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Fluorescent labeling of alkyl chains of phosphocholine lipids by one-pot TMS cleavage-click reaction

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ABSTRACT

A simple click strategy for fluorescent tagging of azide-containing alkyl-chains of phosphocholine lipids is described, based on the easily prepared TMS-alkyne-BODIPY labeling reagent (**1**). Lipid labeling is carried out by one-pot Ag(I)-catalyzed deprotection of **1** followed by *in situ* Cu(I)-mediated cycloaddition of the alkyne-dye to the azide-phospholipid counterpart, under mild hydroalcoholic conditions with high yields. Since the reaction is compatible with the phosphocholine headgroup it was exemplified here by the synthesis of a fluorescent analog of *n*-hexadecylphosphocholine, a leishmanicidal drug (miltefosine). In addition, the preparation of a novel, strongly fluorescent 2-acyl BODIPY dye (**6**) by the unprecedented one-pot Ag(I)-catalyzed TMS-cleavage/alkyne-hydration of **1**, is reported; this compound may also be a convenient labeling reagent for amino-containing biomolecules.

1. Introduction

Fluorescent analogs of natural phospholipids are of great utility in a variety of applications in biology, spanning from the study of lipid traffic and function to the biophysical characterization of lipidic supramolecular assemblies, as membrane bilayers, lipoproteins, etc. Since lipids lack, as a rule, intrinsic fluorophores the original amphipathic structure needs to be modified to produce emitting analogs, as in other biomolecules (proteins, carbohydrates, and polynucleotides) [1]. Fluorescent lipids are frequently synthesized by reaction of a functionalized dye with a complementary group not usually present in the natural compound. In the case of fatty acids and linear-chain phospholipids, the bound emitting label might perturb both the polarity balance of the molecule as well as its original solution conformation, which may be essential traits for the lipid transport and function. In our previous work we wanted to produce emitting analogs of different phospholipids, preserving as much as possible the anticancer or antiparasite activity of the parent molecule (bioorthogonal labeling). It was found that linking a dye to the polar head invariably corrupted the original lipid bioactivity, probably due to changes in charge distribution and microscopic dissociation constants. In contrast, true fluorescent analogs could be produced by attaching specific lipophilic fluorescent groups to a final position of the lipid acyl chain. Different synthetic strategies were used for that purpose, but all of them were based on the same concept: to construct "*de novo*" the complete phospholipid molecule with the pending fluorescent group [2]. The main advantage of this lengthy and expensive approach is, of course, the tight control of the structure of the resulting substituted lipid acyl chain. The discovery [3–5] of the Cu(I)-catalyzed variant of the Huisgen terminal alkyne-azide 1,3-cycloaddition [6] provided a convenient way of linking together polyfunctionalized compounds, because the azide group can be introduced into many types of biomolecules through well-known chemical reactions [7,8]. The azide-alkyne reaction has been successfully applied for the bioorthogonal fluorescent labeling of proteins, nucleic acids and carbohydrates [9]. On the other hand, there are fewer reports of fluorescent labeling of lipids with this technique [10], and most of them are concerned with tagging the lipid polar head [11]. An outstanding exception is the labeling of diacylglicerol derivatives of phosphatidic acid by introducing first an alkyne group at one of the saturated lipid chains by six reaction steps [12]. The alkyne-functionalized lipid was then permeated into cells and treated with an azido-coumarin fluorogenic reactant. In another example [13] phosphatidylserine was tagged at the sn-2 acyl chain with an alkyne group, using also *de novo* synthesis of the glycerolipid.

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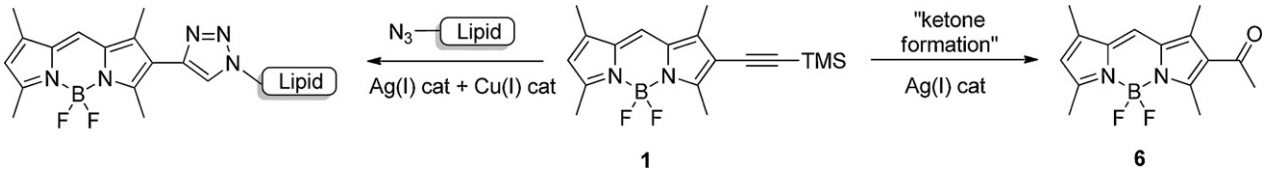


Scheme 1. One-pot Ag(I)-catalyzed deprotection of **1** followed by *in situ* Cu(I)-mediated click reaction and preparation of a novel 2-acyl BODIPY **6** by one-pot Ag(I)-catalyzed TMS-cleavage/alkyne-hydration of **1**.

In this way azide-functionalized dyes could be linked to the acyl chain by "click" conjugation. Here a much simpler strategy for lipid alkyl-chain labeling is reported, in which a terminal azide group is introduced into the lipid alkyl chain, in contrast with the examples mentioned above.

This provided direct access to one-step click conjugation with an alkyne-substituted tetramethyl-borondifluorodipyrromethene fluorescent dye (TM-BODIPY or BDP). In addition, the chemical stability of the azide group, particularly under oxidative conditions, could also commend it as a protective group in alternative multistep synthetic sequences.

The lipophilic BODIPY group is widely used to probe biological systems because of its small size, large absorption coefficient and emission yield, lack of toxicity and very good photostability. In addition, shifting the absorption and emission spectra from the VIS to the NIR is relatively simple, through manipulation of the BODIPY core [14]. In the present case, functionalization of the BODIPY core with an alkyne group at 2 position would be beneficial to preserve the original extended linear chain conformation in the labeled lipid. Thus, we selected for that purpose the 2-susbtituted BDP reagent **1**, which can be easily prepared. Lipid labeling was carried out by one-pot Ag(I)-catalyzed-TMS deprotection/Cu(I)-catalyzed click reaction between the TMS-protected alkyne-BDP (**1**) and its azide-phospholipid counterpart (Scheme 1). The utility of the method is demonstrated here by the synthesis of a fluorescent analog of n-hexadecylphosphocholine (miltefosine), a potent leishmanicidal drug, without previous protection of the phosphocholine head group.

In addition, the preparation of the new 2-acetyl BDP dye by a simple one-pot Ag(I)-catalyzed deprotection-hydration of **1** not previously described is also reported. This new functionalized dye is also of potential utility for direct fluorescent labeling of a wide variety of biomolecules containing amine groups (Scheme 1).

2. Results and discussion

2.1. Preparation of the alkyne-BDP (*1*) labeling reagent

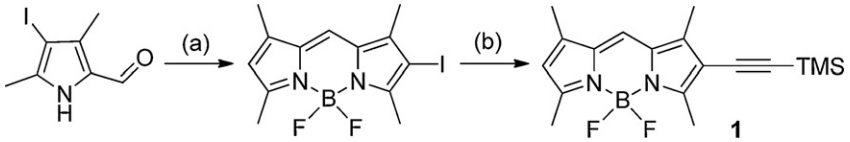
The preparation of the free alkyne-BDP is a lengthy and unproductive procedure [15]. Alternatively, one may take advantage of the easily prepared TMS-protected alkyne-BDP by using the protecting group as a temporary mask. This idea has been successfully developed by Aucagne and Leigh [16] who reported a one-pot procedure for the successive Ag(I) and Cu(I)-mediated chemoselective formation of two triazole linkages in a dialkyne tripeptide. Accordingly, reagent **1** was prepared as described before [17] in two steps: (1) condensation of 4-iodo-3,5-dimethylpyrrole-2-carbaldehyde with 2,4-dimethylpyrrole, and subsequent treatment of the obtained dipyrromethene with boron trifluoride diethyl etherate to yield the corresponding 2-iodinated BDP dye and (2) Sonogashira coupling of the latter compound with trimethylsilylacetylene (Scheme 2).

2.2. Preparation of alkylphosphocholine azide *3*

The azide functional group can be installed in a terminal site of a lipid acyl chain by several synthetic routes, some of them exploiting functionalities already present in the lipid structure. For instance, amine groups can easily be transformed into azides by reaction with diazo transfer reagents [8] (triflyl azide and derivates). A variation of the Mitsunobu reaction also provides simple access to azides from alcohols. In the present case we selected the halide displacement by azide ion, a widely used route to alkyl azides due to its flexibility and the easy access to the corresponding ω-haloalkanol starting material. Accordingly, the alkylphosphocholine azide **3** was obtained by reaction of 7-bromoheptanol with sodium azide in DMSO [18] yielding hydroxyalkylazide **2** which was subjected to 2-chloro-1,3,2-dioxa-phospholane-2-oxide and trimethylamine to introduce the phosphocholine group [2e]. The specific methylene chain length of phosphocholine **3** was selected as to yield a final fluorescent analog (see below) in which the extension of the lipophilic part was close to that of a C16–C18 alkyl chain, based on previous studies on structure–antiparasite activity relationship of alkylphosphocholines [19].

2.3. Click fluorescent lipid labeling

The analog of n-hexadecylphosphocholine with the terminal fluorescent BDP group aligned with the alkyl chain was synthesized by one-pot successive Ag(I)/Cu(I)-mediated reaction between the fluorogenic alkyne-containing BDP reactant **1** and the azide-containing alkylphosphocholine **3**, in hydroalcoholic solution (Scheme 3). Thus, the TMS-protected BDP **1** treated with $AgBF_4$ gave rise to the unprotected alkyne (not isolated) that, after *in situ* reaction with azide **3** in the presence of $CuSO_4$ and sodium ascorbate, yielded the fluorescent phosphatidylcholine **4** in good yield (74%) and purity. Similarly, the click reaction between **1** and **2** yielded the hydroxylated compound **5**, which is required as control lipid in studies of bioactivity of the tagged lipid [2c]. A small amount of a strongly fluorescent by-product was also observed by TLC at the end of the Ag(I)-mediated TMS-cleavage stage of the labeling reaction, identified as 2-acetyl-1,3,5,7-tetramethyl BODIPY (**6**) by



Scheme 2. (a) (1) 2,4-Dimethylpyrrole, $POCl_3$, *n*-pentane, CH_2Cl_2, 0 °C, 5 min; (2) DIPEA, PhMe, 25 °C, 10 min; and (3) $BF_3{\cdot}OEt_2$, 70 °C, 2 h, 35% overall. (b) TMS-acetylene, $PdCl_2(PPh_3)_2$, CuI, PhOH, nBu_4NI, DMF, iPr_2EtN, r.t., 3 h, 82%.