Journal of Natural Products
Vol. 52, No. 5, pp. 1015-1021, Sep-Oct 1989

BIOSYNTHESIS OF ELSAMICIN A, A NOVEL ANTITUMOR ANTIBIOTIC

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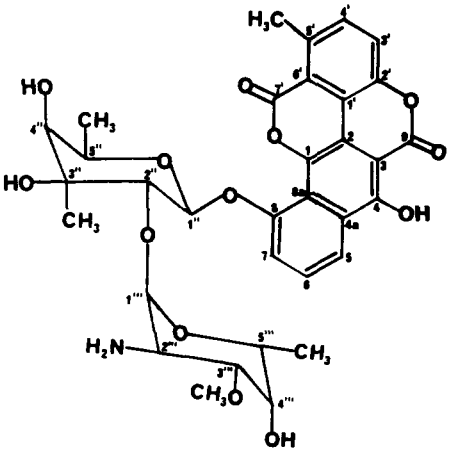
ABSTRACT.—The ^{1}H noise-decoupled ^{13}C-nmr spectrum of elsamicin A [**1**] prepared from the cultures of an unknown actinomycete species (ATCC 39417) supplemented with [1-^{13}C]acetate and [2-^{13}C]acetate showed enrichment of all 19-carbons in the aglycone. In addition, L-[methyl-^{13}C]methionine- and D-[1-^{13}C]glucose-supplemented cultures enriched the carbons of the two methyl groups on the disaccharide moiety and the C-1 carbons of the disaccharide, respectively. The results demonstrated that the aglycone of elsamicin A is derived entirely from acetate and the disaccharide portion is biosynthesized from two units of glucose and methionine.

In our continuing search for fermentation antitumor antibiotics, two new antibiotics, elsamicin A [**1**] and elsamicin B [**2**] were isolated from an unidentified actinomycete strain J907-21 (ATCC 39417) collected in El Salvador. Both elsamicin A and B showed antibacterial activity against Gram-positive bacteria and anaerobic organisms, with elsamicin A being 2–4 times more potent than elsamicin B (1,2). Elsamicin A induces significant prolongation of survival time in mice bearing P-388 leukemia, L1210 leukemia, and B16 melanoma (1,2), while elsamicin B is devoid of antitumor activity.

Structural studies (3) showed that elsamicins A [**1**] and B [**2**] are chemically related to another antitumor antibiotic chartreusin [**3**] (4–6). Chartreusin showed very promising activity in experimental tumor models (6) but did not proceed to clinical study because of its H_2O insolubility and poor pharmacokinetic profile. They share the same aglycone, chartarin, but differ in the sugar moieties. Elsamicin A contains both a novel neutral sugar (6-deoxy-3-*C*-methyl-D-galactose) and a novel amino sugar (2-amino-2,6-dideoxy-3-*O*-methyl-D-galactose). Elsamicin A is remarkably more H_2O-soluble than chartreusin especially under acidic conditions. Both elsamicin B and chartreusin, which lack an amino sugar moiety, are nearly insoluble in H_2O.

Elsamicin A [**1**], which is presently undergoing Phase I clinical trials, has been the focus of active investigation in our laboratories with regard to large scale production



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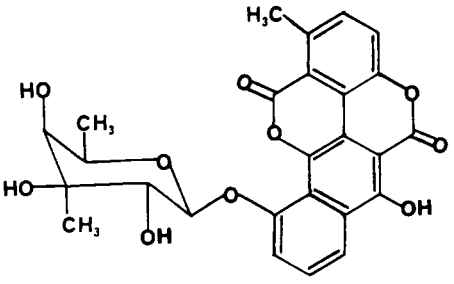

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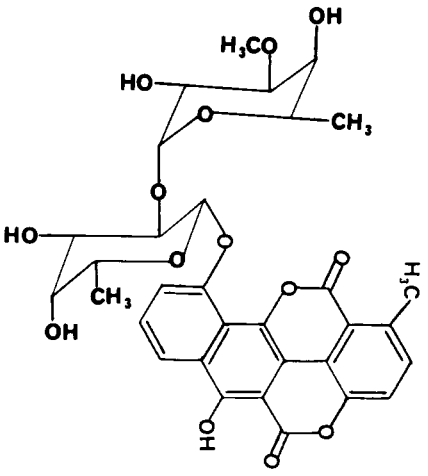

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improvements (7) and its biosynthetic origin. Radiolabeled elsamicin A is in fact required for pharmacokinetic studies and knowledge of its biosynthetic origin can be used to exploit possibilities leading to production optimization and provide the optimal route for the preparation of isotopically labeled drug. In this paper we present evidence indicating that the aglycone of elsamicin A is derived entirely from acetate and that the disaccharide portion is derived from two units of glucose and methionine.

RESULTS AND DISCUSSION

Initial experiments were designed both to identify biosynthetic precursors and to optimize their incorporation into elsamicin A [**1**]. A series of ATCC 39417 cultures were prepared, and ^{14}C-labeled precursors (0.1 μ Ci/flask) were added to these at various times as indicated in Table 1. Elsamicin A was isolated following 7 days incubation by extracting the whole broth with CH_2Cl_2. The concentrated extract was injected into hplc (see Experimental), and the fraction containing elsamicin A was collected and counted for radioactivity. The results, summarized in Table 1, demonstrate that NaOAc, L-methionine, and D-glucose are efficiently incorporated into elsamicin A. The percent incorporation of glucose and acetate into elsamicin A was about 1.25%. L-Methionine was incorporated into elsamicin A at a rate of 6.15%. Based upon these

TABLE 1. ^{14}C-Incorporation of Precursors into Elsamicin A [**1**].

Precursor	Time of Addition (h)	Incorporation (%)
Sodium [1-^{14}C]Acetate	36	0.84
	48	1.27
	60	1.29
	72	1.14
L-[Methyl-^{14}C]Methionine	36	2.33
	48	4.16
	60	3.09
	72	6.15
D-[^{14}C(u)]Glucose	36	0.52
	48	1.18
	60	1.25
	72	0.96

preliminary studies with ^{14}C-labeled precursors, sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, and D-[1-^{13}C]glucose were added to the production culture at 60 h. L-[Methyl-^{13}C]methionine was added at 72 h. Two grams of NaOAc and glucose and 1 g of L-methionine were added to 1 liter of cultures (i.e., 10 flasks). Elsamicin A was purified by the procedures described in the Experimental section. The labeling pattern was deduced using ^{13}C-nmr analysis, and the enriched carbons are given in Table 2. The assignment of ^{13}C resonances was reported by Medley (8).

The data from Table 2 demonstrate that the 19-carbon aglycone moiety was derived entirely from acetate. [1-^{13}C]Acetate enriched 9 carbons of the aglycone by 10–11.5-fold (Figure 1A and Table 2). [2-^{13}C]Acetate enriched 10 carbons of the aglycone by 6–10-fold (Figure 1B and Table 2). There is no enrichment of the carbons of the glycosidic moiety from ^{13}C-labeled acetate. L-[Methyl-^{13}C]methionine significantly enriched only the two carbons (20- and 25-fold) of the methyl groups on the disaccharide portion (Figure 1C and Table 2). The enrichments by ^{13}C-labeled acetates and methionine were large enough to show the labeling pattern unequivocally; therefore, we can specifically label the aglycone and the sugar moiety of elsamicin A by using ^{14}C-labeled acetate and

TABLE 2. Incorporation of ^{13}C-Precursors into Elsamicin A [**1**].

Carbon	ppm	Enrichment Factor			
		[1-^{13}C]Acetate	[2-^{13}C]Acetate	D-[1-^{13}C]Glucose	L-[Methyl-^{13}C]Methionine
C-9	168.7	—	6	1.7	—
C-7′	159.4	10	—	—	—
C-4	159.2	—	6	1.7	—
C-8	151.3	10	—	—	—
C-2′	147.6	—	7.5	1.9	—
C-5′	135.1	11	—	—	—
C-1	133.9	10.5	—	—	—
C-4′	130.9	—	9	1.6	—
C-4a	130.6	10	—	—	—
C-6	123.6	11.5	—	—	—
C-1′	120.8	10	—	—	—
C-5	119.3	—	7.5	1.6	—
C-8a	118.9	—	7.5	1.6	—
C-3′	117.9	10	—	—	—
C-6′	116.8	—	9	1.8	—
C-7	113.3	—	8	2	—
C-2	111.1	—	7	1.8	—
C-1‴	97.6	—	—	5	—
C-1″	96.5	—	—	5	—
C-3	90.9	9	—	—	—
C-2″	79.6	—	—	—	—
C-4‴	77.2	—	—	—	—
C-3″	76.2	—	—	—	—
C-4″	73.3	—	—	—	—
C-5″	69.0	—	—	—	—
C-5‴	66.4	—	—	—	—
C-4‴	65.5	—	—	—	—
3‴-OMe	55.4	—	—	—	25
C-2‴	49.6	—	—	—	—
5′-Me	22.1	—	10	1.6	—
3″-Me	19.6	—	—	—	20
5‴-Me	16.8	—	—	—	—
5″-Me	16.7	—	—	—	—

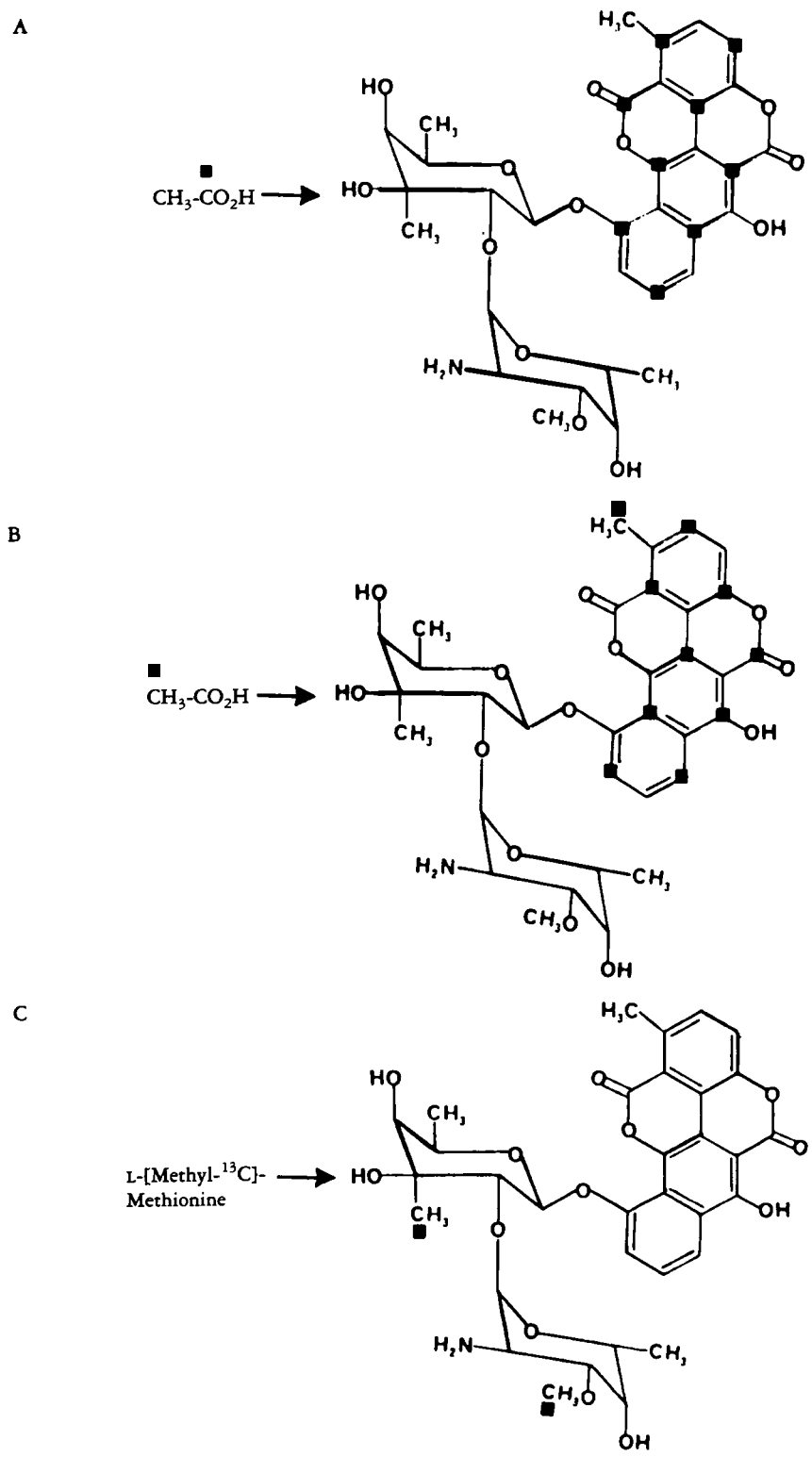

FIGURE 1. ^{13}C enrichment pattern of elsamicin A from cultures supplemented with [1-^{13}C]acetate (A), [2-^{13}C]acetate (B), L-[methyl-^{13}C]methionine (C), and D-[1-^{13}C]glucose (D). Closed square (■) denotes the position of enrichment.

D

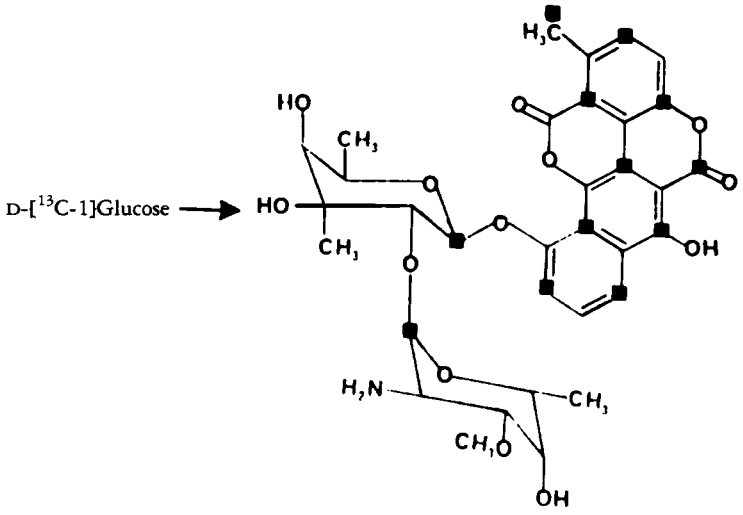

FIGURE 1. Continued.

L-[methyl-^{14}C]methionine, respectively. Alternatively, very high specific activity of elsamicin A can be obtained by labeling both the aglycone and sugar moieties by ^{14}C-labeled acetate and L-[methyl-^{14}C]methionine together.

D-[1-^{13}C]Glucose enriched the two C-1 carbons of the disaccharide as expected (Figure 1D and Table 2). The observed enrichment was about 5-fold. Not unexpectedly, certain carbons of the aglycone were enriched by D-[1-^{13}C]glucose (Figure 1D and Table 2). The enrichment pattern of carbons of the aglycone by this precursor is similar to that by [2-^{13}C]acetate. This observation is consistent with the fact that glucose is the precursor for acetate. When glucose breaks down to form acetate units via pyruvate, the C-1 carbon of glucose corresponds to the C-2 carbon of acetate. Therefore, addition of D-[^{14}C(u)]glucose to elsamicin A fermentation labels both the aglycone and the disaccharide portion of elsamicin A.

The results thus far reported demonstrate that elsamicin A is derived from acetate, D-glucose, and L-methionine. Nineteen carbons of the aglycone, including the methyl group at the 5 position, are derived entirely from acetate. This is consistent with the findings of Canham *et al.* (9) showing that the aglycone of chartreusin is also derived entirely from acetate units. The ^{13}C-enrichment patterns of elsamicin A and chartreusin by [1-^{13}C]acetate and [2-^{13}C]acetate are the same, indicating that the biosynthesis of the aglycone moiety for both antibiotics is via the same route. Canham *et al.* (9) postulated that the aglycone of chartreusin is derived from a single 22-carbon polyketide chain. Cyclization to a benzpyrene-like intermediate followed by ring cleavage and loss of three carbon atoms provides a possible route from the polyketide to the substituted isocoumarin structure of the aglycone.

The disaccharide portion of elsamicin A consists of a neutral sugar (6-deoxy-3-*C*-methyl-D-galactose) and an amino sugar (2-amino-2,6-dideoxy-3-*O*-methyl-D-galactose). Results from our experiment show that these sugars are derived from D-glucose. Although we have not carried out further experiments to show how these sugars are formed, the similarity with the biosynthesis of 6-deoxyhexose and aminodideoxyhexoses of bacterial polysaccharides and lipopolysaccharides provides some information on the probable biosynthetic pathway. The mechanism of the biosynthesis of the 6-deoxyhexose is known. This involves production of an intermediate nucleoside-diphospho-4-keto-6-deoxyhexose from deoxy-thymidine diphospho-(dTDP)-D-glucose by an oxidoreductase (10–15) with subsequent enzymatic reduction of the 4-keto group of

the intermediate to the corresponding nucleotidyl-6-deoxyhexose (16–20). The formation of 6-deoxy-3-*C*-methyl-D-galactose is probably via the following reaction sequence: (1) dTDP-D-glucose is converted to TDP-4-keto-6-deoxy-D-glucose by an oxidoreductase, (2) reduction at C-4 position by a reductase to form TDP-6-deoxy-D-glucose, (3) TDP-6-deoxy-D-galactose is produced from TDP-6-deoxy-D-glucose by a 4-epimerase reaction, (4) subsequent methylation at 3-C position by a methylase using *S*-adenosyl methionine as a methyl donor yields the formation of TDP-6-deoxy-3-*C*-D-galactose. A similar sequence of reactions may be involved in the biosynthesis of 6-deoxy derivatives from 2-amino sugars. 2-Amino-2,6-dideoxy-D-galactose is a common constituent of bacterial polysaccharides (21–26). The biosynthesis of this amino sugar in *Citrobacter freundii* ATCC 10053 has been examined by Barrow (27). He demonstrated that the biosynthesis of 2-amino-2,6-dideoxy-D-galactose is similar to that of 6-deoxyhexose except using glucosamine instead of glucose as the starting precursor. Therefore the formation of 2-amino-2,6-dideoxy-3-*O*-methyl-D-galactose from dTDP-D-glucosamine is probably via the same reaction sequence as described for the formation of 6-deoxy-3-*C*-methyl-D-galactose from dTDP-D-glucose.

Based on our results on the ^{13}C-labeling pattern, we have been able to prepare radiolabeled elsamicin A [**1**] specifically labeled at the aglycone (1.5 μCi/mg) and sugar moiety (2.5 μCi/mg) by using ^{14}C-acetate and L-[methyl-^{14}C]methionine, respectively. Addition of the precursor alone or in combination to the fermentation of elsamicin A may enhance the production of this antibiotic. Because glucose is the precursor for both the aglycone and the disaccharide portions of elsamicin A, the effect of glucose on the production of elsamicin A in fermentation of ATCC 39417 should be determined. Further studies on the precursor-directed biosynthesis of elsamicin A are ongoing.

EXPERIMENTAL

CULTURE OF ATCC 39417.—The elsamicin-A-producing culture, an unidentified actinomycete strain J907-21, has been deposited with the American Type Culture Collection with the accession number ATCC 39417. ATCC 39417 was grown in a medium containing soluble starch 3%, Pharmamedia 1%, peanut meal 0.5%, yeast extract 0.5%, Bacto-liver 0.1%, NaCl 0.3%, $(NH_4)_2SO_4$ 0.1%, and $CaCO_3$ 0.6%, at 28° and 250 rpm on a gyrotary shaker. After 3 days, 5-ml aliquots were transferred to a 500-ml Erlenmeyer flask containing 100 ml of an elsamicin-A-production medium prepared using soluble starch 3%, linseed meal 2.5%, NaCl 0.3%, $(NH_4)_2SO_4$ 0.1%, and $CaCO_3$ 0.6%. The production cultures were incubated at 28° and 250 rpm for 7 days to obtain the maximum titer of 220 μg/ml.

ISOTOPICALLY-LABELED PRECURSORS.—Sodium [1-^{14}C]acetate (59.0 mCi/mmole), L-[methyl-^{14}C]methionine (45.5 mCi/mmole), and D-[^{14}C(u)]glucose (257.6 mCi/mmole) were obtained from New England Nuclear Corporation, Boston. Sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, L-[methyl-^{13}C]methionine, and D-[1-^{13}C]glucose, all 99% enriched, were purchased from Sigma Chemical Company, St. Louis. Precursors were added to cultures as filtered-sterilized solutions at the times indicated. Radioactivity was determined using liquid scintillation counting employing aquasol cocktail and a Beckman counter.

ELSAMICIN A PURIFICATION.—Elsamicin A [**1**] was isolated from 1 liter of culture by extracting the whole broth with an equal volume of CH_2Cl_2. The organic extract was evaporated to dryness. Twenty ml of 0.1 M NH_4OAc (pH 4.0) was added to the dried extract and mixed. The solution was filtered through Whatman No. 1 filter paper. The filtrate was adjusted to pH 7.2 by using 20 ml of 10% $NaHCO_3$, and the solution was extracted twice with an equal volume of CH_2Cl_2. The organic extract was evaporated to dryness. MeOH (5 ml) was used to dissolve the extract and applied on a column (3.0 × 30 cm) of Si gel H, Merck 10-40. The column was eluted with a $CHCl_3$/MeOH mixture with stepwise increase of the MeOH concentration (5–10%). Elsamicin A was eluted at 10% MeOH fractions. The fractions containing elsamicin A were pooled and dried and yielded about 80 mg of elsamicin A. The purified elsamicin A gave a single peak (retention time 4.5 min) by hplc using a C-18 reversed-phase column (μ-Bondapak, 3.9 × 300 mm, Waters Associates). The solvent system was NH_4OAc (0.1 M, pH 4.0)/MeCN (1:1) at a flow rate of 1 ml/min and the detector wavelength set at 266 nm.

NMR SPECTROSCOPY.—^{13}C-nmr spectra were recorded at 35° on a Bruker WM-360 spectrometer

operated in Fourier transform mode under the following conditions: frequency, 90.556 mHz; spectral width, 21,700 Hz; acquisition time, 0.377 sec; delay between acquisitions, 10.0 sec; flip angle, 45°; sample concentration, 100 mg/ml; solvent, DMSO-d_6; solvent volume, 0.6 ml; sample tubes, 5-mm diameter; TMS as internal standard. A spectrum produced from a sample of elsamicin A in a proton decoupled mode under these conditions was identical to the spectrum produced using the inverse gated proton decoupled technique. Hence, these conditions were suitable for quantitating the isotope incorporation in the samples with the advantage of nOe to the ^{13}C resonances using the proton decoupled method.

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Received 13 March 1989