SUPPORTING INFORMATION FOR MICROFILM ADDITION

Catalytic Mechanism of Scytalone Dehydratase:

Site-Directed Mutagenisis, Kinetic Isotope Effects, and Alternate Substrates

Synthesis of N-(3,3-diphenylpropyl)-5-fluoro-2-hydroxybenzamide ***2***. A solution of 2.0 g (12.8 mmol) of 5-fluorosalicylic acid and 2.08 g (12.8 mmol) of carbonyl diimidazole in 30 mL CH_2Cl_2 was stirred 2 h at ambient temperature before 2.7 g (2.08 mmol) of 3,3-diphenyl propanol and 1.4 mL (12.8 mmol) Et_3N were added sequentially. After stirring overnight, the mixture was diluted with CH_2Cl_2 and washed with water and brine. Drying ($MgSO_4$) and removal of solvent gave material which was purified by chromatography on silica gel (9:1 hexanes-ethyl acetate) to give 2.0 g of the title compound as a white solid, mp 143-144 °C. ^{1}H NMR ($CDCl_3$) δ 2.4 (q, J= 6 Hz, 2H), 3.5 (q, J = 6 Hz, 2H), 4.0 (t, J= 7 Hz, 1H), 5.9 (s, br, 1H), 6.45 (d, 1H), 6.9 (m, 1H), 7.1 (m, 1H), 7.2-7.4 (m, 10H), 12.0 (s, 1H).

Synthesis of 3,4-Dihyro-6,8-dihydroxy-1(2H)-2-13C-naphthalenone ***5*** *(*^{13}*C-DHS)*. A solution of 1.4 g (6.2 mmol) of 3.5-dimethoxy-benzenebutanoic-carboxy-13C acid (1) in 50 mL polyphosphoric acid was heated to 120 °C for 90 min, cooled to 80 °C and poured into water. The water was extracted with ethyl acetate which was washed with water and brine. Drying ($MgSO_4$) and removal of solvent was followed by chromatography on silica gel (2:1 hexanes-ethyl acetate) to afford 700 mg of 3,4-dihyro-6,8-dimethoxy-1(2H)-2-^{13}C-naphthalenone as a solid, mp 64-66.5°C. ^{1}H NMR ($CDCl_3$) δ 2.0 (m, 2H), 2.6 (d of t, J = 132, 7 Hz, 2H), 2.9 (m, 2H), 3.85 (s, 3H), 3,9 (s, 3H), 6.3 (2s, 2H). A portion (0.5 g, 2.4 mmol) of this material was dissolved in 20 mL CH_2Cl_2 and

cooled to -78 °C before a solution of 12 mL BBr_3 in CH_2Cl_2 (1.0 M, 12 mmol) was added dropwise. After stirring 2 h cold and warming to room temperature, the solution was quenched with water. The solution was extracted 2 times with ethyl acetate which was washed with brine. Drying ($MgSO_4$) and removal of solvent was followed by chromatography on silica gel (9:1 ethyl acetate-methanol) to afford 240 mg of product as a solid, mp 207-209 °C. ^{1}H NMR ($CDCl_3$) δ 2.4 (m, 2H), 2.65 (d of m, J = 132 Hz, 2H), 2.85 (m, 2H), 5.4 (s, 1H), 6.2 (s, 2H), 12.8 (s, 1H).

Preparation of Mutant Constructs. PT7-4, an SD expression plasmid, was obtained from Dr. Janet Rice of DuPont. Since the expression vector PT7-4 contains two *Nco I* sites at the 5′ and 3′ ends of the coding sequence, it was modified to remove the 3′ site by partial restriction enzyme digestion with *Nco I*, selecting singly cut DNA, blunt-ending the linear fragment with Klenow fragment, and religating with T4 ligase. Clones containing the *Nco I* site at the 5′ end of the cDNA were selected by restriction enzyme analysis and sequencing. PCR reactions were in a buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin using *Taq* DNA polymerase (Promega). Fifteen amplification cycles were carried out in a Perkin Elmer 480 DNA Thermal Cycler under the following conditions: 94 °C, 2 min; 55 °C, 1 min; 72 °C, 1 min. Figure 1 shows the nucleotide and corresponding amino acid sequence for the enzyme.

Y30F, D31N, and Y50F mutants of SD were prepared by megaprimer mutagenisis (2). The complementary mutagenisis primers used for PCR amplification (where the altered codons are underlined) were SCD.Y30F-S: GGG CAG ACA GCT TCG ACT CCA AGG ACT GGG, SCD.Y30F-A: CCC AGT CCT TGG AGT CGA AGC

TGT CTG CCC, SCD.D31N-S: GGG CAG ACA GCT ACA ACT CCA ACT ACT GGG, SCD.D31N-A: CCC AGT CCT TGG AGT TGT AGC TGT CTG CCC, SCD.Y50F-S: GCG CAT TGA CTT CCG CTC CTT CCT CGA C, and SCD.Y50F-A: GTC GAG GAA GGA GCG GAA GTC AAT GCG C. The 5′ and 3′ primers, respectively, were SCD10: CTT TAA GAA GGA GAT ATA CAT ACC ATG GGT TCG CAA G and SCD11: GGG GTC GCC CAG CAC CTG CTT GCT CGA GAC A. Following isolation and purification, the final PCR products and expression vector were separately digested with *Nco I* and *Xho I*, combined and ligated. The Y30F/Y50F double mutant expression vector was prepared by carrying out the PCR reaction with Y50F primers on the Y30F product.

Simpler methods were utilized to construct the other mutants. The common primer (SCD7: GCC GAG TGG GCG TGG CCC TTC ATG GTG ACC) was used to construct the K73 mutants along with the mutant primer, GCC GGC CGA GGA GTT CGT CGG CAG GGT CTC GAG C**XY Z**CA GGT GCT GGG CGA CCC C (in K73A, **XYZ** = GCG; in K73Q, **XYZ** = CAG). The primers for the other constructs are listed below. H85N: GCC GGC CGA GGA GTT CGT CGG CAG GGT CTC GAG CAA GCA GGT GCT GGG CGA CCC CAC CCT CCG CAC GCA GAA CTT CAT CGG CGG C with SCD7. SCD.H110A: CCC CGT GGC CCT TCA TGG TGA CCT CCT TCA TGG TGG TGT CCT TGT ACC TCT GGG CCG GGA CGC with SCD12 (GGA GTT CGT CGG CAT GGT CTC GCG C). SCD.H110N: GGG CGT GGC CCT TCA TGG TGA CCT CCT TCA TGG TGG TGT CCT TGT ACC TCT GGT TCG GGA CGC GC with SCD12. SCD.S129A: GGT ACA AGG ACA CCA CCA TGA AGG AGG TCA CCA

TGA AGG GCC ACG CCC ACG CGG CAA ACC TTC with SCD14 (CCG GAT CCA AAG CTT GAA TTC CAT ACC). SCD.S129T: GGT ACA AGG ACA CCA CCA TGA AGG AGG TCA CCA TGA AGG GCC ACG CCC ACA CGG CAA ACC TTC with SCD14. N131A (with SCD 14): SCD.N131A: GGT ACA AGG ACA CCA CCA TGA AGG AGG TCA CCA TGA AGG GCC ACG CCC ACT CGG C*AG CGC* *T*TC ACT GG. The introduction of the mutant codon in the N131A expression vector created a unique *Eco47 III* site (shown in italics above) which aided in selection of mutants transformants. The PCR products synthesized with the above primer pairs could be introduced as *Xho I-BstE II* (H85N and H110N) and *BstE II-EcoR I* (N131A) fragments into the appropriately cut original expression vector

Figure 1: Nucleotide and corresponding protein sequence of scytalone dehydratase.

Nco I 10

M G S Q V Q K S D E I T
1 *CC ATG GGT* TCG CAA GTT CAA AAG AGC GAT GAG ATA ACC

Dde I 20

F S D Y L G L M T C V Y E
39 TT*C TCA* GAC TAC CTG GGC CTC ATG ACT TGC GTC TAT GAG

30

W A D S Y D S K D W D R L
78 TGG GCA GAC AGC TAC GAC TCC AAG GAC TGG GAT AGG CTG

40 50

R K V I A P T L R I D Y R
117 CGA AAG GTC ATT GCG CCT ACT CTG CGC ATT GAC TAC CGC

60

S F L D K L W E A M P A E
156 TCC TTC CTC GAC AAG CTC TGG GAG GCA ATG CCG GCC GAG

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                                    70
  E   F   V   G   M   V   S   S   K   Q   V   L   G
195 GAG TTC GTC GGC ATG GTC TCG AGC AAG CAG GTG CTG GGC

                80                                  90
  D   P   T   L   R   T   Q   H   F   I   G   G   T
234 GAC CCC ACC CTC CGC ACG CAG CAC TTC ATC GGC GGC ACG

                                        100
  R   W   E   K   V   S   E   D   E   V   I   G   Y
273 CGC TGG GAG AAG GTG TCC GAG GAC GAG GTC ATC GGC TAC

                            110
  H   Q   L   R   V   P   H   Q   R   Y   K   D   T
312 CAC CAG CTG CGC GTC CCG CAC CAG AGG TAC AAG GAC ACC

            120
  T   M   K   E   V   T   M   K   G   H   A   H   S
351 ACC ATG AAG GAG GTC ACC ATG AAG GGC CAC GCC CAC TCG

130                                         140
  A   N   L   H   W   Y   K   K   I   D   G   V   W
390 GCA AAC CTT CAC TGG TAC AAG AAG ATC GAC GGC GTC TGG

                                150
  K   F   A   G   L   K   P   D   I   R   W   G   E
429 AAG TTC GCC GGC CTC AAG CCC GAC ATC CGC TGG GGC GAG

                        160
  F   D   F   D   R   I   F   E   D   G   R   E   T
468 TTC GAC TTT GAC AGG ATC TTT GAG GAC GGA CGG GAG ACC

    170
  F   G   D   K   *
507 TTT GGC GAC AAA TAA ATG CAT GCA TCA TGC GTG CCG GTT

                                        EcoR I

546 ATG GGG AGT GTT ACA TGG GAC GTA TGG AAT TC 567
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