Detection of a Phenylalanine Aminomutase in Cell-Free Extracts of *Taxus brevifolia* and Preliminary Characterization of Its Reaction

Kevin D. Walker† and Heinz G. Floss*

Department of Chemistry, Box 351700
University of Washington, Seattle, Washington 98195-1700

Received February 10, 1998

The biosynthesis of the potent antitumor agent, paclitaxel (Taxol), from the bark of Pacific Yew[1] involves the assembly of two separate building blocks. One is the diterpene, baccatin-III, which is generated from geranylgeranyl diphosphate via the hydrocarbon, taxa-4(5),11(12)-diene,[2] which is subject to extensive further functionalization,[3] and the other is the phenylisoserine side chain which arises from phenylalanine;[4,5] the two components are linked late in the pathway[6] to give rise to taxanes exhibiting antitumor activity. Feeding experiments have shown that the elaboration of the side chain from phenylalanine proceeds via β-phenylalanine rather than cinnamic acid;[4] a key step thus must be a mutase reaction converting α- into β-phenylalanine. In this communication we report the detection of a phenylalanine aminomutase in cell-free extracts of *Taxus brevifolia* tissue and some characteristics of the reaction it catalyzes.

Cell-free extracts of *T. brevifolia* were prepared from stems of young saplings as described by Koepp et al.[2] Six milliliters of extract in HEPES extraction buffer, pH 7.5, representing 1.2 g of tissue, was incubated for 12 h at 30 °C with (*S*)-[U-^{14}C]-phenylalanine (0.11 μmol, 50 μCi, NEN) and 5 mg of *S*-adenosylmethionine (AdoMet).[7] Carrier (*S*)-phenylalanine (5.5 mg) and (*R,S*)-β-phenylalanine (6 mg) were then added; the phenylalanines were isolated as their *N*-benzoyl methyl esters and separated by successive HPLC on PRP-1 (CH_3CN:H_2O, 45:55) and amino/cyano columns (EtOAc:hexane, 40:60). The β-phenylalanine fraction was further diluted with carrier material and recrystallized from different solvents to constant specific radioactivity, which was confirmed by another flash chromatography purification (0.085 μCi, min. 0.17% conversion). Control experiments showed that the substrate contained no detectible radioactive β-phenylalanine and that no product was formed with boiled enzyme.

The configuration of the enzymatically formed product was established as *R* by a similar incubation with 5 μCi of (*S*)-[U-^{14}C]phenylalanine and conversion to the 1(*S*)-camphanate methyl ester. The purified radioactive product cleanly cochromatographed with authentic[8] *N*-[(1′*S*)-camphanoyl]-(3*R*)-β-phenylalanine methyl ester (R_t = 10.4 min), not the 1′*S*,3*S* isomer (R_t = 8.2 min). Thus, the enzyme produces the enantiomer of β-phenylalanine which corresponds in its configuration to the phenylisoserine moiety of paclitaxel[9] (as well as the Winterstein's acid moiety of other taxanes[10]). This finding was important since we had observed that β-phenylalanine extracted from *T. brevifolia* tissue was a mixture of the *R* and *S* isomers, with the unnatural isomer usually predominating.[11] Thus, *T. brevifolia* must contain an enzyme(s) which can epimerize β-phenylalanine.

To gain some insight into the mechanism of the aminomutase reaction, we synthesized (*S*)-[2-^{15}N, ring-$^2H_5$]-, (2*S*,3*R*)-[ring, 3-$^2H_6$]-, and (2*S*,3*S*)-[ring,2,3-$^2H_7$]phenylalanine (90+% e.e., 98+% enriched) by appropriate modifications of the procedure of Fryzuk and Bosnich.[12] These samples were incubated with *T. brevifolia* cell-free extract, the resulting β-phenylalanine samples were derivatized to the *N*-benzoyl methyl esters and analyzed by GC−MS. The molecular ion (*m*/*z* 283) was very weak, but fragment ions at *m*/*z* 210 (loss of CH_2COOCH_3, C-1, and C-2) and *m*/*z* 178 (base peak, loss of benzoate) (Scheme 1) allowed quantitative analysis of the isotope enrichment and distribution, and lesser fragment ions served to confirm the data. The incubation with (*S*)-[2-^{15}N, ring-$^2H_5$]phenylalanine gave product displaying, in addition to unlabeled background, only P + 6 peaks (48%) but no significant P + 5 peaks. This indicates that the nitrogen migrates quantitatively without exchange from the α to the β carbon. Repetition of the experiment with a 1:1 mixture of unlabeled and (*S*)-[2-^{15}N, ring-$^2H_5$]phenylalanine gave the same result, only P + 6 (29%) but no detectible P + 5 peaks, except that the amount of unlabeled β-phenylalanine was increased. Hence the migration of the nitrogen is strictly intramolecular.

The incubation with (2*S*,3*R*)-[ring, 3-$^2H_6$]phenylalanine gave product carrying six atoms of deuterium in both the *m*/*z* 178 (P, 45%; P + 6, 55%; P + 5 and P + 7, both 0%) and the *m*/*z* 210 fragment (P, 44%; P + 6, 56%), indicating that the side-chain deuterium is located at C-3. In contrast, the β-phenylalanine from the incubation with (2*S*,3*S*)-[ring, 2,3-$^2H_7$]phenylalanine contained virtually no deuterium at C-3 (*m*/*z* 210: P, 41%; P + 5, 59%; P + 6, <5%), and the labeling pattern at C-2 was complex (*m*/*z* 178: P, 32%; P + 5, 12%; P + 6, 37%; P + 7, 19%). These data indicate that, in the aminomutase reaction, the pro-3*S* hydrogen of (*S*)-phenylalanine migrates to C-2 of β-phenylalanine, whereas the pro-3*R* hydrogen remains at C-3, and that this migration is at least partly intermolecular. In addition the substrate must undergo some hydrogen exchange at C-2, presumably catalyzed by an α-amino acid racemase. From the configurations of substrate and product it also follows that the departing pro-3*S* hydrogen is replaced by the migrating nitrogen with retention of configuration at C-3 and, because the nitrogen migration is intramolecular, that the substrate must be bound to the enzyme in a syn conformation (Scheme 2).

The enzyme activity reported here seems to be the first example of an aminomutase from a higher plant and the first example of a phenylalanine aminomutase from any source. Several aminomutases have been reported from microorganisms which use different cofactors and seem to operate by different mechanisms;[13] among these, the lysine-2,3-aminomutase from *Clostridium* has been studied in most detail.[19] No statements can be made at this point about the cofactor requirement of the phenylalanine aminomutase from *T. brevifolia*,[7] although B_{12} catalysis seems

* To whom correspondence should be directed.
† Present address: Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340.

(1) For reviews, see: (a) *Taxol: Science and applications*; Suffness, M., Ed.; CRC Press: Boca Raton, FL, 1995. (b) *Taxane anticancer agents: Basic science and current status*; Georg, G. I., Chen, T. T., Ojima, I., Vyas, D. M., Eds.; American Chemical Society, Washington, DC, 1995.

(2) Koepp, A. E.; Hezari, M.; Zajicek, J.; Stofer Vogel, B.; LaFever, R. E.; Lewis, N. G.; Croteau, R. *J. Biol. Chem.***1995**, *270*, 8686−8690.

(3) Cf. Hezari, M.; Croteau, R. *Planta Med.* **1997**, *63*, 291−295.

(4) Fleming, P. E.; Mocek, U.; Floss, H. G. *J. Am. Chem. Soc.* **1993**, *115*, 805−806.

(5) Cf. Floss, H. G.; Mocek, U. Biosynthesis of Taxol. In ref 1a, pp 191−208.

(6) Fleming, P. E.; Knaggs, A. R.; He, X.-G.; Mocek, U.; Floss, H. G. *J. Am. Chem. Soc.* **1994**, *116*, 4137−4138.

(7) Subsequent experiments showed that, at least in the crude cell-free extract, the reaction was not dependent on added AdoMet.

(8) Synthesis: Davis, F. A.; Reddy, R. E.; Szewczyk, J. M. *J. Org. Chem.* **1995**, *60*, 7037.

(9) Wani, M. C.; Taylor, H. L.; Wall, M. E.; Coggon, P.; McPhail, A. T. *J. Am. Chem. Soc.* **1971**, *93*, 2325.

(10) Graf, E.; Boeddeker, H. *Liebigs Ann. Chem.***1958**, *613*, 111−120.

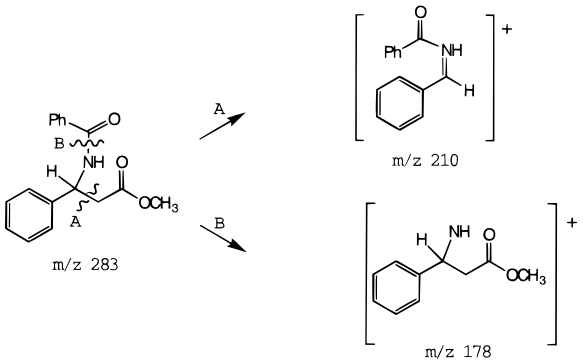
(11) Walker, K. Ph.D. Thesis, University of Washington, 1997.

(12) Fryzuk, M. D.; Bosnich, B. *J. Am. Chem. Soc.* **1978**, *100*, 5491.

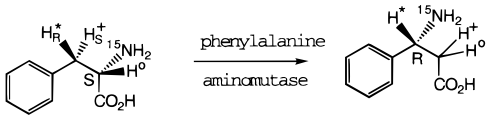
(13) These include lysine-2,3-aminomutase,[14] which uses AdoMet, pyridoxal phosphate (PLP), and an iron−sulfur cluster in catalysis; β-lysine-5,6-aminomutase,[15] arginine-2,3-aminomutase,[16] and leucine-2,3-aminomutase,[17] which all employ cobalamin as a cofactor; and tyrosine-2,3-aminomutase,[18] which uses ATP but does not contain PLP.

S0002-7863(98)00457-0 CCC: $15.00
Published on Web 05/12/1998

Scheme 1. Mass Spectral Fragmentation of *N*-Benzoyl-*β*-phenylalanine Methyl Ester



Scheme 2. Steric Course of the Reaction Catalyzed by Phenylalanine Aminomutase from *Taxus brevifolia*



unlikely since it is generally accepted that higher plants do not contain cobalamin. However, it must be noted that in all the microbial enzymes studied[13] a hydrogen at the migration terminus is replaced by the migrating nitrogen with inversion of configuration, in contrast to the retention of configuration observed in the present work. Thus, the mechanism of the phenylalanine aminomutase from *Taxus* may differ fundamentally from that of the microbial aminomutases.

The stereochemical preference of the phenylalanine aminomutase from *T. brevifolia*, i.e., retention of the pro-3*R* and migration of the pro-3*S* hydrogen of phenylalanine, contrasts with a report by Haslam and co-workers[20] on the incorporation of stereospecifically tritiated phenylalanines into the Winterstein's acid moiety of taxine in *Taxus baccata*. On the basis of the T/^{14}C ratio of the elimination product, cinnamic acid, they reported 91% loss of the pro-3*R* and 33% loss of the pro-3*S* hydrogen in the transformation. This observation led them to suggest, correctly as it turns out, that the formation of Winterstein's acid cannot proceed via cinnamic acid, since the phenylalanine ammonia-lyase (PAL) reaction proceeds with loss of the opposite hydrogen. It is, of course, possible that the aminomutase functioning in the formation of Winterstein's acid in *T. baccata* operates with a different stereochemistry than the one studied here, but more likely the results of Haslam's group[20] reflect the difficulty of analyzing a subtle stereochemical feature of an enzyme reaction in a complex in vivo system.[21]

Acknowledgment. We are grateful to Prof. R. Croteau, Washington State University, and members of his laboratory for advice on the preparation of *T. brevifolia* cell-free extracts and to Mr. Jim Roe for assistance in the mass spectral analyses. This work was supported by NIH Research Grant CA 64483; K.D.W. was the recipient of a fellowship (No. 9119783) from the NSF.

JA980457X

(14) (a) Chirpich, T. P.; Zappia, V.; Costilow, R. N.; Barker, H. A. *J. Biol. Chem.* **1970**, *245*, 1778−1789; Stereochemistry: (b) Aberhart, D. J.; Gould, S. J.; Lin, H.-J.; Thiruvengadam, T. K.; Weiller, B. H. *J. Am. Chem. Soc.* **1983**, *105*, 5461−5470.

(15) (a) Baker, J. J.; Van der Drift, C.; Stadtman, T. C. *Biochemistry* **1973**, *12*, 1054−1063. Stereochemistry: (b) Retey, J.; Kunz, F.; Arigoni, D.; Stadtman, T. C. *Helv. Chim. Acta* **1978**, *61*, 2989.

(16) Prabhakaran, P. C.; Woo, N.-T.; Yorgey, P. S.; Gould, S. J. *J. Am. Chem. Soc.* **1988**, *110*, 5785.

(17) (a) Poston, J. M. *J. Biol. Chem.* **1976**, *251*, 1859−1863. Stereochemistry: (b) Freer, I.; Pedrocchi-Fantoni, G.; Picken, D. J.; Overton, K. H. *J. Chem. Soc., Chem. Commun.* **1981**, 80.

(18) (a) Kurylo-Borowska, Z.; Abramsky, T. *Biochim. Biophys. Acta* **1972**, *264*, 1−10. Stereochemistry: (b) Parry, R. J.; Kurylo-Borowska, Z. *J. Am. Chem. Soc.* **1980**, *102*, 836−837.

(19) Frey, P. A.; Reed, G. H. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1993**, *66*, 1−39.

(20) Platt, R. V.; Opie, C. T.; Haslam, E. *Phytochemistry* **1984**, *23*, 2211.

(21) As a further control we subjected the same samples used to analyze the phenylalanine aminomutase stereochemistry to the action of PAL, resulting in the anticipated loss of one deuterium from (2*S*,3*S*)-[ring,2,3-$^{2}H_{7}$]-phenylalanine and no deuterium loss from (2*S*,3*R*)-[ring,3-$^{2}H_{6}$]phenylalanine.