Antiviral Activities of Methylated Nordihydroguaiaretic Acids. 1. Synthesis, Structure Identification, and Inhibition of Tat-Regulated HIV Transactivation

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Nordihydroguaiaretic acid (NDGA, *meso*-**1**) possesses four phenolic hydroxyl groups. Treatment of NDGA with 0.50−4.1 equiv of dimethyl sulfate and 3.0−6.0 equiv of potassium carbonate in acetone at 56 °C gave nine methylated products. Eight of those mono-, di-, tri-, and tetra-*O*-methylated NDGAs were isolated in pure form, and their structures were identified unambiguously by spectroscopic methods. A preparative amount of tetramethyl NDGA M_4N (**10**) was obtained in 99% yield from NDGA by use of 4.1 equiv of dimethyl sulfate for the methylation. Among the eight different methylated NDGAs (**2**−**6** and **8**−**10**), tetra-*O*-methyl-NDGA (**10**) showed the strongest anti-HIV activity (IC_{50} 11 μM). Chemically synthesized 3′-*O*-methyl-NDGA ((±)-**2**) showed identical anti-HIV activity (IC_{50} 25 μM) to the lignan isolated from Creosote Bush. Lignans with methylated catecholic hydroxyl groups can be produced in large quantities with low cost. At drug concentrations below 30 μM, tetramethyl NDGA (**10**) was a stronger anti-HIV agent than mono- and dimethylated NDGAs.

Background

A plant lignan, 3′-*O*-methylnordihydroguaiaretic acid (Mal.4, 3′-*O*-methyl-NDGA), isolated from *Larrea tridentata*, was found to suppress human immunodeficiency virus type 1 (HIV-1) replication in infected human cells by preventing proviral transcription and HIV Tat-regulated transactivation. The drug targets the nucleotide sequence between −87 and −40 of the HIV long terminal repeat, which includes the Sp1 protein binding sites of its transcription promoter. Mal.4, but not the nonmethylated NDGA, directly and specifically interferes with the interaction between the Sp1 protein and its binding sites when assayed in vitro. Purification and isolation of plant lignans are labor intensive and costly. In anticipation of the possible clinical use of plant lignans in controlling HIV infections in human, we searched for efficient production of a highly active species of methylated NDGA by chemical synthesis with unmethylated NDGA as the parent substrate.

Introduction

Lignans have been isolated from more than 55 families of vascular plants. The earliest report on the occurrence of a lignan dates back to 1945, as described by Waller and Gisvold.[1] It describes the discovery of nordihydroguaiaretic acid (NDGA) in the Creosote Bush (*Larrea divaricta*), a desert medicinal plant. Several lignans have been detected in human urine, plasma, and bile in amounts comparable with thoseof steroid metabolites.[2] Lignans exhibit a wide range of biological properties including antifungal, antimicrobilal, and inhibition of several enzyme activities.[3]

Lignans are dimers of phenylpropanoid linked by central carbons of their side chains followed by oxidative coupling. Dependent upon the way in which oxygen is incorporated into the skeleton, four structural groups of linear lignans have been recognized. These are derivatives of butane, butanolide, tetrahydrofuran, and 3,7-dioxabicyclo[3.3.0]octane. By further cyclization, a larger class of natural cyclolignans are formed through the introduction of a C-7/C-6″ linkage.[4,5]

At least 33 different lignans possess cytostatic or antitumor activities when assayed by a variety of methods in vitro or in vivo. Several lignan species of podophyllum are effective in treatments of venereal warts,[6] influenza A, vaccina viruses, herpes simplex type II,[7] and herpes simplex I.[8] More recently, the lignan NDGA and several other nonsulfhydryl antioxidants have been found to inhibit cytokine-stimulated promoter activity of human immunodeficiency virus (HIV).[9] At high concentrations, however, NDGA inhibits several enzymes. This harmful effect is greatly reduced when one of its catechol hydroxyl groups is blocked or a hydrophilic group is added to the butane backbone in the mid-part of NDGA.[10]

We reported earlier that 3′-*O*-methyl-NDGA (i.e., Mal.4), isolated from Creosote Bush, can specifically block basal HIV transcription, Tat-regulated transactivation, and HIV replication in human cell culture.[11−13] It works by interfering with binding of transcription factor Sp1 to the promoter of the HIV proviral template. The target of Mal.4 has been mapped to nucleotides −87 to −40, the Sp1 binding sites of the HIV long terminal repeat (LTR). Unmodified NDGA, in vitro, does not

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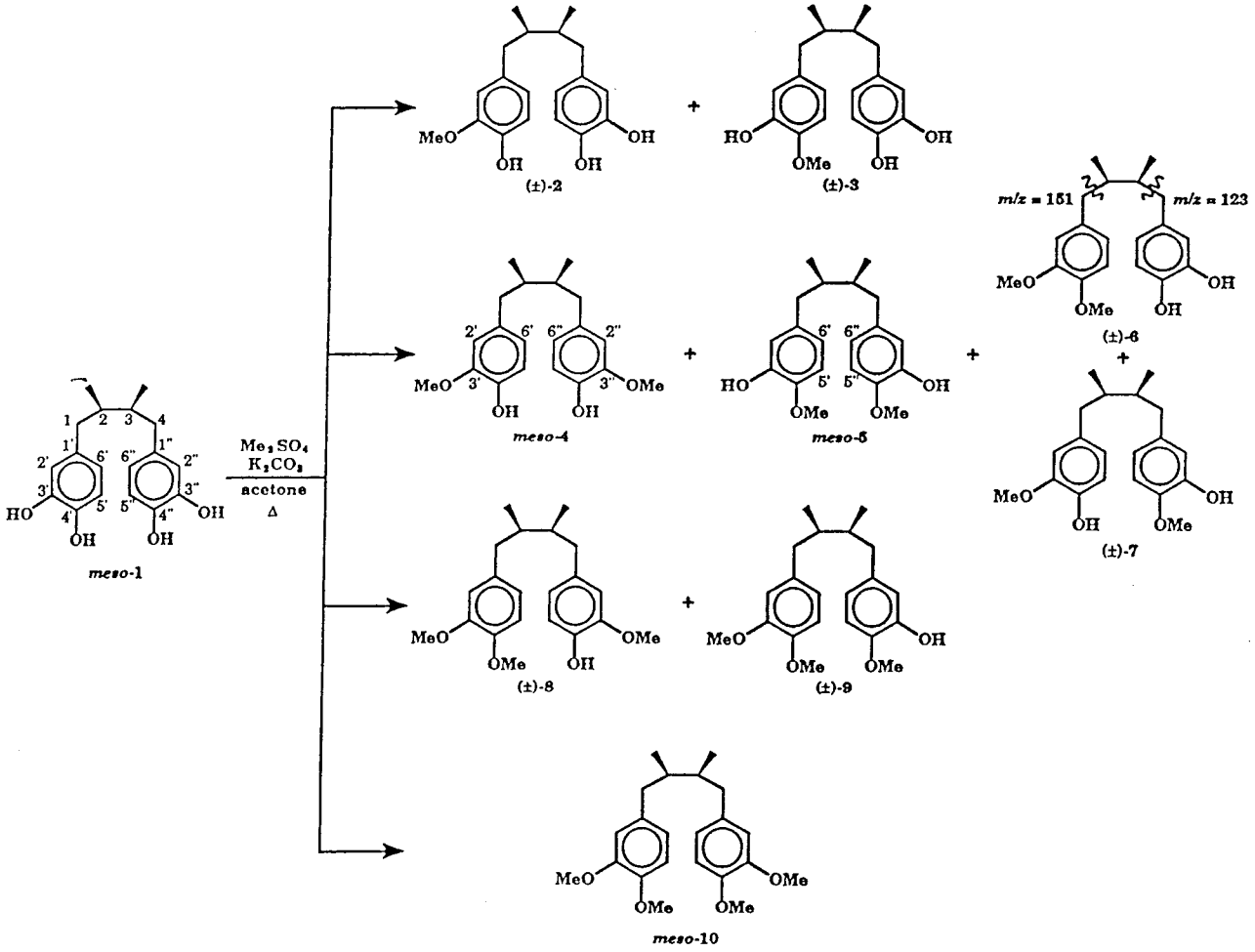
Scheme 1. Reaction of Nordihydroguaiaretic Acid with Dimethyl Sulfate To Give a Mixture of Nine Methylated Products



inhibit HIV transcription and has no effect on Sp1 binding.[13]

To elucidate further the structural specificity of lignans underlying HIV suppression, we synthesized nine different methylated NDGA. The effect of eight methylated NDGAs in their pure form was examined on HIV Tat-regulated transactivation in human epithelial cells (COS cell).

Results

Synthesis of Methylated Nordihydroguaiaretic Acids. Nordihydroguaiaretic acid, a commercially available chemical, bears four phenolic hydroxyl groups at the C-3′, C-4′, C-3″, and C-4″ positions. To obtain methylated NDGAs with various degrees of methylation, we first treated NDGA with 0.50 equiv of dimethyl sulfate and 3.0 equiv of potassium carbonate in acetone at 56 °C (Scheme 1). The two regioisomeric monomethylated NDGAs (i.e., (±)-**2** and (±)-**3**) were obtained in 38% overall yields, along with a mixture of dimethylated NDGAs (i.e., (±)-**4**−**7**) in 9% overall yield (entry 1 in Table 1). Then we improved the overall yields for monomethylated NDGAs to 45% by increasing the equivalents of dimethyl sulfate to 0.70−1.0 equiv and potassium carbonate to 4.9 equiv (entries 2 and 3).

Use of 2.0 equiv of dimethyl sulfate allowed us to obtain tri- and tetramethylated NDGAs as the major products in 40% and 44% yields, respectively. Furthermore, we successfully synthesized the tetramethyl-NDGA (i.e., *meso*-**10**) in an almost quantitative yield by using 4.1 equiv of dimethyl sulfate (entry 5).

Table 1. Methylation of NDGA by Dimethyl Sulfate in the Presence of Potassium Carbonate in Acetone at Reflux for 8.0 h

			yield (%) of methylated NDGA[a]			
entry	Me_2SO_4 (equiv)	K_2CO_3 (equiv)	mono-Me (**2** and **3**)	di-Me (**4**-**7**)	tri-Me (**8** and **9**)	tetra-Me (**10**)
1	0.50	3.0	38	9	0	0
2	0.7	4.9	45	19	0	0
3	1.0	4.9	45	28	10	0
4	2.0	4.9	0	7	40	44
5	4.1	6.0	0	0	0	99

[a] Structures for **2**−**10** are shown in Scheme 1.

Separation and Purification of Methylated Nordihydroguaiaretic Acids. The starting material for methylation, NDGA (i.e., **1**), is a meso compound although it possesses two stereogenic centers. Its methylation may produce nine different methylated compounds (Scheme 1). Among them, methylated NDGAs **2**, **3**, and **6**−**9** should exist in a racemic form, yet **4**, **5**, and **10** would retain the meso characteristic.

We were able to separate and to purify the mixture of methylated products in large quantity (up to 10 g of crude mixture) by means of a medium-pressure liquid chromatography (MPLC) system designed previously.[14] Use of reversed phase silica gel C18 and a mixture of aqueous acetonitrile and methanol as an eluant allowed

Table 2. Carbon-13 Chemical Shifts of Aromatic Nuclei of Pure Methylated NDGAs[a]

	methylated NDGAs								
carbon	**1**	**2**	**3**	**4**	**5**	**6**	**8**	**9**	**10**
1′	144.74	143.45	144.58	*143.46*	144.55	134.75	**134.45**	**134.54**	**134.35**
2′	115.28	111.58	110.53	*111.41*	110.41	112.41	**112.17**	**112.17**	**112.14**
3′	142.89	146.31	135.27	*146.28*	135.17	148.50	**148.65**	**148.65**	**148.62**
3′	132.27	133.91	144.95	*133.72*	145.19	146.83	**146.98**	**146.98**	**146.95**
5′	116.20	114.02	115.27	*113.93*	115.18	121.12	**120.88**	**120.99**	**120.85**
6′	119.49	121.74	120.61	*121.64*	120.40	111.20	**110.96**	**110.99**	**110.93**
1″	*b*	143.37	143.31	*b*	*b*	143.52	*143.52*	144.58	*b*
2″	*b*	115.15	115.12	*b*	*b*	115.08	*111.38*	110.41	*b*
3″	*b*	141.33	141.43	*b*	*b*	141.49	*146.25*	135.17	*b*
4″	*b*	135.08	134.99	*b*	*b*	134.75	*133.69*	145.31	*b*
5″	*b*	116.09	116.06	*b*	*b*	116.03	*113.93*	115.21	*b*
6″	*b*	121.48	121.52	*b*	*b*	121.31	*121.64*	120.37	*b*

[a] The matrices in italics include two sets of close figures in comparison of C-1′ with C-1″, C-2′ with C-2″, C-3′ with C-3″, C-4′ with C-4″, C-5′ with C-5″, and C-6′ with C-6″, respectively; so as the two matrices in underlines. The three matrices in bold include three sets of close figures for C-1′, C-2′, C-3′, C-4′, C-5′, and C-6′, individually. [b] The same values as those listed for 1′−6′ because of the nature of meso compounds.

us to separate a crude mixture to fractions containing only mono-, di-, tri-, or tetramethylated NDGAs. Further purification of the regioisomers with the same extent of methylation in a single fraction required repetitive MPLC operations. Accordingly, we isolated all of the methylated isomers, except **7**, in >99.9% purity for biological tests.

Numerous attempts met with failure for separation of dimethylated product (±)-**7** by use of different separation instruments (including HPLC and preparative GC), techniques, and conditions. With the best of modern separation methods, we obtained (±)-**7** (along with its regioisomers **4** and **5**) in 80% purity.

Structure Identification. By comparing with the literature data related to naturally occurring (+)-**2** and (+)-**3**,[12] we readily identified the pure monomethylated products (±)-**2** and (±)-**3** by spectroscopy. On the basis of the molecular ion peaks in mass spectra and the ^{13}C NMR spectra, we easily distinguished the matrixes of (**4**−**7**), (**8** + **9**), and **10**. The structure of tetra-O-methylated **10** was unambiguously assigned on the basis of the literature data.[15] During its purification, we found that **10** had the longest retention time in MPLC with reversed phase media.

To distinguish *meso*-**4** from other dimethylated NDGAs (i.e., **5**−**7**), we doubly irradiated the protons of the two symmetric CH_3O groups at the C-3′ and the C-3″ positions. Accrued from the nuclear Overhauser effect (NOE), an increase in signal up to 11% was observed for the C-2′ and the C-2″ hydrogens at δ 6.61 ppm. By using the same NOE strategy, we detected a 14% increase for the C-5′ and the C-5″ hydrogens of *meso*-**5** at δ 6.76 ppm. These two hydrogens showed a $^3J_{ortho}$ coupling of 8.0 Hz with the C-6′ and the C-6″ hydrogens of *meso*-**5**. In comparison, the C-2′ and the C-2″ hydrogens of *meso*-**4** showed a much smaller $^4J_{meta}$ coupling (2.0 Hz versus 8.0 Hz of *meso*-**5**) with the C-6′ and the C-6″ hydrogens. The mass spectrum of (±)-**6** exhibited characteristic peaks at *m*/*z* 151 (100%) and 123 (55%), which belonged to the fragments of dimethoxytoluene cation and dihydroxytoluene cation, respectively.

Two 3,4-dimethoxybenzene units exist in *meso*-**10**; two 4-hydroxy-3-methoxybenzene units exist in *meso*-**4**. Compound (±)-**8**, however, contains both types of units. Therefore the chemical shifts of the 12 aromatic C-13 nuclei in (±)-**8** should be consistent with values in combination of *meso*-**4** and *meso*-**10**. Examination of the ^{13}C NMR data listed in Table 2 provided us an avenue to assign the structure of (±)-**8** without ambiguity. Moreover, we used the same method to identify (±)-**9** by comparing its ^{13}C NMR spectrum with those of *meso*-**5** and *meso*-**10**.

After the structures of eight of the nine compounds had been confirmed by process of elimination, the major component in the remaining fraction was identified as dimethylated NDGA (±)-**7**. Consequently we proceeded the biological tests using all of the methylated NDGAs with assured structures.

Effect of Methylated Derivatives of NDGAs on Inhibition of HIV Tat-Regulated Transactivation. The HIV Tat protein, a potent transactivator of HIV proviral transcription, is required for HIV replication.[16] A powerful transactivation assay[17] involving transfection of plasmid constructs was used to test the effect of methylated NDGAs on Tat-regulated secreted alkaline phosphatase (SEAP) production in COS cells as described previously.[11,13,17] The plasmid constructs included a cytomegalovirus (CMV) promoter driven *tat* gene and a HIV LTR promoter driven reporter gene (i.e., SEAP). We have previously shown that compound 3′-*O*-methyl-NDGA, a plant lignan isolated from Creosote Bush is able to inhibit the replication of five different HIV-1 strains (WM, MN, VS, JR-CSF, and III_B) in mitogen-stimulated peripheral blood mononuclear cells by suppressing proviral transcription and Tat-regulated transaction.[13] It is interesting to note that chemically synthesized 3′-*O*-methyl-NDGA showed identical anti HIV transactivation activity (IC_{50} 25 μM). Furthermore, we observed that all of the methylated NDGAs at 100 μM inhibited Tat-regulated SEAP production to above 85% except 3′,3″-dimethyl-NDGA (**4**, 73% only). At low drug concentrations (=30 μM), however, trimethyl-NDGAs (**8** and **9**) and tetramethyl-NDGA (**10**) were more effective than mono- and dimethyl-NDGAs (Table 3). For instance, at 3 μM, tetramethyl-NDGA (**10**) was 11 times more effective than 4′-*O*-methyl-NDGA (**3**) and 3.4 times more effective than 3′-*O*-methyl-NDGA (**2**). Technically, tetramethyl-NDGA (**10**) was much easier to purify. Thus large quantities of this compound can be made at low cost. It remained stable and showed little toxicity in vivo. Mice exhibited no weight loss over

Table 3. Inhibition (%) of HIV Tat-Regulated Transactivation in COS Cells by Different Methylated NDGAs[a]

	concentrations (μM)							
compound	1	3	10	20	30	60	100	IC_{50} (μM)[b]
(±)-**2**	9.0	7.3	20.7	38.5	56.3	83.3	92.3	25.03 ± 2.77
(±)-**3**	5.0	2.3	5.0	27.5	52.0	73.7	91.3	30.81 ± 3.88
meso-**4**	5.5	10.3	22.3	30.5	43.0	63.7	73.7	38.98 ± 9.13
meso-**5**	9.5	8.7	19.0	38.0	52.0	70.0	85.7	29.88 ± 7.22
(±)-**6**	12.5	14.3	20.0	59.0	69.3	87.7	96.0	16.52 ± 1.81
(±)-**8**	14.5	26.7	45.0	50.5	65.0	76.3	87.7	13.66 ± 5.44
(±)-**9**	16.5	27.0	41.7	52.0	66.0	78.3	88.3	14.31 ± 1.39
meso-**10**	18.5	25.3	45.0	61.0	70.0	78.7	90.3	11.09 ± 2.14

[a] All the data represent the average of three experiments. [b] Concentrations exhibiting 50% inhibitory activity (IC_{50}) represent the mean of triplicate determinations with standard deviations. IC_{50} for natural lignan NDGA (+)-**2** is 25 μM.[13]

a 2 week period of intraperitoneal injection of the compound at a concentration of 300 mg/kg of body weight.

Discussion

We succeeded in methylating every hydroxyl group of NDGA. In the analysis of the IC_{50} values of compounds **2**−**10** for their inhibition of HIV Tat-regulated transactivation in human epithelial cells (see Table 3), we found that all of the four compounds with potency (i.e., **6** and **8**−**10**) bear one permethylated catechol moiety. Overton's rule indicates that permeability of a compound is roughly proportional to its lipid solubility.[18] The permethylated catechol moiety of **6** and **8**−**10** possesses good lipophilicity and thus could assist these compounds penetrating the cell membrane. Furthermore, we found that the potency increases for 3′,4′-dimethyl-NDGA derivatives bearing more methyl groups onto the other catechol ring. Thus IC_{50} values drop by the order of **6** > **8** ∼ **9** > **10**. On the other hand, dimethylation of NDGA on different catechol moieties results in products bearing one free hydroxyl group attached to a methoxybenzene ring. The lipophilicity of those compounds, including **4** and **5**, decreases, and thus their potency is significantly weaker than that of **6**, in which the dimethoxy groups are attached to the same benzene ring.

Compounds **2** and **3** are regioisomers. Both have a free catechol moiety, yet the methyl group is attached to the 3′- or the 4′-position. These two compounds exhibit similar biological activity. The IC_{50} values are very close for the racemic compound **2** (i.e., (±)-**2**) and the single enantiomeric (+)-**2**. Thus the activity does not appear to be enantiospecific. The pair of **8** and **9**, both having a dimethoxybenzene unit and possessing similar activity, are also regioisomers. Thus we conclude that the methylation position does not significantly influence the biological activity for NDGA derivatives having an identical aromatic unit.

Proviral transcription is an essential step in the HIV life cycle. Being entirely host-dependent, it utilizes host-transcriptional machinery and transcriptional factors for its promoter activity. Host genes are not subject to the same mutational pressure found for the virus. Mutated viruses, like wild-type viruses, are also active in proviral transcription and require host transcriptional machinery for replication. Thus a drug targeted at this step should still be effective for controlling HIVs carrying reverse transcriptase (RT) or protease mutations. Transcription inhibitors are therefore an entirely separate class of antiviral drugs, which may be used in suppressing mutant viruses that fail to respond to RT or protease inhibitors.

Lignans 3′-*O*-methyl-NDGA (Mal.4) and tetramethyl-NDGA target Sp1 sites in the HIV LTR. In this article it has been shown that tetramethyl-NDGA (M_4N) (**10**) functions as a more effective inhibitor of Tat-regulated transactivation than Mal.4. As discussed earlier, permethylation of the catecholic hydroxyl groups of NDGA resulted in no loss in lignan specificity in targeting HIV transactivation. Our IC_{50} of 11 μM for tetramethyl-NDGA (**10**) is likely an overestimation because lignans bearing more methyl groups were less soluble in culture medium containing only low concentrations of DMSO (0.3%). Several lipophilic solvents for lignans have been tested currently. In addition, synthesis of several fully substituted hydrophilic lignans is in progress. Selective inhibition of HIV and other Sp1-regulated viral or tumor growth by these modified lignans will be accessed once these agents become available.

Significance. Combination therapy with HIV reverse transcriptase and protease inhibitors is able to eliminate a majority of the viruses in infected humans. A small fraction of the remaining viruses, however, continues to replicate and mutate rapidly in AIDS patients.[19] Plant lignan 3′-*O*-methylnordihydroguaiaretic acid (Mal.4) targets HIV at a stage of the viral life cycle that involves no viral DNA synthesis or proteolysis. Thus Mal.4 and its derivatives represent a new class of antiviral drugs differing in mode of action from these viral protein inhibitors.

In the current study, we showed that tetramethyl-NDGA (**10**) can be produced in large amounts with low cost by complete methylation of NDGA with dimethyl sulfate. Protection of all catecholic hydroxyl groups of the compound decreases the possible metabolic side effects found with unprotected NDGA. Because compound **10** maintains its specificity in suppression of HIV Tat-regulated transactivation in COS cells, use of it in concert with other HIV protein inhibitors should help to eliminate HIV mutants that are resistant to the currently available anti-HIV drugs. In addition, methods established by this study can be used to prepare alternative lignans for examination of their anti viral or antitumor potentials. In the following article (Part 2) we showed that 3′-*O*-methyl-NDGA and tetra-*O*-methyl-NDGA can also suppress Sp1-regulated expression of an immediate early ICP_4 gene of HSV-1 and compound **10** is a mutation insensitive inhibitor of Herpes simplex virus (HSV-1) in Vero cells.

Experimental Section

General. Acetone, acetonitrile, ethyl acetate, hexanes, and methanol were purchased from Mallinckrodt Chemical Co. Acetone was dried with 4A molecular sieves and distilled. Ethyl acetate and hexanes were dried and distilled from CaH_2. Acetonitrile and methanol were purchased in HPLC grade and used without further purification. Nordihydroguaiaretic acid was purchased from Fluka Chemical Co. Dimethyl sulfate and potassium carbonate were purchased from Merck Inc. Analytical thin-layer chromatography (TLC) was performed on precoated plates (silica gel 60 F-254), purchased from Merck Inc.

Gas chromatographic analyses were performed on a Hewlett-Packard 5890 series II instrument equipped with a 25-m cross-linked methyl silicone gum capillary column (0.32 mm i.d.). Nitrogen gas was used as a carrier gas, and the flow rate was kept constant at 14.0 mL/min. The retention time t_R was measured under the following conditions: injector temperature 300 °C, isothermal column temperature 280 °C. Gas chromatography and low-resolution mass spectral analyses were performed on a Hewlett-Packard 5890 series II instrument equipped with a Hewlett-Packard 5971A mass selective detector and a capillary HP-1 column. Separations by medium-pressure liquid chromatography (MPLC) were performed on a column packed with reversed phase silica gel C18 (particle size 0.035−0.070 mm), and the flow rate of eluant was kept at 120 mL/h with Jasco model 880-PU intelligent HPLC pump.

Proton NMR spectra were obtained on a Varian Unity-400 (400 MHz) spectrometer by use of chloroform-*d* as the solvent and tetramethylsilane as an internal standard. Carbon-13 NMR spectra were obtained on a Varian Unity-400 (100 MHz) spectrometer by use of chloroform-*d* as the solvent. Carbon-13 chemical shifts are referenced to the center of the $CDCl_3$ triplet (δ 77.0 ppm). Multiplicities are recorded by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; *J*, coupling constant (hertz). Infrared (IR) spectra were measured on a Bomem Michelson Series FT-IR spectrometer. The wavenumbers reported are referenced to the polystyrene 1601 cm^{-1} absorption. Absorption intensities are recorded by the following abbreviations: s, strong; m, medium; w, weak. High-resolution mass spectra (HRMS) were obtained by means of a JEOL JMS-HX110 mass spectrometer.

A Representative Procedure for Methylation of Nordihydroguaiaretic Acid (NDGA). A solution containing NDGA (5.69 g, 18.8 mmol, 1.0 equiv) and potassium carbonate (12.8 g, 92.6 mmol, 4.9 equiv) in acetone (150 mL) was stirred at 25 °C for 10 min. Dimethyl sulfate (2.37 g, 18.8 mmol, 1.0 equiv) was then added into the reaction flask, and the solution was heated to reflux for 8.0 h. The reaction mixture was neutralized by aqueous HCl solution (1.0 N) and extracted with ethyl acetate (4 × 100 mL). The combined organic layers were washed with water (4 × 30 mL) and brine (2 × 20 mL), dried over $MgSO_{4(s)}$, and concentrated under reduced pressure. The crude mixture was separated by MPLC (4.9 cm × 46 cm column, 20% acetonitrile and 30% methanol in water as the eluant) to provide monomethyl-NDGAs (2.69 g, 8.50 mmol) in 45% overall yield, dimethyl-NDGAs (1.75 g, 5.30 mmol) in 28% overall yield, and trimethyl-NDGAs (0.651 g, 1.89 mmol) in 10% overall yield successively. The procedure described above was followed by use of various amount of reagents and the results are summarized in Table 1.

Separations of Methylated NDGAs. A mixture of monomethyl-NDGAs (2.69 g, 8.50 mmol) was sequentially purified twice by MPLC (4.9 cm × 46 cm column, 20% acetonitrile and 20% methanol in water as eluant) to give pure **2** (883 mg, 2.79 mmol) and pure **3** (732 mg, 2.31 mmol) as pale yellow oils. Under similar conditions, dimethyl-NDGAs (1.75 g, 5.30 mmol) were purified by use of 20% acetonitrile and 30% methanol in water as the eluant to give pure **4** (252 mg, 0.763 mmol) and pure **5** (265 mg, 0.802 mmol) as white solids and **6** (427 mg, 1.29 mmol) as a yellow oil. Compound **7** (502 mg, 1.52 mmol) was, however, obtained in only 80% purity contaminated with **4** and **5**. Trimethyl-NDGAs (0.651 g, 1.89 mmol) was purified by use of 20% acetonitrile and 40% methanol in water as eluant to provide pure **8** (195 mg, 0.566 mmol) as needle crystal and pure **9** (203 mg, 0.589 mmol) as a white solid. Purity of compounds **2**−**6**, **8**, and **9** was >99.9%, as determined by gas chromatographic analyses with a capillary column.

(±)-1-(3,4-Dihydroxyphenyl)-(2*R*,3*S*)-dimethyl-4-(4-hydroxy-3-methoxyphenyl)butane (2): GC t_R 7.13 min; TLC R_{xa6} 0.25 ($CHCl_3$/MeOH = 9:1); UV λ_{max} (MeOH) 282 nm (ϵ 5710); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.82 (d, *J* = 6.7 Hz, 3 H, CH_3), 0.83 (d, *J* = 6.7 Hz, 3 H, CH_3), 1.72 (m, 2 H, 2 × CH), 2.25 (dd, *J* = 13.4, 9.3 Hz, 2 H, 2 × ArCH), 2.68 (dd, *J* = 13.4, 5.2 Hz, 1 H, ArCH), 2.71 (dd, *J* = 13.4, 4.7 Hz, 1 H, ArCH), 3.86 (s, 3 H, CH_3O), 5.05 (s, 1 H, OH), 5.19 (s, 1 H, OH), 5.47 (s, 1 H, OH), 6.58 (dd, *J* = 8.0, 2.0 Hz, 1 H, HC(6″)), 6.61 (d, *J* = 1.9 Hz, 1 H, HC(2′)), 6.64 (dd, *J* = 8.0, 1.9 Hz, 1 H, HC(6′)), 6.67 (d, *J* = 2.0 Hz, 1 H, HC(2″)), 6.77 (d, *J* = 8.0 Hz, 1 H, HC(5″)), 6.82 (d, *J* = 8.0 Hz, 1 H, HC(5′)); IR (neat) 3427 (br s, OH), 2965 (m), 1606 (s, C═C), 1515 (s, C═C), 1456 (m), 1269 (m), 1122 (w), 1034 (m), 959 (w), 791 (m) cm^{-1}; MS *m*/*z* (relative intensity) 316 (M^+, 51), 151 (5), 138 (14), 137 (100), 124 (8), 123 (68), 122 (17), 94 (14), 91 (5), 77 (16); HRMS calcd for $C_{19}H_{24}O_4$ 316.1674, found 316.1666. Anal. Calcd for $C_{19}H_{24}O_4$: C, 72.13; H, 7.65. Found: C, 71.88; H, 7.72. Its spectroscopic characteristics are consistent with those of an authentic sample.[11,12]

(±)-1-(3,4-Dihydroxyphenyl)-(2*R*,3*S*)-dimethyl-4-(3-hydroxy-4-methoxyphenyl)butane (3): GC t_R 7.49 min; TLC R_{xa6} 0.23 ($CHCl_3$/MeOH = 9:1); UV λ_{max} (MeOH) 282 nm (ϵ 6020); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.81 (d, *J* = 6.7 Hz, 3 H, CH_3), 0.82 (d, *J* = 6.7 Hz, 3 H, CH_3), 1.72 (m, 2 H, 2 × CH), 2.21 (dd, *J* = 13.4, 9.3 Hz, 1 H, ArCH), 2.24 (dd, *J* = 13.4, 9.3 Hz, 1 H, ArCH), 2.68 (dd, *J* = 13.4, 5.2 Hz, 1 H, ArCH), 2.69 (dd, *J* = 13.4, 4.7 Hz, 1 H, ArCH), 3.87 (s, 3 H, CH_3O), 5.08 (s, 1 H, OH), 5.39 (s, 1 H, OH), 5.59 (s, 1 H, OH), 6.58 (dd, *J* = 8.0, 2.0 Hz, 1 H, HC(6″)), 6.63 (dd, *J* = 8.1, 2.0 Hz, 1 H, HC(6′)), 6.67 (d, *J* = 2.0 Hz, 1 H, HC(2″)), 6.71 (d, *J* = 2.0 Hz, 1 H, HC(2′)), 6.77 (d, *J* = 8.0 Hz, 1 H, HC(5′)), 6.77 (d, *J* = 8.0 Hz, 1 H, HC(5″)); IR (neat) 3406 (br s, OH), 2960 (m), 1592 (m, C═C), 1513 (s, C═C), 1442 (m), 1357 (w), 1272 (s), 1028 (m), 964 (m), 770 (m) cm^{-1}; MS *m*/*z* (relative intensity) 316 (M^+, 100), 314 (9), 138 (21), 137 (88), 124 (7), 123 (41), 122 (8), 58 (9), 43 (28), 15 (8); HRMS calcd for $C_{19}H_{24}O_4$ 316.1674, found 316.1676. Anal. Calcd for $C_{19}H_{24}O_4$: C, 72.13; H, 7.65. Found: C, 72.01; H, 7.70. Its spectroscopic characteristics are consistent with those of an authentic sample.[11,12]

***meso*-1,4-Bis(4-hydroxy-3-methoxyphenyl)-(2*R*,3*S*)-dimethylbutane (4, Dihydroguaiaretic Acid):** mp 87−88 °C (lit.[20] mp 88 °C); GC t_R 5.85 min; TLC R_f 0.58 ($CHCl_3$/MeOH = 9:1); UV λ_{max} (MeOH) 282 nm (ϵ 5910); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.84 (d, *J* = 6.4 Hz, 6 H, 2 × CH_3), 1.75 (m, 2 H, 2 × CH), 2.28 (dd, *J* = 13.2, 9.2 Hz, 2 H, 2 × ArCH), 2.73 (dd, *J* = 13.2, 5.2 Hz, 2 H, 2 × ArCH), 3.85 (s, 6 H, 2 × CH_3O), 5.46 (s, 2 H, 2 × OH), 6.61 (d, *J* = 2.0 Hz, 2 H, HC(2′) + HC(2″)), 6.65 (dd, *J* = 8.0, 2.0 Hz, 2 H, HC(6′) + HC(6″)), 6.82 (d, *J* = 8.0 Hz, 2 H, HC(5′) + HC(5″)); IR (neat) 3466 (br s, OH), 2965 (m), 1612 (w, C═C), 1514 (s, C═C), 1465 (m), 1377 (w), 1268 (m), 1239 (m), 1156 (w), 1037 (w) cm^{-1}; MS *m*/*z* (relative intensity) 330 (M^+, 5), 165 (2), 151 (2), 138 (19), 137 (100), 123 (2), 122 (8), 94 (5), 91 (2), 77 (3); HRMS calcd for $C_{20}H_{26}O_4$ 330.1831, found 330.1833. Anal. Calcd for $C_{20}H_{26}O_4$: C, 72.70; H, 7.93. Found: C, 72.65; H, 8.02. Its spectroscopic characteristics are consistent with those of the same compound reported.[20]

***meso*-1,4-Bis(3-hydroxy-4-methoxyphenyl)-(2*R*,3*S*)-dimethylbutane (5):** mp 125−126 °C; GC t_R 6.45 min; TLC R_{xa6} 0.60 ($CHCl_3$/MeOH = 9:1); UV λ_{max} (MeOH) 282 nm (ϵ 5820); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.82 (d, *J* = 6.8 Hz, 6 H, 2 × CH_3), 1.72 (m, 2 H, 2 × CH), 2.22 (dd, *J* = 13.4, 9.4 Hz, 2 H, 2 × ArCH), 2.69 (dd, *J* = 13.4, 4.6 Hz, 2 H, 2 × ArCH), 3.85 (s, 6 H, 2 × CH_3O), 5.60 (s, 2 H, 2 × OH), 6.62 (dd, *J* = 8.0, 2.0 Hz, 2 H, HC(6′) + HC(6″)), 6.74 (d, *J* = 2.0 Hz, 2 H, HC(2′) + HC(2″)), 6.76 (d, *J* = 8.0 Hz, 2 H, HC(5′) + HC(5″)); IR (neat) 3417 (br s, OH), 2975 (m), 1588 (m, C═C), 1512 (s, C═C), 1456 (m), 1274 (m), 1129 (m), 1023 (m), 804 (m), 770 (m)

cm^{-1}; MS *m/z* (relative intensity) 330 (M^+, 4), 165 (4), 151 (3), 139 (3), 138 (38), 137 (100), 122 (8), 105 (2), 94 (5), 77 (2); HRMS calcd for $C_{20}H_{26}O_4$ 330.1831, found 330.1829. Anal. Calcd for $C_{20}H_{26}O_4$: C, 72.70; H, 7.93. Found: C, 72.78; H, 8.14.

(±)-1-(3,4-Dihydroxyphenyl)-4-(3,4-dimethoxyphenyl)-(2*R*,3*S*)-dimethylbutane (6): GC t_R 7.06 min; TLC R_{xa6} 0.34 ($CHCl_3$/MeOH = 9:1); UV λ_{max} (MeOH) 282 nm (ϵ 5720); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.81 (d, J = 5.8 Hz, 3 H, CH_3), 0.82 (d, J = 5.8 Hz, 3 H, CH_3), 1.72 (m, 2 H, 2 × CH), 2.24 (dd, J = 13.5, 9.4 Hz, 2 H, 2 × ArCH), 2.65 (dd, J = 13.5, 5.6 Hz, 1 H, ArCH), 2.71 (dd, J = 13.5, 4.4 Hz, 1 H, ArCH), 3.82 (s, 3 H, CH_3O), 3.83 (s, 3 H, CH_3O), 5.07 (s, 1 H, OH), 5.25 (s, 1 H, OH), 6.56 (dd, J = 8.0, 2.0 Hz, 1 H, HC(6″)), 6.63 (d, J = 2.0 Hz, 1 H, HC(2′)), 6.65 (d, J = 2.0 Hz, 1 H, HC(2″)), 6.68 (dd, J = 8.0, 2.0 Hz, 1 H, HC(6′)), 6.75 (d, J = 8.0 Hz, 1 H, HC-(5″)), 6.77 (d, J = 8.0 Hz, 1 H, HC(5′)); IR (neat) 3422 (br s, OH), 2958 (m), 1603 (m, C═C), 1515 (s, C═C), 1452 (m), 1261 (m), 1146 (m), 1026 (m), 816 (w), 770 (w) cm^{-1}; MS *m/z* (relative intensity) 330 (M^+, 51), 152 (15), 151 (100), 137 (10), 124 (5), 123 (55), 107 (11), 91 (6), 78 (5), 77 (14); HRMS calcd for $C_{20}H_{26}O_4$ 330.1831, found 330.1832. Anal. Calcd for $C_{20}H_{26}O_4$: C, 72.70; H, 7.93. Found: C, 72.56; H, 8.07.

(±)-1-(3,4-Dimethoxyphenyl)-(2*R*,3*S*)-dimethyl-4-(4-hydroxy-3-methoxyphenyl)butane (8): mp 85−86 °C; GC t_R 5.76 min; TLC R_{xa6} 0.69 ($CHCl_3$/MeOH = 9:1); UV λ_{max} (MeOH) 281 nm (ϵ 5840); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.84 (d, J = 6.8 Hz, 3 H, CH_3), 0.85 (d, J = 6.4 Hz, 3 H, CH_3), 1.76 (m, 2 H, 2 × CH), 2.28 (dd, J = 13.2, 1.6 Hz, 1 H, ArCH), 2.30 (dd, J = 13.2, 1.6 Hz, 1 H, ArCH), 2.73 (dd, J = 13.2, 5.8 Hz, 1 H, ArCH), 2.75 (dd, J = 13.2, 5.8 Hz, 1 H, ArCH), 3.84 (s, 6 H, 2 × CH_3O), 3.85 (s, 3 H, CH_3O), 5.47 (s, 1 H, OH), 6.62 (d, J = 1.6 Hz, 1 H, HC(2″)), 6.64 (d, J = 2.0 Hz, 1 H, HC(2′)), 6.66 (dd, J = 8.0, 1.6 Hz, 1 H, HC(6″)), 6.69 (dd, J = 8.0, 2.0 Hz, 1 H, HC(6′)), 6.78 (d, J = 8.0 Hz, 1 H, HC(5′)), 6.83 (d, J = 8.0 Hz, 1 H, HC(5″)); IR (neat) 3444 (br s, OH), 2957 (m), 1608 (m, C═C), 1515 (s, C═C), 1465 (m), 1377 (w), 1265 (m), 1236 (m), 1153 (m), 1031 (m) cm^{-1}; MS *m/z* (relative intensity) 344 (M^+, 8), 152 (21), 151 (100), 138 (10), 137 (53), 122 (54), 107 (6), 94 (3), 91 (4), 77 (3); HRMS calcd for $C_{21}H_{28}O_4$ 344.1987, found 344.1990. Anal. Calcd for $C_{21}H_{28}O_4$: C, 73.23; H, 8.19. Found: C, 73.30; H, 8.38.

(±)-1-(3,4-Dimethoxyphenyl)-(2*R*,3*S*)-dimethyl-4-(3-hydroxy-4-methoxyphenyl)butane (9): mp 96−97 °C; GC t_R 6.07 min; TLC R_{xa6} 0.71 ($CHCl_3$/MeOH = 9:1); UV λ_{max} (MeOH) 281 nm (ϵ 5650); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.82 (d, J = 6.6 Hz, 3 H, CH_3), 0.84 (d, J = 6.6 Hz, 3 H, CH_3), 1.75 (m, 2 H, 2 × CH), 2.25 (dd, J = 13.5, 9.5 Hz, 1 H, ArCH), 2.29 (dd, J = 13.5, 9.5 Hz, 1 H, ArCH), 2.71 (dd, J = 13.5, 5.0 Hz, 1 H, ArCH), 2.75 (dd, J = 13.5, 4.8 Hz, 1 H, ArCH), 3.86 (s, 6 H, 2 × CH_3O), 3.87 (s, 3 H, CH_3O), 5.57 (s, 1 H, OH), 6.63 (dd, J = 8.0, 2.0 Hz, 1 H, HC(6″)), 6.65 (d, J = 2.0 Hz, 1 H, HC(2′)), 6.69 (dd, J = 8.0, 2.0 Hz, 1 H, HC(6′)), 6.76 (d, J = 8.0 Hz, 1 H, HC(5′)), 6.76 (d, J = 2.0 Hz, 1 H, HC(2″)), 6.78 (d, J = 8.0 Hz, 1 H, HC(5″)); IR (neat) 3487 (br s, OH), 2965 (m), 1590 (m, C═C), 1514 (s, C═C), 1465 (m), 1266 (m), 1146 (m), 1029 (m), 762 (w) cm^{-1}; MS *m/z* (relative intensity) 344 (M^+, 4), 152 (23), 151 (100), 138 (10), 137 (40), 122 (2), 107 (3), 105 (1), 94 (1), 91 (1); HRMS calcd for $C_{21}H_{28}O_4$ 344.1987, found 344.1982. Anal. Calcd for $C_{21}H_{28}O_4$: C, 73.23; H, 8.19. Found: C, 73.45; H, 8.27.

***meso*-1,4-Bis(3,4-dimethoxyphenyl)-(2*R*,3*S*)-dimethylbutane (10).** The representative procedure was followed by use of NDGA (458 mg, 1.51 mmol, 1.0 equiv), potassium carbonate (1.25 g, 9.04 mmol, 6.0 equiv), and dimethyl sulfate (781 mg, 6.19 mmol, 4.1 equiv) in acetone (30 mL). After the reaction mixture was worked up, the residue was purified by column chromatography on silica gel (2.2 cm × 15 cm column, 30% ethyl acetate in hexanes as eluant) to give pure **10** (537 mg, 1.50 mmol) in 99% yield: mp 100−101 °C (lit.[15] mp 100−102 °C); GC t_R 5.80 min; TLC R_{xa6} 0.76 ($CHCl_3$/MeOH = 9:1); UV λ_{max} (MeOH) 280 nm (ϵ 5900); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.85 (d, J = 6.8 Hz, 6 H, 2 × CH_3), 1.78 (m, 2 H, 2 × CH), 2.31 (dd, J = 13.6, 9.2 Hz, 2 H, 2 × ArCH), 2.75 (dd, J = 13.2, 4.6 Hz, 2 H, 2 × ArCH), 3.85 (s, 6 H, 2 × CH_3O), 3.86 (s, 6 H, 2 × CH_3O), 6.65 (d, J = 2.0 Hz, 2 H, HC(2′) + HC(2″)), 6.70 (dd, J = 8.0, 2.0 Hz, 2 H, HC(6′) + HC(6″)), 6.78 (d, J = 8.0 Hz, 2 H, HC(5′) + HC(5″)); IR (neat) 2960 (m), 2931 (m), 1598 (w, C═C), 1515 (s, C═C), 1465 (m), 1262 (m), 1237 (m), 1161 (m), 1146 (m), 1030 (m) cm^{-1}; MS *m/z* (relative intensity) 358 (M^+, 7), 179 (2), 152 (20), 151 (100), 135 (2), 107 (6), 106 (3), 105 (2), 91 (2), 77 (2); HRMS calcd for $C_{22}H_{30}O_4$ 358.2144, found 358.2130. Anal. Calcd for $C_{22}H_{30}O_4$: C, 73.71; H, 8.44. Found: C, 73.69; H, 8.46. Its spectroscopic characteristics are consistent with those of the same compound reported.[15]

The Secreted Alkaline Phosphatase (SEAP) Assay. Materials and methods applied were similar to those described previously.[11,13,17] COS cells were maintained in Iscove's Modified Dulbecco's Medium supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, 0.25 μg/mL Fungizone, and 100 μg/mL streptomycin. The medium used for dilution of compounds and maintenance of cultures during the assay was the same as above. Cultures were maintained in disposable tissue culture labware at 37 °C in a humidified atmosphere of 5% CO_2 in air. Compounds were dissolved as stock solutions in 100% dimethyl sulfoxide (DMSO) at a concentration of 33.3 mM. The stock solutions were diluted with DMSO and the medium to the desired concentration by a 30-s vortex just before addition into the cell culture. The concentration of DMSO was kept at 0.30% in all dilutions. Triplicate cell samples were seeded at a density of ca. 1.5 × 10^5 cell per well in Linbro 24-well flat-bottom culture dishes (17-mm diameter) and incubated 24 h until they reached 50% confluence. The cells were cotransfected by the calcium phosphate procedure with DNA from plasmids pBC12/CMV/t2 (coding for Tat function, 0.20 μg/well) and pBC12/HIV/SEAP (0.40 μg/well). Cells and DNAs were kept in contact for 18 h, after which time the medium was aspirated and replaced by 500 μL of medium containing the test compound. The compound-treated cells were then incubated for an additional 48 h. At the end of the incubation, an aliquot of cell culture medium was removed and SEAP activities were analyzed as described.[18] The samples of culture medium were heated at 65 °C for 5.0 min to inactivate endogenous phosphatases selectively (SEAP is thermal stable). One hundred microliters of 2 × SEAP buffer (1.0 M diethanolamine, pH 9.8; 0.50 mM $MgCl_2$; 10 mM l-homoarginine) were added to 100 μL of the culture medium sample in a 96-well flat-bottom culture dish (Corning). Then, 20 μL of the substrate solution (120 mM *p*-nitrophenyl phosphate dissolved in 1 × SEAP buffer) was dispensed into each well containing the reaction mixture. The absorbance at wavelength 405 nm specific for the hydrolysis product was read at 2.5-min intervals at 37 °C over the course of 60 min on an EL340i microplate reader (Bio-tek Instruments) with 5-s automatic shaking before each reading. The percent inhibition of SEAP expression was calculated at 60 min as previously described.[11,13]

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