Full Paper

Synthesis and Antiprotozoal Evaluation of New N^4-(Benzyl)spermidyl-linked bis(1,3,5-thiadiazinane-2-thiones)

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The synthesis and *in-vitro* antiprotozoal evaluation of novel N^4-(benzyl)spermidyl-linked bis(1,3,5-thiadiazinane-2-thione) (bis-THTT) derivatives from N^4-(benzyl)spermidine is disclosed. Several of the new bis-THTT have *in-vitro* activities against *L. donovani* and *T. cruzi* that are comparable or superior to those of currently employed protozoocidal agents.

Keywords: Protozoocidal activity / Spermidine / 1,3,5-Thiadiazinane-2-thione

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Introduction

Trypanosomatids are parasitic protozoa within the order Kinetoplastida which comprise the causative agents of African sleeping sickness (*Trypanosoma brucei gambiense* and *T. b. rhodesiense*; Chagas' disease (*T. cruzi*), and the different forms of leishmaniasis (*Leishmania donovani*, *L. major*, and *L. tropica*) [1]. At present, chemotherapy against all forms of trypanosomiasis is very limited and unsatisfactory [2].

Leishmaniasis affects twelve million people around the world with an annual death rate of approximately 80 000 people. Several drugs are available for treating leishmaniasis [2, 3]. For example, pentavalent antimonial compounds, such as sodium stibogluconate (pentostam, 2,4:2′,4′-*O*-(oxydistibylidyne)bis[D-gluconic acid]Sb,Sb′-dioxide trisodium salt nonahydrate) (Fig. 1) and meglumine antimoniate (glucantime) are the drugs used in first-line chemotherapy. As second-line drugs, amphotericin B and pentamidine (Fig. 1) are used. However, current treatments against leishmaniasis are usually unsatisfactory due to some limitations including the route of administration of the drugs, their unaffordable cost and toxicity. The chemotherapy for Chagas' disease at hand is still deficient [2]. It is based on two drugs empirically discovered, nifurtimox ((4-([5-nitrofurfurylidene]-amino)-3-methylthiomorpholine-1,1-dioxide), now discontinued, and benznidazole (*N*-benzyl-2-nitro-1-imidazoleacetamide) (Fig. 1). Although both of these compounds are able to cure at least 50% of recent infections as indicated by the disappearance of symptoms, and negativization of parasitemia and serology, they have important drawbacks. For example, (a) selective drug sensitivity on different *T. cruzi* strains; (b) these agents also produce serious side effects including vomiting, anorexia, peripheral neuropathy, allergic dermopathy, *etc.*; long-term treatment is an additional disadvantage [4]. Moreover, these

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Abbreviations: 1,3,5-thiadiazinane-2-thione (THTT); therapeutic index (TI)

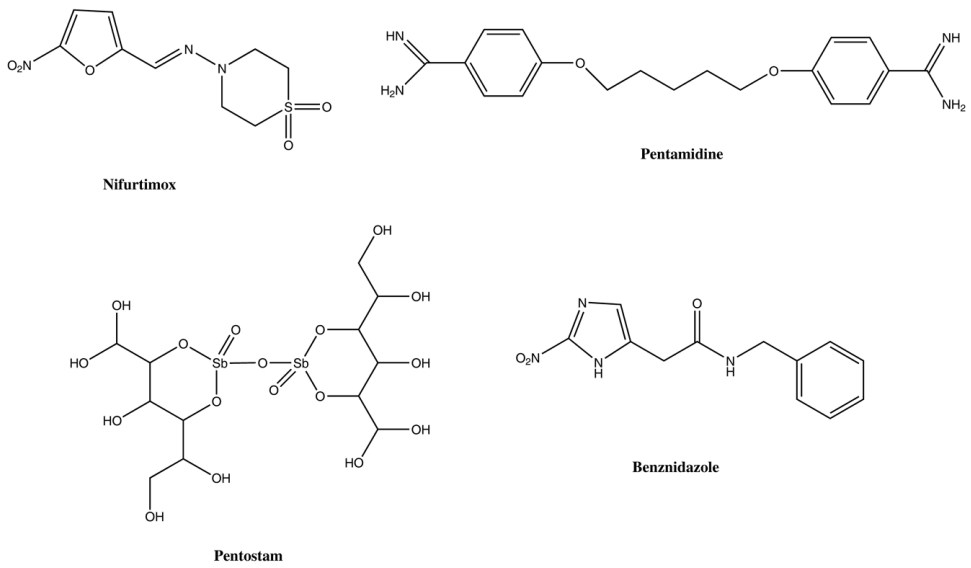


Figure 1. Some of the existing drugs clinically employed for the treatment of trypanosomiasis and leishmaniasis.

compounds are not effective in the chronic stage of the disease. In addition, there are a number of uncertainties concerning gentian violet (*N*-{4-bis[[4-(dimethylamino)-phenyl]methylene]-2,5-cyclohexadien-1-ylidene}*N*-methylmethan-aminium chloride), the only drug available to prevent blood transmission of Chagas' disease, because it is carcinogenic in animals [5]. This drug was empirically discovered for this purpose some decades ago.

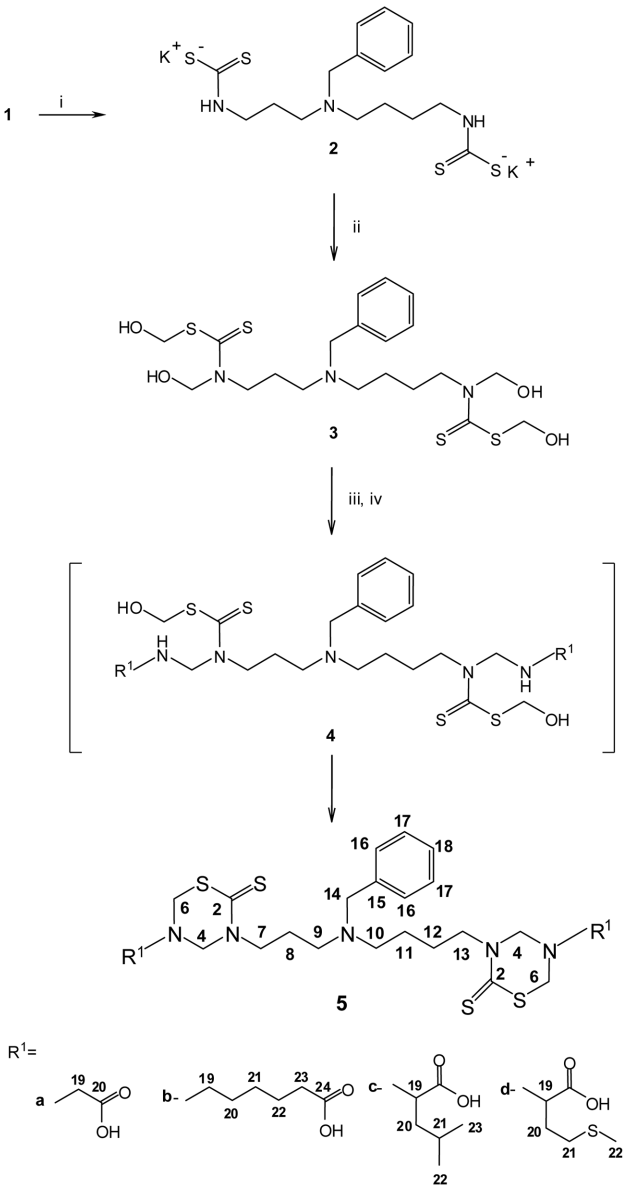
On the other hand, Human African trypanosomiasis or sleeping sickness is caused by two kinetoplastid flagellates: *T. brucei rhodesiense* and *T. brucei gambiense.* The number of new cases yearly is around 500 000 people, while 60 million individuals are at risk. Sleeping sickness is fatal if untreated and has re-emerged in recent years. Four drugs are clinically available for the treatment of this disease: melarsoprol, suramin, pentamidine, and eflornithine [2]. The first three compounds were introduced more than half a century ago, while eflornithine was registered in 1990. However, this compound is only effective when sleeping sickness is caused by *T. b. gambiense*. Most of these compounds are toxic and lack efficacy. Parasite resistance is another important drawback for these drugs [6].

Associated with this devastating panorama is the lack of financial motivation from pharmaceutical companies for carrying out rigorous research programs bearing in mind that all of these diseases are closely related with populations with low economical resources; therefore, efforts to develop new drugs should be achieved largely by academic and governmental institutions [7].

The 1,3,5-thiadiazinane-2-thione (THTT) ring has great potential in medicinal chemistry due to its wide spectrum of antimicrobial activity [8]. Furthermore, the high lipid solubility and ease of enzymatic hydrolysis [9] generally associate with this heterocycle has motivated its use as a biolabile prodrug in the design of drug-delivery systems (DDS). The aforementioned properties and the possibility to attach several structurally distinct substituents to the heterocycle ring to modify either the biological or physico-chemical properties of these compounds, have prompted us to use the thiadiazinane-2-thione as a template in our research program aimed at the development of new antiparasitic compounds [10].

Interestingly, despite the presence of two THTT cores in the same molecular scaffold appear to be very attractive from the point of view of drug design, most of the works reported so far deal with the synthesis and bioactivity of mono-THTT derivatives and only a few papers are devoted to tethered THTT analogs. The preparation of 3,3′-ethylenbis(5-alkyl-1,3,5-thiadiazine-2-thiones) [11] and 2,3-bis(5-alkyl-2-thiono-1,3,5-thiadiazin-3-yl)propionic acid derivatives [12], account for some recent examples of the latter.

Recently, we described the synthesis and antiprotozoal evaluation of two series of alkyl-linked bis[2-thioxo-(1,3,5)thiadiazinan-3-yl]carboxylic acids from 1,6-diaminohexane and 2,2-dimethyl-1,3-propanediamine, respectively [13, 14]. In general, most of the evaluated compounds showed high activity against *T. b. rhodesiense* and those possessing the less hindered alkyl residue bridging



Reagents and conditions: (i) H_2O / KOH (20%), CS_2, r.t.; (ii) HCHO (37%); (iii) H_2N-R^1, buffer phosphate (pH = 7.8); (iv) HCl (7%).

Scheme 1. Synthetic route leading to derivatives **5** from the benzylated spermidine.

both heterocycles displayed the most favored activity / cytotoxicity profiles expressed as therapeutic index (TI).

Encourage by our previous results, we have decided to explore the feasibility of synthesis of new bis-THTT using more complex polyamines as linkage other than the initially reported diamines. Behind this reasoning is the known fact that structures containing protonated polyamines linked to hydrophobic moieties such as Norespermidines-based peptides [15a], alkylpolyaminoguanidines [15b], and spermine and spermidine derivatives bearing benzyl, phenylpropyl and naphthyl groups as substituents [15c] also displayed a potent antiprotozoal activity. Furthermore, the presence of secondary nitrogen in the alkylic chain allows for the exploitation of an additional point of diversity in the overall synthetic scheme. With all this considerations in mind, the following paper deals with preliminary results obtained in the synthesis and protozoocidal effect of novel N^4-(benzyl)spermidyl-linked bis(1,3,5-thiadiazinane-2-thione) derivatives **6.**

Results and discussion

Chemistry

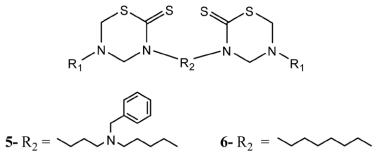
The starting N^4-benzyl polyamine **1** was previously synthesized according to a reported methodology by O'Sullivan *et al*. [16] via a protection-deprotection strategy using ethyl trifluoroacetate as the selective protective group for primary amines in the presence of secondary amines. The synthetic route leading to derivatives **5** from the benzylated spermidine is depicted in Scheme 1.

Reaction of polyamine **1** with CS_2 / OH^- provided the desired bis-dithiocarbamate salt **2**. The formation of this type of intermediate has been confirmed earlier by our group through the isolation of the 6-(dithiocarboxyamino) hexanoic acid upon acidic cleavage of the resin-bound dithiocarbamate analog [17]. Based on experimental facts [18], the condensation of **2** with formaldehyde would likely lead to the corresponding bis(hydroxymethyl-dithiocarbamic acid hydroxymethyl ester) **3** excluding the participation of a methylideneiminium Mannich-type intermediate in the reaction mechanism and most likely driving the heterocyclization pathway via the adduct **4** to form the expected bis-THTT derivatives. A computational study about the probable reaction pathway to the thiadiazinane-2-thione ring from intermediate **3** has been recently disclosed by our group [19]. In this report, it has been proposed that the heterocyclic ring is formed through an intramolecular heterocyclization of the adduct **4** via an S_N2 reaction. Computational calculations also predicted an active role of water in the reaction mechanism which promotes the heterocyclization step. Compounds **5a–d** were obtained as solids in moderate yields and showed satisfactory analytical and spectroscopic data.

Antiprotozoal activity

The *in-vitro* antiprotozoal activity of the new compounds was evaluated against *L. donovani*, *T. cruzi*, *T. b. rhodesiense STIB900*, and *P. falciparum 3D7*. Results are shown in Table 1. Inspection of the data reveals that the former

Table 1. Results for *in-vitro* anti-protozoal activity of N^4-(benzyl)spermidinyl-linked bis-THTT **5** and their hexyl-linked bis-THTT analogs **7**.



Compound	IC$_{50}$ μM[a] (TI)[b]				Toxicity[c] LD$_{50}$ (μM)
	L. donovani	*T. cruzi*	*T. b. rhodesiense*	*P. falciparum*	
5a	5.35 (0.53)	5.04 (0.56)	0.96 (2.97)	6.32 (0.45)	2.85
5b	6.45 (1.05)	8.69 (0.78)	0.95 (7.16)	23.55 (0.29)	6.8
5c	5.85 (0.78)	3.64 (1.25)	1.46 (3.12)	18.08 (0.25)	4.56
5d	5.47 (0.32)	4.23 (0.41)	1.14 (1.53)	8.73 (0.20)	1.75
6a	7.07 (1.2)	– –	0.73 (11.4)	10.97 (0.8)	8.3
6b	6.74 (2.4)	– –	0.36 (44.4)	16.95 (1.0)	16.09
6c	7.26 (7.3)	– –	2.30 (22.8)	14.19 (3.7)	52.54
Pentostan	84.42 (20.43 μgSb^v/mL)				
Benznidazole		4.00			
Pentamidine			2.50×10^{-3}		
Chloroquine				0.84	
Podophylotoxin					0.50×10^{-3}

[a] Compound concentrations of 30.0, 10.0, 3.0, 1.0, 0.3, and 0.1 μg/mL were used in the IC$_{50}$ determinations. All reported values are averages of three independent repeats.

[b] TI (Therapeutic Index) is the ratio of cytotoxicity LD$_{50}$ to the anti-parasite IC$_{50}$ (LD$_{50}$/IC$_{50}$).

[c] Cytotoxicities were assayed against KB cells using podophylotoxin as standard (LD$_{50}$ in μM). Compound concentrations of 300.0, 30.0, and 0.3 μg/mL were used in the LD$_{50}$ determinations. All reported values represent the average of three independent repeats.

two parasites are particularly susceptible to these compounds. In the case of *T. cruzy*, activities ranged between 3.64 – 8.69 μM, very similar to the control drug Benznidazole, which had an IC$_{50}$ of 4.00 μM. Results for *L. donovani* were even more encouraging, revealing that all the bis-THTT were considerably more active than sodium stibogluconate (pentostam). For this parasite, activities were more than one order of magnitude higher than the control. None of the compounds showed a remarkable activity against *T. b. rhodesiense* and *P. falciparum 3D7*, respectively.

In order to compare the protozoocidal profile of the synthesized compounds in relation to the previously reported hexyl-linked bis-THTT analogs **6** the antiprotozoal results of the latter and the calculated therapeutic index (TI) values for both series were also included in Table 1. For comparative purposes, structures **5** and **6** differ only in the nature of their connective backbone (R$_2$). As could be observed in Table 1 for compounds in both series possesing the same aminoacid residue (R$_1$), the displayed protozoocidal profile against the three pasasitic lines assayed was almost the same. However, higher values of therapeutic indexes were found for compounds **6a – c**. Considering that both series exerted a similar protozoal activity it is possible to assume that structures **5** are more cytotoxic than their alkyl tethers analogs.

In the case of the thiadiazinane-2-thiones the mechanisms of biological activity and cytotoxicity are closely related. It has been demonstrated that under physiological conditions the THTT ring undergoes chemical and / or enzymatic degradation to generate highly reactive species such as isothiocyanates, dithiocarbamic acids and their ester derivatives. These degradation products are known for being toxic to mammalian cells and also for their capacity to interact with the thiol groups located at the active site of the cysteine proteinases (CPs), present in most groups of parasitic protozoa [10a, 20] with the consequent inactivation of the enzymatic activity. The latter mechanism was recently supported by the fact that 3-furfuryl-5-substituted-1,3,5-thiadiazinane-2-thione derivatives are more active against intracellular amastigote than promastigote forms of *Leishmania* [10b]. In this case, the difference between the activities of both stages could be correlated with the expression of CPs, which are more abundant in the amastigote stage, where they play an important role [21].

Taking into account the considerations above, the origen of the higher cytotoxicities of compounds **5** does not

seems to solely rely on the degradation product of the THTT ring, since compounds in both series posses the same type and number of heterocyclic structures. On the other hand, it is likely that compounds **5** could also interfere with the polyamine metabolism due to the presence of a N^4-(benzyl)spermidyl moiety. Since THTT derivatives are renown prodrugs, they could be able to traverse the cell membrane of the mammalian cells via a polyamine transporter and to exert their cytotoxic effect upon degradation. Since, at this moment, we do not have conclusive evidences about the latter hypothesis, a more extensive activity / toxicity study will be carried out on these and other polyamine-linked bis-THTT derivatives.

Conclusion

To summarize, we have reported the feasibility of synthesis and the structural elucidation of novel N^4-(benzyl) spermidyl-linked bis-(1,3,5-thiadiazinane-2-thione) derivatives from the corresponding N^4-(benzyl) spermidine. The synthesized compounds showed a potent protozoocidal activity against *T. cruzy* and *L. Donovani* which turns to be comparable or greater than the currently employed chemotherapies. Despite this fact, the novel structures displayed a higher cytotoxicity than previously synthesized alkyl tethers analogs having the same amino acidic residues attached to position N^5 of the heterocyclic ring. It has been hypothesized that increased cytotoxicity could be related with an interference of the polyamine metabolism in mammals.

In light of the results presented here and taking into account those from earlier reports, we are evaluating how the chemical nature of *N*-substituents influences the overall activity / cytotoxicity profile in the spermidyl-linked bis(1,3,5-thiadiazinane-2-thione) series. The synthesis of novel bis-THTT derivatives employing other polyamines as bridging moieties will be also the theme of future investigation in our group.

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The authors have declared no conflict of interest.

Experimental

All reagents were of analytical grade and purified when necessary. Melting points were determined in a Büchi 535 apparatus (Büchi Labortechnik, Flawil, Switzerland) and were not corrected. The progress of the reaction and purity of compounds were monitored by TLC analytical silica gel plates (Merck F_{254}, Merck, Darmstadt, Germany). All NMR experiments were performed at 298K for a solution of 30 mg of compound dissolved in 0.7 mL of DMSO-d_6 on a Bruker AVANCE-500 instrument (Bruker Bioscience, Billerica, MA, USA). Microanalysis was performed in a Perkin–Elmer 2400 CHN (Perkin-Elmer, Norwalk, CT, USA) by the microanalysis service at Complutense University of Madrid.

General procedure for [5-(3-(benzyl{4-[5-(carboxyalkyl)-2-thioxo-1,3,5-thiadiazinan-3-yl]butyl}-aminopropyl)-6-thioxo-1,3,5-thiadiazinan-3-yl]carboxylic acids *5a–d*

To a stirred solution of the previously synthesized ^1N-(3-aminopropyl)-^1N-benzyl-1,4-butanediamine **2** (5 mmol) in 25 mL of water, potassium hydroxide (0.56 g, 10 mmol, as a 20% aqueous solution) and carbon disulfide (0.6 mL, 10 mmol) were added at room temperature. The mixture was subsequently stirred for 4 h. Then, formaldehyde solution 37% (1.6 mL, 20 mmol) was added and the stirring continued for 1 h. The reaction mixture was added dropwise to a suspension of the corresponding amino acid (10 mmol) in a dihydrogen phosphate buffer solution pH = 7.8 (10 mL) and stirred for 2 h. The aqueous solution was passed through a celite pad, cooled in an ice bath and acidified to pH = 3 with 7% hydrochloric acid. The precipitate was filtered, washed several times with small portions of cold ether and dried under vacuum over silica gel for 12 h.

2-(5-{3-(Benzyl[4-(5-carboxymethyl-2-thioxo-1,3,5-thiadiazinan-3-yl)butyl]aminopropyl}-6-thioxo-1,3,5-thiadiazinan-3-yl]acetic acid ***5a***

From glycine (0.95 g, 10 mmol); yield **5a** (0.58 g, 20%); m.p.: 122–123°C; ^{1}H-NMR (DMSO-d_6) δ: 1.54 (m, 4H, H-11, H-12), 1.84 (m, 2H, H-8), 2.56 (br.s, 4H, H-9, H-10), 3.49 (s, 4H, H-19), 3.75 (br. s, 2H, H-14), 3.86 (m, 6H, H-7, H-13), 4.48 (s, 4H, H-6), 4.51 (s, 4H, H-4), 7.28 (m, 1H, H-18), 7.34 (t, 2H, *J* = 7.2 Hz H-17), 7.38 (d, 2H, *J* = 6.8 Hz, H-16), 12.01 (br. s, 2H, COO*H*) ppm; ^{13}C-NMR (DMSO-d_6) δ: 22.39, 22.49, 23.60 (C-8, C-11, C-12), 49.46, 49.87, 50.88, 52.15 (C-7, C-9, C-10, C-13), 50.78, 50.82 (C-19), 57.07 (C-14), 58.01 (C-6), 69.36, 69.49 (C-4), 127.62 (C-18), 128.35 (C-17), 129.53 (C-16), 136.80 (C-15), 170.60, 170.64 (C-20), 190.09, 190.27 (C-2) ppm; Anal. Calcd for $C_{24}H_{35}N_5O_4S_4$: C, 49.21; H, 6.02; N, 11.95; O, 10.92; S, 21.89. Found: C, 49.39; H, 6.1; N, 10.5.

6-[5-(3-(Benzyl{4-[5-(5-carboxypentyl)-2-thioxo-1,3,5-thiadiazinan-3-yl]butyl}aminopropyl)-6-thioxo-1,3,5-thiadiazinan-3-yl]hexanoic acid ***5b***

From 6-amino-*n*-hexanoic acid (1.30 g, 10 mmol); yield **5b** (0.50 g, 14%); m.p.: 106–107°C; ^{1}H-NMR (DMSO-d_6) δ: 1.28 (m, 4H, H-21), 1.48 (m, 12H, H-11, H-12, H-20, H-22), 1.75 (m, 2H, H-8), 2.19 (t, 4H, *J* = 7.2 Hz, H-23), 2.41 (m, 4H, H-9, H-10), 2.61 (m, 4H, H-19), 3.51 (br. s, 2H, H-14), 3.85 (m, 4H, H-7, H-13), 4.43 (m, 8H, H-6, H-4), 7.21 (m, 4H, H-16, H-17), 7.29 (m, 1H, H-18), 12.01 (br. s, 2H, COO*H*) ppm; ^{13}C-NMR (DMSO-d_6) δ: 23.48, 23.56, 23.89 (C-8, C-11, C-12), 24.26 (C-22), 26.01, 26.02 (C-20, C-21), 33.60 (C-23), 40.04, 40.06 (C-19), 49.95, 50.56, 51.08, 52.71 (C-7, C-9, C-10, C13), 57.32, 57.38 (C-6), 57.78 (C-14), 69.38, 69.53 (C-4), 126.73 (C-18), 128.04 (C-17), 128.68 (C-16), 139.53 (C-15), 174.38 (C-24), 190.03,

190.13 (C-2) ppm; Anal. Calcd for $C_{32}H_{51}N_5O_4S_4$: C, 55.06; H, 7.36; N, 10.03. Found: C, 55.29; H, 7.53; N, 10.1.

2-[5-(3-(Benzyl{4-[5-(1-carboxy-3-methylbutyl)-2-thioxo-1,3,5-thiadiazinan-3-yl]butyl}aminopropyl)-6-thioxo-1,3,5-thiadiazinan-3-yl]-4-methylpentanoic acid ***5c***

From L-leucine (830 mg, 10 mmol); yield **5c** (0.55 g, 19%); m.p.: 126 – 127°C; ^{1}H-NMR (DMSO-d_6) δ: 0.88 (m, 12H, H-22, H-23), 1.60 (br. s, 10H, H-12, H-20, H-21), 1.74 (br.s, 2H, H-11), 2.10 (br. s, 2H, H-8), 3.02 (br. s, 4H, H-9, H-10), 3.51 (m, 2H, H-19), 3.92 (m, 4H, H-7, H-13) 4.31 (br. s, 2H, H-14), 4.56 (m, 8H, H-6, H-4,), 7.46 (m, 3H, H-17, H-18), 7.60 (m, 2H, H-16), 12.85 (br. s, 2H, COO*H*) ppm; ^{13}C-NMR (DMSO-d_6) δ: 20.15, 20.79, 23.38 (C-8, C-11, C-12), 21.85, 21.94, 23.09, 23.20 (C-22, C-23), 24.75, 24.79 (C-21), 38.59, 38.63 (C-20), 48.84, 48.89, 50.84, 51.20 (C-7, C-9, C-10, C-13), 55.77, 55.82, 55.87 (C-6, C-14), 60.80, 60.90 (C-19), 67.53, 67.61 (C-4), 128.87 (C-17), 129.56 (C-18), 129.83 (C-15), 131.32 (C-16), 173.29, 173.37 (C-24), 190.95, 191.69 (C-2) ppm; Anal. Calcd for $C_{34}H_{55}N_5O_4S_4$: C, 56.27; H, 7.55; N, 9.65. Found: C, 56.35; H, 7.63; N, 9.79.

2-[5-(3-(Benzyl{4-[5-(1-carboxy-3-methylsulfanylpropyl)-2-thioxo-1,3,5-thiadiazinan-3-yl]butyl}aminopropyl)-6-thioxo-1,3,5-thiadiazinan-3-yl]-4-methyl sulfanyl butanoic acid ***5d***

From L-methionine (1.49 g, 10 mmol); yield **5d** (0.73 g, 20%); m.p.: 118 – 120°C; ^{1}H-NMR (DMSO-d_6) δ: 1.59 (m, 2H, H-12), 1.68 (m, 2H, H-11), 1.97 (m, 4H, H-20), 2.03, 2.04 (s, 6H, H-22), 2.06 (m, 2H, H-8), 2.43 (m, 4H, H-21), 2.90 (br. s, 4H, H-9, H-10), 3.61 (m, 2H, H-19), 3.92 (m, 4H, H-7, H-13), 4.18 (br. s, 2H, H-14), 4.55 (m, 8H, H-6, H-4), 7.42 (m, 3H, H-16, H-18), 7.56 (m, 2H, H-17) ppm; ^{13}C-NMR (DMSO-d_6) δ: 14.67, 14.69 (C-22), 20.72, 21.17, 23.49 (C-8, C-11, C-12), 29.25, 29.29, 29.33 (C-20, C-21), 49.06, 49.18, 50.93, 51.41 (C-7, C-9, C-10, C-13), 55.67, 55.74 (C-6), 56.09 (C-14), 61.10, 61.23 (C-19), 67.65, 67.73 (C-4), 128.74 (C-17), 129.09 (C-18), 130.91 (C-16), 172.59, 172.67 (C-23), 190.86, 191.48 (C-2) ppm; Anal. Calcd for $C_{30}H_{47}N_5O_4S_6$: C, 49.08; H, 6.45; N, 9.54. Found: C, 49.41; H, 6.29; N, 9.70.

Protozoocidal *in-vitro* assays

For the *L. donovani* assay, peritoneal exudate macrophages were infected with *L. donovani* amastigotes and exposed to the compounds for five days. IC_{50} values were determined by comparing the% infection of the test compounds to uninfected controls [22]. Protozoocidal activity was evaluated by exposing bloodstream forms of the parasite to compound for 72 h [23] and antiplasmodial activity was determined in a 72-h assay, exposing infected red blood cells to the compounds and evaluating via uptake of tritiated hypoxanthine $[H]^3$ [24].

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