Some Aspects of the Stereochemistry and Biosynthesis of Asukamycin

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Asukamycin (1), isolated by Ōmura and co-workers from *Streptomyces nodosus* ssp.*asukaensis*,[1,2] is a member of the manumycin family of antibiotics,[3-7] which also includes colabomycin A,[8,9] U-62162,[10] and U-56407[11] in addition to the parent compound, manumycin A (2).[3-7] The recently found alisamycin shows a striking structural similarity to 1 with one double bond missing in the cyclohexyl terminated side chain.[12] The structure of 1 was originally reported as 3 based on spectroscopic data. The absolute configuration at C-4 was deduced by the exciton chirality method, but the configurations at C-5 and C-6 remained undetermined.[2] Earlier studies on the origin of this molecule[13-15] have shown that 1 incorporates three biosynthetically unique moieties, a C_5N unit which arises by intramolecular cyclization of 5-aminolevulinic acid,[13,15] a shikimate-derived[15] cyclohexanecarboxylic acid[16] serving as starter unit of a short polyketide chain, and a mC_7N unit serving as the starter unit of a second polyketide chain. Unlike the mC_7N unit in ansamycin and mitomycin antibiotics,[17,18] the mC_7N unit in 1 and 2 is not derived from the shikimate pathway but is assembled from one C_3 unit derived from glycerol and a C_4 unit closely related to succinic acid.[15] In the biosynthesis of manumycin A (2) by *Streptomyces parvulus* (strain Tü 64) the natural mC_7N unit can be replaced by aminobenzoic acids resulting in aromatic manumycin analogs, when the artificial precursors are fed in unusually high amounts (55 mM) at the beginning of the stationary growth phase.[19,20]

In the present paper we relate the complete stereochemistry of asukamycin, including a minor revision of the original structure, as well as additional information on the mode of incorporation of glycerol into the mC_7N unit of 1. Furthermore, as the first metabolite of precursor-directed biosynthesis with *Streptomyces nodosus* ssp. *asukaensis*, asuka-mABA (4) is presented which is produced upon feeding of *m*-aminobenzoic acid.

Results and Discussion

Stereochemistry. The relative stereochemistry of the mC_7N unit in manumycins A–D was determined using ^1H NMR spectroscopy in pyridine-d_5.[21] Certain ASIS (aromatic solvent induced shift) effects on the olefinic 3-H in the mC_7N unit allowed a distinction between the *cis* and the *trans* configuration of the oxygen functionalities at C-4 and C-5. Asukamycin (1) showed a strong shift effect ($\Delta_H = \delta^{chloroform} - \delta^{pyridine} = -0.74$) and should therefore belong to the group of compounds carrying the 4-OH and the oxirane oxygen on the same side of the cyclohexenone plane (Table I). Combined with the CD spectroscopically deduced center of chirality at C-4[2] this establishes the 4*S*,5*R*,6*S* configuration for 1.

The aromatic solvent also simplified the spin systems of the olefinic protons and allowed an unambiguous determination of the $^3J_{H,H}$ coupling constants. For all signals in 1, which were assigned from a $^1H,^1H$ COSY spectrum, the value of $^3J_{H,H} > 14$ Hz for the larger coupling

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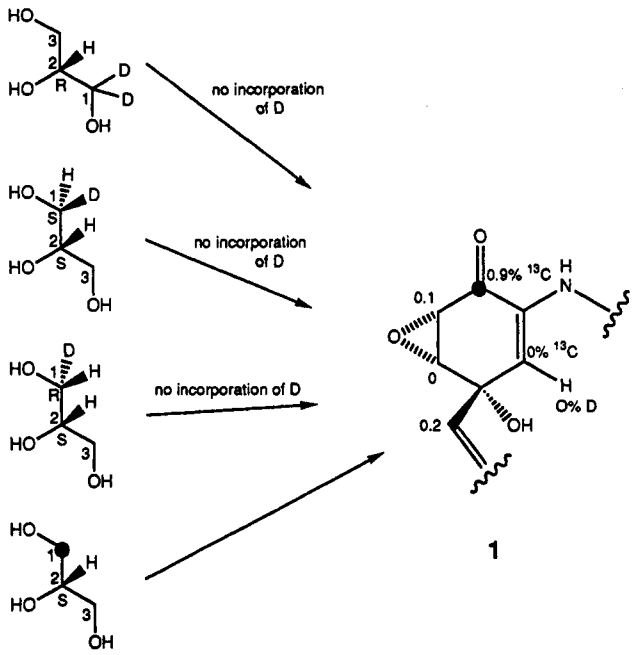

Figure 1. Incorporation of labeled glycerol samples into the mC_7N unit of 1.

Table I. ^1H NMR Chemical Shifts (δ, ppm; J, Hz) of Asukamycin (1) at 500 MHz

hydrogen	pyridine-d_5	chloroform-d_1
3-H	8.16 d (2.7)	7.41 d (2.5)
5-H	4.04 dd (3.8/2.7)	3.71 dd (3.8/2.4)
6-H	3.90 d (3.9)	3.66 d (3.8)
7-H	6.19 d (15.0)	5.85–5.93 m
8-H	7.02 dd (15.0/11.0)	6.59 m
9-H	6.73 dd (14.5/11.0)	6.59 m
10-H	6.51 dd (14.5/11.2)	6.42 m
11-H	7.65 dd (14.5/11.2)	7.33 dd (14.8/11.2)
12-H	6.72 d (14.5)	6.05 d (14.8)
2′-H	6.56 d (14.5)	5.85–5.93 m
3′-H	7.65 dd (14.5/11.2)	7.29 dd (15/11.5)
4′-H	6.33 dd (14.5/11.0)	6.23 dd (15/1.5)
5′-H	6.46 dd (14.5/11.0)	6.56 dd (15/11)
6′-H	6.09 ddd (15.0/11.0/0.8)	6.11 dd (15/11)
7′-H	5.79 dd (15.0/6.5)	5.85–5.93 m
8′-H	1.91 (m)	2.11 m
cyclohexyl-H	1.60 m (4H), 1.50 m (1H), 1.12 m (2H), 1.07 m (3H)	1.1–1.3 m (7H), 1.6–1.7 m (3H)
4″-H_2	2.40 s	2.58 m
5″-H_2	2.40 s	2.58 m
4-OH		3.04
3″-OH		13.58
NH		7.58 (2H)

constant indicated the all-*E* configuration (Table I).[22] Higher order couplings in chloroform-d_1 that were demonstrated by a spin simulation may have earlier[2] led to a misinterpretation of the ^1H NMR signals.[23,24] Possibly, this minor revision may also apply to the structure of antibiotic U-56407 that was elucidated in analogy to 1.[11]

Biosynthesis. [U-$^{13}C_3$]Glycerol is incorporated as an intact unit into carbon atoms 1–3 of the central mC_7N unit of 1 and 2.[15] An attempt was made in the earlier work to establish the orientation of the glycerol moiety in the mC_7N unit, i.e., whether the *pro-R* or the *pro-S* hydroxymethyl group of glycerol becomes C-1 of the mC_7N unit, by feeding (*R*)-[1,1-$^2H_2$]glycerol to a fermentation producing 2. No incorporation of deuterium at C-3 of 2 was observed in this experiment.[15] Reasoning that this might be due to incorporation of the *pro-R* hydroxymethyl group of glycerol into the C-1 rather than the C-3 of 1 we have now prepared glycerol deuterated in the *pro-S* hydroxymethyl group and examined its utilization in 1 biosynthesis. Anticipating that one hydrogen from this position would be incorporated at C-3 of 1, we wanted also to determine whether this came from the *pro-R* or *pro-S* hydrogen at this hydroxymethyl group. We thus synthesized (1*R*,2*S*)- and (1*S*,2*S*)-[1-$^2H_1$]glycerol by reduction of 2,3-isopropylidene-L-[1-$^2H_1$]glyceraldehyde, itself prepared by $LiAl^2H_4$ reduction of methyl 2,3-isopropylidene-L-glycerate (Fluka) and subsequent Swern oxidation, with (*S*)- and (*R*)-Alpine-Borane,[25] respectively. Deuterium NMR analysis of the two samples of 1 obtained upon feeding these precursors to *S. nodosus* ssp. *asukaensis* ATCC 29757 revealed no detectible signal at δ 7.25 ppm, the chemical shift of H-3 of 1 (< 0.5% enrichment). There was also very little deuterium present in other positions of the molecule, e.g., the acetate- and succinate-derived positions. This may reflect the fact that deuterium from this carbon of glycerol, which is oxidized in the conversion to phosphoglyceraldehyde and phosphoglyceric acid, is removed in most metabolic transformations of glycerol. The present experiments combined with the previous one thus indicate that none of the hydroxymethyl hydrogens of glycerol are retained during the incorporation into the mC_7N units of 1 and that the hydrogen at C-3 of 1 originates from a source other than the precursor, glycerol.

To establish the orientation with which glycerol is incorporated into the mC_7N unit of 1 we then synthesized (*S*)-[1-^{13}C]glycerol from [1-^{13}C]mannose via lead tetraacetate cleavage of isopropylidenemannitol. Feeding of this material (50 atom % ^{13}C) gave a sample of 1 whose inverse-gated ^{13}C NMR spectrum revealed incorporation of ^{13}C into many positions. Significantly, C-1 was substantially enriched (0.9%), whereas C-3 showed only natural abundance levels of ^{13}C. Thus, the *pro-S* hydroxymethyl group of glycerol gives rise to the carbonyl group in the mC_7N unit of 1, whereas C-3, but not its attached hydrogen, arises from the hydoxymethyl group which is phosphorylated by glycerol kinase in the metabolism of glycerol.

We have previously speculated on a hypothetical reaction sequence for the assembly of the mC_7N unit of 1 and 2 from its two building blocks.[15] The present results provide additional boundary conditions which will aid in refining the mechanism of this intruiging transformation once additional experimental data become available.

Precursor-Directed Biosynthesis. The feeding of *m*-aminobenzoic acid (mABA) to *Streptomyces nodosus* ssp. *asukaensis* according to the high concentration protocol[19] resulted in formation of a new metabolite, named asuka-mABA (4, 5 mg/L). The HR EI mass spectrum of 4 revealed a formula of $C_{18}H_{18}N_2O_7$ for the molecular-ion peak (*m/z* 310). The ^1H and ^{13}C NMR spectra in dimethyl sulfoxide-d_6 indicated the presence of the C_5N unit and one triene chain as in 1, as well as an aromatic ring. The cyclohexyl residue and the connected triene chain were missing. The structure assigned to asuka-mABA (4) turned out to be identical with the chemical degradation

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Table II. ^{13}C-Enrichments in 1 Biosynthesized from (*S*)-[1-^{13}C]Glycerol

carbon no.	δ_c, ppm	^{13}C enrichment[a] (%)
1	189.0	0.9
2	128.1	0
3	126.5	0
4	70.7	0
5	57.2	0
6	52.5	0.1
7	136.9	0.2
8	131.2	0
9	139.4	0
10	131.6	0.1
11	143.2	0.1
12	121.5	0.1
13	165.6	0
1′	165.3	0
2′	121.3	0
3′	143.5	0.2
4′	127.4	1.0
5′	142.2	nd
6′	127.5	0.3
7′	146.5	1.1
8′	41.0	0.2
9′	32.3	0.2
10′	25.8	0.4
11′	26.0	0.7
12′	25.8	0.4
13′	32.3	0.2
1″	197.9	nd
2″	115.1	0.1
3″	174.5	nd
4″	32.5	0
5″	25.8	0.5

[a] Excess ^{13}C over natural abundance, standard: C-2 = natural abundance.

product of 64-mABA.[19] In contrast to 64-mABA, the corresponding precursor-directed product of the manumycin A producing *Streptomyces parvulus*, the amidically linked side chain is missing. We assume that *m*-aminobenzoic acid interferes with the biosynthesis of the shikimate-derived cyclohexanecarboxylic acid that functions as starter unit of the polyketide chain.[15] However, the production of 4 demonstrates that the high concentration method of precursor-directed biosynthesis can be successfully applied to other *Streptomyces* sp. producing manumycin group antibiotics.

Experimental Section

Fermentations. *S. nodosus* ssp. *asukaensis* ATCC 29757 was maintained and cultivated, and feeding experiments were conducted in a two-stage fermentation protocol as previously described.[15] Labeled glycerol samples (350 mg) were fed to two cultures in two equal portions at 24 and 36 h after inoculation, and the fermentations were harvested 36 h after the second addition. **4** was produced upon precursor-directed biosynthesis by feeding 7.5 g of *m*-aminobenzoic acid to a 45-h-old production culture in a 1-L fermentor.[19] The culture was harvested after 72 h. Detection of metabolites was achieved by TLC on silica gel and on RP8 silica gel. The compounds were visualized by UV absorption at 254 nm.

Asukamycin (1): $C_{31}H_{34}N_2O_7$ (546.62); R_f 0.39 (silica gel, $CHCl_3$/MeOH 9:1)), 0.39 (RP8 silica gel, MeOH/H_2O 85:15)); $[\alpha]_D^2$ +121.5 (c = 0.11, $CHCl_3$); UV (MeCN) λ_{max} (ϵ) 313 (49 400) nm; CD (MeCN) I_{max} ($[\theta]^{20}$) 345 (+72 840), 307 (−97 530) nm; ^1H NMR (500 MHz, $CDCl_3$) see Table I; ^1H NMR (500 MHz, pyridine-d_5) see Table I; ^{13}C NMR (75.5 MHz, $CDCl_3$) see Table II.

(*S*)-[1-^{13}C]Glycerol. D-[1-^{13}C]Mannose (5.50 g, 30.4 mmol, 99 atom % ^{13}C, Los Alamos Stable Isotope Resource) was reduced with $NaBH_4$ to give 5.21 g (28.6 mmol) of crude [1-^{13}C]mannitol,[26] mp 154–156 °C.

The mannitol was treated with 2,2-dimethoxypropane and a trace of *p*-toluenesulfonic acid in DMSO as described by Kierstead *et al.*[27] to give 5.69 g (21.6 mmol) of 1,2;5,6-diisopropylidene-D-[1-^{13}C]mannitol. The subsequent cleavage with lead tetraacetate and $NaBH_4$ reduction of the aldehyde followed the procedure of Baldwin *et al.*[28] and gave 3.27 g (24.8 mmol) of (*S*)-isopropylidene-[1-^{13}C]glycerol (40.8% yield based on mannose): ^1H NMR (300 MHz, $CDCl_3$) δ 1.36 (s, 3H), 1.42 (s, 3H), 3.50–3.60 (m, 1H), 3.68–3.77 (m, 1H), 3.77 (dd, 1H, J = 8.1, 6.5 Hz), 4.02 (dd, 1H, J = 8.1 Hz, 6.5 Hz), 4.18–4.29 (m, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 65.71 (enriched).

The labeled isopropylidene glycerol was hydrolyzed with 10% aqueous acetic acid as described by Baer and Fischer[29] to give (*S*)-[1-^{13}C]glycerol (50 atom % ^{13}C) in quantitative yield: ^{13}C NMR (75.5 MHz, DMSO-*d*6) δ 63.04 (enriched).

(1*R*,2*S*)- and (1*S*,2*S*)-[1-$^2H_1$]Glycerol. Methyl isopropylidene-L-glyceric acid (8.3 g, 51.9 mmol) was added dropwise to a stirred solution of $LiAl^2H_4$ (1.22 g, 29.1 mmol, 98 atom % ^2H) in 80 mL of ether at a rate to maintain gentle reflux. After an additional 1 h of reflux 1 mL of EtOAc was added slowly with ice cooling, followed by 4 mL of 50% aqueous EtOH. The granular precipitate was removed by filtration and washed with ether, and the combined ether solution was dried ($MgSO_4$) and evaporated. The crude residue of isopropylidene [1-$^2H_2$]glycerol (6.42 g) was used directly for the next reaction: GC–MS: m/z (rel intens) 119, $(M - CH_3)^+$ (100), 101 (48), 59 (96); ^1H NMR (300 MHz, DMSO-d_6) δ 1.37 (s, 3H), 1.43 (s, 3H), 3.78 (dd, 1H, J = 8.2, 6.5 Hz), 4.04 (dd, 1H, J = 8.2, 6.5 Hz), 4.23 (t, 1H, J = 6.5 Hz).

Oxalyl chloride (5.55 mL) was dissolved in 90 mL of CH_2Cl_2 and the solution cooled to −78 °C. DMSO (8.5 mL) was added from a syringe under an N_2 atmosphere, and the solution was stirred at −78 °C for 15 min. Crude isopropylidene [1-$^2H_2$]glycerol (4.5 g) in 25 mL of CH_2Cl_2 was then added over a period of 5 min with stirring at −78 °C and stirring was continued for another 20 min. Et_3N (36 mL) was added, and the mixture was stirred for another 20 min and then allowed to warm to room temperature. Water (90 mL) was added and the mixture stirred for 10 min. The organic layer was then separated and the aqueous phase extracted with 3 × 100 mL of ether. The combined organic extract was dried ($MgSO_4$) and evaporated to give 4.05 g of crude isopropylidene[1-$^2H_1$]glyceraldehyde, which was used directly for the next reaction: GC–MS m/z (rel intens) 116, $(M - CH_3)^+$ (61), 101 (100), 86 (34), 61 (42).

To 2.35 g (18.0 mmol) of the above aldehyde was added with ice-bath cooling 36 mL of 0.5 M (*S*)-Alpine-Borane (Aldrich) in THF under a nitrogen atmosphere. The resulting solution was stirred for 2 h at 0 °C and then warmed to room temperature and stirring continued overnight. Acetaldehyde (0.8 mL) was added, and after 15 min of stirring the solvent was evaporated and the residue pumped at 4 Torr and 50 °C for 1 h. It was then taken up in 35 mL of ether and cooled to 0 °C, and 1.24 mL of ethanolamine was added. After being stirred for 30 min at 0 °C the white precipitate formed was filtered off and washed twice with 50 mL of ether. The combined ether solution was washed with 30 mL of water, dried ($MgSO_4$), and evaporated. The residue was chromatographed on a silica gel column (hexane/EtOAc (2:1)) to give 600 mg of isopropylidene (2*R*,3*R*)-[3-$^2H_1$]glycerol of 48% de: GC–MS m/z (rel intens) 118 $(M - CH_3)^+$ (100), 101 (M − CHDOH)$^+$ (50), 73 (18), 72 (27), 61(36), 59 (57), 58 (67); ^1H NMR (300 MHz, DMSO-d_6) δ 1.35 (s, 3H), 1.42 (s, 3H), 3.53–3.58 (m, 0.26H), 3.66–3.70 (m, 0.74H), 3.77 (dd, 1H, J = 8.2, 6.5 Hz), 4.01 (dd, 1H, J = 8.2, 6.5 Hz), 4.20 (dt, 1H, J = 6.5, 4.2 Hz).

Similarly, reduction of 1.68 g of the aldehyde with (*R*)-Alpine-Borane gave 250 mg of isopropylidene (2*R*,3*S*)-[3-$^2H_1$]glycerol of 64% de: ^1H NMR (300 MHz, DMSO-d_6) δ 1.35 (s, 3H), 1.42 (s,

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3H), 3.53–3.58 (m, 0.82H), 3.67–3.70 (m, 0.18H), 3.77 (dd, 1H, J = 8.2, 6.5 Hz), 4.01 (dd, 1H, J = 8.2, 6.5 Hz), 4.21 (q, 1H, J = 6.5 Hz).

Both samples were hydrolyzed as described before to give (1*R*,2*S*)- and (1*S*,2*S*)-[1-$^2H_1$]glycerol, respectively.

***N*-(1-Hydroxy-5-oxocyclopent-1-enyl)-7-(3-aminophenyl)-hepta-2,4,6-trienamide (Asuka-mABA) (4).** 4 was isolated from the ethyl acetate extract of the mycelium by chromatography on silica gel (chloroform/methanol (9:1)), in which 3 mg of 1 eluted from the column before 4. Further purification was achieved on Sephadex LH-20 ($CHCl_3$). 4 is soluble in chloroform/methanol mixtures, DMSO, and pyridine: $C_{18}H_{18}N_2O_3$ (310.13); mp 172 °C; R_f 0.35 (silica gel, $CHCl_3$/MeOH (9:1)), 0.64 (RP8 (silica gel, MeOH/H_2O (85:15)); IR (KBr) ν 3440 (br), 3380, 3260, 1680, 1610, 1550, 1540, 1385, 1150, 1005 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 342 (44 500), 259 (24 600) nm; (MeOH, HCl) λ_{max} (ϵ) 349 (50 000), 254 (11 600) nm; (MeOH, NaOH) λ_{max} (ϵ) 340 (45 300), 259 (26 200) nm; EI MS (70 eV, abundance) *m/e* 310 (M^+, 16, calcd for $C_{18}H_{18}N_2O_3$ and found 310.1317), 198 ($M^+ - C_5H_6NO_2$, 18), 170 ($M^+ - C_6H_6NO_3$, 40), 132 ($M^+ - C_9H_8NO_3$, 48); ^{1}H-NMR (200 MHz, DMSO-d_6) δ 2.09 (s, 2H), 2.48 (s, partly obsc), 4–5.5 (br, 2H), 6.5.–6.95 (m), 7.02 (t, J = 8.2 Hz, 1H), 7.28 (dd, J = 15.0, 11.2 Hz, 1H), 9.91 (s, 1H); ^{13}C-NMR (50.6 MHz, DMSO-d_6) δ 112.2, 114.9, 114.9, 115.8, 121.2, 127.6, 129.4, 129.9, 137.0, 137.3, 141.3, 142.7, 148.2, 166.0.

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Supplementary Material Available: 500-MHz ^{1}H NMR spectrum of 1 (1 page). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.