[A0054]

Prof. Dr. János Rétey, Dipl.-Chem. Alexander Skolaut
Institut für Organische Chemie, Universität Karlsruhe, Richard-Willstätter-Allee, Postfach 6980, D-76128 Karlsruhe, Germany.

3-(2,5-Cyclohexadienyl)-L-alanine (1,4-Dihydro-L-phenylalanine)

Its Synthesis and Behaviour in the
Phenylalanine Ammonia-Lyase Reaction

Alexander Skolaut and János Rétey
E-mail: biochem@ochhades.chemie.uni-karlsruhe.de and skolaut@ochhades.chemie.uni-karlsruhe.de

Abstract   Introduction   Results and Discussion

Conclusion and Acknowledgements   References

These pages are best viewed with Netscape Navigator 4.X.

All comments on this poster should be sent by e-mail to (mailto:ecsoc@listserv.ariz ona.edu) ecsoc@listserv.arizona.edu with **A0054** as the message subject of your e-mail.

3-(2,5-Cyclohexadienyl)-L-alanine (1,4-Dihydro-L-phenylalanine)

Its Synthesis and Behaviour in the Phenylalanine Ammonia-Lyase Reaction

Abstract

The title compound was synthesised by the reduction of L-phenylalanine with lithium in liquid ammonia. Consistent with the Birch rule the main product was 2,5-dihydro-L-phenylalanine. 1,4-dihydro-L-phenylalanine was isolated by HPLC on a reversed phase column and characterised as a minor product of the Birch reduction. Both dihydro-L-phenylalanines were assayed in the phenylalanine ammonia-lyase (PAL) reaction. 2,5-Dihydro-L-phenylalanine was a substrate for PAL with K_m about one third and V_{max} 30 times less than that of the values of the natural substrate L-phenylalanine and converted into the corresponding 2,5-dihydro-cinnamic acid. On the other hand 1,4-dihydro-L-phenylalanine was not converted by PAL. Both isomers were, however, competitive inhibitors of the enzymatic reaction. The K_i value of 1,4-dihydro-L-phenylalanine was 5.1 times higher as with the 2,5-dihydro isomer. These results support the proposed mechanism of the PAL reaction starting with an electrophilic attack of the 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO) prosthetic group at the *ortho* position of the phenyl ring of the substrate.

3-(2,5-Cyclohexadienyl)-L-alanine (1,4-Dihydro-L-phenylalanine)

Its Synthesis and Behaviour in the Phenylalanine Ammonia-Lyase Reaction

Introduction

The phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is one of the best studied plant enzymes. It catalyses a central reaction at the branching point of primary and secondary plant metabolism by converting L-phenylalanine into *trans*-cinnamic acid, the precursor of phenylpropanoids like flavonoids, coumarins, and lignins [1,2]. These substance classes have functions as dyes, UV-absorbing pigments, or chemical signal-transmitters (scheme 1).

Since the discovery of PAL by KOUKOL and CONN in 1961 [3] this enzyme has met great interest. In 1970 HANSON and HAVIR proposed dehydroalanine to be at the active site of PAL [4], which was supported by chemical and mutation studies. A serine residue has been identified as precursor, that is posttranslationally converted into dehydroalanine [5]. HANSON and HAVIR also made a proposal for the mechanism of the PAL reaction (scheme 2) [4,6,7]. In the key step of the mechanism a Michael-type addition of the amino group of L-phenylalanine to the ß-position of dehydroalanine takes place. This was supposed to enhance the leaving ability of the amino group. A 1,3-hydrogen shift and the abstraction of a benzylic proton by an enzymatic base lead then to *trans*-cinnamic acid.

This mechanism was accepted as working hypothesis for almost 25 years. But there were some unanswered questions concerning the proposed ionic intermediate. It was not clear how it could be formed or stabilised, because no base is likely to exist in proteins that is strong enough to abstract a non-activated benzylic proton [8].

SCHUSTER and RÉTEY provided evidence against this mechanism by observing the conversion of 4-nitro-L-phenylalanine with inactivated PAL. The inactivation occurred either by reduction of the prosthetic dehydroalanine with sodium borohydride or by site-directed mutagenesis (Ser202 to Ala202) [8]. Their conclusion was that the nitro group in *para* position can substitute the electrophilic prosthetic group. Consequently SCHUSTER and RÉTEY saw the role of dehydroalanine in the activation of the protons in ß-position of L-phenylalanine to facilitate their abstraction by an enzymatic base and to stabilise the resulting carbanion.

Based on these considerations the following mechanism was proposed: The prosthetic dehydroalanine attacks the *ortho* position of the aromatic ring of L-phenylalanine in a Friedel-Crafts-type reaction [8,9]. In the resulting carbenium ion the protons in ß-position are acidified and the abstraction by an enzymatic base is facilitated. After removal of the pro-*3S*-proton elimination of ammonia, rearomatisation of the ring and regeneration of the prosthetic group occur (scheme 3).

The enzymes PAL and HAL (histidine ammonia-lyase, histidase, EC 4.3.1.3) have a high homology (19-29% sequence identity). Hence it can be concluded that the essential part of the active site of both enzymes has the same structure. The X-ray structure of PAL's "sister enzyme" HAL revealed that the catalytic electrophile was not dehydroalanine but 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO), which is autocatalytically formed from the inner tripeptide Ala-Ser-Gly by cyclisation and elimination of two molecules of water [10] (scheme 4). This modification can be interpreted as an enhancement of the electrophilicity of the prosthetic group by preventing the delocalisation of the nitrogen lone pairs into the 2,3-unsaturated carbonyl system.

To provide further support for the proposed mechanism two different non-aromatic L-phenylalanine derivatives were regarded as useful probes: The first is 2,5-dihydro-L-phenylalanine [3-(1,4-

cyclohexadienyl)-L-alanine], the other 1,4-dihydro-L-phenylalanine [3-(2,5-cyclohexadienyl)-L-alanine] [11]. Of these the 2,5-dihydro isomer is expected to be a substrate: the electrophilic attack generates a carbenium ion next to the side chain. Thereby the ß-protons are made more acidic and can be abstracted by an enzymatic base (scheme 5).

In the case of the 1,4-dihydro isomer an electrophilic attack is also possible. But the generated positive charge is not located next to the side chain, the ß-protons remain non-acidic and the lyase reaction does not take place (scheme 6). If this prediction turns out to be correct, further evidence for the recently proposed mechanism would be provided.

Conclusion and Acknowledgements

We have described for the first time the isolation of 1,4-dihydro-L-phenylalanine as minor product in the Birch reduction of L-phenylalanine. The yield could be improved to 3 % by changing the proton source from *tert.*-butanol to ethanol. Kinetic experiments showed that 2,5-dihydro-L-phenylalanine is a substrate (K_m = 35.5 % of K_m (phe), V_{max} = 3.3 % of V_{max} (phe)) and simultaneously a moderately good competitive inhibitor (K_i = 30.6 µM). 1,4-dihydro-L-phenylalanine was no substrate but a weaker competitive inhibitor (K_i = 157 µM). These results lend further support for the recently proposed mechanism of the PAL reaction [8,9].

Results and Discussion

The chemical synthesis of 2,5-dihydro-L-phenylalanine by Birch reduction has already been reported (scheme 7) [12]. The product was obtained in 43-48% yield after crystallisation but its purity was only 92-95% and it was contaminated by up to 2% L-phenylalanine and 3-5% 3-(1-cyclohexenyl)-L-alanine. In an attempt to obtain 2,5-dihydro-L-phenylalanine in pure form we applied HPLC. The reversed phase separation with a water/methanol system yielded two different fractions. While the second fraction was pure 2,5-dihydro-L-phenylalanine the ^{1}H-NMR-spectrum revealed that the first fraction was not pure L-phenylalanine as first expected, but a mixture thereof with 2,5- and 1,4-dihydro-L-phenylalanine (spectrum 1). This was surprising because it had consistently been reported that the Birch reduction of aromatic compounds bearing an alkyl side chain yields only the product with the side chain at a residual double bond.

BIRCH *et al.* have performed *ab initio* calculations about the preferred site of protonation in the radical anion [13-15]. They concluded that the kinetically favoured site of protonation in a substituted (strong pi-donor substituent and CH_3) benzene radical anion is the *ortho* and/or *meta* position, while the thermodynamically favoured site is the *ortho* and/or *para* position. The calculated energy differences for *ortho* and *para* protonation were so small that a mixture of both resulting products seemed possible. The second protonation occurs normally *para* to the first one. A product derived from the first protonation in *para* position yielding a 1,4-cyclohexadiene with the donor substituent at a reduced ring carbon atom has so far not been reported.

To completely separate and characterise the 1,4-dihydro-L-phenylalanine, a second chromatography was performed. Good separation was obtained by isocratic elution with water. Due to the instability of the dihydro-products with respect to rearomatisation [16,17], the isomers were accompanied by 3-5% rearomatised product after solvent removal.

Samples of the isolated isomers were analysed on a chiral-phase HPLC column to proof that no racemisation took place during Birch reduction and separation. All substances have the L-configuration as deduced from comparison with authentic samples.

To increase the yield of 1,4-dihydro-L-phenylalanine the proton source used in the Birch reduction was changed from *tert.*-butanol to ethanol. As it can be seen in table 1 the total yield slightly decreased from 95.7 to 87.7 % but the amount of the desired 1,4-dihydro product doubled from 1.5 to 3 %. We explain

this as follows: Ethanol is more acidic than *tert.*-butanol which provides faster protonation of the generated radical anion. Because the protonation is irreversible and the second protonation takes place *para* to the first one, it can be concluded that 1,4-dihydro-L-phenylalanine is a kinetically controlled product. A further optimisation of the conditions may improve the yield of this minor product.

In kinetic experiments with PAL the two isomers showed different behaviour. As expected, 2,5-dihydro-L-phenylalanine was a substrate for PAL with smaller K_m and V_{max} values than those for the natural substrate L-phenylalanine (table 2). The K_m value was only one third of the value of L-phenylalanine, V_{max} was 30 times lower. The smaller K_m value corresponds to a higher affinity of the substrate to the active site of PAL. Because the binding pocket is designed to bind the aromatic phenyl ring of L-phenylalanine, it is hydrophobic. An increase in hydrophobicity in this part of the substrate may increase the binding affinity and hence lower the K_m value [18]. The smaller reaction velocity can be explained by the steric difference in the ring system. In contrast to the planar phenyl ring the 1,4-cyclohexadienyl ring adopts a boat-like conformation. This deviation from the plane places the MIO group and the double bond of the substrate in an inappropriate steric position, slowing the reaction rate down. Simultaneously, a more tight binding results in a lower dissociation rate of the product, leading to a decreased V_{max} value. The weaker acidification of the ß-protons may shift the rate limiting step to the C-H-bond cleavage, also leading to a slower reaction.

1,4-dihydro-L-phenylalanine was no substrate for the enzyme as shown by incubation with PAL. HPLC analysis of withdrawn samples were performed after 2, 4, 6 and 20 hours. During the first two hours the concomitant L-phenylalanine was completely converted into *trans*-cinnamic acid. No conversion of 1,4-dihydro-L-phenylalanine was observed during 20 hours (graphs a and b). From these data one can conclude that 1,4-dihydro-L-phenylalanine is no substrate of PAL.

It has been known that 2,5-dihydro-L-phenylalanine is an inhibitor of microbial growth [12,19-21]. This inhibitory effect can be overcome by addition of an equimolar amount of L-phenylalanine. According to these data a competitive inhibition of PAL by 2,5-dihydro-L-phenylalanine can be expected. Therefore inhibition kinetics were performed to determine the K_i value of 2,5-dihydro-L-phenylalanine. The results show a competitive inhibition of PAL with a K_i value of 30.6 µM (table 2). 1,4-Dihydro-L-phenylalanine showed also competitive inhibition with a 5.1 times higher K_i value (157 µM) probably due to weaker binding at the active site. Comparing with other known inhibitors the two dihydro-phenylalanines are moderately good inhibitors. They show stronger inhibition than for example open chain unsaturated analogues or the completely hydrogenated analogue, 3-cyclohexyl-L-alanine (table 3) [18].

References

[1] K. R. Hanson and E. A. Havir *Rec. Adv. Phytochem.* **1978**, *12*, 91.

[2] K. Hahlbrock and D. Scheel *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 347.

[3] J. Koukol and E. E. Conn *J. Biol. Chem.* **1961**, *236*, 2692.

[4] K. R. Hanson and E. A. Havir *Arch. Biochem. Biophys.* **1970**, *141*, 1.

[5] B. Schuster and J. Rétey *FEBS Lett.* **1994**, *349*, 252.

[6] K. R. Hanson, E. A. Havir and C. Ressler *J. Biol. Chem.* **1969**, *244*, 6341.

[7] K. R. Hanson and E. A. Havir in: *Enzymes*, 3rd Ed. **1972**, *7*, 75.

[8] B. Schuster and J. Rétey *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 8433.

[9] J. Rétey *Naturwissenschaften* **1996**, *83*, 439.

[10] T. F. Schwede, J. Rétey and G. E. Schulz *Biochemistry* **1999**, *38*, 5355.

[11] Abbreviation and nomenclature used: under the IUPAC-IUB 1974 recommendations (1975), the compound (2S)-2-amino-3-(1,4-cyclohexadienyl)propanoic acid may be named 3-(1,4-cyclohexadienyl)-L-alanine or 2,5-dihydro-L-phenylalanine. The latter name leads to the abbreviation L-DiHPhe. The compound (E)-3-(1,4-cyclohexadienyl)-2-propenoic acid which could be named as *trans*-3-(1,4-cyclohexadienyl)acrylic acid or as a ring 2,5-dihydro derivative of cinnamic acid.

[12] K. L. Snow, C. Lauinger and C. Ressler *J. Org. Chem.* **1968**, *33*, 1774.

[13] A. J. Birch, A. L. Hinde and L. Radom *J. Am. Chem. Soc.* **1980**, *102*, 3370.

[14] A. J. Birch, A. L. Hinde and L. Radom *J. Am. Chem. Soc.* **1980**, *102*, 4074.

[15] A. J. Birch, A. L. Hinde and L. Radom *J. Am. Chem. Soc.* **1980**, *102*, 6430.

[16] D. G. Hendry and D. Schuetzle *J. Am. Chem. Soc.* **1975**, *97*, 7123.

[17] C. Ressler *J. Org. Chem.* **1972**, *37*, 2933.

[18] K. R. Hanson, E. A. Havir and C. Ressler *Biochemistry* **1979**, *18*, 1431.

[19] U. Fickenscher, W. Keller-Schierlein and H. Zähner *Arch. Mikrobiol.* **1971**, *75*, 346.

[20] U. Fickenscher and H. Zähner *Arch. Mikrobiol.* **1971**, *76*, 28.

[21] D. S. Genghof *Can. J. Microbiol.* **1970**, *16*, 545.

[22] M. Baedeker and G. E. Schulz *FEBS Lett.* **1999**, *457*, 57.

[23] W. P. C. Stemmer, A. Crameri, K. D. Ha, T. M. Brennan and H. L. Heyneker *Gene* **1995**, *164*, 49.

[24] E. Layne *Methods Enzymol.* **1957**, *3*, 447.

[25] A. Zimmermann and K. Hahlbrock *Arch. Biochem. Biophys.* **1975**, *166*, 54.

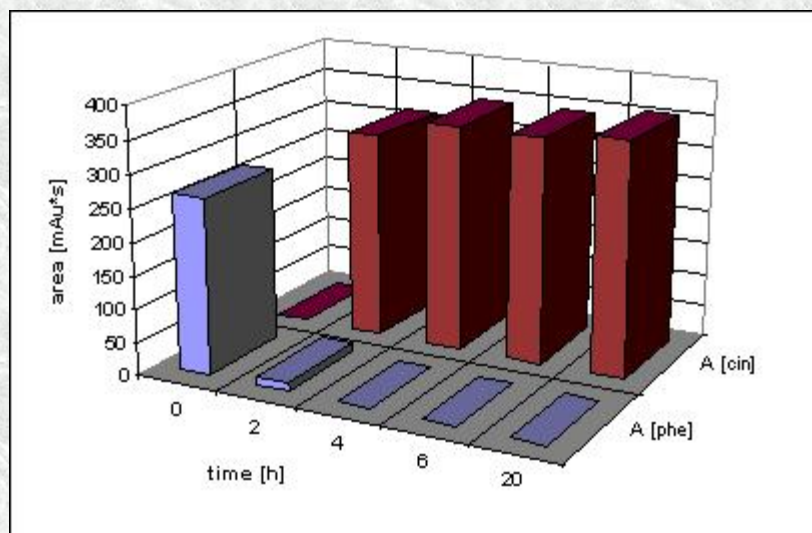**3-(2,5-Cyclohexadienyl)-L-alanine (1,4-Dihydro-L-phenylalanine)**

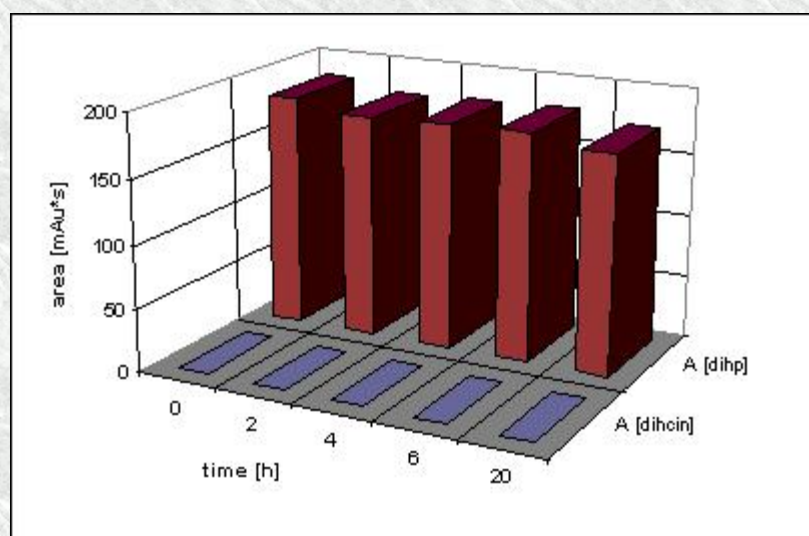**Its Synthesis and Behaviour in the Phenylalanine Ammonia-Lyase Reaction**

Graph:
a) Enzymatic conversion of concomitant L-phenylalanine by incubation with PAL.
b) Incubation of 1,4-dihydro-L-phenylalanine with PAL.
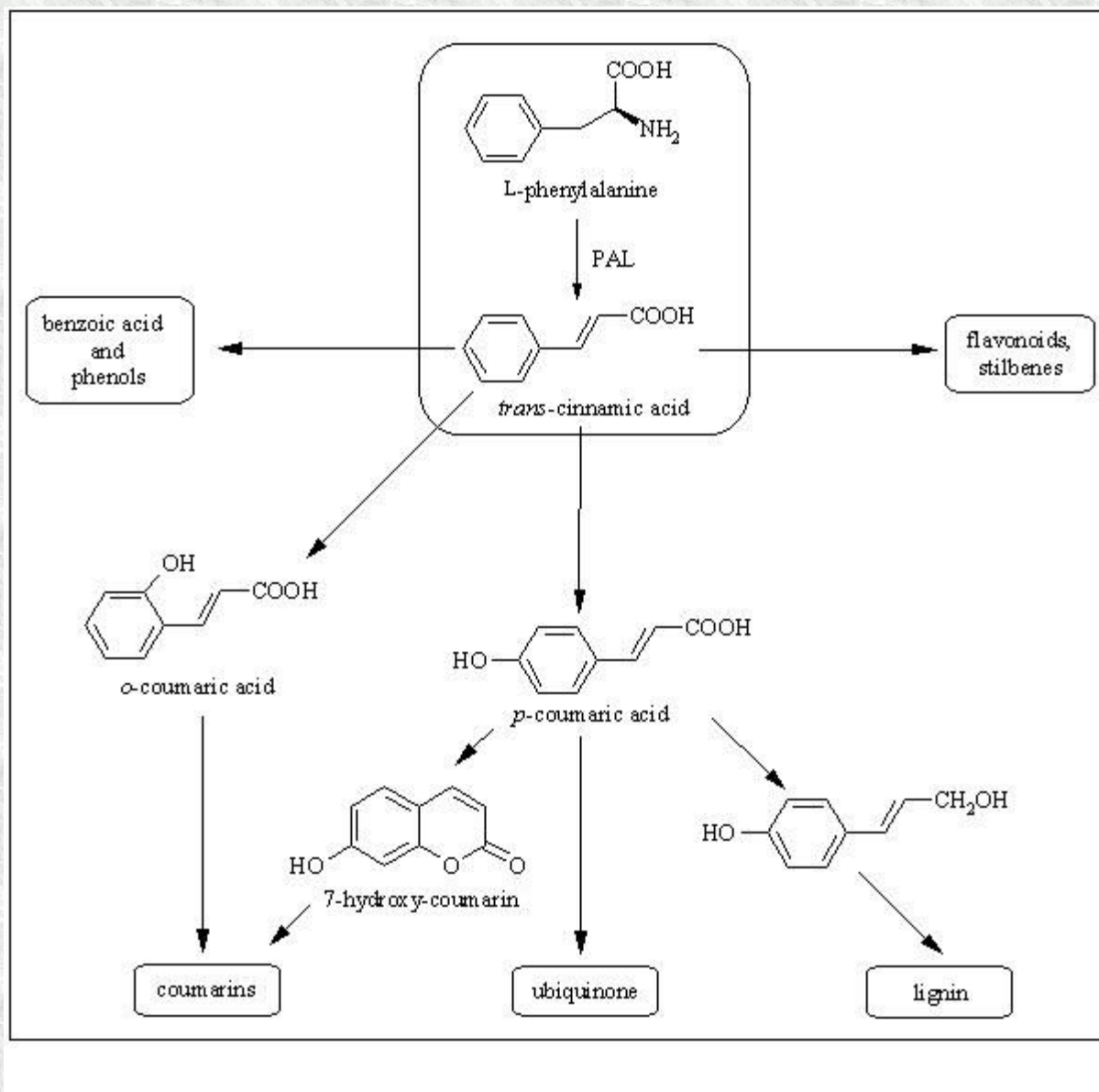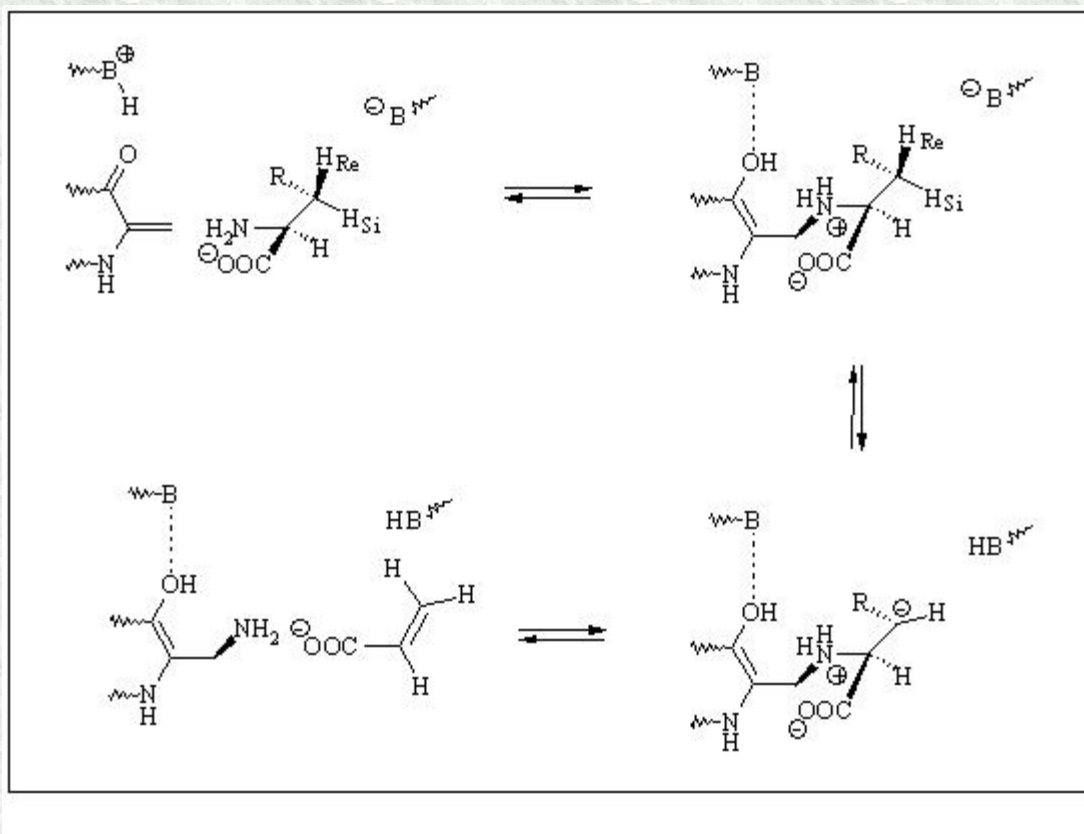
a)

b)

[BACK]

Scheme 1:
Localisation of PAL in plants.

3-(2,5-Cyclohexadienyl)-L-alanine (1,4-Dihydro-L-phenylalanine)

Its Synthesis and Behaviour in the Phenylalanine Ammonia-Lyase Reaction

Scheme 2:
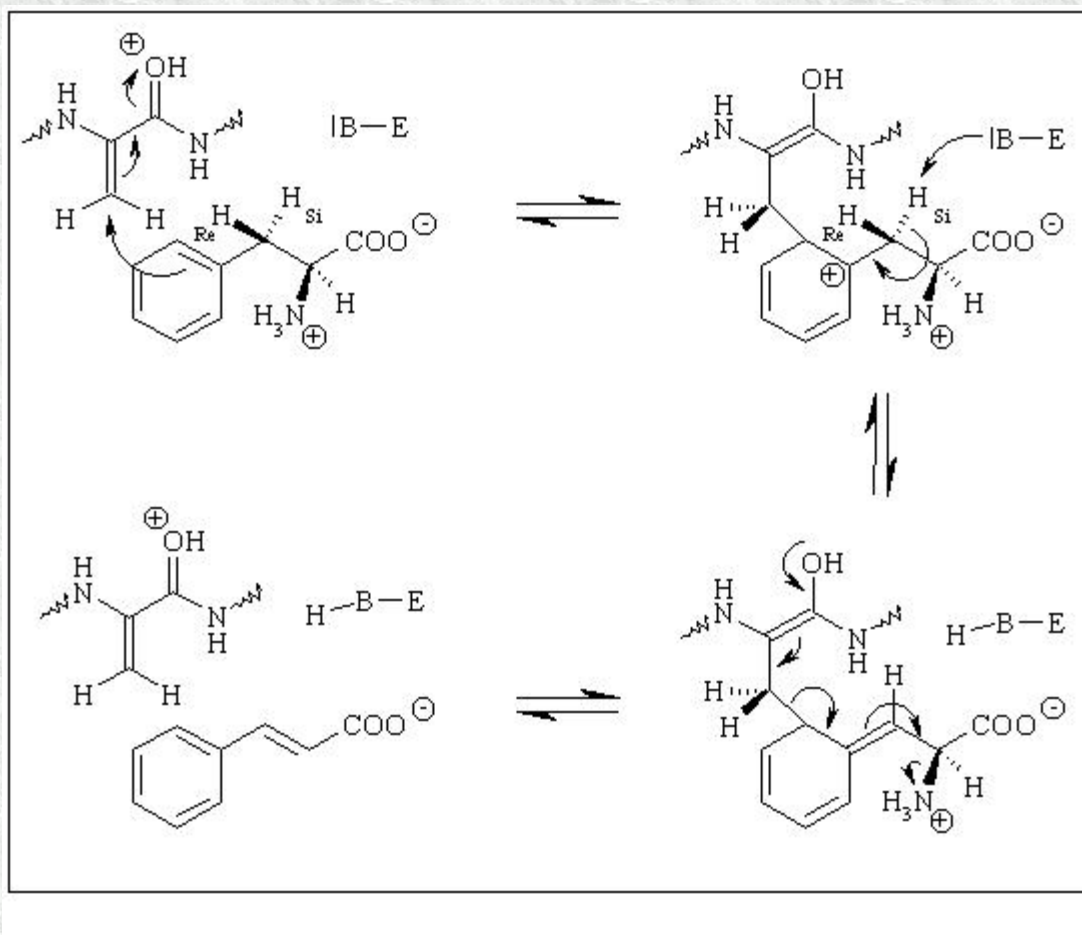**Mechanism of the PAL reaction according to HANSON and HAVIR [4,6,7].**

3-(2,5-Cyclohexadienyl)-L-alanine (1,4-Dihydro-L-phenylalanine)

Its Synthesis and Behaviour in the Phenylalanine Ammonia-Lyase Reaction

Scheme 3:
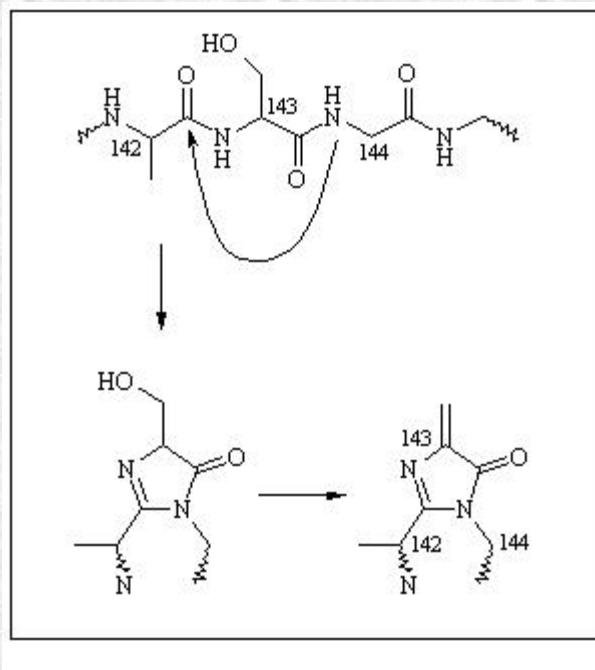## Mechanism of action of PAL as proposed by SCHUSTER and RÉTEY [8,9].

Scheme 4:
Modification of dehydroalanine in HAL: 3,5-dihydro-5-methylidene-4H-imidazol-4-one.

Scheme 5:
Course of the PAL reaction with 2,5-dihydro-L-phenylalanine.

3-(2,5-Cyclohexadienyl)-L-alanine (1,4-Dihydro-L-phenylalanine)

Its Synthesis and Behaviour in the Phenylalanine Ammonia-Lyase Reaction

Spectrum:
^{1}H-NMR-spectrum of the first fraction from the HPLC of the Birch reduction product mixture.
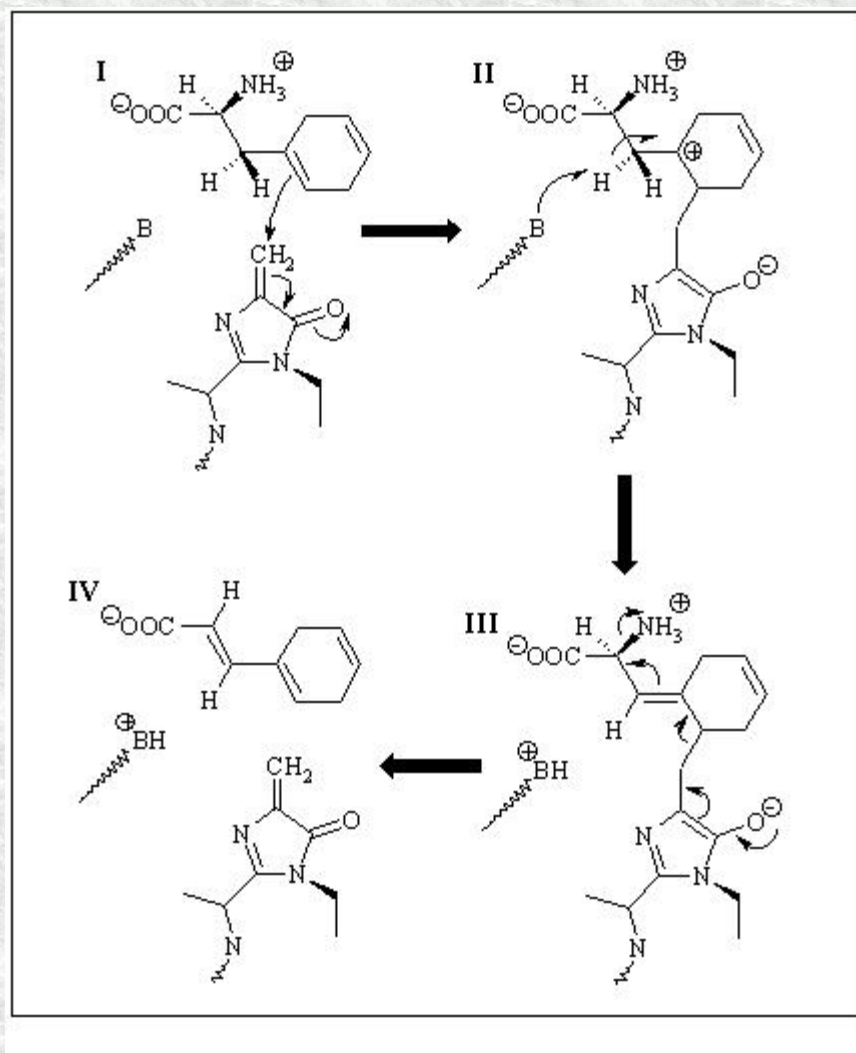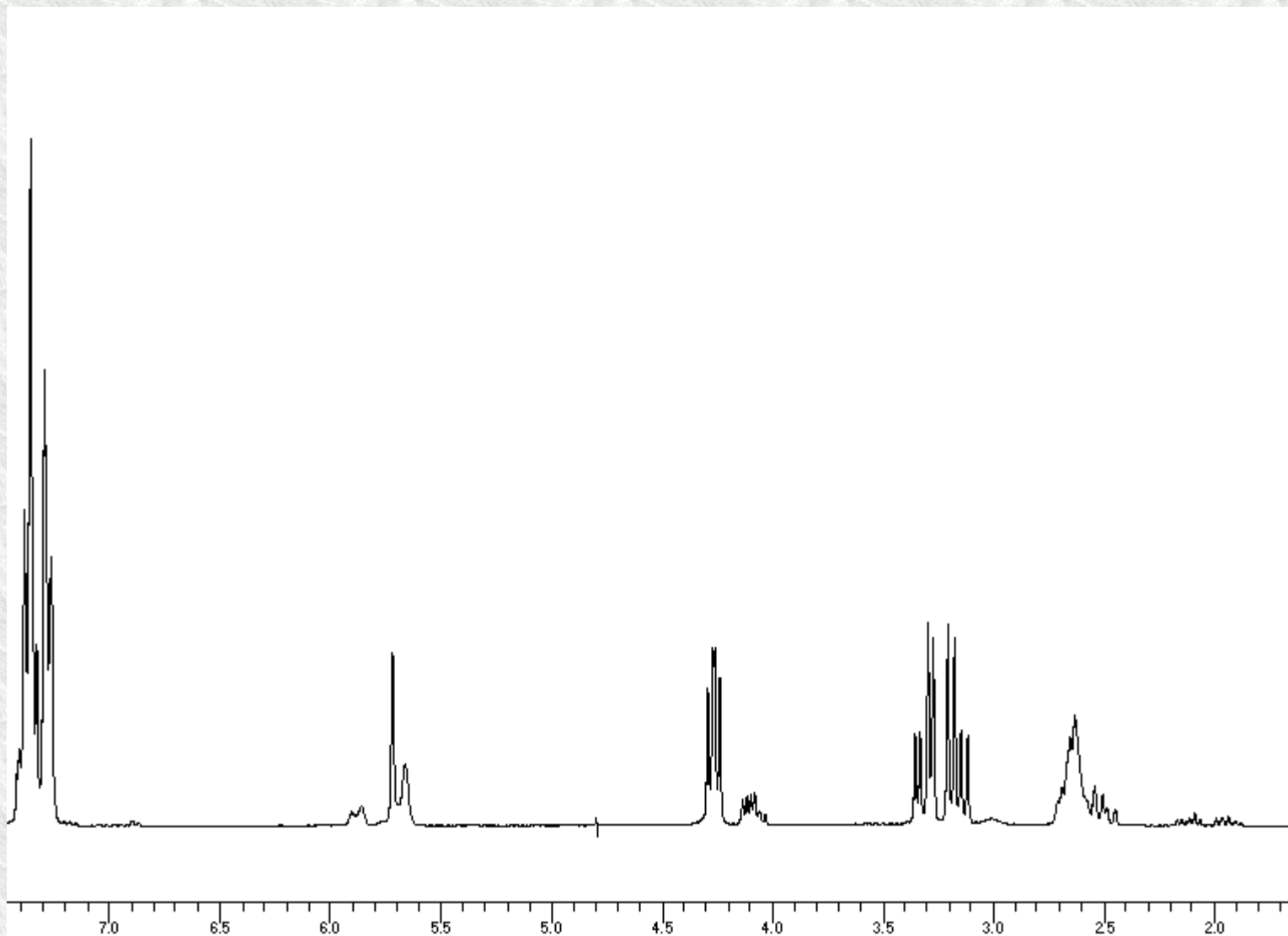

[BACK](#)

3-(2,5-Cyclohexadienyl)-L-alanine (1,4-Dihydro-L-phenylalanine)

Its Synthesis and Behaviour in the Phenylalanine Ammonia-Lyase Reaction

Table 1:

Experimental results of the Birch reduction of L-phenylalanine with different proton sources.

Nº	metal	proton source	yield	2,5-dihydro isomer [a]	1,4-dihydro isomer [a]	L-phenylalanine [a]
1	Li	*tert.*-butanol	92.7 %	96.9 %	1.5 %	1.3 %
2	Li	ethanol	87.7 %	88.8 %	3.0 %	7.8 %
3	Li	CH_3COONa	1.0 %	100.0 %	n.d.	n.d.

a) calculated from ^{1}H-NMR.
n.d. = not detectable

BACK

3-(2,5-Cyclohexadienyl)-L-alanine (1,4-Dihydro-L-phenylalanine)

Its Synthesis and Behaviour in the Phenylalanine Ammonia-Lyase Reaction

Table 2:

Kinetic properties of the dihydro-phenylalanines in the PAL reaction.

substance	K_m [µM]	K_m / K_m (phe)	V_{max}	V_{max} / V_{max} (phe)	K_i [µM]
L-phenylalanine	40.9	1.0	0.244	1.0	-
2,5-dihydro-L-phenylalanine	14.5	0.355	0.0081	0.033	30.6
1,4-dihydro-L-phenylalanine	-	-	-	-	157

3-(2,5-Cyclohexadienyl)-L-alanine (1,4-Dihydro-L-phenylalanine)

Its Synthesis and Behaviour in the Phenylalanine Ammonia-Lyase Reaction

Table 3:

Inhibitors of PAL.

inhibitor	K_i [µM]	K_i / K_m (phe)
3-cyclohexyl-L-alanine [1]	2.3	8.5
(2S,E)-2-amino-4-methylhex-4-enoic acid [1]	30	111
D,L-allylglycine [1]	280	1037
L-3-cyanoalanine [1]	50	185
trans,*trans*-sorbic acid [1]	10	37
(S)-2-aminooxy-3-phenyl-propanoic acid [2]	0.014	0.0003
(R)-(1-amino-2-phenylethyl)phosphonic acid [2]	1.5	0.32
2,5-dihydro-L-phenylalanine [3]	30.6	0.75
1,4-dihydro-L-phenylalanine [3]	157	3.8

1) PAL from maize; K. R. Hanson, E. A. Havir and C. Ressler *Biochemistry* **1979**, *18*, 1431.
2) PAL from buckwheat; J. Zon´ and N. Amrhein *Liebigs Ann. Chem.* **1992**, 625.
3) Recombinant PAL from parsley.