Mechanistic Investigation of Phenylalanine Ammonia Lyase by Using *N*-Methylated Phenylalanines

by **Sandra Viergutz**[a]), **László Poppe***[b]), **Anna Tomin**[b]), and **János Rétey***[a])

[a]) Institute of Organic Chemistry, University of Karlsruhe, Richard-Willstätter-Allee, D-76128 Karlsruhe (phone: +49 721 6083222; fax: +49 721 6084823; e-mail: janos.retey@ioc.uka.de)

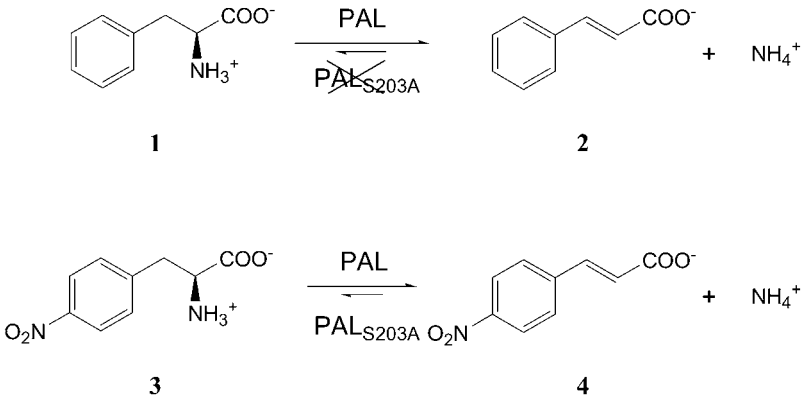
[b]) Institute for Organic Chemistry and Research Group for Alkaloid Chemistry, Budapest University of Technology and Economics, H-1111 Budapest, Gellért tér 4. (phone: +36 1 4632229; fax: +36 1 4633297; e-mail: poppe@mail.bme.hu)

Dedicated to Professor *Duilio Arigoni* on the occasion of his 75th birthday

N-Methyl-L-phenylalanine (**5**), *N*-methyl-4-nitro-L-phenylalanine (**6**), and *N*,*N*-dimethyl-4-nitro-L-phenylalanine (**7** · H^+) were investigated as substrates or inhibitors of phenylalanine ammonia lyase from *Petroselinum crispum*. Whereas the former was a reluctant substrate ($K_m = 6.6$ mM, $k_{cat} = 0.22\ s^{-1}$), no reverse reaction could be detected by using methylamine and (*E*)-cinnamate (**2**). The K_m value for ammonia in the reverse reaction by using (*E*)-cinnamate (**2**) was determined to be 4.4 and 2.6M at pH 8.8 and 10, respectively. The *N*-methylated 4-nitro-L-phenylalanines **6** and **7** showed only strong inhibitory effects ($K_i = 130$ nM and 8 nM, resp.). These and former results are discussed in terms of the mechanism of action of phenyalalanine and histidine ammonia lyases.

Introduction. – Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) is an important plant enzyme that catalyzes the conversion of L-phenylalanine (**1**) into (*E*)-cinnamate (**2**) (*Scheme 1*), which is the precursor of a great variety of phenylpropanoids, such as lignins, flavonoids, and coumarins [1][2]. Because of its central role in plant metabolism, PAL is a potential target for herbicides [2].

Scheme 1. *Reactions of* L-*Phenylalanine* (**1**) *and 4-Nitro*-L-*phenylalanine* (**3**) *with wt-PAL and S203A PAL Mutant*



The related enzyme histidine ammonia lyase (HAL, EC 4.3.1.3) catalyzes a very similar reaction, converting L-histidine into (*E*)-urocanic acid (= 3-(1*H*-imidazol-4-yl)prop-2-enoic acid) [3][4]. In the three decades between the first mechanistically relevant work published in 1969, which assumed that HAL contains dehydroalanine as catalytically essential electrophilic group [3], and the recent discovery of the structure of the electrophilic prosthetic group as 3,5-dihydro-5-methylidene-4*H*-imidazol-4-one (MIO) [5], a large number of mechanistic investigations were published (see ref. cit. in [6][7]). Their results were interpreted mostly in terms of the mechanism proposed by *Hanson* and *Havir* for PAL [8] (*Scheme 2*, *Path I*), and later by the mechanisms proposed by *Rétey* and co-workers for HAL [9] and PAL [10] (*Scheme 2*, *Path II*).

The first proposal for the role of the electrophilic group of HAL [3] and PAL [11] implied a *Michael* addition of the α-NH_2 group of the substrate followed by abstraction of the β-proton and elimination. In the amino–enzyme intermediate, a covalent bond between the prosthetic electrophile and ammonia was supposed. This seemed to be consistent with *Peterkofsky*'s experiments showing a relatively stable amino–HAL intermediate [12]. The latter, in the presence of [^{14}C]urocanate, could be re-converted into [^{14}C]histidine, while $^{15}NH_3$ at 10–60 mM concentrations was not incorporated into the substrate [12]. This led to the erroneous assumption that the HAL and PAL reactions are irreversible. However, it was demonstrated [13] that the histidase reaction can be completely reversed at higher (up to 6M) NH_4^+ concentrations.

Later, a kinetic isotope effect (*ca.* 1%) with [^{15}N]phenylalanine was also interpreted in favor of the *Hanson* mechanism [14]. Since the latter could not explain how the nonacidic β-proton of the substrates can be abstracted by an enzymic base, an alternative mechanism has been proposed for both HAL [9] and PAL [10] in which the aromatic ring is attacked by the prosthetic electrophile in a *Friedel–Crafts*-like reaction. The concomitant electron withdrawal from the ring could significantly activate the β-proton.

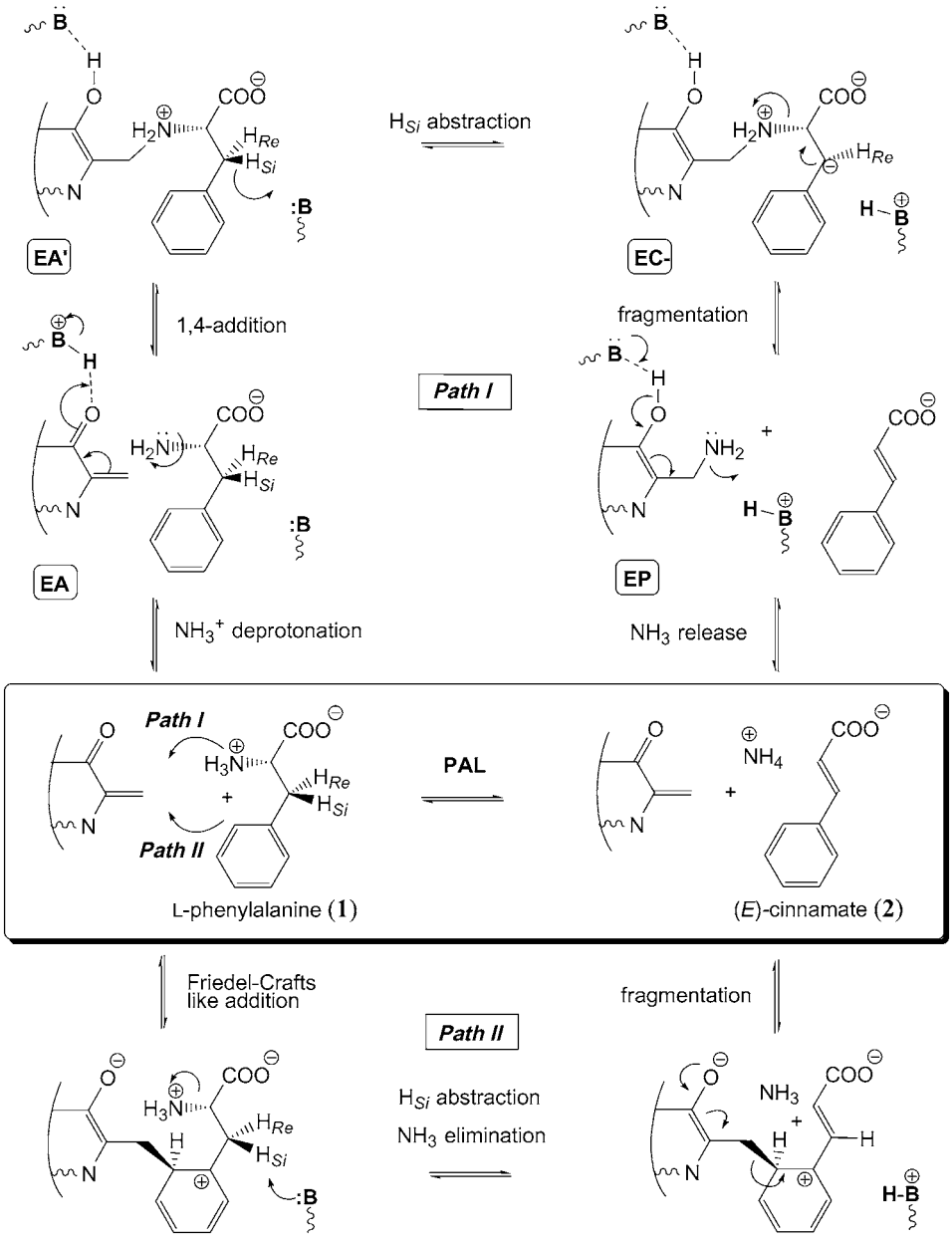
Identification of the electrophilic group as MIO [5] led to the insight that the latter is much more electrophilic than dehydroalanine and, thus, able to attack the aromatic ring. The geometry of MIO prevents delocalization of the N lone pairs into the α,β-unsaturated carbonyl system. Moreover, addition of a nucleophile renders MIO itself aromatic, thus compensating for the transitional abolition of the aromaticity of the substrate phenyl ring.

The strongest argument in favor of the *Friedel–Crafts*-type attack was, however, as follows: It was found that the S203A mutant of PAL, lacking MIO, still catalyzed the reaction. The V_{max} value with L-phenylalanine (**1**) was, however, *ca.* 10^4 times lower than for wild-type (wt) PAL. In contrast, S203A-PAL reacted relatively fast with 4-nitro-L-phenylalanine (**3**) [10] (*Scheme 1*).

Here we describe further experiments which invalidate some arguments in favor of the old mechanism and explain why the PAL reaction is practically irreversible under physiological conditions.

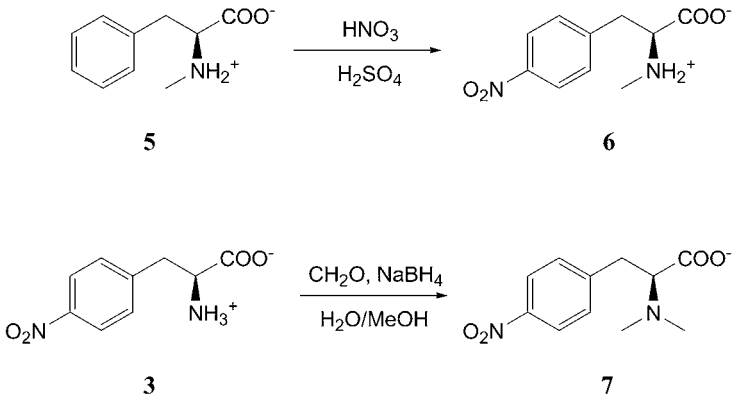
Results. – *Synthesis of* N-*Methyl-4-nitro*-L-*phenylalanine* (**6**) *and* N,N-*Dimethyl-4-nitro*-L-*phenylalanine* (**7**·H^+). One route to the synthesis of **6** and **7** started from the commercially available 4-nitro-L-phenylalanine (**3**) and involved reductive alkylation

Scheme 2. *Mechanisms Proposed by* Hanson *and* Havir [1] *(Path I) and by* Schuster *and* Rétey [10] *(Path II) for the PAL Reaction*



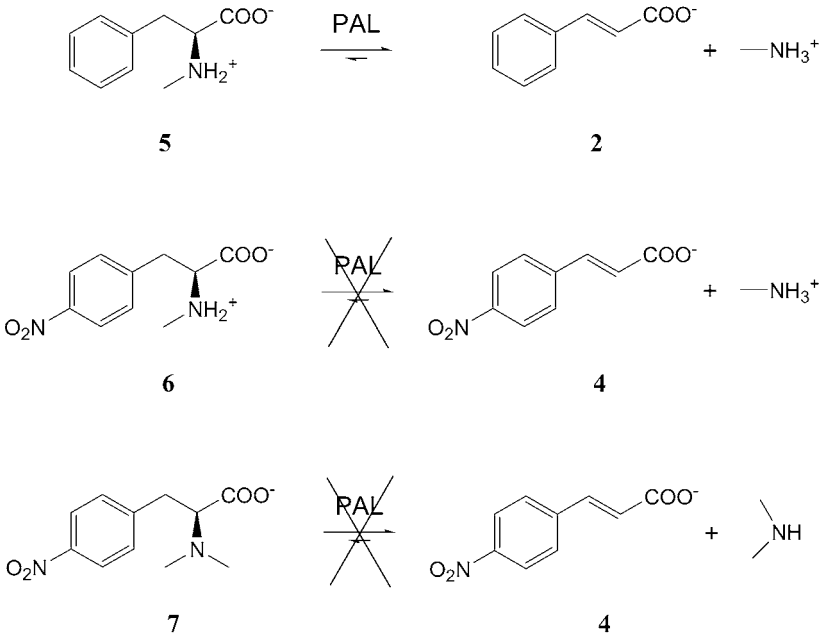
with aqueous formaldehyde solution and $NaBH_4$ (*Scheme 3*). In spite of extensive variation of the reaction conditions (formaldehyde/$NaBH_4$ ratio, reaction time, temperature, as well as solvent composition), only minor amounts of **6** were detected by HPLC; in most cases, the main product was **7**, even at low conversions. Moreover, whereas the retention time of the dimethylated product **7** was significantly longer than that of the educt **3** (reversed-phase HPLC), the retention time of the monomethylated product **6** was only slightly longer. Therefore, for the synthesis of **6**, the commercially available *N*-methyl-L-phenylalanine (**5**) was used as starting material. Although conventional nitration thereof has been published [15][16], no separation from by-products and full spectroscopic data have been described. Besides **6** as major product, *ca.* 10% of a not fully characterized by-product (most likely *N*-methyl-2-nitro-L-phenylalanine as its UV maximum at 274 nm indicates) was also detected by HPLC in the crude reaction product. The final purification of both *N*-methylated 4-nitro-L-phenylalanines **6** and **7** was achieved by prep. reversed-phase HPLC. The ^{1}H- and ^{13}C-NMR spectra as well as HR-MS data of the purified products **6** and **7** were in full agreement with their proposed structures. It is interesting to note that maximal solubility of the monomethylated 4-nitro compound **6** in neutral (pH 6–9) water was only about 2 mM, whereas the dimethylated or non-methylated 4-nitro-L-phenylalanines **7** and **3**, respectively, were soluble up to 20 mM. Acidification increased dramatically the solubility of **6** (over 100 mM in 10% CF_3COOH/H_2O, 50% HCOOH/H_2O, or in concentrated H_2SO_4 solution).

Scheme 3. *Preparation of* N-*Methyl-4-nitro*-L-*phenylalanine* (**6**) *and* N,N-*Dimethyl-4-nitro*-L-*phenylalanine* (**7**·H^+)



Kinetics of the PAL Reaction with N-*Methyl*-L-*phenylalanine* (**5**). Highly purified recombinant PAL [17] was used as catalyst and *N*-methyl-L-phenylalanine (**5**) as substrate (*Scheme 4*). The concentration of the latter was varied from 4 to 40 mM, and formation of (*E*)-cinnamate (**2**) was monitored at 290 nm. The change of absorbance was measured at intervals of 1 min for 5 min. On the basis of the *Michaelis–Menten* and *Lineweaver–Burk* plots, the K_m value was determined to be 6.58 mM and the V_{max} value to be 0.042 U/mg (*Fig. 1*, *Table 1*). The formation of (*E*)-cinnamate (**2**) was also confirmed by HPLC/UV (retention data and peak spectrum (λ_{max} 278 nm) were

Scheme 4. *Kinetic Properties of* N-*Methyl*-L-*phenylalanine* (**5**), N-*Methyl-4-nitro*-L-*phenylalanine* (**6**) *and* N,N-*Dimethyl-4-nitro*-L-*phenylalanine* (**7**·H^+) *with wt-PAL*



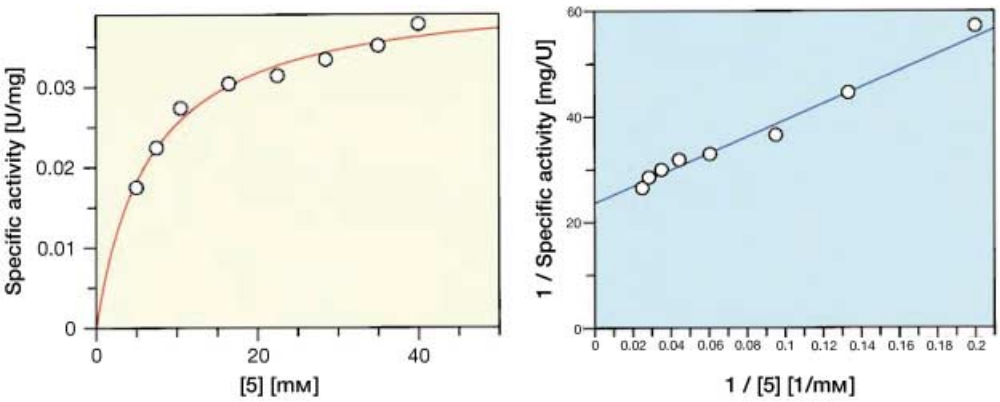


Fig. 1. Michaelis–Menten *and* Lineweaver–Burk *plots for* N-*methyl*-L-*phenylalanine* (**5**) *in its reaction with wt-PAL*

identical with those of authentic **2**) and ^{1}H-NMR spectroscopy (the signals of the isolated product at δ(H) 6.48 (d, $J = 15.9$ Hz, CH (α)), 7.38 (d, $J = 15.9$ Hz, CH(β), 7.41 ($m(t)$, H_m), and 7.58 ($m(dd)$, H_o) matched with the δ(H) of authentic **2** recorded under similar conditions).

Table 1. *Kinetic Constants of* N-*Methyl*-L-*phenylalanine* (**5**), N-*Methyl-4-nitro*-L-*phenylalanine* (**6**) *and* N,N-*dimethyl-4-nitro*-L-*phenylalanine* (**7**·H^+) *with wt-PAL*

	K_m [mM]	V_{max} [U/mg]	k_{cat} [s^{-1}]	K_i [nM]
5	6.6	0.042	0.22	–
6	–	–	–	130
7	–	–	–	8

*Kinetics of the Reverse Reaction; Addition of Ammonia to (*E*)-Cinnamate* (**2**) *Catalyzed by PAL.* Inspite of its biocatalytical usefulness [18][19], the kinetics of the reverse PAL reaction has not yet been quantitatively examined. In particular, the K_m value of ammonia would be of interest. The latter was determined by variation of the ammonia concentration from 0.1 to 6.4M at constant concentration of (*E*)-cinnamate (**2**) (0.15 mM), and the reaction was monitored by the decrease of absorption at 290 nm. The *Michaelis–Menten* and *Lineweaver–Burk* plots of the measurements are shown in *Fig. 2*. The K_m values at pH 8.8 and 10.0 were determined to be 4.4 and 2.6M, respectively (*Table 2*). The V_{max} values differed only slightly at the above pH values, being 0.89 and 0.81 U/mg, respectively.

Table 2. *Kinetic Constants of Ammonia at Different pH in the Ammonia Addition to (*E*)-Cinnamate* (**2**) *Catalyzed by wt-PAL*

pH	K_m [M]	V_{max} [U/mg]	k_{cat} [s^{-1}]
8.8	4.4	0.89	4.62
10.0	2.6	0.81	4.20

Inhibition Kinetics of N-*Methyl-4-nitro*-L-*phenylalanine* (**6**) *and* N,N-*Dimethyl-4-nitro*-L-*phenylalanine* (**7**·H^+) *with wt-PAL.* Apparent inhibition constants (K_i) for *N*-methylated 4-nitrophenylalanines **6** and **7** were determined at different inhibitor concentrations (0.1–2 mM of **6** or **7**) by varying L-phenylalanine (**1**) concentrations (0.5–4 mM). K_i values were 130 mM for **6** and 8 nM for **7**, as obtained from linearization in the *Dixon* plot (*Table 1*).

The binding properties of the *N*-methylated 4-nitro-L-phenylalanines **6** and **7** were also investigated by using the recently constructed homology model of PAL containing L-phenylalanine (**1**) in a *Friedel–Crafts*-like arrangement in its active site [20]. As shown in *Fig. 3*, both *N*-methylated compounds **6** and **7** fit well into the active site in an almost identical steric arrangement as that of **1**: the nitrated aromatic moieties sit in a relatively hydrophobic pocket close to the exocyclic methylene group of MIO, whereas the *N*-methylated amino moieties can occupy the amino binding pocket facing towards Y110 which proved to be the most important residue in catalysis of PAL. The most pronounced but minor difference between the L-phenylalanine (**1**) binding model [20] and those containing *N*-methylated 4-nitro-L-phenylalanines **6** and **7** is the modified orientation of H396, which seems to contribute to H-bonding of the amino moiety of **1**.

Discussion. – *Synthesis of the* N-*Methylated 4-Nitro*-L-*phenylalanines* **6** *and* **7**. The synthesis of *N*-methyl-4-nitro-L-phenylalanine (**6**) is known [15][16], although without

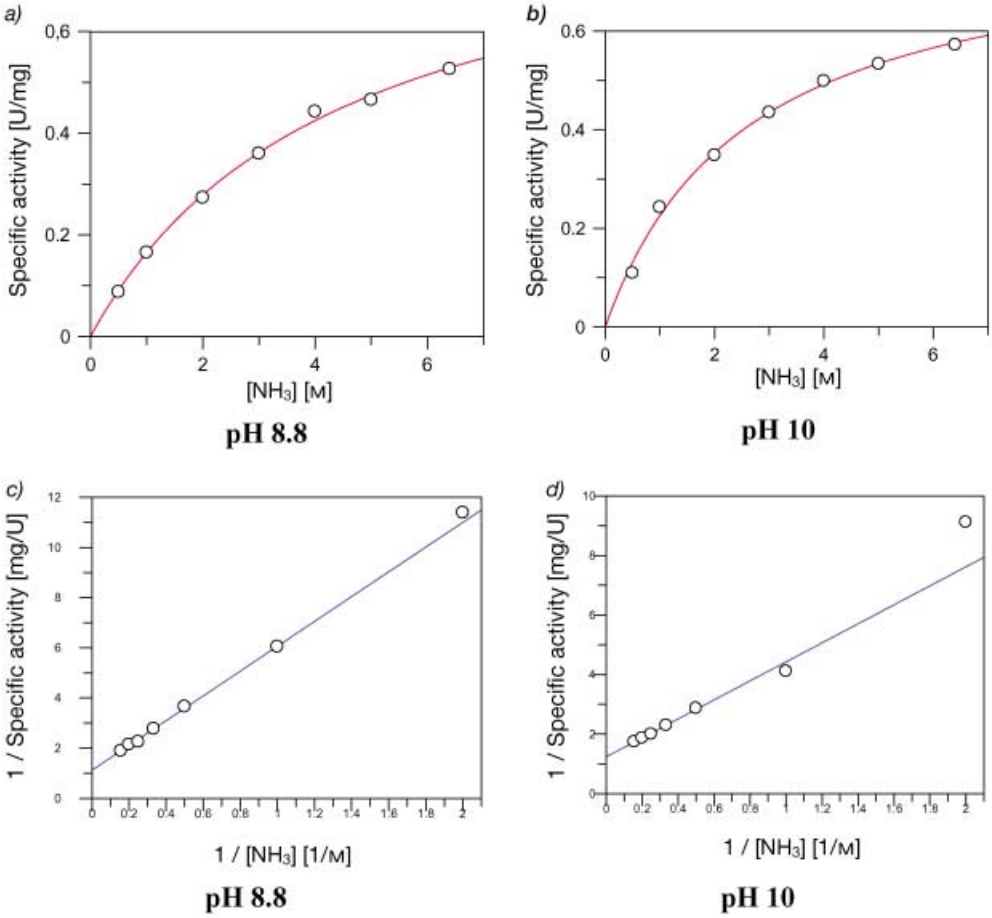


Fig. 2. Michaelis–Menten *and* Lineweaver–Burk *plots for ammonia with wt-PAL* a) c) *at pH 8.8 and* c) d) *at pH 10.0 in the addition reaction of ammonia to* (E)*-cinnamate* (**2**)

details of purification and full spectral data. To the best of our knowledge, *N*,*N*-dimethyl-4-nitro-L-phenylalanine (**7**) has not been prepared yet. Here two synthetic pathways were evaluated, and the HPLC separation of the products as well as full spectroscopic characterization by up-to-date methods are described.

Evaluation of N-*Methyl*-L-*phenylalanine* (**5**) *as Substrate or Inhibitor.* According to the *Hanson* mechanism [8] (*Scheme 2*, *Path I*), the α-amino group of the substrate should attack the prosthetic electrophile of PAL. Therefore, we tested **5** as a substrate. Indeed, **5** was converted into (*E*)-cinnamate (**2**), but its V_{max} value was *ca.* 62 times lower, while its *Michaelis* constant (K_m) 55 times higher than that found for the natural substrate [10]. In the concentration range studied, no significant inhibitory effect was seen as expected from the low binding affinity to PAL.

Noteworthy is the fact that no reverse reaction could be detected with (*E*)-cinnamate (**2**) and molar concentrations of methylamine. Considering the high K_m

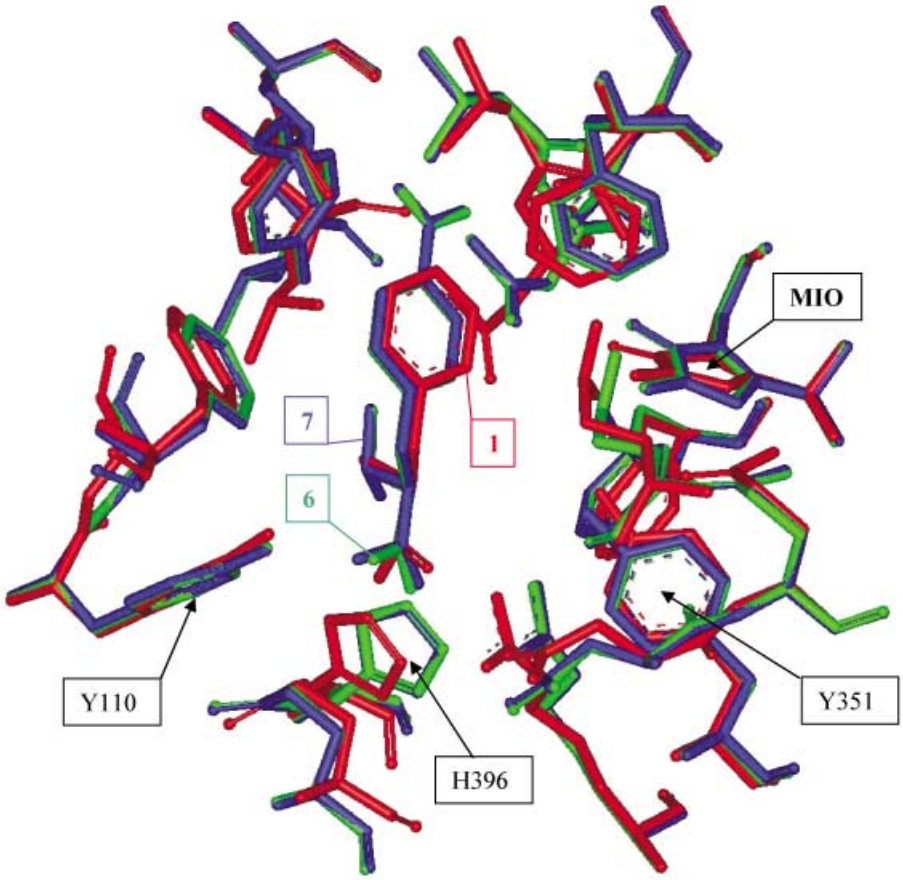


Fig. 3. *Substrate* (**1**: in red) *and inhibitor* (**6**: in green; **7**: in blue) *binding active site models of wt-PAL*

value of ammonia for the reverse reaction, this is understandable. From the large increase of the K_m for **5** as compared to that of L-phenylalanine, one can estimate that the required concentration of methylamine can hardly be realized.

Inhibition of the PAL Reaction by the N-*Methylated 4-Nitro-*L-*phenylalanines* **6** *and* **7**. Interestingly, *N*-methyl-4-nitro-L-phenylalanine (**6**) and *N*,*N*-dimethyl-4-nitro-L-phenylalanine (**7**) were bound stronger ($K_i = 130$ and 8 nM, resp.) than **5** ($K_m =$ 6.6 mM) and behaved as inhibitors but did not serve as substrate. Although this can not fully exclude or favor either the *Hanson* mechanism or the *Friedel–Crafts*-type mechanism, the relatively strong inhibition can be better explained with the latter. Considering the slow but definitive reaction of *N*-methyl-L-phenylalanine (**5**) with wt-PAL, a faster reaction with *N*-methyl-4-nitro-L-phenylalanine (**6**) is expected *via* the *Hanson* route because an electron-withdrawing group at the 4-position of the phenyl ring would enhance the proton abstraction from the **EA′** state (*Scheme 2*, *Path I*) and also stabilize the forming carbocation in the **EC** state.

It was shown that both wt-PAL and the MIO-less S203A-PAL mutant reacted with 4-nitro-L-phenylalanine (**3**) [10] (*Scheme 1*). Whereas the binding ability of **3** was found to be comparable to the substrate L-phenylalanine (**1**) (**3**: $K_m = 0.32$; and **1**: $K_m = 0.17$) [10], it reacts 21 times slower than the natural substrate indicating that the 4-nitro substituent can not fully take over the function of MIO. The slower reaction of **3** can be interpreted in terms of the *Friedel–Crafts*-like mechanism [10] (*Fig. 2*, *Path II*) as follows. Although the electron-withdrawing 4-nitro group acidifies the $H_{Si}-C(\beta)$ bond, it also deactivates the aromatic ring of **3** and, thus, it may not add to the exocyclic methylene of the MIO system. However, the amino function of **3** can be similarly protonated at the active site as that of **1** and, thus, can become a similarly good leaving group. In this context, the inhibitory behavior of the *N*-methylated 4-nitro-L-phenylalanines **6** and **7** can also be rationalized. Molecular modelling indicated that the 4-nitro-bearing aromatic moieties of **6** and **7** fit well into the apolar binding pocket. On the other hand, gas-phase proton affinities of phenylalanine (**1**) and *N*-methyl- and *N*,*N*-dimethylphenylalanine [21] – determined to be 220.3, 223.6, and 224.5 kcal/mol, respectively (relative to the proton affinity of NH_3 (204.0 kcal/mol) – indicate that protonation of the *N*-methylamino and *N*,*N*-dimethylamino moieties are favored over the non-methylated one's. Molecular modelling also imply that the *N*-methylated amino moieties may fit well into the binding pocket. If the *N*-methylamino moieties are strongly anchored to this site (*e.g.*, to Y110) but the aromatic portion does not add to MIO, the enzymic base (presumably Y351) cannot get close enough to $H_{Si}-C(\beta)$. In addition, some effects (*e.g.*, change in interaction with H396) may influence proper protonation and/or positioning of the leaving moiety and, thus, also diminish the elimination.

Why is the PAL Reaction under Physiological Conditions Irreversible? The kinetics of the reverse reaction catalyzed by PAL revealed an unprecedented high K_m value for ammonia, being 4.4M at pH 8.8 and 2.6M at pH 10.0. The dependence on pH indicates that ammonia and not ammonium ion is the active species, since the molarity was calculated from the total amount of both added to the reaction mixture. It is astonishing that PAL works at such a high ammonia concentration. On the other hand, it makes it clear that no reverse reaction occurs under physiological conditions, *e.g.*, at mM concentrations of ammonia.

Although it has been known for some time that enantioselective synthesis of various arylalanines can be achieved by using 6M concentrations of ammonia in the PAL reaction [18][19], the K_m value for ammonia has not yet been determined. To our knowledge, there is only one report on an even higher K_m value for ammonia in the related HAL reaction [22]. However, no experimental details are given in this communication [22].

General Conclusions about the Mechanism of Action of Phenylalanine Ammonia Lyase. – Although the mechanism implying a *Friedel–Crafts*-type attack at the aromatic ring [10] is supported by a number of experiments, there are a few results in the old literature that have been interpreted in favor of the first proposed mechanism implying the addition of the substrate α-amino group to the prosthetic electrophile. The relative stability of the amino–enzyme [12] and the ^{15}N kinetic isotope effects [14], have been discussed in the introduction.

In the paper of *Hermes et al.* [14], which describes the ^{15}N kinetic isotope effects, the problem of the *Hanson* mechanism is formulated as follows: '*For now the facility of enzymes in forming carbanions remains a challenging puzzle*'. The *Friedel–Crafts*-type attack [10] is the adequate solution to this puzzle.

The recent discovery of a MIO-containing tyrosine amino mutase adds a further member to the MIO-enzyme family [23]. Here the ammonia is not released but returned to the β-position of the intermediate (*E*)-4-hydroxycinnamate. Nevertheless, the lyase products were also detected in small amounts [23]. The mechanism of the mutase reaction is formulated [23] as proposed by the Karlsruhe group for PAL. It will be interesting to see which mechanism is used by other tyrosine [24] and phenylalanine [25] α/β aminomutases.

We consider the results presented here in favor of the mechanism involving the *Friedel–Crafts*-like attack of MIO at the aromatic portion of the substrates for more conclusive than the results in older papers [8][14] in favor of the alternative mechanism [8].

We thank Prof. *G. E. Schulz* and *M. Baedeker* (University of Freiburg) for providing us with the new PAL expression system and also Dr. *M. Stieger* (*Hoffmann-La Roche Ltd.*, Basel, Switzerland) for vector pREP4-GroESL carrying the HSP-60 system. The work was supported by the *Deutsche Forschungsgemeinschaft* and the *Fonds der Chemischen Industrie. L. P.* thanks the Hungarian *OTKA Foundation* (T-033112 and M4-041764) for financial support.

Experimental Part

General. L-Phenylalanine (**1**), *N*-methyl-L-phenylalanine (**5**), 4-nitro-L-phenylalanine monohydrate (**3**·H_2O), $NaBH_4$, 36% aq. formaldehyde soln., 40% aq. methylamine soln., and 25% NH_4OH soln. were products of *Fluka* or *Aldrich*. All solvents were HPLC grade from *Merck*. Anal. HPLC: 150 × 3.9-mm, 5-μm *NovaPack-C_{18}* column (*Waters*), *Waters 600E* quaternary gradient pump/*Rheodyne 7725i* injector/*Waters 2996* photodiode-array detector/*Waters Millenium-32* software system or *Hewlett-Packard 1050* gradient pump/autosampler/photodiode-array detector/*Hewlett-Packard ChemStation* for NT software system; t_R in min. Prep. HPLC: 250 × 21.2-mm, 12-μm *Supelcosil-PLC-18* column (*Supelco*), with the same HPLC systems. Melting points: hot-plate method; uncorrected. UV/VIS Spectra: *Cary-3E* spectrometer (*Varian*), λ_{max} in nm, 1-cm quarz cuvettes. NMR Spectra: *Bruker WM-250* or *Bruker DRX-500* spectrometers for ^1H and *Bruker DRX-500* spectrometer operating at 125 MHz for ^{13}C and APT experiments; D_2O solns., unless otherwise stated; δ in ppm, J in Hz. EI-MS and HR-MS: *Finnigan MAT-90* high-resolution instrument; EI (electron ionization) at 70 eV, *m*/*z*(rel. intensity).

N-*Methyl-4-nitro-L-phenylalanine* (**6**). To an ice-cooled and well stirred soln. of *N*-methyl-L-phenylalanine (**5**; 179 mg, 1 mmol) in conc. sulfuric acid (700 μl, 96 %, $d = 1.85$), nitric acid (70 μl, 65%, $d = 1.40$) was added portionwise, and the soln. was stirred at r.t. for 40 min. The resulting yellow mixture was poured into ice-water (7 g) and the pH was set to 6.5 with 25% NH_4OH soln. After cooling to $-5°$, the forming precipitate was filtered off and washed with H_2O (2 × 3 ml). Drying in air (overnight) resulted in apparently dry crude product (165 mg) as light yellow solid. A part (82 mg) of this material was dissolved in 50% aq. formic acid (2 ml) and, after filtration through a 30-kD nitrocellulose membrane, was purified by prep. HPLC (*Supelcosil PLC-18*, isocratic 0.5% HCOOH/H_2O and 10% MeOH/H_2O, flow 5.0 ml/min, λ 280 nm): pure (by HPLC) **6** (32 mg) and a later eluting crude fraction (45 mg). **6**: White solid. M.p. 250–253° (dec.) ([15]: 155° (dec.); [16]: 254–257° (dec.)). Anal. HPLC (0 → 10% *B* in *A* from 0 to 20 min, $A = 0.5\%$ HCOOH/H_2O, $B = 0.5\%$ HCOOH/MeOH flow 1.0 ml/min, λ 275 nm): t_R 10.5, > 98%. UV (0.5% HCOOH/H_2O): 274 ([15]: 270 (8750)). ^1H-NMR (500 MHz, 10% CF_3COOH/D_2O): 2.576 (*s*, Me); 3.205 (*dd*, $J(\alpha,\beta_{Re}) = 7.1$, $J(\beta_{Si},\beta_{Re}) = 14.6$, $H_{Re}-C(\beta)$); 3.293 (*dd*, $J(\alpha,\beta_{Si}) = 5.8$, $J(\beta_{Si},\beta_{Re}) = 14.6$, $H_{Si}-C(\beta)$); 4.149 (*dd*, $J(\alpha,\beta_{Si}) = 5.8$, $J(\alpha,\beta_{Re}) = 7.1$, $H-C(\alpha)$); 7.317 (*d*, $J = 8.7$, 2 H_o), 8.017 (*d*, $J = 8.7$, 2 H_m). ^{13}C-NMR (125 MHz, 10% CF_3COOH/D_2O): 31.75 (MeN); 34.40 ($C(\alpha)$); 61.28 ($C(\beta)$); 124.04 (C_m); 130.36 (C_o); 141.51 (C_p); 147.22 (C_{ipso}), 169.88 (C=O). MS (200°): 42(28), 70(4), 80(100), 89(8), 117(2), 133(16), 137(2), 79(27), 224(1). HR-MS: 224.0788 ($C_{10}H_{12}N_2O_4^+$; calc. 224.0797).

N,N-*Dimethyl-4-nitro-L-phenylalanine* (**7**). To a well stirred soln. of 4-nitro-L-phenylalanine monohydrate (**3**·H_2O; 114 mg, 0.5 mmol) and 35% aq. formaldehyde soln. (185 μl, *ca.* 2 mmol) in 30% MeOH/H_2O (2 ml),

$NaBH_4$ (95 mg, 2.5 mmol) was added portionwise, and the soln. was stirred at r.t. for 3 h. After acidification to pH 2 with conc. HCl soln. and filtration through a 30-kD nitrocellulose membrane, the mixture was separated by prep. HPLC (*Supelcosil PLC-18*, $0 \rightarrow 50\%$ *B* in *A* from 0 to 60 min, $A = 0.5\%$ $HCOOH/H_2O$, $B = 0.5\%$ HCOOH/MeOH, flow 5.0 ml/min, λ 280 nm): pure (by HPLC) **7** (62 mg, 52%). Slightly brownish solid. M.p. 165–170° (dec.). Anal. HPLC ($0 \rightarrow 10\%$ *B* in *A* from 0 to 20 min; $A = 0.5\%$ $HCOOH/H_2O$, $B = 0.5\%$ HCOOH/MeOH, flow 1.0 ml/min, λ 275 nm): t_R 13.7, > 98%. UV (0.5% $HCOOH/H_2O$): 274. ^{1}H-NMR (500 MHz, D_2O): 2.906 (*s*, 2 Me); 3.127 (*dd*, $J(\alpha,\beta_{Re}) = 9.6$, $J(\beta_{Si},\beta_{Re}) = 13.1$, $H_{Re}-C(\beta)$); 3.401 (*dd*, $J(\alpha,\beta_{Si}) = 5.3$, $J(\beta_{Si},\beta_{Re}) = 13.1$, $H_{Si}-C(\beta)$); 3.816 (*dd*, $J(\alpha,\beta_{Si}) = 5.3$, $J(\alpha,\beta_{Re}) = 9.6$, $H-C(\alpha)$); 7.439 (*d*, $J = 8.0$, 2 H_o); 8.120 (*d*, $J = 8.0$, 2 H_m). ^{13}C-NMR (125 MHz, D_2O): 34.46 ($C(\alpha)$); 42.34 (*br*, MeN); 72.19 ($C(\beta)$); 124.33 (C_m); 130.62 (C_o); 143.94 (C_p); 147.17 (C_{ipso}); 171.85 (C=O). MS (140°): 42(9), 56(5), 88(11), 102(100), 147(18), 179(4), 193(29), 238(1). HR-MS: 238.0950 ($C_{11}H_{14}N_2O_4^+$); calc. 238.0954).

Bacterial Strain and Enzyme. Wild-type (wt) PAL was overexpressed in *E. coli* BL21(DE3) cells. The gene coding for phenylalanine ammonia lyase from *Petroselinum crispum* was changed to the codon usage of *E. coli* and cloned in vector pT7-7 followed by a transformation in *E. coli* BL21(DE3) cells containing vector pREP4-GroESL [17]. The *E. coli* BL21 (DE3) cells carrying the plasmids with the gene of wt-PAL was cultured, and wt-PAL was purified as described in detail in [17]. The isolated enzyme was electrophoretically pure as verified by staining with Coomassie Brillant Blue R250, and, therefore, it was possible to measure the turnover numbers (k_{cat}) with the molecular mass M_r of 311.313 for the tetrameric wt-PAL. Determination of protein concentration was carried out according to *Warburg* and *Christian* [26][27], *Murphy* and *Kies* [28], and *Groves et al.* [29]. Bovine serum albumin (BSA) was used as reference protein for the measurements.

Enzyme Kinetics Assays. wt-PAL Activity and inhibition measurements were made spectrophotometrically at 30° in 0.1M *Tris*·HCl (pH 8.8) in 1-cm quartz cuvettes by modification of the method described in [30].

Substrate Activity Assays. wt-PAL (20 µg) was preincubated in buffer (900 µl) at 30° for 5 min. The reaction was started by adding 100 µl of a substrate soln., and absorption data (at 290 nm for reactions yielding (*E*)-cinnamate (**2**); at 340 nm for reactions yielding (*E*)-4-nitrocinnamate (**4**)) were recorded at intervals of 1 min for 5 min, or for less active substrates at intervals of 5 min for 30 min. The assays of *N*-methylated 4-nitro-L-phenylalanines (**6** up to 2 mM; **7** up to 4 mM) with wt-PAL (20 µg) in *Tris*·HCl (pH 8.8; 1 ml) at 30° showed no change in extinction over 30 min.

Substrate Activity of N-*Methyl*-L-*phenylalanine* (**5**) *with wt-PAL.* Formation of (*E*)-cinnamate (**2**) in the reaction of **5** with wt-PAL was determined at 290 nm by recording the extinction at intervals of 1 min for 5 min. Kinetics constants for **5** with wt-PAL were determined from these data (4–40 mM of **5**, 8 data points) according to *Lineweaver* and *Burk* [31] (*Fig. 1*). For the identification of the product of this reaction, **5** (62 mg, 0.35 mmol) was incubated with wt-PAL (400 µg) in 0.1M *Tris*·HCl (pH 8.8) at 37° for 16 h. After concentration of the resulting mixture to 2 ml, the pH was set to 10 by 5M NaOH, the forming precipitate removed by filtration through a 30-kD nitrocellulose membrane, and the filtrate separated by prep. HPLC (*Supelcosil PLC-18*, isocratic 0.5% $HCOOH/H_2O$ and 10% $MeOH/H_2O$ flow 5.0 ml/min, λ 280 nm): t_R 14.1 for **5** (λ_{max} 253 nm) and 30.7 for **2** (λ_{max} 278 nm)). The solvent was evaporated from the product fraction (29–33 min), and the residue (2.5 mg) was redissolved in D_2O and analyzed by ^{1}H-NMR (500 MHz).

*Reaction of (*E*)-Cinnamate* (**2**) *and Methylamine with wt-PAL.* To **2** (5 mM) in methylamine solns. (2 and 4.5M, pH set to 10 with conc. HCl soln.; 1 ml, each), wt-PAL (20 µg in 200 µl 50% glycerol soln.) was added at 30°, and the reaction was monitored at 290 nm. After 1, 8, and 16 h, the mixtures were also analyzed by anal. HPLC (10-µl samples, 0–10% *B* in *A* from 0 to 20 min, $A = 0.5\%$ $HCOOH/H_2O$, $B = 0.5\%$ HCOOH/MeOH, flow 1.0 ml/min, $\lambda = 254$ and 280 nm). No significant decrease of extinction at 290 nm in the UV or peak matching with authentic **5** by HPLC (t_R 6.7 for **5** (λ_{max} 254 nm)) were observed.

*Kinetics Properties of Ammonia at Different pH on Addition of Ammonia to (*E*)-Cinnamate* (**2**) *Catalyzed by wt-PAL.* Consumption of **2** in the reaction of **2** and ammonia with wt-PAL was determined spectrophotometrically at 290 nm by recording the extinction at 1-min intervals for 10 min. Kinetics constants for ammonia with wt-PAL were determined from these data (0.15 mM of **2**, 0.1–6.4M of ammonia, pH 8.8 and 10.0, 7 data points at each pH) according to *Lineweaver* and *Burk* [31] (*Fig. 2*).

Inhibition Kinetics of N-*Methyl-4-nitro*-L-*phenylalanine* (**6**) *and* N,N-*Dimethyl-4-nitro*-L-*phenylalanine* (**7**·H^+) *with wt-PAL.* Apparent inhibition constants (K_i) for *N*-methylated 4-nitrophenylalanines **6** and **7** were determined at different inhibitor concentrations (0.25–1 mM of **6** or **7**) by varying L-phenylalanine (**1**) concentrations (0.5–2 mM; 4–6 data points for each inhibitor concentration): A soln. of **1** (200 µl, 0.5–2 mM calc. to the final 1000-µl volume) was added to a soln. of the inhibitor **6** or **7** (780 µl in 0.1M *Tris*·HCl (pH 8.8), 0.25–1 mM calc. to the final 1000 µl volume), and the resulting mixture was preincubated at 30° for 2 min. The reaction was started by addition of wt-PAL (20 µl of a 50% glycerol soln.) and monitored at 290 nm at intervals

of 1 min for 5 min. Inhibition constants were calculated from the K_i (slope · [L-Phe] · V_{max})/K_m equation (K_i, [L-Phe], K_m [mM]; V_{max} [U/mg]; linear regression in *Dixon* plot; final K_i taken as an average of the particular K_i values of the data sets at different [L-Phe] values).

Inhibitor Fit and Optimization within the Active Site of wt-PAL. Conformations of **6** and **7** resembling best to **1** bound into the wt-PAL model in their zwitterionic state [20] were optimized by PM3 calculations [32], and the L-phenylalanine (**1**) in the substrate-binding wt-PAL model [20] was replaced with these conformations of **6** and **7**. These constructs were used as starting structures of the inhibitor-binding wt-PAL models. The inhibitor-binding models of the active site of wt-PAL were then optimized by MM+ calculations [32] within 10-Å radii around C(α) of the inhibitors **6** and **7**. The outside sphere between 10 and 25 Å of the whole 25-Å-radii portion of the wt-PAL model was kept 'frozen' during the calculations.

REFERENCES

[1] K. R. Hanson, E. A. Havir, *Rec. Adv. Phytochem.* **1978**, *12*, 91.
[2] K. Hahlbrock, D. Scheel, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 347.
[3] I. L. Givot, T. A. Smith, R. H. Abeles, *J. Biol. Chem.* **1969**, *244*, 6341.
[4] R. B. Wickner, *J. Biol. Chem.* **1969**, *244*, 6550.
[5] T. F. Schwede, J. Rétey, G. E. Schulz, *Biochemistry* **1999**, *38*, 5355.
[6] B. Langer, M. Langer, J. Rétey, in 'Advances in Protein Chemistry', Eds. J. P. Klinman and J. E. Dove, Academic Press, New York, 2001, Vol. 58.
[7] L. Poppe, *Curr. Opin. Chem. Biol.* **2001**, *5*, 512.
[8] K. R. Hanson, E. A. Havir, *Arch. Biochem. Biophys.* **1970**, *141*, 1.
[9] M. Langer, A. Pauling, J. Rétey, *Angew. Chem., Int. Ed.* **1995**, *34*, 1464.
[10] B. Schuster, J. Rétey, *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 8433.
[11] D. S. Hodgins, *J. Biol. Chem.* **1971**, *246*, 2977.
[12] A. Peterkofsky, *J. Biol. Chem.* **1962**, *237*, 787.
[13] V. R. Williams, J. M. Hiroms, *Biochim. Biophys. Acta* **1967**, *139*, 214.
[14] J. D. Hermes, P. M. Weiss, W. W. Cleland, *Biochemistry* **1985**, *24*, 2959.
[15] J. Pless, W. Bauer, F. Cardinaux, A. Closse, D. Hauser, R. Huguenin, D. Römer, H.-H. Buescher, R. C. Hill, *Helv. Chim. Acta* **1979**, *62*, 398.
[16] J. E. Rivier, G. Jiang, J. Porter, C. A. Hoeger, A. G. Craig, A. Corrigan, W. Vale, C. L. Rivier, *J. Med. Chem.* **1995**, *38*, 2649.
[17] M. Baedeker, G. E. Schulz, *FEBS Lett.* **1999**, *457*, 57.
[18] S. Yamada, K. Nabe, N. Izuo, K. Nakamichi, I. Chibata, *Appl. Environ. Microbiol.* **1981**, *42*, 773; C. T. Evans, K. Hanna, C. Payne, D. Conrad, M. Misawa, *Enzyme Microb. Technol.* **1987**, *9*, 417; M. Yanaka, D. Ura, A. Takahashi, N. Fukuhara, Jap. Pat. 06-113870, 1994 (*Chem. Abstr.* **1994**, *121*, 155941); W. Liu, US Pat. 5981239, 1999 (*Chem. Abstr.* **1999**, *131*, 321632).
[19] A. Gloge, B. Langer, L. Poppe, J. Rétey, *Arch. Biochem. Biophys.* **1998**, *359*, 1; A. Gloge, J. Zon, Á. Kővári, L. Poppe, J. Rétey, *Chem.–Eur. J.* **2000**, *6*, 3386.
[20] D. Röther, L. Poppe, G. Morlock, S. Viergutz, J. Rétey, *Eur. J. Biochem.* **2002**, *268*, 6011.
[21] S. Campbell, E. M. Marzluff, M. T. Rodgers, J. L. Beauchamp, M. E. Rempe, K. F. Schwinck, D. L. Lichtenberger, *J. Am. Chem. Soc.* **1994**, *116*, 5251.
[22] R. L. Fuchs, J. F. Kane, *J. Bacteriol.* **1985**, *162*, 98.
[23] S. D. Christensen, W. Liu, M. D. Toney, B. Shen, *J. Am. Chem. Soc.* **2003**, *125*, 6062.
[24] P. Spiteller, M. Ruth, F. von Nussbaum, W. Steglich, *Angew. Chem., Int. Ed.* **2000**, *39*, 2754.
[25] K. D. Walker, H. G. Floss, *J. Am. Chem. Soc.* **1998**, *120*, 5333.
[26] O. Warburg, W. Christian, *Biochem. Z.* **1942**, *310*, 384.
[27] E. Layne, *Methods Enzymol.* **1957**, *3*, 447.
[28] J. B. Murphy, M. W. Kies, *Biochim. Biophys. Acta* **1960**, *45*, 382.
[29] W. E. Groves, F. C. Davis, B. H. Sells, *Anal. Biochem.* **1968**, *22*, 195.
[30] A. Zimmermann, K. Hahlbrock, *Arch. Biochem. Biophys.* **1975**, *166*, 54.
[31] H. Lineweaver, D. Burk, *J. Am. Chem. Soc.* **1934**, *56*, 658.
[32] HyperChem 7.0: Hypercube, Inc., 1115 NW 4th Street, Gainesville, FL 32601, USA.

Received August 22, 2003