6-(*p*-Chlorobenzylamino)-3-methylpurine.—A solution of 3-methyl-6-methylthiopurine (XVI, 1 g.) and 3 g. of *p*-chlorobenzylamine dissolved in 50 ml. of 90% ethanol was refluxed for 2 hours and finally cooled and filtered. The white product was washed with methanol and dried at 45° for 10 hours to yield 1.2 g. Recrystallization from methanol gave a pure product that melted at 263–265°.

Anal. Calcd. for $C_{13}H_{12}N_5Cl$: C, 57.1; H, 4.4; N, 25.6. Found: C, 57.3; H, 4.5; N, 25.4.

[CONTRIBUTION FROM THE DIVISION OF PLANT INDUSTRY, C.S.I.R.O., CANBERRA, AUSTRALIA]

Studies on Phytoalexins. V. The Structure of Pisatin from *Pisum sativum* L.[1,2]

BY DAWN R. PERRIN AND W. BOTTOMLEY

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Chemical and physicochemical studies of pisatin and some of its degradation products indicate that pisatin has the chromanocoumarane ring skeleton and is 3-hydroxypterocarpin (IIb).

In the course of a study of the chemical basis of disease resistance in plants, an antifungal substance, named pisatin, was isolated from the pods of garden peas (*Pisum sativum*) which had been inoculated with fungal spores.[3] Some biological properties of pisatin have been discussed elsewhere.[3] No other substance with antifungal activity was detected in the active extracts from which pisatin was isolated, and the activity of pisatin accounted for all the activity in those diffusates. At concentrations around 10^{-4} *M* pisatin possessed antifungal activity toward a wide range of plant fungi.[2,3]

Pisatin has the molecular formula $C_{17}H_{14}O_6$ and is optically active. It is sparingly soluble in water, but is soluble in organic solvents. Its ease of extraction by solvents from aqueous alkaline solutions indicates the absence of phenolic or acidic groups. Pisatin is stable in neutral or alkaline solutions but is very acid labile; for example, when solutions of pisatin in alcohol, acetone or water are made weakly acid and allowed to stand in the cold, pisatin loses a molecule of water and is transformed to anhydropisatin. Anhydropisatin is optically inactive. A comparison of the ultraviolet absorption spectra showed that the conversion of pisatin to anhydropisatin was accompanied by a marked shift to longer wave lengths and an intensification of the absorption maxima, indicating that dehydration had considerably extended the conjugation pathway in the molecule. Both pisatin and anhydropisatin contained one alkoxyl (probably methoxyl) group and both compounds gave a positive color test for a methylene dioxy group.[4] Pisatin could not be methylated using either methyl iodide and potassium carbonate or methyl iodide and silver oxide, and attempts to methylate with methyl sulfate, or to acetylate, resulted in its dehydration to anhydropisatin. Both pisatin and anhydropisatin were extremely resistant to hydrogenation using Adams catalyst.

No chemical evidence could be obtained for the presence of a carbonyl grouping in pisatin or anhydropisatin, nor were any strong bands observed in the 1630–1800 cm.$^{-1}$ region of their infrared spectra (Fig. 1). The band at 3610 cm.$^{-1}$ in the infrared spectrum of pisatin showed the presence of an alcoholic hydroxyl group. An integration of the area under this peak and a comparison with other hydroxyl-containing substances strongly suggested that only one hydroxyl group was present.[5] This band is absent from the infrared spectrum of anhydropisatin. According to Briggs, *et al.*,[6] a methylene dioxy group attached to an aromatic ring gives twelve major bands, but most of these are also given by a methoxyl group; the strong band around 930–940 cm.$^{-1}$ is the only one given by the methylene dioxy group alone. Other workers[7] attribute absorption bands near 1037 and 1165 cm.$^{-1}$ to methylene dioxy groupings. All three bands are present in pisatin (945, 1047, 1163 cm.$^{-1}$) and anhydropisatin (950, 1045, 1140 or 1170 cm.$^{-1}$) and also in the two degradation products identified below as V and VI. The inertness of the two remaining oxygen atoms suggested that they were present in other ether linkages.

When an ethanolic solution of anhydropisatin was exposed to diffuse daylight or mercury 365 mμ light, a phenol was produced with a molecular formula corresponding to the addition of one molecule of ethanol to the anhydropisatin molecule. This phenol was presumably formed by the fission of an aryl ether moiety. On the other hand, if an alcoholic or aqueous solution of pisatin was irradiated briefly with mercury 253.7 mμ light, a substance was formed whose ultraviolet spectrum differed from that of pisatin only by the absence of the maximum at 280 mμ and by a shift of the 309 mμ maximum to 312 mμ. This substance had no anti-fungal activity[3] nor was it dehydrated under the same conditions as those which transformed pisatin into anhydropisatin. The compound could not be isolated because further irradiation or attempted evaporation gave a yellow material having two visible absorption maxima (λ_{max} 425 mμ, log ε 4.0; λ_{max} 550 mμ, log ε 4.2). In strongly acid solutions the color changed to a bright cherry-red, but the solution became yellow once more on dilution or neutralization. The yellow material could be extracted quantitatively

(1) Presented, in part, at the International Symposium on The Chemistry of Natural Products (International Union of Pure and Applied Chemistry), Sydney, Australia, August, 1960.

(2) Part IV of the series: I. A. M. Cruickshank, *Aust. J. Biol. Sci.*, in press, (1962).

(3) I. A. M. Cruickshank and D. R. Perrin, *ibid.*, **14**, 336 (1961).

(4) J. A. Labat, *Bull. soc. chim. biol.*, **15**, 1344 (1933).

(5) E. Spinner, private communication

(6) L. H. Briggs, L. D. Colebrook, H. M. Fales and W. C. Wildman, *Anal. Chem.*, **29**, 904 (1957).

(7) A. Robertson and W. B. Whalley, *J. Chem. Soc.*, 1440 (1954).

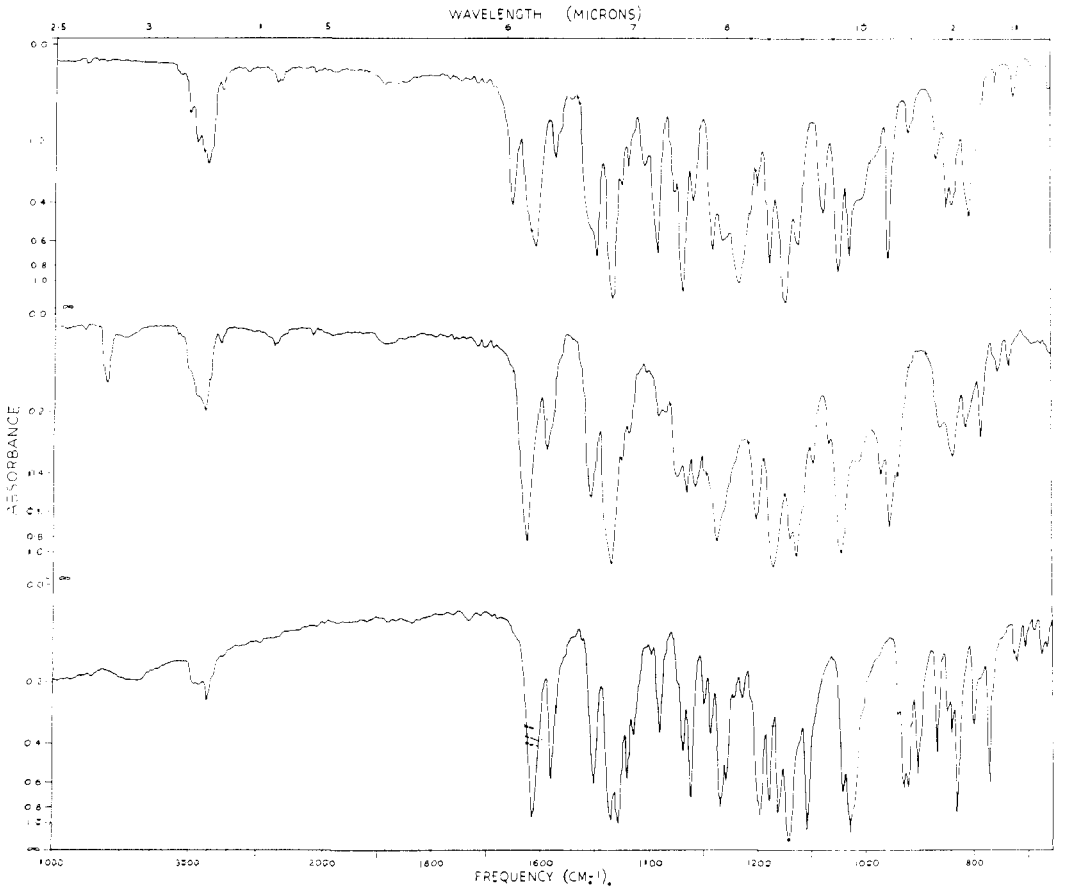

Fig. 1.—Comparison of infrared spectra: anhydropisatin (top), pisatin (middle), pterocarpin (bottom curve). Anhydropisatin and pisatin are composites of solution in CCl_4 and KBr disk; pterocarpin is a KBr disk.

from dilute acid solutions into benzene from which it could be re-extracted as the red form, using concentrated hydrochloric acid. These color changes indicated the existence of a yellow neutral molecule and its red cation. The *pK* of this molecule determined spectrophotometrically was −1.1, corresponding to a concentration of 3.4 *M* hydrochloric acid for half conversion to its cation. These properties are characteristic of the pseudo-base of an isoflavylium salt,[8] a class of compounds which is readily distinguished from flavylium bases, such as the anthocyanidins, which have *pK* values around 4. A positive ferric chloride test showed that the isoflavylium salt contained a phenolic group, but attempts to determine the *pK* of this phenol spectrophotometrically were unsuccessful. Spectral changes observed in a series of alkaline buffers indicated either the formation of a mono- and di-anion or some other kind of reversible change. This was supported by the transition from yellow, to colorless, to light brown as a neutral solution of the pseudo base was made progressively more alkaline, and probably also implies the ionization of the hydroxyl group.

The characterization of this photodegradation product as an isoflavylium pseudo-base accounts for all the unassigned carbon atoms of pisatin and shows that the ring skeleton of pisatin is capable of photochemical transformation into an isoflavylium compound.

There is a marked similarity between the ultraviolet absorption spectrum of anhydropisatin and that of anhydrosophorol (Ia), which is a dehydration product of the isoflavanone, sophorol, isolated by Suginome from the heartwood of the Japanese pagoda tree *Maakia amurensis*.[9] Direct comparison of anhydropisatin with O-methylanhydrosophorol (mixed melting point and ultraviolet spectra) showed the two to be identical, thus confirming the structure Ib for anhydropisatin.

The reduced ring skeleton of anhydrosophorol occurs in the chromanocoumarane, pterocarpin (IIa), isolated from the heartwood of the red sandalwood tree, *Pterocarpus santalinus*.[10,11] The ultraviolet absorption spectra of pisatin and pterocarpin are almost superimposable. The optical rotations of pterocarpin, $[\alpha]^{20.5}_{546}$ − 208°, and pisatin, $[\alpha]^{20}_{578}$ +280°, are also comparable but of opposite sign. The infrared absorption spectra of pisatin and pterocarpin are also closely similar (Fig. 1) except that the hydroxyl group in pisatin leads to the loss of some of the fine structure

(8) W. Baker, *J. Chem. Soc.*, 1593 (1929).

(9) H. Suginome, (a) *J. Org. Chem.*, **24**, 1655 (1959); (b) *Tetrahedron Letters*, No. **19**, 16 (1960).

(10) A. McGookin, A. Robertson and W. B. Whalley, *J. Chem. Soc.*, 787 (1940).

(11) J. B. Bredenberg and J. N. Shoolery, *Tetrahedron Letters*, No. **9**, 285 (1961).

and the appearance of several additional bands. The above properties are consistent with pisatin and pterocarpin having the same ring skeleton and aromatic substitution pattern, leaving only the location of the hydroxyl group of pisatin uncertain.

Pisatin is non-phenolic, so the hydroxyl group must be in the 2-, 3- or 4-position. Since pisatin readily dehydrates to O-methylanhydrosophorol, the hydroxyl is most likely located in the 3- or 4-positions. If the hydroxyl group was in the 4-position, opening of the dihydrofuran ring under the influence of short wave ultraviolet light would give a ketone hydrate (III), which would immediately dehydrate to a relatively stable isoflavanone. However, with the hydroxyl group in the 3-position, opening of the dihydrofuran ring would give an isoflavan-3,4-diol (IV). This could be expected to dehydrate across the 2,3-bond, forming a compound which would behave as the pseudo-base of the isoflavylium cation (V), in a manner somewhat analogous to the formation of flavylium compounds from flavan-3,4-diols.[12] The assignment of the hydroxyl group to carbon 3 also explains the ease of dehydration of pisatin in acid, and the stability in alkaline, solutions. Such reactions are to be expected from a tertiary alcohol with a hydrogen on the β-carbon, particularly when an α phenyl group is present.[13] The hydroxyl group on carbon 3 is also in accord with nuclear magnetic resonance data on pisatin.[14]

The absence from the n.m.r. spectrum of anhydropisatin of any signal corresponding to a proton on a vinyl ether (expected τ-value around 3.76)[15] confirms the ultraviolet spectrophotometric evidence that dehydration does not take place between carbons 2 and 3. This is also supported by the loss of optical activity during dehydration, because the only asymmetric carbon atoms are at positions 3 and 4.

Hence it is concluded that pisatin has structure IIb and is 3-hydroxy-7-methoxy-4′,5′-methylenedioxychromanocoumarane.[16,17] It is suggested that the action of light on anhydropisatin in alcohol leads to fission of the pyran ring with alcoholysis occurring across the 1,2-oxygen–carbon bond to give the phenolic ether VI. The formation of a different product when the reaction is carried out in methanolic solution confirms this interpretation. The ultraviolet absorption spectrum of the alcoholysis product is very similar to that reported for 5,6-dimethoxy-2-(2,4,6-trimethoxyphenyl)-benzofuran (VII).[18] The observed pK of the phenolic

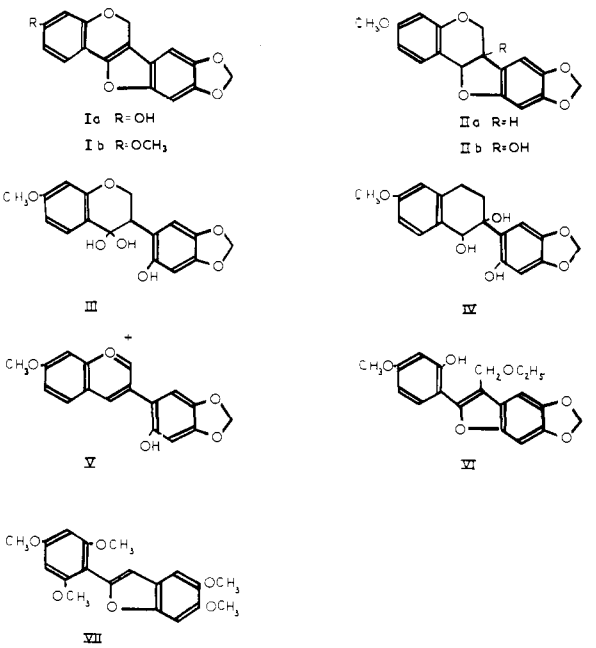

Fig. 2.

ether VI is about 1 pH unit lower than that of phenol itself. Such greater acidity is consistent with this structure because m- methoxyl substitution in phenol lowers the pK by 0.35,[19] and the conjugative effect of the o-substituent would be expected to lower the pK further.

Pisatin appears to be the first naturally-occurring chromanocoumarane or coumarinocoumarone to be characterized with a hydroxyl group in the non-aromatic portion of the molecule.

Grisebach and Ollis[20] have suggested that compounds of the isoflavanoid type usually occur naturally in plants of one family, *Leguminosae*. Indeed the ring structure of pisatin occurs in eight other known natural products, and, with one exception, these substances have all been isolated from plants of same sub-family, Papillionaceae, as *Pisum sativum*.[21]

Biogenetic studies suggest that these substances are derived from a 3-phenylchroman precursor[9b,20] and pisatin may also result from some modification of an isoflavanoid intermediate.

Experimental[22]

Isolation of Pisatin.—The exposed endocarp of seed pods of *Pisum sativum* L. cultivar Greenfeast (140 kg.) was inoculated with spore suspension of *Monilinia fructicola* (Wint.) Honey, and, after incubation at 20° for 40 hours or more, the liquid was removed, centrifuged and extracted with light petroleum (b.p. 55–60°). Full details of the experimental technique used for the production of pisatin, and of the biological assay for antifungal activity have already been re-

(12) W. Bottomley, *Chemistry & Industry*, 516 (1954).

(13) E. S. Gould, "Mechanism and Structure in Organic Chemistry," H. Holt and Co., New York, N. Y., 1959, p. 473.

(14) D. D. Perrin and D. R. Perrin, *J. Am. Chem. Soc.*, **84**, 1922 (1962).

(15) G. V. D. Tiers, unpublished, quoted by H. Conroy in *Adv. Org. Chem.*, **2**, 265 (1960).

(16) The systematic name for this compound is 6a,12a-dihydro-6a-hydroxy - 3 - methoxy - 6H - 1,3 - dioxolo[5,6]benzofuro[3,2 - c][1] benzopyran.

(17) In our preliminary communication (ref. 21), the structure of pisatin was reported to be 3-hydroxy-pterocarpin. Later it was found that the methylene dioxy group was, in fact, in the 4′,5′-position in contrast to the then known structure of pterocarpin (ref. 7). Subsequently the structure of pterocarpin has been revised (ref. 11) and the methylene dioxy group also shown to be in the 4′,5′-position.

(18) T. R. Govindachari, K. Nagarajan and B. R. Pai, *J. Chem. Soc.*, 629 (1956).

(19) F. G. Bordwell and G. D. Cooper, *J. Am. Chem. Soc.*, **74**, 1058 (1952).

(20) H. Grisebach and W. D. Ollis, *Experientia*, **17**, 4 (1961).

(21) D. R. Perrin and W. Bottomley, *Nature*, **191**, 76 (1961).

(22) All melting points are uncorrected. The microanalyses were performed by the Australian Microanalytical Service, C.S.I.R.O., Melbourne. The ultraviolet absorption spectra were measured on either a Hilger and Watts Uvispek spectrophotometer or a Unicam SP700 recording spectrophotometer. The infrared spectra were recorded on a Perkin–Elmer model 21 spectrophotometer. The optical rotations were measured using a Zeiss photoelectric polarimeter over the wave length range 364 to 578 mμ.

ported.[3] The light petroleum extracts were concentrated, and the crude pisatin, which separated on cooling, was purified by repeated recrystallization from light petroleum, aqueous ethanol and finally from light petroleum; yield about 4.5 g., m.p. 72°.

Properties of Pisatin.—In aqueous solution pisatin is stable to heating to 98° (30 minutes) and autoclaving (15 pounds, 20 minutes), its ultraviolet spectrum and biological activity being unchanged. Physical constants are $[\alpha]^{20}_{364}$ mμ +940°, $[\alpha]^{20}_{436}$ mμ +570°, $[\alpha]^{20}_{578}$ mμ +280° (*c* 0.11 in ethanol); λ_{max} in ethanol: 213 mμ (log ε 4.75), 280 mμ (log ε 3.62), 286 mμ (log ε 3.68) and 309 mμ (log ε 3.86); λ_{max} values in ethanol were unchanged by the addition of 0.05 *M* sodium hydroxide. Solubility in 1 ml. at 23° was: water 0.03 mg., light petroleum 0.5 mg., oleyl alcohol 6 mg., carbon disulfide 15 mg., carbon tetrachloride 25 mg., ethanol > 42 mg. and dioxane > 130 mg. Distribution coefficients between light petroleum and water, and water–methanol mixture were: water (2.3:1), 20% methanol (1.7:1), 50% methanol (0.2:1) and 80% methanol (0.02:1); between cyclohexane and water (3.6:1) and oleyl alcohol and water (>100:1).

Anal. Calcd. for $C_{17}H_{14}O_6$: C, 64.96; H, 4.49; O, 30.55; OCH_3, 9.87; active H, 0.32; mol. wt., 314. Found in sample dried below 40° *in vacuo:* C, 64.95; H, 4.61; O, 31.1; OCH_3, 9.76; active H, 0.44; mol. wt., 291.

Anhydropisatin.—Pisatin (350 mg.) was dissolved in acetone (450 ml.), diluted to 500 ml. with water, and concentrated hydrochloric acid (2.5 ml.) added. After standing overnight in the dark, at room temperature, the white precipitate was collected by centrifuging, washed with acetone and then recrystallized from ethanol, yielding anhydropisatin in colorless needles; yield 220 mg., m.p. 179–180° alone or mixed with an authentic specimen of O-methylanhydrosophorol. No optical rotation was observed over the wave length range 405 to 578 mμ (*c* 0.04 in ethanol); λ_{max} in ethanol: 215 mμ (log ε 4.49), 234 mμ (log ε 4.30), 244 mμ (log ε 4.2), 257 mμ (log ε 4.1), 291 mμ (log ε 3.8), 339 mμ (log ε 4.58) and 358 mμ (log ε 4.60). Solubility in 1 ml. at 23° was; water 0.0 mg., carbon disulfide 6.5 mg., carbon tetrachloride 9 mg., ethanol 10 mg., dioxane 733 mg.

Anal. Calcd. for $C_{17}H_{12}O_5$: C, 68.91; H, 4.08; O, 27.00; OCH_3, 10.47; mol. wt., 296. Found in sample dried below 70° *in vacuo:* C, 68.87; H, 4.10; O, 26.8; OCH_3, 10.31; mol. wt., 285.

Irradiation of Anhydropisatin.—Anhydropisatin (100 mg.) was dissolved in ethanol, and the resulting solution allowed to stand in diffuse daylight for 1 hour. The solution was concentrated and, on standing, colorless crystals separated and were subsequently recrystallized from aqueous ethanol; yield 30 mg., m.p. 104°. This substance gave positive color tests for phenols and had a *pK* of 9.1 in 10% ethanol; λ_{max} in ethanol: 214 mμ (log ε 4.67), 269 mμ (log ε 4.18) and 319 mμ (log ε 4.39). The anion had λ_{max} 325 mμ, and was intensely blue-fluorescent in solution.

Anal. Calcd. for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found in sample dried below 40° *in vacuo:* C, 66.21; H, 5.35.

Irradiation of Pisatin.—Pisatin (30 mg.) in aqueous solution was irradiated with 253.7 mμ light until deep yellow. The solution was shaken with benzene, which was then washed with water. The resulting isoflavylium compound was re-extracted into a small volume of concentrated hydrochloric acid, diluted with water, and then re-extracted into benzene. This process was repeated six times. The isoflavylium compound was finally taken into ether, and dried over sodium sulfate. It was then saturated with hydrogen chloride, and a dark red globule separated out from the ether. This was dried *in vacuo*, yielding a deposit of the isoflavylium chloride (8 mg.).

Acknowledgments.—The authors thank Professor A. J. Birch, University of Manchester, for providing a sample of pterocarpin and Professor H. Suginome, Hökkaido University, for a sample of O-methylanhydrosophorol. Thanks are also tendered to Drs. E. Spinner and D. D. Perrin, Department of Medical Chemistry, Australian National University for providing and assisting with the interpretation of the infrared spectra, and for determining dissociation constants, respectively.

[CONTRIBUTION FROM THE DEPARTMENT OF MEDICAL CHEMISTRY, AUSTRALIAN NATIONAL UNIVERSITY, AND THE DIVISION OF PLANT INDUSTRY, C.S.I.R.O., CANBERRA, AUSTRALIA]

The N.m.r. Spectrum of Pisatin

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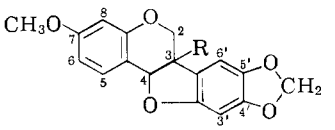
From an analysis of its nuclear magnetic resonance spectrum in carbon tetrachloride, pisatin is assigned the structure Ia. Examination of the aromatic portions of their n.m.r. spectra indicates that in pisatin and pterocarpin the methylenedioxy group is located across positions 4′ and 5′, and hence that pisatin is 3-hydroxypterocarpin.[1]

Pisatin, an antifungal substance of molecular formula $C_{17}H_{14}O_6$, has been isolated from fungal-infected pods of *Pisum sativum*.[2] At an early stage in the investigation, infrared spectra and chemical evidence, including a positive Labat test, showed pisatin to have a methoxyl group, a methylenedioxy group and an alcoholic (non-phenolic) hydroxyl group,[3] but otherwise provided little information about its structure. Subsequently, detailed analysis of n.m.r. spectra, as discussed below, suggested that pisatin is Ia. Examination of the n.m.r. spectrum of pterocarpin (a benzofurobenzopyran derivative found in the heartwood of *Pterocarpus santalinus* L.[4]) strongly supported this conclusion, which was also reached independently from examination of other chemical evidence.[3]



Ia, R = OH
b, R = H

Experimental

The n.m.r. spectra, for which we are indebted to Dr. N. Hayakawa of the Japan Atomic Energy Research Institute, were obtained using a Varian Associates V-4300-C high reso-

(1) J. B. Bredenberg and J. N. Shoolery, *Tetrahedron Letters*, **9**, 285 (1961), have also concluded from n.m.r. spectra that the methylenedioxy group in pterocarpin is located across positions 4′ and 5′, so that pterocarpin has the structure Ib.

(2) I. A. M. Cruickshank and D. R. Perrin, *Nature*, **187**, 799 (1960); *Australian J. Biol. Sci.*, **14**, 336 (1961).

(3) D. R. Perrin and W. Bottomley, *J. Am. Chem. Soc.*, **84**, 1919 (1962).

(4) A. McGookin, A. Robertson and W. B. Whalley, *J. Chem. Soc.*, 787 (1940).