Journal of Natural Products
Vol. 50, No. 1, pp. 108-118, Jan-Feb 1987

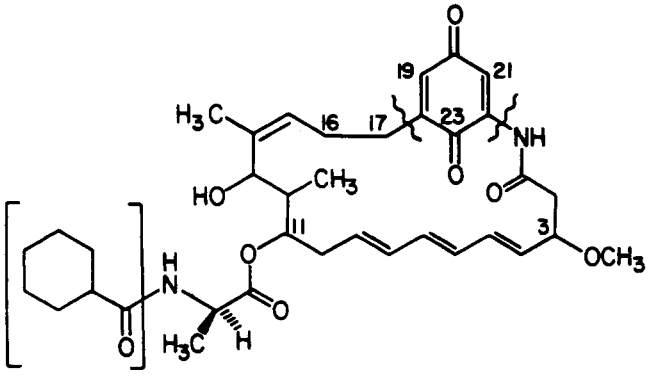
BIOSYNTHESIS OF THE ANSAMYCIN ANTIBIOTIC ANSATRIENIN (MYCOTRIENIN) BY *STREPTOMYCES COLLINUS*[1]

T.S. WU, J. DUNCAN, S.W. TSAO, C.J. CHANG, P.J. KELLER, and H.G. FLOSS*,[2]

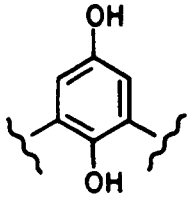
Department of Chemistry, Ohio State University, Columbus, Ohio 43210, and Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, Indiana 47907

ABSTRACT.—The biosynthesis of ansatrienin (mycotrienin) has been studied in radioactive and stable isotope feeding experiments with *Streptomyces collinus* Tü 1892. The *m*-C_7N unit of the ansa ring is efficiently and specifically derived from 3-amino-5-hydroxybenzoic acid; shikimic acid is not incorporated into this part of the molecule but does label the cyclohexanecarboyxlic acid moiety, providing all seven of its carbon atoms. Incorporation of methionine confirms origin of the methoxy group by transmethylation. The D-alanine moiety is derived directly from D-alanine rather than L-alanine. The terminal steps in the conversion of shikimic acid into cyclohexanecarboyxlic acid seem to be sequential reduction of 2,5-dihydrobenzoic acid and cyclohexene-1-carboxylic acid as evidenced by feeding experiments and the detection of a new ansatrienin containing a 1-cyclohexene instead of the cyclohexane moiety.

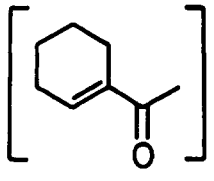
The ansatrienins or mycotrienins represent a small group of novel ansamycin antibiotics that have been isolated from *Streptomyces collinus* (1) and *Streptomyces rishiriensis* (2). They show pronounced activity against fungi and yeasts but little antibacterial activity (1,2). The structures of the two main products, the redox pair ansatrienin A (=mycotrienin I) [**1**] and ansatrienin B (=mycotrienin II) [**2**], were established independently by the groups of Zeeck (3) and Seto and Ōtake (4,5), largely on the basis of



1 ansatrienin A=mycotrienin I



2 ansatrienin B=mycotrienin II



3 ansatrienin A_4

[1]This article commemorates the 50th year of publication of the *Journal of Natural Products* (formerly *Lloydia*).

[2]Member of the Editorial Advisory Board of the *Journal of Natural Products* (*Lloydia*) since 1971.

[3]A. Zeeck, University of Gottingen, personal communication, 1985.

nmr spectroscopy. A minor discrepancy between the structures published by the two laboratories regarding the stereochemistry of the alanine moiety has been resolved in favor of the D configuration reported by the Japanese group.[3] Minor components isolated include the alcohols mycotrienol I and II lacking the cyclohexanecarbonyl-alanine moiety (6), the 22-*O*-methyl ether of mycotrienin II (7), and the congeners ansatrienin A_2 and A_3 carrying a 2- and a 3-methylbutyryl moiety, respectively, in place of the cyclohexanecarbonyl group (8). Closely related are the trienomycins, also isolated from a *Streptomyces* species (7,9-11), which have all the structural features of the ansatrienin B series but lack the 19-hydroxy group. These compounds, interestingly, have cytotoxic but no antimicrobial activity (11). Some microbial transformations of mycotrienin II with *Bacillus megaterium* have also been reported (12).

Several structural features of the ansatrienins are unusual from a biosynthetic point of view and caused us to carry out studies on selected aspects of the mode of formation of these antibiotics. These features are primarily a cyclohexanecarboxamide moiety found for the first time in nature in these compounds and a *m*-C_7N unit which has been encountered in various structural permutations in numerous other ansamycins (13,14) and other antibiotics, e.g., the mitomycins (15) and pactamycin (16). A cyclohexane ring at the terminus of a carbon chain is also found in asukamycin (17) and in some ω-cyclohexyl fatty acids isolated from thermophilic bacteria (18-20). In both those cases, cyclohexanecarboxylic acid most likely serves as starter unit for a polyketide/fatty acid chain.

EXPERIMENTAL

GENERAL METHODS.—^{13}C- and ^1H-nmr spectra were recorded on JEOL PFT-100, Varian XL-200, and Bruker WM-300 nmr spectrometers. Chemical shifts were determined relative to TMS as internal standard or were converted to the TMS scale by reference either to the carbonyl resonance of acetone-d_6 at δ_c=205.6 ppm or to the $CHCl_3$ resonance at δ_H=7.26 ppm as internal standard. Mass spectra were recorded on a Kratos MS-50 mass spectrometer, and gc-ms was carried out on a Finnigan 4021 GC-MS instrument. Preparative tlc was performed on 0.25 mm or 0.5 mm thick 20×20 cm silica gel 60 F-254 plates (E. Merck, supplied through Brinkmann). Radioactivity determinations were carried out by liquid scintillation counting in a Beckman LS 7500 spectrometer using Aquasol as solvent. Counting efficiencies were determined with [^{14}C]toluene as internal standard. Radioactivity on chromatograms was located with a Packard model 7201 radiochromatogram scanner. Sonications were carried out with a Virsonic Model 16-850 sonicator.

CULTURE CONDITIONS.—*S. collinus* strain Tü 1892 (1) was maintained in spore suspension prepared from well-sporulated cultures grown on agar slants on the following medium: yeast extract, 0.4 g; malt extract, 1.0 g; glucose, 0.4 g; agar, 2.0 g; distilled H_2O, 100 ml; pH 7.4. A part of the spore suspension was transferred under sterile conditions to a 500-ml Erlenmeyer flask containing 100 ml of seed medium and incubated for 2 days on a rotary shaker at 28° and 300 rpm. Of the seed culture, 10 ml was then used to inoculate 100 ml of production medium in 500-ml Erlenmeyer flasks, which were again grown for 48-72 h at 28° with rotary shaking at 300 rpm. Both the seed and the production medium contained soy bean meal, full fat, 2.0 g; mannitol, 2.0 g; tap H_2O, 100 ml; pH 7.3. Labeled precursors were fed after 24 h growth in the production medium, and the cultures were harvested 24 h later.

ISOLATION OF ANSATRIENINS.—The cultures were filtered through Celite; the mycelia were collected and suspended in Me_2CO. The suspension was sonicated for 5 min at maximum power setting and filtered, and the process was repeated once more. The combined filtrates were concentrated to remove the Me_2CO, and the crude product was recovered by three extractions with EtOAc. The extracts were dried over Na_2SO_4 and concentrated, and the crude ansatrienin was precipitated with petroleum ether with cooling to 0° overnight. The precipitate was collected, washed with petroleum ether, dissolved in a small volume of EtOAc, and purified by preparative tlc (solvent: EtOAc-C_6H_6, 3:1; ansatrienin A Rf 0.37, B Rf 0.22). The two ansatrienin bands were eluted with EtOAc; the eluate of ansatrienin B was shaken briefly with a few ml of a saturated aqueous solution of $FeCl_3$ to oxidize the hydroquinone to the quinone and combined with the eluate of ansatrienin A. Final purification of ansatrienin A involved hplc on a C_{18} column (Altex, solvent: MeOH-H_2O, 3:1).

GC/MS ANALYSIS OF CYCLOHEXANECARBONYL-ALANINE MOIETY.—A mixture of 0.3-0.5 mg an-

satrienin A and 0.5 mg $NaHCO_3$ in 0.5 ml of MeOH was heated in a sealed vial at 60° for 60-70 h. One drop of 5% HCl was then added, and the mixture was treated with CH_2N_2. After evaporation of the solvent, the residue was taken up in $CHCl_3$ and chromatographed on a 0.25 mm 5×20 cm silica gel plate (solvent, EtOAc-hexane, 1:1). The band of methyl cyclohexanecarbonylalanine was scraped off and eluted with $CHCl_3$. It was then subjected to *gc*-ms analysis using a DB-1 fused silica capillary column with temperature programming from 140-250° at 5°/min.

DEGRADATION OF ANSATRIENIN A.—One mg ansatrienin A in 2 ml 10 N HCl was heated to 90° for 6 h or more. The reaction mixture was extracted with 3×2ml Et_2O; the combined extract was concentrated and cyclohexanecarboxylic acid isolated by tlc on silica gel (solvents; EtOH-H_2O-NH_3, 20:4:1; *n*-PrOH-H_2O, 7:3; or Et_2O). For further degradation, the labeled acid was diluted with carrier material, converted to the dibenzylethylenediamine salt, recrystallized to constant specific radioactivity, and then reconverted to the free acid. The latter, after conversion to the sodium salt, was subjected to the Schmidt degradation (21), isolating cyclohexylamine as the hydrochloride and CO_2 as $BaCO_3$.

LABELED PRECURSORS AND REFERENCE COMPOUNDS.—Commercially available radioactive compounds were obtained from New England Nuclear or Amersham. D,L-2,5-Dihydro[3-^{14}C]phenylalanine (22) and 3-amino-5-hydroxy-[7-^{13}C]benzoic acid (23) were materials prepared in earlier work. 1,4-Dihydro-[7-^{14}C]benzoic acid was prepared by Birch reduction of [7-^{14}C]benzoic acid (24) and 2,5-dihydro-[7-^{13}C]benzoic acid by Diels-Alder reaction of butadiene and [1-^{14}C]propargylic acid (25), which was prepared by carboxylation of sodium acetylide with $^{14}CO_2$. [7-^{14}C]- and [7-^{13}C]cyclohexanecarboxylic acid were prepared by carboxylation of the Grignard reagent from bromocyclohexane (26).

^{13}C, ^{15}N-Labeled *N*-(Cyclohexanecarboxyl)-D- and L-alanine.—D- and L-[^{15}N]alanine were prepared by resolving the racemate (98% ^{15}N) with hog kidney acylase I (Sigma grade II) (27,28). The compounds (96-100% e.e.) were protected as benzyl esters (29) and coupled with [7-^{13}C]cyclohexanecarboxylic acid (99% ^{13}C) using dicyclohexylcarbodiimide in CH_2Cl_2 below −5° (30). Hydrogenolysis over Pd-C gave cyclohexane-[^{13}C]carbonyl-D- and L-[^{15}N]alanine, respectively. L isomer: ^{1}H nmr ($CDCl_3$) δ 1.1-2.2 (m, 11H, cyclohexane ring); 1.40 (dd, 3H, $^3J_{H,H}$=7.2 Hz, $^3J_{N,H}$=2.4 Hz, *CH*$_3$); 4.55 (br, q, 1H, $^3J_{H,H}$=7.2 Hz, alanine-*CH*); 6.35 (ddd, 1H, $^1J_{N,H}$=91.2 Hz, $^3J_{H,H}$=7.2 Hz, $^2J_{C,H}$=4.8 Hz, *NH*); 8.8 (br. s., 1H, *COOH*). Proton broadband decoupled ^{13}C nmr ($CDCl_3$) δ 18.11 (s, CH_3); 25.61 (s, CH_2); 29.40 (s, CH_2); 45.11 (dd, $^1J_{C,C}$=50 Hz, $^2J_{C,N}$=6.3 Hz, cyclohexane-*CH*) 48.08 (d, $^1J_{C,N}$=12.5 Hz, alanine-*CH*); 175.95 (s, *COOH*); 176.88 (d, $^1J_{C,N}$=12.75 Hz, ^{13}*CO*). Ms (*m/z*, relative intensity) 202 (M+1, 4.7); 201 (M, 17.5); 156 (M-COOH, 22.6); 146; 128 (cyclohexyl-^{13}CO-^{15}NH, 7.5); $[\alpha]^{22}D$=+28.34° (*c*=0.854 in MeOH). D isomer: similar nmr and mass spectra, $[\alpha]^{22}D$=−26.0° (*c*=1.008 in MeOH). The enantiomer ratio of both samples was determined by ^{1}H-nmr analysis of the corresponding esters with *S*-(+)-mandelic acid (31,32). The D isomer contained 67% D and 33% L form: the L isomer consisted of 69% L and 31% D enantiomer. Hence, considerable racemization had occurred in the coupling reaction.

Reference samples of methyl *N*-(cyclohexanecarbonyl)-alanine and methyl *N*-(1-, 2-, and 3-cyclohexenecarbonyl)-alanine were prepared by stirring alanine methyl ester and the appropriate acid chloride in $CHCl_3$ in the presence of triethylamine at −40° overnight. All four compounds showed different gc retention times and discernibly different mass spectra.

RESULTS

The biosynthesis of ansatrienin (mycotrienin) was studied in feeding experiments using shake cultures of *S. collinus* Tü 1892. In a series of optimization studies, we determined that feeding of precursors to 24-h-old second stage cultures and harvesting 24 h later gave best results. However, as is evident from the data in Table 1, the yields in this fermentation are extremely variable, rendering quantitative comparisons of incorporation data rather tenuous. The work of Sugita *et al.* (4,5) has demonstated that, as expected, carbon atoms 1-16 of **1**, representing the ansa bridge, are derived from 2 propionate and 6 acetate units by a polyketide-type biosynthesis. The methoxy group attached to C-3 presumably results from an *O*-methylation of the corresponding alcohol. In accordance with this assumption, we observed incorporation of L-[methyl-^{14}C]methionine into the antibiotic (Table 1, expt. 1), although the location of the label was not confirmed by degradation.

The aromatic ring with the attached nitrogen and C-17 represents a *m*-C_7N unit which presumably serves as starter unit for the polyketide assembly. Labeling studies on the *m*-C_7N unit in rifamycin (33,34), mitomycin (15,35), and geldanamycin

TABLE 1. Incorporation of Radioactive Precursors into Ansatrienin A by *Streptomyces collinus* Tü 1892

Expt. No.	Precursor	Spec. Radioact. of precursor (dpm/μmole)	Amount added		Ansatrienin produced			Percentage incorporation	Dilution factor
					Yield		Spec. Radioact.		
			(μmoles/liter)	(dpm/liter)	(μmoles/liter)	(dpm/liter)	(dpm/μmole)		
1	L-[methyl-^{14}C]-methionine	$1.58 \cdot 10^6$	37	$5.9 \cdot 10^7$	10.7	$9.6 \cdot 10^4$	$8.9 \cdot 10^3$	0.16	178
2	D,L-[1,6-^{14}C]-shikimic acid	$8.1 \cdot 10^4$	530	$4.3 \cdot 10^7$	79.5	$1.98 \cdot 10^5$	$2.44 \cdot 10^3$	0.46	33
3	D,L-[3-^{14}C]-phenylalanine	$3.43 \cdot 10^6$	27	$9.44 \cdot 10^7$	21.4	$8.5 \cdot 10^4$	$3.92 \cdot 10^3$	0.09	875
4	[7-^{14}C]benzoic acid	$1.58 \cdot 10^6$	19.5	$3.15 \cdot 10^7$	7.95	$1.67 \cdot 10^4$	$2.1 \cdot 10^3$	0.05	752
5	D,L-2,5-dihydro-[3-^{14}C]phenylalanine	$5.76 \cdot 10^6$	5.5	$3.17 \cdot 10^7$	18.9	$1.67 \cdot 10^5$	$8.7 \cdot 10^3$	0.53	(662)
6	D-[G-^{14}C]shikimate	$4.6 \cdot 10^4$	742	$3.41 \cdot 10^7$	27.0	$3.96 \cdot 10^5$	$1.47 \cdot 10^4$	1.16	3.1
7	2,5-dihydro-[7-^{14}C]-benzoic acid	$5.83 \cdot 10^6$	7.2	$4.21 \cdot 10^7$	103	$1.23 \cdot 10^6$	$1.20 \cdot 10^4$	2.92	(485)
8	[7-^{14}C]cyclohexane-carboxylic acid	n.d.[a,b]	n.d.	$4.75 \cdot 10^7$	69.9	$8.8 \cdot 10^5$	n.d.	1.85	n.d.
9	1,4-dihydro[7-^{14}C]-benzoic acid	$1.11 \cdot 10^7$	1.59	$1.76 \cdot 10^7$	50.8	$6.6 \cdot 10^5$	$1.30 \cdot 10^3$	3.75	(8440)

[a]n.d. = not determined.

[b]Specific radioactivity on the order of 1-5 μC/μmole.

(16,36) have shown patterns of isotope incorporation matching those into shikimic acid and shikimate-derived metabolites, pointing to a shikimate pathway origin of the *m*-C_7N unit. Genetic work also supports this conclusion (37,38). However, no incorporation of shikimic acid nor, in the case of the mitomycins, of dehydroquinic acid was observed. This suggests that either shikimic acid is not able to penetrate to the site of synthesis or the formation of the *m*-C_7N unit branches off from the main shikimate pathway at a very early stage. Rickards and coworkers (39-42) and, independently, Ghisalba and Nüesch (43-45) recognized 3-amino-5-hydroxybenzoic acid as an intermediate in the formation of the *m*-C_7N unit in actamycin, mitomycin, rifamycin, and the ansamitosins.

To establish whether the *m*-C_7N unit of ansatrienin is of the same origin we synthesized 3-amino-5-hydroxybenzoic acid labeled with ^{13}C in the carboxyl group as described by Rickards and co-workers (46). This material, 270 mg, 99% ^{13}C-enriched at C-7, was fed to seven 100-ml cultures of *S. collinus*, and the resulting ansatrienin A (44.9 mg) was analyzed by ^{13}C-nmr spectroscopy, reduced with $NaHSO_3$ to the hydroquinone and reanalyzed. A single, heavily enriched carbon was seen which resonates at 32.2 ppm in ansatrienin B (acetone-d_6) and 29.4 ppm in ansatrienin A ($CDCl_3$). These signals are assigned to C-17 based on chemical shift theory, multiplicity, and chemical shift change upon oxidation/reduction. A specific enrichment at this site of 48% was calculated based on the radioactivity incorporated from simultaneously fed 3-amino-5-hydroxy-[7-^{14}C]benzoic acid. This very high and specific incorporation of 3-amino-5-hydroxybenzoic acid leaves no doubt that the *m*-C_7N unit in ansatrienin, an ansamycin with a benzenoid aromatic moiety, is of similar biosynthetic origin as that in the other compounds mentioned above, including the "naphthalenic" ansamycins, rifamycin and actamycin.

The work of Oshima and Ariga (19) had shown that the cyclohexane ring of the ω-cyclohexyl fatty acids originates from shikimic acid. To determine if this was also true for the cyclohexanecarboxylic acid moiety of ansatrienin, and at the same time to test for incorporation into the *m*-C_7N unit, we fed D,L-[1,6-^{14}C]shikimic acid (Table 1, expt. 2). The results, 0.46% incorporation at a dilution factor of only 33, indicated efficient utilization of shikimate in the biosynthesis of ansatrienin, particularly since only one enantiomer of the precursor is biologically active. Hydrolysis of the labeled ansatrienin and chromatographic analysis of the hydrolysate revealed the presence of only one radioactive peak which co-migrated with authentic cyclohexanecarboxylic acid. We conclude, therefore, that the cyclohexanecarboxylic acid moiety of ansatrienin is biosynthesized from shikimic acid, but that the latter, as in the other cases examined, is not incorporated into the *m*-C_7N unit.

The formation of a completely saturated hydroaromatic ring by a pathway normally producing aromatic compounds is rather curious. Clearly, the cyclohexane ring is not formed by reduction of a benzene ring, as evidenced by the poor incorporation of phenylalanine and benzoic acid (Table 1, expts. 3+4). What little incorporation of radioactivity from these substrates is seen is undoubtedly indirect, i.e., due to metabolic breakdown of the added compound. Consistent with this interpretation, the hydrolysates of these ansatrienin samples showed no radioactivity associated with the cyclohexanecarboxylic acid position on the chromatograms. Another hydroaromatic compound commonly encountered in streptomycetes is 2,5-dihydrophenylalanine (47-50), and in earlier work we had shown (51) that this compound is formed by a variant of the shikimate pathway. We, therefore, tested the possibility that the same route might be operating in *S. collinus* and that 2,5-dihydrophenylalanine is then converted to cyclohexanecarboxylic acid by side-chain degradation and reduction of the double bonds. D,L-2,5-Dihydro-[3-^{14}C]phenylalanine gave only modest incorporation (0.53%) at a

high dilution factor, 667 (Table 1 expt. 5), but the hydrolysate of this ansatrienin sample showed some radioactivity associated with the position of cyclohexanecarboxylic acid on the chromatograms. It is, therefore, possible that some conversion of 2,5-dihydrophenylalanine to cyclohexanecarboxylic acid has occurred, but in view of low efficiency we interpret this to a be a biotransformation not related to the normal biosynthetic pathway or, at best, a minor biosynthetic route. This view is supported by the next experiment discussed below.

Cyclohexanecarboxylic acid may arise from shikimic acid by a direct route, retaining all seven carbon atoms of the precursor, or by a route via C_6C_3 compounds and degradation of the 3-carbon to a 1-carbon side chain, resulting in net loss of the shikimate carboxyl group and incorporation of only the six ring carbons. To distinguish between these two possibilities, we carried out a feeding experiment with [G-^{14}C]shikimic acid (Table 1, expt. 6). The high incorporation and low dilution factor confirms the precursor role of shikimate. The labeled sample from this experiment was diluted with unlabeled material and recrystallized to give about 90 mg of a sample of 0.6 μCi/mmole. Hydrolysis of this material gave cyclohexanecarboxylic acid which was isolated with carrier dilution and purified to constant specific radioactivity via the dibenzylethylenediamine salt. Schmidt degradation of the regenerated acid gave cyclohexylamine, isolated as the hydrochloride, and CO_2, isolated as $BaCO_3$. The radioactivity counted in these two products, corrected for yields, indicated that 82% of the radioactivity of the cyclohexanecarboxylic acid resided in the ring, and 18% was associated with the carboxyl group. The amount of label in the carboxyl carbon is close to the value of 14% expected if the precursor shikimic acid, assuming it was close to uniformly labeled (52), was incorporated intact, i.e., if the carboxyl group of cyclohexanecarboxylic acid orginated from the carboxyl group of shikimate rather than from C-3 of a C_6C_3 precursor.

In order to obtain more information on the sequence of reactions leading from shikimic acid to cyclohexanecarboxylic acid we fed 2,5-dihydro-[7-^{14}C]benzoic acid, reasoning that (a) the conversion sequence presumably involves an alternating series of dehydrations and reductions, (b) conjugated systems require higher energy for reduction and may, therefore, be avoided and (c) the double bond of shikimate may be retained until late in the sequence. The compound gave good incorporation (Table 1, expt. 7) and markedly stimulated ansatrienin production at fairly low concentration. Increased yields of ansatrienin were also observed in the experiments with shikimic acid (Table 1, expt. 2 and 6) and 3-amino-5-hydroxy-[7-^{13}C]benzoic acid (100 μmoles/liter), but much larger amounts of precursor had been added in those cases. The percentage incorporation of 2,5-dihydrobenzoic acid (2.8 and 2.9% in 2 experiments) was on the same order of magnitude as that for [7-^{14}C]cyclohexanecarboxylic acid (Table 1, expt. 8), suggesting initially that the compound may be an intermediate in the biosynthesis. However, it was subsequently observed that 1,4-dihydro-[7-^{14}C]benzoic acid was also efficiently incorporated (Table 1, expt. 9). Obviously, at best one of these compounds is likely to be a biosynthetic intermediate, and the other, or possibly both, can be reduced to cyclohexane carboxylic acid when added to the culture without being a normal pathway intermediate.

Further information on the mode of assembly of the cyclohexanecarbonyl-alanine side chain was sought by feeding D- and L-[^{15}N]alanine and cyclohexanecarbonyl-D- and -L-alanine. The latter precursors were labeled with ^{13}C and ^{15}N in the amide linkage in order to determine whether the acylamino acid is incorporated intact into ansatrienin or only via hydrolysis to the components. Product analysis in each case involved hydrolysis of the isolated and purified ansatrienin, methylation of the resulting cyclohexanecarbonyl-alanine, and gc-ms analysis of the methyl ester. Under electron-im-

TABLE 2. Analysis of Cyclohexanecarbonylalanine Methyl Ester Obtained by Hydrolysis of Ansatrienin Samples from Feeding Experiments with Stable-isotope Labeled Precursors

Expt. No.	Precursors	Mass spectrum of product, % intensity			Isotopic composition of product		
		m/z 154	*m/z* 155	*m/z*156	Unlabeled	Single labeled	Double labeled
10	50 mg D-[^{15}N]alanine +50 mg L-alanine	100(100)[a]	31.13(9.34)	2.26(0.24)	82.1%	17.9%	0%
11	50 mg L-[^{15}N]alanine +50 mg D-alanine	100(100)	18.16(9.34)	1.11(0.24)	91.9%	8.1%	0%
12	50 mg cyclohexane-[^{13}C]-carbonyl-D-[^{15}N]alanine +100 mg cyclohexanecarboxylic acid	100(100)	12.67(9.72)	0.69(0.45)	97.1%	2.9%	0%
13	50 mg cyclohexane-[^{13}C]-carbonyl-L-[^{15}N]alanine +100 mg cyclohexanecarboxylic acid	100(100)	21.23(9.11)	1.42(0.31)	89.2%	10.8%	0%

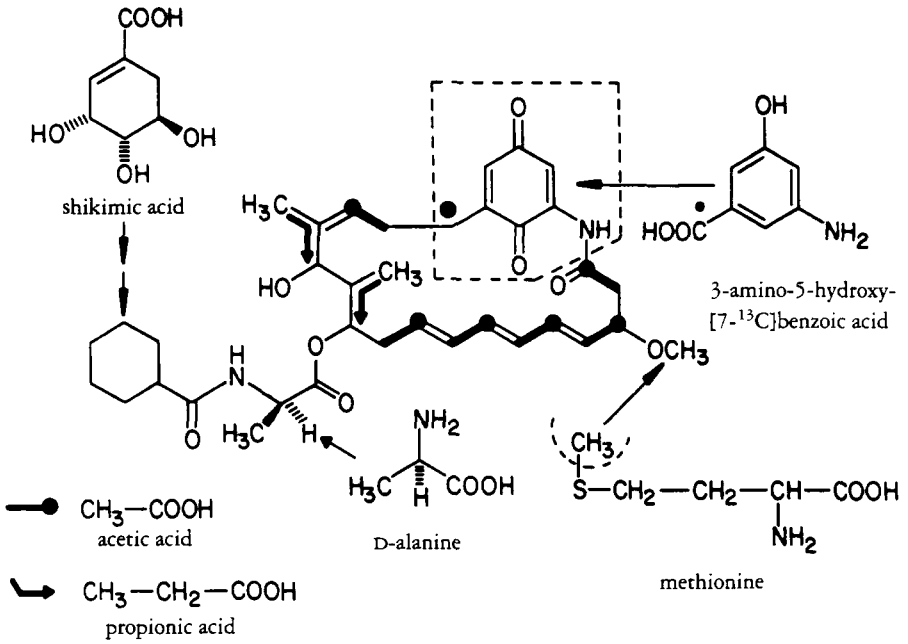
[a]Figures in parenthesis are the data obtained with simultaneously analyzed unlabeled reference compound.

pact ionization the molecular ion was weak (15), and the isotopic analysis was based on the prominent fragment ion at *m/z* 154 resulting from loss of H and $COOCH_3$ via a McLafferty rearrangement. In experiments 10 and 11 (Table 2) were fed (to one 100-ml culture each) 50 mg D-[^{15}N]alanine plus 50 mg L-alanine and 50 mg L-[^{15}N]alanine plus 50 mg D-alanine, respectively. The gc-ms analysis shows more than twice the enrichment in the cyclohexanecarbonyl-alanine moiety after feeding D-[^{15}N]-alanine than after feeding the L isomer. This is consistent with the established D configuration of the alanine moiety of ansatrienin[3] (4,5) and suggests that D-alanine is the immediate precursor. L-Alanine can, however, be converted to the D isomer. Feeding of cyclohexane-[^{13}C]carbonyl-D- and -L-[^{15}N]alanine (Table 2, expts. 12 and 13) showed that neither enantiomer is incorporated as an intact unit, as evidenced by the absence of any doubly labeled species in the product. The observed incorporations of 2.5 and 10%, respectively, result only in singly labeled species and must thus be due to hydrolysis of the added precursor. The much higher incorporation of label from the L isomer undoubtedly reflects the more facile enzymatic hydrolysis of this enantiomer. In fact, since neither isomer was optically pure, the results suggest that the D isomer may not be hydrolyzed and utilized at all and that all the labeling observed is due to hydrolysis of the L enantiomer in each sample and utilization of its hydrolysis products.

In the course of the gc-ms analysis we observed in all ansatrienin hydrolysates a second component of higher retention time than cyclohexanecarbonyl-alanine methyl ester (8 min 20 sec vs. 7 min 10 sec), which gave a molecular ion of two mass units lower molecular weight. This material represents approximately 2-3% of the amount of the parent compound. Inspection of the fragmentation pattern clearly showed that the compound contains a double bond in the cyclohexane ring, and gc-ms comparison with authentic 1-, 2-, and 3-cyclohexenecarbonyl-alanine methyl ester, prepared from the respective cyclohexenecarboxylic acids, identified it as 1-cyclohexenecarbonyl-alanine methyl ester. Hydrogenation of a sample of ansatrienin prior to hydrolysis eliminated this minor component, indicating that the double bond is present in the antibiotic itself and not formed during workup from a precursor, such as a hydroxy compound.

DISCUSSION

The work reported here and the earlier studies of Ōtake, Seto, and coworkers (4,5) establish the building blocks of ansatrienin (mycotrienin) as shown in Scheme 1. A polyketide chain is extended by six acetate and two propionate units. The chain starter



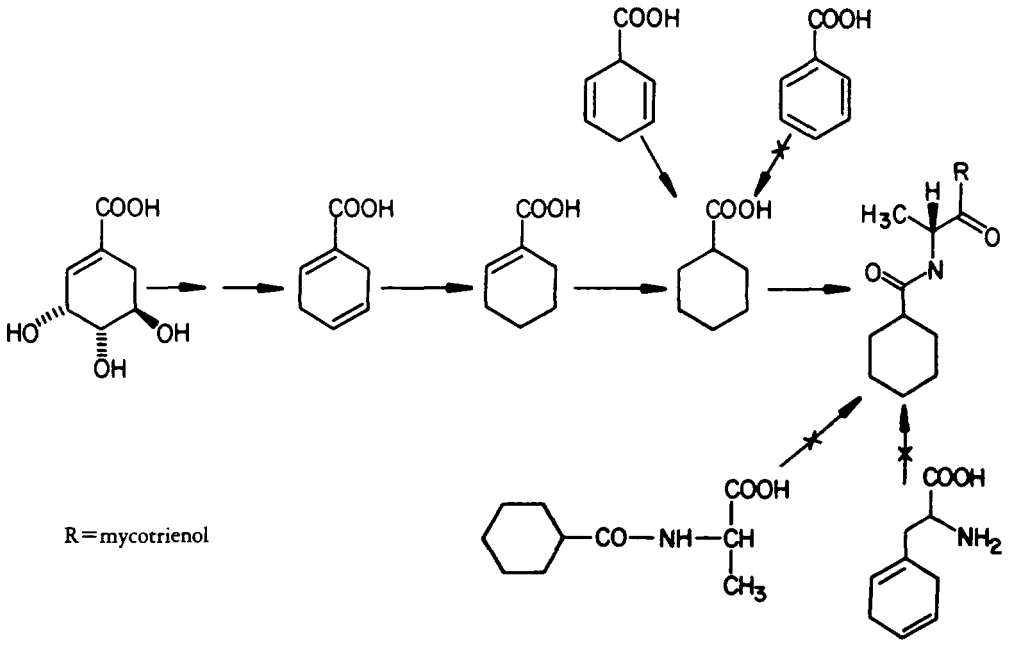
SCHEME 1. Biosynthetic orgin of ansatrienin (mycotrienin)

is a *m*-C_7N unit which, as in other examples (39-45), is derived from 3-amino-5-hydroxybenzoic acid. This formally extends the precursor role of 3-amino-5-hydroxybenzoic acid to a *m*-C_7N unit in a "benzenoid" ansamycin, a finding which does not come as a surprise in view of Rinehart's work on geldanamycin (16,36) and particularly in view of the fact that the trienomycins contain the almost unmodified 3-amino-5-hydroxybenzoic acid moiety. Nevertheless, it was important to verify this fact, since we have recently found that the *m*-C_7N units in other types of compounds, asukamycin/manumycin (unpublished results) and the valienamine moiety of acarbose (23), contrary to the suggestions in the literature (39,53), are not derived from 3-amino-5-hydroxybenzoic acid. Also, Sato and Gould (54) have recently demonstrated a polyketide origin of yet another *m*-C_7N unit in the biosynthesis of kinamycins. It is presumed that the "orientation" of the *m*-C_7N unit in ansatrienin conforms to that established by Rinehart's group (16) for geldanamycin, i.e., C-22 of **1** has the same origin as C-5 of shikimic acid and C-18 corresponds in its origin to C-3 of shikimic acid. This, of course, still leaves unexplained how 3-amino-5-hydroxybenzoic acid is formed by some variant of the shikimate pathway, a question which calls for further investigation. As in other cases, however, shikimic acid itself is not incorporated into the *m*-C_7N unit. Because it does label the cyclohexanecarboxylic acid moiety, the nonincorporation into the *m*-C_7N unit is not due to permeability problems. Rather, one has to conclude that the formation of 3-amino-5-hydroxybenzoic acid branches off at a much earlier stage of the shikimate pathway.

The D-alanine moiety of **1** is evidently derived from free D-alanine as a more immediate precursor than L-alanine. This is in contrast to the situation observed in the biosynthesis of gramicidin S, where L-phenylalanine is the immediate precursor of the D-phenylalanine moiety and epimerization takes place on the enzyme (55). However, it does conform to the observation that the D-valine moiety of boromycin, also a single D-amino acid attached to a non-peptide backbone, is derived from D- rather than L-valine (56). The fact that cyclohexanecarbonyl-alanine is not incorporated as an intact unit

suggests that the side chain is added one component at a time rather than being preassembled.

The formation of cyclohexanecarboxylic acid, a fully saturated hydroaromatic compound, by the shikimate pathway, designed by nature to generate aromatic molecules, is very intriguing. Although 2,5-dihydrophenylalanine is a widely distributed metabolite of streptomycetes, it is evidently not the normal precursor of cyclohexanecarboxylic acid. Rather, formation of the latter seems to involve a more direct route in which all seven carbon atoms of shikimate are retained. Whether this route still proceeds through chorismic acid, or more economically, involves directly dehydrations and reductions of shikimic acid remains to be established. Our efforts to define the late stages of the conversion by feeding 2,5- and 1,4-dihydrobenzoic acid gave ambiguous results. However, the detection in ansatrienin samples of a new minor compound containing the 1-cyclohexene instead of the cyclohexane moiety favors the 2,5- over the 1,4-dihydro compound. The likely biogenetic relationships surrounding the cyclohexanecarboxylic acid moiety are summarized in Scheme 2. The new ansatrienin containing the cy-



SCHEME 2. Precursors of the cyclohexanecarboxylic acid moiety of ansatrienin (mycotrienin)

clohexene moiety [3], which we propose to call ansatrienin A_4, has not yet been isolated as such, but its presence as a minor component follows clearly from the gc-ms analyses and from the fact that it disappears upon hydrogenation of the original ansatrienin sample.

ACKNOWLEDGMENTS

We are indebted to Mr. David Chang and the OSU Campus Chemical Instrument Center for gc/ms analyses, to Professors Axel Zeeck and Hans Zähner for references samples and cultures and for useful advice and discussions, to the Los Alamos Stable Isotope Resource (supported by NIH grant RR 00962) for ^{13}C material, and to the National Institutes of Health for financial support through Research Grants AI 11728 and AI 20264.

LITERATURE CITED

1. W. Weber, H. Zähner, M. Damberg, P. Russ, and A. Zeeck, *Zbl. Bakt. Hyg., I. Abt. Orig.*, **C2**, 122 (1981).
2. M. Sugita, Y. Natori, T. Sasaki, K. Furihata, A. Shimazu, H. Seto, and N. Ōtake, *J. Antibiot.*, **35**, 1460 (1982).
3. M. Damberg, P. Russ, and A. Zeeck, *Tetrahedron Lett.*, 59 (1982).
4. M. Sugita, K. Furihata, H. Seto, N. Ōtake, and T. Sasaki, *Agr. Biol. Chem.*, **46**, 1111 (1982).
5. M. Sugita, T. Sasaki, K. Furihata, H. Seto, and N. Ōtake, *J. Antibiot.*, **35**, 1467 (1982).
6. M. Sugita, Y. Natori, N. Sueda, K. Furihata, H. Seto, and N. Ōtake, *J. Antibiot.*, **35**, 1474 (1982).
7. S. Hiramoto, M. Sugita, C. Andō, T. Sasaki, K. Furihata, H. Seto, and N. Ōtake, *J. Antibiot.*, **38**, 1103 (1985).
8. G. Lazar, H. Zähner, M. Damberg, and A. Zeeck, *J. Antibiot.*, **36**, 187 (1983).
9. I. Umezawa, S. Funayama, K. Okada, K. Iwasaki, J. Satoh, K. Masuda, and K. Komiyama, *J. Antibiot.*, **38**, 699 (1985).
10. S. Funayama, K. Okada, K. Komiyama, and I. Umezawa, *J. Antibiot.*, **38**, 1107 (1985).
11. S. Funayama, K. Okada, K. Iwasaki, K. Komiyama, and I. Umezawa, *J. Antibiot.*, **38**, 1677 (1985).
12. M. Sugita, S. Hiramoto, C. Audo, T. Sasaki, K. Furihata, H. Seto, and N. Ōtake, *J. Antibiot.*, **38**, 799 (1985).
13. K.L. Rinehart and L.S. Shield, *Progr. Chem. Org. Nat. Prod.*, **33**, 231 (1976).
14. W. Wehrli, *Topics Current Chem.*, **72**, 21 (1977).
15. U. Hornemann, J.H. Eggert, and D.P. Honor, *J. Chem. Soc., Chem. Commun.*, 11 (1980) (and references therein).
16. K.L. Rinehart, Jr., M. Potgieter, D.L. Delaware, and H. Seto, *J. Am. Chem. Soc.*, **103**, 2099 (1981).
17. K. Kakinuma, N. Ikekama, A. Nakagawa, and S. Ōmura, *J. Am. Chem. Soc.*, **101**, 3402 (1979).
18. M. DeRosa, A. Gambacorta, L. Minale, and J.D. Bu'Lock, *J. Chem. Soc., Chem. Commun.*, 1334 (1971).
19. M. Oshima and T. Ariga, *J. Biol. Chem.*, **250**, 6963 (1975).
20. K. Suzuki, K. Saito, A. Kawaguchi, S. Okuda, and K. Komagata, *J. Gen. Appl. Microbiol.*, **27**, 261 (1981).
21. H. Simon and H.G. Floss, "Bestimmung der Isotopenverteilung in markierten Verbindungen," Springer-Verlag, Berlin, 1967, p. 23.
22. Y. Takeda, V. Mak, C.C. Chang, C.J. Chang, and H.G. Floss, *J. Antibiot.*, **37**, 868 (1984).
23. U. Degwert, R. van Hülst, H. Pape, R.E. Herrold, J.M. Beale, P.J. Keller, J.P. Lee, and H.G. Floss, *J. Antibiot.*, in press.
24. J.B. Lambert, F.R. Koenig, and J.W. Hamersma, *J. Org. Chem.*, **36**, 2941 (1971).
25. R. Grewe and I. Hinrichs, *Chem. Ber.*, **97**, 443 (1964).
26. C. Baret and L. Pichart, *Bull. Soc. Chim. France*, **18**, 580 (1951).
27. C.G. Baker and H.A. Sober, *J. Am. Chem. Soc.*, **75**, 503 (1953).
28. V.N. Kerr and D.G. Ott, *J. Labelled Comp.*, **15**, 503 (1978).
29. J.E. Shields, W.H. McGregor, and F.H. Carpenter, *J. Org. Chem.*, **26**, 1491 (1961).
30. F. Weygand, A. Prox, L. Schmidhammer, and W. König, *Angew. Chem. Int. Ed. Engl.*, **2**, 183 (1963).
31. D. Parker, *J. Chem. Soc., Perkin II*, 83 (1983).
32. J.K. Whitesell and D. Reynolds, *J. Org. Chem.*, **48**, 3548 (1983).
33. R.J. White and E. Martinelli, *FEBS Lett.*, **49**, 233 (1974).
34. A. Karlson, G. Sartori, and R.J. White, *Eur. J. Biochem.*, **47**, 251 (1974).
35. U. Hornemann, J.P. Kehrer, and J.H. Eggert, *J. Chem. Soc., Chem. Commun.*, 1045 (1974).
36. A. Haber, R.D. Johnson, and K.L. Rinehart, Jr., *J. Am. Chem. Soc.*, **99**, 3541 (1977).
37. O. Ghisalba and J. Nüesch, *J. Antibiot.*, **31**, 202 (1978).
38. O. Ghisalba and J. Nüesch, *J. Antibiot.*, **31**, 215 (1978).
39. J.J. Kibby, I.A. McDonald, and R.W. Rickards, *J. Chem. Soc., Chem. Commun.*, 768 (1980).
40. M.G. Anderson, J.J. Kibby, R.W. Rickards, and J.M. Rothschild, *J. Chem. Soc., Chem. Commun.*, 1277 (1980).
41. K. Hatano, S. Akiyama, M. Asai, and R.W. Rickards, *J. Antibiot.*, **35**, 1415 (1982).
42. A.M. Becker, A.J. Herlt, G.L. Hilton, J.J. Kibby, and R.W. Rickards, *J. Antibiot.*, **36**, 1323 (1983).
43. O. Ghisalba, H. Fuhrer, W. Richter, and S. Moss, *J. Antibiot.*, **34**, 58 (1981).

44. O. Ghisalba and J. Nüesch, *J. Antibiot.*, **34**, 64 (1981).
45. P. Traxler and O. Ghisalba, *J. Antibiot.*, **35**, 1361 (1982).
46. A.J. Herlt, J.J. Kibby, and R.W. Rickards, *Aust. J. Chem.*, **34**, 1319 (1981).
47. T. Yamashita, M. Miyairi, K. Kumugita, K. Shimizu, and H. Sakai, *J. Antibiot.*, **23**, 537 (1970).
48. J.P. Scannell, D.L. Preuss, T.C. Demney, T. Williams, and A. Stempel, *J. Antibiot.*, **23**, 618 (1970).
49. U. Fickenscher, W. Keller-Schierlein, and H. Zähner, *Arch. Mikrobiol.*, **75**, 356 (1971).
50. K. Okabayashi, H. Morishima, M. Hamada, T. Takeuchi, and H. Umezawa, *J. Antibiot.*, **30**, 675 (1977).
51. K. Shimada, D.J. Hook, G.F. Warner, and H.G. Floss, *Biochemistry*, **17**, 3054 (1978).
52. P.O. Larsen, *Biochim. Biophys. Acta*, **141**, 27 (1967).
53. U. Hornemann, J.P. Kehrer, C.S. Nunez, and R.L. Ranieri, *J. Am. Chem. Soc.*, **96**, 320 (1974).
54. Y. Sato and S.J. Gould, *J. Am. Chem. Soc.*, **108**, 4625 (1986).
55. F. Lipmann, *Acc. Chem. Res.*, **6**, 361 (1973).
56. T.S.S. Chen, C.J. Chang, and H.G. Floss, *J. Org. Chem.*, **46**, 2661 (1981).

Received 3 October 1986