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On the Biosynthetic Origin of Methoxymalonyl-Acyl Carrier Protein, the Substrate for Incorporation of "Glycolate" Units into Ansamitocin and Soraphen A

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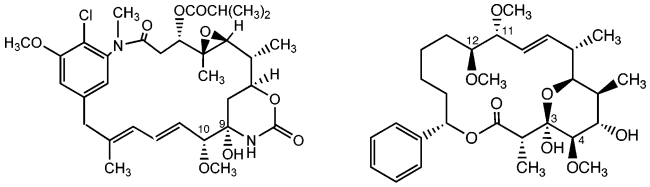
Abstract: Feeding experiments with isotope-labeled precursors rule out hydroxypyruvate and TCA cycle intermediates as the metabolic source of methoxymalonyl-ACP, the substrate for incorporation of "glycolate" units into ansamitocin P-3, soraphen A, and other antibiotics. They point to 1,3-bisphosphoglycerate as the source of the methoxymalonyl moiety and show that its C-1 gives rise to the thioester carbonyl group (and hence C-1 of the "glycolate" unit), and its C-3 becomes the free carboxyl group of methoxymalonyl-ACP, which is lost in the subsequent Claisen condensation on the type I modular polyketide synthases (PKS). D-[1,2-$^{13}C_2$]Glycerate is also incorporated specifically into the "glycolate" units of soraphen A, but not of ansamitocin P-3, suggesting differences in the ability of the producing organisms to activate glycerate. A biosynthetic pathway from 1,3-bisphosphoglycerate to methoxymalonyl-ACP is proposed. Two new syntheses of *R*- and *S*-[1,2-$^{13}C_2$]glycerol were developed as part of this work.

Polyketides represent one of the larger classes of natural products, and many of them are endowed with useful biological activities, such as antibiotic, antitumor, and immunosuppressive properties.[1] Many polyketide antibiotics, particularly of the macrolide and macrolactam families, are assembled on type I modular polyketide synthases (PKS) by repeated chain extension of a starter unit and reductive/dehydratative modification of the newly incorporated unit in each chain extension cycle.[2] The most common substrates for the chain extension reactions are malonyl-CoA for the incorporation of acetate units, 2-methylmalonyl-CoA for the incorporation of propionate units, and less frequently, 2-ethylmalonyl-CoA to incorporate butyrate units. A number of antibiotics, such as ansamitocin P-3 (AP3, **1**, Figure 1)**,**[3] soraphen A (**2**, Figure 1),[4] geldanamycin,[5] leucomycin,[6] FK-506 and FK-520,[7] concanamycin,[8] bafilomycin,[9] aflastatin,[10] oxazolomycin,[11] and tautomycin[12] contain another chain extension unit, called a "glycolate" unit, carrying an oxygen (frequently methylated) at C-2 of a two-carbon chain.



1 **2**

Figure 1. Structures of ansamitocin P-3 (AP3, **1**) from *Actinosynnema pretiosum* ssp. *auranticum* and soraphen A (**2**) from *Sorangium cellulosum*.

In analogy to the other chain extension units, one would expect the substrate for incorporating this "glycolate" unit to

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be 2-hydroxy- or 2-methoxymalonyl-CoA. This was, however, found not to be the case.[9,13] Instead it was discovered that the biosynthetic gene clusters for antibiotics carrying such "glycolate" units contain subclusters of four or five genes which are responsible for synthesizing the substrate for incorporating the "glycolate" unit. In the ansamitocin cluster (*asm*),[14] as well as the FK-520,[15] geldanamycin,[16] concanamycin,[17] oxazolomycin,[11b] and tautomycin[12] clusters, this operon of five genes encodes two oxidoreductases (*asm13* and *asm15*), an acyl carrier protein (ACP) (*asm14*), a methyltransferase (MT, *asm17*), and a protein of unknown function (*asm16*). In the soraphen cluster (*sor*) the *asm16* and *asm17* equivalents are replaced by a single gene, *sorC*, encoding a multifunctional protein with an acyltransferase (AT), an ACP, and a MT domain.[18] Mutational and heterologous expression experiments have demonstrated that *asm13*−*17* are necessary and sufficient for the synthesis of the substrate for incorporation of a "glycolate" unit, deduced to be 2-methoxymalonyl-ACP, and for its delivery to the cognate AT module of a PKS.[13,19] The crystal structure of FkbI (corresponding to Asm15), an acyl-CoA dehydrogenase homologue from the *fkb* methoxymalonate subcluster, supports this notion, predicting that the enzyme uses an ACP rather than a CoA ester as substrate.[20]

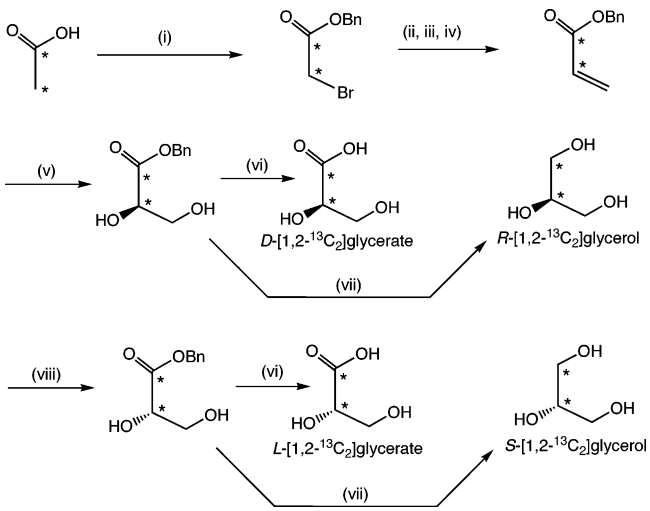
However, the primary metabolism substrate for the synthesis of the methoxymalonyl thioester has remained elusive. Numerous isotopic tracer experiments on several of the antibiotics containing "glycolate" units have tried to explore this question, but no consistent picture has emerged from these studies.[3−10] In this paper we report the results of additional feeding experiments with the ansamitocin producer, *Actinosynnema pretiosum*, and the soraphen producer, *Sorangium cellulosum*, which further narrow the possibilities, pointing toward 1,3-bisphosphoglycerate as the primary substrate, and define the orientation in which this three-carbon substrate is incorporated into the two carbons of the "glycolate" unit.

Results and Discussion

Synthesis of Labeled Precursors. To probe further the origin of the "glycolate" units in AP3 (**1**) and soraphen A (**2**), we aimed to feed the enantiomers of glycerate and stereospecifically labeled glycerol, each carrying labels of two contiguous ^{13}C atoms. Since these were not commercially available, we adapted a route for the synthesis of nonstereospecifically labeled [1,2-$^{13}C_2$]glycerate and -glycerol published by Pitlik and Townsend[21] for their preparation. Starting from glacial [1,2-$^{13}C_2$]acetic acid (99+% ^{13}C), the *R*- and *S*-[1,2-$^{13}C_2$]glycerols were prepared in 63% overall yield as shown in Scheme 1. The asymmetric dihydroxylation[22] of benzyl acrylate gave benzyl glycerates of 75% ee for the D (*R*) and 69% ee for the L (*S*) isomers, as determined by enzymatic phosphorylation of the derived glycerol samples with glycerokinase/ATP and ^{13}C NMR analysis of the ratio of *sn*-[1,2-$^{13}C_2$]- and *sn*-[2,3-$^{13}C_2$]glycerol 3-phosphate in the products (Scheme 2). D-[1,2-$^{13}C_2$]Glycerate and L-[1,2-$^{13}C_2$]-glycerate were derived from the benzyl glycerates by hydrogenolysis, and *R*- and *S*-[1,2-$^{13}C_2$]glycerol, by $LiBH_4$ reduction. Reduction of the benzyl glycerates with $LiBD_4$ also gave the corresponding glycerol samples carrying two deuterium atoms at C-1.

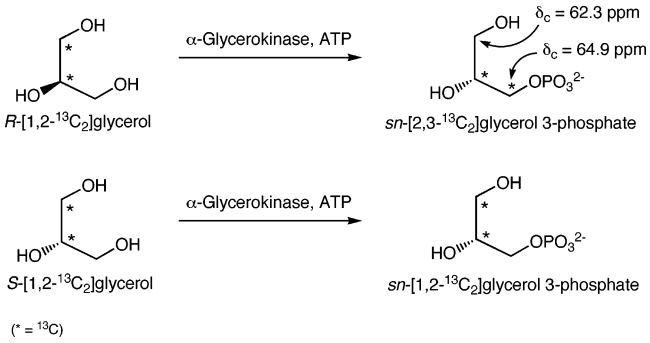
Independent of these syntheses, the group at the Los Alamos Stable Isotope Resource developed a new, more stereoselective route to *R*- and *S*-[1,2-$^{13}C_2$]glycerol (Scheme 3). This route is based on chiral sulfoxides[23] with the key reaction being the asymmetric reduction of a β-keto sulfoxide to a β-hydroxy sulfoxide.[24] The condensation of commercially available *S*-methyl

Scheme 1 [a]



[a] Reagents and conditions: (i) TFA, DMAP, 60 °C, Br_2, then BnOH; 86.5%; (ii) PPH_3, toluene, RT, 3 days, 96%; (iii) Et_2O/H_2O, K_2CO_3, 35 °C, 24 h; (iv) $(CH_2O)n$, Et_2O, reflux 24 h, 91% over two steps; (v) $K_3Fe(CN)_6$, K_2CO_3, $(DHQ)_2$-PHAL, OsO_4, tBuOH/H_2O, 0 °C, 10 h, 83%; (vi) THF/H_2O, Pd/C, H_2, RT, 3.5 h, quantitative; (vii) THF, $LiBH_4$, 0 °C, quantitative; (viii) $K_3Fe(CN)_6$, K_2CO_3, $(DHQD)_2$-PHAL , OsO_4, tBuOH/H_2O, 0 °C, 10 h, 84%.

Scheme 2



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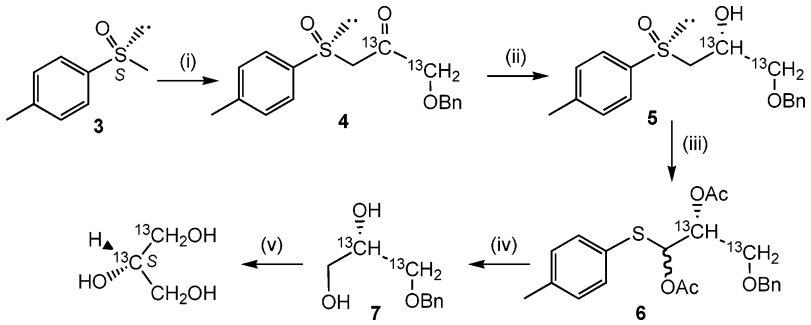
Scheme 3 [a]



[a] Reagents and conditions: (i) LDA, THF, −78 °C − 0 °C, 30 min, ethyl 2*O*-benzyl-[1,2-$^{13}C_2$]glycolate, 0 °C to RT, 2 h; (ii) DIBAL, CH_2Cl_2, −78 °C, 30 min, 62% (over two steps); (iii) NaOAc, Ac_2O, reflux, 15 h, 94%; (iv) $LiAlH_4$, THF, 0 °C to RT, 1.5 h, 91%, (v) 10% Pd/C, H_2 (45 psi), Amberlyst (H^+), EtOH, RT, 4.5 h, 98%.

p-tolyl sulfoxide **3** with ethyl 2-*O*-benzyl-[1,2-$^{13}C_2$]glycolate gave *β*-keto sulfoxide **4**, which was then reduced with DIBAL in THF at −78 °C to form *β*-hydroxy sulfoxide **5** in 62% overall yield from *S*-methyl *p*-tolyl sulfoxide **3** (Scheme 3). Subjecting *β*-hydroxy sulfoxide **5** to Pummerer rearrangement conditions, by heating with acetic anhydride and sodium acetate, gave (2*R*)-1,2-diacetoxy-3-benzyloxy-1-(*p*-tolylthio)-[2,3-$^{13}C_2$]propane **6** in high yield. Reductive deprotection of **6** with lithium aluminum hydride gave benzyl glycerol **7** in 91% yield, hydrogenation of which in the presence of 10% Pd/C catalyst yielded *S*-[1,2-$^{13}C_2$]-glycerol. Substituting *R*-methyl *p*-tolyl sulfoxide for the *S* enantiomer, this route was used to produce *R*-[1,2-$^{13}C_2$]glycerol. The enantiomeric purity of these glycerol samples was again determined by phosphorylation with glycerol kinase and ^{13}C NMR analysis.

[1,2-$^{13}C_2$]Glycerol samples synthesized by the second route, $R = 96\%$ ee, $S = 90\%$ ee, were used in the feeding experiments with the soraphen A producer, whereas all other feeding experiments employed the ^{13}C-labeled glycerol and glycerate samples of lower enantiomeric purity from the first synthesis.

Previous Feeding Experiments. Earlier isotopic tracer experiments had shown that the two carbons of the "glycolate" units of AP3 and soraphen A are not derived from acetate, propionate, or glycine,[3,4] consistent with the results in other systems.[6−10,25,26] This is not due to lack of precursor uptake, as these compounds labeled other parts of the respective product molecules, such as acetate and propionate units in the polyketide chain or, in the case of C-2 of glycine, *O*- and *N*-methyl groups. C-2, and to a lesser extent C-1, of the "glycolate" unit, i.e., C-10 and C-9 of AP3, were, however, somewhat enriched by D-[6-^{13}C]glucose, which also labeled most other positions of the AP3 molecule.[3] This finding is mirrored in low incorporation into the corresponding carbons in geldanamycin[5] and suggests an involvement of carbohydrate metabolism. The incorporation of [U-$^{13}C_6$]glucose into leucomycin to give contiguous labeling of C-3 and C-4 (the "glycolate" unit), in addition to the sugar moieties and the acetate-derived carbons, also supports this suggestion.[6] Feeding experiments with glycerol point to a product of glycolysis as the precursor, showing labeling of C-2 of the "glycolate" units from [2-^{13}C]glycerol in soraphen[4a] and leucomycin[6] and C-1 from [1(3)-^{13}C]glycerol in concanamycin.[8] These observations are somewhat at variance with the labeling pattern from C-6 of glucose, suggesting that the metabolic path from glucose to the "glycolate" unit is complex.

The earliest studies on the origin of the "glycolate" unit, by Rinehart and co-workers on geldanamycin, showed labeling of C-1 of the "glycolate" moiety by C-1 of glycolic acid.[5] This would support a pathway similar to the incorporation of acetic acid into acetate-derived units, i.e., by activation to a thioester followed by carboxylation. Low incorporation of [1-^{13}C]-glycolate into C-1 of the five "glycolate" units of aflastatin[10] has also been reported, but similar experiments in several other systems (leucomycin,[6] concanamycin[8]) gave no incorporation. Permeability barriers have been invoked as a possible explanation for this lack of incorporation. However, the absence of a carboxylase gene in the subclusters of genes known to be necessary and sufficient for the formation of methoxymalonyl-ACP and the strict requirement for two oxidoreductases argues strongly against a process of carboxylation of an activated glycolate. Rather, the utilization of glycolate in some systems must involve an indirect process, possibly via conversion to glycerate.

[1-^{13}C]Glycerate was found to label rather specifically and efficiently C-1 of the "glycolate" units of geldanamycin,[5] although it was not incorporated at all into leucomycin.[6] If glycerate were activated in the geldanamycin producer to the 3-phosphate, it would then enter the glycolytic pathway and could be converted into methoxymalonyl-ACP either directly or via intermediates such as hydroxypyruvate. Alternatively, glycerate could be activated at the carboxyl group and converted into methoxymalonyl-ACP without passing through the glycolytic pathway. If glycerate enters metabolism via the glycolytic pathway, the labeling of C-1 of the "glycolate" units, but not of acetate-derived units, would suggest that the free carboxyl group of methoxymalonyl-ACP, which is lost in the condensation step on the PKS, is derived from the phosphorylated carbon, C-3. This notion is indirectly supported by feeding experiments with [1-^{13}C]erythrose to the FK520 producer, which resulted in labeling of C-1 of the two "glycolate" units, in addition to the shikimate-derived starter unit.[7] Following phosphorylation,

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[1-^{13}C]erythrose would give rise, via the pentose phosphate shunt, to triose phosphates and phosphoglyceric acid (PGA) labeled at C-1. On the other hand, feeding experiments with *R*- and *S*-[1-D_2]glycerol showed incorporation of deuterium from the *R* but not the *S* isomer at C-11 and C-14 of soraphen, carbons representing C-1 of a "glycolate" and C-2 of an acetate unit, respectively. This led Hill et al.[4a] to conclude that the pro-*R* hydroxymethyl group of glycerol, which is phosphorylated by glycerokinase and gives rise to C-3 of triose phosphates and PGA, is retained and labels C-1 of the "glycolate" unit, whereas the pro-*S* hydroxymethyl group, corresponding to C-1 of PGA, is lost. The authors also concluded from the incorporation of deuterium at C-11 of soraphen that the formation of the "glycolate" unit cannot involve a 2-hydroxy- or 2-methoxymalonate intermediate.

New Feeding Experiments. To probe the possible involvement of 3-hydroxypyruvate in the formation of the "glycolate" units, we fed serine to the ansamitocin producer, *Actinosynnema pretiosum*. This compound, which can give rise to hydroxypyruvate by transamination, had not been evaluated as a precursor of "glycolate" units in any of the previous work. Feeding of D,L-[1-^{13}C]serine gave no significant enrichment in any of the carbon atoms of the resulting AP3. D,L-[3-^{13}C]Serine labeled (2−3.5% enrichment) only the three *O*- and *N*-methyl groups and the carbamoyl carbon C-24 of AP3. No significant enrichment at C-9 or C-10 was seen in either of the experiments, ruling out serine or 3-hydroxypyruvate as precursor of the "glycolate" unit. The involvement of the tricarboxylic acid (TCA) cycle in the formation of the "glycolate" unit was probed by feeding [1,2-$^{13}C_2$]succinate. This gave AP3 which showed no significant ^{13}C−^{13}C coupling, only low enrichment and no coupling at C-9 (0.8%) and C-10 (1.2%), but single-carbon enrichment (1.9−2.5%) at each of the three carbons of the three propionate units, at C-24 (2.6%) and to a lesser extent at the three acetate units (1.4−1.6%). The data argue against any prominent role of the TCA cycle in the generation of the "glycolate" units. The labeling pattern of the propionate units points to their formation from succinate via the succinyl-CoA−methylmalonyl-CoA mutase reaction.

In view of the labeling of the "glycolate" unit of AP3 by [6-^{13}C]glucose,[3] we explored the mode of incorporation of this sugar further by feeding D-[1,2-$^{13}C_2$]glucose. The amount of precursor available was small (120 mg), and hence, the overall level of incorporation was quite low. However, coupling was clearly detected between the carbon pairs (C-1/C-2, C-7/C-8, C-11/C-12) of the acetate units, whereas no coupling was detectible between C-9 and C-10, the carbons of the "glycolate" unit. The level of enrichment was too low to determine with any confidence whether there was single-carbon enrichment at either C-9 or C-10. These data suggest metabolism of glucose via the glycolytic pathway and are consistent with, although do not prove, incorporation via a triose phosphate or (phospho)-glycerate, C-2 and C-3 of which give rise to acetate units and C-1 and C-2 possibly to "glycolate" units.

The following experiments were then carried out to probe the role of triose phosphates or (phospho)glycerate further. First, the incorporation of glycerate into AP3 was evaluated by feeding D-[1,2-$^{13}C_2$]glycerate (75% ee) and L-[1,2-$^{13}C_2$]glycerate (69% ee). However, unlike in the case of geldanamycin,[5] no enrichment of, nor coupling between, any carbons of AP3 derived from either enantiomer of glycerate was detected. However, feeding of the same precursors to *Sorangium cellulosum* unequivocally gave intact incorporation into the two "glycolate" units, C-3/4 and C-11/12, of soraphen A (Table 1). Consistent with the low chiral purity of the precursors, incorporation was seen from both samples of glycerate, but the substantially higher enrichment from D-[1,2-$^{13}C_2$]glycerate leaves little doubt that D-glycerate is the true precursor. According to textbook biochemistry, only D-glycerate is phosphorylated by glycerate kinases, and only phospho-D-glycerates are intermediates in glycolysis. The observation of single enrichment in the carbons derived from C-1 of acetate (C-9, C-13 and C-15) and C-2 of phosphoenolpyruvate (C-1′) indicates that D-glycerate enters the glycolytic pathway, presumably via phosphoglycerate.

We then established the utility of glycerol as a precursor of the "glycolate" unit in these systems by feeding [U-$^{13}C_3$]glycerol to both *A. pretiosum* and *S. cellulosum*. The resulting AP3 indeed showed ^{13}C−^{13}C coupling between the carbons of the three acetate units as well as between C-9 and C-10. Thus, glycerol serves effectively as a substrate for the formation of both the acetate and the "glycolate" units in *A. pretiosum*, supporting the involvement of a triose phosphate or phosphoglyceric acid in both processes. Identical results were obtained for the incorporation of [U-$^{13}C_3$]glycerol into soraphen A by *S. cellulosum*.

To elucidate which two carbons of a triose phosphate or phosphoglycerate give rise to the "glycolate" unit, we then determined the orientation of the incorporation of glycerol by feeding to *A. pretiosum* *R*-[1,2-$^{13}C_2$]glycerol (75% ee) and *S*-[1,2-$^{13}C_2$]glycerol (69% ee), each diluted 4-fold with unlabeled glycerol to suppress statistical coupling. In the ^{13}C NMR analysis of the resulting AP3 samples, the average of the signals for C-30, C-31, and C-32, carbons derived from unlabeled valine added to the fermentation, was used as natural abundance reference in the normalization of the spectra. The results, shown in Table 2, reveal that the *R* isomer, in which the labeled hydroxymethyl group is phosphorylated by α-glycerokinase, gives rise to significant coupling between the two carbon atoms of each of the three acetate units, but only a very small amount of coupling between C-9 and C-10. The latter reflects the small amount (12.5%) of *S* isomer present in the precursor. There was, however, significant enrichment of C-10, as well as the three *O*- and *N*-methyl groups and the carbamate carbon. Coupling was also observed in the aromatic moiety between C-16 and C-17 and between C-20 and C-21. This is consistent with the formation from the labeled *R*-glycerol of [2,3-$^{13}C_2$]-phosphoenolpyruvate and [3,4-$^{13}C_2$]erythrose 4-phosphate, which via the aminoshikimate pathway[27] then give rise to the starter unit, 3-amino-5-hydroxybenzoic acid (AHBA) (Figure 2). The *S* isomer of the glycerol, in contrast, gave significant coupling between C-9 and C-10 of AP3, but single enrichments only in the acetate units at the carboxyl-derived carbons. Consistent with the aminoshikimate pathway, coupling was also seen between C-15 and C-16 and C-19 and C-20, as well as enrichment at C-18. In addition, the carbons corresponding to C-1 of the propionate units and the carbamate carbon were enriched (Figure 2). The results thus show that methoxymalonyl-ACP, the precursor of the "glycolate" unit, is derived from a triose phosphate or phosphoglycerate such that C-1 gives rise to the

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Table 1. ^{13}C-Enrichments and $^{13}C-^{13}C$-Couplings in Soraphen A Derived from D- and L-[1,2-$^{13}C_2$]Glycerate and *R*- and *S*-[1,2-$^{13}C_2$]Glycerol (100 MHz; numbers in red indicate substantial enrichments, bold face numbers represent still significant enrichments)

Carbon #	Chemical shift	*L*-[1,2-$^{13}C_2$]Glycerate feeding (referenced on C-2′,6′)				*D*-[1,2-$^{13}C_2$]Glycerate feeding (referenced on C-2′,6′)				*S*-[1,2-$^{13}C_2$]Glycerol feeding (referenced on C-2′,6′)				*R*-[1,2-$^{13}C_2$]Glycerol feeding (referenced on C-4′)			
		% ^{13}C	^{13}C excess			% ^{13}C	^{13}C excess			% ^{13}C	^{13}C excess			% ^{13}C	^{13}C excess		
			Singlet (%)	Doublet (%)	Jcc (Hz)		Singlet (%)	Doublet (%)	Jcc (Hz)		Singlet (%)	Doublet (%)	Jcc (Hz)		Singlet (%)	Doublet (%)	Jcc (Hz)
1	170.45	1.50	0.40	-	-	2.30	**1.20**	-	-	4.88	**3.78**	-	-	3.34	**2.24**	-	-
2	45.63	1.44	0.34	-	-	2.34	**1.24**	-	-	2.79	**1.69**	-	-	2.78	**1.68**	-	-
3	98.93	2.26	0.11	**1.05**	46.8	4.39	0.25	**3.04**	46.8	8.24	0.67	**6.47**	46.8	0.77	0	-	-
4	76.12	2.59	0.02	**1.47**	46.8	6.19	0.47	**4.62**	46.8	13.23	**2.54**	**9.59**	46.8	3.69	**2.59**	-	-
5	67.53	1.33	0.23	-	-	2.08	0.98	-	-	3.25	**2.15**	-	-	2.19	**1.09**	-	-
6	34.30	1.38	0.28	-	-	2.63	**1.53**	-	-	3.68	**2.58**	-	-	3.02	**1.92**	-	-
7	71.28	1.25	0.15	-	-	1.34	0.24	-	-	2.20	**1.10**	-	-	2.61	**1.51**	-	-
8	34.55	1.49	0.39	-	-	2.55	**1.45**	-	-	2.69	**1.59**	-	-	2.59	**1.49**	-	-
9	139.07	2.11	**1.01**	-	-	4.60	**3.50**	-	-	8.98	**7.88**	-	-	3.23	0.04	**2.09**	71.3
10	122.22	1.06	0	-	-	1.48	0.38	-	-	1.49	0.39	-	-	3.49	0.07	**2.32**	71.3
11	84.50	2.78	0.12	**1.56**	42.8	6.30	0.66	**4.54**	42.8	12.42	**1.42**	**9.90**	42.8	0.99	0	-	-
12	82.56	3.62	0.37	**2.15**	42.8	7.75	**1.02**	**5.63**	42.8	14.52	**3.39**	**10.03**	42.8	4.42	**3.32**	-	-
13	31.06	2.27	**1.17**	-	-	5.21	**4.11**	-	-	7.46	**6.36**	-	-	3.39	0.14	**2.15**	32.6
14	23.19	1.07	0	-	-	1.63	0.53	-	-	1.50	0.40	-	-	3.01	0	**1.91**	32.6
15	24.53	2.21	**1.11**	-	-	5.32	**4.22**	-	-	10.09	**8.99**	-	-	3.33	0.12	**2.11**	34.6
16	36.53	1.43	0.33	-	-	2.72	**1.62**	-	-	3.21	**2.11**	-	-	5.14	0.46	**3.58**	34.6
17	71.54	1.52	0.42	-	-	2.57	**1.47**	-	-	4.63	**3.53**	-	-	3.03	**1.93**	-	-
18	11.21	1.39	0.29	-	-	1.84	0.74	-	-	3.16	**2.06**	-	-	2.38	**1.28**	-	-
19	56.81	1.92	0.82	-	-	2.40	**1.30**	-	-	4.05	**2.95**	-	-	3.71	**2.61**	-	-
20	9.75	1.79	0.69	-	-	2.55	**1.45**	-	-	4.24	**3.14**	-	-	3.18	**2.08**	-	-
21	12.11	1.27	0.17	-	-	1.95	0.85	-	-	3.39	**2.29**	-	-	2.43	**1.33**	-	-
22	55.23	1.59	0.49	-	-	1.84	0.74	-	-	3.46	**3.36**	-	-	3.19	**2.09**	-	-
23	57.62	1.41	0.31	-	-	1.95	0.85	-	-	3.94	**2.84**	-	-	3.30	**2.20**	-	-
1′	142.41	2.44	**1.34**	-	-	5.05	**3.95**	-	-	7.87	**6.77**	-	-	3.81	0.38	**2.33**	~57
2′,6′	125.58	1.10	0	-	-	1.10	0	-	-	1.10	0	-	-	1.75	0	**0.65**	~57
3′,5′	128.31	1.23	0.13	-	-	1.69	0.59	-	-	6.35	**1.65**	**3.60**	~57	2.55	0.04	**1.41**	~57
4′	127.32	1.23	0.13	-	-	1.77	0.67	-	-	7.97	0.87	**6.00**	~57	1.10	0	-	-

Table 2. ^{13}C-Enrichments and $^{13}C-^{13}C$-Couplings in Ansamitocin P-3 Derived from *R*- and *S*-[1,2-$^{13}C_2$]Glycerol (150.8 MHz; numbers in red indicate significant enrichments)

Carbon #	Chemical shift	*R*-[1,2-$^{13}C_2$]Glycerol feeding				*S*-[1,2-$^{13}C_2$]Glycerol feeding			
		% ^{13}C	^{13}C Excess			% ^{13}C	^{13}C Excess		
			Singlet (%)	Doublet (%)	Jcc (Hz)		Singlet (%)	Doublet (%)	Jcc (Hz)
1	168.61	2.21	0.14	0.97	53.3	2.15	1.05		
2	32.66	2.65	0.11	1.44	53.9	1.24	0.14		
3	~76.6	buried				buried			
4	60.35	2.26	1.16			1.35	0.25		
5	66.33	2.3	1.2			2.05	0.95		
6	38.72	2.33	1.23			1.37	0.27		
7	74.26	2.55	0.28	1.17	34.7	2.2	1.1		
8	35.78	2.31	0.04	1.17	35.2	1.62	0.52		
9	80.81	0.98	0	Small amount of doublet		2.24	0	1.14	46.4
10	88.38	3.22	1.77	0.35	~48	2.65	0.2	1.35	46.4
11	127.62	2.64	0.37	1.17	71	2.08	0.98		
12	132.7	2.33	0.05	1.18	71	0.93	0		
13	124.37	2.35	1.25			1.93	0.83		
14	140.04	2.52	1.42			1.35	0.25		
15	47.12	1.12	0.02			3.09	0.31	1.68	42.7
16	139.99	3.08	0.09	1.89	58.7	2.66	0.21	1.35	~43
17	121.97	3.03	0	1.96	58.7	1.04	0		
18	142.52	1.36	0.26			1.9	0.8		
19	119.43	1.11	0.01			2.32	0.06	1.16	~74
20	156.02	3.16	0.14	1.92	67.2	3.51	0.5	1.91	~74
21	112.82	2.82	0.04	1.68	66.7	1.03	0		
22	12.14	1.85	0.75			1.3	0.2		
23	14.55	2.41	1.31			1.61	0.51		
24	152.36	3.07	1.97			3.11	2.01		
25	56.53	3	1.9			1.65	0.55		
26	15.75	2.25	1.15			1.18	0.08		
27	35.5	2.72	1.62			1.66	0.56		
28	56.65	2.43	1.33			1.36	0.26		
29	175.72	0.77	0			1.2	0.1		
30	33.81	1.08	0			1.26	0.16		
31	19.96	1.03	0			1.07	0		
32	17.84	1.18	0.08			0.97	0		

thioester carbonyl group, which becomes C-1 of the "glycolate" unit and the phosphorylated C-3 gives rise to the free carboxyl group which is lost in the subsequent Claisen condensation on the PKS.

The same conclusion was reached in the feeding experiments to *Sorangium cellulosum* with the stereochemically purer *R*- and *S*-[1,2-$^{13}C_2$]glycerol samples. The quantitative NMR analysis of the ^{13}C enrichments in the soraphen A samples presented a problem, since the molecule contains no carbon atoms of an external origin which could be used as a *bona fide* reference for the normalization of the spectra and the amounts of sample were too small for chemical derivatization. For the normalization of the soraphen A samples from D- and L-[1,2-$^{13}C_2$]glycerate and *S*-[1,2-$^{13}C_2$]glycerol, we used the signal for C-2′/C-6′ and for the *R*-[1,2-$^{13}C_2$]glycerol-derived sample we used the C-4′ carbon signal. According to biogenetic theory, these atoms are not expected to be labeled by the respective precursors. If they should carry some excess of ^{13}C after all, this would result in an underestimation of the enrichments of the other carbons. Calculations using other carbon atoms as reference gave lower apparent enrichments, i.e., were clearly underestimations. In light of the above, the data shown in Table 1 are minimum values. As summarized in Figure 2, the soraphen A from the *S* configured [1,2-$^{13}C_2$]glycerol sample displayed coupling between C-3 and C-4 as well as C-11 and C-12, the two carbon pairs of each "glycolate" unit, which therefore in this system, too, are derived from the two carbons of glycerol which are not phosphorylated by glycerol kinase. Coupling was also observed between C-3′/C-5′ and C-4′ of the aromatic ring, consistent with its shikimate pathway origin. Strong single-carbon enrichments were seen at C-9, C-13, and C-15, the carbons directly derived from the carboxyl group of acetate, and at C-1′. The absence of coupling between C-1′ and C-17 supports the previously deduced[28] formation of the benzoate starter unit via phenylalanine rather than by direct aromatization of a shikimate-derived C_6C_1 moiety. Since the levels of enrichment were rather high in this experiment, lesser degrees of single-carbon enrichments were seen in many other positions, e.g., the *O*-methyl groups, some of the propionate-derived carbons, C-17, C-4, and C-12, undoubtedly due to more extensive metabolism of the administered precursor. The feeding experiment with *R*-[1,2-$^{13}C_2$]glycerol gave complementary results. Coupling was observed between the two carbon atoms of each acetate-derived chain extension unit, but not within the

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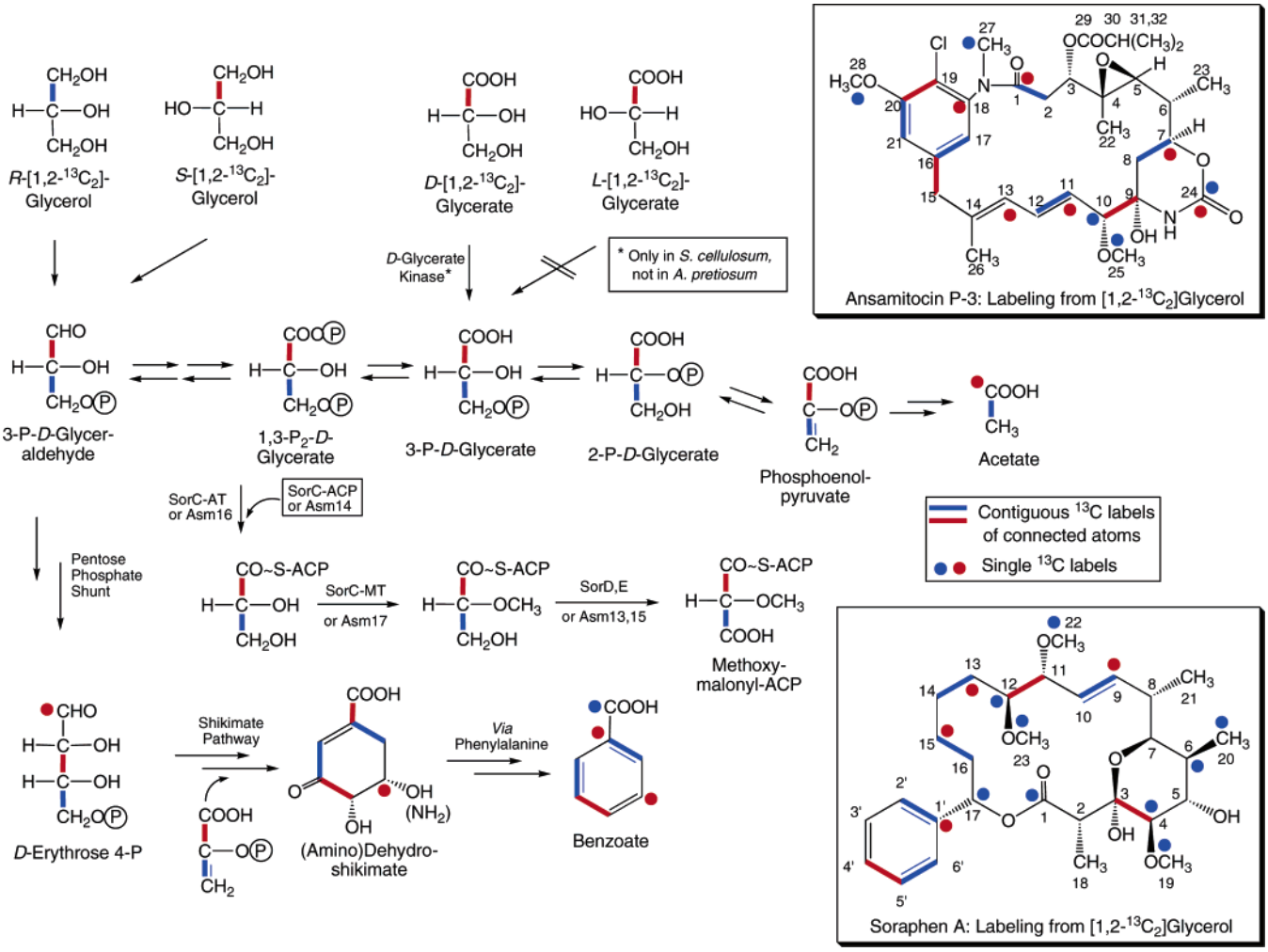

Figure 2. Labeling patterns of ansamitocin P-3, soraphen A, and metabolic intermediates after feeding ^{13}C-labeled glycerols and glycerates, and proposed biosynthesis of methoxymalonyl-ACP, the precursor of "glycolate" units. Heavy bars denote contiguous ^{13}C labels of connected atoms, dots denote single ^{13}C labels.

two "glycolate" units. The latter instead showed single-carbon enrichments at C-4 and C-12. Coupling was also observed in the aromatic moiety between C-1′ and C-2′/C-6′ as well as C-2′/C-6′ and C-3′/C-5′, as predicted by the shikimate pathway. In addition, single-carbon enrichments were observed for C-17, which originates from C-3 of phosphoenolpyruvate, the three *O*-methyl groups, and some of the propionate-derived carbons. These labeling patterns are well within expectations for the metabolism of glycerol, following stereospecific phosphorylation by glycerol kinase, via the glycolytic pathway and its known ancillary reactions (Figure 2).

The results clearly demonstrate that C-1 and C-2 of the "glycolate" units of both AP3 and soraphen A arise from C-1 and C-2, respectively, of a three-carbon glycolytic intermediate. A similar conclusion was reached for the "glycolate" units of concanamycin and bafilomycin.[29] They also disprove the interpretation of Hill et al. of their observation that *R*-[1-D$_2$]-glycerol results in deuterium incorporation at C-11 of soraphen A, i.e., C-1 of the "glycolate" unit.[4a] Since the deuterium in the precursor is attached to a carbon which itself is not incorporated into the "glycolate" unit, the incorporation of the deuterium obviously must represent an indirect process via redox reactions and the cellular pyridine nucleotide pool. This interpretation was considered by the authors but rejected as unlikely. Consequently, their conclusion that the formation of the "glycolate" unit cannot involve hydroxy- or methoxymalonate derivatives as intermediates is also invalid.

Conclusions

All the present and previous results are consistent with and support the formation of "glycolate" units from 2-methoxymalonyl-ACP, which in turn arises from phosphoglyceric acid, phosphoglyceraldehyde, or a closely related glycolytic intermediate. 1,3-Bisphospho-D-glyceric acid is the most likely substrate, based on two lines of evidence. One is the very specific incorporation of [1-^{13}C]glyceric acid into the C-1 carbons of the "glycolate" units of geldanamycin[5] and the intact incorporation of D-[1,2-^{13}C$_2$]glyceric acid into the "glycolate" units of soraphen A demonstrated in the present work. The lack of incorporation of glyceric acid in some systems, such as leukomycin[6] or AP3, probably reflects the ability or inability of a given organism to phosphorylate glyceric acid, i.e., the presence or absence of an active D-glycerate kinase.

(29) Schuhmann T.; Grond S. New Insights into the "Glycolate" Unit Biosynthesis in the Plecomacrolides Bafilomycin A$_1$, B$_1$ and Concanamycin A. Manuscript in preparation.

The other argument relates to the mode of attachment of the carboxyl group of the glycerate unit to the thiol group of the ACP. This could involve 3-phosphoglyceric acid as substrate and a carboxyl activation to the adenylate or the CoA thioester. However, there is little support for such a process at the genetic level. There is no obvious adenylation or other carboxyl-activation gene in the "glycolate" subclusters,[14,15] and Asm16 and SorC, which have been assigned the function of attaching the three-carbon unit to the ACP,[13] show no homology to known carboxyl-activating enzymes. The absence of a dedicated gene encoding a carboxyl-activating enzyme from the methoxymalonate subclusters suggests that the primary metabolism substrate is already activated at the carboxyl group. This points to 1,3-bisphospho-D-glycerate, a normal glycolytic intermediate, which would be formed from glycerol directly via glycerol 1-phosphate and the triose phosphates and, in some organisms only, from D-glycerate via 2- and/or 3-phospho-D-glycerate by the action of a glycerate kinase and glycolytic enzymes. 1,3-Bisphospho-D-glycerate can be transferred to the thiol group of an ACP, Asm14 or the ACP domain of SorC, by Asm16 or the AT domain of SorC, respectively. The necessary cleavage of the phosphate ester group from the resulting 3-phosphoglyceryl-ACP may also be catalyzed by Asm16, which shows at least 25−30% identity at the protein level to members of the IIIC subgroup of the HAD superfamily of aspartate-dependent hydrolases (Interpro IPR010033).[30,31] The functions of many of these enzymes are unknown, but all the characterized members of the HAD III subfamily are phosphatases[31,32] apparently using large substrates (phosphorylated proteins),[33] and the family includes the FkbH protein which shows up to 64% identity at the protein level to Asm16 and its homologues (Figure 3). Whether SorC also has such a function is not clear, since it shows no homology to Asm16 or to known phosphatases; the *sor* cluster also contains no other Asm16 homologue. Another possibility, which, however, we consider less likely, would be a reaction of 3-phosphoglyceraldehyde with the thiol group of the ACP to form a thiohemiacetal, which would then be oxidized to the thioester by one of the dehydrogenases encoded in the methoxymalonate subclusters, possibly Asm15 and SorE. The reverse of this reaction appears to be responsible for a reductive product release mechanism from polyketide/nonribosomal peptide synthetases by reductive cleavage of acyl-ACPs.[34]

The conversion of 1,3-bisphosphoglycerate into methoxymalonyl-ACP involves connecting C-1 to the thiol group of the ACP to become the thioester carbonyl group of methoxymalonyl-ACP. Methylation of the oxygen at C-2 has been proposed as the terminal step of the conversion sequence to methoxymalonyl-ACP.[15] However, we consider it more likely that this methylation occurs at the stage of D-glyceryl-ACP, before the stepwise oxidation of C-3 to a carboxyl group. This suggestion is based on the sequence of the *sorC* gene of the *sor* biosynthetic gene cluster[18] which encodes a trifunctional gene product consisting of a dedicated ACP, an AT domain, and a methyltransferase domain. It seems likely that SorC is responsible not only for the attachment of the glyceryl moiety to the dedicated ACP but also its subsequent *O*-methylation,[13] i.e., that it catalyzes the formation of 2-*O*-methyl-D-glyceryl-ACP from 1,3-bisphosphoglycerate. In analogy we propose that Asm17 acts before Asm13 and Asm15. We thus propose as a working hypothesis the pathway shown in Figure 2 for the formation of methoxymalonyl-ACP and hence of the "glycolate" unit.

The finding that D-glycerate, and thus presumably 1,3-bisphospho-D-glycerate, is the precursor of the "glycolate" unit predicts that the 2-methoxymalonyl-ACP must have *R* configuration at C-2. Since decarboxylative Claisen condensations on the PKS are known to proceed with inversion of configuration,[35] the direct utilization of (2*R*)-methoxymalonyl-ACP without epimerization would result in "glycolate" units of L configuration.[36] A survey of the configurations of known, chiral "glycolate" units shows a majority of them, C-10 of AP3, C-4 of soraphen A, C-6 of geldanamycin, C-4 of leucomycin, C-13 of FK-520/FK-506, C-14 of bafilomycin, C-16 of concanamycin, C-4 of oxazolomycin, and C-23 of tautomycin, to be D configured, whereas a minority, C-12 of soraphen A and geldanamycin and C-15 of FK-520/FK-506, have L configuration. Thus, in at least some of the "glycolate" units the chain extension substrate must have undergone epimerization either before or after its incorporation into the polyketide. In the analogous case of the chain extension by propionate units, only (2*S*)-methylmalonyl-CoA is used as substrate but gives rise to both D and L configured propionate units.[37] Work on module 2 of the erythromycin PKS, which generates a propionate unit with an epimerized methyl-bearing center, has shown that the configuration at this center is controlled by the ketoreductase domain which reduces the adjacent carbonyl group and is stereoselective for only the epimerized substrate.[38] However, the authors stress that different mechanisms may control the methyl stereochemistry in other PKS modules. Such a mechanism can clearly not operate in the formation of the "glycolate" units at C-10 of AP3 and C-4 of soraphen A, because in both cases the adjacent carbonyl group has not been reduced. Substantially more work will be necessary to unravel the complex stereochemical control mechanisms governing these chain extension reactions.

Experimental Section

Materials and General Methods. ^{1}H- and ^{13}C NMR spectra were recorded on Bruker AM-300, DRX-300, ARX-400, DRX-499, and INOVA-600 NMR spectrometers in either $CDCl_3$, D_2O, or [D_6]DMSO as solvent. For determination of ^{13}C enrichments the peak integral from the enriched sample was compared with that of a control sample of natural abundance, using the average of the signals for C-30, C-31, and C-32 (AP3), the C-2′/C-6′ or the C-4′ signal (soraphen A) as natural abundance reference. For HPLC, a Beckman System Gold programmable solvent module was used with a Beckman System Gold diode

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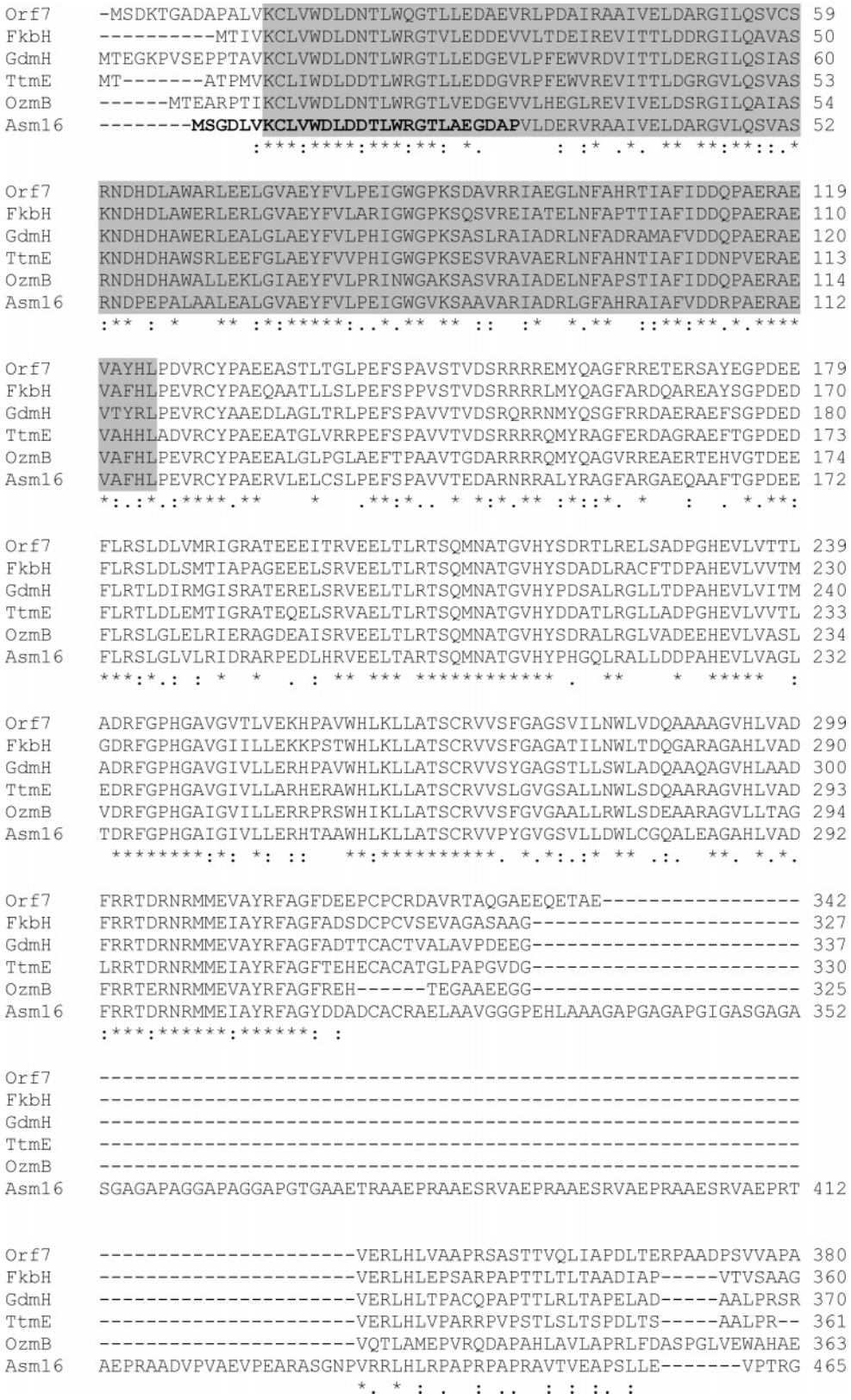

Figure 3. Alignment of Asm16 and its equivalents from the concanamycin (Orf7), FK-520 (FkbH), geldanamycin (GdmH), tautomycin (TtmE), and oxazolomycin (OzmB) biosynthetic gene clusters (GenBank accession numbers: AAF86387 (Asm16), AAZ94395 (Orf7), AAF86387 (FkbH), AAO06922 (GdmH), AAZ08062 (TtmE), ABA39082 (OzmB)). Based on the alignment with its homologues, the translation start of Asm16 was repositioned resulting in an N-terminal extension of 28 amino acids (shown in bold) in comparison to the published sequence (AAF86387). The HAD-superfamily phosphatase subfamily IIIC domain (Interpro IPR010033) is highlighted in gray.

array detector. Fermentations were carried out with shaking in an Adolf Kühner ISF-4-V temperature-controlled shaker cabinet. Chemicals were purchased from Aldrich, biochemicals from Sigma, and D-[1,2-$^{13}C_2$]-glucose, [U-$^{13}C_3$]glycerol, D,L-[1-^{13}C]serine, D,L-[3-^{13}C]serine, and

[1,2-$^{13}C_2$]succinate from Cambridge Isotope Laboratories. Anhydrous [1,2-$^{13}C_2$]acetic acid for the synthesis of [1,2-$^{13}C_2$]glycerate and [1,2-$^{13}C_2$]-glycerol was provided by the Los Alamos Stable Isotope Resource.

Synthesis of Labeled Glycerates and Glycerols via Asymmetric Dihydroxylation. Benzyl [1,2-$^{13}C_2$]Bromoacetate. [1,2-$^{13}C_2$]Acetic acid (10.0 mL, 10.5 g, 170 mmol), trifluoroacetic anhydride (40.0 mL, 59.5 g, 383 mmol) and DMAP (0.213 g, 1.74 mmol, 0.01 equiv) were heated to 60 °C. Bromine (9.40 mL, 29.3 g, 183 mmol, 1.05 equiv) was added at 60 °C at a rate such that the orange color was just maintained (90 min). Stirring was continued at 60 °C for another hour. The reaction mixture was then brought to room temperature and purged with argon for 2 h to remove HBr and unreacted bromine. Benzyl alcohol (100 mL, 966 mmol) was added carefully at room temperature, and the reaction mixture was then heated at 60 °C overnight. After cooling to room temperature it was poured into concentrated $NaHCO_3$ solution (100 mL). Diethyl ether (150 mL) was added, the phases were separated, and the aqueous phase was extracted three more times with ether (100 mL each). The combined organic phases were washed with concentrated $NaHCO_3$ solution (100 mL), concentrated $Na_2S_2O_3$ solution (100 mL), and water (100 mL) and were dried over $MgSO_4$. Chromatography (silica gel, ethyl acetate/hexanes, 5:95) gave the title compound (34.0 g, 147 mmol, 86.5%). ^{1}H NMR: δ 3.88 (dd, $J_{CH2,2-13C} = 153.4$ Hz, $J_{CH2,1-13C} = 4.7$ Hz, CH_2Br), 5.21 (d, $J_{OCH2,1-13C} = 3.3$ Hz, OCH_2), 7.33−7.41 (m, Ar).

Benzyl [1,2-$^{13}C_2$]Acrylate. Benzyl [1,2-$^{13}C_2$]bromoacetate (34.0 g, 147 mmol) and triphenylphosphine (42.6 g, 162 mmol, 1.1 equiv) were added to toluene (350 mL). The reaction mixture was stirred at room temperature for 3 days. The Wittig salt was filtered, washed with toluene, and dried under vacuum. The Wittig salt (69.7 g, 141 mmol) was dissolved in the two-phase system diethyl ether/water (600/300 mL), and K_2CO_3 (19.5 g, 141 mmol, 1.00 equiv) was added over 15 min. The reaction mixture was stirred at 35 °C for 24 h. The layers were separated, the aqueous phase was extracted with diethyl ether (3 × 200 mL), and the combined organic phases were dried over $MgSO_4$. Paraformaldehyde (4.44 g, 148 mmol, 1.05 equiv) was added to the organic phase, and the reaction mixture was heated under reflux for 24 h. The solvent was evaporated, and chromatography (silica gel, 20% ethyl acetate in hexanes) gave the title compound (21.02 g, 87% based on benzyl bromoacetate). ^{1}H NMR (500 MHz): δ 5.21 (d, $J_{OCH2,1-13C} = 3.1$ Hz, CH_2O), 5.85 (ddd, $J_{cis-H,2-13C} = 14.3$ Hz, $J_{cis-H,2-H} = 10.4$ Hz, $J_{cis-H,trans-H} = 1.4$ Hz, *cis*-H), 6.17 (dddd, $J_{2-H,2-13C} = 164.1$ Hz, $J_{2-H,trans-H} = 17.3$ Hz, $J_{2-H,cis-H} = 10.3$ Hz, $J_{2-H,1-13C} = 3.9$ Hz, 2-H), 6.45 (dddd, $J_{trans-H,2-H} = 17.3$ Hz, $J_{trans-H,2-13C} = 7.6$ Hz, $J_{trans-H,1-13C} = 3.4$ Hz, $J_{trans-H,cis-H} = 1.4$ Hz, *trans*-H), 7.30−7.42 (m, Ar).

Benzyl D-[1,2-$^{13}C_2$]Glycerate. $K_3Fe(CN)_6$ (116.1 g, 355.5 mmol, 3 equiv), K_2CO_3 (48.59 g, 355.5 mmol, 3 equiv), $(DHQ)_2$-PHAL (0.9243 g, 1.185 mmol, 0.01 equiv), and $K_2OsO_2(OH)_4$ (0.0877 g, 0.237 mmol, 0.002 equiv) were dissolved in *tert*-butyl alcohol (600 mL) and water (600 mL). Stirring at room temperature produced two clear phases. The mixture was cooled to 0 °C. Benzyl [1,2-$^{13}C_2$]acrylate (19.46 g, 118.5 mmol) was added and the reaction mixture was stirred at 0 °C for 10 h. Na_2SO_3 (177.8 g, 1.411 mol) was added at 0 °C, the reaction mixture was allowed to warm to room temperature and stirred for 1 h. Ethyl acetate (500 mL) was added, and the phases were separated. The aqueous phase was extracted with ethyl acetate (3 × 400 mL). The combined organic phases were dried over $MgSO_4$, and chromatography (silica gel, EtOAc/hexanes, 1:1) gave the title compound (19.47 g, 83%, ee: 75%). ^{1}H NMR (500 MHz): δ 3.42 (br s, 2 × OH), 3.84 (ddd, $J_{ab} = 11.7$ Hz, $J_{3-Ha,2-H} = 4.0$ Hz, $J_{3-Ha,2-13C} = 1.1$ Hz, 3-H_a), 3.87−3.93 (m, 3-H_b), 4.30 (dddd, $J_{2-H,\ 2-13C} = 146.5$ Hz, $J_{2-H,3-Ha} = 4.0$ Hz, $J_{2-H,3-Hb} = 4.0$ Hz, $J_{2-H,1-13C} = 4.0$ Hz, 2-H), 5.20 (dd, $J_{ab} = 12.2$ Hz, $J_{Ha,1-13C} = 3.1$ Hz, OCH_aH_b), 5.24 (dd, $J_{ab} = 12.2$ Hz, $J_{Hb,1-13C} = 3.0$ Hz, OCH_aH_b), 7.25−7.40 (m, Ar).

The same procedure, using $(DHQD)_2$-PHAL as reagent and benzyl [1,2-$^{13}C_2$]acrylate (19.46 g, 118.5 mmol), gave benzyl L-[1,2-$^{13}C_2$]-glycerate (19.66 g, 84%, ee: 69%). ^{1}H NMR was identical to that of D isomer.

Sodium D- and L-[1,2-$^{13}C_2$]Glycerate. Benzyl D-[1,2-$^{13}C_2$]glycerate (1.926 g, 9.73 mmol) was dissolved in THF (14 mL) and water (6 mL). Pd−C (196 mg) was added, and the mixture was stirred vigorously under an atmosphere of H_2 for 3.5 h, with more Pd−C (100 mg) being added after 2 h. When TLC showed no more educt, the reaction mixture was filtered and the filtrate evaporated to dryness. The residue was dissolved in water (10 mL), neutralized with solid $NaHCO_3$ (828 mg, 9.86 mmol), and evaporated to dryness to give sodium D-[1,2-$^{13}C_2$]-glycerate as a colorless glass (1.296 g, 9.95 mmol, 102%). ^{1}H NMR (300 MHz, D_2O): δ 3.66 − 3.95 (m, 3-H_{ab}), 4.29−4.42 (m, 2-H). ^{13}C NMR (75 MHz, D_2O): δ 61.4 (d, $J = 40.1$ Hz, C-3), 70.6 (d, $J = 53.8$ Hz, C-2, enriched), 176.1 (d, $J = 53.8$ Hz, C-1, enriched).

In the same way, benzyl L-[1,2-$^{13}C_2$]glycerate (1.93 g, 9.75 mmol) gave sodium L-[1,2-$^{13}C_2$]glycerate (1.249 g, 9.61 mmol, 98.6%). NMR spectra were identical to those of D sample.

***R*- and *S*-[1,2-$^{13}C_2$]Glycerol.** Benzyl D-[1,2-$^{13}C_2$]glycerate (7.785 g, 39.28 mmol) was dissolved in THF (130 mL). The solution was cooled to 0 °C and stirred, and $LiBH_4$ (2 M in THF, 69.0 mL, 138 mmol, 3.5 equiv) was added dropwise. With continued stirring, the reaction mixture was allowed to warm to room temperature over 30 min and then neutralized with water (50 mL) and 2 N HCl. The organic phase was evaporated, and the aqueous phase was washed with ethyl acetate (3 × 100 mL). Cations were removed from the aqueous phase by passage through a column of ion-exchange resin AG 50 H^+ with water as eluent. The remaining borate in the aqueous phase was removed by evaporation to dryness followed by repeated addition of methanol and evaporation of the resulting borate ester to give *R*-[1,2-$^{13}C_2$]glycerol (3.697 g, 100%). ^{1}H NMR (500 MHz, D_2O): δ 3.36−3.70 (m, 2 × CH_2OH), 3.73−3.94 (m, C*H*OH). ^{13}C NMR (125 MHz, D_2O): δ 62.5 (d, $J = 41$ Hz, C-1), 72.0 (d, $J = 41$ Hz, C-2).

In the same way, benzyl L-[1,2-$^{13}C_2$]glycerate (7.849 g, 39.60 mmol) gave *S*-[1,2-$^{13}C_2$]glycerol (3.727 g, 100%). NMR spectra identical to those of the *R* sample.

Determination of Enantiomeric Purity. *R*-[1,2-$^{13}C_2$]Glycerol (20 mg, 0.21 mmol) was dissolved in water (2 mL). HEPES buffer (500 mg, ca. 10 equiv) and ATP (300 mg, ca. 2.5 equiv) were added, and the solution was adjusted with NaOH (1 and 0.1 N) to pH 7. Glycerol kinase (100 units) was added, and the reaction mixture was gently shaken for 20 h at 37 °C. The reaction mixture was then analyzed by ^{13}C NMR. The ratio of the integral of the signals at 64.9 ppm ($CH_2OPO_3^{2-}$) and 62.3 ppm (CH_2OH) was 1:0.1461, corresponding to 75% ee *R*.

The same procedure was used with *S*-[1,2-$^{13}C_2$]glycerol to give a δ 64.9 vs 62.3 ratio of 0.1826:1, corresponding to 69% ee *S*.

Synthesis of Labeled Glycerols via Chiral Sulfoxides. Ethyl 2-*O*-Benzyl-[1,2-$^{13}C_2$]glycolate. 2-*O*-Benzyl-[1,2-$^{13}C_2$]glycolic acid (8.48 g, 50.4 mmol) was heated to reflux in EtOH (80 mL) in the presence of Amberlyst-[H^+] resin. After 10 h, the reaction mixture was allowed to cool to RT and was filtered through Celite. The Celite was rinsed with EtOH (8 × 25 mL), and the filtrate was concentrated in vacuo. Purification by flash column chromatography (EtOAc/hexane, 1:6) yielded the product as a clear, colorless oil (8.77 g, 89%). ^{1}H NMR ($CDCl_3$): δ 1.27 (t, $J = 7.2$ Hz, 3H), 4.09 (dd, $^1J_{CH} = 143.7$ Hz, $J = 4.5$ Hz, 2H), 4.21 (dq, $J = 7.2$ Hz, 3 Hz, 2H), 4.64 (d, $J = 4.2$ Hz, 2H), 7.27−7.39 (m, 5H). ^{13}C NMR ($CDCl_3$): δ 14.4, 60.9, 67.48 (str, d, $^1J_{CC} = 62.9$ Hz), 73.6, 128.5, 128.1, 128.1, 137.3, 170.6 (str, d, $^1J_{CC} = 62.9$ Hz). IR (neat, cm^{-1}): 2981.4, 2872.8, 1707.9, 1454.1, 1424.7, 1234.2, 1182.5, 1109.7, 1029.2. Anal. Calcd for $C_{15}{}^{13}C_2H_{19}O_3S$: C, 68.35; H, 7.19. Found: C, 68.06; H, 7.02.

***S*-3-Benzyloxy-1-(*p*-tolylsulfinyl)-2-[2,3-$^{13}C_2$]propanone (4).** A 2.5 M solution of *n*-butyllithium in hexanes (5 mL, 12.5 mmol) was added dropwise to a solution of diisopropylamine (1.8 mL, 12.8 mmol) in THF (15 mL), under argon, and was cooled to −78 °C. The reaction

mixture was allowed to reach 0 °C and was stirred for 30 min. The LDA solution was then added to a solution of *S*-methyl *p*-tolyl sulfoxide **3** (0.92 g, 5.97 mmol) in THF (10 mL) at 0 °C. After stirring for 30 min, the sulfoxide anion solution was added to a solution of ethyl 2-*O*-benzyl-[1,2-$^{13}C_2$]glycolate (1.17 g, 5.97 mmol) in THF (20 mL). The reaction mixture was allowed to warm to RT. After stirring for 2 h, the reaction was quenched with saturated NH_4Cl (50 mL), acidified to pH 2 with 5% H_2SO_4, extracted with CH_2Cl_2 (3 × 50 mL), and dried over Na_2SO_4. The solvent was removed in vacuo to give 2.19 g of crude product **4** as a yellow crystalline solid, which was used in the next step without further purification.

(2*R*,*SS*)-3-Benzyloxy-1-(*p*-tolylsulfinyl)-2-[2,3-$^{13}C_2$]propanol (5). A solution of DIBAL in CH_2Cl_2 (1 M, 7.2 mL, 7.2 mmol) was added dropwise to a solution of the crude β-ketosulfoxide **4** in THF (40 mL) at −78 °C. After stirring for 30 min, the reaction was quenched with saturated NH_4Cl (30 mL) and allowed to warm to RT, acidified to pH 4 with 5% H_2SO_4, extracted with CH_2Cl_2 (3 × 50 mL), and dried over Na_2SO_4, and the solvent was removed in vacuo. The crude product (1.85 g) was purified by flash column chromatography (EtOAc/hexanes, 75:25) to give the product **5** as a pale-yellow crystalline solid (1.13 g, 62% from **3**). $[\alpha]^{22}_D$ −166.4 (*c* 1.21, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.42 (s, 3H), 2.79−2.74 (m, 1H), 3.12−3.01 (m, 1H), 3.49 (dm, $^1J_{CH}$ = 141.9 Hz, 2H), 3.65 (br s, 1H), 4.38 (dm, $^1J_{CH}$ = 146.1 Hz, 1H), 4.56−4.59 (m, 2H), 7.25−7.53 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.6, 66.1 (d, $^1J_{CC}$ = 41.8 Hz), 66.2 (str, d, $^1J_{CC}$ = 42.1 Hz), 73.4 (str, d, $^1J_{CC}$ = 42.1 Hz), 74.1, 124.2, 127.9, 128.1, 128.7, 130.3, 141.8. IR (neat, cm^{-1}): 3302, 2859, 1491, 1446, 1359, 1295, 1112, 1079, 1011. Anal. Calcd for $C_{15}{}^{13}C_2H_{20}O_3S$: C, 67.29; H, 6.58. Found: C, 67.52; H, 6.57.

(2*R*)-1,2-Diacetoxy-3-benzyloxy-1-(*p*-tolylthio)-[2,3-$^{13}C_2$]propane (6). A mixture of **5** (1.68 g, 5.49 mmol) and NaOAc (4.51 g, 54.94 mmol) was heated at reflux in Ac_2O (60 mL) for 15 h. The acetic anhydride was removed by azeotropic distillation with toluene (3 × 50 mL). The residue was dissolved in CH_2Cl_2 (25 mL) and filtered to remove the NaOAc. The filtrate was concentrated and purified by flash column chromatography (EtOAc/hexanes, 20:80) to yield a mixture of the two diastereoisomers **6** as a pale-yellow oil (2.02 g, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.99 (s, 3H), 2.03 (s, 3H), 2.05 (s, 3H), 2.08 (s, 3H), 2.32 (s, 3H), 3.68 (dm, $^1J_{CH}$ = 141.8 Hz, 4H), 4.43−4.59 (m, 4H), 5.30 (dm, $^1J_{CH}$ = 147.6 Hz, 2H), 6.25−6.29 (m, 2H), 7.07−7.39 (18H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.0, 21.1, 21.4, 68.2 (str, d, $^1J_{CC}$ = 43.8 Hz), 68.3 (str, d, $^1J_{CC}$ = 43.5 Hz), 69.3, 72.6 (str, d, $^1J_{CC}$ = 43.8 Hz), 72.7 (str, d, $^1J_{CC}$ = 43.5 Hz), 73.5, 128.0, 128.6, 128.6, 130.1, 130.1, 134.0, 134.3, 137.8, 138.9, 139.1, 170.2, 169.3. IR (neat, cm^{-1}): 3030.8, 2860.8, 1744.6, 1492.6, 1453.0, 1369.7, 1207.3, 1017.8. Anal. Calcd for $C_{19}{}^{13}C_2H_{24}O_5S$: C, 65.11; H, 6.20. Found: C, 65.20; H, 6.12.

(2*S*)-1-Benzyloxy-2,3-[1,2-$^{13}C_2$]propanediol (7). $LiAlH_4$ (697 mg, 18.36 mmol) was added to a solution of **6** (1.20 g, 3.06 mmol) in THF (17 mL) at 0 °C. The reaction mixture was allowed to warm to RT. After 1.5 h, the mixture was cooled to 0 °C and cautiously quenched with saturated aqueous NH_4Cl (50 mL). After stirring for 20 min, the mixture was diluted with CH_2Cl_2 (50 mL) and acidified to pH 1 with 5% H_2SO_4. The aqueous fraction was extracted with CH_2Cl_2 (4 × 50 mL), and the combined organics were dried over Na_2SO_4 and concentrated under reduced pressure. The crude oil was purified by flash column chromatography (EtOAc/hexanes, 75:25) to give the desired product **7** as a dark-yellow oil (604 mg, 91%). $[\alpha]^{25}_D$ −2.95 (*c* 3.73, C_6H_6). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.10 (br s, 1H), 2.61 (br s, 1H), 3.28−3.39 (m, 1H), 3.61−3.85 (m, 3H), 3.89 (dm, $^1J_{CH}$ = 139.8 Hz, 1H), 4.56 (d, *J* = 3.6 Hz, 2H), 7.27−7.39 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 64.1 (dd, *J* = 38.1, 4.1 Hz), 70.97 and 71.72 (str, ABq, $^1J_{CC}$ = 41.8 Hz), 73.6, 127.9, 127.9, 128.6, 137.9. IR (neat, cm^{-1}): 3355.2, 3062.5, 3029.9, 2857.6, 1453.1, 1362.9, 1206.1, 1060.0, 1027.9. Anal. Calcd for $C_8{}^{13}C_2H_{14}O_3$: C, 66.28; H, 7.66. Found: C, 66.16; H, 7.73.

***S*-[1,2-$^{13}C_2$]Glycerol.** Compound **7** (515 mg, 2.80 mmol) was dissolved in EtOH (15 mL) in a thick-walled reaction flask; 10% Pd/C (80 mg) and Amberlyst-15 (H^+) resin (50 mg) were added, and the reaction vessel was connected to a Parr hydrogenator, with an H_2 atmosphere of 45 psi. The reaction was complete after shaking for 4.5 h. The reaction mixture was filtered through Celite and rinsed with EtOH, and the solvent was removed in vacuo to yield (*S*)-[1,2-$^{13}C_2$]glycerol as a clear, colorless oil (257 mg, 98%). ^{1}H NMR (300 MHz, D_2O): δ 3.55−3.74 (m, 2H), 3.65 (dm, $^1J_{CH}$ = 142 Hz, 2H), 3.84 (dm, $^1J_{CH}$ = 142 Hz, 1H). ^{13}C NMR (75 MHz, D_2O): δ 62.5 (d, $^1J_{CC}$ = 40.9 Hz), 72.1 (d, $^1J_{CC}$ = 40.9 Hz). IR (neat, cm^{-1}): 3294.1, 2927.2, 2874.8, 1416.2, 1321.0, 1205.4, 1091.3, 1031.6. Anal. Calcd for $C^{13}C_2H_8O_3$: C, 40.41; H, 8.57. Found: C, 40.53; H, 8.72.

By the same procedures, *R*-methyl-*p*-tolyl sulfoxide (1.16 g, 7.53 mmol) gave *R*-[1,2-$^{13}C_2$]glycerol (298 mg, 3.16 mmol) in 42% overall yield.

Biological Experiments. Feeding Experiments with *Actinosynnema pretiosum*. For resting cell experiments, *Actinosynnema pretiosum* ssp. *auranticum* ATCC 31565 was grown on yeast−malt extract agar plates at 28 °C for 4 days and then stored at 0 °C. A loop of *A. pretiosum* was used to inoculate 25 mL of YMG medium (yeast extract 0.4%, malt extract 1.0%, glucose 0.4%, pH adjusted to 7.4 with NaOH before autoclaving) in a 250-mL Erlenmeyer flask with a stainless steel coil and incubated for 24 h at 29 °C with shaking at 225 rpm. One milliliter of this starter culture was used to inoculate each 100 mL of YMG medium in a 500-mL Erlenmeyer flask with a spring, all of which were incubated for 48 h at 29 °C with shaking at 225 rpm. These were then harvested by centrifugation to form a pellet, which was washed three times with 20 mL each of sterile double distilled (DDI) water and then resuspended in 20 mL of DDI water. The suspension was added to a 500-mL Erlenmeyer flask with a spring containing 80 mL of sterile, resting cell medium (20 mL of 0.25 M lactose, 10 mL of 0.05 M $MgCl_2$, 10 mL of 0.05 M sodium isobutyrate, 10 mL of 0.1 M Tris × HCl pH 8.5, and 30 mL of DDI water) and incubated at 29 °C with shaking at 225 rpm. Labeled precursors in the total amounts indicated were added to the number of cultures indicated in three equal portions at times 0, 24, and 48 h and the cultures harvested at 72 h to give the amounts of purified AP3 indicated: D,L-[1-^{13}C]serine, 150 mg, 10 cultures, 2 mg of AP3; D,L-[3-^{13}C]serine, 150 mg, 10 cultures, 9.4 mg of AP3; [1,2-$^{13}C_2$]succinate, 150 mg, six cultures, 5.1 mg of AP3; D-[1,2-$^{13}C_2$]glucose, 120 mg, 10 cultures, 5.8 mg of AP3; [U-$^{13}C_3$]-glycerol, 150 mg, 10 cultures, 3.0 mg of AP3. At the end of the fermentation period the combined cultures were extracted three times with an equal volume of ethyl acetate, the combined extracts were dried, and the solvent was evaporated to give a viscous oil. This residue was dissolved in methanol and passed through a C_{18} cartridge (2.5 cm × 0.5 cm). The eluent was purified by HPLC with UV detection (234 and 280 nm) either on a YMC Pack ODS-AQ column (5 μm, 250 mm × 10 mm) equipped with a YMC guard column (30 mm × 10 mm) with a gradient of water/methanol, 1:1 to 1:9, over 25 min (flow rate 3 mL/min, t_{ret} AP3 = 21.5 min), or on an Alltech Econosil C18 10U column (250 mm × 22.5 mm), also equipped with a guard column, with water/methanol, 2:3 (flow rate 20 mL/min, t_{ret} AP3 = 11 min).

Subsequent experiments used improved fermentation conditions. A single colony from a YMG agar plate of *Actinosynnema pretiosum* ssp. *auranticum* ATCC 31565 was used to inoculate 100 mL of YMG medium in a 500-mL Erlenmeyer flask with coil, which was grown for 48 h at 30 °C with shaking at 220 rpm. Portions of 0.5 mL of this stock culture were mixed with 0.5 mL of sterilized 40% aqueous glycerol and stored at −20 °C. To prepare seed cultures, 1 mL of stock culture was used to inoculate 100 mL of YMG medium in a 500-mL Erlenmeyer flask with coil, which was grown for 48 h at 30 °C with shaking at 220 rpm. Eight mL of seed culture was then used to inoculate each 100 mL of production medium[39] in a 500-mL Erlenmeyer flask with a coil; 12 mL of a filter-sterilized 3% solution of L-valine was added to each flask, and the cultures were grown for 9 days at 30 °C

with shaking at 220 rpm. Labeled precursors were added in the total amounts indicated to the number of cultures indicated in eight equal portions every 24 h starting at day 1 and gave the amounts of purified AP3 indicated: *R*- And *S*-[1,2-$^{13}C_2$]glycerol, 80 mg mixed with 320 mg of unlabeled glycerol, five cultures each, 24.5 and 25.4 mg of AP3, respectively; sodium D- and L-[1,2-$^{13}C_2$]glycerate, 80 mg, five cultures each, 34.3 and 40.7 mg of AP3, respectively. AP3 was isolated by extracting the fermentation broth 3× with an equal volume of ethyl acetate and evaporating the combined, dried extract to dryness. The residue was taken up in 2 mL of chloroform/methanol (9:1) and loaded onto a column (2 cm diameter) of 50 g of neutral aluminum oxide. The column was washed with 100 mL of chloroform, after which AP3 was eluted with chloroform containing 5% methanol. This process was repeated once to obtain pure AP3. The production medium[39] consists of (per liter): 60 g of dextrin, 30 g of maltose, 5.25 g of cotton seed flour (ProFlo), 4.5 g yeast extract, 5 g of $CaCO_3$, 0.5 g of K_2HPO_4, 2 mg of $FeSO_4 \times 7\ H_2O$; the medium is brought to a boil and slowly cooled off before autoclaving.

Feeding Experiments with *Sorangium cellulosum* So ce26. *Sorangium cellulosum* So ce26 Y2 was cultivated in H medium [soybean meal (soyamine 50T Lucas Meyer, Hamburg) 0.2%; glucose (Maizena) 0.2%; starch (Cerestar SF 12618, Cerestar Deutschland Krefeld) 0.8%; yeast extract (Marcor) 0.2%; $CaCl_2 \times 2\ H_2O$ 0.1%; $MgSO_4 \times 7\ H_2O$ 0.1%; ethylenediaminetetraacetic acid iron(III)-sodium salt 0.0008%; HEPES 1.19%; XAD-16 (Rohm and Haas, Frankfurt/M) 2% (w/v)]. The pH of the medium was adjusted to 7.2 with KOH before autoclaving. From a 4-day-old preculture, fresh, 100-mL cultures were inoculated with 10% of the seed culture and incubated in 250-mL Erlenmeyer flasks at 30 °C and 180 rpm on a rotary shaker. One hundred milligrams of each of the respective labeled compounds was dissolved in 6 mL of distilled water and sterilized by filtration. After 3 days, 6 days, and 9 days of cultivation 2 mL of each of the respective labeled compounds was added from the stock solutions above. Fifteen days after the start of the cultivation, the adsorber resin XAD was harvested by sieving and eluted with 100 mL of methanol. After evaporation of the methanol, the resulting aqueous layer was made up to 50 mL and extracted with ethyl acetate (2 × 50 mL). Evaporation of the solvent yielded an oily residue, which was dissolved in 500 μL of 60% methanol and subjected to sequential preparative RP-HPLC (Nucleosil C_{18}, solvent 50% methanol) to yield 1−2 mg of soraphen A.

(39) Srinivasulu, B.; Kim, Y.-J.; Chang, Y.-K.; Shang, G.; Yu, T.-W.; Floss, H. G. Unpublished work (manuscript in preparation).

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