Supramolecular Surfactants: Polymerized Bolaphiles Exhibiting Extraordinarily High Membrane-Disrupting Activity[1]

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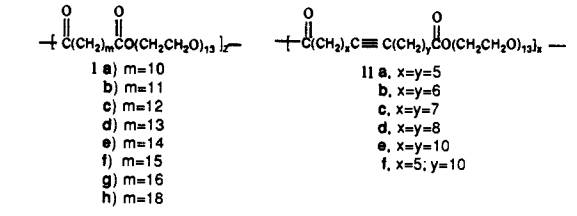
Abstract: Polymerized forms of saturated, olefinic, and acetylenic bolaphiles (double-headed surfactants) have been prepared by condensation of a series of α,ω-dicarboxylic acids with tridecaethylene glycol, and evaluated for their ability to induce the release of 5(6)-carboxyfluorescein (CF) entrapped within large unilamellar vesicles derived from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). Each amphiphilic polymer was found to have substantially greater membrane-disrupting activity than that found for Triton X-100; operationally, one was more than 300 times greater in activity. Comparison of each supramolecular surfactant with its corresponding bolaphile demonstrates, in nearly all cases, that a significant enhancement in membrane-disrupting activity is provided through polymerization, and that the magnitude of this amplification decreases, exponentially, as the length of the hydrophobic segment increases. In the most striking case, the disruptive power of the supramolecular surfactant was at least 3 orders of magnitude greater than its monomeric counterpart. As one goes from saturated to olefinic to acetylenic polyesters, longer hydrophobic segments are required in order to reach a maximum in activity. From a practical standpoint, supramolecular surfactants extend nonionic detergents into a new and uncharted domain in membrane-disrupting activity.

Introduction

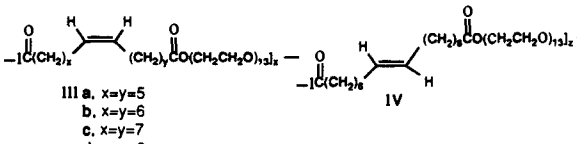
In the preceding paper, we have described the synthesis of four homologous series of saturated and unsaturated bolaphiles (i.e., "double-headed" single-chain surfactants) and have defined their ability to disrupt lipid bilayers made from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC).[3] Our primary interest in such molecules was based, in part, on the belief that certain of these surfactants could selectively alter the structure and biological function of the lipid envelope of certain microorganisms, in the presence of mammalian cells; i.e., they could have practical potential as therapeutic agents. An intriguing extension of this chemistry can be envisaged, if one considers a "polymeric string of bolaphiles" (or "*supramolecular surfactants*") acting as membrane-perturbing agents (Figure 1).[4] In particular, one might expect that such polymers should exhibit enhanced disrupting power due to (i) a stronger affinity toward the lipid membrane via multiple sites of attachment, and (ii) a high local concentration of repeat units (defects) within the membrane; i.e., dilution of the repeating bolaphile units through lateral diffusion would be precluded. If this were the case, then such polymers could serve as valuable additions to our "arsenal" of surfactants, by significantly extending our working range of membrane-disrupting activity.

Do supramolecular surfactants exhibit much greater membrane-disrupting action than their monomeric counterparts, from an *operational* standpoint? Is their disrupting power a sensitive function of the structure and composition of the hydrophobic segment? The primary aim of the work which is described herein was to answer both of these questions by synthesizing polymers I–IV, and by evaluating their membrane-disrupting properties.[5]



I a) m=10
b) m=11
c) m=12
d) m=13
e) m=14
f) m=15
g) m=16
h) m=18

II a, x=y=5
b, x=y=6
c, x=y=7
d, x=y=8
e, x=y=10
f, x=5; y=10



III a, x=y=5
b, x=y=6
c, x=y=7
d, x=y=8
e, x=y=10
f, x=5; y=10

IV

Experimental Section

General Methods. All of the organic diacids that were used in this work were prepared by use of procedures similar to those described in the accompanying paper.[3] Unless stated otherwise, all reagents and chemicals were obtained from Aldrich Chemical Co. and used without further purification.

Tridecaethylene Glycol. 55% Sodium hydride (4.20 g, 96 mmol) was washed four times with 10 mL of THF and then suspended in 50 mL of fresh THF. To this suspension was added 26.0 g (94 mmol) of tetraethylene glycol mono(tetrahydropyranyl ether), dropwise. After the evolution of hydrogen gas ceased, (ca. 0.5 h), 20.0 g (36 mmol) of pentaethylene glycol di-*p*-toluenesulfonate in 15 mL of THF was added dropwise over a period of 0.5 h (Note: sodium *p*-toluenesulfonate precipitates during addition). After the resulting suspension was refluxed for 3 h, an additional portion of NaH (0.6 g. 25 mmol) was added, and the mixture was stirred for 12 h at room temperature. Methanol (10 mL) was then added to destroy any unreacted NaH, and the mixture was filtered. The filtrate was concentrated under reduced pressure, and the unreacted starting material was removed by distillation (180 °C, 0.5 mmHg). The residue was then dissolved in 200 mL of methanol and acidified by adding a few drops of concentrated HCl, containing *p*-toluenesulfonic acid (100 mg). After stirring for 12 h, the solution was concentrated under reduced pressure, redissolved in 100 mL of CH_2Cl_2, and filtered through a short plug of silica gel. The solvent was removed under reduced pressure and the residue was distilled (245 °C, 0.5 mm) in order to remove low molecular weight products. The residue that was left behind was then purified by flash chromatography on silica gel, with CH_2Cl_2/CH_3OH (9/3, v/v) as the eluent, to give 10.05 g of tridecaethylene glycol. A residual color in the product was eliminated by dissolving the polyether in methanol and treating with decolorizing carbon, to give 9.91 g (46%) of tridecaethylene glycol as a white waxy solid: ^1H NMR ($CDCl_3$) δ 3.0 (s, 2 H), 3.6 (m, 52 H). In order to confirm its purity, 0.448 g (0.76 mmol) of the tridecaethylene glycol was esterified with 1.82 mmol of palmitoyl chloride (1 h, 75 °C), and the diester was isolated as a white solid by silica gel chromatography $CHCl_3/CH_3OH$

(1) Supported by PHS Grant AI28220 awarded by the National Institute of Allergy and Infectious Diseases.

(2) On leave from the Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia.

(3) Jayasuriya, N.; Bosak, S.; Regen, S. L. *J. Am. Chem. Soc.*, preceding paper in this issue.

(4) A preliminary account of this work has previously appeared: Regen, S. L.; Jayasuriya, N.; Fabianowski, W. *Biochem. Biophys. Res. Commun.* **1989**, *159*, 566.

(5) Certain poly(ethylenimine) derivatives have been found active in disrupting phospholipid bilayers: Takigawa, D. Y.; Tirrell, D. A. *Macromolecules* **1985**, *18*, 338.

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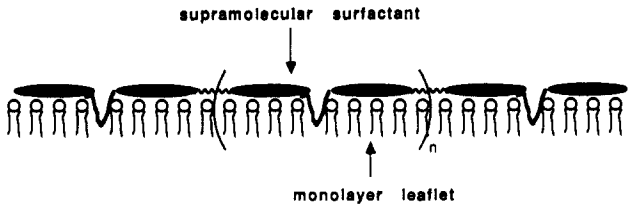

Figure 1. Stylized illustration of a supramolecular surfactant interacting with a monolayer leaflet of a lipid membrane.

(95/5, v/v): ^{1}H NMR ($CDCl_3$) δ 1.2 (m, 54 H), 1.55 (m, 4 H), 2.3 (t, 4 H), 3.6 (m, 48 H), 4.2 (t, 4 H). The expected ratio of palmitoyl/ethylene glycol protons is 62:52; found, 62:51.

Polyester Synthesis. The following general procedure was used for the preparation of each of the polyesters I–IV: An appropriate α,ω-dicarboxylic acid (ca. 0.2 mmol) was refluxed with 4 equiv of $SOCl_2$ for 3 h under a nitrogen atmosphere. The excess $SOCl_2$ was removed under reduced pressure, and the residue was then dissolved in 0.5 mL of CH_2Cl_2. To this solution was added a second solution containing 1 equiv of tridecaethylene glycol plus 1.5 equiv of pyridine in 0.5 mL of CH_2Cl_2. The mixture was then stirred for 12 h at ambient temperature, washed with water (4 × 1 mL), concentrated under reduced pressure, and the residue dried under vacuum [12 h, 23 °C (0.2 mm Hg)]. All saturated polyesters were purified by repeated solubilization–precipitation (3×) with CH_2Cl_2/hexane (0.5 mL/8 mL) and dried under vacuum [12 h, 23 °C (0.2 mmHg)]; isolated yields ranged between 58 and 86%. In the case of the olefinic and acetylenic polymers, purification was carried out with use of silica gel (30 g) column chromatography, eluting first with 200 mL of $CHCl_3/CH_3OH$ (40/1, v/v) and then with 200 mL of $CHCl_3/CH_3OH$ (20/1, v/v). All of the unsaturated polymers were isolated (30–50%) as oils, having an R_f in the range of 0.25 to 0.40 [silica, $CHCl_3/CH_3OH$, 15/1 (v/v)].

Surfactant-Induced Release of Liposome-Encapsulated CF. Approximately 20 mg of POPC in 2 mL of chloroform (obtained from Avanti Polar Lipids as a 10 mg/mL solution) was placed in a test tube (13 × 100 mm) and the chloroform was evaporated under a stream of nitrogen. The resulting thin lipid film was dried under vacuum [12 h, 23 °C (0.2 mmHg)] in order to remove residual solvent and then suspended in 1 mL of a 0.25 M CF solution (pH 7.4) via vortex mixing. The resulting multilamellar dispersion was allowed to equilibrate for 0.5 h and then subjected to five freeze–thaw cycles (−196 °C and 23 °C). After subsequent extrusion (10 times) through two stacked 0.1-μm polycarbonate filters (Nuclepore), the resulting liposomes were purified by gel filtration with a Sephadex G-50 (0.7 × 40 cm) column, with a 10 mM borate buffer (pH 7.4) as the eluant. The vesicle fractions were combined, and the volume was adjusted to ca. 5 mL by using the same buffer. The dispersion was then dialyzed against 200 mL of buffer at 15 °C for 12 h and was allowed to warm to room temperature, just prior to use.

Typically, 10-μL aliquots of the vesicle dispersion were added into each of eight test tubes (6 × 50 mm) containing 90 μL (final concentration of the lipid was ca. 0.5 mM) of a given polymer solution, at appropriate concentrations, and then vortex mixed. After the samples were allowed to incubate for 0.5 h, 25-μL aliquots were diluted in 4 mL of buffer, and the fluorescence was measured. A blank determination was carried out by diluting a 10-μL aliquot of the vesicles with 90 μL of buffer. The total fluorescence was determined by complete disruption of the vesicles with Triton X-100.[6]

Gel Permeation Chromatography. The isocratic liquid chromatograph which was used for all GPC measurements was a modular system consisting of a Waters Model 510 solvent delivery system, a Waters Model U6K injector, and a Waters Model 410 differential refractometer, that was interfaced with a Maxima 820 work station. Ultrastyragel columns (10^3 Å, 500 Å, and 100 Å) were obtained from Waters, and used in series. The column temperature was maintained at 40 °C. The flow rate that was used in all cases was 1 mL/min. Polystyrene standards that were used were purchased from Showa Denko K.K., Japan, (MW: 6.60×10^4, 2.85×10^4, 9.24×10^3, 3.25×10^3, 1.25×10^3) and from Waters (MW 776). Chromatography grade tetrahydrofuran was purchased from Burdick and Jackson and degassed before use. Calibration standards and test solutes were injected as dilute solutions in the eluant. Polystyrene standards were 0.1% (w/v), and the polyesters were 0.1–0.2% (w/v). The injection volume that was used in all cases was 100 μL. All molecular weights that are reported are polystyrene-equivalent molecular weights.

(6) 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.

Scheme I

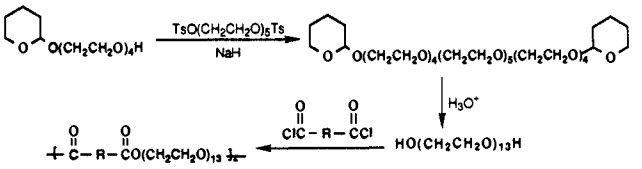

Table I. Supramolecular Surfactants as Membrane-Disrupting Agents

polymer	M_n	M_w	polydispersity index	DP	R_{50}
Ia	4340	6460	1.49	5.5	36.2
Ib	4010	5800	1.45	5.0	53.3
Ic	3890	6000	1.54	4.8	137
Id	4220	7140	1.69	5.1	232
Ie	4000	5780	1.45	4.8	80.6
If	3730	5400	1.45	4.4	44.3
Ig	4690	6980	1.45	5.4	73.6
Ih	5530	8330	1.51	6.2	12.5
IIa	9170	13300	1.44	11.4	7.20
IIb	6110	8990	1.47	7.3	79.0
IIc	10200	19900	1.95	11.8	1310
IId	5770	8700	1.51	6.5	79.0
IIe	6400	9550	1.49	6.7	4.98
IIf	7330	10500	1.43	8.3	154
IIIa	10300	22300	2.16	12.7	208
IIIb	4780	6800	1.43	5.7	228
IIIc	9010	15300	1.70	10.4	131
IIId	4790	6680	1.40	5.4	52.3
IIIe	5620	8240	1.47	5.9	4.25
IIIf	8340	11800	1.41	9.5	122
IV	7560	12400	1.64	9.0	330
Triton X-100	-	-	-	-	4.06

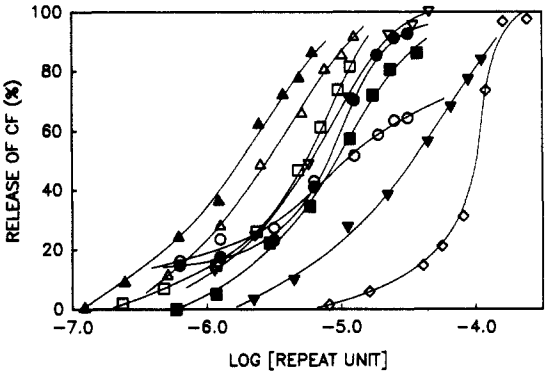

Figure 2. Plot of percent release of CF as a function of repeat unit concentration present in the dispersion for Ia (○), Ib (●), Ic (△), Id (▲), Ie (□), If (■), Ig (▽), Ih (▼), and Triton X-100 (◇).

Results and Discussion

Polymer Synthesis. Polymers I–IV were synthesized by condensing the appropriate α,ω-dicarboxylic acid dichloride with 1 equiv of tridecaethylene glycol (Scheme I). This specific polyether was chosen as the hydrophilic segment in order to prepare polymers that closely matched those bolaphile structures which were previously investigated (tridecaethylene glycol can be thought of as two hexaethylene glycols that are "loosely" coupled via an ethylene glycol bridge). Number average molecular weights (M_n), weight average molecular weights (M_w), and number average degrees of polymerization (i.e., the average number of bolaphiles per polymer chain, DP) were determined by standard size exclusion chromatography and are listed in Table I.

Membrane Disrupting Activity. In order to evaluate its membrane-disrupting properties, each amphiphilic polymer has been examined for its ability to induce the release of 5(6)-carboxyfluorescein (CF) from within large unilamellar vesicles (LUVs) of POPC. The rationale for using this specific liposomal system has been described in the accompanying paper.[3] Figure 2 summarizes the influence that various concentrations of type I sur-

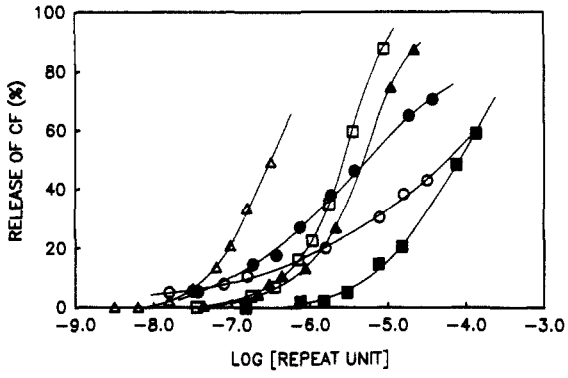

Figure 3. Plot of percent release of CF as a function of repeat unit concentration present in the dispersion for IIa (○), IIb (●), IIc (△), IId (▲), IIe (■), and IIf (□).

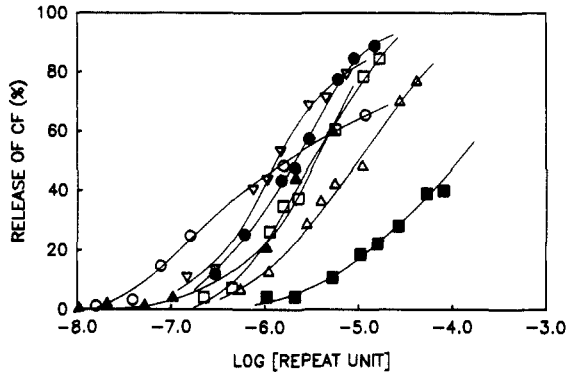

Figure 4. Plot of percent release of CF as a function of repeat unit concentration present in the dispersion for IIIa (○), IIIb (●), IIIc (▲), IIId (△), IIIe (■), IIIf (□), and IV (▽).

factants have on the release of liposome-encapsulated CF. Analogous plots for type II and type III plus type IV polymers are shown in Figures 3 and 4, respectively. What is readily apparent from each of these figures is that *all* of the curves appear to the left of the Triton X-100 curve; i.e., *all* of the polymers tested exhibit significantly greater membrane-disrupting activity than that found for this commonly used nonionic detergent. Because we are presently unable to judge how many bolaphile repeat units of a liposome-bound polymer are in intimate contact with the bilayer and because our primary interest in these surfactants has been to expand our working range of membrane-disrupting activity, we have limited the current investigation, exclusively, to *operational* comparisons. If we define an empirical parameter, R_{50}, as the ratio of lipid/bolaphile repeat unit that is needed to induce the release of 50% of the entrapped CF from a ca. 0.5 mM solution of POPC-liposomes), then plots of R_{50} as a function of the number of carbon atoms (n) which separate the saturated and unsaturated ester carbonyl groups have a striking similarity to those seen for the analogous bolaphiles. In particular, *as one goes from saturated to olefinic to acetylenic polyesters, longer hydrophobic segments are required in order to reach a maximum in activity* (Figure 5). Of all of the polymeric surfactants tested, IIc showed the highest membrane-disrupting activity. Here, only 0.08 mol% of repeat units were required to induce the release of 50% of the entrapped CF. If one assumes a cross-sectional area of POPC equalling 70 Å^2, and a bilayer thickness of 50 Å, then a 1000-Å diameter unilamellar vesicle contains ca. 80 000 lipids. On the basis of the number average degree of polymerization of IIc and on the basis of its R_{50} value, the average number of polymers which are associated with each liposome under these conditions can then be estimated to be ≤ 5.

In order to determine whether or not the stitching together (polymerization) of bolaphiles leads to an amplification of their membrane-disrupting activity, we have compared the R_{50} value measured for each supramolecular surfactant with that of its corresponding bolaphile. As can be readily seen in Figure 6, a

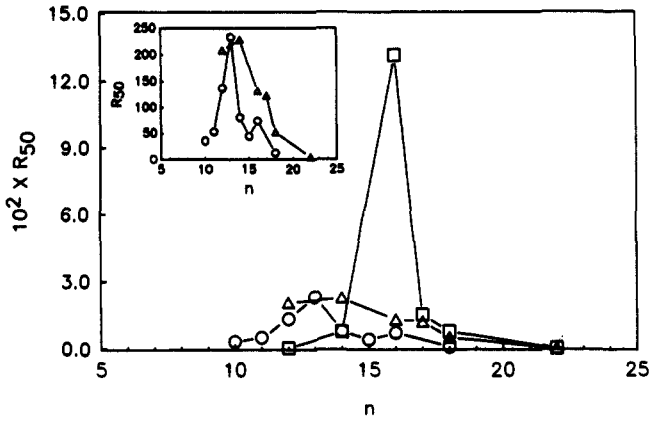

Figure 5. Plot of R_{50} as a function of the number of carbon atoms (n) separating the carbonyl groups: saturated (○), olefinic (△), and acetylenic (□) polymers. Insert shows a comparison of the saturated and olefinic polymers on an expanded scale.

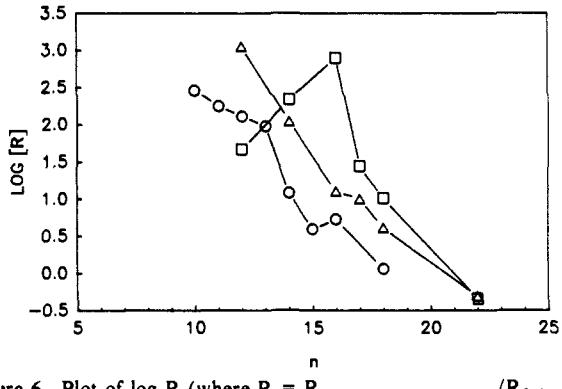

Figure 6. Plot of log R (where R = $R_{50\ \text{supramolecular surfactant}}/R_{50\ \text{bolaphile}}$) versus the number of carbon atoms separating the ester carbonyl groups for saturated (○), olefinic (△), and acetylenic (□) surfactants. All R_{50} bolaphile values that were used were taken directly from our previous work.[3]

plot of the log ($R_{50\ \text{supramolecular surfactant}}/R_{50\ \text{bolaphile}}$) versus the number of carbon atoms separating the ester carbonyl groups (n) establishes that, in nearly all cases, a significant enhancement in activity occurs upon polymerization and that the magnitude of this amplification decreases, exponentially, as the length of the hydrophobic segment increases. In the most striking example, IIIc, the quantity of membrane-bound bolaphile that is needed to induce the release of 50% of the entrapped CF is almost 3 orders of magnitude greater than its polymeric analogue.[7] Each membrane-bound bolaphile unit of IIIc must, therefore, have an effective disrupting power which is at least 3 orders of magnitude greater than its corresponding monomer.

While we do not presently understand the precise origin of this amplification, we believe that the key factors that must be considered are (i) a high local concentration of "bolaphile-like" defects within the bilayer due to covalent linkages, (ii) domains of supramolecular surfactant within the bilayer that are in equilibrium with nonaggregated membrane-bound polymers, (iii) repeat unit defects which are intrinsically more disruptive than those of the free monomer, and (iv) a net reduction in the number of hydroxyl groups per hydrophobic segment. The fact that binding studies have shown that most bolaphiles, when added to aqueous dispersions of POPC, are extensively bound to the membrane (at 50% release), indicates that increased binding of the amphiphilic polymer to the lipid membrane cannot account for the sizeable amplification that is observed in most cases. The exponential

(7) If all of the polymer that is present in the dispersion is bound to the liposomes, then the ratio of membrane-bound bolaphile/membrane-bound supramolecular surfactant which is required for 50% release is 945. If a lesser amount of polymer were bound to the liposomes, then this ratio would increase, proportionally.

decrease in amplification, as the length of the hydrophobic segment is increased, also remains to be clarified. We presently suspect, however, that self-aggregation of the surfactant polymers in solution and/or at the liposomal surface may be an important factor in this regard. Despite these questions which remain to be answered, all of the results that are presented herein, clearly demonstrate that *a very substantial amplification of the membrane-disrupting activity of a bolaphile is possible through polymerization, and that the magnitude of this amplification is a sensitive function of the structure and composition of the hydrophobic segment.* Operationally, one of the supramolecular surfactants (**IIc**) has been found to exhibit a membrane-disrupting activity which was more than 300 times greater than that observed for Triton X-100.

From a practical standpoint, supramolecular surfactants extend nonionic detergents into a new and uncharted domain in membrane-disrupting activity, which should be exploited. It is particularly significant to note that in preliminary in vitro studies, one polyester, having a structure which is very similar to that of Ie, exhibited substantial protection for human CD4+ lymphocytes against HIV-1; i.e., a 100% level of protection was observed when a polymer concentration of 53 μg/mL is used.[4,8] While the precise mechanism of anti-HIV action and the ultimate therapeutic utility of this and related polymers remain to be established, these results, nonetheless, lend strong support to our belief that a careful and systematic examination of supramolecular surfactants (and their corresponding bolaphiles) as antimicrobial agents is warranted.

Studies that are now in progress are aimed at (i) developing a more complete "library" of membrane-disrupting agents, (ii) defining the interactions between such agents and phospholipid membranes, and (iii) searching for correlations that may exist between membrane-disrupting activity and antimicrobial action.

(8) In preliminary in vivo studies, Ic, Id, Ie, and If, when injected intraperitoneally into outbred white mice (up to 50 mg/kg), do not show any signs of toxicity: Jayasuriya, N.; Juliano, R. L.; Regen, S. L. Unpublished results.

Kinetics of the Thermolysis of [*n*.2.2]Propellanes and Related Compounds. Mechanism of the Thermolysis of Bicyclo[2.2.0]hexanes

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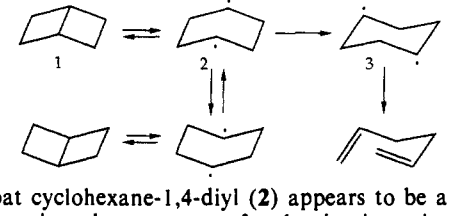
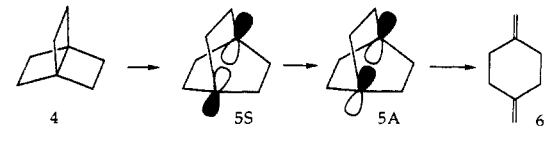
Abstract: The thermolyses of a series of 1,4-bridged bicyclo[2.2.0]hexanes have been studied. With bridges having three or more carbons, the compounds have higher activation energies than for bicyclo[2.2.0]hexane, indicating that the bridge prevents the formation of a chair cyclohexane-1,4-diyl, forcing the reaction to proceed via an orbital symmetry disallowed process. It appears likely that [2.2.2]propellane and its derivatives react via the same mechanism, and the driving force from strain relief appears to be the major factor in reducing its activation energy. The thermolysis of the relatively unstrained [3.3.2]propellane occurs at a significantly higher temperature and leads to a mixture of products which also were found in the thermolysis of 1,5-dimethylenecyclooctane. The thermolysis of the latter at 420 °C formed the propellane. The strain relief in the cleavage of the central bond in this group of propellanes were estimated via a combination of ab initio and molecular mechanics calculations and was found to be correlated with the changes in activation energy. The thermolyses of [3.2.1]- and [4.2.1]propellanes also are reported and were found to be less reactive than expected on the basis of strain energy relief.

The thermolysis of small ring hydrocarbons containing cyclobutane rings has received considerable attention.[1] Bicyclo[2.2.0]hexane (**1**) has been studied both with regard to its kinetics and stereochemistry of ring opening, and a multistep process appears well established.[2-4] The observation of ring inversion proceeding more rapidly than ring opening,[3] along with the stereochemical observations[4] are well accounted for by the process:



The boat cyclohexane-1,4-diyl (**2**) appears to be a required intermediate in order to account for the ring inversion.[3] A key element of the process is the conformational change from the boat of the chair diyl (**3**), and the orbital symmetry allowed cleavage of the latter to the product diene.[4]

The thermolysis of the known [2.2.2]propellane derivative (the *N*,*N*-dimethylamide) leads to the same overall process and has a remarkably low activation energy, E_a = 22 kcal/mol, which allows the reaction to occur at room temperature with a half-life of less than an hour.[5] It has been proposed that the reaction proceeds as follows:[6]



Here, the initial cleavage of the central bond of **4** leads to the symmetric diyl, **5S**. It has little through-space interaction because of the flexibility of the ring system which allows the bridgehead carbons to be fairly far apart. The antisymmetric diyl, **5A**, may now have the lower energy because of the interaction of the bridgehead orbitals with the σ^* orbitals of the ethano bridges.

(1) For a review of cyclobutane thermolyses, see: Berson, J. A. In *Rearrangements in Ground and Excited States*; de Mayo, P., Ed.; Academic Press: New York, 1980; Vol. 1.
(2) Steel, C.; Zand, R.; Hurwitz, P.; Cohen, S. G. *J. Am. Chem. Soc.* **1964**, *76*, 679. Srinivasan, R. *Int. J. Chem. Kinet.* **1969**, *1*, 133.
(3) Goldstein, M. J.; Benzon, M. S. *J. Am. Chem. Soc.* **1972**, *94*, 5119.
(4) Paquette, L. A.; Schwartz, J. A. *J. Am. Chem. Soc.* **1970**, *92*, 3215. Sinnema, A.; Rantwijk, F. v.; De Konig, A. T.; Wijk, A. M. v., Bekkum, H. v. Tetrahedron **1973**, *32*, 364. Wehrli, R.; Bellus, D.; Hansen, H.-J.; Schmid, H. *Chimia* **1976**, *30*, 416.
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