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SYNTHESIS OF NOVEL FLUORESCENTLY LABELED SPHINGOMYELIN DERIVATIVES USEFUL FOR SPHINGOMYELINASE ASSAY

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Abstract. Substrates for the sphingomyelinase enzyme class have been prepared, which contain a fluorescent tag and a biotin moiety. These molecules are useful for development of rapid high throughput assays for sphingomyelinase activity. It was observed that retaining the choline ammonium group (as found in the native sphingomyelinase substrate) in synthetic substrates was critical to retain high cleavage rate by the enzyme.
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Sphingomyelinases are a subclass of phospholipase C enzymes that catalyze the hydrolysis of sphingomyelin to ceramide and phosphocholine (see Figure 1).[1]

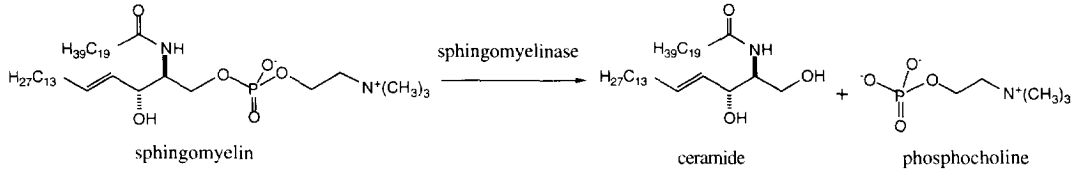


Figure 1

Two mammalian sphingomyelinases, each with unique activity requirements and cellular localization, have been extensively studied. An acidic sphingomyelinase, which has a pH optima for cleavage of 4-5.5, and subcellular localization to the lysosome[2,3] and a neutral sphingomyelinase, which has a neutral pH optima for cleavage, a Mg^{2+} requirement for activity, and localization to the plasma membrane.[4] Human deficiency in acidic

sphingomyelinase manifests itself as the disorder Niemann-Picks Disease.[5] Both acidic and neutral sphingomyelinase have been implicated in signal transduction pathways involving ceramide as a second messenger.[6]

Critical to the study of these enzymes is access to a sensitive, reliable and rapid assay for sphingomyelinase activity. The most commonly used sphingomyelinase assay utilizes sphingomyelin containing ^{14}C labeled choline as a substrate.[2] As the radioactive substrate is cleaved, aliquots of the assay mixture can be removed and partitioned between chloroform/methanol and water. Phosphocholine is water soluble while sphingomyelin and ceramide are extractable into chloroform/methanol. Release of phosphocholine can be monitored by scintillation counting of the separated aqueous phase. Fluorescent tag assays have been performed utilizing sphingomyelin derivatives fluorescently labeled in the ceramide moiety.[7] These assays also require an organic/aqueous partitioning scheme in order to monitor sphingomyelinase activity. Additionally, continuously monitorable sphingomyelinase assays have been developed.[8] In these assays sphingomyelinase cleavage of para-nitrophenyl choline phosphate derivatives to colored phenolic compounds can be directly monitored spectrophotometrically. Unfortunately, para-nitrophenyl choline phosphate derivatives are relatively poor substrates for sphingomyelinases and therefore the sensitivity of this type of assay is low.[9] In order to study the sphingomyelinases more effectively, access to an assay which achieves the sensitivity of the traditional radioactive assay while avoiding the use of a tedious aqueous/organic extraction step was required.

To develop a more efficient sphingomyelinase assay we examined a sphingomyelin derivative that could be attached to a solid support via the ceramide moiety and that contained a fluorescent tag in the phosphocholine moiety. The ceramide portion of this substrate, when cleaved by sphingomyelinase, would be retained on the solid support along with unreacted starting material while fluorescently labeled phosphocholine would go into the solution phase. The solution phase could then be removed and phosphocholine content measured spectrophotometrically. Solid phase attachment of sphingomyelin was envisioned as occurring via a biotin/avidin (or streptavidin) interaction. A generic structure for the desired sphingomyelinase substrate is as shown (see Figure 2).

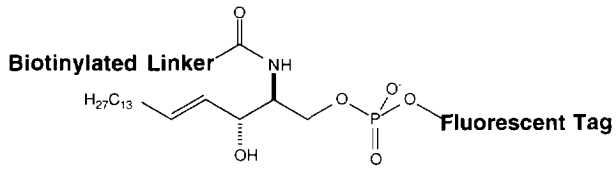
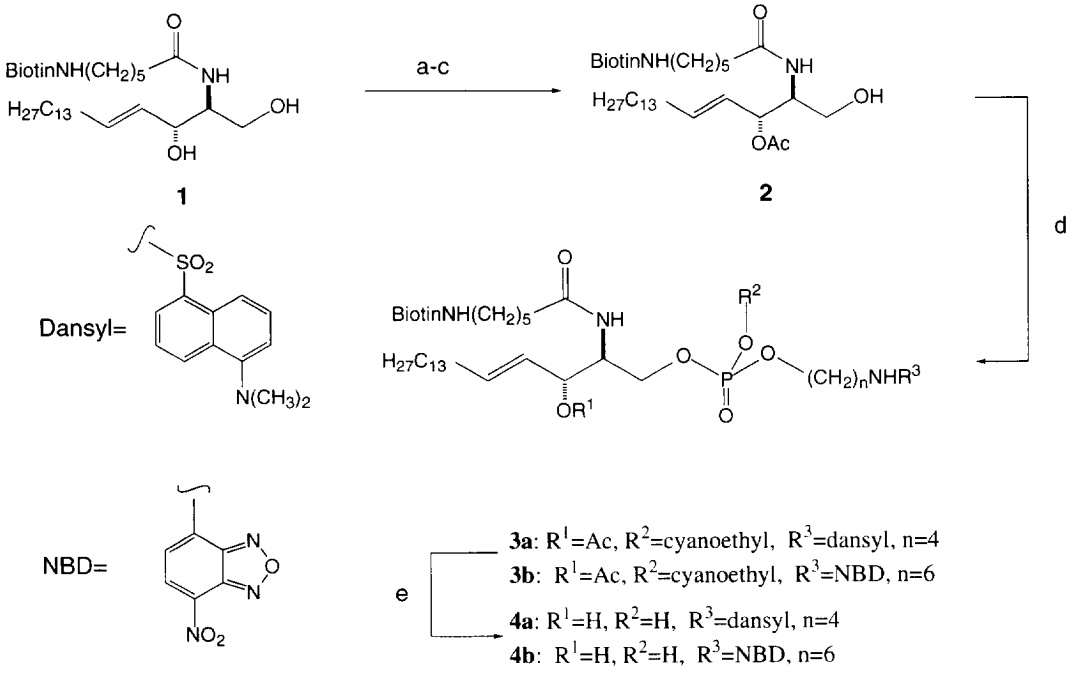


Figure 2

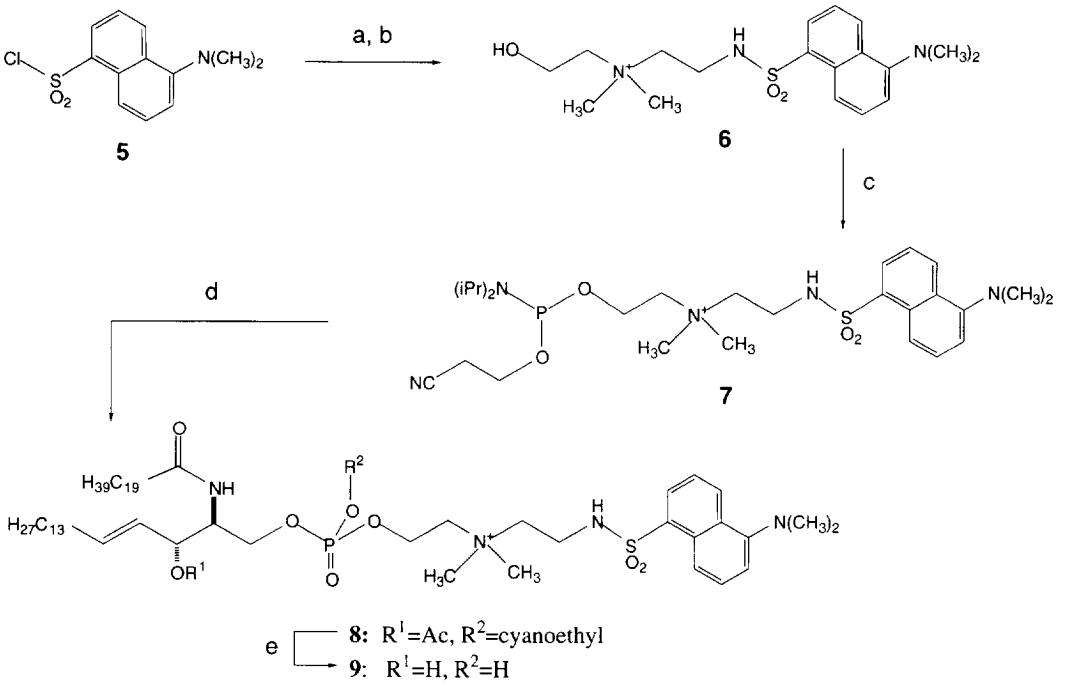
The feasibility of this approach to a rapid, high throughput sphingomyelinase assay was dependent on two key questions. First, would sphingomyelinases recognize and cleave a solid support bound sphingomyelin as

efficiently as sphingomyelin itself, and second, would sphingomyelinases recognize and cleave a sphingomyelin derivative substituted with a fluorescent tag in the phosphocholine moiety. Reports from the literature indicated that cleavage of a solid support bound sphingomyelin by a sphingomyelinase was feasible[10] but no data was available on cleavage of sphingomyelin derivatives bearing a fluorescent tag in the phosphocholine moiety. Therefore we first synthesized a variety of fluorescently tagged sphingomyelins and evaluated them as sphingomyelinase substrates. The syntheses of these compounds are outlined in Schemes 1 and 2.



(a) Dimethoxytrityl chloride, pyridine, 3 h; (b) Acetic anhydride, pyridine, 4 h; (c) Trifluoroacetic acid, 10:1 CH_2Cl_2:MeOH, O °C, 20 min, 70% overall from **1**; (d) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, diisopropylethylamine, 1:1 CH_2Cl_2:CH_3CN, 1 h, then $HO(CH_2)_nNHR^3$, 1-H-tetrazole, 4 h, then t-butylhydroperoxide, THF, overnight, 40%; (e) NH_4OH, dioxane, overnight, 90%.

Scheme 1



(a) *N,N*-dimethylethylene diamine, pyridine, 1 h, RT, 90%; (b) 2-bromoethanol, K_2CO_3, EtOH, reflux, 3 days, 87%; (c) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, $(iPr)_2NEt$, pyridine, 10 min, 44%; (d) 3-O-acetyl ceramide, tetrazole, THF, CH_3CN, 25 min, then I_2, H_2O, pyridine, 10 min, 77%; (e) NH_4OH, dioxane, 60 h, 46%.

Scheme 2

The synthesis of sphingomyelin derivatives outlined in Scheme 1 utilizes chemistry developed originally for solid-phase DNA synthesis.[11] The highly reactive phosphorous III reagents form the key diester linkage in a one pot, stepwise approach. This type of chemistry has been used to prepare sphingomyelin itself.[12] A highly reactive phosphorous reagent is critical to this approach since selectively protected ceramide derivatives like the ones used here (**2**) are known to be extremely unreactive primary alcohols.[13] An important aspect of this scheme is the choice of the protecting group for the ceramide secondary alcohol. Acetate was chosen because it allows for simultaneous deprotection of the ceramide secondary alcohol and the phosphate cyanoethyl protecting group with aqueous ammonia while leaving the sensitive phosphodiester linkage intact. Benzoate was tried as a substitute for acetate since acetate migration from secondary to primary alcohol was somewhat problematic during ceramide detritylation[14] but benzoate was difficult to remove using aqueous ammonia even with heating in a sealed tube.

The synthesis of sphingomyelin derivatives outlined in Scheme 2 utilizes phosphorous III chemistry to prepare the phosphodiester linkage but is done in two discreet steps instead of a one pot approach. While the two step approach is more labor intensive, it does offer an advantage over the one step synthesis in that the yield based on the valuable ceramide reagent is higher and following the progress of the reaction is easier.

With this series of fluorescently labeled compounds in hand, evaluation as sphingomyelinase substrates was performed. Each of the compounds was evaluated as a substrate for human placental acidic sphingomyelinase[15] utilizing the usual substrate ^{14}C-labeled sphingomyelin as a standard. The results of this study are summarized in Table 1.

Cleavage of sphingomyelin derivatives by human placental acidic sphingomyelinase[16]

compound	cleavage rate (nmol/U/h) (n = 3)
^{14}C-sphingomyelin	1.72 ± 0.01
9	1.32 ± 0.16
4a	0.51 ± 0.23
4b	0.29 ± 0.03

Table 1

Compound **9** which was cleaved by sphingomyelinase at 77% of the rate observed for ^{14}C-sphingomyelin, was the best fluorescent substrate. This compound incorporated a positively charged quaternary ammonium group into the fluorescent tag linker. The relative positioning of the ammonium group in compound **9** and the ammonium group in sphingomyelin to the cleavage site is identical, therefore this cleavage rate study suggests that for human placenta acidic sphingomyelinase, the ammonium group of sphingomyelin is important for substrate recognition. Preparation of a compound containing the fluorescent tag of compound **9** and a biotin containing lipid is underway. This compound, along with compounds **4a** and **4b**, will allow for final evaluation of a fluorescent sphingomyelinase assay on solid phase.

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