Synthesis and Lipase Catalyzed Hydrolysis of Thiolesters of 2-, 3- and 4-Methyl Octanoic Acids

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Racemic 2-, 3- and 4-methyloctanoic acids were synthesized. The 2-mercaptoethanol S-esters of these branched acids, and of octanoic acid, were exposed to lipases of the fungi *Candida rugosa* and *Aspergillus niger*. Reactions were followed spectrophotometrically using Ellman's Reagent. Branching at the 3-position retards lipolysis more severely than at either the 2- or 4-positions. Features of the assay method are discussed with reference to titrimetry.
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There is considerable interest in enhancing, and understanding the basis for, the several kinds of selectivity (positional, fatty acid and stereo) exhibited by lipases. Recently we synthesized 1,2- and 1,3-dialkylglycerol ethers and developed methods for analyzing their configurations. These compounds and their esters were viewed as complementary to the triglycerides and would avoid some of the ambiguities inherent in the use of the trifunctional substrates (1). As was the case for the major portion of the literature dealing with lipase stereoselection (2), asymmetry resided only in the alcohol portion of the compounds. At this time far less is known of the effects of chirality in the acid residue, despite the inference that one might develop from the literature that lipases primarily bind the acid.

A survey of the effects of methyl branching on the maximum velocity (V_{max}) of ester hydrolysis catalyzed by porcine pancreatic lipase was limited to commercially available, and racemic, fatty acids (3). Little could be learned from these other than that branching close to the acid carbonyl severely retarded reaction. Nevertheless, α-substitution by halogen or ether oxygen does not preclude reaction; and hydrolysis, esterification, and transesterification methods served as the basis for resolving the enantiomers of such acids (2, 4—8). A recent examination of fungal lipases that had been solubilized in organic solvents by derivatization with polyethylene glycol indicated that 4-methylvaleric acid was as reactive as valeric acid itself in esterification of 1-pentanol in benzene, although 2- and 3-methylvaleric acids were not (9). A selection of configurationally pure methyl branched fatty acids could prove useful for learning more of the details of lipase binding, as well as serving to monitor and guide efforts at protein engineering of these enzymes. However, since such compounds are not available they would have to be synthesized.

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Abbreviations: DTNB, 5,5′-dithio-bis-(2-nitrobenzoic) acid; GLC, gas-liquid chromatography; HMPT, hexamethyl phosphoric triamide; IR, infrared; LDA, lithium diisopropylamide; MS, mass spectra; NMR, nuclear magnetic resonance; V_i, initial velocity; V_{max}, maximum velocity.

Before undertaking the preparation of a series of configurationally pure methyl branched fatty acids, we prepared the more readily obtainable racemic 2-,3- and 4-methyloctanoic acids as model compounds. In addition, it was necessary to select 1) a suitable acid derivative to submit to lipolysis, and 2) a lipase assay. The initial velocities (V_i) of several octanoic acid esters were obtained by titrimetry, and those of several thiolesters were measured both titrimetrically and spectrophotometrically (10, 11) using commercial lipase preparations of the fungi *Candida rugosa* and *Aspergillus niger*. The reactivities of the 2-mercaptoethanol S-esters of the racemic acids were then determined spectrophotometrically.

MATERIALS AND METHODS

Infrared spectra (IR) were obtained on a Perkin-Elmer 1310 Spectrophotometer (Norwalk, CT) using 3% solutions in CCl_4. ^1H Nuclear magnetic resonance spectra (NMR) were obtained with a Varian A-60 spectrometer (Florham Park, NJ) using CCl_4 solutions. Gas liquid chromatography (GLC) was accomplished using a Shimadzu GC-Mini 2 instrument (Columbia, MD) using an SBP-1 column (0.25 mm i.d. × 30 m) fitted with flame ionization detection and employing He as carrier gas with a 50:1 split ratio. Mass spectra (MS) were obtained with a Hewlett-Packard 5995 GC-MS system (Avondale, PA) interfaced with an OV-1 column (0.25 mm i.d. × 12 m). Free fatty acid titrations were performed with a Radiometer titrilab II unit (Westlake, OH), and the spectrophotometric assays were conducted with a Perkin Elmer Model 559 UV-Vis spectrophotometer.

All organic solvents were reagent grade; hexamethyl phosphoric triamide (HMPT), 99%, was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was stored over 13A molecular sieves to remove dimethylamine. Ellman's Reagent, 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB) was obtained from Aldrich Chemical Co. All other chemicals were reagent grade and were obtained from commercial sources.

2-Methyloctanoic acid (2). The general procedure of Pfeffer and Silbert was followed (13) to alkylate the dianion of octanoic acid 1 with methyl iodide producing 2 (92%): b.p. 131-135°C (20 mm) IR,NMR data consistent with assignment (13).

Methyl E-2-octenoate (5). Hexanal (freshly distilled) (104 ml, 0.87 mol), malonic acid (100 g, 0.96 mol), pyridine (dried over KOH) (100 ml) and diisopropylamine (3 ml) were brought together and allowed to stand for 24 hr. The mixture was then heated to 60°C for about 24 hr. The reaction mixture was acidified with cold 6N H_2SO_4 and extracted with ether. The organic phase was washed with water, dried ($MgSO_4$), and concentrated, stripping with benzene to remove residual water azeotropically. The crude acid was esterified with methanol (500 ml) containing H_2SO_4 (1 ml) by heating under

reflux for 16 hr. The mixture was concentrated and worked up in a standard manner, then distilled to give 5 (72% from hexanal): b.p. 88—93°C (20 mm); IR and NMR were consistent with assignment (14).

3-Methyloctanoic acid (3). Cuprous iodide (23.9 g, 0.125 mol) was stirred in dry ether under an inert atmosphere and cooled in an ice bath as methyllithium (180 ml of 1.4 M in ether) was injected. The mixture was stirred for 0.3 hr at 0-5°C, and then was cooled in dry ice-acetone. Compound 5 (19.5 ml, 0.11 mol) was injected, and the resulting mixture was stirred overnight attaining room temperature. The reaction was worked up by adding a solution of 90 ml of sat. NH_4Cl and 10 ml of conc. NH_4OH (slowly). The resulting mixture was filtered by suction through Celite, and the organic phase was isolated and washed several times with water. After drying ($MgSO_4$) and removal of solvent, the product was distilled to give methyl 3-methyloctanoate (56%): b.p. 84-86°C (20 mm); IR, 1740 cm^{-1}. The ester was saponified with 6N KOH:methanol (1:1) by heating under reflux for 2 hr. Product workup was standard and produced the acid 3 (15) (quantitatively): b.p. 93-96°C (0.5 mm) (16); IR 1700 cm^{-1}; NMR, 0.90 (bt, CH_3), 0.95 (unresolved d, CH_3), 1.28 (CH_2 envelope), 2.2 (2H, m, CH_2 C=0), 11.97 (1H, s, CO_2H).

4-Methyl-E-2-octenoic acid (8). Hexanal (freshly distilled) and *t*-butylamine were converted to N-*t*-butylhexanimine, 6, in 78% yield by the general method of Stork and Dowd (16): b.p. 56—62°C (20 mm); IR, 1670 cm^{-1}. Imine 6 was treated with 1.1 equiv of lithium diisopropylamide (LDA) in dry tetrahydrofuran in a manner completely analogous to the alkylation of octanoic acid. Reaction with excess CH_3I followed by the usual workup procedure gave N-*t*-butyl-2-methylhexanimine, 7, in 84% yield: b.p. 60-70°C (20 mm); IR, 1670 cm^{-1}; NMR, 0.94 (m, $2CH_3$), 1.10 (s, *t*-butyl CH_3), 1.3 (CH_2 envelope), 2.2 (m, CH), 7.35 (d, J=5, N=CH). Imine 7 (30 g, 0.178 mol), malonic acid (27.7 g, 0.178 mol) and pyridine (25 mol) were allowed to react as above for the preparation of 5. Acid 8 was obtained in 81% yield: b.p. 140-144°C (20 mm); IR, 1690, 1650 cm^{-1}; NMR, 0.92 (bt, CH_3), 1.08 (d, J=7, CH_3), 1.3 (CH_2 envelope), 5.72 (d, J=16, C=CHC=0), 6.94 (d of d, J=8, 16, HC=CC=0), 11.95 (s, CO_2H). Anal. calcd for: C,69.19; H,10.33; found: C,68.99; H,10.31.

4-Methyloctanoic acid (4). Acid 8 (5.7 g, 36.5 mmol) was hydrogenated in absolute ethanol (30 ml) over 20% Pd/C at 3 atm in a Paar shaking apparatus. The reaction mixure was filtered, diluted with water, and extracted with ether. Continuing a standard workup procedure, the product was distilled giving the acid 4 (quantitatively): b.p. 94-98°C (0.5 mm) (17), IR and NMR were consistent with assignment.

Thiolesters. Compounds 1a, 1b, 1c (Table 1) were prepared by a general procedure of Renard et al. (11) whereby the requisite thiol was treated with one equiv of octanoyl chloride and 2.4 equiv of pyridine in anhydrous ether (ice bath). The reaction product was obtained by a conventional workup procedure, and the hydric thiolesters (containing O-ester) were chromatographed over silica gel (12). Obtained in this manner were: 1a (33%); IR, 1690 cm^{-1}; NMR 0.90 (bt, CH_3), 1.3 (CH_2 envelope), 2.56 (t, J=7, CH_2C=0), 3.02 (t, J=5, CH_2S), 3.68 (t, J=5, CH_2O); MS, m/e 127 ($C_7H_{15}C$=0)$^+$; b.p. 86-89°C (0.6 mm)-rearranges!

1b (24%); m.p. 59-60°C (hexane); IR, 1690 cm^{-1}; NMR, 0.92 (bt, CH_3), 1.3 (CH_2 envelope), 2.62 (bt, CH_2C=0), 3.04 (d, J=5, CH_2S), 3.6 (m, CHOH, CH_2OH).

1c (98%); b.p. 120-123°C (0.45 mm); IR, 1690 cm^{-1}; NMR, 0.90 (m, CH_3), 1.25 (CH_2 envelope + CH_3), 1.30 (s, CH_3), 2.5 (m, CH_2 C=0), 2.96 (d, J=6, CH_2S), 3.4-4.2 (CHOH, CH_2OH); MS, m/3 259 (M-15)$^+$, 127 ($C7H_{15}C$=0)$^+$. The required thiol for 1c was obtained by converting 3-mercapto-1,2-propanediol to a mixture of the two acetonides and separating these by silica gel column chromatography (19).

Compounds 2a, 3a and 4a, the 2-mercaptoethanol S-esters of the branched octanoic acids, were prepared directly from the acids (1 equiv) and dicyclohexylcarbodiimide (1.1 equiv) in methylene chloride. The product was principally S-esterified and was isolated by precipitating dicyclohexylurea with hexane followed by suction filtration. The solvent was removed from the filtrate, and the crude product was chromatographed as before. Each adduct was obtained in 60-70% yield: IR, 1690 cm^{-1}; MS m/e 141 ($C_8H_{17}C$ = 0)$^+$; NMR consistent with structure. All S-esters containing vic-hydroxyl underwent rearrangement to a mixture of O- and S-esters in the injection port (glass liner) of our GLC.

Lipase assays: Titrimetry. Weighed amounts of commercial lipase were allowed to react in an emulsion created by brief sonication of mixtures containing several concentrations of substrate esters in 5 ml of 10% gum arabic. Free fatty acid release was measured by titration with 0.10 N NaOH using "pH stat" mode at pH 7.3. enzyme solutions were prepared in distilled water; substrate concentrations were varied to maximize the initial reaction rate.

Lipase assays: Spectrophotometry. The general procedure of Renard et al. (11) was followed in which solutions of substrate in HMPT (100 μl of 0.0105 M stock), 100 μl of DTNB (Ellman's reagent) in HMPT (20 mg/ml), HMPT (300 μl) and 2.4 ml of 0.05 M Tris buffered at pH 8.0 were brought together in a cuvette. The absorption at 412 mm was immediately monitored

TABLE 1

Initial Velocities of Lipase-Catalyzed Hydrolysis of Octanoate Esters/Thiolesters Determined Titrimetrically[a]

Compound (No.)	*C. rugosa*[b]	*A. niger*[c]
Trioctanoin (11)	8.7	19.0
Methyl octanoate (12)	0.3	2.5
2,2-Dimethyl-4-hydroxymethyl-1,3-dioxolane octanoate (13)	7.7	12.0
S-2-Mercaptoethanol octanoate (1a)	0.4	5.7
S-3-Mercapto-1,2-propanediol octanoate (1b)	0.5	1.5
2,2-Dimethyl-4-mercaptomethyl-1,3-dioxolane octanoate (1c)	3.2	4.7

[a]Titrimetric velocities were obtained on emulsified mixtures (Experimental Methods). Values are μmol fatty acid min^{-1} mg of powder^{-1} ± 5%).

[b]*C. rugosa* lipase was purchased from Enzyme Development Corp., NY.

[c]*A. niger* was a gift from Amano Co., Troy, VA (Lipase-K).

for background hydrolysis. A solution of the enzyme in buffer (100 μl) was added and the new slope calculated. The Ellman's reagent did not react with the enzyme itself.

RESULTS AND DISCUSSION

Synthesis of methyl branched octanoic acids. Octanoic acid, 1, (Fig. 1) was deprotonated with lithium diisopropylamide (LDA), and the resulting dianion was alkylated with methyl iodide to give 2-methyloctanoic acid, 2. Hexanal was condensed with malonic acid in pyridine and then heated to decarboxylate the adduct producing E-2-octenoic acid. The methyl ester of this acid, 5, was alkylated with lithium dimethylcuprate in ether, and the resulting methyl 3-methyloctanoate was saponified to 3-methyloctanoic acid, 3. The N-*t*-butylimine of hexanal, 6, was deprotonated with LDA and methylated to give 7, and this intermediate was condensed directly with malonic acid to give 4-methyl-E-2-octenoic acid, 8. Hydrogenation of 8 over palladium/carbon in ethanol led to 4-methyloctanoic acid, 4.

Synthesis of thiolesters. Thiolesters 1a, b, c were synthesized from the corresponding thiols using octanoyl chloride and pyridine in ether and purifying the product by column chromatography in the case of 1a, b (11) or by distillation for 1c. The thiolesters of 2-mercaptoethanol and the branched acids, namely 2a, 3a and 4a, were prepared using dicyclohexylcarbodiimide in methylene chloride.

Lipase assays. Earlier we had noted that esters of the 1,2-acetonide of glycerol, 2,2-dimethyl-4-hydroxymethyl-1,3-dioxolane, often were hydrolyzed by lipases with rates comparable to those of the corresponding triglycerides (19). The data of Table 1 indicate again that the octanoate ester of the acetonide of glycerol, 13, does react more rapidly than methyl octanoate and comparably to (trifunctional) trioctanoin. The corresponding thiolester 1c similarly reacted more readily than did the other thiolesters examined (except that 1a was slightly more reactive than 1c), though more slowly than 13. An assay of thiolesters has been devised that makes use of Ellman's Reagent at pH 8 to monitor formation of thiolate ion (10); some examples of its utility have been reported (20)(21). The suggested use of HMPT as a cosolvent (11) can provide a homogenous medium that avoids the preparation of emulsions normally required for studies of lipase catalyzed hydrolysis. The reproducibility of such data and the ability to refer with greater certainty to "substrate concentrations" improves the value of kinetic data obtained. Additionally, this methodology would be more sensitive so that precious substrates might be employed sparingly. Implicit are assumptions that 1) candidate substrates do not hydrolyze under these conditions; 2) S to O migration in compounds such as 1a and 1b does not occur during lipolysis—the assay monitors for thiolate ion generated, hence indirectly for free fatty acid, and 3) the same HMPT concentration will maintain a clear solution for a broad range of substrates.

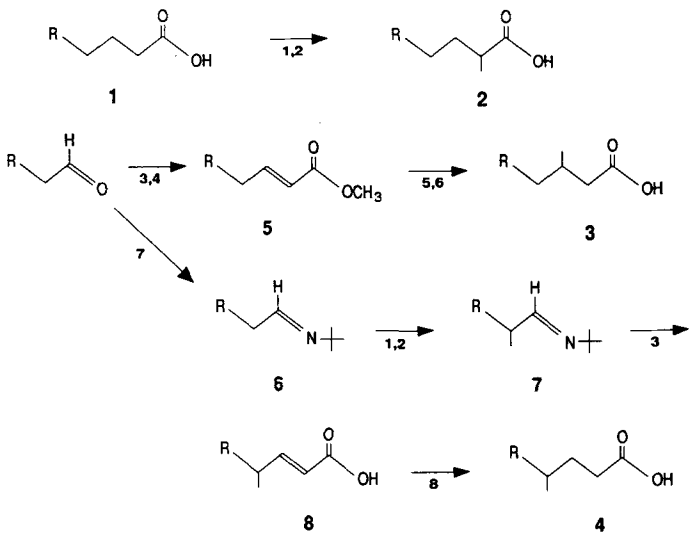

FIG. 1. Synthesis of racemic methyl branched octanoic acids. 1) LDA; 2) CH_3I; 3) malonic acid, pyridine; 4) CH_3OH, H^+; 5) lithium dimethyl cuprate; 6) OH^-; 7) *t*-butylamine; 8) H_2, Pd/C.

The data of Table 2 indicate slower rates of lipolysis in the HMPT containing solutions, with compound 1c faster reacting than 1a and 1b with both lipases. The solution of 1c was cloudy, however, and underwent spontaneous hydrolysis as determined by titrimetry at (the required) pH 8.0. Although solutions of 1a and 1b were homogeneous, a low background hydrolysis was observed as well (about 10%). The values in Table 2 have been corrected for the backgrounds. Although the point was not thoroughly evaluated, synthetic work in our laboratory indicates that S to O migration can occur at high pH, and titrimetry using the HMPT mixtures does not indicate non-enzymatic hydrolysis of compounds 1a and 1b.

The spectrophotometric assay was then performed with the S-mercaptoethanol esters of the branched acids, 2a-4a, (Table 2). The reaction velocities relative to 1a (unbranched analog) showed slower reaction for 2a and 4a, and no reaction at all for 3a. The activity changes are roughly parallel for both commercial lipases.

In summary, we prepared several methyl branched octanoic acids. Several esters and thiolesters of oc-

TABLE 2

Initial Velocities of Lipase-Catalyzed Hydrolysis of Thiolesters by Spectrophotometric Assay (15% HMPT)[a]

Compound	*C. rugosa*[b] V_i	Rel. V_i	*A. niger*[c] V_i	Rel. V_i
1a	0.24 ± 0.01	1.00	0.49 ± 0.06	1.00
1b	0.40 ± 0.01	—	nd[d]	—
1c	0.74 ± 0.01	—	nd[d]	—
2a	0.04 ± 0.01	0.17	0.025 — 0.01	0.05
3a	0	0	0	0
4a	0.13 ± 0.01	0.53	0.09 ± 0.02	0.18

[a]Values given are μmol fatty acid min^{-1} mg of $powder^{-1}$ and are the averaged results of at least three runs corrected for background hydrolysis. Solvents other than HMPT gave these results for 1c (solvent, %, V_i): DMF, 15, 0.55; DMF, 20, 0.60; DMSO, 15, 0.59; DMSO, 20, 0.56; dioxane, 15, 0. All mixtures were heterogeneous.
[b]See Table 1, footnote *b*.
[c]See Table 1, footnote *c*.
[d]Not determined.

tanoic acid itself were hydrolyzed by two commercial lipase preparations, and the 2-mercaptoethanol S-ester was selected as the most conveniently prepared derivative. We then made and assayed the esters of the racemic methyl branched acids. Although faster reacting thiolesters than those originally described (11) are evidently possible, reaction homogeneity may have to be sacrificed. The parallel changes in reactivity of the branched octanoic acid esters observed using two randomly selected (impure) lipases is intriguing, and studies are projected for the corresponding configurationally pure compounds, as well as the remaining methyl branched octanoic acids of the series.

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