Discovery, Isolation, Structure Elucidation, and Biosynthesis of U-106305, a Cholesteryl Ester Transfer Protein Inhibitor from UC 11136

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Abstract: During our screening of fermentation broths, culture UC 11136 was identified as producing potent inhibitor(s) of the *in vitro* cholesteryl ester transfer protein (CETP) reaction. Subsequent chemical isolation work identified two inhibitors of CETP produced by this culture. One of these inhibitors, U-106305, represented a novel CETP inhibitor as well as a structural class of compounds not previously reported from microbial fermentations. The structure of U-106305 was elucidated as *N*-isobutyl-*all-trans*-4,5:6,7:8,9:10,11:12,13:16,17-hexamethylene-(*E*,*E*)-2,14-octadecadienamide by extensive NMR studies. Biogenetically, the backbone of U-106305 was found to derive from nine acetates linked in a head-to-tail fashion, while the cyclopropyl methylene carbons were derived from the methyl group of L-methionine. A biosynthetic pathway is proposed based on these findings.

Introduction

Atherosclerosis is a disease of the arteries in which lipids, especially cholesterol, are progressively deposited into the arterial wall. Continued lipid deposition leads to coronary heart disease (CHD), the major cause of death in the United States and other industrialized countries. Prominent among the risk factors for CHD is high plasma cholesterol levels associated with low-density lipoproteins (LDL).[1] In contrast, high-density lipoprotein (HDL) cholesterol levels have been shown to be inversely related to the incidence of CHD. Several years ago it was discovered that the redistribution of cholesteryl esters, and other neutral lipids, among lipoproteins is facilitated by the action of a plasma protein called cholesteryl ester transfer protein (CETP). Animals that lack this protein have relatively high levels of HDL cholesterol and do not spontaneously develop atherosclerosis.[2] It has been suggested that inhibiting the action of CETP would result in an increase in HDL cholesterol levels, and thus the progression of atherosclerotic disease in man could be slowed. This is supported by the report that individuals who lack CETP have elevated levels of HDL-like particles,[3] and that CETP transgenic mice suffer accelerated atherosclerosis.[4] Therefore, it is reasonable to expect that an *in vivo* inhibitor of CETP will retard the progression of atherosclerosis and be a valuable therapeutic agent.

During the screening of microbial fermentations for CETP inhibitors, UC 11136 was found to produce two inhibitors of CETP activity. One of the components of this fermentation, designated as U-106305, appeared to be a new compound. Therefore, we undertook a series of spectroscopic studies of U-106305 to elucidate the molecular structure of this compound. In this paper we report the discovery, production, purification, structure elucidation, and biosynthesis of U-106305.

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Results and Discussion

Molecular Formula. The molecular weight of U-106305 was deduced to be 407 by the FAB-mass spectrometry measurements. Molecular ion adducts at m/z = 408, 430, and 446 representing $(M + H)^+$, $(M + Na)^+$, and $(M + K)^+$, respectively, were all detected. Results from a high-resolution FAB-MS (HRMS) study of the $(M + H)^+$ ion suggest that $C_{28}H_{41}NO$ is the most likely molecular formula (408.3275 theoretical vs 408.3266 found). In agreement with this, the ^{13}C NMR (CMR) spectrum exhibited 28 resonances (Table 1). Analysis of DEPT experimental results further revealed that these 28 carbons comprise three methyls (two of them being equivalent), seven methylenes, seventeen methines, and one quaternary carbon. The previous analysis accounted for all 40 non-exchangeable protons ($3 \times 3 + 7 \times 2 + 17 \times 1 = 40$). This left only one hydrogen unaccounted for. Comparison between the PMR spectrum run in $CDCl_3$ and $CDCl_3 + D_2O$ revealed that there was an exchangeable proton at 5.3 ppm. Thus, NMR experimental results corroborated results from the high-resolution mass spectrometry (HRMS) measurement.

Functional Group Analysis. U-106305 gives intense IR absorption bands at 1624 and 1558 cm^{-1}, suggesting that U-106305 contains a secondary amide group. This is supported by the quaternary signal at 166 ppm in the CMR spectrum and an exchangeable proton at 5.3 ppm in the PMR spectrum (*vide supra*). From the CMR and PMR spectra, we deduced that U-106305 contains two olefinic groups (C-2 and C-3, C-14 and C-15, see Table 1). We further concluded that, based on the chemical shift values of C-2, C-3, H-2, and H-3, one of the olefinic group is conjugated to the amide carbonyl. This conclusion is also supported by a strong UV absorption band detected at 215 nm with a shoulder at 240 nm in the UV/vis spectrum. No other functional group can be conclusively deduced from spectroscopic evidences. However, the extremely shielded resonances recorded between 0 and 0.5 ppm in the PMR spectrum indicate that U-106305 contains cyclopropyl groups. In support of this is the fact that six methylene carbon resonances were recorded between 7 and 15 ppm in the CMR spectrum (7.6 × 2, 8.0, 11.4, 13.4, 14.8 ppm). Also supporting this conclusion are the proton chemical shift values [ranged

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Table 1. NMR Data of U-106305

C no.	C chemical shift and multiplicities	C enrichment by labeled precursors:[a] [1-^{13}C]acetate/[2-^{13}C]acetate/[*methyl*-^{13}C]-L-methionine	proton chemical shift and coupling constants
21	7.6 (T)	1/1/90	0.07, 0.09 (dt: 8.43, 4.85)
20	7.6 (T)	1/1/90	0.12, 0.16 (dt: 8.39, 4.90)
22	8.0 (T)	1/1/90	0.08 (non first order)
23	11.4 (T)	1/1/90	0.32, 0.34 (dt: 8.20, 4.77)
19	13.4 (T)	1/1/90	0.65 (dt: 8.59, 4.87)
24	14.8 (T)	1/1/90	0.34, 0.43 (dt: 8.33, 4.60)
17	14.8 (D)	5/1/1	0.63 (m)
6	17.9 (D)	1/5/1	0.57 (dq: 13.27, 4.93)
9	18.0 (D)	5/1/1	0.58 (m)
8	18.2 (D)	1/5/1	0.49 (m)
11	18.2 (D)	5/1/1	0.51 (m)
10	18.4 (D)	1/5/1	0.53 (m)
18	18.41 (Q)	1/5/1	1.02 (d: 6.0)
7	18.8 (D)	5/1/1	0.60 (m)
13	20.0 (D)	5/1/1	1.00 (m)
3',3'	20.02 (Q)	1/1/1	0.90 (d: 6.8)
4	20.7 (D)	1/5/1	1.29 (m)
12	21.8 (D)	1/5/1	0.68 (m)
16	22.4 (D)	1/5/1	0.94 (m)
5	24.0 (D)	5/1/1	1.01 (m)
2'	28.5 (D)	1/1/1	1.77 (h: 6.8)
1'	46.7 (T)	1/1/1	3.20 (d: 6.8)
2	120.0 (D)	1/5/1	5.91 (d: 15.2)
14	130.4 (D)	1/5/1	4.98 (dd: 15.5, 7.2)
15	131.0 (D)	5/1/1	4.98 (dd: 15.5, 7.7)
3	148.8 (D)	5/1/1	6.24 (dd: 15.2, 9.8)
1	166.0 (S)	5/1/1	

[a] Enrichments are determined by comparing peak heights of carbon signals to those of non-enriched signals.

between 0.07 and 0.67 ppm (see Table 1)] of those methylene protons identified through 2D C−H correlation experiments.

Determination of Double Bond Equivalence. The molecular formula dictates that U-106305 has nine double bonds or unsaturation equivalents, assuming no symmetry exists in U-106305. Since we have already accounted for three double bonds, it can be concluded that U-106305 has six ring structures. As discussed in the previous paragraph, it is highly likely that these six ring structures are all cyclopropyl groups.

NMR Spectra Analysis and Structure Elucidation. To elucidate the structure of U-106305, we rely heavily on NMR data, particularly the 1D and 2D proton spectra. The 1D PMR and CMR spectral information is summarized in Table 1. With 32 protons located between 0 and 1 ppm of the PMR spectrum, not all of the signals are resolved, even at 600 MHz. This hampered the interpretation of the spectra. However, with the knowledge that there are six cyclopropyl groups in the molecule and by interpretation of the 2D ^{1}H−^{13}C correlation spectrum, we were able to identify all cyclopropyl proton signals and construct the molecular structure by interpretation of the double quantum filtered (DQF) COSY spectrum (see Figure 1).

To begin with, the two equivalent methyls (C-3′) are found to connect to a methine proton (C-2′) which is coupled to an amide-linked methylene group (C-1′). Thus, an isobutylamino substructure defines this spin network. As established previously, the only amide group (C-1) of U-106305 is linked to a double bond (C-2,3). In the aliphatic region of the CMR spectrum, there are a total of 13 methine signals as indicated by the DEPT experimental results. One of these already has been identified as the isobutylamino methine. The remaining 12 methine signals therefore should represent the six cyclopropyl methines. The chemical shift values of these 12 methine carbons are 14.8, 17.9, 18.0, 18.2 × 2, 18.4, 18.8, 20.0, 20.7, 21.8, 22.4, 24.0 ppm, respectively. The 2D ^{1}H−^{13}C correlation spectrum again pinpoints the locations of their respective attached protons in the proton spectrum. However, four of them were found overlapping between 0.50 and 0.55 ppm, making the analysis difficult. Nevertheless, the knowledge of the whereabouts of these protons is very useful for the structure elucidation work as discussed below. The values indicated in Table 1 are based on the 2D DQF COSY spectral results.

Further analysis of the 2D DQF COSY spectrum starting at the β end of the carbonyl-conjugated double bond allowed us to connect it sequentially through three cyclopropyl groups (C-4 through C-9). Along the way, each of the three bridgehead methylene groups was also identified. The methine proton of the third cyclopropyl group at C-9 is among one of the four protons in the 0.50−0.55 ppm region, making it difficult to continue to follow the connectivities in this region. However, the lack of cross peaks outside this region (other than the already identified C-21 methylene) strongly suggests that this proton is further connected to another methine proton (10-H) in this region. The other possibility is that the carbon bearing the proton of the third cyclopropyl group is connected to a quaternary carbon. This possibility can be disregarded based on the fact that the neighbors of the only quaternary carbon in this molecule have all been accounted for. Considering the chemical shift value of this methine proton at C-10, we are

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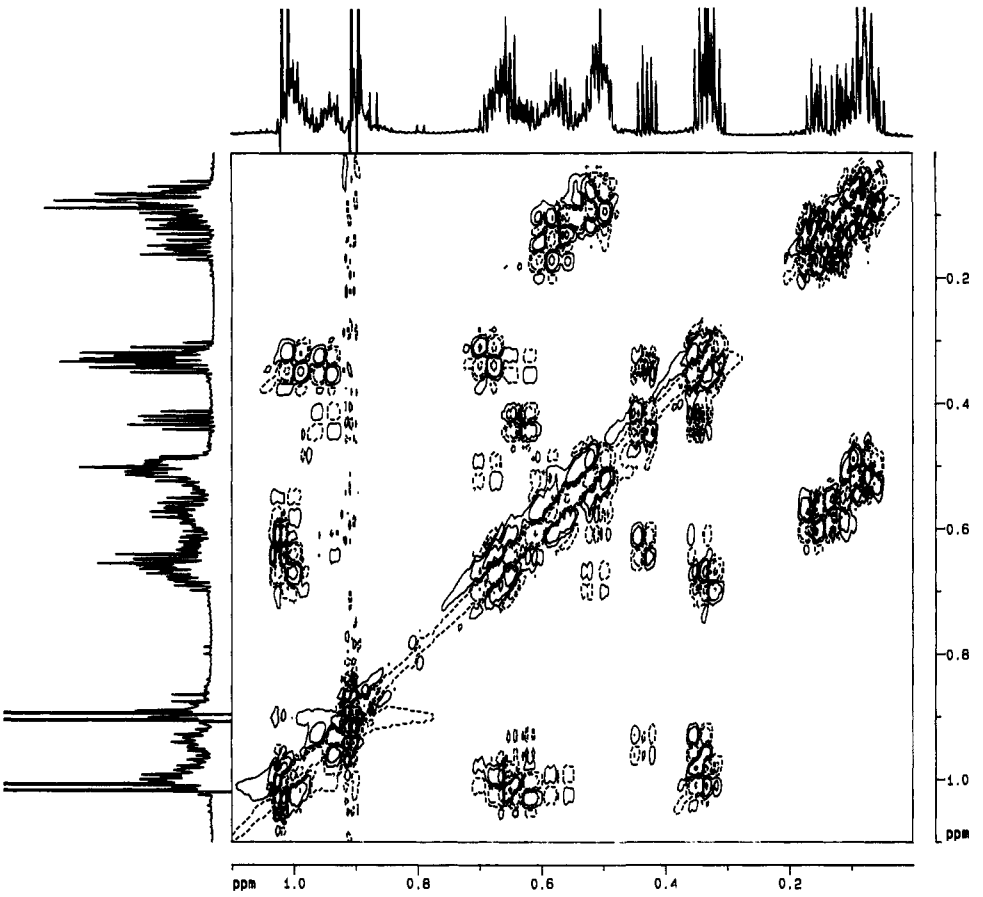

Figure 1. DQF COSY spectrum of U-106305 (0−1 ppm).

forced to deduce that another cyclopropyl group is linked in series to the three cyclopropyl groups already identified.

Starting at the other end, the terminal methyl (C-18) at 1.02 ppm is found to connect to a cyclopropyl group (C-16, -17, -24) which is further connected to the unconjugated double bond (C-14 and -15). This double bond is then traced to other cyclopropyl groups (C-13, -12, -23). The methine proton at C-12 is found to further connect to another methine proton at 0.51 ppm. Again, the chemical shift value of this newly connected methine falls into the 0.5−0.55 ppm region, making the tracing of connectivities of the 2D COSY spectrum difficult to continue. However, the fact that it falls into that region also reveals to us that this methine proton is part of a cyclopropyl group. Thus, we have accounted for all six cyclopropyl groups of this molecule.

It is further deduced that both olefinic groups are *trans*-disubstituted as suggested by the size of the coupling constants (15.2 and 15.5 Hz, see Table 1) of the two pair of olefinic protons. It should be noted that the coupling constant and chemical shift values of the unconjugated olefinic group are obtained by the NMR signal simulation method since these two protons displayed non-first-order character. The simulated and recorded spectra of these two protons are shown in Figure 2. The structure of U-106305 is therefore elucidated as *N*-isobutyl-4,5:6,7:8,9:10,11:12,13:16,17-hexamethylene-(*E*,*E*)-2,14-octadecadienamide, as shown in Figure 3.

To address the question of stereochemistry of U-106305, we analyzed the coupling constants of resolved and partially resolved peaks in the PMR spectrum. As listed in Table 1, all analyzable protons of the cyclopropyl methylenes displayed doublet of triplet patterns with the coupling constants in the neighborhood of 9 and 5 Hz, respectively. It is well established in the literature[14] that in a cyclopropyl system the *cis* vicinal coupling constant is approximately 9 Hz, while the geminal and *trans* vicinal coupling constants are approximately 5 Hz. Thus, the PMR signals of each of the methylene protons of a *trans*-disubstituted cyclopropyl spin system would display the pattern we have observed: a doublet (J = 9 Hz) of a pseudotriplet (J = 5 Hz). On the other hand, the methylene signals of a *cis*-disubstituted cyclopropyl spin system would display two dissimilar signal patterns: one being a pseudotriplet (J = 9 Hz) of doublets (J = 5 Hz), the other being a pseudoquartet (J = 5 Hz). To further support the *trans* configuration, we conducted a 2D NOESY experiment. We observed strong NOE interactions between the following pairs: H-3/H-5, H-13/H-14, and H-16/H-18. These observations strongly support the *trans* relationship for the substituents as indicated by the coupling patterns. As a result of the *trans* substitution, the two geminal protons have nearly identical electronic environments and, therefore, almost identical chemical shift values. It is important to note that only one (C-24) of the six methylenes resolve into two distinct $^1H-^{13}C$ cross peak signals (0.34 and 0.43 ppm). Not surprisingly, the biggest chemical shift difference between the two geminal protons belongs to the terminal cyclopropyl group since the substituents of this particular cyclopropyl group are quite different. The complete structure of U-106305 is therefore elucidated as *N*-isobutyl-*all-trans*-4,5:6,7:8,9:10,11:12,13:16,17-hexamethylene-(*E*,*E*)-2,14-octadecadienamide. Without knowledge of the absolute geometry of each of the chiral centers, U-106305 could adopt any of the 64 (2^6) possible structures. Two such structures are displayed in Figure 4 as an example.

Biosynthesis. On examination of the molecular structure of U-106305, it is apparent that the biosynthesis of the backbone

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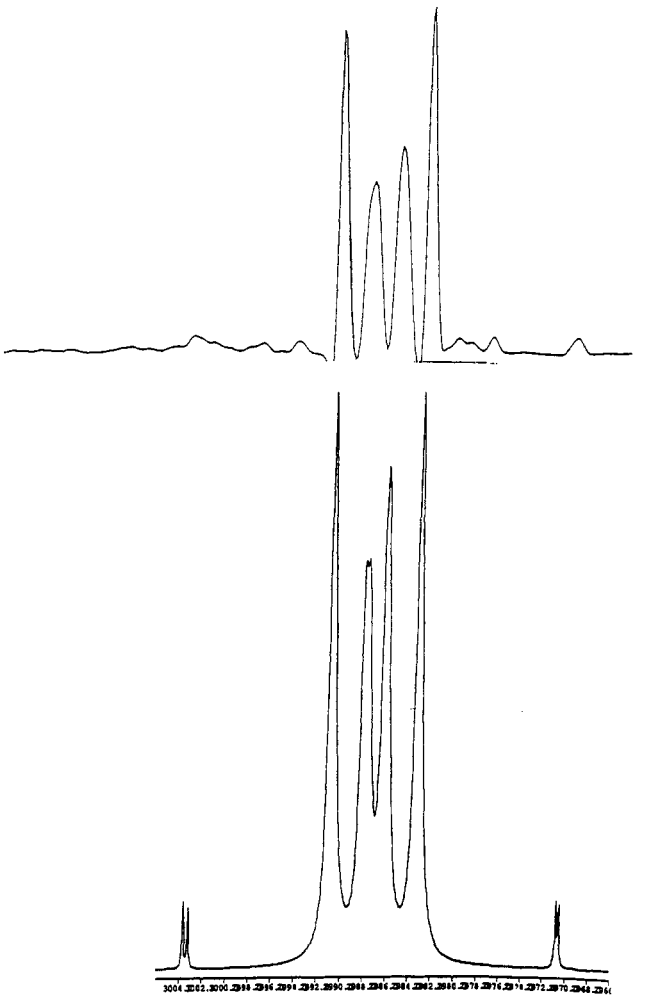

Figure 2. Comparison between the simulated spectrum (lower trace) and the NMR spectrum of U-106305 at the 4.98 ppm region.

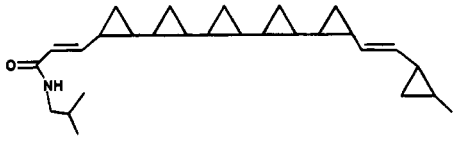

Figure 3. Structure of U-106305.

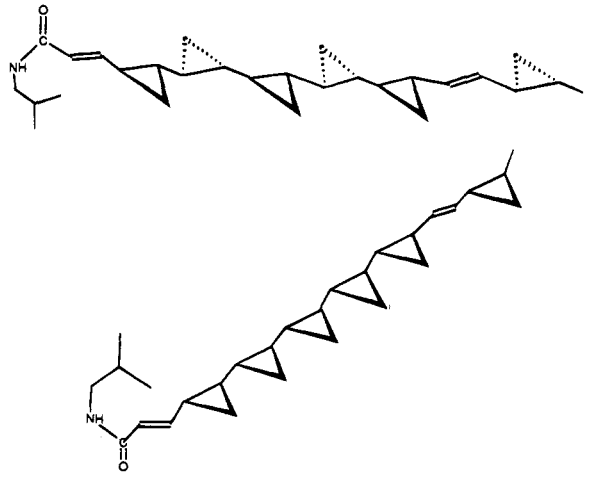

Figure 4. Two possible stereostructures of U-106305.

of this compound is related to the fatty acid biosynthesis pathway. Thus, acetate is the most likely precursor of the backbone carbons. An acetate feeding experiment would then confirm this hypothesis. What is not immediately clear is the origin of the methylene carbons located on the cyclopropyl groups. Since it is most likely they are derived from branched methyl groups, either C_1 or C_3 units could be the biosynthetic precursors. Generally speaking, the origin of the branching methyl grous from L-methionine is typical of fungal metabolism, whereas actinomycetes utilize propionate to provide the extra carbon.[15] The results from the aforementioned acetate experiment theoretically should delineate the biosynthetic process. For example, if the C_3 unit is involved in the biosynthesis, acetate feeding would not account for all backbone carbons. On the other hand, complete labeling of backbone carbons would suggest the involvement of the C_1 unit. We therefore started with the acetate feeding experiment.

The biosynthesis of U-106305 was studied in shake cultures fermented in an inorganic salts medium as described in the Experimental Section. Sterile addition of labeled precursors ca. 24 h after inoculation and harvest ca. 96−120 h later were chosen as standard conditions for these feeding experiments. After filtration, U-106305 was extracted from the broth and purified as described in the Experimental Section.

Experiments were first carried out with sodium [1-^{13}C]acetate. Workup yielded 4.6 mg of pure U-106305. In the 75-MHz CMR spectrum, carbons 1, 3, 5, 7, 9, 11, 13, 15, and 17 were found to be enhanced by ca. a factor of 5 (Table 1). To differentiate overlapped peaks, DEPT and 2D ^{1}H−^{13}C long-range correlation experiments were performed. None of the other carbons were found to be enriched. This result suggested that the backbone of the fatty acid was derived from acetate. Furthermore, these acetates were most likely linked together by the polyketide mechanism in a straightforward head-to-tail fashion as judged by the fact that every other carbon was enriched. If this deduction was true, then we would expect that in a different experiment using C-2 labeled acetate, the enriched carbons would be C-2, -4, -6, -8, -10, -12, -14, -16, and -18. Indeed, when this experiment was performed, the resulting CMR spectrum clearly indicated that was the case. Furthermore, the enhancement factor was also ca. 5:1 (Table 1), in agreement with previous results.

After we demonstrated that the backbone of the fatty acid is derived from acetate in a head-to-tail fashion, we then turn our attention to the biogenesis of methylene carbons of the fatty acid. As argued previously, the most likely candidate for the origin of these methylenes is a C_1 unit, e.g. the methyl group of L-methionine. We therefore carried out the same experiment as described above except employing the [*methyl*-^{13}C]-L-methionine instead of acetate. The resulting CMR spectrum showed six enhanced peaks. Analysis of the peak positions indicates that these peaks correspond to the six methylene carbons. From the peak height measurement, we estimate the enhancement factor is ca. 90 (Table 1). This demonstrated that the methylene carbons of the six cyclopropyl groups are derived from the methyl group of L-methionine. In summary, these results show that the main skeleton of U-106305 consists of nine acetate units with six methionine-derived methyl groups at each apex of the six cyclopropyl groups as shown in Figure 5.

After we elucidated the biogenesis of the carbons of the 24 carbon fatty acid, we can theorize about how these precursors are linked together. As stated previously, the acetates were probably formed by the polyketide mechanism. The methyl group was most likely added to the polyunsaturated polyketide and cyclized as indicated in Scheme 1 (step 4). This mode of

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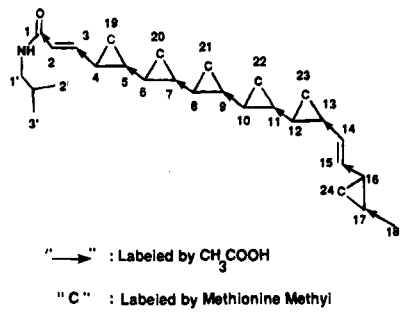


Figure 5. Incorporation of methionine and acetate of U-106305.

addition was proposed previously for biosynthesis of lactobacillic acid.[16] Although the exact structures of the key intermediates between acetate and the C-24 fatty acid are still unknown, the present study allowed construction of a plausible working hypothesis. Condensation of acetate units (via malonate) with incomplete reduction could lead to a C_{18} chain with unsaturation on every carbon except for the two terminal carbon atoms. Six of these unsaturated carbons would then be subjected to methyl addition to form the branched intermediate before cyclization. Futhermore, the fact that acetate failed to enrich this moiety indicated that an alternative carbon source was responsible for this isobutyl group. One possibility was that it originated from valine by way of a decarboxylation reaction. The biosynthesis pathway of this unusual fatty acid is proposed as shown in Scheme 1.

Experimental Section

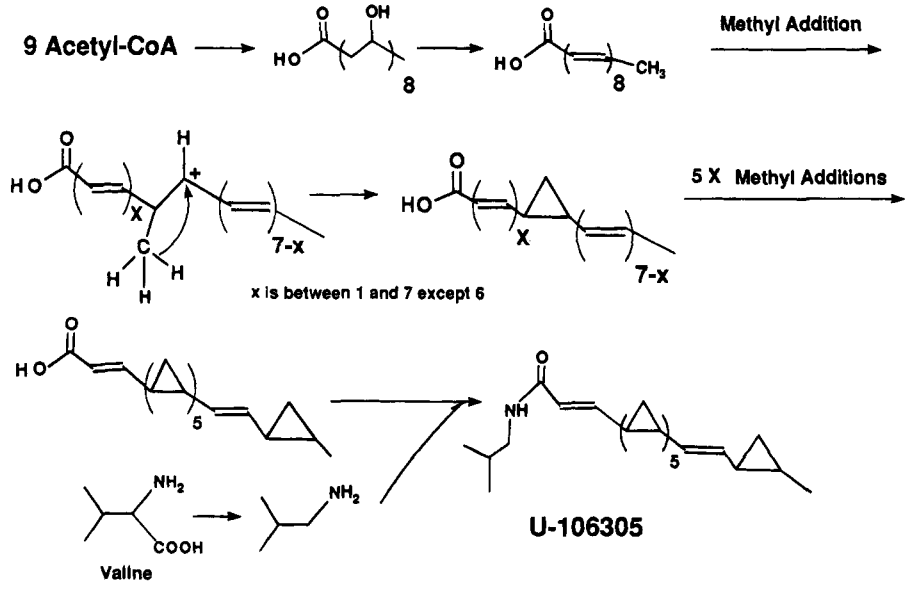
Bacterial Culture. The producing organism, *Streptomyces* sp. UC 11136, was isolated from a soil sample collected in Bolivia. The isolate grew well and produced gray yellow to yellow brown colonies on International *Streptomyces* Project (ISP) media two and six.[5] The growth was poor to fair on ISP media three, four, five, and seven. No aerial hyphae were produced on any of the ISP media except medium seven supplemented with 1 μM vitamin B_{12}, on which some long aerial hyphae were observed without spore formation. Substrate mycelia were not fragmented. Melanoid pigment was produced on ISP medium six but not medium seven. The isolate grew in the temperature range 24–37 °C; but no growth occurred at 45 °C. The organism utilized glucose and inositol as a carbon source but not arabinose, cellulose, fructose, mannitol, raffinose, rhamnose, sucrose, or xylose. The organism contained LL-diaminopimelic acid in whole cell hydrolysates and no diagnostic sugars. The morphological characteristics and the cell wall type place the isolate in the genus *Streptomyces*.[6]

Fermentation Conditions. UC 11136 was obtained from the culture collection of The Upjohn Company on 6 mm diameter agar plugs stored over liquid nitrogen. Three or four plugs of UC 11136 were inoculated into GS-7 medium which contained Cerelose and Pharmamedia each at 25 g/L of tap water. This seed medium was adjusted to pH 7.2 with NH_4OH and autoclaved for 30 min in 100 mL volumes contained in 500 mL wide mouth fermentation flasks. The flasks were inoculated and shaken for 48 h at 250 rpm and 28 °C. The mature seed cultures were employed at a 4% seed rate as the inoculum for the production medium (CBS-33). Medium CBS-33 was prepared using tap water and each liter contained 10 g of Cerelose, 20 g of dextrin, 10 g of cottonseed meal, and 10 g of brewer's yeast. The pH of CBS-33 was adjusted to 7.2 with KOH, and the medium was autoclaved as described above for the GS-7 medium. The inoculated flasks of CBS-33 were shaken as described above for 5 days.

Fermenation Conditions for the ^{13}C-Labeled U-106305. Mature seed fermentations grown as above were used to inoculate a minimal glucose salts medium at a 4 to 5% rate. This minimal medium is a modification of one reported earlier.[7] The minimal salts medium was prepared with tap water and each liter contained glucose, (10 g), salts solution (10 mL), MOPS (3-(*N*-morpholine)propanesulfinic acid) (5 g), and K_2HPO_4 (50 mg). The salts solution contained NaCl (46.7 g), NH_4Cl (10.7 g), Na_2SO_4 (4.26 g), $MgCl_2 \cdot 6H_2O$ (2.03 g), $CaCl_2 \cdot 2H_2O$ (0.29 g), and $ZnCl_2$ (3 mg) per liter of tap water. The production medium was sterilized in 100 mL volumes contained in 500-mL flasks. The inoculated production flasks were shaken at 250 rpm for 24 h at 28 °C. At this time, sterilized ^{13}C-labeled precursors were added to the production fermentations which were continued under the reported conditions for 4 or 5 days. The ^{13}C-labeled precursors included sodium [1-^{13}C]acetate (99%), sodium [2-^{13}C]acetate (99%), and [*methyl*-^{13}C]-L-methionine (98%). Both the labeled acetate and methionine were added as filter sterilized solutions to a concentration of 500 mg per liter.

Preparation of Tritiated LDL. The donor lipoprotein—tritiated LDL—was prepared by adding 250 μcuries of tritiated cholesteryl oleate to 5 mg of cholesterol, 54 mg of L-α-phosphatidyl choline, and 108 mg of sodium taurocholate. The solvents were evaporated and 2 mL of buffer (Dulbecco's PBS) was added followed by gentle agitation to form the emulsion. One-sixth of this was added dropwise to 60 mL of human plasma in a dialysis sac and dialysis at 37 °C was continued for 45 min against 4 L of buffer. Forty-five minutes after the second addition of the same volume of radioactive solution, the buffer was changed. This sequence was repeated two more times after which the dialysis sac was transferred to fresh buffer at 37 °C and a static incubation continued for 18 to 24 h. Usually an overnight dialysis at

Scheme 1. Biosynthesis of U-106305



4 °C followed this. In all instances, the buffer density was adjusted to 1.006 g/mL using sodium chloride.

The tritiated LDL's were isolated by flotation between the densities of 1.019 and 1.063 g/mL. These LDL's were exhaustively dialyzed at 4 °C against argon-purged buffer containing 1 mM EDTA. Storage of the LDL's was under argon at 4 °C. The specific activity of the isolated LDL's was about 8000 DPM per microgram of total cholesterol. The acceptor lipoprotein—HDL—was collected from human plasma. The HDL's were floated between the densities of 1.063 and 1.21 g/mL. These were dialyzed and stored in the same manner as the LDL's.

Preparation of CETP. A two-step procedure was used to obtain CETP from human plasma. First, the plasma's density was adjusted to 1.21 g/mL and the plasma was centrifuged for 24 to 48 h at 200 000 × *g* at 10 °C. The lipoprotein deficient plasma (LPDP) below the floated lipoproteins was collected. About 1 L of this LPDP was processed using 500 mL of Phenyl-Sepharose 4B. Buffer was used to wash the column until the A_{280} was 0.3. Then the CETP fraction was eluted with 0.5 mM EDTA, pH 7.4.

CETP Assay. The assay was conducted in 96-well, V-bottom, microtiter plates. Each well contained 5.0 μg of total cholesterol which was equally contributed by ^{3}H-LDL and HDL. The reaction was initiated by incubation of CETP with a final reaction volume of 170 μL. Static incubation continues for 3 h at 37 °C. Then 40 μL each of 0.1 M $MnCl_2$ and 0.45 M KH_2PO_4 (pH 7.4) were added and mixed. The plate was centrifuged at 1000 × *g* for 10 min to sediment the LDL's. The radioactivity in 100 μL of the supernatant liquid was quantitated to determine the transfer of ^{3}H-cholesteryl oleate to the HDL's. Under these assay conditions, U-106305 at 25 μM inhibited CETP by 50%.

Isolation of U-106305. The fermentation broth (10 L) was filtered and the mycelium was then extracted (2×) with acetone (1/10 and 1/20 broth volumes). The acetone extracts were combined and diluted with water (1/40 broth volume) and the acetone/aqueous solution was extracted with an equal volume of CH_2Cl_2. The aqueous upper layer was discarded. The acetone/CH_2Cl_2 lower layer was concentrated to dryness *in vacuo*. U-106305 was recovered from the mycelium extract by preparative HPLC using a Waters Prep LC 3000 equipped with a Waters variable wavelength 490E UV/VIS detector and a Waters 745B integrator. The separation was performed on three Waters 25 × 100 mm radial compression C-18 (μBondapak) cartridges preceded by a C-18 guard cartridge. Elution was achieved with isocratic acetonitrile (ACN):H_2O (78:22) for the first time 30 min of the separation followed by a linear gradient to 100% ACN at 35.0 min, which was maintained for a total separation time of 45.0 min. Mobile phase flow rate was maintained at 19.5 mL/min. Column temperature was ambient and column eluant was monitored by UV detection at 210 nm. Samples for chromatography were prepared in ACN:H_2O (8:2) at 3.5 mg/mL and 5-mL injections were made manually by injection loop. Under these separation parameters, U-106305 eluted at 27.8 min. Appropriate column fractions, assessed by bioactivities and analytical HPLC, were combined and concentrated to dryness *in vacuo*, weighed (10 mg), and studied by spectroscopic methods. From the NMR and FAB-MS data, it was deduced that a small amount of palmitic acid was present in the sample. The small amount of palmitic acid was subsequently removed from the sample by washing with a sodium bicarbonate solution. The palmitic acid-free sample (9 mg) was then subjected to various biological studies including the CETP assay.

Instrumentation. Proton NMR spectra were recorded on dilute (<20 mg/mL) methanolic (d_4-MeOH, 99.96% D, Cambridge Isotope Laboratories, Cambridge, MA) or chloroform ($CDCl_3$, 99.96% D, CIL, Cambridge, MA) solutions at 300 K (27 °C) using Bruker AMX-500 and AMX-600 spectrometers (Billerica, MA) operating at 500.13 and 600.14 MHz, respectively. Data were processed on Bruker X-32 computers using Bruker UXNMR software vsn.s 920801, 930701, and 930901.3. Sample solution volumes were ca. 500 μL. One-dimensional proton spectra were recorded as free induction decays (fids) of 32K complex points which were zero-filled once, multiplied by a resolution-enhancing Gaussian window (LB = −1 Hz, GB = 0.2 Hz), and Fourier transformed. A polynomial baseline correction was applied after phasing and before peak integration. Residual methanol and chloroform solvent peaks were used as proton references at 3.30 and 7.27 ppm, respectively (49.0 and 77.0 ppm, respectively, as ^{13}C references). Spectrum simulation was performed by employing the computer program NMRII (Calleo Scientific Software, 1300 Miramount Dr., Ft. Collins, CO). All 2D experiments were phase sensitive in both dimensions, using sequential acquisition in F2 and the TPPI method[8] to achieve quadrature detection in F1. Sweep widths were typically 7−9 ppm for proton and 200 ppm for ^{13}C dimensions. Data tables for COSY experiments typically were 2K by 512W, zero-filled once in F2 and twice in F1 to obtain a 2K by 2K hypercomplex spectrum after transform. Sinebell squared window functions shifted $\pi/3$ radians were applied in both F2 and F1 prior to transform. Baseline corrections of the transformed spectra in both F2 and F1 were performed where this was advantageous. Relaxation delays typically were 1.0 s. NOESY spectra[9] used mixing times of 300 ms. Double-quantum filtered COSY spectra[10] and inverse ^{1}H−^{13}C Hetcorrs[11] also were recorded, the latter experiment using GARP decoupling[12] of ^{13}C during acquisition. One-dimensional CMR spectra were obtained on a Bruker AM 300 spectrometer. Standard pulse sequences were used to obtain the broad-band and DEPT[13] spectra. In the biosynthesis study for which the peak intensities were integrated, the reverse gated decoupling method was used to acquire the FID with a 15-s delay between pulses.

Fast-atom bombardment-mass spectrometry (FAB-MS) was performed on a MAT CH-5 spectrometer operating in a positive ion mode. The source and probe temperatures were ambient. Infrared absorption analysis was performed on a Perkin-Elmer (PE) 1750 infrared Fourier transform spectrometer. Spectra were obtained using thin-film techniques on a ZnSe crystal. Ultraviolet absorption analyses were performed on a Hewlett Packard 8450A UV/VIS spectrophotometer. Spectra were recorded in MeOH.

Chemicals and Solvents. All organic solvents used for all chromatographic techniques were of HPLC grade or higher quality. All chemical reagents used for fermentation media were of analytical reagent grade. Tritiated cholesteryl oleate (NET 746) was obtained from DuPont NEN. Sodium [1-^{13}C]acetate (99 atom %), sodium [2-^{13}C]-acetate (99 atom %), and [*methyl*-^{13}C]methionine (98 atom %) were purchased from Cambridge Isotope Laboratories (Andover, MA) and used directly without further purification.

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