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Synthesis, biological evaluation, and molecular modeling of 3,5-substituted-N^1-phenyl-N^4,N^4-di-*n*-butylsulfanilamides as antikinetoplastid antimicrotubule agents

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Abstract—Dinitroanilines are of interest as antiprotozoal lead compounds because of their selective activity against the tubulin of these organisms, but concern has been raised due to the potentially mutagenic nitro groups. Analogues of N^1-phenyl-3,5-dinitro-N^4,N^4-di-*n*-butylsulfanilamide (**GB-II-150**, compound **2b**), a selective antimitotic agent against African trypanosomes and *Leishmania*, have been prepared where the nitro groups are replaced with amino, chloro, cyano, carboxylate, methyl ester, amide, and methyl ketone moieties. Dicyano compound **5** displays IC_{50} values that are comparable to **2b** against purified leishmanial tubulin assembly (6.6 vs 7.4 μM), *Trypanosoma brucei brucei* growth in vitro (0.26 vs 0.18 μM), *Leishmania donovani* axenic amastigote growth in vitro (4.4 vs 2.3 μM), and in vitro toxicity against Vero cells (16 vs 9.7 μM). Computational studies provide a rationale for the antiparasitic order of activity of these analogues and further insight into the role of the substituents at the 3 and 5 positions of the sulfanilamide ring.

1. Introduction

Dinitroaniline herbicides are well known for their activity against protozoan parasites. Chan and Fong first reported the activity of trifluralin (**1a**) against the pathogenic protozoan *Leishmania* in 1990 and showed that this compound bound selectively to partially purified leishmanial tubulin compared to rat brain tubulin.[1] Another dinitroaniline herbicide, oryzalin (**1b**), also displayed antimicrotubule activity against *Leishmania*.[2] These compounds were later shown to possess antiparasitic activity against trypanosomes,[3,4] *Plasmodium*,[5,6] and *Toxoplasma*.[7–9] Given the need for new drug candidates against diseases caused by protozoan parasites,[10,11] the dinitroaniline herbicides are intriguing lead compounds. Replacement of the nitro moieties present in these molecules with other functional groups would be desirable, however, since several nitroaromatic compounds have been shown to be mutagenic.[12–14]

Our laboratory previously demonstrated that N^1-phenyl-3,5-dinitro-N^4,N^4-di-*n*-propylsulfanilamide (**GB-II-5**, compound **2a**), N^1-phenyl-3,5-dinitro-N^4,N^4-di-*n*-butylsulfanilamide (**GB-II-150**, compound **2b**), and other N^1-aryl-3,5-dinitroaniline sulfanilamides possess selective antimicrotubule activity against *Leishmania* and African trypanosomes in vitro and are far superior to **1a** and **1b** in their potency against these parasites.[15–18] Replacement of one of the nitro groups present in **2a** with a hydrogen resulted in a profound loss in antiparasitic and antitubulin activity.[17] A recent computational study by Mitra and Sept suggested that these nitro groups in **2a** serve as hydrogen bond acceptors at a unique binding site occurring on the α-subunit of leishmanial tubulin.[19] These authors also predicted that cyano and methyl ketone groups could act as hydrogen bond acceptors if they were substituted for the nitro groups present in the dinitroaniline sulfanilamides. In the present manuscript, we describe the synthesis and biological activity of analogues of **2b** where the nitro

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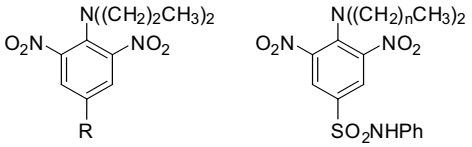
moieties are replaced by several groups with chemical and physical similarities.



1a: R = CF_3
1b: R = SO_2NH_2

2a: n = 2
2b: n = 3

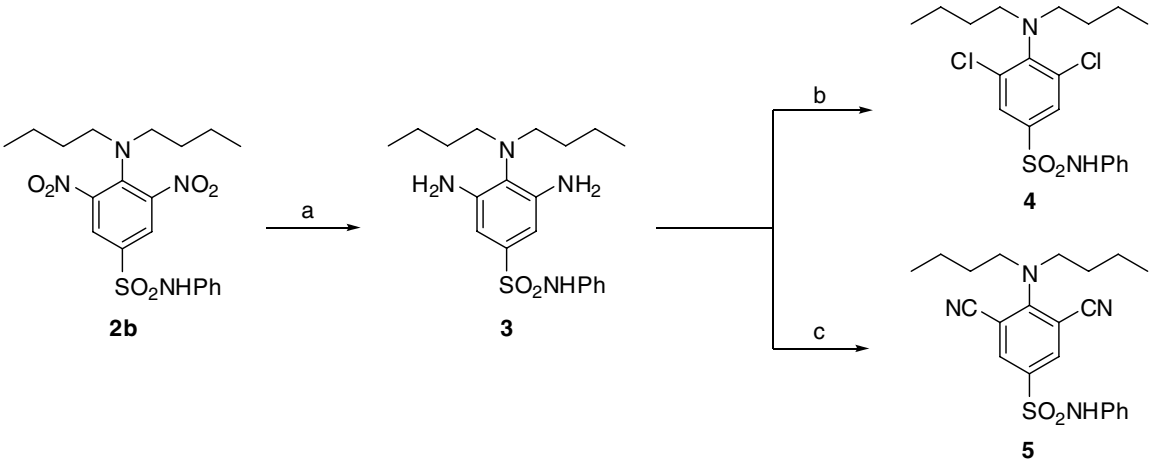
2. Results

2.1. Chemistry

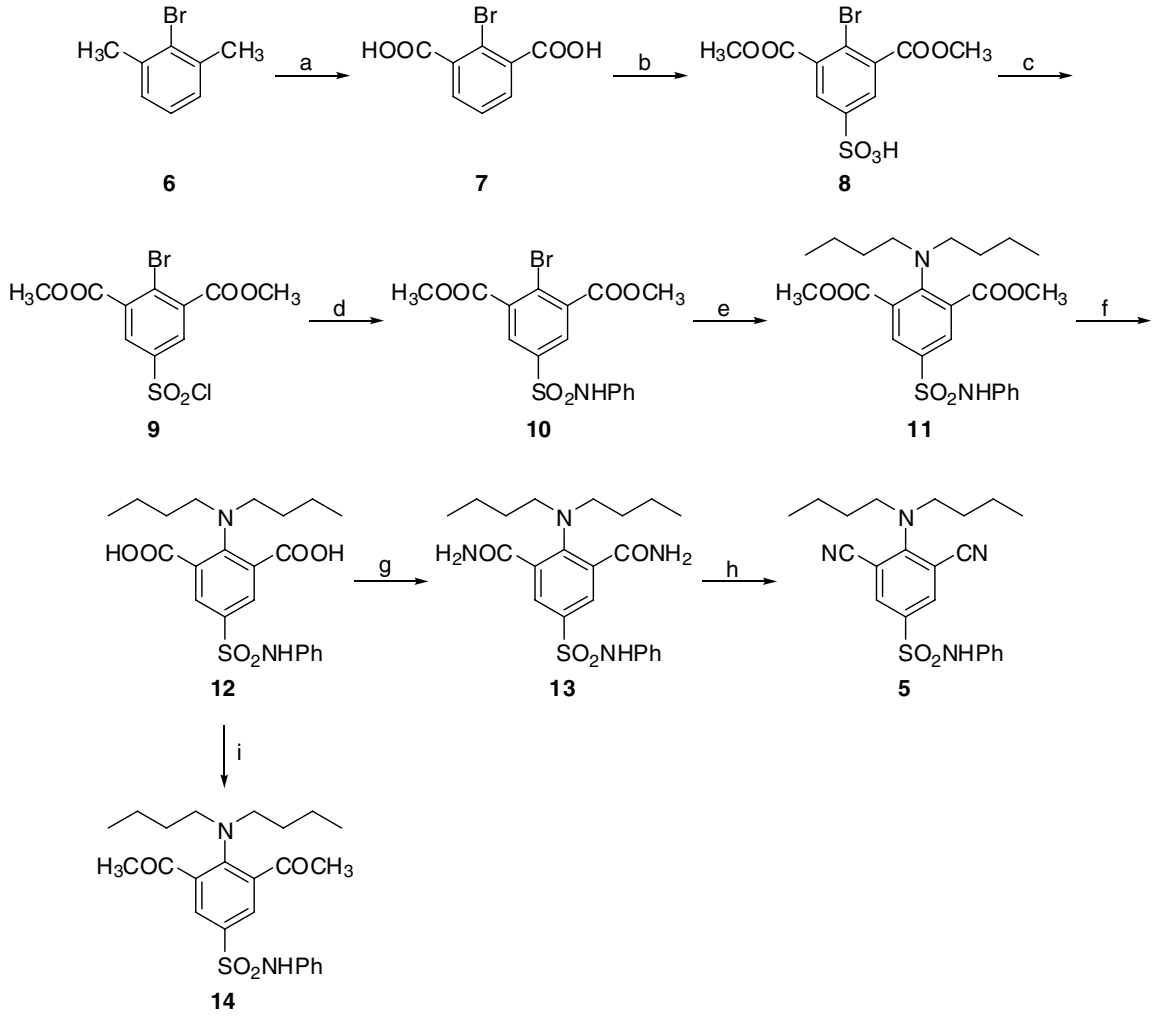
It was proposed that target compounds containing Cl atoms and cyano moieties in place of the nitro groups present in **2b** could be introduced through a strategy culminating with a Sandmeyer reaction. Reduction of **2b** was carried out using H_2 in the presence of 10% Pd–C, which afforded the diamino compound **3** in high yield (Scheme 1). Treatment of **3** with $NaNO_2$ in HCl followed by reaction of the resultant bis-diazonium salt with CuCl afforded the dichloro analogue **4**, albeit in low yield (Scheme 1). All efforts to improve the yield of **4** by changing the reaction conditions were unsuccessful. Target compound **5** was also prepared by this strategy, but in extremely low yield (Scheme 1). The diazotization reaction was carried out using $NaNO_2$ and HCl in a mixture of H_2O/AcOH containing **3** at 0 °C. This solution was then treated with an excess of CuCN and KCN in a mixture of $CHCl_3/H_2O$ at 0 °C[20] followed by heating to 50 °C. These conditions did not reproducibly provide the desired product. Other related approaches, such as preparing the bis-diazonium salt using either *tert*-butyl nitrite or *tert*-butyl nitrite in BF_3 etherate in situ, isolating the bis-diazonium salt prior to reaction with cyanide, or adding 18-crown-6 to complex the potassium ion were unsuccessful. The presence of the bulky dibutylamino groups in the substrate may interfere with the Sandmeyer reaction in this case and may be the cause of the poor reaction yield.

In order to provide a more efficient and reproducible alternative for the preparation of **5** and additional target compounds, another synthetic route was envisioned (see Scheme 2). Permanganate oxidation of commercially available 2-bromo-*m*-xylene (**6**) gave 2-bromoisophthalic acid (**7**),[21] which was sulfonylated with fuming sulfuric acid and esterified using methanol/HCl to afford the sulfonic acid **8**. Treatment of **8** with $POCl_3$ provided the sulfonyl chloride **9**, which was then converted to sulfonamide **10** by reaction with aniline in the presence of pyridine in acetone. An Ullmann reaction[22] between sulfonamide **10** and dibutylamine afforded the desired diester analogue **11**, which was hydrolyzed to yield diacid **12**. The formation of bis-amide **13** was carried out in a one-pot reaction between ammonia and the intermediate bis-acid chloride derivative of **12**, which in turn was prepared by the reaction of **12** with hexachloroacetone in the presence of PPh_3.[23] Attempts to synthesize the bis-amide directly from diester **11** were unsuccessful, resulting in quantitative recovery of the starting material. Bis-amide **13** was found to be an excellent precursor for the target dicyano compound **5**. Dehydration of **13** using $PdCl_2$ in an acetonitrile/water mixture[24] afforded **5** in good and reproducible yield. The synthesis of dicyano compound **5** from bis-amide **13** is an equilibrium process in which the presence of excess acetonitrile favors the formation of **5**.

With target compounds **3**–**5** and **11**–**13** in hand, attention was focused on the preparation of bis-ketone **14**. Initial attempts to synthesize **14** by treating the bis-acid chloride derivative of **12** with dimethyl malonate in the presence of NaH followed by decarboxylation[25] were unsuccessful. Reaction between methyl magnesium bromide and the bis-acid chloride gave compound **14** along with other unidentified side products. The formation of these side products could not be avoided by varying the



Scheme 1. Reagents and conditions: (a) H_2, 10% Pd–C, EtOAc, rt, 98%; (b) 1—$NaNO_2$, concd HCl, H_2O, 0 °C; 2—CuCl, HCl, 0 °C to rt, 23%; (c) 1—$NaNO_2$, concd HCl, H_2O/AcOH, 0 °C; 2—CuCN, KCN, $CHCl_3/H_2O$, 0–50 °C, 12%.



Scheme 2. Reagents and conditions: (a) $KMnO_4$, H_2O, reflux, 66%; (b) 1—fuming H_2SO_4, 170 °C; 2—HCl, MeOH, reflux, 79% after two steps; (c) $POCl_3$, reflux, 70%; (d) aniline, pyridine, acetone, 0 °C–rt, 66%; (e) dibutylamine, K_2CO_3, Cu, dioxane, reflux, 79%; (f) 3 N NaOH, MeOH, reflux, 88%; (g) 1—CCl_3COCCl_3, PPh_3, THF, 0 °C; 2—NH_3/dioxane, rt, 68% after two steps; (h) $PdCl_2$, CH_3CN/H_2O, 70 °C, 71%; (i) 1—CCl_3COCCl_3, PPh_3, THF, 0 °C; 2—MeMgBr/THF, CuBr, THF, 0 °C, 55% after two steps.

reaction temperature, time, and the amount of Grignard reagent. We hypothesize that further reaction of the bis-ketone with methyl magnesium bromide was responsible for the occurrence of the side products. Therefore, it was reasoned that a soft nucleophile such as an organocuprate might lead to more efficient production of the diketone. In accord with this expectation, reaction of the acid chloride with a mixture of MeMgBr and CuBr in THF at 0 °C afforded the target bis-ketone **14** in moderate yield (Scheme 2).

2.2. Biological results

Target compounds **3**–**5** and **11**–**14** were judged against **2b** for their ability to block the assembly of purified tubulin isolated from *Leishmania tarentolae* (see Table 1). Compound **2b** displayed an IC_{50} value of 7.4 μM, which compares favorably with the previously reported IC_{50} values for this compound (6.9 μM[17] and 5.8 μM[26]) in the same assay. Of the new compounds, the two most potent inhibitors of leishmanial tubulin assembly were **5** and **4**, whose IC_{50} values of 6.6 and 9.1 μM are comparable to and slightly higher than that of **2b**. Compounds **3** and **11**–**14** displayed IC_{50} values >100 μM according to the previously defined conditions of the assembly assay, which measures the change in absorbance due to tubulin polymerization at 351 nm after 20-min incubation at 30 °C for samples containing the compound of interest compared to controls.[17,18] However, both **3** and **14** caused a pronounced lag in the assembly of leishmanial tubulin at concentrations $\geqslant$20 μM and thus show a significant effect on the kinetics of polymerization. The effects of **3** and **14** on the kinetics of leishmanial tubulin assembly are dose-dependent, with higher concentrations producing a greater lag in assembly. Similar effects of other antimitotic compounds have been observed previously in our laboratory[16] and by others.[27,28] Leishmanial tubulin assembly was relatively insensitive to **11**–**13** at concentrations up to 100 μM. The effect of **2b**, **5**, **13**, and **14** on parasite tubulin assembly is illustrated in Figure 1. Compounds **2b**, **3**–**5**, and **14** were also examined for their effects on the assembly of purified porcine brain tubulin (Table 1), with **2b**, **4**, **5**, and **14** displaying little influence on the kinetics or extent of polymerization at the highest concentrations tested. Approximately 50% inhibition of porcine tubulin

Table 1. Biological activity of target compounds[a] (μM)

Compound	IC_{50} versus *L. tarentolae* tubulin assembly	IC_{50} versus porcine brain tubulin assembly	IC_{50} versus *L. donovani* axenic amastigotes[a]	IC_{50} versus *T. b. brucei* bloodstream forms	IC_{50} versus Vero cells
2b	7.4 ± 0.2	>40[e]	2.3 ± 0.5	0.18 ± 0.01	9.7 ± 1.1
3	>100[b]	>50	12 ± 2	4.0 ± 0.1	27 ± 4
4	9.1 ± 4.5	>50[e]	11 ± 3	1.6 ± 0.1	19 ± 1
5	6.6 ± 0.7	>50[e]	4.4 ± 0.9	0.26 ± 0.03	16 ± 1
11	>100	NT	>50	>50	NT
12	>100	NT	>50	>50	NT
13	>100	NT	>50	>50	NT
14	>100[b]	>100	30 ± 7	1.6 ± 0.3	26 ± 1
Pentamidine	NT[c]	NT	2.3 ± 0.9	NT	NT
Suramin	NT	NT	NT	0.077 ± 0.001	NT
Podophyllotoxin[d]	NT	NT	NT	NT	0.016 ± 0.006

[a] Activities represent the mean ± standard deviation of at least three independent experiments.
[b] Produces a lag in tubulin assembly; see text and Figure 1.
[c] NT, not tested.
[d] Podophyllotoxin is used here solely as a standard for the cytotoxicity assay. Colchicine-site antimicrotubule agents such as podophyllotoxin have little effect on *Leishmania* tubulin assembly.[37]
[e] Compounds not soluble at higher concentrations.

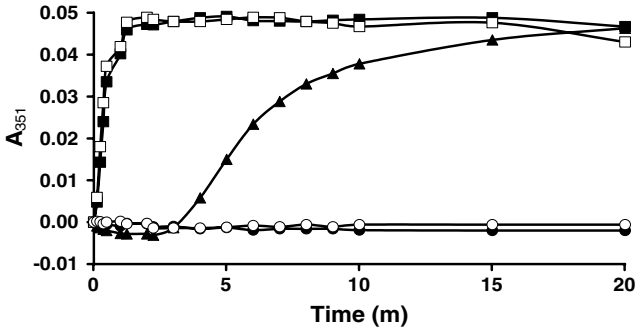


Figure 1. The assembly of 1.5 mg/mL purified tubulin from *Leishmania tarentolae* was assessed as described previously[26] in the absence (open squares) or presence of **5** (closed circles), **13** (closed squares), or **14** (triangles) at 20 μM or **2b** (open circles) at 10 μM. Data shown are from a representative experiment performed on three separate occasions.

assembly was observed in assays containing **3** at a concentration of 100 μM.

Compounds **2b**, **3**–**5**, and **11**–**14** were docked into the proposed dinitroaniline binding pocket on leishmanial α-tubulin[19] using AutoDock[29] and Glide.[30] These molecules bind similarly as shown in Figure 2 and in a manner consistent with the model proposed by Mitra and Sept.[19] Both docking methods show that **4** has the best binding clustering, **2b** and **5** follow, and the remaining compounds display worse binding clustering (see Table 2). While there is some variability between the values determined by AutoDock and Glide, the calculated binding energies of **2b**, **3**–**5**, **11**, **13**, and **14** are comparable. Interestingly, compound **12**, where isosteric replacement of the nitro moieties with carboxylate groups has occurred, shows intermediate binding clustering with

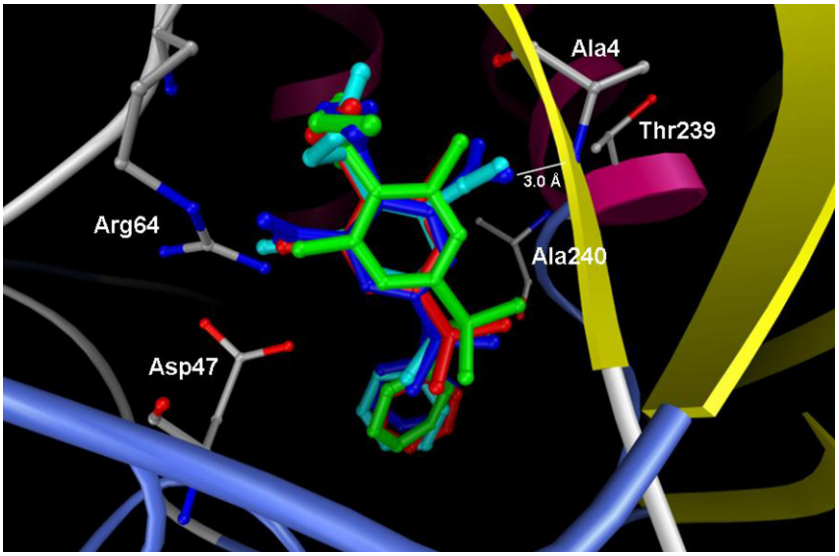


Figure 2. The docked positions of compounds **2b** and **3**–**5** are shown as stick models (**2b**, blue; **3**, green; **4**, red; **5**, cyan), while the tubulin protein is shown as ribbons. Stick models are also shown for Ala4 (which takes part in hydrogen bonding to a nitro group oxygen of compound **2b**), Thr239/Ala240 (which participate in electrostatic interactions with the other oxygen atom of this nitro moiety), and Arg64/Asp47 (which form a salt bridge and interact with the other nitro group of **2b** through the solvent network). This figure was created with the aid of AutoDock.

Table 2. In silico binding energies and binding clustering of target compounds

Compound	Percent binding clustering (AutoDock)	Binding energy (AutoDock, in kcal/mol)	Percent binding clustering (Glide)	Binding energy (Glide, in kcal/mol)
2b	93	−11.0	44	−7.9
3	4	−9.8	3	−8.9
4	96	−10.2	76	−9.9
5	92	−10.7	44	−8.5
11	2	−11.2	2	−7.6
12	33	−7.0	2	−3.2
13	2	−10.3	2	−8.7
14	2	−10.6	2	−7.5

AutoDock but displays poor binding clustering with Glide and shows binding energies that are inferior to all of the other target compounds.

The activities of the target compounds against *Leishmania donovani* axenic amastigotes, *Trypanosoma brucei brucei* bloodstream forms, and African Green Monkey kidney (Vero) cells are shown in Table 1. Consistent with previous reports, **2b** possesses potent in vitro activity against these organisms and displays excellent selectivity against African trypanosomes compared to the mammalian cell line.[17,18] Compound **5** was only 1.4-fold less active than **2b** against *T. b. brucei* and twofold less active than this compound against *L. donovani* while showing 1.6-fold lower toxicity against Vero cells. Compounds **4** and **14** were sixfold less potent than **5** against *T. b. brucei*, while **3** displayed 15-fold lower activity than **5** against this parasite. In terms of antileishmanial activity, **3** and **4** were nearly threefold less active than **5** against *L. donovani*, while **14** was sevenfold less potent in this assay. Compounds **11**–**13** failed to display measurable activity against either *Leishmania* or African trypanosomes at the highest concentrations tested.

3. Discussion

Nitro groups have thus far proven essential for the antiparasitic antimitotic activity of dinitroaniline sulfonamide analogues. Mitra and Sept's binding site model for dinitroaniline sulfonamide **2a** on leishmanial α-tubulin permitted formulation of the first hypothesis regarding the role of these nitro moieties in tubulin binding.[19] According to this model, one of the nitro groups makes several direct interactions with the protein. Electrostatic interactions are observed between one of the oxygen atoms in this moiety and a partially positively charged structural motif consisting of Thr239 and Ala240, while the other oxygen atom of this group accepts a hydrogen bond from the amide NH of Ala4 (see Fig. 2). The second nitro group does not interact directly with α-tubulin; instead, a solvent network connects this moiety to the salt bridge between Asp47 and Arg64. Through docking experiments, Mitra and Sept also showed that an analogue of **2a** bearing cyano groups in place of the nitro moieties displays in silico binding affinity to leishmanial tubulin similar to **2a**, while an analogue possessing acetyl groups in these positions binds more strongly than **2a** to the parasite protein. In this work, we have prepared a series of analogues of **2b**, which is slightly superior to **2a** in in vitro antiparasitic activity and differs in structure from **2a** only in the presence of an additional methylene group in the N^4 dialkyl chains.[17] Replacement of the nitro moieties of **2b** with carboxylate groups (**12**) represents an isosteric substitution. Substitutions of the nitro groups with methyl ester (**11**), amide (**13**), and acetyl (**14**) groups were envisioned as non-classical bioisosteric replacements.[31] Nitrile, methyl ester, amide, and acetyl functional groups present in compounds **5**, **11**, **13**, and **14**, respectively, have similar lipophilic and electronic properties as compared to the nitro group.[32] Evaluation of **3**–**5** and **11**–**14** for their effects on purified leishmanial tubulin assembly now permits refinement of the hypotheses advanced by Mitra and Sept and provides greater insight into the role of these groups in tubulin-ligand interactions.

In accord with previous expectations,[19] dicyano compound **5** displays activity in the leishmanial tubulin assembly assay comparable to that of dinitro lead **2b**. Dichloro compound **4** shows potency similar to **5** in this assay, while **3** and **14** display weaker activity and **11**–**13** are essentially inactive. Refinements to the binding site model[19] through the present docking studies provide a rationale for these observations. Compounds **2b**, **4**, and **5**, the most effective inhibitors of leishmanial tubulin assembly described in this work, also exhibited the best AutoDock and Glide scores of the group. At the positions where nitro groups occur in **2b**, each of these compounds possesses functional groups that can serve as a hydrogen bond acceptor. Figure 2 clearly shows the proposed hydrogen bond formed between a nitro group oxygen atom of **2b** and the amide group of Ala4. Similarly, a Cl atom of **4** and particularly a cyano group of **5** can also serve as hydrogen bond acceptors as pictured in Figure 2. While a hydrogen bond acceptor is required where one of the nitro moieties is located in **2b**, close inspection of the binding site model suggests that activity should be maintained when a different functional group is placed at the other position. Although the second nitro group of **2b** is proposed to interact with the solvent network as mentioned earlier, placement of a bulky hydrophobic group at this subpocket consisting of Trp21, Phe24, Met36, Ile42, and His61 may also permit favorable interactions at the binding site. Individual molecular dynamics simulations will be required to determine whether the salt bridge between Asp47 and Arg64 interacts with the newer analogues through the solvent network.

Inactive compounds **11**–**13** show poor scores in molecular docking studies. Steric considerations are likely to be

responsible in part for the lack of activity of diester **11**, while amide bond resonance limits the availability of the lone pair electrons present on the amino group of **13**. Although the substitution of nitro moieties with carboxylate groups in **12** is an isosteric replacement, the nitro moieties lack a net charge while the carboxylate groups are expected to possess a negative charge at physiological pH. The difference in net charge between **2b** and **12** is likely to be responsible for the lack of activity of the latter molecule. Compounds **3** and **14** also display poor AutoDock and Glide scores but have measurable effects on leishmanial tubulin assembly (albeit weaker than **2b**, **4**, and **5**). As an arylamine, **3** is potentially carcinogenic,[33] but this compound was prepared as a necessary intermediate in the synthesis of **4** and was tested because of the contribution that could be made to the structure–activity relationship. The van der Waals radius of the nitrogen atom (1.55 Å) is 0.2 Å smaller than that of chlorine,[34] suggesting that the lone pair electrons of the amino group of **3** may not be positioned ideally to serve as a hydrogen bond acceptor. In addition, the amino group is most often a hydrogen bond acceptor rather than a donor. One of the oxygen atoms of the nitro group in **2b** is proposed to provide electrostatic interactions with Thr239 and Ala240 at the binding site, which would not be possible for the methyl group of diketone **14**.[19]

Data shown in Table 1 and Figure 1 provide support for the hypothesis that the disruption of microtubule assembly plays a critical role in the antikinetoplastid action of the target molecules. The rank order of these compounds for activity against leishmanial tubulin assembly (**2b**, **5**, **4** > **3**, **14** > **11**–**13**) is very similar to the rank order for activity against *L. donovani* axenic amastigotes in vitro (**2b** > **5** > **3**, **4** > **14** > **11**–**13**) and *T. b. brucei* in vitro (**2b**, **5** > **4**, **14** > **3** > **11**–**13**). Minor variation between antiparasitic potency and activity against leishmanial tubulin could be due to the ability of the compounds to reach their cellular target or, in the case of antitrypanosomal activity, to subtle differences between the ligand susceptibility of leishmanial and trypanosomal tubulin (the amino acid identity between *L. tarentolae* and *T. brucei* tubulin is 94%).[26] Given the potent antitrypanosomal activity and selectivity of compounds **2b** and **5**, further investigation of the effects of these molecules on purified trypanosomal tubulin and the trypanosomal cell cycle is warranted.

In addition to the presence of nitro groups, another barrier to the development of **2a** and **2b** as antikinetoplastid drug candidates is metabolic instability. Neither compound showed significant activity against murine African trypanosomiasis when administered intraperitoneally at 4×20 mg kg^{-1} day^{-1}.[17] Rapid and extensive metabolism of **2b** was later observed, primarily through N^4-alkane oxidation, providing a rationale for the lack of activity of these dinitroaniline sulfanilamides.[35] While the metabolic stability of **5** remains to be investigated, this molecule retains the N^4-dibutyl chain that was the site of extensive metabolism in **2b**. However, a recent SAR investigation revealed that a range of hydrophobic substituents was permitted at the N^4 position in terms of antiparasitic and antitubulin activity,[18] and the tubulin binding site model for **2a** and its analogues[19] indicates that sufficient space is available for the design of molecules containing substituents at N^4 that may be less susceptible to metabolism. The ability to substitute the cyano groups for the potentially mutagenic nitro moiety while retaining activity provides further flexibility in the design of antimitotic antiparasitic molecules. Given the current knowledge of the SAR for this group of molecules and the availability of a binding site model for the generation of new target compounds, the design of new structural classes of antimitotic antikinetoplastid agents with increased metabolic stability may now be possible.

4. Conclusion

Replacement of the nitro groups present in antimitotic antikinetoplastid molecule **2b** with cyano moieties provides a compound (**5**) which displays potent and selective activity against African trypanosomes and *Leishmania*. Activity is also observed when other groups are placed at these positions, although such molecules are less potent than the corresponding nitro- and cyano-containing compounds. This knowledge, together with refinement of the tubulin binding site model for such ligands, represents a point of departure from the dinitroaniline scaffold in the design of selective antitubulin antiparasitics and provides renewed hope for the development of tubulin-targeted drugs against African trypanosomes, *Leishmania*, and perhaps other parasitic protozoans as well.

5. Experimental

5.1. General methods

All reagents and solvents were from the Sigma–Aldrich Corporation (St. Louis, MO) unless otherwise indicated. Nuclear magnetic resonance spectra were obtained at 250 or 300 MHz for ^{1}H and 62.5 or 75 MHz for ^{13}C using instruments from Bruker. Thin layer chromatoraphy was conducted on precoated TLC plates from E. Merck (Darmstadt, Germany). HPLC analysis was carried out using a System Gold Model 127 pump equipped with a Model 166 UV detector (Beckman, Fullerton, CA) and a 10×250 mm Polaris C18-A column (Varian, Palo Alto, CA). Melting points were recorded on a Thomas–Hoover capillary melting point apparatus and are uncorrected.

5.2. N^1-Phenyl-3,5-diamino-N^4,N^4-di-*n*-butylsulfanilamide (3)

To a degassed solution of **2b** (1 g, 2.22 mmol) in EtOAc (44 mL), 10 wt% Pd on activated carbon (133 mg) was added and stirred at rt under an atmosphere of H_2 for 1 day. The reaction mixture was then filtered through Celite and evaporated to afford **3** (850 mg, 98%) as a colorless solid, mp = 85–86 °C. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 7.1 Hz, 6H), 1.19–1.44 (m, 8H), 2.95 (t, J = 7.7 Hz, 4H), 4.01 (bs, 4H), 6.51 (s, 2H), 6.61 (bs, 1H), 7.06–

7.31 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 14.01, 20.48, 31.81, 52.68, 104.24, 121.61, 125.09, 125.15, 129.15, 136.85, 136.88, 146.75; HRMS (ESI) calcd for $C_{20}H_{30}N_4NaO_2S$ $(M+Na)^+$ 413.1987, measured $(M+Na)^+$ 413.1994. Anal. ($C_{20}H_{30}N_4O_2S$) calcd C, 61.51; H, 7.74, N, 14.35; found C, 61.53; H, 7.77; N, 14.15.

5.3. N^1-Phenyl-3,5-dichloro-N^4,N^4-di-*n*-butylsulfanilamide (4)

To a suspension of diamine **3** (200 mg, 0.51 mmol) in a mixture of concd HCl (1.5 mL) and H_2O (0.5 mL), an ice-cold solution of $NaNO_2$ in H_2O (1 mL) was added very slowly over 15 min at 0 °C. To this an ice-cold solution of CuCl (126 mg, 1.26 mmol) in HCl (1 mL) was added dropwise with stirring. The reaction mixture was stirred for 3 h while warming to rt, then was neutralized with 6 N NH_4OH. This mixture was extracted with dichloromethane and washed successively with water, brine and dried. The crude product was then purified by column chromatography using hexanes/EtOAc (10:1) to afford **4** (51 mg, 23%) as a colorless solid, mp = 90–91 °C. ^{1}H NMR ($CDCl_3$) δ 0.86 (t, J = 7.2 Hz, 6H), 1.16–1.42 (m, 8H), 3.17 (t, J = 7.2 Hz, 4H), 6.62 (s, 1H), 7.09–7.34 (m, 5H), 7.65 (d, J = 0.9 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 13.93, 20.16, 30.79, 52.22, 122.00, 125.99, 127.94, 129.52, 134.82, 135.74, 135.83, 149.69. HRMS (ESI) calcd for $C_{20}H_{26}Cl_2N_2O_2S$ $(M+Na)^+$ 451.0990, measured $(M+Na)^+$ 451.0970. Anal. ($C_{20}H_{26}Cl_2N_2O_2S$) calcd C, 55.94; H, 6.10, N, 6.52; found C, 56.11; H, 6.17; N, 6.48.

5.4. 2-Bromoisophthalic acid (7)[21]

To a suspension of 2-bromo-*m*-xylene **6** (13.9 g, 75 mmol) in water (260 mL), $KMnO_4$ (18 g, 113 mmol) was added and refluxed overnight. Another portion of $KMnO_4$ (18 g, 113 mmol) was added and again refluxed for 24 h. A third portion of $KMnO_4$ (18 g, 113 mmol) was added and refluxed for an additional day. The reaction mixture was filtered while hot through Celite and washed with hot water, then the combined aqueous phase was evaporated to about 75 mL and neutralized with 20% HCl. The reaction mixture was then kept at 0 °C overnight and the crystals formed were filtered and dried to afford **7** (12.1 g, 66%) as a white solid, mp = 209–210 °C (literature 218 °C[36]). ^{1}H NMR (DMSO-d_6) δ 7.50–7.71 (m, 3H). 13.61 (s, 2H).

5.5. 4-Bromo-3,5-bis(methoxycarbonyl)benzenesulfonic acid (8)

2-Bromoisophthalic acid **7** (5.0 g, 20.4 mmol) was added to fuming sulfuric acid (26–29%, 12 mL) at rt and heated to 170 °C for 4 h. The reaction mixture was cooled to rt, added to ice, and neutralized with sodium bicarbonate. The crystals formed were filtered, co-evaporated with toluene, and dried. This crude material was then suspended in MeOH (150 mL) and concd HCl (6 mL) was added and refluxed for 24 h. Solvent was removed under reduced pressure, then the residue was co-evaporated with toluene (3×) and dried under vacuum to afford **8** (5.7 g, 79% after two steps), mp = 139–140 °C. ^{1}H NMR (DMSO-d_6) δ 3.88 (s, 6H), 7.94 (s, 2H); HRMS (ESI) calcd for $C_{10}H_9BrO_7S$ $(M)^+$ 351.9252, measured $(M)^+$ 351.9255.

5.6. Dimethyl 2-bromo-5-(chlorosulfonyl)isophthalate (9)

A suspension of **8** (2.08 g, 5.89 mmol) in $POCl_3$ (6.3 mL, from Acros Organics, Morris Plains, NJ) was refluxed for 24 h. This mixture was then cooled to rt, added to ice water, and stirred for 20 min. The crystals formed were filtered, washed with ice water, and dried to afford **9** (1.52 g, 70%) as colorless solid, mp = 98–99 °C.^{1}H NMR ($CDCl_3$) δ 4.03 (s, 6H), 8.35 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 53.51, 127.68, 129.93, 137.06, 142.98, 164.64.

5.7. Dimethyl 2-bromo-5-(phenylsulfamoyl)isophthalate (10)

To a solution of **9** (3.0 g, 8.07 mmol) in acetone (60 mL), pyridine (616 mg, 7.79 mmol, from Mallinckrodt Chemicals, Phillipsburg, NJ) was added at rt, and the solution was cooled to 0 °C. Aniline (725 mg, 7.79 mmol) was then added and the solution was stirred at 0 °C and allowed to warm to rt overnight. Solvent was evaporated and the remaining mixture was extracted using EtOAc. The organic layer was washed with sat. NH_4Cl, dried over anhydrous Na_2SO_4, and evaporated. Purification by silica gel flash column chromatography using hexane/ethyl acetate 5:1 to 4:1 afforded **10** (2.29 g, 69%), mp = 106–107 °C. ^{1}H NMR ($CDCl_3$) δ 3.95 (s, 6H), 6.68 (s, 1H), 7.07–7.34 (m, 5H), 8.04 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 53.22, 122.58, 124.76, 126.45, 129.67, 130.49, 135.32, 136.16, 138.58, 165.35.

5.8. Dimethyl 2-(di-*n*-butylamino)-5-(phenylsulfamoyl)isophthalate (11)

To a solution of **10** (2.26 g, 5.27 mmol) in dioxane (40 mL), K_2CO_3 (870 mg, 6.3 mmol) was added followed by Cu (50 mg, 0.79 mmol) and dibutylamine (2.07 g, 16.0 mmol). The reaction mixture was refluxed overnight. Solvent was evaporated, then the residue was extracted with EtOAc, and washed with sat. NH_4Cl followed by brine and dried using anhydrous $MgSO_4$. Purification by silica gel flash column chromatography (hexane/ethyl acetate 10:1) afforded **11** (1.99 g, 79%) as a yellow viscous liquid. ^{1}H NMR ($CDCl_3$) δ 0.86 (t, J = 7.3 Hz, 6H), 1.19–1.31 (m, 4H), 1.47–1.57 (m, 4H), 3.04 (t, J = 7.4 Hz, 4H), 3.87 (s, 6H), 6.76 (s, 1H), 7.08–7.30 (m, 5H), 8.01 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.81, 19.95, 29.63, 52.58, 52.86, 121.82, 125.54, 126.23, 128.36, 129.41, 133.25, 136.25, 153.36, 167.25; HRMS (ESI) calcd for $C_{24}H_{33}N_2O_6S$ $(M+H)^+$ 477.2059, measured $(M+H)^+$ 477.2061. Anal. ($C_{24}H_{32}N_2O_6S$) calcd C, 60.48; H, 6.77, N, 5.88; found C, 60.65; H, 7.02; N, 5.50.

5.9. 2-(Di-*n*-butylamino)-5-(phenylsulfamoyl)isophthalic acid (12)

To a solution of **11** (518 mg, 1.09 mmol) in MeOH (30 mL), 3 N NaOH (5 mL) was added at rt and the reaction mixture was refluxed for 2 days. Solvent was evaporated; the residue was dissolved in water (5 mL) and neutralized with 2 N HCl. The crystals formed were filtered and dried to afford **12** (430 mg, 88%) as a colorless solid,

mp = 237–238 °C. ^{1}H NMR (DMSO-d_6) δ 0.77 (t, J = 7.2 Hz, 6H), 1.12–1.24 (m, 4H), 1.33–1.42 (m, 4H), 3.10–3.15 (m, 4H), 7.03–7.27 (m, 5H), 7.99 (s, 2H), 10.28 (s, 1H), 14.19 (bs, 2H); ^{13}C NMR (DMSO-d_6) δ 14.07, 19.88, 29.50, 53.28, 120.93, 124.95, 129.35, 129.69, 131.65, 132.36, 137.81, 150.72, 168.05; HRMS (ESI) calcd for $C_{22}H_{28}N_2NaO_6S$ $(M+Na)^+$ 471.1566, measured $(M+Na)^+$ 471.1560. Anal. ($C_{22}H_{28}N_2O_6S$) calcd C, 58.91; H, 6.29, N, 6.25; found C, 58.31; H, 6.09; N, 5.99.

5.10. 2-(Di-*n*-butylamino)-5-(phenylsulfamoyl)isophthalamide (13)

To a suspension of the diacid **12** (50 mg, 0.11 mmol) and triphenylphosphine (58 mg, 0.22 mmol, from Acros Organics, Morris Plains, NJ) in THF (3 mL), hexachloroacetone (30 μL, 0.20 mmol) was added at 0 °C and stirred for 1 h. Then, 0.5 M NH_3 in dioxane (1.32 mL, 0.67 mmol) was added, the reaction mixture was allowed to warm to rt and was stirred for 1 h. Solvent was evaporated and water was added. The product was then extracted with EtOAc, washed with brine, and dried over anhydrous Na_2SO_4. Evaporation of the solvent followed by column chromatography using hexane/EtOAc 3:1 to 2:1 afforded **13** (33.7 mg, 68%) as a colorless solid, mp = 161–162 °C. HPLC (MeOH/H_2O 65:35, 1.0 mL/min, 254 nm) >98%, t_R = 4.09 min. ^{1}H NMR (acetone-d_6) δ 0.84 (t, J = 7.4 Hz, 6H), 1.19–1.32 (m, 4H), 1.45–1.56 (m, 4H), 3.17–3.22 (m, 4H), 6.89 (bs, 2H), 7.07–7.10 (m, 1H), 7.21–7.29 (m, 4H), 7.89 (bs, 2H), 8.01 (s, 2H), 9.02 (s, 1H); ^{13}C NMR (acetone-d_6) 14.27, 21.18, 31.05, 54.47, 121.71, 125.50, 130.15, 130.87, 134.44, 135.32, 138.75, 152.20, 169.61. HRMS (ESI) calcd for $C_{22}H_{30}N_4O_4S$ $(M+H)^+$ 447.2066, measured $(M+H)^+$ 447.2046.

5.11. N^1-Phenyl-3,5-diacetyl-N^4,N^4-di-*n*-butylsulfanilamide (14)

To a solution of diacid **12** (50 mg, 0.11 mmol) and triphenylphosphine (58 mg, 0.22 mmol) in dry THF (3 mL), hexachloroacetone (30 μL, 0.20 mmol) was added at 0 °C. The clear yellow reaction mixture was stirred at 0 °C for 1.5 h to give the corresponding diacyl chloride, which was used as such for next reaction. To a suspension of CuBr (157 mg, 1.11 mmol) in THF (2 mL), methyl magnesium bromide (370 μL, 3.0 M in THF, 1.11 mmol) was added at 0 °C and stirred at room temperature for 1 h, which gave a light yellow green suspension. The reaction mixture was cooled once again to 0 °C, and the solution of the diacyl chloride derivative of **12** in THF (3 mL) was transferred by cannula. The resulting reaction mixture was stirred at 0 °C for 40 min. Water (6 mL) was added, phases were separated, and the aqueous phase was extracted with EtOAc (3× 5 mL). The combined organic layers were washed with aq NH_4Cl (10 mL, 10% w/v), dried over anhydrous Na_2SO_4, and concentrated. Silica gel flash column chromatography of the crude material [hexane/EtOAc (8:1 to 4:1)] gave compound **14** (26.7 mg, 55%) as light yellow solid, mp = 99–100 °C. HPLC (MeOH/H_2O 80:20, 1.0 mL/min, 254 nm), >98%, t_R = 5.24 min. ^{1}H NMR ($CDCl_3$) δ 0.85 (t, J = 7.2 Hz, 6H), 1.14–1.26 (m, 4H), 1.41–1.53 (m, 4H), 2.46 (s, 6H), 2.94 (t, J = 7.8 Hz, 4H), 6.50 (s, 1H), 7.07–7.28 (m, 5H), 7.72 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.76, 20.11, 29.03, 29.58, 54.22, 122.17, 125.71, 129.36, 129.54, 130.30, 135.83, 136.34, 151.13, 200.95; HRMS (ESI) calcd for $C_{24}H_{32}N_2NaO_4S$ $(M+Na)^+$ 467.1980, measured $(M+Na)^+$ 467.1984.

5.12. N^1-Phenyl-3,5-dicyano-N^4,N^4-di-*n*-butylsulfanilamide (5)

The title compound was synthesized with slight modification of the literature procedure.[24] To a solution of bisamide **13** (65 mg, 0.146 mmol) in a mixture of acetonitrile and water (2:1 ratio, 15 mL), $PdCl_2$ (12.5 mg, 0.073 mmol) was added. The resulting yellow solution was stirred at 70 °C for 24 h. Acetonitrile was removed in vacuo and the residue was extracted with CH_2Cl_2 (3× 10 mL) and dried over anhydrous Na_2SO_4. Silica gel flash column chromatography [hexane/EtOAc (10:1)] of the crude material gave compound **5** (42.5 mg, 71%) as white solid, mp = 145–146 °C. ^{1}H NMR ($CDCl_3$)δ 0.91 (t, J = 7.2 Hz, 6H), 1.23–1.39 (m, 4H), 1.55–1.65 (m, 4H), 3.62 (m, 4H), 6.64 (s, 1H), 7.09–7.37 (m, 5H), 7.99 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 13.64, 19.69, 30.09, 52.77, 107.56, 116.15, 122.15, 126.51, 129.76, 130.80, 135.33, 138.11, 158.89; HRMS (ESI) calcd for $C_{22}H_{26}N_4NaO_2S$ $(M+Na)^+$ 433.1674, measured $(M+Na)^+$ 433.1666. Anal. ($C_{22}H_{26}N_4O_2S$) calcd C, 64.36; H, 6.38, N, 13.65; found C, 64.00; H, 6.40; N, 13.13.

5.13. Susceptibility testing of parasites and Vero cells

The susceptibility of *L. donovani* axenic amastigote-like parasites,[16,37] *T. b. brucei* bloodstream forms,[38] and Vero cells[38] to growth inhibition by compounds of interest was assayed as described previously.

5.14. Tubulin assembly assays

Tubulin from *L. tarentolae*[26] and from pig brain[17] was isolated as outlined earlier. Assembly reactions, used to assess the effect of compounds on tubulin polymerization, were carried out as described previously.[17,26]

5.15. AutoDock

AutoDock version 4.0.0[29] was used for docking simulations. The Lamarckian genetic algorithm (LGA) was selected for ligand conformational searching because it has enhanced performance relative to simulated annealing or the simple genetic algorithm. For the ligands, all hydrogens were added, Gasteiger charges[39] were assigned, then non-polar hydrogens were merged. $80 \times 80 \times 70$ 3-D affinity grids centered on the **2b** binding site with 0.375 Å spacing were calculated for each of the following atom types: (a) protein: A (aromatic C), C, HD, N, NA, OA, SA; (b) ligand: C, A, OA, HD, S, N, NA, e (electrostatic), and d (desolvation) using Autogrid4. The ligand's translation, rotation, and internal torsions are defined as its state variables and each gene represents a state variable. LGA adds local minimization to the genetic algorithm, enabling modification of the gene population. The docking parameters were as follows: trials of 100 dockings, population size of 150,

random starting position and conformation, translation step ranges of 2.0 Å, rotation step ranges of 50°, elitism of 1, mutation rate of 0.02, crossover rate of 0.8, local search rate of 0.06, and 50 million energy evaluations. Final docked conformations were clustered using a tolerance of 1.5 Å root-mean-square deviations (RMSD).

5.16. Glide[30]

Ligand preparation: the molecules were built in Maestro Suite and were minimized using the OPLS-2005 forcefield. A maximum of 32 stereoisomers were allowed to generate per ligand. Possible ionization states were assigned at a target pH of 7.0 ± 2.0. Target preparation: the homology modeled structure of *Leishmania* tubulin[19] was prepared for docking using the protein preparation utility provided by Schrodinger LLC. This process optimizes the protonation states and tautomers of His residues as well as the position of hydroxyl and thiol hydrogens. The structure was then subjected to impact minimization with a cut off RMSD of 0.3 Å. Partial atomic charges were assigned according to the OPLS-2005 force field. Molecular Docking: the model was submitted to grid map calculations using a box of 12 Å cube centered on the bound ligand **2b**. The consequent docking has been conducted using the Glide program, with the default settings using the previously prepared ligands. The most stable Glide pose, one per ligand, was retained.

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Supplementary data

^{1}H and ^{13}C NMR spectra for **13** and **14**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.06.042.

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