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Inhibition of *Escherichia coli* tryptophan indole-lyase by tryptophan homologues

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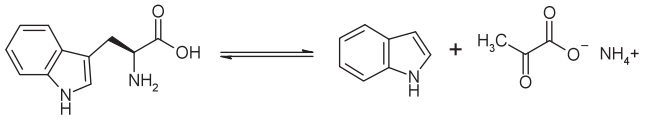
ABSTRACT

We have designed, synthesized and evaluated homotryptophan analogues as possible mechanism-based inhibitors for *Escherichia coli* tryptophan indole-lyase (tryptophanase, TIL, E.C. 4.1.99.1). As a quinonoid structure is an intermediate in the reaction mechanism of TIL, we anticipated that homologation of the physiological substrate, L-Trp would provide analogues resembling the transition state for β-elimination, and potentially inhibit TIL. Our results demonstrate that L-homotryptophan (**1a**) is a moderate competitive inhibitor of TIL, with K_i = 67 μM, whereas L-bishomotryptophan (**1b**) displays more potent inhibition, with K_i = 4.7 μM. Pre-steady-state kinetics indicated the formation of an external aldimine and quinonoid with **1a**, but only the formation of an external aldimine for **1b**, suggesting differences in the inhibition mechanism. These results demonstrate that formation of a quinonoid complex is not required for strong inhibition. In addition, the Trp analogues were evaluated as inhibitors of *Salmonella typhimurium* Trp synthase. Our results indicate that compound **1b** is at least 25-fold more selective toward TIL than Trp synthase. We report that compound **1b** is comparable to the most potent inhibitor previously reported, while displaying high selectivity for TIL. Thus, **1b** is a potential lead for the development of novel antibacterials.

Tryptophan indole-lyase (tryptophanase, TIL, E.C. 4.1.99.1)[1] is a pyridoxal-5′-phosphate (PLP)-dependent bacterial enzyme that catalyzes the reversible hydrolytic cleavage of the C_β—C_γ bond of L-Trp to yield indole and ammonium pyruvate (Eq. (1)) [1]. For many years, this source of indole was considered to be a bacterial metabolic waste product of L-Trp. However, recent reports have suggested that metabolic indole has a critical role in formation of biofilm in



(1)

Escherichia coli [2–4]. In addition, it has been shown that indole is a bacterial signaling molecule that regulates gene expression [5], plasmid stability [6], pathogenicity [7], and antibiotic resistance [8,9]. Since TIL is a bacterial enzyme not found in eukaryotes, inhibition of its activity presents a selective and attractive approach for an antibiofilm and antibacterial treatment. Thus, potent inhibitors of TIL are of interest as possible antibiotics.

In addition to L-Trp, TIL can also catalyze the *in vitro* β-elimination of a series of substrates, including L-serine, *O*-alkyl-L-serines, *S*-alkyl-L-serines, β-chloroalanine, 2,3-diaminopropionate, *O*-acyl-L-serines, and *S*-(*o*-nitrophenyl)-L-cysteine (SOPC), since all of these substrates have a reasonable leaving group suitable for β-elimination [10–12]. However, the cleavage mechanism for its physiological substrate, L-Trp, is intriguing as indole is formally an unactivated aromatic carbon leaving group in this reaction. Nevertheless, previous work from our laboratory [13–18] and that of others [19,20] has provided valuable insights into the catalytic mechanism of TIL (Scheme 1).

The active form of *E. coli* TIL is a tetramer with PLP bound to Lys-270 in the active site through a Schiff's base linkage [21]. Nucleophilic attack by the amino acid ligand generates a *gem*-diamine, which releases the Lys-270 and forms a substrate-bound external aldimine, observed at λ_{max} = 422 nm (Scheme 1) [14]. Previously, it was suggested that an indolenine tautomer of L-Trp was an intermediate in this reaction [22,23]. Further studies in our laboratory

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[1] *Abbreviations used:* PLP, pyridoxal-5′-phosphate; SOPC, *S*-(*o*-nitrophenyl)-L-cysteine; DEAM, diethyl acetamidomalonate; L-Homotryptophan, (2*S*)-4-(3′-indolyl)-2-aminobutanoic acid; L-Bishomotryptophan, (2*S*)-5-(3′-indolyl)-2-aminopentanoic acid; TIL, tryptophan indole-lyase [tryptophanase, EC 4.1.99.1].

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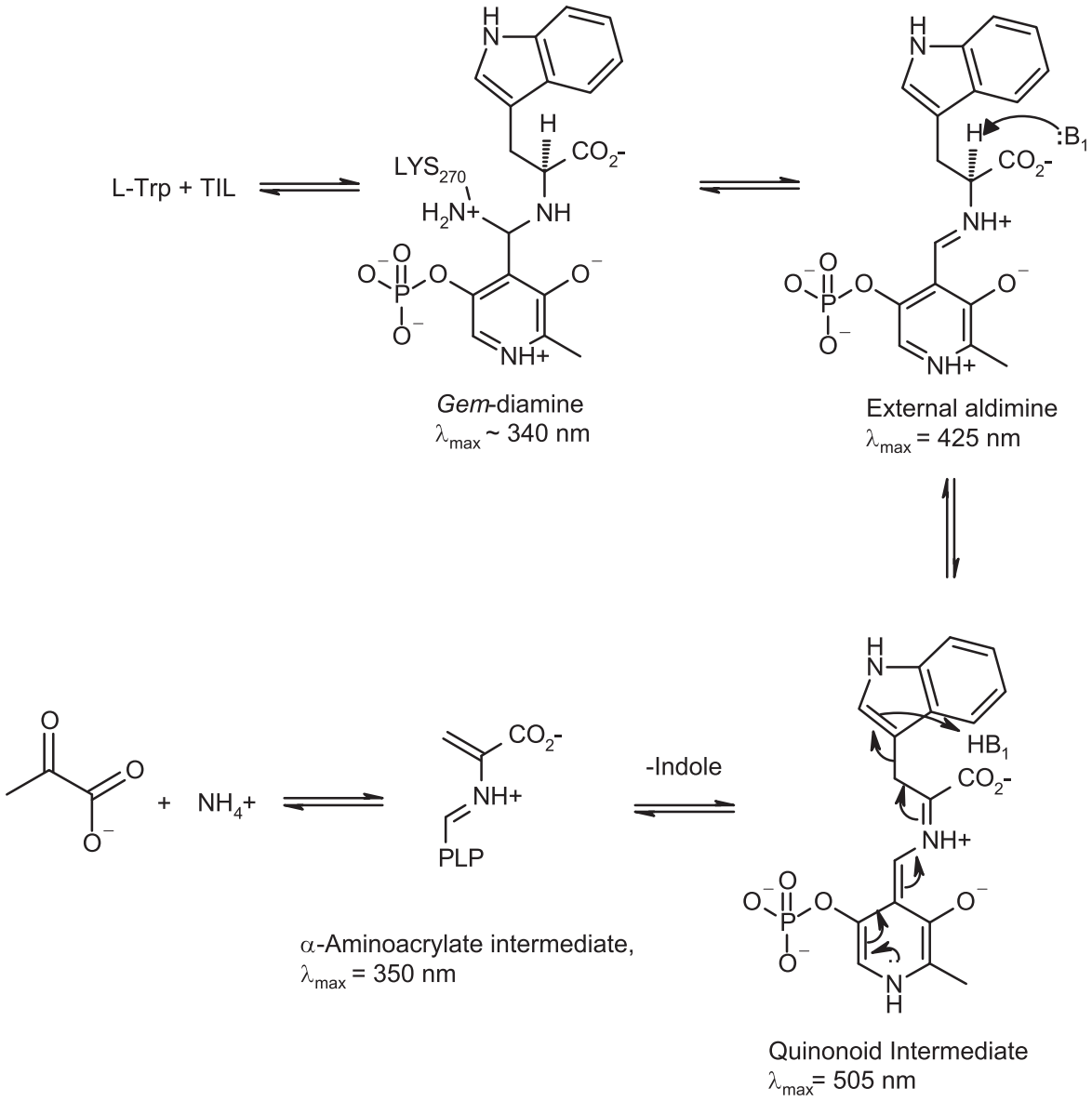


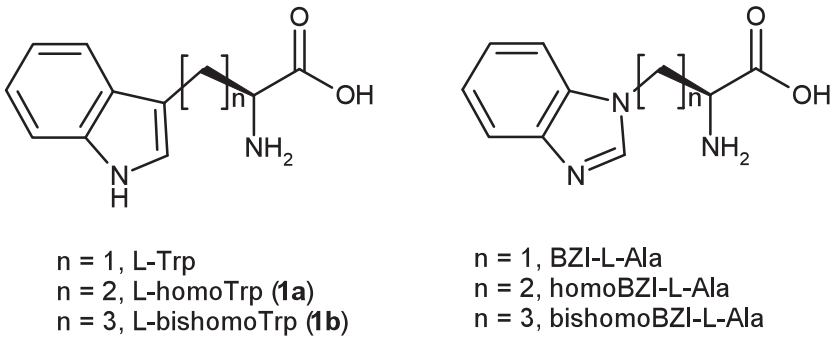
Scheme 1. Proposed mechanism of tryptophan indole-lyase.

suggested that following deprotonation of C_α, the departure of indole was accomplished by C_γ protonation to form a quinonoid intermediate, absorbing at 505 nm followed by C_β—C_γ bond cleavage (Scheme 1) [15]. Decay of the quinonoid intermediate in the presence of benzimidazole, an uncompetitive inhibitor, demonstrated the presence of an aminoacrylate intermediate, with λ_{max} = 345 nm (Scheme 1) [15]. The aminoacrylate intermediate then releases the ammonium pyruvate product. The transition state for indole elimination is expected to have an extended C_β—C_γ bond length. Hence, we proposed that L-homotryptophan (**1a**) and L-bishomotryptophan (**1b**) (Scheme 2) might resemble the transition state for indole elimination, and thus could potentially act as potent competitive inhibitors for TIL [24]. In addition, L-Trp analogues that are potent inhibitors of TIL are also potent inhibitors of Trp synthase, an enzyme widely distributed in plants, fungi and bacteria that catalyzes the last two steps of L-Trp biosynthesis [23,25]. Therefore, we also evaluated the inhibitory activity of **1a** and **1b** toward Trp synthase. We present herein the synthesis, inhibition and pre-steady-state kinetics evaluation of **1a** and **1b** as inhibitors of TIL and Trp synthase.

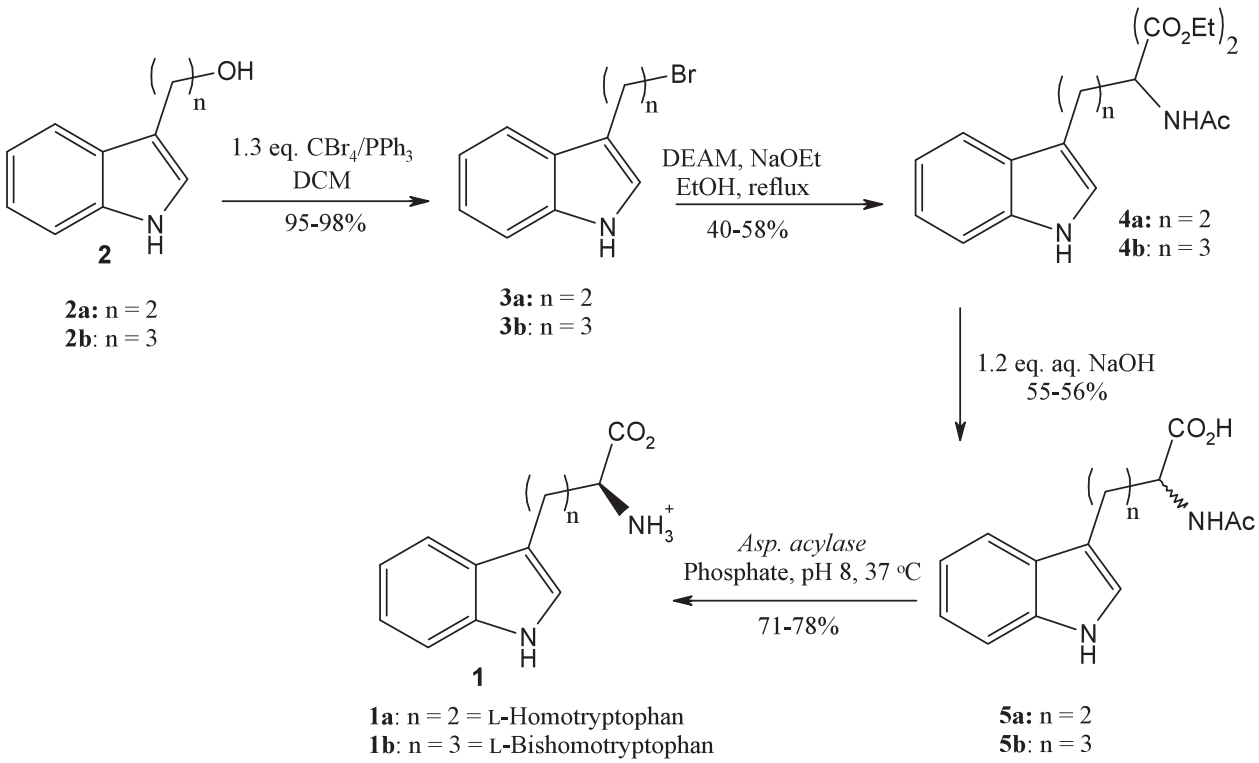
Results and discussion

Synthesis of L-homotryptophan and L-bishomotryptophan

Syntheses of racemates of both **1a** [26] and **1b** [27] were previously reported, but to our knowledge, no attempts at optical resolution of these compounds were reported in the literature. Elegant asymmetric syntheses of **1a** were also reported, using the Schollkopf chiral auxiliary [28] and Larock's heteroannulation [29]. However, both methods for asymmetric synthesis suffered from lengthy procedures to introduce the chiral center. Following a modified procedure, we were able to obtain, in three steps, the racemic *N*-acetyl derivatives, **5a** and **5b**, which can be enzymatically resolved conveniently to yield enantiomeric **1a** or **1b** (Scheme 3). Tryptophol (**2a**) and homotryptophol (**2b**) were selected as starting materials since they can be obtained from com-



Scheme 2. Structures of Trp and AzaTrp homologues.



Scheme 3. Synthesis of L-Trp homologues.

mercial sources. Bromination of compounds **2** using phosphorus tribromide, in our hands, only gave modest yields, consistent with previous reports for this reaction [27]. Alternatively, the Appel's salt is a milder brominating agent for primary alcohols [30]. When bromination of **2** was carried out using the Appel's salt, a clean conversion with high yield was achieved. Alkylation of **3** using 1 eq. of diethyl acetamidomalonate afforded **4** in modest yield [27]. Hydrolysis using 5 eq. of NaOH, as reported by Snyder [27], resulted in an excess amount of salt recovered during the workup and did not provide the decarboxylated product **5**. However, when using 1.2 eq. of NaOH, ester hydrolysis and decarboxylation was achieved in one step to yield the *N*-acetyl derivatives, **5**. Enantioselective hydrolysis by *Aspergillus* acylase under the conditions previously reported for *N*-acetyl-α-amino acids [31] was difficult due to low solubility of **5** in water. Alternatively, when this step was carried out in phosphate buffer at pH 8, we found that compound **5** solubility was greatly enhanced. Hydrolysis of **5a** and **5b** under this condition yielded the L-enantiomers, **1a** and **1b**, which were conveniently recovered through simple filtration, as both product compounds have very low solubility in phosphate buffer. To our knowledge, this is the first report of the resolution of **1a** and **1b** using *Aspergillus* acylase. Our specific rotation for L-homotryptophan (**1a**), +35.7°, is very similar to that reported previously for L-homotryptophan obtained by asymmetric synthesis, +37.5° [28]. The resolution and specific rotation of L-bishomotryptophan (**1b**) has not been reported previously, although the synthesis of DL-bishomotryptophan has been reported [26].

Enzyme inhibition

Our results indicate that **1a** exhibits moderate competitive inhibition against TIL with K_i = 67 ± 12 μM (Table 1). However, by adding an additional methylene between the amino acid and the indole ring, compound **1b** displayed an increase in potency of more than an order of magnitude as an inhibitor of TIL, with K_i = 4.7 ± 0.5 μM (Table 1). Previously, we reported that 2-oxindolyl-L-alanine and 2,3-dihydrotryptophan are potent competitive inhibitors of both TIL (K_i = 2.5 and 4.5 μM, respectively) and tryptophan synthase [24,25]. These compounds remain the most potent

Table 1
Competitive inhibitors of tryptophan indole-lyase.

Compound	K_i (μM)
L-Tryptophan	200[a]
(3*S*)-2,3-Dihydro-L-Trp	2[b]
Oxindolyl-L-alanine	5[c]
(2*S*)-2-Amino-4-(benzimidazol-1-yl)butanoic acid (HomoBZI-Ala)	13.6[d]
(2*S*)-2-Amino-5-(benzimidazol-1-yl)pentanoic acid (BishomoBZI-Ala)	>600[d]
L-Homotryptophan (**1a**)	67 ± 12
L-Bishomotryptophan (**1b**)	4.7 ± 0.5

[a] K_m, from Ref. [12].
[b] From Ref. [36].
[c] From Ref. [35].
[d] From Ref. [33].

inhibitors reported to date for TIL. Parola et al. also reported the evaluation of several L-Trp and anthraquinone derivatives as inhibitors for TIL, with moderate inhibition (K_i = 48–174 μM) [32]. More recently, our group reported the evaluation of several benzimidazole homologues of L-Trp as inhibitors, with the most efficient compound being (*S*)-4-(benzimidazol-1-yl)-2-aminobutanoic acid (Scheme 2), a benzimidazole derivative of homotryptophan, with K_i = 13 μM [34]. To our knowledge, **1b** is the only compound thus far to achieve a K_i for TIL on the same order as 2-oxindolyl-L-alanine and 2,3-dihydrotryptophan. It is interesting to note that from our previous work with benzimidazole derivatives [33], by replacing the C-3 carbon of L-Trp with a nitrogen, the compound was also a substrate for TIL. By extending the C_α position with one methylene, the aza-analogue of homotryptophan showed high efficiency, K_i = 13 μM, as a competitive inhibitor (Table 1). However, further extension with a second methylene to the aza analogue of bishomotryptophan resulted in a great decrease in inhibitory potency. Based on these results, our initial expectation was that **1a** would show greater inhibition than **1b**. Unexpectedly, **1b** was discovered to have a K_i of more than 10-fold lower than **1a**. As indole and benzimidazole are isosteric and isoelectronic, this difference is likely due to the differences in the structure of the N-3 nitrogen in the two rings. In the tryptophan homologue series, the indole nitrogen has a hydrogen on it, while in the azatryptophan homologue series, the benzimidazole nitrogen is an unprotonated imine. Thus, in the tryptophan homologue series, N-3 is an H-bond donor, while in the azatryptophan homologues, N-3 is an H-bond acceptor (Scheme 2).

Tryptophan synthase is also a PLP-dependent enzyme that catalyzes the last two steps of L-Trp biosynthesis. In our experience, L-Trp analogues that are potent inhibitors of TIL also show potent inhibition of Trp synthase [24,25]. However, the diastereomers of 2,3-dihydro-L-tryptophan are stereoselective for inhibition of TIL and Trp synthase. With regard to antibacterial development, an ideal inhibitor is one which efficiently and effectively inhibits TIL but not Trp synthase, as Trp synthase is widely distributed in plants, fungi and bacteria. Assays for compounds **1a** and **1b** with Trp synthase displayed no measurable inhibition at concentrations up to 100 μM, suggesting that both compounds are more selective for TIL than Trp synthase, and compound **1b** is at least 25-fold more selective for TIL.

Pre-steady-state kinetics

Rapid-scanning stopped-flow experiments were conducted to determine the inhibitor–enzyme binding interactions. Pre-steady-state kinetics evaluation demonstrated significant differences in the inhibition mechanism of both compounds. For **1a**, an initial absorbance peak at λ_{max} = 420 nm, followed by a second absorbance peak at λ_{max} = 500 nm, was observed (Fig. 1A). This result is consistent with the accepted mechanism for TIL reaction with L-Trp shown in Scheme 1, in which the initial formation of an external aldimine with λ_{max} = 422 nm and subsequent formation of quinonoid intermediate at λ_{max} = 505 nm are observed. The reaction requires 3 exponentials to obtain an adequate fit. In the fast phase, with $1/\tau_1 = 1.29 \pm 0.02\ s^{-1}$, there is a decrease in the 420 nm peak and an increase at 500 nm, with an isosbestic point at 438 nm (Fig. 1B). In the second phase, with $1/\tau_2 = 0.67 \pm 0.12\ s^{-1}$, and the third phase, with $1/\tau_3 = 0.06 \pm 0.01\ s^{-1}$, there is an increase at 500 nm, but little change in the absorbance at 420 nm, and no isosbestic point. Therefore, we conclude that compound **1a** initially complexes with PLP at the active site to form an external aldimine, which occurs within the dead time of the stopped-flow device, followed by deprotonation of C_α to yield a quinonoid intermediate, which then undergoes two subsequent slow conformational changes (Eq. (2)) [14,15,18]. The rate constant for quinonoid intermediate formation from **1a** is much slower than L-tryptophan, which forms a 505 nm intermediate at about 500 s^{-1} [14,15].

$$\begin{aligned} \mathrm{E} + \text{L-HTrp} &\overset{\text{fast}}{\Longleftrightarrow} \mathrm{E} - \text{L-HTrp}\,(420\,\mathrm{nm}) \\ &\overset{1.29\,s^{-1}}{\Longleftrightarrow} \mathrm{E} - \text{L-HTrp}\,(500\,\mathrm{nm}) \\ &\overset{0.67\,s^{-1}}{\Longleftrightarrow} \mathrm{E} - \text{L-HTrp}\,(500\,\mathrm{nm}) \\ &\overset{0.06\,s^{-1}}{\Longleftrightarrow} \mathrm{E} - \text{L-HTrp}\,(500\,\mathrm{nm}) \end{aligned} \quad (2)$$

With compound **1b**, the absorbance peak at λ_{max} = 422 nm is observed, corresponding to the formation of an external aldimine (Fig. 2A), but the 500 nm absorbance peak of the quinonoid intermediate is not seen. The course of the reaction can be fit with 2 exponentials, with $1/\tau_1 = 16.1 \pm 2.4\ s^{-1}$ and $1/\tau_2 = 0.52 \pm 0.09\ s^{-1}$. In the first phase, there is a red-shift to 422 nm and small increase in intensity of the aldimine peak. In the second phase, there is a further increase in the 422 nm aldimine peak and a very small shoulder at about 500 nm, concomitant with a decrease in absorbance at 338 nm (Fig. 2B). Thus, it appears that the initial rapid reaction of TIL with **1b** forms an equilibrating mixture of *gem*-diamine and external aldimine complexes, and a slow conformational change results in conversion of the *gem*-diamine to the external aldimine. In our previous study of benzimidazole aza-analogues of L-Trp (Scheme 2), we found that the homologues also showed evidence for *gem*-diamine intermediates, which are not seen in the reaction of natural amino acids [33]. Furthermore, homophenylalanine had also been found to exhibit a transient *gem*-diamine intermediate in binding to TIL [34]. Thus, having 2 or more methylenes between the amino acid moiety and the aromatic ring must introduce steric restrictions which slow down the formation of external aldimines.

Mechanistic implications

Interestingly, by extending L-Trp by one additional methylene at the C_α position (**1a**), the reaction is mechanistically similar to that of the physiological substrate, but carbon–carbon cleavage cannot occur. In compound **1a**, the indole ring is positioned on the γ-carbon prohibiting the participation of C_β—C_γ π-electrons in the β-elimination of indole. In the crystal structure of the highly homologous (ca. 40% identity) tyrosine phenol-lyase with L-Ala and pyridine-*N*-oxide bound, which mimics the complex of aminoacrylate and the phenol leaving group, there is a distance of 3.0 Å between the C_β of the Ala quinonoid complex and the C_4 of the aromatic ring [35]. Furthermore, the aromatic ligand is found to be positioned directly over the C_α—C_β bond of the Ala. It is likely that TIL would have a similar geometry for the elimination of indole from L-tryptophan. In the case of **1b**, with the extension of L-tryp-

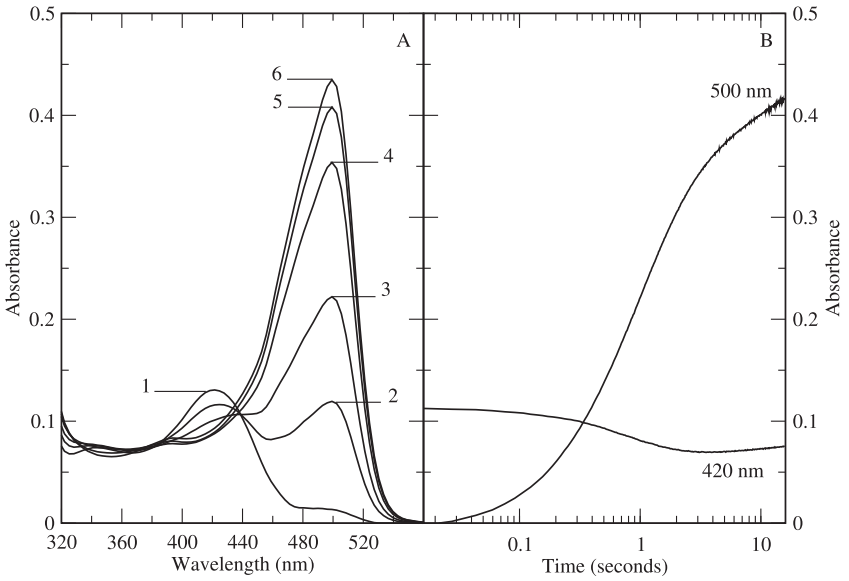


Fig. 1. Rapid-scanning stopped-flow spectra for TIL reaction with L-homoTrp (**1a**). (A) The scans are shown at the following times after mixing: 1, 0.002 s; 2, 0.0345 s; 3, 0.0875 s; 4, 2.81 s; 5, 7.60 s; 6, 15.9 s. (B) Time courses at 420 and 500 nm.

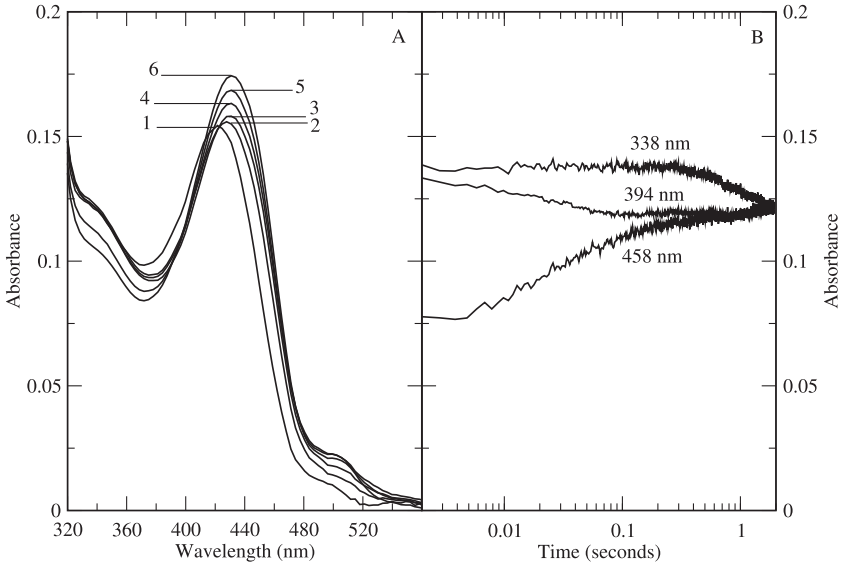


Fig. 2. Rapid-scanning stopped-flow spectra for TIL reaction with L-bishomoTrp (**1b**). (A) The scans are shown at the following times after mixing: 1, 0.002 s; 2, 0.046 s; 3, 0.120 s; 4, 0.261 s; 5, 0.955 s; 6, 1.914 s. (B) Time courses at 338, 394 and 458 nm.

tophan by two additional methylenes, the compound apparently must adopt a different conformation for the external aldimine, possibly with the indole ring of the inhibitor occupying a remote site which the nascent indole product would occupy transiently on its way out. This conformation must not allow the quinonoid intermediate to adopt the planar geometry of N_α—C_α—C_β necessary for deprotonation to occur, which explains why the absorbance peak at 500 nm for the quinonoid intermediate is negligible. Thus, it is likely that **1b** is a byproduct analogue of TIL. All of the other known potent inhibitors of TIL form quinonoid complexes [24,25], but the present results demonstrate that formation of a quinonoid complex is not necessary for potent inhibition.

Conclusion

Recent reports suggest bacterial TIL is a novel attractive target for development of novel antibiotics targeting biofilm formation, so we were interested in the design of mechanism-based inhibitors of TIL. As an alternative to asymmetric syntheses, we developed a modified method of Snyder et al. to conveniently obtain the optically active isomer of compounds **1a** and **1b**. Evaluation of **1a** in inhibition assays indicated that it is a moderate inhibitor for TIL with $K_i = 67$ μM and the presence of a quinonoid intermediate was observed in pre-steady-state evaluation. In contrast, **1b** displayed potent inhibition for TIL with $K_i = 4.7$ μM and formation of a quinonoid intermediate was not observed. No inhibition was observed with Trp synthase for both compounds up to 100 μM, indicating higher selectivity for TIL in comparison with Trp synthase. To our knowledge, L-bishomotryptophan (**1b**) is the first TIL inhibitor with efficiency on the same order as 2-oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan while displaying high selectivity for TIL in preference to Trp synthase.

Materials and methods

Materials

Methylene chloride and ethanol were dried over CaH_2 and Mg, respectively, prior to use. Tryptophol (Acros), homotryptophol (Acros), triphenylphosphine (Aldrich), carbon tetrabromide (Janssen Chimica), diethyl acetamidomalonate ester (DEAM) (Aldrich), *Aspergillus* acylase I (0.43 unit/mg, Aldrich), indole (Aldrich), L-serine (USB Corp.), pyridoxal-5′-phosphate (PLP) (USBiochemical Corp.) and all other reagents (Fisher) were used without further purification. *S*-(*o*-Nitrophenyl)-L-cysteine (SOPC) used in enzyme assays was prepared as previously described [36]. Enzyme assays were performed using distilled deionized water. All NMR data were collected on a 400 MHz Varian Mercury Plus NMR instrument and data were processed by MNova NMR processing software. ESI-MS experiments were performed on a Perkin Elmer Sciex API I Plus.

Synthesis of L-homotryptophan and L-bishomotryptophan

*3-(2-Bromoethyl)-1H-indole (**3a**)*

In a three-neck flask, tryptophol (2.00 g, 1 eq.) and triphenylphosphine (4.23 g, 1.3 eq.) was dissolved in dry CH_2Cl_2 (15 ml). In an addition funnel, carbon tetrabromide (5.35 g, 1.3 eq.) dissolved in dry CH_2Cl_2 (5 ml) was added drop-wise, under inert atmosphere at 0 °C, until the addition was complete. The reaction was allowed to stir at room temperature for an additional 3 h, or until complete disappearance of starting materials by TLC. Solvent was removed under reduced pressure and the residue was purified by column chromatography using hexane and ethyl acetate to yield **3a** as an off-white solid. Yield: 2.64 g (95%). (Appendix) ^{1}H NMR ($CDCl_3$) δ (ppm): 3.32–3.36 (t, 2H), 3.62–3.66 (t, 2H), 7.10 (s, 1H), 7.12–7.16 (t, 1H), 7.19–7.23 (t, 1H), 7.37–7.39 (d, 1H), 7.59–7.61 (d, 1H), 8.04 (s, 1H).

*Diethyl 2-(2-(1H-indol-3-yl)ethyl)-2-acetamidomalonate (**4a**)*

In a three-neck flask, diethyl acetamidomalonate (2.56 g, 1 eq.) was added to a solution of dry ethanol (20 ml) containing dissolved sodium metal (0.271 g, 1 eq.) at 0 °C. The mixture was stirred for an additional 30 min at 0 °C. Compound **3a** (2.64 g, 1 eq.) was then added, and the solution was allowed to reflux under inert atmosphere for an additional 15 h. Solvent was removed under reduced pressure and the residue was purified by column chromatography using hexane and ethyl acetate to yield **4a** as a white solid. Yield: 1.69 g (40%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.18–1.22 (t, 3H), 2.00 (s, 3H), 2.63–2.67 (m, 2H), 2.79–2.83 (m, 2H), 4.07–4.21 (m, 4H), 6.85 (s, 1H), 6.97 (s, 1H), 7.09–7.12 (t, 1H), 7.16–7.20 (t, 1H), 7.33–7.35 (d, 1H), 7.54–7.56 (d, 1H), 8.00 (s, 1H).

*2-Acetamido-4-(1H-indol-3-yl)butanoic acid (**5a**)*

In a round bottom flask, NaOH (0.225 g, 1.2 eq.) was dissolved in aqueous tetrahydrofuran (2:1, THF:H_2O, 45 ml) solution. Then compound **4a** (1.69 g, 1 eq.) was added in and the solution was allowed to reflux for 15 h. The solvent was then removed under reduced pressure and the residue was taken up in water and ethyl acetate. The aqueous layer was acidified to pH 2 using 6 M HCl and extracted with ethyl acetate. The organic layer was washed with water and dried over $MgSO_4$. Removal of solvent under reduced pressure give crude **5a** as a yellow solid which can be further purified by recrystallization in water to give pure **5a** as an off-white needle-shaped solid. Yield: 0.684 g (56%). ^{1}H NMR (DMSO) δ (ppm): 1.87 (s, 3H), 1.98–2.01 (m, 2H), 2.63–2.74 (m, 2H), 4.15–4.20 (m, 1H), 6.93–6.97 (t, 1H), 7.02–7.06 (t, 1H), 7.07 (s, 1H), 7.30–7.32 (d, 1H), 7.48–7.49 (d, 1H), 8.22–8.24 (1H), 12.51 (broad, 1H).

*L-Homotryptophan (**1a**)*

In a 50 ml culture tube, compound **5a** (150 mg) was dissolved in potassium phosphate buffer (9 ml, 100 mM) and the final pH was adjusted to 8 using 6 M NaOH. Then *Aspergillus* acylase I (45 mg) was added to the mixture and it was allowed to incubate overnight at 37 °C, with shaking speed of 250 rpm (C25, New Brunswick Scientific). The resulting mixture was cooled, filtered and washed with cold water to give pure **1a** as a crystalline solid. No additional product was observed after readjusting the pH of the filtrate to 6 using 6 M HCl. Attempts to readjust pH of the filtrate to 8, introducing additional enzyme and longer incubation time also did not result in additional product. Yield: 49.2 mg (78%). Unreacted **5a** can be recovered by acidifying the filtrate to pH 2 using 6 M HCl and extraction using ethyl acetate. ESI-MS M+1 (m/z): 219. ^{1}H NMR (D_2O/NaOD) δ (ppm): 1.72–1.88 (m, 2H), 2.62–2.66 (t, 2H), 3.12–3.16 (t, 1H), 6.97–7.01 (t, 1H), 7.06–7.09 (t, 2H), 7.32–7.34 (d, 1H), 7.53–7.55 (d, 1H). $[\alpha]_D$ = +35.7° (Lit., +37.5° [38]) (CH_3CO_2H, c = 0.28, 25.9 °C).

*L-Bishomotryptophan (**1b**)*

Compound **1b** was obtained following the procedure described above for compound **1a**. Starting with homotryptophol **2b**, bromination using the Appel's salt yielded **3b** as a yellow oil (yield: 98%). Alkylation of **3b** with diethyl acetamidomalonate gave **4b** as an oil (yield: 58%). Ester hydrolysis and decarboxylation of **4b** in aqueous NaOH gave crude **5b** as a yellow solid which upon purification by recrystallization in water gave a white solid (yield: 55%). Enantioselective hydrolysis of **5b** using *Aspergillus* acylase I, as described above, gave **1b** as a white crystalline solid (yield: 71%). ^{1}H NMR (D_2O/NaOD) δ (ppm): 1.45–1.57 (m, 4H), 2.61–2.64 (t, 2H), 3.07–3.10 (t, 1H), 6.96–7.00 (t, 1H), 7.04–7.09 (t, 2H), 7.32–7.34 (d, 1H), 7.53–7.55 (d, 1H). $[\alpha]_D$ = +43.0° (CH_3CO_2H, c = 0.232, 26.3 °C).

Enzymes and enzyme assays

TIL was purified from *E. coli* JM101 containing plasmid *pMD6*, as previously described [37]. Enzyme assays were performed on a Cary 1 UV–visible spectrophotometer equipped with a Peltier temperature-controlled six-cell changer. Enzyme activity was routinely determined as previously described [12], by following the decrease in absorbance of SOPC at 370 nm ($\Delta\varepsilon$ = −1860 M^{-1} cm^{-1}), in 50 mM phosphate buffer, pH 8.0, at 25 °C. Enzyme concentration in solution was estimated spectrophotometrically from the absorbance of the holoenzyme at 278 nm ($A^{1\%}$ = 9.19) [37]. Trp synthase from *Salmonella typhimurium* was purified as previously described [38]. The concentration of Trp synthase was determined from the absorbance at 278 nm ($A^{1\%}$ = 6.0), and enzyme activity were determined by following the increase in absorbance of L-Trp at λ = 290 nm ($\Delta\varepsilon$ = 1800 M^{-1} cm^{-1}) [38].

Enzyme inhibition assays

A typical enzyme inhibition assay contained potassium phosphate (50 mM), PLP (40 μM), with varying concentrations of SOPC, **1a** or **1b** in a total volume of 600 μl. Similarly, Trp synthase assays were performed in phosphate buffer (50 mM) containing PLP (40 μM), indole (0.1 mM), with varying concentration of serine, **1a** or **1b** in a total volume of 600 μl. Experimental velocities from inhibition assays were fitted to Eq. (3) and the inhibition constant, K_i, was calculated using the FORTRAN program, COMPO, of Cleland [39].

$$v = V_{max} * [S]/(K_m^*(1 + [I]/K_i) + [S]) \quad (3)$$

Rapid-scanning stopped-flow experiments

Prior to use, the enzyme was incubated with 0.5 mM PLP for 30 min and subsequently eluted with phosphate buffer (20 mM, pH 8, 0.16 M KCl) through a gel filtration column (PD-10, Pharmacia) to remove excess PLP. Rapid-scanning stopped-flow experiments were performed in the same buffer on an RSM-1000 spectrophotometer (OLIS, Inc.), equipped with a stopped-flow cell mixer compartment of 1 cm path length capable of collecting up to 1000 scans per second with a dead time of 2 ms. The spectra were fitted with Global Works software provided by OLIS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.abb.2014.07.027.

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