JOURNAL OF NATURAL PRODUCTS

Note

pubs.acs.org/jnp

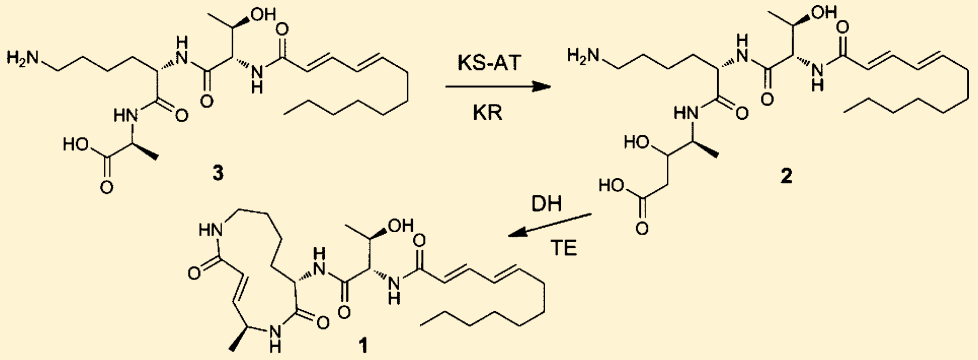
Luminmycins A−C, Cryptic Natural Products from *Photorhabdus luminescens* Identified by Heterologous Expression in *Escherichia coli*

Xiaoying Bian,[†] Alberto Plaza,[†] Youming Zhang,*[,‡] and Rolf Müller*[,†]

[†]Helmholtz Institute for Pharmaceutical Research Saarland, Helmholtz Centre for Infection Research and Department of Pharmaceutical Biotechnology, Saarland University, P.O. Box 15115, 66041 Saarbrücken, Germany

[‡]Gene Bridges GmbH, Building C2 3, Saarland University, 66123 Saarbrücken, Germany

Supporting Information



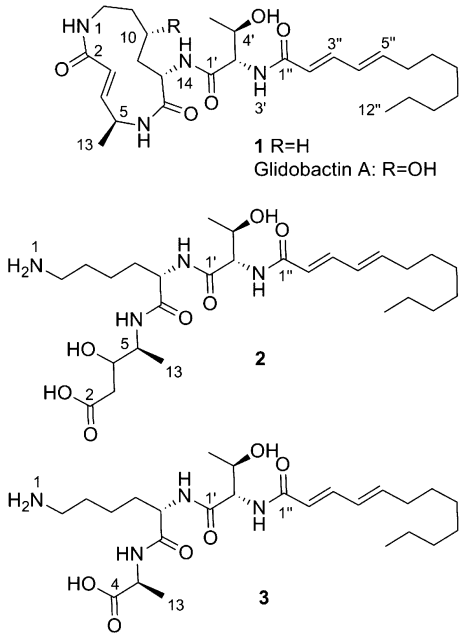
ABSTRACT: The 18 kb "silent" luminmycin biosynthetic pathway from *Photorhabdus luminescens* was cloned into a vector by using the newly established linear−linear homologous recombination and successfully expressed in *Escherichia coli*. Luminmycins A−C (**1**−**3**) were isolated from the heterologous host, and their structures were elucidated using 2D NMR spectroscopy and HRESIMS. Luminmycin A is a deoxy derivative of the previously reported glidobactin A, while luminmycins B and C most likely represent its acyclic biosynthetic intermediates. Compound **1** showed cytotoxicity against the human colon carcinoma HCT-116 cell line with an IC_{50} value of 91.8 nM, while acyclic **2** was inactive at concentrations as high as 100 μg/mL.

The increasing number of bacterial genome sequences have revealed an enormous amount of cryptic or silent secondary metabolite biosynthetic pathways, outnumbering by far the known natural products from these species.[1] Heterologous expression of biosynthetic pathways in a well-characterized host is a rational approach to mine this hidden resource of natural products, especially for slow-growing sources. However, the time-consuming and labor-intense cloning and engineering of large biosynthetic gene clusters restricts the application of this approach.[2] Recently, we reported the full-length RecE and its partner RecT in *E. coli*-mediated linear plus linear homologous recombination, which can circumvent the limitations of cloning large gene clusters without construction and screening of genomic libraries.[3] By using this technique, 10 unknown secondary metabolite biosynthetic gene clusters were directly cloned from the genomic DNA of *Photorhabdus luminescens* subsp. *laumondii* TT01 (DSM15139)[4] into plasmids under inducible promoters. Two of these gene clusters, *plu3263* and *plu1881-plu1877*, were successfully expressed in *E. coli*, and their assigned products were designated as luminmides A/B and luminmycin A (**1**), respectively.[3] It is worth noting that compound **1** was not previously detected in HPLC-MS chromatograms of extracts obtained from *P. luminescens*, demonstrating that the cryptic biosynthetic gene cluster *plu1881*−*plu1877* was activated by heterologous expression.[3] The structure of **1** was proposed on the basis of comparative biosynthetic analysis and HRMS fragmentation with glidobactin A.[3,5,6] Furthermore comparative HPLC/MS analysis between tetracycline-induced and uninduced extracts of *E. coli* strain Nissle 1917[7] containing *plu1881*−*plu1877* indicated the presence of more luminmycin derivatives. However, structure elucidation and purification of these derivatives were hampered by low-yield production in the heterologous host. To improve the yield, tetracycline-inducible promoter P_{tetO} was changed to a constitutive promoter by replacement of tetracycline-inducible repressor gene *tetR* with chloramphenicol-resistant gene cm^R via Red/ET recombination.[8,9]

Luminmycins A−C (**1**−**3**) were absorbed onto Amberlite XAD 16 adsorber resin that had been added to a 12 L batch fermentation of *E. coli* Nissle harboring the luminmycin gene cluster. The products were eluted from the resin with MeOH and further purified by Sephadex LH-20 chromatography and repeated semipreparative RP-HPLC, yielding compounds **1** (3.0 mg), **2** (0.5 mg), and **3** (0.4 mg).

Received: June 25, 2012
Published: August 21, 2012

dx.doi.org/10.1021/np300444e | *J. Nat. Prod.* 2012, 75, 1652−1655



The HRESIMS of **1** showed an ion peak at *m/z* 505.3378 $[M + H]^+$ corresponding to a molecular formula of $C_{27}H_{44}N_4O_5$. Detailed analysis of the 2D NMR data of **1** (see Table S1) in comparison to that of the previous reported glidobactin A[5,6] clearly indicated **1** to be a 10-deoxy derivative of glidobactin A. This result corroborates the proposed structure previously reported.[3] The HRESIMS spectra of **2** and **3** showed major ion peaks at *m/z* 541.3604 $[M + H]^+$ and *m/z* 497.3345 $[M + H]^+$ corresponding to molecular formulas of $C_{27}H_{48}N_4O_7$ and $C_{25}H_{45}N_4O_6$, respectively. These values indicated that the molecular weight of **2** was 36 amu higher than **1**, while **3** was 44 amu lower than **2**. The 2D NMR data for **2** closely resembled those of **1**, differing only by the signals corresponding to the macrocycle ring. In particular **2** showed an additional oxymethine group ($\delta_{C\text{-}4}$ 68.7, $\delta_{H\text{-}4}$ 3.84) and a proton signal at δ 7.63 (2H, m) ascribable to a terminal NH_2 group (Table 1). It was also evident that the olefinic carbons at δ 6.23 and 6.78 in **1** were absent in **2**. HMBC correlations from the methyl protons at δ 1.00 to the carbon resonances at C-4 and from the methylene protons at δ 2.14 and 2.27 to the carbonyl at δ 173.1 established the presence of a 4-amino-3-hydroxypentanoic acid (Ahp) residue (Table 1, Figure 1). Long-range correlations from the methine proton at δ 3.81 and the amide proton at δ 7.55 to the lysine carbonyl at δ 170.8 linked the Ahp residue to the lysine (Figure 1), thus establishing the structure of **2** as a *seco*-hydrated derivative of **1**. The 2D NMR data for **3** closely resembled those of **2** with the exception that resonances belonging to the Ahp were replaced by resonances belonging to an alanine residue (Table S2). The geometries of all of the double bonds in the dodecadienoic acid (Dda) residue of **2** and **3** were established as *E* based on the large $^3J_{H-H}$ values (~15 Hz). Additional evidence confirming the structure of **2** was obtained from ESIMS/MS experiments. Fragmentation of the major ion peak at *m/z* 541 $[M + H]^+$ displays an intense ion at *m/z* 523 $[M + H - H_2O]^+$ and several fragments of small intensity at *m/z* 505 $[M + H - 2H_2O]^+$, 390 $[M + H - H_2O - Ahp]^+$, 346 $[M + H - H_2O - Dda]^+$, 280 $[M + H - Lys - Ahp]^+$, 262 $[M + H - Thr - Dda]^+$, and 244 $[M + H - H_2O - Thr - Dda]^+$. Therefore the MS^2 fragmentation patterns were in total agreement with the structure proposed for **2** by NMR.

Table 1. NMR Spectroscopic Data for Luminmycin B (2)

position	δ_C, type[a]	δ_H (*J* in Hz)[b]	HMBC[c]	ROESY[d]
1-NH		7.63, m	11, 12	11, 12
2	173.1, C			
3	37.9, CH_2	2.14, m	2, 4, 5	4, 5
		2.27, m		
4	68.7, CH	3.84, m	3, 7, 13	3, 13
5	48.0, CH	3.81, m	3, 4, 7,13	3, 13
6-NH		7.55, d (8.5)	5, 7, 13	3, 5, 8, 13
7	170.8, C			
8	51.9, CH	4.26, m	7, 9, 10, 1′	6, 9, 10
9	31.1, CH_2	1.52, m	7, 8, 10, 11	1, 8, 10, 12,14
		1.70, m		
10	21.9, CH_2	1.31, m	8, 9, 11, 12	8, 9, 12,
11	26.3, CH_2	1.51, m	10, 12	1, 8, 10, 12, 14
12	38.4, CH_2	2.74, m	10, 11	1, 10, 11
Me-13	15.7, CH_3	1.00, d (6.8)	4, 5	3, 4, 5, 6
14-NH		7.91, d (8.2)	8, 9, 1′	8, 9, 10, 2′, 4′
1′	169.8, C			
2′	58.2, CH	4.32, dd (4.6, 7.9)	1′, 4′, 5′, 1″	14, 5′
3′-NH		7.95, d (8.1)	2′, 4′, 5′, 1″	4′, 5′, 2″
4′	66.5, CH	3.98, m	1′, 5′	14, 2′, 5′
5′	19.3, CH_3	1.05, d (6.3)	2′, 4′	2′, 3′, 4′
1″	165.5, C			
2″	122.8, CH	6.16, m	1″, 3″, 4″	3′, 3″
3″	139.5, CH	7.00, dd (11.4, 15.1)	1″, 2″, 4″, 5″	4″, 5″
4″	128.4, CH	6.18, m	2″, 3″, 5″, 6″	3″, 6″, 7″
5″	142.0, CH	6.10, m	3″, 4″, 6″, 7″	3″, 6″, 7″
6″	32.1, CH_2	2.13, m	3″, 4″, 7″	5″, 7″
7″	28.2, CH_2	1.38, m	5″, 6″	5″, 6″
8″–9″	28.2, CH_2	1.25, m		
10″	31.1, CH_2	1.24, m	11″, 12″	10″, 11″
11″	21.9, CH_2	1.28, m	10″, 12″	10″, 12″
12″	13.6, CH_3	0.86, t (6.8)	10″, 11″	11″

[a]Recorded at 175 MHz; referenced to residual DMSO-d_6 at δ 39.51 ppm. [b]Recorded at 700 MHz; referenced to residual DMSO-d_6 at δ 2.50 ppm. [c]Proton showing HMBC correlation to indicated carbon. [d]Proton showing ROESY correlation to indicated proton.

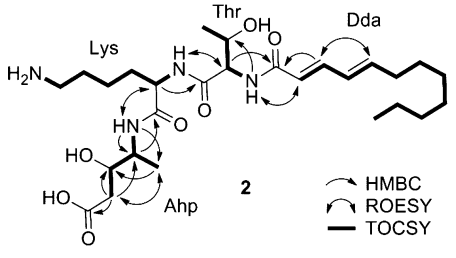


Figure 1. Key correlations of HMBC, ROESY, and TOCSY of luminmycin B (**2**). Lys: lysine; Thr: threonine; Ahp: 4-amino-3-hydroxypentanoic acid; Dda: 2(*E*),4(*E*)-dodecadienoic acid.

The absolute configurations of threonine (Thr), lysine (Lys), and alanine (Ala) were determined as L by MS-detected chromatographic comparison of the derivatives of the acid hydrolysate (6 N HCl, 90 °C, 16 h) of **1** and **3** with L-FDLA (1-fluoro-2,4-dinitrophenyl-5-L-leucinamide)[10,11] and D-FDLA derivatives of amino acid standards (Table S3, Figures S1 and S2). Because these three compounds are biosynthesized by one gene cluster, the absolute configurations of the amino acids in **2**

dx.doi.org/10.1021/np300444e | J. Nat. Prod. 2012, 75, 1652–1655

should be the same as **1** and **3**. Low amounts of **2** prevent the assignment of the configuration at C-4 by NMR or chemical derivatizations. Inspection of Plu1880 indicates that the hydroxy group at C-4 is formed by reduction of a keto group by a ketoreductase (KR) domain. Nevertheless prediction of the configuration at C-4 was hampered by the lack of an LDD motif and a highly conserved typtophan in this KR domain (Figure S3).[12] Compounds **2** and **3** are assumed to be intermediates of the luminmycin assembly line hydrolyzed from the carrier proteins. The possible biosynthetic pathway of **1**−**3** was proposed according to the known luminmycin biosynthesis (Figure S4).[3] Compound **3** is hydrolyzed from the second thiolation (T) domain of Plu1880 directly without the involvement of the last PKS module, leading to the absence of a PKS extender unit (malonyl-CoA). The last PKS module contains functional KR and dehydratase (DH) domains; however it is possible to generate β-hydroxyacyl or β-ketoacyl intermediates by skipping the DH or KR domains.[13] Compound **2** is the hydrolysis product from β-hydroxyacyl-S-T lacking dehydration by DH domain in the last PKS module (Figure S4). Thus, the identification of **2** and **3** supports the previously hypothetical biosynthetic pathway of luminmycin A *in vivo*.

Moreover we evaluated the cytotoxic and antifungal activity of **1** and **2**. Luminmycin A (**1**) is a potent inhibitor of tumor cell proliferation *in vitro*, inhibiting the growth of the tumor cell line HCT-116 with an IC_{50} value of 91.8 nM, which is weaker than that of glidobactin A (IC_{50} 33.3 nM). The acyclic derivative luminmycin B (**2**) was found inactive at concentrations as high as100 μg/mL. Glidobactin A shows antifungal activity against *Candida albicans* with an MIC value of 2 μg/mL, while **1** and **2** show no activity. The loss of cytotoxic and antifungal activity in the open-ring luminmycin B fits with the mechanism of proteasome inhibition, because the α,β-unsaturated carbonyl moiety in the 12-membered-ring system binds covalently to the hydroxy group of the active site amino N-terminal threonine of the proteasome.[14]

In conclusion, we have isolated two new peptides, luminmycins B and C, by heterologous expression of the genes *plu1881*−*plu1877* in *E. coli*. The previously identified luminmycin A was also isolated, and its structure corroborated by NMR. Additionally, luminmycin A shows interesting cytotoxic activity toward a human colon tumor cell line. Thus, expression of a cryptic biosynthetic gene cluster in a heterologous host represents a powerful approach to mine the enormous biosynthetic capabilities of microorganisms.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer polarimeter. UV data were recorded on a NanoDrop 2000C spectrophotometer (Thermo Scientific) using MeOH as solvent. IR data were recorded on a Perkin-Elmer Spectrum 100 FT-IR spectrometer. NMR spectra were recorded on a Bruker Ascend 700 MHz spectrometer equipped with a CryoProbe system. The samples were measured in DMSO-d_6, and the solvent peaks were used as references (δ_C 39.51, δ_H 2.50 ppm). Data acquisition, processing, and spectral analysis were performed with standard Bruker software, TopSpin 3.0. Chemical shifts are given in ppm and coupling constants in Hz. HRESIMS data were recorded on an LTQ-Orbitrap (Thermo Scientific), and molecular formulas were identified by including the isotopic pattern in the calculation (Xcalibur). Analytical RP-HPLC was carried out on an Agilent 1100 HPLC system equipped with a UV diode-array detector and a Bruker Daltonics HCTultra ESI-MS ion trap instrument operating in positive and negative ionization mode at a scan range of m/z = 100−1400, auto MS^n. Chromatographic conditions: Luna RP C_{18} column, 100 × 2 mm, 2.5 μm particle size, and precolumn C_{18}, 8 × 3 mm, 5 μm; solvent gradient (with solvents A [H_2O and 0.1% formic acid] and B [CH_3CN and 0.1% formic acid]) from 5% B at 2 min to 95% B within 20 min, followed by 3 min with 95% B at a flow rate of 0.4 mL/min.

Extraction and Isolation. *E. coli* Nissle strain 1917[7] containing pGB-P_{tetO}-plu1881-plu1877-cm was cultivated in 12 L of M9 medium[15] supplemented with 10 μg/mL chloramphenicol and 2% XAD 16 resin (after 2 days' incubation) at 30 °C for 5 days. The resin was collected by sieving, washed with H_2O twice, and then extracted stepwise with MeOH (5 L). The extract was concentrated under reduced pressure, followed by suspension in MeOH and extraction with *n*-hexane to degrease. The resulting MeOH extract (0.8 g) was fractionated initially on a Sephadex LH-20 column (100 × 2.5 cm) using MeOH as a mobile phase, and 55 fractions were obtained. Fractions containing compounds **1**−**3** were subjected to a semi-preparative reversed-phase HPLC system (Jupiter Proteo C_{12}, 250 × 10 mm, 4 μm, DAD at 254 nm) with isocratic 75% MeOH/H_2O with 0.05% TFA to yield **1** (3.0 mg, t_R = 21.5 min). Compounds **2** (0.5 mg, t_R = 23.9 min) and **3** (0.4 mg, t_R = 27.3 min) eluted with isocratic 60% MeOH.

*Luminmycin A (**1**):* white, amorphous solid; $[\alpha]^{20}_D$ −8.1 (*c* 0.36; MeOH); UV (MeOH) λ_{max} (log ε) 261 nm (4.85); IR (film) ν_{max} 3314, 2925, 1644, 1522, 1213, 999 cm^{-1}; for NMR data see Table S1; HRESIMS m/z 505.3378 $[M + H]^+$ (calcd for $C_{27}H_{45}N_4O_5$, 505.3384).

*Luminmycin B (**2**):* colorless, amorphous solid; $[\alpha]^{20}_D$ −3.4 (*c* 0.06; MeOH); UV (MeOH) λ_{max} (log ε) 261 nm (4.76); IR (film) ν_{max} 3294, 2933, 1671, 1540, 1209, 1140, 1000, 726 cm^{-1}; for NMR data see Table 1; HRESIMS m/z 541.3604 $[M + H]^+$ (calcd for $C_{27}H_{49}N_4O_7$, 541.3596).

*Luminmycin C (**3**):* colorless, amorphous solid; $[\alpha]^{20}_D$ −1.1 (*c* 0.02; MeOH); for NMR data see Table S2; HRESIMS m/z 497.3345 $[M + H]^+$ (calcd for $C_{25}H_{45}N_4O_6$, 497.3334).

Determination of Amino Acid Configurations. Approximately 0.2 mg of **1** and **3** were hydrolyzed with 6 N HCl (0.8 mL) at 90 °C for 16 h. These solutions were evaporated to dryness, and the residue was dissolved in 100 μL of H_2O. To each half-portion (50 μL) were added 1 N $NaHCO_3$ (20 μL) and 1% 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA or D-FDLA in acetone, 100 μL), and the mixture was vortexed and incubated at 37 °C for 60 min. The reaction was quenched by the addition of 2 N HCl (20 μL) and evaporated to dryness. The residues were resuspended in 300 μL of CH_3CN, and about 10 μL of each solution of FDLA derivatives was analyzed by HPLC/MS. The HPLC/MS conditions are the same as that in the general experimental procedure. The retention times of the Marfey-derivatized amino acids are summarized in Table S3 and Figures S1 and S2. Retention times of the FDLA-derivatized authentic standards are L-Thr 13.4 min, D-Thr 14.7 min, L-*allo*-Thr 13.5 min, D-*allo*-Thr 14.4 min, m/z 414.2 $[M + H]^+$; L-Lys (mono-α) 11.5 min, D-Lys (mono-α) 11.2 min, m/z 441.3 $[M + H]^+$; L-Lys (di) 18.7 min, D-Lys (di), 19.2 min, m/z 735.4 $[M + H]^+$; L-Ala 14.6 min, D-Ala 15.5 min, m/z 384.2 $[M + H]^+$.

ASSOCIATED CONTENT

Supporting Information

Supplementary experimental section, NMR spectroscopic data and advanced Marfey's results for **1** and **3**, biosynthetic pathways and copies of 1D and 2D NMR spectra of **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*(Y.Z.) Tel: +49 681 30270236. Fax: +49 681 30270202. E-mail: youming.zhang@genebridges.com. (R.M.) Tel: +49 681

30270201. Fax: +49 681 30270202. E-mail: rom@mx.uni-saarland.de.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

X.B. is indebted to China Scholarship Council (CSC) for a Ph.D. scholarship. We thank J. Herrmann and T. Hoffmann from R.M.'s laboratory for bioactivity tests and high-resolution MS measurements. Research in the laboratory of R.M. was funded by the Deutsche Forschungsgemeinschaft (DFG) and the Bundesministerium für Bildung und Forschung (BMBF). Y.Z. in Gene Bridges was partially funded by the BMBF (MiPro).

■ REFERENCES

(1) Bode, H. B.; Müller, R. *Angew. Chem., Int. Ed.* **2005**, *44*, 6828−6846.
(2) Wenzel, S. C.; Müller, R. *Curr. Opin. Biotechnol.* **2005**, *16*, 594−606.
(3) Fu, J.; Bian, X.; Hu, S.; Wang, H.; Huang, F.; Seibert, P. M.; Plaza, A.; Xia, L.; Muller, R.; Stewart, A. F.; Zhang, Y. *Nat. Biotechnol.* **2012**, *30*, 440−446.
(4) Duchaud, E.; Rusniok, C.; Frangeul, L.; Buchrieser, C.; Givaudan, A.; Taourit, S.; Bocs, S.; Boursaux-Eude, C.; Chandler, M.; Charles, J. F.; Dassa, E.; Derose, R.; Derzelle, S.; Freyssinet, G.; Gaudriault, S.; Medigue, C.; Lanois, A.; Powell, K.; Siguier, P.; Vincent, R.; Wingate, V.; Zouine, M.; Glaser, P.; Boemare, N.; Danchin, A.; Kunst, F. *Nat. Biotechnol.* **2003**, *21*, 1307−1313.
(5) Oka, M.; Nishiyama, Y.; Ohta, S.; Kamei, H.; Konishi, M.; Miyaki, T.; Oki, T.; Kawaguchi, H. *J. Antibiot.* **1988**, *41*, 1331−1337.
(6) Oka, M.; Yaginuma, K.; Numata, K.; Konishi, M.; Oki, T.; Kawaguchi, H. *J. Antibiot.* **1988**, *41*, 1338−1350.
(7) Sonnenborn, U.; Schulze, J. *Microb. Ecol. Health Dis.* **2009**, *21*, 122−158.
(8) Zhang, Y.; Buchholz, F.; Muyrers, J. P.; Stewart, F. A. *Nat. Genet.* **1998**, *20*, 123−128.
(9) Zhang, Y.; Muyrers, J. P. P.; Testa, G.; Stewart, A. F. *Nat. Biotechnol.* **2000**, *18*, 1314−1317.
(10) Fujii, K.; Ikai, Y.; Mayumi, T.; Oka, H.; Suzuki, M.; Harada, K. *Anal. Chem.* **1997**, *69*, 3346−3352.
(11) Fujii, K.; Ikai, Y.; Oka, H.; Suzuki, M.; Harada, K. *Anal. Chem.* **1997**, *69*, 5146−5151.
(12) Keatinge-Clay, A. T. *Chem. Biol.* **2007**, *14*, 898−908.
(13) Fischbach, M. A.; Walsh, C. T. *Chem. Rev.* **2006**, *106*, 3468−3496.
(14) Groll, M.; Schellenberg, B.; Bachmann, A. S.; Archer, C. R.; Huber, R.; Powell, T. K.; Lindow, S.; Kaiser, M.; Dudler, R. *Nature* **2008**, *452*, 755−758.
(15) Sambrook, J.; Russell, D. W. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.