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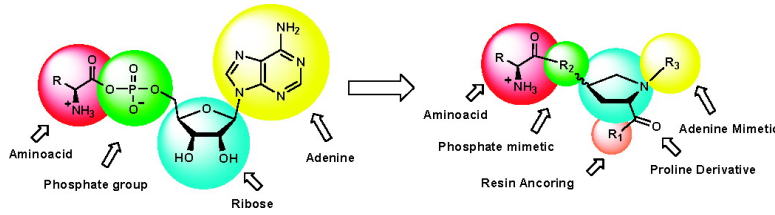

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Solid-Phase Combinatorial Synthesis of a Lysyl-tRNA Synthetase (LysRS) Inhibitory Library

Josep Farrera-Sinfreu,[†,§,‡] Yaiza Español,[†] Renaud Geslain,[†] Tanit Guitart,[†] Fernando Albericio,[†,§] Lluís Ribas de Pouplana,[†,||] and Miriam Royo*[,‡]

Institute for Research in Biomedicine and Combinatorial Chemistry Unit, Barcelona Science Park, University of Barcelona, Josep Samitier 1, 08028-Barcelona, Spain, Department of Organic Chemistry, University of Barcelona, Martí i Franqués 1, 08028-Barcelona, Spain, and Catalan Institution for Research and Advanced Studies (ICREA), Passeig Lluís Companys 23, 08010-Barcelona, Spain

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The solid-phase combinatorial synthesis of a new library with potential inhibitory activity against the cytoplasmic lysyl-tRNA synthetase (LysRS) isoform of *Trypanosoma brucei* is described. The library has been specifically designed to mimic the lysyl adenylate complex. The design was carried out by dividing the complex into four modular parts. Proline derivatives (cis-γ-amino-L-proline or trans-γ-hydroxy-L-proline) were chosen as central scaffolds. After primary screening, three compounds of the library caused in vitro inhibition of the tRNA aminoacylation reaction in the low micromolar range.

Introduction

The continuous emergence of antibiotic-resistant pathogens has led to an intensive search for new anti-infectives.[1] The translation apparatus (including ribosomes, specific elongation and initiation factors, and aminoacyl-tRNA synthetases) represents one of the main pathways targeted by commercial antibiotics. Among these, aminoglycosids, macrolides, and tetracyclines constitute the most represented families. These drugs interact with the 30S or 50S ribosomal subunits and display remarkable degrees of molecular specificity. Nevertheless, these therapeutic agents have a number of limitations, such as lack of oral availability, toxicity in humans, and even resistance.[2]

Among the components of the translational apparatus that have been traditionally hailed as potential antibiotic targets are the aminoacyl-tRNA synthetases (aaRS).[3] These enzymes are responsible for the correct translation of genetic information into protein sequence, which takes place when aaRS catalyze the specific aminoacylation of tRNA molecules with their cognate amino acids. Aminoacyl-tRNA synthetases represent attractive pharmacological objectives because they are essential enzymes of very ancient origin,[4] ensuring that their inhibition can be both specific and effective at eliminating infection.

Trypanosomatids are a family of protozoa that causes disease in livestock and humans. They are characterized by the presence of a single mitochondrion that is physically attached to a single flagellum via a structure known as a kinetoplast. In contrast to the majority of mitochondriate eukaryotes, Trypanosomatids do not code for any tRNA molecules in their mitochondrial genome, and they import these molecules and their cognate aminoacyl-tRNA synthetases from the cytoplasm.[5] Despite the fact that the tRNA population in the mitochondria of Trypanosomatids is assumed to be identical to that of the cytoplasm, these organisms contain some mitochondrial specific aaRS. Among the aaRS that are duplicated in Trypanosoma is lysyl-tRNA synthetase, a situation not found in human cells.[6] For this reason, the development of inhibitors against Trypanosoma aminoacyl-tRNA synthetases constitutes a possible strategy for the development of new therapies against this parasite.

Currently, aaRS inhibition is the mechanism of action of only one commercial antibiotic, the Pseudomonic acid (mupirocin, Glaxo Smithkline) that inhibits the aminoacylation activity of bacterial isoleucyl-tRNA synthetases and shows 8000-fold selectivity versus mammalian homologues.[7] With mupirocin as a model, other synthetase inhibitors have been described (Figure 1).[8] The design of new drugs addressed to the tRNA synthetases has been inspired by the aminoacyl adenylate complex, the mixed anhydride intermediate generated during the aminoacylation reaction. Because this complex is bound more tightly to the enzyme than the substrates (amino acid and ATP), generally by 2 or 3 orders of magnitude, analogues based on the adenylate intermediate could potentially bind as tightly as inhibitors. This hypothesis has been explored by several groups with success.[3b,9] Other inhibitors of these enzymes are natural products that are less structurally related to the complex, such as borrelidin (Thr),[10,11] furanomycin (Ile),[12] granaticin (Leu),[13] indolmycin (Trp),[14] ochratoxin A (Phe),[15] or cispentacin (Pro).[16]

In the present work, the solid-phase combinatorial synthesis of a new library with potential inhibitory activity

* To whom correspondence should be addressed. E-mail: mroyo@pcb.ub.es.
† Institute for Research in Biomedicine.
‡ Combinatorial Chemistry Unit.
§ Department of Organic Chemistry.
|| Catalan Institution for Research and Advanced Studies.

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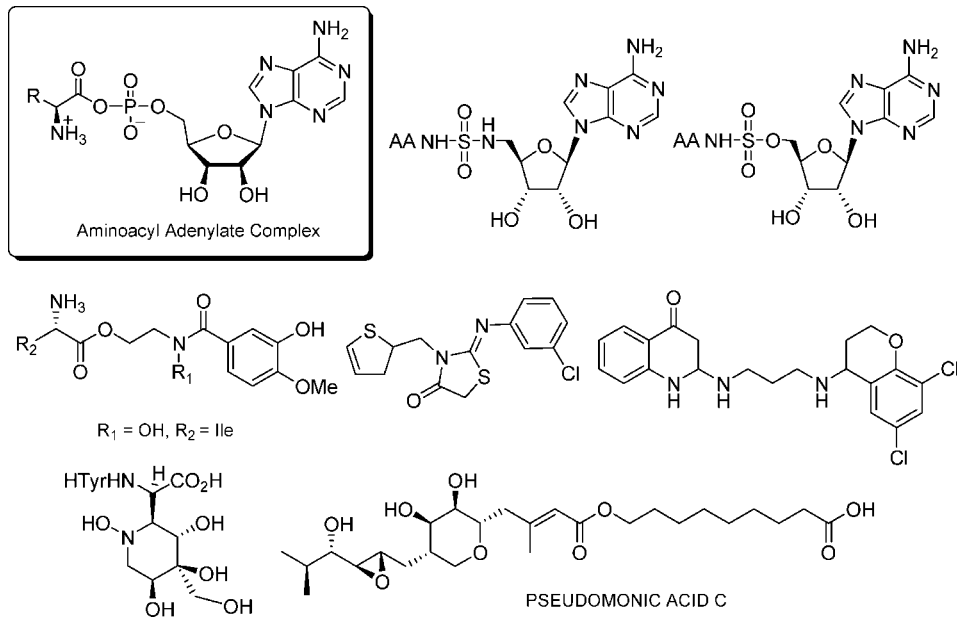


Figure 1. Representative examples of aminoacyl tRNA synthetase inhibitors.[17–21]

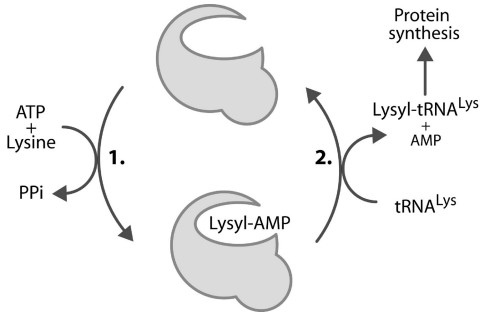


Figure 2. Transfer of lysine onto $tRNA^{Lys}$ catalyzed by LysRS. LysRS (in gray) first activates lysine with ATP. There is formation of lysyl-AMP, an intermediate that remains bound to the catalytic cavity. Next, the lysine moiety is transferred onto the tRNA. After tRNA release, the enzyme is recycled for a new activation−transfer cycle.

against the cytoplasmic lysyl-tRNA synthetase (LysRS) isoform of *Trypanosoma brucei* is described.

Results

Library Design. Lysine, ATP, and tRNA lysine ($tRNA^{Lys}$) are the three substrates of this universal enzyme (Figure 2). The catalytic core of the protein forms a cavity that specifically binds its substrates, places them in a reactive conformation, and promotes covalent binding of one lysine residue onto $tRNA^{Lys}$ during a two-step reaction.[22] First, condensation of lysine and ATP produces lysyl-adenylate, an intermediate molecule that remains tightly bound to the catalytic core. Second, the lysine moiety of the intermediate is transferred onto the tRNA.

The library has been specifically designed to mimic the lysyl adenylate complex. This intermediate is bound 2 or 3 orders of magnitude more tightly to the enzyme than the substrates (amino acid and ATP) and participates in both steps of the reaction, making it a convenient model for the design of potential inhibitors because it can completely block the active site.[23] Our reference molecule (Figure 3A) is formed by a lysine linked to an adenine monophosphate molecule (AMP). For design purposes, the basic structure of this intermediate was divided into four modular parts, and each one was replaced with a set of suitable mimetics covering a predetermined diversity space. To ensure the recognition of the inhibitors by the LysRS, the amino acid moiety was maintained as a lysine. Thus, the final library design contains three points of diversity (Figure 3B). The ribose was replaced by commercially available trifunctional proline derivative cyclic bioisosters (*cis*-γ-amino-L-proline or *trans*-γ-hydroxy-L-proline). Several modifications at the C-terminal part of these proline derivatives, such as carboxylic acid, carboxamide, and hydroxamic acid, were explored (Figure 3C, R_1). The labile acylphosphate group was replaced by distinct stable non -ionizable groups, such as sulfamate,[9a,b,24] hydroxamate,[17,25] and amide[25a] (Figure 3C, R_2). The third point of diversity (Figure 3C, R_3) was the introduction of different aromatic rings at the α-amino position of the proline, as well as two heterocycles that include the natural adenine moiety. The combinations of these four modular parts of the complex gave the different families of inhibitors. In this figure, the numbering of the library is also described.

General Strategy for Solid-Phase Synthesis. The strategy for library preparation depended on the resin and the γ-amino bond, but in general, it is shown in Figure 4. The proline derivate (Alloc-L-Hyp(THP)-OH or Alloc-L-Amp(Fmoc)-OH) was coupled to the resin using common solid-phase reagents. After removal of the protecting group (PG) in the γ-position of the proline, lysine (protected with Boc in both amine positions) was introduced. After that, the α-amino position of the proline was liberated, and the diversity in this point was introduced. Finally, compounds were cleaved from the resin under acidic conditions, giving the final products defined as **5**{R_1,R_2,R_3}.

First Point of Diversity (R_1). R_1 diversity was obtained by using different linkers and resins. Carboxylic acids and carboxamides were obtained using MBHA resin with 3-(4-hydroxymethylphenoxy)propionic acid (AB) and Rink am-

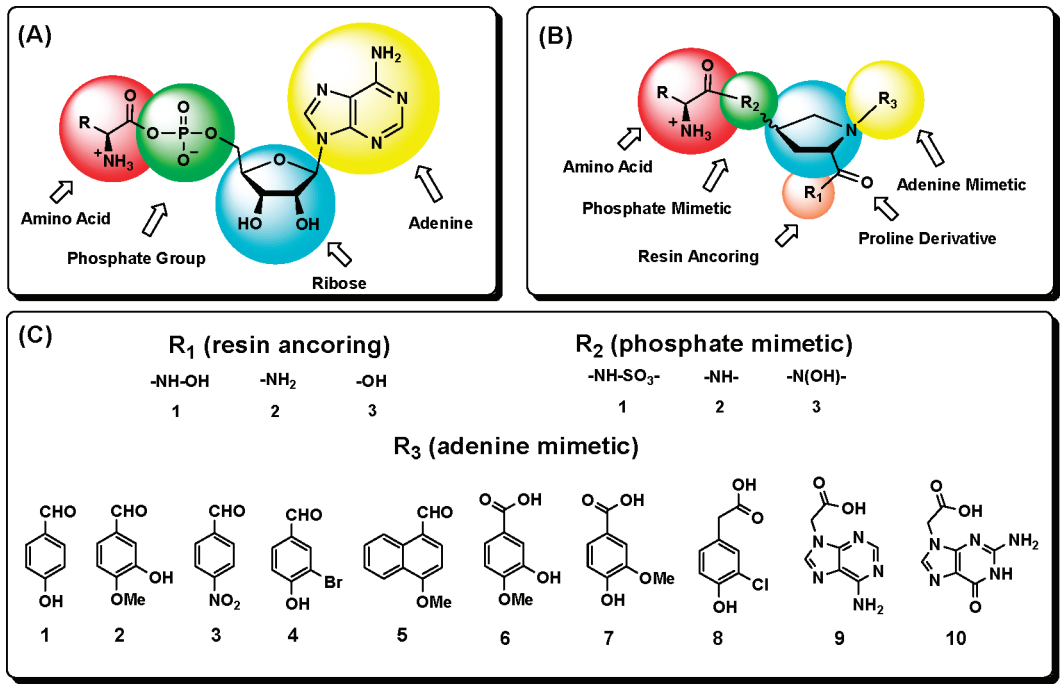


Figure 3. Comparison of (A) the lysyl-adenylate complex and (B) the basic structure of the library. R_1, R_2, and R_3 correspond to the region where diversity has been introduced. (C) Library definition (H-Lys-R_2-Hyp(R_3)-R_1 or H-Lys-R_2-Amp(R_3)-R_1), with all the building blocks used during the synthesis.

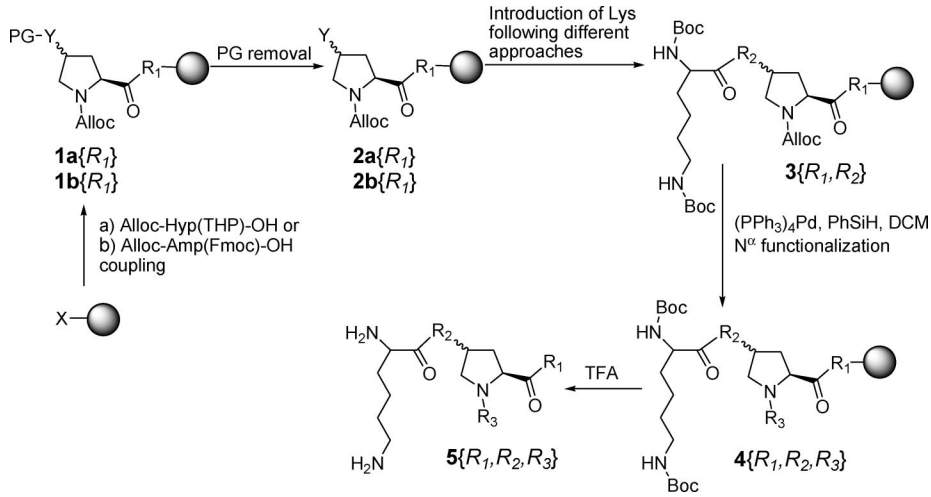


Figure 4. General synthetic strategy for the library preparation.

ide handles, respectively. Hydroxamic acids were synthesized by incorporation of Fmoc-NH-OH into the Cl-TrtCl resin in basic conditions.[26]

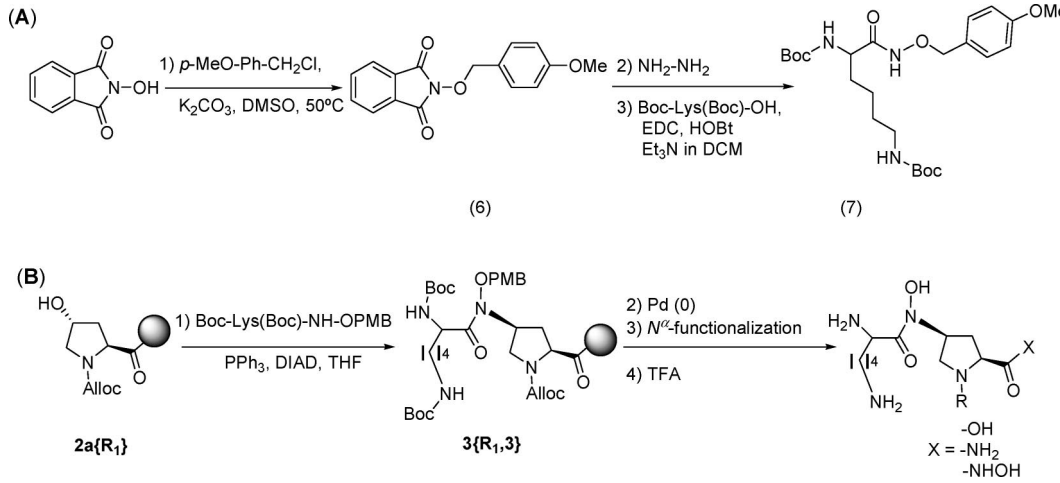
The first step of the synthesis was the coupling of the proline derivative to the resin. Two scaffolds were used for this purpose. The first one was (2*S*,4*R*)-THP-4-hydroxy-1-Alloc-pyrrolidine-2-carboxylic acid [(2*S*,4*R*)-Alloc-Hyp(THP)-OH], which after coupling to the resin gave compounds **1a**{R_1}. This scaffold was used in the synthesis of both sulfamate and hydroxamate peptidomimetics (**5**{R_1,*1*,R_3} and **5**{R_1,*3*,R_3}, respectively). The alcohol of hydroxyproline must be protected to avoid polymerizations. THP was selected as a protecting group because it is compatible with Boc chemistry and orthogonal to the Alloc one.[27]

The second scaffold was (2*S*,4*S*)-Fmoc-4-amino-1-Alloc-pyrrolidine-2-carboxylic acid [(2*S*,4*S*)-Alloc-Amp(Fmoc)-OH], which after coupling to the resin gave compounds **1b**{R_1}. This scaffold was used in the synthesis of the amide linked peptidomimetics (**5**{R_1,*2*,R_3}).

The solid-phase synthesis of hydroxamic acids were carried out by incorporation of the two proline derivatives onto the H_2N-O-ClTrt-resin[28] using HATU (5 equiv) and DIEA (10 equiv) as coupling reagents, obtaining peptidyl resins **2a**{*1*} and **1b**{*1*}.[29] This reaction was monitored with the ninhydrin test. Elimination of the γ-position protecting group of compound **1b**{*1*} gave **2b**{*1*}.

Carboxamides were obtained by coupling the proline derivative [(2*S*,4*R*)-Alloc-Hyp(THP)-OH or (2*S*,4*S*)-Alloc-Amp(Fmoc)-OH] (5 equiv) to the Rink amide resin using DIPCDI (5 equiv) and HOBt (5 equiv) as coupling reagents. The reaction was checked with the ninhydrin test. Finally, the Fmoc and THP protecting groups were removed with piperidine or *p*-TsOH, respectively, obtaining compounds **2a**{*2*} and **2b**{*2*}.

Scheme 1. (A) Synthesis of Boc-Lys(Boc)-NH-OPMB. (B) General Strategy for the Hydroxamate Synthesis on Solid-Phase Supports



Carboxylic acids were obtained by incorporating [(2*S*,4*R*)-Alloc-Hyp(THP)-OH or (2*S*,4*S*)-Alloc-Amp(Fmoc)-OH] (5 equiv) onto 3-(4-hydroxymethylphenoxy)propionyl-MBHA resin using DIPCDI (5 equiv) and catalytic amounts of DMAP (0.5 equiv). After that, THP and Fmoc protecting groups were removed as described above, furnishing compounds **2a**{*3*} and **2b**{*3*}.

Second Point of Diversity (R_2). Solid-Phase Lysine Introduction in the γ-Position of the Proline Ring. Sulfamoylation was carried out following a previously described procedure that involves the use of sulfamoyl chloride in DMA in the absence of base.[30] Boc-Lys(Boc)-OH was then coupled using DIPCDI and DMAP (10%) as coupling reagents. To check the extent of the reaction, a small amount of each resin was cleaved and compounds **3**{R_1,*1*} were verified by analytical MS-HPLC. The hydroxamic compounds **3**{*1*,*1*} were not possible to obtain because of problems with premature cleavage of the compounds during the sulfamoylation resulting from the acidic lability of Cl-Trt resin.

The second linkage evaluated was the amide bond, which was obtained by common solid-phase peptide synthesis protocols to render compounds **3**{R_1,*2*}. DIPCDI and HOBt were used as coupling reagents.

For the hydroxamate derivatives, Boc-L-Lys(Boc)-NH-OPMB was required and was prepared in solution following the strategy described in Scheme 1A. *N*-Hydroxyphtalimide was protected with *p*-methoxybenzyl chloride, and after treatment of the product with hydrazine, Boc-L-Lys(Boc)-OH was coupled to afford the final protected hydroxamic acid **7**.[31] Hydroxamates were then prepared by incorporation of Boc-L-Lys(Boc)-NH-OPMB using Mitsunobu conditions (DIAD, Ph_3P) to the hydroxyproline contaning peptidyl resin **2a**{R_1} to produce products **3**{R_1,*3*} (see Scheme 1B). Unfortunately, synthesis of compound **3**{*1*,*3*} (made on Cl-Trt resin) did not work.

Third Point of Diversity (R_3) and Cleavage. Once the protected lysine was attached to the resin, syntheses continued by the deprotection of the α-amino group of the proline using $Pd(PPh_3)_4/Ph_3SiH$. The aromatic moieties were then introduced via a reductive amination using several aldehydes or via acylation using different carboxylic acids (see Table 1). Alkylations and acylations were carried out on resin using the same conditions described in a previous work with the corresponding aldehyde and $NaBH_3CN$ in the former case and the corresponding carboxylic acid in the presence of DIPCDI and HOBt in the later.[32,33] Adenine and guanine derivatives were used with their amino groups protected with benzhydryloxycarbonyl (Bhoc), which was removed during the cleavage with TFA. Both alkylations and acylations were monitored with the chloranil test.

Table 1. Library Yields[a] and Purities[b] after Purification

	side chain (R_3)									
R_1 and R_2	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
5{*1*,*1*,R_3}										
5{*1*,*2*,R_3}	$^{24}/_{88}$	$^{33}/_{99}$	$^{10}/_{91}$	$^{42}/_{97}$	$^{10}/_{97}$	$^{52}/_{87}$	$^{48}/_{82}$	$^{39}/_{96}$	$^{75}/_{97}$	$^{51}/_{95}$
5{*1*,*3*,R_3}										
5{*2*,*1*,R_3}	$^{1}/_{94}$	$^{5}/_{76}$	$^{2}/_{85}$	$^{3}/_{88}$	$^{1}/_{82}$		$^{4}/_{80}$	$^{6}/_{91}$	$^{19}/_{96}$	$^{8}/_{49}$
5{*2*,*2*,R_3}	$^{9}/_{61}$	$^{13}/_{81}$	$^{4}/_{99}$	$^{6}/_{84}$	$^{9}/_{99}$	$^{59}/_{97}$	$^{15}/_{88}$	$^{33}/_{99}$	$^{65}/_{40}$	$^{43}/_{90}$
5{*2*,*3*,R_3}		$^{3}/_{99}$		$^{1}/_{93}$	$^{3}/_{88}$	$^{46}/_{89}$	$^{36}/_{81}$	$^{53}/_{84}$	$^{88}/_{93}$	$^{52}/_{99}$
5{*3*,*1*,R_3}		$^{3}/_{80}$	$^{3}/_{83}$	$^{8}/_{81}$		$^{1}/_{45}$	$^{3}/_{81}$	$^{4}/_{93}$	$^{32}/_{36}$	$^{9}/_{58}$
5{*3*,*2*,R_3}	$^{26}/_{92}$	$^{26}/_{96}$	$^{28}/_{99}$	$^{23}/_{83}$	$^{15}/_{99}$	$^{36}/_{92}$	$^{32}/_{92}$	$^{18}/_{92}$	$^{46}/_{87}$	$^{15}/_{95}$
5{*3*,*3*,R_3}	$^{5}/_{71}$	$^{12}/_{71}$	$^{14}/_{88}$	$^{15}/_{84}$	$^{16}/_{48}$	$^{17}/_{94}$	$^{19}/_{90}$	$^{21}/_{89}$	$^{29}/_{81}$	$^{27}/_{89}$

[a] Yields were calculated on the basis of the weight of product and the initial functionalization of the resin and are shown in the upper left of each cell. [b] Purities were calculated on the basis of integration of the 220 nm UV absorption of the peak of the expected molecular ion. Purities are shown in the lower right of each cell.

Compounds were finally cleaved from the resin. Products from Cl-Trt resin were cleaved with TFA-DCM (3:97) and, after solvent evaporation, protected compounds were treated with TFA−H_2O (95:5) to remove the Boc protecting groups of the lysine. Products from Rink amide and AB resins were cleaved using TFA−H_2O (95:5) for 2 h. Total removal of *p*-methoxybenzyl *N*-hydroxy protecting group from products **5**{R_1,*3*,R_3} needed longer treatment time with TFA (total 6 h). TFA was removed in a Speed-Vac system, products were then dissolved in water and lyophilized. Overall yields of the crude products ranged between 90% and 100%. High-throughput purifications gave the target products in purities higher than 80% for more than 80% of the library, as determined by HPLC (see Table 1). Compounds were characterized by HPLC with PDA, ELSD, and MS detectors; 20% of the library was also characterized by ^{1}H NMR, and 50% of those compounds were also checked by ^{13}C NMR.

Table 2. In Vitro Impact of the Compounds on the Aminoacylation Reaction[a]

R_1 and R_2	Side Chain (R_3)									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
5{*1,2,R*$_3$}	>100	>100	>100	>100	>100	>100	>100	>100	>100	97
5{*2,1,R*$_3$}	37	88	40	51	78	-	48	0	0	43
5{*2,2,R*$_3$}	78	100	88	>100	>100	>100	86	>100	96	>100
5{*2,3,R*$_3$}	-	37	-	5	56	71	>100	76	77	71
5{*3,1,R*$_3$}	-	51	50	38	-	50	56	61	>100	78
5{*3,2,R*$_3$}	>100	96	88	100	89	94	92	67	62	77
5{*3,3,R3*}	47	22	46	43	21	>100	44	>100	>100	>100

[a] Enzyme activities are expressed as percentage of the activity in the absence of an adenylate analogue. Activities that are strongly affected are shaded in dark gray. Moderate changes are shaded in light gray.

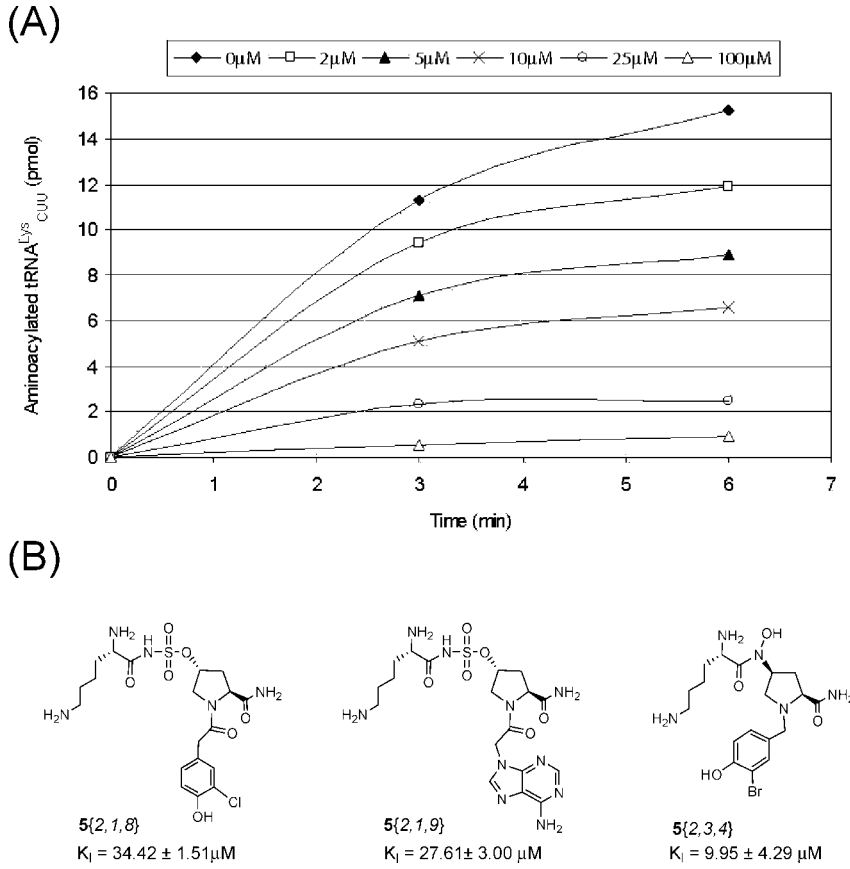


Figure 5. (A) Effect of **5**{*2,3,4*} on the time course of tRNA^{Lys} aminoacylation by LysRS. Time courses were initiated by addition of enzyme and performed in the presence of five different concentrations of compound. (B) Structure of the best inhibitors with their K_i.

In Vitro Screening for Inhibitors of *Trypanosoma brucei* Lysyl-tRNA Synthetase. The inhibitory effects of the library members were analyzed using a simple in vitro tRNA aminoacylation assay.[34] The rate of the reaction was monitored by using radioactive amino acid. tRNA-Bound amino acid was precipitated and separated from soluble nonincorporated amino acid by filtration. *T. brucei* tRNA^{Lys} was prepared by in vitro transcription, overexpressed in *Escherichia coli*, and purified by affinity chromatography. The effect of each lysine-adenylate derivate was analyzed at 100 μM in the presence of nearly saturating concentrations of lysine (Table 2).

Among the 65 compounds tested, there were 15 that had a mild inhibitory effect at 100 μM, and three of these had a strong effect (**5**{*2,1,8*}, **5**{*2,1,9*}, and **5**{*2,3,4*}). The results show that dipeptidomimetics containing an amide bond in the R_2 position are not active. In the comparison of the populations of the sulfamates and the hydroxamates, both families have several moderate inhibitors, 10 and 8, respectively. Interestingly, most of the active compounds display an alkylated proline α-amino group.

Typical K_m values of LysRS for tRNA^{Lys} and lysine are in the low micromolar range, whereas the K_m for ATP tends to be at least 3 orders of magnitude higher.[35] Inhibition constants (K_i) for the compounds that showed strong inhibitory effect (**5**{*2,1,8*}, **5**{*2,1,9*}, and **5**{*2,3,4*}) are, respectively, 34, 28, and 10 μM (Figure 5). With K_i values comparable to the K_m for lysine and tRNA^{Lys}, these molecules can compete with the natural substrates for the access to the catalytic cavity.

Discussion

A library of proline derivatives, using the lysyl adenine monophosphate complex as a model, was prepared on solid-phase support to search for inhibitors of lysyl-tRNA syn-

thetase. The synthetic strategy developed allowed three points of diversity. The first one (C-terminal function) could be easily obtained through the choice of the solid support (hydroxamic acid, carboxylamide, and carboxylic acid).

The introduction of the second point of diversity (sulfamate, hydroxamate, or amide linkage) was successful for carboxylamide and carboxylic acid (Rink-MBHA and AB-MBHA resins, respectively). With these resins, the sulfamate solid-phase synthesis described previously in our group worked fine. The synthesis of hydroxamate analogues using Mitsunobu conditions was also satisfactory and allowed the reduction of excess reagents significantly, obtaining yields that improved those from similar syntheses described in the literature.[27b] However, sulfamates and hydroxamates could not be obtained on Cl-Trt resin. Sulfamates could not be obtained because of premature compound cleavage resulting from resin lability in the strong conditions of the synthesis. In the hydroxamate synthesis, the steric hindrance could be the origin of the low or null incorporation of the lysine residue. This effect was also reported when Mitsunobu conditions were used to modify a residue directly attached to the Cl-Trt resin.[36,37]

Related to the third point of diversity, acylations and alkylations of the α-amino group of the proline worked fine with satisfactory yields. Resin cleavages furnished the final compounds. Crude products from Cl-Trt resin were treated with TFA in solution to remove the Boc protecting groups. Products **5**{*2*−*3*,*3*,R_1} were also exposed for longer time to TFA to completely remove the *p*-methoxybenzyl *N*-hydroxy protecting group.

In general, products were obtained in good purities after purification. Among the different families, the worst results were obtained when compounds with a tertiary amine at the α-position of the proline were synthesized using a Rink amide resin (compounds **5**{*2*,R_2,*1*−*5*}). The main problem in these syntheses was probably the cleavage of the linker during the final acydolitic treatment of the product because of the proximity of a basic point to the linker resin as described Yraola et al.[38]

In terms of inhibition, the combination of carboxamide (R_1) and sulfamate (R_2) apparently provided the most successful compounds. Indeed, seven analogues of the group **5**{*2*,*1*,R_3} had significant inhibitory properties. This group also contained two of the three analogs having a strong inhibitory effect, **5**{*2*,*1*,*8*} and **5**{*2*,*1*,*9*}.

Generally, R_3 substitutions showed no dominant negative effect. Interestingly, the population of inhibitory activities was higher in those series that are alkylated in the α-position of the proline. When proline is acylated in this position, this amide bond tends to adopt a trans configuration, a fact that can fix the orientation of R_3 moiety, preventing conformations that could be better accommodated in the active site. Furthermore, R_3 substitutions *8* and *9* were only effective in combination with carboxamide (R_1) and sulfamate (R_2).

Compounds **5**{R_1,*2*,R_3}, which have the natural phosphate bond substituted by an amide group (R_2), were poor competitors. Because identical analogues with different R_2 substitutions had mid-level or even strong inhibitory effects, it is likely that this amide group designed to mimic the phosphate bond is responsible for the rejection or lack of stabilization of the corresponding molecules.

Unfortunately the effect of the hydroxamic acid substitution (R_1) could not be fully evaluated because groups **5**{*1*,*1*,R_3} and **5**{*1*,*3*,R_3} could not be synthesized. Moreover, compounds **5**{*1*,*2*,R_3} did not show any inhibitory effect.

In conclusion, a library of proline derivatives was synthesized on solid-phase supports. Using the methodology described previously in our group, we easily synthesized sulfamates and hydroxamates by applying a combinatorial approach. Three of the compounds caused inhibition of the tRNA aminoacylation reaction in vitro, in the low micromolar range. Further libraries are being synthesized in our laboratory to increase the inhibitory activities, as well as their membrane permeabilities.

Experimental Section

General Procedures. Solid-Phase Synthesis. Syntheses were performed manually in a polypropylene syringe fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washings between deprotection, coupling, and subsequent deprotection steps were carried out with DMF (5 × 1 min) and DCM (5 × 1 min) using 10 mL of solvent/g of resin each time.

Fmoc Group Removal. The Fmoc group was removed using the following reaction conditions: (i) DMF (5 × 1 min); (ii) piperidine/DMF (2:8) (1 × 1 and 2 × 15 min); (iii) DMF (5 × 1 min).

Alloc Group Removal. Removal of the Alloc group was achieved with $Pd(PPh_3)_4$ (0.1 equiv) in the presence of $PhSiH_3$ (10 equiv) in DCM under Ar (2 × 20 min, 25 °C).

THP Group Removal. Removal of the THP group was achieved with a solution of *p*-TsOH (5 mg/ml) in DCM-MeOH (97:3) 2 × 1 h, preceded by a 3 min washing with the same solution.

Amide Bond Formation. Peptidyl resin **2a**{R_1} was washed with anhydrous DCM (5 × 1 min) and DMF (5 × 1 min). After it was washed, it was treated with Boc-L-Lys(Boc)-OH (5 equiv), DIPCDI (5 equiv), and HOBt (5 equiv) in DMF for 2 h. After the products was washed with DMF (5 × 1 min) and DCM (5 × 1 min), the extension of the coupling was monitored by the ninhydrin test.

Sulfamoylations.[30] Sulfamoyl chloride was obtained in situ by dropwise addition of formic acid (270 μL, 10 equiv) to neat chlorosulfonyl isocyanate (620 μL, 10 equiv) at 0 °C with rapid stirring. Vigorous gas evolution was observed during the addition. DMA was then added (2 mL), and the mixture was stirred at room temperature for one hour. Meanwhile, resins **2a**{*R*1} (1 g, 1 equiv) were preswollen in DMA (5 mL) and were stirred at room temperature for 30 min. The solution of sulfamoyl chloride (10 equiv) in DMA was then added dropwise, and the resins were stirred at room temperature for 3 h. Finally, the resins were filtered and washed with DMA (3 × 1 min), DMF (3 × 1 min), and DCM (3 × 1 min).

General Method for Hydroxamate Synthesis using Mitsunobu Reaction. To the peptidyl resins **2a**{R_1} (1 g) preswollen in anhydrous DCM (5 mL) was added PPh_3 (7 equiv) and Boc-L-Lys(Boc)-OPMB (7 equiv). The mixtures

were shaken until the reagents were completely dissolved. Then, DIAD (7 equiv) was added dropwise at 0 °C, and the mixture was shaken overnight at room temperature. After that, the solvent was removed by suction; the resins were washed with DCM (5 × 1 min) and then were washed twice with DMF, DCM, methanol, and finally, with DCM.

N^{α}-Acylation. After the N^{α}-Alloc group had been removed, acylations of the α-amino groups were carried out using RCOOH (5 equiv), DIPCDI (5 equiv), and HOBt (5 equiv.) in DMF for 2 h at 25 °C. Resins were washed with DMF (5 × 1 min) and DCM (5 × 1 min). Acylations were monitored by the chloranil test.

N^{α}-Alkylation. After the Alloc group had been removed, alkylations of the α-amino group were performed by on-resin reductive amination using RCHO (5 equiv) and $NaBH_3CN$ (5 equiv) in 1% HOAc in DMF for 2 h. After the reductive amination, resins were washed with DMF (5 × 1 min) and DCM (5 × 1 min). The extent of the reactions was monitored with the chloranil test.

Acidolytic Cleavage with TFA. Cl-Trt resins were cleaved with five 30 s washes with TFA/CH_2Cl_2 (3:99). Crude products were then evaporated and treated with TFA/H_2O (95:5) for 2 h at room temperature to remove the protecting groups. Rink amide and MBHA with handle AB resins were cleaved with TFA/H_2O (95:5) for 2 h at room temperature. TFA was evaporated; the compounds were dissolved in H_2O−MeCN and then lyophilized.

HPLC-PDA-ELSD-ESMS Analysis. HPLC analyses were carried out with Waters equipment. The sample was injected into the system with a Waters Alliance 2696 system, which supplied the HPLC gradient, maintaining the column heater set at 40 °C. Flow proceeded from the column through a Waters 996 photodiode array (PDA) detector, which monitored wavelengths from 210 to 400 nm. A wavelength of 220 nm was selected for the analysis of purity. From the PDA, the 1 mL/min flow was split using a flow splitter (0.5 mL/min each) to the Waters 2420 ELS detector (ELSD) and the electrospray Waters Micromass ZQ MS detector. In the ELSD detector, the nitrogen pressure was set at 25 psi; the temperature was set at 40 °C, and the gain was set to 135. The system was controlled by a Micromass−Masslyx 4.0. data system. The first analysis was carried out using a gradient with water (containing 0.1% formic acid) and acetonitrile (containing 0.07% formic acid) and another one using the same solvents containing 0.1% of TFA. The gradient used in the analysis was from 0% of ACN to 40% of ACN in 10 min. The column used was a X-Terra C_{18} 3.5 μm (4.6 × 100 mm).

Purification. All the products were purified in a Waters semipreparative HPLC system. Samples were injected automatically using a Waters 2767 sample Manager, which was used also for the collection. The HPLC gradient was supplied by a Waters 600 Controller System. The sample passed through a Symmetry C_{18} 5 μm (30 × 100 mm) column and, in a make-up pump, the flux was split and just 0.1% went to the Waters 2487 Dual detector and to the electrospray Waters Micromass ZQ MS detector. The other flux went to the collector, which collected the samples when detected. Purifications were carried out using different gradients with water (containing 0.1% formic acid) and acetonitrile (containing 0.1% formic acid). The system was controlled by a Micromass−Masslyx 4.0. data system. Fractions were finally lyophilized.

NMR Spectroscopy. NMR spectra were acquired on a Mercury 400 spectrometer. Compounds were characterized in CD_3OD or D_2O at 25 °C. Twenty percent of the library was analyzed by ^1H NMR spectra, and ∼10% was assigned unequivocally with ^{13}C spectra and the gCOSY and gHSQC experiments. One-dimensional spectra were recorded using different scans depending on the sample concentration (from 16 to 128 scans in the ^1H experiments and were maintained between 2000 and 5000 scans for the ^{13}C experiments). Two-dimensional spectra gCOSY and gHSQC were recorded employing standard pulse sequences.

(2*S*,4*R*)-THP-4-hydroxy-1-Alloc-pyrrolidine-2-carboxylic Acid. 3,4-Dihydro-2*H*-pyrane (3.48 mL, 36.6 mmol) was added dropwise to a stirred solution of (2*S*,4*R*)-4-hydroxy-1-Alloc-pyrrolidine-2-carboxylic acid (5.25 g, 24.4 mmol) and *p*-TsOH (0.47 g, 2.4 mmol) in CH_2Cl_2 (50 mL) at 0 °C. After 5 min, the ice bath was removed, and the mixture was stirred for 2 h at room temperature. After that, the solvent was evaporated; the solid was dissolved in EtOAc (60 mL) and extracted with aqueous 0.2 N KOH (2 × 50 mL). The combined aqueous layers were acidified with 6 N HCl to pH 3−4 and extracted with EtOAc (3 × 50 mL), maintaining the pH between 3 and 4. The combined organic extracts were washed with water, dried with anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The crude was purified by flash chromatography to give the final product (6.6 g, 92%) as a colorless oil.

***N*-Hydroxyphtalimide *p*-methoxybenzylether (6).** *p*-Methoxybenzyl chloride (17.43 mL, 126 mmol) was added dropwise to a solution of *N*-hydroxyphtalimide (10.00 g, 63 mmol) and K_2CO_3 (6.69 g, 48 mmol) in DMSO (70 mL). The reaction mixture was stirred for 24 h, poured into 200 mL of cold water, and kept in ice. Crystals were collected in a Buchner funnel, washed twice with cold water, and dried in the vacuum. Product **6** (17.36 g) was used in the next step without any purification and characterization.

Boc-Lys(Boc)-NH-OPMB (7). Hydrazine (3.31 mL, 67.0 mmol) was added to a suspension of compound **6** (17.36 g, 61.3 mmol) in 200 mL of EtOH. The mixture was stirred overnight. Hydrochloric acid (1.1 equiv) was then added, and a white precipitate appeared. The precipitate was filtered off and washed with EtOH (3 × 50 mL) and H_2O (100 mL). Ethanol from the aqueous mixture was evaporated under vacuum; H_2O (250 mL) was added, and the mixture was make alkaline. A precipitate appeared, and it was extracted with Et_2O (4 × 200 mL), dried with $MgSO_4$, and evaporated. *O*-(4-Methoxy-benzyl)-hydroxylamine (7.25 g, 78%) was obtained as colorless crystals and was used in the next step without purification.

To a stirring solution of Boc-Lys(Boc)-OH (15.90 g, 104 mmol) at room temperature in anhydrous DCM (200 mL) was added HOBt (6.76 g, 50.0 mmol) and EDC (11.73 g, 169 mmol). After 30 min, compound *O*-(4-Methoxy-benzyl)-hydroxylamine (7.00 g, 104 mmol) was added. The mixture was stirred at room temperature overnight, and then the

organic phase was washed with 0.1 M HCl and saturated aqueous $NaHCO_3$ (100 mL), dried with $MgSO_4$, filtered, and concentrated under vacuum. The crude product was purified by flash chromatography to give the protected hydroxamic acid **7** (18.53 g, 85%) as a white solid. ^{1}H NMR [DMSO-d_6, 400 MHz]: δ 1.10−1.40 (m, 22H, 2 × CH_2 and 6 × CH_3), 1.44−1.50 (m, 2H, CH_2), 2.84−2.88 (m, 2H, CH_2), 3.752 (m, 4H, CH_3 and CH), 4.678 (s, 2H, CH_2), 6.745 (t, 1H, NH), 6.920 (d, 2H, 2 × CH, J = 8.8 Hz), 7.307 (d, 2H, 2 × CH, J = 8.8 Hz), 11.056 (s, 1H, NH). ^{13}C NMR [DMSO-d_6, 100 MHz]: δ 22.7 (CH_2), 28.1 (CH_3), 28.2 (CH_3), 29.1 (CH_2), 31.5 (CH_2), 40.0 (CH_2), 52.1 (CH), 55.1 (CH_3), 76.3 (CH_2), 77.3 (C), 77.9 (C), 113.6 (CH), 127.8 (CH), 130.6 (C), 155.2 (C), 155.5 (C), 159.3 (C), 169.0 (C).

5{*1,2,2*}. ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.43−1.50 (m, 2H, CH_2), 1.65−1.70 (m, 2H, CH_2), 1.77−1.90 (m, 2H, CH_2), 2.04−2.01 (m, 1H, CH_2), 2.78−2.85 (m, 1H, CH_2), 2.94 (t, 2H, CH_2, J = 7.6 Hz), 3.52−3.53 (m, 2H, CH_2), 3.82 (t, 1H, J = 6.8 Hz, CH), 4.03−406 (m, 1H, CH), 4.12 (d, 1H, CH_2, J = 12.8 Hz), 4.25 (d, 1H, CH_2, J = 12.8 Hz), 4.43−4.52 (m, 1H, CH), 6.89−6.98 (m, 3H, 3 × CH).

5{*1,2,9*}. ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.52−1.58 (m, 2H, CH_2), 1.68−1.93 (m, 4H, 2 × CH_2), 2.55−2.62 (m, 1H, CH_2), 2.95−2.99 (m, 1H, CH_2), 3.13 (t, 2H, J = 6.0 Hz, CH_2), 3.71−3.73 (m, 1H, CH_2), 3.87 (m, 1H, CH), 4.12 (m, 1H, CH_2), 4.42 (m, 1H, CH), 4.59 (m, 1H, CH), 5.18 (s, 2H, CH_2), 8.07 (s, 1H, CH), 8.19 (s, 1H, CH).

5{*2,1,9*}. ^{1}H NMR [D_2O, 400 MHz]: δ1.31−1.43 (m, 2H, CH_2), 1.54−1.64 (m, 2H, CH_2), 1.76−1.87 (m, 2H, CH_2), 2.13−2.19 (m, 1H, CH_2), 2.55−2.61 (m, 1H, CH_2), 2.89 (t, 2H, CH_2, J = 7.6 Hz), 3.73 (t, 1H, CH, J = 6.4 Hz), 3.94 (dd, 1H, CH_2, J = 12.0 Hz, J = 4.0 Hz), 4.05 (d, 1H, CH_2, J = 12.0 Hz), 4.43 (t, 1H, CH, J = 8.4 Hz), 5.03−5.20 (m, 3H, CH_2 and CH), 7.94 (s, 1H, CH), 8.05 (s, 1H, CH), 8.32 (NH). ^{13}C NMR [D_2O, 100 MHz]: δ 21.4 (CH_2), 26.5 (CH_2), 30.5 (CH_2), 35.5 (CH_2), 39.2 (CH_2), 45.6 (CH_2), 52.8 (CH_2), 55.4 (CH), 59.2 (CH), 79.4 (CH), 118.2 (C), 143.0 (C), 149.4 (CH), 152.8 (CH), 155.7 (C), 167.6 (C), 175.6 (C), 175.8 (C).

5{*2,2,5*}. ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.19−1.28 (m, 2H, CH_2), 1.44−1.67 (m, 4H, 2 × CH_2), 1.81−1.86 (m, 1H, CH_2), 2.57−2.64 (m, 1H, CH_2), 2.79−2.84 (m, 4H, 2 × CH_2), 3.24−3.31 (m, 1H, CH), 3.47 (m, 1H, CH), 3.90 (d, 1H, CH_2, J = 12.4), 3.99 (s, 3H, CH_3), 4.22 (d, 1H, CH_2, J = 12.4), 4.29 (m, 1H, CH), 6.82 (d, 1H, CH, J = 7.6 Hz), 7.36 (d, 1H, CH, J = 7.6 Hz), 7.47 (t, 1H, CH, J = 8.0 Hz), 7.56 (t, 1H, CH, J = 8.0 Hz), 8.25 (d, 1H, CH, J = 8.4 Hz), 8.30 (d, 1H, CH, J = 8.4 Hz), 8.49 (bb, 1H, NH).

5{*2,2,7*}. ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.40 (m, 2H, CH_2), 1.69 (m, 2H, CH_2), 1.78 (m, 2H, CH_2), 1.96−2.02 (m, 1H, CH_2), 2.63−2.67 (m, 1H, CH_2), 2.90 (m, 2H, CH_2), 3.53 (m, 1H, CH_2), 3.67 (m, 1H, CH), 3.89 (m, 4H, CH_3 and CH_2), 4.35 (m, 1H, CH), 4.61 (m, 1H, CH), 6.98 (d, 1H, CH, J = 8.0 Hz), 7.05 (s, 1H, CH), 7.08 (d, 1H, CH, J = 8.0 Hz).

5{*2,3,5*}. ^{1}H NMR [D_2O, 400 MHz]: δ 1.32−1.40 (m, 2H, CH_2), 1.54−1.66 (m, 2H, CH_2), 1.70−1.83 (m, 2H, CH_2), 2.00−2.06 (m, 1H, CH_2), 2.67−2.74 (m, 1H, CH_2), 2.90−2.95 (m, 2H, CH_2), 3.01−3.06 (m, 1H, CH_2), 3.42−3.48 (m, 1H, CH_2), 3.70−3.75 (m, 1H, CH_2), 4.05 (s, 3H, CH_3), 4.10−4.14 (m, 1H, CH_2), 4.27−4.42 (m, 1H, CH), 4.64 (t, 1H, CH, J = 7.6 Hz), 5.13 (bb, 1H, CH), 6.97 (d, 1H, CH, J = 7.8 Hz), 7.48 (d, 1H, CH, J = 7.8 Hz), 7.61 (t, 1H, CH, J = 7.4 Hz), 7.69 (t, 1H, CH, J = 7.4 Hz), 8.24−8.32 (m, 2H, 2 × CH).

5{*2,3,8*}. The NMR spectrum corresponds to a mixture of two conformers in a relative ratio (3:4). ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.41−1.56 (m, 2H, CH_2), 1.66−1.75 (m, 2H, CH_2), 1.79−2.01 (m, 1H, CH_2), 1.93 (m, 1H, CH_2), 2.34 (d, 0.6H, CH_2, J = 14.5 Hz), 2.48 (d, 0.4H, CH_2, J = 14.5 Hz), 2.53−2.61 (m, 0.6H, CH_2), 2.68−2.76 (m, 0.4H, CH_2), 2.92−2.98 (m, 2H, CH_2), 3.50 (d, 1H, CH_2, J = 12.4 Hz), 3.65 (s, 1H, CH_2), 3.69−3.81 (m, 1H, CH_2), 3.93−4.03 (m, 2H, CH and CH_2), 4.64 (d, 0.6H, CH, J = 10.0 Hz), 4.72 (d, 0.4H, CH, J = 10.0 Hz), 5.40−5.44 (m, 1H, CH), 6.84 (d, 0.4H, CH, J = 8.4 Hz), 6.86 (d, 0.6H, CH, J = 8.4 Hz), 6.98 (dd, 0.4H, CH, J = 8.4 Hz, J = 2.0 Hz), 7.03 (dd, 0.6H, CH, J = 8.4 Hz, J = 2.0 Hz), 7.19 (d, 0.4H, CH, J = 2.0 Hz), 7.24 (d, 0.6H, CH, J = 2.0 Hz). ^{13}C NMR [CD_3OD, 100 MHz]: δ 21.6−21.9 (CH_2), 26.7 (CH_2), 29.4−29.5 (CH_2), 34.7−37.2 (CH_2), 39.0−39.1 (CH_2), 39.5−39.8 (CH_2), 52.5 (CH), 52.6−53.1 (CH_2), 58.8−59.3 (CH), 74.4−76.1 (CH), 116.3−116.4 (CH), 120.3−120.4 (C), 126.4−126.7 (C), 128.8−130.6 (CH), 152.1 (CH), 161.7−162.1 (C), 168.7−168.8 (C), 171.6−172.2 (C), 174.6−174.8 (C).

5{*2,3,10*}. The NMR spectrum corresponds to a mixture of two conformers. ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.50−1.59 (m, 2H, CH_2), 1.69−1.76 (m, 2H, CH_2), 1.89−2.01 (m, 1H, CH_2), 1.83−2.01 (m, 1H, CH_2), 2.32 (d, 0.5H, CH_2, J = 14.4 Hz), 2.56−2.63 (m, 1H, CH_2), 2.75−2.83 (m, 0.5H, CH_2), 2.95−3.01 (m, 2H, CH_2), 3.72 (d, 0.5H, CH_2, J = 14.4 Hz), 3.90 (d, 1H, CH_2, J = 14.4 Hz), 4.00−4.04 (m, 1H, CH), 4.16 (dd, 0.5H, CH_2, J = 12.2 Hz, J = 5.2 Hz), 4.67 (d, 1H, CH, J = 8.4 Hz), 4.78−5.02 (m, 3H, CH_2 and CH), 5.44 and 5.53 (two d, 1H, CH, J = 4.4 Hz), 7.97 (s, 1H, CH). ^{13}C NMR [CD_3OD, 100 MHz]: δ 21.6−21.9 (CH_2), 26.7−26.8 (CH_2), 29.34−29.5 (CH_2), 34.6−37.2 (CH_2), 39.1 (CH_2), 44.9 (CH_2), 52.1−53.3 (CH_2), 52.5 (CH), 58.9−59.2 (CH), 74.2−76.1 (CH), 139.2 (C), 154.5 (C), 157.9 (CH), 161.6−167.3 (C), 166.8−167.3 (C), 168.7−168.8 (C), 174.0 (C), 174.5 (C).

5{*3,1,6*}. ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.40−1.48 (m, 2H, CH_2), 1.56−1.65 (m, 2H, CH_2), 1.75−1.84 (m, 2H, CH_2), 2.31−2.37 (m, 1H, CH_2), 2.61−2.67 (m, 1H, CH_2), 2.83−2.87 (m, 2H, CH_2), 3.03−3.08 (m, 1H, CH_2), 3.59−3.64 (m, 1H, CH_2), 3.80 (s, 3H, CH_3), 3.98−4.02 (m, 1H, CH), 4.37−4.42 (m, 1H, CH), 5.39−5.48 (m, 1H, CH), 6.87 (d, 1H, CH, J = 7.6 Hz), 8.00 (d, 1H, CH, J = 7.6 Hz), 7.38 (s, 1H, CH).

5{*3,2,2*}. The ^{1}H NMR spectrum corresponds to a mixture of two conformers in a relative ratio (4:3). ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.43−1.49 (m, 2H, CH_2), 1.65−1.73 (m, 2H, CH_2), 1.79−1.90 (m, 2H, CH_2), 2.22−2.28 (m, 0.5H, CH_2), 2.45 (t, 1H, CH_2), 2.78−2.86 (m, 0.5H, CH_2), 2.91−2.99 (m, 2H, CH_2), 3.18−3.23 (m, 1H, CH_2), 3.70−3.79 (m, 2H, CH_2 and CH), 3.86 and 3.86 (s, 3H, CH_3), 4.04−4.08 (m, 0.5H, CH), 4.15−4.22 (m, 1.5H, 0.5CH and CH_2), 4.33−4.48 (m, 2H, CH and CH_2), 6.95−6.99 (m, 3H, 3 × CH). ^{13}C

NMR [CD_3OD, 100 MHz]: δ 21.7−21.8 (CH_2), 26.9 (CH_2), 30.7−30.8 (CH_2), 34.3−34.7 (CH_2), 39.0 (CH_2), 49.3 (CH), 52.6−53.0 (CH), 55.2−55.2 (CH_3), 56.2−57.6 (CH_2), 58.3 (CH_2), 66.6−67.0 (CH), 111.6 (CH), 116.8−117.1 (CH), 121.9−122.1 (CH), 122.9−123.1 (C), 147.1−149.3 (C), 161.7−162.1 (C), 169.1−169.5 (C), 170.7−171.3 (C).

5{*3,2,7*}. ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.40−1.450 (m, 2H, CH_2), 1.67 (bb, 2H, CH_2), 1.80−1.88 (m, 2H, CH_2), 1.99−2.05 (m, 1H, CH_2), 2.67−2.74 (m, 1H, CH_2), 2.91 (t, 2H, CH_2, J = 7.6 Hz), 3.55 (dd, 1H, CH_2, J = 10.8 Hz, J = 6.8 Hz), 3.78−3.83 (m, 1H, CH), 3.86−3.94 (m, 4H, CH_3 and CH_2), 4.35 (t, 1H, CH, J = 6.4 Hz), 4.58 (t, 1H, CH, J = 7.6 Hz), 6.97 (d, CH, J = 8.4 Hz), 7.04 (s, 1H, CH), 7.01 (d, CH, J = 8.4 Hz). ^{13}C NMR [CD_3OD, 100 MHz]: δ 21.7 (CH_2), 26.8 (CH_2), 30.6 (CH_2), 33.9 (CH_2), 39.052 (CH_2), 49.3 (CH), 52.9 (CH), 54.7 (CH_3), 55.2 (CH_2), 59.2 (CH), 110.9 (CH), 114.5 (CH), 119.494 (CH), 128.2 (C), 146.4 (C), 150.0 (C), 168.7 (C), 170.4 (C).

5{*3,3,3*}. ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.44−1.53 (m, 1H, CH_2), 1.55−1.63 (m, 1H, CH_2), 1.66−1.74 (m, 2H, CH_2), 1.78−1.87 (m, 1H, CH_2), 1.93−2.00 (m, 1H, CH_2), 2.39 (d, 1H, CH_2, J = 14.8 Hz), 2.75−2.83 (m, 1H, CH_2), 2.91−2.99 (m, 2H, CH_2), 3.28−3.33 (m, 1H, CH_2), 3.69 (d, 1H, CH_2, J = 12.8 Hz), 3.85 (dd, 1H, CH, J = 10.0 Hz, J = 4.0 Hz), 3.98 (t, 1H, CH, J = 6.4 Hz), 4.29 (d, 1H, CH_2, J = 13.2 Hz), 4.36 (d, 1H, CH_2, J = 13.2 Hz), 5.40 (t, 1H, CH, J = 5.2 Hz), 7.77 (d, 2H, 2 × CH, J = 8.4 Hz), 8.25 (d, 2H, 2 × CH, J = 8.4 Hz). ^{13}C NMR [CD_3OD, 100 MHz]: δ 22.1 (CH_2), 26.7 (CH_2), 29.6 (CH_2), 35.0 (CH_2), 38.9 (CH_2), 52.6 (CH), 57.6 (CH_2), 59.4 (CH_2), 65.3 (CH), 75.5 (CH), 123.6 (2 × CH), 131.1 (2 × CH), 140.9 (C), 148.4 (C), 168.6 (C), 172.9 (C).

5{*3,3,4*}. ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.45−1.53 (m, 1H, CH_2), 1.55−1.63 (m, 1H, CH_2), 1.66−1.73 (m, 2H, CH_2), 1.78−1.84 (m, 1H, CH_2), 1.93−2.03 (m, 1H, CH_2), 2.54 (d, 1H, CH_2, J = 14.8 Hz), 2.73−2.80 (m, 1H, CH_2), 2.93−3.00 (m, 2H, CH_2), 3.58 (dd, 1H, CH_2, J = 13.2 Hz, J = 4.4 Hz), 3.95−3.99 (m, 2H, CH and CH_2), 4.08 (dd, 1H, CH, J = 10 Hz, J = 2.0 Hz), 4.21 (d, 1H, CH_2, J = 12.8 Hz), 4.41 (d, 1H, CH_2, J = 12.8 Hz), 5.43 (t, 1H, CH, J = 4 Hz), 6.92 (d, 1H, J = 8.4 Hz,), 7.35 (dd, 1H, CH, J = 8.4 Hz, J = 2.4 Hz), 7.72 (d, 1H, CH, J = 2.0 Hz). ^{13}C NMR [CD_3OD, 100 MHz]: δ 23.3 (CH_2), 27.9 (CH_2), 30.8 (CH_2), 35.6 (CH_2), 40.1 (CH_2), 53.9 (CH), 59.2 (CH_2), 60.6 (CH_2), 67.0 (CH), 76.8 (CH), 111.3 (C), 117.5 (CH), 124.3 (C), 132.3 (CH), 136.7 (CH), 157.1 (C), 169.7 (C), 173.1 (C).

5{*3,3,7*}. ^{1}H NMR [CD_3OD, 400 MHz]: δ 1.47 (m, 1H, CH_2), 1.56−1.62 (m, 1H, CH_2), 1.67−1.76 (m, 2H, CH_2), 1.84−1.96 (m, 2H, CH_2), 2.41−2.60 (m, 2H, CH_2), 2.96−3.02 (m, 2H, CH_2), 3.75−3.80 (m, 1H, CH_2), 3.89 (s, 3H, CH_3), 3.94−3.99 (m, 1H, CH_2), 4.14 (dd, 1H, CH, J = 14.8 Hz, J = 6.0 Hz), 4.48 (d, 1H, CH, J = 8.0 Hz), 5.41 (t, 1H, CH, J = 4.4 Hz), 6.91−7.06 (m, 3H, CH). ^{13}C NMR [CD_3OD, 100 MHz]: δ 21.7 (CH_2), 26.8 (CH_2), 29.6 (CH_2), 36.8 (CH_2), 38.8 (CH_2), 52.7 (CH), 55.2 (CH_2), 62.6 (CH), 75.3 (CH), 110.7 (CH), 114.3 (CH), 118.9 (CH), 129.2 (C), 146.3 (C), 149.6 (C), 162.1 (C), 168.7 (C), 171.7 (C).

tRNA preparation. A construct containing a T7 promoter, followed by the gene encoding wild-type $tRNA^{Lys}_{CUU}$, was assembled using six DNA oligonucleotides that were first annealed, and then ligated, between *Hin*dIII and *Bam*HI restriction sites of plasmid pUC19. In vitro transcription using T7 RNA polymerase was performed according to standard protocols. Transcripts were separated on denaturing PAGE, full-length tRNAs were eluted from gel using an electroelution apparatus (Schleicher & Schüll).

Enzyme Cloning and Purification. The 1.7-kbp intronless gene coding for *T. brucei* LysRS (Tb927.8.1600) was amplified by PCR from genomic DNA using Pfu Ultra DNA polymerase and cloned in the vector pET30EK/Lic (Novagen) for bacterial expression of a N-terminal His_6-tagged protein. The correct sequence of the gene was checked by sequencing it entirely. Novablue *E. coli* cells (Novagen) transformed with this construction were grown at 20 °C up to A_{700nm} = 0.3. Protein expression was then induced with 1 mM IPTG for 12 h. Purification on nickel affinity columns was performed using standard procedures.

Aminoacylation Assay. The screening for inhibitory compounds was performed in 96-well filter plates at 37 °C in reaction mixtures containing 100 mM HEPES, pH 7.2, 20 mM KCl, 30 mM $MgCl_2$, 500nM dithiothreitol, 0.1 mg/ml BSA, 5 mM ATP, 3 μM L-[^{3}H] lysine (6000 Ci/mol), 8 μM of in vitro transcribed $tRNA^{Lys}_{CUU}$, and 100 μM of each analog. The reactions were started by the addition of 20 nM of purified LysRS. Aliquots were taken at different time points and quenched into 200 μL of 5% trichloroacetic acid on a multiscreen filter plate (Millipore). The solution was filtered on a Multiscreen HTS vacuum manifold (Millipore), and the retained radioactivity was measured by liquid scintillation with a TopCount NXT Scintillation and Luminescence Counter (Packard).

Inhibitory constants (K_i) were calculated using at least four different concentrations of analog in reaction mixtures containing 3μM L-[^{3}H] lysine (1500 Ci/mol). Aliquots were placed on Whatman 3-mm paper discs and immediately submerged in a solution of 5% trichloroacetic acid. Radioactivity was measured by liquid scintillation. At least three independent experiments were done for each of the analogs **5**{*2,1,8*}, **5**{*2,1,9*}, and **5**{*2,3,4*}.

Abbreviations: Ac, acetyl; Ac_2O, acetic anhydride; Amp, *cis*-4-amino-L-proline or (2*S*,4*S*)-4-amino-pyrrolidine-2-carboxylic acid; Boc, *tert*-butoxycarbonyl; Dab, α,γ-diaminobutyric acid; DCM, dichloromethane; DHP, 3,4-dihydro-2H-pyrane; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; EDC, 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride; ELSD, evaporative light-scattering detector; Fmoc, 9-fluorenylmethoxycarbonyl; (2*S*,4*S*)-Boc-Amp(Fmoc)-OH, (2*S*,4*S*)-Fmoc-4-amino-1-Boc-pyrrolidine-2-carboxylic acid; HOAc, acetic acid; HOBt, 1-hydroxy-1,2,3-benzotriazole; HR-ESI: high-resolution electrospray; Hyp, hydroxyproline; *i*V, *iso*-valeryl; MALDI-TOF, matrix-assisted laser desorption ionization, time-of-flight; MBHA, *p*-methylbenzhydrylamine; MeCN, acetonitrile; MeOH, methanol; MPLC, medium-pressure liquid chromatography; PDA, photodiode array; PhAc, phenylacetyl; RP-HPLC, reversed-phase high-

performance liquid chromatography; SPOS, solid-phase organic synthesis; SPPS, solid-phase peptide synthesis; TBME, *t*-butylmethyl ether; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; THP, tetrahydropyran. Amino acid symbols denote the L configuration.

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Supporting Information Available. HPLC chromatograms and ^{1}H and ^{13}C NMR spectra for selected compounds and the effect of **5**{*2*,*3*,*4*} on the time course aminoacylation of $tRNA^{Lys}$ by LysRS. This material is available free of charge via the Internet at http://pubs.acs.org.

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