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Syntheses of glucose analogues of E5564 as a highly potent anti-sepsis drug candidate

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Abstract—Glucose analogues **5** and **9** of E5564 were synthesized, and their LPS-antagonistic activities were measured. The inhibitory activities (IC_{50}) on LPS-induced TNFα production of these two compounds towards human whole blood cells were 0.06 and 0.83 nM, respectively. Inhibitory doses (ID_{50}) of compounds **5** and **9** on TNFα production induced by coinjection of galactosamine and LPS in C3H/HeN mice in vivo were measured and were 0.55 and <0.20 mg/kg, respectively. And also C3H/HeN mice preinjected with compounds **5** and **9** were protected from lethality induced by coinjection of galactosamine and LPS; out of eight mice preinjected with 1 mg/kg of the compounds, one–six and three of eight mice were protected, respectively.

1. Introduction

Sepsis (septicemia) is a severe illness caused by overwhelming infection of the bloodstream by toxin [such as lipopolysaccharide (LPS)]-producing bacteria and can be very dangerous even when optimally treated. Severe sepsis can lead to widespread organ damage and organ failure, making it one of the most common causes of death in ICU patients. And unfortunately there is no effective medicine for severe sepsis to date. Therefore, the development of an effective drug for sepsis is urgently needed.

Recently, a nontoxic natural lipid A-related compound (RsDPLA)[1] was isolated from *Rhodobacter sphaeroides* by an Eisai group. This compound did not show LPS-agonistic activity towards mouse macrophages, but showed strong LPS-antagonistic activity.[2] Furthermore, the Eisai group found that many RsDPLA-related compounds having an olefinic double bond in their molecules behave as LPS antagonists towards both human and murine macrophages,[2] and developed a compound related to RsDPLA as a potent anti-septicemia drug candidate, E5564 (eritoran),[3] which passed phase II trial successfully.

We were also interested in RsDPLA-related compounds, in which the glucosamine is replaced with glucose analogues at the reducing end or non-reducing end. Therefore, we synthesized some β(1–6)-linked glucosamine–glucose disaccharides[4] and glucose–glucosamine disaccharides.[5] And we reported their activities towards both human blood cells and mice.[4,5] It was proven that these novel synthetic compounds had almost the same or stronger activities towards both human blood cells and murine macrophages than against classic glucosamine–glucosamine-type disaccharides. This result aroused our interest in the biological activities of the glucose–glucose β(1–6)-linked disaccharides of E5564. Consequently, we could synthesize β(1–6)-linked disaccharides **5** and **9**.

This paper describes the synthesis of two compounds **5** and **9**, their LPS-antagonistic activities towards both human whole blood cells[6] and galactosamine-loaded C3H/HeN mice[7] and their improvement of the lethality in C3H/HeN mice[7] (Fig. 1).

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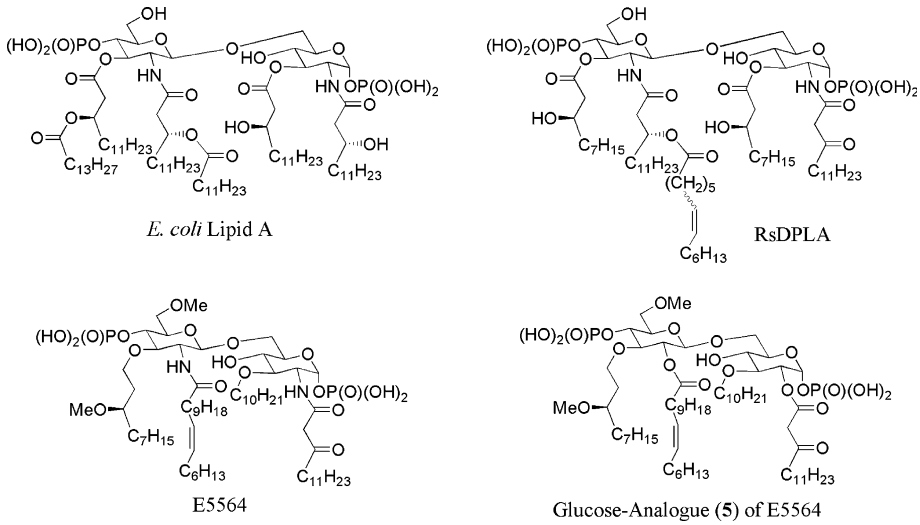


Figure 1. Structures of *Escherichia coli* lipid A, RsDPLA, E5564 and glucose-analogue (**5**) of E5564.

2. Results and discussion

2.1. Synthesis

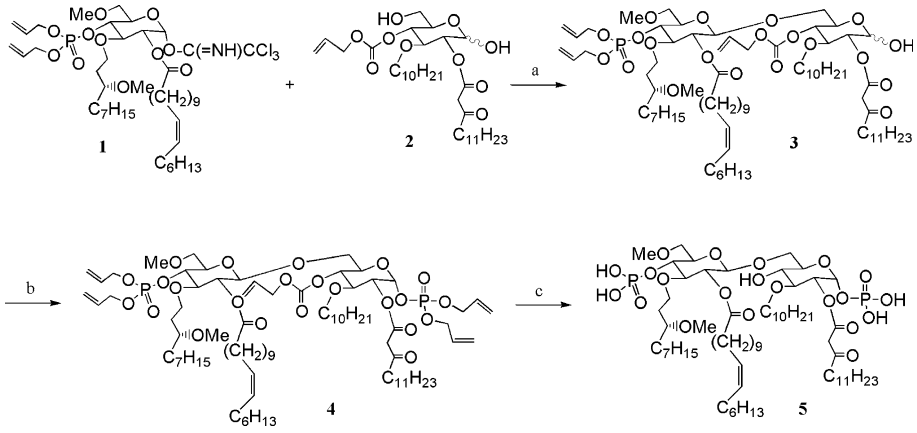
First, glucose-analogue (**5**) of E5564 was synthesized from both reported D-glucoside analogues **1**[5] and **2**[4a] obtained from common starting material, diacetone D-glucose. 2,2,2-Trichloroacetimidoyl 4-*O*-(diallylphosphono)-3-*O*-[(*R*)-3-methoxydecyl]-6-*O*-methyl-2-*O*-[(*Z*)-11-octadecenoyl]-α-D-glucopyranoside **1** and 4-*O*-allyloxycarbonyl-3-*O*-decyl-2-*O*-(3-oxotetradecanoyl)-α,β-D-glucopyranose **2** were coupled using silver trifluoromethanesulfonate (AgOTf) and trimethylsilyl trifluoromethanesulfonate (TMSOTf)[4] in methylene chloride to give β(1–6) disaccharide **3** without detection of α(1–6) disaccharide or orthoester via anomeric oxonium ion by involvement of the C2 acyl group. Treatment of **3** with 2 equiv of diallyl diisopropylphosphoramidite[8] and 5 equiv of 1*H*-tetrazole, and successively with aq 30% H_2O_2 gave phosphate **4** as an α-anomer. We reported recently that the phosphorylation reaction of C2′-amide analogue of **3** gave a 1:1 anomeric mixture at C1-position.[4a] However, this time, only α-anomer **4** was obtained without detection of β-anomer. We could not explain the reason. Treatment of **4** with tetrakis(triphenylphosphine)palladium(0) ($(PPh_3)_4Pd$), triphenylphosphine (Ph_3P), and triethylamine–formic acid (Et_3N–HCOOH) in THF at 50 °C for 16 h gave **5** (Scheme 1).

Second, compound **9** was synthesized from both alcohol **2** and imidate **6**.[5] Treatment of alcohol **2** and imidate **6** with AgOTf and TMSOTf in methylene chloride gave β(1–6) disaccharide **7** without detection of α(1–6) disaccharide. Treatment of **7** with diallyl diisopropylphosphoramidite and 1*H*-tetrazole, and successively with aq 30% H_2O_2 gave phosphate **8** as an α-anomer without detection of β-anomer. Treatment of **8** with $(PPh_3)_4Pd$, Ph_3P and Et_3N–HCOOH in THF at 55 °C for 16 h gave **9** (Scheme 2).

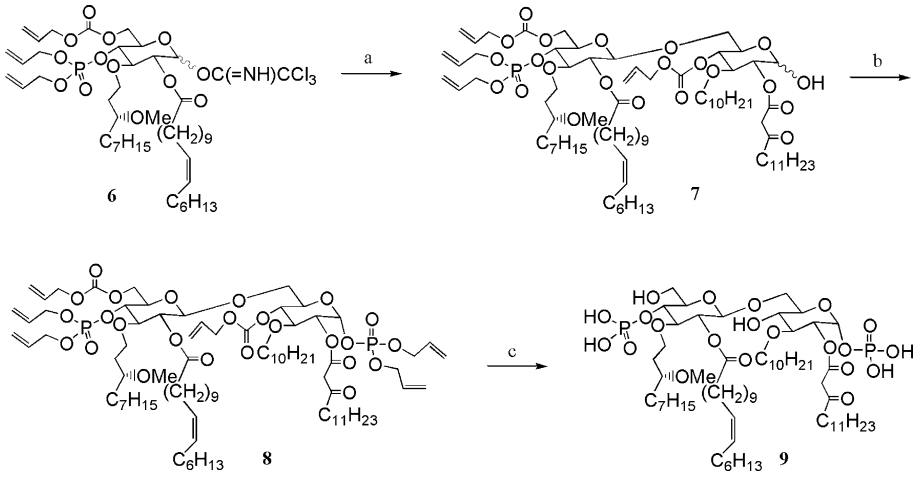
Thus, we could synthesize the two β(1–6)-linked disaccharides **5** and **9**.

2.2. Biological activity

The inhibitory activity on LPS-induced TNFα production, LPS-antagonistic activity, of two synthetic compounds **5** and **9** was investigated in vitro using human whole blood cells by comparison with E5564. The IC_{50}



Scheme 1. Reagents and conditions: (a) AgOTf, TMSOTf, MS4A, CH_2Cl_2 rt, 16 h, 65%; (b) 2 equiv of *i*-$Pr_2NP(OCH_2CH{=}CH_2)_2$, 5 equiv of 1*H*-tetrazole, Na_2SO_4 CH_2Cl_2 rt, 30 min, then aq 30% H_2O_2, THF, 0 °C, 30 min, 79%; (c) $Pd(PPh_3)_4$, PPh_3, Et_3N, HCOOH, THF, N_2, 50 °C, 16 h, 54%.



Scheme 2. Reagents and conditions: (a) **2**, AgOTf, TMSOTf, MS4A, CH_2Cl_2, rt, 16 h, 86%; (b) 2 equiv of *i*-$Pr_2NP(OCH_2CH{=}CH_2)_2$ 5 equiv of 1*H*-tetrazole, Na_2SO_2, rt, 30 min, then aq 30% H_2O_2, CH_2Cl_2–THF, 0 °C, 30 min, 76%; (c) $Pd(PPh_3)_4$, PPh_3, Et_3N, HCOOH, THF, N_2, 55 °C, 16 h, 48%.

values (nM) of compounds **5**, **9** and E5564 towards human whole blood cells were 0.06, 0.83 and 2.94–8.24 nM, respectively. The activity of compound **5** towards human whole blood cells was fifty times stronger than that of E5564.

Inhibitory doses (ID_{50}) of compounds **5** and **9** on TNFα production induced by coinjection of galactosamine and LPS in C3H/HeN mice were measured by comparison with E5564. The values of these two compounds and E5564 were 0.55, <0.20 and 1.82–3.21 mg/kg, respectively. Compound **9** was much stronger than E5564.

Moreover, C3H/HeN mice preinjected with compounds **5** and **9** and E5564 were protected from lethality induced by coinjection of galactosamine and LPS.[7] Out of eight mice preinjected with 1 mg/kg of compounds **5** and **9**, and E5564, one–six, three, and one–six of eight mice were protected, respectively. Compounds **5** and **9** were approximately equivalent to that of E5564.

3. Conclusion

Thus, we could synthesize two glucose analogues of E5564, compounds **5** and **9**. As a result, it was proven that these novel synthetic compounds were effective towards human whole blood cells, inhibition of TNFα production in C3H/HeN mice and survival rate for LPS of C3H/HeN mice. These two compounds were more active than the classic glucosamine–glucosamine-type disaccharides.[9] The effective dose of compound **5** for human whole blood cells was at least reduced to approximately one-fiftieth of that of E5564. Therefore, we believe that compound **5** should be a potent drug candidate for severe sepsis.

4. Experimental

4.1. General procedure

^1H NMR spectra were recorded with JEOL-GSX 400 and JNM-ECT 500 spectrometers using tetramethylsilane (TMS) as an internal standard. IR absorption spectra were measured with an IR A-2 spectrophotometer, and mass spectra were obtained with a JMS-700 mass spectrometer. Separation of compounds by column chromatography was done with silica gel 60 (230–400 mesh ASTM) under a slightly elevated pressure (111–182 kPa) for easy elution. Commercially available anhydrous THF and dichloromethane were used for the reactions. DMF and pyridine were dried by storage over 4 Å molecular sieves.

4.1.1. 4-*O*-Allyloxycarbonyl-3-*O*-decyl-6-*O*-{4-*O*-(diallylphosphono)-3-*O*-[(*R*)-3-methoxydecyl]-6-*O*-methyl-2-*O*-[(*Z*)-11-octadecenoyl]-β-D-glucopyranosyl}-2-*O*-(3-oxotetradecanoyl)-α,β-D-glucopyranose (3). To a solution of trichloromethylimidoyl 4-*O*-(diallylphosphono)-3-*O*-[(*R*)-3-methoxydecyl]-6-*O*-methyl-2-*O*-[(*Z*)-11-octadecenoyl]-α-D-glucopyranoside **1** (260 mg, 0.279 mmol) and 4-*O*-allyloxycarbonyl-3-*O*-decyl-2-*O*-(3-oxotetradecanoyl)-D-glucopyranose **2** (175 mg, 0.279 mmol) in CH_2Cl_2 (8 mL) was added MS 4A (500 mg) under nitrogen. After the mixture was stirred for 30 min at room temperature, AgOTf (180 mg, 0.701 mmol) and TMSOTf (20 mg, 0.090 mmol) were added to this mixture, which was stirred for 16 h at room temperature under nitrogen, and diluted with CH_2Cl_2. The solution was washed with aq satd $NaHCO_3$ and brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated in vacuo to give a mixture, which was chromatographed on a silica gel column. Elution with cyclohexane/EtOAc (1:1) gave **3** (240 mg, 65%) as a gum. IR ν_{max} (film) 3326 (w), 2926, 2856, 1754, 1720, 1650 (w), 1629 (w), 1465 cm^{-1}. 400 MHz ^1H NMR ($CDCl_3$) δ 0.88 (12H, *t*, *J* = 6.6 Hz, fatty acids terminal CH_3 ×4), 1.26 (60H, br s), 1.40–1.74 (10H, m), 1.99–2.04 (4H, m, C2′-side chain C10–H_2 and C13–H_2), 2.19 (0.5H, t, *J* = 6.5 Hz, C2-side chain C4–H_2 ×1/4), 2.32–2.36 (2H, m, C2′-side chain C2–H_2), 2.54 (1.5H, *t*, *J* = 6.5 Hz, C2-side chain C4-H_2 ×3/4), 3.22 (1H, m, C3′-side chain C3-H), 3.26 (3H, s, C3′-side chain C3–OCH_3), 3.39 (3H, s, C6′–OCH_3), 3.46–3.90 (14H, m, C3-H, C5-H, C6–H_2, C3-side chain C1–H_2, C3′–H, C5′–H, C6′–H_2, C3′-side chain C1–H_2, and containing 2H, s, at 3.48 ppm, C2-side chain C2–H_2), 4.26 (1H, *t*, *J* = 9.0 Hz, C4-H), 4.40 (1H, q,

J = 9.4 Hz, C4′-H), 4.51–4.67 (8H, m; allylic O–CH_2 ×2, C1′-H, C1–H, and containing 2H, d, J = 5.9 Hz, at 4.64 ppm, C4–O–CO–OCH_2), 4.77 (1H, dd, J = 3.5, 9.8 Hz, C2-H), 4.85 (1H, m, OH), 4.94 (1H, *t*, J = 7.6 Hz, C2′–H), 5.24–5.40 (8H, m, allyl olefinic 6H, C2′-side chain olefinic C11–H and C12–H), 5.89–5.97 (3H, m, allyl olefinic 3H). FABMS (positive-ion) *m*/*z*, 1421 $[M+Na]^+$.

4.1.2. Diallylphosphono 4-*O*-allyloxycarbonyl-3-*O*-decyl-6-*O*-[4-*O*-(diallylphosphono)-3-*O*-[(*R*)-3-methoxydecyl]-6-*O*-methyl-2-*O*-[(*Z*)-11-octadecenoyl]-β-D-glucopyranosyl]-2-*O*-(3-oxotetradecanoyl)-α-D-glucopyranoside (4). To a solution of **3** (240 mg, 0.171 mmol) in CH_2Cl_2 (8 mL) were added Na_2SO_4 (0.5 g), 1*H*-tetrazole (60 mg, 0.855 mmol) and diallyl diisopropylphosphoramidite (84 mg, 0.342 mmol). After stirring for 30 min at room temperature under nitrogen, the reaction mixture was chromatographed directly on a silica gel short column. Elution with cyclohexane/EtOAc (3:2) gave phosphite (260 mg), which was diluted with THF (8 mL). To this phosphite solution, aq 30% H_2O_2 (0.55 mL) was added. After stirring for 30 min at 0 °C, the reaction mixture was diluted with EtOAc, washed with aq 10% $Na_2S_2O_3$, aq satd $NaHCO_3$ and brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated in vacuo to give a mixture, which was chromatographed on a silica gel column. Elution with cyclohexane/EtOAc (1:1) gave **4** (211 mg, 79%, R_f = 0.458) as a viscous oil. IR ν_{max} (film) 2927, 2856, 1757, 1721, 1650 (w), 1464 cm^{-1}. 400 MHz ^1H NMR ($CDCl_3$) δ 0.88 (12H, *t*, J = 6.6 Hz, fatty acids terminal CH_3 ×4), 1.25 (60H, br s), 1.40–1.71 (10H, m), 1.99–2.02 (4H, m, C2′-side chain C10–H_2 and C13–H_2), 2.18 (0.5H, t, J = 6.5 Hz, C2-side chain C4–H_2 ×1/4), 2.33, 2.40 (2H, m, C2′-side chain C2–H_2), 2.54 (1.5H, t, J = 6.5 Hz, C2-side chain C4–H_2 ×3/4), 3.20 (1H, m, C3′-side chain C3–H), 3.26 (3H, s, C3′-side chain C3–OCH_3), 3.38 (3H, s, C6′–OCH_3), 3.44 (2H, s, C2-side chain C2–H_2), 3.44–4.08 (12H, m; C3–H, C5–H, C6–H_2, C3-side chain C1–H_2, C3′–H, C5′–H, C6′–H_2 and C3′-side chain C1–$_2$), 4.30 (1H, q, J = 9.4 Hz, C4′–H), 4.39 (1H, m, C4–H), 4.44–4.65 (11H, m; allylic O–CH_2 ×4, C1′–H and containing 2H, d, J = 5.5 Hz, at 4.64 ppm, C4–O–CO–OCH_2), 4.75 (1H, *t*, J = 9.4 Hz, C2′-H), 4.84 (1H, dd, J = 3.4, 10.2 Hz, C2-H), 5.21–5.42 (12H, m; allyl olefinic 10H, C2′-side chain olefinic C11–H and C12–H), 5.80 (1H, dd, J = 3.5, 6.6 Hz, C1–H), 5.89–5.99 (5H, m, allyl olefinic 5H). FABMS (positive-ion) *m*/*z*, 1581 $[M+Na]^+$. HRFABMS calcd for $C_{82}H_{144}O_{23}P_2Na$: 1581.9471. Found: 1581.9447.

4.1.3. Phosphono 3-*O*-decyl-6-*O*-[3-*O*-[(*R*)-3-methoxydecyl]-6-*O*-methyl-2-*O*-[(*Z*)-11-octadecenoyl]-4-*O*-phosphono-β-D-glucopyranosyl]-2-*O*-(3-oxotetradecanoyl)-α-D-glucopyranoside (5). To a solution of **4** (100 mg, 0.064 mmol) in dry THF (5 mL) were added PPh_3 (11 mg, 0.042 mmol), Et_3N (43 mg, 0.425 mmol), HCOOH (36 mg, 0.782 mmol) and $Pd(PPh_3)_4$ (11 mg, 0.010 mmol) in this sequence. The solution was stirred for 16 h at 50 °C under nitrogen and concentrated in vacuo to give a mixture, which was chromatographed on a DEAE–cellulose (Whatman ion-exchange cellulose, wet 6 g) column. The column was prepared by preliminary consecutive washing with 60 mL each of aq 0.5 M HCl, H_2O, aq 0.5 M NaOH and H_2O, 24 mL of aq 1 M AcOH, 60 mL each of H_2O, aq 0.05 M $AcONH_4$, $CHCl_3$/MeOH/H_2O (2:3:1), and finally $CHCl_3$/MeOH (2:1). The column was eluted with 5 mL each of $CHCl_3$/MeOH (2:1), then 0.05 M $AcONH_4$ in $CHCl_3$/MeOH/H_2O (2:3:1). Six fractions containing **5** were collected. To this solution were added further $CHCl_3$ (5 mL) and aq 0.15 M HCl (10 mL), and the mixture was shaken well to adjust to pH 2–3. The lower $CHCl_3$ layer was separated and then concentrated in vacuo to give **5** (46 mg, 54%) as a wax. IR ν_{max} (KBr) 3505 (w), 2925, 2854, 1747, 1719, 1653 (w), 1466 cm^{-1}. 400 MHz ^1H NMR ($CDCl_3$ + CD_3OD, 5:1) δ 0.87–0.90 (12H, m, fatty acids terminal CH_3 ×4), 1.27 (60H, br s), 1.40–1.76 (10H, m), 1.99–2.04 (4H, m, C2′-side chain C10–H_2 and C13–H_2), 2.19 (0.5H, t, J = 7.5 Hz, J = 6.5 Hz, C2-side chain C4–H_2 ×1/4), 2.32–2.42 (2H, m, C2′-side chain C2–H_2), 2.57 (1.5H, t, J = 7.5 Hz, C2-side chain C4–H_2 ×3/4), 3.26–3.98 (21H, m; C3–H, C6–H_2, C3-side chain C1–H_2, C3′-H, C5–H, C6′–H_2 and C3′-side chain C3–H, C3′-side chain C1–H_2, C5′–H, and containing two 3H, s, at 3.30 and 3.41 ppm, C3′-side chain C3–OCH_3 and C6′–OCH_3, respectively, and 2H, s, at 3.52 ppm, C2-side chain C2–H_2), 4.18 (1H, q, J = 10.2 Hz, C4′–H), 4.51 (1H, d, J = 7.9 Hz, C1′–H), 4.71 (1H, dd, J = 3.2, 10.2 Hz, C2–H), 4.88 (1H, dd, J = 8.0, 8.9 Hz, C2′–H), 5.35 (2H, m, C2′-side chain olefinic C11–H and C12–H), 5.68 (1H, dd, J = 3.1, 7.4 Hz, C1–H). FABMS (negative-ion) *m*/*z*, 1313 $[M-H]^-$, 1335 $[M-2H+Na]^-$. Anal. Calcd for $C_{66}H_{124}O_{21}P_2 \cdot 2.5H_2O$: C, 58.27; H, 9.56; P, 4.55. Found: C, 58.43; H, 9.39; P, 4.51.

4.1.4. 4-*O*-Allyloxycarbonyl-3-*O*-decyl-6-*O*-{6-*O*-allyloxycarbonyl-4-*O*-(diallylphosphono)-3-*O*-[(*R*)-3-methoxydecyl]-2-*O*-[(*Z*)-11-octadecenoyl]-β-D-glucopyranosyl}-2-*O*-(3-oxotetradecanoyl)-α,β-D-glucopyranose (7). To a solution of 2,2,2-trichloroacetimidoyl 6-*O*-allyloxycarbonyl-4-*O*-(diallylphosphono)-3-*O*-[(*R*)-3-methoxydecyl]-2-*O*-[(*Z*)-11-octadecenoyl]-α,β-D-glucopyranoside **6** (340 mg, 0.344 mmol) and diol **2** (216 mg, 0.344 mmol) in CH_2Cl_2 (10 mL) was added MS 4A (620 mg) under nitrogen. After the mixture was stirred for 30 min at room temperature, AgOTf (222 mg, 0.864 mmol) and TMSOTf (20 mg, 0.090 mmol) were added to this mixture, which was stirred for 16 h at room temperature under nitrogen, and diluted with CH_2Cl_2. The solution was washed with aq satd $NaHCO_3$ and brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated in vacuo to give a mixture, which was chromatographed on a silica gel column. Elution with cyclohexane/EtOAc (3:2) gave **7** (436 mg, 86%) as a gum. IR ν_{max}(film) 3500–3200, 2927, 2856, 1754, 1650 (w), 1461 cm^{-1}. 400 MHz ^1H NMR ($CDCl_3$) δ 0.88 (12H, t, J = 6.6 Hz, fatty acids terminal CH_3 ×4), 1.26 (60H, br s), 1.40–1.48 (4H, m), 1.56–1.72 (6H, m), 1.99–2.04 (4H, m, C2′-side chain C10–H_2 and C13–H_2), 2.19 (0.4H, *t*, J = 7.4 Hz, C2-side chain C4–H_2 ×1/5), 2.32–2.40 (2H, m, C2′-side chain C2–H_2), 2.52–2.58 (1.6H, t, J = 7.4 Hz, C2-side chain C4–H_2 ×4/5), 3.23 (1H, m, C3′-side chain C3–H), 3.26 (3H, s, C3′-side chain C3–OCH_3), 3.46–3.90 (12H, m; C3–H, C5–H, C6–H_2, C3-side chain C1–H_2, C3′–H, C5′–H, C3′-side

chain C1–H_2, and containing 2H, s, at 3.49 ppm, C2-side chain C2–H_2), 4.23 (1H, *t*, *J* = 8.8 Hz, C4–H), 4.34–4.40 (2H, m, C6′–H_2), 4.40 (1H, q, *J* = 9.5 Hz, C4′–H), 4.56–4.64 (10H, m, allylic O–CH_2 ×2, C1′–H, C1–H, C6′–O–CO–OCH_2 and C4–O–CO–OCH_2), 4.77 (1H, dd, *J* = 3.1, 9.8 Hz, C2–H), 4.93 (1H, t, *J* = 7.4 Hz, C2′–H), 5.23–5.41 (10H, m; allyl olefinic 8H, C2′-side chain olefinic C11–H and C12–H), 5.88–5.98 (4H, m, allyl olefinic 4H). FABMS (positive-ion) *m*/*z*, 1491 $[M+Na]^+$. HRFABMS calcd for $C_{79}H_{137}O_{22}PNa$: 1491.9237. Found: 1491.9266.

4.1.5. Diallylphosphono 4-*O*-allyloxycarbonyl-3-*O*-decyl-6-*O*-{6-*O*-allyloxycarbonyl-4-*O*-(diallylphosphono)-3-*O*-[(*R*)-3-methoxydecyl]-2-*O*-[(*Z*)-11-octadecenoyl]-β-D-glucopyranosyl}-2-*O*-(3-oxotetradecanoyl)-α-D-glucopyranoside (8). To a solution of **7** (424 mg, 0.288 mmol) in CH_2Cl_2 (12 mL) were added Na_2SO_4 (0.8 g), 1*H*-tetrazole (101 mg, 1.442 mmol) and diallyl diisopropylphosphoramidite (141 mg, 0.577 mmol). After stirring for 30 min at room temperature under nitrogen, the reaction mixture was chromatographed directly on a silica gel short column. Elution with cyclohexane/EtOAc (3:2) gave phosphite (493 mg), which was diluted with THF (10 mL). To this phosphite solution, aq 30% hydrogen peroxide (1 mL) was added. After stirring for 20 min at 0 °C, the reaction mixture was diluted with EtOAc, which was washed with aq 10% $Na_2S_2O_3$, aq satd $NaHCO_3$ and brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated in vacuo to give a mixture, which was chromatographed on a silica gel column. Elution with cyclohexane/EtOAc (3:2) gave **8** (355 mg, 76%) as an oil. IR ν_{max} (film) 2927, 2856, 1754, 1721 (w), 1650 (w), 1461 cm^{-1}. 400 MHz ^1H NMR ($CDCl_3$) δ 0.86–0.89 (12H, m, four fatty acids terminal CH_3 ×4), 1.25 (60H, br s), 1.37–1.46 (4H, m), 1.56–1.75 (6H, m), 1.99–2.02 (4H, m, C2′-side chain C10–H_2 and C13–H_2), 2.16–2.20 (0.4H, t, *J* = 7.6 Hz, C2-side chain C4–H_2 ×1/5), 2.28–2.47 (2H, m, C2′-side chain C2–H_2), 2.51–2.56 (1.6H, *t*, *J* = 7.6 Hz, C2-side chain C4–H_2 ×4/5), 3.20 (1H, m, C3′-side chain C3–H), 3.25 (3H, s, C3′-side chain C3–OCH_3), 3.45–4.06 (14H, m; C3–H, C5–H, C6–H_2, C3-side chain C1–H_2, C3′–H, C5′–H, C3′-side chain C1–H_2 and containing 2H, s, at 3.46 ppm, C2-side chain C2–H_2), 4.22–4.30 (2H, m, C4–H and C6′–H), 4.41–4.47 (2H, m, C4′–H and C6′–H), 4.53–4.65 (14H, m, allylic O–CH_2 ×6, C1–H and C1′–H), 4.70–5.03 (2H, m, C2–H and C2′–H), 5.24–5.41 (14H, m, allyl olefinic 12H and C2′-side chain olefinic C11–H and C12–H), 5.80 (1H, dd, *J* = 3.3, 7.0 Hz, C1–H), 5.88–5.99 (6H, m, allyl olefinic 6H). FABMS (positive-ion) *m*/*z*, 1651 $[M+Na]^+$. HRFABMS calcd for $C_{85}H_{146}O_{25}P_2Na$: 1651.9526. Found: 1651.9546.

4.1.6. Phosphono 3-*O*-decyl-6-O-[3-*O*-[(*R*)-3-methoxydecyl]-2-*O*-[(*Z*)-11-octadecenoyl]-4-*O*-phosphono-β-D-glucopyranosyl]-2-*O*-(3-oxotetradecanoyl)-α-D-glucopyranoside (9). To a solution of **8** (210 mg, 0.129 mmol) in dry THF (10 mL) were added PPh_3 (22 mg, 0.084 mmol), Et_3N (86 mg, 0.850 mmol), HCOOH (72 mg, 1.564 mmol) and $Pd(PPh_3)_4$ (22 mg, 0.020 mmol) in this sequence. The solution was stirred for 16 h at 55 °C under nitrogen and concentrated in vacuo to give a mixture, which was chromatographed on a DEAE–cellulose (Whatman ion-exchange cellulose, wet 9 g) column. The column was prepared by preliminary consecutive washing with 90 mL each of aq 0.5 M HCl, H_2O, aq 0.5 M NaOH and H_2O, 24 mL of aq 1 M AcOH, 90 mL each of H_2O, aq 0.05 M $AcONH_4$, $CHCl_3$/MeOH/H_2O (2:3:1) and finally $CHCl_3$/MeOH (2:1). The column was eluted with 5 mL each of $CHCl_3$/MeOH (2:1), then 0.05 M $AcONH_4$ in $CHCl_3$/MeOH/H_2O (2:3:1). Six fractions containing **9** were collected. To this solution were added further $CHCl_3$ (5 mL) and aq 0.15 M HCl (10 mL), and the mixture was shaken well to adjust to pH 2–3. The lower $CHCl_3$ layer was separated and concentrated in vacuo to give **9** (80 mg, 48%) as a wax. IR ν_{max} (KBr) cm^{-1}. 400 MHz ^1H NMR ($CDCl_3$ + CD_3OD, 4:1) δ 0.88 (12H, t, *J* = 6.6 Hz, fatty acids terminal CH_3 × 4), 1.20–1.28 (60H, br s), 1.24–1.75 (10H, m), 1.99–2.04 (4H, m, C2′-side chain C10–H_2 and C13–H_2), 2.19 (0.4H, *t*, *J* = 7.2 Hz, C2-side chain C4–H_2 ×1/5), 2.31–2.38 (2H, m, C2′-side chain C2–H_2), 2.57 (1.6H, t, *J* = 7.2 Hz, C2-side chain C4–H_2 ×4/5), 3.30 (3H, s, C3′-side chain C3–OCH_3), 3.32 (1H, m, C3′-side chain C3–H), 3.37–4.04 (15H, m, C3–H, C4–H, C5–H, C6–H_2, C3-side chain C1–H_2, C3′–H, C5′–H, C6′–H_2, C3′-side chain C1–H_2 and C2-side chain C2–H_2), 4.23 (1H, m, C4′–H), 4.50 (1H, d, *J* = 7.8 Hz, C1′–H), 4.70 (1H, dd, *J* = 3.4, 9.3 Hz, C2–H), 4.87 (1H, t, *J* = 8.4 Hz, C2′–H), 5.35 (2H, m, C2′-side chain olefinic C11–H and C12–H), 5.67 (1H, dd, *J* = 3.5, 7.4 Hz, C1–H).

4.2. Methods for measurement of biological activity

4.2.1. Production of TNFα by human whole blood cells[6]

4.2.1.1. Materials. Lipopolysaccharide (LPS, *Escherichia coli* 026:B6), human tumour necrosis factor alpha (TNFα) immunoassay kit and 96-well assay plates were purchased from Sigma, BioSource International, Inc. and Corning Inc. (Cat. No. 3956), respectively.

4.2.1.2. Whole blood TNFα production. Fresh blood was collected aseptically in the presence of heparin by venipuncture from healthy adult volunteers. The subjects did not have any apparent inflammatory conditions and had taken no drugs for at least 7 days prior to blood collection. Written informed consent was obtained from all volunteers before the experiment. In each well of the 96-well assay plates, 360 μL aliquots of blood were mixed with 20 μL of LPS solution (200 ng/mL) dissolved in PBS in the presence (for test sample) or absence (for positive control samples) of test compound (dissolved in 10% DMSO/PBS solution). For the negative control samples, the same amount of blood was cultured with PBS and a test compound solution. After 6 h of incubation at 37 °C, the plates were centrifuged at 490*g* for 15 min, and the plasma was collected and stored at −20 °C. The concentrations of TNFα in the plasma were measured with commercially available immunoassay kits.

4.2.1.3. Statistical analysis. The percentage inhibition of TNFα production was calculated by the following formula: [1 − (concentration of TNFα in the test sample-concentration of TNFα in the negative control sample)/(concentration of TNFα in the positive control

sample-concentration of TNFα in the negative control sample)] × 100. The suppressive activity of each test compound is expressed as the 50% inhibitory concentration (IC_{50}) of the test compound, the concentration at which the test compound suppresses TNFα production by 50%. The IC_{50} was calculated from the percentage inhibition using the SAS System for Windows. The results are expressed as the mean IC_{50} of triplicate experiments.

4.2.2. Production of TNFα and lethality in C3H/HeN mice coinjected with galactosamine and LPS[7]

4.2.2.1. Materials: animals. Male C3H/HeN mice were purchased from Charles River Japan (Tokyo, Japan). All mice were used at the age of 7 weeks and housed at Sankyo Laboratories (Tokyo, Japan) with free access to standard rodent chow diet.

4.2.2.2. Reagents. Lipopolysaccharide (LPS, from *E. coli* O26:B6) and D-galactosamine (GalN) were purchased from Sigma (St. Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kits of murine TNFα were from R&D Systems (Minneapolis, MN).

4.2.2.3. TNFα production. Naïve C3H/HeN mice (five per group) were intravenously injected with the test compound dissolved in 0.1% triethylamine/saline solution (0.2, 1.0, and 5.0 mg /10 mL/kg), and immediately after, mice were intravenously injected with a mixture of LPS (0.05 mg/10 mL saline/kg) and GalN (1 g/10 mL saline/kg). Mice were injected with vehicle (0.1% triethylamine/saline solution) and saline for negative control samples, and with vehicle and LPS/GalN for positive control samples. One hour after injection, venous blood was collected under ether anaesthesia with heparinized syringes fitted with 23-gauge needles from the abdominal vein and centrifuged at 4 °C for 3 min at 13,230*g* to obtain the plasma. Plasma was stored at −30 °C before measuring TNFα levels by ELISA. The concentrations of TNFα of mouse plasma were measured using ELISA according to the manufacturer's instructions.

4.2.2.4. Statistical analysis. The percentage inhibition of TNFα production was calculated by the following formula: [1 − (concentration of TNFα in the test sample-concentration of TNFα in the negative control sample)/(concentration of TNFα in the positive control sample-concentration of TNFα in the negative control sample)] × 100. The suppressive activity of each test compound is expressed as the 50% inhibitory dose (ID_{50}) of the test compound, the dose at which the test compound suppresses TNFα production by 50%. The ID_{50} was calculated from the percentage inhibition using the SAS System for Windows (version 5).

Lethality: Naïve C3H/HeN mice (eight per group) were intravenously injected with the test compound solution dissolved in 0.1% triethylamine/saline solution (1.0 mg/10 mL/kg), and immediately after, intravenously injected with a mixture of LPS (0.05 mg/10 mL saline/kg) and GalN (1 g/10 mL saline/kg). Mice were injected with vehicle (0.1% triethylamine/saline solution) and LPS/GalN for control samples. Deaths occurring up to 3 days following administration were recorded.

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References and notes

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8. In this phosphorylation reaction, when 5 equiv of diallyl diisopropylphosphoramidite and 15 equiv of 1*H*-tetrazole were used, compound **4** was obtained in 57% yield and one-third weight unknown by-product (MS; $m/z = 1741$ $[M+Na]^+$), which was different from the corresponding β-anomer. The by-product lacked the 3-ketoester structure on the C-2 position but another phosphono group was added. This suggests to be enol phosphate in the 3-ketoester moiety of **4**.
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