Biosynthesis of the Manumycin Group Antibiotics

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Abstract: The biosynthesis of the manumycin group antibiotics manumycin (**1**) and asukamycin (**2**) was studied in *Streptomyces parvulus* Tü 64 and *Streptomyces nodosus* ssp. asukaensis ATCC 29757 by using radioactive and stable isotope tracer techniques and high-field NMR spectroscopy. The results have demonstrated that the central, multifunctional mC_7N unit typical of this group of antibiotics, which serves as the starter unit for a short polyketide chain, is biosynthesized from a C_4 Krebs cycle and a C_3 triose phosphate pool intermediate by a new pathway, distinct from the shikimate, polyketide, or pentose phosphate routes leading to other mC_7N units in nature. The C_5 unit in both **1** and **2** arises by a novel intramolecular cyclization of 5-aminolevulinic acid, and a cyclohexane ring and the adjacent carbon in **2** arise from the seven carbon atoms of shikimic acid. The side chains of both antibiotics represent typical polyketide-derived moieties, differing with respect to their combinations of starter and elongation units. Results of isotopically labeled precursor feedings and precursor-directed biosynthesis experiments, in combination, allow predictions to be made about the biosynthetic assembly of the manumycin group antibiotics.

Manumycin (**1**)[1-5] and asukamycin (**2**)[6,7] (Figure 1) are two members of the manumycin group of antibiotics, also represented by colabomycin A,[8,9] U-62162,[10] and U-56407.[11] The biological activity of manumycin as the parent antibiotic was tested in a wide variety of organisms; it is active against Gram-positive bacteria, fungi, and L-1210 leukemia stem cells but shows no activity against Gram-negative bacteria or yeasts.[3] In addition, **1** exhibits inhibitory activity against polymorphonuclear leukocyte elastase.

The manumycin group antibiotics contain as a central structural element a multifunctional mC_7N unit. This moiety, consisting of a six-membered carbocyclic ring bearing one carbon and one nitrogen atom in a meta disposition, is quite common in nature, where it appears in many different forms.[12,13] It usually occurs in a benzo- or naphthoquinone structure, as found in rifamycins,[14] mitomycins,[15] and ansamitocins,[16] or it may be aromatic, as in pactamycin.[17] In these cases biosynthesis of the mC_7N unit has been shown to proceed through a branch of the shikimic acid pathway,[13] with 3-amino-5-hydroxybenzoic acid (AHBA) or 3-aminobenzoic acid (ABA) as proximate precursors. Similar mC_7N units are found in validamycin and acarbose as well as in kinamycin; these have been shown to arise via a sedoheptulose from the pentose phosphate pathway[18,19] and via polyketide metabolism from acetate,[20] respectively. Besides the mC_7N unit, the manumycin group antibiotics contain a variety of other interesting structural elements of different biosynthetic origins, notably the 2-amino-3-hydroxycyclopent-2-enone (C_5N) unit also found in other antibiotics, e.g., reductiomycin,[21] moenomycin,[22,23] and bafilomycin B_1.[24] Attached to these units are hydrocarbon side chains in various states of unsaturation. In addition, **2** carries a very unusual substitution at the terminus of one of the side chains, a saturated cyclohexane ring. This structure has previously been found only in the ansatrienins[25,26] and in the cyclohexyl fatty acids, which are constituents of the cell membrane of acidophilic, thermophilic bacteria.[27] **1** and **2** differ from each other in the stereochemistry of their mC_7N units and in the nature of their side chains.[5,7] Because of the similarities between these two antibiotics, it was of interest to study their biosyntheses in parallel. Some of our results have been published in preliminary communications.[28-32] In the present paper we provide a complete account of this work.

Experimental Section

Materials. Cultures of the producing strains were obtained from Prof. H. Zähner, Tübingen, FRG (*Streptomyces parvulus* Tü 64), and the American Type Culture Collection, Rockville, MD (*Streptomyces nodosus* ssp. asukaensis ATCC 29757). Radioactive precursors were ob-

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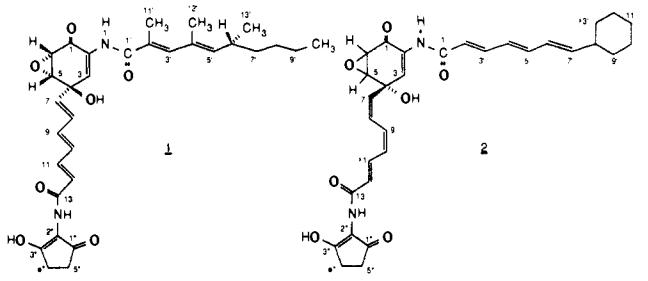

Figure 1. Structures of manumycin (1) and asukamycin (2).

tained from the following sources: D-[G-^{14}C]shikimic acid (19.7 mCi/mmol), [7-^{14}C]benzoic acid (56.6 mCi/mmol), and [5-^{14}C]-5-aminolevulinic acid (49.0 mCi/mmol) from New England Nuclear; [2-^{14}C]-glycine (56 mCi/mmol) and [1(4)-^{14}C]succinic acid (118 mCi/mmol) from Amersham; [1(3)-^{14}C]glycerol (50 mCi/mmol) from ICN. 1,4-Dihydro[7-^{14}C]benzoic acid (5.0 mCi/mmol) was prepared by Birch reduction of [7-^{14}C]benzoic acid[33] and 2,5-dihydro[7-^{14}C]benzoic acid (2.7 mCi/mmol) by Diels–Alder reaction of butadiene and [1-^{14}C]-propiolic acid,[34] which was prepared by carboxylation of sodium acetylide with $^{14}CO_2$. [7-^{14}C]Cyclohexanecarboxylic acid was prepared by carboxylation of the Grignard reagent from bromocyclohexane.[35] Radioactive precursors were added to the cultures as dilutions of the high-specific-activity materials. The following stable isotopes were supplied by Cambridge Isotopes: sodium [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetate (99%), sodium [1-^{13}C,$^{18}O_2$]acetate (99% ^{13}C, 96% ^{18}O), and L-[*methyl*-^{13}C]methionine (97%). Sodium [1-^{13}C]propionate (99%), sodium [1(3)-$^{13}C_1$]- and [2-^{13}C]malonate (90%), sodium [$^2H_4$]malonate (99%), L-[3-^{13}C]serine (99%), L-[2,3-$^{13}C_2$]serine (99%), [2-^{13}C,^{15}N]glycine (99% ^{13}C, 99% ^{15}N), L-[*amido*-^{15}N]glutamine (99%), and $^{18}O_2$ gas (90%) were supplied by Merck and Co. [2-^{13}C,2,2-$^2H_2$]Malonate (90% ^{13}C, 99% ^{2}H) was prepared by exchange of [2-^{13}C]malonate (Merck) with D_2O in a sealed tube. L-[2,3-$^{13}C_2$]Serine was obtained from the Los Alamos Stable Isotope Resource and L-[*methyl*-^{13}C]methionine (99%) from Cambridge Isotopes, Inc. The [U-$^{13}C_3$]glycerol was synthesized from K^{13}CN and [1,2-$^{13}C_2$]acetic acid (both 99%), provided by the Los Alamos Stable Isotope Resource, via diethyl malonate and diethyl 2-acetoxymalonate-.[36,37] 3-Amino-5-hydroxy[7-^{13}C]benzoic acid (AHBA) was synthesized from Cu^{13}CN (99%) as described by Rickards and co-workers,[38] and 3-amino[7-^{13}C]benzoic acid (ABA) was prepared by analogous chemistry from 3-nitrochlorobenzene and Cu^{13}CN.[18] [1,1-$^2H_2$]Glycerol (90%) was synthesized from 2,3-isopropylideneglyceric acid by reduction with Li-AlD_4.

NMR Spectroscopy. ^{13}C and ^{1}H NMR spectra were acquired in $CDCl_3$ at 305 K at a field strength of 4.7 T on a Varian XL-200 instrument, at 7.1 T on an IBM AF-300 instrument, or at 11.8 T on a Bruker AM-500 instrument. Deuterium and ^{15}N NMR spectra and all two-dimensional experiments were performed on the IBM spectrometer. ^{15}N chemical shifts were calculated by reference to an external standard of saturated NH_4Cl. Deuterium NMR spectra were referenced to an external standard of $CDCl_3$. Chemical shifts are reported in ppm relative to tetramethylsilane (TMS). Two-dimensional NMR spectra were acquired with the IBM AF-300 spectrometer and processed with the Aspect 3000 computer with array processor. Line broadening was often observed in the lines for C1″, C3″, C4″, and C5″, leading in some cases to complete disappearance of the signals. We attribute these effects to tautomeric changes occurring very rapidly on the NMR time scale, resulting in nearly complete coalescence and hence corresponding line broadening. Treatment with Chelex 100 and preparation of the samples in a rigorously dry atmosphere could sometimes yield sharper lines.

Fermentation. Manumycin: *S. parvulus* (Tü 64) was grown on slants of yeast–malt extract agar (YM), which were incubated at 28 °C and stored in sealed, screw-cap tubes at 4 °C until used. Spore mass was amplified prior to fermentation by growing on YM agar contained in 500-mL Roux bottles. For manumycin production, Tü 64 was cultivated with a single-stage protocol in a medium consisting of the following: soybean meal, 2%, and mannitol, 2%, in deionized water, adjusted to pH 7.0 prior to autoclaving. A part of the spore contents of a Roux bottle was inoculated into each 100-mL volume of medium contained in a 1-L nonbaffled, foam-plugged Erlenmeyer flask. Cultures were incubated at 28 °C with reciprocal shaking at 280 rpm. Labeled precursors were typically added to the fermentation at 36–40 h, and cultures were harvested at 72 h. The fermentation under $^{18}O_2$ atmosphere was conducted in a closed vessel as previously described.[39] A culture was grown under standard conditions in an Erlenmeyer flask (500 mL) equipped with a side arm. At 42 h the flask was connected to a closed system containing a mixture of N_2 and $^{18}O_2$ (50:50), and 8 mmol/L nonlabeled glycerol was added to enhance yield.[31] The gas mixture was pumped continuously from a reservoir into the fermentation vessel, and the output from the flask was directed into a KOH solution (40% in water) to trap the CO_2 produced.

Asukamycin: Cultures of *S. nodosus* ssp. asukaensis (ATCC 29757) were grown on slants of yeast–malt extract agar (Difco), which were incubated at 28 °C. Slants were stored at 4 °C in sealed, screw-cap tubes prior to use. Asukamycin was produced according to a two-stage fermentation protocol in the following media: (seed stage) glucose, 4%, soluble starch, 2.4%, peptone, 1%, yeast extract, 1%, $CaCO_3$, 0.5%, deionized water, to 1 L, pH adjusted to 7.0 before autoclaving; (production stage) glucose, 2%, soybean meal, 2%, NaCl, 0.5%, deionized water, to 1 L, pH adjusted to 7.0 prior to autoclaving. Both stages were conducted in 500-mL triply baffled, cotton-plugged Erlenmeyer flasks, each containing 100 mL of medium. The spore content of one slant was used to inoculate each seed culture, which was incubated with shaking at 300 rpm on a New Brunswick G25 shaker at 28 °C for 48 h. At this time, the seed cultures were pooled and used to provide a 10% inoculum into each production flask. Production cultures were incubated under the above conditions. Labeled precursors were typically added as filtration-sterilized solutions at 24 h, and the cultures were harvested at 72 h.

Isolation and Purification. Manumycin: The fermentation broth was adjusted to pH 4.5 with 2 M HCl and centrifuged at 3000 rpm for 30 min, and the supernatant was discarded. The mycelial pellet was treated repeatedly with acetone until extraction of manumycin was complete, and the extracts were pooled. After evaporation of acetone, the remaining aqueous residue was extracted with chloroform. The organic layer was dried with anhydrous Na_2SO_4 and concentrated to an oily crude product, which was chromatographed twice on a Sephadex LH-20 (Pharmacia) column (80 × 2.5 cm, $CHCl_3$). Manumycin-containing fractions were pooled, concentrated, and dried under vacuum prior to NMR analysis.

Asukamycin: The fermentation broth was centrifuged at 5000 rpm for 15 min in a Sorvall centrifuge. The supernatant was extracted with three 150-mL portions of ethyl acetate/2-propanol (9:1), and the mycelial pellet was extracted with acetone repeatedly until TLC (silica gel GF-254, benzene/acetone 3:2) indicated that no asukamycin remained in the cell mass. Extracts were pooled, dried with anhydrous Na_2SO_4, and concentrated to an oil on a rotary evaporator. The crude extract was dissolved in a small amount of chloroform and applied to 1.0-mm preparative TLC plates (silica gel GF-254, Brinkmann, 25 mg of extract per plate), which were developed once with benzene/acetone (3:1) and then once with benzene/acetone (3:2). The asukamycin-containing band (R_f 0.4) was located by UV, scraped off, and eluted with chloroform. The chloroform extract was concentrated and chromatographed on a Sephadex LH-20 column (80 × 2.5 cm, CH_2Cl_2). The band containing pure asukamycin was collected and concentrated to dryness on a rotary evaporator. Samples were dried under vacuum prior to NMR analysis. The yield of **1** after purification averaged 60 mg/L, while that of **2** was typically 25 mg/L.

Feeding Experiments with Labeled Precursors. Feeding experiments were carried out in the normal production media for both strains. In general, precursors were administered to the fermentation at a time at which production of the antibiotics was just detectable in the medium (manumycin after 36–40 h, asukamycin after 24–36 h). Pulse feeding protocols were followed in most cases with the manumycin strain to increase the incorporation rate, with administration of the precursor at 44, 48, 52, 56, 60, 64, and 68 h into the production phase. Asukamycin feedings were conducted with single doses of precursor. Precursors were added as sterile aqueous solutions, adjusted to pH 7.0, in the amounts indicated. Experiments on **1**: sodium [1-^{13}C]- and [2-^{13}C]acetate, 20.3 mmol/L; sodium [1,2-$^{13}C_2$]acetate, 35.7 mmol/L; sodium [$^2H_3$]acetate,

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Table I. Carbon-13 Distribution in **1** and **2** Biosynthesized from Singly Labeled Precursors

carbon no.	δ_c, ppm[c]		relative ^{13}C abundances[a]							
			[1-^{13}C]-acetate		[2-^{13}C]-acetate		[1(3)-^{13}C]-malonate (**2**)	[2-^{13}C]-malonate (**2**)	[1-^{13}C]-propionate **1**	L-[3-^{13}C]-serine **1**
	1	**2**	**1**	**2**	**1**	**2**				
1	189.0	189.0	1.2	1.2	1.3	2.0	1.4	2.0	1.1	0.8
2	128.0	128.1	0.9	1.0	1.0	1.0	1.0	1.2	1.2	0.9
3	126.6	126.5	1.1	1.8	1.3	1.5	0.6	1.0	1.4	0.8
4	71.2	70.7	0.7	1.1	1.9	3.7	1.0	2.5	1.1	3.0
5	57.4	57.2	1.2	1.1	2.3	2.6	1.1	1.7	1.3	2.4
6	52.8	52.5	2.6	2.5	1.9	2.0	1.3	1.5	6.9	1.8
7	136.6	136.9	2.8	2.2	2.0	2.0	1.1	1.2	5.4	1.6
8	131.4	131.2	1.1	1.0	2.1	2.4	0.9	5.9	1.5	2.5
9	139.6	139.4	2.9	2.0	1.3	2.1	6.2	1.0	1.3	1.0
10	131.7	131.6	1.0	1.0	2.0	2.8	0.6	5.5	1.5	2.7
11	143.4	143.2	2.8	2.4	1.4	2.0	5.6	1.0	1.5	0.9
12	121.7	121.5	1.1	0.9	2.0	3.1	0.9	8.3	1.3	2.6
13	165.6	165.6	2.1	1.3	1.0	1.5	6.5	1.2	1.3	0.9
1′	168.0	165.3	1.8	1.3	1.7	1.7	8.5	1.3	14.9	2.2
2′	128.2	121.3	0.9	1.0	2.1	2.1	1.2	8.2	0.9	2.5
3′	140.3	143.5	1.0	1.3	1.8	0.7	6.5	1.1	10.4	1.8
4′	129.9	127.4	1.1	1.1	2.3	2.0	1.0	8.7	0.9	2.7
5′	142.8	142.2	1.8	1.3	2.0	1.2	4.6	0.8	10.8	1.8
6′	32.8	127.5	0.8	1.1	2.1	5.0	1.6	2.1	0.7	2.4
7′	37.0	146.5	2.7	0.8	1.5	1.2	0.7	0.9	0.8	0.9
8′	29.8	41.0	0.9	0.8	1.7	1.3	0.7	1.4	0.9	2.2
9′	22.8	32.3	2.5	1.0	1.6	1.4	1.0	1.1	1.1	1.0
10′	14.1	25.8	0.9	1.0	2.1	1.1	1.0	1.1	0.9	2.3
11′	14.0	26.0	0.9	1.0	2.1	1.3	1.1	1.3	0.9	2.5
12′	16.5	25.8	0.9	1.0	2.6	1.1	1.0	1.1	0.9	2.4
13′	20.7	32.3	1.0	1.0	2.2	1.1	1.1	1.1	0.8	2.5
1″	197.5	197.9	nd[b]	1.9	nd	0.6	1.0	nd	nd	nd
2″	115.2	115.1	1.1	1.0	1.0	1.0	1.1	1.0	1.0	1.1
3″	174.5	174.5	nd	1.8	nd	0.5	1.1	nd	nd	nd
4″	32.2	32.5	1.0	1.0	nd	1.3	1.0	nd	nd	nd
5″	25.7	25.8	0.9	0.9	0.8	0.8	1.0	nd	nd	nd

[a] Relative to the abundance of C2″ = 1.0. [b] nd = not detected. [c] Referenced to $CDCl_3$.

29.4 mmol/L; sodium [1-^{13}C]propionate, 18.8 mmol/L; [1,4-$^{13}C_2$]-succinate, 10.3 mmol/L; [1,2-$^{13}C_2$]succinate, 14.1 mmol/L; [2-^{13}C,2,2-$^{2}H_2$]malonate, 7.5 mmol/L; [2,2-$^{2}H_2$]malonate, 19.2 mmol/L; L-[3-^{13}C]serine, 7.9 mmol/L; L-[2,3-$^{13}C_2$]serine, 5.7 mmol/L; [U-$^{13}C_3$]-glycerol, 5.4 mmol/L; [1,1-$^{2}H_2$]glycerol, 5.9 mmol/L; [2-^{13}C,^{15}N]glycine, 21.7 mmol/L; L-[*amido*-^{15}N]glutamine, 5.4 mmol/L; sodium [1-^{13}C,$^{18}O_2$]acetate, 23.0 mmol/L; [7-^{13}C]-3-amino-5-hydroxybenzoic acid, 2.6 mmol/L; [7-^{13}C]-*m*-aminobenzoic acid, 3.2 mmol/L; L-[*methyl*-^{13}C]methionine, 0.7 mmol/L. Experiments on **2**: sodium [1-^{13}C]- and [2-^{13}C]acetate, 25 mmol/L; sodium [1,2-$^{13}C_2$]acetate, 16.6 mmol/L; [2-^{13}C,$^{2}H_3$]acetate, 25 mmol/L; [1,4-$^{13}C_2$]succinate, 5.1 mmol/L; [2-^{13}C,2,2-$^{2}H_2$]malonate, 3.0 mmol/L; [U-$^{13}C_3$]glycerol, 5.4 mmol/L; [2-^{13}C,^{15}N]glycine, 6.5 mmol/L; [2-^{13}C]- and [1(3)-$^{13}C_1$]malonate, 3.0 mmol/L; [7-^{13}C]-3-amino-5-hydroxybenzoic acid, 2.6 mmol/L; [7-^{13}C]-*m*-aminobenzoic acid, 3.2 mmol/L; L-[*methyl*-^{13}C]methionine, 0.7 mmol/L. Radioactive precursors were diluted with nonlabeled carrier material and added to the fermentation in single doses following filter sterilization. Radioactivity in the starting materials and isolated antibiotics was determined with a Beckman LS-7000 liquid scintillation counter using Aquasol-2 LSC cocktail (New England Nuclear).

Results

NMR Assignments. Unequivocal assignments for all of the proton and ^{13}C signals were made for both antibiotics. Initial proton assignments were made for the side chains by analysis of coupling patterns, multiplicity, and chemical shift parameters and by NOE difference spectra and selective proton homodecoupling experiments. Proton connectivity maps were constructed from 2D COSY, 2D NOESY, and 2D double- and triple-quantum spectra. These experiments allowed proton sequences to be determined in a straightforward manner, and the resulting assignments were used to confirm ^{13}C resonance positions through 2D heteronuclear correlation spectroscopy. In some cases, congestion in the olefinic regions was relieved by making use of solvent-induced shift changes caused by changing from chloroform to benzene or pyridine. A complete identification of all coupling constants allowed unequivocal descriptions of side-chain stereochemistries to be made. Multiplicities of ^{13}C signals were determined with edited DEPT analyses, and proton signals were correlated to carbons through one-bond couplings by 2D heteronuclear correlation experiments. Quaternary carbon signals were assigned from 2D COLOC spectra obtained at tuning values of 4 and 6 Hz or by the use of single-frequency proton-decoupled, gated-decoupled ^{13}C NMR spectra. ^{15}N signals in the ^{15}N NMR spectra were assigned on samples obtained from the [^{13}C,^{15}N]-glycine feeding experiments in both cases, by identification of the ^{13}C-coupled ^{15}N signal. A tabulation of the ^{13}C NMR signal assignments for **1** and **2** is given in Table I. Details of the NMR work will be the subject of a separate communication.

Biosynthesis. Our working hypothesis for the biosynthesis of the side chain units of **1** and **2** envisioned polyketide-type pathways, differing in the nature of the chain starter and extension units. In the case of **1**, the "upper" side chain (C1′–C13′) was predicted to arise from an acetate starter unit that is elongated by condensation with one malonyl-CoA and three methylmalonyl-CoA ("propionate") units. For **2**, in the upper chain (C1′–C13′) the starter unit was presumed to be cyclohexylcarbonyl-CoA, possibly derived from the shikimate pathway, with chain extension by three molecules of malonyl-CoA. The "lower" side chains (C7–C13) in both cases appeared to be classically polyketide- (malonyl-CoA) derived, with the notable differences being the all-trans stereochemistry of **1** and the cis, cis, trans geometry of **2**. The starter unit of this portion in both cases seemed to be the mC_7N unit. In accordance with the hypothesis of Rickards et al.[40] that the manumycin group antibiotics are actually incomplete or open-chain ansamycins,[41] we initially expected that the mC_7N units of **1** and **2** would originate from the shikimate pathway. The biosynthesis of the C_5N unit was thought to represent a novel, intramolecular

(40) Becker, A. M.; Rickards, R. W.; Brown, R. F. C. *Tetrahedron* **1983**, *39*, 4189.

(41) Wehrli, W. *Ansamycins Chemistry, Biosynthesis and Biological Activity*; Topics in Current Chemistry Series; Springer Verlag: Berlin, 1977; pp 21–49.

Table II. Incorporation of Radioactive Precursors into Asukamycin (2)

precursor	sp. act. of precursor, dpm/μmol	amount of precursor		yield of 2 (1)[a]		sp. act. of isolated 2 (1),[a] dpm/μmol	incorpn, %
		μmol/L	dpm/L	μmol/L	dpm/L		
D-[G-^{14}C]shikimate	4.3×10^7	0.33	1.42×10^7	84.2	4.22×10^4	5.02×10^2	0.297
[7-^{14}C]cyclohexanecarboxylic acid	4.4×10^7	0.67	2.95×10^7	102.6	1.59×10^6	1.55×10^4	5.38
1,4-dihydro[7-^{14}C]benzoic acid	1.1×10^7	1.35	1.50×10^7	58.7	1.72×10^5	2.93×10^3	1.15
2,5-dihydro[7-^{14}C]benzoic acid	5.8×10^6	5.3	3.00×10^7	56.0	1.25×10^5	2.24×10^3	0.42
[7-^{14}C]benzoic acid	1.6×10^6	18.5	2.92×10^7	58.1	4.95×10^3	85	0.017
[1(3)-^{14}C]glycerol	9.34×10^2	21930	2.05×10^7	12.6	3.19×10^3	253	0.156
[2-^{14}C]glycine	2.6×10^3	8070	2.09×10^7	24.9	1.38×10^4	553	0.066
[5-^{14}C]-5-ALA·HCl	2.03×10^4	1000	2.03×10^7	27.9	1.37×10^3	49	0.007
[5-^{14}C]-5-ALA·HCl	9.70×10^3	2090	2.03×10^7	24.4	2.86×10^3	110	0.013
[5-^{14}C]-5-ALA·HCl	4.00×10^3	5050	2.03×10^6	8.15	7.09×10^2	87	0.0034
[1,4-$^{14}C_2$]succinic acid	6.5×10^3	6000	3.06×10^7	41.0	1.64×10^3	40	0.007
[1,4-$^{14}C_2$]succinic acid into 1	5.1×10^3	6000	3.06×10^7	130.7	1.46×10^4	112	0.048

[a] Experiment with manumycin producer.

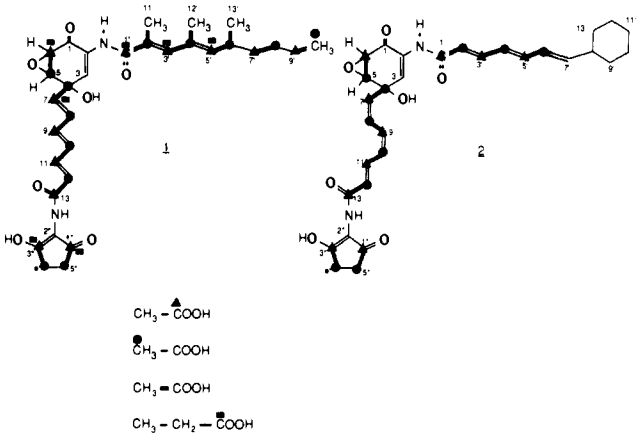

Figure 2. Labeling patterns in 1 and 2 from [^{13}C]acetate and [^{13}C]-propionate.

cyclization of 5-aminolevulinic acid, which otherwise in nature, i.e., in the biosynthesis of porphyrins and corrins, undergoes exclusively an intermolecular cyclization to yield a pyrrole.

As an initial probe, incorporation of ^{14}C-labeled precursors provided information to guide experiments with stable isotopes. These experiments were conducted chiefly with **2**, and the data are presented in Table II.

Acetate Feeding Experiments. Sodium acetate was deemed the best initial precursor for feeding experiments since it had a chance of incorporation into most of the structural elements of **1** and **2**, regardless of the metabolic pathways involved. Feeding of [1-^{13}C]- and [2-^{13}C]acetate labeled the positions of the side chains as depicted in Figure 2. These patterns are consistent with the polyketide hypothesis as outlined above. The enrichments were higher when pulse feeding protocols were employed, as in the manumycin case, than when single doses were administered (Table I). It is evident that there was a high degree of turnover of acetate in the TCA cycle, which gave rise to significant scrambling of label from [2-^{13}C]acetate and resulting statistical spin coupling in the ^{13}C NMR spectrum of **1**. Label from [2-^{13}C]acetate was found to be distributed into all carbon atoms of the three presumably propionate-derived units. Unexpectedly, label from [1-^{13}C]acetate was found in carbon atoms 6 and 7 of the mC_7N unit, while label from the [2-^{13}C]acetate was distributed over C4, C5, C6, and C7. The remaining three carbon atoms of this multifunctional ring showed no enrichments from either precursor. In the C7–C13 segment of both **1** and **2**, the normal labeling pattern for polyketide biosynthesis was found, demonstrating that this unit is assembled from three molecules of acetate (malonate). Consistent with the 5-aminolevulinate hypothesis for the C_5N unit, in which the initial precursors of the ring would be glycine and succinate, the building blocks of 5-aminolevulinate, label from [1-^{13}C]- and [2-^{13}C]acetate was distributed into carbon atoms 1″, 3″, 4″, and 5″ in patterns conforming to those expected for molecules of succinate derived via the TCA cycle. Further information about the incorporation of intact acetate moieties was obtained from experiments with [1,2-$^{13}C_2$]acetate. In such an experiment with a multiply labeled precursor, whenever the bond connecting the two isotopes is cleaved during biosynthesis, metabolic dilution with unlabeled substrate causes a loss of coupling, except for the small amount of coupling introduced by statistical recombination. Only in those instances where there is intact incorporation of the precursor will strong spin–spin coupling be observed in the final product. In **1**, we found intact acetate incorporation at C10′/C9′, C8′/C7′, C5/C6, C4/C7, C8/C9, C10/C11, C12/C13, C1″/C5″, and C3″/C4″ (Figure 2). Coupling was also detected in the propionate-derived units of the upper side chain, but this was limited to only statistical spin–spin coupling. In **2**, intact incorporation of acetate was detected in C1′/C2′, C3′/C4′, C5′/C6′, C5/C6, C4/C7, C8/C9, C10/C11, C12/C13, C1″/C5″, and C3″/C4″. No incorporation of acetate was observed in the cyclohexane ring. In neither antibiotic was label from acetate found in C1, C2, or C3. A list of labeled carbon atoms with coupling constants at various positions in **1** and **2** is compiled in Table III. These experiments strongly suggest that Rickard's hypothesis for the origin of the mC_7N units of the manumycin-type antibiotics from the shikimate pathway is not valid. In fact, the labeling pattern from acetate does not correspond to any previously observed pathway for the biosynthesis of mC_7N units.

Feeding Experiments with Possible Aromatic Precursors. To further confirm our result suggesting nonparticipation of the shikimate pathway in the biosynthesis of the mC_7N unit of **1** and **2**, experiments were conducted with [*carboxy*-^{13}C]AHBA and [*carboxy*-^{13}C]ABA. These two compounds are the mC_7N unit precursors of the ansamycins,[14] ansamitocins,[16] and mitomycin[15] and of pactamycin,[17] respectively. At moderate concentrations (ca. 3 mmol/L), no incorporation of label was detected into the mC_7N units of **1** or **2**. In higher concentrations, Tü 64 produced manumycin analogues through a novel mode of directed biosynthesis, in which the normal mC_7N unit is replaced by aromatic compounds.[42,43]

Feeding Experiments with Propionate and Methionine. The results of the acetate experiments with **1** led us to investigate the formation of the methyl-branched side chain. Feeding of sodium [1-^{13}C]propionate resulted in enrichments of ca. 10–14% in carbon atoms C1′, C3′, and C5′ of **1**, supporting the notion that the methyl branches arise from methylmalonyl-CoA chain extension units (Figure 2). Surprisingly, enrichments of ca. 6% were detected at C6 and C7, as well as in C1″ and C3″ (Table I). These results are consistent with our previous data implicating the involvement of succinate in both the mC_7N unit and the C_5N unit. Obviously, this is the effect of a highly active methylmalonyl-CoA mutase/methylmalonyl-CoA racemase pathway, which can equilibrate label between propionate and succinate. The incorporation

(42) Thiericke, R.; Zeeck, A. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2123.
(43) Thiericke, R.; Langer, H.-J.; Zeeck, A. *J. Chem. Soc., Perkin Trans. 1* **1989**, 851.

Table III. ^{13}C–^{13}C Patterns in **1** and **2** from Multiply Labeled Precursors

carbon no.	δ_c, ppm 1	δ_c, ppm 2	J_{cc}, manumycin (**1**) [1,2-$^{13}C_2$]-acetate	[2,3-$^{13}C_2$]-serine	[U-$^{13}C_3$]-glycerol	[1,2-$^{13}C_2$]-succinate	J_{cc}, asukamycin (**2**) [1,2-$^{13}C_2$]-acetate	[U-$^{13}C_3$]-glycerol
1	189.0	189.0			$^1J_{cc}$ = 52.2; $^2J_{cc}$ = 8.1			$^1J_{cc}$ = 54.3; $^2J_{cc}$ = 6.3
2	128.0	128.1			$^1J_{cc}$ = 52.2 (J_{12}); $^1J_{cc}$ = 75.0 (J_{23})			$^1J_{cc}$ = 52.3 (J_{12}); $^1J_{cc}$ = 74.6 (J_{23})
3	126.6	126.5			$^1J_{cc}$ = 75.0; $^2J_{cc}$ = 8.1			$^1J_{cc}$ = 74.6; $^1J_{cc}$ = 6.3
4	71.2	70.7	48.8	49.1	48.9	49.0	48.0	47.2
5	57.4	57.2	24.7	24.8	24.1	25.0	23.5	23.3
6	52.8	52.5	24.7	24.8	24.1	25.0	23.3	23.3
7	136.6	136.9	48.8	49.1	48.9	49.0	48.0	47.2
8	131.4	131.2	56.2	56.2	56.0		56.6	56.4
9	139.6	139.4	56.2	56.2	56.0		56.6	56.4
10	131.7	131.6	56.6	56.4	56.6		55.8	56.0
11	143.4	143.2	56.6	56.4	56.6		55.8	56.0
12	121.7	121.5	67.5	67.7	67.4		66.4	66.0
13	165.6	165.6	67.5	67.7	67.4		66.4	66.0
1′	168.9	165.3	62.8		62.7[a]		66.4	66.3
2′	128.2	121.3	62.8 (C1′–C2′); 45.1 (C2′–C11′)[a]		62.7 (C1′–C2′); 45.5 (C2′–C11′)[a]		66.4	66.3
3′	140.3	143.5	54.1[a]		53.8[a]		53.5	53.5
4′	129.9	127.4	54.1 (C3′–C4′); 43.2 (C4′–C12′)[a]		53.8 (C3′–C4′); 42.5 (C4′–C12′)[a]		53.6	53.3
5′	142.8	142.2	44.2[a]		43.2[a]		53.0	54.0
6′	32.8	127.5	44.2 (C5′–C6′); 34.7 (C6′–C13′)[a]	43.2[a]	43.2 (C5′–C6′); 34.6 (C6′–C13′)[a]		53.0	54.1
7′	37.0	146.5	34.7	35.1	35.1			$^1J_{cc}$ = 42.6; $^2J_{cc}$ = 1.8
8′	29.8	41.0	34.7	35.1				$^1J_{cc}$ = 42.6, 40.0
9′	22.8	32.3	34.9	34.8	34.9			$^1J_{cc}$ = 34.5; $^2J_{cc}$ = 1.8
10′	14.1	25.8	34.9	34.8	34.9		34.5, 34.0	
11′	14.0	26.0	45.1 (to C2′)[a]		45.5 (to C2′)[a]		34.0, 1.8	
12′	16.5	25.8	43.2 (to C4′)[a]		42.5 (to C4′)[a]			
13′	20.7	32.3	34.7 (to C6′)[a]		34.6 (to C6′)[a]			
1″	197.5	197.9	45.1	45.6	45.6[a]	46.0	45.6	45.6
2″	115.2	115.1						
3″	174.5	174.5	39.7	38.7	38.7[a]	39.5	40.1	40.1
4″	32.2	32.5	39.7	38.7	38.7[a]	39.5	40.1	40.1
5″	25.7	25.8	45.1	45.6	45.6[a]	46.0	45.6	45.6

[a] Statistical coupling.

of label from propionate via this route would be expected to give equal enrichments of about 50% of those observed in the propionate units, due to the symmetrical nature of succinate (if free succinic acid is formed in the biosynthetic pools). The differential labeling at C6 (6.9%) and C7 (5.4%) suggests, however, that the mC_7N unit is derived directly from succinyl-CoA. To further rule out the involvement of the one-carbon pool in the formation of the methyl branches and to assess possible participation of this pathway in the formation of the two nitrogen-containing ring systems, experiments with L-[*methyl*-^{13}C]methionine were conducted with both **1** and **2** (data not shown). No incorporation of label from methionine was observed into either antibiotic. There was, however, a drastic inhibitory effect on the biosynthesis of both compounds.

Feeding Experiments with Succinate. Experiments with [1-(4)-^{14}C]succinate produced specific incorporations into **1** of 2.2% and into **2** of 0.61% (Table II). The radiolabeled compounds were not degraded to locate the label. Instead, further experiments with [1,4-$^{13}C_2$]succinate revealed, as expected, enrichments of C6, C7, C1″, and C3″ in both **1** and **2** (Figure 3, Table IV). In accord with our supposition about the involvement of methylmalonyl-CoA mutase/racemase, enrichments in **1** were detected at C1′, C3′, and C5′. An experiment was carried out to investigate the intact incorporation of [1,2-$^{13}C_2$]succinate into **1**. Intact incorporation of the precursor was evidenced by spin–spin coupling between C5/C6, C4/C7, C1″/C5″, and C3″/C4″ (Figure 3). Due to an extremely efficient incorporation of label, statistical coupling was observed between C4 and C5 and between C4″ and C5″. Smaller degrees of coupling were also seen in the propionate-derived moieties of the upper side chain of **1** and at both C9′ and C10′.[44]

(44) The enrichment in the malonyl-CoA-derived carbons of the triene chain and C-7′ and C-8′ was not statistically significant, suggesting substantial dilution of ^{13}C at the malonyl-CoA level.

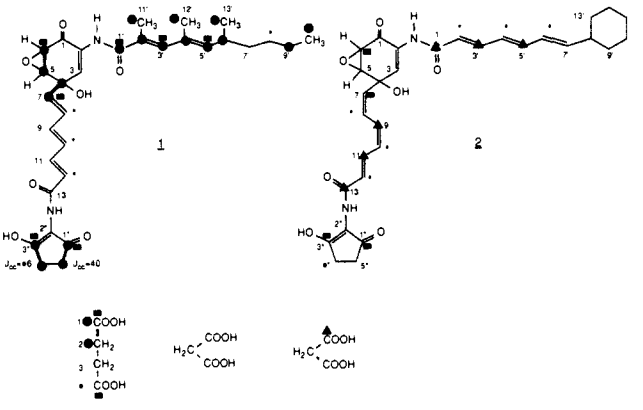

Figure 3. Distribution of label in **1** and **2** from [^{13}C]succinate and [1(3)-$^{13}C_1$]- and [2-^{13}C]malonate.

Table IV. Carbon-13 Distribution in the mC_7N Units of **1** and **2** Labeled from Succinate Precursors

carbon no.	δ_c, ppm[b] 1	δ_c, ppm[b] 2	relative ^{13}C abundances[a] [1,4-$^{13}C_2$]-succinate 1	2	$^1J_{cc}$, Hz, [1,2-$^{13}C_2$]-succinate 1	2
1	189.0	189.0	1.2	1.0	nc[c]	nc
2	128.0	128.1	1.1	1.0	nc	nc
3	126.6	126.5	1.4	1.0	nc	nc
4	71.2	70.7	1.3	1.0	49.0	47.5
5	57.4	57.2	1.3	1.0	25.0	22.0
6	52.8	52.5	7.4	2.3	25.0	22.0
7	136.6	136.9	7.7	2.3	49.0	47.5

[a] Relative to the abundance of C2″ = 1.0. [b] Referenced to $CDCl_3$. [c] Not coupled.

Notably, addition of succinate to the medium stimulated production of **1** by Tü 64 approximately 2-fold. A corresponding effect was not observed in the asukamycin-producing strain.

Feeding Experiments with Malonate. Experiments with [2-^{13}C]malonic acid were conducted on both **1** and **2**. These experiments discriminated perfectly the malonate-derived units in the side chains of both antibiotics, labeling C8′, C8, C10, and C12 in **1** and C2′, C4′, C6′, C8, C10, and C12 in **2** (Figure 3, Table I). An additional experiment with [1(3)-^{13}C$_1$]malonate provided the expected label in C9, C11, C13, C1′, C3′, and C5′ of **2**. To probe the feasibility of feeding experiments with chirally labeled malonate[45] with these organisms, experiments were performed with [2-^{13}C,2,2-^{2}H$_2$]malonate. Incorporation of ^{13}C was identical with that found in the earlier experiment, but complete washout of deuterium occurred. In the manumycin-producing strain, where better incorporation rates were typically observed, an additional feeding was done with higher levels (19.23 mmol/L) of [2,2-^{2}H$_2$]malonate. As previously observed, loss of deuterium label from malonate was complete. Therefore, further experiments probing the stereochemistry of incorporation of hydrogen labels from C2 of malonate seem to be impossible with both strains.

Feeding Experiments with Serine. Serine is an obvious candidate to be the precursor of the C1/C2/C3 segment of the mC$_7$N unit of **1** and **2**. This amino acid possesses a nitrogen atom at C2 and carbon centers at C1 and C3 that are functionalized for potential carbon–carbon bond formation. An experiment with L-[U-^{14}C]serine (data not shown) provided an appreciable specific incorporation (2.5%) into **1**. No degradation of the sample was done to locate the label. As with succinate, this experiment (performed with 6.7 mmol/L unlabeled carrier) also gave a 2-fold enhancement of production of **1**. An experiment with L-[3-^{13}C]serine produced nearly the same labeling pattern as [2-^{13}C]acetate (Table I), demonstrating that the labeled precursor had been extensively metabolized. To obtain better sensitivity, a feeding experiment was performed with L-[2,3-^{13}C$_2$]serine. The labeling pattern was identical with that observed with [1,2-^{13}C$_2$]acetate with the exception of a much higher enrichment at C2″. No incorporation of label was detected in C1–C3 of the mC$_7$N unit of **1**. These labeling patterns are easily rationalized. On the one hand, conversion of serine to glycine by serine hydroxymethyltransferase results in appearance of label from C2 of serine into C2 of glycine. On the other hand, through the action of serine dehydratase, serine can form pyruvate, channeling label from C2 and C3 of serine into C1 and C2 of acetate.

Feeding Experiments with [U-^{13}C$_3$]Glycerol. Since none of the previous experiments had provided labeling of the C1/C2/C3 segment of the mC$_7$N units in **1** and **2**, we turned to [U-^{13}C$_3$]-glycerol as a more general precursor. Glycerol has been, in our hands, a very useful precursor to map out biosynthetic pathways since it can be synthesized inexpensively, typically provides more efficient incorporation rates than uniformly labeled glucose, and produces more readily analyzable coupling patterns in NMR spectral analysis. As expected from the known metabolism of glycerol through glycolysis and the TCA cycle, a general acetate labeling pattern was observed in both **1** and **2**. Careful examination of the proton-decoupled ^{13}C NMR spectrum, however, also revealed definite incorporation into the C1/C2/C3 segment of the mC$_7$N units of both antibiotics. Intact incorporation of glycerol was evident from the presence of satellites, indicating doubly coupled spin systems in both **1** and **2** (Figure 4). The spin systems were of the AMX type, demonstrating both one- and two-bond coupling constants (Table III). Intact incorporation of glycerol was proven unequivocally by ^{13}C homodecoupling experiments and by identification of the connectivities in the respective 2D INADEQUATE[46] experiments (see supplementary material). Moreover, calculation of the chemical shift positions of the carbon lines for C1, C2, and C3 on the basis of AMX systems with the

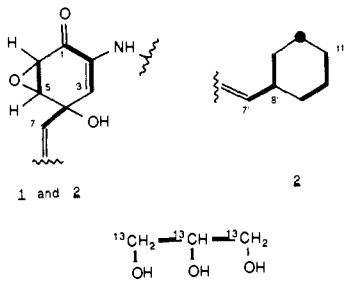

Figure 4. ^{13}C-Labeling patterns in the mC$_7$N units of **1** and **2** and the cyclohexane unit of **2** from [U-^{13}C$_3$]glycerol.

measured chemical shifts allowed exact reconstructions of the spectra. These data indicate that the C1–C3 segment of the mC$_7$N units of **1** and **2** arises in some way from the triose pool, but not via serine.

The notion that the formation of the cyclohexane ring and C7′ of **2** involves the shikimic acid pathway was confirmed by the observation of the expected shikimate-type labeling pattern from [U-^{13}C$_3$]glycerol (Figure 4). Observation of the broad-band proton-decoupled ^{13}C NMR spectrum clearly showed the presence of two doubly coupled spin systems (C7′/C8′/C9′, C11′/C12′/C13′) and a single enriched, noncoupled signal for C10′. This coupling pattern reveals that all seven carbon atoms of this moiety arise directly from shikimate, as opposed to a possible route via aromatic amino acids in which only the six ring carbons arise from shikimate and C7′ comes from another molecule of glycerol via C3 of phosphoenolpyruvate. It should be noted, however, that unlike the case of ansatrienin,[47] ^{13}C-labeled shikimate gave very poor incorporation, probably because of poor uptake into the cells.[48]

In an attempt to determine the orientation of incorporation of glycerol, a chirally labeled version, [1,1-^{2}H$_2$]glycerol, was fed to Tü 64. Analysis of the proton and deuterium NMR spectra of **1** from this experiment revealed no deuterium incorporation at C3 of the mC$_7$N unit. This result is inconclusive because two explanations are possible. Either carbon 1 of glycerol, bearing the deuterium, has become C1 of the mC$_7$N units, which does not carry any hydrogen, or the deuterium may have been washed out during earlier metabolic reactions.

Origin of the Nitrogen Atoms and Biosynthesis of the C$_5$N Unit. Initial experiments with [2-^{14}C]glycine were conducted with the asukamycin-producing strain.[28] As expected for biosynthesis of the C$_5$N unit from 5-aminolevulinic acid (5-ALA), and in agreement with the result of the serine feeding experiments, a very high specific incorporation (21.3%) of label from this precursor was found in **2**. The antibiotic was not degraded to locate the label, but consistent with expectations [1-^{14}C]glycine was incorporated 9 times less efficiently (data not shown). In order to determine whether both C2 and the nitrogen of glycine were incorporated into **1** and **2** as an intact unit, experiments were performed with [2-^{13}C,^{15}N]glycine.[31] Analysis of **1** by ^{13}C NMR spectroscopy demonstrated a general incorporation of ^{13}C label into all carbon atoms of manumycin except the C1/C2/C3 moiety of the mC$_7$N unit. This situation perhaps results from conversion of glycine to acetate by glycine reductase, scrambling carbon label into both carbon atoms of acetate, and transferral of ^{15}N label either to the nitrogen pool as ammonium ion or directly from glycine to an amino acid such as glutamate that could participate in transamination reactions with a mC$_7$N unit precursor. Some of the free ^{15}N efficiently labeled the mC$_7$N unit nitrogen (Figure 5), as evidenced by a 14.6-Hz statistical coupling observable in 25% of the signal for C2 in the ^{13}C NMR spectrum. Statistical coupling of ^{13}C to ^{15}N was also observable at C1′ and C13. In the C$_5$N unit, a strong 17.6-Hz coupling of ^{13}C to ^{15}N was observed in **1**, indicating that C2″ and the adjacent nitrogen atom are derived from the same molecule of glycine. An identical result

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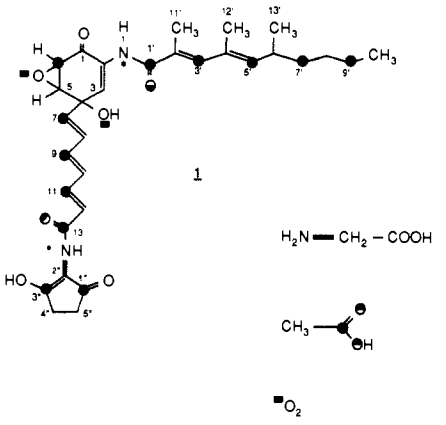

Figure 5. Labeling patterns in **1** from [^{13}C,^{15}N]glycine, [2-^{13}C,$^{18}O_2$]-acetate, and $^{18}O_2$.

was found in the C_5N unit of **2** (Figure 6). In the latter case, however, general labeling of the carbon pool from glycine was not observed. The results were further confirmed by analysis of the ^{15}N NMR spectra of both antibiotics, reflecting the same percentages of heteronuclear coupling that were observed in the ^{13}C NMR spectra. These experiments also provided unequivocal assignments for the ^{15}N chemical shifts of **1** and **2** (for C2″–N, **1**, δ_N = 125.4, **2**, δ_N = 125.1; for C2–N, **1**, δ_N = 115.3, **2**, δ_N = 114.6).

Intact incorporation of the ^{13}C and ^{15}N label from [2-^{13}C,^{15}N]glycine into the C_5N unit is consistent with biosynthesis of this moiety from 5-aminolevulinate, which is formed from one molecule of succinyl-CoA and one of glycine by 5-aminolevulinate synthase. During this pyridoxal phosphate mediated reaction, carbon 1 of glycine is lost by decarboxylation and C2 is incorporated into the product. Four separate feeding experiments were conducted with [5-^{14}C]-5-aminolevulinic acid hydrochloride with the asukamycin-producing organism. The radioactive precursor was diluted with unlabeled carrier, providing levels of precursors from 0.2 to 1 mmol/L. The resulting **2** showed a maximum specific incorporation of label of 2.18% at the highest concentration of carrier (Table II). Extensive decomposition of the precursor during the fermentation was noted, resulting in formation of copious amounts of brown pigments.

Origin of the Oxygen Atoms in the mC$_7$N Unit. Since the preceding data had indicated that the manumycin group antibiotics contain a biosynthetically novel type of mC$_7$N unit, it was of interest to determine the origin of the oxygen atoms in this multifunctional moiety. Experiments were conducted with the manumycin-producing strain Tü 64 to obtain this information.[31] Incorporation of [1-^{13}C,$^{18}O_2$]acetate yielded **1**, whose ^{13}C NMR spectrum showed ^{13}C enrichments up to 17% per labeled carbon atom, displaying the same labeling pattern as described above (Figure 5). C13 and C1′ exhibited upfield ^{18}O-induced isotope shifts ($\Delta\delta$ = 24 and 29 ppb, respectively), indicating the intact incorporation of ^{13}C–^{18}O assemblies from the carboxyl group of acetate into these positions. This result was as expected for the polyketide-derived side chains. Presumably C1″ and C3″ were also enriched, but extreme signal broadening made observation of these lines impossible. Interestingly, there was no incorporation of ^{18}O from acetate into the mC$_7$N unit. To obtain further information on the biogenetic origin of the oxygens of this unit, a fermentation was conducted in a closed system under an atmosphere of 50% $^{18}O_2$ and 50% N_2. The field desorption mass spectrum of the resulting **1** showed, in addition to a molecular ion at *m/e* 550 (14%), other peaks at *m/e* 552 (70%), 554 (100%), and 556 (14%), indicating the incorporation of up to two ^{18}O atoms into the antibiotic. The locations of these ^{18}O labels in **1** were established by natural abundance ^{13}C NMR; upfield isotope-shifted peaks were observed for C4 ($\Delta\delta$ = 19 ppb), C5 ($\Delta\delta$ = 29 ppb), and C6 ($\Delta\delta$ = 24 ppb), demonstrating the incorporation of ^{18}O from $^{18}O_2$ into the hydroxy group at C4 as well as at the oxirane oxygen atom.

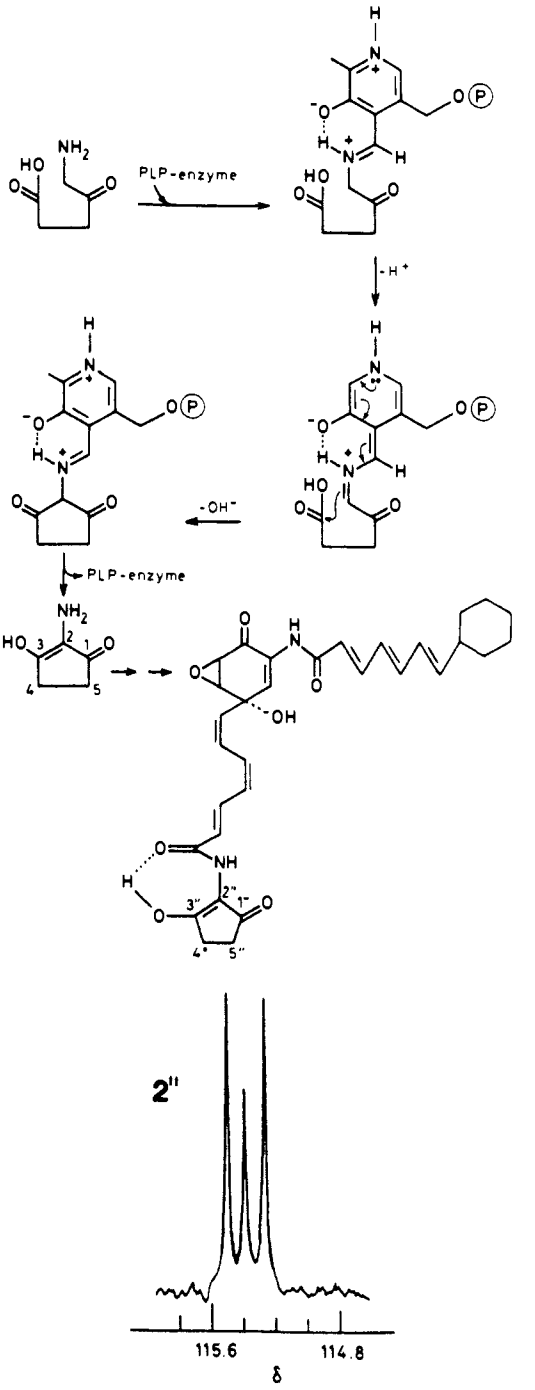

Figure 6. Model for assembly of C_5N units in **2**, with the ^{13}C NMR spectrum resulting from feeding of [^{13}C,^{15}N]glycine. J_{CN} = 17.6 Hz from HOOC–$^{13}CH_2$–$^{15}NH_2$.

Discussion

In combination, the results of our parallel experiments with manumycin and asukamycin have provided a nearly complete picture of the biosynthetic origin of both antibiotics. Data obtained from precursor-directed biosynthesis experiments with **1** provide additional insights into the substrate specificities of the enzymes involved and the sequence of the biosynthetic steps.[42,43] Our working hypothesis that separated the structural elements into biosynthetically different entities has now been confirmed. It appears that several distinct enzymatic processes are involved in the biosyntheses of **1** and **2**. The polyketide-derived upper side chains of **1** and **2** are initiated by different starter units, acetyl-CoA in the case of **1** and a shikimic acid derived precursor, probably cyclohexylcarbonyl-CoA, in **2**. From our work on ansatrienin biosynthesis, it is known that shikimate-derived cyclohexanecarboxylic acid is efficiently incorporated into the corresponding

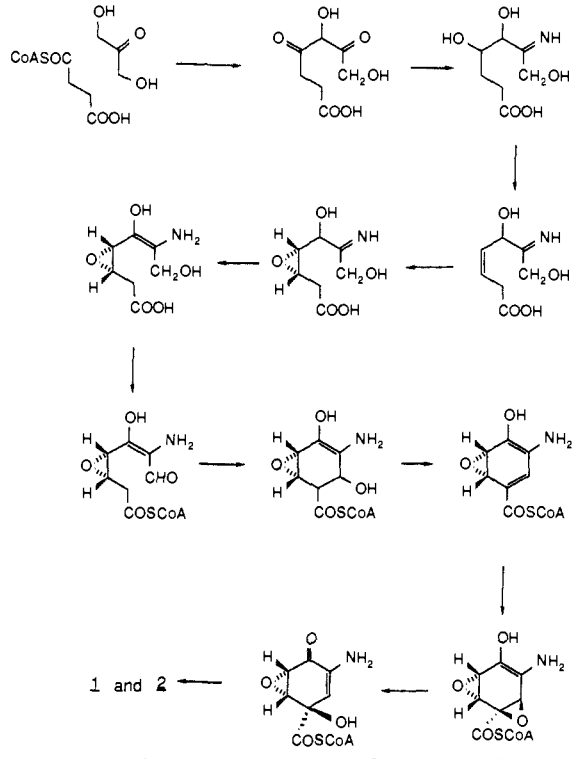

Figure 7. Proposed pathway for assembly of mC_7N units in the manumycin group antibiotics.

unit in *Streptomyces collinus*. Further elongation of the upper chains in **1** and **2** is another point of divergence. In the biosynthesis of **1**, the acetate starter unit is elongated by condensation with one molecule of malonyl-CoA and three molecules of methylmalonyl-CoA. The elongation of the corresponding chain in **2** is simpler, involving condensation of the cyclohexanecarbonyl starter unit with only three molecules of malonyl-CoA. Experiments with unnatural side-chain precursors in Tü 64 provided no new compounds, suggesting a high degree of specificity for the starter unit and for the all-trans product. For example, attempts to replace the starter unit of **1** by a variety of compounds such as propionate, lysine, isoleucine, or six-membered ring systems added in high concentrations failed.

The starter unit for the lower side chain of the manumycin group antibiotics is the mC_7N unit. From the feeding experiments, we now know that the mC_7N moieties in **1** and **2** represent a departure from the biosynthesis of most other types of mC_7N units encountered in nature. This unusual biosynthetic pathway derives a four-carbon unit closely related to succinate from the TCA cycle and a three-carbon unit from the triose pool. In the biosynthetic assembly of the multifunctional ring system, carbon–carbon bond formation occurs between carbons 1 and 6 and carbons 3 and 4 of the antibiotic. The glycerol-derived three-carbon portion is not formed via serine. This fact also rules out pyruvate as the precursor of the three-carbon moiety, since serine efficiently labels pyruvate. Both the oxirane and the hydroxyl oxygen atoms of the mC_7N unit are derived from atmospheric oxygen. The introduction of the oxygen atoms apparently occurs via oxygenases, and in the case of manumycin, the opposite facial positions of these two oxygen atoms preclude the participation of a dioxygenase and dictate that two separate monooxygenase reactions must occur. Due to the fact that the stereochemistry of the oxirane ring in asukamycin is not defined, this statement cannot be extended to **2** and the other manumycin group antibiotics with certainty.

Our proposed scheme for the biosynthesis of the mC_7N units, incorporating all of the constraints noted above, is depicted in Figure 7. The most plausible precursors appear to be succinyl-CoA and dihydroxyacetone (or its phosphate). Carbon–carbon bond formation may occur first between C1 and C6, but initial formation of a C3–C4 bond is also a possibility. At present, we can say little about the sequence of introduction of the oxygen and nitrogen atoms or about the timing of attachment of the upper side chain or assembly of the lower one. The proposed scheme has the advantage that it allows for all possible oxygen stereochemistries encountered in the manumycin group and involves no aromatic intermediates. The lack of incorporation of aromatic precursors, particularly 3-amino-5-hydroxybenzoic acid, and the data from the precursor-directed biosynthesis experiments[42,43] argue against the participation of aromatic intermediates in the pathway. However, because of the fact that the biosynthetic enzymes can accommodate functionalized aromatic compounds in lieu of the natural substrates and produce manumycin analogues, we can infer that a nearly planar precursor is required for initiation of the lower polyketide chain. The precursor-directed biosynthesis studies provided insights that could not be obtained from stable isotope feeding experiments. There is a requirement for a carbonyl function at C7, which presumably must be activated as the CoA ester prior to elongation by condensation with malonyl-CoA. The biosynthetic enzymes dictate that the carbonyl function must be in a meta disposition relative to the amino group, since ortho- and para-substituted aminobenzoic acids were not elaborated by the organism into manumycin analogues containing the entire carbon skeleton.

Our working hypothesis had envisioned the biosynthesis of the structurally unique C_5N moiety through a novel intramolecular cyclization of 5-aminolevulinic acid. The experimental results clearly support the formation of the C_5N unit from 5-aminolevulinic acid. The patterns of incorporation of glycine and succinate into **1** and **2** are entirely consistent with their established role as precursors of 5-aminolevulinic acid. Feeding experiments with [5-^{14}C]-5-aminolevulinic acid hydrochloride provided a maximum specific incorporation of 2.18% into **2**. The intermediate 5-aminolevulinic acid is a difficult precursor to use in biosynthetic studies, since it decomposes during the fermentation, thereby becoming unavailable for biosynthetic utilization. This difficulty is reflected in the low specific incorporations. Because the antibiotic was not degraded to locate the label, it was impossible to state unequivocally that 5-aminolevulinate was incorporated only into the C_5N unit. The question was, however, settled in subsequent work with another antibiotic, reductiomycin.[49] Feeding experiments with acetate, glycine, succinate, and 5-aminolevulinate showed that the C_5N unit of reductiomycin has the same origin as that of **1** and **2**, but incorporation rates into reductiomycin were typically higher and more consistent than those into **1** and **2**. A feeding experiment with [4,5-$^{13}C_2$]-5-aminolevulinic acid gave reductiomycin in which the C_5N unit was enriched in carbon atoms 1, 2, and 3 in a ratio 1:2:1 and showed spin–spin coupling between carbons 1 and 2 in half the molecules and between carbons 2 and 3 in the other half, as expected from the tautomeric behavior of the ring system. These data proved unequivocally that the working hypothesis for the biosynthesis of the C_5N unit is indeed correct.

It is proposed that intramolecular ring closure of 5-aminolevulinate is facilitated by formation of an α-carbanion equivalent, possibly through the intermediacy of a Schiff base with a pyridoxal phosphate cofactor. As shown in Figure 6, accompanied by the loss of a molecule of water, the normal pyridoxal phosphate electron cascade would bring about ring closure to give the 2-amino-3-hydroxycyclopent-2-enone unit.

As demonstrated by our work, the structural elements of the manumycin group antibiotics derive from several separate biosynthetic pathways. It is clear that the upper side chain is biosynthesized as a separate entity, as is the mC_7N unit, which serves as a starter unit for the elongation process that generates the lower side chain. Presumably, formation of the lower chain is contingent upon the presence of the mC_7N unit at the enzyme before any elongation can occur. The time course of the biosynthesis probably depends on the construction, *in* parallel, of the upper side-chain unit and the mC_7N unit precursor. From the results of our experiments with precursor-directed biosynthesis, we are able to

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make further predictions about biosynthetic processes leading to manumycin group antibiotics. There appear to be two separate enzymes, or enzyme systems, that are essential for the incorporation of the natural mC_7N unit as a central starter unit. One of them, an amide synthase, is responsible for the connection of the upper side chain to the C2 amino group. The other activates the mC_7N unit carboxyl group (CoA transferase) for the subsequent chain-extension process via the polyketide pathway. The amide synthase and the CoA transferase act in a noncoupled fashion. It seems very likely that the amide synthase and/or the polyketide synthase have tight substrate specificities, since only a few artificial precursors were acceptable to them. The fact that feeding experiments with 3-hydroxybenzoic acid produced only a lower chain elaborated analogue demonstrates that an amino group is necessary for attachment of the upper chain carboxylic acid. The enzyme involved appears to be able to synthesize only amides but not esters. The polyketide synthase involved in lower chain formation is highly specific in its action, and termination of elongation appears to be under strict control of the synthase or, less likely, of a second amide synthase that is responsible for connecting the activated trienecarboxylic acid to the C_5N unit. While the precursor-directed biosynthesis studies were conducted only with the manumycin producer, due to the correspondence of all the isotope feeding data for **1** and **2**, it is likely that these predictions can be extended to other members of the manumycin group of antibiotics.

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Supplementary Material Available: Pulse-decoupled ^{13}C NMR spectra and 2D INADEQUATE spectra of **1** and **2** labeled from [U-$^{13}C_3$]glycerol (5 pages). Ordering information is given on any current masthead page.

Stereochemistry of Reduction of Methylenetetrahydrofolate to Methyltetrahydrofolate Catalyzed by Pig Liver Methylenetetrahydrofolate Reductase

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Abstract: Methyltetrahydrofolate; chirally labeled with deuterium and tritium in the methyl group, was synthesized from L-(3*R*)- or L-(3*S*)-[3-^{3}H]serine, tetrahydrofolate, and NADPH by coupling the reactions of pig liver methylenetetrahydrofolate reductase and serine hydroxymethyltransferase in deuterated aqueous buffer. The stereochemistry at the N^5 methyl group of the methyltetrahydrofolate products was determined by chemical degradation of methyltetrahydrofolate to methylamine and conversion to acetate. L-(3*R*)-[3-^{3}H]Serine is converted to (*S*)-acetate, which is derived from (*methyl-R*)-methyltetrahydrofolate, and L-(3*S*)-[3-^{3}H]serine is converted to (*R*)-acetate. The reaction proceeds with ~75% stereospecificity. The results indicate that reduction of the methylene group of methylenetetrahydrofolate takes place with addition of hydrogen to the more sterically accessible face of the pteridine; this is the same face from which methylenetetrahydrofolate dehydrogenase abstracts a hydride equivalent and it is the face attacked by enzyme-bound dUMP in the thymidylate synthase reaction (Slieker, L. J.; Benkovic, S. J. *J. Am. Chem. Soc.* **1984**, *106*, 1833-1838). To determine the degree to which racemization of methylenetetrahydrofolate was occurring in solution during the coupled synthesis of methyltetrahydrofolate, the stereochemical course of the serine hydroxymethyltransferase reaction was investigated under the same conditions in a coupled reaction with methylenetetrahydrofolate dehydrogenase, as initially described by Tatum and co-workers (Tatum, C. M.; Benkovic, P. A.; Benkovic, S. J.; Potts, R.; Schleicher, E.; Floss, H. J. *Biochemistry* **1977**, *16*, 218-220). In contrast to the results reported earlier, we observed greater than 95% stereospecificity in this coupled reaction, suggesting that little or no racemization associated with the production of methylenetetrahydrofolate from serine occurred under the conditions employed for synthesis of methyltetrahydrofolate.

Methylenetetrahydrofolate reductase is a flavoprotein that catalyzes the NADPH-dependent reduction of CH_2-H_4folate to CH_3-H_4folate according to eq 1.

$$\text{NADPH} + \text{CH}_2\text{-H}_4\text{folate} \rightarrow \text{NADP}^+ + \text{CH}_3\text{-H}_4\text{folate} \quad (1)$$

Previous studies have shown that the reaction proceeds via a ping-pong kinetic mechanism in which $NADP^+$ release from the enzyme precedes the binding of CH_2-H_4folate.[1,2] The enzyme catalyzes the transfer of the 4*S* hydrogen of NADPH to the flavin coenzyme, FAD.[3] Reduction of CH_2-H_4folate in deuterated aqueous buffer occurs with the incorporation of one deuteron from solvent into the methyl group of CH_3-H_4folate.[4,5] It is not known

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