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Immunobiologically Active Lipid A Analogs Synthesized According to a Revised Structural Model of Natural Lipid A

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Synthetic lipid A analogs which have two amide-bound and two ester-bound (*R*)-3-hydroxytetradecanoyl groups at the C-2 and -2′ and C-3 and -3′ positions of β(1-6)glucosamine disaccharide mono- or diphosphates showed high activities in most in vitro assays, and the lethality of a diphosphate derivative to galactosamine-treated mice was almost comparable to that of natural lipid A. The pyrogenicity and Shwartzman induction activity of the synthetic analogs, however, were much less than those of natural lipid A.

Endotoxic lipopolysaccharide (LPS) is a main cell surface component of gram-negative bacteria that shows many strong immunobiological activities (9, 15). Kusumoto and colleagues (7) have carried out synthetic studies in attempts to determine the chemical structure and structure-activity relationships of lipid A, which carries most of the immunobiological activities of LPS (9, 15). On the basis of the structure proposed for *Salmonella*-type lipid A (9, 15), they synthesized 13 acylated derivatives of β(1-6)glucosamine disaccharide with or without phosphate groups at C-1 and -4′ (7). Some of these compounds, especially 1,4′-diphosphates with two amide-bound (*R*)-3-hydroxytetradecanoyl or tetradecanoyl groups at C-2 and -2′ and three ester-bound tetradecanoyl groups at C-3, -4, and -6′, were active in most assays of immunobiological activity (6). However, the potencies of these compounds were much less than those of natural lipid A products in these assays (6).

During these studies, evidence was obtained that the proposed structure of lipid A should be revised, with respect to the linkage site, to a polysaccharide moiety through 3-deoxyl-D-*manno*-octulosonate. Namely, using the two-dimensional nuclear magnetic resonance technique, Imoto et al. (3) demonstrated that the disaccharide backbone of a highly purified preparation of *Escherichia coli* lipid A was acylated at the 3- and 3′-hydroxy groups, as well as having two amide groups, and that the 6′-hydroxy position was free. This conclusion was supported by the finding of Strain et al. (12) that the so far accepted 3-deoxyl-D-*manno*-octulosonate linkage site at C-3′ of the disaccharide in LPS of *E. coli* D31m4 is not consistent with ^{13}C chemical shift data and also by the finding of Takayama et al. (13) that a novel glycolipid that accumulates as a very early precursor in the biosynthesis of lipid A by a temperature-sensitive *E. coli* mutant contains two (*R*)-3-hydroxytetradecanoyl groups, one attached as an amide at C-2 and the other attached as an ester at C-3.

Imoto et al. (M. Imoto, H. Yoshimura, M. Yamamoto, T. Shimamoto, S. Kusumoto, and T. Shiba, Tetrahedron Lett., in press) then synthesized the β(1-6)glucosamine disaccharide and its phosphates (compounds 403 to 406, Fig. 1), which contain 4 mol of (*R*)-3-hydroxytetradecanoyl groups at positions C-2, -2′, -3, and -3′. Compound 406 was expected to correspond to the lipid A precursor isolated by Lehmann (8), which has the same biological activities as lipid A, but is less active than lipid A. The monosaccharide precursors (compounds 402, 401, and 407, Fig. 1) were also synthesized.

Compounds 403 to 405 were all synthesized from a common disaccharide intermediate, i.e., allyl 2-acylamino-6-*O*-(3-*O*-acyl-2-acylamino-2-deoxy-β-D-glucopyranosyl)-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside, in which the acyl moiety was an (*R*)-3-benzyl-oxytetradecanoyl group. This intermediate was obtained by coupling a peracetyl oxazoline derivative with allyl 2-acetamido-3-*O*-benzoyl-4-*O*-benzyl-2-deoxy-β-D-glucopyranoside and then introducing 3-benzyloxyacyl moieties stepwise, according to the principle described previously (4). Removal of protecting groups afforded the dephospho derivative 403, whereas their removal after introduction of phosphate moieties gave 404, 405, and 406. The acyl monosaccharide phosphate (compound 401), corresponding to the above precursor (13), and its dephospho and lauroyl analogs (402 and 407) were prepared from allyl 2-acetamido-2-deoxy-β-D-glucopyranoside. Details of these syntheses are to be published (Imoto et al., in press; S. Kusumoto, M. Yamamoto, and T. Shiba, submitted for publication).

The immunobiological activities of the compounds synthesized (disaccharide derivatives 403 to 406 and monosaccharide derivatives 401, 402, and 407) and of compounds 316 and 317 described previously (6) were examined. For tests, the compounds and natural lipid A (2) prepared from *E. coli* O8:K27 F515 (Re mutant) for comparison were dissolved or suspended as homogeneously as possible at 2 mg/ml in distilled water (pyrogen free for clinical use; Ohtsuka Seiyaku Co. Ltd, Tokyo, Japan) supplemented with 0.1% (vol/vol) triethylamine (Wako Pure Chemicals, Osaka, Japan). This stock solution was diluted to an appropriate concentration with pyrogen-free distilled water, physiological saline, or culture medium.

In both the conventional Pre Gel test and the Toxicolor

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TABLE 1. Activation of clotting enzyme cascade of an amoebocyte lysate of the horseshoe crab, *T. tridentatus*, and of the complement system of human serum by synthetic lipid A analogs and related compounds

Analog or compound	Activation as determined by[a]:		Complement activation[c]	
	Pre Gel test (minimal effective dose [ng per test])	Toxicolor test (equivalents [ng/mg] of reference LPS[b])	ED_{50}[d] (μg/ml)	Inhibition by 10 mM EGTA–5 mM $MgCl_2$
Disaccharide derivative				
403	10^2	0.9	Inactive	
404	10^{-2}	2.7×10^6	<40 and >20	Complete
405	1	3.2×10^4	<400 and >200	Complete
406	10^{-1}	4.8×10^5	>400	Complete
316	$\geq 10^5$	1.0×10	Inactive	
317	10^4	Not detected	Inactive	
Monosaccharide derivative				
402	$\geq 10^4$	Not detected	Inactive	
401	10	3.0×10	Inactive	
407	10^2	9.0×10^0	Not done	
Natural product				
Lipid A[e]	10^{-2}	5.9×10^6	<10	Almost complete
LPS	10^{-1}	1.0×10^6	Not done	

[a] The Pre Gel and Toxicolor (recently developed quantitative method [11], using a chromogenic substrate) tests were performed, using reagents from Seikagaku Kogyo Co., Tokyo, Japan, according to the instructions of the manufacturer.

[b] The reference LPS, prepared from *E. coli* O111:B4, was purchased from Difco Laboratories, Detroit, Mich. In the Toxicolor test, 1 ng of this LPS was equivalent to 2.9 endotoxin units of USP reference standard endotoxin from *E. coli* O113.

[c] Complement activation was determined by a modification of the method of Meyer (5) by measuring the decrease of hemolytic activity (50% hemolytic complement) of pooled, fresh adult human serum.

[d] ED_{50} of complement activation is defined as the effective dose causing 50% reduction of hemolytic activity of test complement serum.

[e] The reference lipid A specimen was prepared from *E. coli* O8:K27 (Re mutant) F515.

TABLE 2. Stimulatory effects on murine lymphocytes, guinea pig macrophages, and human adult peripheral PMNLs of synthetic lipid A analogs and related compounds[a]

Analog or compound	Effects on murine splenocytes				Effects on peritoneal guinea pig macrophages[b]			Enhancement on migration of human adult PMNLs[c]
	Enhancement of [^{3}H]TdR uptake[d]			PBA[e] BALB/c	Inhibition of [^{3}H]TdR uptake	Enhancement of [^{14}C]glucosamine uptake	Spreading[f]	
	BALB/c nu/nu	C3H/HeN	C3H/HeJ					
Disaccharide derivative								
403	1.0 (10)	0.8 (10)	1.1 (0.1)	1.3 (10)	15	1.3	−	2.1 (0.1)[g]
404	5.0 (1.0)[g]	8.1 (0.1)[g]	1.0 (0.1)	3.0 (10)[g]	94[g]	3.7[h]	++	4.0 (0.01)[g]
405	3.3 (10)[g]	3.5 (1.0)[g]	1.1 (0.1)	1.4 (10)[h]	93[g]	3.2[h]	++	2.9 (0.1)[g]
406	3.6 (10)[g]	9.8 (0.1)[g]	1.2 (0.1)	2.1 (10)[g]	92[g]	4.1[h]	++	3.5 (0.1)[g]
316	0.8 (10)	0.9 (10)	1.0 (0.1)	1.9 (10)	33	1.9	−	1.3 (0.01)
317	0.8 (1.0)	0.7 (0.1)	0.8 (0.1)	ND	0	1.2	−	1.3 (0.01)
Monosaccharide derivative								
402	1.0 (10)	0.7 (10)	0.8 (0.1)	ND	5	1.0	−	1.6 (0.01)[h]
401	0.8 (1.0)	1.0 (0.1)	0.6 (0.1)	1.2 (10)	56[h]	0.7	−	1.6 (0.01)[h]
407	1.1 (1.0)	1.3 (0.1)	0.8 (0.1)	ND	86[h]	1.6	±	1.6 (0.01)[h]
Natural product lipid A	3.1 (1.0)[g]	9.1 (0.1)[g]	0.7 (0.1)	6.3 (10)[g]	96[g]	4.5[h]	++	3.2 (0.01)[g]

[a] Data are expressed as stimulation indices in assays on lymphocytes and PMNLs and as percent inhibition of [^{3}H]TdR uptake and the extent of spreading in assays on macrophages. Values in parentheses are doses causing the highest stimulation within the concentration range examined: 0.1 to 10 μg/5 × 10^5 cells (mitogenicity), 1 and 10 μg/ml (polyclonal B cell activation), 10 μg/ml (macrophages), and 0.001 to 0.1 μg/ml (PMNLs). ND, Not determined.

[b] Stimulatory effects on macrophages were examined by using adherent exudate cells of a Hartley guinea pig (male; ca. 400 g [body weight]; Nihon Rabbit Co., Osaka, Japan) whose peritoneal cavity had been irritated by injection of liquid paraffin, as described previously (10, 14).

[c] Enhancement of human peripheral PMNL migration was examined as described previously (6), using a 48-well chemotaxis chamber from Neuro Probe (Cabin John, Md.).

[d] The assay on mitogenicity (increase of [^{3}H]TdR uptake) was made in mice of strains BALB/c nu/nu (male; 6 weeks old; Clea Japan, Osaka, Japan), C3H/HeN (female; 10 weeks old; Charles River Japan, Osaka, Japan), and C3H/HeJ (female; 14 weeks old; a gift from Takeshi Nakano, Department of Laboratory Animal Science, Scholl of Medicine, Kitasato University, Sagamihara, Kanagawa, Japan) as described in a previous paper (6), except that the cells were cultured in the presence of bovine serum albumin (Fraction V; Sigma Chemical Co., St Louis, Mo.) at an equal concentration to that of the test compound and in the absence of fetal bovine serum.

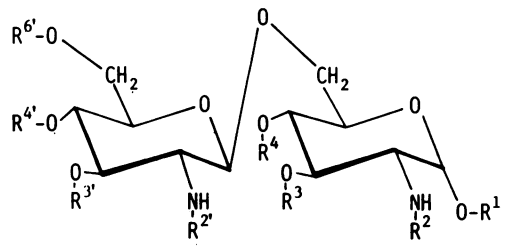
[e] Polyclonal B cell activation (PBA) was determined in BALB/c mice (male; 8 weeks old; Charles River Japan) as described previously (6).

[f] Percentage of spreading was arbitrarily graded as follows: (cells spread on microtray surface/total cells counted) × 100. ++, ≥70%; +, 40 to 70%; ±, 10 to 40%; −, <10%.

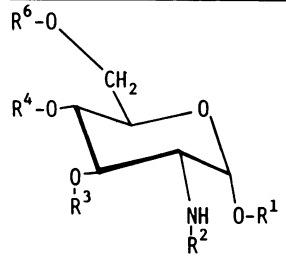
[g] Significantly different from control ($P < 0.01$).

[h] Significantly different from control ($P < 0.05$).

Disaccharide derivative



Monosaccharide derivative



Compound No	R^2	$R^{2'}$	R^3	$R^{3'}$	R^4	$R^{4'}$	$R^{6'}$	R^1
403	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	H	H	H	H
404	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	H	P	H	H
405	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	H	H	H	P
406	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	H	P	H	P
316	C_{14}-OH	C_{14}-OH	C_{14}	H	C_{14}	H	C_{14}	P
317	C_{14}-OH	C_{14}-OH	C_{14}	H	C_{14}	P	C_{14}	P

Compound No	R^1	R^2	R^3	R^4	R^6
402	H	C_{14}-OH	C_{14}-OH	H	H
401	P	C_{14}-OH	C_{14}-OH	H	H
407	P	C_{12}	C_{12}	H	H

FIG. 1. Chemical structures of synthetic lipid A analogs and related compounds. Symbols: P, $PO(OH)_2$; C_{14}-OH, (*R*)-3-hydroxytetradecanoyl; C_{12}, dodecanoyl.

test (11) on the activation of the clotting enzyme cascade of the horseshoe crab, *Tachypleus tridentatus*, (*R*)-3-hydroxytetradecanoyl derivatives of phosphorylated disaccharide, especially 404, showed strong activity (Table 1). All other compounds showed much less activity than natural lipid A. Table 1 also shows that compounds 404 to 406, especially 404, caused the activation (the consumption of hemolytic activity) of human serum complement (5), mainly via the classical pathway which is blocked by chelation with EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid]. The activity of 404 was somewhat weaker than that of the reference lipid A.

Table 2 summarizes data on the stimulatory effects of the compounds on lymphocytes, polymorphonuclear leukocytes (PMNLs), and macrophages. Compounds 404 to 406, unlike 316 and 317, stimulated murine B lymphocytes (increasing their uptake of thymidine [TdR] [6], and causing polyclonal B cell activation [6]), human adult peripheral PMNLs (enhancing their migration [6]), and guinea pig peritoneal macrophages (inhibiting their TdR uptake [10], increasing their glucosamine uptake [10], and enhancing their spreading on a solid surface [14]). None of these active compounds increased TdR uptake by splenocytes of C3H/HeJ strain, a low responder to lipid A, indicating that these mitogenicities were specific to the lipid A structure.

Results of in vivo assays are shown in Table 3. Compounds 404 and 406 showed lethal toxicity on C57BL/6 mice which were rendered highly susceptible to LPS and lipid A by intraperitoneal injection of galactosamine (1). The toxicity of 406 was comparable to that of natural lipid A, but the toxicities of 404 and 405 were weaker. None of the other compounds were lethal at doses of 1,000 ng per mouse. In

TABLE 3. Lethal toxicity to mice, pyrogenicity in rabbits, and ability to prepare for provocation of hemorrhagic reaction by LPS in rabbit skin (Shwartzman reaction) of synthetic lipid A analogs and related compounds

Analog or compound	Lethal toxicity[a] (LD_{50} [ng per mouse])	Pyrogenicity[b] (minimum effective dose [μg/kg per rabbit])	Induction of Shwartzman reaction[c] (minimum effective dose [μg per site per rabbit])
Disaccharide derivative			
403	$>10^{3.5}$	ND	>80
404	$10^{2.2}$	>10 and <31.6	≥80
405	$>10^{2.6}$	>10 and <31.6	>80
406	$10^{1.4}$	>10 and <31.6	≥80
316	$>10^{3.5}$	ND	ND
317	$>10^{3.5}$	ND	>80
Monosaccharide derivative			
402	$>10^{3.5}$	ND	ND
401	$>10^{3.5}$	ND	ND
407	$>10^{3.5}$	ND	ND
Natural product lipid A	$10^{1.1}$	>0.1 and <0.316	>1.6 and <8

[a] Lethal toxicity was determined in galactosamine (16 mg per mouse)-loaded C57BL/6 mice (male; 8 weeks old; Charles River Japan) as described by Galanos et al. (1). LD_{50}, 50% lethal dose.

[b] Pyrogenicity was tested as described in Japanese Pharmacopoeia, using Japanese domestic white rabbits (2.0 to 2.5 kg [body weight]). ND, Not done.

[c] The Shwartzman reaction was examined by the conventional technique, with provocation by intravenous injection of 100 μg of an LPS specimen (from *E. coli* O127:B8; Difco Laboratories) per kg 18 h after intracutaneous injection of 1.6, 8, 40, or 80 μg of test compound per 0.2 ml per site. ND, Not done.

the pyrogenicity test, the entire 400 series except the dephospho derivative 403 showed activity, but the minimum pyrogenic doses of 404 to 406 (31.6 μg/kg) were ca. 100 times that of lipid A. We also tested another in vivo activity characteristic of LPS and lipid A, namely, the ability to induce a Shwartzman reaction in rabbit skin upon subsequent intravenous injection of LPS. None of the compounds showed activity at a dose of 80 μg per site under conditions in which natural lipid A was active at a dose of 8.0 μg per site.

Thus β(1-6)glucosamine disaccharide derivatives with (*R*)-3-hydroxytetradecanoyl groups at positions C-2 and -2′ (amide bound) and C-3 and -3′ (ester bound) and phosphates at C-1, C-4′, or both showed strong immunobiological activities, similar to those of natural lipid A, in most in vitro assays. In in vivo assays, however, these synthetic compounds were less active than lipid A in inducing a Shwartzman reaction, although compound 406 had as high a lethal toxicity as did lipid A in mice pretreated with galactosamine.

Since the activities of compounds 404 to 406, which have the revised lipid A structure, were much higher than those of compounds 316 and 317, which have the lipid A structure proposed originally, the presence of an ester-bound (*R*)-3-hydroxytetradecanoyl group at C-3′ or the absence of an acyl group at C-6′, or both, seems to increase greatly the activities (at least the in vitro activities) of the molecule. The present findings also provide additional support for the proposed acylation pattern of the disaccharide backbone.

We have now synthesized lipid A analogs with almost comparable in vitro activities to those of natural lipid A. Further work is necessary to obtain a compound with identical in vivo activities to those of natural lipid A.

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