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Bio-orthogonal Phosphatidylserine Conjugates for Delivery and Imaging Applications

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Abstract

The syntheses of phosphatidylserine (PS) conjugates are described, including fluorescent derivatives for potential cellular delivery and bioimaging applications. Installation of terminal functional groups (amine, thiol, or alkyne) onto the *sn*-2 chain provides reactive sites for bio-orthogonal conjugation of cargo with suitably protected PS derivatives. An amine-containing PS forms amide bonds with peptidic cargo, a thiol derivative is designed for conjugation to cargo that contain α-halo carbonyls or Michael acceptors, and the terminal alkyne PS analog permits 'click' conjugation with any azide-tagged molecule. This latter conjugation method is quite versatile as it can be performed without PS headgroup protection, in aqueous media, and with acid-labile cargo.

Introduction

Phosphatidylserine (PS) is emerging as a biologically important phospholipid that is associated with a wide range of cell signaling events.[1] There is good evidence that the plasma membranes of most mammalian cells contain an active transport system that pumps PS to the inner membrane surface.[2] There is also strong evidence that macrophages can engulf cells,[3] liposomes,[4] and viruses[5] that have surface exposed PS. We wish to determine if these endogenous PS transport systems can deliver PS-conjugates into cells, and whether they can be used for delivery and imaging applications in the same way that vitamin receptors are used to deliver conjugates of folate, B12, biotin, etc.[6]

This report describes our entry into this project, that is, the preparation of appropriate PS-conjugates for cell delivery experiments. We decided that the cargo should not be attached to the PS head group because this will likely interfere with the molecular recognition and that a more prudent point of attachment was the terminus of one of the phospholipid acyl chains.[7,8] Therefore, the synthetic challenge was to devise mild and general methods of attaching various types of molecular cargo to the end of a PS acyl chain. The methods had to be compatible with the PS head group, which contains both nucleophilic amine and electrophilic carboxyl sites.

The de novo synthesis of PS analogues has not been investigated in great detail. The few reported routes are lengthy,[9] target specific,[10] or plagued with stereochemical complications,[11] thus they are unsuited for the rapid preparation of PS scaffolds. Here we describe the preparation of three classes of PS derivatives that are capable of *efficient, bio-orthogonal conjugation* to the end of the *sn*-2 acyl chain (Scheme 1). First, a primary amine version (PS-amine) allows formation of amide bonds to peptidic cargo. Second, a thiol group (PS-thiol) can be utilized for selective addition to α-halo carbonyls or conjugate

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addition to Michael acceptors. Finally, a terminal alkyne (PS-alkyne) provides direct access to 'click' conjugation with any functionalized azide. The latter two methods exploit orthogonal reactivity patterns, which means that the conjugation can be achieved in the presence of the unprotected PS headgroup.

Results and Discussion

Synthesis of Protected PS from PC Precursors

The general synthetic strategy is to make the more tractable zwitterionic phosphatidylcholine (PC) precursor and then convert the headgroup to PS. The PC derivatives (**2a**,[12] **2b**,[13] and **2c**[14]) were prepared from readily available lyso-PC (**1**) by reacting the *sn*-2 hydroxyl group with an appropriately functionalized fatty acid. This potentially troublesome acylation was carried out in consistently high yield by using ultrasonication and glass beads according to the procedure of Hajdu (Scheme 2).[14]

Initially, we attempted an enzyme-mediated transphosphatidylation of the PC head group to the unprotected PS using phospholipase D (PLD) and excess serine.[15] However, in our hands, this approach gave consistently poor yields (0-5%) of desired product even when the most active[16] commercial PLD (obtained from *Streptomyces species*) was employed. In most cases, the corresponding phosphatidic acid (PA) hydrolysis products (**3a-c)** were obtained in very high yield. Therefore, we decided to take advantage of this outcome and synthetically convert **3a-c** into the doubly protected PS derivatives **4a-c**, which can be subsequently deprotected using mild acid (vide infra). This synthetic route is slightly longer but it has several important advantages. Compared to unprotected PS derivatives, which aggregate extensively in organic solvents,[11b,16] the protected PS compounds (**4a-c**) are much easier to purify and characterize by NMR spectroscopy.

Conjugation Reactions with PS-amine

The PS-amine precursor **4c** has two orthogonally protected amines, and selective deprotection of the N-Fmoc amine allows for coupling with amine-reactive cargo (i.e. polypeptides, activated esters, acid chlorides, etc.). The utility of this methodology is showcased by the preparation of the biotin-PS conjugate **6** (Scheme 3). Treatment of **4c** with DBU selectively liberates the terminal primary amine of the *sn*-2 acyl chain allowing for *in situ* amide bond conjugation to afford protected biotin conjugate **5**. Subsequent headgroup deprotection with TFA cleanly gives the desired PS derivative **6** as a single TLC spot that is stained by both Dittmer's[17] and ninhydrin reagents (indicating the presence of phosphate and primary amine, respectively). Although straightforward, this amide conjugation protocol has several potential limitations. First, a reactive acylating agent is required for conjugation. Thus, problems in chemoselectivity may result when polyfunctionalized substrates are used. Also, this method requires TFA deprotection of the PS headgroup *after* cargo attachment to the lipid scaffold. Therefore, this conjugation method is not suitable for acid-labile cargo.

Conjugation Reactions with PS-thiol

The PS-thiol precursor **4b** was prepared and used to react with thiol-active groups. Examples of electrophilic functional groups that react preferentially with thiols (even in the presence of free amines and carboxylates) include α-halo carbonyls and maleimides, both of which are commonly found in commercially available bioconjugation precursors. Scheme 4 illustrates a two-step, one-pot thiol conjugation protocol. Treatment of PS-thiol precursor **4b** with TFA, in the presence of $(i\text{-}pr)_3SiH$, affords the globally deprotected PS-thiol intermediate, which is *immediately* reacted with either α-bromoacetophenone or *N*-ethylmaleimide (NEM) to afford conjugate **7** or **8**, respectively. Both targets were prepared in moderate yield, and ultrasonication was necessary for optimal reactivity, as this

apparently deaggregates the deprotected PS derivatives. Of note, the freshly generated PS-thiol loses its reactivity upon chromatographic purification or prolonged storage. Thus, it should be used immediately *in situ* after generation.

Conjugation Reactions with PS-alkyne

PS-alkyne derivative **4a** was specifically constructed as a substrate for the well-known Cu(I) catalyzed cycloaddition reaction with suitably functionalized azides.[18] This method of 'click' bioconjugation is attractive because it is completely atom economical, tolerant of virtually all other chemical functionality, and compatible with aqueous reaction conditions.

Shown in Scheme 5 are the straightforward syntheses of several examples of azide-tagged cargo. Azides **11**, **12**,[19] and **13**[20] all react smoothly with PS-alkyne precursor **4a** to give the corresponding nitrobenzofurazan- (NBD) (**24**), biotin- (**23**), and pyrene- functionalized (**22**) triazoles in good yields (Scheme 6). This conjugation reaction was optimized by using 50 mol% $CuSO_4$, sodium ascorbate, and a biphasic $CHCl_3/H_2O$ solvent system. The products were isolated in high purity by simple liquid extraction followed by flash chromatography. Removal of the acid-labile protecting groups with TFA cleanly affords the corresponding PS conjugates (**25**, **26**, and **27**) in high yield.

We envision that in some cases, the conjugated molecular cargo may be sensitive to the acidic conditions required for PS headgroup deprotection, so we evaluated the click conjugation chemistry with the deprotected PS alkyne **28** (Scheme 7). Initially, very low yields (< 5%) were obtained when the previously described 'click' conditions were employed. However, consistently higher product yields (45-90%) were obtained when the reactions were carried out with ultrasonication. In fact, **25**, **26**, and **27** were also prepared directly from **28** using this methodology in similar yields as reported in Scheme 6. Removal of copper salts from these unprotected PS reaction products is necessary,[21] in contrast to their protected derivatives, and is efficiently accomplished by treatment of the crude reaction mixtures with silica-thiol resin, and simple filtration.

Scheme 7 highlights additional examples that were prepared *directly* from the deprotected PS-alkyne **28**. Ester **29** was the first derivative whose synthesis required this direct methodology. While ester-azide **16** successfully underwent cycyloaddition with protected PS-alkyne precursor **4a** (reaction not shown, 81% yield), subsequent TFA-mediated PS headgroup deprotection resulted in ester cleavage,[22] rendering **29** a previously unattainable target. Labile amino-acid derivative **21** was also directly 'clicked' with PS scaffold **28** resulting in D-ala-D-ala PS conjugate **30**, demonstrating that this method is ideal for acid-sensitive cargo. A final example is the production of fluorescent coumarin conjugate **32**, which is suitable for microscopic imaging studies. In summary, direct 'click' conjugation is a robust, bio-orthogonal method for preparing PS conjugates from *potentially any* azide-functionalized cargo, including molecules that are acid-labile.

Conclusions

Synthetic PS precursors with reactive amine, thiol, and alkyne groups at the end of the *sn*-2 acyl chain are prepared and conjugated with a wide variety of cargo molecules. Direct reaction of alkyne **28** with azide-functionalized cargo is the broadest in scope and most applicable to widespread production of PS conjugates, although all of the conjugation methods have utility depending on the exact conditions. The products of this versatile and mild synthetic chemistry allow us to broadly investigate PS-mediated cellular delivery and the results will be presented in due course.

Experimental Section

Each general method of PS conjugation is illustrated below with one example. Detailed experimental procedures and spectral data for all products are provided in the supporting information along with chemical structures showing the counter cation for each anionic phospholipid.

General Procedure for PLD Hydrolysis of PC Derivative

Phospholipase D (ca. 0.5 mg, from *Streptomyces chromofoscus*) was added to a stirring solution of PC **2a** (1.52 mmol) in a biphasic buffer/$CHCl_3$ (140/100 mL) system, and the reaction was allowed to stir overnight at 40 °C. The layers were separated and the aqueous layer extracted with 2:1 $CHCl_3$:MeOH (x 3). All organic layers were combined and washed with water (x 3), dried over Na_2SO_4 and concentrated to dryness. The residue was purified using column chromatography (65:25:4 $CHCl_3$:MeOH:H_2O eluent system) to afford the PA product **3a** (91% yield).

General Procedure for the Conjugation of PS-amine with NHS Ester

To a solution of protected amine **4c** (0.04 mmol) in $CHCl_3$ (10 mL), was added DBU (0.67 mmol) and the solution was stirred for 30 mins at ambient temperature. A solution of biotin NHS ester (0.06 mmol, in 10 mL DMF) was then added and the mixture allowed to stir for another 16 h. The reaction was then concentrated under reduced pressure and the crude residue purified using flash chromatography (65:30:5 $CHCl_3$:MeOH:H_2O eluent system) to give the conjugated amide **5** in 67% yield. The PS headgroup was then deprotected by dissolving in CH_2Cl_2/TFA (2:1) and allowing to stir at rt for 12 h. The reaction was then concentrated to a crude residue which was taken up in 2:1 $CHCl_3$:MeOH and washed with saturated $NaHCO_3$ and water. The organic layer was then concentrated and purified using flash chromatography (65:25:4 $CHCl_3$:MeOH:H_2O eluent system) to afford the PS biotin conjugate **6** in 85% yield.

General Procedure for the Conjugation of PS-thiol with Maleimide or α-Halo Ketone

A solution of protected thiol **4b** (0.110 mmol) and $(i\text{-Pr})_3SiH$ (0.220 mmol) in CH_2Cl_2 (6 mL) and TFA (2 mL) was allowed to stir at ambient temperature for 12 h. The reaction was then concentrated to a crude residue which was taken up in 2:1 $CHCl_3$:MeOH and washed with saturated $NaHCO_3$ and water. The organic layer was then concentrated to dryness. The residue was taken up in dry $CHCl_3$ (3 mL), alpha-bromoacetophenone (0.110 mmol) and Cs_2CO_3 (0.240 mmol) were added, and the resulting suspension was placed in a sealed vial and warmed in an ultrasonicating water bath at 40 °C for 12 h. The reaction mixture was loaded directly onto a silica column, which was eluted with $CHCl_3$, then 65:25:4 $CHCl_3$:MeOH:H_2O to afford the conjugated product **7** in 42% yield.

General Procedure for the Conjugation of PS-alkyne with Functionalized Azide

A mixture of alkyne **28** (0.076 mmol), azide **13** (0.091 mmol), $CuSO_4 \cdot 5\ H_2O$ (0.038 mmol), and sodium ascorbate (0.076 mmol) in 1:1 $CHCl_3$/H_2O (10 mL) was placed in a sealed vial and warmed in an ultrasonicating water bath at 40 °C for 3 h. The organic layer was then separated and the aqueous layer extracted with 2:1 $CHCl_3$:MeOH (x 3). All organics were combined, dried over Na_2SO_4, concentrated and purified using flash chromatography (0-20% MeOH in $CHCl_3$ then 65:25:4 $CHCl_3$:MeOH:H_2O). The fractions that contained product were combined and thiol-derived silica gel added followed by vigorous stirring for 30 min. The resin was then removed by suction filtration and the filtrate concentrated to afford conjugated product **29** in 55% yield.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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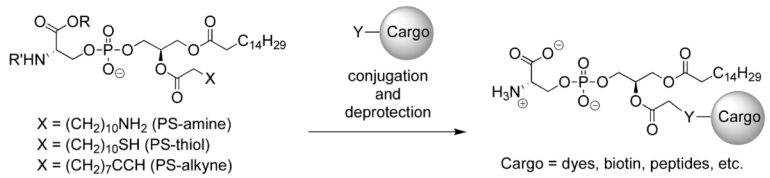
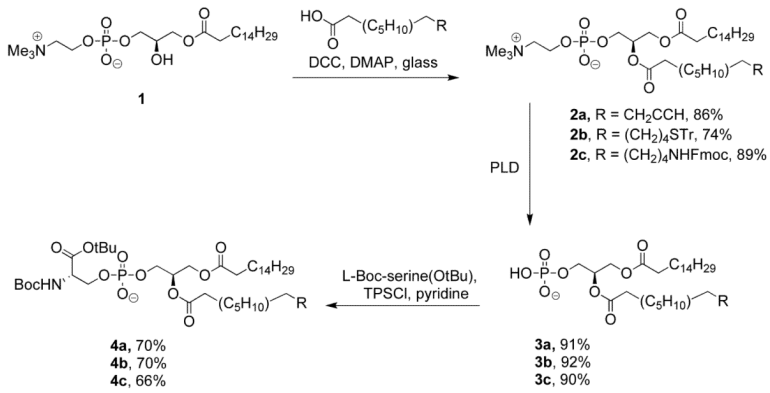


Scheme 1.



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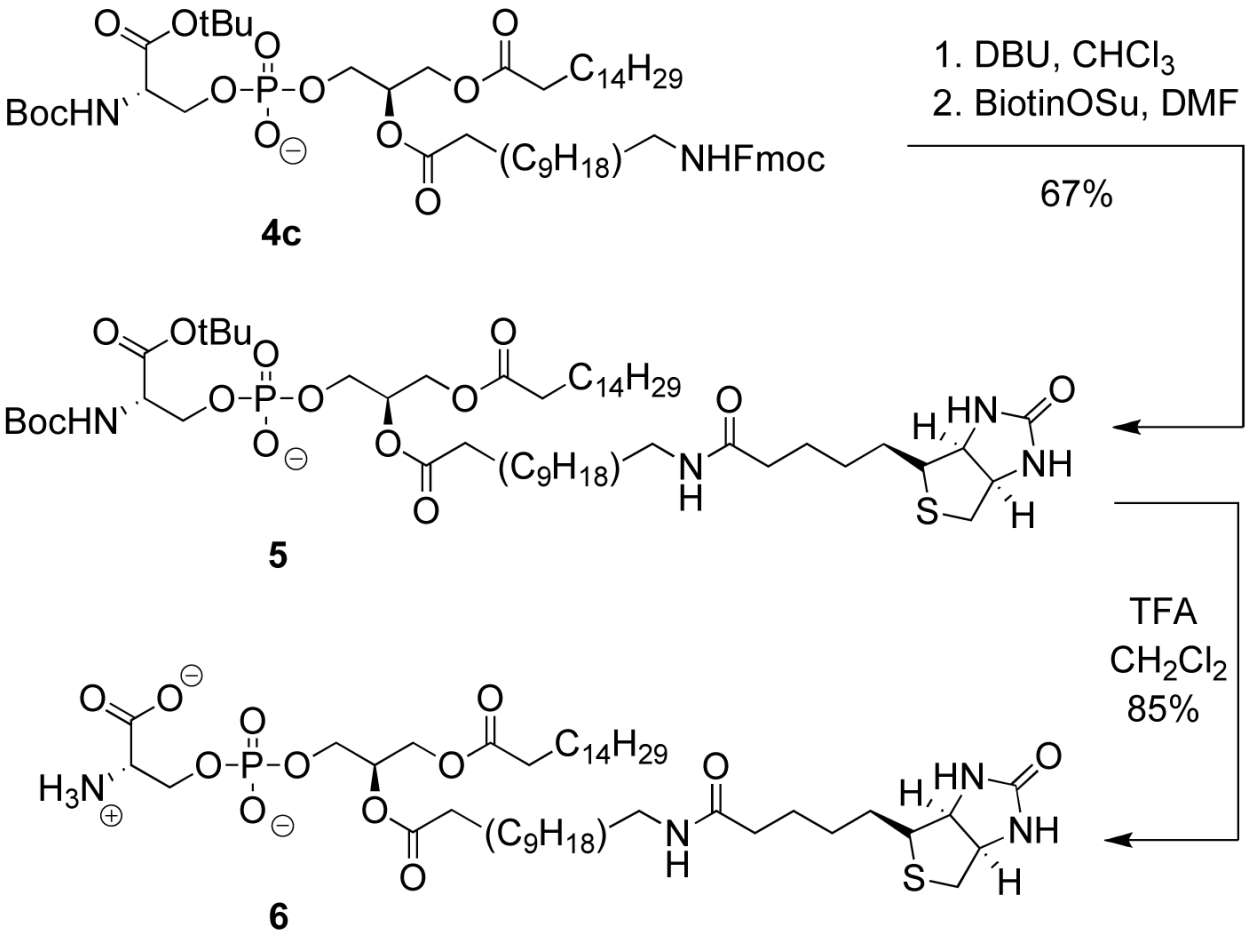

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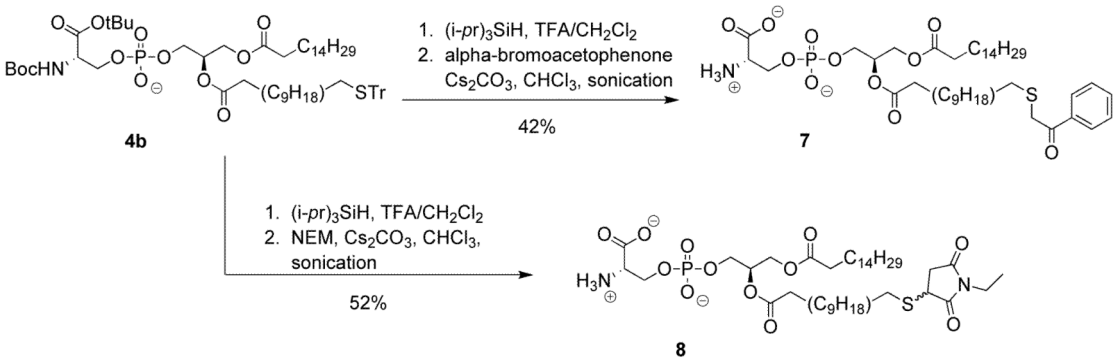

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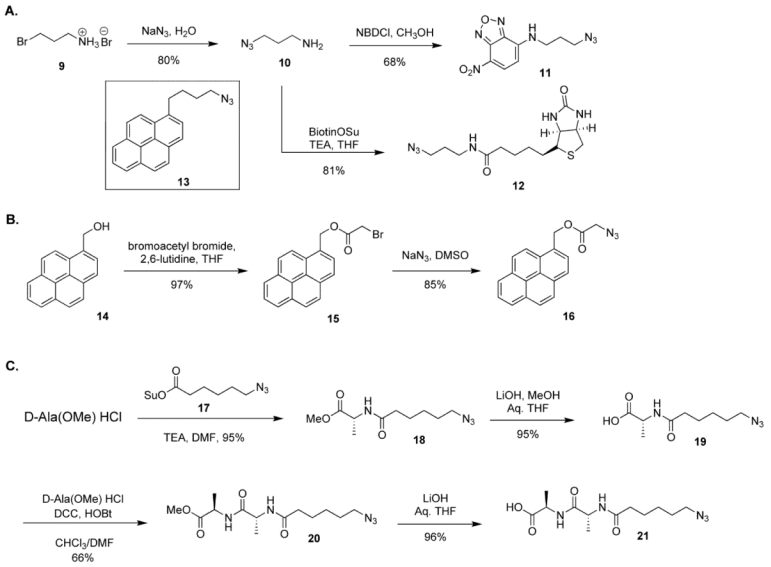

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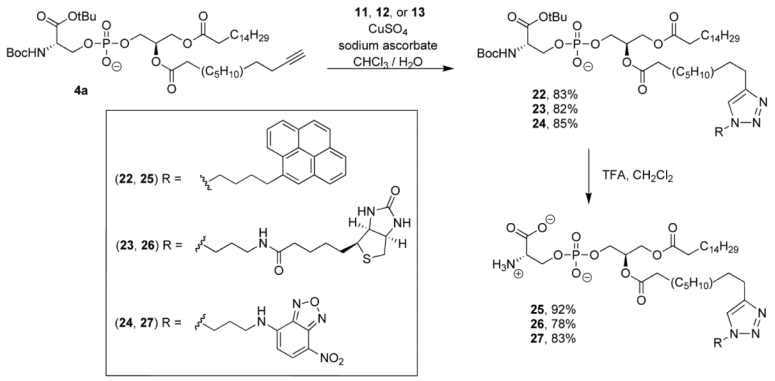

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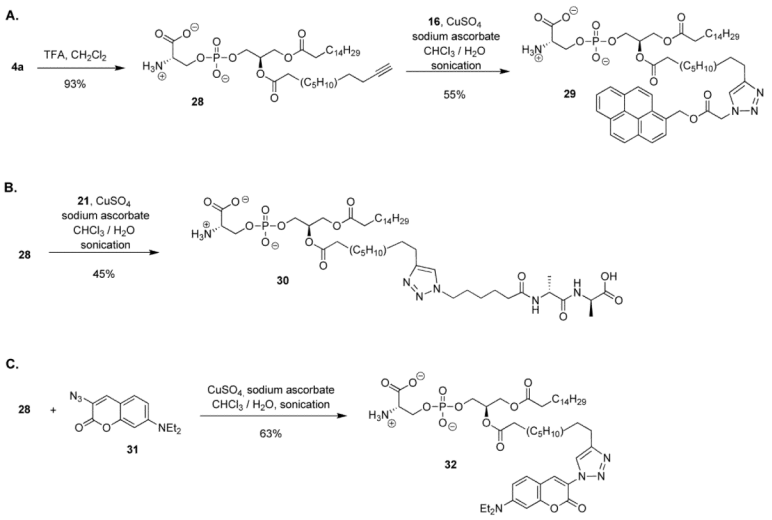

Scheme 7.