ORGANIC LETTERS

2011
Vol. 13, No. 12
2996–2999

Plantazolicin A and B: Structure Elucidation of Ribosomally Synthesized Thiazole/Oxazole Peptides from *Bacillus amyloliquefaciens* FZB42

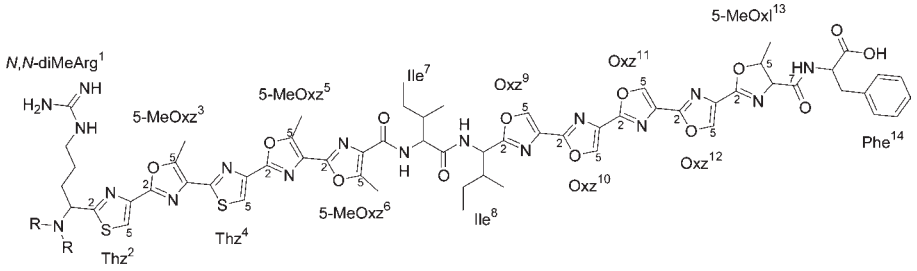
Bahar Kalyon,[†] Soleiman E. Helaly,[†,‡] Romy Scholz,[§] Jonny Nachtigall,[†] Joachim Vater,[†] Rainer Borriss,[§] and Roderich D. Süssmuth*[,†]

Institut für Chemie, Technische Universität Berlin, 10623 Berlin, Germany, Department of Chemistry, Faculty of Science, South Valley University, Aswan 81528, Egypt, and Institute of Biology/Bakteriengenetik, Humboldt Universität zu Berlin, 10115 Berlin, Germany

suessmuth@chem.tu-berlin.de

Received March 28, 2011

ABSTRACT



Plantazolicin A, R=CH_3
Plantazolicin B, R=H

The structures of the ribosomally synthesized peptide antibiotics from *Bacillus amyloliquefaciens* FZB42, plantazolicin A and B, have been elucidated by high resolving ESI-MSMS, 2D $^{1}H-^{13}C$-correlated NMR spectroscopy as well as $^{1}H-^{15}N$-HMQC/$^{1}H-^{15}N$-HMBC NMR experiments. ^{15}N-labeling prior to the experiments facilitated the structure determination, unveiling a hitherto unusual number of thiazoles and oxazoles formed from a linear 14mer precursor peptide. This finding further extends the number of known secondary metabolites from *B. amyloliquefaciens* and represents a new type of secondary metabolites from the genus Bacillus.

The bacterium *Bacillus amyloliquefaciens* FZB42, which is highly related to the Gram-positive model organism *Bacillus subtilis*, promotes plant growth.[1] Following complete sequencing and annotation of its genome,[2] it was found that a considerable part of the genomic DNA of *B. amyloliquefaciens* was dedicated to the biosynthesis of various secondary metabolites. These genes comprise the biosynthesis of nonribosomally synthesized lipocyclodepsipeptides, fengycin,[3] surfactin,[4] and bacillomycin[5] as well as the siderophore bacillibactin.[6] Likewise, based on the genomic data, genes coding for three different polyketide synthase gene clusters could be assigned to the biosynthetic

[†] Technische Universität Berlin.
[‡] South Valley University.
[§] Humboldt Universität zu Berlin.

(1) Idriss, E. E.; Makarewicz, O.; Farouk, A.; Rosner, K.; Greiner, R.; Bochow, H.; Richter, T.; Borriss, R. *Microbiology* **2002**, *148*, 2097–2109.
(2) Chen, X.-H.; Koumoutsi, A.; Scholz, R.; Eisenreich, A.; Schneider, K.; Heinemeyer, I.; Morgenstern, B.; Voss, B.; Hess, W. R.; Reva, O.; Junge, H.; Voigt, B.; Jungblut, P. R.; Vater, J.; Süssmuth, R.; Liesegang, H.; Strittmatter, A.; Gottschalk, G.; Borriss, R. *Nat. Biotechnol.* **2007**, *25*, 1007–1014.
(3) Vanittanakom, N.; Löffler, W.; Koch, U.; Jung, G. *J. Antibiot.* **1986**, *39*, 888–901.
(4) (a) Arima, K.; Kakinuma, A.; Tamura, G. *Biochem. Biophys. Res. Commun.* **1968**, *31*, 488–494. (b) Peypoux, F.; Bonmatin, J. M.; Wallach, J. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 553–563.
(5) Besson, F.; Peypoux, F.; Michel, G.; Delcambe, L. *Eur. J. Biochem./FEBS* **1977**, *77*, 61–67.
(6) Budzikiewicz, H.; Bössenkamp, A.; Taraz, K.; Pandey, A.; Meyer, J.-M. *Z. Naturforsch., C: J. Biosci.* **1997**, *52*, 551–554.

10.1021/ol200809m
Published on Web 05/13/2011

products[7] bacillaene,[8] difficidine,[9] and macrolactin[10] which display marked antibacterial or antifungal activity, respectively. The biosynthesis of the above-mentioned nonribosomally synthesized peptides is essentially dependent on the expression of a 4′-phosphopantheinyltransferase (Sfp).[11] Inactivation of the Sfp from *B. amyloliquefaciens* by mutagenesis led to the discovery of a compound, named plantazolicin, which was biosynthetically assigned to be of ribosomal origin.[12] The corresponding biosynthesis gene cluster (*pzn* cluster) consists of 12 genes, coding for the prepropeptide PznA, and the trimeric PznBCD protein complex (cyclodehydratase (C), dehydrogenase (B) and docking/scaffolding protein (D)) encoding posttranslational modifications. This set is complemented by the methyltransferase PznL and PznE, putatively the corresponding protease, which displays homologies to a Zn-dependent protease. While the amino acid sequence of the prepropeptide was known, extensive posttranslational modifications hampered initial attempts for structure elucidation.

In this contribution we describe the fermentation and isolation of plantazolicin A (**1a** and **1b**), and together with its desmethyl analogue plantazolicin B (**2**), we present the structure elucidation by ESI-MS/MS and 2D NMR experiments. The strain was grown on plates containing a glucose minimal medium (see Supporting Information).[13] Compound **1a** was obtained as a white solid. The molecular ions of m/z 1336.48 $[M + H]^+$ and m/z 668.74 $[M + 2H]^{2+}$ in the ESI-MS spectrum of **1a** revealed the molecular mass of 1335.47 g/mol. The exact molecular mass of **1a** (m/z 1336.47612 $[M + H]^+$) derived from the high-resolution Orbitrap-ESI-MS spectrum (m/z calcd 1336.47804 $[M + H]^+$, Δm −1.027 ppm) gave a molecular formula of $C_{63}H_{69}O_{13}N_{17}S_2$. In accordance with the information of the prepropeptide PznA, extensive posttranslational modification of the precursor peptide H_2N-RCTCTTIISSSSTF-OH, i.e. dehydratation (−180 Da = 10 × H_2O), dehydrogenation (−18 Da = 9 × H_2), and methylations (+28 Da = 2 × CH_2) had to be assumed.

1D and 2D NMR data of **1a** (including ^{1}H NMR, ^{1}H−^{1}H-COSY, ^{1}H−^{1}H-TOCSY, ^{1}H−^{13}C-HSQC, ^{1}H−^{13}C-HMBC, see Supporting Information, Figures S3−S7) turned out to be inconclusive, because of the reduced number of protons and the highly repetitive occurrence of structurally similar heterocyclic systems. Therefore, in order to aquire additional NMR-spectral information we decided to perform ^{15}N-labeling of the plantazolicin peptide by feeding ^{15}N-labeled ammonium sulfate under the above-mentioned media conditions.[14] The isolation yielded ^{15}N-labeled plantazolicin (**1b**) displaying the expected mass shift of 17 amu (1353.42566 $[M + H]^+$, Δm −0.891 ppm; $C_{63}H_{69}O_{13}{}^{15}N_{17}S_2$) compared to unlabeled peptide **1a**. Subsequently, ^{1}H−^{15}N-HMQC and ^{1}H−^{15}N-HMBC NMR spectra of **1b** were recorded (see Supporting Information, Figures S8−S13), and combined with 2D ^{1}H−^{13}C NMR data, they revealed the presence of the following substructures: an α-*N*,α-*N*-dimethylargininyl residue (*N*,*N*-diMeArg), two thiazole rings (Thz), three 5-methyloxazole rings (MeOxz), two isoleucines (Ile), four oxazole rings (Oxz), one 5-methyloxazolidine ring (MeOxl), and one phenylalanine (Phe).

The characteristic spin system of the arginyl residue *N*,*N*-diMeArg[1] could be easily identified. Interestingly, the α-amino group (δ_N 30.7) was methylated twice (δ_H 2.26, 6H). ^{1}H−^{13}C-HMBC correlations from these geminal methyl groups of *N*,*N*-diMeArg[1] to C-2 (δ_C 172.6) of Thz[2] as well as ^{1}H−^{15}N-HMBC correlations from these methyl groups to N-3 (δ_N 312.1) of Thz[2] established the *N*-terminal end of **1a** and **1b**. In the course of the subsequent structure elucidation, correlations of the ^{1}H−^{15}N-HMBC experiment turned out to be crucial for the establishment of the sequence of heterocycles and their placement in the peptide. Hence, an HMBC correlation from H-5 of Thz[2] at δ_H 8.41 to N-3 of MeOxz[3] at δ_N 250.0 connected Thz[2] with MeOxz[3]. Furthermore, the ^{1}H−^{15}N-HMBC correlation from the methyl group 5-CH_3 (δ_H 2.83) of MeOxz[3] to N-3 of Thz[4] at δ_N 303.7 confirmed the connectivity between MeOxz[3] and Thz[4]. The methyl group 5-CH_3 of MeOxz[5] (δ_H 2.75) showed an HMBC correlation with N-3 of MeOxz[5] (δ_N 249.5) and N-3 of MeOxz[6] (δ_N 246.9). The methyl group 5-CH_3 (δ_H 2.64) of MeOxz[6] showed a correlation to N-3 of the same ring. Connectivities linking Thz[4] with MeOxz[5] could not be established. However, the above data, together with the biosynthetic logic deduced from the precursor peptide, could establish the mainly heteroaromatic sequence *N*,*N*-diMeArg[1]-Thz[2]-MeOxz[3]-Thz[4]-MeOxz[5]-MeOxz[6].

The amino acid sequence of the precursor peptide of plantazolicin A (H_2N-RCTCTTIISSSSTF-OH) features two Ile residues located *C*-terminally of MeOxz[6] and *N*-terminally of Oxz[9] in plantazolicin A. HMBC correlations from the α-proton of Ile[7] at δ_H 4.48 to the amide nitrogen of Ile[7] (δ_N 113.9) and to the amide nitrogen of Ile[8]

(7) (a) Chen, X.-H.; Vater, J.; Piel, J.; Franke, P.; Scholz, R.; Schneider, K.; Koumoutsi, A.; Hitzeroth, G.; Grammel, N.; Strittmatter, A. W.; Gottschalk, G.; Süssmuth, R. D.; Borriss, R. *J. Bacteriol.* **2006**, *188*, 4024–4036. (b) Schneider, K.; Chen, X.-H.; Vater, J.; Franke, P.; Nicholson, G.; Borriss, R.; Süssmuth, R. D. *J. Nat. Prod.* **2007**, *70*, 1417–1423. (c) Chen, X. H.; Koumoutsi, A.; Scholz, R.; Schneider, K.; Vater, J.; Süssmuth, R.; Piel, J.; Borriss, R. *J. Biotechnol.* **2009**, *140*, 27–37.

(8) (a) Butcher, R. A.; Schroeder, F. C.; Fischbach, M. A.; Straight, P. D.; Kolter, R.; Walsh, C. T.; Clardy, J. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 1506–1509. (b) Moldenhauer, J.; Götz, D.; Albert, C.; Bischof, S.; Schneider, K.; Süssmuth, R. D.; Engeser, M.; Gross, H.; Bringmann, G.; Piel, J. *Angew. Chem., Int. Ed.* **2010**, *49*, 1465–1467.

(9) (a) Wilson, K. E.; Flor, J. E.; Schwartz, R. E.; Joshua, H.; Smith, J. L.; Pelak, B. A.; Liesch, J. M.; Hensens, O. D. *J. Antibiot.* **1987**, *40*, 1682–1691. (b) Zimmerman, S. B.; Schwartz, C. D.; Monaghan, R. L.; Pelak, B. A.; Weissberger, B.; Gilfillan, E. C.; Mochales, S.; Hernandez, S.; Currie, S. A.; Tejera, E. *J. Antibiot.* **1987**, *40*, 1677–1681.

(10) (a) Gustafson, K.; Roman, M.; Fenical, W. *J. Am. Chem. Soc.* **1989**, *111*, 7519–7524. (b) Nagao, T.; Adachi, K.; Sakai, M.; Nishijima, M.; Sano, H. *J. Antibiot.* **2001**, *54*, 333–339.

(11) Koumoutsi, A.; Chen, X. H.; Henne, A.; Liesegang, H.; Hitzeroth, G.; Franke, P.; Vater, J.; Borriss, R. *J. Bacteriol.* **2004**, *186*, 1084–1096.

(12) Scholz, R.; Molohon, K. J.; Nachtigall, J.; Vater, J.; Markley, A. L.; Süssmuth, R. D.; Mitchell, D. A.; Borriss, R. *J. Bacteriol.* **2011**, *193*, 215–224.

(13) Spizizen, J. *Proc. Natl. Acad. Sci. U.S.A.* **1958**, *44*, 1072–1078.

(14) (a) Chan, J. K.; Moore, R. N.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc.* **1983**, *105*, 3334–3336. (b) Noguchi, H.; Harrison, P. H.; Arai, K.; Nakashima, T. T.; Trimble, L. A.; Vederas, J. C. *J. Am. Chem. Soc.* **1988**, *110*, 2938–2945.

(δ_N 118.6) as well as correlations from the α-proton (δ_H 4.93) of Ile^8 to the amide nitrogen of Ile^8 and N-3 Oxz^9 (δ_N 248.5) prove the sequence MeOxz^6-Ile^7-Ile^8-Oxz^9. Even though no NMR connectivities have been found to link MeOxz^6 with Ile^7, this assignment was further corroborated by ESI-Orbitrap-MSMS spectra of **1a** and **1b** (Figure 2). These show a characteristic fragment peak at m/z 679.25769, $C_{31}H_{39}O_4N_{10}S_2^+$, Δm −2.175 ppm (^{15}N-labeled **1a**, at m/z 689.22854, $C_{31}H_{39}O_4{}^{15}N_{10}S_2^+$, Δm −1.417 ppm) assigned to the N-terminal part of the structure: *N*,*N*-diMeArg^1-Thz^2-MeOxz^3-Thz^4-MeOxz^5-MeOxz^6-Ile^7.

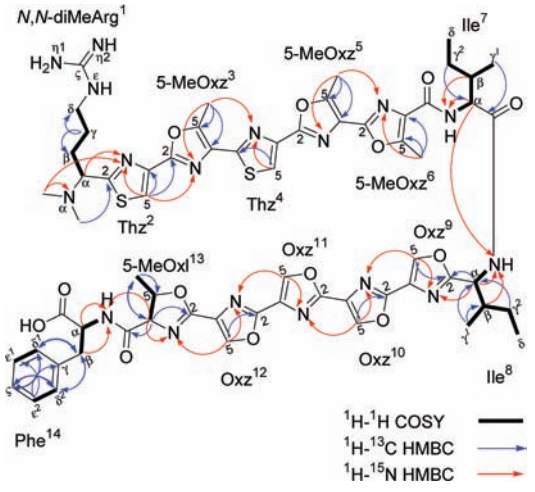


Figure 1. Structures of plantazolicin A (**1a**) and ^{15}N-labeled plantazolicin A (**1b**) and 2D NMR correlations.

A further sequence was established as follows: ^{1}H–^{15}N-HMBC correlations were assigned from H-5 of Oxz^9 at δ_H 8.96 to N-3 of Oxz^{10} at δ_N 246.8, as well as from H-5 Oxz^{10} at δ_H 9.08 to N-3 of Oxz^{10} and N-3 of Oxz^{11} (δ_N 247.2). Furthermore, HMBC correlations from H-5 of Oxz^{11} at δ_H 9.11 to N-3 of Oxz^{11} (δ_N 247.2) and N-3 of Oxz^{12} (δ_N 250.1) revealed a stretch of four oxazoles from Oxz^9 to Oxz^{12}. Additionally, HMBC correlations from H-5 of Oxz^{12} at δ_H 8.81 to N-3 of MeOxl^{13} at δ_N 222.1 established the connectivity of this series of aromatic heterocycles to oxazolidine MeOxl^{13}. Finally, HMBC correlations from H-4 (δ_H 4.23) of MeOxl^{13} to the amide nitrogen of Phe^{14} at δ_N 122.0 revealed the connectivity between MeOxl^{13} and the *C*-terminal Phe^{14}. Accordingly, a characteristic fragment peak at m/z 630.23022, $C_{31}H_{32}O_8N_7^+$, Δm −0.742 ppm (^{15}N-labeled, at m/z 637.20820, $C_{31}H_{32}O_8{}^{15}N_7^+$, Δm −2.718 ppm) from the Orbitrap-MS-MS spectra of **1a** and **1b** (Figure 2) were assigned to the *C*-terminal part of the structure (Ile^8-Oxz^9-Oxz^{10}-Oxz^{11}-Oxz^{12}-MeOxl^{13}-Phe^{14}) (Figure 1). The full structure of plantazolicin **A** is depicted in Figure 1.

HPLC-ESI-Orbitrap-MS of the methyltransferase knockout mutant RS33[12] revealed the mass signal of the desmethyl derivative plantazolicin B (**2**), at m/z 1308.43863 $[M + H]^+$ (m/z calcd 1308.44674 $[M + H]^+$, Δm −5.780 ppm, $C_{61}H_{65}O_{13}N_{17}S_2$) corresponding to a loss of two methyl groups (Figure 2). The assignment of MS/MS spectra in comparison with those of **1a** and **1b** independently confirmed the placement of the methyl groups at the *N*-terminus (see Supporting Information, Figures S1, S2).

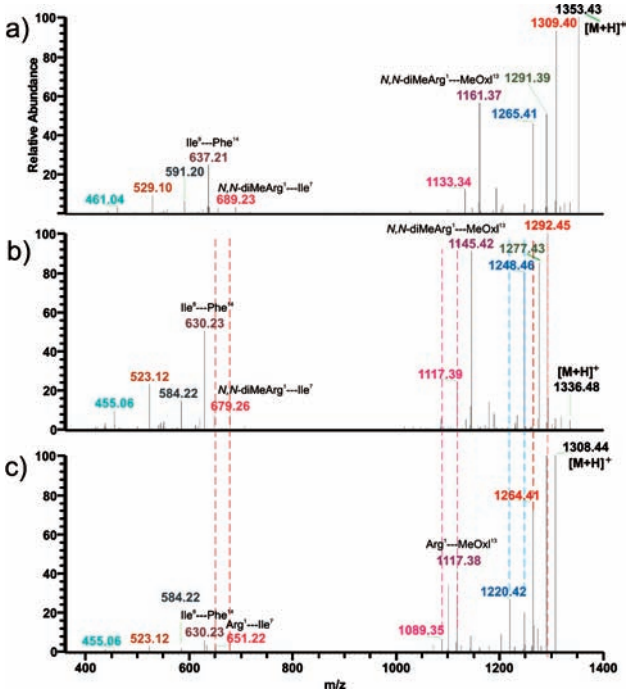


Figure 2. Orbitrap-ESI-MS-MS spectra of (a) ^{15}N-labeled plantazolicin A (**1b**) with Δm = +17 amu compared to (b) plantazolicin A (**1a**) and (c) the desmethyl plantazolicin B (**2**). Dashed lines symbolize Δm = 28 amu between **1a** and **2**.

Plantazolicin A (**1a** and **1b**) which shows antibacterial activity against closely related Gram-positive bacteria[12] and its desmethyl analogue plantazolicin B (**2**) represent an unusual type of thioazole/oxazole-containing peptide antibiotics. From a structural perspective, many other ribosomally synthesized thiazole/oxazole-containing peptides, e.g. microcin B17 synthesized by *E. coli*,[15] thiopeptides

(15) (a) Bayer, A.; Freund, S.; Nicholson, C. N.; Jung, G. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1336–1339. (b) Bayer, A.; Freund, S.; Jung, G. *Eur. J. Biochem.* **1995**, *234*, 414–426.

(16) (a) Morris, R. P.; Leeds, J. A.; Naegeli, H. U.; Oberer, L.; Memmert, K.; Weber, E.; LaMarche, M. J.; Parker, C. N.; Burrer, N.; Esterow, S.; Hein, A. E.; Schmitt, E. K.; Krastel, P. *J. Am. Chem. Soc.* **2009**, *131*, 5946–5955. (b) Wieland Brown, L. C; Acker, M. G.; Clardy, J.; Walsh, C. T.; Fischbach, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 2549–2553. (c) Liao, R.; Duan, L.; Lei, C.; Pan, H.; Ding, Y.; Zhang, Q.; Chen, D.; Shen, B.; Yu, Y.; Liu, W. *Chem. Biol.* **2009**, *16*, 141–147. (d) Kelly, W. L.; Pan, L.; Li, C. *J. Am. Chem. Soc.* **2009**, *131*, 4327–4334.

(17) Schmidt, E. W.; Nelson, J. T.; Rasko, D. A.; Sudek, S.; Eisen, J. A.; Haygood, M. G.; Ravel, J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7315–7320.

(18) Shin-ya, K.; Wierzba, K.; Matsuo, K.; Ohtani, T.; Yamada, Y.; Furihata, K.; Hayakawa, Y.; Seto, H. *J. Am. Chem. Soc.* **2001**, *123*, 1262–1263.

(19) Stein, T. *Mol. Microbiol.* **2005**, *56*, 845–857.

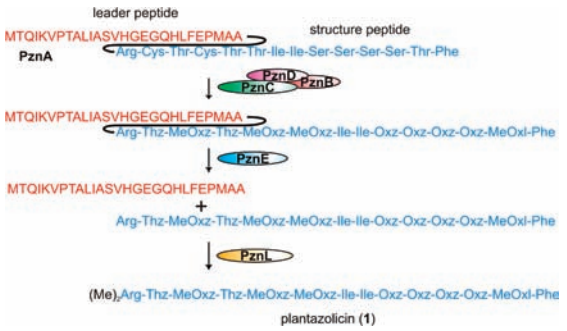


Figure 3. Biosynthetic model of plantazolicin biosynthesis. The prepropeptide PznA is processed by the putative protein complex PznC/B/D; after subsequent proteolysis by PznE, the methytransferase PznL performs *N*,*N*-dimethylation of the *N*-terminus. Proteins for export, for immunity, and with regulatory function have not been considered. Oxz = oxazole, Thz = thiazole, MeOxz = 5-methyloxazole, MeOxl = 5-methyloxazolidine.

of the micrococcin-type,[16] and the patellamides from cyanobacteria exist.[17] A unique structural feature of plantazolicin A is the adjacency of two pentaheterocyclic moieties that mainly confer a planar structure to the peptide and are reminiscent of telomerase inhibitor telomestatin.[18]

The herein determined structure of plantazolicin A also has implications for the biosynthetic assembly by post-translationally acting proteins of the *pzn* gene cluster. The leader peptide of prepropeptide PznA likely serves as a recognition motif by the posttranslational biosynthesis machinery, represented by the PznC/B/D complex[12] assigned to perform cyclodehydration and dehydrogenation reactions. Remarkably, the heterocycle MeOxl[13], nearest to the *C*-terminus, is not oxidized and remains in the oxazolidine form. We hypothesize that the *N*,*N*-dimethylation by methyltransferase PznL must occur subsequently to proteolytic processing by the protease PznE (Figure 3). Future experiments will have to be performed in order to assign enzyme functions on a biosynthetic level. In summary, the structure of plantazolicin represents a milestone in the assignment and structure elucidation of unknown metabolites from *B. amyloliquefaciens* FZB42. Simultaneously, plantazolicin extends the collection of metabolites known from bacteria of the genus Bacillus.[19]

Acknowledgment. Dr. Peter Schmieder (FMP Leibnitz Institut for Molecular Pharmacology, Berlin) is kindly acknowledged for performing the ^{15}N NMR experiments. S.E.H. would like to thank the Egyptian government for a scholarship at TU Berlin. The project was supported by a grant from the Cluster of Excellence "Unifying Concepts in Catalysis" coordinated by the Technische Universität Berlin and the German Ministry of Education and Research BMBF (GenomikPlus No. 0313805).

Supporting Information Available. The experimental section contains data on fermentation, mass spectrometry and NMR spectroscopy. This material is available free of charge via the Internet at http://pubs.acs.org.