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ISOLATION AND IDENTIFICATION OF "DIAZEPAM-LIKE" COMPOUNDS FROM BOVINE URINE

KIN-CHUN LUK,* LORRAINE STERN, MANFRED WEIGELE,

Chemical Research Department, Hoffmann-LaRoche, Inc., Nutley, NJ

ROBERT A. O'BRIEN, and NENA SPIRT

Pharmacology II Department, Hoffmann-LaRoche, Inc., Nutley, NJ 07110

ABSTRACT.—Attempts to isolate the putative endogenous ligand for the benzodiazepine receptor from bovine urine resulted in the identification of three isoflavans: equol (**1**), 3′,7-dihydroxyisoflavan (**2**), and 4′-hydroxy-7-methoxyisoflavan (**9**), as "diazepam-like" compounds. 3-Chloro-9H-carbazole (**17**) was found to enhance the binding of diazepam in the benzodiazepine receptor binding assay. Pinosylvine monomethyl ether (**18**), indigo (**20**), and indirubin (**21**) were isolated as inactive compounds.

Many drugs acting on the central nervous system are believed to do so *via* membrane-bound receptor sites. In fact, specific receptors for the benzodiazepine-type anxiolytic agents have been demonstrated in synaptosomal preparations obtained from the brains of a number of mammalian species (1-5). The logical corollary, that there should also exist endogenous ligands for such receptors (6), has stimulated many attempts to find endogenous "diazepam-like" compounds (DLCs) (7-18).

Several groups have reported on the isolation of inosine, hypoxanthine, and nicotinamide from brain tissues, and on the interaction of these substances with the benzodiazepine receptor (7-15). However, the concentrations of these compounds, which cause 50% inhibition of specific ^{3}H-diazepam binding (IC_{50}) in a benzodiazepine receptor binding assay (BRBA) (3), are relatively high (*ca.* 1 mM). Thus, it is unlikely that any of them represents the natural ligand for the benzodiazepine receptor. Additional workers have presented their evidence for the existence of DLCs in brain tissues without identifying structural details (11, 16-18). The isolation of β-carboline-3-carboxylic acid ethyl ester from human urine and various mammalian brain tissues has been reported (19, 20). However, the utilized isolation procedure makes it likely that this potent BRBA inhibitor (IC_{50} 7 nM) is an artifact.

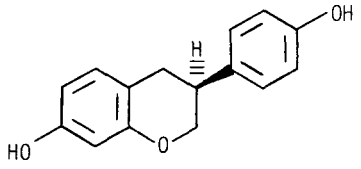
The present report describes our own attempts to isolate and identify ligands for the benzodiazepine receptor from bovine urine. For the purposes of this account, substances that competitively inhibit ^{3}H-diazepam binding in the BRBA (3) shall be termed DLCs.

RESULTS AND DISCUSSION

During the course of this study, four compounds active in the BRBA, compounds A through D, were isolated, together with an inactive compound E, and two pigments, F and G. Of the four active compounds, three (A-C) were DLCs, while compound D increased the binding of diazepam.

Bovine urine was collected and extracted with a neutral, nonionic resin, Amberlite XAD-2 (21), in a batchwise operation to adsorb most of the organic substances. The adsorbed material was washed off the resin with chloroform-methanol to provide a crude extract. With this procedure it was possible to obtain crude material from a large volume of urine more conveniently than with a column-type operation (22). The crude extract was chromatographed, monitoring with BRBA (3) (see the Experimental Section), to give the various pure compounds.

Compound A was shown to be *S*-(−)-equol (**1**) by spectroscopic data. Equol was first isolated from mare's urine (23). Its structure was elucidated in 1935 (24), while more recently its absolute configuration was determined to be *S* (25-27).



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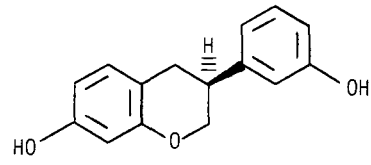

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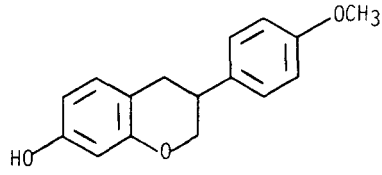

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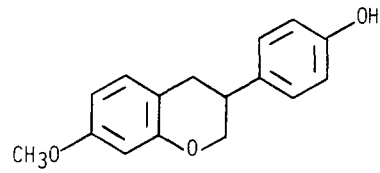

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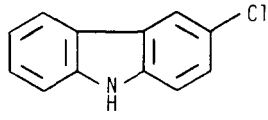

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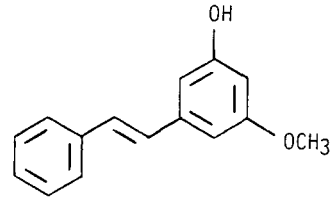

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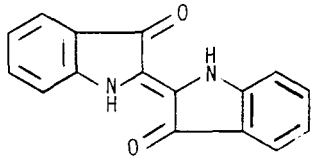

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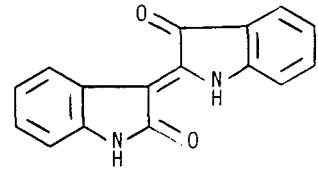

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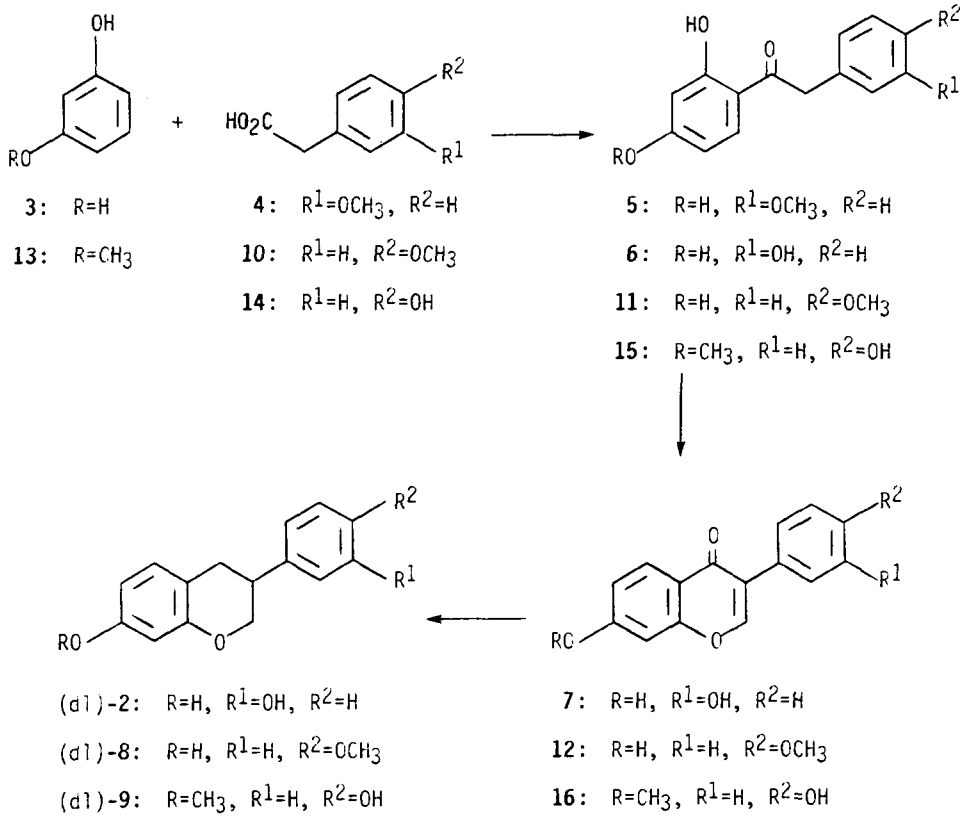
Compounds B and C were shown to be two new isoflavans. Compound B was isomeric with equol. The only difference between its proton-nmr spectrum and that of equol was the replacement of the *para*-substitution pattern of ring B of equol by a *meta*-substitution pattern. Thus, compound B was assigned the structure of 3',7-dihydroxyisoflavan (**2**). This assignment was confirmed by total synthesis (see Scheme 1, **3**+**4**↦**5**↦**6**↦**7**↦**2**). Inasmuch as the isolated **2** and *S*-(−)-equol (**1**) have virtually identical ord spectra, the absolute configuration of these compounds must be the same.

From spectroscopic data compound C was identified as a monomethyl ether of equol. Unambiguous syntheses (**3**+**10**↦**11**↦**12**↦**8**; **13**+**14**↦**15**↦**16**↦**9**) of both possible monomethyl ethers, **8** and **9**, allowed the assignment of structures **9** to C. This is so, despite the fact that biosynthetic relationships (28-30) would favor structure **8**. Since only a small amount of **9** was isolated, its chirality was not studied.



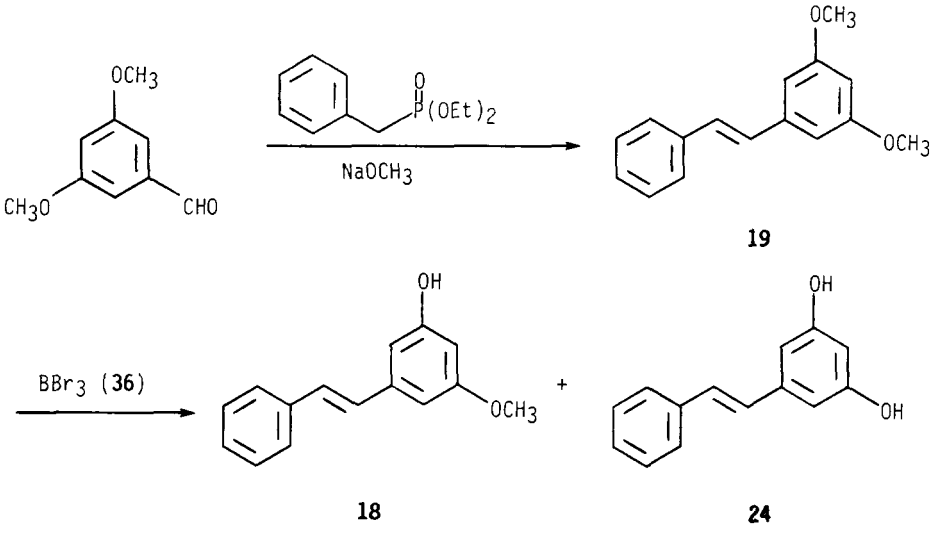
SCHEME 1. Synthesis of Isoflavans

Compound D, which enhanced the binding of diazepam in the BRBA, was obtained only in a very small amount. Spectroscopic examination suggested the material to be 3-chloro-9H-carbazole (**17**). This was confirmed by comparison with a synthetic sample of **17** (31), which was identical in all respects to D, including its activity in the BRBA.

Compound E was also isolated only in very small amounts. A sample of it, which was judged to be more than 90% pure by hplc, also enhanced diazepam binding in the BRBA. Spectroscopic data identified compound E as pinosylvine monomethyl ether (**18**), which was first isolated from the heartwood of pine (32). In order to confirm this structure and to obtain enough material for biological studies, **18** was synthesized via 3,5-dimethoxystilbene (**19**) according to scheme 2. The synthetic pinosylvine monomethyl ether (**18**) and compound E were identical spectroscopically and chromatographically, but synthetic **18** was totally devoid of activity in the BRBA. Thus, the originally observed activity was not due to **18** but to some minor impurity. Because only minute amounts were available, attempts to identify this enhancement factor had to be abandoned.

Pigments F and G were isomeric compounds. Their isolation was easily followed by monitoring their color: compound F is blue, while G is reddish-purple. They were identified as indigo (**20**) and indirubin (**21**), respectively, by their spectroscopic data. These structure assignments were confirmed by comparing compound F with a commercial sample of indigo and compound G with a sample of synthetic indirubin (33). Both indigo and indirubin had been isolated from mammalian urine before (34).

The IC_{50} values of the various isoflavans and isoflavones in BRBA are summarized in table 1. Although the isolated isoflavans are inhibitors of diazepam binding in the



SCHEME 2. Synthesis of Pinosylvine Monomethyl Ether

BRBA 10-20 times more potent than inosine, hypoxanthine, and nicotinamide, they presumably originate in the diet and are, thus, precluded from consideration as endogenous ligands for the benzodiazepine receptor. At concentrations of 0.5 and 1.0 mM in the BRBA, 3-chloro-9H-carbazole (**17**) caused an increase in the amount of ^{3}H-diazepam binding to 143% and 172% of the control, respectively. To the best of our knowledge carbazole derivatives have not been isolated from mammalian tissues before, and the origin of this compound is mysterious. On the other hand, it is interesting that the action of **17** in the BRBA is similar to that of γ-aminobutyric acid (GABA), and the GABAmimetic drugs (35-44), (1-ethyl)-4-(isopropylidene-hydrazino)-1H-pyrazolo-[3,4-b]-pyridine-5-carboxylic acid ethyl ester hydrochloride, and (4-butylamino)-1-ethyl-1H-pyrazolo[3,4-b]pyridine-5-carboxylic acid ethyl ester (45). Further biological studies with **17** are underway and will be reported elsewhere.

TABLE 1. Activity of isoflavans, isoflavones, and some standard compounds in the benzodiazepine receptor binding assay (3).

Compound	Approximate IC_{50} (M)
Diazepam	5×10^{-9}
S-(−)-Equol (**1**)	80×10^{-6}
dl-3′,7-Dihydroxyisoflavan (dl-2)	45×10^{-6}
dl-4′-Hydroxy-7-methoxyisoflavan (**9**)	1.3×10^{-3}
dl-7-Hydroxy-4′-methoxyisoflavan (**8**)	$>1.5\times10^{-3}$
Formononetin (**12**)	$>700\times10^{-6}$
4′-Hydroxy-7-methoxyisoflavone (**16**)	1.1×10^{-3}
3′,7-Dihydroxyisoflavone (**7**)	140×10^{-6}
Inosine	900×10^{-6} (12)
Hypoxanthine	700×10^{-6} (12)
Nicotinamide	3.9×10^{-3} (12)

From hindsight, the usefulness of urine as a starting material for the isolation of endogenous receptor ligands is questionable. It is likely that exogenous compounds are encountered as well, and furthermore, endogenous materials may have undergone metabolic changes before excretion. Caution must also be exercised in interpreting re-

sults obtained when using competitive binding assays (*e.g.*, receptor binding assay, radioimmunoassay), as a means of tracking progress of the isolation. Until one has the pure substance, it is not possible to know whether the observed activity is due to a potent compound or the presence of a large quantity of a marginally active substance. To be absolutely certain, one should, whenever possible, compare the biological activity of an isolated material with that of synthetic substance to confirm that the activity resides in the assigned structure and not in a minor contaminant. Lastly, to give real meaning to activity data obtained from receptor binding assays, a ligand should also possess significant *in vivo* pharmacological activity (agonist or antagonist). The DLCs reported here did not have such activity.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were taken on a Kofler hot stage melting point apparatus and are uncorrected. Infrared (ir) spectra were recorded on a Digilab FTS 14 spectrometer. Mass spectra (ms) were obtained on a Varian MAT CH5 spectrometer. Proton nmr spectra were obtained with a Varian XL-100 instrument and are reported in ppm downfield from internal tetramethylsilane. Ultraviolet (uv) spectra were recorded on a Cary-14 spectrophotometer and optical rotatory dispersion (ord) spectra were measured on a Jasco J-20 spectrophotometer.

ISOLATION OF METABOLITES.—Female bovine urine was collected through implanted indwelling Foley catheters connected with plastic urine collection bags. After collection, the pH was adjusted to between 9.0 and 9.5 with concentrated ammonium hydroxide, and the urine was allowed to stand at room temperature overnight. The precipitate formed was removed by filtration. Amberlite XAD-2 resin was added to this filtrate (20 g resin/liter of urine), and the suspension stirred at room temperature for 24 h. The resin was then collected by filtration, washed with distilled water, and extracted in a Soxhlet extractor with methanol-chloroform mixture (4:1, v/v) overnight. On concentrating the organic extract, a black oily residue of about 190 mg/liter of urine was obtained. This residue was then suspended in methanol (20 g/100 ml) and filtered to give a methanol soluble fraction and a residue. The residue (about 100 mg, from several batches) was chromatographed on a silica gel column (100 g silica gel, 5% ethyl acetate in chloroform as solvent). The two colored bands were collected and evaporated to dryness to give indigo (**20**, 4 mg) and indirubin (**21**, 12 mg), identical in all respects with authentic materials.

The methanol soluble fraction was chromatographed on a Sephadex LH20 column (5 cm×190 cm, approximately 1 kg of resin) using methanol as solvent and chromatographing about 20 g each time, with a flow-rate of 4 ml/min and 24 ml/tube. Every fifth tube from the LH20 column was analyzed by BRBA using 20 μl of the tube.

Benzodiazepine receptor binding assays were performed according to the modified procedure of H. Möhler and T. Okada (3), using 50 mM Tris buffer, pH 7.4, instead of Krebs buffer. The region that was active in the BRBA, approximately tubes 200 to 330, was analyzed for the presence of equol, (**1**), by analytical hplc using a μBondapak-C_{18} reverse phase column, 3.9 mm id×30 cm, Waters Associates, 1.5 ml/min flow-rate, using a 15-min linear gradient from 50% H_2O in CH_3OH to 100% CH_3OH, monitoring at 254 nm with a uv detector. [When the fractions were allowed to stand, equol (**1**) crystallized above the liquid level in the test-tubes of some fractions. This crude material was used as a chromatographic standard for analyzing the various fractions].

Contents of the test tubes were combined into three major fractions: fraction IIIA, containing equol (**1**) (approximately tubes 260-300); fraction IIIB (approximately 200-259); and fraction IIIC (approximately 301-330). Each of the fractions were concentrated to give solid residues: (fraction IIIA: 1.3 g, IIIB: 0.5 g, and IIIC: 0.3 g).

Fraction IIIA (1.3 g) was chromatographed on a C_{18} reverse phase column (1″ od×4′, Separations Lab) using a 130-min linear methanol-water (1:1, v/v) to methanol gradient, with a flow-rate of 20 ml/min and collecting 5 min/fraction. [The gradient was generated as follows: 2 two liter flasks were connected with a stopcock. Flask A was equipped with a magnetic stirrer and contained 1.2 liters of methanol-water (1:1, v/v). Flask B contained 1.4 liters of methanol. The solvent gradient was generated by opening the stopcock and allowing pure methanol to flow into flask A and was mixed while solvent was being continuously pumped out from flask A.] After 20 fractions the solvent was switched to methanol. A total of 30 fractions (IIIA-1 to IIIA-30) were collected. Fractions IIIA-4 to IIIA-8 were combined and concentrated. The residues obtained from two runs were combined and recrystallized from aqueous methanol to give 2.2 g of a crystalline material, which was a mixture of 49:1 (by hplc) equol (**1**) and 3′,7-dihydroxyisoflavan (**2**). This mixture, when redissolved in methanol, treated with charcoal, and fractionally crystallized from aqueous methanol, gave 1.96 g of pure equol (by hplc).

S-(−)-*Equol* (**1**).—Recrystallized from chloroform as white needles, mp 189°; ir (KBr) 3540, 3420, 1615, 1595, 1515, 1210, 1155 cm^{-1}; ms *m/z* (rel. int.) 242 (M^+, 55), 135 (27), 123 (82), 120 (100), 107 (35), 91 (23); nmr ($CDCl_3$+DMSO-d_6) δ 2.86 (m, 2, Ar-CH_2-), 3.06 (m, 1, ArC*H*-), 3.88 (dd, 1, J=10 and 10, OCH_2), 4.22 (dd, 1, J=10 and 4, OCH_2), 6.35 (s, 1, Ar*H*), 6.42 (m, 1, Ar*H*), 6.80 (A_2 part of A_2B_2 system, 2, Ar*H*), 6.84 (m, 1, Ar*H*), 7.06 (B_2 part of A_2B_2 system, 2, Ar*H*), 8.44 (s, 2, O*H*); λ max (CH_3OH) 203 nm (ε 92440), 225 (sh, 28310), 280 (5080), 283 (5080), 289 (sh, 3750), (0.1 N HCl) 221 (17670), 280 (4550), (0.1 *N* KOH) 240 (22290), 294 (6730); ord (CH_3OH) 292 nm ([Φ] −3500), 282 (0), 273 (3000), 255 (1000), 242 (3250), 237 (0), 208 (−50,000), 204 (0); $[\alpha]_D$−22.78° (CH_3CH_2OH; C 0.74).

Anal. calcd for $C_{15}H_{14}O_3$. C, 74.36; H, 5.82. Found: C, 74.42; H, 5.81.

The mother liquor from the fractional crystallization of equol (**1**) was evaporated to dryness and chromatographed using a μBondapak-C_{18} reverse phase column [methanol-water (9:11, v/v), 1.5 ml/min flow-rate, approximately 1 mg residue/run, total of 40 runs] to give equol (**1**, 31.3 mg, retention time 14.4 min), and 3′,7-dihydroxyisoflavan (**2**, 8.4 mg, retention time 17.2 min).

3′,7-Dihydroxyisoflavan (**2**).—Crystallized from chloroform-hexanes, mp 126-129°; ir (KBr) 3320, 1617, 1600, 1508, 1153, 838, 785, 694 cm^{-1}; high resolution ms found 242.0957 (M^+) [calcd for $C_{15}H_{14}O_3$ 242.0944]; nmr ($CDCl_3$) δ 2.92 (m, 2, ArCH_2), 3.17 (m, 1, Ar-C*H*), 3.97 (m, 1, O-CH_2CH), 4.30 (m, 1, O-CH_2CH), 4.80 (br s, 2, O*H*), 6.40 (m, 2, Ar*H*), 6.60-7.00 (m, 4, Ar*H*), 7.10-7.30 (m, 1, ArH); λ max (CH_3OH) 222 nm (sh, ε 18760), 276 (sh, 4860), 282 (5630), and 290 (sh 3490); ord (CH_3OH) 295 nm ([Φ] −3250), 278 (0), 270 (1837), 258 (1555), 241 (3533), 237 (0), 215 (−28264).

Fractions IIIA-10 through IIIA-13 were combined and concentrated to give a residue of 86 mg, which was redissolved in 2 ml of methanol and chromatographed by hplc (μBondapak C_{18} column CH_3OH-H_2O (3:2, v/v), 1 ml/min flow-rate with 20 μl injection each). Eight fractions were collected. 4′-Hydroxy-7-methoxyisoflavan (**9**) was eluted out with a retention time of 19.7 min, while pinosylvine monomethyl ether (**18**) was eluted at 23.8 min.

4′-Hydroxy-7-methoxyisoflavan (**9**) *0.3 mg from 27 injections.*—The data are: ir ($CHCl_3$) 3600, 1620, 1585, 1520, 1510, 1160 cm^{-1}; ms *m/z* (rel. int.) 256 (M^+, 38), 137 (100), 120 (38); nmr ($CDCl_3$) δ 2.92 (m, 2, Ar-CH_2), 3.09 (m, 1, Ar-C*H*), 3.76 (s, 3, OCH_3), 3.95 (m, 1, OCH_2), 4.28 (m, 1, OCH_2), 4.69 (s, 1, O*H*), 6.45 (m, 2, Ar*H*), 6.7-7.2 (m, 5, Ar*H*); λ max (CH_3OH) 224 nm (ε 19600), 279 (5150), 283 (5080), and 289 (sh, 4100).

Pinosylvine monomethyl ether (**18**) *0.9 mg from 27 injections.*—The data are: ir ($CHCl_3$) 3600, 1640, 1610, 1598, 1150 cm^{-1}; ms *m/z* 226 (M^+, 100), 211 (16), 194 (20); nmr ($CDCl_3$) δ 3.75 (s, 3, OCH_3), 5.30 (s, 1, O*H*), 6.32 (m, 1, Ar*H*), 6.57 (m, 1, Ar*H*), 6.63 (m, 1, Ar*H*), 6.97 (s, 2, C*H*=C*H*), and 7.2-7.5 (m, 5, Ar*H*); λ max (CH_3OH) 211 nm (ε 24680), 228 (sh, 18530), 234 (sh, 17650), 300 (28890), and 308 (28640).

Fraction IIIC (1.0 g) was chromatographed as in the case of fraction IIIA to give fractions IIIC-1 to IIIC-30. Fraction IIIC-13 was evaporated to dryness to give 19.7 mg of a solid residue. This residue was chromatographed on a Bondapak-C_{18} reverse phase column (⅜″ od×8′, methanol-water, 3:2 v/v, 6 ml/min flow-rate and 3.5 min/tube). Tubes 69 to 81 were combined and evaporated to dryness to give 1.36 mg of a residue, which was redissolved in 1 ml of methanol and chromatographed using a μBondapak-C_{18} reverse phase column (3.9 mm, od×30 cm, Waters Associates, acetonitrile-water 11:9, v/v, 1.5 ml/min flow-rate, 90 μl per run) to give 3-chloro-9-H-carbazole (**17**, 0.47 mg, retention time 13.1 min).

3-Chloro-9H-carbazole (**17**).—The data are: ir (KBr) 3410, 1475, 1450, 1440, 1275, 1245, 814, 747 cm^{-1}; ms *m/z* (rel. int.) 203 (33), 201 (M^+, 100), 166 (67); nmr ($CDCl_3$) δ 7.18-7.50 (m, 5), 7.90-8.15 (m, 3); λ max (CH_3OH) 215 nm (ε 2030), 223 (2010), 229 (2075), 236 (2200), 246 (1230), 260 (1120), 291 (sh, 600), 297 (865), 319 (sh, 135), 330 (180), and 343 (150).

SYNTHESES.—1-(2,4-Dihydroxyphenyl)-2-(3-methoxyphenyl)ethanone (**5**). Boron trifluoride gas was bubbled into a suspension of resorcinol (5.5 g, 50 mM) and *m*-methoxyphenylacetic acid (8.31 g, 50 mM) in 1,2-dichloroethane (50 ml) with constant stirring magnetically. After a gain of 5 g in weight, the gas inlet tube was removed, and the reaction mixture was heated at 60° for 2 h under an argon atmosphere. The reaction mixture was then cooled to room temperature, poured into water (400 ml) containing sodium acetate trihydrate (25 g), and stirred at room temperature for 30 min. This suspension was extracted with EtOAc (2×400 ml). Each of the EtOAc layers was washed with saturated aqueous $NaHCO_3$, then combined and dried with $MgSO_4$. After removing the solvent under reduced pressure, the residue was dissolved in CH_3OH, treated with activated charcoal, and crystallized by adding water. These crystals were then recrystallized from CH_3OH-CH_2Cl_2-hexanes to give **5** (8.18 g, 63% yield); colorless plates: mp 109-110°; ir ($CHCl_3$) 3585, and 1632 cm^{-1}; ms *m/z* (rel. int.) 258 (M^+, 8), 137 (100), 121 (3), 109 (3); nmr ($CDCl_3$) δ 3.75 (s, 3, OCH_3), 4.13 (s, 2, CH_2CO), 5.71 (br s, 1, O*H*, D_2O exchangeable), 6.35 (m, 2, Ar*H*), 6.80 (m, 3, Ar*H*), 7.23 (m, 1, Ar*H*), 7.71 (m, 1, Ar*H*), 12.62 (s, 1, O*H*); λ max (CH_3OH) 213 nm (ε 26300), 238 (sh, 8000), 280 (15900), 317 (9350) and λ max (0.1 *N* KOH) 253 nm (sh, 8400), 280 (sh, 4500), 334 (18750).

Anal. calcd for $C_{15}H_{14}O_4$: C, 69.76; H, 5.46. Found: C, 69.81; H, 5.52.

1-(2,4-Dihydroxyphenyl)-2-(3-hydroxyphenyl)ethanone (**6**).—Methyl ether **5** (3.76 g, 14.5 mM) was dissolved in glacial acetic acid (80 ml) and concentrated HBr (20 ml) and the mixture heated at reflux under an argon atmosphere for 4 h. After cooling to room temperature, the reaction mixture was poured into water (400 ml) and extracted twice with ether (400 ml each). Each of the ether layers was washed with saturated aqueous $NaHCO_3$ and then combined and dried ($MgSO_4$). The solvent was removed under reduced pressure and the residue crystallized from aqueous methanol to give **6** (3.14 g, 89% yield): mp 214-216°; ir (KBr) 3250, 1633 cm^{-1}; ms *m/z* (rel int.) 244 (M^+, 6), 137 (100); nmr ($CDCl_3$+DMSO-d_6) 4.05 (s, 2, CH_2CO), 6.35 (m, 1, Ar*H*), 6.38 (m, 1, Ar*H*), 6.73 (m, 3, Ar*H*), 7.10 (m, 1, Ar*H*), 7.65 (m, 1, Ar*H*), 8.59 (s, 1, O*H*), 9.84 (s, 1, O*H*), 12.65 (s, 1, O*H*); λ max (CH_3OH) 212 nm (ε 23150), 230 (sh, 9800), 278 (14600), 315 (8200).

Anal. calcd for $C_{14}H_{12}O_4$: C, 68.84; H, 4.95. Found: C, 68.58; H, 4.96.

1-(2,4-Dihydroxyphenyl)-2-(4-methoxyphenyl)ethanone (**11**).—A magnetically stirred suspension of resorcinol (5.52 g, 50 mM) and *p*-methoxyphenylacetic acid (8.30 g, 50 mM) in $BF_3 \cdot Et_2O$ (50 ml) was heated at 100° under an argon atmosphere for 1 h. (By then, the reactants had completely dissolved to form a red solution.) After cooling to room temperature, the reaction mixture was poured into water (500 ml) containing NaOAc·3 H_2O (69 g). This aqueous suspension was extracted with CH_2Cl_2 (2×500 ml), and each CH_2Cl_2 layer was washed with saturated aqueous $NaHCO_3$, combined, and dried ($MgSO_4$). The solvent was removed under reduced pressure, and the residue crystallized from $CHCl_3$ to give, in two crops, crystalline **11** (9.83 g, 77%); white crystals: mp 153-155°; ir (KBr) 3360, 1618, 1243 cm^{-1}; ms *m/z* (rel. int.) 258 (M^+, 9), 137 (100), 121 (13); nmr ($CDCl_3$+DMSO-d_6) 3.74 (s, 3, OCH_3), 4.08 (s, 2, $COCH_2$), 6.37 (m, 2, Ar*H*), 6.84 (m, 2, Ar*H*), 7.15 (m, 2, Ar*H*), 7.66 (m, 1, Ar*H*), 9.85 (s, 1, O*H*), 12.63 (s, 1, O*H*); λ max (CH_3OH) 213 nm (ε 24400), 217 (sh, 24150), 229 (sh, 16200), 239 (sh, 8000), 278 (16050), 318 (9400).

Anal. calcd for $C_{15}H_{14}O_4$: C, 69.76; H, 5.46. Found: C, 69.52; H, 5.46.

1-(2-Hydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl)ethanone (**15**).—BF_3 gas was bubbled into a suspension of *m*-methoxyphenol (**13**, 6.20 g, 50 mM) and *p*-hydroxyphenylacetic acid (**14**, 7.60 g, 50 mM) in 1,2-dichloroethane (50 ml) with magnetic stirring until the gain in weight was 5.74 g. The gas inlet tube was removed and the reaction mixture heated at 80° under an argon atmosphere for 2 h. After cooling at room temperature, the reaction mixture was poured into water (500 ml) containing NaOAc·3 H_2O (25 g) and extracted with EtOAc (2×500 ml). Each of the EtOAc layers was washed with saturated aqueous $NaHCO_3$ (500 ml), then combined and dried ($MgSO_4$). The solvent was evaporated under reduced pressure, and the residue was chromatographed on silica gel (600 g) using first EtOAc-CH_2Cl_2 (1:9, v/v), then EtOAc-CH_2Cl_2 (3:7, v/v) as solvent. A by-product, 1-(2-hydroxy-4-methoxyphenyl)-2-(4-ethoxyphenyl)ethanone (**22**), eluted off first, followed by the desired **15**, then by 1-(2-methoxy-4-hydroxyphenyl)-2-(4-hydroxyphenyl)ethanone (**23**).

Compound **22**.—White crystals (0.32 g, 2%) from dichloromethane-hexanes: mp 95-97°; ir ($CHCl_3$) 1630 cm^{-1}; ms *m/z* (rel. int.) 286 (M^+, 9), 151 (100), 107 (12); nmr ($CDCl_3$) δ 1.37 (t, 3, *J*=6, OCH_2CH_3), 3.79 (s, 3, OCH_3), 3.97 (q, 2, *J*=6, OCH_2CH_3), 4.10 (s, 2, $COCH_2Ar$), 6.41 (m, 2, Ar*H*), 6.84 (m, 2, Ar*H*), 7.14 (m, 2, Ar*H*), 7.71 (m, 1, Ar*H*), 12.70 (s, 1, O*H*); λ max (CH_3OH) 213 nm (ε 24000), 217 (23800), 228 (sh, 18800), 238 (sh, 8730), 276 (16300), 317 (8270).

Anal. calcd for $C_{17}H_{18}O_4$: C, 71.31; H, 6.34. Found: C, 71.18; H, 6.52.

Compound **15**.—White crystals (6.08 g, 47%) from dichloromethane-hexanes: mp 151-154°; ir (KBr) 3470, 3300, 1635, 1232 cm^{-1}; ms *m/z* (rel. int.) 258 (M^+, 5), 151 (100), 108 (9). 107 (14), 95 (15), 77 (17); nmr ($CDCl_3$) δ 3.78 (s, 3, OCH_3), 4.08 (s, 2, OCH_2), 4.82 (s, 1, O*H*), 6.41 (m, 2, Ar*H*), 6.76 (m, 2, Ar*H*), 7.09 (m, 2, Ar*H*), 7.70 (m, 1, Ar*H*), 12.68 (s, 1, O*H*); λ max (CH_3OH) 212 nm (ε 25300), 227 (sh, 17100), 275 (16600), 316 (8550).

Compound **23**.—White crystals (2.96 g, 23%) from dichloromethane-hexanes: mp 147-151°; ir (KBr) 3300, 1650, 1640 cm^{-1}; ms *m/z* (rel. int.) 258 (M^+, 2), 151 (100); nmr ($CDCl_3$+DMSO-d_6) δ 3.80 (s, 3, OCH_3), 4.10 (s, 2, OCH_2), 6.41 (m, 2, Ar*H*), 6.73 (m, 2, Ar*H*), 7.00 (m, 2, Ar*H*), 7.63 (m, 1, Ar*H*), 8.06 (s, 1, O*H*), 9.29 (s, 1, O*H*); λ max (CH_3OH) 211 nm (ε 2000), 224 (17600), 270 (11320), 304 (8300).

Formononetin (**12**).—A suspension of **11** (5.16 g, 20 mM) in *t*-butoxy-bis(dimethylamino)methane (46) (50 ml) was heated at 50° overnight; by then, the suspended material had completely dissolved. After cooling to room temperature, the reaction mixture was poured into water (100 ml) and acidified with concentrated HCl. The precipitate so formed was collected and recrystallized from 95% ethanol in two crops to give **12** (4.30 g, 75%) as white needles: mp 260-263°; ir (KBr) 3140, 1638, 1621, 1608, 1597 cm^{-1}; ms *m/z (rel. int.)* 268 (M^+, 100), 253 (17), 225 (9), 132 (69), 117 (14), 63 (13); nmr (DMSO-d_6) δ 3.75 (s, 3, OCH_3), 6.90 (m, 4, Ar*H*), 7.47 (m, 2, Ar*H*), 7.93 (m, 1, Ar*H*), 8.29 (s, 1, O-C*H*=), and 10.50 (br s, 1, O*H*); λ max (CH_3OH) 240 nm (sh, ε 25700), 249 (28450), 260 (sh, 25700), 301 (10920).

Anal. calcd for $C_{16}H_{12}O_4$: C, 71.63; H, 4.51. Found: C, 71.44; H, 4.70.

(dl)-7-Hydroxy-4'-methoxyisoflavan (**8**).—Formononetin (**12**, 1.34 g, 5 mM) was hydrogenated over 10% Pd-C (250 mg) in glacial acetic acid (250 ml) at room temperature and atmospheric pressure overnight. The catalyst was removed by filtration and washed with methanol. The combined filtrate was evaporated under reduced pressure and the residue recrystallized from chloroform-hexanes to give racemic **8** (1.18 g, 92%) as white crystals: mp 160-161°; ir ($CHCl_3$) 3410, 1610, 1595, 1515 cm^{-1}; ms *m/z* (rel. int.) 256 (M^+, 30), 134 (100); nmr ($CDCl_3$) δ 2.89 (m, 2, ArCH_2) 3.10 (m, 1, Ar$CH$$CH_2$), 3.76 (s, 3, O$CH_3$), 3.93 (m, 1, O$CH_2$CH), 4.25 (m, 1, O$CH_2$CH), 4.75 (br s, 1, O*H*), 6.36 (m, 2, Ar*H*), 6.90 (m, 3, Ar*H*); λ max (CH_3OH) 225 nm (ε 20420), 278 (sh, 4200), 283 (4820), 289 (sh, 3220).

Anal. calcd for $C_{16}H_{16}O_3$: C, 74.98; H, 6.29. Found: C, 74.67; H, 6.39.

3',7-Dihydroxyisoflavone (**7**).—To the solution of ethanone **6** (1.0 g, 4 mM) in DMF (5 ml) was added *t*-butoxy-bis(dimethylamino)methane (46) (5 ml). The reaction mixture was stirred at room temperature for 1 h then poured into water (40 ml). After acidifying with concentrated HCl, the precipitate formed was collected and recrystallized from aqueous ethanol to give **7** (0.93 g, 89%) as white prisms: mp 286-288°; ir (KBr) 3350, 3145, 1620, 1585, 1445, 1385, 1280, 1245 cm^{-1}; ms *m/z* (rel. int.) 254 (M^+, 100), 253 (75), 226 (13), 225 (10), 197 (13), 137 (43), 136 (19), 118 (23), 108 (19), 79 (14); nmr (DMSO-d_6) δ 6.66-7.60 (m, 6, Ar*H*), 7.95 (d, 1, *J*=8, Ar*H*), 8.30 (s, 1, O-C*H*=), 9.36 (br s, 1, O*H*), 10.70 (br s, 1, O*H*); λ max (CH_3OH) 218 nm (ε 32400), 245 (23600), 295 (11200), 307 (sh, 10000).

Anal. calcd for $C_{15}H_{10}O_4$: C, 70.86; H, 3.96. Found: C, 70.69; H, 4.15.

(dl)-3',7-Dihydroxyisoflavan (**2**).—Isoflavone **7** (500 mg, 1.97 mM) was dissolved in glacial HOAc (50 ml) and hydrogenated over 10% Pd-C (150 mg) at atmospheric pressure and room temperature overnight. The catalyst was then filtered off and the residue washed with CH_3OH. The combined filtrate was evaporated to dryness under reduced pressure to give a white solid, which was crystallized from aqueous methanol to give **2** (408 mg, 86%) as white needles: mp 145-147°; with spectroscopic data, except ord, identical to that of the natural product.

Anal. calcd for $C_{15}H_{14}O_3$: C, 74.36; H, 5.82. Found: C, 74.20; H, 5.81.

4'-Hydroxy-7-methoxyisoflavone (**16**).—To the solution of ethanone **15** (3.0 g, 11.6 mM) in DMF (15 ml) was added *t*-butoxy-bis(dimethylamino)methane (15 ml), and the mixture was stirred at room temperature under an argon atmosphere for 45 min. The reaction mixture was then poured into water (120 ml) and the aqueous solution was acidified with concentrated HCl. The precipitate formed was collected and recrystallized from aqueous ethanol to give **16** (2.82 g, 91%) as white needles: mp 225-227°; ir (KBr) 3220, 1626 cm^{-1}; ms *m/z* (rel. int.) 268 (M^+, 100), 267 (54), 151 (65), 118 (22); nmr ($CDCl_3$+DMSO-d_6) δ 3.87 (s, 3, OCH_3), 6.80-7.04 (m, 4, Ar*H*), 7.36 (m, 2, Ar*H*), 7.89 (s, 1, O-C*H*=), 8.14 (d, 1, *J*=9, Ar*H*), 8.76 (s, 1, O*H*); λ max (CH_3OH) 235 nm (sh, 20900), 248 (24100), 260 (24300), 300 (sh, 9700).

Anal. calcd for $C_{16}H_{12}O_4$: C, 71.63; H, 4.51. Found: C, 71.62; H, 4.59.

(dl)-4'-Hydroxy-7-methoxyisoflavan (**9**).—Isoflavone **16** (1.34 g, 5 mM) was hydrogenated over 10% Pd-C in glacial acetic acid (250 ml) overnight. The reaction mixture was then filtered and the residue washed with methanol. The combined filtrate was evaporated to dryness under reduced pressure, and the residue recrystallized from chloroform-hexanes to give racemic **9** (1.11 g, 87%): mp 150-151°; with spectroscopic properties identical to that of the isolated natural product.

Anal. calcd for $C_{16}H_{16}O_3$: C, 74.98; H, 6.29. Found: C, 74.70; H, 6.29.

3,5-Dimethoxystilbene (**19**).—NaH (50% in oil, 8.8 g, 185 mM) was washed twice with pentane under an argon atmosphere, then suspended in dry THF (30 ml). To this suspension was added dropwise a solution of diethyl benzylphosphonate (47) (38.4 g, 168 mM) in THF (70 ml), with external cooling in an ice bath to maintain the reaction temperature below 15°, followed dropwise by methanol (6 ml, 148 mM). The reaction mixture was then allowed to warm to room temperature and stirred for 90 min. To this was then added a solution of 3,5-dimethoxybenzaldehyde (16.62 g, 100 mM) in THF (80 ml) dropwise, again with cooling in an ice bath. When all the aldehyde had been added, the ice bath was removed and the mixture stirred at room temperature for 42 h. Water (200 ml) was then added, followed by anhydrous K_2CO_3 (10 g), and the two layers were separated. The aqueous layer was extracted with ether (3×400 ml). The combined ether THF layers were dried ($MgSO_4$), the solvent was removed under reduced pressure and the residue dissolved in CH_3OH, treated with charcoal, and then crystallized from aqueous CH_3OH to give **19** as white needles (19.6 g, 82%): mp 55-56°; ir ($CHCl_3$) 2965, 2945, 1636, 1593, 1154 cm^{-1}; ms *m/z* (rel. int.) 240 (M^+, 100), 165 (32); nmr ($CDCl_3$) δ 3.77 (s, 6, OCH_3), 6.38 (t, 1, *J*=2, Ar*H*), 6.65 (d, 2, *J*=2, Ar*H*), 7.03 (s, 2, C*H*=C*H*), 7.02-7.56 (m, 5, Ar*H*); λ max (CH_3OH) 211 nm (ε 25200), 227 (19000), 235 (sh, 18400), 298 (29600), 306 (29300) and 325 (sh, 15500).

Anal. calcd for $C_{16}H_{16}O_2$: C, 79.97; H, 6.71. Found: C, 80.15; H, 6.77.

Demethylation of **19**.—To a solution of **19** (16.68 g, 69 mM) in CH_2Cl_2 (250 ml) at room temperature was added BBr_3 (16.56 g, 66 mM) in CH_2Cl_2 (50 ml) dropwise. The mixture was heated at reflux for 3 h, then stirred at room temperature overnight. Water (200 ml) was added, and the mixture was partitioned between saturated aqueous NaCl (400 ml) and CH_2Cl_2-CH_3OH (9:1, v/v, 200 ml). The two layers were separated, and the aqueous layer further extracted with CH_2Cl_2-CH_3OH (9:1, v/v, 2×300 ml). The or-

ganic layers were washed with saturated aqueous NaCl (200 ml) before combining and dried ($MgSO_4$). After removing the solvent under reduced pressure, the residue was chromatographed on the Waters' Prep 500 Chromatograph (two silica gel cartridges) using EtOAc-hexanes (3:17, v/v) as solvent to give **18** and **24**.

Compound **18**.—A colorless oil (5.48 g, 34.9%) which solidified on standing; ir ($CHCl_3$) 3600, 1640, 1610, 1598 cm^{-1}; ms *m/z* (rel. int.) 226 (M^+, 100), 225 (40), 165 (40); nmr ($CDCl_3$) δ 3.75 (s, 3, OCH_3), 5.30 (s, 1, O*H*), 6.32 (m, 1, Ar*H*), 6.57 (m, 1, Ar*H*), 6.63 (m, 1, Ar*H*), 6.97 (s, 2, $-CH=CH-$), 7.20-7.52 (m, 5, Ar*H*); λ max (CH_3OH) 211 nm (ε 24680), 228 (sh, 18530), 234 (sh, 17650), 300 (28890), 308 (28640).

Anal. calcd for $C_{15}H_{14}O_2$: C, 79.62; H, 6.24. Found: C, 79.39; H, 6.13.

Compound **24**.—Crystallized from CH_3OH-H_2O as white needles (5.37 g, 36.5%): mp 154-156°; ir (KBr) 3400, 3320, 1637, 1615, 1600, 1590, 1520, 750, 690 cm^{-1}; ms *m/z* (rel. int.) 212 (M^+, 100), 211 (43), 165 (37); nmr ($CDCl_3$+DMSO-d_6) δ 6.34 (t, 1, J=2, Ar*H*), 6.54 (d, 2, J=2, Ar*H*), 6.97 (s, 2, $-CH=CH-$), 7.10-7.50 (m, 5, Ar*H*), 8.15 (s, 2, O*H*); λ max (CH_3OH) 211 nm (ε 23800), 223 (17850), 235 (sh, 16800), 299 (28850), 308 (28600).

Anal. calcd for $C_{14}H_{12}O_2$: C, 79.22; H, 5.70. Found: C, 79.30; H, 5.65.

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