

# Directed Evolution Experiments Reveal Mutations at Cycloartenol Synthase Residue His477 that Dramatically Alter Catalysis

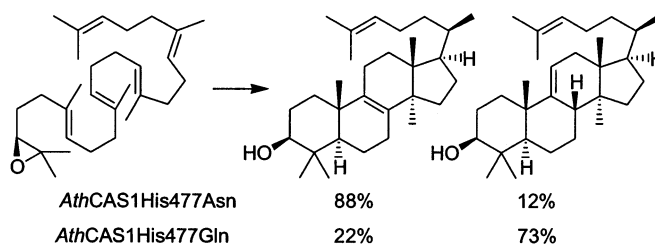
Michael J. R. Segura, Silvia Lodeiro, Michelle M. Meyer, Akash J. Patel, and Seiichi P. T. Matsuda\*

Department of Chemistry and Department of Biochemistry and Cell Biology,  
Rice University, Houston, Texas 77005

matsuda@rice.edu

Received September 28, 2002

## ABSTRACT



Cycloartenol synthase cyclizes and rearranges oxidosqualene to the protosteryl cation and then specifically deprotonates from C-19. To identify mutants that deprotonate differently, randomly generated mutant cycloartenol synthases were selected in a yeast lanosterol synthase mutant. A novel His477Asn mutant was uncovered that produces 88% lanosterol and 12% parkeol. The His477Gln mutant produces 73% parkeol, 22% lanosterol, and 5%  $\Delta^7$ -lanosterol. These are the most accurate lanosterol synthase and parkeol synthase that have been generated by mutagenesis.

Oxidosqualene cyclases convert oxidosqualene to a large family of  $C_{30}H_{50}O$  triterpene alcohols and  $C_{30}H_{52}O_2$  triterpene diols.<sup>1</sup> Cyclization reactions are initiated by protonating the epoxide, and ring diversity is then generated through alternative cyclization and rearrangement of the resultant carbocation. Olefin positioning is then achieved by specific deprotonation. How oxidosqualene cyclases promote specific rearrangement and deprotonation of cationic intermediates remains unclear, and a full understanding will require identifying the catalytically relevant enzyme residues and determining how their structures influence cyclization, rearrangement, and deprotonation. We have approached this problem by generating mutant enzymes that acquire new

product profiles and we present here experiments that convert cycloartenol synthase to quite accurate lanosterol synthase and parkeol synthase enzymes.

Cycloartenol synthase cyclizes oxidosqualene (1) to cycloartenol (2), the pentacyclic sterol precursor in plants<sup>2</sup> and some protists (Scheme 1).<sup>3</sup> Lanosterol synthase is a related enzyme that cyclizes the same precursor through similar intermediates to lanosterol (3), an isomeric tetracyclic sterol precursor in fungi and most animals.<sup>4</sup> These enzymes catalyze similar cyclizations and rearrangements; they gener-

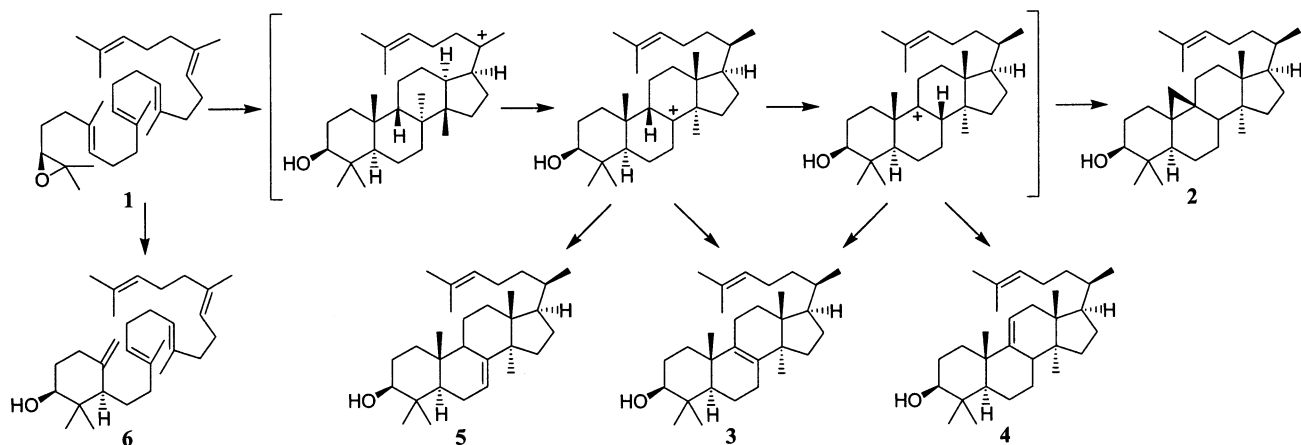
(2) Ehrhardt, J. D.; Hirth, L.; Ourisson, G. *Phytochemistry* **1967**, 6, 815–821.

(3) (a) Rees, H. H.; Goad, L. J.; Goodwin, T. W. *Biochim. Biophys. Acta* **1969**, 176, 892–894. (b) Anding, C.; Brandt, R. D.; Ourisson, G. *Eur. J. Biochem.* **1971**, 24, 259–263. (c) Raederstorff, D.; Rohmer, M. *Biochem. J.* **1985**, 231, 609–615. (d) Raederstorff, D.; Rohmer, M. *Eur. J. Biochem.* **1987**, 164, 427–434. (e) Nes, W. D.; Norton, R. A.; Crumley, F. G.; Madigan, S. J.; Katz, E. R. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, 87, 7565–7569.

\* To whom correspondence should be addressed.

(1) Matsuda, S. P. T. In *Biochemical Principles and Mechanisms of Biosynthesis and Biodegradation of Polymers*; Steinbüchel, A., Ed.; Wiley-VCH: Weinheim, Germany, 1998; pp 300–307.

**Scheme 1.** Cyclization of Oxidosqualene in CAS1 Mutants



ate different products by abstracting different protons. We have described directed evolution<sup>5</sup> approaches to find mutations that allow either *Arabidopsis thaliana* cycloartenol synthase (*AthCAS1*)<sup>7</sup> or *Dictyostelium discoideum* cycloartenol synthase (*DdiCAS1*)<sup>7</sup> to biosynthesize lanosterol. After finding several catalytically relevant positions, we generated site-specific mutants of both *AthCAS1* and *Saccharomyces cerevisiae* lanosterol synthase to explore how varying the residue at these positions altered the triterpene product profile.<sup>8</sup> These experiments established that Tyr410 and Ile481 (*AthCAS1* numbering) are essential for accurate cycloartenol biosynthesis. With hopes of identifying additional catalytically relevant positions, we generated libraries of randomly mutated *AthCAS1* derivatives by amplifying a plasmid encoding *AthCAS1* in the mutagenic *E. coli* strain XL1-Red (Stratagene). We then selected individuals from this mutant library that make lanosterol by their ability to genetically complement the yeast lanosterol synthase mutant SMY8.<sup>9</sup> Because lanosterol is an essential precursor to the yeast sterol ergosterol, SMY8 is sterol dependent when it expresses a cycloartenol synthase but gains sterol independence when the cycloartenol synthase acquires mutations that allow lanosterol biosynthesis. Yeast transformants were selected on synthetic complete medium lacking uracil and supplemented with heme and ergosterol as described.<sup>9</sup> Mutant

cycloartenol synthase derivatives that acquired the ability to biosynthesize lanosterol were identified by replica-plating transformants to the corresponding plates containing galactose but lacking ergosterol.<sup>10</sup> Because it was conceivable that strains could gain ergosterol independence by means other than evolving lanosterol synthase activity, we conducted a secondary screen for lanosterol biosynthesis. Yeast ultimately converts lanosterol into ergosterol, which we assayed by GC after TMS-derivatization. Strains that accumulated ergosterol were assumed to biosynthesize lanosterol, and their cyclase genes were investigated further.

Plasmids were recovered from positive strains and sequenced; each had a single mutation. SMY8 was retransformed with each individual mutant construct, and the transformants were rescreened to ensure that the sterol-independent phenotypes were plasmid-linked. Two of the plasmids that complement most effectively (from 7500 *AthCAS1* transformants) had His477Asn and Tyr532His mutations.

The randomly generated His477Asn mutant was expressed in the yeast strain LHY4 essentially as described.<sup>5a</sup> An *in vitro* assay with oxidosqualene generated milligram amounts of lanosterol and parkeol (**4**), which were identified as follows. The alcohol products were derivatized to the more volatile trimethylsilyl (TMS) ethers, which GC resolved as two components that comigrated with TMS-derivatized standards of lanosterol (88% by FID detection) and parkeol (12%). GC-MS analysis of these samples confirmed the structural assignments and did not reveal any other signals with masses consistent with being oxidosqualene cyclization products. Silica gel chromatography readily separated the triterpene alcohol fraction from other components. The product assignments were further confirmed by <sup>1</sup>H NMR analyses of this partially purified product mixture. Distinctive chemical shifts for parkeol ( $\delta$  0.647, 0.737, and 5.224 ppm) and lanosterol ( $\delta$  0.687 and 0.810 ppm) angular methyl groups and vinyl protons confirm the structural assignments. Although NMR typically provides less accurate quantitation

(4) (a) Corey, E. J.; Russey, W. E.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1966**, *88*, 4750–4751. (b) van Tamelen, E. E.; Willett, J. D.; Clayton, R. B.; Lord, K. E. *J. Am. Chem. Soc.* **1966**, *88*, 4752–4754.

(5) (a) Hart, E. A.; Hua, L.; Darr, L. B.; Wilson, W. K.; Pang, J.; Matsuda, S. P. T. *J. Am. Chem. Soc.* **1999**, *121*, 9887–9888. (b) Meyer, M. M.; Xu, R.; Matsuda, S. P. T. *Org. Lett.* **2002**, *4*, 1395–1398.

(6) Corey, E. J.; Matsuda, S. P. T.; Bartel, B. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11628–11632.

(7) Godzina, S. M.; Lovato, M. A.; Meyer, M. M.; Foster, K. A.; Wilson, W. K.; Gu, W.; de Hostos, E. L.; Matsuda, S. P. T. *Lipids* **2000**, *36*, 249–255.

(8) (a) Herrera, J. B. R.; Wilson, W. K.; Matsuda, S. P. T. *J. Am. Chem. Soc.* **2000**, *122*, 6765–6766. (b) Joubert, B. M.; Hua, L.; Matsuda, S. P. T. *Org. Lett.* **2000**, *2*, 339–341. (c) Matsuda, S. P. T.; Darr, L. B.; Hart, E. A.; Herrera, J. B. R.; McCann, K. E.; Meyer, M. M.; Pang, J.; Schepmann, H. G. *Org. Lett.* **2000**, *2*, 2261–2263. (d) Meyer, M. M.; Segura, M. J. R.; Wilson, W. K.; Matsuda, S. P. T. *Angew. Chem.* **2000**, *112*, 4256–4258; *Angew. Chem., Int. Ed.* **2000**, *39*, 4090–4092.

(9) Corey, E. J.; Matsuda, S. P. T.; Baker, C. H.; Ting, A. Y.; Cheng, H. *Biochem. Biophys. Res. Commun.* **1996**, *219*, 327–331.

(10) Lovato, M. A.; Hart, E. A.; Segura, M. J. R.; Giner, J.-L.; Matsuda, S. P. T. *J. Biol. Chem.* **2000**, *275*, 13394–13397.

than GC-FID, strikingly close agreement with integration of the peak areas of both angular methyl groups (88:12) and vinyl protons confirms the quantitation. This agreement also provides reassurance both that no comigrating compound distorted the GC values and that no additional product went unnoticed in the GC-FID and GC-MS experiments.

The previous high watermark for converting cycloartenol synthase into a lanosterol synthase was the Tyr410Thr Ile481Val double mutant that produces 75% lanosterol and 25%  $9\beta$ -lanosta-7,24-dien-3 $\beta$ -ol ( $\Delta^7$ -lanosterol, **5**).<sup>8a</sup> Although the known native lanosterol synthases encode not asparagine but glutamine (fungi and trypanosomes) or cysteine (mammals) at this position, the His477Asn mutant biosynthesizes lanosterol quite cleanly. Hypothesizing that a derivative encoding the native glutamine residue might increase lanosterol production further, we generated and assayed the *AthCAS1* His477Gln mutation. We were surprised to find that this mutant is a parkeol synthase. The triterpene alcohols were converted to TMS ethers. GC-MS revealed a major peak with retention time and mass spectral characteristics indistinguishable from TMS-parkeol. Only one other peak with the appropriate mass appeared, and it had a retention time and mass spectral characteristics consistent with TMS-lanosterol. GC-FID provided a tentative ratio of 73:27 parkeol:lanosterol. <sup>1</sup>H NMR of a partially purified sample confirmed the presence of parkeol ( $\delta$  0.647, 0.737, and 5.224 ppm) and lanosterol ( $\delta$  0.687 and 0.810 ppm), but integration of the 0.647 and 0.687 signals provided a 77:23 ratio. The reason for this discrepancy was readily apparent from the <sup>1</sup>H NMR spectra; signals at  $\delta$  5.556, 1.020, and 1.017 ppm revealed the presence of  $\Delta^7$ -lanosterol. Integration of the H-7 proton of  $\Delta^7$ -lanosterol relative to the H-11 proton of parkeol showed that  $\Delta^7$ -lanosterol made up 5% of the total product mixture. This compound went undetected in the GC analyses because its TMS ether comigrates with that of lanosterol in diverse GC conditions. Combining this figure with the GC quantitation of 73% parkeol and 27%  $\Delta^8$ -lanosterol +  $\Delta^7$ -lanosterol isomers provides a product yield of 73% parkeol, 22% lanosterol, and 5%  $\Delta^7$ -lanosterol. Integrating the <sup>1</sup>H NMR signals of H-7 ( $\Delta^7$ -lanosterol), H-11 (parkeol), and H-24 (side-chain representative of total product) provides a product ratio of 71% parkeol, 24% lanosterol, and 5%  $\Delta^7$ -lanosterol. Again, these numbers are in close agreement.

The Tyr532His mutant corresponds to the *DdiCAS1* Tyr481His mutant uncovered in one of our previous selections.<sup>5b</sup> In that study, the *AthCAS1* Tyr532His mutant was generated by site-specific mutagenesis and was shown to produce no cycloartenol but a mixture of 45% lanosterol (**3**), 31% parkeol (**4**), and 24% achilleol A (**6**).<sup>5b</sup> Because Tyr532 is strictly conserved between the known examples of both cycloartenol synthase and lanosterol synthase, this position is not part of the catalytic difference between native cycloartenol and lanosterol synthase. The Tyr532His mutation allows lanosterol biosynthesis not by altering residues that directly participate in deprotonation but by some indirect mechanism. The formation of significant amounts of the monocyclic triterpene alcohol achilleol A in the mutant

establishes that Tyr532 facilitates (but is not essential for) tetracycle formation.

While our analytical and structure determination efforts were underway, a report appeared<sup>11</sup> that describes a mutant selection essentially identical to those we have published previously<sup>5a,b</sup> and explored further here. In addition to mutations at the Tyr410, Ala469, and Ile481 positions that we have described previously, His477Tyr and Tyr532His mutants were found to complement a lanosterol synthase mutant. These enzymes were reported to produce primarily lanosterol (and an unquantified amount of achilleol A in the case of the Tyr532His) on the basis of genetic behavior coupled with TLC analysis.<sup>11</sup> Unfortunately, neither genetic complementation nor TLC can provide evidence that lanosterol is the major product. Relatively little lanosterol is necessary for genetic complementation of a lanosterol synthase mutant; the *SceERG7* Thr384Tyr Val454Ile double mutant genetically complements despite making only 13% lanosterol.<sup>8d,12</sup> Although yeast requires a trace of ergosterol to spark cell growth, a wide variety of sterols are serviceable membrane components.<sup>13</sup> Both parkeol and cycloartenol can be converted into usable membrane sterols, and either can genetically complement a yeast lanosterol synthase mutant when supplemented with only 1% lanosterol.<sup>13</sup> Silica gel TLC does not provide even a meaningful qualitative indication of product structure because most oxidosqualene cyclase products comigrate (including cycloartenol, lanosterol, parkeol,  $\Delta^7$ -lanosterol, and a wide variety of pentacyclic compounds). Although incubating with radiolabeled substrate and identifying products by chromatographic comigration is a standard and appropriate approach in many areas of enzymology, most triterpene alcohols have  $3\beta$ -OH groups in sterically similar environments, and they are not trivial to separate even using HPLC. Determining the composition of products formed by oxidosqualene cyclases requires multiple orthogonal techniques that should include NMR and either GC-MS or LC-MS.

Having a quantitative product profile of both the Gln and Asn mutants allows us to draw conclusions regarding the role of His477. His477 is strictly conserved in the known cycloartenol synthases (Figure 1). Both the Asn and Gln mutations abolish cycloartenol biosynthesis, consistent with a specific role for His477 in cyclopropyl ring formation. The *AthCAS1* His477Gln mutant is the most accurate known parkeol synthase for which sequence information is available.<sup>26</sup> Although Gln differs from Asn only by having an additional methylene that slightly increases steric bulk and shifts the polar moiety, the His477Gln mutant has radically different catalytic properties from the His477Asn mutant. The Asn mutant produces lanosterol more accurately than any described cycloartenol synthase mutant. Asn induces lanosterol formation more effectively than Gln in the *AthCAS1* background, but Gln is preferred in native lanosterol synthases. This finding is a vivid illustration that a

(11) Wu, T. K.; Griffin, J. H. *Biochemistry* **2002**, *41*, 8238–8244.

(12) The His477Gln mutant was also shown to genetically complement the lanosterol synthase mutation in *SMY8*.

(13) Nes, W. D.; Janssen, G. G.; Crumley, F. G.; Kalinowska, M.; Akihisa, T. *Arch. Biochem. Biophys.* **1993**, *300*, 724–733.

|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 | 96 | 97 | 98 | 99 | 100 | 101 | 102 | 103 | 104 | 105 | 106 | 107 | 108 | 109 | 110 | 111 | 112 | 113 | 114 | 115 | 116 | 117 | 118 | 119 | 120 | 121 | 122 | 123 | 124 | 125 | 126 | 127 | 128 | 129 | 130 | 131 | 132 | 133 | 134 | 135 | 136 | 137 | 138 | 139 | 140 | 141 | 142 | 143 | 144 | 145 | 146 | 147 | 148 | 149 | 150 | 151 | 152 | 153 | 154 | 155 | 156 | 157 | 158 | 159 | 160 | 161 | 162 | 163 | 164 | 165 | 166 | 167 | 168 | 169 | 170 | 171 | 172 | 173 | 174 | 175 | 176 | 177 | 178 | 179 | 180 | 181 | 182 | 183 | 184 | 185 | 186 | 187 | 188 | 189 | 190 | 191 | 192 | 193 | 194 | 195 | 196 | 197 | 198 | 199 | 200 | 201 | 202 | 203 | 204 | 205 | 206 | 207 | 208 | 209 | 210 | 211 | 212 | 213 | 214 | 215 | 216 | 217 | 218 | 219 | 220 | 221 | 222 | 223 | 224 | 225 | 226 | 227 | 228 | 229 | 230 | 231 | 232 | 233 | 234 | 235 | 236 | 237 | 238 | 239 | 240 | 241 | 242 | 243 | 244 | 245 | 246 | 247 | 248 | 249 | 250 | 251 | 252 | 253 | 254 | 255 | 256 | 257 | 258 | 259 | 260 | 261 | 262 | 263 | 264 | 265 | 266 | 267 | 268 | 269 | 270 | 271 | 272 | 273 | 274 | 275 | 276 | 277 | 278 | 279 | 280 | 281 | 282 | 283 | 284 | 285 | 286 | 287 | 288 | 289 | 290 | 291 | 292 | 293 | 294 | 295 | 296 | 297 | 298 | 299 | 300 | 301 | 302 | 303 | 304 | 305 | 306 | 307 | 308 | 309 | 310 | 311 | 312 | 313 | 314 | 315 | 316 | 317 | 318 | 319 | 320 | 321 | 322 | 323 | 324 | 325 | 326 | 327 | 328 | 329 | 330 | 331 | 332 | 333 | 334 | 335 | 336 | 337 | 338 | 339 | 340 | 341 | 342 | 343 | 344 | 345 | 346 | 347 | 348 | 349 | 350 | 351 | 352 | 353 | 354 | 355 | 356 | 357 | 358 | 359 | 360 | 361 | 362 | 363 | 364 | 365 | 366 | 367 | 368 | 369 | 370 | 371 | 372 | 373 | 374 | 375 | 376 | 377 | 378 | 379 | 380 | 381 | 382 | 383 | 384 | 385 | 386 | 387 | 388 | 389 | 390 | 391 | 392 | 393 | 394 | 395 | 396 | 397 | 398 | 399 | 400 | 401 | 402 | 403 | 404 | 405 | 406 | 407 | 408 | 409 | 410 | 411 | 412 | 413 | 414 | 415 | 416 | 417 | 418 | 419 | 420 | 421 | 422 | 423 | 424 | 425 | 426 | 427 | 428 | 429 | 430 | 431 | 432 | 433 | 434 | 435 | 436 | 437 | 438 | 439 | 440 | 441 | 442 | 443 | 444 | 445 | 446 | 447 | 448 | 449 | 450 | 451 | 452 | 453 | 454 | 455 | 456 | 457 | 458 | 459 | 460 | 461 | 462 | 463 | 464 | 465 |  |
|--|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
|--|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|

residue with optimal features for catalysis in one background may interact differently with the reaction intermediate or neighboring amino acid residues when its environment changes.

A model derived from the squalene-hopene cyclase<sup>27</sup> crystal structure positions *AthCAS1* His477 outside of the

(14) Morita, M.; Shibuya, M.; Lee, M.-S.; Sankawa, U.; Ebizuka, Y. *Biol. Pharm. Bull.* **1997**, *20*, 770–775

(15) Kushi, T.; Shibuya, M.; Ebizuka, Y. *Eur. J. Biochem.* **1998**, *256*, 238–244.

(16) Hayashi, H.; Hiraoka, N.; Ikeshiro, Y.; Kushiro, T.; Morita, M.; Shibuya, M.; Ebizuka, Y. *Biol. Pharm. Bull.* **2000**, 23, 231–234.

(17) Hayashi, H.; Hiraoka, N.; Ikeshiro, Y.; Yazaki, K.; Tanaka, S., Kushiro, T.; Shibuya, M.; Ebizuka, Y. *Plant Physiol.* **1999**, *121*, 1384.

cycloartenol synthase active site.<sup>11</sup> However, deprotonation is more strongly influenced by the residue at 477 than any other position that has been studied to date. Subtle changes in the amino acid structure alter catalysis with an influence that is consistent with residue 477 being located within the active site. The physical location of His477 will not likely be resolved without a crystal structure, but if experiments should ever confirm the modeled position of His477, this residue would have unprecedented catalytic importance for a residue outside of the active site.

**Acknowledgment.** We thank William K. Wilson for expert advice on NMR. M.J.R.S. is a Houston Livestock Show and Rodeo Scholar, holds a Schlumberger Foundation Fellowship, and was supported by an NIH training grant (T32-GM08362). M.M.M. was an American Society of Pharmacognosy Undergraduate Fellow. This research was funded by the NSF (MCB-0209769) and the Robert A. Welch Foundation (C-1323).

OL0269897

(18) Haralampidis, K.; Bryan, G.; Qi, X.; Papadopolou, K.; Bakht, S.; Melton, R.; Osbourn, A. E. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 13431–13436.

(19) Buckner, F. S.; Ngyuen, L. N.; Joubert, B. M.; Matsuda, S. P. T. *Mol. Biochem. Parasitol.* **2000**, *110*, 399–403.

(20) Joubert, B. M.; Buckner, F. S.; Matsuda, S. P. T. *Org. Lett.* **2001**, 3, 1957–1960.

(21) Corey, E. J.; Matsuda, S. P. T.; Bartel, B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2211–2215.

(22) (a) Buntel, C. J.; Griffin, J. H. *J. Am. Chem. Soc.* **1992**, *114*, 9711–9713. (b) Roessner, C. A.; Min, C.; Hardin, S. H.; Harris-Haller, L. W.; McCollum, J. C.; Scott, A. I. *Gene* **1993**, *127*, 149–150.

(23) Abe, I.; Naito, K.; Takagi, Y.; Noguchi, H. *Biochim. Biophys. Acta* **2001**, *1522*, 67–73.

(24) (a) Kusano, M.; Shibuya, M.; Sankawa, U.; Ebizuka, Y. *Biol. Pharm. Bull.* **1995**, *18*, 195–197. (b) Abe, I.; Prestwich, G. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9274–9278.

(25) (a) Baker, C. H.; Matsuda, S. P. T.; Liu, D. R.; Corey, E. J. *Biochem. Biophys. Res. Commun.* **1995**, 213, 154–160. (b) Sung, C.-K.; Shibuya, M.; Sankawa, U.; Ebizuka, Y. *Biol. Pharm. Bull.* **1995**, 18, 1459–1461.

(26) Parkeol synthase activity has been observed in sea cucumbers, but the responsible cDNA has not yet been cloned; see: Kerr, R. G.; Chen, Z. *J. Nat. Prod.* **1995**, 58, 172–176.

(27) (a) Wendt, K. U.; Lenhart, A.; Schulz, G. E. *J. Mol. Biol.* **1999**, 286, 175–187. (b) Wendt, K. U.; Poralla, K.; Schulz, G. E. *Science* **1997**, 277, 1811–1815.