BIOSYNTHESIS OF THIOPEPTIDE ANTIBIOTIC A10255: INCORPORATION OF ISOTOPICALLY-LABELED PRECURSORS

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The biosynthetic origin of antibiotic A10255 was investigated using ^{14}C- and ^{13}C-labeled amino acids. DL-[1-^{13}C]Serine labeled 15 of the 17 amino acid residues present in A10255G. These included the oxazole, thiazole, dehydroalanine, masked glycine, masked alanine and pyridine moieties. The same 15 residues labeled by serine were labeled by [2-^{13}C]glycine, apparently by conversion of the glycine to [2,3-^{13}C]serine. Formation of the pyridine ring occurred *via* a C3 to C3 condensation of two serines. The results indicated origin of the masked alanine from alanine; the masked glycine from glycine; the thiazole residues from cysteine; and the threonine, masked dehydrobutyrine, masked dehydronorvaline and masked dehydroleucine residues from threonine. L-[CH_3-^{13}C]Methionine labeled the methyl carbon of the masked dehydronorvaline moiety in factor B and the two methyl carbons of the masked dehydroleucine moiety in factor E. The results demonstrate that A10255 originates exclusively from amino acids in a manner similar to the closely related thiopeptide antibiotics nosiheptide and thiostrepton.

A10255, a thiopeptide antibiotic complex shown to promote growth and alleviate acidosis in ruminants, is produced by *Streptomyces gardneri*[1]. A10255 contains a cyclic peptide core with an amino acid side chain[2]. This compound is structurally similar to a known family of antibiotics whose members include berninamycin, sulfomycin I, micrococcin, nosiheptide, thiostrepton, thioxamycin and GE2270[3~7]. A10255 shares several structural features common to the thiopeptide family. These include thiazole rings, oxazole rings, several unusual dehydro-amino acids and a unique pyridine ring structure. Previous reports have demonstrated that these unusual compounds originate from various amino acids[5,8~11].

In addition to several minor components, the complex contains major factors A10255G, A10255B and A10255E (Fig. 1). Factors G, B and E are identical except for the alkyl substituents at R located in one of the oxazole-containing moieties. Factor G contains a masked dehydrobutyrine residue (methyl group at R) while factor B contains a masked dehydronorvaline residue (ethyl group at R) and factor E contains a masked dehydronorleucine residue (isopropyl group at R). This paper reports studies investigating the origin of the individual A10255 residues by using isotopically-labeled precursors and analysis of the labeling pattern in the various factors.

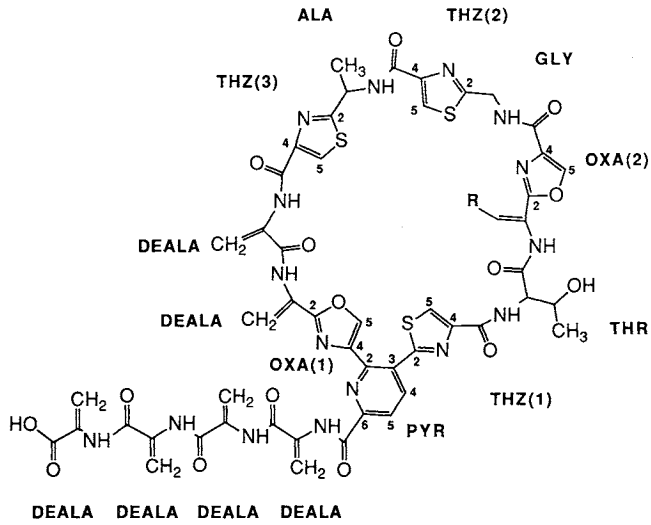
Materials and Methods

Chemicals

Radiolabeled precursors were obtained from the following sources: L-[*U*-^{14}C]serine, L-[*U*-^{14}C]alanine and [*U*-^{14}C]glycine from New England Nuclear; L-[*U*-^{14}C]cysteine from Amersham; L-[CH_3-^{14}C]methionine and L-[*U*-^{14}C]threonine from ICN. Stable isotopes were obtained as follows: DL-[3-^{13}C]Alanine, DL-[3-^{13}C]cysteine, [2-^{13}C]glycine, DL-[2-^{13}C]serine, L-[1-^{13}C]threonine and L-[CH_3-^{13}C]methionine from Cambridge Isotopes Labs; DL-[1-^{13}C]serine from ICN and DL-[3-^{13}C]serine from MSD Isotopes.

Fig. 1. Structure of A10255.

Abbreviations: ALA, masked alanine; DEALA, dehydroalanine; GLY, masked glycine; OXA, oxazole; PYR, pyridine; THR, threonine; THZ, thiazole.



Factor	R=	Residue
A10255G	CH_3–	Dehydrobutyrine
A10255B	CH_3CH_2–	Dehydronorvaline
A10255E	$\begin{matrix}CH_3\\CH_3\end{matrix}$>CH–	Dehydroleucine

Organism

Stock cultures of strain NRRL 18260, a nitrosoguanidine-induced mutant of *S. gardneri*, were maintained in the vapor phase of liquid nitrogen.

Culture Conditions

Seed cultures, fermentation flasks and incubation conditions were as described elsewhere[12]. Except as noted, the defined fermentation medium (GGA) used for production consisted of glucose 0.5%, glycerol 3.0%, NH_4Cl 0.32%, Na_2SO_4 0.2%, $FeCl_3$ 0.0062%, $ZnCl_2$ 0.0019%, $MgCl_2 \cdot 6H_2O$ 0.03%, KH_2PO_4 0.1% and $CaCO_3$ 3.0%. The GGA medium was supplemented with additional NH_4Cl 0.054% for incorporation studies using L-[1-^{13}C]threonine. For studies with factors B and E, the medium was supplemented with $CoCl_2 \cdot 6H_2O$ 0.00005%.

Incorporation Studies

Isotopically-labeled substrates were added to cultures grown in GGA medium as noted above. For radiolabeling experiments, cells were grown in 50 ml of broth in 250 ml flasks for 72 hours. They were then transferred to 50 ml flasks (10 ml/flask) and 7.5 μCi of ^{14}C-substrate was added. The ^{14}C-substrate addition was repeated 24 hours later. At each addition, nonlabeled amino acid at a final concentration of 2.0 mM was also added (L-[*U*-^{14}C]cysteine was at 1.0 mM). Twenty-four hours after the final addition, the cells were collected by centrifugation, extracted with methanol (a volume equivalent to the original volume of whole broth) and the extract assayed for A10255 levels and radioactivity. The individual factors were isolated by reversed phase HPLC as described below and their specific radioactivity was determined.

All ^{13}C-compound labeling experiments were done as follows except where noted. A 0.225 M solution of the ^{13}C-compound was prepared in deionized water at pH 7.0 and filter-sterilized. One ml of this solution was added to each of 10 flasks (containing 45 ml of culture broth) at 72 and 96 hours. For L-[CH_3-^{13}C]methionine, 0.5 ml of solution was added to each of 16 flasks at 72 and 96 hours. A

Table 1. Incorporation ratios for the enrichment of A10255G with ^{13}C-amino acids.

Carbon assignment[a]	Shift	Relative ^{13}C-enrichment (enriched/natural) in A10255G[b]				
		DL-[1-^{13}C]-Serine	DL-[3-^{13}C]-Cysteine	DL-[3-^{13}C]-Alanine	[2-^{13}C]-Glycine	L-[1-^{13}C]-Threonine
Oxazole (1)						
2	158.01	2.5				
4	139.20				7.1	
5	140.62				9.0	
Pyridine						
2	146.83	3.2				
3	130.14				5.6	
4	141.26				12.4	
5	121.40				9.0	
6	149.41				3.8[c]	
6-C=O	161.52	3.2				
Thiazole (1)						
2	163.59	3.9				
4	149.41				3.8[c]	
5	127.08		5.3		4.2	
4-C=O	160.12	2.7				
Threonine						
CH–N	57.94					
CH–O	67.26					
CH_3	20.22					
C=O	168.89					12.7
Masked dehydrobutyrine/oxazole (2)						
C=	123.66					
CH=	128.70					
CH_3	13.41					
2	159.42					15.1
4	136.04				14.4	
5	141.94				11.9	
4-C=O	160.24	4.6				
Masked glycine/thiazole (2)						
CH_2	40.12				3.7[d]	
2	168.71	4.0				
4	148.73				2.1	
5	124.92		5.5		3.2	
4-C=O	159.94	2.9				
Masked alanine/thiazole (3)						
CH	46.49				2.0	
CH_3	20.60			2.5	1.9	
2	172.80	4.0				
4	148.04				2.7	
5	125.83		5.6		3.8	
4-C=O	158.91	2.3				
Dehydroalanine						
C=	136.25				5.9	
	136.20				5.9	
	134.20				8.7	
	133.81				9.3	
	133.20				10.0	
	128.99				7.9	
CH_2=	110.77				11.8	
	110.22				13.3	
	109.85				10.6	

Table 1. (Continued)

Carbon assignment[a]	Shift	Relative ^{13}C-enrichment (enriched/natural) in A10255G[b]				
		DL-[1-^{13}C]-Serine	DL-[3-^{13}C]-Cysteine	DL-[3-^{13}C]-Alanine	[2-^{13}C]-Glycine	L-[1-^{13}C]-Threonine
CH_2=	109.35				14.1	
	106.12				13.3	
	104.65				12.0	
C=O	164.80	3.3				
	163.00	4.6				
	162.79	3.5				
	162.61	4.2				
	162.53	5.3				

[a] NMR assignment values for A10255G.
[b] Natural abundance = 1.0. Only values greater than 1.6 are shown. Solvent was DMSO-d_6.
[c] Since both the pyridine C6 and thiazole (1) C4 exhibit identical chemical shifts, this value is based on the assumption that both are enriched.
[d] Value may have been affected by interference from solvent peak.

Table 2. Incorporation ratios for the enrichment of A10255B with ^{13}C-amino acids.

Carbon assignment[a]	Shift	Relative ^{13}C-enrichment (enriched/natural) in A10255B[b]			
		L-[1-^{13}C]-Threonine	L-[CH_3-^{13}C]-Methionine	DL-[2-^{13}C]-Serine	DL-[3-^{13}C]-Serine
Oxazole (1)					
2	158.10				
4	139.23			2.5	
5	140.68				3.5
Pyridine					
2	146.88				
3	130.21			2.5	
4	141.32				3.4
5	121.47				3.1
6	149.37			2.6	
6-C=O	161.57				
Thiazole (1)					
2	163.67				
4	148.79			2.6	
5	127.22				3.2
4-C=O	160.17				
Threonine					
CH–N	57.94				2.5
CH–O	67.37				3.3
CH_3	20.32				3.5
C=O	169.17	14.7			
Masked dehydronorvaline/oxazole (2)					
C=	122.36				1.8
CH=	135.26				2.9
CH_2	20.74				2.3[c]
CH_3	12.93		5.8		2.6
2	159.46	14.7			
4	136.14			3.9	
5	142.05				4.1
4-C=O	160.32				

Table 2. (Continued)

Carbon assignment[a]	Shift	Relative ^{13}C-enrichment (enriched/natural) in A10255B[b]			
		L-[1-^{13}C]-Threonine	L-[CH_3-^{13}C]-Methionine	DL-[2-^{13}C]-Serine	DL-[3-^{13}C]-Serine
Masked glycine/thiazole (2)					
CH_2	40.24			4.2	
2	168.87				
4	148.79			2.2	
5	124.98				3.0
4-C=O	160.01				
Masked alanine/thiazole (3)					
CH	46.53			3.8	
CH_3	20.74				2.3[c]
2	172.88				
4	148.05			2.1	
5	125.94				2.7
4-C=O	158.96				
Dehydroalanine					
C=	136.33			3.9	
	136.29			3.8	
	134.23			3.1	
	133.82			3.0	
	133.28			3.2	
	129.07			3.0	
CH_2=	110.98				3.1
	110.92				4.1
	110.49				3.2
	109.46				3.6
	106.23				4.1
	104.73				3.4
C=O	164.90				
	163.07				
	162.84				
	162.68				
	162.61				

[a] NMR assignment values for A10255B.

[b] Natural abundance = 1.0. Only values greater than 1.6 are shown. Solvent was DMSO-d_6.

[c] Since both the masked dehydronorvaline CH_2 and the masked alanine CH_3 exhibit identical chemical shifts, this value is based on the assumption that both are enriched.

0.113 M solution of DL-[3-^{13}C]cysteine at pH 4.0 was prepared and 1.5 ml added to each of 10 flasks at 72 and 96 hours. All fermentations were terminated 24 hours after the final addition and A10255 was isolated as described below. Yields of purified factors from the precursed fermentations were as follows: DL-[1-^{13}C]serine, 34 mg of factor G; [2-^{13}C]glycine, 43 mg of factor G; DL-[3-^{13}C]alanine, 60 mg of factor G; DL-[3-^{13}C]cysteine, 16 mg of factor G; L-[CH_3-^{13}C]methionine, 59 mg of factor B and 33 mg of factor E; L-[1-^{13}C]threonine (factor G fermentation), 54 mg of factor G; L-[1-^{13}C]threonine (factors B and E fermentation), 54 mg of A10255B and 14 mg of A10255E; DL-[2-^{13}C]serine, 40 mg of A10255 B and 21 mg of A10255 E; DL-[3-^{13}C]serine, 37 mg of A10255B and 26 mg of A10255E.

A10255 Assay and Isolation

A10255 was extracted from the cell mass with aqueous methanol and assayed by HPLC as previously described[1]. For isolation of ^{14}C-labeled A10255, the HPLC system was modified to employ a 3.9 × 300 mm μBondapak C_{18} column (Millipore/Waters) with a mobile phase composed of acetonitrile - water (45 : 55) containing 1% acetic acid (w/v).

The following procedure was used to isolate ^{13}C-labeled A10255 from the mycelial cell mass for

Table 3. Incorporation ratios for the enrichment of A10255E with ^{13}C-amino acids.

Carbon assignment[a]	Shift	Relative ^{13}C-enrichment (enriched/natural) in A10255E[b]			
		L-[1-^{13}C]-Threonine	L-[CH_3-^{13}C]-Methionine	DL-[2-^{13}C]-Serine	DL-[3-^{13}C]-Serine
Oxazole (1)					
2	158.00				
4	139.09			1.8	
5	140.54				1.8
Pyridine					
2	146.77				
3	130.11			3.3	
4	141.22				1.7
5	121.36				1.9
6	149.33[c]			2.5	
6-C=O	161.42				
Thiazole (1)					
2	163.51				
4	149.30[c]			2.5	
5	127.16				2.0
4-C=O	159.98				
Threonine					
CH–N	57.75				
CH–O	67.27				1.7
CH_3	20.21				2.6
C=O	169.33	20.3			
Masked dehydroleucine/oxazole (2)					
C=	121.01				
CH=	140.30				
CH	26.49				
CH_3	21.68		9.4		
CH_3	21.86		9.4		
2	159.35	19.3			
4	136.02			1.8	
5	141.91				2.0
4-C=O	160.18				
Masked glycine/thiazole (2)					
CH_2	40.16				
2	168.89				
4	148.67			1.7	
5	124.80				1.9
4-C=O	159.89				
Masked alanine/thiazole (3)					
CH	46.38			2.9	
CH_3	20.61				2.6
2	172.88				
4	147.88			2.0	
5	125.86				1.8
4-C=O	158.81				
Dehydroalanine					
C=	136.23			2.1	
	136.19			2.6	
	134.12			2.1	
	133.68			1.8	
	133.24			2.3	
	129.00			1.8	
CH_2=	110.98				2.1

Table 3. (Continued)

Carbon assignment[a]	Shift	Relative ^{13}C-enrichment (enriched/natural) in A10255E[b]			
		L-[1-^{13}C]-Threonine	L-[CH_3-^{13}C]-Methionine	DL-[2-^{13}C]-Serine	DL-[3-^{13}C]-Serine
CH_2=	110.70				2.1
	109.58				1.9
	109.18				2.8
	106.08				2.4
	104.58				2.0
C=O	164.72				
	162.91				
	162.67				
	162.55				
	162.42				

[a] NMR assignment values for A10255E.
[b] Natural abundance = 1.0. Only values greater than 1.6 are shown. Solvent was DMSO-d_6.
[c] Assignments may be reversed.

^{13}C NMR analysis. The cell mass was collected by centrifugation (1,000 × g, 15 minutes at 4°C). A10255 was extracted by using a volume of 85% aqueous acetone equal to the harvest broth volume and stirring for 15 minutes at room temperature. The extract was collected by centrifugation, acetone was removed by vacuum evaporation and the aqueous solution lyophilized. The dried, crude A10255 was dissolved in tetrahydrofuran - 1.5% acetic acid (50 : 50) and the individual factors were isolated utilizing a Waters LC 3000 HPLC system (Millipore/Waters) with a 50 × 350 mm Dynamax C_{18} column (Rainin). A10255 was eluted with acetonitrile - THF - 1.5% acetic acid (25 : 10 : 65) at a flow rate of 40 ml/minute. The effluent was monitored at 280 nm using a Waters model 481 LC spectrophotometer (Millipore/Waters). The column fractions were concentrated by vacuum evaporation, which resulted in precipitation of A10255. This precipitate was washed twice in deionized water (37,000 × g, 10 minutes) and dried by lyophilization. The samples of factor E labeled with DL-[2-^{13}C]serine and DL-[3-^{13}C]serine were rechromatographed as described for the crude A10255 extracts in order to remove residual factor B.

NMR Analysis

Most of the resonances of the ^{13}C NMR spectrum in DMSO-d_6 were assigned using one bond and long range 2D heteronuclear correlations. The dehydroalanine resonances were assigned only to the structural type (*e.g.*, C=O, C= and CH_2)[2] and were not specifically determined.

Spectra of natural abundance and enriched samples were obtained and free induction decay values (FIDs) were processed under identical experimental conditions. This included acquisition parameters of a 15,151 Hz spectral width, a 90 degree pulse width (16.4 μseconds), no relaxation delay, 32K of memory and 30,000 transients. The FIDs were processed with 1 Hz of Lorentzian line broadening.

Although enrichment was determined by subtracting the natural abundance spectrum from the enriched spectrum, enrichment sites in every case could be identified by inspection. The enrichment ratios (enriched/natural) were determined by normalizing the intensities of the various resonances to either the threonine CH-O resonance or the masked dehydrobutyrine C= resonance. A ratio of enrichment to natural abundance is shown in Table 1 for factor G, Table 2 for factor B and Table 3 for factor E.

Results

Initial experiments with ^{14}C-amino acids (Table 4) provided information regarding the possible biosynthetic origin of A10255 and helped to determine the conditions used for ^{13}C-precursor incorporation experiments. Optimum incorporation occurred upon the addition of ^{14}C-precursors at 72 and 96 hours followed by harvest at 120 hours (data not shown). Titers at harvest were 420 ~ 570 μg/ml. For studies

Table 4. Incorporation of ^{14}C-labeled amino acids into A10255.

Amino acid	Specific activity (μCi/mmol)		Molar specific incorporation (%)
	Amino acid	A10255[a]	
L-[*U*-^{14}C]Alanine	375	36	9.6
[*U*-^{14}C]Glycine	375	239	63.7
L-[*U*-^{14}C]Serine	375	102	27.2
L-[*U*-^{14}C]Cysteine	750	102	13.6
L-[*U*-^{14}C]Threonine	375	122	32.5
L-[CH_3-^{14}C]-Methionine[b]	375	89	23.7
L-[*U*-^{14}C]Leucine	375	0[c]	0[c]

[a] Total A10255 (methanol extract of cell mass). The concentration of A10255 was 0.34~0.46 mM (420~570 μg/ml).
[b] See Table 5 for the L-[CH_3-^{14}C]methionine incorporation data on the individual factors of A10255.
[c] No radioactivity detected in this sample.

Table 5. Incorporation of L-[CH_3-^{14}C]methionine into A10255 factors[a].

Factor	A10255 level (μg/ml)[b]	Molar specific activity (μCi/mmol)	Molar specific incorporation (%)
A10255G	28	5	2.4
A10255B	278	99	26.4
A10255E	116	40	19.2
Total	422	89	23.7

[a] Cultures were grown in the GGA medium supplemented with $CoCl_2 \cdot 6H_2O$ 0.00005%.
[b] Methanol extract of culture broth.

investigating the synthesis of factors B and E, cobalt, which is essential for their biosynthesis[12], was added to the GGA medium. The relatively low incorporation of L-[*U*-^{14}C]alanine suggested that it was a precursor of the single masked alanine residue of A10255 and not a precursor of the five dehydroalanine residues. As predicted[8], L-[*U*-^{14}C]serine was significantly incorporated into A10255. Serine is a likely precursor of the oxazole rings and dehydroalanine residues and, as a source of cysteine, would also serve as a precursor of the thiazole rings. Although addition of cysteine to fermentations of A10255 suppressed total synthesis (data not shown), incorporation from L-[*U*-^{14}C]cysteine was observed. The significant incorporation of [*U*-^{14}C]glycine and L-[*U*-^{14}C]threonine suggested these amino acids were directly incorporated and also served as precursors for other residues. Leucine was not incorporated. Addition of L-[CH_3-^{14}C]methionine to cobalt-supplemented fermentations demonstrated low incorporation into factor G but high incorporation into factors B and E (Table 5). This suggested that methionine was serving as a methyl donor for the biosynthesis of factors B and E.

Results from the ^{14}C-amino acid incorporation demonstrated the likely precursors of the A10255 molecule. Labeling studies using ^{13}C-amino acids were then conducted to determine the exact positions within A10255 that were derived from these amino acids. For studies with factor G, the precursors added included DL-[1-^{13}C]serine, DL-[3-^{13}C]cysteine, [2-^{13}C]glycine, DL-[3-^{13}C]alanine and L-[1-^{13}C]threonine. For studies with factors B and E, the precursors added included DL-[2-^{13}C]serine, DL-[3-^{13}C]serine, L-[CH_3-^{13}C]methionine and L-[1-^{13}C]threonine. The enrichment ratios (enriched/natural) were determined by normalizing the intensities of the various resonances to a resonance in both spectra.

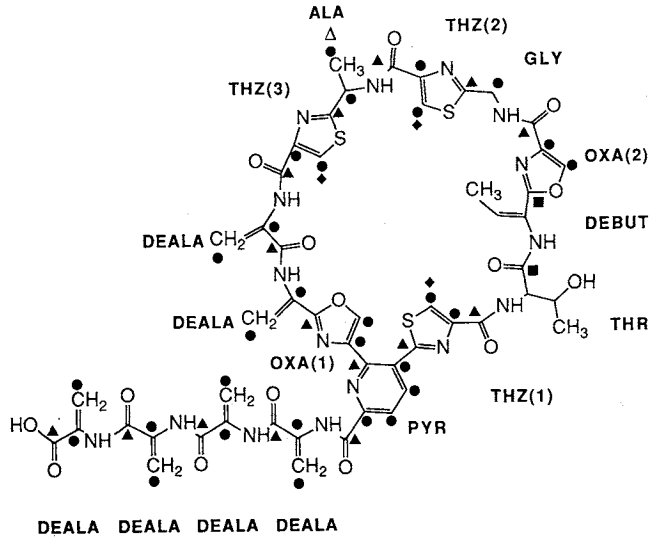
Supplementing the A10255 fermentation with DL-[1-^{13}C]serine enriched 15 of the 17 amino acid residues in factor G (Table 1 and Fig. 2). Only the threonine and masked dehydrobutyrine residues were not enriched. The dehydroalanine residues were labeled at the carbonyl position. All thiazole residues were labeled at both C2 and the C4-carbonyl. The oxazole (1) C2 and the oxazole (2) C4-carbonyl were labeled. Enrichment of the pyridine ring at the C2 and C6-carbonyl indicated formation of this residue *via* a C3 to C3 condensation of two serines. Both the masked alanine and masked glycine were labeled at their corresponding thiazole C2 position.

Because addition of cysteine to fermentations of A10255 decreased antibiotic synthesis, the amount of DL-[3-^{13}C]cysteine fed to the culture was reduced. Nevertheless, addition of cysteine resulted in labeling

Fig. 2. Sites in A10255G labeled by ^{13}C-amino acids.

Abbreviations: ALA, masked alanine; DEALA, dehydroalanine; GLY, masked glycine; OXA, oxazole; PYR, pyridine; THR, threonine; THZ, thiazole and DEBUT, masked dehydrobutyrine.

Enrichment from: ● [2-^{13}C]Glycine, ▲ DL-[1-^{13}C]serine, ◆ DL-[3-^{13}C]cysteine, △ DL-[3-^{13}C]-alanine, ■ L-[1-^{13}C]threonine.



of the three thiazole residues at their C5 position. This suggested cysteine as the direct precursor of the thiazole residues, even though serine also served as a precursor through its conversion to cysteine.

As expected based on the ^{14}C results, DL-[3-^{13}C]alanine labeled only the masked alanine CH_3. This supported the conclusion that serine, and not alanine, served as the primary precursor of the dehydroalanine residues.

Because the A10255 factors contain a masked glycine residue, [2-^{13}C]glycine was fed to determine its involvement in biosynthesis. The NMR analysis unexpectedly indicated that the same 15 residues of factor G shown to be labeled by serine were also enriched by glycine. With the exception of the masked glycine CH_2, all of these residues were labeled at pairs of adjacent carbon atoms. These included the dehydroalanines C= and CH_2=, the C4 and C5 of both the oxazole and thiazole residues, the masked alanine CH and CH_3 and the pyridine C3, C4, C5 and C6. These data, in conjunction with the L-[1-^{13}C]serine labeling results, suggested that [2-^{13}C]glycine was converted to [2,3-^{13}C]serine, which was then incorporated into A10255 in a manner similar to L-[1-^{13}C]serine. The labeling pattern produced in the pyridine ring by enriched glycine further demonstrated that this ring residue was formed *via* a C3 to C3 condensation of two serines.

Addition of L-[1-^{13}C]threonine resulted in enrichment of the threonine carbonyl and the oxazole C2 which corresponds to the masked dehydrobutyrine carbonyl of factor G.

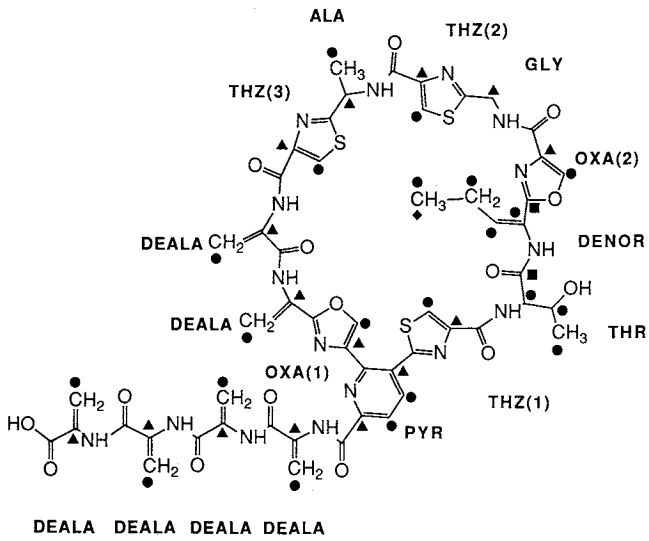
The results of these experiments indicated that, with the exception of the threonine and masked dehydrobutyrine residues, factor G can be derived exclusively from serine or, indirectly, from glycine by conversion of the latter to serine.

Since factors G, B and E are identical except for the alkyl substituents located in the oxazole (2) moiety (Fig. 1), the data obtained from incorporation studies with factor G were also applicable to factors

Fig. 3. Sites in A10255B labeled by ^{13}C-amino acids.

Abbreviations: ALA, masked alanine; DEALA, dehydroalanine; GLY, masked glycine; OXA, oxazole; PYR, pyridine; THR, threonine; THZ, thiazole and DENOR, masked dehydronorvaline.

Enrichement from: ■ L-[1-^{13}C]Threonine, ◆ L-[CH_3-^{13}C]methionine, ▲ DL-[2-^{13}C]serine, ● DL-[3-^{13}C]serine.



B and E. Incorporation studies with factors B and E were done to determine: 1) origin of the threonine and the masked dehydronorvaline/dehydroleucine residues, 2) origin of the ethyl and isopropyl groups of the masked dehydronorvaline/dehydroleucine residues and, 3) the role of serine in the biosynthesis of A10255. In order to obtain adequate amounts of factors B and E, it was necessary to use cobalt-supplemented fermentations. Since these fermentations produce primarily factors B and E[12], amounts of factor G obtained were insufficient for NMR analysis.

Addition of L-[1-^{13}C]threonine resulted in enrichment of the threonine C=O in factors B and E (Tables 2 and 3; Figs. 3 and 4). Likewise, the oxazole (2) C2 of both factor B and E was enriched. This corresponded to both the masked dehydronorvaline carbonyl of factor B and masked dehydroleucine carbonyl of factor E. No further enrichment by threonine occurred. These results are identical to those seen with factor G enrichment by L-[1-^{13}C]threonine.

The ^{14}C-labeling experiments demonstrated significant incorporation of L-[CH_3-^{14}C]methionine into factor B (Table 5). Further studies were done to determine the possible origin of the ethyl group of factor B (masked dehydronorvaline residue) and the isopropyl group of factor E (masked dehydroleucine residue). Cobalt-supplemented fermentations of A10255 were fed L-[CH_3-^{13}C]methionine and factors B and E were analyzed. Enrichment occurred at the masked dehydronorvaline CH_3 (ethyl group) of factor B and at both masked dehydroleucine CH_3 carbons (isopropyl group) of factor E, demonstrating that methionine could serve as a methyl donor for these factors. However, whether methionine is directly involved in methylation of factor G to form factors B or E, or is indirectly involved *via* formation of the masked dehydronorvaline/dehydroleucine residues is unknown.

Based on the enrichment results with [2-^{13}C]glycine, it was evident that some fraction of the glycine was converted to serine. This suggested the involvement of hydroxymethyltransferase in the biosynthesis

Fig. 4. Sites in A10255E labeled by ^{13}C-amino acids.

Abbreviations: ALA, masked alanine; DEALA, dehydroalanine; GLY, masked glycine; OXA, oxazole; PYR, pyridine; THR, threonine; THZ, thiazole and DELEU, masked dehydroleucine.

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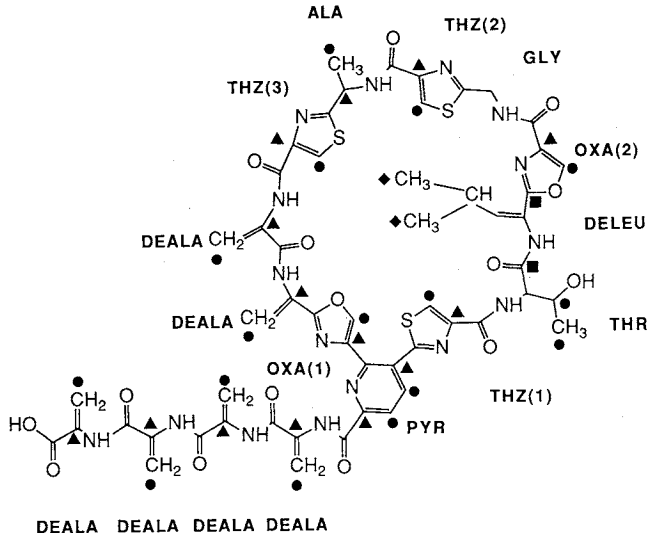

of A10255. Due to the action of this enzyme and other metabolic processes, serine may be converted to other precursors of the A10255 factors. Therefore, studies with both DL-[2-^{13}C] and DL-[3-^{13}C]serine were conducted to determine alternate roles for serine in the biosynthesis of A10255. Factors B and E were chosen for this study because they are identical to factor G except for the additional alkyl groups. Thus, results could be extrapolated to include factor G and also provide more information on biosynthesis of the methylated factors. Addition of DL-[2-^{13}C]serine enriched the C4 of all the oxazole and thiazole residues, the C3 and C6 positions of pyridine, the dehydroalanine C= and the masked alanine CH. The masked glycine CH_2 in factor B, but not factor E, was enriched. These data supported the results from studies with DL-[1-^{13}C]serine which demonstrated derivation of these residues from serine. Additions of DL-[3-^{13}C]serine enriched the C5 of all oxazole and thiazole residues, the C4 and C5 positions of pyridine, the dehydroalanine CH_2 and the masked alanine CH_3 of factors B and E. The masked glycine was not enriched in either factor. Further enrichment by the DL-[3-^{13}C]serine occurred at the threonine CH-O and CH_3 in both factors B and E and at the threonine CH–N in factor B only. In addition, the masked dehydronorvaline residue of factor B was enriched at C=, CH=, CH_2 and CH_3 positions.

Discussion

Isotope incorporation studies and NMR analyses have shown that the thiopeptide antibiotic A10255 was derived exclusively from amino acids. Enrichment studies with DL-[1-^{13}C]serine indicated that 15 of the 17 residues of factor G were derived from serine. These included the masked alanine, masked glycine, pyridine, dehydroalanine, oxazole and thiazole residues. Although DL-[3-^{13}C]alanine labeled the masked alanine residue, the dehydroalanine residues were not enriched by this amino acid or by cysteine. This is further evidence that dehydroalanine is derived by dehydration of serine. The antibiotics nosiheptide and berninamycin both also contain dehydroalanine residues that are derived from serine, not alanine[8,10].

Likewise, formation of the thiazole residues from cysteine (or serine), as seen with A10255, has also been demonstrated for these antibiotics.

Origin of the oxazole rings from serine, as established in this study, has also been demonstrated for virginiamycin M_1[9]. A similar mechanism has been proposed for berninamycin, where the 5-methyloxazoles of this cyclic peptide are derived from threonine[10].

Based on the incorporation results obtained with serine and glycine, the pyridine residue is formed *via* a C3 to C3 condensation of two serines, most likely following the conversion of serine to dehydroalanine. This is in agreement with the biosynthesis of the pyridine moiety in nosiheptide[8] and the piperidine moiety of thiostrepton[5]. In both these antibiotics, the unusual ring residues are formed by a C3 to C3 condensation of two serines.

The indirect incorporation of glycine into A10255 by its conversion to serine is a biosynthetic process which has been reported for other secondary metabolites. Incorporation studies with the β-lactam antibiotic nocardicin A demonstrated enrichment of two adjacent carbons within the β-lactam ring by [2-^{13}C]glycine[13]. Because the β-lactam unit is derived from serine, it was concluded that glycine was converted to L-[2,3-^{13}C]serine, which was then incorporated into nocardicin. When virginiamycin M_1 was labeled with [1-^{13}C]glycine, enrichment occurred at C22, a position derived from serine[9]. Again, glycine had been converted to [1-^{13}C]serine. With A10255, it is likely that the formation of serine from glycine occurred *via* the glycine cleavage pathway, which forms CO_2, NH_4 and ^{13}C-methylenetetrahydrofolate. Thereafter, serine hydroxymethyltransferase could catalyze the reaction of ^{13}C-methylenetetrahydrofolate with [2-^{13}C]glycine to form [2,3-^{13}C]serine[14].

L-[1-^{13}C]Threonine added to cultures of *S. gardneri* was directly incorporated into the A10255 factors. Furthermore, enrichment of the oxazole (2) C2 corresponded to the C1 of the masked dehydrobutyrine, dehydronorvaline and dehydroleucine residue of factors G, B and E, respectively. This indicated that formation of the oxazole (2) was through condensation of a serine with either a threonine residue or a threonine derivative. Thiostrepton feeding studies using [1-^{13}C]threonine also demonstrated direct incorporation of threonine and labeling of the C1 of dehydrobutyrine[11]. The high specific incorporation of L-[CH_3-^{14}C]methionine into factors B and E, coupled with enrichment of the CH_3 carbons of the dehydronorvaline and dehydroleucine residues of factors B and E by L-[CH_3-^{13}C]methionine, suggested that the masked dehydronorvaline and dehydroleucine residues are formed by postinsertional methylation. Alternatively, these residues could be synthesized from threonine prior to insertion into the peptide. However, L-[*U*-^{14}C]leucine, a possible precursor of the masked dehydroleucine residue of factor E, was not incorporated. This supports the hypothesis of postinsertional methylation. Previous studies[12] identified cobalt as the critical element controlling biosynthesis of the methylated factors, apparently *via* a cobalamin coenzyme. Cobalamin is primarily involved in methylation reactions and thus may function as a coenzyme for a methylase catalyzing the formation of factors B and E from factor G. Although data from this study and the previous studies also support this pathway, direct conversion of factor G to the methylated factors has not been demonstrated.

As expected, both DL-[2-^{13}C] and DL-[3-^{13}C]serine were incorporated into the same 15 residues as DL-[1-^{13}C]serine. This further supports the fact that serine serves an essential role as a precursor of A10255. Although the masked glycine residue in factor B was labeled by DL-[2-^{13}C]serine, this residue was not enriched in factor E. Because synthesis of factor E terminates early in the fermentation (data not shown), amounts of factor E necessary to observe enrichment may not have been produced following addition of the DL-[2-^{13}C]serine. The labeling of several threonine carbons in both factors B and E, as well as the masked dehydronorvaline residue of factor B, by DL-[3-^{13}C]serine was an unexpected result. Serine is not considered a precursor of threonine or of dehydronorvaline. However, serine can be converted to acetyl coenzyme A (acetyl-CoA) *via* dehydration and deamination to pyruvate with loss of the C1 carbon as CO_2[15]. Thus, the DL-[3-^{13}C]serine may have been converted to [2-^{13}C]acetyl-CoA, which subsequently entered the tricarboxylic acid (TCA) cycle. Condensation of the [2-^{13}C]acetyl-CoA with oxaloacetate would form citrate labeled at the CH_2 position. After one cycle, ^{13}C-labeled oxaloacetate would be formed[15]. Since oxaloacetate is a precursor of threonine[14], the labeled carbons would appear in threonine. Several courses of the TCA cycle would result in a source of multiple-labeled oxaloacetate and hence, multiple-labeled threonine and dehydronorvaline. The lack of enrichment in all carbons of these residues may have been due to loss of labeled carbons as CO_2 or to a slow rate of threonine formation from serine

via acetyl-CoA. Certain carbons in viginiamycin M_1 that are derived from acetate units are also derived from serine *via* its transformation to acetyl-CoA[9]. These investigators suggested that incorporation from [3-^{13}C]serine occurred at a slower rate than from [2-^{13}C]acetate due to the slow rate of serine conversion to acetyl-CoA. The fact that threonine was not enriched by DL-[1-^{13}C]serine or DL-[2-^{13}C]serine was possibly due to loss of the ^{13}C as CO_2. Conversion of pyruvate to acetyl-CoA results in loss of the pyruvate C1 in the form of CO_2. Likewise, upon entering the TCA cycle, the acetyl-CoA C1 (*i.e.*, serine C2) would eventually be lost as CO_2 due to decarboxylation reactions[15]. Labeling of the CH_3 (ethyl group) of dehydronorvaline by DL-[3-^{13}C]serine suggests that the C3 of serine was transferred to methionine *via* N^5-methyltetrahydrofolate[14].

This study has demonstrated the biosynthetic origin of A10255 from amino acids. Results concerning the origin of the oxazole, thiazole, pyridine and dehydroalanine residues are in agreement with the findings reported for other closely-related thiopeptide antibiotics. Furthermore, this study demonstrates the ability of certain precursors to contribute their atoms indirectly by their conversion to other intermediates that are then incorporated into the secondary metabolites.

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