Common Principles in Macrolactone (Marginolactone) Biosynthesis. Studies on the Desertomycin Family

Marion Zerlin and Ralf Thiericke*

Hans-Knöll-Institut für Naturstoff-Forschung e. V., Beutenbergstrasse 11, 07745 Jena, Germany

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Administration of sodium [^{13}C]-labeled acetates and sodium [1-^{13}C]propionate to cultures of *Streptoverticillium baldacii* subsp. *netropse* (strain FH-S 1625) proved the polyketide origin of the 42-membered macrolactone in oasomycin B (**1**), a member of the desertomycin family. In a series of feeding different amino acids as presumptive polyketide starters ornithine was found to embody the initiator of the oasomycin biosynthesis, in which oxidative deamination, decarboxylation, and CoA-activation steps are involved. Resulting from the feeding of D,L-[1-^{13}C]glucose, the D-mannose located at C22 of **1** derives from glucose *via* isomerization at C2 during carbohydrate metabolism. A fermentation in an [$^{18}O_2$]-enriched atmosphere proved the oxidative deamination reaction during the macrolactone initiation. In addition, an oxygenase introduces the OH group at C22, which is used as the connecting site for the post-polyketide mannosylation step. Consequently, the glycosylation reaction as well as other characteristics of the desertomycin family's biosynthesis illuminate unexpected analogies and common principles in the formation of a number of related macrocyclic lactones. Due to the obvious biosynthetic relationships we introduce the term "marginolactones" for this class of *actinomycetes* macrolactones.

Introduction

In the large group of macrocyclic lactones[1] biosynthetic studies have already been highlighted in several cases, e.g., for macrolides[2] or for polyene antibiotics.[3] Besides these targets of detailed research, however, little is known about polyhydroxylated macrolactones of the nonpolyene type. As biosynthetic relationships should today be included in a modern, general classification system of antibiotics,[4] we apply this principle of rule on the large group of macrolactones. Herein, the "marginolactones" can be subdivided as a separate class of macrocyclic lactones, grouped by their close structural and, furthermore, their biosynthetic relationships described below. The term **marginolactone** arises from the common structural element of these metabolites, the **macrolactone** ring of more than 31 carbon atoms. In addition, all members of this group of antibiotics usually bear a side chain in the α-position to the lactone carbonyl with a terminal amino or guanidino functionality as the second typical characteristic. On the basis of our biosynthetic studies on oasomycin B (**1**), which are obviously representative of the marginolactones, the origin of the side chains is derived from the amino acid **argin**ine or the related **o**rnithine. In this paper, we further illuminate interesting biosynthetic analogies, which argue for the necessity of the separate classification of the marginolactones as distinct from other macrocyclic lactones.

Members of the marginolactones, exclusively produced by various *actinomycetes* strains, are the monazomycins A and B[5] with a 48-membered lactone ring. After the recently described 60-membered quinolidomicins[6] they represent the second largest, natural lactones in general. Besides the α-D-mannosylated monazomycins and the 42-membered desertomycins,[7–9] as well as the 36-membered primycin[10] containing a D-arabinose moiety, the majority of marginolactones bear no glycosidic structural elements. In some of the members (e.g., the azalomycins[11]), the guanidino moiety in the side chain is singly or 2-fold methylated. Another frequent structural characteristic is a 6-membered hemiketal cyclic integrated in the macrolactone ring, for example in scopafungin (niphimycin),[12] copiamycin (niphithricin),[13] neocopiamycin,[14] guanidylfungin A and B,[15] RP-63834,[16] the malolactomycins,[17] and the amycins.[18]

Our chemical screening project[19,20] supplied us with marginolactone producing organisms, e.g., *Streptomyces baldacii* subsp. *netropse* (strain FH-S 1625). In the metabolite pattern of this strain we discovered the

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(1) Bérdy, J.; Aszalos, A.; Bostian, M.; McNitt, K. L. *Handbook of Antibiotic Compounds*; CRC Press: Boca Raton, FL, 1980–1987; Vols. I–XIV.

(2) O'Hagan, D. *Nat. Prod. Rep.* **1992**, 447.

(3) Herbert, R. B. *The Biosynthesis of Secondary Metabolites*; Chapman and Hall, London, 1989.

(4) (a) Rohr, J.; Zeeck, A. In *Biotechnology Focus II*; Finn, R. K., Präve, P., Eds.; Hanser Publishers: Munich, 1989; p 251. (b) Rohr, J.; Thiericke, R. *Nat. Prod. Rep.* **1992**, *9*, 103.

(5) Kuo, M.-S.; Yurek, D. A.; Laborde, A. L.; Truesdell, S. E.; Nielsen, J. W.; Argoudelis, A. D.; Baczynskyj, L. *J. Antibiot.* **1990**, *43*, 438.

(6) (a) Hayakawa, Y.; Shin-ya, K.; Furihata, K.; Seto, H. *J. Am. Chem. Soc.* **1993**, *115*, 3014. (b) Hayakawa, Y.; Shin-ya, K.; Furihata, K.; Seto, H. *J. Antibiot.* **1993**, *46*, 1563.

(7) Uri, J.; Bognar, I.; Bekesi, V. B. *Nature* **1958**, *182*, 401.

(8) Dinya, Z.; Sztaricskai, F.; Horvath, G. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 534.

(9) Bortolo, R.; Spera, S.; Cassani, G. *J. Antibiot.* **1992**, *45*, 1016.

(10) Fehr, T.; Jain, R. C.; de Mayo, P.; Motl, O.; Szilagyi, I.; Baczynskyj, L.; Gracey, D. E. F.; Holland, H. L.; MacLean, D. B. *J. Chem. Soc., Perkin Trans. 1* **1974**, 836.

(11) Iwasaki, S.; Namikoshi, M.; Sasaki, K.; Fukushima, K.; Okuda, S. *Chem. Pharm. Bull.* **1982**, *30*, 4006.

(12) Gassmann, P.; Hagmann, L.; Keller-Schierlein, W. *Helv. Chim. Acta* **1984**, *67*, 696.

(13) Fukushima, K.; Arai, T. *J. Antibiot.* **1982**, *35*, 1480.

(14) Arai, T.; Uno, J.; Horimi, I.; Fukushima, K. *J. Antibiot.* **1984**, *37*, 103.

(15) Takesako, K.; Beppu, T. *J. Antibiot.* **1984**, *37*, 1161.

(16) Fréchet, D.; Danzer, M.; Debu, F.; Monegier du Sorbier, B.; Reisdorf, D.; Snozzi, C.; Vuilhorgne, M. *Tetrahedron* **1991**, *47*, 61.

(17) Koshino, H.; Kobinata, K.; Uzawa, J.; Uramoto, M.; Isono, K.; Osada, H. *Tetrahedron* **1993**, *49*, 8827.

(18) Grabley, S.; Hammann, P.; Raether, W.; Wink, J.; Zeeck, A. *J. Antibiot.* **1990**, *43*, 639.

(19) Bach, G.; Breiding-Mack, S.; Grabley, S.; Hammann, P.; Hütter, K.; Thiericke, R.; Uhr, H.; Wink, J.; Zeeck, A. *Liebigs Ann. Chem.* **1993**, 241.

(20) Grabley, S.; Granzer, E.; Hütter, K.; Ludwig, D.; Mayer, M.; Thiericke, R.; Till, G.; Wink, J.; Philipps, S.; Zeeck, A. *J. Antibiot.* **1992**, *45*, 56.

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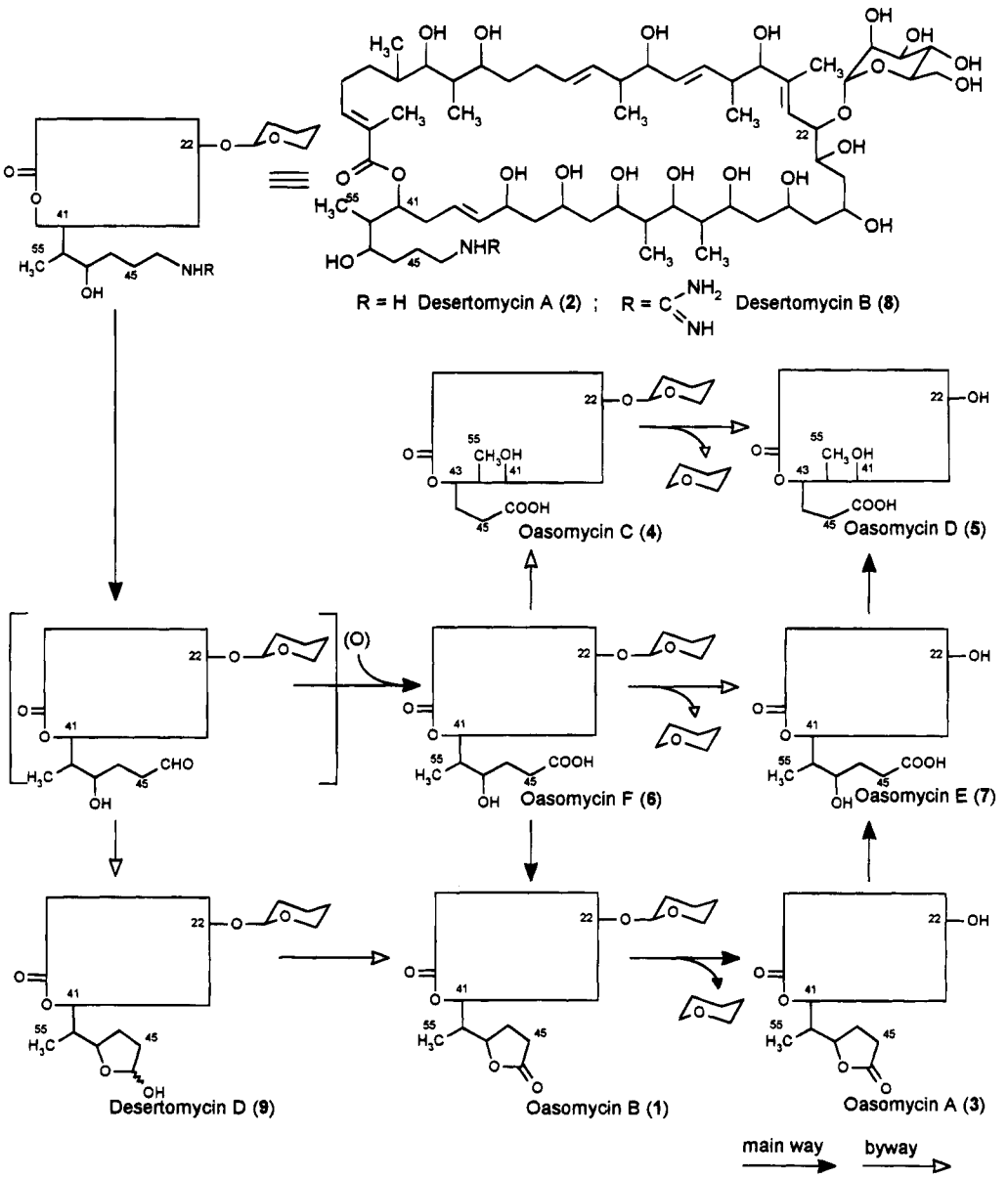
Scheme 1. Biosynthetic Relationships in the Desertomycin Family



already known desertomycin A (**2**)[7] as well as the new oasomycins A–F.[21,22] These structures, which usually bear a 42-membered macrolactone ring as a typical element, had been elucidated in analogy to **2**.[23] The oasomycin C (**4**) and its aglycon D (**5**) are the first representatives of 44-membered macrolactones. The oasomycins and desertomycins vary in the side chain located at C41 or C43, respectively, as well as in the presence of an α-linked D-mannose moiety attached to 22-OH. *In vitro* testing using a HEP-G2 cell assay showed oasomycin A (**3**) to be an inhibitor of *de novo* cholesterol biosynthesis. Desertomycin A (**2**) exhibits broad antibacterial and selective antifungal activities,[24] which has been observed for most of the marginolactones, too.[10–18]

Because of these structural characteristics, the metabolites of the desertomycin family embody useful model substances to study the biosynthesis of marginolactones in detail. Our first investigations focused on the late stage of biosynthesis based on a detailed analysis of the fermentation time-course of the producing organism FH-S 1625, pH-static fermentations, and *in vitro* conversion of the oasomycins. These results had already revealed a complete picture of the biosynthetic relationships in the desertomycin family (Scheme 1).[22] Desertomycin A (**2**), the first detectable metabolite in the biosynthetic sequence, undergoes an oxidative deamination leading to oasomycin F (**6**), which is converted into the main product oasomycin B (**1**) *via* lactonization of the side chain. In the later fermentation, enzymatic demannosylation results in the aglycon oasomycin A (**3**). Further nonenzymatic, alkaline-catalyzed reactions lead to a successive opening of the γ-lactone and the macrolactone moiety, followed by an unexpected relactonization to a 44-membered macrolactone ring [oasomycin E (**7**) and D (**5**)].

In this paper, we describe feeding experiments with labeled precursors offering insight into the early stages of the biosynthesis of the desertomycin family. Besides the oxygenation pattern, e.g., the diol at C22/C23, the

(21) Grabley, S.; Kretzschmar, G.; Mayer, M.; Philipps, S.; Thiericke, R.; Zeeck, A. *Liebigs Ann. Chem.* **1993**, 573.
(22) Mayer, M.; Thiericke, R. *J. Chem. Soc., Perkin Trans. 1* **1993**, 2525.
(23) Bax, A.; Aszalos, A.; Dinya, Z.; Sudo, K. *J. Am. Chem. Soc.* **1986**, *108*, 8054.
(24) Uri, I. V. *Acta Microbiol. Hung.* **1986**, *33*, 271.

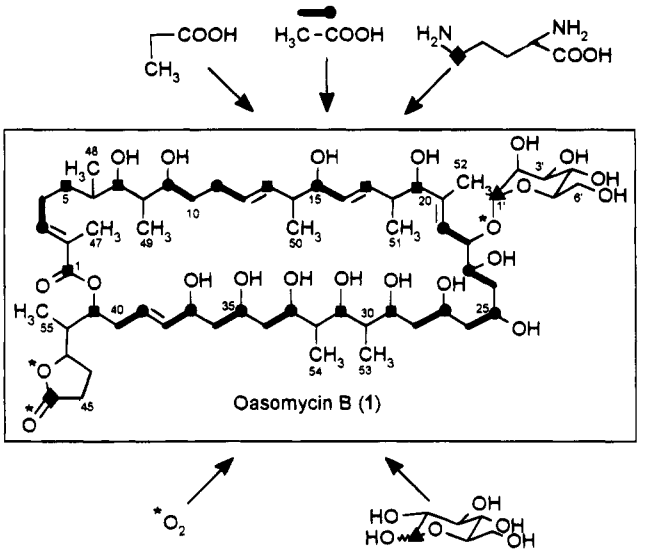


Figure 1. Positional and bond-labeling patterns of oasomycin B (**1**) biosynthesized from [^{13}C]- and [^{18}O]-labeled precursors.

origin of the polyketide starter unit, obviously located in the side chain, attracted our main interest to identify the initiator for the macrolactone biosynthesis. As a consequence of our studies, the results allow prediction of common principles in the formation of a number of macrolactones, which can be—from the biosynthetic point of view—combined into the marginolactone group.

Results

Our working hypothesis for the biosynthesis of the carbon skeleton of the desertomycin family envisioned a similar polyketide-type pathway as in the case of monazomycin,[25] guanidylfungin A,[26] and azalomycin F_{4a},[27] resulting from the already reported feeding experiments with acetate as C_2- and propionate as C_3-building blocks. Considering the variety of the metabolite pattern of the producing organism *Streptoverticillium baldacii* subsp. *netropse* (strain FH-S 1625) during fermentation,[22] biosynthetic studies were carried out in small fermentation scales in Erlenmeyer flasks (600–800 mL), which allowed sufficient yields for detailed NMR analysis of the main product of the early fermentation stage, oasomycin B (**1**). In combination with the information from the fermentation curves, we enforced pulse feeding experiments (between 8 and 18 h) to incorporate the precursors successfully within the time of the producing optimum of the glycosylated metabolite oasomycin B (**1**).[22]

Acetate Feeding Experiments. Feeding of sodium [1-^{13}C]acetate, a typical polyketide precursor, labeled 12 positions of oasomycin B (**1**) as depicted in Figure 1. However, for the signals of C27, C33, and C37 with nearly identical chemical shifts with the NMR solvent DMSO-$_6$ (δ_{C27} = 66.7, δ_{C33} and δ_{C37} = 66.8), the corresponding ^{13}C NMR spectra of **1** in CD_3OD were utilized. This resulted in a signal splitting suitable to determine the specific incorporation rates in these positions (Table 1). Besides these highly enriched carbon atoms (specific incorporation: 6.5–16.4), lower enrichments were found for eight further signals (C1, C5, C7, C13, C17, C19, C29, C31) with incorporation rates between 0.6 and 1.6. Labeled carbon atoms in β-position to methyl groups inserting at the macrolactone ring indicated the indirect incorporation of acetate *via* propionate as a C_3-building block for polyketide chain elongation. Obviously, acetyl CoA is metabolized in the TCA cycle, leading to succinyl CoA as a substrate for methylmalonyl CoA mutase. The formed methylmalonyl CoA embodies the activated C_3 unit, which is used as a biosynthetic building block in **1**.

Whereas single-labeled ^{13}C-acetate gave information about the direction of the acetate incorporation, intact incorporation of the C_2 units was examined with sodium [1,2-$^{13}C_2$]acetate (Figure 1). We found intact acetate building blocks in C3/C4, C9/C10, C11/12, C15/16, C21/22, C23/24, C25/26, C35/36, and C39/40 deduced from their strong spin–spin coupling (see Table 1). However, for the remaining acetate units C27/28, C33/34, and C37/38 high enrichments were detectable, although $^1J_{C-C}$ coupling constants could not be determined unambiguously because of the signal overlapping. In addition, statistical coupling was observed for neighboring, acetate-enriched carbon atoms. As it is evident that there is a high degree of turnover of acetate in the TCA cycle, the label of sodium [1,2-$^{13}C_2$]acetate was found to be distributed into the eight presumably propionate-derived units of the macrolactone as well. In contrast to the [1-^{13}C]-acetate feeding, another C_3-building block (C55/C42/C41), located in the side chain of **1**, could be identified. Because of the significant ^{13}C-enrichment and signal splitting of C55 caused by the [1,2-$^{13}C_2$]acetate scrambling, we concluded the origin of this methyl group to be also from propionate. No incorporation of acetate was observed in the α-D-mannose (C1′–C6′) as well as in the γ-lactone moiety (C43–C46), whose origin obviously is independent of the acetate metabolism.

Feeding Experiments with Propionate. Resulting from the acetate experiments, a feeding of sodium [1-^{13}C]-propionate promised to clear the origin of the remaining carbon atoms of the macrolactone. Surprisingly, we observed less yield of oasomycin B (**1**) from this incorporation experiment, indicating a possible catabolite repression of the oasomycin biosynthesis. However, the isolated sample was unambiguously proved to be **1** by FAB-MS spectroscopy. Fortunately, ^{13}C NMR spectra analysis of **1** was possible, because all signals of enriched C-atoms were observable, whereas a number of the signals with natural ^{13}C abundance could not clearly be identified from the base line. This led us to choose C16 as a reference signal for determining the specific incorporation rates. As expected, C1, C5, C7, C13, C17, C19, C29, and C31 with specific incorporations of 1.5–2.7 reflected their origin from the carboxyl group of propionate (Figure 1). Consequently, an insertion of the methyl branches of the macrocyclic ring *via* the C_1-pool (e.g., *S*-adenosylmethionine) could be excluded. In addition, lower signal enrichment than the other eight carbon atoms was detectable for C41 (specific incorporation: 0.8), which points out the already assumed origin of the methyl-branched side chain from propionate (Table 1). Because of the low enrichment in the position C41, it is not surprising that during the indirect incorporation of propionate after feeding sodium [1-^{13}C]acetate the label of this carbon atom was not detectable.

Incorporation Studies with D,L-[1-^{13}C]Glucose. To study the origin of the sugar moiety in oasomycin B (**1**), D,L-[1-^{13}C]glucose was fed to the strain FH-S 1625. The significant enrichment (specific incorporation: 6.9, Table 1) at C1′ of the α-D-mannose unit of **1** proved the direct

(25) Nakayama, H.; Furihata, K.; Seto, H.; Otake, N. *Tetrahedron Lett.* **1981**, *22*, 5217.
(26) Takesako, K.; Beppu, T. *J. Antibiot.* **1984**, *37*, 1170.
(27) Iwasaki, S.; Sasaki, K.; Namikoshi, M.; Okuda, S. *Heterocycles* **1982**, *17*, 331.

Table 1. Chemical Shifts, Specific Incorporation of Different [^{13}C]-Labeled Precursors, and J_{C-C} Coupling Constants of Oasomycin B (1) (125.7 MHz, DMSO-d_6)

carbon no.	δ (ppm)	specific incorporation [1-^{13}C] acetate	[1-^{13}C] propionate[c]	[1-^{13}C] glucose	[5-^{13}C] glutamic acid	$^1J_{C-C}$ (Hz) [1,2-$^{13}C_2$] acetate	carbon no.	δ (ppm)	specific incorporation [1-^{13}C] acetate	[1-^{13}C] propionate	[1-^{13}C] glucose	[5-^{13}C] glutamic acid	$^1J_{C-C}$ (Hz) [1,2-$^{13}C_2$] acetate
1	166.4	0.7	2.1	0	1.3	70.8[d]	32	40.4	−0.2	2.0	0.4	n.d.	32.0[d]
2	126.9	−0.6	n.d.	0	−0.4	70.8[d]	33	66.8	13.3[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]
3	142.5	6.5	n.d.	0	−0.2	41.0	34	42.2	0	n.d.	1.0	n.d.	n.d.
4	26.1	−0.5	n.d.	0.9	0	41.0/37.5[d]	35	63.8	13.1	n.d.	−0.6	0.2	45.0
5	32.9	0.8	2.6	−0.2	1.0	35.0[d]	36	45.7	−0.4	n.d.	0.7	0.1	45.0
6	34.3	−0.4	n.d.	−0.3	0	n.d.	37	66.8	13.0[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.
7	74.6	0.6	1.5	−0.4	1.2	n.d.	38	138.2	−0.8	n.d.	0.6	−0.2	n.d.
8	41.4	−0.5	n.d.	0.2	0.4	n.d.	39	122.4	6.0	n.d.	n.d.	−0.2	n.d.
9	72.0	8.1	n.d.	−0.8	0	40.0	40	32.4	−0.6	n.d.	0.6	−0.2	35.0
10	32.1	−0.5	n.d.	0.6	0	40.0	41	73.0	−0.5	0.8	n.d.	0.7	n.d.
11	28.9	13.7	n.d.	−0.5	−0.4	43.5/35.0[d]	42	39.9	−0.1[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]
12	129.5	−0.2	n.d.	1.3	0.1	43.5/70.0[d]	43	80.4	−0.7	n.d.	n.d.	−0.3	
13	132.6	1.1	2.7	0.3	0.8	70.0[d]/45.0[d]	44	24.8	−0.7	n.d.	n.d.	−0.3	
14	42.2	−0.3	n.d.	−0.4	n.d.	45.0[d]	45	28.2	−0.7	n.d.	n.d.	−0.3	
15	74.4	14.9	−0.5	−0.4	0.3	48.5	46	176.7	−0.2	n.d.	n.d.	0.7	
16	131.0	−0.1	0	1.2	0.1	48.5/75.0[d]	47	12.1	−0.4	n.d.	0	−0.1	33.0[d]
17	132.7	1.6	3.6	0.2	1.3	75.0[d]/45.0[d]	48	12.2	0.1	n.d.	0.4	0	35.0[d]
18	39.4	0.1[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	49	11.2	−0.5	n.d.	−0.5	0.1	35.0[d]
19	80.7	0.6	2.4	−0.3	1.1	45.0[d]	50	15.2	0	n.d.	−0.3	0	36.5[d]
20	143.2	−0.1	n.d.	n.d.	0.1	71.0[d]	51	16.6	−0.3	n.d.	−0.3	0	36.0[d]
21	122.2	16.4	n.d.	n.d.	0.3	50.0/71.0[d]	52	11.7	−0.4	n.d.	−0.4	−0.1	40.0[d]
22	73.7	−0.3	0.5	1.1	0.3	50.0	53	9.4	0.2	n.d.	0.4	0	38.5[d]
23	69.4	14.4	0.2	−0.5	0.4	47.5	54	10.4	0	n.d.	0	0	35.5[d]
24	40.8	−0.3	n.d.	1.7	0.5	47.5	55	9.6	−0.6	n.d.	−0.6	−0.2	38.0[d]
25	63.4	13.2	n.d.	−0.6	0.5	45.0	1′	95.9	0	n.d.	6.9	0.1	
26	45.6	0	n.d.	1.8	0.3	45.0	2′	70.6	0.3	n.d.	−0.5	0.2	
27	66.7	14.0[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	3′	70.9	0.3	−0.3	−0.2	0.3	
28	42.3	−0.3	n.d.	1.2	n.d.	n.d.	4′	67.2	0.3	n.d.	−0.2	0.8	
29	72.8	1.3	2.7	0.3	1.3	35.0[d]	5′	73.5	0.4	n.d.	−0.4	0.4	
30	39.4	0.2[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	6′	61.2	0.1	n.d.	0.5	0.2	
31	70.7	0.8	n.d.	−0.2	1.4	32.0[d]							

[a] Signal referenced to C54 (CD_3OD/D_2O). [b] Signals overlapping. [c] Signal referenced to C16. n.d. = not determined. [d] Statistical $^1J_{C-C}$-coupling.

incorporation of D-glucose (Figure 1), which seemed to be turned into D-mannose *via* glucose phosphate isomerase. Presumably, after activation to UDP mannose the glycosidic bond with the hydroxy group at C22 is formed by a mannosyl-transferase. In addition, 12 further signals showed low signal enhancements in the ^{13}C NMR spectra of **1** (Table 1). Considering the labeling pattern, these enrichments could be assigned to the methyl group (C2) of each acetate polyketide building block. This label transfer from [1-^{13}C]glucose is explainable by its degradation *via* the Embden–Meyerhof–Parnas pathway and an oxidative decarboxylation of pyruvate leading to [2-^{13}C]acetate. The rapid metabolism of added precursors, especially of acetate or glucose, supports the results from the fermentation time course,[22] as the metabolites of the desertomycin family are produced during the logarithmical growth phase. Because of the high turnover in the primary metabolism the isotopic labels of universal precursors are subjected to the observed scrambling.

Origin of the Polyketide Starter. The previous results revealed the four carbon atoms of the γ-lactone of oasomycin B (**1**) to be the polyketide starter unit. Considering the biosynthetic relationships in the desertomycin family deduced from the fermentation time curves and pH-static fermentations,[22] desertomycin A (**2**) with a terminal amino group in its side chain obviously represents the direct oasomycin precursor (Scheme 1). Consequently, the polyketide starter had to be sought in the amino acid pool, as depicted in Figure 2. Aspartic acid and 4-aminobutyric acid as C_4N building blocks could act as possible initiators. After a CoA activation step, the chain elongation with the first propionate unit (C55/C42/C41) is conceivable *via* their carboxyl groups. In an analogous reaction, glutamic acid could be incorporated, presupposing a decarboxylation of the α-carboxy function during polyketide biosynthesis. Including the fact that desertomycin B (**8**)[8] with a terminal guanidino functionality in the side chain was detected as a minor metabolite in strain *Streptomyces flavofungini*, arginine or ornithine promised to be further, plausible starters for the biosynthesis of the desertomycin family.

Feeding Experiments with D,L-[1-^{13}C]Aspartic Acid. From the feeding with D,L-[1-^{13}C]aspartic acid oasomycin F (**6**) and desertomycin D (**9**)[9] were isolated as the main fermentation products in addition to an unusually low yield (2 mg/L) of oasomycin B (**1**). Obviously, a pH fluctuation (pH of the added solution 7.0, harvest: pH 6.5) prevented the lactonization of the carboxy group in the side chain of **6** leading to oasomycin B (**1**), in analogy to the results from the pH-static (pH = 8) fermentations.[22] The formation of desertomycin D (**9**) could be explained by an incomplete oxidation and acetalization of the hypothetic aldehyde intermediate (Scheme 1). As no label incorporation of D,L-[1-^{13}C]aspartic acid into both samples, oasomycin F (**6**) and desertomycin D (**9**), was observed, this amino acid could be excluded as the oasomycin polyketide starter.

Feeding Experiments with [^{15}N]Glutamic Acid and [^{15}N]-4-Aminobutyric Acid. Feedings with [^{15}N]-labeled precursors could only be carried out using desertomycin A (**2**) as a target for incorporation studies, because the [^{15}N]-label in **1** got lost during biosynthesis. In parallel, continuous feedings of [^{15}N]glutamic acid and [^{15}N]-4-aminobutyric acid at a constant pH value (pH = 5), in which we can direct the metabolite pattern of the

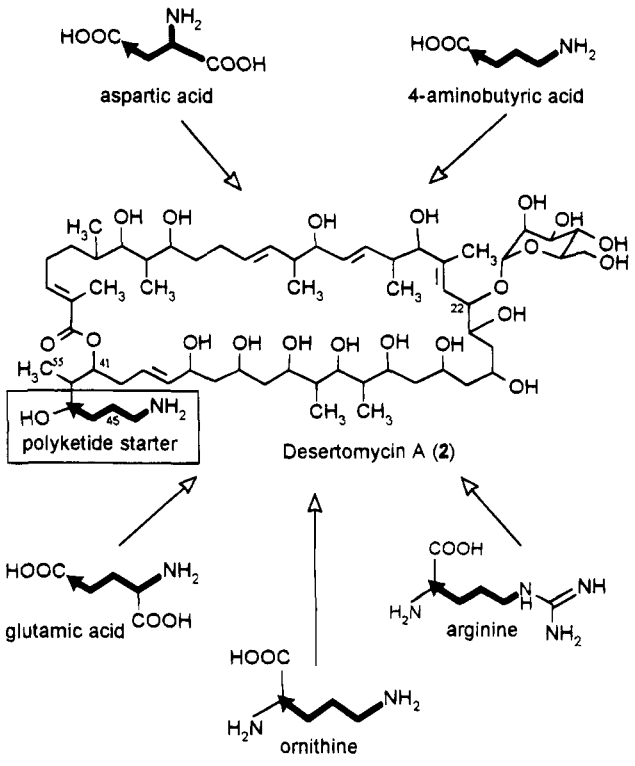


Figure 2. Hypothetical polyketide starters of the desertomycin family.

strain FH-S 1625 to an exclusive production of desertomycin A (**2**),[22] gave us sufficient amounts of **2** for the determination of the [^{15}N]-enrichments by recording [^{15}N]-emission spectra.[28] The similar [^{15}N]-enrichments of **2** in both samples (7.3% from the 4-aminobutyric acid feeding and 6.9% from the glutamic acid feeding) did not allow a definite clarification of a direct or indirect incorporation of the [^{15}N]-label in **2**. Glutamic acid could directly be decarboxylated into the biosynthetic related 4-aminobutyric acid, explaining the observed, nearly identical incorporation rates. However, considering the high turnover in the primary metabolism, a scrambling of the fed precursors *via* the nitrogen pool seems more reasonable.

Feeding Experiments with L-[5-^{13}C]Glutamic Acid. An incorporation experiment with L-[5-^{13}C]glutamic acid promised to clarify the results of the [^{15}N]-labeled precursor feedings. Assuming a C–C bond formation between the γ-carboxy group of glutamic acid activated as a CoA-ester with the first propionate building block (Figure 2), the [^{13}C]-label was to be expected in position C43 of desertomycin A (**2**) or oasomycin B (**1**). As shown by the ^{13}C NMR analysis of the received oasomycin B (**1**) sample, no enrichment was observed at that particular carbon atom (Table 1). In contrast, a low signal enhancement with a specific incorporation of 0.7 was detected at C46, indicating an unexpected incorporation direction into the γ-lactone moiety of **1**. Consequently, glutamic acid did not represent the direct polyketide starter of the oasomycin biosynthesis, but seemed to be transformed into amino acids of the glutamate family during anabolism. Herein, the members ornithine and arginine had already been discussed as possible starter building blocks (Figure 2).

In addition, further weak enrichments were found for nine carbon atoms of the macrolactone ring and the methyl-branched side chain of **1**. As the resulting labeling pattern was identical to that of the sodium [1-^{13}C]propionate experiment, a transformation of glutamic acid into propionate or methylmalonyl CoA is obvious. Deamination of glutamic acid leads to 2-oxoglutarate, which is turned into succinyl CoA *via* the TCA cycle. An enzymatic isomerization into methylmalonyl CoA delivers the activated C_3-building block for polyketide biosynthesis. Thus, the label in 5-position of glutamic acid was transferred into the carboxyl group of propionate.

Table 2. Chemical Shifts and Specific Incorporation of the Side Chain Signals (C41–C46 and C55) of Desertomycin D (9) Enriched from D,L-[5-^{13}C]Ornithine (75 MHz, DMSO-d_6)

carbon no.	δ (ppm)	D,L-[5-^{13}C]ornithine
41	73.8	0.1
42	41.4	n.d.
43[a]	79.7; 77.4[a]	−0.3; −0.3[a]
44	31.9	0.1
45	27.0	−0.4
46[a]	97.3; 97.0[a]	20.5; 18.7[a]
55	10.2	0

[a] Diastereomeric mixture.

Incorporation Studies with D,L-[5-^{13}C]Ornithine. The previous results suggested ornithine or the related arginine to represent the polyketide starter of the desertomycin family. Therefore, D,L-[5-^{13}C]ornithine was fed as a suitable precursor, resulting in a significant yield (60 mg/L) of the main fermentation metabolite desertomycin D (**9**), in analogy to the aspartic acid experiment (see above). Remarkably, the observed ^{13}C-NMR spectrum of the isolated sample of **9** showed significant signal enhancements of the hemiacetal C46 in both stereoisomers (specific incorporation: 18.7/20.5, Table 2). These high incorporation rates left no doubt about the utilization of ornithine as a substrate in the oasomycin formation. Considering that ornithine is the direct precursor in the metabolic pathway of arginine, we intended a feeding of [^{13}C]-labeled arginine. However, the activation of ornithine/arginine obviously involves a deamination of the α-amino group and a decarboxylation step of these amino acids before they are used as a starter of the desertomycin family's biosynthesis.

Origin of the Oxygen Atoms. Feeding of Sodium [1-^{13}C,$^{18}O_2$]Acetate. Because oxygen atoms in the polyketide biosynthesis frequently originate from their C_2- or C_3-building blocks, the hydroxy groups at C9, C15, C23, C25, C27, C33, C35, and C37 in oasomycin B (**1**) should derive from the carboxy group of acetate. In the ^{13}C NMR spectrum of **1**, isolated from the sodium [1-^{13}C,$^{18}O_2$]acetate incorporation experiment, 12 carbon atoms showed significant [^{13}C]-enrichments. Besides the identical labeling pattern to the sodium [1-^{13}C]acetate feeding (Figure 1), unfortunately, no [^{18}O]-label was detectable, indicating a loss of the oxygen label during metabolic processes and/or polyketide biosynthesis. Considering the high turnover of acetate at the time of the oasomycin production,[22] an [^{18}O]-label exchange in the primary metabolism is not surprising. Consequently, we did not decide to continue with further experiments using [^{13}C,^{18}O]-labeled precursors, e.g., sodium [1-^{13}C,$^{18}O_2$]-propionate.

Fermentation in an [$^{18}O_2$]-Enriched Atmosphere. Our main interest focused on the origin of the glycosidic oxygen atom at C22, which is typically not located at a C1 position of an acetate or propionate building block (Figure 1). As we postulated its origin from molecular

(28) Wutzke, K. D.; Heine, W.; Plath, C.; Müller, M.; Uhlemann, M. *Pediatr. Res.* **1992**, *31*, 95.

Table 3. [$^{18}O_2$]-Isotope-Induced Shifts in the ^{13}C NMR Spectra of Oasomycin B (1) Enriched from [$^{18}O_2$]Gas

carbon no.	δ ($^{13}C-^{16}O$) (ppm)	δ ($^{13}C-^{18}O$) (ppm)	$\Delta\delta$ (ppm)	^{18}O:^{16}O
22	73.684	73.664	0.020	45:55
43	80.308	80.275	0.033	25:75
46	176.616	176.596	0.020	22:78
1′	95.926	95.910	0.016	48:52

oxygen, a fermentation, conducted in a closed system under an atmosphere of 20% [$^{18}O_2$] and 80% N_2, was carried out. A first indication of a successful [^{18}O]-incorporation was received from the FAB mass spectrum of the isolated oasomycin B (**1**) sample. Besides the molecule ion *m/z* ($M + Na^+$) = 1212 (3.9) further peaks (*m/z* = 1214 (21.8), 1216 (54.1), and 1218 (19.7) were detectable. Consequently, three oxygen atoms of **1** should derive from molecular oxygen. The position of [^{18}O]-incorporation was determined by observation of [^{18}O]-isotope induced shifts of ^{13}C signals. As expected, the signals assigned to C22 ($\Delta\delta_C$ = 0.020 ppm) and C1′ ($\Delta\delta_C$ = 0.016 ppm) with nearly identical incorporation rates (Table 3) were found to be clearly enriched. Furthermore [^{18}O]-label was observed for the ring oxygen atom of the γ-lactone (Figure 1), leading to α-isotope shifts at C43 ($\Delta\delta_C$ = 0.033) and C46 ($\Delta\delta_C$ = 0.020). Deduced from the mass spectra analysis, another remaining [^{18}O]-incorporation should be detectable in the ^{13}C NMR spectrum of **1**, which obviously is to be found at the carbonyl oxygen of the γ-lactone at C46 (Figure 1). Because of the signal overlapping with [$^{18}O_2$]-isotope-induced shifts deriving from the neighboring ring oxygen, the incorporation rate could not be determined unambiguously.

Discussion

The Biogenesis of the Desertomycin Family. Together, the results of our feeding experiments have provided a detailed picture of the biosynthetic origin of oasomycin B (**1**). The polyketide biosynthesis is probably initiated by ornithine or the related arginine, whose carbon skeleton is located in the γ-lactone side chain (C43 to C46) of **1**. Therefore, we presume that the activation of these amino acids involves an oxidative deamination introducing an oxygen atom from molecular O_2 of the formed intermediate.[29] This reaction explains the [^{18}O]-incorporation into the lactone ring oxygen of the later formed metabolite **1**, deduced from the fermentation in [$^{18}O_2$]-enriched atmosphere. After an oxidative decarboxylation the activated CoA-building block initiates polyketide biosynthesis using methylmalonyl CoA (located as the methyl group C55 and C41/C42 in **1**) as a substrate in the first elongation step. In summary, 12 C_2 (acetate) and nine C_3 (propionate) units are used as building blocks to form the macrolactone moiety of **1**.

The characteristic oxygenation pattern and the existence of four double bonds illuminate the enzymatic processes at the level of the oasomycin polyketide synthase, obviously of a PKS I-type.[30] In analogy to the already investigated polyketide synthase of *Saccharomyces erythrea*,[31] we assumed that the oasomycin-PKS consists of individual modules, which catalyze the condensation, reduction, as well as elimination steps. It is most likely that in a number of modules, after the condensation of the C_2 or C_3 units, a reduction of the formed β-ketoacyl intermediate leads to the hydroxy groups of the macrolactone ring of **1**. Five modules involve an additional elimination reaction, which lead to the *E*-configured double bounds at C2/C3, C12/C13, C16/C17, C20/C21, and C38/C39. Complete enzymatic reductions are presented at C4/C5 and C10/C11. In the final elongation module the presumably CoA-activated polyketide chain is linked to a 42-membered lactone ring *via* the OH group of the propionate building block.

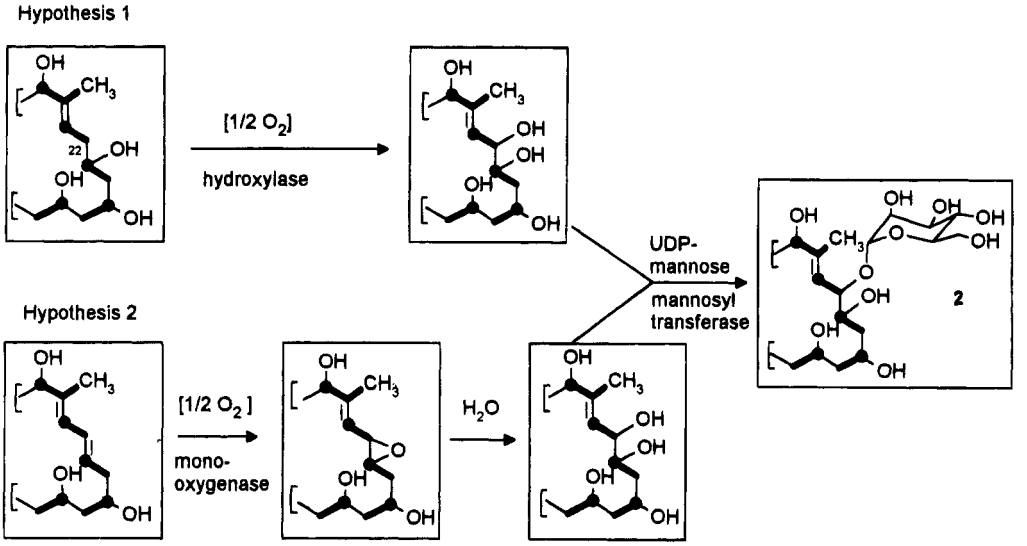
This formed 42-membered polyketide intermediate is attacked by an oxygenase at position 22 introducing a hydroxy group from molecular oxygen. For the mechanism of this substitution two pathways are plausible (Scheme 2). Usually, a direct introduction of OH groups is catalyzed by hydroxylases.[32] On the other hand, monooxygenases are described to form an epoxide intermediate, which requires a previous existing double bond at C22/C23, respectively.[33] The following stereocontrolled opening of the epoxide leads to a *trans*-diol, bearing the oxygen from molecular O_2 at 22-OH. Consequently, in this pathway the neighboring oxygen (23-OH) should derive from water instead of from the acetate precursor. As the coupling constants at 22-H/23-H could not be determined from the complex ^1H NMR spectrum of **1**, the configuration in these centers of chirality is still unknown. On the other hand, the sodium [1-^{13}C,$^{18}O_2$]-acetate feeding did not give information about the origin of 23-OH from acetate. Experiments to isolate the hypothetic oasomycin precursor using oxygenase inhibitors,[34] which may illustrate the mechanism of the oxygenation, are still in progress.

The last step of the biosynthetic pathway of the oasomycins involves a glycosylation reaction using α-D-mannose as a substrate. Remarkably, the OH group at C22 is favored for the mannosyltransferase, indicating that this position seemed to be spatially exposed because of the folding of the 42-membered macrolactone ring.

From this biosynthetic sequence—the combination of polyketide biosynthesis, oxygenase, and glycosyltransferase-catalyzed reactions—desertomycin A (**2**) is the first detectable metabolite in the culture broth of the producing organism *Streptomyces baldacii* subsp. *netropse* (FH-S 1625). As desertomycin D (**9**) and all oasomycins obviously derive from **2** (Scheme 1) *via* enzymatically catalyzed and nonenzymatically post-polyketide modifications,[22] the biosynthetic studies on oasomycin B (**1**) are representative for these members of the desertomycin family. In addition, our results allowed us to postulate two hypotheses about the origin of desertomycin B (**8**) from *Streptomyces flavofungini* bearing a terminal guanidino group in the side chain of the macrolactone. In analogy to desertomycin A (**2**) **8** seems to represent an early fermentation product. Considering that the oasomycin-PKS uses arginine as the ultimate polyketide starter, the formation of the primary amine in **2** is explainable by a hydrolysis of the guanidino group in **8** *via* a guaninobutyrase.[29] However, as we could not detect desertomycin B (**8**) in the fermentation broth of strain FH-S 1625 we assume, that the oasomycin-PKS of the desertomycin B (**8**)-producing organism *Streptomyces flavofungini*[8] accepts ornithine as well as arginine as

(29) Gottschalk, G. *Bacterial Metabolism*; Springer Verlag: Berlin, 1985.
(30) Hopwood, D. A.; Sherman, D. H. *Ann. Rev. Gen.* **1990**, *24*, 37.
(31) Donadio, S.; Staver, M. J.; McAlpine, J. B.; Swanson, S. J.; Katz, L. *Science* **1991**, *252*, 675.
(32) Komagata, D.; Shimada, H.; Murakawa, S.; Endo, A. *J. Antibiot.* **1989**, *42*, 407.
(33) Mayer, M.; Thiericke, R. *J. Chem. Soc., Perkin Trans. 1* **1993**, 495.
(34) Oikawa, H.; Murakami, Y.; Ichihara, A. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2955.

Scheme 2. Proposed Mechanism of the Glycosylation of the Desertomycin A Aglycon



polyketide starters, leading to a parallel appearance of both metabolites during fermentation.

Common Principles in Marginolactone Biosynthesis. The described results from the biosynthesis of the desertomycin family could be transferred to a number of structurally related macrolactones, the marginolactones monazomycin A and B,[5,25] the malolactomycins,[17] the azalomycins,[11,27] guanidylfungin A and B,[15,26] primycin,[10] scopafungin,[12] copiamycin,[13] neocopiamycin,[14] as well as amycin A and B.[17] Consequently, we are able to formulate the following common principles in marginolactone biosynthesis (Figure 3): (1) All marginolactones derive from the polyketide pathway with ornithine or arginine as polyketide starters. (2) The starter amino or guanidino group can be methylated, e.g., in primycin. (3) The CoA-activated polyketide starters are usually elongated by acetate or propionate building blocks. (4) The lengths of the polyketide chains and the sequence of the C_2 or C_3 units are individually determined by the different polyketide synthases of the particular producing organism. (5) In general, the linkage to the macrolactone ring is formed by the hydroxy group of the first propionate unit, which is preserved during the polyketide biosynthesis. (6) Each metabolite of the marginolactone group bears one hydroxy group (in the middle region between A_7 and A_{10}), which obviously is introduced from molecular oxygen [e.g., 22-OH of oasomycin B(**1**)]. (7) In the aglycons of some marginolactones, e.g., the azalomycins, guanidylfungins, and amycins, the oxygen atoms obviously deriving from molecular O_2 are found as a hydroxy group located at the 6-membered hemiketal ring. (8) Glycosylated marginolactones (the monazomycins, the desertomycin family, and primycin) bear this particular oxygen atom as the glycosidic O-atom of their mannose or arabinose residues.

In addition, a comparison of the acetate/propionate sequences of the different marginolactones (Figure 3) lead to unexpected homologies. Beginning with the propionate unit responsible for lactone formation (*P), identical successions could be deduced for the 36-membered guanidylfungin as well as for scopafungin. The copiamycin biosynthesis deviates only in the absence of one acetate building block (after P_3) and the terminal propionate. In comparison to the acetate/propionate sequence of azalomycin F_{4a}, the positions of P_3 and A_4 are exchanged. In addition, the desertomycin family shows analogies to the described metabolites in the regions between A_5 and A_7 as well as between P_5 and P_9 of the macrolactone, indicating evolutionary relationships for all the different polyketide synthases. However, in the primycin biosynthesis an unusual C_6-unit[35] is used as a terminator for the PKS. Besides the obviously evolutionary related polyketide synthases of different *actinomycetes* strains furthermore the post-polyketide modifications—oxygenation and glycosylation reactions—reflect analogous processes for the functionalization of polyhydroxylated macrolactones of the marginolactone group.

Experimental Section

Culture. *Streptoverticillium baldacii* subsp. *netropse* (strain FH-S 1625, deposited in the German Culture Collection: DSM 5990) was grown on agar slants containing medium A (2% soybean meal, degreased; 2% mannitol, 1.5% agar, pH = 7.2 prior to sterilization). Liquid cultures were performed in medium B (medium A omitting agar). A storage of the strain was performed in glycerol (50%) at −20 °C.

Fermentation. Fermentations of strain FH-S 1625 were carried out in two steps, using the glycerol-containing storage mixture (3 mL) to inoculate 300 mL Erlenmeyer flasks containing 100 mL of medium B. The flasks were cultivated on a rotary shaker (180 rpm) for 3 days at 30 °C. These cultures (inoculation volume 3%) were used to inoculate a 1 L fermenter or 300 mL Erlenmeyer flasks containing 100 mL of medium B.

Feeding Experiments. Most feeding experiments were performed in cultures of Erlenmeyer flasks as described above. Labeled precursors (Table 4), which had been dissolved in 20 mL of sterile water and adjusted to pH = 7.0 by the addition of 2 M HCl or 2 M NaOH, were added to the culture broth in pulse feedings at 8, 10, 12, 14, and 16 h after inoculation. Usually, the cultures were harvested at 60 h.

Fermentation under [$^{18}O_2$]-Enriched Atmosphere. The fermentation under [$^{18}O_2$]-enriched atmosphere was carried out in a closed vessel as previously described.[36] Cultures were grown under standard conditions described above in 250 mL Erlenmeyer flasks containing 100 mL of medium B. After 8 h of cultivation N_2 was pumped into the flasks to remove oxygen. The following cultivation was carried out in an atmosphere of [$^{18}O_2$] and N_2 (1:4). [$^{18}O_2$] gas (3.7 L) was pumped continuously from a reservoir into the fermentation vessel. The output from the flasks was directed into a KOH solution (5 M in water) to trap the CO_2 produced. During

(35) Thiericke, R.; Rohr, J. *Nat. Prod. Rep.* **1993**, *10*, 265.
(36) Udvarnoki, G.; Henkel, T.; Machinek, R.; Rohr, J. *J. Org. Chem.* **1992**, 1274.

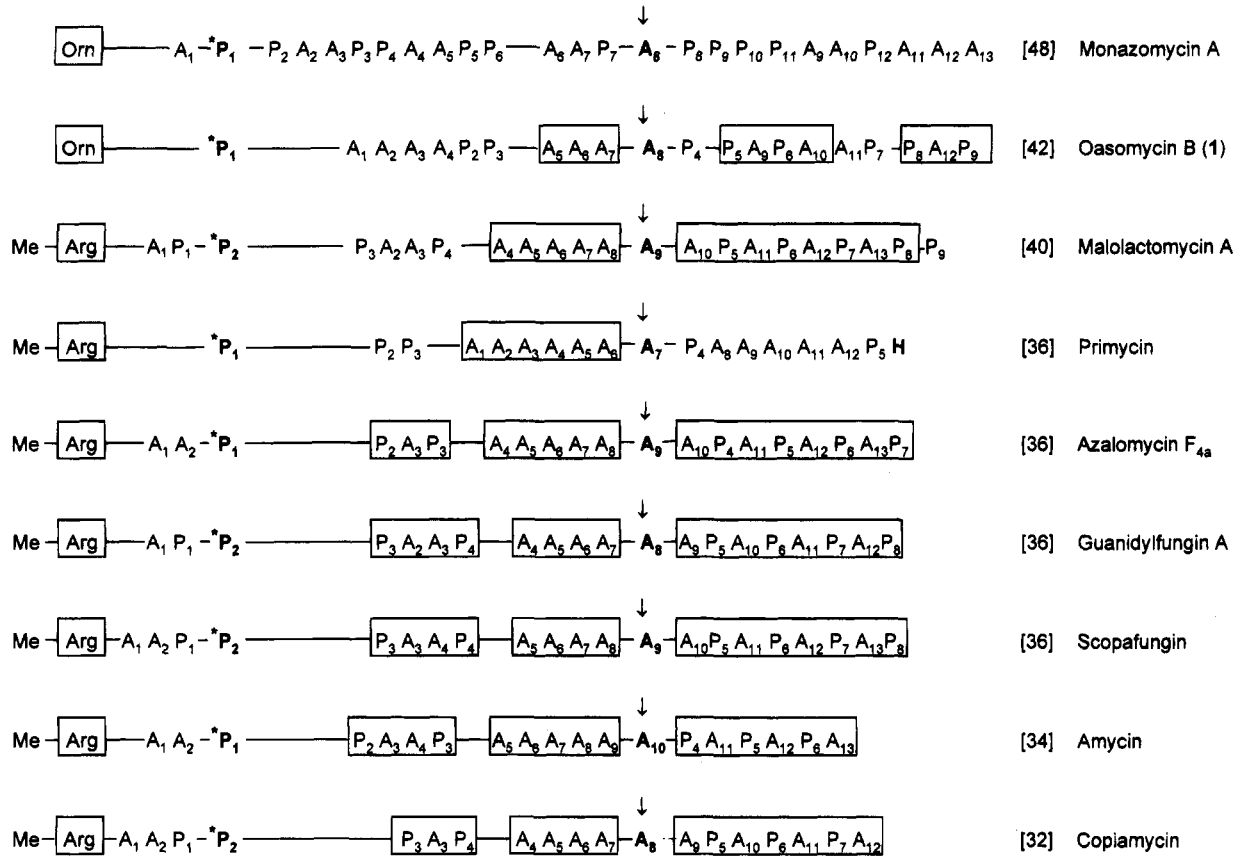


Figure 3. Illustration of the common principles in the biosynthesis of the marginolactones. Key: Me = methyl group, A = acetate, P = propionate, Arg = arginine, Orn = ornithine, H = C_6-building block, *P = connecting site for the formation of the macrolactone, ↓ = O-atom from molecular O_2, [ ] = macrolactone size, □ = homologies in the sequences of the polyketide building blocks.

Table 4. Labeled Precursors Used for Biosynthetic Studies of the Desertomycin Family

precursor	amount (mmol/L)	yield (mg/L) 1	2	9	6
[1-^{13}C]acetate	18	40			
[1,2-$^{13}C_2$]acetate	10	45			
[1-^{13}C]propionate	8	4			
D,L-[1-^{13}C]glucose	3	33			
D,L-[1-^{13}C]aspartic acid	5	5		13	35
L-[5-^{13}C]glutamic acid	3	35			
D,L-[5-^{13}C]ornithine	3			60	
[1-^{13}C,$^{18}O_2$]acetate	12	50			
[$^{18}O_2$]gas	91	33			
[^{15}N]-4-aminobutyric acid	8		40		
L-[^{15}N]glutamic acid	6		58		

fermentation under the [$^{18}O_2$]-enriched atmosphere (40 h) the oxygen consumption was found to be 95–125 mL/h. The cultures were harvested after 48 h.

Feeding Experiments with [^{15}N]-Labeled Precursors. The feedings with [^{15}N]-labeled precursors were carried out in a 1-L fermenter containing 600 mL of medium B (200 rpm, 30 °C, aeration 5.0 L/min). Between 8 and 21 h, 8 mmol of [^{15}N]-4-aminobutyric acid or 6 mmol of L-[^{15}N]glutamic acid dissolved in 100 mL of sterile water (pH 5.5) were continuously added to the fermentation vessel using a tubing pump (8 ml/h). During the feeding time the pH value of the culture broth was controlled by a pH-electrode and maintained at pH = 5.5 by the addition of 0.667 M citric acid or 2 M NaOH. The cultures were harvested after 60 h.

Isolation and Purification Procedures. The fermentation broth (400–800 mL) of each feeding experiment was centrifugated (4000 rpm, 10 min), and the supernatant was adsorbed on Amberlite XAD-16 (column 20 × 6 cm). The column was washed with 200–400 mL of distilled water and eluted with 400–800 mL of methanol. The eluate was evaporated to a watery residue and lyophilized (yields about 1–2 g). The mycelium was extracted four times with 50 mL of acetone using an ultrasonic bath for 15 min. The filtered organic layers were combined and evaporated to dryness yielding about 750 mg/L of a red amorphous powder. Both obtained crude products were combined and chromatographed in aliquots of 500 mg on Sephadex G-25 SF (column: 30 × 4 cm, methanol–water, 1:1). Repeated gel permeation chromatography of the obtained enriched product on Sephadex LH-20 (column: 200 × 4 cm) in methanol–water (9:1) and in methanol yielded pure, white powders of **1**, **2**, as well as **6** and **9** (for yields, see Table 4). In some cases repurification using HPLC chromatography on RP-18 was necessary (see Materials).

NMR Experiments. ^{13}C-NMR spectra of labeled oasomycin samples were recorded at 125.7 MHz in DMSO-d_6 or CD_3OD. Chemical shifts are expressed in δ values, and J values in Hz, with tetramethylsilane (TMS) as internal standard.

[^{15}N]-Determination. An emission-spectrometric [^{15}N]-analysis system, which makes use of the isotopic shifts in the emission spectrum of nitrogen, was employed.

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