Published in final edited form as:
J Am Chem Soc. 2009 July 1; 131(25): 8742–8743. doi:10.1021/ja9025566.

Bacterial AmpD at the Crossroads of Peptidoglycan Recycling and Manifestation of Antibiotic Resistance

Mijoon Lee, **Weilie Zhang**, **Dusan Hesek**, **Bruce C. Noll**, **Bill Boggess**, and **Shahriar Mobashery**
Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

Shahriar Mobashery: mobashery@nd.edu

Bacterial peptidoglycan, the major constituent of the cell wall, is a polymer comprised of repeating units of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). An unusual peptide stem is appended to the NAM unit, which is the site of cross-linking among neighboring peptidoglycan strands in the mature cell wall.[1] In the course of the normal bacterial growth, more than 50% of the parental peptidoglycan is recycled.2,3 The recycling is initiated by the action of lytic transglycosylases, which convert the NAM unit to the 1,6-anhydromuramyl moiety, a process that results in liberation of the novel disaccharide **1** from the polymeric cell wall (Figure 1).[4] The peptide in the cell wall is processed by a number of enzymes, and it is typically comprised of three to five amino acids in the mature cell wall.[5] On liberation of **1**, it is internalized by the membrane protein AmpG. Once in the cytoplasm, compound **1** serves as the substrate for NagZ, which converts it to **2**. Compound **2**, in its various peptide forms, is believed to be an important player both in entry into the ensuing peptidoglycan recycling events and in an induction event that leads to the expression of β-lactamase, a key β-lactam antibiotic resistance enzyme.[6,7]

The tripeptide and tetrapeptide forms of the peptide stem are prevalent in the mature peptidoglycan, because of the action of penicillin-binding proteins (PBPs) and certain bacterial cell wall endopeptidases. Hence, when **1** is internalized and processed by NagZ, the variants **2a** and **2b** predominate. On the other hand, when bacteria are exposed to β-lactam antibiotics, which inhibit PBPs, the result is the predominance of the form **2c**.[3,8] The enzyme AmpD would appear to be central to the fate of compounds **2**. It has been proposed to be a peptidase that removes the peptide stem from the saccharide scaffolding of **2**, initiating the recycling process.[9] The enzyme is specifically implicated in processing of the tripeptide variant **2a**, which makes it a player in the early stages of commitment to recycling.[3,9] Binding of **2a** to the dimer of the gene regulator AmpR is proposed to induce transcription of the gene for β-lactamase, unleashing resistance against β-lactam antibiotics. The notion is that exposure of the bacterium to the antibiotic would influence cell wall fragmentation and the recycling events, which down the line lead to induction of the resistance gene to counter the challenge by the antibiotic.[3,10]

The studies of these biochemical events have been held back by the lack of availability of authentic substrate(s) for AmpD. We describe herein the synthesis of a set of metabolites involved in cell wall recycling. Furthermore, we document that AmpD, as a gatekeeper for many of these biochemical events, is competent to turn over not merely the tripeptidyl analogue **2a** but also the full-length peptide derivative **2c**. Hence, it is also the key catalyst in the reversal of induction of resistance, once the challenge of the antibiotic is no longer present.

Correspondence to: Shahriar Mobashery, mobashery@nd.edu.

Supporting Information **Available:** Experimental procedures of cloning, enzyme purification, kinetics, mass spectrometry, syntheses of the new compounds, and crystallographic information file (CIF). This material is available free of charge via the Internet at http://pubs.acs.org.

We cloned the *ampD* gene from *Citrobacter freundii* (ATCC6879) into the pET24a(+) vector under the T7 promoter by PCR amplification of the chromosomal gene. *Escherichia coli* BL21 (DE3) cells were transformed with the vector for expression of the protein. The protein was purified to homogeneity in three chromatographic steps (see Supporting Information).

As indicated earlier, the major difficulty in studies of AmpD has been the lack of authentic substrate(s) for characterization of the enzyme.[9,11] This has been an obstacle, since the putative substrates are inherently complex[12,13] and they have not been available for characterization of the AmpD reaction. We have addressed this paucity of information in this study. We synthesized compounds **1** (pentapeptide), **2a, 2c, 4a**, and **4b** for characterization of the reaction of AmpD. We prepared **1, 2a**, and **2c** to explore the specificity of AmpD for its proposed 1,6-anhydromuramyl-containing substrate(s). Whereas the cytoplasmic AmpD would not come in contact with species **4a** and **4b**, we prepared these two compounds to explore the selectivity or specificity of AmpD for the intermediates containing the 1,6-anhy-dromuramyl moiety found in **1, 2a**, and **2c**.

The synthesis of compound **1** is reported elsewhere.[14] Compound **6**, an important intermediate in the syntheses of compounds **2a** and **2c**, was prepared from 4-benzyl D-glucal (**5**) (Scheme 1).[14,15] Compound **6** was poised to receive the requisite protected peptide at its carboxylic acid, which then was put through deprotection to furnish the desired target compounds **2a** and **2c**. We also confirmed the structure of 1,6-anhydromuramic acid derivative by determination of the X-ray crystal structure of **11**. The methyl ester of anhydromuramic acid **3** (compound **11**) keeps a typical anhydropyranose structure, where all substituents are in axial positions. The details of synthetic steps leading to compounds **2a** and **2c** are given in the Supporting Information. The approach to synthesis of **4a** and **4b**, and related compounds, has been described earlier.[16]

We subsequently investigated whether **2a** and **2c** would be processed by AmpD. The analysis by LC/MS revealed that AmpD hydrolyzed both compounds at the lactyl amide bond to generate a peptide **12a** (or **12b**) and the corresponding 1,6-anhydromuramyl moiety **3** (Scheme 2). Authentic synthetic samples of the peptides and of the 1,6-anhydromuramyl moiety **3** confirmed the structure assignments for the products of the AmpD reaction (see Supporting Information).

The quantitative analysis by nonlinear regression of the data for either the consumption of the substrate or the formation of the products reveals that compounds **2a** and **2c** were turned over by AmpD with k_{cat}/K_m values of $(6.9 \pm 0.4) \times 10^4$ and $(2.2 \pm 0.3) \times 10^4$ M^{-1} s^{-1}, respectively (Table 1). In essence, the enzyme does not discriminate between the two substrates (a mere difference of 3-fold on k_{cat}/K_m). It is interesting to note that **1** (R = pentapeptide) is also turned over by AmpD, but in terms of k_{cat}/K_m, it is 300-fold worse as a substrate than **2a**. But, more importantly since its K_m is in the millimolar range, it is likely that AmpD would not experience saturation with this compound *in vivo*. This observation, compounded by the fact that the k_{cat} value is also considerably attenuated for this substrate, indicates that it is likely that **1** is not turned over *in vivo*.

A more striking finding is that compounds **4a** and **4b** are not substrates for AmpD. Evolution of the AmpD function as a peptidase has clearly been driven by the atypical structure of the peptide, which includes features such as D-Ala, D-Glu, *meso*-diaminopimelate, and a peptide bond through the side chain of D-Glu. But it also has evolved to recognize the structurally distinct 1,6-anhydromuramyl moiety. This moiety is comprised of the sterically encumbered bicyclo system, with all its substituents in the axial positions, which is in sharp contrast to the muramyl ring found in the peptidoglycan (and in **4a** and **4b**) with its all-equatorial substituents.[17]

In summary, AmpD is capable of turning over 1,6-anhydromuramyl species **2a** and **2c** equally well. The importance of this finding is two-fold. AmpD is the gatekeeper for entry into the peptidoglycan recycling events by its turnover of **2a**. Equally importantly, since it has the ability to turn over **2c**—the resultant predominant species after exposure of bacteria to the β-lactam antibiotics involved in antibiotic resistance gene expression[3,8]—AmpD is the catalyst that reverses the recruitment of bacterial resources in induction of β-lactamase, the antibiotic resistance enzyme. It is likely that **1**, the immediate product of fragmentation of peptidoglycan, does not experience effective turnover *in vivo*, if at all. Furthermore, AmpD has evolved to recognize both the atypical peptidoglycan peptide stem and the 1,6-anhydromuramyl moiety; hence it is a peptidase with a unique function in bacterial physiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Suvorov, M.; Fisher, JF.; Mobashery, S. Bacterial Cell Wall: Morphology and Biochemistry. In: Goldman, E.; Green, LH., editors. Practical Handbook of Microbiology. 2nd. CRC Press; 2008. p. 153-183.
2. de Pedro MA, Donachie WD, Höltje JV, Schwarz H. J Bacteriol 2001;183:4115–4126. [PubMed: 11418550]
3. Park JT, Uehara T. Microbiol Mol Biol Rev 2008;72:211–227. [PubMed: 18535144]
4. Suvorov M, Lee M, Hesek D, Boggess B, Mobashery S. J Am Chem Soc 2008;130:11878–11879. [PubMed: 18700763]
5. Glauner B. Anal Biochem 1988;172:451–464. [PubMed: 3056100]
6. Jaeger T, Mayer C. Cell Mol Life Sci 2008;65:928–939. [PubMed: 18049859]
7. Wiedemann B, Pfeifle D, Wiegand I, Janas E. Drug Resist Updat 1998;1:223–226. [PubMed: 16904404]
8. Uehara T, Park JT. J Bacteriol 2008;190:3914–3922. [PubMed: 18390656]
9. Jacobs C, Joris B, Jamin M, Klarsov K, Vanbeeumen J, Mengin-lecreulx D, Vanheijenoort J, Park JT, Normark S, Frère JM. Mol Microbiol 1995;15:553–559. [PubMed: 7783625]
10. Jacobs C, Frère JM, Normark S. Cell 1997;88:823–832. [PubMed: 9118225]
11. Généreux C, Dehareng D, Devreese B, Van Beeumen J, Frère JM, Joris B. Biochem J 2004;377:111–120. [PubMed: 14507260]
12. Kawasaki A, Karasudani Y, Otsuka Y, Hasegawa M, Inohara N, Fujimoto Y, Fukase K. Chem—Eur J 2008;14:10318–10330.
13. Kubasch N, Schmidt RR. Eur J Org Chem 2002:2710–2726.
14. Hesek D, Lee M, Zhang W, Noll BC, Mobashery S. J Am Chem Soc 2009;131:5187–5193. [PubMed: 19309146]
15. Paulsen H, Himpkamp P, Peters T. Liebigs Ann Chem 1986:664–674.
16. Hesek D, Suvorov M, Morio KI, Lee M, Brown S, Vakulenko SB, Mobashery S. J Org Chem 2004;69:778–784. [PubMed: 14750804]
17. Meroueh SO, Bencze KZ, Hesek D, Lee M, Fisher JF, Stemmler TL, Mobashery S. Proc Natl Acad Sci USA 2006;103:4404–4409. [PubMed: 16537437]

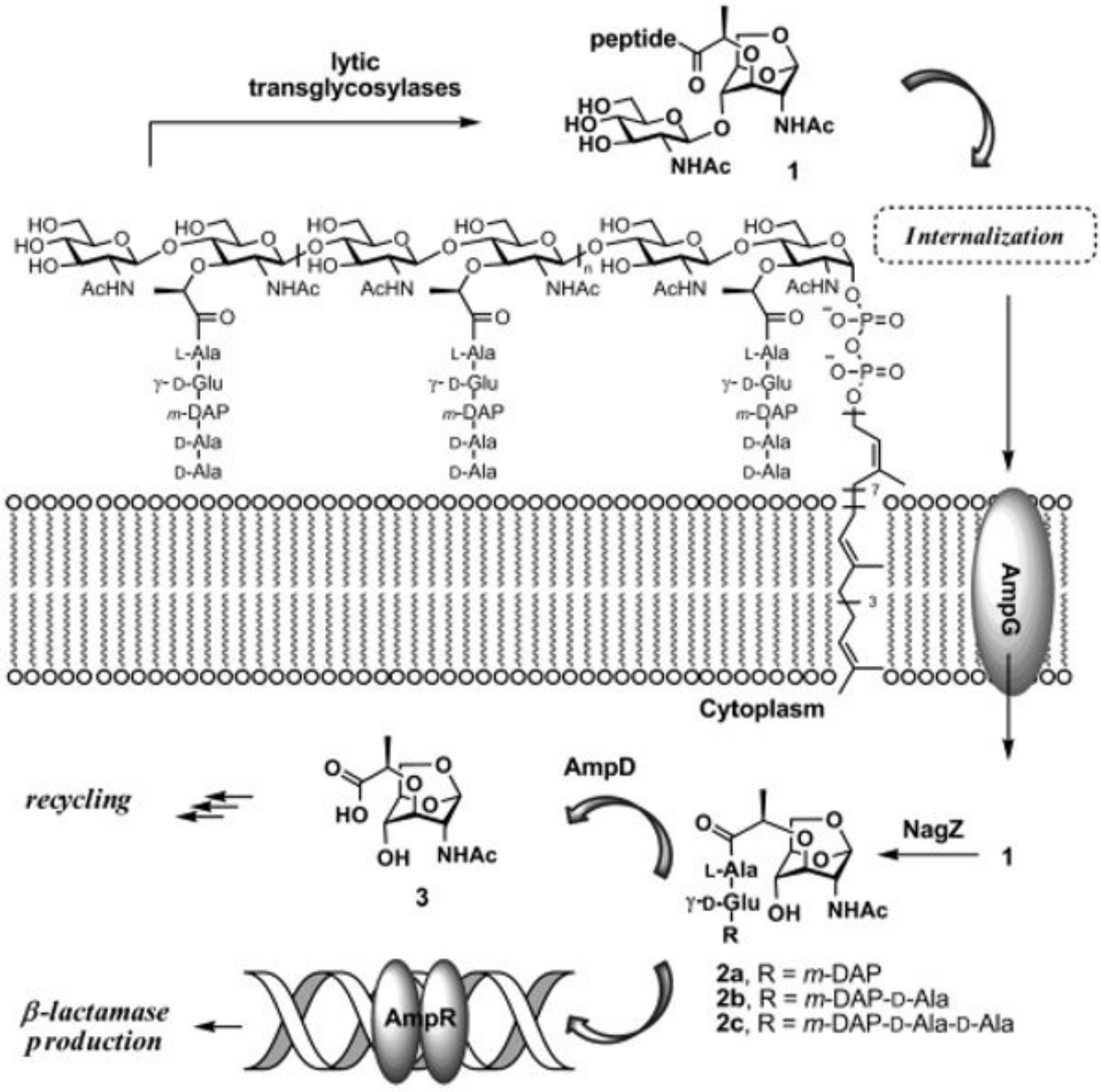
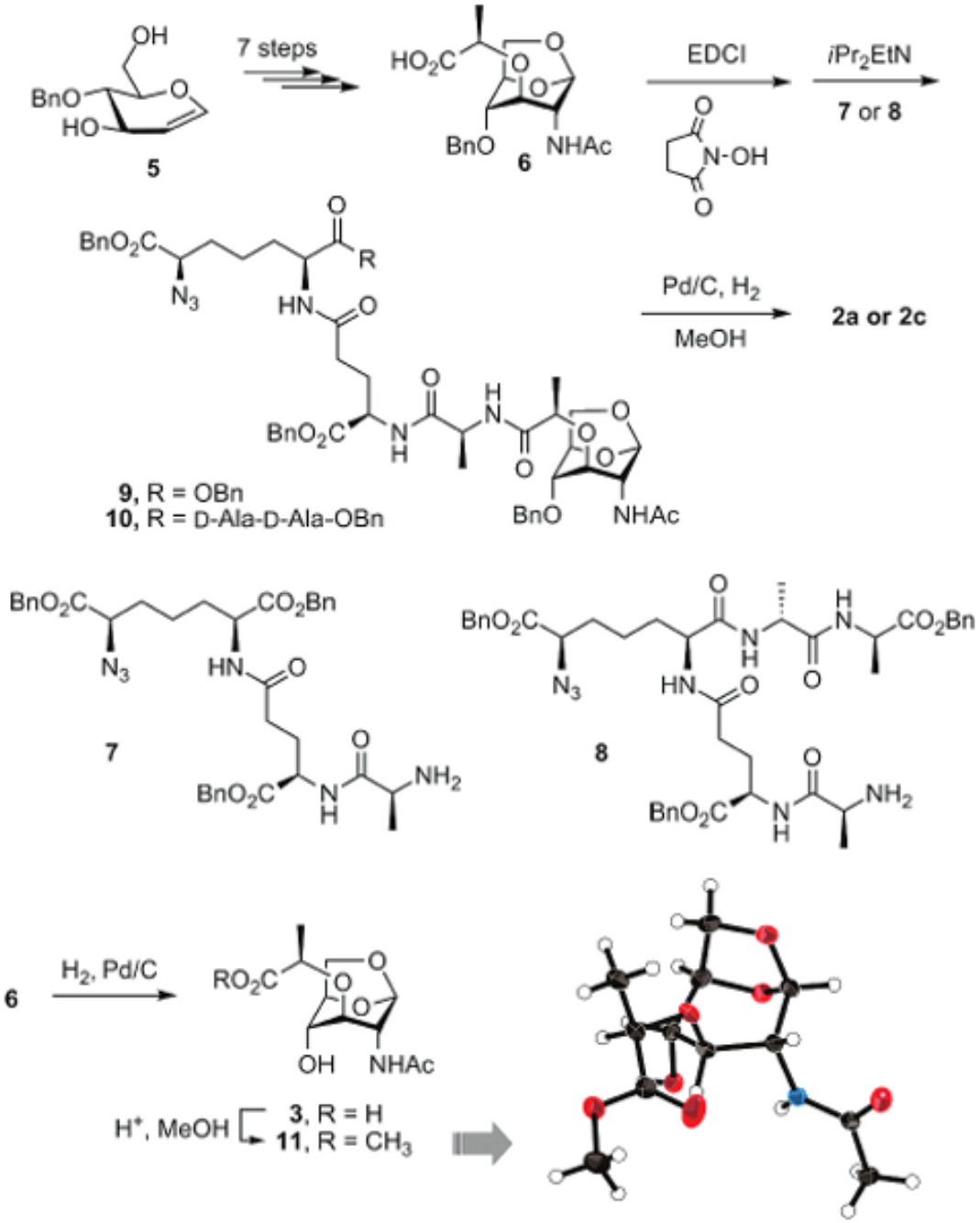
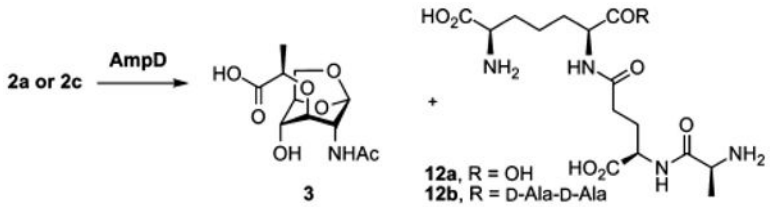


Figure 1.
Peptidoglycan fragmentation leads to the formation of **1**, which is transported across plasma membrane to initiate its recycling and the gene induction for production of β-lactamase.



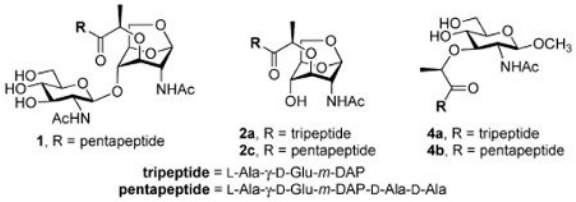
Scheme 1.



Scheme 2.

Table 1

Kinetic Parameters for Turnover of Substrates by AmpD at pH 7.0



	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
1[14]	0.4 ± 0.1	1760 ± 210	$(2.3 \pm 0.1) \times 10^2$
2a	25 ± 2.0	360 ± 10	$(6.9 \pm 0.4) \times 10^4$
2c	11 ± 1	500 ± 60	$(2.2 \pm 0.3) \times 10^4$