Biosynthesis of Esperamicin A_1, an Enediyne Antitumor Antibiotic

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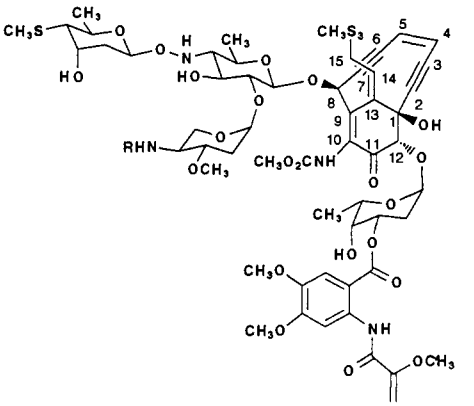
Abstract: Biosynthetic studies on esperamicin A_1 (esp A_1) were carried out by examining the incorporation of singly and doubly ^{13}C-labeled acetates, L-[*methyl*-^{13}C]methionine and $Na_2{}^{34}SO_4$, by cultures of *Actinomadura verrucosospora* MU-5019. The acetate incorporation results show that the C_{15} bicyclic enediyne core of esp A_1 is derived from head-to-tail condensation of seven acetate units and the uncoupled carbon attached to the trisulfide unit is derived from the C2 of acetate. The L-[*methyl*-^{13}C]methionine incorporation result shows that the *S*-methyl groups of the trisulfide and the thiosugar and the *O*-methyl groups of the aminosugar, the aromatic chromophore, and the carbamate moiety are derived from L-methionine via *S*-adenosylmethionine. Using $Na_2{}^{34}SO_4$ as the sole sulfur source in the fermentation and by mass spectrometric analysis, we have demonstrated that all four sulfur atoms in esperamicin A_{1c} (esp A_{1c}) can be derived from $Na_2{}^{34}SO_4$. On the basis of the ^{13}C-labeled acetate-enrichment pattern, the enediyne ring moiety of esp A_1 may be derived from an octaketide with the loss of the C1 of the end acetate unit. The acetate-enrichment pattern of the enediyne moiety of esp A_1 is in good agreement with that of dynemicin A (DNM-A). The two carbons comprising the yne moieties of esp A_1 and DNM-A are derived from separate acetate units. The corresponding carbons in chromophore A of neocarzinostatin (NCS Chrom A) are derived from the same acetate units. This may suggest that enediyne cores of esp A_1 and DNM-A are biosynthesized from a common precursor while NCS Chrom A is biosynthesized via a different process.

Esperamicin A_1 (esp A_1, Figure 1), an extremely potent antitumor antibiotic, was isolated from cultures of *Actinomadura verrucosospora*. The isolation and the elucidation of the structure of esp A_1 have been reported.[1] Esp A_1 consists of a bicyclic core to which are attached a trisaccharide and a substituted 2-deoxy-L-fucose with an aromatic chromophore attached to the sugar 3 position. The C_{15} bicyclic core contains the enediyne, an allylic trisulfide, and a bridgehead enone. Detailed structure–activity studies have established that the interaction of these three functionalities results in a bioreductively activated, highly efficient DNA strand scission.[2] The mechanism of action of esp A_1 and several related compounds, calicheamicin,[3] neocarzinostatin,[4] dynemicins,[5] and kedarcidin,[6] has been reported. It appears that these compounds are capable of cleaving DNA via direct carbon radical abstraction of deoxyribose hydrogen atoms. The presence of the enediyne function in these compounds is a prerequisite to their potent activities. The biosyntheses of the neocarzinostatin chromophore[7] (NCS Chrom A) and dynemicin A[8] (DNM-A) have recently been reported. The C_{14} dienediyne moiety of NCS Chrom A is proposed to be derived from degradation of C18 oleate via the oleate–crepenynate pathway rather than by *de novo* synthesis from acetate.[7] Tokiwa et al.[8] have proposed that DNM-A is derived from an octaketide precursor with a loss of a two-carbon unit from the carboxylate end to yield the heptaketide intermediate. In this paper, we report the pattern of incorporation of various ^{13}C-labeled precursors into esp A_1. A possible biosynthetic pathway of the C_{15} enediyne core of esp A_1 is



Esperamicin A_1 R = $CH(CH_3)_2$

Esperamicin A_{1b} R = CH_2CH_3

Esperamicin A_{1c} R = CH_3

Figure 1. Structures of esperamicin A_1, esperamicin A_{1b}, and esperamicin A_{1c}.

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Table I. ^{13}C NMR Assignments of [1-^{13}C]- and [2-^{13}C]Acetate-Labeled Diacetylesperamicin A_1 and $^1J_{cc}$ of [1,2-$^{13}C_2$]Acetate-Labeled Diacetylesperamicin A_1

carbon	δ, ppm	[1-^{13}C]acetate (relative intensity)	[2-^{13}C]acetate (relative intensity)	[1,2-$^{13}C_2$]acetate	
				$^1J_{^{13}C1-^{13}C2}$	satellites
1	77.7	4.5	1	79.9	42.5, 45.1
2	99.9	1	3.0	80.2	186
3	84.2	4.2	1	89.4	187
4	126.8	1	3.8	90.3	72.2
5	123.5	4.1	1	89.7	72.1
6	88.9	1	3.5	89.8	
7	98.1	4.2	1	74.5	165
8	72.5	1	4.7	73.6	45.0
9	148.8	4.3	1	77.0	
10	134.4	1	3.0	76.7	52.2, 53.6
11	194.8	2.0	1	45.2	broad
12	84.3	1	3.3	45.5	obscured
13	136.7	1	4.7	80.7	44.0, 45.0
14	131.8	5.8	1	80.5	45.4
15	41.0	1	6.0		45.2

presented. We also report the labeling pattern of esp A_1 and esp A_{1c} by L-[*methyl*-^{13}C]methionine and $Na_2{}^{34}SO_4$, respectively.

Results and Discussion

Incorporation of ^{13}C-Labeled Acetate. The major problem in elucidating the biosynthesis of esp A_1 is due to its low production in the fermentation. The estimated titer of esp A_1 by the parent strain in the original production medium was about 0.05 μg/mL.[9] Through extensive media development and strain improvement studies, the titer of esp A_1 was increased to 25–30 μg/mL in the production medium H946 by the new strain *A. verrucosospora* MU-5019.[9] With the improvement by this mutant strain, feeding experiments can be carried out on a 5–10-L scale to obtain sufficient ^{13}C-enriched esp A_1 for NMR studies. Incorporation experiments were carried out by feeding 0.2% [1-^{13}C]acetate, [2-^{13}C]acetate, and [1,2-$^{13}C_2$]acetate to the cultures of *A. verrucosospora* MU-5019. Twenty-five to thirty milligrams of pure ^{13}C-enriched esp A_1 can be isolated from a 10-L fermentation. Esp A_1 is very soluble in $CDCl_3$ but has limited solubility in CH_3OH. $CDCl_3$ was used as the solvent for ^{13}C-enriched esp A_1 in the initial NMR analysis. The signals of C8, C9, and C10 of the enediyne ring were difficult to quantitate because they were broadened in $CDCl_3$. The signal of C1 of the enediyne ring was also buried underneath the $CDCl_3$ signal. In order to obtain accurate integration of the signals for the above carbons, ^{13}C-enriched esp A_1 was first converted to its diacetyl derivative and CD_3OD was used as the solvent for NMR analysis. Sharp signals for all 15 carbons of the enediyne ring of the diacetyl-esp A_1 were obtained in the NMR spectrum and are shown in Table I. Figure 2 summarizes the ^{13}C-labeling pattern of esp A_1 from the [^{13}C]acetate supplemented cultures. Upon feeding the culture with [1-^{13}C]acetate, we have demonstrated that C1, C3, C5, C7, C9, C11, and C14 of the enediyne ring were enriched (Figure 2a). Peak intensity enhancements at these seven carbons signals were 2.0–5.8-fold (Table I). Using [2-^{13}C]acetate, we have demonstrated that C2, C4, C6, C8, C10, C12, C13, and C15 were enriched (Figure 2b). Peak intensity enhancements at these eight carbon signals were 3.0–6.0-fold (Table I). We have clearly shown that all of the 15 carbons of the enediyne ring portion of esp A_1 were derived from acetate. In order to determine the connectivity of the carbon units of the enediyne ring, doubly enriched [1,2-$^{13}C_2$]acetate was fed to the esperamicin-producing culture. The incorporation pattern of [1,2-$^{13}C_2$]acetate was confirmed by matching of $^1J_{cc}$ values as shown in Table I and Figure 2c. The $^1J_{cc}$ coupling constants clearly showed that seven

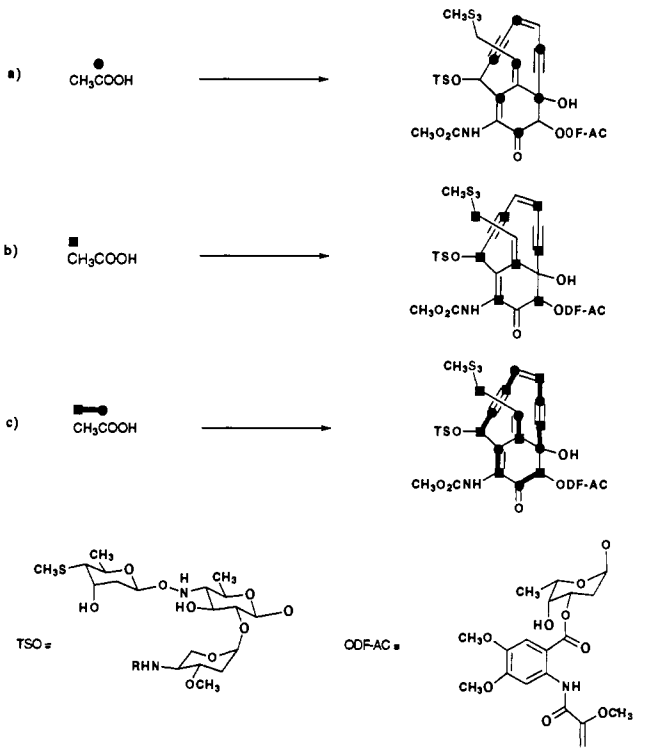

Figure 2. [^{13}C]Enrichment pattern of esperamicin A_1 from cultures of *A. verrucosospora* MU-5019 supplemented with (a) sodium [1-^{13}C]-acetate, (b) sodium [2-^{13}C]acetate, and (c) sodium [1,2-$^{13}C_2$]acetate.

pairs of carbons were coupled to each other with C15 being the only carbon that was not coupled.

There are only four possible folding patterns for a linear C_{15} unit to yield the enediyne ring system of the esperamicins as shown in Scheme I. On the basis of the result of the connectivities of the carbons in Figure 2c, folding patterns a and d can be eliminated from consideration. From Table I, C14 and C15 have the highest peak intensity enhancements at 5.8–6.0-fold while C11 and C12 have the least peak intensity enhancements at 2.0–3.3-fold. It is reasonable to assume that C11 and C12 may be the chain termination unit while C15 is part of the starter acetate unit. If this is true, the C_{15} chain of the enediyne of esp A_1 can be derived from an octaketide with the loss of the C1 of the acetate unit (Scheme I). The C_{15} chain can then be folded as in path c, and further reaction (path f) would lead to the formation of the enediyne ring of esp A_1. This pathway is therefore favored over the alternate possibility in Scheme I (paths b and e). Tokiwa et al.[8] have demonstrated that DNM-A is biosynthesized from

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Scheme I. Possible Folding Patterns of the C_{15} Enediyne Ring of Esperamicin A_1

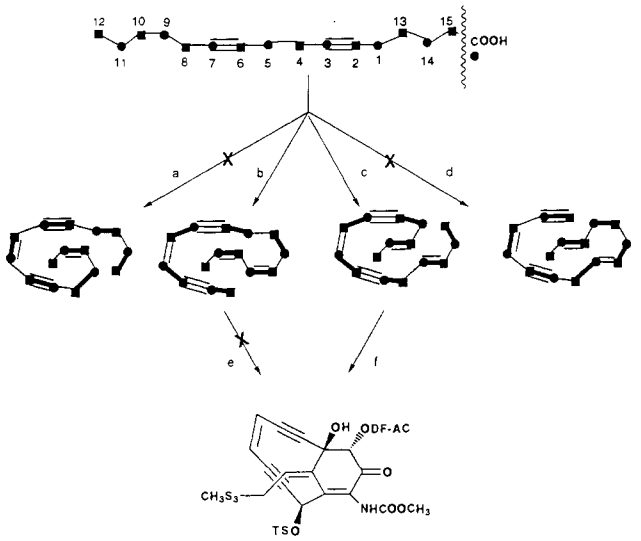

Table II. Effect of Cerulenin and Sodium Oleate on Esperamicin A_1 Production by *A. verrucosopora* MU-5019

culture condition[a]	[esperamicin A_1][b] (μg/mL)	% inhibition
control (no addition)	23.3	
0.5 mM cerulenin	8.8	62.2
0.5 mM cerulenin + 0.1% sodium oleate	8.2	64.8
0.5 mM cerulenin + 0.5% sodium oleate	6.7	71.3
1.0 mM cerulenin	0	100
1.0 mM cerulenin + 0.1% sodium oleate	0	100
1.0 mM cerulenin + 0.5% sodium oleate	0	100

[a] Cerulenin and/or sodium oleate were added to the culture at day 4 of the fermentation. [b] The titers of esperamicin A_1 were determined at day 14 of the fermentation.

two heptaketide chains, which form the enediyne ring and anthraquinone moiety, respectively. Both the enediyne and anthraquinone moieties are derived from seven head-to-tail coupled acetate units. They further proposed a biosynthetic scheme involving a common octaketide intermediate for the formation of the enediyne ring of the esperamicin/calicheamicin/dynemicin class of antibiotics. Our [^{13}C]acetate enrichement data of the enediyne ring of esp A_1 support the above hypothesis. The labeling patterns of the diyne moieties of esp A_1 and DNM-A are the same. The two carbons comprising the respective yne moieties are derived from separate acetate units.

Hensens et al.[7] have proposed that the C_{14} dienediyne chain of NCS Chrom A is derived from degradation of oleate via the oleate–crepenynate pathway for polyacetylenes rather than by *de novo* synthesis from acetate. Hensens et al.[7] further postulated that the C_{15} enediyne ring of esperamicin/calicheamicin could similarly be derived via the oleate–crepenynate pathway. Our data on esp A_1 production from cultures of *A. verrucosospora* MU-5019 supplemented with cerulenin and sodium oleate are at variance with the above hypothesis. Addition of cerulenin (0.5 and 1.0 mM) to the cultures of *A. verrucosospora* MU-5019 before the onset of esperamicin synthesis significantly inhibited esp A_1 production (Table II). Cerulenin specifically inhibits the β-ketoacyl–acyl carrier protein synthase of fatty acid and polyketide biosynthesis[10] and has no effect on the preformed fatty acid. If esp A_1 is derived from the oleate–crepenynate pathway,

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Table III. Effect of L-Methionine on the Production of Esperamicin A_1 by *A. verrucosospora* MU-5019

[L-methionine][a]	[esperamicin A_1] (μg/mL)[b]	% inhibition
0	21.8	
0.02%	12.7	41.7
0.05%	5.1	76.6
0.10%	0	100
0.20%	0	100

[a] L-Methionine was added to the culture at day 4 of the fermentation. [b] The titers of esperamicin A_1 were determined at day 14 of the fermentation.

adding sodium oleate to the cerulenin supplemented culture should provide the precursor for esp A_1 and obviate the repression of esp A_1 synthesis by cerulenin. We did not observe any improvement in esp A_1 production when adding sodium oleate (0.1 and 0.5%) to the cerulenin supplemented cultures (Table II). The [^{14}C]-acetate labeling patterns of esp A_1 in the cultures with or without cerulenin at the active production phase have demonstrated that esp A_1 is synthesized *de novo* from acetate.[11] Furthermore, the two carbons of the yne moieties of NCS Chrom A are derived from the same acetate units,[7] indicating that the biosynthetic pathway of the C_{14} dienediyne ring structure of NCS Chrom A is different from those of esp A_1 and DNM-A.

Incorporation of L-[*methyl*-^{13}C]Methionine. Most biological methylation in bacteria involves L-methionine, the methyl group of which is activated by *S*-adenosylation with ATP. Numerous reports[12] have shown that L-methionine inhibits the production of antibiotics even though L-methionine is the precursor (methyl donor) of the antibiotics. Table III shows the effect of L-methionine on the production of esp A_1 by *A. verrucosospora* MU-5019. L-Methionine, at a concentration as low as 0.02%, inhibited the production of esp A_1 by 41.7%. At 0.1% L-methionine concentration, no esp A_1 can be detected in the fermentation even though there was no effect on growth of the organism and pH of the fermentation. Experiments with radiolabeled anthramycin showed that reduced yields of anthramycin production when L-methionine was added to the culture of *Streptomyces refuineus* were due to the interaction of anthramycin with the reactive metabolites produced in the L-methionine-supplemented cultures.[12e] The thiol of L-methionine or its reactive metabolites generated in the fermentation may react with the trisulfide of esp A_1 and convert esp A_1 to its aromatized derivative. Inhibition of DNM-A production was observed when more 0.04% L-methionine was added to the culture of *Micromonospora chersina*.[8] The above findings indicated that we cannot add a high initial concentration of L-[*methyl*-^{13}C]methionine to the culture of *A. verrucosospora* for the esp A_1 labeling study. Consequently, L-[*methyl*-^{13}C]-methionine was added to the culture on two different days to yield a final concentration of 0.05%. Even though the production of esp A_1 was significantly decreased (56.7%), adequate amounts (6.5 mg) of ^{13}C-enriched esp A_1 were obtained for NMR analysis. Figure 3 shows the ^{13}C-enrichment pattern of esp A_1 isolated from the L-[*methyl*-^{13}C]methionine feeding study. The carbons of seven methyl groups were clearly enriched by 2.6–3.2-fold when L-[*methyl*-^{13}C]methionine was fed into the culture. We also observed low enrichment of the three carbons of the isopropyl group of the aminosugar. Several examples where formation of an ethyl group from two methyl groups of L-methionine have been reported. These include the hydroxyethyl side chains of

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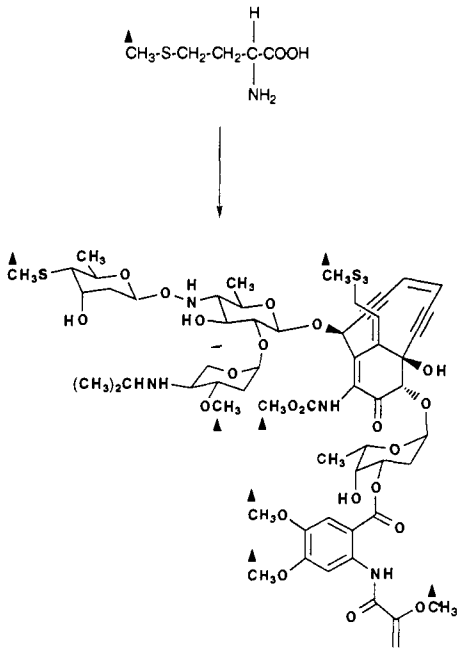

Figure 3. [^{13}C]Enrichment pattern of esperamicin A_1 from culture of *A. verrucosospora* MU-5019 supplemented with L-[*methyl*-^{13}C]methionine.

thienamycin[13] and pectamycin[14] and the C24 ethyl group of certain plant sterols.[15] However, formation of an *N*-isopropyl group from the methyl of L-methionine has not been reported. The possible incorporation of the methyl of L-methionine into the *N*-isopropyl group of esp A_1 is supported by our results from a study of blocked mutants. Two blocked mutants of esp A_1, DG-111-10-6 and DG-108-9-3, were isolated which do not produce any esp A_1. They each produce one major product of the fermentation, esp A_{1b} and esp A_{1c} (Figure 1), respectively,[16] which differ from esp A_1 only in the *N*-alkyl substituent of the aminosugar. The presence of these blocked mutants may suggest that the formation of esp A_{1c} (methyl), esp A_{1b} (ethyl), and esp A_1 (isopropyl) involves the sequential addition of methyl groups at the nitrogen and *N*-methyl sites of the aminosugar. Similar blocked mutants of the related enediyne antitumor antibiotic calicheamicin have recently been reported.[17] This aspect of the work will be further investigated.

Biosynthetic Origin of Sulfur. Esp A_1 contains four sulfur atoms. It contains a thiomethyl sugar in the trisaccharide moiety and an allylic trisulfide in the bicyclic core. Understanding the biosynthesis of the allylic trisulfide is very important in light of what is known of the mechanism of action of the esperamicins. Studies have shown that the thiolate anion, generated by the reduction of the trisulfide group, undergoes subsequent interaction with the bridgehead enone and the enediyne moiety results in highly efficient DNA strand scission.[2] Feeding ^{35}S-labeled precursors to the culture, we were able to detect the incorporation of $Na_2{}^{35}SO_4$, L-[^{35}S]cysteine and L-[^{35}S]methionine into esp A_1 in the complex medium H946 (Table IV). The rates of incorporation of these three radiolabeled precursors were very low (0.019–0.095%). In an attempt to increase the rate of incorporation of ^{35}S-precursors into esperamicins, the above experiment was repeated by growing the culture in a defined medium. Defined medium DF-15, using sodium sulfate (0.2%) as the sole source of sulfur, supports the production of esp A_{1c} but not esp A_1 in the fermentation.[18] Adding $Na_2{}^{35}SO_4$ into this defined medium yielded 1.7% incorporation of radioactivity into esp A_{1c} (Table IV). L-[^{35}S]methionine and L-[^{35}S]cysteine were also incorporated into esp A_{1c} at about the same efficiency as $Na_2{}^{35}SO_4$ (Table IV). When L-methionine (0.2%) or L-cysteine (0.2%) was substituted for sodium sulfate as the sole sulfur source in the defined medium DF-15, no esperamicins were detected even though the growth of the organism and the pH of the fermentation were the same as those in medium DF-15. The above data suggested that the sulfur from L-methionine or L-cysteine can only be incorporated into either the thiomethyl sugar or the trisulfide moiety but cannot provide all four sulfur atoms. Since sulfur from L-methionine has been shown to be efficiently incorporated into the thiosugar moieties in actinomycete fermentations,[19] this may suggest that the allylic trisulfide cannot be derived from L-methionine or L-cysteine.

Table IV. Incorporation of ^{35}S-Precursors into Esperamicins in the Complex (H946) and Defined (DF-15) Media[a]

	% incorporation of radioactivity in esp A_1 and esp A_{1c}	
precursors	medium H946	medium DF-15
L-[^{35}S]methionine	0.095	1.9
L-[^{35}S]cysteine	0.019	1.2
$Na_2{}^{35}SO_4$	0.034	1.7

[a] ^{35}S-Precursors (1 mCi) were added to the growing cultures of *A. verrucosospora* MU-5019 at day 4 and day 2, in medium H946 and medium DF-15, respectively. The major product of esperamicins produced in medium H946 and medium DF-15 was esp A_1 and esp A_{1c}, respectively.

An experiment was set up to test whether all four sulfur atoms of esperamicin can be derived from Na_2SO_4. $Na_2{}^{34}SO_4$ was used to label esp A_{1c} and the incorporation of the ^{34}S isotope evaluated by MS. Comparison of the MS fragments of the natural esp A_{1c} (^{32}S) with those of the labeled species (^{34}S) allowed for rapid quantitation and location of the incorporated ^{34}S atoms (Figure 4). [^{34}S]Esp A_{1c} was prepared by growing the culture in defined medium DF-15 using $Na_2{}^{34}SO_4$ as the sole sulfur source. The full scan FAB mass spectrum of [^{34}S]esp A_{1c} showed a molecular weight of 1304 Da, 8 mass units higher than that for native esp A_{1c}, suggesting the incorporation of four atoms of ^{34}S. The presence of the four ^{34}S atoms was confirmed by high resolution FAB-MS ($[M+H]^+$ m/z 1305.3708, calcd for $C_{57}H_{77}N_4O_{22}{}^{34}S_4$ 1305.3743). The location of the labeled atoms was determined by comparison of the substructures of native and labeled compounds (Figure 4). The ions observed at m/z 1185 and 1187 result from the neutral loss of the allylic trisulfide from the protonated molecular ions of the native (m/z 1297) and labeled (m/z 1305) compounds, respectively. The 118 Da neutral loss of the allylic trisulfide from the labeled compound confirmed that all three sulfur atoms of the trisulfide were present as ^{34}S atoms. The difference of 2 Da observed for the trisaccharide fragments confirmed the presence of ^{34}S in the thiosugar.

Conclusion

We have demonstrated that the C_{15} bicyclic enediyne ring structure of esp A_1 is derived from head-to-tail condensation of seven acetate units and the uncoupled carbon is derived from C2 of acetate. The two carbons of the yne moieties of esp A_1 are derived from separate acetate units and are in good agreement with those of DNM-A.[8] On the basis of the ^{13}C-labeled acetate

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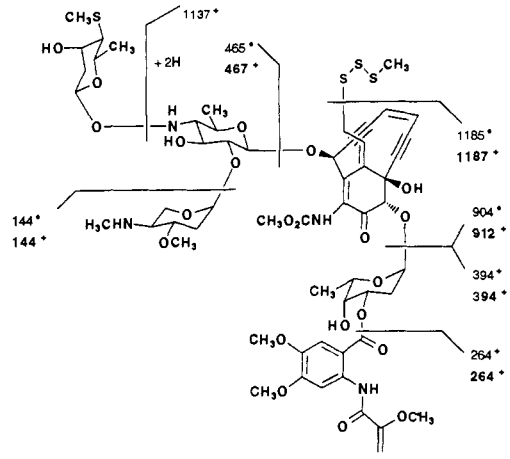

^{32}S - Esperamicin A_{1c} Accurate mass = 1296.3860, $C_{57}H_{75}N_4O_{22}S_4$, 2.0 ppm

^{34}S-Esperamicin A_{1c} Accurate mass = 1304.3630, $C_{57}H_{75}N_4O_{22}{}^{34}S_4$, 2.6 ppm

Figure 4. Summary of ions observed in the full-scan FAB mass spectra of [^{32}S] esperamicin A_{1c} (MW 1296) and [^{34}S] esperamicin A_{1c} (MW 1304). The molecular ions of [^{34}S] esperamicin A_{1c} are in bold type.

enrichment pattern, we propose that the enediyne ring moiety of esp A_1 is derived from an octaketide with the loss of C1 of the end acetate unit as shown in Scheme I. Our data on the production of esp A_1 from cultures of *A. verrucosospora* supplemented with cerulenin and sodium oleate rule out the possibility that the enediyne moiety of esp A_1 is derived from the oleate–crepenynate catabolic pathway. Since the labeling pattern of the two carbons of the yne moieties of NCS Chrom A[7] is different from those of esp A_1 and DNM-A, this may suggest that the enediyne cores of esp A_1 and DNM-A are biosynthesized from a common precursor while NCS Chrom A is biosynthesized via a different process.

The L-[*methyl*-^{13}C]methionine incorporation results show that the *S*-methyl groups of the trisulfide and the thiosugar groups and the *O*-methyl groups of the aminosugar, the aromatic chromophore, and the carbamate moieties are derived from L-methionine via *S*-adenosylmethionine. Further work will be carried out to confirm the formation of the isopropyl group of the aminosugar from L-methionine. Understanding the mechanism of inhibition of esperamicin production by L-methionine may help us to design a way to relieve this inhibitory effect and control the formation of esp A_1, esp A_{1b}, and esp A_{1c} in the fermentation.

Initial studies carried out in our laboratory using $Na_2{}^{34}SO_4$ as the sole sulfur source in the fermentation have demonstrated that all four sulfur atoms in esp A_{1c} can be derived from $Na_2{}^{34}SO_4$. The above method requires only microgram quantities of ^{34}S-labeled esperamicin. We thus hope to use a combination of ^{34}S-precursors, blocked mutants of sulfur metabolism of *A. verrucosospora*, and FAB-MS to further study the biosynthesis of sulfur in esperamicin. Information concerning sulfur metabolism in actinomycetes is scarce, and it would therefore be valuable to explore the biosynthetic pathway leading from sulfate to the allylic trisulfide and the thiosugar in esperamicn from *A. verrucosospora*.

Experimental Section

Materials. Sodium salts of 90% enriched [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetic acid were purchased from Merck Isotopes. $Na_2{}^{34}SO_4$ was obtained from Icon Company. L-[^{35}S]Methionine, L-[^{35}S]cysteine, and $Na_2{}^{35}SO_4$ were purchased from Du Pont NEN Research Products.

Microorganism. *A. verrucosospora* strain MU-5019 was used in this study. Strain MU-5019 was maintained as a cryopreserved culture stored at −80 °C. To prepare a cryopreserved culture, strain MU-5019 was grown on slants for 2–3 weeks until uniform production of spores was obtained. The surface growth of the slant culture was transferred into a 500-mL Erlenmeyer flask, containing 100 mL of the vegetative medium consisting of 2% starch, 0.5% glucose, 1% Pharmamedia, 1% yeast extract, and 0.2% $CaCO_3$. This vegetative culture was incubated at 28 °C for 96 h on a rotary shaker set at 250 rpm. The vegetative culture was mixed with an equal volume of cryoprotective solution consisting of 10% sucrose and 20% glycerol. Four-milliliter portions of this mixture were transferred to sterile cryogenic tubes (5-mL capacity) and were frozen in a dry ice–acetone bath. The frozen vegetative cultures were stored at −80 °C until use.

Media and Culture Conditions. A vegetative culture of strain MU-5019 was prepared by transferring 4 mL of the cryopreserved culture to a 500-mL Erlenmeyer flask containing 100 mL of a vegetative medium. The vegetative culture was incubated at 28 °C and 250 rpm on a rotary shaker. After 96 h, 8-mL aliquots were transferred to 500-mL Erlenmeyer flasks containing 100 mL of esperamicin-producing medium. Two production media were used in this study. A complex medium, H946, was prepared using 6% cane molasses, 2% starch, 2% fish meal, 0.01% $CuSO_4{\cdot}5H_2O$, 0.2% $CaCO_3$, and 0.00005% NaI. A defined medium, DF-15, consisted of 4% sucrose, 0.2% NH_4Cl, 0.2% Na_2SO_4, 0.1% K_2HPO_4, 0.1% $MgCl_2$, 0.1% NaCl, 0.2% $CaCO_3$, 0.0001% $MnCl_2$, 0.0001% $ZnCl_2$, 0.0001% $FeCl_2$, and 0.00005% NaI. The production cultures were incubated at 28 °C and 250 rpm on a rotary shaker.

Extraction and Analytical Method. The production of esp A_1 and esp A_{1c} was monitored by HPLC using a C-18 reverse-phase column (Novapak, 3.9 × 150 mm^2, Waters Associates). The solvent system was 0.05 M ammonium acetate (pH 4.5)/CH_3OH/CH_3CN (1:1:1) at a flow rate of 1 mL/min with the detector wavelength set at 254 nm. The fermentation extracts for HPLC assay were prepared by extracting the fermentation broth with an equal volume of ethyl acetate. The ethyl acetate extracts were concentrated 10-fold. Twenty-five to fifty microliters of the extracts were used for HPLC analysis. The amounts of esp A_1 and esp A_{1c} in the extracts were determined by comparison with authentic standards.

[^{13}C]Acetate Incorporation. The time of addition of [^{13}C]acetate was carefully determined by monitoring the production of esp A_1 during 2–5 days after inoculation of strain MU-5019 to the production medium. When the culture produced about 0.1–0.2 μg/mL esp A_1 (usually between 4–5 days), [^{13}C]acetate was added to the culture to yield the final concentration of 0.2%. The production of esp A_1 was then monitored daily. When the rate of production of esp A_1 dropped to about 2–3 μg mL^{-1} day^{-1}, ^{13}C-labeled esp A_1 was extracted from the culture. The titer of esp A_1 at the time of harvest was about 18–20 μg/mL.

L-[*methyl*-^{13}C]Methionine Incorporation. The first portion of L-[*methyl*-^{13}C]methionine was added to the fermentation to yield a concentration of 0.025% when the production of esp A_1 was about 0.1–0.2 μg/mL. The second portion of [^{13}C]methionine was added to the culture 24 h after the first addition to yield a final concentration of 0.05%.

Purification of Biosynthetically ^{13}C-Labeled Esp A_1. Culture broth (10 L) was extracted with an equal volume of ethyl acetate. After the extract was dried with anhydrous sodium sulfate, it was evaporated to dryness under reduced pressure. The dried extract was dissolved in 60 mL of $CHCl_3$ and mixed with 20 g of silica gel (LiChroprep Si 60, 40–63 μm, Merck). The solvent was removed to absorb the esperamicins onto the silica gel. This was placed on top of a silica gel column comprising 100 g of silica gel in a 150-mL sintered glass funnel. The column was washed sequentially with 2 L of hexane, 3 L of toluene, 2 L of CCl_4, 2 L of CH_2Cl_2, 3 L of $CHCl_3$, and 1 L of $CHCl_3$/CH_3OH (9:1). The $CHCl_3$/CH_3OH (9:1) eluate, containing the esperamicin complex, was dried over anhydrous sodium sulfate and concentrated *in vacuo*. This residue was then dissolved in 30 mL of $CHCl_3$ and mixed with 10 g of silica gel. The solvent was removed, and the silica gel containing the esperamicin complex was applied onto the top of a column of silica gel comprising 50 g of silica gel in a 60-mL sintered glass funnel. The column was successively developed using 1-L aliquots of increasing concentrations of acetone in hexane. Esperamicin complex was eluted in the 50% acetone fraction. The acetone fraction was treated with anhydrous sodium sulfate and concentrated *in vacuo*. The enriched esperamicin complex was further purified by reverse-phase chromatography using a Prep System Gold LC/system (Beckman) fitted with a Radial-Pak Cartridge (μBondapak C_{18}, 8 × 100 mm^2, Waters Associates). The solvent system was 0.05 M ammonium acetate/CH_3OH/CH_3CN (3:2:5), and the detector wavelength was set at 320 nm. The flow rate was 10 mL/min. Esp A_1 was eluted at near 17 min. The fractions containing esp A_1 were combined and concentrated *in vacuo* to remove CH_3OH and CH_3CN. The aqueous mixture was extracted twice with an equal volume of CH_2Cl_2. The organic

extract was then evaporated to dryness under reduced pressure to yield 25–30 mg of pure [^{13}C]esp A_1.

Purification of ^{34}S-Labeled Esp A_{1c}. The production culture (100 mL) using medium DF-15 containing $Na_2{}^{34}SO_4$ was extracted with 100 mL of ethyl acetate. The extract was evaporated to dryness under reduced pressure. The dried extract was dissolved in 3 mL of $CHCl_3$. The solution was applied to a column containing 10 g of silica gel (Lichroprep Si60, 40–63 μm, Merck) previously equilibrated with 100 mL of hexane. The column was washed sequentially with 200 mL of hexane, 100 mL of hexane/acetone (9:1), 40 mL of hexane/acetone (4:1), 40 mL of hexane/acetone (7:3), 40 mL of hexane/acetone (3:2), 40 mL of hexane/acetone (1:1), and 40 mL of acetone. Esperamicins were recovered from the last three fractions, which were combined and evaporated to dryness. The extract was then dissolved in 0.5 mL of DMSO, and five aliquots of 0.1 mL each were injected into a C-18 reverse-phase column (Novapak, 3.9 × 300 mm^2, 4 μm, Waters Associates) using the solvent system of 0.05 M ammonium acetate (pH 4.5)/CH_3OH/CH_3CN (1:1:1) at a flow rate of 2 mL/min. Fractions containing esp A_{1c} were collected and dried under nitrogen. About 200 μg of esp A_{1c} was recovered from HPLC.

Conversion of Esp A_1 to Diacetyl-esp A_1. A solution of esp A_1 (30 mg) in dry methylene chloride (5 mL), cooled to 0 °C, was treated with acetic anhydride (100 μL) and 4-(dimethylamino)pyridine (5 mg). Reaction was carried out for 16 h at 4 °C. The reaction was monitored by TLC on silica gel using 33% acetone in methylene chloride (esp A_1, R_f = 0.25; diacetyl-esp A_1, R_f = 0.74). After the reaction was completed, the mixture was filtered through a layer of silica gel (3 mL), which was then washed with methylene chloride. The products were eluted from the silica gel layer with 10 mL of acetone. The solution was concentrated *in vacuo* to a small volume and subjected to purification by preparative TLC (Kieselgel 60 F_{254}S, 20 × 20 × 0.05 cm^3 plate, Merck) using 33% acetone in methylene chloride. The major product diacetyl-esp A_1 (24 mg, 80% yield) was eluted from the plate with acetone. The major byproduct triacetyl-esp A_1 (5.3 mg, 17.6% yield), with R_f of 0.84, was also isolated.

^{13}C NMR Spectroscopy. The NMR experiments were performed either on a Bruker WM 360wb spectrometer or on a Bruker AM 500 spectrometer. ^{13}C-Enriched esp A_1 samples were dissolved in deuterated methanol under argon. Broad-band proton decoupled, 'gated', and one-dimensional INADEQUATE ^{13}C spectra were obtained on the enriched samples using delays optimized for $^1J_{CC}$ coupling of 83 or 166.7 Hz at ambient temperature. The carbon–carbon coupling constants were tabulated using the broad-band decoupled as well as the INADEQUATE spectra. All the chemical shifts are referenced to external TMS.

Mass Spectrometry. Low- and high-resolution mass spectrometric analyses were performed on a Kratos MS50 mass spectrometer in the positive-ion mode using fast atom bombardment (FAB) ionization. The instrument was equipped with a saddle-field FAB gun (Ion Tech, Teddington, UK) operating at 8 KeV with xenon as the primary atom beam. *m*-Nitrobenzyl alcohol (NBA) was used as the matrix. Accurate mass measurements were obtained by peak matching with a cesium iodide saturated glycerol solution as the reference.