Biosynthesis of the Antitumor Antibiotic Sparsomycin

Ronald J. Parry,* Yan Li, and Elizabeth Eudy Gomez

Contribution from the Department of Chemistry, Rice University, Houston, Texas 77251.
Received November 18, 1991

Abstract: The biosynthesis of the antitumor antibiotic sparsomycin (**1**) has been investigated by administration of isotopically labeled precursors to *Streptomyces sparsogenes* var. *sparsogenes*. These studies indicated that the dithioacetal moiety (**2**) of sparsomycin is derived from L-cysteine via the intermediacy of *S*-methylcysteine and *S*-(methylthiomethyl)cysteine, with reduction of the carboxyl group of *S*-(methylthiomethyl)cysteine preceding attachment of the uracil moiety (**3**). Investigations of the origin of the uracil moiety (**3**) of sparsomycin revealed that it is derived from L-tryptophan by a process that involves loss of the side chain and oxidative ring opening of both rings of the amino acid. The intermediacy of *N*-formylanthranilic acid in this process was ruled out. Investigations of the terminal stage in the biosynthesis of the uracil moiety (**3**) demonstrated that it resembles the conversion of inosine-5′-monophosphate into xanthosine-5′-monophosphate.

Sparsomycin (**1**) (Scheme I) is a unique antibiotic isolated from the fermentation broth of *Streptomyces sparsogenes* var. *sparsogenes*[1] and *Streptomyces cuspidosporus*.[2] The structure of sparsomycin was elucidated in 1970 by Wiley and MacKellar,[3] while the absolute configuration was deduced by Ottenheijm and co-workers in 1981.[4] Three total syntheses of sparsomycin have been reported in the literature.[5] Sparsomycin exhibits antibiotic activity against a variety of gram-negative and gram-positive bacteria, and it shows potent antitumor activity against KB human epidermoid carcinoma cells in vitro.[6] The biological activity of sparsomycin is undoubtedly the result of its ability to inhibit the peptide bond-forming step of protein biosynthesis.[7] This inhibition appears to occur by reversible binding of sparsomycin to the ribosomal A site of the peptidyl transferase.[8] Although the clinical use of sparsomycin is precluded due to drug-related retinopathy, a number of more potent analogues have been prepared which are currently in clinical trial.[8]

The unusual structure of sparsomycin and its potentially important biological activity stimulated us to investigate the biosynthesis of this interesting compound. The results of these studies will now be summarized.[9]

Results

From the viewpoint of biosynthetic investigations, the sparsomycin molecule can be formally divided into two fragments, the monooxo-dithioacetal moiety (**2**) and the uracil moiety (**3**) (Scheme I). The biosynthesis of each of these fragments will be discussed separately.

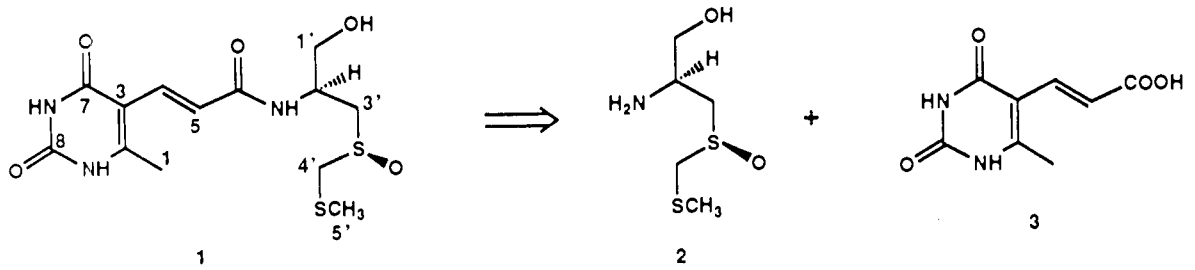
Origin of the Dithioacetal Moiety (2). The structure of the monooxo-dithioacetal moiety suggests that L-methionine and L-cysteine are likely to serve as primary precursors. Administration of (*methyl*-^{13}C)-L-methionine to *S. sparsogenes* yielded antibiotic that was labeled at both C-4′ and C-5′ (Table I, expt 1), thereby demonstrating that both of these carbon atoms are derived from the methyl group of methionine. Also noteworthy was the fact that the C-methyl group (C-1) of sparsomycin was not labeled in this experiment. Administration of (3-^{13}C)-DL-cysteine, synthesized by the method of Gasparini et al.,[10] produced sparsomycin that exhibited the expected enrichment at C-3′ (Table I, expt 2). However, enrichment also appeared at C-4′ of the antibiotic, an observation suggesting that some of the labeled cysteine might be converted into (3-^{13}C)serine which could then label the C_1 pool. Administration of (1-^{13}C)-DL-serine yielded antibiotic that showed the anticipated enrichment at C-1′ of the moiety **2** (Table I, expt 3). This result demonstrates that C-1′ is derived from the carboxyl group of serine and implies that C-1′ to C-3′ of **2** are derived from an intact L-cysteine unit. This conclusion is further supported by the specific incorporation of (2,3-$^{13}C_2$)-DL-serine into **2** described below.

The timing of the reduction of the cysteine carboxyl group versus the formation of the monooxo-dithioacetal moiety was next examined. L- and D-(4-^{13}C)-*S*-(methylthiomethyl)cysteine (**4** and **5**) were synthesized[5a,11] for this purpose (Scheme IIa). Both enantiomers were prepared since the absolute configuration at C-2′ of the antibiotic corresponds to that of D-cysteine. Administration of **4** and **5** to *S. sparsogenes* yielded antibiotic that exhibited high levels of enrichment at the expected position (Table I, expts 4 and 5). The monooxo-dithioacetal moiety therefore appears to be formed before reduction of the cysteine derived carboxyl group, and the epimerization of the asymmetric center present at C-2 of the amino acid can occur at a late stage. Additional information on the timing of carboxyl group reduction was sought by evaluation of L- and D-(4-^{13}C)-*S*-(methylthiomethyl)cysteinol (**6** and **7**) as precursors. These compounds were prepared from **4** and **5** (Scheme IIb). Administration of these two labeled alcohols followed by NMR analysis of the resulting sparsomycin revealed that both of the compounds served as specific precursors, although the incorporation figures were considerably lower than those observed with the amino acids **4** and **5** (Table I, expts 6 and 7). The specific incorporation of the L-isomer was somewhat surprising and raised concerns that the incorporation of **6** and **7** might take place via oxidation back to the corresponding amino acids, **4** and **5**. These concerns were allayed by administration of (1-$^2H_2$)-**6** and (1-$^2H_2$)-**7**, which were prepared by utilizing sodium borodeuteride to reduce the methyl esters of unlabeled **4** and **5** to the corresponding alcohols. Examination of the sparsomycin derived from deuterated **6** and **7** by ^{2}H NMR revealed that, in both cases, deuterium was present at C-1′ of the sparsomycin skeleton (Table I, expts 8 and 9). Consequently, one can conclude that **6** and **7** are incorporated into sparsomycin without prior oxidation to the amino acids **4** and **5**. The specific incorporation of **6** and **7** suggests that reduction of the cysteine derived carboxy group precedes

(1) Argoudelis, A. D.; Herr, R. R. *Antimicrob. Agents Chemother.* **1962**, 780.
(2) Higashide, E.; Hasegawa, T.; Shibata, M.; Mizuno, K.; Akaike, H. *Takeda Kenkyusho Nempo* **1966**, *25*, 1; *Chem. Abstr.* **1967**, *66*, 54238.
(3) Wiley, P. F.; MacKellar, F. A. *J. Am. Chem. Soc.* **1970**, *92*, 417. Wiley, P. F.; Mackellar, F. A. *J. Org. Chem.* **1976**, *41*, 1858.
(4) Ottenheijm, H. C. J.; Liskamp, R. M. J.; Helquist, P.; Lauher, J. W.; Shekhani, M. S. *J. Am. Chem. Soc.* **1981**, *103*, 1720.
(5) (a) Ottenheijm, H. C. J.; Liskamp, R. M. J.; van Nispen, S. P. J. M.; Boot, H. A.; Tijhuis, M. W. *J. Org. Chem.* **1981**, *46*, 3273. (b) Helquist, P.; Shekhani, M. S. *J. Am. Chem. Soc.* **1979**, *101*, 1057. (c) Hwang, D. R.; Helquist, P.; Shekhani, M. S. *J. Org. Chem.* **1985**, *50*, 1264.
(6) Owen, S. P.; Dietz, A.; Camiene, G. W. *Antimicrob. Agents Chemother.* **1962**, 772.
(7) Goldberg, I. H. *Antibiotics*; Hahn, F. E., Ed.; Springer-Verlag: New York, 1979; Vol. 1, pp 264-271.
(8) Lazaro, E.; Felix, A. S.; van den Broek, L. A. G. M.; Ottenheijm, H. C. J.; Ballesta, J. P. G. *Antimicrob. Agents Chemother.* **1991**, *35*, 10.
(9) Portions of this work have been the subject of a preliminary communication: Parry, R. J.; Eudy, E. E. *J. Am. Chem. Soc.* **1988**, *110*, 2316.
(10) Gasparini, G. M.; Pichat, L.; Baret, C. *Bull. Soc. Chim. Fr.* **1965**, 794.
(11) Bohme, H.; Fischer, H.; Frank, R. *Ann.* **1949**, *563*, 54.

0002-7863/92/1514-5946$03.00/0

Scheme I



Scheme II

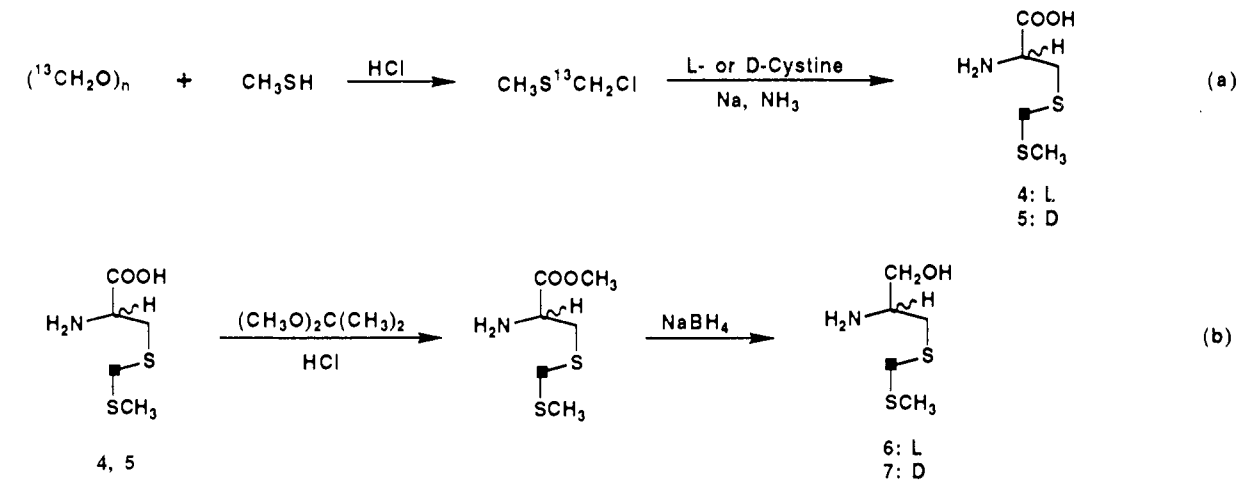


Table I. Incorporation of Precursors into the Dithioacetal Moiety (**2**) of Sparsomycin

expt	precursor ($^3H/^{14}C$)	% enrichment	labeling pattern
1	(*methyl*-^{13}C)-L-methionine	2.7 (C-4′) 6.7 (C-5′)	C-4′, C-5′
2	(3-^{13}C)-DL-cysteine	1.0 (C-3′) 0.6 (C-4′)	C-3′, C-4′
3	(1-^{13}C)-DL-serine	2.5	C-1′
4	(4-^{13}C)-*S*-(methylthiomethyl)-L-cysteine	84	C-4′
5	(4-^{13}C)-*S*-(methylthiomethyl)-D-cysteine	72	C-4′
6	(4-^{13}C)-*S*-(methylthiomethyl)-L-cysteinol	1.3	C-4′
7	(4-^{13}C)-*S*-(methylthiomethyl)-D-cysteinol	6.7	C-4′
8	(1-$^2H_2$)-*S*-(methylthiomethyl)-L-cysteinol	4.6	C-1′
9	(1-$^2H_2$)-*S*-(methylthiomethyl)-D-cysteinol	3.8	C-1′
10	(*methyl*-^{13}C)-*S*-methyl-L-cysteine	0.3	C-4′
11	(*methyl*-^{13}C)-*S*-methyl-D-cysteine	0.6	C-4′
		1.1 (C-4′)[a] 0.6 (C-5′)[a]	C-4′, C-5′
12	[*methyl*-^{14}C,3-^{3}H]-*S*-methyl-L-cysteine ($^3H/^{14}C$ = 5.05)		$^3H/^{14}C$ = 40
13	(2-^{13}C)glycine	0.5 (C-4′) 2.2 (C-5′)	C-4′, C-5′
14	(2,3-$^{13}C_2$)-DL-serine	0.9 (C-2′) 0.9 (C-3′) 1.4 (C-4′) 2.7 (C-5′)	C-2′, C-3, $^1J_{CC}$ = 38 Hz C-4′, C-5′
15	(*methyl*-$^2H_3$,*methyl*-^{13}C)-L-methionine	3.9	C-5′ (upfield shift = 0.68 ppm)

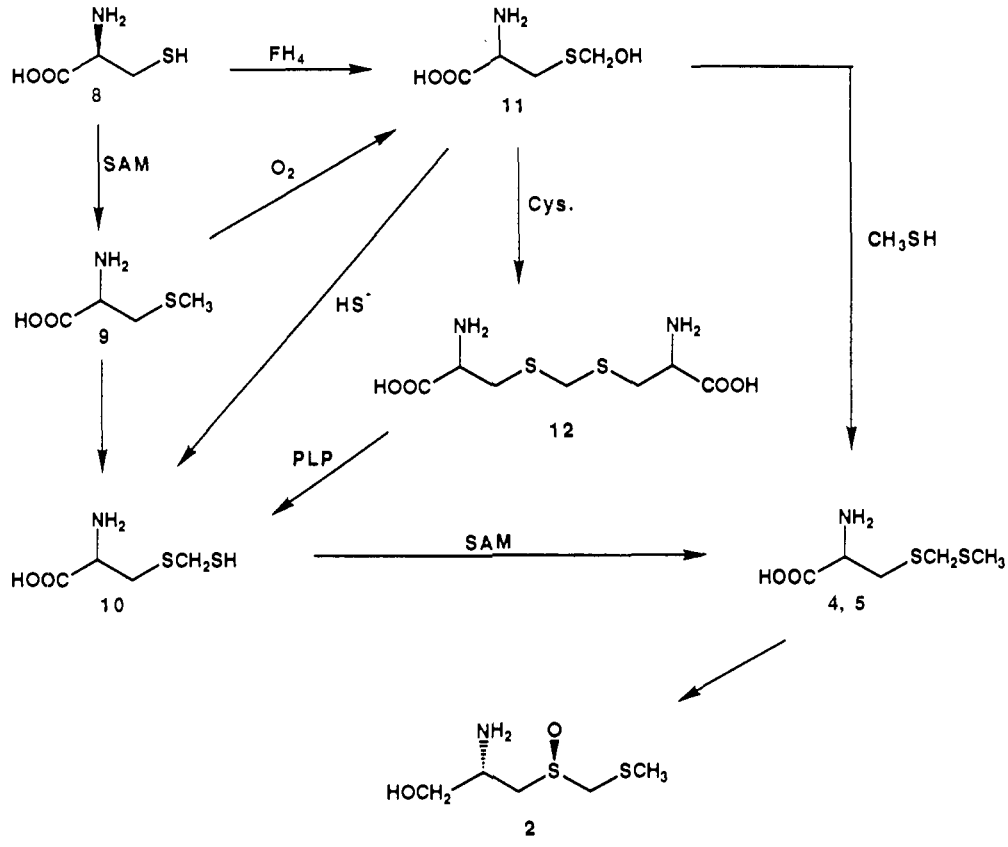
[a] Results from a second incorporation experiment using an improved strain of *S. sparsogenes*.

coupling of the uracil moiety to the cysteine derived amino group.

Mechanism of Formation of the Dithioacetal Group. The results of the precursor incorporation experiments just discussed strongly support the hypothesis that L-cysteine (**8**) is converted into sparsomycin via the intermediacy of L- and/or D-*S*-(methylthiomethyl)cysteine (**4** and **5**). A crucial question with respect to sparsomycin biosynthesis therefore concerns the mechanism of the conversion of cysteine into *S*-(methylthiomethyl)cysteine. Scheme III summarizes the most plausible routes that can be envisioned for this transformation. One route would proceed via methylation of cysteine in a reaction using *S*-adenosylmethionine (SAM) as a cofactor to yield *S*-methylcysteine (**9**). *S*-Methylcysteine could then be converted into *S*-(methylthiomethyl)cysteine in two ways. The first way would be by direct insertion of sulfur into the *S*-methyl group to generate the dithiohemiacetal **10**, subsequent methylation of which would then produce **4** or **5**. The postulated sulfur insertion reaction would presumably be related to the sulfur insertion reactions encountered in the biosynthesis of biotin, lipoic acid, and isopenicillin N.[12] The second way would proceed by hydroxylation of the *S*-methyl group of *S*-methylcysteine to yield the monothiohemiacetal **11**. Reaction of **11** with hydrosulfide and SAM or with methanethiol would then yield **4** or **5**. Alternatively, **11** could react with cysteine to yield djenkolic acid (**12**), an amino acid that occurs naturally in *Pithecolobium lobatum* and *Albizzia lophanta*.[13] The djenkolic acid could

(12) Parry, R. J. *Tetrahedron* **1983**, *39*, 1215.

Scheme III



fragment by a pyridoxal mediated process to form **10** and then be converted to **4** or **5**.

The second route that can be envisioned for the conversion of cysteine into *S*-(methylthiomethyl)cysteine would proceed by reaction of cysteine with methylene-tetrahydrofolate to form the monothiohemiacetal **11**. Once formed, **11** could then be converted to **4** or **5** in the manner already described.

Evaluation of the pathways outlined in Scheme III was initiated by precursor incorporation experiments with L- and D-(*methyl*-^{13}C)-*S*-methylcysteine, which were synthesized by reduction of L- and D-cystine with sodium in liquid ammonia followed by alkylation with (^{13}C)methyl iodide.[39] Administration of the two enantiomeric forms of *S*-methylcysteine indicated that they are both specific precursors of sparsomycin since they each label the antibiotic at the expected position (C-4′) (Table I, expts 10 and 11). However, in one of two experiments utilizing D-*S*-methylcysteine as a precursor, some enrichment was also present at C-5′. This result indicated that some demethylation of *S*-methylcysteine might be taking place in vivo. This suspicion was confirmed by means of a double-label experiment which was carried out with [*methyl*-^{14}C, 3-^{3}H]-L-*S*-methylcysteine. The sparsomycin derived from this precursor exhibited an 8-fold increase in the tritium to carbon-14 ratio (Table I, expt 12). Since this observation raised concerns about the intact incorporation of *S*-methylcysteine into sparsomycin, an isotope dilution experiment was carried out in an attempt to verify that *S*-methylcysteine was truly an intermediate in the biosynthesis of the dithioacetal moiety. [U-^{14}C]-L-Cystine was administered to 300 mL of the *S. sparsogenes* fermentation and the mycelium isolated after 10 h. The washed mycelium was sonicated in the presence of carrier L-*S*-methylcysteine, and the amino acid was then reisolated from the centrifuged sonicate by ion-exchange and chromatography on cellulose. The purified L-*S*-methylcysteine was converted into its *N*-acetyl-*p*-bromophenacyl ester which was first purified chromatographically and then recrystallized to constant specific activity. The final incorporation figure (0.02%) suggests that L-*S*-methylcysteine is present in *S. sparsogenes*.

Additional evidence favoring the intermediacy of *S*-methylcysteine in the biosynthesis of the dithioacetal moiety (**2**) was obtained by evaluation of the alternate route to **2** shown in Scheme III, that which proceeds by reaction of cysteine with methylene-tetrahydrofolate. If the C-4′ methylene group of sparsomycin is derived from the folate pool, then administration of (2-^{13}C)glycine or (3-^{13}C)serine to the fermentation should lead to preferential labeling of C-4′ over C-5′, since C-2 of glycine and C-3 of serine serve as direct sources of the C_1 unit of methylenetetrahydrofolate.[14,15] In the event, administration of (2-^{13}C)glycine led to sparsomycin exhibiting a significantly higher level of enrichment at C-5′ than at C-4′ (Table I, expt 13). The incorporation behavior of (2,3-$^{13}C_2$)-DL-serine[16] was then examined. A doubly ^{13}C labeled form of serine was utilized in order to enhance the sensitivity of the NMR analysis, since an earlier incorporation experiment with (3-^{13}C)-DL-serine had yielded sparsomycin which failed to show enrichment in the cysteine derived portion of **2**.[9] Incorporation of the doubly-labeled serine into sparsomycin gave results that were consistent with those observed in the glycine feeding; i.e., a higher level of ^{13}C enrichment was found at C-5′ of the dithioacetal moiety than at C-4′ (Table I, expt 14). In addition, ^{13}C–^{13}C coupling was observed between C-2′ and C-3′ of **2** in accordance with the expectation that cysteine should be derived from serine in *S. sparsogenes*. The last experiment that was carried out in order to evaluate a possible folate origin for C-4′ of the dithioacetal moiety was to administer (5-^{13}C)-(2*S*,8*S*)-djenkolic acid, which was prepared from D-cysteine and (^{13}C)-formaldehyde.[17] No incorporation into sparsomycin was observed with this precursor (data not shown).

(13) van Veen, A. G.; Hyman, A. *J. Recl. Trav. Chim.* **1935**, *54*, 493. Gmelin, G.; Hasenmaier, G.; Strauss, G. *Z. Naturforsch.* **1957**, *12B*, 687.

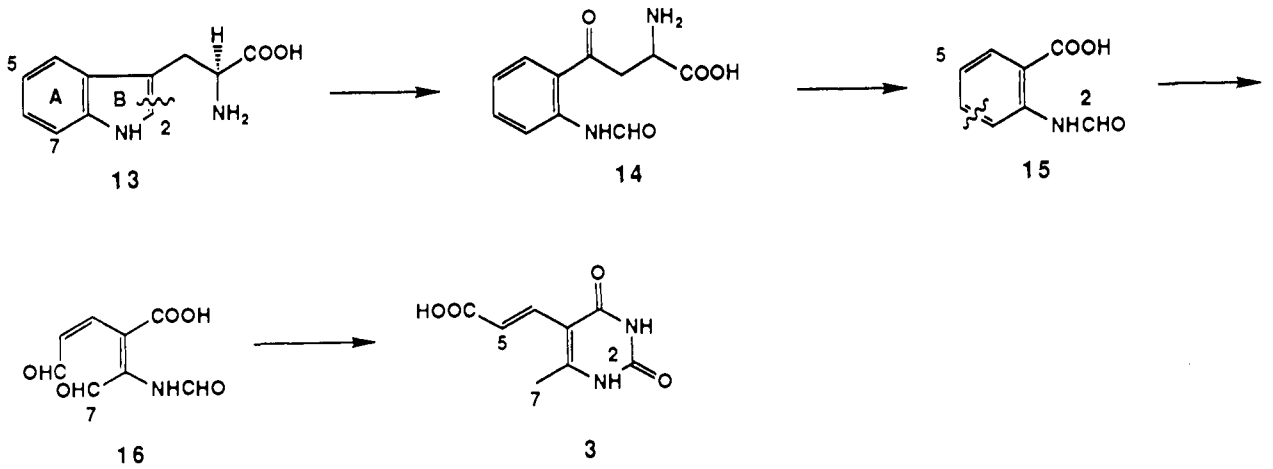
(14) Kochi, H.; Kikuchi, G. *J. Biochem.* **1974**, *75*, 1113.

(15) Yoshida, T.; Kikuchi, G. *J. Biochem.* **1972**, *72*, 1503.

(16) Prepared from (^{13}C)formaldehyde and (2-^{13}C)diethyl acetamidomalonate by the method of King: King, J. A. *J. Am. Chem. Soc.* **1947**, *69*, 2738.

(17) Armstrong, M. D.; du Vigneaud, V. *J. Biol. Chem.* **1947**, *168*, 373.

Scheme IV



Since the weight of the evidence appeared to favor *S*-methylcysteine as an intermediate in the biosynthesis of the dithioacetal moiety (**2**), experiments were carried out whose goal was to determine the number of hydrogens removed from the methyl group of *S*-methylcysteine as the result of the formation of the dithioacetal group. (*methyl*-^{13}C,*methyl*-$^{2}H_3$)-*S*-Methyl-D-cysteine and (*methyl*-^{13}C,*methyl*-$^{2}H_3$)-L-methionine were synthesized from (^{13}C,$^{2}H_3$)methyl iodide for this purpose. Administration of the doubly-labeled *S*-methylcysteine gave a surprising result: no enrichment was observed when the resulting sparsomycin was examined either by ^{13}C NMR or by ^{2}H NMR (data not shown). Similar behavior was encountered with the doubly-labeled methionine: no ^{13}C or deuterium enrichment was present at C-4′ of the antibiotic. However, the sparsomycin derived from methionine did exhibit ^{13}C and deuterium enrichment at C-5′. Furthermore, an examination of the ^{13}C NMR spectrum while simultaneously decoupling both protons and deuterons confirmed the presence of three deuterium atoms at C-5′ of the antibiotic (Table I, expt 15).

Origin of the Uracil Moiety (3). Another important question to be answered with respect to sparsomycin biosynthesis concerns the origin of the uracil moiety (**3**). After some negative preliminary experiments examining this question, it occurred to us that this moiety could be derived from L-tryptophan in the manner shown in Scheme IV. This hypothetical pathway begins with the familiar[18] oxidative cleavage of L-tryptophan (**13**) to *N*-formylkynurenine (**14**). Loss of the amino acid side chain from **14** could then give *N*-formylanthranilic acid (**15**). Oxidative cleavage of the *N*-formylanthranilic acid to **16** followed by several additional steps would finally generate the uracil moiety (**3**). This hypothesis predicts that C-8 of sparsomycin should be derived from the *N*-formyl group of *N*-formylanthranilic acid which would in turn be derived from C-2 of tryptophan, while C-5 of the antibiotic should correspond to C-5 of tryptophan.

The pathway outlined in Scheme IV was first evaluated by administration of [5-^{3}H]-L-tryptophan to the sparsomycin fermentation. Since radioactive sparsomycin was obtained in this experiment (data not shown), a double-label experiment was carried out with [5-^{3}H,U-^{14}C]-L-tryptophan and the isotopic ratio of the resulting antibiotic was determined (Table II, expt 1). The biosynthetic hypothesis predicts complete retention of tritium and retention of eight carbon atoms of tryptophan. Since the precursor exhibited a ratio of 5.45, the antibiotic should display a ratio of 7.49, if eight carbons are retained. Should seven carbons of tryptophan be retained, the predicted ratio would be 8.56. The experimental value (7.96) fell between the two theoretical values, and so it does not allow one to decide whether seven or eight carbon atoms of tryptophan have been incorporated into sparsomycin. Nevertheless, the data clearly support the hypothesis that tryptophan is a specific precursor of sparsomycin. Additional support for the specific incorporation of tryptophan into the uracil moiety of sparsomycin was obtained from two experiments. In the first of these experiments, (2-^{13}C)-DL-tryptophan was synthesized by a combination of literature procedures[19] and administered to *S. sparsogenes*. The results of this experiment were highly satisfying, as a strong enrichment was apparent at C-8 of the antibiotic (Table II, expt 2). The second experiment utilized (5-$^{2}H_1$)-DL-tryptophan[20] as a precursor. The ^{2}H NMR spectrum of the antibiotic isolated in this experiment exhibited a high deuterium enrichment at 7.17 ppm, thereby demonstrating that C-5 of sparsomycin is derived from C-5 of trytophan (Table II, expt 3).[21] The data obtained from these two experiments are completely consistent with the biosynthetic hypothesis shown in Scheme IV.

Table II. Incorporation of Precursors into the Uracil Moiety (**3**) of Sparsomycin

expt	precursor (^{3}H/^{14}C)	% enrichment[a]	labeling pattern
1	[U-^{14}C,5-^{3}H]-L-tryptophan (^{3}H/^{14}C = 5.45)	0.16	^{3}H/^{14}C = 7.96
2	(2-^{13}C)-DL-tryptophan	22	C-8
3	(5-$^{2}H_1$)-DL-tryptophan	6	C-5
4	(3,5-$^{2}H_2$)-*N*-formyl-anthranilic acid	38	C-5
5	(*formyl*-^{13}C,5-$^{2}H_1$)-*N*-formylanthranilic acid	39 (C-5) (^{2}H)	C-5, C-1′, C-2′, C-3′
		0.9 (C-1′) (^{13}C)	C-4′, C-5′
		0.6 (C-2′) (^{13}C)	
		0.9 (C-3′) (^{13}C)	
		0.8 (C-4′) (^{13}C)	
		2.8 (C-5′) (^{13}C)	
6	(*carboxyl*-^{13}C,3,5-$^{2}H_2$)-*N*-formylanthranilic acid	20.9 (C-5) (^{2}H)	C-5, C-5′
		0.3 (C-5′) (^{13}C)	
7	(2-^{13}C)indole	26	C-8
8	(*indole*-$^{2}H_5$)-DL-tryptophan	47 (C-4)	C-4, C-5
		47 (C-5)	
9	(2′-^{13}C)-(*E*)-3-(4′-hydroxy-6′-methyl-5′-pyrimidinyl)-acrylic acid	7	C-8

[a] The figure for expt 1 represents the percent ^{14}C incorporation.

Mechanism of Formation of the Uracil Moiety (3). Investigations of the mechanism of conversion of tryptophan into the uracil moiety (**3**) were begun with evaluation of the potential intermediacy of *N*-formylanthranilic acid (**15**). (3,5-$^{2}H_2$)-*N*-Formylanthranilic acid was synthesized for this purpose from

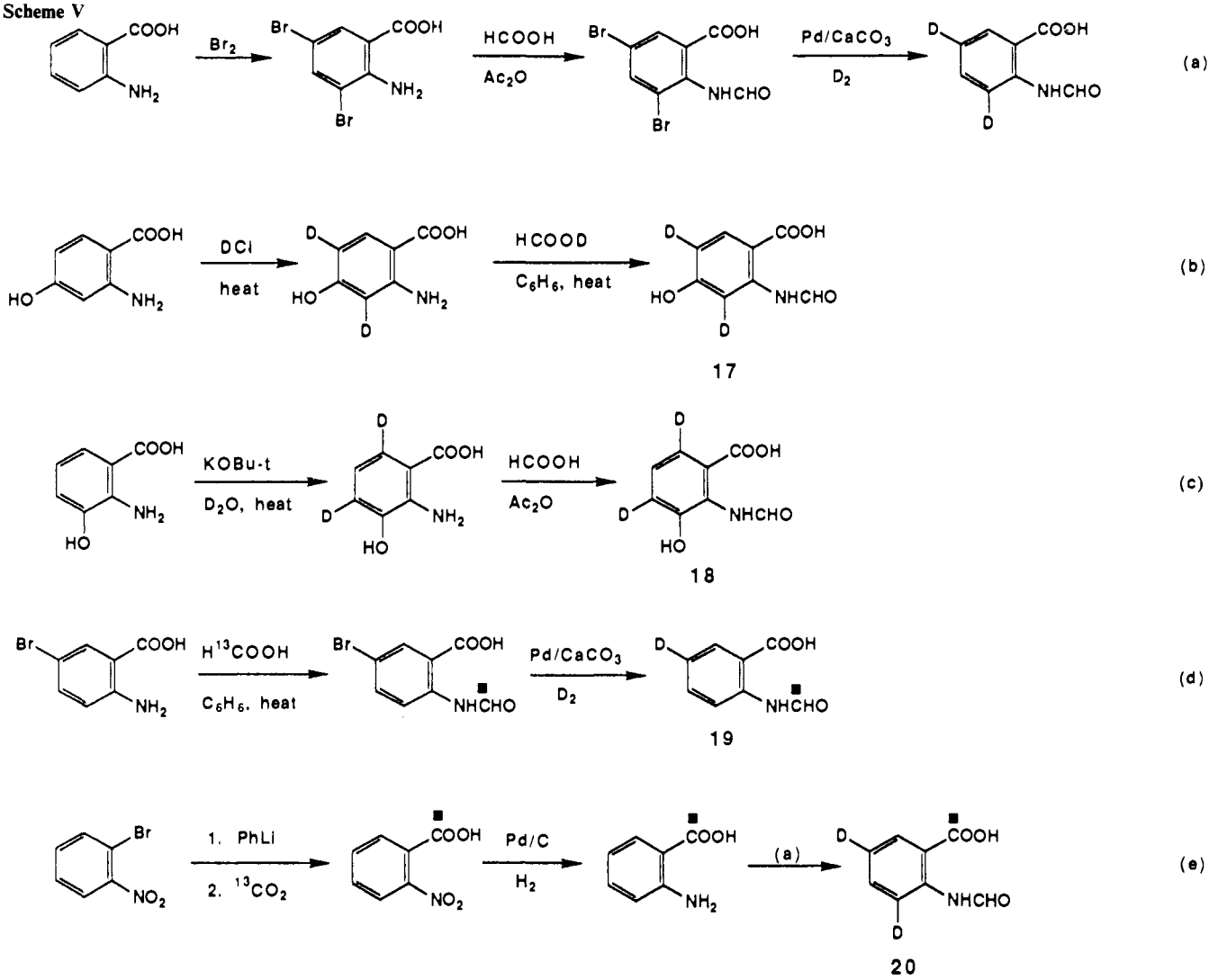
(18) Metzler, D. E. *Biochemistry*; Academic Press: New York, 1977; pp 867–869.

(19) Krimen, L. I.; Savage, J.; Yates, P. *Org. Synth.* **1970**, *50*, 1. Hurd, C. D.; Roe, A. S. *J. Am. Chem. Soc.* **1939**, *61*, 3355. Tyson, F. T.; Shriner, R. L.; Tilford, C. H. *Organic Syntheses*; Wiley: New York, 1955; Vol. III, p 479. Heidelberger, C. *J. Biol. Chem.* **1949**, *179*, 139. Albertson, N. F.; Archer, S.; Suter, C. M. *J. Am. Chem. Soc.* **1945**, *67*, 36.

(20) Birkofer, L.; Hempel, K. *Chem. Ber.* **1963**, *96*, 1373.

(21) The resonance position of the corresponding proton in sparsomycin is at 7.11 ppm.

Scheme V



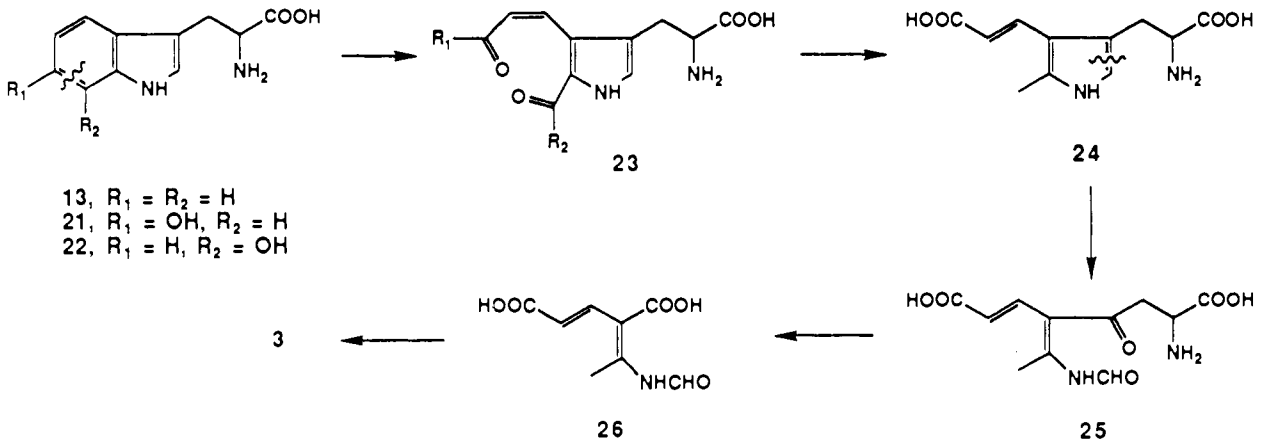
anthranilic acid as shown in Scheme Va. Administration of this precursor to the fermentation yielded sparsomycin whose ^2H NMR spectrum revealed that significant deuterium enrichment was present at C-5 of the antibiotic (Table II, expt 4). However, no enrichment was present at C-1, the other site of expected labeling. Nevertheless, this experiment demonstrated that *N*-formylanthranilic acid was specifically incorporated into sparsomycin. We therefore proceeded to examine the oxidative ring-cleavage of **15** which must occur for its conversion to the uracil moiety. Since the aromatic ring of **15** is electron deficient, it appeared likely that activation for the ring cleavage would be provided by hydroxylation prior to cleavage. The most plausible site for hydroxylation of **15** appeared to be C-4, since cleavage would then directly generate the carboxyl group present in **3**, but hydroxylation at C-3 also remained a possibility. (3,5-$^2H_2$)-4-Hydroxy-*N*-formylanthranilic acid (**17**) was synthesized from 4-hydroxyanthranilic acid[22] as shown in Scheme Vb and administered to the fermentation in the usual way. Examination of the resulting sparsomycin by ^2H NMR failed to show any enrichment (data not shown). This result was verified by synthesis and administration of (*formyl*-^{13}C)-4-hydroxy-*N*-formylanthranilic acid, which was easily prepared from 4-hydroxyanthranilic acid by treatment with (^{13}C)formic acid in refluxing benzene. This form of 4-hydroxy-*N*-formylanthranilic acid also failed to label sparsomycin at the expected position (C-8), but ca. 0.6% enrichment appeared at C-4′, suggesting that the C_1 pool had been labeled by deformylation. The negative results with 4-hydroxy-*N*-formylanthranilic acid led us to evaluate 3-hydroxy-*N*-formylanthranilic acid. (4,6-$^2H_2$)-3-Hydroxy-*N*-formylanthranilic acid (**18**) was prepared as shown in Scheme Vc. Administration of this precursor to *S. sparsogenes* also gave unlabeled sparsomycin (data not shown).

The lack of incorporation of hydroxylated forms of *N*-formylanthranilic acid into sparsomycin as well the deformylation observed when (*formyl*-^{13}C)-4-hydroxy-*N*-formylanthranilic acid was used as a precursor raised concerns as to whether *N*-formylanthranilic acid was truly an intermediate on the pathway from tryptophan to the uracil **3**. If *N*-formylanthranilic acid were deformylated by *S. sparsogenes* to give anthranilic acid, then the anthranilic acid could be converted to tryptophan by the normal biosynthetic pathway leading to this amino acid.[23] The status of *N*-formylanthranilic acid as a sparsomycin precursor was therefore reevaluated. Two experiments were carried out for this purpose. For the first experiment, (*formyl*-^{13}C,5-$^2H_1$)-*N*-formylanthranilic acid (**19**) was synthesized (Scheme Vd) and used as a precursor. Intact incorporation of this precursor was expected to label sparsomycin with ^{13}C at C-8 and with deuterium at C-5. In the event, the antibiotic formed from this precursor was found to carry a deuterium label at C-5, but no ^{13}C label was present at C-8. Curiously, some ^{13}C labeling appeared to be present at C-1′, -2′, -3′, -4′, and -5′, a result which must presumably be due to labeling of the C_1 pool (Table II, expt 5). The labeling pattern observed with **19** confirmed our suspicions that *N*-formylanthranilic acid was being deformylated before incorporation into sparsomycin. Unequivocal proof was obtained by the second experiment which utilized (1-^{13}C,3,5-$^2H_2$)-*N*-formyl-

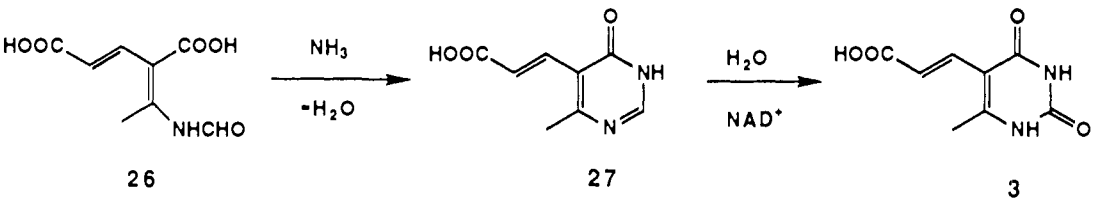
(22) Tamura, K.; Yamagata, K. *Nippon Kagaku Zasshi* **1964**, *85*, 72.

(23) Voet, D.; Voet, J. G. *Biochemistry*; Wiley: New York, 1990; p 723.

Scheme VI



Scheme VII



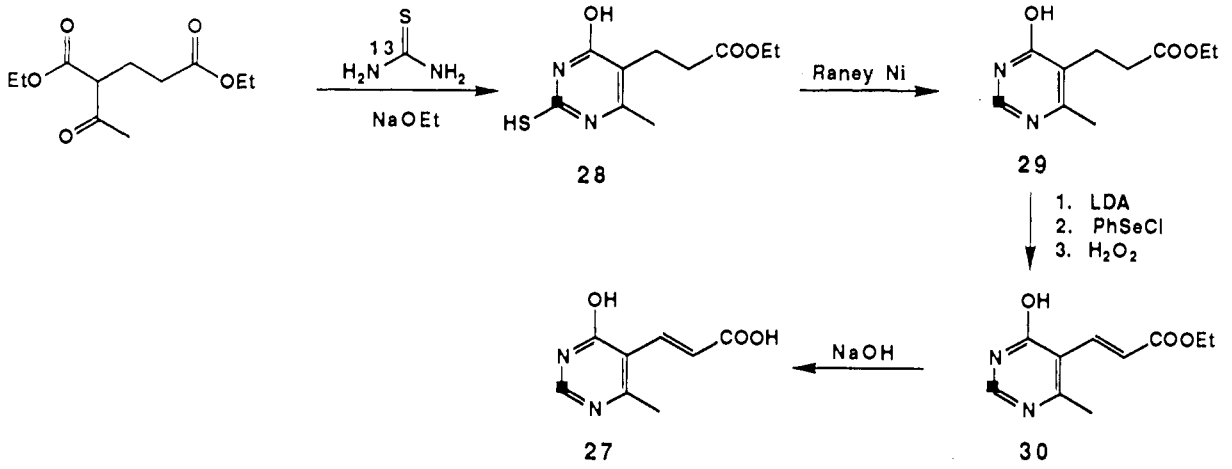
anthranilic acid (**20**), synthesized according to Scheme Ve, as a precursor. The rationale for this experiment was provided by the fact that incorporation of *N*-formylanthranilic acid by deformylation and conversion back to tryptophan should result in the loss of the label ^{13}C-labeled carboxyl group.[23] The labeling pattern in the sparsomycin derived from this precursor was exactly as predicted: strong deuterium enrichment was present at C-5 of the antibiotic, but no ^{13}C enrichment was apparent at C-7; the small amount of ^{13}C enrichment which appeared at C-5′ was attributed to labeling of the C_1 pool by labeled CO_2 (Table II, expt 6).

The results from the precursor incorporation experiments with multiply-labeled forms of *N*-formylanthranilic acid provided convincing evidence that this compound does not lie on the pathway from tryptophan to **3** and that the biosynthetic hypothesis shown in Scheme IV cannot be correct. It was therefore necessary to devise an alternative biosynthetic hypothesis consistent with these observations. Such a hypothesis is outlined in Scheme VI. The principal distinction between the revised pathway and the preceding one is that the timing for the oxidative cleavage of rings A and B of tryptophan has been reversed; i.e., ring A is cleaved before ring B. In its simplest version, this pathway proceeds by cleavage of ring A of L-tryptophan (**13**), 6-hydroxy-L-tryptophan (**21**), or 7-hydroxy-L-tryptophan (**22**) to yield the intermediate **23**. A cis/trans isomerization of the acrylic acid side chain and adjustment of the oxidation levels of both side chains would then yield the pyrrole derivative **24**. Oxidative cleavage of the pyrrole ring would next produce **25** which, by analogy with the reaction catalyzed by kynureninase,[24] could lose its amino acid side chain to produce **26**. Additional steps would then be required to convert **26** into **3** (vide infra). Although the loss of the amino acid side chain is postulated to occur at a late stage in Scheme VI, it could certainly occur at an earlier stage through a process mimicking the reaction catalyzed by tryptophanase.[25]

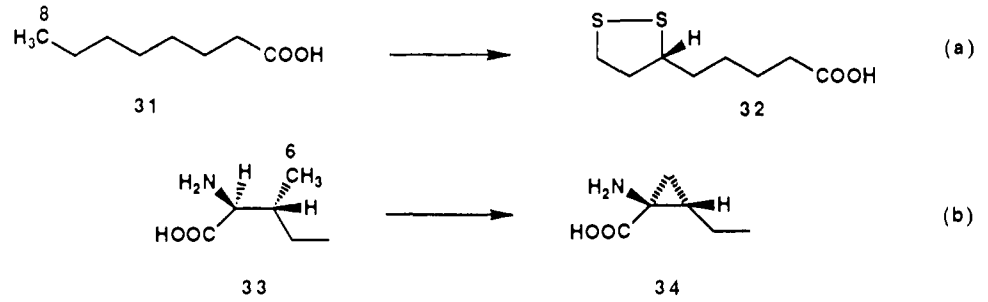
Investigation of the pathway shown in Scheme VI was first carried out by evaluation of (2-^{13}C)-6-hydroxy-DL-tryptophan as a precursor. The labeled amino acid was synthesized from 4-benzyloxy-2-nitrobenzaldehyde[26] which was condensed with (^{13}C)nitromethane to give the corresponding nitrostyrene.[26] The latter compound was then cyclized to (2-^{13}C)-6-benzyloxyindole.[26] The labeled 6-benzyloxyindole was finally converted into the labeled tryptophan by standard methods.[27] Administration of the (2-^{13}C)-6-hydroxy-DL-tryptophan to *S. sparsogenes* failed to yield labeled sparsomycin (data not shown). This suggested that the first step in the biosynthetic pathway from tryptophan might involve the tryptophanase-catalyzed loss of the amino acid side chain to give indole which could then be hydroxylated to give 6-hydroxyindole. Administration of commercial (2-^{13}C)indole to the sparsomycin fermentation did indeed yield sparsomycin labeled in the expected position (Table II, expt 7). However, the interpretation of this observation is ambiguous, since it could indicate either that indole lies beyond tryptophan on the pathway to **3** or that indole is converted back to tryptophan by tryptophan synthetase[28] before incorporation into **3**. Administration of (6-^{13}C)-6-hydroxyindole, obtained by catalytic debenzylation of (6-^{13}C)-6-benzyloxyindole, did not clarify this ambiguity, since the 6-hydroxyindole was not incorporated into sparsomycin (data not shown). Since activation of ring A of tryptophan for cleavage could also be achieved by hydroxylation at C-7, we attempted to obtain evidence for this possibility. Commercial (*indole*-$^2H_5$)-L-tryptophan was administered to *S. sparsogenes* and the isolated antibiotic examined by ^{2}H NMR. The NMR analysis revealed that deuterium was present at C-4 and C-5, but absent from C-1 (Table II, expt 8). This labeling pattern is completely consistent with the intermediacy of 7-hydroxytryptophan or 7-hydroxyindole, since C-1 of sparsomycin should be derived from C-7 of the indole nucleus. It is also consistent with the earlier observation that (3,5-$^2H_2$)-*N*-formylanthranilic acid labels sparsomycin only at C-5 (Table II, expt 4). Accordingly, (2-^{13}C)-7-hydroxyindole and (2-^{13}C)-7-hydroxy-DL-tryptophan were synthesized from 3-benzyloxy-2-nitrobenzaldehyde[29] by reaction with (^{13}C)nitromethane to generate the labeled β-nitrostyrene which was then cyclized to give (2-^{13}C)-7-benzyloxyindole.[27] The labeled 7-benzyloxyindole was then transformed into (2-^{13}C)-7-benzyloxy-DL-tryptophan using the methodology employed for the 6-benzyloxy derivative. Both the labeled tryptophan and labeled indole derivatives were finally converted into the labeled 7-hydroxy

(24) Tanizawa, K.; Soda, K. *J. Biochem.* **1979**, *86*, 1199.
(25) Vederas, J. C.; Schleicher, E.; Tsai, M.-D.; Floss, H. G. *J. Biol. Chem.* **1978**, *253*, 5350.
(26) Suvorov, N. N.; Fedotova, M. V.; Ogareva, O. B.; Balasheva, E. G. *J. Gen. Chem. USSR (Engl. Transl.)* **1960**, *30*, 3118. Suvorov, N. N.; Fedotova, M. V.; Ogareva, O. B.; Balasheva, E. G. *Ibid.* **1962**, *32*, 5579.
(27) Ek, A.; Witkop, B. *J. Am. Chem. Soc.* **1954**, *76*, 5579.
(28) Dunn, M. F.; Aguilar, V.; Brzovic, P.; Drewe, W. F., Jr.; Houben, K. F.; Leja, C. A.; Roy, M. *Biochemistry* **1990**, *29*, 8598.
(29) Just, G.; Zamboni, R. *Can. J. Chem.* **1978**, *6*, 2725.

Scheme VIII



Scheme IX



compounds by catalytic hydrogenolysis. Unfortunately, administration of the (2-^{13}C)-7-hydroxy-DL-tryptophan and (2-^{13}C)-7-hydroxyindole to *S. sparsogenes* failed to produce labeled sparsomycin (data not shown).

Some insight into the mechanism of formation of the uracil **3** from tryptophan was gained by investigation of the final stage of the pathway. A hypothetical route from the putative intermediate **26** (Scheme VI) to the uracil **3** is shown in Scheme VII. On the basis of the analogy provided by the conversion of inosine-5′-monophosphate (IMP) to xanthosine-5′-monophosphate (XMP),[30] it appeared plausible that **26** could cyclize to the pyrimidine derivative **27** and that the latter compound could then react with water and NAD^+ to form the uracil **3**. This hypothesis was tested by the synthesis and administration of (8-^{13}C)-**27**. The synthesis of labeled **27** is delineated in Scheme VIII. Ethyl (2′-^{13}C)-3-(2′-mercapto-4′-hydroxy-6′-methyl-5′-pyrimidyl)-propionate (**28**) was obtained by condensation of diethyl 2-acetylglutarate with (^{13}C)thiourea in the presence of sodium ethoxide.[31] Desulfurization of **28** to the pyrimidine **29** was then accomplished by refluxing with Raney nickel in aqueous solution. Treatment of **29** with LDA followed by phenylselenyl chloride afforded the phenylseleno derivative which was purified chromatographically and then converted into the unsaturated ester **30** by oxidation with hydrogen peroxide. Finally, alkaline hydrolysis of the ester group converted **30** into the desired compound, (8-^{13}C)-**27**. Administration of (8-^{13}C)-**27** to *S. sparsogenes* yielded sparsomycin showing a significant level of enrichment at C-8 (Table II, expt 9). The last step in the conversion of tryptophan into the uracil **3** therefore appears to be mechanistically similar to the formation of XMP from IMP.

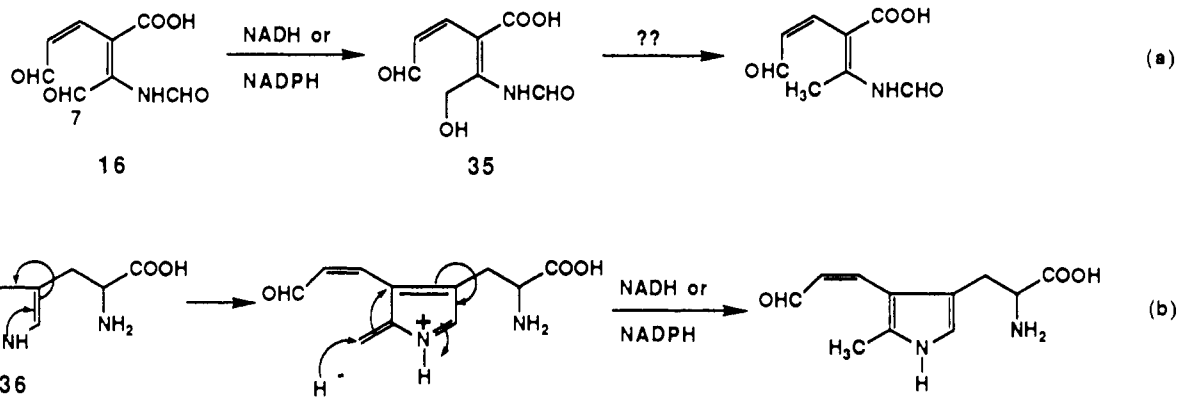
Discussion

The investigations of the biosynthesis of the dithioacetal moiety (**2**) of sparsomycin establish that the primary building blocks for this fragment of the antibiotic are L-cysteine and L-methionine. The very high levels of incorporation observed with L- and D-*S*-(methylthiomethyl)cysteine demonstrate that the dithioacetal moiety is biosynthesized before reduction of the cysteine carboxyl group and before attachment of the uracil moiety (**3**). Three conclusions can be drawn from the specific incorporation of ^{13}C- and ^{2}H-labeled forms of L- and D-*S*-(methylthiomethyl)cysteinol: (1) reduction of the carboxyl group occurs before attachment of the uracil moiety, (2) the formation of the sulfoxide occurs after carboxyl group reduction, and (3) the epimerization of the asymmetric center at C-2 can occur at a very late stage. However, it must be noted that the incorporation levels observed for the cysteinols are considerably lower than those of the corresponding amino acids. This may reflect the absence of an active transport system for these compounds, or it may indicate that these compounds are not true intermediates. The specific incorporation of (1-$^{2}H_2$)-L-*S*-(methylthiomethyl)cysteinol also raises the question of the mechanism of epimerization of this compound to the D form. This might occur via intermediacy of the 2-oxo derivative.

The results of our efforts to determine the mechanism of formation of the dithioacetal group appear to support the intermediacy of *S*-methylcysteine, although the data are not entirely unambiguous. Precursor incorporation experiments with (*methyl*-^{13}C)-L-methionine, (*methyl*-^{13}C)-*S*-methylcysteine, (2-^{13}C)-glycine, and (2,3-$^{13}C_2$)-DL-serine certainly favor the hypothesis that C-4 of the dithioacetal moiety is derived from the methyl group of methionine rather than the folate pool. The intermediacy of *S*-methylcysteine is also supported by the results of an isotope dilution experiment. If *S*-methylcysteine is involved in the formation of the dithioacetal moiety, this suggests that the biosynthesis of **2** may be related to the biosynthesis of biotin, lipoic acid, and isopenicillin N.[12] On the other hand, it is clear from the results of an incorporation experiment with doubly-labeled *S*-methylcysteine that a significant degree of catabolism takes place when this compound is administered to *S. sparsogenes*. This catabolism could occur by hydroxylation[32] of the *S*-methyl group with subsequent loss of formaldehyde or by a pyridoxal-phosphate-mediated loss of methanethiol.[33] In either case, label from the *S*-methyl

(30) Gilbert, H. J.; Lowe, C. R.; Drabble, W. T. *Biochem. J.* **1979**, *183*, 481.
(31) Morreal, C. E.; Hall, J. H.; Eskivs, K. *J. Med. Chem.* **1967**, *10*, 1176.
(32) See, for example: Bernhardt, F.-H.; Pachowsky, H.; Staudinger, H. *Eur. J. Biochem.* **1975**, *57*, 241.
(33) Kiick, D. M.; Phillips, R. S. *Biochemistry* **1988**, *27*, 7339. Kiick, D. M.; Phillips, R. S. *Ibid.* **1988**, *27*, 7333. Ishiwata, K.-I.; Nakamura, T.; Shimada, M.; Makiguchi, N. *J. Ferm. Bioeng.* **1989**, *67*, 167.

Scheme X



group could ultimately be incorporated into sparsomycin. The lack of incorporation of (*methyl*-^{13}C,*methyl*-$^{2}H_3$)-*S*-methylcysteine into sparsomycin can be attributed to the operation of a significant isotope effect associated with the functionalization of the *S*-methyl group. It is noteworthy that a large isotope effect apparently accompanies the transformation of [8-^{3}H]octanoic acid (**31**) into lipoic acid (**32**) (Scheme IXa).[12] Furthermore, in a potentially related process, the incorporation of a mixture of (6-^{13}C,6-$^{2}H_3$)-DL-isoleucine and alloisoleucine (**33**) into coronamic acid (**34**) (Scheme IXb) was found to be greatly reduced in comparison to that observed with a mixture of (6-^{13}C)-DL-isoleucine and alloisoleucine.[34] The apparent magnitude of such an isotope effect could be increased in the case of *S*-methylcysteine if the catabolism of this precursor does not involve a deuterium isotope effect. Final proof for the intermediacy of *S*-methylcysteine in the biosynthesis of the dithioacetal (**2**) may require experiments at the cell-free level.

The studies of the biosynthesis of the uracil moiety (**3**) provide compelling evidence that this portion of sparsomycin is derived from L-tryptophan. However, experiments with doubly-labeled forms of *N*-formylanthranilic acid demonstrate that this compound is not an intermediate lying between tryptophan and **3**. These results suggest two alternative routes for the conversion of tryptophan into **3**. One possibility is that the hypothesis in Scheme IV should be modified to show cleavage of ring A prior to the loss of the amino acid side chain of tryptophan. In this case *N*-formylkynurenine (Scheme IV, **14**) or hydroxylated forms of **14** might be the substrates for ring cleavage rather that *N*-formylanthranilic acid. The second possibility is that ring A of tryptophan undergoes oxidative cleavage prior to the cleavage of ring B. This is the process outlined in Scheme VI. The failure of hydroxylated forms of tryptophan and indole to serve as sparsomycin precursors indicates that if Scheme VI is correct, then either tryptophan or indole must be the substrates for the oxidative opening of ring A. While a decision between a modified version of Scheme IV and Scheme VI cannot be made at this time, Scheme VI appears more compelling, since it can more readily account for the formation of the C-methyl group present in the uracil moiety **3**. A troublesome feature of any hypothesis in which ring B is cleaved prior to ring A is that it is difficult to envision the mechanism whereby the aldehyde group derived from C-7 of tryptophan is reduced to a methyl group. For example, reduction of the hypothetical intermediate **16** (Scheme IV) to the alcohol **35** (Scheme Xa) bears ample biochemical precedent, but a mechanism for removal of the hydroxyl group from **35** is not apparent. The removal of hydroxyl groups in biological systems typically proceeds by β-elimination of a β-hydroxy ester or ketone followed by 1,4-addition of hydride to the resulting α,β-unsaturated ester or ketone. Such a mechanism cannot be employed to reduce alcohol **35**. In contrast, the hypothesis outlined in Scheme VI completely alleviates this difficulty, since the intact pyrrole ring in the putative intermediate **36** can be used to facilitate removal of the hydroxyl group (Scheme Xb).

Since neither 7-hydroxytryptophan nor 7-hydroxyindole is a precursor of sparsomycin, the loss of the proton from C-7 of tryptophan during sparsomycin biosynthesis must be due to processes other than hydroxylation. One possibility is that the protons present at the C-7 derived carbon atom of one of the intermediates leading from tryptophan to the uracil moiety are sufficiently acidic to undergo exchange with the medium.[35] Support for this hypothesis is provided by our serendipitous observation that, on long standing, a solution of sparsomycin in D_2O exhibits exchange of the C-1 hydrogens for deuterium.

Summary

Investigations using the sparsomycin producing organism *S. sparsogenes* var. *sparsogenes* have demonstrated that the dithioacetal moiety (**2**) of the antibiotic is constructed from L-cysteine and the *S*-methyl group of methionine. *S*-(Methylthiomethyl)cysteine and *S*-methylcysteine appear to be intermediates. The uracil moiety (**3**) of sparsomycin was shown to be derived from L-tryptophan by a process that involves loss of the side chain and oxidative cleavage of both rings of the amino acid. Since it was found that *N*-formylanthranilic acid is not an intermediate in the formation of the uracil moiety, it appears likely that ring A of tryptophan is cleaved prior to ring B. The terminal step in the biosynthesis of the uracil moiety of sparsomycin was shown to be related to the conversion of inosine-5′-monophosphate to xanthosine-5′-monophosphate. A more comprehensive understanding of the processes involved in the biosynthesis of sparsomycin is likely to require studies at the cell-free level.

Experimental Section

General Methods. Proton nuclear magnetic resonance spectra were taken on either an IBM AF300 (300 MHz) or Bruker AC250 (250 MHz) spectrometer. Chemical shifts are given in parts per million downfield from tetramethylsilane (0.0 ppm) for spectra taken in $CDCl_3$. In other organic solvents, the solvent itself was used as the reference. In D_2O the water signal (4.80 ppm) was used as reference. Carbon-13 NMR spectra were taken on the same two instruments at 75.45 MHz and 62.89 MHz, respectively. Methanol (49.00 ppm) was used as internal reference when D_2O was the solvent. Deuterium NMR spectra were obtained with the IBM AF300 at 46.07 MHz. Deuterium-depleted water was used both as solvent and as reference. *t*-BuOH was added as a standard for calculation of ^{2}H-enrichment (25 μL of *t*-BuOH contains 0.38 mmol of ^{2}H atoms). Mass spectra were run on Finnigan 3300 and CEC 111021-110B mass spectrometers. Infrared spectra were recorded on a Nicolet 205 FT-IR spectrophotometer. All melting points were

(34) Parry, R. J.; Lin, M.-T.; Walker, A. E.; Mhaskar, S. *J. Am. Chem. Soc.* **1991**, *113*, 1849.

(35) An alternative, but less likely, explanation is that the C-methyl group of the uracil moiety **3** is not derived from L-tryptophan. In principle, this possibility could be evaluated by Kuhn–Roth oxidation of sparsomycin derived from (U-^{14}C)-L-tryptophan, but in practice the results of such an experiment are not likely to be meaningful. If we assume that eight carbon atoms of tryptophan are incorporated into the uracil moiety, then we would expect only 12% of the total radioactivity to be present in the methyl group of the derived acetic acid. Because of the errors inherent in the Kuhn–Roth experiment and because of the likelihood of some nonspecific incorporation of radioactivity from tryptophan, the appearance of a small amount of radioactivity in the methyl group would be difficult to interpret unambiguously.

taken on a Fisher-Johns melting point apparatus and are uncorrected. Preparative thin layer chromatography was accomplished using 0.75-mm layers of Merck silica gel, Type PF-254. Analytical TLC was carried out using glass plates precoated with Cellulose F or Merck silica gel, Type 60, F-254. Cellulose plates were visualized with short wavelength ultraviolet light or by spraying with a 2% solution of ninhydrin in ethanol and heating. Silica gel plates were visualized with short wavelength ultraviolet light or with iodine vapor. Flash chromatography was carried out using silica gel, Type 60A, 230–400 mesh as the stationary phase. Cellulose column chromatography utilized Merck microcrystalline cellulose. Gas chromatography was performed on a Hewlett-Packard 5710A machine using a 10% SE-30 glass column (6 ft, 2 mm i.d.). Medium pressure liquid chromatography (MPLC) was accomplished using a column (11 mm i.d. × 48 cm) of Baker reversed-phase octadecylsilane bonded to silica gel (average particle diameter of 40 μm). Solvent was delivered with a Rainin Model B-100-S Eldex pump. The column output was monitored with an ISCO Model UA-5 absorbance detector with an ISCO Type 6 optical unit set at 310 nm, 2-mm flow cells, and a built-in recorder. Fractions were automatically collected with either an ISCO Model 273 or a Retriever II fraction collector. Antibiotic production was monitored by high pressure liquid chromatography (HPLC) on a Whatman octadecylsilane reversed-phase column (4.6 mm i.d. × 15 cm, 5 μm) preceded by a Micro-Guard ODS-5S precolumn using either a Spectraphysics SP Model 8700 solvent delivery system or an Altex 110A pump. Monitoring was carried out at 302 nm using either an ISCO Model UA-5 Absorbance Monitor or a Hitachi Model 100-40 UV detector equipped with a Kipp & Zonen BD 40 recorder. Preparative HPLC was carried out with an Altex octadecylsilane reversed-phase column (10 mm i.d. × 25 cm, 10 μm) preceded by a microguard ODS-5S precolumn and using a Spectraphysics SP Model 8800 ternary HPLC pump equipped with a Spectraphysics Spectra Chrom 200 programmable UV detector set to 302 nm and a Spectraphysics SP 4400 integrator. Measurement of pH was accomplished with an Orion 611 pH meter equipped with a combination electrode. Large-scale centrifugation was carried out on a Sorvall RC-5B refrigerated centrifuge, and small-scale centrifugation was accomplished with a Beckman GPR centrifuge. Spore suspensions were prepared on a Vortex-Genie mixer. Slants were grown in either a Scientific Products Model B7001-3 water bath, or a Scientific Products Model IC-62 incubator. Shake cultures were incubated in a New Brunswick Model G-25R rotary shaker. Lyophilization was done on an FTS MAXI-DRY system. The radiochemical purity of precursors was evaluated with a Berthold LB 22832 automatic TLC linear analyzer, interfaced with an Apple IIE computer. Samples for liquid scintillation counting were weighed on a Perkin-Elmer AD-2 autobalance and were counted with a Beckman Model LS 3801 liquid scintillation counter.

Materials. An authentic sample of sparsomycin was obtained as a gift from Dr. Paul Wiley of the Upjohn Co. Ingredients used in initial *S. sparsogenes* fermentations were also obtained from Upjohn. Distiller's grains and solubles were obtained from Sigma Chemical Co.; Pharmamedia and Proflo oil were obtained from Traders Protein. Radiolabeled compounds were purchased from Amersham Corp. and Dupont NEN Research Products. Scintillation cocktails were purchased from Dupont NEN. Precursors labeled with stable isotopes were purchased from Cambridge Isotopes, Merck Isotopes, or Aldrich Chemical Co.

Organism and Fermentation. The producing organism, *S. sparsogenes* var. *sparsogenes*, was obtained either from the Upjohn or from the ATCC (No. 25498). The production level of the original strain was very low (ca. 2 mg/L), and it could be improved to ca. 20 mg/L by one round of strain selection. The organism was maintained on Bennett's agar slants at 30 °C and preserved either by lyophilization in mist. desiccans[36] or by inoculation onto sterile soil. The early precursor incorporation experiments were carried out with the original strain, using the production medium reported by the Upjohn group. Later experiments utilized the improved strain and a modified production medium consisting of 1.2% glucose, 1% dextrin, 1% distiller's grains and solubles, 1% Pharmamedia, and 18 drops of Proflo oil per liter. The pH was adjusted to 7.2 with 1 N NaOH. A typical fermentation consisted of 1.2 L of production medium distributed equally between eight 1-L Erlenmeyer flasks closed with foam plugs. These flasks were inoculated with 10 mL each of a 3-day-old inoculum prepared from the same medium. Both the inoculum and the production phase of the fermentation were carried out at 32 °C. Under these conditions, production of sparsomycin began after 3 to 4 days and peaked after 9 or 10 days. The production of sparsomycin was monitored by HPLC using a C_{18} reverse-phase column and a solvent mixture consisting of water/methanol (85:15).

Isolation of Sparsomycin. Sparsomycin was isolated by a modification of the procedure of Argoudelis and Herr.[1] The fermentation broth from individual flasks was combined and each flask was rinsed with water. The rinses were added to the fermentation broth. The pH was then adjusted to 3.0 with concentrated H_2SO_4, whereupon a copious precipitate formed. The mixture was filtered with the aid of acid-washed Celite and the pH of the filtrate adjusted to 8.0 with 1 N NaOH, causing the solution to darken. Twenty grams of Darco charcoal and 30.0 g of acid-washed Celite were then added and the resulting mixture was stirred mechanically for 2 h. The mixture was filtered, the charcoal cake was washed with water, and it was then washed with 100 mL of water/acetone (80:20, pH = 8.0). The carbon cake was then suspended in 800 mL of acetone/water (50:50, pH = 2.5), and stirred by hand for 1 h. The mixture was filtered, and the bright yellow filtrate was adjusted to pH 6.2 with 1 N NaOH. The acetone was removed under reduced pressure, and remaining aqueous solution was then lyophilized. The resulting orange solid was stirred with 25 mL of methanol at 40 °C for 30 min. The mixture was filtered, and an additional 20 mL of fresh methanol was added to the residue; stirring was continued at 40 °C for 20 min. The mixture was again filtered and the combined filtrates were evaporated to give an orange solid. McIlvaine's buffer (pH 6.0) was then prepared by mixing 0.2 M aqueous Na_2HPO_4 solution and 0.1 M aqueous citric acid solution in a 12.6:7.4 ratio of volumes. This buffer solution was shaken with an equal volume of methyl ethyl ketone, and the mixture was separated into two layers, an "aqueous layer" and an "organic layer". The orange solid was dissolved in 10 mL of the "aqueous layer". This solution was then extracted eight times with the "organic layer" using 100 mL for each extraction. The combined organic layers were evaporated under reduced pressure to give a small amount of yellow oil. The oil was then chromatographed on the MPLC column described under "general methods" using a mixture of 0.01 M aqueous KH_2PO_4 (pH 5.4) and methanol (86:14), as the solvent. The column output was monitored at 310 nm, and 3-mL fractions were collected at a flow rate of 1.5 mL/min. Sparsomycin was typically found in fractions 20–40. These fractions were combined and concentrated to give a small volume of light yellow oil. The oil was rechromatographed on the same MPLC column with water/methanol (86:14) as solvent. The column was monitored and fractions were collected as described above. Sparsomycin was usually found in fractions 35–60. These fractions were combined and concentrated in vacuo to an aqueous solution which was then lyophilized to give an off-white solid. The solid was dissolved in a small amount of methanol, and the solution was transferred to a tared Craig tube. The methanol solution was concentrated to approximate 0.5 mL by evaporation in a stream of nitrogen. Dry ether was then added to precipitate the sparsomycin. The solid was collected by centrifugation, the liquid was decanted, and solid was dried in vacuo over P_2O_5 at room temperature for 1 h. About 25–30 mg of sparsomycin were obtained. At this point, the material was usually sufficiently pure for ^{13}C NMR and ^2H NMR spectrometry. In the event that the material was not pure enough, preparative HPLC was performed. The column used was the one described under "general methods". Twenty milligrams of sparsomycin was dissolved in 4 mL of water, and 40 μL of this solution was injected each time and the chromatography was monitored at 302 nm. The sparsomycin eluted at 23 min when the stepwise gradient shown in Table III was employed.

Table III. Gradient Solvent System for Preparative HPLC[a]

time (min)	solvent A (%)	solvent B (%)	flow rate (mL/min)
0.0	100	0.0	3
20	75	25	3
25	75	25	3
35	100	0.0	3

[a] Solvent A, water; solvent B, methanol.

Administration of Labeled Compounds to *S. Sparsogenes*. The labeled precursor was dissolved in 16 mL of distilled water and 1 mL added to each of eight 1-L flasks through a sterile Millipore filter. The remainder of the precursor was added in the same fashion, after 24 h had elapsed. In those cases in which the precursor was insoluble in water, a minimum amount of DMSO was used to dissolve it. The timing for administration was determined by daily HPLC monitoring of the fermentation, with the first feeding occurring immediately after the appearance of the antibiotic. In experiments employing precursors labeled with stable isotopes, 250–300 mg of the labeled precursor was used in each precursor incorporation experiment.

Synthesis of Labeled Precursors. (2,3-$^{13}C_2$)-DL-Serine. (2-^{13}C)Diethyl acetamidomalonate (1.02 g, 4.67 mmol) was suspended in a solution of 20% aqueous (^{13}C)formaldehyde (200 mg, 6.45 mmol) in 1.0 mL of water. The clear solution which was formed after the addition of 66 μL (0.066 mmol) of 1 N NaOH was stirred for 5.25 h. Aqueous NaOH solution (1.4 N, 7.0 mL, 9.8 mmol) was then added, and stirring was

(36) Norris, J. R.; Ribbons, D. W. *Methods in Microbiology*; Academic Press: New York, 1970; Vol. 3A, p 131.

continued for 46 h. Glacial acetic acid (2.0 mL, 35 mmol) was added, and the solution was refluxed for 1 h. The solvent was removed in vacuo, and 10.3 mL of concentrated HCl was added. The mixture was refluxed for 1 h, and it was then concentrated under reduced pressure to give a yellow oil. The oil was refluxed for 15 min with 15 mL of absolute ethanol. The white solid which precipitated was collected by filtration and refluxed for 15 min with another 15 mL of absolute ethanol. The mixture was filtered and the solid collected was subjected to ethanol extraction once more. The combined ethanol extracts were concentrated in vacuo to give a yellow oil. Concentrated HCl (2.34 mL) was added, and the mixture was refluxed for 1 h. The solution was then refluxed for additional 30 min with 25 mg of acid-washed charcoal. The charcoal was removed by filtration and washed three times with 3-mL portions of hot water; the combined filtrate and washings were refluxed for 30 min with a fresh 25-mg portion of acid-washed charcoal. The charcoal was washed with three 3-mL portions of hot water, and the combined filtrate and washings were concentrated in vacuo. The concentrated solution was then loaded onto a column of 23 g of Amberlite IRA-45 (weakly basic ion exchange resin, free base form, 16–50 mesh, 15 mm × 18 cm). The column was eluted with water, and eluate was checked for the presence of serine with ninhydrin. All the serine was contained in the first 200 mL of water. The aqueous solution was lyophilized, and the white solid remaining was recrystallized from water/ethanol (2:5). White crystals (317.5 mg, 3.02 mmol, 65% yield) of (2,3-$^{13}C_2$)-DL-serine were obtained. The ^{1}H NMR of the product was consistent with that expected and the product exhibited TLC behavior identical with that of authentic serine (R_f = 0.18 on cellulose developed with 25:4:10 1-butanol/acetic acid/water (BAW). The ^{13}C NMR spectrum (75.45 MHz) confirmed the locations of the label since it showed two enriched doublets at 60.3 ppm and 56.5 ppm (J = 37.7 Hz).

(4-^{13}C)-*S*-(Methylthiomethyl)-L-cysteine. A. (*methylene*-^{13}C)-Chloromethylmethyl Sulfide. In a 50-mL three-necked flask was placed 0.5 g (16.4 mmol) of ^{13}C-labeled paraformaldehyde and 2 mL of CH_2Cl_2. One neck was equipped with a condenser bearing a $CaCl_2$ drying tube, and the drying tube was connected to a NaOH reservoir to absorb excess HCl. The other two necks were stopped with rubber septa. The flask was immersed in an ice–$CaCl_2$–dry ice bath to maintain the temperature at −10 to −15 °C, and 1 mL (0.87 g, 18 mmol) of CH_3SH was transferred to the flask from a calibrated tube through a long needle. After the CH_3SH had been transferred, HCl gas, which was dried with concentrated H_2SO_4, was bubbled into the mixture. The mixture was stirred at −15 °C for 3 h until all the solids were dissolved. Anhydrous $CaCl_2$ (1.0 g) was then added and the flask was stopped with a rubber septum. A needle was inserted into the rubber septum, and the flask was left at room temperature for 24 h. The mixture was next extracted with CH_2Cl_2 several times. The combined CH_2Cl_2 extracts were dried over anhydrous $CaCl_2$ and the CH_2Cl_2 was distilled off. The residue was analyzed by GC (SE-30 column) and three peaks were found which were identified as CH_2Cl_2, $CH_3S^{13}CH_2Cl$, and $CH_3S^{13}CH_2SCH_3$. From the ^{1}H NMR spectrum, the ratio of these three compounds was CH_2Cl_2:$CH_3S^{13}CH_2Cl$:$CH_3S^{13}CH_2SCH_3$ = 11.8%:54.9%:33.3% (mol:mol:mol). A total of 994 mg (10.2 mmol, 62%) of $CH_3S^{13}CH_2Cl$ was obtained: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.63 (d, $^1J_{CH}$ = 149.7 Hz, 2 H, $^{13}CH_2$), 2.16 (d, $^3J_{CH}$ = 4.9 Hz, 3 H, CH_3).

B. (4-^{13}C)-*S*-(Methylthiomethyl)-L-cysteine (4). L-Cystine (241 mg, 1 mmol) was placed in a 50-mL three-necked flask containing a stir bar, and NH_3 (20 mL) was then distilled into the flask with the aid of a dry ice condenser. Small pieces of sodium (105 mg, 4.56 mmol) were slowly added with stirring. A blue color appeared which lasted for more than 10 min. The mixture of CH_2Cl_2, $CH_3S^{13}CH_2Cl$, and $CH_3S^{13}CH_2SCH_3$ (300 mL, 205 mg, 2.1 mmol of $CH_3S^{13}CH_2Cl$) was added and the blue color disappeared immediately. The resulting mixture was stirred for 15 min under the dry ice condenser, and the NH_3 was then allowed to evaporate. The residue was dissolved in water, and the aqueous solution extracted three times with ether. The pH of the aqueous solution was then adjusted to 5, and the acidic solution was concentrated to dryness. The residue was recrystallized from H_2O/EtOH to yield 159 mg (0.87 mmol, 44%) of white shiny solid. The product exhibited an R_f of 0.45 on a cellulose plate developed with BAW (4:1:1) and was visualized with a 2% alcoholic solution of ninhydrin: ^{1}H NMR (D_2O, 300 MHz) δ 3.74 (d, $^1J_{CH}$ = 152.4 Hz, 2 H, $^{13}CH_2$), 3.95 (m, 1 H, C-2), 3.18 (m, 2 H, C-3), 2.16 (d, $^3J_{CH}$ = 5.0 Hz, 3 H, SCH_3); the ^{13}C NMR spectrum (75.45 MHz) showed enrichment at 36.8 ppm.

(4-^{13}C)-*S*-(Methylthiomethyl)-D-cysteine (5). This compound was synthesized from D-cysteine and (*methylene*-^{13}C)chloromethylmethyl sulfide in 45% yield by the same procedure used to prepare (4-^{13}C)-*S*-(methylthiomethyl)-L-cysteine. It was characterized in the same way.

(4-^{13}C)-*S*-(Methylthiomethyl)-D-cysteinol. A. (4-^{13}C)-*S*-(Methylthiomethyl)-D-cysteine Methyl Ester Hydrochloride. A mixture of 0.51 g (2.80 mmol) of (4-^{13}C)-*S*-(methylthiomethyl)-D-cysteine in 30 mL of 2,2-dimethoxypropane containing 3 mL of concentrated HCl was stirred at room temperature for 18 h. The solution darkened considerably. The solvent was evaporated in vacuo and the dark residue was dissolved in 2 mL of MeOH. Ethyl acetate (50 mL) was then added and the mixture was left in a refrigerator overnight. An off-white residue precipitated which was collected by filtration, washed with EtOAc, and dried in vacuo to yield 0.62 g (2.7 mmol, 96% yield) of product, with R_f = 0.79 on silica gel developed with *sec*-BuOH/NH_4OH (5:2): ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.79 (bs, 2 H, NH_2), 4.34 (m, 1 H, C-2), 3.75 (s, 3 H, OCH_3), 3.81 (d, 2 H, $^1J_{CH}$ = 152.3 Hz, C-4), 3.13 (m, 2 H, C-3), 2.10 (d, $^3J_{CH}$ = 4.8 Hz, 3 H, SCH_3); ^{13}C NMR (DMSO-d_6, 62.89 MHz) 37.3 ppm (enriched).

B. (4-^{13}C)-*S*-(Methylthiomethyl)-D-cysteinol (7). (4-^{13}C)-*S*-(Methylthiomethyl)-D-cysteine methyl ester hydrochloride (0.62 g, 2.66 mmol) was dissolved in 7 mL of 1:1 ethanol/water. This solution was added dropwise to a solution of 716 mg (18.9 mmol) of $NaBH_4$ in 7 mL of ethanol/water (1:1) at 0 °C. The resulting mixture was stirred at 0 °C for 4 h and then stirred at room temperature overnight. The insoluble material was filtered off and the filtrate was concentrated in vacuo to remove the ethanol. The remaining aqueous solution was extracted with chloroform four times. The combined chloroform extracts were dried over Na_2SO_4, and then taken to dryness in vacuo. The colorless oil which remained was dried in vacuo to yield 365 mg (2.2 mmol, 82% yield) of product, with R_f = 0.65 on silica gel developed with *sec*-BuOH/NH_4OH (5:2). This compound was treated with an aqueous HCl solution to convert it to its hydrochloride salt, mp 45–46 °C: ^{1}H NMR (unlabeled compound) (D_2O, 250 MHz) δ 3.77 (s, 2 H, C-4), 3.58 (m, 2 H, C-1), 3.05 (m, 1 H, C-2), 2.89 (q, 1 H, J = 13.5 Hz, J = 5.1 Hz, J = 5.1 Hz, C-3), 2.61 (q, 1 H, J = 13.6 Hz, J = 7.8 Hz, J = 7.9 Hz, C-3), 2.19 (s, 3 H, SCH_3); ^{13}C NMR (unlabeled compound) (D_2O, 62.89 MHz) 64.7, 50.8, 37.0, 34.2, 13.7 ppm; MS m/z 55, 75, 87, 136, 167 (M^+); HRMS m/z (M^+, $C_5H_{13}NOS_2$) calcd 167.043 845, obsd 167.0434; ^{1}H NMR (labeled) (D_2O, 250 MHz) δ 3.75 (d, 2 H, $^1J_{CH}$ = 151.8 Hz, C-4), 3.56 (m, 2 H, C-1), 3.02 (m, 1 H, C-2), 2.84 (m, 1 H, C-3), 2.59 (m, 1 H, C-3), 2.17 (d, $^3J_{CH}$ = 4.93 Hz, 3 H, SCH_3); ^{13}C NMR (labeled) (D_2O, 62.9 MHz) 36.9 ppm (enriched).

(4-^{13}C)-*S*-(Methylthiomethyl)-L-cysteinol. A. (4-^{13}C)-*S*-(Methylthiomethyl)-L-cysteine Methyl Ester Hydrochloride. This compound was synthesized from (4-^{13}C)-*S*-(methylthiomethyl)-L-cysteine and 2,2-dimethoxypropane in 96% yield by the same procedure used to prepare (4-^{13}C)-*S*-(methylthiomethyl)-D-cysteine methyl ester hydrochloride.

B. (4-^{13}C)-*S*-(Methylthiomethyl)-L-cysteinol (6). This compound was synthesized by reduction of (4-^{13}C)-*S*-(methylthiomethyl)-L-cysteine methyl ester hydrochloride with $NaBH_4$ in 72% yield by the same procedure used to prepare (4-^{13}C)-*S*-(methylthiomethyl)-D-cysteinol.

(1-^{2}H$_2$)-*S*-(Methylthiomethyl)-L-cysteinol. This compound was synthesized by reduction of *S*-(methylthiomethyl)-L-cysteine methyl ester hydrochloride with $NaBD_4$ in 96% yield by the same method used to prepare (4-^{13}C)-*S*-(methylthiomethyl)-D-cysteinol: ^{1}H NMR (D_2O, 300 MHz) δ 3.75 (s, 2 H, C-4), 3.01 (m, 1 H, C-2), 2.84 (q, 1 H, J = 13.5 Hz, J = 5.1 Hz, J = 5.1 Hz, C-3), 2.56 (q, 1 H, J = 13.6 Hz, J = 7.9 Hz, J = 7.8 Hz, C-3), 2.17 (s, 3 H, SCH_3); ^{13}C NMR (D_2O, 75.45 MHz) 64.0 (quintet), 50.9, 37.1, 34.3, 13.8 ppm.

(1-^{2}H$_2$)-*S*-(Methylthiomethyl)-D-cysteinol. This compound was synthesized by reduction of *S*-(methylthiomethyl)-D-cysteine methyl ester hydrochloride with $NaBD_4$ in 85% yield by utilizing the same method that was employed to synthesize the (4-^{13}C)-labeled compound.

(*methylene*-^{13}C)-D,D-Djenkolic Acid. A mixture of 1.98 g (12.6 mmol) of D-cysteine hydrochloride monohydrate, 3.5 mL of 7 N HCl, and 1.0 g (6.45 mmol) of a 20% aqueous solution of (^{13}C)formaldehyde was warmed to 70 °C with stirring, then cooled to room temperature and stirred for 24 h. The solution was then neutralized with 6 N NaOH, whereupon a white solid precipitated. The solid was collected by filtration and resuspended in 10 mL of water; concentrated NH_4OH was added dropwise until all the solid was dissolved. Sodium cyanide (0.155 g) was added, the solution was stirred for 0.5 h, and it was then neutralized with AcOH. A white solid precipitated which was collected by filtration and dried in vacuo to yield 0.98 g (3.8 mmol, 61%) of product. TLC analysis (TBuK = *tert*-butyl alcohol/water/methyl ethyl ketone/diethylamine = 80:80:40:8) showed only one spot with R_f of 0.50 on a cellulose plate. This was the same as that exhibited by an authentic sample. The compound could be recrystallized by suspending it in 15 mL of boiling water, adding 6 N HCl until all solid dissolved, and then carefully neutralizing with 6 N NaOH at the boiling point. A white solid precipitated after cooling which consisted of very pure djenkolic acid: ^{1}H NMR (D_2O, 300 MHz) δ 3.92–3.41 (d, $^1J_{CH}$ = 152.4 Hz, 2 H, $^{13}CH_2$), 3.37 (m, 2 H, C-2), 3.84 (m, 4 H, C-3).

(*methyl*-^{13}C)-*S*-Methyl-D-cysteine. Liquid NH_3 was distilled from Na and condensed with a dry ice condenser into a three-necked flask containing 408 mg (1.7 mmol) of D-cystine. Small pieces of sodium (180

mg, 7.83 mmol) were added causing a blue color to appear which lasted for 15–20 min. (*methyl*-^{13}C)Methyl iodide (240 μL, 3.75 mmol) was added, whereupon the blue color faded. The mixture was stirred in liquid NH_3 for 45 min, the condenser was then removed, and the NH_3 was allowed to evaporate. The residue was dissolved in water, the solution was concentrated, and its pH was adjusted to 5. Some insoluble material was filtered off. The solution was then concentrated to dryness and the residue was recrystallized from H_2O/EtOH, yielding 344 mg (2.47 mmol, 73%) of white crystals. The product showed the same R_f value (0.31, BAW = 12:3:5) as an authentic sample on a cellulose plate and exhibited spectral data consistent with that expected.

(*methyl*-^{13}C)-*S*-Methyl-L-cysteine. This compound was prepared from L-cystine and (^{13}C)methyl iodide by employing the same protocol as that used to obtain the corresponding D-amino acid. The yield was comparable.

(*methyl*-^{13}C,$^{2}H_3$)-*S*-Methyl-D-cysteine. This compound was synthesized from D-cystine and (^{13}C,$^{2}H_3$)methyl iodide utilizing a procedure similar to that employed for the preparation of the (*methyl*-^{13}C)-labeled D-amino acid. The yield was approximately the same.

(*methyl*-^{14}C)-*S*-Methyl-L-cysteine. This compound was prepared in the standard fashion from L-cystine and [*methyl*-^{14}C]methyl iodide in 47% yield and 19% radiochemical yield. The radiochemical purity of the recrystallized compound was found to be ca. 99% by radioscanning of a thin-layer chromatogram.

(3-^{3}H)-*S*-Methyl-L-cysteine. This compound was synthesized in the usual manner from (3,3′-^{3}H)-L-cystine and unlabeled methyl iodide. The chemical and radiochemical yields were ca. 42%. The recrystallized compound exhibited a radiochemical purity of ca. 99% as determined by radioscanning of a thin-layer chromatogram.

Isotopic Trapping of *S*-Methyl-L-cysteine. A. Reisolation of *S*-Methyl-L-cysteine. Ten microcuries of (U-^{14}C)-L-cystine was administered to 300 mL of a 3-day-old fermentation broth of *S. sparsogenes*. After 10 h the fermentation broth was centrifuged at 9000 rpm for 20 min. The mycelium was rinsed twice with deionized water and centrifuged using the same conditions after each washing. The mycelium was then suspended in 50 mL of deionized water, and 107 mg of *S*-methyl-L-cysteine was added. The mixture was sonicated at output control 6, duty cycle 90%, for 5–8 min and the resulting mixture was centrifuged at 12000 rpm for 20 min. The supernatant was decanted and the cellular debris was rinsed with deionized water and centrifuged under the same conditions. The supernatants were combined, concentrated to 100 mL in vacuo, and acidified to pH 2 with 1 N HCl. The precipitate was filtered off with the aid of acid-washed Celite. The filtrate was neutralized to pH 7, concentrated to 100 mL and then applied to an Amberlite IR-120 plus strongly acidic ion-exchange column. The column was rinsed with 1 L of deionized water and then eluted with 300 mL of 2 N NH_4OH solution. The eluate was concentrated to 100 mL, and then passed through an IRC-50 weakly acidic ion-exchange column, which was subsequently rinsed with water. The combined eluate and rinse was concentrated and lyophilized to yield 241 mg of white solid. The solid was further purified by column chromatography on cellulose, using BAW (12:3:5) as the solvent. The fractions containing *S*-methylcysteine were combined, the butanol and acetic acid were removed in vacuo, and the aqueous solution was lyophilized to yield 113.4 mg of *S*-methylcysteine. The product was recrystallized from aqueous ethanol several times to constant specific activity. The radioactive *S*-methyl-L-cysteine was then converted into the *N*-acetyl-*p*-bromophenacyl ester.

B. *N*-Acetyl-*S*-methyl-L-cysteine. In a 10-mL flask was placed 56 mg (0.42 mmol) of *S*-methyl-L-cysteine, and 1 mL of 1 N NaOH solution was added to dissolve the solid. The solution was cooled to 0–5 °C and Ac_2O (70 μL, 0.74 mmol) was added dropwise with a syringe. The solution was stirred for 0.5 h in an ice bath and then stirred at room temperature for 2 h. The mixture was passed through a Dowex 50 × 8 (50–100 mesh) cation exchange column. The column was rinsed with water (50 mL) and the eluate was concentrated to dryness to give a residue which was dried in vacuo to yield 69.4 mg (0.39 mmol, 94%) of white solid: ^{1}H NMR (D_2O, 90 MHz) δ 2.16 (s, 3 H), 2.24 (s, 3 H), 3.10 (m, 2 H).

C. *N*-Acetyl-*O*-*p*-bromophenacyl-*S*-methyl-L-cysteine. A mixture of 54 mg (0.30 mmol) of *N*-acetyl-*S*-methyl-L-cysteine, 52 mg of K_2CO_3, 100 mg (0.36 mmol) of 2,4′-dibromoacetophenone, 25 mg of benzo-18-crown-6, and 10 mL of distilled, anhydrous acetonitrile was heated to 50 °C with stirring under argon for 30 min. Heating was stopped, and the mixture was filtered to remove the insoluble material. The filtrate was evaporated and the residue was purified by preparative TLC, using ethyl acetate/chloroform (1:1) as solvent (R_f = 0.54). The white solid obtained was further purified by recrystallization from ethyl acetate/petroleum ether to give 67 mg (0.18 mmol, 59%) of product, mp 121–122 °C: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 2.06 (s, 3 H, CH_3), 2.16 (s, 3 H, SCH_3), 3.10 (m, 2 H, C-3), 4.94 (m, 1 H, C-2), 5.36 (q, 2 H, J = 16.4 Hz, OCH_2), 7.70 (q, J = 8.5 Hz, 4 H, Ph-H); ^{13}C NMR ($CDCl_3$, 62.89 MHz) 170.5, 132.3, 129.1, 66.4, 51.5, 36.2, 23.1, 16.1 ppm; MS m/z 43, 61, 90, 101, 155 (157), 183 (185), 314 (316), 373 (375) (M^+); HRMS m/z (M^+, $C_{14}H_{16}BrNO_4S$) calcd 372.998 31, observed 372.998 05.

(2-^{13}C)-DL-Tryptophan. A. (*formyl*-^{13}C)Formic Acetic Anhydride. Sodium (^{13}C)formate (3.1 g, 44.3 mmol) and 2.6 mL of dry ether were placed in an ice-cold, dry 50-mL three-necked flask fitted with an addition funnel, a thermometer, and a condenser protected with drying tube. Freshly distilled acetyl chloride (2.75 mL, 38.7 mmol) was added dropwise via an addition funnel to the stirred reaction mixture over the course of 10 min. The reaction mixture was then stirred for 5.5 h; an ice bath was used as necessary to keep the mixture at approximately room temperature. The mixture was then filtered and the white solid washed with a little ether. The ether was then removed from the combined filtrate and wash; the residue was distilled at 40 °C (39 mm) to yield a colorless distillate (1.97 g, 57%) that was used immediately.

B. (*formyl*-^{13}C)-*N*-Formyl-*o*-toluidine. Freshly prepared (*formyl*-^{13}C)formic acetic anhydride (1.97 g, 22.1 mmol) was placed in a dry, ice-cooled 25-mL round-bottom flask, and freshly distilled *o*-toluidine (2.30 mL, 21.4 mmol) was added dropwise with stirring. After the addition was complete, the reaction mixture was concentrated in vacuo and the residue was dissolved in ethyl acetate. The solution was washed twice with 10% aqueous $NaHCO_3$; it was then dried and evaporated in vacuo to give a white solid which was purified by bulb-to-bulb distillation at 174 °C (12 mm). The yield of labeled *N*-formyl-*o*-toluidine was 2.36 g (81%), mp 50–54 °C (lit.[19] 62 °C); its spectral properties were in agreement with the assigned structure.

C. (2-^{13}C)Indole. A dry 100-mL three-necked flask was fitted with a reflux condenser protected by a drying tube and the system flushed with dry nitrogen. Dry *tert*-butyl alcohol (20.8 mL, 221 mmol) and potassium metal (0.96 g, 24.6 mmol) were added, and the mixture gently warmed with stirring under nitrogen until all of the potassium had dissolved. (*formyl*-^{13}C)-*N*-Formyl-*o*-toluidine (2.25 g, 16.6 mmol) was added and the condenser replaced with a distillation head. The mixture was then heated in a Wood's metal bath and the temperature slowly increased until all of the excess *tert*-butyl alcohol had distilled. The residue was then heated slowly under a nitrogen atmosphere to 350 °C, and then held at 350–360 °C for a period of 20 min. The dark reaction mixture was allowed to cool to room temperature under N_2, and water (10 mL) was then added. The mixture was extracted several times with ether, the combined ether extracts dried, and the solvent removed in vacuo. The resulting dark oil was chromatographed on silica gel using hexane/ethyl acetate (9:1) to give indole as a light green oil. Two successive bulb-to-bulb distillations of this oil (121 °C (5 mm)) yielded (2-^{13}C)indole as a colorless solid (0.59 g, 30%), mp 50–51 °C (lit.[19] 52.5 °C).

D. (2-^{13}C)Gramine. (2-^{13}C)Gramine was prepared from (2-^{13}C)indole using the procedure of Heidelberger.[19] The yield was 95%.

E. Ethyl α-Carbethoxy-α-acetamido-β-[3-(2-^{13}C)indolyl]propionate. The title compound was synthesized from (2-^{13}C)gramine in 43% yield using the procedure of Albertson et al.[19]

F. (2-^{13}C)-DL-Tryptophan. Ethyl α-carbethoxy-α-acetamido-β-[3-(2-^{13}C)indolyl]propionate was converted into (2-^{13}C)-DL-tryptophan in 54% yield using Heidelberger's procedure.[19]

(5-$^{2}H_1$)-DL-Tryptophan. Methanolic potassium hydroxide (9 mL, 3%), freshly prepared palladium on $CaCO_3$ (800 mg, 10% Pd), and 5-bromo-DL-tryptophan (800 mg) were placed in a 50-mL three-necked flask which was purged with deuterium gas. A balloon attached to the flask via a needle and septum was filled with D_2 and the mixture stirred under D_2 for 2 h at room temperature. At the end of the reaction period, the catalyst was filtered off and washed well with water. The combined filtrate and washings were adjusted to pH 7.0 with 0.1 N HCl. The water was then removed in vacuo and the residue recrystallized from hot water to yield (5-$^{2}H_1$)-DL-tryptophan (0.28 g, 49%): ^{1}H NMR (DCl–D_2O, 300 MHz) δ 3.40 (2 H, m), 4.32 (1 H, t, J = 6.0 Hz), 7.20 (1 H, d, J = 12.0 Hz), 7.26 (1 H, s), 7.46 (1 H, d, J = 12 Hz), 7.62 (1 H, s); ^{13}C NMR (DCl–D_2O, 75.5 MHz) triplet at 119.3 ppm.

(3,5-$^{2}H_2$)-*N*-Formylanthranilic Acid. A. 3,5-Dibromoanthranilic Acid. Anthranilic acid (6.94 g, 50.7 mmol) dissolved in 44 mL of MeOH was stirred at 40–50 °C, while 5.40 mL (104.6 mmol) of bromine was introduced through an addition funnel. After a few minutes, a large amount of light yellow solid precipitated. The mixture was stirred for 3 h at 40–50 °C. After this time, the color faded and the mixture was cooled to room temperature. The light yellow solid was collected by filtration and dried in vacuo, to yield 10.6 g (36.0 mmol, 71%) of product, mp 240–240.5 °C (lit.[37] 236–238 °C). The product displayed an R_f of 0.48 on silica gel developed with hexane/ethyl acetate (1:1) containing 2% acetic acid: ^{1}H NMR (acetone-d_6, 90 MHz) δ 7.98 (d, J = 2.7 Hz, 1 H), 7.76 (d, J = 2.7 Hz, 1 H).

(37) Anilin, B.; Sida-Fabrich, A.-G. *Chem. Abstr.* **1966**, *65*, 2176g.

B. 3,5-Dibromo-*N*-formylanthranilic Acid. A mixture of 0.905 g (3.07 mmol) of 3,5-dibromoanthranilic acid, 0.34 g of sodium formate, and 50.4 mL of 96% formic acid was stirred at 0 °C. Acetic anhydride (16.8 mL) was added dropwise during an hour. The mixture was then stirred at room temperature for 12 h. After stirring overnight, the solution became clear. Water was then added and the solution was concentrated in vacuo. A white precipitate formed which was collected by filtration and dried in vacuo, to yield 0.83 g (2.56 mmol, 83%) of white solid. The product displayed an R_f of 0.15 on silica gel developed with hexane/ethyl acetate (1:1) containing 2% acetic acid, mp 192–193 °C: ^{1}H NMR (acetone-d_6, 90 MHz) δ 8.40 (s, 1 H), 8.00 (s, 1 H); ^{13}C NMR (DMSO-d_6, 75.45 MHz) 166.9, 160.6, 136.6, 136.6, 132.5, 119.1 ppm; MS m/z 29, 57, 88, 141 (143), 168 (170), 224 (226), (246) 248 (250), (275) 277 (279), (293) 295 (297), (321) 323 (325) (M^+); HRMS m/z (M^+, $C_8H_5Br_2NO_3$) calcd 320.863 585, 322.861 545, obsd 320.862 87, 322.861 28.

C. (3,5-$^2H_2$)-*N*-Formylanthranilic Acid. A mixture of 1.38 g (4.30 mmol) of 3,5-dibromo-*N*-formylanthranilic acid, 1.22 g of 10% Pd/$CaCO_3$, and 12 mL of a 2% solution of KOH in MeOD was stirred under deuterium gas at room temperature and 1 atm for 3 h. The catalyst was removed by filtration and washed with water. The filtrate was concentrated in vacuo, and the pH of the remaining aqueous solution was adjusted to 3.0. A white solid precipitated which was collected by filtration to yield 0.42 g (59%) of product which exhibited an R_f of 0.56 on silica gel developed with ethyl acetate/methanol (4:1) containing 2% acetic acid, mp 166 °C (lit.[38] 167 °C): ^{1}H NMR (acetone-d_6, 300 MHz) δ 9.03 (s, 1 H, CHO), 8.52 (s, 1 H, NH), 8.03 (s, 1 H, C-6), 7.57 (s, 1 H, C-4); ^{13}C NMR (acetone-d_6, 75.45 MHz) 170.0, 161.3, 140.8, 134.7, 132.4, 123.9 (t), 121.3 (t), 116.8 ppm.

(3,5-$^2H_2$)-*N*-Formyl-4-hydroxyanthranilic Acid. A. (3,5-$^2H_2$)-4-Hydroxyanthranilic Acid. 4-Hydroxyanthranilic acid[22] (0.49 g, 3.22 mmol) was dissolved in a mixture of 15 mL of D_2O and 4.3 mL of 35% DCl. The solution was stirred under N_2 at 50–60 °C for 3.5 h. The solution was then neutralized with a solution of NaOD in D_2O to pH 6, and concentrated in vacuo. The residue was extracted with CH_3OD. The methanol solution was evaporated, leaving 0.75 g of a light yellow solid, which contained a considerable amount of NaCl. Since it was difficult to remove the NaCl, and it did not interfere with the next reaction, no further purification was carried out. The product showed the same TLC behavior as an authentic sample: ^{1}H NMR (acetone-d_6 + D_2O, 300 MHz) δ 7.61 (s, 1 H).

B. (3,5-$^2H_2$)-*N*-Formyl-4-hydroxyanthranilic Acid (17). A mixture of 0.108 g (0.70 mmol) of (3,5-$^2H_2$)-4-hydroxyanthranilic acid, 2 mL of distilled benzene, and 0.14 mL (0.160 g, 3.45 mmol) of 95% HCOOD was refluxed at 70 °C in a flask equipped with a Dean–Stark trap for 4 h. The purpose of the Dean–Stark trap was to remove the D_2O present in the 95% HCOOD. By the end of the reaction period, a solid had precipitated. The solid was collected by filtration and washed with a little EtOAc to yield 0.087 g (0.48 mmol, 69%) of an off-white solid. The product displayed an R_f of 0.80 on silica gel developed with ethyl acetate containing 2% acetic acid, mp 244 °C (dec): ^{1}H NMR (unlabeled compound) (CD_3CN + D_2O, 300 MHz) δ 6.57–6.60 (d, J = 8.7 Hz, 1 H, C-5), 7.90–7.93 (d, J = 8.8 Hz, 1 H, C-6), 8.04 (s, 1 H, C-3), 8.39 (s, 1 H, NH), 8.87 (s, 1 H, CHO); ^{13}C NMR (unlabeled compound) (DMSO-d_6, 75.45 MHz) 106.9, 107.1, 110.6, 113.4, 142.2, 161.2, 162.5, 169.4 ppm; MS m/z 29, 52, 107, 135, 153, 181 (M^+); HRMS m/z (M^+, $C_8H_7NO_4$) calcd 181.034 85, obsd 181.036 76; ^{1}H NMR (labeled compound) (CD_3CN + D_2O, 300 MHz) δ 7.91 (s, 1 H, C-6), 8.38 (s, 1 H, NH), 8.85 (s, 1 H, CHO); ^{13}C NMR (labeled compound) (DMSO-d_6, 75.45 MHz) 106.8 (t), 107.6, 110.6 (t), 133.2, 142.0, 161.1, 162.3, 169.4 ppm.

(*formyl*-^{13}C)-*N*-Formyl-4-hydroxyanthranilic Acid. This compound was synthesized from 4-hydroxyanthranilic acid and (^{13}C)formic acid in 71% yield by the same procedure used to prepare (3,5-$^2H_2$)-*N*-formyl-4-hydroxyanthranilic acid. The ^{13}C NMR spectrum of the product exhibited enrichment at 162.3 ppm.

(4,6-$^2H_2$)-*N*-Formyl-3-hydroxyanthranilic Acid. A. (4,6-$^2H_2$)-3-Hydroxyanthranilic Acid. 3-Hydroxyanthranilic acid (108 mg, 0.71 mmol) was placed inside a thick-walled screw-capped tube, the tube was flushed with N_2, and 2 mL of D_2O was added. The solid did not dissolve. Potassium *tert*-butoxide (154 mg, 1.37 mmol) was added and the cap was screwed on tightly. All of the solids dissolved and the solution became brown in color. The solution was heated at 100 °C for 3 days, then cooled to room temperature and acidified to pH 6 with 6 N HCl. A large amount of white solid precipitated which was collected by filtration and dried in vacuo, to yield 65 mg (0.42 mmol, 64%) of product. The product showed the same TLC behavior as the unlabeled authentic sample. The ^{1}H NMR spectrum exhibited a singlet at δ 6.68 indicating that two aromatic protons had exchanged with deuterium.

B. (4,6-$^2H_2$)-*N*-Formyl-3-hydroxyanthranilic Acid (18). A mixture of 381 mg (2.46 mmol) of (4,6-$^2H_2$)-3-hydroxyanthranilic acid, 1.91 g of sodium formate and 41.2 mL of 96% formic acid was stirred under N_2 at −5 to 0 °C, while 13.7 mL of Ac_2O was added dropwise over an hour. After the addition of Ac_2O, the mixture was stirred at room temperature for 12 h. Water was then added to the solution and all of the solvent evaporated. The residue was extracted with MeOH several times. The combined MeOH extracts were evaporated, leaving a brown gum. Purification was accomplished by chromatography on a CC-4 silica gel column. Elution was first carried out with ethyl acetate, then with a mixture of methanol and ethyl acetate (30:70). This yielded 269 mg (1.47 mmol, 60%) of off-white solid. The product exhibited an R_f of 0.50 on silica gel developed with ethyl acetate containing 2% acetic acid, mp 154–155 °C: ^{1}H NMR (unlabeled compound) (CD_3CN + D_2O, 300 MHz) δ 8.80 (s, 1 H, CHO), 8.25 (s, 1 H, NH), 7.55–7.52 (d, J = 7.3 Hz, 1 H, C-6), 7.16 (m, 2 H, C-4 + C-5); ^{13}C NMR (unlabeled compound) (CD_3OD, 75.45 MHz) 167.6, 162.8, 127.5, 125.3, 123.6, 123.3, 121.6 ppm; MS m/z 29, 52, 79, 107, 119, 135, 153, 181 (M^+); HRMS m/z (M^+, $C_8H_7NO_4$) 181.034 85, obsd 181.037 67; ^{1}H NMR (labeled compound) (CD_3CN + D_2O, 300 MHz) δ 8.85 (s, 1 H, CHO), 8.25 (s, 1 H, NH), 7.16 (s, 1 H, C-5); ^{13}C NMR (labeled compound) (CD_3OD, 75.45 MHz) 167.5, 162.9, 127.4, 125.1, 123.5, 123.2 (t), 121.9 (t) ppm.

(*carboxyl*-^{13}C,3,5-$^2H_2$)-*N*-Formylanthranilic Acid. A. (*carboxyl*-^{13}C)-2-Nitrobenzoic Acid. 2-Bromonitrobenzene from Aldrich Chemical Co. contains 20% water. This was removed by addition of ethanol to dissolve the wet solid, evaporation of the solvent, and drying in vacuo for 3 h. An ^{1}H NMR spectrum of the compound revealed that only a trace of water was left. In a 100-mL three-necked flask was placed 3.10 g (15.3 mmol) of the dry 2-bromonitrobenzene. One neck was equipped with an addition funnel containing 40 mL of freshly distilled THF, while one of the other necks was connected to another 50-mL three-necked flask via a stopcock. The third neck was stopped with a rubber septum. The second 50-mL flask, which contained 2.0 g (10.1 mmol) of $Ba^{13}CO_3$, was equipped with an addition funnel containing 7 mL of concentrated H_2SO_4. The whole system was flushed with N_2 for 30 min; then the THF was added and the solid dissolved to form a yellow solution. The flask containing the yellow solution was cooled to −100 °C in a liquid N_2–isooctane bath. A solution of phenyllithium in hexane (1.8 M, 7 mL, 12.6 mmol) was transferred by syringe to the dropping funnel and then added dropwise over 10 min to the 2-bromonitrobenzene solution. The mixture was allowed to stir for an additional 45 min, and then the N_2 flow was stopped. The stopcock connecting the two three-necked flasks was opened and H_2SO_4 was slowly added dropwise to the $Ba^{13}CO_3$, generating $^{13}CO_2$. The flask containing the organolithium derivative was stirred for 2 h at −100 °C. Nitrogen was then admitted and 50 mL of 10% of NaOH was added slowly. After the addition, the mixture was allowed to come to room temperature gradually. A very dark solution was obtained. The two layers were separated and the aqueous layer was washed with ether (4–6×). It was then acidified with concentrated HCl. The yellow acidic solution was cooled to room temperature and extracted with ether three times. The yellow ether solution was dried over Na_2SO_4, and then evaporated to afford 1.65 g (9.8 mmol, 97%) of a light brown solid. The product displayed an R_f of 0.70 on silica gel developed with ethyl acetate/methanol (1:1) which was identical with the R_f of unlabeled material. The compound was used without additional purification.

B. (*carboxyl*-^{13}C)Anthranilic Acid. A mixture of 1.65 g (9.8 mmol) of crude (*carboxyl*-^{13}C)-2-nitrobenzoic acid, 0.25 g of 10% palladium on charcoal, and 80 mL of ethanol was hydrogenated on a Parr shaker at room temperature and 2 atm for 30 min. The catalyst was removed and the solvent was evaporated to yield 1.19 g (8.6 mmol, 88%) of light yellow solid. The product had an R_f of 0.65 on silica gel developed by methanol which was identical with that exhibited by an authentic sample. The ^{1}H NMR spectrum was identical with that of an authentic sample. The ^{13}C NMR spectrum exhibited a high level of enrichment at 170.8 ppm which was the position of the carboxyl group.

C. (*carboxyl*-^{13}C,3,5-$^2H_2$)-*N*-Formylanthranilic Acid (20). This compound was synthesized in 29% yield from (*carboxyl*-^{13}C,3,5-$^2H_2$)-anthranilic acid by the same procedure as that used to prepare (3,5-$^2H_2$)-*N*-formylanthranilic acid. The product displayed the same R_f value (0.45) as an authentic sample on a silica gel TLC plate developed with ethyl acetate containing 2% acetic acid: ^{1}H NMR (acetone-d_6, 300 MHz) δ 9.05 (s, 1 H, CHO), 8.59 (s, 1 H, NH), 8.11 (d, J = 3.8 Hz, 1 H, C-6), 7.61 (s, 1 H, C-4); ^{13}C NMR (acetone-d_6, 75.45 MHz) 169.9 ppm (enriched).

(*formyl*-^{13}C,5-$^2H_1$)-*N*-Formylanthranilic Acid. A. (*formyl*-^{13}C)-5-Bromo-*N*-formylanthranilic Acid. A mixture of 0.98 g (4.5 mmol) of 5-bromoanthranilic acid, 1 g (21.3 mmol) of 95% H^{13}COOH, and 10 mL

(38) Spiteller, G.; Grupe, A. *J. Chromatogr.* **1981**, *226*, 301.
(39) du Vigneaud, V.; Loring, H. S.; Craft, H. A. *J. Biol. Chem.* **1934**, *105*, 481.

of benzene was refluxed for 8 h under an argon atmosphere. A white solid precipitated. The mixture was filtered and the solid was washed with hexane followed by ethyl acetate. After drying in vacuo, 1.04 g (4.24 mmol, 93%) of product was obtained which displayed an R_f of 0.35 on silica gel developed with ethyl acetate/methanol (4:1) containing 2% acetic acid, mp 225–226 °C: ^{1}H NMR (unlabeled compound) (acetone-d_6, 250 MHz) δ 9.15 (s, 1 H, CHO), 8.70 (d, J = 9.0 Hz, 1 H, C-3), 8.62 (bs, 1 H, NH), 8.19 (d, J = 2.5 Hz, 1 H, C-6), 7.77 (dd, J = 8.9 Hz, J = 2.5 Hz, 1 H, C-4); ^{13}C NMR (DMSO-d_6, 300 MHz) 167.6, 161.1, 138.9, 136.7, 133.4, 122.5, 119.6, 114.4 ppm; MS m/z 170, 197 (199), 215 (217), 243 (245) (M^+); HRMS m/z (M^+, $C_8H_6BrNO_3$) calcd 242.953 08, obsd 242.952 96; ^{1}H NMR (labeled compound) (acetone-d_6, 250 MHz): δ 8.96 (s, 1 H, CHO), 8.72–8.69 (d, J = 7.5 Hz, 1 H, C-3), 8.28 (s, 1 H, NH), 8.19 (d, J = 2.0 Hz, C-6), 7.77 (dd, J = 2.0 Hz, J = 7.6 Hz, 1 H, C-4); ^{13}C NMR (DMSO-d_6, 300 MHz) 160.9 ppm (enriched).

B. (*formyl*-^{13}C,5-^{2}H$_1$)-*N*-Formylanthranilic Acid (19). This compound was synthesized by hydrogenation of (*formyl*-^{13}C)-5-bromo-*N*-formylanthranilic acid with deuterium gas using the same procedure employed to prepare (3,5-^{2}H$_2$)-*N*-formylanthranilic acid and was characterized in the same way: ^{1}H NMR (acetone-d_6, 250 MHz) δ 9.00 (s, 1 H, CHO), 8.75–8.72 (d, J = 8.4 Hz, 1 H, C-3), 8.20 (s, 1 H, NH), 8.12 (s, 1 H, C-6), 7.63–7.60 (d, J = 8.0 Hz, 1 H, C-4); ^{13}C NMR (acetone-d_6, 250 MHz) 160.9 ppm (enriched).

(2-^{13}C)-6-Hydroxytryptophan. A. (β-^{13}C)-4-Benzyloxy-2,β-dinitrostyrene. A solution of 1.30 g (23.2 mmol) of KOH in 2.25 mL of water and 22.5 mL of ethanol was added dropwise with vigorous mechanical stirring to a suspension of 2.90 g (11.3 mmol) of 4-benzyloxy-2-nitrobenzaldehyde[26] and 1.00 g (14.3 mmol) of (^{13}C)nitromethane in 47 mL of ethanol, cooled to −5 °C. The KOH solution was added over 60 min after which time the mixture was held for 1.5 h at −5 °C with stirring. The mixture was then treated with dilute HCl (2.1 mL concentrated HCl in 26.25 mL of water) and cooled to −1 °C; 105 mL of water was added. The resulting cloudy solution was extracted with ether three times. The yellow ether extract was dried over Na_2SO_4 and evaporated to yield an orange gum. The residue was refluxed for 10 min with 19 mL of acetic anhydride and 3.75 g of sodium acetate, and then poured into water. The resulting cloudy solution was extracted twice with EtOAc. The EtOAc extract was washed with saturated $NaHCO_3$ solution and saturated NaCl solution, then dried over Na_2SO_4, filtered, and evaporated, to yield 2.40 g (7.97 mmol, 71%) of yellow needles, mp 134–135 °C (lit.[26] 134–135 °C). The product displayed an R_f of 0.44 on silica gel developed with ethyl acetate/hexane (1:3): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.42–8.49 (dd, J = 13.5 Hz, $^3J_{CH}$ = 7.5 Hz, 1 H, C-2′), 7.73–7.74 (d, J = 2.6 Hz, 1 H, C-3), 7.54–7.57 (d, J = 8.7 Hz, 1 H, C-6), 7.38–7.44 (m, 5 H, Ph-H), 7.07–7.76 (dd, $^1J_{CH}$ = 192.8 Hz, J = 13.4 Hz, 1 H, C-1′), 5.20 (s, 2 H, Ph-CH_2); ^{13}C NMR ($CDCl_3$, 75.45 MHz) 161.1, 149.4, 138.5 (enriched), 134.9, 134.6, 130.3, 128.7, 128.5, 127.3, 120.6, 117.8, 111.4, 70.8 ppm.

B. (2-^{13}C)-6-Benzyloxyindole. Iron filings (4.30 g, 76.8 mmol) were added with vigorous mechanical stirring to a solution of 1.30 g (4.32 mmol) of (β-^{13}C)-4-benzyloxy-2,β-dinitrostyrene dissolved in a mixture of 10.4 mL of ethanol, 10.4 mL of acetic acid, and 3 mL of water. The mixture was warmed to 50 °C and kept at that temperature for 30 min. After cooling to room temperature, the mixture was then treated with sodium bisulfite (16 g in 120 mL) solution; the product was extracted five times with ether. The combined ether extract was washed with sodium bicarbonate solution and dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo, leaving a dark brown residue. Purification by silica gel flash chromatography, using ethyl acetate/hexane (1:3) as solvent, yielded 0.752 g (3.36 mmol, 75%) of off-white crystalline compound, mp 114–116 °C (lit.[26] 115–118 °C). The product exhibited an R_f of 0.49 on silica gel developed by ethyl acetate/hexane (1:3). The product can be further purified by recrystallization from benzene/hexane: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.99 (bs, 1 H, NH), 7.35–7.56 (m, 6.5 H, Ph-H + C-4 + C-2), 6.76–6.78 (t, 0.5 H, C-2), 6.90–6.93 (m, 2 H, C-5 + C-7), 6.49–6.51 (m, 1 H, C-3), 5.12 (s, 2 H, Ph-CH_2); ^{13}C NMR ($CDCl_3$, 75.45 MHz) 155.3, 137.2, 136.2, 128.3, 127.3, 127.6, 127.3, 122.9 (enriched), 122.1, 121.05, 110.4, 102.7, 95.7, 70.4 ppm.

C. (2-^{13}C)-6-Hydroxy-DL-tryptophan. (2-^{13}C)-6-Benzyloxyindole was converted into (2-^{13}C)-6-hydroxy-DL-tryptophan in an overall yield of 74% using the procedure of Ek and Witkop:[27] ^{13}C NMR (D_2O, 75.45 MHz) 123.8 (enriched) ppm.

(2-^{13}C)-6-Hydroxyindole. Catalytic debenzylation of (2-^{13}C)-6-benzyloxyindole to (2-^{13}C)-6-hydroxyindole was carried out using the procedure of Ek and Witkop.[27] Since the product was somewhat unstable, it was used without further purification: ^{13}C NMR ($CDCl_3$, 62.89 MHz) 122.8 (enriched) ppm.

(2-^{13}C)-7-Hydroxy-DL-tryptophan. A. 3-Benzyloxy-2-nitrobenzaldehyde. A mixture of 2.30 g (13.7 mmol) of crude 3-hydroxy-2-nitrobenzaldehyde,[29] 1.67 mL (2.40 g, 14.0 mmol) of distilled benzyl bromide, and a solution of 2.03 g (19.2 mmol) of sodium carbonate in 7 mL of water and 70 mL of ethanol was heated to reflux for 3 h. The alcohol was then evaporated, and the residue was diluted with water. An orange solid precipitated which was collected by filtration, washed with water, and dried in vacuo to yield 3.12 g (12.1 mmol, 89%) of product, mp 76–78 °C (lit.[29] 77–79 °C). The product exhibits an R_f of 0.51 on silica gel developed with ethyl acetate/hexane (1:2): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.94 (s, 1 H, C*H*O), 7.55–7.32 (m, 8 H, C_6H_3 + C_6H_5), 5.23 (s, 2 H, Ph-CH_2).

B. (β-^{13}C)-3-Benzyloxy-2,β-dinitrostyrene. A solution of 1.26 g (22.5 mmol) of KOH in 2.2 mL of water and 22 mL of ethanol was added dropwise with vigorous mechanical stirring to a suspension of 3.10 g (12.1 mmol) of 3-benzyloxy-2-nitrobenzaldehyde and 1.00 g (16.1 mmol) of (^{13}C)nitromethane in 56 mL of ethanol cooled to −5 °C. The KOH solution was added over 40 min, after which time the mixture was held for 45 min at −5 °C with stirring. The mixture was then treated with 1 N HCl (27 mL) and cooled to −1 °C; 80 mL of water was added. A light yellow solid precipitated which was collected by filtration, washed with water, and dried in vacuo. The solid was then refluxed for 2 h with 27 mL of acetic anhydride and 3.6 g of sodium acetate, and then poured into water. Yellow solid precipitated which was collected by filtration, washed with water, and dried in vacuo to yield 3.05 g (10.1 mmol, 84%) of product, mp 135–138 °C (lit.[27] 135–138 °C). The product displayed an R_f of 0.48 on silica gel developed with ethyl acetate/hexane (1:2): ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.89 (m, 1 H), 7.50–7.09 (m, 7 H), 5.23 (s, 1 H, Ph-CH_2); ^{13}C NMR ($CDCl_3$, 62.89 MHz) 150.5, 140.5 (enriched), 134.8, 131.7, 128.8, 127.3, 119.4, 117.7, 68.5 ppm.

C. (2-^{13}C)-7-Benzyloxyindole. Iron filings (3.4 g, 60.7 mmol) were added with vigorous mechanical stirring to a solution of 1.00 g (3.32 mmol) of (β-^{13}C)-3-benzyloxy-2,β-dinitrostyrene dissolved in a mixture of 25 mL of ethanol, 25 mL of acetic acid, and 3 mL of water. The mixture was warmed to 60 °C and was kept at that temperature for 5 h. After cooling to room temperature, the mixture was then treated with sodium bisulfite (20%, 200 mL) solution and the product was extracted three times with ether. The combined ether extracts were washed with sodium bicarbonate solution, dried over anhydrous sodium sulfate, and then filtered and concentrated in vacuo to leave a dark brown residue. Purification by silica gel flash chromatography, using ethyl acetate/hexane (1:2) as solvent, yielded 0.520 g (2.32 mmol, 66%) of pink solid, mp 65–67 °C (lit.[27] 67–68 °C). The product exhibited an R_f of 0.80 on silica gel developed by ethyl acetate/hexane (1:2): ^{1}H NMR ($CDCl_3$, 250 MHz) δ 8.45 (bs, 1 H, NH), 7.55–7.39 (m, 5.5 H), 7.31 (d, 1 H, J = 7.96 Hz), 7.06 (t, 1 H, J = 7.81 Hz, J = 7.86 Hz), 6.81 (t, 0.5 H, half C-2), 6.75 (d, 1 H, J = 7.69 Hz), 6.56 (m, 1 H, C-3), 5.23 (s, 2 H, Ph-CH_2); ^{13}C NMR ($CDCl_3$, 75.45 MHz) 123.7 (enriched) ppm.

D. (2-^{13}C)-7-Hydroxy-DL-tryptophan. (2-^{13}C)-7-Benzyloxyindole was converted into (2-^{13}C)-7-hydroxy-DL-tryptophan in an overall yield of 68% using the procedure of Ek and Witkop:[27] ^{13}C NMR (D_2O, 75.45 MHz) 128.0 (enriched) ppm.

(2-^{13}C)-7-Hydroxyindole. (2-^{13}C)-7-Hydroxyindole was synthesized by catalytic debenzylation[27] of (2-^{13}C)-7-benzyoxyindole in 96% yield: ^{13}C NMR ($CDCl_3$, 62.89 MHz) 124.1 (enriched) ppm.

(2′-^{13}C)-(*E*)-3-(4′-Hydroxy-6′-methyl-5′-pyrimidinyl)acrylic Acid. A. Ethyl (2′-^{13}C)-3-(2′-Mercapto-4′-hydroxy-6′-methyl-5′-pyrimidinyl)-propionate (28). Sodium (310 mg, 13.5 mmol) was dissolved in 25 mL of ethanol at 0 °C to form sodium ethoxide solution. To this solution, (^{13}C)thiourea (1.00 g, 13.0 mmol) and diethyl 2-acetylglutarate (2.90 mL, 13.2 mmol) were added. The resulting mixture was refluxed for 2 h. A large amount of white solid precipitated. Water (100 mL) was added to the reaction mixture. A clear solution was obtained which was neutralized with acetic acid. The solution was stored in a refrigerator overnight, whereby white crystals were formed which were collected by filtration and washed with water. The crystals were dried in vacuo to yield 1.67 g (6.85 mmol, 53%) of product, mp 192–193 °C (lit.[31] 192–193 °C). The product exhibited an R_f of 0.87 on silica gel plate developed with EtOAc/MeOH (9:1): ^{1}H NMR (CD_3CN, 250 MHz) δ 1.20 (t, 3 H, $-OCH_2CH_3$), 2.15 (s, 3 H, CH_3), 2.41 (m, 2 H, CH_2), 2.58 (m, 2 H, CH_2), 4.08 (q, 2 H, $-OCH_2$); ^{13}C NMR (CD_3CN, 62.89 MHz) 174.86 (enriched) ppm.

B. Ethyl (2′-^{13}C)-3-(4′-Hydroxy-6′-methyl-5′-pyrimidinyl)propionate (29). A mixture of ethyl (2′-^{13}C)-3-(2′-mercapto-4′-hydroxy-6′-methyl-5′-pyrimidinyl)propionate (1.66 g, 6.83 mmol) in 20 mL of water and 2 mL of 50% Raney nickel slurry (Aldrich) was refluxed for 2 h. The mixture was cooled to room temperature and the catalyst was removed by filtration. The filtrate was evaporated in vacuo and the residue was extracted with ethanol three times. The ethanol extracts were combined and evaporated, and then dried in vacuo to afford 1.15 g (5.40 mmol, 80%) of off-white solid, mp 150–152 °C. The product showed an R_f of 0.46 on silica gel plate developed with EtOAc/MeOH (9:1): ^{1}H

NMR (unlabeled compound) (CD_3OD, 250 MHz) δ 8.01 (s, 1 H, C-2′), 4.10 (q, 2 H, $-OCH_2$), 2.80 (m, 2 H, CH_2), 2.53 (m, 2 H, CH_2), 2.34 (s, 3 H, CH_3), 1.21 (t, 3 H, $-OCH_2CH_3$); ^{13}C NMR (unlabeled compound) (CD_3CN, 75.45 MHz) 173.7, 162.3, 161.1, 146.6, 124.4, 61.1, 32.7, 22.3, 21.6, 14.5 ppm; MS m/z 42, 123, 136, 164, 210 (M^+); HRMS m/z (M^+, $C_{10}H_{14}N_2O_3$) calcd 210.100 42, obsd 210.100 15; ^{13}C NMR (labeled) (CD_3CN, 75.45 MHz) 146.6 (enriched) ppm; MS m/z 124, 137, 165, 211 (M^+).

C. Ethyl (2′-^{13}C)-(*E*)-3-(4′-Hydroxy-6′-methyl-5′-pyrimidinyl)acrylate (30). Isopropylamine (1.66 mL, 11.9 mmol) was dissolved in 15 mL of freshly distilled THF and the solution was cooled to −78 °C under N_2. A solution of *n*-butyllithium in hexane (1.86 M, 6.4 mL, 11.9 mmol) was added to the cooled solution and the mixture was stirred at −78 °C for 15 min to generate LDA solution. Ethyl (2′-^{13}C)-3-(4′-hydroxy-6′-methyl-5′-pyrimidinyl)propionate (1.14 g, 5.40 mmol) dissolved in 80 mL of THF was added dropwise to the LDA solution at −78 °C. After the addition, the mixture was stirred at −78 °C for 1 h. A solution of phenylselenyl chloride (2.11 g, 10.8 mmol) in 20 mL of THF was then added dropwise to the cooled enolate solution; stirring was continued for another 30 min at low temperature. The mixture was then gradually warmed to room temperature. Water was added to quench the reaction and the two layers were separated. The aqueous layer was neutralized with 1 N HCl to pH 6 and extracted several times with ethyl acetate. The organic layers were combined, dried over anhydrous sodium sulfate, and evaporated in vacuo to yield a dark gum. The dark gum was purified by silica gel flash chromatography, with elution using EtOAc/MeOH (9:1). The fractions containing the product were combined and evaporated to yield a yellow gum (1.07 g). The yellow gum was dissolved in 8 mL of ethyl acetate, and hydrogen peroxide (1.66 mL, 30%, 14.6 mmol) as well as water (8 mL) were added. The mixture was stirred at room temperature for 1 h. Sodium sulfite solution was added to decompose the excess hydrogen peroxide (monitored by potassium iodide–starch paper). The aqueous solution was extracted with ethyl acetate several times. The EtOAc extracts were combined and dried over anhydrous sodium sulfate and evaporated to afford 0.56 g (2.7 mmol, 50%) of crude product. The product could be further purified by silica gel flash chromatography eluted with a solution of EtOAc/MeOH (9:1). The product exhibited an R_f of 0.61 on a silica gel plate developed with EtOAc/MeOH (9:1), mp 194–196 °C: ^1H NMR (unlabeled compound) (CD_3CN, 250 MHz) δ 1.28 (t, 3 H, $-OCH_2CH_3$), 2.45 (s, 3 H, CH_3), 4.21 (m, 2 H, $-OCH_2$), 7.26–7.20 (d, 1 H, J = 15.7 Hz), 7.64–7.58 (d, 1 H, J = 15.7 Hz), 8.03 (s, 1 H, C-2′); ^{13}C NMR (CD_3CN, 75.45 MHz) 170.0, 165.5, 162.3, 149.3, 136.8, 123.3, 61.7, 21.8, 14.6 ppm; MS m/z 135, 163, 179, 208 (M^+); HRMS m/z (M^+, $C_{10}H_{12}N_2O_3$) calcd 208.084 77, obsd 208.084 38; ^1H NMR (labeled compound) (CD_3CN, 250 MHz) δ 1.26 (t, 3 H, $-OCH_2CH_3$), 2.44 (s, 3 H, CH_3), 4.19 (m, 2 H, $-OCH_2$), 7.24–7.19 (d, 1 H, J = 15.7 Hz), 7.63–7.57 (d, 1 H, J = 15.7 Hz), 8.00 (d, 1 H, $^1J_{CH}$ = 205.8 Hz, C-2′); ^{13}C NMR (labeled compound) (CD_3CN, 75.45 MHz) 148.60 (enriched) ppm.

D. (2′-^{13}C)-(*E*)-3-(4′-Hydroxy-6′-methyl-5′-pyrimidinyl)acrylic Acid (27). Ethyl (2′-^{13}C)-(*E*)-3-(4′-hydroxy-6′-methyl-5′-pyrimidinyl)acrylate (335 mg, 1.60 mmol) was dissolved in 15 mL of MeOH, and 1.65 mL of 4 N NaOH as well as 10 mL of water were added. The solution was stirred at 40 °C for 8 h. The mixture was evaporated and the aqueous solution was washed twice with chloroform. The aqueous solution was then acidified to pH 5 with 1 N HCl and evaporated in vacuo. The residue was extracted with MeOH several times. The MeOH extracts were combined and evaporated to yield a light brown solid, mp 275 °C (dec). The product displayed an R_f of 0.35 on silica gel plate developed with a solution of EtOAc/MeOH (9:1) containing 3% acetic acid: ^1H NMR (unlabeled compound) (D_2O, 300 MHz) δ 2.61 (s, 3 H, CH_3), 7.09–7.15 (d, 1 H, J = 15.7 Hz), 7.57–7.62 (d, 1 H, J = 15.7 Hz), 9.01 (s, 1 H, C-2′); ^{13}C NMR (unlabeled compound) (D_2O, 75.45 MHz) 149.3, 134.3, 125.1, 17.3 ppm; UV λ^{H_2O} 302 (ε = 4181); MS m/z 135, 180 (M^+); HRMS m/z (M^+, $C_8H_8N_2O_2$) calcd 180.053 47, obsd 180.053 34; ^1H NMR (labeled compound) (D_2O, 300 MHz) δ 2.52 (s, 3 H, CH_3), 6.99–7.05 (d, 1 H, J = 15.7 Hz), 7.61–7.66 (d, 1 H, J = 15.7 Hz), 8.26 (d, J_{CH} = 206.6 Hz, 1 H, C-2′); ^{13}C NMR (labeled compound) (D_2O, 75.45 MHz) 148.6 (enriched) ppm.

Acknowledgment. We are pleased to acknowledge support of this work by PHS Grant No. CA 25142 awarded by the National Cancer Institute and by The Robert A. Welch Foundation (C-729).

A Novel Approach to the Synthesis of Morphine Alkaloids: The Synthesis of (*d*,*l*)-Thebainone-A

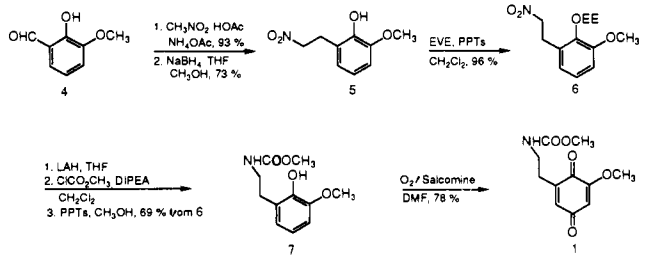
Marcus A. Tius*,[1] and Michael A. Kerr

Contribution from the Department of Chemistry, 2545 The Mall, University of Hawaii, Honolulu, Hawaii 96822. *Received November 18, 1991*

Abstract: A nonconventional approach to the preparation of morphinans has been applied to the total synthesis of thebainone-A and β-thebainone-A. Noteworthy features of this synthesis are the regiospecificity of the Diels–Alder reaction to form **9** and of the enolization–hydroxylation of **11**, the unusual aromatization of **14** as well as the selectivity of the intramolecular Michael addition of the amine to form **41**. This route offers access to several alkaloid skeleta which are related to morphine as well as demonstrating a new approach to the synthesis of aromatic molecules.

A long-standing interest in the synthesis of aromatic rings from nonaromatic precursors led to a consideration of the total synthesis of morphine alkaloids.[2] Conventional wisdom holds that the retrosynthetic disconnection of aryl or arylalkyl C–C bonds is not strategic.[3] We felt that this was no longer invariably true and that the development in recent years of efficient methods for the assembly of regiospecifically substituted benzene rings[4] might open new pathways for total synthesis. One could consider a synthesis

Scheme I



(1) Fellow of the Alfred P. Sloan Foundation, 1988–1991.

(2) (a) Tius, M. A.; Ali, S. *J. Org. Chem.* **1982**, *47*, 3163. (b) Tius, M. A.; Thurkauf, A.; Truesdell, J. *Tetrahedron Lett.* **1982**, 2819. (c) Tius, M. A.; Thurkauf, A.; Truesdell, J. *Tetrahedron Lett.* **1982**, 2823. (d) Tius, M. A.; Savariar, S. *Synthesis* **1983**, 467. (e) Tius, M. A ; Thurkauf, A. *J. Org. Chem.* **1983**, *48*, 3839. (f) Tius, M. A.; Gomez-Galeno, J. *Tetrahedron Lett.* **1986**, 2571. (g) Tius, M. A.; Thurkauf, A. *Tetrahedron Lett.* **1986**, 4541.

(3) Corey, E. J.; Cheng, X. In *The Logic of Chemical Synthesis*; Wiley-Interscience: New York, 1989; p 38.

0002-7863/92/1514-5959$03.00/0