Vaccines

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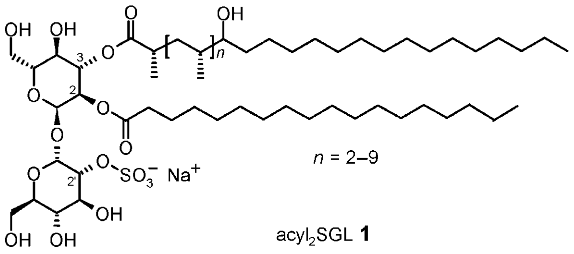
Synthesis of Diacylated Trehalose Sulfates: Candidates for a Tuberculosis Vaccine**

Julie Guiard, Anthony Collmann, Martine Gilleron, Lucia Mori, Gennaro De Libero, Jacques Prandi, and Germain Puzo*

Tuberculosis remains a major world-wide health problem and results in the loss of almost 2 million lives annually, with the majority of deaths occurring in the developing world.[1] Despite the discovery of active antibiotics in the 1960s and the production of the Bacillus Calmette–Guérin (BCG) vaccine in the early 20th century, tuberculosis is still not under control. The increasing incidence of human immunodeficiency virus (HIV) epidemics and the emergence of multidrug-resistant strains of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, impair the eradication of the disease. New therapeutic approaches and the development of new vaccines to fight tuberculosis are thus urgently needed.

Recently, a diacylated sulfoglycolipid, acyl$_2$SGL (**1**),[2] was characterized and identified as a new mycobacterial antigen able to stimulate populations of CD1b-restricted human T lymphocytes during infection with *M. tuberculosis*. Furthermore, these acyl$_2$SGL-specific activated T cells were shown to: 1) release interferon-γ (IFN-γ), 2) recognize *M. tuberculosis* infected antigen-presenting cells, and 3) kill intracellular mycobacteria in vitro.[2] In light of these properties, sulfoglycolipid **1** seemed to be a promising candidate for the development of a new tuberculosis vaccine. Compound **1** is specific to the *M. tuberculosis* species and is not present in *M. bovis* BCG, the species used in the current vaccine. The structure of acyl$_2$SGL encompasses an α,α-D-trehalose core, which is esterified at the 2-position with a palmitic (or stearic) acid, esterified at the 3-position with a hydroxyphthioceranoic acid, and O-sulfated at the 2′-position. Hydroxyphthioceranoic acids are a family of complex dextrorotatory fatty acids specific to the *Mycobacterium* genus, which contain a hydroxy group and methyl groups arranged in a 2,4,6 pattern.[3] All methyl-substituted stereocenters are of the L series,[3] whereas the configuration of the hydroxy-substituted carbon atom has not been assigned.



acyl$_2$SGL **1**

Compound **1** was isolated in tiny amounts (about 1 mg L^{-1}) from cultures of *M. tuberculosis*. Its low availability limits its further development as a potential tuberculosis vaccine. We therefore devised a synthetic route to this class of diacylated sulfated trehalose compounds and hypothesized that the hydroxyphthioceranoic acid, which is not readily available from natural sources, might be replaced by simpler fatty acids. Herein we report the preparation of various sulfoglycolipid (SGL) analogues of the natural compound acyl$_2$SGL (**1**) in which the hydroxyphthioceranoic acid has been replaced by less complex acids. Some of these analogues were able, like the natural product, to activate the acyl$_2$SGL-specific T-cell clone with the production of (IFN-γ). Of the utmost importance, it was found that small modifications to the structure of the hydroxyphthioceranoic acid substituent can modulate the immunogenicity of the analogues. Recent interest in the synthesis of mycobacterial sulfoglycolipids has led to the preparation of a tetraacylated trehalose sulfate.[4,5]

The elaboration of the trisubstituted α,α-D-trehalose core of the sulfoglycolipids on the basis of the pioneering synthetic studies of Goren and co-workers, and Baer and Wu was straightforward.[3,6,7] The known compound 4,6,4′,6′-dibenzylidene α,α-D-trehalose (**2**)[8] was acylated selectively at the 2-position (or 2′-position) by using palmitoyl chloride (or another acyl chloride) in pyridine to give a trehalose derivative **3** in 45 % yield (Scheme 1). This direct acylation reaction avoided the dibutylstannylene procedure,[6] which involves toxic tin derivatives and which was found to be difficult to carry out on a larger scale. The use of the bifunctional reagent 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (TIPSCl$_2$) enabled the selective protection of the 2′- and 3′-positions of **3** in one step and gave alcohol **4** in good yield (60 %). Owing to the steric crowding around the alcohol functionality of **4**, the nucleophilicity of the oxygen atom was low, and the esterification of compound **4** with fatty-acid derivatives proved to be difficult. The best yields of **5** (up to

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[*] J. Guiard, Dr. M. Gilleron, Dr. J. Prandi, Dr. G. Puzo
Département des Mécanismes Moléculaires des Infections Mycobactériennes, Institut de Pharmacologie et de Biologie Structurale du CNRS, UMR 5089, Université de Toulouse III
205 route de Narbonne, 31077 Toulouse Cedex (France)
Fax: (+33) 5-61-17-59-94
E-mail: Jacques.Prandi@ipbs.fr

A. Collmann, Dr. L. Mori, Prof. Dr. G. De Libero
Experimental Immunology, Department of Biomedicine
University Hospital Basel
Hebelstrasse 20, 4031 Basel (Switzerland)

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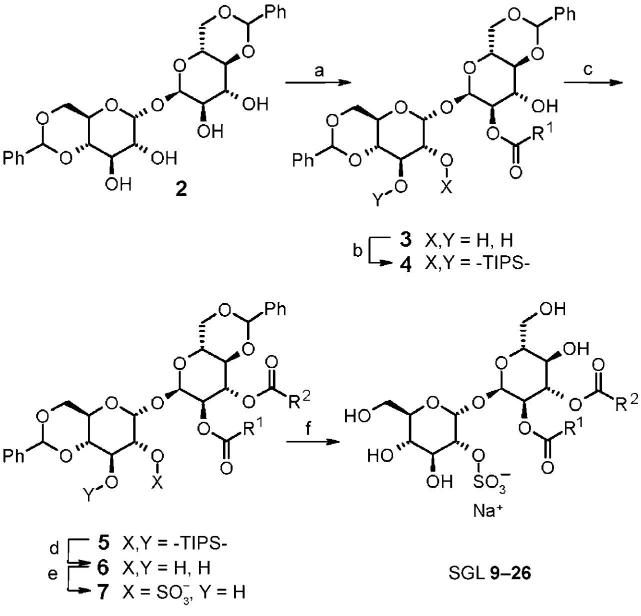
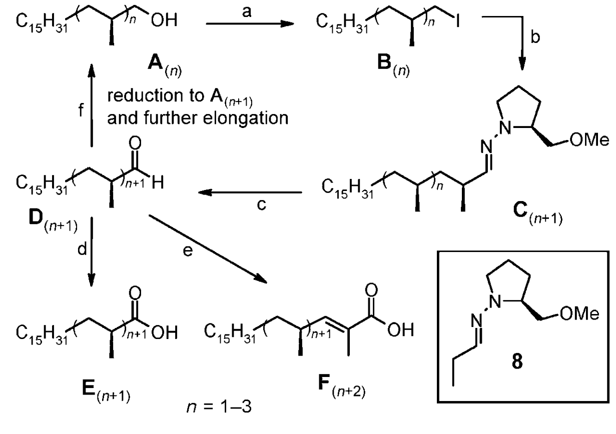


Scheme 1. General synthesis of sulfoglycolipids **9–26**: a) R^1COCl, pyridine, DMAP, room temperature; b) $TIPSCl_2$ (1.2 equiv), pyridine, room temperature, 48 h; c) R^2COCl (2.0 equiv), pyridine, DMAP, or R^2COOH (2.0 equiv), DCC (2.0 equiv), toluene, microwave irradiation; d) 1 M nBu_4NF in THF, room temperature, 24 h; e) SO_3·pyridine (1.5 equiv), DMF, room temperature, 24 h; f) 1.7% aqueous $H_2SO_4/CHCl_3/MeOH$, room temperature, 24 h. R^1 and R^2 groups are shown in Table 1. DCC = dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine, TIPS = tetraisopropyldisiloxane.

68%) were observed when the free fatty acid was activated with dicyclohexylcarbodiimide in toluene under microwave irradiation, but this method precluded the recycling of the fatty acid. When precious chiral polymethyl fatty acids were used, despite the lower acylation yields (26–54%), the reaction was carried out more classically in pyridine after activation of the acid by conversion into the acid chloride through treatment with oxalyl chloride. The 2′- and 3′-positions of diester **5** were unmasked with 1M tetrabutylammonium fluoride in THF to afford diol **6** in high yield (> 90%), provided that the slightly basic commercial solution had been neutralized to pH 6.5 (pH paper) with trifluoroacetic acid before use. Without this precaution, some scrambling of the acyl groups on the two glucose units of the trehalose was observed. Selective 2′-O-sulfation of diol **6** with the SO_3·pyridine complex in anhydrous *N,N*-dimethylformamide (DMF) at room temperature gave a mixture of 2′-O- and 3′-O-monosulfated trehalose derivatives. The observed regioselectivity was 5:1 in favor of the desired 2′-*O*-sulfate, and the regioisomers were separated readily by chromatography on silica gel. Compound **7** was isolated in 40–50% yield. The synthesis of the SGLs was completed by removal of the benzylidene protecting groups under carefully controlled acidic conditions (chloroform/methanol/1.7% aqueous H_2SO_4 60:40:8 (v/v/v), room temperature), and the final products were isolated in almost quantitative yield. All compounds in Table 1 were prepared by this route.

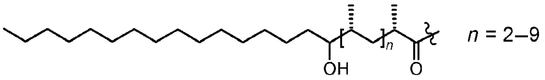
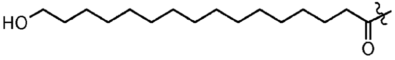
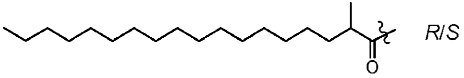
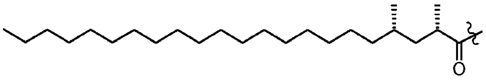
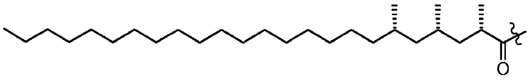
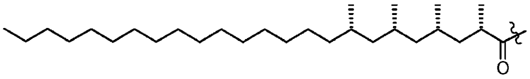
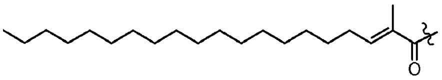
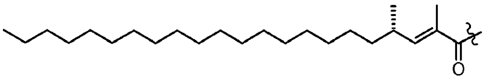
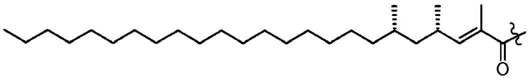
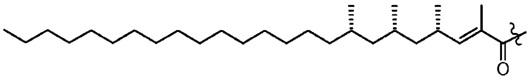
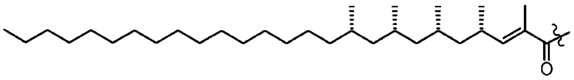
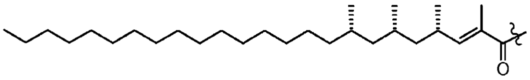
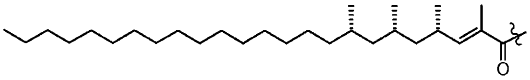
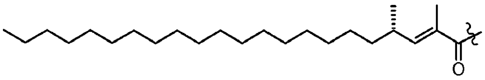
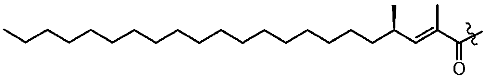
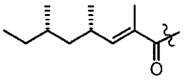
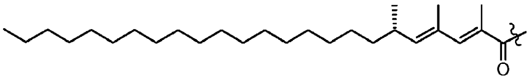
Preliminary testing of the antigenic properties of sulfoglycolipids **9–11**, which contain linear fatty acids with chain lengths from C_{16} to C_{30}, showed that they were unable to activate T cells specific for the acyl$_2$SGL complexes (data not shown). As the only difference between these compounds and the natural antigen is the nature of the 3-*O*-acyl group, these data emphasized the crucial role of the hydroxyphthioceranoyl group for CD1b presentation, T-cell-receptor (TCR) engagement, and T-lymphocyte activation. We therefore prepared polymethyl chiral fatty acids and incorporated them into the synthesis of the sulfoglycolipids. Hydroxyphthioceranoic and phthioceranoic acids belong to the deoxypolypropionate family of natural products. Numerous approaches to the stereoselective construction of the 1,3,5…-polymethyl pattern on the basis of enolate alkylation,[9–11] conjugate addition,[12–14] or other methods[15–17] have been reported. Recently, the first synthesis of phthioceranoic acid was completed by using an iterative catalytic asymmetric 1,4-conjugate addition of a Grignard reagent to an unsaturated thioester.[18] As the synthesis of these polymethyl fatty acids is linear and use of a cycle of reactions, the number of steps involved, the reaction times, and the number of chromatographic purification steps are important parameters for the efficiency of the synthetic route. For these reasons, we chose the asymmetric alkylation of hydrazone enolates derived from (*S*)-1-amino-2-methoxymethylpyrrolidine (SAMP) with iodides, a method developed by Enders and co-workers,[19,20] as the whole cycle of four reactions can be carried out in less than three days with only two chromatographic steps (Scheme 2).



Scheme 2. General synthesis of polymethylated fatty acids: a) $\mathbf{A}_{(n)}$ (1.0 equiv), PPh_3 (1.2 equiv), I_2 (1.3 equiv), imidazole (3.1 equiv), toluene, reflux, 1 h; b) **8** (2.0 equiv), LDA (2.0 equiv), THF, 0°C, 1 h, then $\mathbf{B}_{(n)}$ (1.0 equiv), −50→−25°C, overnight; c) 4 M HCl, petroleum ether, room temperature, 3 h; d) Jones reagent, acetone, 0°C, 30 min; e) (carbethoxyethylidene)triphenylphosphorane (1.2 equiv), CH_2Cl_2, room temperature, overnight, then 3 M KOH, H_2O/EtOH, reflux; f) BH_3·THF complex (1.7 equiv), THF, 0°C→RT, overnight. LDA = lithium diisopropylamide.

The starting material used was the long-chain alcohol $\mathbf{A}_{(1)}$, which was obtained in 98% yield after the reduction with BH_3·THF of (2*S*)-2-methyloctadecanoic acid $\mathbf{E}_{(1)}$[21] in THF. The treatment of alcohol $\mathbf{A}_{(1)}$ under conditions developed by Garegg and Samuelsson (PPh_3, imidazole, I_2 in toluene)[22] gave iodide $\mathbf{B}_{(1)}$ in 93% yield. This iodide was treated with the

Table 1: Synthetic analogues of **1**.

Product	R^1CO	R^2CO
1	palmitoyl/stearoyl	
9	palmitoyl	palmitoyl
10	palmitoyl	
11	palmitoyl	triacontanoyl
12	palmitoyl	
13	palmitoyl	
14	palmitoyl	
15	palmitoyl	
16	palmitoyl	
17	palmitoyl	
18	palmitoyl	
19	palmitoyl	
20	palmitoyl	
21	octanoyl	
22	tetracosanoyl	
23		palmitoyl
24	palmitoyl	
25	palmitoyl	
26	palmitoyl	

lithium enolate of SAMP propanal hydrazone (**8**)[23] in THF to give the 2*S* alkylated product $\mathbf{C}_{(2)}$ in good yield (72 %). Owing to the very low solubility of the long-chain iodides $\mathbf{B}_{(n)}$ at −78 °C in THF, alkylation reactions had to be performed at −50 °C with warming to −25 °C, and erosion of the optical purity of the products occurred to a small extent. The diastereomeric ratio for each alkylation was 94:6. The key aldehyde $\mathbf{D}_{(2)}$ was obtained almost quantitatively after acidic hydrolysis of hydrazone $\mathbf{C}_{(2)}$ in a petroleum ether/aqueous HCl biphasic mixture at room temperature. This aldehyde could then be oxidized quantitatively with the Jones reagent[24] to the saturated acid $\mathbf{E}_{(2)}$ or submitted to a Wittig reaction with (carbethoxyethylidene)triphenylphosphorane followed by saponification to give the unsaturated acid $\mathbf{F}_{(3)}$

in 80–85 % yield. Aldehyde $\mathbf{D}_{(2)}$ could also be reduced with the $BH_3{\cdot}THF$ complex in THF to alcohol $\mathbf{A}_{(2)}$ in quantitative yield and the whole cycle repeated with comparable overall yield for the introduction of additional methyl groups.

This procedure is short and efficient (only four steps and 60 % overall yield for each cycle) and can be scaled up easily. All polymethyl fatty acids prepared by this route are presented in Table 1 (third column). They were used for the synthesis of the corresponding sulfoglycolipids **13**–**26** according to the general procedure described herein.

Sulfoglycolipids **9**–**26** were tested for their ability to activate the $acyl_2SGL$-specific T cells by following the reported procedure.[2] Human dendritic cells that express the CD1b protein were incubated with varying amounts of the antigen, and the T-lymphocyte clone was added. The amount of IFN-γ released by the T cells was quantified, and dose-dependent curves were obtained (Figure 1 a). As also observed with the natural product, the maximum activity was usually observed for an antigen concentration of around 3 $\mu g\,mL^{-1}$. More importantly, it was found that the level of T-lymphocyte activation was highly dependent on the length, the position, and the structure of the fatty-acid residues connected to the trehalose core. When the hydroxyphthioceranoic acid moiety was replaced by linear fatty acids (products **9**–**11**, data not shown) or with saturated fatty acids containing one or two methyl groups (compounds **12** and **13**), no T-cell activation was observed (Figure 1 b). When this fatty-acid chain was substituted with additional methyl groups (compounds **14** and **15**), a steady increase in the immunogenicity of the sulfolipids was observed. A similar relationship between the immunogenicity and the number of methyl groups was observed when a chiral polymethylated α,β-unsaturated fatty acid was used in place of the hydroxyphthioceranoic acid moiety (see compounds **16**–**20**). However, with these unsaturated acids, there was no further improvement of the antigenic activity upon the introduction of a further methyl group once four methyl groups had been incorporated in the fatty-acid chain (compounds **19** and **20**).

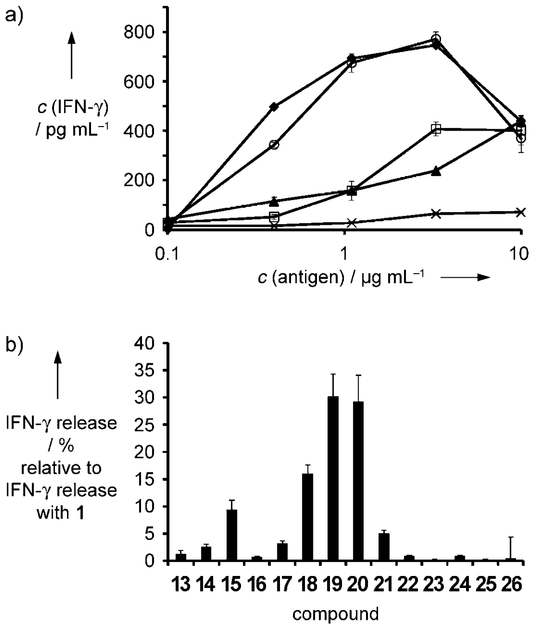


Figure 1. IFN-γ release by the T-cell clone. a) Response curves for compounds **14**, **15**, and **18**–**20**: × **14**, ▲ **15**, □ **18**, ○ **19**, ◆ **20**. b) Relative intensity of IFN-γ release for analogues **13**–**26** compared to that for **1**, for a fixed antigen concentration of 3.3 $\mu g\,mL^{-1}$.

Other structural variations on the SGLs gave more insight into the parameters which are crucial for the antigenic activity of these analogues. The length and the respective positions of the fatty acyl chains at the 2- and 3-positions of the trehalose unit were found to be very important. Shortening of the 2-*O*-acyl chain of the SGL from a palmitoyl (in **19**) to an octanoyl group gave compound **21**, the potency of which was greatly diminished with respect to that of **19**. An even more pronounced effect on the activity was observed when the 3-*O*-acyl group was shortened from a trimethyltetracosanoyl chain to a trimethyloctanoyl chain (compare the results for **18** and **25**). Lengthening of the 2-*O*-acyl chain from a palmitoyl (in **19**) to a tetracosanoyl group (in **22**) was also deleterious to the T-cell-stimulatory activity. The immunogenicity was also lost when the two acyl groups in **17** were permutated, as in **23**. Finally, the introduction of further unsaturation at the γ,δ-position of the 3-*O*-fatty acid (in **26**) and the use of a methylated fatty acid with an *R* stereocenter (in **24**) gave inactive products.

A general and convenient synthesis of 2,3-di-*O*-acyl-2′-*O*-sulfate trehalose derivatives has been developed. This route enables the preparation of numerous analogues of the natural *M. tuberculosis* antigen sulfoglycolipid **1**, with the incorporation of polymethylated chiral fatty acids in place of the complex hydroxyphthioceranoic acid moiety. Although none of the synthetic compounds were as potent as the natural sulfoglycolipid **1**, some of these sulfoglycolipids showed promising T-lymphocyte-activation properties. All active analogues have a saturated or monounsaturated polymethylated fatty acid with stereocenters of the *S* configuration at the 3-position of the trehalose core. These synthetic sulfoglycolipids are available in large amounts, and their protective effects against tuberculosis infection might be investigated further. The synthesis of analogues with other variations of the fatty-acid structure is underway in an attempt to gain a better understanding of the factors that influence their interaction with CD1 proteins[25,26] and their recognition by the TCR.

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Keywords: antigens · fatty acids · glycolipids · tuberculosis · vaccines

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[1] http://www.who.int/tb/en/.

[2] M. Gilleron, S. Stenger, Z. Mazorra, F. Wittke, S. Mariotti, G. Böhmer, J. Prandi, L. Mori, G. Puzo, G. De Libero, *J. Exp. Med.* **2004**, *199*, 649 – 659.

[3] M. B. Goren, O. Brokl, B. C. Das, E. Lederer, *Biochemistry* **1971**, *10*, 72 – 81.

[4] F. Lin, L. H. van Halbeek, C. R. Bertozzi, *Carbohydr. Res.* **2007**, *342*, 2014 – 2030.
[5] C. D. Leigh, C. R. Bertozzi, *J. Org. Chem.* **2008**, *73*, 1008 – 1017.
[6] H. H. Baer, X. Wu, *Carbohydr. Res.* **1993**, *238*, 215 – 230.
[7] A. Liav, M. B. Goren, *Carbohydr. Res.* **1984**, *127*, 211 – 216.
[8] H. H. Baer, B. Radatus, *Carbohydr. Res.* **1984**, *128*, 165 – 174.
[9] A. Abiko, S. Masamune, *Tetrahedron Lett.* **1996**, *37*, 1081 – 1084.
[10] A. G. Myers, B. H. Yang, H. Chen, L. McKinstry, D. J. Kopecky, J. L. Gleason, *J. Am. Chem. Soc.* **1997**, *119*, 6496 – 6511.
[11] M. O. Duffey, A. LeTiran, J. P. Morken, *J. Am. Chem. Soc.* **2003**, *125*, 1458 – 1459.
[12] D. R. Williams, A. L. Nold, R. J. Mullins, *J. Org. Chem.* **2004**, *69*, 5374 – 5382.
[13] R. Des Mazery, M. Pullez, F. Lopez, S. R. Harutyunyan, A. J. Minnaard, B. L. Feringa, *J. Am. Chem. Soc.* **2005**, *127*, 9966 – 9967.
[14] B. ter Horst, B. L. Feringa, A. J. Minnaard, *Chem. Commun.* **2007**, 489 – 491.
[15] T. Novak, Z. Tan, B. Liang, E.-i. Negishi, *J. Am. Chem. Soc.* **2005**, *127*, 2838 – 2839.
[16] E.-i. Negishi, Z. Tan, B. Liang, T. Novak, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 5782 – 5787.
[17] S. Hanessian, S. Giroux, V. Mascitti, *Synthesis* **2006**, 1057 – 1076.
[18] B. ter Horst, B. L. Feringa, A. J. Minnaard, *Org. Lett.* **2007**, *9*, 3013 – 3015.
[19] A. A. Birkbeck, D. Enders, *Tetrahedron Lett.* **1998**, *39*, 7823 – 7826.
[20] D. Enders, J. Tiebes, N. De Kimpe, M. Keppens, C. Stevens, G. Smagghe, O. Betz, *J. Org. Chem.* **1993**, *58*, 4881 – 4884.
[21] G. S. Besra, D. E. Minnikin, P. R. Wheeler, C. Ratledge, *Chem. Phys. Lipids* **1993**, *66*, 23 – 34.
[22] P. J. Garegg, B. Samuelsson, *J. Chem. Soc. Chem. Commun.* **1979**, 978 – 980.
[23] D. Enders, H. Eichenauer, *Tetrahedron Lett.* **1977**, *18*, 191 – 194.
[24] K. Bowden, I. M. Heilbron, E. R. H. Jones, B. C. L. Weedon, *J. Chem. Soc.* **1946**, 39 – 45.
[25] G. De Libero, L. Mori, *Trends Immunol.* **2006**, *27*, 485 – 492.
[26] A. de Jong, E. Casas Arce, T.-Y. Cheng, R. P. van Summeren, B. L. Feringa, V. Dudkin, D. Crich, I. Matsunaga, A. J. Minnaard, D. B. Moody, *Chem. Biol.* **2007**, *14*, 1232 – 1242.