3-Alkyl-6-Chloro-2-pyrones: Selective Inhibitors of Pancreatic Cholesterol Esterase

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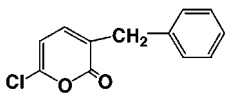
A series of 3-alkyl-6-chloro-2-pyrones with cyclohexane rings tethered to the 3-position was synthesized. The tether ranged from 0 to 4 methylene units. Inhibition of pancreatic cholesterol esterase by this series of pyrones was markedly dependent upon the length of the tether. Dissociation constants as low as 25 nM were observed for 6-chloro-3-(1-ethyl-2-cyclohexyl)-2-pyranone. This class of cholesterol esterase inhibitors functioned as simple competitive inhibitors of substrate binding rather than as suicide substrates or active site inactivators. Trypsin and chymotrypsin were not strongly inhibited by this class of pyrones. Selectivities for cholesterol esterase were greater than 10^3. This is in contrast to 3-aryl-6-chloro-2-pyrones which are nonselective, irreversible inactivators of serine hydrolases. Thus, replacement of the 3-aryl group by an appropriately tethered 3-alkyl ring can produce highly selective inhibitors of cholesterol esterase. A second series of halogen-containing esters was prepared in which cholesterol was esterified with α-haloacyl halides. These haloesters were simple substrates of cholesterol esterase with no evidence of irreversible inactivation.

Bile salt-stimulated cholesterol esterase (CEase), also known as bile salt-stimulated lipase, pancreatic carboxyl ester lipase, pancreatic lysophospholipase, nonspecific lipase, and pancreatic CEase, functions in the hydrolysis of dietary cholesterol esters as well as other dietary esters. CEase has been suggested to have a critical role in determining the bioavailability of dietary cholesterol by catalyzing both the hydrolysis of cholesterol esters and the transport of free cholesterol across the brush border of the intestinal villi.[1,2] Recent gene knockout studies, however, suggest that CEase primarily is involved in hydrolysis of cholesterol esters. Knockout mice exhibited a dramatically impaired ability to absorb cholesterol derived from cholesterol esters but exhibited normal absorption of free cholesterol.[3]

CEase is a classical serine hydrolase in terms of its catalytic triad of S194, H435, and D320 (numbered for rat CEase) and the presence of an α/β-hydrolase fold. Inhibitors of serine hydrolases, such as 3-benzyl-6-chloro-2-pyrone (**1**), inactivate CEase and prevent absorption of cholesterol esters in animal studies,[4] which is in agreement with results from gene knockout studies,



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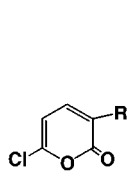
both of which argue for an important role of CEase in digestion of cholesterol esters. 3-Aryl-6-chloro-2-pyrones such as **1** were developed as potential mechanism-based inactivators of serine proteinases.[5] Their use as inactivators of CEase is limited due to lack of selectivity.

In the present study, we have addressed the question of development of selective inhibitors of CEase based upon the 6-chloro-2-pyrone backbone. A series of 3-alkyl-6-chloro-2-pyrones of general structure **2** was synthesized in which the 3-substituent included an aliphatic rather than aromatic ring. This approach was designed to test the hypothesis that replacement of aromatic groups in the 3-position of **1** with aliphatic groups would lead to the development of selective inhibitors of CEase compared to chymotrypsin. A second series of halogenated esters of general structure **3** was synthesized in



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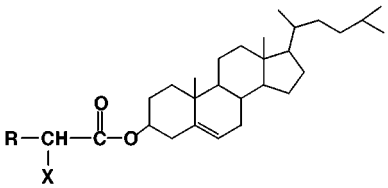

3

which cholesterol was esterified with short or long chain fatty acids containing chloro or bromo groups at the 2-position. Both series **2** and **3** have the potential to function as (1) conventional substates of CEase where both acylation and deacylation of the active site serine are facile reactions; (2) pseudosubstrates where acylation is followed by slow deacylation; (3) suicide inhibitors where formation of the acyl enzyme is followed by nucleophilic attack by some enzyme reactive group at the halogenated carbon to form an irreversible modification and inactivation of CEase; (4) conventional competitive inhibitors; or (5) irreversible inactivators where binding to the active site is followed by covalent modification of CEase in a reaction that is not part of the catalytic cycle.

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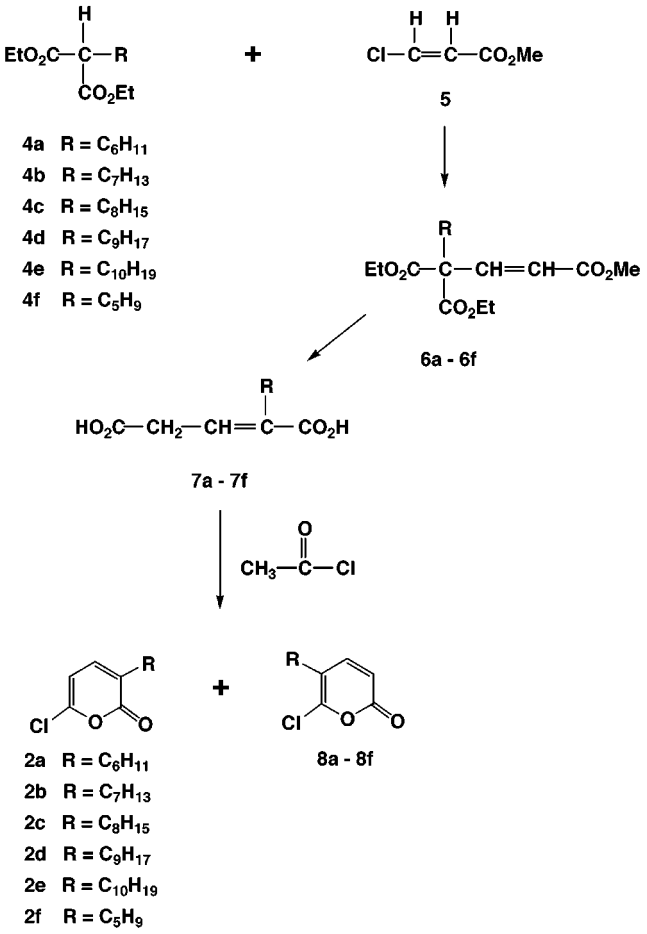
Scheme 1



Chemical Syntheses

The synthesis of 3-alkyl-6-chloro-2-pyrones (**2**) was accomplished as described in Scheme 1. The appropriately substituted diethyl malonate compounds were synthesized by published procedures. The resulting diesters were reacted with sodium hydride to form the sodium salts of compounds **4a**−**f** and then heated with methyl *cis*-2-chloroacrylate (**5**) in tetrahydrofuran. The resulting triesters **6a**−**f** were saponified, acidified, and decarboxylated to give substituted pentenedioic acids (glutaconic acids) **7a**−**f** as mixtures of synthetically equivalent isomers. Reflux of the mixture of isomers with acetyl chloride formed the 3-alkyl-6-chloro-2-pyrones **2a**−**f** with minor amounts of the 5-alkyl-6-chloro-2-pyrones **8a**−**f**; the isomers are thermally interchangeable with the equilibrium generally favoring the 3-substituted pyrones.

The synthesis of halogenated cholesterol esters was carried out as described in Scheme 2. Cholesterol (**9**) was reacted with an α-haloacyl halide (**10a**−**g**) in methylene chloride containing triethylamine to form esters **11a**−**g**. Halides **10a**−**g** are either commercially available or were prepared from the corresponding α-haloacid by reaction with thionyl chloride, except for α-chloropalmitoyl chloride (**10d**) and α-chlorohexanoyl chloride (**10c**) which were synthesized by treatment of the corresponding carboxylic acids with thionyl chloride and *N*-chlorosuccinimide.

Scheme 2

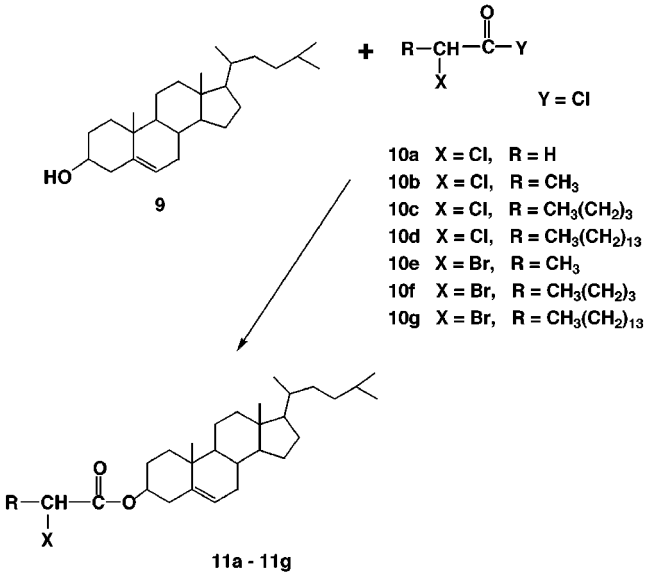
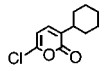
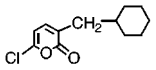
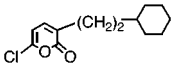
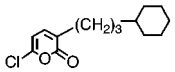
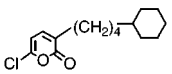
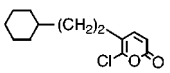


Table 1. Inhibition of Cholesterol Esterase by 6-Chloro-2-pyrones

	inhibitor	K_i (μM)
2a		2.3
2b		2.2
2c		0.025
2d		0.06
2e		0.58
2f		0.04
8c		0.058

Inhibition of Pancreatic CEase, Chymotrypsin, and Trypsin by 3-Alkyl-6-chloropyrones and Cholesteryl-α-haloesters

Inhibition of CEase by 6-chloropyrones containing a tethered cyclohexane ring at the 3-position is highly dependent upon the length of the tether. For 3-cyclohexyl-6-chloropyrone (**2a**) where the cyclohexane ring is attached to the pyrone ring without a spacer, K_i = 2.3 μM (Table 1) and is not altered by introduction of a single methylene spacer (**2b**, K_i = 2.2 μM). However, introduction of a 2-methylene spacer improves binding 100-fold; for **2c**, K_i = 0.025 μM. Lengthening the spacer to three or four methylenes (**2d** and **2e**) increases the K_i values. Thus, in the tethered cyclohexane series, the 2-methylene spacer produces the most potent inhibitor. In all cases, inhibition is competitive as shown by the double reciprocal plots in Figure 1 for **2c**. The inhibition of CEase by **2c** was also analyzed by Dixon plots and by Straus-Goldstein plots for high-affinity inhibitors; the results were the same.

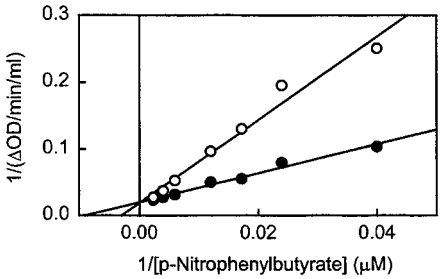


Figure 1. Double reciprocal plot of the inhibition of CEase by **2c** (0.1 μM, open circles) with *p*-nitrophenylbutyrate as substrate demonstrates that inhibition is competitive, K_i = 0.025 μM.

The structural features of alkyl-6-chloropyrones that are potent inhibitors of CEase are more complicated than suggested by the tethered cyclohexane series. Compound **2f** in which a cyclopentane ring is attached directly to the 3-position of the pyrone ring is almost as strong an inhibitor as **2c**; (for **2f**, K_i = 0.040 μM). Likewise, **8c**, the positional isomer of **2c** in which the tethered cyclohexane ring is attached to the 5-position, is a good inhibitor, K_i = 0.058 μM. It is apparent, therefore, that inhibition of CEase by alkyl-6-chloropyrones is amenable to wide variation in the nature and location of the alkyl groups.

The inhibition of CEase by **2a**−**f**, **8c** was analyzed using *p*-nitrophenylbutyrate as substrate in an assay system that included the bile salt taurocholate. In the absence of bile salt, the Michaelis constant of *p*-nitrophenylbutyrate increased from 64 to 175 μM. Likewise, the K_i values of **2c** increased from 0.025 μM in the presence of bile salt to 0.090 μM in the absence of bile salt.

The possibility that **2a**−**f**, **8c** are substrates of CEase or pseudosubstrates was examined using **2c** as a representative inhibitor. CEase and excess **2c** were incubated at neutral pH for up to 6 h with samples examined periodically by TLC for formation of 2-(2-cyclohexylethyl)glutaconic acid (**7c**), the expected product of the hydrolysis of **2c**. No evidence for product formation was obtained. Likewise, separation of CEase from **2c** after several hours of incubation provided active enzyme, suggesting that **2c** is neither a substrate nor a pseudosubstrate where deacylation is slow. We conclude, therefore, that the substituted pyrones are simple competitive inhibitors of CEase.

Inhibition of trypsin and chymotrypsin by **2a**−**f** was examined to determine the extent of selectivity for CEase. This series of pyrones did not inhibit trypsin at inhibitor concentrations up to 250 μM. Inhibition of chymotrypsin was competitive. The most potent inhibitor of chymotrypsin was **2c**; K_i = 50 μM compared to K_i = 0.025 μM for inhibition of CEase by **2c**. Thus, selectivity for CEase is greater than 10^3.

The second series of haloesters, compounds **11a**−**g**, was examined as potential suicide substrates or irreversible active site inhibitors of CEase. These cholesterol esters were incubated with CEase, and samples were examined by TLC. These esters were substrates of CEase with no evidence of inactivation of CEase.

Discussion

The concept that interference with the absorption of cholesterol may represent a useful approach to the problem of hypercholesterolemia has a long history. Plant sterols such as campesterol and sitosterol have long been known to reduce plasma cholesterol by inhibiting cholesterol absorption.[6] This appears to result from competition with cholesterol for incorporation into micelles, although other absorption steps may also be involved. However, large doses of sitosterol are required for modest reductions in plasma cholesterol. Although interest in plant sterols decreased somewhat as new approaches to treatment of hypercholesterolemia were developed, such as introduction of the statins, the use of agents that reduce cholesterol absorption has reached commercial success. Sitostanol, a 5-α-saturated derivative of sitosterol, and its esters have been reported to be more effective hypocholesterolemic agents than sitosterol.[7] Sitostanol-ester margarine for treatment of hypercholesterolemia[8] is now available commercially. Recently, it has been shown that margarines enriched with oils containing either sitosterol-esters or sitostanol-esters were equally effective in lowering LDL-cholesterol,[9] which suggests that margarines containing a wide range of plant sterol-esters will likely be developed.

Other approaches to interference with cholesterol absorption with a long history include the use of the nonabsorbable aminoglycoside antibiotic neomycin, which appears to inhibit cholesterol absorption by forming complexes with cholesterol that are excreted,[10] and use of the bile salt binder cholestyramine, an anion exchanger that indirectly alters cholesterol levels by limiting the resorption of cholesterol-derived bile salts. All of these approaches suffer to some extent from the sizable quantities of therapeutic agents that must be administered. The development of selective inhibitors of cholesterol absorption that are targeted at specific enzymes would appear to be a more attractive approach. More recent studies of cholesterol absorption have identified a scavenger receptor class B type I that is present in the brush border membrane. This receptor appears to function as a conduit for entry of a variety of lipids into the mucosal cells. Apolipoprotein A-I interacts with this receptor to inhibit lipid entry, suggesting that this receptor may be a selective target for therapeutic intervention.[11]

Digestion of dietary lipid prior to absorption primarily involves gastric lipase and the three pancreatic enzymes triglyceride lipase, phospholipase A_2, and CEase. The broad specificity of CEase suggests that this hydrolase participates in the hydrolysis of triglycerides as well as catalyzing the hydrolysis of cholesterol esters. However, the activity of triglyceride lipase in pancreatic juice appears to be much greater than CEase activity, at least in rat, which suggests that selective inhibition of CEase will not interfere with digestion of triglycerides. It is noteworthy that in some species of fish CEase also functions as triglyceride lipase.[12]

The possible dual roles of CEase in hydrolysis of cholesterol esters as well as in transport of cholesterol into mucosal cells of the small intestine have been studied extensively. Rat pancreatic CEase enhances sterol transfer in small unilamellar vesicles that contain negatively charged phospholipids, independent of esterase activity.[13] CEase binds to membrane-associated heparin on the intestinal brush border. This appears to involve a proline-rich 11 amino acid repeat in the

C-terminal portion of CEase. The binding of human CEase to membrane-bound heparin increases the enzyme activity, whereas soluble heparin is a potent inhibitor.[14] The results from gene knockout studies, however, do not support a role for CEase in the absorption of cholesterol.[3] This may reflect differences between human and mouse CEase. Most species that have been analyzed contain 2−4 repeat units while human CEase contains 16 proline-rich repeats. The inhibitory properties of heparin depend on the size of the repeat, and it has been suggested that this could regulate cholesterol uptake.[14] Thus the role of CEase in absorption of cholesterol remains controversial. A recent report of cholesterol uptake by human intestinal cells identified pancreatic phospholipase A_2 as the major cholesterol transport protein.[15] This was shown to be due to phospholipase-induced modification of the micellar lipid composition rather than to direct transport of cholesterol. It was suggested that CEase may act similarly to enhance cholesterol uptake, which would argue against a direct transport function.

6-Chloro-2-pyrones and the related 3-chloroisocoumarins were initially developed as irreversible inactivators of serine proteases.[5,16] Although it was suggested that this class of pyrones operated as mechanism-based (suicide) inactivators, subsequent studies suggested that inactivation is due to slow turnover of the acylated enzymes.[17] The most extensive structure−activity studies of 6-chloro-2-pyrones with alkyl or aryl substituents at positions 3, 4, or 5 were carried out by Boulanger and Katzenellenbogen who demonstrated that both binding and inactivation of chymotrypsin are markedly dependent on the nature and location of the substituent.[18] Chymotrypsin is inactivated by 3-benzyl-6-chloro-2-pyrone through formation of a fairly stable acyl enzyme intermediate.[17] This same pyrone inactivates CEase even more rapidly than chymotrypsin,[4] suggesting that 3-aryl-substituted 6-chloro-2-pyrones are poor choices for development of selective inhibitors of CEase. By comparison, 3-ethyl- or 3-butyl-6-chloro-2-pyrone inhibit chymotrypsin poorly but reversibly (K_i = 785 and 1980 μM, respectively).[18] These results with chymotrypsin are in agreement with the present study where the 3-alkyl-6-chloro-2-pyrones in Table 1 were generally poor, reversible inhibitors of chymotrypsin. Thus the selectivity that was attained in the development of 3-alkyl-6-chloro-2-pyrones as inhibitors of CEase resulted from a combination of weak inhibition of chymotrypsin and potent inhibition of CEase. Weak inhibition of chymotrypsin by 3-alkyl-6-chloro-2-pyrones is markedly different from the inhibition of chymotrypsin by 5-alkyl-6-chloro-2-pyrones. For example, 5-ethyl- or 5-butyl-6-chloro-2-pyrone are strong inhibitors of chymotrypsin (K_i = 5.6 and 3.9 μM, respectively).[18] This suggests that 3-alkyl-6-chloro-2-pyrones are more promising than 5-alkyl-6-chloro-2-pyrones as selective inhibitors of CEase.

The development of mechanism-based inhibitors of CEase includes boronic and borinic acids, arylhaloketones, aryl phosphates and phosphonates, and aryl and cholesteryl carbamates.[19] The most extensive molecular recognition studies have been carried out with carbamates,[20] which are transient inhibitors, or pseudosubstrates, wherein rapid acylation (carbamylation) of the active site serine is followed by slow deacylation. Kinetic studies of CEase with carbamates allowed SAR to be developed that individually describe the binding, acylation, and deacylation steps of the CEase catalytic cycle and suggest a role for bile salts in conformational modulation of both the fatty acid and steroid binding sites to increase the hydrophobicity of these sites, at least in the case of porcine CEase.[20]

In the present study, we focused on the development of 3-substituted 6-chloro-2-pyrones with a tethered cyclohexane ring attached to the 3-position. The size of the tether ranged from 0 to 4 methylene units (structures **2a**−**e**). In addition, 6-chloro-2-pyrone with a cyclopentane ring attached at the 3-position was synthesized (**2f**) in order to compare a five-membered ring substituent with a six-membered ring substituent. Inhibition of CEase was compared with inhibition of trypsin and chymotrypsin to gain some insight into selectivity. The results (Table 1) suggest that replacement of 3-aryl substituents as in **1** with 3-alkyl substituents as in **2a**−**f** results in the formation of compounds that are highly selective for CEase. The results also indicate that the compounds in Table 1 are simple competitive inhibitors of substrate binding. There was no indication that CEase was inactivated through covalent modification. This is surprising in view of the reported rapid inactivation of CEase by 3-benzyl-6-chloro-2-pyrone.[4] It is anticipated that further modification of the structures of **2** will lead to the development of potent inhibitors that will irreversibly modify and inactivate CEase.

Molecular modeling studies of CEase predicted that CEase would have a relatively open active site for substrate binding, unlike the situation with a number of lipases where access to the active site requires the movement of a hinge-like surface loop.[21] Two recent crystal structure studies of bovine CEase support the conclusion that movement of a hinge-like surface loop is not required for activity.[22,23] Interestingly, bovine CEase crystallized in the absence of detergent or bile salts adopts a conformation in which the highly conserved C-terminal hexapeptide is lodged in the active site, with distortion of the oxyanion hole away from a productive binding mode.[23] In the presence of bile salts, CEase adopts a conformation with an open active site and a productive orientation of the oxyanion hole; one molecule of bile salt is located near the active site while a second is complexed at a remote site.[22] These results suggest that part of the mechanism of bile salt activation of CEase involves displacement of the C-terminal peptide and conformational change leading to formation of a competent active site. The fact that CEase is active with small water soluble substrates such as *p*-nitrophenylbutyrate in the absence of bile salts may indicate that some substrates are capable of displacing the C-terminal peptide from the active site. Inhibitor **2c** inhibits CEase both in the presence and absence of bile salts; however, K_i increases from 0.025 μM in the presence of bile salts to 0.090 μM in the absence of bile salts which suggests that some energy is being expended to displace the C-terminal peptide from the active site when bile salts are absent.

In summary, the present study has identified 3-alkyl-6-chloro-2-pyrones as selective inhibitors of CEase.

These inhibitors represent promising lead compounds for development of irreversible inhibitors of CEase as potential drugs for treatment of hypercholesterolemia.

Experimental Section

Chemical Synthesis. Reagent quality solvents were used without further purification. Melting points were determined on a VWR Scientific Electrothermal capillary melting point apparatus and are uncorrected. Infrared spectra were obtained on a FT-IR Bomem MB-100 spectrophotometer. NMR spectra were recorded on a Bruker AC250 NMR spectrometer in $CDCl_3$, unless otherwise stated. Chemical shifts are in ppm (δ) relative to TMS. Solvents and other chemicals are reagent grade. The α-bromoacyl chlorides and α-chloroacyl chlorides that were not readily available were synthesized by reported procedures.[24] *cis*-3-Chloroacrylic acid was made from propiolic acid[25] and then was esterified to form the methyl ester (**5**).[26] The diethyl 2-alkylmalonates (**4a**−**f**) were prepared according to published procedures.[27,28]

6-Chloro-3-(1-ethyl-2-cyclohexyl)-2-pyranone (2c) and 6-Chloro-5-(1-ethyl-2-cyclohexyl)-2-pyranone (8c). Diethyl 2-(2-cyclohexylethyl)malonate (9.02 g, 33.4 mmol) was added dropwise to a solution containing pentane-washed sodium hydride (0.80 g, 33.4 mmol) in 100 mL of tetrahydrofuran. After the mixture was stirred for 10 min, methyl *cis*-2-chloroacrylate (4.03 g, 33.4 mmol) was added, and the resulting mixture was heated under reflux for 14 h. After cooling, water was added and the mixture was extracted with ether. The ether extracts were combined, washed with saturated sodium chloride, dried over magnesium sulfate, filtered, and evaporated to afford the triester as an oil (10.5 g, 89%). The oil was dissolved in 20 mL of ethanol, and 150 mL of water containing sodium hydroxide (10 g, 250 mmol) was added. The mixture was refluxed for 8 h, cooled, and adjusted to pH 1 with concentrated hydrochloric acid. After foaming ceased, the mixture was refluxed for 1 h, cooled, and extracted with ether. The ether extracts were washed with saturated sodium chloride, dried over magnesium sulfate, filtered, and evaporated to give a mixture of 2-ethylcyclohexyl glutaconic acids, 7.92 g (99%), as an oily solid. Trituration of the mixture with ethyl acetate afforded a buff solid which was recrystallized from ethyl acetate to give *E*-2-(2-cyclohexylethyl)-2-pentenedioic acid as white crystals: mp 117−118 °C; ^{1}H NMR (DMSO-d_6) δ 0.87−1.75 (m, 13 H), 2.20 (t, 2H), 3.15 (d, 2H), 6.71 (t, 1H), 12.34 (s, 2H). Evaporation of the filtrate and recrystallization from chloroform gave *Z*-2-(2-cyclohexylethyl)-2-pentenedioic acid as a white solid: mp 166−167 °C; ^{1}H NMR (DMSO-d_6) δ 0.87−1.75 (m, 13H), 2.20 (t, 2H), 3.39 (d, 2H), 6.00 (t, 1H), 12.34 (s, 2H). Assignment of *E* and *Z* isomers was based upon previous reports of NMR data.[29] The mixture of glutaconic acids (7.92 g, 33.0 mmol) and acetyl chloride (80 mL) was refluxed for 3 days. The cooled solution was concentrated to give a dark oil (7.92 g, 85%) that was flash chromatographed on silica gel with ethyl acetate/hexane to give 2.00 g (25%) of **2c** as white crystals: mp 33−34 °C; ^{1}H NMR δ 0.86−1.77 (m, 13H), 2.44 (t, 2H), 6.16 (d, 1H, J = 6.97 Hz), 7.17 (d, 1H, J = 6.99 Hz). An analytical sample was obtained by sublimation at 80 °C (0.1 Torr). Anal. ($C_{13}H_{17}ClO_2$) C, H. Compound **8c** (0.9 g, 12%) was eluted second from the column and obtained as an oil: ^{1}H NMR δ 1.16−1.66 (m, 13H), 2.41 (t, 2H), 6.23 (d, 1H, J = 9.3 Hz), 7.29 (d, 1H, J = 9.4 Hz). Assignment of 3 and 5 isomers was based upon previous reports of NMR data.[30]

6-Chloro-3-cyclohexyl-2-pyranone (2a). In the same manner as for **2c** diethyl 2-cyclohexylmalonate (10.5 g, 43.3 mmol) was added to sodium hydride in tetrahydrofuran followed by methyl *cis*-2-chloroacrylate. Saponification and decarboxylation of the resulting triester (13.5 g, 96%) gave a mixture of 2-cyclohexyl glutaconic acids as an oily solid (8.00 g, 87%). Reaction with acetyl chloride and chromatography of the resulting oil gave 2.00 g (22%) of **2a** as white crystals: mp 69−70 °C; ^{1}H NMR δ 1.14−1.92 (m, 10H), 2.60 (br t, 1H), 6.16 (d, 1H, J = 7.07 Hz), 7.02 (d, 1H, J = 7.10 Hz). Anal. ($C_{11}H_{13}ClO_2$) C, H.

6-Chloro-3-methylcyclohexyl-2-pyranone (2b). In the same manner as for **2c** diethyl 2-methylcyclohexylmalonate (8.50 g, 33.2 mmol) was added to sodium hydride in tetrahydrofuran followed by methyl *cis*-2-chloroacrylate. Saponification and decarboxylation of the resulting triester (10.5 g, 93%) gave a mixture of 2-methylcyclohexyl glutaconic acids (>90% *E* by NMR) as an oil which solidified to a buff solid (6.90 g, 92%). Trituration with ethyl acetate followed by recrystallization from ethyl acetate gave a white solid of *E*-2-(2-cyclohexylmethyl)-2-pentenedioic acid (**7b**): mp 140−142 °C; ^{1}H NMR (DMSO-d_6) d 0.86−1.60 (m, 11H), 2.10 (d, 2H), 3.19 (d, 2H), 6.81 (t, 1H), 12.4 (s, 2H). Assignment of the isomer was based upon previous reports of NMR data.[29] Reaction with acetyl chloride and chromatography of the resulting oil gave 1.90 g (25%) of **2b** as white crystals: mp 58−60 °C; ^{1}H NMR δ 0.81−1.80 (m, 11H), 2.45 (d, 2H), 6.16 (d, 1H, J = 6.95 Hz), 7.05 (d, 1H, J = 7.00 Hz). Anal. ($C_{12}H_{15}ClO_2$) C, H.

6-Chloro-3-(1-propyl-3-cyclohexyl)-2-pyranone (2d). In the same manner as for **2c** diethyl 2-(3-propylcyclohexyl)-malonate (9.50 g, 33.4 mmol) was added to sodium hydride in tetrahydrofuran followed by methyl *cis*-2-chloroacrylate. Saponification and decarboxylation of the resulting triester (11.5 g, 93%) gave a mixture of 2-propylcyclohexyl glutaconic acids (>90% *E* by NMR) as a semisolid (9.79 g, 93%): ^{1}H NMR (DMSO-d_6) δ 0.75−1.70 (m, 15H), 2.15 (t, 2H), 3.22 (d, 2H), 6.73 (t, 1H). Reaction with acetyl chloride and chromatography of the resulting oil gave 1.50 g (18%) of **2d** as white waxy crystals: mp 31−32 °C; ^{1}H NMR δ 0.80−1.71 (m, 15H), 2.40 (t, 2H), 6.15 (d, 1H, J = 7.00 Hz), 7.05 (d, 1H, J = 7.03 Hz). An analytical sample was obtained by sublimation at 80−90 °C (0.1 Torr). Anal. ($C_{14}H_{19}ClO_2$) C, H.

6-Chloro-3-(1-butyl-4-cyclohexyl)-2-pyranone (2e). In the same manner as for **2c** diethyl 2-(4-butylcyclohexyl)-malonate (7.50 g, 25.1 mmol) was added to sodium hydride in tetrahydrofuran followed by methyl *cis*-2-chloroacrylate. Saponification and decarboxylation of the resulting triester (8.80 g, 92%) gave a mixture of 2-butylcyclohexyl glutaconic acids as an oily solid (5.50 g, 82%). Reaction with acetyl chloride and chromatography of the resulting oil gave 1.20 g (18%) of **2e** as a waxlike solid: ^{1}H NMR δ 0.75−1.72 (m, 17H), 2.42 (t, 2H), 6.14 (d, 1H, J = 6.96 Hz), 7.05 (d, 1H, J = 6.97 Hz) Anal. ($C_{15}H_{21}ClO_2$) C, H.

6-Chloro-3-cyclopentyl-2-pyranone (2f). In the same manner as for **2c** diethyl 2-cyclopentylmalonate (11.0 g, 48.2 mmol) was added to sodium hydride in tetrahydrofuran followed by methyl *cis*-2-chloroacrylate. Saponification and decarboxylation of the resulting triester gave a 50/50 mixture (indicated by NMR) of 2-cyclopentyl glutaconic acids (9.00 g, 94%). Reaction with acetyl chloride and chromatography of the resulting oil gave 2.10 g (22%) of **2f** as a clear oil: ^{1}H NMR δ 1.32−2.05 (m, 8H), 2.95 (br p, 1H), 6.16 (d, 1H, J = 7.08 Hz), 7.09 (d, 1H, J = 7.04 Hz). Anal. ($C_{10}H_{11}ClO_2$) C, H.

Cholesteryl 2-Chloropalmitate (10d). Cholesterol (2.00 g, 5.20 mmol) was dissolved in methylene chloride, and triethylamine (1.45 mL, 10.4 mmol) and 2-chloropalmitoyl chloride (1.76 mL, 5.70 mmol) were added to the mixture. The reaction was allowed to stir overnight at which time TLC indicated complete disappearance of starting material. The methylene chloride reaction mixture was first washed twice with dilute HCl then twice with saturated sodium chloride and dried over magnesium sulfate. The mixture was then filtered, and the solvent was removed by rotary evaporation. The resulting solid was purified on a silica gel column. Elution with methylene chloride gave 2.73 g (80%) of **10d** as a white solid: mp 52−53 °C; IR 1740 (carbonyl) cm^{-1}; ^{1}H NMR δ 0.65−2.39 (m, 72H), 4.20 (t, 1H), 4.62 (m, 1H), 5.37 (br d, 1H). Anal. ($C_{43}H_{75}BrO_2$) C, H.

Cholesteryl 2-Chlorohexanoate (10c). Cholesterol (2.00 g, 5.2 mmol), triethylamine (1.45 mL, 10.4 mmol), and 2-chlorohexanoyl chloride (0.96 mL, 5.7 mmol) were combined in methylene chloride. The same procedure as that used for **10d** was followed to afford 2.23 g (83%) of **10c** as white needles: mp 67−69 °C; IR 1740 (carbonyl) cm^{-1}; ^{1}H NMR δ 0.65−2.38

(m, 52H), 4.20 (t, 1H), 4.65 (m, 1H), 5.37 (br d, 1H). Anal. ($C_{33}H_{55}ClO_2$) C, H.

Cholesteryl 2-Chloropropionate (10b). Cholesterol (1.00 g, 2.60 mmol), triethylamine (0.73 mL, 5.20 mmol), and 2-chloropropionyl chloride (0.36 mL, 2.90 mmol) were combined in methylene chloride. The same procedure as that used for **10d** was followed to afford 0.99 g (80%) of **10b** as white needles: mp 123−125 °C; IR 1740 (carbonyl) cm^{-1}; ^1H NMR δ 0.65−2.38 (m, 46H), 4.33 (q, 1H), 4.64 (m, 1H) 5.36 (br d, 1H). Anal. ($C_{30}H_{49}ClO_2$) C, H.

Cholesteryl 2-Chloroacetate (10a). Cholesterol (2.00 g, 5.20 mmol), triethylamine (1.45 mL, 10.4 mmol), and chloroacetyl chloride (0.64 mL, 5.70 mmol) were combined in methylene chloride. The same procedure as that used for **10d** was followed to afford 1.92 g (80%) of **10a** as a white solid: mp 161−162 °C (lit.[31] mp 160−161 °C); IR 1740 (carbonyl) cm^{-1}; ^1H NMR δ 0.65−2.48 (m, 43H), 4.02 (s, 2H), 4.68 (m, 1H), 5.37 (br d, 1H). Anal. ($C_{29}H_{47}ClO_2$) C, H.

Cholesteryl 2-Bromopalmitate (10g). Cholesterol (2.00 g, 5.20 mmol), triethylamine (1.45 mL, 10.4 mmol), and 2-bromopalmitoyl chloride (2.00 mL, 5.70 mmol) were combined in methylene chloride. The same procedure as that used for **10d** was followed to afford 3.10 g (85%) of **10g** as a white solid: mp 50−52 °C; IR 1740 (carbonyl) cm^{-1}; ^1H NMR δ 0.65−2.38 (72H), 4.15 (t, 1H), 4.64 (m, 1H), 5.37 (br d, 1H). Anal. ($C_{43}H_{75}BrO_2$) C, H.

Cholesteryl 2-Bromohexanoate (10f). Cholesterol (2.00 g, 5.20 mmol), triethylamine (1.45 mL, 10.4 mmol), and 2-bromohexanoyl bromide (1.47 mL, 5.7 mmol) were combined in methylene chloride. The same procedure as that used for **10d** was followed to afford 2.50 g (85%) of **10f** as white needles: mp 60−62 °C; IR 1740 (carbonyl) cm^{-1}; ^1H NMR δ 0.65−2.38 (m, 52H), 4.15 (t, 1H), 4.62 (m, 1H), 5.37 (br d, 1H). Anal. ($C_{33}H_{55}BrO_2$) C, H.

Cholesteryl 2-Bromopropionate (10e). Cholesterol (2.00 g, 5.20 mmol), triethylamine (1.45 mL, 10.4 mmol), and 2-bromopropionyl bromide (1.23 mL, 5.7 mmol) were combined in methylene chloride. The same procedure as that used for **10d** was followed to afford 1.97 g (100%) of **10e** as white needles: mp 123−124 °C; IR 1740 (carbonyl) cm^{-1}; ^1H NMR δ 0.66−2.40 (m, 46H), 4.33 (q, 1H), 4.65 (m, 1H) 5.36 (br d, 1H). Anal. ($C_{30}H_{49}BrO_2$) C, H.

Enzyme Assays and Kinetics. CEase (porcine), chymotrypsin (bovine), and trypsin (porcine) were from Sigma. CEase was assayed in 0.1 M Hepes, pH 7, containing 6 mM taurocholate and 1 mM *p*-nitrophenylbutyrate, at 405 nm. Inhibitors of CEase were generally analyzed by double reciprocal and Dixon plots and, for high-affinity inhibitors such as **2c**, by Straus-Goldstein plots. Kinetic analysis of Michaelis constants and k_{cat} values was carried out by nonlinear regression analysis with the Enzfitter program. Dissociation constants of inhibitors were determined by linear regression analysis of the double reciprocal, Dixon, and Straus-Goldstein plots. Chymotrypsin was assayed using the same substrate and buffer as for CEase. Trypsin was assayed in 0.05 M Tris, pH 7.4, with 0.4 mM *p*-nitrophenyl-*p′*-guanidino benzoate at 405 nm.

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