Synthesis and Enzymatic Evaluation of Conformationally Defined Carnitine Analogs

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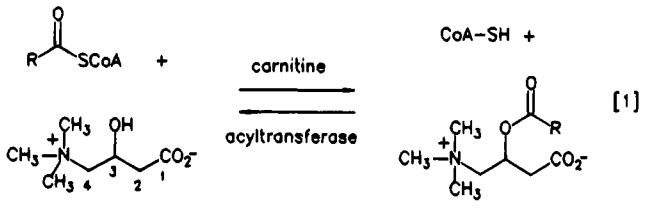
Carnitine (**1**, 3-hydroxy-4-trimethylammoniobutyrate) is essential as a donor and acceptor of acyl groups in cellular metabolism. The major solution conformation of carnitine about C3–C4 contains the gauche relationship between the trimethylammonium and hydroxy groups, while two of the three low-energy, staggered conformations about C2–C3 are significantly populated. For studies of carnitine's protein binding sites, we designed conformationally defined cyclohexyl carnitine analogs (2-hydroxy-3-trimethylammoniocyclohexanecarboxylate) which all contain the favored carnitine conformation about the C3–C4 bond. Of the four possible diastereomers for these analogs, we synthesized three (**2–4**) that contain different carnitine conformations about C2–C3. Diastereomers **2** and **3** were prepared via the major diastereomeric products resulting from reduction of ethyl 5-chloro-3-nitrosalicylate (**7**). Compound **4**, which could not be obtained in practical quantities by the above method, was prepared stereoselectively via opening of the epoxide of 3-(benzyloxycarbonylamino)cyclohexene (**23**) with Et_2AlCN. Compounds **2–4** were not substrates for pigeon breast carnitine acetyltransferase but were weak, competitive inhibitors with K_i values from 2.9 to 4.1 mM. In contrast, compounds **2** and **4** were not inhibitors of neonatal rat cardiac myocyte CPT-2, while compound **3** was a modest competitive inhibitor (K_i 5.3 mM). These results suggest differences between the carnitine binding sites of CAT and CPT-2.

Several different carnitine acyltransferases catalyze the reversible synthesis of *O*-acylcarnitines from (*R*)-carnitine (**1**) and an acyl-coenzyme A ester (see eq 1). Among these, carnitine acetyltransferase (CAT) is selective for the transfer of short chain acyl groups and is important for maintaining the CoASH/acetyl-SCoA ratio.[1] Carnitine octanoyltransferase (COT) and carnitine palmitoyltransferases 1 and 2 (CPT-1 and CPT-2) are selective for transferring medium and long chain acyl groups, respectively, and the latter are essential for the entry of fatty acids into mitochondria prior to β-oxidation.[2,3]



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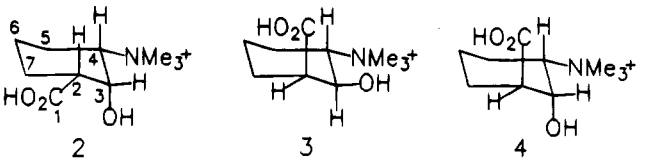
In spite of considerable interest in the properties and structure of the carnitine acyltransferases, very few reports have addressed differences in the carnitine binding sites of these enzymes. Most of the known potent inhibitors are either bisubstrate or fatty acid analogs. As part of a study to compare and elucidate the topographies for carnitine binding sites on these enzymes, we designed cyclic carnitine analogs **2–4** which contain defined spatial relationships between the quaternary ammonium, hydroxyl, and carboxylate moieties. Here we describe the syntheses of these cyclohexylcarnitine analogs and their effects on pigeon breast CAT and neonatal rat cardiac myocyte CPT-2.



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Results and Discussion

Chemistry. Carnitine is a conformationally flexible molecule which may present key groups to the binding site on carnitine acyltransferases with different spatial relationships. The conformational distribution of carnitine in solution has been determined[4] using ^1H NMR techniques, and the results are summarized in Figure 1. Three low energy conformations are possible about C2–C3 and, similarly, three low-energy conformations are possible about C3–C4. About C3–C4, conformer A is predominant in solution and places the quaternary ammonium and hydroxy groups gauche to each other. (This is consistent with the "gauche effect" observed in related compounds such as choline.)[5] Gandour *et al.*[1b]

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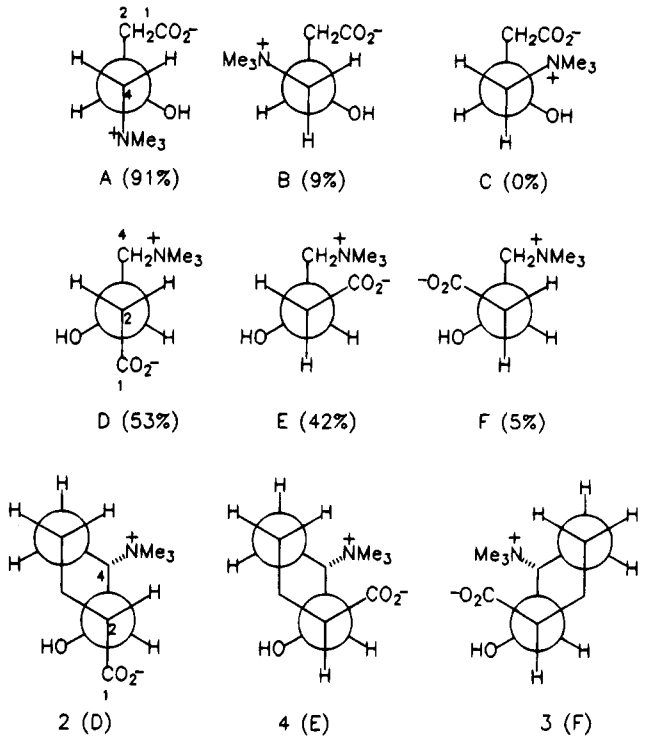


Figure 1. Solution conformations for carnitine (A–F) and their relative populations (%) as compared to the cyclohexyl carnitine analogs **2–4**. Note that **2–4** all maintain carnitine conformation A about C3–C4 while varying the conformation about C2–C3.

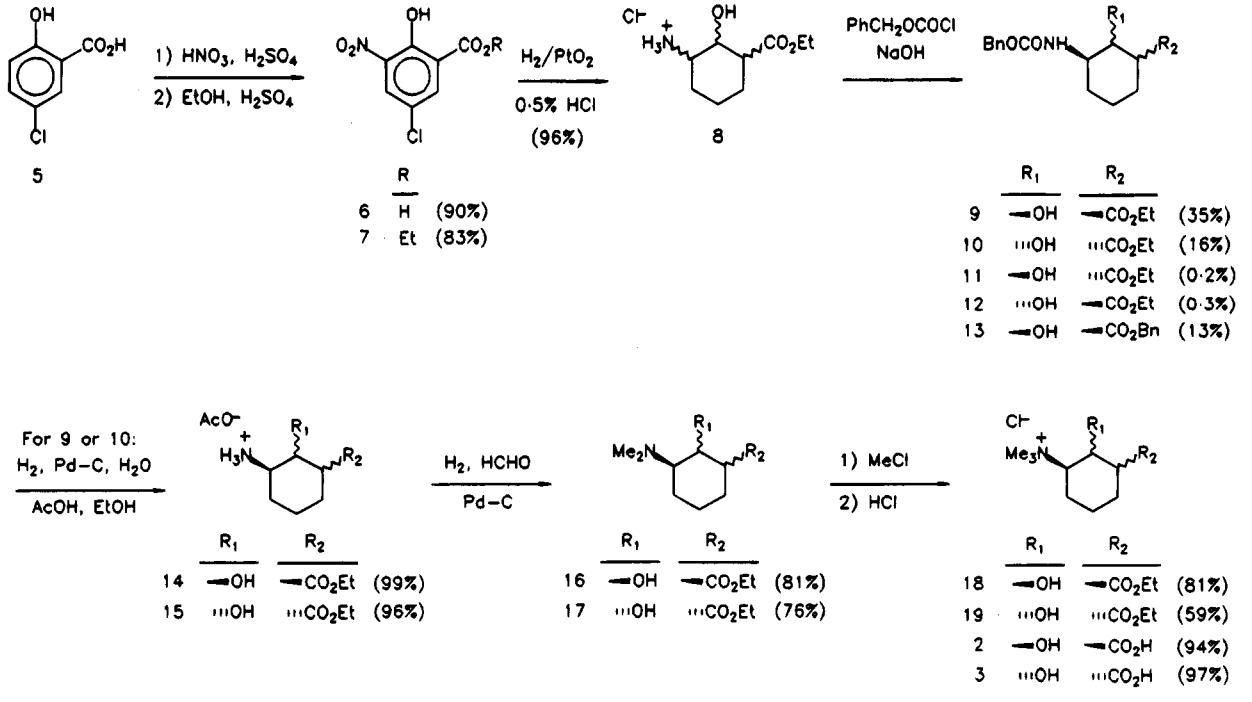
concluded that conformer A is the active conformation of carnitine as a substrate for carnitine acyltransferases. However, the active conformation about C2–C3 has not been determined, and two of the three low-energy staggered conformations are significantly populated in solution. We thus designed a series of conformationally defined carnitine analogs (**2–4**, Figure 1) which use a cyclohexane ring to control stereochemistry. Compounds **2–4**, which represent three of the four possible diastereomers, all maintain conformation A about C3–C4, but the conformation about C2–C3 varies as indicated in Figure 1.

The first synthetic approach to **2–4** involved the reduction of an appropriately substituted benzenoid system. This was anticipated to produce a mixture of the possible cyclohexyl diastereomers, but the method offered a straightforward entry into the desired system and could potentially provide useful yields of more than one target. The approach is summarized in Scheme 1. To avoid separating the two products produced by nitration of salicylic acid,[6] 5-chlorosalicylic acid (**5**) was alternatively nitrated to give 5-chloro-3-nitrosalicylic acid (**6**).[7] This was esterified to give **7**,[8] which was hydrogenated to provide the cyclohexylammonium salt **8** as a mixture of diastereomers. This mixture was not characterized but was reacted with benzyl chloroformate to give a mixture of cyclohexyl diastereomers **9–12** as well as the transesterification product **13**. Separation on a silica flash chromatography column gave pure **9** (35%) and **13** (13%). Diastereomers **10–12** (16%, 0.2%, and 0.3% yields, respectively) were then purified by HPLC on silica.

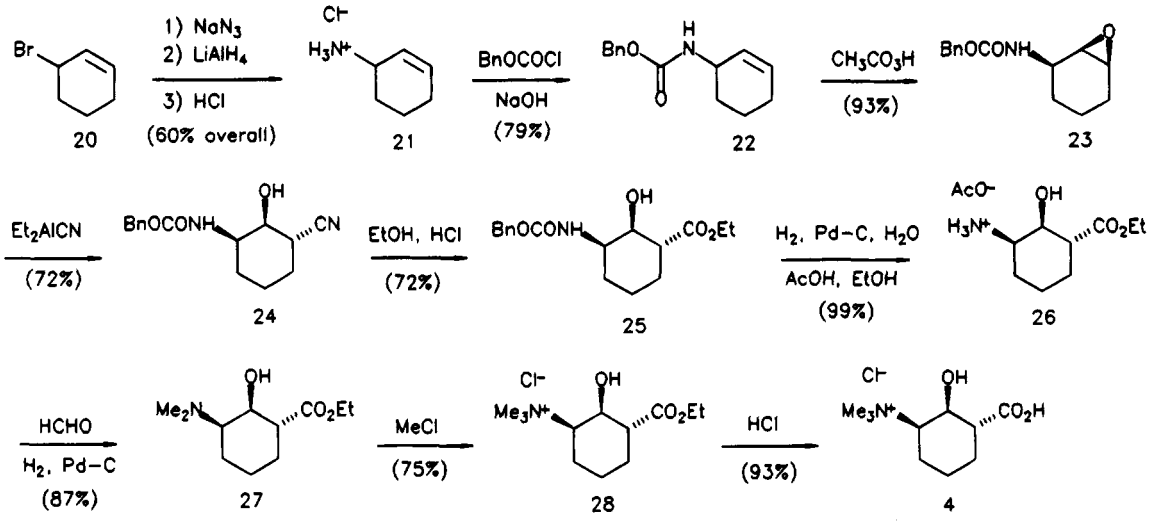
Diastereomers **9** and **10** were each reacted as detailed in Scheme 1 according to procedures developed in our laboratory[9] for the synthesis of carnitine. (Note that **13** contains the same stereochemistry as **9** and, while not performed here, could presumably be carried on to provide additional **2**.) Thus **9** and **10** were deprotected[10] to give the ammonium acetate salts **14** and **15**, respectively. Reductive alkylation under Eschweiler–Clarke conditions[11] then provided the dimethylamino compounds **16** and **17**. Quaternization with chloromethane, followed by hydrolysis of the ester, provided the final products **2** and **3**.

Since the synthetic approach in Scheme 1 could not provide usable quantities of target **4**, the stereoselective synthesis detailed in Scheme 2 was developed. In this approach 3-aminocyclohexene (**21**)[12] was synthesized

Scheme 1



Scheme 2



from 3-bromocyclohexene (**20**) via reaction with NaN_3 followed by reduction.[13] The amine was protected as the benzyloxycarbonyl derivative to give **22**, which was epoxidized with peracetic acid.[12] This stereoselectively provided the *cis*-epoxide **23** (93% yield). Epoxide **23** underwent regioselective ring opening with diethylaluminum cyanide[14] to give **24** (72% yield). Nitrile **24** was converted to the ester **25** via the imino ether,[15] and **25** was converted to product **4** as previously described for diastereomers **9** and **10** in Scheme 1.

The relative configurations of compounds **2**, **3**, and **4** were determined from the ^1H NMR coupling constants between H2, H3, and H4. The bulky trimethylammonium group must occupy the equatorial position, locking **2**–**4** into a single chair conformation for which H4 is axial. The region of the ^1H NMR spectrum (recorded at 400 MHz) containing resonances for H2–H4 is shown in Figure 2, and the residual splittings between H2, H3, and H4 that were determined by single frequency off-resonance decoupling experiments (recorded at 300 MHz) are summarized in Table 1.

Carnitine exhibits characteristic chemical shifts[3] for H2 (2.5 ppm), H3 (4.6 ppm), and H4 (3.4 ppm), and these were used for preliminary assignments of the comparable proton resonances in **2**–**4**. The preliminary assignments were subsequently confirmed by decoupling experiments. For compound **2**, the irradiation of H3 resulted in the collapse of H4 into a doublet of doublets with a large splitting ($J_{4,5a}$ = 12.6 Hz) and a small splitting ($J_{4,5e}$ = 3.4 Hz), as expected for axial H4. In the fully coupled spectrum, H3 appears as a broad singlet, revealing only small couplings (gauche) to H4 and H2. Since H4 is axial, H3 is equatorial. The irradiation of H3 collapsed H2 to a doublet of doublets with a large splitting ($J_{2,7a}$ = 13.0 Hz) and a small splitting ($J_{2,7e}$ = 4.3 Hz); thus H2 is axial.

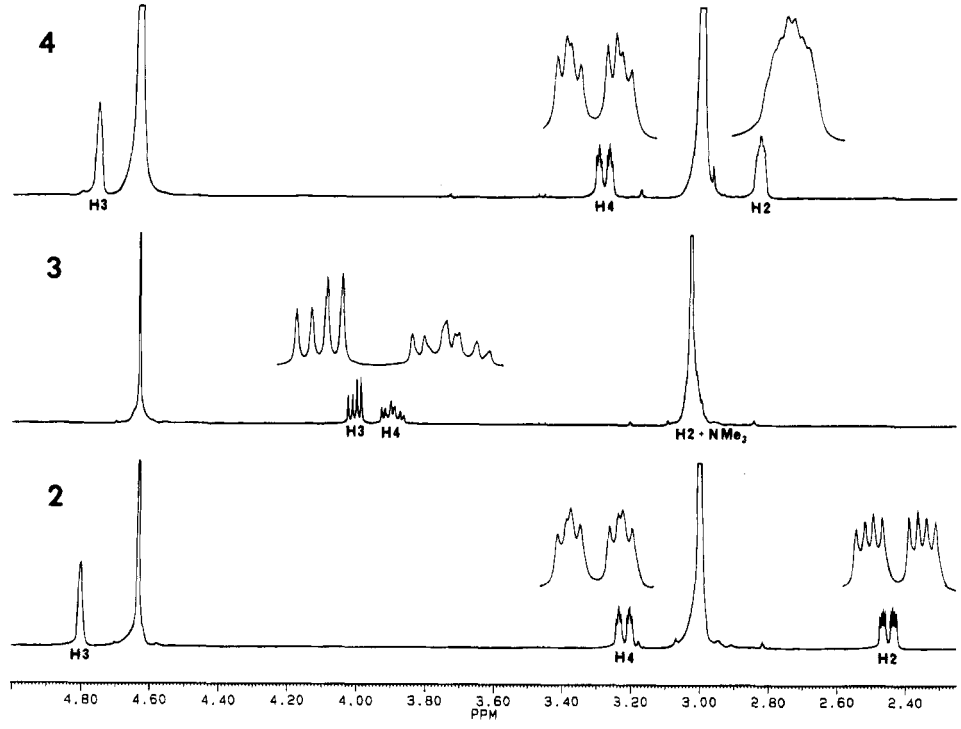


Figure 2. ^1H NMR (400 MHz in D_2O) spectral regions containing the H2, H3, and H4 resonances for compounds **2**–**4**.

Table 1. Selected Residual Splittings for H2, H3, and H4 in Compounds 2–4 Obtained from ^{1}H NMR Decoupling Experiments at 300.1 MHz in D_2O at 40 °C

compound	proton	δ (ppm)	$^3J_{H,H}$ (Hz)[a]
2	H2	2.45	$J_{2,3} < 2$
			$J_{2,7a} = 13.0$
			$J_{2,7e} = 4.3$
	H3	4.80	$J_{3,4} < 2$
	H4	3.22	$J_{4,5a} = 12.6$
			$J_{4,5e} = 3.4$
3	H2	*b*	$J_{2,3} = 5.1$[c]
	H3	4.00	$J_{3,4} = 10.1$
	H4	3.89	
4	H2	2.83	$J_{2,3} = 2.5$
	H3	4.75	$J_{3,4} = 2.9$
	H4	3.27	$J_{4,5a} = 12.6$
			$J_{4,5e} = 3.7$

[a] Unless otherwise noted, residual $J_{2,3}$ was obtained by irradiating H4 and observing H3; residual $J_{3,4}$ by irradiating H2 and observing H3; residual $J_{2,7}$ by irradiating H3 and observing H2; residual $J_{4,5}$ by irradiating H3 and observing H4. [b] Masked by the quaternary ammonium methyl resonance at 3.05 ppm. [c] Coupling constant was determined from the fully coupled spectrum.

Upon irradiation of H2 or H4, H3 remained a broad singlet ($J_{2,3}$ and $J_{3,4} < 2$ Hz).

Similar decoupling experiments were performed to establish the relative configurations of **3** and **4**. For compound **3**, the irradiation of H2 resulted in the collapse of H3 to a doublet with a residual splitting of 10.1 Hz ($J_{3,4}$), removing a coupling with $J = 5.1$ Hz ($J_{2,3}$). Thus H3, like H4, is axial, and H2 is equatorial. For compound **4**, H3 is a broad singlet with only small couplings. Thus H3 is equatorial (gauche to H4). The irradiation of H3 collapsed H2 into a broadened doublet with a residual splitting of 4.4 Hz ($J_{2,7}$); splitting of H2 by the second H7 proton was less than 3 Hz. Thus H2 is equatorial. The irradiation of H2 or H4 collapsed H3 into a doublet, revealing a residual $J_{3,4} = 2.9$ Hz and $J_{2,3} = 2.5$ Hz, respectively. The additional residual splittings in Table 1 were obtained in an analogous manner.

Enzyme Kinetics Assays. Racemic **2–4** were evaluated as alternate substrates and inhibitors of purified pigeon breast carnitine acetyltransferase (CAT) in the forward direction (acylcarnitine formation). They were also evaluated as inhibitors of mitochondrial carnitine palmitoyltransferase 2 (CPT-2) in cultured neonatal rat cardiac myocytes (in the direction of acylcarnitine formation). The results are summarized in Table 2.

None of the racemic cyclohexyl carnitine analogs **2–4** were substrates for pigeon breast CAT at concentrations up to 10 mM. Compounds **2–4** were weak competitive inhibitors in the forward direction, and the observed K_i values were similar. In all cases the K_i values were much larger than the K_m for L-carnitine (0.3 mM). Similarly, compounds **2** and **4** were not inhibitors of CPT-2 at concentrations up to 5 mM, and compound **3** was a weak competitive inhibitor.

Table 2. Kinetic Constants for Conformationally Defined Carnitine Analogs with Selected Carnitine Acyltransferases[a]

compound	K_i, mM, CPT-2[b] (type inhib)	K_i, mM, CAT[c] (type inhib)
(*R,S*)-**2**	no effect	4.1 (competitive)
(*R,S*)-**3**	5.3 (competitive)	3.7 (competitive)
(*R,S*)-**4**	no effect	2.9 (competitive)

[a] All assays were in the forward direction (acylcarnitine formation) and used varying concentrations of L-carnitine (K_m 0.3 mM for CAT and 0.2 mM for CPT-2). None of the compounds had substrate activity for CAT at concentrations up to 10 mM. [b] Assayed in cultured neonatal rat cardiac myocytes. [c] Assayed using purified pigeon breast CAT.

These results reveal that cyclohexyl carnitine analogs **2–4** bind much less tightly than L-carnitine to the carnitine binding site on pigeon breast CAT and neonatal rat cardiac myocyte CPT-2. Possible reasons for this result are that (1) none of the low-energy solution conformations of carnitine contained in these rigid compounds represents the bound conformation for carnitine or (2) the extra steric bulk provided by the cyclohexyl ring residues interferes with binding. Other evidence from our laboratory is consistent with the latter possibility.[16]

Of significance was the observation that compounds **2** and **4**, which inhibit CAT with comparable potency, do not inhibit CPT-2. This result suggests that pigeon breast CAT is less selective than neonatal rat myocyte CPT-2 in binding to carnitine analogs and that there are differences between the carnitine binding sites of CAT and CPT-2 which may potentially be utilized for selective inhibitor design. Furthermore, the observation that only racemic **3** inhibits CPT-2, even though the binding is roughly 10-fold less potent than that for racemic carnitine, suggests that **3** may most closely represent the bound conformation of carnitine to CPT-2.

Experimental Section

Melting points are uncorrected. Elemental analyses were performed at Atlantic Microlabs of Atlanta, GA. Flash chromatographic separations were carried out on Baker silica gel (40 μm), and TLC was performed on Fisher brand silica gel GF plates (0.2 mm layer, 5 × 10 cm). HPLC separations were performed using a Rainin Dynamax silica column on a Rainin Rabbit HPLC system.

For the enzyme kinetics assays, pigeon breast CAT, L-carnitine, acetyl-CoA, palmitoyl-CoA, fatty acid free bovine serum albumin (BSA), 3-(morpholino)propanesulfonic acid (MOPS), *N*-(2-hydroxyethyl)piperazine-*N*′-2-(ethanesulfonic acid) (HEPES), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma. All other chemicals were of analytical or reagent grade. The enzyme kinetics data were analyzed using the computer program ENZFITTER (distributed by Sigma Chemical Co.). Lineweaver–Burk plots were directly derived from K_m and k_{cat} values obtained by nonlinear regression analysis of the [substrate] vs rate curve. K_i values were then determined from a plot of [inhibitor] vs apparent K_m.

5-Chloro-3-nitrosalicylic Acid (6). 5-Chlorosalicylic acid (**5**, 15.9 g, 92.1 mmol) was dissolved in concd H_2SO_4 (60 mL) with stirring in an ice bath. To this solution was added dropwise over 0.5 h a mixture of concd HNO_3 (5.2 mL) and concd H_2SO_4 (12.4 mL), maintaining the temperature below 10 °C. The reaction mixture was stirred for an additional 6 h

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at rt and was then poured onto crushed ice. The resulting yellow precipitate was filtered, washed with ice cold water, and then dried *in vacuo*. The yellow solid **6** (18.1 g, 90.5%) was recrystallized from ethanol–water to afford long yellow needles: mp 168–169 °C (lit.[7] mp 165–167 °C).

Ethyl 5-Chloro-3-nitrosalicylate (7). To a solution of **6** (9.90 g, 45.6 mmol) in EtOH (100 mL) was added concd H_2SO_4 (20 mL). Benzene (100 mL) was added to the reaction mixture, and it was heated at reflux for 48 h with azeotropic removal of water. The mixture was concentrated on a rotary evaporator, and the residue was dissolved in EtOAc (150 mL). The resulting solution was washed with 5% $NaHCO_3$ (2 × 100 mL) followed by brine (2 × 100 mL). The organic layer was dried (Na_2SO_4) and concentrated on a rotary evaporator to give **7** (9.30 g, 83.2%) as a yellow solid: mp 88–90 °C (lit.[8] mp 90–91 °C).

Ethyl 3-(Benzyloxycarbonylamino)-2-hydroxycyclohexanecarboxylate (9–12) and Benzyl 3-(Benzyloxycarbonylamino)-2-hydroxycyclohexanecarboxylate (13). Compound **7** (3.69 g, 15.0 mmol) was mixed with 0.5% aqueous HCl (75 mL) and PtO_2 (0.50 gm) in a hydrogenation pressure bottle. The mixture was placed on a Parr shaker (55 psi H_2) for 24 h. The catalyst was filtered and washed with hot water (10 mL). The filtrate was evaporated on a rotary evaporator, and the residue was dried *in vacuo* to give crude **8** (3.22 g, 95.9%) as a white solid: mp 151–152.5 °C. Crude **8** was dissolved in water (50 mL) and chilled to 0–5 °C in an ice bath with stirring. The pH of the solution was raised to 8.0 using 5% NaOH. Benzyl chloroformate (7.14 g, 41.9 mmol) was added in five portions over a period of 45 min, and after each portion 5% NaOH was added to adjust the pH to 8.0. The reaction mixture was kept cold and alkaline (pH ≈8; 5% NaOH added as necessary) for an additional 2 h, and the mixture was then stirred at rt for 12 h. This was extracted with EtOAc (3 × 50 mL), the combined extracts were dried (Na_2SO_4), and the solution was concentrated *in vacuo* to give an oily residue (3.75 g) containing a mixture of **9–12** and **13**. The crude product was chromatographed on a flash silica column (35% EtOAc/hexane) to first provide **13** (0.700 g, 13.4%) as a white solid (R_f 0.55, 40% EtOAc/hexane): mp 119–121 °C; IR (KBr) 3100–3550, 1680 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.21–1.89 (m, 6 H), 2.39–2.58 (m, 1 H), 3.01–3.14 (br s, 1 H, O*H*), 3.47–3.67 (m, 1 H), 4.17–4.29 (m, 1 H), 5.03–5.20 (two s, 4 H), 5.26–5.38 (d, 1 H, N*H*), and 7.25–7.42 (br s, 10 H); MS *m/z* 383 (M^+).

Anal. Calcd for $C_{22}H_{25}NO_5$: C, 68.91; H, 6.57; N, 3.65. Found: C, 68.77; H, 6.62; N, 3.59.

Further elution gave **9** (1.63 g, 35.3%) as a white solid (R_f 0.50, 40% EtOAc/hexane), which was recrystallized from hexane, followed by an oily mixture of **10–12** (0.895 g).

For **9**: mp 94.5–95.5 °C; IR (KBr) 3500–3300, 1720, 1660 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.08–1.86 (m, 9 H), 2.25–2.57 (m, 1 H), 3.06–3.36 (br s, 1 H, O*H*), 3.41–3.71 (m, 1 H), 4.02–4.30 (m, 3 H), 5.02–5.22 (s, 2 H), 5.30–5.57 (d, 1 H, N*H*), and 7.35 (s, 5 H); MS *m/z* 321 (M^+).

Anal. Calcd for $C_{17}H_{23}NO_5$: C, 63.54; H, 7.21; N, 4.36. Found: C, 63.52; H, 7.21; N, 4.38.

The mixture of **10–12** (0.895 g) was separated on a Rainin Dynamax silica HPLC column (21.4 mm × 250 mm, 60 Å, 50% EtOAc/hexane) using a flow rate of 8 mL/min to give **10** (0.775 g, 16.8%, t_R = 52 min), which crystallized upon standing at rt during 3 days: mp 78–79 °C; IR (neat) 3520–3300, 1730–1680 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.10–2.18 (m, 9 H), 2.71–2.90 (m, 1 H), 3.32–3.55 (m, 1 H), 3.62–3.80 (br s, 1 H, O*H*), 3.81–3.97 (m, 1 H), 4.06–4.28 (q, 2 H), 4.75–4.98 (m, 1 H, N*H*), 5.15 (s, 2 H), and 7.41 (s, 5 H); MS *m/z* 321 (M^+).

Anal. Calcd for $C_{17}H_{23}NO_5$; C, 63.54; H, 7.21; N, 4.36. Found: C, 63.39; H, 7.26; N, 4.34.

Further elution with 50% EtOAc/hexane gave a mixture of **11** and **12** (43 mg, t_R = 61.5 min). This mixture was then chromatographed on the same HPLC column using 60% Et_2O/hexane to give, as oils, **11** (10 mg, 0.2%, t_R = 32.2 min) and **12** (15 mg, 0.3%, t_R = 37.3 min).

For **11**: IR (neat) 3700–3150, 1710–1650 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.20–2.05 (m, 9 H), 2.43–2.60 (m, 1 H), 3.99–4.11 (m, 2 H), 4.10–4.23 (q, 2 H), 4.91–5.20 (m, 3 H), and 7.39 (s, 5 H); MS *m/z* 321 (M^+).

For **12**: IR (neat) 3700–3100, 1700, 1680 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.23–2.15 (m, 9 H), 2.34–2.47 (m, 1 H), 3.41–3.65 (m, 2 H), 4.11–4.26 (q, 2 H), 4.80–4.95 (m, 1 H, N*H*), 5.08–5.14 (s, 1 H), and 7.35 (s, 5 H); MS *m/z* 321 (M^+).

Ethyl 3-Ammonio-2-hydroxycyclohexanecarboxylate, Acetate (14 and 15). Ethanol (5 mL), water (5 mL), acetic acid (5 mL), and 10% Pd–C (300 mg) were placed on an atmospheric hydrogenator and stirred at rt. After 2 h a solution of **9** (1.00 g, 3.11 mmol) or **10** (0.760 g, 2.37 mmol) in ethanol (5 mL) was added. The reaction mixture was then stirred for 24 h at rt. The catalyst was filtered and washed with hot water, and the filtrate was evaporated *in vacuo* to give **14** (0.766 g, 99.6%) or **15** (0.565 g, 96.6%) as white solids which were recrystallized from acetone.

For **14**: mp 136–138 °C; IR (KBr) 3500–2500, 1700 cm^{-1}; ^1H NMR (D_2O) δ 1.10 (t, 3 H), 1.13–1.69 (m, 6 H), 1.75 (s, 3 H), 2.21–2.66 (m, 1 H), 3.06–3.28 (m, 1 H), 3.89–4.16 (m, 2 H), and 4.27 (br s, 1 H).

Anal. Calcd for $C_{11}H_{21}NO_5$: C, 53.43; H, 8.56; N, 5.66. Found: C, 53.53; H, 8.56; N, 5.71.

For **15**: mp 151–152.5 °C; IR (KBr) 3400–2400, 1710 cm^{-1}; ^1H NMR (D_2O) δ 1.02–1.16 (t, 3 H), 1.20–1.60 and 1.76–1.98 (m, 6 H), 3.46–3.70 (m, 2 H), 3.92–4.12 (q, 2 H).

Anal. Calcd for $C_{11}H_{21}NO_5$: C, 53.43; H, 8.56; N, 5.66. Found: C, 53.34; H, 8.51; N, 5.71.

Ethyl 3-(*N,N*-Dimethylamino)-2-hydroxycyclohexanecarboxylate (16 and 17). Compound **14** or **15** (0.780 g, 3.16 mmol) was dissolved in water (5 mL) in a hydrogenation pressure bottle. Then 10% Pd/C (300 mg) was added followed by 0.6 mL of a 36% formaldehyde solution (212 mg of formaldehyde, 6.92 mmol). This was placed on a Parr shaker (50 psi H_2) for 24 h. The mixture was filtered, and the catalyst was washed with hot water. The filtrate was cooled in an ice bath, saturated with Na_2CO_3, and extracted with $CHCl_3$ (3 × 50 mL). The combined extracts were dried (Na_2SO_4) and concentrated *in vacuo* to give **16** (0.550 g, 81.1%) or **17** (0.520 g, 76.7%) as a colorless oil.

For **16**: IR (neat) 3450, 1740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.10–1.30 (t, 3 H), 1.40–1.90 (m, 6 H), 2.20–2.60 (br s, 9 H), 3.80–4.40 (m, 3 H).

Anal. Calcd for $C_{11}H_{21}NO_3$: C, 61.37; H, 9.83; N, 6.51. Found: C, 61.65; H, 9.89; N, 6.36.

For **17**: IR (neat) 3450, 1740 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.10–1.30 (t, 3 H), 1.40–1.80 (m, 6 H), 2.30 (s, 6 H), 3.10–3.30 (br s, 3 H), 3.60–3.80 (dd, 1 H), and 4.00–4.40 (q, 2 H).

Anal. Calcd for $C_{11}H_{21}NO_3$: C, 61.37; H, 9.83; N, 6.51. Found: C, 61.16; H, 9.75; N, 6.35.

Ethyl 2-Hydroxy-3-(trimethylammonio)cyclohexanecarboxylate, Chloride (18 and 19). A solution of **16** (0.550 g, 2.60 mmol) or **17** (0.285 g, 1.32 mmol) in anhydrous acetone (10 mL for **16** and 6 mL for **17**) was placed in a pear-shaped pressure bottle. The mixture was cooled to −78 °C and CH_3Cl gas was bubbled through the solution for 20 min. The bottle was capped, and the solution was stirred at rt for 48 h. During this time a white precipitate formed. The solid was filtered and washed with anhydrous acetone (10 mL) to give **18** (0.590 g, 81.0%) or **19** (0.210 g, 59.6%).

For **18**: mp 192.5–195 °C; IR (KBr) 3500–3200, 1720 cm^{-1}; ^1H NMR (D_2O) δ 1.02–1.15 (t, 3 H), 1.15–1.97 (m, 6 H), 2.40–2.53 (m, 1 H), 2.90 (s, 9 H), 3.15–3.28 (m, 1 H), 3.87–4.22 (m, 2 H), and 4.80 (s, 1 H).

Anal. Calcd for $C_{12}H_{24}ClNO_3$; C, 54.23; H, 9.10; N, 5.27; Cl, 13.34. Found: C, 54.32; H, 9.11; N, 5.26; Cl, 13.41.

For **19**: mp 184–186 °C; IR (KBr) 3500–3200, 1720 cm^{-1}; ^1H NMR (D_2O) δ 1.10–1.20 (t, 3 H), 1.30–2.30 (m, 6 H), 3.00–3.10 (s, 10 H), 3.90–4.00 (m, 1 H), and 4.00–4.10 (m, 3 H).

Anal. Calcd for $C_{12}H_{24}ClNO_3$: C, 54.23; H, 9.10; N, 5.27; Cl, 13.34. Found: C, 54.21; H, 9.05; N, 5.30; Cl, 13.30.

2-Hydroxy-3-(trimethylammonio)cyclohexanecarboxylic Acid, Chloride (2 and 3). A solution of **18** (0.400 g, 1.50 mmol) or **19** (0.225 g, 0.847 mmol) in concd HCl (20 mL for **18** and 10 mL for **19**) was heated at reflux for 2 h. The reaction mixture was cooled to rt and concentrated *in vacuo*

to yield **2** (0.340 g, 94.9%) or **3** (0.195 g, 97.0%) as white solids which were washed on a filter with anhydrous acetone.

For **2**: mp 248–249 °C; IR (KBr) 3500–2700, 1710 cm^{-1}; ^1H NMR (D_2O) δ 1.10–2.01 (m, 6 H), 2.33–2.52 (m, 1 H), 3.02 (s, 9 H), 3.12–3.39 (m, 1 H), and 4.81 (s, 1 H); ^{13}C NMR (D_2O) δ 20.1, 20.5, 22.8, 48.0, 52.4, 65.7, 74.5, 176.7.

Anal. Calcd for $C_{10}H_{20}ClNO_3$: C, 50.52; H, 8.48; N, 5.89; Cl, 14.91. Found: C, 50.61; H, 8.53; N, 5.85; Cl, 14.99.

For **3**: mp 207–208 °C; IR (KBr), 3500–2700, 1710 cm^{-1}; ^1H NMR (D_2O) δ 1.10–2.10 (m, 6 H), 2.90–3.00 (s, 10 H), 3.80–3.90 (m, 1 H), 3.90–4.00 (m, 1 H); ^{13}C NMR (D_2O) δ 23.6, 28.1, 28.9, 51.3, 55.9, 72.8, 76.4, 180.1.

Anal. Calcd for $C_{10}H_{20}ClNO_3$: C, 50.52; H, 8.48, N, 5.89; Cl, 14.91. Found: C, 50.43; H, 8.51; N, 5.91; Cl, 14.84.

3-Aminocyclohexene, Hydrochloride (21). A solution of 3-bromocyclohexene (**20**) (10 g, 62 mmol) in CCl_4 (100 mL) was added to a solution of NaN_3 (14.2 g, 0.206 mol) in water (100 mL). The heterogeneous mixture was stirred vigorously for 48 h at rt. The CCl_4 layer was separated, and the aqueous layer was extracted with CCl_4 (3 × 50 mL). The combined organic layers were dried (Na_2SO_4) and concentrated *in vacuo* to give 3-azidocyclohexene (7.32 g, 95.8%) as a colorless oil, which was utilized without additional purification.

To a suspension of $LiAlH_4$ (6.40 g, 168 mmol) in anhydrous ether (25 mL) was added dropwise a solution of 3-azidocyclohexene (7.32 g, 590 mmol) in anhydrous ether (25 mL). The reaction mixture was stirred at reflux for 48 h. After cooling, water (25 mL) was added carefully, followed by 15% KOH (25 mL) and additional water (25 mL). The white precipitate was filtered and thoroughly washed with ether. The ether layer was separated, and the aqueous layer was extracted with ether (3 × 25 mL). The combined ether layers were dried (Na_2SO_4) and concentrated *in vacuo* to a volume of about 50 mL. [In one preliminary procedure, the free amine was isolated for comparison to literature data: bp 65–66 °C (53 mm); lit.[12] bp 139–140 °C (756 mm).] Anhydrous HCl gas was bubbled through the solution to yield a white precipitate which was filtered and dried *in vacuo* to give **21** (5.01 g, 63.1%): mp 150–155 °C.

3-(Benzyloxycarbonylamino)cyclohexene (22). A solution of **21** (2.1 g, 16 mmol) in water (30 mL) was cooled in an ice bath, and 5% NaOH was added to adjust the pH to 10. Benzyl chloroformate (5.3 g, 31 mmol) was added in four portions during 30 min with stirring. The pH of the reaction mixture was maintained near 10 by adding additional 5% NaOH solution periodically. The reaction mixture was stirred overnight at rt and then extracted with EtOAc (3 × 50 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated *in vacuo* to give an oil (3.39 g). This was chromatographed (10% EtOAc/hexane) on a flash silica column to give **22** (R_f 0.31, 10% EtOAc/hexane) as a white solid (2.9 g, 79%): mp 51–53 °C; IR (KBr) 3400–3200, 1680 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.47–2.03 (m, 6 H), 4.15–4.27 (m, 1 H), 4.68–4.81 (m, 1 H), 5.10 (s, 2 H), 5.57–5.87 (m, 2 H), and 7.32 (s, 5 H); MS *m/z* 231 (M^+).

Anal. Calcd for $C_{14}H_{17}NO_2$: C, 72.70; H, 7.41; N, 6.06. Found: C, 72.69; H, 7.40; N, 6.00.

2-(Benzyloxycarbonylamino)-7-oxabicyclo[4.1.0]heptane (23). To a solution of **22** (5.00 g, 21.6 mmol) in $CHCl_3$ (200 mL) was added a solution of 32% peracetic acid (6.25 mL, 2.00 g of CH_3CO_3H, 26.3 mmol). The reaction mixture was maintained at 0–5 °C for 48 h. Excess acid was neutralized by adding 10% $NaHCO_3$ (75 mL) and the $CHCl_3$ layer was separated. The aqueous layer was extracted with $CHCl_3$ (2 × 50 mL). The combined $CHCl_3$ layers were dried (Na_2SO_4) and evaporated *in vacuo* to give **23** (5.0 g, 93%) as a colorless oil. This was crystallized from cold hexane to give a white solid: mp 72–74 °C; IR (KBr) 3300, 1680 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.20–1.87 (m, 6 H), 3.21–3.32 (br s, 2 H), 3.98–4.17 (m, 1 H), 5.11 (s, 2 H), 5.14–5.27 (m, 1 H) and 7.34 (s, 5 H); MS *m/z* 247 (M^+).

Anal. Calcd for $C_{14}H_{17}NO_3$: C, 67.99; H, 6.93; N, 5.66. Found: C, 67.78; H, 7.00; N, 5.56.

3-(Benzyloxycarbonylamino)-2-hydroxycyclohexanecarbonitrile (24). To a solution of epoxide **23** (4.00 g, 16.2 mmol) in toluene (60 mL) was added a solution of Et_2AlCN (1 M in toluene, 45.0 mL, 45.0 mmol). The reaction mixture was stirred at rt under N_2 for 12 h. It was then poured into an ice-cold solution of 2 N KOH (150 mL) and extracted with $CHCl_3$ (3 × 75 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and concentrated *in vacuo* to give **24** (3.2 g, 72%) as a white solid which was recrystallized from EtOAc/hexane: mp 108–109.5 °C; IR (KBr) 3600–3100, 2200, 1660 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.54–2.03 (m, 6 H), 2.80–2.90 (m, 1 H), 3.41–3.57 (br s, 1 H, O*H*), 3.89–4.08 (m, 2 H), 5.05–5.22 (m, 3 H), and 7.32 (s, 5 H); ^{13}C NMR ($CDCl_3$) δ 156.7, 136.0, 128.6, 120.2, 69.7, 67.2, 50.5, 33.0, 26.8, 24.4, 20.1; MS *m/z* 274 (M^+).

Anal. Calcd for $C_{15}H_{18}N_2O_3$: C, 65.68; H, 6.61; N, 10.21. Found: C, 65.59; H, 6.61; N, 10.25.

Ethyl 3-(Benzyloxycarbonylamino)-2-hydroxycyclohexanecarboxylate (25). Nitrile **24** (3.0 g, 10.9 mmol) was dissolved in anhydrous ethanol (40 mL), and anhydrous HCl gas was bubbled through the solution for 45 min. The reaction mixture was maintained near −20 °C for 4 days. The product was dissolved in water (50 mL), which turned milky, and storage at rt for 18 h resulted in the separation of an oil. This mixture was extracted with EtOAc (3 × 50 mL), the organic extracts were dried (Na_2SO_4), and the solution was concentrated *in vacuo* to give crude **25** as an oil (3.2 g, 90%). This was chromatographed on a flash silica column (40% EtOAc/hexane) to give pure **25** (R_f 0.30, 40% EtOAc/hexane) as an oil (2.53 g, 72.1%): IR (neat) 3700–3100, 1700, 1670 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.23–1.30 (t, 3 H), 1.33–2.02 (m, 6 H), 2.45–2.56 (m, 1 H), 3.97–4.08 (m, 2 H), 4.12–4.23 (q, 2 H), 4.93–5.16 (m, 3 H), and 7.32 (s, 5 H); ^{13}C NMR ($CDCl_3$) δ 174.2, 157.0, 136.2, 128.6, 128.2, 71.1, 67.0, 60.8, 51.0, 46.1, 28.2, 26.2, 19.6, 14.2.

Anal. Calcd for $C_{17}H_{23}NO_5$: C, 63.54; H, 7.21; N, 4.36. Found: C, 63.39; H, 7.26; N, 4.41.

Ethyl 3-Ammonio-2-hydroxycyclohexanecarboxylate, Acetate (26). A mixture of 10% Pd/C (300 mg), ethanol (5 mL), water (5 mL), and acetic acid (5 mL) was stirred at rt under 1.0 atm of hydrogen gas. After 2 h, a solution of **25** (1.00 g, 3.11 mmol) in ethanol (5 mL) was added. This reaction mixture was hydrogenated for 24 h at rt. The catalyst was filtered and washed with hot water, and the filtrate was concentrated *in vacuo* to give crude **26** as an oil (0.766 g, 99.0%). This was crystallized from EtOAc/hexane to give pure **26** (0.710 g, 92.3%) as a white solid: mp 105–106 °C; IR (KBr) 3500–2500, 1700 cm^{-1}; ^1H NMR (D_2O) δ 1.07 (t, 3 H), 1.31–1.78 (m, 6 H), 1.81 (s, 3 H), 2.51–2.62 (m, 1 H), 3.37–3.42 (m, 1 H), 3.95–4.06 (m, 3 H).

Anal. Calcd for $C_{11}H_{21}NO_5$: C, 53.43; H, 8.56; N, 5.66. Found: C, 53.40; H, 8.60; N, 5.61.

Ethyl 3-(*N,N*-Dimethylamino)-2-hydroxycyclohexanecarboxylate (27). Compound **26** (0.500 g, 2.02 mmol) was dissolved in deionized water (5 mL) in a hydrogenation pressure bottle. To this was added 10% Pd/C (300 mg) followed by 36% formaldehyde (0.50 mL, 0.18 g of formaldehyde, 5.9 mmol). The mixture was shaken on a Parr shaker (50 psi H_2) for 24 h. The mixture was filtered and the catalyst was washed with hot deionized water. The filtrate was cooled in an ice bath and saturated with sodium carbonate. This mixture was then extracted with $CHCl_3$ (3 × 25 mL), the combined extracts were dried (Na_2SO_4), and the solution was concentrated *in vacuo* to give **27** as a colorless oil (0.380 g, 87.3%): IR (neat) 3600–3200, 1720 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.22–1.30 (t, 3 H), 1.31–1.91 (m, 6 H), 2.17–2.27 (m, 1 H), 2.31 (s, 6 H), 2.90–2.97 (m, 1 H), 3.43–3.58 (br s, 1 H, O*H*), 4.10–4.20 (q, 2 H), 4.34–4.39 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 173.4, 65.1, 63.3, 60.3, 44.7, 42.7, 24.3, 21.5, 21.4, 14.2.

Anal. Calcd for $C_{11}H_{21}NO_3$: C, 61.37; H, 9.83; N, 6.51. Found: C, 61.33; H, 9.77; N, 6.43.

Ethyl 2-Hydroxy-3-(trimethylammonio)cyclohexanecarboxylate, Chloride (28). A solution of **27** (0.350 g, 1.62 mmol) in anhydrous acetone (10 mL) was placed in a pear-shaped pressure bottle. The mixture was cooled to −78 °C and CH_3Cl gas was bubbled through the solution for 10 min. The bottle was capped and the solution was stirred at rt for 48 h. A white solid separated during this time. The solid was filtered and washed with anhydrous acetone (10 mL) to give **28** (0.325 g, 74.9%): mp 159–161 °C; IR (KBr) 3500–3200,

1720 cm^{-1}; ^1H NMR (D_2O) δ 1.04–1.12 (t, 3 H), 1.15–1.93 (m, 6 H), 2.79–2.85 (m, 1 H), 2.98 (s, 9 H), 3.16–3.29 (m, 1 H), 3.96–4.07 (q, 2 H), and 4.74–4.78 (m, 1 H); ^{13}C NMR (D_2O) δ 174.1, 72.8; 64.6, 62.2, 52.3, 48.1, 21.6, 20.4, 20.1, 13.3.

Anal. Calcd for $C_{12}H_{24}ClNO_3$: C, 54.23; H, 9.10; N, 5.27; Cl, 13.34. Found: C, 53.95; H, 9.16; N, 5.24; Cl, 13.27.

2-Hydroxy-3-(trimethylammonio)cyclohexanecarboxylic Acid, Chloride (4). A solution of **28** (0.325 g, 1.22 mmole) in 10% HCl (20 mL) was heated at reflux for 5 h. The reaction mixture was cooled to rt and concentrated to dryness *in vacuo* to yield a white solid. This was triturated with anhydrous acetone (10 mL) and filtered to give **4** (0.270 g, 92.8%), which was recrystallized from ethanol/ether: mp 237–238 °C; IR (KBr) 3500–2700, 1710 cm^{-1}; ^1H NMR (D_2O) δ 1.16–1.93 (m, 6 H), 2.78–2.85 (m, 1 H), 2.99 (s, 9 H), 3.23–3.32 (m, 1 H), and 4.73–4.76 (br s, 1 H); ^{13}C NMR (D_2O) δ 177.8, 72.6, 64.7, 52.2, 47.8, 21.6, 20.4, 20.1.

Anal. Calcd for $C_{10}H_{20}ClNO_3$: C, 50.52; H, 8.48; N, 5.89; Cl, 14.91. Found: C, 50.63; H, 8.52; N, 5.90; Cl, 14.97.

Enzyme Kinetics Assays. Pigeon Breast CAT. The procedure was essentially identical to the method that we previously described.[17] Briefly, the reaction mixture, which contained 100 mM MOPS, 10 mM EDTA, 0.25 mM DTNB, 0.2 mM acetyl-CoA, and 0.5 unit of enzyme in a volume of 1 mL was incubated at 25 °C for 1 min. The reaction was initiated by adding L-carnitine (0.04–0.80 mM final concentrations), and the change in thiolate absorbance at 412 nm was monitored. The alternate substrate activities for **2–4** were measured at 10 mM concentration in the absence of L-carnitine. The inhibitory activities for **2–4** were determined at concentrations of 2.5–10 mM. For the latter, the inhibitor was added to the reaction mixture at appropriate concentration and incubated for 1 min at 25 °C, and the reaction was then initiated by the addition of L-carnitine (0.04–0.80 mM final concentration).

Cultured Neonatal Rat Cardiac Myocyte CPT-2 Assay. The details of the CPT-2 assay have been described.[17] Briefly, neonatal rat cardiac myocytes were isolated and cultured in 35 mm, 12-well plates (2×10^5 cells/well) as previously described.[18] The mitochondrial membranes of these cells were permeabilized with 0.16% Triton X-100 to inactivate CPT-1 and to express latent CPT-2. After removal of the permeabilization medium from each well, a solution (0.5 mL) containing 10 mM HEPES (pH 7.0) and 1% BSA was added. Palmitoyl-CoA was present at a final concentration of 60 μM. Increasing concentrations of L-[^{14}C]carnitine (specific activity = 2000–3000 dpm/nmol) were then added to a series of cell cultures (0.08–0.40 mM final concentration) to initiate the reaction. Under these conditions initial rates of [^{14}C]palmitoylcarnitine formation were linear. The K_i of **2–4** for CPT-2 was determined by adding the inhibitor to a final concentration of 5 mM in each well. After 20 min of gentle shaking at 37 °C, the reaction was stopped with butanol-saturated 0.73 M HCl, and the amount of radioactive product in the butanol extract was determined by scintillation counting.

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