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A comparative evaluation of monomethoxy substituted *o*-diarylazoles as antiproliferative microtubule destabilizing agents

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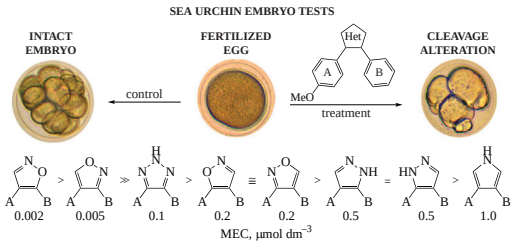
Monomethoxy substituted *o*-diarylazoles with isoxazole, triazole, pyrazole, or pyrrole linkers were synthesized and evaluated for antimitotic antitubulin activity in a sea urchin embryo model. Structure–activity relationship study revealed that only isoxazole heterocycle together with the unsubstituted phenyl ring next to the heteroatom endowed the molecule with appropriate configuration to exhibit antiproliferative effect by microtubule destabilizing mode of action.



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Cytostatic agents that affect structure or dynamics of microtubules attract considerable attention in the development of novel antitumor agents.[1–3] A characteristic feature of natural molecules, such as colchicine, combretastatin A-4 (CA4), steganacin, and podophyllotoxin is the presence of a trialkoxybenzene moiety essential for the interaction with the colchicine site of tubulin.[4–7] Structure–activity relationship (SAR) studies of *cis*-restricted CA4 analogues with pyrazole, isoxazole, 1,2,3-triazole, and pyrrole bridges and various substituents in benzene rings revealed that the most active molecules contained the 3,4,5-trimethoxyphenyl fragment.[8–12] However, replacing of one or more methoxy groups of the 3,4,5-trimethoxyphenyl motif in CA4 derivatives with other substituents also yielded highly cytotoxic molecules capable of inhibiting tubulin polymerization due to interaction with the colchicine binding site.[13] Furthermore, compounds with considerable antitubulin activity featuring unsubstituted and/or monomethoxy substituted benzene rings were also found in other classes of tubulin-targeting substances.[14–18] These molecules exhibited antimitotic microtubule destabilizing effect at minimal effective concentration (MEC) of 1–10 nM in a sea urchin embryo model (Figure 1).

Recently *o*-diarylisoxazoles containing the only one methoxy group were found to be potent tubulin-targeting antimitotics. Docking studies assumed that the specific relative configuration of isoxazole and *p*-methoxyphenyl fragments was responsible for the robust interaction with the colchicine binding site of tubulin.[19] Biological evaluation of the isoxazole derivatives also revealed their significant cytotoxicity *in vitro* against human cancer cells in NCI60 screen and high antimitotic microtubule destabilizing activity *in vivo* in a sea urchin embryo model compared to that of CA4.[19] Considering this, we decided to study antiproliferative antitubulin activity of *o*-diarylisoxazoles featuring benzene ring with different position of methoxy group or with another substituent as well as analogues linked by other N-heterocycles such as 1,2,3-triazole, pyrazole, and pyrrole.

General synthetic routes are presented in Schemes 1 and 2. Starting nitrostilbenes **1a**–**h** were obtained according to a

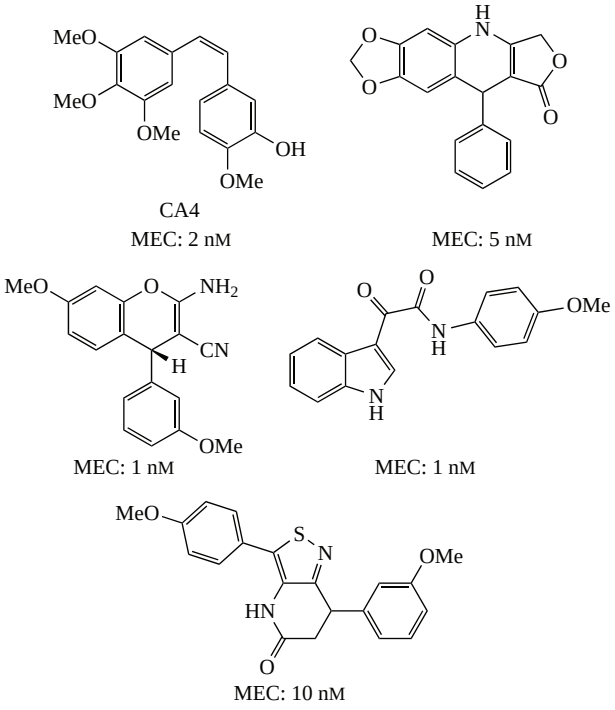
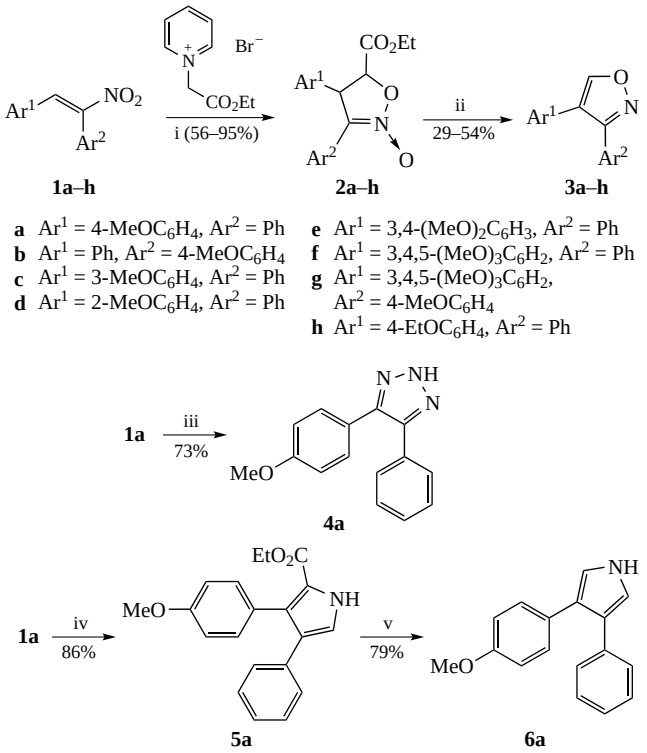
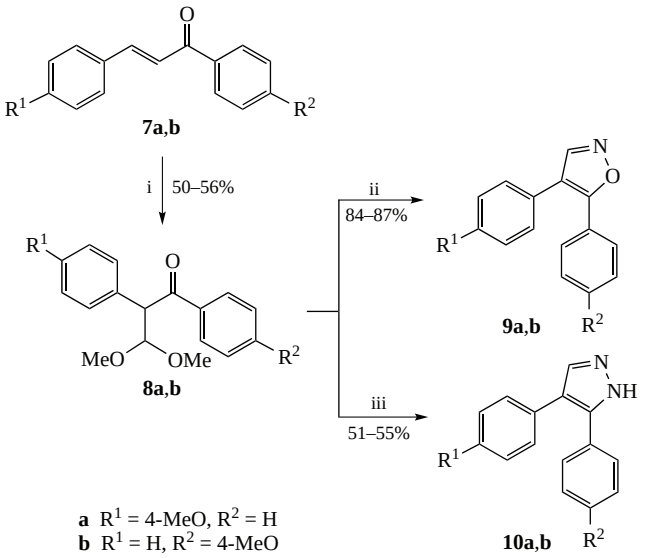


Figure 1 Structures of combretastatin A-4 (CA4) and microtubule destabilizing antimitotics with unsubstituted and/or monomethoxy substituted benzene rings. MEC: minimal effective concentration for antimitotic antitubulin effect in the phenotypic sea urchin embryo assay.



Scheme 1 *Reagents and conditions*: i, NEt_3, MeCN, room temperature, 1 h; ii, NaOH (2%), EtOH/H_2O, 60 °C, 6 h; iii, NaN_3, TsOH, DMF, 60 °C, 1 h; iv, $C^-{\equiv}N^+CH_2CO_2Et$, K_2CO_3, EtOH, room temperature, 12 h; v, NaOH (aq.), reflux.

previously published protocol.[19] The synthesis of methoxy substituted *o*-diarylisoxazolines **2a**–**h** from nitrostilbenes **1a**–**h** was carried out using an improved synthetic procedure described earlier (see Scheme 1).[12,20] The target *o*-diarylisoxazoles **3a**–**h** were obtained through one-pot recyclization–decarboxylation–cyclization sequence of **2a**–**h**.[20] Diaryl-1,2,3-triazole **4a** was synthesized by 1,3-dipolar cycloaddition of nitrostylbene **1a** with hydrazoic acid formed *in situ* according to published procedure.[21] Ethyl 3,4-diarylpyrrole-2-carboxylate **5a** was prepared using an improved Barton–Zard reaction.[22] Pyrrole **6a** was obtained by one-pot hydrolysis and decarboxylation under basic conditions.[12] Monomethoxy substituted 4,5-diaryl-isoxazoles **9** and *o*-diarylpyrazoles **10** were synthesized by



Scheme 2 *Reagents and conditions*: i, $PhI(OAc)_2$, H_2SO_4, MeOH, room temperature, 4 h; ii, $NH_2OH{\cdot}HCl$, EtOH, reflux, 2 h; iii, N_2H_4, conc. HCl, reflux, 2 h.

oxidative rearrangement of available chalcones **7** to afford dimethylacetals **8**[19] followed by cyclization with hydroxylamine or hydrazine, respectively (see Scheme 2).

Biological activity of the synthesized *o*-diarylazole series **3**, **4**, **6**, **9** and **10** was studied in the sea urchin embryo model.[12] The *in vivo* phenotypic sea urchin embryo assay allows for facile identification of antimitotic molecules with microtubule destabilizing mode of action, which can be verified by a specific pattern of embryo motility, spinning on the bottom of the vessel. For compounds with moderate antimitotic effect that failed to induce embryo spinning, the appearance of tuberculate arrested eggs indicate targeting tubulin as well. Effects of novel compounds **3c**–**e**,**h**, **4a**, **6a**, and **10a**,**b**, and previously reported isoxazole derivatives **3a**,**b**,**f**,**g**, and **9a**,**b**[19,20] were compared to assess the significance of the substituent position in benzene ring and the structure of N-heterocycle bridge for antimitotic antitubulin activity. The results are presented in Table 1.

o-Diarylazoles **3a**,**b**,**g**,**h**, and **9a** markedly inhibited cleavage and induced embryo spinning, providing evidence for their antimitotic microtubule destabilizing mode of action. Less active compounds **3c**,**e**, **4a**, **9a**, and **10a**,**b** also may be considered as antitubulin agents, because at 2 μM concentration they caused formation of tuberculate arrested eggs.

As reported previously, relocation of 4-methoxyphenyl fragment next to the isoxazole heteroatom significantly attenuated the activity: **3a** *versus* **3b** and **9a** *versus* **9b**.[19] Therefore, compounds with unsubstituted phenyl ring next to the N atom were selected for SAR study. A displacement of the methoxy group to 3-position (**3c**) markedly decreased the effect, whereas 2-methoxy substitution (**3d**) resulted in activity loss. The introduction of additional methoxy groups to 3- and 3,5-positions (compounds **3e** and **3f**, respectively) noticeably reduced the potency as well. In contrast, isoxazole **3g** with 3,4,5-trimethoxyphenyl and 4-methoxyphenyl rings retained antimitotic antitubulin activity, although a little less than that of monomethoxy substituted isoxazole **3a**. The replacement of 4-methoxy group with 4-ethoxy substituent caused a tenfold decrease in activity (**3a** *versus* **3h**).

Table 1 Effect of *o*-diarylazoles series **3**–**5**, **8**, and **9** on sea urchin embryos.[a]

Compound	MEC/μmol dm^{-3}		
	Cleavage alteration	Cleavage arrest	Embryo spinning
CA4	0.002	0.01	0.5
3a[b]	0.005	0.05	0.1
3b[b]	0.2	2	2
3c	1	2 TE[c]	>5
3d	>4	>4	>4
3e	0.1	2 TE[c]	>4
3f[d]	4	>4	>4
3g[d]	0.01	0.1	1
3h	0.05	0.5	0.5
4a	0.1	2 TE[c]	>4
6a	1	>4	>4
9a[b]	0.002	0.02	0.1
9b[b]	0.2	2 TE[c]	>5
10a	0.5	2 TE[c]	>4
10b	0.5	2 TE[c]	>5

[a]The sea urchin embryo assay was conducted as described previously (ref. 12). Fertilized eggs and hatched blastulae were exposed to 2-fold decreasing concentrations of compounds. Duplicate measurements showed no differences in MEC (minimal effective concentration) values. [b]Data from ref. 19. [c]TE: tuberculate eggs typical of microtubule destabilizing agents. [d]Data from ref. 20.

Next, an impact of different N-heterocycles on the antimitotic microtubule destabilizing activity of monomethoxy substituted *o*-diarylazoles was assessed. Of all tested compounds only isoxazoles **3a** and **9a** displayed antimitotic potency comparable with that of the parent CA4. Triazole **4a** and pyrazoles **10a**,**b** exhibited moderate effect. Nevertheless, they may be considered as weak microtubule destabilizing agents since the arrested eggs acquired the tuberculate shape. Pyrrole derivative **6a** was identified as the weakest compound with antiproliferative effect at 1 μM concentration. The antimitotic activity in the series of *ortho*-substituted (4-methoxyphenyl)(phenyl)azoles decreased in the following order: **9a** (4,5-diarylisoxazole) > **3a** (3,4-diarylisoxazole) ≫ **4a** (triazole) > **3b** (3,4-diarylisoxazole) ≈ **9b** (4,5-diarylisoxazole) > **10a** (pyrazole) = **10b** (pyrazole) > **6a** (pyrrole) (see Online Supplementary Materials, Figure S1). Thus, the most active antimitotic compounds featured the isoxazole bridge and the unsubstituted phenyl ring next to the heteroatom. The data obtained suggest that in monomethoxy substituted *o*-diarylazoles only the isoxazole heterocycle provides proper configuration for the successful interaction with the colchicine binding site of tubulin.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2022.01.039.

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