Thioester analogues of peptidoglycan fragment MurNAc-L-Ala-γ-D-Glu as substrates for peptidoglycan hydrolase MurNAc-L-Ala amidase

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MurNAc-L-amidase is one of a family of peptidoglycan hydrolases which catalyses the breakdown of bacterial peptidoglycan. Analogues of the peptidoglycan fragment MurNAc-L-Ala-γ-D-Glu containing *S*-thiolactic acid in place of L-alanine were synthesised as thioester substrates for this enzyme. Triphenylmethanethiol was used to develop a stereoselective synthesis of *S*-thiolactic acid, which was elaborated synthetically into MurNAc-dipeptide analogues. MurNAc-*S*-thioacetyl-*N*-propylamide **13** and MurNAc-*S*-thiolactyl-2*R*-alaninamide **16** were found not to be substrates for recombinant MurNAc-L-Ala amidases CwlA from *Bacillus subtilis* and Ply21 from bacteriophage TP21, however, turnover of tripeptide thioester *S*-propionylthiolactyl-γ-D-Glu-L-Lys-OMe **21** was observed using amidase Ply21. Therefore, recognition of the amino acid at position 3 of the pentapeptide sidechain appears to be important for enzymatic turnover.

Introduction

The peptidoglycan layer of bacterial cell walls, consisting of *N*-acetylmuramic acid (MurNAc)-GlcNAc polysaccharide cross-linked *via* L-Ala-γ-D-Glu-X-D-Ala-D-Ala (where X = L-Lys or m-DAP, *meso*-diaminopimelic acid) pentapeptide sidechains, provides much of the strength and rigidity of bacterial cell envelopes.[1,2] The biosynthesis of peptidoglycan is known to be essential for bacterial growth, and the inhibition of this pathway is the mechanism of action of a number of antibacterial agents, including the penicillin family of β-lactams, and the vancomycin group of glycopeptides.[1,2] During cell growth and cell division, a family of peptidoglycan hydrolase enzymes is required in order to effect the local breakdown of peptidoglycan, concomitant with the synthesis of new peptidoglycan.[3] The peptidoglycan hydrolases can be divided into two groups: the glycosidases, which hydrolyse the polysaccharide backbone; and the peptidases, which hydrolyse the peptide sidechain.[3] The molecular enzymology of the peptidoglycan hydrolases has been little studied, and is hindered by the lack of small molecule substrates for their assay. The cellular consequences of inhibition of these enzymes is also unclear, the only existing inhibitor being bulgecin A, an inhibitor of lytic glycosidase, which is known to augment the antibacterial effects of penicillin.[4]

A well-proven strategy for the assay of protease enzymes utilises synthetic substrates containing a thioester linkage in place of the scissile amide bond. Cleavage of the thioester functional group liberates a thiol, which can be reacted *in situ* with a chromogenic thiol reagent such as 5,5′-dithiobis(2-nitrobenzoic acid)† (DTNB), giving a colour change at 412 nm.[5] This manuscript describes the synthesis and assay of thioester-containing substrates for MurNAc-L-Ala amidase, which catalyses the hydrolysis of the amide bond between the MurNAc lactyl sidechain and the L-alanine residue at position 1 of the pentapeptide sidechain (see Fig. 1).

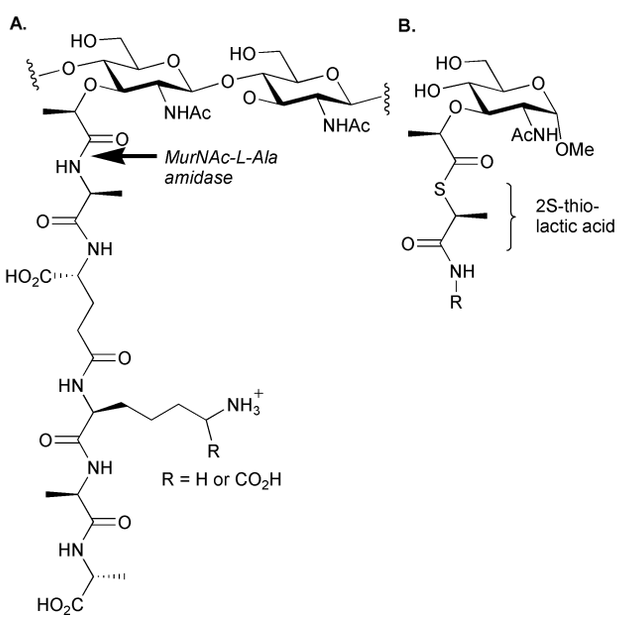

Fig. 1 A. Structure of peptidoglycan repeating unit, showing site of hydrolytic cleavage by MurNAc-L-Ala amidase. R = H (L-Lys) or CO_2H (*meso*-diaminopimelic acid); **B**. General structure of MurNAc-*S*-thiolactyl thioester substrates designed for MurNAc-L-Ala amidase.

MurNAc-L-amidase enzyme activities have been purified from *Escherichia coli* K12,[6] *Bacillus subtilis*,[7,8] *Bacillus megaterium*,[9] and *Staphylococcus aureus*.[10] The *cwlA* gene encoding MurNAc-L-Ala amidase in *B. subtilis* 168 has been cloned and expressed in *E. coli*.[11,12] This enzyme is also found as a bacteriolytic enzyme in certain bacteriophages: notably, the bacteriophage T7 lysozyme is a bifunctional RNA polymerase/MurNAc-L-Ala amidase.[13,14] Recently the *ply* genes encoding MurNAc-L-Ala amidases have been identified in bacteriophage 12826 and TP21, which infect *Bacillus cereus*.[15]

Little is known about the substrate requirements of MurNAc-L-Ala amidase, beyond the requirement for a divalent metal ion,

† The IUPAC name for 5,5′-dithiobis(2-nitrobenzoic acid) is 5-[(3-carboxy-4-nitrophenyl)disulfanyl]-2-nitrobenzoic acid.

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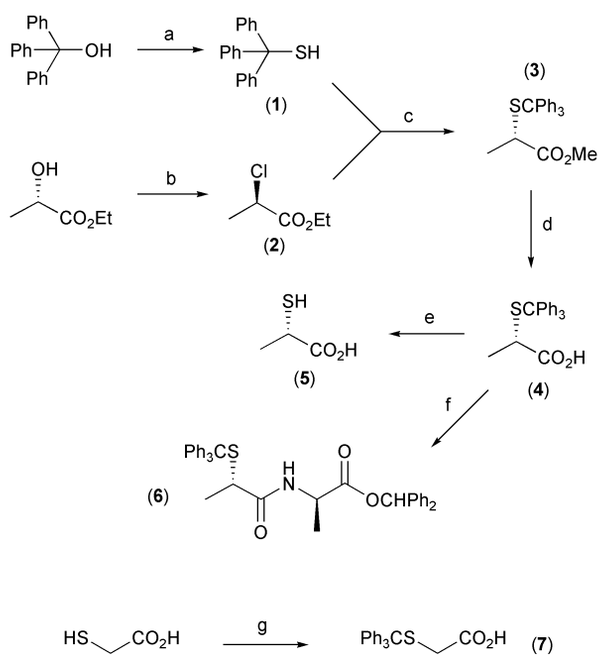
usually Mg^{2+} for the bacterial enzymes,[6–12] and Zn^{2+} for the bacteriophage T7 enzyme.[13,14] Using radiolabelled peptidoglycan fragments, van Heijenoort *et al.* have previously shown that MurNAc-L-Ala-γ-D-Glu-m-DAP-D-Ala-D-Ala and MurNAc-L-Ala-γ-D-Glu-m-DAP are substrates for the *E. coli* enzyme.[6] It was anticipated that, in common with most peptidase enzymes, the specificity of the enzyme would be largely determined by the groups immediately adjacent to the site of cleavage.[5] In order to undertake molecular studies of this enzyme, a reliable continuous enzyme assay was required. Therefore, the synthesis of thioester analogues of MurNAc-L-Ala-γ-D-Glu was undertaken, in which the L-alanine residue at position one is replaced with *S*-thiolactic acid (see Fig. 1).

Results

Stereoselective synthesis of protected *S*-thiolactic acid

S-Thiolactic acid has previously been synthesised using thioacetic acid, a noxious liquid whose thioester linkage is prone to base-catalysed cleavage.[16] We have investigated the use of triphenylmethanethiol (**1**) for the stereoselective introduction of sulfur, and have found that it is a convenient and versatile reagent for this purpose (a communication describing this part of the work has previously been published[17]). Triphenylmethanethiol (**1**) can be prepared in 97% yield by passage of hydrogen sulfide gas through an acidic solution of triphenylmethanol,[18] and is isolated as an odourless white solid which is stable to extended storage at room temperature.

Triphenylmethanethiol (**1**) was reacted with ethyl (2*R*)-2-chloropropionate (**2**), readily prepared from ethyl *S*-lactate,[16] in the presence of sodium methoxide–methanol to give the methyl ester **3** resulting from transesterification. Alkaline hydrolysis of **3** in 0.5 M sodium hydroxide in 1 : 1 water–dioxane, conditions known to effect the hydrolysis of *S*-tritylcysteine derivatives without racemisation,[19] gave the acid product **4** in 97% after column chromatography (see Scheme 1).



Scheme 1 Synthesis of *S*-thiolactic acid derivatives from triphenylmethanethiol. a, H_2S, AcOH–H_2SO_4, 97%; b, $SOCl_2$, DMF(cat), 77%; c, NaOMe, MeOH, 100%; d, 0.5 M NaOH, H_2O–dioxane (1 : 1), 97%; e, CF_3COOH, Et_3SiH, 39%, f, DCC, HOBT, D-Ala-$OCHPh_2$, 100%; g, Ph_3COH, $BF_3{\cdot}OEt_2$, AcOH, 70%.

In order to determine the stereochemical purity of **4**, the trityl group was deprotected using trifluoroacetic acid–triethylsilane to give *S*-thiolactic acid (**5**). Analysis by ^1H NMR spectroscopy in the presence of chiral shift reagent tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato]europium(III) gave no splitting of the δ 1.3 ppm CH_3 doublet, whereas a sample of racemic thiolactic acid under the same conditions gave two doublets. DCC–HOBT coupling of acid **4** with D-alanine diphenylmethyl ester gave a single diastereomer of a highly crystalline derivative **6**, whose X-ray crystal structure was determined (see Fig. 2). The structure establishes the

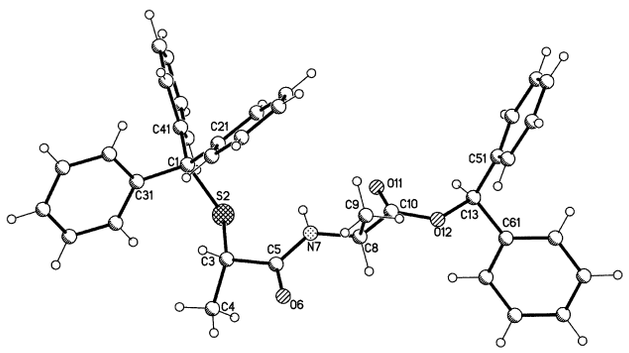


Fig. 2 X-Ray crystal structure of *S*-trityl (*S*)-thiolactyl-(2*R*)-alanyl diphenylmethyl ester **6**, used to elucidate the configuration of the *S*-thiolactyl chiral centre (C-3 on picture).

S configuration of the thiolactyl C-2 centre, thus establishing that the reaction of triphenylmethanethiol (**1**) with ethyl (2*R*)-2-chloropropionate occurs with inversion of configuration.

S-Tritylmercaptoacetic acid (**7**), lacking the methyl sidechain, was also prepared in 70% yield by reaction of mercaptoacetic acid and triphenylmethanol in glacial acetic acid.[20] Both derivatives were subsequently used for the synthesis of MurNAc thioesters.

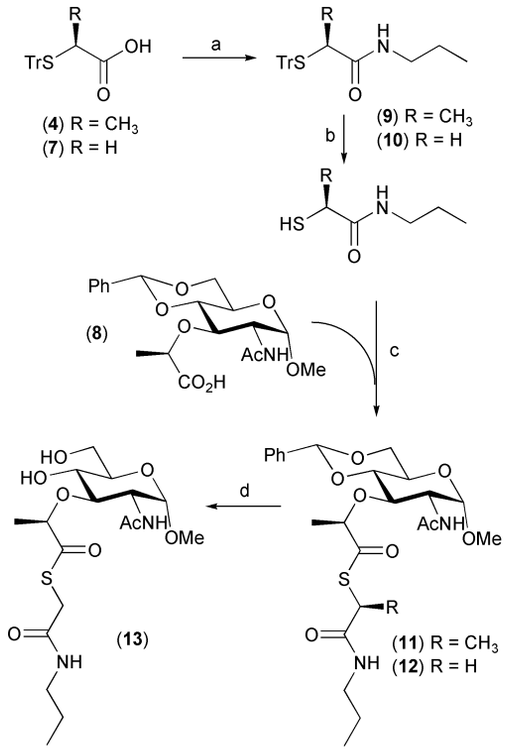
Synthesis of MurNAc-*S*-thiolactyl-X thioesters

N-Acetylmuramic acid contains an 2*S*-lactyl ether at the C-3 position of *N*-acetyl D-glucosamine, and has been prepared previously from *N*-acetyl D-glucosamine either *via* a furanose oxazoline derivative,[21] or *via* a 4,6-protected pyranose derivative.[22–24] The latter method was found to be amenable for synthesis of muramic acid derivatives. Thus, the 4,6-benzylidene α-methyl glycoside derivative of *N*-acetyl-D-glucosamine was prepared,[22,23] and was alkylated with 2*S*-chloropropionic acid using NaH–dioxane to give the protected muramic acid **8**.[24]

Two thioesters were first synthesised in which the *S*-thiolactic acid was coupled to *n*-propylamine, as shown in Scheme 2. Trityl-protected acids **4** and **7** were coupled to *n*-propylamine, using carbonyldiimidazole‡ as coupling agent, to give the corresponding amides **9** and **10** in 66% and 92% yields respectively. Amides **9** and **10** were de-tritylated by treatment with trifluoroacetic acid and triethylsilane, and immediately coupled with protected muramic acid **8** using DCC–DMAP, to give the protected thioesters **11** and **12**, in 40% and 20% yield respectively. The presence of the thioester linkage was confirmed by the appearance of ^{13}C NMR signals at 205.0 and 202.3 ppm respectively, and the observation of parent ions at *m*/*z* 525.5 and 511.3 by electrospray mass spectrometry.

Several methods were attempted for the deprotection of the benzylidene protecting group present in **11** and **12**. Treatment with 60% acetic acid–water resulted in cleavage of the thioester linkage. Hydrogenation using Pd/C was unsuccessful, probably due to the presence of the thioester. Reduction with Na–NH_3 led to decomposition. However, the method of Szarek *et al.*, involving treatment with 1% iodine in refluxing methanol for

‡ The IUPAC name for carbonyldiimidazole is di(1*H*-imidazol-1-yl)-methanone.

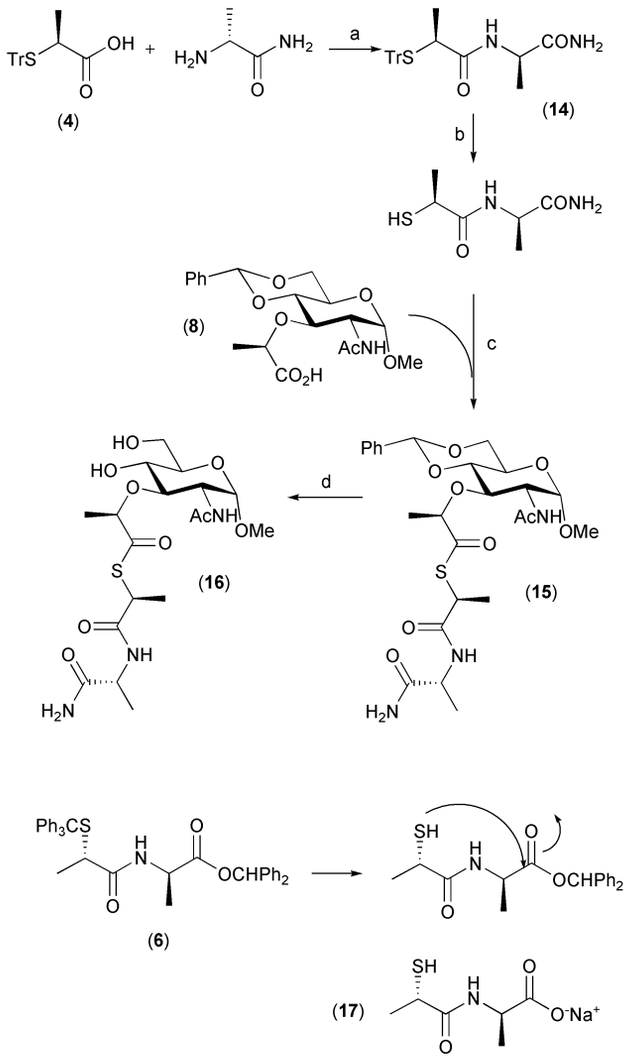


Scheme 2 Synthesis of MurNAc-*S*-thioacetyl-*N*-propylamide **13**. a, $CH_3CH_2CH_2NH_2$, carbonyldiimidazole, CH_2Cl_2, 66% (**9**), 92% (**10**); b, CF_3COOH, Et_3SiH; c, DCC, DMAP, DMF, 40% (**11**), 20% (**12**); d, 1% I_2–MeOH, 75%.

2 hours,[25] was successful for the deprotection of thioester **12**, to give the MurNAc thioester **13** in 75% yield. Thioester **13** was characterised by NMR spectroscopy, and showed parent ion *m*/*z* 423 by matrix-assisted laser desorption ionisation (MALDI) mass spectrometry. Deprotection of thioester **11** was not successful *via* this method, for reasons that are not clear, although the reaction was carried out on a very small scale. Thioester **13** was used for biological testing.

Synthetic studies were continued towards two further thioesters, which mimicked the carboxylate sidechain of γ-D-glutamic acid at position 2 of the pentapeptide sidechain, by addition of a D-alanine residue. Since both γ-D-Glu and γ-D-Gln are found in bacterial peptidoglycan,[1,2] a thioester terminating in D-alanine amide was also synthesised, as shown in Scheme 3. D-Alanine amide was synthesised by reaction of D-alanine with thionyl chloride in dry methanol, followed by addition of 25% ammonia solution, to give the amide in 24% yield. D-Alanine amide was coupled to trityl-protected acid **4**, using DCC and HOBT in THF, to give the amide **14** in 33% yield. Deprotection with trifluoroacetic acid and triethylsilane, followed by coupling of the thiol to protected muramic acid **8**, gave the protected thioester **15** in 62% yield. The thioester carbonyl group was observed by ^{13}C NMR spectroscopy at 202.81 ppm, and an (M + Na) peak at *m*/*z* 576 was observed by electrospray mass spectrometry. Deprotection of the benzylidene protecting group was achieved using 1% I_2–MeOH to give MurNAc thioester **16** (*m*/*z* 488 (M + Na)) in 38% yield after chromatography.

It was anticipated that the D-alanine-containing thioester could be synthesised using the acid-labile diphenylmethyl group to protect the D-alanine carboxy. D-Alanine diphenylmethyl ester was synthesised by reaction of Boc-D-alanine with benzophenone hydrazone and Oxone, in 64% yield, followed by selective removal of the Boc group using 3 M HCl–MeOH. As shown in Scheme 1, coupling to trityl-protected acid gave derivative **6**, whose X-ray crystal structure was determined. Unfortunately, attempts to detritylate **6** and couple to protected muramic acid **8** were all unsuccessful, perhaps due to



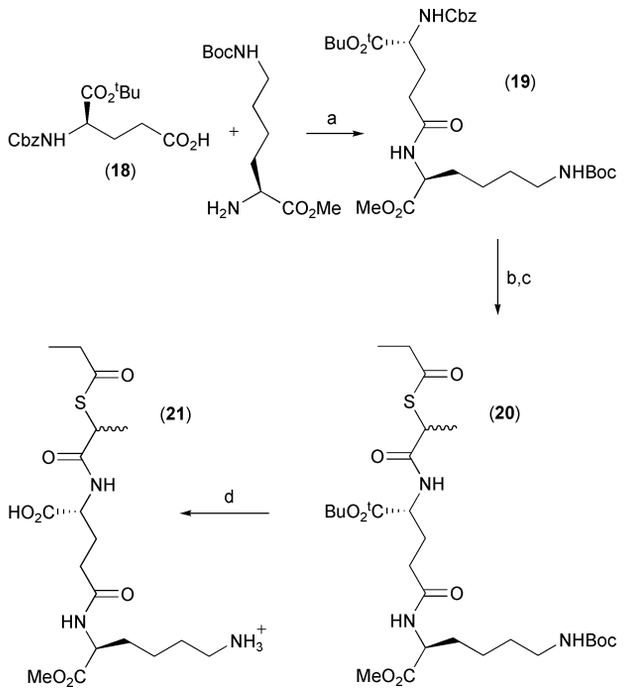
Scheme 3 Synthesis of MurNAc-*S*-thiolactyl-2*R*-alaninamide **16**. a, DCC, HOBT, THF, 33%; b, CF_3COOH, Et_3SiH; c, DCC, HOBT, THF, 62%; d, 1% I_2–MeOH, 40%.

intramolecular cyclisation of the free thiol to form a 6-membered cyclic thioester (shown in Scheme 3). Attempts to couple the sodium salt (**17**) onto **8** gave only a small amount of coupled product, as observed by NMR spectroscopy, which was insufficient for deprotection. Therefore, only thioester **16** was used for biological evaluation.

Synthesis of *S*-propionyl thiolactyl-γ-D-Glu-L-Lys tripeptide analogue

For reasons explained below, a thioester analogue mimicking the L-Ala-γ-D-Glu-L-Lys tripeptide, but lacking the MurNAc sugar, was also synthesised, using solution phase peptide synthesis methods, as shown in Scheme 4.

Following the procedures of Bavetsias *et al.*, orthogonally protected Cbz-D-glutamic acid α-*tert*-butyl ester γ-methyl ester was synthesised in three steps from D-glutamic acid, and the γ-methyl ester hydrolysed under alkaline conditions to give Cbz-D-glutamic acid α-*tert*-butyl ester (**18**).[26] **18** was coupled onto L-Lys(N^{ε}-Boc)-OMe using DCC–HOBT, to give the protected D-Glu-Lys dipeptide (**19**) in 22% yield. The *N*-terminal Cbz group was deprotected by hydrogenation with $PdCl_2$ as catalyst, and the product was coupled onto racemic *S*-propionylthiolactic acid using DCC–HOBT, to give protected tripeptide **20** in 30% yield. The Boc and *tert*-butyl ester protecting groups were removed by treatment with trifluoroacetic acid, to give the tripeptide thioester **21**, which was used for biological evaluation.



Scheme 4 Synthesis of *S*-propionyl-thiolactyl-γ-D-Glu-L-Lys-OMe **21**. a, DCC, HOBT, CH_2Cl_2, 22%; b, $H_2/PdCl_2$, 30%; c, *S*-propionyl-thiolactic acid, DCC, HOBT, CH_2Cl_2, 30%; d, CF_3COOH, CH_2Cl_2, 100%.

Expression and assay of MurNAc-L-Ala amidase

Two MurNAc-L-Ala amidases whose genes had previously been over-expressed in *Escherichia coli* were studied: CwlA from *Bacillus subtilis* 168 (gift of Dr Simon Foster, University of Sheffield),[12] which was expressed from plasmids pSFP102 (native protein) and Pet24d (*N*-terminal Hex_6 fusion protein); and the Ply21 amidase from bacteriophage TP21 (gift of Professor Martin Loessner, Technische Universität München), which was expressed from plasmid pBCPL21.[15]

Cell extract from *E. coli* JM109/pSFP102 (expressing CwlA) was purified by G-75 Sephadex gel filtration, giving a soluble fraction in which the desired 30 kDa CwlA protein could be discerned by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis. Cell extract from *E. coli* BL21/Pet24d (expressing His_6-CwlA) showed high levels of the desired 30 kDa CwlA protein, which was pelleted by centrifugation, indicating that it is insoluble. Treatment of the pellet with 8 M urea gave a fraction containing CwlA as the major band by SDS-polyacrylamide electrophoresis, which could be dialysed into buffer containing 3 M urea without precipitation. Cell extract from *E.coli* JM109/pBCPL21 contained high levels of a 29 kDa protein corresponding to amidase Ply21. This enzyme was also pelleted by centrifugation, but growth of the producing strain at 25 °C yielded a soluble fraction after centrifugation containing Ply21 as the major band by SDS-polyacrylamide electrophoresis.

In order to verify the activity of the expressed proteins, two biological assays were carried out. The first involves the treatment of a growing culture of *Bacillus subtilis* with a sample of protein, and observation of a decrease in A_{600} due to lysis of growing cell walls.[15] Using this assay, a clear decrease in A_{600} could be observed upon addition of 0.5 mg Ply21 or 1 mg egg white lysozyme (which also functions as a peptidoglycan hydrolase), but not for CwlA. The second assay, developed by Leclerc *et al.*,[27] involves the analysis of the purified protein on an SDS-polyacrylamide gel which had been pre-treated with intact cell walls obtained from *Bacillus subtilis*. Treatment of the gel with 0.1% Triton X-100 (to re-nature the enzyme), followed by treatment with 0.1% methylene blue gives a blue colouration in the presence of peptidoglycan, but a clear zone in regions of the gel where an active peptidoglycan hydrolase is present. Using this assay, a clear zone was observed at the predicted M_r of 29 kDa for the Ply21 enzyme (Fig. 3), but no clear zone was

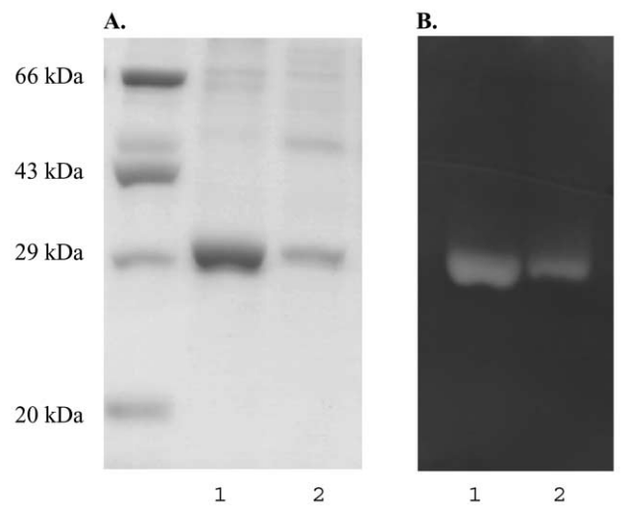


Fig. 3 Renaturing SDS-PAGE assay of Ply21 amidase from bacteriophage PL21. **A**. SDS-polyacrylamide gel stained with Coomassie blue, showing Ply21 amidase (29 kDa). **B**. Duplicate SDS-polyacrylamide gel, containing *B. subtilis* cell walls, renatured and stained as described in Experimental section. Clear zone indicates peptidoglycan hydrolase activity. Lane 1, sample of Ply21 amidase from JM109/pBCPL21 grown at 37 °C, insoluble fraction, re-suspended in 8 M urea. Lane 2, sample of Ply21 amidase from JM109/pBCPL21 grown at 25 °C, soluble fraction.

observed for CwlA, hence the activity of the CwlA amidase is undetectable in our hands.

Each of the two MurNAc-L-Ala enzymes were assayed for hydrolysis of the thioester substrates, by coupling with chromogenic thiol reagent DTNB. Assays were carried out in both stopped format (adding DTNB at a fixed time-point) and continuous format (with DTNB present throughout). For each of the MurNAc-containing thioesters **13** and **16**, no time-dependent turnover could be detected with either enzyme, relative to control assays lacking either enzyme or substrate, even at extended reaction times with high protein concentrations.

In order to test whether recognition of the amino acid at position 3 is important for enzymatic turnover, the *S*-propionyl-thiolactyl-γ-D-Glu-L-Lys thioester substrate **21** was subsequently synthesised, and assayed *versus* amidase Ply21. Time-dependent release of thiol was observed using a DTNB stopped assay (see Table 1), indicating that **21** is a substrate

Table 1 Turnover of thioester **21** by amidase Ply21 in DTNB coupled assays, as observed at 412 nm

		+ thioester **21**	− thioester **21**	Rate of turnover of **21** (nmol product (mg protein)$^{-1}$ min^{-1})
Stopped assay[a]	t = 6 min	0.100 AU	0.00 AU	39.3
	t = 10 min	0.163 AU	0.00 AU	38.4
Continuous assay[b]		0.0071 AU min^{-1}	0.0014 AU min^{-1}	0.44

[a] Stopped assay contained 0.15 mg (ml protein)$^{-1}$, 200 µl aliquot withdrawn for A_{412} determination. Mean value from duplicate assays quoted. [b] Continuous assay contained 1.15 mg (ml protein)$^{-1}$. Mean value from duplicate assays quoted.

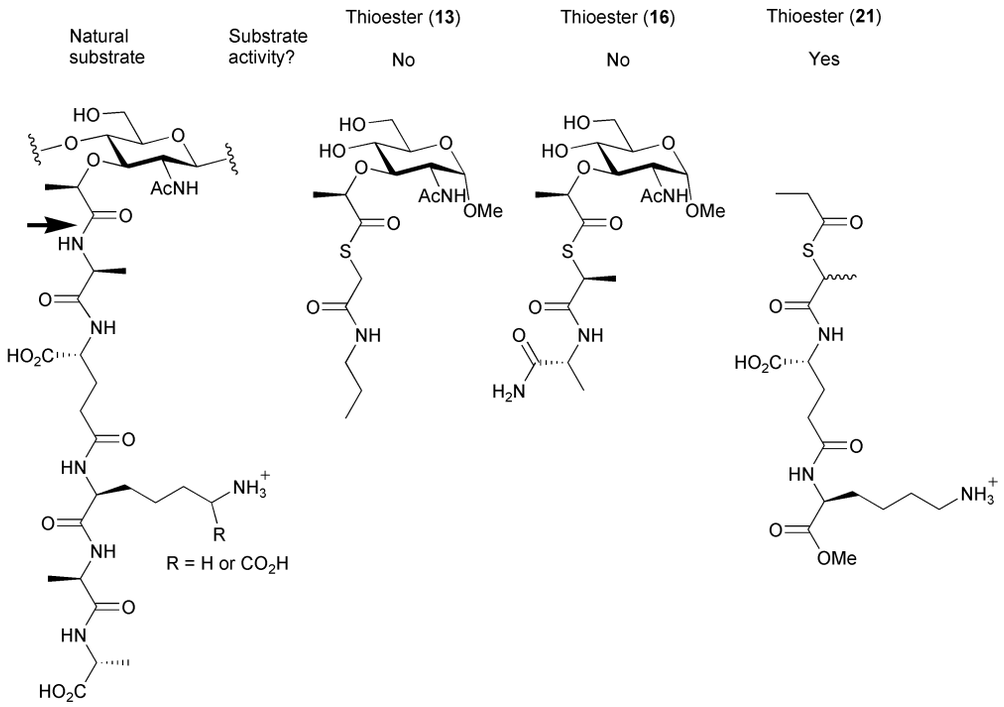


Fig. 4 Summary of biological activity shown by thioesters **13**, **16**, and **21** as substrates for MurNAc-L-Ala amidase.

for this enzyme. From the activity over a 10 minute assay, a specific activity of 39 nmol product min^{-1} (mg protein)$^{-1}$ can be calculated (see Table 1). Much lower activity was measured in a continuous assay format (0.44 nmol min^{-1} (mg protein)$^{-1}$), implying that the enzyme is inactivated by treatment with DTNB.

Conclusions

Although a number of peptidoglycan hydrolase activities have been identified biologically,[3] and a number of corresponding genes identified, little is known about the molecular details of their action, or the consequences of inhibition. This is partly due to the lack of a convenient *in vitro* enzyme assay. In this paper we describe the synthesis of three thioester-containing analogues of the MurNAc-peptide substrate for MurNAc-L-Ala amidase.

Biological testing of these thioesters was complicated by the lack of an independent quantitative assay, since a negative result could be interpreted either as lack of activity as substrate, or lack of active enzyme present in the assay. Using two independent biological assays, we have established that the expressed Ply21 amidase is active as a peptidoglycan hydrolase,[15] but shows no turnover with thioesters **13** and **16**. This disappointing series of observations suggested that the enzyme required the recognition of functional groups beyond position 2 of the pentapeptide sidechain. Since ^{3}H-MurNAc-L-Ala-γ-D-Glu-m-DAP has previously been shown to be a substrate for *E.coli* MurNAc-L-Ala amidase,[6] it seemed plausible that recognition of the amino acid at position 3 is important for enzymatic turnover. Hence we subsequently synthesised the tripeptide thioester analogue **21**, and we have established that **21** is indeed a substrate for Ply21 amidase, the first synthetic substrate for this class of enzyme. These observations are summarised in Fig. 4.

The recognition of a functional group distant from the site of cleavage is unusual for peptidase enzymes, which normally show selectivity for the residue preceding (P1 site) or following (P1′ site) the site of cleavage.[5] In this case, it appears that MurNAc-L-Ala amidase recognises the amino acid *three* residues after the cleavage site (P3′ site). The practical consequences of this observation are that substrates (and presumably potential inhibitors) for this enzyme will need to encompass at least a tripeptide in size. Perhaps this unusual selectivity reflects the fact that the natural substrate for MurNAc-L-Ala amidase is a physically inaccessible polysaccharide, rather than a monosaccharide-peptide. It is hoped that the availability of synthetic substrates for this class of enzymes will enable enzymologists to study the detailed function of these enzymes, and the cellular consequences of their inhibition.

Experimental

Materials

Triphenylmethanethiol (**1**) was prepared by the method of Balfe *et al.*,[18] by passage of hydrogen sulfide through an acidic solution of triphenylmethanol in acetic acid, in 97% yield, δ_H (300 MHz, $CDCl_3$) 7.34–7.24 (15H, m), 3.11 (1H, s) ppm; δ_C (75 MHz, $CDCl_3$) 147.3, 129.5, 128.0, 127.0, 63.0 ppm. Ethyl (2*R*)-2-chloropropionate (**2**) was prepared from ethyl *S*-lactate, using the method of Hof and Kellogg.[16] Boc-D-alanine diphenylmethyl ester was prepared by reaction of Boc-D-alanine with benzophenone hydrazone and Oxone,[28] in 64% yield. D-Alanine diphenylmethyl ester was prepared by treatment of Boc-D-alanine diphenylmethyl ester with 3 M HCl in methanol, in 67% yield; mp 61–62 °C; δ_H (300 MHz, $CDCl_3$) 7.34 (10H, m), 6.90 (1H, s), 3.69 (1H, q, J = 6.9 Hz), 2.11 (2H, s), 1.41 (3H, d, J = 6.9 Hz) ppm; *m/z* (ES^+) 256.2 (MH^+, 100%).

S-Tritylmercaptoacetic acid **7** was prepared by the method of Alarabi *et al.*,[20] in 70% yield. *N*-Acetyl-D-glucosamine was prepared from D-glucosamine by the method of Inouye *et al.*[29] Oxazoline **8** was prepared from *N*-acetyl-D-glucosamine by the method of Mack *et al.*[21] α-Methyl-D-glucosamine was synthesised by treatment of GlcNAc with Dowex 50W in refluxing methanol, according to Galemo and Horton,[22] in 74% yield, and was converted to the 4,6-benzylidene derivative by treatment with benzaldehyde and $ZnCl_2$, using the method of Neuberger.[23] Protected muramic acids **8** were prepared by alkylation with 2*S*-2-chloropropionic acid, according to Jeanloz *et al.*[24] Cbz-D-glutamic acid α-*tert*-butyl ester (**18**) was prepared from D-glutamic acid in four steps using the procedures of Bavetsias *et al.*[26] *S*-Propionylthiolactic acid was prepared by acylation of racemic thiolactic acid with propanoyl chloride in the presence of triethylamine (2 eq.), in 88% yield.

δ_H (300 MHz, $CDCl_3$) 4.25 (1H, q, J = 7.4 Hz), 2.61 (2H, q, J = 7.5 Hz), 1.53 (3H, d, J = 7.4 Hz), 1.19 (3H, t, 7.5 Hz) ppm. All other chemicals and biochemicals were purchased from Sigma-Aldrich.

Plasmids pSFP102 and Pet24d containing the *Bacillus subtilis cwlA* gene were gifts of Dr Simon Foster (University of Sheffield).[12] Plasmid pBCPL21 containing the *ply* gene from bacteriophage TP21 was a gift of Professor Martin Loessner (Technische Universität München).[15] SDS-polyacrylamide electrophoresis was carried out using a Bio-Rad Mini-Protean apparatus, according to the manufacturer's instructions. UV–visible spectrophotometry was carried out using a Cary 1 spectrophotometer.

Methyl (2*R*)-*S*-tritylthiolactate 3

To methanol (10 ml) at 0 °C under nitrogen was added sodium (12 mg, 0.5 mmol), which was stirred until the sodium had dissolved (5–10 min). Triphenylmethanethiol **1** (0.138 g, 0.5 mmol) was added and the solution was allowed to warm to room temperature, and stirring continued for 1 h. Ethyl (2*R*)-2-chloropropanoate **2** (0.068 g, 0.5 mmol) in methanol (5 ml) was added and the mixture was allowed to stir at room temperature overnight. The solution was concentrated under reduced pressure to give an oil with solid precipitate, which was washed through a filter with dichloromethane (100 ml), and the filtrate was concentrated under reduced pressure to give a viscous yellow oil. The crude product was purified by silica column chromatography (30% Et_2O–petroleum ether R_f 0.37) to give **3** as a colourless oil (87 mg, 48% yield). IR (liquid film, cm^{-1}) 3057 (s), 2949 (m), 1736 (s), 1595 (m), 1489 (s); δ_H (300 MHz, $CDCl_3$) 7.48 (6H, d, J = 7.7 Hz), 7.34–7.21 (9H, m), 3.51 (3H, s, $–COOCH_3$), 3.02 (1H, q, J = 7.4 Hz, –C*H*STr–), 1.25 (3H, d, J = 7.4 Hz, $–CH_3$) ppm; δ_C (75 MHz, $CDCl_3$) 174.2, 144.5, 129.8, 128.1, 127.0, 68.3, 52.3, 42.5, 18.9 ppm; *m*/*z* (ES^+) 243 (100%, Ph_3C^+); $[a]_D^{24}$ −3.9 (*c* 6.0, CH_2Cl_2).

(2*S*)-*S*-Tritylthiolactic acid 4

To methyl ester **3** (0.16 g, 0.44 mmol) was added a solution of 50% 0.5 M sodium hydroxide (10 ml)–50% 1,4-dioxane (10 ml). The solution was allowed to stir at room temperature for 1 h. When TLC indicated the disappearance of the starting material, 0.5 M HCl (10 ml) was added until the solution became acidic. The solution was washed with diethyl ether (4 × 20 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to give a viscous oil. The product was purified by silica column chromatography (50% diethyl ether–petroleum ether, R_f 0.29) to afford **4** as a white solid (0.149 g, 97% yield). Mp 139–140 °C; IR (Nujol mull, cm^{-1}) 2968 (s), 2880 (s), 2841 (s), 1710 (m), 1456 (s); δ_H (300 MHz, $CDCl_3$) 7.47 (6H, d, J = 7.4 Hz), 7.32–7.19 (9H, m), 3.04 (1H, q, J = 7.4 Hz, –C*H*STr), 1.21 (3H, d, J = 7.4 Hz, $–CH_3$) ppm; δ_C (75 MHz, $CDCl_3$) 179.6, 144.3, 129.8, 128.2, 127.1, 68.4, 42.6, 18.7 ppm; *m*/*z* ($APCI^-$) 347.2 (M − 1, 11%); HRMS (FAB^-) 347.1105 ($C_{22}H_{19}O_2S$ requires 347.1101); $[a]_D^{24}$ −3.9 (*c* 60, CH_2Cl_2).

(2*S*)-Thiolactic acid 5

To (2*S*)-*S*-tritylthiolactic acid **4** (0.137 g, 0.4 mmol) in trifluoroacetic acid (2 ml) at 0 °C was added triethylsilane (80 µl, 0.5 mmol) dropwise. Immediate colour change to a colourless solution was observed, and a white precipitate was formed. The ice bath was removed, and hexane (10 ml) and water (10 ml) were added. The water layer was separated, washed with hexane (3 × 10 ml), filtered and concentrated under reduced pressure to give **5** as a colourless liquid (16 mg, 39% yield), which was identical to authentic material. δ_H (300 MHz, D_2O) 3.42 (1H, q, J = 7.0 Hz), 1.28 (3H, d, J = 7.0 Hz) ppm; δ_C (75 MHz, D_2O) 178.3, 38.4, 23.4 ppm.

(2*S*)-*S*-Tritylthiolactyl-2*R*-alanine diphenylmethyl ester 6

To a solution of acid **4** (0.697 g, 2 mmol) in dry THF (20 ml) under nitrogen was added dicyclohexylcarbodiimide (0.454 g, 2.2 mmol) and hydroxybenzotriazole (0.297 g, 2.2 mmol) and the solution was stirred at room temperature for 5 min. D-Alanine diphenylmethyl ester (0.500 g, 2 mmol) in THF (10 ml) was added and the mixture allowed to stir at room temperature overnight under nitrogen. The solution was filtered to remove the precipitated urea, washed through with ethyl acetate and concentrated under reduced pressure to give a white solid. The crude product was purified by silica column chromatography (50% diethyl ether–petroleum ether, R_f 0.20) to afford **6** as a white solid (1.190 g, 100%). A sample of **6** was re-crystallised by vapour diffusion (isopropanol (propan-2-ol)–water) to give single crystals suitable for X-ray diffraction (see data below; structure shown in Fig. 2). IR (Nujol mull, cm^{-1}) 3071 (m), 2992 (s), 1754 (m), 1678 (w), 1586 (m); δ_H (300 MHz, $CDCl_3$, 2 rotamers) 7.43–7.12 (25H, m), 6.89 & 6.82 (1H, s, $–CHPh_2$), 6.70 & 6.50 (1H, d, J = 6.7 Hz, NH), 4.30 & 4.15 (1H, dq, J = 7.1, 6.7 Hz, –C*H*NCH_3), 2.99 (1H, q, J = 7.6 Hz, –C*H*SCH_3), 1.42 & 1.39 (3H, d, J = 7.6 Hz, CH_3CS), 1.27 & 1.20 (3H, d, J = 7.1 Hz, CH_3CN) ppm; δ_C (75 MHz, $CDCl_3$) 172.1, 171.6, 144.5, 144.3, 139.8, 139.7, 129.9, 129.6, 128.8, 128.8, 128.7, 128.4, 128.2, 128.2, 128.1, 127.3, 127.2, 127.1, 127.0, 78.0, 68.3, 48.6, 48.6, 44.6, 44.4, 20.2, 18.5, 17.9 ppm; *m*/*z* (ES^+) 342 (60%, M − Tr).

N-Propyl-(2*S*)-*S*-tritylthiolactamide 9

To acid **4** (0.400 g, 1.1 mmol) in dichloromethane (10 ml) was added *N,N′*-carbonyldiimidazole (0.185 g, 1.2 mmol) in dry glassware under nitrogen. The solution was stirred at room temperature for 3 h. *n*-Propylamine (90 µl, 1.1 mmol) was added and stirring continued overnight at room temperature. Water (10 ml) was added, and the solution was extracted with dichloromethane (3 × 10 ml). The combined organic layers were dried ($MgSO_4$), and concentrated under reduced pressure to give a yellow solid. The product was purified by silica column chromatography (Et_2O, R_f 0.52) to give **9** as a white solid (0.282 g, 66% yield). Mp 122–124 °C; IR (Nujol mull, cm^{-1}) 3262 (m), 2919 (s), 1644 (s), 1557 (s); δ_H (300 MHz, $CDCl_3$) 7.41 (6H, d, J = 7.4 Hz), 7.29–7.17 (9H, m), 5.95 (1H, br s, NH), 3.01 (1H, q, J = 7.7 Hz, –C*H*SCH_3), 2.90 (1H, ddt, J = 13.2, 7.0 and 6.2 Hz, –C*H*H′N), 2.65 (1H, ddt, J = 12.9, 7.0 and 5.5 Hz, –CH*H′*N), 1.43 (3H, d, J = 7.7 Hz, –CHSC*H*$_3$), 1.27 (2H, m, $–CH_2–$), 0.78 (3H, t, J = 7.4 Hz, $–CH_2CH_3$) ppm; δ_C (75 MHz, $CDCl_3$) 172.2, 144.4, 129.4, 128.1, 127.0, 68.2, 44.7, 41.4, 22.3, 20.3, 11.3 ppm; *m*/*z* (ES^+) 243.3 (Ph_3C^+, 100%), 390.6 (MH^+, 5%).

N-Propyl-*S*-trityl-2-mercaptoacetamide 10

To acid **7** (4.51 g, 13.5 mmol) in dichloromethane (30 ml) at room temperature under nitrogen was added a solution of *N,N′*-carbonyldiimidazole (2.40 g, 14.8 mmol) in dichloromethane (30 mL). The solution was stirred at room temperature for 3 h. *n*-Propylamine (1.11 ml, 13.5 mmol) was added and the solution was stirred at room temperature for 24 h. The solution was washed with 0.5 M HCl (20 ml) and saturated ammonium chloride solution (20 ml), dried (Na_2SO_4) and concentrated under reduced pressure to give **10** as a white solid (4.664 g, 92% yield). Mp 141–142 °C; IR (Nujol mull, cm^{-1}) 3259 (w), 2921 (s), 1634 (m), 1558 (m); δ_H (300 MHz, $CDCl_3$) 7.44 (6H, d, J = 7.4 Hz), 7.34–7.22 (9H, m), 6.07 (1H, br s, NH), 3.15 (2H, s, $–COCH_2S$), 2.93 (2H, q, J = 6.6 Hz, $–CH_2N$), 1.36 (2H, m), 0.84 (3H, t, J = 7.4 Hz, $–CH_3$) ppm; δ_C (75 MHz, $CDCl_3$) 168.0, 144.2, 129.6, 128.3, 127.2, 68.0, 41.6, 36.1, 22.6, 11.5 ppm.

N-Propyl-(2*S*)-*S*-{2*R*-2-*O*-[*O*-α-methyl-4′,6′-*O*-benzylidene-2′-acetamido-2′-deoxy-D-glucopyranos-3′-yl]propionyl}thiolactamide 11

To amide **9** (0.233 g, 0.6 mmol) in trifluoroacetic acid (5 ml) at

0 °C was added triethylsilane (128 μl, 0.8 mmol) dropwise. Immediate colour change to a colourless solution was observed and a white precipitate was formed. The ice bath was removed, and hexane (10 ml) and water (10 m) were added. The water layer was separated, washed with hexane (3 × 10 ml), filtered and concentrated under reduced pressure to give the free thiol as a colourless liquid (98 mg, quant.). δ_H (300 MHz, D_2O) 3.34 (1H, q, J = 7.0 Hz), 2.92 (2H, t, J = 7.0 Hz), 1.28 (2H, tq, J = 7.4, 7.0 Hz), 1.22 (3H, d, J = 7.0 Hz), 0.66 (3H, t, J = 7.4 Hz) ppm; δ_C (75 MHz, D_2O) 180.3, 44.4, 40.1, 24.8, 23.9, 13.6 ppm.

To acid **8** (79 mg, 0.2 mmol) in dry DMF (10 ml) at 0 °C under nitrogen was added *N*,*N*-dimethyl-4-aminopyridine (30 mg) and dicyclohexylcarbodiimide (41 mg, 0.2 mmol). The solution was stirred at 0 °C for 10 min. The above thiol (26 mg, 0.2 mmol) in DMF (5 ml) was added and stirring continued at 0 °C under nitrogen for 5 minutes. The pink mixture was allowed to warm to room temperature and stirring was continued overnight. Water (10 ml) and dichloromethane (10 ml) were added to the yellow solution, and the dichloromethane layer was washed with water (6 × 10 ml), dried ($MgSO_4$), and concentrated under reduced pressure to give a yellow solid. The crude product was purified by silica column chromatography (EtOAc, R_f 0.20) to afford thioester **11** as a white solid (45.2 mg, 40% yield). Mp 194–195 °C; IR (Nujol mull, cm^{-1}) 3284 (m), 2971 (s), 2879 (m), 1684 (m), 1649 (s), 1560 (m); δ_H (300 MHz, $CDCl_3$, 2 rotamers) 7.41 (5H, m), 6.33 (1H, br, NH), 6.24 & 6.14 (1H, br, NH), 5.58 (1H, s, –CHPh), 4.88 & 4.80 (1H, d, J = 3.3 Hz, H-1′), 4.57 & 4.54 (1H, q, J = 7.0 Hz, –C*H*OCH_3), 4.30–3.68 (7H, m), 3.38 (3H, s, –OCH_3), 3.20 (2H, m, –CH_2N), 2.10 & 2.08 (3H, s, –$COCH_3$), 1.71 (2H, m, –CH_2–), 1.50 (3H, d, J = 6.6 Hz, –CHSC*H*$_3$), 1.40 & 1.39 (3H, t, J = 7.4 Hz, –CHOC*H*$_3$), 0.91 & 0.90 (3H, t, J = 7.4 Hz, –CH_2C*H*$_3$) ppm; δ_C (75 MHz, $CDCl_3$) 205.0, 171.5, 170.4, 137.0, 129.3, 128.5, 126.0, 101.6, 99.2, 99.0, 83.3, 82.4, 76.5, 69.1, 62.5, 55.5, 53.1, 52.8, 40.6, 40.4, 29.8, 22.9, 22.8, 20.2, 16.9, 16.4, 11.4 ppm; *m*/*z* (ES^+) 525.5 (MH^+, 100%).

N-Propyl-(2*S*)-*S*-[2*R*-2-*O*-{*O*-α-methyl-4′,6′-*O*-benzylidene-2′-acetamido-2′-deoxy-D-glucopyranos-3′-*O*-yl}propionyl]thioacetamide 12

To amide **10** (0.162 g, 0.43 mmol) in trifluoroacetic acid (2 mL) at 0 °C was added triethylsilane (83 μL, 0.52 mmol) dropwise. Immediate colour change to a colourless solution was observed and a white precipitate was formed. The ice bath was removed, and hexane (20 ml) and water (20 ml) were added. The water layer was separated and washed with hexane (3 × 20 ml), the aqueous layer was passed through Celite and concentrated under reduced pressure to give the free thiol as a yellow oil (0.071 g, quant.). δ_H (300 MHz, D_2O) 2.82 (2H, s), 2.74 (2H, t, J = 7.0 Hz), 1.10 (2H, m), 0.47 (3H, t, J = 7.4 Hz) ppm; δ_C (75 MHz, D_2O) 176.1, 43.9, 29.5, 24.0, 12.8 ppm; *m*/*z* (ES^+) 265.1 (MH^+, 100%). To acid **8** (100 mg, 0.3 mmol) in dry DMF (10 ml) at 0 °C under nitrogen was added *N*,*N*-dimethyl-4-aminopyridine (30 mg) and dicyclohexylcarbodiimide (62 mg, 0.3 mmol). The solution was stirred at 0 °C for 10 min. The above thiol (40 mg, 0.3 mmol) in dichloromethane (5 ml) was added, and stirring continued at 0 °C under nitrogen for 5 minutes. The pink mixture was allowed to warm to room temperature and stirring was continued overnight. Water (10 ml) and dichloromethane (10 ml) were added to the yellow solution. The organic layer was washed with water (6 × 10 ml), dried ($MgSO_4$) and concentrated under reduced pressure to give a yellow solid. The crude product was purified twice by silica column chromatography (EtOAc, R_f 0.12) to give thioester **12** as a white solid (30.3 mg, 20% yield). IR (Nujol mull, cm^{-1}) 3282 (m), 2896 (s), 2852 (s), 1681 (w), 1644 (m), 1556 (w); δ_H (300 MHz, $CDCl_3$) 7.47–7.38 (5H, m), 6.30 (1H, d, J = 7.4 Hz, NH), 6.16 (1H, br, NH), 5.58 (1H, s, –CHPh), 4.83 (1H, d, J = 3.5 Hz, H-1′), 4.59 (1H, q, J = 7.0 Hz, –C*H*OCH_3), 4.29–3.69 (6H, m), 3.54 (2H, AB quartet, J = 14.9 Hz, –$COCH_2$S), 3.38 (3H, s, –OCH_3), 3.21 (2H, q, J = 6.5 Hz, –CH_2N), 2.08 (3H, s, –$COCH_3$), 1.52 (2H, sextet, J = 7.2 Hz, –CH_2–), 1.41 (3H, d, J = 7.0 Hz, –CHOC*H*$_3$), 0.91 (3H, t, J = 7.4 Hz, –CH_2C*H*$_3$) ppm; δ_C (75 MHz, d_6-DMSO) 202.3, 169.6, 167.0, 137.6, 128.9, 128.3, 126.0, 100.4, 98.9, 82.2, 81.0, 77.0, 68.0, 62.5, 54.8, 52.7, 40.8, 31.9, 22.9, 22.3, 19.3, 11.4 ppm; *m*/*z* ($APCI^+$) 511.3 (MH^+, 100%); HRMS (FAB^+) 511.2114 ($C_{24}H_{35}N_2O_8S$ requires 511.2111).

N-Propyl-(2*S*)-*S*-[2*R*-2-*O*-{*O*-α-methyl-2′-acetamido-2′-deoxy-D-glucopyranos-3′-*O*-yl}propionyl]thioacetamide 13

Thioester **12** (29 mg, 57 μmol) was dissolved in a 1% iodine–methanol solution (10 ml), and the mixture was heated to reflux for 2 h. When TLC indicated the disappearance of the starting material, sodium thiosulfate (1 g) was added to the deep red solution. The resulting colourless solution was filtered, and the solid washed with methanol. The solution was concentrated under reduced pressure to give a white residue, which was extracted with isopropanol (5 ml), filtered and concentrated under reduced pressure. The white residue was dissolved in water (2 ml) and lyophilised overnight to afford **13** as a white solid (18 mg, 75% yield). δ_H (400 MHz, D_2O) 4.60 (1H, d, J = 3.6 Hz, H-1′), 4.31 (1H, q, J = 6.9 Hz, –C*H*OCH_3), 3.78–3.36 (8H, m), 3.22 (3H, s, –OCH_3), 3.02 (2H, t, J = 6.8 Hz, –CH_2N), 1.86 (3H, s, –$COCH_3$), 1.37 (2H, tq, J = 7.4, 7.2 Hz, –CH_2–), 1.24 (3H, d, J = 6.9 Hz, –CHOC*H*$_3$), 0.73 (3H, t, J = 7.5 Hz, –CH_2C*H*$_3$) ppm; δ_C (100 MHz, D_2O) 177.1, 175.5, 99.4, 81.5, 78.4, 73.0, 71.0, 61.8, 56.6, 54.2, 43.1, 39.2, 23.5, 23.0, 19.7, 12.0 ppm; *m*/*z* (MALDI) 423 (MH^+).

S-Trityl-2*S*-thiolactyl-2*R*-alanine amide 14

To acid **4** (0.348 g, 1 mmol) in dry THF (10 ml) under nitrogen was added dicyclohexylcarbodiimide (0.227 g, 1.1 mmol) and hydroxybenzotriazole (0.149 g, 1.1 mmol), and the mixture was stirred at room temperature for 5 min. D-Alanine amide (88 mg, 1 mmol) in THF (5 ml) was added, and the mixture stirred at room temperature overnight under nitrogen. The solution was filtered to remove the precipitated urea, washed with ethyl acetate, and concentrated under reduced pressure to give a white solid. The crude product was purified by silica column chromatography (1% Et_3N–EtOAc, R_f 0.26) to afford **14** as a white solid (0.137 g, 33% yield). IR (Nujol mull, cm^{-1}) 3172 (m), 1692 (s), 1661 (m), 1586 (m); δ_H (300 MHz, $CDCl_3$) 7.39 (6H, d, J = 7.7 Hz), 7.28–7.16 (9H, m), 6.36 (1H, br s, NH), 6.16 (1H, d, J = 6.7 Hz, NH), 5.46 (1H, br s, NH), 4.08 (1H, dq, J = 7.0, 6.7 Hz, –C*H*NCH_3), 2.96 (1H, q, J = 7.2 Hz, –C*H*SCH_3), 1.37 (3H, d, J = 7.4 Hz, –CHSC*H*$_3$), 1.09 (3H, d, J = 7.0 Hz, –CHNC*H*$_3$), ppm; δ_C (75 MHz, $CDCl_3$) 174.1, 173.1, 144.4, 128.3, 128.2, 127.2, 68.4, 48.7, 44.3, 20.1, 17.4 ppm.

(2*S*)-*S*-[2*R*-2-*O*-{α-Methyl-4′,6′-*O*-benzylidene-2′-acetamido-2′-deoxy-D-glucopyranos-3′-*O*-yl}propionyl]thiolactyl-2*R*-alaninamide 15

To a solution of **14** (50 mg, 0.12 mmol) in trifluoroacetic acid (2 ml) at 0 °C was added triethylsilane (44 μl, 0.28 mmol), to give a clear solution with a white precipitate. Hexane (10 ml) and water (10 ml) were added; the water layer was separated, washed with hexane (3 × 10 ml), filtered, and freeze-dried to afford the free thiol as a white solid (25 mg, quant.). δ_H (300 MHz, D_2O) 4.14 (1H, q, J = 7.0 Hz), 3.50 (1H, q, J = 7.0 Hz), 1.34 (3H, d, J = 7.0 Hz), 1.28 (3H, q, J = 7.4 Hz) ppm; δ_C (75 MHz, D_2O) 180.4, 179.1, 52.3, 38.9, 23.2, 19.2 ppm; *m*/*z* (ES^+) 177.0 (MH^+, 30%).

To a solution of acid **8** (47 mg, 0.12 mmol) in THF (10 ml) in dry glassware was added dicyclohexylcarbodiimide (27 mg, 0.13 mmol) and hydroxybenzotriazole (18 mg, 0.13 mmol). The

solution was stirred at room temperature under nitrogen for 5 min, then a solution of the above thiol (21 mg, 0.12 mmol) in THF (5 ml) was added, and stirring continued overnight at room temperature. The precipitated urea was filtered off, and the solvent was removed under reduced pressure to give the solid product, which was purified by silica column chromatography to give thioester **15** as a white solid (41 mg, 62% yield). Mp 194–196 °C; IR (Nujol mull, cm^{-1}) 3284 (m), 2900 (s), 2854 (s), 1748 (m), 1689 (w), 1651 (m); δ_H (300 MHz, d_6-DMSO) 8.18 (1H, d, J = 7.7 Hz), 8.00 (1H, d, J = 8.8 Hz), 7.42–7.37 (5H, m), 7.23 (2H, s, NH), 7.02 (1H, s, NH), 5.72 (1H, s, –CHPh), 4.61 (1H, d, J = 3.7 Hz, H-1′), 4.32 (1H, q, J = 6.6 Hz, –C*H*OCH_3), 4.24–3.63 (9H, m), 3.32 (3H, s, –OCH_3), 1.83 (3H, s, –$COCH_3$), 1.31 (3H, d, J = 7.0 Hz, –CHOC*H*$_3$), 1.31 (3H, d, J = 7.0 Hz, –CHSC*H*$_3$), 1.17 (3H, d, J = 7.0 Hz, –CHNC*H*$_3$) ppm; δ_C (75 MHz, d_6-DMSO) 202.8, 173.8, 170.2, 137.5, 128.8, 128.1, 125.9, 100.3, 98.8, 82.1, 80.8, 77.0, 67.9, 62.4, 54.7, 52.7, 48.1, 40.9, 25.3, 22.9, 19.3, 18.2 ppm; *m*/*z* (FAB^+) 554 (MH^+, 8%), 576 (M + Na, 100%); HRMS (FAB^+) 554.2172 ($C_{25}H_{36}N_3O_9S$ requires 554.2173).

(2*S*)-*S*-[2*R*-2-*O*-{α-Methyl-2′-acetamido-2′-deoxy-D-gluco-pyranos-3′-*O*-yl}propionyl]thiolactyl-2*R*-alaninamide 16

To thioester **15** (26.3 mg, 48 μmol) was added 1% iodine in methanol (2 ml), and the solution heated to reflux for 2 h. After cooling, sodium thiosulfate was added until the solution became clear. The mixture was filtered, washed through with methanol and concentrated under reduced pressure to give a white residue. The residue was extracted with isopropanol, filtered, and concentrated under reduced pressure. The residue was then extracted with water, filtered and freeze-dried overnight. The white residue was purified by silica column chromatography (70% ^{i}PrOH–20% H_2O–10% MeOH, R_f 0.57) to afford thioester **16** as a white solid (8.6 mg, 38% yield). δ_H (300 MHz, D_2O) 4.69 (1H, d, J = 2.6 Hz, H-1′), 4.23 (1H, q, J = 5.3 Hz, –C*H*OCH_3), 4.02–3.40 (8H, m), 3.21 (3H, s, –OCH_3), 1.85 (3H, s, –$COCH_3$), 1.32 (3H, d, J = 5.6 Hz, –CHOC*H*$_3$), 1.25 (2 × 3H, d, J = 5.3 Hz) ppm; δ_C (75 MHz, D_2O) 177.1, 176.2, 172.3, 95.9, 77.6, 75.6, 69.8, 67.8, 58.5, 53.2, 51.3, 47.9, 39.9, 20.1, 16.6, 14.6, 14.1 ppm; *m*/*z* (MALDI) 488 (M + Na, 10%).

Cbz-D-Glu(α-^{t}Bu)-γ-L-Lys($N^ε$-Boc)-OMe 19

To a solution of Cbz-D-glutamic acid α-*tert*-butyl ester (0.615 g, 1.85 mmol) and triethylamine (0.28 ml, 2.0 mmol) in dry dichloromethane (20 ml) at 0 °C was added dicyclohexylcarbodiimide (0.47 mg, 2.28 mmol). After stirring for 5 min, hydroxybenzotriazole (30 mg) was added, followed by L-lysine($N^ε$-Boc) methyl ester (0.60 g, 2.2 mmol) and triethylamine (0.31 ml, 2.2 mmol). The reaction was stirred for 24 h at room temperature. The precipitated urea was filtered off, and the filtrate was evaporated at reduced pressure. The residue was dissolved in ethyl acetate (50 ml), and the solution was washed with water (2 × 10 ml), 5% potassium hydrogen sulfate solution (2 × 10 ml) and 5% sodium bicarbonate solution (2 × 10 ml), sat. sodium chloride solution (2 × 10 ml). The organic layer was dried (Na_2SO_4) and evaporated at reduced pressure. The product was purified by silica column chromatography (EtOAc, R_f 0.48) to give dipeptide **19** as a colourless oil (0.235 g, 22% yield). δ_H (300 MHz, $CDCl_3$) 7.26 (5H, s, Ar), 6.72 (1H, d, J = 7.7 Hz, NH), 6.47 (1H, d, J = 7.4 Hz, NH), 5.61 (1H, d, J = 8.3 Hz, NH), 5.03 (2H, s, –CH_2Ph), 4.48 (1H, m, $C_αH$), 4.26 (1H, m, $C_αH$), 3.64 (3H, s, –OCH_3), 3.11 (2H, m, –CH_2N), 2.35–1.6 (8H, m), 1.38 (9H, s, ^{t}Bu), 1.36 (9H, s, ^{t}Bu), 1.25 (2H, m) ppm; δ_C (75 MHz, $CDCl_3$) 173.3, 172.5, 171.4, 156.8, 156.4, 136.6, 128.9, 128.6, 128.5, 82.9, 79.3, 67.5, 54.2, 52.8, 52.5, 40.4, 32.8, 32.1, 29.9, 29.7, 28.8, 28.4, 22.9 ppm.

S-(Propionyl)thiolactyl-D-Glu(α-^{t}Bu)-γ-L-Lys($N^ε$-Boc)-OMe 20

Dipeptide **19** (93 mg, 0.16 mmol) was dissolved in 1 : 1 dry THF–methanol, to which palladium(II) chloride catalyst (15 mg) was added. The reaction mixture was purged with hydrogen gas, and stirred under an atmosphere of hydrogen for 16 h at room temperature. Solvent was evaporated under reduced pressure, and the product was purified by silica column chromatography (EtOAc, R_f 0.51) to give the deprotected dipeptide (21 mg, 30%). δ_H (300 MHz, $CDCl_3$) 4.38 (1H, m, $C_αH$), 4.28 (1H, m, $C_αH$), 3.62 (3H, s, –OCH_3), 3.04 (2H, m, –CH_2N), 2.35–1.6 (8H, m), 1.41 (9H, s, ^{t}Bu), 1.36 (9H, s, ^{t}Bu), 1.20 (2H, m) ppm.

S-(Propionyl)thiolactic acid (6 mg, 0.035 mmol) was dissolved in dry dichloromethane (5 ml), to which was added 5 μl triethylamine (0.035 mmol) and dicyclohexylcarbodiimide (8 mg, 0.039 mmol). Hydroxybenzotriazole (5 mg, 0.035 mmol) was added, then the deprotected D-Glu-L-Lys dipeptide (15 mg, 0.034 mmol), and triethylamine (5 μl, 0.035 mmol). The reaction was stirred for 16 h at room temperature. The precipitated urea was filtered off, and the solvent evaporated under reduced pressure. The residue was dissolved in ethyl acetate (10 ml), and the solution was washed with water (5 ml), 5% potassium hydrogen sulfate solution (5 ml), 5% sodium bicarbonate solution (5 ml), and sat. sodium chloride solution (5 ml). The organic layer was dried (Na_2SO_4) and evaporated at reduced pressure. The product was purified by silica column chromatography (EtOAc, R_f 0.56) to give tripeptide **20** as a white powder (6 mg, 30% yield). δ_H (300 MHz, $CDCl_3$, 2 diastereoisomers) 7.05 (1H, br s, NH), 6.96 (1H, br s, NH), 4.57 (1H, m, $C_αH$), 4.10 (1H, m, $C_αH$), 3.76 & 3.75 (3H, 2 × s, –OCH_3), 3.52 (1H, m, –$CHSCH_3$), 3.12 (2H, m, –CH_2N), 2.64 (2H, q, J = 7.0 Hz, –$COCH_2CH_3$), 2.25 (2H, m), 2.0–1.6 (6H, m), 1.48 & 1.47 (9H, 2 × s, ^{t}Bu), 1.42 & 1.41 (9H, 2 × s, ^{t}Bu), 1.5–1.3 (3H, 2 × d, –CHSC*H*$_3$), 1.21 (3H, t, J = 7.0 Hz, –$COCH_2CH_3$), 1.2–1.0 (2H, m) ppm.

S-(Propionyl)thiolactyl-D-Glu-γ-L-Lys-OMe 21

Tripeptide **20** (6 mg) was dissolved in a mixture of trifluoroacetic acid (1 ml) and dichloromethane (1 ml). The reaction was stirred for 2 h at room temperature, then the solvent was evaporated at reduced pressure. The residue was washed with diethyl ether, then evaporated at reduced pressure to give tripeptide **21** as a pale green solid (6 mg, 100%). δ_H (300 MHz, $CDCl_3$, 2 diastereoisomers) 7.3–7.1 (4H, m, NH), 4.45 (1H, m, $C_αH$), 4.28 (1H, m, $C_αH$), 3.65 (3H, s, –OCH_3), 3.44 (1H, m, –C*H*SCH_3), 3.0–2.8 (2H, m), 2.56 (2H, q, J = 7.0 Hz, –$COCH_2CH_3$), 2.25 (2H, m), 2.0–1.5 (6H, m), 1.37 & 1.32 (3H, 2 × d, J = 7.5 Hz, –CHSC*H*$_3$), 1.15 (3H, t, J = 7.0 Hz, –$COCH_2CH_3$), 1.0–0.9 (2H, m) ppm; *m*/*z* (ES^+) 434.2 (MH^+, 45%); HRMS (FAB^+) 434.1973 ($C_{18}H_{32}N_3O_7S$ requires 434.1961).

Expression of MurNAc-L-Ala amidases

Escherichia coli strain JM109/pSFP102 (expressing CwlA) was grown with aeration at 37 °C in Luria broth containing 100 μg ml^{-1} ampicillin; BL21/Pet24d (expressing CwlA) was grown with aeration at 25 °C in Luria broth containing 0.5% glucose and 30 μg ml^{-1} kanamycin; and JM109/pBCPL21 (expressing Ply21) was grown at 25 °C with aeration in Luria broth containing 100 μg ml^{-1} ampicillin. Induction of each culture was carried out by addition of isopropylthio-D-galactoside (IPTG, 0.5 mM) at A_{595} 0.6, then growth was continued for a further 5 h. Cells were harvested by centrifugation (6000*g*, 10 min), and were re-suspended in 50 mM Tris buffer pH 7.5 containing 20 mM $MgCl_2$, and cells lysed by sonication. Cell debris was removed by centrifugation (10 000*g*, 20 min), to give the crude cell extracts, which were examined by SDS polyacrylamide electrophoresis. The soluble protein fraction was obtained by

further centrifugation (100 000*g*, 30 min), which in the case of Ply21 yielded soluble enzyme. In the case of CwlA, the pellet was re-suspended by treatment with 8 M urea, followed by dialysis *vs*. 3 M urea (1 l).

Renaturing SDS-PAGE assay for peptidoglycan hydrolase activity

The procedure of Leclerc *et al.* was employed,[27] using bacterial cell walls from *Bacillus cereus* W23 (prepared using the method of Mintz *et al.* [30]). The SDS-PAGE gel was prepared in the presence of 0.1% (w/v) cell walls. After electrophoresis, the gel was incubated in 1% (v/v) Triton X-100 for 3 × 30 min at 20 °C, then in 0.1% Triton X-100 for 16 h at 37 °C. The gel was then stained with 0.1% (w/v) methylene blue in 0.01% KOH. Bands containing peptidoglycan hydrolase activity appeared as a clear zone, against a dark blue background (see Fig. 3).

A_{600} assay for peptidoglycan hydrolase activity

A culture of *Bacillus subtilis* W23 was grown at 37 °C to mid-log phase (OD600 0.6–1.0). An aliquot of culture (0.5 ml) was mixed with 100 mM Tris buffer pH 7.5 containing 20 mM $MgCl_2$ (0.5 ml), to which was added a sample of protein (0.5–1 mg), and the absorbance at 600 nm monitored over a 5 min assay. Addition of 1 mg lysozyme gave a decrease of 0.2 AU over 5 min; addition of 0.5 mg Ply21 gave a decrease of 0.05 AU over 5 min.

DTNB assays of thioester substrates for MurNAc-L-Ala amidase

Assays (1.0 ml total volume) containing 50 mM Tris buffer pH 7.5, 20 mM $MgCl_2$, thioester (0.1 mM), and protein samples (0.1–1.5 mg protein). Continuous DTNB assays were carried out in the presence of 0.1 mg ml^{-1} DTNB, and the assay monitored continuously at 412 nm (ε 14150 $M^{-1}cm^{-1}$). Stopped assays were carried out by withdrawal of an aliquot (200 µl), dilution to 1.0 ml with water, and addition of an aliquot of 20 mg ml^{-1} DTNB solution (5 µl), followed by measurement of A_{412}. Control assays lacking enzyme showed deviation of <0.005 AU over 10 min.

Crystal data for 6 [17]

$C_{38}H_{35}NO_3S$, M = 585.73, triclinic, a = 8.6803(6), b = 9.1105(6), c = 10.6271(8) Å, U = 773.11(9) $Å^3$, T = 180(2) K, space group $P\bar{1}$, Z = 1, μ = 0.143 mm^{-1}, 5048 reflections measured, 4151 unique reflections (R_{int} 0.0482), R indices (all data) $R1$ = 0.0618, $wR2$ = 0.1178. The configuration of the C-3 chiral centre was determined by comparison with the known chirality of C-8, and independently by refinement of a $\delta f''$ multiplier (refined value 0.10(10)).

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