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α-Substituted Malonester Amides: Tools To Define the Relationship between ACAT Inhibition and Adrenal Toxicity

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We prepared a series of α-substituted malonester amides that were evaluated for their ability to inhibit acyl-CoA:cholesterol *O*-acyl transferase activity in vitro and to lower plasma total cholesterol levels in a variety of cholesterol-fed animal models. Compounds of this series were also useful in examining the relationship between adrenal toxicity and ACAT inhibition. One compound from this series, **9f**, was a potent inhibitor of ACAT in both the microsomal and cellular assays. It was also bioavailable as determined by both a bioassay and a HPLC−UV assay. This compound was evaluated in both guinea pig and dog models of adrenal toxicity and compared to tetrazole amide **15**. In the most sensitive species, the dog, both of these compounds achieved good plasma levels; however, compound **9f** caused adrenal necrosis, whereas compound **15** had no effect on the adrenal gland. This adds to the growing body of evidence that the adrenal toxicity observed with ACAT inhibitors may not be mechanism related.

Introduction

Inhibition of ACAT activity in monocyte-macrophages of the arterial wall is an area of intense interest in the field of experimental atherosclerosis prevention, since direct inhibition of arterial ACAT may reduce the formation of macrophage-enriched fatty streaks and contribute to plaque stabilization by direct lipid depletion.[1] Our continuing research has primarily focused on ACAT inhibitors that possess the necessary structural features to achieve sufficient plasma levels of active drug to inhibit the arterial enzyme.[2] However, issues surrounding the toxic effect of ACAT inhibitors on the adrenal gland continue to be pervasive in this field.[3] There is still no conclusive data as to whether the toxic effects on the adrenal gland are due to the mechanism of action of these drugs. In addition, if these toxicities are associated with ACAT inhibition, then the goal of selectively inhibiting ACAT in the artery wall (a desirable peripheral target) without inhibiting ACAT in the adrenal gland (an undesirable peripheral target) may be difficult one to achieve in a therapeutic agent.

In this paper, we describe the synthesis and structure−activity relationships of a series of α-substituted malonester amides and describe our efforts to address the adrenal toxicity issues surrounding the identification of the best candidates for long-term antiatherosclerotic studies.

Chemistry

The unsubstituted malonester amides (**1**) were previously synthesized by the acylation of suitable anilines (**2**) with ethyl malonyl chloride (**3**), followed by hydrolysis and re-esterification with a desired alcohol.[4] However, attempts to repeat this procedure for the synthesis of certain α-substituted malonester amides failed. Thus, attempted monohydrolysis of diethyl phenylmalonate (**4a**) with alcoholic base followed by acidification led to decarboxylation to yield the acid (**5a**).[5] However, this route was successful for compounds **7b**,**c** and **14a**,**b** (Scheme 1).

An alternative route (Scheme 2) to the majority of target compounds avoided this problem in most cases. Deprotonation of ethyl phenylacetate (**6**) with LDA at −78 °C followed by quenching with an aryl isocyanate gave the malonester amides (**7**). Hydrolysis, followed by acidification with 1 M HCl gave the acids (**8**) which were then esterified with a suitable alcohol, using DCC in dichloromethane at 0 °C, to yield the target compounds (**9a**−**p**). Interestingly, hydrolyses of esters of type **7** proved capricious, since decarboxylations occurred on random occasions when Ar = 2,4,6-trimethoxyphenyl or the α-substituent was 2-pyridyl. To synthesize these analogues, the formation of the desired ester was the first step, thus avoiding the troublesome alkaline hydrolysis, acidification, and re-esterification (Scheme 3). Thus, the acids (**10**) were converted to the esters (**11**) by esterification with a suitable alcohol using DCC in dichloromethane at 0 °C. Deprotonation followed by quenching with an aryl isocyanate gave the desired compounds (**12a**−**e**).

Results and Discussion

Biological Methods. Compounds prepared were initially tested for ACAT inhibition in hepatic microsomes isolated from cholesterol-fed rats (LAI screen).[6] Using murine IC-21 macrophages (obtained from the American Type Culture Collection, Rockville, MD) as a model system for a cell type found in arterial lesions,

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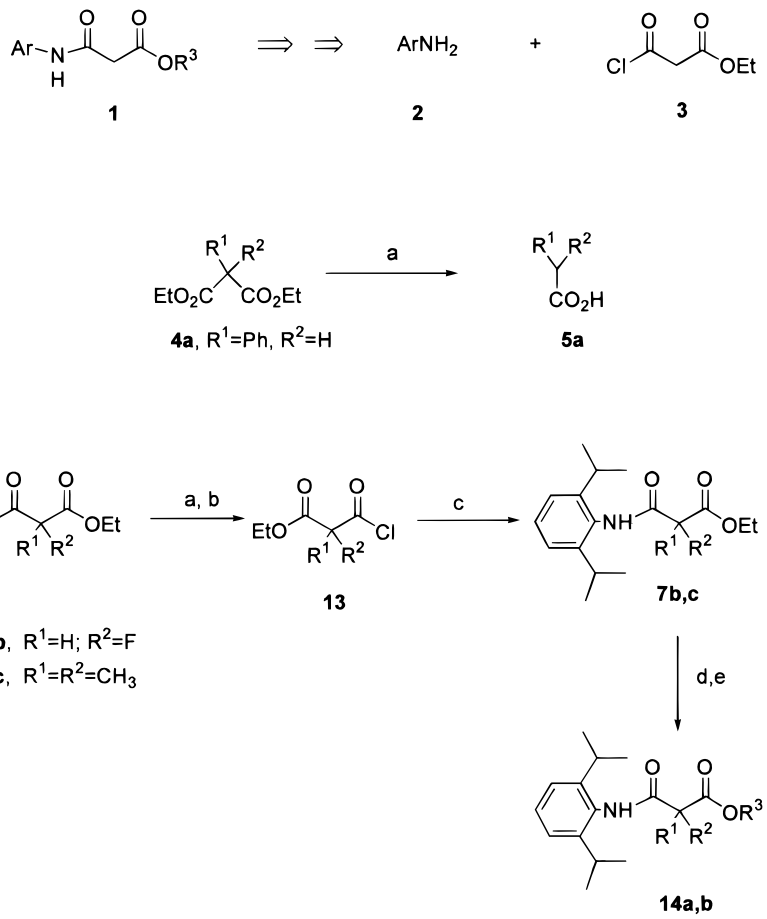
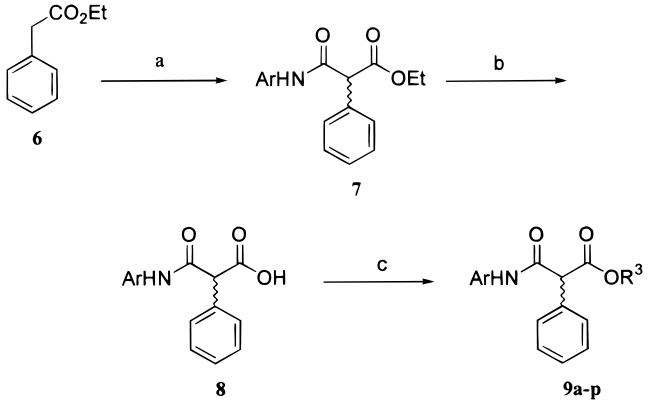
Scheme 1. Method A[a]



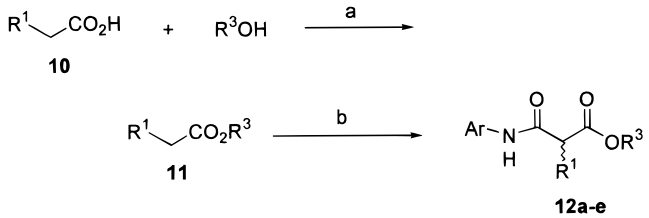
[a] Reagents: (a) KOH, EtOH, then 1 M HCl; (b) C_6H_4-1,2-$(COCl)_2$; (c) 2,6-diisopropylphenyl aniline, Et_3N, CH_2Cl_2; (d) NaOH, MeOH then 1 M HCl; (e) DCC, CH_2Cl_2, R^3OH.

Scheme 2. Method B[a]



[a] Reagents: (a) LDA, ArNCO; (b) NaOH, MeOH/THF then 1 M HCl; (c) DCC, CH_2Cl_2, R^3OH.

Scheme 3. Method C[a]

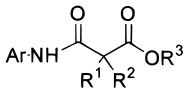


[a] Reagents: (a) DCC, CH_2Cl_2, R^3OH; (b) LDA, ArNCO.

selected compounds were also evaluated in a cellular assay that measured the ability of the compounds to inhibit ACAT in a cell type found in atherosclerotic lesions.[7] Acute in vivo activity (APCC) was measured in rats (male Sprague−Dawley strain, 200−225 g, Charles River, Portage, MI) dosed with the test compound orally as suspensions in water containing carboxymethylcellulose (1.5%) and Tween-20 (0.2%). After dosing, the ability of the compound to prevent the rise in plasma total cholesterol (TC) after the consumption of a high-fat, high-cholesterol meal [known as the PCC diet and consisting of peanut oil (PO) (5.5%), cholesterol (C) (1.5%), cholic acid (CA) (0.5%)] was assessed. The TC concentration was then measured and reported as a percent change from controls.[7] This same diet was also used in a chronic screen (PCC), with the rats receiving both drug and diet simultaneously for 7 days.[8] In a second chronic model (CPCC), hypercholesterolemia was first established (1 week, PCC diet) and then the compounds were coadministered with diet for 1 week. Efficacy in this model is defined as (1) the ability to reduce non-HDL-C (high-density lipoprotein-cholesterol), defined as the amount of cholesterol in apo B containing lipoproteins, and (2) also the ability to elevate the diet-induced low levels of HDL-C.[7]

Efficacy in cholesterol-fed dogs (CDOG) [female Beagles, 6−9 kg (Marshall Labs, Northrose, NY)] was also assessed. Oral dosing (capsule) of the test substance was begun when the plasma TC concentration

Table 1. Physical and Biological Properties of the α-Substituted Malonester Amides



compd no.	Ar	R^1	R^2	R^3	method	formula[b]	mp (°C)	in vitro[c] IC_{50} (μM)	acute in vivo (%ΔTC)[d] 30 mg/kg	3 mg/kg
7a	2,6-(*i*-Pr)$_2$Ph	Ph	H	CH_2CH_3	B	$C_{23}H_{29}NO_3$	175−177	>5	−23	
7b	2,6-(*i*-Pr)$_2$Ph	F	H	CH_2CH_3	A	$C_{17}H_{24}FNO_3$	116−119	>1	1	
7c	2,6-(*i*-Pr)$_2$Ph	CH_3	CH_3	CH_2CH_3	A	$C_{19}H_{29}NO_3$	126−128	>1	−24**	
9a	2,6-(*i*-Pr)$_2$Ph	Ph	H	$(CH_2)_2CH_3$	B	$C_{24}H_{31}NO_3$[e]	148−150	>1	−20*	
9b	2,6-(*i*-Pr)$_2$Ph	Ph	H	$(CH_2)_6CH_3$	B	$C_{28}H_{39}NO_3$	108−110	0.050	−71***	−47***
9c	2,6-(*i*-Pr)$_2$Ph	Ph	H	$(CH_2)_7CH_3$	B	$C_{29}H_{41}NO_3$	120−122	0.013	−56***	−46**
9d	2,6-(*i*-Pr)$_2$Ph	Ph	H	$(CH_2)_8CH_3$	B	$C_{30}H_{43}NO_3$	124−126	0.012	−67***	−53***
9e	2,6-(*i*-Pr)$_2$Ph	Ph	H	$(CH_2)_9CH_3$	B	$C_{31}H_{45}NO_3$	104−105	0.013	−58***	−27***
9f	2,6-(*i*-Pr)$_2$Ph	Ph	H	$(CH_2)_{11}CH_3$	B	$C_{33}H_{49}NO_3$	102−104	0.010	−63***	−29**
9g	2,6-(*i*-Pr)$_2$Ph	Ph	H	$(CH_2)_{13}CH_3$	B	$C_{35}H_{53}NO_3$	99−101	0.016	−49***	
9h	2,6-(*i*-Pr)$_2$Ph	Ph	H	$(CH_2)_{15}CH_3$	B	$C_{37}H_{57}NO_3$	89−91	0.037	−26	
9i	2,6-(*i*-Pr)$_2$Ph	Ph	H	$(CH_2)_{17}CH_3$	B	$C_{39}H_{61}NO_3$	78−81	0.200	−25	
9j	2,6-(*i*-Pr)$_2$Ph	Ph	H	$CH(CH_3)(CH_2)_4CH_3$	B	$C_{28}H_{39}NO_3$	104−105	0.040	−67***	−61***
9k	2,6-(*i*-Pr)$_2$Ph	Ph	H	$CH(CH_3)(CH_2)_9CH_3$	B	$C_{33}H_{49}NO_3$	102−104	0.013	−37***	
9l	2,6-(*i*-Pr)$_2$Ph	Ph	H	$CH(CH_3)(CH_2)_{11}CH_3$	B	$C_{35}H_{53}NO_3$	94−97	0.022	−61***	−46***
9m	2,4,6-(OMe)$_3$Ph	Ph	H	$CH(CH_3)(CH_2)_{11}CH_3$	B	$C_{32}H_{47}NO_6$[f]	84−86	0.030	−59***	−48***
9n	2,6-(*i*-Pr)$_2$Ph	Ph	H	$C(CH_3)_2(CH_2)_3CH_3$	B	$C_{28}H_{39}NO_3$[g]	79−82	0.011	−72***	−65***
9o	2,6-(*i*-Pr)$_2$Ph	Ph	H	$C(CH_3)_2(CH_2)_{11}CH_3$	B	$C_{36}H_{55}NO_3$[h]	89−91	0.012	−26*	
9p	2,6-(*i*-Pr)$_2$Ph	Ph	H	$C(CH_3)_2(CH_2)_{13}CH_3$	B	$C_{37}H_{57}NO_3$[i]	98−101	0.072	−37*	
12a	2,4,6-(OMe)$_3$Ph	Ph	H	$(CH_2)_{11}CH_3$	C	$C_{30}H_{43}NO_6$	93−95	0.013	−51***	−51***
12b	2,4,6-*i*-Pr)$_3$Ph	Ph	H	$(CH_2)_{11}CH_3$	C	$C_{36}H_{55}NO_3$	124−126	0.056	−21**	
12c	2,6-(*i*-Pr)$_2$Ph	2-Py[a]	H	$(CH_2)_{11}CH_3$	C	$C_{32}H_{48}N_2O_3$	44−47	0.008	−43**	
12d	2,4,6-(*i*-Pr)$_3$Ph	2-Py[a]	H	$(CH_2)_{11}CH_3$	C	$C_{35}H_{54}N_2O_3$[j]	97−99	0.180	−9	
12e	2,4,6-(OMe)$_3$Ph	Ph	H	$C(CH_3)_2(CH_2)_{11}CH_3$	C	$C_{33}H_{49}NO_6$	52−54	0.020	NT	
14a	2,6-(*i*-Pr)$_2$Ph	F	H	$(CH_2)_{11}CH_3$	A	$C_{27}H_{44}FNO_3$	oil	0.032	−36**	
14b	2,6-(*i*-Pr)$_2$Ph	CH_3	CH_3	$CH(CH_3)(CH_2)_{11}CH_3$	A	$C_{31}H_{53}NO_3$[k]	oil	0.021	NT	

[a] 2-Pyridyl. [b] C, H, N analysis within 0.4% of theoretical values unless otherwise noted. [c] In vitro ACAT inhibition determined in rat liver microsomes. [d] Denotes percent change in total cholesterol (TC) in cholic acid (0.5%)−cholesterol (1.5%)−peanut oil (5.5%)−fed rats (APCC screen). See ref 7 for complete protocol. Significantly different from control using the unpaired Student's two-tailed *t*-test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. NT = not tested. [e] C: calcd, 75.56; found, 76.88. N: calcd, 3.67; found, 4.14. [f] N: calcd, 2.59; found, 3.41. [g] N: calcd, 3.20; found, 4.52. [h] N: calcd, 2.55; found, 2.99. [i] N: calcd, 2.48; found, 3.32. [j] C: calcd, 75.09; found, 74.63. [k] C: calcd, 76.33; found, 75.64.

plateaued at 300 mg/dL after initiating a PCC diet (same composition as described above for the rats). This is about 2-fold higher than the levels found in chow-fed dogs. Efficacy was expressed as the percent change in plasma TC before and after treatment.[6,7]

Oral bioavailability was assessed using two methods. Rabbits (Male New Zealand Whites, 1.2−1.5 kg, Kuipers Farms, Gary, IN) were dosed (in the daily meal containing 3% peanut oil and 3% coconut oil) with the test compound at 25 and 50 mg/kg. Blood samples were obtained predose and at intervals up to 24 h. The corresponding plasma samples were assayed, following solvent extraction, by an HPLC−UV assay. A similar assay was developed for chow-fed dogs. The limit of quantitation for both assays was 20 ng/mL. A rabbit bioassay (ABIO) assessed oral bioactivity by incubating extracts of plasma, from animals dosed with the inhibitors, with rat liver microsomes. The ability of the extract to inhibit the ACAT reaction was assessed, and the data are expressed as relative percent inhibition.[9] Adrenal toxicity was evaluated in both chow-fed guinea pigs (male Hartley strain, 500−600 g, Charles River, Portage, MI) and dogs. For guinea pigs, the drugs were dissolved or suspended in oleic acid to facilitate absorption and dosed at 100 mg/kg for 2 weeks. Toxicity was defined by the incidence, severity, and complexity of adrenal histopathologic alterations in the zona fasciculata.[10] For dogs, the drug was administered as bulk drug in capsule at a dose of 100 mg/kg for 2 weeks.[11]

Results and Discussion

Structure−Activity Relationships. In previous studies with a series of fatty acid anilides[8] or the unsubstituted malonester amides,[4] the retention of significant ACAT inhibitory activity depended upon the incorporation of the 2,6-diisopropylphenyl or 2,4,6-trimethoxyphenyl substitution pattern into the anilide moiety. Thus, both of these substitution patterns (and the 2,4,6-triisopropylphenyl pattern) were used during the course of this study and it was shown that all three anilides gave compounds that were potent inhibitors of ACAT activity in vitro (Table 1). For example, compounds **9f**, **9l**, **9m**, and **12a** showed comparable potency in vitro and equivalent efficacy in the acute in vivo model. However, compound **12b**, although of comparable potency, was much less efficacious in vivo, probably due to its very high lipophilicity.

Like the unsubstituted malonester amides,[4] varying the chain length of the alkyl esters, in general, led to increased in vitro potency and in vivo efficacy. In the unsubstituted malonester amides, the optimum chain length was C_{12} (dodecyl); however, in the α-substituted series the optimal length was any length between C_8 (octyl, **9c**) and C_{14} (tetradecyl, **9g**). All of these compounds (**9c**−**g**) showed both equivalent potency at inhibiting ACAT activity in vitro and equivalent efficacy in vivo at 30 mg/kg. Potency differences were noted in vivo, since at the lower dose of 3 mg/kg, the more

Table 2. Efficacy Data for Selected Compounds in the Chronic Cholesterol-Fed Rat Model[a]

compd no.	dose (mg/kg)	%Δ non HDL-C	%Δ HDL-C
9b	10	−21	2
9e	10	21	−5
9f	30	−80***	148***
9g	30	−57**	28
9j	10	−18	−1
9k	30	−64**	97***
9l	30	−75***	215***
9m	30	−85***	272***
12a	30	−71*	127**

[a] For experimental details, see ref 6. Significantly different from control using the unpaired two-tailed *t*-test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Table 3. Biological Activity Summary for Compound **9f**

test	**9f**
LAI (IC_{50}, μM)	0.01
MAI2 (IC_{50}, μM)	0.02
APCC (%Δ TC)[a,c]	-63^{30}, -29^{3}
PCC-OA (%Δ non HDL-C)[a,c]	-54^{1} (+29% Δ HDL)
PCC-CMC (%Δ non HDL-C)[a,c]	-54^{1} (+23% Δ HDL)
CPCC (%Δ non HDL-C)[a,c]	-80^{30} (+148% Δ HDL)
CDOG (%Δ TC)[a,c]	-43^{10}
plasma levels (ng/mL)[a,b]	$69-131^{25}$, $75-140^{50}$
ABIO (% inhibition)[a,b]	99^{25}, 100^{50}

[a] Dose is superscript (mg/kg). [b] Determined in rabbits. [c] Unless otherwise noted, the changes were significantly different from control, $p < 0.05$ using analysis of variance followed by Fisher's multiple range test.

lipophilic compounds **9f** and **9g** were less effective. Although not as potent as compounds **9c**−**g**, the heptyl ester (**9b**) was the most effective compound in the acute in vivo screen. Compounds outside of this range (C_7−C_{14}) were less active both in vitro and especially in vivo [e.g. C_3 (**9a**), C_{16} (**9h**), and C_{18} (**9j**)]. Branching the alkyl chain gave equivocal results. By comparing compounds with the same number of carbon atoms in the alkyl ester moiety, it was possible to show both an advantage for branching (compare **9g** to **9l**) and a disadvantage for branching (compare **9f** to **9k**). Addition of another methyl group, as in **9o**, at the branchpoint of **9l** had a detrimental effect on in vivo activity. Interestingly, the 2-methyl-2-hexyl (**9n**) and 2-heptyl (**9j**) esters, like the 1-heptyl ester (**9b**), were among the most effective agents both in vitro and in vivo.

Replacing the α-phenyl in **9f** by the α-2-pyridyl (as in **12c**) or the α-fluoro (as in **14a**) led to significant decreases in in vivo activity, despite potent in vitro activity.

Several of the more active compounds in Table 1 were then evaluated in the clinically relevant, chronic cholesterol-fed rat model of preestablished hypercholesterolemia (Table 2). In this model, the compounds with the shorter alkyl ester chains (**9b** and **9e**) were not as effective as initially observed in the acute in vivo screen. This observation was also noted for compound **9j**, a compound previously shown to possess a desirable profile both in vitro and in in vivo. The lack of correlation between the acute and chronic in vivo screens cannot be explained at this time, although it may relate to the ability of the compounds to reach the liver and prevent the production of cholesteryl ester-rich lipoproteins. The best activity in the chronic model was observed with alkyl side chains longer than 10 carbon atoms. Compound **9f** was very effective, giving a 80% decrease in plasma non-HDL-C and a 148% increase in HDL-C. The 2,4,6-trimethoxyphenyl analogue (**12a**) was comparable to **9f**. Compound **9g**, with the longer tetradecyl side chain, had little effect on increasing plasma HDL levels. In contrast to compound **9g**, compounds with 14 carbon atoms that were branched, showed good efficacy. Thus, both compounds **9l** and **9m** lowered plasma non-HDL-C by 75% and 85%, respectively, and elevated HDL-C by 215% and 272%, respectively. These compounds suffered from the significant drawback of existing as a pair of inseparable diastereomers.

After a consideration of all the biological and chemical data, compound **9f** was chosen as a model compound for further pharmacological evaluation.

As shown in Table 3, compound **9f** is a potent ACAT inhibitor in both the microsomal LAI assay ($IC_{50} = 0.01$ μM) and in the cellular MAI2 assay ($IC_{50} = 0.02$ μM). This latter observation is important in that it shows that the compound can cross cell membranes and inhibit ACAT activity in a cell type found in atherosclerotic lesions. In addition to the in vivo activities described previously, compound **9f** was evaluated in a number of other cholesterol-fed animal models. In the chronic version (PCC) of the APCC screen, it lowers plasma non-HDL-C to the same extent irrespective of whether an oleic acid vehicle (PCC-OA) or an aqueous vehicle is employed (PCC-CMC). This is a very important point since, typically, such lipophilic inhibitors only show efficacy when dosed in an oil vehicle.[12] In addition, the compound also produced cholesterol lowering (43% at 10 mg/kg) in the cholesterol-fed dog model. This model reflects the situation faced in the clinic, where bulk drug is dosed in soft gelatin capsules. No emesis was noted in this study.

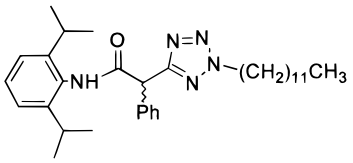
It was also determined if plasma drug levels capable of inhibiting arterial ACAT can be achieved on oral dosing. This was measured using two methods. The first was a direct measurement, by an HPLC−UV assay, of plasma levels achieved in the rabbit after oral dosing. At doses of 25 and 50 mg/kg, plasma levels of 69−131 and 75−140 ng/mL were achieved, respectively. On the basis of macrophage ACAT inhibition (MAI2) results (IC_{50} = 20 nM at 10 ng/mL), there is more than sufficient drug to inhibit arterial macrophage ACAT. In addition, in the bioassay (ABIO) there was almost complete inhibition of ACAT in this assay at the doses tested (25 and 50 mg/kg), thus suggesting the presence of active drug (or active metabolite) in the plasma.

Adrenotoxic Comparison with the α-Substituted Tetrazole Amides. Compound **9f** is a potent inhibitor of ACAT and a potent hypocholesterolemic agent in two rodent models of hypercholesterolemia, both acutely and chronically. We also showed that this compound inhibited cholesterol esterification in murine IC-21 macrophages and was bioactive in rabbits. The major hurdle facing bioavailable ACAT inhibitors is adrenal toxicity. We have shown that compound **9f** is bioavailable and capable of inhibiting ACAT in the tissue of interest (macrophage in the artery wall); we must now determine that it is possible to have a beneficial effect in one peripheral tissue (artery wall) without having a nonbeneficial effect in another peripheral tissue (adrenal gland).

The key issue requiring resolution is whether the adrenal toxicity observed with a number of ACAT inhibitors is due to inhibition of cholesterol esterification in that tissue (i.e., mechanism-based) or is unrelated to enzyme inhibition. Some evidence suggests that the adrenal toxicity observed is unrelated to ACAT inhibition. The first report of toxicity, in beagle dogs, with an ACAT inhibitor (PD 132301-2) revealed the now characteristic adrenal lesion consisting of adrenocortical degeneration with necrosis in the zona fasciculata and reticularis.[13] Further mechanistic studies in guinea pig adrenocortical cells in culture, however, suggested that the observed cytotoxicity was not a result of ACAT inhibition but of ATP depletion resulting from direct inhibition of mitochondrial respiration.[14] Shortly thereafter, reports of adrenal toxicity with other potent bioavailable ACAT inhibitors appeared in the literature. Thus, RP 73163 given to guinea pigs at doses of 100, 300, and 1000 mg/kg for 3 weeks produced dose-related histopathologic changes in the adrenal cortex consisting of diffuse coarse vacuolation of zona fasciculata cells similar to that observed in dogs with PD 132301-2. These authors also concluded that the adrenal toxicity was not related to ACAT inhibition, since the pharmacologically inactive enantiomer of RP 73163 also caused similar toxic effects.[15] Further mechanistic studies suggested that both RP 73163 and its enantiomer were metabolized to a common product which was an inhibitor of adrenal cholesterol 17α-reductase activity. The authors speculated that this inhibition of cholesterol 17α-reductase activity may be the source of the coarse vacuolation produced by RP 73163, although ACAT inhibition could not be ruled out.[16] It was recently disclosed that FR 145237, a potent ACAT inhibitor possessing direct antiatherosclerotic activity at the artery wall,[17] also produced adrenal toxicity in normal rabbits after a single iv dose.[18] Surprisingly, FR 145237 did not induce adrenal toxicity in low-density lipoprotein receptor deficient WHHL rabbits, despite producing adrenal drug concentrations equivalent to those found in the normal rabbits. These authors speculated that, because of the structural similarity between FR 145237 and PD 132301-2, the toxicity induced by FR 145237 may not be due to ACAT inhibition but to inhibition of mitochondrial respiration. Another study in rabbits demonstrated severe cellular damage with XP767 at doses between 10 and 100 mg/kg; however, a structurally related analogue, XR920, produced no adrenal changes, despite having similar in vitro potency to XP767.[19] Long-term incubations of potent ACAT inhibitors (SaH 58-035 and CP-113,818) with cholesterol-enriched mouse peritoneal macrophages led the authors to conclude that ACAT inhibition in these cells increased cell toxicity due to the build-up of intracellular free cholesterol concentrations. This was supported by the observations that cell toxicity paralleled the increase in intracellular free cholesterol concentrations and that removal of free cholesterol by the addition of extracellular cholesterol "acceptors" or by blocking intracellular sterol transport relieved the ACAT inhibitor-induced toxicity. Thus, it was proposed that ACAT inhibition in vivo could induce cell death by destabilization of the plasma membrane upon cholesterol enrichment, unless sufficient cholesterol acceptors were present.[20]

Despite these findings of adrenal toxicity with systemically available ACAT inhibitors, the recent results in ACAT-deficient transgenic mice would tend to support the position that the observed toxicity is not related to ACAT inhibition, since these animals developed normally and had no evidence of adrenal dysfunction although their adrenal cholesteryl esters were markedly reduced.[21]

The adrenal glands of guinea pigs given compound **9f** at doses of 10−100 mg/kg had minimal to mild increases in coarse vacuolation and reduced fine vacuolation of zona fasciculata cortical cells. There was no evidence of adrenal cortical necrosis at any dose. The beagle dog has been reported to be the most sensitive species for evaluating the adrenal effects of ACAT inhibitors.[22] Thus, compound **9f** was evaluated in this model and compared with the tetrazole amide **15**, a



15

potent bioavailable ACAT inhibitor previously shown to be nontoxic to the adrenal gland in guinea pigs.[9] Adrenal toxicity was observed in dogs given compound **9f** at 100 mg/kg for 3 weeks. This toxicity was characterized by zonal degeneration and necrosis of zona fasciculata cortical cells. There was also less widespread degeneration and necrosis of zona reticularis cortical cells. These changes often resulted in substantial thinning and/or near complete collapse of these zones. The inner portion of the zona reticularis at the corticomedullary junction persisted. Collapsed stroma and minor infiltrates of small mononuclear inflammatory cells remained in regions of substantial cortical loss. These changes were not observed in dogs administered compound **15** at the same dose for the same duration of treatment (Figure 1). Plasma drug blood levels for compounds **9f** and **15** were determined by HPLC. Both compounds achieved blood levels far in excess of the IC_{50} necessary to inhibit macrophage ACAT. These levels were highly variable and entirely consistent with the very lipophilic nature of the compounds. For compounds **9f** and **15**, blood levels of 200−690 and 240−310 ng/mL were achieved, respectively. Thus, even though both compounds achieved blood levels adequate to inhibit ACAT in the periphery, only one compound (**9f**) was shown to be adrenotoxic.

Thus, we have demonstrated in the most sensitive species (dog) that it is possible to identify potent ACAT inhibitors, such as **15**, that are bioavailable and not adrenotoxic.

Conclusions

We have identified a series of α-substituted malonester amides that were shown to be potent ACAT inhibitors in vitro and hypocholesterolemic agents in vivo. Compound **9f** was shown to be a potent inhibitor of both microsomal and cellular ACAT and was particularly effective in a rodent model of preestablished hypercholesterolemia. Despite its lipophilicity, com-

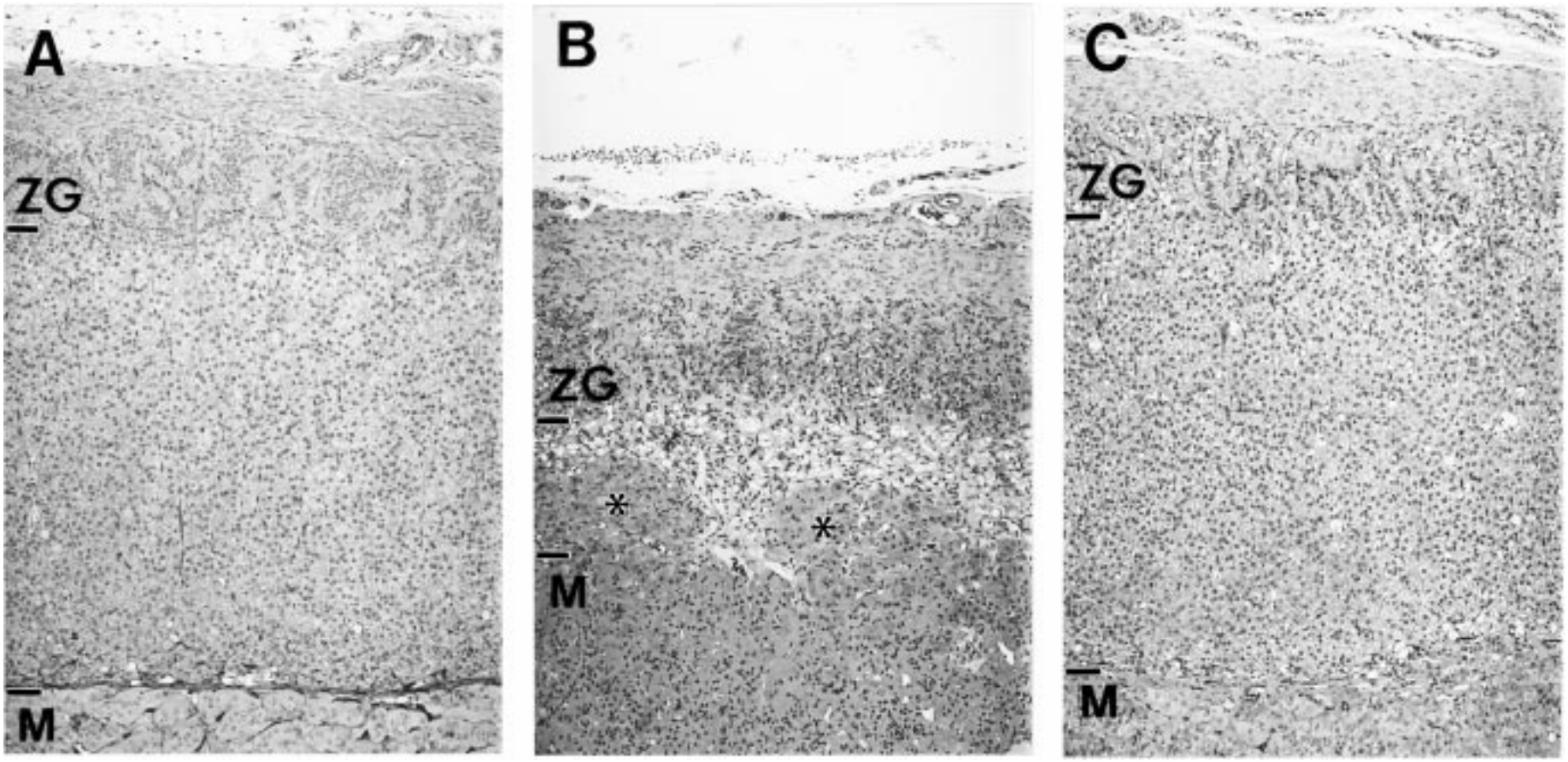


Figure 1. Adrenotoxic comparison between compounds **9f** and **15**: Adrenal cortex from an untreated dog (A) and dogs given compounds **9f** (B) and **15** (C) at 100 mg/kg for 2 weeks. Notice the collapse of the zona fasciculata and reticularis (between hash marks) in the **9f**-treated dog (B). Collapsed stroma and islands of cortical cells (asterisks) remain in this region. The adrenal cortex of the compound **15**-treated dog is (C) comparable to the untreated dog. Zona glomerulosa (ZG) and adrenal medulla (M) are indicated. Normal and collapsed zona fasciculata/reticularis is shown as an area between side hash marks. Hematoxylin and Eosin stain. Magnification of A, B, and C = 95×.

pound **9f**, was readily absorbed and plasma levels, capable of inhibiting ACAT in the periphery, were easily attained. This compound was then used as a pharmacological tool to define the relationship between adrenal toxicity and ACAT inhibition. Consequently, it was shown to be toxic to the adrenal gland of beagle dogs. In the same study, compound **15**, a structurally unrelated potent ACAT inhibitor, achieved similar plasma drug levels and was shown to be nontoxic to the adrenal glands. This finding would tend to support the argument that adrenal toxicity is unrelated to inhibition of ACAT activity.

Experimental Section

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. All organic extracts were dried over $MgSO_4$ except when otherwise noted. Column chromatography was performed on Merck silica gel 60 (230−400) mesh. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra were determined on either a Bruker 250 MHz, Varian XL 300 MHz, or Varian Unity 400 MHz instrument. Chemical shifts (δ) are expressed as parts per million (ppm) downfield from internal tetramethylsilane. Elemental analyses for carbon, hydrogen, and nitrogen were performed on a Perkin-Elmer 240C elemental analyzer and are within 0.4% of theory unless noted otherwise. HPLC analyses of heparinized dog plasma samples were analyzed with a Waters 600E HPLC equipped with a 717 plus autosampler and 490E programmable multiwavelength detector. The column used was a reverse phase Altima C_{18} 5 μM column. The mobile phase used was 100% acetonitrile at a flow rate of 1.0 mL/min with a detection wavelength of 214 nM.

General Procedure for Preparing α-Substituted Malonester amides. Method B: Synthesis of (±)-Benzeneacetic Acid, α-[[[2,6-Bis(1-methylethyl)phenyl]amino]carbonyl]-, Dodecyl Ester (9f). (a) (±)-Benzeneacetic Acid, α-[[[2,6-Bis(1-methylethyl)phenyl]amino]carbonyl]-, Ethyl Ester (7a). To a THF solution (40 mL) of diisopropylamine (22.53 mL, 0.16 mol) at −40 °C under nitrogen with stirring was added *n*-BuLi (100.5 mL, 1.6 M in hexanes, 0.1607 mol). After 10 min, the resulting LDA solution was cooled to −78 °C and a solution of ethyl phenyl acetate (**6**, 24 g, 0.146 mol) in THF (150 mL) was added. A yellow suspension resulted that was stirred at −78 °C for an additional 30 min. To this was then added a THF (80 mL) solution of 2,6-diisopropylphenyl isocyanate (31.24 g, 0.146 mol) dropwise. The resulting mixture was stirred at −78 °C for 3 h before quenching with 1 N HCl (120 mL). Ethyl acetate (300 mL) was added and the organic layer separated. This was washed with water (2 × 200 mL), brine (200 mL), and dried. Filtration, concentration in vacuo, and recrystallization from ethyl acetate/hexane gave **7a** (42.25 g, 78%) as a white solid: mp 175−177 °C, ^{1}H NMR ($CDCl_3$) δ 8.0 (s, 1H), 7.05−7.5 (m, 8H), 4.75 (s, 1H), 4.2−4.4 (m, 2H), 2.85 (br m, 2H), 1.3 (tr, 3H), 1.09 (s, 6H), 1.06 (s, 6H) ppm. Anal. ($C_{23}H_{29}NO_3$) C, H, N.

(b) (±)-Benzeneacetic Acid, α-[[[2,6-Bis(1-methylethyl)phenyl]amino]carbonyl] (8, Ar = 2,6-diisopropylphenyl). To a methanol (750 mL)/THF (100 mL) solution of **7a** (33.55 g, 0.0913 mol) was added 1 N aqueous NaOH (91.3 mL, 0.0913 mol). The resulting solution was stirred overnight at room temperature. It was then concentrated in vacuo and the resulting residue was redissolved in water (500 mL). This was then washed with ethyl ether (2 × 200 mL) and the aqueous layer was cooled in ice water and acidified with 1 N HCl. A white solid precipitated, which was filtered and air-dried to yield **8** (Ar = 2,6-diisopropylphenyl) (32.12 g, 82%) as a white solid: mp 201−203 °C; ^{1}H NMR ($CDCl_3$) δ 8.7 (s, 1H), 7.0−7.5 (m, 8H), 4.60 (s, 1H), 2.75 (br m, 2H), 1.09 (s, 6H), 1.06 (s, 6H) ppm. Anal. ($C_{21}H_{25}NO_3 \cdot 0.5\ H_2O$) C, H, N.

(c) (±)-Benzeneacetic Acid, α-[[[2,6-Bis(1-methylethyl)phenyl]amino]carbonyl]-, Dodecyl Ester (9f). To a dichloromethane solution (1.3 L) of **8** (40 g, 0.1178 mol) was added 1-dodecanol (21.96 g, 0.1178 mol) at 0 °C under nitrogen with stirring. To this was added dicyclohexylcarbodiimide (25.53 g, 0.124 mol). An immediate precipitate resulted and the resulting suspension was allowed to warm to room-temperature overnight. This was then filtered and the solution was concentrated in vacuo. The residue was flash chromatographed (eluting with a gradient of 10−25% ethyl acetate/hexane) to give 44.97 g (75%) of **9f** as a white solid: mp 102−104 °C; ^{1}H NMR ($CDCl_3$) δ 8.0 (s, 1H), 7.5 (d, 2H), 7.4 (m, 3H), 7.3 (tr, 1H), 7.1 (d, 2H), 4.70 (s, 1H), 4.2 (m, 2H), 2.90 (br m, 2H), 1.7 (m, 2H), 1.3 (s, 18H), 1.05 (s, 12H), 0.95 (tr, 3H) ppm. Anal. ($C_{33}H_{49}NO_3$) C, H, N.

Compounds **9a**−**e** and **9g**−**p** were synthesized according to the procedures described in method B above and exemplified for compound **9f**; their physical and biological properties are listed in Table 1.

Alternative Procedure for Preparing selected α-Substituted Malonester Amides. Method C: Synthesis of (±)-Benzeneacetic Acid, α-[[(2,4,6-Trimethoxyphenyl)amino]carbonyl]-1,1-dimethyltridecyl Ester (12e). Benzeneacetic Acid, 1,1-Dimethyltridecyl Ester (11, R^1 = Ph, R^3 = $C(CH_3)_2(CH_2)_{11}CH_3$). To a dichloromethane (100 mL) solution of phenylacetic acid (3.5 g, 0.0213 mol) at 0 °C under an inert nitrogen atmosphere with stirring was added 2-methyl-2-tetradecanol (4.87 g, 0.0213 mol). After 10 min, dicyclohexylcarbodiimide (4.84 g, 0.0234 mol) was added. An immediate precipitate resulted and the resulting suspension was allowed to warm to room-temperature overnight. This was then filtered and the solution was concentrated in vacuo. The residue was flash chromatographed (eluting with 5% ethyl acetate/hexane) to give 2.0 g (27%) of the title compound as a low melting solid: ^{1}H NMR ($CDCl_3$) δ 7.2−7.3 (m, 5H), 3.51 (s, 2H), 1.7 (m, 2H), 1.4 (s, 6H), 1.2 (m, 20H), 0.9 (tr, 3H) ppm. Anal. ($C_{23}H_{38}O_2$) C, H, N.

(±)-Benzeneacetic Acid, α-[[(2,4,6-Trimethoxyphenyl)amino]carbonyl]-1,1-dimethyltridecyl Ester (12e). To a THF solution (20 mL) of diisopropylamine (1.01 mL, 0.0072 mol) at −40 °C under an inert nitrogen atmosphere with stirring was added *n*-BuLi (3.6 mL, 2.01 M in hexanes, 0.0072 mol). After 15 min, a THF solution (10 mL) of benzeneacetic acid, 1,1-dimethyltridecyl ester (1.5 g, 0.00656 mol), was added dropwise. This mixture was cooled to −78 °C for 30 min and a THF solution (10 mL) of 2,4,6-trimethoxyphenyl isocyanate[23] (1.37 g, 0.00656 mol) was added dropwise. After stirring for 3 h at −78 °C, the reaction was quenched by the addition of 1 N HCl (10 mL). The solution was warmed to room temperature and partitioned between ethyl acetate and water. The organic layer was separated, washed with water (1 × 100 mL) and brine (1 × 100 mL), and dried. After filtration and concentration, flash chromatography (eluting with a gradient of 25−50% ethyl acetate/hexane) gave 0.87 g (24%) of **12e** as a white solid: mp 52−54 °C; ^{1}H NMR ($CDCl_3$) δ 8.0 (br s, 1H), 7.2−7.7 (m, 5H), 6.1 (s, 6H), 4.6 (s, 1H), 3.8 (s, 9H), 1.7 (m, 2H), 1.4 (s, 6H), 1.2 (m, 20H), 0.9 (tr, 3H) ppm. Anal. ($C_{33}H_{49}NO_6$) C, H, N.

Compounds **12a**−**d** were synthesized according to the procedures described in method C above and exemplified for compound **12e**; their physical and biological properties are listed in Table 1.

Alternative Procedure for Preparing selected α-Substituted Malonester Amides. Method A: Synthesis of (±)-Propanoic Acid, 3-[[2,6-Bis(1-methylethyl)phenyl]amino]-2-fluoro-3-oxo-, Dodecyl Ester (14a). (a) (±)-Propanoic Acid, 3-[[2,6-Bis(1-methylethyl)phenyl]amino]-2-fluoro-3-oxo, Ethyl Ester (7b). Monoethyl fluoromalonate[24] (10.37 g, 0.068 mol) and phthaloyl dichloride (13.84 mL, 0.096 mol) were heated together at 100 °C for 2.5 h. After cooling,

the resulting solid was distilled (70−80 °C/15 mmHg) to yield the corresponding acid chloride (4.43 g, 44%), which was used in the next step without any further purification.

To a dichloromethane solution (120 mL) of 2,6-diisopropylaniline (2.97 mL, 0.0206 mol) and triethylamine (3.6 mL, 0.0257 mol) at 0 °C was added the acid chloride (3.93 g, 0.0257 mol), obtained in the previous step, under an inert nitrogen atmosphere with stirring. The resulting red solution was stirred at 0 °C for 1 h and then quenched by the addition of 1 N HCl (20 mL). The organic layer was separated, washed with water (1 × 100 mL) and brine (1 × 100 mL), and dried. After filtration and concentration, the residue was flash chromatographed (eluting with 10% ethyl acetate/hexane) to yield 3.46 g (55%) of **7b** as a white solid: mp 116−119 °C; ^{1}H NMR ($CDCl_3$) δ 7.58 (br s, 1H), 7.1−7.4 (m, 3H), 5.2 (d, 1H, J_{H-F} = 49 Hz), 4.4 (q, 2H), 3.0 (hept, 2H), 1.4 (tr, 3H), 1.2 (m, 12H) ppm. Anal. ($C_{17}H_{24}FNO_3$) C, H, N.

(b) (±)-Propanoic Acid, 3-[[2,6-Bis(1-methylethyl)-phenyl]amino]-2-fluoro-3-oxo-, Dodecyl Ester (14a). To a methanol (70 mL) solution of **7b** (3.0 g, 0.0097 mol) was added 1 N aqueous NaOH (9.7 mL, 0.0097 mol). The resulting solution was stirred at room temperature for 2 h. It was then concentrated in vacuo and the resulting residue was redissolved in water (50 mL). This was washed with ethyl acetate (2 × 20 mL) and the aqueous layer was cooled in ice water and acidified with 1 N HCl. This was extracted with ethyl acetate and the resulting organic extract was dried. Filtration and concentration gave the corresponding acid (2.5 g, 92%): mp 139−141 °C; ^{1}H NMR ($CDCl_3$) δ 7.90 (br s, 1H), 7.2−7.4 (m, 3H), 5.5 (d, 1H, J_{H-F} = 47 Hz), 2.9 (hept, 2H), 1.2 (m, 12H) ppm. Anal. ($C_{15}H_{20}FNO_3$) C, H, N.

To a dichloromethane solution (100 mL) of the acid obtained above (0.7 g, 0.0025 mol) was added 1-dodecanol (0.56 mL, 0.0025 mol) at 0 °C under nitrogen with stirring. To this was added dicyclohexylcarbodiimide (0.56 g, 0.0027 mol). The resulting suspension was allowed to warm to room temperature overnight. This was then filtered and the filtrate was partitioned between dichloromethane and water. The organic layer was separated, washed with water (1 × 50 mL) and brine (1 × 50 mL), and dried. After filtration and concentration, the residue was flash chromatographed (eluting with a gradient of 10−25% ethyl acetate/hexane) to give 0.44 g (39%) of **14a** as a viscous oil: ^{1}H NMR ($CDCl_3$) δ 7.6 (br s, 1H), 7.2−7.4 (m, 3H), 5.4 (d, 1H, J_{H-F} = 49 Hz), 4.2 (tr, 2H), 3.0 (hept, 2H), 1.7 (m, 2H), 1.3 (m, 30H), 0.9 (tr, 3H) ppm. Anal. ($C_{27}H_{44}FNO_3$) C, H, N. Compounds **7c** and **14b** were synthesized according to the procedures described in method A above and exemplified for compounds **7b** and **14a**; their physical and biological properties are listed in Table 1.

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