Investigations of the Biosynthesis and Structural Revision of Landomycin A†

Susanne Weber, Carola Zolke, and Jürgen Rohr*,‡

Institut für Organische Chemie der Universität, Tammannstr. 2, D-37077 Göttingen, Germany

John M. Beale*

Division of Medicinal and Natural Products Chemistry, University of Texas, College of Pharmacy, Austin, Texas 78712

Received March 24, 1994®

Feeding experiments with [1-^{13}C]-, [1,2-$^{13}C_2$]-, and [1-^{13}C,$^{18}O_2$]acetate as well as a fermentation under an ^{18}O-enriched atmosphere resulted in information regarding the biosynthetic origin of all carbon and of the oxygen atoms of the landomycin A aglycon moiety as well as of the direction of the incorporation of the decaketide chain. Only two of the six oxygens in landomycinone seem to originate from the acetate building blocks. This raises questions about current hypotheses for the formation of multicyclic aromatic polyketides. Some unusual prearomatic deoxygenation steps early in the biosynthesis have been proposed. As an additional result of the biosynthetic investigations, the structure of landomycin A and thus of all other landomycins were revised concerning the position of the phenol–glycosidically linked deoxysaccharide chain from **2** to **1**.

The angucycline antibiotics have become an ever-growing group of bioactive natural products with unusual structures and unique chemical reactions.[1] The group has attracted several recent innovative synthetic approaches.[2] Also, the investigations of the biosyntheses of certain members have yielded particularly unexpected results, such as interesting rearrangements (vineomycin B_1, kinamycins, PD 116198) or partially nonenzymatic chromophore enlargements through amino acids (urdamycins).[1]

Landomycin A (**1**), the largest member of the angucycline family,[1b] shows an interesting antitumor activity and is currently under investigation by the National Cancer Institute (Bethesda, MD).[3] The structure of **1** consists of two extraordinary features, the naphthazarine chromophore-containing aglycon moiety (unique among the angucyclines) and the phenol–glycosidically linked hexadeoxysaccharide chain.[1b]

Investigations of polyketide biosynthetic pathways have undergone a renaissance during the past 6 years. For macrocyclic and polyether antibiotics, i.e., metabolites assembled by a PKS (polyketide synthase) type-1,[4] it is widely accepted that considerable functionalization of partially reduced polyketides appears as a result of an assembly process resembling incomplete fatty acid biosynthesis; i.e., the (partial) reduction steps occur immediately after each condensation of a polyketide building block during the assembly process. In contrast, for the aromatic, multicyclic polyketides, i.e., those whose biosyntheses are catalyzed by a PKS type-2, participation of an unmodified or nearly unmodified nonreduced polyketide precursor (poly-β-keto-CoA ester) is still the current hypothesis.[5] Biosynthetic investigations, for example, on the frequently occurring angucyclinone aquayamycin, as well as genetic engineering experiments confirmed this hypothesis widely.[6] For certain multicyclic oligoketides, e.g., for the anthracyclines and the actinorhodins, only one prearomatic reduction of the hypothetical decaketide and octaketide (containing 10 and eight C=O groups, respectively) into an OH group has been discussed[5] in context with the first cyclization (first cyclase reaction). There is still disagreement over whether this reduction happens during or after the assembly of the polyketide chain. Recently, Gould et al. could prove another prearomatic reduction step during the biosynthesis of the angucyclinone PD 116740.[6c]

Feeding experiments with $^{18}O_2$ in addition to those with ^{18}O-labeled biosynthetic building blocks which can be evaluated with NMR spectroscopy using the ^{13}C{^{18}O} isotope shift method may provide suggestions regarding earlier biosynthetic steps.[7] This may lead to novel hypotheses which can be probed by further biosynthetic

† This publication is dedicated to Professor Dr. Hans Zähner on the occasion of his 65th birthday.

‡ To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* June 15, 1994.

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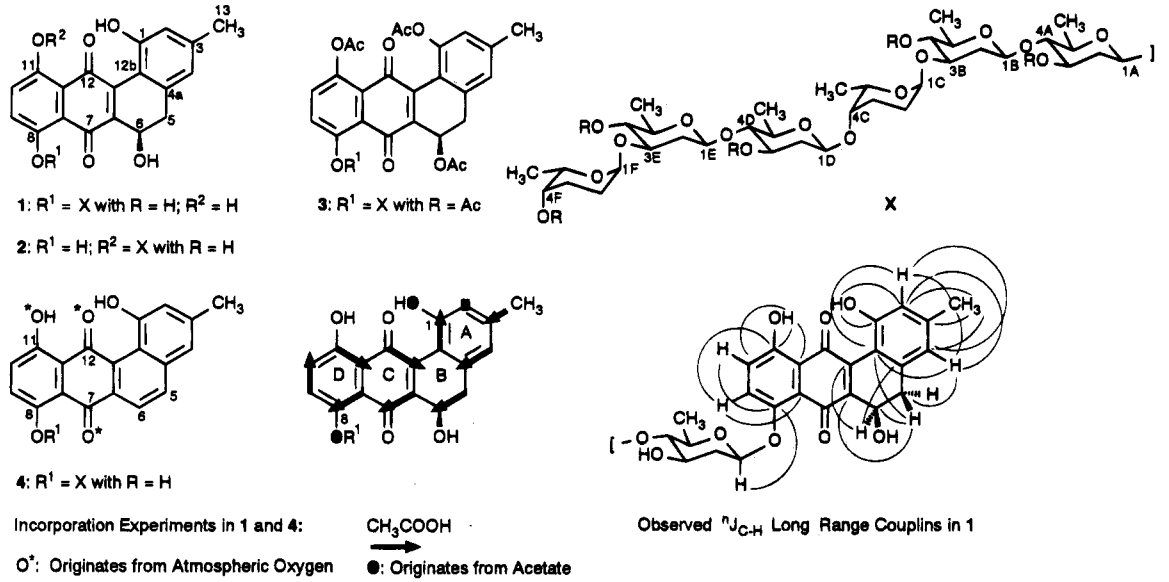
Scheme 1. Structures, Biosynthetic Incorporation Pattern, and Observed Long Range Couplings of Landomycin A (1) and the Discussed Derivatives



investigations. Our studies on the lysolipins have shown that feeding experiments with ^{18}O-labeled precursors are also of interest for the biosynthesis of multicyclic aromatic polyketides. In this way it is possible to prove individually whether oxygen atoms connected directly to a carbon deriving from a carboxyl group of acetate were indeed incorporated *via* the polyketide building block or not.[8] Alternatively, oxygen atoms, especially those connected to other carbons, stem either from atmospheric oxygen or from water. In both cases a certain functionality is a prerequesite for the enzymatic introduction of the oxygen, for example, a double bond for an epoxidation step. This functionality can either be provided during the polyketide assembly process (through reduction steps) or after the PKS reaction. The application of the ^{18}O technique to other angucyclinones and comparison with known examples of this group in general and to the landomycins in particular seemed promising, since the latter antibiotics carry six oxygen atoms in their aglycon structures, and their origin might reveal mechanistic features of the biosynthesis.

Experimental Section

Cultivation and Fermentation. *Streptomyces cyanogenus* (strain S-136, DSM 5087) was incubated on agar disks (21% agar, 10% malt extract, 4% glucose, 4% yeast extract, 1% $CaCO_3$) for 6 days at 28 °C. Then the strain was precultivated in 250 mL of triply baffled Erlenmeyer flasks, each containing 100 mL of a glucose–soy peptone–$CaCO_3$–$CoCl_2$ medium (2%, 1%, 0.2%, 0.0001%; pH adjusted to 7.2 before autoclaving), with rotary shaking at 250 rpm and 28 °C. The cultures were inoculated directly from the agar disks. One hundred mL of the preculture was used to inoculate a 1-L fermentor with the same medium. The fermentor was operated with an aeration rate of 1.5 L/min at 28 °C and 500 rpm for 60 h.

Isolation. The cultures were filtered with Celite (100 g/L), and the filtrate was defatted by extraction with *n*-pentane (3×, 200 mL each). Extraction with ethyl acetate (3×, 500 mL each) and evaporation of the combined organic layers to dryness gave the crude product which was purified in portions of 100–200 mg by chromatography on silica gel (first column, 30 × 3 cm, $CHCl_3$–MeOH (9:1); second column, 30 × 3 cm, $CHCl_3$–MeOH (95:5 → 9:1), gradient) and on Sephadex LH 20 (third column, 100 × 2.5 cm, $CHCl_3$) to yield 60–90 mg/L of pure landomycin A as a dark red solid.

Feeding Experiments with Labeled Precursors: (a) Feeding of Sodium [1-^{13}C]Acetate, [1,2-$^{13}C_2$]Acetate, and [1-^{13}C,$^{18}O_2$]Acetate, Respectively. Each labeled substrate (12, 12, and 18.4 mmol/L culture, respectively) was divided into four portions. Immediately before the feeding, each portion was dissolved in 25 mL of sterile water and added at 10, 12 , 14, and 16 h after inoculation to the growing culture of *S. cyanogenus* S-136 (fermentor with 1 L of culture), yielding 63.9, 84.4, and 87.5 mg, respectively, of **1**. **(b) Fermentation of a 300-mL Culture in an $^{18}O_2$-Containing Atmosphere.** The fermentation was carried out in a closed system using a previously described apparatus,[6a,7e] here attached to three 250-mL Erlenmeyer flasks, each containing 100 mL of the culture. The fermentation was started with normal air, and the consumed oxygen was replaced by $^{16}O_2$ during the first 6 h. The fermentation atmosphere in the apparatus was then replaced by a mixture of 80% N_2 and 20% oxygen (50% ^{18}O, total volume: 7 L); consumed oxygen was replaced by $^{18}O_2$ (50% ^{18}O). Because of a late discoloration of this culture, harvesting was delayed until 96 h. Yield: 37 mg of 5,6-anhydrolandomycin A (**4**).

Labeled Compounds. Sodium [1-^{13}C]- and [1,2-$^{13}C_2$]-acetate (each 99% ^{13}C), $^{18}O_2$ (50% ^{18}O) was obtained from CIL (Cambridge Isotope Laboratories, Cambridge, MA). The [1-^{13}C, $^{18}O_2$]acetate was prepared from [1-^{13}C]acetate and $H_2{}^{18}O$ (97% ^{18}O, obtained from Isotec Inc., Miamisburg, OH).[9]

NMR Experiments. The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 200, 300, or 500 MHz and at 50.3, 75.4, or 125.7 MHz, respectively. The ^{13}C NMR signals of the aglycon moiety of landomycin A (**1**) were assigned with a variety of 2D homonuclear and heteronuclear correlation experiments, namely H,H-COSY, C,H-COSY, APT (attached proton test), COLOC (correlation spectroscopy *via* long range coupling), HMBC (hetero multiple bond connectivity), and INADEQUATE (incredible natural abundance double quantum transfer experiment); see also the text. The observed

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Table 1. ^{13}C NMR Data for Landomycinone A (1)[a]

no.	δ		no.	δ	
C-1	155.1 (s)	(68.0)	C-8	150.6 s	(74.4)
C-2	120.0 (d)	(−)[b]	C-9	132.4 d	(59.5)
C-3	143.6 s	(43.6)	C-10	126.7 d	(59.1)
C-4	123.7 d	(62.1)	C-11	159.5 s	(65.1)
C-4a	136.6 s	(61.8)	C-11a	114.8 s	(65.0)
C-5	36.3 t	(37.3)	C-12	192.9 s	(52.7)
C-6	62.0 d	(37.2)	C-12a	138.7 s	(52.8)
C-6a	146.7 s	(52.8)	C-12b	113.2 s	(68.3)
C-7	182.2 s	(52.9)	C-13	21.1 q	(43.7)
C-7a	119.0 s	(74.4)			

[a] δ in ppm relative to internal TMS in $CDCl_3$ at 50.2 MHz ($^1J_{C-C}$ in hertz, from the feeding experiment with [1,2-$^{13}C_2$]acetate), multiplicities from the APT (attached proton test) experiment. [b] Singlet due to the decarboxylation of the last acetate unit.

Table 2. Upfield Shifts (Δδ $^{13}C-^{18}O$ in ppm) Observed in Landomycin A (1) and 5,6-Anhydrolandomycin A (4), Respectively, after Feeding [1-^{13}C,$^{18}O_2$]Acetate (for 1) and from the Fermentation with $^{18}O_2$ (for 4) at 125.7 MHz in $CDCl_3$ (^{18}O Enrichments in %[a])

no.	1	4
C-1	0.01 (30)	
C-6		
C-7		0.02 (72)
C-8	0.01 (26)	
C-11		0.01 (70)
C-12		0.02 (74)

[a] ^{18}O Enrichment = $(I_{^{13}C-^{18}O}/I_{^{13}C-^{16}O} + I_{^{13}C-^{18}O})100\%$.

$^nJ_{C-H}$ couplings (n = 2, 3) are summarized in Scheme 1. To detect the ^{18}O upfield shifts of the directly attached carbon atoms, the broadband decoupled ^{13}C NMR spectra of **1** and **4**, respectively, were recorded at 125.7 MHz. The upfield shifts were of the expected magnitude for ^{18}O-isotope shifted ^{13}C (Table 2).

Results

The specific incorporation rates[10] obtained from the feeding experiments with the different labeled acetates ranged from 3 to 6% per site. [1-^{13}C]Acetate labeled nine carbons, namely C-1, C-3, C-4a, C-6, C-7, C-8, C-10, C-11a, and C-12a. The feeding experiment with [1,2-$^{13}C_2$]-acetate labeled all carbons of the aglycon moiety of **1**. From the $^1J_{C-C}$ coupling constants (Table 1) and the 2D $^{13}C,^{13}C$-INADEQUATE NMR spectrum nine intact acetate-derived units could be identified unambigously, C-4/C-4a, C-5/C-6, C-6a/C-7, C-7a/C-8, C-9/C-10, C-11/C-11a, C-12/C-12a, C-12b/C-1, and C-13/C-3. C-2 appeared as a singlet due to decarboxylation of the tenth acetate building block. The findings are in agreement with the expected single chain polyketide assembly as was previously found for all but one of the studied angucyclinones.[1c,11] The upfield shifts observed in the proton noise decoupled ^{13}C NMR spectra of **1** after feeding with [1-^{13}C,$^{18}O_2$]acetate were in the expected magnitude (Table 2) and indicate that only two oxygens in landomycin A (**1**) are derived from acetate, namely 1-O and 8-O. Three of the other oxygen atoms (7-O, 11-O, and 12-O) in **1** originate from atmospheric oxygen, as the fermentation with $^{18}O_2$ showed (Table 2). The product obtained in this fermentation was 5,6-anhydrolandomycin A (**4**) instead of **1**. Thus, the source of one oxygen atom (6-O) remains unclear (see Discussion). The results of the feeding experiments with the ^{13}C- and ^{18}O-labeled precursors are depicted comprehensively in Scheme 1.

Discussion

Our current work resulting in the revision of the structure of landomycin A (from **2** into **1**) presents a good example of the value of biosynthetic studies to confirm structure elucidations. Revision has only been possible due to the biosynthetic studies on its aglycon moiety. The more important result, nevertheless, is the surprising biogenetic origin of some of the oxygen atoms in the landomycin aglycon, which raises general questions of the biosynthesis of multicyclic, aromatic polyketides (type-2 polyketides).

For the biosynthetic studies, the signals of the ^{13}C NMR spectrum of the landomycin aglycon had to be reassigned unambigously. They were previously assigned on the basis of chemical shifts and, so far as possible, in analogy to the octaacetyl derivative **3**. The assignments of the carbon signals of **1** could be achieved by several 1- and 2D homonuclear and heteronuclear NMR experiments in agreement with the results of the incorporation experiments with the ^{13}C-labeled acetates. Especially the 2D-INADEQUATE experiment after feeding [1,2-$^{13}C_2$]acetate allowed the unambiguous pairwise assignment of two carbons deriving from the same acetate unit. Several assignments[1b] had to be corrected. For the structure revision (see below) especially the long range $^nJ_{C-H}$ (n = 2, 3) couplings of 11-OH (with C-10, C-11, and C-11a) and of 1A-H (with C-8) were important. All $^2J_{C-H}$ and $^3J_{C-H}$ couplings found in the landomycin A aglycon moiety are summarized in Scheme 1. An opposite anellation of ring D of landomycin A can be excluded, although theoretically still possible from the NMR results, because then the biosynthesis would require the scission and reconnection of two bonds (C-7/C-7a and C-11a/C-12), a very unplausible process.[12]

The fermentation experiment under an ^{18}O-enriched atmosphere yielded 5,6-anhydrolandomycin A (**4**) instead of landomycin A (**1**). Even though a dehydration due to the partially acidic pH value during the fermentation cannot be absolutely ruled out, it is more likely that **4** is an immediate biosynthetic precursor of **1**, because a lack of oxygen in the closed-system apparatus used for the fermentation under ^{18}O-enriched atmosphere as opposed to a "normal" fermentation could have developed. Thus, the oxygenase performing the introduction of an oxygen atom at C-6 may have operated insufficiently. Additionally, the proton noise decoupled ^{13}C NMR spectrum of **1** from the feeding experiment with [1-^{13}C,$^{18}O_2$]acetate exhibited no upfield ^{18}O isotope shift for C-6. Although it can never be completely excluded that the ^{18}O-label might be lost from exchange or that the ^{18}O upfield isotope shift was too small to be detectable, it is more likely that 6-OH of **1** is not derived from acetate and therefore must originate either from atmospheric oxygen

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(12) According to the NMR data, all carbons of the rings A, B, and C on the one hand and all carbons within ring D on the other hand can be unequivocally assigned. Thus, also the three intact acetate units in ring D are unequivocally incorporated. The anellation of ring D to the ABC fragment is ambiguous, since in both alternative structures (**1** and **2**) no $^3J_{C-H}$ coupling is possible from the one structural fragment into the other, e.g., from any of the protons of ring D to one of the quinone carbonyls of ring C. This ambiguity had led to the wrong structure **2**.[1b] If the alternative structure **2** was correct, the three intact acetate units of ring D would be incorporated "against" the direction of the rest of the polyketide chain.

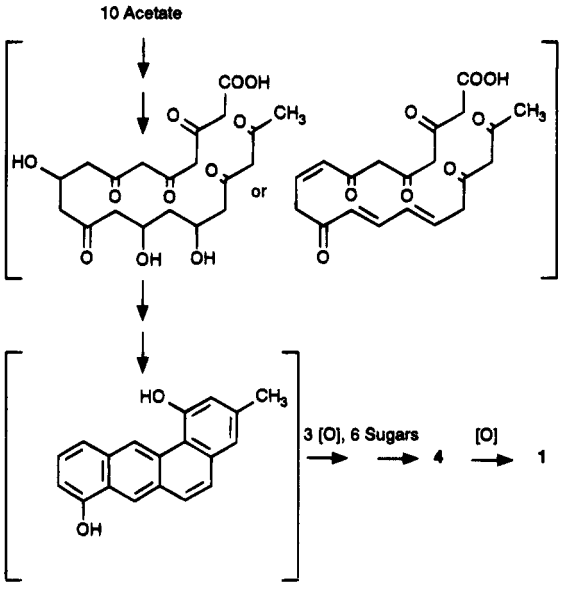

Figure 1. Proposed biosynthesis of landomycin A (**1**).

or from water. The derivation from atmospheric oxygen is more likely in accord with the hypothesis that **4** is an immediate precursor of **1** (Figure 1). Due to the cost factor of the experiment, we could not repeat the fermentation in an $^{18}O_2$-enriched atmosphere. A recent study on the angucyclinone PD 116740 also proved a prearomatic deoxygenation step at the 6-position during its early biosynthesis. Therefore, the oxygen at C-6 in PD 116740 has to be introduced later, preferably through an oxygenase.[6c]

The fact that only 1-O and 8-O of landomycin A (**1**) were proven to stem from the polyketide building block acetate, while the quinone oxygen 7-O originates from atmospheric oxygen, is unexpected and intriguing. Since the PKS also catalyzes the intramolecular aldol reactions of the ring formations during the biosynthesis of the aromatic, multicyclic polyketides,[5] an almost unmodified polyketide (poly-β-keto ester) chain is required. This view of the type-2-polyketides has to be reconsidered. The results described here and the studies on lysolipin[8] and on PD 116740[6c] are already three examples of prearomatic reduction steps probably occurring during the assembly of the polyketide chain leading to such multicyclic aromatic compounds. On the other hand, some intramolecular aldol condensation steps are then disadvantaged because of the lowered CH acidity. Our conclusive hypothesis for the biosynthesis of landomycin A is depicted in Figure 1.

Acknowledgment. The authors would like to thank the Deutsche Forschungsgemeinschaft (Grant No. Ro 676/4-1 and Ro 676/5-1), the Fonds der Chemischen Industrie (Grants Nos. 635050 and 160094), and NATO (Grant No. 88368) for financial support of this work.