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Synthesis and Biological Evaluation of CTP Synthetase Inhibitors as Potential Agents for the Treatment of African Trypanosomiasis

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Acivicin analogues with an increased affinity for CTP synthetase (CTPS) were designed as potential new trypanocidal agents. The inhibitory activity against CTPS can be improved by increasing molecular complexity, by inserting groups able to establish additional interactions with the binding pocket of the enzyme. This strategy has been pursued with the synthesis of α-amino-substituted analogues of Acivicin and N1-substituted pyrazoline derivatives. In general, there is direct correlation between the enzymatic activity and the in vitro anti-trypanosomal efficacy of the derivatives studied here. However, this cannot be taken as a general rule, as other important factors may play a role, notably the ability of uptake/diffusion of the molecules into the trypanosomes.

Introduction

Human African trypanosomiasis (HAT) is a neglected tropical disease endemic to sub-Saharan Africa. HAT is caused by an unicellular protozoan belonging to the class of zooflagellates and transmitted by the bite of tsetse flies to their mammalian hosts. The disease is characterized by an initial stage in which parasites proliferate in the hemolymphatic system, and a second stage in which the parasites have reached the central nervous system.[1,2] Whereas symptoms are relatively nonspecific in the first stage, the second stage is marked by progressive neurological dysfunction, including the breakdown in sleep–wake patterns that lend the disease its common name of sleeping sickness. Two subspecies are responsible for causing the sickness in humans: *Trypanosoma brucei* (*T. b.*) *gambiense* and *T. b. rhodesiense*. While *T. b. gambiense* affects countries in western and central Africa, causing a chronic form of the disease, *T. b. rhodesiense* is confined to eastern and southern Africa and causes an acute illness within a few weeks of infection.

Chemotherapy is the main way to control this disease, as there are no effective vaccines, and current treatment depends on the causative subspecies and the stage of the disease.[3] The main drawbacks of currently available treatment are poor efficacy, poor pharmacokinetic properties, cost, and increasing drug resistance.[1–3] Recent efforts have focused on finding optimal therapeutic regimens and on development of combination therapy with drugs already registered or those used to treat related diseases. To overcome the difficulties encountered in the control of HAT, the development of new therapeutic tools is urgent and efforts have been made to identify new molecular targets.[4]

CTP synthetase (CTPS), a glutamine amidotransferase (GAT) responsible for the de novo synthesis of cytidine triphosphate (CTP), was suggested to be a potential drug target for the treatment of HAT.[5] CTPS is the rate-limiting enzyme in the synthesis of cytosine nucleotides, which play an important role in various metabolic processes and provide the precursors necessary for the synthesis of RNA and DNA. CTPS is expressed both in humans and parasites, however, *T. brucei* seems to be more susceptible to CTPS inhibition due to low rate of the de novo synthesis and to the lack of the salvage pathways for cytosine or cytidine.[5]

Acivicin (Figure 1), an antibiotic isolated from the fermentation broths of *Streptomyces sviceus*, acts as a covalent inhibitor of several GATs, including CTPS.[6] Interestingly, its CTPS inhibi-

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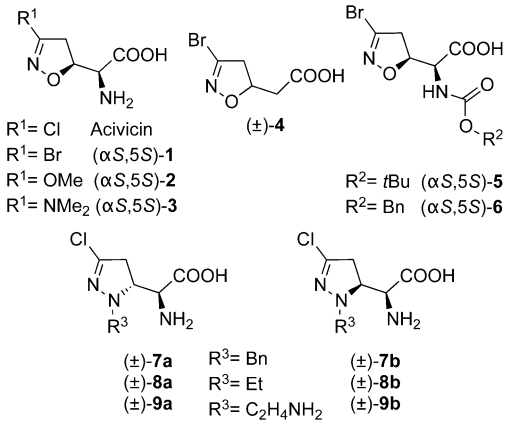


Figure 1. Structure of model and target compounds.

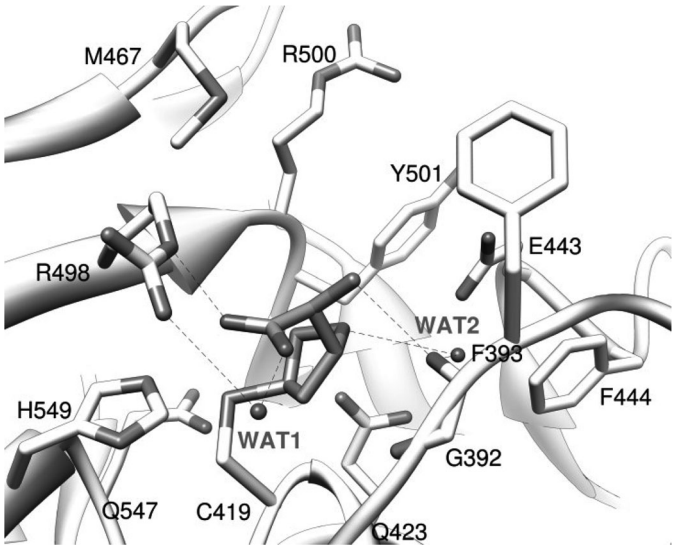


Figure 2. Binding mode of Acivicin into the CTPS catalytic site represented as transparent white ribbons. The ligand and the interacting residues are shown in grey and white sticks, respectively. Hydrogen bonds are represented with dashed black lines. All hydrogen atoms were removed for clarity.

tory activity has been correlated to the observed trypanocidal activity in bloodstream *T. brucei* cell cultures.[5]

The target of this project is the study of the structure–activity relationship of Acivicin and the design and synthesis of new analogues characterized by an increased affinity for CTPS as potential new trypanocidal agents.

Acivicin binds to the glutaminase domain of CTPS mimicking the natural substrate L-Gln. The enzyme is irreversibly inactivated due to the formation of a covalent adduct produced by nucleophilic attack of the thiol group of a Cys residue to the C3 of the isoxazoline nucleus, with displacement of the chlorine atom.[7] We previously reported that substituting the 3-chloro group with bromo to give 3-Br-Acivicin led to a threefold increase of the inhibitory potency against the target enzyme CTPS. Interestingly this translated into a twelvefold increase in the in vitro anti-trypanosomal activity, while leaving unaffected the toxicity against mammalian cells.[8] The observed increased activity against the enzyme is in accordance with the proposed mechanism of action.[7]

As an extension of our previous work intended at investigating the role of the C3 substituent of Acivicin, we have now prepared and tested the 3-methoxy analogue **2**, and compound **3**, which, at variance with the other compounds, should behave as a glutamine mimic without having a good leaving group at the C3 position, thus possibly inhibiting the enzyme in a noncovalent manner.

Furthermore, we have prepared the des-amino analogue of Br-Acivicin (±)-**4** to test the importance of the α-amino group on the biological activity, as our analysis of the crystal structure of *T. brucei* CTPS glutaminase domain in complex with Acivicin (PDB ID: 2W7T, Figure 2) shows that such a group is not directly involved in an ionic interaction with the binding pocket but establishes a charge reinforced hydrogen bond with the Gly392 backbone carbonyl oxygen atom. An additional aim of this project was to design Acivicin analogues with an increased affinity for *T. brucei* CTPS.

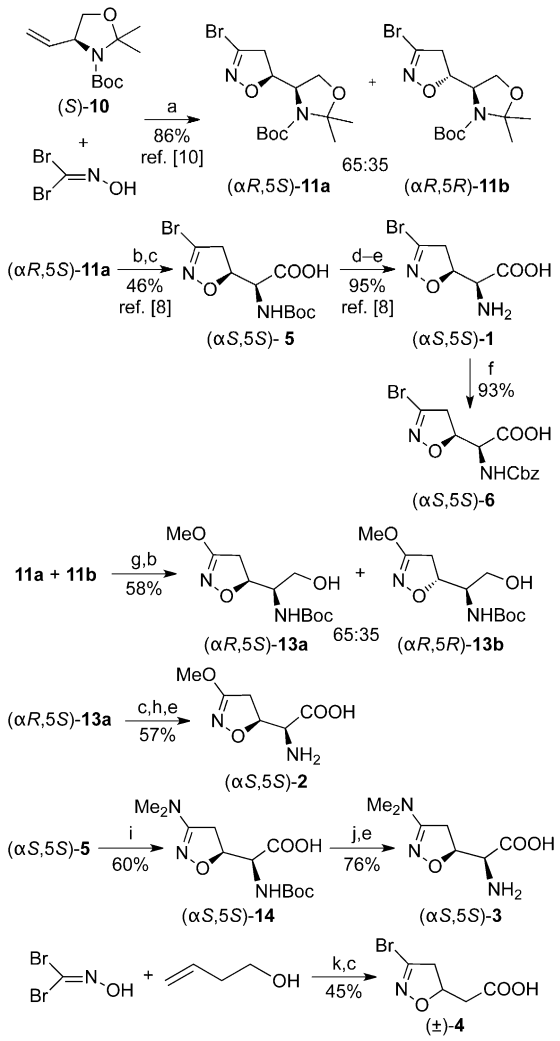
A typical medicinal chemistry approach to enhance the affinity for a target enzyme is to increase the molecular complexity, by inserting groups able to establish additional interaction with the binding pocket of the enzyme. In this line, the α-amino group of Br-Acivicin, which as we said does not seem to be involved in an ionic interaction, was exploited to design carbamates **5** and **6**. These derivatives, in addition to hydrogen bonding, may establish further hydrophobic or electronic interactions with the enzyme, thus reinforcing the binding. Moreover, we identified in the glutamine binding site two amino acid residue, that is, Phe393 and Glu443, that could be the target of additional interactions. To this aim, the isoxazoline nucleus of Acivicin was replaced by a pyrazoline ring, which represents a more versatile scaffold thanks to the ease of functionalization of the N1 position (compounds **7**–**9**) and allows decoration of the dihydroisoxazole fragment in a fragment-based drug discovery perspective.

Results and Discussion

Chemistry

As for the synthesis of Acivicin and Br-Acivicin, previously reported by us,[8,9] the key-step for the synthesis of the isoxazoline analogues was a 1,3-dipolar cycloaddition of bromonitrile oxide, generated in situ by base-promoted dehydrohalogenation of the stable precursor dibromoformaldoxime, to (*S*)-3-(*tert*-butoxycarbonyl)-2,2-dimethyl-4-vinyloxazolidine **10** (Scheme 1), producing the mixture of diastereomers **11a** and **11b**.[10] Compound (α*S*,5*S*)-**5** was obtained as previously described,[8] being a synthetic intermediate of Br-Acivicin **1**, while derivative (α*S*,5*S*)-**6** was simply prepared by treating compound (α*S*,5*S*)-**1** with benzylchloroformate in a mixture of tetrahydrofuran and water in the presence of $NaHCO_3$ (Scheme 1).

To obtain derivative **2**, we treated the mixture of cycloadducts **11a** and **11b** with a MeOH suspension of K_2CO_3 at 50 °C. The acetonide function was removed by treating with a mixture of acetic acid and water (5:1 *v/v*) at 40 °C and the diastereo-



Scheme 1. Synthesis of derivatives **2**–**6**. *Reagents and conditions:* a) $NaHCO_3$, EtOAc; b) AcOH/H_2O (5:1 *v/v*), 40 °C; c) $NaIO_4$, $RuO_2{\cdot}H_2O$, $CCl_4/H_2O/CH_3CN$; d) HBr/AcOH; e) Amberlite IR 120H, 1 N NH_4OH; f) CbzCl, $NaHCO_3$, H_2O/THF; g) K_2CO_3, MeOH, 50 °C; h) 30% TFA, CH_2Cl_2; i) 2 N $(Me)_2NH$, THF, 75 °C; j) 4 N HCl, dioxane; k) $NaHCO_3$, EtOAc, MW, 80 °C.

meric alcohols **13a** and **13b** were separated by flash chromatography. The sole *erythro* cycloadduct (α*R*,5*S*)-**13a** was oxidized to the corresponding carboxylic acid by treating with ruthenium(IV) oxide in the presence of sodium periodate in a mixture of water, acetonitrile and carbon tetrachloride.[11] Finally, the *tert*-butoxycarbonyl (Boc) protection was cleaved with a 30% solution of trifluoroacetic acid in dichloromethane, to give the final amino acid (α*S*,5*S*)-**2**, which was obtained in its zwitterionic form after ion-exchange chromatography, using Amberlite IR 120H and eluting the product with a 1 N aqueous solution of ammonia.

Derivative **3** was prepared from (α*S*,5*S*)-**5** treating with 2 N dimethylamine in tetrahydrofuran in a sealed vial and heating at 75 °C for 16 h. When the reaction was completed, the mixture was cooled at 0 °C to allow the precipitation of dimethylamine hydrobromide, which was easily removed by filtration. Finally, Boc-deprotection with 4 N hydrochloric acid in dioxane followed by ion-exchange chromatography using Amberlite IR 120H afforded derivative (α*S*,5*S*)-**3** as a zwitterion.

Derivative (±)-**4** was prepared by 1,3-dipolar cycloaddition of bromonitrile oxide, generated in situ by base-promoted dehydrohalogenation of the stable precursor dibromoformaldoxime, to commercially available 3-buten-1-ol. The reaction was carried out under microwave irradiation, heating for 1 h at 80 °C. The primary alcohol was oxidized to the corresponding carboxylic acid (±)-**4** using ruthenium(IV) oxide and sodium periodate in a biphasic system, as described above (Scheme 1).

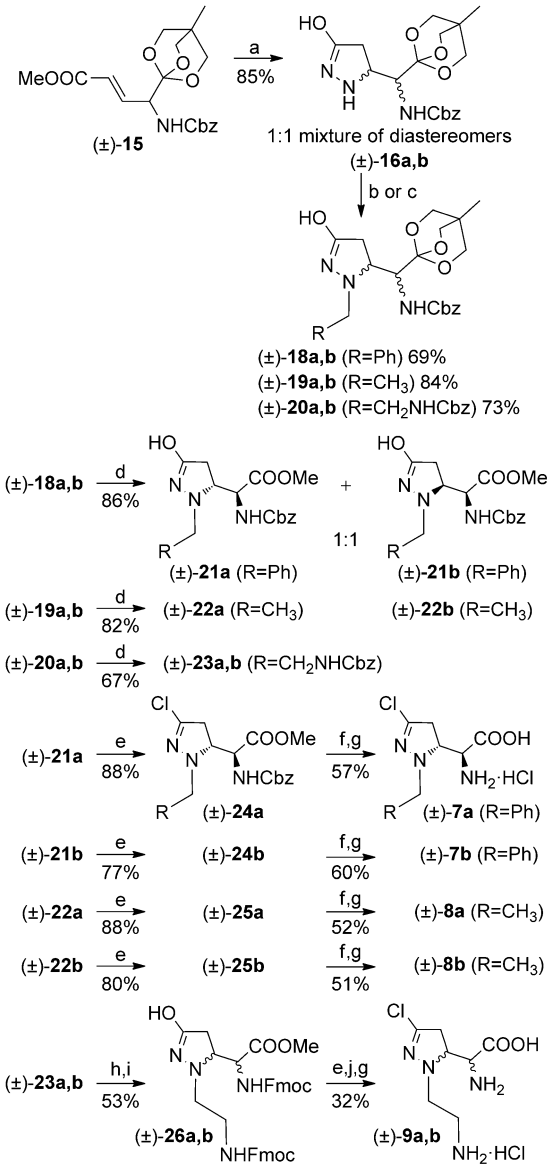
For the synthesis of derivatives **7**–**9**, the pyrazoline nucleus was generated in a one-pot procedure, through the condensation of hydrazine to the α,β-unsaturated ester (±)-**15**, followed by intramolecular cyclization. The α-carboxylate group of alkene (±)-**15** had to be appropriately protected to avoid its participation into the cyclization reaction. In detail, the protective group chosen for the carboxylic acid was an *ortho*-ester, that is, the trioxabicyclo[2.2.2]octane ester (OBO-ester).[12] This is an attractive protecting group for the carboxylic acid because it is highly stable in basic reaction media and does not react toward nucleophiles. Moreover, it can be easily removed in mild acidic conditions to give the free acids or, through a transesterification reaction, conveniently converted into the corresponding methyl ester. On the other hand, the amino group of (±)-**15** was protected as a benzylcarbamate, which guarantees a good stability in the subsequent steps of the planned reaction sequence, both in basic and in mild acidic conditions (Scheme 2).

The desired ester (±)-**15** was obtained as described,[13] and then reacted with hydrazine hydrate, in ethanol at reflux, to afford key intermediate (±)-**16a**,**b**, which was obtained as a 1:1 mixture (based on ^{1}H NMR spectrum) of two racemic diastereomers, inseparable by column chromatography at this step. Selective alkylation of the more nucleophilic N1 nitrogen was accomplished by treating (±)-**16a**,**b** with benzyl bromide in the presence of potassium carbonate and a catalytic amount of sodium iodide, under reflux, affording the mixture of diastereomers (±)-**18a**,**b**.[14] At this stage, the OBO ester was converted into the corresponding methyl ester through a two-step procedure involving treatment with pyridinium *para*-toluene sulfonate (PPTS) in a solution of methanol and water followed by transesterification with methanol and potassium carbonate.[15] The methyl esters (±)-**21a** and (±)-**21b** could finally be separated by silica gel column chromatography.

Due to low reactivity of the N1 in the nucleophilic substitution, for the synthesis of derivatives **19** and **20** a different approach had to be followed. Indeed the selective N1 functionalization was achieved through a reductive amination reaction, using acetaldehyde/$NaBH_4$ or Cbz-glycinal **17**/$NaBH_4$, respectively.

As described before for the N1-benzyl derivatives, the next stages involved the two-step transesterification of the OBO ester to methyl ester followed by chromatographic separation of the two diastereomers (±)-**22a** and (±)-**22b**, while in the case of (±)-**23a**,**b** the separation was unsuccessful.

Because ^{1}H NMR spectra did not allow the secure assignment of the relative configuration to the couple of diastereo-



Scheme 2. Synthesis of derivatives **7**–**9**. *Reagents and conditions:* a) $N_2H_4{\cdot}H_2O$, EtOH, reflux; b) BnBr, K_2CO_3, NaI, CH_3CN, reflux; c) CH_3CHO or $CbzNHCH_2CHO$ (**17**), $NaBH_4$, MeOH; d) i. PPTS, $MeOH/H_2O$; ii. K_2CO_3, MeOH; e) $POCl_3$, CH_3CN, reflux; f) BCl_3, CH_2Cl_2; g) i. Ambersep 900-OH, H_2O/dioxane, ii. 0.05 N HCl; h) 33% HBr, AcOH; i) Fmoc-*N*-hydroxysuccinimide ester, Na_2CO_3, H_2O/dioxane; j) piperidine, CH_2Cl_2.

mers (±)-**22a** and (±)-**22b**, we performed an X-ray crystallographic analysis on compound **22a**. Derivative (±)-**22a** turned out to possess the (α*S*,5R**) configuration; consequently the (α*S*,5S**) configuration was assigned to diastereomer (±)-**22b**. Subsequently, we assigned the relative stereochemistry to derivatives (±)-**21a** and (±)-**21b** by comparing their ^{1}H NMR spectra, in particular by comparing the coupling constant of the α-amino acidic proton.

The remaining reaction sequence was accomplished on the single diastereomers. The first step concerned the halogenation reaction at C3 of the heterocycle, which was performed by reacting derivatives (±)-**21a**, (±)-**21b**, (±)-**22a** and (±)-**22b** with phosphoryl chloride in acetonitrile at reflux; the corresponding 3-chloro derivatives were obtained in good yields.

The final amino acids (±)-**7a**, (±)-**7b**, (±)-**8a** and (±)-**8b** were obtained after deprotection of the amine, by treatment with BCl_3 in dichloromethane, and hydrolysis of the methyl ester. Because conventional hydrolysis with aqueous sodium hydroxide led to the partial replacement of the chlorine with the hydroxy group, this step was performed using a polymer-supported base Ambersep 900-OH. The carboxylic acid formed upon hydrolysis was bound by the resin and subsequently released by washing the resin with 0.05 N HCl (Scheme 2).

In the case of derivatives (±)-**23a**,**b**, due to the presence of two *N*-Cbz-protected functions, the synthetic protocol described above had to be slightly modified. The two Cbz groups could simultaneously be removed only by treatment with 33% HBr in acetic acid. Unfortunately, such a treatment caused the partial Cl/Br exchange. For such a reason, we removed these two groups before the chlorination step and then conveniently replaced them by two Fmoc groups, which could be subsequently cleaved in mild basic conditions.

Treatment of (±)-**26a**,**b** with $POCl_3$ in acetonitrile at reflux, followed by deprotection of the amino acid function, afforded final derivative (±)-**9a**,**b** still as a mixture of diastereomers, as separation was not possible at any stage of the synthetic procedure.

Biology

The first step of the biological evaluation concerned the assessment of the inhibitory activity of all new synthesized compounds in comparison with Acivicin and Br-Acivicin **1**, against recombinant CTPS from *T. b. brucei*. The results are listed in Table 1.

Subsequently, the compounds were tested as trypanocidal agents, estimating the cytotoxicity against the trypanosomes and against human HeLa cells. Where possible, the selectivity index (SI) was calculated. This parameter expresses the ratio between EC_{50} against human cells and EC_{50} against trypanosomes. Finally, all derivatives under study were converted into their corresponding methyl esters and re-tested against bloodstream form *T. b. brucei* (strain 427). These experiments were carried out to evaluate whether the observed differences between enzymatic and cellular activity could be somehow related to an impaired uptake of the derivatives into the target cells. Due to the hydrophilic nature of Acivicin (calculated $\log D = -2.84$ at pH 7.4), it is reasonable to hypothesize that its uptake into the cells takes place via amino acid transporters located on the plasma membrane. On the other hand, increasing the lipophilicity could facilitate the penetration through a passive diffusion mechanism.

Discussion

As previously observed with Br-Acivicin **1**, the nature of the leaving group at the C3 of the isoxazoline ring plays an important role in determining the inhibitory efficacy toward CTPS.[8] In fact, while **1** was threefold more potent than Acivicin as

Table 1. Inhibitory activity of Acivicin and derivatives **1**–**9** (and corresponding methyl esters) against recombinant *T. b. brucei* CTPS and their cytotoxicity against *T. b. brucei* and HeLa cells.

Compound	*T. b. brucei* CTPS IC_{50} [μM]	*T. b. brucei* EC_{50} [μM]	HeLa EC_{50} [μM]	SI[a]
Acivicin	0.320 ± 0.025	0.450 ± 0.010	16.3	36
Acivicin-COOMe	–	22.4 ± 1.3	41.9 ± 3.1	2
1	0.098 ± 0.010	0.038 ± 0.013	15.1 ± 1.2	397
1-COOMe	–	2.1 ± 0.5	12.5 ± 1.6	6
2	> 500	> 100	> 500	> 5
2-COOMe	–	> 500	> 500	–
3	> 500	> 100	> 500	> 5
3-COOMe	–	29.8 ± 1.7	> 500	> 17
4	> 500	> 100	> 500	> 5
4-COOMe	–	156.6 ± 13.2	> 500	> 3
5	0.043 ± 0.003	3.4 ± 0.4	4.6 ± 0.5	1.4
5-COOMe	–	14.8 ± 0.5	43.4 ± 2.5	3
6	1.740 ± 0.400	> 100	410 ± 39	–
6-COOMe	–	117.7 ± 5.0	39.1 ± 3.1	0.3
7a	> 500	56.2 ± 4.3	> 500	> 10
7a-COOMe	–	20.7 ± 1.0	> 500	> 24
7b	> 500	> 500	> 500	–
7b-COOMe	–	> 500	> 500	–
8a	0.100 ± 0.004	89.8 ± 7.8	> 500	> 5
8a-COOMe	–	10.0 ± 0.5	> 500	> 50
8b	> 500	> 500	> 500	–
8b-COOMe	–	> 500	> 500	–
9a,**b**	0.125 ± 0.002	23.7 ± 3.9	> 500	> 23
9a,**b**-COOMe	–	19.3 ± 0.6	> 500	> 26

[a] SI = selectivity index [EC_{50}(HeLa)/EC_{50}(*T. b. brucei*)]

a CTPS inhibitor, and 12-fold more efficacious as an anti-trypanosomal agent, derivative **2**, in which the halogen was replaced by a methoxy was not able to inhibit the enzyme up to 500 μM concentration. Also the insertion of a dimethylamine residue at the C3 position (compound **3**), mimicking the natural substrate L-Gln, produced a complete loss of the inhibitory potency against the enzyme, though a modest trypanocidal activity was detected (EC_{50} = 29.8 μM) when the amino acid was converted into the corresponding methyl ester.

Concerning the modifications involving the α-amino acid group, while the absence of the amino group (compound **4**) produced an inactive derivative, the conversion of the amine into a carbamate gave rise to quite interesting results. While the *N*-Cbz protected derivative **6** was fivefold weaker than Acivicin and 17-fold weaker than Br-Acivicin as a CTPS inhibitor, on the contrary, the *N*-Boc protected derivative **5** showed an excellent inhibitory activity against *T. b. brucei* CTPS (IC_{50} = 0.043 μM), being twice more potent than Br-Acivicin. The in vitro trypanocidal activity was not as good as expected. In fact, though compound **5** is provided with low micromolar anti-trypanosomal activity (EC_{50} = 3.4 μM), this is two order of magnitude weaker than that of Br-Acivicin (EC_{50} = 0.038 μM). We hypothesized that such a result could be related to decreased uptake, due to loss of the amino acidic character, which may hamper the active transport into the target cell. Conversion of derivative **5** into its more lipophilic methyl ester did not increase the trypanocidal activity. However, methyl ester may not be the ideal prodrug, as intracellular enzymatic hydrolysis may not occur at a satisfactory rate; thus more suitable prodrugs could be designed.

To rationalize the structure–activity relationship data (SARs) of the newly discovered compounds the X-ray structure of the covalent Acivicin–CTPS adduct (PDB ID: 2W7T, Figure 2) was used to run covalent docking calculations on compounds **5** and **6**. As represented in Figure 2, the covalent Acivicin–CTPS adduct is stabilized by several ligand–enzyme interactions and by the presence of several water molecules. In particular, one of them (WAT 1 in Figure 2) further reinforces the Coulombic interaction established by the Acivicin carboxylate group and R498 side chain by donating and accepting two hydrogen bonds with the aforementioned groups. In addition, another ordered water molecule mediates the ligand–enzyme interactions by donating two hydrogen bonds to the oxygen of the Acivicin isoxazoline ring and to G392 backbone CO. From this analysis it is clear that covalent and noncovalent ligand–enzyme interactions and water molecules all have important roles in CTPS inhibition. Therefore, in our analysis we decided to include these factors in docking calculations of compound **5** and **6**. In particular covalent docking calculations were attained by employing the AutoDock 4.2 (AD4) software and calculating a peculiar grid map for the site of attachment of the covalent ligand (C419 sulfur atom). In particular, a Gaussian function was constructed with zero energy at the site of attachment and steep energetic penalties at surrounding areas. This allowed the AD4 simulation to place the ligand atom that forms the covalent bond within the Gaussian well.[16]

Interestingly, a further improvement of the AD4 methodology has been recently reported in a pioneering work by Forli and Olson. These authors have developed a new force field and hydration docking method that allows for the automated prediction of waters mediating the binding of ligands with target proteins with no prior knowledge of the apo- or holoprotein hydration state.[17] Both methodologies, covalent and hydrated ligand docking, were applied in our inspection. With the aim of testing the method performances, Acivicin was docked itself in the enzyme site revealing that the lowest energy and also most populated conformation calculated by AD4 was virtually superimposable with the one detected in the X-ray structure. These encouraging results prompted the docking of **5** and **6**. Analysis of the results achieved for these compounds revealed that in both cases the ligand isoxazoline core was placed in the same binding position adopted by Acivicin (Figure 3a and b) so as to allow the Coulombic interaction of the ligand carboxylate group and R498. Interestingly, in both cases the hydrated covalent docking predicted the presence of two water molecules that should mediate almost the same ligand–enzyme interactions detected in the X-ray Acivicin–CTPS structure. The main difference between the binding poses predicted for **5** and **6** resided in the position of the *N*-α-substituents. Indeed while the *N*-Boc substituent of **5** is projected toward the outer part of the enzyme active site taking contacts with M467 and F393, in **6** the *N*-Cbz substituent is placed in a rather hydrophilic cleft were unfavorable interactions take place with E443. This should lead to a rather unsta-

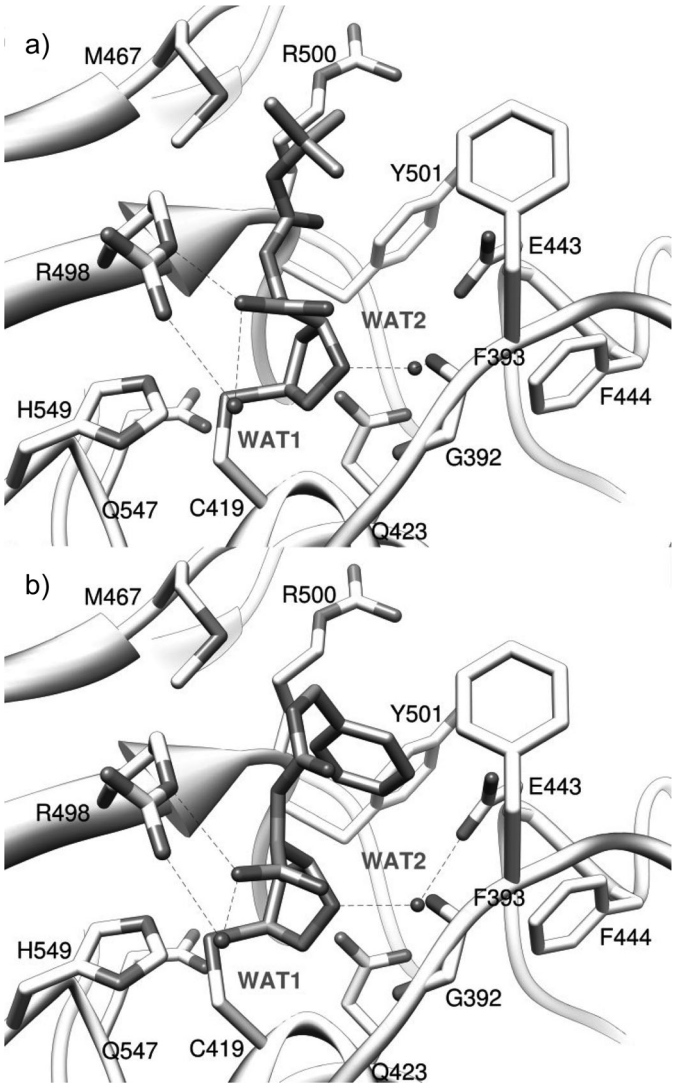


Figure 3. Binding mode of a) **5** and b) **6** into the CTPS catalytic site represented as transparent white ribbons. The ligands and the interacting residues are shown in grey and white sticks, respectively. Hydrogen bonds are represented with dashed black lines. All hydrogen atoms were removed for clarity.

ble covalent adduct thus negatively influencing CTPS inhibitory potency of **6**.

The results obtained with the pyrazoline analogues are more difficult to interpret. In fact, two of these derivatives, notably the *N*-ethyl derivative (±)-**8a** and the *N*-aminoethyl derivative (±)-**9a**,**b** displayed an excellent sub-micromolar inhibition against CTPS, being about threefold more potent than Acivicin. It must be noted that the *N*-aminoethyl derivative (±)-**9a**,**b** was preliminarily tested as a diastereomeric mixture, thus testing the single stereoisomer may actually give an even lower IC_{50} value. However, no molecular modeling studies were attempted for these compounds. In fact, if for the halogen substituted isoxazoline ring of **5** and **6** we could assume the same reactivity and same covalent adduct formation displayed by Acivicin, no experimental data are available for the pyrazolines **8** and **9** (**a** and **b**) to support our modeling studies.

Disappointingly, despite their excellent CTPS inhibitory potency, superior to that of Acivicin, both derivatives showed a trypanocidal activity significantly lower than Acivicin. Upon conversion into the corresponding methyl esters the trypanocidal activity was improved, more significantly in the case of derivative **8a**, the activity of which increased about ninefold. This may indicate that the pyrazoline analogues have a decreased affinity for the amino acid transporters responsible for Acivicin uptake. However, the activity of the methyl esters is still too weak relative to the very good inhibitory activity against CTPS of the free amino acids. Possible additional explanations may involve a not efficient regeneration of the drug from its methyl ester prodrug, or the instability of the molecule in the intracellular medium.

Concerning the selectivity index, from a drug discovery point of view, ideally a selectivity index greater than 200 would be required to be encouraged to move lead compounds forward. Rather surprisingly, only Br-Acivicin has a notable selectivity index (SI 397), which is drastically decreased upon esterification, whereas pyrazoline derivatives show a modest increase in trypanocidal activity and selectivity upon esterification, for example, compound **8a**.

Conclusions

The present research project was focused on the study of the structure–activity relationship of Acivicin. In summary, the results obtained have shown that while substituting the 3-chloro with a 3-bromo increased the inhibitory potency of Acivicin against the target enzyme CTPS threefold, the substitution with a 3-methoxy group produced a complete loss of the activity, confirming the important role played by the leaving group in the C3 position of the isoxazoline ring. In this respect it would be highly interesting to test the 3-iodo analogue of Acivicin; however attempts to synthesize such derivative have failed so far.

Another important finding of the present research project is that the inhibitory activity against CTPS can be increased by applying a molecular complication approach, that means inserting groups able to establish additional interaction with the binding pocket of the enzyme. This can be realized either functionalizing the nitrogen of the amino acid moiety, or replacing the isoxazoline ring with a pyrazoline and inserting the selected substituents at the N1 position.

The parallel analysis of the enzymatic activity and the in vitro anti-trypanosomal activity of the derivatives in this study, leads to the conclusion that an increased inhibitory activity toward CTPS may produce a great increase of the anti-trypanosomal activity, as was the case for Br-Acivicin, but this cannot be taken as a general rule, as other important factors may play a role, notably the ability of the molecules to penetrate into the target cells.

Future efforts will be devoted to understanding the reasons behind the decreased anti-trypanosomal activity of the potent CTPS inhibitors identified in the present work and to their optimization, in particular through the design of suitable prodrugs

able to guarantee a good penetration into the cells followed by an efficient release of the active drug.

Experimental Section

Materials and methods

All reagents were purchased from Sigma. Enantiomerically pure Acivicin and Br-Acivicin, used as standard in the biological assays, were prepared as previously described.[8,9] Dibromoformaldoxime (DBF) was prepared according to a published procedure.[18] The diastereomeric mixture of cycloadducts **11a** and **11b** was prepared as previously described[10] from DBF, and (*S*)-3-(*tert*-butoxycarbonyl)-2,2-dimethyl-4-vinyloxazolidine (*S*)-**10**.[19] Compound (α*S*,5*S*)-**5** was prepared as previously described.[8] Alkene (±)-**15** was obtained according to a reported method.[13] *N*-Cbz-glycinal **17** was prepared according to a published procedure.[20]

^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) are expressed in Hz. Rotary power determinations were carried out using a Jasco P-1010 spectropolarimeter, coupled with a Haake N3-B thermostat. TLC analyses were performed on commercial silica gel 60 F254 aluminum sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution or ninhydrin. Melting points were determined on a model B 540 Büchi apparatus and are uncorrected. MS analyses were performed on a Varian 320-MS triple quadrupole mass spectrometer with ESI source. Microanalyses (C, H, N) of new compounds were within ±0.4% of theoretical values.

For biological assays, the final amino acids were converted into their corresponding methyl esters, by adding an ethereal solution of diazomethane (3×20 μL aliquots) to the dried compound, while on ice. After 30 min the samples were allowed to warm to room temperature and left to evaporate to dryness in a fume hood. Electrospray mass spectrometry was used to confirm 100% conversion.

Synthesis of (*S*)-(benzyloxycarbonylamino)-2-((*S*)-3-bromo-4,5-dihydroisoxazol-5-yl)acetic acid [(α*S*,5*S*)-6]: To a stirred solution of (α*S*,5*S*)-**1** (55 mg, 0.25 mmol) in a 1:1 mixture of H_2O and THF (8 mL) was added $NaHCO_3$ (62 mg, 0.75 mmol). After the gas evolution finished, benzyl chloroformate (39 μL, 0.28 mmol) was added dropwise and the reaction was stirred at room temperature for 1 h. After disappearance of the starting material, the organic solvent was evaporated under reduced pressure and the aqueous phase was washed with Et_2O (1×4 mL), made acidic with 2 N HCl and extracted with EtOAc (3×5 mL). The organic extract was dried over anhydrous Na_2SO_4, and the solvent evaporated to give compound (α*S*,5*S*)-**6** (83 mg, 93% yield) as a hygroscopic white foam; R_f: 0.35 (CH_2Cl_2/MeOH, 95:5 + 1% AcOH); $[\alpha]_D^{20}$: +167 (*c* = 0.2 H_2O); ^{1}H NMR ($CDCl_3$): δ = 3.18–3.58 (m, 2H), 4.58 (dd, *J* = 3.6, 8.2, 1H), 4.85–4.98 (m, 1H), 5.12 (s, 2H), 5.92 (br d, *J* = 7.7, 1H), 7.25–7.40 (m, 5H), 8.90 ppm (br s, 1H); ^{13}C NMR ($CDCl_3$): δ = 44.2, 56.6, 67.9, 81.9, 128.4, 128.7, 128.9, 135.9, 138.7, 156.4, 171.7 ppm; MS: 357.0 $[M+H]^+$; Anal. calcd for $C_{13}H_{13}BrN_2O_5$: C 43.72, H 3.67, N 7.84, found: C 43.90; H 3.80, N 7.98.

Synthesis of *tert*-butyl (*R*)-2-hydroxy-1-((*S*)-3-methoxy-4,5-dihydroisoxazol-5-yl)ethylcarbamate [(α*R*,5*S*)-13a]: a) The mixture of diastereomers **11a**,**b** (800 mg, 2.29 mmol) was dissolved in dry MeOH (12 mL) and K_2CO_3 (1.0 g, 7.23 mmol) was added. The reaction was heated at 50 °C for 2 h. The volatiles were removed under vacuum and the residue was dissolved in EtOAc (10 mL) and washed with H_2O (2×10 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated to dryness, obtaining the crude product (625 mg, 91%) that was directly submitted to the next step. b) The crude intermediate obtained from the previous step (625 mg, 2.08 mmol) was treated with a 5:1 mixture of AcOH/H_2O (15 mL) and the solution was stirred at 40 °C for 48 h. The solvent was evaporated and the residue was dissolved in EtOAc (10 mL) and washed with H_2O (2×5 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. The crude material was purified by column chromatography (cyclohexane/EtOAc 1:1, then EtOAc) to obtain the desired compound (α*R*,5*S*)-**13a** (227 mg) as a yellow oil; R_f: 0.55 (cyclohexane/EtOAc, 2:8); $[\alpha]_D^{20}$: +9.61 (*c* = 1.0 $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ = 1.42 (s, 9H), 2.70–2.80 (m, 1H), 2.90–3.08 (m, 2H), 3.60–3.94 (m, 3H), 3.81 (s, 3H), 4.65 (ddd, *J* = 8.0, 8.0, 16.8, 1H), 5.20 ppm (br d, *J* = 7.7, 1H); ^{13}C NMR ($CDCl_3$): δ = 28.5, 35.7, 54.18, 57.5, 61.4, 80.1, 80.7, 156.2, 168.5 ppm; MS: 261.2 $[M+H]^+$.

Synthesis of (*S*)-2-amino-2-((*S*)-3-methoxy-4,5-dihydroisoxazol-5-yl)acetic acid [(α*S*,5*S*)-2]: a) Compound (α*R*,5*S*)-**13a** (227 mg, 0.87 mmol) was dissolved in a mixture of H_2O (2.9 mL), CH_3CN (1.9 mL) and CCl_4 (1.9 mL). $NaIO_4$ (744 mg, 3.48 mmol) and a catalytic amount of $Ru_2O{\cdot}H_2O$ (2.3 mg) were added and the suspension was vigorously stirred at room temperature until disappearance of the starting material. After 45 min, CH_2Cl_2 (10 mL) and H_2O (10 mL) were added. The organic layer was separated and evaporated under reduced pressure. The residue was dissolved in EtOAc (5 mL), the organic layer was extracted with a 10% aqueous solution of K_2CO_3 (3×5 mL) and the aqueous phase was made acidic with 2 N HCl and newly extracted with EtOAc (3×5 mL). The organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and the solvent evaporated to yield the desired carboxylic acid (174 mg, 73% yield) as a white solid, which was used without further characterization in the next step. b) The carboxylic acid obtained from the previous step (174 mg, 0.63 mmol) was dissolved in CH_2Cl_2 (1.6 mL) and TFA (0.48 mL, 6.30 mmol) was added dropwise at 0 °C. The reaction was stirred for 2 h. The volatiles were removed under reduced pressure and the residue was dissolved in H_2O and submitted to cation exchange chromatography using Amberlite IR-120H. The acidic solution was slowly eluted onto the resin, and then the column was washed with H_2O until the pH was neutral. The compound was eluted off the resin with 1 N aq. NH_3 and the product-containing fractions (detected with ninhydrin stain on a TLC plate) were combined. The solvent was freeze-dried to give compound (α*S*,5*S*)-**2** (87 mg, 80% yield) as a white solid; mp: dec >170 °C; R_f: 0.28 (BuOH/H_2O/AcOH, 4:2:1); $[\alpha]_D^{20}$: +78 (*c* = 0.5 H_2O); ^{1}H NMR (D_2O): δ = 3.07 (dd, *J* = 8.0, 17.1, 1H), 3.16 (dd, *J* = 10.5, 17.1, 1H), 3.70 (s, 3H), 3.89 (d, *J* = 3.6, 1H), 5.04 ppm (ddd, *J* = 3.6, 8.0, 10.5, 1H); ^{13}C NMR (D_2O): δ = 33.8, 56.2, 58.0, 79.2, 169.5, 170.3 ppm; MS: 175.0 $[M+H]^+$; Anal. calcd for $C_6H_{10}N_2O_4$: C 41.38, H 5.79, N 16.09, found: C 41.08, H 5.62, N 15.81.

Synthesis of (*S*)-2-amino-2-((*S*)-3-(dimethylamino)-4,5-dihydroisoxazol-5-yl)acetic acid [(α*S*,5*S*)-3]: a) In a sealed vial, compound (α*S*,5*S*)-**5** (300 mg, 0.93 mmol) was treated with a 2 N solution of dimethylamine in THF (10 mL) and the reaction mixture was heated at 75 °C until disappearance of the starting material (16 h). The volatiles were removed under reduced pressure and the residue was dissolved in EtOAc (5 mL) and extracted with 0.1 N NaOH (3×5 mL). The aqueous phase was made acidic with 1 N HCl and extracted with EtOAc (4×5 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum, obtaining compound (α*S*,5*S*)-**14** (160 mg, 60% yield), as a white foam; R_f: 0.16 (CH_2Cl_2/MeOH, 95:5 + 1% AcOH). ^{1}H NMR ($CDCl_3$): δ = 1.45 (s,

9H), 2.82 (s, 6H), 3.19 (dd, J=10.5, 15.8, 1H), 3.38 (dd, J=5.3, 15.8, 1H), 4.25–4.35 (m, 1H), 4.70–4.82 (m, 1H); 5.65 (br d, J=7.4, 1H), 7.95 ppm (br s, 1H); ^{13}C NMR ($CDCl_3$): δ=28.5, 37.2, 39.7, 56.3, 80.5, 80.8, 155.8, 162.5, 171.5 ppm; MS: 288.3 $[M+H]^+$. b) Compound ($\alpha S,5S$)-**14** obtained from the previous step (120 mg, 0.42 mmol) was treated with a 4 N solution of HCl in dioxane (10 mL) at 0 °C. The reaction was stirred at room temperature for 2 h. The volatiles were removed under vacuum and the residue was dissolved in H_2O and submitted to cation exchange chromatography using Amberlite IR-120H. The acidic solution was slowly eluted onto the resin, and then the column was washed with H_2O until the pH was neutral. The compound was eluted off the resin with 1 N aq. ammonia, and the product-containing fractions (detected with ninhydrin stain on a TLC plate) were combined. The solvent was freeze-dried to give compound ($\alpha S,5S$)-**3** (60 mg, 76% yield) as a white solid; mp: dec >193 °C; R_f: 0.19 (BuOH/H_2O/AcOH, 4:2:1); $[\alpha]_D^{20}$: +105 (c=0.5 H_2O); ^{1}H NMR (D_2O): δ=2.69 (s, 6H), 3.13 (dd, J=7.7, 16.5, 1H), 3.13 (dd, J=11.5, 16.5, 1H), 3.81 (d, J=3.9, 1H), 4.85 ppm (ddd, J=3.9, 7.7, 11.5, 1H); ^{13}C NMR (D_2O): δ=35.6, 38.8, 56.0, 77.9, 164.1, 170.7 ppm; MS: 188.1 $[M+H]^+$; Anal. calcd for $C_7H_{13}N_3O_3$. C 44.91, H 7.00, N 22.45, found: C 45.10, H 7.22, N 22.70.

Synthesis of 2-(3-bromo-4,5-dihydroisoxazol-5-yl)acetic acid [(±)-4]: a) Solid $NaHCO_3$ (0.84 g, 10.0 mmol) and DBF (0.40 g, 2.0 mmol) were added to a 5% solution of 3-buten-1-ol (0.14 g, 2.00 mmol) in EtOAc (2.8 mL). The reaction mixture was heated in the microwave at 80 °C for 1 h, and the progress of the reaction was monitored by TLC (cyclohexane/EtOAc 8:2). Few drops of H_2O were added and the solvent was decanted. The organic phase was washed with H_2O (2×10 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. Purification of the crude material by column chromatography (cyclohexane/EtOAc 1:1) afforded the desired cycloadduct (237 mg, 61% yield), as a yellow oil. R_f: 0.26 (cyclohexane/EtOAc 1:1); ^{1}H NMR ($CDCl_3$): δ=1.80–2.03 (m, 2H), 2.59 (br s, 1H), 2.94 (dd, J=8.5, 17.3, 1H), 3.32 (dd, J=10.2, 17.3, 1H), 3.72–3.79 (m, 2H), 4.84 ppm (dddd, J=5.2, 8.5, 8.5, 10.2, 1H); ^{13}C NMR ($CDCl_3$): δ=37.5, 47.0, 59.2, 80.25, 138.1 ppm. b) The product obtained in the previous step (237 mg, 1.22 mmol) was dissolved in a mixture of H_2O (4.0 mL), CH_3CN (2.7 mL) and CCl_4 (2.7 mL). $NaIO_4$ (1.04 g, 4.88 mmol) and a catalytic amount of $Ru_2O{\cdot}H_2O$ (2.5 mg) were added and the suspension was vigorously stirred at room temperature. After disappearance of the starting material (45 min), CH_2Cl_2 (10 mL) and H_2O (10 mL) were added. The organic layer was separated and evaporated under reduced pressure. The residue was dissolved in EtOAc (5 mL), the organic layer was extracted with a 10% aqueous solution of K_2CO_3 (3×5 mL) and the aqueous phase was made acidic with 2 N HCl and newly extracted with EtOAc (3×5 mL). The organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and the solvent evaporated to yield the desired product (±)-**4** (185 mg, 73% yield) as a white solid; crystallized from hexane/EtOAc as white prisms; mp: 82–83 °C; R_f: 0.40 (CH_2Cl_2/MeOH, 95:5+1% AcOH); ^{1}H NMR ($CDCl_3$): δ=2.54 (dd, J=7.3, 16.8, 1H), 2.90 (dd, J=6.0, 16.8, 1H), 3.00 (dd, J=7.3, 17.4, 1H), 3.45 (dd, J=10.4, 17.4, 1H), 5.05 ppm (dddd, J=6.0, 7.3, 7.3, 10.4, 1H); ^{13}C NMR ($CDCl_3$): δ=39.2, 46.8, 77.5, 137.6, 175.3 ppm; MS: 207.8, 205.8 $[M-H]^-$; Anal. calcd for $C_5H_6BrNO_3$: C 28.87, H 2.91, N, 6.73, found: C 29.10, H 3.05, N 6.90.

Synthesis of benzyl (3-hydroxy-4,5-dihydro-1*H*-pyrazol-5-yl)(4-methyl-2,6,7-trioxabicyclo[2.2.2]octan-1-yl)methylcarbamate [(±)-16a,b]: To a solution of compound (±)-**15** (5.18 g, 13.75 mmol) in EtOH (110 mL) was added hydrazine monohydrate (3.33 mL, 68.73 mmol) and the reaction mixture was held at reflux for 3 h. The solvent was removed under vacuum and the crude was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 95:5 to 90:10 gradient) to give an inseparable mixture of the two diastereomers (±)-**16a,b** (4.41 g, 85% yield, 1:1 mixture of diastereomers), as a white foam; R_f: 0.55 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR ($CDCl_3$): δ=1.79 (s, 3H), 2.32 (dd, J=7.4, 16.8, 0.5H), 2.48–2.60 (m, 1.5H), 3.88 and 3.90 (s, 6H), 4.00–4.18 (m, 2H) 5.09 (s, 2H), 5.20 (br d, J=8.5, 0.5H), 5.54 (br d, J=9.6, 0.5H), 7.30–7.42 ppm (m, 5H); MS: 378.2 $[M+H]^+$.

Synthesis of benzyl (1-benzyl-3-hydroxy-4,5-dihydro-1*H*-pyrazol-5-yl)(4-methyl-2,6,7-trioxabicyclo[2.2.2]octan-1-yl)methylcarbamate [(±)-18a,b]: Compound (±)-**16a,b** (1.02 g, 2.7 mmol) was dissolved in CH_3CN (50 mL). Benzyl bromide (0.32 mL, 2.7 mmol), K_2CO_3 (373 mg, 2.7 mmol) and NaI (41 mg, 0.27 mmol) were added to the solution. The reaction mixture was held at reflux for 90 min. After disappearance of the starting material, the solvent was evaporated under vacuum and the residue was diluted with EtOAc (50 mL). The organic layer was washed with 3% aq. NH_4Cl (50 mL), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The product (±)-**18a,b** (870 mg, 69% yield) was isolated by column chromatography (silica gel, cyclohexane/EtOAc, 1:1, 100% EtOAc gradient) and was used in the next step without further characterization.

Synthesis of benzyl (*S)-(methoxycarbonyl)((*R**)-1-benzyl-4,5-dihydro-3-hydroxy-1*H*-pyrazol-5-yl)methylcarbamate [(±)-21a] and benzyl (*S**)-(methoxycarbonyl)((*S**)-1-benzyl-4,5-dihydro-3-hydroxy-1*H*-pyrazol-5-yl)methylcarbamate [(±)-21b]:** a) To a solution of (±)-**18a,b** (870 mg, 1.9 mmol) in MeOH (12 mL) and bi-distilled H_2O (1.7 mL), a catalytic amount of PPTS (47 mg, 0.19 mmol) was added. The reaction mixture was stirred for 1 h at room temperature until completion. After evaporation of MeOH under reduced pressure, the residue was dissolved in EtOAc (5 mL) and washed with bi-distilled H_2O (2×5 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was dissolved in MeOH (18 mL) and K_2CO_3 (54 mg, 0.4 mmol) was added to the solution. The reaction mixture was stirred for 3 h at room temperature. The solvent was evaporated in vacuo and the residue was dissolved in EtOAc (5 mL) and washed with 3% aq. NH_4Cl (10 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated to dryness. Purification via column chromatography (silica gel, eluent: EtOAc) allowed the separation of the two diastereomers. (±)-**21a**: 322 mg (43% yield); crystallized from EtOAc/hexane as a foamy white solid; mp: 123–125 °C; R_f: 0.66 (EtOAc); ^{1}H NMR ($CDCl_3$): δ=2.32 (d, J=17.3, 1H), 2.80 (dd, J=9.4, 17.3, 1H), 3.58 (s, 3H), 3.72 (d, J=12.7, 1H), 3.80 (d, J=12.7, 1H), 3.87–3.98 (m, 1H), 4.46 (dd, J=2.5, 9.4, 1H), 5.05 (d, J=12.1, 1H), 5.13 (d, J=12.1, 1H), 5.70 (d, J=9.4, 1H), 7.18–7.40 (m, 10H), 7.78 ppm (s, 1H); ^{13}C NMR ($CDCl_3$): δ=31.8, 52.8, 58.4, 61.9, 64.8, 67.6, 128.3, 128.4, 128.7, 128.8, 128.9, 129.9, 135.4, 136.3, 157.2, 170.5, 173.8 ppm; MS: 398.2 $[M+H]^+$; Anal. calcd for $C_{21}H_{23}N_3O_5$: C 63.46, H 5.83, N 10.57, found: C 63.66, H 5.95, N 10.74; (±)-**21b**: 325 mg (43% yield); crystallized from EtOAc/hexane as a foamy white solid; mp: 108–110 °C; R_f: 0.51 (EtOAc); ^{1}H NMR ($CDCl_3$): δ=2.47 (d, J=17.1, 1H), 2.80 (dd, J=9.1, 17.1, 1H), 3.68 (s, 3H), 3.63–3.75 (m, 1H), 3.79 (d, J=12.5, 1H), 3.84 (d, J=12.5, 1H), 4.27 (dd, J=4.1, 8.1, 1H), 4.97 (d, J=12.3, 1H), 5.04 (d, J=12.3, 1H), 5.67 (d, J=8.1, 1H), 7.20–7.40 ppm (m, 11H); ^{13}C NMR ($CDCl_3$): δ=31.6, 52.8, 56.6, 63.5, 64.5, 67.3, 128.3, 128.5, 128.7, 128.8, 129.0, 129.8, 135.5, 136.3, 155.7, 170.4, 173.8 ppm; MS: 398.2 $[M+H]^+$; Anal. calcd for $C_{21}H_{23}N_3O_5$: C 63.46, H 5.83, N 10.57, found: C 63.63, H 5.94, N 10.71.

Synthesis of benzyl (*S)-(methoxycarbonyl)((*R**)-1-benzyl-3-chloro-4,5-dihydro-1*H*-pyrazol-5-yl)methylcarbamate [(±)-24a]:**

$POCl_3$ (0.85 mL, 9.3 mmol) was added to a stirred solution of compound (±)-**21 a** (370 mg, 0.93 mmol) in CH_3CN (40 mL). The reaction mixture was held at reflux for 3 h, until complete conversion was observed by TLC analysis. After removal of the solvent under vacuum, the residue was dissolved in EtOAc (50 mL) and the solution was added dropwise to a crushed ice solution (30 mL). The organic layer was separated and washed with H_2O (50 mL) and brine (50 mL). The pooled organic layers were dried over Na_2SO_4 filtered and evaporated. The obtained reaction crude was purified by column chromatography on silica gel (eluent: cyclohexane/EtOAc 8:2, then 7:3) to obtain the desired product (±)-**24 a** (342 mg, 88% yield); crystallized from diisopropyl ether as white prisms; mp: 124–126 °C; R_f: 0.30 (cyclohexane/EtOAc, 8:2); ^{1}H NMR ($CDCl_3$): δ = 2.86 (dd, J = 10.6, 17.6, 1H), 2.98 (dd, J = 11.7, 17.6, 1H), 3.74 (d, J = 14.4, 1H), 3.79 (s, 3H), 4.10 (dd, J = 10.6, 11.7, 1H), 4.26 (d, J = 14.4, 1H), 4.46 (d, J = 9.4, 1H), 5.16 (s, 1H), 5.63 (d, J = 9.4, 1H), 7.20–7.41 ppm (m, 10H); ^{13}C NMR ($CDCl_3$): δ = 41.8, 53.0, 55.5, 59.1, 63.9, 67.6, 128.1, 128.3, 128.5, 128.7, 128.8, 129.8, 135.6, 136.2, 143.1, 157.0, 171.1 ppm; MS: 416.1 $[M+H]^+$; Anal. calcd for $C_{21}H_{22}ClN_3O_4$: C 60.65, H 5.33, N 10.10, found: C 60.42, H 5.26, N 9.97.

Synthesis of (*S)-2-amino-2-((*R**)-1-benzyl-3-chloro-4,5-dihydro-1*H*-pyrazol-5-yl)acetic acid hydrochloride [(±)-7 a]:** a) A 1 M solution of BCl_3 in CH_2Cl_2 (1.64 mL, 1.64 mmol) was added dropwise to a solution of (±)-**24 a** (342 mg, 0.82 mmol) in CH_2Cl_2 (30 mL) cooled at −10 °C. The reaction mixture was allowed to warm up to room temperature and stirred for 2 h. After complete conversion, the reaction was quenched by the addition of 0.2 N HCl (17 mL). The aqueous layer was separated, made basic with aq. K_2CO_3 (pH 8) and extracted with EtOAc (3×30 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated under vacuum. Purification via column chromatography (silica gel, EtOAc) afforded the free amine, which was immediately dissolved in a mixture of dioxane (9.8 mL) and bi-distilled H_2O (4.5 mL). Ambersep 900-OH resin (300 mg) was added. The reaction mixture was stirred for 4 h, until disappearance of the starting material. The resin was filtered and washed with dioxane, bi-distilled H_2O and Et_2O to remove impurities. The product was then eluted off the resin with 0.05 N HCl and the product-containing fractions (detected with ninhydrin on a TLC plate) were combined and concentrated in vacuo to obtain the desired hydrochloride amino acid (±)-**7 a** (142 mg, 57% yield), as a white solid; mp: dec > 141 °C; R_f: 0.62 (butanol/H_2O/AcOH, 4:2:1); ^{1}H NMR (D_2O): δ = 2.85 (dd, J = 10.0, 18.5, 1H), 3.17 (dd, J = 12.3, 18.5, 1H), 3.75 (d, J = 14.2, 1H), 3.91 (d, J = 2.2, 1H), 4.13 (ddd, J = 2.2, 10.0, 12.3, 1H), 4.20 (d, J = 14.2, 1H), 7.15–7.35 ppm (m, 5H); ^{13}C NMR (D_2O): δ = 41.2, 55.0, 58.8, 62.0, 128.3, 128.8, 130.0, 135.4, 146.0, 171.2 ppm; MS: 268.1 $[M+H]^+$; Anal. calcd for $C_{12}H_{15}Cl_2N_3O_2$: C 47.38, H 4.97, N 13.81, found: C 47.03, H 5.00, N 13.71.

Synthesis of benzyl (*S)-(methoxycarbonyl)((*S**)-1-benzyl-3-chloro-4,5-dihydro-1*H*-pyrazol-5-yl)methylcarbamate [(±)-24 b]:** Compound (±)-**24 b** was synthesized following the procedure reported for (±)-**24 a** starting from intermediate (±)-**21 b**. (±)-**24 b**: colorless oil, 77% yield; R_f: 0.26 (cyclohexane/EtOaAc, 8:2); ^{1}H NMR ($CDCl_3$): δ = 2.82 (dd, J = 11.5, 16.9, 1H), 3.10 (dd, J = 11.5, 16.9, 1H), 3.74 (s, 3H), 3.76–3.84 (m, 1H), 4.07 (d, J = 14.0, 1H), 4.31 (d, J = 14.0, 1H), 4.44–4.52 (m, 1H), 5.11 (s, 2H), 5.51 (d, J = 6.3, 1H), 7.22–7.40 ppm (m, 10H); ^{13}C NMR ($CDCl_3$): δ = 40.5, 53.1, 55.0, 58.9, 65.9, 67.5, 128.0, 128.1, 128.2, 128.3, 128.7, 129.8, 135.8, 136.2, 142.1, 156.0, 170.3 ppm; MS: 416.2 $[M+H]^+$; Anal. calcd for $C_{21}H_{22}ClN_3O_4$: C 60.65, H 5.33, N 10.10, found: C 60.75, H 5.40, N 10.23.

Synthesis of (*S)-2-amino-2-((*S**)-1-benzyl-3-chloro-4,5-dihydro-1*H*-pyrazol-5-yl)acetic acid hydrochloride [(±)-7 b]:** Compound (±)-**7 b** was synthesized following the procedure reported for (±)-**7 a** starting from intermediate (±)-**24 b**. (±)-**7 b**: white solid, 60% yield mp: dec > 156 °C; R_f: 0.58 (butanol/H_2O/AcOH, 4:2:1); ^{1}H NMR (D_2O): δ = 2.82 (dd, J = 11.4, 18.2, 1H), 3.01 (dd, J = 11.4, 18.2, 1H), 3.83 (d, J = 4.1, 1H), 4.05 (ddd, J = 4.1, 11.4, 11.4, 1H), 4.17 (q, J = 13.8, 2H), 7.21–7.39 ppm (m, 5H); ^{13}C NMR (D_2O): δ = 38.3, 53.3, 58.4, 63.2, 128.5, 129.1, 129.8, 135.4, 145.5, 169.8 ppm; MS: 268.1 $[M+H]^+$; Anal. calcd for $C_{12}H_{15}Cl_2N_3O_2$: C 47.38, H 4.97, N 13.81, found: C 47.01, H 5.02, N 13.70.

Synthesis of benzyl (1-ethyl-3-hydroxy-4,5-dihydro-1*H*-pyrazol-5-yl)(4-methyl-2,6,7-trioxabicyclo[2.2.2]octan-1-yl)methylcarbamate [(±)-19 a,b]: To a solution of compound (±)-**16 a,b** (1.67 g, 4.42 mmol) in MeOH (100 mL) was added acetaldehyde (0.41 mL, 6.84 mmol) at room temperature. After 30 min, $NaBH_4$ (245 mg, 6.61 mmol) was added and the reaction mixture was allowed to stir for an additional hour. After disappearance of the starting material, the solvent was evaporated under reduced pressure, the residue was dissolved in EtOAc (50 mL) and washed with a 10% solution of NH_4Cl (3×50 mL). The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure to give the product (±)-**19 a,b** (1.52 g, 84% yield), as a white foam; R_f: 0.28 (CH_2Cl_2/MeOH 95:5); MS: 406.2 $[M+H]^+$. The product was used in the next step without further characterization.

Synthesis of benzyl (*S)-(methoxycarbonyl)((*R**)-1-ethyl-4,5-dihydro-3-hydroxy-1*H*-pyrazol-5-yl)methylcarbamate [(±)-22 a] and benzyl (*S**)-(methoxycarbonyl)((*S**)-1-ethyl-4,5-dihydro-3-hydroxy-1*H*-pyrazol-5-yl)methylcarbamate [(±)-22 b]:** a) PPTS (93 mg, 0.37 mmol) was added to a solution of (±)-**19 a,b** (1.52 g, 3.70 mmol) in a mixture of MeOH (32 mL) and bi-distilled H_2O (3.9 mL). The reaction mixture was stirred for 90 min at room temperature, until disappearance of the starting material. After evaporation of the organic solvent under reduced pressure, the residue was dissolved in EtOAc (20 mL) and washed with H_2O (3×20 mL). The organic layer was dried over Na_2SO_4 and concentrated under vacuum. The residue was dissolved in MeOH (34 mL) and K_2CO_3 (94 mg, 0.68 mmol) was added to the solution. The reaction mixture was stirred for 1 h at room temperature. After disappearance of the starting material, the solvent was evaporated under vacuum and the residue was dissolved in EtOAc (20 mL). The organic layer was washed with 3% aq. NH_4Cl (20 mL), brine (20 mL) and dried over Na_2SO_4, filtered and concentrated to dryness. The crude was purified by column chromatography (CH_2Cl_2/*i*PrOH, 97:3), to obtain the two diastereomers. (±)-**22 a**: 513 mg (41% yield); crystallized from EtOAc/hexane as a white foam; mp: 94–96 °C; R_f: 0.49 (CH_2Cl_2/*i*PrOH 97:3); ^{1}H NMR ($CDCl_3$): δ = 1.00 (t, J = 7.1, 3H), 2.35 (dd, J = 2.5, 17.3, 1H), 2.68 (q, J = 7.1, 2H), 2.92 (dd, J = 9.6, 17.3, 1H), 3.72 (s, 3H), 3.75–3.85 (m, 1H), 4.41 (dd, J = 2.5, 9.6, 1H), 5.09 (d, J = 12.4, 1H), 5.12 (d, J = 12.4, 1H), 5.65 (d, J = 9.6, 1H), 7.25–7.40 (m, 5H), 8.57 ppm (br s, 1H); ^{13}C NMR ($CDCl_3$): δ = 12.2, 31.9, 52.8, 55.0, 58.4, 62.4, 67.6, 128.2, 128.4, 128.7, 136.3, 157.2, 170.7, 174.0 ppm; MS: 336.1 $[M+H]^+$; Anal. calcd for $C_{16}H_{21}N_3O_5$: C 57.30, H 6.31, N 12.53, found: C 57.05, H 6.22, N 12.40; (±)-**22 b**: 515 mg (41% yield); colorless oil; R_f: 0.35 (CH_2Cl_2/*i*PrOH 97:3); ^{1}H NMR ($CDCl_3$): δ = 1.00 (t, J = 7.1, 3H), 2.49 (d, J = 17.3, 1H), 2.72 (q, J = 7.1, 2H), 2.92 (dd, J = 9.1, 17.3, 1H), 3.53–3.62 (m, 1H), 3.72 (s, 3H), 4.29 (dd, J = 4.7, 8.0, 1H), 5.10 (s, 2H), 5.90 (d, J = 8.0, 1H), 7.20–7.40 (m, 5H), 8.40 ppm (br s, 1H); ^{13}C NMR ($CDCl_3$): δ = 12.4, 31.7, 52.8, 54.7, 56.6, 63.9, 67.4, 128.4, 128.5, 128.8, 136.3, 156.0, 170.6, 174.2; MS: 336.1 $[M+H]^+$; Anal. calcd for $C_{16}H_{21}N_3O_5$: C 57.30, H 6.31, N 12.53, found: C 57.09, H 6.26, N 12.41.

Synthesis of (*S)-2-amino-2-((*R**)-3-chloro-1-ethyl-4,5-dihydro-1*H*-pyrazol-5-yl)acetic acid hydrochloride [(±)-8 a]:** a) $POCl_3$

(0.98 mL, 10.74 mmol) was added to a stirred solution of compound (±)-**22 a** (360 mg, 1.07 mmol) in CH_3CN (52 mL). The reaction mixture was held at reflux for 3 h, until complete conversion was observed by TLC analysis. After removal of the solvent in vacuo, the residue dissolved in EtOAc (50 mL) and the solution was added dropwise to a crushed ice solution (30 mL). The organic layer was separated and washed with H_2O (50 mL) and brine (50 mL). The pooled organic layers were dried over Na_2SO_4 filtered and evaporated. The obtained reaction crude was purified by column chromatography on silica gel (eluent: cyclohexane/EtOAc 8:2, then 7:3) to obtain (±)-**25 a** (332 mg, 88% yield) as a colorless oil; R_f: 0,54 (cyclohexane/EtOAc 7:3); ^{1}H NMR ($CDCl_3$): $\delta = 1.12$ (t, $J = 7.0$, 3H), 2.68 (dq, $J = 7.0$, 13.5, 1H), 2.85 (dq, $J = 7.0$, 13.5, 1H), 2.93 (dd, $J = 11.3$, 17.3, 1H), 3.10 (dd, $J = 11.3$, 17.3, 1H), 3.75 (s, 3H), 4.05 (ddd, $J = 1.7$, 11.3, 11.3, 1H), 4.45 (dd, $J = 1.7$, 9.6, 1H), 5.14 (s, 2H), 5.56 (brd, $J = 9.6$, 1H), 7.38 (s, 5H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 12.0$, 41.7, 50.1, 52.9, 55.0, 65.5, 67.6, 128.3, 128.5, 128.8, 136.2, 142.7, 156.9, 171.1 ppm; MS: 354.1 $[M+H]^+$; Anal. calcd for $C_{16}H_{20}ClN_3O_4$: C 54.32, H 5.70, N 11.88, found: C 54.50, H 5.82, N 12.05. b) A 1 M solution of BCl_3 in CH_2Cl_2 (1.88 mL, 1.88 mmol) was added dropwise to a solution of compound (±)-**25 a** (332 mg, 0.94 mmol) in CH_2Cl_2 (35 mL) cooled at −10 °C. The reaction mixture was allowed to warm up to room temperature and stirred for 2 h. After complete conversion, the reaction was quenched by the addition of 0.2 N HCl (20 mL). The aqueous layer was separated, made basic with aq. K_2CO_3 (pH 8) and extracted with EtOAc (3×30 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated under vacuum. Purification via column chromatography (silica gel, cyclohexane/EtOAc 1:1 then EtOAc) afforded the desired amine which was directly dissolved in a mixture of dioxane (3.4 mL) and bi-distilled H_2O (3.4 mL). Ambersep 900-OH resin (500 mg) was added. The reaction mixture stirred for 40 min, until disappearance of the starting material. The resin was filtered off and washed with dioxane, bi-distilled H_2O and Et_2O to remove impurities. The product was then eluted off the resin with 0.05 N HCl and the product-containing fractions (detected with ninhydrin on a TLC plate) were combined and concentrated in vacuo to obtain the desired hydrochloride amino acid (±)-**8 a** (118 mg, 52% yield), as a white solid; mp: >162 °C dec; R_f: 0.25 (butanol/H_2O/AcOH 4:2:1); ^{1}H NMR (D_2O): $\delta = 0.98$ (t, $J = 7.0$, 3H), 2.70 (dq, $J = 7.0$, 13.5, 1H), 2.90 (dd, $J = 10.8$, 18.5, 1H), 2.98 (dq, $J = 7.0$, 13.5, 1H), 3.35 (dd, $J = 10.8$, 18.5, 1H), 3.80 (d, $J = 2.1$, 1H), 4.12 ppm (ddd, $J = 2.1$, 10.8, 10.8, 1H); ^{13}C NMR (D_2O): $\delta = 10.5$, 41.2, 49.7, 54.6, 62.4, 145.8, 170.9 ppm; MS: 206.0 $[M+H]^+$; Anal. calcd for $C_7H_{13}Cl_2N_3O_2$: C 34.73, H 5.41, N 17.36, found: C 34.47, H 5.46, N 17.20.

Synthesis of (*S)-2-amino-2-((S*)-3-chloro-1-ethyl-4,5-dihydro-1*H*-pyrazol-5-yl)acetic acid hydrochloride [(±)-8 b]:** Compound (±)-**8 b** was synthesized following the procedure reported for (±)-**8 a** starting from intermediate (±)-**22 b**. (±)-**25 b**: colorless oil; 80% yield; R_f: 0.41 (cyclohexane/EtOAc 7:3); ^{1}H NMR ($CDCl_3$): $\delta = 1.20$ (t, $J = 6.9$, 3H), 2.82 (dq, $J = 6.9$, 13.2, 1H), 2.85 (dd, $J = 11.6$, 17.1, 1H), 3.05 (dd, $J = 12.1$, 17.1, 1H), 3.09 (dq, $J = 6.9$, 13.2, 1H), 3.79 (s, 3H), 3.75–3.85 (m, 1H), 4.50–4.60 (m, 1H), 5.10 (s, 2H), 5.60–5.70 (m, 1H), 7.35–7.60 ppm (m, 5H); ^{13}C NMR ($CDCl_3$): $\delta = 12.4$, 40.1, 49.5, 53.1, 54.7, 67.1, 67.6, 128.4, 128.6, 128.8, 136.2, 141.7, 156.0, 170.4 ppm; MS: 354.1 $[M+H]^+$; Anal. calcd for $C_{16}H_{20}ClN_3O_4$: C 54.32, H 5.70, N 11.88, found: C 54.02, H 5.59, N 11.65. (±)-**8 b**: white solid; 51% yield mp: >179 °C dec.; R_f: 0.48 (butanol/H_2O/AcOH 4:2:1); ^{1}H NMR (D_2O): $\delta = 1.00$ (t, $J = 7.1$, 3H), 2.77–2.88 (m, 1H), 2.84 (dd, $J = 7.0$, 13.4, 1H), 2.98–3.10 (m, 1H), 3.04 (dd, $J = 7.0$, 13.4, 1H), 3.98–4.10 ppm (m, 2H); ^{13}C NMR (D_2O): $\delta = 11.1$, 38.0, 48.5, 53.4, 63.5, 145.1, 170.7 ppm; MS: 206.0 $[M+H]^+$; Anal. calcd for $C_7H_{13}Cl_2N_3O_2$: C 34.73, H 5.41, N 17.36, found: C 34.38, H 5.48, N 17.15.

Synthesis of benzyl (1-(2-benzyloxycarbonylaminoethyl)-3-hydroxy-4,5-dihydro-1*H*-pyrazol-5-yl)(4-methyl-2,6,7-trioxabicyclo-[2.2.2]octan-1-yl)methylcarbamate [(±)-20 a,b]: A solution of *N*-Cbz-glycinal **17** (2.3 g, 11.91 mmol) in MeOH (18 mL) was added to a solution of (±)-**16 a,b** (1.5 g, 3.97 mmol) in MeOH (30 mL) at room temperature. After 1 h, $NaBH_4$ (0.4 g, 11.91 mmol) was added and the reaction was stirred for an additional hour. After disappearance of the starting material, the solvent was evaporated under reduced pressure, the residue was dissolved in EtOAc (50 mL) and washed with a 10% solution of NH_4Cl (3×50 mL). The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. Purification via column chromatography (silica gel, cyclohexane/EtOAc 1:1, 100% EtOAc) afforded the desired product (±)-**20 a,b** (1.6 g, 73% yield), as a white foam; R_f: 0.46 (EtOAc); MS: 555.2 $[M+H]^+$. The product was used in the next step without further characterization.

Synthesis of benzyl (methoxycarbonyl)(1-(2-benzyloxycarbonylaminoethyl)-4,5-dihydro-3-hydroxy-1*H*-pyrazol-5 yl)methylcarbamate hydrochloride [(±)-23 a,b]: a) PPTS (73 mg, 0.29 mmol) was added to a solution of (±)-**20 a,b** (1.6 g, 2.89 mmol) in a mixture of MeOH (25 mL) and bi-distilled H_2O (3.1 mL). The reaction mixture was stirred for 2 h at room temperature, until disappearance of the starting material. After evaporation of the organic solvent under reduced pressure, the residue was dissolved in EtOAc (20 mL) and washed with H_2O (3×20 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was dissolved in MeOH (30 mL) and K_2CO_3 (100 mg, 0.72 mmol) was added to the solution. The reaction mixture was stirred for 2 h at room temperature. After disappearance of the starting material, the solvent was evaporated under vacuum and the residue was dissolved in EtOAc (20 mL). The organic layer was washed with 3% aq. NH_4Cl (20 mL), brine (20 mL) and dried over Na_2SO_4, filtered and concentrated to dryness. The product (±)-**23 a,b** (945 mg, 67% yield, 1:1 mixture of diastereomers) was isolated after column chromatography (silica gel, eluent: EtOAc), as white foam; R_f: 0.43 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR ($CDCl_3$): $\delta = 2.30$–2.50 (m, 1H), 2.65–3.00 (m, 3H), 3.07–3.20 (m, 1H), 3.25–3.45 (m, 1H), 3.50–3.62 (m, 0.5H), 3.65 and 3.68 (s, 3H), 3.75–3.85 (m, 0.5H), 4.30–4.38 (m, 0.5H), 4.42–4.50 (m, 0.5H), 5.02–5.20 (m, 4H), 5.08–5.12 (m, 0.5H), 5.25–5.32 (m, 0.5H); 5.60 (brd, $J = 8.2$, 0.5H), 5.88 (brd, $J = 8.2$, 0.5H), 7.25–7.40 (m, 10H), 8.05 (bs, 0.5H), 8.20 ppm (bs, 0.5H). MS 485.2 $[M+H]^+$.

Synthesis of methyl 2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-2-(1-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)ethyl)-3-hydroxy-4,5-dihydro-1*H*-pyrazol-5-yl)acetate [(±)-26 a,b]: a) A solution of compound (±)-**23 a,b** (660 mg, 1.36 mmol) in a 33% solution of hydrobromic acid in AcOH (5 mL) was stirred for 30 min at room temperature. After the disappearance of the starting material monitored by TLC, Et_2O was added and a white solid precipitate from the solution. The solid was filtered, washed with MeOH and dried under vacuum to afford the desired amine which was directly dissolved in a 1:1 mixture of H_2O and dioxane (50 mL). After cooling with an ice bath, solid Na_2CO_3 (664 mg, 6.26 mmol) and Fmoc *N*-hydroxysuccinimide ester (918 mg, 2.72 mmol) were added. The reaction mixture was stirred overnight at room temperature and then the organic solvent was evaporated. The residue was diluted with H_2O (50 mL) and EtOAc (50 mL). The aqueous phase was extracted with EtOAc (3×50 mL) and the combined organic layers were dried over Na_2SO_4 and evaporated in vacuo. The crude was purified by column chromatography (silica gel, eluent: EtOAc) to give the product (±)-**26 a,b** (476 mg, 53% yield, 1:1 mixture of dia-

stereomers) as a white foam; R_f: 0.55 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (DMSO): $\delta = 1.90–2.02$ (m, 1H), 2.60–3.15 (m, 5H), 3.45–3.70 (m, 4H), 3.98–4.05 (m, 1H), 4.10–4.40 (m, 6H), 7.22–7.42 (m, 8H), 7.55–7.70 (m, 4H), 7.82–7.90 ppm (m, 4H); MS: 661.4 $[M+H]^+$.

Synthesis of 2-amino-2-(1-(2-aminoethyl)-3-chloro-4,5-dihydro-1*H*-pyrazol-5-yl)acetic acid monohydrochloride [(±)-9a,b]: a) $POCl_3$ (0.66 mL, 7.26 mmol) was added to a stirred solution of compound (±)-**26a**,**b** (476 mg, 0.72 mmol) in CH_3CN (18 mL). The reaction mixture was held at reflux for 2 h, until complete conversion was observed by TLC analysis. After removal of the solvent under vacuum, the residue dissolved in EtOAc (20 mL) and the solution was added dropwise to a crushed ice solution (30 mL). The organic layer was separated and washed with H_2O (50 mL) and brine (50 mL). The pooled organic layers were dried over Na_2SO_4 filtered and evaporated. The obtained reaction crude was purified by column chromatography on silica gel (eluent: cyclohexane/EtOAc 7:3, then 1:1). The product obtained was dissolved in CH_2Cl_2 (20 mL) and treated with piperidine (1.64 mL, 28 mmol) and stirred for 15 min at room temperature. The solvent was evaporated under reduced pressure and the residue was dissolved in bi-distilled H_2O (30 mL) and washed with Et_2O (3×15 mL). The aqueous layer was evaporated in vacuo; EtOH was added and the precipitated was filtered off under vacuum to yield the free amine, directly submitted to next step. The intermediate obtained from the previous step was dissolved in a mixture of dioxane (5 mL) and bi-distilled H_2O (5 mL). Ambersep 900-OH resin (500 mg) was added. The reaction mixture stirred for 40 min, until disappearance of the starting material. The resin was filtered off and washed with dioxane, bi-distilled H_2O and Et_2O to remove impurities. The product was then eluted off the resin with 0.05 N HCl and the product-containing fractions (detected with ninhydrin on a TLC plate) were combined and concentrated in vacuo to obtain the desired amino acid (±)-**9a**,**b** monohydrochloride (60 mg, 32% yield, 1:1 mixture of diastereomers), as a white solid; ^{1}H NMR (D_2O): $\delta = 2.80–3.40$ (m, 6H), 3.75–4.10 ppm (m, 2H); MS: 221.0 $[M+H]^+$; Anal. calcd for $C_7H_{14}Cl_2N_4O_2$: C 32.70, H 5.49, N 21.79, found: C 32.33, H 5.55, N 21.54.

Single crystal X-ray diffraction analysis of benzyl (*S)-(methoxycarbonyl)((*R**)-1-ethyl-4,5-dihydro-3-hydroxy-1*H*-pyrazol-5-yl)methylcarbamate (±)-22a:** A single crystal suitable for the X-ray diffraction analysis has been obtained by slow crystallization from a 1:1 mixture of EtOAc and hexane at $T = 4\,^\circ$C. A 100% complete sphere of data has been collected at room temperature up to a 2ϑ Bragg angle of 52.7° using graphite-monochromated $Mo_{K\alpha}$ radiation on a three-circle Bruker Smart Apex diffractometer equipped with a CCD area detector. The measured structure factor amplitudes have been corrected for beam anisotropy effects by using the program SADABS.[21] The substance crystallizes in the non-centrosymmetric orthorhombic $Pca2_1$ space group, with eight formulae in cell and the following cell parameters (Å, Å^3): $a = 14.6960(6)$, $b = 9.3401(9)$, $c = 25.4311(15)$, $V = 3490.7(4)$. The structure has been solved by direct methods and refined within the spherical atom approximation implemented in the software SHELX,[22] giving final agreement factors $R1(F) = 0.0405$ for 4154 unique data with $I > 2\sigma(I)$, goodness-of-fit as high as 1.032 and greatest Fourier residues as low as ± 0.20 e Å^{-3}. The asymmetric unit is made up by a pair of symmetry-independent enantiomers, with the corresponding stereocenters showing αS^*, $5R^*$ (and αR^*, $5S^*$) relative configurations. Some structural disorder affects the methyl ester carbonyl of one of the two molecules, with occupation coefficients of the Oxygen atom as high as 0.49(2) and 0.51(2). Interestingly, in the solid state only the pyrazolidinone N–NH–C=O form of the dihydropyrazole ring is present. CCDC 885136 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Covalent hydrated docking

The new version of the docking program AutoDock (version 4.2, AD4),[23] as implemented through the graphical user interface called AutoDockTools (ADT), was used to dock Acivicin, **5** and **6**. Ligand structures were built using the builder in the Maestro package of Schrödinger Suite 2012 and optimized using a version of MacroModel also included. The constructed compounds were converted into AD4 format files using ADT, hydrated using the wet.py suite and edited to include the correct atom type for the ligand atom that forms the covalent bond with the enzyme C419 sulfur atom. ADT was also used to convert the receptor in the AD4 format file. The docking area was centered around the putative binding site. A set of grids of 60×60×60 Å with 0.375 Å spacing was calculated around the docking area for the ligand atom types using AutoGrid4. An additional grid map was calculated for the water molecules using the mapwater.py suite. For each ligand, 100 separate docking calculations were performed. Each docking calculation consisted of 10 million energy evaluations using the Lamarckian genetic algorithm local search (GALS) method. The GALS method evaluates a population of possible docking solutions and propagates the most successful individuals from each generation into the subsequent generation of possible solutions. A low-frequency local search according to the method of Solis and Wets is applied to docking trials to ensure that the final solution represents a local minimum. All dockings described in this paper were performed with a population size of 250, and 300 rounds of Solis and Wets local search were applied with a probability of 0.06. A mutation rate of 0.02 and a crossover rate of 0.8 were used to generate new docking trials for subsequent generations, and the best individual from each generation was propagated over the next generation. The docking results from each of the 100 calculations were clustered on the basis of root-mean square deviation (RMSD) (solutions differing by < 2.0 Å) between the Cartesian coordinates of the atoms and were ranked on the basis of free energy of binding (ΔG_{AD4}). The top-ranked compounds were visually inspected for good chemical geometry. Because AD4 does not perform any structural optimization and energy minimization of the complexes found, a molecular mechanics/energy minimization (MM/EM) approach was applied to refine the AD4 output. The computational protocol applied consisted of the application of 100 000 steps of the Polak–Ribiére conjugate gradients (PRCG) or until the derivative convergence was 0.05 kJ mol^{-1}. All complexes pictures were rendered employing the UCSF Chimera software.[24]

CTPS enzyme assays

T. b. brucei CTPS was expressed recombinantly and purified as described elsewhere.[5c] A coupled spectrophotometric assay was used consisting of a final volume of 200 μL containing: 50 mM MOPS pH 7.6, 150 mM KCl, 12 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 1 mM phosphoenolpyruvate, 0.5 mM NADH, 1.5 units of pre-mixed pyruvate kinase and lactate dehydrogenase, 300 μM GTP, 1 mM UTP, 750 μM L-glutamine and 25 μg of purified *T. b. brucei* CTPS.[5c] Prior to assaying various concentrations of test compounds were pre-incubated with *T. b. brucei* CTPS at room temperature, assays were started with addition of pre-incubated CTPS to reaction mix

(substrates, buffer, coupled enzymes and test compounds) and decreasing absorbance at λ 341 nm was monitored.

In vitro toxicity assays for T. b. brucei *and HeLa mammalian cells*

T. b brucei (strain 427) was cultured to the optimum density of 1–2×10^6 cells mL^{-1} in HMI-9 supplemented with 10% fetal calf serum (FCS) under environmental conditions of 37 °C and 5% CO_2. Solutions of test compounds were prepared in culture media at stock concentration of 200 μM and diluted serially (1:2) across the 96-well, flat-bottom solid white plates to give a total of 11 decreasing concentrations (100 μL $well^{-1}$). The last well of each series was left blank, that is, "drug free" (negative control). Cells were prepared at the concentration of 4×10^4 cells mL^{-1} and was added to each well of the respective compound series (100 μL $well^{-1}$). Plates were incubated at 37 °C/5% CO_2 for 48 h prior to addition of Alamar Blue solution (20 μL $well^{-1}$, 0.49 mM in 1× PBS, pH 7.4) followed by a further 24 hour. Assay end points were measured fluorimetrically with the fluorescence spectrometer (FluoStar, BMG LabTech, Germany) and Optima program set at λ excitation 544 nm and λ emission 590 nm. Data were analyzed using Prism 5.0 software to obtain EC_{50} values. Experiment was performed in duplicate and repeated three times. Similar Alamar Blue assay was carried out with HeLa cells, cultured in DMEM supplemented with 10% FCS and 2 mM L-glutamine. HeLa cells were plated at initial cell concentration of 3×10^5 cells mL^{-1} (100 μL $well^{-1}$) and incubated with test compounds for 16 h prior to addition of Alamar Blue solution.

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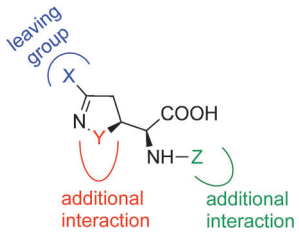
Increasing molecular complexity is a tool to improve the activity of CTPS inhibitors: New potent inhibitors of CTPS were designed based on the Acivicin scaffold as potential anti-trypanosomal agents. Compounds that can establish additional interactions with CTPS have greater inhibitory efficacy.



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Synthesis and Biological Evaluation of CTP Synthetase Inhibitors as Potential Agents for the Treatment of African Trypanosomiasis

