Development of a New Antileishmanial Aziridine-2,3-Dicarboxylate-Based Inhibitor with High Selectivity for Parasite Cysteine Proteases

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ABSTRACT

Leishmaniasis is one of the major neglected tropical diseases of the world. Druggable targets are the parasites' cysteine proteases (CPs) of clan CA, family C1 (CAC1). In previous studies we identified two peptidomimetic compounds, the aziridine-2,3-dicarboxylates **13b** and **13e**, out of a series of inhibitors of the cathepsin L (CL) subfamily of the papain clan CAC1. Both displayed antileishmanial activity *in vitro*, while not showing cytotoxicity against host cells. In further investigations the mode of action was characterized in *Leishmania major* (*L. major*). It was demonstrated that aziridines **13b** and **13e** mainly inhibited the parasitic cathepsin B (CB)-like CP C (CPC) and additionally mammalian CL. Although they induced cell death in *Leishmania* promastigotes and amastigotes *in vitro*, the induction of a pro-leishmanial T helper type 2 (Th2) response caused by host CL inhibition was observed *in vivo*.

Therefore, we describe in the present study the synthesis of a new library of more selective peptidomimetic aziridine-2,3-dicarboxylates discriminating between host and parasite CPs. They are based on **13b** and **13e** as lead structures. One of the most promising compounds of this series is **s9**, showing selective inhibition of the parasite CPs *Lma*CatB of *L. major* (a CB-like enzyme, also named: *L. major* CPC) and *Lm*CPB2.8 of *Leishmania mexicana* (*L. mexicana*) (a CL-like enzyme), while not affecting mammalian CL and CB. It displayed excellent leishmanicidal activity against *L. major* promastigotes (IC_{50} = 37.4 μM) and amastigotes (IC_{50} = 2.3 μM).

In summary, we demonstrated with **s9** a new selective aziridine-2,3-dicarboxylate which might be a good candidate for future *in vivo* studies.

INTRODUCTION

Leishmaniasis is one of the 17 neglected tropical diseases (NTDs) assigned by the World Health Organization (WHO). NTDs affect one billion people worldwide (1). The primary occurrence is in low-income countries in sub-Saharan Africa, Asia, and Latin America, but the Mediterranean countries of Europe are also concerned (2). Among the NTDs, there is the group of „most neglected diseases“, affecting the poorest, mainly rural, areas, including leishmaniases, sleeping sickness (African trypanosomiasis), and Chagas' disease (3). These three NTDs have the highest rates of death. However, the NTD drug discovery pipeline is almost empty, thus leading to a lack of efficient and safe drugs (2, 4). Because of the climate warming and tourism, incidence of leishmaniasis is also reported in states around the Mediterranean Sea (1).

Leishmaniasis is caused by more than 20 species of protozoan parasites belonging to the genus *Leishmania*. The parasites' life cycle is characterized by two morphological stages, the extracellular flagellated promastigotes occurring in the insect vector and the intracellular aflagellated amastigotes in the mammalian host. The promastigotes are transmitted by an insect bite into the skin of the host, where they are internalized by macrophages, dendritic cells, neutrophils and fibroblasts, and differentiate into amastigotes residing and replicating in parasitophorous vacuoles of these phagocytes. The parasites disseminate through the lymphatic and vascular systems. During the blood meal of an (uninfected) sandfly, amastigotes are transmitted back from the infected mammalian host to the insect vector and differentiate again to promastigotes (5, 6).

The clinical outcome of leishmaniasis depends on both the complex interactions between the virulence characteristics of the infecting species and the type of immune response of the host. There are three clinical forms: the cutaneous, the mucocutaneous, and the visceral leishmaniasis (6).

Concerning the treatment of leishmaniasis, it is obvious that new drugs must circumvent the limitations of currently established chemotherapies; i.e. toxicity, long courses

of treatment, frequently parenteral administration, high costs in endemic countries, and the emergence of resistance. Therefore, it is not only important to test and apply combinations of existing drugs for avoiding resistance, but also to develop new potential leishmanicidal compounds with alternative mechanisms as well as vaccination strategies (7, 8).

Cysteine proteases of parasites like *Plasmodia*, *Trypanosoma*, and worms are druggable targets for developing a new promising strategy for chemotherapy based on protease inhibition (9-12). Therefore, the identification and synthesis of highly selective protease inhibitors might be a promising way for the treatment of such infections in future. In recent years, we have been working on the development of inhibitors of papain-like CPs belonging to the CAC1 family (13-18). These proteases may represent attractive targets because of their key roles in parasite infections (9-12). The *L. major* genome encodes a total of 65 CPs, grouped into four clans (CA, CD, CF, PC(C)) and 13 families. Leishmanial CPs belonging to the CAC1 family are the lysosomal CL-like enzymes CP A (CPA) and CP B (CPB), as well as the CB-like enzyme CPC (19). They share some homology with the related mammalian enzymes; however, substrate specificity is different. While human CB accepts Arg in P2 position (benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarine (Cbz-Arg-Arg-AMC) is a quite good substrate), the leishmanial homolog CPC does not, and prefers Phe in that position, comparable to human and leishmanial CL enzymes (22). In case of *Leishmania* CPs it was shown that cysteine proteases are essential for parasite growth, differentiation, pathogenicity, and virulence (19-21). However, it is yet not fully understood to which extent the additional inhibition of related host cathepsins may have an anti-infective effect or in contrast may even support the infection (25-27). Therefore, it is necessary to develop inhibitors selective for *Leishmania* cysteine proteases.

In previous studies, we identified two peptidomimetic aziridine-2,3-dicarboxylate-based inhibitors - Boc-(*S*)-Leu-(*R*)-Pro-(*S*,*S*)-Azi(OBn)$_2$ (compound **13b**) and Boc-(*R*)-Leu-(*S*)-Pro-(*S*,*S*)-Azi(OBn)$_2$ (compound **13e**) - out of a series of inhibitors of CL and CL-like CPs

104 (15, 16, 23, 24), which exert excellent antileishmanial activity. Both aziridines targeted the
105 leishmanial CB-like enzyme *Lma*CatB (*L. major* CPC), as documented with a biotin-tagged
106 derivative of **13b** (24). The inhibitor **13b** induced an accumulation of undigested debris in
107 autophagy-related lysosome-like vacuoles in *L. major* followed by parasite cell death (24). An
108 *in vivo* experiment was carried out, using the BALB/c mouse model of *L. major* infection.
109 After application of **13b**, a weak exacerbation of the infection was observed; this was
110 characterized by a significantly increased secretion of the Th2 cell cytokine interleukin 4 by
111 murine splenic cells. This effect was observed probably caused by inhibition of murine CL
112 (data not shown). This is in accordance with studies of the Katanuma group, indicating that
113 inhibition of human CL results in the potentiation of Th2-type immune responses and, thus,
114 leads to an exacerbation of the inflammation (25-27). The studies also showed that CB-
115 specific inhibitors can switch T-cell development from Th2- to Th1-type immune responses in
116 mice resulting in an amelioration of the infection. In summary, there is an urgent need for
117 inhibitors which selectively inhibit the CL-like parasite CPs and do not affect the mammalian
118 equivalents.

119 There is no X-ray structure available of leishmanial papain-like CPs, making the
120 development of selective inhibitors a matter of "trial and error" by synthesis and testing of a
121 broad variety of related inhibitors. Therefore, we extended our study by synthesizing a series
122 of aziridine-2,3-dicarboxylates based on **13b** and **13e** as lead structures. This series comprises
123 structural isomers (**s11-s14**), derivatives with ethyl ester moieties (**s1-s8**), a derivative with an
124 extended peptide chain (**s15**), and derivatives with non-proteinogenic amino acids within the
125 peptide sequence in order to improve hydrolytic stability (β-Ala in **s21**, α-aminoisobutyric
126 acid (Aib) in **s22**, norvaline (Nva), norleucine (Nle), cyclohexylglycine (Chg),
127 cyclohexylalanine (Cha), phenylglycine (Phg) in **s26-s30** and **s32**). The influence of the
128 configuration of the three membered aziridine ring (*R,R* or *S,S*) on affinity and selectivity was
129 investigated for most of the structural isomers (**s16-s19),** and for the lead compounds **13b** and

13e (**s9**, **s10**). Additionally, the Leu residue in **13b** was replaced by other neutral amino acids (Gly in **s20**, Ala in **s23**, Val in **s24**, Ile in **s25**, Phe in **s31**, Trp in **s33**). On the other hand, the Pro residue in **13b** was replaced by the amino acids Orn in **s34**, (NO_2)Arg in **s35** and nipecotic acid (Nip) in **s38**, the latter containing brominated benzyl esters at the aziridine ring in order to improve the properties for X-ray diffraction studies of enzyme inhibitor complexes.

The compounds were tested against mammalian CL and CB, the recombinantly expressed CB-like protease *Lma*CatB (*L. major* CPC), and the recombinantly expressed CL-like protease *Lm*CPB2.8 from *L. mexicana*.

Furthermore, selected compounds were tested for their ability to inhibit proteolytic acticity in *L. major* promastigote lysates. This was done with the compounds alone, and in combination with the standard cysteine protease inhibitors E64 and CA074, in order to evaluate to which extent the proteolytic activity is further decreased by addition of the aziridine-based cysteine protease inhibitors.

The most promising compounds were analyzed for their ability to inhibit the growth and viability of *L. major* promastigotes and amastigotes *in vitro*, and for cytotoxicity against the macrophage cell line J774.1.

MATERIALS AND METHODS

Syntheses. The synthesis of the potential inhibitors was performed as depicted in Fig. 1. The preparation was carried out through fragment coupling of the Boc-protected dipeptides or amino acid to the *trans*-configured aziridine-2,3-dicarboxylates.

Diethyl and dibenzyl aziridine-2,3-dicarboxylates either in (*S*,*S*)-, or in (*R*,*R*)-configuration were prepared stereoselectively as described before (28). In the same manner, (2*S*,3*S*)-bis(4-bromobenzyl) aziridine-2,3-dicarboxylat was synthesized as building block for

s38 from (4-bromophenyl)methanol and L-(+)-tartaric acid as starting material. Dipeptides for fragment coupling were synthesized by using standard peptide coupling procedures (29). Racemic Nip as building block for compounds **s36**, **s37**, **s38** was synthesized by microwave-assisted hydrogenation of nicotinic acid (30). *N*-acylation of the aziridines with Boc-protected fragments or dipeptides was accomplished via propylphosphonic anhydride (PPA) as coupling reagent (31). General structures of compounds **s1**-**s37** and the structure of compound **s38** are presented in Fig 2.

Table 1 summarizes all synthesized *N*-acylated aziridine-2,3-dicarboxylates. Detailed analytical data can be found in the supplementary material.

Cloning and site-directed mutagenesis of *Lma*CatB (*L. major* CPC). The coding sequence of *Lma*CatB was cloned from extracted genomic DNA of *L. major* (clinical isolate MHOM/IL/81/FE/BNI) into the vector pGAPZαA (Invitrogen, Darmstadt, Germany) for expression in the yeast *Pichia pastoris* (*P. pastoris*). *Lma*CatB was amplified by PCR as pro-form according to Chan *et al.* (32) with the sense primer 5'-AGAGAGGCTGAAGCTAAGCCGAGTGACTTTCCGCTTC-3' and the antisense primer 5'-ATGATGGTCGACGGCCTCCTGCGCGGGTATGCCAG-3' for expression with a hexahistidine tag and 5'-ATGATGGTCGACGGCCTACTCCTGCGCGGGTATGCCAG-3' with a stop codon for expression without the tag. The purified PCR product was cloned into pGAPZαA using sequence and ligation-independent cloning (33) and the resulting construct was used for transformation of *Escherichia coli* XL1-blue cells. Transformants were selected on LB agar plates containing 25 µg/ml Zeocin™ (InvivoGen, Toulouse, France) and verified using colony PCR. Plasmids isolated from individual clones were sequenced in both directions (Seqlab, Goettingen, Germany) with pGAP forward and 3'AOX1 primers. A sufficient amount of plasmid was linearized for transformation of *P. pastoris* by digestion for 2 h at 37 °C with the restriction enzyme AvrII. *P. pastoris* X-33 cells were transformed by

electroporation (Gene Pulser MXcell, Bio-Rad, Munich, Germany) with the linearized plasmids at 1,500 V, 400 Ω and 25 µF. *P. pastoris* colonies were selected on YPD agar plates containing 100-200 µg/ml Zeocin™. Colony PCR did not produce reliable results with *P. pastoris* cells; therefore, genomic DNA was extracted for verification of proper integration of the construct into the *P. pastoris* genome.

Expression, purification, and activation of *Lma*CatB (*L. major* CPC). Recombinant *P. pastoris* clones were screened for expression in small scale cultures (5 ml YPD) after 24 h, 48 h, and 72 h at 30 °C. Genes under the GAP promoter of pGAPZαA are transcribed constitutively and the expressed proteins are secreted into the medium. The expressed protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot using murine anti-His antibodies. After 72 h expression, the supernatant from the cultures was harvested by centrifugation at 5,000 g for 15 min, followed by vacuum filtration through a glass microfiber filter (Whatman; grade GF/A, commercially available from Sigma-Aldrich) to remove residual *P. pastoris* cells. The pH was adjusted to 8.0 by addition of Tris/HCl to a final concentration of 10 mM. Subsequently, the supernatant was loaded on an XK16 column packed with Q Sepharose Fast Flow resin (GE Healthcare, Freiburg, Germany). Bound protein was eluted in a concentration gradient between buffer A (10 mM Tris/HCl, pH 8.0) and buffer B (10 mM Tris/HCl (pH 8.0),1 M NaCl). Fractions containing the recombinant protein were determined by SDS-PAGE, pooled, and concentrated by ultrafiltration in a 10 kDa cut-off concentrator (Vivaspin 20, Sartorius AG, Goettingen, Germany). The two major bands on the gel at 35 and 43 kDa were confirmed as *Lma*CatB by ESI-LC/MS mass spectrometry (LTQ Orbitrap; Thermo Scientific, Darmstadt, Germany) from their peptides after digestion with trypsin. As the final purification step, the protein was loaded onto a size exclusion chromatography column (Superdex XK26/60; GE Healthcare) equilibrated with 20 mM sodium citrate (pH 5.0), and 250 mM NaCl. The protein-containing

fractions were concentrated and the buffer exchanged into activation buffer (100 mM sodium citrate (pH 5.0), 100 mM NaCl, 10 mM dithiothreitol (DTT), 1 mM EDTA). The protein was then incubated for 24 h at 4 °C to convert any remaining pro-form enzyme into the mature form by releasing its *N*-terminal pro-peptide. Finally, the buffer was exchanged into storage buffer (10 mM sodium citrate (pH 5.0), 1 mM DTT and 1 mM EDTA) and aliquots of *Lma*CatB were flash frozen in liquid nitrogen and stored at -80 °C.

Parasites. The virulent *L. major* isolate (strain: MHOM/IL/81/FE/BNI) was maintained by continuous passages in female BALB/c mice (Government of Lower Franconia (Germany), permission number: 55.2-2531.01-26/12). Promastigotes were isolated from BALB/c mice lesions and finally grown in blood agar cultures at 27 °C, 5% CO_2, and 95% humidity.

Enzyme assays with recombinantly expressed *Leishmania* proteases and mammalian proteases. Activity assays were carried out as described previously (24, 34, 35). *Lm*CPB2.8 was recombinantly expressed as described previously (36). CL and CB were purchased (Calbiochem, Schwalbach, Germany). The fluorimetric substrate Cbz-Phe-Arg-AMC was purchased from Bachem (Bubendorf, Switzerland). The assay buffer for CL and CB was 50 mM Tris (pH 6.5), 5 mM EDTA, 200 mM NaCl, 0.005% polyoxyethyleneglycol dodecyl ether (Brij 35). The enzyme buffer for CL and CB was 50 mM Tris (pH 6.5), 5 mM EDTA, 200 mM NaCl, 2 mM DTT. For *Lm*CPB2.8 and *Lma*CatB the assay buffer consisted of 50 mM phosphate buffer (pH 6.5), 5 mM EDTA. The enzyme buffer for *Lm*CPB2.8 and *Lma*CatB consisted of 50 mM phosphate buffer (pH 6.5), 5 mM EDTA, 5 mM DTT.Substrates and inhibitor stock solutions were prepared in dimethyl sulfoxide (DMSO) and diluted with assay buffer (final DMSO concentration of 7.5%). A Varian Cary Eclipse

fluorescence spectrophotometer (Varian, Darmstadt, Germany) with 96-well plates was used: λ_{ex} = 380 nm and λ_{em} = 460 nm.

Fluorometric assays for inhibition of proteolytic activity of promastigote lysates. For preparation of promastigote lysates, stationary-phase *L. major* promastigotes were harvested from blood-agar plates and washed twice with phosphate-buffered saline (PBS) and pelleted by centrifugation at 3,000 × g for 10 min. Afterwards, the pelleted cells were resuspended in acidic sodium acetate buffer (pH 5.5). Finally, the promastigotes were disrupted by freezing in liquid nitrogen and thawing at 37 °C for three times, followed by centrifugation at 700 × g for 15 min at 4 °C. Supernatant was aliquoted in fresh tubes and stored at -20 °C until use. Final protein concentrations of these lysates were determined with a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Pittsburgh, PA). The assay buffer for *L. major* promastigote lysates was 200 mM sodium acetate, 1 mM EDTA, 0.05% Brij 35, 0.5 mM DTT. As fluorimetric substrate also Cbz-Phe-Arg-AMC (see above) was used.

Determination of K_i values. The hydrolysis of the substrate was monitored over 10 min in the presence of inhibitor. The K_i^{app} values were calculated using the following equation: $y = v_0/1+([I]/K_i^{app})^s$ (2-parameter logistics), with y as enzyme activity (dF/min increase of fluorescence over time as a result of substrate hydrolysis), v_0 as enzyme activity in absence of inhibitor, [I] as inhibitor concentration, and s as the Hill coefficient. Correction to zero substrate concentration necessary for competitive inhibitors was done by considering substrate concentrations and affinity of the substrate to the target enzyme (K_m values) using the equation: $K_i = K_i^{app}/(1+[S]/K_m)$ (37) with [S] = 6.25 µM and K_m = 6.5 µM for CL, [S] = 100 µM and K_m = 150 µM for CB, [S] = 10.0 µM and K_m = 5.0 µM for *Lm*CPB2.8 and

[S] = 25.0 µM and K_m = 7.0 µM for *Lma*CatB. The GraFit software (38) was used to calculate the K_i^{app} values.

Half maximal inhibitory concentration (IC_{50}) value determination for *L. major* promastigotes and amastigotes. The IC_{50} values of compounds against *L. major* promastigotes were determined by the Alamar Blue assay, as described previously (23, 39). Stationary-phase promastigotes were seeded into 96-well plates at a density of 1×10^7 ml^{-1} in RPMI medium without phenol red with 10% fetal calf serum (FCS), in the absence or presence of increasing concentrations of compounds. Parasites were then incubated for 24 h at 27 °C, 5% CO_2, and 95% humidity. Following the addition of 20 µl of ready to use Alamar Blue solution (Trinova Biochem, Gießen, Germany) per well, the plates were incubated again and the optical densities measured after 48 h.

The amastigote drug screening assay against intracellular amastigotes recently described (39) was applied to determine the IC_{50} values of compounds against *L. major* amastigotes. Bone marrow-derived macrophages (BMDM) were generated and infected with luciferase-transgenic *L. major* promastigotes at a ratio of 1:15 as recently described (39). Compounds were added to BMDM 24 h after infection when the differentiation of promastigotes into amastigotes was completed. Control BMDM were incubated for the same amount of time in phenol red-free RPMI medium with 10% FCS and 1% DMSO. Then, BMDM were incubated at 37 °C, 5% CO_2, and 95% humidity for further 24 h. After cell lysis with a luciferin-containing buffer, the IC_{50} values of the compounds used against *L. major* amastigotes were determined by the resulting luminescence.

Promastigote staining after treatment with inhibitor s9. Promastigotes with a cell density of $10^8 \times ml^{-1}$ were treated with 100 µM **s9** for 180 min at 27 °C. Control cultures were incubated in 0.5% DMSO-containing RPMI medium. Cells were harvested and transferred to

microscopic slides by centrifugation for 5 min and 1,500 rpm using the cytospin® 3 Shadon (Thermo Electron Corporation, Waltham, MA, USA). Parasites were fixed and stained using the Diff-Quik kit (Medion Dignostics, Duedingen, Switzerland) according to the manual.

TEM of s9-treated *L. major* amastigotes. BMDM were generated and infected with promastigotes at a ratio of 1:15 as recently described (39). After 24 h of coculture the complete differentiation from the extracellular promastigotic stage to the intracellular amastigotic stage was observed (39). Finally, amastigote-infected macrophage cultures were incubated in RPMI medium containing 0.5% DMSO in control cultures or in RPMI medium containing **s9** at a concentration of 10 μM. After incubation for 30 min or 60 min, **s9**-treated amastigote-infected and control macrophages were subjected to TEM as recently described (24).

RESULTS

Aziridine-2,3-dicarboxylate-based inhibitors selectively inhibited parasite CPs. In fluorescence enzyme assays, the inhibitory effects of the potential inhibitors were evaluated against human CL and CB, the CB-like protease *Lma*CatB from *L. major* (*L. major* CPC), and the CL-like enzyme *Lm*CPB2.8 from *L. mexicana* (Table 1).

In contrast to the lead compounds **13b** and **13e,** which were active against human CL in the single digit micromolar range, most of the new compounds showed no or only weak inhibition of CL and CB (i.e., K_i > 10 μM). Exception was **s35** being active against CB (K_i = 5.4 μM). In agreement with earlier studies on CPs (15), most of the compounds containing ethyl ester moieties, namely compounds **s1**-**s8**, did not or only weakly inhibit the enzymes. Only compounds **s5** and **s8** showed weak inhibition of *Lma*CatB (*L. major* CPC). The structural isomers and the stereoisomers of **13b** (**s9**-**s14**, **s16**-**19**) inhibited the CL-like enzyme

*Lm*CPB2.8 and most of them also inhibited the CB-like enzyme *Lma*CatB (*L. major* CPC). Interestingly, a better selectivity between mammalian and parasite enzymes was achieved on the one hand with the stereoisomers of **13b** and **13e**, namely **s9** and **s10**, which were *R,R*-configured at the aziridine ring, and with the *S,S*-configured structural isomers **s11**-**s14**, on the other hand. Since **s9** turned out to be the most selective inhibitor concomitantly displaying antileishmanial activity against promastigotes (IC_{50} = 37.4 μM against *L. major*, Table 1), the compound was further modified by exchanging the amino acid (*S*)-Leu yielding the compounds **s15**, **s20**-**s35**. Elongation of the amino acid sequence of **s9** yielded the tripeptide derivative **s15**, which was a quite good inhibitor of the parasites' proteases maintaining the antileishmanial activity against *L. major* promastigotes (IC_{50} = 34.2 μM, Table 1). From these compounds, only those with lipophilic or bulky groups showed considerably improved inhibition (Phe in **s31**: IC_{50} = 1.7 μM, hPhe in **s32**: IC_{50} = 1.5 μM, Table 1). Interestingly, these compounds do not inhibit the cathepsin B-like *L. major* enzyme CPC (*Lma*CatB) but only the cathepsin L-like protease *Lm*CPB2.8. The exchange of the (*R*)-Pro residue in **s9** against (*R*)-Orn(Boc) and (*R*)-Arg(NO_2) (**s34** and **s35**) resulted in two strong inhibitors of the parasites' protease *Lma*CatB (*L. major* CPC) which may be explained by its preference for amino acids with Arg in P1 position. Compounds with a Nip residue, **s36**-**s38**, were quite good inhibitors of *Lm*CPB2.8 with selectivity over *Lma*CatB making the brominated compound a good candidate for co-crystallization with the target enzyme.

Selective inhibitors of the parasite CPs displayed highly significant antileishmanial activity *in vitro*. The anti-parasite activities of seleced inhibitors were evaluated against *L. major* promastigotes (Table 1), and, for the most promising inhibitors also against *L. major* amastigotes (Table 2) (24). Since previous studies showed that diethyl esters were not active in cell assays (15), probably due to poor membrane permeability, only the dibenzyl esters were tested. The cytotoxicity against host cells was determined using the

336 macrophage cell line J774.1 (Table 1). We recently demonstrated (24) that the broad-
337 spectrum inhibitor E-64 (40, 41), the CB-selective inhibitors CA074 (43), and CA074ME
338 (43), and paromomycin have no or only weak effects against promastigotes. The IC_{50} values
339 of **13b** and **13e** against promastigotes were comparable to those of pentamidine and
340 miltefosine. Only amphotericin B was more effective against *L. major* promastigotes (24).
341 Within the series of the new dibenzylesters, the compounds **s9**, **s15**-**s19**, **s23**-**s25**, **s28**, and **s31**
342 showed inhibitory potency against *L. major* promastigotes (Table 1). The IC_{50} values are in
343 the same range like those of **13b**, **13e**, pentamidine, and miltefosine (24) (Table 1).
344 Inhibitor **s25** displayed the best inhibition of growth and viability of *L. major*
345 promastigotes (IC_{50} = 9.8 μM, Table 1). At the concentrations used, none of the tested
346 compounds was cytotoxic against the macrophage cell line J774.1 (Table 1). With compound
347 **s9**, the changes in morphology of the promastigotes were studied. Rounding of *L. major*
348 promastigotes after treatment with **s9** for 180 min was observed before cell death induction
349 (see Fig. 2 SM of Supplementary Material, images 3 - 4).
350 Selected compounds, namely **13b**, **13e**, **s9**, **s17**, **s24**, **s25**, together with the epoxides E-
351 64d (the cell-permeable prodrug form of E64c which is similarly active to E64), CLIK-148
352 (CL-selective inhibitor), and CA074ME were additionally tested for their antileishmanial
353 activity against *L. major* amastigotes (Table 2). All aziridine-based inhibitors displayed high
354 antileishmanial activity with IC_{50} values in the low micromolar range, in contrast to the
355 epoxide-based inhibitors E-64d, CLIK-148, and CA074ME (Table 2). This is in agreement
356 with the previous results with the aziridines which showed better effects on amastigotes than
357 on promastigotes (24). With IC_{50} values > 250 μM for **s17**, **s24**, and **s25** on macrophages, the
358 selectivity indices are excellent ($SI_{\mathbf{s17}}$ = 156, $SI_{\mathbf{s24}}$ = 114, $SI_{\mathbf{s25}}$ = 125), matching the
359 identification criteria for hits of protozoan diseases of the WHO (44, 45).
360

Aziridine-2,3-dicarboxylate-based inhibitor s9 showed similar enzyme inhibition of *L. major* promastigote protein lysates like E-64. For further evaluation, the highly selective compound **s9** (Table 1) was chosen to characterize its potential to inhibit leishmanial CPs in promastigote protein lysates. With this inhibitor, fluorescence proteinase activity assays with protein lysates, obtained from stationary-phase promastigotes, were performed. For comparison, the standard CP inhibitors E-64, CLIK-148, and CA074, as well as the lead aziridine-based inhibitors **13b** and **13e** were included. Proteinase activities were determined by proteolytic cleavage of substrate Cbz-Phe-Arg-AMC. Protein lysates were incubated with either DMSO or with the inhibitors in a first incubation step, and in a second step the incubation with DMSO followed. The residual proteolytic activity after treatment with E-64 was 3.2%, after treatment with the CB-selective inhibitor CA074 20.1%, and after treatment with the CL-selective CLIK-148 8.9% (Fig. 3A). Compounds **13b** and **13e** provoked only moderate inhibition (residual activity after treatment with **13b** = 47.0%, **13e** = 61.6%) (Fig. 3A). For both inhibitors, it was demonstrated previously that they reduced specifically the activity of the CB-like enzyme CPC in protein lysates of *L. major* promastigotes (24). This result was confirmed in the present study with recombinantly expressed *Lm*CPB2.8 (Table 1).

Treatment with **s9** resulted in a residual enzyme activity of 5.6% which was comparable to E-64 (Fig. 3A). The result clearly showed that **s9** caused additional inhibitory effects compared to its isomers **13b** and **13e**. For detailed analyses of the selectivity of the inhibitors, protein lysates were furthermore preincubated in a first incubation step with E-64 (broad spectrum CP inhibitor, inhibition of leishmanial CPA, CPB, CPC) and CA074 (CB-selective CP inhibitor, inhibition of leishmanial CPC) (Fig. 3B). In the second incubation step, protein lysates were incubated with DMSO, **13b**, **13e**, or **s9**. In the case of **13b** and **13e** no further effect on activity after preincubation with E-64 and CA074 was observed (Fig. 3B), which clearly confirmed that only CPC is affected. However, there was a significant decrease

of activity after additional incubation with **s9** for preincubation with CA074. These data suggested that **s9** might inhibit not only the CB-like CPC of *L. major* but also the CL-like CPA and/or CPB of *L. major*.

CP inhibitor s9 induced an accumulation of lysosome-like vacuoles followed by cell death in amastigotes. TEM studies were performed to analyze how cell death was induced in *L. major* amastigotes by **s9** (see Fig. 3 SM of Supplementary Material). We described recently that treatment with the aziridine-based inhibitor **13b** resulted in cell death, characterized by an inhibition of digestion in lysosome-like vacuoles, and hallmarked by an accumulation of debris in these organelles (24). Based on this fact we expected a similar phenotype in **s9**-treated amastigotes. An accumulation of lysosome-like vacuoles in **s9**-treated amastigotes was observed (see Fig. 3 SM of Supplementary Material, images 4 - 6) compared to control macrophages after 30 min (data not shown) and 60 min (see Fig. 3 SM of Supplementary Material, images 1 - 3) of incubation. Such lysosome-like vacuoles have been described to contain CPA, CPB, and CPC. Surprisingly, the phenotype was slightly different from that induced by **13b** (24) in terms of the vacuoles which were more numerous in **s9**-treated compared to **13b**-treated amastigotes. Finally, cell death of amastigotes was observed after 60 min of treatment with **s9** (see Fig. 3 SM of Supplementary Material, images 7 - 9).

DISCUSSION

CPs of parasites are attractive targets for developing new leishmanicidal drugs. *Leishmania* species express the CL-like proteases CPA and CPB, and the CB-like enzyme CPC. We previously identified two aziridine-2,3-dicarboxylate-based inhibitors **13b** and **13e** with antileishmanial activity (23, 24). Since inhibition of host cell CL may lead to compensation of the positive effects caused by inhibition of *Leishmania* cathepsins, the aim of

413 the present study was the development of inhibitors selective for *Leishmania* enzymes. Using
414 **13b** as lead structure, a second series was synthesized and is presented in this study. The
415 series contains structural isomers, stereoisomers, derivatives with ethyl ester moiety, and
416 derivatives with non-proteinogenic amino acids within the peptide sequence. In most cases,
417 the compounds of this second series showed selective inhibition of the parasites' CPs, while
418 the mammalian proteases CL and CB were not affected. Since no X-ray structure of
419 *Leishmania* papain-like CPs has been published so far, docking studies to identify possible
420 binding modes and to explain the selectivity would only be possible on homology models,
421 which is a rather uncertain method. In previous studies, we suggested possible binding modes
422 for CL- and CB-selective aziridine-based inhibitors (15). We also performed docking studies
423 with the related parasite enzyme cruzain from *Trypanosoma cruzi* (unpublished data) which in
424 principle are in agreement with the previous findings. According to these results, aziridines
425 consisting of at least two large, hydrophobic moieties interact with the hydrophobic S_2- and/or
426 S_1' binding pockets of a CL-like enzyme, while the other residues (*N*-terminal protecting
427 group, second benzyl ester) are widely solvent exposed during the binding process and do
428 have defined contacts to amino acids of the protein. Based on these findings, two of the three
429 hydrophobic residues of the aziridine-2,3-dicarboxylate-based inhibitors (two benzyl esters,
430 one hydrophobic amino acid side chain) are involved in binding, whereby the proline residue
431 and the configuration of the aziridine ring determine the relative position of these groups to
432 each other. Hence, different binding modes are theoretically possible depending on the ligand
433 and target enzymes. Interestingly the structural isomers of **13b** and **13e** with (*R,R*)-configured
434 aziridine ring (**s16**-**s19**) are not selective between mammalian and parasitic enzymes while
435 those with the (*S,S*)-configured ring (**s11**-**s14**) are highly potent and selective inhibitors of
436 *Leishmania* enzymes, especially of *Lma*CatB (*L. major* CPC). However, these compounds do
437 not affect the growth of *L. major* promastigotes. In contrast, the less selective inhibitors **s16**-
438 **s19** display anti-leishmanial activity in the same range as **13b**, **13e**, and **s9**. The compounds

active against promastigotes are also active against the amastigotes with even better IC_{50} values. Fortunately, the compounds do not show cytotoxicity against host cells.

To elucidate the antileishmanial activity of **s9** which combines selective inhibition of both leishmanial enzymes with good anti-leishmanial activity against both promastigotes and amastigotes, fluorescence protease activity assays with lysates of *L. major* promastigotes were carried out. Compound **s9** produces a stronger inhibition of the leishmanial enzyme activity than **13b** and **13e** after a first incubation step with the inhibitors and a second incubation step with DMSO. After preincubation of the lysates with the broad spectrum CP inhibitor E-64, or with the CB-selective inhibitor CA074, a significant further reduction of the proteolytic activity was observed after incubation with **s9**, in contrast to incubation with **13b** or **13e**. This clearly demonstrates that **s9** targets additional proteases compared to **13b** or **13e**, and also to E64. This may also explain why the phenotype of amastigotes after treatment with **s9** is slightly different from that observed after treatment of amastigotes with **13b** (24).

To sum up, the present study extended our previous knowledge about aziridine-2,3-dicarboxylate-based inhibitors with leishmanicidal activity as potential targets. We achieved exclusive selectivity in inhibition between the parasites' CPs and the related mammalian proteases. Furthermore, we identified a new lead structure with highly selective inhibitor **s9**.

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FIGURE LEGENDS

FIG 1 General synthesis of *N*-acylated *trans*-aziridine-2,3-dicarboxylates. Reagents and conditions: (*i*) PPA, ethyl acetate or dimethylformamide, 1 h 0 °C, 24 h room temperature **s1**-**s37**); (*ii*) PPA, triethylamine, ethyl acetate, 1 h 0 °C, 4 h 40 °C, 7 d room temperature (**s38**).

FIG 2 Structures of the synthesized *N*-acylated *trans*-aziridine-2,3-dicarboxylates **s1**-**s37** and structure of the dibromo derivative **s38**.

FIG 3 Assay for proteolytic activity of promastigote protein lysates. (A and B) Protein lysates that had been obtained from stationary-phase promastigotes were preincubated in a first incubation step (1st Inc.) with DMSO, 200 µM E-64, 200 µM CA074, 200 µM CLIK-148, 200 µM compound **13b**, 200 µM compound **13e**, or 200 µM compound **s9**. In a second incubation step (2nd Inc.), protein lysates were incubated with either DMSO, 200 µM compound **13b**, 200 µM compound **13e**, and 200 µM compound **s9**. Proteinase activities were determined by proteolytic degradation of the fluoropeptide Cbz-Phe-Arg-AMC.

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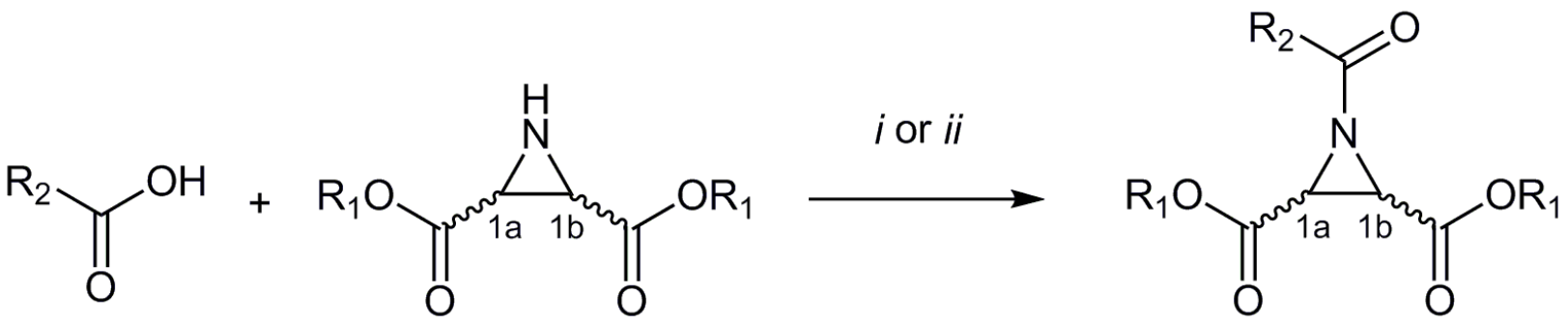
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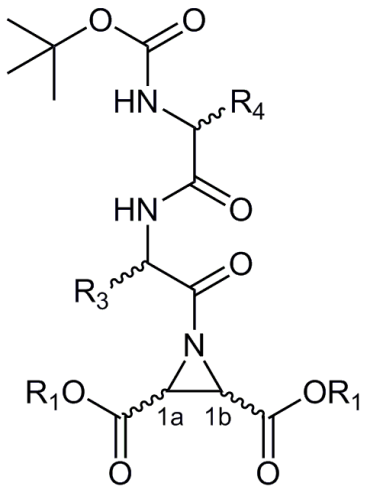

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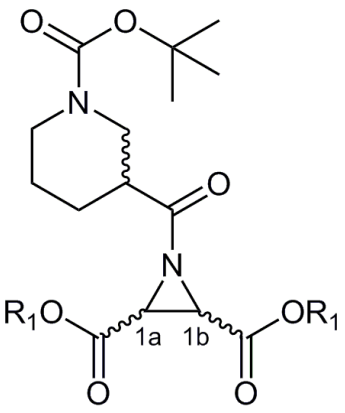

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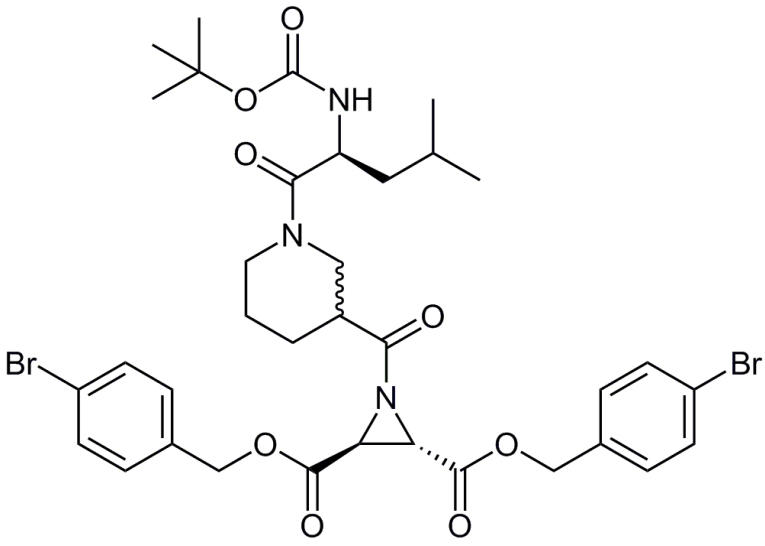

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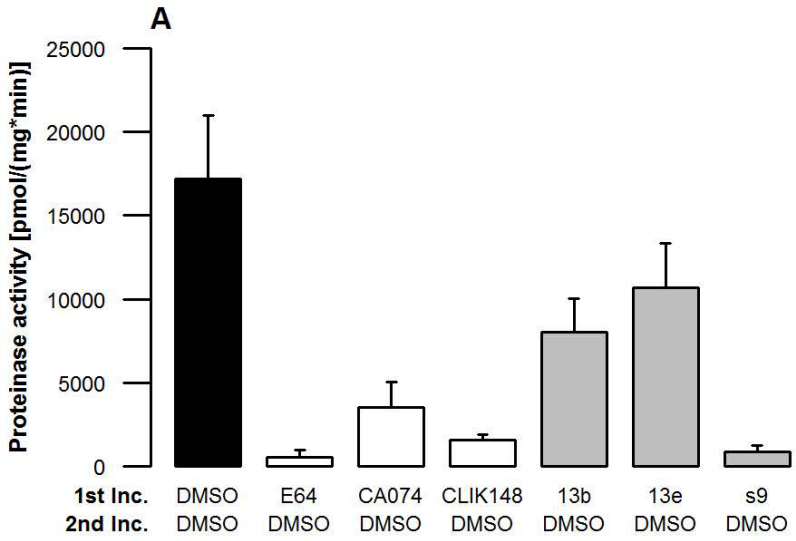
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Proteinase activity [pmol/(mg*min)]
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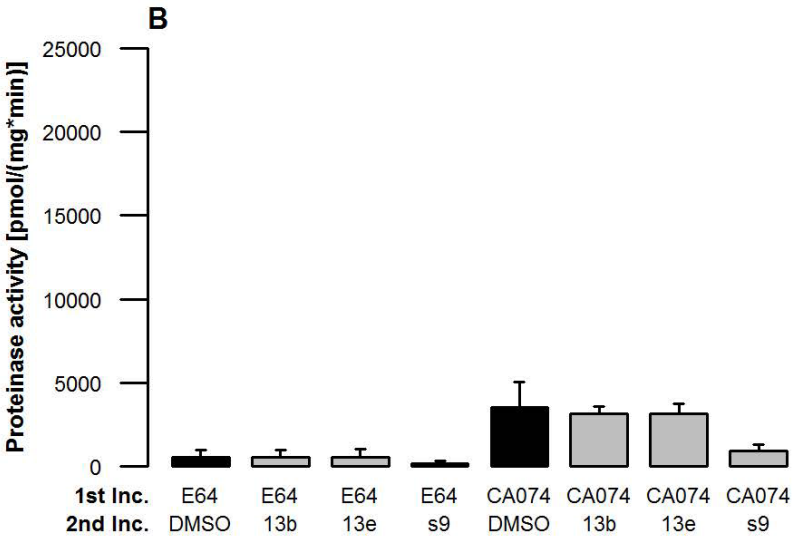
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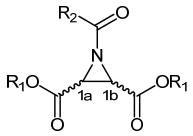
TABLE 1 Inhibition of CL, CB, *Lm*CPB2.8 and *Lma*CatB, and antileishmanial activity against *L. major* promastigotes and cytotoxicity of *trans*-aziridine-2,3-dicarboxylates **13b**, **13e**, **s1**-**s38**.



No.	R_2	R_1	1a, 1b	CL K_i [μM]	CB K_i [μM]	*Lm*CPB2.8 K_i [μM]	*Lma*CatB K_i [μM]	*L. m.* (p) IC_{50} [μM]	Cyt. (J774.1) IC_{50} [μM]
13b	Boc-(*S*)-Leu-(*R*)-Pro	Bn	*S*,*S*	6.0 ± 0.8 (14)	ni (14)	1.7 ± 0.2	nd	33.4 ± 2.5	> 100
13e	Boc-(*R*)-Leu-(*S*)-Pro	Bn	*S*,*S*	4.0 ± 0.2 (14)	ni (14)	2.1±0.4	nd	47.0 ± 11.8	> 100
s1	Boc-(*S*)-Leu-(*R*)-Pro	Et	*S*,*S*	ni	ni	ni	ni	nd	nd
s2	Boc-(*R*)-Leu-(*S*)-Pro	Et	*S*,*S*	ni	ni	ni	ni	nd	nd
s3	Boc-(*S*)-Leu-(*R*)-Pro	Et	*R*,*R*	ni	ni	ni	ni	nd	nd
s4	Boc-(*R*)-Leu-(*S*)-Pro	Et	*R*,*R*	ni	ni	ni	ni	nd	nd
s5	Boc-(*S*)-Pro-(*S*)-Leu	Et	*S*,*S*	ni	ni	ni	15.2 ± 0.1	nd	nd
s6	Boc-(*R*)-Pro-(*S*)-Leu	Et	*S*,*S*	ni	ni	ni	ni	nd	nd
s7	Boc-(*S*)-Pro-(*R*)-Leu	Et	*S*,*S*	ni	ni	ni	ni	nd	nd
s8	Boc-(*R*)-Pro-(*R*)-Leu	Et	*S*,*S*	ni	ni	ni	10.3 ± 0.3	nd	nd
s9	Boc-(*S*)-Leu-(*R*)-Pro	Bn	*R*,*R*	ni	ni	3.8 ± 0.1	18.2 ± 0.3	37.4	68
s10	Boc-(*R*)-Leu-(*S*)-Pro	Bn	*R*,*R*	ni	ni	3.4 ± 0.2	ni	95	> 100
s11	Boc-(*S*)-Pro-(*S*)-Leu	Bn	*S*,*S*	ni	ni	4.3 ± 0.1	0.69 ± 0.02	> 100	87
s12	Boc-(*R*)-Pro-(*S*)-Leu	Bn	*S*,*S*	ni	ni	4.1 ± 0.1	0.82 ± 0.08	> 100	> 100
s13	Boc-(*S*)-Pro-(*R*)-Leu	Bn	*S*,*S*	ni	ni	4.0 ± 0.01	0.72 ± 0.01	> 100	89
s14	Boc-(*R*)-Pro-(*R*)-Leu	Bn	*S*,*S*	ni	ni	4.6 ± 0.1	1.5 ± 0.1	> 100	> 100
s15	Boc-Gly-(*S*)-Leu-(*R*)-Pro	Bn	*R*,*R*	ni	26.4 ± 4.9	8.1 ± 1.5	4.8 ± 0.1	34.2	> 100
s16	Boc-(*S*)-Pro-(*S*)-Leu	Bn	*R*,*R*	14.2 ± 0.1	29.2 ± 1.1	2.6 ± 0.2	17.9 ± 7.7	32.6	> 100
s17	Boc-(*R*)-Pro-(*S*)-Leu	Bn	*R*,*R*	19.7 ± 4.1	44.0 ± 2.1	2.9 ± 0.2	ni	40.7	> 250
s18	Boc-(*S*)-Pro-(*R*)-Leu	Bn	*R*,*R*	ni	24.6 ± 7.9	3.1 ± 0.1	ni	47.2	> 100
s19	Boc-(*R*)-Pro-(*R*)-Leu	Bn	*R*,*R*	70.9 ± 0.3	ni	4.6 ± 2.5	17.3 ± 1.1	41.0	> 100
s20	Boc-Gly-(*R*)-Pro	Bn	*R*,*R*	53.1 ± 8.7	ni	ni	7.0 ± 0.2	> 100	> 100

s21	Boc-β-Ala-(*R*)-Pro	Bn	*R*,*R*	ni	ni	ni	6.2 ± 0.2	> 100	> 100
s22	Boc-Aib-(*R*)-Pro	Bn	*R*,*R*	ni	ni	ni	ni	> 100	> 100
s23	Boc-(*S*)-Ala-(*R*)-Pro	Bn	*R*,*R*	ni	ni	25.4 ± 1.6	ni	46.1	> 100
s24	Boc-(*S*)-Val-(*R*)-Pro	Bn	*R*,*R*	51.5 ± 1.6	54.6 ± 1.5	4.3 ± 0.3	ni	34.8	> 250
s25	Boc-(*S*)-Ile-(*R*)-Pro	Bn	*R*,*R*	ni	ni	ni	8.7 ± 0.2	9.8	> 250
s26	Boc-(*S*)-Nva-(*R*)-Pro	Bn	*R*,*R*	ni	ni	ni	ni	nd[*p]	> 100
s27	Boc-(*S*)-Nle-(*R*)-Pro	Bn	*R*,*R*	ni	ni	2.8 ± 0.04	ni	nd[*p]	> 100
s28	Boc-(*S*)-Chg-(*R*)-Pro	Bn	*R*,*R*	ni	ni	ni	ni	31.3	> 100
s29	Boc-(*S*)-Cha-(*R*)-Pro	Bn	*R*,*R*	ni	ni	ni	ni	nd[*p]	> 100
s30	Boc-(*S*)-Phg-(*R*)-Pro[a]	Bn	*R*,*R*	ni	ni	10.3 ± 0.6	20.6 ± 8.9	> 100	> 100
s31	Boc-(*S*)-Phe-(*R*)-Pro	Bn	*R*,*R*	ni	ni	1.7 ± 0.1	ni	48.0	> 100
s32	Boc-(*S*)-hPhe-(*R*)-Pro	Bn	*R*,*R*	ni	ni	1.5 ± 0.5	ni	> 100	> 100
s33	Boc-(*S*)-Trp-(*R*)-Pro	Bn	*R*,*R*	ni	ni	2.7 ± 0.4	ni	> 100	> 100
s34	Boc-(*S*)-Leu-(*R*)-Orn(Boc)	Bn	*R*,*R*	ni	ni	nd	1.5 ± 0.2	> 100	42.5
s35	Boc-(*S*)-Leu-(*R*)-Arg(NO_2)	Bn	*R*,*R*	ni	5.4 ± 0.1	nd	1.1 ± 0.1	> 100	> 100
s36	Boc-(*S*/*R*)-Nip[b]	Bn	*S*,*S*	ni	83.9 ± 28.2	1.5 ± 0.1	ni	> 100	> 100
s37	Boc-(*S*/*R*)-Nip[c]	Bn	*R*,*R*	75.9 ± 8.7	80.3 ± 24.0	1.8 ± 0.4	ni	> 100	> 100
s38	Boc-(*S*)-Leu-(*S*/*R*)-Nip	*p*Br-Bn	*S*,*S*	ni	21.2 ± 1.6	0.6 ± 0.02	67.0 ± 6.5	> 100	> 100

[a] diastereomeric ratio *dr* = 1:0.59, [b] diastereomeric ratio *dr* = 1:0.59, [c] diastereomeric ratio *dr* = 1:0.55; ni = no inhibition, nd = not deterrmined, nd[*p] = not determined because of precipitation, *L. m.* (p) = *Leishmania major* promastigotes, Cyt. = Cytotoxicity, J774.1 = macrophage cell line. AMCA, 2-(7-amino-4-methyl-2-oxo-2H-chromen-3-yl)acetic acid. Hxa, amino hexanoic acid. Nip, nipecotic acid. CB, cathepsin B. CL, cathepsin L.

TABLE 2 Antileishmanial activity against *L. major* amastigotes of *trans*-aziridine-2,3-dicarboxylates **13b**, **13e**, **s9**, **s17**, **s24**, **s25** and of standard inhibitors

No.	*L. major* IC_{50} [μM]
13b	2.2 ± 1.5
13e	2.7 ± 0.7
s9	2.3 ± 0.6
s17	1.6 ± 0.3
s24	2.2 ± 0.6
s25	2.0 ± 0.6
E-64d	39.8 ± 11.3
CLIK-148	> 100
CA074ME	> 100