tons), 7.65–7.88 (m, 2 aromatic protons); mass spectrum (70 eV) *m/e* (rel intensity) 449 (3.4), 448 (10), 447 (4.5), 446 (10), 369 (31), 368 (100), 376 (50), 366 (11), 365 (12), 363 (12), 353 (29), 352 (20), 351 (15), 291 (10), 290 (9), 289 (11), 191 (29), 190 (25), 189 (48), 184 (15), 183 (11), 182 (13), 175 (27), 164 (10); uv ($CHCl_3$) 271 nm (ε 3340), 281 (2180). Recrystallization from benzene–hexane gave an analytical sample.

Anal. Calcd for $C_{29}H_{19}Br$: C, 77.86. Found: C, 77.60; H, 4.47; Br, 17.76.

Removal of solvents from the filtrate and addition of small amounts of cold ether and benzene followed by filtration and benzene and ether washings gave an additional 0.25 g (12.5%) of product, mp 259–262° dec. The total yield of **6b** was 1.75 g (87.5%).

Registry No.—**1a,** 15080-14-5; **1b,** 15156-60-2; **1c,** 15080-12-3; **1d,** 55043-36-2; **2,** 15080-13-4; **3a,** 55043-37-3; **3b,** 55043-38-4; **3c,** 55043-39-5; **4,** 55043-40-8; **5,** 55043-41-9; **6a,** 55043-42-0; **6b,** 55043-43-1; anthraquinone, 84-65-1; 9,10-dibromoanthracene, 523-27-3; 9-chloroanthracine 716-53-0; 9-chloromethylanthracene, 24463-19-2; bromine, 7726-95-6.

References and Notes

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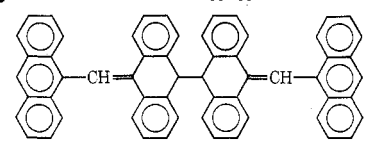
(3) The structure of the substance of mp 382–395° dec previously assigned structure **1a**[7] has been briefly reinvestigated. The observations that its infrared spectrum closely resembles that of **3a** between 1400 and 1500 cm^{-1}, that its mass spectrum shows the highest mass at *m/e* 367 instead of 368, and that its uv spectrum is essentially the same as that of **3a** with nearly the same extinction coefficients on a weight basis, suggest a dimeric structure $C_{58}H_{38}$ as follows.



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Polyphenolic Acids of *Lithospermum ruderale* Dougl. ex Lehm. (Boraginaceae). 1. Isolation and Structure Determination of Lithospermic Acid[1a]

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A structure is proposed for lithospermic acid ($C_{27}H_{22}O_{12}$, **1a**), the major polyphenolic acid of *Lithospermum ruderale* and several other plant species of the families, Boraginaceae and Labiatae. Chromatography on Sephadex of aqueous extracts of the plant yields the dipotassium salt of **1a,** together with salts of lesser constituents which include (*R*)-3-(3,4-dihydroxyphenyl)lactic acid (**2a**), 2-(3,4-dihydroxyphenyl)-3-carboxy-4-(2-carboxy-*trans*-vinyl)-7-hydroxycoumaran (**3a**), and rosmarinic acid (**4a**). Structures were deduced from spectral studies of the salts, the free acids, and also the methylated derivatives produced by the action of diazomethane on the free acids or dimethyl sulfate on the salts.

This paper describes our isolation of lithospermic acid, $C_{27}H_{22}O_{12}$, the principal polyphenolic acid constituent of roots of the plant *Lithospermum ruderale* Dougl. ex Lehm. (Boraginaceae; common names, gromwell, puccoon) and the structure elucidation of this acid and of three closely related plant constituents.

Interest in the chemical constituents of *Lithospermum ruderale* was stimulated by the report in 1941 of Train et al.[2] that certain Indians of Nevada use the plant to make a contraceptive tea. Extracts from at least six species of the genus *Lithospermum* have been found to inactivate gonadotropins.[3] The most readily cultivated of these species, *Lithospermum officinale* L., long known in European herbal medicine,[4,5] has been shown to produce a pituitary hormone blocking effect[5] both in vitro and in vivo.

The plants *Lycopus europaeus* and *Lycopus virginicus*, although members of the family Labiatae, were reported to have antihormonal activities remarkably similar to those of *Lithospermum* species.[6]

Lithospermic acid[7] has been implicated[8,9] in the hormonal inhibitory mechanisms, presumably as the chemical precursor of the active inhibitory substances produced by oxidative processes involving air- or enzyme-catalyzed reactions. It has been recognized[9-11] as a constituent of *Lycopus europaeus, Lycopus virginicus, Lithospermum ruderale, Lithospermum officinale,* and *Symphytum officinale* (common name, comfrey). More recently, *Anchusa officinalis* and *Echium vulgare* were added to the growing number of Boraginaceae which have antigonadotropic activity in animals and also contain lithospermic acid.[12] Reviews of contraceptive plant species have been published.[13,14]

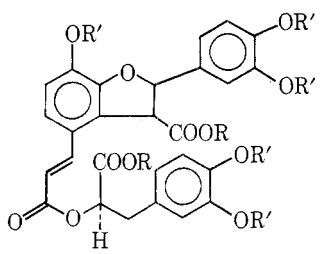
von Seemann and Grant[15] isolated fractions of *Lithospermum ruderale* as amorphous powders having antigonadotropic activity. They characterized the materials as polyphenolic acid salts, possibly flavanoids, which gave reactions characteristic of phlobatannins, and observed that the active principle(s) in the plant extract could be precipitated by acids and redissolved at pH above 5. A number of phytochemical investigations failed to identify the specific antihormonal compound(s) of *Lithospermum* species.[16]

In 1963, Johnson et al.[7] reported the isolation of a polyphenolic acid from roots of *Lithospermum ruderale* which they named lithospermic acid and assigned the structure of 2-hydroxy-2,4-bis(3,4-dihydroxyphenyl)butenoic acid, $C_{16}H_{14}O_7$. The amorphous, water-soluble acid was said to melt at 130–131° and the structure was supported by analyses for several crystalline derivatives, including a "pen-

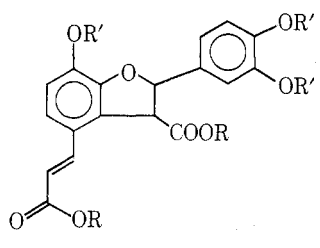
tamethyl" derivative. Schmiechen and Gibian[17] described the synthesis of the *O*-methyl derivative corresponding to the racemate of the proposed lithospermic acid structure. Since their pentamethyl compound behaved very differently than the derivative of natural lithospermic acid, it was concluded that the proposed structure was not correct. The same conclusion was reached by Wagner et al.,[9] who isolated lithospermic acid from *Lycopus europaeus* and *Symphytum officinale*, prepared several of the derivatives reported by Johnson et al., and made a direct comparison with a specimen of the acid supplied by those workers.

Since we had achieved some concentration of inhibiting constituents of *L. ruderale*[1f] in earlier column chromatography on cellulose, we evolved a more effective separation procedure involving extracts of the root on columns of Sephadex. On elution with water, polymeric materials, followed by carbohydrates, preceded the polyphenolic carboxylate salts responsible for the antihormonal effects. One chromatographic fraction contained almost exclusively the salts of lithospermic acid in quantities up to 3% of the weight of the dry roots.

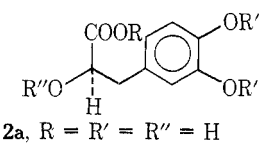
From a study of the salts, the liberated carboxylic acid, and methylated derivatives, we have deduced that lithospermic acid, the principal polyphenolic acid, is a dicarboxylic acid having structure **1a**. In addition, *L. ruderale* contains lesser quantities of the two formal hydrolysis products of **1a**, (*R*)-3-(3,4-dihydroxyphenyl)lactic acid (**2a**) and 2-(3,4-dihydroxyphenyl)-3-carboxy-4-(2-carboxy-*trans*-vinyl)-7-hydroxycoumaran (**3a**). Also found in the plant was the caffeyl ester of (*R*)-**2a**, rosmarinic acid (**4a**).



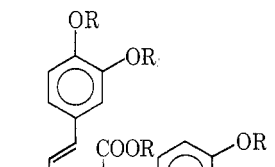
1a, R = R′ = H
b, R = Me, R′ = H
c, R = R′ = Me



3a, R = R′ = H
b, R = Me, R′ = H
c, R = R′ = Me



2a, R = R′ = R″ = H
b, R = Me; R′ = R″ = H
c, R = R′ = Me; R″ = H
d, R = R′ = Me; R″ = Ac
e, R = R″ = H; R′ = Me



4a, R = H
b, R = Me

Fractionation of Polyphenolic Acids. The polyphenolic acids of *Lithospermum ruderale* have been found in roots, leaves, seeds, and stems in varying amounts, and are extractable with water as nearly neutral salts over a range of pH, and as the free carboxylic acids at pH 2. In practice, the roots are a much better source of these materials owing to the ease with which the extracts may be handled. The direct extraction of powdered root materials with water under an inert atmosphere gives a solution (pH ~7) containing up to 40% of the dry weight of the plant material. If these solutions are allowed to stand in air, they darken and begin to deposit amorphous materials in a few hours. If such solutions are lyophilized immediately, however, the resulting dry powders may be stored without significant change for years.

Table I
Separation of Whole Root Extracts of *L. ruderale* on Sephadex G-50

Group[a]	Fractions	Appearance of lyophilized powder	Wt, g	Percent
Insoluble pellet		Removed by centrifugation	0.69	3.5
A	1–6	Light yellow	0.73	3.6
B	7–8	Brown, crisp	0.73	3.6
C	9–12	Brown, crisp	7.77	38.9
D	13–14	Dark brown, crisp	4.30	21.5
E	15	Medium brown, fluffy	1.41	7.1
F	16–22 (–28)	Light yellow, fluffy	3.27	16.4
Remaining on column			1.10	5.5
Total weight of extract powder			20.0[b]	100

[a] Representative samples of groups A–E were assayed by ^{13}C NMR, and the following conclusions were drawn. That portion of A soluble at pH 8.2 and groups B–D are almost exclusively carbohydrates. The principal carbohydrate in group C is sucrose. Group E contains carbohydrates similar to group D and in addition some polyphenols. A chemical separation of group E resulted in Ea, Eb, and Ec, the last consisting of the water-soluble carbohydrate. Ea consists of polyphenolate salts whose parent acids are precipitated in aqueous acid; it appears to be oligomeric material with very broad carbon resonances corresponding in chemical shift to the resonances observed for **1a**. Fraction Eb contains an unknown ester carboxylate salt, ca. 5% of salts of **3a**, and a trace of salts of **1a**. [b] This weight of lyophilized powder represents the total weight extracted in three extractions of approximately 50 g of finely ground plant roots.

After much preliminary exploratory work,[1] we found that reconstituted aqueous extracts could be efficiently separated by chromatography on Sephadex G-50 and G-25 with water as an eluent. While these adsorbents allow enzymes and carbohydrates to pass rapidly through, they selectively adsorb the polyphenolic carboxylates. Smaller carbohydrates pass through in intermediate fractions. When eluted, the polyphenolic carboxylic acid salts are stable in aqueous solution, apparently as a result of having been separated efficiently from plant enzymes which can catalyze their chemical transformation.[9]

The progress of the chromatographic separation on Sephadex G-50 (Table I) was readily observed in a series of colored bands which reproducibly formed on the column. The fractions containing the catechol acids (groups E and F) were identified by the precipitates formed with lead acetate or ferric chloride solutions. Group F of Table I is of particular interest as a source of lithospermic acid. It was further fractionated on Sephadex G-25 into five fractions which are summarized in Table II. Group E was separated by chemical methods into three subfractions (Table I, footnote *a*). The segregation of these fractions was routinely carried out after lyophilization. To aid this process, spot tests on filter paper and infrared spectroscopy (Table II) were found to be useful criteria. The use of ^{13}C NMR gives, however, the clearest fingerprints of the components present in the individual fractions (see footnote *a*, Tables I and II).

The reported[15] insolubility of biologically active constituents of *Lithospermum ruderale* in water at low pH was followed up by acidification of group E material (Table I), centrifugation of the precipitated material, dissolution of the separated solid in water at pH 5.8, and lyophilization to give Ea as a powder. The acidified aqueous filtrate was extracted with ethyl acetate to give a product containing principally an unidentified ester carboxylic acid. This lat-

Table II
Fractionation of Polyphenolic Acid Salts (Group F, Table I) on Sephadex G-25

Fraction[a]	Wt, g	Percent	Carbonyl absorption in infrared, λ (μ)[b]		
F-1	3.92	19.5	5.78 (s)	5.97 (w)	6.28 (s)
F-2	3.27	16.3	5.78 (s)	5.95 (m)	6.28 (vs)
F-3	8.72	43.5		5.91 (m)	6.28 (vs)
F-4	1.20	6.0		5.91 (m)	6.28 (vs)
F-5	0.15	0.7			
Remaining on column	2.80	13.9			
	20.1	100			

[a] ^{13}C NMR analysis of representative samples of fractions F1–F4 allowed the following conclusions. Fraction F1 was very similar to group E material. Fraction F2 was quite complex but contained up to ca. 30% of salts of **1a** and ca. 10% of **3a**. Fraction F3 contained salts chiefly of **1a** admixed with ca. 10% of **3a**. Fraction F4 contained principally salts of **1a**, but it also contained up to ca. 20% of salts of **4a** and some carbohydrate (15%). [b] Relative intensities: vs, very strong; s, strong; m, medium; w, weak.

ter was dissolved in water, neutralized with base, and lyophilized to give fraction Eb. Ec consisted of the plant material still remaining in solution (carbohydrate).

Since the biological actions affecting pituitary hormones have always been associated with the phenolic fractions of the plant, both in our own[1b–f] and others' reports,[8,9] and not at all with the large carbohydrate fractions, our attention has been directed to the identification of the constituents of fractions E and F. Experiments with animals involving these plant fractions will be reported elsewhere.

Characterization of Lithospermic Acid. Fraction F3, which represents approximately 3% of the dry root weight of *Lithospermum ruderale*, consists principally of the dipotassium salt of **1a**. While the organic anion is not exclusively that of **1a**, the contaminants, **3a** (ca. 10%) and **4a** (<2%), do not interfere with the interpretation of ir and NMR data. The liberation of lithospermic acid from its salts is readily accomplished by acidification, and the very water-soluble **1a** is readily extracted into ethyl acetate. To aid in the interpretation of the spectral properties of **1a**, authentic rosmarinic acid (**4a**) was isolated by the procedure[18a] of Scarpati and Oriente from *Rosmarinus officinalis*.[18b,c]

The published[7,9] infrared spectra for lithospermic acid are virtually identical with the ir spectrum (KBr) of our fraction F3 except that the carbonyl region of the latter is split, revealing the internal α,β-unsaturated ester band at 5.91 μ, while the two carboxylate anions have their stretching bands centered at 6.28 and 7.3 μ. The infrared spectrum of sodium rosmarinate shows the α,β-unsaturated ester band also at 5.91 μ, indicating the structural similarity of **1a** and **4a**. In addition, the rest of the ir spectrum of Na-**4a** is virtually identical with that of F3. Further evidence for the structural similarity of **1a** and **4a** was obtained from NMR measurements.

The carbonyl region of the ^{13}C NMR spectrum of Na-**4a** (in D_2O) showed an α,β-unsaturated ester carbon at 168.6 ppm and a carboxylate anion at 177.2 ppm. The spectrum of F3 showed a similar ester carbon at 168.3 ppm and carboxylate anions at 176.9 and 178.8 ppm, the latter arising from C-19 (see the numbering system in Table III). That the second carboxylate peak is C-19 rather than C-9 (as would be required by the alternative structure having the dihydroxyphenyllactate group esterified to C-19) was further established by observing that the carboxylate resonance of several model cinnamate salts falls in the range of 175.3–176.3 ppm.[19] A full analysis of the ^{13}C NMR spectrum of **1a**, **4a**, and the model compounds will be published.[20]

The striking similarity of the ^1H NMR spectra of lithospermic acid (**1a**) and rosmarinic acid (**4a**) in Table III is immediately apparent. Each molecule contains two benzene rings bearing three protons in a 1,2,4 relationship. This arrangement gives rise to the distinctive ^1H NMR coupling pattern of two doublets and one doublet of doublets for each ring. The resonances were assigned to specific hydrogens on the basis of known ring coupling constants ($J_{ortho} \simeq 8$, $J_{meta} \simeq 2$, $J_{para} \simeq 0$ Hz) and on their relative chemical shifts. The chemical shifts of H-5, -13, -16, and -17 remained almost the same for both compounds. The two protons H-2 and H-6 in **4a** ortho to the unsaturated side chain and deshielded by its anisotropy are in **1a** diminished to only one (further) deshielded proton, H-6, with a simplified coupling pattern. This indicates that C-2 is the site of fusion of the additional C_6–C_3 biogenetic unit in **1a**. The protons H-23, -26, and -27 fall at intermediate chemical shifts in **1a**. The only ambiguity in these data arises from the almost equal chemical shifts and coupling constants of H-13 and H-23 in **1a**, which do not allow them to be differentiated.

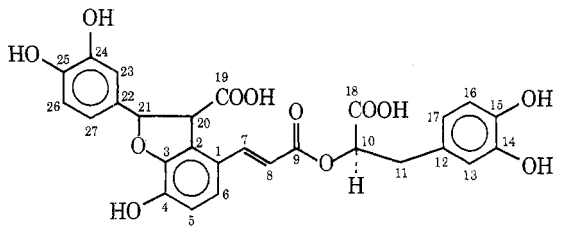
The aryllactate side chain (H-10 and 11) exhibited similar ABX coupling patterns in compounds **1a** and **4a**, differing only in the overlapping of two of the eight lines in the AB portion of the spectrum of **1a** due to a smaller $\Delta\delta_{AB}$. The chemical shift of H-10 in esters **1a** and **4a** is a full 0.7 ppm deshielded from the chemical shift of the analogous proton in the free aryllactic acid, **2a**. This difference allows the ready observation of the presence of **2a** as a contaminant of **1a** or **4a**.

The unique coupling constant of 4.8 Hz for H-20 and H-21 on the coumaran ring appears in both **1a** and salt F3. Early workers pointed out that the proton coupling constant in a 2,3-disubstituted coumaran (dihydrobenzo[*b*]furan) ring is dependent not only on the configuration of the protons but also on the nature of the other substituents at those positions.[21] A more reliable criterion for the assignment of stereochemistry in 3-substituted 2-arylcoumarans, where the 3 substituent contains hydrogen, is the shielding (cis) or lack of shielding (trans) of the protons in the 3 substituent by the aromatic ring.[22]

Esterification of **1a** with MeOH–2,2-dimethoxypropane–HCl gave a mixture in approximately 3:1 ratio of two dimethyl esters, **1b** and **3b**, which were soluble in dilute aqueous bicarbonate (apparently through phenolic ionization). Diester **3b**, which apparently arose from transesterification of **1b** with the methanol solvent, was identified by its NMR spectrum in the mixture and by its parent ion in the mass spectrum.

The ^1H NMR spectrum (acetone-d_6) showed three new methyl resonances at δ 3.72, 3.69, and 3.65. From the intensities of these resonances, it was judged that the major diester, **1b**, has resonances at δ 3.69 and 3.65, while the minor diester, **3b**, has resonances at δ 3.72 and 3.69 (overlapped by **1b**). Since none of these resonances is markedly

Table III
^{1}H NMR Spectra (220 MHz) of Lithospermic Acid and Rosmarinic Acid in Acetone-d_6[a]



1a

Proton[b]	Lithospermic acid (1a) δ (multiplicity, J, Hz)	Rosmarinic acid (4a) δ (multiplicity, J, Hz)
H-11 (2 H)	3.03 (2 q, 5, 8, 14)	3.03 (2 q, 5, 8, 14)
H-20	4.47 (d, 4.8)	
H-10	5.15 (dd, 5, 8)	5.17 (dd, 5, 8)
H-21	5.94 (d, 4.8)	
H-8	6.32 (d, 16)	6.23 (d, 16)
H-17	6.61 (dd, 2, 8)	6.60 (dd, 2, 8)
H-16	6.69 (d, 8)	6.69 (d, 8)
H-27	6.72 (dd, 2, 8)	
H-26	6.77 (d, 8)	
H-23	6.81[c] (d, 2)	
H-13	6.82[c] (d, 2)	6.79 (d, 2)
H-5	6.83 (d, 8)	6.79 (d, 8)
H-6	7.21 (d, 8)	6.96 (dd, 2, 8)
H-2		7.09 (d, 2)
H-7	7.80 (d, 16)	7.50 (d, 16)

[a] The use of deuterioacetone as a ^{1}H NMR solvent is important in obtaining the resolution of the aromatic resonances. The ^{1}H NMR spectrum of the salt **1a** (fraction F3) in D_2O shows a much more compact aromatic proton region in which not even H-6 is resolved. [b] The 18 carbon atoms shared in common in the structure of acids **1a** and **4a** are numbered in a consecutive sequence of 1–18; atoms numbered 19–27 represent the additional phenylpropanoid unit contained in lithospermic acid which is condensed as shown in the formula. [c] Overlapping resonances; assignments may be reversed.

shielded, we have assigned a trans configuration to the methoxycarbonyl and the aryl substituents on the coumaran ring in **1b** and thus in **1a.** The absolute configurations of the asymmetric carbons in the coumaran nucleus have not yet been determined.

The ultraviolet spectra of the free lithospermic acid were in general agreement with those already published,[7,9] and consistent with the presence of conjugated unsaturation of the type contained in **1a.** Quantitative data are presented in the Experimental Section. Ultraviolet spectra for the salts of **1a** showed the same major features with some variations in detail.

Further information about the configuration and composition of the polyphenolic acids was obtained on fully methylated derivatives. The total absence of O-methylated compounds in the aqueous extracts of *L. ruderale* (by ^{1}H and ^{13}C NMR criteria) makes it clear that all of the methyl groups subsequently found in the methylated derivatives were experimentally introduced into unmethylated natural products.

Methylation of Polyphenolic Acids with Diazomethane. At the same time that the separations on Sephadex (Tables I and II) were being carried out, an alternative isolation of the polyphenolic acids was undertaken by a modification of a published procedure.[7] Root powders of *L. ruderale* were extracted with aqueous HCl, and the dissolved polyphenolic carboxylic acids were taken up in organic solvents. Ethyl acetate was found to be a much more efficient solvent than ether[7] for the removal of polyphenolic acids from aqueous solution.

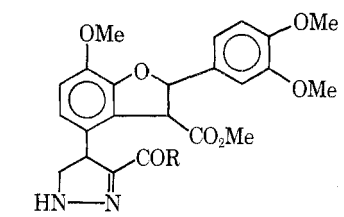
The extracted solutions of these acids were permethylated with excess diazomethane. A complicating reaction in the use of diazomethane was subsequently shown to be the cycloaddition of this reagent to the olefinic bond of the substituted cinnamic esters in these mixtures. The cinnamates were principally transformed into the corresponding pyrazoline derivatives.[26-29] The chemical changes were accompanied by changes in the ultraviolet absorption spectra—in particular, decreases in absorption above 300 nm. In spite of the formation of nitrogen heterocycles, this procedure yielded valuable early indications of the nature of the polyphenolic acids present in the plant.

The aryllactic acid **2a** was converted by diazomethane into methyl (*R*)-3-(3,4-dimethoxyphenyl)lactate [(*R*)-**2c**], which was isolated in 3% yield by repeated column chromatography. The identity of (*R*)-**2c** was established by spectral comparison with synthetic (*RS*)-**2c.** Subsequently, the absolute configuration was established to be *R* by the undepressed melting point of the ester mixed with an authentic sample[18b] of (*R*)-**2c** prepared as a derivative following the saponification of rosmarinic acid.

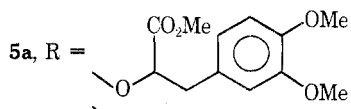
Methyl aryllactate (*R*)-**2c** was acetylated to (*R*)-**2d,** and the acetyl derivative was hydrolyzed to the aryllactic acid (*R*)-**2e.** Resolution of (*RS*)-**2e** to give (*R*)-**2e** was accomplished. The CD spectra of (*R*)-**2c** and (*R*)-**2e** are comparable in sign and intensity but opposite in sign to spectra of (*S*)-3,4-dihydroxyphenylalanine (L-dopa), (*S*)-3-phenyllactic acid,[30] and methyl (*S*)-3-phenyllactate.[30] The CD spectrum of (*R*)-**2c** from *L. ruderale* is identical with that of the authentic sample[18b] in confirmation of the previous assignment.[18c]

In the chromatographic separations used to fractionate diazomethane-methylated polyphenolic acids, the methyl aryllactate (*R*)-**2c** was eluted from a Florisil column with benzene–ether mixtures. About 70% of the methylated product, the material containing nitrogen heterocyclic derivatives, was more strongly held on the column and was eluted with ether–ethyl acetate mixtures. These latter materials were rechromatographed and were characterized by osmometric molecular weights (mol wt) in the range of 500–1200.

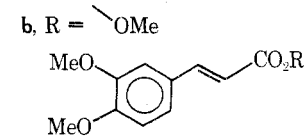
Mass spectra of these fractions show parent ions at *m/e* 470 and 472 for which the pyrazoline structures **5b** and **6a** can be written. These are the products expected from **3a** and **4a** under the reaction conditions. At higher temperatures in the mass spectrum, a major ion at *m/e* 650 was observed, apparently formed from the molecular ion of **5a** (*m/e* 678) by loss of nitrogen.[27]



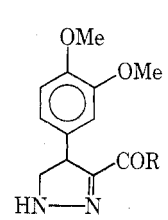
5a, R =



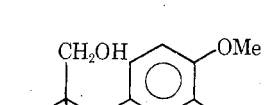
b, R =



7a, R = H
b, R = Me



6a, R = same as in **5a**
b, R = same as in **5b**



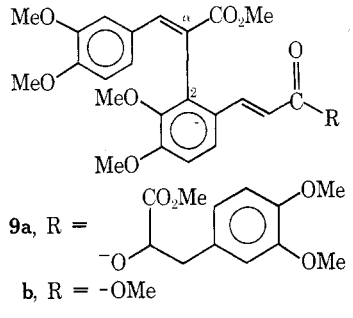
(*R*)-**8**

As a model for the diazomethane cycloadditions, the reaction of methyl 3,4-dimethoxycinnamate (**7b**) with diazomethane was observed to give in 65% yield a crystalline pyrazoline derivative, **6b.** The reaction of pentamethyl rosmarinate (**4b**) with diazomethane gave the pyrazoline **6a** (*m/e* 472) and further transformation products.[31]

Saponification of the complex permethylated product mixture, separation of the acids, remethylation of the acids with diazomethane, and separation by chromatography yielded additional amounts of (*R*)-**2c.** Treatment of the complex mixture with lithium aluminum hydride produced (*R*)-3-(3,4-dimethoxyphenyl)-1,2-propanediol [(*R*)-**8**], which was crystallized after chromatography. For identification, (*RS*)-**8** was synthesized by reduction of (*RS*)-**2c.**

Since **2c** and diol **8** have the same origin, their absolute configurations should be the same. The CD spectrum of a solution of **8** and $Ni(acac)_2$ in CCl_4 has a positive induced exciton-split type Cotton effect centered at 306 nm [$\lambda°$ ($\Delta\epsilon°$) 316 (+3.6) and 297 (−3.6)], establishing the absolute configuration as *R*.[33] Moreover, the ^1L_b and ^1L_a bands of (*R*)-**8** are both positive, while in (*S*)-3-phenyl-2-amino-1-propanol both of these bands are negative.[30]

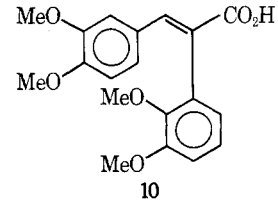
Base-Catalyzed Methylation of Fraction F3. In another procedure, fraction F3 (Table II) was permethylated in refluxing acetone with dimethyl sulfate and potassium carbonate, and the neutral methylated material was chromatographed on a series of columns (either Florisil or nearly neutral alumina), with benzene–ether–ethyl acetate as increasingly polar eluting solvents. Fractions were monitored by TLC. One compound, $C_{35}H_{38}O_{12}$, was found repeatedly in the middle fractions and accounted for ca. 25% of the weight of methylated products. This substance gave clean mass spectral and ^1H NMR data, from which the structure **9a** was deduced.



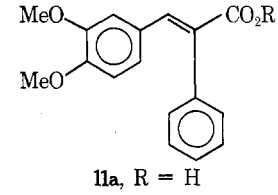
From the ^1H NMR spectrum it is concluded that **9a** is an octamethyl derivative, where a heptamethyl derivative would be expected from the uncomplicated methylation of **1a.** Thus, one additional oxygen function which was not already present as a phenol or a carboxyl group in **1a** must have been methylated under these basic conditions. The anomaly is explained as a consequence of base-catalyzed opening of the coumaran ring in **1a,** initiated by abstraction of H-20 (cf. Table III), which generates concomitantly a new phenolic function (susceptible to methylation) and a new *trans*-caffeyl functional unit. Since opening of the coumaran ring is formally an isomerization, we shall refer to **9a** as octamethyl isolithospermate. The sequence of the methylation reactions under basic conditions is not known, but it seems likely that the ring-opening reaction occurs late in the sequence. Certainly esterification of carboxylate C-19 precedes abstraction of the α hydrogen, H-20.

Accompanying **9a,** and present chiefly in chromatographic fractions immediately preceding it, were minor amounts of several other derivatives of **1a** and **3a.** These included the heptamethyl derivative, **1c,** and two derivatives, **3c** and **9b,** lacking the aryllactate group (the latter being replaced with the methyl ester function). Also present in very minor amounts was pentamethyl rosmarinate (**4b**). The presence of all of these compounds was first revealed by mass spectra; confirmatory ^1H NMR spectra were subsequently obtained for **4b** and **9b.**

To aid in the interpretation of the array of spectral data obtained from chromatographically similar methylated derivatives, the synthesis of several model compounds proved expedient: 3,4-dimethoxycinnamic acid (**7a**), its methyl ester **7b,** and the aryllactates (*RS*)-**2c,** (*RS*)-**2e,** and (*RS*)-**4b.** The latter was synthesized by the reaction of **7a** with *p*-toluenesulfonyl chloride in pyridine, followed by the addition of (*RS*)-**2c.** Two further model compounds, **10** and **11a,** were prepared by the Perkin condensation of appropriately substituted phenylacetic acids with 3,4-dimethoxybenzaldehyde.[34] The ethyl ester **11b** was prepared from the acid **11a.**



10

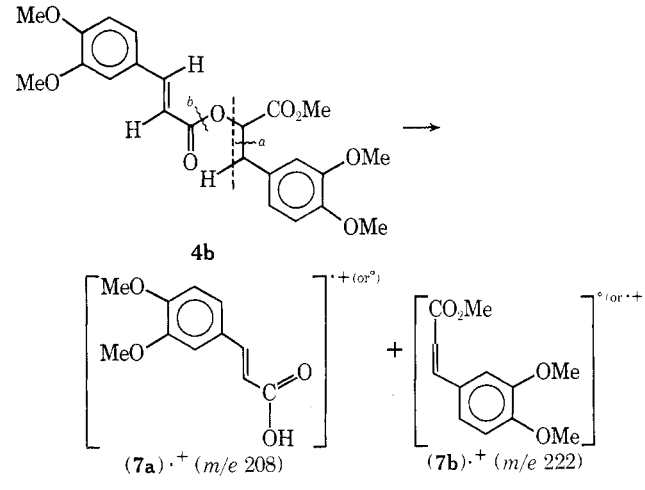


11a, R = H
b, R = Et

Mass Spectra. Table IV shows the fragmentation patterns of the five model compounds, **4b, 7a, 7b, 2e,** and **2c.**

For pentamethyl rosmarinate (**4b**), two strong ion intensities in the mass spectrum arise from a McLafferty rearrangement of the molecular ion (*m/e* 430) into two fragments, *m/e* 222 and 208, according to Scheme I. Other

Scheme I
Mass Spectral Fragmentation of Pentamethyl Rosmarinate (4b)



major fragmentation pathways include cleavage at bond a to yield the dimethoxybenzyl cation at *m/e* 151 and possible cleavage at bond b to produce the acylonium ion at *m/e* 191. Alternatively, the latter ion could arise from further cleavage of (**7a**)·+ and (**7b**)·+ as is observed in the mass spectra of their parent compounds (Table IV).

The base peaks of the mass spectrum of octamethyl isolithospermate (**9a**) shown in Table V are produced in a direct cleavage of the molecular ion to give the 3,4-dimethoxybenzyl cation (*m/e* 151) and in a McLafferty rearrangement to give the methyl 3,4-dimethoxycinnamate ion, (**7b**)·+ (*m/e* 222). These same two ions were the dominant ones in the mass spectrum of pentamethyl rosmarinate (**4b**), confirming that **9a** is an *O*-acyl derivative of methyl 3-(3,4-dimethoxyphenyl)lactate (**2c**). The McLafferty rear-

Table IV
Mass Spectra of Model Compounds

	Rel intensities at 70 eV for model compounds[a,b]				
Ion *m/e*	4b	7a[c]	7b[d]	2e	2c
430	4.3				
399	0.6				
240	<1				10.2
226	0			13.7	0
222	100		100	0	1.3
208	23	100	2.0	0.6	0.6
207	4.6	0	16	0	0
191	25	4.9	47	0	0
181	1.7	0	0	1.3	2.6
165	1.1	4.3	1.3	0.5	0.6
163	8.2	3.5	9.4	0	0
151	56	0	0	100	100

[a] Determined in a Varian MAT-CH-7 mass spectrometer. [b] Intensities expressed as percentage of the strongest ion intensity. [c] Also observed: *m/e* 193 (19). [d] For complete mass spectrum, see ref 35.

Table V
Mass Spectrum of Octamethyl Isolithospermate (9a)

Ion[a] *m/e*	Rel abundance,[b] %	Mass found[c]	Mass calcd	Molecular formula
650	35	650.251	650.236	$C_{35}H_{38}O_{12}$
428	31	428.143	428.147	$C_{23}H_{24}O_8$
410	3			
396	4			
382	13			
368	9			
351	38	351.121	351.123	$C_{21}H_{19}O_5$
222	100	222.089	222.089	$C_{12}H_{14}O_4$
151	100	151.076	151.076	$C_9H_{11}O_2$

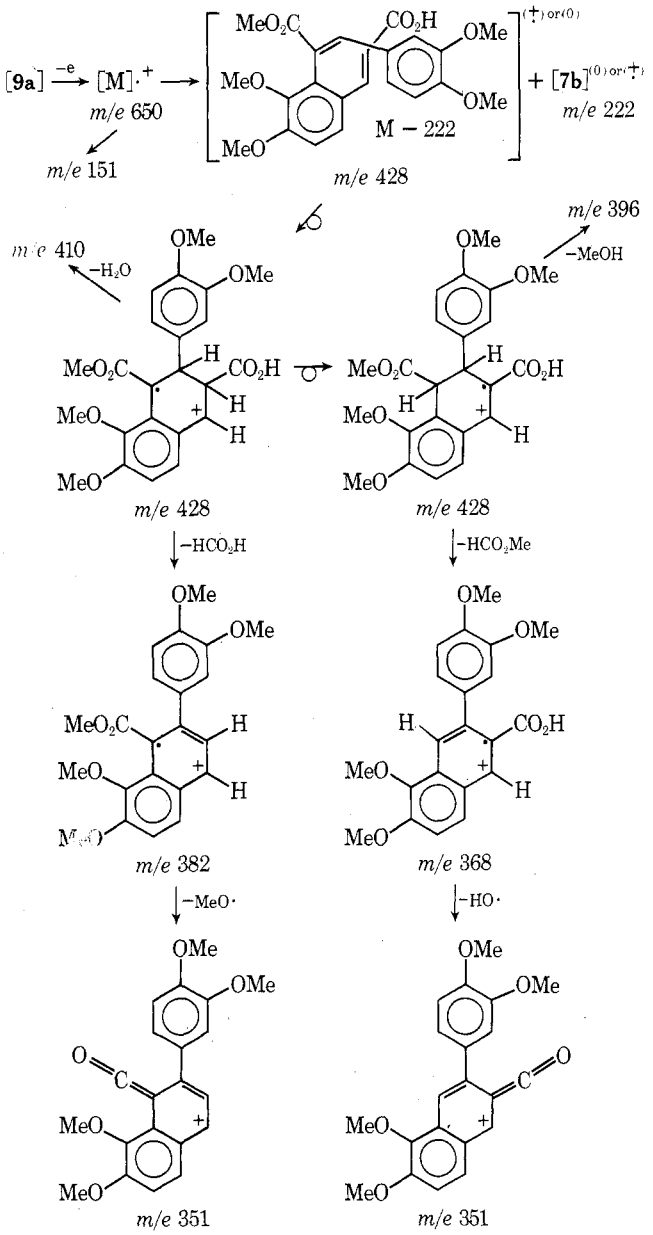
[a] Also observed: *m/e* 337 (4), 324 (5), 323 (4), 309 (6), 298 (8), 246 (7), 191 (10), 181 (20). [b] Determined in a Varian CH-7 mass spectrometer. [c] Determined in an AEI MS-9 mass spectrometer by peak matching technique.

rangement of the parent ion (*m/e* 650) also gives the *m/e* ion 428 (metastable ion at *m/e* 282), which decays through a series of ions to a stabilized ion at *m/e* 351. Possible structures for these ions are presented in Scheme II.

The apparent loss of the elements of HCOOMe and of HCOOH is a characteristic feature of the spectrum of **9a** which is not observed in the model compounds, and is best explained by the postulated cyclization of two ortho-situated unsaturated side chains to form a six-membered ring which can extrude small substituents to achieve additional stabilization. Such an interaction of side chains can only occur with the ortho placement of the vinyl groups of **9a,** and is one piece of evidence in the assignment of the 2,α-bicaffeate structure to **9a.** An alternative 5,α structure would be ruled out by this logic, as well as from the ^{1}H NMR data.

One chromatographic fraction of methylated derivatives (31 mg out of 5.8 g taken for the separation) consisted of an almost equal mixture of **4b** and **9b.** The NMR peaks of **9b** (reported in Table VI) in the spectrum of this sample were readily distinguished from those of **4b.** The mass spectrum of this mixture showed, in addition to the characteristic ions of **4b** [*m/e* 430, 222 (100), and 208], the molecular ion of **9b** [*m/e* 442 (33)] and two principal daughter ions [*m/e* 382 (19) and 351 (40)] in good agreement with the expected fragmentation of **9b** by the same pathway as **9a** (cf. Scheme II).

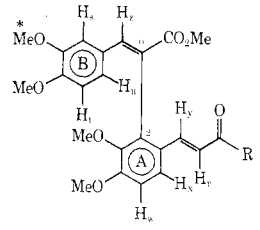
Scheme II
Fragmentation Patterns in the Mass Spectrum of Octamethyl Isolithospermate (9a)



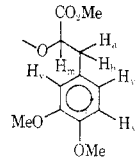
^{1}H NMR Spectra. The ^{1}H NMR spectra of octamethyl isolithospermate (**9a**) and the hexamethyl 2,α-bicaffeate (**9b**) are summarized in Table VI and compared with the two synthetic model compounds, **10** and **11b.** The methoxy region of **9a,** reproduced in Figure 1, shows eight distinct *O*-methyl groups, six of which appear as doubled resonances. We attribute the doubling of these resonances to restricted rotation about the bond joining the 2,α positions of the bicaffeyl subunit.[36] If an approximately equal distribution of two rotational forms exists about this bond, the presence of the asymmetric center in the aryllactyl ester portion of the molecule would mean that **9a** is a mixture of nearly equal amounts of two diastereoisomers. The doubling of the methyl hydrogen resonances disappears upon full catalytic hydrogenation of the olefinic unsaturation. It is also not characteristic of **9b,** which lacks the aryllactate asymmetric center and would therefore be a simple racemic pair as a consequence of the restricted rotation.

That the caffeyl moiety containing phenyl ring B in esters **9a** and **9b** has the stereochemistry of an α-phenyl-*trans*-cinnamic ester (i.e., that the phenyl rings A and B

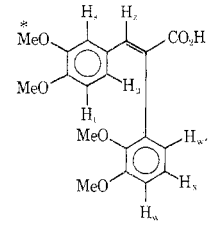
Table VI
^{1}H NMR (HR-220) Spectra of Compounds 9a, 9b, 10, and 11b in $CDCl_3$



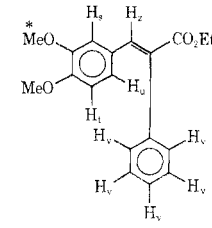
9b, R = OMe



9a, R = aryllactate



10



11b

Proton δ^a (multiplicity, J)		δ^b (multiplicity, J, $\Delta\delta$)	δ^c (multiplicity, J)	δ^d (multiplicity, J)
H_a, H_b		3.02 (m, 2 H)		
H_m		5.21 (4d, 5, 7, 3)		
*Me	3.41 (s)	3.42 (2 s, 0, 3)	3.39 (s)	3.29 (s)
H_r	6.17 (d, 16)	6.20 (2 d, 16, 1)		
H_s	6.39 (d, 2)	6.39 (2 d, 2, 4)	6.50 (d, 2)	6.30 (d, 2)
H_t	6.65 (d, 8)	6.65 (d, 8, 0)	6.66 (d, 8)	6.61 (d, 8)
H_u	Obscured by compound **4b**	Included in H_v multiplet	6.83 (dd, 2, 8)	6.75 (dd, 2, 8)
H_v		6.59–6.80 (m, 4 H)		7.14–7.36 (m, 5 H)
H_w	6.93 (d, 8)	6.93 (d, 8, 0)	6.71 (dd, 2, 8)	
$H_{w'}$		—	6.90 (dd, 2, 8)	
H_x	7.44 (d, 8)	7.45 (d, 8, 0)	7.03 (t, 8)	
H_y	7.48 (d, 16)	7.54 (2 d, 16, 1)		
H_z	7.93 (s)	7.93 (2 s, 0, 1)	7.85 (s)	7.71 (s)
$CHCl_3$	7.22 (internal)	7.21	7.20	

[a] Other OMe singlet resonances: δ 3.59, 3.64, 3.71, and 3.77; one OMe resonance obscured by compound **4b**. [b] Other OMe resonances, cf. Figure 1; δ 3.62 (d, $\Delta\delta$ = 3 Hz), 3.67 (d, $\Delta\delta$ = 3 Hz), 3.72 (d, $\Delta\delta$ = 1.5 Hz), 3.79 (d, $\Delta\delta$ = 1.5 Hz), 3.81 (s), 3.83 (d, $\Delta\delta$ = 3 Hz), 3.90 (s). [c] Other OMe singlet resonances: δ 3.69, 3.77, and 3.81. [d] Other resonances: δ 1.27 (t, J = 7 Hz, CH_2CH_3), 3.72 (s, OCH_3), 4.17 (q, J = 7 Hz, CH_2CH_3).

and the connecting double bond comprise a *cis*-stilbene subunit) is established by the chemical shift of vinyl hydrogen H_z in these compounds as compared with the vinyl proton in model compounds **10** and **11b.** Not only has the α-phenyl-*trans*-cinnamic stereochemistry been established as being more thermodynamically stable than the cis,[34b,37] but the chemical shifts of the vinyl hydrogen have been well documented as an unambiguous means of distinguishing the trans from the cis series.[29]

An additional effect of the trans stereochemistry of the caffeate subunit created by the opening of the original coumaran ring is the marked shielding of the ortho H_s and the meta CH_3O resonances in ring B. In the four compounds in Table VI, H_s is shielded by 0.5–0.7 ppm compared with the corresponding proton in **4b** (H-2; cf. Experimental Section). Similarly, the meta *CH_3O group in ring B experiences a shielding of 0.4–0.6 ppm with reference to a comparable CH_3O in **4b.**

The two ortho hydrogens on ring A (H_x and H_w) exhibit a 0.5-ppm difference in their chemical shifts in both compounds **9a** and **9b.** This difference is due in part to the deshielding of H_x by the anisotropic effect of the unsaturated substituent ortho to it on the ring. The identity of the unsaturated group which deshields H_x was revealed by the following hydrogenation experiment.

Octamethyl isolithospermate (**9a**) rapidly consumed 1 mol of hydrogen in ethyl acetate at room temperature over 10% palladium on carbon catalyst, and only quite slowly consumed a second mole of hydrogen. A mass spectrum of the product of such a hydrogenated reaction mixture which had not yet absorbed 2 mol revealed that a mixture of dihydro-**9a** and tetrahydro-**9a** had been produced (*m/e* 652:654 = 3:2). The ^{1}H NMR spectrum of this mixed product showed that, as expected, the trisubstituted double bond conjugated with ring B was the more slowly hydrogenated, since the protons H_z (δ 7.85) and H_s (δ 6.42) and the shielded meta methoxyl group (starred in Table VI) in dihydro-**9a** were clearly visible in the ^{1}H NMR spectrum, while the aromatic hydrogens in phenyl ring A (H_x and H_w) had shifted completely into the aromatic multiplet at δ 6.6–6.9. The conclusion is that the disubstituted double bond which is rapidly and fully hydrogenated must be located ortho to H_x. That H_x and H_w are ortho to each other follows from their coupling constants.

Relationship of Lithospermum Constituents to Known Plant Principles. Since the discovery of rosmarinic acid (**4a**) in 1958,[18bc] that compound has become recognized as one of the most widely distributed naturally occurring derivatives of caffeic acid.[12,38–41] Its biogenesis has been recently studied.[42]

The structure which we have proposed for lithospermic acid, **1a,** incorporates a molecule of rosmarinic acid to which is condensed a third catecholpropanoid unit. The coexistence of **1a** and **4a** in *Lithosperum ruderale* suggests that **4a** may be a precursor of **1a.**

The condensation of two *p*-hydroxyphenylpropenoid units to give a 2-aryl-3-substituted coumaran nucleus is a well-documented process in plant biochemistry. In the laboratory, models of this reaction are found in the oxidative coupling of isoeugenol[21] to dehydrodiisoeugenol (**12a**) and of coniferyl alcohol[43] to **12b.** These condensations have been cited as models of reactions which occur in lignin formation. Typically, the coumarans produced in this way have the trans arrangement of the 2,3 substituents on the 2,3-dihydrobenzo[*b*]furan nucleus.[24,44,45]

Similar self-condensations of cinnamic acid derivatives have also been observed to occur through both chemical and enzymatic oxidative coupling reactions. Ferulic acid ethyl ester has yielded coumaran **12c,**[46] and a complex amide of *p*-coumaric acid has been similarly dimerized to

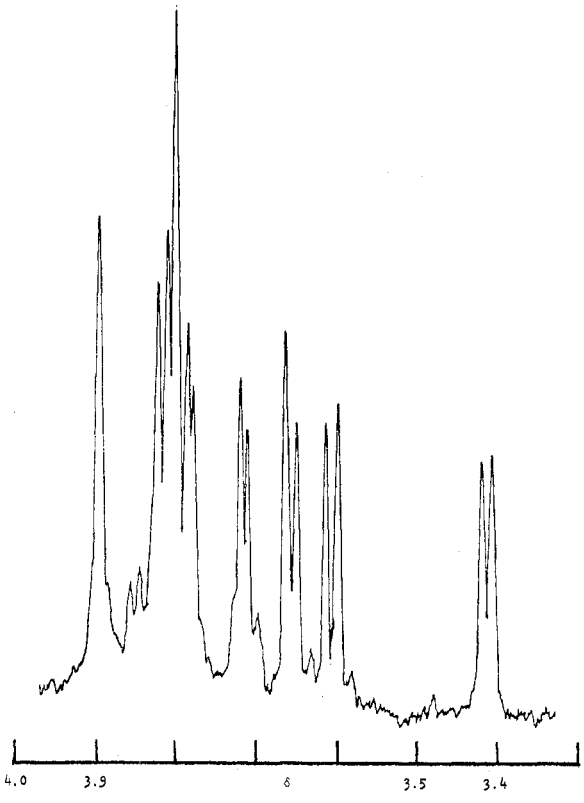
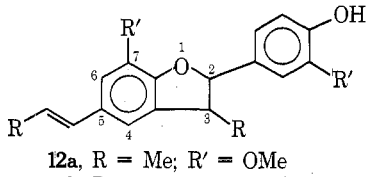


Figure 1. Proton resonances (220 MHz) of the *O*-methyl groups in octamethyl isolithospermate (**9a**).

hordatine A (aglucone) (**12d**), a member of the family of optically active antifungal factors isolated from barley coleoptiles.[47]



12a, R = Me; R′ = OMe
b, R = CH_2OH; R′ = OMe
c, R = CO_2Et; R′ = OMe
d, R = CONHR″; R′ = H

In all of the above oxidative dimerizations, the coumaran produced has its unsaturated side chain attached at C-5. This is in agreement with the radical coupling mechanisms by which the reactions are envisioned to proceed. Only a phenolic function para to a vinyl group is mechanistically suited to undergo this type of coumaran formation.

If lithospermic acid is formed by conjugation of a catecholpropanoid unit with rosmarinic acid, then the formation of the coumaran nucleus must in this case involve the 3-hydroxy (meta) group of the rosmarinate unit and formation of the new carbon–carbon bond occurs at the open ortho position, resulting in the placement of the unsaturated side chain on C-4 of the product coumaran. Data from ^1H NMR, ^{13}C NMR, and mass spectra are all consistent with the assignment of the 2,3,4,7-tetrasubstituted coumaran structure to lithospermic acid. Thus, the latter appears to be a unique example of a class of natural coumaran derivatives for which previous oxidative coupling mechanisms involving *p*-hydroxystyrene derivatives cannot be readily adapted.

The ability of lithospermic acid to inhibit or block the action of pituitary peptide hormones has been reported to require[8,9] its prior oxidation by air or phenoloxidases. The nature of the oxidation and the mechanism(s) of hormone blocking are still unknown. One must consider the possibility that *o*-benzoquinones may be implicated. The oxidative coupling of two or more lithospermate units would be a facile process, and probably occurs. We have observed that plant fractions E and F1 (Tables I and II) contain oligomeric materials which are insoluble in water at low pH but spectroscopically similar to lithospermic acid. Further chemical studies are being directed to these materials, which have shown inhibitory responses in animal endocrine experiments.

Experimental Section

General. Melting points were taken in open glass capillaries in a Mel-Temp apparatus and are uncorrected. Analyses were carried out by Alfred Bernhardt, Max Planck Institut, Mülheim (Ruhr), Germany, or Midwest Microlab Inc., Indianapolis, Ind. Infrared (ir) spectra were measured on Perkin-Elmer Infracord Models 137B or 137G. Ultraviolet (uv) and circular dichroism (CD) spectra were recorded with a Durrum–Jasco ORD/UV/CD-5 incorporating the SS-10 modification. Uv data are represented as λ_{max} (ϵ), sh = shoulder, and i = inflection. CD data are represented as λ_{max} ($\Delta\epsilon$) where $\Delta\epsilon = [\theta]/3300$. ^1H NMR spectra were recorded on Varian HR-220, HA-100, A-60, and EM-360 spectrometers. Mass spectra (MS) were obtained at 70 eV on a Varian MAT CH-7 mass spectrometer. Absolute masses were determined on a AEI MS-9 mass spectrometer. Optical rotations were measured with a Rudolph polarimeter, Model 80, in a 2-dm cell. Osmometric molecular weights were determined in a Mechrolab Model 301A vapor pressure osmometer in boiling benzene. Anhydrous $MgSO_4$ was routinely used as a drying agent.

Extraction of Water-Soluble Constituents of *Lithospermum ruderale* Roots. Roots of *L. ruderale* Dougl. ex Lehm. were collected by Mr. J. H. Coleman near Missoula, Mont., in the summer of 1959 and air dried. The woody roots were brushed free of soil and were ground in a Wiley Laboratory mill using a 2-mm mesh sieve. In a typical extraction, 100 g of the powder was stirred vigorously with 1 l. of distilled water under N_2 for 30 min, then filtered through a heavy cotton towel in a Büchner funnel. The solid cake was stirred vigorously with two additional 1-l. portions of water for 30 min each and filtered as before. Since the dark brown, opaque filtrates clogged ordinary filters, each of the three solutions (812, 881, and 943 ml, respectively) was clarified by centrifugation at 2800 rpm for 20 min at 7°. The water was removed by lyophilization during 3 days, and the dried powder preparations weighed 25, 11, and 4 g, respectively.

The powders have a flaky, gold-bronze appearance and a crisp, friable texture. They may be stored indefinitely in a thoroughly dry state and can be almost completely redissolved in water. Reconstituted solutions should, in general, be centrifuged to ensure adequate flow rates through any chromatographic medium.

Separation of Root Extracts on Sephadex G-50. A quantity of 20.0 g of lyophilized root-extract powder was dissolved in 100 ml of deoxygenated, distilled water and centrifuged for 1 hr at 35,000 rpm, and the supernatant solution was transferred to a chromatographic column (5.5 × 78 cm) containing 200 g of coarse grade Sephadex G-50 packed and washed according to the manufacturer's directions. The pellet removed by centrifugation weighed 0.7 g. Elution with water was followed by the development of colored bands; the first solute appeared after 540 ml of water, after which fractions of ca. 100 ml were collected, combined, and lyophilized.

The reproducibly colored bands were taken as the principal criterion for the combination of fractions into groups A–F. Treatment of aliquots of the solutions with neutral lead acetate or 1% aqueous ferric chloride gave voluminous precipitates (brown or green) only with groups E and F. After lyophilization, ir (KBr) spectra show significant aromatic ring stretching bands (6.6–6.8 μ) for groups A, E, and F, but not for groups B, C, and D. Of all the groups, only A would not readily redissolve in water; however, much of the group A material could be dissolved at pH 8.2.

Fractionation of Polyphenolic Acid Salts on Sephadex G-25. Group F material (20.1 g) was dissolved in 400 ml of water and chromatographed on 1 kg of Sephadex G-25-course prepared in a 8.5 × 80 cm glass column according to the manufacturer's directions. Portions of 100 ml were collected and lyophilized directly. Fractions F1–F5 were segregated on the basis of TLC on silica gel with *tert*-butyl alcohol–acetic acid–water (3:1:1) and filter paper

spot tests. In both assays, fractions F2 and F3 gave a blue-white fluorescence under uv light which became blue-green after exposure to ammonia vapors. Fractions F1, F4, and F5 appeared yellow under uv light with or without ammonia. Fraction F2 was distinguished from F3 by the absence of the 5.78-μ band in the ir spectrum of the latter (cf. Table II). Fraction F3 was further characterized as follows: uv (MeOH) (ϵ calcd using mol wt 614) 335 nm (sh, 12,700), 309 (14,500), 289 (14,800), 254 (16,400), 230 (i, 22,000); ^{1}H NMR (100 MHz, D_2O, internal acetone δ 2.23) δ 3.11 (m, 2 H, H-11), 4.25 (d, J = 4.8 Hz, H-20), 4.7–5.2 (HOD and H-10), 5.86 (d, J = 4.8 Hz, H-21), 6.22 (d, J = 16 Hz, H-8), 6.7–7.1 (m, 8 H, aromatic), 7.61 (d, J = 16 Hz, H-7).

Anal. Calcd for $C_{27}H_{20}K_2O_{12}\cdot H_2O$: C, 51.26; H, 3.51; K, 12.36; O, 32.87. Found: C, 51.47, 51.68; H, 4.19, 3.96; K, 10.1, 10.1; O, 34.1, 34.0.

Fractionation of Group E. Group E powder (2.13 g) was dissolved in 100 ml of water (pH 6.0), and the pH was lowered to 2 by the dropwise addition of 2 N HCl. Below pH 4, a precipitate appeared. This precipitate was collected by centrifugation and was resuspended in 75 ml of water with vigorous stirring (pH 3). The pH of the solution was adjusted to 5.6 by the addition of 1.49 ml of 1 N KOH. Lyophilization gave 523 mg of a dark brown powder (fraction Ea).

The supernatant from the centrifugation was acidified further (pH 1.5) and extracted with four 60-ml portions of ethyl acetate. The dried extract was concentrated to an oil which was redissolved in 40 ml of water to give a solution of pH 2.7. The pH was adjusted to 5.8 by the addition of 1.78 ml of 1 N KOH, and the solution was lyophilized to give 566 mg of a brown powder (fraction Eb): ir (KBr) 5.76 (s, ester C=O), 6.3 μ (vs, CO_2^-). Neutralization and lyophilization of the aqueous solution remaining after the ethyl acetate extraction gave lumpy brown material containing much KCl (fraction Ec).

Liberation of Compound 1a from F3. A solution of F3 (350 mg) in 20 ml of water (pH 4.9) was acidified to pH 1.6 with 1 ml of 2 N HCl. A few milligrams of a red-brown precipitate were removed by filtration. The clear brown solution was extracted with four 20-ml portions of ethyl acetate, and the extract was dried and concentrated to a brown film. The film was twice dissolved in 5 ml of MeCN and stripped to leave 290 mg of crude **1a** containing a little residual MeCN (^{1}H NMR in Table III). The sample was dissolved in 4 ml of water and lyophilized to an off-white, amorphous powder: uv (MeOH) (ϵ calcd using mol wt 538) 335 nm (sh, 12,000), 310 (14,600), 289 (14,700), 255 (15,700), 225–230 (i, 24,000).

Esterification of 1a. To a solution of 90 mg of **1a** in 2 ml of MeOH and 2 ml of 2,2-dimethoxypropane was added 4 drops of concentrated HCl, and the solution was stirred at room temperature for 3 days. The solvents were removed under reduced pressure. The residue was dissolved in ethyl acetate, and the solution was washed three times each with water and dilute $NaHCO_3$. The bright yellow bicarbonate washed solution was immediately added to 2 N HCl and the gummy precipitate was mostly dissolved during three extractions with CH_2Cl_2. Concentration of the solvent gave a mixture of **1b** and **3b** (ca. 3:1, 50 mg) which would not redissolve in CH_2Cl_2: ^{1}H NMR (100 MHz, acetone-d_6) for **1b** (partial) δ 3.65 and 3.69 (2 s, 6 H, CO_2CH_3), 4.50 (d, J = 5 Hz, H-20), 5.15 (2 d, J = 6, 7 Hz, H-10), 5.92 (d, J = 5 Hz, H-21), 6.29 (d, J = 16 Hz, H-8), 7.22 (d, J = 8 Hz, H-6), 7.74 (d, J = 16 Hz, H-7); for **3b** (partial) 3.69 and 3.72 (2 s, 6 H, CO_2CH_3), 4.48 (d, J = 5 Hz, H-20), 5.89 (d, J = 5 Hz, H-21), 6.28 (d, J = 16 Hz, H-8), 7.20 (d, J = 8 Hz, H-6), 7.70 (d, J = 16 Hz, H-7).

Polyphenolic Acids. A. Direct Isolation from Roots. Whole root powder (100 g) was vigorously stirred with 400 ml of 0.5 N HCl at room temperature for 20 min. The root cake was filtered on cotton cloth and saved for separate extraction. The clear filtrate was extracted with six portions of 100 ml each of peroxide-free ethyl ether. From the dried ether extracts, removal of the solvent left 1.86 g of friable brown material (fraction I). The aqueous solution was then extracted with five portions of 100 ml each of ethyl acetate, from which removal of the solvent left 1.19 g of straw-colored product (fraction II). The root cake was also stirred with 200 ml of ethyl acetate for 15 min and filtered; a second similar extraction with ethyl acetate was combined with the first. Removal of the solvent from the combined root cake extracts left a dark brown, friable material weighing 3.33 g (fraction III).

B. Methylation with Diazomethane. Fraction I (1.26 g) in 15 ml of ether and 15 ml of MeOH was treated with ethereal diazomethane prepared[48a] from 5.0 g of N-methylnitrosourea and was allowed to stand at room temperature for 3.5 hr. Excess diazomethane was destroyed with dilute HCl. The entire mixture was washed with 1 N NaOH, and ethyl acetate was added to dissolve some suspended solid. The dried organic solution yielded 1.12 g of friable, brown, methylated product.

In a similar way and in similar proportions but with a 20-hr reaction time, fractions II and III materials yielded 4.09 g of neutral product. In a larger run, 21.4 g of neutral methylated product was obtained from 22.2 g of combined extract fractions II and III.

C. Separation of (*R*)-2c. On a column (3 × 78 cm) containing 300 g of Florisil (60–100 mesh) packed in benzene was placed 26.0 g of combined neutral products from the methylation of polyphenolic acids II and III. Elution with solvent mixtures of steadily increasing polarity (benzene–ether–ethyl acetate) allowed nearly quantitative recovery of methylated materials.

The initial materials eluted with benzene (1.6 g) were not further investigated, but the subsequent 6.6 g eluted with ether (and increasing amounts of ethyl acetate in ether) gave, after four further column separations, 0.836 g of a viscous liquid. Distillation at 100–140° (bath) (0.02 mm) and two recrystallizations from MeOH gave 0.318 g of (*R*)-**2c**, mp 66°, $[\alpha]^{22}D$ −19.6° (c 0.9, 95% EtOH). Admixture with authentic[18c] (*R*)-**2c** showed no depression of melting point: ir, same as literature;[18c] uv (hexane) 280 nm (ϵ 2800), 231 (8650), 200 (78,000); CD (MeOH) 288 (+0.025), 276 (−0.120), 231 (−2.23), 214 (−1.93), 200 (ca. +9); ^{1}H NMR (60 MHz, $CDCl_3$) δ 2.96 (d, $\Delta\delta$ = 6.5 Hz) and 2.99 (d, $\Delta\delta$ = 4.5 Hz) (J_{gem} unobserved, 2 H, $ArCH_2$), 3.76 (s, CO_2CH_3), 3.84 [s, 6 H, $Ar(OCH_3)_2$], 4.40 (2 d, J = 4.5, 6.5 Hz, 1 H, CHOH), 6.76 (s, C_6H_3).

Anal. Calcd for $C_{12}H_{16}O_5$: C, 59.99; H, 6.71; O, 33.30; $3CH_3O$, 38.75; mol wt, 240.3. Found: C, 60.06; H, 6.41; O, 33.13; CH_3O, 38.28; mol wt, 241.

Further elution of the initial chromatography column with ether and ethyl acetate brought off a 12.7-g fraction (mol wt 1055), 50% of the material recovered, and two subsequent fractions totaling 4.7 g.

D. Characterization of Pyrazolines. The major (12.7 g) fraction from the preceding chromatogram was rechromatographed on Florisil; elution with benzene and ether brought off only traces of free **2c**. Subsequent elution with ethyl acetate gave the major fraction, 8.0 g (mol wt 854). The mass spectrum of this material changed significantly as the inlet probe temperature of the CH-7 instrument was increased. At all probe temperatures (137–207°) an ion m/e 240 (4.2–5.8) corresponding to **2c** and ions m/e 222 (38–51), 181 (11–38), and 151 (100) were present. At 160–170° parent ions m/e 470 (2.9–4.8) and 472 (0.7–0.8) corresponding to **5b** and **6a** were observed. The former showed a fragment ion m/e 411 (2.8–4.7), metastable m/e 359.5 (calcd for 470 → 411: m/e 359.5), while the latter showed a fragment ion m/e 250 (5.1–8.9, M − 222, McLafferty). At temperatures of 171–207° the mass spectrum showed a weak parent ion corresponding to **5a**, m/e 678 (0.6–0) with fragment ions m/e 650 (11–1.2), 636 (0–2.7), 618 (0.5–3.1), 590 (1.2–6.4), and 351 (24–32). The large fragment ions correspond to the loss of 28 (N_2), 42 (CH_2N_2?), 60 (N_2 and MeOH, the latter loss occurring by the opening of the coumaran ring with reclosure to a lactone), and 88 (N_2, MeOH, and C=O; or N_2 and HCOOMe). All of these large fragment ions show McLafferty rearrangements to ions at F − 222.

E. Saponification of Pyrazolines. A 2.0-g portion of the rechromatographed fraction (mol wt 854) was refluxed with 2.0 g of KOH in 20 ml of 50% aqueous EtOH under N_2 for 15 hr. After removal of the EtOH under reduced pressure, the alkaline solution was extracted with ethyl acetate, acidified with dilute HCl, and reextracted with ethyl acetate. The acids were reesterified with diazomethane and worked up to yield 1.29 g of neutral product. Chromatography on Florisil and distillation of the early chromatography fractions as before gave, after one recrystallization from ether, 340 mg of (*R*)-**2c**, mp 62–63°. Later chromatographic fractions were examined by MS and were found to contain some **5b** (m/e 470.175; exact mass calcd for $C_{26}H_{26}N_2O_8$, 470.169), **6b** (m/e 264, etc.), and other products. Hydrolyzable adducts **5a** and **6a** were not observed.

F. Reduction of Pyrazolines. Another portion of the rechromatographed fraction (mol wt 854, 2.0 g) was treated with 0.5 g of $LiAlH_4$ in 25 ml of THF. After an initial vigorous reaction, the solution was refluxed for 5 hr. The cooled reaction mixture was cautiously treated with ethyl acetate, water, and dilute HCl. The aqueous solution was extracted with three portions of ethyl acetate. The dried extract yielded 2.1 g of brown liquid on concentration. Chromatography on Florisil and elution with benzene–ether gave early fractions which crystallized from ligroin–ether. Four recrystallizations from benzene–ether gave shining needles of (*R*)-**8**: mp 86.5–87°; ^{1}H NMR (60 MHz, $CDCl_3$) δ 2.66 (d, J = 7 Hz,

$ArCH_2$), 3.02 (s, broad, 2 OH, moves with D_2O), 3.55 (m, 3 H, $OCHCH_2O$), 3.84 [s, 6 H, $Ar(OCH_3)_2$], 6.76 (s, C_6H_3).

Anal. Calcd for $C_{11}H_{16}O_4$: C, 62.25; H, 7.60. Found: C, 62.06; H, 7.38.

Acetylation of (*R*)-2c. (*R*)-**2c** (60 mg) was treated in 1 ml of pyridine and 2 ml of ether with 0.25 ml of acetyl chloride and 0.30 ml of acetic anhydride, at first in an ice bath, then at room temperature for 5 hr. The product was washed until neutral and distilled at 120–130° (bath) (0.02 mm) to yield 54.3 mg of viscous liquid (*R*)-**2d:** ^{1}H NMR (60 MHz, $CDCl_3$) δ 2.08 (s, $COCH_3$), 3.02 (d, $\Delta\delta$ = 2 Hz) and 3.13 (s) (J_{gem} unobserved, 2 H, $ArCH_2$), 3.72 (s, CO_2CH_3), 3.86 (s, 6 H, $ArOCH_3$), 5.20 (2 d, J = 6, 8 Hz, 1 H, CHOAc), 6.76 (s, C_6H_3).

Anal. Calcd for $C_{14}H_{18}O_6$: C, 59.56; H, 6.43. Found: C, 60.11; H, 6.43.

Saponification of (*R*)-2d. (*R*)-**2d** (56 mg) was heated under reflux in an N_2 atmosphere for 2 hr with 140 mg of KOH in 2 ml of 95% EtOH. The mixture was acidified with 2 *N* HCl and extracted with ethyl acetate, yielding 44.8 mg of brown gum. Treatment in MeOH with charcoal and passage through a short column of alumina, followed by crystallization from benzene–hexane, yielded **2e** in two crystalline forms: long, colorless needles, mp 93–93.5°, and cotton-like material, mp 101.5–102.5°. After standing for 10 years, the long needles melted at 101.5–102.5°. The mixture melting point with synthetic (*R*)-**2e** was undepressed with both samples.

Methyl 3-(3,4-Dimethoxyphenyl)lactate [(*RS*)-2c]. Methyl 3-(3,4-dimethoxyphenyl)glycidate[49] (34 g, mp 63–64°) was hydrogenated in 300 ml of ethyl acetate over 1.6 g of 5% palladium on carbon at 3 atm. After 3 hr the solution was filtered and concentrated to an oil. The oil was dissolved in 50 ml of ethyl acetate–hexane (3:2), and it crystallized slowly after seeding [with crystals of (*RS*)-**2c** obtained by methylation of acid (*RS*)-**2e** with diazomethane] to give 24.3 g (70%) of (*RS*)-**2c:** mp 46.5–48° (lit.[18c] mp 54–55°); ir and ^{1}H NMR same as those of (*R*)-**2c.**

Methyl 2-Acetoxy-3-(3,4-dimethoxyphenyl)propanoate [(*RS*)-2d]. Crude (*RS*)-**2c** from the hydrogenation of 12.2 g of methyl 3-(3,4-dimethoxyphenyl)glycidate was dissolved in 15 ml of pyridine and 15 ml of acetic anhydride was added with chilling. After standing overnight the solution was concentrated at 10 mm, and the residue was dissolved in ether and washed with 2 *N* HCl, water, saturated $NaHCO_3$, and water. The oil obtained after drying and concentration of the solution crystallized from 18 ml of MeOH to give 11.1 g (73%) of (*RS*)-**2d:** mp 57–58°; ^{1}H NMR same as that of (*R*)-**2d;** MS *m/e* (rel intensity) 282 (18), 251 (1), 222 (100), 207 (6), 191 (11), 181 (3), 151 (89); exact mass, 282.1102 (calcd for $C_{14}H_{18}O_6$, 282.1105).

3-(3,4-Dimethoxyphenyl)lactic Acid [(*RS*)-2e]. Crude (*RS*)-**2c** (25 g) was dissolved in 50 ml of chilled MeOH, and a solution of 5.8 g of NaOH in 100 ml of water was added portionwise with stirring. After being stirred overnight at room temperature, the solution was concentrated under reduced pressure, and more water was added. The alkaline solution was washed twice with CH_2Cl_2, acidified to pH 1 with concentrated HCl, and extracted three times with ethyl acetate. The solvent was removed, and the solid product was taken up in a minimum of boiling MeCN. On cooling, (*RS*)-**2e** precipitated in large crystals (13 g, 57%): mp 121–123° (lit.[18c] mp 123–124°); ^{1}H NMR (100 MHz, $CDCl_3$) δ 3.02 (octet) [2.97 (d, $\Delta\delta$ = 7 Hz) and 3.07 (d, $\Delta\delta$ = 4 Hz), J_{gem} = 14 Hz, 2 H, $ArCH_2$], 3.82 (s, 6 H, $ArOCH_3$), 4.45 (2 d, J = 4, 7 Hz, CHOH), 6.77 (s, C_6H_3).

Resolution of 2e. A solution of 5.2 g of (*RS*)-**2e** in 105 ml of warm MeCN was treated with 2.8 g of (−)-α-methylbenzylamine (Aldrich). After chilling and rewarming of the solution, crystallization ensued to give 3 g of the salt, mp 137–141°. Five recrystallizations from MeCN gave 2.03 g of constant-melting crystals, mp 143–145.5°, $[\alpha]^{26}D$ + 29.4° (*c* 2.0, MeOH).

The salt (178 mg) was dissolved in dilute HCl, and the solution was extracted four times with ethyl acetate. After drying, the solvent was removed and the residue was solidified. The chilling of a toluene solution gave cottony crystals of (*R*)-**2e:** mp 102–103°; $[\alpha]^{26}D$ +29.0° (*c* 1.3, $CHCl_3$); uv (MeOH) 285 nm (ϵ 2590), 279 (3100), 230 (10,500); CD (MeOH) 287 (+0.04), 271 (−0.04), 233 (−1.90), 214 (−1.23), 196 (ca. +14).

Pentamethyl Rosmarinate [(*RS*)-4b]. A solution of 5.22 g (25.1 mmol) of **7a** and 6.08 g (31.9 mmol) of *p*-toluenesulfonyl chloride in 60 ml of dry pyridine under N_2 was warmed to 80°. The solution colored red-orange on warming, and an orange acylpyridinium salt precipitated while the temperature was maintained for 3 hr. The thick slurry was cooled and 0.46 g (6.8 mmol) of imidazole was added. After 30 min the temperature was 30°; 4.80 g (20 mmol) of (*RS*)-**2c** in 15 ml of pyridine was added. After stirring for 19 hr the solution was poured into 250 ml of 2 *N* HCl. The mixture was extracted four times with ether. The ether layer was washed with 2 *N* HCl and water, and was dried, filtered, and concentrated to give 8.7 g of brown oil. Crystallization from ether–isopropyl ether during 2 days gave 5.48 g of **4b,** mp 81–82.5°. A second crop from ether (0.99 g) brought the overall yield to 77%. Three recrystallizations from ethyl acetate–hexane gave an analytical sample: mp 82–83°; ir (KBr) 5.77, 5.84 (C=O), 6.15 μ (C=C); uv (MeOH) 325 nm (ϵ 22,900), 296 (16,700), 286 (16,100), 260 (5200), 233 (22,000), 217 (21,600); ^{1}H NMR (220 MHz, $CDCl_3$) δ 3.15 (m, CH_2), 3.72, 3.82, and 3.83 (3 s, 9 H, OCH_3), 3.88 (s, 6 H, OCH_3), 5.34 (2 d, J = 5, 8 Hz, CH), 6.30 (d, J = 16 Hz, H-8), 6.72–6.80 (m, H-13, -16, -17), 6.83 (d, J = 8 Hz, H-5), 7.01 (d, J = 2 Hz, H-2), 7.06 (dd, J = 2, 8 Hz, H-6), 7.62 (d, J = 16 Hz, H-7).

Anal. Calcd for $C_{23}H_{26}O_8$: C, 64.17; H, 6.09. Found: C, 64.31; H, 6.17.

3-Methoxycarbonyl-4-(3,4-dimethoxyphenyl)-2-pyrazoline (6b). To a chilled solution of 16 g (67 mmol) of **7b** in 100 ml of ether was added 175 ml of ca. 0.1 *M* ethereal diazomethane[48b] in portions in an ice bath. The reaction flask, fitted with a drying tube, was allowed to stand at 0° for 2 hr, and was stored at room temperature for 12 hr. The excess diazomethane was destroyed by the dropwise addition of 12% acetic acid in ether. The white precipitate was filtered to give 11.6 g (65%) of **6b:** mp 111–114°; ir (KBr) 2.95 (s, NH), 5.86 (s, C=O), 6.42 μ (m); uv (MeOH) 287 nm (ϵ 11,700), 248 (2300), 225 (10,000); ^{1}H NMR (60 MHz, $CDCl_3$) δ 3.83 (s, CO_2CH_3), 3.96 (s, 6 H, $ArOCH_3$), 3.9–4.6 (m, 4 H, $CHCH_2$, NH), 6.90 (s, C_6H_3); MS *m/e* (rel intensity) 264 (81), 236 (16), 205 (16), 204 (31), 177 (26), 176 (100); exact mass, 264.1117 (calcd for $C_{13}H_{16}N_2O_4$, 264.1110).

3-(3,4-Dimethoxyphenyl)-1,2-propanediol [(*RS*)-8]. A solution of 0.5 g of (*RS*)-**2c** in 18 ml of ether was added dropwise to 0.20 g of $LiAlH_4$ in 7 ml of ether and the slurry was refluxed for 2 hr. The reaction mixture was decomposed with wet $(NH_4)_2SO_4$ and water, concentrated, extracted three times with ethyl acetate, and concentrated to 0.33 g of a viscous liquid. Distillation at 145–155° (bath) (0.03 mm) gave a liquid which was chromatographed on alumina (III) with ether–ethyl acetate–EtOH. Crystals from later chromatographic fractions gave on recrystallization from benzene–ether (*RS*)-**8,** mp 63–64°, ir and ^{1}H NMR identical with those of (*R*)-**8.**

Anal. Calcd for $C_{11}H_{16}O_4$: C, 62.25; H, 7.60. Found: C, 62.25; H, 8.01.

Base-Catalyzed Methylation of F3. Five grams of F3 was stirred at reflux in 600 ml of dry acetone with 21 ml of freshly distilled dimethyl sulfate and 35 g of anhydrous K_2CO_3. After 18 hr the solid F3 had nearly all dissolved, and the solution was filtered. The solid was washed with acetone, and the combined filtrate was concentrated. The resulting oil was agitated with water at room temperature for several hours and extracted with ethyl acetate. The extract was washed with $NaHCO_3$ solution and with water, dried, and then evaporated under reduced pressure to a neutral oil (5.8 g). Chromatographic fractionation on Florisil (50 g) with benzene–ether–ethyl acetate as eluents was followed by refractionation on both Florisil and alumina (II) columns. Substance **9a** was found in many of the fractions. A sample of ca. 90% purity by ^{1}H NMR criteria had the following spectral properties: ir (KBr) 5.69 (m) and 5.85 (s, ester C=O), 6.12 μ (w, C=C); uv (MeOH) (ϵ calcd using mol wt 650) 315 nm (29,200), 288 (sh, 23,500), 263 (11,100), 233 (31,500); ^{1}H NMR, see Table VI.

The material, a mixture of disastereomers, is not crystalline, but forms a flaky solid on being freed from solvent.

Anal. Calcd for $C_{35}H_{38}O_{12}$: C, 64.61; H, 5.89; O, 29.51; mol wt, 650.7. Found: C, 65.34; H, 5.73; O, 29.02; mol wt, 623, 650.

α-(2,3-Dimethoxyphenyl)-*trans*-3,4-dimethoxycinnamic Acid (10). A solution of 6.33 g (32.6 mmol) of 2,3-dimethoxyphenylacetic acid and 5.34 g (32.2 mmol) of 3,4-dimethoxybenzaldehyde in 12 ml of acetic anhydride–triethylamine (2:1) was refluxed for 7 hr. The reaction mixture was hydrolyzed with water and concentrated HCl and was extracted thoroughly with CH_2Cl_2. The combined organic extracts were washed three times with 2 *N* NaOH and the basic solution was washed twice with CH_2Cl_2. Acidification and reextraction into CH_2Cl_2 gave after drying and concentration an oil which crystallized spontaneously. Recrystallization from 50 ml of MeCN gave, on chilling overnight and partial evaporation of the solvent, two crops of **10,** 3.52 g (31%), mp 186–190°. Several other runs of this condensation in more dilute solutions of acetic anhydride gave significantly lower yields of **10.** Three recrystallizations from MeCN gave the analytical sample: mp 189–191°; uv (MeOH) 315 nm (ϵ 18,000), 288 (16,700), 218 (sh, 27,000).

Anal. Calcd for $C_{19}H_{20}O_6$: C, 66.26; H, 5.86. Found: C, 66.33; H, 5.86.

α-Phenyl-*trans*-3,4-dimethoxycinnamic Acid (11a). After being refluxed (ca. 160°) for 15 hr, a solution of 33 g (0.02 mol) of 3,4-dimethoxybenzaldehyde and 40 g (0.29 mol) of phenylacetic acid in 125 ml of acetic anhydride–triethylamine (4:1) was cooled to 90° while 100 ml of water was added slowly at that temperature.[34a] The mixture was cooled slowly to room temperature, and the precipitate was filtered and washed twice with 80% acetic acid. The crude product was boiled in acetic acid (in which it was only slightly soluble), filtered, and washed with MeCN to give after air drying 36 g (60%) of **11a:** mp 229–231° (lit.[50] mp 228°); uv (MeOH) 315 nm (ε 18,000), 293 (15,300), 238 (sh, 13,500), 213 (sh, 21,600).

Ethyl α-Phenyl-*trans*-3,4-dimethoxycinnamate (11b). A mixture of 2.04 g (7.2 mmol) of acid **11a** in 3.5 ml of thionyl chloride and 15 ml of $CHCl_3$ was refluxed until all of the solid had dissolved (3 hr). The volatiles were removed under reduced pressure, and 5 ml of EtOH was added. After the exothermic reaction, the solution was filtered, chilled, and scratched to give **11b,** 1.68 g (75%), in two crops: mp 86–87.5°; ir (melt) 5.86 (C=O), 6.19 μ (C=C); exact mass, 312.138 (calcd for $C_{19}H_{20}O_4$, 312.1361).

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Registry No.—**1a,** 54814-35-6; **1b,** 54844-34-7; (*R*)-**2c,** 54814-41-4; (*R,S*)-**2c,** 54910-31-5; (*R*)-**2d,** 54910-35-9; (*RS*)-**2d,** 54814-42-5; (*R*)-**2e,** 54844-37-0; (*RS*)-**2e,** 54910-33-7; (*R*)-**2e** α-methylbenzylamine, 54844-38-1; **3b,** 54814-36-7; **4a,** 20283-92-5; (*R*)-**4b,** 54814-43-6; (*RS*)-**4b,** 54910-34-8; **6b,** 54814-37-8; **7a,** 14737-89-4; **7b,** 30461-77-9; (*R*)-**8,** 54910-32-6; (*RS*)-**8,** 54844-36-9; **9a,** 54844-35-8; **9b,** 54814-38-9; **10,** 54814-39-0; **11a,** 36854-32-7; **11b,** 54814-40-3; methyl 3,4-(dimethoxyphenyl)glycidate, 39829-15-7; (−)-α-methylbenzylamine, 2627-86-3; *p*-toluenesulfonyl chloride, 98-59-9; 2,3-dimethoxyphenylacetic acid, 90-53-9; 3,4-dimethoxybenzaldehyde, 120-14-9; benzeneacetic acid, 103-82-2.

References and Notes

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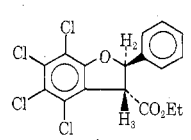

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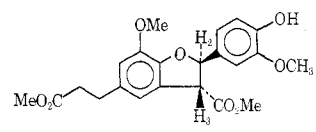
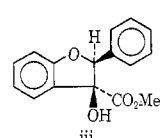


ii

was based on the lack of strong shielding of either methoxycarbonyl group, δ 3.67 and 3.80 ($CHCl_3$).[24] In an analysis of the ^1H NMR spectrum of a diastereomeric mixture of iii and iv,[25] the *O*-methyl resonance



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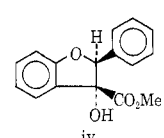

iv

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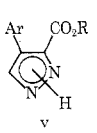
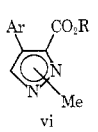


v



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Structure Determination of the *N*-Methyl Isomers of 5-Amino-3,4-dicyanopyrazole and Certain Related Pyrazolo[3,4-*d*]pyrimidines

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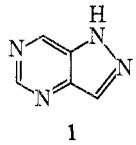
The position of N substitution of certain substituted 4-aminopyrazolo[3,4-*d*]pyrimidine derivatives has been studied by chemical and spectroscopic techniques and has resulted in the assignment of structures to the pyrazole precursors of these compounds. The more abundant pyrazole resulting from treatment of tetracyanoethylene with methylhydrazine [identical with the single pyrazole isomer originally isolated by the same condensation procedure, C. L. Dickinson, J. K. Williams, and B. C. McKusick, *J. Org. Chem.*, **29**, 1915 (1964), which was not characterized definitively with respect to the position of the N substituent] has thus been assigned as 3-amino-4,5-dicyano-1-methylpyrazole on the basis of its conversion to a pyrazolo[3,4-*d*]pyrimidine identical with authentic 4-amino-2-methylpyrazolo[3,4-*d*]pyrmidine, rather than with the authentic 1-methyl isomer. The assigned structure has been verified by X-ray crystallographic determination of 3-amino-4,5-dicyano-1-methylpyrazole. Because 3-amino-4,5-dicyano-1-methylpyrazole would not be expected to be the more abundant pyrazole on the basis of previous work, a mechanism is proposed which accounts for its formation. Also studied was the position of tautomeric equilibrium in 3-amino-4,5-dicyanopyrazole. A consideration of the ^{13}C NMR spectrum of 3-amino-4,5-dicyanopyrazole, relative to those of 5-amino-3,4-dicyano-1-methylpyrazole and 3-amino-4,5-dicyano-1-methylpyrazole, as well as the *N*-acetyl derivatives of all three, indicated that the major tautomer was 5-amino-3,4-dicyano-1*H*-pyrazole. A comparison of the ultraviolet spectrum of this pyrazole with those of the two methylated isomers led to the same conclusion.

The considerable biological and medicinal activities of substituted pyrazolo[4,3-*d*]pyrimidines (1) and pyrazolo[3,4-*d*]pyrimidines (2) as adenine analogs and antagonists has



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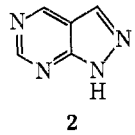

2

contributed to the interest in the pyrazoles from which they are derived synthetically. Of special concern for many years has been a description of the position of N substitution in such pyrazoles. This information is usually not available by simple consideration of the reaction scheme by which a pyrazole is synthesized and appropriate methods for differentiation between such isomeric species are frequently less than obvious.

The question of the position of tautomeric equilibrium in pyrazoles which are not N substituted (e.g., **14a** ⇌ **14b**) has also been the subject of several studies,[1-3] as has the existence of individual tautomers as discrete substances.[4,5] Although the position of tautomeric equilibrium has been determined for several compounds by the use of molecular refractions or NMR spectroscopy, and the use of ultraviolet spectroscopy could be envisioned along similar lines, there is no well-established general method for such determinations.

This report is concerned with the chemical and spectroscopic determination of the position of N substitution in the isomeric N-methylated 5-amino-3,4-dicyanopyrazoles and compounds derived therefrom, as well as with the position of tautomeric equilibrium in these pyrazoles.

Results and Discussion

The difficulty in assigning correct structures to N-1- or N-2-substituted pyrazolo[3,4-*d*]pyrimidines can be attributed directly to the lack of available structural information concerning their pyrazole precursors. Substantial effort was expended in early studies in an attempt to prepare and characterize pyrazoles of authentic structure, including methods involving ring closures,[6,7] alkylations,[8,9] and selective dealkylations.[8,10,11] Subsequent studies, however, have rendered questionable many of the preparations of "authentic" samples. In addition, although at least three NMR studies have dealt with the problem of differentiating between isomeric pyrazoles,[2,3,12] the reported methods are not applicable in the present case.

A consideration of the mechanistic routes suggested in the literature for the formation of related pyrazoles illustrates the source of structural ambiguity in the formation of **3** and **4** from methylhydrazine and tetracyanoethylene. One might, for example, envision formation of the compound assigned structure **3** by conjugate addition of the more nucleophilic substituted nitrogen of methylhydrazine