Selectivity Paradigm in Lipase Reactions: Correlating Actual and Observed Regioselectivity in Hydrolysis of Unsymmetrical Diacetates†

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The first simple method using labeling technique has been developed to confirm the actual and observed selectivities in the enzymatic hydrolysis of unsymmetrical diacetates and to measure the enzyme selectivity efficiency. The simple method consists of enzymatic hydrolysis of unsymmetrical diacetate followed by labeling of the hydroxyacetate formed with CD_3COOD/DCC and enzymatic rehydrolysis of the labeled compound under the identical set of reaction conditions to estimate the amount of label retained by ^1H NMR spectroscopy. The amount of label lost directly indicates the extent of regioselective action of the enzyme.

A large number of meso and unsymmetrical vicinal diacetates with a wide range of structural diversities have been enzymatically hydrolyzed to obtain chiral molecules.[1] The enzymatic conversions of these diacetates have been carried out at acidic, neutral, or basic pH depending upon the enzyme used. Since intramolecular acyl migrations under various reaction conditions are well documented,[2,3] a major unresolved problem is the unambiguous determination of the actual and observed regioselectivities in enzymatic hydrolyses. For example, the literature reports on enzymatic hydrolysis of di- and triesters of glycerol reveal varying observations, viz., (i) the enzyme directly recognizes the primary acetate,[4] (ii) the enzyme directly recognizes the secondary acetate,[5] (iii) the enzyme first recognizes the primary acetate followed by in situ intramolecular migration to yield the secondary alcohol derivative,[4a] (iv) the formation of a multiple point attachment of the substrate with enzyme,[6] and (v) the recognition of the primary acetate in antibody-mediated hydrolysis[7] of the *p*-nitro-substituted derivative of **7**. To delineate the mechanisms of hydrolysis and migrations in enzymatic reactions, we present here a simple unambiguous protocol using labeling technique to validate the actual and observed selectivities, along with a measure of enzyme selectivity efficiency.

Recently[8] we noticed a remarkable chemo-, regio-, and enantioselective enzymatic hydrolysis of (±)-diacetate **1** using AmanoPS and pig liver acetone powder (PLAP) to obtain chiral precursors of clinically used (+)-diltiazem in very good yields and optical purities. The product analysis[8] of (2*S*,3*R*)-hydroxyacetate **2** revealed that the enzymes AmanoPS and PLAP are nearly 100% chemoselective and about 98−99% enantioselective in their action with substrate (±)-**1**. We were keen to confirm the nearly 100% regioselective action of these enzymes, as theoretically, the hydroxyacetate **2** can form directly or partially/completely via **5**, through in situ intramolecular acyl migration. It is necessary to establish or rule out such a possibility to account for the observed high regiospecificity, and a literature search revealed a lack of any direct and unambiguous method to correlate the actual and observed specificity in enzymatic hydrolysis of vicinal diacetates. Our present strategy consists of an enzymatic hydrolysis of unsymmetrical diacetate, followed by labeling with CD_3COOD/DCC and enzymatic rehydrolysis[9] of the labeled compound under the identical set of reaction conditions to estimate the amount of label retention by ^1H NMR spectroscopy. The amount of label lost will directly indicate the extent of regioselective action of the enzyme. As depicted in Scheme 1, the (±)-diacetate **1** was enzymatically hydrolyzed using AmanoPS at its optimum pH (7.0), to obtain a mixture of (2*S*,3*R*)-hydroxyacetate **2** and unreacted (2*R*,3*S*)-

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(7) Ikeda, K.; Achiwa, K. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 225. The ^1H NMR data reported by the authors for the *p*-nitro derivative of **8** fit in well with the alternate structure, i.e., the *p*-nitro derivative of **9**, and our method will be directly useful to confirm actual and observed selectivities in this example.

(8) Desai, S. B.; Argade, N. P.; Ganesh, K. N. *J. Org. Chem.* **1996**, *61*, 6730.

(9) The isotopic effect on rate and selectivity of enzymatic hydrolysis has not been taken into account in this method. The labeled compounds were characterized by using ^1H NMR and mass spectral data, while the quantitative estimation of the label was done using ^1H NMR data (relative integrations of methyl group from −OAc).

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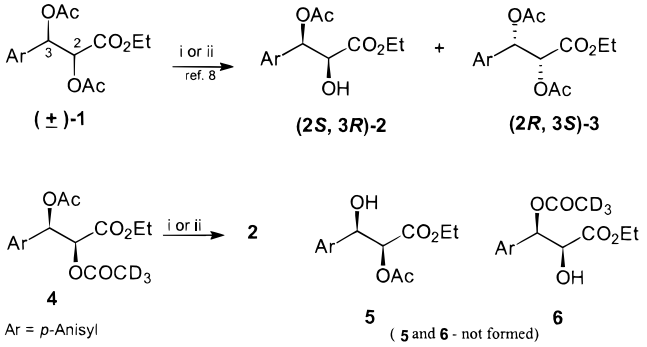
Scheme 1[a]



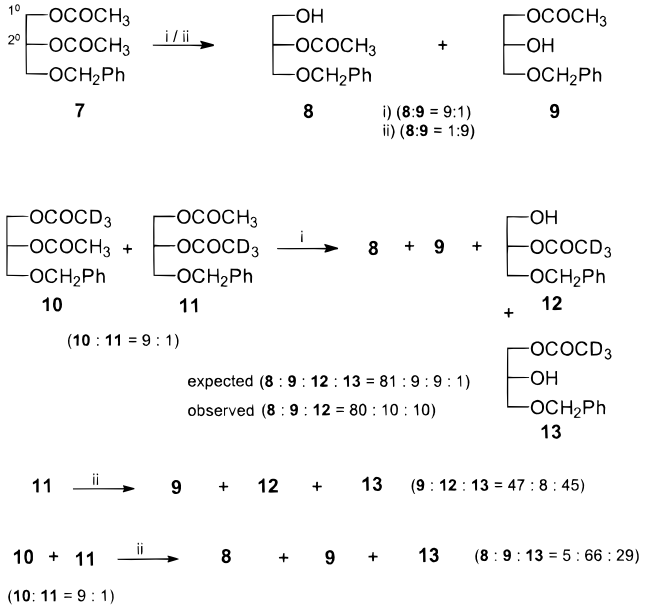
(±)-1 (2S, 3R)-2 (2R, 3S)-3 2

[a] (i) AmanoPS, C_6H_6/petroleum ether (2:1), 50 mM sodium phosphate buffer (pH 7.0), 25 °C, 2 h. (ii) PLAP, C_6H_6/petroleum ether (2:1), 50 mM sodium phosphate buffer (pH 8.0), 25 °C, 2 h.

diacetate **3**. The chromatographically purified (2*S*,3*R*)-hydroxyacetate **2** (98% ee) was reacylated using CD_3-COOD and DCC as a coupling reagent to obtain **4**.[9] The 2*S*-labeled diacetate **4** was resubjected to enzymatic hydrolysis using AmanoPS under the respective previous set of reaction conditions. The exclusive product formed in this experiment[10] was **2** in 75% yield and nearly 100% ee. The fact that the labeled compound **6** was not obtained proves that the compound **2** is formed from **4** by direct hydrolysis. This implies that **1** → **2** conversion is also by direct hydrolysis and not via migration in the initially formed intermediate **5**. Thus the true and observed regioselectivity of the hydrolysis with AmanoPS at pH 7 with the present substrate is the same, and the enzyme is nearly 100% regioselective in its action. In a second example to demonstrate the validity of our method, we chose the glyceroldiacetate (±)-**7**,[11,12a] which was subjected to biphasic enzymatic hydrolysis using AmanoPS at pH 7.0 (Scheme 2). This reaction furnished a product mixture of **8** and **9**[12] (9:1, 55% yield) in 2 h. In control experiments without enzyme AmanoPS at pH 7.0, the diacetate (±)-**7** remained unreacted, proving the absence of any accompanying chemical hydrolysis. The mixture of hydroxyacetates **8** and **9** thus obtained[13] was monolabeled with CD_3COOD/DCC to yield a mixture of **10** and **11** in 9:1 proportion. If **9** (10%) is formed from **8** via in situ intramolecular acyl migration, then the expected ratio of the products **8**+**9**+**12**+**13** from the **9**+**10** (9:1) mixture will be 81:9:9:1. This mixture of monolabeled diacetates on AmanoPS-catalyzed hydrolysis yielded a mixture of **8**, **9**, and **12** in approximately 80: 10:10 proportion. These observed results indicate that in the enzymatic hydrolysis of both **7** and **10**+**11** the enzyme AmanoPS recognizes the primary acetate in the substrate in nearly 100% regioselective (*sn*-1 specific) manner and 10% of **9** is formed by intramolecular acyl migration.

To examine whether these observations are general for lipase hydrolysis, we studied the enzymatic hydrolysis of (±)-**1** and (±)-**7** with PLAP at its optimum pH (8.0). The optically pure monolabeled diacetate **4** obtained via the PLAP-catalyzed hydrolysis of (±)-**1** on rehydrolysis with PLAP at pH 8.0 also revealed a complete loss of

Scheme 2[a]



[a] (i) AmanoPS, C_6H_6/petroleum ether (2:1), 50 mM sodium phosphate buffer (pH 7.0), 25 °C, 2 h. (ii) PLAP, C_6H_6/petroleum ether (2:1), 50 mM sodium phosphate buffer (pH 8.0), 25 °C, 2 h.

label, confirming that the actual and observed selectivity is the same. In a second set of experiments, the diacetate (±)-**7** was subjected to biphasic enzymatic hydrolysis using PLAP[14] at pH 8.0, and the reaction upon 25% conversion (2 h)[15] gave a mixture of **8** and **9** but in 1:9 proportion (reversal of AmanoPS experiment). In control experiments without enzyme PLAP at pH 8.0, the diacetate (±)-**7** remained unreacted, proving the absence of any accompanying chemical hydrolysis. The chromatographically purified compound **9** on coupling with CD_3-COOD/DCC gave the secondary labeled diacetate **11**, which was resubjected to enzymatic hydrolysis using PLAP at pH 8.0. The reaction upon 25% completion (2 h) yielded a mixture of **9, 12**, and **13** in 47:8:45 ratio. Similarly the primary labeled mixture of diacetates **10**+**11** (9:1) on PLAP-catalyzed hydrolysis at pH 8.0 furnished again a mixture of **8, 9**, and **13** in 5:66:29 proportion. These results clearly indicate an intramolecular acyl migration in PLAP-induced hydrolysis of both **11** and **10**+**11**. In the absence of PLAP, the mixture of hydroxyacetates **8**+**9** (9:1) at pH 8.0 showed very slow intramolecular acyl migration to yield **9** (24 h, 60%). Hence in PLAP-catalyzed hydrolysis of (±)-**7**, the observed intramolecular acyl migration seems to be a synergic effect of enzyme and pH.

The observed difference in the amount of label retained in **9**+**12**+**13** and **8**+**9**+**13** mixtures in the above-mentioned two experiments is interesting. It is possible that during the formation and cleavage of an unsymmetrical intermediate of substrate with the enzyme PLAP the

(10) The rate of enzymatic hydrolysis for (2*S*,3*R*)-**4** was relatively faster (24 h) than for (±)-**1** (156−180 h).

(11) Menger, F. M.; Chen, X. Y.; Brocchini, S.; Hopkins, H. P.; Hamilton, D. *J. Am. Chem. Soc.* **1993**, *115*, 6600.

(12) (a) Herradon, B.; Cueto, S.; Morcuende, A.; Valverde, S. *Tetrahedron Assym.* **1993**, *4*, 845. (b) Argazzoni, F.; Maconi, E.; Potenza, D.; Scolastico, C. *Synthesis* **1989**, 225.

(13) The ^1H NMR spectral data of the crude reaction products and column-purified products revealed that in both examples there was no intramolecular acyl migration during silica gel column chromatography.

(14) The AmanoPS- and PLAP-catalyzed hydrolysis of (±)-**7** was nonstereoselective; see: Taterie, N. H.; Bailey, R. A.; Kates, M. *Arch. Biochem. Biophys.* **1958**, *78*, 319.

(15) PLAP-catalyzed hydrolysis of (±)-**7** was arrested when the corresponding diol formation was detected upon TLC (~25% conversion, 2 h).

amount of label retained may depend on its position (primary or secondary) in the substrate. These results (Scheme 2) convincingly indicate that in AmanoPS-catalyzed hydrolysis of (±)-**7**, although the observed regioselectivity from the final product composition appears to be 90%, the actual *sn*-1 selectivity is ~100%, since 10% of **9** is a result of intramolecular acyl migration during the hydrolysis. In PLAP-catalyzed hydrolysis of (±)-**7** at pH 8.0, the observed regioselectivity is very high (~90%) and the actual selectivity differs markedly (intramolecular acyl migration/multiple point attachment and hydrolysis).

In summary, we have demonstrated with the above two examples the first simple method to correlate the actual and observed regioselectivity in enzymatic hydrolysis of unsymmetrical diacetates. By suitable manipulations in reaction conditions (enzyme type, solvent system, pH range, and temperature), it may be possible to obtain both isomers with high regioselctivity by completely preventing or forcing the acyl migration (Scheme 2). This strategy may also be useful for assessing the actual and observed regioselectivities in enzymatic hydrolysis of polyacylated systems including sugars[2k] and stereoselectivities in meso diacetates by exclusively labeling the pro-*R* or pro-*S* acetate.

Experimental Section

DCC and CD_3COOD (99.5%) were obtained from Aldrich Chemical Co. The substrate diacetates (±)-**1**,[8] (±)-**7**,[11,12a] and (2*S*,3*R*)-hydroxyacetate **2**[8] were prepared as reported before. The biphasic enzymatic hydrolyses of (±)-**1** and (−)-**4** with AmanoPS (800 U) and PLAP (20 U) were carried out using known[8] procedures. The activity of lipase powder has been expressed in terms of units: 1 unit corresponds to micromoles of butyric acid (estimation by GC) liberated from glyceryl tributyrate per minute per milligram of enzyme powder.[16] The term usual workup refers to extraction with ethyl acetate, washing the organic layer with water and brine, drying of the organic layer over Na_2SO_4, and concentration in vacuo. Column chromatographic purification was done on ACME silica gel.

AmanoPS-Catalyzed Biphasic Hydrolysis of 7. A solution of diacetate **7** (1 mmol) in petroleum ether/benzene (2:1) (20 mL) was added to a suspension of AmanoPS (125 mg) in 50 mM sodium phosphate buffer (10 mL), pH 7.0, at 25 °C. After 2 h, the reaction mixture was filtered through Celite, and usual workup followed by silica gel column chromatographic removal of unreacted diacetate (elution with 20% ethyl acetate/petroleum ether) furnished **8**+**9** (9:1) as a thick oil in 55% yield. In the spectral data only signals due to the major component **8** have been listed below. **8**: IR (neat) ν_{max} 3440, 1730 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 2.12 (s, 3H), 3.67 (d, J = 5.5 Hz, 2H), 3.82 (d, J = 5.5 Hz, 2H), 4.56 (d, J = 2.5 Hz, 2H), 5.05 (quin, J = 5.5 Hz, 1H), 7.20−7.45 (m, 5H); ^{13}C NMR ($CDCl_3$, 50 MHz): 20.8, 62.0, 68.7, 73.2 (2C), 127.4, 127.6, 128.2, 137.6, 170.6; MS (*m*/*e*) 225, 207, 154, 137, 117, 105, 91.

Similarly **10**+**11** upon AmanoPS-catalyzed hydrolysis furnished a mixture of **8**+**9**+**12** as a thick oil in 80:10:10 proportion.

(16) Dupus, C.; Corre, C.; Boyaval, P. *Appl. Environ. Microbiol.* **1993**, *59*, 4004.

PLAP-Catalyzed Biphasic Hydrolysis of 7. A solution of diacetate **7** (5 mmol) in petroleum ether/benzene (2:1) (150 mL) was added to a suspension of PLAP (500 mg) in 50 mM sodium phosphate buffer (75 mL), pH 8.0, at 25 °C, and the pH was maintained at 8 using auto-stat with 0.1 M NaOH solution. At the end of 2 h the reaction mixture was filtered through Celite, and usual workup followed by silica gel column chromatographic purification (elution with 20% ethyl acetate/petroleum ether) first gave pure **9** as a thick oil (20% yield) and then a mixture of **8** and **9** in 5% yield. **9**: IR (neat) ν_{max} 3440, 1720 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 2.08 (s, 3H), 2.60 (bs, 1H), 3.45−3.62 (m, 2H), 3.97−4.10 (m, 1H), 4.08−4.26 (m, 2H), 4.58 (s, 2H), 7.20−7.48 (m, 5H); ^{13}C NMR ($CDCl_3$, 50 MHz) 21.0, 65.8, 68.9, 71.2, 73.6, 128.0 (2C), 128.7, 138.0, 178.3.

Similarly **11** upon PLAP-catalyzed hydrolysis furnished a mixture of **9**+**12**+**13** as a thick oil in 47:8:45 proportion, while **10**+**11** (9:1) upon PLAP-catalyzed hydrolysis furnished a mixture of **8**+**9**+**13** as a thick oil in 5:66:29 proportion.

Labeling of Hydroxyacetate-2 with CD_3COOD/DCC. To a stirred solution of hydroxyacetate **2** (1 mmol), CD_3COOD (72 mg, 1.2 mmol), and a catalytic amount of DMAP in EDC (5 mL) was added a solution of DCC (247 mg, 1.2 mmol) in EDC (2 mL) in a dropwise fashion at room temperature. The reaction mixture was further stirred at room temperature for 45 min and then filtered through Celite, the residue was washed with EDC, and the organic layer was concentrated in vacuo. The residue on usual workup followed by silica gel column chromatographic purification (elution with 10% ethyl acetate/petroleum ether) gave the corresponding monolabeled compound **4** in 90−95% yield as a thick oil. **4**: IR (neat) ν_{max} 1740, 1730, 1720 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 1.18 (t, J = 7.0 Hz, 3H), 2.10 (s, 3H), 3.80 (s, 3H), 4.15 (q, J = 7.0 Hz, 2H), 5.30 (d, J = 4.4 Hz, 1H), 6.22 (d, J = 4.4 Hz, 1H), 6.88(d, J = 8.8 Hz, 2H), 7.32 (d, J = 8.8 Hz, 2H); MS (*m*/*e*) 327, 264, 222, 179, 151, 137, 121.

Similarly **8**+**9** (9:1) gave a mixture of **10**+**11** (9:1) as a thick oil in 90−95% yield. **10**+**11**: ^1H NMR ($CDCl_3$, 200 MHz) δ 2.06 (s, 0.3H), 2.10 (s, 2.7H), 3.60 (d, J = 5 Hz, 2H), 4.12−4.25 (dd, J = 12 and 7 Hz, 1H), 4.30−4.42 (dd, J = 12 and 5 Hz, 1H), 4.54 (d, J = 2 Hz, 2H), 5.23 (quin, J = 6 Hz, 1H), 7.20−7.45 (m, 5H); MS (*m*/*e*) 270, 210, 162, 137, 91.

Similarly **9** gave **11** as a thick oil in 90−95% yield. **11**: ^1H NMR ($CDCl_3$, 200 MHz) δ 2.06 (s, 3H), 3.60 (d, J = 5 Hz, 2H), 4.12−4.26 (dd, J = 12 and 7 Hz, 1H), 4.28−4.40 (dd, J = 12 and 5 Hz, 1H), 4.56 (d, J = 2 Hz, 2H), 5.16−5.30 (m, 1H), 7.25−7.45 (m, 5H); MS (*m*/*e*) 270, 207, 181, 162, 120, 91.

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Supporting Information Available: ^1H NMR spectra of **2**, **4**, **7**, **8**+**9**, **9**, **10**+**11**, **11**, **8**+**9**+**12**, **9**+**12**+**13**, and **8**+**9**+**13**, ^{13}C NMR spectra of **7**, **8**+**9**, and **9**, mass spectra of **2**, **4**, **8**+**9**+**12**, **10**+**11, 11**, and **9**+**12**+**13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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