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1,6-AnhMurNAc derivatives for assay development of amidase AmiD

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ABSTRACT

Various peptidoglycan fragments were synthesized from two anhydro-muramic acid derivatives protected with a Bn or a PMB group at the 4th position, in homogenate phase or on a solid support. In order to facilitate HPLC detection, a chromophoric group was attached to the peptide chain. The periplasmic amidase sAmiD of *Escherichia coli* was used to cleave the amide bond between the lactyl group of the MurNAc and the α-amino group of L-Ala where the peptide chain was at least a dipeptide (L-Ala-γ-D-Glu) amidated by benzylamine on the γ-carboxyl group of D-Glu. In the presence of a tripeptide chain (L-Ala-γ-D-Glu-L-Lys) or a tetrapeptide chain (L-Ala-γ-D-Glu-*m*-A_2pm-D-Ala) higher hydrolysis rates were observed. We have also demonstrated that the presence of TNB on the ε-amino group of L-Lys only has a small influence on the hydrolysis capacity of sAmiD.

1. Introduction

Peptidoglycan is an essential macromolecule in the bacterial cell wall, found outside the cytoplasmic membrane of almost all bacteria. The main functions of peptidoglycan are preserving cell integrity by withstanding the internal osmotic pressure and maintaining the cell shape. This macromolecule consists of linear heteroglycan chains cross-linked by short peptide chains.[1,2] The heteroglycan chains are composed of alternating β-(1-4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues. In Gram-negative bacteria, MurNAc is linked through the lactyl amide bond to L-alanyl-γ-D-glutamyl-*meso*-diaminopimelyl-D-alanine (L-Ala-γ-D-Glu-*m*-A_2pm-D-Ala). In some Gram-positive bacteria, *m*-A_2pm is replaced by L-Lys.

Both cell growth and cell division require continuous remodelling of peptidoglycan. Up to 60% of the parental peptidoglycan can be recycled for further peptidoglycan biosynthesis. Up to now, nine enzymes have been discovered that are specifically involved in this recycling process.[3] Degradation by lytic transglycosidases leads to the production of GlcNAc-anhMurNAc-tri(tetra)peptides. The liberated peptidoglycan fragments (muropeptides) are transported from the periplasm into the cycloplasm and recycled for further peptidoglycan biosynthesis. *N*-Acetylmuramyl-L-alanine amidases (MurNAc-L-Ala amidases) specifically cleave the amide bond between the lactyl group of MurNAc and the α-amino group of L-alanine, the first amino acid of the peptide chain. The cytosolic amidase AmpD of *Escherichia coli* specifically catalyses the hydrolysis of 1,6-anhydroMurNAc-tri(tetra)peptide after it has been transported into the cytosol by the permease AmpG. Four periplasmic amidases have been identified in *E. coli*: AmiA, AmiB, AmiC and AmiD. AmiD was first detected in an ampDnagZ double mutant where a significant amount of the disaccharide GlcNAc-anhMurNAc accumulates in addition to the expected GlcNAc-anhMurNAc-tripeptide, suggesting that another AmpD-like enzyme must exist in *E. coli*.[4] The AmpD-like lipoprotein AmiD requires Zn^{2+} for its activity. It is a member of the amidase_2 family (PF01510). The AmiD amidase is, in contrast to AmiA, B and C, not involved in cell separation. AmiD catalyses the hydrolysis of various substrates such as anhMurNAc-L-Ala-γ-D-Glu-*m*-A_2pm and GlcNAc β(1,4)-anhMurNAc-L-Ala-γ-D-Glu-*m*-A_2pm-D-Ala (**TCT**) (Fig. 1). Interestingly, AmiD does not have a strict specificity for 1,6-anhydromuropeptides[5,6] and is an interesting target to study substrate specificity on synthetic anhMurNAc and MurNAc derivatives. Up to now, the lack of authentic fully characterized substrates has limited the study of this enzyme. AmiD is a periplasmic lipoprotein anchored in the outer membrane through a modified N-terminal cysteine. A truncated form of AmiD (sAmiD) has been cloned in the production vector pBAD, overproduced, purified, characterized[5] and crystallized.[7] In this paper we describe the synthesis of

Abbreviations: Ac_2O, anhydride acetic; Bn, benzyl; BOC, *tert*-butoxycarbonyl; BOP, benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate; Cbz, carboxybenzoyl; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; NMM, *N*-methylmorpholine; MS, mass spectrometry; PMB, *para*-methoxybenzyl; PyBOP, benzotriazol-1-yl-oxy-tris-pyrrolidino-phophonium hexafluorophoshate; TBTU, *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TNB, trinitrobenzene; TNBSA, trinitrobenzensulfonic acid.

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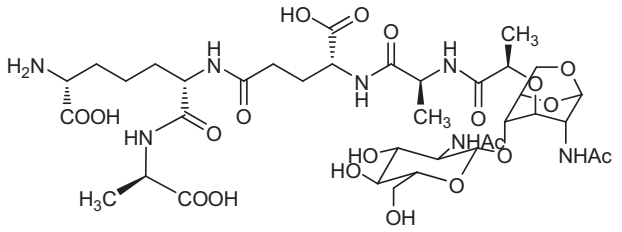


Figure 1. Structure of TCT.

1,6-anhydro-muramic acid derivatives and their biological evaluation with sAmiD in order to develop a simple biological assay for this important enzyme.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of anhydro-muramic acid derivatives containing a (peptidic) chain of variable length (compounds 6, 7, 8, 11, 20, 27, 28) in homogenate phase

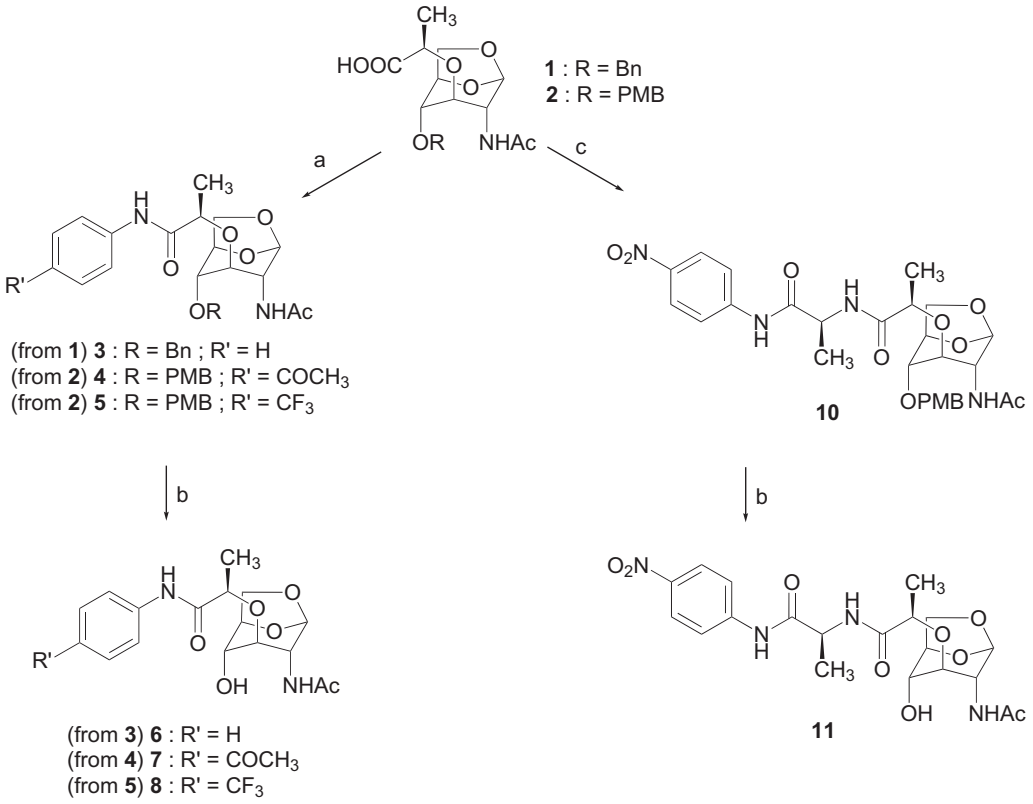
Various anhydro-muramic acid derivatives containing a peptidic chain of variable length have been synthesized in homogenate phase using similar synthetic methods, such as Kawasaki et al.[8] and Mobashery and co-workers.[9,10] The precursor **1**[8–10] or **2**[8–10] is coupled in the presence of an activating agent such as TBTU or PyBOP with various aromatic amines (Scheme 1: compounds **6**, **7**, and **8**), modified amino acids such as L-Ala-*p*-nitroanilide **9** (commercially available) (Scheme 1: compound **11**) and peptide chains containing two (compound **20**) or three amino acids (compound **27**) (Scheme 2). The final step is a complete deprotection to obtain the molecules, which were subsequently used for biological experiments. The compound **28** with a trinitrobenzene (TNB) chromophoric ring on the ε-amino group of L-Lys was also prepared from **27** (Scheme 2).

2.1.2. Solid phase synthesis of anhydro-muramic acid derivatives containing a peptide chain of three or four amino acids (compounds 27 and 29)

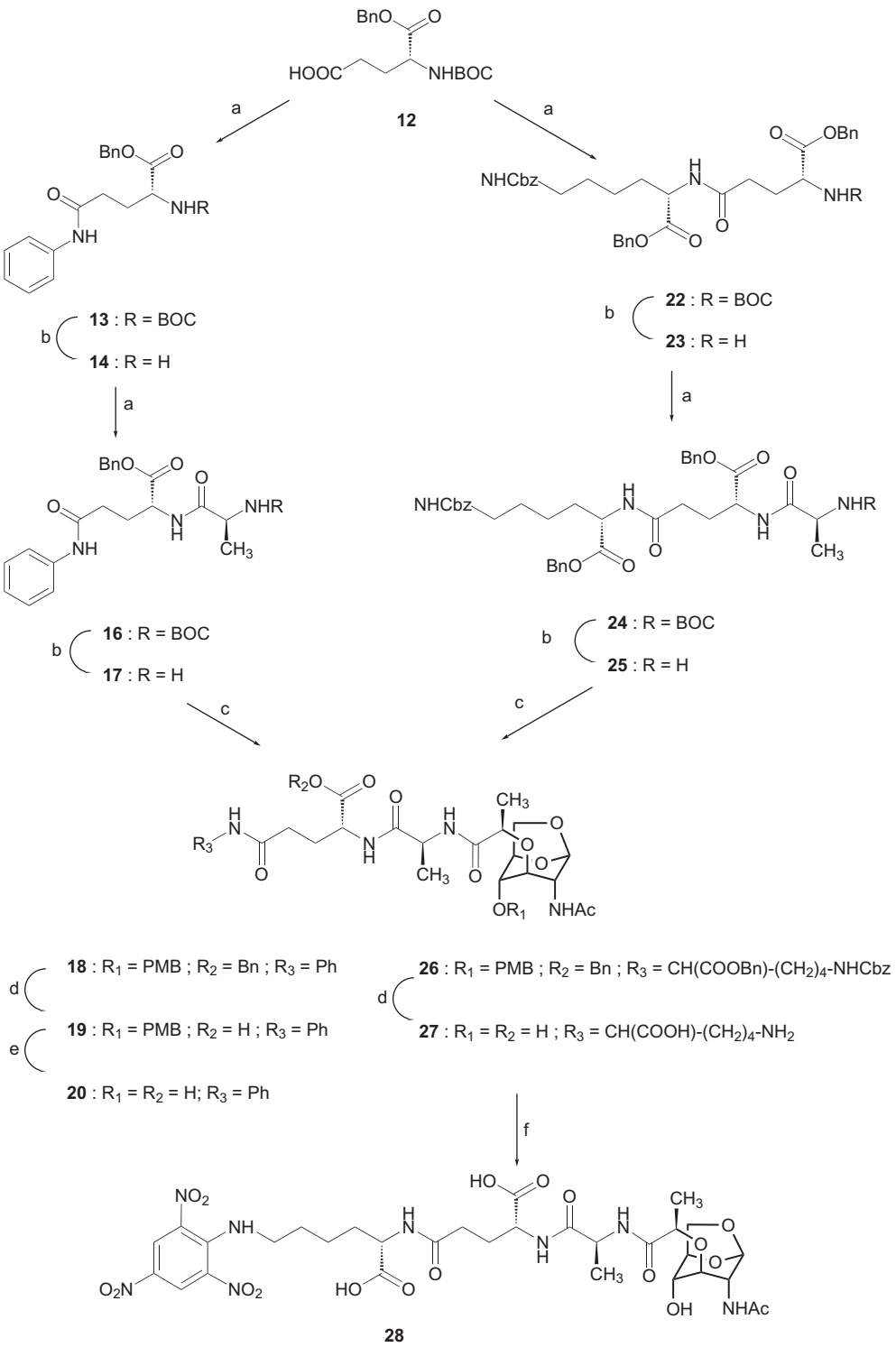
Syntheses of glycopeptides on solid phase are not very common in the literature. Synthesis on solid supports (Scheme 3) is simple, fast and requires small volumes of solvents and compounds. Moreover, the possible side products and excess reagents are removed by simple washing, which facilitates the purification of the desired compound.

The synthesis on solid supports of *N*-acetylglucosamine derivatives containing a peptide chain has been presented by Kumar et al.[11] and the synthesis on solid supports of a pentapeptide by Chowdhury and Boons.[12] According to these literatures, compound **27** was also synthesized by polymeric support synthesis using the Wang-Fmoc-L-Lys(Boc) resin, Fmoc-protected amino acids, and the properly protected anhydro-muramic acid derivative **2**. The peptide chain was elongated on solid support and **2** was coupled by an acid carboxylic function to produce the resin-bound glycopeptide. A TFA treatment of the resin and a complete deprotection of the glycopeptide produced the compound **27** with 40% yield for all steps ($n = 2$). Compound **27** was previously obtained in four steps in homogenate phase with a global yield of 6.3%. It was clear that the solid support synthesis produced a better yield (in a better time).

Following the same strategy, compound **29** was obtained from the Wang-Fmoc-D-Ala resin. The *meso*-diaminopimelic acid prepared by Teller[13] was first coupled, followed by the



Scheme 1. Reagents and conditions: (a) **1**, CH_2Cl_2, BOP, NMM, rt, 1 h, N_2 and then aniline, rt, N_2, 48 h, 50% for **3**; **2**, DMF, BOP, NMM, rt, 1 h, N_2 and then *para*-aminoacetophenone or *para*-trifluoromethylaniline, rt, N_2, 48 h, 25% for **4**, 40% for **5**; (b) **3**, MeOH, Pd/C 10%, H_2, rt, 6 h, 12% for **6**; **4** or **5**, CH_2Cl_2–H_2O, DDQ, 0 °C to rt, N_2, 24 h, 20% for **7**, 31% for **8**, 10% for **11**; (c) **2**, DMF, BOP, NMM, rt, 1 h, N_2 and then L-Ala-*para*-nitroanilide hydrochloride **9**, rt, N_2, 48 h, 73%.



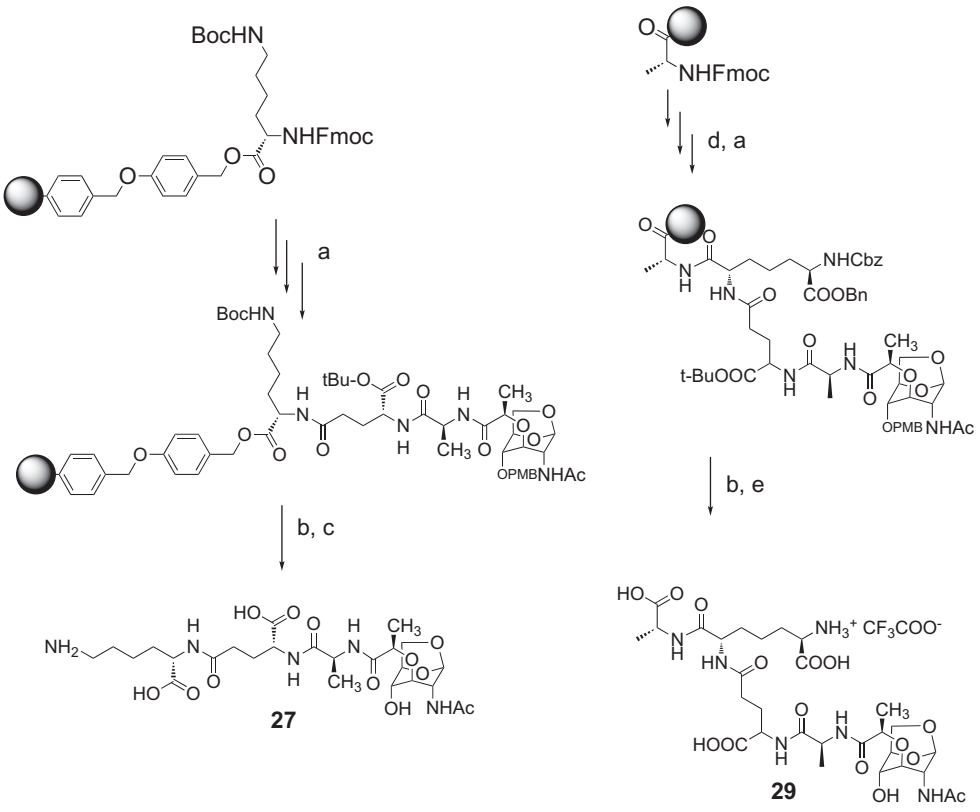
Scheme 2. Reagents and conditions: (a) CH_2Cl_2, DIEA, TBTU, rt, 1 h and then desired amine is added (aniline, NHBOC-L-Ala **15** or H-Lys(Z)-OBzl·HCl **21**, rt, 1 h, 75% for **13**, 75% for **16**, 98% for **22**, 60% for **24** (two steps); (b) CH_2Cl_2, TFA, rt, 0.5 h, N_2, 71% for **14**, 74% for **17**; (c) **2**, CH_2Cl_2, DIEA, PyBOP or TBTU, rt, 1 h and then **17** or **25**, rt, 1 h, 51% for **18** and 18% for **26** (two steps); (d) EtOH, Pd/C 10%, H_2, rt, 6 h, 68% for **18**, MeOH, Pd/C 10%, H_2, 35 °C, 72 h, 60% for **27**; (e) CH_2Cl_2–H_2O, DDQ, 0 °C to rt, N_2, 24 h, 10%; (f) carbonate buffer (0.1 N), pH 9.5, TNBSA, 35 °C, 2 h, 97%.

Fmoc-D-Glu-OtBu, the Fmoc-L-Ala and finally the derivative **2**. A TFA treatment of the resin and a complete deprotection of the glycopeptide produced compound **29** with 51% yield for all steps ($n = 1$).

Here we show the feasibility of rapidly and automatically coupling the properly protected anhydro-muramic acid derivative **2** to peptides of various lengths which are still bound to the resin. After cleavage from the resin and final deprotection, a large variety of 1,6-AnhMurNAc glycopeptides could be obtained with this method.

2.2. Biological experiments

Eight anhydro-muramic derivatives (**6**, **7**, **8**, **11**, **20**, **27**, **28** and **29**) with various aromatic residues or various peptide chains were



Scheme 3. Reagents and conditions: (a) piperidine then Fmoc-D-Glu-OtBu, HOBt; piperidine then Fmoc-L-Ala, HOBt; piperidine then **2**, HOBt; (b) TFA, 2 h; (c) MeOH, Pd/C 10%, H_2, 35 °C, 72 h, 40% for all steps; (d) piperidine then *meso*-diaminopimelic acid, HOBt; (e) THF, TFA 0.1%, Pd/C 10%, H_2, rt, 2 d, 51% for all steps.

Table 1
The derivatives were incubated with various concentrations of sAmiD in 20 mM sodium phosphate buffer containing 48 nM $ZnCl_2$ at 30 °C for various incubation times *t*

Compound	*c* (μM)	*t* (min)	sAmiD (μM)	(sAmiD) * *t* (μM min)	Product (%)
TCT (GlcNAc β-(1,4)-anhMurNAc-tetraeptide (with A_2pm))	28	40	0.04	1.6	74
6	1000	120	3.9	464	0
7	250	120	3.9	464	0
8	1000	120	3.9	464	0
11	1000	120	3.9	464	0
20 (anhMurNAc-dipeptide-Bnz)	1000	120	3.9	464	9

The solutions were analyzed by HPLC.

synthesized from the anhydro-muramic acid derivatives **1** and **2** previously presented. These derivatives were used for biological studies with sAmiD.

2.2.1. Hydrolysis studies

Hydrolysis of the various compounds catalysed by sAmiD was studied and the results are summarized in Table 1. sAmiD was not able to catalyse the cleavage of the amide bond between the lactyl group of MurNAc and the amino group of substituted aromatic rings (**6**, **7** and **8**). No hydrolysis of **11** containing the first amino acid L-alanine and a *para*-nitrophenyl residue was observed. The anhMurNAc-dipeptide derivative **20**, the anhyMurNAc-tripeptide derivatives **27** and **28**, the anhyMurNAc-tetrapeptide derivative **29**, and GlcNAc β(1,4)-anhMurNAc-tetrapeptide **TCT** (Fig. 1) were hydrolysed by sAmiD (Table 2).

The reaction mixtures of hydrolysates of **20**, **27**, **28** and **29** with sAmiD were analysed by HPLC and MS. AnhMurNAc with a theoretical mass of 275.1 g/mol ((M+1) = 276) was found for **27** and **29** but it was not possible to observe their non-substituted peptide chains by HPLC-analysis. On the other hand, the peptide chains (which contain chromophoric groups) of the anhMurNAc derivatives **20** and **28** were detected with theoretical masses of [M+1] = 293 and [M+1] = 558, respectively.

2.2.2. Kinetic studies

Compounds **20**, **27**, **28** and **29** were used for kinetic studies. A linear relationship between the initial rates and substrate concentrations up to 1 mM was observed. The hydrolysis of these compounds at a concentration of 0.1 mM was studied and curves monitoring first order decrease of the substrate concentration over the incubation time were obtained (Fig. 2).

TCT exhibits a burst kinetic (Fig. 3),[5] characterized by a rapid phase preceding a linear phase. Similar kinetics have been found with MurNAc-L-Ala-γ-D-Glu-mA_2pm and MurNAc-L-Ala-γ-D-Glu-mA_2pm-D-Ala-D-Ala.[5] The biphasic kinetic curves have been explained by the rapid and reversible inactivation of sAmiD by the substrate, which can be described by the kinetic scheme shown in Figure 4. The sAmiD-dependent product formation in these burst kinetics obeys Eq. (1):

$$P = v_{ss} * t - (v_{ss} - v_i)(1 - e^{-kt})/k \quad (1)$$

Table 2
The derivatives were incubated with various concentrations of sAmiD in 20 mM sodium phosphate buffer containing 48 nM $ZnCl_2$ at 30 °C for various incubation times *t*

Compound	*c* (μM)	v_i (μM min^{-1})	sAmiD (E_0) (μM)	v_i/E_0 (min^{-1})
TCT (GlcNAc β-(1,4)-anhMurNAc-tetraeptide (with A_2pm))	28	1.6	0.04	40
20 (anhMurNAc-dipeptide-Bnz)	100	0.2	15.4	0.013 (0.004[a])
27 (anhMurNAc-tripeptide (with Lys))	100	3.4	0.77	4.4 (1.2[a])
28 (anhMurNAc-tripeptide (with ε-TNBS-Lys))	100	1.4	0.34	4.1 (1.1[a])
29 (anhMurNAc-tetrapeptide (with A_2pm))	100	0.5	0.77	0.65 (0.18[a])

The solutions were analyzed by HPLC.
[a] Estimated v_i/E_0 at a substrate concentration of 28 μM. For these compounds a linear relationship between initial rates and substrate concentrations up to 1 mM was observed.

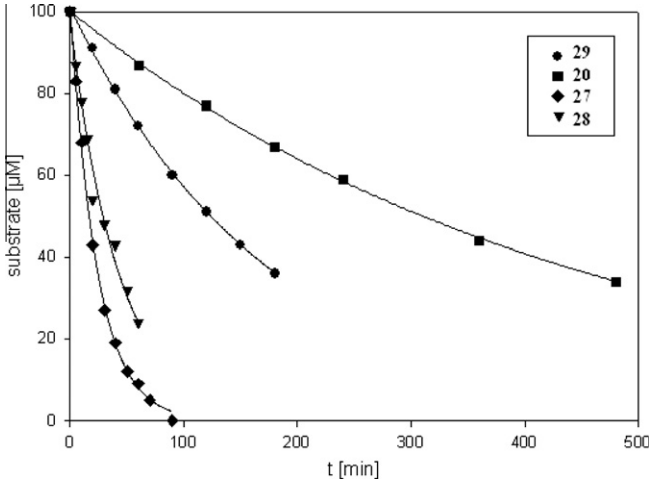


Figure 2. Hydrolysis time courses of **20**, **27**, **28** and **29**, **20**, **27**, **28** and **29** (100 μM) were hydrolysed with various enzyme concentrations (**20**: 7.7 μM, **27**: 0.7 μM, **28**: 0.3 μM and **29**: 0.7 μM) in 20 mM sodium phosphate buffer containing 48 nM $ZnCl_2$ at 30 °C. The reactions were stopped after various incubation times and analysed as described in Section 4.29.

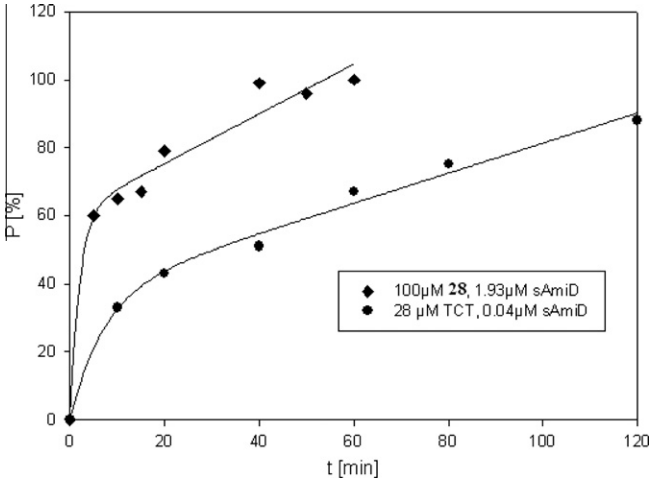


Figure 3. Hydrolysis time courses of **TCT** (28 μM) and **28** (100 μM) in the presence of sAmiD (0.04 μM or 1.93 μM). The biphasic behaviour is characterized by a rapid phase preceding a linear phase, a typical burst reaction. The curve fitting was obtained using Eq. (1). **TCT**: v_{ss} = 0.12 μM min^{-1}, v_{ii} = 1.6 μM min^{-1}, k = 0.14 min^{-1}. **28**: v_{ss} = 0.73 μM min^{-1}, v_{ii} = 31.4 μM min^{-1}, k = 0.5 min^{-1}.

where the concentration (p) of product at time t is given a function of v_i, v_{ss} and k. v_i and v_{ss} represent the initial and the stationary rates. k is the burst constant. The burst constant k increases with initial substrate concentration S. If the initial concentration of substrate is too low, the v_i/v_{ss} ratio is not far from 1 and no burst is observed. Conversely, if the amount of enzyme is too low, it becomes

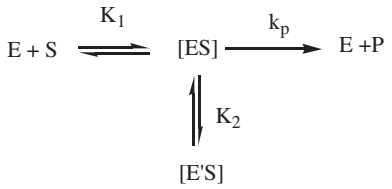


Figure 4. Kinetic scheme for the substrate-induced inactivation mechanism of sAmiD (Pennartz et al.). E, native enzyme; E′, inactivated or poorly active form of the enzyme induced by the substrate; S, substrate; P, product; [ES] and [E′S] substrate-enzyme complexes. K_1 and K_2 represent dissociation constants. k_p is the rate constant characterizing the hydrolytic decay of [ES]. This kinetic scheme can be used because the conversion of E′ to E is fast and the E↔E′ equilibrium completely displaced toward E.

difficult to distinguish a burst from a slow down due to substrate depletion.[14] For example, **28** exhibited burst kinetics in the presence of a high sAmiD concentration (1.9 μM) (Fig. 3), while in the presence of a lower sAmiD concentration (0.3 μM) no burst kinetic behaviour was found (Fig. 2). Thus as postulated by Waley[14] a branched-pathway mechanism as described in Figure 4 may prevail even when no burst is observed. This is probably due to the fact that one of the above conditions is not fulfilled (sufficient substrate and enzyme concentration).

In order to compare data, initial rates for all substrates were calculated from various curves (**20**, **27**, **28** and **29**) (Fig. 2) and **TCT** (Fig. 3). The v_i/E_0 values (Table 2) show that:

- **TCT** is about 33 times more active than the anhyMurNAc-tripeptide **27** and about 222 times more active than anhyMurNAc-tetrapeptide **29**, which contains the same peptide chain as **TCT**, showing that sAmiD prefers substrates containing GlcNAc-anhMurNAc to substrates containing only anhMurNAc, as already described.[5] Thus, GlcNAc-anhMurNAc-tripeptide was described as 100-fold more active than anhMurNAc-tripeptide.[6]
- The presence of TNB on the ε-amino group of L-Lys (compound **28**) appears to have little influence on the hydrolysis capacity of sAmiD, such that nearly identical v_i/E_0 values were observed for **28** and **27** (Table 2).
- The anhMurNAc derivative **20** containing a dipeptide chain was 10,000 times less active than **TCT** and the v_i/E_0 value of **20** was +/− 300 times smaller than those of **27** and **28**. Previously, other MurNAc-dipeptides (MurNAc-L-Ala-D-Glu, MurNAc-D-Ala-D-Glu and MurNAc-L-Ala-L-Glu) studied by Pennartz et al.[5] were found not to be substrates of sAmiD. Here we show that the elongation of the peptide chain by addition of a benzylamine group bound to the γ-carboxyl group of D-Glu allowed the recognition and hydrolysis of **20** by sAmiD.

In conclusion, a good substrate of sAmiD should contain anhMurNAc as a carbohydrate moiety with a tripeptide extension. It is possible to replace m-A_2pm by Lys, which facilitates synthesis. In agreement with crystallographic data showing that the

side-chain amino group of lysine projects into a solvent,[7] the addition of a chromophoric residue like TNB has no influence on the substrate specificity and allows easy monitoring of the hydrolysis reaction in complex samples by HPLC using a PDA-detector.

2.2.3. Inhibition studies of sAmiD

Amidases play an essential role in the bacterial life cycle and are considered as appropriate drug targets in the development of antibiotics. Thus, amidase inhibitors have been proposed as antibacterial agents.[15] Inhibition studies were performed with several derivatives that were not substrates of sAmiD (**6**, **7**, **8** and **11**). These compounds were incubated for 20 min at 30 °C with sAmiD (39 nM). The residual activity of the amidase was determined by measuring the initial rate of the hydrolysis of 29 μM **TCT**. No inhibition was observed with 100 μM of the anhMurNAc derivatives **6**, **7**, **8**, **11** and **20**. In the presence of **20** at concentrations of 0.5 mM and 1 mM residual activities of 83% and 64% (respectively) were detected, demonstrating the competition between the natural substrate **TCT** and **20**. Furthermore, after a pre-incubation of 30 min at 30 °C of 1 mM **1**, **6**, **7**, **8** and **11** with sAmiD, the residual activity of sAmiD was determined by measuring the initial rate of the hydrolysis of 1 mM **20**, which is 10,000 less active than **TCT** (Table 2). Despite the fact that these conditions could facilitate the detection of an inhibition, no such phenomenon was observed.

Our results illustrate that anhMurNAc **1** and anhMurNAc derivatives containing an amide bond between the lactyl group of MurNAc and the amino group of substituted aromatic rings (**6**, **7** and **8**) or the α-amino group of L-alanine of the L-alanine-para-nitrophenyl residue **11** (Table 1) were neither substrates nor inhibitors of sAmiD. For binding, the enzyme requires an anhMurNAc derivative containing at least a dipeptide chain amidated by benzylamine on the γ-carboxylgroup of D-Glu (Section 2.2.1).

3. Conclusion

Using the solid support strategy, we have shown that it was possible to attach compound **2** with peptides of various lengths still bound to the resin. After a TFA treatment, the glycopeptide obtained is completely deprotected to produce the desired 1,6-AnhMurNAc derivatives containing peptide side chains of various lengths. With this strategy, we have prepared compounds **27** and **29** containing three and four amino acids (respectively) in the peptide side chains. We have also shown that the solid support synthesis of **27** produces a better yield (40%) than in homogenate phase (6.3%).

Biological studies with sAmiD showed that anhMurNAc-L-Ala-γ-D-Glu-L-Lys **28** with TNB as chromophoric group on the ε-amino group of L-Lys is a good candidate for the development of a novel sensitive enzyme assay of sAmiD.

4. Experimental

All chemicals and reagents were either purchased puriss. p.a. from commercial suppliers. Melting points (Mp) were determined with Büchi Melting Point-B545 and are uncorrected. For analytical thin layer chromatography (TLC), plastic silica gel plates (Macherey-Nagel Polygram SIL G/UV_{254}) were used, and compounds were viewed by irradiation with UV light and/or by pulverizing of a solution of ethanol/anisaldehyde/sulfuric acid (v/v/v: 90:5:5), each followed by heating. The HPLC chain consists of a pump (Waters 600) and an UV detector (PDA Waters 996), (200 and 400 nm). The analytical HPLC analyses were performed on a XTerra RP18 column (4.6 × 150 mm, 3.5 μm) whereas the semi-preparative purifications were performed with a semi-preparative XTerra RP18 column (7.8 × 300 mm, 7 μm). Preparative HPLC was performed at a pressure of 50 bars on HPLC NOVASEP LC50.500.VE150 with 250 g of silica gel Merck (0.015–0.040 mm). Column chromatography was performed with silica gel 0.060–0.200 mm from Acros. ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature on Bruker 250 or 400 MHz apparatus. Chemical shifts are given in δ relative to tetramethylsilane as an internal standard, the coupling constants *J* are given in hertz. Assignment of peaks was based upon HMBC, HSQC and COSY experiments. The abbreviations are as follows: s = singulet, d = doublet, t = triplet, q = quadruplet, m = multiplet. MS analysis was made on a TSQ 7000 Thermoquest Finnigan device equipped with an electrospray source (ES). The co-solvent (injected in 200 μl/min) is a mixture 50:50: H_2O/MeOH containing 0.1% of HCOOH or HAc. The automated solid phase peptide synthesizer was a PS3 from Protein Technologies used in Fmoc strategy employing HBTU as the coupling agent.

4.1. General procedure for peptide coupling with BOP and NMM (Procedure A)

Under a nitrogen atmosphere, NMM (2.2 equiv) and BOP (1 equiv) were added at 0 °C to a stirred solution of the free acid (1 equiv) in dry DMF (approximately 0.08 M per component). After 1 h, the amine (1 equiv) was added. The reaction was stirred under a nitrogen atmosphere at rt for 48 h. The solvent was concentrated in vacuum and the residue was coevaporated several times with toluene in order to remove traces of DMF. The residue is diluted with CH_2Cl_2 and the organic phase was washed with three portions of saturated NH_4Cl, dried ($MgSO_4$), filtered and concentrated in a vacuum to produce an oil which was purified by preparative HPLC.

4.2. General procedure for peptide coupling with TBTU and DIEA (Procedure B)

DIEA (2.2 equiv) was added at room temperature to a stirred solution of the free acid (1 equiv) in dry CH_2Cl_2 (approximately 0.08 M per component). After 1 h, the amine (1 equiv) was added and the reaction was stirred for another 1 h. The organic phase was washed with three portions of saturated NH_4Cl, dried ($MgSO_4$), filtered and concentrated in a vacuum to produce a solid or oil. Sometimes, purification by preparative HPLC was necessary.

4.3. General procedure for PMB-cleavage by DDQ (Procedure C)

Under a nitrogen atmosphere, DDQ (1.1 equiv) was added at 0 °C to a stirred solution of starting material in a mixture of CH_2Cl_2/H_2O (20:1 v/v) (approximately 0.05 M for starting material). After 24 h, CH_2Cl_2 (10 v) was added and the organic phase was washed with saturated $NaHCO_3$ and brine, dried ($MgSO_4$), filtered and concentrated in a vacuum to produce an oil which was purified with a semi-preparative in reverse phase HPLC.

4.4. General procedure for BOC-cleavage (Procedure D)

Under a nitrogen atmosphere, the starting material was stirred for 30 min in a mixture of TFA/CH_2Cl_2 (1:1 v/v) (approximately 0.1 M for starting material). The solvents were concentrated in a vacuum and the residue was diluted with saturated Na_2CO_3 (5 v). The desired product was extracted with CH_2Cl_2 and the organic phase was dried ($MgSO_4$), filtered and concentrated in a vacuum to produce an oil which was used without further purification

4.5. General procedure for glycopeptide solid phase synthesis (Procedure E)

The glycopeptides were prepared by the solid phase peptide synthesis strategy on a PS3 automated peptide synthesizer

(Protein Technologies, Inc., Tucson, AZ) using *N*-α-fluorenylmethoxycarbonyl (Fmoc)-based chemistry on Wang-Fmoc-L-Lys(Boc) resin or Wang-Fmoc-D-Ala resin. These resins and *N*-α-Fmoc amino acids (0.4 mmol) were purchased from Iris Biotech GmbH, Marktredwitz, Germany. The side chain of Glu was protected with the t-butyl derivative (*t*Bu). Compound **2** (0.4 mmol) was used as an amino acid. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (Iris Biotech GmbH) (0.4 mmol) and *N*-methylmorpholine 0.4 M in *N*,*N*-dimethylformamide (DMF) (3 ml, 0.4 mmol) were used as coupling and activating reagents, respectively. Fmoc deprotection at each step was carried out using 20% piperidine/DMF (12 ml). The glycopeptides attached to the resin beads were washed subsequently with DMF, EtOH, and CH_2Cl_2. The side-chain protecting groups were removed, and the glycopeptides were cleaved from the resin using TFA/anisole/water (6 ml; 10:1:1) for 3 h at room temperature. The uncharged resin was separated from the solution by filtration. The crude glycopeptides were precipitated with Et_2O and used directly in the next step.

4.6. 2-Acetamido-1,6-anhydro-4-*O*-benzyl-2-deoxy-3-*O*-{[(2*R*)-*N*-phenylpropionamide]-2-yl}-β-D-glucopyranose (3)

Compound **12** and aniline were coupled according to Procedure A (DMF was replaced by CH_2Cl_2). Purification by preparative HPLC (petroleum/AcOEt: 34:66–0:100) afforded **3** as a white solid (50% yield). TLC (AcOEt/petroleum: 2:1): R_f = 0.4. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.32 (1H, s, NH-amide), 7.67–7.65 (2H, m, ArH-amide), 7.39–7.29 (7H, m, ArH-amide and ArH PMB), 7.11–7.08 (1H, m, ArH PMB), 6.05 (1H, d, NH NHAc, J = 8.0), 5.51 (1H, s, H-1), 4.73–4.70 (2H, m, H-5 and –CH_2-phenyl), 4.63 (1H, d, –CH_2-phenyl, J = 11.6), 4.31 (1H, d, H-6$_{endo}$, J = 7.0), 4.23 (1H, q, H-lactyl, J = 6.6), 4.12 (1H, d, H-2, J = 8.0), 3.89 (1H, dd, H-6$_{exo}$, J = 5.0 and 7.0), 3.49 (1H, s, H-3), 3.27 (1H, s, H-4), 1.94 (3H, s, CH_3 NHAc), 1.43 (3H, d, lactyl-CH_3, J = 6.6). HPLC (CH_3CN/H_2O: 40:60, 0.7 ml/min): t_R = 20 min. MS (ES+): m/z = 441 (M+1), 463 (adduct Na^+).

4.7. 2-Acetamido-3-*O*-{[(2*R*)-*N*-4-acetylphenylpropionamide]-2-yl}-1,6-anhydro-2-deoxy-4-*O*-*p*-methoxybenzyl-β-D-glucopyranose (4)

Compound **2** and *p*-aminoacetophenone were coupled according to Procedure A. Purification by preparative HPLC (AcOEt) afforded **4** as a white solid (25% yield). TLC (AcOEt): R_f = 0.6. Mp: 145 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.58 (1H, s, NH-amide), 7.91 (2H, d, ArH-amide, J = 8.9), 7.78 (2H, d, ArH-amide, J = 8.9), 7.25 (2H, d, ArH PMB, J = 8.6), 6.91 (2H, d, ArH PMB, J = 8.6), 6.09 (1H, d, NH NHAc, J = 8.0), 5.52 (1H, s, H-1), 4.69–4.62 (2H, m, –CH_2-phenyl and H-5), 4.57 (1H, d, –CH_2-phenyl, J = 11.6), 4.30 (2H, m, H-6$_{endo}$ and H-lactyl), 4.10 (1H, d, H-2, J = 8.0), 3.92 (1H, dd, H-6$_{exo}$, J = 5.0 and 7.0), 3.55 (1H, s, H-3), 3.48 (1H, s, H-4), 2.57 (3H, s, CH_3–CO-Ar), 1.94 (3H, s, CH_3 NHAc), 1.43 (3H, d, lactyl-CH_3, J = 6.6). HPLC (CH_3CN/H_2O: 40:60, 0.5 ml/min): t_R = 18.0 min. MS (ES+): m/z = 513 (M+1). MS (ES–): m/z = 511 (M–1).

4.8. 2-Acetamido-1,6-anhydro-4-*O*-benzyl-2-deoxy-3-*O*-{[(2*R*)-*N*-4-(2,2,2-trifluoroacetyl)phenylpropionamide]-2-yl}-β-D-glucopyranose (5)

Compound **2** and *p*-trifluoromethylaniline were coupled according to Procedure A. Purification by preparative HPLC (AcOEt) afforded **5** as a white solid (40% yield). TLC (AcOEt): R_f = 0.77. Mp: 154 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.56 (1H, s, NH-amide), 7.78 (2H, d, ArH-amide, J = 8.9), 7.54 (2H, d, ArH-amide, J = 8.9), 7.25 (2H, d, ArH PMB, J = 8.6), 6.91 (2H, d, ArH PMB, J = 8.6), 6.09 (1H, d, NH NHAc, J = 8.0), 5.51 (1H, s, H-1), 4.67–4.62 (2H, m, –CH_2-phenyl and H-5), 4.54 (1H, d, –CH_2-phenyl, J = 11.6), 4.29–4.24 (2H, m, H-lactyl and H-6$_{endo}$), 4.12 (1H, d, H-2, J = 8.0), 3.89 (1H, dd, H-6$_{exo}$, J = 5.0 and 7.0), 3.82 (3H, s, –OCH_3), 3.55 (1H, s, H-3), 3.48 (1H, s, H-4), 1.95 (3H, s, CH_3 NHAc), 1.43 (3H, d, lactyl-CH_3, J = 6.6). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 170.9, 169.7, 159.8, 141.3, 129.5, 126.5 (7), 119.2 (2), 114.2 (2), 100.8, 76.1, 75.5, 74.4 (2), 71.5, 65.7, 55.4, 47.3, 23.2, 17.2. HPLC (CH_3CN/H_2O: 60:40, 0.7 ml/min): t_R = 7.5 min. MS (ES+): m/z = 539 (M+1). MS (ES–): m/z = 597 (adduct HAc).

4.9. 2-Acetamido-1,6-anhydro-2-deoxy-3-*O*-{[(2*R*)-*N*-phenylpropionamide]-2-yl}-β-D-glucopyranose (6)

Catalytic Pd/C (10%) (45 mg) was added to a solution of **3** (35.8 mg, 0.08 mmol) in MeOH (20 ml). A hydrogen atmosphere (two bars) was maintained for 6 h. The mixture was filtered through Celite, and the catalyst was washed with hot MeOH. After concentration of the filtrate, the residual colourless oil was crystallized under cooling to afford **6** as a white solid (12% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.34 (1H, s, NH-amide), 7.67 (2H, d, ArH-amide, J = 7.6), 7.29 (2H, t, ArH-amide, J = 7.6), 7.08 (1H, t, ArH-amide, J = 7.6), 6.43 (1H, d, NH NHAc, J = 8.0), 5.51 (1H, s, H-1), 4.58 (1H, d, H-5, J = 5.0), 4.30 (1H, d, H-6$_{endo}$, J = 7.0), 4.28–4.23 (1H, q, H-lactyl, J = 6.6), 4.10 (1H, d, H-2, J = 8.0), 3.86 (1H, dd, H-6$_{exo}$, J = 5.0 and 7.0), 3.82–3.73 (2H, m, H-4 and –OH), 3.51 (1H, s, H-3), 1.98 (3H, s, CH_3 NHAc), 1.45 (3H, d, lactyl-CH_3, J = 6.6). HPLC (CH_3CN/H_2O: 40:60, 0.7 ml/min): t_R = 3 min. MS (ES+): m/z = 373 (adduct Na^+), 414 (adduct $CH_3CN + Na^+$).

4.10. 2-Acetamido-3-*O*-{[(2*R*)-*N*-4-acetylphenylpropionamide]-2-yl}-1,6-anhydro-2-deoxy-β-D-glucopyranose (7)

Compound **4** was deprotected according to Procedure C. Purification by semi-preparative reverse phase HPLC (CH_3CN/H_2O: 40:60) produced **7** as a white solid (20% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.52 (1H, s, NH-amide), 7.91 (2H, d, ArH-amide, J = 8.9), 7.78 (2H, d, ArH-amide, J = 8.9), 6.33 (1H, d, NH NHAc, J = 8.0), 5.54 (1H, s, H-1), 4.62 (1H, d, H-5, J = 5.0), 4.35–4.29 (2H, m, H-lactyl and H-6$_{endo}$), 4.10 (1H, d, H-2, J = 8.0), 3.89 (1H, dd, H-6$_{exo}$, J = 5.0 and 7.0), 3.82–3.73 (2H, m, H-4 and –OH), 3.53 (1H, s, H-3), 2.57 (3H, s, CH_3–CO-Ar), 1.99 (3H, s, CH_3 NHAc), 1.48 (3H, d, lactyl-CH_3, J = 6.6). HPLC (CH_3CN/H_2O: 40:60, 05 ml/min): t_R = 4.5 min. MS (ES+): m/z = 393 (M+1). MS (ES–): m/z = 451 (adduct HAc).

4.11. 2-Acetamido-1,6-anhydro-2-deoxy-3-*O*-{[(2*R*)-*N*-4-(2,2,2-trifluoroacetyl)phenylpropionamide]-2-yl}-β-D-glucopyranose (8)

Compound **5** was deprotected according to Procedure C. Purification by semi-preparative reverse phase HPLC (CH_3CN/H_2O: 30:70) produced **8** as a white solid (31% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.55 (1H, s, NH-amide), 7.78 (2H, d, ArH-amide, J = 8.9), 7.54 (2H, d, ArH-amide, J = 8.9), 6.24 (1H, d, NH NHAc, J = 8.0), 5.54 (1H, s, H-1), 4.62 (1H, d, H-5, J = 5.0), 4.35–4.29 (2H, m, H-lactyl and H-6$_{endo}$), 4.11 (1H, d, H-2, J = 8.0), 3.89 (1H, dd, H-6$_{exo}$, J = 5.0 and 7.0), 3.82–3.73 (2H, m, H-4 and –OH), 3.54 (1H, s, H-3), 2.00 (3H, s, CH_3 NHAc), 1.48 (3H, d, lactyl-CH_3, J = 6.6). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 171.5, 170.4, 141.3, 126.2 (4), 119.3 (2), 100.1, 79.0, 76.5, 76.2, 69.8, 65.1, 47.7, 23.1, 17.5. HPLC (CH_3CN/H_2O: 60:40, 0.7 ml/min): t_R = 3.6 min. MS (ES+): m/z = 419 (M+1). MS (ES–): m/z = 415 (M–1), 477 (adduct HAc).

4.12. 2-Acetamido-1,6-anhydro-2-deoxy-4-*O*-*p*-methoxybenzyl-3-*O*-{(2*R*)-propionyl-[L-alanine-*p*-nitroanilide]-2-yl}-β-D-glucopyranose (10)

Compound **2** and L-Ala-*p*-nitroanilide hydrochloride **9** were coupled according to Procedure A. Purification by preparative HPLC (AcOEt/MeOH: 9:1) produced **10** as a white solid (73% yield). TLC (AcOEt/MeOH: 9:1): R_f = 0.7. Mp: 61.6 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.65 (1H, s, NH-Ar), 8.16 (2H, d, ArH, J = 9.2), 8.04 (1H, d, NH-Ala, J = 6.3), 7.68 (2H, d, ArH, J = 9.2), 7.26 (2H, d, ArH, J = 8.6), 6.90 (2H, d, ArH, J = 8.6), 6.09 (1H, d, NH NHAc, J = 8.0), 5.43 (1H, s, H-1), 4.62–4.59 (3H, m, 1H CH_2-phenyl, H-Ala and H-5), 4.53 (1H, d, 1H CH_2-phenyl, J = 11.6), 4.17–4.15 (2H, m, H-lactyl and H-6$_{endo}$), 4.03 (1H, d, H-2, J = 8.0), 3.81 (3H, s, –OCH_3), 3.79 (1H, m, H-6$_{exo}$), 3.48 (1H, s, H-3), 3.40 (1H, s, H-4), 1.96 (3H, s, CH_3 NHAc), 1.48 (3H, d, –CH_3 Ala, J = 7.0), 1.37 (3H, d, lactyl-CH_3, J = 6.6). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 174.0, 171.1, 169.9, 159.9, 144.4, 143.4, 129.5 (2), 129.1, 124.9 (2), 119.2 (2), 114.3 (2), 100.6, 76.4, 75.7, 75.6, 74.3, 71.5, 65.3, 55.4, 50.0, 47.4, 23.2, 17.7, 16.7. HPLC (CH_3CN/H_2O: 60:40, 0.7 ml/min): t_R = 4.3 min. MS (ES−): m/z = 585 (M−1), 645 (adduct HAc).

4.13. 2-Acetamido-1,6-anhydro-2-deoxy-3-*O*-{(2*R*)-propionyl-[L-alanine-*p*-nitroanilide]-2-yl}-β-D-glucopyranose (11)

Compound **10** was deprotected according to Procedure C. However, the reaction was stirred for 48 h. Purification by semi-preparative reverse phase HPLC (CH_3CN/H_2O: 40:60) produced **11** as a white solid (10% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.66 (1H, s, NH-Ar), 8.16 (2H, d, ArH, J = 8.6), 8.06 (1H, d, NH-Ala, J = 6.3), 7.69 (2H, d, ArH, J = 8.6), 6.25 (1H, d, NH NHAc, J = 8.0), 5.46 (1H, s, H-1), 4.60–4.58 (2H, m, H-Ala and H-5), 4.25–4.23 (2H, m, H-lactyl and H-6$_{endo}$), 4.02 (1H, d, H-2, J = 8.0), 3.79–3,49 (3H, m, H-6$_{exo}$ and H-4 and OH), 3.07 (1H, s, H-3), 2.01 (3H, s, CH_3 NHAc), 1.48 (3H, d, –CH_3 Ala, J = 7.0), 1.40 (3H, d, lactyl-CH_3, J = 6.9). HPLC (CH_3CN/H_2O: 40:60, 0.5 ml/min): t_R = 5.4 min. MS (ES+): m/z = 467 (M+1). MS (ES−): m/z = 465 (M−1).

4.14. α-Benzyl-*N*-(*tert*-butyloxycarbonyl)-*N*-δ-phenyl-D-glutamine (13)

Compound **12** and aniline were coupled according to Procedure B. Purification by preparative HPLC (CH_2Cl_2/AcOEt: 1:1) produced **13** as a white solid (75% yield). The spectroscopic data were identical to these reported previously.[16]

4.15. α-Benzyl-*N*-δ-phenyl-D-glutamine (14)

Compound **13** was deprotected according to Procedure D (71% yield). The spectroscopic data were identical to these reported previously.[17]

4.16. α-Benzyl-*N*-α-(*N*-*tert*-butyloxycarbonyl-L-alanyl)-*N*-δ-phenyl-D-glutaminate (16)

Compound **14** and NHBOC-L-Ala **15** (commercially available from IRIS) were coupled according to Procedure B, and yielded **16** as a white solid (75% yield). Mp: 129 °C. TLC (CH_2Cl_2/AcOEt: 1:1): R_f = 0.56. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.42 (1H, s, NH-C_6H_5), 7.58–7.29 (10H, m, ArH), 7.07 (1H, d, NH BOC, J = 4.3), 6.99 (1H, d, NH-Glu, J = 7.0), 5.15 (2H, d, CH_2 Bn, J = 11.6), 4.66 (1H, d, H-Glu, J = 7.0), 4.17 (1H, m, H-Ala), 2.36 (2H, m, CH–CH_2-Glu), 1.95 (2H, m, CH–CH_2–CH_2-Glu), 1.45 (9H, s, –$C(CH_3)_3$), 1.37 (3H, d, –CH_3 Ala, J = 7.0). HPLC (CH_3CN/H_2O: 60:40, 0.7 ml/min): t_R = 6.7 min. MS (ES+): m/z = 484 (M+1). MS (ES−): m/z = 482 (M−1), 542 (adduct HAc).

4.17. α-Benzyl-*N*-α-L-alanyl-*N*-δ-phenyl-D-glutaminate (17)

Compound **16** was deprotected according to Procedure D to produce **17** (74% yield). TLC (CH_2Cl_2/AcOEt: 1:1) R_f = 0.05. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.66 (1H, s, NH–C_6H_5), 8.15 (1H, d, NH-Glu, J = 7.0), 7.60–7.07 (10H, m, ArH), 5.22–5.12 (2H, d, CH_2 Bn, J = 11.6), 4.66 (1H, d, H-Glu, J = 7.0), 3.53 (1H, m, H-Ala), 2.36 (2H, m, CH–CH_2-Glu), 2.02 (2H, m, CH–CH_2–CH_2-Glu), 1.37 (3H, d, –CH_3 Ala, J = 7.0). MS (ES+): m/z = 384 (M+1). MS (ES−): m/z = 382 (M−1), 442 (adduct HAc).

4.18. 2-Acetamido-1,6-anhydro-2-deoxy-4-*O*-*p*-methoxybenzyl-3-*O*-{(2*R*)-propionyl-[L-alanyl-(α-benzyl-*N*-δ-phenyl-glutaminate)]-2-yl}-β-D-glucopyranose (18)

Compounds **17** and **2** were coupled according to Procedure B to produce **18** as a white solid. However, PyBOP was used instead of TBTU (51% yield). TLC (AcOEt/MeOH: 10:1): R_f = 0.55. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.66 (1H, s, NH-C_6H_5), 7.96 (1H, d, NH-Ala, J = 6.3), 7.57–7.55 (2H, m, ArH), 7.31–7.22 (10H, m, ArH), 6.95 (1H, d, NH-Glu, J = 7.0), 6.90 (2H, d, ArH PMB, J = 8.6), 6.06 (1H, d, NH NHAc, J = 8.0), 5.39 (1H, s, H-1), 5.22–5.12 (2H, d, CH_2 Bn, J = 11.6), 4.61 (1H, d, H-Glu, J = 7.0), 4.60 (1H, d, 1H CH_2 PMB, J = 11.6), 4.57 (1H, d, H-5, J = 5.0), 4.51 (1H, d, 1H CH_2 PMB, J = 11.6), 4.39 (1H, m, H-Ala), 4.17 (1H, d, H-6$_{endo}$, J = 7.0), 4.11 (1H, q, H-lactyl, J = 6.6), 4.01 (1H, d, H-2, J = 8.0), 3.79 (3H, s, CH_3 PMB), 3.72 (1H, dd, H-6$_{exo}$, J = 5.0 and 7.0), 3.46 (1H, s, H-3), 3.39 (1H, s, H-4), 2.35–2.30 (4H, m, 2 × CH_2 Glu), 1.92 (3H, s, CH_3 NHAc), 1.37 (3H, d, –CH_3 Ala, J = 7.0), 1.31 (3H, d, lactyl-CH_3, J = 6.6). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 174.4, 173.4, 172.5, 171.2, 170.6, 159.0, 139.1, 136.0, 130.2 (2), 129.9–129.0 (7), 124.8, 120.7 (2), 115.0 (2), 101.4, 77.2, 76.8, 76.3, 75.1, 72.3, 68.4, 66.0, 56.3, 52.4, 50.6, 48.2, 34.2, 29.9, 24.1, 18.7, 18.0. HPLC (CH_3CN/H_2O: 60:40, 0.7 ml/min): t_R = 6.7 min. MS (ES+): m/z = 761 (M+1), 783 (adduct Na^+). MS (ES−): m/z = 759 (M−1), 819 (adduct HAc).

4.19. 2-Acetamido-1,6-anhydro-2-deoxy-4-*O*-*p*-methoxybenzyl-3-*O*-{(2*R*)-propionyl-[L-alanyl-(*N*-δ-phenyl-glutaminate)]-2-yl}-β-D-glucopyranose (19)

Catalytic Pd/C (5%, wet) (120 mg) was added to a stirred solution of **18** (496 mg, 0.65 mmol) in EtOH (25 ml). A hydrogen atmosphere (2.5 bars) was maintained for 6 h. The mixture was filtered through Celite, and the catalyst was washed with hot EtOH. The filtrate was concentrated in a vacuum to yield **19** as a colourless oil (68% yield). TLC (AcOEt/MeOH + HCOOH: 9:1): R_f = 0.30. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 8.84 (1H, s, NH-C_6H_5), 7.96 (1H, d, NH-Ala, J = 6.3), 7.57–7.55 (2H, m, ArH), 7.31–7.22 (5H, m, ArH), 7.03 (1H, d, NH-Glu, J = 7.0), 6.86 (2H, d, ArH, J = 8.6), 6.10 (1H, d, NH NHAc, J = 8.0), 5.36 (1H, s, H-1), 4.55–4.46 (4H, m, H-Glu, H-5 and 2H CH_2 PMB), 4.36 (1H, m, H-Ala), 4.16 (1H, d, H-6$_{endo}$, J = 7.0), 4.07 (1H, q, H-lactyl, J = 6.6), 3.96 (1H, d, H-2, J = 8,0), 3.78 (3H, s, CH_3 PMB), 3.69 (1H, dd, H-6$_{exo}$, J = 5.0 and 7.0), 3.43 (1H, s, H-3), 3.38 (1H, s, H-4), 2.41–2.29 (4H, m, 2 × CH_2 Glu), 1.92 (3H, s, CH_3 NHAc), 1.37 (3H, d, –CH_3 Ala, J = 7,0), 1.30 (3H, d, lactyl-CH_3, J = 6.6). HPLC (CH_3CN/H_2O: 60:40, 0.7 ml/min): t_R = 2.1 min. MS (ES+): m/z = 671 (M+1). MS (ES−): m/z = 669 (M−1).

4.20. 2-Acetamido-1,6-anhydro-2-deoxy-3-*O*-{(2*R*)-propionyl-[L-alanyl-(*N*-δ-phenyl-glutaminate)]-2-yl}-β-D-glucopyranose (20)

Compound **19** was deprotected according to Procedure C. Purification by preparative HPLC (AcOEt/MeOH + HCOOH 0.1%: 82:18) followed by a semi-preparative reverse phase HPLC (CH_3CN/H_2O: 20:80) yielded **20** as a white solid (10% yield). TLC (AcOEt/MeOH:

9:2): R_f = 0.45. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 9.21 (1H, s, NH-C_6H_5), 7.96 (1H, d, NH-Ala, J = 6.3), 7.47–7.20 (5H, m, ArH), 6.98 (1H, d, NH-Glu, J = 7.0), 6.06 (1H, d, NH NHAc, J = 8.0), 5.32 (1H, s, H-1), 4.46 (2H, m, H-Glu and H-5), 4.30 (1H, m, H-Ala), 4.15 (1H, d, H-6_{endo}, J = 7.0), 4.06 (1H, d, H-lactyl, J = 6.6), 3.89 (1H, d, H-2, J = 8,0), 3.70 (1H, dd, H-6_{exo}, J = 5.0 and 7.0), 3.61–3.49 (2H, m, H-4 and –OH), 3.32 (1H, s, H-3), 2.27 (4H, m, 2 × CH_2 Glu), 1.90 (3H, s, CH_3 NHAc), 1.34 (3H, d, lactyl-CH_3, J = 6.6), 1.28 (3H, d, –CH_3 Ala, J = 7,0). HPLC (CH_3CN/H_2O: 20:80, 0.7 ml/min): t_R = 5.5 min. MS (ES+): m/z = 551 (M+1), 573 (adduct Na^+). MS (ES−): m/z = 549 (M−1).

4.21. Benzyl *N*-(*N*-*tert*-butyloxycarbonyl-*O*-benzyl-γ-D-glutamyl)-*N*-ε-benzyloxycarbonyl-L-lysine (22)

Compound **21** H-Lys(Z)-OBzl·HCl (commercially available from IRIS) and **12** were coupled according to Procedure B, and produced **22** as a white solid after crystallization in $CHCl_3$–petroleum (98% yield). TLC (petroleum/AcOEt: 1:1): R_f = 0.4. Mp: 109 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 7.36–7.24 (15H, s, ArH), 6.73 (1H, d, NH-Lys, J = 7.0), 5.33 (1H, d, NHBOC, J = 4.3), 5.20 (6H, m, 3 × –CH_2-Bn), 4.88 (1H, s, –NHZ), 4.61 (1H, d, H-Lys, J = 7.0), 4.43 (1H, d, H-Glu, J = 7.0), 3.14 (2H, d, –CH_2-NHZ, J = 5.0), 2.31–1.95 (4H, m, 2 × –CH_2-Glu), 1.86–1.67 (2H, m, –CH–CH_2-Lys), 1.55–1.25 (13H, m, –CH–CH_2–CH_2–CH_2–CH_2-NHZ and –C$(CH_3)_3$). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 172.1 (2), 156.6, 155.9, 136.6, 135.4, 135.2, 128–110 (15), 80.1, 67.2, 67.0, 66.5, 52.9, 52.2, 40.5, 32.2, 29.3, 28.9, 28.3 (3), 27.7, 22.3. HPLC (CH_3CN/H_2O: 60:40, 0.7 ml/min): t_R = 16 min. MS (ES+): m/z = 690 (M+1), 712 (adduct Na^+).

4.22. Benzyl *N*-((*N*-*tert*-butyloxycarbonyl-L-alanyl)-*O*-benzyl-γ-D-glutamyl)-*N*-ε-benzyloxycarbonyl-L-lysine (24)

Compound **22** was deprotected according to Procedure D and coupled with NHBOC-L-Ala **15** according to Procedure B to produce **24** as a white solid after crystallization in $CHCl_3$–petroleum (60% yield). Mp: 115 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 7.35 (17H, s, ArH, NH-Lys and NH-Glu), 5.21–5.09 (7H, m, 3 × –CH_2-Bn and NH-CBZ), 5.01 (1H, d, NH-Ala, J = 6.3), 4,56 (2H, d, H-Lys and H-Glu, J = 7.0), 4.11 (1H, m, H-Ala), 3.20–3.08 (2H, m, –CH_2-NHZ Lys), 2.25–2.06 (4H, m, 2 × –CH_2-Glu), 1.83–1.75 (2H, m, –CH–CH_2-Lys), 1.53 (2H, m, –CH_2–CH_2-NHZ Lys), 1.46 (9H, s, –C$(CH_3)_3$), 1.34 (3H, d, –CH_3 Ala, J = 7.0), 1.29 (2H, m, –CH_2–CH_2–CH_2-NHZ Lys). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 173.5, 172.6, 171.8, 171.3, 156.5, 155.6, 136.5, 135.3, 135.2, 128.5, 128.1 (15), 80.2, 67.2, 67.1, 66.6, 53.8, 52.4, 51.6, 40.4, 31.8, 30.9, 29.2, 28.2 (3), 27.7, 22.5, 17.9. HPLC (CH_3CN/H_2O: 75:25, 0.7 ml/min): t_R = 6.0 min. MS (ES+): m/z = 761 (M+1), 783 (adduct Na^+).

4.23. 2-Acetamido-1,6-anhydro-2-deoxy-4-*O*-*p*-methoxybenzyl-3-*O*-{(2*R*)-propionyl-[L-alanyl-(γ-D-glutamyl α-benzyl ester)-(*N*-ε-benzyloxycarbonyl-L-lysine α-benzyl ester)]-2-yl}-β-D-glucopyranose (26)

Compound **24** was deprotected according to Procedure D and coupled with **2** according to Procedure B to produce **26** as a white solid after purification by manually chromatography (AcOEt/MeOH: 9:1) (18% yield). TLC (AcOEt/MeOH: 9:1): R_f = 0.64. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm 7.90 (1H, d, NH-Glu, J = 7.0), 7.51 (1H, d, NH-Lys, J = 7.0), 7.35–7.29 (15H, s, ArH); 7,28 (1H, d, NH-Ala, J = 6.3), 7.28 (2H, d, ArH PMB, J = 8.6), 6.93 (2H, d, ArH PMB, J = 8.6), 6.10 (1H, d, NH NHAc, J = 8.0), 5.40 (1H, s, H-1), 5.21–5.09 (7H, m, 3 × –CH_2-Bn and NH-CBZ), 4.63 (3H, m, H-5 and CH_2-PMB), 4.53 (1H, d, H-Lys, J = 7.0), 4.32 (2H, m, H-Ala and H-Glu), 4.16–4.09 (2H, m, H-6_{endo} and H-lactyl), 4.02 (1H, d, H-2, J = 8.0), 3.83 (3H, s, –OMe), 3.76 (1H, dd, H-6_{exo}, J = 5.0 and 7.0), 3.47 (1H, s, H-3), 3.42 (1H, s, H-4), 3.14 (2H, m, –CH_2-NHZ Lys), 2.30–2.17 (4H, m, 2 × –CH_2-Glu), 1.93 (3H, s, CH_3 NHAc), 1.83–1.75 (2H, m, –CH–CH_2-Lys), 1.49 (2H, m, –CH_2–CH_2-NHZ Lys), 1.38–1.33 (8H, m, –CH_3 Ala, CH_2–CH_2–CH_2–NHZ Lys and CH_3-lactyl). ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 173.1–169.8 (6), 159.6, 156.5, 136.6, 135.4 (3), 129.9–127.8 (17), 114.2 (2), 100.4, 76.1, 75.7, 75.5, 74.1, 71.3, 67.1, 67.0, 66.4, 65.1, 55.8, 52.4, 51.6, 49.1, 47.3, 40.5, 31.7, 31.0, 29.3, 26.9, 23.1, 22.6, 17.7, 17.1. HPLC (CH_3CN/H_2O: 75:25, 0.7 ml/min): t_R = 5.1 min. MS (ES+): m/z = 1039 (M+1), 1061 (adduct Na^+).

4.24. *N*-Acetyl-1,6-anhydromuramyl-L-ala-γ-D-Glu-L-Lys (27) (homogenate phase)

Catalytic Pd/C (10%) (30 mg) was added to a stirred solution of **26** (118 mg, 0.11 mmol) in MeOH (30 ml). A hydrogen atmosphere (six bars) was maintained for 72 h at 35 °C. The mixture was filtered through Celite, and the catalyst was washed with hot MeOH. The filtrate was concentrated in a vacuum and the residue was purified by semi-preparative reverse phase HPLC (CH_3CN/H_2O: 50:50) to produce **27** as a white solid (60% yield). ^{1}H NMR (D_2O, 400 MHz) δ ppm (NH_2, 2 × COOH, 4 × NH–, OH-4 not detected) 5.40 (1H, s, H-1), 4.60 (1H, d, H-5, J = 5.0), 4.29 (1H, m, H-Ala), 4.22–4.08 (4H, m, H-lactyl, H-6_{endo}, H-Lys and H-Glu), 3.84 (1H, s, H-2), 3.79 (1H, s, H-4), 3.72 (1H, dd, H-6_{exo}, J = 5.0 and 7.0), 3.34 (1H, s, H-3), 2.90 (2H, m, –CH_2-NH_2 Lys), 2.40–2.07 (4H, m, 2 × –CH_2-Glu), 1.94 (3H, s, CH_3 NHAc), 1.78–1.58 (4H, m, –CH–CH_2-Lys and –CH_2–CH_2-NHZ Lys), 1.35–1.29 (8H, m, –CH_3 Ala, CH_2–CH_2–CH_2-NHZ Lys and CH_3-lactyl). HPLC (CH_3CN/H_2O: 50:50, 0.7 ml/min): t_R = 2.5 min. MS (ES+): m/z = 604 (M+1).

4.25. *N*-Acetyl-1,6-anhydromuramyl-L-ala-γ-D-Glu-L-Lys (27) (on solid support)

The glycopeptide was produced according to General Procedure E and the final deprotection was performed according to Section 4.24 (above) with a global yield of 40%. For spectroscopy data see Section 4.24.

4.26. *N*-Acetyl-1,6-anhydromuramyl-L-ala-γ-D-Glu-[*N*-ε-(2,4,6-trinitrophenyl)-L-lysine] (28)

TNBSA (1 M in MeOH, Aldrich) (200 μl, 0.2 mmol) was added to a stirred solution of **27** (49 mg, 0.081 mmol) at 37 °C in a carbonate buffer (0.1 N) to pH 9.5. After 1.5 h, a novel addition of TNBSA was performed (200 μl, 0.2 mmol) and the mixture was stirred for 1.5 h again. The solvent was concentrated in a vacuum and the residue was purified by semi-preparative reverse phase HPLC (CH_3CN/H_2O: 50:50) to produce **28** as a red solid (97% yield). ^{1}H NMR (MeOD, 400 MHz) δ ppm (2 × COOH, 5 × NH-, OH-4 not detected) 9.14 (2H, s, ArH), 5.49 (1H, s, H-1), 4.69 (1H, d, H-5, J = 5.0), 4.39–4.12 (5H, m, H-lactyl, H-6_{endo}, H-Lys, H-Glu and H-Ala), 3.93 (1H, s, H-2), 3.89 (1H, s, H-4), 3.82 (1H, dd, H-6_{exo}, J = 5.0 and 7.0), 3.44 (1H, s, H-3), 3.12 (2H, s, –CH_2-NH_2 Lys), 2.40–2.11 (4H, m, 2 × –CH_2-Glu), 2.02 (3H, s, CH_3 NHAc), 1.98–1.58 (4H, m, –CH–CH_2-Lys and –CH_2–CH_2-NHZ Lys), 1.43–1.29 (8H, m, –CH_3 Ala, CH_2–CH_2–CH_2-NHZ Lys and CH_3-lactyl). HPLC (CH_3CN/H_2O: 50:50, 0.7 ml/min): t_R = 2.5 min. MS (ES+): m/z = 815 (M+1). MS (ES−): m/z = 813 (M−1).

4.27. *N*-Acetyl-1,6-anhydromuramyl-L-ala-γ-D-Glu-meso-DAP-D-Ala trifluoroacetate salt (29)

The glycopeptide was produced according to General Procedure E and the final deprotection was performed as follows. Catalytic Pd/C (10%) (10 mg) was added to a stirred solution of previously

synthesized glycopeptide (63 mg, 6.69 10^{-2} mmol) in THF (15 ml) and 0.1% of TFA. A hydrogen atmosphere (six bars) was maintained for 48 h at rt. The mixture was filtered through Celite, and the catalyst was washed with hot THF. The filtrate was concentrated in a vacuum and the residue was precipitated in a mixture of TFA/Et_2O and collected by centrifugation (51% yield). ^{1}H NMR (D_2O, 400 MHz) δ ppm: 5.46 (1H, s, H-1), 4.65 (1H, d, J = 6.0, H-5), 4.35 (3H, m, 2 × H-Ala, H-DAP), 4.25 (2H, m, H-6_{endo}, H-DAP), 4.15 (1H, m, H-lactyl), 3.97 (1H, t, J = 6.0, H-Glu), 3.90 (1H, s, H-2), 3.84 (1H, s, H-4), 3.78 (1H, m, H-6_{exo}), 3.39 (1H, s, H-3), 2.36 (2H, m, $COCH_2$-Glu), 2.23 (2H, m, $COCH_2CH_2$-Glu), 1.72–1.98 (7H, m, CH_3 NHAc, $CH_2CH_2CH_2$ *DAP*), 1.47 (2H, m, $CH_2CH_2CH_2$ DAP), 1.40 (m, 9H, CH_3-lactyl, CH_3-L-Ala, CH_3-D-Ala). RMN ^{13}C (D_2O, 100 MHz) δ: 173.6–176.8 (8), 100.5, 79.1, 76.5, 76.4, 68.6, 65.7, 54.2, 53.6, 52.4, 50.1, 50.0, 49.2, 31.9, 31.1, 30.8, 27.2, 22.3, 21.3, 18.6, 17.4, 16.65. MS (ES+): m/z = 719 (M+1).

4.28. HPLC analysis for biological experiments

The following protocol was used: solvent A: water (Millipore quality) containing 0.05% or 0.1% TFA and solvent B: CH_3CN containing 0.05% or 0.1% TFA. Flow: 0.7 ml/min, gradient: 0–2 min: 100% A, 2–4 min: 0–10% B, 4–34 min: 10–70% B, 34–39 min: 70% B, 39–40 min: 30–100% A, 40–60 min: 100% A. The injection volumes were 10–100 μl.

4.29. Hydrolysis of anhMurNAc derivatives

The anhMurNAc derivatives were dissolved in water (concentration: **6**, **20**, **27**, **28** and **29**: 10 mM, **7**: 250 μM, **8**: 2.5 mM, **11**: 1 mM). The compounds were incubated for various incubation times with various enzyme concentrations (Table 1) in 20 mM sodium phosphate buffer containing 48 nM $ZnCl_2$ at 30 °C. The reaction was stopped by addition of 1:10 of the total assay volume of H_3PO_4 (1:100 dilution with water). The solution was analysed by HPLC as described previously.

4.30. Kinetic studies

Kinetic studies were performed with **20**, **27**, **28** and **29**. Various concentrations of the compounds were incubated with the following enzyme concentrations (**20**: 3.9 μM, **27**: 0.7 μM, **28**: 0.3 μM and **29**: 0.7 μM) in 20 mM sodium phosphate buffer containing 48 nM $ZnCl_2$ at 30 °C. The reactions were stopped after various incubation times (**20**: 2 h, **27** and **28**: 10 min; **29**: 30 min) and analysed as described previously.

100 μM of **20**, **27**, **28** and **29** were hydrolysed with various enzyme concentrations (**20**: 7.7 μM, **27**: 0.7 μM **28**: 0.3 μM and **29**: 0.7 μM) in 20 mM sodium phosphate buffer containing 48 nM $ZnCl_2$ at 30 °C. The reaction was stopped after various incubation times (Fig. 2) and analysed as described previously.

4.31. Inhibition studies

Inhibition studies were performed with compounds **1**, **6**, **7**, **8**, **11** and **20**. The anhMurNAc derivatives were dissolved in water (**1**, **6** and **11**: 10 mM, **20**: 1 mM, **8**: 2.5 mM) or in DMF (**7**: 100 mM). The presence of 1% DMF in the assay had no influence on the activity of sAmiD. The compounds (100 μM) were incubated in the presence of 39 nM sAmiD in 20 mM sodium phosphate buffer containing 48 nM $ZnCl_2$ at 30 °C for 20 min (total volume: 94 μl). The residual activity of sAmiD was determined by adding 6 μl **TCT** (0.5 mM). The hydrolysis was stopped after 20 min by addition of 10 μl H_3PO_4 (1:100 dilution with water). One hundred microlitres of the solutions were injected onto the HPLC column and analysed as described previously.

Furthermore, compounds **1**, **6**, **7**, **8**, **11** (1 mM) were incubated in the presence of 3.9 μM sAmiD in 20 mM sodium phosphate buffer containing 48 nM $ZnCl_2$ at 30 °C for 30 min (volume total: 50 μl). The residual activity of sAmiD was determined by adding 1 mM **20**. The hydrolysis of **20** was stopped after 2 h and the solution (50 μl) was analysed by HPLC as described previously.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.010.

References and notes

1. Coyette, J.; van der Ende, A. *FEMS Microbiol. Rev.* **2008**, *32*, 147.
2. Vollmer, W.; Blanot, D.; de Pedro, M. A. *FEMS Microbiol. Rev.* **2008**, *32*, 149.
3. Park, J. T.; Uehara, T. *Microbiol. Mol. Biol. Rev.* **2008**, *72*, 211.
4. Cheng, Q.; Li, H.; Merdek, K.; Park, J. T. *J. Bacteriol.* **2000**, *182*, 4836.
5. Pennartz, A.; Genereux, C.; Parquet, C.; Mengin-Lecreulx, D.; Joris, B. *Antimicrob. Agents Chemother.* **2009**, *53*, 2991.
6. Uehara, T.; Park, J. T. *J. Bacteriol.* **2007**, *189*, 5634.
7. Kerff, F.; Petrella, S.; Mercier, F.; Sauvage, E.; Herman, R.; Pennartz, A.; Zervosen, A.; Luxen, A.; Frere, J.-M.; Joris, B.; Charlier, P. *J. Mol. Biol.* **2010**, *397*, 249.
8. Kawasaki, A.; Karasudani, Y.; Otsuka, Y.; Hasegawa, M.; Inohara, N.; Fujimoto, Y.; Fukase, K. *Chem. Eur. J.* **2008**, *14*, 10318.
9. Hesek, D.; Lee, M.; Zhang, W.; Noll, B. C.; Mobashery, S. *J. Am. Chem. Soc.* **2009**, *131*, 5187.
10. Lee, M.; Zhang, W.; Hesek, D.; Noll, B. C.; Boggess, B.; Mobashery, S. *J. Am. Chem. Soc.* **2009**, *131*, 8742.
11. Kumar, S.; Roychowdhury, A.; Ember, B.; Wang, Q.; Guan, R.; Mariuzza, R. A.; Boons, G.-J. *J. Biol. Chem.* **2005**, *280*, 37005.
12. Chowdhury, A. R.; Boons, G.-J. *Tetrahedron Lett.* **2005**, *46*, 1675.
13. Teller, N. Centre de Recherches du Cyclotron, Faculté des Sciences. Thèse de doctorat: Université de Liège, 2009.
14. Waley, S. G. *Biochem. J.* **1991**, *279*, 87.
15. Lutzner, N.; Patzold, B.; Zoll, S.; Stehle, T.; Kalbacher, H. *Biochem. Biophys. Res. Commun.* **2009**, *380*, 554.
16. Rosowsky, A.; Bader, H.; Radike-Smith, M.; Cucchi, C. A.; Wick, M. M.; Freisheim, J. H. *J. Med. Chem.* **1986**, *29*, 1703.
17. Ryan, J. W.; Chung, A. U.S. Patent No. 641,395, 1989.