Second Generation, Orally Active, Antimalarial, Artemisinin-Derived Trioxane Dimers with High Stability, Efficacy, and Anticancer Activity

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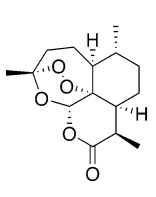
In only two steps and in 63% overall yield, naturally occurring 1,2,4-trioxane artemisinin (**1**) was converted into C-10-carba trioxane conjugated diene dimer **4**. This new dimer was then transformed easily in one additional 4 + 2-cycloaddition step into phthalate dimer **5**, and further modification led to bis-benzyl alcohol dimer **7** and its phosphorylated analogues **8** and **9**. Bis-benzyl alcohol dimer **7** is the most antimalarially active in vitro, 10 times more potent than artemisinin (**1**). Bis-benzyl alcohol dimer **7** is approximately 1.5 times more orally efficacious in rodents than the antimalarial drug sodium artesunate and is about 37 times more efficacious than sodium artesunate via subcutaneous administration. Both dimers **5** and **7** are thermally stable neat even at 60 °C for 24 h. Phthalate dimer **5** is very highly growth inhibitory but not cytotoxic toward several human cancer cell lines; both dimers **5** and **7** very efficiently and selectively kill human cervical cancer cells in vitro in a dose-dependent manner with no cytotoxic effects on normal cervical cells.

Introduction

Some monomeric 1,2,4-trioxanes such as natural artemisinin (**1**) have not only excellent antimalarial activities[1,2] but also significant anticancer activities.[3−6] Recently, efforts to explore the molecular mechanism of action of these monomeric 1,2,4-trioxanes toward tumor cells have established a correlation between a trioxane's potency and mRNA gene expression, cell doubling time, and the portion of cells in different cell cycle phases.[7,8] Also, the importance of transferrin[9−11] for selective cytotoxicity of monomeric 1,2,4-trioxanes toward cancer cells was studied[12,13] to support the hypothesis that the higher iron uptake by rapidly proliferating cancer cells[14,15] is likely responsible for the anticancer activity of trioxanes such as artemisinin. Some dimeric trioxanes as well have very high anticancer activities.[16−21] For example, three-carbon atom linked trioxane dimer alcohol **2** is almost as growth inhibitory toward aggressive prostate cancer C2H cells as the clinically used anticancer agent gemcitabine (Gemzar).[21] We report here design, synthesis, and preliminary biological evaluation of a new series of hydrolytically stable, C-10 nonacetal, four-carbon atom linked trioxane dimers including phthalate **5** and its derivatives **6**−**9**.



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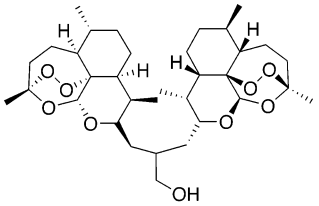

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Results

Scheme 1 illustrates our two-step synthesis of trioxane dimer conjugated diene **4** in overall 63% yield from artemisinin (**1**) via formation of two new carbon−carbon bonds, using the linker 2,3-bis(trimethylsilylmethyl)-1,3-butadiene.[22,23] Our original design of this diene dimer **4** was based on its anticipated ease of preparation; an analogous successful double coupling of an allylic bis-silane with dihydroartemisinin acetate (**3**) proceeds smoothly.[18] Diene dimer **4** was expected to be desirable due to the well-known propensity of 1,3-dienes to enter easily into Diels−Alder cycloadditions[24,25] leading to rigid six-membered carbocycles. Being a conjugated diene, dimer **4** did indeed undergo a 4 + 2 (Diels−Alder) cycloaddition with dimethyl acetylenedicarboxylate followed by dichlorodicyanoquinone (DDQ) oxidation[26] to give phthalate dimer **5**. Bis-ester **5** was hydrolyzed into phthalic acid **6** and separately was reduced into bis-benzyl alcohol **7**. Bis-benzyl alcohol **7** was phosphorylated into bis-phosphate **8** and separately into cyclic phosphate **9** (Scheme 1). Importantly, none of these reactions destroyed the crucial peroxide pharmacophore in trioxane dimers **5**−**9**.

X-ray crystallography of crystalline phthalate diester **5** shows that the two peroxide units in this trioxane dimer are oriented in opposite directions. Whether this structural feature affects the mechanism of action of this dimer remains to be determined. All of the aromatic four-carbon linked dimers **5**−**9** are thermally stable even upon accelerated aging in the absence of solvent at 60 °C for 24 h; less than 5% decomposition was observed by ^{1}H NMR spectroscopy. Of these new trioxane dimers, phthalic acid **6** is by far the most soluble in aqueous pH 7.4 buffer solution (≈14 mg/mL) at 25 °C. As C-10 nonacetal analogues of artemisinin (**1**), all of these trioxane dimers **5**−**9** are hydrolytically stable for at least 4 days in pH 7.4 buffer at 25 °C.

Using our standard assay,[27] we determined the antimalarial potencies of these dimers in vitro against chloroquine-sensitive *Plasmodium falciparum* (NF 54) parasites (Table 1). Except for water-soluble phthalic acid dimer **6**, all of the other dimers in

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Scheme 1

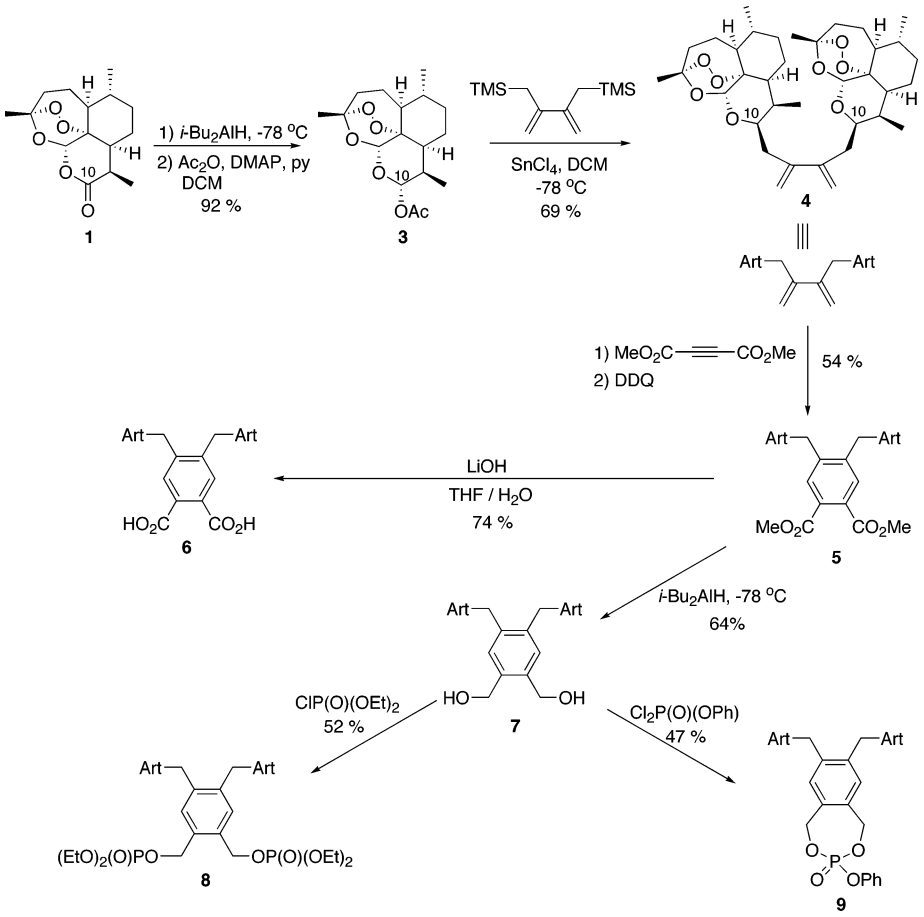

Table 1. Antimalarial Activities in Vitro[a]

trioxane dimer	IC_{50} (nM)
4	2.9
5	1.6
6	360
7	0.77
8	3.0
9	3.7
artemisinin	6.6

[a] The standard deviation for each set of quadruplicates was an average of 7.8% (≤18%) of the mean. R^2 values for the fitted curves were ≥0.967. Artemisinin activity is the mean ± standard deviation of the concurrent control ($n = 6$).

Table 1 are considerably more potent antimalarials than natural artemisinin (**1**, $IC_{50} = 6.6 \pm 0.76$ nM). Bis-benzyl alcohol dimer

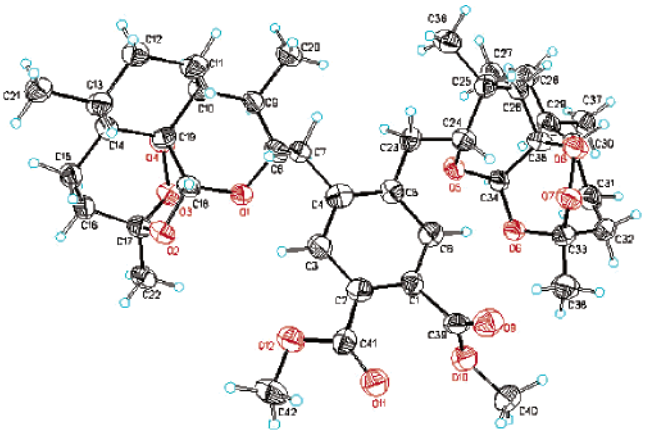

Figure 1. X-ray structure of phthalate dimer **5**.

7 stands out as the most potent, being approximately 10-times more antimalarially active than artemisinin (**1**).

As measured in mice according to a published protocol involving single administration at a dose of 3, 10, or 30 mg/kg, either subcutaneously (sc) or orally (po),[28] bis-ester dimer **5** has sc $ED_{50} = 0.71$ mg/kg and diol dimer **7** has sc $ED_{50} = 0.06$ mg/kg and po $ED_{50} = 2.6$ mg/kg. Under these test conditions, the clinically used monomeric trioxane sodium artesunate has sc $ED_{50} = 2.2$ mg/kg and po $ED_{50} = 4.0$ mg/kg. Thus, these two dimers **5** and **7** are approximately 3−37 times more efficacious than the antimalarial drug sodium artesunate administered sc, and diol dimer **7** is approximately 1.5 times more efficacious than sodium artesunate administered po. Neither overt toxicity nor behavioral modification was observed in the mice due to drug administration.

Preliminary growth inhibitory activities at nanomolar to micromolar concentrations, measured in vitro as described previously using a diverse panel of 60 human cancer cell lines in the National Cancer Institute's (NCI's) Development and Therapeutic Program[29] showed phthalate dimer **5** to be extremely selective and highly potent at inhibiting the growth of only nonsmall cell lung carcinoma HOP-92 cells, melanoma SK-MEL-5 cells, and breast cancer BT-549 cells. Employing a tetrazolium salt (XTT)-based calorimetric proliferation assay (Roche Diagnostics, Mannheim, Germany) and using a modified version of a recently reported protocol for in vitro evaluation of the growth inhibitory activity of DHA toward the human cervical cancer cell line HeLa ($IC_{50} = 5-10$ μM),[30] we have found unexpectedly but importantly that trioxane phthalate dimer **5** ($IC_{50} = 500$ nM) is approximately 10−20 times more potent than trioxane monomer DHA and that trioxane diol dimer **7**

(IC_{50} = 46.5 nM) is approximately 110−220 times more potent than DHA, without being toxic to primary normal cervical cells. Cell growth was inhibited in a dose-dependent manner.

In conclusion, new C-10 nonacetal trioxane dimers **5**−**9**, easily prepared in good overall yields in only three to five steps from artemisinin, are stable and biological promising new chemical entities. The most potent and selective two dimers in this series, diester **5** and especially diol **7**, deserve further preclinical evaluation as potential drug candidates for effective chemotherapy of malaria and cancer.[31]

Experimental Section

Synthesis of Trioxane Butadiene Dimer 4. A solution of dihydroartemisinin acetate (DHA acetate, **3**) (835 mg, 2.56 mmol) and the bis-silane butadiene linker (346 mg, 1.53 mmol, 0.6 equiv) in dichloromethane (45 mL) was cooled to −78 °C. Tin(IV) tetrachloride (1 M solution in CH_2Cl_2, 1.53 mL, 1.53 mmol, 0.6 equiv, diluted in 4 mL of dichloromethane and precooled to −78 °C) was added quickly to the reaction mixture. The reaction was stirred at −78 °C for a further 45 min at which time TLC analysis confirmed complete consumption of starting material. Distilled water (3 mL) was then added, and the reaction was allowed to warm to room temperature. Distilled water (10 mL) and dichloromethane (30 mL) were added, and organics were extracted with dichloromethane (3 × 20 mL), dried ($MgSO_4$), and concentrated in vacuo to give a yellow solid. Gradient column chromatography on silica eluting with 5−10% ethyl acetate/hexanes isolated trioxane butadiene dimer **4** as a white solid (541 mg, 0.88 mmol, 69%). Mp = 68−72 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.35 (s, 2H), 5.19 (s, 2H), 5.16 (s, 2H), 4.48 (ddd, J = 9.6, 6.0, 3.2 Hz, 2H), 2.73−2.63 (m, 2H), 2.55−2.26 (m, 6H), 2.06−1.96 (m, 2H), 1.95−1.86 (m, 2H), 1.86−1.77 (m, 2H), 1.69−1.58 (m, 4H), 1.52−1.20 (m, 14H including singlet at 1.39), 0.96 (d, J = 6.0 Hz, 6H), 0.91 (d, J = 7.2 Hz, 6H), 0.98−0.86 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.07, 113.58, 103.00, 89.16, 81.05, 72.74, 52.23, 44.36, 37.45, 36.64, 34.44, 33.79, 30.46, 25.98, 24.85, 24.73, 20.15, 13.00; IR (film, cm^{-1}) 2991, 2950, 2909, 2854, 1366, 1085, 1044, 872, 729; HRMS(ES) m/z calcd for $C_{36}H_{54}O_8Na$ (M + Na) 637.3711, found 637.3683; $[\alpha]_D^{23.6}$ 65.9 ($CHCl_3$, c = 0.28).

Synthesis of Phthalate Dimer 5. To a solution of trioxane butadiene dimer **4** (235 mg, 0.382 mmol) in anhydrous benzene (12.0 mL) was added dimethylacetylene dicarboxylate (0.094 mL, 0.764 mmol, 2.0 equiv). Then, the reaction mixture was heated to 80−85 °C for 18 h, at which time TLC analysis showed full consumption of starting material. The reaction mixture was cooled to room temperature and treated with dichlorodicyanoquinone (DDQ) (43.4 mg, 0.191 mmol, 0.5 equiv) and heated to 80−85 °C for 20 min. Brine (15 mL) and ethyl ether (30 mL) were added, and organics were extracted with ethyl ether (3 × 30 mL), dried ($MgSO_4$) and concentrated in vacuo to give a yellow sticky solid. Gradient column chromatography on silica eluting with 20−30% ethyl acetate/hexanes isolated bis-trioxane **5** as a white solid (157 mg, 0.208 mmol, 54%). Mp = 108−111 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.62 (s, 2H), 5.40 (s, 2H), 4.56−4.49 (m, 2H), 3.85 (s, 6H), 3.10 (dd, J = 14.8, 9.6 Hz, 2H), 2.82−2.70 (m, 4H), 2.35−2.25 (m, 2H), 2.05−1.82 (m, 6H), 1.74−1.62 (m, 5H), 1.45−1.21 (m, 13H, including singlet at 1.29), 1.00−0.81 (m, 14H, including two doublets at 1.00 (J = 7.6 Hz) and 0.97 (J = 6.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.39, 142.99, 129.95, 129.48, 103.10, 89.17, 81.01, 75.47, 52.41, 52.16, 44.30, 37.45, 36.57, 34.46, 32.83, 30.81, 25.78, 24.75, 20.16, 13.16; HRMS (EI, m/z) for $C_{42}H_{58}O_{12}Na$ calcd 777.3820, found 777.3824; IR (film, cm^{-1}) 2932, 2866, 1726, 1443, 1389, 1284, 1238, 1120, 1054, 988; $[\alpha]_D^{24.5}$ = 112.8 ($CHCl_3$, c = 0.51).

Synthesis of Bis-acid 6. Bis-trioxane phthalate bis-ester **5** (5.3 mg, 7.0 μmol) was dissolved in tetrahydrofuran (0.7 mL) and distilled water (0.3 mL) and treated with lithium hydroxide monohydrate (5.9 mg, 0.14 mmol, 20 equiv). The reaction mixture was stirred for 18 h, at which time TLC analysis showed full consumption of starting material. Hydrochloric acid (0.3%) (10 mL) and ethyl ether (10 mL) were added. Then, aqueous layer was acidified with 10% hydrochloric acid (upon addition white precipitates were shown) and extracted with ethyl acetate (3 × 20 mL), dried ($MgSO_4$), and concentrated in vacuo. Flash column chromatography on silica eluting with 40% ethyl acetate/hexanes (2% acetic acid) to isolated bis-trioxane phthalic acid **6** as a white solid (3.8 mg, 5.2 μmol, 74%). mp = 139−140 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.81 (s, 2H), 5.45 (s, 2H), 4.51−4.45 (m, 2H), 3.20−3.02 (m, 2H), 2.82−2.70 (m, 4H), 2.35−2.25 (m, 2H), 2.05−1.82 (m, 6H), 1.74−1.62 (m, 5H), 1.45−1.21 (m, 14H, including singlet at 1.43), 1.00−0.81 (m, 14H, including two doublets at 1.00 (J = 6.8 Hz) and 0.95 (J = 6.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.66, 143.13, 131.85, 129.48, 103.42, 89.08, 80.91, 75.81, 52.22, 44.40, 37.32, 36.51, 34.47, 30.71, 29.68, 25.68, 24.70, 20.17, 13.30; HRMS (EI, m/z) for $C_{40}H_{54}O_{12}Na$ calcd 7749.3507, found 749.3527; IR (film, cm^{-1}) 3200(br), 2952, 2919, 2879, 1712, 1462, 1383, 1277, 1146, 1047, 994; $[\alpha]_D^{24.4}$ = 79.2 ($CHCl_3$, c = 0.11).

Synthesis of Bis-benzylic Alcohol 7. A solution of bis-trioxane phthalate bis-ester **5** (22.3 mg, 0.030 mmol) in dichloromethane (2.0 mL) was cooled to −78 °C. Diisobutyl aluminum hydride (1.5 M solution in CH_2Cl_2, 0.2 mL, 0.30 mmol, 10 equiv) was added slowly dropwise to the reaction mixture. The reaction was stirred at −78 °C for further 30 min at which time TLC analysis confirmed complete consumption of starting material. Distilled water (0.5 mL) was then added, and the reaction was allowed to warm to room temperature. Distilled water (5 mL) and dichloromethane (15 mL) were added, and organics were extracted with dichloromethane (2 × 20 mL), dried ($MgSO_4$) and concentrated in vacuo to give a yellow oil. Gradient column chromatography on silica eluting with 70−80% ethyl acetate/hexanes isolated bis-trioxane bis-benzyl alcohol **7** as a white solid (13.2 mg, 0.019 mmol, 64%). Mp = 128−130 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.24 (s, 2H), 5.43 (s, 2H), 4.64 (s, br, 4H), 4.49−4.41 (m, 2H), 3.22 (s, br, 2H), 2.95 (dd, J = 15.2, 10.0 Hz, 2H), 2.80−2.68 (m, 4H), 2.35−2.25 (m, 2H), 2.05−1.61 (m, 11H), 1.45−1.21 (m, 13H, including singlet at 1.32), 1.00−0.81 (m, 14H, including apparent triplet at 0.98 (J = 8.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.68, 137.17, 131.12, 103.24, 89.05, 81.01, 76.12, 63.99, 52.28, 44.47, 37.43, 36.58, 34.48, 31.96, 30.74, 25.89, 24.80, 24.71, 20.20, 13.32; HRMS (EI, m/z) for $C_{40}H_{58}O_{10}Na$ calcd 721.3922, found 721.3917; IR (film, cm^{-1}) 3401, 2949, 2875, 1454, 1377, 1205, 1188, 1124, 1090, 1042, 1013, 941, 877, 825, 735; $[\alpha]_D^{23.5}$ = 63.7 ($CHCl_3$, c = 0.10).

Synthesis of Bis-trioxane Bis-ethyl Phosphate 8. To a solution of bis-trioxane bis-benzyl alcohol **7** (20.0 mg, 0.029 mmol) in anhydrous dichloromethane (2.0 mL) were added pyridine (0.012 mL, 0.143 mmol, 5.0 equiv) and diethyl chlorophosphate (0.020 mL, 0.143 mmol, 5.0 equiv) at 0 °C. The reaction mixture was stirred for 30 min at 0 °C and slowly warmed to room temperature for 1 h, at which time TLC analysis showed full consumption of starting material. Brine (5 mL) and dichloromethane (10 mL) were added and organics were extracted with dichloromethane (2 × 20 mL), dried ($MgSO_4$), and concentrated in vacuo to give a sticky solid. Flash column chromatography on silica eluting with 2% methanol/dichloromethane isolated bis-trioxane bis-phosphate **8** as a white foam (14.4 mg, 0.015 mmol, 52%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.28 (s, 2H), 5.43 (s, 2H), 5.12 (d, J = 7.6 Hz, 4H), 4.41−4.36 (m, 2H), 4.12−4.00 (m, 8H), 3.06 (dd, J = 15.2, 9.6 Hz, 2H), 2.81−2.70 (m, 4H), 2.38−2.25 (m, 2 H), 2.01−1.85 (m, 6H), 1.73−1.60 (m, 4H), 1.53−1.19 (m, 26H, including singlet at 1.30), 1.00−0.81 (m, 14H, including doublet at 0.99 (J = 7.6 Hz) and doublet at 0.97 (J = 6.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 140.16, 132.09, 132.02, 131.16, 103.18, 88.84, 80.99, 66.52, 66.47, 63.80, 63.75, 52.32, 44.56, 37.38, 36.58, 34.51, 32.35, 30.72, 25.96, 24.71, 20.20, 16.14, 16.07, 13.45; HRMS (EI, m/z) for $C_{48}H_{76}O_{16}P_2Na$ requires 993.4501, found 993.4503l; IR (film, cm^{-1}) 2965, 2932, 2879, 2860, 1390, 1271, 1027, 974; $[\alpha]_D^{24.6}$ = 96.1 ($CHCl_3$, c = 0.04).

Synthesis of Bis-trioxane Cyclic Phosphate 9. To a solution of bis-trioxane bis-benzyl alcohol **7** (20.0 mg, 0.029 mmol) in anhydrous dichloromethane (2.0 mL) were added pyridine (0.010

mL, 0.129 mmol, 4.5 equiv) and phenyl dichlorophosphate (0.013 mL, 0.086 mmol, 3.0 equiv) at room temperature. The reaction mixture was stirred for 18 h, at which time TLC analysis showed full consumption of starting material. Brine (5 mL) and dichloromethane (10 mL) were added, and organics were extracted with dichloromethane (2 × 20 mL), dried ($MgSO_4$), and concentrated in vacuo to give a sticky solid. Flash column chromatography on silica eluting with 40% ethyl acetate/hexanes isolated bis-trioxane cyclicphosphate **9** as a white solid (11.2 mg, 0.013 mmol, 47%). Mp = 130−133 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.38−7.32 (m, 3H), 7.30−7.25 (m, 2H), 7.22−7.15 (m, 1H), 5.42 (s, 1H), 5.41 (s, 1H), 5.38−5.27 (m, 2H), 5.22−5.11 (m, 2H), 4.60−4.55 (m, 1H), 4.51−4.47 (m, 1H), 2.98−2.91 (m, 2H), 2.79−2.69 (m, 4H), 2.36−2.26 (m, 2H), 2.05−1.81(m, 6H), 1.71−1.60 (m, 6H), 1.45−1.20 (m, 13H, including singlets at 1.31 and 1.30), 1.00−0.81 (m, 14H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 140.09, 140.05, 132.61, 132.47, 130.54, 130.12, 129.82, 125.09, 119.80, 119.75, 103.08, 102.99, 89.45, 89.22, 81.01, 75.26, 74.81, 69.16 (d, J = 4.5 Hz), 69.09 (d, J = 4.5 Hz), 52.19, 52.10, 44.29, 44.19, 37.45, 36.57, 34.41, 32.23, 32.06, 30.84, 25.91, 24.82, 24.76, 24.73, 20.15, 20.13, 13.10, 12.97; HRMS (EI, m/z) for $C_{46}H_{61}O_{10}PNa$ calcd 859.3793, found 859.3793; IR (film, cm^{-1}) 2929, 2881, 1498, 1444, 1389, 1295, 1193, 1125, 1085, 1017, 1010, 935, 738; $[\alpha]_D^{24.1}$ = 28.6 ($CHCl_3$, c = 0.45).

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Supporting Information Available: X-ray crystallographic data for compound **5**. ^{1}H and ^{13}C NMR spectra of compounds **5** and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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