$Na_2S_2O_3$. Relative peroxide value against vitamin E (POV) was calculated.

Inhibition of Lipid Peroxide Formation (m-LPO). This was investigated by a method similar to that described by Malvy et al.[22] (rat liver microsomes, ferrous sulfate/cysteine).

Effects on Hyperlipoperoxidemia and Hyperlipidemia (ALLOXAN). Male BALB/c mice were used at the age of 8 weeks. The animals were fasted for 18 h, after which 75 mg/kg of alloxan was administered intravenously. Each of the test compounds was administered orally at a dose of 300, 200, 100, 50, or 10 mg/kg body weight 30 min before and 24 and 30 h after administration of alloxan. Blood was collected from an incision in the cervical region 48 h after administration of alloxan. The collected amount of blood was 100 or 200 μL. Then whole blood was diluted 10 or 20 times with a saline solution and centrifuged (3000 rpm, 10 min) to determine lipid content.

LPO was measured by the TBA method.[5] CHOL and TG were measured according to the enzyme method. A Determiner TC (a registered trade mark of Kyowa Medix) kit was used to measure CHOL, and a Triglyceride Measuring Agent (GPO-*p*-chlorophenol color developing method) (Wako Pure Chemical Industries) kit was used for TG.

As a control, the procedure was repeated, except that no test compound was administered.

Effects on Hyperglycemia (KK-MICE). Male KK-mice were housed in individual cages at the age of 8 weeks. They were used for the experiment when their body weight was more than 40 g at the age of about 4–5 months.

Test compound was finely suspended in 0.5% (carboxymethyl)cellulose saline (vehicle). Each of the test compounds was administered orally at a dose of 150 or 50 mg/kg body weight 18 h before blood sampling. Blood was collected from the tail vein in a heparinized hematocrit tube and then centrifuged and plasma was separated to measure blood glucose. Plasma glucose level was determined by a glucose analyzer (Mitsubishi Kasei Co., Ltd, Model-101).

As a control, the same test was done simultaneously after administration of the vehicle.

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Registry No. 19, 62516-91-0; **20**, 62-56-6; **25**, 97322-69-5; **27**, 97322-87-7; **28**, 700-13-0; **29**, 112109-69-0; **30**, 112109-70-3; **31**, 112136-00-2; **32**, 112109-68-9; **33**, 112109-67-8; **34**, 112109-65-6; **35a**, 112109-66-7; **35b**, 112109-74-7; **37**, 79907-49-6; **38**, 107188-55-6; **39**, 118070-84-1; **40a**, 107188-58-9; **40b**, 118070-83-0; **41**, 107188-37-4; **42**, 97322-68-4; **43**, 97323-08-5; **44**, 97322-88-8; **45**, 97322-94-6; **46**, 97323-07-4; **47**, 97322-98-0; **48**, 97322-92-4; **49**, 97322-97-9; **50**, 97322-91-3; **51**, 97323-01-8; **52**, 97323-02-9; **53**, 97323-00-7; **54**, 97322-93-5; **55**, 97322-99-1; **56**, 97322-96-8; **57**, 97323-04-1; **58**, 97323-05-2; *p*-chloronitrobenzene, 100-00-5; 1,3-dibromopropane, 27072-47-5; pivaloyl chloride, 3282-30-2.

Studies on Bioactive Compounds. 13.[1] Synthesis and Lack of Growth-Inhibitory Properties of Cyclohexane-1,2,4-triol 1,2-Diesters, Which Resemble Ring C of the Phorbol Ester Molecule

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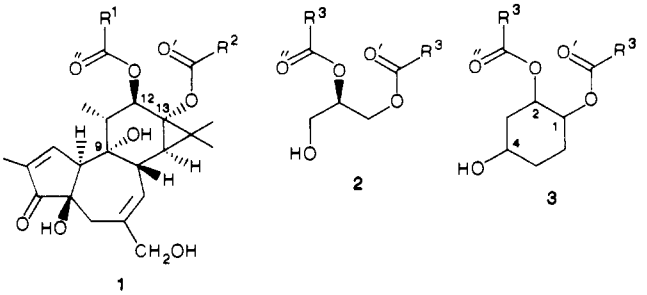
It has been suggested that ring C of biologically active phorbol esters is an essential structural feature of the pharmocophore which confers activity on these compounds. In this study the hypothesis has been tested that compounds which resemble ring C of the phorbol ester molecule mimic the ability of phorbol esters to inhibit cell growth at nontoxic concentrations. All four diastereoisomers of (±)-1,2-di-*O*-octanoylcyclohexane-1,2,4-triol have been prepared from cyclohexen-4-ol and tested for growth-inhibitory and cytotoxic properties. The phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate inhibited the growth of A549 human lung carcinoma cells by 50% at a concentration of 0.2 nM and exerted cytotoxicity at concentrations of >1 μM. Diacylglycerols are the physiological ligands and activators of protein kinase C, the receptor via which phorbol esters are thought to mediate their effects. The diacylglycerols 1-oleoyl-2-acetylglycerol and 1,2-dioctanoylglycerol and the cyclohexanetriol diesters inhibited the growth of A549 cells only at concentrations of 10^{-5} to 10^{-4} M, at which they were also cytotoxic. A computer-assisted analysis of the goodness of fit between the cyclohexanetriol diesters and ring C of the phorbol moiety revealed possible energetic grounds for conformational dissimilarities. The results suggest that activation of protein kinase C alone is probably not sufficient to reproduce phorbol ester induced growth arrest in A549 cells and that the cyclohexanetriol diesters may lack pivotal elements of the phorbol ester pharmacophore.

The multitude of recent studies on the mechanism by which tumor-promoting phorbol esters, of which 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 1; see Scheme I) is the most potent derivative, exert their pleiotropic effects in biological systems have left many intriguing questions unanswered. There is now little doubt about the contention that the ability of these compounds to bind to their receptor, the ubiquitous calcium and phospholipid-dependent enzyme protein kinase C (pkC), plays a pivotal role in the generation of their biological effects.[2,3] However it is not clear whether the diverse responses to TPA, such as, for example, induction or mitogenesis,[4] inhibition of growth,[5] and induction or inhibition of differentiation,[6] are

Scheme I



1 2 3

R^1 = Me, R^2 = $Me(CH_2)_{12}$, R^3 = $Me(CH_2)_6$

(1) For paper 12, see: Cunningham, B. D. M; Lowe, P. R.; Threadgill, M. D. *J. Chem. Soc., Perkin Trans. 2*, in press.
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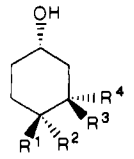
Scheme II



3a: $R^1 = R^4 = H$, $R^2 = R^3$ = octanoyloxy
b: $R^2 = R^3 = H$, $R^1 = R^4$ = octanoyloxy
c: $R^1 = R^3 = H$, $R^2 = R^4$ = octanoyloxy
d: $R^2 = R^4 = H$, $R^1 = R^3$ = octanoyloxy

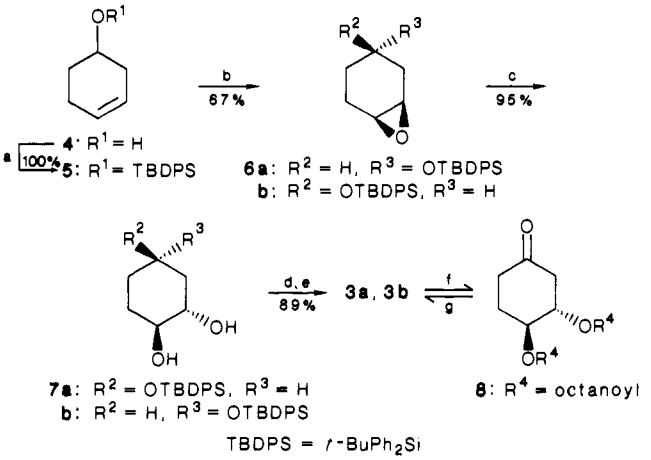
caused by the same or by different biochemical events. Extensive structure–tumor promotory activity studies have defined the importance of several structural features of the phorbol ester molecule for tumor promotion.[4] The ability of TPA to activate pkC has been suggested to be linked to the diester portion of the molecule which bears a structural resemblance to diacylglycerols (e.g. dioctanoylglycerol, DiC_8, **2**), the physiological ligands of pkC.[2] However, it is unclear to what extent the structure–activity relationships valid for the tumor-promotory efficacy hold for other biological effects of phorbol esters.

We have been particularly interested in the ability of phorbol esters to cause growth inhibition at nontoxic concentrations.[7] This property suggests that the interactions between phorbol esters and some of their biochemical targets might be exploited for the therapeutic intervention with proliferative diseases. As part of a program of rational drug design we wished to determine relationships between chemical structure and growth-inhibitory activity associated with phorbol esters with the ultimate aim to exploit this property therapeutically. The structural simplicity of the diacylglycerols suggests that, firstly, only a few of the functional groups present in phorbol esters are involved in the binding interaction with the receptor protein and that, secondly, these functionalities lie close to each other. One candidate partial structure for consideration consists of the hydroxyl group at C-9 and the two acyloxy groupings at C-12 and C-13 which are disposed around ring C of the phorbol esters. On the basis of this hypothesis, we report here the synthesis of cyclohexane-1,2,4-triol 1,2-diesters, **3**, as stripped-down versions of this ring. These compounds also mimic a diacylglycerol molecule upon which severe conformational restrictions have been imposed. The degree of similarity of the newly synthesized cyclohexanetriol esters to ring C of phorbol esters has been assessed by computer-assisted modeling. Furthermore the ability of the cyclohexanetriol derivatives to inhibit cell growth or to exert cytotoxicity has been tested with the A549 human lung carcinoma cell line, which is exquisitely sensitive to the growth-inhibitory potential of TPA.[7]

Chemistry

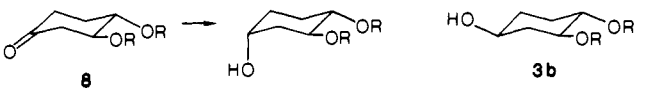
The stereochemistry of the oxygen substituents about the C ring of phorbols corresponds to that of the 1*R*,2*R*,4*S* isomer of cyclohexane-1,2,4-triol. The synthetic target chosen was the 1,2-dioctanoate derivative **3a** in view of the superior pkC activating potency of 1,2-dioctanoylglycerol (DiC_8) as compared to other glycerol diesters.[8] However,

Scheme III[a]



[a] (a) TBDMSCl, imidazole, DMF; (b) mcpba, CH_2Cl_2; (c) $Mo(CO)_6$, H_2O, dioxane; (d) octanoyl chloride, pyridine, $CHCl_3$; (e) TBAF, THF; (f) PCC, CH_2Cl_2; (g) $NaBH_4$, EtOH, or K-Selectride, THF.

Table I. Reduction of Ketone 8



R = octanoyl

reducing agent	ratio 3a:3b[a]
$NaBH_4$	15:85
L-Selectride	92:8

[a] Determined by GLC.

all four diastereoisomers of this target (Scheme II) are of interest as a structure–activity relationship is being sought. All are potentially available from a suitably protected 4-cyclohexenol (**4**); the *tert*-butyldiphenylsilyl derivative **5** was chosen as it was hoped that the bulky protecting group would encourage diastereoselectivity in reactions at the double bond, despite its remoteness. The required cyclohexenol was synthesized in racemic form from the mixed cyclohexane-1,4-diols.[9] The chiral material is not available; however, it is known that, at least in the case of diacylglycerols, the presence of the unnatural isomer in assays does not affect the activity of the natural isomer;[10] therefore an enantiospecific synthesis was not attempted at this stage.

After silylation (Scheme III), epoxidation of **5** (mcpba) yielded the two epoxides **6a** and **6b** in a ratio of 11:5, respectively. The stereochemistry of the products, which were not separated, was determined from their ^1H NMR spectra and confirmed by the unambiguous synthesis of isomer **6b** via the *tert*-butyl hydroperoxide/$Mo(CO)_6$ epoxidation of cyclohexenol **4**.[11] No attempt was made to separate the epoxide isomers as it was expected that the hydrolysis of either would produce largely diol **7a**. The preferred mode of trans-diaxial ring opening should be that which maintains the bulky silyloxy substituent in an equatorial conformation, i.e. attack at C-1 for isomer **6a** and at C-2 for isomer **6b**. Examination of the literature did, however, reveal a similar case where this appeared not

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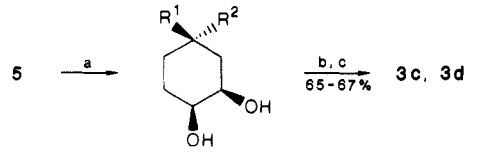
Scheme IV[a]



7c: R^1 = OTBDPS, R^2 = H (28%)
d: R^1 = H, R^2 = OTBDPS (36%)

[a] (a) OsO_4, *N*-methylmorpholine *N*-oxide, Me_2CO, H_2O; (b) octanoyl chloride, pyridine, $CHCl_3$; (c) TBAF, THF.

Table II. Growth-Inhibitory Properties and Cytotoxicities of TPA, Mezerein, Diacylglycerols, and Dioctanoylcyclohexanetriols

compd	growth inhibition: IC_{50},[a] μM	cytotoxicity: LC_{50},[b] μM
TPA	0.002 (0.0001, 0.0004)[c]	>1
OAG	70 (60, 81)	75 (67, 82)
DiC_8	44 (35, 53)	56 (42, 68)
3a	43 (27, 66)	36 (30, 41)
3b	58 (50, 69)	46 (43, 49)
3c	21 (17, 24)	36 (30, 41)
3d	49 (47, 53)	31 (9, 47)

[a] Concentration which caused half-maximal growth inhibition determined by cell counting. [b] Concentration which caused half-maximal LDH release. [c] Values were obtained by linear regression analysis of the linear portion of the concentration–response curve constructed with values measured at at least five concentrations in each of four separate experiments. Values in parentheses are the 90% confidence limits.

to have been the case.[12] Hydrolysis of the mixed epoxides yielded the two *trans*-diols **7a** and **7b** in a ratio of 9:2, respectively. The two isomers were inseparable by TLC, flash chromatography, or HPLC, and the stereochemistries were determined by ^1H NMR: the downfield shifts of protons involved in 1,3-diaxial interactions with oxygen functionalities proved particularly informative in this and other analyses.[13] Octanoylation of the mixed diols followed by deprotection yielded the two target isomers **3a** and **3b**, which were now readily separable by flash chromatography. Both isomers could be oxidized to give the same ketone **8**. The results of the reduction of **8** with sodium borohydride and K-Selectride (Aldrich) provide further support for the original stereochemical analysis of the epoxide opening reaction (Table I).[14]

The second pair of target isomers were approached (Scheme IV) via the cis-hydroxylation of **5**, which gave diols **7c** and **7d** in a ratio of 7:6 as established by HPLC. In this case the diols could be separated by careful flash chromatography. The relative stereochemistries of the products were again deduced by ^1H NMR. Dioctanoylation of **7c** followed by desilylation gave target isomer **3c**. Similar treatment of **7d** led to the final target isomer **3d**.

Biological Properties

The growth-inhibitory and the cytotoxic potentials of the cyclohexanetriol diesters were investigated in the human A549 lung carcinoma cell line and compared with those of TPA and the diacyglycerols OAG and DiC_8. The effect of the compounds on cell growth was assessed by cell counting (Table II) and by measurement of DNA synthesis after exposure to the test compounds (results not shown). Both methods furnished virtually identical IC_{50} values. Cytotoxicity was quantified by assaying the leakage of lactate dehydrogenase (LDH) from cells into the culture medium. Table II shows that, in accordance with a previous report,[7] TPA inhibited cell growth at concentrations below 1 nM, whereas cytotoxicity was only evident at micromolar concentrations. Neither the diacylglycerols nor any of the cyclohexanetriol esters inhibited cell growth at noncytotoxic concentrations. They also failed to elicit the change in cell morphology (result not shown) which accompanied phorbol ester induced growth inhibition.[7]

Studies involving the repeated addition of DiC_8 (100 μM) to cultures revealed that its cytotoxicity was dependent on cell density. At densities of 2×10^3 or 1×10^4 cells/mL LDH leakage was 56 ± 4% and 54 ± 14%, respectively (n = 4), of total enzyme release achieved by detergent, whereas at cellular concentrations of 0.5×10^5 or 9×10^5 cells/mL LDH release was decreased to 16 ± 18% and 12 ± 8%, respectively (n = 4).

The cytotoxicity of the cyclohexane triol diesters showed little variation.

Table III. Energetic Penalties Associated with the Fitting of the Cyclohexanetriol Diacetates to the Low-Energy Conformation of Phorbol Diacetate

compd	energy, kcal: unrestrained	fitted	difference
3a	58.7	63.4	4.7
3b	57.9	112.4	54.5
3c	68.6	79.8	11.2
3d	67.1	107.1	40.0

Computer-Assisted Modeling

In a preliminary assessment of the degree of resemblance between the cyclohexanetriol diesters and ring C of the phorbol ester molecule, the spatial relationship between the relevant oxygen atoms was investigated. Attempts were made to superimpose the O-1, O-2, O-4 oxygens of the cyclohexane-1,2,4-triol diesters over the O-13, O-12, and O-9 oxygens, respectively, of the phorbol ester molecule. Both chair conformations of each isomer were examined, and the goodness of fit, defined as the minimum mean separation between corresponding oxygen atoms in the two molecules, was determined (results not shown). Derivatives **3a** and **3d** exhibited the best fit, with a minimum mean separation between correlated atoms of 0.36 Å. In view of the biological results, a more detailed computer-aided conformational analysis of the diol diester portion of both the cyclohexanetriol diesters and TPA was undertaken. Analysis of the phorbol ester revealed a single low-energy conformation of the diester moiety. The atoms that are hypothesized as playing a key role in the hydrogen bonding of the molecule to the enzyme are the carbonyl oxygens O′ and O″ and the hydroxyl hydrogen OH-9 (see Scheme I). Each cyclohexanetriol diester isomer was minimized subject to the restraints that the separations between the corresponding atoms, O′, O″, and OH-4, lay within 0.1 Å of the values for the phorbol ester. Table III lists, for each conformation, the difference in energy between the conformation thus found and the unconstrained minimum-energy conformation. The conformational analysis of the phorbol ester revealed a particularly short distance (3.00 Å) between O′ and OH-9, and it seems possible that the 13-acetyl and C9-hydroxyl groups interact via hydrogen bonding. It is the difficulty that the cyclohexanetriol diesters have in adopting a conformation with a correspondingly short O′ to OH-4 separation that mainly accounts for the large energy differences between the constrained and unconstrained conformational minima that

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are observed, and particularly when O′ and OH-4 are attached to opposite faces of the cyclohexane ring (isomers **3b** and **3d**).

Discussion

Recently two models have been advanced[15,16] which explain in terms of common structural features the resemblance in biological properties between TPA and other tumor promoters and pkC activators. Both models attempt to establish the relationship in space between various functional groups in these compounds. The guiding hypothesis in the chemical synthesis conducted in this project is based on the original proposal[3] that the vicinal diester moiety is a molecular feature crucial for the activity of both phorbol esters and diacylglycerols, and that the pharmacophore common to TPA and other activators of pkC contains two hydrogen bond acceptors (the diester carbonyl oxygens), one hydrogen bond donor (an alcoholic hydroxyl) and a hydrophobic region, suitably disposed.[8] The cyclohexane part of the TPA molecule (ring C) was chosen as the basic structural skeleton, and the four novel stereoisomeric 1,2,4-cyclohexanetriol diesters **3a–d** were synthesized.

When tested in cultures of A549 carcinoma cells, the newly synthesized compounds were found to lack the growth-inhibitory properties of TPA at nontoxic concentrations. This result suggests that either the structural similarity with phorbol esters achieved in these compounds is inconsequential for activity or, alternatively, that the hypothesis which guided the design of the cyclohexanetriol diesters does not take important elements of the phorbol ester pharmacophore into account.

The latter alternative is a distinct possibility as shown by the analysis of the goodness of fit in which the orientation and position of the carbonyl groups were taken into consideration. This analysis revealed that considerable energetic penalties could result from the cyclohexanetriol diesters adopting conformations resembling those of phorbol esters, which could explain the lack of biological similarity. The cyclohexanetrol diesters share the lack of growth-inhibitory properties in A549 cells with the diacylglycerols OAG and DiC_8, which were used in this study as representatives of the physiological ligands and activators of pkC. It could be argued that the concentrations of diacylglycerols to which the cells were exposed in these experiments were too low to elicit a growth-inhibitory response, as diacylglycerols are known to undergo rapid metabolism in cells.[17,18] However this explanation seems unlikely as the diacylglycerols were added at 6 hourly intervals at concentrations in the 10^{-4} M range. At such concentrations diacylglycerols have been reported to stimulate pkC.[8,19] Furthermore in a preliminary experiment the ability of the diacylglycerol kinase inhibitor R 59022[20] to influence the effect of DiC_8 on A549 cell growth was studied. Inhibition of this enzyme should increase the biochemical stability of diacylglycerols. The presence of the inhibitor at concentrations as high as 45 μM did not render nontoxic but pkC-activating amounts of DiC_8 growth inhibitory. These results suggest that activation of pkC alone may not be sufficient to trigger growth arrest in A549 cells.

The concentrations at which diacylglycerols were cytotoxic against these cells were of an order of magnitude, which is tolerated by HL-60 myeloid leukemia cells.[17,21–23] The reason for this discrepancy in sensitivity might be associated with the relationship between cytotoxicity and cell density which was observed here. HL-60 cells are maintained in suspension cultures in which cell densities are obtained which far surpass those reached in cultures in cells grown, like those of the A549 line, as monolayers.

The cytotoxicities determined for the cyclohexanetriol diesters were similar to those of DiC_8 and OAG. It seems likely that the cytotoxicity caused by these compounds is the consequence of nonspecific cellular damage rather than receptor-mediated processes. That the cytotoxicity of these molecules is influenced by their lipophilicities is borne out by the finding that of two 1,4-cyclohexanediol monoesters synthesized for comparative purposes the tetradecanoyl derivative was significantly more cytotoxic than the octanoyl ester (result not shown). We are currently investigating if the cyclohexanetriol diesters are capable of binding to the phorbol ester receptor or of activating pkC. Preliminary results show that the cyclohexanetriol diesters at concentrations in the 10^{-5} M range compete with phorbol dibutyrate for phorbol ester binding sites on purified pkC. The affinity of the cyclohexanetriol diesters to the phorbol ester receptor appears to be 1 order of magnitude lower than that for dioctanoylglycerol (results not shown). Therefore the failure of the cyclohexane derivatives to arrest A549 cell growth at nontoxic concentrations may well be linked to their inability to activate pkC.

Experimental Section

Chemistry. Melting points were determined in open capillaries with an Electrothermal apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Varian EM360A, Bruker WH300, and WH400 spectrometers. ^{13}C NMR spectra were recorded on the latter two instruments. IR spectra were recorded on a Perkin-Elmer 1310 spectrophotometer. Mass spectra were recorded on a VG Micromass 12 instrument. Elemental analyses were performed by Butterworth Laboratories Ltd., Middlesex (U.K.) and Elemental Microanalysis, Okehampton (U.K.). TLC was performed on fluorescent silica gel coated aluminum-backed plates (Merck Art. 5554); spots were visualized with UV light or by spraying with dodecamolybdophosphoric acid. Flash chromatography was performed with May and Baker Sorbisil C-60 flash silica and dry column chromatography[24] with Merck silica gel 60H (Art. 7736). HPLC was performed on a Waters instrument fitted with a Merck LiChrosorb Si 60 column using a solvent system of 5% methanol in chloroform and GLC on a Pye Unicam instrument fitted with a 2.5-m glass column packed with 3% SP2100 DOH on Supelcoport operating at 200 °C. Ether and THF were freshly distilled from sodium; ethyl acetate and hexanes (both HPLC grade) were used as received. Dichloromethane and chloroform were dried just prior to use by passage through a column of active basic alumina.

Dioctanoylglycerol (DiC_8). DiC_8 was prepared in racemic form according to standard literature procedures.[25,26]

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4-[(*tert*-Butyldiphenylsilyl)oxy]cyclohexene (5). *tert*-Butyldiphenylsilyl chloride (4.31 g, 16 mmol) was added to a solution under nitrogen of 4-cyclohexenol (4)[9] (1.4 g, 14 mmol) and imidazole (2.14 g, 31 mmol) in DMF (25 mL). After 24 h the solution was poured into water (200 mL) and extracted with 3:1 hexanes–ether (200 mL). After drying (Na_2SO_4) and evaporation of the solvent, dry column chromatography[24] (eluant 0–12% ether in hexanes) led to the isolation of **5** (4.83 g, ~100%) as an oil: NMR ($CDCl_3$) δ 1–2 (6 H, m), 1.05 (9 H, s, Me_3C), 3.6 (1 H, m, H-4), 5.5 (2 H, m, H-1, H-2), 7.3–7.7 (10 H, m, Ph_2Si); MS (EI, 70 eV), m/z 279 (M^+ – *t*-Bu). Anal. ($C_{22}H_{28}OSi$) C, H.

3-[(*tert*-Butyldiphenylsilyl)oxy]-7-oxabicyclo[4.1.0]heptanes 6a and 6a. To a solution of **5** (2.0 g, 5.9 mmol) in dichloromethane (40 mL) was added in portions 85% *m*-chloroperbenzoic acid (1.54 g, 7.6 mmol). After stirring for 18 h, the solution was washed with 1 M Na_2CO_3 and brine and dried (Na_2SO_4). After evaporation of the solvent, flash chromatography (20% ether in hexanes) led to the recovery of starting material **5** (170 mg, 8.5%) and isolation of the mixed epoxides **6a** and **6b** as an oil (1.34 g, 67% based on recovered starting material): NMR ($CDCl_3$) δ 1.03 and 1.05 (9 H, 2 s, ratio 5:11, Me_3C), 1.3–2.2 (6 H, m), 2.95 (~0.7 H, m, H-1, H-6, minor isomer), 3.1–3.2 (~1.4 H, m, H-1, H-6, major isomer), 3.55 (~0.3 H, m, H-3, minor isomer), 3.85 (~0.7 H, m, H-3, major isomer), 7.3–7.65 (10 H, m, Ph_2Si); MS (EI, 70 eV), m/e 295 (M^+ – *t*-Bu). Anal. ($C_{22}H_{28}O_2Si$) C, H.

4-*O*-(*tert*-Butyldiphenylsilyl)cyclohexane-1,2,4-triols 7a and 7b. A solution of the mixed epoxides **6a** and **6b** (1.28 g, 3.6 mmol) and $Mo(CO)_6$ (~5 mg, 0.02 mmol) in dioxane (12 mL) and water (1.5 mL) was heated at 95 °C for 24 h.[27] After evaporation of the solvents, the residue was taken up in ether, washed with brine, and dried (Na_2SO_4). HPLC analysis revealed a single peak (retention time 5.8 min). After evaporation of the solvent, flash chromatography (eluant 33% hexanes in ethyl acetate) gave the monoprotected triols **7a** and **7b** as an oil (1.27 g, 95%): NMR ($CDCl_3$) δ 1.03 and 1.05 (9 H, 2 s, ratio 2:9 Me_3C), 1.2–2.0 (6 H, m), 3.35 (~0.7 H, ddd, J = 11.5, 9, 4.5 Hz, H-1, major isomer), 3.6–3.7 (~0.6 H, m, H-1, H-2, minor isomer), 3.85 (~0.3 H, m, H-4, minor isomer), 3.95 (~0.7 H, ddd, J = 11.5, 9, 4.5 Hz, H-2, major isomer), 4.1 (~0.7 H, m, H-4, major isomer), 7.3–7.65 (10 H, m, Ph_2Si).

(±)-(1*R,2*R**,4*S**)-1,2-Di-*O*-octanoylcyclohexane-1,2,4-triol (3a) and the (±)-1*R**,2*R**,4*R** Isomer 3b.** Octanoyl chloride (1.67 g, 10 mmol) was added dropwise to a solution of the mixed monoprotected triols **7a** and **7b** (1.27 g, 3 mmol) in dry chloroform (30 mL) containing pyridine (1.08 g, 14 mmol). After 48 h the solution was washed with 1 M H_2SO_4, 1 M Na_2CO_3, and brine and dried (Na_2SO_4). After evaporation of the solvent, the residue was taken up in dry THF (20 mL) and tetrabutylammonium fluoride solution (1 M in THF; 6 mL, 6 mmol) was added. After 42 h the solvent was removed under reduced pressure, the residue partitioned between ether and water, and the organic layer dried (Na_2SO_4). After evaporation of the solvent, dry column chromatography[24] (hexane–ethyl acetate gradient) led to the isolation of the mixed diesters **3a** and **3b** (1.17 g, 89%). Flash chromatography (eluant 33% hexane in ether) led to the isolation of pure **3a** (770 mg, 58%) as a waxy solid and pure **3b** (140 mg, 11%) as an oil, in addition to mixed fractions (130 mg, 10%). Isomer **3a**: mp 36–38 °C (hexanes); NMR ($CDCl_3$) δ 0.85 (6 H, t, J = 7 Hz, 2 *Me*), 1.2–1.3 (16 H, m), 1.5–2.1 (11 H, m), 2.25 (4 H, m), 4.05 (1 H, m, H-4), 4.8 (1 H, ddd, J = 7.8, 7.8, 5.0 Hz, H-1), 5.15 (1 H, ddd, J = 7.8, 7.8, 4.2 Hz, H-2); MS (EI, 70 eV), m/z 367 (M^+ – OH). Anal. ($C_{22}H_{40}O_5$) C, H.

Isomer **3b**: NMR ($CDCl_3$)3 δ 0.85 (6 H, t, J = 7 Hz, 2 *Me*), 1.2–1.3 (16 H, m), 1.3–2.4 (m, 15 H), 3.8 (1 H, m, H-4), 4.85 (2 H, m, H-1, H-2); MS (EI, 70 eV), m/z 367 (M^+ – OH). Anal. ($C_{22}H_{40}O_5$) C, H.

Oxidation/Reduction of Alcohols 3a and 3b. To a mixture of alcohols **3a** and **3b** (ratio 6:1; 200 mg, 0.52 mmol) in dichloromethane (10 mL) were added first powdered 4-Å molecular sieve (300 mg) and then pyridinium chlorochromate (280 mg, 1.3 mmol). After 30 min the solution was diluted with ether (15 mL) and filtered through a pad of silica topped with Celite. Evaporation of the solvents afforded (±)-(3*R**,4*R**)-3,4-bis(octanoyloxy)cyclohexanone (**8**), which was used immediately in the reduction procedures: NMR ($CDCl_3$) δ 0.85 (6 H, t, J = 6.5 Hz), 1.2–1.3 (16 H, m), 1.5–1.7 (4 H, m), 2.05 (1 H, m), 2.1–2.6 (8 H, m), 2.8 (1 H, ddd, J = 15.3, 4.6, 1.1 Hz), 5.1 (1 H, ddd, J = 5.5, 5.5, 3.4 Hz), 5.25 (1 H, ddd, $J \approx 5, 5, 5$ Hz). Reductions were performed at room temperature with 3 equiv of reducing agent in ethanol ($NaBH_4$) or THF (K-Selectride). When the reactions were complete as judged by TLC, they were quenched with saturated NH_4Cl and extracted with ether. The ether layers were then analyzed by GLC (retention times: 73 min (**3b**), 77 min (**3a**)).

4-*O*-(*tert*-Butyldiphenylsilyl)cyclohexane-1,2,4-triols 7c and 7d. A solution of alkene **5** (780 mg, 2.32 mmol) in acetone (16 mL) was added to a solution of *N*-methylmorpholine *N*-oxide monohydrate (530 mg, 3.94 mmol) and OsO_4 (1 crystal) in 50% aqueous acetone (12 mL). After 18 h the acetone was removed by evaporation under reduced pressure and the residue partitioned between water and ether. HPLC analysis revealed two peaks in a ratio of 6:7 in order to elution (retention times: 4.1 and 5.0 min). After evaporation of the solvent, flash chromatography (eluant 5% MeOH in $CHCl_3$) led to the isolation of first diol isomer **7c** (240 mg, 28%) and then mixed fractions (150 mg, 18%) and finally isomer **7d** (310 mg, 36%), all as ols. Isomer **7c**: NMR ($CDCl_3$) δ 1.08 (s, 9 H, Me_3C), 1.25 (1 H, m), 1.5–1.65 (3 H, m), 1.95 (1 H, m), 2.2 (1 H, m), 2.45 (1 H, d, J = 9 Hz), 3.45 (1 H, m, H-4), 3.8 (1 H, br, O*H*), 3.85 (1 H, m, H-2), 4.0 (1 H, m, H-1), 7.3–7.7 (10 H, m, Ph_2Si); MS (EI, 70 eV), m/e 313 (M^+ – *t*-Bu). Anal. ($C_{22}H_{30}O_3Si$) C, H.

Isomer **7d**: NMR ($CDCl_3$) δ 1.05 (9 H, s, Me_3C), 1.4 (1 H, m), 1.6–2.0 (7 H, m), 3.85 (1 H, m, H-1), 4.1 (2 H, m, H-2, H-4), 7.3–7.7 (10 H, m, Ph_2Si); MS (EI, 70 eV), m/e 313 (M^+ – *t*-Bu). Anal. ($C_{22}H_{30}O_3Si$) C, H.

(±)-(1*R,2*S**,4*S**)-1,2-Di-*O*-octanoylcyclohexane-1,2,4-triol (3c).** To a stirred solution of diol **7c** (230 mg, 0.62 mmol) in dry $CHCl_3$ (4 mL) were added first pyridine (196 mg, 0.20 mL, 2.42 mmol) and then octanoyl chloride (305 mg, 0.32 mL, 1.86 mmol). After 8 h the solution was poured into water and extracted with hexanes–ether (3:1). After drying (Na_2SO_4), evaporation of the solvent, and flash chromatography (eluant 10% ether in hexanes), the protected diester was immediately taken up in dry THF (3 mL) and tetrabutylammonium fluoride solution (1 M in THF; 0.9 mL, 0.9 mmol) was added. After 18 h the solvent was evaporated; flash chromatography of the residue led to the isolation of diester **3c** (160 mg, 67%) as an oil: NMR ($CDCl_3$) δ 0.8 (6 H, t, J = 7 Hz, 2 Me), 1.0–2.4 (31 H, m), 3.7 (1 H, m, H-4), 4.8–5.2 (2 H, m, H-1, H-2); MS (EI, 70 eV), m/z 367 (M^+ – OH). Anal. ($C_{22}H_{40}O_5$) C, H.

(±)-(1*R,2*S**,4*R**)-1,2-Di-*O*-octanoylcyclohexane-1,2,4-triol (3d).** Treatment of diol **7d** (310 mg, 0.84 mmol), essentially as described for isomer **7c**, led to the isolation of diester **3d** (210 mg, 65%) as an oil: NMR ($CDCl_3$) δ 0.85 (6 H, t, J = 6.5 Hz, 2 Me), 1.2–1.3 (16 H, m), 1.4–2.1 (11 H, m), 2.3 (4 H, m), 4.1 (1 H, m, H-4), 5.0 (1 H, m, H-1), 5.25 (1 H, m, H-2); MS (EI, 70 eV), m/z 367 (M^+ – OH). Anal. ($C_{22}H_{40}O_5$) C, H.

Biochemistry. TPA, OAG, NADH, and sodium pyruvate were purchased from Sigma Chemical Co. Tissue culture reagents were purchased from Gibco Ltd. TPA and the diacylglycerols were stored at −20 °C. Stock solutions were prepared in dimethyl sulfoxide (in the case of DiC_8, OAG, and the racemic cyclohexanetriol diesters immediately prior to use). The final concentration of dimethyl sulfoxide in the culture medium did not exceed 0.5%. Dimethyl sulfoxide was added to control cultures and this concentration did not affect cell growth.

Cell Culture and Measurement of Cell Growth. Human-derived A549 lung carcinoma cells were obtained from the American Type Culture Collection and routinely cultured as described previously.[7] In the experiments in which the effect of compounds on cell growth was assessed cells ((2–5) × 10^4/well) were seeded in six-well multidishes (35-mm diameter, Nunclon U.K.) and incubated with 3 mL of Nutrient Hams F12 medium supplemented with 10% fetal calf serum and different concentrations of the test compound in a humidified incubator gassed with 95% air/5% CO_2. Cells and drugs were incubated for 4–5 days after which control cultures reached confluence. Medium and test compounds were replaced every 2 days. DiC_8 or OAG was added

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three times daily at 6 hourly intervals for 3 days. At the end of the incubation period, cells were counted with either a hemocytometer or a Coulter Counter, Model ZM, after detachment of cells from the dish by short incubation with a solution of trypsin (0.1%) in versene. The IC_{50} values shown in Table II are the concentrations of test compounds which inhibited cell growth by 50%.

Cytotoxicity Assay. Cells (1×10^5/well) were incubated for 24 h with the test compound and with medium supplemented with only 1% fetal calf serum, as larger serum concentrations interfered with the LDH assay. Diacylglycerols were added 3 times at 6 hourly intervals. The supernatant was removed and briefly centrifuged at 1000 rpm at the end of the incubation or when it was replaced with fresh medium and agent. The supernatant was kept on ice until assayed. The activity of LDH in the cell supernatant was measured spectrophotometrically as described by Leathwood and Plummer[28] with a Beckman DU-7 spectrophotometer. The amount of maximally releasable LDH was measured in the supernatant of control cells lysed by 1% Triton X-100 immediately before the assay. The LC_{50} values shown in Table II are the concentrations of test compounds which caused 50% of the maximal LDH leakage.

Computer-Assisted Modeling. Molecular modeling was performed on a DEC8650 processor using the CHEM-X graphics package developed and distributed by Chemical Design Ltd., Oxford, U.K. The coordinates for phorbol were obtained from the published crystal structure;[29] the acetyl group coordinates and those for cyclohexane were obtained from the CHEM-X fragments database. Structural modification and conformational analysis was performed by using the supplied routines. Briefly, the energy of each molecule as a function of rotations about each carbon–oxygen single bond of the diester moiety was calculated and minima in this (4-dimensional) conformational space were located. Each was then subjected to a full molecular mechanics minimization, subject to interatom distance restraints if required, in order to determine the lowest energy conformation. For the cyclohexanetriol diesters, both chair conformations of the ring were considered separately as start points.

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Registry No. **3a**, 117918-47-5; **3b**, 117918-48-6; **3c**, 117918-52-2; **3d**, 117918-53-3; **4**, 72137-22-5; **5**, 117918-43-1; **6a**, 117918-44-2; **6b**, 118013-64-2; **7a**, 117918-45-3; **7b**, 117918-46-4; **7c**, 117918-50-0; **7d**, 117918-51-1; **8**, 117918-49-7; $CH_3(CH_2)_6COCl$, 111-64-8; phorbol, 17673-25-5.

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New Brain Perfusion Imaging Agents Based on ^{99m}Tc-Bis(aminoethanethiol) Complexes: Stereoisomers and Biodistribution

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In developing new brain perfusion imaging agents, we prepared ^{99m}Tc complexes of racemic mixtures of bis(aminoethanethiol) (BAT) derivatives containing an *N'*-benzylpiperazinyl (BPA) side chain. Due to the presence of a chiral center, a mixture of diastereomers (syn and anti) following chelation with the ^{99m}Tc (no-carrier-added) was obtained. The neutral and lipid-soluble ^{99m}Tc-BPA-BAT (^{99m}Tc, $T_{1/2}$ = 6 h) isomers were separated. The syn and anti isomers of carrier-added ^{99}Tc-BPA-BAT (^{99}Tc, $T_{1/2} = 2 \times 10^5$ years) were also synthesized, separated, and crystallized. The X-ray crystallography of ^{99}Tc-BPA-BAT showed the syn and anti conformations (in relationship with the central $TC(=O)N_2S_2$ core). Despite a similarity in the partition coefficients for the two isomers, the syn isomer showed a higher in vivo brain uptake and longer brain retention in rats (2.77 and 1.08% dose/organ at 2 and 15 min) than that of the corresponding anti isomer (0.57 and 0.27% dose/organ at 2 and 15 min). This information is important and should be taken into consideration when new ^{99m}Tc-labeled brain perfusion imaging agents are being designed.

Neutral and lipid-soluble compounds labeled with γ-emitting isotopes are potentially useful as brain perfusion imaging agents in nuclear medicine.[1-9] Due to the superior physical characteristics of ^{99m}Tc ($T_{1/2}$ = 6 h, 140 keV) for nuclear medicine imaging, there is a growing interest in the development of ^{99m}Tc-labeled brain perfusion imaging agents. Two types of ^{99m}Tc ligands have been studied extensively, namely, propylenediamine oxime (PnAO)[10-14] and bis(aminoethanethiol) (BAT).[15-22] Both ligands are known to form a neutral $Tc^{III}O$ complex with a pyramidal center core. Introduction of any single substituent on the ligand will generally produce epimers with relationship to the pyramidal center core. One of the derivatives of PnAO, ^{99m}Tc-HMPAO, is currently used in nuclear medicine clinics as a regional brain perfusion imaging agent.

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