Generation of Novel Landomycins M and O through Targeted Gene Disruption

Andriy Luzhetskyy,[a] Lili Zhu,[b] Miranda Gibson,[b] Marta Fedoryshyn,[a] Clemens Dürr,[a] Carsten Hofmann,[a] Dirk Hoffmeister,[a] Bohdan Ostash,[c] Cynthia Mattingly,[d] Val Adams,[d] Victor Fedorenko,[c] Jürgen Rohr,[b] and Andreas Bechthold*[a]

Two genes from Streptomyces cyanogenous *S136 that encode the reductase LanZ4 and the hydroxylase LanZ5, which are involved in landomycin A biosynthesis, were characterized by targeted gene inactivation. Analyses of the corresponding mutants as well as complementation experiments have allowed us to show that LanZ4 and LanZ5 are responsible for the unique C-11-hydroxylation that occurs during landomycin biosynthesis. Compounds accumulated by the lanZ4/Z5 mutants are the previously described landomycin F and the new landomycins M and O.*

Introduction

Streptomyces cyanogenus S136 is the producer of landomycins A, B, and D (Scheme 1).[1] These compounds consist of an angucycline polyketide backbone decorated with a sugar side chain that contains different numbers of deoxysugars. Landomycins are characterized by an unusual spectrum of antitumor properties; in particular, they act against anthracycline-resistant tumor cells and prostate gland adenoma. The antitumor activity of landomycins depends on the length of the sugar chain.[2] Landomycin A, the largest member of the landomycins with a hexasaccharide side chain, is the most active compound.

The generation of novel landomycins with changes in the angucycline moiety can open the way to further examination of landomycins' structure–activity relationships with regard to both their glycosylation and aglycon patterns. Oxygenases catalyze a variety of reactions that lead to a vast structural diversity in polyketides. This makes oxygenases particularly attractive for combinatorial biosynthesis.[3] Recently, we obtained new landomycins that are produced by *S. globisporus* 1912 and which lack the C-11 hydroxyl group.[2] Interestingly, landomycin F, which contains two sugars in the side chain, was approximately threefold less active against the MCF-7 breast cancer cell line than its corresponding 11-hydroxy analogue, landomycin D.[2a] Thus, as in doxorubicin,[4] the 11-hydroxyl group seems to be important for antitumor activity.

In order to obtain further 11-deoxylandomycin derivatives and especially 11-dehydroxylandomycin A we focused our research on *lanZ4* and *lanZ5* of *S. cyanogenus* S136, which are very similar to *lndZ4* and *lndZ5* from *S. globisporus* 1912. We took advantage of a mutant in which an integrated plasmid prevents the transcription of *lanZ4* and *lanZ5* by a polar effect. Novel landomycin derivatives obtained in our studies all lacked the 11-hydroxyl group of the angucycline backbone.

Results and Discussion

Generation and characterization of a *S. cyanogenus* mutant

The analysis of the landomycin biosynthetic gene cluster revealed that *lanGT3*, *lanZ2*, *lanZ3*, *lanGT4*, *lanZ4*, and *lanZ5* are all cotranscribed from the same promoter [3] As the integration of a plasmid into the transcription unit might cause polar effects on downstream genes,[5] we carefully analyzed a *lanGT3* mutant obtained after integration of pOJΔGT3 into the chromosome[6] (Figure 1). After culture of this mutant in soytone glucose medium, 18 mg of the major compound, which was later identified as the recently described landomycin F,[2a] was isolated. Landomycin F displayed the typical landomycin-like UV-visible spectrum but with a significant hypsochromic shift in the third λ_{max} of about 40 nm. The NMR analysis showed that this compound contains only two sugars and lacks the 11-hydroxyl group, and was also identical to landomycin F with respect to all other NMR data (Figure 1).[2a] The structure of landomycin F could only be explained by a polar effect of the

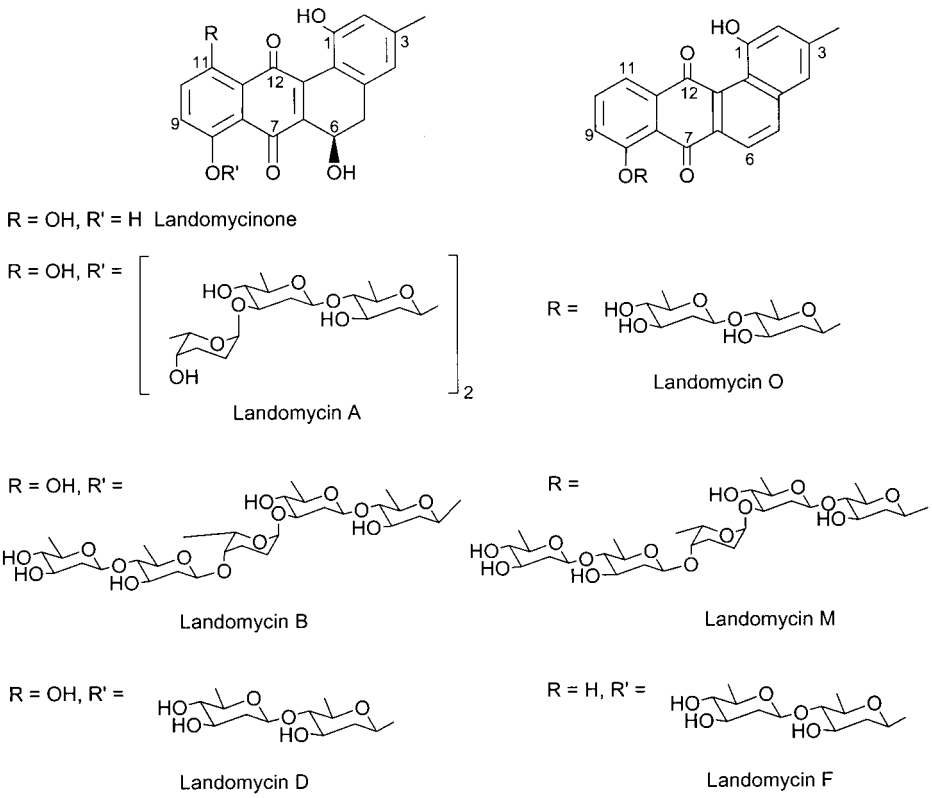
[a] *Dr. A. Luzhetskyy, M. Fedoryshyn, C. Dürr, C. Hofmann, Dr. D. Hoffmeister, Prof. Dr. A. Bechthold*
Albert-Ludwigs-Universität
Institut für Pharmazeutische Wissenschaften
Stefan-Meier-Strasse 19, 79104 Freiburg (Germany)
Fax: (+49) 0761-203-8383
E-mail: andreas.bechthold@pharmazie.uni-freiburg.de

[b] *Dr. L. Zhu, M. Gibson, Prof. Dr. J. Rohr*
Department of Pharmaceutical Sciences, College of Pharmacy
University of Kentucky
725 Rose Street, Lexington, KY 40536-0082 (USA)

[c] *Dr. B. Ostash, Prof. Dr. V. Fedorenko*
L'viv National University, Department of Genetic and Biotechnology
Grushevskyy st. 4, 79005 L'viv (Ukraine)

[d] *C. Mattingly, Prof. Dr. V. Adams*
Department of Pharmacy Practice and Science
College of Pharmacy University of Kentucky
725 Rose Street, Lexington, KY 40536-0082 (USA)

 DOI: 10.1002/cbic.200400316 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 1. *Structures of landomycin family members. The inactivation of the genes* lanZ4 and lanZ5 *led to the production of landomycins F, M, and O; the last two are novel compounds.*

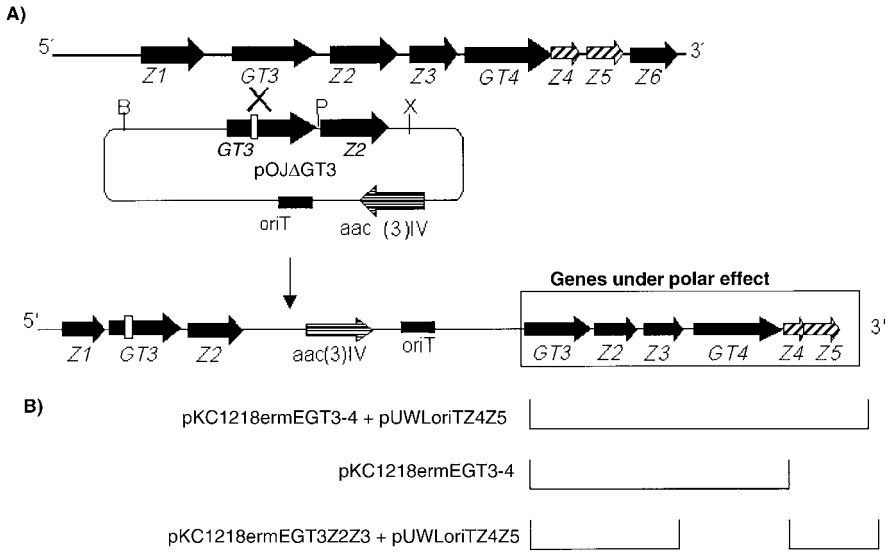


Figure 1. *A) Generation of the* S. cyanogenus *pOJΔGT3 mutant by single-crossover integration of plasmid pOJΔGT3. B, P, and X indicate the* BamHI, PstI, *and* XbaI *restriction sites, respectively. B) DNA fragments used for complementation experiments of* S. cyanogenus *pOJΔGT3 strain by using different combinations of genes, which were inactivated in this mutant.*

integrated plasmid on genes located downstream to the integration site (Figure 1).

Generation of other 11-deoxylandomycin derivatives by complementation experiments

Complementation of the *S. cyanogenus* pOJΔGT3 mutant was achieved by the expression of the *lanGT3*, *lanZ2*, *lanZ3*, *lanGT4*, *lanZ4*, and *lanZ5* genes (plasmids pKC1218ermGT3-4 and pUWLoriTZ4Z5) in *S. cyanogenus* pOJΔGT3, which gave rise to the wild-type production.

To demonstrate the function of *lanZ4* and *lanZ5* and to generate novel landomycin derivatives that lack the OH-group at the 11-position, additional complementation experiments were performed.

1. *LanZ4* and *lanZ5* (pUWLoriTZ4Z5) were used to transform *S. cyanogenus* pOJΔGT3, leading to the production of landomycin D. This indicates that LanZ4 and LanZ5 are indeed responsible for the hydroxylation at position 11.
2. When *lanGT3*, *lanZ2*, *lanZ3*, *lanZ4*, and *lanZ5* (pKC1218ermEGT3Z2Z3, pUWLoriTZ4Z5), but not *lanGT4*, were introduced into *S. cyanogenus* pOJΔGT3, again production of landomycin D was observed. From this result we concluded that LanGT4, which was shown to be an L-rhodinosyltrasferase[7] is responsible for the attachment of the third sugar during landomycin A biosynthesis.
3. The expression of *lanGT3*, *lanZ2*, *lanZ3*, and *lanGT4* (pKC1218ermEGT3-4) in *S. cyanogenus* pOJΔGT3 leads to the production of landomycins F, M, and O, which all lack the OH-group at position 11.
4. When both landomycins D and F were fed to a PKS mutant of *S. cyanogenus S136*,[8] landomycins A and B were produced. These data indicate that hydroxylation at the 11-position is not dependent on the length of the side chain and may occur at different stages during landomycin A biosynthesis.

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Structural elucidation of the new landomycins

All of the above mentioned landomycins were identified by TLC, HPLC, and HPLC/MS. The structures of the new landomycins M and O were further determined by NMR spectroscopy, mass spectrometry, and UV/Vis spectroscopy.

Landomycins O, M, and F can be easily distinguished from the other landomycins because of their light orange color, which differs from the dark red color of the wild-type landomycins. This observation was reflected in the hypsochromic shift found in the UV/Vis spectra of the orange compounds.

After culture of the *S. cyanogenus* mutant strain, two major products as well as a small quantity of a third compound were confirmed by UV spectroscopy and mass spectrometry to be a well-known antibiotic, tetrangulol, based upon TLC and HPLC-MS. Both of the major compounds are orange and possess very similar UV spectra. Compared with compound **1**, the second one displayed a much larger protonated molecular ion peak ($m/z = 939$) in the positive mode APCI-MS spectrum, and they were later shown to possess different sugar moieties of varying length attached to the same aglycon. The chemical structures of these two novel angucyclines were elucidated by NMR and MS spectra. Both compounds exhibit some of the representative NMR signals of a tetracyclic landomycin-type aglycon, except for the 6- and 11-positions. The ^{1}H NMR data (Tables 1 and 2) of the new compounds showed an ABC system in ring D and an AB system in ring B. This was further confirmed by 2D NMR correlation spectroscopy (H,H-COSY). There are obvious couplings between 5-H and 6-H as well as those between 10-H and 9/11-H, while the hydroxy signals normally observable for the wild-type strain landomycins were missing. Moreover, the comparison with two known angucycline antibiotics landomycins B and D revealed that the new compounds contain the same sugar moieties. The NMR spectra of landomycin M (^{13}C NMR, see Table 2 and ^{1}H NMR, see Table 1) showed the presence of five *O*-glycosidic hexopyranoses, for example, five anomeric C signals ($\delta = 91.9$–100.7) and five anomeric protons ($\delta = 4.52$–5.46). While landomycin O only displayed the NMR signals of two sugar moieties (^{1}H NMR, see Table 1 and ^{13}C NMR, see Table 2).

The structures of the new landomycins M and O are unexpected. One could speculate that these compounds appear due to the labile nature of the 6-OH group, which can easily get lost during the fermentation or the work-up procedure. It is also possible that the 6-hydroxylation in landomycin A biosynthesis is negatively impacted by the loss of the hydroxyl group at position C-11, which might play a role as a linker that stabilizes the post-PKS enzyme cluster.

Table 1. *^{1}H NMR signals of landomycins O and M in [D_6]DMSO at 400 MHz (δ in ppm relative to TMS).*

Position H-atom	Landomycin O δ [ppm], multiplicity, *J* [Hz]	Landomycin M δ [ppm], multiplicity, *J* [Hz]
1-OH[a]	10.66, brs	10.67, brs
2-H	6.97, d, 1.5	6.97, d, 1.5
3-CH_3	2.44, s	2.44, s
4-H	7.35, d, 1.5	7.35, d, 1.5
5-H	8.09, d, 8.5	8.09, d, 8.5
6-H	8.18, d, 8.5	8.18, d, 8.5
9-H	7.58, dd, 8, 1	7.58, dd, 8, 1
10-H	7.82, ddd, 8, 8, 1	7.82, ddd, 8, 8, 1
11-H	7.74, dd, 8, 1	7.74, dd, 8, 1
1A-H	5.46, dd, 8, 1.5	5.46, dd, 10, 2
2A-H_a	1.71, ddd, 12, 12, 8	2.0, ddd, 12, 12, 10
2A-H_e	2.65, ddd, 12, 5, 1.5	2.76, ddd, 12, 5, 2
3A-H	3.62, ddd, 12, 9, 5	3.54, m
3A-OH[a]	5.05, brs	n.a.
4A-H	3.10, dd, 9, 9	3.12, dd, 8, 8
5A-H	3.35, m	3.2–3.5, m[b]
5A-CH_3	1.22, d, 6	1.16, d, 6
1B-H	4.65 dd, 8, 1.5	4.69, dd, 10, 2
2B-H_a	1.37, ddd, 12, 12, 8	1.7–2.0, ddd, 12, 12, 10
2B-H_e	2.08, ddd, 12, 5, 1.5	2.44, ddd, 12, 5, 2
3B-H	3.60, ddd, 12, 9, 5	3.2–3.5, m
3B-OH[a]	4.96, brs	–
4B-H	2.79, dd, 9, 9	3.12, dd, 8, 8
4B-OH[a]	4.75, brs	n.a.
5B-H	3.30, m	3.2–3.5, m[b]
5B-CH_3	1.20, d, 6	1.23, d, 6
1C-H	–	4.95, brs
2C-H_a[b]	–	1.6–2.0, m
2C-H_e[b]	–	1.8–2.2, m
3C- H_a[b]	–	1.6–2.0, m
3C- H_e[b]	–	2.2–2.4, m
4C-H	–	3.50, brs
5C-H	–	4.14, m
6C-CH_3	–	1.03, d, 6
1D-H	–	4.52, dd, 10, 2
2D-H_a[b]	–	1.6–2.0, m
2D-H_e[b]	–	2.3–2.4, m
3D-H[b]	–	3.2–3.6, m
4D-H	–	2.95, dd, 8, 8
5D-H[b]	–	3.2–3.6, m
5D-CH_3	–	1.04, d, 6
1E-H	–	4.57, dd, 10, 2
2E-H_a[b]	–	1.6–2.0, m
2E-H_e[b]	–	2.3–2.4, m
3E-H[b]	–	3.2–3.6, m
4E-H	–	2.95, dd, 8, 8
5E-H[b]	–	3.2–3.6, m
5E-CH_3	–	1.17, d, 6

[a] Exchangeable in D_2O; n.a.: not assigned other OH-signals: 5.11, 5.04, 4.89, 4.74, 4.59 (brs). [b] Overlapped and complex.

Anticancer assays

The antitumor assays performed against common lung and breast cancer cell lines (the standard sulforhodamine B assay was used) reveal that the loss of both the 11- and 6-OH groups in landomycins O and M render the molecules inactive (see Table 3).

Experimental Section

General genetic manipulation: Standard molecular-biology procedures were performed as described.[6] Isolation of *Escherichia coli* DNA, DNA restriction, DNA modification such as the filling-in-sticky ends, and Southern hybridization were performed by following the protocols provided by the manufacturers of kits, enzymes, and reagents (Amersham Pharmacia, Boehringer Mannheim, Promega, and Stratagene).

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 2. ^{13}C *NMR signals of landomycins O and M in* $[D_6]$*DMSO at 100 MHz.*

Landomycin C atom	O δ [ppm]	M δ [ppm]	C atom	O δ [ppm]	M δ [ppm]
C-1	154.54	155.73	C-1B	100.05	100.11
C-2	120.2	120.2	C-2B	30.73	35.78
C-3	135.11	135.09	C-3B	71.91	71.88
C-3-CH_3	21.17	21.17	C-4B	76.33	75.63
C-4	121.73	121.9	C-5B	68.25	69.98
C-4a	134.18	134.18	C-5B-CH_3	17.87	17.8
C-5	137.4	137.37	C-1C	–	91.97
C-6	120.5	120.52	C-2C	–	24.05
C-6a	137.43	137.43	C-3C	–	24.0
C-7	180.55	180.52	C-4C	–	73.80
C-7a	119.29	119.29	C-5C	–	65.14
C-8	140.38	140.38	C-5C-CH_3	–	17.01
C-9	132.86	132.86	C-1D	–	100.70
C-10	122.28	122.23	C-2D	–	38.5
C-11	155.71	154.56	C-3D	–	71.88
C-11a	117.68	117.68	C-4D	–	87.05
C-12	186.78	186.78	C-5D	–	68.25
C-12a	134.62	134.64	C-5D-CH_3	–	17.96
C-12b	116.43	116.43	C-1E	–	99.87
C-1A	96.6	96.62	C-2E	–	36.6
C-2A	30.73	38.1	C-3E	–	70.15
C-3A	70.14	73.02	C-4E	–	76.32
C-4A	86.38	86.40	C-5E	–	69.62
C-5A	70.0	68.63	C-5E-CH_3	–	17.78
C-5A-CH_3	17.65	17.83			

Table 3. Anticancer activity assays of the new landomycins M and O in comparison with landomycins A, D, and F.

Landomycin (number of sugars; hydroxy groups)	IC_{50} [μM] H460 (lung)	IC_{50} [μM] MCF-7 (breast)
A (6; 6-OH, 11-OH)	10.4 ± 6.0	2.6 ± 0.4
D (2; 6-OH, 11-OH)	105.3 ± 46.2	5.6 ± 1.8
F (2; 6-OH)	111.0 ± 57.5	15.9 ± 3.0
M (5)	67.8 ± 4.1	53.2 ± 0.7
O (2)	26.4 ± 14.3	46.7 ± 9.8

Intergeneric conjugation between *E. coli* and *S. cyanogenus*: Intergeneric conjugation between *E. coli* and *S. cyanogenus* was performed as described earlier.[6,9]

Construction of gene inactivation and complementation plasmids: The plasmid pOJΔGT3 that was used for *lanGT3* inactivation was constructed as described in ref. [6]. For the construction of the complementation plasmid, pKC1218ermEGT3Z2Z3, a 4.2 kb *Bam*HI-*Sph*I fragment of the landomycin biosynthetic gene cluster that contained *lanGT3*, *lanZ2*, and *lanZ3* was ligated into the expression vector pIAGO[10] to yield pIAGOermEGT3Z2Z3. After restriction of pIAGOermEGT3Z2Z3 with *Hind*III and *Eco*RI, a 4.5 kb fragment that contained the *erm*E promoter was ligated into pKC1218[5] to yield pKCermEGT3Z2Z3. Finally, an *aad*A cassette obtained from pHP45Ω, which confers resistance to spectinomycin, was subcloned into the *Eco*RI site of pKCermEGT3Z2Z3 to yield pKC1218-ermEGT3Z2Z3.

A 5.4 kb *Bam*HI-*Stu*I fragment from cosmid H2-26[8] that contained *lanGT3*, *lanZ2*, *lanZ3*, and *lanGT4* was ligated into the *Bam*HI-*Eco*RV sites of pKC1218ermE[2] to yield plasmid pKCermEGT3-4. pKC1218-ermEGT3-4 was constructed by integration of the spectinomycin resistance cassette (*aadA*) into the *Eco*RI site of pKCermEGT3-4.

A *Mun*I and an *Eco*RI restriction site were introduced upstream and downstream to *lanZ4* and *lanZ5* by PCR. Amplified fragments were cloned into pMUN2[7] restricted by *Mun*I and *Eco*RI to create plasmid pMUNlanZ4Z5. A *Hind*III–*Xba*I fragment from pMUNlanZ4Z5 that carried *lanZ4* and *lanZ5* was ligated into pUWLoriT to yield pUWLoriTZ4Z5.

HPLC-MS: HPLC/MS was performed by using the Waters Alliance 2695 system, which was equipped with a Waters 2996 photodiode array detector and a Micromass ZQ2000 mass spectrometer that used an APCI probe (solvent A: H_2O; solvent B: acetonitrile; flow rate = 0.5 mL min^{-1}; 0–20 min 50% A and 50% B to 100% B, (linear gradient), 20–23 min 100% B, 23–25 min 100% B to 50% A and 50% B (linear gradient). The system was equipped with a Waters Symmetry C-18 column, 4.6 × 50 mm, particle size 5 μm, maintained at 23 °C. The UV detection wavelength was 451 nm. Retention time was 10.0 min for lamdomycin O and 12.0 min for landomycin M.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) grant Bechthold 5–1 to A.B., and by a grant from the Kentucky Lung Cancer Research Foundation to J.R. and V.A.

Keywords: antitumor agents · biosynthesis · glycosylation · hydroxylation · landomycin

[1] a) T. Henkel, J. Rohr, J. Beale, L. Schwenen, *J. Antibiot.* **1990**, 43, 492–503; b) S. Weber, C. Zolke, J. Rohr, J. M. Beale, *J. Org. Chem.* **1994**, *59*, 4211–4214.

[2] a) B. Ostash, U. Rix, L. L. Remsing Rix, T. Liu, F. Lombo, A. Luzhetskyy, O. Gromyko, C. Wang, A. F. Braña, C. Méndez, J. A. Salas, V. Fedorenko, J. Rohr, *Chem. Biol.* **2004**, *11*, 547–555; b) T. Crow, B. Rosenbaum, R. Smith, Y. Guo, K. Ramos, G. Sulikowski, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1663–1666.

[3] U. Rix, C. Fischer, L. L. Remsing, J. Rohr, *Nat. Prod. Rep.* **2002**, *19*, 542–580.

[4] S. Filippini, M. M. Solinas, U. Breme, M. B. Schluter, D. Gabellini, G. Biamonti, A. L. Colombo, L. Garofano, *Microbiology* **1995**, 141, 1007–1016.

[5] T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater, D. A. Hopwood, *Practical Streptomyces Genetics* John Innes Foundation, Norwich, **2000**.

[6] A. Luzhetskyy, T. Liu, M. Fedoryshyn, B. Ostash, V. Fedorenko, J. Rohr, A. Bechthold, *ChemBioChem* **2004**, *5*, 1567–1570.

[7] A. Trefzer, C. Fischer, S. Stockert, L. Westrich, E. Künzel, U. Girreser, J. Rohr, A. Bechthold, *Chem. Biol.* **2001**, *8*, 1239–1252.

[8] U. von Mulert, A. Luzhetskyy, C. Hofmann, A. Mayer, A. Bechthold, *FEMS Microbiol. Lett.* **2004**, 230, 91–97.

[9] A. Luzhetskyy, M. Fedoryshyn, D. Hoffmeister, A. Bechthold, V. Fedorenko, *Visnyk Lviv Univ.* **2002**, *29*, 62–68.

[10] E. Fernández, U. Weißbach, C. Sánchez Reillo, A. F. Braña, C. Méndez, J. Rohr, J. A. Salas, *J. Bacteriol.* **1998**, 180, 4929–4937.

Received: September 7, 2004

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim