Communications to the Editor

Self-Cleaving Ortho Ester Lipids: A New Class of pH-Vulnerable Amphiphiles

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Received November 29, 1999

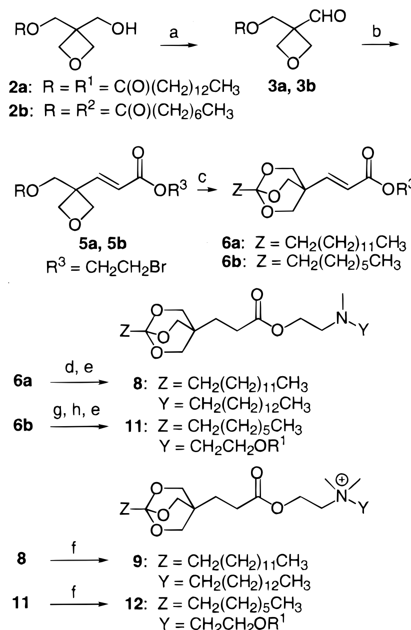
The potential of liposomes to function as carriers of pharmaceutical agents,[2] and more recently as vehicles for polynucleotide delivery,[3] continues to stimulate the development of more effective lipid-based delivery systems. A principal area of liposome research has been the design of the "trigger" for the liposome to release its payload. Various parameters to initiate release have been used including low pH, ionic strength, light, and enzymatic activity.[4] Among these approaches, the most widely used triggering mechanism is the use of pH-sensitive lipids to effect liposome permeability in acidic medium.

Past developments of pH-sensitive liposomes have focused principally in the area of anionic liposomes.[5] Recently, the hydrolysis of pH-sensitive functionality within the lipid framework has been used to destabilize liposomes. Investigations by Thompson[6] have demonstrated the viability of this approach by showing that plasmalogen-derived liposomes degrade on exposure to aqueous acid. Plasmalogens contain a pH-sensitive vinyl ether linkage that readily undergoes hydrolysis to the corresponding lysolipid. To extend this strategy, we have applied the ortho ester functionality to engineer liposome assemblies that are highly susceptible to mild acid conditions. The ortho ester is among the most acid-sensitive functional groups,[7] yet no ortho ester lipid has been constructed for the purpose of imparting pH-sensitivity to liposomes.[8] We report here the first examples of ortho ester amphiphiles and their functions in liposomes. We also demonstrate application of these novel pH-sensitive liposomes as potential carriers of small molecules and polynucleotides.

3,3-Bis(hydroxymethyl)oxetane (**1**)[9] was mono-esterified using either myristoyl chloride or octanoyl chloride to afford esters **2a** and **2b** and followed by Swern oxidation to give the corresponding aldehydes **3a** and **3b** (Scheme 1). Subsequent olefination[10] using phosphonate **4** afforded diesters **5a** and **5b** in 79 and 74% yield, respectively. Rearrangement of the oxetane to the ortho ester was accomplished according to the method of Corey[11] by treatment with catalytic BF_3 etherate. We were gratified to find that the adjacent α,β-unsaturated ester in **5a** and **5b** did not interfere with the 2,6,7-trioxabicyclo[2.2.2]octane (OBO) formation and that esters **6a** and **6b** were obtained in 67 and 65% yield, respectively.[12] Attachment of the ortho ester moiety onto lipid frameworks was achieved using a transesterification procedure,[13] wherein amino alcohols **7** and **10** were first treated with *n*-BuLi and then reacted with the 2-bromoethyl esters **6a** and **6b**. This approach proved to be the most secure means for attaching the sensitive ortho ester-containing side chains (~65% yield). In the case of **6b**, a second acyl chain is conveniently attached after the transesterification procedure. Hydrogenation of the α,β-unsaturated ester moiety under mild basic conditions provided lipids **8** and **11**, and amine quaternization gave the cationic ortho ester lipids **9** and **12**.

The susceptibility of the ortho ester construct to mild acid conditions was assessed using lipids **8** and **9** as representative examples (Scheme 2). As expected, exposure of **8** to a pH 4.5

Scheme 1[a]



[a] (a) i. $(COCl)_2$, DMSO, CH_2Cl_2, −78 °C; ii. *i*-Pr_2NEt, 0 °C. (b) $(EtO)_2P(O)CH_2C(O)OCH_2CH_2Br$ (**4**), Et_3N, LiBr, THF, 0 °C to rt. (c) $BF_3{\cdot}Et_20$ (0.25 equiv), CH_2Cl_2, 0 °C to rt. (d) i. *n*-BuLi, $HOCH_2CH_2N(Me)$-$CH_2(CH_2)_{12}CH_3$ (**7**), THF, 0 °C; ii. **6a**, 0 °C to rt. (e) H_2, 10% Pd/C (0.2 equiv), C_6H_6, Et_3N, rt, 90−92%. (f) CH_3I. (g) i. *n*-BuLi, $(HOCH_2CH_2)_2NCH_3$ (**10**), THF, 0 °C; ii. **6b**, 0 °C to rt. (h) Myristoyl chloride, Et_3N, CH_2Cl_2, 0 °C to room temperature, 88%.

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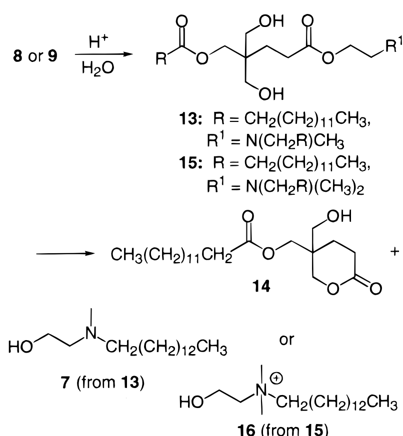
10.1021/ja994149q CCC: $19.00 © 2000 American Chemical Society
Published on Web 03/02/2000

Scheme 2



buffer solution at 38 °C resulted in complete hydrolysis of the ortho ester functionality. In addition, the subsequent lactonization of diol **13** effectively excised the aminium ion headgroup to give lactone **14** and amino alcohol **7** in near quantitative yield. The analogous process of liberating an alcohol of interest by designing lactone formation as the release feature has been previously demonstrated by Crimmins.[14] In agreement with our design concept, diol **13**, the initial hydrolysis product, was not detected in the product mixture after a 12 h exposure. We examined ortho ester lipid **9** under identical conditions and found that this lipid was equally vulnerable toward self-cleavage, providing lactone **14** and ammonium salt **16**. The lipid disassembly process as shown in Scheme 2 occurs more readily than the previously documented acid-catalyzed plasmalogen hydrolysis.[6a] In comparison, the hydrolysis and self-cleavage of **8** and **9** were complete within 12 h, whereas the plasmalogen enol ether hydrolysis required 30 h to effect 50% cleavage of a lipid side chain under similar conditions.[15]

To determine whether vesicles composed of ortho ester lipid **9** would release liposome contents in response to a decrease in pH, we examined the entrapment and release of calcein.[6b] Escape of calcein from within a liposome is accompanied by an increase in fluorescence emission as the free calcein in bulk solution is dequenched. Measurement of the changes in fluorescence intensity thereby can serve as an indicator of liposome integrity. We prepared liposomes using ortho ester lipid **9** by formulation of **9** with dioleoylphosphatidylethanolamine (DOPE). Hydration of the lipid mixture (~1 mM in PBS) followed by brief sonication afforded vesicles ranging in size from 150 to 400 nm.[16] When the lipid hydration step is performed using a 50 mM solution of calcein, the ortho ester lipid vesicles form with concomitant entrapment of calcein. Exposure of the calcein-loaded liposomes to pH 3.5 buffer for 10 min resulted in increased fluorescence emission indicative of liposome rupture and release of calcein. Comparison of the measured fluorescence emission to the maximum emission, determined by treatment of the calcein liposomes with Triton X-100,[6b] revealed that roughly 40% of the entrapped calcein was released from the ortho ester liposomes within the short time period examined. To confirm that the liposome rupture and calcein release are a consequence of the pH-vulnerability of the ortho ester lipid, we performed a control experiment using an analogous cationic diester lipid, DOTAP.[17] Calcein-loaded DOTAP−DOPE liposomes were prepared and subjected to identical acid conditions. In this instance, we observed no change in fluorescence intensity. Thus, our premise of effecting liposome disassembly by triggering the pH-induced hydrolysis and self-cleavage of a constituent ortho ester lipid has been proven.

Another practical application of cationic lipids has been to bind DNA for the purpose of facilitating intracellular delivery of the polynucleotide to mammalian cells.[18,19] To probe whether the ortho ester construct of lipids **9** and **12** is sufficiently robust to mediate a gene transfer process, we performed a transfection experiment.[20] The widely used transfection lipids DOTAP and DC-cholesterol[21] were tested in this experiment for reference purposes. The data (not depicted) indicate that slight structural variations in the ortho ester lipid construct do have an impact on overall transfection activity. Whereas lipid **12** displayed activity only slightly better than the transfection standards, lipid **9** was significantly more active (~10-fold) than DOTAP and DC-cholesterol in facilitating delivery of luciferase to NIH 3T3 cells. Although preliminary, this result is encouraging and suggests there may be an advantage in integrating the ortho ester functionality into the lipid framework. There are several possibilities for the noted improvement in transfection activity of **9** over DOTAP, including a potentially decreased toxicity. Another hypothesis stems from work by Hughes[19a] and considerations by Bally et al.[22] who have speculated that the detergent-like properties of certain lipids aid the escape of DNA from endosomal compartmentalization.[23] Indeed, the hydrolysis product of lipid **9** is ammonium ion **16**, a single-chain amphiphile prone to exhibit detergent behavior. In contrast, the single-chain amphiphile liberated by hydrolysis of ortho ester lipid **12** might be further degraded through ester cleavage into metabolites devoid of detergent properties. The fate of ortho ester lipids in an endosomal environment is the subject of ongoing investigations.

In conclusion, we have engineered an ortho ester construct to effect lipid headgroup cleavage in response to mild acid-induced hydrolysis. When the ortho ester lipids are formulated into liposome aggregates, the tandem sequence of ortho ester hydrolysis and headgroup cleavage triggers liposome rupture and release of entrapped contents. These observations illustrate the potential in using ortho ester lipids for pH-mediated release of a liposome payload. Moreover, the acute sensitivity of the ortho ester linkage to hydrolysis at pH 4.5 falls within the acidification range of endosomes and points toward cellular delivery applications that proceed via endocytosis. Thus, the present approach to pH-triggered liposome disassembly provides a new tactic for the delivery of pharmaceutical agents.

Acknowledgment. Support of this work by the Cystic Fibrosis Foundation is gratefully acknowledged. Thanks are due to Dr. Michael J. Bennett for performing DNA transfection analyses.

Supporting Information Available: Synthetic and characterization details for all compounds and experimental protocols for liposome studies (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA994149Q

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