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FULL PAPER

Exploring the Promiscuous Enzymatic Activation of Non-natural Polyketide Extender Units *In Vitro* and *In Vivo* for Monensin Biosynthesis

Marius Grote[a] and Frank Schulz*[a]

Abstract: The incorporation of new-to-nature extender units into polyketide synthesis harbours an important source for diversity yet is restricted by a limited availability of suitably activated building blocks *in vivo*. We here describe a straightforward workflow for the biogenic activation of commercially available new-to-nature extender units. Firstly, the substrate scope of a highly flexible malonyl co-enzyme A synthetase from *Streptomyces cinnamonensis* was characterized. The results were matched by *in vivo* experiments in which said extender units were accepted by both the polyketide synthase and the accessory enzymes of the monensin biosynthetic pathway. The experiments gave rise to a series of predictable monensin derivatives via the exploitation of the innate substrate promiscuity of an acyltransferase and downstream enzyme functions.

Keywords: malonyl-CoA synthetase • acyltransferase • monensin • antibiotics • polyketide synthase

Introduction

Polyketides represent a large group of natural products with a variety of biological and therapeutic activities.[1] Their scaffolds are synthesized by repetitive decarboxylative Claisen condensations of thioester-activated building blocks on an enzymatic machinery called polyketide synthases (PKS). PKS act as molecular assembly lines, consisting of a number of modules and specifically select co-enzyme A (CoA)-activated malonic acid derivatives for the elongation of the growing polyketide. The degree of reduction after each elongation step in the case of the common type I PKS is specified by the catalytic domains present in the reductive loop of each module.[2]

While polyketides exhibit an overall large structural and functional diversity, the variety of extenders available remains limited. In most cascades, malonyl-CoA and methylmalonyl-CoA, sometimes also ethylmalonyl-CoA, are specifically recruited by acyltransferase (AT) domains.[3]

In order to incorporate new-to-nature extender units, the gatekeeper AT domains must possess a significant substrate promiscuity, or flexibility must be introduced by suitable enzyme engineering approaches.[4]

An interesting system is the biosynthetic pathway towards monensin A and B, two main secondary metabolites produced by *Streptomyces cinnamonensis*.[5] This polyether antibiotic transports mono and divalent cations across cell membranes and has coccodiostatic, antifungal and various further biological activities.[6] Its biosynthesis proceeds via a type I PKS, in which *cis*-acting AT act as gatekeepers and select extender units specifically. An exceptional case is the monensin AT_5 ($monAT_5$), which accepts both ethyl- and methylmalonyl-CoA as building blocks, giving rise to monensin A and B. Previous experiments in a variant of *S. cinnamonensis* suggest that $monAT_5$ is highly promiscuous and accepts a variety of extender unit analogues into the biosynthesis of the new-to-nature shunt product premonensin (Figure 1).[4g, 7]

The suitable activation of artificial extender units is a prerequisite for their introduction into PKS assembly lines. In addition to the canonical extender units (*vide supra*), rare CoA-activated extender units are accessible by reductive carboxylation of α/β-unsaturated CoA-linked precursors.[3, 8] However, others are only available by enzymatic modification of ACP-bound intermediates.[9]

Numerous approaches to introduce new-to-nature building blocks not supplied by the producing organism were investigated in previous studies.[10]

While direct supplementation of CoA-activated malonate derivatives is unsuitable due to its limited membrane permeability and synthetic accessibility, *N*-acetylcysteamine esters (SNAC esters), acting as a CoA-mimic, have been successfully used in feeding experiments.[11] However, significant synthetic effort ahead of the fermentation, toxicity in fermentations and inferior activation compared to CoA-thioesters have limited their application.[7b, 11a, 11b, 12]

An alternative to the use of SNAC are non-activated diesters of malonate derivatives, which are stable and can be supplied to fermentations without requiring significant synthetic efforts. However, before their acceptance into the biosynthetic assembly line, both ester hydrolysis and activation must be enzymatically achieved *in vivo*.

The first enzyme capable of fusing CoA and malonate by ATP hydrolysis, termed malonyl-CoA synthetase (MatB), was identified in 1984.[13] Subsequently, a homologous enzyme from *Rhizobium trifolii* was described.[14] The MatB from *R. trifolii* was engineered by directed evolution to identify a substrate promiscuous variant by the use of which non-natural building blocks have been incorporated in simplified polyketides and into the complex natural product kirromycin.[4d-f, 15]

We recently introduced a new MatB-type enzyme from the monensin producer *S. cinnamonensis* and demonstrated its *in vivo* activity on a small number of different malonic acid derivatives, outcompeting artificial SNAC activation.[7b]

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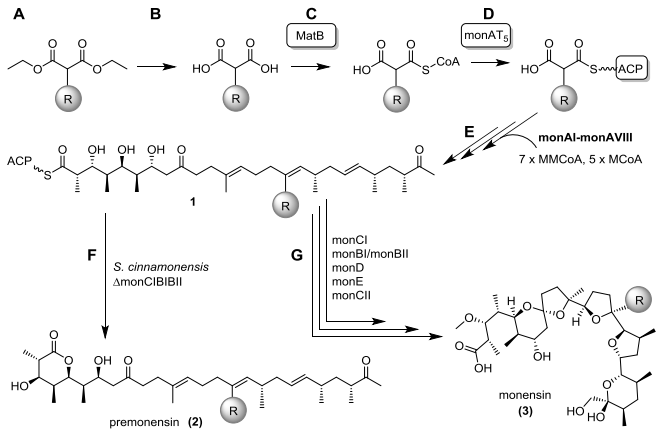


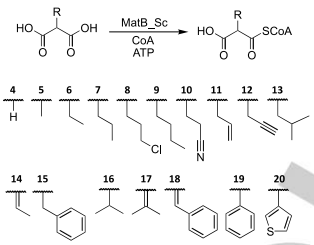
Figure 1. Schematic overview of reaction steps using malonate diester derivatives in fermentations. A. Compounds with a wide variety of side chains can be supplemented to the medium. B/C. Uptake and unspecific hydrolysis provide the non-native extender units for activation by the promiscuous MatB. D/E. Elongation of a growing polyketide by modules monAI to monAVIII complete the biosynthesis of the polyketide backbone of monensin. The nonspecific monAT5 domain accepts the activated malonate derivative and recruits it for the elongation step of the growing polyketide. This leads to the incorporation of a new side chain in a defined position of the growing polyketide. F. A shunt product premonensin (**2**) is produced in a mutant strain devoid of a post-PKS acting epoxidase monCI.[16] G. Post-PKS processing of the polyketide backbone is orchestrated by six enzymes (monCI: epoxidase; monB: epoxide hydrolase; monD: hydroxylase; monE: methylase; monCII: thioesterase) in a cascade of reactions (see Fig. 5).

Here, we investigate the substrate scope of this enzyme, henceforth termed MatB_Sc, for the activation of a broad variety of new-to-nature malonate derivatives. Furthermore, we explore the flexibility of the monensin assembly line and its extensive post-PKS processing machinery regarding the nature of the biosynthetic building block activated.

Results and Discussion

In vitro characterization of the malonyl-CoA synthetase from *S. cinnamonensis*

We recently reported on the MatB_Sc from *S. cinnamonensis*.[7b] A biocatalytic assay with purified MatB_Sc was performed to further explore its substrate scope. Conversion was first confirmed by HPLC-HRMS and the characteristic MS^2 fragmentation pattern of the Coenzyme A conjugate obtained (Fig. 2).



R	ion formula $[M+H]^+$	m/z calc.	m/z meas.	error ppm	mSigma
4	$C_{24}H_{39}N_2O_{19}P_3S$	854.1229	854.1229	-0.0	16
5	$C_{25}H_{41}N_2O_{19}P_3S$	868.1395	868.1385	-1.1	16
6	$C_{26}H_{43}N_2O_{19}P_3S$	882.1542	882.1548	-0.7	11
7	$C_{27}H_{45}N_2O_{19}P_3S$	896.1698	896.1712	-1.6	6
8	$C_{27}H_{44}ClN_2O_{19}P_3S$	930.1309	930.1325	-1.7	15
9	$C_{28}H_{43}N_2O_{19}P_3S$	910.1855	910.1846	0.9	7
10	$C_{27}H_{42}N_4O_{19}P_3S$	907.1494	907.1523	-3.1	52
11	$C_{27}H_{43}N_2O_{19}P_3S$	894.1542	894.1553	-1.2	12
12	$C_{27}H_{41}N_2O_{19}P_3S$	892.1385	892.1386	-0.0	65
13	$C_{28}H_{43}N_2O_{19}P_3S$	910.1855	910.1888	-3.7	10
14	$C_{26}H_{41}N_2O_{19}P_3S$	880.1385	880.1382	0.4	67
15	$C_{31}H_{45}N_2O_{19}P_3S$	944.1698	944.1675	2.4	72
16-20	no conversion				

Figure 2. Compounds tested in the substrate screening using MatB_Sc from *S. cinnamonensis*. Substrate acceptance was monitored by direct product detection via HRMS/MS^2. HRMS data for the main H^+-adduct are shown in the table enclosed, and further adducts, characteristic fragmentation pattern, isotopic pattern and MS^2 data are shown in the SI (Table S1/2, Fig. S1/2). **4-6** and **11-12** were detected in an earlier report on this enzyme.[7b] Additional data were obtained for **8-16** in Michaelis-Menten kinetics (Fig. 3). For **17-19**, conversion was too low for detection in 5,5'-dithiobis-(2-nitrobenzoic acid) assays. Calc: calculated; meas: measured; mSigma: match factor between theoretical and measured isotopic pattern based on isotope signal intensities.[17]

The substrate screening revealed a significant promiscuity of MatB_Sc. Building blocks with varying side chain length, saturation, and even heteroatom composition were accepted. Side chains were not accepted with a branch or cyclic substituent in the α-position, while an isobutyl-substituted substrate was cleanly converted. Remarkably, product **14** was clearly identified by $HRMS^2$, even though the acid's properties deviate strongly from the other substrates in the sp2-configuration at C-2. Based on this initial screening, kinetic data were obtained using a colorimetric 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) assay of residual CoA. Initial velocity was calculated for different substrate concentrations with fixed ATP and CoA concentrations by using the linear range of CoA consumption. Figure 3 illustrates the Michalis-Menten constants of malonates **4-12** obtained. Compounds **13-15** were not converted with significant reaction rates in the colorimetric assay, identifying these as very poor substrates.

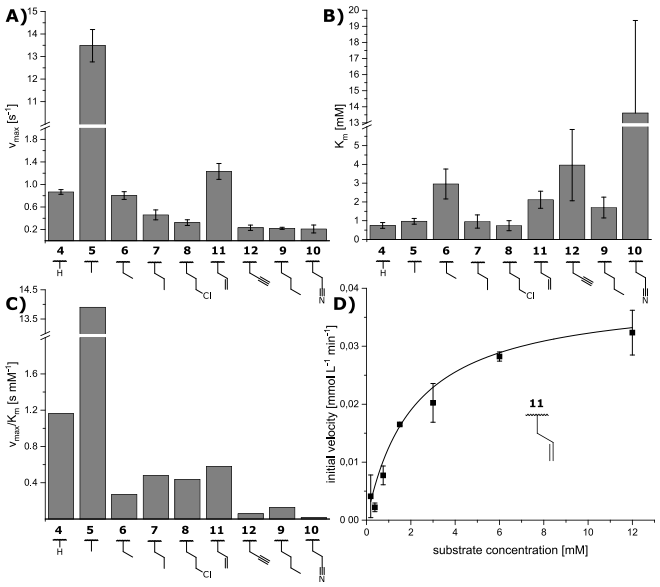
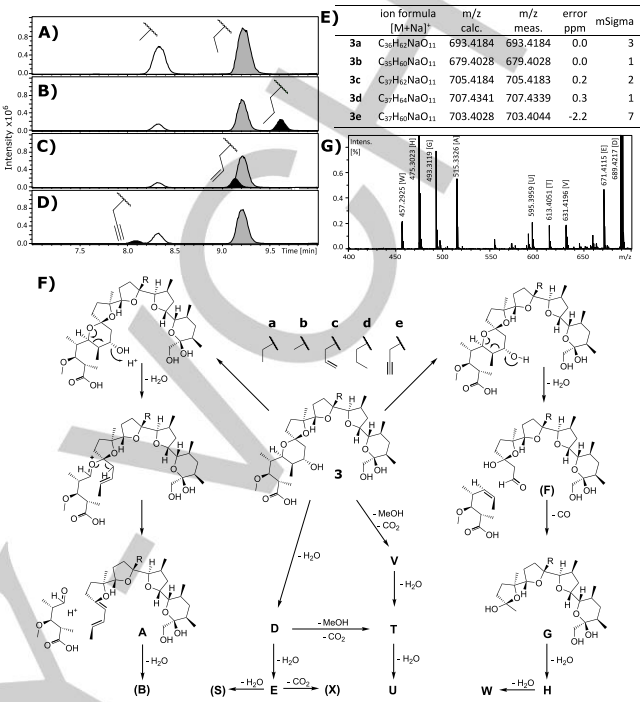


Figure 3. Michaelis-Menten analysis for the CoA activation of different malonate derivatives derived from initial velocities with different substrate concentrations ranging from 0.2 – 12 mM, and fixed ATP (2.5 mM) and CoA (1.0 mM) concentrations. Error bars represent the values calculated by fitting in OriginPro 2017G with standard parameters. **A**. Maximal reaction rate for substrates **4**-**12**. **B**. The corresponding Michaelis constant. **C**. Catalytic efficiency as represented by the v_{max}/K_m ratio. **D.** Representative fit of initial velocities with allyl-malonate as a substrate. Error bars represent the standard deviation of three replicates of each malonate concentration. See figure S5 for additional curve fittings.

Our combined data show the flexibility of MatB_Sc in the activation of different malonic acid derivatives. In contrast to studies on MatB-type enzymes characterized previously, methylmalonate was identified as the preferred substrate, with a 10-fold higher catalytic efficiency. This speaks against a natural function in malonic acid metabolism, as previously suggested for *R. trifolii* MatB.[4d, 14b, 18] The enzyme reveals a broad specificity profile. Suchlike activity was only reached with *R. trifolii* MatB after its extensive mutagenesis, the reason for which is not yet identifiable due to low sequence identity between the two enzymes. However, mutagenesis of *R. trifolii* MatB abolished its activity with malonic acid. Furthermore, the engineered enzyme accepts α-branched substrates, which are not accepted by MatB_Sc.[4d, 15a]

Use of the malonyl-CoA synthetase in the engineered biosynthesis of monensin derivatives

In order to explore the *in vivo* applicability of MatB_Sc, five malonate derivatives (2-allyl, 2-propyl, 2-propargyl, 2-butyl and 2-(3-chloropropyl)) were supplemented to a fermentation culture of *S. cinnamonensis* ATCC15413, assuming a co-expression of MatB_Sc and the monensin PKS, as observed in the case of analogous premonensin fermentations.[7b] Five replicates were analysed independently to allow a computer-assisted analysis using the MetaboScape software suite for the determination of new metabolites.[19] HPLC-HRMS and MS^2 analysis suggested the incorporation of propyl-, allyl- and propargyl-malonate into monensin (Fig. 4). The results indicate that a poor but significant *in vitro* efficiency can suffice to achieve incorporation of the new-to-nature building block into the PKS assembly line.[7b]



	ion formula [M+Na]⁺	m/z calc.	m/z meas.	error ppm	mSigma
3a	$C_{36}H_{62}NaO_{11}$	693.4184	693.4184	0.0	3
3b	$C_{35}H_{60}NaO_{11}$	679.4028	679.4028	0.0	1
3c	$C_{37}H_{62}NaO_{11}$	705.4184	705.4183	0.2	2
3d	$C_{37}H_{64}NaO_{11}$	707.4341	707.4339	0.3	1
3e	$C_{37}H_{60}NaO_{11}$	703.4028	703.4044	-2.2	7

Figure 4. Identification of monensin A (**3a**) and B (**3b**), and three new derivatives (**3c**-**3e**). See SI (Fig. S4-8, Table S3/4) for full MS and MS^2 data. Each compound was found with the $[M+NH_4]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+2Na-H]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$ and $[M+H-3H_2O]^+$ adducts with an average error of 1.1 ppm (< 0.5 ppm for the main adduct $[M+Na]^+$); furthermore the isotope patterns found experimentally match the sum formulas calculated. **A-D.** Extracted ion chromatograms of the adduct $[M+Na]^+$ ± 0.005 m/z of fermentations without supplementation (A), with propyl-malonate (B), allyl-malonate (C) and propargyl-malonate (D). **E.** MS data of the main adduct $[M+Na]^+$. **F.** Characteristic fragmentation pattern of the main adduct of the different monensin derivatives in full accord with Lopes et al.[20] Fragments with letters in brackets have been recorded with very low intensities. **G.** Exemplary MS^2 spectrum of propyl-monensin with accurate masses (Δ < 3.9 ppm) of characteristic fragment ions. Each mass is assigned to a specific fragment, as shown in **F**.

Butyl- and chloropropyl-substituted monensin derivatives were not identified, in spite of the previous identification of the butyl-premonensin shunt product in the mutant strain *S. cinnamonensis* A495, indicating a potential limitation in the post-PKS processing towards the polyether product derivative.[4g, 7b, 16, 21]

Promiscuity of the post-PKS processing enzymes in monensin biosynthesis

The feeding experiments mentioned above reveal a remarkable flexibility of the post-PKS machinery, particularly as the new-to-nature side chains introduced in these experiments are located at one of the epoxidation sites. Liquid chromatography–mass spectrometry analysis identified mass ions and fragmentation patterns consistent with monensin post-PKS shunt products in the case of the fermentations in the presence of propyl-, allyl- and

propargyl-malonic acid (Fig. 5; see Fig. S9-S13 and Table S5-S8 for chromatograms and detailed MS/MS^2 data).

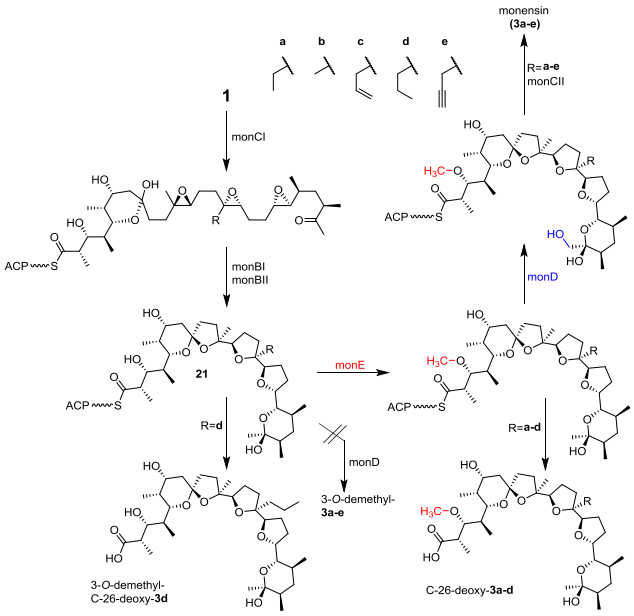


Figure 5. After backbone synthesis, the polyketide is modified still bound to the PKS. The enzymatic cascade is started by epoxidation of compound **1** by monCI, followed by polyether formation orchestrated by monBI and monBII. In the case of C-16 propyl (d), the 3-O-demethyl-C-26-deoxy monensin was set free. The cascade succeeds by methylation of O-3 through monE. Another shunt product, C-26-deoxy monensin, was identified for residues a-d, but not for propargyl (e). Shunt products lacking methylation but carrying the hydroxylation (3-O-demethyl-monensin) were not detected. The hydroxylase monD oxidises C-26 before the fully processed monensin derivatives are set free by the thioesterase monCII.

After formation of the polyether **21**, C-3-*O*-methylation and C-26-hydroxylation take place to yield the final monensin derivative (Fig. 5).[22] The corresponding C-3-*O*-demethyl and C-26-deoxy monensin derivatives were previously accessed through the deletion or inhibition of post-PKS enzymes in *S. cinnamonensis*.[21d, 22c] The corresponding C-26-deoxy derivatives were identified in our control fermentation of monensin A and B. In the case of the modified fermentations yielding a derivative with a non-native allyl- or propyl-substituent at C-16, the analogous derivatives were also detected, however, at an increased relative abundance (Fig. S9). No analogous post-PKS derivatives were identifiable in the fermentation of propargyl-monensin (**3e**), which may be attributed to the low intensity of propargyl-monensin.

Upon the incorporation of propyl malonic acid as an extender unit, a missing hydroxylation at C-26 was frequently accompanied by a missing methylation at position C-3-O; the C-3-*O*-demethyl-derivatization with the full hydroxylation pattern was not found. This result suggests that the hydroxylation by monD precedes the methylation by monE, which would contradict earlier studies on the assembly line.[21a, 22c, 23] This finding might be explained through different acceptance of the intermediates by the post-PKS enzymes. No accumulated intermediates were found for the incorporation of butyl- or 3-chloropropyl-malonic acid.

The data show a remarkable promiscuity of the entire monensin biosynthetic pathway, particularly monCI, monBI and monBII regarding substitution at C-16. At the same time, comparably subtle modifications at the side chain lead to significant variations in the late-stage post-PKS processing via monD and monE, as the conversion of allyl- and propyl-substituted monensin intermediates differs from control experiments and previous accounts.[21a, 22c, 23]

Conclusions

In summary, the experiments presented demonstrate the feasibility of the recently described MatB_Sc from *S. cinnamonensis* for the engineered biosynthesis of polyketide derivatives through the exploitation of promiscuous AT domains. Furthermore, the data reveal a strong promiscuity of the monensin assembly line, starting with the acceptance of different extender units in module 5, downstream processing of the nascent polyketide chain and a pronounced flexibility of the post-PKS machinery, which is largely congruent with the selectivity profile of monAT$_5$.

The introduction of non-natural extender units into polyketide biosynthesis is an underexploited strategy towards the biosynthetic derivatization of reduced polyketides and the new results show that revealing inbuilt enzymatic promiscuity should be considered. The enzyme MatB_Sc described here provides a valuable tool for the *in vivo* activation of synthetic extender units towards this end. Its heterologous expression in other interesting polyketide producers is currently under investigation.

Experimental Section

Expression and purification of MatB_Sc: The enzyme was prepared as previously described.[7b]

Synthesis of malonic ester derivatives: Diethyl esters of all studied malonic acid derivatives apart from 2-propargylmalonic acid[24] were purchased from Alfa Aesar and used as supplied. The free acids for activity measurements were prepared as follows: Four equivalents of LiOH were dissolved in 10 mL of water and 1 g of the corresponding diethyl ester was added to 10 mL methanol. The suspension was stirred at 25 °C for 2 h. Solvent was removed *in vacuo*, residual water was acidified to pH = 1 with HCl and extracted with 20 mL EtOAc. Removal of the solvent yielded the products as white powders with a yield of 50 – 90 %.

Biocatalysis and DTNB assay of MatB_Sc: An amount of 2.5 mM ATP, 1 mM CoA, 10 mM $MgCl_2$, 0.1-1.0 µM enzyme and malonate derivatives

(0.2-12 mM) were incubated in 50 mM HEPES, pH = 7.5. Aliquots (10 µL) were removed at different time points (2 – 30 min after adding the malonate derivative) and mixed with 90 µL of a 0.7 mM solution of DTNB. E_{412nm} was determined over a period of 5 min or until constant values were reached. Linearity and stability of the reaction determining the remaining CoA were proven using a calibration curve obtained from CoA dilutions. Initial velocities in the linear range of conversion were determined by linear regression. The velocity of three replicates was determined separately. Michalis-Menten kinetics were calculated using OriginPro 2017G (b9.4.0.220) using standard parameters and weighted values of the three replicates.

HPLC-HRMS/MS² analysis of *in vitro* biocatalysis: HPLC-HRMS analysis of the *in vitro* reaction to determine the CoA activation of different malonate derivatives was carried out as described previously.[7b]

Fermentation and sample preparation for HPLC-HRMS/MS²: *S. cinnamonensis* ATCC 15413 was grown in tryptic soy broth at 30 °C for 48 h. Next, 15 mL SM-16 medium (20.9 g L^{-1} MOPS, 10 g L^{-1} L-proline, 20 g L^{-1} glucose, 0.5 g L^{-1} NaCl, 2.10 g L^{-1} K_2HPO_4, 0.25 g L^{-1} EDTA, 0.49 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 0.029 g L^{-1} $CaCl_2 \cdot 2\ H_2O$) was inoculated (10 % v/v) and grown at 30 °C in 250-mL flasks equipped with metal springs (180 rpm, Multitron Standard 3-stack system, 50 mm throw, Infors HT). The 10 mM malonate acid ester derivatives were supplemented 24 h after inoculation. After a further four days, culture broth was extracted with one equivalent EtOAc. An amount of 0.5 mL of the organic phase was dried and the solid residue dissolved in 0.5 mL acetonitrile. An amount of 3 µL of each sample was analysed by HPLC-HRMS/MS². Five replicates were independently fermented and analysed for each compound and control.

HPLC-HRMS/MS² analysis of fermentation extracts of *S. cinnamonensis*: HPLC-HRMS analysis was run on an Ultimate 3000 HPLC System (consisting of a pump, autosampler, column oven and UV detector) coupled to a compact mass spectrometer (BRUKER DALTONIK GmbH, Life Sciences, Bremen, Germany) using the standard electrospray ionization source. All solvents were LC-MS grade (Chromasolv). A NUCLEODUR C18 Isis column (Macherey&Nagel), with a length of 25 cm, particle size of 1.8 µm and inner diameter of 2 mm, was used for chromatographic separation. Further details are described in the supplementary information (Fig. S14/S15).

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Figure 1. Schematic overview of reaction steps using malonate diester derivatives in fermentations. A. Compounds with a wide variety of side chains can be supplemented to the medium. B/C. Uptake and unspecific hydrolysis provide the non-native extender units for activation by the promiscuous MatB. D/E. Elongation of a growing polyketide by modules monAI to monAVIII complete the biosynthesis of the polyketide backbone of monensin. The nonspecific monAT5 domain accepts the activated malonate derivative and recruits it for the elongation step of the growing polyketide. This leads to the incorporation of a new side chain in a defined position of the growing polyketide. F. A shunt product premonensin (**2**) is produced in a mutant strain devoid of a post-PKS acting epoxidase monCI. G. Post-PKS processing of the polyketide backbone is orchestrated by six enzymes (monCI: epoxidase; monB: epoxide hydrolase; monD: hydroxylase; monE: methylase; monCII: thioesterase) in a cascade of reactions (see Fig. 5).

Figure 2. Compounds tested in the substrate screening using MatB_Sc from *S. cinnamonensis*. Substrate acceptance was monitored by direct product detection via HRMS/MS^2. HRMS data for the main H^+-adduct are shown in the table enclosed, and further adducts, characteristic fragmentation pattern, isotopic pattern and MS^2 data are shown in the SI (Table S1/2, Fig. S1/2). **4-6** and **11-12** were detected in an earlier report on this enzyme. Additional data were obtained for **8-16** in Michaelis-Menten kinetics (Fig. 3). For **17-19**, conversion was too low for detection in 5,5'-dithiobis-(2-nitrobenzoic acid) assays. Calc: calculated; meas: measured; mSigma: match factor between theoretical and measured isotopic pattern based on isotope signal intensities.

Figure 3. Michaelis-Menten analysis for the CoA activation of different malonate derivatives derived from initial velocities with different substrate concentrations ranging from 0.2 – 12 mM, and fixed ATP (2.5 mM) and CoA (1.0 mM) concentrations. Error bars represent the values calculated by fitting in OriginPro 2017G with standard parameters. **A**. Maximal reaction rate for substrates **4**-**12**. **B**. The corresponding Michaelis constant. **C**. Catalytic efficiency as represented by the v_{max}/K_m ratio. **D.** Representative fit of initial velocities with allyl-malonate as a substrate. Error bars represent the standard deviation of three replicates of each malonate concentration. See figure S5 for additional curve fittings.

Figure 4. Identification of monensin A (**3a**) and B (**3b**), and three new derivatives (**3c**-**3e**). See SI (Fig. S4-8, Table S3/4) for full MS and MS^2 data. Each compound was found with the $[M+NH_4]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+2Na-H]^+$, $[M+H-H_20]^+$, $[M+H-2H_20]^+$ and $[M+H-3H_20]^+$ adducts with an average error of 1.1 ppm (< 0.5 ppm for the main adduct $[M+Na]^+$); furthermore the isotope patterns found experimentally match the sum formulas calculated. **A-D.** Extracted ion chromatograms of the adduct $[M+Na]^+$ ± 0.005 m/z of fermentations without supplementation (A), with propyl-malonate (B), allyl-malonate (C) and propargyl-malonate (D). **E.** MS data of the main adduct $[M+Na]^+$. **F.** Characteristic fragmentation pattern of the main adduct of the different monensin derivatives in full accord with Lopes et al. Fragments with letters in brackets have been recorded with very low intensities. **G.** Exemplary MS^2 spectrum of propyl-monensin with accurate masses (Δ < 3.9 ppm) of characteristic fragment ions. Each mass is assigned to a specific fragment, as shown in **F**.

Figure 5. After backbone synthesis, the polyketide is modified still bound to the PKS. The enzymatic cascade is started by epoxidation of compound **1** by monCI, followed by polyether formation orchestrated by monBI and monBII. In the case of C-16 propyl (d), the 3-O-demethyl-C-26-deoxy monensin was set free. The cascade succeeds by methylation of O-3 through monE. Another shunt product, C-26-deoxy monensin, was identified for residues a-d, but not for propargyl (e). Shunt products lacking methylation but carrying the hydroxylation (3-O-demethyl-monensin) were not detected. The hydroxylase monD oxidises C-26 before the fully processed monensin derivatives are set free by the thioesterase monCII

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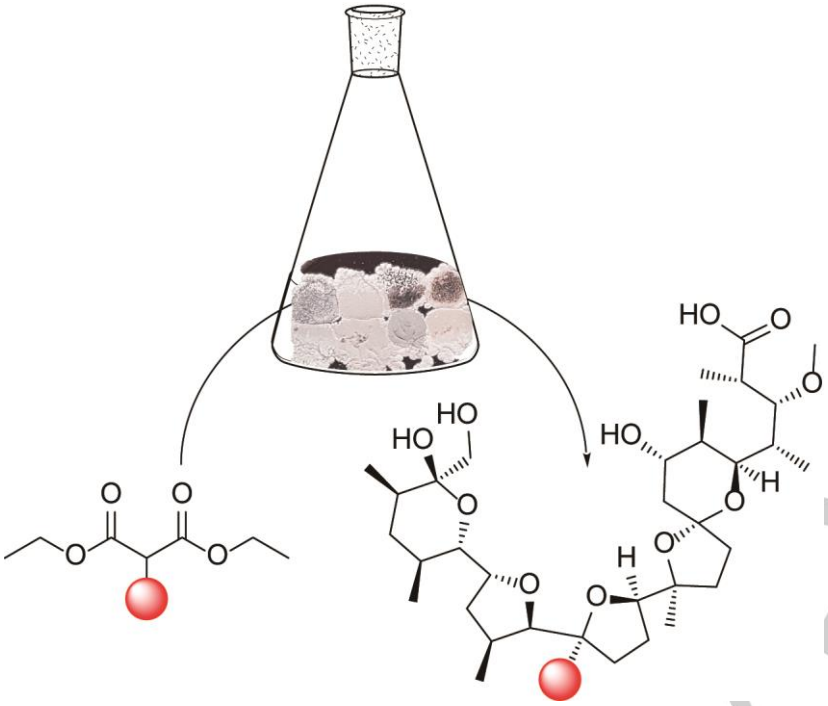

New-to-nature extender units in polyketide biosynthesis require thioester activation for their incorporation into natural product derivatives. Here, a new enzyme for the in vivo activation of a broad variety of extender units is investigated and its use for the targeted derivatization of the polyether monensin is studied.