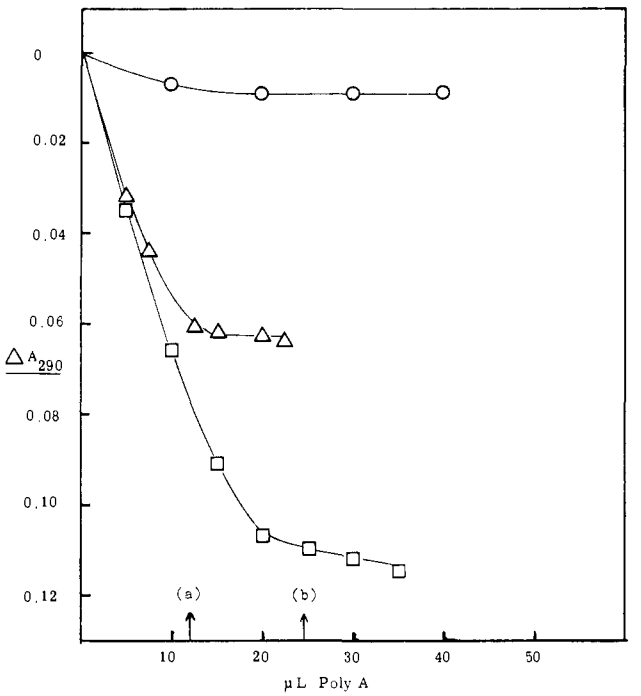

Figure 3. Decrease in absorbance at 290 nm of a solution of ethidium bromide (16 μM) (○), compound **1** (5 μM) (△), and compound **1** (5 μM) + ethidium bromide (14 μM) (□) in 925 μL of Tris buffer (0.01 M, pH 6.8) 0.1 M in NaCl on addition of 5-μL increments of an aqueous solution of poly-A (380 μM). The absorbances have been corrected for volume changes and the small absorbance of poly-A at 290 nm. At the arrows, the A (in poly-A)/d*T* (in **1**) ratio is (1) 0.5 and (2) 1.0.

ester of thymidylyl-(3′-5′)-thymidine ($dT_{Et}T$) (60 μM) has no effect on the spectrum of ethidium bromide (30 μM) in a solution of poly-A (60 μM) at 0 °C, either in the presence or absence of NaCl (0.1 M). Furthermore, spectral data show little or no interaction of **1** with poly-G, poly-C, and poly-U. We therefore believe that the spectral shifts exhibited on addition of poly-A to aqueous solutions of **1** demonstrate formation of a complex stabilized both by specific Watson-Crick base pairing and by an interaction involving a phenanthridinium group favorably positioned by covalent attachment to the thymidine nucleotide. Work is being continued with the objectives of further defining the nature of the interaction of **1** with poly-A and, by extending the oligonucleotide chain, of developing more effective and versatile recognition-delivery systems.

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Preparation and Spectral Properties of Lipophilic Fluorescein Derivatives: Application to Plasma Low-Density Lipoprotein

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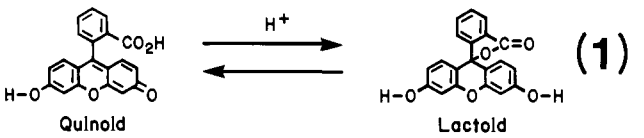
Carrier systems for delivery of analytical probes and pharmacologically active agents in a biological milieu have gained prominence in many applications.[1] However, limitations[2] in the carriers and their ability to shield the transported substance from the surroundings have spurred continued interest in improved systems. Recently, low-density lipoprotein (LDL), the major cholesterol-transport protein in human plasma, has been exploited[3] as a selective and rapid vehicle for delivery of certain classes[4] of lipophilic compounds to mammalian cells in tissue culture. This powerful technique satisfies many of the major objectives[2] for carriers by sequestering compounds inside the delipidated core of LDL during an ordered sequence[5] of binding at a specific cell surface receptor, endocytic internalization, and fusion with lysosomes wherein the LDL core contents are liberated. Molecules are incorporated into the LDL core most readily when they are attached to cis-unsaturated fatty acid esters of cholesterol. We report herein the preparation and some spectral properties of several lipophilic fluorescein derivatives specifically designed[4] for reconstitution into LDL. As a consequence of their unique structure and fluorescence characteristics, these probes are valuable tools[6] in the study of LDL and cholesterol metabolism.

The facile equilibrium (reaction 1) between fluorescein's



(1)

fluorescent quinoid and nonfluorescent lactoid tautomers is reflected in its well-known pH dependent fluorescence.[7] To ensure maximum fluorescence following release from the LDL core into a lysosomal environment[8] (pH ~4), we sought to lock the fluorophore into its quinoid form by esterifying the carboxyl. All attempts, however, to esterify fluorescein with cholesteryl ricinoleate (**1**) using a wide variety of carboxyl activating reagents resulted in complex product mixtures.[9] In contrast, 3-*O*-methylfluorescein (**2**) is esterified readily with **1** using diethyl azodicarboxylate/triphenylphosphine furnishing **3** (78%) as a bright yellow oil[10] [NMR ($CDCl_3$) δ 0.62-2.12 (70 H, complex m), 2.32 (2 H, t, J = 7 Hz), 3.88 (3 H, s), 4.36-5.02 (2 H, m), 5.12-5.44 (3 H, m), 6.38-7.96 (6 H, m), 7.16-7.34 (1 H, m),

(1) For recent reviews, consult: Gregoriadis, G., Ed. "Drug Carriers in Biology and Medicine"; Academic Press: New York, 1979. Gregoriadis, G. *Nature (London)* **1977**, *265*, 407-411.

(2) Gregoriadis, G. In "Liposomes in Biological Systems"; Gregoriadis, G., Allison, A. C., Eds.; Wiley: New York, 1980; Chapter 2. Yoshicami, S.; Nöll, G. N. *Science (Washington, D.C.)* **1978**, *200*, 1393-1394.

(3) Krieger, M.; Goldstein, J. L.; Brown, M. S. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5052-5056. Krieger, M.; Brown, M. S.; Faust, J. R.; Goldstein, J. L. *J. Biol. Chem.* **1978**, *253*, 4093-4101.

(4) Some structural guidelines for compounds to be reconstituted into LDL have been suggested. Krieger, M.; McPhaul, M. J.; Goldstein, J. L., Brown, M. S. *J. Biol. Chem.* **1979**, *254*, 3845-3853.

(5) For a review of LDL and its relation to atherosclerosis and cholesterol metabolism, see: Goldstein, J. L.; Brown, M. S. *Annu. Rev. Biochem.* **1977**, *46*, 897-930.

(6) Naphthalene and pyrene containing sterol probes are restricted in some applications because of a requirement for fluorescence excitation in the UV range. The hydrophobic probe dioleyl fluorescein (DOF) is excited in the visible region; however, reconstituted LDL preparation of DOF displays receptor-independent uptake by cells. For a study using DOF and a pyrene bisnorcholenic ester as well as a discussion concerning the need for refinements in fluorescent probes, see: Krieger, M.; Smith, L. C.; Anderson, R. G. W.; Goldstein, J. L.; Kao, Y. J.; Pownall, H. J.; Gotto, A. M., Jr.; Brown, M. S. *J. Supramol. Struct.* **1979**, *10*, 467-478. Naphthalene: Kao, Y. J.; Soutar, A. K.; Hong, K.-Y.; Pownall, H. J.; Smith, L. C. *Biochemistry* **1978**, *17*, 2689-2696. For a recent example using the nitrobenzoxadiazole fluorophore, consult: Craig, I. F.; Via, D. P.; Mantulin, W. W.; Pownall, H. J.; Gotto, A. M., Jr.; Smith, L. C. *J. Lipid Res.* **1981**, *22*, 687-696.

(7) Martin, M. M.; Lindquist, L. *J. Lumin.* **1975**, *10*, 381-390. Fothergill, J. E. In "Fluorescent Protein Tracing", 3rd ed.; Nairn, R. C., Ed.; Williams and Wilkins Co.: Baltimore, 1969; Chapter 2.

(8) Vaes, G. In "Lysosomes and Storage Diseases"; Hers, H. G., Van Hoof, F., Eds.; Academic Press: New York, 1973; Chapter 2.

(9) The chemistry of fluorescein has been reviewed. Wawzonek, S. In "Heterocyclic Compounds"; Elderfield, R. C., Ed.; Wiley: New York, 1951; Vol. 2, Chapter 13.

(10) Satisfactory IR, NMR, and elemental analysis data were obtained on chromatographically homogeneous samples unless otherwise noted. Mass spectral data were obtained for compounds with M_r 1000.

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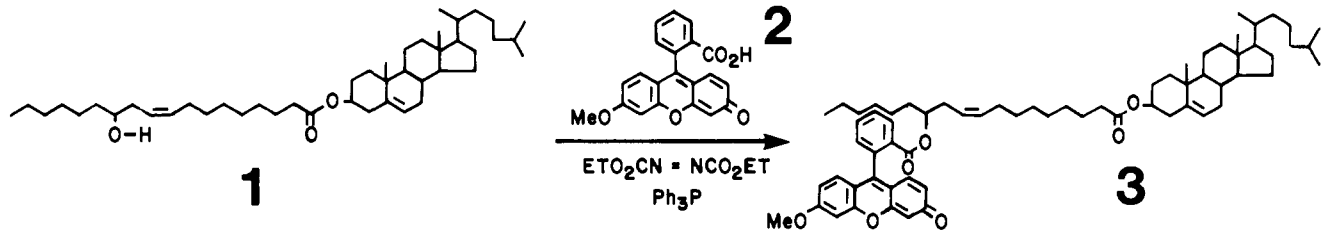
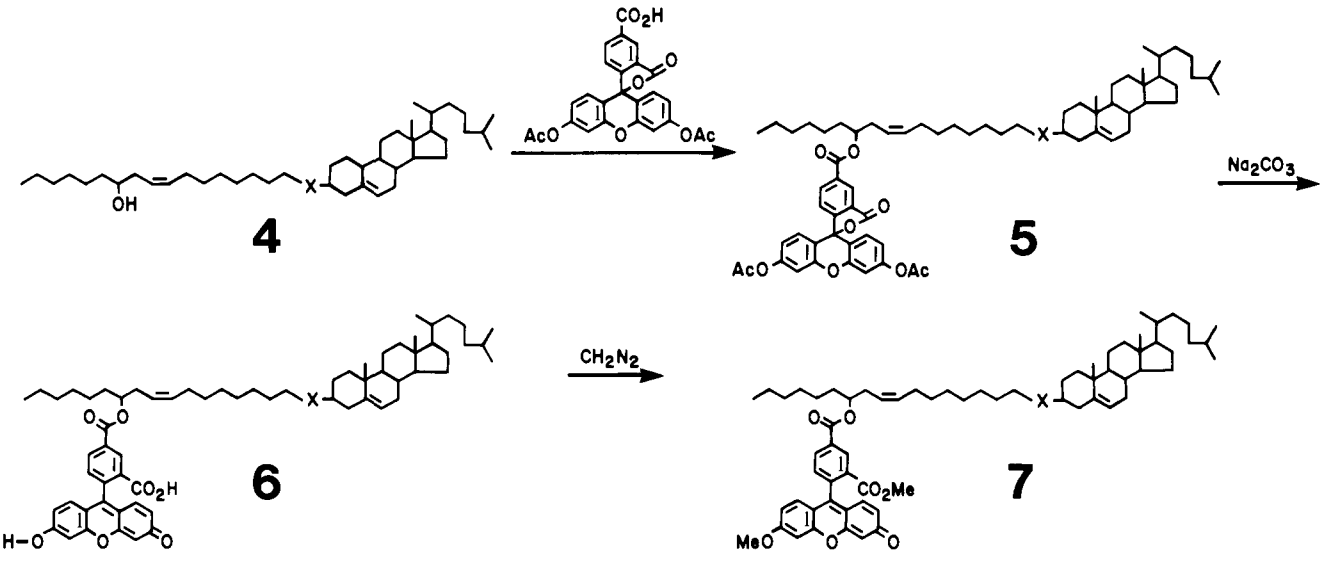


Scheme I



a: X = $-CH_2OCO_2-$
b: X = $-CO_2-$
c: X = $-CH_2O-$

Table I. Spectral Properties of Fluorescein and Derivatives

compd	λ_{max},[a] nm	$\epsilon_{max} \times 10^3$ ETOH	$\epsilon_{max} \times 10^3$ 5% HOAc/ETOH	relative fluorescence[b] (relative quantum yield[c]) ETOH	relative fluorescence[b] (relative quantum yield[c]) 5% HOAc/ETOH
fluorescein	499[d]	94[d]	<1	290[d] (1[d])	3
6[e]	507[d]	84[d]	<1	163[d] (0.97[d])	1
7[e]	458	20	20	38 (0.12)	39 (0.13)
3	455	28	28	55 (0.13)	57 (0.13)

[a] Measured in 95% ETOH at 5×10^{-6} M on a Cary 118 with 95% ETOH blank. [b] Perkin Elmer MPF-44A in the energy mode (uncorrected): excitation 458 nm, slit width 1 nm; emission 530 nm, slit width 6 nm. Fluorescence maximum for 7 at 524 nm and 3 at 515 nm in 95% ETOH; fluorescein at 516 nm and 6 at 530 nm in 0.1 M KOH/ETOH. [c] Excitation 458 nm, slit width 1 nm; emission 530 nm, slit width 6 nm. Extinction coefficient ($\times 10^3$) at 458 nm in ETOH and 5% HOAc/ETOH, respectively: fluorescein, 19, <1; 6, 11, <1; 7, 20, 20; 3, 28, 28. [d] Measured in 0.1 M KOH/ETOH to enhance absorption and fluorescence. The spectra of 7 and 3 are unaffected by added base. [e] Analogues a–c have virtually identical spectra.

7.54–7.80 (2 H, m), 8.12–8.30 (1 H, m); IR ($CHCl_3$) 1720, 1645 cm^{-1}].

An alternative approach to quinoid fluorescein derivatives uses a masked fluorescein (Scheme I). Esterification of 5(6)-carboxydiacetylfluorescein[11] with **1** using dicyclohexylcarbodiimide or diethyl azodicarboxylate/triphenylphosphine yields **5b** (77–84%) which is hydrolyzed quantitatively (Na_2CO_3, 30 h) to acid **6b**, isolated as a mixture of 5- and 6-carboxy isomers separable by chromatography (SiO_2, 2:1 ether/hexane, $R_f \sim 0.14$ and 0.16). Treatment of **6b** with excess ethereal CH_2N_2 and chromatography (SiO_2, ether, two elutions, $R_f \sim 0.36$) affords bright yellow ester **7b** (64%) [NMR ($CDCl_3$) δ 0.62–2.64 (72 H, complex m), 3.64 and 3.66 (3 H, s, isomeric OCH_3), 3.91 (3 H, s), 4.40–4.82 (1 H, m), 4.98–5.25 (1 H, m), 5.26–5.63 (3 H, m), 6.44–7.06 (6 H, m), 7.34–8.85 (3 H, m); IR ($CHCl_3$) 1730, 1645 cm^{-1}]. The corresponding carbonate and ether, **7a** and **7c**, respectively, were prepared analogously from **4a** (made from ricinoleyl alcohol, cholesteryl chloroformate, pyridine, 0 °C, 85%) and **4c** (prepared from 12-*O*-tetrahydropyranylricinoleyl tosylate, sodium cholesterylate, and then 0.1 N HCl, 82%; mp 52–53 °C).

The salient spectral properties of fluorescein and its derivatives are shown in Table I. In basic ETOH, the λ_{max} of acid **6** is slightly red shifted relative to fluorescein, and its absorption and fluorescence intensities are lower. Absorption and fluorescence for both are reduced dramatically in 5% HOAc/ETOH. In comparison, esters **3** and **7** are less fluorescent under neutral and basic conditions, but, importantly, are approximately 40–50 times more fluorescent in acidic ETOH. Experiments show **3** and **7** are readily reconstituted into LDL and retain their fluorescence in cellular lysosomes after delivery to mammalian cells.[12,13] Ad-

(11) Available from Molecular Probes, Inc., Plano, TX, as a mixture of 5 and 6-carboxy isomers. Although the isomers could be resolved by TLC and NMR spectroscopy, the mixture was used without separation in subsequent steps. For convenience, only one isomer is shown.

(12) Specific receptor-dependent incorporation of these probes into mammalian cells using reconstituted LDL and their concentration in the perinuclear region has been confirmed by fluorescence microscopy. When used in conjunction with a fluorescence-activated cell sorter, human lymphocyte populations with differing levels of LDL receptor activity may be distinguished and quantified. The details of these experiments and their implications in the study of the LDL pathway and cholesterol metabolism will be reported elsewhere.

(13) This research was supported by USPHS Research Grant P01-HL-20948 from the National Institutes of Health. M.K. was the recipient of a USPHS Postdoctoral Fellowship, HL-05657. Professor Julian Peterson and Dr. Peter Roome are thanked for their assistance in recording absorption spectra.

ditionally, they provide valuable insight into the structural range suitable for incorporation into LDL. It is expected that the compounds described here will be useful adjuncts in other areas where highly fluorescent, lipophilic probes are required.

A Stereospecific Synthesis of Highly Substituted Tetrahydrofurans

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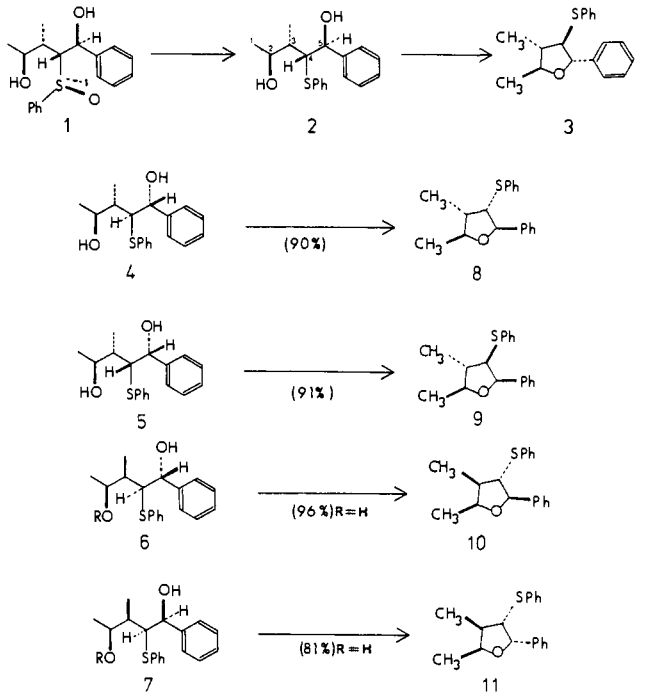
In recent years, keen interest has developed in the preparation of complex dihydro- and tetrahydrofurans, many of which possess valued biological properties.[1] Numerous representative substances display challenging stereochemical arrangements about the heterocyclic ring demanding stereoselective methodologies for construction.[2] Several elegant studies have led to the total syntheses of ionophore antibiotics.[3] Our investigations toward related natural products have uncovered a remarkably facile transformation of acyclic precursors to tetrasubstituted tetrahydrofurans with complete stereospecificity.

We have recently reported stereoselective condensations of α-lithiosulfinyl carbanions with aldehydes demonstrating useful methodology for construction of 1,3-asymmetric relationships in acyclic systems.[4] Stereochemical features of a principal sulfoxide adduct **1**, resulting from condensation with benzaldehyde, have been confirmed by X-ray crystallography,[4] and reduction with borane in tetrahydrofuran at 22 °C (24 h) gave the phenyl sulfide **2** (96%). Treatment of **2** with dimethyl sulfate (CH_2Cl_2, 0 °C, 10 min, under N_2) cleanly afforded cyclization to the tetrahydrofuran **3** in 85% yield. Other methylating agents such as methyl triflate, methyl fluorosulfonate (added at −78 °C to a methylene chloride solution of sulfide with warming to 0 °C), and methyl iodide (benzene at reflux) also provided ring closure. Likewise, the isomeric sulfides **4**, **5**, **6** (R = H), and **7** (R = H) were individually submitted to the reaction conditions, yielding tetrahydrofurans **8–11**, respectively.[5]

Although spectroscopic data supported the products, it is widely recognized that coupling constants of vicinal protons in these heterocycles often fail to offer a completely reliable basis for stereochemical assignments. Dehydration of the acyclic sulfides could reasonably occur with loss of either hydroxyl group (at C-2 or C-5). One might anticipate preferred loss of the benzylic alcohol owing to a more favorable stabilization of charge in transition state intermediates. However, our experiments also demonstrated the stereospecific cyclization of benzylic ethers **6** (R = $CH_2C_6H_5$) and **7** (R = $CH_2C_6H_5$), under the usual conditions of *S*-methylation, affording tetrahydrofurans **10** (70%) and **11** (72%), respectively, as previously obtained from their corresponding alcohols.[6] Similar dehydration processes have been



previously reported. For example, 1,4-butanediol is smoothly converted into tetrahydrofuran upon reaction with a diaryldialkoxysulfurane, $Ph_2S(OC(CF_3)_2Ph)_2$ (10–20 min at room temperature).[7] Stereospecific epoxide formation is observed from 1,2-diols under these conditions. More recently, the acid-catalyzed ring closure of *rac*- and *meso*-2,5-hexanediol has been shown to proceed with inversion of configuration, affording *cis*- and *trans*-2,5-dimethyltetrahydrofuran, respectively.[8] The stereochemical features of our products were unambiguously assigned by X-ray crystallography of the sulfone of cyclic ether **11**, thus demonstrating net retention of configuration at each of the four asymmetric carbon centers.[9]

In addition, the phenyl ring (originally derived from benzaldehyde) may be replaced by an alkyl substituent without affecting the course or ease of the reaction. Condensation of the α-lithiosulfinyl carbanion of 1-[2(*S*)-methyl-3(*S*)-hydroxy]butyl phenyl (*R*)-sulfoxide with 3-methylbutanal gave two major adducts which were chromatographically separated (silica gel) and reduced with borane in tetrahydrofuran. Treatment of each of these

(1) For a review: Semple, J. E.; Joullié, M. M. *Heterocycles* **1980**, *14*, 1825.

(2) Some recent developments not included in ref 1: Amouroux, R.; Folefoc, G.; Chastrette, F.; Chastrette, M. *Tetrahedron Lett.* **1981**, *22*, 2259. Suzuki, H.; Yashima, H.; Hirose, T.; Takahashi, M.; Moro-oka, Y.; Ikawa, T. *Ibid.* **1980**, *21*, 4927. Ireland, R. E.; Vevert, J.-P. *J. Org. Chem.* **1980**, *45*, 4260. Bartlett, P. A.; Jernstedt, K. K. *Tetrahedron Lett.* **1980**, *21*, 1607.

(3) Ireland, R. E.; Thaisrivongs, S.; Wilcox, C. S. *J. Am. Chem. Soc.* **1980**, *102*, 1155. Fukuyama, T.; Akasaka, K.; Karanewsky, D. S.; Wang, C. L. J.; Schmid, G.; Kishi, Y. *Ibid.* **1979**, *101*, 260–262. Collum, D. B.; McDonald, J. H., III; Still, W. C. *Ibid.* **1980**, *102*, 2119, 2120.

(4) Williams, D. R.; Phillips, J. G.; Huffman, J. C. *J. Org. Chem.* **1981**, *46*, 4101. Stereochemical arrangements, as represented by phenyl sulfides **1** and **6**, are readily available.

(5) All compounds are racemic, and purified samples were characterized by infrared, nuclear magnetic resonance, and mass spectral analysis. Infrared and mass spectral data are unexceptional and not useful for isomer identification of these substances. The ^1H NMR spectra were recorded on a 220-MHz instrument in $CDCl_3$ (0.1% Me_4Si) solutions. Partial characterization is as follows. Ether **3**: ^1H NMR δ 7.23 (10 H), 4.82 (d, J = 9 Hz, 1 H), 3.92 (m, 1 H), 3.03 (dd, J = 11 Hz, J = 9 Hz, 1 H), 1.84 (m, 1 H), 1.30 (d, J = 7 Hz, 3 H), 1.11 (d, J = 7 Hz, 3 H); mass spectrum, *m/e* 284.8 (18.3%) M^+, 283.8 (85.8%), 177.8 (100%), 109.9 (63.1%), 68.9 (51.9%). Ether **8**: ^1H NMR δ 7.25 (10 H), 4.85 (d, J = 5 Hz, 1 H), 3.91 (m, 1 H), 3.70 (dd, J = 8 Hz, J = 5 Hz, 1 H), 2.19 (m, 1 H), 1.41 (d, J = 6 Hz, 3 H), 1.11 (d, J = 7 Hz, 3 H). Ether **9**: ^1H NMR δ 7.23 (10 H), 5.22 (d, J = 7.5 Hz, 1 H), 3.66 (m, 2 H), 1.87 (m, 1 H), 1.43 (d, J = 7 Hz, 3 H), 1.09 (d, J = 6 Hz, 3 H). Ether **10**: ^1H NMR δ 7.25 (10 H), 4.67 (d, J = 7.5 Hz, 1 H), 4.32 (m, 1 H), 3.09 (t, J = 7 Hz, 1 H), 2.30 (m, 1 H), 1.27 (d, J = 6 Hz, 3 H), 1.05 (d, J = 7 Hz, 3 H). Ether **11**: ^1H NMR δ 7.13 (10 H), 5.18 (d, J = 8 Hz, 1 H), 4.23 (m, 1 H), 4.06 (t, J = 8 Hz, 1 H), 2.69 (m, 1 H), 1.34 (d, J = 6 Hz, 3 H), 1.11 (d, J = 7 Hz, 3 H).

(6) Benzyl ethers **6** and **7** were obtained by condensation of the α-lithiosulfinyl carbanion available from 1-[2(*R*)-methyl-3(*S*)-benzyloxy]butylphenyl(*R*)-sulfoxide (LDA, THF, −78 °C) with benzaldehyde.

(7) Martin, J. C.; Franz, J. A.; Arhart, R. J. *J. Am. Chem. Soc.* **1974**, *96*, 4604.

(8) Molnár, Á.; Felföldi, K.; Bartók, M. *Tetrahedron* **1981**, *37*, 2149.

(9) Full details of the X-ray crystallographic study of the sulfone derived from tetrahydrofuran **11** will be published elsewhere and are also available from the Indiana University Chemistry Library. Request Molecular Structure Center Report 81053.

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