MedChemComm

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/medchemcomm

MedChemComm

RSCPublishing

CONSICE ARTICLE

Synthesis of Cyclohexapeptides as Antimalarial and Anti-trypanosomal Agents

Cite this: DOI: 10.1039/x0xx00000x

S. Peña,[a] C. Fagundez,[a] A. Medeiros,[b,c] M. Comini,[b] L. Scarone,[a] D. Sellanes,[a] E. Manta,[a] J. Tulla-Puche,[d,e] F. Albericio,[d,e,f,g] L. Stewart,[h] V. Yardley[h] and G. Serra.[a]*

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Cyclohexapeptides analogs of natural products were obtained in very good yields by a combination of solid-phase peptide synthesis, for the linear peptide and solution cyclization. The activities against *Plasmodium falciparum* K1, *Trypanosoma brucei brucei* and murine macrophages (cell line J774) of these novel compounds and azolic macrocycles, previously reported by us, were evaluated. Seven macrocycles showed submicromolar activities against *Plasmodium falciparum* K1 and a high selectivity (SI > 125) for the parasite. In addition, two compounds displayed one digit micromolar EC_{50} against *T. brucei brucei* and satisfactory selectivity (SI 82 and 95). Preliminary structure activity relationships are presented.

Introduction

Parasitic tropical diseases such as Human African Trypanosomiasis (HAT) and malaria affect almost 300 million people every year.

Malaria is a life-threatening illness caused by *Plasmodium* parasites that are transmitted to human through the bite of an infected female *Anopheles* mosquito. *P. falciparum* is the most deadly of the four species that cause malaria.

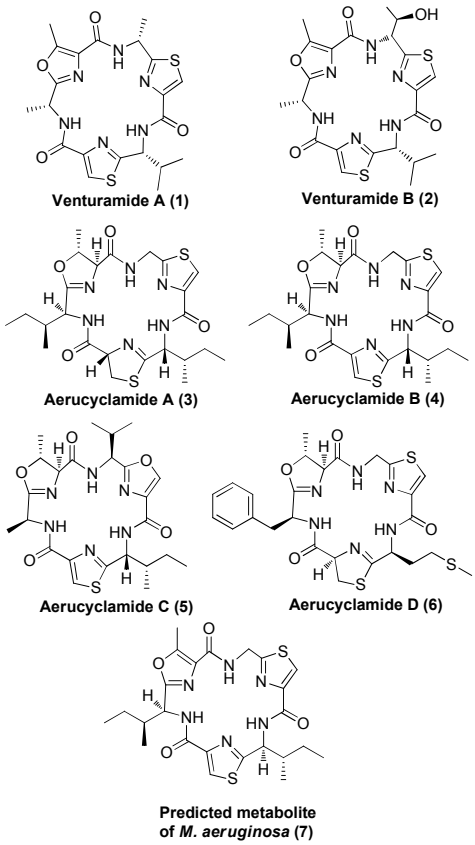


Figure 1. Examples of natural azole cyclohexapeptides with antimalarial or/and antitrypanosomal activities

[a] E-mail: gserra@fq.edu.uy; Fax:+ 598 29241906; Tel.: +598 29290290. Cátedra de Química Farmacéutica, (DQO), Facultad de Química, Universidad de la República, Gral. Flores 2124, Montevideo, CP 11800, Uruguay.

[b] Group Redox Biology of Trypanosomes, Institut Pasteur de Montevideo, Mataojo 2020, CP 11400, Montevideo, Uruguay.

[c] Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, Gral. Flores 2125 CP 11800, Montevideo, Uruguay.

[d] Institute for Research in Biomedicine (IRB Barcelona), Baldiri Reixac 10, 08028-Barcelona, Spain.

[e] Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028-Barcelona, Spain.

[f] Department of Organic Chemistry, University of Barcelona, 08028-Barcelona, Spain.

[g] School of Chemisty & Physics, University of KwaZulu-Natal, 4001-Durban, South Africa

[h] Faculty of Infectious & Tropical Disease, LSHTM, Keppel Street, London, WC1E 7HT, UK.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

Medicinal Chemistry Communications Accepted Manuscript

According to the World Health Organization 2012 report, approximately 3.3 billion people were at risk of malaria in 2011, principally in the sub-Saharan region.[1] Indeed, this region of Africa is also endemic to HAT or sleeping sickness, which is caused by *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. Both subspecies are transmitted by the bite of an infected tsetse fly (*Glossina Genus*) and have a fatal outcome if left untreated. The current statistics in Africa's endemic countries report about 60 million people at risk with ~10,000 new cases per year.[2] Moreover, and though not pathogenic to humans, the closely related subspecies *T. brucei brucei* produces a highly disabling disease in cattle, known as Nagana, which impacts heavily in the economy and nutritional status of the rural communities affected.[3]

Present-day chemotherapy for these diseases is not effective due to a limited number of useful drugs, poor efficacy, undesirable route of administration, emergence of drug resistance, side effects and unacceptable toxicity.[4] In the case of malaria, drug resistance is a major problem, since recently artemisinin resistance has been detected in the south-east Asia.[1,5]

With billions of people at risk and the unsuccessful current chemotherapy, there is an urgent need for discovering safe and effective antimalarial and anti-trypanosomal drugs. For these reasons, during the last decade, several research groups have been involved in the isolation, design and synthesis of antiparasitic compounds.[6]

Natural products are excellent models for the development of potential antiparasitic chemotherapy. The structural diversity is a source of inspiration for drug discovery and the preparation of analogs as simplified, synthetically more accessible and stable models are broadly described in the literature. According to Newman and Cragg's revision, approximately 71 % of new approved drugs from 1981 to 2010, were derived or inspired from natural products.[7]

Several azolic cyclopeptides displaying anti-plasmodial or anti-trypanosomal activity were identified over the last years.[8] Gerwick and co-workers have isolated antimalarial venturamides A (**1**) and B (**2**) from the marine cyanobacterium *Oscillatoria sp.*; which show IC_{50} = 8.2 μM and 5.2 μM against *P. falciparum* W2 chloroquine-resistant strain, respectively, and mild cytotoxicity to mammalian Vero cells (IC_{50} = 86 and 56 μM, respectively), Figure 1.[9] Gademann and co-workers have isolated aerucyclamides A (**3**), B (**4**), C (**5**) and D (**6**) from the cyanobacterium *Microcystis aeruginosa* PCC 7806.[10] Aerucyclamide B (**4**) and its predicted metabolite (**7**),[11] show promising antiplasmodial activity towards *P. falciparum* K1 chloroquine-resistant strain with IC_{50} of 0.7 μM and 0.18 μM, respectively).[10, 12] Aerucyclamide C (**5**) is the most active of the aerucyclamides against *T. b. rhodesiense* (IC_{50} = 9.2 μM). In addition, aerucyclamides display a large selectivity for the parasites over a cell line of L6 rat myoblast and the analog (**7**) shows 555-fold selectivity for *P. falciparum* than against the murine macrophages.[10, 12]

Interestingly, several non azolic- cyclopeptides displayed antiparasitic activity. Mahafacyclin B (**8**) and chevalierin A (**9**),

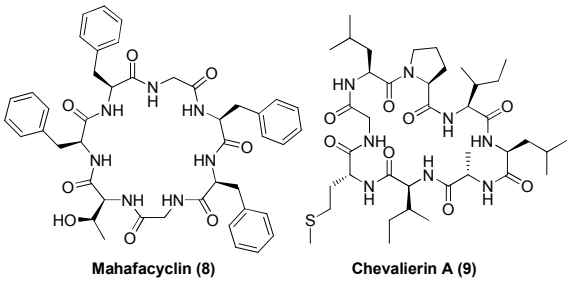


Figure 2. Non-azolic cyclopeptides with antimalarial activity

Figure 2, cyclic peptides isolated from the latex of *Jatropha* species, showed IC_{50}= 2.2 and 8.9 μM, respectively, against *P. falciparum*.[13] Cyclopeptides possess a number of desirable properties that make them promising candidates for the discovery of novel drug molecules. In general, they present structural features to favor bioactive conformations, selectivity to the receptors and metabolic stability. In addition, the absence of C- and N-terminal ionizable groups and the internal hydrogen bonded conformations allow them to cross membranes.[14]

As part of a search for compounds with antimalarial or anti-trypanosomal activity, recently we reported the syntheses of aerucyclamide B,[15] and azolic analogs.[12,16] Based on the relevant biological activities showed by aerucyclamides,[10] their oxa(thia)zole analogs,[12, 16] and several natural cyclopeptides,[13] we emabarked on the synthesis of new cyclohexapeptides analogs. These compounds, containing cysteine or threonine/serine amino acids, which are biomimetic precursors of thiazoline/thiazole or oxazoline/oxazole, respectively,[17] were prepared and evaluated to study the influence of the azole rings and of the open precursors on biological activity. The thiol or hydroxyl groups of Cys or Thr/Ser, respectively, of these macrocycles remained protected in order to maintain the number of ionizable and polar groups present in the azolic analogs and to avoid undesired reactions.

In this work, seven novel macrocycles were obtained from hexapeptides prepared by solid phase peptide synthesis (SPPS) methodology. These compounds and seven other macrocycles previously obtained by us, the predicted metabolite of *M. aeruginosa* (**7**),[12] the precursor of aerucyclamide B (**10**),[15] the fluorous derivative (**11**),[15] synthetic aerucyclamide B,[15] and the aerucyclamides analogs (**12-14**),[16] Figure 3, were evaluated against *Plasmodium falciparum* K1, *Trypanosoma brucei brucei* and murine macrophages (cell line J774).

Results and discussion

For the preparation of the macrocycles **15-21** (Figure 4), we followed the general procedure showed in Scheme 1. SPPS was used to accelerate access to the desired linear hexapeptide precursors. The linear sequence was build-up on a 2-chlorotrityl chloride-resin (CTC-resin) which allowed the cleavage of the peptide under very mildly acidic conditions in the presence of other acid-labile protecting groups and minimizes the formation of diketopiperazines due to the large volume of the chlorotrityl

Medicinal Chemistry Communications Accepted Manuscript

This journal is © The Royal Society of Chemistry 2014

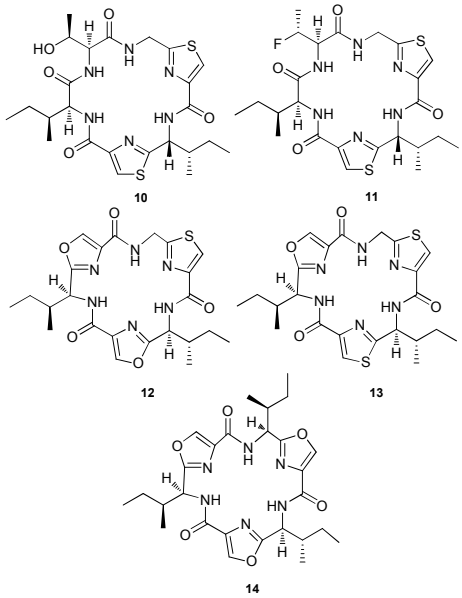


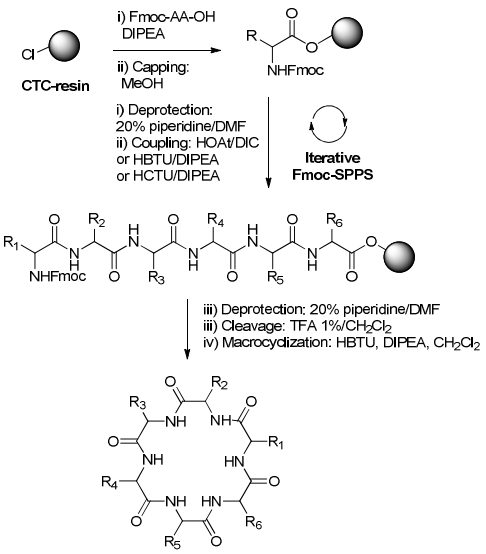
Figure 3. Azole cyclohexapeptides previously synthesized

groups.[18]

The first coupling between the resin and the Fmoc-AA-OH was performed with an excess of *N,N′*-diisopropylethylamine (DIPEA) and the unreacted reactive sites were capped with MeOH.[19]

The hexapeptide precursors of compounds **15** and **16** were obtained through activation with *N,N′*-diisopropyl carbodiimide (DIC) and 1-hydroxy-7-azabenzotriazole (HOAt) in dimethylformamide (DMF). In the case of the hexapeptide precursors of compounds **17-19** the growing chain was enabled using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluoro- phosphate *N*-oxide (HBTU) and DIPEA in DMF. The amide bond formation between the peptide sequence and Fmoc-L-Cys(Trt)-OH was carried on with *N*-[(1*H*-6-chloro-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluoro- phosphate *N*-oxide (HCTU) and DIPEA since HCTU is more reactive and effective than HBTU.[20] Iterative Fmoc-strategy SPPS was followed in order to reach the target sequence in each hexapeptide. After the final Fmoc-deprotection with 20% piperidine in DMF, the desired hexapeptide was cleaved from the resin with 1% TFA in CH_2Cl_2. Under these conditions, the *tert*-butyl and trityl groups remained intact.[21]

For the synthesis of the hexapeptide precursors of compounds **20** and **21**, Fmoc-protected thiazole (Fmoc-Thz-OH) **22** was obtained from Boc-Gly-OH using Hantzsch's methodology[22] and then the N^{α}-Boc group was removed and replaced by N^{α}-Fmoc (Scheme 2).[23] The attachment of Fmoc-Thz-OH to the CTC-resin was conducted in CH_2Cl_2 using DIPEA to furnish resin bound. The Fmoc-Thz was incorporated directly into the Fmoc-strategy SPPS and the desired sequence was subsequently assembled as previously described.



Scheme 1. General procedure of SPPS Fmoc-strategy and macrocyclization

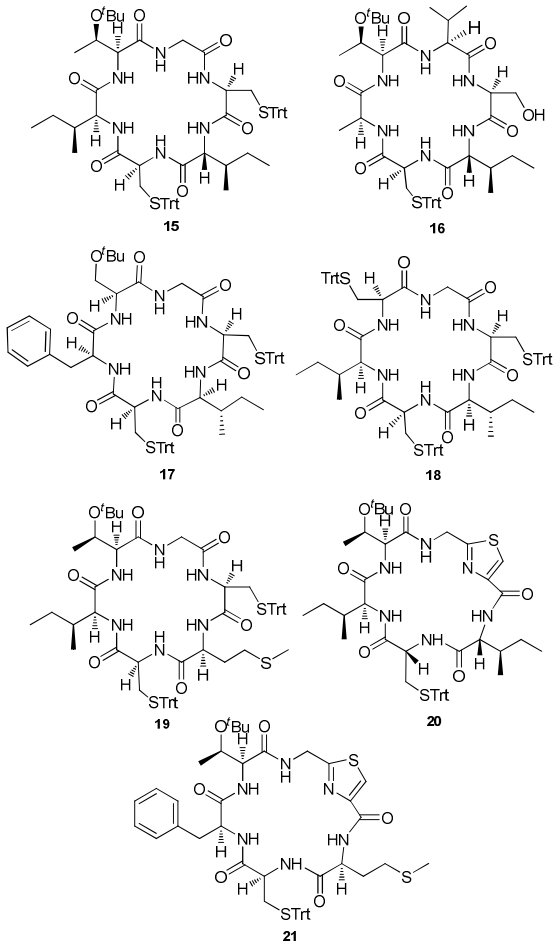
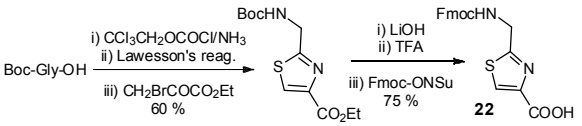


Figure 4. Cyclohexapeptides synthesized in this work

Medicinal Chemistry Communications Accepted Manuscript



Scheme 2. Synthesis of Fmoc-protected thiazole **22**.

Once the hexapeptides were obtained in suitable purity, macrocyclization was performed in high dilution conditions (1-5 mM) by activation with HBTU affording the desired compounds in rather good yields (40-83%).

Despite the fact that hexapeptides precursors of **17**, **18** and **19** are composed by all L-amino acids, relative good yields in the macrolactam formation were obtained (Table 1).[24] In these cases the selection of the cyclization point could facilitate the reaction since in the open intermediates the amino group is derived from Gly and consequently gave the synthetic advantages of non-epimerization and non-steric hindrance.[25] Product **20** was obtained in very good yield (83%) since the presence of the turn-inducing thiazole constraint favors the reaction.[26] Compound **21** was obtained from a hexapeptide precursor with a Met at the NH-termini point in moderate yield (40%).

Table 1. Synthesis and yield (Y %) of the macrocyclic analogs

Entry	Hexapeptide precursor	Compound	Y (%)
1	H_2N-Gly-L-Cys(Trt)-D-Ile-L-Cys(Trt)-L-Ile-L-Thr(^{t}Bu)-OH	**15**	59
2	H_2N-D-Ile-L-Cys(Trt)-L-Ala-L-Thr(^{t}Bu)- L-Val-L-Ser-OH	**16**	55
3	H_2N-L-Cys(Trt)-L-Ile-L-Cys(Trt)-L-Phe-L-Ser(^{t}Bu)-Gly-OH	**17**	48
4	H_2N-L-Cys(Trt)-L-Ile-L-Cys(Trt)-L-Ile-L-Cys(Trt)-Gly-OH	**18**	66
5	H_2N-L-Cys(Trt)-L-Met-L-Cys(Trt)-L-Ile-L-Thr(^{t}Bu)-Gly-OH	**19**	54
6	H_2N-D-Ile-D-Cys(Trt)-L-Ile-L-Thr(^{t}Bu)- Gly-Thz-OH	**20**	83
7	H_2N-L-Met-L-Cys(Trt)-L-Phe-L-Thr(^{t}Bu)-Gly-Thz-OH	**21**	40

The novel synthesized macrocycles **15-21** and compounds **7, 10-14** were evaluated *in vitro* as antimalarial and antitrypanosomal agents. The antimalarial activities were determined against the chloroquine-resistant K1 strain of *P. falciparum* by using the [^{3}H]-hypoxanthine incorporation method reported by Desjardins *et al*.[27] The anti-trypanosomal activities were evaluated against the infective form of *T. b. brucei*, the etiologic agent of Nagana disease in cattle, which is a suitable laboratory model of the subspecies *rhodesiense* that cause HAT. Selectivity of compounds was evaluated against murine macrophages (cell line J774) and calculated for each parasite. The results are summarized in Table 2.

The most active compounds against the resistant strain *P. falciparum* K1, showing EC_{50} lower than 0.2 μM, are macrocycles **7, 10, 17** and **20**. In addition, they were not toxic to murine macrophages showing selectivity indexes higher than 500, (entries 1, 2, 9 and 12, Table 2). Compounds **7**, **10**, **17**, **20** and **21** are more potent against *P. falciparum* K1 than the most active of the aerucyclamides (**4**, EC_{50} = 0.7 μM),[10] but they are less potent that the antimalarials artemisinin and artesunate, EC_{50} =0.02 and 0.003 μM, respectively.

Comparing the EC_{50} of the compounds containing three heterocycles, first it is worthy to note that removal of the methyl group of the oxazole in **7** impacts negatively in the biological activity by decreasing 3-folds the potency towards *P. falciparum* and increasing at least 2-folds the cytotoxicity against macrophages of compound **13** (compare entries 1 and 5). In addition, the substitution of one of the thiazole rings of compound **13** by its isostere oxazole (compound **12**) has a negative effect on the antiplasmodium activity, decreasing it in almost six times. In contrast, the activity is enhanced five times if the Gly-Thiazole moiety of **12** is changed by an L-Ile-Oxazole (compound **14**, see entries 4 and 6).

By comparison of the EC_{50} for compound **7** and **10** (entries 1 and 2), it could be concluded that the presence of the 5-methyloxazole ring and L-Ile instead of L-allo-Thr and D-allo-Ile, respectively, have the same effect in the antiplasmodium activity. In contrast, synthetic aerucyclamide B containing an oxazoline, is ten times less active than the β-hydroxyamide **10** (entries 2 and 14). These results suggest that more flexible cyclohexapeptides containing at least two azoles, such as **10**, could display good antimalarial properties as well as the tris-azoles ones. Notably, the substitution of the alcohol group presents in **10** by fluorine (compound **11**) decreased by almost 140 times the parasiticidal activity (entries 2 and 3).

One of the most active compounds evaluated in this work, cyclopeptide **20** differing from **15** in the presence of Gly-Thz residue instead of its precursor Gly-L-Cys(Trt) and in the configuration of one Cys(Trt) residue, is ten times more active than **15** against *P. falciparum* K1 (entries 7 and 12, Table 2). It is worthy to note that only one of the non azolic cyclopeptides (**17**) showed EC_{50} lower than 0.2 μM as the most active macrocycles containing heterocycles (**7**, **10** and **20**). Notably, the substitution of the residue L-Phe-L-Ser(^{t}Bu) in **17** by L-Ile-L-Cys(Trt) present in **18** decreased the antiplasmodium activity six times (entries 9 and 10). Similarly, compound **19** differing from **15** in the presence of L-Met instead of D-Ile is at least two times more active than compound **15**.

The most potent compounds against *T. b. brucei* ($EC_{50} \leq 3$ μM), are macrocycles **15, 19**, **20** and **21** (Table 2). They are less active than methylene blue (EC_{50} against *T.b. brucei*= 0.4 μM) but more potent and selective than nifurtimox (EC_{50}= 13 μM, SI= 13). Compounds **15** and **19** are the most active (EC_{50} 1.06 and 2.1 μM, respectively) and selective (SI 82 and 95, respectively) derivatives against trypanosoma. The higher EC_{50} values against *T. b. brucei* were obtained for the macrocycles containing two or three azole heterocycles (**7**, **10**, **11**, **12, 13** and **14**). A remarkable effect is observed by substitution of L-Thr(^{t}Bu) and D-Ile by L-Cys(Trt) and L-Ile respectively (see

Medicinal Chemistry Communications Accepted Manuscript

This journal is © The Royal Society of Chemistry 2014

MedChemComm RSCPublishing

CONSICE ARTICLE

Table 2. Biological activities of macrocycles. Values represent the mean ± SD (n=2).

		EC_{50} (μM)				
Entry	Compound	*P. falciparum K1*(μM)[a]	Infective *T. b. brucei*[b]	Murine Macrophage[c]	Selectivity Index 1[d]	Selectivity Index 2[e]
1	**7**	0.18 ± 0.02[12]	>25	>100	> 556	>4
2	**10**	0.18 ± 0.01	14.5 ± 1.2	>100	> 588	>7
3	**11**	24.8*	>25	ND	ND	ND
4	**12**	3.7 ± 0.1	>25[16]	ND	ND	ND
5	**13**	0.6 ± 0.2	11.4 ± 0.2[16]	48.8 ± 0.1	79	4
6	**14**	0.80 ± 0.02	>25[16]	>100	>125	>4
7	**15**	1.97 ± 0.08	1.06 ± 0.01	87.4±3.8	44	82
8	**16**	1.00 ± 0.02	23.8 ± 1.5	>100	>100	>4
9	**17**	0.19 ± 0.02	7.3 ± 0.2	202**	1063	28
10	**18**	1.2 ± 0.3	>25	>200	>172	>4
11	**19**	0.8 ± 0.2	2.1 ± 0.1	199***	258	95
12	**20**	0.19 ± 0.03	3.0 ± 0.1	>100	>526	>33
13	**21**	0.41 ± 0.02	2.8 ± 0.2	>100	>244	>36
14	**Synthetic Aerucyclamide B**	1.84± 0.3	ND	ND	ND	ND

[a] Control: chloroquine: EC_{50} = 0.47 μM, Artemisinin: EC_{50} = 0.02 μM, Artesunate: EC_{50} = 0.003 μM.[b] Control: methylene blue: EC_{50} = 400 nM[28]; Nifurtimox: EC_{50}= 13 μM.[c] Nifurtimox: EC_{50} = 170 μM,[d] EC_{50} macrophage / EC_{50} plasmodium. [e] EC_{50} macrophage / EC_{50} trypanosomes, SI Nifurtimox= 13. ND: not determined. * Estimated value. **Estimated value from 49 ± 4 % of grow inhibition at 200 μM. ***Estimated value from 52 ± 4 % of grow inhibition at 200 μM.

entries 7 and 10); compound **18** resulted in more than 25 times less active than **15**. Interestingly, all cyclohexapeptides containing two or three azoles, with the exception of compound **11**, displayed a marked species- specificity towards killing *P. falciparum* (average EC_{50} of 1.09 μM for compounds **7**, **10**, **12**, **13** and **14**) rather than *T. b. brucei* (average EC_{50} >20 μM). Importantly, cyclohexapeptides with only one thiazole (compounds **20** and **21**) exhibited satisfactory mutual anti-parasitic activities and selectivity indexes.

Conclusions

In summary, seven novel macrocycles were obtained in very good yield combining solid phase and solution phase procedures. Several of the compounds evaluated in this work, show increased antimalarial and anti-trypanosomal activities compared to their natural products analogs, although they are less active than artemisinin and artesunate.

With respect to the evaluation against *P. falciparum* K1, we can conclude that: a) a thiazole instead of L-Cys in the macrocycle increases the potency; b) replacement of 5-methyloxazole by its corresponding β-hydroxyamide does not affect bioactivity; c) the more flexible non-azolic macrocyles (compounds **15**, **16**, **18** and **19**) are less potent than the azolic ones; d) the chemical nature of the aminoacidic residues seems to be critical for the activity of non azolic-cyclohexapeptides; L-Met and the dipeptide L-Phe-L-Ser(^{t}Bu) increase the potency of this compound class.

The evaluation of the anti-trypanosomal activity revealed that the presence of two or three heterocycles in the macrocycles impairs the biological activity. The most active compound is a non azolic- cyclohexapeptide (**15**) showing 82-fold selectivity for the parasite than against murine macrophages. Cyclohexapeptides containing only one thiazole (**20** and **21**) showed satisfactory cytotoxic profiles against both protozoan parasites. Compounds with this property are highly required for multi-disease intervention in multi-endemic areas as it is the case of Malaria and HAT in Africa.

Considering that these cyclohexapeptides are a new class of compounds that have demonstrated anti-plasmodial and/or anti-trypanosomal activities, the data reported here provide a valuable basis for further optimization.

Medicinal Chemistry Communications Accepted Manuscript

Experimental section

Chemistry

SPPS general procedure: The synthesis was done in a plastic syringe equipped with teflon filters attached to a vacuum manifold to enable rapid removal of reagents and solvents. The 2-chlorotritylchloride resin (loading =1.0 mmol/g; 100 mg) was washed with CH_2Cl_2 (5 × 30 sec), DMF (5 × 30 sec) and then with CH_2Cl_2 (5 × 30 sec). A solution of Fmoc-AA-OH (1 mmol/g of resin) in minimal CH_2Cl_2 and DIPEA (7 eq.) was gently shaken for 1 h, then an extra 3 eq. of DIPEA was added and shaking was continued for 5 min. MeOH (0.08 mL/ g of resin) was then added in order to cap unreacted functional groups on the resin; the mixture was then shaken for 20-30 min. The resin was filtered, and washed with CH_2Cl_2 and DMF (5 × 30 sec. each). The N-terminus was deprotected using 20% piperidine in DMF (2 × 5 min and 1 x 10 min). The resin was then washed with DMF, CH_2Cl_2 and DMF (5 × 30 sec. each). Fmoc-AA-OH (3 eq.) was pre-activated by vigorous shaking for 4 min in the presence of DIC (3 eq.) and HOAt (3 eq.) in minimal DMF, and then poured onto the resin and the resulting mixture was gently shaken for 1-2 h. After the coupling was completed, the resin was washed with CH_2Cl_2 and DMF (5 × 30 sec. each). Deprotection and coupling cycles were repeated with the appropriate amino acids to provide the desired compound.

The peptide was cleaved from the resin by treatment with 1% TFA in CH_2Cl_2 for 2-3 minutes at room temperature followed by filtration and collection of the filtrate in water. The treatment was repeated 4-5 times. Solvents were removed in vacuo and the crude peptide was washed twice with CH_2Cl_2, cooled with N_2 liquid and lyophilized to render the desired hexapeptide.

General procedure for macrocyclization: Macrocyclization reactions were performed in diluted conditions (1-5 mM) using HBTU (2 eq.), DIPEA (3 eq.), 4-DMAP (catalytic) in dried CH_2Cl_2 at room temperature during 3-5 days. Then solvent was evaporated *in vacuo*. The crude was redissolved in EtOAc, washed with HCl 5%, saturated aqueous $NaHCO_3$, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude was purified by flash chromatography to obtain the macrocycle.

Cyclo-[Gly-L-Cys(Trt)-D-Ile-L-Cys(Trt)-L-Ile-L-Thr(^{t}Bu)] ***(15)***

The trifluoroacetate salt of H_2N-Gly-L-Cys(Trt)-D-Ile-L-Cys(Trt)-L-Ile-L-Thr(^{t}Bu)-OH was obtained following the general SPPS procedure. Lyophilization yielded 1.25 g (98% yield) of the hexapeptide as a white solid. The purity (89%) was determined by HPLC (linear gradient: 70 to 100% acetonitrile (0.036% TFA) in H_2O (0.045% TFA) over 8 min; flow rate = 1.0 mL/min; t_R = 5.08 min). mp: 159-160 ºC. α_D = - 8.25 (c 0.667, MeOH:CH_2Cl_2, (1:1)). HRMS calculated for $C_{66}H_{81}N_6O_8S_2$ ($[M+H]^+$) 1149.5557 observed 1149.55652

Macrocyclization reaction was performed following the general procedure (dilution 5 mM, 3 days), starting from the trifluoroacetate salt of the amino acid H_2N-Gly-L-Cys(Trt)-D-Ile-L-Cys(Trt)-L-Ile-L-Thr(^{t}Bu)-OH (100 mg, 0.079 mmol). Further purification by flash chromatography, the desired macrocycle was obtained (53 mg, 0.046 mmol) in 59% yield.

White solid, mp: decompose above 210 ºC, Rf = 0.3 (AcOEt:EP, 3:2), α_D = + 10,7 (c 0.9, CH_2Cl_2), ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.76 (d, J = 6.7 Hz, 3H), 0.88 - 0.96 (m, 9H), 1.07 (d, J = 6.4 Hz, 3H), 1.11 - 1.15 (m, 1H), 1.21 (s, 9H), 1.23 - 1.26 (m, 1H), 1.45 - 1.55 (m, 2H), 1.62 - 1.67 (m, 1H), 2.19 - 2.27 (m, 1H), 2.37 (dd, J_1 = 12.6 Hz, J_2 = 4.4 Hz, 1H), 2.40 (dd, J_1 = 13.1 Hz, J_2 = 5.4 Hz, 1H), 3.07 (dd, J_1 = 12.5 Hz, J_2 = 5.8 Hz, 1H), 3.12 (dd, J_1 = 13.3 Hz, J_2 = 9.7 Hz, 1H), 3.34 (dd, J_1 = 17.0 Hz, J_2 = 2.6 Hz, 1H), 3.49 (t, J = 7.5 Hz, 1H), 3.67 (ddd, J_1 = 3.5 Hz, J_2 = 5.8 Hz, J_3 = 9.1 Hz, 1H), 4.05 - 4.17 (m, 4H), 4.61 (dd, J_1 = 17.6 Hz, J_2 = 9.9 Hz, 1H), 5.83 (d, J = 6.3 Hz, 1H), 6.25 (dd, J_1 = 2.7 Hz, J_2= 9.6 Hz, 1H), 6.62 (d, J = 6.3 Hz, 1H), 7.09 (d, J = 9.1 Hz, 1H), 7.16 (d, J = 2.01 Hz, 1H), 7.18 - 7.34 (m, 19H), 7.39 - 7.44 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 10.3, 10.7, 15.5, 16.0, 18.4, 24.7, 25.5, 28.3 (3C), 28.9, 31.2, 32.4, 35.4, 42.5, 51.7, 53.2, 56.8, 60.3, 61.9, 65.5, 66.9, 67.2, 74.9, 126.8 (3C), 127.1 (3C), 128.0 (6C), 128.2 (6C), 129.4 (6C), 129.6 (6C), 144.2 (2C), 144.6 (2C), 146.9 (2C), 169.1, 169.9, 172.1, 172.8, 173.3, 173.4. IR ν(cm^{-1}) 1381, 1543, 1640, 2854, 2927, 2966, 3059, 3286, 3402. HRMS calculated for $C_{66}H_{78}N_6NaO_7S_2$ ([M+Na]+) 1153.5271 observed 1153.5226.

Fmoc-Gly-Thz-COOH ***(22)****:*

Compound NH-Boc-Gly-Thz-COOH (7.09 mmol, 1.83 g) (obtained as we reported previously)[13,16] was treated with TFA/CH_2Cl_2 (1:1, v/v, 52 mL) for 1 h and then concentrated under reduced pressure. The trifluoroacetate salt was dissolved in dioxane-H_2O (3:2, 130 mL) and then $NaHCO_3$ (1.2 eq.) was added followed by Fmoc-OSu (2.0 eq.) in dioxane (40 mL) and stirred for 16 h. The dioxane was removed under reduced pressure, 10% aqueous $KHSO_4$ (10% aq.) was added until pH 3 and then the mixture was extracted in $CHCl_3$:i-PrOH (3:1). Organic layer was washed with brine and dried with $MgSO_4$. The solvent was removed under reduced pressure, affording the title compound as a white solid (1.99 g, 74%).

^{1}H NMR (400 MHz, DMSO) δ ppm 4.23 (t, J = 6.73 Hz, 1H), 4.37 (d, J = 6.79 Hz, 2H), 4.45 (d, J = 5.94 Hz, 2H), 7.31 (t, J = 7.36 Hz, 2H), 7.40 (t, J = 7.39 Hz, 2H), 7.68 (d, J = 7.42 Hz, 2H), 7.88 (d, J = 7.49 Hz, 2H), 8.34-8.24 (m, 2H).

Cyclo-[Gly-Thz-L-D-Ile-D-Cys-L-Ile-L-Thr(^{t}Bu)] ***(20)****:*

The trifluoroacetate salt of H_2N-D-Ile-D-Cys-L-Ile-L-Thr(^{t}Bu)-Gly-Thz-OH was obtained following the general SPPS procedure. Lyophilization yielded 470 mg (95% yield) of the hexapeptide as a white solid. The purity (100 %) was determined by HPLC (linear gradient: 50 to 100% acetonitrile (0.036% TFA) in H_2O (0.045% TFA) over 8 min; flow rate = 1.0 mL/min; t_R = 3.69 min). mp 189-191 ºC. α_D = + 9.29 (c 0.53, DMSO). HRMS calculated for $C_{47}H_{63}N_6O_7S_2$ ($[M+H]^+$) 887.41956 observed 887.41942. Macrocyclization reaction was performed following the general procedure (dilution 1mM, 5

Medicinal Chemistry Communications Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2014

days), starting from the trifluoroacetate salt of the amino acid H_2N-D-Ile-D-Cys-L-Ile-L-Thr(^{t}Bu)-Gly-Thz-OH (50 mg, 0.05 mmol). Further purification by flash chromatography, the desired macrocycle was obtained (36 mg, 0.041 mmol) in 83% yield.

White solid, mp: 136-137 ºC, Rf = 0.5 (AcOEt:EP, 4:1). α_D = + 34.8 (c 1.27, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84 (s, 9H), 0.85 - 0.93 (m, 12H), 0.99 (d, J = 6.7 Hz, 3H), 1.05 (d, J = 6.2 Hz, 3H), 1.22 - 1.29 (m, 2H), 1.39 - 1.45 (m, 2H), 1.86 – 1.94 (m, 1H), 1.95 – 2.01 (m, 1H), 2.65 (dd, J_1 = 12.5 Hz, J_2 = 7.3 Hz, 1H), 2.81 (dd, J_1 = 12.6 Hz, J_2 = 6.3 Hz, $1H_{17}$), 4.08 - 4.15 (m, 2H), 4.25 (dd, J_1 = 4.5 Hz, J_2 = 5.5 Hz, $1H_2$), 4.29 (dd, J_1 = 2.1 Hz, J_2 = 6.3 Hz, 1H), 4.37 (dd, J_1 = 4.5 Hz, J_2 = 15.4 Hz, 1H), 4.48 (dd, J_1 = 7.6 Hz, J_2 = 9.6 Hz, 1H), 4.97 (dd, J_1 = 7.8 Hz, J_2 = 15.4 Hz, 1H), 6.34 (d, J = 5.4 Hz, 1H), 6.79 (d, J = 6.9 Hz, 1H), 6.87 (d, J = 6.3 Hz, 1H), 7.21 – 7.33 (m, 9H), 7.39 – 7.42 (m, 6H), 7.63 (dd, J_1 = 4.7 Hz, J_2 = 7.7 Hz, 1H), 7.78 (d, J = 9.8 Hz, 1H), 7.90 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) 11.3, 11.7, 15.5, 15.9, 21.1, 24.6, 24.8, 27.9 (3C), 33.5, 36.4, 37.7, 39.5, 52.8, 59.1, 59.5, 65.6, 67.4, 74.6, 79.2, 123.5, 127.0 (3C), 128.2 (6C), 129.4 (6C), 144.1 (3C), 149.4, 161.2, 165.8, 170.4, 170.6, 170.7, 171.2. IR film ν(cm^{-1}) 1080, 1188, 1267, 1356, 1449, 1491, 1499, 1640, 2876, 2930, 3057, 3309, 3325, 3337. HRMS calculated for $C_{47}H_{60}N_6NaO_6S_2$ ([M+Na]+) 891.3913 observed 891.3925.

Biology

In vitro antimalarial activity

Parasite cultures. The K1 strain of *P. falciparum* originating from Thailand that is resistant to chloroquine and pyrimethamine, but sensitive to mefloquine was used. The cultures are naturally asynchronous (65-75% ring stage) and are maintained in continuous log phase growth in RPMI1640 medium supplemented with 5% washed human A+ erythrocytes, 25 mM HEPES, 32 nM $NaHCO_3$, and AlbuMAXII (lipid-rich bovine serum albumin) (GIBCO, Grand Island, NY) (CM). All cultures and assays are conducted at 37°C under an atmosphere of 5% CO_2 and 5% O_2, with a balance of N_2.

Drug sensitivity assays. Stock drug solutions are prepared in 100% DMSO (dimethylsulfoxide) at 20 mM. The compound is further diluted to the appropriate concentration using complete medium RPMI1640 supplemented with 15 nM cold hypoxanthine and AlbuMAXII. Assays are performed in sterile 96-well microtitre plates, each plate contains 100 µl of parasite culture (0.5% parasitemia, 2.5% hematocrit). Each drug is tested in triplicate and parasite growth compared to control and blank (uninfected erythtocytes) wells. After 24 h of incubation at 37°C, 3.7 Bq of [^{3}H]hypoxanthine is added to each well. Cultures are incubated for a further 24 h before they are harvested onto glass-fiber filter mats. The radioactivity is counted using a Wallac Microbeta 1450 scintillation counter. The results are recorded as counts per minute (CPM) per well at each drug concentration, control and blank wells. Percentage inhibition is calculated from comparison to blank and control wells, and EC_{50} values calculated using Prism™.

Screen. The K1 line is used. The compound is diluted three-fold over at 12 different concentrations with an appropriate starting concentration. The IC_{50} is determined by a sigmoidal dose response analysis using Prism™. For each assay, the IC_{50} value for the parasite line is determined against the known anti-malarials chloroquine and artesunate, plus other standard compounds appropriate for the assay.

In vitro anti-trypanosomal activity.

Materials. Chemical reagents were purchased from Sigma or ROCHE. Medium and consumables for cell culture were purchased from Invitrogen and Greiner, respectively.

Parasites. The infective form of *T. brucei brucei* strain 427, cell line 449 (encoding one copy of the tet-represor protein: Pleo^R) and cell line Grx1-roGFP$_2$ (encoding a redox sensitive biosensor; Sardi and Comini, unpublished) were aerobically cultivated in a humidified incubator at 37ºC with 5% CO_2 in HMI-9 medium supplemented with 10% (v/v) fetal calf serum (FCS), 10 U/ml penicillin, 10 µg/ml streptomycin. Cell line 449 was also supplemented with 0.2 µg/ml phleomycin and Grx1-roGFP$_2$ with 0.2 µg/ml phleomycin, 5 µg/mL hygromycin and induced with 1 µg/ml tetracycline.

Murine macrophages. The *J*774 mouse macrophage cell line was cultivated in a humidified 5% CO_2/95 % air atmosphere at 37ºC in DMEM medium supplemented with 10% (v/v) FCS, 10 U/ml penicillin and 10 µg/ml streptomycin.

Cytotoxicity assays. Stock solutions (10 mM) of the test compounds were prepared using dimethylsulfoxide (DMSO) as solvent and then diluted in culture medium to obtain the specific experimental concentrations (5 and 25 µM for screening and between 0.025 to 25 µM and 0.1 to 100 µM for the determination of EC_{50} against parasites and macrophages, respectively). Controls included compound vehicle (DMSO) at final concentrations up to 0.25 % (v/v) and culture medium (growth control). Each condition was tested in duplicate or triplicates. The cytotoxic effect of the compounds towards trypanosomes and macrophages was evaluated by cell counting with a Neubauer chamber or flow cytometry and by colorimetric assay of cell viability with a tetrazolium salt (WST-1 reagent), respectively. If required, the drug concentration range was adjusted to allow a more accurate determination of the EC_{50} value. The methodological procedure is essentially as described previously.[16]

Data Evaluation. Living parasites were counted in both hemi-chambers with a light microscope. The cell density in the case of flow cytometry was calculated using the following formula: cell/mL= A/B X C/D, where A= number of cell events, B= number of bead events, C= assigned bead count of the lot (beads/5 µL) and D= volume of sample (250 µL).

For each compound concentration, cytotoxicity was calculated according to the following equation: Cytotoxicity (%) = (experimental value - DMSO control)/(growth control - DMSO control) × 100. For assays involving trypanosomes and murine macrophages, the input value is the mean (n=2) of the cell

Medicinal Chemistry Communications Accepted Manuscript

densities and corrected absorbance at 450 nm (A_i^{c450}), respectively. The data were plotted as percentage cytotoxicity versus drug concentration. EC_{50} values were obtained from dose response curves fitted to a sigmoidal equation (Boltzmann model) or extrapolated from non-linear fitting plots.

ACKNOWLEDGMENT

This work was supported by Grants from ANII (FCE2720, FCE 6919), CSIC Grupos (Universidad de la República) and PEDECIBA (Uruguay) and CICYT (CTQ2012-30930), the Generalitat de Catalunya (2009SGR 1024) (Spain).
The authors acknowledge a PhD fellowship from ANII (Agencia Nacional de Investigación e Innovación) (Stella Peña) and an internship supporting by ANII and PEDECIBA. MAC acknowledges the support of ANII (grant Innova Uruguay, agreement DCI-ALA/2007/19.040 between Uruguay and the European Commission and grant FMV 2617).

Notes and references

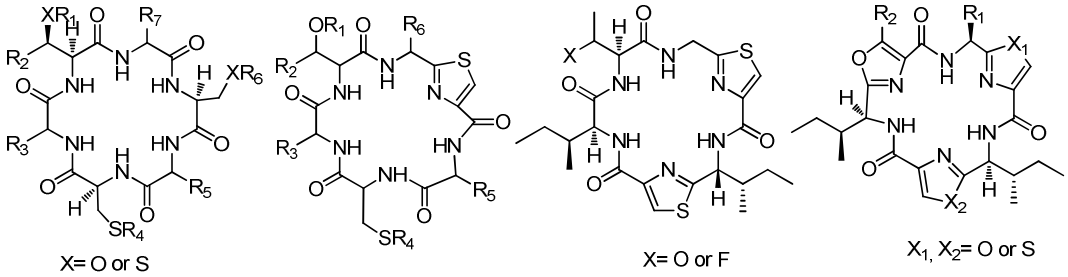
1 World Malaria Report. Geneva, World Health Organization, 2012. http://www.who.int/malaria/publications/world_malaria_report_2012/en/index.html
2 World Health Organization. Research Priorities for Chagas Disease, Human African Trypanosomiasis and Leishmaniasis. Technical Report of the TDR Disease Reference Group on Chagas Disease, Human African Trypanosomiasis and Leishmaniasis. Technical Report Series no. 975 (2012). ISBN 978 92 4 120975 5. http://www.who.int/tdr/publications/research_priorities/en/index.html
3 A. Cavalli and M. L. Bolognesi, *J. Med. Chem*., 2009, **52**, 7339–7359.
4 C. J. Bacchi, *Interdiscip. Perspect. Infect. Dis*., 2009, ID 195040. A.H. Fairlamb, *Trends Parasitol*., 2003, **19**, 488–494.
5 T. N. C. Wells, P. L. Alonso, and W. E. Gutteridge, *Nat. Rev. Drug Discov*., 2009, **8**, 879–891; C. Amaratunga, S. Sreng, S. Suon, E. S. Phelps, K. Stepniewska, P. Lim, C. Zhou, S. Mao, J. M. Anderson, N. Lindegardh, H. Jiang, J. Song, X. Su, N. J. White, A. M. Dondorp, T. J. C. Anderson, M. P. Fay, J. Mu, S. Duong, and R. M. Fairhurst, *Lancet Infect. Dis*., 2012, **12**, 851–858.
6 K. Kaur, M. Jain, T. Kaur, and R. Jain, *Bioorg. Med. Chem.,* 2009, **17**, 3229–3256; E. L. Flannery, A. K. Chatterjee, and E. a Winzeler, *Nat. Rev. Microbiol.*, 2013, **11**, 849–62; S. Dandapani, E. Comer, J. R. Duvall, and B. Munoz, *Future Med. Chem.*, 2012, **4**, 2279–2294; D. Steverding and K. M. Tyler, *Expert Opin. Investig. Drugs*, 2005, **14**, 939–955.
7 D. J. Newman and G. M. Cragg, *J. Nat. Prod*., 2012, **75**, 311–335.
8 K. Gademann and J. Kobylinska, *Chem. Rec.*, 2009, **9**, 187–198.
9 R. G. Linington, J. Gonzalez, L.-D. Ureña, L. I. Romero, E. Ortega-Barría, and W. H. Gerwick, *J. Nat. Prod.*, 2007, **70**, 397–401.
10 C. Portmann, J. F. Blom, K. Gademann, and F. Jüttner, *J. Nat. Prod.*, 2008, **71**, 1193–1196; C. Portmann, J. F. Blom, M. Kaiser, R. Brun, F. Jüttner, and K. Gademann, *J. Nat. Prod.*, 2008, **71**, 1891–1896.
11 N. Ziemert, K. Ishida, P. Quillardet, C. Bouchier, C. Hertweck, N. T. de Marsac, and E. Dittmann, *Appl. Environ. Microbiol.*, 2008, **74**, 1791–1797.
12 S. Peña, L. Scarone, E. Manta, L. Stewart, V. Yardley, S. Croft, and G. Serra, *Bioorg. Med. Chem. Lett.*, 2012, **22**, 4994–4997.
13 C. Baraguey, A. Blond, F. Cavelier, J.-L. Pousset, B. Bodo, and C. Auvin-Guette, *J. Chem. Soc. Perkin Trans. 1*, 2001, 2098–2103; C. Baraguey, C. Auvin-guette, A. Blond, F. Cavelier, F. Lezenven, J. Pousset, and B. Bodo, *J. Chem. Soc., Perkin Trans. 1*, 1998, 3033–3040.
14 E. M. Driggers, S. P. Hale, J. Lee, and N. K. Terrett, *Nat. Rev. Drug Discov*., 2008, **7**, 608–624; J. S. Davies, *J. Pept. Sci.*, 2003, **9**, 471–501.
15 S. Peña, L. Scarone, E. Manta, and G. Serra, *Tetrahedron Lett.*, 2013, **54**, 2806–2808.
16 S. Peña, L. Scarone, A. Medeiros, E. Manta, M. Comini, and G. Serra, *Med. Chem. Comm*, 2012, **3**, 1443-1448.
17 R. S. Roy, a M. Gehring, J. C. Milne, P. J. Belshaw, and C. T. Walsh, *Nat. Prod. Rep.*, 1999, **16**, 249–263.
18 K. Barlos, D. Gatos, and W. Schafer, *Angew. Chem. Int. Ed. Engl*. 1991, **30**, 590–593.
19 López-Macià, J. C. Jiménez, M. Royo, E. Giralt, and F. Albericio, *J. Am. Chem. Soc.*, 2001, **123**, 11398–11401.
20 C. A. Hood, G. Fuentes, H. Patel, K. Page, M. Menakuru, and J. A. E. H. Park, *J. Pept. Sci.*, 2008, **14**, 97–101; O. Marder and F. Albericio, *Chim. Oggi*, 2004, **35**, 6–11.
21 A. Isidro-Llobet, M. Alvarez, and F. Albericio, *Chem. Rev.*, 2009, **109**, 2455–2504.
22 A. Hantzsch. *Ann. Chem.,* 1888, **249**, 54-65.
23 R. E. Thompson, K. Jolliffe, and R. J. Payne, *Org. Lett.*, 2011, **13**, 680–683.
24 The presence of D-amino acids in the sequence uses to favour the cyclization reaction.
25 C. J. White and A. K. Yudin, *Nat. Chem.*, 2011, **3**, 509–524.
26 N. Sayyadi, D. Skropeta, and K. A. Jolliffe, *Org. Lett.*, 2005, **7**, 5497–5499; R. Black, V. Dungan, R. Li, P. Young, and K. Jolliffe, *Synlett*, 2010, **4**, 551–554; R. E. Thompson, K. A. Jolliffe, and R. J. Payne, *Org. Lett.*, 2011, **13**, 680–683; D. Skropeta, K. A. Jolliffe, and P. Turner, *J. Org. Chem.*, 2004, **69**, 8804–8809.
27 R. E. Desjardins, C. J. Canfield, D. E Haynes and J. D. Chulay, *Antimicrob. Agents Chemother*. 1979, **16**, 710-718.
28 K. Buchholz, M.A Comini, D. Wissenbach, R.H Schirmer, R. L. Krauth-Siegel and S. Gromer, *Mol Biochem. Parasitol*. 2008, **160**, 65-69.

.

Medicinal Chemistry Communications Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2014

Azolic and non -azolic ciclohexapeptides were obtained and/or evaluated as promising antimalarials and/or anti-trypanosomals agents.



Medicinal Chemistry Communications Accepted Manuscript