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Synthesis and Antitumor Activity of *N*-Acetylmuramyl-L-alanyl-D-isoglutamine 6-Phosphate and Its Lipophilic Derivatives

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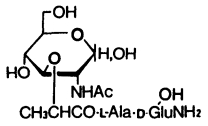
6-Phosphate of *N*-acetylmuramyl-L-alanyl-D-isoglutamine and its lipophilic derivatives, i.e., 6-(octadecyl hydrogenphosphate) and 6-(2-docosyltetracosyl hydrogenphosphate) were synthesized after the structural feature of mycobacterial cell walls for the purpose to modify and possibly to enhance the immunostimulating activity of the muramyl dipeptide which is the minimum requisite for the activity. As expected, the latter more lipophilic phosphate showed a high activity in a tumor regression test.

As already described,[1,2] *N*-acetylmuramyl-L-alanyl-D-isoglutamine (**1**) is the minimum partial structure of bacterial peptidoglycan required for the expression of the immunostimulating activity. Our successive study demonstrated that lipophilic 6-*O*-acyl derivatives of the muramyl dipeptide which were synthesized in imitation of the structural feature of mycobacterial cell walls exhibit an antitumor activity characteristic of these natural cell walls.[3,4] However, the potency of the synthetic derivatives to inhibit the growth of tumor was still much less significant than the natural cell walls. In this respect, we continue synthetic studies towards derivatives of the muramyl dipeptide with more potent activities. One direction is syntheses of compounds with longer glycan structures.[5-7] As the second approach for the same purpose, we describe in this paper synthesis and antitumor activities of 6-*O*-phosphorylated derivatives of the muramyl dipeptide **1**.[8]



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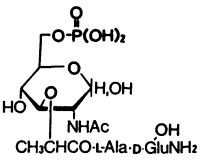

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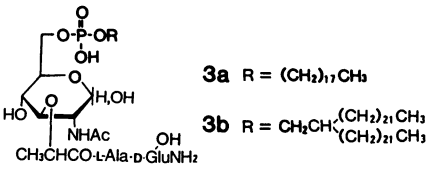
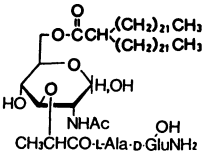


3a R = $(CH_2)_{17}CH_3$

3b R = $CH_2CH\langle^{(CH_2)_{21}CH_3}_{(CH_2)_{21}CH_3}$



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In cell walls of mycobacteria and related species, the 6-hydroxyl groups of muramic acid residues are bound through phosphodiester structures to arabinogalactan. It is further linked to mycolic acid, which is α-branched β-hydroxylated fatty acid with more than forty carbon atoms and thus render the cell wall highly lipophilic.[9] Since the 6-*O*-acylmuramyl dipeptides described above correspond to an oversimplified model of cell wall, both the phosphate and the polysaccharide parts being omitted and natural mycolic acid or analogous synthetic fatty acids being directly bound to the muramic acid residue, we were interested in testing the effect of the phosphate moiety on the biological activity of the molecule of the muramyl dipeptide. Especially, we anticipated that the simultaneous presence of the polar phosphate and the lipophilic part in the same molecule might have a significant effect. This consideration prompted us to synthesize *N*-acetylmuramyl-L-alanyl-D-isoglutamine 6-phosphate (**2**) and its lipophilic derivatives, i.e., the octadecyl derivative **3a** and the 2-docosyltetracosyl derivative **3b**. In the structures of **3a** and **3b**, the polysaccharide part is still lacking but the phosphodiester function is retained, the fatty acids present in the cell walls being replaced with long chain alcohols which are directly bound to the phosphate group.[10] In order to test the effect of different lipophilicity, two alcohols, i.e., 1-octadecanol and 2-docosyl-1-tetracosanol, were used. These two particular alcohols were chosen by taking into account the fact that 6-*O*-acylmuramyl dipeptides containing the fatty acids with the corresponding carbon chains showed characteristic activities. Namely, 6-*O*-stearoylmuramyl dipeptide showed the highest adjuvant activity among the acyl derivatives prepared,[11] and 6-*O*-(2-docosyltetracosanoyl)muramyl dipeptide **4** had a strong tumor suppressive activity.[4]

For the synthesis of 6-phosphate **2**, a known 4,6-*O*-benzylidene derivative of *N*-acetylmuramic acid α-benzyl glycoside (**5**) was prepared by slight modification of the reported conditions.[12] An improved yield was obtained at the formation of 2-carboxyethyl ether by reducing the amount of sodium hydride and by employing a lower reaction temperature. Benzyl esterification of **5** with phenyldiazomethane followed by acidic hydrolysis of the benzylidene group afforded **7** which is feasible for 6-*O*-phosphorylation.

When **7** was treated with diphenyl hydrogenphosphate[13] and 2,4,6-triisopropylbenzenesulfonyl chloride (TPS chloride) in pyridine, the desired 6-(diphenyl phosphate) **8** was obtained as a single product. The position of the phosphate moiety was confirmed by ^{13}C NMR, where a signal of methylene carbon (C-6) was observed which couples with ^{31}P of the phosphate group. After selective hydrogenolysis of the benzyl ester of **8**, the product was coupled with L-alanyl-D-isoglutamine benzyl ester to give the protected *N*-acetylmuramyl dipeptide 6-phosphate **9**, which was identified with the compound obtained via an alternative route described below. Deprotection of **9** was effected by catalytic hydrogenolysis in two steps, first with palladium black then with platinum oxide. Lyophilization from water gave the product **2** as colorless powder which gave a satisfactory result in elemental analysis. On treatment with diazomethane, **2** gave a trimethyl ester. Two of the methyl groups are bound to the phosphate residue as judged from the observation of through-three-bond H–P coupling in the ^1H NMR spectrum. Therefore, the presence of phosphomonoester structure in **2** was unequivocally assured, a possibility of the presence of a cyclic phosphodiester at 4 and 6 positions being thus excluded. The third methyl group was introduced at the carboxyl group of the isoglutamine residue.

Alternatively, the 6-phosphate **2** could be also prepared by phosphorylation after the dipeptide had been coupled with muramic acid. A partially protected derivative of muramyl dipeptide **10** prepared as an intermediate in our previous synthesis of 6-*O*-acyl muramyl dipeptide[14] was phosphorylated with the same reagents as above to give the 6-(diphenyl phosphate) which was identical with **9** obtained above. However, the yield of **9** was low (52%) because the undesired competitive dehydration occurred at the amide function of the isoglutamine residue to form a nitrile **11**. Consequently, the route described first was more favorable.

For the synthesis of lipophilic derivatives, **3a** and **3b**, the corresponding alkyl phenyl hydrogenphosphates were first prepared. Condensation of phenyl dihydrogenphosphate with 1-octadecanol or 2-docosyl-1-tetracosanol which had been prepared from methyl 2-docosyltetracosanoate by lithium aluminum hydride reduction was effected with dicyclohexylcarbodiimide (DCC) in pyridine. In case of the former alcohol, the reaction proceeded readily to give octadecyl phenyl hydrogenphosphate (**12a**) in a good yield. In contrast, the formation of the alkyl phenyl hydrogenphosphate was slow in case of the latter branched higher alcohol and more than 50% of the starting alcohol was recovered even after 70 h.

Octadecyl phenyl hydrogenphosphate was then treated with TPS chloride and muramic acid benzyl ester **7** in the same way as described for the synthesis of **2** to give a syrupy product **13a**, which was characterized by means of ^1H NMR and FD-MS. Hydrogenolysis of the benzyl ester of **13a** followed by condensation with the dipeptide benzyl ester afforded the protected muramyl dipeptide 6-(docosyl phenyl phosphate) **14a** as a solid after chromatographic purification. Hydrogenolysis in two steps followed by purification with a column of Bio-gel P-2 gave the muramyl dipeptide **3a** having octadecyl hydrogenphosphate at 6-position. Since the product gave a poorly resolved ^1H NMR spectrum due to aggregation, the structure was further characterized as its dibenzyl ester.

The 6-(2-docosyltetracosyl hydrogenphosphate) **3b** was also prepared in the same way. Like as the formation of the alkyl phenyl hydrogenphosphate **12b** described above, the yields of its condensation with muramic acid and the successive reaction with dipep-

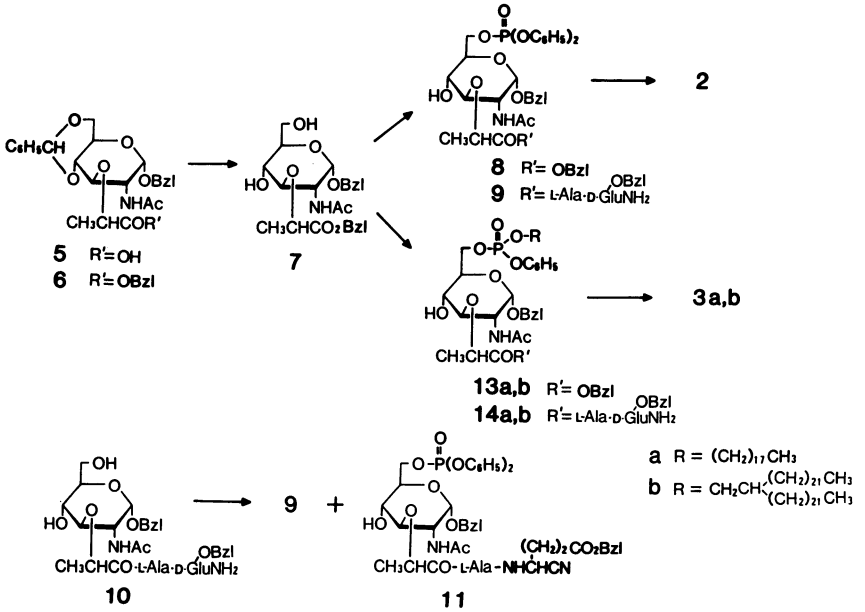

Table 1. Autitumor Effect of Synthetic Muramyl Dipeptide Derivatives on Meth-A Fibrosarcoma

Compound		Suppression Test[a]				Regression test[a]		
		Dose	Viability[b]	Suppression[c]	T/C[d]	Dose	Regression[c]	T/C[d]
		μg/mouse	%		%	μg/mouse		%
6-Phosphate	(**2**)	100	80	0/9	91.4			
6-(Octadecyl hydrogenphosphate)	(**3a**)	6.25	67	2/10	30.9			
6-(2-Docosyltetracosyl hydrogenphosphate)	(**3b**)	100	73	1/10	30.9	100	0/10	67.4
6-*O*-(2-Docosyl-tetracosanoate)	(**4**)	100	71	1/9	42.0	100	0/10	100.1
Nocardia CWS		100	96	2/9	32.1	100	0/8	59.2
Conrol		—	93	0/10	100	—	0/14	100

a) See the experimental part. b) The value represents the proportion of living/total cells before inoculation. c) Number of cured mice/number of treated mice. d) See Ref. 16 in the text.

tide were low presumably owing to a steric hindrance of the bulky alkyl group. The final product was also characterized after having been converted into the corresponding dibenzyl ester with phenyldiazomethane.

The antitumor activity of the synthetic compounds were first examined in the suppression test in which one can evaluate the ability of each compound to suppress the growth of tumor when tumor cells and the compound to be tested were simultaneously applied to animals.[15] Mice were inoculated intradermally with a mixture of the specified amount of the tumor cells and test materials given in Table 1. The weight of tumor was measured and compared with that of the control group where mice were given the tumor cells alone. The compound which gives smaller T/C value[16] is assumed to have higher suppression activity. The results are summarized in Table 1. While the hydrophilic 6-phosphate **2** showed no effect, both of the lipophilic derivatives, **3a** and **3b**, exhibited supression comparable to that obtained with *Nocardia* cell wall skeleton (CWS) which is known to have a potent antitumor activity.[17] 6-*O*-(2-Docosyltetracosanoyl)-muramyl dipeptide **4** of 6-*O*-acyl type prepared previously[4] also showed a similar activity in this test system. It should be noted here that the octadecyl derivative **3a** could not be used in higher doses because this compound kills the tumor cells by direct action probably due to its detergent-like character as shown by the decrease of viable tumor cells (Table 1). Tumor regression activity[18] of muramyl dipeptide 6-(2-docosyltetracosyl hydrogenphosphate) **3b** was then tested in comparison with *Nocardia* CWS and 6-*O*-(2-docosyltetracosanoyl)muramyl dipeptide **4**. In this test, growth inhibition with the compound is evaluated on the tumor which was previously inoculated and already grown up in mice. Therefore this system enables more strict estimation of the antitumor activity under conditions similar to actual immunotherapy. The result shown in Table 1 clearly indicate that compound **3b** prepared in this study shows high activity comparable to the natural cell wall preparation. On the contrary, 6-*O*-acyl type derivative **4** showed no effect in this regression test. Since **4** contains a carbon chain with the same length as that of **3b**, the difference in the biological activities could be attributed to the presence of the phosphate function in **3b**. As mentioned in the introduction of this paper, it could be speculated that simultaneous existence of both the phosphate moiety and the long alkyl chain would give a character like a phospholipid to the molecule and enhance the interaction with the membrane of host animals cells, resulting in expression of high biological activity.

Experimental

All melting points are uncorrected. Unless otherwise stated, ^{1}H and ^{13}CNMR spectra were measured at 100 and 22.5 MHz, respectively, for $CDCl_3$ solutions. FD mass spectra were obtained with a JEOL JMS-01SG mass spectrometer equipped with silicon emitter. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. Column chromatography were carried out with Kieselgel 60 (0.063—0.2 mm), E. Merck.

Benzyl 2-Acetamido-4,6-*O*-benzylidene-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-α-D-glucopyranoside (5). Sodium hydride (60% oil dispersion, 3.0 g, 75 mmol) was added to a solution of benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside[19] (15.0 g, 37.6 mmol) in dry dioxane (750 ml). The mixture was kept at 60°C with stirring. (*S*)-2-Chloropropionic acid (16.4 g, 151 mmol) was added to the mixture and stirring was continued at 60°C. Soon after addition of the acid, a large amount of small crystals separated out which made efficient stirring difficult. Stirring again became possible within 10 min. Then NaH (7.5 g, 188 mmol) was again added. Stirring was continued at the same temperature for further 40 min. After addition of water (800 ml), the mixture was washed with hexane and then acidified with 6M[††] HCl. The separated crystals were collected by filtration, washed thoroughly with water, and dried. Recrystallization was effected from $CHCl_3$; Yield 14.8 g (84%); mp 230—234°C decomp.

Benzyl 2-Acetamido-4,6-*O*-benzylidene-3-*O*-[(*R*)-1-(benzyloxycarbonyl)ethyl]-2-deoxy-α-D-glucopyranoside (6). To a solution of **5** (4.70 g, 10.0 mmol) in $CHCl_3$ (470 ml) was added a solution of phenyldiazomethane in hexane. After

††1 M=1 mol dm^{-3}.

usual work-up, the product was recrystallized from acetone; yield 5.51 g (98%); mp 178—181 °C. Found: C, 68.31; H, 6.22; N, 2.57%. Calcd for $C_{32}H_{35}NO_8$: C, 68.44; H, 6.28; N, 2.49%.

Benzyl 2-Acetamido-3-*O*-[(*R*)-1-(benzyloxycarbonyl)ethyl]-2-deoxy-α-D-glucopyranoside (7). A suspension of **6** (5.00 g, 8.90 mmol) in 60% AcOH (250 ml) was heated in a boiling water bath with shaking for 20 min. After usual work-up, the product was recrystallized from $CHCl_3$–ether–hexane; yield 3.75 g (89%); mp 120—123 °C (with sintering at 115 °C). Found: C, 63.10; H, 6.58; N, 3.02%. Calcd for $C_{25}H_{31}NO_8$: C, 63.41; H, 6.60; N, 2.96%.

Benzyl 2-Acetamido-3-*O*-[(*R*)-1-(benzyloxycarbonyl)ethyl]-2-deoxy-α-D-glucopyranoside 6-(Diphenyl Phosphate) (8). A mixture of **7** (500 mg, 1.05 mmol), diphenyl hydrogenphosphate (528 mg, 2.11 mmol), and TPS chloride (639 mg, 2.11 mmol) in dry pyridine (5 ml) was stirred at room temperature for 13 h. Diphenyl hydrogenphosphate (264 mg, 1.05 mmol) and TPS chloride (320 mg, 1.05 mmol) was added, and the mixture was stirred at room temperature for additional 19 h. After addition of water, the solvent was evaporated in vacuo. The residue was dissolved in $CHCl_3$, washed with water, and dried ($MgSO_4$). The product was isolated by column chromatography on silica gel (50 g, $CHCl_3$–MeOH 50:1) as colorless syrup; yield 674 mg (91%); ^{1}H NMR δ=7.1—7.5 (21H, aromatic H and NH); ^{13}C NMR δ=67.75 ($J_{P\text{-}O\text{-}C}$=6.1 Hz, C-6).

Benzyl L-Alanyl-D-isoglutaminate *N*-Acylated with Benzyl 2-Acetamido-2-deoxy-3-*O*-[(*R*)-1-carboxyethyl]-α-D-glucopyranoside 6-(Diphenyl Phosphate) (9). a) From **8**. Compound **8** (280 mg, 0.40 mmol) was dissolved in THF (8 ml) and hydrogenolyzed at room temperature in the presence of Pd-black for 2 h. The catalyst was filtered off and the solvent was evaporated in vacuo. The residue was again dissolved in THF (3 ml). L-Alanyl-D-isoglutamine benzyl ester trifluoroacetate (200 mg, 0.47 mmol), *N*-hydroxysuccinimide (HONSu) (55 mg, 0.48 mmol), triethylamine (65 μl, 0.47 mmol) and DCC (98 mg, 0.48 mmol) were added to the solution at 0 °C with stirring. The mixture was stirred overnight, being allowed to warm up to room temperature during this period. After usual work-up, the syrupy product obtained was subjected to column chromatography on silica gel (45 g, $CHCl_3$–MeOH 20:1) and recrystallized from $CHCl_3$–ether–hexane; yield 256 mg (71%); mp 106—108 °C. It was identical with the product obtained by the following method b).

b) From **10**. To a suspension of benzyl L-alanyl-D-isoglutaminate *N*-acylated with benzyl 2-acetamido-2-deoxy-3-*O*-[(*R*)-1-carboxyethyl]-α-D-glucopyranoside (**10**)[14] (336 mg, 0.50 mmol) in pyridine (3 ml) were added diphenyl hydrogenphosphate (250 mg, 1.0 mmol) and TPS chloride (302 mg, 1.0 mmol). The mixture had been stirred at room temperature for 3 h and worked up as usual. The crude product was subjected to silica-gel column chromatography (48 g). Elution with a mixture of $CHCl_3$–MeOH (10:1) first gave the nitrile derivative **11**, which was recrystallized from $CHCl_3$–ether; yield 100 mg (23%); mp 75—77 °C; IR (KBr) 2250 cm^{-1} (CN). Found: C, 60.67; H, 5.92; N, 6.16%. Calcd for $C_{45}H_{51}N_4O_{13}P$: C, 60.94; H, 5.80; N, 6.32%.

Further elution with the same solvent afforded **9** which was recrystallized from $CHCl_3$–ether: yield 235 mg (52%); mp 107—110 °C; $[\alpha]_D^{22}$ +57.2° (*c* 0.50, $CHCl_3$). Found; C, 59.29; H, 5.99; N, 6.03%. Calcd for $C_{45}H_{53}N_4O_{14}P\cdot 0.5H_2O$: C, 59.14; H, 5.96; N, 6.13%.

L-Alanyl-D-isoglutamine *N*-Acylated with 2-Acetamido-2-deoxy-3-*O*-[(*R*)-1-carboxyethyl]-D-glucose 6-Phosphate (2). Compound **9** (90 mg, 0.10 mmol) was dissolved in AcOH (5 ml) and stirred under atmospheric pressure of H_2 in the presence of Pd-black for 7 h. After catalyst had been filtered off, PtO_2 was added to the filtrate. The mixture was stirred under H_2 (5 kg cm^{-2}) at room temperature for 14 h. The catalyst was removed by filtration and the solvent was removed in vacuo. The residue was again dissolved in water. The solution was filtered through a Milipore filter (pore size 0.45 μm) and subjected to lyophilization to give colorless powder: yield 60 mg (quantitative); $[\alpha]_D^{22}$ +45.9° (*c* 0.51, MeOH). Found: C, 36.91; H, 6.04; N, 9.07; P, 4.73%. Calcd for $C_{19}H_{33}N_4O_{14}P\cdot 2.5H_2O$: C, 36.96; H, 6.20; N, 9.07; P, 5.02%.

The compound was dissolved in MeOH and treated with an ethereal solution of CH_2N_2. After evaporation of the solvent, the product was directly subjected to ^{1}H NMR measurement: (in $CDCl_3$–CD_3OD) δ=3.81 (6H, d, $J_{P\text{-}O\text{-}C\text{-}H}$= 11 Hz, $PO(OCH_3)_2$), 3.60 (3H, s, $COOCH_3$).

Octadecyl Phenyl Hydrogenphosphate (12a). A solution of 1-octadecanol (3.00 g, 11.1 mmol), phenyl dihydrogenphosphate (2.32 g, 13.3 mmol) and DCC (2.75 g, 13.3 mmol) in pyridine (30 ml) was stirred at room temperature for 13 h. Phenyl dihydrogenphosphate (0.97 g, 5.5 mmol) and DCC (1.14 g, 5.5 mmol) were added and the mixture was stirred for further 37 h. After usual work-up, the product was recrystallized from MeOH; yield 3.80 g (80%); mp 56—58 °C. Found: C, 67.71; H, 10.14%. Calcd for $C_{24}H_{43}O_4P$: C, 67.58; H, 10.16%.

Benzyl 2-Acetamido-3-*O*-[(*R*)-1-(benzyloxycarbonyl)ethyl]-2-deoxy-α-D-glucopyranoside 6-(Octadecyl Phenyl Phosphate) (13a). TPS chloride (1.28 g, 4.22 mmol) was added to a solution of the muramic acid benzyl ester **7** (1.00 g, 2.11 mmol) and octadecyl phenyl hydrogenphosphate (**12a**) (1.80 g, 4.22 mmol) in pyridine (20 ml). After the mixture had been stirred at room temperature for 23 h, the solvent was evaporated in vacuo and the residue subjected to silica-gel column chromatography (90 g, $CHCl_3$–MeOH 50:1) to give a syrupy product: yield 1.33 g (72%); FD-MS: *m/z* 882 $(M+H)^+$.

Benzyl L-Alanyl-D-isoglutaminate *N*-Acylated with Benzyl 2-Acetamido-2-deoxy-3-*O*-[(*R*)-1-carboxyethyl]-α-D-glucopyranoside 6-(Octadecyl Phenyl Phosphate) (14a). Compound **13a** (500 mg, 0.567 mmol) was dissolved in THF (15 ml) and hydrogenolyzed in the presence of Pd-black catalyst at room temperature for 45 min. After the catalyst had been filtered off, the solvent was once evaporated in vacuo and the residue was again dissolved in THF (5 ml). L-Alanyl-D-isoglutamine benzyl ester trifluoroacetate (300 mg, 0.711 mmol), HONSu (82 mg, 0.712 mmol), triethylamine (96 μl, 0.69 mmol), and DCC (140 mg, 0.679 mmol) were added to the solution cooled to 0 °C. The mixture was stirred overnight and worked up as usual. The product was purified by column chromatography on silica gel (50 g, $CHCl_3$–MeOH 10:1) and lyophilized from dioxane: yield 235 mg (38%); ^{1}H NMR δ=7.1—7.5 (15H, aromatic H), 5.09 (4H, s, $C_6H_5CH_2$–), 1.90 (3H, s, CH_3CO), 1.2—1.5 (32H, broad, $-CH_2$).

L-Alanyl-D-isoglutamine *N*-Acylated with 2-Acetamido-2-deoxy-3-*O*-[(*R*)-1-carboxyethyl]-D-glucose 6-(Octadecyl Hydrogenphosphate) (3a) and Its Dibenzyl Ester. Compound **14a** (50 mg, 4.6 μmol) was dissolved in MeOH (5 ml). After a few drops of AcOH and Pd-black catalyst had been added, the

mixture was stirred under H_2 atmosphere (5 kg cm^{-2}) at room temperature for 48 h. The catalyst was filtered off, and the solvent was evaporated in vacuo. The residue was again dissolved in MeOH (5 ml) and the solution stirred under H_2 (5 kg cm^{-2}) for 3 h after addition of PtO_2. The product was dissolved in water and purified by a column of Bio-gel P-2 (100—200 mesh, 17 ml). Lyophilization from AcOH afforded colorless powder; yield 27 mg (71%); $[\alpha]_D^{22}$ +25.1° (*c* 1.00, $CHCl_3$–MeOH 5:1). Found: C, 51.83; H,8.26; N, 6.79%. Calcd for $C_{37}H_{69}N_4O_{14}P \cdot 2H_2O$: C, 51.62; H, 8.55; N, 6.51%.

Compound **3a** obtained from **14a** (0.10 mmol) was dissolved in $CHCl_3$, washed once with 1M HCl and then treated with phenyldiazomethane. The product was purified by preparative TLC on silica gel and lyophilized from benzene to give the dibenzyl ester; yield 35 mg; ^{1}H NMR; δ=7.2—7.4 (10H, aromatic H), 5.08 (4H, $C_6H_5CH_2$). Found: C, 58.74; H, 7.98; N, 5.42; P, 2.52%. Calcd for $C_{51}H_{81}N_4O_{14}P \cdot 2H_2O$: C, 58.83; H, 8.23; N, 5.38; P, 2.97%.

2-Docosyl-1-tetracosanol. A mixture of 2-docosyltetracosanoic acid (5.00 g, 7.38 mmol) and concd H_2SO_4 (0.5 ml) in MeOH (50 ml) and CH_2ClCH_2Cl (50 ml) was heated under reflux for 5 h. After usual work-up, recrystallization from acetone afforded methyl 2-docosyltetracosanoate; yield 4.94 g (97%); mp 68—70°C.

To a suspension of this methyl ester (4.94 g, 7.15 mmol) in anhyd ether (200 ml) and anhyd THF (100 ml), a suspension of $LiAlH_4$ (271 mg, 7.15 mmol) in anhyd ether (75 ml) was added dropwise with stirring during 30 min. The mixture was heated under reflux for 3 h, $LiAlH_4$ (540 mg, 14.3 mmol) being added after 1.5 h. After usual work-up, the product was recrystallized from acetone; yield 4.46 g (94%); mp 65—67°C.

2-Docosyltetracosyl Phenyl Hydrogenphosphate (12b). DCC (1.66 g, 8.04 mmol) was added to a mixture of 2-docosyl-1-tetracosanol (4.46 g, 6.72 mmol) and phenyl dihydrogenphosphate (1.40 g, 8.04 mmol) in pyridine (50 ml). The mixture was stirred at room temperature for 55 h. Phenyl dihydrogenphosphate (1.17 g, 6.72 mmol) and DCC (1.38 g, 6.72 mmol) were added to the mixture and stirring was continued for further 16 h. After the mixture had been worked up as described for **12a**, the product and the unchanged 2-docosyl-1-tetracosanol were separated by means of silica-gel column chromatography (225 g, $CHCl_3$–MeOH 20:1 to 10:1).

2-Docosyl-1-tetracosanol recovered was recrystallized from acetone; 2.47 g (recovery 55%).

The product eluted from the column was dissolved in $CHCl_3$ and washed with 1M HCl and water. The residue obtained by evaporation of the solvent was recrystallized from acetone; yield 2.05 g (37%); mp 57—60°C. Found: C, 75.94; H, 12.06%. Calcd for $C_{52}H_{99}O_4P$: C, 76.23; H, 12.18%.

Benzyl 2-Acetamido-3-*O*-[(*R*)-1-(benzyloxycarbonyl)ethyl]-2-deoxy-α-D-glucopyranoside 6-(2-Docosyltetracosyl Phenyl Phosphate) (13b). TPS chloride (640 mg, 2.11 mmol) was added to a solution of muramic acid benzyl ester (**7**) (500 mg, 1.05 mmol) and **12b** (1.73 g, 2.11 mmol) in anhyd pyridine (15 ml). The mixture was stirred at room temperature for 48 h and worked up as described for **13a**. The product was isolated by column chromatography on silica gel (50 g, $CHCl_3$–MeOH 50:1) as colorless syrup; yield 550 mg (41%); FD-MS *m/z* 1175 $(M+H)^+$.

Benzyl L-Alanyl-D-isoglutaminate *N*-Acylated with Benzyl 2-Acetamido-3-*O*-[(*R*)-1-carboxyethyl]-α-D-glucopyranoside 6-(2-Docosyltetracosyl Phenyl Phosphate) (14b). Compound **13b** (545 mg, 0.427 mmol) was dissolved in THF (20 ml) and hydrogenolyzed in the presence of Pd-black catalyst for 1 h. The product was dissolved without purification in THF (7 ml). L-Alanyl-D-isoglutamine benzyl ester trifluoroacetate (270 mg, 0.641 mmol), HONSu (74 mg, 0.64 mmol), triethylamine (90 μl, 0.65 mmol) and DCC (106 mg, 0.514 mmol) were added to the solution. The mixture was stirred at room temperature for 17 h, worked up as described for **14a**. Silica-gel column chromatography (50 g, $CHCl_3$–MeOH 20:1) followed by lyophilization from dioxane afforded colorless powder; yield 233 mg (37%); $[\alpha]_D^{22}$ +34.0° (*c* 0.51, $CHCl_3$). Found: C, 67.92; H, 9.47; N, 3.88%. Calcd for $C_{85}H_{141}N_4O_{14}P$: C, 68.01; H, 9.67; N, 3.73%.

L-Alanyl-D-isoglutamine *N*-Acylated with 2-Acetamido-2-deoxy-3-*O*-[(*R*)-1-carboxyethyl]-D-glucose 6-(2-Docosyltetracosyl Hydrogenphosphate) (3b) and Its Dibenzyl Ester. Compound **14b** (60 mg, 41 μmol) was dissolved in a mixture of THF (20 ml)–MeOH (10 ml)–AcOH (1 ml) and hydrogenolyzed in the presence of Pd-black under H_2 (7 kg cm^{-2}) for 48 h. After removal of the catalyst, PtO_2 was added to the filtrate and again hydrogenolyzed at 7 kg cm^{-2} for 12 h. The solvent was evaporated in vacuo, the residue was dissolved in $CHCl_3$–MeOH (10:1) and filtered through a Fluoropore filter disk (pore size 0.45 μm) to remove small particles of insoluble materials. After evaporation of the solvent in vacuo, the residue was lyophilized from a mixture of benzene–AcOH to give **3b** as colorless powder; yield 48 mg (quantitative); $[\alpha]_D^{21}$ +18° (*c* 0.12, $CHCl_3$–MeOH 5:1).

The product (27 mg) was dissolved in $CHCl_3$ (10:1, 30 ml), washed with 1M HCl and treated with phenyldiazomethane. Purification by preparative TLC on silica gel followed by lyophilization from dioxane afforded the dibenzyl ester as colorless powder; yield 12.2 mg. Found: C, 67.29; H, 10.03; N, 4.06; P, 1.92%. Calcd for $C_{79}H_{137}N_4O_{14}P \cdot 0.5H_2O$: C, 67.45; H, 9.89; N, 3.98; P, 2.20%.

Tumor Suppression and Regression Tests. Antitumor effects of the muramyl dipeptide derivatives against Meth-A fibrosarcoma were evaluated by two distinct test systems using syngeneic BALB/c male mice (5 week-old, purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan). The suppression test was conducted according to the method of Yamamura et al.[15]: Briefly, a mixture of 2×10^5 tumor cells and a test compound in phosphate-buffered saline was inoculated intradermally into the right flank of mice. In the regression test,[18] mice were injected the same number of the tumor cells as above on day 0 and then given intratumor injection of a test compound seven times every other days from day 7. In both tests, mice were sacrificed three weeks after tumor cell inoculation and tumor growth inhibition was measured.[16]

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10) We first attempted to use 2-aminoethanol as a linkage between a long chain carboxylic acid and the phosphate moiety (see Ref. 8 above). However, when a 2-(acylamino)-ethyl phenyl hydrogenphosphate was subjected to coupling with the 6-hydroxyl group of muramic acid, the desired product could not be isolated in a pure state. It decomposed spontaneously losing the acyl moiety. Therefore we connected long-chain alcohols directly to the phosphate moiety in the present study.

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13) When dibenzyl hydrogenphosphate was used in place of diphenyl hydrogenphosphate, the desired 6-(dibenzyl phosphate) could not be isolated. TLC analysis of the reaction mixture indicated the disappearance of the starting material **7** and the presence of a polar product which was assumed to have a phosphate function because of the positive reaction against Dittmer-Lester reagent. This result could be explained by assuming that the desired product was once formed but decomposed because of the instability of the benzyl ester function in the phosphotriester.

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