Natural Products

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Antiterminator-Mediated Unveiling of Cryptic Polythioamides in an Anaerobic Bacterium**

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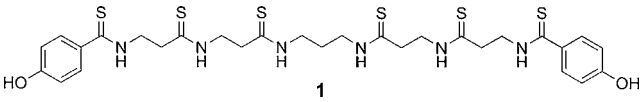
The genus *Clostridium* comprises a highly heterogeneous array of obligate anaerobic organisms that inhabit diverse ecological niches, ranging from soil to human intestines. These microorganisms have been extensively studied, not only because of their ability to produce useful solvents, but also because some species produce harmful protein toxins such as botulinum toxin.[1,2] However, despite the large body of knowledge on clostridia, until recently no secondary metabolites have been isolated from these or any other strict anaerobes. Yet, mining the sequenced genomes of *Clostridium* species has revealed a widespread occurrence of secondary metabolism genes, in particular polyketide synthase and non-ribosomal peptide synthetase genes.[3,4] We have shown that the encoded pathways remain silent under standard laboratory conditions.[4] Obviously, such cryptic biosynthesis genes are only activated in the presence of particular stimuli.[5,6] This is plausible, because secondary metabolite pathways require much ATP-bound energy, a rare commodity in the anaerobic world. We recently discovered that the addition of aqueous soil extract to a *C. cellulolyticum* culture activates an otherwise silent metabolic pathway, leading to a wholly unprecedented type of polythioamide named closthioamide (**1**; Scheme 1).[7] Apart from its exceptional structural features, closthioamide is highly active against a variety of bacteria, such as vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA), and is the first antibiotic from a strictly anaerobic bacterium.[7] However, the variable nature of soil constrains constant production rates and hampers the complicated fermentation of these organisms. As a result, various minor closthioamide congeners evaded isolation and full characterization, and biosynthetic studies were unapproachable. Herein we present a new strategy using an antiterminator gene to trigger a cryptic biosynthetic pathway and disclose the structures and antibacterial activities of seven novel polythioamide congeners. Besides revealing structure–activity relationships, we used synthetic closthioamide analogues as probes to gain an initial insight into the biogenetic relationships of the natural polythioamides.



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Scheme 1. Structure of closthioamide (**1**), the first antibiotic from a strictly anaerobic bacterium, *Clostridium cellulolyticum*.[7]

An established approach for the targeted induction of secondary metabolite biosynthesis is the manipulation of pathway-specific regulatory genes.[5,8–10] However, as the closthioamide biosynthesis gene locus still remains obscure, this approach was out of reach. As an alternative, we focused on regulatory elements that could be involved in a more global activation of secondary metabolism.[6–11] When analyzing the *C. cellulolyticum* genome, we noted the presence of a putative antiterminator gene, *nusG*. N-utilizing factor G (NusG) is an essential protein in *E. coli* that can increase the overall rate of transcription. NusG has the ability to decrease the occupancy of some of the paused RNA-polymerase (RNAP) complexes by promoting the forward translocation of RNAP.[12] NusG also enables RNAP to read through transcription-terminating ρ-dependent sites (Figure 1 A), especially in *rrn* operons, and plays a role in translation owing to the presence of the Kyprides–Onzonis–Woese (KOW) motif it shares with ribosomal protein families.[13] Increasing the processivity of RNA polymerase aids in the efficient synthesis of the corresponding transcripts and produces polycistronic mRNA, which results in elevated protein production. Thus, increasing the *nusG* expression rate could potentially activate secondary metabolism in *C. cellulolyticum*.

To generate a *C. cellulolyticum* mutant overexpressing the antiterminator gene, *nusG* was PCR-amplified from genomic DNA, subcloned and sequenced. It was then cloned downstream of the strong and constitutive promoter P*thl* into the high-copy vector pSOS95,[14] yielding *E. coli–Clostridium* shuttle plasmid pSB050, which was introduced into *C. cellulolyticum*. The successful transformation was verified by PCR and plasmid re-isolation and restriction analysis.[15] To test the ability of pSB050 to promote overexpression of the *nusG* gene, reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) experiments were performed using total RNA isolated from logarithmically growing cultures of

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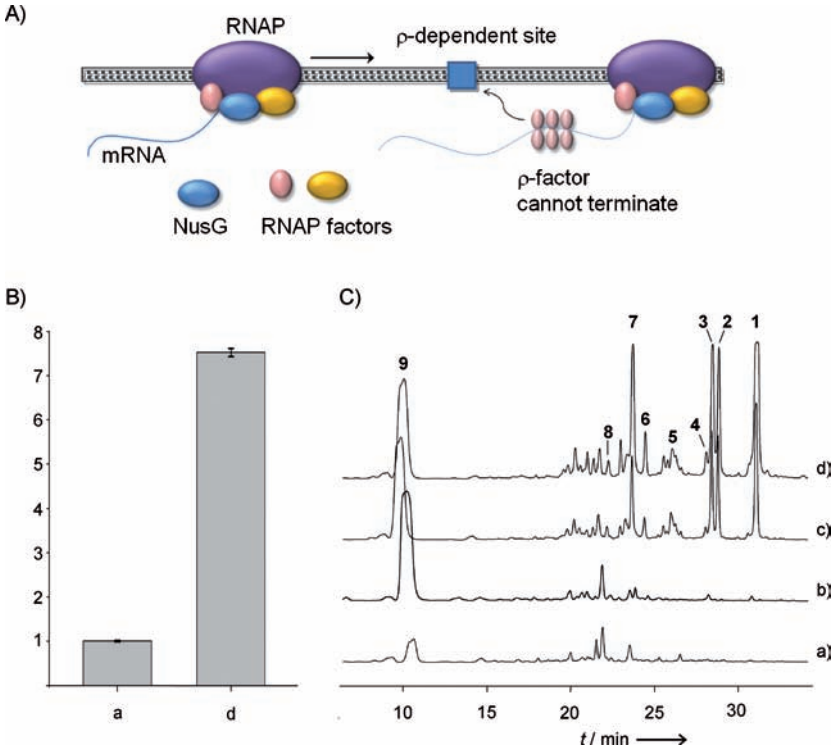


Figure 1. A) Simplified model of NusG antiterminator function. RNAP = RNA polymerase. B) Relative quantity of the mRNA level determined by RT-qPCR of *nusG* in *C. cellulolyticum*/pSB050 (d) vs. wild-type (a). Constitutive expression of 16S rRNA was used for normalization; data of wild-type were set as 1. C) HPLC profiles of extracts from cultures of a) *C. cellulolyticum* wild-type; b) *C. cellulolyticum*/pSOSzero (negative control); c) wild-type induced with soil extract; d) *C. cellulolyticum*/pSB050 overexpressing *nusG*. UV detection at 270 nm.

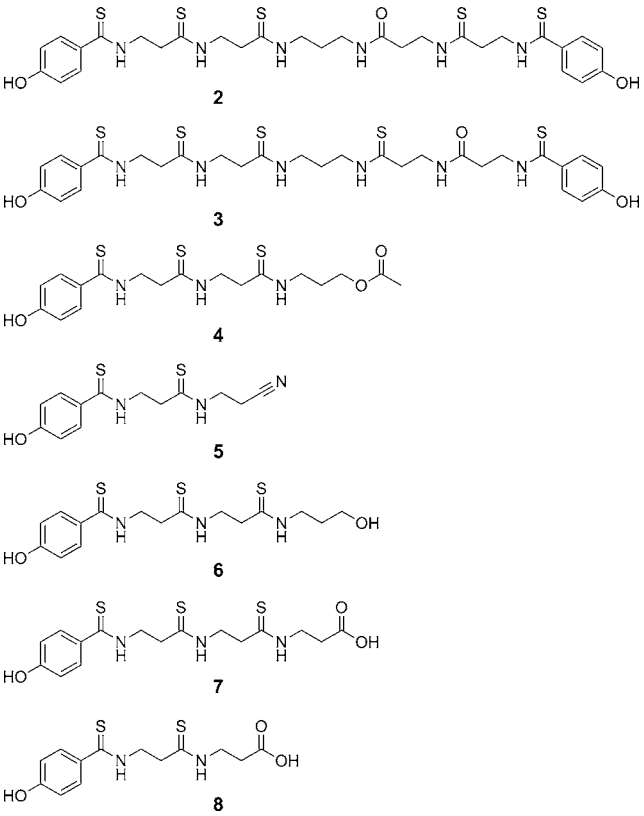
both wild-type and *C. cellulolyticum*/pSB050. One-step RT-qPCRs were performed using a 16S rRNA gene as an internal standard for the calculation of expression levels. We obtained relative expression levels for each mRNA sample using the $2^{-\Delta\Delta Cq}$ method with the wild-type culture as a calibration culture (Figure 1).[16] Indeed, the expression level of *nusG* was substantially higher in the *C. cellulolyticum*/pSB050 strain, in comparison to the wild-type (Figure 1B).

To investigate the effect of *nusG* expression, both the wild-type and the mutant were grown in strictly anaerobic batch cultures in 1 L fermenters with 400 mL of modified CM3 medium[7] and metabolite production was monitored by HPLC-HRMS. Intriguingly, the metabolic profile of the mutant was significantly altered (Figure 1C, trace d). We observed a peak pattern comparable to the wild-type treated with soil extract (Figure 1C, trace c),[7] and HR ESI-MS measurements corroborated the successful production of the antibiotic closthioamide **1** in the mutant. To exclude the possibility of any secondary effects from the plasmid, we constructed a strain containing an empty vector (*C. cellulolyticum*/pSOSzero) by deleting the *Sal*I expression cassette in pSOS95.[17] The absence of any thioamides in the broth of this strain (Figure 1C, trace b) showed that the induction of closthioamide biosynthesis correlates with the overexpression of *nusG*. Notably, whereas a NusG-like antiterminator has been shown to be essential for myxovirescin (TA) biosynthesis,[11] this is the first case where the overexpression of a *nusG* homologue triggers an otherwise silent biosynthetic pathway (Figure 1C, trace a).

In addition to the known closthioamide and ample amounts of hydroxybenzoate (**9**), HPLC-MS and HR-MS analyses of the mutant broth revealed the presence of closthioamide congeners with varying numbers of sulfur atoms. With this constitutive producer strain in hand, we were able to explore the nature of these minor components. The crude extracts of 8 L (10×0.8 L) of the fermentation broth containing the NusG overproducing strain were subjected to open column chromatography followed by a final purification by preparative HPLC to provide closthioamide **1** (18.73 mg) as well as seven novel compounds, **2** (3.58 mg), **3** (3.51 mg), **4** (1.49 mg), **5** (0.85 mg), **6** (1.24 mg), **7** (0.96 mg), and **8** (0.77 mg).

HRMS data provided the molecular formula for **2** and **3** ($C_{29}H_{38}O_3N_6S_5$), indicating that one sulfur atom is replaced by oxygen in these compounds. The ^{1}H and ^{13}C NMR spectra of **2** and **3** are very similar to those of **1** except for the presence of one signal for an amide carbonyl carbon at C11 (δ169.9) in **2** and at C8 (δ173.9) in **3**. The HMBC correlations of H9 (δ3.65), H10 (δ2.41), and H12 (δ3.05) to the carbonyl carbon C11 (δ169.9) in **2** and the correlations of H9 (δ3.57), H7 (δ2.64), and H6 (δ4.00) to C8 (δ173.9) in **3** reveal the positions of the amide functions (Scheme 2). Thus, **2** and **3** represent regioisomeric oxa analogues of closthioamide (Scheme 2).

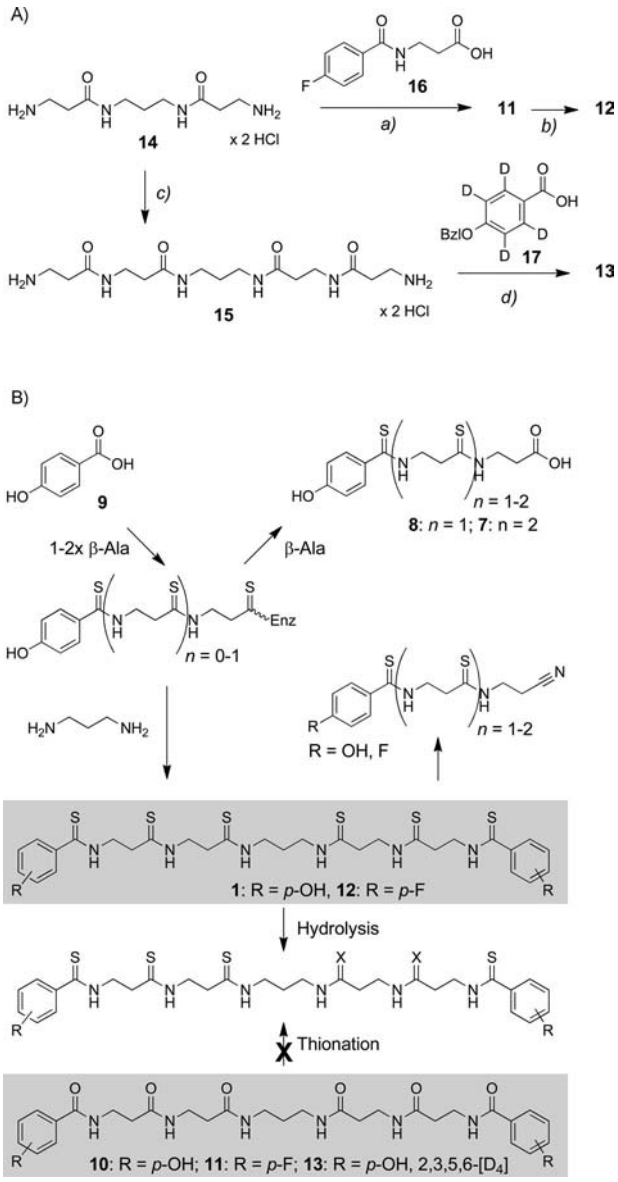
The molecular formulas of **4** ($C_{18}H_{25}N_3O_3S_3$), **6** ($C_{16}H_{23}N_3O_2S_3$), and **7** ($C_{16}H_{21}N_3O_3S_3$) suggested that these metabolites are truncated non-symmetrical congeners. The ^{13}C NMR spectra of **4**, **6**, and **7** are similar to the spectrum obtained for **1**, indicating that these compounds share the same substructures, and 2D NMR spectral analysis (^{1}H–^{1}H COSY, HSQC, and HMBC) indeed revealed the expected *p*-hydroxythiobenzoyl-β-thioalanyl-β-thioalanyl sequence for these three compounds. However, according to ^{1}H and ^{13}C NMR spectra, thioamides **4**, **6**, and **7** feature different residues in place of a diaminopropane unit. In the ^{13}C NMR spectrum of **6**, a signal for a hydroxymethylene carbon at 58.5 ppm (C1) was observed. The ^{1}H–^{1}H COSY (H1 to C3-NH) and HMBC (H3 to C1, C2, and C4) correlations showed that **6** bears a 3-aminopropanol unit. The ^{13}C NMR data for **4** differed from those for **6** in two signals for methyl and carbonyl carbons (δ173.0; C2), and the HMBC correlations of C2 to methyl protons (H1) and hydroxymethylene protons (H3) revealed that **4** is an acetyl ester of **6**. In contrast, compound **7** has a carbonyl carbon (C1) resonating at 175.4 ppm, which is part of a β-Ala moiety, according to ^{1}H–^{1}H COSY and HMBC correlations. HRMS and ^{13}C NMR data of **5** and **8** indicated that these two metabolites feature only two thioamide groups. As for the other polythioamides, *p*-



Scheme 2. Structures of closthioamides B–H (**2**–**8**).

(hydroxy)thiobenzoyl and β-thioalanyl units were deduced from 2D NMR data. Compound **8** proved to be the shorter analogue of **7**, composed of only two β-Ala building blocks. The NMR data for **5** were similar to those for **8**, yet the quaternary carbon (C1) resonating at 119.3 ppm, the HRMS data, and a characteristic band at 2328 cm^{-1} in the IR spectrum revealed that **5** represents a dithioamide variant with a terminal nitrile moiety (Scheme 2).

Considering the scarcity of currently known natural products featuring thioamide groups (5 out of >170000), the prevalence of thioamides produced by this anaerobic bacterium is impressive. The structural kinship of these *Clostridium*-derived thioamides is highly suggestive of a common biosynthetic origin. A particularly intriguing question is whether the oxa analogues **2** and **3** were precursors of the hexathioamide **1** or vice versa. To pinpoint the timing of thionation and to elucidate the course of metabolite formation, we synthesized chemical polyamide and polythioamide probes equipped with fluoro and deuterium labels (**11**–**13**, Scheme 3). In brief, a convergent peptide coupling strategy enabled the efficient assembly of the hexaamide backbone of difluoroclosamide (**11**) from *bis*-amide **14**. In conjunction with the terminal β-alanine moieties, the *p*-fluorobenzamide residues were introduced to give **11** in good overall yield (84%). Thionation of the hexaamide **11** by Lawesson's reagent in pyridine afforded the corresponding difluoroclosthioamide **12** in good yield (65%). For the synthesis of deuterated closamide **13**, a different approach was chosen that allows the late incorporation of the costly [D_4]-*p*-hydroxybenzoic acid. First, bis(amide) **14** was symmetrically



Scheme 3. A) Outline of the synthesis of labeled probes **11**–**13**. B) Summary of labeling experiments and model for the biosynthesis of closthioamides. a) EDC, HOBt, Hünig's Base, DMF, RT, 19.5 h, 86%; b) Lawesson's reagent, pyridine, 100°C, 22 h, 65%; c) 1. Boc-β-Ala-OH, EDC, HOBt, Hünig's Base, DMF, RT, 42 h, 80%; 2. MeOH, dioxane, HCl (3.0 M), RT, sonication, 30 min; directly converted; d) 1. EDC, HOBt, Hünig's Base, DMF, 40°C, 15 h, 91%; 2. H_2, Pd/C (12 mol%), acetic acid, 40–50°C, sonication, 3.5 h, 96%. EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, HOBt = hydroxybenzotriazole.

elongated by two β-alanine units, deprotection then gave tetraamide **15**. Finally, amide coupling was used to introduce the benzyl-protected [D_4]-*p*-hydroxybenzoyl residue, and ultrasound-mediated hydrogenolysis afforded [D_8]-closamide (**13**) in excellent yield (96%).

We then monitored growing cultures of *C. cellulolyticum*/pSB050 by HPLC-HRMS (Orbitrap) with and without supplementation of fluorinated or deuterated probes. When administering [D_8]-closamide, neither single nor multiple thionation could be observed. Conversely, after addition of

fluoro-labeled hexathioamide **12** to the *C. cellulolyticum*/pSB050 culture, we observed the slow exchange of sulfur for oxygen, resulting in the formation of oxa analogues. These results indicate that thionation does not take place after the polyamide backbone has been assembled, but rather concomitant with chain elongation. As to the truncated thioamides, no fluorinated analogues of **4**, **6**, or **7** could be detected. Thus, we could rule out that these compounds are degradation products, but rather result from a diverging pathway. A plausible scenario would be that **7** results from an erratic third elongation of the (thio)amide backbone. The carboxyl of the third β-alanine unit would then undergo reduction and acetylation to yield **6** and **4**. The fusion to an aminopropanol linker in lieu of the diaminopropane unit is likewise conceivable, and it is also possible that the diaminopropane unit could undergo transamination to form the hydroxy and carboxy substituents. The metabolite production curve (see the Supporting Information) suggests that dithioamide **8** is a biosynthetic intermediate that was hydrolyzed instead of being ligated to the central diaminopropane unit of **1**. As traces of fluorinated **8** could be detected, it appears that the acid might also result from hydrolytic cleavage of closthioamide. We also observed the formation of the fluoro analogue of **5**, which indicates that the nitrile moiety derives from transformation of the thioamide group. Such transformations, through a biosynthetic mechanism involving consecutive oxygenations of the thioamide sulfur atom to form the corresponding nitrile and sulfur dioxide, have been reported.[18,19] HRMS data also point to another nitrile, corresponding to the truncated monothioamide (see the Supporting Information). Taken together, our results strongly suggest that closthioamide is assembled stepwise from hydroxybenzoate (**9**), and one, two, or three β-alanine units with concomitant thionation of the intermediates. The final product would be obtained by the fusion of two of these precursors with a diaminopropane linker (Scheme 3B).

Finally, all new compounds were subjected to antibacterial assays.[20] Compared to hexathioamide **1**, which is highly active against pathogens such as MRSA and VRE,[7] the activities of oxa analogues **2** and **3** are significantly less pronounced. This observation is in line with the complete loss of activity of closamide (**10**).[7] We also observed dramatically reduced activities of the truncated closthioamide congeners **4**, **5**, **6**, and **8**, implying that the symmetrical polythioamide backbone is crucial for antibiotic action against *E. coli*, MRSA, and VRE (Supporting Information, Table S3).

In summary, we have successfully employed an antiterminator gene, *nusG*, for the induction of a cryptic biosynthetic pathway. We thus generated a mutant that can be harnessed for the sustainable production of a potent polythioamide antibiotic. This mutant allowed the isolation of seven novel thioamide metabolites (**2**–**8**), which more than doubles the number of currently known natural products featuring this rare functional group. Notably, closthioamides B–H (**2**–**8**) are also the second to eighth known secondary metabolites from a strictly anaerobic bacterium. Furthermore, through synthesis and application of deuterium- and fluoro-labeled probes we have gained an initial insight into the biogenetic relationship of the polythioamides. Biological profiling of the new di- and polythioamides gave valuable information on the structure–activity relationships of this new type of antibiotic and revealed the importance of the symmetric hexathioamide backbone for biological activity. To the best of our knowledge, this is the first report of both the genetic manipulation of an anaerobic microorganism to stimulate secondary metabolism and the use of an antiterminator to induce the production of cryptic natural products. We believe that this approach is generally applicable and of high value for the discovery and sustainable production of new biologically active, yet cryptic, natural products. In particular, this method may represent another avenue to explore the untapped biosynthetic potential of neglected and difficult to handle bacteria, such as strict anaerobes.

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[1] H. Bahl, P. Dürre, *Clostridia: Biotechnology and Medical Applications*, Wiley-VCH, Weinheim, **2001**.
[2] C. J. Paredes, K. V. Alasker, E. T. Papoutsakis, *Nat. Rev. Microbiol.* **2005**, *3*, 969.
[3] H. Seedorf, W. F. Fricke, B. Veith, H. Brüggemann, H. Liesegang, A. Strittmatter, W. Buckel, J. Hinderberger, C. Hagemeier, R. K. Thauer, G. Gottschalk, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 2128.
[4] S. Behnken, C. Hertweck, *PlosOne* **2012**, *7*(1), e29609.
[5] J. M. Winter, S. Behnken, C. Hertweck, *Curr. Opin. Chem. Biol.* **2011**, *15*, 22.
[6] K. Scherlach, C. Hertweck, *Org. Biomol. Chem.* **2009**, *7*, 1753.
[7] T. Lincke, S. Behnken, K. Ishida, M. Roth, C. Hertweck, *Angew. Chem.* **2010**, *122*, 2055; *Angew. Chem. Int. Ed.* **2010**, *49*, 2011.
[8] S. Bergmann, J. Schümann, K. Scherlach, C. Lange, A. A. Brakhage, C. Hertweck, *Nat. Chem. Biol.* **2007**, *3*, 213.
[9] C. Corre, G. L. Challis, *Nat. Prod. Rep.* **2009**, *26*, 977.
[10] K. Ishida, T. Lincke, S. Behnken, C. Hertweck, *J. Am. Chem. Soc.* **2010**, *132*, 13966.
[11] Y. Paitan, E. Orr, E. Z. Ron, E. Rosenberg, *FEMS Microbiol. Lett.* **1999**, *170*, 221.
[12] K. M. Herbert, J. Zhou, R. A. Mooney, A. L. Porta, R. Landick, S. M. Block, *J. Mol. Biol.* **2010**, *399*, 17.
[13] J. Liu, H. Pei, S. Mei, J. Li, L. Zhou, H. Xiang, *Biochem. Biophys. Res. Commun.* **2008**, *371*, 573.
[14] S. B. Tummala, N. E. Welker, E. T. Papoutsakis, *Appl. Environ. Microbiol.* **1999**, *65*, 3793.
[15] C. Tardif, H. Maamar, M. Balfin, J. P. Belaich, *J. Ind. Microbiol. Biotechnol.* **2001**, *27*, 271.
[16] K. J. Livak, T. D. Schmittgen, *Methods* **2001**, *25*, 402.
[17] S. Perret, H. Maamar, J. P. Belaich, C. Tardif, *Mol. Microbiol.* **2004**, *51*, 599.
[18] A. G. Dodge, J. E. Richman, G. Johnson, L. P. Wackett, *Appl. Environ. Microbiol.* **2006**, *72*, 7468.
[19] T. A. Vannelli, A. Dykman, P. R. Ortiz de Montellano, *J. Biol. Chem.* **2002**, *277*, 12824.
[20] F. Kloss, T. Lincke, C. Hertweck, *Eur. J. Org. Chem.* **2011**, 1429–1431.

