Phytochemistry, Vol. 27, No. 3, pp. 711–714, 1988.
Printed in Great Britain.

0031 9422/88 $3.00 + 0.00
© 1988 Pergamon Journals Ltd.

NOVEL PHENYLALANINE ANALOGUES AS PUTATIVE INHIBITORS OF ENZYMES ACTING ON PHENYLALANINE

JERZY ZOŃ and BERND LABER*

Institute of Organic and Physical Chemistry, Technical University of Wroclaw, PL-50-370 Wroclaw, Poland; *Chair of Plant Physiology, Ruhr-University Bochum D-4630 Bochum, F.R.G.

(*Received* 5 *June* 1987)

Key Word Index—*Fagopyrum esculentum*; Polygonaceae; *Vigna radiata*; Leguminosae; phenylalanine ammonia-lyase; aromatic amino acid transaminase; (±)-2-aminomethyl-3-phenylpropanoic acid; (*E*)-2-aminomethyl-3-phenylpropenoic acid; enzyme inhibitors.

Abstract—Two analogues of phenylalanine, (±)-2-aminomethyl-3-phenylpropanoic acid and (*E*)-2-aminomethyl-3-phenylpropenoic acid, were synthesized and found to inhibit buckwheat phenylalanine ammonia-lyase (PAL) competitively with K_i values of 16.5 and 1.3 μM, respectively. They interfered, however, only weakly with light-induced anthocyanin synthesis in etiolated buckwheat seedlings, indicating negligible inhibition of PAL *in vivo*. In addition, (±)-2-aminomethyl-3-phenylpropanoic acid was a mixed type inhibitor of phenylalanine transamination ($K_i = 0.93$ mM) catalysed by an aminotransferase preparation from mungbean shoots. The two phenylalanine analogues inhibited neither rat liver phenylalanine hydroxylase nor soybean phenylalanyl *t*RNA synthetase.

INTRODUCTION

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyses the formation of (*E*)-cinnamic acid, the central precursor for the biosynthesis of a large number of phenylpropanoid compounds in higher plants [1]. The search for specific inhibitors of this enzyme as tools for the study of secondary plant metabolism has been of continuing interest [2].

The most powerful inhibitor of PAL known todate is the *O*-hydroxylamine analogue of (*S*)-phenylalanine, (*S*)-2-aminooxy-3-phenylpropanoic acid (AOPP) which inhibits buckwheat PAL with a K_i/K_m ratio of 0.00003 [3]. It is proposed that AOPP acts as a transition state analogue in the deamination reaction [4]. AOPP also strongly inhibits tyrosine decarboxylase (EC 4.1.1.25) from *Syringa vulgaris* (K_i/K_m *ca* 0.00004) [5], while the inhibition of an aromatic amino acid transaminase (EC 2.6.1) from mung bean shoots is less pronounced ($K_i/K_m = 0.003$) [3].

The three enzymatic reactions reported to be inhibited by AOPP, i.e. deamination, decarboxylation, and transamination of phenylalanine and/or tyrosine, have in common the attack of the amino group of the aromatic amino acid on an electrophilic acceptor at the active site of the respective enzyme. Moreover, according to postulated mechanisms, the three enzymes catalyse the formation of carbanion intermediates [6, 7]. Both the presence of a nucleophilic group as well as structural similarity to either the substrate or product of the respective reaction should therefore be requirements for potential new inhibitors of these enzymes. Here, we report the synthesis and evaluation of two β-amino acid analogues of phenylalanine, (±)-2-aminomethyl-3-phenylpropanoic acid (**1**) and (*E*)-2-aminomethyl-3-phenylpropenoic acid (**2**), as putative inhibitors of phenylalanine deamination and transamination.

*Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

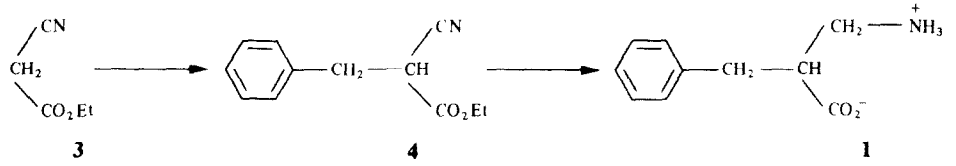
Syntheses of inhibitors

(±)-2-Aminomethyl-3-phenylpropanoic acid (**1**) was synthesized by the route shown in Scheme 1. Alkylation of ethyl cyanoacetate (**3**) under phase transfer catalysis conditions gave the cyano ester **4**. After hydrogenation of **4** and subsequent hydrolysis, the free β-amino acid **1** was obtained. The synthesis of the hydrochloride of **1** had previously been achieved via diethylphthalimidomethyl-benzylmalonate [8].

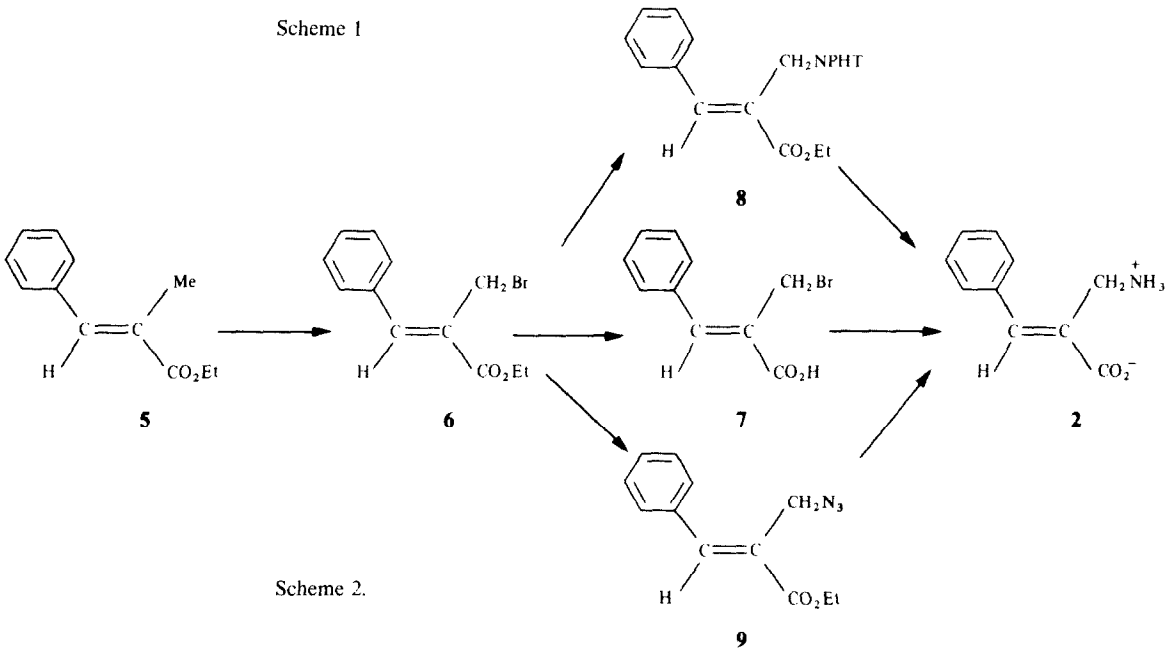
The stereoselective synthesis of ethyl-(*E*)-2-methyl-3-phenylpropenoic acid (**5**) in a Wittig–Horner reaction [9] suggested a route for the synthesis of (*E*)-2-aminomethyl-3-phenylpropenoic acid (**2**). Thus, bromination of **5** in the allylic position gave the bromo ester **6** which, after hydrolysis to **7**, was reacted with ammonia to provide the unsaturated β-amino acid **2** (Scheme 2). The synthesis of **2** was also accomplished via either compound **8** or **9** (Scheme), but these two routes were found to be less satisfactory. X-ray analysis confirmed the structure of **2** as that of (*E*)-2-aminomethyl-3-phenylpropenoic acid (Glowiak, T., personal communication).

Inhibition of PAL and of anthocyanin formation

The effect of the two compounds (**1**, **2**) on the reaction catalysed by buckwheat PAL was evaluated by standard kinetic analysis. Double reciprocal plots of initial velocities vs variable substrate concentration (30–1000 μM)



Scheme 1



Scheme 2.

clearly indicated linear competitive inhibition by both compounds (**1**: app. $K_i = 16.5\ \mu M$, $K_i/K_m = 0.37$; **2**: app. $K_i = 1.3\ \mu M$, $K_i/K_m = 0.029$). As **1** was used as the racemic compound, a lower apparent K_i-value for the (*S*)-enantiomer can be assumed on the basis of the observation that the (*S*)-enantiomers of the isosteric *O*-hydroxylamine and hydrazine analogues of phenylalanine are more potent inhibitors of PAL than the corresponding (*R*)-enantiomers [10]. A comparison of the three isosteres as PAL inhibitors reveals the following order of potency: $-CH_2-NH_2 < -NH-NH_2 < -O-NH_2$ (present result and [10]) indicating a positive correlation between the inhibitory potency of a compound and the basicity of the amino group.

The aminomethyl derivative (**2**) of cinnamic acid is a more potent inhibitor of PAL than (*E*)-cinnamic acid itself (app. $K_i = 16.5\ \mu M$). It had previously been shown [11] that α-methylcinnamic acid inhibits PAL from sweet potato and yeast less effectively than cinnamic acid. Steric hindrance by the methyl of α-methylcinnamic acid is obviously overcome by the aminomethyl group of **2**.

The efficiency of a PAL inhibitor *in vivo* can be evaluated by measuring the inhibition of light-induced anthocyanin synthesis in buckwheat hypocotyls [2]. With both compounds no inhibition was observed at concentrations up to 0.1 mM, and *ca* 2 mM concentrations were required to produce 50% inhibition. As the phosphonic analogue of phenylalanine [(*R*)-(1-amino-2-phenylethyl)phosphonic acid] inhibits buckwheat PAL with an apparent K_i value of 1.5 μM, which is slightly larger than that of **2**, but inhibits anthocyanin formation 50% at *ca* 50 μM concentration [12], we conclude that the two β-amino acids (**1**, **2**) are not delivered to the catalytic site of PAL (due to factors, such as poor uptake and transport, or to metabolic inactivation). By coupling **2** to 6-aminohexanoic acid-activated-Sepharose 4B through the aminomethyl group it was hoped to produce an affinity matrix for PAL. However, the matrix did not bind PAL (Leubner, G., personal communication).

Inhibition of phenylalanine transamination

While several plant aminotransferases have been shown to catalyse the transamination of phenylpyruvate and 4-hydroxyphenylpyruvate [13], their involvement in the biosynthesis of the corresponding aromatic amino acids is questionable and their function is thought to be in the further metabolism of the aromatic amino acids [14]. We studied the effect of **1** and **2** on the transamination of phenylalanine by extracts from mungbean shoots which exhibit an aminotransferase activity with broad substrate specificity [15, 16]. While **2** did not inhibit the transamination of phenylalanine at all, **1** affected both the apparent K_m and V_{max} of the enzyme, features characteristic of mixed inhibition [17]. An apparent K_i-value of 0.93 mM was calculated from a replot of the slopes of the Lineweaver–Burk diagram vs inhibitor concentration, while a secondary plot of the intercepts with the $1/v$ axis vs inhibitor concentration gave an apparent K_{ii}-value of 28 mM (app. K_m for phenylalanine = 0.75 mM).

Effects on phenylalanine hydroxylase and phenylalanyl-tRNA synthetase

Phenylalanine hydroxylation (in animals), as well as phenylalanine activation for protein synthesis (in all organisms) represent two important metabolic reactions of this amino acid in which, contrary to the reactions investigated above, no substituent is removed from C-2 of the side chain. As it is known that rat liver phenylalanine hydroxylase requires an unmodified alanyl side chain attached to an aromatic ring for a compound to act either as a substrate or as an inhibitor [18], it was not surprising that neither **1** nor **2** inhibited the enzyme. Likewise, both compounds were neither activated by, nor did they inhibit the activation of phenylalanine by soybean phenylalanyl *t*RNA synthetase.

EXPERIMENTAL

Ethyl (±)-2-*cyano*-3-*phenylpropanoate* (**4**). A suspension of benzyl chloride (60 ml, 0.5 mol), dry K_2CO_3 (144 g, 1.04 mol), ethyl cyanoacetate (**3**) (125 ml, 1.04 mol) and tetrabutylammonium bromide (1 g) was stirred for 5 hr at 20°. The reaction mixture was cooled and, after the addition of H_2O to dissolve K_2CO_3, exhaustively extracted with CH_2Cl_2. The CH_2Cl_2 was washed with H_2O and brine, dried over $MgSO_4$ and evapd. The residue, after vacuum distillation, gave (**4**) (51 g, 50%) as oil: bp 132–140° (2.5 mm Hg); GC (2% DEGS on Chromosorb G, 1 m glass column, 180°, N_2 at 13 ml/min) R_t 9 min, single peak; ^{1}H NMR ($CDCl_3$, TMS, 100 MHz): δ 1.22 (3H, *t*), 3.1–3.3 (2H, *m*), 3.6–3.8 (1H, *m*), 4.20 (2H, *q*), 7.30 (5H *br s*).

(±)-2-*Aminomethyl*-3-*phenylpropanoic acid* (**1**). A suspension of dry NaOAc (16 g, 0.20 mol) freshly distilled Ac_2O (210 ml, 2.2 mol), **4** (40.7 g, 0.20 mol) and Raney Ni of W-2 activity (7.5 g) was hydrogenated twice for 12 hr at 50° and an initial hydrogen pressure of 300 psi, filtered, diluted with H_2O and kept at room temp. for 1.5 hr. An equal vol. of conc HCl was added, the mixture refluxed for 12 hr and evapd. The residue was dissolved in H_2O and extracted with $CHCl_3$. The aq. phase was evapd, the residue dissolved in H_2O and after charcoal treatment the filtrate was adjusted to pH *ca* 5.5 by the addition of pyridine, whereupon white crystals of crude **1** pptd. The crystals (23 g) were dissolved in 0.5 M HCl, decolourized with charcoal and crystallized by the addition of pyridine. After washing with H_2O, EtOH and hexane, recrystallization from 0.5 M HCl–pyridine gave 14 g (39%) of **1** as a white solid: mp 237–238°; (found: C, 66.86; H, 7.45; N, 7.68; $C_{10}H_{13}NO_2$ requires: C, 67.02; H, 7.31; N, 7.82); IR ν_{max}^{KBr} cm^{-1}: 700, 750, 840, 1170, 1290, 1390, 1405, 1450, 1570, 2100–3100; ^{1}H NMR (D_2O–DCl, HMDSO, 100 MHz): δ 3.1–3.5 (5H, *m*), 7.5 (5H, *br s*).

Ethyl (E)-2-*methyl*-3-*phenylpropenoate* (**5**) was prepared according to [10].

Ethyl (E)-2-*bromomethyl*-3-*phenylpropenoate* (**6**). *N*-Bromosuccinimide (10.3 g, 57.8 mmol), **5** (11.3 g, 57.8 mmol), dry CCl_4 (60 ml) and a catalytic amount of 2,2′-azobis (2-methylpropionitrile) were refluxed for 0.5 hr, filtered and evapd to dryness. After distillation the residue gave 13.1 g (84%) **6** [bp 126–130° (2 mm Hg), n_D^{20} 1.5808], which was solidified and crystallized from hexane to give 10.1 g (65%) of pure **6**: mp 42–44°; ^{1}H NMR ($CDCl_3$, TMS, 100 MHz): δ 1.36 (3H, *t*), 4.12–4.44 (4H, *m*), 7.2–7.6 (5H, *m*), 7.81 (1H, *s*).

(E)-2-*Bromomethyl*-3-*phenylpropenoic acid* (**7**). 5.4 g (20 mmol) of **6** and 20 ml 48% HBr were refluxed for 10 hr. After evaporation to dryness, the residue was dissolved in toluene and concd under red. pres. The crude product was crystallized from toluene to give 3.8 g (80%) of **7**: mp 160–162°; IR ν_{max}^{KBr} cm^{-1}: 930, 1220, 1280, 1300, 1420, 1440, 1670; ^{1}H NMR ($CDCl_3$, TMS, 100 MHz): δ 4.46 (2H, *s*), 7.3–7.7 (5H, *m*), 8.00 (1H, *s*), 11.0 (1H, *br s*).

(E)-2-*Aminomethyl*-3-*phenylpropenoic acid* (**2**). 2.41 g (10 mmol) of **7** were dissolved at room temp. in 250 ml of a satd methanolic NH_3 soln and kept at room temp. for 5 days. After evapn under red. pres. the residue was dissolved in H_2O, reduced to dryness again, dissolved in 0.5 M HCl and decolourized by charcoal treatment. After filtration, pyridine was added to incipient turbidity (pH *ca* 4). The crystals were washed with H_2O, EtOH and hexane. Recrystallization from dil HCl–pyridine as described above gave 0.99 g (50%) analytically pure **2**: mp 235–237° (decomp.); (found: C, 67.52; H, 6.19; N, 8.02; $C_{10}H_{11}NO_2$ requires: C, 67.78; H, 6.26; N, 7.91); IR ν_{max}^{KBr} cm^{-1}: 710, 758, 778, 1300, 1360, 1440, 1515, 1565, 1645, 2650, 2770, 2850; ^{1}H NMR (D_2O–DCl, HMDSO, 100 MHz): δ 4.20 (2H, *s*), 7.8 (5H, *s*), 8.38 (1H, *s*).

Extraction and assay of enzymes and anthocyanin. PAL was extracted from the hypocotyls of illuminated buckwheat seedlings and assayed as described in [19]. An aromatic amino acid aminotransferase was extracted and assayed as in [15]. Rat liver phenylalanine hydroxylase was purified by the procedure given in [20], as modified in [21], and assayed as in [20]. Phenylalanine *t*RNA-synthetase was extracted from soybean cotyledons and purified *ca* × 400 according to [22]. The enzyme was assayed as in [22] using *t*RNA-Phe isolated from soybean cotyledons [23]. The anthocyanin content of excised illuminated buckwheat hypocotyls was determined as described previously [12].

Acknowledgements—We thank T. Glowiak (Wroclaw) and G. Leubner (Bochum) for X-ray analysis and phenylalanine *t*RNA-synthetase measurements, respectively. One of the authors (J. Z.) is grateful to Prof. P. Mastalerz for his encouragement, and both authors appreciate the assistance of Prof. N. Amrhein in the preparation of the manuscript. Work in Wroclaw was supported by grant CPBR 313.6.1.3, while the work in Bochum was supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn (to Prof. N. Amrhein).

REFERENCES

1. Hanson, K. R. and Havir, E. A. (1981) in *Secondary Plant Products* (Conn, E. E., ed.) Vol. 7 of *The Biochemistry of Plants. A Comprehensive Treatise* (Stumpf, P. K. and Conn, E. E., eds), p. 577–625. Academic Press, New York.
2. Amrhein, N. (1986) *Recent Adv. Phytochem.* **20**, 83.
3. Amrhein, N. and Gödeke, K. H. (1977) *Plant Sci. Letters* **8**, 313.
4. Hanson, K. R. (1981) *Arch. Biochem. Biophys.* **211**, 575.
5. Chapple, C. C. S., Walker, M. A. and Ellis, B. E. (1986) *Planta* **167**, 101.
6. Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, p. 777–827. Freeman, San Francisco.
7. Hermes, J. D., Weiss, P. M. and Cleland, W. W. (1985) *Biochemistry* **24**, 2959.
8. Chaturvedi, N. C., Park, W. K., Smeby, R. R. and Bumpus, F. M. (1970) *J. Med. Chem.* **13**, 177.
9. Hülskämper, L. and Weyerstahl, P. (1981) *Chem. Ber.* **114**, 746.
10. Holländer, H., Kiltz, H.-H. and Amrhein, N. (1979) *Z. Naturforsch.* **34c**, 1162.
11. Sato, T., Kiuchi, F. and Sankawa, U. (1982) *Phytochemistry* **21**, 845.
12. Laber, B., Kiltz, H.-H. and Amrhein, N. (1986) *Z. Naturforsch.* **41c**, 49.

13. Wightman, F. and Forest, J. C. (1978) *Phytochemistry* **17**, 1455.
14. Bonner, C. A. and Jensen, R. A. (1985) *Arch. Biochem. Biophys.* **238**, 237.
15. Gamborg, O. L. and Wetter, L. R. (1963) *Can. J. Biochem. Physiol.* **41**, 1733.
16. Gamborg, O. L. (1965) *Can. J. Biochem.* **43**, 723.
17. Dixon, M. and Webb, E. C. (1979) *Enzymes* 3rd Edn, pp. 339–341. Academic Press, New York.
18. Kaufman, S. (1978) *Methods Enzymol.* **53**, 278.
19. Scherf, H. and Zenk, M. H. (1967) *Z. Pflanzenphysiol.* **56**, 203.
20. Shiman, R., Gray, D. W. and Pater, A. (1979) *J. Biol. Chem.* **254**, 11300.
21. Gottschall, D. W., Dietrich, R. F. and Berkovic, S. J. (1982) *J. Biol. Chem.* **257**, 845.
22. Swamy, G. S. and Pillay, D. T. N. (1980) *Plant. Sci. Letters* **20**, 99.
23. Rafalski, A. J., Barciszewski, J., Gulewicz, K., Twardowski, T. and Keith, G. (1977) *Acta Biochim. Polon.* **24**, 301.