Inhibition of Diamino Pelargonic Acid Aminotransferase, an Enzyme of the Biotin Biosynthetic Pathway, by Amiclenomycin: A Mechanistic Study

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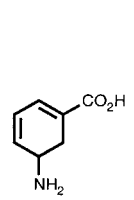
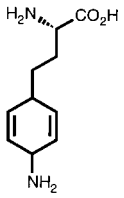
Dedicated to Professor *Duilio Arigoni* on the occasion of his 75th birthday

The mechanism of action of amiclenomycin (**1a**), a naturally occuring inhibitor of diaminopelargonic acid aminotransferase, has been established. The enzyme catalyzes the formation of an aromatic adduct between the inhibitor and pyridoxal-5′-phosphate. The structure of the adduct, determined by mass spectrometry, is in agreement with the reported X-ray crystal structure. Kinetic parameters, characteristic of k_{cat} inhibitors, have been observed, with a K_I value of 2 μM and a k_{inact} value of 0.4 min^{-1}. The irreversibility of the inactivation observed, in spite of the absence of covalent bond between the inhibitor and the protein, reveals the high affinity of the adduct for the active site. Two other *cis*-1-amino-4-substituted-cyclohexa-2,5-dienes, **3a** and **4a**, were also found to efficiently inhibit the enzyme. The *trans*-isomers were either much less potent (**1b**) or inactive (**3b** and **4b**). The aminocyclohexadiene moiety, which is, apparently, responsible for the inhibition, could constitute an original pharmacophore for the design of new herbicides.

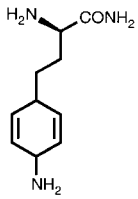
Introduction. – The enzymes of the biotin biosynthetic pathway, which is unique to plants and microorganisms, represent attractive targets for the discovery of new herbicides and eventually new antibiotics. Several inhibitors have been designed and synthesized, but, until now, only one naturally occuring inhibitor, amiclenomycin (=(2*S*)-2-amino-4-(4-aminocyclohexa-2,5-dienyl)butanoic acid; ACM; **1a**), has been discovered (for a recent review, see [1]). It is particularly active, as an antibiotic, against mycobacteria [2]. Although **1a** has been isolated both as the free amino acid and as a residue in di- and tripeptides, there is evidence that the active form is the free amino acid [3].



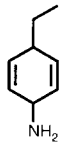
Gabaculine



1a : *cis*
1b : *trans*



3a : *cis*
3b : *trans*

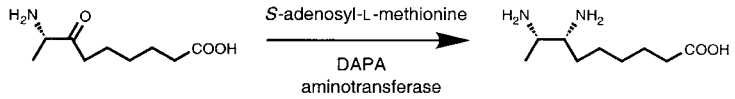


4a : *cis*
4b : *trans*

It has been established by *Hotta et al.* [4] that the target of **1a** is 7,8-diamino-pelargonic acid (=7,8-diaminononanoic acid; DAPA) aminotransferase (*S*-adenosyl-

methionine-8-amino-7-oxononanoate transaminase, EC 2.6.1.62), the second enzyme of the pathway, which catalyzes the reaction shown in *Scheme 1*. Interestingly, it is the only transaminase known that employs *S*-adenosyl-L-methionine (SAM) as an amino-group donor. These authors observed that the enzyme was inactivated during preincubation with ACM. However, they reported that 50% of the activity was restored after extensive dialysis, indicating that no covalent bond was present, at least to this extent, in the enzyme–inhibitor complex. This was tentatively interpreted as evidence for tight binding of amiclenomycin to the enzyme, but no real proof to support this claim was given.

Scheme 1. *The Reaction Catalyzed by DAPA Aminotransferase*



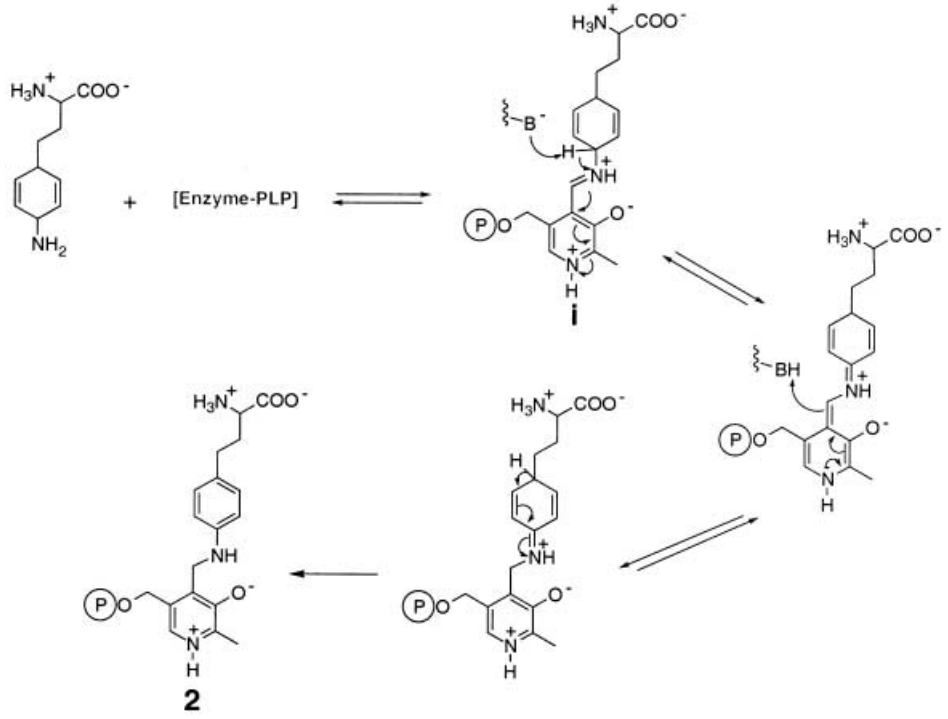
Some time later, the inhibition of GABA-aminotransferase [5] and other pyridoxal-5-phosphate (PLP) enzymes [6] by gabaculine (= 5-aminocyclohexa-1,3-diene-1-carboxylic acid) was described. In that case, the mechanism of inhibition has been well-established [5][7]: the enzyme catalyzes the formation of an aromatic adduct with PLP, whose affinity for the protein is such that the inhibition appears to be irreversible, and the complex can be dissociated only after denaturation of the enzyme.

The structural analogy between **1a** and gabaculine, namely the presence of an aminocyclohexadiene moiety, prompted us to postulate that the mechanisms of action of the two compounds could be similar. *Scheme 2* describes a mechanism that leads to the formation of the aromatic adduct **2**.

We have already reported data concerning the inactivation of DAPA amino-transferase by **1a**, together with the X-ray structure of the inactivated enzyme [8], and the time-dependent inactivation reported by *Hotta et al.* [4] was confirmed. Interestingly, we found that the *cis*-isomer **1a**, corresponding to the natural product, was a much-more-efficient inhibitor than *trans*-**1b**. The X-ray structure reveals, as expected, the presence of an adduct between ACM and PLP that is bound noncovalently to the protein. Coordinates are consistent with the postulated aromatic adduct **2**, although it is not straightforward, at that resolution, to differentiate an aromatic ring from a cyclohexa-2,5-diene (like intermediate **i** in *Scheme 2*), which is expected to be planar [9]. We present here definitive proof of the adduct's structure based on mass spectrometry data. We have also reconsidered the reversibility of the inactivation, which indeed has not been observed in our hands. Furthermore, we have extended this study by examining the inhibiting potency of ACM analogs, (2*R*)-2-amino-4-(4-aminocyclohexa-2,5-dienyl)butanamide (**3**) and 4-ethylcyclohexa-2,5-dieneamine (**4**).

Results. – 1. *Assay of* Escherichia coli *DAPA Aminotransferase.* The enzyme was expressed in *Escherichia coli* BL21(DE3)/pT7bioA and purified as previously described [10]. A bioassay for DAPA aminotransferase, as initially described by *Stoner* and *Eisenberg* [11], allowed detection of DAPA at the pmol level with a bioA(–) strain of *E. coli*, bioA109. We had at our disposal a different bioA(–) strain, *E. coli* C268, which was, unfortunately, not sensitive enough towards DAPA for this

Scheme 2. *Mechanism Postulated for Inhibition of DAPA Aminotransferase by* **1a**



study, the lower limit for detection being 80 pmol under the standard assay conditions. However, we observed that *E. coli* C268 was much more sensitive to dethiobiotin (DTB), since 2 pmol could be detected. Thus, we used this strain for a coupled assay with DTB synthetase, the sensitivity of which was suitable for our study. Attempts to replace this bioassay with chromatographic detection of dansylated DAPA were unsuccessful due to insufficient sensitivity ($\geq$25 pmol) of our fluorimetric equipment.

The commercially available SAM that we used is a 80:20 mixture of (*S*,*S*)- and (*R*,*S*)-diastereoisomers according to its ^{1}H-NMR spectrum [12]. It should be pointed out that the reagent contains traces of DTB and biotin (detected by autobiography) that must be removed with avidin since they interfere with the determination of DAPA in the bioassay.

This work has been carried out with racemic 7-keto-8-aminopelargic acid (=8-amino-7-oxononanoic acid; KAPA) as the substrate. We confirmed that only the (*S*) enantiomer, which has the same configuration of the corresponding C-atom in biotin, is a substrate of the enzyme. We also had at our disposal small amounts of enantiomerically pure (*R*)- and (*S*)-KAPA [13], which we tested separately as substrates. It is clear from *Fig. 1* that (*R*)-KAPA is not a substrate for DAPA aminotransferase. The small activity observed with this sample, which quickly reaches a plateau, may be due to minor contamination with (*S*) enantiomer. Indeed, under the same assay conditions, racemic KAPA indicates only half the concentration of DTB indicated by (*S*)-KAPA.

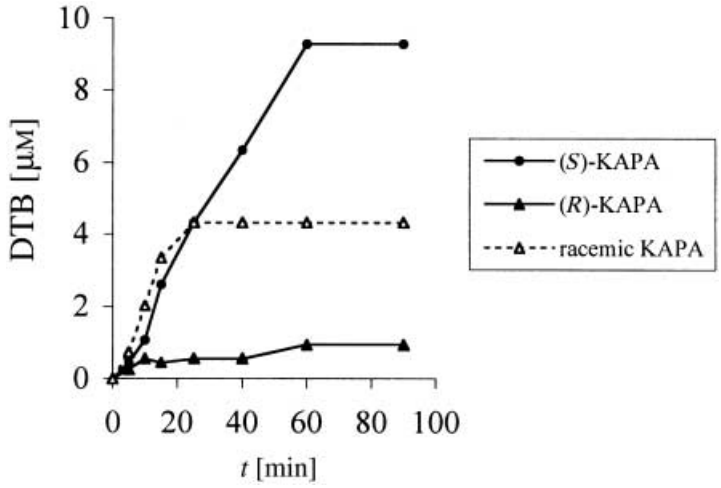


Fig. 1. *Conversion of KAPA to DTB* via *DAPA by DAPA aminotransferase and DTB synthetase.* The reaction was carried out under the standard assay conditions (see *Exper. Part*). The DTB formed from racemic KAPA (△), (*R*)-KAPA (▲), and (*S*)-KAPA (●) was quantified by the standard disc bioassay procedure.

Fig. 1 also shows that, since the initial velocities are comparable, the (*R*) isomer does not inhibit the reaction.

We had already studied the growth response of *Saccharomyces cerevisiae* [13] to (*R*)-, (*S*)-, and (*RS*)-KAPA and found that both enantiomers were active, with the (*S*)-isomer being the more potent. We envisaged that the culture conditions might give rise to racemization: the *in vitro* test with purified enzyme described here shows that racemization probably does occur. Indeed, there has been some confusion in the literature regarding the enantiomeric purity of KAPA samples used in assays. In early reports, KAPA was prepared according to *Suyama* and *Kaneo* [14] from L-alanine: *Eisenberg* and *Star* [15] concluded that the synthesis gave (*S*)-KAPA, whereas the configuration was discussed neither by *Izumi et al.* [16] nor by *Hotta et al.* [4]. We assume that KAPA prepared this way is racemic, since the last step includes refluxing in concentrated HCl, *i.e.*, strongly racemizing conditions (we have shown that refluxing KAPA for 15 min in 6M DCl/D_2O leads to full exchange of the three H-atoms at C(6) and C(8) [17]). Another synthesis of KAPA has been published by *Nudelman et al.* [18], also from L-alanine, and the authors claimed that they obtained optically pure (*S*)-KAPA · HCl (m.p. 133–134°, $[\alpha]_D = -4.46$ ($c = 0.23$, MeOH)). As this product is also obtained after refluxing for 2 to 4 h in 4M HCl, it is certainly racemic product containing an unknown impurity. Indeed, the data we reported [13] for the HCl salt of the optically pure (*S*) enantiomer are quite different (m.p. 108.3–108.4°, $[\alpha]_D = +48.1$ ($c = 1.0$, MeOH)). On the other hand, the melting point for the racemate: 139–140°, is similar to the one reported by *Nudelman et al.* [18]. We, thus, assume that the kinetic parameters reported in the literature have all been calculated for racemic KAPA.

2. *Kinetic Characterization.* The *E. coli* DAPA aminotransferase was first studied by *Stoner* and *Eisenberg* [11], who drew the following main conclusions: the enzyme exhibits 'bi-bi ping-pong' kinetics typical of PLP-dependent aminotransferases, and is

inhibited by high concentrations of KAPA ($K_I = 25$ μM). Careful kinetic analysis by the constant-ratio method gave true k_{cat} (0.13 s^{-1}) and K_M values (0.2 mM for SAM (very likely a mixture of diastereoisomers) and 1.2 μM for KAPA (as the racemate)). Analogous results, although not so complete, were obtained by *Izumi et al.* with the enzyme of *Brevibacterium divaricatum* [16].

Our results are quite similar to those of *Eisenberg: Fig. 2* shows the inhibition by high concentrations of KAPA and the parallel lines characteristic of a ping-pong mechanism. We deduced apparent kinetic parameters from the curve corresponding to the highest SAM concentration (1 mM) of K_M^{app} (KAPA) = 1 μM and $k_{cat} = 0.08$ s^{-1}.

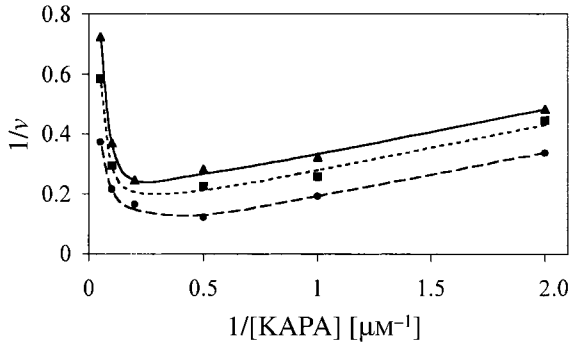


Fig. 2. *Double-reciprocal plot of initial velocity* vs. *KAPA concentration*. Racemic KAPA at 0.5, 1.0, 2.0, 4.0, 10.0, and 20.0 μM and SAM at 0.167 mM (▲), 0.25 mM (△), and 1.0 mM (●) were used in the assay. The DTB formed was quantified by the standard disc bioassay procedure (see *Exper. Part*). Velocity (v) is expressed as 10^{-2} μM/min/mg of enzyme.

Recently, after the present work was completed, *Eliot et al.* [19] reported another kinetic study based on a HPLC assay. They confirmed the inhibition by excess KAPA and estimated $k_{cat} = 0.013$ s^{-1}, K_M (SAM) = 0.15 mM (the K_M of KAPA could not be accurately measured because the HPLC-based assay was not sensitive enough). Thus, all these values are in good agreement, except for the k_{cat} determined by *Eliot et al.* [19].

3. *Inhibition by* **1a** *and Related 1-Aminocyclohexa-2,5-dienes.* When we started this work, natural amiclenomycin was no longer available, and there were also doubts regarding its stereochemistry, which was tentatively postulated to be *trans.* To obtain the compound and also to eliminate the stereochemical ambiguity, we achieved the synthesis of both *cis*- and *trans*-isomers, **1a** and **1b**, respectively [20], and we concluded that the natural product was indeed the *cis*-isomer. In the course of this study, two other pairs of *cis*- and *trans*-aminocyclohexadienes, **3a**,**b** [20] and **4a**,**b** [9], were obtained and investigated as inhibitors.

3.1. *Time-Dependent Inhibition.* All compounds were incubated with the enzyme for various amounts of time, and, after 30-fold dilution, the residual activity was measured (*Fig. 3*). We observed in each case stronger inhibition with the *cis*-isomers under conditions of a *ca.* fourfold excess of the inhibitor, at which concentration almost no inhibition was observed with the *trans*-isomers (the data reported were obtained with a *ca.* 15-fold excess of the *trans*-compounds). The evolution of the UV/VIS spectra during the incubation confirms the conclusions deduced from the activity measurements (see below).

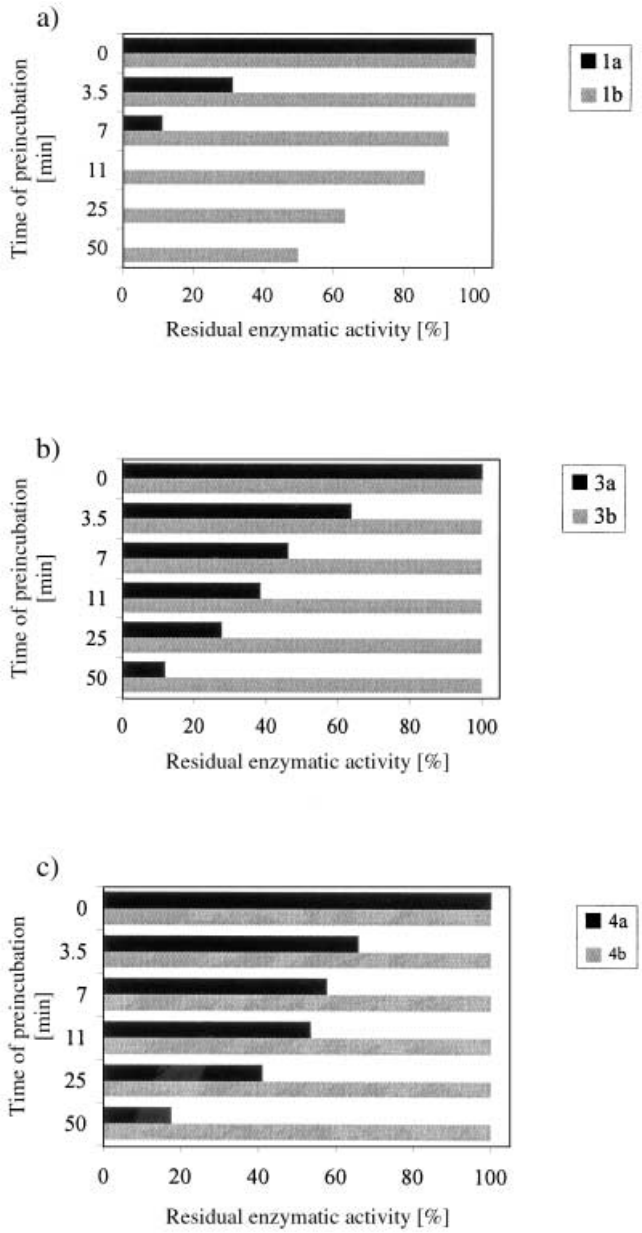


Fig. 3. *Residual activity* [%] *of DAPA aminotransferase after preincubation with aminocyclohexadienes.* DAPA Aminotransferase (13.5 pmol, 0.38 μM) was preincubated with *a*) **1a**,**b**, *b*) **3a**,**b**, or *c*) **4a**,**b**. *cis*-Isomers (■) at 1.4 μM, *trans*-isomers (■) at 5.7 μM. Enzyme activity quantified as DTB formed in the standard assay as described in the *Exper. Part*.

Fig. 3 shows also that the nature of the side chain has only a moderate influence on the inhibition efficiency. The amino amide **3a** differs from **1a** by replacement of the α-amino acid moiety with an α-amino amide and also in the configuration of the α-C-atom (the L-acids were obtained by enzymatic resolution of the racemic amides, and **3a** and **3b** correspond to the residual D-amides). However, the reactivities of the *cis*-compounds **1a** and **3a** are similar, and the *trans*-compounds are both much less active. Furthermore, the inhibition potency of compound **4a**, which contains no side-chain functionality, is similar to that of **3a**. The most-important structural feature influencing inhibition is, thus, the aminocyclohexadiene ring, and the same mechanism is probably operating in all cases.

3.2. *Reversibility of Inactivation*. We also checked the reversibility of inactivation with **1a** as described by *Hotta et al.* [4]. We did not detect any reversibility after dialysis for 72 h of a 100% inactivated enzyme sample, whereas these authors observed about 50% recovery of activity after dialysis for 20 h starting from a 90% inactivated DAPA aminotransferase sample.

3.3. *Inactivation Kinetics*. Having proved the irreversibility of the inactivation, we carried out a more-complete investigation of the kinetics. The results shown in *Fig. 4* clearly demonstrate the time-dependent inhibition characteristic of mechanism-based inhibitors. A double-reciprocal plot of k_{obs} *vs.* ACM concentration (*Inset*) gave $k_{inact} = 0.4\ min^{-1}$ and $K_I = 2\ \mu M$.

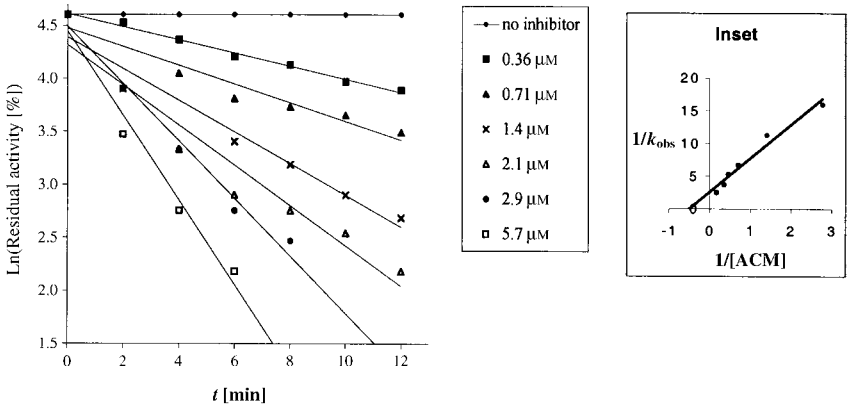


Fig. 4. *Time-dependent inactivation of DAPA aminotransferase by* **1a**. Concentrations of **1a** (0.36, 0.71, 1.4, 2.1, 2.9, and 5.7 μM) as shown. Residual enzyme activity quantified as DTB formed in the standard assay as described in the *Exper. Part*. Kinetic parameters $K_I = 2\ \mu M$ and $k_{inact} = 0.4\ min^{-1}$ were determined (see *Inset*) by reploting $1/k_{obs}$ (min) *vs.* 1/[ACM] [μM^{-1}].

3.4. *Determination of the Structures of the* **1a**-*PLP Adducts*. As we expected, in each case, the formation of aromatic adducts, we synthesized the adducts, as previously described for gabaculine [21], by heating compounds **1**, **3**, and **4** with PLP and analyzed them by UV/VIS spectroscopy and mass spectrometry. The detailed analysis, published elsewhere [22], provides reference data for the identification of the enzyme-bound products. The enzyme was first saturated with PLP and then incubated with a large excess (14 equiv.) of inhibitor. To eliminate, to the extent possible, all compounds that

might invalidate the results, especially the mass spectrometry results, the inactivated enzyme was quickly purified by gel filtration.

3.4.1. *UV/VIS Spectroscopy.* The UV/VIS spectra of enzyme inactivated with the different aminocyclohexadienes under identical conditions are given in *Fig. 5*. The data confirm the relative reactivities of the different inhibitors deduced from *Fig. 3: Fig. 5,a* shows the disappearance of the internal aldimine absorption of the native enzyme at 420 nm and the appearance of a new band at 323 nm, attributed to the pyridoxamine structure of the adduct after treatment of the enzyme with **1a**. When the synthetic adducts were prepared by heating PLP with the different amino-cyclohexadienes, a secondary reaction was always observed, namely the dephosphorylation of PLP leading to the formation of pyridoxal hemiacetal. This compound presented a UV/VIS spectrum that interferes with that of the pyridoxamines [22]. There is no reason for such decomposition of PLP during the enzymatic assay, and, in *Fig. 5*, the absorption at 323 nm reflects the concentrations of pyridoxamines. The fragmentations at *m*/*z* 150 and 168, characteristic of the hemiacetal, are indeed not visible on the mass spectra (*Fig. 6*, *left panels*).

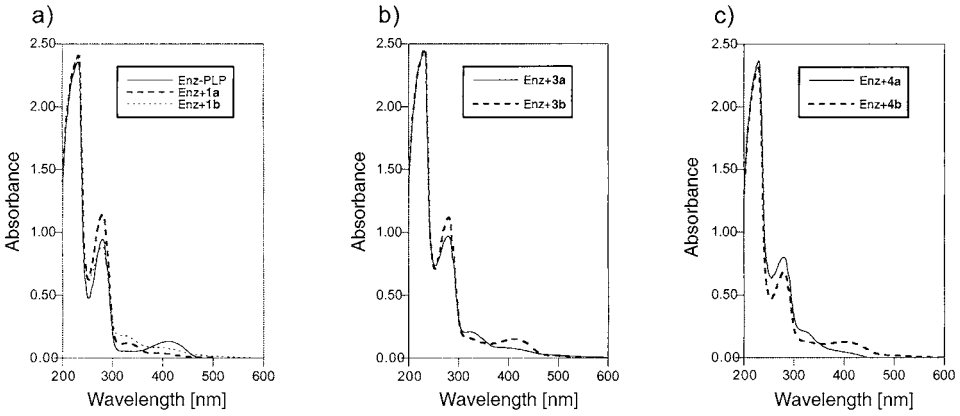


Fig. 5. *UV/VIS Spectra of PLP-bound DAPA aminotransferase after incubation with* a) **1a**,**b**, b) **3a**,**b**, *or* c) **4a**,**b**. DAPA Aminotransferase was preincubated with PLP at room temperature for 15 min. Excess amino-cyclohexadiene (14 equiv. per active site of enzyme) was added. After incubation for 30 min at 37°, the protein was separated from excess inhibitor and PLP. The spectrum of the enzyme in its PLP form is also shown in *a*).

It appears clear that, after incubation with *trans*-**1b** (*Fig. 5,a*), the reaction is not complete, since some aldimine is still present. The time-dependence of the evolution of these spectra has already been reported [8]. *Fig. 5,b* shows also that the amide **3a** is more reactive than **3b**. After incubation with compound **4b** (*Fig. 5,c*), the internal aldimine is conserved, and no clear band is observed at *ca.* 320 nm, in agreement with the absence of inactivation revealed in *Fig. 3,c*.

3.4.2. *Mass Spectrometry.* Enzyme inactivated by each of the six compounds **1**, **3**, and **4** was analyzed by mass spectrometry. The protein fractions isolated by gel filtration were injected directly into an ion-trap spectrometer. In *Fig. 6* (*left panels*), the spectra obtained after treatment with the *cis*-isomers are shown. In all three cases, the quasi-

molecular ions of the aromatic adducts were detected, at *m/z* 426 (**1a**), 425 (**3a**), and 353 (**4a**). The collision-induced-dissociation (CID) product spectra (*Fig. 6*, *right panels*) of these ions displayed fragment ions identical to those observed for the related synthetic samples [22]. When enzyme was incubated with the *trans*-isomers (not shown), the quasi-molecular ions of the adducts were visible as well, except in the case of **4b**. MS-MS Spectra confirmed the structures. Even in the case of **4b**, the spectra issued from the more-sensitive CID technique [22] at *m/z* 353 revealed the presence of the aromatic adduct. Under the conditions used, we could not observe protein. Further experiments are in progress to identify the adduct within the protein and try to evaluate the energy of noncovalent interactions.

Discussion. – *E. coli* DAPA aminotransferase was purified as already described [10] and assayed *via* a sufficiently sensitive coupled assay with DTB synthetase involving microbiological determination of DTB [16].

The substrates used were racemic KAPA and commercial SAM. In this work, we have answered the question of which stereoisomer of KAPA acts as a substrate for DAPA aminotransferase: this point had certainly not been definitively settled before. Careful analysis of the published data brought us to the conclusion that, in previous studies in which this point was not addressed or in which it was claimed that the single stereoisomer (*S*)-KAPA was used, the substrate had, indeed, been racemic KAPA, since the compound had been obtained by a chemical synthesis involving a strongly racemizing step. Having at our disposal for the first time authentic samples of the two enantiomers [13], we could show that only the (*S*)-isomer, in which the corresponding C-atom is configured as in biotin, is a substrate of the pure enzyme and that the (*R*)-enantiomer does not inhibit the reaction. However, we calculated the K_M value for racemic KAPA to allow comparison with the literature data. Accordingly, the concentrations of SAM are given for the commercial sample we used, although we confirmed by NMR [12] that it is a 80:20 mixture of (*S*,*S*) and (*R*,*S*) isomers, whereas *Eliot et al.* [19] recently demonstrated that only the natural (*S*,*S*) diastereoisomer is a substrate of the enzyme. Again, in all the preceeding papers, commercially available SAM was used.

We found the kinetic behavior of the enzyme in good agreement with that reported in the first study of *Stoner* and *Eisenberg* [11]: the enzyme exhibits ping-pong kinetics, classical for aminotransferases, and is inhibited by excess KAPA. *Stoner* and *Eisenberg* determined true kinetic parameters by means of the constant-ratio method. We did not repeat this work, but the values we deduced from the curve for the highest concentration of SAM (1 mM) were very similar to those found by *Eisenberg* at saturating concentrations, respectively 1 μM and 1.2 μM for the K_M of KAPA, and 0.08 s^{-1} and 0.13 s^{-1} for k_{cat}. In another recent investigation of this enzyme, *Eliot et al.* [19] determined the K_M of SAM, again in accordance with that found by *Eisenberg*, respectively 0.15 mM and 0.2 mM, but reported a k_{cat} value of 0.013 s^{-1}, not at all in line with *Eisenberg*'s results.

Time-dependent inhibition of DAPA aminotransferase by **1a**, already reported by *Hotta et al.* [4], was confirmed as shown in *Fig. 3*,*a*, however, we did not observe reversibility of the inactivation described by these authors, in spite of a more-extensive dialysis step. This absence of reversibility allowed us to carry out a more-careful kinetic

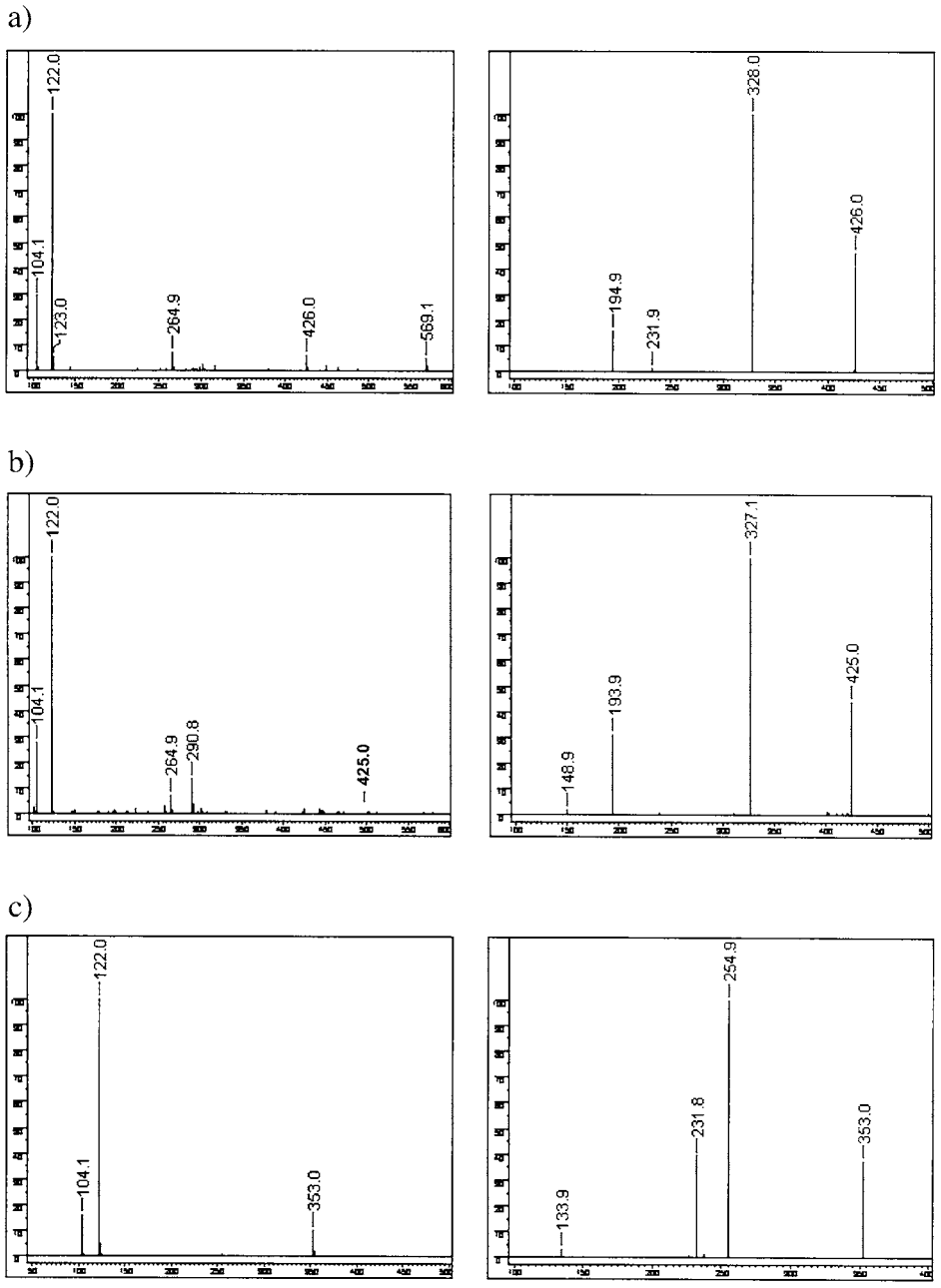


Fig. 6. *ESI-MS* (*left panels*) *of the protein fraction after gel-filtration chromatography of DAPA amino-transferase inactivated with* a) **1a**, b) **3a**, *or* c) **4a**. Samples eluted from gel-filtration columns were injected in the spectrometer without any dilution. CID-MS (*right panels*) of the *m*/*z* 426 (**1a**, *a*), 425 (**3a**, *b*), and 353 (**4a**, *c*) ions from ESI-MS.

analysis, described in *Fig. 4*. This kinetic picture is clearly characteristic of k_{cat} inhibitors, with a K_I value of 2 μM and k_{inact} value of 0.4 min^{-1}.

Hotta et al. [4] also previously reported that inactivation is slowed by the substrate KAPA, and they suggested that ACM occupies the KAPA binding site, and that it is a slow, tight-binding inhibitor. But, at that time, no experimental proof was available. Shortly thereafter, a natural inhibitor of GABA aminotransferase, gabaculine, was discovered [5]. Its mechanism of action, which is very well-established [5][7], is related to its aminocyclohexadienic structure: it forms first a *Schiff* base with PLP, which aromatizes easily by abstraction of the acidic proton adjacent to the conjugated diene. The aromatic adduct has strong affinity for the enzyme, and the complex dissociates only after denaturation of the protein.

Due to the structural analogy between gabaculine and ACM, another amino-cyclohexadiene, it was tempting to postulate for ACM a similar mechanism of action, which is depicted on *Scheme 2*. We could demonstrate that the expected aromatic adduct **2** was, indeed, formed when the enzyme was incubated with **1a**. When the inactivated enzyme was analyzed by ESI-MS, the complex dissociated and the aromatic adduct was clearly detected. A reference compound synthesized by heating **1a** with PLP was also studied by mass spectrometry [22]. The adduct issued from the inactivated enzyme displays exactly the same fragmentations as the reference compound on the CID-product spectrum of the quasi-molecular peak. Furthermore, the X-ray structure of the inactivated enzyme [8] clearly shows the presence in the active site of an adduct between ACM and PLP, with a planar six-membered ring in the ACM moiety, consistent with the aromatic adduct identified by mass spectrometry. The structure [8] reveals that, as expected, the ACM moiety of **2** (*Scheme 2*) binds in a similar fashion to the binding of the substrate KAPA. Its hydrophobic carbon skeleton superposes well on the aliphatic C chain of KAPA, and the terminal carboxy group interacts similarly with Arg-391. The high stability of the enzyme-aromatic-adduct complex can be rationalized by the presence of this salt bridge, several H-bonds, and hydrophobic and π-π stacking interactions with the residues of the substrate-binding cleft (Tyr-17, Trp-52, Trp-53, Tyr-144, Ala-217, and Phe-393). Interestingly, although the conformations of the adducts in the two subunits of the homodimeric KAPA synthase are somewhat different, the noncovalent interactions are preserved. These structural data have been discussed in detail elsewhere [8].

We have observed that the *trans*-isomer **1b** also inhibits the enzyme, although with much lower efficiency, as shown in *Figs. 3*,*a* and *5*,*a*. The same aromatic adduct has, however, been detected in the inactivated enzyme by mass spectrometry (data not shown) and X-ray crystallography [8], but, consistent with the kinetic and UV/VIS data, in much lower amounts. In the MS, the molecular peak was hardly detectable, but the CID-product spectrum of the *m*/*z* 426 ions revealed the same fragments as those observed after inactivation with **1a**. When the crystals of enzyme were soaked with **1b** [8], the aromatic adduct was detected in the active site of one subunit, but the occupancy was low, and it was almost negligible in the second one. Differences in kinetic behavior between the two isomers have been discussed on the basis of the 3D structure: according to the proposed mechanism (*Scheme 2*), the first step of the formation of **2** is the transimination by ACM of the internal aldimine between PLP and Lys-274. Tautomerisation of the *Schiff* base involves abstraction of H−C(4) of ACM,

most likely by Lys-274, the only base in close proximity (*Fig. 7*). Completion of aromatization requires the removal of H–C(1). Although there is no obvious proximal basic residue positioned to abstract H–C(1), it may be that this proton is sufficiently acidic to be abstracted by one of the H_2O molecules visible in the structure. The X-ray structure is, of course, that of the complex obtained after reaction, which is why the two isomers **1a** and **1b** have been modeled in the active site. Whereas **1a** can be positioned in an orientation suitable for the formation of the adduct, this is not the case for **1b** (for a more-complete discussion, see [8]).

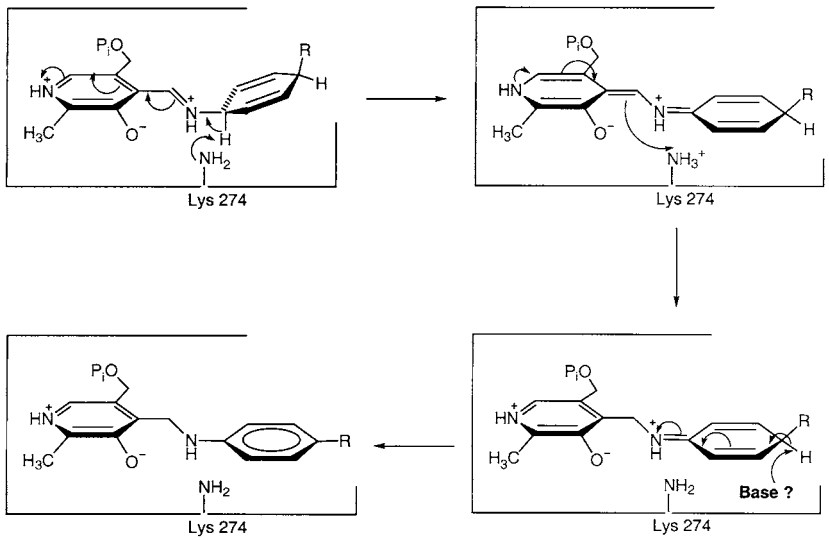


Fig. 7. *Proposed mechanism for formation of the covalent aromatic adduct* **2** *between ACM and PLP in the active site of DAPA aminotransferase.*

The other aminocyclohexadienes, **3a** and **4a**, also inactivate the enzyme, but more slowly, also leading to the corresponding aromatic adducts. Again, the *cis*-isomers are more efficient than the *trans*-isomers. Although no detailed kinetic analysis was carried out with these compounds, it seems reasonable to assume that the modification of the side chain, *i.e.*, the loss of interactions due to the L-α-amino acid, increased the K_M value and possibly modified the geometry of the complex, making one or both proton abstractions less favorable and, thus, decreasing the rate of formation of the adduct. Whether it is also conceivable that the complexes lacking the favorable interactions of the side chain are less stable and that the inactivation is, in that case, reversible has not yet been investigated.

Conclusions. – We have demonstrated the mechanism of action of amiclenomycin, a natural inhibitor of DAPA aminotransferase, which appears similar to that of gabaculine, an inhibitor of several other aminotransferases. The time-dependent inactivation of the enzyme is not due to covalent binding of the inhibitor to the protein, but to the formation of an aromatic adduct with PLP that binds very tightly to the enzyme, making the inactivation quasi-irreversible.

The L-α-amino acid moiety of ACM is not absolutely required, since other aminocyclohexadienes having a α-D-amino amide or without any functionality in the side chain, although less potent, also inactivate the enzyme. In all cases, the rate of inactivation is higher with the *cis*- than with the *trans*-isomers, behavior that can be rationalized on the basis of the X-ray structure.

The specificity of ACM towards DAPA aminotransferase *vs.* other aminotransferases should be studied.

Inhibitors of biotin biosynthesis generally present antibiotic properties, and **1a** was, indeed, isolated in the course of antibiotic screening, but they are often reversed in complex media or *in vivo* by traces of biotin. On the other hand, their herbicidal properties, which appear to be more promising [23], of **1a** remain to be tested. Should aminocyclohexadienes prove effective as herbicides, production by fermentation should be considered, the synthetic approach being probably too complex for production of an herbicide. However, the moderate influence of the side chain on the inhibition potency allows us to postulate that simple and more-accessible aminocyclohexadienes such as **4a** could also be sufficiently effective.

Experimental Part

General. SAM was purchased from *Sigma*. (*R*)- and (*S*)-KAPA were obtained as described in [13], *rac*-KAPA was obtained as described in [17]. UV/VIS Spectra: *Uvikon 930* apparatus. ESI- (pos. mode) and CID-MS: *Bruker Esquire-3000* ion-trap mass spectrometer under low-energy conditions.

Production and Purification of DAPA Aminotransferase and DTB Synthetase. DAPA aminotransferase was overexpressed in *E. coli* BL21(DE3)/pT7bioA. Purification of the enzyme was according to the procedures outlined in [10]. DTB Synthetase was obtained by overexpression in *E. coli* BL21(DE3)/pbioCD as described in [24]. The enzyme was then partially purified in one step on a *Q-Sepharose* column. The isolated sample did not show any DAPA aminotransferase activity.

Preparation of E. coli *C268 bioA(−) Suspension*. LB Medium (100 ml) was inoculated with *E. coli* C268 and incubated overnight at 37°. Cells were centrifuged in 10 ml aliquots at 2000 rpm for 10 min; the pellet was washed twice with 2 ml modified *Vogel–Bonner* agar-free medium and suspended in 1 ml of the same medium with 1 ml of 50% glycerol. Aliquots (2 ml) were stored at −80°, ready to use.

Standard Disc Bioassay. DTB Concentrations were quantified in a microbiological assay with modified *Vogel–Bonner* minimal medium. A sterile soln. (200 ml) containing anh. K_2HPO_4 (2 g), citric acid monohydrate (0.4 g), anh. $MgSO_4$ (20 mg), NH_4Cl (172 mg), $Na_2HPO_4 \cdot 12\ H_2O$ (1.16 g), thiamine HCl (4 mg), casamino acids (0.4 g), agar (3 g) was supplemented with 4 ml of a 250 g l^{-1} glucose soln., 0.5 ml of a 35 g ml^{-1} triphenyltetrazolium chloride soln. and a suspension of *E. coli* C268 bioA(−) strain (2 ml). The addition of triphenyltetrazolium chloride allowed easier measurement of the growth diameter. The medium, poured on a plate (20 × 20 cm), was dried for 2 h and samples pipetted on 6-mm diameter paper discs beside a range of authentic DTB samples, from 2–80 pmol. After overnight incubation at 37°, pink growth discs appeared. Measurement of the diameters allowed quantification of DTB by comparison with those of the authentic DTB samples.

Purification of SAM. A sol. of the *p*-toluenesulfonate salt of SAM (8 mg) in H_2O (163 µl) was incubated for 15 min at 4° in the presence of 1.5 units of avidin (1 unit binds, 1 µg of biotin). Avidin was removed by ultrafiltration (*Microcon YM-30*, 13000 g, 10 min) at 4°. Aliquots of the filtrate were diluted with H_2O to 50 mM (based on absorbance measured at 260 nm, $\varepsilon = 15400\ cm^{-1}\ M^{-1}$).

Standard Enzymatic Assay. The assay was carried out in a 100 µl final volume containing (unless otherwise stated) 20 mM potassium phosphate buffer pH 8.0, 10 mM ATP, 50 mM $NaHCO_3$, 10 mM $MgCl_2$, DTB synthetase (390 ng), 0.1 mM PLP, various concentrations of *rac*-KAPA, purified SAM, and DAPA aminotransferase. The reaction was initiated by adding enzyme after preincubation of the reagent mixture for 1 min at 37°. Addition of 25 µl of 15% (*w*/*v*) CCl_3COOH soln. stopped the reaction and DTB was quantified by the standard disc bioassay procedure.

Evaluation of the Reversibility of DAPA Aminotransferase Inactivation by **1a**. A soln. of DAPA aminotransferase (0.43 μg (9.0 pmol), 35 μl final volume) in 50 mM *Tris* buffer, pH 8.0, containing 10 mM 2-(sulfanyl)ethanol and 1.4 μM amiclenomycin, was incubated at 37° for 30 min, or until inactivation of the enzyme was complete. The residual activity in aliquots of 3.5 μl (*i.e.*, 0.043 μg enzyme) was tested with the standard assay. Inactivated enzyme was then diluted to 300 μl with 10 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM PLP and dialyzed against the same buffer. Aliquots were withdrawn at time intervals to test the activity in the standard assay. No recovered activity was detected even after 72-h dialysis. Control experiments (enzyme incubated without inhibitor) carried out under the same conditions showed a constant activity over 72 h. All experiments were conducted in duplicate.

Inactivation of DAPA Aminotransferase by **1**, **3**, *and* **4**. A soln. of DAPA aminotransferase (0.64 μg, 13.5 pmol) in 50 mM Tris buffer pH 8.0 containing 10 mM 2-sulfanylethanol and aminocyclohexadiene (1.4 μM *cis*-isomer **1a**, **3a**, or **4a**; 5.7 μM *trans*-isomer **1b**, **3b**, or **4b**) was preincubated at 37°. Residual activity was tested at different times by adding 3.5 μl samples of the reaction into the standard assay (100 μl total volume, resulting in a 30-fold dilution of the preincubation mixture). The DTB formed was then quantified. A control experiment without inhibitor carried out under the same conditions showed constant activity.

Kinetics of DAPA Aminotransferase Inactivation by **1a**. Inactivation experiments were conducted as described above. The aminotransferase (1.7 μg) was preincubated with inhibitor at 0.36, 0.71, 1.4, 2.1, 2.9, and 5.7 μM.

Preparation of DAPA Aminotransferase Samples for UV/VIS and MS Analysis. A soln. (270 μl) of DAPA aminotransferase (28.6 nmol, 1.35 μg) in 50 mM Tris buffer pH 8.0 containing 10 mM D,L-dithiothreitol (DTT) and 0.37 mM PLP was incubated for 15 min at r.t. The aminocyclohexadiene was added (400 nmol, 8 μl), and the mixture was incubated at 37° for 30 min. Excess inhibitor and PLP were removed by gel-filtration chromatography on a PD10 column eluted with 1 mM *Tris* buffer pH 7.5. The final enzyme concentration was 0.8 mg ml^{-1}. UV/VIS and MS of the protein were recorded on these samples without any dilution.

MS: Enzyme samples were introduced into the ESI source by infusion with a syringe pump (120 μl/h) without any org. solvent, unlike the previous method described in [22] used to identify authentic adducts. Addition of an org. solvent, which generally favors the formation of ions, was omitted here since it induced precipitation of the protein. Conditions for ESI source were as follows: source temperature 250°; drying gas (N_2) flow rate 7 ml/min; nebulizing gas 7 psi; capillary voltage 4000 V; capillary offset 70 V; skimmer 27 V; octopole RF 120 Vpp. For MS-MS experiments, the quasi-molecular ions displayed in the ESI-MS were selected by means of the broad-band isolation method. These selected ions, in a second step, could be excited by collision with He in the trap cell and then decomposed. Resonant-excitation conditions were chosen (0.77 V, isolation width 1 Th). All mass spectra were averages of 2 min accumulation.

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