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Bioorganic & Medicinal Chemistry Letters 17 (2007) 624–627
Bioorganic & Medicinal Chemistry Letters

Identification of the benzodiazepines as a new class of antileishmanial agent

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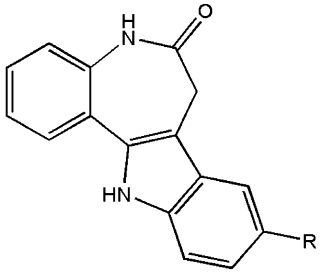
Received 10 October 2006; revised 31 October 2006; accepted 1 November 2006
Available online 6 November 2006

Abstract—The continual increase in drug resistance; the lack of new chemotherapeutic agents; the toxicity of existing agents and the increasing morbidity with HIV co-infection mean the search for new antileishmanial agents has never been more urgent. We have identified the benzodiazepines as a structural class for antileishmanial hit optimisation, and demonstrated that their in vitro activity is comparable with the clinically used drug, sodium stibogluconate, and that the compounds are not toxic to macrophages.

Leishmaniasis, which threatens approximately 350 million people in 88 countries, was the cause of death of 59,000 people in 2002, and of the 1.5–2 million new cases reported, one-third were the life-threatening form of leishmaniasis.[1] The continual increase in drug resistance, the lack of new chemotherapeutic agents and the toxicity of existing agents means that new drugs are required. A recent study by Meijer and coworkers[2] demonstrated that paullones **1**, previously identified as antitumour agents through their inhibition of cyclin-dependent kinases, completely inhibit the growth of *Leishmania mexicana* promastigotes in vitro. Whilst paullones offer a promising starting point for chemical investigation,[2] we considered their non-selectivity and multi-step, low yielding synthesis[3] a disadvantage for development of structurally related compounds with increased antiparasitic efficacy. Consequently, we prepared a series of synthetically amenable benzodiazepines and pyrrolobenzodiazepines structurally related to the paullone nucleus to probe for activity. Our aim was to chemically modify the basic benzodiazepine skeleton and optimise for antileishmanial activity in a macrophage amastigote infection model,[4] rather than activity against the promastigote stage, so that we could test for antiparasitic activity against the host-relevant form, potential cytotoxicity and ability to enter the macrophage in the same assay.[5,6]

We report here the short, efficient synthetic route and the results of the biological testing for antileishmanial activity. Whilst modifications were made to probe the effects of structural changes, low cost economics and synthetic efficiency were also important factors in selecting compounds for synthesis.



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To establish whether tricyclic or tetracyclic systems similar to paullones exhibited antileishmanial activity, compounds **3**–**5** were prepared in a one-step reaction[7] by condensation of isatoic anhydride **2** and the appropriate amino acid derivative in DMSO at 100 °C (Scheme 1).

Keywords: Antileishmanial agents; Amastigote; Benzodiazepine; Pyrrolobenzodiazepine; Paullone.
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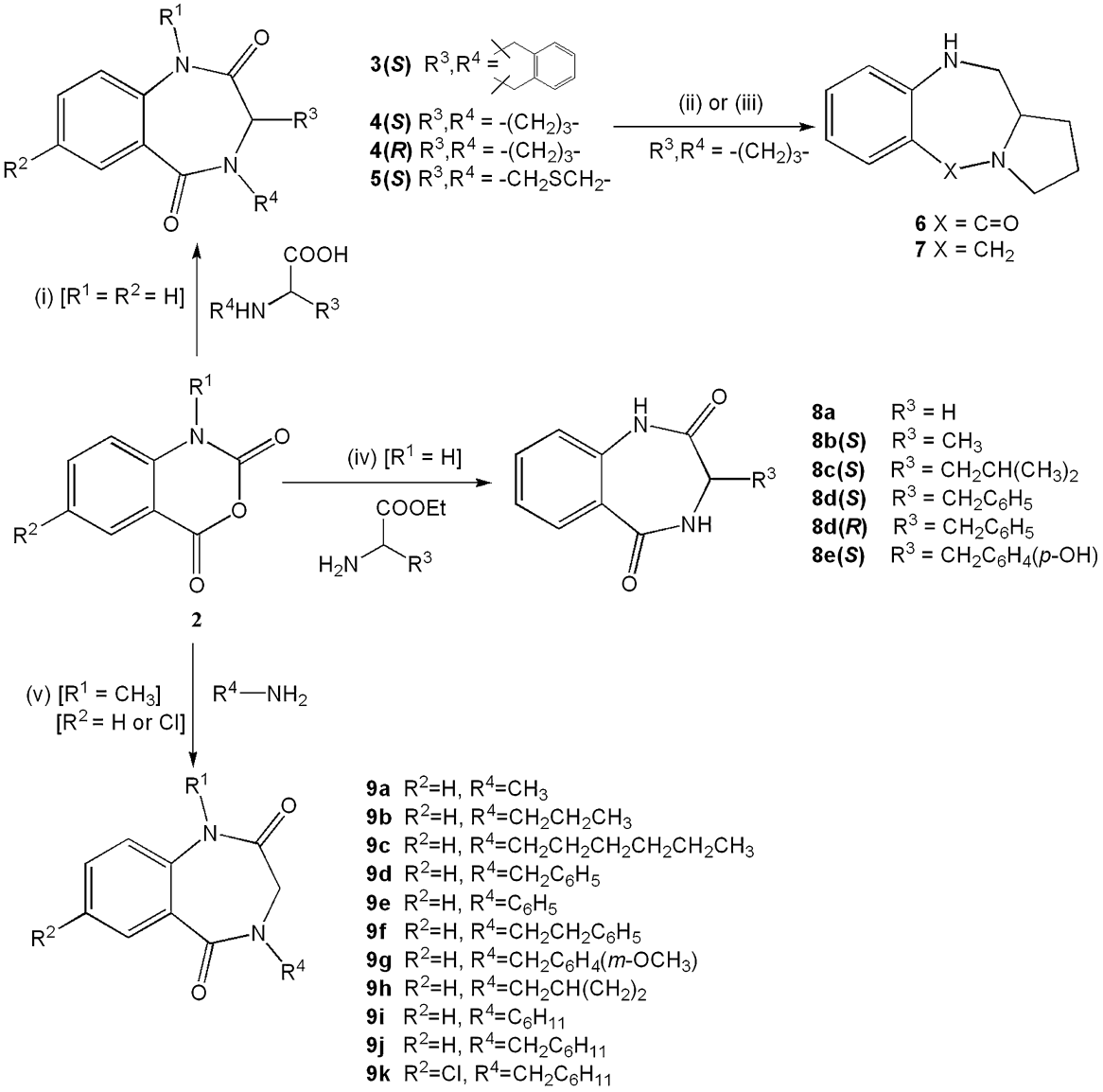
doi:10.1016/j.bmcl.2006.11.004



Scheme 1. Simple and affordable route to the pyrollobenzodiazepines and the benzodiazepine diones. Reagents and conditions: (i) 1—DMSO, 100 °C (2) H_2O, 0 °C; (ii) $NaBH_4$, TFA, glyme, reflux; (iii) $LiAlH_4$, THF, reflux; (iv) 1—pyridine, reflux; 2—H_2O, 0 °C; (v) 1—CH_2Cl_2, KPhos, pH 7.0, $BrCOCH_2Br$, 5 °C, DBU.

The effects of chirality on activity were investigated with both enantiomers of proline. The compounds were isolated by precipitation of the cooled reaction mixture using iced water. To establish whether the hydrogen-bond acceptors of the amide carbonyls were necessary for activity, the secondary amide of dione **4** was selectively reduced to the corresponding secondary amine using sodium borohydride and trifluoroacetic acid in glyme to give **6** in a quantitative yield. Both amides in dione **3** were reduced with lithium aluminium hydride to give the diamine **7**.[8] Reductions were performed on both the *R*- and *S*-isomers of **4** to produce enantiomerically pure compounds. In order to establish whether a tricyclic system was an activity requirement, proline was replaced with a series of amino acid ester hydrochlorides,[9] which in refluxing pyridine, produced benzodiazepine-2,5-diones **8** with different hydrophobic substituents (or no substituent in the case of glycine) at C_3, whilst leaving N_4 free as the secondary amide. Finally, to investigate the role of substituents at N_4, a high yielding, 'one-pot' reaction between *N*-methyl isatoic anhydride, an appropriate primary amine and bromoacetyl bromide in the presence of DBU was used to produce a series of N_4-substituted benzodiazepine-2,5-diones **9**.[10]

The antileishmanial activity of all compounds against a clinically derived strain of *Leishmania donovani* (antimony-sensitive 200016)[4] is shown in Table 1. In addition, the activity of the clinically used drug sodium stibogluconate is also shown for comparison.

These data indicate that whilst the tetracycle ***3(S)*** is inactive, the tricyclic pyrrolobenzodiazepine-2,5-diones are more effective antileishmanial agents than sodium stibogluconate at the concentrations tested, with no evidence of toxicity against the host macrophage cells. Activity appears to be independent of chirality in the tri-

cyclic 2,5-diones. Replacement of the pyrrolidine with a thiazolidine ring **5**(***S***) not only abolished activity but gave rise to some evidence of cellular host toxicity characterised by unusual cellular growth and vacuole formation in the macrophages (data not shown).

Reduction of the secondary amide to the corresponding amine retained activity for the *S*-enantiomer **6**(***S***), whilst there was lower suppression rate of the parasites for the corresponding *R*-isomer **6**(***R***), albeit at a level comparable with sodium stibogluconate. Reduction of both amides to the diamine produced divergent activity between the enantiomers, with suppression levels maintained for ***7(R)***, but rendered not significant with **7**(***S***). Given that the physicochemical properties of the enantiomers are the same, we can only surmise that disparities in activity are consequences of differential affinities for a target or carrier protein. It would appear therefore that chirality becomes important in the absence of the 5-oxo group. The loss of H-bond acceptor capacity in this region does not seem to be necessary for substantial antileishmanial activity.

The data clearly show that when the tricyclic system is trimmed down to a benzodiazepine-2,5-dione, a bulky substituent at position C_3 is required, with the benzyl derivatives **8d**(***S***) and **8e**(***S***) producing suppression similar to that of the clinically used drug, sodium stibogluconate, at comparable concentrations. The results for the two stereoisomers of **8d** suggest that stereochemical factors may be important when a bulky group is present at C_3. Comparing the activity of **3**(***S***) with **8d**(***S***) suggests a degree of flexibility at C_3 is required when an aromatic substituent is present.

For the N_4-substituent series, the benzyl derivatives **9d** and **9g** retain activity in common with the phenylaniline derivative **8d**(***S***), although a single methylene spacer between the benzodiazepine nucleus and the aromatic group is essential, with the phenyl **9c** and phenylethyl **9f** analogues demonstrating no significant antileishmanial activity. Again, comparison with **3**(***S***) and **8d**(***S***) alludes to flexibility in this region of the molecule, rather than the common conformation that **3**(***S***) offers between **9** and **8d**(***S***).

When the N_4-substituent is aliphatic, the antileishmanial activity profile is inconclusive. The inactivity of the *N*-methyl **9a** and nor analogue **8a** suggests that a degree of bulkiness is required at N_4. However, whilst the *N*-propyl derivative **9b** has suppressive activity equivalent to sodium stibogluconate, the longer chain *N*-hexyl **9c** and methylcyclopropyl **9h** derivatives are inactive. The activity of the conformationally restricted cyclohexyl derivative **9i** is statistically not significant, yet the bulky, but more flexible methylcyclohexyl derivative **9j** was the first compound to instigate cell death of the host macrophages.

The toxicity of compound **9j** prompted us to investigate the role of an electron-withdrawing substituent in the aromatic ring (**9k**), based on the development of the anxiolytic benzodiazepines as therapeutic agents, where a 7-chloro or nitro substituent was incorporated to block aromatic hydroxylation by the P450 system. Aromatic hydroxylation can also yield toxic metabolites,[11] which may be responsible for the toxicity of **9j** at higher concentrations.

Comparing the activity of the parent compound **9j** with its analogue **9k** suggests that the introduction of a 7-chloro aromatic substituent overcomes the debilitating effect of the N_4-methylcyclohexyl group. However, systematic metabolism studies using rat-derived liver microsomes and hepatocytes demonstrated identical biotransformation routes; N-demethylation, hydroxylation, and O-glucuronidation. Although both compounds produced a marked decrease in cellular glutathione levels in macrophages, there was neither a significant effect on macrophage viability (leakage of lactate dehydrogenase) nor glutathione reductase activity at the concentrations used (50 and 100 μM).

The LD_{50} for compound **9k** when determined against *L. donovani* 200016 was 115 μM (37 μg/ml) and produced a 73 ± 5% mean parasite suppression. At this concentration, **9j** was inactive (and non-toxic, as confirmed

Table 1. A comparison of the antileishmanial activity of synthesized compounds and the antileishmanial drug sodium stibogluconate[13]

Compound	Mean parasite suppression (%) and standard error	Compound	Mean parasite suppression (%) and standard error
Na stibogluconate	59.4 ± 2.8*	**8d*(R)***	23.0 ± 7.0^{NS}
3(***S***)	25.0 ± 11^{NS}	**8e**(***S***)	64.4 ± 16.0*
4(***S***)	70.3 ± 2.1*	**9a**	Inactive
4(R)	85.4 ± 0.6*	**9b**	72.0 ± 16.0*
5(***S***)	33.0 ± 8.0[a,*]	**9c**	Inactive[a]
6(***S***)	70.6 ± 1.2*	**9d**	51.0 ± 8.0*
6(R)	61.7 ± 2.2*	**9e**	Inactive
7(***S***)	23.0 ± 6.0^{NS}	**9f**	19.6 ± 6.0^{NS}
7(R)	67.8 ± 16.0*	**9g**	54.6 ± 9.0*
8a	19.0 ± 7.5^{NS}	**9h**	Inactive
8b(***S***)	28.0 ± 17.0^{NS}	**9i**	26.4 ± 6.0^{NS}
8c(***S***)	Inactive	**9j**	Toxic
8d(***S***)	53.5 ± 8.2*	**9k**	85.0 ± 5.0*

[a] Some evidence of host cell toxicity.

* The statistical significance was assessed using ANOVA and Fisher's PLSD in Statview 5.01. Results highlighted with an asterisk indicate significance at 99% or greater ($p = 0.01$, NS, not significant).

by the metabolism studies), which implies a potency enhancing effect for the 7-substituent. This could possibly be due to enhanced cellular penetration through improved lipophilicity, or an improved pharmacodynamic interaction with the target (so far unidentified), given there are no differences in their metabolism by the macrophage host system.

In conclusion, we have identified a promising new hit for the treatment of leishmaniasis. The 1,4-benzodiazepine-2,5-dione **9k** has an amastigote suppression efficacy comparable with the clinically used sodium stibogluconate and is non-toxic in our model. **9k** demonstrates efficacy at a concentration of 37 μg/ml, whereas sodium stibogluconate has plasma concentrations of 10–20 μg/ml after clinical dosing.[12] We have therefore designed a detailed programme of further optimisation to enhance the activity of **9k**. In future, we hope to report on our compound's progression through preclinical studies and confirm its economical therapeutic efficacy in the treatment of this devastating and currently neglected disease.

Acknowledgment

GO-D and MD were supported by grants from the Governments of Ghana and Vietnam, respectively.

Supplementary data

Experimental procedures and analysis data are available on-line. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.11.004.

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13. Bone marrow-derived macrophages were infected with *L. donovani* antimony-sensitive strain 200016[9] promastigotes, using a 20:1 parasite: host cell ratio. Twenty-four hours later cells were treated with medium alone (controls), a synthesized compound (100μg/ml) or sodium stibogluconate solution (70 μg/ml Sb^{v}/ml); using four replicates/treatment. Seventy-two hours post-treatment cells were fixed, stained with 10% v/v aqueous Giemsa stain and the mean percentage of cells infected determined, using 200 randomly selected cells. The mean percentage suppression ± SE was calculated using the relevant mean control value. A Mann–Whitney *U* test using the control data and test compound data was carried out to determine whether a compound caused significant parasite killing, and a $p < 0.05$ was considered significant.