Sciences Foundation for support of this work. J. H. is a Whitaker Health Sciences Fund graduate fellow. P.L. is grateful to the National Science Foundation for a Presidential Young Investigator Award and to Merck, Pfizer, ICI, General Electric, and Hoechst-Celanese for providing matching funds. We thank Charles Burkins and Paul Matsudaira of the Whitehead Protein Sequencing Facility for providing sequence information.

Syntheses, Structures, and Enzymatic Evaluations of Hemiacylcarnitiniums, a New Class of Carnitine Acyltransferase Inhibitors

Richard D. Gandour,*[†] Noelle L. Blackwell,[†,‡] William J. Colucci,[†,§] Chang Chung,[⊥,∥] Loran L. Bieber,[⊥] Rona R. Ramsay,[●] Eric P. Brass,[▽] and Frank R. Fronczek[†]

Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana 70803-1804, Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824, Department of Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, California 94121, Department of Biochemistry/Biophysics, University of California, San Francisco, California 94143, and Department of Medicine, Division of Clinical Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106

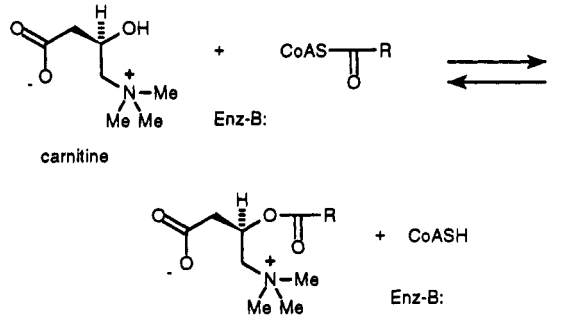
Received August 27, 1991 (Revised Manuscript Received February 11, 1992)

The syntheses of (2*S*,6*R*)-6-(carboxymethyl)-2-hydroxy-2,4,4-trimethylmorpholinium chloride (hemiacetylcarnitinium, HAC), (2*S*,6*R*)-6-(carboxymethyl)-2-ethyl-2-hydroxy-4,4-dimethylmorpholinium bromide (hemipropanoylcarnitinium, HPrC), and (2*S*,6*R*)-6-(carboxymethyl)-2-hydroxy-4,4-dimethyl-2-phenylmorpholinium chloride monohydrate (hemibenzoylcarnitinium, HBC) are described. The crystal structure of HAC is reported and compared with crystal structures of HPrC, HBC, carnitine·HCl, acetylcarnitine·HCl, and acetylcarnitine·HCl·H_2O. HAC, HPrC, and HBC inhibit carnitine acetyltransferase (CAT) activity from multiple biological sources. The best inhibitor, HAC, has K_i of 69 ± 5 μM with rat liver peroxisomal CAT. HAC binds more strongly than the natural substrate (and isomer), acetylcarnitine. HAC also strongly inhibits, K_i = 92 ± 14 μM, CAT from rat heart mitochondria. HPrC inhibits pigeon breast CAT with a K_i of 200 ± 30 μM. HBC inhibits pigeon breast CAT, rat heart mitochondrial CAT, rat liver mitochondrial CAT, and rat liver peroxisomal carnitine octanoyltransferase (COT), with values of K_i of 3500 ± 500, 2400 ± 70, 1670 ± 70, and 1100 ± 100 μM, respectively. Racemic 6-(carboxymethyl)-2-hydroxy-2-pentadecyl-4,4-dimethylmorpholinium bromide (hemipalmitoylcarnitinium, HPC) strongly inhibits (K_i = 2 ± 0.3 μM) beef liver mitochondrial CPT_i. In summary, hemiacylcarnitiniums show promise as a general class of carnitine acyltransferase inhibitors.

The carnitine acyltransferases comprise a family of proteins that differ with respect to subcellular localization, substrate specificity, and sensitivity to inhibitors.[1] The molecular basis for this protein diversity remains unexplained, but the recent mappings of multiple distinct gene sequences[2-4] confirm the cellular synthesis of distinct polypeptides. Insufficient data are available to address the question of active site homology among these enzymes. The evaluation of putative active-site-directed inhibitors against a spectrum of carnitine acyltransferases reveals similarities and differences among these enzymes, and it provides information that is needed to design specific inhibitors for each protein.

Carnitine acetyltransferase (CAT), carnitine octanoyltransferase (COT), and carnitine palmitoyltransferase (CPT) catalyze acyl transfer between carnitine and coenzyme A (CoASH). CAT, CPT, and carnitine–acylcarnitine translocase regulate the transport of activated acyl groups across the inner mitochondrial membrane, while peroxisomes and microsomes contain COT activity. The three-dimensional structures of these proteins are unknown.

Conformationally rigid inhibitors can probe the structure of enzymic binding sites. This idea originates in medicinal



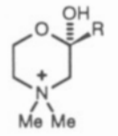
chemistry;[5,6] e.g., hemicholiniums[7] 1 are analogues of choline. Similarly, we make carnitine analogues, which we term hemiacylcarnitiniums,[8,9] 2. Racemic hemiacetyl-

[†] Louisiana State University.
[‡] Present address: Marathon Oil Inc., Garyville, LA.
[§] Present address: Ethyl Technical Center, Baton Rouge, LA.
[⊥] Michigan State University.
[∥] Present address: KKPC, Ltd. Res. Inst., Yeocheon-City, Korea.
[●] University of California.
[▽] Case Western Reserve University.

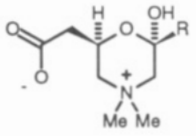
(1) Bieber, L. L. *Annu. Rev. Biochem.* **1988**, *57*, 261-283.
(2) Bloisi, W.; Colombo, I.; Caravaglia, B.; Giardini, R.; Finocchiaro, G.; DiDonato, S. *Eur. J. Biochem.* **1990**, *189*, 535-546.
(3) Chatterjee, B.; Song, C. S.; Kim, J. M.; Roy, A. K. *Biochemistry* **1988**, *27*, 9000-9006.
(4) Woeltje, K. F.; Esser, V.; Weis, V. C.; Sen, A.; Cox, W. F.; McPhaul, M. J.; Slaughter, C. A.; Foster, D. W.; McGarry, J. D. *J. Biol. Chem.* **1990**, *265*, 10720-10725. Finocchiaro, G.; Taroni, F.; Rocchi, M.; Martin, A. L.; Colombo, I.; Tarelli, G. T.; DiDonato, S. *Proc. Natl. Acad. Sci., U.S.A.* **1991**, *88*, 661-665.
(5) Mathison, I. W.; Solomons, W. E.; Morgan, P. H.; Tidwell, R. R. In *Principles of Medicinal Chemistry*, 3rd ed.; Foye, W. O., Ed.; Lea & Fibeger: Philadelphia, 1989; pp 49-77.
(6) Taylor, P.; Insel, P. A. In *Principles of Drug Action: the Basis of Pharmacology*, 3rd ed.; Pratt, W. B., Taylor, P., Eds.; Churchill Livingstone: New York, 1990; pp 23-43.
(7) Long, J. P.; Schueler, F. W. *J. Am. Pharm. Assoc.* **1954**, *43*, 79-85. Schueler, F. W. *J. Pharmacol. Expt. Therap.* **1955**, *115*, 127-143. Schueler, F. W. *J. Am. Pharm. Assoc.* **1956**, *45*, 197-199.

0022-3263/92/1957-3426$03.00/0

carnitinium[8] (HAC, **2**, R = CH_3) and racemic hemipalmitoylcarnitinium[9] (HPC, **2**, R = $(CH_2)_{14}CH_3$) inhibit pigeon breast CAT and rat liver mitochondrial CPT_i,[10] respectively.

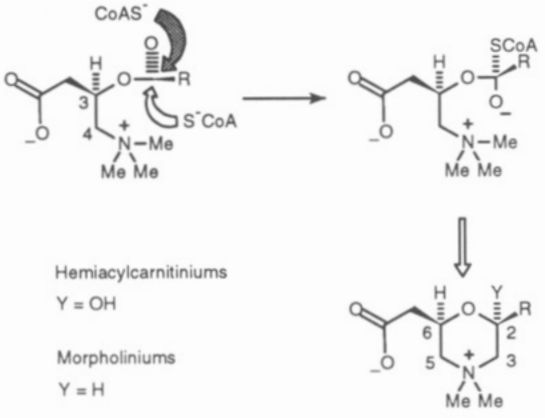


1



2

Inhibitor Design. The design rationale[11] for these conformationally rigid analogues emanates from solution[12] and single-crystal X-ray[13] structural studies, which show that carnitine and acetylcarnitine strongly prefer a gauche (−) conformation about the N–C4–C3–O torsion angle. The N–C5–C6–O torsion angle in this morpholinium ring is locked in a gauche conformation. Furthermore, these analogues resemble the tetrahedral intermediate that we have proposed[13] for CAT catalysis.



In forming such a tetrahedral intermediate, the thiolate should approach the acyloxy from the less-congested side (carboxylate on carnitine "folded" back). This attack vector (gray arrow) is on the *Re* face of the ester, presuming that the acyloxyl group is in the most stable conformation.[13] This *R* configuration of the tetrahedral intermediate can adopt a conformation that favors intramolecular electrostatic catalysis.[14] The developing negative charge on the carbonyl oxygen would be stabilized by the positively charged trimethylammonium group. The enzymes also recognize the configuration at C3 of (acyl)carnitine during the acyl transfer. A reaction-intermediate analogue must mimic both of these configurations.

A covalent bond (C2–C3) in the morpholinium ring replaces this electrostatic interaction. A six-membered ring was chosen because of ease of synthesis and fewer conformations, although a seven- or eight-membered ring might better structurally match the putative tetrahedral intermediate. Conformational considerations in six-membered rings dictate that the carboxymethyl and the alkyl chain are cis diequatorial in the most stable conformation. Substituent Y represents a possible location for coenzyme A.

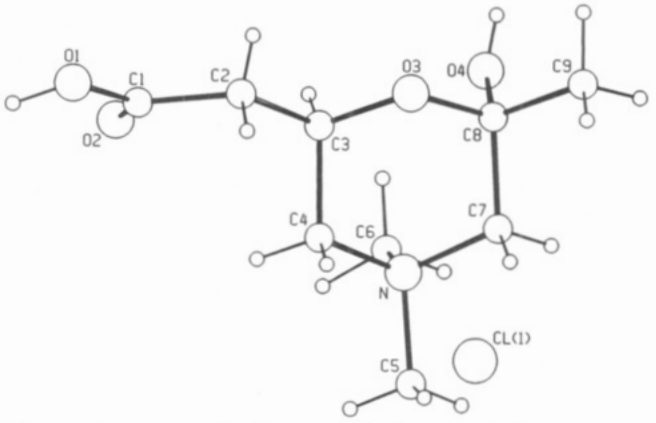
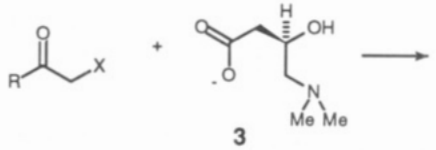


Figure 1. ORTEP drawing of 6-(carboxymethyl)-2-hydroxy-2,4,4-trimethylmorpholinium chloride (hemiacetylcarnitinium, HAC).

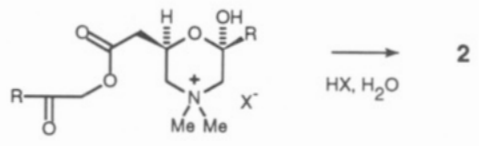
To test the feasibility of the design, we have synthesized morpholiniums[15] (Y = H) and hemiacylcarnitiniums (2-hydroxymorpholiniums, Y = OH). Herein, we describe the syntheses and structures of HAC, hemipropanoylcarnitinium (HPrC, **2**, R = CH_2CH_3), and hemibenzoylcarnitinium (HBC, **2**, R = Ph). Then, we report the inhibition constants for these compounds and racemic HPC with various carnitine acyltransferases from different sources.

Results

Syntheses of Hemiacylcarnitiniums. Synthesizing hemiacylcarnitiniums requires norcarnitine, **3**, which we make in high yield by demethylating carnitine with thiophenol in 2-(*N*,*N*-dimethylamino)ethanol.[16] Chloroacetone, 1-bromo-2-butanone, and chloroacetophenone react with **3** to produce the corresponding morpholinium ester hemiketals, **4** (not purified), which hydrolyze in the



3



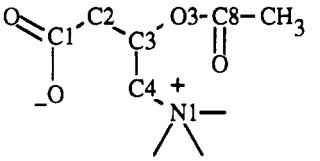
2

4

corresponding aqueous hydrogen halide to give HAC, HPrC, and HBC, respectively. The highly favored ring closure only gives an axial hydroxyl, which arises from the anomeric effect,[17] the gauche effect, and steric effects. We only detect the hemiketal form in NMR and IR spectra.

(8) Gandour, R. D.; Colucci, W. J.; Brady, P. S.; Brady, L. J. *Biochem. Biophys. Res. Commun.* **1986**, *138*, 735–742.
(9) Gandour, R. D.; Colucci, W. J.; Stelly, T. C.; Brady, P. S.; Brady, L. J. *Arch. Biochem. Biophys.* **1988**, *267*, 515–520.
(10) CPT_i (or CPT-II or CPT-B) is the enzyme located on the inner mitochondrial membrane. CPT_o (or CPT-I or CPT-A) is the enzyme located on the outer mitochondrial membrane. Whether these are the same protein remains a confusing issue. For a complete update on the confusion, see: *Current Concepts in Carnitine Research*; Carter, A. L., Ed., CRC Press: Boca Raton, 1992; pp 129–178.
(11) Gandour, R. D. *J. Inclusion Phenom. Mol. Recognit. Chem.* **1989**, *7*, 39–51.
(12) Colucci, W. J.; Gandour, R. D., Mooberry, E. S. *J. Am. Chem. Soc.* **1986**, *108*, 7141–7147.
(13) Gandour, R. D.; Colucci, W. J.; Fronczek, F. R. *Bioorg. Chem.* **1985**, *13*, 197–208.
(14) Asknes, G.; Prue, J. E. *J. Chem. Soc.* **1959**, 103–107.
(15) Colucci, W. J.; Gandour, R. D.; Fronczek, F. R.; Brady, P. S.; Brady, L. J. *J. Am. Chem. Soc.* **1987**, *109*, 7915–7916.
(16) Colucci, W. J.; Turnbull S. P., Jr.; Gandour, R. D. *Anal. Biochem.* **1987**, *162*, 459–462.
(17) Kirby, A. J. *The Anomeric Effect and Related Stereoelectronic Effects at Oxygen*; Springer-Verlag: Berlin, 1983; pp 3–32.

Table I. Comparison of Structural Parameters of Hemiacylcarnitiniums, Carnitine, and Acetylcarnitine



structure	N1–C4–C3–O3 (deg)	C1–C2–C3–C4 (deg)	C4–C3–O3 (deg)	N1–C4–C3 (deg)	C3–O3 (Å)	O3–C8 (Å)	C8–O4 (Å)
HAC	−57.0 (2)	−72.3 (2)	110.2 (2)	112.0 (3)	1.428 (2)	1.429 (3)	1.395 (3)
HPrC	−56.8 (4)	179.8 (3)	110.1 (2)	112.0 (2)	1.426 (4)	1.431 (4)	1.396 (4)
HBC(A)·1/2H_2O	−60.3 (3)	−167.1 (3)	110.0 (2)	110.7 (2)	1.430 (4)	1.428 (3)	1.392 (4)
HBC(B)·1/2H_2O	−57.1 (3)	−66.1 (4)	109.9 (2)	112.1 (2)	1.442 (4)	1.412 (3)	1.411 (4)
Carn·HCl	−66.4	−166.2	111.3 (7)	116.8 (7)	1.42 (1)	n/a[a]	n/a
AcCarn·HCl	−88.0	−71.4	105.0 (4)	115.9 (4)	1.470 (7)	1.352 (6)	1.195 (5)
AcCarn·HCl·H_2O	−83.6	−77.1	106.8 (2)	116.4 (2)	1.451 (2)	1.349 (2)	1.203 (2)

[a] Not applicable.

Table II. Inhibition Constants of Hemiacylcarnitiniums with Various Carnitine Acyltransferases

inhibitor	enzyme	substrate	substrate K_m μM, ± SE[a]	inhibitor K_i μM, ± SE
HAC				
	rat liver peroxisomal CAT	(*R*)-acetylcarnitine	290 ± 20[b]	69 ± 5[b]
		(*R*)-carnitine	143[c]	
	rat heart mitochondrial CAT	CoASH	130 ± 20[b]	92 ± 14[b]
HPrC				
	pigeon breast CAT	(*R*)-carnitine	120[d]	200 ± 30[b]
		(*R*)-acetylcarnitine	350[d]	
HBC				
	pigeon breast CAT	(*R*)-carnitine	120[d]	3500 ● 500[b]
	rat heart mitochondrial CAT	CoASH	120 ± 10[b]	2400 ± 70[b]
	rat liver peroxisomal CAT	(*R*)-acetylcarnitine	280 ± 10[b]	1670 ± 70[b]
	beef liver peroxisomal COT	(*R*)-carnitine	200[e]	1100 ± 100[b]
HPC				
	beef liver mitochondrial CPT_i	(*R*)-carnitine	2000[e]	2.0 ± 0.3[b]
	rat liver mitochondrial CPT_i	(*R*)-carnitine	200[f]	5.1 ± 0.7[f]
		(*R*)-palmitoylcarnitine	30[f]	1.6 ± 0.6[f]

[a] Standard error. [b] This work. [c] Reference 25. [d] Reference 23. [e] Reference 29. [f] Reference 9.

Single-crystal X-ray analyses have confirmed the structures.

Comparison of Crystal Structures. Figure 1 shows the crystal structure of HAC. Table I lists selected structural parameters from HAC, HPrC,[18] HBC·1/2H_2O,[19] carnitine·HCl,[20] acetylcarnitine·HCl,[21] and acetylcarnitine·HCl·H_2O.[22] The N1–C3–C4–O3 torsion angle is smaller in the cycles than in the open-chain compounds, especially acetylcarnitine. The cycle enforces a torsion angle of approximately −60°. In solution, carnitine slightly prefers anti for the C1–C2–C3–C4 torsion angle, but acetylcarnitine prefers gauche (−).[12] The cycles show no preference between anti and gauche (−) in this torsion angle. HBC has a molecule in each conformation in the crystal. The steric and electrostatic repulsions that favor gauche (−) in acetylcarnitine are absent in the cycles. The N1–C4–C3 bond angle is smaller in the cycles, where it is nearly tetrahedral. The C4–C3–O3 bond angles are similar to that in carnitine but larger than those in the acetylcarnitines. The C3–O3 bond distances in the cycles are shorter than that in acetylcarnitine, but O3–C8 is longer in the cycles than in acetylcarnitine. In the latter, C8 is an sp^2-hybridized carbon, which gives a shorter distance. In summary, the cycles have more idealized values for structural parameters than do carnitine and acetylcarnitine.

Enzymatic Assays. Hemiacylcarnitiniums inhibit carnitine acyltransferases. Table II displays inhibition constants (K_i) for hemiacylcarnitiniums as competitive inhibitors in either the forward (acylCoA + carnitine →) or the reverse direction (acylcarnitine + CoASH →), varying one substrate with the other saturating. In order to compare the binding of inhibitors with that of substrates, we assume that values of K_m approximate dissociation constants (K_s); e.g., K_m (120 μM)[23] and K_s (115 μM)[24] of (*R*)-carnitine for pigeon breast CAT are almost identical.

1. **HAC.** HAC binds better than (*R*)-carnitine or (*R*)-acetylcarnitine to CAT. In rat liver peroxisomal CAT, HAC binds 2-fold better than (*R*)-carnitine[25] and 4-fold better than (*R*)-acetylcarnitine. In rat heart mitochondrial CAT,[26] HAC binds better than CoASH. In pigeon breast CAT, HAC (K_i = 59 μM)[27] binds 2-fold better than (*R*)-carnitine[23] and 6-fold better than (*R*)-acetylcarnitine.[23]

(18) Blackwell, N. L.; Fronczek, F. R.; Gandour, R. D. *Acta Crystallogr.* **1989**, *C45*, 1089–1091.
(19) Blackwell, N. L.; Fronczek, F. R.; Gandour, R. D. *Acta Crystallogr.* **1990**, *C46*, 1164–1166.
(20) Tomita, K.-i.; Urabe, K.; Kim, Y. B.; Fujiwara, T. *Bull. Chem. Soc. Jn.* **1974**, *47*, 1988–1993.
(21) Sass, R. L.; Werness, P. *Biochem. Biophys. Res. Commun.* **1973**, *55*, 736–742.
(22) Destro, T.; Heyda, A. *Acta Crystallogr., Sect. B* **1977**, *B77*, 504–509.
(23) Chase, J. F. A.; Tubbs, P. K. *Biochem. J.* **1966**, *99*, 32–40.
(24) Tipton, K. F.; Chase, J. F. A. *Biochem. J.* **1969**, *115*, 517–521.
(25) Markwell, M. A. K.; Tolbert, N. E.; Bieber, L. L. *Arch. Biochem. Biophys.* **1976**, *176*, 479–488.
(26) Chung, C.; Chung, C. D.; Bieber, L. L. *Prot. Expr. Purif.* **1991**, *2*, 426–431.
(27) For a review of CAT see: Colucci, W. J.; Gandour, R. D. *Bioorg. Chem.* **1988**, *16*, 307–334.

HAC does not inhibit rat or beef liver peroxisomal COT or rat or beef liver mitochondrial CPT_i.

2. HPrC. HPrC inhibits pigeon breast CAT with a K_i that is 3-fold larger than that of HAC (K_i = 59 μM).[27] This result parallels the decreased activity of pigeon breast CAT with (*R*)-propanoylcarnitine compared to (*R*)-acetylcarnitine.[28]

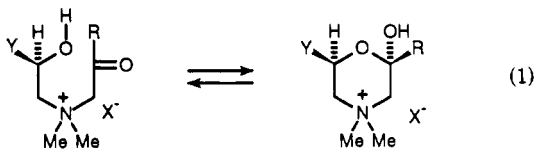
3. HBC. HBC weakly inhibits one COT and three CATs. HBC binds to the three CATs less tightly than do the substrates. HBC inhibits beef liver peroxisomal COT more effectively than it does the three CATs. It does inhibit rat liver CAT better than pigeon breast and rat heart CAT, both of which are proteins from muscle. HBC does not inhibit rat liver peroxisomal COT, rat liver mitochondrial CPT_i, and beef liver mitochondrial CPT_i.

4. HPC. Racemic HPC strongly inhibits beef liver mitochondrial CPT_i with (*R*)-carnitine as the competing substrate and decanoylCoA as the saturating substrate. Racemic HPC binds better than the substrates.[29] The inhibition is better than that for rat liver mitochondrial CPT_i[9] with palmitoylCoA as the saturating substrate. The K_i values vary for the rat liver enzyme depending on which substrate is saturating.

Discussion

Hemiacylcarnitiniums competitively inhibit carnitine acyltransferases with K_i's in the same range as the K_m's or K_s's of the substrates, (*R*)-carnitine and (*R*)-acylcarnitine. HAC binds to CAT better than (*R*)-carnitine and (*R*)-acetylcarnitine, and HPC binds to CPT_i much better than the (*R*)-carnitine and (*R*)-palmitoylcarnitine. This suggests that the hemiacylcarnitiniums compete well with substrates for the active site.

Ring–Chain Tautomerism. The hemiacylcarnitiniums presumably bind to the enzymes as cyclic structures, but the evidence is circumstantial. A fraction of the compound in solution might be in the open form (eq 1), but none of



(1)

1 Y = H

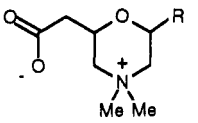
2 Y = $CH_2CO_2^-$

the open form is seen by NMR. Furthermore, NMR studies also indicate that the six-membered ring strongly favors the conformation that is seen in the crystal. Axial and equatorial signals are resolved, e.g., HBC. We cannot completely exclude selective binding of the open form, which might react with CoASH or the thiol of the cysteine in the active site[27] to form a hemithioketal. (We are designing compounds to explore this possibility.)

Equilibrium constants for hemiaroylcholiniums (**1**, R = Aryl) range from 1.4 to 176 for electron-donating to electron-withdrawing substituents.[30] The pK_a values of the hemiketal hydroxyl range from 12.0 to 10.0 in the same series. We estimate that hemiacylcarnitiniums have equilibrium constants of ca. 1000 for this tautomerization and pK_a values around 11 for the hydroxyl.

The hemiketal carbon of hemiacylcarnitiniums and the hemiortho thioester carbon of the putative tetrahedral intermediate are chiral centers. The 2*S* configuration of a hemiacylcarnitinium is the same relative configuration as the proposed tetrahedral intermediate. Our design predicts that of the four possible stereoisomers the 2*S*,6*R* enantiomer will inhibit most strongly. Because the hydroxy is more stable in the axial position, formation of the hemiketal gives only one pair of enantiomers (2*R*,6*S*:2*S*,6*R*). This stereoselective reaction excludes the formation of the 2*S*,6*S*:2*R*,6*R* pair, which has equatorial hydroxys. Unfortunately, we cannot use hemiacylcarnitiniums to determine the configuration of the reaction center by comparing the values of K_i of the four stereoisomers. (We are designing new morpholiniums to test the chirality of the tetrahedral intermediate.)

Comparison of Hemiacylcarnitiniums with Other Inhibitors. HAC strongly inhibits CAT compared to other competitive inhibitors.[27] (Acetylamino)carnitine inhibits pigeon breast CAT and rat liver CAT with values of K_i of 24 and 130 μM, respectively.[31] (Acetylamino)-carnitine competitively inhibits CAT because acetyl transfer to CoASH is endergonic. Other morpholiniums inhibit pigeon breast CAT;[15] e.g., racemic **5** (R = CH_3) has



5

a K_i of 1080 μM. Comparison of inhibition by HAC to that by **5** (R = CH_3) suggests that a hydroxyl group improves binding. The (*S*)-(carboxymethyl)-CoA-(*R*)-carnitine ester, which forms during the CAT-catalyzed reaction of (*R*)-(bromoacetyl)carnitine and CoASH, inhibits pigeon breast CAT most effectively.[32] This bisubstrate analogue[33] binds to CAT with an estimated K_i less than the product (0.012 μM) of the individual K_m's.

Enzymic Selectivity. The selectivity of inhibition by the hemiacylcarnitiniums suggests that this class of inhibitors might be useful for comparing the various carnitine acyltransferases. Inhibition of pigeon breast CAT by HAC, HPrC, and HBC resembles the pattern of chain-length specificity of acylcarnitines as substrates for this enzyme.[28] HAC does not inhibit COT or CPT_i. HBC weakly inhibits the CATs and beef liver peroxisomal COT. HBC inhibits the liver enzymes better than those from muscle (pigeon breast or heart). Racemic HPC strongly inhibits both rat and beef liver CPT_i, but not CAT.

Summary and Conclusions

After structural studies[12] on carnitine and acetylcarnitine as well as an analysis of a possible mechanism for acyl transfer,[13] we have designed and synthesized effective inhibitors of CAT and CPT_i. The exact mode of action of these inhibitors, however, remains to be demonstrated. Racemic HPC is currently the most potent inhibitor of purified rat liver CPT_i and beef liver CPT_i. HAC and racemic HPC bind more strongly than their respective natural substrates (and isomers), the acylcarnitines. Because they lack a CoASH fragment, hemiacylcarnitiniums have values of K_i that are too large to claim these inhibitors as reaction-intermediate analogues. Nevertheless, they work well, if only as acylcarnitine (substrate) analogues. Our ultimate goal is to construct a bisubstrate reaction-intermediate analogue. The morpholinium framework

(28) Chase, J. F. A. *Biochem. J.* **1967**, *104*, 510–518.
(29) Ramsay, R. R. Unpublished results.
(30) Sørensen, P. E.; McClelland, R. A.; Gandour, R. D. *Acta Chem. Scand, Ser. A* **1991**, *A45*, 558–566.
(31) Jenkins, D. L.; Griffith, O. W. *J. Biol. Chem.* **1985**, *260*, 14748–14755.
(32) Chase, J. F. A.; Tubbs, P. K. *Biochem. J.* **1969** *111*, 225–235.
(33) Byers, L. D. *J. Theor. Biol.* **1978**, *74*, 501–512.

provides a basis for elaborating our design.

Experimental Section

Hexane was distilled from CaH_2, Et_2O from sodium benzophenone ketyl, and THF from Na and K. *i*-PrOH was distilled from CaO and stored over 5-Å sieves. MeOH was distilled and stored over 4-Å sieves. Reagent-grade acetone, MeCN, and chloroacetone and absolute EtOH were used as received. 1-Bromo-2-butanone (Aldrich) was recrystallized at low temperature. 2-Chloroacetophenone was recrystallized from hot MeOH using decolorizing carbon. Sodium (*R*)-norcarnitine was prepared by the demethylation of (*R*)-carnitine (Sigma Tau) and recrystallized from hot *i*-PrOH. Racemic HPC was prepared previously.[9] FAB MS samples were suspended in glycerol. MicAnal Organic Microanalysis of Tuscon, AZ, and Oneida Research Services of Whitesboro, NY, did the elemental analyses. The optical rotations were recorded at Tulane University School of Medecine.

Vapor-diffusion crystallizations were carried out by dissolving the compound in a solvent in an oversized beaker or test tube, placing this container in a sealed jar containing a volatile cosolvent, and allowing the assembly to stand for several d. Alternatively, crystallizations on a larger scale were more conveniently done by placing the solution in a single-neck round-bottom flask and attaching a sealed cosolvent reservoir.

(2*S*,6*R*)-6-(Carboxymethyl)-2-hydroxy-2,4,4-trimethylmorpholinium Chloride (Hemiacetylcarnitinium, HAC). Sodium (*R*)-norcarnitine (3.38 g, 20.0 mmol) was dissolved in hot *i*-PrOH (175 mL), and chloroacetone (5.55 g, 60.0 mmol) was added. After 12 h of reflux, NaCl (1.125 g) was collected by filtration. The filtrate was concentrated to a thick oil, which was triturated with Et_2O and dried under vacuum. A solution of the resulting solid in 2 M HCl (60 mL) was refluxed for 12 h. Concentrating the acidic solution gave a yellow glassy solid, which was triturated with MeCN (4 × 220 mL). The resulting white solid was dried (3.68 g, 77%). The solid was recrystallized from EtOH/MeOH (190 mL/10 mL) by vapor diffusion with Et_2O. After several d, large colorless needles formed; mp 200 °C dec: ^{1}H NMR (100 MHz, ref to DSS in D_2O) δ 4.9 (m, HCO), 3.75–3.15 (m, CH_2N^+), 3.46 (s, CH_3N^+), 3.22 (s, CH_3N^+), 2.80, 2.60 (dd,dd, CH_2COO), 1.48 (s, CCH_3); IR (KBr) 3462–2662 (b, OH), 1722 (s, C=O) cm^{-1}; $[\alpha]_D^{22.5}$ +42.9° (*c* 0.884, H_2O). Anal. Calcd for $C_9H_{18}ClNO_4$: C, 45.10; H, 7.57; N, 5.84. Found: C, 45.20; H, 7.84; N, 5.60.

(2*S*,6*R*)-6-(Carboxymethyl)-2-ethyl-2-hydroxy-4,4-dimethylmorpholinium Bromide (Hemipropanoylcarnitinium, HPrC). Sodium (*R*)-norcarnitine (1.0 g, 5.9 mmol) was dissolved in hot *i*-PrOH (100 mL), and 1-bromo-2-butanone (1.78 g, 12.0 mmol) was added. The solution was refluxed for 48 h. The precipitated NaBr (0.56 g, 92%) was filtered. Concentrating the filtrate gave a yellow oily solid, which was triturated with anhyd Et_2O (3 × 20 mL) to remove excess bromo ketone. After drying overnight, a flaky yellow solid (2.09 g) formed. The ^{1}H NMR spectrum suggested the desired ester hemiketal. The yellow solid (2.09 g) was dissolved in 2 N HBr (50 mL) and stirred in the dark for 6 d. Evaporating the H_2O produced an orange oil, which was washed with MeCN several times to form a yellow solid (0.97 g). This solid was recrystallized drom anhyd EtOH by vapor diffusion with anhyd Et_2O. Off-white crystals (0.43 g, 24% yield) were collected; mp 157–158 °C: ^{1}H NMR (100 MHz, ref TSS in D_2O) δ 4.9 (m, HCO), 3.75–3.15 (m, CH_2N^+), 3.46 (s, NCH_{3ax}), 3.22 (s, NCH_{3eq}), 2.80, 2.60 (dd, dd, CH_2COO), 1.74 (q, CH_2), 0.93 (t, CH_3); ^{13}C NMR (100 MHz, ref TSS in CD_3CN) δ 171 (*C*OOH), 90 (*C*2), 65, 62 (*C*3,*C*5), 60 (*C*6), 57 (NC_{eq}), 51 (NC_{ax}), 38 (CH_2COOH), 31 (CH_2CH_3), 5 (CH_2CH_3); IR (KBr) 3500–2700 (b, OH), 1750 (s, C=O) cm^{-1}; MS *m/e* 218.0 (100), 174 (60), 116 (40); daughters of 218.0 218.0 (50), 116.0 (100), 102.9 (25), 85.0 (70), 71.0 (25), 58.1 (40), 46.2 (5). Anal. Calcd for $C_{10}H_{20}BrNO_4$ C, 40.28; H, 6.76; N, 4.70. Found C, 39.96; H, 6.85; N, 4.48.

(2*S*,6*R*)-6-(Carboxymethyl)-2-hydroxy-4,4-dimethyl-2-phenylmorpholinium Chloride Monohydrate (Hemibenzoylcarnitinium, HBC). Sodium (*R*)-norcarnitine (1.0 g, 5.9 mmol) was dissolved in hot *i*-PrOH (150 mL), and 2-chloroacetophenone (2.77 g, 18.0 mmol) was added. The solution was refluxed for 48 h and NaCl precipitated. Filtering the salt and concentrating the filtrate gave an oily solid, which was triturated with anhyd Et_2O (3 × 20 mL) to produce a glassy orange solid. After being dried overnight, the orange solid (2.5 g) was dissolved in 2 N HCl (100 mL) and refluxed for 48 h. Concentration of the solution gave an oil, which was washed repeatedly with MeCN. The viscous oil was dried for 12 h, and a yellow solid formed (1.45 g). The solid was washed with anhyd Et_2O to remove residual MeCN and with acetone to remove color. Recrystallizing by vapor diffusion with Et_2O from MeOH resulted in white crystals (0.210 g, 15% yield); mp 189–189.5 °C: ^{1}H NMR (200 MHz, ref TMS in 50:50 DMSO-d_6:C_6D_6) δ 7.70–7.40 (m, Ar), 4.90 (m, HCO), 3.90 (d, C5-H_{eq}), 3.70 (d, C3-H), 3.60 (t, C5-H_{ax}), 3.50 (NCH_{3ax}), 3.30 (t, C3-H), 3.20 (s, NCH_{3eq}), 2.80, 2.60 (dd, dd, CH_2COO). ^{13}C NMR (200 MHz, ref TMS in 50:50 DMSO) δ 171 (*C*OOH), 141 (*C*1), 129 (*C*4′), 128, 126 (*C*2′,*C*3′), 95 (*C*2), 65, 62 (*C*3, *C*4), 61 (*C*5), 57 (NC_{eq}), 51 (NC_{ax}), 38 (CH_2COOH); IR (neat) 3500–2700 (b, OH), 1732 (s, C=O) cm^{-1}; MS *m/e* 266.2 (>100), 164.0 (100), 129.9 (40); daughters of 266.2 265.7 (100), 206.1 (5), 164.0 (95), 130.0 (5), 119.1 (15), 103.0 (25), 91.1 (70), 84.1 (40), 58.2 (5); $[\alpha]_D^{20}$ +41.5° (*c* 8.1, MeOH). Anal. Calcd for $[C_{14}H_{20}NO_4]Cl \times 1/2H_2O$:[19] C, 54.11; H, 6.81; N, 4.51. Found C, 54.07; H, 6.84; N, 4.47.

Enzymatic Assays. East Lansing. Kinetic analyses were performed using a kinetic analyzer and calculations with the Tankin Program.[34,35] Initial velocity was measured by monitoring the appearance of product, acetylCoA, at 232 nm (ϵ = 4.5 μmol/cm^3) at rt. The values of K_i and K_m were calculated by linear least-squares analysis of [inhibitor] vs observed K_m. Conditions and results for each analysis follow.

1. HAC and HBC Inhibiting Rat Liver Peroxisomal CAT. Conditions for the assays were as follows: 50 mM $KHPO_4$ buffer, pH = 7.5; [CoASH] = 400 μM; [(*R*)-acetylcarnitine] ranged from 69 to 1670 μM (seven values); and 10 μL of purified rat liver peroxisomal CAT.[25]

For [HAC] = 0, 77.5, and 155 μM HAC, observed values of K_m were 257, 674, and 905 μM, respectively, and observed values of V_{max} were 28.7, 27.4, and 31.2 nmol mL^{-1} min^{-1}, respectively. These data gave K_i = 69 ± 5 μM and K_m = 290 ± 20 μM.

For [HBC] = 0, 1500, and 3000 μM HBC, observed values of K_m were 281, 513, and 777 μM, respectively, and observed values of V_{max} were 30.7, 31.7, and 22.8 nmol mL^{-1} min^{-1}, respectively. These data gave K_i = 1670 ± 70 μM and K_m = 280 ± 10 μM.

2. HAC and HBC Inhibiting Rat Heart Mitochondrial CAT. Conditions for the assays were as follows: 50 mM $KHPO_4$ buffer; pH = 7.5; [(*R*)-acetylcarnitine] = 31 mM; [CoASH] ranged from 24 to 240 μM (seven values); and 20 μL of purified rat heart mitochondrial CAT.[36]

For [HAC] = 0, 62.2, 124.4, and 311 μM, observed values of K_m were 120, 213, 353, and 573 μM, respectively. These data gave K_i = 92 ± 14 μM and K_m = 130 ± 20 μM.

For [HBC] = 0, 360, and 1080 μM, observed values of K_m were 119, 130, and 170 μM, respectively. These data gave K_i = 2400 ± 70 μM and K_m = 120 ± 10 μM.

San Francisco. Assays were performed as described by Ramsay and Tubbs,[37] and linear least-squares analyses of Dixon plots were used to calculate the values of K_i.

1. HBC Inhibiting Beef Liver Peroxisomal COT. Conditions for the assays were as follows: 20 mM $KHPO_4$ buffer; pH = 7.4; [decanoylCoA] = 50 μM (K_m = 1 μM);[29] [aldrithiol] = 125 μM, [(*R*)-carnitine] = 100, 200, and 1000 μM; purified beef liver peroxisomal COT;[38] and [HBC] ranging from 100 to 1000 μM (seven values). A value of K_i = 1100 ± 100 μM was found.

2. HBC Inhibiting Pigeon Breast CAT. Conditions for the assays were as follows: 20 mM $KHPO_4$ buffer; pH = 7.4; [acetylCoA] = 300 μM (K_m = 34 μM);[23] [aldrithiol] = 125 μM, [(*R*)-carnitine] = 200 and 1000 μM (K_m = 120 μM);[23] pigeon breast CAT (Sigma); and [HBC] ranging from 100 to 1000 μM (seven values). A value of K_i = 3500 ± 500 μM was found.

3. HPC Inhibiting Beef Liver Mitochondrial CPT$_i$. Conditions for the assays were as follows: 20 mM $KHPO_4$ buffer;

(34) LeBlond, D. J.; Ashendel, C. L.; Wood, W. A. *Anal. Biochem.* **1980**, *104*, 355–369.
(35) LeBlond, D. J.; Ashendel, C. L.; Wood, W. A. *Anal. Biochem.* **1980**, *104*, 370–385.
(36) Fiol, C. J.; Bieber, L. L. *J. Biol. Chem.* **1984**, *259*, 13084–13088.
(37) Ramsay, R. R.; Tubbs, P. K. *FEBS Lett.* **1975**, *45*, 21–25.
(38) Ramsay, R. R.; Derrick, J. P.; Friend, A. S.; Tubbs, P. K. *Biochem. J.* **1987**, *244*, 271–278.

pH = 7.4; [decanoylCoA] = 50 μM (K_m = 10 μM);[29] [aldrithiol] = 125 μM, [(R)-carnitine] = 1000, 2500, and 1000 μM; purified beef liver mitochondrial CPT_i;[39,40] and [HPC] ranging from 0.5 to 20 μM (six values). A value of K_i = 2 ± 0.3 μM was found.

Cleveland. HPrC Inhibiting Pigeon Breast CAT. Conditions for the assays were as follows:[41] 0.1 mL DTNB (1 mM in 1 M TRIS, pH = 8.1); 25 μL of 8-mM acetylCoA; 10 μL of pigeon breast CAT (88 μg/mL, Sigma); [(R)-carnitine] = 75, 750, 1500, and 3000 μM; [HPrC] = 0, 25, 50, 100, 500, 1000, 2500, and 5000 μM; and total volume = 1 mL. Velocities (μmol/min/mg·protein) were measured and gave the following results for the respective concentrations of HPrC: 75 μM (R)-carnitine: 15.0, 13.0, 11.4, 8.9, 4.2, 2.7, -, 0.78; 750 μM (R)-carnitine: 42.4, 39.4, 41.4, 33.1, 25.1, 18.7, -, 6.0; 1500 μM (R)-carnitine: 43.4, -, 39.4, 40.4, 35.1, 37.7, 18.7, 8.6; and 3000 μM (R)-carnitine: 53.1, 109.6, 54.8, 93.5, 63.5, 45.4, -, 10.7. K_i = 200 ± 30 μM from Dixon plots and was confirmed by transformed double-reciprocal plots (i.e., $K_{m\,apparent}/V_{max}$ vs [HPrC]).

Acknowledgment. We thank Sigma Tau for a gift of (R)-carnitine. R.D.G. thanks Monsanto for financial support. We thank NIH for supporting this work through grants: GM42016 to R.D.G., DK18427 to L.L.B., DK36069 to E.P.B., DK41572 and HL16251 to R.R.R. We thank Mr. Robert Hendricks for preparing (R)-norcarnitine and Professor Coy for use of the micropolarimeter. We thank the reviewers for many helpful comments and suggestions.

Supplementary Material Available: X-ray data for HAC (7 pages). Ordering information is given on any current masthead page.

(39) Clarke, P. R. H.; Bieber, L. L. *J. Biol. Chem.* **1981**, *249*, 9861-9868.
(40) Ramsay, R. R. *Biochem. J.*, **1988**, *249*, 239-245.
(41) Fritz, I. B.; Schultz, S. K. *J. Biol. Chem.* **1965**, *240*, 2188-2192.

Application of Protease-Catalyzed Regioselective Esterification in Synthesis of 6′-Deoxy-6′-fluoro- and 6-Deoxy-6-fluorolactosides

Shaopei Cai, Senitiroh Hakomori, and Tatsushi Toyokuni*

The Biomembrane Institute and University of Washington, 201 Elliott Ave. West, Seattle, Washington 98119

Received January 17, 1992

Subtilisin-catalyzed esterification of methyl 4-O-β-D-galactopyranosyl-β-D-glucopyranoside (methyl β-lactoside) (**1**) with 2,2,2-trichloroethyl butyrate (**3**) distinguished between the two primary hydroxyl groups of **1**, yielding exclusively the 6′-O-monobutyryl derivative **6** from which 6′-deoxy-6′-fluoro- and 6-deoxy-6-fluorolactosides (**22** and **29**, respectively) were efficiently synthesized. A key feature in the synthesis of **22** was the use of the 2,4,6-trimethylbenzoyl (mesitoyl) group to protect the remaining free hydroxyl groups. A mesitoate ester, in addition to being inert to the condition that hydrolyzed a butyrate ester, could be easily cleaved by reduction with AlH_3 without hydrogenolysis of a C–F bond. The steric bulk of a mesitoyl group suppressed the C-4′ → C-6′ acyl migration during the fluorination with (diethylamino)sulfur trifluoride (DAST). The success in the synthesis of **29** depended on the choice of solvent employed for the DAST fluorination. With diglyme the desired 6-fluoro derivative **28** was the only product, whereas the use of CH_2Cl_2 yielded 6-O-methyl-β-lactosyl fluoride **30** concomitantly through a C-1 → C-6 migration of the methoxyl group.

During the past few decades it has become clear that cell surface carbohydrates play a major role in cell interaction processes, including the determinants for A, B, O, H, etc., groupings in human blood,[1] the immunological response to carbohydrate antigens,[2] and a variety of cell adhesion phenomina.[3,4] Metastasis is the process by which tumor cells spread into healthy body tissues resulting in the major cause of death in human malignancies. Extensive investigations on the biochemical events making up this process have suggested a possible involvement of cell surface carbohydrates in the metastatic process such as tumor cell aggregation and adhesion of tumor cells to endothelial cells.[4,5]

As part of our program aimed at developing carbohydrate-based agents that effectively prevent metastatic spread of tumor cells by blocking the cognitive interactions among tumor cells and between tumor and host cells,[6] we have demonstrated that methyl 4-O-β-D-galactopyranosyl-β-D-glucopyranoside (methyl β-lactoside)[7] (**1**) and its trivalent and polyvalent derivatives[8] dramatically suppress the formation of metastatic lung colonies in mice injected with mouse B16 melanoma cells. For elucidation of the structural requirements for this inhibition process and to discover more effective inhibitors, a facile access to structural analogues of **1** was required. Since intro-

(1) Recent reviews: (a) Watkins, W. M. *Pure Appl. Chem.* **1991**, *63*, 561. (b) Von dem Borne, A. E. G. K.; Kuijpers, R. W. A. M. *Colloq. INSERM* **1991**, *206*, 219.
(2) Recent reviews: (a) Hakomori, S. *Adv. Cancer Res.* **1989**, *52*, 257. (b) Garegg, J. *Chem. Br.* **1990**, *26*, 669. (c) Singhal, A.; Hakomori, S. *BioEssays* **1990**, *12*, 223.
(3) Recent reviews: (a) Sharon, N.; Lis, H. *Science* **1989**, *246*, 227. (b) Brandley, B. K.; Swiedler, S. J.; Robbins, P. W. *Cell* **1990**, *63*, 861. (c) Winkelhake, J. L. *Glycoconjugate J.* **1991**, *8*, 381.
(4) Recent reviews of tumor cell lectins: (a) Gabius, H.-J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 1267. (b) Raz, A.; Lotan, R. *UCLA Symp. Mol. Cell. Biol. New Ser.* **1988**, *78*, 237. (c) Gabius, H.-J.; Gabius, S. *Naturwissenschaften* **1990**, *77*, 505.
(5) Recent papers: (a) Walz, G.; Aruffo, A.; Kolanus, W.; Bevilacqua, M.; Seed, B. *Science* **1990**, *250*, 1132. (b) Gabius, S.; Schirrmacher, V.; Franz, H.; Joshi, S. S.; Gabius, H.-J. *Int. J. Cancer* **1990**, *46*, 500. (c) Miyake, M.; Hakomori, S. *Biochemistry* **1991**, *30*, 3328. (d) Takada, A.; Ohmori, K.; Takahashi, N.; Tsuyuoka, K.; Yago, A.; Zenita, K.; Hasegawa, A.; Kannagi, R. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 713.
(6) Inhibition of liver tumor cell colonization in mice by blocking hepatic lectin with D-galactose or arabinogalactan has been reported: Beuth, J.; Ko, H. L.; Schirrmacher, V.; Uhlenbruck, G.; Pulverer, G. *Clin. Expl. Metastasis* **1988**, *6*, 115.
(7) Oguchi, H.; Toyokuni, T.; Dean, B.; Ito, H.; Otsuji, E.; Jones, V. L.; Sadozai, K. K.; Hakomori, S. *Cancer Commun.* **1990**, *2*, 311.
(8) Dean, B.; Oguchi, H.; Cai, S.; Otsuji, E.; Tashiro, K.; Hakomori, S.; Toyokuni, T. *Carbohydr. Res.* In press.

0022-3263/92/1957-3431$03.00/0