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SYNTHESIS AND BIOLOGICAL EVALUATION OF A NEW CLASS OF VACCINE ADJUVANTS: AMINOALKYL GLUCOSAMINIDE 4-PHOSPHATES (AGPs)

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Abstract: A novel series of acylated ω-aminoalkyl 2-amino-2-deoxy-4-phosphono-β-D-glucopyranosides (aminoalkyl glucosaminide 4-phosphates) was synthesized and screened for immunostimulant activity. Several of these compounds enhance the production of tetanus toxoid-specific antibodies in mice and augment vaccine-induced cytotoxic T cells against EG.7-ova target cells.

Lipid A, the active principle of bacterial lipopolysaccharide (LPS, endotoxin), is a potent adjuvant for protein and carbohydrate antigens and induces both humoral (antibody) and cell-mediated (T-cell) responses in animal models.[1,2] However, the profound pyrogenicity and lethal toxicity of lipid A have precluded its use in human vaccines.[2] Currently, the only adjuvant licensed for human use in the USA is a group of aluminum salts known as alum. But alum is not without side effects and enhances humoral immunity only.[1] The recognition that cell-mediated responses—particularly the induction of cytotoxic T lymphocytes (CTLs)—are crucial for generating protective immunity against many viral diseases and cancer has stimulated considerable interest in the discovery of non-toxic derivatives of lipid A which amplify both antibody and T-cell responses.[1,2]

It has been shown that the toxic effects of *Salmonella minnesota* R595 lipid A (**1**) can be ameliorated by selective hydrolysis of the 1-phosphono and (*R*)-3-hydroxytetradecanoyl groups.[3] Known as monophosphoryl lipid A (MPL®) immunostimulant, *S. minnesota* lipid A modified in this way is an effective adjuvant in prophylactic and therapeutic vaccines.[4] However, due to the inherent heterogeneity of the cognate LPS and incomplete chemoselectivity in the hydrolytic steps, MPL® immunostimulant comprises several less highly acylated compounds in addition to the major, hexaacyl component **2**.[4]

Various subunit derivatives of bacterial lipid A have also been prepared with the aim of improving toxicity/bioactivity profiles.[5,6] However, synthetic analogs of the reducing and non-reducing glucosamine moieties of lipid A containing up to five fatty acid residues typically exhibit low biological activity, supporting the tenet that a β-1,6-diglucosamine unit bearing six fatty acids is prerequisite to maximal immunostimulant activity.[6]

In the course of our own structure–activity studies on lipid A, however, we have identified a new class of potent monosaccharide adjuvants containing six fatty acids and structurally related to MPL component **2**. Here we report the synthesis and biological activity of a series of hexaacyl ω-aminoalkyl 2-amino-2-deoxy-4-phosphono-β-D-glucopyranosides (aminoalkyl glucosaminide 4-phosphates, AGPs) possessing general structure **3**. Several of these compounds exhibit significant adjuvant activity in mice immunized with tetanus toxoid vaccine and augment vaccine-induced CTLs against EG.7-ova target cells.

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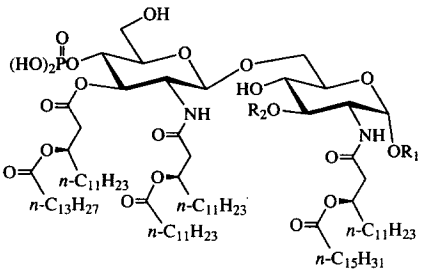
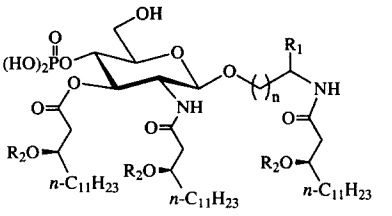


1 $R_1 = PO_3H_2$, R_2 = (*R*)-3-hydroxytetradecanoyl
2 $R_1 = R_2 = H$



3

We speculated that glycosidically linking the non-reducing (and structurally more conserved)[6] sugar moiety of lipid A to a conformationally flexible N-acylated aminoalkyl (aglycon) residue would permit assimilation and energetically favored close packing[7] of the six fatty acyl chains contained in MPL component **2**, as well as provide a structural motif more amenable to systematic structure–activity investigation. Conformational energy calculations of lipid A molecules and X-ray investigations in the solid state suggest that tight packing of six fatty acids in a hexagonal array may play an essential role in the bioactivity of lipid A-like molecules.[7]

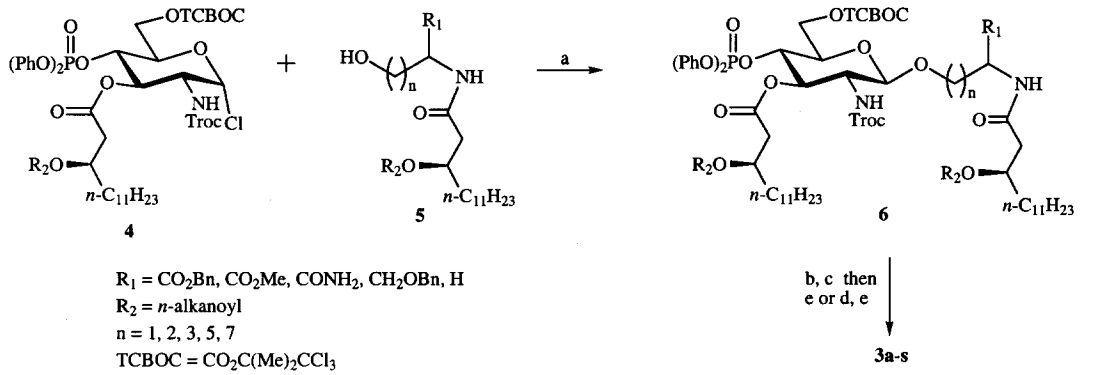
Since both the spacial arrangement and chain length of fatty acyl residues appear to be important determinants of immunostimulant activity in lipid A and other bacterial amphiphiles,[7,8] we prepared a series of AGP derivatives **3** in which the length of both the normal fatty acid chains R_2 and the aminoalkyl spacer unit was varied. We also examined modifications to the aglycon R_1 substituent, anticipating that physicochemical properties like solubility and aqueous stability would also be important to adjuvanticity. The known immunostimulating ability of naturally occurring *N*-(3-acyloxyacyl)amino acids such as serine-derived flavolipin[9] made the evaluation of seryl β-*O*-glycosides (**3**, $R_1 = CO_2H$, n = 1) of particular interest. Since the fatty acid dissimilitude displayed by MPL component **2** and certain lipid A molecules does not appear essential to bioactivity,[6,10] the AGPs were constructed with three identical (*R*)-3-*n*-alkanoyloxytetradecanoyl residues.

Chemistry

The AGPs shown in Table 1 were synthesized by a convergent method similar to what we employed in our recent synthesis of the major constituents (e.g., compound **2**) of MPL® immunostimulant and a related series of disaccharides.[10,11] Compounds **3a–s** were assembled from the glycosyl chlorides **4**[10] and glycosyl acceptors **5** using the *N*-2,2,2-trichloroethoxycarbonyl (Troc) method for stereoselective β-glycosylation (Scheme 1). The serine-based glycosyl acceptors (**5**, $R_1 = CO_2Bn$, CO_2Me, $CONH_2$; n = 1) were prepared by N-acylation of the corresponding serine ester or amide with the appropriate (*R*)-3-alkanoyloxytetradecanoic acid[12] in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDC·Mel) or 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ). The serinol glycosyl acceptors (**5**, $R_1 = CH_2OBn$, n = 1) were obtained by $NaBH_4$-H_2SO_4 (*in situ*-BH_3) reduction[13] of D- or L-serine *O*-benzyl ether and subsequent EDC-mediated N-acylation. The aminoalkanol acceptors (**5**, R_1 = H, $n \geq 1$) were prepared by the BOP/HOBt (benzotriazolo-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate/1-hydroxybenzotriazole) method. Koenigs–Knorr coupling of glycosyl chlorides **4** with **5** in 1,2-dichloroethane at room temperature in the presence of silver triflate gave the β-glycosides **6**. Reductive cleavage of the trichloroethyl-based protecting groups, N-acylation with the

requisite (*R*)-3-alkanoyloxytetradecanoic acid in the presence of EEDQ, and subsequent hydrogenolysis of the benzyl (compounds **3a–f**, **3k**, and **3l**) and phenyl protecting groups provided the desired AGPs **3a–s**, which were isolated and analyzed as triethylammonium salts after chromatographic purification on silica gel.

Scheme 1



Reagents and conditions: (a) AgOTf, 4 Å sieves, $ClCH_2CH_2Cl$; (b) Zn, AcOH, 60 °C; (c) (*R*)-3-*n*-alkanoyloxytetradecanoic acid, EEDQ, CH_2Cl_2; (d) H_2/Pd-C or $Pd(OH)_2$, AcOH, THF; (e) PtO_2, 4.9 kg/cm^2 H_2, AcOH, THF.

Biological Testing

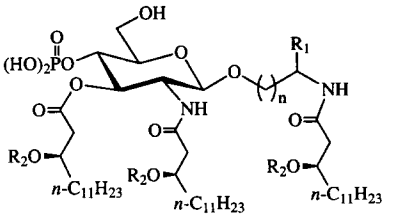
The AGPs **3a–s** were first compared with MPL® immunostimulant and compound **2** for their ability to induce nitric oxide synthase (iNOS) in murine macrophage and cytokine production in human peripheral monocytes (Table 1). The induction of iNOS in activated macrophage correlates directly with macrophage cytotoxicity presumably in part via the destructive action of the radical nitric oxide on microbial DNA and membranes.[14] The production of the proinflammatory cytokines TNF-α and IL-1β by activated monocytes is known to enhance normal host resistance to infection and induce a cascade of other mediators whose functions include T-cell activation and antibody synthesis.[15] Toxicity was evaluated as the minimal pyrogenic dose (MPD) in a standard three-rabbit USP pyrogen test.

As Shown in Table 1, compounds containing a carboxyl group in the aglycon unit (**3a–f**) are pyrogenic at a dose of 2.5 μg/kg in the rabbit pyrogen test irrespective of R_2, whereas **3g–s**, MPL®, and **2** are non-pyrogenic at this dose. The minimum pyrogenic dose (MPD) for **3c**, however, is at least ten times lower than that for **3e**, indicating a significant dependence of the fever response on R_2 for seryl compounds. Masking or removing the carboxyl group increases the MPD to 10 μg/kg or greater. Since it is known that removing the labile anomeric phosphate of lipid A or replacing it with a stable isostere reduces endotoxic effects,[4,16] these data suggest that the carboxyl group of serine-based AGPs is bioisosteric with the 1-phosphate moiety of lipid A.

iNOS induction shows a profound dependence on both fatty acid and aglycon chain length, reaching a maximum when R_2 is decanoyl in the seryl series of compounds (**3c**, **3i**, and **3n**), and when n = 2 in the aminoalkyl series (**3p**). The iNOS response is affected to a lesser extent by the nature of R_1 and the stereochemistry of the aglycon portion, but is abolished when R_2 is hexanoyl (**3a**, **3h**, and **3m**) and when n = 7 (**3s**). Compounds **3i** and **3n** both exhibit greater iNOS activity than MPL® and disaccharide **2** and concomitant low pyrogencity, indicating that the structural requirements for these two bioactivities may be different.

Table 1. Comparison of Immunostimulant Activities

3a–s



Compd[a]	R_1	R_2	n	Minimum Pyrogenic Dose[b] (μg/kg)	iNOS Induction[c] ED_{50} (ng/mL)	Relative *Ex Vivo* Cytokine Induction[d] TNF-α	IL-1β
3a	CO_2H (*S*)	*n*-$C_5H_{11}CO$	1	(≤2.5)[e]	>10,000	ND[f]	ND[f]
3b	CO_2H (*S*)	*n*-$C_7H_{15}CO$	1	(≤2.5)[e]	33	ND[f]	ND[f]
3c	CO_2H (*S*)	*n*-$C_9H_{19}CO$	1	(≤0.06)[e]	0.06	>6.0	23
3d	CO_2H (*S*)	*n*-$C_{11}H_{23}CO$	1	(≤2.5)[e]	0.9	3.6	2.0
3e	CO_2H (*S*)	*n*-$C_{13}H_{27}CO$	1	0.6	3	1.7	4.8
3f	CO_2H (*R*)	*n*-$C_9H_{19}CO$	1	(≤2.5)[e]	0.3	1.5	0.6
3g	CO_2Me (*S*)	*n*-$C_{13}H_{27}CO$	1	10	67	1.1	1.1
3h	$CONH_2$ (*S*)	*n*-$C_5H_{11}CO$	1	(>10)[g]	>10,000	ND[f]	ND[f]
3i	$CONH_2$ (*S*)	*n*-$C_9H_{19}CO$	1	10	0.3	3.5	6.2
3j	$CONH_2$ (*S*)	*n*-$C_{13}H_{27}CO$	1	10	20	1.1	0.9
3k	CH_2OH (*R*)	*n*-$C_{13}H_{27}CO$	1	10	9	3.5	4.9
3l	CH_2OH (*S*)	*n*-$C_{13}H_{27}CO$	1	(>10)[g]	150	ND[f]	ND[f]
3m	H	*n*-$C_5H_{11}CO$	1	10	>10,000	ND[f]	ND[f]
3n	H	*n*-$C_9H_{19}CO$	1	10	0.5	1.3	1.9
3o	H	*n*-$C_{13}H_{27}CO$	1	10	100	0.3	0.3
3p	H	*n*-$C_{13}H_{27}CO$	2	10	8	2.3	3.5
3q	H	*n*-$C_{13}H_{27}CO$	3	10	51	1.0	0.3
3r	H	*n*-$C_{13}H_{27}CO$	5	(>10)[g]	160	ND[f]	ND[f]
3s	H	*n*-$C_{13}H_{27}CO$	7	(>10)[g]	>10,000	ND[f]	ND[f]
2				10	14	1.2	0.7
MPL®				5	2	1.0	1.0

[a]Compounds **3a–s** gave satisfactory analytical and spectroscopic data. [b]Febrile responses were determined in a standard 3-rabbit USP pyrogen test using New Zealand White rabbits injected in the marginal ear vein with solutions of the test samples prepared initially at 100 μg/mL in 10% aq. EtOH and then diluted with D_5W to the desired concentrations just prior to injection. The minimum pyrogenic dose (MPD) was determined as the dose at which the total temperature rise for three rabbits was ≥1.2 °C. [c]ED_{50} values represent the concentration required for half-maximal response in an *in vitro* system using peritoneal exudate cells from *Propionibacterium acnes*-primed mice.[17] For details, see ref 10. [d]Human whole blood was stimulated with 10 μg/mL of the test sample and analyzed for cell-associated TNF-α and IL-1β by a sandwich ELISA.[18] For details, see ref 10. [e]Pyrogenic at this dose; MPD not determined. [f]ND = not detected; relative cytokine induction ≤0.05. [g]No significant febrile response at this dose; MPD not determined.

The induction of proinflammatory cytokines TNF-α and IL-1β generally parallels iNOS responses, showing both a striking chain length (R_2 and "n") and stereochemical dependence (cf. **3k** and **3l**). For the most part, compounds only moderately active or lacking activity in the iNOS model do not show any ability to elaborate cytokines in human monocytes. Similarly, the known[19] monosaccharide GLA-47 and *N*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-L-serine, corresponding to the sugar and aglycon portions of compound **3e**,

respectively, are devoid of immunostimulant activity in both these models (data not shown). Our results indicate that the AGP motif can constitute a potent, low-toxicity biomimetic of lipid A.

The ability of certain AGP derivatives to enhance antibody response to tetanus toxoid vaccines as well as augment vaccine-induced CTLs against EG.7-ova target cells (an ovalbumin gene-transfected cell line which expresses the ovalbumin CTL epitope)[20] was evaluated in two separate murine models (Table 2). Several of the AGPs induce higher titers of tetanus toxoid-specific antibodies and/or greater CTL responses to ovalbumin than compound **2** or MPL®, but the requirements for adjuvanticity appear to be less strict than those for other endotoxic activities. No significant pattern relating adjuvant activity and AGP structure is evident among compounds that are strong inducers of iNOS and proinflammatory cytokines. Compounds **3c** and **3i** exhibit the highest total Ig responses when used as adjuvants for tetanus toxoid vaccine, whereas short-chain derivative **3a** shows virtually no adjuvant activity in this model, consistent with its lack of potency in the iNOS and cytokine models. Compounds **3c**, **3l**, and **3p** produce more complement fixing IgG2a and IgG2b antibodies than MPL®, suggesting these AGPs (as well as **3o** and **3q**, which exhibited IgG2a production comparable to **3c** in a separate experiment—data not shown)[21] may participate in antibody-dependent cellular cytotoxicity, which is thought to be important for the clearance of infectious pathogens.[22] Several of the compounds eliciting high levels of the complement fixing subclasses in the tetanus toxoid model also induce much stronger CTL activity against EG.7-ova target cells than MPL®, reflecting their ability to potentiate T helper (Th)-1 type responses with different antigens. Given the role of TNF-α and IL-1 in inflammatory and fever responses,[23,24] the significant adjuvant activity of **3l** in both murine models despite its markedly weaker ability to induce iNOS, proinflammatory cytokines and the fever response *vis-à-vis* diastereomeric **3k** is particularly noteworthy.

Table 2. Comparison of Adjuvant Activities

Compd	R_1	R_2	n	Tetanus Toxoid Vaccine[a] Total Ig	IgG1	IgG2a	IgG2b	Ova CTL Response[b] % Cytotoxicity
3a	CO_2H (*S*)	*n*-$C_5H_{11}CO$	1	1.1	1.3	0.3	0.9	NT[c]
3c	CO_2H (*S*)	*n*-$C_9H_{19}CO$	1	18.9	4.2	6.8	2.8	58
3e	CO_2H (*S*)	*n*-$C_{13}H_{27}CO$	1	3.6	1.9	2.1	0.9	14
3i	$CONH_2$ (*S*)	*n*-$C_9H_{19}CO$	1	8.9	2.6	1.7	3.3	65
3k	CH_2OH (*R*)	*n*-$C_{13}H_{27}CO$	1	3.8	2.2	4.3	1.6	21
3l	CH_2OH (*S*)	*n*-$C_{13}H_{27}CO$	1	4.0	3.4	3.8	2.4	34
3n	H	*n*-$C_9H_{19}CO$	1	NT[c]	NT[c]	NT[c]	NT[c]	36
3o	H	*n*-$C_{13}H_{27}CO$	1	NT[c]	NT[c]	NT[c]	NT[c]	59
3p	H	*n*-$C_{13}H_{27}CO$	2	4.7	2.2	5.8	6.1	38
3q	H	*n*-$C_{13}H_{27}CO$	3	NT[c]	NT[c]	NT[c]	NT[c]	58
2				NT[c]	NT[c]	NT[c]	NT[c]	31
MPL®				5.7	2.5	3.4	1.6	37
Control				1.0	1.0	1.0	1.0	10

[a]C57BL/6 × DBA/2F_1 mice (8 per group) were immunized with 0.2 mL of 2.5% oil–water emulsions containing 50 μg of test articles and 0.2 μg of tetanus toxoid. A second immunization was administered 21 days post primary. Serum samples were collected 14 days after the second injection and evaluated by ELISA analysis; values given represent experimental test titers divided by oil–water Control titer. Control mice received oil–water vehicle containing tetanus toxoid only. For details, see ref 10. [b]C57BL/6(H-2_b) mice (6 per group) were immunized on day 0 and again on day 14 with 0.2 mL of 2.5% oil–water emulsions containing 25 μg of ovalbumin and the test articles. On day 21 spleens were tested for the presence of ovalbumin-specific CTL against ^{51}Cr-labeled E.G7-ova target cells.[20] Percent specific lysis (cytotoxicity) was then determined; effector/target ratio is 50:1. [c]NT = not tested.

In summary, we have demonstrated that substituting the reducing sugar of MPL structure **2** with various *N*-[(*R*)-3-*n*-alkanoyloxytetradecanoyl]aminoalkyl aglycon units produces a new class of potent monosaccharide adjuvants: aminoalkyl glucosaminide 4-phosphates **3**. Certain AGPs not only enhance humoral and cell-mediated responses in murine models but also exhibit low pyrogenicity in rabbits. Studies are currently underway to evaluate the adjuvant activity of these and related AGPs in infectious models.

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References and Notes

1. Rudbach, J. A.; Johnson, D. A.; Ulrich, J. T. In *Theory and Practical Application of Adjuvants*; Steward-Tul, D. E. S., Ed; Wiley: Chichester, UK, 1995; pp 287–313.
2. Johnson, A. G. *Clin. Microbiol. Rev.* **1994**, *7*, 277.
3. Myers, K. R.; Truchot, A. T.; Ward, J.; Hudson, Y.; Ulrich, J. T. In *Cellular and Molecular Aspects of Endotoxin Reactions*; Nowotny, A., Spitzer, J. J., Ziegler, E. J., Eds; Endotoxin Research Series, Vol. 1; Elsevier: Amsterdam, 1990; pp 145–156.
4. Ulrich, J. T.; Myers, K. R. In *Vaccine Design: The Subunit and Adjuvant Approach*, Powell, M. F., Newman, M. J., Eds.; Plenum: New York, 1995; pp 495–524.
5. Fukase, K.; Fukase, Y.; Oikawa, M.; Liu, W.-C.; Suda, Y.; Kusumoto, S. *Tetrahedron* **1998**, *54*, 4033 and references cited therein.
6. Quereshi, N.; Takayama, K. In *The Bacteria,* Iglewski, B. H., Clark, V. L., Eds.; Academic: New York, 1990; Vol. 11, pp 319–338.
7. Seydel, U.; Labischinski, H.; Kastowsky, M.; Brandenburg, K. *Immunobiol.* **1993**, *187,* 191.
8. Werner, G. H.; Jollès, P. *Eur. J. Biochem.* **1996**, *242*, 1.
9. Kawai, Y.; Akagawa, K. *Infect. Immun.* **1989**, *57,* 2086. For revised structure, see: Shiozaki, M.; Deguchi, N.; Mochizuki, T.; Wakabayashi, T.; Ishikawa, T.; Haruyama, H.; Kawai, Y.; Nishijima, M. *Tetrahedron* **1998**, *54*, 11861.
10. Johnson, D. A.; Keegan, D. K.; Sowell, C. G.; Livesay, M. T.; Johnson, C. L.; Taubner, L. M.; Harris, A.; Myers, K. R.; Thompson, J. D.; Rhodes, M. L.; Ulrich, J. T.; Ward, J. R.; Yorgensen, Y. M.; Cantrell, J. L.; Brookshire, V. G. *J. Med. Chem.*, submitted.
11. Johnson, D. A.; Sowell, C. G.; Keegan, D. S.; Livesay, M. T. *J. Carbohydr. Chem.* **1998**, *17*, 1421.
12. Keegan, D. S.; Hagen, S. R.; Johnson, D. A. *Tetrahedron: Asymmetry* **1996**, *7*, 3559.
13. Abiko, A.; Masamune, S. *Tetrahedron Lett.* **1992**, *33,* 5517.
14. Hibbs, J. B., Jr.; Taintor, R. R.; Vavrin, Z.; Rachlin, E. M. *Biochem. Biophys. Res. Commun.* **1988**, *157,* 87.
15. DeForge, L. E.; Kenney, J. S.; Jones, M. L.; Warren, J. S.; Remick, D. G. *J. Immunol.* **1992**, *148*, 2133.
16. Kusama, T.; Soga, T.; Ono, Y.; Kumzawa, E.; Shioya, E.; Nakayama, K.; Uoto, K.; Osada, Y. *Chem. Pharm. Bull.* **1991**, *39,* 3244.
17. Gustafson, G. L.; Rhodes, M. J.; Hegel, T. In *Bacterial Endotoxins: Lipopolysaccharides from Genes to Therapy*; Levin, J.; Alving, C. R.; Munford, R. S.; Redl, H., Eds; Progress in Clinical and Biological Research, Vol. 392; Wiley-Liss: New York, 1995; pp 567–579.
18. Nerad, J. L.; Griffiths, J. K.; Van der Meer, J. W. M.; Endres, S.; Poutsiaka, D. D.; Dinarello, C. A.; Cannon, J. G. *J. Leukocyte Biol.* **1992**, *52,* 687.
19. Kiso, M.; Tanaka, S.; Fujita, M.; Fujishima, Y.; Ogawa, Y.; Ishida, H.; Hasegawa, A. *Carbohydr. Res.* **1987**, *162*, 127.
20. Moore, M. W.; Carbone, F. R.; Bevan, M. J. *Cell* **1988**, *55*, 777.
21. In this second experiment, compound **2** and MPL® induced comparable tetanus toxoid-specific antibody responses, while compound **3c** elicited IgG antibody titers (of all isotypes) two or more times greater than both **2** and MPL®.
22. Snapper, C. M.; Finkelman, F. D. In *Fundamental Immunology,* 4th ed.; Paul, W. E., Ed.; Lippincott-Raven: Philadelphia, 1999; pp 831–861.
23. Beutler, B; Cerami, A. *Ann. Rev. Immunol.* **1989**, *7*, 625.
24. Durum, S. K.; Schmidt, J. A.; Oppenheim, J. J. *Ann. Rev. Immunol.* **1985**, *3*, 263.