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# Biosynthetic Origin of the Methoxyl Extender Unit in Bafilomycin and Concanamycin using Stereospecifically Labeled Precursors

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**Abstract** The microbial macrolides bafilomycin $A_1$, $B_1$ and concanamycin A from *Streptomyces* spp. are potent and specific inhibitors of V-ATPases. The question of the biosynthetic origin of the two uncommon “glycolate units” of each of the macrolide structures was addressed by feeding experiments with stereospecifically $^{13}$C-labeled precursors. Our studies clearly indicate that glycerol is a source for the methoxylated $C_2$-units and determines the orientation of the incorporation. Products from the carboxylic acid pool or TCA cycle are ruled out as key precursors. The data suggest the action of a glycerol kinase and point to phosphoglycerate as an intermediate in their biosynthesis. However, glycerate itself is not accepted as a precursor. We present the likely biosynthetic pathway and show the value of stereospecifically labeled presursors as an important tool for biosynthetic investigations.

**Keywords** biosynthesis, stereospecific isotopic labeling, macrolides, natural products, polyketides

## Introduction

The sequencing of the human genome and the genomes of numerous pathogens has revealed a huge number of genes with as yet unknown functions. The demand for small, drug-like molecules as possible inhibitors of the respective gene products and hence for new tools to study the function of these proteins has steadily risen. The plecomacrolides bafilomycin and concanamycin (Fig. 1) are secondary metabolites, which have been isolated from certain *Streptomyces* strains [1]. Their ability to selectively inhibit vacuolar-type ATPases (V-ATPases) even at nanomolar concentrations has made them useful tools in studying the mode of action of these enzymes and their function in cell metabolism [2~5]. The fundamental role which V-ATPases play in regulating the pH gradient at osteoclast membranes has made bafilomycin $A_1$ (**1a**) and concanamycin A (**2**) promising lead structures for the development of new osteoporosis therapeutics [6, 7]. However, their application is still impeded by their pronounced cytotoxicity. Intense semi-synthetic structure-activity-relationship (SAR) investigations, mainly focussing on **1a**, have so far not resulted in a significant decrease of this critical drawback [6~9].

The biosynthesis of bafilomycins (**1**) and concanamycin A (**2**) has been revealed by feeding experiments using $^{13}$C-labeled precursors [1, 10]. Besides several common PKS I chain elongation steps using acetate and propionate extender units, the biosynthesis also involves two chain extension steps which use an as yet unidentified precursor arising from the carbohydrate pool. The so called “glycolate units” which occur in the final assembled metabolites **1** and **2** as methoxylated two-carbon units have also been found in some other microbial secondary metabolites, such as ansamitocin [11], soraphen [12, 13], FK506 [14], geldanamycin [15, 16], tautomycin [17], oxazolomycin [18], and leucomycin [19]. Experiments with $^{13}$C-labeled D,L-glycerate and glycolate gave enrichments in the respective extender units of geldanamycin [15] but similar investigations for leucomycin [19] and **2** [1] did not confirm these precursors. In the cases of **1**, **2** and ansamitocin feeding experiments revealed that

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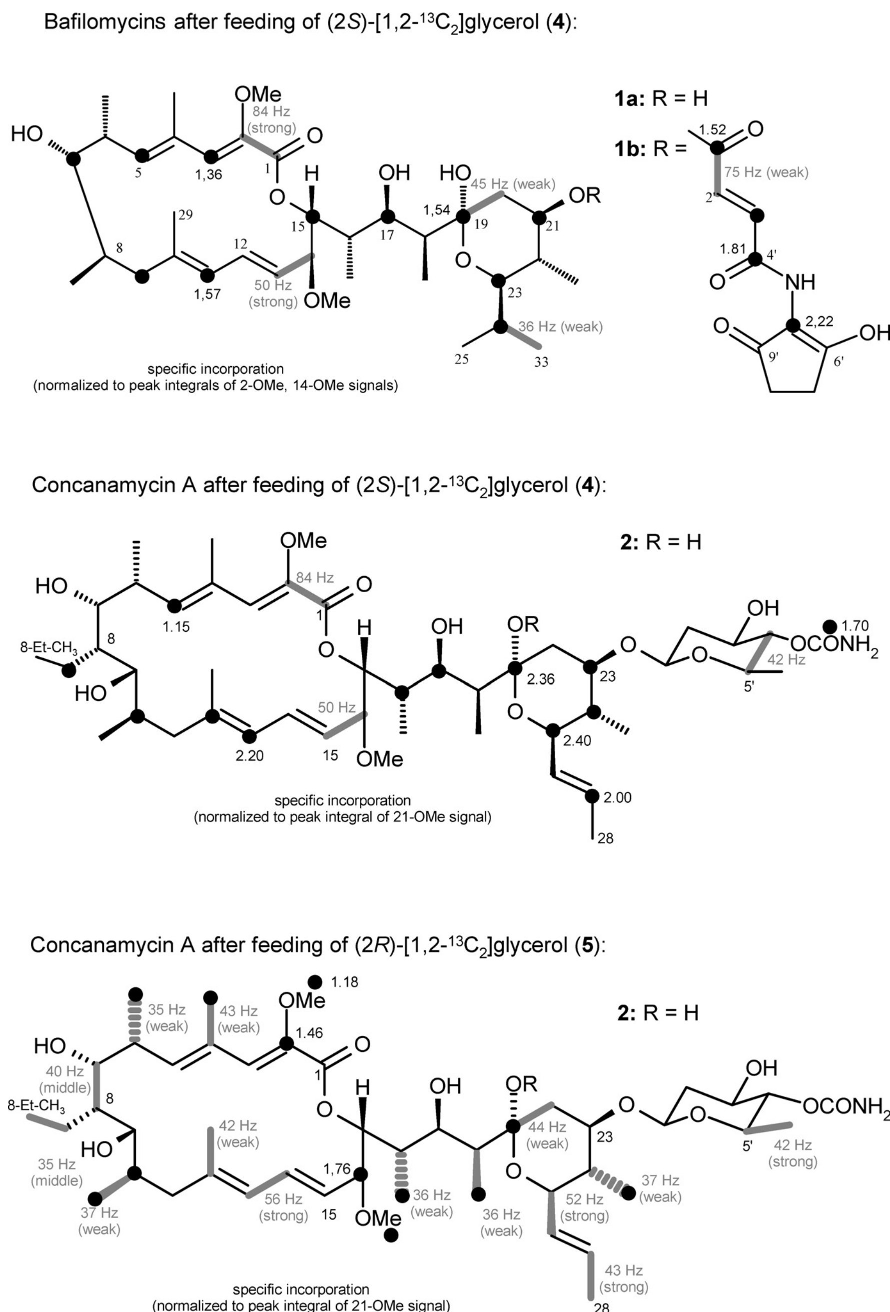


**Fig. 1** Structures of the plecomacrolides bafilomycin $A_1$ (**1a**), $B_1$ (**1b**) and concanamycin A (**2**).

Highlighted bonds indicate the position of intact incorporation.

hydroxymalonyl- and methoxymalonyl-CoA presumably are not the direct precursors for these units [10, 20]. According to sequence homologies of the FK506 gene cluster, a three carbon intermediate of the glycolytic pathway is assumed to be transformed into a glyceryl-ACP, followed by two oxidation steps and a methylation to give methoxymalonyl-ACP as a substrate for the PKS [14].

Key remaining questions are, which two carbons of a glycerol-derived $C_3$-building block and what intermediate of the glycolytic pathway provide the methoxyl $C_2$-unit. A degradation to glycolate followed by carboxylation prior to acceptance by the PKS, similar to the common mechanism for acetate units, is unlikely based on indications from the biosynthetic gene clusters (*e.g.* common module organization, presence of two oxidoreductases, but the lack of a carboxylase) [11, 13, 14, 16～18]. A methoxymalonyl

extender molecule must undergo decarboxylation during the Claisen condensation on the PKS, therefore careful choice of $^{13}$C-labeled precursors could distinguish between potential $C_3$-intermediates and define the orientation of their incorporation into the polyketides. The finding that [1-$^{13}$C]glycerate labels the C-1 atoms of the methoxyl extender units, but not the acetate units in geldanamycin [15], would suggest that the C-1 atom is retained as the thioester activated carboxyl group of a methoxymalonyl extender unit, and that the C-3 atom is lost. However, this indication is in contrast to $^{2}$H-labeling experiments with soraphen [12], and the incorporation of glycerate was not observed for leucomycin [19]. Glycerate could either be activated to the 3-phosphate analogue and enter the glycolytic pathway, or the C-1 carboxyl group could be directly activated and transformed into a thioester.

## Results

To probe how the unusual extender unit arises from the glycolytic pathway, we examined the incorporation of asymmetrically labeled glycerol, making use of the feature of glycerol kinase to produce stereospecifically only L-glycerol 3-phosphate (**3**) using ATP (Fig. 2A) [21].

The strong incorporation of [U-$^{13}C_3$]glycerol into the methoxyl $C_2$-extender unit carbon atoms of compounds **1** and **2** indicates a close biosynthetic connection [10]. To adress the question which two of the three carbon atoms of glycerol and hence of three-carbon glycolytic intermediates give rise to the unusual extender units, we decided to feed doubly labeled, chiral (2*S*)-[1,2-$^{13}C_2$]glycerol (**4**) and–in separate experiments–(2*R*)-[1,2-$^{13}C_2$]glycerol (**5**) [22] to the organisms producing bafilomycin $A_1$ (**1a**), bafilomycin $B_1$ (**1b**) and concanamycin A (**2**), respectively. Glycerol kinase would convert **4** into [1,2-$^{13}C_2$]-*sn*-glycerol 3-phosphate (**6**), whereas the other enantiomer **5** would lead to the formation of [2,3-$^{13}C_2$]-*sn*-glycerol 3-phosphate (**7**, see Fig. 2B). If glycerol kinase is indeed involved in the biosynthesis of the $C_2$-precursor, feeding of only one of the glycerol enantiomers should lead to an intact incorporation of two labeled carbon atoms into the methoxyl extender units. The $^{13}$C NMR spectra show strong and distinct spin–spin coupling for the bafilomycins (**1**) only between C-1/C-2 and C-13/C-14, and for **2** only between C-1/C-2 and C-15/C-16 after feeding of **4**, thus indicating intact incorporation (Tables 1, 2) [23]. Conversely, the administration of **5** to the producing strains only led to single enrichment of the methoxylated carbon atoms of the $C_2$-units of interest, that is C-2 and C-14 of **1a** and **1b** and C-2 and C-16 in **2**.

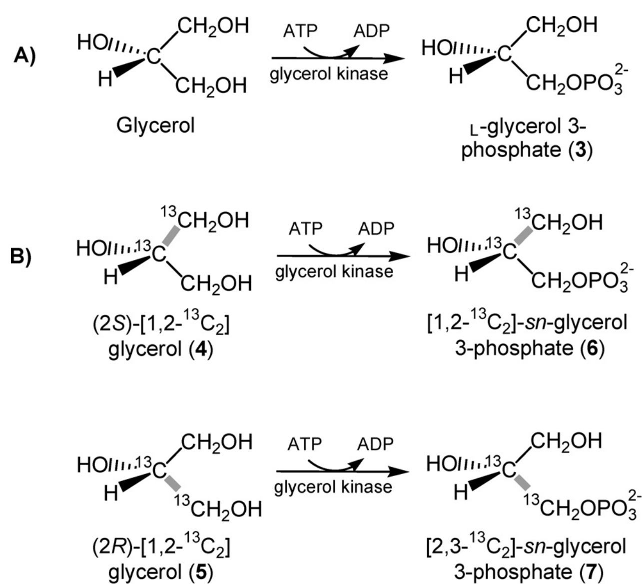


**Fig. 2** A) The enzyme glycerol kinase produces exclusively L-glycerol 3-phosphate (**3**) from glycerol. B) Stereospecifically labeled glycerol (**4**, **5**) enantiomers as substrates of glycerol kinase.

Additionally, the 2-deoxy-D-rhamnose moiety of **2** was labeled in positions C-4′/C-5′ after feeding of **4** and in C-5′/C-6′ after administration of **5**. Using **4**, **1b** showed very weak coupling for C-19/C-20, C-24/C-33 and C-1′/C-2′ due to a small amount of *R*-isomer present in the precursor. Feeding of **5** led to strong coupling in the acetate-derived units of **1** and **2**, and in the isobutyrate unit of **1**. Furthermore, significant incorporation into the propionate-derived units could be observed *via* coupling in 2- and 3-position of propionate, which occurs according to the direct conversion of isobutyrate to propionate retaining the intact coupling [24]. Enantiomer **5** led to spin–spin coupling between C-7/C-8 and 8-Et-$CH_2$/8-Et-$CH_3$ in the butyrate derived unit of **2**. In an attempt to quantify the $^{13}$C incorporation into both labeled **1** and **2** better, both compounds were subjected to derivatization, because the [$^{13}C_2$]glycerol precursors **4** and **5** presumably label the macrolides **1** and **2** such that no atom is suitable for referencing in both feeding experiments. But only methylation of **2** readily gave a quantitative reaction to afford 21-*O*-methyl **2** (R=Me) which was also analyzed by NMR with the C-atom 21-OMe as reference [2]. The carbon atoms 2-OMe and 14-OMe were used as reference for **4**-derived **1**, because they are not labeled from **4** according to the common methionine metabolism and the results for **2**. For **5**-derived **1** the carbon atoms C-1 and C-13 were chosen as reference [23]. If they show some $^{13}$C-label, it would lead to lower incorporation of the other C-atoms.

**Table 1** Incorporation and $^{13}C$ NMR coupling constants from $^{13}C$ NMR spectra for the 21-methyl ether of concanamycin A (**2**, R=Me) (150.8 MHz, $CD_2Cl_2 : CD_3OD$, 2 : 1) after feeding of the $^{13}C_2$-labeled glycerol enantiomers **4** and **5**

| C-Atom | Chemical shift (ppm) | Precursor **4** | | | Precursor **5** | | |
|---|---|---|---|---|---|---|---|
| | | Specific incorporation (%) [23] | | | Specific incorporation (%) [23] | | |
| | | Singlet | Doublet | $J_{CC}$ (Hz) | Singlet | Doublet | $J_{CC}$ (Hz) |
| 1 | 166.6 | 0.16 | 1.06 | 84 | 0.18 | | |
| 2 | 141.5 | **0.85** | 1.27 | 84 | 1.46 | | |
| 2-OMe | 60 | −0.35 | | | 1.18 | | |
| 3 | 132.3 | 0.55 | | | **0.73** | | |
| 4 | 131.5 | 0.47 | | | 0.69 | 0.75 | 43 |
| 4-Me | 13.9 | 0.09 | | | 1.22 | 0.53 | 43 |
| 5 | 141.4 | 1.15 | | | **0.89** | | |
| 6 | 34.7 | −0.02 | | | −0.35 | 0.98 | 35 |
| 6-Me | 16.7 | 0.10 | | | 1.10 | 0.48 | 35 |
| 7 | 75.2 | **0.87** | | | 0.52 | 0.41 | 40 |
| 8 | 43.6 | 0.24 | | | −0.41 | 0.37 | 40 |
| 8-Et-$CH_2$ | 22.50 | 1.08 | | | 0.09 | 0.87 | 35 |
| 8-Et-$CH_3$ | 11.60 | 0.23 | | | 0.12 | 0.45 | 35 |
| 9 | 79.4 | **0.72** | | | **0.89** | 0.33 | 38 |
| 10 | 34.9 | 1.05 | | | 0.99 | 0.52 | 37 |
| 10-Me | 21.7 | **0.77** | | | 1.29 | 0.61 | 37 |
| 11 | 45.3 | 0.43 | | | 0.47 | 0.31 | 41 |
| 12 | 142.7 | 1.13 | | | **0.82** | 0.46 | 42 |
| 12-Me | 16 | −0.10 | | | **0.89** | 0.52 | 42 |
| 13 | 122.8 | 2.20 | | | 0.08 | 0.88 | 56 |
| 14 | 133.8 | 0.55 | | | −0.18 | 1.22 | 56 |
| 15 | 126.5 | 0.63 | 1.05 | 50 | −0.31 | | |
| 16 | 82.3 | **0.86** | 1.02 | 50 | 1.76 | | |
| 16-OMe | 55.7 | −0.13 | | | 1.08 | | |
| 17 | 75.7 | 0.63 | | | 0.45 | | |
| 18 | 38.1 | 1.04 | | | 0.47 | 0.50 | 36 |
| 18-Me | 10 | 0.49 | | | 1.15 | 0.45 | 36 |
| 19 | 69.5 | 0.99 | | | **0.73** | | |
| 20 | 37.8 | **0.70** | | | 0.47 | 0.30 | 36 |
| 20-Me | 7.2 | 0.14 | | | 1.24 | 0.50 | 36 |
| 21 | 103.6 | 2.36 | | | 0.09 | 0.77 | 44 |
| 21-OMe | 46.4 | 0.00 | | | 0.00 | | |
| 22 | 35.7 | **0.72** | | | 0.42 | 0.59 | 44 |
| 23 | 73.2 | 1.81 | | | −0.41 | | |
| 24 | 40.2 | 1.04 | | | **0.73** | 0.64 | 37 |
| 24-Me | 13.2 | 0.18 | | | 1.18 | 0.42 | 37 |
| 25 | 76.9 | 2.40 | | | 0.42 | 0.96 | 52 |
| 26 | 130.3 | 0.16 | | | −0.13 | 1.15 | 52 |
| 27 | 130.1 | 2.00 | | | 0.40 | 1.48 | 52 |
| 28 | 17.8 | −0.58 | | | 0.36 | 1.20 | 52 |
| 1′ | 95.6 | 0.49 | | | −0.28 | | |
| 2′ | 39.6 | 0.22 | | | −0.47 | | |
| 3′ | 69 | 0.46 | | | −0.36 | | |
| 4′ | 78.6 | 0.21 | 0.51 | 42 | −0.32 | | |
| 5′ | 69.8 | 0.28 | 0.48 | 42 | −0.30 | 1.02 | 42 |
| 6′ | 17.4 | −0.27 | | | 0.12 | 1.09 | 42 |
| $CONH_2$ | 157.9 | 1.70 | | | 0.62 | | |

$^{13}C_2$-Glyerate feedings gave no incorporation. Reference atom: 21-OMe. Strong couplings and single enrichments are indicated by gray shading, still significant enrichments by bold numbers [23].

**Table 2** Incorporation and coupling constants from selected $^{13}C$ NMR data for bafilomycin $A_1$ (**1a**) and $B_1$ (**1b**) (150.8 MHz, $CD_2Cl_2$) after feeding of the $^{13}C_2$-labeled glycerol enantiomer **4**

| C-Atom | Chemical shift (ppm) | Specific incorporation (%) Doublet | $J_{CC}$ (Hz) |
|---|---|---|---|
| of **1a** | | | |
| 1 | 167.6 | 1.04 | 84 |
| 2 | 141.4 | 1.02 | 84 |
| 13 | 127.1 | 0.93 | 50 |
| 14 | 82.6 | 1.03 | 50 |
| of **1b** | | | |
| 1 | 167.6 | 2.13 | 84 |
| 2 | 141.4 | 1.81 | 84 |
| 13 | 127.1 | 2.12 | 50 |
| 14 | 82.6 | 1.97 | 50 |
| 1′ | 164.6 | 0.73 | 75 |
| 2′ | 133.5 | 1.18 | 75 |
| 3′ | 133.3 | 1.07 | 66 |
| 4′ | 163.8 | 0.89 | 66 |

$^{13}C_2$-Glyerate feedings gave no incorporation. Reference: Ø of C-2-OMe, C-14-OMe. Strong couplings are indicated by gray shading [23]. All other results for **1a**, **1b** are accordant to concanamycin A (**2**) with little variations.

After the glycerol feeding experiments, the remaining question is whether triose phosphates or phosphoglycerates are incorporated as such or *via* free glycerate, taking into account that the final methoxymalonate unit is not phosphorylated. Using glycerate as a putative precursor in feeding experiments would reveal if this molecule can enter metabolism towards the biosynthesis of the methoxyl extender unit. Feeding studies with D-[1,2-$^{13}C_2$]glycerate and L-[1,2-$^{13}C_2$]glycerate, respectively, were performed with [$^{13}C$-methyl]methionine or [U-$^{13}C_3$]glycerol experiments running simultaneously as controls. The $^{13}C$ NMR spectra of isolated **1a**, **1b** and **2** from both glycerate enantiomers did not show either any single enrichments or coupling between any carbons. However, [$^{13}C$-methyl]methionine strongly labeled the methoxyl carbon atoms (2-OMe/14-OMe) of both **1a** and **1b**, and also **2** was strongly labeled in the control experiment, thus ruling out effects from the cultivation process. These data suggest that free D- or L-glycerate, added to the producing strains, or their metabolic products can not be utilized as precursors for macrolide production. This is in contrast to results for the “glycolate units” of soraphen and geldanamycin [12, 15].

## Discussion

The incorporation of only the (2*S*)-isomer **4** into both carbon atoms of the methoxylated $C_2$-units of **1a**, **1b** and **2** is consistent with the involvement of glycerol kinase in the assimilation of glycerol. It shows unequivocally that only carbon atoms 1 and 2 of *sn*-glycerol are used for the formation of the unusual extender units, whereas C-3, the carbon which is phosphorylated by glycerol kinase, is lost during transformation to the directly used extender molecule (Fig. 3). Glycerate, which is added to growing strains, must also be phosphorylated by kinases to enter metabolism; and generally only D-glycerates occur as natural intermediates and are the only form accepted by the kinases. The lack of enrichments in **1** and **2** after feeding of labeled glycerate emphasizes that the cells of the microbial producers are unable to phosphorylate these precursors, given the prerequisite of a successful uptake of glycerates.

Our sequence data of the biosynthetic gene cluster of **2** from strain *Streptomyces* sp. Gö22/15 [25] and those of other metabolites with methoxyl extender units suggest a plausible pathway based on the observed sequence homologies [11, 13, 14, 16～18]. The concanamycin gene sequence data from *Streptomyces* sp. Gö 22/15 named *cocI*, *J*, *K*, *G*, *H*, the respective enzymes of which presumably refer to the methoxyl extender unit, are almost identical to those published for the concanamycin producer *S. neyagawaensis* [26]. The following set of five distinct enzymes presumably mediates the formation of methoxymalonyl-ACP as the direct biosynthetic precursor: an acyl carrier protein (ACP) (*e.g.* FkbJ, Asm14, Con-ORF2*), two dehydrogenases (*e.g.* FkbK/FkbI, Asm Asm 15, Con-ORF1*/3*, respectively), a methyltransferase (*e.g.* FkbG, Asm17, Con-ORF12*) and a gene with hitherto unknown function (*e.g.* FkbH, Asm 16, Con-ORF4*). In the course of our work, it has interestingly become apparent that the amino acid sequence alignment of CocH (and Con-ORF4*, FkbH, GdmH, respectively) reveals homologies to the phosphatase subgroup of the haloacid dehalogenases (HAD) superfamily. The magnesium-dependent phosphatases all contain a highly conserved motif (Table 3) [27～29].

This motif is part of the critical loop I, which is crucial for function, as was shown by X-ray analysis and mutation experiments [27～29]. Indeed, in a paper just published by Shen, Kelleher and coworkers it is shown for oxazolomycin that the gene product OzmB with the HAD enzymatic activity binds D-1,3-bisphosphoglycerate and generates glyceryl-ACP [30]. We conclude that CocH and its analogues mediate the dephosphorylation and transfer to an

**Table 3** Aligned sequences of CocH and the respective amino acids from other HAD phosphatases comprising the strictly conserved motif (DLDXT, X=N for Streptomycetes, Y for various eukaryotic MDP-1, E for various eucaryotic eyes absent-factors)

| CocH | MSD-KTGADAPAMVKCLVWDLDNTLWQ-GTLLED- 32 |
|---|---|
| Con-ORF4* | MSD-KTGADAPALVKCLVWDLDNTLWQ-GTLLED- 32 |
| GdmH | MTEGKPVSEPPTAVKCLVWDLDNTLWR-GTLLED- 33 |
| FkbH | MT----------IVKCLVWDLDNTLWR-GTVLED- 23 |
| Mouse-MDP-1 | ---------MTRLPKLAVFDLDYTLWP---FWVD- 22 |
| Mouse-eya3 | KRKADASSSQDSELERVFLWDLDETIIIFHSLLT--234 |
| Homo s.-eya1 | GRNNNPSPPPDSDLERVFIWDLDETIIVFHSLLT--300 |
| | . : ::****:*: ::* |

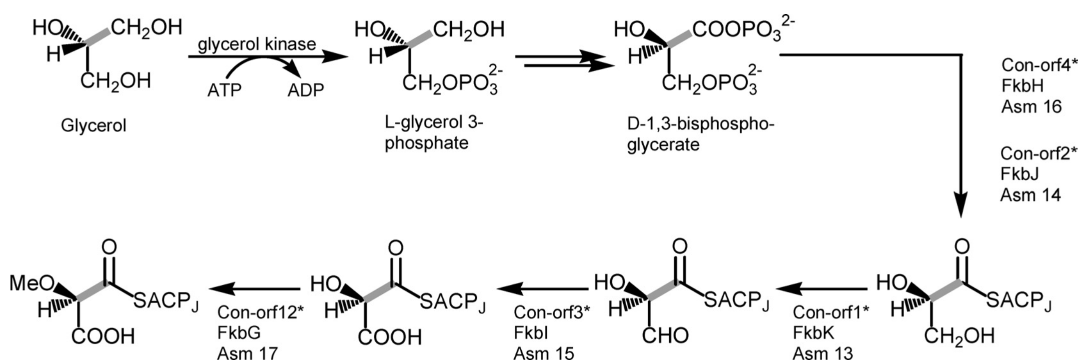


**Fig. 3** Proposed pathway for the biosynthesis of the methoxyl $C_2$-extender unit precursor.

Highlighted bonds indicate position of labeling from the (2*S*) enantiomer **4**. Proposed enzyme activities from sequence data homologies of concanamycin (Con), FK520 (Fkb) and ansamitocin (Asm) [11, 13, 14, 16～18].

ACP of a triose intermediate in the biosynthetic pathway of the methoxyl extender unit (Fig. 3). The genes for soraphen biosynthesis in myxobacteria show a slightly different organization, with SorC resembling the function of FkbH and FkbG [13, 14, 20]. All data indicate that the common primary metabolism transforms L-glycerol 3-phosphate into 3-phospho-D-glycerate and 1,3-phospho-D-glycerate. The latter intermediate formally enters secondary metabolism as it is loaded onto the acyl carrier protein and dephosphorylated (Fig. 3). The resulting ACP-intermediate is processed by the two further oxidation steps [11, 13, 14, 16～18]. Methylation of the 2-hydroxyl group may be an earlier or the final step of methoxymalonyl-ACP biosynthesis.

The intact incorporation of **5** into all acetate derived units of compounds **1** and **2** can be explained by the formation of pyruvate from **5** *via* the glycolytic pathway and further transformation into acetyl coenzyme A [31]. Feeding of **5** also led to moderate single incorporations and incorporations of intact $C_2$-units into propionate units in **1** and **2** with intact units providing their C-2 and C-3 atoms, which can be explained by the known conversion of isobutyryl CoA into propionyl CoA [24, 31]. The labeling of the butyrate-derived unit of **2** after feeding of **5** is observed due to its biosynthetic formation from acetyl CoA and malonyl CoA [1]. The 2-deoxy-D-rhamnose moiety is labeled from **4** and **5** as expected by the gluconeogenesis [1, 22, 32]. Interestingly, the dihydroxyacetone phosphate-derived moiety of the sugar (C-1′/C-2′/C-3′), one of the biosynthetic building blocks from gluconeogenesis, is not labeled by the administered glycerol. Since primary metabolism shows dihydroxyacetone phosphate and phosphoglyceraldehyde in equilibration, $C_6$-sugars would reflect identical labeling patterns for their $C_3$-precursors. However, desoxy sugar biosynthesis of **2** shows that dihydroxyacetone phosphate must be derived from a different, endogenous pool which is not labeled from exogenously added [$^{13}C_3$]glycerol and which might not be an equilibrating dihydroxyacetone phosphate- phosphoglyceraldehyde pool [32].

The study presented herein points out a detailed biosynthetic pathway for the formation of the methoxyl extender unit. The biosynthetic steps also underline and complement some earlier insights into the five biosynthetic steps which were deduced from the respective genes of the biosynthetic gene clusters of the various antibiotics (like **2**)

[11, 13, 14, 16~18]. The feeding experiments with **4** and **5** yielded significantly labeled **1a**, **1b** and **2**. D- and L-[1,2-$^{13}C_2$]glycerate gave rise to **1** and **2**, which did not show any $^{13}C$ labeling. The data obtained give evidence that the formation of the methoxyl extender unit starts from glycerol with the involvement of glycerol kinase and includes 1,3-bisphosphoglycerate from primary metabolism. Exogenously added $^{13}C$-labeled glycerate is not phosphorylated by the primary metabolism of the investigated streptomycetes strains Gö 3822-14F and Gö 22/15 and therefore does not represent a biosynthetic precursor. An alternative biosynthetic route, in which the respective thioester carbonyl group of methoxymalonyl-ACP (C-1 of the methoxyl extender unit) comes from C-3 of a phosphoglycerate or a similar glycolytic intermediate can be clearly ruled out from the obtained $^{13}C$-labeling patterns. All conclusions of this work are in accordance with results from feeding experiments using stereospecifically $^{13}C$-labeled glycerols and glycerates to the ansamitocin producer, but are different from the biosynthesis of geldanamycin and soraphen in terms of glycerate utilization [15, 22]. Thus, enzymes from both primary and secondary metabolism work on the formation of the unusual methoxylated extender unit. The biosyntheses of **1** and **2** with the distinct feature of the methoxyl extender unit represent an important example for the complex interaction of the primary and secondary metabolism in streptomycetes. The pool of diverse scaffolds thus created represents the basis for the observed variety of biological activities, which can be used as valuable tools in biochemical and pharmaceutical research.

## Experimental

### Materials

(2*S*)-[1,2-$^{13}C_2$]Glycerol (**4**, >99% $^{13}C$, >95% *ee*) and (2*R*)-[1,2-$^{13}C_2$]glycerol (**5**, >99% $^{13}C$, >95% *ee*) were provided by the Los Alamos Stable Isotope Resource, sodium (2*S*)-[1,2-$^{13}C_2$]glycerate (>99% $^{13}C$, >69% *ee*) and sodium (2*R*)-[1,2-$^{13}C_2$]glycerate (>99% $^{13}C$, >75% *ee*) by H. G. Floss and C. Grünanger, University of Washington. To avoid NMR signals arising from statistical coupling, the labeled glycerol enantiomers were diluted with a four-fold amount of unlabeled glycerol prior to the feeding experiments. Column chromatography: Silica gel 60 (<0.063 mm, Macherey-Nagel), Sephadex LH-20 (Pharmacia), Lobar RP-18 (Merck). TLC: Silica gel 60 $F_{254}$ plates (Merck, 0.2 mm). Staining reagent: One ml of anisaldehyde in 85 ml methanol, 5 ml of concentrated sulphuric acid and 10 ml of acetic acid. Fermentation: Braun Biostat B (2 liters, 250 rpm, 28°C).

### NMR Analysis

$^{13}C$ NMR analysis was performed on a Varian Inova 600 spectrometer with proton decoupling. In the case of compound **2**, all spectra were recorded at −20°C in $CD_2Cl_2/CD_3OD$ 2 : 1 with methanol as internal standard, while in the case of compounds **1a** and **1b** ambient temperature and $CD_2Cl_2$ as solvent were chosen. $^{13}C$ chemical shifts were assigned according to the literature [1, 10].

### Fermentation Media

Medium A: Oat bran (20 g), tap water (1 liter), pH was adjusted to 7.8 prior to sterilization. Medium B: Starch (20 g), glucose (2.0 g), casein peptone (2.0 g), $K_2HPO_4$ (1.0 g), NaCl (1.0 g), $MgSO_4 \times 7H_2O$ (0.5 g), trace element solution (5.0 ml), distilled water (1 liter), pH was adjusted to 7.0 prior to sterilization. Trace element solution: see ref. 32.

### Cultivation and Precursor Feeding

The concanamycin-producing strain *Streptomyces* sp. Gö 22/15 was cultivated at 28°C in a 2-liter fermentor containing 540 ml of medium A. Fermentations were started by adding 60 ml of a 48-hour preculture and stirred for 72 hours at 250 rpm using an aeration of 3.3 vvm. Sterile solutions of the $^{13}C$-labeled glycerol enantiomers (1.1 mM final concentration) and $^{13}C$ sodium glycerates (4.0 mM final concentration) were pumped continuously into the culture broth from the 34th to the 48th hour of incubation. The bafilomycin-producing strain *Streptomyces griseus* Gö 3822-14F was fermented for 56 hours on a linear shaker in two 1 liter Erlenmeyer flasks each containing 250 ml of medium B at 28°C. $^{13}C$-labeled precursors (2.2 mM final concentration) were administered continuously from the 30th to the 42th hour of cultivation.

### Compound Isolation and Purification

The culture broth of strain Gö 22/15 was centrifuged and the supernatant was discarded. The mycelium was extracted three times with 500 ml each of acetone. Evaporation of the solvent yielded crude extract (~1.0 g). To isolate compound **2**, the crude extract was subjected to chromatography using silica gel (gradient chloroform/methanol 95 : 5 to 90 : 10). The concanamycin A fraction (detected by TLC) was further purified on RP-18 *via* MPLC (eluent acetone/water 75 : 25) to yield 4.0 to 8.0 mg/liter of **2**. In the case of the bafilomycin-producing strain 3822-14F, the culture broth was centrifuged and the mycelium discarded. The supernatant was extracted three times with 1 liter each of ethyl acetate and the solvent of the combined organic

phases was evaporated. The crude extract (~200 mg) was subjected to chromatography on silica gel (eluent cyclohexane/ethyl acetate 60 : 40). Final purification of **1a** and **1b** (detection *via* TLC) containing fractions was achieved by chromatography on Sephadex LH-20 (eluent: acetone or dichloromethane) to yield 4.0 mg/liter of **1a** and 10 mg/liter of **1b**.

### Methylation

For methylation of **2** from the $^{13}C_2$-glycerol feeding experiments, the pure labeled compounds were diluted with pure unlabeled **2** to give 10 mg each (in detail: 7.0 mg (8.1 $\mu$mol) of labeled **2** from **4** gave a final 12.4 $\mu$mol of **2**. 3.9 mg (4.5 $\mu$mol) of **2** from **5** gave a final 12.1 $\mu$mol of **2**). This allowed for convenient derivatization, purification and NMR analysis. Methylation was performed as described in the literature and afforded the pure 21-*O*-methyl **2** ($R{=}CH_3$) in 69% yield as purified compounds [1]. Incorporation rates were calculated considering the dilution factors of 1.53 and 2.69 for 21-OMe-**2** from feeding **4** and **5**, respectively.

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