Natural Products

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Synthesis and Structural Characterization of Three Unique *Helicobacter pylori* α-Cholesteryl Phosphatidyl Glucosides**

*Huy Q. Nguyen, Ryan A. Davis, and Jacquelyn Gervay-Hague**

Abstract: *Steryl glycosides produced by bacteria play important biological roles in the evasion and modulation of host immunity. Step-economical syntheses of three cholesteryl-6-O-phosphatidyl-α-D-glucopyranosides (αCPG) unique to Helicobacter pylori have been achieved. The approach relies upon regioselective deprotection of per-O-trimethylsilyl-α-D-cholesterylglucoside at C6 followed by phosphoramidite coupling. Global TMS ether deprotection in the presence of oxygen and subsequent deprotection of the cyano ethyl phosphoester afforded the target compounds in 16–21% overall yield starting from D-glucose. The structures of these natural products were determined using a combination of 2D NMR methods and mass spectrometry. These robust synthesis and characterization protocols provide analogues to facilitate glycolipidomic profiling and biological studies.*

It has been 30 years since Marshall and Warren isolated and characterized *Helicobacter pylori* bacteria from patients with chronic gastritis.[1] Although the gram-negative bacterium infects half of the world population, most people are asymptomatic. However, patients who do show symptoms are left with a gambit of illnesses ranging from peptic ulcers to gastric carcinomas.[2] Extensive scientific efforts have contributed to understanding the role *H. pylori* plays in these illnesses. One area of research is the synthesis and chemical characterization of the biomolecules produced by this pathogen.

Steryl glycosides are an intriguing class of bacteria-derived immune modulators. Bacteria do not produce steroids and the mechanisms they use to confiscate cholesterol from the host are not well understood. Currently, the three known cholesteryl glucosides isolated from *H. pylori* are αCG (**1**), αCAG (**2**), an analogue of αCG that is acylated with tetradecanoic acid at C6, and αCPG (**3**), a group of C6-phosphorylated derivatives (Figure 1).[2a,b,3] The lipid portion on the phosphatidyl glycerol unit varies in composition as identified in lyso-CPG analogues isolated from *H. pylori*.[2a,b,3,4] Together, these α-cholesteryl glucosides make up approximately 25% of the total lipid content of *H. pylori*, and can make up as much as 33% of the total lipid content in other *Helicobacter* species.[2a,b,3]

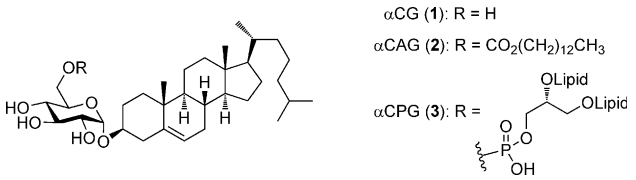

Figure 1. Structures of *H. pylori* cholesteryl glucosides. Lipid (length of carbon chain/number of unsaturated bonds) C14:0, C16:0, C18:1, C19:0 (cyclopropyl).

To date, biological studies involving α-cholesteryl glucosides have mostly relied upon mixtures from natural sources, making it difficult to determine the independent roles of each constituent.[2e,f] Moreover, biological studies have mainly utilized TLC R_f values and/or mass spectrometry to characterize the components of the *H. pylori* glycolipid profile.[2a,b,f,3–5] Recently, αCG (**1**) and αCAG (**2**) were synthesized and fully characterized by NMR spectroscopy and mass spectrometry, thus making pure samples of these compounds available to the biological community for the first time.[6] The exact structures of naturally occurring αCPG analogues (**3**) have yet to be defined, as neither TLC nor MS can readily distinguish the diversity of isomers resulting from esterification of the phosphatidyl moiety.[2a,b,3]

Given the growing importance of understanding the biological significance of *H. pylori* and related bacterial immunomodulators, a synthetic campaign focused on developing step-economical syntheses of αCPG analogues (**3a**–**c**, Figure 2) was initiated. The target compounds are composed of three structural units, including a cholesteryl aglycon, a sugar core, and a phosphate ester at C6 with variable glycerol ester side chains. These structural units present several synthetic challenges, including: 1) regioselective phosphorylation of the C6-hydroxy group,[7] 2) avoidance of phosphite acetal formation as seen in previous research,[8] 3) installment of the phosphatidyl group while avoiding acyl migration on the glycerol unit, and 4) regioselective oxidation

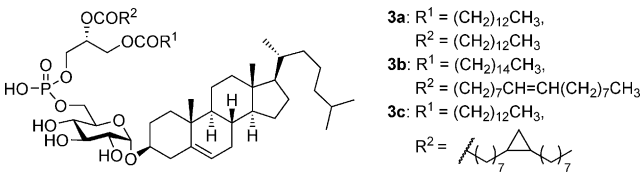

Figure 2. Structures of *H. pylori* α-cholesteryl phosphatidyl glucosides.

[*] H. Q. Nguyen, R. A. Davis, Prof. J. Gervay-Hague
Department of Chemistry, University of California, Davis
One Shields Ave, Davis, CA 95616 (USA)
E-mail: jgervayhague@ucdavis.edu

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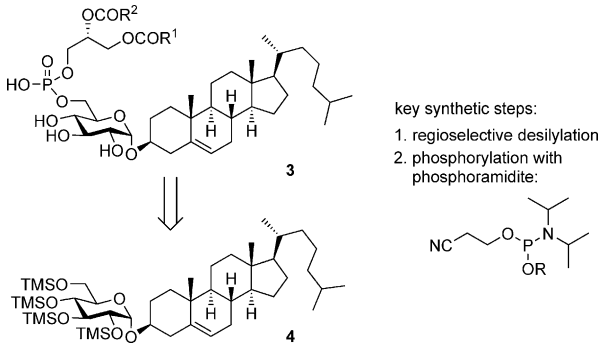
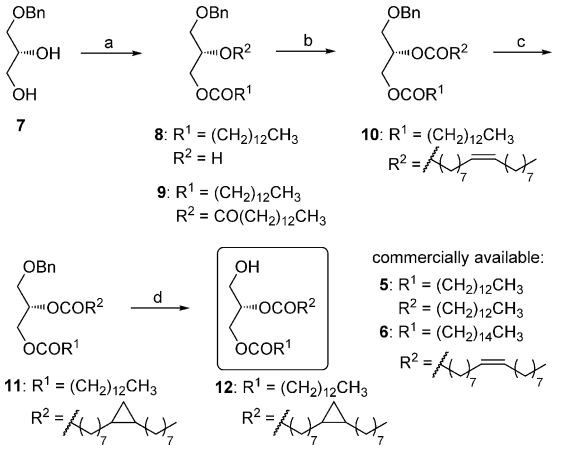


Figure 3. Retrosynthesis strategy for the preparation *H. pylori* αCPGs.

of the phosphorus atom without oxidizing the double bond of cholesterol.[9] We envisioned starting the synthetic route with per-*O*-TMS αCG (**4**, Figure 3)[10] because of its ready availability in two steps from glucose and its compatibility with the required criteria. Regioselective deprotection of the C6 ether followed by condensation with an appropriate phosphoramidite would achieve the desired synthesis in a convergent manner.

Three different fatty acids are required to address the syntheses of glycerol esters. Although 1,2-dimyristoyl-*sn*-glycerol (**5**) and 1-palmitoyl-2-oleoyl-*sn*-glycerol (**6**) are commercially available, we wanted to establish a generalized synthesis of various diacyl glycerols for future library development. Thus we began our studies with the esterification of 1-*O*-benzyl-*sn*-glycerol (**7**) using myristic acid in the presence of DCC and DMAP to afford **8** in 70% yield along with 23% of the double-addition product (**9**).[11] Compound **8** was then condensed a second time with oleic acid to form **10**. Diacylglycerol **11** was synthesized by cyclopropanation of **10** via a Simmons–Smith carbene utilizing Zn/Cu and diiodomethane (Scheme 1). Presumably, this reaction gave a mixture of cyclopropyl diastereoisomers, which were not distinguishable by chromatography or NMR spectroscopy, and thus no



Scheme 1. Synthesis of diacylglycerols. Reaction conditions: a) myristic acid, DCC, DMAP, CH_2Cl_2, 0°C→RT, 15 h, **8**: 70%, **9**: 23%; b) oleic acid, DCC, DMAP, CH_2Cl_2, RT, 15 h, 67%; c) Zn/Cu, CH_2I_2, Et_2O, reflux, 15 h, 62%; d) $Pd(OH)_2/C$, H_2 (1 atm), CH_2Cl_2, MeOH, RT, 2 h, 90%.

attempt was made to independently characterize the cyclopropyl isomers. The benzyl ether in compound **11** was removed by hydrogenation to afford the desired glycerol **12**, which was characterized by NMR spectroscopy.

A DEPT NMR experiment was utilized to identify the *sn*-2 ^{13}C (δ 70.6 ppm), and the corresponding ^1H NMR shift for the *sn*-2 C-H (δ 5.17 ppm) was assigned using HSQC NMR experiments. Afterwards, COSY NMR experiments revealed the ^1H NMR shift for both the *sn*-1 (δ 4.36 and 4.17 ppm) and *sn*-3 (δ 3.51 ppm) methylene protons. Likewise, HMBC NMR experiments showed *sn*-1 CH_2 correlation with the fatty ester carbonyl ^{13}C (δ 173.2 ppm), thus distinguishing *sn*-1 from *sn*-3 (Figure 4).

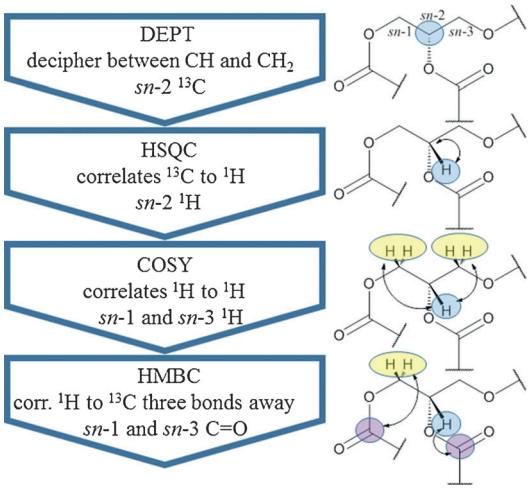
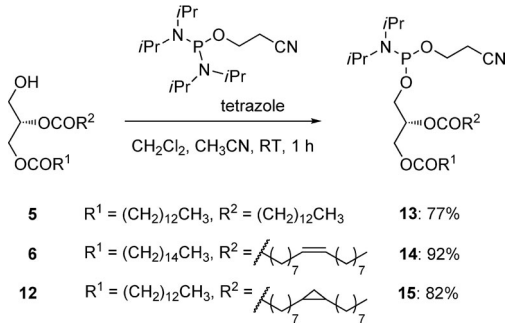


Figure 4. NMR experiments utilized to determine ester locations.

The next step in the synthesis was the conversion of commercially available 1-palmitoyl-2-oleoyl-*sn*-glycerol to the phosphoramidite needed to couple to cholesteryl glucose. This was achieved by first activating 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphordiamidite with tetrazole and its displacement with 1-palmitoyl-2-oleoyl-*sn*-glycerol **6** to afford the phosphoramidite **14** in 92% yield as a 1:1 mixture of diastereomers (Scheme 2).[12] The same strategy was utilized to prepare phosphoramidite **13** from **5**[11a] and **15** from **12** (Scheme 2).



Scheme 2. Synthesis of phosphoramidites **13**–**15**.

Regioselective phosphatidylation at the C6 position was initiated with the synthesis of compound **4**, which was achieved using our glycosyl iodide one-pot glycosylation protocol starting from per-*O*-TMS glucose.[10] In this manner, we could generate per-*O*-silylated αCG (**4**) and then regioselectively desilylate the primary ether at C6 using ammonium acetate in dichloromethane and methanol to afford the free alcohol (**16**, Scheme 3).[13] The synthetic route to αCPG through compound **16** avoids the formation of a phosphite acetal between the hydroxy groups at C4 and C6 of unprotected αCG(**1**) and any acyl migration event that could occur.[8] Freshly prepared phosphoramidites **13**–**15** were then coupled to **16** using tetrazole as the promoter (Scheme 3). Utilizing three molar equivalents of tetrazole was key to the success of the coupling reaction, as smaller amounts of tetrazole led to diminished yields (< 10 %). Even under these conditions, the yields were lower than desired, presumably because of steric congestion around the phosphoramidite.[14] Subsequent introduction of O_2 and DOWEX H^+ resin resulted in the oxidation of the phosphorus atom with concomitant deprotection of the TMS ethers. Finally, the cyanoethyl protecting group was removed with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). After purification, αCPGs **3a**–**3c** were obtained in 16–21 % yield starting from D-glucose (Scheme 3).

Extensive NMR analysis of the αCPG compounds revealed the phosphatidyl group to be attached to the C6′ positions. In the ^{13}C NMR spectrum of **16**, C6′ appears as a singlet at δ 61.9 ppm (Table 1). After phosphatidylation with **13**–**15**, the C6′ was shifted downfield and became a doublet because of the coupling with the phosphorus atom (J = 3.5–6.8 Hz). Also of note, C4′, C3′, and C2′ remained as singlets, indicating that the phosphate is not attached at these positions. After coupling with the phosphoramidites, a mixture of diastereomers (**17**–**19**) originating from the phosphorus center is formed. The diastereomeric mixture is observed in the ^{31}P and ^{13}C NMR spectra with C3′, C5′, C6′, *sn*-1-C, *sn*-2-C, and *sn*-3-C carbon resonances appearing as duplicates.

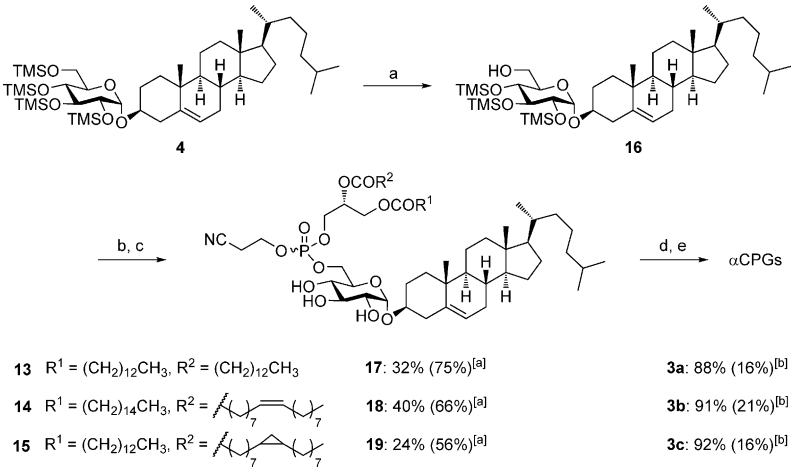
Table 1: Selected ^{13}C NMR resonances (in ppm) of compounds **16**–**19** and **3a**–**3c**.

	C2′	C3′	C4′	C5′	C6′
16[a]	74.2	75.1	72.4	73.0	61.0
17[b]	71.8	73.9, 73.9	69.3	70.3, 70.2	67.5 (d, J = 5.4 Hz), 67.4 (d, J = 5.4 Hz)
18[c]	75.9	74.4	72.1	72.8, 72.7	69.3 (d, J = 6.8 Hz)
19[b]	72.5	74.4	70.3	71.1, 71.0	68.5 (d, J = 5.3 Hz), 68.3 (d, J = 3.5 Hz)
3a[d]	72.4	73.4	69.2	71.6 (d, J = 3.2 Hz)	64.4 (d, J = 5.6 Hz)
3b[e]	72.7	73.9	68.9	72.1 (d, J = 2.6 Hz)	64.2 (d, J = 6.8 Hz)
3c[f]	72.8	73.9	69.9	71.9 (d, J = 5.3 Hz)	64.9 (d, J = 6.4 Hz)

NMR solvents: [a] C_6D_6. [b] $CDCl_3$/MeOD = 5:1. [c] C_5D_5N/MeOD = 5:1. [d] $CDCl_3$/MeOD/TEA = 5:1.5:0.5 (0.1 M). [e] $CDCl_3$/DBU/CD_3COOD = 5:0.8:0.2. [f] $CDCl_3$/MeOD 1:1.

Different deuterated solvents were required to solubilize each sample for NMR investigations, which resulted in chemical shift fluctuations. Nevertheless, the ^{13}C NMR shifts for **3a**–**3c** were all quite similar. Furthermore, HMBC NMR experiments indicated that the *sn*-2-CH and *sn*-1-CH_2 from αCPG compounds **17**–**19** and **3a**–**3c** were correlated with the carbonyl carbon atom of the corresponding fatty acids, thus providing evidence that acyl migration did not occur and the phosphorus atom remained attached to the *sn*-3-CH_2.

A synthetic protocol has been developed for the preparation of three different αCPG analogues (**3a**–**c**) associated with *H. pylori* immune modulation. Glycosylation of per-*O*-silylated glucose proceeds efficiently and with high α-selectivity because of the armed nature of the per-*O*-silyl donors.[10] Selective deprotection of the primary ether and subsequent condensation with a highly functionalized phosphoramidite followed by concomitant oxidation and deprotection afforded the desired analogues. Importantly, we have established a modular approach to preparing these natural products that is amenable to library development. The glycosyl iodide glycosylation is versatile, allowing various cholesterol analogues as well as other lipids to be attached to different carbohydrate cores.[15] Likewise, we have demonstrated that the phosphoramidite chemistry is compatible with biologically relevant functional groups such as olefins and cyclopropanes. In light of the recent discovery that *H. pylori* enzymes are promiscuous and readily incorporate a variety of cholesterol analogues,[16] this modular platform offers accessibility to various phosphatidyl glycolipids to study their biological properties and also to aid in the discovery of new analogues.



Scheme 3. Synthesis of αCPG (**3a**–**c**). Reaction conditions: a) NH_4OAc, CH_2Cl_2, MeOH, RT, 20 h, 93 %; b) **13**, **14,** or **15**, tetrazole, CH_2Cl_2, CH_3CN, RT, 40 h; c) O_2 (1 atm), MeOH, DOWEX H^+, RT, 2 h; d) DBU, CH_2Cl_2, RT, 3 min; e) HOAc. [a] Yields based on recovered αCG. [b] Total yields from glucose.

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