Synthesis of 6-*O*-Acyl Derivatives of Immunoadjuvant Active *N*-Acetylmuramyl-L-alanyl-D-isoglutamine[1]

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Seven acyl derivatives of *N*-acetylmuramyl-L-alanyl-D-isoglutamine, which had previously been shown to be a minimum structure required for the immunoadjuvant activity, were synthesized in order to evaluate the significance of lipophilicity on the adjuvant activity and on the possible antitumor effect. Among them, are included 6-*O*-docosanoyl, stearoyl, lauroyl, octanoyl, butyryl, acetyl, and 4,6-di-*O*-acetyl derivatives. Some of these compounds were found to serve an improvement in the administration method to living body.

Synthetic studies by us[2] as well as Merser *et al.*[3] had established that a minimum structure required for the "immunoadjuvant activity" of bacterial cell walls is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (**1**), which corresponds to a common building unit of the cell wall peptidoglycans. The synthetic muramyl dipeptide (**1**) enhanced distinctly both humoral and cellular immunities of living bodies towards various antigens. Since that time, it became possible to discuss the immunopotentiating activity in a sense of molecular level. Meanwhile, we have already reported a synthesis of the labelled compound of **1** for the purpose of an elucidation of the action mechanism of such unique molecule.[4] Relationships between structure and activity of the muramyl peptides were also investigated by syntheses of various analogs of **1**.[2,5]

Our synthetic effort was next brought in focus on the following two subjects, namely, i) an improvement in the administration method of the muramyl dipeptide (**1**) which exhibited the adjuvant activity in the injection only in an emulsified form of water in mineral oil; ii) a possible appearance of an antitumor activity in derivatives of the muramyl peptides on an extended line of the adjuvant activity. It has long been known that even bacterial cell walls must be injected in a form of water in mineral oil emulsion for test of the adjuvant activity.[6] Synthetic muramyl dipeptide (**1**) also requires such physical form. These facts may indicate that the physical state predominates over the chemical entity in this biological action. Furthermore, the mineral oil emulsion causes a severe tissue reaction like the skin swelling in tested animals and may not be applicable to human beings. Therefore, a derivative of the muramyl peptide which can be used in a form other than emulsion to the living bodies has been desired not only for a clarification of physical character of the adjuvant active material, but also for the therapeutic purpose.[7] It could be assumed that the immunoactive substance must be anyhow transported to a lipophilic region in lymph system. A role of the mineral oil in the emulsion may be related to an importance of the lipophilicity in this action, although it is not quite clear.[8] Therefore, we anticipated that the use of the mineral oil could be avoided if enough lipophilicity is given to the molecule of muramyl dipeptide (**1**).

On the other hand, an antitumor effect of BCG cell wall has been understood on the basis of the immunoadjuvant activity.[9] Although the distinct adjuvant activity was observed in *N*-acetylmuramyl-L-alanyl-D-isoglutamine (**1**) as mentioned before, it did not show any antitumor activity so far.[10] One of the big differences between BCG cell wall and **1** itself must be in a lack of the lipophilicity in the latter, since the BCG cell wall is covered with a long-chain fatty acid, *i.e.*, mycolic acid, on the surface. Now, an assumption is made that the lipophilicity in the molecule may be required for the antitumor effect, if the muramyl peptide moiety is also essential to this activity.

In these respects, we planned to prepare several acyl derivatives of the muramyl dipeptide (**1**) for investigation of the biological activities. For the synthesis, the primary hydroxyl group at C-6 of muramic acid moiety in **1** was chosen for introduction of various acyl groups.[11]

Two alternative pathways for the synthesis of 6-*O*-acyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamines (**2**) were tested in terms of 6-*O*-stearoyl derivative (**2e**) to elucidate whether the muramic acid moiety should be first condensed with the acyl group (route A) or with the peptide moiety (route B).

The synthesis *via* route A was carried out by an introduction of stearoyl group to benzyl *N*-acetyl-α-muramide (**3**) prior to coupling with the peptide moiety. Selective stearoylation of the primary hydroxyl group in **3** was performed by use of mixed anhydirde with trifluoroacetic acid[12] though in a rather low yield. Thus, two monostearoyl products were isolated from the reaction mixture after silica gel column chromatography. From elemental analyses and spectral data, the slower moving product was assumed to be the desired 1-α-*O*-benzyl-6-*O*-stearoyl-*N*-acetylmuramic acid (**4**) while the faster one, without free carboxyl group in it, was determined to be an intramolecular ester (**5**) of the former. The 6- rather than 4-*O*-acyl structure for the compound **4** was deduced by its conversion into **5** by treatment with dicyclohexylcarbodiimide. Use of stearoyl chloride in the above acylation did not improve the yield of **4**. Condensation of stearoyl muramic acid (**4**) with L-alanyl-D-isoglutamine benzyl ester[2b] with dicyclohexylcarbodiimide–*N*-hydroxysuccinimide gave the protected stearoyl muramyl dipeptide (**7e**), *i.e.*, 1-α-*O*-benzyl-6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine benzyl ester, in a good yield. Hydrogenolytic deprotection of **7e** in acetic acid afforded 6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (**2e**).

Synthesis of the stearoyl derivative (**2e**) *via* route B

Table 1. Physical properties and analytical data of protected *O*-acyl-*N*-acetylmuramyl dipeptides obtained *via* route B

Compd	Yield from **6** (%)	Mp (°C)	$[\alpha]_D^{23}$ a)	Molecular formula	Found (Calcd) C%	H%	N%
7b	52	174—175	+72.8°	$C_{37}H_{50}O_{12}N_4 \cdot 1/2H_2O$	59.18 (59.11	6.80 6.84	7.52 7.45)
7cb)	66	175—177	+67.3°	$C_{41}H_{58}O_{12}N_4$	61.27 (61.64	7.30 7.32	7.00 7.01)
7d	55	172—175	+62.4°	$C_{45}H_{66}O_{12}N_4$	63.14 (63.20	7.85 7.78	6.40 6.55)
7f	58	171.5—173.5	+54.4°	$C_{55}H_{86}O_{12}N_4$	66.17 (66.37	8.69 8.71	5.43 5.63)

a) *c* 0.5 in $CHCl_3$. b) Recrystallized from acetone-ether.

Table 2. Physical properties and analytical data of *O*-acyl-*N*-acetylmuramyl dipeptides (**2a—f, 9**)a)

Compd	Mp (dec, °C)	$[\alpha]_D^{16}$ b)	R_fc)	Partitiond) coefficient $H_2O/CHCl_3$	Molecular formula	Found (Calcd) C%	H%	N%
2a	132—134	+38.6°	0.14	148	$C_{21}H_{34}O_{12}N_4 \cdot 1/2H_2O$	46.14 (46.40	6.31 6.49	10.17 10.31)
2b	105—107	+35.6°	0.18	56	$C_{23}H_{38}O_{12}N_4 \cdot 1/2H_2O$	48.47 (48.33	6.76 6.88	9.62 9.80)
2c	66— 72	+32.6°	0.22	45	$C_{27}H_{46}O_{12}N_4 \cdot H_2O$	51.22 (50.93	7.39 7.60	8.85 8.80)
2d	107—110	+35.6°	0.29	14 (13)	$C_{31}H_{54}O_{12}N_4 \cdot H_2O$	54.00 (53.74	7.94 8.15	8.07 8.09)
2e	133—136	+29.3°	0.35	27 (2)	$C_{37}H_{66}O_{12}N_4 \cdot 2H_2O$	55.99 (55.90	8.57 8.88	7.09 7.05)
2f	153—155	+37.0° e)	0.49	—	$C_{41}H_{74}O_{12}N_4 \cdot 1/2H_2O$	59.52 (59.75	9.28 9.18	6.54 6.80)
9	111—116	+31.2°	0.17	(55)	$C_{23}H_{36}O_{13}N_4 \cdot 1/2H_2O$	46.86 (47.17	6.17 6.37	9.55 9.57)

a) These values are obtained for substances isolated by lyophilization. b) *c* 0.5 in H_2O, 2 days after dissolution. c) Silica gel G, $CHCl_3$-CH_3OH-CH_3CO_2H (100 : 20 : 1). d) See experimental section. e) In THF-H_2O (10 : 1).

was then examined starting from the protected muramyl dipeptide,[2b] *i.e.*, 4,6-*O*-benzylidene-1-α-*O*-benzyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine benzyl ester. The benzylidene group of this compound was selectively removed in the usual manner with 60% acetic acid to afford 1-α-*O*-benzyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine benzyl ester (**6**), which was then treated with stearoyl chloride in a mixture of pyridine and tetrahydrofuran (THF). For the selective acylation of the primary hydroxyl group, a short reaction time and use of large excess of the chloride were preferable. When the reaction was stopped just after the disappearance of the starting material in the reaction mixture, **7e** was obtained as a practically sole product. The structure of **7e** was identified with the compound prepared above *via* route A by means of NMR and TLC. It was then converted into free stearoyl muramyl dipeptide (**2e**) similarly as described above.

Ample solubilities of 6-*O*-stearoyl derivative (**2e**) thus obtained both in chloroform and in water may indicate a detergent-like property of this compound, and actually its aqueous solution showed vigorous foaming in a procedure of evaporation *in vacuo*.[13] Since **2e** showed enough adjuvant activity in a preliminary test, we then synthesized various acyl derivatives of **1**, *i.e.*, acetyl, butyryl, octanoyl, lauroyl, and docosanoyl derivatives, in order to compare the effect of the acyl groups on the activity.

For the syntheses of these acyl derivatives, we adopted the route B rather than A, since the muramyl dipeptide derivative (**6**) can be used as a common synthetic intermediate in the former route and the intramolecular ester formation, which was observed in the route A, could be avoided by a prior introduction of the peptide moiety. On acetylation of **6** with acetic anhydride, 6-*O*-monoacetate (**7a**) was obtained at 0 °C and 4,6-di-*O*-acetate (**8**) at above room temperature. The protected 6-*O*-butyryl, octanoyl, lauroyl, and docosanoyl derivatives (**7b—d, f**) were prepared by use of the corresponding acid chlorides on the muramyl dipeptide (**6**) in a similar manner to that described for the stearoyl derivative (**7e**). Hydrogenolysis of **7a—d, f** and **8** afforded pure 6-*O*-acyl and 4,6-di-*O*-acetyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (**2a—d, f** and **9**) in quantitative yields, respectively. The physical properties and results of elemental analyses of the protected and free acyl muramyl dipeptides are summarized in Tables 1 and 2.

As shown in Table 2, the R_f values of the compounds increase with the carbon number of the acyl groups, indicating the augmentation in lipophilicity in this order. However, the partition coefficients of the series

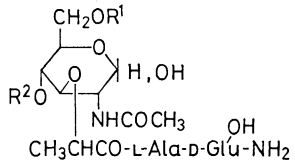
of the compounds between water and chloroform showed a peculiar phenomenon at the stearoyl derivative (**2e**). In Table 2, the values obtained by extraction from their aqueous solutions with chloroform are shown without parentheses, and those obtained reversely by extraction from chloroform solutions with water were indicated in parentheses. Whereas both values were practically equal in the case of lauroyl derivative (**2d**), the stearoyl derivative (**2e**) gave different values depending on the procedure used. This fact may suggest that **2e** could exist as micelles of different type of association either in chloroform or in water, thus resulting in the unusual behavior on extraction. The following observation also supports this assumption. In the NMR spectrum of **2e** in chloroform-*d*, no distinct signals were observed at all in the regions of protons due to the sugar peptide moiety around at δ 3—5, while those of fatty acid moiety were clearly recognized. However, when methanol-d_4 was added to the same solution, the former signals appeared instantaneously and a spectrum expected from the structure was finally obtained. This indicates that methanol could destroy the micellar structure of **2e** in chloroform, where the fatty acid moiety can interact with the solvent or can fluoctuate freely whereas the sugar peptide are rigidly fixed. The partition coefficient of docosanoyl derivative (**2f**) could not be determined by the above extraction procedure because of facile emulsion formation of the two phases. This might indicate the most effective detergent activity of this compound of all the acyl derivatives prepared.

The adjuvant activities of the 6-*O*-acyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (**2a**—**f**, **9**) thus obtained were investigated by Kotani *et al.* of our collaborative group.[14] Thus, 6-*O*-acetyl and 6-*O*-stearoyl derivatives (**2a** and **2e**) have activities comparable to that of the original *N*-acetylmuramyl dipeptide (**1**) while the other derivatives showed somewhat less activities when administered in a form of water in oil emulsion. The relatively high activity of **2e** seems to reflect an adequate balance of lipophilicity and hydrophilicity in the molecule, although the details of this relation are not yet clear. Furthermore, Kotani *et al.* found that highly lipophilic congeners, **2e** and **2f**, can manifest their adjuvant activities even if they are applied in liposomes to animals.[14] This finding will give an ample hope for the therapeutic use of these substances in the future.

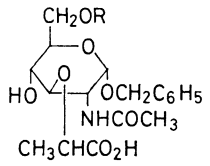
Unfortunately, no antitumor activity was found even in **2e**, which showed the maximum adjuvant activity among those prepared in this study. However, we further extended the study along this line and finally reached the synthesis of 6-*O*-mycoloyl derivative of the muramyl dipeptide (**1**)[15] which exhibited a distinct antitumor activity in the animal test. The results were already reported in a preliminary form.[16]

Experimental

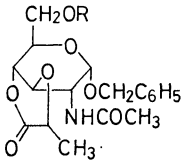
Melting points are uncorrected. All fatty acids were of commercial sources and purified by fractional distillation of their methyl esters if necessary. The purities of the acids were guaranteed over 99.9% by means of gas chromatography.



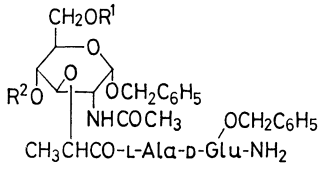
1 $R^1=R^2=H$
2a—**f** R^1=acyl, $R^2=H$
9 $R^1=R^2=CH_3CO$



3 R=H
4 R=stearoyl



5 R=stearoyl



6 $R^1=R^2=H$
7a—**f** R^1=acyl, $R^2=H$
8 $R^1=R^2=CH_3CO$

2 and **7**
a: R^1=acetyl, **b**: R^1=butyryl,
c: R^1=octanoyl, **d**: R^1=lauroyl,
e: R^1=stearoyl, **f**: R^1=docosanoyl.

Acid chlorides were prepared by refluxing the corresponding acids in thionyl chloride followed by distillation as usual. Silica gel 60 (0.063—0.2 mm) and G, Merck, were used for column chromatography and TLC, respectively.

Benzyl N-*Acetyl-α-muramide* (**3**). Benzyl 4,6-*O*-benzylidene-*N*-acetyl-α-muramide[17] (2.0 g, 4.2 mmol) was heated in 60% aqueous acetic acid (150 ml) at 100 °C for 35 min. After evaporation of the solvent *in vacuo*, benzene and ethanol were added to the residue and agin evaporated *in vacuo*. The residual colorless solid was recrystallized from ethyl acetate; yield, 1.35 g (84%); mp 156—157 °C; $[\alpha]_D^{22}$ +163° (*c* 0.5, methanol), (lit,[18] mp 160—161 °C; $[\alpha]_D^{20}$ +168° (*c* 1.25, methanol)).

Found: C, 56.22; H, 6.56; N, 3.66%. Calcd for $C_{18}H_{25}O_8N$: C, 56.39; H, 6.57; N, 3.65%.

1-α-O-Benzyl-6-O-stearoyl-N-acetylmuramic Acid (**4**). A mixture of stearic acid (0.90 g, 3.2 mmol) and trifluoroacetic anhydride (1.0 ml) was heated at 50 °C for 15 min and then dissolved in anhydrous THF (2 ml). This solution was added with pyridine (2 ml) to a stirred solution of **3** (1.0 g, 2.6 mmol) in anhydrous THF (8 ml) at −14 °C. After 35 min, the mixture was poured into 10% aqueous K_2CO_3 solution (25 ml) and set aside for 10 min. The solution was brought to pH 4 by addition of 6 M HCl and extracted with $CHCl_3$ (25 ml×2). The $CHCl_3$ layer was dried ($MgSO_4$), and evaporated *in vacuo*. The residue was subjected to silica gel column chromatography. Elution with benzene–ethyl acetate (1:1 v/v) gave a mixture of stearic acid and the intramolecular ester (**5**). Recrystallization from ether afforded pure **5**; yield, 0.45 g (27%); mp 122—123 °C.

Found: C, 67.92; H, 9.15; N, 2.24%. Calcd for $C_{36}H_{57}O_8N$: C, 68.43; H, 9.09; N, 2.22%.

Successive elution with $CHCl_3$–methanol–acetic acid (20:1:0.05 v/v) afforded a syrup, which was dissolved in 0.3 M aqueous NaOH (4 ml) and acidified with 1 M HCl to give **4** as a waxy solid; yield, 0.43 g (25%); mp 62—65 °C; $[\alpha]_D^{22}$ +61.6° (*c* 0.5, $CHCl_3$).

Found: C, 66.70; H, 9.39; N, 1.96%. Calcd for $C_{36}H_{59}O_9N$: C, 66.53; H, 9.15; N, 2.16%.

Conversion of **4** *into* **5**. Dicyclohexylcarbodiimide (16 mg, 0.076 mmol) was added to a solution of **4** (48 mg,

0.074 mmol) in anhydrous THF (2 ml) and the mixture was stirred at room temperature overnight. *N,N'*-Dicyclohexylurea was filtered off, the solvent was evaporated *in vacuo* and the residue was recrystallized from ether; yield, 44 mg (94%); mp 121—123 °C. This product was identified with the intramolecular ester (**5**) obtained above by means of IR, NMR and TLC ($CHCl_3$–methanol–acetic acid 30:1:0.3 v/v).

*1-α-O-Benzyl-*N*-acetylmuramyl-*L*-alanyl-*D*-isoglutamine Benzyl Ester (**6**).* 1-α-*O*-Benzyl-4,6-*O*-benzylidene-*N*-acetylmuramyl-L-alanyl-D-isoglutamine benzyl ester[2b] (2.72 g, 3.6 mmol) was heated in 60 % aqueous acetic acid (270 ml) on a boiling water bath for 30 min. After the procedure as described for **3**, the product was recrystallized from methanol–ether; yield, 1.77 g (71%); mp 221.5—223 °C (dec); $[\alpha]_D^{22}$ +93.6° (*c* 0.5, methanol).

Found: C, 58.42; H, 6.55; N, 8.27%. Calcd for $C_{33}H_{44}O_{11}N_4 \cdot 1/2CH_3OH$: C, 58.42; H, 6.73; N, 8.14%.

*1-α-O-Benzyl-6-O-stearoyl-*N*-acetylmuramyl-*L*-alanyl-*D*-isoglutamine Benzyl Ester (**7e**).* *i) From **4** (*via *route A*)*: To an ice-cooled solution of **4** (125 mg, 0.19 mmol) in anhydrous THF (5 ml), *N*-hydroxysuccinimide (45 mg, 0.39 mmol) and dicyclohexylcarbodiimide (39 mg, 0.19 mmol) were added. After the mixture had been stirred in an ice bath for 2 h, *N,N'*-dicyclohexylurea formed was filtered off. The filtrate was again cooled, L-alanyl-D-isoglutamine benzyl ester hydrochloride[2b] (66 mg, 0.19 mmol) and triethylamine (0.027 ml, 0.22 mmol) were added, and the final mixture was stirred overnight. After the usual work-up the product was recrystallized from ethyl acetate; yield, 122 mg (68%); mp 167—169 °C; $[\alpha]_D^{15}$ +58.9° (*c* 1, $CHCl_3$).

Found: C, 64.97; H, 8.35; N, 5.96%. Calcd for $C_{51}H_{78}O_{12}N_4$: C, 65.21; H, 8.37; N, 5.97%.

*ii) From **6** (route B)*: Stearoyl chloride (3.0 g, 10 mmol) in anhydrous THF (30 ml) was added to a solution of **6** (1.34 g, 2.0 mmol) in pyridine (100 ml) and anhydrous THF (90 ml) with stirring, The reaction mixutre was kept at 12 °C and further amount of stearoyl chloride (4.2 g, 14 mmol) was added within 3 h. The remaining chloride was destroyed with addition of ice. After evaporation of the solvent *in vacuo*, the mixture was diluted with water and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with 1 M HCl and aqueous NaCl solution. It was then dried ($MgSO_4$) and evaporated *in vacuo*. The residue was extracted with hot hexane to remove excess stearic acid. The insoluble material was subjected to silica gel column chromatography. Elution with $CHCl_3$–methanol (20:1 v/v) followed by recrystallization from methanol afforded **7e**, which was identical with the sample obtained above in (i) by means of NMR and TLC ($CHCl_3$–methanol 9:1 v/v); yield, 1.03 g (55%); mp 174.5—175.5 °C.

Found: C, 65.13; H, 8.40; N, 5.86%.

*6-O-Stearoyl-*N*-acetylmuramyl-*L*-alanyl-*D*-isoglutamine (**2e**).* A solution of **7e** (253 mg, 0.27 mmol) in acetic acid (20 ml) was hydrogenolyzed at room temperature in the presence of palladium black. After the catalyst had been filtered off and washed with water, the filtrate and the washing were combined and then subjected to lyophilization to afford colorless solid; yield, 204 mg (95%); mp 133—136 °C (dec); $[\alpha]_D^{16}$ +29.3° (*c* 1, H_2O, 2 days after dissolution).

Found: C, 55.99; H, 8.57; N, 7.09%. Calcd for $C_{37}H_{66}O_{12}N_4 \cdot 2H_2O$: C, 55.90; H, 8.88; N, 7.05%.

*6-O-Acetyl-1-α-O-benzyl-*N*-acetylmuramyl-*L*-alanyl-*D*-isoglutamine Benzyl Ester (**7a**).* Acetic anhydride (0.20 ml, 2.1 mlmo) was added to a stirred solution of **6** (200 mg, 0.30 mmol) in pyridine (4 ml) under ice-cooling. After 1 h, ice was added and the mixture was worked up as usual. Recrystallization was effected from methanol; yield, 130 mg (59%); mp 184—185 °C; $[\alpha]_D^{22}$ +72.3° (*c* 0.5, $CHCl_3$).

Found: C, 57.14; H, 6.58; N, 7.65%. Calcd for $C_{35}H_{46}O_{12}N_4 \cdot H_2O$: C, 57.36; H, 6.60; N, 7.65%.

*4,6-Di-O-acetyl-1-α-O-benzyl-*N*-acetylmuramyl-*L*-analyl-*D*-isoglutamine Benzyl Ester (**8**).* Acetic anhydride (0.20 ml, 2.1 mmol) was added to a stirred solution of **6** (160 mg, 0.24 mmol) in pyridine (3 ml) under ice-cooling. The mixture was then stirred at 45 °C for 3.5 h, worked up as usual and the product was recrystallized from ethanol; yield, 118 mg (65%); mp 219—220 °C; $[\alpha]_D^{22}$ +78.7° (*c* 0.5, $CHCl_3$).

Found: C, 58.87; H, 6.45; N, 7.45%. Calcd for $C_{37}H_{48}O_{13}N_4$: C, 58.72; H, 6.39; N, 7.40%.

*6-O-Acyl-1-α-O-benzyl-*N*-acetylmuramyl-*L*-alanyl-*D*-isoglutamine Benzyl Ester (**7b—d, f**).* These compounds were prepared from the corresponding acid chlorides and **6** in similar manners to that described for **7e** (route B). Selective monoacylation with excess of butyryl, octanoyl and lauroyl chlorides were better carried out under ice-cooling, whereas reaction with stearoyl and docosanoyl chlorides were performed at 12—15 °C. The latter two chlorides were not dissolved sufficiently in a mixture of THF and pyridine at low temperature. In all cases, the products were pure enough after single recrystallization from ethyl acetate, so that no chromatographic purification was needed. The yields, physical constants and results of elemental analyses of the products are summarized in Table 1.

*6-O-Acyl and 4,6-Di-O-acetyl-*N*-acetylmuramyl-*L*-alanyl-*D*-isoglutamine (**2a—d, f, and 9**).* These compounds were prepared by catalytic hydrogenolysis of **7a—d, f** and **8** in acetic acid followed by lyophilization as described above for **2e**. The yields, physical constants and results of elemental analyses are summarized in Table 2.

*Determination of Partition Coefficients of **2a—f** and **9**.* The compound **2a—f** and **9** (2 mg each) were dissolved in water (5.0 ml) in a test tube (1.2×15.5 cm), respectively. After $CHCl_3$ (5.0 ml) had been added, the tube was stoppered, gently rotated upside down 50 times, and then set aside overnight. The aqueous phase was withdrawn with a pipette. The $CHCl_3$ layer was once evaporated *in vacuo*, and the residue was dissolved in an exact amount of water. The relative amounts of the substances in aqueous and $CHCl_3$ layer were determined colorimetrically by modified Morgan-Elson method[19] described for *N*-acetylglucosamine. In some cases the substances were first dissolved in $CHCl_3$ and then extracted with water. The results are summarized in Table 2.

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