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New tryptophanase inhibitors: Towards prevention of bacterial biofilm formation

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Abstract

Tryptophanase (tryptophan indole-lyase, Tnase, EC 4.1.99.1), a bacterial enzyme with no counterpart in eukaryotic cells, produces from L-tryptophan pyruvate, ammonia and indole. It was recently suggested that indole signaling plays an important role in the stable maintenance of multicopy plasmids. In addition, Tnase was shown to be capable of binding Rcd, a short RNA molecule involved in resolution of plasmid multimers. Binding of Rcd increases the affinity of Tnase for tryptophan, and it was proposed that indole is involved in bacteria multiplication and biofilm formation. Biofilm-associated bacteria may cause serious infections, and biofilm contamination of equipment and food, may result in expensive consequences. Thus, optimal and specific factors that interact with Tnase can be used as a tool to study the role of this multifunctional enzyme as well as antibacterial agents that may affect biofilm formation. Most known quasi-substrates inhibit Tnase at the mM range. In the present work, the mode of Tnase inhibition by the following compounds and the corresponding Ki values were: S-phenylbenzoquinone-L-tryptophan, uncompetitively, 101 μM; α-amino-2-(9,10-anthraquinone)-propanoic acid, non-competitively, 174 μM; L-tryptophane-ethylester, competitively, 52 μM; N-acetyl-L-tryptophan, noncompetitively, 48 μM. S-phenylbenzoquinone-L-tryptophan and α-amino-2-(9,10-anthraquinone)-propanoic acid were newly synthesized.

Keywords: *L-tryptophan, indole, quasi-substrates, inhibition*

Introduction

Tryptophanase (tryptophan indole-lyase, Tnase, EC 4.1.99.1) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme. It is a multifunctional enzyme that catalyses α,β-elimination and β-replacement reactions of L-tryptophan and a number of other β-substituted amino acids. The quaternary structure of Tnase consists of 4 identical 52 kDa subunits; each binds one molecule of PLP *via* an aldimine bond with a lysine residue. Its holo (active) form exhibits a pH-dependent absorption and CD spectra with maxima at 420 and 337 nm typical for covalently bound PLP [1–3]. In addition, it requires certain monovalent cations (K^+, NH_4^+, TI^+) for activity and for tight PLP binding [4–6]. The enzyme undergoes a reversible inactivation followed by dissociation into dimers or monomers, depending on the bacteria species, after incubation for several hours at 2°C [7]. The enzymatic reaction with tryptophan is shown in Scheme 1.

A class of biofilm regulatory molecules known as "cell-to-cell signaling molecules" or CCSMs was identified. Among them are N-acylhomoserine lactones, peptides, and quinolones [9]. Recently it was suggested that indole is a CCSM at the site of infection both in *Escherichia coli* and *Vibrio cholera*, [10–17]. The prokaryotic enzyme Tnase is widely distributed in pathogenic Gram-negative bacteria, including *E. coli*, *Shigella flexeneri*, *V. cholera*, *Haemophilus influenzae*, and *Clostridium tetani*, with no counterpart in eukaryotic cells. *Escherichia coli* are a very efficient colonizer of a wide variety of surfaces. Recent experiments demonstrated that the *tnaA* gene, which

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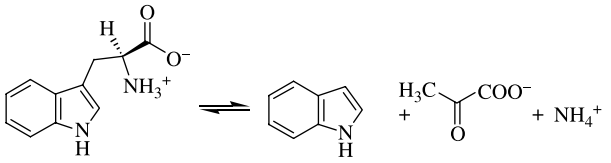


Scheme 1. The enzymatic reaction of Tnase with tryptophan results in pyruvate, ammonia and indole production [8].

encodes Tnase, is important for biofilm formation in *E. coli* and in several other species of pathogenic bacteria [10–17]. It was reported that indole can restore biofilm phenotype in *tnaA* mutants [18]. On the other hand, it was recently reported that indole signaling plays an important role in the stable maintenance of multicopy plasmids. In addition, Tnase was shown to be capable of binding Rcd, a short RNA molecule involved in resolution of plasmid multimers. Binding of Rcd increases the affinity of Tnase for tryptophan, suggesting that Tnase serves as a multifunctional enzyme [19].

The most efficient inhibitor of Tnase known hitherto, oxindolyl-L-alanine, (Ki = 5μM) inhibits both the formation of indole and the creation of biofilm [20,11]. Other quasi-substrates of Tnase such as alanine and phenylalanine inhibit the enzyme in the mM concentrations range [21].

We now report on our finding of four inhibitors of Tnase, which inhibit at sub-milli to micro molar concentrations. The compounds were chosen based on mechanistic rationales, i.e., N-acetyl-L-tryptophan and S-phenyl benzoquinone-L-tryptophan, solubility, i.e., L-tryptophan-ethylester, and a combination of known inhibitory characteristics with active site targeting, i.e., α-amino-2-(9,10-anthraquinone)-propanoic acid. Here we describe the synthesis of two new compounds S-phenylbenzoquinone-L-tryptophan and α-amino-2-(9,10-anthraquinone)-propanoic acid.

Experimental

Materials

N-acetyl-L-tryptophan and L-tryptophan-ethylester were purchased from Sigma-Aldrich.

Protein expression and purification

E. coli wild-type (*wt*) Tnase was expressed and purified as described before [22].

Synthesis of inhibitors

α-Amino-9,10-dihydro-9,10-dioxo-2-anthracenepropanoic acid. The L isomer of this compound was prepared in 3 stages starting from 2-anthracenepropanoic acid according to the method of Matsubara et al. [23]: mp 230-231°C (lit mp 229–230°C).

^{1}H-NMR (DMSO-d_6): δ = 8.23 ppm (m, 2H, aromatic CH), δ = 8.14 ppm (d, J = 7.25 Hz, 1H, aromatic CH) δ = 7.95 ppm (d, J = 1.2 Hz, 1H, CH aromatic), δ = 7.73 ppm (m, 2H, aromatic CH), δ = 7.45 ppm (dd, J = 7.5 Hz, 1.2 Hz, 1H, aromatic CH), δ = 6.65 ppm (2H, bs, NH_2,), δ = 4.2 ppm (m, 1H, chiral CH), δ = 3.4 ppm (m, 2H, CH_2).

MS. (FAB) m/z (%)296 (40)MH^+223(27).

S-phenylbenzoquinone-L-tryptophan. To a hot stirring solution of 2-phenylthio-1,4-benzoquinone (1.08 gr, 5 mmol) in 95% ethanol (50 mL) an aqueous solution (20 mL) of tryptophan (1.02 gr, 5 mmol) was added drop wise [24]. The mixture was left to stir at room temperature for 24 h. Within this time, the obtained hydroquinonic product was re-oxidized to the quinonic end product. The solvents were evaporated *in vacuo* and the crude product was purified on a silica gel column using a gradient of dichloromethane/ethanol. Dark red micro-crystals were obtained (1.67 gr, 80% yield). Mp 223-225°C (dec).

In the HR-MS (CI in CH_4) the compound loses HCOOH, thus we obtain: *m/z* (M^+-HCOOH) 372.0934, calc. for $C_{22}H_{16}O_2N_2S$, 372.0932.

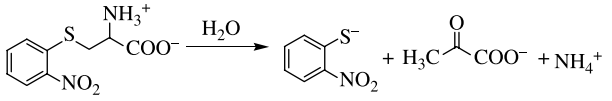
^{1}H-NMR (DMSO-d_6): δ = 10.3 ppm (s, 1H, COOH), δ = 7.5 ppm (s, 5H, aromatic CH), δ = 7.48 ppm (d, J = 8 Hz, 1H, aromatic CH), δ = 7.2-7.3 ppm (d, J = 8 Hz aromatic, 1H, CH), δ = 7.0 ppm (s, 1H, indolic CH), δ = 7.0 ppm (t, J = 8 Hz, 1H, aromatic CH), δ = 6.9-7.0 ppm (t, J = 8 Hz, 1H, aromatic CH) δ = 5.4 ppm (s, 1H, quinonic CH), δ = 5.2-5.3 ppm (s, 1H, quinonic CH), δ = 3.3 ppm (m, 1H, chiral CH), δ = 2.8 ppm (m, 2H, CH_2).

IR(KBR): OH (carboxylic) (3346 cm^{-1}), CH aromatic (3025, 2823 cm^{-1}), C=O (carboxylic and quinonic) (1651 cm^{-1}, 1598 cm^{-1}, 1553 cm^{-1}).

Kinetic measurements

The activity of Tnase was determined spectrophotometrically with the use of S-(*o*-nitrophenyl)-L-cysteine (SOPC) as a substrate [25]. In the presence of Tnase SOPC undergoes *α,β*-elimination reaction as shown bellow in Scheme 2.

SOPC has a maximum absorption band at 370 nm with a molar absorption coefficient ε = 2700M^{-1} cm^{-1} and the product *o*-nitrothiophenolate has a maximum absorption band at 412 nm with a molar absorption coefficient ε = 1860 M^{-1} cm^{-1}. All analyses were done



Scheme 2. The *α,β*-elimination reaction of SOPC results in the production of *o*-nitrothiophenolate, pyruvate and ammonia.

in potassium phosphate buffer (50 mM, pH = 7.8) (KPB) using 0.06-0.6 mM SOPC. The reaction mixture and the enzyme solution are kept at 25°C for ten minutes. Then, 1 mL of the reaction mixture is placed in a cuvette, and 10 μL of the enzyme solution (0.3-0.5 mg protein/mL, specific activity 40-47 $\mu mol \times min^{-1} mg^{-1}$) is added. The reaction is followed for 1 min. The reaction is carried out at [S] ≫ Km (Km = 0.06 mM) and therefore, V = Vmax, thus, the velocity of the reaction is not dependent on the concentration of the substrate (zero order kinetics). One activity unit of Tnase is defined as the amount of enzyme needed to break down 1 μmol of the substrate SOPC to its products in one minute at 25°C (1 unit = 1 $\mu mol \times min^{-1}$). The specific activity of Tnase is defined according to the following equation:

$$\text{Specific activity} = \Delta A_{370\,nm} \times V_t / 1.86 \times V_e \times C$$

where, $\Delta A_{370\,nm}$ is the change in absorption during a period of 1 minute (O.D. 0.2-0.5); V_t – the total volume of the reaction mixture in mL; V_e – the volume of protein taken for analysis, in mL, C - enzyme concentration, and the difference between the molar extinction coefficients of SOPC and its degradation products at pH = 7.8 ($1860\,M^{-1} \times cm^{-1} = 1.86\ mL \times \mu mole^{-1} \times cm^{-1}$) [25].

The measurements were done with an 8453A Hewlett Packard spectrophotometer connected to a UC-F-10 Julabo thermostated bath (± 0.1°C).

All kinetic experiments were repeated three to four times (results show the average; standard deviation amounted to less than 5%).

Results

A Michaelis-Menten fit for S-phenylbenzoquinone-L-tryptophan calculated with non-linear regression, as a function of SOPC concentration is shown in Figure 1. All calculations were done with Calidagraph (version 4). Table I summarizes the values calculated with non-linear regression for the inhibitors N-acetyl-L-tryptophan, S-phenylbenzoquinone-L-tryptophan, tryptophan-L-ethylester and α-Amino-2-(9,10-anthraquinone)-propanoic acid; R (correlation factor) was found to be >0.99 for all. Table I presents the suggested mode of Tnase inhibition by each inhibitor and the corresponding Ki values. All four inhibitors are shown to be reversible, whether competitive, non-competitive, or uncompetitive (Figures 1–4). Lineweaver-Burk plots of the specific activity as a function of SOPC concentration resulted in similar inhibition characteristics.

Discussion

Several crystal structures of Tnase were reported. The structure of *E. coli* apo enzyme was solved in two crystal forms [26,27] and the structure of a highly homologous

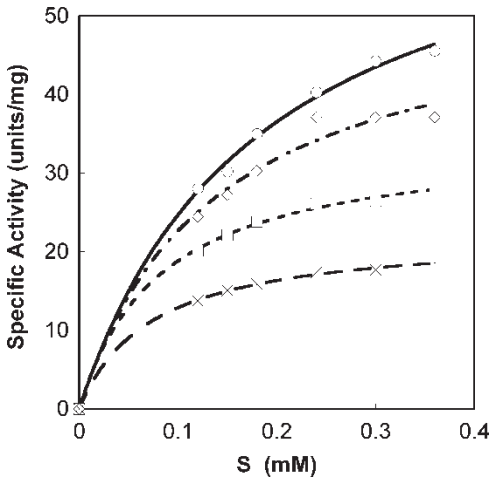


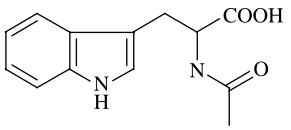
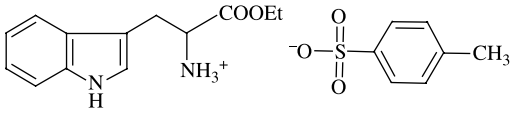
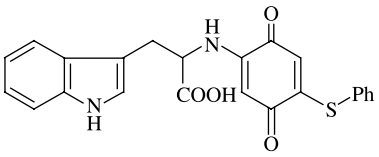
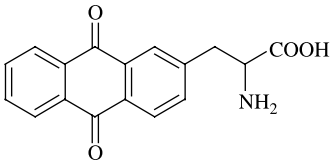
Figure 1. Michaelis-Menten plot of Tnase with S-phenyl-benzoquinone tryptophan. SOPC at different concentrations was used as a substrate, as described in the experimental section. Analyses were done with Calidagraph version 4. Measurements were performed in the absence, ○, and the presence of the inhibitor at a concentration of 47.5 μM, ◇; 95 μM, □; 190 μM, X.

Tnase from *P. vulgaris* was solved in the holo form [28]. The three structures hold significant deviations in the relative orientation of the 'large' and 'small' domains and, as a consequence, show variations in the width of the catalytic site cleft and the geometry of the cofactor binding site. The conformational changes of Tnase molecule are supposed to be of functional importance [26], in a way similar to tyrosine-phenol lyase [29]. The flexibility of Tnase makes a structure based design of specific inhibitors a challenging task. Here we present our finding of four Tnase inhibitors. Two of the inhibitors, N-acetyl-L-tryptophan and S-phenyl benzoquinone-L-tryptophan were rationalized based on the known mechanism of Tnase catalysis. It is suggested that designing tryptophan derivatives with enhanced detachment of the α-proton (the proton attached to the chiral carbon) will result in the acceleration of the initial step of the catalytic mechanism (Scheme 3, the conversion of (2) to (3) [21]).

In this step the α-proton of the complexed L-tryptophan (2) is detached by a catalytic amino acid, which serves as a base in the Tnase active site, giving rise to structure (3). The present results suggest that S-phenyl benzoquinone-L-tryptophan is uncompetitive inhibitor while N-acetyl-L-tryptophan is noncompetitive. L-tryptophan-ethylester was chosen as a possible inhibitor due to its decreased polarity which enables it to cross non polar barriers (e.g., the cell membrane) better than the free acid [30,31]. Since a competitive inhibition is observed with L-tryptophan-ethylester, it is proposed that this inhibitor binds to the active site of the enzyme.

A variety of quinones are known to act as inhibitors of various metabolic paths in the cell. Some natural quinones and some specially designed synthetic quinones are known to serve as antibacterial, antiviral,

Table I. Kinetics parameters of the Tnase inhibitors.

Inhibitor	Ki (mM)	Suggested type of inhibition	Structure
N-acetyl tryptophan	48 ± 3	noncompetitive	
L-tryptophan-ethylester (p-toluene sulfuric acid salt)	52 ± 0.1	competitive	
S-phenylbenzoquinone tryptophan	101 ± 2	uncompetitive	
α-Amino-2-(9,10-anthraquinone)-popanoic acid	174 ± 9	noncompetitive	

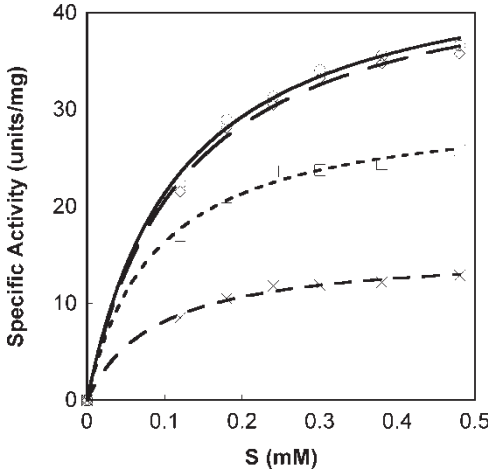


Figure 2. Michaelis-Menten plot of Tnase with N-acetyl-tryptophan. SOPC was used as a substrate at different concentrations. Measurements were performed in the absence, ○, and the presence of the inhibitor at a concentration of 15.5 μM, ◇; 31 μM, □; 62 μM, X.

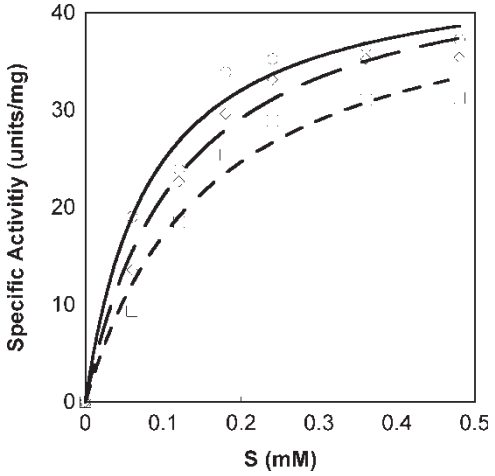


Figure 3. Michaelis-Menten plot of Tnase with p-Toluene sulfonic acid salt of L-tryptophan-ethylester. SOPC was used as a substrate at different concentrations. Measurements were performed in the absence, ○, and the presence of the inhibitor at a concentration of 26 μM, ◇; 52 μM, □.

and even anti-cancer agents [32–35]. Tryptophanase can bind several amino acids; therefore, we designed and prepared in a 4-stage synthesis an anthraquinone that is covalently attached to the side chain of alanine, α-amino-2-(9,10-anthraquinone)-propanoic acid. This compound showed most likely a non-competitive inhibition with a Ki of 174 μM. Such a noncompetitive inhibition may suggest that it does not enter the active site. Instead, it may either sterically block the entrance of the substrate or induce conformational changes in Tnase which prevent binding of the substrate to the active site. Line-weaver-Burk analysis led to similar conclusions regarding the mode of inhibition.

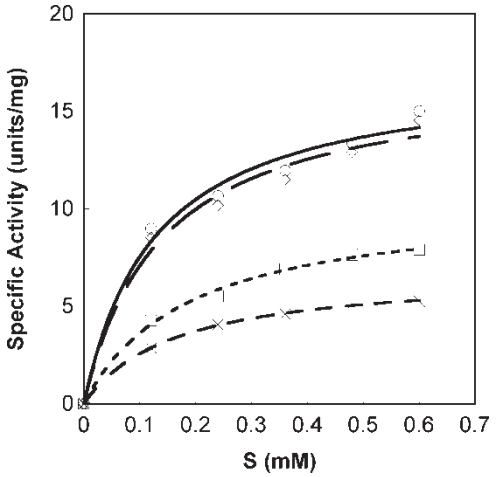
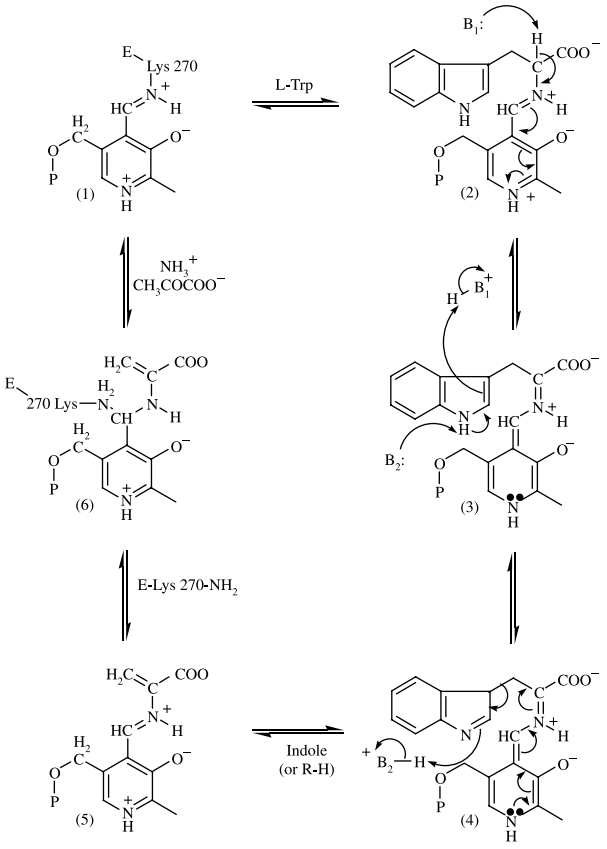


Figure 4. Michaelis-Menten plot of Tnase with α-Amino-2-(9,10-anthraquinone)-propanoic acid. SOPC was used as a substrate at different concentrations. Measurements were performed in the absence, ○, and the presence of the inhibitor at a concentration of 55 μM, ◇; 110 μM, □; 220 μM, X.

It was suggested that indole can act as an extracellular signaling molecule that activates the *astD*, *tnaB*, and *gabT* genes in a concentration-dependent manner. To date, there is no direct evidence that *E. coli* produces any of the N-acyl homoserine lactone signaling molecules commonly used in other Gram-negative bacteria. Therefore, *E. coli* may have evolved to utilize alternative signals, such as the accumulation of certain metabolites. Signaling via metabolites may allow cells to fine-tune the regulation of target genes in response to changing environmental conditions [10].



Scheme 3. The mechanism of catalysis of Tnase as proposed by Snell & Mari [21].

In conclusion, we evaluated four new inhibitors of tryptophanase with Ki values between 48 μM and 174 μM. The compounds were chosen based on mechanistic rationales, solubility and a combination of known inhibitory effects. Thus, optimal and specific factors that interact with Tnase can be used as a decorous tool to study the role of this multifunctional enzyme.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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