The Structures of Two Naphthoquinone Pigments from an Actinomycete[1]

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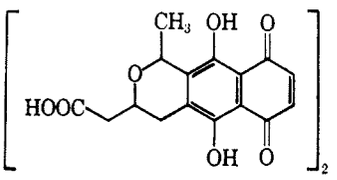
Two pigments from a strain of nonsporulating *Streptomyces*, no. 12396, have been identified as 5,8-dihydroxy-2,7-dimethoxy-1,4-naphthoquinone (**2**) and 2,7-dimethoxy-5-hydroxy-1,4-naphthoquinone (**3**). The red pigment, **2**, was previously known as a degradation product of spinochrome M. It inhibits the growth of Gram-positive bacteria and fungi *in vitro*. The orange pigment, **3**, was previously unknown and is antibiotically inactive.

Most natural naphthoquinones are plant products; a few are found in sea urchins;[2] and some are elaborated by microorganisms.[2a,3,4] Excluding the K vitamins all microbial naphthoquinones have been isolated from fungi except actinorhodin (**1**) which is produced by *Streptomyces* coelicolor.[5]



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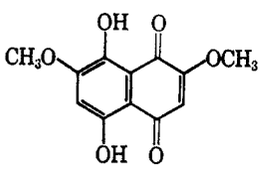
On the basis of its cell-wall analysis[6] nonsporulating actinomycete 12396, received from Dr. Enrique Tejera, Caracas, Venezuela, has been tentatively identified as a strain of streptomyces. It produced two major pigments when grown on 6% aqueous Pablum; one was dark red, the other orange. Some pigment production occurred also in wheat and oat extracts, but not in a variety of other media although growth of the organism was excellent.

The pigments were extracted from whole broth with chloroform then separated and purified by column chromatography on silicic acid eluting with chloroform. They were not well resolved by paper chromatography or thin layer chromatography and formed mixed crystals which melted sharply. Mixtures of the two pigments in solution were assayed by absorption spectrophotometry: the dark red one at its 550-mμ peak where the orange one had no absorption; the orange one at its 435-mμ maximum correcting for the small absorption of the dark red one at this wavelength.

The dark red pigment (80–100 mg/l. from acidified whole broth) melted at 275–6°, gave a positive ferric chloride test, was reversibly decolorized by sodium hydrosulfite, and was insoluble in sodium bicarbonate solution. It showed maxima at 285, 308, 480, 512, and 550 mμ; the infrared spectrum contained no free hydroxyl bands and no carbonyl absorption lower than 6.3 μ. Elementary analysis and a molecular weight determination indicated molecular formula $C_{12}H_{10}O_6$, and functional group analyses showed two O-methyl groups, no C-methyl groups, and two active hydrogen atoms. Attempted methylation with dimethyl sulfate gave recovered starting material; reductive acetylation furnished a leucotetraacetate, $C_{20}H_{20}O_{10}$.

The analyses, ultraviolet spectrum and color tests pointed to a dihydroxydimethoxynaphthoquinone structure; furthermore the insolubility in bicarbonate,[7] unsuccessful methylation, and infrared spectrum[8] indicated strong hydrogen bonding for all hydroxyl and carbonyl functions. Thus, the dark red pigment is a dimethoxy-5,8-dihydroxy-1,4-naphthoquinone (a dimethoxynaphthazarin). The known 2,3-dimethoxynaphthazarin melted at 133°.[9] Since in solution naphthazarins exist as mixtures of rapidly interconverting tautomers the 2,3 and 6,7 isomers are identical.[7,10] 2,6- and 2,7-dimethoxy naphthazarins were reported to melt at 295–296 and 235–236°, respectively;[11] however, the published nmr spectrum of the 2,7 isomer,[11] **2** (Scheme I), agreed with that of our pigment

SCHEME I



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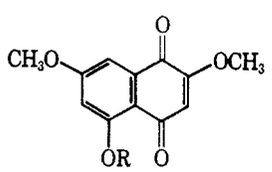

3, R = -H

4, R = -CH_3

in showing two strongly hydrogen-bonded hydroxyl groups, at δ 12.7 and 13.1. This **2** had been obtained by methanolic hydrogen chloride treatment of 2-acetyl-3,6-dihydroxynaphthazarin (spinochrome M), a pigment of sea urchin spines. A sample of this **2**, after purification melted at 274° and was completely identical with our **2**.[11a]

The orange pigment (10–20 mg/l. from whole broth adjusted to pH 8) had mp 266–268°, gave a positive ferric chloride test, was reversibly decolorized by sodium hydrosulfite, and was insoluble in sodium bicarbonate solution. It showed maxima at 261, 302,

(1) This work was supported by the National Science Foundation GB-3130.

(2) (a) R. H. Thomson, "Naturally Occurring Quinones," Academic Press Inc., New York, N. Y., 1957, p 55; (b) for recent work, see J. Gough and M. D. Sutherland, *Tetrahedron Letters*, 269 (1964); C. W. J. Chang, R. E. Moore, and P. J. Scheuer, *ibid.*, 3557 (1964).

(3) R. H. Thomson, "Chemistry and Biochemistry of Plant Pigments," T. W. Goodwin, Ed., Academic Press Inc., New York, N. Y., 1965, p 309.

(4) M. W. Miller, "The Pfizer Handbook of Microbial Metabolites," McGraw-Hill Book Co., Inc., New York, N. Y., 1961, p 248.

(5) H. Brockmann, W. Müller, and K. Van der Merve, *Naturwissenschaften*, **49**, 131 (1962).

(6) B. Becker, M. P. Lechevalier, and H. A. Lechevalier, *Appl. Microbiol.*, **13**, 236 (1965).

(7) See ref 2a, pp 102, 103, 112, 121.

(8) H. Brockmann and B. Franck, *Naturwissenschaften*, **42**, 45 (1955).

(9) C. Kuroda, *J. Sci. Res. Inst.* (Tokyo), **46**, 188 (1952); *Chem. Abstr.* **48**, 6411a (1954).

(10) See L. F. Fieser, "Organic Chemistry," 2nd ed, D. C. Heath and Co., Boston, Mass., 1950, pp 754–756.

(11) C. W. J. Chang, R. E. Moore, and P. J. Scheuer, *J. Am. Chem. Soc.*, **86**, 2959 (1964).

(11a) NOTE ADDED IN PROOF.—S. Natori, Y. Kumada, and H. Nishikawa [*Chem. Pharm. Bull.* (Tokyo), **13**, 633 (1965)] prepared **2** by methylation of 2,5,7,8-tetrahydroxynaphthoquinone, mompain, from *Helicobasidium mompa*. They reported 260–262° as the melting point of **2** and demonstrated its identity with the **2** from spinochrome M.

and 435 mμ; the infrared spectrum was similar to that of **2** but contained carbonyl bands at 6.0 and 6.2 μ. Elementary and functional group analyses indicated an empirical formula of $C_{10}H_4O_3(OCH_3)_2$; a satisfactory molecular weight determination could not be obtained because of poor solubility. Reductive acetylation furnished a leucotriacetate, $C_{18}H_{18}O_8$. Thus, the orange pigment is 2,7-dimethoxy-5-hydroxy-1,4-naphthoquinone, **3**. The arrangement of substituents was chosen on biosynthetic grounds, by analogy to **2** and because of the similarity of the ultraviolet spectrum to that of flaviolin trimethyl ether, **4**. The structure was proved by vigorous methylation of **3** to **4** whose melting point and spectra were identical with published values;[12] furthermore it was completely identical with an authentic specimen of **4**.

Compound **2** had modest activity against some Gram-positive bacteria and some fungi; **3** was inactive toward all microorganisms used. Both were nontoxic at 600 mg/kg when administered intraperitoneally to mice.

Experimental Section[13]

5,8-Dihydroxy-2,7-dimethoxy-1,4-naphthoquinone (2). A. From Actinomycete No. 12396.—This organism was maintained on yeast extract–glucose agar slants, transferred every 4–6 weeks and when well grown stored at 5°. For production a week old slant was used to inoculate two flasks of yeast extract–glucose broth,[14] 50 ml/250 ml flask. After 2 days at 215 rpm[15] and 28° the resulting whole broth was inoculated at 5% into 6% aqueous Pablum,[14] 75 ml/250 ml flask. After 10 days at 28° and 215 rpm the resulting whole broth (3 l.) was adjusted to pH 4.0 with sulfuric acid and filtered by gravity. The wet mycelium was ground in a Waring Blendor then shaken overnight with chloroform (1.5 l.) and sufficient water to make two layers. The red organic layer was separated and after a second extraction the conbined chloroform extracts were concentrated and applied to a silicic acid column (150 g). Chloroform elution of the main dark red band, evaporation of the chloroform, and trituration of the residue with hexane gave reddish black crystals of **2** which after recrystallization from acetic acid–water then chloroform–hexane melted at 275–276°. Pure **2** gave a blue color with alcoholic ferric chloride, was reversibly decolorized with sodium hydrosulfite, was insoluble in water, concentrated hydrochloric acid, and 5% sodium bicarbonate, but dissolved in dilute aqueous sodium hydroxide (purple solution): λ_{max}^{Nujol} 3.25 (w), 3.7–3.9, 6.3, 6.45, 7.85, 8.2, 8.35, 8.5, 8.95, 9.2, 10.0, 10.55, 11.4, 12.3, 13.0 μ; $\lambda_{max}^{CHCl_3}$ 285, 308, 480, 512, 550 mμ (ε 8625, 9450, 7125, 8500, 5500). The nmr spectrum in deuterochloroform showed 6 methoxyl group hydrogens at δ 3.90, 2 aromatic hydrogens at δ 6.40, and 2 hydrogen-bonded phenolic hydrogens at δ 12.67 and 13.08 which disappeared when D_2O was added.

Anal. Calcd for $C_{12}H_{10}O_6$: C, 57.61; H, 4.03; mol wt, 250; 2 O–CH_3, 24.8; active hydrogen, 0.80. Found: C, 57.09, 57.19; H, 3.93, 3.92; N, 0.0; mol wt 243 (by osmometry in chloroform solution); O–CH_3, 24.9; C–CH_3, 0.4; active hydrogen, 0.66.

B. Reactions.—Acetylation of **2** (85 mg) was carried out with acetic anhydride and 1 drop of sulfuric acid on the steam bath for 30 min. After the usual work-up, the crude product was chromatographed on silicic acid (5 g) eluting with chloroform. The orange solid from the main band (66 mg) melted over a range (200–250°) which was not narrowed by recrystallization or further chromatography.

Attempted methylation of **2** with dimethylsulfate and potassium carbonate in refluxing acetone for 30 hr gave only recovered starting material.

Reductive acetylation of **2** (114 mg) was accomplished with acetic anhydride (4 ml) and zinc dust (210 mg) refluxing 2 hr. After additional acetic anhydride (2 ml), acetic acid (2 ml), and refluxing (1 hr) the straw colored solution was decanted and the residue washed with hot acetic acid. The combined acid solutions poured into water furnished a crude product (118 mg, mp 238–242°) which was chromatographed on a 30-g silica column eluting with 5% methanol in chloroform. After recrystallization from ethanol the yield of white crystals, mp 250–252°, was 52 mg: λ_{max}^{EtOH} 245, 313 mμ (ε 62600, 6720); λ_{min} 270 mμ; λ_{max}^{Nujol} 5.77, 6.22 μ.

Anal. Calcd for $C_{20}H_{20}O_{10}$: C, 57.14; H, 4.80; Ac, 41.0. Found: C, 56.80; H, 4.78; Ac, 43.6.

C. From Spinochrome M.—Because the vial of **2** was completely crushed in transit it was extracted with chloroform, the solution filtered, taken to dryness, and the residue (in C_6H_6) chromatographed on a 2-g column of acid-washed silicic acid[11] eluting with benzene. The purified material (1 mg, mp 240–50°) after recrystallization from dioxane (0.3 ml.)–water (0.3 ml) melted at 273–4° and weighed 0.5 mg. The mixture melting point was **2** from part A was 273–5° and the 2 products had identical paper chromatographic behavior: R_f values 0.24 and 0.33 in solvents A and B, 3.5 cm in solvent C after 24 hr.

2,7-Dimethoxy-5-hydroxy-1,4-naphthoquinone (3).—Chloroform elution of the orange band as for the red band above gave orange crystals of **3** which after recrystallization from dioxane–water melted at 266–268°. Pure **3** gave a brown color with alcoholic ferric chloride, was reversibly decolorized with sodium hydrosulfite, was insoluble in water, concentrated hydrochloric acid, and 5% sodium bicarbonate but dissolved in dilute aqueous sodium hydroxide (red-purple solution): λ_{max}^{Nujol} 3.25 (w), 3.7–3.9, 6.0, 6.2, 6.35, 7.85, 8.1, 8.3, 8.6, 8.95, 9.2, 9.75, 10.1, 10.5, 11.4, 11.5, 11.92, 12.5, 13.2 μ; $\lambda_{max}^{CHCl_3}$ 261, 302, 435 mμ (ε 15,200, 11,800, 4300). The nmr spectrum in deuterochloroform showed methoxy group hydrogens at δ 3.90.

Anal. Calcd. for $C_{12}H_{10}O_5$: C, 61.54; H, 4.30; 2 O–CH_3 26.5. Found: C, 61.32; H, 4.41; N, 0.0; O–CH_3, 26.1.

In order to obtain greater quantities of **3** whole broth from pilot plant fermentations was adjusted to pH 8 and extracted with chloroform. Under these conditions very little **2** but all of the **3** and much oil was extracted. Separation of **3** from the oil was accomplished by repeated column chromatography on silicic acid eluting with chloroform.

Reductive acetylation of **3** as of **2** gave tan crystals, mp 161–162° after recrystallization from methanol then chloroform: λ_{max}^{EtOH} 242, 295, 303 mμ (ε 45,760, 9220, 9220).

Anal. Calcd for $C_{18}H_{18}O_8$: C, 59.67; H, 5.01; Ac, 35.6. 35.6. Found: C, 58.95; H, 5.15; Ac, 36.56.

2,5,7-Trimethoxy-1,4-naphthoquinone (Flaviolin Trimethyl Ether, 4).—A mixture of **3** (35 mg), anhydrous potassium carbonate (3 g), dimethyl sulfate (3 ml), dry acetone (15 ml), and dry dimethylformamide (5 ml) was refluxed 10.5 hr. The acetone was removed in a stream of air, the mixture poured into water, the solution adjusted to pH 3, then extracted with chloroform. After washing, the chloroform solution was applied to a 40-g silicic acid column. Elution with chloroform followed by 1% methanol in chloroform brought down a broad orange band of unreacted **3** followed by an intense narrow dark band. Extrusion of the column and extraction of this dark band with methanol gave **4**, 15 mg after recrystallization from ethanol–water: mp 196–199°; λ_{max}^{EtOH} 214, 260, 296, 416 mμ (ε 33800, 14200, 9800, 2700); lit.[12] mp 186–187°, 191°; λ_{max} 215, 262, 296, 414, or 419 mμ (ε 34700, 16200, 11500, 3160). An authentic sample of **4** melted at 197–199°; the mixture melting point was undepressed. The **2** products had identical paper chromatographic behavior: R_f values 0.34 and 0.36 in solvents A and B, 3.5 cm in solvent C after 24 hr.

Antimicrobial Assay.—Assays were carried out as previously described.[16] Neither **3** nor **2** showed any activity against *Es-*

(12) B. D. Astill and J. C. Roberts, *J. Chem. Soc.*, 3302 (1953).

(13) Melting points were determined using the Kofler Micro hot stage. Analyses were by George Robertson, Florham Park, N. J., and Alfred Bernhardt, Mülheim, Germany. Paper chromatography (descending method) used Schleicher and Schuell No. 2497 (fully acetylated) paper to which a leader strip of Whatman No. 1 paper had been sewed. Solvent systems employed were A, toluene–ethanol–water (4:17:1); B, butanol–acetic acid–water (4:1:1); C, ethanol–water (1:1). Mallinckrodt silicic acid 100 mesh (powder) was used for all column chromatography. Ultraviolet and infrared spectra were measured on a Cary Model 14 recording spectrophotometer and a Perkin-Elmer Infracord respectively. Nmr data were recorded on a Varian Associates Model A-60 spectrophotometer using tetramethylsilane as the internal standard.

(14) For details of media preparation, see N. N. Gerber and H. A. Lechevalier, *Appl. Microbiol.*, **13**, 935 (1965).

(15) Rotary action shaker, Model V, New Brunswick Scientific Co., New Brunswick, N. J.

(16) N. N. Gerber and M. P. Lechevalier, *Biochemistry*, **3**, 598 (1964).

cherichia coli 54, *Proteus vulgaris* 73, or *Pseudomonas aeruginosa* 77. The minimum inhibitory concentration of **2** in μg/ml for the following organisms is given in parentheses; **3** was either inactive as 50 μg/ml or was not tested, (−). An asterisk means static activity only. *Sarcina lutea* 14 (3), *Staphylococcus aureus* 15 (7−), *Corynebacterium fimi* 22 (3), *Candida albicans* 204 (4), *Saccharomyces cerevisiae* 216 (6), *Hansenula anomala* (8*−), *Aspergillus niger* 13 (9*−), *Penicillium notatum* 40 (9−), *Trichophyton mentagrophytes* 171 (2), *Mycobacterium smegmatis* 607 (2), *M. rhodochrous* 271 (2), *Micropolyspora brevicatena* 1086W/F (2*−), *Microellobosporia cinerae* 3855 (2), *Microbispora rosea* 3748 (2−), *Nocardia asteroides* 3409 (2.5), *N. madurae* 1091 (2−), *Actinoplanes sp.* W13 (2), *Streptosporangium roseum* W48 (2−).

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Vat Dyes from Benzimidazo[1,2-*b*]isoquinolin-5(12H)-one

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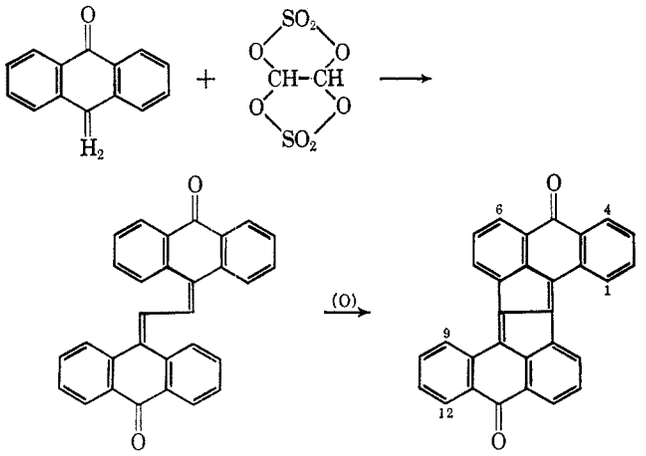
Research and Development Division Publication No. 392, Jackson Laboratory, Organic Chemicals Department, E. I. du Pont de Nemours and Company, Wilmington, Delaware

A benzimidazoisoquinolinone, prepared by Bistrzycki and Fassler[1] in 1923, is shown to be benzimidazo[1,2-*b*]-isoquinolin-5(12H)-one (II). Several routes to the corresponding dione (V) are described. The preparation and properties of heterocyclic vat dyes, analogs of acedianthrones, are reported.

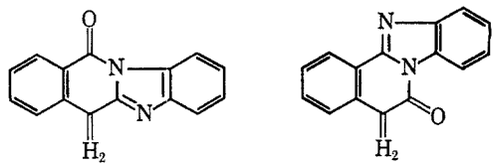
Aceanthryleno[2,1-*a*]aceanthrylene-5,13-dione (I) is the parent compound of an important class of vat dyes known as the acedianthrones. It is also noteworthy as an example of that uncommon class of compounds which contains the pentalene ring system. The method of preparation of I suggests that analogous structures, possibly useful as vat dyes, might be prepared from compounds which show a similar structural relationship



I

between a carbonyl group, a methylene group, and an aromatic ring. This paper describes the preparation of heterocyclic analogs of I from an intermediate possessing these structural features, benzimidazo[1,2-*b*]-isoquinolin-5(12H)-one (II).

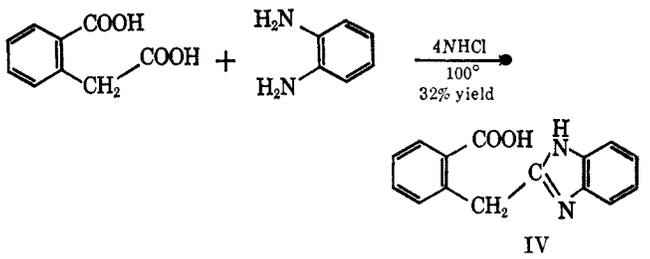
The fusion of *o*-phenylenediamine with homophthalic anhydride has been reported[1] to give a benzimidazoisoquinolinone of structure II or III.



II III

(1) A. Bistrzycki and K. Fassler, *Helv. Chim. Acta*, **6**, 527 (1923).

This ambiguity made it desirable to prepare II by an unequivocal route. First α-(2-benzimidazolyl)-*o*-toluic acid (IV) was prepared from *o*-phenylenediamine and homophthalic acid. Structure IV is assigned on the



IV

basis of the different reactivities of the two carboxyl groups of homophthalic acid. It is reported that benzoic acid and *o*-phenylenediamine heated at 100° in the presence of 4 *N* hydrochloric acid give traces of 2-phenylbenzimidazole,[2] whereas phenylacetic acid and *o*-phenylenediamine under the same conditions give 2-benzylbenzimidazole in 50–60% yield.[3]

Further evidence for structure IV is given by the similarity of the ultraviolet spectrum of IV with that of other 2-benzylbenzimidazoles (Table I). By comparison, the 2-phenylbenzimidazoles exhibit completely different spectra.

The cyclization of IV to II, was carried out in acetic anhydride at 60°. Evidence that the cyclized compound has structure II rather than III was provided by its conversion to the corresponding dione, which was identical with a sample of benzimidazo[1,2-*b*]isoquinoline-5,12-dione (V) prepared by the unambiguous route shown in Scheme I.

Comparison of the physical properties of II with those of the acetic acid soluble fraction of Bistrzycki's product[1] suggests that Bistrzycki's product also has structure II. The presence of a small amount of III in the reaction mixture of Bistrzycki is not excluded.

(2) M. A. Phillips, *J. Chem. Soc.*, 2395 (1928).

(3) G. K. Hughes and F. Lions, *J. Proc. Roy. Soc. N. S. Wales*, **71**, 221 (1938).