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Phosphatidylcholine-Derived Bolaamphiphiles via Click Chemistry

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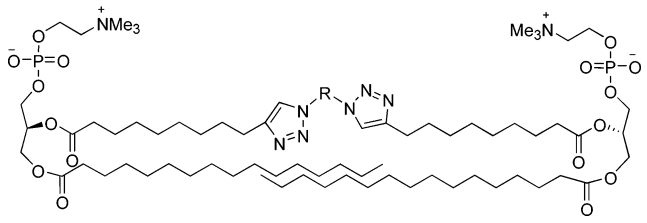
ABSTRACT



The copper-catalyzed azide alkyne cycloaddition is employed to modify phosphatidylcholine precursors with *sn*-2 acyl chains containing terminal alkyne or azide groups. Although the reactions are conducted as biphasic dispersions, the yields are essentially quantitative. Bolaamphiphiles are formed by simply clicking together two phosphatidylcholine alkyne precursors to a central bisazide scaffold. The chemistry introduces polar 1,4-triazole units into the lipophilic region of the bilayer membrane, and the bolaamphiphiles do not form stable vesicles.

Bolaamphiphiles are amphiphilic compounds, containing two hydrophilic headgroups connected by a hydrophobic spacer.[1] One of the best-known families of naturally occurring bolaamphiphiles are the archae lipids. A typical structure, shown in Figure 1, has a hydrocarbon chain that is connected by ether linkages to two zwitterionic phosphocholine headgroups.[2] These membrane-spanning, polar lipids rigidify the bilayer membrane and allow the thermophilic archae to endure temperatures in excess of 80 °C and low pH.[3] Because it is difficult to produce these natural products in large amounts, several groups have investigated simplified synthetic mimics and found that they also have membrane-stabilizing properties. Synthetic bolaamphiphiles with membrane-spanning macrocycles,[4] or unbranched hydrocarbon chains,[5] linking two phosphoglycerol headgroups are known to form membranes with increased melting temperatures, decreased permeation of ions, and small polar molecules and increased ordering of the membrane components.[1] In addition, Langmuir−Blodgett bilayers containing high concentrations of bolaamphiphile have diminished lateral membrane diffusion.[6] Because of these membrane-stabilizing properties,

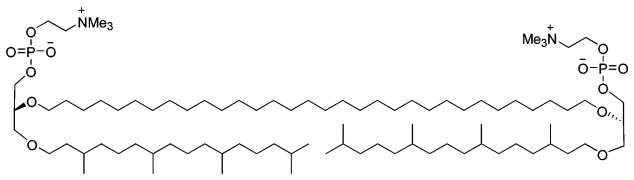


Figure 1. Structure of archae phospholipid, L-32-Phy.

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(2) (a) Yamauchi, K.; Sakamoto, Y.; Moriya, A.; Yamada, K.; Hosokawa, T.; Higuchi, T.; Kinoshita, M. *J. Am. Chem. Soc.* **1990**, *112*, 3188−3191. (b) Shinoda, W.; Shinoda, K.; Baba, T.; Mikami, M. *Biophys. J.* **2005**, *89*, 3195−3202.

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(5) (a) Menger, F. M.; Wong, Y.-L. *J. Org. Chem.* **1996**, *61*, 7382−7390. (b) Wang, G.; Hollingsworth, R. I. *J. Org. Chem.* **1999**, *64*, 4140−4147. (c) Kai, T.; Sun, X.-L.; Faucher, K. M.; Apkarian, R. P.; Chaikof, E. L. *J. Org. Chem.* **2005**, *70*, 2606−2615. (d) Kohler, K.; Forster, G.; Hauser, A.; Dobner, B.; Heiser, U. F.; Zeithe, F.; Rechter, W.; Steiniger, F.; Dreschsler, M.; Stettin, H.; Blume, A. *J. Am. Chem. Soc.* **2004**, *126*, 16804−16813.

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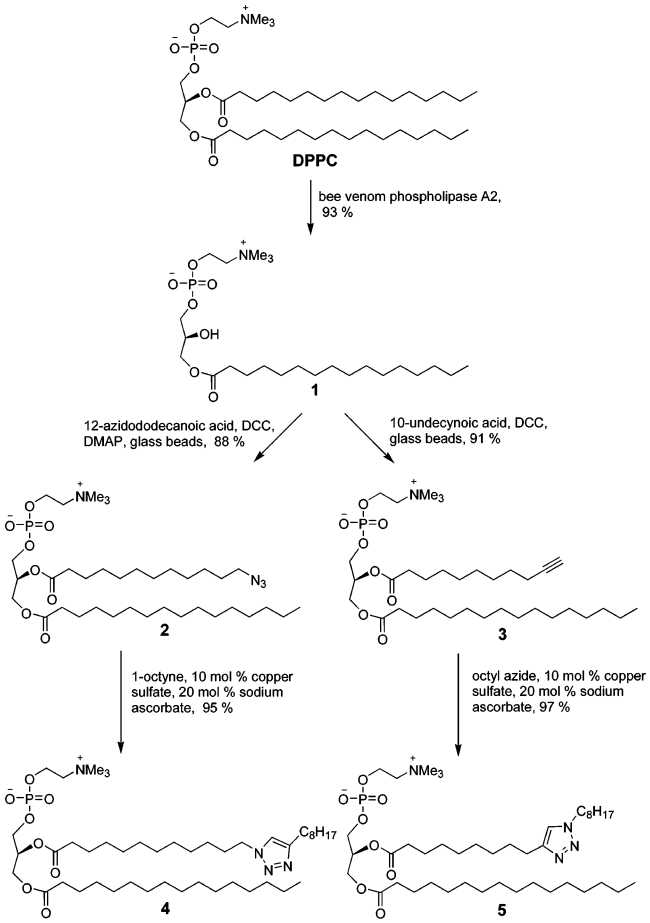
bolaamphiphiles are attractive building blocks for membrane assembly in various drug delivery and biotechnology applications. Conversely, many bolaamphiphiles have been *designed* to act as membrane disruptors and ion transporters.[7] Typically, these molecular designs incorporate polar functionality into the central region of a spacer that is otherwise hydrophobic. Whatever the specific functional objective, the bolaamphiphile synthesis is always a major technical challenge, especially if the molecule contains sensitive functionality such as that found in the headgroups of the common, naturally occurring phospholipids.

Our recent interest in the effects of bolaamphiphiles on membrane structure and function[8] has lead us to develop a method of producing them in large amounts from readily available starting materials. An obvious family of potential building blocks that have preinstalled polar headgroups are the lysophospholipids, with lysophophosphatidylcholine as the archetype example. Acylation of the *sn*-2 hydroxyl in lysophophosphatidylcholine with a suitably functionalized acyl chain gives a phosphatidylcholine precursor that can be dimerized to produce a bolaamphiphile. The chemistry to produce symmetric couplings includes olefin metathesis[9] and Glaser oxidation,[4a] whereas asymmetric cross-couplings have been achieved using the Wittig reaction, nucleophilic substitution, conjugate addition, and the Diels−Alder reaction.[5c,6] The latter reactions have also been conducted in preformed liposomal membranes. All of these synthetic procedures have drawbacks: either the yields are not extremely high or the chemistry is not compatible with all types of biomolecular functional groups. In an effort to improve the synthesis, we have investigated the [3+2] azide alkyne cycloaddition, a member of a larger class of reactions known as Huisgen 1,3-dipolar cycloadditions.[10,11] The copper-catalyzed azide alkyne cycloaddition has emerged in recent years as the most popular reaction in the series known as click chemistry.[12] The chemistry is compatible with a broad array of biological functionalities, and it is finding increasing employment in many areas of chemical technology. We report here that click chemistry is compatible with the phosphatidylcholine headgroup and that the coupling yields are essentially quantitative which greatly facilitates purification of the bolaamphiphile products.

The synthesis starts with the lysophosphatidylcholine **1**, which is easily prepared in gram quantities by an enzymatic cleavage of the *sn*-2 chain of dipalmitoylphosphatidylcholine (DPPC) using bee venom phospholipase A_2 (Scheme 1).[13]

Scheme 1. Synthesis of Phosphatidylcholine Derivatives



Acylation of the newly generated secondary alcohol was achieved in high yield and without acyl chain migration by employing the conditions described by Hajdu and coworkers.[14] Briefly, a suspension of compound **1** was sonicated for 5 h over glass beads with a 5-fold excess of either 12-azidododecanoic acid or undecynoic acid, DCC, and DMAP. The reaction is believed to take place on the glass surface. By adding more glass surface area to the flask, the reaction time is drastically reduced from days to hours. Upon the completion of the reaction, the DMAP was removed using Dowex 8X ion-exchange resin. The product was purified by column chromatography to give phosphatidylcholine derivatives **2** and **3** in 88% and 91% yield, respectively.

The azide derivative **2** and the terminal alkyne **3** react smoothly under the aqueous solvent conditions described by

(6) Halter, M.; Nogata, Y.; Dannenberger, O.; Sasaki, T.; Vogel, V. *Langmuir* **2004**, *20*, 2416−2423.

(7) Examples of bolaamphiphiles as ion channels include: Cameron, L. M.; Fyles, T. M.; Hu, C.-W. *J. Org. Chem.* **2002**, *67*, 1548−1553. Goto, C.; Yamamura, M.; Satake, A.; Kobuke, Y. *J. Am. Chem. Soc.* **2001**, *123*, 12152−12159. Leevy, W. M.; Huettner, J. E.; Pajewski, R.; Schlesinger, P. H.; Gokel, G. W. *J. Am. Chem. Soc.* **2004**, *126*, 15747−15753. Gokel, G. W.; Murillo, O. *Acc. Chem. Res.* **1996**, *29*, 425−432 and references therein. For bolaamphiphiles as membrane disruptors, see: Naka, K.; Sadownik, A.; Regen, S. L. *J. Am. Chem. Soc.* **1993**, *115*, 2278−2286.

(8) Forbes, C. C.; DiVittorio, K. M.; Smith, B. D. *J. Am. Chem. Soc.* **2006**, *128*, 9211−9218.

(9) (a) Patwardhan, A. P.; Thompson, D. H. *Langmuir* **2000**, *16*, 10340−10350. (b) Meglio, C. D.; Rananavare, S. B.; Svenson, S.; Thompson, D. H. *Langmuir* **2000**, *16*, 128−133.

(10) Huisgen, R. *Angew. Chem., Int. Ed.* **1963**, 565−632.

(11) For a more recent review on 1,3-dipolar cycloadditions: Gothelf, K. V.; Jorgenson, K. A. *Chem. Rev.* **1998**, *98*, 863−909.

(12) (a) Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radic, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 1053−1057. (b) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596−2599. (c) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004−2021.

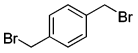
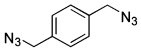
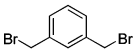
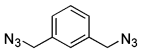
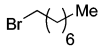
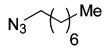
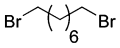
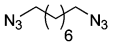
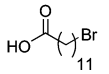
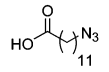
(13) Pluckthun, A.; Dennis, E. A. *J. Phys. Chem.* **1981**, *85*, 678−683.

(14) Rosseto, R.; Hajdu, J. *Tetrahedron Lett.* **2005**, *46*, 2941−2944.

Sharpless and co-workers[12] to produce the 1,4-triazole products **4** and **5** in 95−97% yield. The high yields are quite remarkable because the reaction mixtures are thick biphasic dispersions from start to finish. The same reactions were repeated using a copper wire as a catalyst instead of copper sulfate/sodium ascorbate, and the products **4** and **5** formed in the same very high yield. Thus, click chemistry is an effective way to conjugate molecular cargo to the end of a phospholipid acyl chain.[15]

To use this chemistry to form bolaamphiphilic structures, various organic bisazides were synthesized from the corresponding dibromides.[16] Briefly, the dibromides were allowed to react with NaN_3 in DMSO for 1−4 h. The reaction was quenched with water, and the bisazide products were extracted into ether in 95−98% yield (Table 1) and used without further purification.

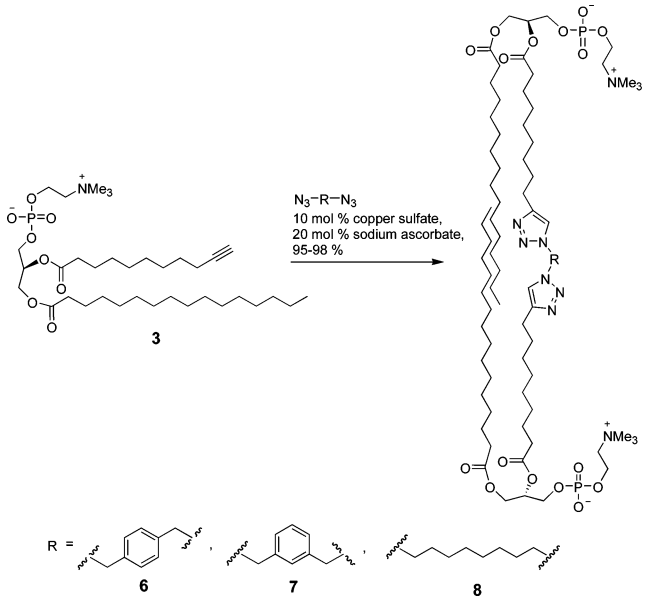
Table 1. Synthesis of Bisazides

starting material	product	reaction time (h)	yield (%)
		1	98
		1	98
		4	99
		4	97
		4	95

To form bolaamphiphiles, 2 equiv of the terminal alkyne **3** was stirred at room temperature with 1 equiv of the corresponding bisazide along with copper sulfate/sodium ascorbate in water. The biphasic reactions were complete after 30 h, and the corresponding bolaamphiphile products, **6**−**8**, were isolated by simple extraction in >95% yield (Scheme 2).

The membrane assembly properties of bolaamphiphiles **6**−**8** were evaluated in a number of different ways. First, thin films composed of 100% bolaamphiphile were dispersed in aqueous solutions of the fluorescent dye, carboxyfluorescein. Various dispersion methods were tried (freeze−thaw, sonication), but there was no evidence that the carboxyfluorescein could be encapsulated inside stable vesicles. A similar conclusion was reached after incorporation of the amphiphilic fluorescent probe NBD−PC. Sonicated dispersions of the bolaamphiphile with preincorporated NBD−PC were treated with the chemical quencher sodium dithionite,[17] which

Scheme 2. Synthesis of Bolaamphiphiles



destroyed all of the NBD−PC fluorescence, indicating that none of the probe was protected from the external added quencher. Typically, about half of the NBD−PC probe is protected if it is incorporated into both sides of enclosed vesicle membranes.[17] Finally, we analyzed aqueous dispersions of bolaamphiphiles **6**−**8** by transmission electron microscopy and observed that they form fibers and sheets (Figure 2).[18]

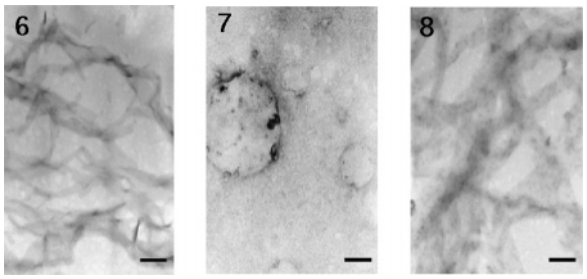


Figure 2. Transmission electron microscopy of bolaamphiphiles **6**−**8**. In each case, the scale bar is 200 nm.

The data suggest that bolaamphiphiles **6**−**8** do not favor vesicle formation, a result that is not surprising considering the propensity of bolaamphiphiles to form planar membranes[5d] and the polar nature of the two 1,4-triazole units. The molecular dipole of a 1,4-triazole is larger than an amide bond,[19] and it is unlikely that a 1,4-triazole will prefer the

(15) Click chemistry has recently been used to conjugate molecular cargo to the headgroup of phospholipids: (a) Musiol, H.-J.; Dong, S.; Kaiser, M.; Bausinger, R.; Zumbusch, A.; Bertsch, U.; Moroder, L. *Chem. Bio. Chem.* **2005**, *6*, 625−628. (b) Cavalli, S.; Tipton, A. R.; Overhand, M.; Kros, A. *Chem. Commun*. **2006**, 3193−3195. (c) Hassane, F. S.; Frisch, B.; Schuber, F. *Bioconjugate Chem*. **2006**, *17*, 849−854.

(16) Alvarez, S. G.; Alvarez, M. T. *Synthesis* **1997**, 413−414.

(17) Jiang, H.; O'Neil, E. J.; DiVittorio, K. M.; Smith, B. D. *Org. Lett.* **2005**, *7*, 3013−3017.

(18) Differential scanning calorimetry measurements were made in water. Cooling at a scan rate of 1° C/min produced a transition temperature of 40.2 ± 0.5 °C for a sample of para-isomer **6** (2.5 mg/mL) and no detectable transition temperature between 2 and 90 °C for meta-isomer **7** under the same conditions.

hydrophobic core of a bilayer membrane, unless it is anchored by a highly lipophilic tail.[20] Indeed, the more likely expectation with bolaamphiphiles **6**−**8** is that they should be vesicle-destabilizing molecules. This hypothesis was tested by measuring the ability of each bolaamphiphile to induce leakage from preformed vesicles. Experimentally, this was achieved by encapsulating carboxyfluorescein (50 mM) inside vesicles composed of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and cholesterol in a 8:2 ratio (25 μM total lipid concentration). This water-soluble dye is self-quenching, and fluorescence intensity increases as it leaks from vesicles into the external solution. In separate experiments, 20 μL aliquots of the bolaamphiphiles in ethanol were added to 3 mL dispersions of vesicles. At low bolaamphiphile concentrations of 1 μM, there was only very slow dye leakage over many hours, but as shown in Figure 3, there

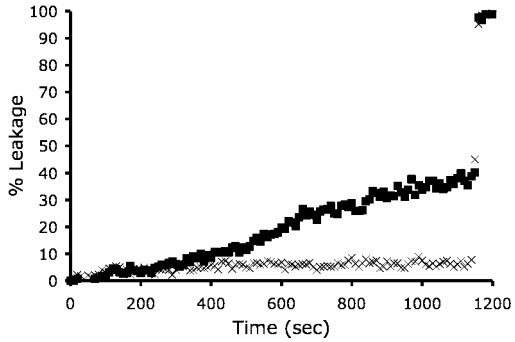


Figure 3. Leakage induced upon addition of bolaamphiphile to vesicles containing carboxyfluorescein (50 mM). To 25 μM of 8:2 POPC/cholesterol vesicles in TES buffer (5 mM TES, 100 mM NaCl, pH 7.4) was added 10 μM of para-isomer **6** (■) or meta-isomer **7** (×) at 50 s, and the vesicles were lysed by addition of Triton X-100 at 1150 s. Repeating the experiment with amphiphiles **4**, **5**, and **8** yielded the same result as **7**, that is, no leakage.

was a remarkable difference in leakage rates when the bolaamphiphile concentration was raised to 10 μM. After an induction period of several minutes, the bolaamphiphile para-isomer **6** induced large amounts of leakage, whereas the corresponding meta-isomer **7** had no measurable membrane disruption effect. It is important to realize that these two isomers of MW 1508 are highly flexible molecules that differ in atom connectivity at one location by only a few angstroms. Indeed, the structures are so similar that it is very difficult to rationalize why there is such a large difference in their membrane disruption abilities. Experiments with amphiphiles **4** and **5** and bolaamphiphile **8** produced the same result as **7**, that is, no leakage. Thus, it appears that the bolaamphiphile para-isomer **6** has an unusual preference to adopt a molecular conformation or a multimeric aggregate that disrupts the integrity of the vesicle membrane.[21]

In conclusion, we report that the copper-catalyzed azide alkyne cycloaddition, which is increasingly referred to as click chemistry, is a reaction that is compatible with the functionality in a phosphatidylcholine headgroup and that molecular cargo can be readily attached to the end of a phosphatidylcholine acyl chain. Furthermore, two phospholipids can be clicked to a bifunctional core molecule to produce bolaamphiphiles in large scale, high yield, and high purity. The synthetic processes described here should enable the conjugation of phosphatidylcholine to a wide range of solid supports[22] and solution-state scaffolds.[23] However, the chemistry introduces polar 1,4-triazole units into the lipophilic region of the phospholipid structure, which may produce molecules that disrupt the packing integrity of bilayer membranes, as seen with one of the bolaamphiphiles described here.

Acknowledgment. We thank William E. Archer for assistance with the electron microscopy. This work was supported by the National Institutes of Health.

Supporting Information Available: Synthetic procedures, spectroscopic data, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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(20) Menger, F. M.; Keiper, J. S.; Cran, K. L. *J. Am. Chem. Soc.* **2002**, *124*, 11842−11843.

(21) The leakage induced by **6** is not due to the sample addition procedure because the other amphiphiles have no effect under the same addition conditions. Furthermore, preincorporation of **6** at 10 μM prior to vesicle formation produces extremely leaky vesicles, which suggests that insertion of **6** into the vesicle membrane is not the process that causes the leakage.

(22) Rozkiewicz, D. I.; Janczewski, D.; Verboom, W.; Ravoo, B. J.; Reinhoudt, D. N. *Angew. Chem., Int. Ed.* **2006**, *45*, 5292−5296.

(23) Lin, P.-C.; Ueng, S.-H.; Tseng, M.-C.; Ko, J.-L.; Huang, K.-T.; Yu, S.-C.; Adak, A. K.; Chen, Y.-J.; Lin, C.-C. *Angew. Chem., Int. Ed.* **2006**, *45*, 4286−4290.