



Regiocomplementary O-Methylation of Catechols by Using Three-Enzyme Cascades

Jutta Siegrist,^[a] Simon Aschwanden,^[b] Silja Mordhorst,^[a] Linda Thöny-Meyer,^[b, d] Michael Richter,^{*,[b, c]} and Jennifer N. Andexer^{*,[a]}

S-Adenosylmethionine (SAM)-dependent enzymes have great potential for selective alkylation processes. In this study we investigated the regiocomplementary O-methylation of catechols. Enzymatic methylation is often hampered by the need for a stoichiometric supply of SAM and the inhibitory effect of the SAM-derived byproduct on most methyltransferases. To counteract these issues we set up an enzyme cascade. Firstly, SAM was generated from L-methionine and ATP by use of an archaeal methionine adenosyltransferase. Secondly, 4-O-methylation of the substrates dopamine and dihydrocaffeic acid was achieved by use of SaFC from the saframycin biosynthesis pathway in 40–70% yield and high selectivity. The regiocomplementary 3-O-methylation was catalysed by catechol O-methyltransferase from rat. Thirdly, the beneficial influence of a nucleosidase on the overall conversion was demonstrated. The results of this study are important milestones on the pathway to catalytic SAM-dependent alkylation processes.

Selective methylation processes are of great interest for syntheses of bioactive substances.^[1–4] Methylation of drug molecules has often been described as very important for their efficiency and bioactivity.^[5,6] In nature, chemo-, regio- and stereo-selective methylations are mainly accomplished through S-adenosylmethionine (SAM)-dependent methyltransferases (MTs) that provide exceptional access to C-, N-, O- and S-methylated compounds as well as to halomethanes.^[1,7] Many of these MTs can be generally used for new alkylation reactions by using artificial SAM analogues.^[1,8–10] In contrast, methylation in classical

organic synthesis is usually achieved by using methylation agents such as methyl iodide, dimethyl sulfate or dimethyl carbonate. These, to a large extent, do not react selectively, are toxic and often have to be used in organic solvents.^[11]

For exploitation of the synthetic diversity provided by SAM-dependent enzymes the main bottleneck is the lack of cofactor supply systems or, ultimately, efficient cofactor regeneration systems.^[12–14] The stoichiometric supply of the cofactor SAM is economically unreasonable. Additionally, SAM is not very stable under aqueous conditions even at neutral pH and at ambient temperature.^[15] One way to overcome these limitations is the integration of the MT in a multistep enzyme cascade involving a SAM-producing methionine adenosyltransferase (MAT, EC 2.5.1.6) by using free or immobilised enzymes or whole-cell preparations.^[16,17] These SAM supply systems are based on the inexpensive starting materials L-methionine and ATP. The modularity of cascades using isolated enzymes has the potential to combine various compatible enzymes, also with non-natural substrates.

The SAM produced in situ can subsequently be used by any selected MT. S-Adenosylhomocysteine (SAH), the side product of SAM-dependent methylation reactions, is a potent inhibitor of many MTs and needs to be removed from the reaction (Scheme 1). In nature, SAH is mainly decomposed by SAH hydrolase (EC 3.3.1.1), leading to L-homocysteine and adenosine. However, the equilibrium of this reaction lies on the starting material side,^[18] thus rendering the enzyme less useful if no coupling steps are present. In addition to chemical methods such as oxidation or capturing of the homocysteine thiol group, a methylthioadenosine/SAH nucleosidase (MTAN; EC 3.2.2.9) can be used to break down SAH, yielding adenine and S-ribosylhomocysteine. In comparison with SAH hydrolase, the MTAN-catalysed reaction has the advantage of being irreversible and thus drives the reaction to completion.^[19] Reported examples of the use of in situ SAM synthesis with the aid either of MATs or of chlorinases (starting from 5'-chlorodeoxyadenosine and L-methionine) coupled to subsequent enzymatic steps include: fluorination reactions,^[20] the tandem N-methylation of teicoplanin^[21] and the alkylation of rebeccamycin^[10] and rapamycin.^[2]

The aim of this study was to establish a modular enzyme cascade including SAM synthesis for the selective O-methylation of catechols. This involved setting up the necessary analytical tools for process optimisation. Catechol moieties can be found in many natural and synthetic bioactive molecules, drug metabolites, aromas and lignin-derived compounds, as well as

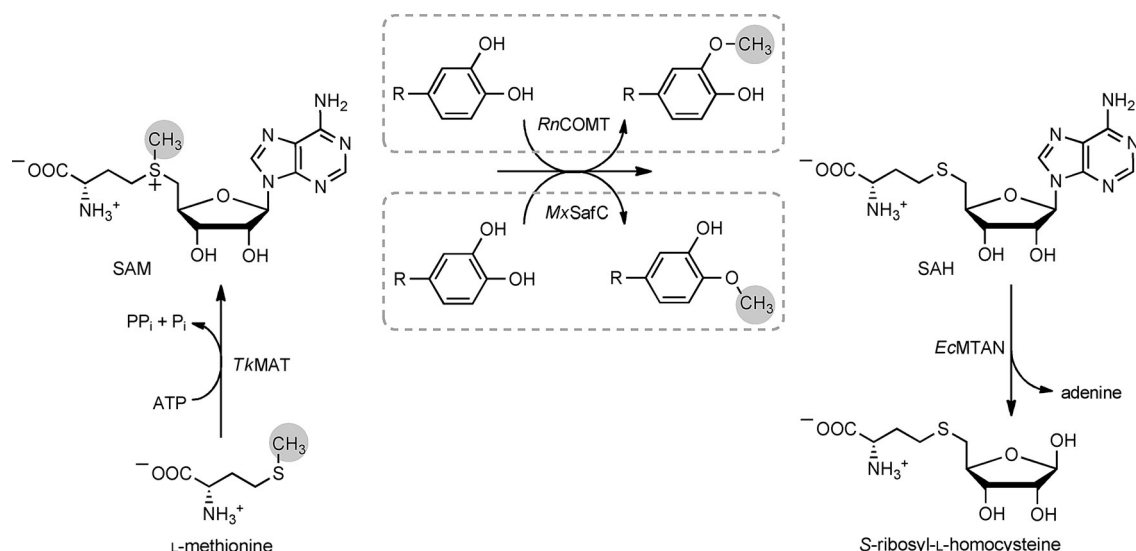
[a] J. Siegrist, S. Mordhorst, Dr. J. N. Andexer
Institute of Pharmaceutical Sciences, University of Freiburg
Albertstrasse 25, 79104 Freiburg (Germany)
E-mail: jennifer.andexer@pharmazie.uni-freiburg.de

[b] S. Aschwanden, Prof. Dr. L. Thöny-Meyer, Dr. M. Richter
Laboratory for Biointerfaces
Empa, Swiss Federal Laboratories for Materials Science and Technology
Lerchenfeldstrasse 5, 9014 St. Gallen (Switzerland)

[c] Dr. M. Richter
Present address:
Fraunhofer Institute for Interfacial Engineering and Biotechnology (IGB)
Branch BioCat
Schulgasse 11a, 94315 Straubing (Germany)
E-mail: michael.richter@igb.fraunhofer.de

[d] Prof. Dr. L. Thöny-Meyer
Present address: AVSV
Blarerstrasse 2, 9001 St. Gallen (Switzerland)

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cbic.201500410>.



Scheme 1. Three-enzyme methylation cascade. The linear three-step model cascade consists of the MAT-catalysed synthesis of SAM from ATP and L-methionine, the subsequent MT reaction leading to selectively methylated catechols and the use of an MTAN to avoid inhibition of the MTs by the side product SAH.

in mediators of laccases and materials with surface binding properties.^[22–25]

Recently, the structural and functional characterisation of a variety of MATs from thermophilic Archaea has been achieved. These enzymes are promising candidates for biotechnological use, due to their enhanced stability.^[26–29] We chose the MAT from *Thermococcus kodakarensis* (TkmAT) that was characterised in earlier studies by our working groups^[27] to evaluate its suitability for application in methylation cascades.

Catechol O-MTs (COMTs; EC 2.1.1.6) are found in many biosynthetic pathways and are involved in the metabolism and detoxification of a variety of natural and synthetic catechols. Many eukaryotic COMTs such as the human and rat COMTs predominantly methylate the hydroxy group in the *meta*-position.^[30,31] The regioselectivity seems to be mainly caused by substitution patterns.^[31–33] Only a few COMTs that transfer the methyl group mainly to the *para*-position in substituted catechols have been described.^[34,35] One example is SafC from *Myxococcus xanthus* (MxSafC). Because of their complementary regioselectivity, we chose MxSafC and COMT from *Rattus norvegicus* (RnCOMT) as model enzymes for the methylation of a range of catechol substrates [dopamine, dihydrocaffeic acid and 3,4-dihydroxybenzoic acid (3,4-DHB)]. The first set of assays was carried out with dopamine because this compound has been shown to be a substrate for both RnCOMT^[31] and MxSafC.^[34] To achieve effective removal of SAH from the reaction mixture, MTAN from *Escherichia coli* (EcMTAN) was used.^[36]

All enzymes were expressed in *E. coli* and purified by Ni-NTA affinity chromatography (Figure S1 in the Supporting Information). A buffer system containing 100 mM HEPES, 200 mM KCl and 20 mM MgCl₂ (pH 8.0) was identified as the most suitable solvent for the enzyme cascade (Figure S2). The magnesium ions present in the buffer are necessary for the activity of the MAT used (which also requires potassium ions), and have also been shown to be involved in the coordination of the catechol

substrate in COMTs.^[30,37] All UV-active cascade substrates, intermediates and products could be separated by HPLC (for experimental procedures see the Supporting Information). The results clearly show that MxSafC is a genuine COMT, because no activity could be detected in a reaction mixture also containing EDTA (Figure S3). In contrast, caffeoyl MTs and the 4-OMTs from the papaverin pathway, which both also accept catechol substrates, are not magnesium-dependent.^[38]

In the next step, the influence of the feedback inhibitor SAH on the two MTs was investigated by assaying both enzymes with and without the addition of EcMTAN. At concentrations of 5 mM dopamine, RnCOMT produced up to 10% *meta*-methylated dopamine after 20 min; the addition of EcMTAN increased the conversion by 10–20%. For MxSafC the degree of conversion was nearly doubled to up to 10% after 20 min when EcMTAN was added (Figure S4). As described earlier, MxSafC is highly selective for the dopamine *para*-hydroxy group (<3% *meta*-methylated dopamine was detected),^[34] whereas transformations with RnCOMT lead to both regioisomers in a *meta/para* ratio of 5:1. Earlier studies had found a similar ratio for RnCOMT (7:1).^[39]

To ensure that the cascade would proceed as required and not be hampered by unexpected enzyme side activities or inhibition issues, the three enzymes were added in a sequential fashion (10 min intervals). All substrates (ATP, L-methionine and dopamine) were present in stoichiometric amounts from the beginning; samples were taken after each step and analysed by HPLC (Figure 1 for MxSafC; for RnCOMT see Figure S5).

After this proof of concept, the cascade was tested in a one-pot reaction by addition of all three enzymes at once. After 30 min reaction time, the simultaneously performed transformations showed similar levels of conversion and *meta/para* ratios as in the sequential mode. In reactions without ATP, L-methionine and catechol substrate or without enzymes, no methylated products were found (Figure S6). For MxSafC, enzy-

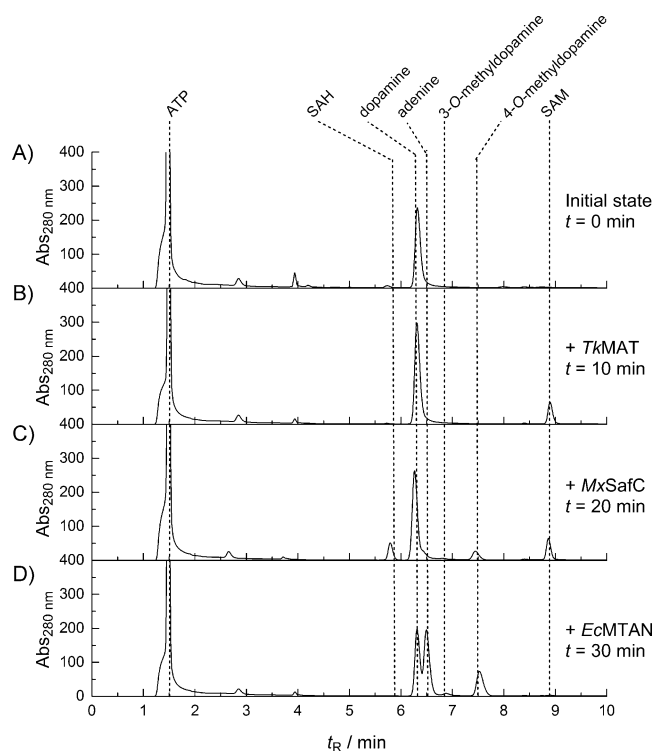


Figure 1. Stepwise addition of enzymes (TkMAT, MxSafC, EcMTAN). HPLC traces of all cascade steps. A) Initial state (substrates). B) SAM production. C) Methylation step, leading to additional peaks due to methylated product and side product SAH. D) SAH degradation with EcMTAN; all SAH produced is immediately converted into adenine, while the amount of product (4-O-methyldopamine) increases.

matic conversion into the *para*-methylated product (up to 40% conversion after 90 min) was detected; only traces of *meta*-methylated product were found (Figure 2). Use of longer incubation times led to oxidation followed by precipitation of the oxidation products. This could possibly be avoided by running the assays under nitrogen, use of a different pH or by adding antioxidants, and will be tested in future experiments. As can be seen from the isolated reactions, RnCOMT is not as strictly regioselective as MxSafC; here, approximately 40% *meta*-methylated and 10% *para*-methylated product were found at 50% conversion after 90 min (Figure 2).

To evaluate the general applicability of the cascade, dihydrocaffeic acid and 3,4-DHB were tested as additional substrates for both enzymes. In the case of dihydrocaffeic acid, MxSafC also shows a strong preference for the *para* position (5% *meta*- and 65% *para*-methylated product were produced after 16 h). For RnCOMT, dihydrocaffeic acid was a poor substrate, resulting in a low product yield and no significant regioselectivity; however, in earlier studies this compound was reported to be a better substrate than dopamine, with the *meta* position being favoured.^[31]

Surprisingly, MxSafC strongly favoured methylation of the *meta*-hydroxy group of the substrate 3,4-DHB, yielding 64% *meta*- and 7% *para*-methylated product after 16 h. Similar results, although with lower regioselectivity (59% *meta*, 31%

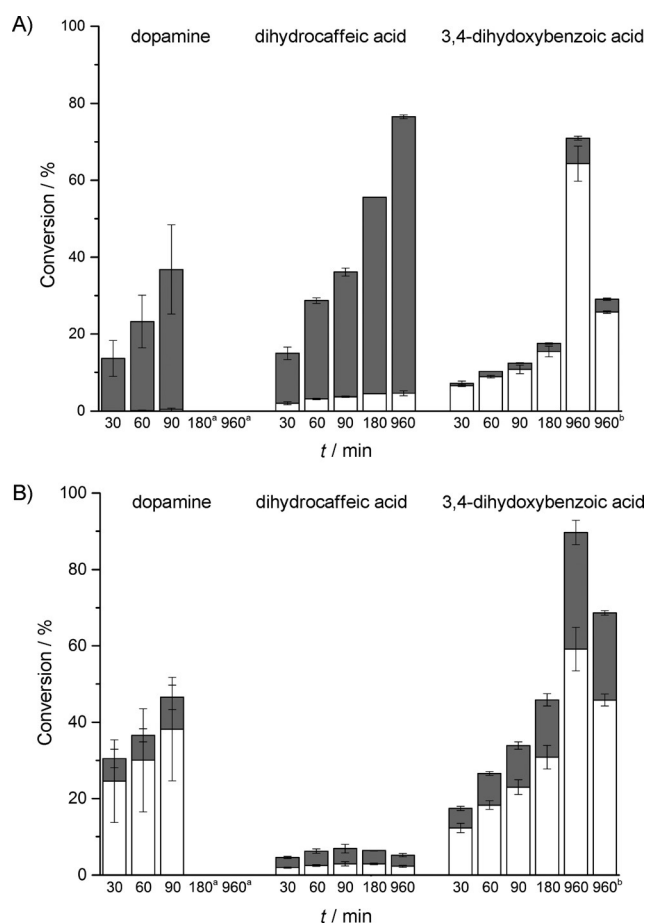


Figure 2. Three-enzyme, one-pot cascade reaction giving *meta* (□) and *para* (■) products. A) Cascade with MxSafC. B) Cascade with RnCOMT. All enzymes (TkMAT, MxSafC/RnCOMT, EcMTAN) and substrates (ATP, L-methionine, catechol substrate, each 5 mM) were present from the start. Samples were taken after 30, 60, 90, 180 and 960 min. [a] The reaction had to be terminated due to oxidation of the substrate. [b] Control reaction without EcMTAN.

para), were obtained with RnCOMT (Figure 2). A control reaction without the addition of EcMTAN confirmed the benefits and importance of the SAH degradation step, as already shown in test reactions with dopamine (Figures 2 and S4).

The regioselectivity strongly depends on the nature of the catechol side chain, whereas MxSafC generally seems to be the more selective enzyme. The molecular reasons for the inverted regioselectivity in the case of 3,4-DHB are currently being investigated in our laboratories. Detailed knowledge about the differences between the two COMTs will be the starting point for the design of variants with altered and/or improved regioselectivity. Such variants would also avoid the necessity for further product separation steps.

In summary, use of a multi-enzyme cascade based on an SAM-forming MAT, a catechol O-MT and the SAH-degrading MTAN led to methylation of various catechol substrates either in the *meta*- or in the *para*-position. The regioselective methylation serves as a proof of concept for methylation by SAM-dependent MTs in a three-enzyme, one-pot reaction. This SAM supply system is generally applicable for a broad range of further SAM-dependent enzymes. It could facilitate and enable

new approaches for many challenging reactions such as stereoselective methylation or radical SAM reactions that are difficult to accomplish by chemical synthesis. The methylation cascade presented here and other studies in this area represent a step towards the application of SAM-dependent enzymes in chemical synthesis.

Acknowledgements

The authors thank Prof. Eric W. Schmidt (University of Utah, USA) for the donation of the MxSafC expression plasmid and Prof. Michael Müller (University of Freiburg, Germany) for critically reading the manuscript. We also thank Dr. Annette Erb and Dr. Simone Bläsi for help with cloning and expression of TkMAT and EcMTAN, as well as Dr. Volker Lorbach (CS Chromatography, Langenwehe, Germany) for his support with method development and the donation of the Multohigh 100 SCX HPLC column. This project is in part funded by the Research Training Group 1976 (Deutsche Forschungsgemeinschaft).

Keywords: biotransformations • cofactors • methyltransferases • multi-enzyme reactions • S-adenosylmethionine

- [1] A.-W. Struck, M. L. Thompson, L. S. Wong, J. Micklefield, *ChemBioChem* **2012**, *13*, 2642–2655.
- [2] B. J. C. Law, A.-W. Struck, M. R. Bennett, B. Wilkinson, J. Micklefield, *Chem. Sci.* **2015**, *6*, 2885–2892.
- [3] E. Fossati, A. Ekins, L. Narcross, Y. Zhu, J.-P. Falgoutyret, G. A. W. Beaudoin, P. J. Facchini, V. J. J. Martin, *Nat. Commun.* **2014**, *5*, 3283.
- [4] L. A. Wessjohann, J. Keim, B. Weigel, M. Dippe, *Curr. Opin. Chem. Biol.* **2013**, *17*, 229–235.
- [5] E. J. Barreiro, A. E. Kümmerle, C. A. M. Fraga, *Chem. Rev.* **2011**, *111*, 5215–5246.
- [6] H. Schönherr, T. Cernak, *Angew. Chem. Int. Ed.* **2013**, *52*, 12256–12267; *Angew. Chem.* **2013**, *125*, 12480–12492.
- [7] D. K. Liscombe, G. V. Louie, J. P. Noel, *Nat. Prod. Rep.* **2012**, *29*, 1238–1250.
- [8] H. Stecher, M. Tengg, B. J. Ueberbacher, P. Remler, H. Schwab, H. Griengl, M. Gruber-Khadjawi, *Angew. Chem. Int. Ed.* **2009**, *48*, 9546–9548; *Angew. Chem.* **2009**, *121*, 9710–9712.
- [9] C. Dalhoff, G. Lukinavicius, S. Klimasauskas, E. Weinhold, *Nat. Chem. Biol.* **2006**, *2*, 31–32.
- [10] S. Singh, J. Zhang, T. D. Huber, M. Sunkara, K. Hurley, R. D. Goff, G. Wang, W. Zhang, C. Liu, J. Rohr, S. G. van Lanen, A. J. Morris, J. S. Thorson, *Angew. Chem. Int. Ed.* **2014**, *53*, 3965–3969; *Angew. Chem.* **2014**, *126*, 4046–4050.
- [11] M. Selva, A. Perosa, *Green Chem.* **2008**, *10*, 457–464.
- [12] M. Richter, *Nat. Prod. Rep.* **2013**, *30*, 1324–1345.
- [13] H. Zhao, W. A. van der Donk, *Curr. Opin. Biotechnol.* **2003**, *14*, 583–589.
- [14] H. K. Chenault, E. S. Simon, G. M. Whitesides, *Biotechnol. Genet. Eng. Rev.* **1988**, *6*, 221–270.
- [15] L. W. Parks, F. Schlenk, *J. Biol. Chem.* **1958**, *230*, 295–305.
- [16] E. Ricca, B. Brucher, J. H. Schrittwieser, *Adv. Synth. Catal.* **2011**, *353*, 2239–2262.
- [17] V. Köhler, N. J. Turner, *Chem. Commun.* **2015**, *51*, 450–464.
- [18] M. Turner, X. Yang, D. Yin, K. Kuczera, R. Borchardt, P. L. Howell, *Cell Biochem. Biophys.* **2000**, *33*, 101–125.
- [19] N. Parveen, K. A. Cornell, *Mol. Microbiol.* **2011**, *79*, 7–20.
- [20] N. Iwai, Y. Kitahara, T. Kitazume, *J. Mol. Catal. B* **2011**, *73*, 1–4.
- [21] J. M. Lipson, M. Thomsen, B. S. Moore, R. P. Clausen, J. J. La Clair, M. D. Burkart, *ChemBioChem* **2013**, *14*, 950–953.
- [22] R. Reiss, J. Ihssen, L. Thöny-Meyer, *BMC Biotechnol.* **2011**, *11*, 9.
- [23] E. Faure, C. Falentin-Daudré, C. Jérôme, J. Lyskawa, D. Fournier, P. Woisel, C. Detrembleur, *Prog. Polym. Sci.* **2013**, *38*, 236–270.
- [24] S. Hajdok, J. Conrad, H. Leutbecher, S. Strobel, T. Schleid, U. Beifuss, *J. Org. Chem.* **2009**, *74*, 7230–7237.
- [25] E. H. Hansen, B. L. Möller, G. R. Kock, C. M. Bünner, C. Kristensen, O. R. Jensen, F. T. Okkels, C. E. Olsen, M. S. Motawia, J. Hansen, *Appl. Environ. Microbiol.* **2009**, *75*, 2765–2774.
- [26] M. Porcelli, C. Ilisso, E. De Leo, G. Cacciapuoti, *Appl. Biochem. Biotechnol.* **2015**, *175*, 2916–2933.
- [27] J. Schlesier, J. Siegrist, S. Gerhardt, S. Blaes, A. Erb, M. Richter, O. Einsle, J. N. Anderson, *BMC Struct. Biol.* **2013**, *13*, 22.
- [28] D. E. Graham, C. L. Bock, C. Schalk-Hihi, Z. J. Lu, G. D. Markham, *J. Biol. Chem.* **2000**, *275*, 4055–4059.
- [29] F. Wang, S. Singh, J. Zhang, T. D. Huber, K. E. Helmich, M. Sunkara, K. A. Hurley, R. D. Goff, C. A. Bingman, A. J. Morris, J. S. Thorson, G. N. Phillips, Jr., *FEBS J.* **2014**, *281*, 4224–4239.
- [30] J. Vidgren, L. A. Svensson, A. Liljas, *Nature* **1994**, *368*, 354–358.
- [31] C. R. Creveling, N. Morris, H. Shimizu, H. H. Ong, J. Daly, *Mol. Pharmacol.* **1972**, *8*, 398–409.
- [32] M. T. I. W. Schüsler-Van Hees, G. M. J. Beijersbergen Van Henegouwen, *Pharm. Weekbl. Sci.* **1982**, *4*, 176–182.
- [33] G. Firnau, S. Sood, R. Pantel, S. Garnett, *Mol. Pharmacol.* **1981**, *19*, 130–133.
- [34] J. T. Nelson, J. Lee, J. W. Sims, E. W. Schmidt, *Appl. Environ. Microbiol.* **2007**, *73*, 3575–3580.
- [35] M. B. Kilgore, M. M. Augustin, C. M. Starks, M. O'Neil-Johnson, G. D. May, J. A. Crow, T. M. Kutchan, *PLoS ONE* **2014**, *9*, e103223.
- [36] K. A. Cornell, W. E. Swarts, R. D. Barry, M. K. Riscoe, *Biochem. Biophys. Res. Commun.* **1996**, *228*, 724–732.
- [37] J. Zhang, H. J. Kulik, T. J. Martinez, J. P. Klinman, *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 7954–7959.
- [38] K. Zhang, M.-W. Bhuiya, J. R. Pazo, Y. Miao, H. Kim, J. Ralph, C.-J. Liu, *Plant Cell* **2012**, *24*, 3135–3152.
- [39] C. R. Creveling, N. Dalgard, H. Shimizu, J. W. Daly, *Mol. Pharmacol.* **1970**, *6*, 691–696.

Manuscript received: August 13, 2015

Accepted article published: October 6, 2015

Final article published: November 6, 2015