Note

An Improved Synthesis of Ceramide for Constructing α-Galactosyl Ceramide Analogs

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In spite of numerous synthetic routes to ceramide analogs, relatively few reports of the direct coupling of the unprotected phytosphingosine with the activated palmitic acid via amide bond formation are available. After purification by HPLC, the chromatogram indicated some impurities had not been removed during previous column chromatography. With the pure ceramides in hand, derivatization with the amino group for constructing libraries could be realized.

Keywords: HPLC; Phytoshingosine; Ceramide; Libraries; Cancer vaccine.

As a member of the glycosphingolipid family, alpha galactosyl ceramide (α-GalCer) has recently received great attention for its critical role in the mammalian immune system.[1,2] A phase I clinical trial conducted by Nieda et al. for treatment of 12 patients with metastatic malignancies reported that after receiving a dose of α-GalCer-pulsed dendritic cells, an increase in Serum IFN-γ was observed.[3] Further, the serum tumor markers were significantly decreased in two patients with adenocarcinoma, suggesting the antitumor effect of α-GalCer-pulsed DC. It is well established that α-GalCer exerts a high specificity for CD-1d molecules, a key component of antigen-presenting cells responsible for mediating numerous cytokines via innate as well as adaptive mechanisms. The recent crystallography structure for complex of α-GalCer and CD-1d defined the molecule architecture and thus paved an avenue for future pharmaceutical design.[4,5] Potential modifications included an introduction of a functional group such as a methyl group onto the acyl chains, and variation of the glycan sequences at the glycan moieties (Fig. 1). To provide enough of the key intermediate, it is important to develop a practical preparation for the lipid part.

At present, numerous approaches for constructing the ceramide moieties have been reported, such as semi-total syntheses starting from L-serine via chirality-installed Garner's aldehyde[6] and the preparation from chiral-rich glycan such as galactose,[7] lyxose,[8] mannofuranose,[9] 2-deoxy galactose,[10] and allosamine.[11] Of these, Garner's-aldehyde route represents an elegant example, since all of the intermediates obtained from reduction, Swern oxidation, Wittig reaction, and the amide coupling are of acceptable to good yields. This route met our requirement for synthesizing a sufficient amount of multigrams of ceramide for derivatization.

Indeed, in our hands, the Wittig reaction and the subsequent dihydroxylation could be feasibly performed and the yields were satisfactory. We, however, encountered some problems during the acid-promoted ring opening (step for transformation of compound **1** to compound **2**). For example, both methods of utilizing HCl-[12] and TFA-[13] promoted ring cleavage have been documented, yet incomplete deprotection was observed for the case of HCl. Whereas this was partly overcome by raising the reaction

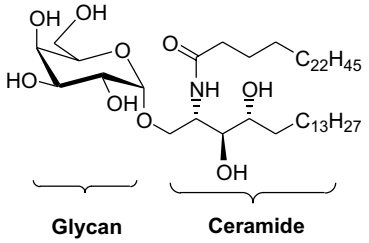

Fig. 1. Alpha-galactosyl ceramide.

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temperature, no adequate staining conditions were available for TLC monitoring. Purification by column chromatography also failed to provide a sufficiently pure compound. The same problem occurred in the subsequent coupling with acid too. Further purification using HPLC coupled with a C-18 column was complicated by the solubility problem, since the product tended to adsorb onto the nonpolar solid support. Attempt to decrease the polarity by introducing acetyl groups did improve the chromatographic purification; nevertheless, we were not able to obtain a sufficient amount of pure product.

Optimization could be achieved by replacing a C-18 column with a silica-based column coupled with a refractive-index detector. The HPLC chromatogram (detected by RI) showed an additional two peaks, indicating that some impurities were likely not completely removed from the previous steps. This might be the reason why only a few reports adopt the current coupling protocol without adequate protection with these secondary alcohols. To the best of our knowledge, only commercial amine was employed for this coupling as reported by Kim[14] and Wang-Peng.[15]

Thus, with the pure ceramide in hand, derivations with the primary or secondary alcohols with amine groups are performable. Furthermore, the construction of libraries via amide bond formation with various carboxylic acids might be possible as developed recently by the individual groups of Wong[16] and Bertozzi.[17] According to their methodology, the chemical synthesis and bioassay are performed in a one-pot manner, which require all protecting groups to be removed before amide-bond formation. The subsequent *in situ* construction of compound libraries and the relevant bioassay experiment are in progress.

EXPERIMENTAL SECTION

N-(2S,3S,4R)-1,3,4-*O*-triacetyloctadecan-2-yl palmitamide (4)

A mixture of compound **1** (26 mg, 0.06 mmol) and aqueous TFA (0.5 mL, 95%) was stirred at rt for 20 min. The starting material **1** (R_f = 0.82) was consumed, while the product **2** (R_f = 0) was detected on TLC (EtOAc/*n*-hexane 7:3). After the removal of the volatile solvents through four times coevaporation with toluene, a white solid (24 mg, 0.08 mmol) adhering on the inner flask was obtained.

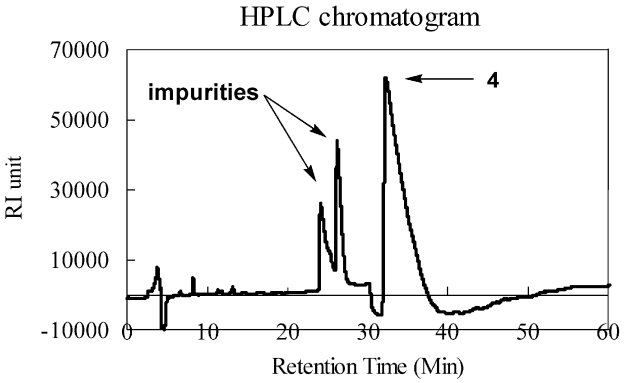
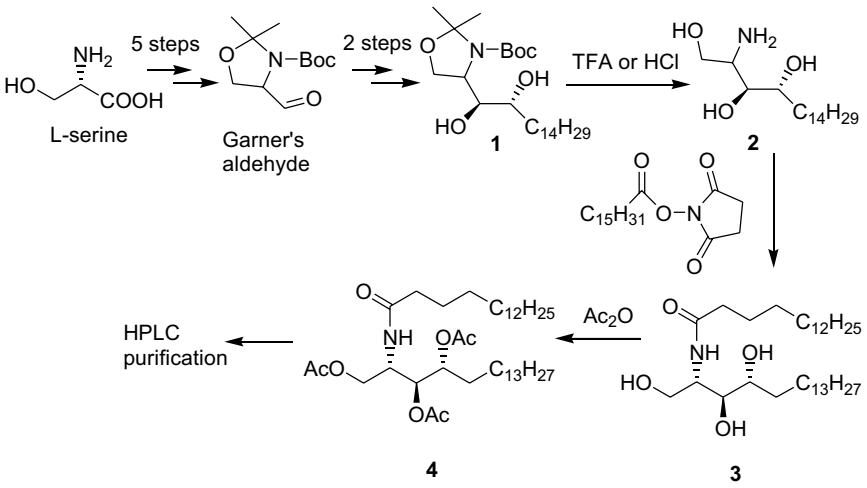


Fig. 2. HPLC chromatogram of alpha galactosyl ceramide **4** using RI detector .

Scheme I Synthesis and purification of ceramide analog **4**



The deprotected product **2** was dissolved in a solution of NEt_3 and THF (1 mL, V/V 2:3). To this solution was added the solution of NHS-ester (32 mg, 0.09 mmol) in THF (0.6 mL), prepared from palmitic acid according to Kim's procedure. It was stirred under gentle reflux at 80 °C for 12 h. After cooling to rt, a solution of DMAP (72 mg, 0.53 mmol), triethyl amine (168 μL, 1.2 mmol) and acetic anhydride (178 μL, 1.9 mmol) in THF (1 mL) was added. It was refluxed for 1 h followed by cooling to rt and a pale yellow solution was obtained. The starting material **3** ($R_f = 0$) was consumed, while the product **4** ($R_f = 0.21$) was detected on TLC (EtOAc/*n*-hexane 1:4) by staining with the vapor of I_2. The reaction was terminated by the addition of cold water followed by extraction with EtOAc (3 × 3 mL). The organic layer was washed with satd. $NaHCO_3$ (aq), 5% HCl and aqueous NaCl, and was dried by Na_2SO_4 followed by filtering through celite. After concentration under reduced pressure, the residue was purified with column chromatography using EtOAc/*n*-hexane 1:5 to provide the colorless oil-like product **4** in 49% yield (42 mg). This was further purified with normal-phase HPLC (Agillent 1100), equipped with a sample loop (500 μL), semipreparative column (9.4 × 250 mm ZORBAX SIL, 5 μm material), eluents: EtOAc/*n*-hexane 1:5, flow rate: 3 mL/min and detection of Refractive Index. A white-oil product corresponding to the retention time of 32.5 min in the chromatogram was obtained in 51% yield (20 mg). Anal. $C_{40}H_{75}NO_7$ calcd mass: 682.0 amu, ESI+Q-TOF MS, M = 681.6 (*m/z*), $[M+H]^+ = 682.4$; ^{1}H-NMR (500 MHz, C_6D_6) δ 0.85 (t, J = 7.0 Hz, 6H), 1.22-1.26 (m, 46H), 1.61-1.64 (m, 6H), 2.02 (s, 6H), 2.05 (s, 3H), 2.18 (t, J = 7.5 Hz, 2H), 3.96 (dd, J = 2.0, J = 11.5 Hz, 1H), 4.27 (dd, J = 4.5, J = 11.5 Hz, 1H), 4.46 (m, 1H), 4.90 (d, J = 9.5 Hz, 1H), 5.08 (dd, J = 2, J = 8.3 Hz, 1H), 5.91 (d, J = 9.5 Hz, 1H); ^{13}C-NMR (125 MHz, C_6D_6) δ 14.1 (CH_3), 20.7 (CH_3), 20.8 (CH_3), 21.0 (CH_3), 22.7 (CH_2), 25.5 (CH_2), 25.6 (CH_2), 28.0 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 31.9 (CH_2), 36.7 (CH_2), 47.4 (CH), 62.9 (CH_2), 71.9 (CH), 73.0 (CH), 170.1 (C), 170.9 (C), 171.1 (C), 172.8 (C).

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