Peptide Liposomes from Amphiphilic Amino Acids

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Abstract: The synthesis and characterization of amphiphilic amino acids containing either cysteine or homocysteine are described. Upon ultrasonication, these lipids form liposomes. The spherical structures were confirmed by electron microscopy and by the entrapment of 6-carboxyfluorescein. In addition, these lipids can form giant liposomes which are detectable by light microscopy. The amino acid liposomes can be converted into peptide liposomes by adding a water-soluble carbodiimide to the liposomal solution or by sonication of the lipids in a carbodiimide-containing buffer. The isolated reaction products show typical trans amide bands in the IR at about 1660 and 1530 cm^{-1} indicative of peptide formation. Finally, the purified and isolated peptides are able to form liposomes and giant vesicles in mixtures with cholesterol.

Introduction. Routes to Polymeric Liposomes

During the last years, the interest in synthetic bilayer-forming lipids increased rapidly. However, liposomes from synthetic compounds lack the stability of those from natural occurring mixtures. To overcome this problem, polymerizable amphiphiles functionalized with, e.g., vinyl, methacryl, diacetylene, and isocyano moieties have been synthesized. Their properties have already been reviewed.[1,2] Nevertheless, the development of other types of polymeric liposomes is still a challenge for chemists. Recently, bilayer-forming amphiphilic polymers were described by Kunitake[3,4] and Elbert:[5] polymeric liposomes can be obtained directly from prepolymerized lipids by ultrasonication, avoiding any polymerization in the liposome. Aliev,[2,6] Regen,[7] and Takada[8] used an alternative approach. Their "liposomes in a net"[6] are liposomes coated with polymers either attached to the bilayer via ionic forces or by insertion of hydrophobic anchors.

The first polymeric liposomes prepared by a polycondensation reaction were described by Folda et al.[9] Their report describes the aminolysis of amino acid esters in liposomes. Although the reaction time is long no complete conversion can be achieved. One of the problems with these vesicles was the fact that the amide head groups were not hydrophilic enough to avoid precipitation.

In this publication, an alternative way to realize peptide liposomes is described. As shown in Figure 1, amino acid liposomes were prepared by ultrasonication of amphiphilic amino acids with one additional hydrophilic unit at the polar head group. The conversion to peptide liposomes was achieved by using water-soluble carbodiimides as condensating agents. The amphiphilic peptides obtained by this method are able to form liposomes by themselves. The synthesis of the amino acids, the conversion to peptides, and the properties of amino acid and peptide liposomes are discussed below. These peptide liposomes should have the advantage of being biodegradable.

Results and Discussion

The structures of the synthesized amphiphilic amino acids are summarized in Table I. With the exception of compound **1**, they are aminodicarboxylic acids with cysteine or homocysteine head groups prepared by the thiol addition of the amino acids to maleic mono- or diesters.

Table I. Structure of the Amphiphilic Amino Acids

AMPHIPHILIC AMINO-ACIDS

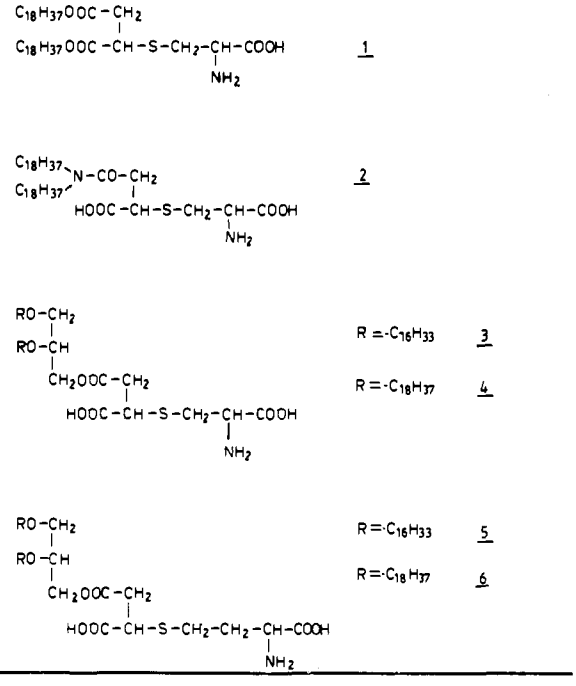

Table II. Phase-Transition Temperatures of 3-6 (as Hydrochlorides) and of Peptides from 3-6 (Obtained by DSC Measurements of Lamellar Phases)

	PHASE-TRANSITION TEMPERATURES	
compound	monomer	peptide from
3	334 K	328 K
4	344 K	337 K
5	340 K	329 K
6	355 K	338 K

Liposomes from Amphiphilic Amino Acids. Upon ultrasonication in water or buffer, these amino acids form liposomes. The spherical structure was confirmed by electron microscopy. Compounds **3–6** also form giant vesicles which can be seen in the light microscope. Figure 2 shows a typical preparation of giant liposomes from compound **5**. 6-Carboxyfluorescein can be entrapped into the liposomes above the self-quench concentration (50 mM). After removal of the dye from the external compartment of the vesicles by GPC, the increased fluorescence upon addition of Triton-X-100 proved the presence of intact vesicles.

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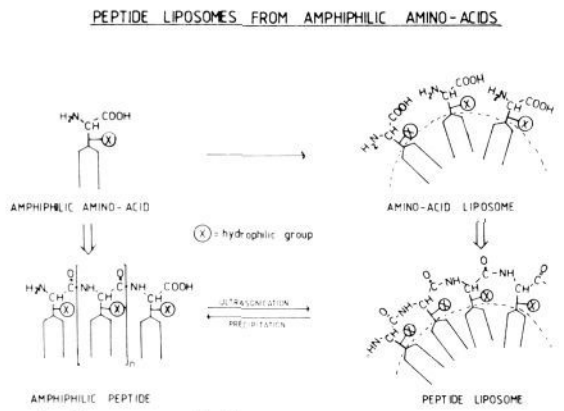

Figure 1. Routes to peptide liposomes.

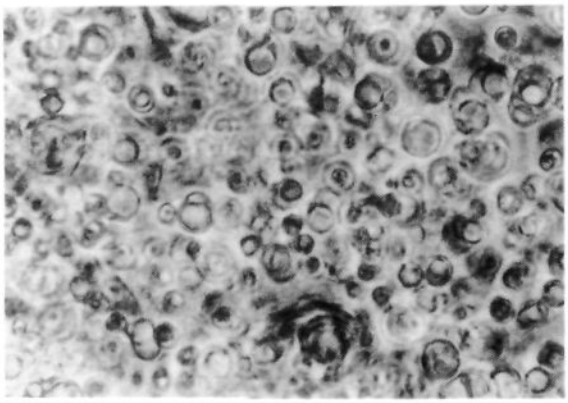

Figure 2. Giant liposomes from the monomeric amino acid **5** (0.067 M phosphate buffer, pH 6.5). $\phi \sim 10\ \mu m$.

The phase-transition temperatures of the hydrochlorides of **3–6** in swollen lamellar phases were determined by DSC measurements (solvent: pure H_2O). The values are given in Table II.

The homocysteine derivatives **5** and **6**, while having only one more CH_2 group than **3** and **4**, show higher phase-transition temperatures than their cysteine analogues. It is assumed that the decoupling of the hydrophobic chains[5] from the amino acid part of the hydrophilic head group is more efficient in the case of the homocysteine lipids **5** and **6**. This allows a better chain packing, and hence a higher phase-transition temperature is observed.

Condensation in Liposomes and Characterization of the Amphiphilic Peptides. To induce a polycondensation in liposomes, the lipids were sonicated in a carbodiimide-containing buffer solution at pH 6.5. *N*-Cyclohexyl-*N'*-[β-(*N*-methylmorpholino)ethyl]carbodiimide–*p*-toluenesulfonate was used as the water-soluble condensating agent. The amphiphilic amino acid **1** with no additional hydrophilic unit in the head group doesn't form liposomes in the presence of the carbodiimide. The solution remains turbid during sonication. After 10 min, a greasy material, but no liposomes, can be detected. Furthermore the addition of the carbodiimide to a liposomal solution of **1** causes precipitation. The precipitation may be due to the fact that the amide bond formed during condensation is not hydrophilic enough to keep the peptide liposome in solution. As to be expected from this result, the amphiphilic amino acids with an additional hydrophilic unit in the head group 2–6 form peptide liposomes which remain in solution (see Figure 1). To estimate the influence of the carbodiimide on the stability of the liposomes, the time-dependent turbidity change of the liposomal solution at 400 nm was measured by varying the molar ratio of carbodiimide to lipid. Figure 3 shows the curves for compound **3** for example. The concentration was always 1 mg/mL, pH 6.5.

Without carbodiimide, the turbidity does not change for over 1 week. After this period, no precipitate was found and liposomes could be detected by EM. The peptide liposomes formed by

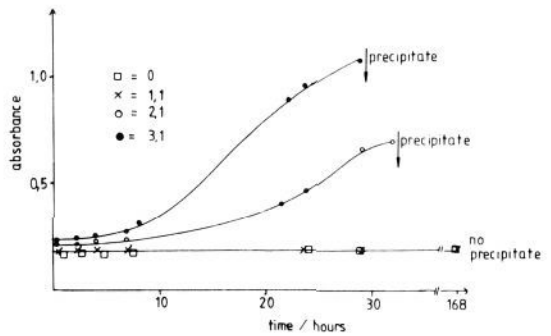

Figure 3. Time-dependent turbidity change of liposomes from **3** (c = 1 mg/mL) at λ = 400 nm (0.067 M phosphate buffer, pH 6.5); (□) no carbodiimide. Molar ratio of carbodiimide to amphiphilic amino acid: (X) 1.1; (○) 2.1; (●) 3.1.

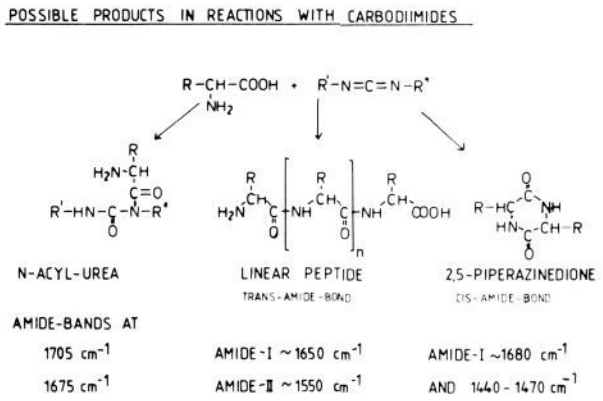

Figure 4. Possible reaction products of amino acids with carbodiimides and their characteristic IR vibration bands.

ultrasonication of the lipid in a carbodiimide-containing buffer stay in solution over the same period up to a molar ratio of carbodiimide to lipid of about 1.5. At higher carbodiimide concentrations, the turbidity increased, and after 24 h, the liposomes precipitated. Consequently, in all the following experiments, we used a carbodiimide to lipid ratio of about 1.5. The peptide formation seems to occur very fast since directly after sonication almost no starting material can be detected by TLC.

The condensation experiments in liposomes were carried out as described in the Experimental Section, and the condensation products were characterized by IR spectroscopy. For comparison, possible reaction products and their characteristic IR vibration bands are given in Figure 4.

In addition to the products in Figure 4, the lactam can be formed which also shows a typical vibration pattern similar to the 2,5-piperazinedione. All the compounds investigated (**2–6**) exhibit absorption bands of about 1660 and 1530 cm^{-1}, indicating the formation of a trans amide bond which is only possible for a linear peptide. No additional band between 1400 and 1500 cm^{-1} which could be due to the lactam or 2,5-piperazinedione formation was found. As an example, the IR spectra of **5** as hydrochloride before and after the condensation is shown in Figure 5.

Vapor pressure osmometry of this product gave a $\overline{DP}$ of about 4. We have not yet determined which of the two carboxylic groups of the amino acids **2–6** form the peptide bond.

Liposomes from Preprepared Peptides. The fact that liposomes can be prepared from prepolymerized and especially hydrophilic spacer group containing lipids[3-5] prompted an attempt to prepare liposomes from the isolated oligo-amino acids. It was found that the peptides obtained by the condensation of amino acid liposomes are able to form liposomes upon ultrasonication. In addition from the peptides obtained from **3–6**, giant liposomes could be prepared in mixtures with cholesterol. These giant liposomes were characterized by light microscopy. An example is shown in Figure 6. The ability to form liposomes from prepolymerized lipids allows comparison of the properties of amino acid and peptide liposomes

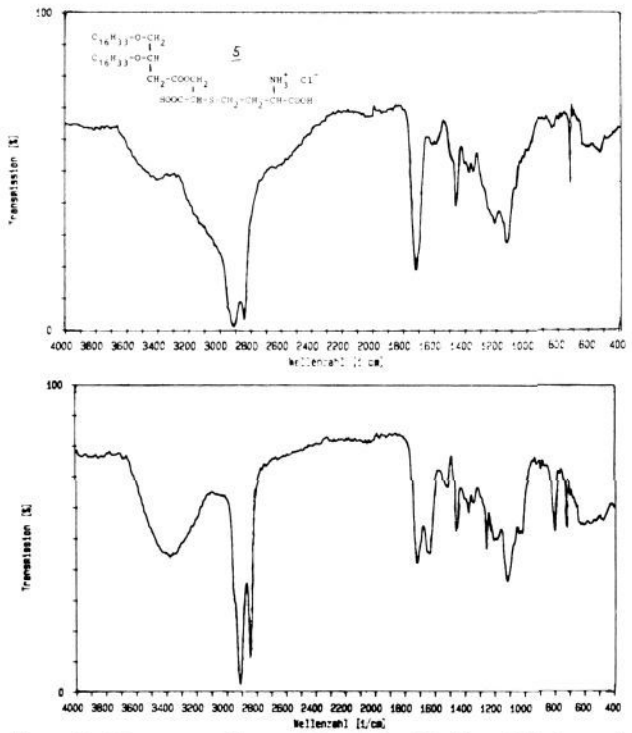

Figure 5. IR spectra of (a, top) **5** as hydrochloride and (b, bottom) peptide obtained from **5**.

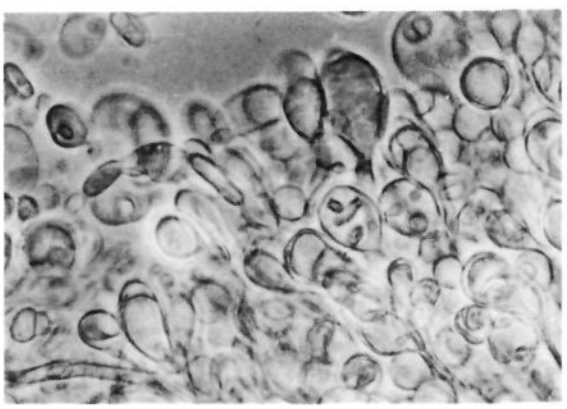

Figure 6. Giant liposomes of a mixture from the oligopeptide of **5** with cholesterol (30 mol % cholesterol, pH 6.5), $\phi \sim 15$ μm.

without the potential complication of the carbodiimide or the urea. In initial experiments, the stability against Triton-X-100 was investigated by using 6-carboxyfluorescein as a marker. We found no difference in the release behavior between amino acid and peptide liposomes. This is in agreement with data reported by Juliano et al.,[10] who observed no difference in the behavior of monomeric vs. polymeric liposomes toward Triton-X-100. The phase-transition temperatures of the peptides in swollen lamellar phases are given in Table II. All the peptides show lower phase-transition temperatures than their monomeric analogues. Interestingly, the peptides obtained from the cysteine and homocysteine derivatives with comparable chain lengths have about the same phase-transition temperatures, whereas the corresponding monomers differ drastically.

Experimental Section

General Methods. Melting points were determined with a Büchi melting point apparatus and are uncorrected. Infrared spectra were obtained on a Beckman IR-4220 spectrometer. The compounds were transferred into a KBr pellet at a concentration of about 5 wt %. The wavenumbers of the bands are reported in inverse centimeters. Nuclear magnetic resonance spectra (NMR) were recorded on a Bruker AM 400 (400 MHz) spectrometer. The chemical shifts are given in parts per million (ppm) (δ) relative to tetramethylsilane. The microanalysis were performed by Microanalysis Laboratories Universität Mainz.

Preparation of Liposomes. The liposomes were prepared by ultrasonication of a 1 mg/mL aqueous dispersion of the lipids in phosphate buffer (0.067 M), pH 6.5. The sonication was carried out by using a Branson sonifier Model B 15 for 10–15 min at 80 °C.

The giant liposomes were prepared according to the procedure given by Hub et al.[11] A Zeiss microscope equipped with a Nikon camera was used for the characterization of the giant vesicles.

Differential Scanning Calorimetry (DSC). The lipid (2–3 mg) and 20–30 μL of water were placed into small pans and allowed to hydrate overnight at 80 °C. The thermal behavior was investigated in a DSC-2C (Perkin-Elmer) by using a heating rate of 2.5 K/min.

Condensation in Liposomes and Purification of the Peptides. The liposomes were sonicated in a carbodiimide-containing buffer, pH 6.5 (0.067 M). The lipid concentration was 1 mg/mL and the molar ratio of carbodiimide to lipid was 1.5. The liposomal solution was allowed to stand at room temperature for 24 h. After this time, the liposomes were destroyed by adding 2 N HCl. The mixture was diluted with water and centrifuged. The water was decanted and the residue resuspended 3 times in water and centrifuged to get rid of the carbodiimide and the urea. The material was dissolved in chloroform, separated from the water, dried over anhydrous Na_2SO_4, and filtered. The solvent was evaporated, and the material was collected.

Materials. D,L-Homocysteine, hexadecanol, octadecanol, and *N,N*-dioctadecylamine were purchased from Fluka. L-Cysteine was obtained from Sigma. *N*-Cyclohexyl-*N'*-[β-(*N*-methylmorpholino)ethyl]carbodiimide–*p*-toluenesulfonate was purchased from Merck Schuchardt. All solvents used were analytical grade. The 1,2-dialkylglycerol ethers were prepared by alkylation of the 3-*O*-tetrahydropyranylglycerol ether[12] with the corresponding alkyl methanesulfonates followed by deprotection using 2 N HCl. The melting points agree with that reported in the literature.[13,14]

The *N,N*-dioctadecylmaleamic acid was prepared according to Tundo et al.[15]

Synthesis of the Maleic Esters. Maleic Acid Dioctadecyl Ester. Maleic anhydride (4.9 g, 50 mmol) 27.05 g (0.1 mmol) of octadecanol, and 1.5 g of *p*-toluenesulfonic acid in chloroform were refluxed for 8 h during which time the reaction water was collected in a trap. The mixture was then extracted first with saturated $NaHCO_3$ solution and then with water. The organic phase was dried over anhydrous Na_2SO_4 and filtered. After evaporation of the solvent, the residue was recrystallized from acetone containing a trace of methanol: yield 19.1 g (61%); mp 48–51 °C. Anal. Calcd for $C_{40}H_{76}O_4$: C, 77.36; H, 12.33. Found: C, 77.10; H, 12.81.

General Procedure for the Preparation of the Maleic Monoesters. To a 3 mmol solution of 1,2-*O*-(dialkyloxy)glycerol in toluene was added 3 mmol of maleic anhydride and 3 mmol of triethylamine and the mixture stirred for 4 h at room temperature. The toluene was evaporated and the residue dissolved in CH_2Cl_2. The solution was extracted with 2 N H_2SO_4 followed by water, dried, and filtered. After evaporation of the solvent, the crude product was recrystallized from acetone.

Maleic Acid (2,3-*O*-Dihexadecyloxy)glycerol Monoester: yield 95%; mp 47–48 °C. Anal. Calcd for $C_{39}H_{74}O_6$: C, 73.30; H, 11.67. Found: C, 72.98; H, 11.81.

Maleic Acid (2,3-*O*-Dioctadecyloxy)glycerol Monoester: yield 96%; mp 60–61 °C. Anal. Calcd for $C_{43}H_{82}O_6$: C, 74.30; H, 11.89. Found: C, 73.74; H, 11.31.

Synthesis of the Amino Acids 1–6. General Procedure. Cysteine or homocysteine (1 mmol) dissolved in 1 mL of 0.1 N NaOH was added to a hot 2-propanol solution of the maleic ester (or maleamic acid) (1 mmol). Water was added until a clear solution was obtained. The mixture was stirred at 50 °C for 2–6 h. The extent of the reaction was monitored by TLC (CH_2Cl_2/MeOH = 9/1). Upon completion of the reaction, 2 N HCl was added; the mixture was cooled to room temperature and then poured into 300 mL of water. The precipitate was filtered by suction and washed extensively with water. After drying, the amino acids were recrystallized from $CHCl_3$/ether. For the conversion to the

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hydrochlorides, the amino acids were dissolved in hot chloroform, and dry HCl was bubbled through the solution for 20 min. When the solution cooled, dry ether was added, and the precipitate was collected.

The nucleophilic attack of the sulfur-containing amino acids on the double bond of the maleic esters generates an asymmetric C atom. Because cysteine and homocysteine are either pure optical antipodes or a racemic mixture, a mixture of diastereomers was obtained by this reaction. Therefore, the ^{1}H NMR spectra of the resulting compounds show a complicated coupling pattern of the amino acid and the succinic acid protons such that the exact assignment is not possible without decoupling experiments.

***S*-[1,2-Bis(octadecyloxycarbonyl)ethyl]cysteine (1)**: yield 85%; mp 130 °C; ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 6 H, $-CH_3$), 1.25 (s, 60 H, CH_2), 1.58 (s, 4 H, CH_2CH_2OOC), 4.0-4.2, (2t, 4 H, CH_2OOC), 2.7-3.95 (m, 6 H, no assignment possible); IR (KBr) 1740 (C=O ester), 1720 (C=O, acid). Anal. Calcd for $C_{43}H_{81}NO_6S$: C, 69.78; H, 11.03; N, 1.89; S, 4.33. Found: C, 68.72; H, 10.86; N, 2.01; S, 4.05.

***S*-(1-Carboxy-2-[(*N*,*N*-dioctadecylamino)carbonyl]ethyl)cysteine (2)**: yield 68%; mp 148 °C; ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 6 H, CH_3), 1.25 (s, 60 H, CH_2), 1.85 (s, 4 H, CH_2CH_2NCO), 2.0-3.6 (m, 10 H, no assignment possible); IR (KBr) 1715 (C=O acid), 1620 (C=O, amide). Anal. Calcd for $C_{43}H_{84}N_2O_5S$: C, 69.31; H, 11.36; N, 3.75; S, 4.30. Found: C, 67.82; H, 10.53; N, 3.90; S, 4.70.

***S*-[1-Carboxy-2-([2,3-bis(hexadecyloxy)propoxy]carbonyl)ethyl]cysteine (3)**: yield 67%; mp 190-195 °C; ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 6 H, CH_3), 1.23 (s, 52 H, CH_2), 1.52 (s, 4 H, CH_2CH_2O), 2.1-3.9 (m, 13 H, no assignment possible), 4.0-4.3 (m, 2 H, CH_2OOC); IR (KBr) 3600-2400 (NH_3^+, OH), 3000-2800 (CH_2), 1720 (C=O, ester, acid), 1625, 1580 (NH_3^+). Anal. Calcd for $C_{42}H_{81}O_8NS$: C, 66.36; H, 10.74; N, 1.84; S, 4.22. Found: C, 64.46; H, 9.47; N, 1.90; S, 4.32.

***S*-[1-Carboxy-2-([2,3-bis(octadecyloxy)propoxy]carbonyl)ethyl]cysteine (4)**: yield 50%; mp 134 °C; ^{1}H NMR ($CDCl_3$) and IR (KBr) similar to **3**. Anal. Calcd for $C_{46}H_{89}O_8NS$: C, 68.36; H, 10.10; N, 1.73; S, 3.97. Found: C, 67.92; H, 10.51; N, 1.96; S, 3.82.

***S*-[1-Carboxy-2-([2,3-bis(hexadecyloxy)propoxy]carbonyl)ethyl]homocysteine (5)**: yield 40%; mp 165 °C; ^{1}H NMR ($CDCl_3$) 0.85 (t, 6 H, CH_3), 1.23 (s, 52 H, CH_2), 1.52 (s, 4 H, CH_2CH_2O), 2.1-3.9 (m, 15 H, no assignment possible), 4.0-4.3 (m, 2 H, CH_2OOC); IR (KBr) similar to **3**. Anal. Calcd for $C_{43}H_{83}O_8NS$: C, 66.71; H, 10.81; N, 1.81; S, 4.14. Found: C, 65.70; H, 10.7; N, 1.70; S, 4.18.

***S*-[1-Carboxy-2-([bis(2,3-octadecyloxy)propoxy]carbonyl)ethyl]homocysteine (6)**: yield 54%; mp 164 °C; ^{1}H NMR ($CDCl_3$) similar to **5**; IR (KBr) similar to **3**. Anal. Calcd for $C_{47}H_{81}O_8NS$: C, 68.65; H, 10.17; N, 1.70. Found: C, 66.97; H, 10.93; N, 1.79.

Registry No. 1 (isomer 1), 99355-64-3; **1** (isomer 2), 99355-65-4; **2** (isomer 1), 99355-66-5; **2** (isomer 2), 99355-67-6; **3**, 99355-68-7; **4**, 99355-69-8; **5**, 99355-70-1; **6**, 99355-71-2; maleic anhydride, 108-31-6; octadecanol, 112-92-5; maleic acid dioctadecyl ester, 7516-70-3; maleic acid (2,3-*o*-dihexadecyloxy)glycerol monoester, 99355-72-3; malic acid (2,3-*o*-dioctadecyloxy)glycerol monoester, 99355-73-4; *N*,*N*-dioctadecylmaleamic acid, 82798-00-3; L-cysteine, 52-90-4; D,L-homocysteine, 454-29-5; 1,2-*o*-(dihexadecyloxy)glycerol, 6076-35-3; 1,2-*o*-(dioctadecyloxy)glycerol, 6076-38-6.

Evidence for a Competing Condensation Reaction in the Alloxan Synthesis of Flavins: Synthesis and Crystal and Molecular Structures of 7-Chloro-8-methylalloxazine and 7,10-Dimethyl-8-[(2-hydroxyethyl)thio]isoalloxazine

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Abstract: The reaction of 2-amino-5-chloro-*N*,4-dimethylaniline with alloxan monohydrate in boric acid-acetic acid mixtures has been shown to produce 7-chloro-8-methylalloxazine (III) in addition to the expected 8-chlorolumiflavin (I). The position of heteroatom substitution in III and I has been verified by optical and ^{1}H NMR studies and by single-crystal X-ray diffraction structure determinations of III·DMF and of a derivative of I, 8-[(2-hydroxyethyl)thio]lumiflavin, IV·H_2O: III·DMF, monoclinic, space group $P2_1/c$ with a = 8.764 (4) Å, b = 16.030 (8), c = 11.047 (6) Å, β = 95.00 (2)°, V = 1546 Å^3, Z = 4, 2480 reflections with $I > 2\sigma(I)$, R_F = 0.056, R_{wF} = 0.048; IV·H_2O, triclinic, space group $P\bar{1}$ with a = 9.194 (2) Å, b = 9.848 (3) Å, c = 11.821 (4) Å, α = 87.50 (2)°, β = 119.46 (3)°, γ = 58.16 (2)°, V = 722.6 Å^3, Z = 2, 1799 reflections with $I > 2\sigma(I)$, R_F = 0.047, R_{wF} = 0.050. Both structures were solved by direct methods. III·DMF is the first structurally characterized alloxazine and exhibits bond lengths and angles consistent with those expected for the alloxazine tautomer. Reaction of other substituted 2-amino-*N*-methylanilines with alloxan was examined, and formation of an alloxazine with reversed substitution compared to that of the isoalloxazine also obtained was observed in all cases. These results strongly suggest that substituted 2-amino-*N*-methylanilines can condense with alloxan in either of two orientations, only one of which produces the isoalloxazines. The other orientation produces an N(5)-alkylalloxazinium intermediate that spontaneously dealkylates to give the observed alloxazine. No evidence was obtained for formation of an alloxazine via N(10)-dealkylation of the isoalloxazine under the conditions examined.

Because of the presence of the isoalloxazine nucleus in riboflavin and its coenzyme derivatives FMN and FAD[2] and the importance of flavin-dependent enzymes in biology,[3] much effort has been devoted to chemical synthesis of riboflavin analogues as model systems for mechanistic studies and as biological agonists or antagonists of the natural coenzyme.[4-6] Reasonably facile syntheses of isoalloxazines have been reported: from 2-amino-*N*-alkylanilines and alloxan, alloxantin, isodialuric acid, or 5-halobarbituric acid;[4] from *N*-alkylanilines and violuric acid;[4] from 2-arylazoanilines and barbituric acid;[4] from N-substituted 5,6-diaminouracils and dimeric biacetyl;[4] from 5-amino-6-(alkylamino)pyrimidines and *o*-benzoquinones;[4] from 6-(*N*-alkyl-

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(1) Alfred P. Sloan Foundation Fellow, 1981-1985.

(2) Abbreviations: FMN, flavin mononucleotide or riboflavin-5-monophosphate; FAD, flavin adenine dinucleotide or riboflavin adenosine diphosphate.

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