Structure-Activity Relationship

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Effect of the Peptide Moiety of Lipid II on Bacterial Transglycosylase**

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The rise of antibiotic-resistant bacteria, such as VRE (vancomycin resistant *Enterococcus*), MRSA (methicillin resistant *Staphylococcus aureus*), MDR-TB, and XDR-TB (multidrug resistant and extensively drug-resistant *tuberculosis*) has stimulated the development of new antibiotics and new strategies to tackle the problem of antibiotic resistance.[1] In bacterial cell-wall biosynthesis, the bifunctional enzyme on the surface, such as *E. coli* PBP1b, possesses two catalytic domains for transpeptidase and transglycosylase activity.[1,2] The transpeptidase responsible for the cross-linking of peptidoglycans has long been a target for antibiotic discovery and development.[1,2] In contrast, no antibiotics, except moenomycin used in animal feeds,[2] have been developed to target the transglycosylase (TGase), which catalyzes the polymerization of Lipid II to form a peptidoglycan (Figure 1).

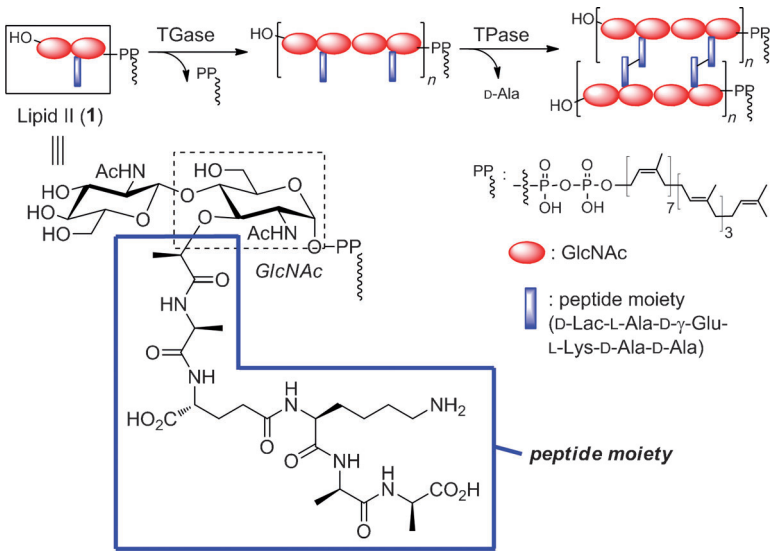

Figure 1. Formula of Lipid II (**1**) for bacterial transglycosylase (TGase) and transpeptidase (TPase).

Because TGase is essential for bacteria and does not have a eukaryotic counterpart, it is thought to be an attractive target for antibiotic discovery and development.[3]

Lipid II (**1**) consists of a disaccharide, a pyrophosphate, an undecaprenol lipid tail, and an oligopeptide (D-lactyl-L-alanyl-γ-D-glutamyl-*meso*-diaminopimelyl (or L-lysyl-D-alanyl-D-alanine) moieties (Figure 1). Notably, the first GlcNAc linked to lactic acid to form a ubiquitous component in cell walls is also called MurNAc (*N*-acetylmuramic acid), which is only found in bacteria.[4] Currently, little information is available regarding the interaction of Lipid II with TGase, presumably because of difficulties in the synthesis and modification of Lipid II (**1**).[5–7]

In this report, we systematically study how the various Lipid II constituents interact with TGases. Although the peptide moiety in Lipid II is for the transpeptidation process,[2,3] the last cross-linking step between neighboring peptidoglycan chains catalyzed by transpeptidase, the function of the peptide moiety in Lipid II towards TGase is still unclear. We have recently reported a method for the synthesis of Lipid II and derivatives,[8] and the X-ray crystal structure of MRSA TGase in complex with a Lipid II analogue to elucidate the mechanism of Lipid II polymerization.[9] Herein, we describe the preparation of Lipid II analogues with varying peptide moieties for evaluation as TGase substrates, as an effort toward development of new antibiotics.

Five analogues **1**–**5** were designed (Figure 2). Compounds **1**–**3** contain a peptide chain (R) prepared by sequential removal of the two amino acids from the end of the peptide stem; **4** has no peptide chain, but a methyl group instead; and **5** was prepared from **1** by adding the fluorophore, 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl (NBD-X), to the ε-NH_2 group of the lysine residue.

Our synthetic strategy for **1**–**3** is depicted in Scheme 1. The lactyl group was installed at the C3 hydroxy group of **6**[8] to obtain **7** in 72% yield. The anomeric thiol group in **7** was removed and the product subjected to phosphorylation to give **8** as a single diastereomer. Deprotection of the C4 OTBS group in **8**, followed by ester hydrolysis gave the acid intermediate, which was individually coupled with peptides and subsequently debenzylated to give **12**. Finally, conjugation of **12** with an undecaprenyl phosphate (C55P), and global deprotection gave the corresponding **1**–**3** in 31–48% yields over two steps.[8] Compound **4** was directly prepared from **6**

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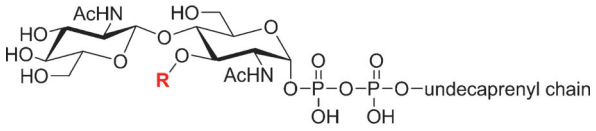
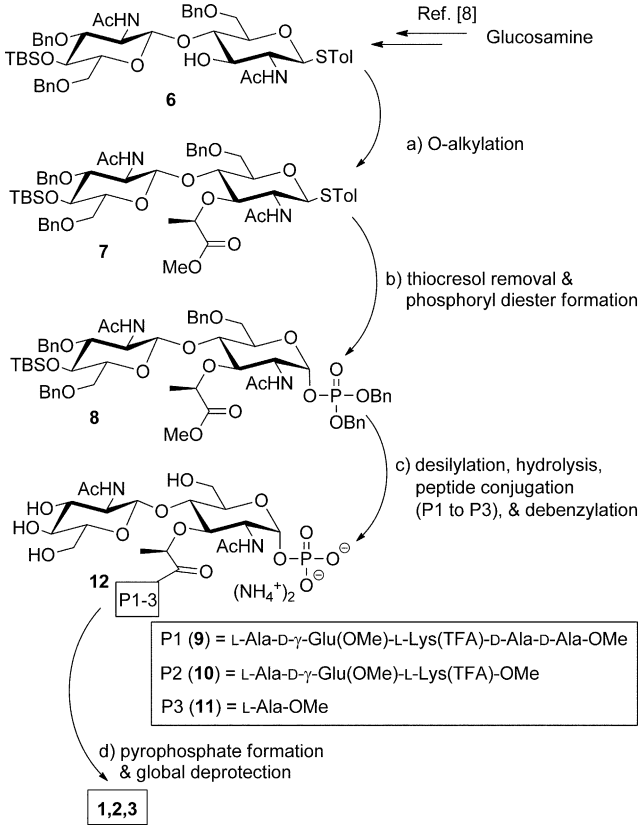


1, R = D-Lac-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala
2, R = D-Lac-L-Ala-D-γ-Glu-L-Lys
3, R = D-Lac-L-Ala
4, R = Me
5, R = D-Lac-L-Ala-D-γ-Glu-L-Lys(ε-NBD-X)-D-Ala-D-Ala

Figure 2. Formulas of Lipid II-based molecules (**1**–**5**).



Scheme 1. General synthetic route of Lipid II analogues (**1**–**3**). Reagents and conditions: a) NaH, (*S*)-methyl lactate triflate, tetrahydrofuran (THF), 0 °C, 2 h, 72%; b) 1. *N*-iodosuccinimide (NIS), acetone/H_2O, 0 °C to RT, 0.5 h; 2. $iPr_2NP(OBn)_2$, 1*H*-tetrazole, CH_2Cl_2, 0 °C to RT, 3 h; 3. *t*BuOOH, −50 °C, 1 h, 73%; c) 1. tetra-*n*-butylammonium fluoride (TBAF), THF, RT, 2 h; 2. LiOH, MeOH/H_2O, 0 °C to RT, 0.5 h; 3. benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), *N*,*N*-diisopropylethylamine (DIEA), **9**–**11**, dimethylformamide (DMF), RT, 0.5 h; 4. H_2, $Pd(OH)_2$, MeOH, RT, 24 h, 41-55%; d) 1. undecaprenyl phosphoroimidazolidate, 1*H*-tetrazole, DMF, 24 h; 2. LiOH, MeOH/H_2O, RT, 1 h, 31–48%. TBS = *tert*-butyldimethylsilyl; Tol = tolyl group.

with MeI by way of O-alkylation, followed by similar transformations in Scheme 1. Compound **5** was prepared from **1** by conjugation with a fluorescent reagent (NBD-X).[5c]

With these valuable Lipid II analogues in hand, our preliminary binding study entailed the use of surface plasmon resonance (SPR) analysis to determine the binding affinity of

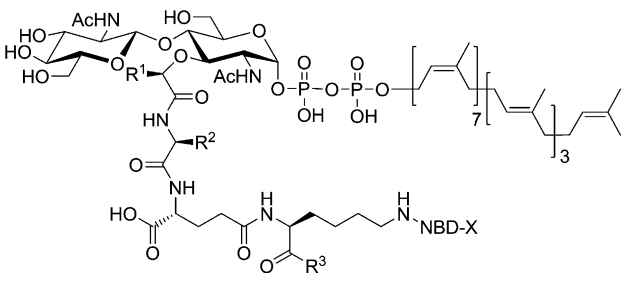
Table 1: Determined dissociation constant (K_D) of **1**–**5** toward TGase.[a]

Compound	K_D [μM]
1	232 ± 46
2	240 ± 37
3	479 ± 62
4	–[b]
5	259 ± 33

[a] Experiments were performed in triplicate at 25 °C, and the K_D values were determined by using steady-state affinity. [b] No binding detected.

1–**5** toward TGase. The steady-state affinity analysis (Table 1) showed that **1** and **2** have similar binding affinity with K_D values of 2.3×10^{-4} M (**1**) and 2.4×10^{-4} M (**2**). Compound **3** ($K_D = 4.8 \times 10^{-4}$ M) showed a twofold decrease in affinity compared to **1**. Surprisingly, no binding signal was observed for **4**, even at a concentration of 2 mM. Compound **5** showed a similar binding affinity to **1**. These findings revealed that 1) the terminal D-Ala-D-Ala moiety is not essential for substrate binding; 2) changing the peptide moiety in Lipid II to a simple methyl group dramatically reduces the binding affinity; and 3) the NBD tag in **5** does not affect the binding affinity with TGase, and therefore this fluorescent probe can be used for mechanistic and inhibition studies.

Next, we determined the substrate specificity of TGase. Lacking an ε-NH_2 residue for conjugation with a fluorophore, **3** and **4** were directly evaluated by thin layer chromatography (TLC) analysis. The results showed that **3** is a weak TGase substrate after 48 hours incubation, but **4** is not a substrate (see Supporting Information). These results demonstrated that the minimum structure requirement for the peptide moiety in Lipid II as a TGase substrate is the D-Lac-L-Ala moiety. The fluorescent Lipid II analogues **5** and **13** (prepared from **1** and **2**, respectively) were evaluated in a quantitative TGase activity assay[10] (Figure 3). As illustrated in Table 2, the kinetic behaviors, such as the K_M and k_{cat} values of **5** and **13**, were rather similar, indicating that the D-Ala-D-Ala moiety in **5** does not provide a significant interaction with TGase during transglycosylation. Because of the similar binding properties of **1** and **2**, as well as the similar kinetic data of the corresponding fluorescent probes **5** and **13**, **13** was chosen as a standard template to further investigate the role of the D-Lac-L-Ala moiety in Lipid II-based analogues.



5 (from **1**), R^1 = Me, R^2 = Me, R^3 = D-Ala-D-Ala
13 (from **2**), R^1 = Me, R^2 = Me, R^3 = OH
14, R^1 = Me, R^2 = H, R^3 = OH
15, R^1 = H, R^2 = Me, R^3 = OH
16, R^1 = H, R^2 = H, R^3 = OH

Figure 3. Lipid II analogues (**5**, **13**–**16**) with an NBD fluorophore for activity evaluation.

Table 2: Kinetic parameters of **5** and **13–16** toward TGase.[a]

Compound	k_{cat} [$\times10^{-2}$ s^{-1}]	K_M [μM]	k_{cat}/K_M [$M^{-1}s^{-1}$]
5	24 ± 7	4.2 ± 1.3	5.7×10^4
13	25 ± 4	4.6 ± 1.2	5.4×10^4
14	8.5 ± 1.3	90.2 ± 8.4	9.4×10^3
15	5.5 ± 1.7	151.8 ± 13.1	3.6×10^3
16[b]	–	–	–

[a] Experiments were performed in 0.085% decyl-PEG, 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 10% dimethylsulfoxide (DMSO), 10% MeOH, and 2 μM TGase at 25 °C and repeated in triplicate. [b] No activity was observed for **16**.

Table 3: Thermodynamic binding parameters for **17–20** toward TGase, determined by ITC.[a]

	K_d [μM]	ΔH [kcal mol^{-1}]	$-T\Delta S$ [kcal mol^{-1}]	ΔG [kcal mol^{-1}]
17	15 ± 2	−4.09 ± 0.03	−2.51 ± 0.40	−6.60 ± 0.065
18	17 ± 2	−3.71 ± 0.2	−2.81 ± 0.14	−6.53 ± 0.057
19	420 ± 59	−0.17 ± 0.004	−4.80 ± 0.064	−4.62 ± 0.068
20[b]	–	–	–	–

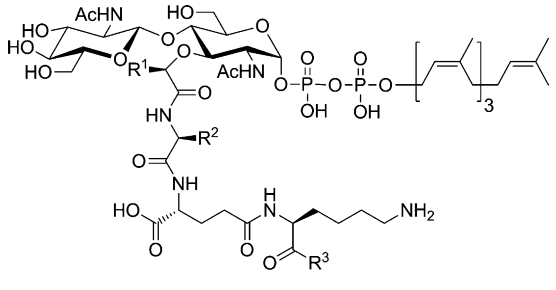
[a] ITC titrations were performed in 1 mM *n*-dodecyl-β-D-maltoside (DDM), 20 mM Tris-HCl (pH 8.0), 30 mM NaCl at 25 °C and repeated in triplicate. [b] No activity was observed for **20** (2 mM).

Several molecules were prepared using a similar synthetic pathway and labeled with the NBD fluorophore at the ε-NH_2 residue to give fluorescent probes **14–16** (Figure 3).

Interestingly, **14** (R^1 = Me, R^2 = H) and **15** (R^1 = H, R^2 = Me) were relatively poor substrates for TGase, with K_M values of 90.2 μM and 151.8 μM, respectively, more than 20-fold weaker than that of **13** (R^1 = Me, R^2 = Me; Table 2). Compound **16** (R^1 = H, R^2 = H) was not a substrate for TGase, even after a longer incubation time (48 hours).

These kinetic results indicated that the first two methyl groups on D-Lac-L-Ala in Lipid II are essential for the substrate activity. Isothermal titration calorimetry (ITC) analysis was then used to investigate quantitatively how these methyl groups affect binding. Our initial attempts using Lipid II (**1**) as a model were unsatisfactory because of an unexpectedly large exothermic enthalpy. Presumably a non-specific hydrophobic interaction with the undecaprenyl lipid chain (C55) contributed to the enthalpy during titration.[11] To resolve this dilemma, a shorter tetraprenyl-chain lipid (C20), was chosen to reduce the interference from the lipid yet retain the substrate activity.[6d] Using a similar synthetic route to that depicted in Scheme 1, compounds **17–20** bearing a C20 tetraprenyl chain were prepared for the ITC experiments (Figure 4).[12]

The titration profiles were readily fitted to a model of one set of binding sites, giving the corresponding thermodynamic parameters as shown in Table 3. C20-Lipid II analogues **17** and **18** had similar enthalpic (ΔH) and entropic (ΔS) energies as well as the binding affinity (K_d). The loss of the Gibbs free energy of binding ($\Delta\Delta G$) was less than 0.1 kcal mol^{-1}, again suggesting that the terminal two amino acids, D-Ala-D-Ala, do not significantly contribute to the interaction between Lipid II and TGase. These results are consistent with our previous SPR and kinetic studies in Tables 1 and 2.



17, R^1 = Me, R^2 = Me, R^3 = D-Ala-D-Ala
18, R^1 = Me, R^2 = Me, R^3 = OH
19, R^1 = Me, R^2 = H, R^3 = D-Ala-D-Ala
20, R^1 = H, R^2 = Me, R^3 = D-Ala-D-Ala

Figure 4. Lipid II analogues (**17–20**) with a tetraprenyl lipid chain.

To our surprise, when L-alanine was converted to glycine in Lipid II-based molecules (**17** versus **19**), the enthalpy of binding (ΔH) was less favorable and the free energy change (ΔG) was from −6.5 kcal mol^{-1} to −4.6 kcal mol^{-1}. Moreover, no obvious binding was observed with **20**, in which D-lactate had been changed to glycolate. From a structural point of view, though only one methyl group was removed (**17** versus **19**), the free energy loss ($\Delta\Delta G$ ca. 2.0 kcal mol^{-1}) was much more than the expected contribution of a methyl group (approximately 0.8 kcal mol^{-1}) according to an estimate of the hydrophobic interaction.[13] Presumably, removal of the side chain (methyl group) not only affects the hydrophobic interaction, but also causes a conformational change of the peptide moiety.

In conclusion, a variety of Lipid II analogues with different peptide moieties have been synthesized and evaluated for binding with TGase. Our results demonstrate that the first two positions of Lipid II, D-lactate and L-alanine, especially the methyl groups, are essential for substrate binding and activity toward TGase. This D-Lac-L-Ala moiety in Lipid II greatly contributes to the interaction with TGase, perhaps enabling a proper conformation for enzyme recognition. The last two amino acids (D-Ala-D-Ala) do not contribute to the interaction between Lipid II and TGase, and the fluorophore tag at the ε-NH_2 group of the lysine residue does not affect the binding affinity. Thus, the structurally simplified **13** is not only a fluorescent substrate for TGases but also a surrogate of Lipid II for the kinetic study and characterization of various TGases. Importantly, the results in this work will provide valuable information for the design of Lipid II-based inhibitors and FRET-based substrates.

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[1] a) D. Kahne, C. Leimkuhler, L. Wei, C. Walsh, *Chem. Rev.* **2005**, *105*, 425–448, and references therein; b) M. A. Fischbach, C. T.

Walsh, *Science* **2009**, *325*, 1089 – 1093; c) D. Yong, M. A. Toleman, C. G. Giske, H. S. Cho, K. Sundman, K. Lee, T. R. Walsh, *Antimicrob. Agents Chemother.* **2009**, *53*, 5046 – 5054; d) E. Marshall, *Science* **2008**, *321*, 364; e) J. Cohen, *Science* **2006**, *313*, 1554; f) S. T. Cole, P. M. Alzari, *Science* **2005**, *307*, 214 – 215.

[2] a) J. Halliday, D. McKeveney, C. Muldoon, P. Rajaratnam, W. Meutermans, *Biochem. Pharmacol.* **2006**, *71*, 957 – 967; b) L. L. Silver, *Curr. Opin. Microbiol.* **2003**, *6*, 431 – 438; c) C. Goffin, J. M. Ghuysen, *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 1079 – 1093; d) C. Walsh, *Nature* **2000**, *406*, 775 – 781; e) P. Welzel, *Angew. Chem.* **2007**, *119*, 4910 – 4914; *Angew. Chem. Int. Ed.* **2007**, *46*, 4825 – 4829.

[3] a) B. de Kruijff, V. van Dam, E. Breukink, *Prostaglandins Leukotrienes Essent. Fatty Acids* **2008**, *79*, 117 – 121; b) T. K. Ritter, C.-H. Wong, *Angew. Chem.* **2001**, *113*, 3616 – 3641; c) T.-J. R. Cheng, M.-T. Sung, H.-Y. Liao, Y.-F. Chang, C.-W. Chen, C.-Y. Huang, L.-Y. Chou, Y.-D. Wu, Y. Chen, Y.-S. E. Cheng, C.-H. Wong, C. Ma, W.-C. Cheng, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 431 – 436; d) H.-W. Shih, K.-T. Chen, S.-K. Chen, C.-Y. Huang, T.-J. R. Cheng, C. Ma, C.-H. Wong, W.-C. Cheng, *Org. Biomol. Chem.* **2010**, *8*, 2586 – 2593; e) T.-J. R. Cheng, Y.-T. Wu, S.-T. Yang, K.-H. Lo, S.-K. Chen, Y.-H. Chen, W.-I. Huang, C.-H. Yuan, C.-W. Guo, L.-Y. Huang, K.-T. Chen, H.-W. Shih, Y.-S. E. Cheng, W.-C. Cheng, C.-H. Wong, *Bioorg. Med. Chem.* **2010**, *18*, 8512 – 8529.

[4] a) E. Schönbrunn, S. Sack, S. Eschenburg, A. Perrakis, F. Krekel, N. Amrhein, E. Mandelkow, *Structure* **1996**, *4*, 1065 – 1075; b) S. Biarrotte-Sorin, A. P. Maillard, J. Delettre, W. Sougakoff, M. Arthur, C. Mayer, *Structure* **2004**, *12*, 257 – 267; c) T. E. Benson, M. S. Harris, G. H. Choi, J. I. Cialdella, J. T. Herberg, J. P. Martin, E. T. Baldwin, *Biochemistry* **2001**, *40*, 2340 – 2350; d) T. E. Benson, C. T. Walsh, J. M. Hogle, *Biochemistry* **1997**, *36*, 806 – 811; e) J. Singh, A. Rivenson, M. Tomita, S. Shimamura, N. Ishibashi, B. S. Reddy, *Carcinogenesis* **1997**, *18*, 833 – 841.

[5] a) A. L. Lovering, L. H. de Castro, D. Lim, N. C. J. Strynadka, *Science* **2007**, *315*, 1402 – 1405; b) Y. Q. Yuan, D. Barrett, Y. Zhang, D. Kahne, P. Sliz, S. Walker, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 5348 – 5353; c) M.-T. Sung, Y.-T. Lai, C.-Y. Huang, L.-Y. Chou, H.-W. Shih, W.-C. Cheng, C.-H. Wong, C. Ma, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 8824 – 8829.

[6] a) X. Y. Ye, M. C. Lo, L. Brunner, D. Walker, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2001**, *123*, 3155 – 3156; b) C. Fraipont, F. Sapunaric, A. Zervosen, G. Auger, B. Devreese, T. Lioux, D. Blanot, D. Mengin-Lecreulx, P. Herdewijn, J. Van Beeumen, J. M. Frere, M. Nguyen-Disteche, *Biochemistry* **2006**, *45*, 4007 – 4013; c) C.-Y. Liu, C.-W. Guo, Y.-F. Chang, J.-T. Wang, H.-W. Shih, Y.-F. Hsu, C.-W. Chen, S.-K. Chen, Y.-C. Wang, T.-J. R. Cheng, C. Ma, C.-H. Wong, J.-M. Fang, W.-C. Cheng, *Org. Lett.* **2010**, *12*, 1608 – 1611; d) D. L. Perlstein, T. S. A. Wang, E. H. Doud, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2010**, *132*, 48 – 49.

[7] a) J. Nakagawa, S. Tamaki, S. Tomioka, M. Matsuhashi, *J. Biol. Chem.* **1984**, *259*, 3937 – 3946; b) S. Mahapatra, T. Yagi, J. T. Belisle, B. J. Espinosa, P. J. Hill, M. R. McNeil, P. J. Brennan, D. C. Crick, *J. Bacteriol.* **2005**, *187*, 2747 – 2757.

[8] H.-W. Shih, K.-T. Chen, T.-J. R. Cheng, C.-H. Wong, W.-C. Cheng, *Org. Lett.* **2011**, *13*, 4600 – 4603.

[9] C.-Y. Huang, H.-W. Shih, L.-Y. Lin, Y.-W. Tien, T.-J. Cheng, W.-C. Cheng, C.-H. Wong, C. Ma, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 6496 – 6501.

[10] a) M. Terrak, M. Nguyen-Disteche, *J. Bacteriol.* **2006**, *188*, 2528 – 2532; b) H. Heaslet, B. Shaw, A. Mistry, A. A. Miller, *J. Struct. Biol.* **2009**, *167*, 129 – 135; c) B. Schwartz, J. A. Markwalder, S. P. Seitz, Y. Wang, R. L. Stein, *Biochemistry* **2002**, *41*, 12552 – 12561; d) D. Barrett, C. Leimkuhler, L. Chen, D. Walker, D. Kahne, S. Walker, *J. Bacteriol.* **2005**, *187*, 2215 – 2217.

[11] M. Fernandez-Vidal, S. H. White, A. S. Ladokhin, *J. Membr. Biol.* **2011**, *239*, 5 – 14.

[12] Compound **17** was also converted to an NBD-linked fluorescent probe **S10**, which was confirmed as a TGase substrate (see Supporting Information).

[13] a) C. N. Pace, B. A. Shirley, M. McNutt, K. Gajiwala, *FASEB J.* **1996**, *10*, 75 – 83; b) C. N. Pace, *Energ. Biol. Macromol.* **1995**, *259*, 538 – 554; c) K. E. Van Holde, W. C. Johnson, P. S. Ho in *Principles of physical biochemistry*, 2nd ed., Pearson/Prentice Hall, Upper Saddle River, NJ, **2006**. pp. 131 – 161, and references therein.

