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Synthesis and Structural Characterization of Three Unique *Helicobacter pylori* α-Cholesteryl phosphatidyl glucosides

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Abstract

Steryl glycosides produced by bacteria play important biological roles in the evasion and modulation of host immunity. Step economy syntheses of three cholesteryl-6-O-phosphatidyl-α-D-glucopyranosides (αCPG) unique to H. pylori have been achieved. The approach relies upon regioselective C-6 deprotection of per-O-trimethylsilyl-α-D-cholesterylglucoside 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 rigorously 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.

Keywords

total synthesis; immunomodulator; glycosyl iodide

It has been 30 years since Marshall and Warren isolated and characterized *H. pylori* 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. An area of research that continues to inform this pursuit is the synthesis and chemical characterization of the biomolecules produced by this pathogen.

Steryl glycosides are an intriguing class of bacterial 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**), a C-6 analogue acylated with tetradecanoic acid (αCAG (**2**)), and a group of C-6 phosphorylated derivatives collectively referred to as αCPG (**3**) (**Figure 1**).[2a, 2b, 3] The lipid portion on the phosphatidyl glycerol unit varies in composition as

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identified from lyso-CPG analogues isolated from *H. pylori*.[2a, 2b, 3-4] Together, these α-cholesteryl glucosides make up approximately 25% of the total lipid content of *H. pylori* and in other *Helicobacter* species can be as high as 33% of the total lipid content.[2a, 2b, 3]

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, 2f] Moreover, biological studies have mainly relied upon TLC R_f values and/or mass spectrometry to characterize the components of the *H. pylori* glycolipid profile.[2a, 2b, 2f, 3-5] Recently, αCG (**1**) and αCAG (**2**) were synthesized and fully characterized by NMR and mass spectrometry 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, 2b, 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**) was initiated. The target compounds are composed of three structural units including a cholesteryl aglycon, a sugar core, and a C-6 phosphate ester with variable glycerol ester side chains, which together present several synthetic challenges including: (1) regioselective phosphorylation of the C6-hydroxyl[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 of phosphorus without oxidizing the double bond of cholesterol.[9] As highlighted in **Figure 3**, we envisioned beginning with per-*O*-TMS αCG (**4**)[10] because of its ready availability in two steps from glucose and its compatibility with the required criteria. Regioselective deprotection of the C-6 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 glycerol ester syntheses. 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 studies began with 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 di-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 Simmons-Smith carbene formation utilizing Zn/Cu and diiodomethane (**Scheme 1**). Presumably this reaction proceeded to form a mixture of cyclopropyl diastereoisomers, which were not distinguishable by chromatography or NMR and thus no 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.

A DEPT NMR experiment was utilized to identify the *sn*-2 ^{13}C (δ 70.6 ppm) and the corresponding ^{1}H shift for the *sn*-2 C-H (δ 5.17 ppm) was assigned using HSQC NMR experiments. Afterwards, COSY NMR experiments revealed the ^{1}H 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 (173.2 ppm) thus distinguishing *sn*-1 from *sn*-3 (**Figure 4**).

The next step in the synthesis was 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 displacing with 1-palmitoyl-2-oleoyl-*sn*-glycerol **6** to afford the glycerol-phosphoramidite compound (**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**).

Regioselective phosphatidylation at the C-6 position was initiated with the synthesis of compound **4**, which was achievedusing 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 C-6 primary ether 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 phosphite acetal formation between the C4 and C6 hydroxyl 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 any ratio less than 3 led to diminished yields (<10%). Even under these conditions, the yields were lower than desired presumably due to steric congestion about the phosphoramidite.[14] Subsequent introduction of O_2 and DOWEX H^+ resin achieved phosphorous oxidation 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 purificiation, αCPGs (**3a**-**3c**) were afforded in 16-21% yield starting from D-glucose (**Scheme 3**).

Extensive NMR analysis of the αCPG compounds revealed the phosphatidyl group to be on the C-6' position of αCG. In the ^{13}C NMR spectrum of **16**, the C-6' appears as a singlet at δ 61.9 ppm (**Table 1**). After phosphatidylation with **13**-**15**, the C-6' shifts downfield and becomes a doublet due to phosphorous coupling (*J*=3.5-6.8 Hz). Also of note, the C-4', C-3', and C-2' remain as singlets indicating the phosphate is not attached at these positions. After coupling with the phosphoramidites, a mixture of diastereomers (**17**-**19**) originating from the phosphorous is formed. The diastereomeric mixture is observed in the ^{31}P NMR and in ^{13}C NMR with C-3', C-5', C-6', *sn*-1-C, *sn*-2-C, and *sn*-3-C carbon peaks appearing as duplicates. 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 the *sn*-2-CH and *sn*-1-CH_2 from αCPG compounds **17**-**19** and **3a**-**3c** were correlated with the carbonyl carbon of the corresponding fatty acids providing evidence that acyl migration did not occur and the phosphorous remained attached to the *sn*-3-CH_2.

A synthetic protocol has been developed for making three different αCPG analogs (**3a-c**) associated with *H. pylori* immune modulation. Glycosylation of per-*O*-silylated glucose

proceeds efficiently and with high α-selectivity due to the armed nature of the per-*O*-silyl donors.[10] Selective deprotection of the primary ether and subsequent condensation with a highly functionalized phosphamidite followed by concomitant oxidation and deprotection afforded the desired analogs. 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 analogs as well as other lipids to be incorporated into 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 analogs,[16] this modular platform offers accessibility to various phosphatidyl glycolipids not only to study their biological properties but to also aid in the discovery of new analogs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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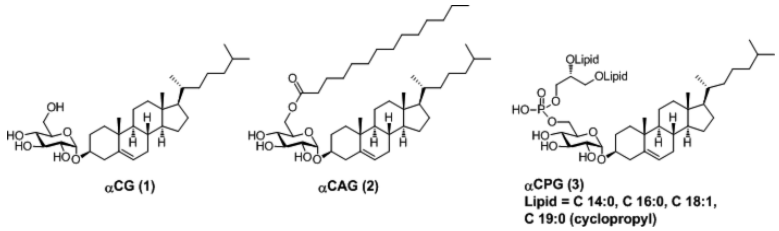


Figure 1.
Structures of *H. pylori* cholesteryl glucosides.

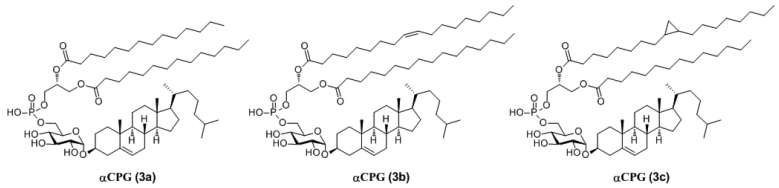


Figure 2.
Structures of *H. pylori* α-cholesteryl phosphatidyl glucosides.

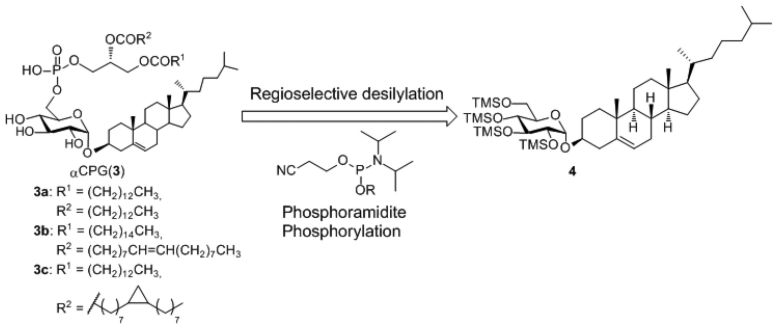
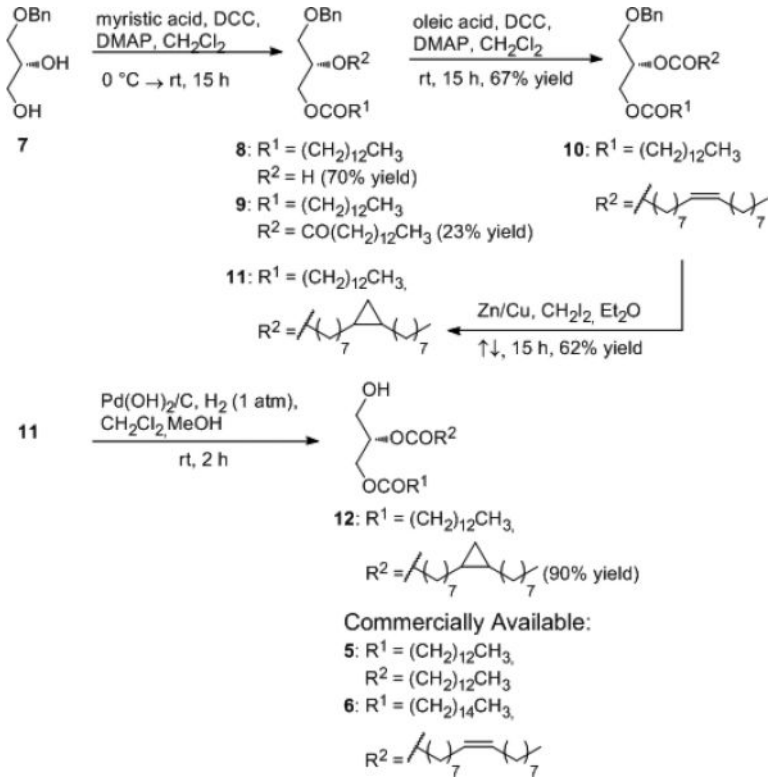


Figure 3.
Retrosynthesis strategy for making *H. pylori* α*CPG*.



Scheme 1.
Synthesis of diacylglycerols.

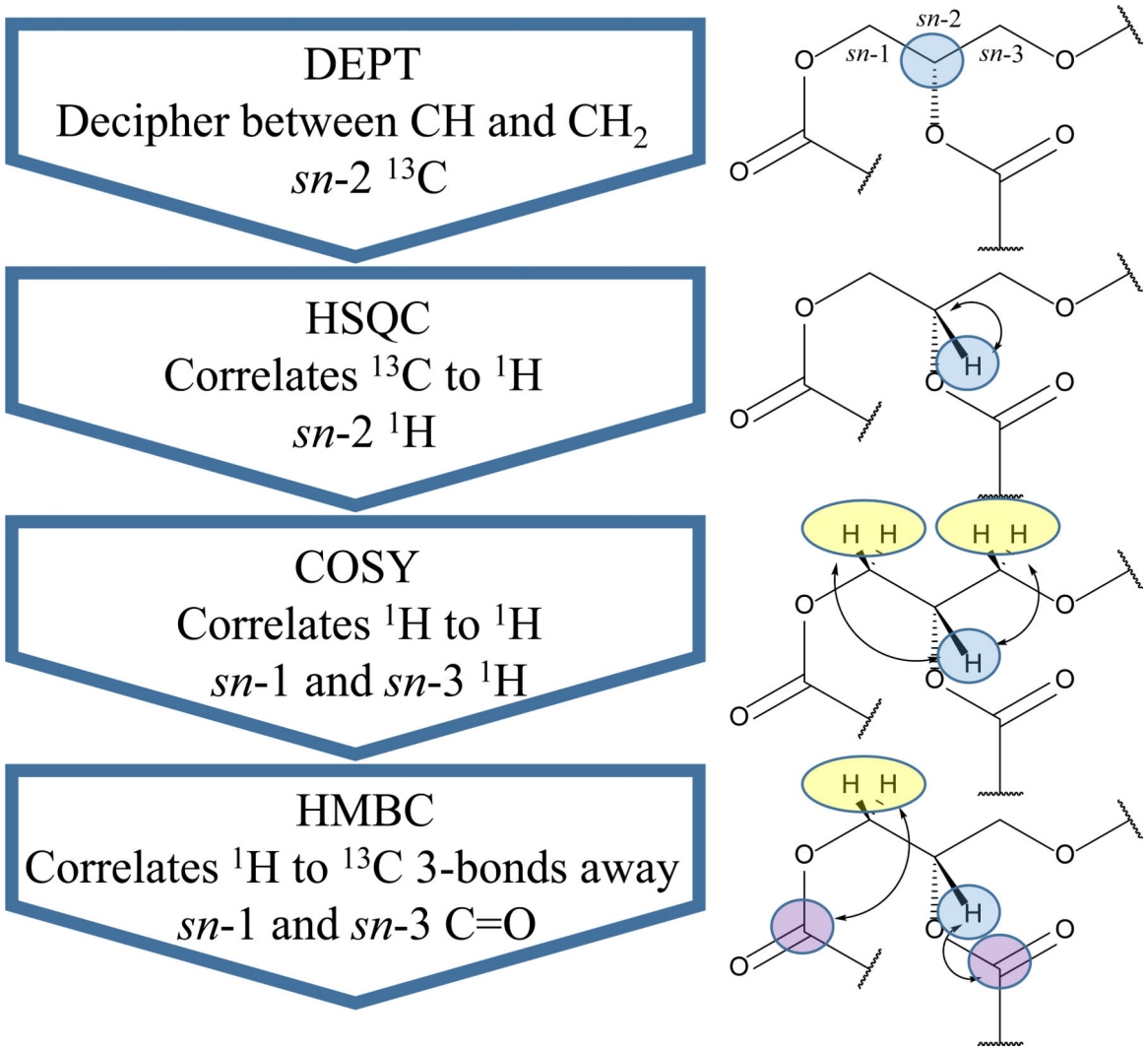
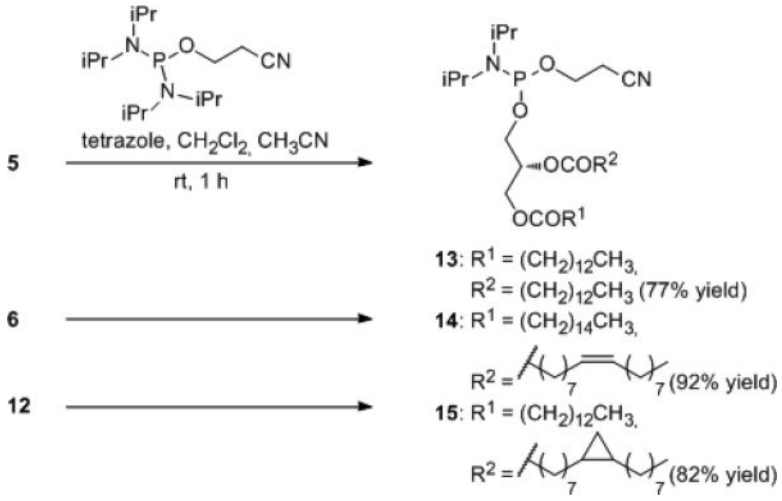
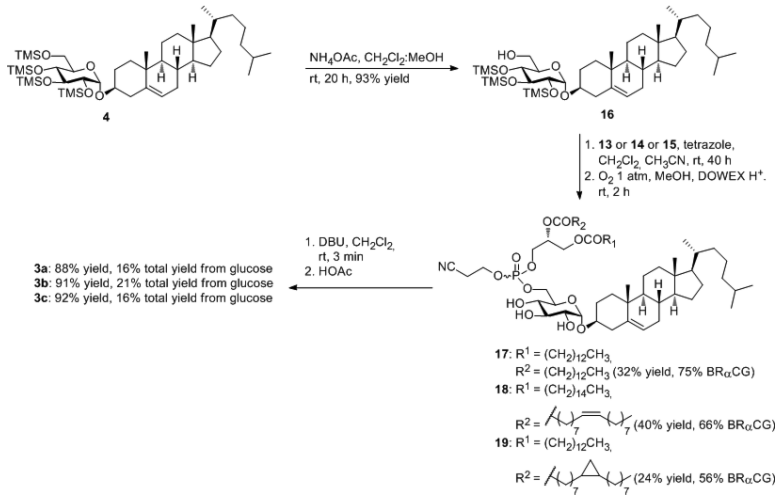


Figure 4.
NMR experiments utilized to determine ester locations.



Scheme 2.

Synthesis of phosphoramidites **13-15**.



Scheme 3.

Synthesis of αCPG(**3a-c**).

Table 1

^{13}C NMR of 16-19, 3a-3c.

	C-2′ (PPm)	C-3′ (PPm)	C-4′ (PPm)	C-5′ (PPm)	C-6′ (PPm)
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 0.1M 5:1.5:0.5

[e] $CDCl_3$:DBU:CD_3COOD 5:0.8:0.2

[f] $CDCl_3$:MeOD 1:1.