3-Amino-5,5-dimethylhexanoic Acid. Synthesis, Resolution, and Effects on Carnitine Acyltransferases

Ahsraf Saeed,† Jeanie B. McMillin,‡ Paul E. Wolkowicz,§ and Wayne J. Brouillette*,†

Departments of Chemistry and Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294, and Department of Pathology, University of Texas Medical School, University of Texas Health Science Center, Houston, Texas 77030

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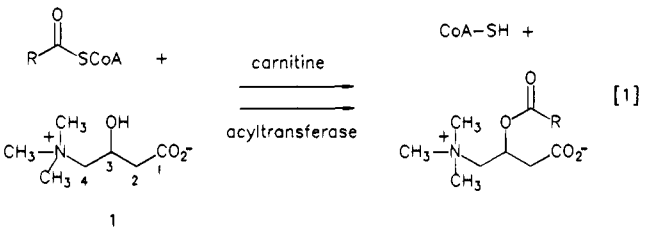
The selective inhibition of individual carnitine acyltransferases may be useful in the therapy of diabetes and heart disease. Aminocarnitine (**3**) is a weak competitive inhibitor (K_i = 4.0 mM) for carnitine acetyltransferase (CAT), although the *N*-acetyl derivative **4** is about 165 times more potent (K_i = 0.024 mM) than **3**. Compound **3** is also a potent competitive inhibitor for carnitine palmitoyltransferases 1 and 2 (CPT-1 and CPT-2) (IC_{50} for CPT-2 = 805 nM). We synthesized 3-amino-5,5-dimethylhexanoic acid (**7**) and its *N*-acetyl derivative (**8**) as isosteric analogs of **3** and **4** that lack the quaternary ammonium positive charge. Like **3** and **4**, compounds **7** and **8** were competitive inhibitors of CAT with significantly different potencies, but in this case, **8** (K_i = 25 mM) was 10 times *less* potent than **7** (K_i = 2.5 mM). *R*-(−)-**7** and *S*-(+)-**7** were stereoselective inhibitors of CAT (K_i = 1.9 and 9.2 mM, respectively). Racemic **7** was a weak competitive inhibitor of CPT-2 (K_i = 20 mM) and had no effect on CPT-1. These results are consistent with differences among the carnitine-binding sites on carnitine acyltransferases that may be useful in selective inhibitor design. Furthermore, the data suggest that the quaternary ammonium positive charge of carnitine may be important for the proper orientation of carnitine and its analogs in the binding site.

Introduction

Carnitine acyltransferases reversibly transfer C_2–$>C_{20}$ acyl groups of acyl-CoAs to the β-hydroxyl group of (*R*)-carnitine (**1**). Enzymes from this class, which differ in kinetic preferences for different chain length acyl-CoAs, are localized in mitochondria, peroxisomes, endoplasmic reticulum, sarcoplasmic reticulum, and the plasma membrane.[1–3] Carnitine palmitoyltransferases



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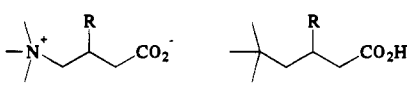
1 and 2 (CPT-1 and CPT-2), which have kinetic preferences for C_{6-8}–C_{18} acyl-CoAs,[2] play an essential role in the mitochondrial β-oxidation of long chain fatty acids.[2,3] In addition, carnitine acyltransferases appear to be important for modulating the cellular acyl-CoA/CoA ratio. For example, carnitine acetyltransferase (CAT), which has kinetic preference for C_2–C_6 acyl-CoAs,[1] has been shown to buffer myocardial acetyl-CoA levels in reconstituted systems.[1,3]

The determination of relative substrate specificities and the development of specific inhibitors for individual carnitine acyltransferases have been of considerable recent interest, since inhibitors of CPT-1 have potential therapeutic applications in the treatment of diabetes[4] or cardiovascular disease.[5]

The most potent inhibitors of carnitine acyltransferases reported to date are fatty acid analogs (such as the coenzyme A esters of 2-tetradecylglycidic acid[6] and etomoxir[7]) or acylcarnitine analogs (such as hemiacetylcarnitinium[8] and hemipalmitoylcarnitinium).[9] Among the simple carnitine analogs thus far described, only aminocarnitine (**3**) and its *N*-acyl derivatives are potent carnitine acyltransferase inhibitors.[10–13] As summarized in Table 1, racemic **3** is a poor substrate and a modest inhibitor of pigeon breast CAT. However, its *N*-acetyl derivative (**4**) is a potent CAT inhibitor with a K_i approximately 12 times lower than the K_m for (*R*)-acetylcarnitine (**2**).[12]



R		
OH	1	5
OAc	2	6
NH_2	3	7
NHAc	4	8

The development of carnitine analogs as probes to determine differences between carnitine-binding site topographies for different carnitine acyltransferases has been of recent interest in our laboratory. We previously described a carnitine analog (**5**) that contains a *tert*-butyl group in place of carnitine's trimethylammonium group.[14,15] Compound **5** is isosteric with carnitine but lacks the quaternary ammonium positive charge. As shown in Table 1, racemic **5** and its *O*-acetyl derivative (**6**) were relatively poor competitive inhibitors of CAT. We hoped to determine whether, as in the case of **2** versus **4**, the binding potency of **6** with CAT could be significantly enhanced through conversion of the *O*-

† Department of Chemistry, University of Alabama at Birmingham.
‡ Department of Pathology, University of Texas Health Science Center.
§ Department of Medicine, University of Alabama at Birmingham.
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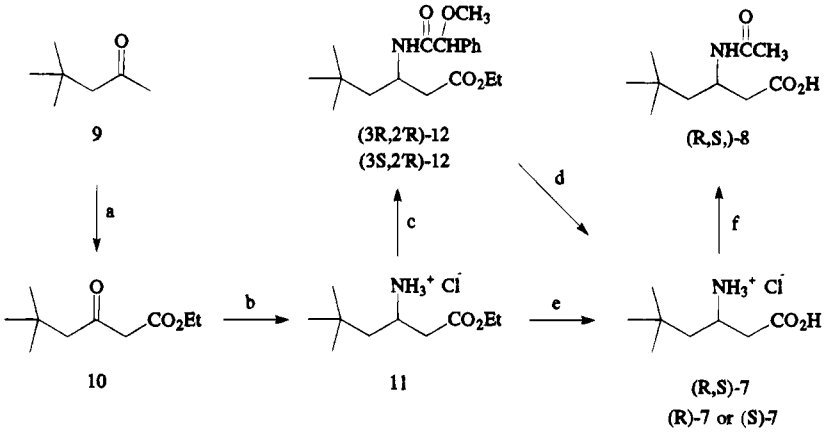
Scheme 1



[a] (a) Reference 14; (b) $NH_4^+ {}^-OAc$, $NaCNBH_3$; (c) (*R*)-(−)-α-methoxyphenylacetic acid, DCC; (d) concentrated HCl; (e) 18% HCl; (f) acetic anhydride, NaOH.

acetyl moiety to an *N*-acetyl group. Here we describe the synthesis, resolution, and enzyme kinetic behavior for the uncharged aminocarnitine analogs **7** and **8**.

Chemistry

The racemic target compounds **7** and **8** were synthesized from 4,4-dimethyl-2-pentanone (**9**) as summarized in Scheme 1. Thus **9** was acylated with NaH and diethyl carbonate, as previously reported[14] for the synthesis of **5**, to give keto ester **10** in 79% yield. Compound **10** underwent reductive amination with ammonium acetate and $NaCNBH_3$ to give amine **11**, which was isolated as the hydrochloride salt in 77% yield. The hydrolysis of **11** in 18% HCl then provided racemic **7** in 98% yield. Compound **7** was deprotonated and reacted with acetic anhydride to provide an 82% yield of racemic **8**.

The resolution of **7**, as described in Scheme 1, was accomplished by reaction of the intermediate amino ester **11** with (*R*)-(−)-α-methoxyphenylacetic acid and DCC to form a mixture of diastereomeric amide derivatives (**12**). These were separated on a flash silica gel column to give pure 3*S*,2'*R*-**12** (91% yield) and 3*R*,2'*R*-**12** (60% yield). The stereochemical assignments were determined from relative chemical shifts of the *tert*-butyl group resonance in the ^1H NMR spectrum, as previously reported[14] for the analogous precursors to the enantiomers of **5**. The direction of the chemical shift difference in amides, however, is opposite to that observed for esters.[16] These were each hydrolyzed in concentrated HCl to give *S*-**7** (60% yield) and *R*-**7** (75% yield), respectively.

Enzyme Kinetics Studies

The results of enzymatic evaluation for **7** and **8** are summarized in Figures 1− 4 and in Table 1. Racemic **7**, each of its enantiomers, and racemic **8** were each evaluated as in vitro inhibitors or alternate substrates for purified pigeon breast CAT. None of these compounds were substrates for CAT at concentrations up to 10 mM. As shown in Figures 1−3, all were competitive inhibitors of CAT as measured against L-carnitine. The K_i for racemic **7** was 2.6 mM. *R*-**7** and *S*-**7** were stereoselective CAT inhibitors with K_i's of 1.9 and 9.2 mM, respectively. The racemic *N*-acetyl derivative **8** (K_i = 25 mM) was a much poorer competitive inhibitor of CAT than **7**.

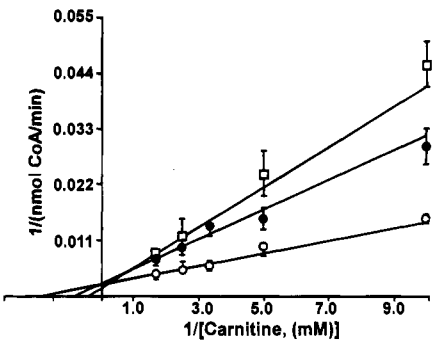


Figure 1. Competitive inhibition of pigeon breast CAT by racemic **7** at 0.00 (○), 5.00 (●), and 10.00 (□) mM final concentrations. Each data point is the mean from two to five separate determinations.

Table 1. Kinetic Constants for Carnitine and Synthetic Analogs with Selected Carnitine Acyltransferases

	pigeon breast CAT		rat liver CPT	neonatal rat cardiac myocyte CPT K_i, mM		
compd	K_m, mM	K_i, mM	inhibition, %	CPT-1	CPT-2	ref
R-**1**	0.3	–	–	0.1	0.1	12
R-**2**	0.3	–	–	–	–	12
R,S-**3**	3.8	4.0	64 (5 μM)	–	–	12
R,S-**4**	–	0.024	15 (5 μM)	–	–	12
R,S-**5**	–	8.3		3.6	2.8	15
S-(−)-**5**	–	7.5	–	1.4	2.2	15
R-(+)-**5**	–	20.3	–	no effect	6.7	15
R,S-**6**	–	4.1	–	–	–	15
R,S-**7**	–	2.6	–	no effect	20	
R-(−)-**7**	–	1.9	–	–	–	
S-(+)-**7**	–	9.2	–	–	–	
R,S-**8**	–	25	–	–	–	

In contrast to its effects on CAT, racemic **7** did not affect CPT-1 in concentrations up to 5 mM, and it was a weak competitive inhibitor of CPT-2 (K_i = 20 mM; see Figure 4).

Discussion

The K_m values for L-carnitine [(*R*)-(−)-carnitine; **1**] and its *O*-acetyl derivative (**2**) reveal that they bind to pigeon breast CAT with comparable affinity.[17,18] The carnitine analog, D,L-aminocarnitine (**3**), is a poor substrate and weak competitive inhibitor of CAT. The K_m and K_i values of 3.8 and 4.0 mM, respectively, are about 13-fold higher than the K_m for L-carnitine.[12] In contrast, D,L-(acetylamino)carnitine (**4**), while not a

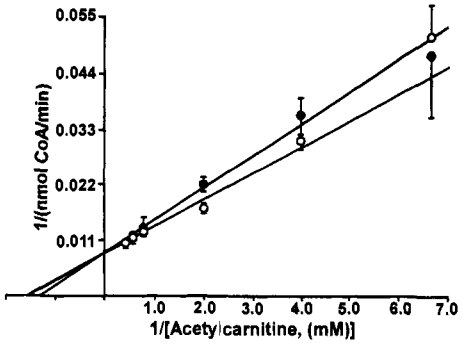


Figure 2. Competitive inhibition of pigeon breast CAT by racemic **8** at 0.00 (○) and 5.00 (●) mM final concentrations. Each data point is the mean from two to five separate determinations.

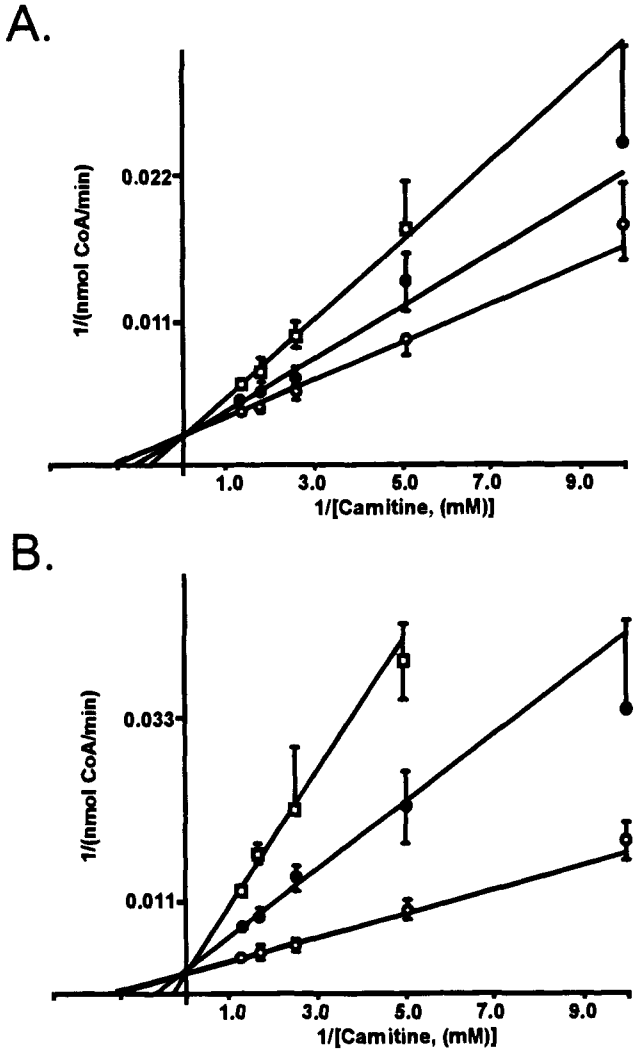


Figure 3. Competitive inhibition of pigeon breast CAT by *S*-(+)-**7** (A) and *R*-(−)-**7** (B) at 0.00 (○), 5.00 (●), and 10.00 (□) mM final concentrations. Each data point is the mean from three to five separate determinations.

substrate for CAT, is one of the most potent competitive inhibitors of CAT reported to date.[12] The K_i (24 μM) for racemic **4** is about 12 times smaller than the K_m (0.3 mM) for L-acetylcarnitine (**2**). Assuming that acetylcarnitine and (acetylamino)carnitine orient in the carnitine-binding site of CAT in a similar manner, this observation suggests that the amide functionality of **4** likely undergoes additional favorable interactions with the protein (such as an additional H-bond donation

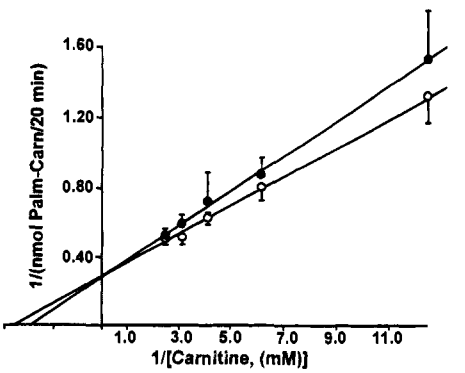


Figure 4. Competitive inhibition of CPT-2 in cultured neonatal rat cardiac myocytes by racemic **7** at 0.00 (○) and 5.00 (●) mM final concentrations. Each data point is the mean from five separate determinations. Note that **7** had no effect on CPT-1 at a concentration of 5 mM.

made possible by the amide NH of **4**). In contrast to CAT, aminocarnitine is an extremely potent reversible inhibitor of CPT. The possibility that this inhibition results from enzymatic conversion to the potent inhibitor (palmitoylamino)carnitine appears unlikely.[12,13]

We previously synthesized[14] and evaluated[15] *tert*-butylcarnitine analog **5** and its *O*-acetyl derivative (**6**) as *isosteric* analogs of carnitine and acetylcarnitine which lack the quaternary ammonium positive charge. Compound **5** was found to be a poor binder of pigeon breast CAT (see Table 1), and the K_i (8.3 mM) of racemic **5** for CAT was approximately 28 times larger than the K_m for (*R*)-carnitine (**1**). Furthermore, **5** was not a substrate for CAT in the forward direction, and the acetyl derivative **6**, similarly a poor competitive inhibitor (K_i = 4.1 mM), was not a substrate in the reverse direction. These observations demonstrated the importance of the positive charge on carnitine for efficient binding to the active site and revealed that this charge is essential for enzymic catalysis.

Since conversion of aminocarnitine to (acetylamino)carnitine dramatically enhances its binding affinity for CAT, we wished to evaluate the same structural changes in the *tert*-butyl carnitine analogs. We thus synthesized the amino (**7**) and *N*-acetylamino (**8**) analogs of **5** and **6**, respectively. Unlike aminocarnitine, **7** was not a substrate for CAT up to 10 mM concentration. This is consistent with our proposal that the carnitine positive charge is essential for enzymic catalysis. Interestingly, the K_i of compound **7** for CAT is comparable to that reported for aminocarnitine (**3**) (Table 1).

If **7** and **8** bind to the carnitine site of CAT in the same orientation as aminocarnitine (**3**; K_i = 4.0 mM) and (acetylamino)carnitine (**4**; K_i = 0.024 mM), then the acetylamino derivative **8** should be a more potent inhibitor of CAT activity than amine **7**. However, **8** (K_i = 25 mM) was found to be a *much* poorer competitive inhibitor of CAT than **7** (K_i = 2.6 mM). These results suggest that **4** and **8** may orient differently in the carnitine-binding site of CAT.

Further support of this proposal is provided by studies on the stereoselectivity of binding for compound **7** to CAT. (*S*)-(+)-Carnitine (D-carnitine) is not a substrate for pigeon breast CAT, but it is a relatively good competitive inhibitor. The K_m and K_i values for (*R*)- and (*S*)-carnitine, respectively, are essentially identical, revealing that both bind equally well to the carnitine

site on CAT.[17,18] We previously described[15] the first example of stereoselective binding of a carnitine analog to CAT in that, unlike carnitine, *S*-(−)-**5** (K_i = 7.5 mM) and *R*-(+)-**5** (K_i = 20.3 mM) had different K_i values. In the present study, even greater stereoselectivity was observed in that *R*-(−)-**7** (K_i = 1.9 mM) was a more potent competitive inhibitor than *S*-(+)-**7** (K_i = 9.2 mM). The enantiomers, as for the racemate, were not substrates for CAT up to 10 mM concentration.

Studies of amine **7** with CPT demonstrated similar effects. While aminocarnitine (**3**) is a potent inhibitor of CPT,[12,13] amine **7** had no effect on CPT-1 at concentrations up to 5 mM and was a very poor competitive inhibitor of CPT-2.

The results described here further refine our previous hypothesis that the quaternary ammonium positive charge in carnitine is essential for enzymic catalysis. This necessity could arise in at least two ways: (1) the absence of carnitine's positive charge may prevent the stabilization of a negative charge proposed[8] to develop in the transition state and/or (2) while carnitine's positive charge contributes to, but is not essential for, binding to the carnitine-binding site on CAT or CPT, it appears to be necessary for the proper alignment of functional groups in the catalytic center that gives rise to tight binding and substrate activity. While we cannot currently address the first possibility, the studies described here are consistent with the latter.

Experimental Section

Chemistry. General Procedures. Melting points were obtained on an Electrothermal melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Varian EM 360 (60 MHz) and GE 300-WB FT-NMR (300 MHz) spectrometers. IR spectra were recorded on a Beckmann Acculab-1 spectrometer, and optical rotations were obtained on a Perkin-Elmer 141 polarimeter in 1 dm cells of 1 mL capacity. Flash chromatography used Baker silica gel (40 μm), and TLC was performed on Fisher brand silica gel GF plates (0.2 mm layer, 5 × 10 cm). Elemental analyses were provided by Atlantic Microlabs of Atlanta, GA.

(*R,S*)-3-Amino-5,5-dimethylhexanoic Acid, Hydrochloride (7). A solution of amino ester **11** (500 mg, 2.24 mmol) in 18% HCl (20 mL) was heated at reflux for 5 h. The mixture was concentrated to dryness on a rotary evaporator. The solid residue was washed out of the flask with anhydrous acetone and filtered. The collected white solid was dried under vacuum to give **7** (435 mg, 97.7%): mp 233−234 °C; IR (KBr) 1700 (CO), 2500−3400 (NH and OH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.75 (s, 9 H, $C(CH_3)_3$), 1.32−1.50 (m, 2 H, CH_2CMe_3), 2.46−2.71 (m, 2 H, CH_2CO), 3.45−3.56 (m, 1 H, CHN). Anal. ($C_8H_{18}ClNO_2$) C, H, Cl, N.

***S*-(+)-7 and *R*-(−)-7.** A suspension of (3*S*,2′*R*)-**12** (100 mg, 0.300 mmol) or (3*R*,2′*R*)-**12** (200 mg, 0.610 mmol) in concentrated HCl (10 mL) was heated at reflux for 24 h with stirring. The mixture was concentrated to dryness on a rotary evaporator, and the residue was triturated with anhydrous acetone (5 mL) to give *S*-**7** (35 mg, 60%), mp 189−194 °C, $[\alpha]^{25}_D$ = +21.0° (c 0.0280, water); or *R*-**7** (89 mg, 75%), mp 190−192 °C, $[\alpha]^{25}_D$ = −20.3° (*c* 0.0265, water), as white solids. All spectra were identical to those for the racemate.

(*R,S*)-3-Acetamido-5,5-dimethylhexanoic Acid (8). To a solution of racemic **7** (250 mg, 1.28 mmol) in water (15 mL) were added solid NaOH (150 mg) and a solution of 10% $NaHCO_3$ (10 mL). The reaction mixture was cooled to 0−5 °C in an ice bath, and a solution of acetic anhydride (0.250 mL, 0.270 g, 2.64 mmol) in acetonitrile (6 mL) was added. The reaction mixture was stirred at 0−5 °C for 1 h and at room temperature for 12 h. The mixture was concentrated to dryness on a rotary evaporator, the residue was dissolved in water (30 mL), and the solution was washed with ethyl acetate (2 × 25 mL). The aqueous layer was acidified (pH 2) with 10% HCl and extracted with ethyl acetate (3 × 25 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated to give racemic **8** (210 mg, 81.7%) as a colorless oil: IR (neat) 1640 and 1710 (amide and ester C=O), 2500−3500 (OH and NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.95 (s, 9 H, $(CH_3)_3C$), 1.35−1.65 (m, 2 H, CH_2CMe_3), 1.98 (s, 1 H, CH_3CO), 2.43−2.68 (m, 2 H, CH_2CO), 4.31−4.45 (m, 3 H, CHN), 6.49−6.65 (d, 1 H, NH). Anal. ($C_{10}H_{19}NO_3$) C, H, N.

Ethyl 3-Amino-5,5-dimethylhexanoate (11). A solution of ethyl 3-oxo-5,5-dimethylhexanoate (**10**) (1.00 g, 5.38 mmol), prepared as previously described,[14] ammonium acetate (4.10 g, 53.2 mmol), and sodium cyanoborohydride (0.338 g, 5.40 mmol) in methanol (16 mL) was stirred at room temperature for 48 h. Concentrated HCl was added until the reaction mixture was pH 2. The mixture was concentrated under vacuum, the residue was dissolved in water (10 mL), and the solution was extracted with ether (3 × 20 mL). Solid KOH was added to the aqueous layer until the pH was 10, and this solution was extracted with ether (3 × 20 mL). The ether layers were combined, dried (Na_2SO_4), and concentrated to give the free amine **11** (0.855 g, 85.0%) as a colorless oil. This was dissolved in anhydrous ether (5 mL), and HCl gas was bubbled through the solution to give a white fluffy solid. The solid was filtered, washed with anhydrous ether on the filter, and dried under vacuum to give the hydrochloride salt **11** (0.920 g, 76.7%): mp 135−136 °C; IR (KBr) 1710 (CO), and 3360 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.95 (s, 9 H, $C(CH_3)_3$), 1.05−1.45 (t, 3 H, CH_3CH_2), 1.49−1.70 (m, 2 H, CH_2CMe_3), 2.20−2.40 (m, 2 H, CH_2CO), 3.05−3.60 (m, 1 H, CHN), 3.85−4.30 (q, 2 H, CH_3CH_2). Anal. ($C_{10}H_{22}ClNO_2$) C, H, Cl, N.

(3*R*,2′*R*)- and (3*S*,2′*R*)-Ethyl 3-(2-Methoxy-2-phenylacetamido)-5,5-dimethylhexanoate (12). A solution of free amine **11** (0.525 g, 2.80 mmol) and (*R*)-(−)-α-methoxyphenylacetic acid (0.466 g, 2.81 mmol) in methylene chloride (40 mL) was chilled to 0 °C, and DCC (0.854 g, 4.16 mmol) was added. The reaction mixture was stirred at 0 °C for 1 h and at room temperature for 14 h. The mixture was filtered to remove DCU, and the filtrate was concentrated to dryness on a rotary evaporator to provide the colorless oil **12** (1.35 g) as a mixture of diastereomers. The crude oil was flash chromatographed on a silica gel column (5 × 15 cm) using ethyl acetate−hexane (25:75) as eluent to give 3*S*,2′*R*-**12** (424 mg, 91.0%, R_f 0.35) which solidified on standing at room temperature for 2 days: mp 65.5−66.5 °C; IR (KBr) 3500−3100 (NH), 1710 and 1660 (ester and amide C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.95 (s, 9 H, $(CH_3)_3$), 1.20 (t, 3 H, CH_3), 1.39−1.65 (m, 2 H, CH_2CMe_3), 2.41−2.48 (d, 2 H, CH_2COOEt), 3.35 (s, 3 H, OCH_3), 4.00−4.15 (m, 2 H, CH_2Me), 4.25−4.40 (m, 1 H, CH), 4.55 (s, 1 H, CHOMe), 7.06−7.16 (d, 1 H, NH), 7.28−7.46 (m, 5 H, aromatic); $[\alpha]^{25}_D$ = −61.0° (*c* 0.0203, methanol). Anal. ($C_{19}H_{29}NO_4$) C, H, N.

Continued elution gave 255 mg of mixed diastereomers followed by pure 3*R*,2′*R*-**3** (284 mg, 60.4%, Rf 0.30) as an oil: IR (neat) 3500−3100 (NH), 1710 and 1660 (ester and amide C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.80 (s, 9 H, $(CH_3)_3$), 1.10−1.60 (m, 5 H, CH_3 and CH_2CMe_3), 2.40−2.55 (d, 2 H, CH_2COOEt), 3.30 (s, 3 H, OCH_3), 3.85−4.40 (m, 3 H, CH_2Me and CH), 4.50 (s, 1 H, CHOMe), 6.95−7.40 (m, 6 H, NH and aromatic); $[\alpha]^{25}_D$ = +27.5° (c 0.0151, methanol). Anal. ($C_{19}H_{29}NO_4$) C, H, N.

Enzyme Assays. General. Pigeon breast CAT, L-carnitine, D,L-acetylcarnitine, CoASH, acetyl-CoA, palmitoyl-CoA, 3-(*N*-morpholino)propanesulfonic acid (MOPS), fatty acid free bovine serum albumin (BSA), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma. All other chemicals were of analytical or reagent grade.

Carnitine Acetyltransferase Assay. Kinetic assays for the effects of **7** and **8** on pigeon breast CAT in the forward or reverse direction were performed according to the previously reported procedure.[15] For the forward reaction, the assay mixture contained 100 mM MOPS, 10 mM EDTA, 0.25 mM DTNB, 0.20 mM acetyl-CoA, and 0.5 unit of enzyme, in the presence or absence of inhibitor, in a final volume of 1 mL at 25 °C. The enzyme reaction was initiated by the addition of varying concentrations of L-carnitine, and the initial rate was

determined by monitoring the change in thiolate absorbance at 412 nm. The initial rate for the reverse reaction was determined by monitoring the change in absorbance at 232 nm (due to the thioester bond). The assay mixture contained 100 mM MOPS, 5 mM EDTA, 0.25 mM CoASH, and 0.5 unit of enzyme, in the presence or absence of inhibitor, in a volume of 1 mL at 25 °C. The reaction was initiated by the addition of varying concentrations of D,L-acetylcarnitine (see Figures 1–3).

Carnitine Palmitoyltransferase 1 and 2 Assay. Kinetic assays for the effects of **7** on CPT-1 and CPT-2 were performed in cultured neonatal rat cardiac myocytes according to a reported procedure.[15] Briefly, the rate of incorporation of [^{14}C]-carnitine into [^{14}C]palmitoylcarnitine was determined for myocytes treated with 10 μM digitonin (for CPT-1) or 0.16% Triton X-100 (for CPT-2). The assay medium (0.5 mL), which contained 10 mM HEPES (pH 7.0) and 1% BSA, was added to detergent-treated cultured cells (2×10^5 cells/well). Palmitoyl-CoA was present at a final concentration of 75 μM. The reaction, in the presence or absence of inhibitor, was initiated by adding increasing concentrations of [1-^{14}C]-L-carnitine. The initial rate of the reaction at 25 °C was determined by scintillation counting of the radiolabeled palmitoylcarnitine that was selectively extracted into butanol (see Figure 4).

Kinetic Data Analysis. The enzyme kinetic data were analyzed using the computer program ENZFITTER (distributed by Sigma Chemical Co.), which utilizes nonlinear regression analysis (Marquart algorithm) of the enzyme velocity curve to provide apparent K_m values. The K_i values were determined from a plot of apparent K_m versus [inhibitor].

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