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Water-Soluble Substrates of the Peptidoglycan-Modifying Enzyme *O*-Acetylpeptidoglycan Esterase (Ape1) from *Neisseria gonorrheae*

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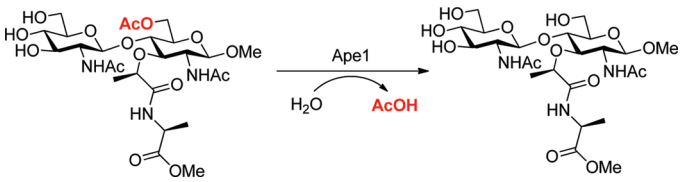

Peptidoglycan is the component of the bacterial cell wall that is essential for maintaining the shape and rigidity of the cell. As such, its polymeric structure, consisting of alternating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), is also a target for the action of host defense enzymes, such as lysozymes. Many bacteria have developed methods of masking their cell wall from these environmental dangers through the addition of aglycon moieties that prevent recognition or sterically hinder the degradative action of exogenous enzymes that would otherwise prove detrimental to the cell. Peptidoglycan acetyl-transferases (Pat's) and *O*-acetylpeptidoglycan esterases (Ape's) are the enzymes responsible for the controlled addition and removal of acetate onto the C-6 hydroxyl group of MurNAc residues in peptidoglycan. Studies on Ape1, an *O*-acetylpeptidoglycan esterase found in *Neisseria gonorrheae*, have suggested that this enzyme is essential for bacterial viability and thus presents an attractive target for antibacterial design. Previous studies on Ape1 have been hindered by the fact that Ape1's natural substrate is an insoluble polymer. In this paper we outline the design, synthesis, and testing of the water-soluble di- and monosaccharide substrate analogues **1** and **2**. Both **1** and **2** serve as substrates of Ape1 with k_{cat}/K_M values of $(5.1 \pm 1.7) \times 10^3\ M^{-1}\ s^{-1}$ and $(3.1 \pm 0.8) \times 10^3\ M^{-1}\ s^{-1}$, respectively. It was determined that the substitution of the GlcNAc residue in compound **1** with an *O*-benzyl group in compound **2** did not significantly decrease the enzyme's affinity for the monosaccharide. These findings are important as they demonstrate that the catalytic prowess of Ape1 is not dependent on its binding to a polymeric substrate. This ensures that small molecule transition state/intermediate analogues can also capture the transition state binding energy of Ape1 and potentially serve as potent inhibitors. The synthetic route to compounds **1** and **2** could readily be modified to allow for the installation of a wide variety of functional groups at the MurNAc C-6 position in both the mono- and disaccharide scaffolds. This will serve as a general method for the construction of Ape1 substrates and inhibitors.

Introduction

Peptidoglycan is an essential component of the bacterial cell wall found in both Gram-positive and Gram-negative bacteria.[1,2] Peptidoglycan allows eubacteria to maintain their shape, and its rigid, polymeric structure protects the cell from lysis due to high osmotic pressure. Its structure is comprised of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues connected via β-(1→4)-glycosidic linkages (Figure 1). Each MurNAc residue possesses a D-lactate group at the C-3 position that

(1) Park, J. T. The Murein Sacculus. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*; Neidhardt, F. C., Curtis, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Resnikoff, W. S., Riley, M., Schaechter, M., Umbarger, H. E., Eds.; American Society for Microbiology: Washington, DC, 1996; pp 48−57.

(2) Vollmer, W.; Blanot, D.; De Pedro, M. A. *FEMS Microbiol. Rev.* **2008**, *32*, 149–167.

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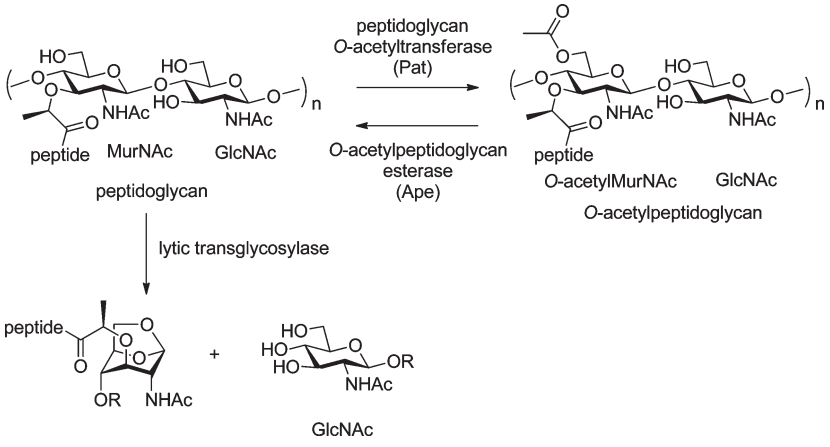


FIGURE 1. Peptidoglycan *O*-acetylation/deacetylation and the action of lytic transglycosylase.

bears a peptide side chain. Cross-linking of neighboring strands through their respective stem peptides then gives rise to the three-dimensional structure of peptidoglycan.[3]

Peptidoglycan is known to be an important factor governing virulence and toxicity by aiding in many pathogenic bacteria's ability to persist within the host. Because of its essential nature, significant research effort has focused on the design and discovery of molecules that inhibit its biosynthesis, as evidenced by the vast number of antibacterial compounds that target the cell wall. For example, the β-lactam penicillin binds to and irreversibly inhibits the penicillin-binding proteins (PBPs) of target cells, the biosynthetic enzymes that are responsible for the insertion and cross-linking of new peptidoglycan strands in the cell wall.[3] Bacteria, however, have rapidly developed resistance to both the natural host mechanisms of defense and virtually all known antibiotics, thus necessitating the discovery of new methods for combating infection and virulence.[4] As a response to invading pathogenic bacteria, hosts will release lysozymes as a first line of defense; lysozyme's mode of action involves hydrolysis of the glycosidic linkage between the GlcNAc and MurNAc residues in bacterial peptidoglycan. As a way to combat these host defenses, bacteria have evolved methods of masking their peptidoglycan through various covalent modifications. One such modification is the *O*-acetylation of the C-6 hydroxyl group on the MurNAc residues of peptidoglycan (Figure 1).[5] This modification has been shown to interfere with the action of lysozyme on peptidoglycan substrates by preventing binding. While this modification effectively blocks lysozymal degradation, it also interferes with the action of bacterial autolysins, most notably the lytic transglycosylases (LTs). Unlike lysozyme, these LTs cleave the β-(1→4) linkages nonhydrolytically to form 1,6-anhydromuramoyl residues and have a strict requirement for a free C-6 hydroxyl group on the MurNAc residues for activity (Figure 1).[3,6] Because the LTs play an important role in the metabolism of peptidoglycan during cell growth and division, the function of bacterial secretion systems, and the insertion of bacterial appendages, a mechanism for the controlled addition and removal of *O*-acetyl groups on peptidoglycan must exist so that these activities may continue.[3,6]

In *Neisseria gonorrheae*, the *O*-acetylation and deacetylation of MurNAc residues in peptidoglycan are governed by a set of enzymes encoded in the *poa* (peptidoglycan *O*-acetylation) gene cluster.[7] Homologous *poa* gene clusters have also been identified in other pathogenic bacteria including *Neisseria menningitidis*, *Proteus mirabilis*, and species of *Helicobacter*, *Campylobacter*, *Bacillus*, and *Treponema*.[7] Two peptidoglycan *O*-acetyltransferases (PatA and PatB) have been proposed to translocate acetate from the cytoplasm to the periplasm and finally onto peptidoglycan.[8] In an opposing fashion, the *O*-acetylpeptidoglycan esterase (Ape1) has been shown to remove acetate from *O*-acetylated peptidoglycan to allow for the action of LTs and other autolysins involved in peptidoglycan metabolism (Figure 1).[9,10] Previous studies have shown that both of these enzymes play an important and perhaps essential role in the metabolism of peptidoglycan.[9] Further characterization of the Pat's and Ape's found in pathogenic bacteria will help to determine their viability as antibacterial targets.

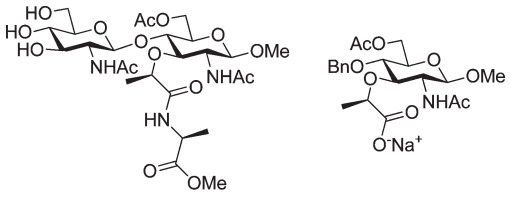
Ape1 belongs to the CE-3 family of carbohydrate esterases and possesses a catalytic triad of Asp-His-Ser that is conserved in many serine proteases/esterases.[10] Ape1 from *N. gonorrheae* was previously expressed in and purified from *Escherichia coli*. Two *N*-terminally processed forms of the wild-type protein were generated and were found to be localized predominately to the perisplasm and the outer membrane, areas where the maintenance of peptidoglycan would be required.[9] The cellular localization of Ape1 makes it an attractive antibacterial target, as inhibitors would not have to pass through the cytoplasmic membrane to reach their target. Perhaps more importantly, Ape1 deletion strains lacked viability, suggesting that Ape1 is an essential enzyme in many pathogenic bacteria.[9]

Ape1 activity was demonstrated using *O*-acetylated peptidoglycan samples isolated from bacteria, but these experiments were complicated by the fact that the substrate is an insoluble polymer and required sonication for even dispersal.[9] In order to obtain the kinetic parameters k_{cat} and K_M of Ape1,

(3) Holtje, J. V. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 181–203.
(4) Davies, J.; Davies, D. *Microbiol. Mol. Biol. Rev.* **2010**, *74*, 417–433.
(5) Clarke, A. J.; Dupont, C. *Can. J. Microbiol.* **1992**, *38*, 85–91.
(6) Koraimann, G. *Cell. Mol. Life Sci.* **2003**, *60*, 2371–2388.
(7) Weadge, J. T.; Pfeffer, J. M.; Clarke, A. J. *BMC Microbiol.* **2005**, *5*, 49.
(8) Moynihan, P. J.; Clarke, A. J. *J. Biol. Chem.* **2010**, *285*, 13264–13273.
(9) Weadge, J. T.; Clarke, A. J. *Biochemistry* **2006**, *45*, 839–851.
(10) Weadge, J. T.; Clarke, A. J. *Biochemistry* **2007**, *46*, 4932–4941.

it was necessary to use either of the highly activated esters *p*-nitrophenyl acetate or α-naphthyl acetate as model substrates. Although these data provide some sense of Ape1's catalytic efficiency, many esterases would be expected to nonspecifically hydrolyze these highly labile acetate esters. In order to obtain more meaningful kinetic data, analysis of enzyme activity with a soluble, unactivated substrate that more closely resembles peptidoglycan is required.

To this end we designed the 6-*O*-acetylated disaccharide and monosaccharide peptidoglycan analogues **1** and **2** that we predicted would serve as water-soluble substrates of Ape1.



1 **2**

Since Ape1 acts on a polymeric substrate, it is likely that both binding and catalysis is influenced by the number of sugar residues present in the substrate. Consequently, Ape1's ability to catalyze the deacetylation of truncated fragments of *O*-acetylated peptidoglycan could be significantly impaired. A marked dependency on the rate of reaction as a function of the number of monomer units in the substrate has been reported for other deacetylases that operate on polysaccharides.[11] The synthesis and testing of both the mono- and disaccharide substrate analogues will serve to identify whether Ape1 is able to accept truncated substrates and, if so, examine whether there is a strict requirement for the presence of the GlcNAc moiety. These substrates will also serve to validate the use of mono- and disaccharide fragments as the basic scaffold for the design of small-molecule transition state/intermediate analogues of Ape1 and act as benchmarks for future experiments involving the testing of these compounds as potential antibacterials.

Results and Discussion

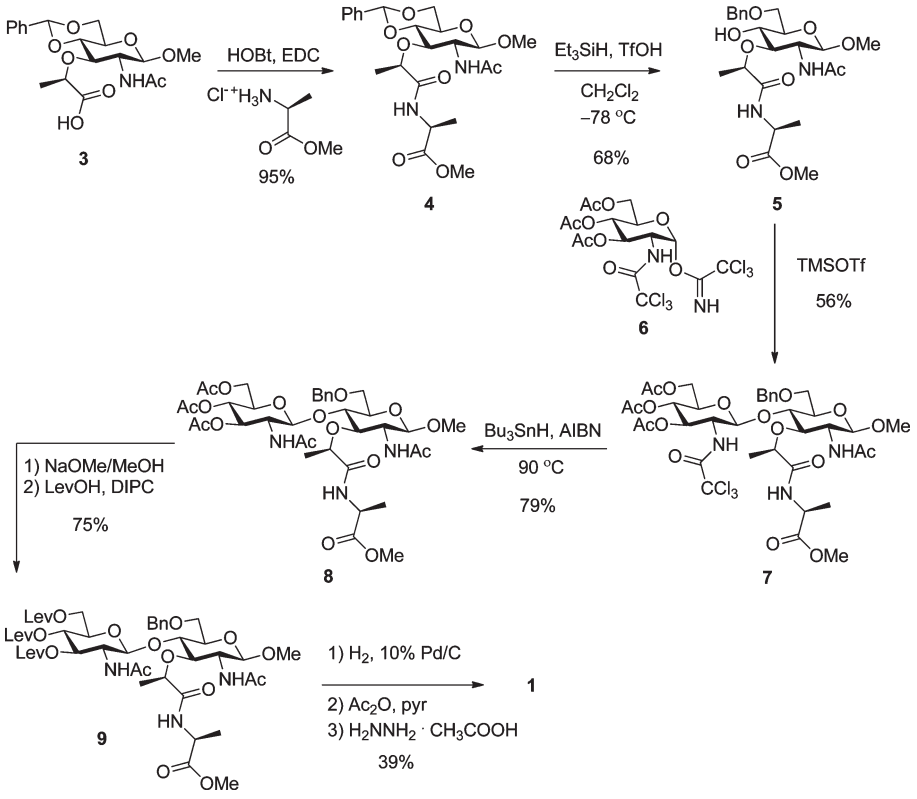
We chose to start with the relatively complex disaccharide substrate **1** to maximize our chances of observing a high level of activity with Ape1. This substrate was designed to serve as a benchmark to which other monosaccharide substrates could be compared. The core structure of **1** is a β-(1→4)-linked GlcNAc-MurNAc disaccharide that is elaborated with an *O*-acetyl ester at the C-6 position of the MurNAc moiety and an L-alanine methyl ester appended to the lactyl side chain. The β-methoxy group at the anomeric carbon of MurNAc was chosen to mimic the β-linkages found in peptidoglycan, and it was anticipated that the three free hydroxyl groups on the GlcNAc residue would increase the water solubility of the final compound. It is important to note that the presence of the β-linked GlcNAc at the C-4 position of MurNAc also serves to prevent any potential migration of the 6-*O*-acetyl ester.[12]

The truncated monosaccharide substrate **2** was designed to further examine the contributions of different structural elements toward binding. The GlcNAc moiety at O-4 has been replaced by a hydrophobic *O*-benzyl ether that is designed to occupy the GlcNAc binding pocket in the enzyme active site and also serves to prevent migration of the 6-*O*-acetyl ester. In addition, the L-alanine moiety has been deleted from the lactyl side chain to test whether it is a critical recognition element for the enzyme. Because compound **2** lacks the free hydroxyl groups contained in the GlcNAc residue of disaccharide **1**, the lactate was left as a free carboxylate to confer greater water solubility. The testing of compounds **1** and **2**, both bearing an unactivated acetate ester at the C-6 position of the MurNAc residue, will provide valuable information with regard to the structural elements required for binding. The results of these tests will also shape future strategies in the design and synthesis of Ape1 substrates and inhibitors.

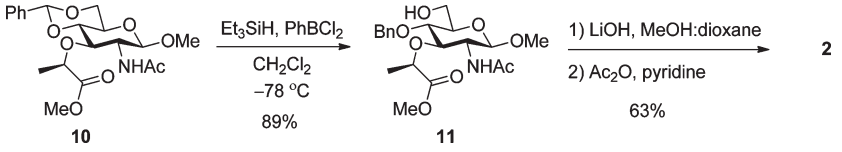
Synthesis of Disaccharide 1. The synthesis of oligosaccharides containing D-glucosamine and, more commonly, its *N*-acetyl derivative GlcNAc has been the subject of significant research effort as a result of their prevalence in glycoconjugates.[13] Glycoside bond formation using GlcNAc donors is complicated, however, by the formation of an oxazolinium intermediate via neighboring-group participation of the 2-acetamido group. This participation ensures the formation of the β-anomeric linkage in coupling reactions. However, the oxazolinium system is also relatively stable, resulting in weak glycosyl donor properties.[13,14] Several methods have been developed to increase the electrophilicity of GlcNAc donors, while still preferentially forming the β-linkage in the coupled products.[15] These methods rely primarily on increasing the electron-withdrawing character of the *N*-acyl substituents. This serves to decrease the stability of the oxazolinium system and increases the rate of nucleophilic attack by a glycosyl acceptor. For the synthesis of disaccharide **1**, we chose to mask the 2-acetamido group as a 2-trichloroacetamido group in our GlcNAc donor system (compound **6**, Scheme 1). This trichloroacetamido group can be reduced back to the corresponding acetamido group using tributyltin hydride and AIBN after the coupling has been completed.[14] Although the synthesis of related peptidoglycan fragments has been previously reported,[16] the requirement of the 6-*O*-acetate ester on the MurNAc moiety of disaccharide **1** necessitates a number of changes to our synthetic strategy, as *O*-acetate esters are traditionally employed as protecting groups in oligosaccharide synthesis. In order to selectively *O*-acetylate at the C-6 position on the MurNAc residue, the hydroxyl groups at C-3, C-4, and C-6 on the GlcNAc moiety must be masked until the *O*-acetate ester is installed. We chose to protect the three hydroxyl groups on the GlcNAc moiety as *O*-levulinate esters after coupling because they can be selectively removed in the presence of an *O*-acetate ester. The orthogonal protecting group strategy detailed within also allows for the installation of different functional groups at the C-6 position of MurNAc, a feature that is very attractive for the future development of small molecule inhibitors of Ape1.

(11) Hekmat, O.; Tokuyasu, K.; Withers, S. G. *Biochem. J.* **2003**, *374*, 369–380.
(12) Chang, K. Y.; Wu, S. H.; Wang, K. T. *Carbohydr. Res.* **1991**, *222*, 121–129.
(13) Schmidt, R. R. *Angew. Chem., Int. Ed.* **1986**, *25*, 212–235.
(14) Blatter, G.; Beau, J. M.; Jacquinet, J. C. *Carbohydr. Res.* **1994**, *260*, 189–202.
(15) Dullenkopf, W.; Castro-Palomino, J. C.; Manzoni, L.; Schmidt, R. R. *Carbohydr. Res.* **1996**, *296*, 135–147.
(16) Hesek, D.; Lee, M.; Morio, K.; Mobashery, S. *J. Org. Chem.* **2004**, *69*, 2137–2146.

SCHEME 1. Synthesis of Disaccharide Substrate 1



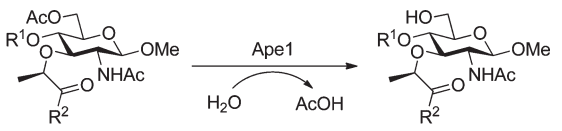
SCHEME 2. Synthesis of Monosaccharide Substrate 2



The known 4,6-*O*-benzylidene-protected MurNAc derivative **3** was prepared from *N*-acetylglucosamine using a combination of literature methods (Scheme 1).[17,18] Since peptidoglycan monomers are linked in a β-(1→4) manner, the methyl glycoside at C-1 was installed in the β-configuration. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt)-mediated coupling of the methyl ester of L-alanine to **3** gave compound **4** in good yield. A regioselective reductive ring opening of the benzylidene acetal with Et_3SiH and TfOH was then used to generate the 6-*O*-benzyl ether **5**.[19] The yield of this selective reduction was lower than expected, most likely due to the presence of the L-alanine attached to the C-3 lactate group and its interaction with TfOH. Reactions involving the selective reduction of the MurNAc derivatives lacking the L-alanine side chain gave significantly higher yields (data not shown). The donor, **6**, was synthesized using literature methodology starting from D-glucosamine.[14] As discussed previously, the 2-trichloroacetamido group was incorporated to mask the 2-acetamido moiety and to ensure successful coupling. The MurNAc acceptor **5** was coupled with donor **6** using TMSOTf as a promoter. The modest yield of disaccharide **7** can be partially accounted for by the lack of nucleophilicity of the C-4 hydroxyl group in acceptors possessing an unmasked 2-acetamido group.[20] Although previous strategies involving masking of the 2-acetamido group in the acceptor have been reported,[16] we chose to leave the group intact to minimize the number of manipulations required after the disaccharide coupling step. The trichloroacetamido functionality of disaccharide **7** was then reduced with tributyltin hydride/AIBN to unmask the 2-acetamido group on the "GlcNAc" portion of disaccharide **8**.[14] To generate the final product **1**, a protecting group for the hydroxyl groups at C-3, C-4, and C-6 of the GlcNAc moiety that could be selectively cleaved in the presence of an *O*-acetate ester was required. Accordingly, the *O*-acetate groups on compound **8** were replaced with *O*-levulinate esters through a two-step procedure involving deprotection with NaOMe, and a subsequent *N*,*N*′-diisopropylcarbodiimide (DIPC)-mediated coupling of the free hydroxyls with levulinic acid to give disaccharide **9**.

(17) Hesek, D.; Suvorov, M.; Morio, K.; Lee, M.; Brown, S.; Vakulenko, S. B.; Mobashery, S. *J. Org. Chem.* **2004**, *69*, 778–784.
(18) Cai, Y.; Ling, C. C.; Bundle, D. R. *Org. Lett.* **2005**, *7*, 4021–4024.
(19) Sakagami, M.; Hamana, H. *Tetrahedron Lett.* **2000**, *41*, 5547–5551.
(20) Crich, D.; Dudkin, V. *J. Am. Chem. Soc.* **2001**, *123*, 6819–6825.

TABLE 1. Kinetic Parameters for the Ape1-Catalyzed Hydrolysis of Synthetic Substrates 1 and 2



1: R^1 = GlcNAc, R^2 = L-Ala methyl ester
2: R^1 = Bn, R^2 = O^-Na^+

substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
1	2.36 ± 0.18	0.46 ± 0.10	$(5.1 \pm 1.7) \times 10^3$
2	1.24 ± 0.07	0.39 ± 0.08	$(3.1 \pm 0.8) \times 10^3$

With the proper protecting groups now in place, the acetate ester was introduced selectively at the C-6 position of the MurNAc residue through the hydrogenolysis of the benzyl ether and acetylation of the newly formed primary hydroxyl group. Deprotection of the *O*-levulinate esters was accomplished selectively in the presence of the *O*-acetate ester with the use of hydrazine acetate, generating disaccharide substrate **1**. The removal of the *O*-levulinate esters was complete almost immediately after addition of the hydrazine acetate, and no corresponding product lacking the C-6 *O*-acetyl ester was detected.

Synthesis of Monosaccharide 2. The synthesis of monosaccharide substrate **2** was accomplished in three steps from the protected MurNAc derivative **10** (Scheme 2).[21] The known compound **10** was prepared from compound **3** using TMS-diazomethane to protect the carboxylic acid as a methyl ester. The protection of the free carboxylate as a methyl ester was required to increase solubility in methylene chloride during the subsequent regioselective reductive ring opening of the benzylidene acetal with Et_3SiH and $PhBCl_2$. 4-*O*-Benzyl ether **11** was generated in good yield (89%), leaving a primary hydroxyl at C-6 for further functionalization. The methyl ester was hydrolyzed with LiOH in a mixture of MeOH and dioxane to give the free carboxylic acid, and the crude compound was *O*-acetylated at the C-6 position. A subsequent exchange from the triethylamine salt to the sodium salt gave monosaccharide substrate **2** in a 63% yield.

Testing of Compounds 1 and 2 with Ape1. The esterase activity with the alternate substrates **1** and **2** was determined by incubating varying concentrations of the compounds with a truncated form of the Ape1 enzyme lacking the *N*-terminal signal peptide.[10] The enzymatic reactions were terminated with the addition of H_2SO_4, and the amount of acetate released was quantified using a coupled enzymatic assay. It was determined that Ape1 follows Michaelis−Menten kinetics when acting on both the mono- and disaccharide substrates. The parameters k_{cat}, K_M, and k_{cat}/K_M were determined for both substrates, and the results are outlined in Table 1.

Both **1** and **2** served as substrates of Ape1, and their k_{cat}/K_M values were on the same order of magnitude as those previously reported for the highly activated *p*-nitrophenyl acetate ($K_M = 0.51 \pm 0.21$ mM, $k_{cat}/K_M = 2.0 \times 10^4$ M^{-1} s^{-1}).[10] Each substrate analogue bears an unactivated acetate ester at the C-6 position of the MurNAc residue, and their acceptance as substrates of Ape1 demonstrates that binding to a polymeric substrate is not a strict requirement for enzymatic activity. The disaccharide, **1**, possesses a β-(1→4)-linked GlcNAc moiety, as well as a methyl ester-protected L-alanine appended to its lactyl side chain. Its more complex design, with the inclusion of these recognition elements, was pursued with the goal of obtaining a substrate with a high level of activity. Indeed, compound **1** served as a slightly better substrate than **2**, but the small magnitude of this difference was perhaps the more interesting finding. Because Ape1 acts on polymeric *O*-acetylated peptidoglycan, it is not unreasonable to assume that disaccharide substrates would bind more tightly in the Ape1 active site than the corresponding monosaccharide units. Replacing the GlcNAc moiety with an *O*-benzyl ether at the C-4 position of MurNAc, however, results in only a minimal decrease in the rate of Ape1 hydrolysis of the acetate ester. It is likely that the movement of the benzyl group from bulk water into the relatively hydrophobic pocket of the GlcNAc binding site is favored by the hydrophobic effect. This entropic driving force could compensate for any loss of enthalpic interactions due to the lack of the GlcNAc moiety. Compound **2** also lacks the L-alanine that was appended to the lactyl side chain in compound **1**. The small decrease in activity with this substrate also suggests that the peptides normally attached to the lactyl moiety are not required for binding in the Ape1 active site. These findings provide important information regarding the recognition elements required for productive binding and bode well for the future development of substrates and inhibitors of Ape1. The synthesis of monosaccharide **2** is more efficient and requires fewer synthetic transformations than that of disaccharide **1**, so the future development of Ape1 inhibitors will focus on monosaccharide scaffolds.

Conclusion

We have successfully synthesized and tested water-soluble mono- and disaccharide substrate analogues of the *O*-acetylpeptidoglycan esterase Ape1. Both **1** and **2** served as substrates of Ape1, and interestingly, the substitution of the GlcNAc residue for an *O*-benzyl ether at the C-4 position of MurNAc did not greatly affect the activity on substrate **2**. These results validate the use of both MurNAc monosaccharides and MurNAc-GlcNAc disaccharides as templates for Ape1 substrate and inhibitor design. This is an important finding as it demonstrates that the catalytic prowess of Ape1 is not dependent on its binding to a polymeric substrate. This ensures that small molecule transition state/intermediate analogues can also capture the transition state binding energy of Ape1 and potentially serve as potent inhibitors.

Future efforts toward the design and synthesis of potential inhibitors of Ape1 are aided by the abundance of literature available outlining mechanism-based strategies for the inhibition of serine proteases and esterases.[22] The conserved catalytic triad of Asp-His-Ser found in this class of enzymes provides a target that has a very well-characterized mechanism of action. Our synthetic route to monosaccharide **2** introduces the *O*-acetyl group in the late stages of the synthesis and allows for the installation of different functional groups at the C-6 position. Therefore, the synthesis of a wide variety of potential inhibitors of Ape1 could be easily accomplished

(21) Jeanloz, R. W.; Walker, E.; Sinay, P. *Carbohydr. Res.* **1968**, *6*, 184–196.

(22) Powers, J. C.; Asgian, J. L.; Ekici, Ö. D.; James, K. E. *Chem. Rev.* **2002**, *102*, 4639–4750.

using this synthetic scheme. Testing of these compounds for bacteriocidal activity will eventually serve to determine Ape1's viability as an antibacterial target.

Experimental Section

Compounds **3** and **6** were prepared by published literature methods and their identities confirmed by MS and NMR spectroscopy.[14,17]

Methyl 4,6-*O*-Benzylidene-β-D-*N*-acetylmuramylpyranoside-L-alanine Methyl Ester (4). Compound **3** (2.9 g, 7.34 mmol) was dissolved in 160 mL of anhydrous DMF, and HOBt (1.35 g, 8.81 mmol), EDC (1.69 g, 8.81 mmol), and 1.25 mL of NEt_3 were added. The resultant mixture was allowed to stir for 5 min at rt before L-alanine methyl ester hydrochloride (1.23 g, 8.81 mmol) and an additional 1.25 mL of NEt_3 were added.The reaction was allowed to stir overnight at room temperature under an argon atmosphere, after which it was judged complete by TLC analysis (9:1 CH_2Cl_2/MeOH). The mixture was diluted with CH_2Cl_2 and washed consecutively with 5% $KHSO_4$, sat. $NaHCO_3$, sat. NaCl, and H_2O. The organic layer was separated, dried over $MgSO_4$, and filtered. The solvents were removed *in vacuo*, and the solid was purified via silica gel chromatography (40:1 CH_2Cl_2/MeOH) to give product **4** (3.37 g, 7.02 mmol, 95%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.48−7.34 (m, 5H), 7.18 (d, 1H, J = 6.8 Hz), 6.09 (d, 1H, J = 8.0 Hz), 5.55 (s, 1H), 4.78 (d, 1H, J = 8.0 Hz), 4.47 (qd, 1H, J = 7.2 Hz), 4.36 (dd, 1H, J = 4.8 Hz, 10.4 Hz), 4.19 (q, 1H, J = 6.8 Hz), 4.13 (dd, 1H, J = 9.2 Hz), 3.79 (dd, 1H, J = 10.4 Hz), 3.76 (s, 3H), 3.61 (dd, 1H, J = 9.2 Hz), 3.54−3.47 (m, 5H), 1.99 (s, 3H), 1.43 (d, 3H, J = 7.2 Hz), 1.39 (d, 3H, J = 6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 173.7, 173.1, 171.3, 137.2, 129.3, 128.5 (2 × C), 126.1 (2 × C), 101.6, 101.5, 82.0, 78.6, 78.2, 68.9, 66.0, 57.7, 57.2, 52.7, 48.2, 23.9, 19.6, 18.3; HRMS (ESI) m/z calcd for $[C_{23}H_{32}N_2O_9Na]^+$, 503.2006, found 503.1994.

Methyl 6-*O*-Benzyl-β-D-*N*-acetylmuramylpyranoside-L-alanine Methyl Ester (5). Molecular sieves (4 Å) were added to a solution of compound **4** (247 mg, 0.514 mmol) in 40 mL of distilled CH_2Cl_2, and the mixture stirred under an argon atmosphere for 1 h. The reaction flask was cooled to −78 °C, and Et_3SiH (0.25 mL, 1.54 mmol) was added, followed 5 min later by TfOH (0.15 mL, 1.75 mmol). The mixture was maintained at −78 °C while stirring and was judged complete after 1.5 h by TLC analysis (9:1 CH_2Cl_2/MeOH). NEt_3 (2 mL) and MeOH (2 mL) were added, and the solution was diluted with $CHCl_3$ and filtered through a pad of Celite. The filtrate was washed consecutively with sat. $NaHCO_3$, sat. NaCl, and H_2O. The organic layer was dried over $MgSO_4$ and filtered, and the solvents were removed *in vacuo*. The residue was purified via silica gel chromatography (CH_2Cl_2, then 39:1 CH_2Cl_2/MeOH, then 9:1 CH_2Cl_2/MeOH) to give compound **5** (170 mg, 0.353 mmol, 68%) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.38−7.24 (m, 5H), 6.90 (d, 1H, J = 8.8 Hz), 4.61−4.53 (m, 2H), 4.46−4.37 (m, 2H), 4.25 (q, 1H, J = 6.8 Hz), 3.86−3.70 (m, 4H), 3.70 (s, 3H), 3.66−3.46 (m, 3H), 3.43 (s, 3H), 2.64 (br s, 1H), 1.94 (s, 3H), 1.42 (d, 3H, J = 6.8 Hz), 1.38 (d, 3H, J = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 173.9(6), 173.5(3), 171.5, 138.0, 128.6 (2 × C), 127.9(3), 127.8(7) (2 × C), 101.9, 82.2, 77.0, 74.6, 73.8, 71.5, 70.5, 56.7, 54.8, 52.6, 48.3, 23.6, 19.6, 17.6; HRMS (ESI) m/z calcd for $[C_{23}H_{34}N_2O_9Na]^+$, 505.2162, found 505.2166.

Methyl 3,4,6-Tri-*O*-acetyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl-(1→4)-6-*O*-benzyl-β-D-*N*-acetylmuramylpyranoside-L-alanine Methyl Ester (7). To a solution of acceptor **5** (330 mg, 0.684 mmol) and donor **6** (666 mg, 1.13 mmol) in 20 mL of distilled CH_2Cl_2 was added 4 Å molecular sieves, and the resultant mixture was stirred under argon atmosphere for 1 h. The mixture was cooled to 0 °C, and TMSOTf (0.19 mL, 1.03 mmol) was added. After stirring for 1 h at 0 °C, the ice bath was removed, and the mixture was warmed to rt. The reaction was judged complete by monitoring the disappearance of acceptor **5** by TLC analysis (EtOAc) and was subsequently quenched with 0.4 mL of NEt_3. The mixture was diluted with CH_2Cl_2 and filtered through a pad of Celite, and the solvents removed *in vacuo*, leaving a dark brown foam. Silica gel chromatography (EtOAc, then 9:1 EtOAc/MeOH) gave compound **7** (355 mg, 0.388 mmol, 56% based on acceptor) as a white solid. ^{1}H NMR (400 MHz, MeOD) δ ppm 7.44−7.28 (m, 5H), 5.40 (dd, 1H, J = 9.2 Hz, 10.4 Hz), 4.98 (dd, 1H, J = 9.6 Hz), 4.86 (obscured by solvent, 1H), 4.67 (dd, 1H, J = 11.6 Hz), 4.64 (dd, 1H, J = 11.6 Hz), 4.39 (q, 1H, J = 7.2 Hz), 4.33 (dd, 1H, J = 4.0 Hz, 12.4 Hz), 4.27 (d, 1H, J = 8.4 Hz), 4.23 (q, 1H, J = 6.8 Hz), 4.02−3.94 (m, 2H), 3.89−3.76 (m, 4H), 3.73 (s, 3H), 3.58−3.45 (m, 3H), 3.43 (s, 3H), 2.02−1.96 (m, 9H), 1.92 (s, 3H), 1.46−1.41 (m, 6H); ^{13}C NMR (100 MHz, MeOD) δ ppm 174.1, 173.2, 172.2, 170.9, 170.3, 170.0, 163.0, 138.5, 128.2 (2×C), 127.6(4) (2×C), 127.5(7), 102.1, 98.6, 92.5, 79.5, 78.0, 75.0, 74.4, 73.0, 71.5, 71.4, 68.8, 68.3, 61.4, 56.5, 55.7, 54.8, 51.6, 48.0, 21.8, 19.5, 19.3 (2 × C), 17.8, 16.3; HRMS (ESI) m/z calcd for $[C_{37}H_{50}N_3Cl_3O_{17}Na]^+$, 936.2104, found 936.2111.

Methyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→4)-6-*O*-benzyl-β-D-*N*-acetylmuramylpyranoside-L-alanine Methyl Ester (8). Disaccharide **7** (270 mg, 0.295 mmol) and AIBN (10 mg, 0.059 mmol) were dissolved in 10 mL of DMA, and the resultant solution was stirred at 90 °C for 1 h with argon gas bubbling directly through the mixture. Tributyltin hydride (0.63 mL, 2.36 mmol) and additional AIBN (3 mg, 0.0183 mmol) were added to the reaction mixture, and the stirring was continued at 90 °C. The reaction progress was monitored for the disappearance of starting material by TLC analysis (9:1 CH_2Cl_2/MeOH) and was judged complete after approximately 2 h. The solvents were removed *in vacuo*, and the residue purified by silica gel chromatography (EtOAc, then 19:1 EtOAc/MeOH, then 9:1 EtOAc/MeOH) to give compound **8** (190 mg, 0.234 mmol, 79%) as a white solid. ^{1}H NMR (400 MHz, MeOD) δ ppm 7.45−7.29 (m, 5H), 5.20 (dd, 1H, J = 9.2 Hz, 10.4 Hz), 4.94 (dd, 1H, J = 9.6 Hz), 4.73−4.59 (m, 3H), 4.40 (q, 1H, J = 7.2 Hz), 4.32−4.20 (m, 3H), 4.00−3.90 (m, 2H), 3.88−3.79 (m, 4H), 3.73 (s, 3H), 3.53−3.40 (m, 3H), 3.44 (s, 3H), 2.01−1.96 (m, 9H), 1.93 (s, 3H), 1.91 (s, 3H), 1.44−1.39 (m, 6H); ^{13}C NMR (100 MHz, MeOD) δ ppm 174.2, 173.3, 172.4, 172.2, 170.9(7), 170.5(2), 170.0(0), 138.5, 128.3 (2×C), 127.7(1) (2×C), 127.6(3), 102.3, 99.5, 79.3, 77.7, 75.1, 74.7, 73.0, 72.4, 71.4, 68.8(2), 68.4(7), 61.5, 55.7, 54.6(9), 54.5(5), 51.7, 48.0, 21.8(5), 21.6(2), 19.5(0), 19.3(3), 19.2(9), 17.7, 16.3; HRMS (ESI) m/z calcd for $[C_{37}H_{54}N_3O_{17}]^+$, 812.3453, found 812.3436.

Methyl 2-Acetamido-2-deoxy-3,4,6-tri-*O*-levulinoyl-β-D-glucopyranosyl-(1→4)-6-*O*-benzyl-β-D-*N*-acetylmuramylpyranoside-L-alanine Methyl Ester (9). Disaccharide **8** (42 mg, 0.052 mmol) was dissolved in 10 mL of distilled MeOH in a flame-dried round-bottom flask, and NaOMe was added (108 mg, 2.0 mmol). The reaction mixture was stirred at rt under an argon atmosphere for 20 min, after which it was judged complete by TLC analysis (9:1 CH_2Cl_2/MeOH). The reaction was neutralized with Amberlite IR120(H^+), filtered, and concentrated *in vacuo*. The residue was dissolved in 9:1 CH_2Cl_2/MeOH, filtered through a silica gel plug, and concentrated *in vacuo* to give the deacetylated product (30 mg) that was used without further purification.

Levulinic acid (0.015 mL, 0.144 mmol) and DMAP (17 mg, 1.38 mmol) were dissolved in 5 mL of distilled CH_2Cl_2, and the mixture was cooled to 0 °C under an argon atmosphere. DIPC (0.023 mL, 0.144 mmol) was added, and the mixture was stirred for 5 min before a solution of the deacetylated disaccharide (30 mg) in 2 mL of CH_2Cl_2 was added. The mixture was allowed to warm to rt and was stirred overnight under an argon atmosphere,

after which TLC analysis (9:1 CH_2Cl_2/MeOH) confirmed the disappearance of the starting material. The solution was diluted with EtOAc and filtered through a plug of silica; the plug was then flushed with 200 mL of 9:1 CH_2Cl_2/MeOH, and the eluent concentrated *in vacuo*. The residue was subjected to silica gel chromatography (40:1 CH_2Cl_2/MeOH then 20:1 CH_2Cl_2/MeOH) to give the levulinate-protected disaccharide **9** (37.8 mg, 0.0386 mmol, 75%) as a white solid. ^{1}H NMR (400 MHz, MeOD) δ ppm 7.44−7.29 (m, 5H), 5.21 (dd, 1H, J = 9.2 Hz, 10.4 Hz), 4.92 (dd, 1H, J = 9.2 Hz, 10.4 Hz), 4.71−4.58 (m, 3H), 4.41 (q, 1H, J = 7.2 Hz), 4.28−4.19 (m, 3H), 4.01 (dd, 1H, J = 1.6 Hz, 12 Hz), 3.93 (dd, 1H, J = 8.4 Hz), 3.88−3.78 (m, 4H), 3.73 (s, 3H), 3.52−3.42 (m, 3H), 3.43 (s, 3H), 2.84−2.66 (m, 6H), 2.58−2.42 (m, 6H), 2.16−2.12 (m, 9H), 1.93 (s, 3H), 1.92 (s, 3H), 1.43−1.37 (m, 6H); ^{13}C NMR (100 MHz, MeOD) δ ppm 207.8, 207.6, 207.5, 174.1, 173.3, 172.6, 172.4(6), 172.4(1), 172.1, 171.9, 138.5, 128.3 (2×C), 127.8 (2×C), 127.6, 102.3, 99.6, 79.0, 77.4, 75.3, 74.7, 73.2, 72.3, 71.7, 69.1, 68.9, 62.1, 55.6, 54.7, 54.2, 51.6, 48.0, 47.7, 37.5, 37.2(8), 37.2(4), 28.4, 28.2, 27.7(8), 27.7(2), 27.6(8), 21.7(7), 21.7(2), 17.8, 16.3; HRMS (ESI) m/z calcd for $[C_{46}H_{65}N_3O_{20}Na]^+$, 1002.4059, found 1002.4034.

Methyl 2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-6-*O*-acetyl-β-D-*N*-acetylmuramylpyranoside-L-alanine Methyl Ester (1). Disaccharide **9** (22 mg, 0.022 mmol) was dissolved in 10 mL of distilled MeOH, and 10% Pd/C (20 mg) was added. The reaction flask was placed under a H_2 atmosphere (1 atm), and the reaction was stirred at rt until TLC analysis (9:1 CH_2Cl_2/MeOH) indicated that the starting material was completely consumed and the hydrogenolysis was complete. The mixture was filtered through a pad of Celite, and the solvents were removed *in vacuo*. The crude residue was carried on without further purification.

The crude debenzylated disaccharide was dissolved in 5 mL of distilled pyridine and cooled to 0 °C before acetic anhydride (0.5 mL, 5.3 mmol) was added. The reaction mixture was allowed to warm to rt and was stirred under a blanket of argon. After 2.5 h, TLC analysis (9:1 CH_2Cl_2/MeOH) showed the disappearance of the starting material and the appearance of a new spot at a higher R_f. Toluene was added, and the mixture was co-evaporated *in vacuo*; this procedure was repeated three times to ensure all of the residual pyridine was removed from the reaction mixture. Acetylation of the disaccharide was confirmed by MS and ^{1}H NMR spectroscopy (data not shown), and the crude product was used without further purification.

The crude 6-*O*-acetylated disaccharide was dissolved in 5 mL of distilled pyridine, and hydrazine acetate (12 mg, 0.13 mmol) was added. The reaction mixture was monitored by TLC analysis (9:1 CH_2Cl_2/MeOH) for the disappearance of starting material and was judged complete after 10 min. The reaction was diluted with toluene, and the mixture was co-evaporated to remove any residual pyridine. Silica gel chromatography (20:1 CH_2Cl_2/MeOH then 9:1 CH_2Cl_2/MeOH) gave compound **1**, which was dissolved in water, frozen, and lyophilized to produce a white solid (5.6 mg, 0.0088 mmol, 39%). ^{1}H NMR (400 MHz, D_2O) δ ppm 4.55 (m, 1H), 4.48−4.35 (m, 4H), 4.19 (dd, 1H, J = 5.2 Hz, 12.0 Hz), 3.98−3.92 (m, 2H), 3.84−3.72 (m, 4H), 3.79 (s, 3H), 3.64−3.52 (m, 2H), 3.48 (s, 3H), 3.44−3.38 (m, 2H), 2.17 (s, 3H), 2.06 (s, 3H), 1.98 (s, 3H), 1.46 (d, 3H, J = 6.8 Hz), 1.41 (d, 3H, J = 6.8 Hz); ^{13}C NMR (100 MHz, D_2O) δ ppm 175.3, 175.1, 174.8, 174.4, 173.9, 102.0, 100.5, 79.4, 78.3, 76.4, 74.9, 73.6, 72.8, 70.4, 62.8, 61.3, 57.2, 56.1, 54.6, 53.2, 48.7, 22.3(7), 22.3(0), 20.4, 18.3, 16.1; HRMS (ESI) m/z calcd for $[C_{26}H_{43}N_3O_{15}Na]^+$, 660.2592, found 660.2582.

Methyl 2-Acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-((*R*)-1-(methoxycarbonyl)ethyl)-β-D-glucopyranoside (10). Compound **3** (138 mg, 0.349 mmol) was dissolved in 72 mL of an 8:1 mixture of MeOH/toluene. TMS-diazomethane (2 M solution in diethyl ether) was added dropwise to the reaction mixture until the yellow color in the solution persisted. The solution was stirred at rt for 1.5 h, after which the reaction was judged complete by TLC analysis (6:3:1 EtOAc/MeOH/H_2O). The solvents were removed *in vacuo*, and the crude methyl ester was subjected to silica gel chromatography (CH_2Cl_2 then 9:1 CH_2Cl_2/MeOH), affording compound **10** as a white solid (134 mg, 0.328 mmol, 94%). MS and ^{1}H NMR spectroscopy were used to confirm the identity of the known compound.[21]

Methyl 2-Acetamido-4-*O*-benzyl-2-deoxy-3-*O*-((*R*)-1-(methoxycarbonyl)ethyl)-β-D-glucopyranoside (11). The known methyl ester-protected MurNAc derivative **10** (170 mg, 0.415 mmol) was dissolved in distilled CH_2Cl_2, and 4 Å molecular sieves were added. The resultant mixture was stirred under an argon atmosphere for 1 h and was subsequently cooled to −78 °C. Et_3SiH (0.33 mL, 2.08 mmol) was first added, followed 5 min later by $PhBCl_2$ (0.27 mL, 2.08 mmol), and the reaction was stirred at −78 °C. The reaction was monitored for the disappearance of starting material by TLC analysis (9:1 CH_2Cl_2/MeOH) and was quenched with 1 mL each of NEt_3 and MeOH after it was judged complete. The mixture was then diluted with $CHCl_3$ and washed with saturated $NaHCO_3$. The organics were dried over $MgSO_4$, filtered, and then concentrated *in vacuo*. The crude product was purified via silica gel chromatography (40:1 CH_2Cl_2/MeOH) to give compound **11** (152 mg, 0.37 mmol, 89%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.38−7.28 (m, 5H), 6.93 (d, 1H, J = 5.6 Hz), 4.71 (m, 2H), 4.57 (q, 1H, J = 6.8 Hz), 4.41 (d, 1H, J = 7.2 Hz), 3.87 (m, 1H), 3.76−3.59 (m, 4H), 3.71 (s, 3H), 3.48 (s, 3H), 3.37−3.31 (m, 1H), 2.32 (br s, 1H), 2.02 (s, 3H), 1.35 (d, 3H, J = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 175.2, 171.9, 137.7, 128.7(7) (2×C), 128.2(2), 127.9(8) (2×C), 103.0, 79.4(0), 79.2(0), 75.5 (2 × C), 75.1, 61.6, 57.1, 55.7, 52.2, 23.7, 19.3; HRMS (ESI) m/z calcd for $[C_{20}H_{29}NO_8Na]^+$, 434.1791, found 434.1782.

Methyl 2-Acetamido-6-*O*-acetyl-4-*O*-benzyl-3-*O*-((*R*)-1-carboxyethyl)-2-deoxy-β-D-glucopyranoside Monosodium Salt (2). Compound **11** (152 mg, 0.369 mmol) was dissolved in 25 mL of 1:1 dioxane/MeOH, and 1 M LiOH was added until the solution reached a pH of 10. The reaction mixture was left to stir overnight at rt, after which it was judged complete by TLC analysis (6:3:1 EtOAc/MeOH/H_2O). The mixture was neutralized by the addition of Amberlite IR-120(H^+), filtered, and then concentrated *in vacuo*. The crude solid was redissolved in H_2O and washed with CH_2Cl_2, and the aqueous layer was concentrated *in vacuo* and used without further purification.

The crude carboxylic acid was dissolved in 20 mL of distilled pyridine, and the resulting solution was cooled to 0 °C. Acetic anhydride (1.0 mL, 10.6 mmol) was added, and the mixture was allowed to warm to rt before being left to stir overnight under an argon atmosphere. After TLC analysis of the reaction mixture (6:3:1 EtOAc/MeOH/H_2O) confirmed the disappearance of starting material, toluene was added, and the solvents were co-evaporated *in vacuo*. The addition of toluene and its removal *in vacuo* was repeated several times to ensure the removal of all residual pyridine. The residue was redissolved in CH_2Cl_2, excess NEt_3 was added, and the resultant solution evaporated *in vacuo*. This procedure was repeated twice to generate the triethylammonium salt of the 6-*O*-acetylated ester and was confirmed by ^{1}H NMR spectroscopy (data not shown). The triethylammonium salt was dissolved in H_2O and passed through a 10 mL column of Amberlite IR-120(Na^+); fractions containing product were pooled, frozen, and lyophilized to generate the sodium salt **12** as a white, fluffy solid (108 mg, 0.234 mmol, 63%). ^{1}H NMR (400 MHz, MeOD) δ ppm 7.37−7.26 (m, 5H), 4.83 (d, 1H, J = 10.8 Hz), 4.61 (d, 1H, J = 10.8 Hz), 4.42 (q, 1H, J = 6.8 Hz), 4.38−4.28 (m, 2H), 4.16 (dd, 1H, J = 3.2 Hz, 11.6 Hz), 3.74−3.48 (m, 4H), 3.41 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H), 1.37 (d, 3H, J = 6.8 Hz); ^{13}C NMR (100 MHz, MeOD) δ ppm 176.8, 172.9, 171.3, 138.0, 128.3 (2×C), 127.7(7) (2×C), 127.7(1), 102.8,

80.8, 78.8, 77.2, 74.7, 73.1, 62.8, 55.7(7), 55.2(4), 22.0, 19.5, 18.6; HRMS (ESI) m/z calcd for $[C_{21}H_{28}NO_9]^-$, 438.1764, found 438.1773.

Bacterial Strains and Growth Media. The sources of plasmids and bacterial strains used in this study are listed with their respective genotypic descriptions in the Supporting Information. *Escherichia coli* Rosetta(λDE3)pLysS, used for protein expression, was maintained on LB agar containing 35 μg/mL chloramphenicol. For expression of high levels of Ape1 (previously Ape1a),[10] cells were always freshly transformed with pACJW16 using standard procedures and grown in SuperBroth (5 g NaCl, 20 g yeast extract and 32 g tryptone/L) containing 35 μg/mL chloramphenicol and 50 μg/mL kanamycin at 37 °C with agitation. All reagents were from Sigma unless otherwise noted.

Production and Purification of Ape1. For the overexpression of Ape1, *E. coli* Rosetta(λDE3)pLysS cells transformed with pACJW16 were grown in SuperBroth at 37 °C to an OD_{600} of ~0.6 and then induced for a minimum of 3 h at 18 °C with the addition of IPTG to a final concentration of 1 mM. Cells were isolated by centrifugation (5000*g*, 15 min, 4 °C), and pellets were stored at −20 °C until needed.

To purify His_6-Ape1, cell pellets were thawed and resuspended in a minimal volume of lysis buffer [50 mM $NaPO_4$ and 500 mM NaCl (pH 8.0)]. Lysozyme (1 mg/mL), RNase A (10 mg/mL), and DNase I (5 mg/mL) were added to aid in lysis, and EDTA-free protease inhibitor tablets (1 per 15 mL of suspension) were added to prevent protein degradation. The suspension was incubated at 4 °C for 1 h before being subjected to lysis in a French pressure cell (four passes at 18 000 psi). The resulting cell lysates were clarified by centrifugation (20,000*g*, 20 min, 4 °C), and 1 mL of Ni^{2+}NTA-agarose was added for every 10 mL of cleared lysate. The mixture was incubated overnight at 4 °C with shaking before being applied to a 15 mL disposable plastic column. Unbound proteins were allowed to flow through, and the matrix was washed with approximately 30 column volumes of lysis buffer. The matrix was subsequently washed with approximately 20 column volumes of wash buffer containing 20 mM imidazole and a further 10 column volumes of wash buffer containing 30 mM imidazole. Bound Ape1 was recovered by batch elution in 10 mL of wash buffer containing 150 mM imidazole. The eluted protein was then dialyzed for 16 h against 2 × 4 L of 25 mM sodium phosphate buffer (pH 7.0).

His_6-tagged Ape1 was further purified by cation-exchange chromatography on MonoS. Protein was applied to the column following its equilibration in running buffer (25 mM $NaPO_4$, pH 7.0) at a flow rate of 0.7 mL/min. Elution of protein from the column was accomplished by increasing the ionic strength of the running buffer using a linear gradient of 0−1 M NaCl over 30 min. Eluted Ape1 was then collected and dialyzed against 6 L of 50 mM sodium phosphate buffer, pH 7.0 for 16 h at 4 °C.

Enzyme Assays. For the routine detection of acetyl esterase activity, 2 mM *p*-nitrophenylacetate (*p*NP-acetate) in 50 mM sodium phosphate buffer (pH 6.5) was used as a substrate in a 96-well microtiter plate as previously described.[9,10]

The Michaelis−Menten parameters of Ape1 were determined for **1** and **2** using concentrations ranging from 0.05 to 4 mM in 50 mM sodium phosphate buffer pH 6.5. Reaction mixtures, performed in triplicate, were initiated by the addition of substrate and incubated at 25 °C. Reactions were terminated by acidifying with H_2SO_4 to a final concentration of 60 mM. Samples of substrate incubated in the absence of enzyme were used as controls for the spontaneous release of any acetate. Quantification of released acetate was performed using the Megazyme Acetic Acid Assay kit (Megazyme International Ireland, Ltd., Wicklow, Ireland). Plots of initial reaction velocities as a function of substrate concentration were analyzed by nonlinear regression using Microcal Origin 7.5, assuming a one-site binding model.

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Supporting Information Available: ^1H and ^{13}C NMR spectra of new compounds and kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org