Biosynthesis of the Unusual Amino Acid (4*R*)-4-[(*E*)-2-Butenyl]-4-methyl-L-threonine of Cyclosporin A: Enzymatic Analysis of the Reaction Sequence Including Identification of the Methylation Precursor in a Polyketide Pathway†

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ABSTRACT: 3(*R*)-Hydroxy-4(*R*)-methyl-6(*E*)-octenoic acid, the C9-backbone of the unusual amino acid (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine (Bmt), is biosynthesized as a coenzyme A thioester from acetyl-CoA, malonyl-CoA, NADPH, and *S*-adenosylmethionine via a polyketide pathway. Here we present detailed enzymatic studies about the basic assembly process. After attachment of the activated building units to Bmt polyketide synthase the intermediates remained enzyme-bound throughout the cycle. Premature cutoff of biosynthesis led to the release of the intermediates from the enzyme, either as coenzyme A thioesters or, in the case of reactive C8-intermediates, as lactones. Enzyme-bound 3-oxo-4-hexenoic acid, the condensation product of the second elongation cycle, could be identified as the exclusive substrate for the introduction of the methyl group. Part of the biosynthesis including the first elongation cycle, the second condensation reaction, and the methylation step was shown to follow a processive mechanism. All activated intermediates of this processive part could be introduced into the correct pathway at the respective steps, whereas 2-methyl-3-oxo-4-hexenoyl-CoA and all following methylated intermediates were not able to enter the cycle any more. Obviously, the region of Bmt polyketide synthase responsible for this latter part of the biosynthetic pathway is inaccessible for externally supplied coenzyme A thioesters. Butyryl-CoA was recognized by Bmt polyketide synthase with an efficiency comparable to that of crotonyl-CoA and processed to 3-hydroxy-4-methyloctanoyl-CoA, the saturated analog of the natural basic assembly product, indicating a relaxed specificity of Bmt polyketide synthase with respect to the starter unit.

Cyclosporins, produced by the fungus *Tolypocladium niveum*, are a family of hydrophobic cyclic undecapeptides with a remarkable spectrum of biological activities (von Wartburg & Traber, 1988). Since 1983, the main metabolite cyclosporin A has been the major drug used to prevent the rejection of organ transplants (Borel, 1989; Feutren, 1989). Cyclosporins are biosynthesized via a non-ribosomal pathway by an extraordinarily large multienzyme called cyclosporin synthetase (Lawen & Zocher 1990; Schmidt et al., 1992; Weber et al., 1994; Leitner et al., 1994). Two out of the eleven constituent amino acids of cyclosporins are supplied by separate pathways: D-alanine, which is derived from L-alanine by a specific racemase (Hoffmann et al., 1994; Kocher et al., 1994), as well as the unusual amino acid (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine (Bmt).[1]

Incorporation studies with ^{13}C-labeled acetates (Kobel et al., 1983) and with ^{13}C-labeled glucose (Senn et al., 1991) proved the polyketide origin of the Bmt backbone. Further detailed incorporation experiments as well as enzymatic studies with cell free extracts of *T. niveum* revealed that the basic assembly reaction, performed by Bmt polyketide synthase, ends at the stage of 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoyl-CoA (Offenzeller et al., 1993), which needs at least two or three additional transformation reactions for its conversion to the end product Bmt (Figure 1). Acetyl-CoA, malonyl-CoA, NADPH, and *S*-adenosylmethionine have been identified as the substrates of the basic assembly reaction. However, further features such as the processive or non-processive nature of the reaction sequence as well as the timing of the methylation step remained obscure. Knowledge of these details is not only of basic interest in polyketide science but also essential to assess the prospects of successfully obtaining structural variants of Bmt and hence novel cyclosporins by introducing cycle intermediates and structural relatives thereof into the biosynthetic pathway.

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[1] Abbreviations: ACP, acyl carrier protein; AdoMet, *S*-adenosylmethionine; Bmt, (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine; Br-DMC, 4-bromomethyl-6,7-dimethoxycoumarin; CoA, coenzyme A; cpm, counts per minute; FAB, fast atom bombardment, HPLC, high-pressure liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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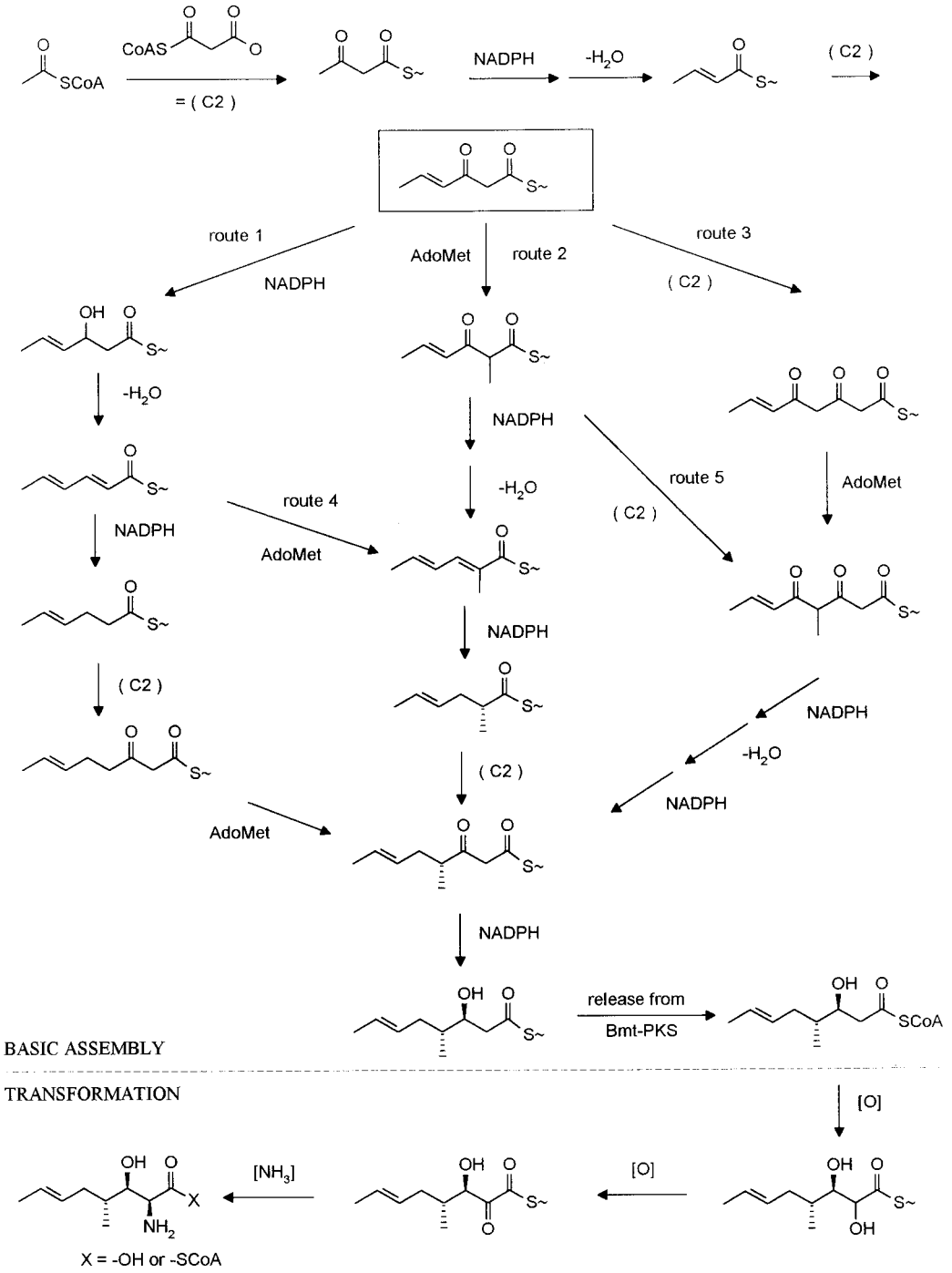

FIGURE 1: Possible routes of the biosynthetic pathway of Bmt. The basic assembly reaction, leading to 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoyl-CoA, takes place in an enzyme-bound form (symbolized as −S∼). Our results exclude a pathway via route 1 as well as routes 3 and 4 and support a biosynthesis model including route 2 or route 5.

Here we present the results of extensive studies about the various reactivities of Bmt polyketide synthase and for the first time a reliable identification of the direct methylation precursor in a basic assembly reaction.

EXPERIMENTAL PROCEDURES

Fungal Strains and Media. T. niveum, strain NRRL 8044, was used. Culture conditions for *in vivo* experiments were as described by Offenzeller et al. (1993), and those for *in vitro* experiments were as described by Lawen et al. (1989).

Labeled Precursors and Chemicals. Sodium [1-^{13}C,^{2}H$_3$]-acetate (99% ^{13}C, 99% ^{2}H) and sodium [2-^{13}C,^{2}H$_3$]acetate (97% ^{13}C, 97% ^{2}H) were purchased from Aldrich Chemical Co. All radiochemicals were obtained from NEN. 3(*R*)-Hydroxy-4(*R*)-methyl-6(*E*)-octenoyl-CoA, 3-oxohexanoyl-CoA, 2(*E*)-hexenoyl-CoA, 4(*E*)-hexenoyl-CoA, 2,4-(*E*,*E*)-hexadienoyl-CoA (= sorbyl-CoA), and 2(*R*)-methyl-4(*E*)-hexenoyl-CoA were synthesized as described below. All other coenzyme A thioesters and coenzyme A, sodium salt, were supplied by Sigma. Chemicals for organic synthesis were obtained from Fluka.

Organic Synthesis of Postulated Intermediates of the Biosynthetic Pathway. The coenzyme A thioesters of 3-oxohexanoic acid, 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoic acid, 2(*R*)-methyl-4(*E*)-hexenoic acid, and 4(*E*)-hexenoic acid were synthesized by activation of the carboxyl group with *N*,*N*'-carbonyldiimidazole according to Kawaguchi et al. (1981). The 3-keto group of 3-oxohexanoic acid had to be

protected prior to the activation reaction; therefore the keto function of the 3-oxohexanoic acid ethyl ester was converted to the ketal with ethylene glycol/*p*-toluenesulfonic acid (Elix & Whitton., 1989). Hydrolysis of the ester with KOH yielded the ethylene ketal of 3-oxohexanoic acid which was subjected to thioesterification with coenzyme A. After purification the product was deprotected by heating in tetrahydrofuran/acetone/*p*-toluenesulfonic acid (Al-Arif & Blecher, 1971).

The coenzyme A thioesters of 2(*E*)-hexenoic acid and 2,4-(*E*,*E*)-hexadienoic acid were obtained by activation of the carboxyl group with isobutyl chloroformate/triethylamine (Lai et al., 1991).

All coenzyme A thioesters were purified by HPLC (Lai et al., 1991) and characterized by FAB-MS as well as ^{1}H- and ^{13}C-NMR spectroscopy.

3(*R*)-Hydroxy-4(*R*)-methyl-6(*E*)-octenoic acid was prepared as described (Offenzeller et al., 1993). 2(*R*)-Methyl-4(*E*)-hexenoic acid was synthesized according to Deyo et al. (1988) as well as according to Evans and Weber (1986). 4(*E*)-Hexenoic acid ethyl ester, which was prepared according to Petrzilka (1978), was subjected to alkaline hydrolysis to obtain the free 4(*E*)-hexenoic acid.

Preparation of Cell-Free Extracts and Partial Purification of Bmt Polyketide Synthase. These procedures were performed as described (Offenzeller et al., 1993).

Enzyme Assays and Incorporation Studies. Products of *in vitro* enzyme reactions were analyzed either as coenzyme A thioesters or after alkaline hydrolysis as free carboxylic acids (in the case of radioactive labeling of the products) or their respective Br-DMC derivatives (see below). In the case of analysis of the reaction products as free acids or as their Br-DMC derivatives the enzyme reactions were essentially performed as described earlier (Offenzeller et al., 1993) with enzyme fractions eluted from a Sephacryl S-300 HR column (Offenzeller et al., 1993). Radioactive labeling of the products was achieved by addition of 18.5 kBq of *S*-[*methyl*-^{14}C]AdoMet (2.2 GBq/mmol). In the case of subsequent analysis of the coenzyme A thioesters the *in vitro* reactions were carried out under the same conditions, but in a total volume of 100 μL and stopped by heating to 90 °C for 5 min. For the incorporation studies 200 μM of the respective substance as a coenzyme A thioester was added to the reaction mixture instead of 200 μM of acetyl-CoA.

TLC Analysis of Radioactively Labeled in Vitro Products. Radioactively labeled *in vitro* products were hydrolyzed by addition of KOH, extracted from the reaction mixtures after acidification, and analyzed as free carboxylic acids by TLC, as described by Offenzeller et al. (1993).

HPLC Analysis of Br-DMC Derivatives. Carboxylic acids, obtained in reaction mixtures after alkaline hydrolysis and acidification, were extracted and converted to Br-DMC derivatives as described earlier (Offenzeller et al., 1993). The Br-DMC derivatives were separated by HPLC (Offenzeller et al., 1993) and detected by fluorescence (excitation 343 nm, emission 423 nm).

HPLC Analysis of Coenzyme A Thioesters. Coenzyme A thioesters, obtained in reaction mixtures without further treatment, were subjected to liquid chromatography according to Norwood et al. (1990), with minor modifications. Separation was carried out with an HP 1050 liquid chromatograph, equipped with an HP 1040 diode array detector, on a 150 mm × 4.1-mm inner diameter analytical column packed with 3 μm Nucleosil 100-C_{18}. A 20-μL aliquot of the reaction mixture was injected. Separation of the compounds was achieved with 0.2 M sodium acetate, pH 6.9, as eluent A and acetonitrile as eluent B, at a flow rate of 1.0 ml/min with the following profile: 8% B for 3 min, followed by a linear gradient to 35% B in 20 min, to 55% B from 20 to 21 min, isocratic elution with 55% B from 21 to 23 min and finally a gradient from 55% to 8% B within another minute. Coenzyme A-containing compounds were monitored by UV absorbance at 260 nm.

HPLC Analysis of Lactones. Liquid chromatography was performed as described above with minor modifications. For the separation of the lactones the solvents were delivered in a linear gradient from 0% to 35% B in 20 min, followed by isocratic elution with 55% B from 20 to 23 min and another linear gradient from 55% to 0% B from 23 to 24 min. Lactones were detected at 295 nm.

LC−MS Analysis of the Br-DMC Derivatives of the Carboxylic Acids. This procedure was performed as described (Offenzeller et al., 1993).

LC−MS Analysis of the Coenzyme A Thioesters and of the Lactones. Liquid chromatography was carried out at ambient temperature on an RP-18, Spherisorb 3-μm (PhaseSep) material packed into a stainless steel 100 × 2.0-mm inner diameter column, at a flow rate of 200 μL/min. Separation of coenzyme A thioesters as well as of lactones was achieved as described above. The total HPLC effluent was split 1:20 (Accurate, LC-Packings) before deliverance to the pneumatically assisted electrospray (ion spray) interface. Ion spray ionization mass spectrometry was performed on the Perkin Elmer/SCIEX API triple-quadrupole mass spectrometer in the positive ion mode. The orifice voltage was maintained at 70 V, and the first RF-only quadrupole (RO) was maintained at 30 V. The interface temperature was kept at 50 °C. High-purity dry nitrogen gas was used as curtain gas at a flow rate of 0.8 L/min. Air was used as nebulizing gas for the ion spray interface at a flow rate of 1.2 L/min.

In Vivo Incorporation of Labeled Acetates and Isolation of Cyclosporin A from Fermentation Broth. *In vivo* incorporation of labeled precursors was performed according to Offenzeller et al. (1993), and isolation of cyclosporins was performed according to Senn et al. (1991). From 500 mL of culture broth (precursor [1-^{13}C,$^{2}H_3$]acetate) and from 300 mL of culture broth (precursor [2-^{13}C,$^{2}H_3$]acetate), 20 mg and 12 mg of cyclosporin A, respectively, were isolated.

^{13}C- and ^{2}H-NMR Analysis of Cyclosporins. ^{13}C-NMR spectroscopy was carried out as described (Senn et al., 1991). ^{2}H-NMR spectra were recorded in $CHCl_3$ (puriss.) at 25 °C on a Bruker DMX-500 spectrometer using a 5-mm broadband probe. The natural abundance $CDCl_3$ signal was taken as internal reference (7.26 ppm).

RESULTS

Products and Byproducts Are Released from Bmt Polyketide Synthase as Coenzyme A Thioesters. All intermediates of the basic assembly pathway (Figure 1) remain enzyme-bound throughout the entire biosynthetic cycle, as will be shown in the following sections. Since the exact nature of this binding remains to be elucidated, the intermediates—with the exception of the externally supplied coenzyme A thioesters—are denoted as carboxylic acids. However, release of 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoic acid, the end product of the basic assembly process, as a coenzyme A thioester is

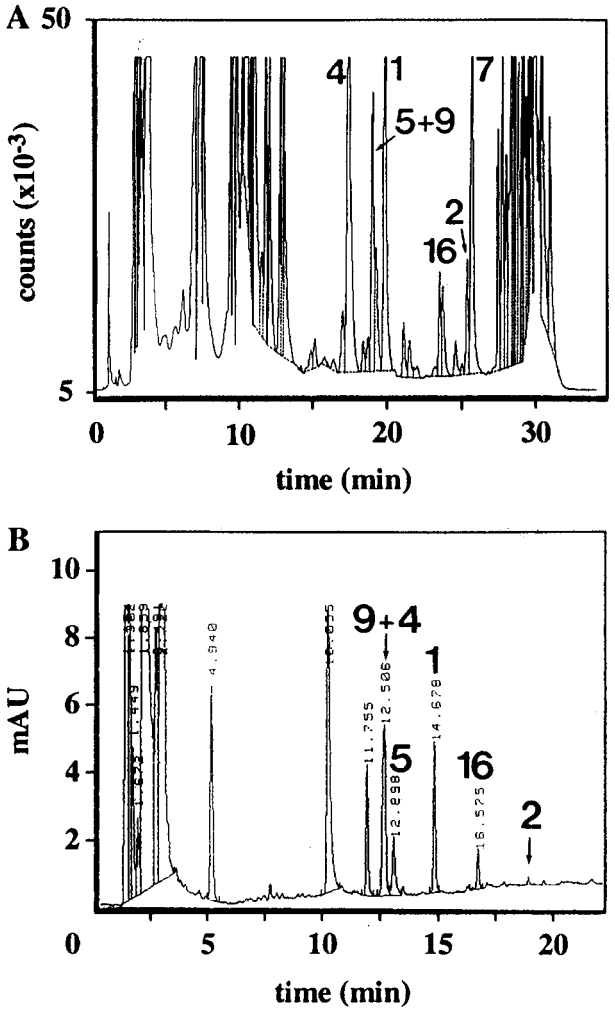

FIGURE 2: HPLC analysis of *in vitro* products of Bmt polyketide synthase. Reactions were carried out with enzyme fractions eluted from Sephacryl S-300 HR as described under Experimental Procedures. Reaction products were analyzed by the coenzyme A thioester method (panel B, mAU are milli absorbance units at 260 nm) as well as after hydrolysis of the thioesters and subsequent derivatization of the free carboxylic acids with Br-DMC by fluorescence (panel A, counts are relative absorbance units), both methods outlined under Experimental Procedures. Only those peaks were given a compound number (listed in Table 1) which were identified by a reference sample and/or by mass determination. Octanoyl-CoA (peak 7 in A) served as an internal standard.

necessary for re-entrance of Bmt polyketide synthase into another round of the cycle (Offenzeller et al., 1993). Hence this end product is depicted in Figure 1 as coenzyme A thioester. The analysis of a representative *in vitro* reaction mixture was carried out by HPLC separation and detection of all acyl compounds having been released from the enzyme as coenzyme A thioesters (Figure 2B, coenzyme A thioester method) as well as by HPLC separation and detection of all carboxylic acids obtained after alkaline treatment of the reaction products (Figure 2A, carboxylic acid method; compound numbers listed in Table 1). Besides the end product (compound **1**), the non-methylated analog 3(*R*)-hydroxy-6(*E*)-octenoyl-CoA (compound **4**) is released from the enzyme as a coenzyme A thioester. Some additional compounds can be detected in the reaction mixtures, for example, compounds **5** [4(*E*)-hexenoic acid] and **16** (2,4,6-octatrienoic acid). They obviously originate from *in vitro* bypass reactions catalyzed by the enzyme, since they cannot serve as intermediates in the biosynthetic pathway (compare Figure 1 and Table 1). Compound **2**, earlier identified as

Table 1: Correlation of Peaks in HPLC Analysis with Chemical Structures[a]

compd no.	substance (acyl-)	compd no.	substance (acyl-)
1	3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoyl-	**13**	2-methylhexenoyl-
2	4(*R*)-methyl-2,6-(*E*,*E*)-octadienoyl-	**14**	3-hydroxy-4-methyloctanoyl-
3	4(*R*)-methyl-6(*E*)-octenoyl-	**15**	2-hexenoyl-
4	3(*R*)-hydroxy-6(*E*)-octenoyl-	**16**	2,4,6-octatrienoyl-
5	4(*E*)-hexenoyl-	**17**	butyryl-
6	6(*E*)-octenoyl-	**18**	3-hydroxyoctanoyl-
7	octanoyl-	**19**	2,4-octadienoyl-
8	hexanoyl-	**20**	3-octenoyl-
9	sorbyl-	**21**	3-oxohexanoyl-
10	2-methyl-3-oxohexanoyl-	**22**	4-hydroxy-6-propyl-2-oxopyran
11	2-methyl-3-hydroxyhexanoyl-	**23**	4-hydroxy-5-methyl-6-propyl-2-oxopyran
12	2-methylhexanoyl-		

[a] Compounds **1**−**7** are also described in Offenzeller et al. 1993). The table comprises all substances which were detected in *in vitro* reaction mixtures in course of the experiments. The compounds were numbered chronologically irrespective of their identification either as a Br-DMC derivative or as a coenzyme A thioester. The identity of the compounds was verified by their elution behavior in HPLC and by LC−MS analysis as described under Experimental Procedures.

4(*R*)-methyl-2,6-(*E*,*E*)-octadienoic acid, most likely is derived from compound **1** by activities contaminating the enzyme preparation (Offenzeller et al., 1993). It should be noted that these (and also some other non-explicitly described) *in vitro* byproducts of Bmt polyketide synthase as well as the products of contaminating activities (Offenzeller et al., 1993) detected in the course of the experiments varied in their amounts due to slight batch to batch differences in the purity of the enzyme preparation used. Interestingly, no compounds were detected in these reaction mixtures which could eventually serve as biosynthetic intermediates, with the exception of sorbyl-CoA (compound **9**). However, since in the following experiments sorbyl-CoA was excluded as a methylation precursor, the appearance of this compound in the *in vitro* reaction mixtures most likely reflects yet another bypass reaction of Bmt polyketide synthase.

The First Elongation Cycle Follows a Processive Mechanism. In Figure 1, a processive mechanism for the first elongation cycle, i.e., conversion of the C atoms to their final oxidation state prior to the next elongation step, is postulated. To test this hypothesis, incorporation studies with acetoacetyl-CoA and crotonyl-CoA were performed. Radioactively labeled reaction products were hydrolyzed and subjected to thin-layer chromatography as described (Offenzeller et al., 1993). Table 2 shows the incorporation amounts of different starter units into the end product 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoyl-CoA, expressed as the counts per minute originating from radioactively labeled methyl groups at C4. Incorporation amounts of crotonyl-CoA were rather low but significantly above background indicating that the first elongation cycle indeed follows a processive mechanism. Formation of small amounts of product in the absence of any starter substance resulted from the ability of the enzyme to decarboxylate malonyl-CoA to acetyl-CoA (data not shown), a mechanism already known from fatty acid synthases, 6-methylsalicylic acid synthase, and chalcone synthase (Dimroth et al., 1976; Kreuzaler et al., 1978).

To analyze the specificity of Bmt polyketide synthase with respect to the starter unit, incorporation of butyryl-CoA in

Table 2: Incorporation Studies with Different Starter Molecules[a]

starter substance (100 μM)	cpm	incorporation amount (%)
acetyl-CoA	11275	100
−	935	8.2
acetoacetyl-CoA	7315	65
crotonyl-CoA	3565	32
butyryl-CoA	4317	38

[a] Enzyme fractions eluted from Sephacryl S-300 HR [50 μL, containing 50 μg of total protein; refer to Offenzeller et al. 1993)] were incubated in a total volume of 500 μL with different starter substances (200 μM in each case) in the presence of 100 mM KH_2PO_4, pH 7, 150 μM malonyl-CoA, 200 μM NADPH, 200 μM AdoMet, and 18.5 kBq *S*-[*methyl*-^{14}C]AdoMet (2.2 GBq/mmol). The resulting products were hydrolyzed and separated by TLC as described (Offenzeller et al., 1993). Cpm are the counts per minute originating from radioactively labeled methyl groups at the C4 atom of the respective products. Since the starter molecules and the C4 methyl groups are incorporated into the end product in an equimolar ratio, the incorporation amount of the C4 methyl group directly reflects the *in vitro* incorporation efficiency of the starter molecules compared to that of the natural starter substance acetyl-CoA (the incorporation amount of the latter defined as 100%).

comparison to crotonyl-CoA was tested. Indeed, butyryl-CoA (compound **17**) could be processed along the correct biosynthetic pathway and elongated to 3-hydroxy-4-methyl-octanoyl-CoA (compound **14**), the saturated analog of 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoyl-CoA (compound **1**), as shown in Figures 3A and B. Incorporation amounts were slightly higher than for crotonyl-CoA (Table 2). Regarding this high amount of turnover of butyryl-CoA and the incorporation of the methyl group exclusively into the analogous position of the backbone, it can be assumed that the observed reactions are carried out by Bmt polyketide synthase itself rather than by another activity contaminating the enzyme preparation.

Methylation Takes Place in the Second Elongation Cycle. To address the question whether methylation takes place in the second or third elongation cycle comparative incorporation studies with C4 and C6 units were performed. In a first approach butyryl-CoA (compound **17**) and hexanoyl-CoA (compound **8**) were chosen, which represent the readily available saturated analogs to the thioesters of the putative biosynthetic intermediates crotonic acid and 4-hexenoic acid shown in Figure 1, and which are just as well recognized by the enzyme as already demonstrated for butyryl-CoA (Figure 3A and Table 2).

Hexanoyl-CoA (compound **8**) was elongated and reduced to 3-hydroxyoctanoyl-CoA (compound **18**) which dehydrated to 3-octenoyl-CoA (compound **20**) as well as to 2-octenoyl-CoA, the latter having been quantitatively reduced to octanoyl-CoA (compound **7**). Beyond that, hexanoyl-CoA could not be transformed to any methylated product by Bmt polyketide synthase (Figure 3C). In contrast, butyryl-CoA (compound **17**) yielded, as already mentioned, the expected end product 3-hydroxy-4-methyloctanoyl-CoA (compound **14** in Figure 3A) in addition to some unmethylated products (compound **8**, **18**, and **19**). This was a first hint that methylation might take place in the second cycle prior to the transformation of the condensation product to the reduced C6-backbone. In this case the candidates for methylation substrates would be either 3-oxohexanoic acid [compound **21**, in analogy to the cycle intermediate 3-oxo-4-(*E*)-hexenoic acid; see Figure 1, route 2] or 2-hexenoic acid (compound **15**, in analogy to 2,4-hexadienoic acid = compound **9**; Figure 1, route 4). To distinguish between these two possible routes,

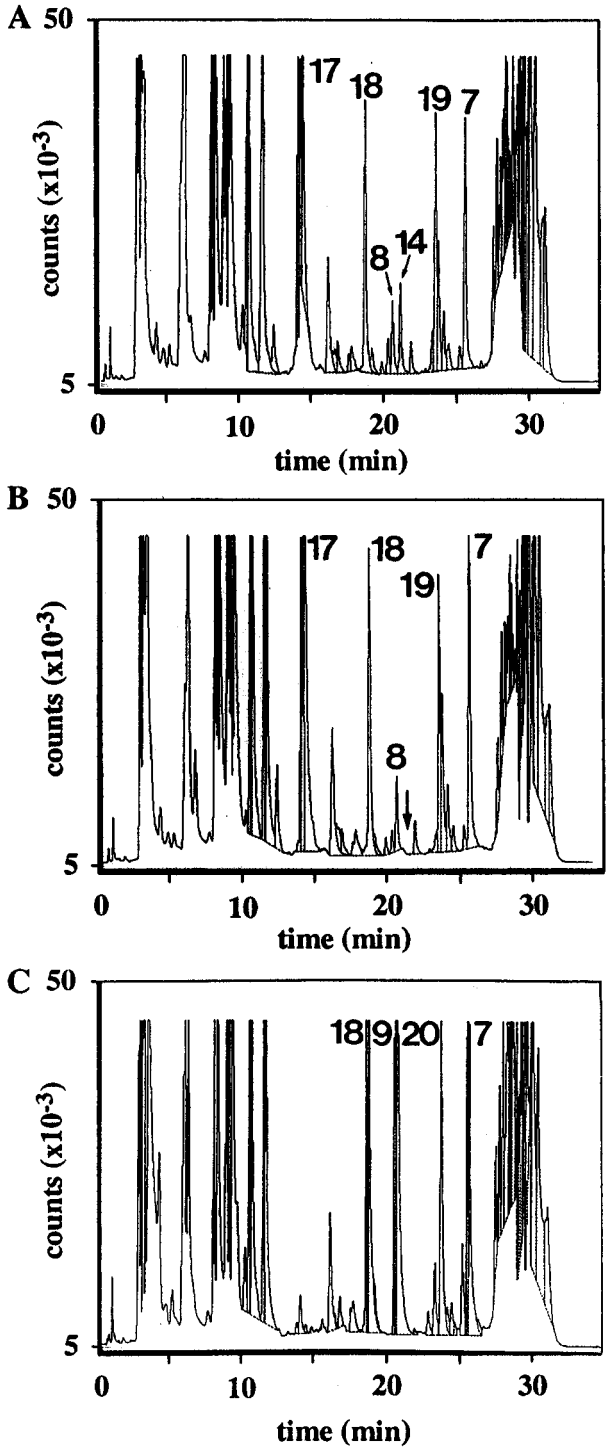

FIGURE 3: Comparative incorporation studies with butyryl-CoA and hexanoyl-CoA. Products derived from reaction mixtures containing either 200 μM butyryl-CoA (A and B) or 200 μM hexanoyl-CoA (C) as a starter molecule were analyzed after hydrolysis of the thioesters and derivatization of the resulting carboxylic acids with Br-DMC by the fluorescence method, as described under Experimental Procedures. Counts are relative absorbance units. Reaction mixtures contained all substrates necessary for the biosynthesis of 3-hydroxy-4-methyloctanoyl-CoA [saturated analog to 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoyl-CoA], but the methyl group donor AdoMet was omitted from the mixture analyzed in B. Incorporation of butyryl-CoA (compound **17**) yielded 3-hydroxy-4-methyloctanoyl-CoA (compound **14** in A, absent in the AdoMet free control B, see arrow), whereas hexanoyl-CoA (compound **8**) was elongated to non-methylated products only (C). For identity of the compounds confer Table 1. Octanoyl-CoA (compound **7**) was used as an internal standard in the analysis of the reaction mixtures (A) and (B), whereas it could be detected as a main product in mixture (C).

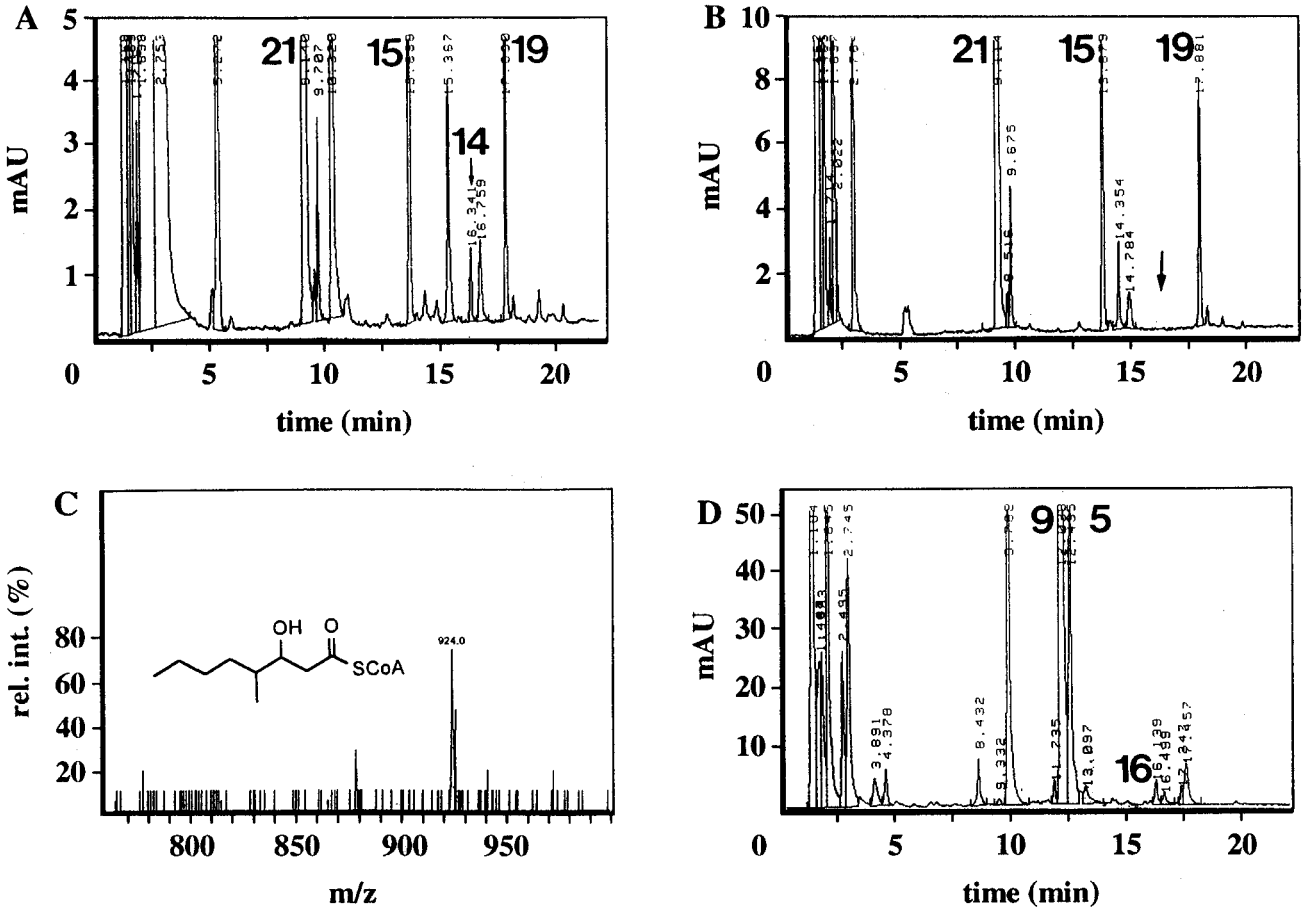

FIGURE 4: Comparative incorporation studies with 3-oxohexanoyl-CoA and sorbyl-CoA. Products derived from reaction mixtures containing either 200 μM 3-oxohexanoyl-CoA (panel A, AdoMet-free control in B) or 200 μM sorbyl-CoA (D) as a starter molecule were analyzed by the coenzyme A thioester method as described under Experimental Procedures. mAU are milli absorbance units at 260 nm. Whereas 3-oxohexanoyl-CoA (compound **21**) was elongated to the correct end product 3-hydroxy-4-methyloctanoyl-CoA (compound **14** in A, absent in the AdoMet-free control B, see arrow), sorbyl-CoA (compound **9**) yielded only non-methylated products (D). Compound **14** was identified as 3-hydroxy-4-methyloctanoyl-CoA by mass analysis ($[M + H]^+ = 924.0$, rel int = relative intensity). For identity of the compounds refer to Table 1.

the corresponding coenzyme A thioesters were tested as starter units in the *in vitro* system. Since the thioester of 3-oxo-4(*E*)-hexenoic acid is unstable and Bmt polyketide synthase accepts the saturated compound as well, 3-oxohexanoyl-CoA was used in the following incorporation studies.

As shown in Figure 4, 3-oxohexanoyl-CoA (compound **21**) yielded several acyl thioesters when incubated with all necessary substrates for the biosynthesis of 3-hydroxy-4-methyloctanoyl-CoA (i.e., *S*-adenosylmethionine, malonyl-CoA, and NADPH). Besides some unmethylated byproducts (compounds no. **15** and **19**) one methylated peak (compound **14**, Figure 4A) was found which could be identified unequivocally by mass analysis as 3-hydroxy-4-methyloctanoyl-CoA (Figure 4C: $[M + H]^+ = 924.0$), the saturated analog of the natural end product 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoyl-CoA. The reasons for the low yield of the correct end product (about 20% to 30% compared to an *in vitro* reaction starting with acetyl-CoA) have been investigated and are discussed below.

The failure of the second possible candidate for the methylation step, 2,4-hexadienoic acid (the respective coenzyme A thioester in analogy to fatty acid biosynthesis supplied in its 2,4-(*E*,*E*)-configuration = sorbyl-CoA, compound **9**), to yield 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoyl-CoA or any other methylated product (Figure 4D) pointed toward 3-oxo-4(*E*)-hexenoic acid as the direct methylation substrate in the biosynthetic cycle (Figure 1, route 2). However, the possibility that 2,4-hexadienoic acid could be a correct intermediate of the pathway, if present as a 2,4-(*Z*,*E*)-isomer, could not be excluded by this experiment.

Identification of 3-Oxo-4(E)-hexenoic Acid As the Direct Methylation Substrate. To confirm the role of 3-oxo-4(*E*)-hexenoic acid or of its analog 3-oxohexanoic acid, respectively, as a direct methylation precursor and to find the reasons for the low incorporation rates of the corresponding coenzyme A thioesters into the end product, we dissected the single biosynthesis steps by specifically omitting and/or adding successively the individual substrates.

3-Oxohexanoyl-CoA (compound **21**), when incubated with the methyl group donor *S*-adenosylmethionine in the absence of NADPH and malonyl-CoA, gave 2-methyl-3-oxohexanoyl-CoA in a good yield (compound **10**, Figure 5A; mass analysis in Figure 5B, $[M + H]^+ = 894.2$), whereas no methylated product could be found after incubation of sorbyl-CoA with *S*-adenosylmethionine (data not shown). This result was taken as a proof that methylation takes place prior to reduction of the keto function.

The High Reducing Potential of NADPH Prevents Efficient in Vitro Processing of β-Oxocarboxylic Acids Along the Biosynthetic Pathway. In the presence of both *S*-adenosylmethionine and NADPH, 3-oxohexanoyl-CoA (compound **21**) was readily reduced and dehydrated to 2-hexenoyl-CoA (compound **15**) and further reduced to hexanoyl-CoA (compound **8**), respectively, in an enzyme-dependent manner,

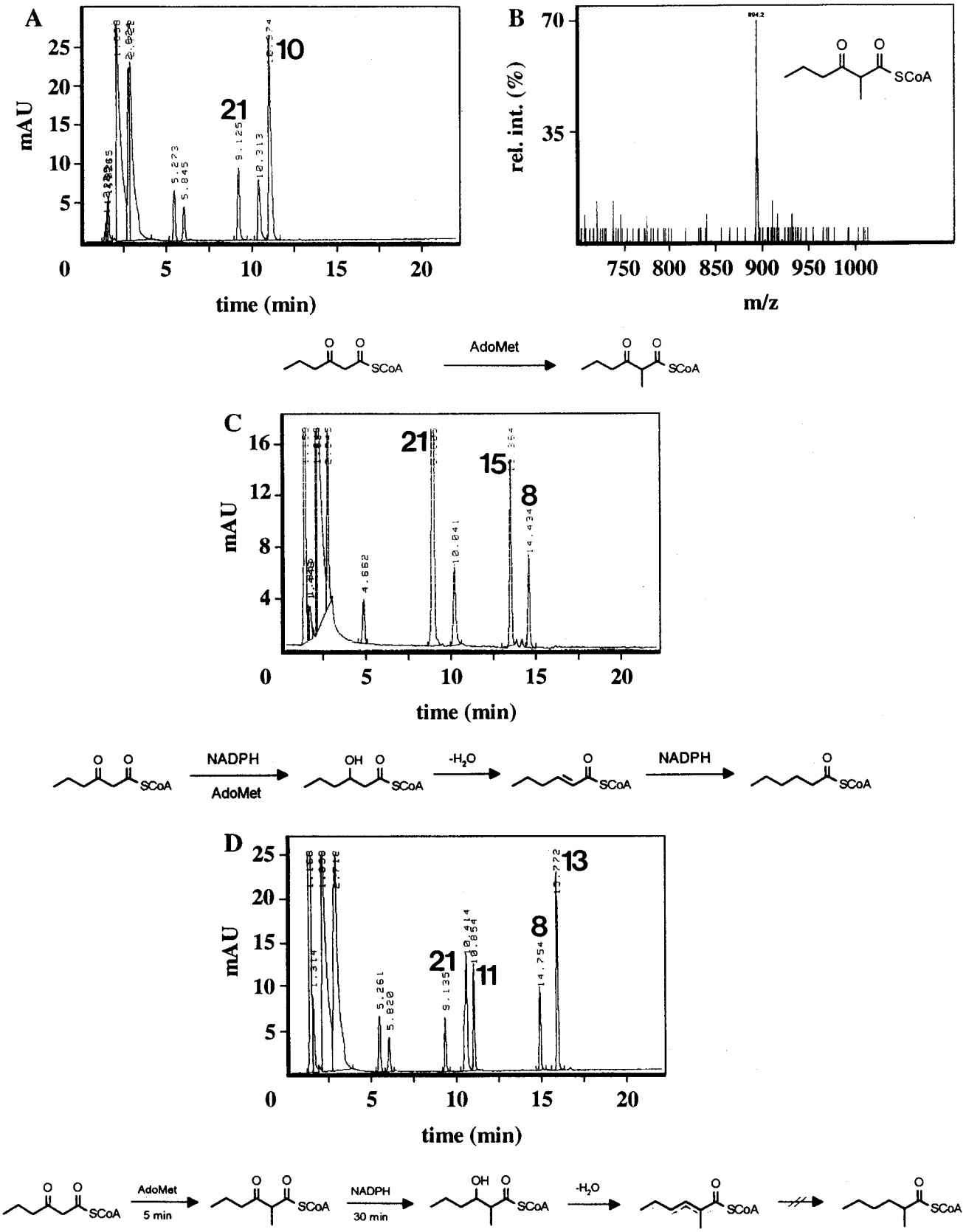

FIGURE 5: Analysis of the biosynthetic route by dissection of the single reactions. Educts and main products of the reactions are summarized below the respective chromatograms. Enzyme reactions were carried out as described under Experimental Procedures and the resulting products were analyzed by the coenzyme A thioester method (mAU are milli absorbance units at 260 nm). A. 200 μM of 3-oxohexanoyl-CoA (compound 21), incubated with AdoMet. B. identification of compound 10 in (A) as 2-methyl-3-oxohexanoyl-CoA by mass analysis ($[M + H]^+ = 894.2$, rel int = relative intensity). C. simultaneous incubation of 200 μM 3-oxohexanoyl-CoA with AdoMet and NADPH. D. Consecutive incubation of 200 μM 3-oxohexanoyl-CoA with (1) AdoMet for 5 min and (2) NADPH for another 30 min. Whereas 3-oxohexanoyl-CoA (compound **21**) was methylated in the absence of NADPH to 2-methyl-3-oxohexanoyl-CoA in a good yield (compound **10** in A), the presence of NADPH led to a rapid reduction of the β-oxo compound to 3-hydroxyoctanoyl-CoA with subsequent dehydration to 2-hexenoyl-CoA (compound **15** in C) and enoyl reduction to hexanoyl-CoA (compound **8** in C) thus preventing an efficient methylation reaction. Already methylated 3-oxohexanoyl-CoA was analogously reduced to 2-methyl-3-hydroxyoctanoyl-CoA (compound **11** in D) with subsequent dehydration to 2-methylhexenoyl-CoA (compound **13** in D).

whereas methylated products in reasonable amounts could not be detected (Figure 5C). Obviously reduction of 3-oxohexanoyl-CoA is so rapid that even in the presence of *S*-adenosylmethionine only a very small amount of precursor is available for the correct pathway, explaining the low incorporation rates into the end product found in the experiment described in Figure 4A.

If NADPH was added to 3-oxohexanoyl-CoA (compound **21**) after a 5-min preincubation period with *S*-adenosylmethionine, the 2-methyl-3-oxohexanoyl-CoA (compound **10**) initially formed was, in analogy to 3-oxohexanoyl-CoA, readily and quantitatively reduced to 2-methyl-3-hydroxyhexanoyl-CoA (compound **11**) which subsequently dehydrated to 2-methylhexenoyl-CoA (compound **13**, Figure 5D). No 2-methylhexanoyl-CoA could be detected in these reactions.

To circumvent the high reducing potential of NADPH on 3-oxocarboxylic acid thioesters and hence to get a higher yield of correct end product in a complete *in vitro* reaction mixture, incubation of 3-oxohexanoyl-CoA with the substrates was carried out in a consecutive manner: after 30 min of incubation with the methyl group donor and malonyl-CoA, NADPH was added. The end product 3-hydroxy-4-methyloctanoyl-CoA (compound **14**) was formed, but the yield was just as high as under simultaneous incubation of all substrates (HPLC data not shown). Apparently no enzyme-bound stabilized intermediate exists, which could have been transformed to the correct end product after addition of NADPH.

Premature Cutoff of Biosynthesis Leads to Irreversible Release of the Intermediates from the Enzyme. To further clarify this finding, radioactive chase experiments were performed. In the first experiment 3-oxohexanoyl-CoA was preincubated with radioactively labeled *S*-adenosylmethionine and with malonyl-CoA followed by the addition of NADPH simultaneously with an excess of unlabeled *S*-adenosylmethionine. 3-Hydroxy-4-methyloctanoyl-CoA was produced in these incubation mixtures, but, as reflected by the lower amount of radioactively labeled methyl groups at C4, rather due to synthesis starting from 3-oxohexanoyl-CoA than to transformation of an enzyme-bound already methylated intermediate (compound **14**, corresponds to spot o in Figure 6, lanes 5 and 6). The thin-layer chromatogram additionally revealed the formation of a more polar product (spot k) which had previously escaped detection by HPLC analysis. This product was no thioester because its chromatographic behavior was not changed after alkaline hydrolysis (data not shown). Considering a well-known reactivity of 6-methylsalicylic acid synthase (Dimroth et al., 1990; Spencer & Jordan, 1992) which leads to formation of a triacetolactone in the absence of NADPH, an analogous reactivity was postulated for Bmt polyketide synthase. Indeed, by eluting the substance causing spot k from thin-layer chromatograms and subjecting it to HPLC as well as to mass analysis it could be identified as a mixture of the lactones 4-hydroxy-5-methyl-6-propyl-2-oxopyran (compound **23**, Figures 7A and C) and its unmethylated analog 4-hydroxy-6-propyl-2-oxopyran (compound **22**, Figures 7A and B).

In the second experiment, preincubation of 3-oxohexanoyl-CoA with radioactively labeled *S*-adenosylmethionine alone was followed by simultaneous addition of malonyl-CoA and an excess of unlabeled *S*-adenosylmethionine. The lactones described above were formed but, again, were due to synthesis starting from 3-oxohexanoyl-CoA rather than to elongation of an enzyme-bound methylated intermediate (Figure 6, lanes 2 and 3).

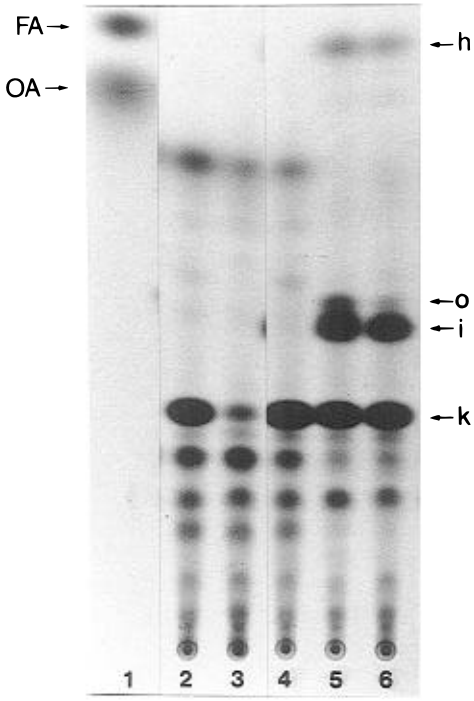

FIGURE 6: TLC analysis of ^{14}C-labeled *in vitro* products of Bmt polyketide synthase with 3-oxohexanoyl-CoA as a starter molecule. Enzyme reactions were carried out as described under Experimental Procedures. Products were labeled by the addition of 18.5 kBq of *S*-[*methyl*-^{14}C]AdoMet (2.2 GBq/mmol) to each reaction mixture, subjected to TLC after alkaline hydrolysis as described by Offenzeller et al. (1993), and visualized by autoradiography. For identification analogously synthesized products lacking the radioactive label were eluted from thin-layer chromatograms and subjected to HPLC analysis. Markers used: FA = fatty acids (mixture of stearic and palmitic acid), OA = octanoic acid. Preincubation of 3-oxohexanoyl-CoA with AdoMet and malonyl-CoA for 5 min predominantly yielded the lactones 4-hydroxy-5-methyl-6-propyl-2-oxopyran and 4-hydroxy-6-propyl-2-oxopyran (spot k, lane 4, corresponding to compounds **23** and **22**; for a detailed analysis of the lactones see Figure 7). After addition of NADPH the expected products 3-hydroxy-4-methyloctanoic acid (spot o, 4200 cpm, corresponding to compound **14**) and 2-methyl-3-hydroxyhexanoic acid (spot i, 17 400 cpm, corresponding to compound **11**) were formed (lane 5). Additionally 2-methylhexenoic acid, the dehydration product of 2-methyl-3-hydroxyhexanoic acid (spot h, corresponding to compound **13**) was detected. The same products were found under "chase" conditions with a 10-fold excess of unlabeled AdoMet (lane 6) but, as outlined for 3-hydroxy-4-methyloctanoic acid (spot o = compound **14**), with a lower specific labeling rate (1700 cpm) indicating the origin of the substance from 3-oxohexanoyl-CoA after addition of NADPH. Preincubation of 3-oxohexanoyl-CoA with AdoMet alone and addition of malonyl-CoA 5 min later yielded also the lactones causing spot k (lane 2, 40 000 cpm). Addition of malonyl-CoA under 10-fold excess of unlabeled AdoMet (lane 3) yielded 10 000 cpm of spot k, indicating again synthesis of the products starting from 3-oxohexanoyl-CoA is not influenced by the sequence of addition of the further substrates.

These results show that in the absence of any of the biosynthesis substrates Bmt polyketide synthase releases the respective intermediate in a bypass reaction, either as a coenzyme A thioester, or in the case of an intermediate with a C8-backbone containing 3,5-dioxo-groups, as a lactone. Neither of the compounds derived from 3-oxohexanoyl-CoA could re-enter the cycle, showing that the biosynthetic reaction sequence, at least in the second and

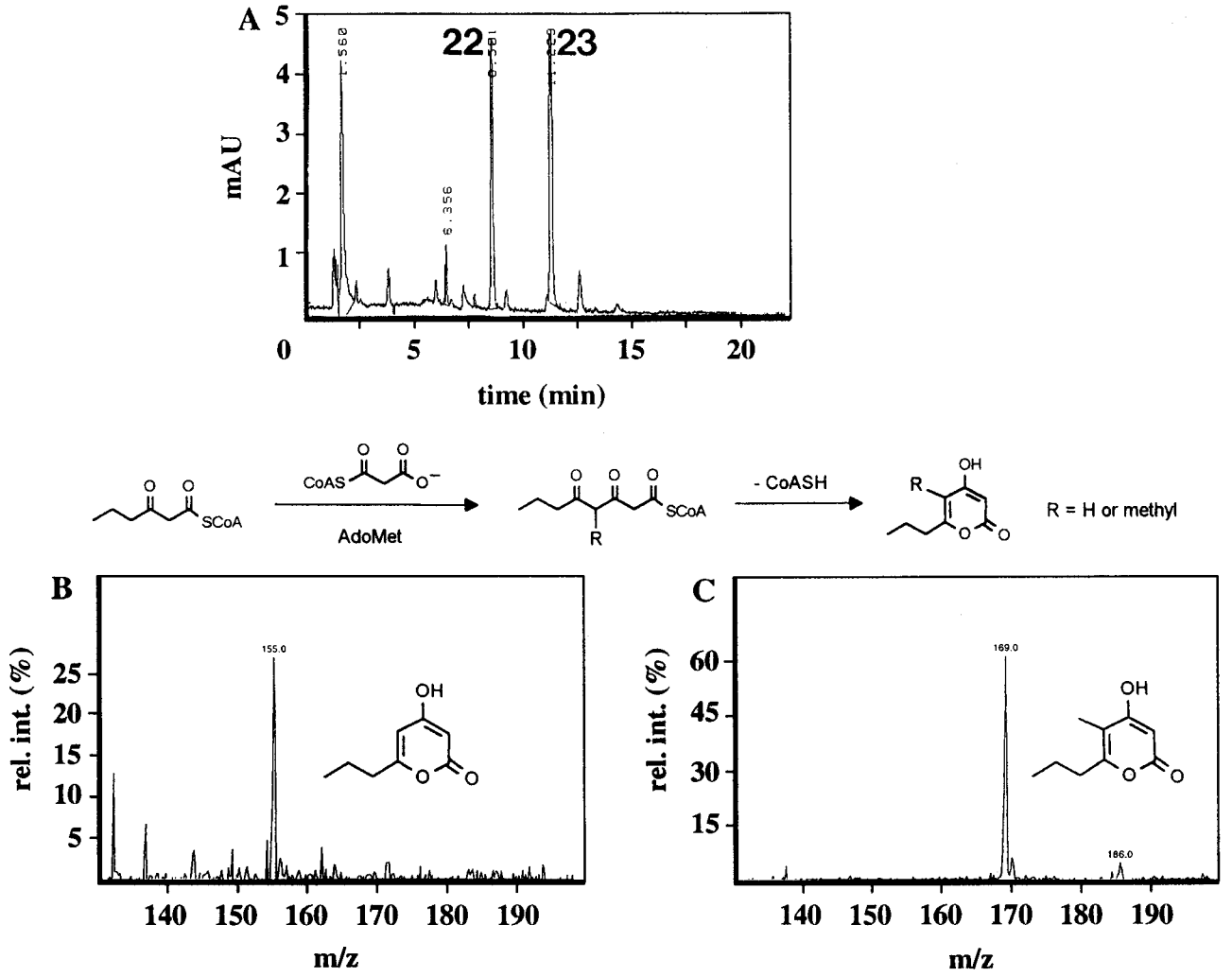

FIGURE 7: Identification of the product causing spot k in Figure 6 as a mixture of lactones. The product causing spot k in Figure 6 was biosynthesized without the radioactive label, eluted from thin-layer chromatograms, and subjected to HPLC analysis (for details see Experimental Procedures). Two peaks were detected at 295 nm (A) and identified by mass analysis as 4-hydroxy-5-methyl-6-propyl-2-oxopyran (compound **23**, mass analysis in C, $[M + H]^+ = 169.0$) and as the non-methylated variant 4-hydroxy-6-propyl-2-oxopyran (compound **22**; mass analysis in B, $[M + H]^+ = 155.0$).

third elongation cycles including the methylation step, takes place on enzyme-bound intermediates in an area of the enzyme which is inaccessible for externally supplied coenzyme A thioesters.

Hence it was not possible to increase the yield of correctly methylated end product with 3-oxohexanoyl-CoA as starter unit by consecutive addition of the biosynthesis substrates.

In Vivo Methylation also Takes Place in the Second Elongation Cycle. To support the *in vitro* result that methylation takes place on 3-oxo-4(*E*)-hexenoic acid, *in vivo* incorporation experiments using [1-^{13}C,^{2}H$_3$]- and [2-^{13}C,^{2}H$_3$]-acetate as precursors with subsequent NMR analysis of the isolated fermentation end product cyclosporin A were performed. According to the mechanism of a head-to-tail condensation of four acetate units (Kobel et al., 1983; Offenzeller et al., 1993), deuterium is expected in the terminal methyl group (C8) and at the olefinic double bond in position 6. The lack of deuterium at C4 would be a strong hint for a biosynthetic parthway via route 2 in Figure 1 and hence in accordance with the *in vitro* results. However, it should be mentioned that, due to the notorious lability of H atoms adjacent to carbonyl groups in the course of the assembly reaction, routes 1 and 4 cannot be excluded definitely by this finding. The proposed transformation process for introduction of the amino function (Figure 1) would lead to the elimination of deuterium at C2.

In the experiment with [1−^{13}C,^{2}H$_3$]acetate, the ^{1}H-decoupled ^{13}C-NMR spectrum of the cyclosporin A sample showed an incorporation rate about 20-fold above natural abundance. The signal of the olefinic carbon C7 was as expected (Figure 8A). For C3, the ^{13}C resonance at 74.69 ppm was not accompanied by a β-isotope shifted signal [most frequent values about 0.1 ppm upfield, cf. Vederas (1987)], indicating absence of deuterium at the neighboring carbon atom C4 (Figure 8B). In the proton-decoupled ^{2}H-NMR spectrum, deuterium signals were observed for the terminal methyl group and the vinyl position revealing 11% retention of deuterium at C6 (not shown). Conclusions regarding potential deuterium at C4 were not possible due to signal overlapping with the resonance of deuterium at C8.

Using a cyclosporin A sample obtained from [2-^{13}C,^{2}H$_3$]-acetate as precursor, the ^{13}C-NMR spectrum confirmed the loss of deuterium at C4 as well as at C2; for the corresponding resonances at 35.97 and 58.74 ppm, respectively, no isotope-shifted signals (triplets of intensity 1:1:1) were detectable in the range 0.3−0.6 ppm upfield due to the carbon nuclei being directly attached to deuterium [data not shown, cf., Hansen (1983)].

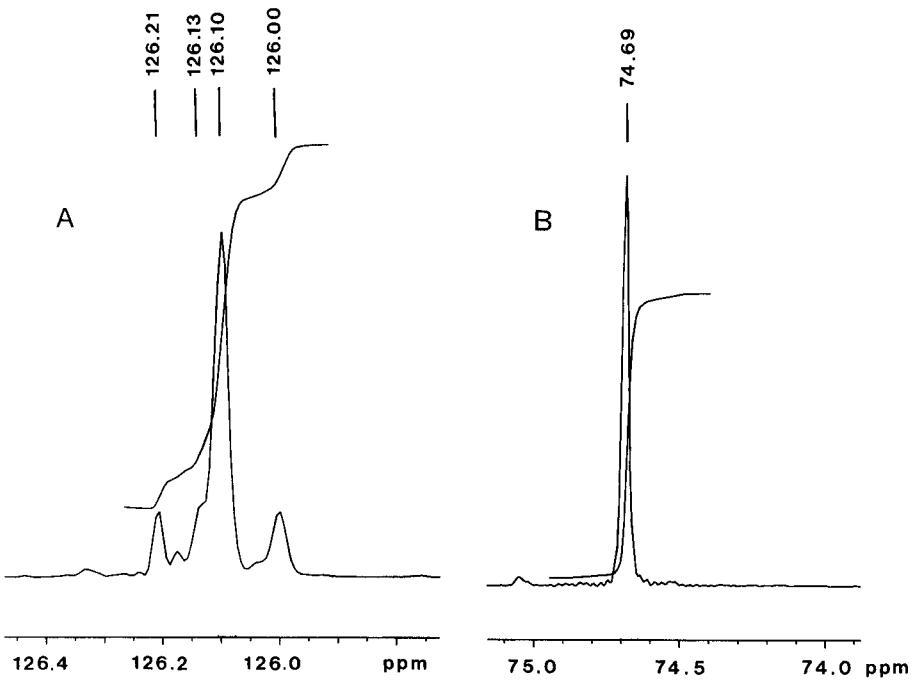

FIGURE 8: $^{13}C\{^1H\}$-NMR spectrum of cyclosporin A (precursor [1-^{13}C,$^2H_3$]acetate) in $CDCl_3$. A, expanded region for C7: the ratio between signal intensity for the natural abundance and isotope-shifted lines reflects the deuterium distribution at C8 of CH_3:CH_2D:CHD_2:CD_3 as 9:12:65:14. B. No β-isotope shifted signal can be observed for the C3 atom, indicating absence of deuterium at the neighboring carbon atom C4.

DISCUSSION

The results presented here describe comprehensive enzymatic studies of a polyketide pathway including a methylation step. The goal of the experiments was to answer three questions: (1) Which intermediate serves as a methylation precursor? (2) Is biosynthesis accomplished in a processive or rather a non-processive manner? And (3) is it possible to introduce cycle intermediates and maybe structural relatives thereof into the pathway with the aim of obtaining structural variants of the C9-backbone of (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine?

For the first time, a direct methylation precursor in the course of a basic assembly reaction, namely, enzyme-bound 3-oxo-4-hexenoic acid, could be unequivocally identified by *in vitro* methods. Methylation by Bmt polyketide synthase obviously is a strictly specific reaction taking place exclusively in the second elongation cycle and only with a β-oxocarboxylic acid as a substrate. This finding could be confirmed by *in vivo* incorporation studies using ^2H- and ^{13}C-labeled acetates as precursors and is in line with the current concept that methylation in fungal polyketide pathways most likely takes place in α-position to a carbonyl group (O'Hagan, 1991). It should be mentioned that already in 1983 Kobel et al. (1983) excluded methylation on a double bond (e.g., on 2,4-hexadienoic acid) based on the intact incorporation of methyl groups from [*methyl*-$^2H_3$]methionine at position C4 of Bmt.

Since Bmt polyketide synthase seems to contain all enzymatic activities in a single polypeptide chain (unpublished data), an overall reaction mechanism comparable to that of 6-methylsalicylic acid synthase or of macrolactone synthases, respectively, could be expected. 6-Methylsalicylic acid synthase has a structure analogous to mammalian fatty acid synthase (Beck et al., 1990; Wang et al., 1990): one polypeptide harbors all necessary catalytic domains, in one copy each; several identical polypeptides are arranged to the active enzyme complex which uses the individual domains iteratively. Enzymatic *in vitro* studies with purified 6-methylsalicylic acid synthase point toward a non-processive mechanism for this enzyme since the dehydration step between the two C atoms involved in the second condensation reaction takes place in the third elongation cycle concomitantly with the cyclization reaction (Schorr et al., 1994).

In contrast, a processive mechanism has been suggested for the biosynthesis of macrolactones. Cloning and sequencing of the respective genes, especially those from *Streptomyces erythrea* encoding the enzymes for synthesis of the parent macrolide for erythromycin, 6-deoxyerythronolide B, revealed a modular structure of this enzyme type with the number of modules corresponding to the number of elongation cycles (Donadio et al., 1991; Donadio & Katz, 1992; Bevitt et al., 1992; MacNeil et al., 1992). Each module harbors all catalytic domains necessary to obtain the final oxidation state of the involved C atoms and each can be manipulated leading to the respective structural variant of the end product (Donadio et al., 1991, 1992, 1993). This modular enzyme structure is in good agreement with the idea of a processive mechanism which arose from a series of studies in which intact *in vivo* incorporation of potential intermediates with a C4-, C6-, or C8-backbone has been demonstrated [reviewed in Katz and Donadio (1993); for more recent results, cf. Dutton et al. (1994) and Hailes et al. (1994)] as well as from studies in which potential biosynthetic intermediates could be isolated from fermentation broths (Katz & Donadio, 1993). Obviously, polyketide synthases, at least those of the macrolactone type, are able to take over activated intermediates and to process them along the correct pathway. Very recently, cell-free enzymatic synthesis of 6-deoxyerythronolide B has been demonstrated (Pieper et al., 1995).

Bmt polyketide synthase seems to act neither analogously to 6-methylsalicylic acid synthase nor to the hitherto

described macrolactone synthases. Two results presented here support the idea of a processive (Figure 1, route 2) or a mixed mechanism (first cycle processive, second and third cycles non-processive: Figure 1, route 5), which, however, is not comparable to the macrolactone type as outlined below.

The first result pointing toward a processive mechanism was the significant incorporation rate of crotonyl-CoA and butyryl-CoA into 3(*R*)-hydroxy-4(*R*)-methyl-6(*E*)-octenoyl-CoA and 3-hydroxy-4-methyloctanoyl-CoA, respectively. Additionally, acetoacetyl-CoA could not be elongated in the presence of malonyl-CoA and *S*-adenosylmethionine to a lactone like crotonyl-CoA (data not shown), which strongly suggested that acetoacetyl-CoA needs to be reduced prior to the next elongation step. Methylation of enzyme-bound 3-oxo-4(*E*)-hexenoic acid as the first cycle intermediate with a sufficiently nucleophilic C atom was the second result which supported the idea of an overall processive mechanism. It seems very unlikely that the first five steps of the pathway should obey a processive mechanism whereas the remaining reactions would deviate from this rule. Nevertheless, as shown in Figure 6, enzyme-bound 2-methyl-3-oxohexanoic acid could be elongated by malonyl-CoA so that both possibilities, an overall processive mechanism via route 2 or a mixed-type mechanism via routes 2 and 5, have to be considered.

The third question, concerning the introduction of possible cycle intermediates or structural relatives thereof into the pathway when supplied as coenzyme A thioesters, could not be answered definitively. In contrast to macrolactone synthases which are able to incorporate intermediates at least *in vivo* (see above), the respective ability of Bmt polyketide synthase is limited. The last intermediate along the biosynthetic route which can unambiguously be taken over from coenzyme A by Bmt polyketide synthase and incorporated into the basic assembly end product is 3-oxo-4(*E*)-hexenoic acid. As shown by the chase experiments, 2-methyl-3-oxohexanoic acid and all following methylated intermediates were unable to re-enter the cycle once released from the enzyme as a coenzyme A thioester. Attempts to elongate synthetically prepared 2(*R*)-methyl-4(*E*)-hexenoyl-CoA also failed (data not shown). The inability of the enzyme to be loaded by already methylated intermediates made it impossible to finally distinguish between a processive or a non-processive route in the second and third elongation cycles (route 2 or 5 in Figure 1, respectively). Obviously the correct biosynthetic process occurs in an enzyme-bound manner becoming inaccessible to externally supplied coenzyme A thioesters in the course of the reaction.

Attempts to circumvent this problem and to analyze the individual steps in the second and the third elongation cycle by successive addition of the substrates failed because Bmt polyketide synthase releases its *in situ* intermediates in bypass reactions as soon as one of the substrates is missing. The nature of the bypass reaction depends on the chemical structure of the respective intermediates: C8-backbones containing 3,5-dioxo groups are cyclized to a lactone, similarly as described of 6-methylsalicylic acid synthase (Dimroth et al., 1970; Spencer & Jordan, 1992), whereas the other compounds are released as coenzyme A thioesters.

Taking into account these results, it seems not very likely to obtain reasonable amounts of structural variants of (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine by offering modified intermediates to the enzyme. However, to answer this question definitely, appropiate *in vivo* experiments have to be performed.

Further conclusive interpretations of the *in vitro* results were impeded by a broad range of side reactions of Bmt polyketide synthase performed on free coenzyme A thioesters. Thioesters of β-oxocarboxylic acids for instance were readily reduced in the presence of NADPH to yield the thioesters of β-hydroxy acids which subsequently dehydrated to the respective enoyl compounds. Thioesters of non-methylated carboxylic acids were elongated by malonyl-CoA and subsequently transformed in a fatty acid synthase-like manner, i.e., keto group reduction, dehydration, and enoyl reduction.

As demonstrated, enzymatic *in vitro* studies are a valuable tool to analyze the individual steps of a polyketide pathway, but were, in this particular case, not sufficient to answer all questions due to the nature of the enzyme. Structural studies on the homogeneously purified enzyme, comprehensive genetic analysis, and comparison with other polyketide synthases will be necessary to elucidate further details of the pathway.

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