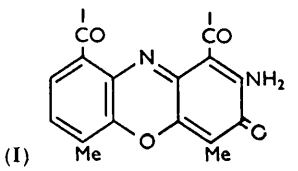
513. *The Chemistry of Mould Metabolites. Part II.* A Partial Structure for Polystictin.*

By G. W. K. Cavill, P. S. Clezy, and J. R. Tetaz.

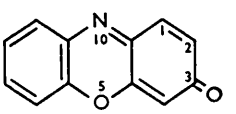
Degradative and spectroscopic studies on polystictin (cinnabarin), a red pigment isolated from *Coriolus sanguineus* (Fr.), show the presence of a phenoxazone nucleus. The quinonoid ring is substituted by an acidic hydroxyl and an amino-group, and the benzenoid ring is believed to contain the uncharacterised moiety ($C_2H_2O_2$). A 1-amino-2-hydroxyphenoxazin-3-one structure is proposed for the chromophore.

Polystictin, $C_{14}H_{10}O_5N_2$, a red pigment isolated from the wood-rotting fungus *Coriolus sanguineus*,[1,2] has been identified with cinnabarin, isolated from *Trametes cinnabarina*.[3] Previous work [2] indicated the presence of an acidic hydroxyl group, an amide group, possibly an ether link, and an unsaturated nucleus containing a quinonoid system and heterocyclic nitrogen. These observations are now supported by degradative evidence,[4] but the amide group is modified to a vinylogous amide system, $\cdot CO\cdot C{:}C\cdot NH_2$.

Polystictin or *O*-methylpolystictin with potassium permanganate or with alkaline hydrogen peroxide yields oxalic acid and ammonia.[5] Such complete breakdown is characteristic of highly substituted quinones but attempts to convert polystictin into a stable derivative of the quinol, suitable for oxidation, failed. Hydrogenation (palladium catalyst) of *O*-methylpolystictin gives a dihydro-derivative, which is rapidly re-oxidised in air. A trace of a reduced methylated substance, insufficient for degradation, was isolated on treatment of *O*-methylpolystictin with dimethyl sulphate in alkaline solution containing an excess of zinc dust.



(I)



(II)

Fusion of polystictin with a mixture of zinc dust, zinc chloride, and sodium chloride was attempted in expectation that the quinone would be converted into the parent carbocyclic (or heterocyclic) system.[6] However, a yellow oil was isolated which, on chromatography, gave an orange substance (H) in $<0{\cdot}1\%$ yield, with other, unidentified products. The ultraviolet absorptions of polystictin and its derivatives (see Table) are similar to that of actinomycin,[7,8] which has the chromophore (I), and the changes in absorption of actinomycin caused by cold aqueous alkali are also shown by *O*-methylpolystictin.[9] The ultraviolet absorption of product H is very similar to that of phenoxazin-3-one (II), although insufficient was available for determination of accurate ε values; and the R_F values of product H and phenoxazin-3-one, in various solvent systems, are identical: hence product H is considered to be phenoxazin-3-one. The infrared spectrum (" Nujol "

* Part I, *J.*, 1953, 525.

[1] Report Nat. Health Med. Res. Council Australia, 1946, p. 12; Lemberg, *Austral. J. Exp. Biol. Med. Sci.*, 1952, **30**, 271.
[2] Cavill, Ralph, Tetaz, and Werner, *J.*, 1953, 525.
[3] Gripenberg, *Acta Chem. Scand.*, 1951, **5**, 590.
[4] Cf. Cavill and Tetaz, *Chem. and Ind.*, 1956, 986.
[5] Cf. Tetaz, Ph.D. Thesis, N.S.W. University of Technology, 1955.
[6] Cf. Clar, *Ber.*, 1939, **72**, 1645.
[7] Brockmann and Muxfeldt, *Chem. Ber.*, 1956, **89**, 1397; *Angew. Chem.*, 1956, **68**, 69.
[8] Angyal, Bullock, Hanger, and Johnson, *Chem. and Ind.*, 1955, 1295.
[9] Personal communication from Professor S. J. Angyal.

mull) of product H, whilst showing no bands in the 3 μ region attributable to hydroxyl or amino-groups (cf. polystictin),[2] shows strong absorption at 1644 and 1620 cm.$^{-1}$; these bands, also present in the spectrum of phenoxazin-3-one, are assigned to >C=O and >C=N– groups. Isolation of a quinonoid product by zinc dust degradation is unusual.

The hydroxyl group in polystictin was previously placed [2] *peri* to a carbonyl system on the basis of colour reactions. However, the strong absorption at 3505 cm.$^{-1}$ in polystictin,

Ultraviolet absorption spectra of polystictin and related compounds.

Compound *	$\lambda_{max.}$ (mμ)	log ε	$\lambda_{max.}$ (mμ) †	log ε	$\lambda_{max.}$ (mμ)	log ε	$\lambda_{max.}$ (mμ)	log ε
Polystictin (EtOH)	225		~270		—		430 445—450	
(dioxan)	234	4·2	~260	3·9	—		430 455	4·0 4·0
Actinomycin (*a*) [7]	238		Infl.		—		425 443	
(*b*) [8]	232—236	4·50			—		443—444	4·33
2-Hydroxyphenoxazin-3-one	223	4·20	~260		—		400	4·09
2-Aminophenoxazin-3-one	237	4·17	268	3·90	—		421—436	4·10
O-Methylpolystictin	234	4·65	265	4·30	310—315	3·59	434	4·55
O-Acetylpolystictin	232	4·61	260	4·30	305	3·53	433	4·39
N-Acetyl-*O*-methylpolystictin	236	4·46	265	4·13	310	3·42	434	4·37
Phenoxazin-3-one	245	4·13	263	3·89	348	3·99	449	3·94
Product H	247		~260—270		350		445—450	
Triacetylanhydrodihydro-polystictin	245	4·52	—		320	3·48	433	3·97
Triacetyldihydro-*O*-methyl-polystictin	235	4·57	—		315	3·55	405	3·86
Reduced methylated poly-stictin	235		—		330		398	
Diacetyl*iso*polystictin	225	4·38	260	4·11	Infl.		393	4·24
Diacetyl-*O*-methyl*iso*poly-stictin	237	4·48	245 265	4·41 4·09	Infl.		404	4·40

* In ethanol (95%) unless otherwise stated.
† Inflexions.

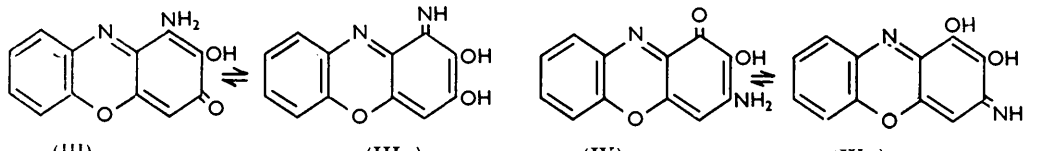
attributable to a weakly hydrogen-bonded, or a non-bonded, hydroxyl group is not shown by *peri*-hydroxyquinones,[10] and hence the hydroxyl group is now placed on the quinonoid ring of the phenoxazone, in which position it also accommodates the known acidity of polystictin.

The formation of ammonia on alkaline hydrolysis of polystictin, together with the subsequent evolution of carbon dioxide on acidification, suggested the presence of an amide (or imide) grouping, an observation supported by infrared spectroscopic data; but we failed to prove the presence of an amide group by dehydration to the nitrile (cf. terramycin [11]). The ready liberation of ammonia on hydrolysis, by 2N-hydrochloric acid, of polystictin and *O*-methylpolystictin, together with the isolation of an acidic methylated substance ($C_{15}H_{11}O_6N$) as a hydrolytic by-product of the methylation of polystictin,[2] indicates the presence of an aminoquinonoid or similar system. That is, polystictin may contain a vinylogous rather than a simple amide. Further, acetylation of the amino-group explains the conversion of *O*-methylpolystictin into *N*-acetyl-*O*-methylpolystictin and, more significantly, explains the formation of triacetyldihydro- rather than diacetyldihydro-compounds on reductive acetylation of polystictin and its derivatives.[2]

The orientation of the amino- and hydroxy-substituents on the phenoxazone nucleus is shown by reductive acetylation. In the conversion of polystictin and *O*-acetylpolystictin into triacetylanhydrodihydropolystictin, and of *O*-methylpolystictin and *N*-acetyl-*O*-methylpolystictin into triacetyldihydro-*O*-methylpolystictin, two acetyl groups are

[10] Flett, *J.*, 1948, 1441.
[11] Hochstein, Stephens, Conover, Regna, Pasternack, Gordon, Pilgrim, Brunings, and Woodward, *J. Amer. Chem. Soc.*, 1953, **75**, 5455.

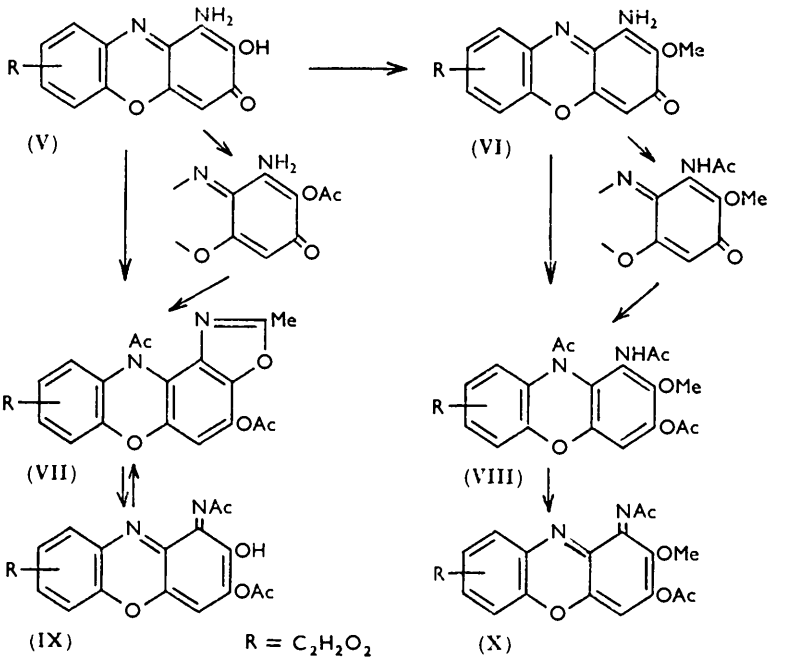
involved in the reductive acetylation of the phenoxazone nucleus and the third is present on the amino-group. The anhydro-compound obtained from polystictin and from *O*-acetylpolystictin is thus an oxazole, formed by cyclisation of the acetamido-substituent with an adjacent hydroxyl (or acetate) group. As *O*-methylpolystictin and its *N*-acetyl derivative do not form an anhydro-compound, the hydroxyl group involved in the above



(III) (IIIa) (IV) (IVa)

cyclisation must be that originally present in polystictin. Further, the original amino-substituent, thus placed *ortho* to the acidic hydroxyl group on the phenoxazone nucleus, cannot be adjacent to the quinone carbonyl group (a potential hydroxyl group). Hence the chromophore of polystictin is represented by structure (III) or (IV).

It is most likely that the pyruvic acid (0·4 mol.), obtained by the action of hot 25% sodium hydroxide solution on polystictin, arises from the breakdown of this trisubstituted quinonoid ring.[12]



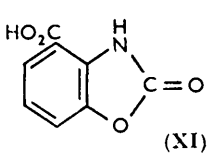
(V) (VI) (VII) (VIII) (IX) (X) R = $C_2H_2O_2$

Whilst the isolation of phenoxazin-3-one (II), on zinc dust distillation, supports the *para*-quinonoid structure (III) for the chromophore of polystictin, the *ortho*-structure (IV) cannot be excluded; a compound of type (IV) could be degraded *via* the tautomeric dihydroxy-*para*-quinone imine (IVa). However, the striking similarity in the ultraviolet absorption spectra of polystictin, of actinomycin (cf. I), and of 2-amino- and 2-hydroxy-phenoxazin-3-one strongly supports the *para*-quinonoid structure (III). Polystictin is

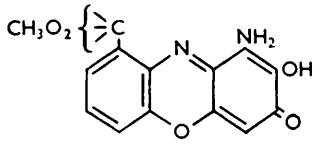
[12] Cf. Asano and Yamaguti, *J. Pharm. Soc. Japan*, 1940, **60**, 585.

then represented by (V; R = $C_2H_2O_2$), *O*-methylpolystictin by (VI), and the reduced acetylated derivatives by (VII) and (VIII), respectively.

Oxidation of triacetylanhydrodihydro- (VII) and of triacetyldihydro-*O*-methylpolystictin (VIII) with nitrous acid in dilute hydrochloric acid yields an acidic diacetyl*iso*polystictin (IX) (with the re-addition of the elements of water) and a neutral diacetyl-*O*-methyl*iso*polystictin (X), respectively. The acetyl group lost during this oxidation of the reduced acetylated derivatives (VII and VIII) must be that attached to the ring-nitrogen atom (cf. Brockmann and Franck [13]) and hence the new quinonoid compounds (IX and X), which retain two acetyl groups, are formulated as derivatives of the tautomeric *iso*polystictin (IIIa). These reactions require the amino-group to be *ortho* (or *para*) to the ring-nitrogen atom and hence provide additional confirmation of the *meta*-relation of the amino-group to the quinone-carbonyl group. Diacetyl*iso*polystictin (IX), which is also obtained as a by-product during the working-up of the triacetylanhydrodihydro-derivative (VII), should be identical with Gripenberg's cinnabarin *leuco*acetate A[3] (compare m. p. and acidity). However, cinnabarin *leuco*acetate A was acetylated to yield the *leuco*acetate B, identical with triacetylanhydrodihydropolystictin (VII), whereas the compound (IX) requires reductive acetylation to reform the oxazole (VII).



(XI)



(XII)

An uncharacterised moiety ($C_2H_2O_2$), substituted on the benzene ring, appears not to have been involved in any of the above reactions. Gripenberg, Honkanen, and Patoharju [14] reported the isolation of benzoxazolone-4-carboxylic acid (XI) on permanganate oxidation of cinnabarin (polystictin) (cf. the analogous formation of 7-methylbenzoxazolone-4-carboxylic acid from actinomycin [8]). Thus the $C_2H_2O_2$ fragment is attached at the 9-position on the phenoxazone nucleus, and polystictin is represented by the partial structure (XII).

Experimental

Light petroleum had b. p. 60—80°. Alumina refers to aluminium oxide of grade H from Peter Spence. Neutralised alumina is prepared by washing grade H alumina with methanol–acetic acid (9 : 1), triturating it with hot methanol until all washings are neutral, and drying it at 150—200°. Carbon, hydrogen and nitrogen microanalyses are by Dr. E. Challen and Mr. D. Weeden of this University, additional microanalyses by C.S.I.R.O. Microanalytical Laboratory (Melbourne), and infrared spectra by Mr. I. H. Reece.

Molecular Weight of Polystictin and its Derivatives.—Low solubility of polystictin, and many of its derivatives, in organic solvents hinders molecular-weight determination. No satisfactory determination has been possible for polystictin. However, an ebullioscopic estimation on *O*-methylpolystictin (28·15 mg.) in chloroform (5 ml.) gave a b. p. elevation of 0·055° (Found : *M*, 254. Calc. for $C_{15}H_{12}O_5N_2$: *M*, 300). Isothermic microdistillation [15] of triacetyldihydro-*O*-methylpolystictin, in acetone, was undertaken because of a wide discrepancy in the results of Rast determinations (Found : *M*, 454. Calc. for $C_{21}H_{20}O_8N_2$: *M*, 428). These results confirm the formula $C_{14}H_{10}O_5N_2$ for the parent compound. In agreement with Lemberg,[1] we have found Rast determinations on triacetylanhydrodihydropolystictin (Gripenberg's[3] cinnabarin *leuco*acetate B) to be unreliable.

Oxidation of O-*Methylpolystictin with Potassium Permanganate.*—*O*-Methylpolystictin (0·3 g.)

[13] Brockmann and Franck, *Angew. Chem.*, 1956, **68**, 68.
[14] Gripenberg, Honkanen, and Patoharju, *Chem. and Ind.*, 1956, 1505.
[15] Niederl, Kasanof, Kisch, and Subba Rao, *Mikrochem.*, 1949, **34**, 132.

and potassium permanganate (1 g.) were dissolved in acetone (300 ml.) and kept for 48 hr. at 0—10°. The precipitated manganese dioxide was then filtered off and, after trituration with saturated sodium hydrogen carbonate solution, rejected. The alkaline washings, after acidification with 2N-hydrochloric acid, were extracted with chloroform. Removal of solvent gave a buff solid (11 mg.), which was shown by paper chromatography [16] to be a mixture of unidentified acids. The aqueous solution remaining after chloroform extraction was evaporated; the residue, sublimed at 160—170°/1 mm., gave oxalic acid (20 mg.), m. p. and mixed m. p. 98—99°. A further sublimate (6 mg.), m. p. >320°, gave a positive test with Nessler's reagent, indicating ammonium chloride.

Oxidation of Polystictin with Alkaline Hydrogen Peroxide Solution.—Polystictin (0·2 g.) in 0·3% aqueous sodium hydroxide solution (60 ml.) was treated with 30% hydrogen peroxide (2 ml.) and kept for 1 week at room temperature, hydrogen peroxide (1 ml.) being added every second day. The colour changed from violet to pale yellow and the pH from 10—11 to 8. No basic products were isolated from the alkaline solution. After acidification with hydrochloric acid, the solution was continuously extracted with ether. Paper chromatography of this extract with ethyl methyl ketone–cineole–53% w/v formic acid (50 : 50 : 36),[17] and ethanol–water–ammonia (*d* 0·88) (80 : 16 : 4) [16] as solvent systems revealed a mixture of acids. The spots were developed with silver nitrate.[17] One had R_F 0·51, identical with that of oxalic acid.[17]

Hydrogenation of O-*Methylpolystictin.*—*O*-Methylpolystictin (100 mg.) in acetic acid (15 ml.) was reduced over palladium–charcoal for 35 min. at N.T.P., 0·84 mol. of hydrogen being absorbed. The amber solution, after removal of catalyst, was neutralised with excess of sodium hydrogen carbonate solution, then extracted with chloroform (2 × 100 ml.). The orange-brown solid obtained gave *O*-methylpolystictin (64 mg.), m. p. 234—240° (decomp.), from ethanol (Found: C, 60·2; H, 4·0; N, 9·65. Calc. for $C_{15}H_{12}O_5N_2$: C, 60·0; H, 4·0; N, 9·3%).

Reductive Methylation of O-*Methylpolystictin.*—*O*-Methylpolystictin (0·1 g.) and zinc dust (3 g.) were suspended in cold aqueous 2N-sodium hydroxide (10 ml.) and shaken with dimethyl sulphate (2 ml.) for 15 min. After filtration, the yellow solution was extracted with chloroform (60 ml.), and the aqueous layer rejected. Removal of solvent gave a brown oil (44 mg.), purified by chromatography on alumina from light petroleum. The yellow band, eluted with chloroform, yielded a yellow substance (4 mg.), m. p. 188—190° (decomp.) (from benzene–light petroleum), which gave the green colour in concentrated sulphuric acid characteristic of the reduced acetylated derivatives of polystictin.[2]

Zinc Dust Fusion of Polystictin.—Polystictin (20 mg.), zinc dust (200 mg.), zinc chloride (400 mg.), and sodium chloride (20 mg.) were fused together at 230—240° for 10 min., with stirring. The products of 85 fusions were combined and digested with N-hydrochloric acid (1 l.) for 1 hr. The remaining amorphous brown solid was filtered off, washed with water, dried at 60°, and extracted (Soxhlet) with chloroform for 24 hr. Similarly, the acid filtrate (and washings) were continuously extracted with ether. The combined product of the two extractions, a yellow oil (100 mg.), was taken up in benzene (50 ml.) and chromatographed on neutralised alumina. An orange-red band eluted with benzene–chloroform (3 : 1) gave a product, purified by distillation at 120—150°/0·15 mm. This substance was further resolved on treatment with hexane. A small quantity of fine red needles (*ca.* 1 mg.) remained, whilst the yellow hexane solution slowly deposited product H (*ca.* 1 mg.), as orange prisms. The red substance, and other fractions obtained during chromatography, are being investigated.

The R_F values of product H and phenoxazin-3-one were identical in 5% aqueous pyridine (R_F 0·53), in *iso*propanol–water–90% formic acid (25 : 65 : 10) (R_F 0·81), and in acetone–water–90% formic acid (10 : 80 : 10) (R_F 0·61), the horizontal technique being used. The spots were detected by their colour and by their orange-pink fluorescence in ultra-violet light.

Acid Hydrolysis of Polystictin and of O-*Methylpolystictin.*—A suspension of polystictin (50 mg.) in 2N-hydrochloric acid (25 ml.) was heated under reflux for 4 hr. On cooling, the mixture was filtered to remove an intractable black precipitate. The red filtrate was diluted with water (100 ml.), the colour changing to pale yellow. A sample of this solution (10 ml.), made alkaline with 2N-sodium hydroxide, gave an orange-yellow precipitate with Nessler's reagent.

O-Methylpolystictin was similarly hydrolysed, ammonia again being detected in the filtrate.

[16] Long, Quayle, and Stedman, *J.*, 1951, 2197.
[17] Anet and Reynolds, *Austral. J. Chem.*, 1955, **8**, 267.

Acetylation of O-*Methylpolystictin.*—*O*-Methylpolystictin (50 mg.), acetic anhydride (6 ml.), and pyridine (1 ml.) were refluxed for 7 min. The red solution was then poured on ice, and the orange-red solid (55 mg.) precipitated was dissolved in chloroform (200 ml.). This extract, after treatment with 2N-hydrochloric acid (2 × 10 ml.) to remove starting material, gave N-*acetyl*-O-*methylpolystictin*, finally isolated as red needles, m. p. 232° (decomp.) (from benzene) (Found: C, 60·0; H, 4·6; N, 8·4; Ac, 12·7. $C_{17}H_{14}O_6N_2$ requires C, 59·7; H, 4·1; N, 8·2; Ac, 12·7%).

Reductive Acetylation of N-*Acetyl*-O-*methylpolystictin.*—*N*-Acetyl-*O*-methylpolystictin (35 mg.), acetic anhydride (5 ml.), pyridine (2 drops), and an excess of zinc dust were heated together for 5 min. The mixture, on cooling, was poured on ice, and the resultant yellow solid filtered off. Repeated recrystallisation from light petroleum–benzene gave triacetyldihydro-*O*-methylpolystictin (28 mg.) as yellow needles, m. p. and mixed m. p. 157°. (This compound is described in Part I [2] as the product of reductive acetylation of *O*-methylpolystictin.)

Reductive Acetylation of O-*Acetylpolystictin.*—*O*-Acetylpolystictin (25 mg.), reductively acetylated as described for polystictin,[2] gave triacetylanhydrodihydropolystictin (15 mg.), as yellow needles, m. p. 201° (from benzene) (Found: C, 60·2; H, 3·8. Calc. for $C_{20}H_{16}O_7N_2$: C, 60·6; H, 4·1%).

Nitrous Acid Oxidations of Reduced Acetylated Derivatives.—(*a*) To triacetylanhydrodihydropolystictin (0·3 g.) in 90% dioxan (15 ml.) at 0—5°, 10N-hydrochloric acid (1 ml.) was added, then 30% sodium nitrite solution (1 ml.), giving a red colour; gas was evolved. The precipitate (0·21 g.) gave *diacetyliso*polystictin, orange-red needles, m. p. 211—212° (decomp.) (from alcohol) (Found: C, 58·1; H, 4·0; N, 7·7; Ac, 25·8. $C_{18}H_{14}O_7N_2$ requires C, 58·4; H, 3·8; N, 7·6; Ac, 23·4%), soluble in cold saturated sodium hydrogen carbonate solution and recovered therefrom on acidification.

Diacetyl*iso*polystictin (100 mg.), on reduction as above, gave triacetylanhydrodihydropolystictin (76 mg.) as yellow needles, m. p. and mixed m. p. 197—198° (from benzene).

(*b*) Similarly, triacetyldihydro-*O*-methylpolystictin (200 mg.) in 90% alcohol (20 ml.), with 10N-hydrochloric acid (4 ml.), and 10% sodium nitrite solution (4 ml.) gave an orange-red product (140 mg.), from which *diacetyl*-O-*methyliso*polystictin (25 mg.) was obtained as orange needles, m. p. 209° (decomp.) (Found: C, 59·4; H, 4·4; N, 7·0; Ac, 27·0. $C_{19}H_{16}O_7N_2$ requires C, 59·4; H, 4·2; N, 7·3; Ac, 22·5%), from benzene or ethyl acetate.

Alkaline Hydrolysis of Polystictin.—Polystictin (0·63 g.) in 25% sodium hydroxide solution (50 ml.) was steam-distilled. No volatile base could be detected in 1 hr. The alkaline solution was filtered, then extracted with chloroform to give a trace of brown gum (0·015 g.). After acidification with 5N-sulphuric acid, the aqueous solution was treated with an excess of 2 : 4-dinitrophenylhydrazine sulphate solution, and the yellow precipitate was washed and dried (0·26 g.). Pyruvic acid 2 : 4-dinitrophenylhydrazone, m. p. and mixed m. p. 218°, was isolated as yellow-orange plates on recrystallisation from benzene [Found: N, 20·7%; *M* (Rast), 272. Calc. for $C_9H_8O_6N_4$: N, 20·9%; *M*, 268].

Synthesis of Phenoxazinones.—Phenoxazin-3-one [18] [m. p. 203° (decomp.); yield 23%], and its 2-amino- [19] [m. p. 254—256° (decomp.); yield 38%] and 2-hydroxy-derivative [20] [m. p. 260—270° (decomp.); yield 1%] were prepared as described in the literature. Phenoxazin-3-one and 2-aminophenoxazin-3-one were purified by chromatography on neutralised alumina, followed by vacuum-sublimation at 120—140°/0·1 mm. and 195—205°/0·15 mm., respectively. 2-Hydroxyphenoxazin-3-one was purified by vacuum-sublimation at 170—200°/0·2 mm.

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[18] Kehrmann and Saager, *Ber.*, 1902, **35**, 341.
[19] Fischer and Jones, *Ber.*, 1894, **27**, 2782.
[20] Diepolder, *Ber.*, 1902, **35**, 2816.