(Hatano, S., Ishikawa, H., & Sato, H., Eds.) pp 225-237, University of Tokyo Press, Tokyo.
Eisenberg, E., & Kielley, W. W. (1974) *J. Biol. Chem. 249*, 4742-4748.
Fabiato, A., & Fabiato, F. (1979) *J. Physiol. (Paris) 75*, 479-494.
Fairbanks, G. T., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606-2617.
Frearson, N., & Perry, S. V. (1975) *Biochem. J. 151*, 99-107.
Gerthoffer, W. T., & Murphy, R. A. (1983) *Am. J. Physiol. 245*, C271-C277.
Gorecka, A., Aksoy, M. O., & Hartshorne, D. J. (1976) *Biochem. Biophys. Res. Commun. 71*, 325-331.
Gratecos, D., & Fischer, E. H. (1974) *Biochem. Biophys. Res. Commun. 58*, 960.
Hartshorne, D. J., & Mrwa, U. (1982) *Blood Vessels 19*, 1-18.
Hoar, P. E., Kerrick, W. G. L., & Cassidy, P. S. (1979) *Science (Washington, D.C.) 204*, 503-506.
Ikebe, M., Aiba, T., Onishi, H., & Watanabe, S. (1978) *J. Biochem. (Tokyo) 83*, 1643-1655.
Ikebe, M., Ogihara, S., & Tonomura, Y. (1982) *J. Biochem. (Tokyo) 91*, 1809-1812.
Kendrick-Jones, J., Lehman, W., & Szent-Gyorgyi, A. G. (1970) *J. Mol. Biol. 54*, 313-326.
Lehrer, S. S., & Morris, E. P. (1984) *J. Biol. Chem. 259*, 2070-2072.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Marchalonis, J. J. (1969) *Biochem. J. 113*, 299-305.
Martin, J. B., & Doty, D. M. (1949) *Anal. Chem. 21*, 965-967.
Mikawa, T., Nonomura, Y., Hirata, M., Ebashi, S., & Kakuchi, S. (1978) *J. Biochem. (Tokyo) 84*, 1633-1636.
Nag, S., & Seidel, J. C. (1983) *J. Biol. Chem. 258*, 6444-6449.
Nag, S., Nath, N., Carlos, A., & Seidel, J. C. (1983) *Biophys. J. 45*, 44a.
Perrie, W. T., Smillie, L. B., & Perry, S. V. (1973) *Biochem. J. 135*, 151-164.
Persechini, A., & Hartshorne, D. J. (1981) *Science (Washington, D.C.) 213*, 1383-1385.
Rees, D. D., & Frederiksen, D. W. (1981) *J. Biol. Chem. 256*, 357-364.
Sanders, C., & Smillie, L. (1984) *Can. J. Biochem. Cell Biol. 62*, 443-448.
Sellers, J. R., Chuck, P. B., & Adelstein, R. S. (1983) *J. Biol. Chem. 258*, 14181-14188.
Sherry, J. M. F., Gorecka, A., Aksoy, M. O., Dabrowska, R., & Hartshorne, D. J. (1978) *Biochemistry 17*, 4417-4418.
Sobieszek, A. (1977) in *Biochemistry of Smooth Muscle* (Stephens, N. L., Ed.) pp 413-443, University Park Press, Baltimore.
Sobieszek, A., & Small, J. V. (1977) *J. Mol. Biol. 112*, 559-576.
Somlyo, A. P., Somlyo, A. V., Shuman, H., & Endo, M. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 2883-2890.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866-4871.
Williams, D. L., Jr., Green, L. E., & Eisenberg, E. (1984) *Biochemistry 23*, 4150-4155.
Yamaguchi, M., Ver, A., Carlos, A., & Seidel, J. C. (1984) *Biochemistry 23*, 774-779.
Yang, Y. Z., Korn, E. D., & Eisenberg, E. L. (1979) *J. Biol. Chem. 254*, 2084-2088.

Chemical Synthesis and Immunological Activities of Glycolipids Structurally Related to Lipid A†

Daniel Charon,‡ Richard Chaby,* Agnès Malinvaud, Michelle Mondange,‡ and Ladislas Szabó

Equipe de Recherche No. 55 du Centre National de la Recherche Scientifique, Institut de Biochimie, Université de Paris Sud, 91405 Orsay, France

Received July 25, 1984

ABSTRACT: Complete chemical syntheses of a number of monosaccharides derived from 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-D-glucopyranose and structurally related to the hydrophobic moiety (lipid A) of several bacterial endotoxins are described. Selected humoral (complement activation) and cellular (mitogenicity and induction of interleukin 1 production) in vitro activities of a lipid A preparation obtained from the *Bordetella pertussis* endotoxin were compared with those of ten of these monosaccharides and with those of previously synthesized, analogous disaccharides. Results show that each of these in vitro activities of the lipid A preparation can be efficiently induced by at least one of the monosaccharide derivatives.

The isolated lipidic moiety (lipid A) of endotoxins obtained from Gram-negative bacteria expresses a great number of the in vivo and in vitro biological activities of the intact molecule (Galanos et al., 1977). Because of significant species variations, intrinsic microheterogeneity, occlusion of other membrane components, and unavailability of suitable techniques of purification, the exact structure of the natural lipid region is unknown. However, chemical analyses (Rosner et al., 1979; Wollenweber et al., 1982) revealed that this material contains a β-1,6-linked D-glucosamine disaccharide substituted by phosphate groups and by ester- and amide-bound fatty acids. In some lipid A preparations ethanolamine (Mühlradt et al.,

† This work was supported by the Institut National de la Santé et de la Recherche Médicale and the Fondation pour la Recherche Médicale Française.

‡ Present address: Equipe No. 55 du C.N.R.S., Centre d'Etudes Pharmaceutiques, Université de Paris Sud, 92290 Châtenay-Malabry, France.

0006-2960/85/0424-2736$01.50/0

Table I: Nomenclature of the Synthetic Glycolipids Used in Immunological Tests

	substituents[a]						
	R_1	R_2	R_3	R_4	R_4'	R_6	denomination
monosaccharides	H	H	H	H		H	M1
	Myr	Myr	Myr	Myr		Myr	M2
	Bzl	H	Myr-OH	H		H	M3
	H	H	Myr-OH	H		H	M4
	Pr	Myr	H	PO_3^{2-}		H	M5
	Pr	Myr	Myr-OMyr	PO_3^{2-}		H	M6
	Pr	Myr	H	PO_3^{2-}		Myr	M7
	Pr	Ac	H	PO_3^{2-}		Myr	M8
	Pr	H	H	PO_3^{2-}		Myr	M9
	Pr	Myr-OH	H	PO_3^{2-}		Myr	M10
disaccharides	Bzl	Ac	H	H	H	H	D1
	H	H	H	H	PO_3^{2-}	H	D2

[a] Abbreviations: Ac = acetyl; Bzl = benzyl; Myr = tetradecanoyl; Myr-OH = (3*R*)-3-hydroxytetradecanoyl; Myr-OMyr = (3*R*)-3-(tetradecanoyloxy)tetradecanoyl; Pr = propyl.

1977), 4-amino-L-arabinose (Volk et al., 1970), D-glucosamine (Hase & Rietschel, 1977), or D-arabinose (Tharanathan et al., 1978) has also been detected.

On the basis of these studies, glycolipids derived from glucosamine disaccharides have been synthesized by several groups of investigators (Inage et al., 1981; Van Boeckel et al., 1983; Charon & Szabó, 1983) and examined for their biological activities (Kotani et al., 1983; Kumuzawa et al., 1984; Matsuura et al., 1984; Yasuda et al., 1984; Galanos et al., 1984). "Endotoxic" activities are, however, not restricted to disaccharide derivatives of glucosamine and have been observed for glycolipids derived from monosaccharides. Thus, Behling et al. (1976) found that *N*-palmitoyl-D-glucosamine was active in B-cell mitogenesis and that *N*-lauroyl-D-glucosamine exhibited a strong adjuvant effect. More recently, mitogenicity and complement activation were observed by Kotani et al. (1983) with *N*-[(3*R*)-3-(tetradecanoyloxy)tetradecanoyl]-D-glucosamine, and Matsuura et al. (1984) reported that the same compound carrying 3-*O*-tetradecanoyl and 4-*O*-phosphoryl groups was very effective both in the *Limulus* amoebocyte lysate gelation test and for induction of interferon and tumor necrosis factor activities. It also appears from recent papers (Vogel et al., 1984) that incomplete lipid A structures of natural origin can express some of the activities of the complete molecule. Thus, a diacylated monosaccharide (lipid X) that accumulates in phosphatidylglycerol-deficient mutants of *Escherichia coli* was found to induce B-lymphocyte proliferation and maturation (Raetz et al., 1983).

In this report, the chemical syntheses of a series of glucosamine monosaccharide derivatives are described; some of their in vitro immunological activities were examined and compared to those of synthetic disaccharide structures and to those of a lipid A preparation isolated from *Bordetella pertussis*.

Materials and Methods

Chemical Syntheses

Compounds used for immunological studies were prepared as summarized in Scheme I. A detailed description of the synthetic work is available as supplementary material (see paragraph at end of paper regarding supplementary material).

Immunological Methods

Materials Used for Immunological Tests. The endotoxin extracted (Le Dur et al., 1980) from *Bordetella pertussis* was incubated (4 mg/mL) with acetic acid (11 mM, pH 3.4) at 100 °C for 1 h. The precipitated crude glycolipid recovered by centrifugation (16000*g*, 30 min) was lyophilized. Lipid A-1 was extracted from this material with a mixture of toluene/methanol (15/10 v/v) containing ammonium acetate (7.7 mg/mL). The soluble material was dried under vacuum, suspended in water, and lyophilized to remove ammonium acetate. The lipid A-1 preparation although devoid of detectable amounts of 3-deoxy-2-octulosonate (thiobarbiturate assay) contained 1.4% w/w of neutral sugars; all of the fatty acids previously identified (Le Dur et al., 1980) in the intact endotoxin [gas–liquid chromatography/mass spectroscopy (GLC/MS) analysis] have been found to be present in the preparation.

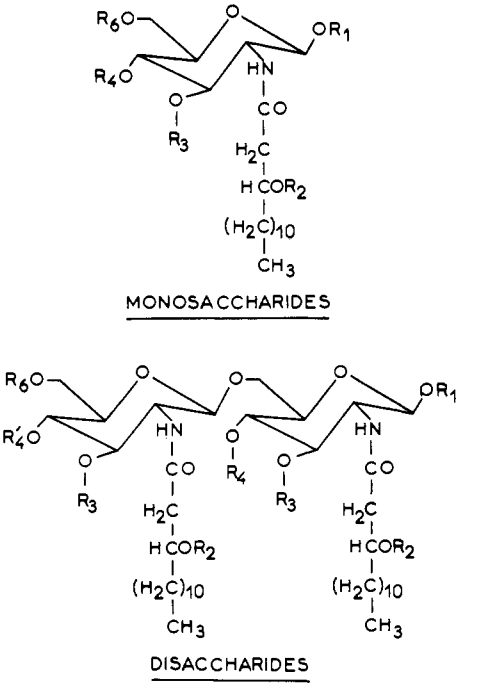
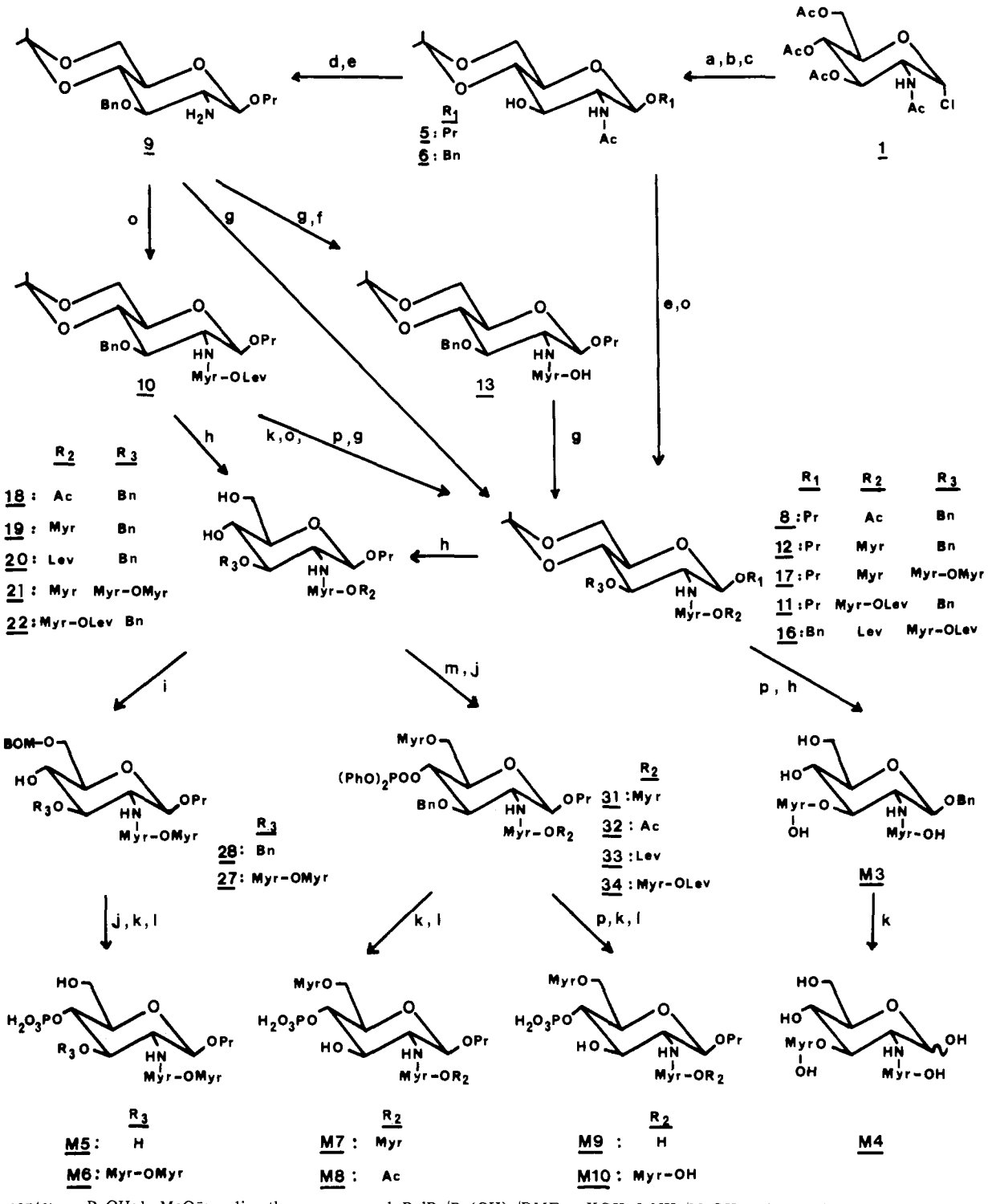


FIGURE 1: Compounds used in immunological tests; their nomenclature is given in Table I.

The structures and denominations of compounds used in immunological assays are given in Figure 1 and Table I. All compounds tested were complexed with bovine serum albumin (BSA): compounds (1 mg) were sonicated for 1 min at 18 °C in a mixture of 0.15 M NaCl (20 μL) and triethylamine (5 μL). Solutions (1 mL) of BSA (0.5 mg/mL) in 0.15 M NaCl were then added, and the mixtures were dried under vacuum. The products, redissolved in water, were dried again to achieve complete removal of triethylamine. The complexes were then sterilized by exposure to ultraviolet light, sonicated for 2 min in apyrogenic water (1 mL), and stored at −30 °C until used.

Cell Preparations. BALB/c (IFFA-CREDO, Saint Germain sur l'Arbresle, France) and C3H/HeJ (CSEAL, Orléans,

Scheme I[a]



[a] Reagents: a, R_1OH; b, MeO^-; c, dimethoxypropane; d, BzlBr/$Ba(OH)_2$/DMF; e, KOH; f, NH_3/MeOH; g, fatty acid anhydride; h, trifluoroacetic acid; i, $BzlOCH_2Br$; j, $(PhO)_2POCl$; k, H_2/Pd; l, H_2Pt; m, myristoyl chloride; n, HO(Myr-O-Myr-OLev)/DCC; o, HO(Myr-OLev)/DCC/DMAP; p, H_2NNH_2. Abbreviations: Ac, acetyl; Bn, benzyl; BOM, benzyloxymethyl; DCC, dicyclohexylcarbodiimide; DMAP, *p*-(dimethylamino)pyridine; DMF, dimethylformamide; Lev, levulinoyl (4-oxopentanoyl); Myr, tetradecanoyl; Myr-OH, (3*R*)-3-hydroxytetradecanoyl.

France) male mice, 5–6 weeks old, were killed by cervical dislocation, and their spleens and thymuses were removed aseptically. Spleen cell and thymocyte suspensions were prepared by teasing the tissue with toothed forceps. Viability of cells was determined by eosin (0.1%) exclusion. Cell clumps and debris were removed by gravity sedimentation for 5 min. Rabbit monocytes were obtained from New Zealand rabbits, 3–4 months old (CNRZ, Jouy en Josas, France). Rabbit blood (25 mL), obtained by cardiac puncture and directly collected on heparin, was adjusted to 50 mL with minimal essential medium (MEM). Fractions of the diluted blood (25 mL) were layered on a Percoll solution (15 mL, density 1.09 g/mL) and centrifuged at 500*g* for 30 min at 15 °C. The cells present in the interface layer were depleted of platelets by centrifugation (500*g*, 15 min) over a Percoll solution of lower density (1.06 g/mL). The cells obtained in the pellet were washed in MEM (500*g*, 10 min) and resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum. The number

of monocytes was estimated by nonspecific esterase (NSE) staining (Tucker et al., 1977) (Technicon Instrument Corp., Tarrytown, NY). Monocytes were allowed to attach to plastic by incubation of the cell suspension (100 μL, 10^6 NSE^+ cells/mL) for 1 h at 37 °C in tissue culture plates (Nunclon delta, 96 wells, Kamstrup, Denmark). Monocyte-enriched cells were obtained after removal of nonadherent cells by four successive washings with MEM (150 μL per well).

Assay for Mitogenic Activity. The culture medium was RPMI-1640 supplemented with L-glutamine (2 mM), streptomycin (100 μg/mL), penicillin (100 IU/mL), and 2-mercaptoethanol (5×10^{-5} M). A suspension of spleen cells from BALB/c mice (0.1 mL, 5×10^5 cells) was placed in wells of flat-bottom tissue culture plates. Quadruplicate solutions of compounds complexed with BSA (0.1 mL), diluted with medium to appropriate concentrations, were added to the wells. The plates were incubated at 37 °C in an atmosphere of 5% CO_2 in air for 48 h. [^{3}H]Thymidine (0.5 μCi, 25 Ci/mmol) was added to the wells for 18 h before harvesting.

Assay for Interleukin 1 (IL-1) Secretion. The plastic-adherent monolayers of monocyte-enriched cells (10^5 cells/well in Nunclon delta plates, 96 wells) were cultured (5% CO_2 in air, 37 °C) in RPMI-1640 (100 μL), with or without the inducer (50 μg/mL). After 24 h, IL-1 activities of the culture supernatants were assayed by measuring the enhancement of the mitogenic response of thymocytes from C3H/HeJ mice to concanavalin A (Con A). Thymocytes from C3H/HeJ mice (10^6 cells/well) were incubated for 65 h at 37 °C in RPMI-1640 supplemented with L-glutamine and antibiotics (200 μL) and containing the culture supernatant to be tested (25 μL), Con A (0.1 μg), fetal calf serum (10 μL), and 2-mercaptoethanol (2×10^{-5} M). [^{3}H]Thymidine (0.5 μCi, 25 Ci/mmol) was added 7 h before harvesting. DNA synthesis was measured by the mean counts per minute (cpm) incorporated in six replicate microcultures.

Activation of the Complement System. VBS^{2+} and EGTA buffers were used. VBS^{2+} is an isotonic veronal–NaCl buffer (VBS) containing 0.15 mM Ca^{2+} and 0.88 mM Mg^{2+}. EGTA buffer is VBS containing ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (10 mM) and $MgCl_2$ (7 mM). Guinea pig complement was from the Institut Mérieux (Lyon, France). Human complement was obtained from healthy donors. Sera were prepared by keeping blood for 1 h at room temperature, separating the serum, and storing it in aliquots at −30 °C. Sensitized sheep erythrocytes (EA) were obtained by incubation of the cells (15 min, 4 °C) with rabbit anti-sheep erythrocyte serum sufficient to give optimal hemolysis with guinea pig complement. Compounds to be tested were complexed with BSA (50% by weight) and suspended in physiological saline.

Activation of the complement system was measured after incubation (60 min, 37 °C) of 20-μL portions of diluted (1:5 in 0.15 M NaCl) guinea pig serum with test materials (5 μL, 1 mg/mL). The reaction mixtures were diluted (1:2 in VBS^{2+}). Portions (25 μL) of the diluted mixtures were adjusted to 2 mL with VBS^{2+} containing 10^8 sensitized sheep erythrocytes and incubated at 37 °C for 60 min with frequent shaking.

Activation of the alternative pathway of the complement was measured after incubation (90 min, 37 °C) of 40-μL portions of diluted (1:2 in EGTA buffer) human serum with test materials (10 μL, 1 mg/mL); the reaction mixtures were then incubated (30 min, 37 °C) with rabbit erythrocytes (50 μL, 1.4×10^8 cells) with frequent shaking. In both assays of complement activation, after hemolysis with complement, the erythrocyte suspensions were centrifuged (5 min, 1000*g*), and the optical densities of the supernatants, measured at 415 nm, were compared to those of the calibration curve obtained with various amounts of complement alone. The assays were carried out in triplicate with each compound to be tested.

Results

Chemical Syntheses. Monosaccharide M1 was obtained by treatment of a supersaturated solution of glucosamine with 1 molar equiv of the mixed anhydride prepared from (3*R*)-3-hydroxytetradecanoic acid and ethyl chloroformate in tetrahydrofuran (THF) (Alberston, 1962); a yield of 50% was routinely obtained. Treatment of M1 with tetradecanoic anhydride (Inouye et al., 1956) then gave the per-O-acylated derivative M2. Syntheses of the disaccharides D1 and D2 have been described previously (Charon & Szabó, 1983). All other compounds were synthesized as shown in Scheme I.

Derivatives of propyl β-D-glucosaminide were obtained by condensation of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (Horton, 1972) with 1-propanol under Helferich conditions. De-O-acylation was accomplished by treatment with sodium methoxide and de-N-acylation with 4 N KOH in ethanol. Free amino groups were quantitatively acylated by treatment with the required fatty acid anhydride, using 1.3 equiv of the reagent. Isopropylidene groups were introduced by condensation with 2,2-dimethoxypropane and removed under mild conditions with 0.1 N trifluoroacetic acid in a mixture of $CHCl_3$/MeOH/H_2O. The primary hydroxyl group was either selectively acylated with myristoyl chloride (1.2 equiv) at 4 °C or temporarily blocked by a benzyloxymethyl group.

Phosphorylation, at position 4, was performed with diphenyl phosphorochloridate in THF at 60 °C, with imidazole as the acid acceptor. Deprotection was accomplished by hydrogenolysis catalyzed first by Pd on charcoal to remove benzyl and benzyloxymethyl groups and then by Pt to remove phenyl groups. The phosphate derivatives obtained were isolated either as free acids or as their ammonium salts. They were purified by column chromatography on silica gel, followed by gel filtration on LH20, if necessary.

The two disaccharides (D1, D2) and the ten monosaccharides (M1–M10) represented on Figure 1 and Table I were complexed with bovine serum albumin (BSA) when used in immunological tests.

Mitogenic Activities. The glycolipids synthesized were presented to spleen cells of BALB/c mice as complexes with BSA, and the mitogenic effects were compared to that induced by lipid A-1 complexed with BSA by the same procedure. A preliminary experiment indicated that the response induced by lipid A-1/BSA complex reached its peak level at the dose of 12.5 μg/mL. Results in Table II show that higher concentrations of synthetic compounds were required for the induction of an optimal response. However, when the highest responses of the various compounds were examined, some of the synthetic monosaccharides appeared significantly mitogenic (M2, M5, M9, M10) whereas others did not (M4). The maximum thymidine uptake induced by compound M5 was even higher than that obtained with lipid A-1. On the other hand, the two disaccharides synthesized induced a significant inhibition of DNA synthesis. When tested on the spleen cells of the lipopolysaccharide-nonresponsive C3H/HeJ mouse strain, the synthetic compounds all induced a thymidine incorporation lower than that observed when the cells were cultured with BSA alone (results not shown).

Induction of Interleukin 1 (IL-1) Secretion. The culture supernatants of rabbit monocytes (10^5 NSE^+ cells), incubated

Table II: Stimulation of [^{3}H]Thymidine Incorporation into Splenic Cells[a] from BALB/c Mice by Synthetic Glycolipids (Mean cpm ± SD)

compd[b]	concn (μg/mL) 12.5	25	50
BSA	1000 ± 100	1200 ± 180	1300 ± 210
lipid A-1	6400 ± 730	2100 ± 520	840 ± 80
M1	2600 ± 460	3400 ± 390	2800 ± 340
M2	1700 ± 270	2900 ± 510	5800 ± 810
M3	1200 ± 150	1700 ± 90	2700 ± 450
M4	1700 ± 640	1600 ± 690	1400 ± 340
M5	2200 ± 230	4000 ± 710	6800 ± 580
M6	1000 ± 130	1900 ± 330	3100 ± 180
M7	1000 ± 90	1500 ± 360	2300 ± 690
M8	2300 ± 320	3000 ± 630	3100 ± 530
M9	2500 ± 120	3300 ± 1070	4400 ± 590
M10	2200 ± 700	3900 ± 1040	3200 ± 1230
D1	480 ± 20	160 ± 40	80 ± 20
D2	200 ± 20	70 ± 40	100 ± 20

[a] 5×10^5 cells per well. [b] Compounds were complexed with BSA (50% by weight) as described under Materials and Methods.

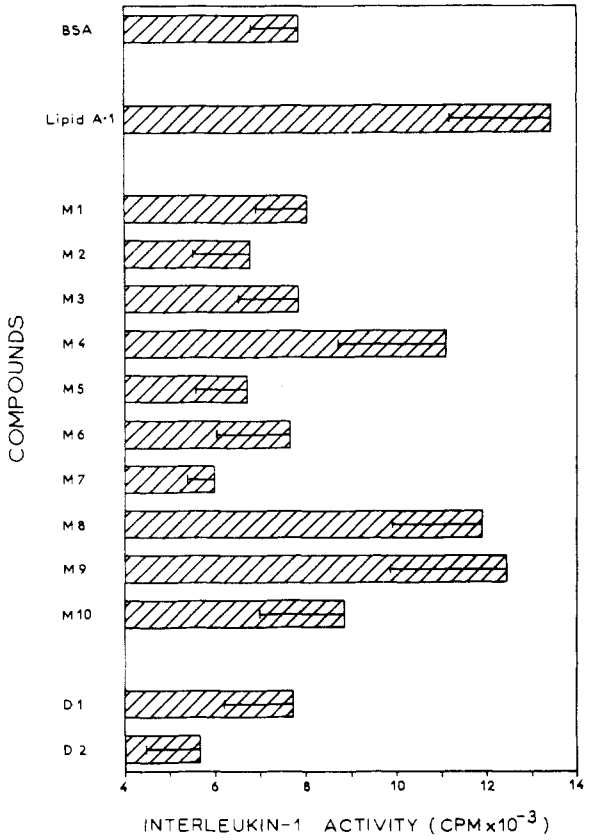


FIGURE 2: Secretion of interleukin 1 (IL-1) by rabbit monocytes, induced by the glycolipids. Monocytes (10^5 cells) were cultured with the inducer (5 μg) for 24 h, and IL-1 activity of the culture supernatant was measured as described under Materials and Methods. Compounds were complexed with BSA (50% by weight).

for 24 h with glycolipids (5 μg) complexed with BSA (2.5 μg), were assayed for their capacity to enhance the proliferative response of C3H/HeJ thymocytes (10^6 cells) to concanavalin A (0.1 μg). Mean values of two separate experiments (six replicates each), carried out with monocytes of two distinct rabbits, are given in Figure 2. Although a rather high IL-1 activity was induced by culture supernatants of monocytes incubated with BSA alone (7849 vs. 1489 cpm), a significant increase of activity was observed (13480 cpm) when the lipid A-1/BSA complex was used as inducer. Among the various glycolipids tested, only three compounds (M8, M9, and M4) appeared significantly active in this assay; activities exhibited

Table III: Activation of the Complement System by Synthetic Glycolipids

compd[a]	complement consumption[b] guinea pig serum[c] (μL/5 μg)	human serum[d] (μL/10 μg)
BSA	0	0.5 ± 1.5
lipid A-1	2.14 ± 0.02	3.3 ± 0.5
M1	1.04 ± 0.38	1.3 ± 0.3
M2	1.08 ± 0.16	1.1 ± 0.8
M3	0.65 ± 0.29	0.6 ± 0.2
M4	1.04 ± 0.32	4.8 ± 0.5
M5	0.32 ± 0.20	5.2 ± 1.2
M6	0.72 ± 0.09	0.9 ± 0.9
M7	0.54 ± 0.14	4.5 ± 0.7
M8	0.90 ± 0.12	4.4 ± 0.6
M9	0.48 ± 0.08	12.1 ± 0.6
M10	0.32 ± 0.16	7.7 ± 0.7
D1	0.54 ± 0.08	2.2 ± 0.3
D2	1.68 ± 0.10	0

[a] Compounds were complexed with BSA (50% by weight) as described under Materials and Methods. [b] Complement consumption is measured by the decrease of the hemolytic activity of the serum and expressed as the volume of serum (μL) inactivated by a given amount of the compound to be tested (5 or 10 μg). [c] The hemolytic assay was carried out in VBS^{2+} buffer by using sheep erythrocytes (SRBC) sensitized with a rabbit anti-SRBC serum. [d] The hemolytic assay (alternative complement pathway) was carried out in EGTA buffer with unsensitized rabbit erythrocytes.

by the other compounds were similar to that of BSA alone.

Activation of the Complement System. Consumption of guinea pig complement during incubation of sera with synthetic glycolipids was measured by the decrease of hemolysis of sheep erythrocytes sensitized with a rabbit anti-sheep erythrocyte serum. Results (Table III) indicate that the highest activity is exhibited by the disaccharide D2 (79% of the activity of lipid A-1). The monosaccharides M1, M2, M8, and M4 were significantly active (about half of the activity of lipid A-1), whereas all of the other glycolipids tested were scarcely active. Activation of human complement via the alternative pathway (Platts-Mills & Ishizaka, 1974) was measured by the decrease of the hemolytic activity of human serum after incubation with the test specimens and assayed with unsensitized rabbit erythrocytes in the presence of EGTA. Results in Table III indicate that the anticomplementary activity of lipid A-1 via the alternative pathway is moderate, as compared to activities exhibited by a number of other synthetic monosaccharide derivatives (M4, M5, and M7–M10).

Discussion

In this work, mainly conventional synthetic procedures were used. However, some of the reaction steps had to be optimized to obtain the specified derivatives.

De-N-acetylation was a key step in our syntheses. Indeed, all compounds, from M3 to M10, were obtained from a free amino derivative resulting from the cleavage of an acetamido group. While acidic methods were excluded because of the presence of the acetal group, treatment with anhydrous or hydrated hydrazine (Hanessian, 1972), alone or with hydrazine sulfate, gave in our hands poor yields. On the contrary, treatment with 4 M potassium hydroxide (Gross & Jeanloz, 1967) in 95% alcohol at 125 °C gave amino derivatives in excellent yields. All the other blocking groups were stable under these conditions. The desired *N*-acyl groups were introduced by using the appropriate acyl anhydride, as different trials to acylate the amino group with mixed anhydrides, activated esters or acids, and carbodiimides were usually found to be less satisfactory: they either gave low yields or compounds difficult to purify. The 4- and 6-positions were blocked

with an isopropylidene rather than a benzylidene group, because the former was found to be more easily cleaved under mild acidic conditions.

Although (3*R*)-3-(benzoyloxy)tetradecanoic acid can be prepared (Diolez et al., 1985), the use of levulinic acid as a temporary protecting group (Van Boom & Burgers, 1978) for the alcoholic function of (3*R*)-3-hydroxytetradecanoic acid was preferred. Indeed, removal of levulinate esters requires very mild conditions that do not affect either *O*-acyl or phosphotriester groups present in the molecules.

The benzyloxymethyl group (Hindsgaul et al., 1982) was chosen for the specific protection of the primary alcohol function in the presence of a free hydroxyl on C4 because it could be introduced with a high degree of selectivity, and in addition, it is cleaved concomitantly with benzyl ethers during the final deprotection procedure.

Phosphorylation of the 4-hydroxyl groups was another key step. In order to reduce the final deprotection to a simple hydrogenolysis of benzyl groups catalyzed by Pd on charcoal, phosphorylation was attempted with dibenzyl phosphorochloridate. Although this reagent has been already used with success when the hydroxyl group was metalated (Inage et al., 1982), in our case the reaction was difficult to perform because it was necessary to use low temperatures, perfectly anhydrous conditions, and exactly 1 equiv of butyllithium. The classical diphenyl phosphorochloridate reagent was found to be more satisfactory despite the fact that removal of both phenyl groups (H_2/Pt) was generally slow. If, instead of pyridine (which led in some cases to erratic results and generally to low yields of products difficult to purify), imidazole was used as the acid acceptor and dried tetrahydrofuran as the solvent, the reaction was virtually quantitative. The position of the diphenyl phosphate group was ascertained by ^{1}H NMR: phosphorylation was accompanied by a downfield shift of 1.2 ppm for H4, and an H–P coupling constant of 9 Hz could be observed, showing that no acyl migration occurred.

It is generally thought that biological activities of lipid A preparations are strongly influenced by the micellar nature of the dispersion. In order to study biological activities of lipid A analogues, different groups of investigators dispersed their synthetic compounds in aqueous media by a number of procedures such as sonication (Kotani et al., 1983), formation of complexes with BSA (Kumazawa et al., 1984; Matsuura et al., 1984), incorporation into liposomes (Yasuda et al., 1984), or formation of triethylammonium salts (Galanos et al., 1984). In our hands, a direct sonication of the synthetic compounds was ineffective and gave unstable dispersions in most cases; furthermore, treatment with triethylamine alone, to form the corresponding salts, was inapplicable since some of the specimens to be tested were nonphosphorylated. We also preferred to avoid the use of liposomes because the ratio of externally available to internally trapped molecules in such multilamellar systems is difficult to estimate and also because a preferential uptake of the liposomes by macrophages during phagocytic processes is likely to occur. For these reasons we decided to prepare stable suspensions of the compounds to be tested by complexing them with BSA. In the present study, various synthetic mono- and disaccharides derived from glucosamine were analyzed in several in vitro test systems and compared to a lipid A preparation (lipid A-1) isolated from the *Bordetella pertussis* endotoxin and similarly complexed with BSA. The results obtained in the different immunological tests are summarized in Table IV.

Induction of mitogenic acitivity by the monosaccharides apparently requires the presence of unsubstituted hydroxyl groups at position 3 and at least at one other position. This agrees with the positive responses of spleen cells to compounds M5, M9, M10, and M1 and their low responses to compounds M3, M4, and M7. It should be noted that this result is not in agreement with the apparent requirement of a 3-hydroxytetradecanoate group at position 3 of a natural lipid A precursor (lipid X) for the expression of its mitogenic activity (Raetz et al., 1983). The significant mitogenicity of compound M1 indicates that the presence of a phosphate group at position 4 is not required. However, the strong activity of the peracylated compound M2 cannot be explained by the above criteria and is likely to be due to a completely different mechanism. The disaccharides tested were not mitogenic and appeared as efficient inhibitors of spleen cell proliferation. Although we noticed that this was not the consequence of an intrinsic cytotoxicity of the compounds (results not shown), the precise explanation of this inhibitory effect remains to be found.

Table IV: Summary of in Vitro Immunological Activities[a] of Natural and Synthetic Glycolipids

		complement activation		
inducer[b]	mitogenicity	guinea pig	human[c]	IL-1 secretion
lipid A-1	++	++	+	++
M1	+	+	±	
M2	++	+	±	
M3				
M4		+	+	+
M5	++		+	
M6	±			
M7			+	
M8	±	+	+	+
M9	+		++	+
M10	+		++	±
D1			±	
D2		++		

[a] Symbols: ++, strongly active, +, significantly active, and ±, moderately active. The absence of symbol denotes scarcely active or inactive compounds. [b] Compounds were complexed with BSA (50% by weight) as described under Materials and Methods. [c] Via the alternative pathway.

It has been demonstrated (Galanos et al., 1971) that lipopolysaccharides and lipid A are efficient activators of the complement system. Our results indicate that among the synthetic compounds assayed, the most potent activator of guinea pig complement is the disaccharide D2 (79% of lipid A-1 activity). The presence of a substitutent at position 1 reduces this activity (M4 and D2 vs. M3 and D1, respectively). On the other hand, the presence of a fatty acid at position 3 enhances the complement activation (M5 vs. M6). Some of the synthetic monosaccharides (M9 and M10) strongly activated human complement via the alternative pathway. The presence of a fatty acid substituent at position 3 enhances the alternative pathway activation induced by nonphosphorylated glucosamine derivatives (M1 vs. M4) but reduces those induced by 4-O-phosphorylated compounds (M5 vs. M6).

Interleukin 1 (IL-1), previously known as lymphocyte activating factor (LAF), is a mediator that regulates immunological (T-lymphocyte responses) (Maizel et al., 1981) and nonimmunological (endogenous pyrogen) functions of macrophages (Murphy et al., 1980). Although bacterial endotoxins are known to be potent stimulants of IL-1 release from monocytes and macrophages (Gery & Waksman, 1972), the influence of synthetic analogues of lipid A upon IL-1 secretion has not yet been investigated. Our results indicate that three of the synthetic monosaccharide derivatives (M4, M8 and M9) induce significant IL-1 release. Two of the active compounds

(M8 and M9) had very similar structures: an unsubstituted hydroxyl group at position 3 and phosphate and tetradecanoate substituents at positions 4 and 6, respectively. Substitution of the hydroxyl group of the *N*-(3*R*)-3-hydroxymyristoyl residue with a fatty acid (compounds M7 and M10) abolished the IL-1 inducing effect. The closely related structures of the active compounds M8 and M9 recall the substitution pattern initially proposed (Rosner et al., 1979; Gmeiner et al., 1971) for the nonreducing glucosamine unit of lipid A. It is noteworthy that compound M6, which fulfills exactly the structure requirement of the nonreducing glucosamine in the most recently proposed structure of lipid A (Wollenweber et al., 1982; Strain et al., 1983), was inactive in this test. The third synthetic compound inducing IL-1 release (M4) has a structure very similar to that of the reducing sugar moiety of lipid A.

These results show that induction of at least those in vitro immunological activities that were examined is not correlated with a single optimal structure, since the most active compounds in a given test are not the best inducers of the other activities (Table IV). This observation indicates that chemically synthesized substructures of lipid A are sufficient to induce a given activity of the natural compound and could advantageously replace the latter in biological studies.

ACKNOWLEDGMENTS

We are indebted to the Direction of the Institut Mérieux (Lyon) and to G. Ayme, R. Donikian, and D. Schulz, who prepared and supplied the pertussis extracts. We thank Dr. Annick Le Dur for the preparation of lipid A-1 and M. H. Sarda for her competent secretarial assistance.

SUPPLEMENTARY MATERIAL AVAILABLE

Detailed description of the syntheses of the compounds used (17 pages). Ordering information is given on any current masthead page.

REFERENCES

Alberston, N. F. (1962) *Org. React. (N.Y.) 12*, 194–195.
Behling, U. H., Campbell, B., Chang, C.-M., Rumpf, C., & Nowotny, A. (1976) *J. Immunol. 117*, 847–851.
Charon, D., & Szabó, L. (1983) *Carbohydr. Res. 111*, C13–C15.
Charon, D., Mondange, M., & Szabó, L. (1984) *J. Chem. Soc., Perkin Trans. 1*, 2291–2295.
Diolez, C., Mondange, M., Sarfati, S. R., Szabó, L., & Szabó, P. (1984) *J. Chem. Soc., Perkin Trans. 1*, 275–279.
Diolez, C., Sarfati, S. R., & Szabó, L. (1985) *J. Chem. Soc., Perkin Trans. 1*, 57–60.
Galanos, C., Rietschel, E. T., Lüderitz, O., & Westphal, O. (1971) *Eur. J. Biochem. 19*, 143–152.
Galanos, C., Lüderitz, O., Rietschel, E. T., & Westphal, O. (1977) *Int. Rev. Biochem. 14*, 239–335.
Galanos, C., Lehmann, V., Lüderitz, O., Rietschel, E. T., Westphal, O., Brade, H., Brade, L., Freudenberg, M. A., Hansen-Hagge, T., Lüderitz, T., Mc Kenzie, G., Schade, U., Strittmatter, W., Tanamoto, K., Zahringer, U., Imoto, M., Yoshimura, H., Yamamoto, M., Shimamoto, T., Kusomoto, S., & Shiba, T. (1984) *Eur. J. Biochem. 140*, 221–227.
Gery, I., & Waksman, B. H. (1972) *J. Exp. Med. 136*, 143–155.
Gmeiner, J., Simon, M., & Lüderitz, O. (1971) *Eur. J. Biochem. 21*, 355–356.
Gross, P. H., & Jeanloz, R. W. (1967) *J. Org. Chem. 32*, 2759–2763.
Hanessian, S. (1972) *Methods Carbohydr. Chem. 6*, 208–214.
Hase, S., & Rietschel, E. T. (1977) *Eur. J. Biochem. 75*, 23–24.
Hasegawa, A., & Fletcher, H. G., Jr. (1973) *Carbohydr. Res. 29*, 209–222.
Hindsgaul, O., Norberg, T., Le Pendu, J., & Lemieux, R. U. (1982) *Carbohydr. Res. 109*, 109–142.
Horton, D. (1972) *Methods Carbohydr. Chem. 6*, 282–285.
Inage, M., Chaki, H., Kusumoto, S., & Shiba, T. (1981) *Tetrahedron Lett. 22*, 2281–2284.
Inage, M., Chaki, H., Kusumoto, S., & Shiba, T. (1982) *Chem. Lett.*, 1281–1284.
Inouye, Y., Onodera, K., Kitaoka, S., & Hirano, S. (1956) *J. Am. Chem. Soc. 78*, 4722–4724.
Kotani, S., Takada, H., Tsujimoto, M., Ogawa, T., Mori, Y., Sakuta, M., Kawasaki, A., Inage, M., Kusumoto, S., Shiba, T., & Kasai, N. (1983) *Infect. Immun. 41*, 758–773.
Kumazawa, Y., Matsuura, M., Nakatsuru-Watanabe, Y., Fukumoto, M., Nishimura, C., Homma, J. Y., Inage, M., Kusumoto, S., & Shiba, T. (1984) *Eur. J. Immunol. 14*, 109–114.
Le Dur, A., Chaby, R., & Szabó, L. (1980) *J. Bacteriol. 143*, 78–88.
Maizel, A. L., Mehta, S. R., Ford, R. J., & Lachman, L. B. (1981) *J. Exp. Med. 153*, 470–475.
Matsuura, M., Kojima, Y., Homma, J. Y., Kubota, Y., Yamamoto, A., Kiso, M., & Hasegawa, A. (1984) *FEBS Lett. 167*, 226–230.
Mühlradt, P. F., Wray, V., & Lehmann, V. (1977) *Eur. J. Biochem. 81*, 193–203.
Murphy, P. A., Simon, P. L., & Willoughby, W. F. (1980) *J. Immunol. 124*, 2498–2501.
Parquet, M., & Sinaÿ, P. (1971) *Carbohydr. Res. 18*, 195–202.
Platts-Mills, T. A. E., & Ishizaka, K. (1974) *J. Immunol. 113*, 348–358.
Raetz, C. R. H., Purcell, S., & Takayama, K. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4624–4628.
Rollin, P., & Sinaÿ, P. (1977) *J. Chem. Soc., Perkin Trans. 1*, 2513–2517.
Rosner, M. R., Tang, J., Barzilay, I., & Khorana, H. G. (1979) *J. Biol. Chem. 254*, 5906–5917.
Strain, S. M., Fesik, S. W., & Armitage, I. M. (1983) *J. Biol. Chem. 258*, 13466–13477.
Tharanathan, R. N., Weckesser, J., & Mayer, M. (1978) *Eur. J. Biochem. 84*, 385–394.
Tucker, S. B., Pierre, R. Y., & Jordon, R. E. (1977) *J. Immunol. Methods 14*, 267–269.
Van Boeckel, C. A. A., Hermans, J. P. G., Westerduin, P., Oltvoort, J. J., Van Der Marel, G. A., & Van Boom, J. H. (1983) *Recl. Trav. Chim. Pays-Bas 102*, 438–449.
Van Boom, J. H., & Burgers, P. M. J. (1978) *Recl. Trav. Chim. Pays-Bas 97*, 73–80.
Vogel, S. N., Madonna, G. S., Wahl, L. M., & Rich, P. D. (1984) *J. Immunol. 132*, 347–353.
Volk, W. A., Galanos, C., & Lüderitz, O. (1970) *Eur. J. Biochem. 17*, 223–229.
Wollenweber, H., Brady, K. W., Lüderitz, O., & Rietschel, E. T. (1982) *Eur. J. Biochem. 124*, 191–198.
Yasuda, T., Kanegasaki, S., Tsumita, T., Tadakuma, T., Ikewaki, N., Homma, J. Y., Inage, M., Kusumoto, S., & Shiba, T. (1984) *Eur. J. Biochem. 140*, 245–248.