Inhibitors of Acyl-CoA:Cholesterol *O*-Acyl Transferase (ACAT) as Hypocholesterolemic Agents. CI-1011: An Acyl Sulfamate with Unique Cholesterol-Lowering Activity in Animals Fed Noncholesterol-Supplemented Diets

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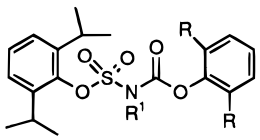
We have previously described the design and synthesis of a new class of ACAT inhibitors which possess very different physicochemical properties when compared to the more "typical" inhibitors, which have been uniformly lipophilic and possess little, if any, aqueous solubility.[1] From this new series, compound **1** was shown to have a log *P* of 2.98 and an aqueous solubility of 21 mg/mL over a pH range of 6.87−9.85. In addition to these novel physicochemical properties and despite weak ACAT inhibition *in vitro*, compound **1** was one of the most effective lipid-regulating agents thus far discovered. However, despite this impressive efficacy, continued pharmacological evaluation was complicated by pH dependent stability of **1**. The corresponding free acid



1, R= *i*-Pr, R^1=Na
2, R= *i*-Pr, R^1=H
5, R= Me, R^1=H
6, R= *t*-Bu, R^1=H

(**2**) of **1** degraded rapidly in neutral aqueous solution. Conversion to the sodium salt (**1**) prevented this degradation. Solution stability studies in acidic aqueous media have shown that **1** degrades into two products identified as 2,6-diisopropylphenol **3** and sulfamate **4**.[2] The problems associated with this acidic degradation are compounded by the fact that compounds **1**, **3**, and **4** induced various hepatic cytochrome P450 isozymes.[3]

In an effort to overcome the inherent chemical instability of **1**, a study was initiated to identify compounds with equivalent (or better) *in vitro* and *in vivo* activities but greater solution stability, especially at pH <7. This paper describes the resulting discovery of [[2,4,6-tris-(1-methylethyl)phenyl]acetyl]sulfamic acid, 2,6-bis(1-methylethyl)phenyl ester (**7**, CI-1011). This compound is highly stable in acidic or basic solution and displays excellent *in vivo* efficacy in standard cholesterol-fed rat models. Of particular note, however, was the finding that **7** potently reduced plasma cholesterol in chow-fed rats and in rabbits fed a cholesterol-free, casein-containing diet characterized by both hepatic overproduction of apo B-containing lipoproteins and delayed lipoprotein clearance.

A consideration of degradation products **3** and **4** indicated that hydrolysis of the carbamate group in **1** was the most likely explanation for the acid-catalyzed degradation. A two-pronged strategy was initiated to stabilize the C(O)−O bond of the carbamate. The first strategy employed was the steric protection of the C(O)−O bond by bulky flanking groups on the aryl ring of the carbamate phenol. As can be seen from Table 1, compounds **2** (isopropyl flanking groups) and **5**[4] (methyl flanking groups) degraded rapidly in neutral solution with only 37% and 20% of **2** and **5**, respectively, remaining after 24 h. The 2,6-di-*tert*-butyl compound (**6**)[4] and sodium salt (**1**) were perfectly stable under these conditions over 24 h. However, in acidic solution (Table 1), **1** degraded at the same rate as the free acid (**2**) while **6** showed only minimal degradation (<4%) under these conditions. Thus, we were able to stabilize the aryl carbamate in **1** by the incorporation of bulky 2,6-di-*tert*-butyl flanking groups.

An alternative strategy was to replace the phenolic oxygen of the carbamate by a methylene group. A retrosynthetic analysis of a prototype compound **7** indicated that bond disconnection of the amide yielded two synthons readily synthesized from commercially available starting materials. The synthetic route to **7** is shown in Scheme 1. The requisite acid chloride **8** was prepared in five steps from commercially available 2,4,6-triisopropylbenzoyl chloride (**9**). Hydride reduction of **9** gave the alcohol in 98% yield, which was then treated with PBr_3 in ether to give the corresponding bromide in 97% yield. Nucleophilic displacement of the bromide by cyanide (KCN/DMSO), acidic hydrolysis, and subsequent acid chloride formation gave the required acid chloride **8** in 50% overall yield. The known sulfamate **4** was prepared according to the method of Hedayatullah.[5] Compounds **4** and **8** were then coupled under basic conditions to give **7** in 80% yield.

This compound was completely stable in solution over a broad range of pH values (4.2−12) and temperatures (25−60 °C) over extended periods of time (up to 72 h).

ACAT inhibition, by **7**, was demonstrated using both microsomal and cellular assays. In the microsomal assay, using livers from cholesterol-fed rats, **7**, like compound **1**, was found to be a weak ACAT inhibitor with an IC_{50} value of 12.0 μM. However, it was subsequently found that the IC_{50} values obtained for this compound are highly dependent on the concentration of microsomes used in the assay. Thus, the IC_{50} of this compound is 0.7 μM when 0.2 mg/mL of microsomes are used instead of the standard 1 mg/mL. Restoring the membrane concentration by adding reconstituted lipids back to the media gave an IC_{50} value close to 12.0 μM. In retrospect, this finding is not surprising since other authors have speculated that inhibitors of membrane-bound enzymes (such as ACAT) reach the enzyme

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Table 1. Solution Degradation Studies

compd no.	% unchanged at pH 0.4[a]					% unchanged at pH 6.4[b]				
	0 h	2 h	4 h	8 h	24 h	0 h	2 h	4 h	8 h	24 h
1	100	92	83	68	25	100	100	100	100	100
2	95	88	80	66	23	95	87	85	74	37
5	93	71	57	37	5	94	79	65	48	20
6	100	100	99	98	97	100	100	99	99	98

[a] pH 0.4 = 50% 1 M HCl/50% acetonitrile. [b] pH 6.4 = 50% water/50% acetonitrile.

through the membrane and not the aqueous volume surrounding the membrane.[6] Thus, activity of a membrane-bound inhibitor should depend on both the total inhibitor concentration and the amount of membrane available for inhibitor adsorption. The amount of inhibitor bound to the membrane can be determined at various membrane concentrations from a single dose–response curve if the inhibitor concentration is expressed in terms of moles of inhibitor per mole of membrane (mol %).[7]

Such an analysis showed that ACAT was inhibited 50% when the microsomal membrane contained 3.9 mol % **7**. In addition to this observation, it has been shown that this compound also binds avidly to bovine serum albumin (BSA) which is present in the assay at a concentration of 50 μM. Thus, as with the membrane binding, the IC_{50} values are overestimated because of the BSA binding. At 20 μM BSA, the IC_{50} in IC-21 macrophages was 0.76 μM; this value decreased to 60 nM when no BSA was in the assay. In order to determine this directly, the efflux of [^{3}H]-**7** from IC-21 cells was determined in the presence and absence of BSA (20 μM). It was determined that 90% of [^{3}H]-**7** remained associated with the cells in the absence of BSA, while in the presence of BSA, only 3% of [^{3}H]-**7** remained cell-associated. In addition, in IC-21 macrophages, if the total inhibitor concentration in the assay sample is considered, the IC_{50} values for **7** and CI-976, a reference ACAT inhibitor,[8] were 3.3 vs 0.3 μM, respectively. However, when [^{3}H]-**7** and [^{14}C]CI-976 were used to determine ACAT inhibition on the basis of cell-associated ACAT inhibitor, it was shown that cellular ACAT was inhibited 50% when cells contained 54 pmol of CI-976/mg of cell protein or 14 pmol of **7**/mg of cell protein. Thus, on the basis of cell-associated ACAT inhibition, **7** is 3.5-fold more potent than CI-976. These latter results may be a more realistic measure of relative inhibitory strength than determinations based on the total inhibitor concentration in the sample since ACAT can only be inhibited by the presence of inhibitor within the cell. Collectively, these data indicate that **7** is indeed a potent ACAT inhibitor and that the previous microsomal data is more a measure of the affinity of **7** for BSA than of ACAT inhibition.[7]

Table 2. Dose–Response for **7** in Cholesterol-Fed Rats: Acute Screen[a]

treatment group	dose (mg/kg)	plasma cholesterol (mg/dL)	% change
PCC controls		183 ± 14	
chow controls		76 ± 5	−58
7	0.1	124 ± 15[b]	−32
7	0.3	81 ± 2[c]	−56
7	1	72 ± 5[c]	−60
7	3	66 ± 5[c]	−64
7	10	51 ± 3[c,d]	−72

[a] Data are mean ± SEM, n = 5/group. [b] $p < 0.05$. [c] $p < 0.01$ vs controls (t-test). [d] Significantly lower than chow controls, p = 0.0024 (t-test).

As has been done with most ACAT inhibitors, efficacy for **7** was demonstrated in several, now standard, cholesterol-fed rat models. Acute efficacy measured the ability of a single dose of **7** to prevent the overnight rise in dietary cholesterol induced by a single high fat, high cholesterol meal. As shown in Table 2, **7** is remarkably

Scheme 1

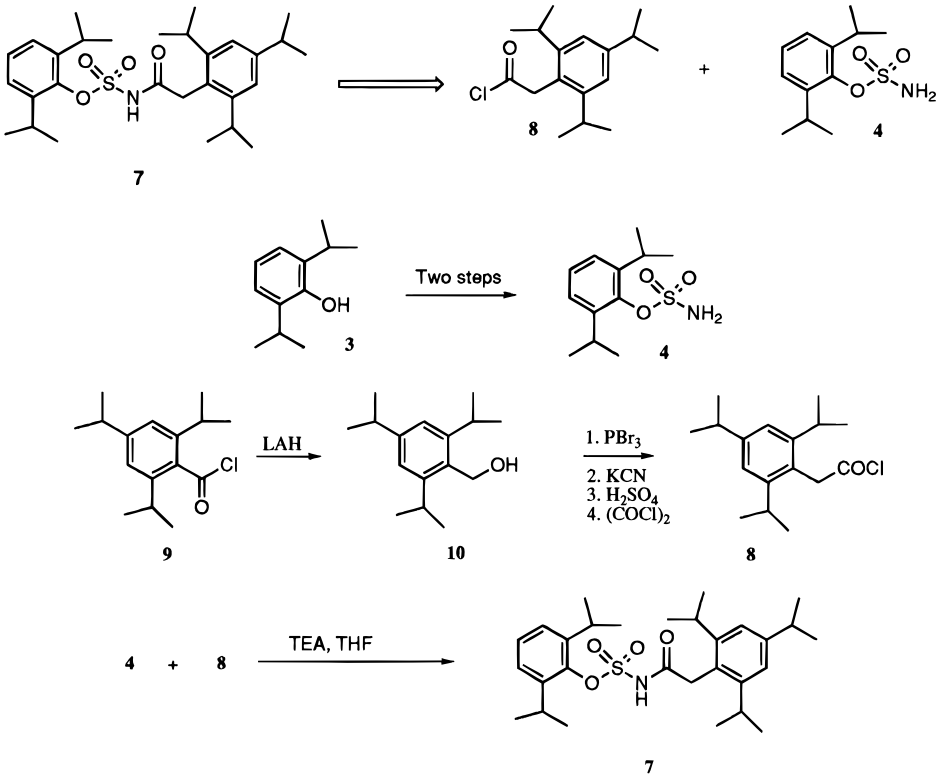

Table 3. Dose−Response for **7** in Cholesterol-Fed Rats: Chronic Screen[a]

exp no.	treatment group	dose (mg/kg)	TC	%Δ	HDL-C	%Δ	non-HDL-C	%Δ
1	PCC controls		222 ± 37		14 ± 1		208 ± 38	
	chow controls		62 ± 2[b]	−72	29 ± 1[b]	+108	33 ± 2[b]	−84
	7	0.1	131 ± 10[c]	−41	16 ± 1	+17	114 ± 9[c]	−45
	7	0.3	90 ± 6[b]	−60	20 ± 1[b]	+42	70 ± 7[b]	−66
	7	1	86 ± 8[b]	−61	25 ± 2[b]	+76	61 ± 9[b]	−70
	7	3	92 ± 8[b]	−59	33 ± 2[b]	+140	59 ± 8[b]	−72
	7	10	69 ± 6[b]	−69	36 ± 4[b]	+155	33 ± 5[b]	−84
2	PCC controls		535 ± 59		17 ± 1		518 ± 59	
	chow controls		84 ± 3[b]	−84	46 ± 3[b]	+178	39 ± 1[b]	−93
	7	0.001	551 ± 69	+3	16 ± 1	−3	535 ± 68	+3
	7	0.01	619 ± 60	+16	15 ± 2	−12	604 ± 60	+17
	7	0.03	334 ± 25[c]	−38	15 ± 1	−12	320 ± 25	−38
	7	0.1	323 ± 38	−40	18 ± 1	+7	306 ± 39[c]	−41

[a] Values are the mean ± SEM, $n = 6$/group. [b] $p < 0.01$ vs PCC controls (*t*-test). [c] $p < 0.05$.

potent in this model with efficacy observed at the lowest dose tested (0.1 mg/kg) and improving with increasing dose. The calculated ED_{50} in this screen was 0.4 mg/kg. For comparison, it can be noted that the ED_{50} values for the ACAT inhibitors CI-976, CL 277082,[9] DuP-128, and **1**[1] are 77, 16, 15, and 2 mg/kg, respectively. At the highest dose tested (10 mg/kg), the resultant plasma cholesterol level was significantly lower than the chow control group. Compound **7** was also capable of reducing total and non-HDL-C and elevating HDL-C after multiple oral doses in rats with preestablished dyslipidemia (Table 3). The ED_{50} value for non-HDL-C reduction was 0.09 mg/kg. The ED_{50} values for the ACAT inhibitors, CL 277082,[8] DuP-128,[1] and **1**,[1] are 17, 17, and 1.1 mg/kg, respectively. The lowest effective dose for HDL-C elevation was 0.3 mg/kg. For comparison, the lowest effective dose for gemfibrozil for elevating HDL-C was 30 mg/kg.[10]

The relevancy of the cholesterol-fed rat in predicting the human response to ACAT inhibitors has been called into question by the finding that CL 277082[11] and DuP-128[12] had little or no effect on cholesterol absorption or plasma cholesterol in normolipidemic volunteers. The same question may pertain to other cholesterol-fed animal models; e.g., these compounds, as well as **7**,[13] also lower plasma cholesterol effectively in cholesterol-fed hamsters[14,15] and rabbits.[16] Therefore, in order to provide some degree of assurance that **7** is preclinically unique and of potential clinical utility, we now report efficacy for **7** in two alternative, non-cholesterol-fed animal models in which drugs (HMG-CoA reductase inhibitors and fibrates) known to reduce LDL cholesterol in humans are active, namely, normal, chow-fed rats and casein-fed rabbits. We believe that efficacy in both models provides suggestive evidence for the extent of liver drug exposure and consequent inhibition of hepatic ACAT, VLDL secretion, and/or "direct" LDL production.

With respect to ACAT inhibitors, we previously reported that CL 277082 failed to lower plasma cholesterol in normal chow-fed rats but that it did reduce plasma triglycerides.[9] When compared to CL 277082 and DuP-128 in this model, **7** was the only compound that lowered plasma cholesterol (Table 4). However, all compounds reduced plasma triglycerides, with **7** being the most potent. We are unaware of any previous reports of ACAT inhibitors lowering plasma cholesterol in chow-fed rats at these doses.

In the second model, rabbits were fed a cholesterol-free, purified diet which contains casein as the sole protein source. The resulting hyperlipidemia observed

Table 4. Effect of **7** in Normal, Chow-Fed Rats[a]

treatment group	dose (mg/kg)	plasma TC (mg/dL)	%Δ	plasma TG (mg/dL)	%Δ
controls		59 ± 4		169 ± 10	
7	3	49 ± 3	−16	95 ± 8[b]	−44
7	10	44 ± 3[b]	−25	75 ± 13[b]	−55
7	30	41 ± 4[b]	−30	58 ± 10[b]	−66
CL 277082	3	53 ± 2	−9	127 ± 8[b]	−25
CL 277082	10	57 ± 3	−3	125 ± 6[b]	−26
CL 277082	30	50 ± 2	−15	78 ± 10[b]	−54
DuP-128	3	67 ± 10	+15	116 ± 16[b]	−31
DuP-128	10	58 ± 2	−2	131 ± 6[b]	−22
DuP-128	30	55 ± 3	−7	107 ± 13[b]	−37

[a] Values are the mean ± SEM, $n = 6$/group TC = total cholesterol; TG = triglycerides. [b] Significantly different from controls, $p < 0.05$ (ANOVA).

Table 5. Comparison of ACAT Inhibitors in Rabbits Fed a Cholesterol-Free Casein Diet[a]

treatment group	dose (mg/kg)	pretreatment plasma cholesterol (mg/dL)	posttreatment plasma cholesterol (mg/dL)	mean percent change (%)
controls		242 ± 25	396 ± 44	+64 ± 6
7	1	215 ± 25	184 ± 35	−15 ± 14[b]
7	3	250 ± 40	171 ± 35	−33 ± 6[c]
7	10	209 ± 28	118 ± 20	−37 ± 14[c]
CL 277082	1	227 ± 21	284 ± 36	+52 ± 9
CL 277082	3	231 ± 32	342 ± 53	+31 ± 18
CL 277082	10	251 ± 38	320 ± 72	+33 ± 33
DuP-128	1	223 ± 21	304 ± 36	+43 ± 18
DuP-128	3	254 ± 36	237 ± 58	+4 ± 27
DuP-128	10	252 ± 372	210 ± 40	−12 ± 20

[a] See ref 17 for composition of diet. [b] $p < 0.10$. [c] $p < 0.05$.

is of purely endogenous origin.[17] The LDL cholesterol increases severalfold (>85% of total cholesterol), due in large part to the hypersecretion of apoB-containing lipoproteins which are enriched in ACAT-derived cholesteryl esters.[17,18] The clearance of LDL is also delayed in this model.[18] The role of ACAT in the etiology of the endogenous hypercholesterolemic condition was confirmed by administering CI-976, a potent ACAT inhibitor, which decreased LDL cholesterol by 43% at 50 mg/kg without affecting biliary cholesterol absorption.[17] When **7** was compared to CL 277082 and DuP-128 in this model, only **7** lowered plasma cholesterol concentrations (Table 5). Plasma triglycerides are typically low in this animal model and unaffected by ACAT inhibitors. Preliminary evidence suggests that **7** decreases the direct secretion of LDL by the liver and also enhances LDL clearance in this animal model.[19] This latter finding is unexpected for an ACAT inhibitor but supports the view that the hepatic concentration of

ACAT-derived cholesteryl esters in the liver may determine LDL receptor number.[20]

In conclusion, we have identified a compound **7**, which displays potent ACAT inhibition in cellular assays where the activity is determined on the basis of cell-associated ACAT inhibitor. In the typical microsomal assay, the potency of **7** is highly dependent on the amount of microsomal membrane and albumin present in the assay. When the concentrations of both of these assay components are decreased, the IC_{50} also decreases. A similar finding has recently been reported that also shows the dependence of the IC_{50} on the lipid composition of the assay system.[21]

The compound is very efficacious in a variety of cholesterol-fed animal models; however, efficacy in these models has not yet been translated into efficacy in human trials. HMG-CoA reductase inhibitors and fibrates are two classes of hypolipidemics that are efficacious in noncholesterol-fed animal models and are known to reduce LDL cholesterol in man. Thus, in order to provide some level of confidence in the clinical utility of this compound, it was evaluated in two noncholesterol-fed animal models, namely, normal, chow-fed rats and casein-fed rabbits. Compound **7** was highly efficacious when evaluated in these models, whereas two previous drug candidates, CL 277082 and DuP-128, which failed in the clinic, did not show any LDL lowering. The relationship between efficacy, for **7**, in noncholesterol-fed animal models and plasma cholesterol lowering in humans is currently under investigation in clinical trials.

Supporting Information Available: Full experimental details (3 pages). Ordering information is given on any current masthead page.

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