# Modulation of Cholesterol Esterase's Diastereoselectivity by Chiral Auxiliaries. The Very Large Effect of a Dihydroxy Bile Salt on the Hydrolysis of α-Tocopheryl Acetates[1]

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In a preliminary communication[3] we reported that an unprecedented modulation of an enzyme's diastereoselectivity could be induced by "minor" changes in the structure of a "chiral auxiliary" which probably does not itself undergo chemical change. The enzyme was cholesterol esterase,[4] CE (bovine, BCE, and porcine, PCE), the chiral auxiliaries were the three 3α,7α,12α-trihydroxy bile salts, cholate, glycocholate, and taurocholate, and the substrates were 2*R*,4′*R*,8′*R*-(*RRR*-) and 2*S*,4′*R*,8′*R*-(*SRR*-)α-tocopheryl acetates (α-TOAc), which were hydrolyzed at 37 °C to the corresponding diastereomeric tocopherols (α-TOH).[5] Problems caused by the less than rigorous purities of both the commercial CEs and the commercial bile salts led us to repeat all our original kinetic measurements and to study the diastereoselectivities of a much wider variety of CE/bile salt/α-TOAc systems.[6] During this work we explored the possibility that dihydroxy bile salts could also activate CE[7] and, more importantly, could also modulate its diastereoselectivity for the hydrolyses of (*RRR*)- and (*SRR*)-α-TOAc. Preliminary experiments[14] with crude BCE showed that only the 3α,7α-dihydroxy bile salts activated the enzyme toward the hydrolyses of (*RRR*)- and (*SRR*)-α-TOAc, the 3α,12α- and 3α,7β-dihydroxy bile salts (and monohydroxy bile salts) being almost without effect.

Chiral discrimination by the CE/bile salt combination is most readily determined by measuring the initial rates of hydrolysis, $V_i$, of the diastereomeric α-tocopheryl acetates and calculating the ratio of these rates, $V_i^{RRR}/V_i^{SRR}$. This can be done either using each diastereomeric acetate separately (i.e., in two noncompetitive experiments) or, with the help of deuterium labeling, using equal concentrations of the two diastereomers together (i.e., in a single competitive experiment). The latter experiment is, of course, far more significant if the bile salt modulating effect on CE-catalyzed reactions is to be exploited in organic syntheses. The formation of α-TOH follows pseudo-first-order kinetics, and excellent straight lines are obtained by converting to a semilogarithmic type of plot:

$$\ln\{([\alpha\text{-TOAc}]_0 - [\alpha\text{-TOH}]_t)/[\alpha\text{-TOAc}]_0\} = C_t$$

or, since the initial concentration of acetate, $[\alpha\text{-TOAc}]_0$, was 100 μM in each experiment,[15]

$$\ln\{(100 - [\alpha\text{-TOH}_t])/100\} = C_t$$

where $[\alpha\text{-TOH}]_t$ is in μM and $C = -V_i$ (M s$^{-1}$)$/[\alpha\text{-TOAc}]_0$ (M) $= -V_i/10^{-4}$ (s$^{-1}$). Thus, these semilog plots yield $V_i$, the values of which at 37 °C using carefully purified PCE (MW 58 kDa (monomer), 340 kDa (oligomer))[6] activated and modulated by three 3α,7α,12α-trihydroxy bile salts and by three 3α,7α-dihydroxy bile salts (all of which had also been carefully purified by recrystallization)[16] are given in Table 1.[17] It can be seen from these data that two of the 3α,7α-salts, viz., chenodeoxycholate and taurochenodeoxycholate, were very much less effective activators of PCE than the corresponding 3α,7α,12α-salts, viz., cholate and taurocholate. However, glycochenodeoxycholate (GCDC) was not only just as effective as glycocholate (GC) in activating PCE, but also it produced an outstanding diastereoselectivity in the *competitive* hydrolysis of (*RRR*)- and (*SRR*)-α-TOAc's. The formation of (*RRR*)- and (*SRR*)-α-TOH as a function of time during the noncompetitive and competitive hydrolyses of (*RRR*)- and (*SRR*)-α-TOAc at 37 °C catalyzed by pure PCE and modulated by GC and GCDC under our "standard" conditions[3,6,15] are shown in the top two panels in Figure 1, and the corresponding semilogarithmic plots are shown in the two bottom panels. In the competitive experiment with GC there is a dramatic, 10-fold reduction in $V_i^{RRR}$ relative to its value in the noncompetitive experiment, whereas there is no change in $V_i^{SRR}$ between the two experiments.[18] As a consequence, there is a complete loss of stereoselectivity in the GC competitive experiment. In sharp contrast, with the dihydroxy bile salt, GCDC, there is little or no change in the initial rates of hydrolysis of the two diastereomeric acetates between the noncompetitive

**Table 1.** Initial Rates, $V_i \times 10^8$ M s$^{-1}$, for the Noncompetitive and (in Parentheses) Competitive Hydrolysis of (*RRR*)- and (*SRR*)-α-TOAc Catalyzed by Pure PCE and Modulated by Three Pure Trihydroxy Bile Salts and Three Pure Dihydroxy Bile Salts under "Standard" Experimental Conditions[15] at 37 °C[a]

| bile salt (hydroxylation) | $10^8 V_i$/M s$^{-1}$ [b] *RRR* | *SRR* | $V_i^{RRR}/V_i^{SRR}$ [c] |
|---|---|---|---|
| cholate (3α,7α,12α) | 3 (2) | 12 (9) | 0.25 (0.22) |
| chenodeoxycholate (3α,7α) | 0.03 (0.02) | 0.04 (0.04) | 0.75 (0.5) |
| glycocholate (3α,7α,12α) | 1.0 (0.1) | 0.1 (0.1) | 10 (1) |
| glycochenodeoxycholate (3α,7α) | 0.8 (0.8) | 0.07 (0.09) | 11 (9) |
| taurocholate (3α,7α,12α) | 4 (0.4) | 0.9 (0.9) | 4 (0.4) |
| taurochenodeoxycholate (3α,7α) | 0.03 (0.03) | 0.03 (0.02) | 1 (1.5) |

[a] $V_i$ is only $7 \times 10^{-12}$ M s$^{-1}$ in the absence of PCE. [b] Individual rate measurements are reproducible to ±5%. [c] Errors for noncompetitive selectivity ratios are ±10% and for competitive selectivity ratios are ±5%.

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(4) EC 3.1.1.13.
(5) 2*R*,4′*R*,8′*R*-α-Tocopherol is natural vitamin E; the (*SRR*) diastereomer is present in synthetic vitamin E.
(6) These results, including those which repeat and largely confirm our initial work,[3] will be reported in a full paper.
(7) Literature evidence[8-13] regarding the activation of CEs by dihydroxy bile salts is rather confusing, possibly because of the use of impure bile salts.
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(14) Under essentially "standard" noncompetitive experimental conditions,[15] the yields of (*RRR*)- and (*SRR*)-α-TOH formed after 24 h of incubation with commercial, nonpurified 3α,7α-dihydroxy bile salts were, respectively, 8 and 21% with chenodeoxycholate, 84 and 36% with GCDC, and 30 and 25% with taurochenodeoxycholate.
(15) "Standard" experimental conditions:[3,6] 40 mM bile salt, 2 mM *l*-dimyristoylphosphatidylcholine (*l*-DMPC), 100 μM (*RRR*)-α-TOAc-$d_6$ or (*SRR*)-α-TOAc-$d_3$ in noncompetitive experiments and 100 μM (*RRR*)-α-TOAc-$d_6$ plus 100 μM (*SRR*)-α-TOAc-$d_3$ in competitive experiments, together with the CE in 50 mM Tris buffer. The temperature was normally 37 °C. The amount of CE employed was adjusted so as to give an approximate initial rate of hydrolysis of (*SRR*)-α-TOAc in the cholate-modulated reaction (which is the fastest) of $5 \times 10^{-8}$ M s$^{-1}$.[3,6] Aliquots of 100 μL were removed at intervals and "quenched" in 0.5 M SDS (0.2 mL), following which 50 μL of an ethanol solution containing α-TOAc-$d_9$ and α-TOH-$d_9$ (130 μM each) was added to provide internal standards, and then 500 μL of ethanol and 500 μL of heptane were added. The heptane extracts were analyzed by GC-MS, and the absolute yields of (*RRR*)-α-TOH-$d_6$ and (*SRR*)-α-TOH-$d_3$ were obtained.
(16) The GC used in the earlier work[3] was very impure.
(17) Rather similar data were obtained with crude, commercial PCE.
(18) We have attributed this difference in the selectivities found in the competitive and noncompetitive experiments to competitive inhibition of the enzyme by (*SRR*)-α-TOAc (or possibly its phenol).[3]

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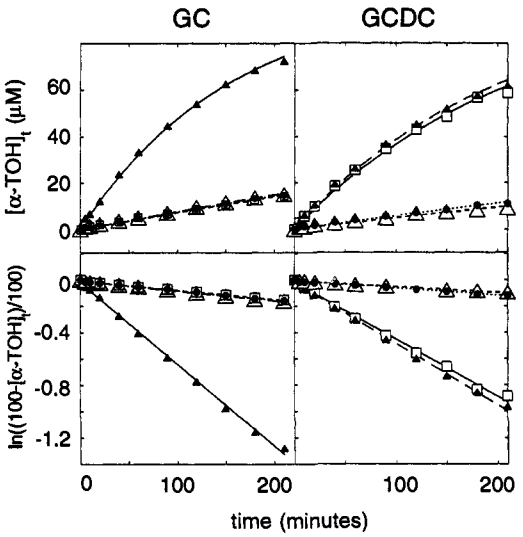


**Figure 1.** Formation of (*RRR*)- and (*SRR*)-α-TOH as a function of time during the pure glycocholate (GC)- and pure glycochenodeoxycholate (GCDC)-modulated, pure PCE-catalyzed hydrolyses of the diastereomeric acetates at 37 °C under noncompetitive and competitive "standard" conditions[15] (two top panels). Semilogarithmic plots of $\ln(100-[\alpha\text{-TOH}]_t/100)$ versus time for the same reactions (two bottom panels). Key: ▲ and △, (*RRR*)- and (*SRR*)-α-TOH, respectively, in the noncompetitive hydrolyses experiments; □ and ●, (*RRR*) and (*SRR*)-α-TOH, respectively, in the competitive hydrolyses experiments. The experimental points have not been drawn all the same size simply for reasons of clarity.

and competitive experiments, and there is, therefore, a uniquely high competitive chiral selectivity.

In view of the strong activating effect of GCDC on PCE (an effect of comparable magnitude to that of the "traditional" trihydroxy bile salts), we were very surprised to discover that this bile salt had a negligible ability to activate the BCE-catalyzed hydrolysis of either of the diastereomeric acetates during 3 h of incubation under standard conditions.[15,19] This is the first firm kinetic evidence which demonstrates that these two enzymes can act differently. That is, in all our earlier experiments,[3,6] in which the enzymes were activated and modulated by one of the 3α,7α,-12α-trihydroxy bile salts, the absolute and relative rates of hydrolysis of (*RRR*)- and (*SRR*)-α-TOAc catalyzed by BCE and PCE were found to be sufficiently similar that any small differences were attributed to experimental error. Other workers have also reported that BCE and PCE show the same substrate specificity and kinetic properties.[20,21] Indeed, cholesterol esterases have appeared to be kinetically interchangeable to the extent that the source of the enzyme is sometimes undefined.[22] Of course, BCE and PCE are not structurally identical.[23] However, it would appear that only when these two enzymes are activated by GCDC does a truly dramatic difference between them become obvious.

Intrigued by the anomalous ability of the GCDC/PCE system to maintain a very large diastereoselectivity under competitive conditions and hoping to increase the magnitude of this discrimination, we explored the effect of temperature on $V_i^{RRR}/V_i^{SRR}$ with this bile salt and, for comparison, with GC.[29] The GCDC/PCE and GC/PCE systems were catalytically active at temperatures from 21 to 50 °C but were inactive at 60 °C, presumably because the enzyme became denatured. For both bile salts, the initial rates of hydrolysis in the noncompetitive and in the competitive experiments showed only a small temperature dependence, increasing by roughly a factor of 2 between 21 °C and 50 °C (see supplementary material), which corresponds to an effective activation energy of about 5 kcal/mol. The stereoselectivity for the GCDC-modulated competitive hydrolyses remained high over this temperature range, with a trend to lower values as the temperature was decreased. The activity of the enzyme is, however, reduced substantially at these low temperatures. For example, in the competitive experiment, the values of $V_i^{RRR}$ were reduced by factors of 14 at 10 °C and 47 at 2 °C relative to the 37 °C value. The hydrolyses of the diastereomeric α-TOAc's are presumed to involve α-TOAc solubilized in mixed bile salt/dimyristoylphosphatidylcholine (DMPC) micelles,[15] with the water-soluble enzyme reacting at the micelle's surface, i.e., at the micelle/CE interface. We presume that these micelles undergo a phase change at a temperature between 21 and 10 °C with a consequent fairly large reduction in reactivity of the bile salt/DMPC/CE system but without major change in diastereoselectivity (i.e., $V_i^{RRR}/V_i^{SRR}$ decreases from 11 at 50 °C through 9 at 37 °C, 8 at 21 °C, ca. 6 at 10 °C, to ca. 5 at 2 °C, see supplementary material).[30]

The spectacular diastereoselectivity achieved in the GCDC-modulated PCE-catalyzed competitive hydrolysis of (*RRR*)- and (*SRR*)-α-TOAc's is made all the more remarkable by the fact that the bond which is cleaved is separated by six bonds from the stereocenter. Despite considerable effort,[6] we still do not know how the structure of the bile salt can have such a profound influence on the diastereoselectivity of the PCE-catalyzed competitive hydrolysis of (*RRR*)- and (*SRR*)-α-TOAc, i.e., at 37 °C $V_i^{RRR}/V_i^{SRR}$ can be varied from a high of ca. 9 with GCDC to a low of ca. 0.22 with cholate (see Table 1). Such a dramatic 40-fold change in an enzyme's chiral selectivity is without precedent—and, as yet, explanation!

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**Supplementary Material Available:** Kinetic data at 21, 37, and 50 °C (Table 2) and structures (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(19) We attribute the earlier positive results with BCE[14] to impurities in the commercial GCDC (probably one or more trihydroxy bile salts).

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(23) PCE and BCE consist of several isoforms with monomeric molecular weights (in kDa) reported to be 80,[24] 45,[25] 74 and 83,[26] for PCE; and 65,[27] 67, and 72[28] for BCE. PCE and BCE undergo bile salt-induced aggregation, PCE having been observed as a dimer[24,25] and BCE plus cholate as a hexamer.[6]

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(29) Changes in the nature of the colipid (*l*-DMPC under standard conditions)[15] and in the trihydroxy bile salt/colipid ratio have been found to have little or no effect on $V_i^{RRR}/V_i^{SRR}$.[6]

(30) In the absence of PCE, the initial rates at 10 and 2 °C are less than 3% of the slower of the two enzyme-catalyzed reactions (*SRR*-α-TOAc) so that the calculated diastereoselectivities of 6 and 5 do not need a "correction" even at these low temperatures.