Biosynthesis of Capreomycin. 2. Incorporation of L-Serine, L-Alanine, and L-2,3-Diaminopropionic Acid[1]

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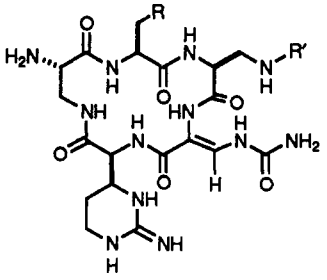
The biosynthesis of the antitubercular antibiotic capreomycin has been studied by administration of isotopically labeled (^{13}C and ^{2}H) precursors to *Streptomyces capreolus* A250. These studies indicated that the 2,3-diaminopropionate (Dap) moiety of capreomycin is derived from serine *via* a dehydroalanyl intermediate. The very high incorporation of labeled serine specifically into the serine moiety of capreomycin IA, **1**, and incorporation of labeled alanine into the alanine moiety of capreomycin IB, **2**, revealed that neither **1** nor **2** is converted into the other. Deuteriated 2,3-diaminopropionic acid was incorporated specifically into the Dap and $\Delta^{2,3}$-ureido-Dap residues, demonstrating that it is an intermediate in the biosynthesis of capreomycin and revealing that the pathway involves prepeptide assembly modification of serine.

The antitubercular antibiotic capreomycin is a strongly basic cyclic peptide isolated from *Streptomyces capreolus* A250 by Herr *et al.* in 1960.[2] In general, capreomycin is a second-line therapeutic agent employed in combination with other antitubercular drugs. In particular, it may be used in place of streptomycin where either the patient is sensitive to or the strain of *Mycobacterium tuberculosis* is resistant to streptomycin.[3-5] Four components, designated IA, IB, IIA, and IIB (**1**, **2**, **3**, and **4**, respectively), have been shown to be produced by *S. capreolus*.[6] The clinical agent contains primarily **1** and **2**. The structures of the capreomycins were established through their similarity to viomycin[7] and subsequently confirmed by total synthesis.[8]



1: R = OH, R' = β-Lysine
2: R = H, R' = β-Lysine
3: R = OH, R' = H
4: R = H, R' = H

We recently reported mechanistic details of the conversion of arginine, **5**, to the L-capreomycidine moiety of capreomycin and showed that **5a** yielded **1a** and **2a**, as shown in Scheme I.[9] We now present results of further investigations into the origins of the remaining four amino acid residues of the cyclic pentapeptide cores of **1** and **2**.

Results and Discussion

Incorporation of Protein Amino Acids. β-Lysine has been shown to be derived by a lysine-2,3-aminomutase reaction in *Streptomyces*[10] and in *Clostridia*.[11] Therefore, attention was now focused on the remaining four residues of the cyclic "core" of **1** and of **2**. Three-carbon amino acids were the logical potential precursors. Administration of L-[U-^{14}C]-serine to fermentations of *S. capreolus* yielded a 2.1% incorporation for **1** and an 1.6% incorporation for **2**, whereas administration of either L-[U-^{14}C]-alanine or L-[U-^{14}C]-cystine yielded less than 1% incorporation into either **1** or **2**. These results indicated that serine was the most likely precursor not only for the serine residue in **1** but also for the 2,3-diaminopropionate (Dap, **6**) residues of **1** and **2**.

Cultures of *S. capreolus* were then fed DL-[1-^{13}C]-serine (**7a**) to obtain positional information on the origin of the residues that make up capreomycins. The full proton and ^{13}C assignments had been previously obtained for both **1** and **2** from a series of 1D and 2D NMR experiments.[12] Table I gives the specific enrichments that were determined from the ^{13}C NMR spectra of the derived **1b** and **2b**, normalized to the C-1 resonance of the natural abundance spectrum, and Scheme I shows the major sites of enrichment. Enrichments (average 1.0%) at C-10, C-17, and C-34 clearly revealed that serine is a primary precursor for the Dap residues and the $\Delta^{2,3}$-ureido-Dap (referred to as "modified Dap") residue. The dramatically higher enrichment at C-30 (12.1%) of **1b** confirmed that **7a** was incorporated directly into the serine residue. These results suggested that the capreomycin pathway involved prepeptide assembly modification rather than postpeptide assembly modification, since approximately the same en-

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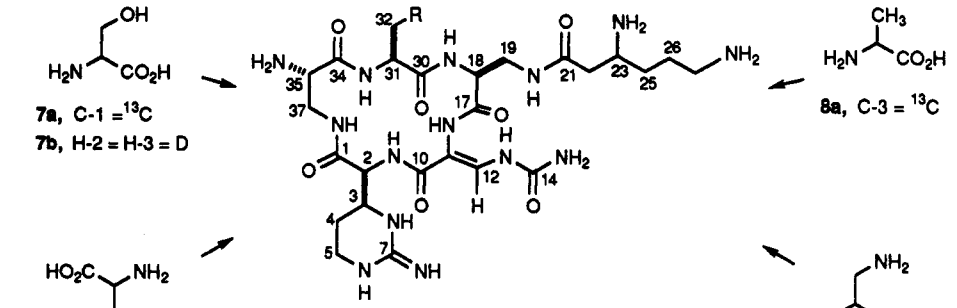
Scheme I



7a, C-1 = ^{13}C
7b, H-2 = H-3 = D

8a, C-3 = ^{13}C

5, H-2 = H-3 = H-5 = H
5a, H-2 = H-3 = H-5 = D

6, H-2 = H-3 = H
6a, H-2 = H-3 = D
6b, H-2 = D, H-3 = H

1a, R = OH, H-3 = H-5 = D
1b, R = OH, C-10 = C-17 = C-30 = C-34 = ^{13}C
1c, R = OH
1d, R = OH, H-12 = H-19 = H-31 = H-32 = H-37 = D
1e, R = OH, H-12 = H-19 = H-37 = D
1f, R = OH, H-18 = H-35 = D

2a, R = H, H-3 = H-5 = D
2b, R = H, C-10 = C-17 = C-30 = C-34 = ^{13}C
2c, R = H, C-32 = ^{13}C
2d, R = D, H-12 = H-19 = H-37 = D
2e, R = H, H-12 = H-19 = H-37 = D
2f, R = H, H-18 = H-35 = D

Table I. ^{13}C Enrichments in Capreomycins Derived from DL-[1-^{13}C]Serine

	^{13}C chemical shift (ppm)		rel ^{13}C enrichment[a] (%) (in excess over natural abundance)	
carbon	1	2	1b	2b
7	155.87	155.84	0.2	0.4
10	168.79	168.61	0.9	1.2
14	157.92	157.92	0.8	0.8
17	173.13	173.33	1.3	*
21	173.53	173.33	0.6	*
30	173.71	177.03	12.1	1.7
34	168.63	168.13	0.8	0.7

[a] C-1 (δ 172.5) was used as reference. * The peaks of C-17 and C-21 in spectrum of **2b** are overlapped with a total of 0.6% enrichment.

richment at all four carbonyl positions should result if serine were incorporated directly into each. In contrast to **1b**, similar enrichments at all four equivalent positions of **2b** were observed (0.7–1.7%). This result appeared to rule out the possibility that capreomycin IA, **1**, is derived by hydroxylation of capreomycin IB, **2**. Incorporation of the labeled serine into the other residues could be interpreted by the metabolic pathways shown in Scheme II (e.g., for **1b**: the guanido-group C-7, 0.2%; the ureido-group C-14, 0.8%; the β-lysine residue C-21, 0.6%).

Since **2b** was derived indirectly from serine, DL-[3-^{13}C]-alanine (**8a**) was tested next and yielded **1c** and **2c** with the enrichments shown in Table II. The 3.0% enrichment at C-32 of **2c** in comparison with significantly lower enrichments at other enriched sites (0.6–1.6%) was consistent with the direct incorporation of **8a** into **2c**. No enrichment was observed at Dap or modified Dap residues. Again, secondary incorporation was observed (see Table II) in the capreomycidine, modified Dap, and β-lysine residues and could be rationalized by the metabolic pathways shown in Scheme III.

In order to determine the mechanism for conversion of serine to Dap, DL-[2,3,3-$^2H_3$]-serine (**7b**) was fed next and yielded **1d** and **2d**. These were analyzed by ^2H NMR spectroscopy in deuterium-depleted water, and FIDs were acquired at 298 K. Since the resonance frequency for H-31 (δ 4.84) overlapped with the small remaining solvent signal,

Scheme II

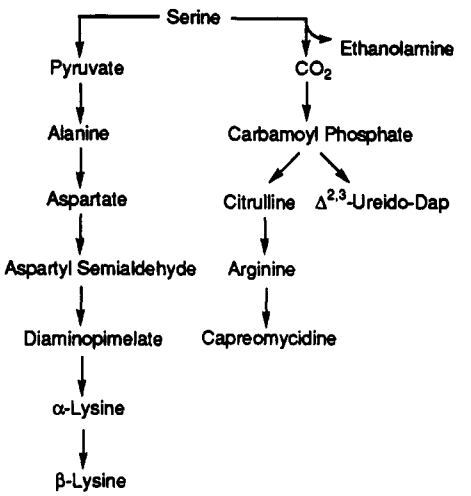


Table II. ^{13}C Enrichments in Capreomycins Derived from DL-[3-^{13}C]Alanine

	^{13}C chemical shift (ppm)		rel ^{13}C enrichment[a] (%) (in excess over natural abundance)	
carbon	1	2	1c	2c
1	172.52	172.40	1.1	0.7
2	56.60	56.48	0.9	0.7
4	24.35	24.24		1.6
7	155.87	155.84	0.3	0.3
14	157.92	157.92	0.8	0.7
23	49.83	49.87		0.6
26	24.46	24.45		0.8
32	63.80	19.63		3.0

[a] C-25 (δ 30.6) was used as reference.

a second FID was acquired at 345 K with a presaturation pulse to diminish the residual water resonance. The spectrum of **2d** (Figure 1b) at 345 K clearly showed resonances for H-12 (δ8.07), H-19 (δ3.86 and δ3.71), H-26 (δ1.82), H-32 (δ1.44), and H-37 (δ4.15 and δ3.29). No deuterium was observed at H-18 (δ4.43) or H-35 (δ4.34). Retention of the β-hydrogens and loss of the α-hydrogen would be consistent with involvement of a dehydroalanyl

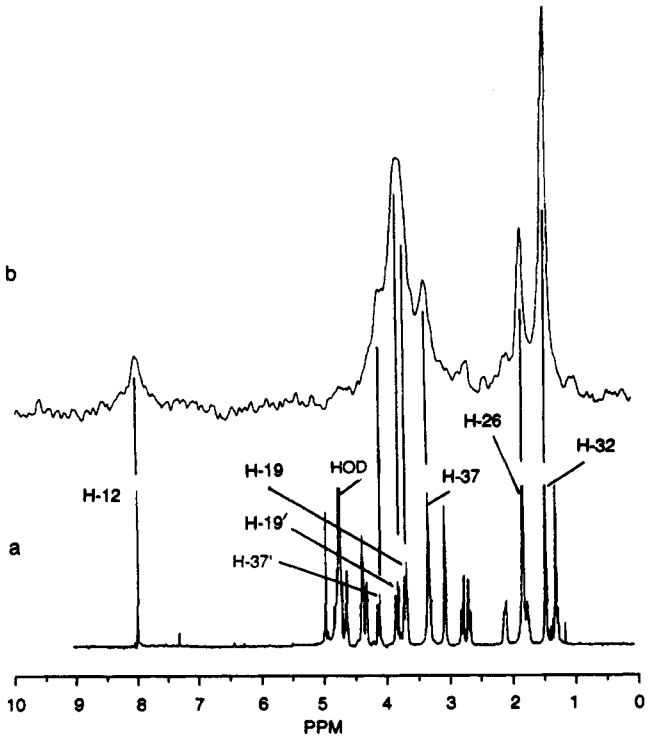
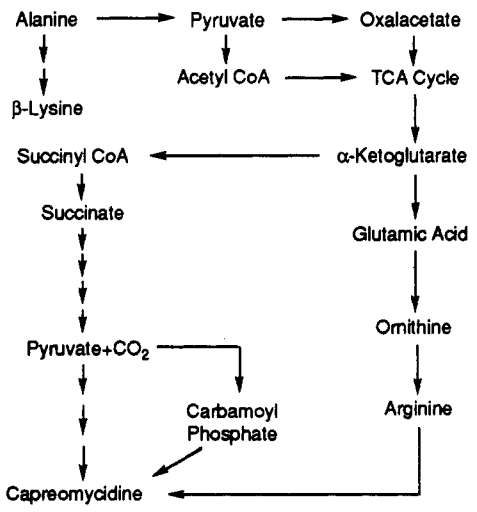


Figure 1. NMR spectra of **2d**. (a) 400-MHz ^1H NMR spectrum in D_2O at 298 K. (b) 61.4-MHz ^2H NMR spectrum in ^2H-depleted H_2O at 345 K showing deuterium enrichment at H-12, H-19, H-26, H-32, and H-37; residual HOD signal (δ 4.4) was suppressed by inversion recovery water suppression pulse.

Scheme III



residue in the conversion to **6**. The ^2H NMR spectrum of **1d** at 345 K was almost the same, except for an additional intense resonance at H-31 (δ4.84), which again was consistent with direct incorporation of serine at this residue.

Incorporation of 2,3-Diaminopropionic Acid (6a). Results from the serine feedings clearly indicated conversion to **6**, prior to peptide assembly. To test this directly, DL-[2,3,3-$^2H_3$]-aspartic acid was prepared[13] and converted to DL-[2,3,3-$^2H_3$]-Dap, **6a**, *via* a Schmidt reaction.[14] The deuterium enrichments of **6a** were 98% at H-2 and 80% at H-3, as measured from the ^2H NMR spectrum, relative to internal 2-methyl-2-propanol (natural abundance) as a standard. A sample was fed to *S. capreolus*, and the derived metabolites, **1e** and **2e**, were analyzed by ^2H NMR spectroscopy. Both spectra clearly showed deuterium at H-12, H-19, and H-37 (cf Figure 2b for **2e**). By comparison with the spectra of **1d** and **2d**, deuterium enrichments at H-18 and H-35 were also recognizable. No enrichments were observed for the serine or alanine residue of **1e** or **2e**, respectively. Enrichments of 25% at H-12 and an average of 14% for each of the other sites in **2e** were obtained again by comparison with the signal from 2-methyl-2-propanol that had been added as a standard. The values for **1e** were 16% and 5%, respectively. 2,3-Diaminopropionate is clearly an intermediate in the biosynthesis of capreomycin and is formed prior to the formation of the cyclic pentapeptide.

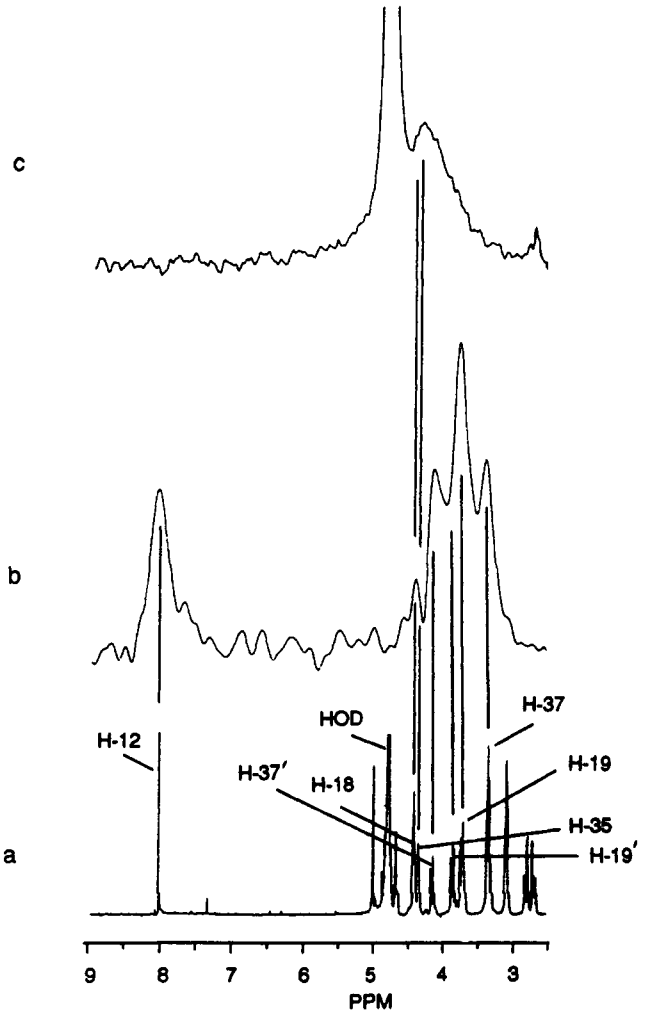


Figure 2. Partial NMR spectra of **2e** and **2f**. (a) 400-MHz ^1H NMR spectrum of **2** in D_2O at 298 K. (b) 61.4-MHz ^2H NMR spectrum of **2e** in ^2H-depleted H_2O at 345 K showing deuterium enrichment at H-12, H-19, and H-37; residual HOD (δ 4.4) was suppressed by inversion recovery water suppression pulse. (c) 61.4-MHz ^2H NMR spectrum of **2f** in ^2H-depleted H_2O at 298 K showing deuterium enrichment at H-18 and H-35.

In order to more clearly define the fate of H-2 of **6**, [2-^2H]-Dap (**6b**) was prepared by acid-catalyzed exchange of **6** at elevated temperature[15] and then fed to *S. capreolus*. The ^2H NMR spectra of the derived antibiotics were expected to be easier to interpret since the additional

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resonances derived from H-3 of **6** would be missing. The ^2H NMR spectra of **1f** and **2f** thus obtained exhibited the expected resonances (cf Figure 2c for **2f**) but contained less deuterium at H-18 and H-35 than expected from the previous feeding. However, these positions were subsequently shown to suffer slow exchange under the fermentation conditions. A sample of **2** was shaken in D_2O at pD 8.4, 30 °C, 250 rpm for 7 days, to simulate the fermentation; both ^1H and ^2H NMR spectra of the sample after freeze–drying and desalting indicated ca. 50% deuteriation at H-2, H-18, and H-35. A portion of this was back-exchanged in H_2O under the same conditions; the resulting sample was now ca. 30% deuteriated at each of the three sites. Thus, absolute levels of apparent incorporations would vary between experiments more than typically observed just from the vagaries of two different biological experiments.

Conclusions

We have established that 1 equiv of serine and alanine are incorporated directly into **1** and **2**, respectively. However, three of the remaining residues of each core cyclic pentapeptide are indirectly derived from serine via 2,3-diaminopropionic acid, **6**. The apparent β-elimination/replacement mechanism revealed by incorporation of the deuteriated amino acids is consistent with previous studies on the biosynthesis of malonomicin[16] in particular and on pyridoxal phosphate-dependent β-replacement reactions[17] in general. However, the clear incorporation of added **6** into the Dap and modified Dap residues is consistent with a prepeptide assembly modification of serine rather than a postpeptide assembly modification (e.g., simple dehydration) such as presumably results in the dehydroalanyl residues of berninamycin[18] and nosiheptide.[19]

In contrast with the incorporation of **6**, results from our previous study[9] of arginine incorporation are consistent with postpeptide assembly modification to yield an *N*-acyldehydroarginyl residue that was then cyclized. The $\Delta^{2,3}$-ureido-Dap residue most reasonably also comes from postpeptide assembly modifications of a peptide Dap residue. Although a few examples of ribosomal biosynthesis of a secondary metabolic peptide have been reported,[20] essentially all microbial peptide antibiotics so far studied at the cell-free level have been shown to be formed by a nonribosomal mechanism.[21,22] The latter involves multifunctional/multienzyme complexes. With some exceptions, most of these appear to involve a pantotheine arm, presumably for covalent attachment of a growing peptide. Although a number of the peptides studied have had an unusual amino acid starter unit, the succeeding residues have generally been either intact protein amino acids or ones that have undergone relatively simple modifications such as α-epimerization or N-methylation within the peptide synthetase complex.[20]

The capreomycins present an opportunity to study the mechanisms and relationships of extensive pre- and postpeptide assembly modification, in addition to the peptide assembly process itself. This will be reported in due course.

Experimental Section

General. *S. capreolus* A250 was obtained from the Eli Lilly Co. *Bacillius subtilis* ATCC 6633 was obtained from Becton Dickinson Microbiology Systems. Radioactivity measurements were carried out using a Beckman Model LS 7800 liquid scintillation counter with automatic quench correction and external standarization to yield disintegrations per minute. L-[U-^{14}C]-Serine, L-[U-^{14}C]-alanine, and L-[U-^{14}C]-cystine were purchased from ICN; DL-[1-^{13}C]-serine and DL-[3-^{13}C]-alanine were purchased from Cambridge Isotope Laboratories. Enrichments in ^2H NMR spectra were determined by cutting and weighing traces of the relevant signals from the capreomycin and the *t*-BuOH (25 μL) that had been included as a chemical shift reference and for deuterium quantitation.

Fermentation of *S. capreolus* A250 and Isolation of 1 and 2. *S. capreolus* was stored as previously described.[9] Fermentation conditions and workup to afford pure **1** and **2** were also described previously.[9]

Synthesis of DL-[2,3,3-$^2H_3$]-2,3-Diaminopropionic Acid (6a). DL-Aspartic acid (2.66 g, 20 mmol), NaOH (1.60 g, 40 mmol), pyridoxal hydrochloride (0.41 g, 2 mmol), and $Al_2(SO_4)_3 \cdot 18H_2O$ (0.33 g, 0.5 mmol) were washed into a 50-mL flask with D_2O (10 mL). The sample was vortexed vigorously and lyophilized. Et_3N (1.4 mL, 10 mmol) and D_2O (20 mL) were added to the sample and the mixture heated at 50 °C for 2 days in an oil bath covered with aluminum foil to minimize photodecomposition of the pyridoxal. The mixture was cooled, and the contents were washed into a 500-mL flask containing water (400 mL), sodium oxalate (0.54 g, 4 mmol), 3-methylpyrazole (0.81 mL, 10 mmol), and 1 M HCl (35 mL, 35 mmol), and the resultant mixture was then loaded onto a Dowex 50W-X8 column (H^+ form, 100–200 mesh, 2.5 × 24 cm). After the column was washed with water, the sample was eluted with 0.1 M NH_4OH. Ninhydrin-positive fractions were pooled, rotary evaporated, redissolved in water, and reevaporated to dryness. The residue was dissolved in 25% DCl (16 mL) and added to a flask which was then evacuated and sealed. After being heated for 8 days at 125 °C, the mixture was cooled, diluted with water, and evaporated to dryness *in vacuo*. The sample was redissolved in water and taken to dryness to remove excess HCl, yielding DL-[2,3,3-$^2H_3$]-aspartic acid (0.80 g, 30%): mp 301 °C dec (lit.[23] mp >300 °C dec); ca. 98% and 97% deuteriated at H-2 and H-3, respectively, as determined from the ^1H and ^2H NMR spectra.

DL-[2,3,3-$^2H_3$]-Aspartic acid (0.77 g, 5.66 mmol) was placed in a 250-mL three-necked flask and dissolved in 20% fuming sulfuric acid (5 mL) with cooling followed by the addition of dry chloroform (15 mL). The mixture was heated to reflux, and NaN_3 (0.80 g, 12.32 mmol) was added in small amounts with efficient stirring over 3 h. The mixture was stirred for a further 4 h and then cooled in an ice bath, and the chloroform layer was poured off. The thick pastelike residue was poured onto crushed ice (ca. 30 g) and diluted to 100 mL. The solution was then passed through a Dowex 50W-X8 column (H^+ form, 100–200 mesh, 2.5 × 11 cm) and washed first with 150 mL of 0.5 M HCl and then with water to neutrality. The adsorbed **6a** was eluted with 10% ethanolic ammonia (4.0 M), and the ninhydrin-positive fractions were combined and evaporated to a small volume (ca. 2 mL). Ethanol (95%, 20 mL) was added slowly, and most of **6a**·HCl precipitated. The precipitate was filtered, washed with 95% ethanol (10 mL), and dried. The filtrate was concentrated to ca. 5 mL, and more **6a**·HCl was recovered. The combined yield was

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0.41 g (69%) as a white powder: mp 234 °C dec (lit.[24] 236–237 °C dec); ^{2}H NMR, 98% deuteration at H-2 and 80% at H-3.

Synthesis of DL-[2-^{2}H]-Diaminopropionic Acid (6b). A solution of DL-diaminopropionic acid monohydrochloride (2.00 g, 19 mmol), D_2O (20 mL), and DCl (35% in D_2O, 1 mL) was placed into a pressure tube, sealed, and heated in an oil bath at 125 °C for 24 h. After cooling, the solvent was removed *in vacuo*, and the resulting residue was dried under vacuum for 2 h. Next, the residue was dissolved in water (50 mL), concentrated to a small volume (ca. 3 mL), and precipitated in absolute ethanol (100 mL). After the residue stood at 4 °C for 4 h, the precipitate was collected by filtration, washed with ethanol, and dried under vacuum to give 1.94 g (96%) of DL-[2-^{2}H]-Dap-HCl: mp 234 °C dec; ^{1}H NMR (D_2O, 300 MHz) δ 4.07 (t, <0.03H, J = 7.1 Hz), 3.50 (s, 2H); ^{2}H NMR, 97% deuteration at H-2.

Incorporation of L-[U-^{14}C]-Serine, L-[U-^{14}C]-Alanine, and L-[U-^{14}C]-Cystine. A single seed culture was used to inoculate three production broths. Aqueous solutions (10 mL each) of L-[U-^{14}C]-serine (10.73 μCi), L-[U-^{14}C]-alanine (11.02 μCi), and L-[U-^{14}C]-cystine (11.98 μCi) were each fed in three equal portions to a separate 200-mL production culture at 12, 32, and 56 h after inoculation. After a total of 6 days, bioassay[9] of the fermentations using *B. subtilis* ATCC 6633 as the test organism indicated ca. 480 mg of total capreomycins for each culture. Workup and purification yielded 113.5 mg of IA (1.36×10^6 dpm/mmol, 2.1% incorporation based on half of the bioassay mass) and 177.0 mg of IB (1.56×10^6 dpm/mmol, 1.6% incorporation based on half of the bioassay mass) from the L-[U-^{14}C]-serine feeding. Similarly, 78.5 mg of IA (3.54×10^5 dpm/mmol, 0.5% incorporation) and 70.8 mg of IB (6.07×10^5 dpm/mmol, 0.9% incorporation) from the L-[U-^{14}C]-alanine feeding and 133.7 mg of IA (3.42×10^5 dpm/mmol, 0.5% incorporation) and 107.2 mg of IB (4.54×10^5 dpm/mmol, 0.6% incorporation) from the L-[U-^{14}C]-cystine feeding were obtained.

Incorporation of DL-[1-^{13}C]-Serine (7a). DL-[1-^{13}C]-Serine (**7a**) (350 mg) was fed in thirds to a 200-mL production culture at 12, 32, and 56 h after inoculation with a seed culture. Bioassay indicated 490 mg of total capreomycins. This was worked up as usual, and 340 mg of crude product was obtained after methanol precipitation. Only the center fractions from each peak obtained from the Amberlite CG-50 were combined, and pure **1b** (79.9 mg) and **2b** (45.4 mg) were collected after the final desalting on DEAE Sepharose CL-6B. A portion of each (40.0 mg) and *t*-BuOH (25 μL) were dissolved in D_2O (0.5 mL) and the ^{13}C NMR spectra (75.5 MHz) obtained with sweep width of 18 518 Hz, 64 K data points, 30° pulse width, 1.769 s acquisition time, and 36 378 scans (**1b**) or 25 600 scans (**2b**). Each FID was transformed with 3.0-Hz line broadening.

Incorporation of DL-[3-^{13}C]-Alanine (8a). A mixture of DL-[3-^{13}C]-alanine (**8a**) (445 mg) and L-[U-^{14}C]-alanine (9.52 μCi) in water (10 mL) was pulse-fed to a 200-mL production culture as described above. After workup and purification, the center fractions yielded 74.7 mg of pure **1c** (1.59×10^5 dpm/mmol, 0.3% incorporation) and 118.5 mg of pure **2c** (3.35×10^5 dpm/mmol, 0.7% incorporation). The ^{13}C NMR spectra were acquired with 22 050 scans for **1c** and 18 450 scans for **2c**.

Incorporation of DL-[2,3,3-$^2H_3$]-Serine (7b). A mixture of DL-[2,3,3-$^2H_3$]-serine (**7b**) (440 mg) and L-[U-^{14}C]-serine (8.06 μCi) in water (10 mL) was pulse-fed to a 200-mL production broth as described above. After workup and purification, 152.2 mg of pure **1d** (2.63×10^5 dpm/mmol, 0.8% incorporation) and 135.3 mg of pure **2d** (4.57×10^5 dpm/mmol, 1.5% incorporation) were obtained. Samples of each (20.0 mg of **1d** and 31.7 mg of **2d**) in deuterium-depleted water (0.5 mL) plus *t*-BuOH (25 μL) were analyzed by ^{2}H NMR (61.4 MHz) at 298 and 345 K, as previously described.[9] A total of 36 860 scans at 298 K and 14 679 scans at 345 K was acquired for **1d** and 54 180 scans at 298 K and 12 288 scans at 345 K for **2d**.

Incorporation of DL-[2,3,3-$^2H_3$]-Diaminopropionic Acid (6a). DL-[2,3,3-$^2H_3$]-Diaminopropionic acid (**6a**) (410 mg) in water (10 mL) was pulse-fed to a 200-mL production broth as described above. After workup and purification, 47.3 mg of pure **1e** and 32.6 mg of pure **2e** were obtained. Each sample in deuterium-depleted water (0.5 mL) plus *t*-BuOH (25 μL) was analyzed by ^{2}H NMR (61.4 MHz) at 298 and 330 K, as previously described.[9] A total of 1892 scans at 298 K and 11 264 scans at 330 K was acquired for **1e** and 2183 scans at 298 K and 13 312 scans at 330 K for **2e**.

Incorporation of DL-[2-^{2}H]-Diaminopropionic Acid (6b). DL-[2-^{2}H]-Diaminopropionic acid (**6b**) (800 mg in 30 mL water, pH was adjusted to 7 with Na_2CO_3 prior to feeding) was fed in four equal portions to a 200-mL production broth as described above. The workup was carried out without the methanol precipitation step. After purification with Amberlite CG-50 column (NH_4^+ form, 100–200 mesh, 1.0 × 42.5 cm), 62.3 mg of **1f** and 54.0 mg of **2f** were obtained. Samples of each (26.6 mg of **1f** and 54.0 mg of **2f**) in deuterium-depleted water (0.5 mL) plus *t*-BuOH (50 μL) were analyzed by ^{2}H NMR (61.4 MHz) at 298 K; 36 115 scans were acquired for **1f** and 27 500 scans for **2f**.

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