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LITHOSPERMUM RUDERALE: PARTIAL CHARACTERIZATION OF THE PRINCIPAL POLYPHENOL ISOLATED FROM THE ROOTS*

GESTUR JOHNSON, S. G. SUNDERWIRTH, H. GIBIAN,† A. W. COULTER and F. X. GASSNER

Chemistry and Endocrine Sections, Colorado State University, Fort Collins, Colorado

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Abstract—Lithospermic acid from *L. ruderale* roots is a polyphenolic carboxylic acid with an empirical formula believed to be $C_{16}H_{14}O_7$. The molecule has two 1,2,4-trisubstituted benzene rings, each containing *o*-dihydroxy groupings; and an olefinic grouping which is conjugated with one of the rings. The last oxygen atom is probably present as a tertiary alcohol. Optical activity indicates that the molecule contains one or more asymmetric centres. The structure of this molecule is discussed.

INTRODUCTION

THE roots of *Lithospermum ruderale* contain a phenolic acid (lithospermic acid) which has not previously been reported in Nature. The compound itself is biologically inactive but on oxidation a polymer is produced which shows anti-ovulatory activity in the hen, and inhibits the effects of exogenous gonadotropin in the immature female rat.[1] Because of these biological effects it was felt that the chemical nature of lithospermic acid, the precursor to the active polymer, should be characterized.

RESULTS

Lithospermic acid (I) is an off-white hygroscopic powder which is extremely soluble in water or ether. All attempts to purify it by crystallization have failed. The positive optical rotation of (I) ($[\alpha]_D^{23} = +122$, H_2O, $C = 0{\cdot}2$) indicates that the molecule contains one or more asymmetric centers. The presence of a free carboxyl group in (I) is indicated by the facts that the compound is acidic and forms a methyl ester (II). Upon heating with acid, (I) yields no carbohydrate, showing that it is not a glycoside.

The presence of an *o*-dihydroxy grouping in (I) was indicated by a positive reaction to Arnow's test,[2] by the green colour it gave with ferric chloride, and by its ease of oxidation using peach polyphenolase.[3] (I) failed to give a positive test with *p*-dimethylaminobenzaldehyde which, according to Kirby *et al.*,[4] indicates that it is not a catechin. This was further substantiated by consideration of the ultraviolet absorption spectral data. Catechins show absorption maxima at 280 mμ, while (I) has absorption peaks at 255, 285, 300–310 and a shoulder at 330 mμ (Fig. 1). Absorption in the 300–310 mμ region is typical of structures having a carbonyl or an olefin conjugated with a benzene ring.[5] However, a

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† Present address: Schering, A.G., Berlin-West, Germany.

[1] F. X. GASSNER, W. JÖCHLE, GESTUR JOHNSON, M. L. HOPWOOD and S. G. SUNDERWIRTH. (In preparation.)
[2] L. E. ARNOW, *J. Biol. Chem.* **118**, 531 (1937).
[3] Crude polyphenolase oxidase prepared in this laboratory by acetone precipitation of a cold water extract of peaches.
[4] K. S. KIRBY, E. KNOWLES and T. WHITE, *J. Soc. Leather Trades' Chemists* **35**, 338 (1951).
[5] R. F. PATTERSON and HAROLD HIBBERT, *J. Am. Chem. Soc.* **65**, 1862 (1943).

negative response to 2,4-dinitrophenylhydrazine by (I) shows that it does not contain a carbonyl group.

On acetylation, (I) yields a tetraacetate (III) which has a neutralization equivalent of 481, corresponding to a molecular weight of 313 for the free acid. This tetraacetate was esterified to form an acetylated methyl ester (IV), the nuclear magnetic resonance spectrum[6] of which indicates that it has a *trans* olefinic grouping.

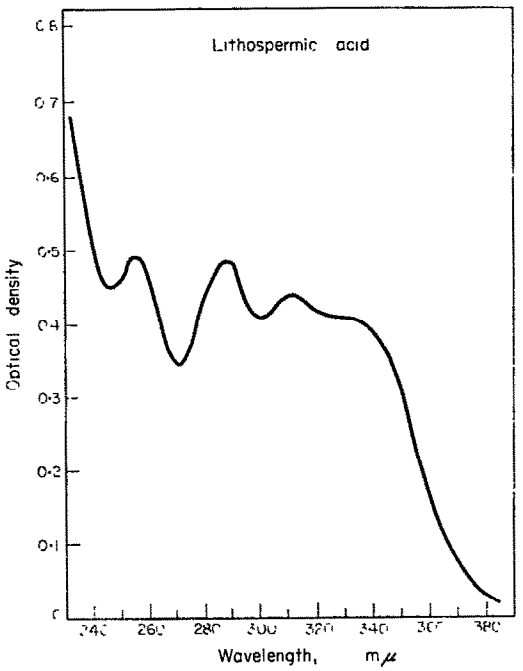

FIG. 1. ULTRAVIOLET ABSORPTION SPECTRUM FOR LITHOSPERMIC ACID (I).

Pentamethyl lithospermic acid (V) was prepared using mild methylating conditions; the usual methylation procedures proved too vigorous and only methylated breakdown products were obtained. The ultraviolet spectrum of (V) maintained the same characteristics as that of (I), showing that no structural rearrangement occurred during methylation. The NMR spectrum of (V) also indicates the presence of a *trans* olefinic grouping.

Chromatographically pure (I) was submitted to mild alkaline fusion, and yielded five breakdown products (Fig. 2). Three of these were identified by paper chromatography as catechol, caffeic acid and protocatechuic acid. The two unidentified breakdown products (compounds A and B) also contained *o*-dihydroxy groupings. More drastic fusion of (I) yielded only protocatechuic acid.

When (I) was refluxed with aqueous 5N sodium hydroxide with exclusion of air, compound A was the main product, with lesser amounts of compound B, and some unchanged (I). This indicates that (I) is not one of the caffeic or other hydroxycinnamic acid esters so often encountered in plant extracts.[7-11]

[6] Determined by Varian Associates, Palo Alto, California.
[7] J. JANET CORNER, J. B. HARBORNE, S. G. HUMPHRIES and W. D. OLLIS, *Phytochemistry* **1**, 73 (1962).
[8] J. B. HARBORNE and J. JANET CORNER, *Biochem. J.* **81**, 242 (1961).
[9] M. L. SCARPATI and G. ORIENTE, *Tetrahedron* **4**, 43 (1958).
[10] LUIGA PANIZZI and MARIA LUISA SCARPATI, *Nature* **174**, 1062 (1954).
[11] MARIA L. SCARPATI and GIOVANNI ORIENTE, *Ricerca sci.* **28**, 2329 (1958).

FIG. 2. BREAKDOWN PRODUCTS OBTAINED BY MILD ALKALINE FUSION OF LITHOSPERMIC ACID. 1 is caffeic acid, 2 is protocatechuic acid, 3 is catechol, 4 is unknown A, and 5 is unknown B.

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Pentamethyl lithospermic acid (V) was also subjected to alkaline fusion, and the breakdown products were identified as 3,4-dimethoxycinnamic acid, caffeic acid, veratric acid and protocatechuic acid. When (V) was vigorously oxidized with alkaline potassium permanganate, only veratric acid was formed.

The infrared spectrum of the free acid (I) (Fig. 3) and its methyl ester (II) are almost identical. This is somewhat unusual since the spectra of acids and their corresponding

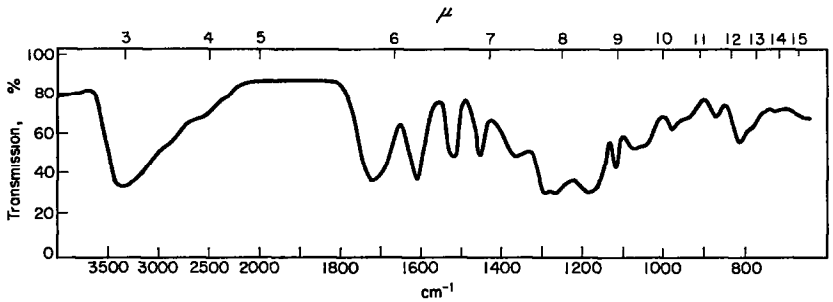

FIG. 3. INFRARED SPECTRUM OF LITHOSPERMIC ACID (I).

esters usually show significant differences in the location of the C = O (1700–1750 cm^{-1}) and C–O (1100–1300 cm^{-1}) regions. It is possible that the phenolic absorptions are so predominant that they mask other changes in structure. The phenyl rings give rise to the band at 1517 cm^{-1} and also contribute to the 1610 cm^{-1} band. Conjugation of the ethylenic bond also contributes to the 1610 cm^{-1} band. If this bond were unconjugated, a medium absorption band between 1625 and 1675 cm^{-1} would be found. The out-of-plane H-bending bands at 865 cm^{-1} and 809 cm^{-1} may be attributed to 1,2,4-tri-substitution of the benzene rings. The *trans* configuration of the olefinic bond accounts for the out-of-plane H-bending band at 980 cm^{-1}. Typical C–O and O–H bands appear at 1370 and 1175 cm^{-1}.

Preparations of the pentamethyl derivative (V) gave a better IR spectrum (Fig. 4) since the phenolic hydroxyl groups of (I) and (II) are masked. Table 1 shows the observed absorption bands and their assignment.

TABLE 1. INFRARED ANALYSIS OF PENTAMETHYL LITHOSPERMIC ACID (V)

Band cm^{-1}	Assignment
3450	water from KBr
2940	C–H stretch
2840	C–H stretch
1735	ester carbonyl
1715	acid carbonyl (possibly trace of carboxyl)
1615	aromatic
1600	aromatic
1520	aromatic
1450	CH_2
1430	*Tert* OH
1375 (shoulder)	CH_3 and/or *Tert* OH
1300–1200	–C–O–R, ester
1150	–C–O–C–, ester
1085	OH
1030	?
980	*Trans*–CH=CH–
855	1,2,4-substitution
810	–CH=CH–
765	1,2,4-substitution

DISCUSSION

The elementary analyses of compounds (I) through (V) indicate that the empirical formula for (I) is $C_{16}H_{14}O_7$. The presence of two 1,2,4-substituted benzene rings with *o*-dihydroxy groupings in (I) seems well established. A carboxyl group is also present in the molecule. The remaining oxygen is believed to be present in the form of an alcoholic

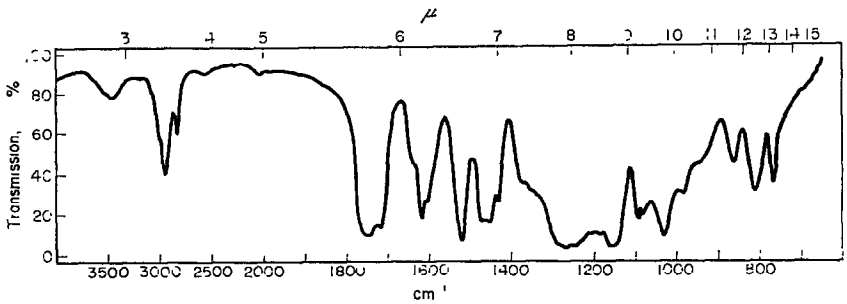

FIG. 4. INFRARED SPECTRUM OF PENTAMETHYL LITHOSPERMIC ACID (V).

group, and the failure of this group to be methylated or acetylated indicates that it may be tertiary. The molecule contains one or more asymmetric carbon atoms, and a *trans* olefinic grouping conjugated with one of the benzene rings.

A possible arrangement for these functional groups is shown in Fig. 5. However, the synthesis of the pentamethyl derivative of the compound with this structure produced a compound which was not identical to (V). The preparation of this synthetic pentamethyl compound will be the subject of a subsequent publication.[12]

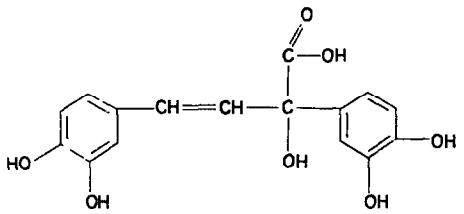

FIG. 5. POSSIBLE ARRANGEMENT OF FUNCTIONAL GROUPS PRESENT IN (I).

Although the structure shown in Fig. 5 is apparently not the correct one for (I), the functional groups shown are undoubtedly present and must have an arrangement which is similar to that shown.

EXPERIMENTAL

Lithospermic acid (I)

Pure lithospermic acid was obtained in the following manner: the dried, ground roots (500 g) were vigorously stirred for 15 min with 2500 ml of water at 25°. During this period the pH was maintained at 2·0 by addition of dilute HCl. The mixture was filtered, and the filtrate extracted with 10×100 ml of peroxide-free diethyl ether. After drying (Na_2SO_4), the ether was removed, and the resulting syrup dried *in vacuo*. (Yield: 7·0–10·0 g.) The crude product was purified using counter-current distribution between diethyl ether and water (mobile phase). After 31 transfer, 2·0 g of the crude compound yielded 0·9–1·1 g of

[12]R. SCHMIFCHEN and H. GIBIAN. *Ann. Chem. Liebigs.* In press.

chromatographically pure lithospermic acid (I) (m.p. 130–131°) which was present in tubes 8–15 (Found: C,59·1; H,4·70. $C_{16}H_{14}O_7$ required: C,60·4; H,4·43%). The R_f values of (I) are: (*a*) 0·43 in 5% acetic acid, (*b*) 0·72 in butanol/acetic acid/water (40:10:19), (*c*) 0·62 in 80% ethanol, (*d*) 0·73 in 70% *t*-butyl alcohol. (I) gives an orange-tan color when sprayed with diazotized sulfanilic acid, and a dark yellow color with Arnow's reagent.

Methyl ester of lithospermic acid (II)

The methyl ester (II) was prepared by refluxing 1·0 g of (I) with 25 ml of absolute methanol containing 0·4 ml of conc HCl for 4 hr. After removal of the methanol, the syrup was taken up in ether which was then washed with water. The water washings were extracted once with ether, and the combined ethereal solution dried (Na_2SO_4) and concentrated to a syrup which was dried *in vacuo*. (Yield: 0·6 g; m.p. 102–104°.) (Found: C, 59·98; H, 5·13; OCH_3, 9·61. $C_{17}H_{16}O_7$ required: C, 61·44; H, 4·85; OCH_3, 9·34%.)

Tetraacetate of lithospermic acid (III)

(I) (0·2 g) was acetylated in pyridine (2·0 ml) with acetic anhydride (0·8 g). The mixture was heated at 95° for 5 min, poured into 50 ml of ice water, acidified with HCl, and filtered. The crystals were washed with cold 2% HCl and dried (m.p. 94–98°). (Found: C, 58·5; H, 4·52; acetyl, 34·0. $C_{24}H_{22}O_{11}$ required: C, 59·3; H, 4·56; acetyl, 34·6%.)

Acetylated methyl ester (IV)

(II) (0·3 g) was acetylated in pyridine (7·0 ml) with acetic anhydride (3·0 ml). The mixture was heated at 95° for 25 min and poured into ice water. After stirring for 15 min, the crystals were collected, and recrystallized twice from ethanol. (Yield: 0·17 g; m.p. 76–78°.) (Found: C, 60·24; H, 4·93; OCH_3, 6·30; acetyl, 34·98. $C_{25}H_{24}O_{11}$ required: C, 60·0; H, 4·83; OCH_3, 6·20; acetyl, 34·39%.)

Pentamethyl lithospermic acid (V)

(I) (2·0 g) was methylated under reflux in anhydrous acetone (50 ml) containing K_2CO_3 (20·0 g) and dimethyl sulfate (16 ml) for 17 hr. The K_2CO_3 was removed on the centrifuge, washed twice with acetone, and the combined solutions concentrated under reduced pressure to a syrup, which was taken up in hot methanol–water and decolorized with carbon. The product formed upon cooling was recrystallized three times from methanol-water. (Yield: 1·15 g; m.p. 67–69°.) (Found: C, 64·00; H, 6·07; OCH_3, 36·96. $C_{21}H_{24}O_7$ required: C, 64·94; H, 6·23; OCH_3, 39·95%.)

Potassium hydroxide fusion of (I) *and* (V)

(I) (0·20 g) was fused on a hot plate with 0·5 g of KOH in 1 ml of water until a thick paste was obtained. The mixture was acidified with HCl and extracted with ether. The ether was removed, and the residue subjected to two-dimensional paper chromatography using butanol/acetic acid/water (40:10:19) followed by 5% acetic acid. The compounds present were located by spraying with Arnow's reagent, diazotized sulfanilic acid and Folin-Denis reagent. They were identified in the usual manner by using known markers. Confirmation was obtained using one dimensional paper chromatography with the following solvents: 15% acetic acid; 10% formic acid; formic acid/*t*-butyl alcohol/water (2:3:15); and phenol saturated with water.

(V) was degraded, and the breakdown products identified as described above.

Degradation of I *with aqueous alkali*

(I) (50 mg) was refluxed for 30 min with 15 ml of 5 N NaOH under hydrogen. The solution was acidified and extracted with ether (4×25 ml). The ether extracts were dried, and concentrated to a syrup, which was taken up in ethanol and chromatographed as before.

Permanganate oxidation of V

(V) (0·02 g) was oxidized with a solution of 0·3 g of $KMnO_4$ and 0·11 g of NaOH in 2 ml of water, at 110–115° for 1·5 hr. The permanganate solution was added continuously until the colour was not discharged. Excess permanganate was destroyed with a concentrated aqueous solution of $NaHSO_3$. The mixture was centrifuged, and the supernatant acidified and extracted with ether. The ether extract was concentrated under reduced pressure to a residue which was recrystallized from hot water. The melting point of the material (180–181°) was not depressed by admixture with an authentic sample of veratric acid, and its R_f values in butanol/acetic acid/water, 5% acetic acid and 80% ethanol were the same as those of the latter compound.

Absorption spectra

The infrared analyses were carried out using a Perkin-Elmer Model No. 21 Spectrophotometer and a Beckman Model IR–5. The ultraviolet absorptions were obtained using a Beckman Model DU, and a Bausch and Lomb Spectronic 505.

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