THE PREPARATION OF CARBON-14 LABELED AVERMECTIN B_{1a}

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SUMMARY

Carbon-14 labeled avermectin B_{1a} was synthesized by a fermentation process using sodium [1-^{14}C]propionate as the precursor, then isolated and purified by preparative HPLC. The total amount of purified product obtained in this preparation was 225 mg with a specific activity of 16.4 μCi/mg. The radiopurity was 99+% by TLC and HPLC analyses. The labeled positions were determined by carbon-13 NMR analysis of [^{13}C]avermectin A_{1a} which was biosynthesized with sodium [1-^{13}C]propionate. The labeled atoms were determined to be C-3, C-7, C-11, C-13 and C-23. However, EI/MS indicated this product was a mixture of five single carbon-13 labeled compounds. The overall radiochemical yeild of [^{14}C]avermectin B_{1a} biosynthesis was estimated to be 0.69%.

Key words: avermectins, biosynthesis, [^{13}C]avermectin B_{1a}, [^{14}C]avermectin B_{1a}, pesticide.

INTRODUCTION

Avermectin B_{1a} is one of the major avermectins (Figure 1) produced by the actinomycete Streptomycetes avermetilis (1). It is active at extremely low dosage

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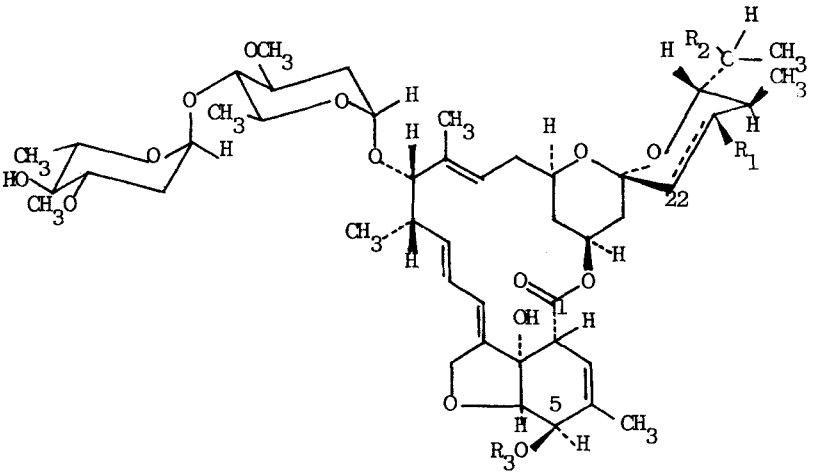
Figure 1. Structure of the Avermectins.



AVERMECTIN	R_1	R_2	R_3
A_1a		C_2H_5	CH_3
A_1b		CH_3	CH_3
A_2a	OH	C_2H_5	CH_3
A_2b	OH	CH_3	CH_3
B_1a		C_2H_5	H
B_1b		CH_3	H
B_2a	OH	C_2H_5	H
B_2b	OH	CH_3	H

Where R_1 is absent, the double bond (⋯⋯) is present.
Both sugars are o-L-oleandrose.

against a wide variety of nematode and arthropod parasites, apparently by virtue of of its action on the mediation of neurotransmission by Γ-aminobutryic acid. In addition, it exhibits excellent activity in controlling different phytophagus pests on field crops and citrus (2), and red imported fire ants (3). The carbon-14 labeled avermectin B_1a is needed for various metabolism studies and environmental chemistry studies.

EXPERIMENTAL

MATERIALS

[^{14}C]Carbon dioxide was purchased from Amersham Corp. Sodium [1-^{14}C]propionate was prepared by Dr. R. Ellsworth of Merck Sharp & Dohme Research Laboratories. Sodium [1-^{13}C]propionate was obtained from MSD Isotopes. All solvents used for high performance liquid chromatographic separations were HPLC grade, used without further purification. The plates used for TLC were E. Merck (Sil GF, 0.25 mm thick). Autoradiography was achieved on ARO X-ray film (Kodak). HPLC columns were made by Whatman.

Biosynthesis of Carbon-13 Avermectins

The cultural procedures are described as follow: One ml of vegetative material growth of MA 5192 (Streptomycetes avermitilis) was added to each of four flasks (250 ml Erlenmeyer flasks with triple baffles) containing 25 ml of seed medium. The flasks were incubated at 28° C for 16 hours in a New Brunswick gyrorotary shaker at an agitation rate of 220 rpm. The growth from the seed flasks were pooled. One ml was used to inoculate a series of 34 flasks containing 20 ml of a glucose-yeast extract peptonized milk medium in each 250 ml Erlenmeyer flask. The flasks were incubated at 28° C at an agitation speed of 220 rpm in a New Brunswick Environmental Shaker. After a 48 hour incubation, 1 ml of sodium [1-^{13}C]propionate solution (1.25 mg/ml) was added to each flask and the flasks returned to the shaker; 24 hours later, another 1 ml of sodium [1-^{13}C]propionate solution was added to each flask and the incubation allowed to continue for another 96 hours.

Biosynthesis of Carbon-14 Avermectins

The fermentation procedures were the same as described in the biosynthesis of carbon-13 avermectins. Sodium [1-^{14}C]propionate was synthesized by a standard Grignard method for labeled carboxylic acids (4).

Isolation of Avermectins

The culture was carefully transferred from the Erlenmeyer flasks to several 250 ml centrifuge bottles. The bottles were centrifuged with a Beckman centrifuge (Model J2-21) at 15,000 rpm for 15 minutes. The supernatants were decanted and discarded. The cells were washed once with distilled water by resuspending the cells in the bottles, followed by centrifugation. The wet cake (cells) from the bottles was slurried in 80% acetone/water (v/v). Then the slurry was filtered through a medium porosity sintered glass funnel with a layer of Celite filter aid. The cake was discarded and the filtrate was extracted twice with an equal volume of methylene chloride. The extracts were combined and dried over a sufficient amount of anhydrous sodium sulfate, filtered, followed by a cake wash of methylene chloride. The filtrate was concentrated under reduced pressure to dryness with a rotary evaporator. The residue was redissolved in approximately 50 ml of methylene chloride, followed by the addition of approximately 20 g of silica gel powder. The silica gel was filtered with a medium porosity sintered glass funnel under reduced pressure. The silica gel was then eluted with approximately 150 ml of ethyl acetate, followed by an approximately 100 ml wash of the same solvent. The silica gel was finally washed with methanol/ethyl acetate (50:50, v/v). The bulk of the avermectins was extracted into the ethyl acetate fraction.

Purification of Avermectin $B_{1}a$

The ethyl acetate fraction which contained the mixture of [^{14}C]avermectins, was concentrated by rotary evaporation and redissolved in methanol. A semipreparative high performance liquid chromatographic method, consisting of a normal phase and reverse phase HPLC system (5), was used to purify avermectin $B_{1}a$. The total amount of purified [^{14}C]avermectin $B_{1}a$ prepared was 225 mg with a specific activity of 16.4 μCi/mg. The radiopurity was found to be 99^{+}% as determined by analytical HPLC and TLC analyses.

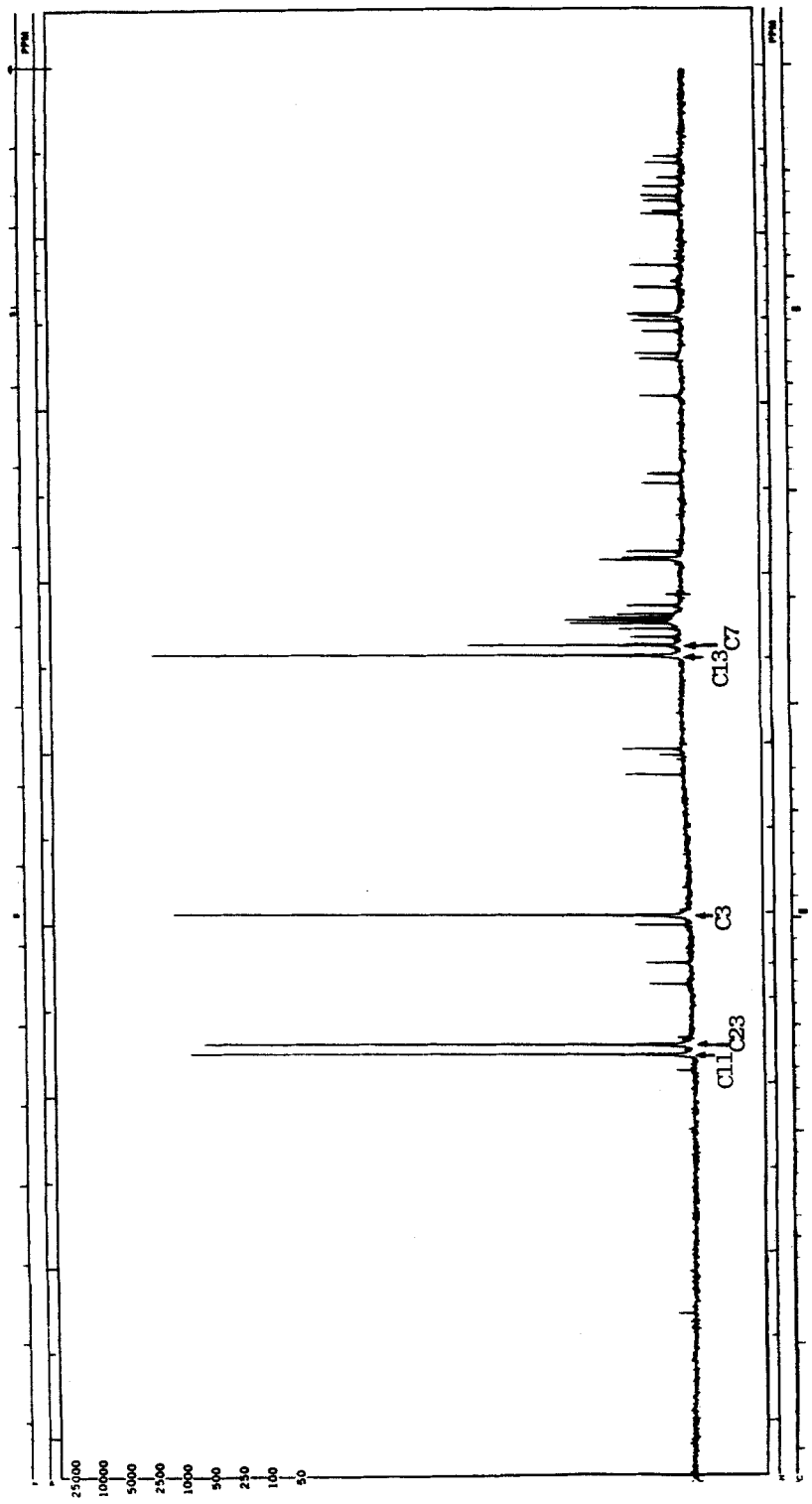


Figure 2. Carbon-13 NMR Spectrum of [^{13}C]Avermectin A_1a

RESULTS AND DISCUSSION

The biosynthetic incorporation of ^{13}C-enriched precursors was investigated by G. Albers-Schonberg et al. (6). In this study, sodium [1-^{13}C]propionate was used as precursor in order to (a) determine the labeling positions in the avermectins, (b) estimate the extent of incorporation, and (c) serve as a pilot run for [^{14}C]-avermectin biosynthesis.

The first pure fraction of [^{13}C]avermectins isolated was [^{13}C]avermectin $A_{1}a$. Subsequently, this fraction was used for carbon-13 NMR and EI/MS analysis. The carbon-13 NMR spectrum of [^{13}C]avermectin $A_{1}a$, a major avermectin component, is shown in Figure 2. Results indicated that the chemical shifts of the enriched carbon atoms were 80.6, 82.0, 118.4, 136.1, and 137.6 ppm. The position were interpreted as C-7, C-13, C-3, C-23, and C-11, respectively, by comparing with the carbon-NMR spectrum of unlabeled avermectin $A_{1}a$ (6). The intensities of all enriched carbon peaks were approximately the same except the smaller peak at 80.6 ppm (C-7), because it is a non-protonated carbon. The approximate ratio of the enriched vs. natural abundant carbon peak was estimated to be 8. Since the labeled positions are common in both avermectin $A_{1}a$ and $B_{1}a$, it should suffice to conclude that avermectin $B_{1}a$ is also labeled at positions of C-3, C-7, C-11, C-13 and C-23. The partial electron impact mass spectrum of [^{13}C]avermectin $A_{1}a$ assignments of its major ions (6) is shown in Figure 3. The base peak in the spectrum of unlabeled avermectin $A_{1}a$ is m/z 305; its isotope peak at m/z 306 (principally due to ^{13}C, 1.1% natural abundance x 19 carbons) is calculated to be 20.9%. For [^{13}C]avermectin $A_{1}a$, the isotope peak at m/z 306 was observed to have an intensity of 36.1% or 15.2% excess intensity for the two singly labeled ^{13}C species (C-13 and C-23) and a ratio of labeled vs. unlabeled species of 1:8. This ratio is in accordance with the carbon-NMR data. We conclude from the appropriate peaks that m/z 306 is a mixture of two singly labeled species and the ion of m/z 581 is a mixture of five singly labeled species.

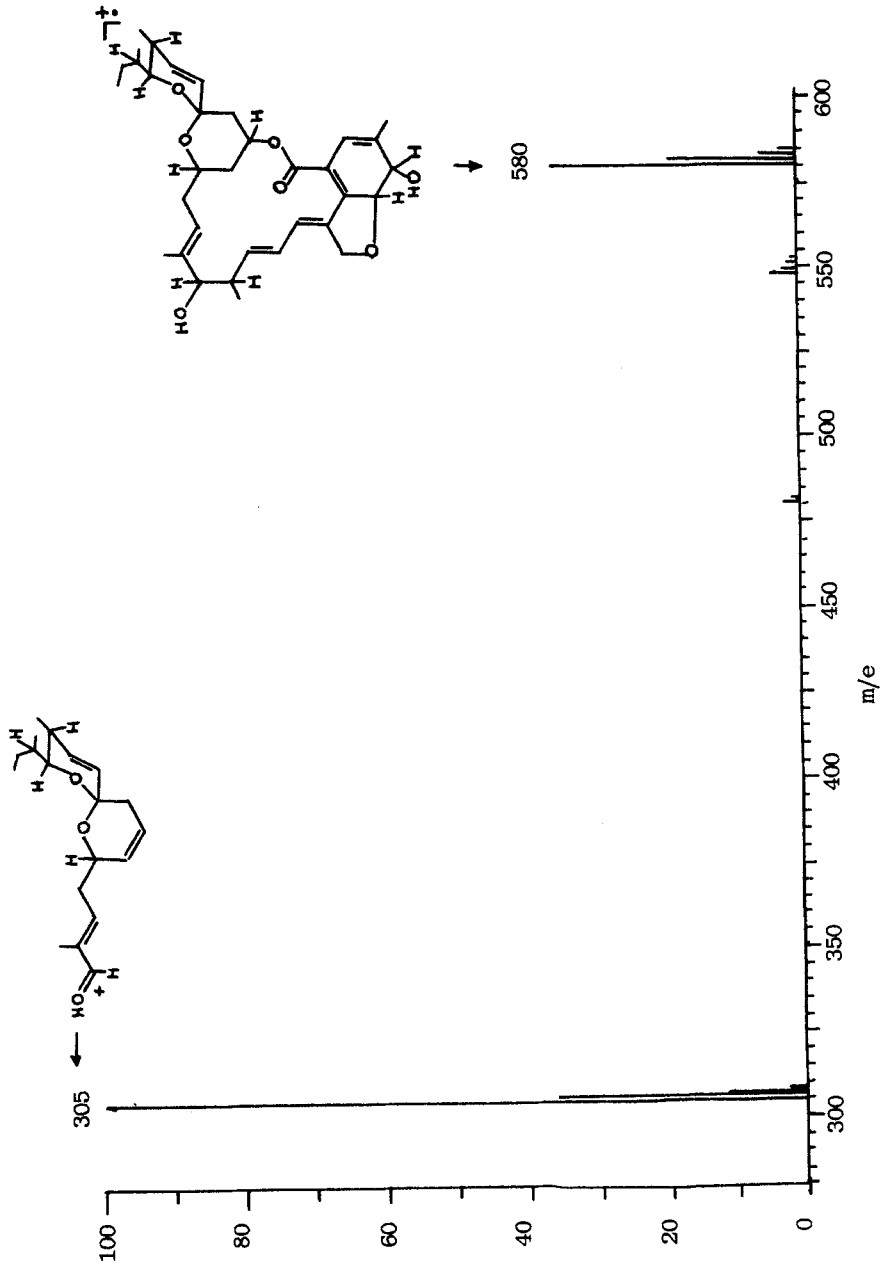


Figure 3. EI/MS Spectrum of [^{13}C]Avermectin A_1a

Table I. [^{14}C]Avermectins Recovered from the Crude Extracts*

[^{14}C]Avermectin		CH_2Cl_2 Extract	EtOAc Extract
A_1		0.983 mCi	2.660 mCi
A_2		0.849	3.285
B_1		0.435	3.898
B_2		0.183	2.144
	Total	2.450 mCi	11.987 mCi

*Insignificant amount of radioactivity was recovered from the EtOAc/MeOH extract. Therefore, this extract is not tabulated.

The biosynthesis of [^{14}C]avermectins was carried out with the same procedures of those of [^{13}C]avermectins. The crude extracts were analyzed by TLC/radioassay. Results are shown in Table 1. The crude yield of [^{14}C]avermectins by this fermentation process was estimated to be 5%. The overall radiochemical yield of [^{14}C]avermectin B_1a biosynthesis was estimated to be 0.69%. Approximately 225 mg of purified [^{14}C]avermectin B_1a was obtained. The chemical and radioactive purity were ascertained by TLC/radioassay and HPLC/radioassay. The radiopuirty was 99+% and the specific activity was 16.4 μCi/mg.

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