Organic & Biomolecular Chemistry

Dynamic Article Links

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 7659

www.rsc.org/obc

COMMUNICATION

Impact of sugar stereochemistry on natural killer T cell stimulation by bacterial glycolipids†

Shenglou Deng,[a] Jochen Mattner,[b,c] Zhuo Zang,[a] Li Bai,[d] Luc Teyton,[e] Albert Bendelac[d] and Paul B. Savage*[a]

Received 28th July 2011, Accepted 30th August 2011
DOI: 10.1039/c1ob06276j

Natural killer T (NKT) cells recognize glycolipids produced by *Sphingomonas* bacteria, and these glycolipids contain C6-oxidized sugars, either glucuronic acid or galacturonic acid, linked to ceramides. Glycolipids with *gluco* stereochemistry are the most prevalent. Multiple studies have demonstrated that galactosylceramides are more potent stimulators of NKT cells than their glucose isomers. To determine if this stereoselectivity is retained in the context of the C6-oxidized sugars found in bacterial glycolipids, we prepared two sets of *gluco* and *galacto*-glycolipids oxidized at their C6 positions and compared their NKT stimulatory properties. In the context of carboxylic acid groups at C6, *gluco* stereochemistry gave the more potent responses. We also prepared bacterial glycolipids containing more complex ceramide groups to determine if these chains impact NKT cell responses.

Natural killer T (NKT) cells are a subset of T cells and play a key role in regulating immune responses.[1–3] This regulation impacts responses to infection, tumor growth, and autoimmune diseases. In addition, NKT cell stimulation has been shown to improve the efficacy of vaccines.[4] Consequently, there has been considerable interest in the means by which these cells can be stimulated to produce the cytokines that in turn control responses of other aspects of the immune systems in higher organisms. NKT cells are stimulated *via* presentation of glycolipids by CD1d, and a limited number of glycolipids and lipid polyols have been identified as antigens for NKT cells.[5] These include α-glycosylceramides,[6] α-glycuronosylceramides,[7,8] α-galactosyldiacylglycerols,[9] isoglobo-side iGb3,[10] and threitolceramides.[11]

The first identified NKT cell antigens were isolated from a marine sponge,[12] and while potent stimulators of NKT cells, these glycolipid are not considered "natural" antigens. Structure–activity studies of the sponge-derived glycolipids, in the context of anti-tumor properties, led to the generation of α-galactosylceramide KRN7000[13] (Fig. 1). This glycolipid has been the primary antigen used in elucidating antigen presentation by CD1d and NKT cell responses.

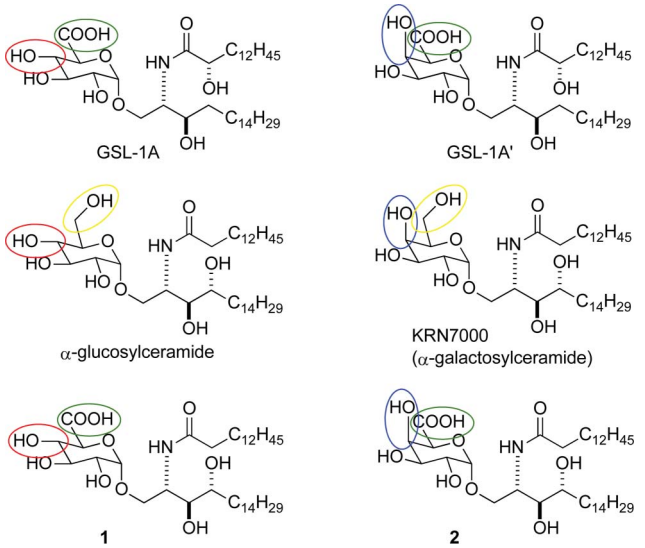


Fig. 1 Structures of glycolipids from *Sphingomonas* (GSL-1A and GSL-1A′), NKT cell agonists α-glucosylceramide, KRN7000 and glycolipids **1** and **2**.

The first natural, exogenous antigens for NKT cells were identified among the membrane components of the *Sphingomonadaceae* family of bacteria (including *Sphingomonas* spp.).[7,8] The bacteria in this family are Gram-negative (possess two lipid bilayers); however, their outer membrane is not made up of the lipopolysaccharide found in many Gram-negative bacteria (*e.g.*, *Escherichia coli*). In its place, bacteria in this family produce a group of glycuronosylceramides including GSL-1A and GSL-1A′ (Fig. 1).[14]

Sphingomonas bacteria are ubiquitous. They are found in water sources,[15] on household items,[16] and are one of the most prevalent airborne forms of life.[17] Consequently, humans are routinely exposed to these bacteria. There is evidence that these

[a]*Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT, 84602, USA*
[b]*Department of Immunobiology, Cincinnati Children's Hospital, Cincinnati, OH, 45229, USA*
[c]*Mikrobiologisches Institut-Klinische Mikrobiologie, Immunologie und Hygiene, Universitätsklinikum Erlangen and Friedrich-Alexander Universität Erlangen-Nürnberg, Wasserturmstr. 3/5, D-91054 Erlangen, Germany*
[d]*Howard Hughes Medical Institute, Committee on Immunology, Department of Pathology, University of Chicago, Chicago, IL, 60637, USA*
[e]*Department of Immunology, Scripps Research Institute, La Jolla, CA, 92037, USA*

† Electronic supplementary information (ESI) available: Experimental procedures for preparation of GSL-1B, GSL-1C, **1** and **2**. ^{1}H and ^{13}C NMR spectra of **1**, **2**, GSL-1B and GSL-1C. Methods of measuring cytokine production stimulated by CD1d presentation of glycolipids to NKT cells. See DOI: 10.1039/c1ob06276j

bacteria modulate immune responses[18] and that glycolipids from *Sphingomonas* can trigger allergic asthma.[19]

To better understand the NKT cell stimulatory properties of the GSLs produced by *Sphingomonas*, we[20] and Kronenberg *et al.*[21] reported the synthesis and NKT cell stimulatory activity of some of the glycolipids produced by this type of bacteria. Both studies showed that sugars appended on GSL-1A decrease activity significantly, and that GSL-1A and GSL-1A′ were less stimulatory than KRN7000. Kronenberg *et al.* also showed that GSL-1A is somewhat less stimulatory than GSL-1A′.

α-Galactosylceramide is a much more potent stimulator of NKT cells than α-glucosylceramide (Fig. 1).[6] Among *Sphingomonas* bacteria, GSL-1A, with *gluco* stereochemistry, is generally more predominant than GSL-1A′, which has *galacto* stereochemistry (Fig. 1).[14,22] If one of the major roles of NKT cells is surveillance for *Sphingomonas* and related bacteria, it would be expected that the more predominant glycolipid (GSL-1A (*gluco* stereochemistry)) would be a more potent stimulator than the less common isomer (GSL-1A′ (*galacto* stereochemistry)). Among the *Sphingomonas* glycolipids, C6 in the sugars is oxidized, and we questioned if oxidation at this position might alter recognition of *gluco vs*. *galacto* selectivity in NKT cell stimulation. To answer this question, we prepared GSL-1A, GSL-1A′ and glycolipids **1** and **2** (Fig. 1). KRN7000 and the *Sphingomonas* glycolipids differ in the ceramide portions of the molecules, and ceramide structure has been shown to play a role in NKT cell responses to glycolipids. Compounds **1** and **2** contain the glucoronic and galacturonic acids found in GSL-1A and GSL-1A′ and the ceramide found in KRN7000. These compounds proved useful in dissecting the impact of the acid groups in the sugars on NKT cell responses.

To determine the full impact of differences in the ceramide portions of glycolipids from *Sphingomonas* on NKT cell stimulation, we also prepared GSL-1B and GSL-1C (Fig. 2), in which the sphinganine chain is elongated and modified as compared to GSL-1A.[14] These two glycolipids are produced by bacteria but in lower amounts as compared to GSL-1A. The syntheses of GSL-1B and GSL-1C and NKT cell stimulatory activities of these two compounds have not been reported.

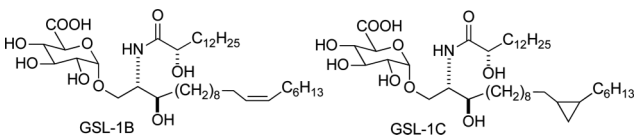
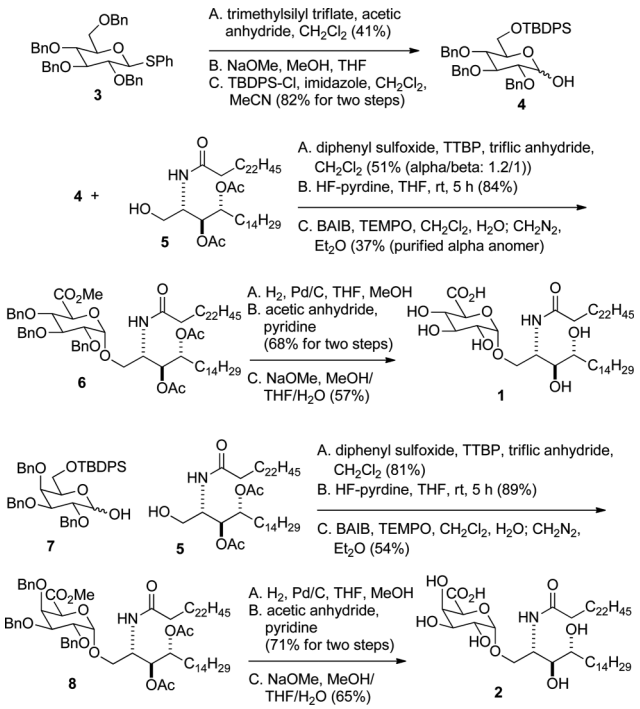


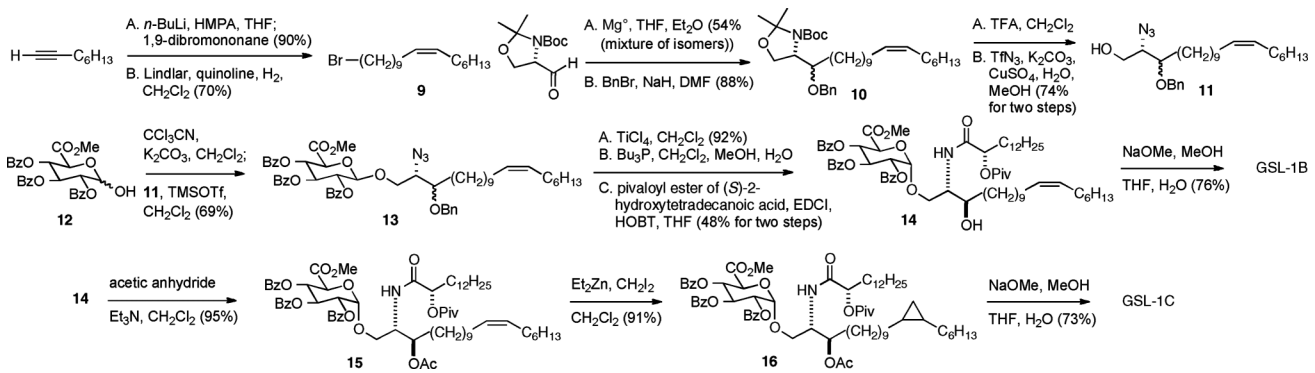
Fig. 2 Structures of GSL-1B and GSL-1C.

Multiple syntheses of GSL-1A and GSL-1A′ have been reported (for examples see ref. 19,20,22 and 23). α-Glycoside bond formation with glucuronic and galacturonic acid derivatives is typically complicated by low anomeric selectivities. Pilgrim and Murphy[23] reported an ingenious means of generating the α-anomer in high yields in the synthesis of GSL-1A and GSL-1A′. This group formed the corresponding β anomer in good yield, then used chelation-induced anomerization to give the α anomer in very high yield. Alternatively, GSL-1A can be formed in reasonable yield by oxidizing glucose to glucuronic acid after glycoside bond formation.[24] We used the latter approach for the glycolipids **1** and **2** (Scheme 1) and the former approach for synthesis of GSL-1B and GSL-1C (Scheme 2).



Scheme 1 Synthesis of glycolipids **1** and **2**.

Oxidation at C6″ after glycoside bond formation required preparation of C6-orthogonally protected glycoside donor **4** (Scheme 1). To access this compound, we used thioglycoside **3**



Scheme 2 Synthesis of GSL-1B and GSL-1C.

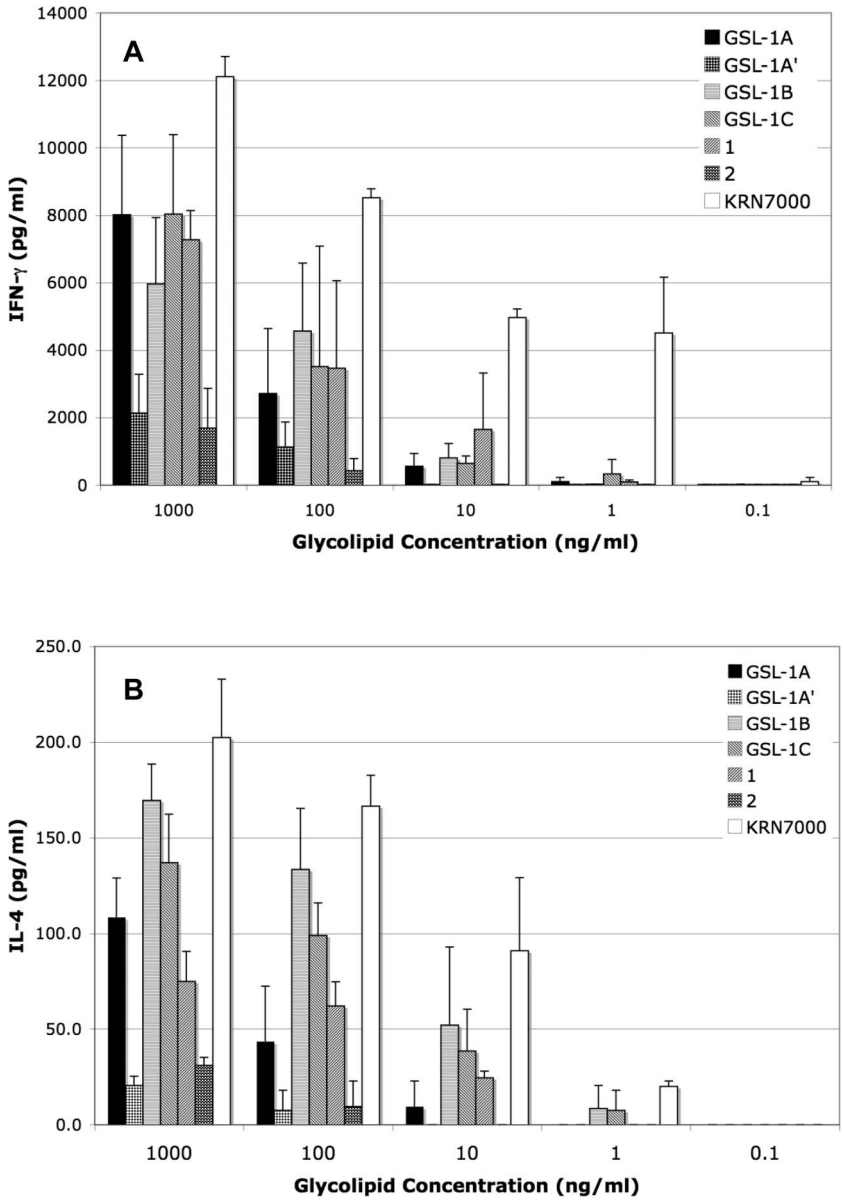


Fig. 3 Cytokine production by splenocytes in response to the indicated glycolipids.

and treated it with TMS-triflate in acetic anhydride to give the corresponding 1,6-diacetate. Though the yield of this reaction is moderate, the starting material is simple to prepare in large scale, and purification of the product is straightforward. Removal of the acetates and selective protection at C6″ gave **4** in good overall yield. Surprisingly, coupling of **4** with **5** gave the corresponding glycoside with little anomeric selectivity. The anomers were not separated but were carried forward through deprotection at C6″, oxidation and ester formation giving **6**. At this point, separation of the α and β anomers was easily accomplished. Reductive deprotection was followed by peracylation to provide a compound that was easily chromatographable. The final deprotection gave **1**. For the synthesis of **2** (Scheme 1), compound **7** was prepared as described for **4**. The remaining steps paralleled those used in the preparation of **2**.

Synthesis of the sphinganine for GSL-1B and GSL-1C began with 1-octyne (Scheme 2). Deprotonation and reaction with 1,9-dibromononane followed by reduction with Lindlar's catalyst gave **9**. Grignard formation and reaction with Garner's aldehyde gave the corresponding alcohol as a mixture of diastereomers that were difficult to separate. Consequently, the mixture was carried on through benzylation (giving **10**), liberation of the amine and alcohol at C1 and azide formation yielding **11**. Glycoside formation with **12** gave **13** in reasonable yield with good anomeric selectivity. Isomerization, giving the α anomer, azide reduction, and amide formation gave **14**, which was readily separated from the minor diastereomer. Treatment with sodium methoxide in the presence of a small amount of water gave GSL-1B. Preparation of GSL-1C required protection of the alcohol at C3 in **14** followed by cyclopropane formation using Simmons–Smith chemistry. In the identification of GSL-1C, the relative stereochemistry of the cyclopropyl group was not determined.[14] The diastereomers formed from cyclopropanation of **16** proved to be inseparable,

and in the NMR spectra of **16**, individual diastereomers were not observed. Deprotection of **16** gave GSL-1C.

Stimulation of NKT cells by glycolipids depends upon presentation by the protein CD1d on antigen presenting cells. Dendritic cells are the primary presenters of glycolipids to NKT cells,[1] and these and NKT cells are found in relatively high abundance in the spleen. Murine (B6 mice) splenocytes were used to measure NKT cell stimulation by the synthesized glycolipids. Stimulation is quantified in terms of cytokine release, and proinflammatory and immunomodulatory cytokines are typified by IFN-γ and IL-4, respectively.[20] The relative amounts of these cytokines released by NKT cells determine the types of immune responses that follow stimulation. Cytokine release was measured using quantitative ELISA.

As expected, stimulation of proinflammatory cytokine IFN-γ by KRN7000 occurred at low concentrations, and as has been observed previously, stimulation by glycolipids from *Sphingomonas* required higher concentrations (Fig. 3A). Nevertheless, substantial responses were observed at 100 ng mL^{-1}. Interestingly, the ceramide structure of the glycolipids had only a minor effect on NKT cell stimulation (compare GSL-1A, GSL-1B, GSL-1C, and **1**). The greatest impact on IFN-γ release was the sugar stereochemistry; compounds with *gluco* stereochemistry were more stimulatory than those with *galacto* stereochemistry (compare GSL1A with GSL1A′ and **1** with **2**).

The same trend was seen with IL-4 release (Fig. 3B); that is, among the *Sphingomonas* glycolipids, we found glycolipids with *gluco* stereochemistry to be more stimulatory than *galacto*. Glycolipids with shorter acyl chains than those found in KRN7000 are associated with higher amounts of IL-4 release, in relation to IFN-γ release.[25] Comparison of the impact of the ceramide in **1** and that found in GSL-1B and GSL-1C suggest that there may be a greater propensity for IL-4 production with the latter two glycolipids due to their relatively short acyl chains.

Few "natural" antigens for NKT cells have been identified, and an understanding of how these antigens impact human health requires elucidation of relative stimulatory properties of glycolipids found in sources of these antigens. We have demonstrated that the more predominant glycolipid found in *Sphingomonas* spp. (GSL-1A) stimulates stronger NKT cell responses than its isomer (GSL-1A′) and that the stereoselective recognition of these glycolipids is independent of ceramide structure. GSL-1B and GSL-1C are less predominant than GSL-1A, but both stimulate NKT cell responses comparable to GSL-1A. These results shed additional light on structural requirements for NKT cell stimulation and may prove useful in the development of novel NKT cell antigens and adjuvants.

Acknowledgements

Financial support from the National Institutes of Health (NIAID, P01 AI053725) is gratefully acknowledged. We thank Michael E. Fusakio for aiding in measuring cytokine release from splenocytes.

References

1 A. Bendelac, P. B. Savage, P. B. and L. Teyton, *Annual Rev. Immunol.*, 2007, **25**, 297–336.
2 A. Balato, D. Unutmaz and A. A. Gaspari, *J. Invest. Dermatol.*, 2009, **129**, 1628–1642.
3 M. Kronenberg and Y. Kinjo, *Curr. Opin. Immunol.*, 2009, **21**, 391–396.
4 C. Guillonneau, J. D. Mintern, F. X. Hubert, A. C. Hurt, G. S. Besra, S. Porcelli, I. G. Barr, P. C. Doherty, D. I. Godfrey and S. J. Turner, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 3330–3335.
5 P. B. Savage, A. Bendelac and L. Teyton, *Chem. Soc. Rev.*, 2006, **35**, 771–779.
6 T. Kawano, J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki and M. Tanguchi, *Science*, 1997, **278**, 1626–1629.
7 Y. Kinjo, D. Wu, G. Kim, G.-W. Xing, M. A. Poles, D. D. Ho, M. Tsuji, K. Kawahara, C.-H. Wong and M. Kronenberg, *Nature*, 2005, **434**, 520–525.
8 J. Mattner, K. L. DeBord, R. D. Goff, C. Cantu, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, K. Hoebe, O. Schneewind, N. Ismail, D. Walker, B. Buetler, L. Teyton, P. B. Savage and A. Bendelac, *Nature*, 2005, **434**, 525–529.
9 Y. Kinjo, E. Tupin, D. Wu, M. Fujio, R. Garcia-Navarro, M. R. Benhnia, D. M. Zajonc, G. Ben-Manachem, G. D. Ainge, G. F. Painter, A. Khurana, K. Hoebe, S. M. Behar, B. Buetler, I. A. Wilson, Tsuji, T. J. Sellati, C.-H. Wong and M. Kronenberg, *Nature Immunol.*, 2006, **7**, 978–986.
10 D. Zhou, J. Mattner, C. Cantu III, N. Yin, Y. Gao, Y. Sagiv, K Hudspeth, Y. Wu, S. Teneberg, Wang, R. Proia, S. B. Levery, P. B. Savage, L. Teyton and A. Bendelac, *Science*, 2004, **306**, 1786–1789.
11 J. D. Silk, M. Salio, B. G. Reddy, D. Shepherd, U. Gileadi, J. Brown, S. H. Masri, P. Pozella, G. Ritter, G. S. Besra, E. Y. Jones, R. R. Schmidt and V. Cerundolo, *J. Immunol.*, 2008, **180**, 6452–6456.
12 T. Natori, Y. Koezuka and T. Higa, *Tetrahedron Lett.*, 1993, **34**, 5591–5592.
13 M. Morita, K. Motoki, K. Akimoto, T. Natori, T. Sakai, E. Sawa, K. Yamaji, Y. Koezuka, E. Kobayashi and H. Fukushima, *J. Med. Chem.*, 1995, **38**, 2176–2187.
14 K. Kawahara, H. Kuraishi and U. Zähringer, *J. Industrial Microbiol. & Biotech.*, 1999, **23**, 408.
15 M. S. Giao, N. F. Azevedo, S. A. Wilks, M. J. Vieira and C. J. Keevil, *BMC Microbiol.*, 2011, **11**, 57.
16 S. T. Kelly, U. Theisen, L. T. Angenent, A. S. Amand and N. R. Pace, *Applied Environ. Microbiol.*, 2004, **70**, 4187–4192.
17 C. Fahlgren, G. Bratbak, R. A. Sandaa, R. Thyrhaug and U. L. Zweifel, *Aerobiologia*, 2011, **27**, 107–120.
18 J. Mattner, P. B. Savage, P. Leung, S. S. Oertelt, V. Wang, O. Trivedi, S. T. Scanlon, L. Teyton, J. Hart, W. M. Ridgway, L. S. Wicker, E. M. Gershwin and A. Bendelac, *Cell Host Microbe*, 2008, **3**, 304–315.
19 E. H. Meyer, S. Goya, O. Akbari, G. J. Berry, P. B. Savage, M. Kronenberg, T. Nakayama, R. H. DeKruyff and D. T. Umetsu, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 2782–2787.
20 X. Long, S. Deng, Z. Zang, J. Mattner, D. Zhou, N. McNary, R. D. Goff, L. Teyton, A. Bendelac and P. B. Savage, *Nature Chem. Biol.*, 2007, **3**, 559–564.
21 Y. Kinjo, B. Pei, S. Sufali, R. Raju, S. K. Richardson, M. Imamura, M. Fujio, D. Wu, A. Khurana, K. Kawahara, C.-H. Wong, A. R. Howell, P. H. Seeberger and M. Kronenberg, *Chem. Biol.*, 2008, **15**, 654–664.
22 T. Naka, N. Fujiwara, E. Yabuuchi, M. Doe, K. Kobayashi, Y. Kato and I. Yano, *J. Bacteriol.*, 2000, **182**, 2660–2663.
23 W. Pilgrim and P. V. Murphy, *Org. Lett.*, 2009, **11**, 939–942.
24 N. Okamoto, O. Kanie, Y.-Y. Huang, E. Fujii, H. Watanabe and M. Shimamura, *Chem. Biol.*, 2005, **12**, 677–683.
25 R. D. Goff, Y. Gao, J. Mattner, D. Zhou, N. Yin, C. Cantu III, L. Teyton, A. Bendelac and P. B. Savage, *J. Am. Chem. Soc.*, 2004, **126**, 13602–13603.

