213. Synthesis and Spectroscopic Characterization of N^2-(*N*-Acetyl-6-*O*-stearoyl-α-D-muramoyl)-L-alanyl-D-isoglutamine

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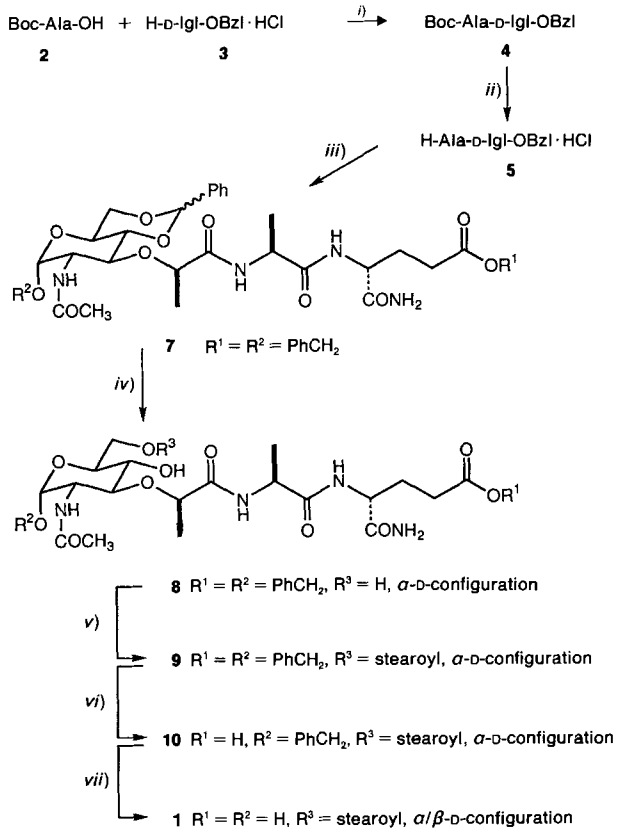
Title compound **1** was synthesized by a published route which had to be modified (seven steps from readily obtainable starting materials). Characterization of **1** was achieved by spectroscopic means (FAB-MS, ^{1}H-NMR, including 2D-COSY). Furthermore, commercially available reference material purchased for comparison, was unequivocally established to be **10**, *i.e.* incompletely deprotected **1**.

Introduction. – Immunoadjuvant activity of mycobacterial cells was first described by *Freund* in 1956 [1]. *N*-Acetyl-D-muramoyl-L-alanyl-D-isoglutamine (so-called muramyl dipeptide, MDP) as part of a larger cell-wall species was later found to be the minimal adjuvant-active structure [2] [3] and was first synthesized by *Merser et al.* [4]. Subsequently, numerous MDP analogs were prepared and tested for activity (*cf.* reviews [5–7] and ref. cit. therein; for some recent papers, see [8–11]).

In the course of our work, we needed reasonable amounts of N^2-(*N*-acetyl-6-*O*-stearoyl-D-muramoyl)-L-alanyl-D-isoglutamine (**1**), a lipophilic analog of MDP. At first sight this seemed to be a rather easy task, because the synthesis had already been published [12], and reference material was commercially available. One step, however, did not yield the desired intermediate as claimed in the published synthesis. After appropriate modification of this step and termination of the synthesis, we obtained a product with the same TLC behaviour as the reference. Neither of the two, however, showed further analytical data consistent with the structure of **1**! We, therefore, decided to investigate the problem in more depth, especially with regard to ^{1}H-NMR characterization of **1** and some of its precursors which, to the best of our knowledge, had not yet been published (for papers dealing with spectroscopy or showing detailed spectroscopic data of MDP and analogs (other than **1**), see [14] [15] (MS) and [16] [17] (NMR)).

Results and Discussion. – Synthesis of **1** was performed according to *Kusumoto et al.* [12] [13] with some modifications (*cf. Scheme 1*), starting with the appropriate amino-acid derivatives **2** and **3** (Igl = isoglutamine = 4-amino-4-carbamoylbutanoic acid). It led *via* **4** and **5**, and using *N*-acetyl-1-*O*-benzyl-4,6-*O*-benzylidene-α-D-muramic acid (= 2-acetamido-1-*O*-benzyl-4,6-*O*-benzylidene-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-α-D-glucopyranose; **6**), to the fully protected MDP **7** in three steps of classical peptide chemistry. Subsequent cleavage of the benzylidene group with AcOH/H_2O yielded the key intermediate **8**. Next, *Kusumoto*'s [12] acylation procedure (reaction of **8** with a five-fold

Scheme



i) Mixed anhydride in AcOEt/DMF. *ii*) AcOH, HCl in AcOEt. *iii*) **6**, as *i*). *iv*) 60% AcOH/H_2O. *v*) Stearic anhydride, 4-(dimethylamino)pyridine in DMF/CH_2Cl_2. *vi*) AcOH, Pd, 4 h. *vii*) AcOH, Pd, 52 h.

excess of stearoyl chloride (= octadecanoyl chloride = SteCl) in THF/pyridine) was repeated. Instead of the desired compound **9**, a more lipophilic product was obtained. Obviously, this was the 4,6-di-*O*-stearoyl analog of **9** (M_r(monoisotopic) 1204.8; FAB-MS: 1205 ($[M + H]^+$); elemental analysis in accordance with $C_{69}H_{112}N_4O_{13}$; details not shown), formed by 'overacylation' under the given reaction conditions. By changing to less forced conditions (1.5 equiv. of stearic anhydride/4-(dimethylamino)pyridine in DMF/CH_2Cl_2), compound **9** could then be easily prepared. Finally, two remaining protecting groups had to be removed by hydrogenolysis. This was effected in AcOH with Pd black as catalyst. After a reaction time of 4 h, all **9** had disappeared and a new product had been formed which co-migrated on TLC with the reference sample. Workup and purification by FC gave a compound which was believed to be target compound **1** (see *Exper. Part*). But neither elemental analysis nor FAB-MS matched theory for the compound synthesized or the reference material! More detailed analysis (UV and ^{1}H-NMR) showed both compounds to be the benzyl ether **10**. Hydrogenolysis, therefore, only

cleaved the benzyl ester without affecting the benzyl-ether function (for other examples of chemoselective deprotection of benzyl esters in the presence of benzyl ethers, see [18])[1]). Benzyl ether **10** was finally resubmitted to hydrogenolysis for 52 h (AcOH/Pd black). Under these conditions, **10** was transformed into a more polar compound with analytical data as expected for **1**.

The ^{1}H-NMR data of **1** and its precursors **8**–**10** are compiled in the *Table*. The assignment of the chemical shifts is based on 2D-COSY experiments, except for **9**, which shows a poorly resolved spectrum, probably due to slow conformational changes. FAB-Mass spectra confirm the structures.

The ^{1}H-NMR spectrum of **1** clearly shows the presence of two isomers (*cf. Fig.*), which are easily identified to be the expected mixture of epimers at the anomeric center. Both the major and the minor component are unequivocally assigned by the chemical shifts (4.95 and 4.44 ppm for α-D- and β-D-**1**, resp.) and the coupling constants (3.4 and 8.3 Hz for α-D- and β-D-**1**, resp.) of the signals for the H at the anomeric C-atom (H–C(1)(Mur)) in each isomer. Noteworthy is the resolved J of 6.3 Hz between H–C(1)(Mur) and the corresponding OH–C(1) in β-D-**1**, whereas in the case of α-D-**1** as well as in the other α-D-configurated compounds, only broadened lines for H–C(1) are observed. The ratio α-D-**1**/β-D-**1** is estimated to be *ca.* 6:1. Surprisingly the corresponding signals for *Me*CHO–C(3)(Mur) and *Me*CH(2) (Ala) of all muramyl-dipeptide derivatives investigated nearly coincide, irrespective of the solvent used or the variations in structure.

The FAB-MS of **1** and its synthetic precursors also merit a brief discussion. The FAB-MS normally show a prominent peak for the protonated compound accompanied occasionally, especially in the case of peptides in the 3-nitrobenzyl alcohol (NBA) matrix, by a peak of higher mass corresponding to the $[M + Na]^+$ cation [22]. Although no Na ions are added, these compounds obviously tend to associate with the ubiquitous Na ions and accumulate preferentially at the surface of the matrix. This frequently encountered behaviour is observed in the case of **8** (signal intensity of $[M + Na]^+$ *ca.* 17% of the intensity of $[M + H]^+$). Compounds **1**, **9**, and **10**, however, show peak intensities for $[M + Na]^+$ which clearly exceed the one for $[M + H]^+$, the latter being an extreme case, since the $[M + H]^+$ peak is completely absent (*cf. Exper. Part*). In addition, a peak corresponding to $[M + K]^+$ is observed. The anal. data for the compounds allow only for a minor contamination of the samples with alkali ions, and a blank spectrum of the matrix containing [18]crown-6 ether shows only traces of Na ions. Contrary to a recent report [22] dealing with other peptidic compounds, we conclude that the alkali-ion contamination of the samples in this investigation clearly shows a beneficial effect on the signal intensities.

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Experimental Part

General. Chemicals and solvents were from *Aldrich* or *Fluka*, unless stated otherwise, and 3.8N HCl in AcOEt, stearic anhydride, Boc-Ala-OH (**2**), H-D-Igl-OBzl·HCl (**3**), and *N*-acetyl-1-*O*-benzyl-4,6-*O*-benzylidene-α-D-muramic acid (**6**) from *Bachem Feinchemikalien AG*, Bubendorf, Switzerland. The reference sample of **1** (which, however, was **10**) was commercially available (US origin). Flash chromatography (FC): according to [19]; *Baker* silica gel *60* (32–62 μm). HPLC: $(Et_3NH)_3PO_4$ (pH 2.25) and 0.1% CF_3COOH buffer systems according to [20]; anal.: *LDC/Milton Roy* with *C18*/5 μm silica gel in 4.6 × 250-mm columns (*Bakerbond 7104-00* or *Vydac 218TP54*); prep.: *Waters LC 3000* with 5 × 30-cm cartridges (*Vydac 218TPB* 15–20 μm). TLC: *Merck* (Art. *5719*) precoated plates; solvent systems: *A*, $CHCl_3$/MeOH 9:1; *B*, $CHCl_3$/MeOH/32% aq. AcOH 15:4:1; *C*, $CHCl_3$/MeOH/AcOH 90:8:2; *D*, $CHCl_3$/MeOH/AcOH 85:10:5; visualization of spots by UV, ninhydrine, or chlorine-tolidine [21]. M.p.: *Büchi 512*; not corrected. Optical rotation: *Perkin-Elmer-241* polarimeter. UV Spectra (MeOH): *Shimadzu UV-160*; in nm (ε). NMR: *Bruker AM-360*. MS: *VG Micromass 70/70E*; FAB (fast-atom bombardment): Ar-atoms and 3-nitrobenzylalcohol matrix. Elemental analyses: *Heraeus CHN-O-RAPID*.

Benzyl N^2-*[(*tert-*Butoxy)carbonyl]*-L-*alanyl*-D-*isoglutaminate* (**4**). To a chilled (0°) soln. of **2** (23.65 g, 125 mmol) in AcOEt (380 ml) and DMF (20 ml) was added *N*-methylmorpholine (15.36 ml, 137.5 mmol). The soln. was

[1]) Thus, **10** could act as valuable intermediate whenever elongation of the peptide chain is desired.

Table. *^{1}H-NMR Data of Muramyl-Dipeptide Derivatives* **1** *and* **8–10**

	α-D-**1** ((D_6)DMSO)	β-D-**1** ((D_6)DMSO)	**8** ((D_6)DMSO)	**9** ($CDCl_3$)	**10** ($CDCl_3$)
CO_2H (Igl)	12.1 (very. br.)	[a])	–	–	[c])
PhCH_2OOC	–	–	5.06 (*s*)	5.10 (*d*, *J* = 12.3), 5.06 (*d*, *J* = 12.3)	–
NH (Igl)	8.18 (br.)	8.13 (br.)	8.14 (*d*, *J* = 8.3)	7.36 (br.)	7.72 (very br.)
NH (Mur)	8.07 (*d*, *J* = 7.4)	7.94 (*d*, *J* = 8.0)	8.09 (*d*, *J* = 8.3)	6.39 (*d*, *J* = 8.5)	6.84 (very br.)
NH (Ala)	7.63 (*d*, *J* = 7.0)	7.50 (*d*, *J* = 6.8)	7.57 (*d*, *J* = 7.0)	7.17 (*d*, *J* = 6.3)	6.53 (very br.)
NH_2 (Igl)	7.30 (*s*), 7.04 (*s*)	[a]), 7.09 (*s*)	7.35[b]), 7.09 (*s*)	6.82 (br.), 5.81 (br.)	[c])
OH–C(1) (Mur) or PhCH_2O–C(1)	6.72 (br.)	6.74 (*d*, *J* = 6.3)	4.66 (*d*, *J* = 12.5), 4.43 (*d*, *J* = 12.5)	4.67 (*d*, *J* = 11.7), 4.46 (*d*, *J* = 11.7)	4.68 (*d*, *J* = 12), 4.45 (*d*, *J* = 12)
OH–C(4) (Mur)	5.49 (br.)	[a])	5.25 (very br.)	3.94 (br.)	[c])
H–C(1) (Mur)	4.95 (*d*, *J* = 3.4)	4.44 (*dd*, *J* = 8.3, 6.3)	4.73 (*d*, *J* = 3.5)	4.94 (*d*, *J* = 3.7)	4.89 (br.)
H–C(2) (Ala)	4.28[b])	[a])	4.25 (*quint.*, *J* = 7.0)	4.16 (*m*)	4.32 (br.)
MeC*H*O–C(3) (Mur)	4.26[b])	[a])	4.26 (*q*, *J* = 6.7)	4.16 (*m*)	4.32 (br.)
H_A–C(6) (Mur)	4.27[b])	4.30[b])	3.65 (*d*, *J* = 10.0)	4.39 (*dd*, *J* = 12.2, 4.8)	4.32 (br.)
H–C(2) (Igl)	4.14 (*m*)	[a])	4.18 (*ddd*, *J* = 8.9, 8.3, 4.9)	4.35 (*dd*, *J* = 8.6, 4.6)	4.17 (br.)
H_B–C(6) (Mur)	4.04 (*dd*, *J* = 11.8, 5.7)	4.02[b])	*ca.* 3.5[b])	4.25 (*dd*, *J* = 12.2, 2.0)	4.32 (br.)
H–C(5) (Mur)	3.81(*ddd*, *J* = 10.3, 5.0, 2.0)	3.37 (obsc.)	*ca.* 3.5[b])	3.79 (*ddd*, *J* = 9.7, 4.7, 2.0)	3.84 (br.)
H–C(2) (Mur)	3.68 (*ddd*, *J* = 10.8, 7.4, 3.4)	3.51 (obsc.)	3.80 (*ddd*, *J* = 10.7, 8.3, 3.5)	4.12 (*ddd*, *J* = 10.2, 8.5, 3.7)	4.17 (br.)
H–C(3) (Mur)	3.46 (*dd*, *J* = 10.8, 8.8)	[a])	*ca.* 3.5[b])	3.53 (*dd*, *J* = 10.2, 8.8)	3.53 (br.)
H–C(4) (Mur)	3.25 (*dd*, *J* = 10.3, 8.8)	[a])	3.29 (*t*, *J* = 8.6)	3.44 (*ddd*, *J* = 9.7, 8.8, 4.2)	3.45 (br.)
CH_2(2) (Ste)	2.28 (*t*, *J* = 7.3)	[a])	–	2.35 (*dd*, 8.4, 6.9)	2.32 (*m*)
CH_2(4) (Igl)	2.19 (*t*, *J* = 7.7)	[a])	2.34 (*t*, *J* = 7.9)	2.51 (*dt*, *J* = 17.1, 7.4) 2.40 (*dt*, *J* = 17.1, 6.7)	2.32 (*m*)
H_A–C(3) (Igl)	1.94 (*m*)	[a])	2.00 (*m*)	2.14 (*m*)	2.04 (*m*)
*A*cNH–C(2) (Mur)	1.78 (*s*)	1.77 (*s*)	1.77 (*s*)	1.89 (*s*)	1.91 (*s*)
H_B–C(3) (Igl)	1.69 (*m*)	[a])	1.74 (*m*)	1.95 (*m*)	1.88[b])
CH_2(3) (Ste)	1.50 (*m*)	[a])	–	1.61 (*m*)	1.60 (*m*)
Me–C(2) (Ala)	1.24[b])	[a])	1.19 (*d*, *J* = 7.0)	1.37 (*d*, *J* = 7.0)	1.34 (br.)
(CH_2) (Ste)	1.22 (br., 28 H)	[a])	–	1.22 (br., 28 H)	1.22 (br., 28 H)
*Me*CHO–C(3) (Mur)	1.21[b])	[a])	1.23 (*d*, *J* = 6.7)	1.35 (*d*, *J* = 6.7)	1.34 (br.)
Me (Ste)	0.84 (*t*, *J* = 7.0)	[a])	–	0.86 (*t*, *J* = 7.0)	0.87 (*t*, *J* = 7.0)
Ph	–	–	7.2–7.4 (*m*, 10 H)	7.2–7.4 (*m*, 10 H)	7.2–7.4 (*m*, 5 H)

[a]) Resonance covered by the corresponding signal of the major isomer.
[b]) Resonance covered by other signals, chemical shift deduced from 2D-spectrum.
[c]) Resonance not observed due to chemical exchange.

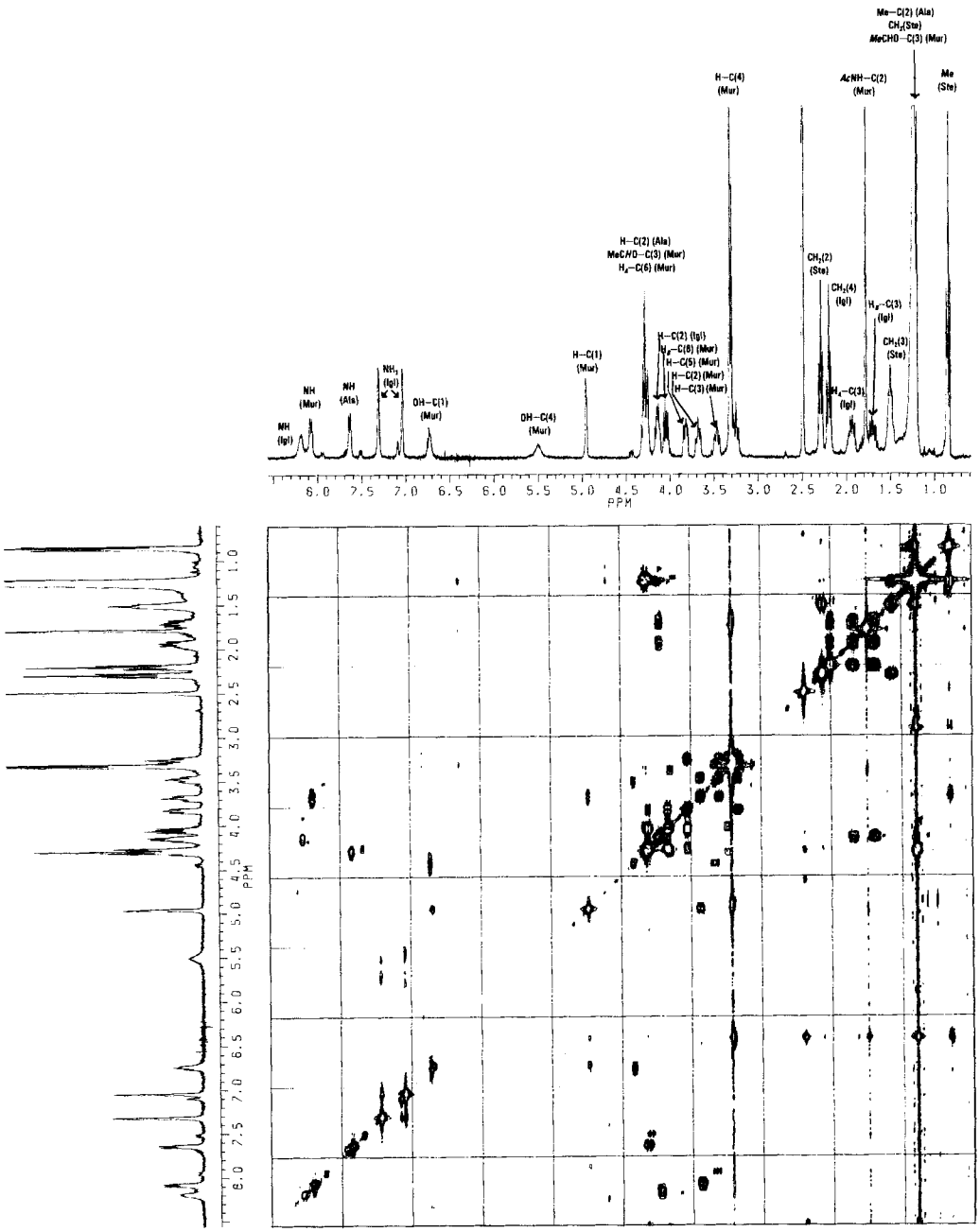

Figure. *2D-COSY(90) of* **1** *in* (D_6)*DMSO at 360 MHz.*
Assignments given only for the major α-D-isomer.

further cooled to −25° and isobutyl chloroformate (17.97 ml, 137.5 mmol) added. After stirring for 5 min, a freshly prepared soln. of **3** (34.1 g, 125 mmol) and *N*-methylmorpholine (13.96 ml, 125 mmol) in DMF (100 ml) was added. Stirring was continued for 2 h without cooling. At that time the mixture was diluted with 700 ml of AcOEt, subsequently washed with H_2O, cold 0.5N aq. HCl, 5% aq. Na_2CO_3 soln., and brine, and dried (Na_2SO_4). The resulting soln. was concentrated *in vacuo* to a smaller volume, diluted with Et_2O (700 ml), and stored in the refrigerator overnight. The crystallized product was collected, washed with Et_2O, and recrystallized from AcOEt/Et_2O: 37.7 g (74%) of **4**. Colorless solid. TLC (*A*): R_f 0.52. M.p. 139–140° ([13]: 137.5–138.5°). $[\alpha]_D^{24} = -9.1$ ($c = 1$, MeOH; [13]: $[\alpha]_D^{28} = -8.1$ ($c = 1$, MeOH)). Anal. calc. for $C_{20}H_{29}N_3O_6$ (407.47): C 58.95, H 7.17, N 10.31; found: C 58.88, H 7.18, N 10.42.

Benzyl L-*Alanyl*-D-*isoglutaminate Hydrochloride* (**5**). To a soln. of **4** (32.6 g, 80 mmol) in AcOH (125 ml), 3.8N HCl in AcOEt (125 ml) was added. After 2 h (TLC (*A*): reaction complete), the soln. was evaporated and the oil dissolved in H_2O and concentrated again. This procedure was repeated twice with H_2O and 3 times with abs. EtOH. The remaining residue was crystallized from abs. EtOH/Et_2O: 27.0 g (97%) of **5**. White solid. TLC (*B*): R_f 0.25. M.p. 154–156° ([13]: 149.5–151°). $[\alpha]_D^{23} = 10.4$ ($c = 2$, EtOH; [13]: $[\alpha]_D^{28} = 10.8$ ($c = 2.1$, EtOH)). Anal. calc. for $C_{15}H_{21}N_3O_4 \cdot HCl$ (343.78): C 52.40, H 6.45, N 12.22; found: C 52.18, H 6.70, N 11.95.

Benzyl N^2-(N-*Acetyl*-1-O-*benzyl*-4,6-O-*benzylidene*-α-D-*muramoyl*)-L-*alanyl*-D-*isoglutaminate* (**7**). A soln. of **6** (5.66 g, 121 mmol) in AcOEt/DMF 6:4 (100 ml) was cooled to 0°: *N*-Methylmorpholine (1.47 ml, 13.2 mmol) was added, the mixture further cooled to −25°, and isobutyl chloroformate (1.74 ml, 13.2 mmol) added. After 5 min of activation time, a freshly prepared soln. of **5** (4.13 g, 12 mmol) and *N*-methylmorpholine (1.33 ml, 12 mmol) in AcOEt/DMF 2:1 (30 ml) was added and stirring continued at r.t. overnight. During this time, the product partly precipitated. Precipitation was completed by adding H_2O (500 ml). The product was filtered off, washed with H_2O (5×) and Et_2O (2×), and dried to constant weight in a desiccator: 8.72 g (95%) of **7**. White solid. TLC (*C*): R_f 0.30. M.p. 235–238° (dec.; [13]: 226.5–227.5° (dec.)). $[\alpha]_D^{25} = 90.4$ ($c = 1$, DMF; [13]: $[\alpha]_D^{25} = 88.5$ ($c = 2.1$, DMF)). Anal. calc. for $C_{40}H_{48}N_4O_{11}$ (760.78): C 63.14, H 6.36, N 7.36; found: C 63.04, H 6.50, N 7.34.

Benzyl N^2-(N-*Acetyl*-1-O-*benzyl*-α-D-*muramoyl*)-L-*alanyl*-D-*isoglutaminate* (**8**). A suspension of **7** (4.18 g, 5.5 mmol) in 60% AcOH/H_2O (400 ml) was heated for 30 min on a boiling H_2O bath (→clear soln.; TLC (*C*): no **7** left). The solvent was evaporated and the crude **8** (3.25 g, 88%) purified by prep. HPLC (CF_3COOH buffer): 2.19 g (60%; no reprocessing of side fractions) of **8**. White lyophilisate. TLC (*B*): R_f 0.45. Anal. HPLC ($(Et_3NH)_3PO_4$ buffer): 94%. $[\alpha]_D^{22} = 92.2$ ($c = 0.26$, MeOH; [12]: $[\alpha]_D^{22} = 93.6$ ($c = 0.5$, MeOH)). UV: 258(350). ^{1}H-NMR ((D$_6$)DMSO): *Table*. FAB-MS: 695.5 (15, $[M + Na]^+$), 673.5 (90, $[M + H]^+$), 566.5 (29, $[M - PhCH_2O]^+$), 437 (22), 409 (11), 380 (23), 363 (24), 329 (19), 301 (21), 289 (23), 237 (36), 192 (84), 186 (58), 144 (98), 126 (100).

Benzyl N^2-(N-*Acetyl*-1-O-*benzyl*-6-O-*stearoyl*-α-D-*muramoyl*)-L-*alanyl*-D-*isoglutaminate* (**9**). A soln. of **8** (0.86 g, 1.28 mmol) in DMF (15 ml) was mixed with a soln. of stearic anhydride (1.06 g, 1.92 mmol) and 4-(dimethylamino)pyridine (31 mg, 0.26 mmol) in CH_2Cl_2 (26 ml) and stirred overnight (TLC (*C*): no **8** left). The mixture was evaporated and the remaining oil purified by FC (*C*): 0.79 g (66%) of **9**. White powder. TLC (*C*): R_f 0.48. $[\alpha]_D^{15} = 59.5$ ($c = 1$, $CHCl_3$; [12]: $[\alpha]_D^{15} = 58.9$ ($c = 1$, $CHCl_3$)). UV: 258(377). ^{1}H-NMR ($CDCl_3$): *Table*. FAB-MS: 961.4 (29, $[M + Na]^+$), 939.5 (12, $[M + H]^+$), 831.6 (25, $[M - PhCH_2O]^+$), 704 (13), 596 (10), 568 (14), 497 (8), 471 (13), 453 (33), 435 (18), 380 (58), 363 (52), 339 (16), 267 (68), 237 (100), 234 (70). Anal. calc. for $C_{51}H_{78}N_4O_{12}$ (939.13): C 65.21, H 8.37, N 5.97; found: C 65.08, H 8.49, N 6.30.

N^2-(N-*Acetyl*-1-O-*benzyl*-6-O-*stearoyl*-α-D-*muramoyl*)-L-*alanyl*-D-*isoglutamine* (**10**). A mixture of **9** (0.25 g, 0.27 mmol) and Pd black (25 mg) in AcOH (10 ml) was hydrogenated (r.t./1 atm) for 4 h (TLC (*D*) monitoring). The catalyst was filtered off, the filtrate lyophilized, and the crude **10** (0.24 g) purified by FC (*D*): 185 mg (92%) of **10**. White lyophilisate. TLC (*D*): R_f 0.50. UV: 258(203). ^{1}H-NMR ($CDCl_3$): *Table*. FAB-MS: 887.5 (11, $[M + K]^+$), 871.6 (100, $[M + Na]^+$), 742 (12, $[M - PhCH_2O]^+$), 698 (8), 568 (13), 471 (11), 453 (25), 340 (17). Anal. calc. for $C_{44}H_{72}N_4O_{12} \cdot H_2O$ (867.06): C 60.95, H 8.60, N 6.46; found: C 60.75, H 8.77, N 6.80.

The reference sample for **1** showed anal. data as above. Its structure, therefore, is **10**, not **1**!

N^2-(N-*Acetyl*-6-O-*stearoyl*-α-D-*muramoyl*)-L-*alanyl*-D-*isoglutamine* (**1**). A mixture of **10** (150 mg, 0.17 mmol) and Pd black (75 mg) in AcOH (15 ml) was hydrogenated (52 h) and worked up as described in the preceding step. Purification by FC (*B*) yielded 60 mg (46%) of **1**. White lyophilisate. TLC (*B*): R_f 0.43, 0.49 (2 anomers). UV: no absorption above 250. ^{1}H-NMR ((D$_6$)DMSO): *Table*. FAB-MS: 797.6 (8, $[M + K]^+$), 781.5 (100, $[M + Na]^+$), 759.7 (6, $[M + H]^+$), 741.5 (45, $[M - OH]^+$), 471 (24), 453 (26), 392 (43), 328 (40). Anal. calc. for $C_{37}H_{66}N_4O_{12} \cdot 1.5\ H_2O$ (785.95): C 56.54, H 8.85, N 7.13; found: C 56.43, H 8.71, N 7.15.

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