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Potential adjuvants for synthetic vaccines. I. Modulators of macrophage activation

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Abstract

The synthesis and biological activities of several glycopeptidolipids derived from muramyl dipeptide (MDP) are described. In combination with gamma interferon (INFγ) MDP derivatives **3c** and **3d** carrying glycyrrhetinic acid as rigid β-aglycone synergise the action of the lymphokine in the release of H_2O_2 by activated human macrophages, while MDP derivatives **3a** and **6a** carrying the more flexible diphytanyl glycerol β-aglycone inhibit it.

Keywords: Adjuvant; MDP; Glycyrrhetinic acid; Diphytanyl glycerol; Interferon γ; Macrophage; Pd deallylation

1. Introduction

In 1985 we embarked on a programme aimed at the synthesis of potential immunomodulators that might be used as adjuvants [1a] in the formulation of an antimalarial synthetic vaccine candidate. Although the mechanisms by which the adjuvants exert their activity are not clear, the result is a mounting effect in the immunological response to the antigen with which it is 'combined'. Currently, the only adjuvant that is licenced for use in human vaccines is alum (aluminium hydroxide), but alum is certainly less than ideal for a defined subunit vaccine. Our basic idea was to incorporate in a hybrid structure, the 'cocktail' of elements, chemical as well as physicochemical, that appeared (from the wealth of published data in the area of vaccines and subunit vaccine constructs [1b]) to be involved in the potentiation of the immunological response to such weak synthetic antigens. At this point it must be stressed that part of these seemingly 'necessary' structural elements were identified from work done with antigens of different origins, including subunit antigens, killed or attenuated viruses, bacteria and parasites. Thus our concept was based on a rather heterogeneous basis. Furthermore, there is little consensus on how to control specific immune responses in this area [2], despite the recent explosion of published information in this field. Consequently, the design of target molecules is largely dependent on intuition and on untested suppositions. Our hybrid structure should then be built on muramyl dipeptide (MDP, *N*-acetyl-D-muramoyl-L-alanyl-D-isoglutamine), which accounts for a major portion of the adjuvant effect of complete Freund's adjuvant

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[3–5], onto which lipophilic as well as hydrophilic substituents would be incorporated. The resulting molecule was expected to form liposomes [6] or immunostimulating complex (ISCOM)-like particles [7], and so might also be used as a 'carrier' for the synthetic antigen. The main cellular target of such a construct was, therefore, the macrophage (Mϕ), first line of cellular defence against infection and antigen-presenting cell, although some data indicate that the adjuvant effects of MDP might also be mediated through its direct action on helper T cells [8,9] or B cells [10–12]. The incorporation of mannose residues was also contemplated in order to target the phagocytic cells via their mannose receptors [13]. Furthermore, results showing that gamma interferon (INFγ) has a potent inhibitory effect on liver stages of the malaria parasite [14] led us to test our compounds for their potential to modulate the oxidative burst of 'activated' macrophages, alone and in combination with INFγ. Recent experimental evidence showing that INFγ is also a powerful adjuvant [15–16] underlines the potential contribution that chemically well-defined compounds might make to the potentiation of the adjuvant effects of this lymphokine.

In the present work, we report the synthesis of some of the compounds constructed along the lines aforementioned and present data on the modulation of the oxidative burst of human macrophages by the compounds alone and in combination with INFγ. The aim of synthesising derivatives carrying the β-aglycone **a** was to increase chain fluidity (when compared to **b** and **c**) by incorporation of methyl branches [21]. *Archeabacteria* have perfected this means of increasing chain fluidity, i.e. they have the peculiarity of only incorporate phytanyl residues in their polar lipids. The goal of synthesising compounds carrying the β-aglycones **b** and **c** has already been delineated in [17]. The free acids **4e** and **5** should allow further coupling, for example, with the synthetic vaccine.

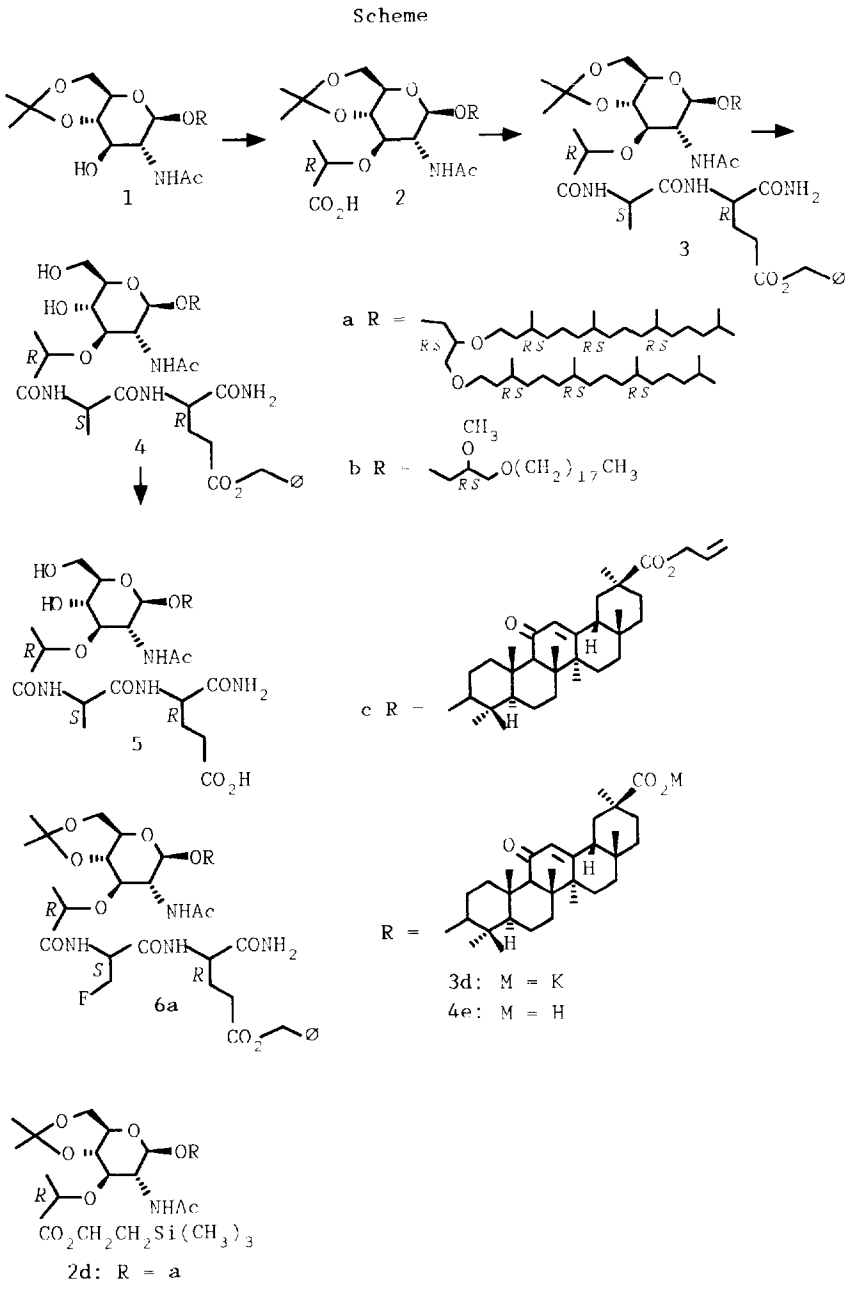
2. Chemistry

The sugar building block **1** (**R**=**H**) as well as **1b**, **1c**, **2b**, **2c**, **3b**, **3c**, **4b** and **4c** were synthesised as described [17]. The synthesis of the other compounds is depicted in the Scheme. The racemic aglycone **a** was prepared in three steps [18] starting from phytyl alcohol and (*R*,*S*)-1-*O*-benzylglycerol following standard conditions. Though the alkylation of the alkoxide of **1a** with (*S*)-2-chloropropionic acid was a smooth and a quantitative one, formation of gels at neutral pH impeded partial purification during work up of the reaction mixture; this behavior was also observed with **2b** and **2c** [17]. Nevertheless, **2a** was obtained in 80% yield as a hard glass-like paste after flash chromatography of the crude product. Assignment of the ^{1}H-NMR signals of **2a** was not possible due to their poor resolution and extensive line broadening. Unambiguous characterisation of the acid was possible after esterification with 2-(trimethylsilyl)-ethanol following the procedure already described [19] and the 2-(trimethylsilyl)ethyl ester of **2a** (which we needed in another context) was obtained in 91% yield as a colourless oil. Consequently, for the following batches, we simply relied on the chromatographic purity of **2a**. Subsequently, **2a**, **2b** and **2c** where subjected to our peptidation protocol [19] to furnish the protected glycopeptides **3a**, **3b** and **3c** in over 80% isolated yield. Cleavage of the acetonide with trifluoroacetic acid (TFA) as described [17] furnished **4a**, **4b** and **4c** quantitatively. Catalytic hydrogenation of **4a** and **4b** in ethyl acetate and MeOH over 10% Pd/C afforded quantitatively **5a** and **5b**. The acid **5a** was obtained as a wax compound and, as for **2a**, ^{1}H-NMR spectroscopy was of little help because very broad peaks were observed, probably the result of the association of the compound in solution. Therefore, **5a** was better characterised after derivatisation to its allylester [17,20]. Pd(0) catalysed deallylation of **3c** in the presence of potassium caproate [20] gave quantitatively **3d** which in turn was acidified with TFA to furnish quantitatively **4e**.

3. Biological results and discussion

Compounds **3b**, **4b** and **5a** alone (Table 1) activate the oxidative burst of in vitro cultured human Mϕ. However, these compounds are less efficient than INFγ and induce, at high concentration

Scheme



(10^{-5} M), an enhancement of H_2O_2 release which is, respectively 83%, 55% and 61% of the possible enhancement induced by INFγ (Table 1). At very low concentration (10^{-9} M), **5b** and **4e** are, nevertheless, more efficient than INFγ (Table 1); they induce an enhancement of H_2O_2 release which is, respectively 173% and 139% of the possible enhancement. In combination with INFγ (Table 2) compounds **3c** and **3d** are definitely synergising the action of the lymphokine at all concentrations tested. They might be of interest in situations were INFγ signals macrophages to release reactive oxy-

Table 1
% maximum release of H_2O_2 after stimulation with PMA[a] induced by compounds alone

Compound	Concentration (M)							
	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	Control[b]	INFγ[c]
3a		2		12		0	100	329
3b	0		0		83		55	130
3c	4		5		0		55	130
3d	1		0		0		55	130
4a	10		11		22		100	345
4b	16		67		55		55	130
4c	0		0		13		55	130
5a	15		21		61		100	345
5b	173		71		23		55	130
4e	139		0		0		55	130
6a		4		5		0	100	329

[a]Phorbol myristate acetate (PMA).
[b]H_2O_2 release of non-activated macrophages.
[c]H_2O_2 release by 100 U/ml INFγ.

gen species and boost antimicrobial activity directed against microorganisms susceptible to reactive oxygen intermediates, i.e. against the intra-erythrocytic forms of *Plasmodium falciparum* [22].

Interestingly, **3a** and **6a** alone (Table 1), as well as in combination with INFγ (Table 2), are inhibiting the activation of macrophage oxidative burst. These compounds might act as anti-inflammatory agents by the inhibition of the production of macrophage-derived oxygenated mediators of inflammation, which are likely to contribute to local tissue destruction. The other compounds alone and in combination with INFγ exibited weak activities only or were inactive.

To summarize, in series **3**, compounds **3c** and **3d** with the rigid aglycone synergise the INFγ-mediated oxidative burst of human macrophages, whilst compounds **3a** and **6a** with a more flexible aglycone, containing phytanyl residues, inhibit the

Table 2
% maximum release of H_2O_2 induced by compounds in combination with INFγ (100 U/ml)

Compound	Concentration (M)							
	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	Control[a]	INFγ[b]
3a		78		48		40	100	329
3b	99		89		76		55	284
3c	150		153		154		55	284
3d	120		124		146		55	284
4a	82		118		149		100	345
4b	119		47		53		55	284
4c	103		101		106		233	455
5a	101		94		149		100	345
5b	97		73		70		233	455
4e	86		93		89		233	455
6a		89		52		41	100	296

[a]H_2O_2 release of non-activated macrophages.
[b]H_2O_2 release by 100 U/ml INFγ.

action of the lymphokine. The present data do not reveal which factors are involved in the activity of both classes of compounds, i.e. whether it is the flexibility or the rigidity of the aglycones or something else.

4. Experimental

Unless stated otherwise, procedures used throughout this work were similar to those previously described [23]. In NMR spectra only signals characteristic for identification are given. The FAB-MS normally show a prominent peak for the protonated compound accompanied in the cases of **3e**, **4e**, as well as in the case of the 2-(trimethylsily)ethyl ester of **2a**, by a peak of higher mass corresponding to the $[M + Na]^+$ cation. In addition, a peak corresponding to $[M + K]^+$ is also observed in the case of **4e**. Although no Na and K anions are added, these compounds tend to associate with ubiquitous Na and K ions and accumulate preferentially at the surface of the matrix [24]. Boc-L-ala-D-isoglutamine benzyl ester (Bachem). NaH (Fluka, 80% suspension in mineral oil). HBTU: *O*-benzotriazolyl-N,N,N′,N′,-tetramethyluronium hexafluorophosphate (Fluka).

4.1. Macrophage oxidative burst activation

The activation of the macrophages by the compounds alone and in combination with INFγ was assessed by measuring their H_2O_2 release according to Pick and Mizel [25]. Table 1 shows the % of maximum release of H_2O_2 at different concentrations of the compounds. The % maximum release refers to the releasing capacity of Mϕ activated by 100 U/ml INFγ, as seen in different experimental groups. The H_2O_2 released by non-activated Mϕ is considered. Each value is calculated as follows:

$$\%\ \text{maximum release} = \frac{\text{release sample} - \text{release control}}{\text{release after 100 U/ml INF}\gamma - \text{release control}}$$

4.2. Compounds

The compounds have been dissolved in DMSO (dimethyl sulfoxide) to have a stock solution of 10^{-2} M and then diluted in medium with 20% of serum. They have been tested in the range of concentration between 10^{-9} M and 10^{-4} M.

4.3. Macrophages

All the tests have been performed on human monocyte-derived macrophages. Monocytes have been purified from peripheral blood counter-flow elutriation. Cells were plated and cultured for 2 days before treatment.

4.4. Macrophage activation

After 3 days with the compounds, the Mϕ were tested for their capacity to produce H_2O_2 under stimulation with phorbol myristate acetate (PMA). Activated Mϕ showed an enhanced oxidative burst (produced higher levels of H_2O_2) in comparison to untreated Mϕ taken as negative controls. The positive controls were Mϕ activated by 100 U/ml of INFγ.

4.5. Modulation of macrophage activation

Mϕ were incubated 3 days with the compounds and with INFγ (100 U/ml) to see whether synergism or antagonism with INFγ can be demonstrated. The parameters to evaluate Mϕ activation were the amounts of H_2O_2 released under PMA stimulation. The controls were represented by untreated Mϕ, by INFγ-treated Mϕ and by compound-treated Mϕ.

4.6. (RS)-2,3-Bis[[(all-RS)-3,7,11,15-tetramethylhexadecyl]oxy]propyl-2-acetamido-3-O-[(R)-1-carboxyethyl]-2-deoxy-4,6-O-isopropylidene-β-D-glucopyranoside (**2a**)

This step was performed in a flame dried apparatus. To a stirred solution of **1a** (2.9g, 3 mmol) in 50 ml toluene-DMF (1:1) was added NaH (0.097g, 3 mmol) portion-wise at 10°C under argon. After a few min, the light-coloured solution was treated with a solution of (*S*)-2-chloropropionic acid (0.33 g, 3 mmol) in 6 ml toluene-DMF (2:1) which was added dropwise over 5 min. Immediately afterwards, the stirred solution was rapidly treated with NaH (0.097 g, 3 mmol). The reaction was allowed to warm up to room temperature and stirring was continued overnight. The beige-coloured solution was quenched with cold water and concentrated under vacuum to remove most of the toluene, and the residue was extracted

with ethyl ether. Formation of gels at this stage were only partially broken by addition of solid sodium chloride. The ethyl ether extracts were backwashed with water The combined aqueous phase was acidified under cooling to pH ≈2 with aqueous citric acid and extracted with EtOAc. The combined EtOAc extracts were washed with a 10% aqueous solution of NaCl, dried, filtered and evaporated. The crude product was rapidly purified by flash chromatography on silica gel using chloroform-MeOH (9.5–0.5) as eluent to give **2a** (2.32 g, 2.4 mmol, 80% yield) as a colourless oil, which turned to a hard-sticky and glassy paste after drying under high vacuum: FAB-MS: 968 $(M + H)^+$, 316 $(M-glycerine)^+$. IR (film) cm^{-1} 3333, 2730, 1722, 1623, 1581, 1113. **2a** was fully characterised after conversion to the following 2-(trimethylsilyl)ethyl ester:

4.6.1. (RS)-2,3-Bis[[(all-RS)-3,7,11,15-tetramethylhexadecyl]oxy]propyl-2-acetamido-2-deoxy-4,6-O-isopropylidene-3-O-[(R)-1-[[2-(trimethylsilyl)ethoxy]carbonyl]ethyl]-β-D-glucopyranoside. To a stirred solution of **2a** (0.37g, 0.38 mmol) in 30 ml CH_2Cl_2 was added in sequence under argon at 20°C, 4-dimethylaminopyridine (DMAP) (0.046 g, 0.38 mmol), HBTU (0.144 g, 0.38 mmol) and 2-(trimethylsilyl)ethanol (0.05 ml, 0.34 mmol). The solution was stirred at 20°C for 5 h. Evaporation of the solvent under reduced pressure followed by flash chromatography of the crude product gave the 2-(trimethylsilyl)ethyl ester as a colourless oil (0.37 g, 0.34 mmol, 91%): (Partial) ^{1}H-NMR (400 MHz, $CDCl_3$, mixture of diastereomers) δ ~6.38 and 6.42 (2 × m, 2 × —NH— of I and II), ~4.54 (dd, J = 8.5 Hz H_1, of I and II), 4.36 (q, J = 7 Hz, lactyl—CH), 1.953 and 1.959 (2 × s, 2 × CH_3-CON of I and II), 2.88 (d, lactyl—CH_3), 0.0 (s, $(CH_3)_3Si$—). FAB-MS: 1068 $(M + H)^+$, 1090 $(M + Na)^+$.

4.7. (RS)-2,3-Bis[[(all-RS)-3,7,11,15-tetramethylhexadecyl]oxy]propyl-2-acetamido-3-O-[(R)-1-[[(S)-1-[[(R)-3-[(benzyloxy)carbonyl]-1-carbamoylpropyl]carbamoyl]ethyl]carbamoyl]ethyl]-2-deoxy-4,6-O-isopropylidene-β-D-glucopyranoside (**3a**)

Boc-L-ala-D-isoglutamine benzyl ester (0.45 g, 1.1 mmol) was dissolved under stirring in 3 ml trifluoroacetic acid at −10°C (ice-MeOH bath). After 30 min the acid was rapidly removed under reduced pressure at 20°C. Benzene was added and rapidly evaporated. This treatment was repeated twice to remove most of the acid. The residue was dissolved in 20 ml CH_2Cl_2 and treated with NEt_3 (0.3 ml, 2.2 mmol). This solution was immediately added under stirring to a solution of **2a** (1.06 g, 1.09 mmol), HBTU (0.42 g, 1.1 mmol), NEt_3 (0.15 ml, 1.1 mmol) and DMAP (0.012 g, 0.1 mmol) in 20 ml CH_2Cl_2 and 10 ml CH_3CN. The mixture was stirred at room temperature for 5 h. The solvent was removed under reduced pressure and without further purification the crude product was flash chromatographed (EtOAc, silicagel) to give **3a** as a thick oil (1.16 g, 92 mmol, 84%): (Partial) ^{1}H-NMR (400 MHz, $CDCl_3$, 1:1 mixture of diastereomers) δ 0.75–0.9 (m, 30H, 10 × —CH_3), ~2.04 and ~2.15 (2 × m, 2H, —$COCH_2$—CH_2—), 1.959 and 1.96 (2 × s, 3H, CH_3CON— of I and II), ~2.50 and ~2.56 (2 × m, 2H, —CO—CH_2—), 4.1 (m, lactyl—CH—), 4.65 and 4.7 (2 × d, J = 6.5 Hz and J = 8.5 Hz, $1H_1$, of I and II), 5.125 (AB-spectrum, J_{AB} = 8.5 Hz, 2H, —CO_2CH_2—), 5.74 and 6.93 (2 × 2s, NH of I and II, $CONH_2$), 6.32 and 6.44 (2 × d, NH of I and II), 7.35 (m, H_{phenyl}, NH, NH of I), 8.08 (d, NH of II). FAB-MS: 1257 $(M + H)^+$, 1279 $(M + Na)^+$.

4.8. (RS)-2,3-Bis[[(all-RS)-3,7,11,15-tetramethylhexadecyl]oxy]propyl-2-acetamido-3-O-[(R)-1-[[(S)-1-[[(R)-3-(benzyloxy)carbonyl]-1- carbamoylpropyl]cabamoyl]ethyl]carbamoyl]ethyl]-2-deoxy-β-D-glucopyranoside (**4a**)

Cleavage of the acetonide **3a** (0.251g, 0.2 mmol) following the protocol described in [17] furnished **4a** quantitatively as a wax (0.24g, 0.2 mmol): (Partial) ^{1}H-NMR δ ~0.84 (m, 30H, 10 × CH_3), 1.96 (s, 3H, CH_3CON), ~2.0 and ~2.16 (2 × m, 2H, $COCH_2$—CH_2—), ~2.5 (m, 2H, $COCH_2$—), 5.5 (AB-spectrum, J = 12 Hz, 2H, —CO_2CH_2—). FAB-MS: 1218 $(M + H)^+$.

4.9. (RS)-2,3-Bis[[(all-RS)-3,7,11,15-tetramethylhexadecyl]oxy]propyl-2-acetamido-3-O-[(R)-1-[[(S)-1-[[(R)-1-carbamoyl-3-carboxypropyl]carbamoyl]ethyl]carbamoyl]ethyl]-2-deoxy-β-D-glucopyranoside (**5a**)

To a stirred suspension of 1 g prereduced 10%

Pd/C in MeOH (20 ml) and AcOEt (20 ml) was added a solution of **4a** (1.2 g, 0.1 mmol) in 10 ml MeOH-AcOEt (1:1). The stirred mixture was hydrogenated at atmospheric pressure for 3h. The catalyst was removed by filtration. The filtrate was evaporated and the residue was flash chromatographed on silica gel ($CHCl_3$-MeOH, 8:2) to give quantitatively **5a** (1.1g, 0.1 mmol). FAB-MS: 1128 $(M + H)^+$.

The acid was better characterised after derivatisation to the following allylester, according to the procedure described in [17]:

4.9.1. (RS)-2,3-Bis[[(all-RS)-3,7,11,15-tetramethylhexadecyl]oxy]propyl-2-acetamido-3-O-[-(R)-1-[[(S)-1-[[(R)-3-(allyloxy)carbonyl]-1-carbamoylpropyl]carbamoyl]ethyl]carbamoyl]ethyl-]-2-deoxy-β-D-glucopyranoside (Partial) ^{1}H-NMR (250 MHz, $CDCl_3$) δ 4.56 (m, 2H, $—CO_2CH_2—$), 5.24 and 5.31 (2 × d, J = 10 Hz, J = 17 Hz, $CH_2=$), 5.88 (ddt, 1H, —CH=). FAB-MS: 1189 $(M + Na)^+$.

4.10. (RS)-2,3-Bis[[(all-RS)-3,7,11,15-tetramethylhexadecyl]oxy]propyl-2-acetamido-3-O-[(R)-1-[[(S)-1-[[(R)-3-[(benzyloxy)carbonyl]-1-carbamoylpropyl]carbamoyl]-2-fluoroethyl]carbamoyl]ethyl]-2-deoxy-4,6-O-isopropylidene-β-D-glucopyanoside (**6a**)

Boc-L-fluoroalanine-D-isoglutamine benzyl ester (the synthesis of this peptide will be published elsewhere) (0.37 g, 1 mmol) and **2a** were treated as described for **3a**. **6a** was isolated as a wax in 85% yield: (Partial) ^{1}H-NMR (400 MHz, $CDCl_3$, 1:1 mixture of diastereomers) δ 1.25 (d, 3H, CH_3—lactyl), 1.95 (s, CH_3CON), 2.07 and 2.25 (2 × m, 2H, $COCH_2—CH_2—$), 2.5 and 2.64 (2 × m, 2H, $CO—CH_2—$), 4.18 (m, 1H, lactyl—CH), 4.68 (m, H_1), 5.11 and 5.13 (2 × AB-spectrum, $—CO_2CH_2—$ of I and II), 7.35 (m, H_{phenyl}). FAB-MS: 1276 $(M + H)^+$.

4.11. 3β-[[2-Acetamido-3-O-[(R)-1-[[(S)-1-[[(R)-3-[(benzyloxy)carbonyl]-1-carbamoylpropyl]carbamoyl]ethyl]carbamoyl]ethyl]-2-deoxy-4,6-O-isopropylidene-β-D-glucopyranosyl]oxy]-4,4-dimethyl-11-oxo-12-oleanen-30-oic acid potassium salt (**3d**)

A solution of **3c** (0.445 g, 0.4 mmol) in 20 ml CH_2Cl_2 and 20 ml EtOAc was treated sequentially under stirring at 20°C with a solution of 1.2 mg palladium (II) acetate in 4.5 μl (4.2 mg, 0.025 mmol) $(EtO)_3P$ and 2 ml (4 mmol) of a 2 N solution of potassium 2-ethyl caproate in EtOAc. Addition of 40 ml Et_2O precipitated the crystalline K salt **3d**. The colourless crystals were filtered, thoroughly washed with Et_2O and dried to give 410 mg (0.37 mmol, 83%) of **3d**: (Partial) ^{1}H-NMR (400 MHz, d–MeOH) δ 0.76, 0.81, 0.96, 1.08, 1.11, 1.12, 1.37, 1.39, 1.51 (9 × s, 9 × CH_3), 1.32 (d, J = 6.5 Hz, CH_3—lactyl), 1.38 (d, J = 7 Hz, CH_3—alanyl), 1.92 (s, CH_3CON), 2.40 (s, H_9), 2.68 (m, $H_{1\,eq}$), 4.5 (d, J = 8.5 Hz, $H_{1'}$), 5.12 (s, $—CO_2CH_2—$), 5.68 (s, =CH—). FAB-MS: 1114 $(M + H)^+$.

4.12. 3β-[[2-Acetamido-3-O-[(R)-1-[[(S)-1-[[(R)-3-[(benzyloxy)carbonyl]-1-carbamoylpropyl]carbamoyl]ethyl]carbamoyl]ethyl]-2-deoxy-β-D-glucopyranosyl]oxy]-4,4-dimethyl-11-oxo-2-oleanen-30-oic acid (**4e**)

A stirred solution of **3d** (50mg, 0.045 mmol) in 20 ml CH_2Cl_2 was treated dropwise at 20°C with 0.5 ml TFA. After 2h the reaction mixture was evaporated to dryness. The residue was washed with water, taken up in 0.5 ml MeOH. Addition of Et_2O precipitated 45 mg of **4e** as colourless crystals (0.04 mmol, 88%): ^{1}H-NMR (Partial) (400 MHz, d–MeOH) δ 0.77, 0.82, 0.97, 1.122, 1.126, 1.16, 1.40 (7 × s, 7 × CH_3), 1.355 and 1.38 (2 × d, CH_3—lactyl and CH_3—alanyl), 1.89 (s, CH_3CON), 2.43 (s, H_9), 2.68 (m, $H_{1\,eq}$), 5.12 (s, $—CO_2CH_2—$), 5.56 (s, —CH=). FAB-MS: 1036 $(M + H)^+$, 1057 $(M + Na)^+$, 1073 $(M + K)^+$.

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