxy-2-heptyl-4-hydroxyquinoline *N*-oxide (VII; $\text{R} = \text{[CH}_2\text{]}_6 \cdot \text{CH}_3$) being obtained in colourless needles (0.25 g.), m.p. 173° (decomp.). (Found: C, 67.3; H, 6.95; N, 5.0. $\text{C}_{17}\text{H}_{21}\text{O}_4\text{N}$ requires C, 67.3; H, 6.9; N, 4.6%.) A crude preparation (500 mg.) of the acid was heated at 160° under N_2 until bubbling ceased. The product, after trituration with ethyl acetate and removal of a higher-melting impurity by crystallization from acetone and methanol, had m.p. 158° undepressed by material made by method (b). The final yield was 14 mg.

Synthesis of 2-n-nonyl-4-hydroxyquinoline N-oxide. Methyl 3-oxododecanoate (20 g.) was condensed, as described above, with *o*-nitrobenzoyl chloride, and the product was hydrolysed with dioxan-sulphuric acid by boiling for 18 hr. An ethereal extract of the hydrolysed product was shaken with saturated aqueous cupric acetate, when copper 1-(2-nitrophenyl)dodecane-1:3-dionate separated; a portion crystallized from ethanol in blue-grey needles, m.p. 149.5–150.5° [Found: N, 4.1. ($\text{C}_{18}\text{H}_{24}\text{O}_4\text{N})_2\text{Cu}$ requires N, 4.0%], the rest was decomposed by shaking with ether and dilute sulphuric acid. After evaporation of the ether the residue was crystallized from light petroleum (b.p. 40–60°) to give 1-(2-nitrophenyl)dodecane-1:3-dione (V; $\text{R} = \text{[CH}_2\text{]}_9 \cdot \text{CH}_3$) (8.7 g.). A sample after two more crystallizations formed plates, m.p. 44°. (Found: C, 67.7; H, 7.5; N, 4.4. $\text{C}_{18}\text{H}_{25}\text{O}_4\text{N}$ requires C, 67.7; H, 7.8; N, 4.4%.) The diketone (7.6 g.) was reduced with SnCl_2 , as described above; but the reaction mixture was left overnight before dilution and became very dark. The product was purified by three crystallizations from ethanol; the *N*-oxide (IV; $\text{R} = \text{[CH}_2\text{]}_9 \cdot \text{CH}_3$) formed colourless leaflets (2.5 g.), m.p. 148–149°. (Found: C, 75.0; H, 8.6; N, 5.1. $\text{C}_{18}\text{H}_{25}\text{O}_2\text{N}$ requires C, 75.3; H, 8.7; N, 4.9%.) The ultraviolet absorption in 0.001N-NaOH was identical with that of the heptyl analogue (see Fig. 2).

2-n-Undecyl-4-hydroxyquinoline N-oxide. Methyl 3-oxotetradecanoate (11.95 g., m.p. 29–30°), purified by low-temperature crystallization from methanol, was condensed as described above with *o*-nitrobenzoyl chloride, and the product was hydrolysed by boiling for 17 hr. with dilute sulphuric acid and dioxan. Copper 1-(2-nitrophenyl)-tetradecane-1:3-dionate was prepared as described above. A sample crystallized from ethanol in blue-grey needles, m.p. 150–151° (Found: N, 4.1. $\text{C}_{20}\text{H}_{28}\text{O}_4\text{N}_2$ requires N, 3.7%); from the remainder, 1-(2-nitrophenyl)tetradecane-1:3-dione (V; $\text{R} = \text{[CH}_2\text{]}_{10} \cdot \text{CH}_3$) was prepared as above and was crystallized from light petroleum (b.p. 40–60°): yield, 4 g., m.p. 53–54°. A sample on further crystallization formed almost colourless plates, m.p. 53.5–54.5°. (Found: C, 69.1, 69.2; H, 8.2, 8.3; N, 4.4. $\text{C}_{20}\text{H}_{28}\text{O}_4\text{N}$ requires C, 69.2; H, 8.4; N, 4.0%.) The diketone (3 g.) was reduced with SnCl_2 as described for the heptyl analogue; however, the chloroform extract of the diluted mixture was thoroughly shaken with dilute sulphuric acid before washing with water. In this way, emulsions caused by separation of tin oxides were avoided. The product on crystallization from ethanol gave leaflets (1.8 g.), m.p. 144–146°. Two further crystallizations gave the *N*-oxide (IV; $\text{R} = \text{[CH}_2\text{]}_{10} \cdot \text{CH}_3$) in colourless shining leaflets, m.p. 148.5–149.5°. (Found after drying at 80°: C, 76.4; H, 8.8; N, 4.8. $\text{C}_{20}\text{H}_{28}\text{O}_2\text{N}$ requires C, 76.2; H, 9.2; N, 4.4%.)

Fig. 2. Ultraviolet absorption spectrum of two synthetic 2-alkylhydroxyquinoline *N*-oxides in 0.001N-NaOH. ■, 2-n-Nonyl-4-hydroxyquinoline *N*-oxide; ●, 2-n-heptyl-4-hydroxyquinoline *N*-oxide.

Oxidation of the antagonist. Chromatograms of the type used to analyse the reduced antagonist would not be expected to separate unsaturated or branched-chain components from the *n*-alkyl series. Accordingly, the antagonist itself was oxidized for examination of the resulting fatty acids by a more sensitive method. In an early oxidation only octanoic and heptanoic acids were identified, and this led to the surmise that one of the major components had an unsaturated nonenyl side chain (Cornforth & James, 1954). Later and more precise results gave no support to this hypothesis, which is now withdrawn.

The antagonist (12 mg.) was dissolved by warming with water (2 ml.) and saturated aqueous Na_2CO_3 (0.1 ml.). To the cooled solution 1% KMnO_4 (1.7 ml.) was added dropwise. Interaction was rapid at first. After 3 hr. the slight excess of reagent was destroyed and the manganese dioxide was removed. The yellow filtrate was acidified and extracted with ether. The acidic product was esterified in the usual manner with ethereal diazomethane. After removal of solvent the esters were ready for vapour-phase chromatography. Synthetic 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide was oxidized by the same procedure for purposes of comparison.

The gas columns used to separate the methyl esters were 4 ft. long and had as stationary phase Apiezon M vacuum stopcock grease (James & Martin, 1956). The separations were carried out at 197°, the esters being applied in ethanolic solution with a micropipette. The zones leaving the column were detected by the gas density meter (Martin & James, 1956). Other conditions are shown in the legend to Fig. 3.

The model experiment with the 2-*n*-heptyl compound yielded not only the expected methyl octanoate, but also a considerable amount of methyl heptanoate and traces of hexanoate and valerate (Fig. 3, curve A). This non-homogeneous oxidation under mild conditions of an alkyl

side chain suggests that care should be taken in the interpretation of similar degradations.

Analysis of the mixture of acids produced by oxidation of the antagonist is shown in Fig. 3, curve B. The acids identified were *n*-pentanoic, *n*-hexanoic, *n*-heptanoic, *n*-octanoic (the major component), *n*-nonanoic, *n*-decanoic (the next largest component), *n*-undecanoic, *n*-dodecanoic and *n*-tridecanoic acids. These results, taken in conjunction with the oxidation of the synthetic *n*-heptyl compound, indicate the presence of *n*-heptyl, *n*-nonyl and *n*-undecyl side chains, with traces of higher homologues.

The question of unsaturated components remains unsettled. Certainly the purity of the 2-nonyl-4-hydroxyquinoline from reduced antagonist was improved by partial oxidation, and we have observed that the melting point of the antagonist is raised about 10° after partial ozonolysis (which produces a little aldehydic material) or oxidation with peracetic acid. However, unsaturated components must be present, if at all, in minor amount.

In an early oxidation of the natural antagonist (87 mg.) the manganese dioxide after drying was extracted twice with boiling ethanol. On concentration of the extract long needles (8 mg.) separated. These were insoluble in water and in aqueous acids and alkalis; on heating they darkened from 250° without melting. (Found: C, 79.0; H, 8.4; N, 6.0. $\text{C}_{35}\text{H}_{40}\text{O}_4\text{N}_2$ requires C, 79.0; H, 8.3; N, 5.8%.) From the composition and properties it is suggested that this product is a 1:1-diquinolyl (VIII). Homogeneity of the side chain is not proved, but the analysis suggests that the heptyl side chain predominates.

(VIII)

Fig. 3. Curve A. Separation by gas-liquid chromatography of methyl esters of the fatty acids resulting from the oxidation of synthetic 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. Peaks in order of appearance: (1) air introduced in loading the sample and ethanol; (2) methyl *n*-pentanoate; (3) methyl *n*-hexanoate; (4) methyl *n*-heptanoate; (5) methyl *n*-octanoate. Column length, 4 ft.; temperature, 197°; nitrogen pressure, 13 cm. Hg; nitrogen-flow rate, 15 ml./min. Stationary phase, Apiezon M vacuum stopcock grease.

Curve B. Separation, under the same conditions, of the methyl esters of fatty acids produced by the oxidation of the crude antagonist. Peaks in order of appearance: (1) air introduced in loading the sample and ethanol (the solvent in which the sample was dissolved); (2) methyl *n*-hexanoate; (3) methyl *n*-heptanoate; (4) methyl *n*-octanoate; (5) methyl *n*-nonanoate; (6) methyl *n*-decanoate; (7) methyl *n*-undecanoate; (8) methyl *n*-dodecanoate; (9) methyl *n*-tridecanoate.

DISCUSSION

The occurrence of *N*-oxides in nature is by no means rare; a review is given by Culvenor (1953). The antagonist resembles in its composition and melting point the Pyo II of Hays *et al.* (1945), and it is possible that this product is also a mixture of 2-alkyl-4-hydroxyquinoline *N*-oxides.

As shown above, the antagonist contains as its principal components 2-heptyl-, 2-nonyl- and 2-undecyl-4-hydroxyquinoline *N*-oxide, the proportions diminishing in that order. Of these three, the nonyl compound has the highest activity

are of course not excluded. The infrared spectrum of the natural mixture, as a paste in medicinal paraffin (Nujol) or in carbon disulphide solution, showed no band in the range 3100–3500 cm.⁻¹. This recalls the similar behaviour of 1-nitroso-2-naphthol (Amstutz, Hunsberger & Chessick, 1951). Another tautomeric form (XII) was suggested for the 2-methyl compound by McCluskey (1922), because a dibenzoate could be obtained. The latter, however (if it is not a benzoic acid salt of a monobenzoate), could be the 2-benzoxymethyl compound (XIII) formed by a rearrangement recently reported for quinaldine *N*-oxide (Pachter, 1953).

against dihydrostreptomycin. A mixture of the three synthetic *N*-oxides in the ratio (w/w) 63·7 heptyl:33·7 nonyl:2·6 undecyl (a proportion suggested by the results of degradation of the natural antagonist) had activity of the same order as the natural mixture against dihydrostreptomycin (Lightbown & Jackson, 1956). The structure of the known Pyo compounds and of these *N*-oxides suggests a plausible mode of biosynthesis. Condensation of anthranilic acid with a β -keto acid intermediate in the synthesis or degradation of fatty acids could lead (IX), with loss of carbon dioxide and water, directly to a Pyo compound, from which the *N*-oxide can be presumed to arise by oxidation.

An origin of this kind would explain why the side chains of the main components, both in the Pyo compounds and in the *N*-oxides, have odd numbers of carbon atoms, since the β -keto acids (containing three additional carbons) would have, predominantly, even numbers. The presence of small amounts of even-number side chains, such as the 2-octyl compound, which may be inferred from analysis of the reduced antagonist, is not inconsistent with this mode of biosynthesis, for traces of odd-number fatty acids have recently been shown to occur regularly in natural mixtures (Hansen, Shorland & Cook, 1955; James & Martin, 1956).

The products are written as 4-hydroxyquinoline *N*-oxides (IV), but tautomeric structures (X, XI)

SUMMARY

1. A chemical examination has been made of a crystalline product from *Pseudomonas pyocyanea* having the property of antagonizing the antibacterial action of dihydrostreptomycin.

2. It is concluded that the product is a mixture containing as major components 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide and 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide in the approximate proportion 2:1, along with a small proportion of 2-*n*-undecyl-4-hydroxyquinoline *N*-oxide. Traces of 2-*n*-octyl-4-hydroxyquinoline *N*-oxide and of higher homologues may also be present.

3. 2-*n*-Heptyl-, 2-*n*-nonyl- and 2-*n*-undecyl-4-hydroxyquinoline *N*-oxides have been synthesized. Of these, the 2-*n*-nonyl compound has the highest activity against dihydrostreptomycin.

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Inhibition of Cytochrome Systems of Heart Muscle and Certain Bacteria by the Antagonists of Dihydrostreptomycin: 2-Alkyl-4-Hydroxyquinoline N-Oxides

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Antagonism of streptomycin activity by culture filtrates of *Pseudomonas aeruginosa* was first reported by Sureau, Arquie, Boyer & Saviard (1948). The factor responsible for the antagonism was isolated (Lightbown, 1954) and was shown by Cornforth & James (1954, 1956) to be a mixture of 2-alkyl-4-hydroxyquinoline N-oxides. The present paper describes an investigation of some of the biological properties of these compounds; part of this work has already been briefly reported (Lightbown & Jackson, 1954; Jackson & Lightbown, 1955).

METHODS AND MATERIALS

Assay of dihydrostreptomycin antagonist activity. A modified cup-plate diffusion method (Lightbown, 1954) was employed. Cups containing the solutions of the quinoline N-oxides were placed on plates of deep-seeded nutrient agar containing a concentration of dihydrostreptomycin five times that sufficient to prevent growth of the test organism in the absence of the antagonist. After incubation, sharply defined zones of growth appeared around the cups and the comparative potencies of the samples could be calculated from the diameters of these zones, as in a normal antibiotic cup-plate assay. With large assay plates accommodating 36 cups arranged in a 6 × 6 Latin square fiducial limits of ±5 to 10% were regularly obtained for potency ratios derived from a single plate.

Bacterial strains. *Bacillus subtilis* I.C.I. strain (N.C.T.C. 8236), *Bacillus pumilus*, Mill Hill strain (N.C.T.C. 8241) and typical laboratory strains of *Staphylococcus aureus*, *Escherichia coli* and *Proteus vulgaris* were used in experiments.

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Bacterial growth curves. Nutrient broth (200 ml.) in a 2 l. conical flask was inoculated and incubated, with shaking, for 3 hr. Samples (10 ml.) were then transferred to separate 250 ml. conical flasks. These smaller flasks had special side arms constructed to fit the carrier of the absorptiometer, so that rapid measurements of optical density could be made by tipping the contents of the flask into the side arm. Aeration of all cultures was effected by shaking at 180 oscillations/min., amplitude of stroke 1 in. Optical-density measurements were made at 650 mμ.

Bacterial suspensions and cell-free preparations. Bacteria were grown in nutrient broth in shaken culture or on nutrient agar for 18 hr. at 36°. The cells were collected and washed three times with distilled water by centrifuging. Dry weights were determined by heating at 110° to constant weight, and opacity/dry-weight relationships were measured with a photoelectric absorptiometer.

Cell-free preparations were prepared by shaking bacterial suspensions (density 10–20 mg./ml.) with ballotini beads, size 14, in a Mickle disintegrator. Shaking was continued for 40 min., or until microscopical examination showed the presence of only occasional whole cells. Unbroken cells and large fragments were removed by centrifuging at 800 g in an angle head for 15 min. and the supernatant fluids collected.

Heart-muscle succinic-oxidase preparations were prepared from fresh pig heart by the method of Slater (1949a), except that the minced and washed muscle was homogenized in a high-speed blender for 2 min., instead of grinding with sand. Fat-free dry weights of these preparations were determined by the method of Slater (1949a).

Spectroscopic examination of the different preparations was carried out with either a Zeiss or Beck low-dispersion micro-spectroscope and a 150 c.p. Pointolite.

Reagents. Cytochrome c was obtained from L. Light and Co. Ltd.; the methylene blue was the 'biologically tested' grade obtained from British Drug Houses Ltd. The p-