Design, Synthesis, and Activity of Membrane-Disrupting Bolaphiles[1]

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Abstract: Four new classes of bolaphiles ("double-headed" single-chain surfactants) have been prepared, via condensation of an homologous series of linear saturated, olefinic, and acetylenic α,ω-dicarboxylic acids with hexaethylene glycol, and evaluated for their ability to induce the release of 5(6)-carboxyfluorescein (CF) entrapped within large unilamellar vesicles derived from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). Operationally, the membrane-disrupting activity of the bolaphiles has been found to vary by a factor of ca. 100, when the total number of carbon atoms which separate the carboxylate moieties range from 12 to 24; the most active bolaphile is approximately three times more active than Triton X-100. When the affinity of the bolaphiles toward POPC membranes is considered, the intrinsic membrane-disrupting activity varies by a factor of ca. 230. A "loop" model is proposed to account for the general trends in bolaphile activity that are observed, where the *depth of loop penetration* and *loop width* are presumed to be the key factors involved in determining membrane-disrupting activity. The potential utility of these surfactants as antimicrobial agents, and as anti-HIV agents in particular, is briefly discussed.

Introduction

Single-chained surfactant molecules are commonly used to disrupt biological membranes in order to isolate peripheral and integral proteins. Currently, it is believed that much of their disruptive power derives from a mismatch between their *intrinsic geometry* and that of the lamellar-forming phospholipids.[3-7] In particular, most single-chained surfactants are thought to be conical in shape and self-assemble into spherical micelles in order to maximize intermolecular hydrophobic interactions. In contrast, double-chained phospholipids are believed to be more cylindrical in character and prefer to aggregate as tightly packed planar arrays (Figure 1). Thus, when a sufficient quantity of single-chain surfactant becomes incorporated into a lipid bilayer, a lamellar to micellar phase transition should be thermodynamically driven by the hydrophobic effect.

Although single-chain surfactants have been extensively investigated over the past several years, analogous bolaphiles (molecules bearing a polar head group at each end of a hydrophobic segment) have received limited attention.[8] Recently, we have become interested in "double-headed" surfactants as membrane-disrupting agents for two reasons. First, from a theoretical standpoint, we hypothesized that the effective geometry of a bolaphile might be adjustable via appropriate molecular design. One can imagine, for example, that a bolaphile having a fully saturated aliphatic segment might favor the formation of a "hydrocarbon loop" upon insertion into a monolayer leaflet.[9] Depending on the size of the loop, its alignment with the lamellar phase could lead to membrane destabilization to varying degrees (Figure 2). Moreover, one might imagine that the introduction of an acetylenic or olefinic moiety, positioned either symmetrically or asymmetrically within the hydrophobic unit, could be used to modulate the surfactant's looped character and hydrophobicity (Figure 3). Acetylenic and olefinic groups that are symmetrically disposed, for example, would be expected to increase the linearity of the central region of the hydrocarbon unit, and thus increase the loop's width; at the same time, they should reduce the lipophilicity of the loop. Placement of an acetylenic or cis-olefinic moiety, asymmetrically within the hydrophobic segment, could lead to "narrow" and "twisted" loops, respectively. Exactly how disruptive each of these hypothetical bolaphiles would be toward a phospholipid membrane, however, cannot be predicted with certainty.

The second reason for our interest in bolaphiles as membrane-disrupting agents was more pragmatic in nature. We have recently hypothesized that the lipid envelope of certain microorganisms may possess "windows of vulnerability", and that such windows could serve as targets for chemotherapy.[10] We have further suggested that it would be highly desirable to create classes of "tunable" membrane-disrupting agents, and to explore, systematically, their in vitro and in vivo antimicrobial properties. Consider, for example, the case of the human immunodeficiency virus (HIV) particle (Figure 4).[11] If one ignores its inner core, then the HIV virion has a striking resemblance to that of a 1000-Å diameter unilamellar liposome; both exist as uniform microspheres that are derived largely or entirely from phospholipids. One key difference, however, is that the former bears the peripheral glycoprotein, gp 120 (appearing as large "knobs" on the HIV surface), which recognizes and binds to the CD4 receptor sites that are located on the surface of T-lymphocyte cells. While the lipid envelope of HIV resembles the lipid membrane found in human erythrocyte ghosts, there is at least one clear difference; i.e., the lipid/protein ratio is significantly lower for the former.[12] Can advantage be taken of subtle differences that exist in lipid/protein ratios, lipid packing, and membrane fluidity between a mammalian

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(7) The geometric model, which has become widely used for rationalizing aggregate structure, has been criticized by some researchers: Gruner, S. M. In *Liposomes: From Biophysics to Therapeutics*; Ostro, M. J., Ed.; Marcel Dekker: New York, 1987; p 1.

(8) One leather ball attached to each end of a string forms a sling called a "bola". By analogy, amphiphiles having a polar head group at each end of a hydrophobic segment have been termed: "bolaform amphiphiles" (Jeffers, P. M.; Daen, J. *J. Phys. Chem.* **1965**, *69*, 2368), "bolaamphiphiles" (Fuhrhop, J.-H.; Mathiewu, J. *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 100), "bolions" (Morawetz, H.; Kandaniem, A. Y. *J. Phys. Chem.* **1966**, *70*, 2995), and "bolytes" (Fuhrhop, J.-H.; David, H.-H.; Mathiew, J.; Liman, U.; Winter, H.-J.; Boekema, E. *J. Am. Chem. Soc.* **1986**, *108*, 1785). While bolaamphiphile has become the more popular terminology (Fuhrhop, J.-H.; Fritsch, D. *Acc. Chem. Res.* **1986**, *19*, 130), we prefer to adopt the abbreviated and more readily pronounceable term, "bolaphile".

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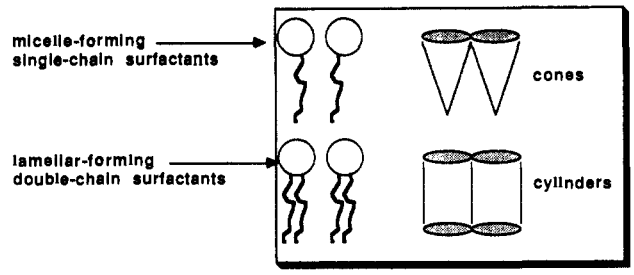


Figure 1. Stylized illustration of molecular geometry of single- and double-chain surfactants.

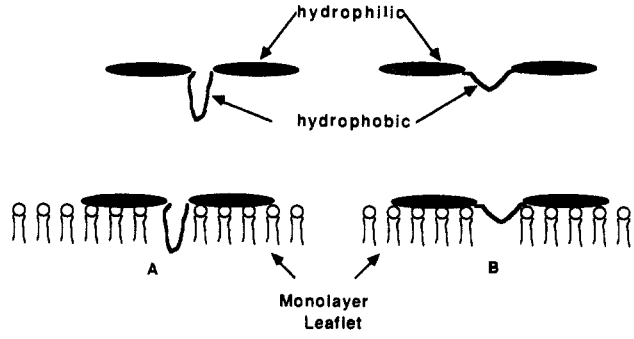


Figure 2. Stylized illustration of bolaphiles having a long (A) or a short (B) hydrophobic segment, interacting with a phospholipid monolayer leaflet.

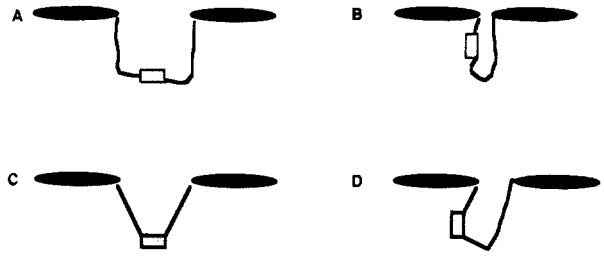


Figure 3. Stylized illustration of bolaphiles having a symmetrical acetylenic (A), an asymmetrical acetylenic (B), a symmetrical olefinic (C), and an asymmetrical olefinic (D) unit.

cell membrane and the lipid envelope of a microorganism, from a therapeutic standpoint? Can, for example, a membrane-disrupting agent be found which selectively perturbs the (i) conformation, (ii) dynamic properties, and/or (iii) lateral distribution of gp 120 (or the integral protein, gp 41, to which it is attached), and can such a perturbation inhibit the binding and/or fusion of HIV with T-lymphocyte cells?[13] Can membrane-disrupting agents be devised which will provide the basis for a viable chemotherapeutic treatment of acquired immune deficiency syndrome (AIDS) or any other viral, bacterial, or fungal infection? While these questions are intriguing, and are of obvious practical importance, appropriate *experimentation* is needed in order to clearly answer them. Motivated by both theoretical and practical considerations, we have thus initiated a program which is aimed at creating *novel classes of membrane-perturbing agents which exhibit a broad range of activity.*

In this paper we describe the synthesis of four new classes of bolaphiles (I, II, III, and IV) in which the structure and composition of the central hydrophobic segment has been systematically varied, on the basis of the above rationale. We also define the membrane-disrupting properties of each of these surfactants, using a model membrane derived from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). In an accompanying paper,

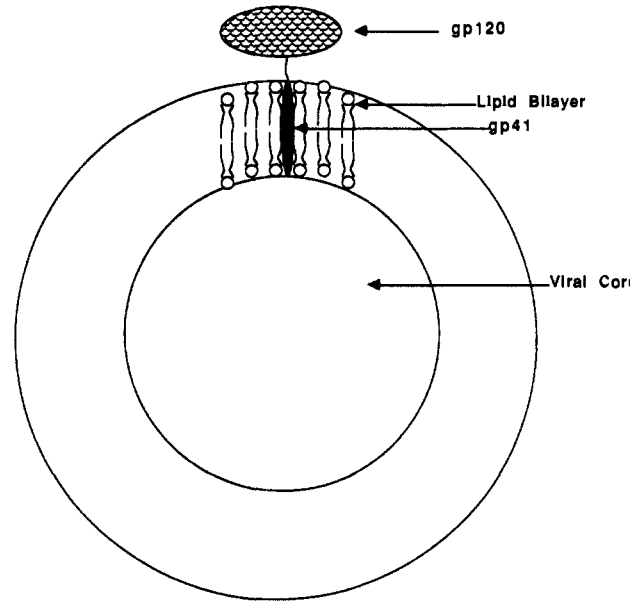
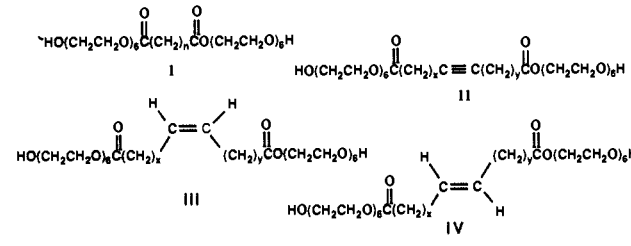


Figure 4. Stylized illustration of an HIV particle showing the outer lipid bilayer membrane, gp 120, gp 41, and the viral core. The HIV particle has diameter of ca. 1000 Å.

we report the synthesis and membrane-disrupting properties of polymeric analogues of these surfactants.



Experimental Section

General Methods. Unless stated otherwise, all reagents and chemicals were obtained from Aldrich Chemical Co. and used without further purification. 1,15-Pentadecanedioic acid was obtained from Farchan Laboratories (Gainesville, FL). Prior to use, the 1,13-tridecanedioic and 1,16-hexadecanedioic acid were recrystallized from methyl ethyl ketone. 1,18-Octadecanedioic acid, 1,20-eicosanedioic acid, and 1,22-docosanedioic acid were prepared by using procedures similar to those previously described in the literature.[14] 1,17-Heptadecanedioic acid[15] was prepared by reduction of 1,15-pentadecanedioic acid with diborane in THF, followed by conversion of the diol to the corresponding alkyl dichloride ($SOCl_2$), nitrile formation (via cyanide displacement), and acid-catalyzed hydrolysis. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids (Birmingham, AL) as a chloroform solution and was used directly. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl. Methylene chloride was dried by distillation over P_2O_5; dimethyl sulfoxide was available as an anhydrous grade (Aldrich) and used directly. House-deionized water was purified by using a Millipore Milli-Q filtering system containing one carbon and two ion-exchange stages. Liposome dispersions were normally prepared in 10 mM borate buffer (pH 7.4) containing 140 mM NaCl and 2 mM NaN_3 (we will refer to this buffer throughout this manuscript as simply borate buffer). Chloroform and methanol used for chromatography were HPLC-grade (Burdick & Jackson). ^1H NMR, IR, and UV spectra were recorded on JEOL FX 90Q, Perkin-Elmer 283 and Perkin-Elmer Lambda 5 spectrometers, respectively. Chemical shifts are reported relative to tetramethylsilane. Elemental analyses were performed by Midwest Microlab, Indianapolis, IN. All vesicle extrusions were carried out with a Lipex Biomembrane apparatus (Vancouver, British Columbia). Chromatographic separations were carried out by using precoated Merck 0.25-mm silica gel 60 TLC plates (with fluorescent indicator) and Merck 70-230 ASTM silica gel. Dynamic light scattering measurements were carried out by using a Nicomp 270 sub-

(13) For a general discussion of strategies for antiviral therapy in AIDS, see: Yarchoan, R.; Mitsuya, H.; Broder, S. *Sci. Am.* **1988**, *259*, 110 and references cited therein.

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micrometer particle analyzer, equipped with a helium–neon laser (632.8 nm, scattering angle of 90°) and a computing autocorrelator. Samples were filtered by using a 0.45-μm HV4 Millipore filter prior to analysis. All differential scanning calorimetry (DSC) runs were performed with use of a Microcal MC-2 instrument. This calorimeter (Microcal, Inc., Amherst, MA) is equipped for microcomputer control of data acquisition and analysis.

ω-Bromo Alcohols. 6-Bromohexanol, 7-bromoheptanol, and 9-bromononanol were prepared by heating a mixture of 25 g of the corresponding organic diols with 125 mL of 48% HBr plus 40 mL of water for 48 h at 80 °C. Periodic extraction (every 8 h) with 200 mL of toluene, followed by concentration of the combined extracts afforded crude ω-bromo alcohols. These ω-bromo alcohols were then dissolved in 300 mL of ethyl ether, dried over anhydrous Na_2CO_3, and concentrated under reduced pressure. 6-Bromohexanol [bp 105–106 °C (5 mm); lit.[16] bp 105–106 °C (15 mm)] and 7-bromoheptanol [bp 111–112 °C (5 mm); lit.[17] bp 111–112 °C (4 mm)] were purified by distillation; 9-bromononanol [mp 29–31 °C lit.[18] mp 31.5–33 °C] was purified by recrystallization from ethyl ether. Thin layer chromatography (silica, $CHCl_3/CH_3OH$, 15/1, v/v) confirmed the absence of the corresponding dibromide in each case: diols (R_f = 0.3); bromoalcohols (R_f = 0.6); dibromides (R_f = 0.9).

Hexaethylene Glycol Mono(triphenylmethyl ether). To a mixture of 10.0 g (35.5 mmol) of hexaethylene glycol and 3.63 g (36.0 mmol, 5.0 mL) of triethylamine in 60 mL of CH_2Cl_2 was added 9.2 g (33.0 mmol) of trityl chloride. After stirring for 4 h at room temperature, the mixture was washed once with 50 mL of ice cold water, dried over anhydrous $CaCl_2$, and concentrated under reduced pressure. The residual oil was purified by chromatography on silica gel with CH_2Cl_2/CH_3OH (95/5, v/v) as an eluting solvent to give 7.83 g (45%) of the monotritylated product as a viscous pale yellow liquid having ^1H NMR ($CDCl_3$) δ 2.70 (br s, 1 H), 3.25 (dd, 2 H), 3.6 (m, 22 H), 7.3 (m, 15 H). Anal. Calcd for $C_{31}H_{40}O_7$: C, 70.97; H, 7.68. Found: C, 70.45; H, 7.66.

Tetrahydropyran-Protected α,ω-Bromo Alcohols (1). In a typical preparation, 22.3 g (0.1 mol) of 9-bromo-1-nonanol was dissolved in 100 mL of anhydrous diethyl ether and then cooled to 0 °C. After addition of 10 g (0.12 mol) of 2,3-dihydro-2*H*-pyran, plus 3 drops of phosphorous oxychloride, the mixture was stirred for 30 min at 0 °C and then 2 h at room temperature. The progress of the reaction was monitored by TLC (silica, hexanes/ether, 5/1, v/v). The tetrahydropyranyl adduct had an R_f of 0.75; the starting bromo alcohol had an R_f of 0.1. When all of the bromo alcohol was consumed, 0.2 g of solid anhydrous sodium carbonate was added, and the mixture was stirred at room temperature for an additional 10 min. The mixture was then poured into 100 mL of ice/water, and the protected ether was isolated by extraction with ethyl ether (3 × 150 mL). The etheral layer was washed with water (5 × 100 mL), dried with anhydrous sodium carbonate, and concentrated under reduced pressure. Subsequent purification by column chromatography (basic alumina, hexanes) afforded 21.5 g (70%) of 9-bromo-1-[(tetrahydropyran-2′-yl)oxy]nonane (**1d**), having ^1H NMR and IR spectra identical with those previously reported.[19]

By use of similar procedures, the following protected alcohols were prepared: 6-bromo-1-[(tetrahydropyran-2′-yl)oxy]hexane (**1a**, 89%); 7-bromo-1-[(tetrahydropyran-2′-yl)oxy]heptane (**1b**, 70%); 8-bromo-1-[(tetrahydropyran-2′-yl)oxy]octane (**1c**; 70%); 11-bromo-1-[(tetrahydropyran-2′-yl)oxy]undecane (**1e**; 75%). The ^1H NMR and IR spectra of each of these protected ethers agreed with those previously reported.[19]

Tetrahydropyran-Protected Acetylenic Alcohols (2). In a typical preparation, 50 mL of anhydrous DMSO (purged with nitrogen) was added over a 10 min period to 6.3 g (0.061 mol) of lithium acetylide–ethylenediamine complex, with cooling (0 °C), while being maintained under a nitrogen atmosphere. The mixture was stirred for an additional 10 min, followed by a dropwise addition (30 min) of 9-bromo-1-[(tetrahydropyran-2′-yl)oxy]nonane (**1d**; 14.0 g, 0.0455 mol). The mixture was then stirred for 3 h at 0 °C, followed by stirring for 12 h at room temperature, and then poured into 300 mL of ice/water. Extraction with ethyl ether (300, 150, and 150 mL), followed by washing with water (8 × 100 mL), drying over anhydrous sodium carbonate, and concentration (under reduced pressure) afforded a crude product which was purified by chromatography (basic alumina, hexanes) to give 9.4 g (82%) of 11-[(tetrahydropyran-2′-yl)oxy]undecan-1-yne (**2d**), having the expected ^1H NMR and IR spectra.[20]

By use of similar procedures, the following protected alcohols were prepared: 8-[(tetrahydropyran-2′-yl)oxy]octan-1-yne (**2a**; 87%),[21] 9-[(tetrahydropyran-2′-yl)oxy]nonan-1-yne (**2b**; 87%),[22] 10-[(tetrahydropyran-2′-yl)oxy]decan-1-yne (**2c**; 76%),[21] and 13-[(tetrahydropyran-2′-yl)oxy]tridecan-1-yne (**2e**; 82%).[23]

Dihydroxyacetylenes (4). In a typical preparation, 11-[(tetrahydropyran-2′-yl)oxy]undecan-1-yne (**2d**; 10.0 g, 0.04 mol) was dissolved in 50 mL of anhydrous tetrahydrofuran, which was maintained under a nitrogen atmosphere. After the resulting solution was cooled to 0 °C, 18 mL of 2.6 M (0.0468 mol) *n*-butyllithium in hexane was added to it dropwise, with rapid stirring. After the mixture was stirred for 10 min at 0 °C, hexamethylphosphoramide (40 mL) was then added, followed by (i) 15 min of additional stirring, and (ii) the dropwise addition (1.5 h) of 14.0 g (0.0455 mol) of 9-bromo-1-[(tetrahydropyran-2′-yl)oxy]-nonane. The mixture was maintained at 0 °C for 2 additional h, stirred for 12 h at room temperature, and poured into 200 mL of ice/water. Extraction with diethyl ether (300, 100, and 100 mL), followed by washing with water (8 × 100 mL), drying (anhydrous sodium carbonate), and concentration under reduced pressure afforded 16.0 g of crude product. This product was then subjected to column chromatography (350 g of silica, hexanes/diethyl ether, 8/1, v/v) to give 12.0 g (62%) of 1,20-bis[(tetrahydropyran-2′-yl)oxy]eicosan-10-yne (**3d**). Deprotection was carried out by dissolving 12.0 g of **3d** in 150 mL of methanol, adding 5 drops of concentrated HCl, and stirring the mixture for 12 h at room temperature. The mixture was then poured into 300 mL of water, and the crude acetylenic diol was extracted with chloroform (200, 100, and 100 mL), washed with 4 × 100 mL of water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. Chromatographic purification (400 g of silica, $CHCl_3$) and subsequent recrystallization from hexane afforded 7.0 g (88%) of 10-eicosyne-1,20-diol (**4d**).

By use of similar procedures, the following acetylenic alcohols were prepared: 7-tetradecyne-1,14-diol (**4a**; 61%), 8-hexadecyne-1,16-diol (**4b**; 56%), 9-octadecyne-1,18-diol (**4c**; 51%), 12-tetracosyne-1,24-diol (**4e**; 40%), and 7-nonadecyne-1,19-diol (**5f**; 74%).

Acetylenic α,ω-Dicarboxylic Acids (5). In a typical preparation, 10-eicosyne-1,20-diol (**4d**; 3.70 g, 0.011 mol) was suspended in 400 mL of acetone. After the mixture was cooled to 0 °C, 50 mL of a chromic acid solution (0.055 mol of CrO_3 dissolved in 49 mL of 17% H_2SO_4) was added, and the reaction was stirred at 0 °C for 30 min and then for 20 min at room temperature. The mixture was poured into 400 mL of water, and the crude acetylenic diacid was recovered by extraction with diethyl ether (4 × 100 mL). After washing with water (8 × 100 mL), drying with anhydrous sodium sulphate, concentrating the diacid under reduced pressure, and drying (2 h, 50 °C (0.05 mm)), the crude product was suspended in 50 mL of diethyl ether at room temperature. Subsequent addition of 40 mL of a 1 M etheral solution of diazomethane, stirring for 5 min, and concentration under reduced pressure afforded 3.80 g of crude dimethyl ester of 10-eicosyne-1,20-dioic acid as an oil, which was further purified by chromatography (silica, hexanes/diethyl ether, 7/1, v/v). The pure diester (3.20 g, 79%) showed the expected ^1H NMR and IR spectra. This diester was either hydrogenated as described below, for the preparation of **6d**, or hydrolyzed to the corresponding diacid by using the following procedure: After 1.20 g (0.0033 mol) of 10-eicosyne-1,20-dioic acid dimethyl ester was dissolved in 20 mL of methanol, a solution of 20 mL of CH_3OH/H_2O (9/1, v/v) containing 1.0 g (0.015 mol) of potassium hydroxide (85%) was then added. The mixture was heated to 60 °C for 20 min, cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in 50 mL of water and the solution of the potassium salt of 10-eicosyne-1,20-dioic acid was then neutralized by addition of 11 mL of 5% HCl. Extraction with diethyl ether (3 × 200 mL), washing with water (6 × 50 mL), drying over anhydrous sodium sulfate, and concentration under reduced pressure afforded 1.10 g of crude diacid; recrystallization from cyclohexane afforded 1.0 g (89%) of 10-eicosyne-1,20-dioic acid (**5d**).

By use of similar procedures, the following acetylenic dimethyl esters, and acetylenic diacids were prepared: 7-tetradecyne-1,14-dioic acid dimethyl ester (78%); 7-tetradecyne-1,14-dioic acid (**5a**, 68%); 8-hexadecyne-1,16-dioic acid dimethyl ester (69%); 8-hexadecyne-1,16-dioic acid (**5b**; 73%); 9-octadecyne-1,18-dioic acid dimethyl ester (73%); 9-octadecyne-1,18-dioic acid (**5c**; 82%); 12-tetracosyne-1,24-dioic acid dimethyl ester (84%); 12-tetracosyne-1,24-dioic acid (**5e**; 91%); 7-nonadecyne-1,19-dioic acid dimethyl ester (60%); 7-nonadecyne-1,19-dioic acid (**5f**, 75%).

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(21) Rossi, R. *Synthesis* **1981**, 359.
(22) Bjorkling, F.; Torbjorn, N.; Unelius, R. *Synth. Commun.* **1985**, *15*, 463.
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Cis-Olefinic α,ω-Dicarboxylic Acids (6). In a typical preparation, the dimethyl ester of 10-eicosyne-1,20-dioic acid (2.0 g, 0.0054 mol) was dissolved in 25 mL of ethyl acetate. After addition of 5 mg of Lindlar catalyst (5% Pd on $CaCO_3$), the mixture was subjected to hydrogenation at 1 atm (room temperature), and the amount of hydrogen uptake carefully monitored. The reaction was stopped when 0.95 equiv of hydrogen was consumed, the catalyst was removed by filtration, and the solution was concentrated under reduced pressure. Analysis by TLC (silica gel, and 4% $AgNO_3$ on silica gel, hexane/diethyl ether, 4/1) revealed a trace amount of starting material plus one hydrogenated product. Purification of the latter by chromatography (silica, hexanes/ether (10/1, v/v)) afforded 1.8 g (90%) of *cis*-10-eicosene-1,20-dioic acid dimethyl ester. Saponification of this ester, using procedures similar to those used for the basic hydrolysis of the dimethyl esters of the corresponding acetylenic diesters, yielded the desired *cis*-10-eicosene-1,20-dioic acid (**6d**; 89%).

By use of similar procedures, the following cis-olefinic α,ω-diacids were prepared: *cis*-7-tetradecene-1,14-dioic acid (**6a**; 83%); *cis*-8-hexadecene-1,16-dioic acid (**6b**; 82%); *cis*-9-octadecene-1,18-dioic acid (**6c**; 90%); *cis*-12-tetracosene-1,24-dioic acid (**6e**; 95%); *cis*-7-nonadecene-1,19-dioic acid (**6f**; 80%).

***trans*-8-Hexadecene-1,16-dioic Acid.** A solution of lithium aluminum hydride (25 mL of a 0.5 M solution in diglyme, 12.5 mmol) was added to 1.38 g (3.27 mmol) of 1,16-bis[(tetrahydropyran-2′-yl)oxy]hexadecan-8-yne (**3b**), and the mixture maintained at 140 °C for 65 h, under a nitrogen atmosphere.[24] The solution was then cooled to room temperature and quenched with ca. 200 g of ice. The organic product was then extracted with hexanes (3 × 200 mL), dried with anhydrous sodium carbonate, and concentrated under reduced pressure to give 0.98 g of crude product. Analysis by thin layer chromatography (silica, hexanes/diethyl ether (4/1, v/v)) revealed the presence of starting material (R_f 0.42) and a new compound (R_f 0.45). Purification of the latter by column chromatography (silica, hexanes/diethyl ether (20/1,v/v)) afforded 0.89 g (65%) of pure *trans*-1,16-bis[(tetrahydropyran-2′-yl)-oxy]-8-hexadecene as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.20–1.80 (m, 32 H), 1.96 (m, 4 H), 3.20–4.03 (m, 2 H), 4.56 (m, 2 H), 5.37 (m, 2 H). Anal. Calcd for $C_{26}H_{18}O_4$: C, 73.54; H, 11.39. Found: C, 73.37; H, 11.33. Subsequent removal of the dihydropyran protecting groups, under acidic conditions (using procedures similar to those described above for the synthesis of the dihydroxyacetylenes), afforded 0.50 g (93%) of *trans*-8-hexadecene-1,16-diol, which was purified by recrystallization from $CHCl_3$/methanol 1/5, v/v: mp 62–63 °C; ^{1}H NMR ($CDCl_3$) δ 1.20–1.80 (m, 22 H), 2.00 (m, 4 H), 3.64 (t, 4 H), 5.38 (m, 2 H). Anal. Calcd for $C_{16}H_{32}O_2$: C, 74.94; H, 12.58. Found: C, 75.26; H, 12.65. Finally, oxidation of 0.45 g (1.75 mmol) of this trans-diol, followed by esterification with diazomethane and saponification, using procedures similar to those described above for the corresponding cis analogues, afforded 0.36 g (79%) of pure *trans*-8-hexadecene-1,16-dioic acid, which was recrystallized from cyclohexane: mp 111–112.5 °C; ^{1}H NMR ($CDCl_3$) δ 1.20–1.80 (m, 16 H), 1.98 (m, 4 H), 2.37 (t, 4 H), 4.00–6.00 (m, 2 H), 5.36 (m, 2 H). Anal. Calcd for $C_{16}H_{28}O_4$: C, 67.57; H, 9.92. Found: C, 67.71; H, 10.06.

Synthesis of Type I Bolaphiles. The appropriate saturated dicarboxylic acid (0.5–0.7 mmol) was refluxed with 4 equiv of $SOCl_2$ for 3 h. The excess of $SOCl_2$ was then removed under reduced pressure and the diacid dichloride dissolved in 2 mL of CH_2Cl_2. The resulting solution was then added to 2.5 mL of CH_2Cl_2 containing 2.2 equiv of hexaethylene glycol mono(triphenylmethyl ether) and 2 equiv of triethylamine. After the mixture was stirred for 12 h at room temperature, 5 mL of methanol and 25 mg of *p*-toluenesulfonic acid were added, and the solution stirred for an additional 0.5–1 h (NOTE: longer reaction times result in significant transesterification.) The mixture was then extracted three times with 2 mL of water, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was then purified by chromatography on silica gel with CH_2Cl_2/CH_3OH (95/5, v/v) as the eluting solvent. When this general procedure was used, yields of I ranged between 57–74%. Analytically pure compounds were obtained by recrystallization from diethyl ether.

Synthesis of Type II, III, and IV Bolaphiles. To a benzene solution of the appropriate unsaturated diacid (ca. 1 mmol of diacid in 3 mL of benzene) was added 1.5 mL (2.4 g; 20 mmol) of thionyl chloride. The mixture was refluxed under a nitrogen atmosphere for 2 h, cooled to room temperature, and then concentrated by first blowing a stream of nitrogen over the mixture, in order to remove most of the volatile solvent, and then placing the oily residue under reduced pressure (1 h, 23 °C, 0.05 mmHg). The acid dichloride was then dissolved in 4.7 mL of anhydrous dichloromethane, to give a final volume of ca. 5.0 mL. A portion of this acid dichloride (3 mL, 0.6 mmol) was added to a second solution comprised of hexaethylene glycol mono(triphenylmethyl ether) (0.63 g, 1.20 mmol), pyridine (0.3 mL, 3.0 mmol), and 5 mL of anhydrous dichloromethane, that was prepared under a nitrogen atmosphere. The resulting solution was stirred under nitrogen for 12 h at ambient temperature, and then concentrated by evaporation of solvent using a stream of nitrogen. The crude product was purified by column chromatography [silica, $CHCl_3/CH_3OH$, 70/1, v/v)], affording the protected bolaphiles in isolated yields that ranged between 65 and 80%. Deprotection of the protected bolaphile was accomplished by dissolving it (ca. 0.4 mmol) in 20 mL of diethyl ether, adding 1 mL of methanol plus several crystals of *p*-toluenesulfonic acid, and stirring the resulting mixture under refluxing conditions for 30 min. The product mixture was then concentrated under reduced pressure and the residue purified by column chromatography (silica, $CHCl_3/CH_3OH$, 50/1, v/v) affording pure bolaphiles in isolated yields ranging between 35 and 55%.

Surfactant-Induced Release of Liposome-Encapsulated CF. Large unilamellar vesicles were prepared from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) by using standard procedures.[25] Typically, 2 mL of a chloroform solution, containing 20 mg of POPC, was placed in a test tube (13 × 100 mm) and the chloroform evaporated under a stream of nitrogen. After further drying (12 h, 23 °C, 0.05 mmHg), the resulting film was suspended in 1 mL of a 0.25 M solution of 5(6)-carboxyfluorescein (CF, pH 7.4),[26] via vortex mixing. The resulting multilamellar vesicle dispersion was allowed to equilibrate for 0.5 h, subjected to five freeze–thaw cycles (liquid nitrogen), extruded (10 times) through a 0.1-μm polycarbonate filter (Nuclepore Company), and purified via gel filtration on a Sephadex G-50 column (0.7 × 40 cm), with use of a borate buffer as the eluant. Those fractions which contained large unilamellar vesicles, prepared by this extrusion technique (LUVETs), were combined, and the final volume was adjusted to ca. 5 mL, by adding further buffer solution. Finally, this liposomal dispersion was dialyzed against 200 mL of borate buffer (12 h, 15 °C), and allowed to reach room temperature, just prior to use.

An aliquot (10 μL) of the above vesicle dispersion was added to each of eight test tubes (6 × 50 mm), which contained 90 μL of a given bolaphile solution (the final concentration of lipid was ca. 0.5 mM), and the resulting suspension was agitated by vortex mixing for ca. 10 s. After allowing the mixture to incubate for 0.5 h at 23 °C, 25-μL aliquots were withdrawn and diluted with 4 mL of borate buffer. The fluorescence was then determined with use of a Turner fluorimeter (Model 112). A blank value was determined by treating 10-μL aliquots of the original vesicle dispersion with 90 μL of buffer, in the absence of detergent. A total fluorescence value was determined by complete disruption of the vesicles, with 90 μL of a buffer solution which was 80 mM in Triton X-100.

Estimation of Affinity of Bolaphiles toward POPC-Membranes. Multilamellar liposomes of POPC were prepared by dispersing 20 mg of the lipid in 1 mL of borate buffer via vortex mixing. After the dispersion was allowed to remain at room temperature for 0.5 h, it was then centrifuged (Eppendorf centrifuge, 15 min). The supernatant was subsequently removed with a pipet, and the vesicle pellet was resuspended in 1 mL of buffer. This procedure was repeated twice. A stock vesicle dispersion was prepared by resuspending the vesicles in 4 mL of buffer and then analyzed for phosphorus content.[27] Aliquots (100 μL) of this dispersion were added into 1.5-mL Eppendorf centrifuge tubes which contained 900 μL of bolaphile. The specific concentrations of bolaphile that were used in these experiments were identical with that which was found to cause the release of 50% of the CF which was entrapped within a ca. 0.5 mM liposomal dispersion of POPC. The dispersion was allowed to equilibrate for 1 h at ambient temperature and then centrifuged for 20 min. The supernatant was removed by using a pipet and centrifuged once again for 20 min. The resulting supernatant was then analyzed for non-liposomal bound bolaphile.

Release of Liposomal-Encapsulated CF as an Analytical Method for the Determination of Free Bolaphile Concentration. Large unilamellar vesicles (1000-Å diameter), containing 0.25 M CF, were prepared by use of procedures similar to those described above for the surfactant-induced release experiments. The final lipid concentration that was present in the stock solution was ca. 1 mg/mL. A 10-μL aliquot of this dispersion was added into each of eight test tubes containing 4 mL of a varying concentration of bolaphile. After each tube was incubated for 0.5 h at ambient temperature, the percentage of released CF was determined by

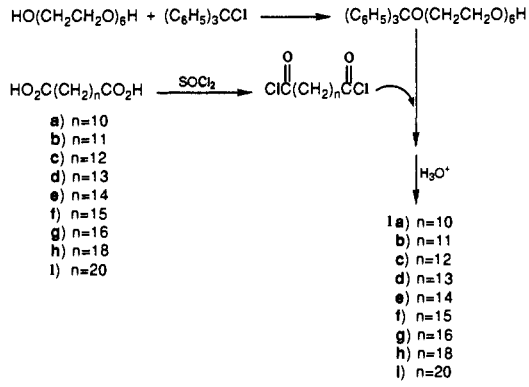
(24) Magoon, E. F.; Slaugh, L. H. *Tetrahedron* **1967**, *23*, 4509.

(25) Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R. *Biochim. Biophys. Acta* **1985**, *812*, 55. Olson, F.; Hunt, C. A.; Szoka, F. C.; Vail, W. J.; Papahadjopoulos, D. *Biochim. Biophys. Acta* **1979**, *557*, 9.

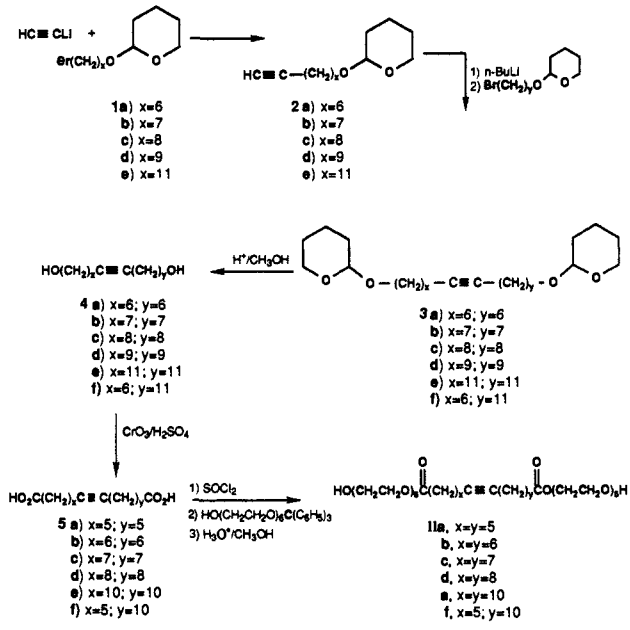
(26) Weinstein, J. N.; Ralston, E.; Leserman, L. D.; Klausner, R. D.; Dragsten, P.; Henkart, P.; Blumenthal, R. In *Liposome Technology*; Gregoriadis, G., Ed.; CRC Press, Inc.: Boca Raton, FL, 1984; Vol. III, p 183.

(27) Regen, S. L.; Kirszenstejn, P.; Singh, A. *Macromolecules* **1983**, *16*, 335.

Scheme I



Scheme II

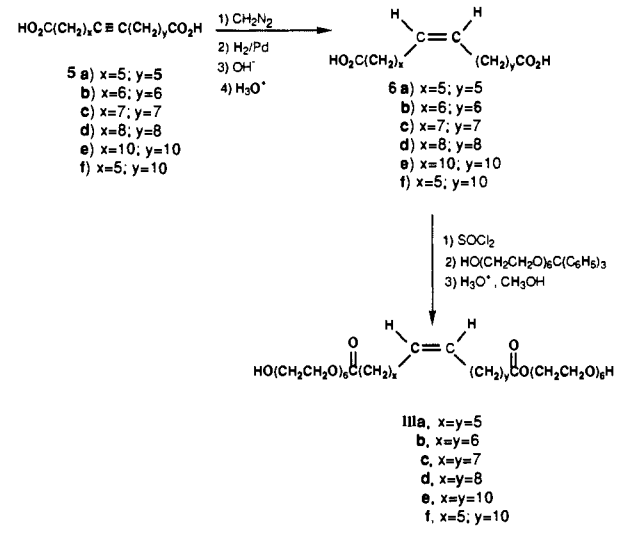


analysis of the sample's fluorescence. A calibration curve was obtained by plotting the percentage of released CF vs log (bolaphile concentration). An aliquot of the supernatant from the binding experiment was diluted in buffer to give a final volume of 4 mL. To this solution was added a 10-μL aliquot of the liposomal dispersion containing CF, and the mixture was allowed to incubate for 0.5 h. Analysis of released CF was then measured directly by fluorescence. The concentration of bolaphile was determined from the previously constructed calibration curve.

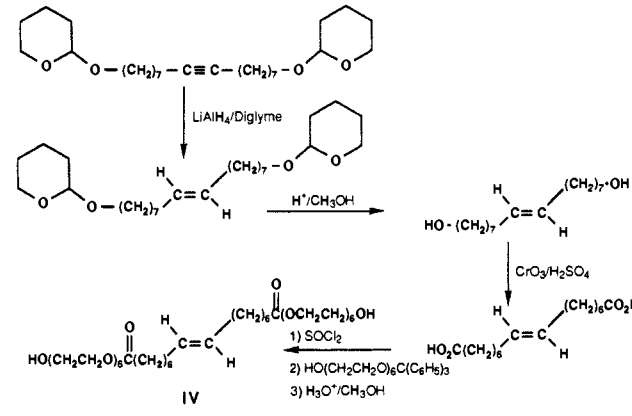
Results and Discussion

Bolaphile Synthesis. Schemes I–IV summarize the synthetic routes that have been used to prepare bolaphiles I, II, III, and IV, respectively. Conversion of each saturated α,ω-dicarboxylic acid into its corresponding acid chloride, followed by condensation with hexaethylene glycol mono(triphenylmethyl ether) and subsequent hydrolysis afforded the corresponding type I bolaphile. Acetylene-bearing dicarboxylic acids that were needed as precursors for type II bolaphiles were synthesized via (i) alkylation of lithium acetylide by the appropriate tetrahydropyranyl-protected α,ω-bromo alcohol (**1**), (ii) alkylation of the resulting acetylene (**2**) with the requisite tetrahydropyranyl-protected α,ω-bromo alcohol, (iii) deprotection, and (iv) oxidation of the resulting hydroxyl groups. All cis-olefinic diacids (**6**) that were used to prepare bolaphiles III were obtained via hydrogenation of the methyl esters of **5**, followed by saponification. While direct hydrogenation of **5** also proved possible, product mixtures were generally more complex than those produced from the corresponding dimethyl ester; in addition, isolated yields of **6** were considerably lower. For purposes of comparison, a trans isomer of one of the bolaphiles (IV) was also synthesized by use of analogous procedures (Scheme IV).

Scheme III



Scheme IV



Surfactant-Induced Release of Encapsulated CF. In order to evaluate the membrane-disrupting properties of each bolaphile, we have examined their ability to induce the release of 5(6)-carboxyfluorescein (CF) entrapped within large unilamellar vesicles (LUVs) of POPC.[28-30] Such methods have been extensively used to determine the membrane-disrupting activity of synthetic and natural surfactants.[28] In essence, when the effective concentration of liposome-encapsulated CF is relatively high (e.g. >0.05 M), its fluorescence intensity is negligible due to self-quenching. As the molecule is released from the liposome interior into the bulk aqueous phase, its effective concentration decreases and the fluorescence of the dispersion increases.[31] Thus, the percentage of CF that is released from the liposomes can be calculated from eq 1, where I_x refers to the intensity associated

$$I\,(\%) = 100[I_a - I_b]/[I_x - I_b] \qquad (1)$$

with the release of 100% of the dye (determined via addition of excess (Triton X-100); I_b and I_a represent the fluorescence intensities before and after incubation with a given bolaphile, respectively.

Although egg phosphatidylcholine (egg PC) has frequently been used as a model membrane for investigating the disrupting properties of detergent molecules,[28] we chose to employ vesicles derived from POPC. Unlike egg PC, which is a complex mixture of phosphatidylcholines, having saturated fatty acids largely in the *sn*-1 position and unsaturated acids in the *sn*-2 position, POPC

(28) Ruiz, J.; Goni, F. M.; Alonso, A. *Biochim. Biophys. Acta* **1988**, *937*, 127.
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(31) Weinstein, J. N.; Ralston, E.; Leserman, L. D.; Klausner, R. D.; Dragsten, P.; Henkart, P.; Blumenthal, R. In *Liposome Technology*; Gregoriadis, G., Ed.; CRC Press, Inc.: Boca Raton, FL, 1984, Vol. III, p 183–204.

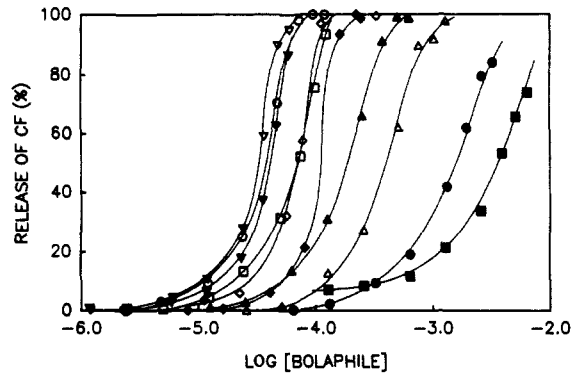

Figure 5. Plot of percent release of CF as a function of surfactant concentration present in the dispersion for bolaphiles Ia (■), Ib (●), Ic (△), Id (▲), Ie (□), If (○), Ig (▽), Ih (▼), Ii (◇), and Triton X-100 (◆).

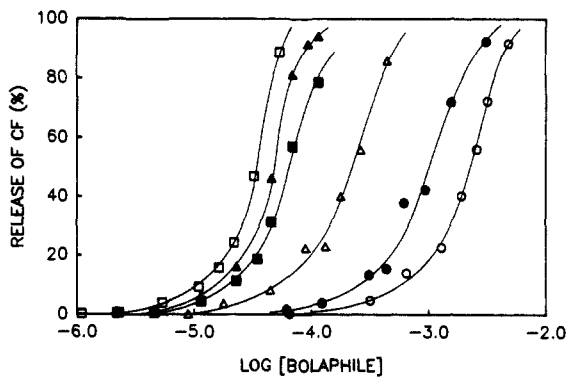

Figure 6. Plot of percent release of CF as a function of surfactant concentration present in the dispersion for bolaphiles IIa (○), IIb (●), IIc (△), IId (▲), IIe (□), and IIf (■).

is a single pure compound. Moreover, the fact that its gel to liquid–crystalline phase transition lies well below room temperature,[32] insures the presence of a biologically-relevant fluid phase at ambient temperature. In this study, we have also chosen Triton X-100, a commonly used nonionic detergent, to serve as our benchmark for calibrating the membrane-disrupting activity of each bolaphile.

Membrane Disrupting Activity. Figure 5 sumarizes the membrane-disrupting properties of type I bolaphiles, as shown by plotting the percentage of CF that is released as a function of bolaphile concentration. Analogous plots for type II and type III plus type IV bolaphiles are presented in Figures 6 and 7, respectively. In order to simplify a comparison of membrane-disrupting activity among the bolaphiles, we define an empirical parameter, (R_{50}), as the ratio of lipid/surfactant that is needed to release 50% of the entrapped CF from a ca. 0.5 mM solution of POPC-liposomes. Figure 8 summarizes the observed R_{50} values for each class of surfactant, as a function of the total number of carbon atoms (n) separating the ester carbonyl groups.

In the saturated and olefinic series of bolaphiles, a maximum activity is observed at n = 16 and n = 18, respectively. For the case of the acetylenic bolaphiles, the disrupting activity was found to increase, continuously with increasing size of the hydrophobic segment, for the range of bolaphiles that were studied. Removal of only two hydrogen atoms from type I bolaphiles, yielding cis-olefinic analogues, resulted in a *net reduction* in disrupting activity per carbon atom, when n was equal to or less than 17. With longer bolaphiles, however, a "cross-over" occurs; i.e., the disrupting activity of the olefinic bolaphiles were higher than the saturated analogues. Placement of a cis double bond asymmetrically within the hydrocarbon chain, did not significantly alter

(32) The T_m value for POPC is −5 °C: DeKrujff, B.; Demel, R. A.; Slotboom, A. J.; Van Deenen, L. L. M.; Rosenthanl, A. F. *Biochim. Biophys. Acta* **1973**, *307*, 1.

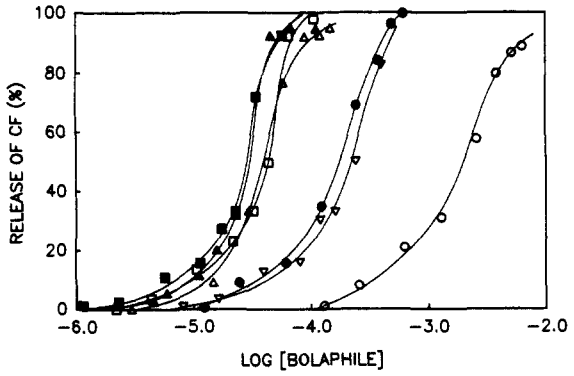

Figure 7. Plot of percent release of CF as a function of surfactant concentration present in the dispersion for bolaphiles IIIa (○), IIIb (●), IIIc (△), IIId (▲), IIIe (□), IIIf (■), and IV (▽).

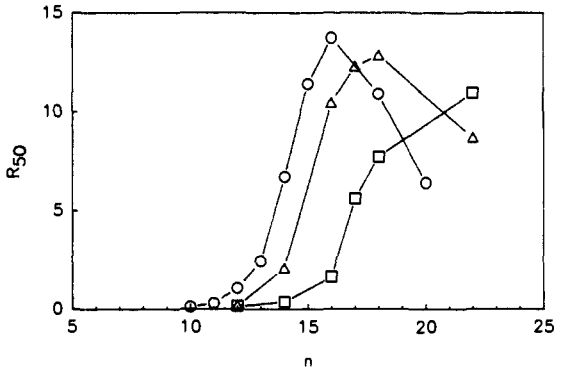

Figure 8. Plot of R_{50} as a function of the number of carbon atoms (n) separating the carbonyl groups: saturated (○), olefinic (△), and acetylenic (□) bolaphiles.

the surfactant's disrupting action from that which would be expected on the basis of its overall chain length; i.e., IIIf, having a total of 17 carbons separating the carboxylic acid moieties, exhibted an activity which lies in between that observed for n = 16 (IIIc) and n = 18 (IIId). In addition, the trans-olefinic bolaphile (IV) was very similar in activity with that found for its cis isomer (i.e., structure IIIb). Thus, both the positioning of the olefinic moiety within the hydrophobic segment, and its geometry, are of lesser importance in defining the bolaphile's membrane-disruptive power, than its overall length. Further removal of two hydrogen atoms from each hydrophobic segment, yielding acetylenic bolaphiles, leads to a further reduction in membrane-disrupting activity per carbon atom when n is ≤19; for a longer bolaphile (i.e., n = 22), however, the disrupting activity was found to be greater than for both the saturated and olefinic analogues. Here also, placement of the acetylene moiety asymmetrically within the hydrocarbon segment does not produce any significant deviation in activity from that which would be expected on the basis of the overall length of the hydrophobic chain; i.e., IIf, having a total of 17 carbons separating the carboxylic acid moieties, exhibited an activity which lies in between that observed for n = 16 (IIc) and n = 18 (IId). On a molar basis, the most active membrane-disrupting bolaphile that was prepared was Ig, which showed an activity which was approximately 3 times greater than Triton X-100. Overall, the R_{50} values for the entire series of bolaphiles that have been prepared extend over the range 0.125–13.8.

Bolaphile Structure–Membrane-Disrupting Activity Relationships. While R_{50} values are of primary importance to us from a *practical standpoint* (i.e., to serve as a basis for comparison with antimicrobial activity), they are defined on an operational basis and cannot be used to draw conclusions regarding structure–activity relationships. In particular, R_{50} values are a function of the *intrinsic* disruptive power of the bolaphile *and* the affinity of the bolaphile toward the lipid membrane. In an effort to examine the intrinsic disruptive power of each bolaphile, we have estimated their binding to POPC membranes (at 50% release) and have

Table I. Disrupting Activity and Affinity of Bolaphiles toward Phospholipid Membranes Derived from 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)

bolaphile	R_{50}	IR_{50}	membrane-bound bolaphile (%)
Ia	0.125	0.143	87
Ib	0.301	0.338	89
Ic	1.07	1.18	90
Id	2.45	3.25	75
Ie	6.73	9.53	71
If	11.4	17.6	65
Ig	13.8	16.4	84
Ih	10.9	11.2	98
Ii	6.43	7.68	83
IIa	0.155	0.198	98
IIb	0.372	0.537	87
IIc	1.66	5.14	41
IId	7.76	24.8	39
IIe	11.0	14.3	97
IIf	5.62	~	–
IIIa	0.186	0.270	86
IIIb	2.09	2.90	91
IIIc	10.5	32.5	41
IIId	12.9	17.9	91
IIIe	8.71	11.5	96
IIIf	12.3	19.4	80
IV	1.62	2.74	74
Triton X-100	4.06	6.53	62

defined a corresponding IR_{50} value as the *intrinsic ratio* of lipid/membrane-bound bolaphile under such conditions. Experimentally, 0.5 mM multilamellar vesicle (MLV) dispersions of POPC have been incubated with appropriate concentrations of bolaphile and subsequently removed, (along with adsorbed bolaphile), via centrifugation. Analysis of the residual bolaphile in the supernatant, then allows one to estimate the percentage of membrane-bound bolaphile (Table I). It should be noted, however, that these IR_{50} values assume: (i) that equilibria between membrane-bound and free bolaphiles for *centrifuged* (pelleted) liposomes are similar (or directly proportional) to that found for MLVs in the *dispersed* state, (ii) that the bolaphiles readily diffuse across multibilayers of POPC, and (iii) that solubilization of the lipid membrane is negligible.

In order to measure dilute bolaphile concentrations, we have used the surfactant-induced release of CF from LUVs of POPC, itself, as an analytical method. Specifically, we have developed calibration curves similar to that shown in Figures 5–7 and have used them to estimate the concentration of bolaphile in the supernatant of pelleted MLV dispersions (see Experimental Section). Under the experimental conditions which we have employed, we have observed that the supernatant, in all cases, contains <10% of the total lipid present in the initial MLV dispersion (phosphorus analysis). In preliminary differential scanning calorimetry (DSC) studies, we have found that the perturbation of bolaphile Ie on the gel to liquid–crystalline phase transition for 1000-Å diameter unilamellar vesicles of dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) is virtually identical with that which is found for analogous multilamellar dispersions, using the same lipid/surfactant ratio.[33] Moreover, the endotherms observed for the latter are the same, regardless of whether the bolaphile is added to a preformed MLV dispersion, or whether it is initially present in the lipid mixture, prior to liposome formation. These results infer that this bolaphile can readily diffuse across multibilayers of DPPC in the gel phase (during the DSC measuement), and strongly suggest that Ie (as well as closely related bolaphiles) should readily diffuse across a POPC membrane, which exists in the liquid crystalline phase.

Table I and Figure 9 summarize all of the IR_{50} and R_{50} data that have been obtained. Qualitatively, the picture that emerges from the IR_{50} values is similar to that which comes from the R_{50} data. A maximum in IR_{50} is seen for all three classes of bolaphiles. In addition, the total range of these values extends from 0.143 to 32.5. Within the range of n = 10–14, the disruptive power of the surfactants decreases as one goes from saturated to olefinic to acetylenic hydrophobic segments. Beyond n = 14, however, there is a reversal; the inherent disruptive power of the bolaphile increases as one goes from saturated to olefinic to acetylenic hydrophobic segments. For the unsaturated bolaphiles, membrane-disrupting activity remains more dependent on the surfactant's overall length than on the positioning of either the acetylenic or olefinic moiety within the hydrophobic segment. Also, the cis- and trans-olefinic bolaphiles exhibit similar membrane-disrupting activity.

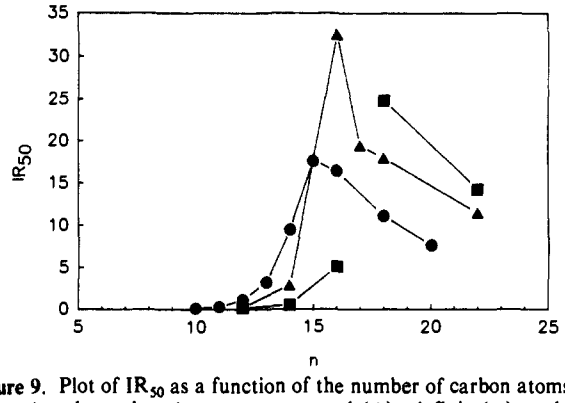

Figure 9. Plot of IR_{50} as a function of the number of carbon atoms (n) separating the carbonyl groups: saturated (●), olefinic (▲), and acetylenic (■) bolaphiles. The IR_{50} value for IIf could not be accurately determined due to its very low affinity toward the POPC-membrane.

A model which accounts for the general "upward and to the right" shift of the IR_{50} vs n curves, as one goes from saturated to olefinic to acetylenic bolaphiles (Figure 9) can be envisaged, if one assumes that each bolaphile forms a loop within the outer monolayer leaflet of the lipid membrane, and that the defects created by the bolaphile depend on the loop's *depth of penetration* and *width*. For the saturated bolaphile series, a maximum in membrane-disrupting activity was observed when n = 15 (If). If the shape of each membrane-bound bolaphile corresponds to a "stretched loop", then the maximum depth that If can penetrate into a POPC-based membrane is approximately one-half of one monolayer. If one further assumes that such a depth is optimal for inducing the leakage of CF, using the unsaturated bolaphiles, then the introduction of a cis- or trans-olefinic moiety in the center of the hydrophobic segment (or a cis-double bond positioned, asymmetrically) should decrease the effective depth of penetration of the loop (for a given value of n), and require a greater number of total carbons atoms (n) in order to reach the optimum depth. Similarly, symmetrically positioned acetylenic groups should further decrease the depth of penetration, due to the linear arrangement of *four* central carbon atoms, and thus require even a greater n value for optimum activity. The increase in maximum activity, as one goes from saturated to olefinic to acetylenic bolaphiles, can also be explained in terms of a wider loop which is more efficient in perturbing the bilayer.

While we suspect that the above model is overly simplistic, it, nonetheless, accounts for the general trends that have been observed, and provides a basis for the rational design of "second-generation" bolaphiles. Efforts that are currently in progress are being aimed at (i) obtaining more detailed insight into these structure–activity relationships, (ii) exploring the influence of structure and composition of a bolaphile's head groups on its membrane-disrupting activity, (iii) creating new and more active classes of bolaphiles, and (iv) systematically evaluating the antimicrobial properties of each double-headed surfactant. Results of these efforts will be reported in due course.

Supplementary Material Available: Tables of ^1H NMR data, melting points, and elemental analyses for all new compounds reported in this work (7 pages). Ordering information is given on any current masthead page.

(33) The T_m value for the main transition of unperturbed DPPC membranes (41.6 °C) was increased upon addition of the bolaphile: Jayasuriya, N.; Regen, S. L. Unpublished results.