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Facile Preparation of an Orthogonally Protected, pH-Sensitive, Bioconjugate Linker for Therapeutic Applications

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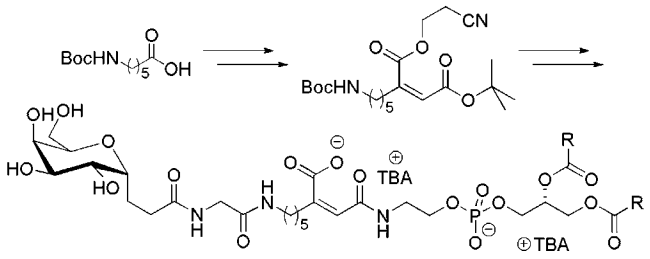
ABSTRACT



We describe the facile, three-step synthesis of an orthogonally protected, pH-sensitive linker (8), based on maleic acid, and report its application to the preparation of a pH-sensitive phospholipid (20) for potential use in drug and gene delivery. In addition, we highlight the benefits of our linker over the use of the commercially available *cis*-aconitic anhydride (4).

Since the first reports in the 1980s of implementing cationic liposomes as vectors (gene-delivery vehicles) for non-viral gene therapy,[1] a whole array of cationic lipids have been created to try to improve the efficacy of these early vectors. An alternative approach has been to attempt to harness the intrinsic fusogenic[2,3] properties of the naturally occurring lipid dioleoylphosphatidylethanolamine (DOPE, **1**), a lipid that invariably leads to enhanced gene delivery and expression (transfection).[4−7]

In 1995, Drummond and Daleke reported the reversible modification of the amino group of DOPE using a range of cyclic acid anhydrides, for potential applications in monitoring transmembrane aminophospholipid transport and membrane fusion.[8] Cyclic, unsaturated anhydrides form pH-sensitive maleamates upon reactions with amines. Stable at high pH, these compounds recyclize at low pH, causing intramolecular cleavage of the amide bond, thereby regenerating both the cyclic anhydride and the amine (Scheme 1).[9,10]

Upon cellular uptake of cationic liposome/DNA complexes (lipoplexes), there is a concomitant drop in pH, suggesting the aforesaid modification to DOPE may also be beneficial for the purpose of gene therapy by masking, and thereby harnessing, the zwitterionic and fusogenic nature of DOPE.

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(1) Felgner, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrop, J. P.; Ringold, G. M.; Danielsen, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7413−7417.
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(10) Bender, M. L. *J. Am. Chem. Soc.* **1957**, *79*, 1258−1259.

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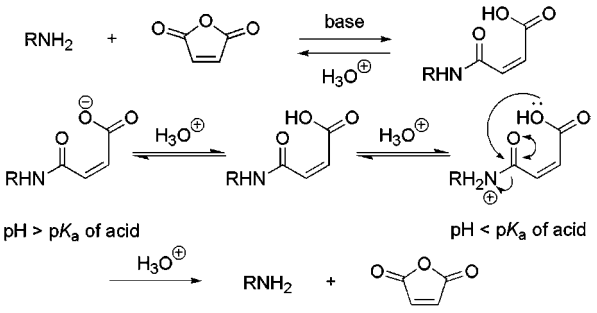
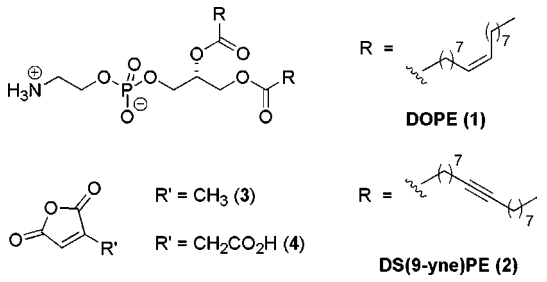
Scheme 1



Drummond et al. discovered that reaction with citraconic anhydride (**3**) generated the most acid-labile DOPE product. For gene therapy applications, however, it is necessary to further stabilize the now anionic DOPE molecule through the conjugation of a highly hydrophilic group (via an acid-labile linker) such as a sugar molecule or poly(ethylene-glycol) (PEG), in order that ion-pairing with cationic lipids, required to bind the anionic DNA, of the vector formulation does not lead to self-fusion.[11]



Analogous drug−polymer conjugates have been prepared in two steps with *cis*-aconitic anhydride (**4**). First, the amino group of the drug is reacted with the anhydride of **4** to form a cis-α,*β*-unsaturated amide bond; then, the *γ*-carboxylic acid is coupled to the polymer, in the presence of the freed α- or *β*-conjugated acid.[12] However, such conjugates have been poorly characterized. As shown in Scheme 1, the importance of the characterization of amine-**4** conjugates is paramount, since the geometry of the *cis*-carboxylic acid is a prerequisite for pH sensitivity. In addition, **4** is known to spontaneously isomerize to propene-1,2,3-tricarboxylic acid-1,3-anhydride, leading to undesired trans products upon coupling with amines.[13] Furthermore, Brocchini et al. have reported that decarboxylation, double-bond isomerization (positional and geometrical), and hydrolysis reactions have all been observed during the coupling of an amine to **4**.[14] The readiness with which **4** undergoes such isomerizations, which will abolish

(11) Hafez, I. M.; Maurer, N.; Cullis, P. R. *Gene Ther.* **2001**, *8*, 1188−1196.
(12) Shen, W. C.; Ryser, H. J. P. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 1048−1054.
(13) Remenyi, J.; Balazs, B.; Toth, S.; Falus, A.; Toth, G.; Hudecz, F. *Biochem. Biophys. Res. Commun.* **2003**, *303*, 556−561.
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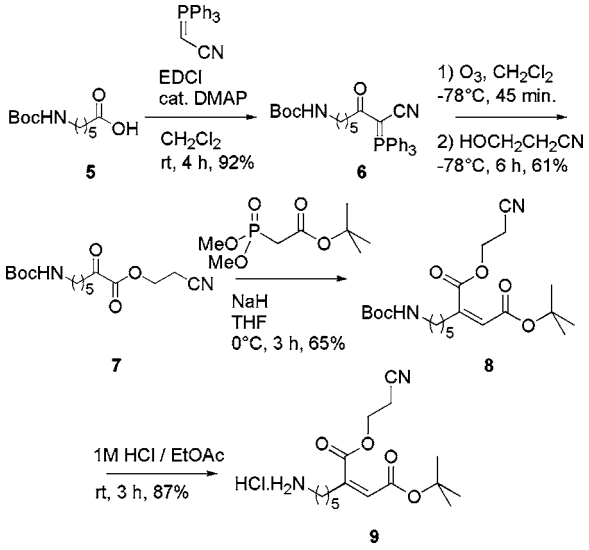
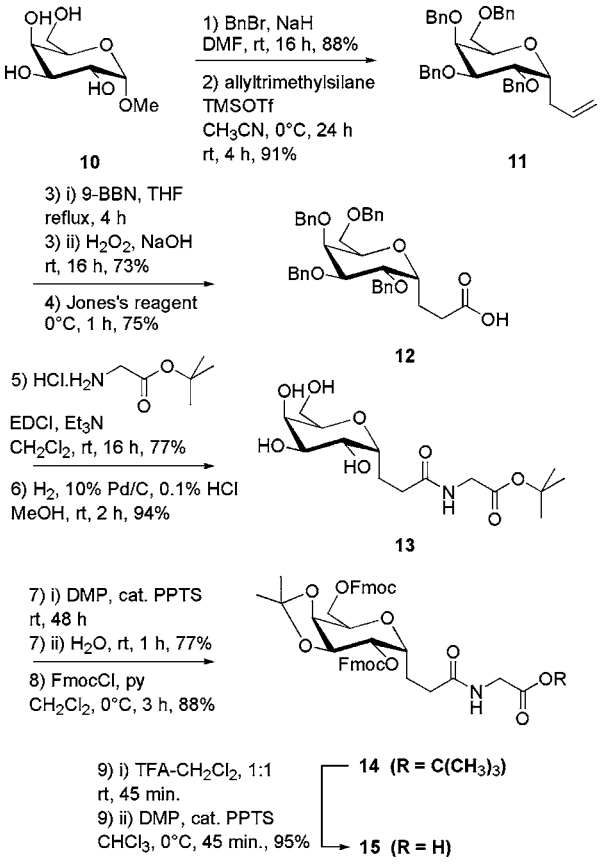
Scheme 2



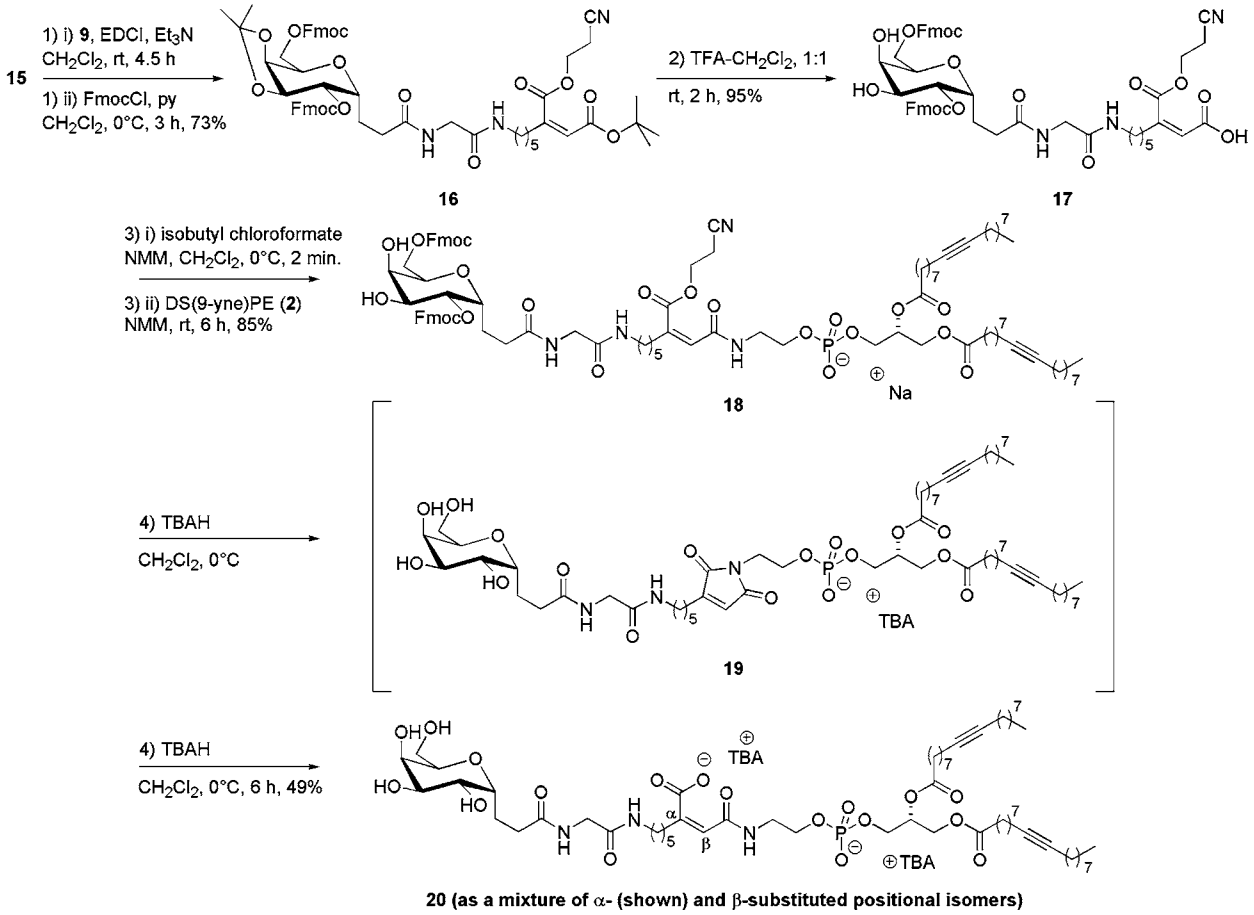
the desired pH sensitivity of the maleamate products and thereby prevent liberation of the drug or lipid, may be due to an unusually low pK_a of the methylene group α- to the free, *γ*-acid.

In this Letter, we report a de novo and facile, three-step route to an acid-labile linker moiety (**8**; Scheme 2), which

Scheme 3



Scheme 4



may have less tendency to undergo cis/trans and positional isomerizations, due to an increased spacer between the maleic anhydride functionality and the linker (acid or amine) functionality. The application of this moiety to the reversible modification of an alkynoyl analogue of DOPE (DS(9-yne)-PE, **2**) into an anionic, pH-sensitive, stabilized lipid, suitable for incorporation into cationic liposomes, is also described.

First, **5** was coupled in excellent yield to (triphenylphosphoranylidene)acetonitrile, thereby furnishing cyano keto phosphorane **6**. Subsequent ozonolysis of **6** followed by quenching of the intermediate vicinal diketo nitrile with cyanoethanol afforded α-keto cyanoethyl ester **7** in 61% yield. This methodology, developed by Wasserman et al., allows for the simple and swift preparation of α-keto acids, esters, and amides, compounds that may have important medical applications as potent inhibitors of proteases.[15]

Hitherto, ozonolysis of cyano keto phosphoranes followed by quenching with an alcohol has only been reported with large excesses of methanol or benzyl alcohol to generate the α-keto methyl and benzyl esters, respectively. We were able to perform the reaction with just 1.3 equiv of cyanoethanol, which therefore did not hinder purification and suggests that the quenching reaction may be feasible for alcohols in general. Treatment of **7** under Horner−Wadsworth−Emmons conditions with *tert*-butyl *P*,*P*-dimethylphosphonoacetate afforded almost exclusively (*Z*)-isomer **8** (as confirmed by ^{1}H NMR and NOE experiments[16]), in good yield (65%). Then, removal of the *tert*-butyl ester of **8** in the presence of the Boc group was achieved with a 1 M solution of hydrogen chloride gas in dry EtOAc in excellent yield, giving **9** as its HCl salt.[17]

The stabilizing moiety to which **9** would be coupled was prepared in nine steps (Scheme 3) from methyl α-D-galactopyranoside (**10**), a sugar that has a particular tropism for hepatocytes.[18] Such a feature may afford desirable targeting properties. Briefly, **10** was fully benzylated then allylated to generate the readily functionalizable alkene **11**.[19] Hydroboration of **11** with 9-BBN led to the desired anti-Markovnikov borane, which upon treatment with alkaline peroxide, followed by oxidation with Jones's reagent, furnished acid **12**[20] in good yield. Next, to improve the hydrophilic properties of the acyclic portion of **12**, glycine

(15) Wasserman, H. H.; Ho, W.-B. *J. Org. Chem.* **1994**, *59*, 4364−4366.

(16) ^{1}H NMR of alkene region of **8** shows a triplet (^4J 1.4 Hz) at δ_H 5.77 ($CDCl_3$); the less than 1% trans impurity shows a triplet at δ_H 6.82 ($CDCl_3$). These observations are in agreement with similar functionalities in the literature: Hosseine Massoudi, H.; Cantacuzene, D.; Wakselman, C.; Bouthier de la Tour, C. *Synthesis* **1983**, *12*, 1010−1012. The NOE spectrum of **8** is available in Supporting Information.

(17) Gibson, F. S.; Bergmeier, S. C.; Rapoport, H. *J. Org. Chem.* **1994**, *59*, 3216−3218.

(18) Kichler, A. *J. Gene Med.* **2004**, *6*, S3−S10.

(19) Giannis, A.; Sandhoff, K. *Tet. Lett.* **1985**, *26*, 1479−1482.

tert-butyl ester was coupled, which also installed a desirable protecting group for the acid functionality. Subsequent debenzylation gave deprotected *C*-pyranoside **13** in excellent yield (94%). Next, treatment of **13** with 2,2-dimethoxypropane (DMP), based on conditions by Liptak et al.,[21] afforded the corresponding 3,4-*O*-isopropylidene almost exclusively; then, reaction with FmocCl in pyridine gave the fully protected sugar **14** in very good yield.[22] Finally, removal of the *tert*-butyl ester of **14** in TFA/CH_2Cl_2 also led to rapid cleavage of the acetal protecting group, but this was easily remedied by post-treatment with DMP and catalytic PPTS, furnishing **15** as a white powder in an overall yield of 20% for nine steps.

Acid-labile linker **9** and sugar **15** were conjugated under standard EDCI peptide coupling conditions to give fully protected compound **16** (Scheme 4). Ester **16** was then subjected to a 1:1 mixture of TFA and CH_2Cl_2 for a prolonged period (>2 h), to ensure removal of both the *tert*-butyl ester and the acetal protecting groups, affording **17** in 95% yield. Next, a more phase-stable, synthetic analogue of DOPE, DS(9-yne)PE (**2**),[23] was coupled to acid **17** via activation with isobutyl chloroformate and *N*-methyl morpholine (NMM) (carbodiimides, CDI, and HBTU were unsuccessful in promoting this reaction, reflecting the deactivated nature of the conjugated acid) to give **18** in good yield (85%).[24]

Subsequent deprotection of the base-labile Fmoc and cyanoethyl groups of **18** with Et_3N led to cyclization of the maleamate derivative, generating imide **19**, as detected by ^{1}H NMR and mass spectrometry. Indeed, we discovered that a range of nonhydrolytic bases such as DBU in CH_2Cl_2 and TBAF in DMF all led to imide **19**. However, the use of a 40% (w/v) aqueous solution of tetra-*n*-butylammonium hydroxide (TBAH) allowed the rapid removal of the protecting groups, followed by in situ cyclization (as monitored by mass spectrometry), and then reopening of the imide all with minimal hydrolysis of the oleate esters, giving **20** as a mixture of equally acid-labile positional isomers in 49% yield. The apparently inevitable cyclization of maleamate **18** to imide **19** may prove to be serendipitous since these findings suggest that the pH sensitivity of the acid-labile maleamic acid moiety of **20** may be "protected" during synthetic modifications as its corresponding cyclic imide.

In conclusion, building on the work of Wasserman, we have developed an efficient synthetic route to the preparation of a fully and orthogonally protected, acid-labile linker (**8**) and highlighted its use with reference to the synthesis of a pH-sensitive lipid (**20**) for use in drug and gene delivery applications. The problems of geometrical and positional isomerizations of the maleamate C−C double-bond functionality upon reaction of **4** with an amine have been addressed and solved. While the deprotection of **18** led ultimately to **20** as two positional isomers, these isomers are positional merely in the alkyl substitution of the maleamate. The all-important *cis*-carboxylate remains intact in both isomers; pH sensitivity has not been compromised upon undesired cyclic imide formation. Preliminary in vitro biological data of CDAN:DOPE[7] cationic liposomes incorporating **20** are encouraging, facilitating the preparation of temporarily lower-charged (through pH sensitivity), and therefore more stabilized, lipoplexes that are still competent at transfection. Finally, the syntheses of other lipids incorporating the acid-labile moiety are ongoing in our laboratory.

Supporting Information Available: We thank Mitsubishi Chemical Corporation and IC-Vec, Ltd., for funding.

Supporting Information Available: Experimental procedures and full characterizations for compounds **5**−**18** and **20**, ^{1}H NMR and *m*/*z* for **19**, and NOESY NMR spectrum for **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) Liptak, A.; Nanasi, P.; Neszmelyi, A.; Wagner, H. *Carbohydr. Res.* **1980**, *86*, 133−136.

(22) To the best of our knowledge, this is the first report of a di-Fmoc-protected pyranoside.

(23) *A Dialkynoyl Analogue of DOPE Has a Higher L_α/H_{II} Phase Transition Temperature—Implications for Cationic Liposome-Based Gene Delivery*; Fletcher, S.; Jorgensen, M. R.; Miller, A. D. Submitted.

(24) If **18** is left in solution for an extended period of time (>8 h), cyclization to di-Fmoc-protected **19** occurs, as observed by the change in the ^{1}H NMR spectrum (vicinal proton: δ_H 5.91 → 6.20 ($CDCl_3$)).