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Communications to the Editor

2-Azetidinones as Inhibitors of Cholesterol Absorption

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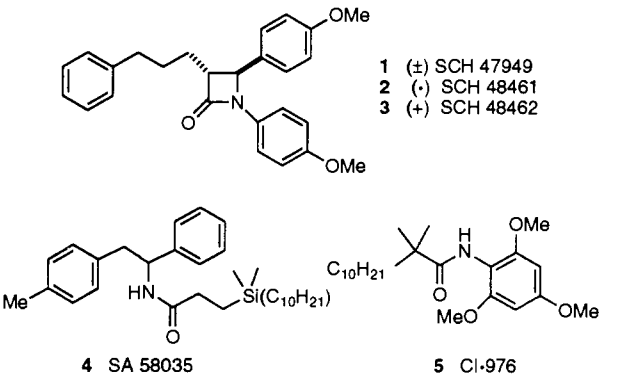
Introduction. Atherosclerotic coronary heart disease (CHD) is the major cause of death and cardiovascular morbidity in the western world.[1] Risk factors for CHD include hypertension, diabetes millitus, family history, male gender, cigarette smoking, and serum cholesterol.[2] Pharmacological reduction of serum cholesterol levels has been linked to reduction in CHD mortality as well as reversal of the atherosclerotic disease as indicated by the degree of occlusion of coronary arteries.[3] The two significant sources for cholesterol are endogenously synthesized cholesterol and exogenous or dietary cholesterol.[4] Efforts to inhibit the absorption of dietary cholesterol have primarily focused on the inhibition of acyl-CoA: cholesterol acyltransferase (ACAT), the major enzyme associated with cholesterol esterification.[4] Inhibition of this enzyme blocks absorption of intestinal cholesterol and may also inhibit cholesteryl ester deposition in the vascular wall in the form of fatty streaks associated with atherosclerotic plaque.[5] Figure 1 shows examples of two known classes of ACAT inhibitors, SA 58035 (**4**) and CI-976 (**5**).[4] As a part of a broad chemical program to discover novel ACAT inhibitors that are particularly active in *in vivo* models of cholesterol absorption, we investigated conformationally restricted compounds related to the above structures. Characterization of the biological activity of a series of conformationally restricted analogs, represented by **1**, suggests that although they are potent inhibitors of cholesterol absorption, they act on an as yet undiscovered mechanism fundamental to the absorption of intestinal cholesterol.



4 SA 58035 5 CI-976

Figure 1. Cholesterol absorption inhibitors.

Results and Discussion. We initially sought to explore the conformational preference of the phenethyl amide class of ACAT inhibitors (e.g., SA 58035) by bridging the two carbons of the ethyl linkage and the nitrogen atom in the simplest ring possible—a 2-azetidinone. It was felt that this ring system would provide a sufficiently rigid scaffold upon which to purposefully vary the substitution pattern and its orientation in three dimensions. These compounds could be prepared via the ester enolate–imine condensation[6] followed by epimerization or stereoselective alkylation as outlined generically in Scheme 1 (see the supplementary material for experimental details). Our first target was the diphenyl derivative, **6**, shown in Scheme 2.

Our products were all tested in two principal biological screens. The first was an *in vitro* ACAT assay measuring inhibition of the esterification of cholesterol with oleic acid. The second was an *in vivo* assay that measures the inhibition of the rise in plasma cholesterol levels and hepatic cholesteryl ester accumulation as a marker for cholesterol absorption in a cholesterol-fed hamster model over 1 week.[7] Although the desired compound **6** showed no activity in our assays, a second product, **7**, arising from stereoselective acylation of the initial adduct, did show activity. This disubstituted derivative was a reasonable ACAT inhibitor and showed modest effects *in vivo* at 100 mg/kg/day (see Table 1).

This initial lead was pursued by preparing a series of C-3 mono- and disubstituted 2-azetidinones designed to mimic the increased lipophilicity of our lead structure at this site. The best overall substitution pattern at C-3 for *in vivo* activity appeared to be concurrent ethyl and phenylpropyl disubstitution; however, there was poor

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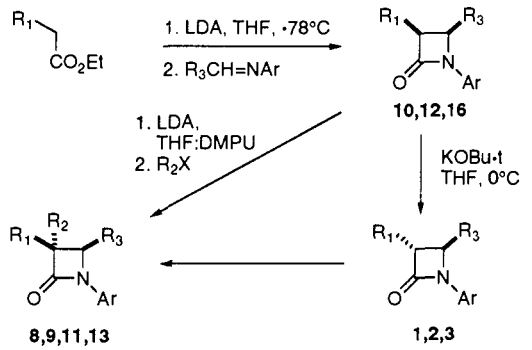
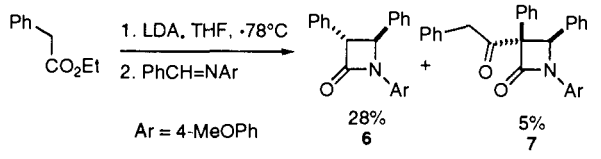
Scheme 1. Preparation of 2-Azetidinones



Scheme 2. Discovery of Initial Lead Compound, 7



correlation with our *in vitro* ACAT assay. Our attention was next focused on improving the *in vitro* activity of our compounds. Since these compounds bore structural similarity to the Warner Lambert ACAT inhibitor, **5** (CI-976), a number of compounds with 2,4,6-trimethoxyphenyl at nitrogen (**11–13**) were prepared. This change produced derivatives that were much more active *in vitro* but were less active *in vivo*. The apparent dichotomy of these two assays led us to follow our SAR based primarily on the results of our *in vivo* cholesterol absorption assay.

The remaining site for our initial SAR development was C-4. Several compounds lacking substitution at C-4 (**14**, **15**) were prepared using the Overman route shown in Scheme 3,[8] and a complete loss of *in vivo* activity was noted. Having clearly demonstrated the importance of aryl substitution at this site, we decided to introduce polar functionality that had benefited our *in vivo* profiles in other previous ACAT programs.[9] Thus both diastereomers of 1,4-bis(4-methoxyphenyl)-3-ethyl-3-(3-phenylpropyl)-2-azetidinone were targeted.

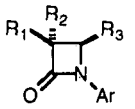
The first compounds prepared with this polar modification (**16** and **1**) were tested and proved to be very active in our *in vivo* hamster assay. Our new lead structure, **1** (SCH 47949), was prepared by the ester enolate–imine condensation of ethyl 5-phenylvalerate with *N*-(4-methoxybenzylidene)anisidine, followed by epimerization. This route results in a 4:1 mixture of trans and cis azetidinones, respectively, which were readily separable by SiO_2 chromatography. Interestingly, while both the *cis*- (**16**) and *trans*-2-azetidinones were active *in vivo*, the trans derivative, **1**, was much more potent (see Table 1). The ED_{50} for this compound in our hamster assay was ~7 mg/kg/day.

The trans racemate was resolved into its two enantiomers via chiral chromatography on a Chiralcel OD column, and the results of biological testing are shown in Table 1. Although the (+) enantiomer, **3**, was a 2-fold better ACAT inhibitor (IC_{50} = 11 μM vs 26 μM for **2**), the (−) enantiomer, **2** (SCH 48461), possessed dramatically greater *in vivo* activity. The ED_{50} for **2** was ~2 mg/kg/day for inhibition of the rise in hepatic cholesteryl esters in our hamster cholesterol absorption assay. This inhibitory effect on cholesterol absorption has subsequently been shown in a variety of species including nonhuman primates.[10] The dose–response curves in the hamster assay for the racemate **1**, **2**, and CI-976 are shown in Figure 2. The difference in *in vivo* activity for enantiomers **2** and **3** are clearly not reflected in the *in vitro* ACAT activity, although selective metabolism *in vivo* remains a possibility. In data presented elsewhere, it has been shown that this class of compounds blocks absorption of free cholesterol, whereas ACAT inhibitors block the subsequent esterification of intracellular cholesterol.[10] Thus, this class of inhibitors blocks an earlier step in the absorption of dietary cholesterol than do ACAT inhibitors. This evidence coupled with the general dichotomy of ACAT *in vitro* and *in vivo* results suggests a novel mechanism of action for these compounds. We are currently investigating the mechanism of action of this agent and are pursuing the development of **2** as a drug candidate.

Experimental Section: Chemistry. All melting points were taken on a Thomas-Hoover melting point apparatus and are uncorrected. Chromatography was performed over Universal Scientific or Selecto Scientific flash silica gel 32–63 mesh. HPLC was performed on a Rainin HPLX system using Rainin Dynamax 60A analytical or preparative SiO_2 columns at flow rates of 1.5 and 33 mL/min, respectively, for solvent mixtures as described in the Experimental Section. Chiralcel OD columns were purchased from Chiral Technologies Inc., Exton, PA. ^1H NMR spectra were determined with a Varian VXR 200 or Gemini 300-MHz instrument using Me_4Si as an internal standard. IR were obtained on a Perkin-Elmer 727B series IR spectrophotometer or on a Nicolet MX-1 FTIR. Elemental analyses were within ±0.4% of the theoretical values unless noted.

***rel*-(3*R*,4*R*)-1,4-Bis(4-methoxyphenyl)-3-(3-phenylpropyl)-2-azetidinone (16): Method A.** LDA was freshly prepared by dissolving 23.96 mL (17.39 g, 172 mmol) of diisopropylamine in 230 mL of dry THF at −78 °C under nitrogen and adding 103.9 mL (166 mmol, 1.6 M in hexanes) of *n*-BuLi. This cold solution was stirred at −78 °C for 1 h followed by the addition of 32.58 g (158 mmol) of the 5-phenylvaleric acid ethyl ester in 195 mL of dry THF over ~1 h, keeping the reaction temperature below −65 °C. The reaction was stirred for 1 h at −78 °C, and then 38.13 g (158 mmol) of *N*-(4-methoxybenzylidene)anisidine in 350 mL of dry dichloromethane was added. The reaction mixture was allowed to slowly come to room temperature, and the precipitate that forms was dissolved. The reaction mixture was stirred for 16 h at room temperature. The mixture was partitioned between 1.2 L of 1 N aqueous HCl and 1 L of ether. The ether layer was washed with 300 mL of 1 N HCl. The acid layers were combined and extracted with 1 L of ether. The ether extracts were combined, dried ($MgSO_4$), and concentrated in vacuo. The residue (35.08 g, 55%) was crystallized from ~200 mL of ethyl acetate–hexane (1:1) to give the desired *cis*-2-azetidinone as off-white crystals (32.05 g, 51%): mp 90–93 °C; IR (NaCl, cm^{-1}) 2900, 1726, 1593, 1491, 1432, 1370, 1278, 1227, 1155, 910, 800; ^1H NMR (300 MHz, $CDCl_3$) δ 7.28–7.11 (m, 7H, 7H, ArH) 6.99 (d, *J* = 7, 2H, ArH) 6.87 (d, *J* = 9, 2H, ArH) 6.79 (d, *J* = 9, 2H, ArH) 5.11 (d, *J* = 6, 1H, CHN) 3.83 (s, 3H, OCH_3) 3.75 (s, 3H, OCH_3) 3.52 (dt, 1H, *J* = 9, 6, CHC=O) 2.49–2.36 (m, 2H, $PhCH_2$) 1.62–1.24 (m, 4H, CH_2CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 167.27, 159.25, 155.53, 141.51, 130.96, 128.05, 127.92, 127.86, 126.40, 125.30, 117.99, 113.83, 113.65, 57.35, 54.81,

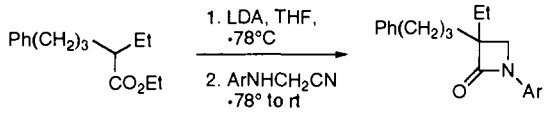
Table 1. ACAT and Cholesterol Absorption Inhibition Activity of 2-Azetidinones



compd no.	R_1	R_2	R_3	Ar	microsomal ACAT assay: % inh ACAT, concn (μM)	microsomal ACAT assay: IC_{50} (μM)	hamster cholesterol absorption assay: % redn HCE[a,f]	hamster cholesterol absorption assay: % redn PC[b]	hamster cholesterol absorption assay: dose (mg/kg/day)
7	Ph	$PhCH_2CO$	Ph	PMP[c]	83, 25	7.5	26	10	100
8	Et	$Ph(CH_2)_3$	Ph	PMP	33, 10		29	12	50
9	Ph	$Ph(CH_2)_3$	Ph	PMP	84, 10	6.0	16	11	50
10	$Ph(CH_2)_3$	H	Ph	PMP	28, 10		0	0	50
11	Et	$Ph(CH_2)_3$	Ph	2,4,6-TMP[d]	42, 10	12	0	0	50
12	$Ph(CH_2)_3$	H	Ph	2,4,6-TMP	61, 10		-20[e]	0	50
13	$Ph(CH_2)_3$	Et	Ph	2,4,6-TMP	82, 10	1.7	31	0	50
14	$Ph(CH_2)_3$	Et	H	2,4,6-TMP	38, 10		-29[e]	7	50
15	$Ph(CH_2)_3$	Et	H	PMP	-36, 10[e]		0	10	50
16	$Ph(CH_2)_3$	H	PMP	PMP	51, 10		55	17	10
1 (±)	H	$Ph(CH_2)_3$	PMP	PMP	33, 10	18	60	21	10
2 (-)	H	$Ph(CH_2)_3$	PMP	PMP	30, 20	26	93	43	10
3 (+)	H	$Ph(CH_2)_3$	PMP	PMP		11	0	10	50
CI-976					72, 10	6	33	0	10

[a] HCE = Hepatic cholesteryl esters. [b] PC = Plasma cholesterol. [c] PMP = *p*-Methoxyphenyl. [d] 2,4,6-TMP = 2,4,6-Trimethoxyphenyl. [e] Indicates stimulatory in this assay. [f] ED_{50}'s in this assay 16 = 11.5 mg/kg/day, 1 = 7.1 mg/kg/day, 2 = 2.2 mg/kg/day, CI-976 = 16 mg/kg/day.

Scheme 3. Synthesis of C-4 Unsubstituted 2-Azetidinones



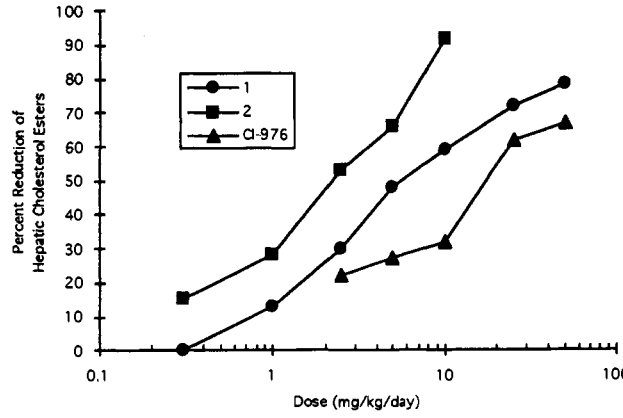


Figure 2. Effect of 1, 2, and CI-976 on the accumulation of hepatic cholesteryl esters of cholesterol-fed hamsters.

54.64, 54.03, 34.99, 28.18, 24.26; FABMS *m/z* 402 (M + 1), 252. Anal. ($C_{26}H_{27}NO_3$) C, H, N.

***rel*-(3*R*,4*S*)-1,4-Bis(4-methoxyphenyl)-3-(3-phenylpropyl)-2-azetidinone (1, SCH 47949): Method B.** The racemic *cis*-2-azetidinone, 16, (32.05 g, 79.8 mmol) was dissolved in 500 mL of THF. KOBu-*t* (1.79 g, 16.0 mmol) was added, and the mixture was stirred at 0 °C for 1.5 h. The reaction mixture was partitioned between 600 mL of 1 N aqueous HCl and 1.2 L of ether. The aqueous layer was extracted with 400 mL of ether. The ether layers were combined, dried ($MgSO_4$), and concentrated in vacuo to give 32.0 g of a mixture of *cis*- and *trans*-2-azetidinone (2.7:1). The pure *trans*-2-azetidinone, 1, was isolated via silica gel HPLC eluting with 10% ethyl acetate–hexane. Crystallization from ethyl acetate–hexane gave white crystals: mp 96.0–97.5 °C; IR (NaCl, cm^{-1}) 2933, 1742, 1463, 1454, 1388, 1297, 1175, 1032, 830; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.31–7.16 (m, 9H, ArH) 6.90 (d, J = 9, 2H, ArH) 6.78 (d, J = 9, 2H, ArH) 4.56 (d, J = 2, 1H, CHN) 3.81 (s, 3H, OCH_3) 3.74 (s, 3H, OCH_3) 3.08–3.06 (m, 1H, CHC=O) 2.65 (t, J = 7, 2H, CH_2Ph) 1.98–1.81 (m, 4H, CH_2CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 167.07, 159.40, 155.58, 141.44, 131.07, 129.68, 128.08, 128.05, 126.83, 125.56, 117.84, 114.13, 113.85, 60.29, 59.91, 54.82, 54.70, 35.06, 28.27, 27.73; FABMS *m/z* 402 (M + 1), 252. Anal. ($C_{26}H_{27}NO_3$) C, H, N.

(3*R*,4*S*)-1,4-Bis(4-methoxyphenyl)-3-(3-phenylpropyl)-2-azetidinone (2, SCH 48461). The racemate was completely resolved on a preparative Chiralcel OD column eluting with 90% hexane:2-propanol, 50 mL/min (analytical: 1 mL/min, R_t = 10.23 min for 2, R_t = 12.70 min for 3) to give 2 as a white solid: mp 46.5–48.0 °C; $[\alpha]_D$ = -19.3° (*c* 6.23 mg/mL MeOH). Anal. ($C_{26}H_{27}NO_3 \cdot {}^1/_4H_2O$) C, H, N.[11]

Biology: Chemicals and Radiochemicals. All reagents and solvents were HPLC grade and obtained from Fisher. [9,10-^{3}H]Oleoyl-CoA (2 Ci/mM) was prepared according to the method of Bishop and Hajra.[12] Ready-Safe scintillation cocktail was purchased from Beckman (Fullerton, CA).

Diets. Rodent chow pellets containing 0.5% cholesterol (w/w) were prepared by Research Diets, Inc. (New Brunswick, NJ).

Plasma Lipid Determinations. Plasma cholesterol levels were determined by a commercial modification of the cholesterol oxidase method which was available in a kit form (Wako Pure Chemicals Industries, Ltd., Osaka, Japan).[13]

Hepatic Lipid Determination. Samples of liver were extracted for neutral lipid analysis by the method of Folch *et al.*[14] Hepatic neutral lipid composition was determined subsequently using a HPLC method which has been described previously.[7]

Microsomal Enzyme Assays. Assays for acyl-CoA: cholesterol acyltransferase (ACAT; EC 2.3.1.26) activity were performed using the incubation conditions described by Tabas with some modifications.[15] The incubation mixtures contained 90 μM bovine serum albumin (essentially fatty acid free) and 12.5 μg of rat hepatic microsomal protein in a buffer containing 0.1 M potassium phosphate and 2 mM dithiothreitol (pH 7.4) in a total volume of 50 μL. Exogenous compound was introduced into the incubations using small volumes (1 μL) of

concentrated stock solutions in dimethyl sulfoxide (DMSO). After a preincubation at 37 °C for 15 min, [^{3}H]-oleoyl-CoA (10 μM final concentration, 1 μCi per incubation mixture) was added, and incubations were continued at 37 °C for 15 min. Assays were terminated by the addition of a 15-μL aliquot of each incubation to a silica gel G thin-layer chromatographic (TLC) plate. The plates were allowed to dry for several minutes and then developed using a solvent containing petroleum ether–diethyl ether–acetic acid (90:10:1, v/v/v). Regions corresponding to the migration position of authentic cholesteryl oleate were scraped and the radioactivity was quantified by liquid scintillation spectrometry.

Experimental *in Vivo* Protocols. Unless indicated, all animals used in these studies were allowed access to food (chow pellets) and water *ad libitum*. All animals were housed, treated, and cared for according to NIH guidelines for humane treatment of laboratory animals and the Animal Welfare Act in a program accredited by the American Association for Accreditation of Laboratory Animal Care.

Screening Assay. The hypercholesterolemic Golden Syrian hamster was utilized as the primary *in vivo* screening model for the evaluation of hypocholesterolemic compounds. Male Golden Syrian hamsters (Charles River Labs, Wilmington, MA), weighing between 100 and 125 g, were fed rodent chow and provided water *ad libitum*. Treatment protocols consisted of feeding chow which had been supplemented with 0.5% cholesterol for 7 days. During this period the animals were gavaged once daily with test compounds dissolved in 0.2 mL of corn oil. On the last day the animals were sacrificed with blood and liver samples taken for lipid analyses.

Acknowledgment. We would like to thank Dr. Sundeep Dugar for many helpful comments and suggestions during the course of this work. We would similarly like to thank Dr. Brian Salisbury, Dan McGregor, Lizbeth Hoos, April Smith, and Dr. Tom Fevig for their hard work and encouragement. We would also like to thank Dr. Joel Berger and Dr. Ashit Ganguly for their support of this work.

Supplementary Material Available: Procedural examples for the synthesis of compounds **6–15** as well as analytical data (4 pages). Ordering information is given on any current masthead page.

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