Evidence for the Combined Participation of a C_{10} and a C_{15} Precursor in the Biosynthesis of Moenocinol, the Lipid Part of the Moenomycin Antibiotics

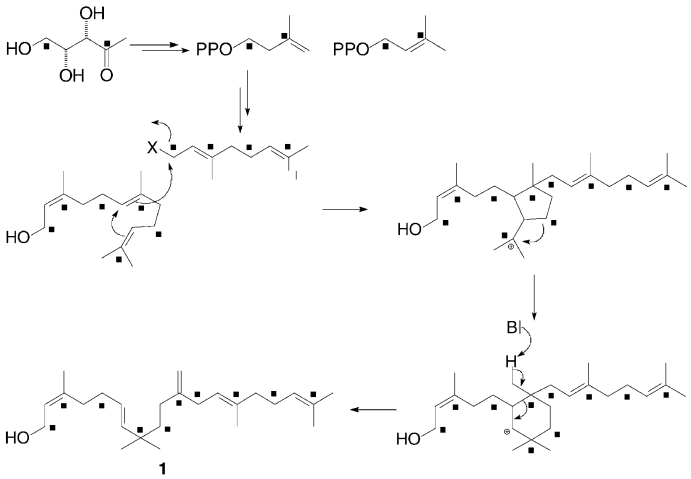
Ines Neundorf,[a] Christian Köhler,[a] Lothar Hennig,[a] Matthias Findeisen,[a] Duilio Arigoni,*[b] and Peter Welzel*[a]

Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday.

Upon feeding of [2-^{13}C,4-^{2}H]-1-deoxy-D-xylulose to Streptomyces ghanaensis, *the deuterium label was retained exclusively at positions C-7 and C-17 in the moenocinol part of the moenomycin antibiotics. This result vindicates the hypothesis that the C_{25} structure of moenocinol is assembled from a C_{10} and a C_{15} precursor, each of which requires for its formation the involvement of a dimethylallyl diphosphate starter unit.*

Introduction

The lipid (moenocinol) part **1** of the moenomycin antibiotics has been found to be completely isoprenoid and to be assembled via the nonmevalonate pathway.[1, 2] On the basis of a set of labeling experiments (*Streptomyces ghanaensis*), the mechanism depicted in Scheme 1 has been proposed to account for the formation of **1** from two terpenoid precursors. In particular, a labeling experiment with 1-deoxy-D-xylulose ^{13}C-labeled at positions 2 and 5 (see ■ in Scheme 1) provided clear evidence for the assembly of the carbon skeleton of **1** as indicated in Scheme 1.[3] The mechanistic rationale implies that two dimethylallyl starter units are involved in the biosynthetic process, an assumption that we attempted to prove independently by exploiting the potential of the branching signature that is characteristic for the nonmevalonate pathway.



Scheme 1. *Mechanism of the formation of **1** from two terpenoid precursors.*

The formation of isopentenyl diphosphate (IPP; **5 a**, Scheme 2) and dimethylallyl diphosphate (DMAPP, **6 a**) from mevalonic acid diphosphate occurs sequentially, that is, the mevalonic acid diphosphate is first converted into IPP, which is then rearranged under the influence of an isomerase (Idi) by addition of a proton to C-4 and removal of a proton from C-2.[4] In contrast, in the nonmevalonate pathway a branching is responsible for the isomerase-independent formation of a 6:1 mixture of IPP and DMAPP from 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate (**3 a**).

The branching was first postulated based on feeding experiments with precursors that are processed to give intermediate **3 b** with a deuterium label at the 2-position[5, 6] and was later proven by genetic methods[7] as well as by ^{13}C NMR spectroscopy[8] and HPLC–MS results,[9] respectively, by using the recombinant purified enzyme IspH (LytB) and reducing agents.

[a] *Prof. Dr. P. Welzel, Dr. I. Neundorf, Dipl. Biochem. C. Köhler, Dr. L. Hennig, Dr. M. Findeisen*
Fakultät für Chemie und Mineralogie, Universität Leipzig
Johannisallee 29, 04103 Leipzig (Germany)
Fax: (+49) 341-973-6559
E-mail: welzel@chemie.uni-leipzig.de

[b] *Prof. Dr. D. Arigoni*
Laboratorium für Organische Chemie
Eidgenössische Technische Hochschule Zürich
Hönggerberg HCI H307
8093 Zürich (Switzerland)
Fax: (+41) 1-632-1154
E-mail: arigoni@org.chem.ethz.ch

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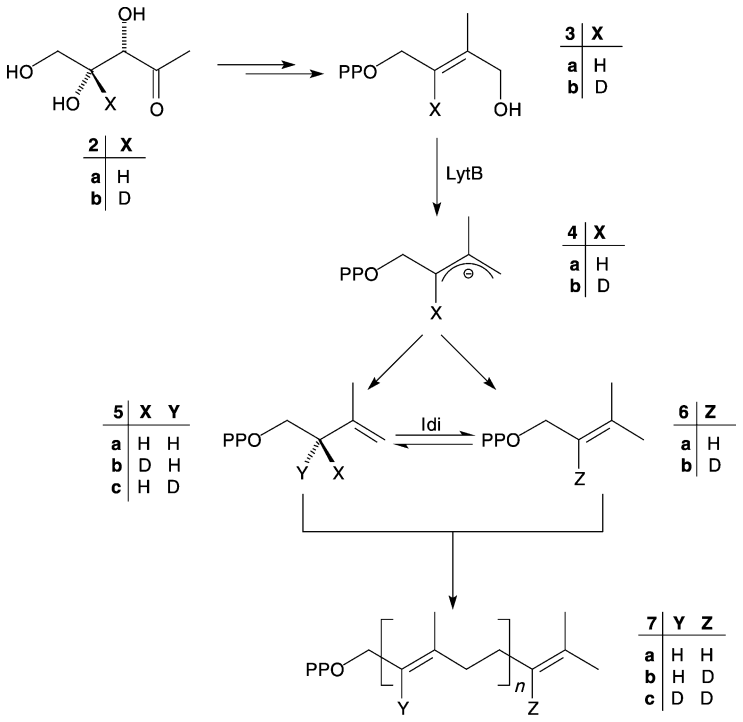


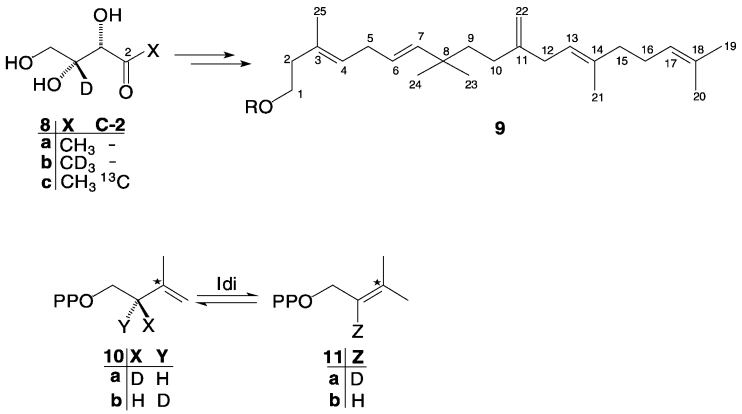
Scheme 2. *The formation of IPP (**5a**) and DMAPP (**6a**) from mevalonic acid diphosphate occurs sequentially.*

Subsequent work demonstrated that **3a** is converted to IPP and DMAPP by two one-electron reduction steps and mechanistic schemes involving protonation of an allylic anion intermediate have been suggested.[9–11] The available evidence requires that the hydrogen ligand introduced at C-2 of IPP in the last reduction step resides in the enantiotopic *Si* half-space.[12, 13] If a precursor such as **2b** is used, monodeuterated IPP and DMAPP (**5b** and **6b**, respectively) are formed. Poulter has shown that in *Escherichia coli*, as in yeast and higher eukaryotes, both the isomerase[14] and the prenyltransferase[15] (specifically the farnesyl diphosphate synthase) remove the 2_{Re} proton (X in **5a**) of IPP. Depending on the presence and activity of the isomerase, different deuterium labeling patterns of the terpenes derived from **5b** and **6b** can be expected and have indeed been found. In the case of *E. coli* the isomerase is dispensable and of low activity.[16] This means that the allyl starter unit retains the deuterium label, whereas IPP looses it in the elongation step, and the resulting terpenes display a deuterium label solely in the starter unit (**7b**).[5, 6] When DMAPP is isomerized to IPP before the elongation step occurs, terpenes **7a** (arising from the equilibrium **5b** → **6a** ⇄ **5a**) and **7c** (arising from **6b** ⇄ **5c**) are to be expected. This situation has been found in *Catharantus roseus*,[17] *Zymomonas mobilis*[11] and tobacco BY-2 cells.[13] In intermediate cases, the isomerase and the prenyltransferase compete for **5b** with comparable efficiency and all three types of terpenes (**7a**, **7b**, **7c**) are formed. This situation was identified for *Eucalyptus globulus* (biosynthesis of cineol).[12] It should be stressed that the expected **7c**, which is formed only to a minor extent, has not actually been identified experimentally in all cases (see below). Clearly, whether and to what extent equilibration of IPP and DMAPP precedes their incorporation into terpenes is not predictable (unless, of course, the absence of the isomerase can be demonstrated) and the issue has to be settled in each case by appropriate experimentation.

If the situation in *S. ghanaensis* is similar to that in *E. coli* and *E. globulus*, the suggested involvement of two starter units in the biosynthesis of moenocinol could be proven. This problem is the subject of this publication.

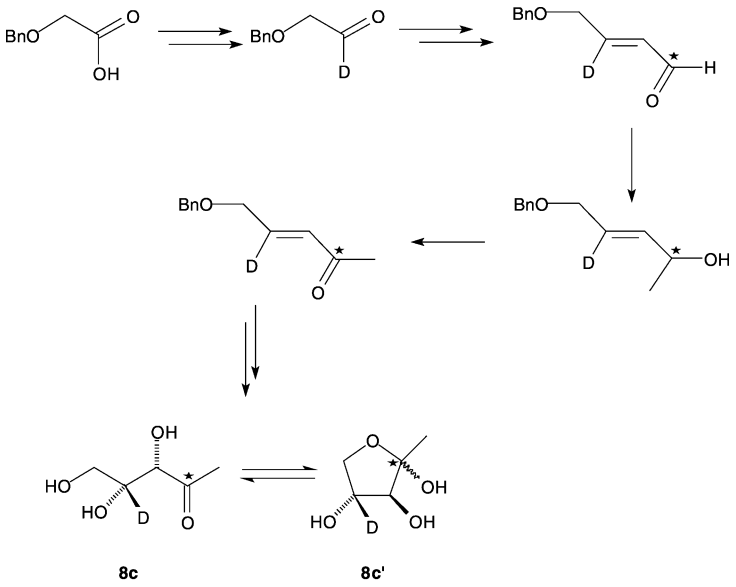
Results and Discussion

We have studied the fate of the 2-H_{Re} proton of IPP and the 2-H proton of DMAPP during the formation of moenocinol by feeding experiments using isotopomers of 1-deoxy-D-xylulose containing an ^2H label at C-4 (see formula **8** in Scheme 3).[18] In the course of these studies a number of severe problems arose. One of these problems could be expected: to produce sufficient amounts of moenomycin a rather rich fermentation medium has to be used with the effect that the incorporation rates of labeled precursors are quite low (1 – 3 %).[1] Furthermore, as a result of aggregate formation, moenomycin gives very poor ^1H NMR spectra at concentrations higher than 5×10^{-4} mol L^{-1} [19] and since, in addition, ^2H NMR signals are inherently broader than ^1H NMR signals, it turned out that ^2H NMR spectroscopy was of very limited value for the localization of the deuterium label in the moenocinol unit of moenomycin after feeding of the ^2H-labeled precursors **8a**,[5] **8b**[6] (prepared by the Giner route[20]), and (in a model experiment) [6,6′-$^2H_2$]-D-glucose, from which terpenes are formed via a tetra-deuterated 1-deoxy-D-xylulose-5-phosphate.[18] The second problem was more unexpected: We have previously found that the wild-type strain of *S. ghanaensis* produces such small amounts of moenomycin that it is of no use for biosynthetic experiments. We employed, therefore, the semi-producing strain H2 (obtained by continuous industrial efforts at strain optimization), which was kindly given to us by BC Biochemie (Frankfurt). We have worked with this strain in our



Scheme 3. *For the labeling pattern of **9**, see the text.*

laboratory for several years (stored as frozen culture at −80 °C, and, when necessary, processed by using precultures, as described previously).[1] However, in the course of the work described herein, the line was found to be genetically unstable.[21] In a number of experiments the amount of produced moenomycin was fairly low and the moenocinol unit was only labeled in the geraniol-derived part.[18] To overcome these obstacles a further set of feeding experiments was performed 1) with a new batch of the H2 strain ($H2_n$, kindly supplied by BC Biochemie) and 2) by using the double labeling strategy in which a ^{13}C reporter nucleus is placed two bonds away from the site that carries the deuterium label[22] (for a successful application of this strategy in a related case, see ref. [12]).



Scheme 4. *Synthesis of* ***8c****.*

Synthesis of the labeled precursor 8c

Compound **8c** was prepared by our previously reported synthetic pathway[1b] (Scheme 4), which combines features of the Giner[20] and Kennedy[23] methods (18% overall yield after 11 steps).[18] 1-Deoxy-D-xylulose can exist in an open-chain and two cyclized forms. In different experiments the NMR spectra revealed the presence of the three forms of **8c**/**8c'** in varying ratios. No attempt was made to achieve equilibration. We used the samples for the feeding experiments as they were obtained after purification.

Feeding experiments

Feeding experiments with compounds **8a**, **8b**, and **8c**, and isolation of the moenomycin complex were performed as described previously.[1, 18] HPLC and ESI-MS of the complex revealed the presence of moenomycins A, A_{12}, C_1, C_3, C_4.[24] It was unnecessary to separate the mixture since both ^1H and ^{13}C NMR spectra furnished a single set of signals for the lipid part. The signals were assigned as described by Donnerstag et al.[19] and Kurz et al.[25] In the [2-^{13}C,4-^2H]-1-deoxy-D-xylulose (**8c**) experiment, the precursor was fed to *S. ghanaensis* $H2_n$ to reach a final concentration of 0.6 mol L^{-1}. The moenomycin sample obtained was beautifully pure and gave very clean NMR spectra. For a quantitative analysis, the ^{13}C NMR spectra of unlabeled and labeled moenomycin samples were recorded under strictly identical conditions (inverse gated decoupling). The enrichments were calculated by comparing the corresponding signals (referenced to C-2 of moenomycin unit A[1]) of labeled and unlabeled moenomycin by a known procedure[26] that disclosed ^{13}C enrichments in the following positions of **9** (see Scheme 3): C-3 (2%), C-8 (3%), C-11 (3%), C-14 (2%), C-18 (2%). Two of the ^{13}C-enriched signals, namely those of C-8 and C-18, were accompanied by up-field-shifted satellites ($\Delta\delta = 102.6$ and 87.5 ppb, respectively; see Figure 1 and Table 1) originating from the ^2H β-shift.

The C-18 signals could be integrated with confidence. The main signal represents the natural background ^{13}C signal (see Scheme 5, **A'**) and the ^{13}C signal generated from the proffered precursor without ^2H at C-17 (Scheme 5, formula **A**, * indicates

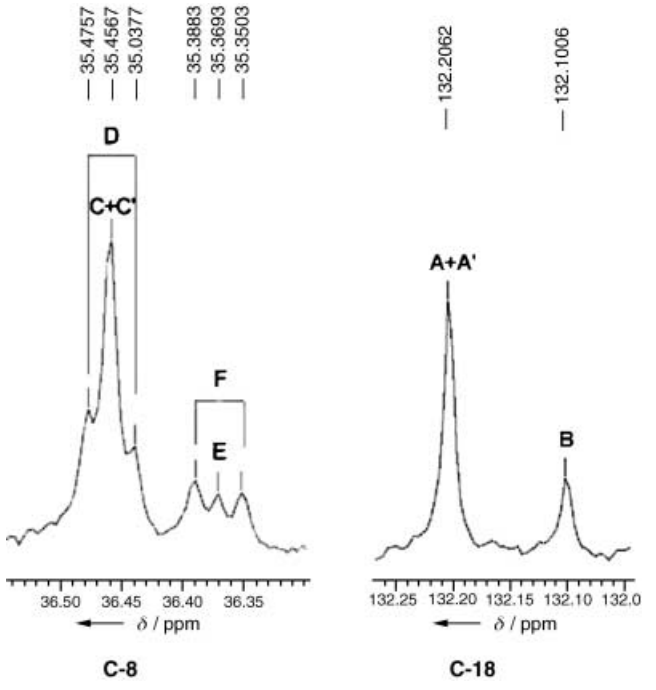
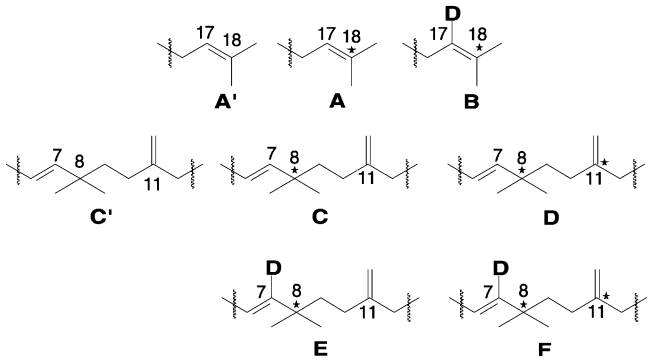


Figure 1. *Part of the ^{13}C NMR spectrum of moenocinol showing the signals of C-8 (left) and C-18 (right). The spectrum shows results from the feeding experiment with* ***8c*** *(see also Scheme 5).*

^{13}C label), whereas the satellite corresponds to the isotopomer **B**, which has a ^{13}C label at C-18 and an ^2H label at C-17. The ratio of the integrals of the satellite and the nonshifted signal (after correction for the natural abundant background signal[1]) was 41:59, which indicates that 59% of the ^2H label was washed out from the doubly labeled precursor **8c** in the DMAPP starter unit in the course of the formation of the moenocinol framework.

*Table 1. Data for the ^{13}C NMR (CD_3OD) signals of C-8 and C-18 of moenocinol after the feeding experiment with **8c**.[27]*

Position	Isotopomer	δ Value	J (C,C) [Hz]	Isotopic shift [ppb]
C-8	**C′**	36.45		
	C	36.45		
	D	36.45	3.8	
	E	36.37		β-(^{2}H-C) = −87.5
	F	36.37	3.8	β-(^{2}H-C) = −87.5
C-18	**A′**	132.21		
	A	132.21		
	B	132.10		β-(^{2}H-C) = −102.6



***Scheme 5.** Isotopomers of **9** after the feeding experiment with **8c**.*

The C-8 signal cluster was more complicated. We assume that it originates from isotopomers **C′** and **C** (unshifted singlet), **D** (unshifted doublet, $^3J_{8,11} = 3.8$ Hz),[28] **E** (shifted singlet), and **F** (shifted doublet, $^3J_{8,11} = 3.8$ Hz). The ratio of the integrals of the satellites and the nonshifted signals (after correction for the natural abundant background signal) was 48:52. If one compares the signals of **F** (which stems from two labeled precursor units) with that of **E** (stemming from the assembly of a labeled and an unlabeled precursor unit of endogenous origin), a ratio of about 2:1 may be roughly estimated, in agreement with previous results.[3]

Conclusions

The spectroscopic results reported here indicate that sizable amounts of the deuterium label of the precursor **8c** are retained in two of the five C_5 units from which moenocinol is assembled. According to the tenets of the nonmevalonate pathway discussed in detail in the introduction, these two building blocks must derive from the DMAPP starter unit **11a** generated directly in the enzymatic reduction of **3b** (with a ^{13}C label at C-3). In addition, the location of these units within the framework of moenocinol demonstrates that the C_{25} compound is indeed assembled from a C_{10} and a C_{15} precursor by the route outlined in Scheme 1.

The apparent partial losses of deuterium detected at C-7 and C-17 (52 and 59%, respectively, compared to the starting material) are fairly similar to those previously observed for the biosynthesis of cineol in *E. globulus*[12] and confirm the participation in the biosynthetic process of deuterium-free DMAPP (**11b**) generated by subsequent isomerization of preformed IPP (**10a**). At the same time, of course, when the isomerase is operating in the opposite direction a certain amount of preformed DMAPP (**11a**) will be converted into labeled IPP (**10b**) resulting in retention of the deuterium label in the elongation step. No signals corresponding to such a labeling pattern could be detected in the study described herein. This fact, however, does not necessarily contradict the argument. By assuming an approximate 6:1 ratio of IPP and DMAPP in the mixture generated in the bifurcation step of their biosynthesis (as is known to be the case for the *E. coli* reductase) and keeping in mind that the equilibrium constant of the isomerization process[10] dictates a value of 7:3 for the ratio of the rate constants $k_{(IPP)}/k_{(DMAPP)}$, it is possible to estimate that while the specific deuterium content in the DMAPP pool **11a**/**11b** drops from 1.0 to 0.5 D, the fraction of molecules in the IPP pool that retains the deuterium label in the elongation step (**10b**) will increase only from 0 to about 2%.

In practical terms, this argument implies that, in the feeding experiment with **8c**, deuterium-shifted ^{13}C satellites diagnostic for the incorporation of IPP units of type **10b** will hardly be detectable as their intensities ought to be reduced by a factor of about 25 with respect to those of corresponding satellites associated with C_5 units derived from the original starter groups.[29]

Experimental section

Instruments: NMR spectroscopy: Gemini 200 and Gemini 2000 (Varian, ^1H NMR 200 MHz, ^{13}C NMR 50 MHz), Gemini 300 (Varian, ^1H NMR 300 MHz, ^{13}C NMR 75 MHz), DRX 400 (Bruker, ^1H NMR 400 MHz, ^{13}C NMR 100 MHz, ^2H NMR 61 MHz), DRX-600 (Bruker, ^1H NMR 600 MHz, ^{13}C NMR 150 MHz, ^2H NMR 92 MHz); Mass spectrometry: EI-MS: VG-12-250 (Vacuum Generators, 70 eV), ESI-MS: FT-ICR MS Apex II (Bruker Daltonics); optical rotation: Polartronic (Carl Zeiss Jena, sodium D line, 0.5-dm cell); preparative medium pressure chromatography was performed on self-packed columns (65-g RP18 material, 40–63 μm, LiChroprep® (Merck) or Polygoprep 60-50 C18 (Macherey Nagel)). Fermentation experiments were performed in a gyrotary shaker (Thermoshaker; Gerhardt) at 37 °C and 160 rpm. Sterile work was performed in a cleanbench (InterMed Nunc).

[2-^{13}C,4-^{2}H]-1-Deoxy-D-xylulose (8c): ^1H NMR (200 MHz, CD_3OD): $\delta = 1.32–1.45$ (furanose; CH_3 signals), 2.25 (open; d, 3H, $^2J_{1\text{-}C2} = 6.1$ Hz, CH_3-1), 3.50–4.18 (m, CH_2-5 of open and furanose forms, 3-H of furanose forms), 4.22 (d, 1H, $^2J_{3\text{-}C2} = 3.5$ Hz, supposedly 3-H of open form) ppm; ^{13}C NMR (50 MHz, CD_3OD): open form: $\delta = 26.44$ (d, $J_{1-2} = 41.0$ Hz, C-1), 63.81 (d, $^3J_{5-2} = 1.8$ Hz, C-5), 78.51 (d, $J_{3-2} = 41.3$ Hz, C-3), 212.18 (C-2) ppm; furanose forms: $\delta = 16.88$ (d, $J_{1-2} = 49.1$ Hz, C-1), 19.17 (d, $J_{1-2} = 49.1$ Hz, C-1), 71.36–73.58 (C-5 signals), 82.64–85.60 (C-3 signals), 105.96–110.55 (C-2) ppm. IR (film): 1676, 1527, 1350, 1201, 1136, 1093 cm^{-1}; $C_4{}^{13}CH_9{}^2HO_4$ (136.14, 136.06), EI-MS (%): $m/z = 136.0$ $[M]^{+\bullet}$ (2), 119.0 $[M - H_2O]^{+\bullet}$ (5), 91.9 (10), 73.9 (50), 61.9 (100); $[\alpha]_D^{20} = +36.2$ (5.0, H_2O).

Culture of *S. ghanaensis* and feeding experiments: For nutrient solutions, fermentation, and incorporation experiments, see refs. [1, 18].

Isolation of the moenomycins: Cells were separated from the medium by centrifugation. The centrifugate was concentrated (rotary evaporator) and the residue was stirred with an ice-cold 80:20 methanol/water mixture for 2 h and then centrifuged. Cell disintegration was achieved by sonication in an ice-cold 80:20 methanol/water mixture. After centrifugation the centrifugates from both fractions were combined and methanol was evaporated. After setting the pH value to 7.5, the aqueous solution was extracted with three portions of 1-butanol. Solvent evaporation from the aqueous phase, taking up the residue in 4:6 acetonitrile/buffer ($KH_2PO_4 \cdot 3\,H_2O$ (13.1 g), KH_2PO_4 (0.3 g), and water, final volume: 1 L, pH value adjusted to 7.5), and medium pressure LC (RP18, solvent: acetonitrile/buffer as described above) gave a fraction that was desalted by solid phase extraction (RP18, first water then 1:1 acetonitrile/water). Acetonitrile removal by distillation and subsequent lyophilization provided the moenomycin complex.

NMR experiments: a) ^{2}H NMR: The pure desalted moenomycin mixture was dissolved in CH_3OH (0.7 mL) and filtered into a 5-mm NMR tube.[30] b) ^{13}C NMR: The pure desalted moenomycin mixture (unlabeled and labeled samples) was dissolved in CD_3OD (0.7 mL) and filtered into a 5-mm NMR tube. The spectra were acquired by using an inverse gated decoupling experiment. The number of scans was 35 000. Incorporations were calculated according to the method described by Scott et al.[26] For results, see the main text.

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