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EFFECT OF A MARINE ALGAL CONSTITUENT ON THE GROWTH OF LETTUCE AND RICE SEEDLINGS

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ABSTRACT.—The dibromocatechol α-*O*-methyllanosol [**1**], a methyl ether of lanosol, was isolated from both the MeOH and the CH_2Cl_2 extracts of *Odonthalia washingtoniensis* and *Odonthalia floccosa*. Its structure was confirmed through spectroscopic methods and through its synthesis. An investigation of its biological activities revealed that it exhibits a stimulating effect on the growth and elongation of certain terrestrial plants both in the in vivo and the in vitro systems.

Seaweeds have been reported from Roman times as a source of an agricultural fertilizer and are still used today in some areas of the world. While some of their utility is no doubt due to organic nutrients and inorganic minerals, algal constituents have been isolated that exhibit specific control over terrestrial plant growth. Both gibberellins and cytokinins have been isolated from algal sources. During our continuing search for natural products as a resource for biologically active compounds, we examined the extract of the red algae *Odonthalia washingtoniensis* Kylin and *Odonthalia floccosa* (Esper) Falk (Rhodophyta) for the presence of phytoactive components. Preliminary bioassays using lettuce and rice plants revealed that the MeOH extract of *O. washingtoniensis* and *O. floccosa* possessed potent growth stimulatory activity. We report the isolation, identification, and biological activity of α-*O*-methyllanosol (3,4-dibromo-5-methoxymethyl-1,2-benzenediol) [**1**].

RESULTS AND DISCUSSION

ISOLATION AND IDENTIFICATION OF A PLANT GROWTH STIMULATOR.—The MeOH and/or the CH_2Cl_2 extracts from the algae gave the biologically active compound which was recrystallized from C_6H_6 as colorless needles (mp 130°). The mass spectrum indicated it to be a dibromo compound, $C_8H_8O_3Br_2$ ($[M]^+$ at 310, 312, 314; rel. int. ratio 1:2:1). The ^{13}C-nmr spectrum indicated a penta-substituted benzene with absorptions at δ 145.1 (s), 143.8 (s), 129.2 (s), 114.6 (d), 113.5 (s), and 113.0 (s), a methoxy group at δ 57.7 (q), and a benzylic methylene group at δ 74.0 ppm (t). The ^1H-nmr spectrum showed the signals for an aromatic proton at δ 6.95 (s, 1H), a methoxy group at δ 3.32 (s, 3H), and a benzylic methylene group at δ 4.38 ppm (s, 2H). The 10.72% nOe of the aromatic proton, observed when the benzylic methylene group was irradiated at δ 4.38 ppm, indicated a close spatial relationship of these two groups. In spite of the possible proton exchange with the solvent (CD_3OD), the two signals from the two hydroxy protons were observed at δ 10.1 (s) and δ 9.58 ppm (s).

This implies a 1,2-relationship of the two hydroxy groups with a strong intramolecular hydrogen bond keeping the protons from exchanging with the solvent. From these spectra this compound was identified as α-*O*-methyllanosol, a compound previously isolated from other red algae [e.g., Odonthaliaceae (1,2), Polysiphoniaceae (3), and Rhodomelaceae (1,2,4)].

Because MeOH was used as the extraction solvent, the methyl group of **1** may have come from the solvent. However, the same compound was isolated in a better yield from the CH_2Cl_2 extract without using MeOH. Therefore α-*O*-methyllanosol of both *O. washingtoniensis* and *O. flocossa* is not an artifact, at least during the process of isolation.

3,4-Dibromo-5-methoxymethyl-1,2-benzenediol [**1**] was previously synthesized by Matsumoto and Kagawa (5). However, the experimental details were not reported. The interesting biological activities of **1** on plant growth prompted us to develop a more efficient synthetic method for the preparation of **1** and its derivatives.

SYNTHESIS OF [**1**].—An attempt to brominate 5-bromo-4-hydroxy-3-methoxybenzaldehyde [**2**] (6,7) with 2.1 equiv of Br_2, refluxing HOAc and using iron powder as a catalyst gave 5,6-dibromo-4-hydroxy-3-methoxybenzaldehyde [**3**] in a low yield of 27% (Figure 1). Neither a large excess of Br_2 nor an extended reaction time improved the yield. While the yield of **3** was low, it was seen as an improvement over the conventional reaction sequence of methylation, nitration, diazotization, and the Sandmeyer reaction. The structure of **3** was confirmed in part by the observation of an nOe enhancement of the C-6 aromatic proton signal (δ 7.44 ppm, 18.4%) when the methoxy methyl proton (δ 4.00 ppm) was irradiated. The ^{1}H-nmr spectrum of **3** showed the characteristic signals at δ 4.00 (OMe), 6.12 (OH), and 10.22 ppm (CHO) in agreement with the proposed structure. The ir spectrum confirmed the presence of a hydroxyl group (3200 cm^{-1}). Demethylation of **3** with BBr_3 gave an unstable catechol, which on immediate reaction with $MeOCH_2Cl$ and NaH gave a protected aldehyde **4**. Reduction of **4** with $NaBH_4$ followed by etherification with MeI and NaH gave **5**, which gave **1** when hydrolyzed with acid. The synthetic product **1** was shown to be identical with the natural compound by direct comparison of physical and spectral properties. Several derivatives of **1** were also synthesized, according to the scheme shown in Figure 2, in order to further investigate their potential for similar biological activity.

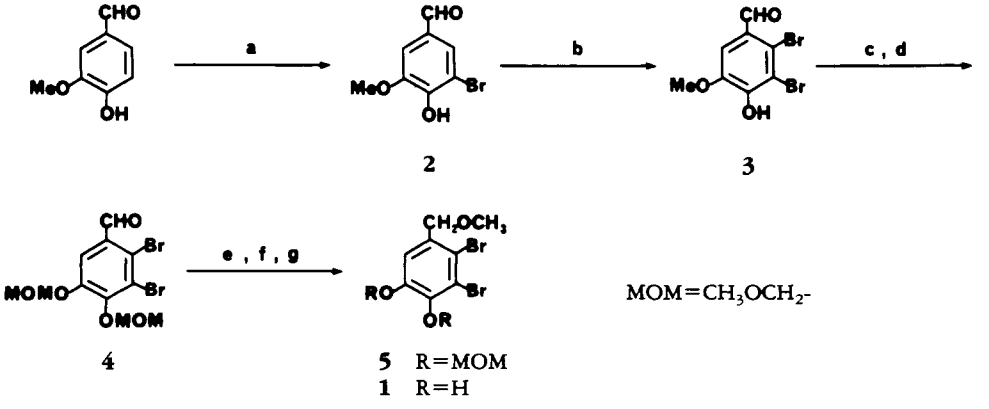

FIGURE 1. Scheme of the synthesis of **1**. (a) Br_2 (1 equiv)/HOAc/reflux; (b) BR_2 (2.1 equiv)/HOAc/Fe/reflux; (c) $BBr_3/CH_2Cl_2/-50°$ to ambient temperature; (d) $MeOCH_2Cl/NaH/THF/0°$; (e) $NaBH_4/EtOH/0°$; (f) MeI/NaH/THF/0° to ambient temperature; (g) concentrated HCl/MeOH/ambient temperature.

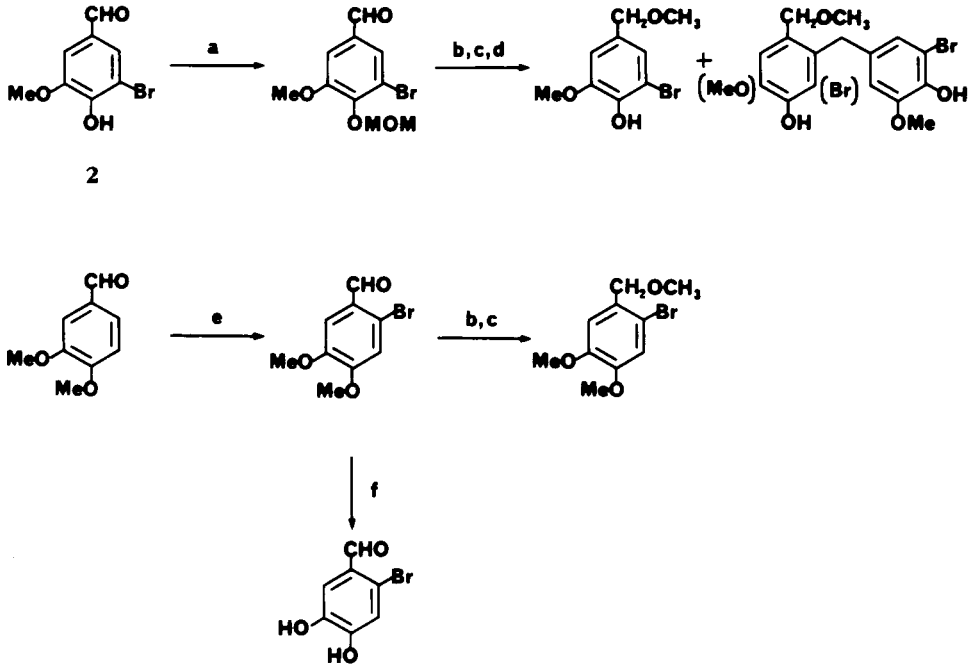

FIGURE 2. Scheme of the synthesis of several derivatives of **1**. (a) $MeOCH_2Cl/NaH/THF/Et_3N$; (b) $NaBH_4/EtOH$; (c) MeI/NaH/THF; (d) concentrated HCl/MeOH; (e) $Br_2/CHCl_3$; (f) BBr_3/CH_2Cl_2.

GROWTH STIMULATING EFFECT OF α-*O*-METHYLLANOSOL ON TERRESTRIAL PLANTS.—The presence of α-*O*-methyllanosol was observed to have little effect on rice seedlings even at the concentration of 100 ppm. However, as exhibited in Figure 3, the growth of lettuce hypocotyl and root were stimulated at this concentration. The overall appearance of greater health among the seedlings exposed to α-*O*-methyllanosol was striking. These seedlings were much larger than the seedlings in the control group and they appeared to be normal in all proportions. No inappropriate elongation was apparent in either the hypocotyl or the root of the lettuce seedlings. In addition, the hypocotyls of the exposed seedlings were of a much richer and deeper green color and the roots had more root hairs than the control group.

This is the first report on the effect of α-*O*-methyllanosol on terrestrial plants, although other related halogenated phenolics have been used as herbicides (8). It is well known that lettuce seedlings are inactive to indoleacetic acid (IAA) but active to gibberellins (GAs). Although the structure of α-*O*-methyllanosol seems to resemble synthetic auxins such as 2,4-D the results from the growth experiments with lettuce seedlings show gibberellin-like activity. Further work is now in progress to elucidate the mechanism of this growth regulator.

There are several possible explanations for the effects that α-*O*-methyllanosol has on plants. α-*O*-Methyllanosol may be a new class of plant growth regulators showing gibberellin-like activity. It is possible that some phenolic substances may be involved as algal growth regulators. The free benzyl alcohol lanosol, its sulfonate, and other simple phenols have been observed both to stimulate and to depress the growth of marine algae. Specifically, it has been reported that lower concentrations of lanosol strongly stimulated the growth of red algae (9). The presence of excreted phenolics has been implicated as necessary for the normal morphology and completion of the life cycle in algal species of *Ulva* and *Monostroma* (10). There is also the potential that these or similar

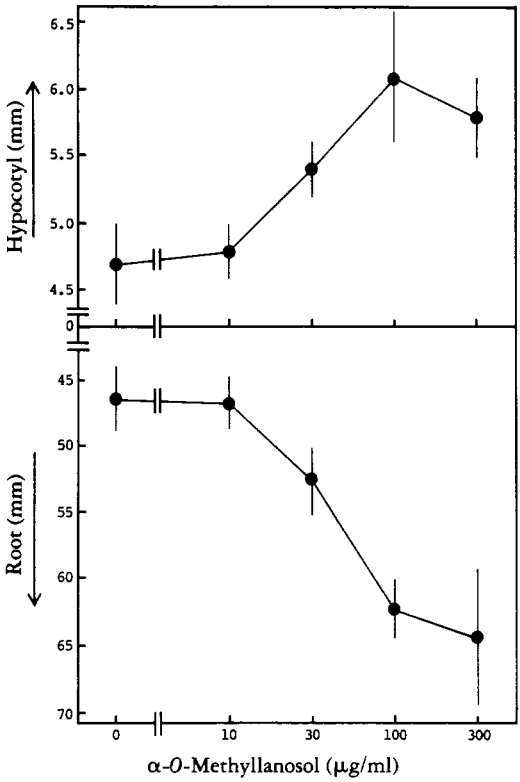

FIGURE 3. Effect of α-*O*-methyllanosol [**1**] on the growth of lettuce seedlings. Vertical lines represent standard error.

compounds may be used as antifouling agents. Many simple phenols exhibit antibacterial and antifungal activity, and it has been suggested that simple phenols may be involved in regulating the growth of epiphytes and parasites in algae (11).

The bioassay shows that the plant stimulation activity of IAA is ten times stronger than that of **1**. However, when **1** was exposed to the cultured cells of lettuce at 0.2 μg/ml, 38 ± 4% of the cells in the treated population had abnormal enlargement and elongation of the cells; this was not observed in the control population (Figure 4) (12).

EXPERIMENTAL

PLANT GROWTH EXPERIMENTS.—Growth experiments were carried out according to the method of Kamisaka (9). Seeds of lettuce (*Lactuca sativa* L., cv. Grand Rapids) were germinated on 2 layers of filter paper, moistened with distilled H_2O, and kept for 2 days under continuous fluorescent light (3000 lux at plant level) at 25.0 ± 0.5°. Rice seeds (*Oryza sativa* L., cv. Norin 20) were allowed to germinate in the dark at 30 ± 0.5°. After 2 days, 10 seedlings selected for uniformity were placed on 2 layers of filter paper in a 9-cm Petri dish containing 4 ml of test solution. Seedlings were allowed to grow under the same light and temperature conditions used for their germination. After 3 days of cultivation the lengths of the lettuce hypocotyl, the rice shoot, and their roots were measured and an average was taken of 30 seedlings from 3 Petri dishes.

INITIATION OF CELL CULTURE.—The cells used for this study were taken from an established cell line derived from the roots of *L. sativa* cv. Grand Rapids (Salinas M.T. Lot number 14620-105021), initiated and grown on Murashige and Skoog (MS) medium supplemented with 2 mg/liter of IAA and of kinetin. The roots from 2-day-old seedlings were surface-sterilized with 0.5% sodium hypochlorite (10% Clorox) for 20 min followed by incubation on the medium at 25° for 20 days with a 12-h photoperiod (3000 lux at plant level). The calluses were subcultured every 3 weeks, using MS media supplemented with 2 mg/liter of IAA and of kinetin in 8.0 g/liter agar.

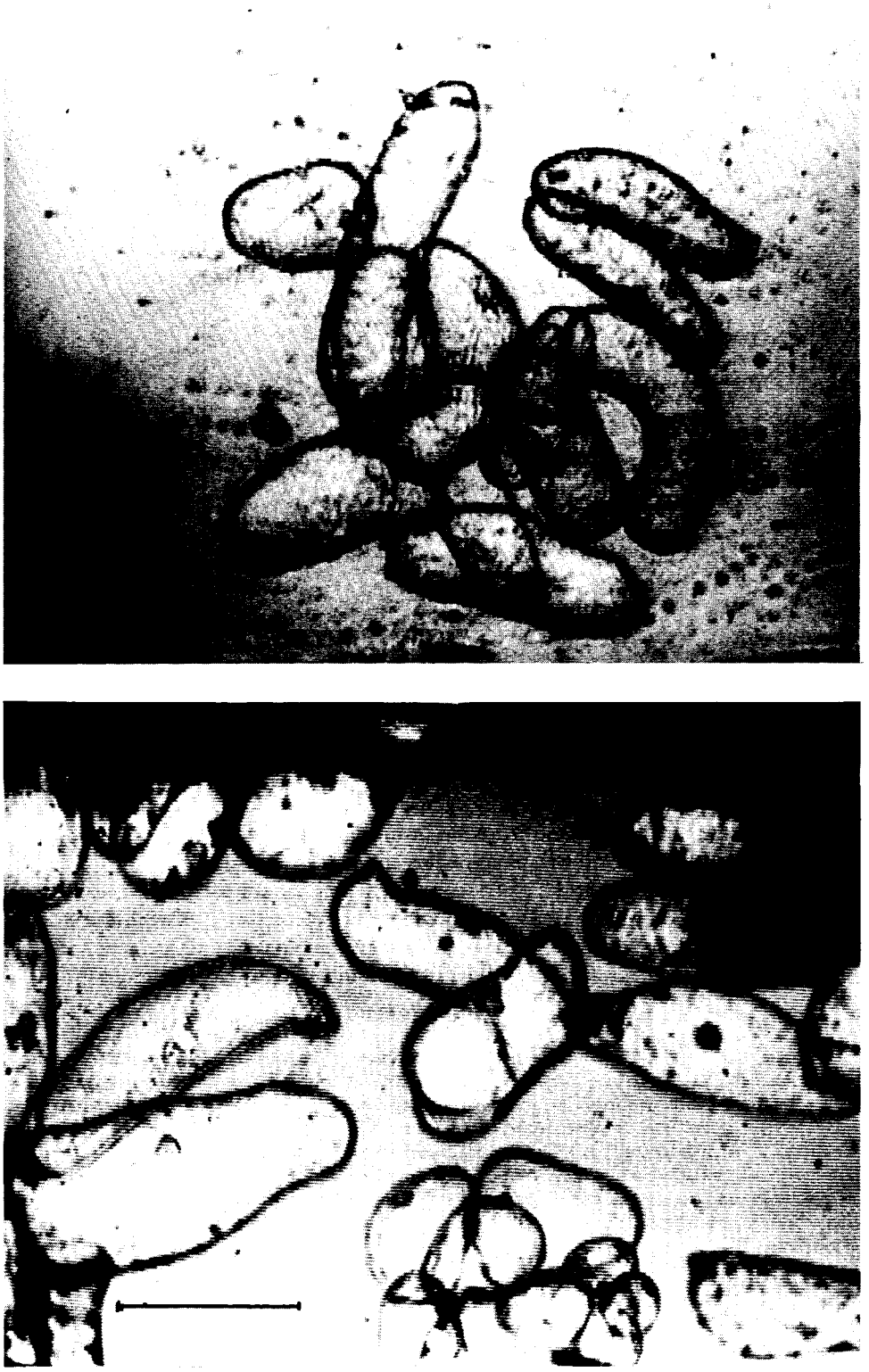

FIGURE 4. The cultured cells of lettuce (a) in the control population; (b) in the population treated with 0.2 μg/ml of α-*O*-methyllanosol [**1**].

BIOASSAY OF THE CELL CULTURE.—The test compound at final concentrations of 0.2, 2, 20, and 200 μg/ml (prepared by serial dilution; **1** was solubilized in MeOH) was incorporated into the media (MS, 0.8% Difco agar solidified). The control contained the same medium composition and was exposed to a 12-h photoperiod for 7 days. The tissues were always grown in agar-containing medium.

CELL MORPHOLOGICAL STUDIES.—The cells were introduced into a flask containing MS liquid medium, and a drop of the medium containing cells was placed on a microscope at 100 × magnification. The images of the cells were displayed on a TV monitor with the aid of a microscope and TV camera.

Each experiment had 8 replicates, and all experiments were repeated at least 3 times.

GENERAL EXPERIMENTAL PROCEDURE.—Melting points are uncorrected. Ir spectra were acquired on either a Perkin-Elmer 1310 ir or a Nippon Bunko A-100 ir Spectrometer. All nmr spectra were acquired on either a Nicolet GE 300 (300 MHz for ^1H, 75 MHz for ^{13}C), a JEOL FX-200 (200 MHz for ^1H, 50 MHz for ^{13}C), or a Hitachi R-600L (60 MHz for ^1H) spectrometer with signals reported in ppm downfield from internal TMS. High-resolution eims and fabms were acquired on a JEOL JMS-HX 100 spectrometer, and low resolution ms were taken on a JEOL DX 303 spectrometer. The chromatographic column adsorbents used were E. Merck Si gel (Type 60, particle size 0.040–0.063 mm) and Fuji Si gel (KC-2, 100–200 mesh). All reactions were monitored by tlc carried out on 0.25 mm E. Merck Si gel plates (60 F254) or on Macherey-Nagel Polygram SIL G/UV254 plates. Uv light, 7% phosphomolybdic acid in EtOH, or 1.5% vanillin in EtOH/concentrated H_2SO_4 was used as visualizing agent. THF was distilled from sodium benzophenone ketyl. CH_2Cl_2 was distilled from P_2O_5.

PLANT MATERIAL.—The red algae *O. washingtoniensis* and *O. floccosa* were collected at low tide from beaches in the vicinity of Trinidad Head, California. Within 2 h of collection the samples were placed in 3-liter bottles, covered with MeOH or CH_2Cl_2, and stored at ambient temperature.

EXTRACTION AND ISOLATION.—The above-mentioned material of *O. washingtoniensis* was extracted with MeOH. The aqueous MeOH solution was condensed by evaporation and extracted with CH_2Cl_2. The CH_2Cl_2 extract was separated on Si gel cc using $CHCl_3$ as eluent. The collected fractions were evaporated and recrystallized with C_6H_6. Finally, colorless prisms of **1** (241 mg from 560 g of *O. washingtoniensis*) were obtained. The direct extraction with CH_2Cl_2 of the algae followed by the same separation procedure gave **1** in a 50% yield of crude extract. The structure was confirmed with ^1H nmr and eims and was identified as the α-*O*-methyl ether of lanosol previously isolated from *O. floccosa* (1).

α-*O-Methyllanosol*.—Mp 130°; ^1H nmr (CD_3OD, 300 MHz) δ 3.32 (3H, s), 4.38 (2H, s), 6.95 (1H, s), 9.58 (1H, s), 10.10 (1H, s); ^{13}C nmr (CD_3OD, 75 MHz) δ 145.1 (s), 143.8 (s), 129.2 (s), 114.6 (d), 113.5 (s), 74.0 (t), 57.7 (q); low resolution eims (70 eV) *m/z* $[M]^+$ 310, 312, and 314 (rel. int. ratio 1:2:1), 279, 281 and 283 (rel. int. ratio 1:2:1), 231 and 233 (rel. int. ratio 1:1), 201 and 203 (rel. int. ratio 1:1).

SYNTHESIS OF **1**.—*5-Bromo-4-hydroxy-3-methoxybenzaldehyde* [**2**].—To a solution of vanillin (3.04 g, 0.02 mol) in 40 ml of $CHCl_3$ was added a solution of Br_2 (3.19 g, 0.02 mol) in 10 ml of $CHCl_3$. After the mixture had been refluxed for 1 h, the solvent was removed under reduced pressure, and the residue was recrystallized from EtOH to give 3.50 g of 5-bromovanillin (76%), mp 158–160°.

5,6-Dibromo-4-hydroxy-3-methoxybenzaldehyde [**3**].—To a solution of vanillin (0.938 g, 0.004 mol) in 5 ml of HOAc was added BBr_3 (1.3 g, 0.0081 mol) and iron powder (0.1 g). After the mixture had been refluxed for 1 h, the solvent was removed under reduced pressure. The residue was washed with $CHCl_3$. The remaining solid was recrystallized from C_6H_6-EtOAc (9:1) to give 0.335 g of pure 5,6-dibromovanillin: 27%; mp 234–235°.

2,3-Dibromo-4,5-bis(methoxymethoxyloxy)benzaldehyde [**4**].—To a solution of **3** (50 mg, 0.016 mmol) in 3 ml of dry CH_2Cl_2 at −50° was added a solution of BBr_3 (3 equiv) in CH_2Cl_2. The mixture was gradually warmed to ambient temperature over a period of 1 h and quenched with H_2O. The mixture was extracted with CH_2Cl_2, and the organic layer was washed with brine, dried with $MgSO_4$, and concentrated to give 56 mg of a green solid. The solid, without further purification, was added in 3 ml of THF to a slurry of NaH (19 mg of 60% oil dispersion, 0.47 mmol, washed twice with Et_2O) at 0° under an argon atmosphere. After the mixture had been stirred for 15 min, $MeOCH_2Cl$ (0.035 ml, 0.47 mmol) was added at 0° and stirred for 15 min at the same temperature (1). The resulting mixture was diluted with CH_2Cl_2, washed with brine, dried with $MgSO_4$, and concentrated to give a brown oil. The crude oil was purified by chromatography [SiO_2; elution with C_6H_6-EtOAc (99:1)] to give colorless needles: 22 mg (37% from **3**); mp 66.5–69.0°; ir ($CHCl_3$) 1690, 1575, 1030 cm^{-1}; ^1H nmr (60 MHz, $CDCl_3$) δ 3.49 (s, 3H), 3.60 (s, 3H), 5.29 (s, 2H), 7.67 (s, 1H), 10.14 ppm (s, 1H).

3,4-Dibromo-5-methoxymethyl-1,2-benzenediol [**1**].—To a stirred solution of **4** (22 mg, 0.057 mmol) in

EtOH (1 ml) was added $NaBH_4$ (1 mg) at 0°. After 5 min of stirring, H_2O was added and the mixture was extracted with CH_2Cl_2, dried with $MgSO_4$, and concentrated to give an alcohol as a colorless oil (20 mg). A solution of this alcohol in THF (0.9 ml) was added to an NaH dispersion at 0° (3 mg, 60% oil dispersion, 0.08 mmol, washed twice with Et_2O). After 15 min of stirring an excess of MeI (20 mg) was added. The resulting mixture was stirred an additional 1.25 h at ambient temperature, after which H_2O was added. The mixture was extracted with CH_2Cl_2, washed with brine, dried with $MgSO_4$, and evaporated to give a viscous yellow oil (20 mg). The oil residue was dissolved in MeOH (2 ml), treated with concentrated HCl (1 ml) and stirred at ambient temperature for 20 min. The solvent was removed in vacuo, and the oily residue was taken up into CH_2Cl_2. The organic layer was washed with H_2O, dried with $MgSO_4$, and concentrated to give **1** as colorless crystals: 12 mg (70% from **4**); mp 112–114°; ir (Nujol) 3470, 3170, 1575, 920, 860, 810 cm^{-1}; ^1H nmr (60 MHz, $CDCl_3$) δ 3.48 (s, 3H), 4.50 (s, 2H), 7.38 ppm (s, 1H); fabms (matrix: glycerol) *m/z* (%) 310.8918 (50.90), 312.8898 (100.00), 314.8879 (49.61).

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