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A convenient synthesis of *cis*-restricted combretastatin analogues with pyrazole and isoxazole cores

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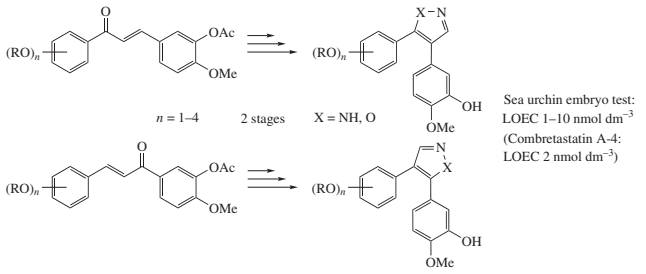
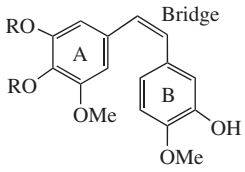
A series of combretastatin analogues, diarylpyrazoles and diarylisoxazoles, have been synthesized and evaluated for their antimitotic tubulin-binding activity using the phenotypic sea urchin (*Paracentrotus lividus*) embryo assay. One pyrazole analogue and four isoxazole analogues have been identified as potent antimitotic agents comparable with combretastatins A-2 and A-4, with the lowest observable effective concentration of 1–10 nmol dm^{-3} for cleavage alteration of the test embryos.

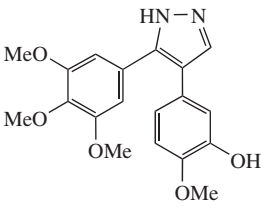


Microtubule-targeting antimitotic compounds affect cell division and cause apoptosis, thereby attracting a considerable attention as potential antitumor agents. Unfortunately, clinical application of these compounds is limited because of undesirable systemic toxicity and multiple drug resistance acquired by malignant cells. Therefore, there is an ongoing need to design molecules displaying both better therapeutic window and an ability to overcome multidrug resistance.

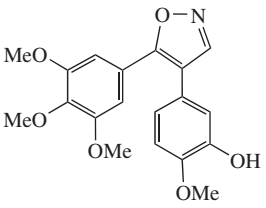
Conformationally restricted analogues of natural cytostatic stilbens, combretastatin A-2 (CA2) and combretastatin A-4 (CA4),



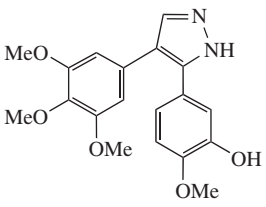
CA2: R + R = CH_2
CA4: R = Me



1a

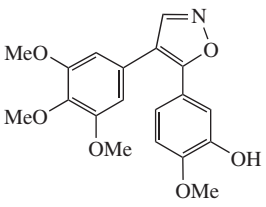


2a



1b

Combretapyrazoles



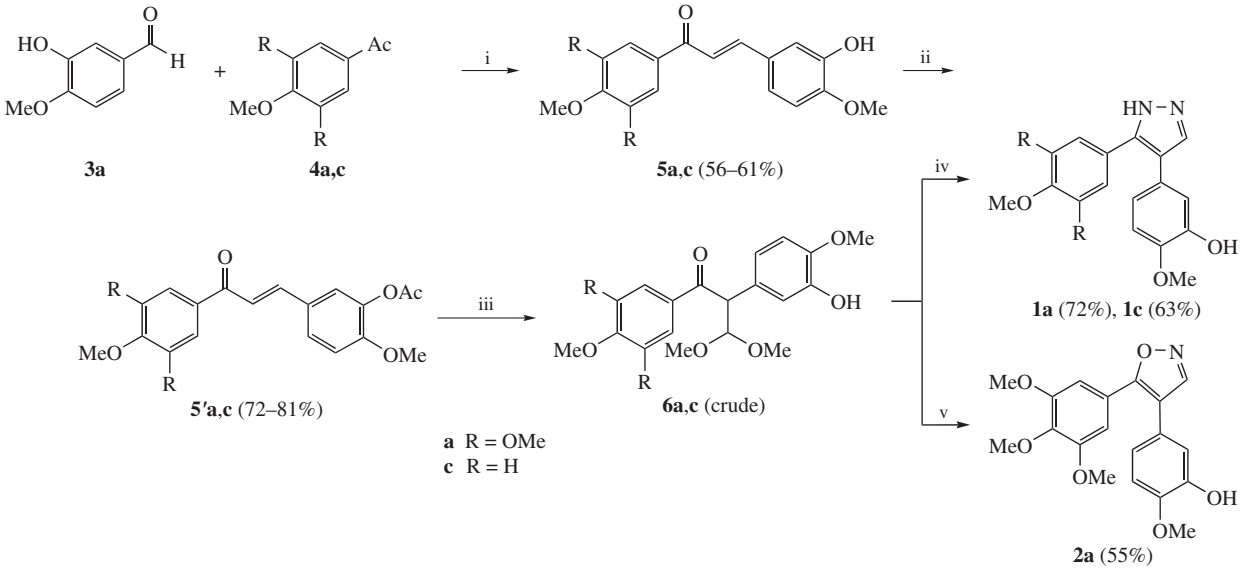
2b

Combretaisoxazoles

are considered to be of particular interest for anticancer drug design. The *cis*-configuration of the ethene linker in combretastatins is essential for the specific interaction with the colchicine binding site of tubulin.[1,2] Isosteric replacement of the labile *cis*-double bond in the parent combretastatins by five-membered N-containing heterocycle provides non-isomerizable and metabolically stable combretazole structures with pronounced antimitotic tubulin-binding activity both *in vitro* and *in vivo*.[3–14] Among known combretazoles 4,5(3,4)-diarylpyrazoles and 4,5-diarylisoxazoles are the most potent.[3]

The preparation of structural analogues of CA4, combretapyrazoles **1a** and **1b**[4] as well as combretaisoxazoles **2a**[5,6] and **2b**[5], has been published, but the reported synthetic routes are rather complicated. In addition, the known data on compound **2b** lack detailed experimental procedure, melting point value and spectral characteristics.[5] Furthermore, there exists a significant difference (700-fold) in published values of cytotoxicity for compounds **1a** and **1b** towards human umbilical vein endothelial cells, HUVEC. Diarylpyrazole **1b** inhibits the growth of HUVEC with GI_{50} value of 7 nmol dm^{-3}, whereas its close analogue **1a** is inactive up to 5 μmol dm^{-3} concentration.[4] This difference does not correspond to the structural similarity for compounds **1a**/**1b** towards analogous molecule of combretastatin A-4 containing a double bond instead of pyrazole rings. Such variation of biological activity for **1a** and **1b** may be explained either by erroneous determination of chemical structure(s) or by the presence of bioactive impurities in the synthesized samples.[4] To clarify this matter, we analyzed both synthetic schemes and the analytical data for these compounds. Specifically, we assumed that the cycloaddition of sydnones to silylated acetylenes[4] may yield an alternative intermediate silylated pyrazole (or a mixture of respective isomers) affording the inactive 3,5-diarylpyrazole derivative.

In general, the presence of OH group in the benzene ring hampers the synthesis of target structures **1** and **2** and requires introduction and removal of protecting benzylic group at the



Scheme 1 *Reagents and conditions:* i, NaOH, EtOH, room temperature, 24 h; ii, AcCl, pyridine, CH_2Cl_2, room temperature, 3 h; iii, $PhI(OAc)_2$, MeOH, H_2SO_4, room temperature, 8 h; iv, EtOH, $N_2H_4 \cdot 2HCl$, reflux, 1 h; v, EtOH, $NH_2OH \cdot HCl$, reflux, 2 h.

hydrogenation stage. Accordingly, we proposed a convenient synthetic protocol for the known and new pyrazole- and isoxazole-based analogues of CA2 and CA4 that included iodosodiacetate-mediated rearrangement of easily accessible chalcones **5a**,**c** into dimethylketals **6a**,**c** followed by their *in situ* cyclization to provide the target pyrazoles (Scheme 1).

The protective acetyl group was easily introduced (step ii) and removed (step iii) during the rearrangement of chalcones **5** into dimethylketals **6**. Importantly, it was not necessary to isolate the intermediate compounds **6**. Instead, the reaction mixture was treated with NH_2NH_2 or NH_2OH to result in pyrazoles **1a**,**c** or isoxazole **2a**.

The corresponding isomeric diarylpyrazole **1b** and its analogues **1d**,**e** as well as isoxazole-based analogues **2b**,**d**–**f** were obtained *via* similar approach from the respective benzaldehydes and 3-acetoxy-4-methoxyacetophenone **4b**, which in turn could be readily synthesized from guaiacol.[15] The protective acetate groups were successfully removed during the isomerization of chalcone acetates **5′** (Scheme 2).†

In our experiments, melting points for compounds **1a** (171–173 °C) and **1b** (232–234 °C) did not match the reported values (198–200 °C and 90–93 °C, respectively).[4] Furthermore, the NMR signal of the NH group from pyrazole ring of product **1a** in $CDCl_3$ was absent in the published data.[4] Conversely, our ^{1}H NMR spectra of compound **1a** in DMSO-d_6 showed signals for two chemically distinct NH groups in the pyrazole ring at 12.9 and 13.1 ppm, suggesting the presence of two NH-tautomers. In addition, the positions of aryl rings in product **1a** were unambiguously determined by NOESY, COSY, HSQC, and HMBC spectra (see Online Supplementary Materials).

The synthesized compounds were evaluated for their biological activity using the phenotypic sea urchin embryo assay.[3] This assay allowed for rapid evaluation of both antiproliferative and microtubule destabilizing effects. Specific change of embryo swimming pattern, namely spinning on the bottom of the vessel instead of normal forward movement near the surface of the seawater, indicated microtubule destabilizing action.

As shown in Table 1, the majority of combretazoles caused pronounced cleavage alteration/arrest and embryo spinning suggesting strong antimitotic microtubule destabilizing activity. Effects of **1a** and **2a**,**b**,**d**,**f** were comparable to those of the parent combretastatins CA2 and CA4. Antiproliferative activity

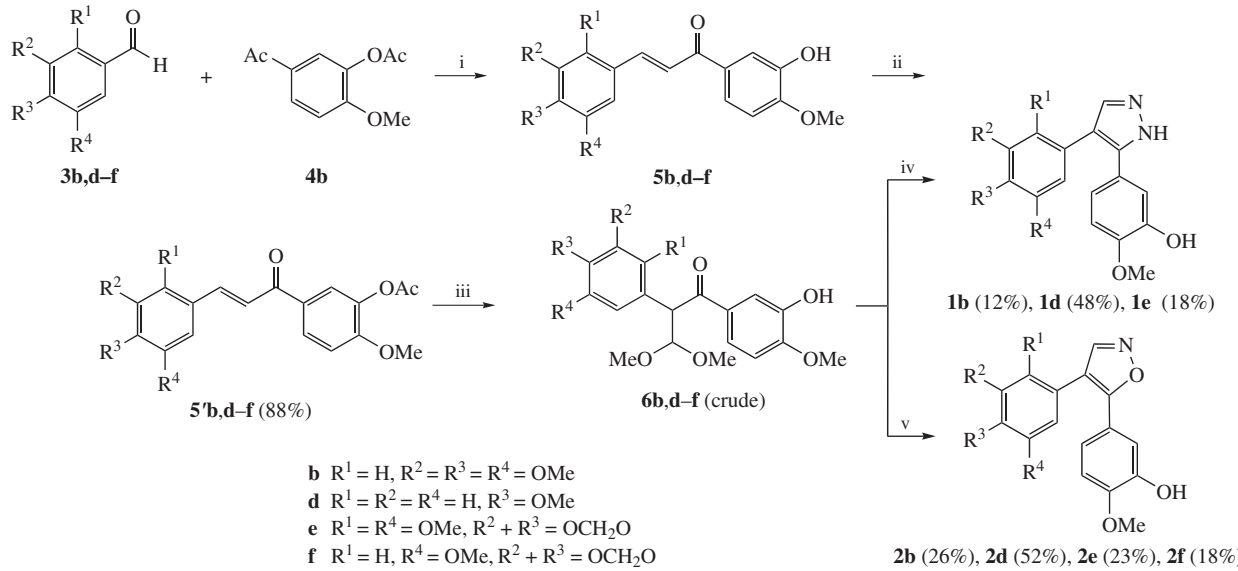
† *General procedure for the synthesis of chalcones* **5**. NaOH (60 mmol) was added to a vigorously stirred solution of benzaldehyde **3** (20 mmol) and arylacetophenone **4** (20 mmol) in EtOH (150 ml) at +5 °C (ice bath). The reaction mixture was stirred at room temperature for 24 h, concentrated *in vacuo*, the residue was treated with distilled water (130 ml), neutralized with 15% HCl and extracted with CH_2Cl_2 (3×80 ml). The combined organic extracts were washed with brine (2×80 ml), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting residue was recrystallized from EtOAc–light petroleum (1 : 1) and dried to afford chalcone **5**.

General procedure for the synthesis of chalcone acetates **5′**. A mixture of acetyl chloride (1.18 g, 15 mmol) and pyridine (1.19 g, 15 mmol) in absolute CH_2Cl_2 (15 ml) was added by small portions to a stirred solution of chalcone **5** (10 mmol) and pyridine (0.79 g, 10 mmol) in absolute CH_2Cl_2 (50 ml) at 20 °C. The reaction mixture was stirred at room temperature for 3 h, then diluted with water (50 ml), and the organic layer was separated. The aqueous layer was extracted with CH_2Cl_2 (30 ml). The combined organic extracts were washed with water (3×50 ml), dried over anhydrous Na_2SO_4 and evaporated. The residue was recrystallized from EtOAc–light petroleum (1 : 1) and dried to afford chalcone acetate **5′**.

General procedure for the synthesis of dimethylketals **6** *with simultaneous removal of acetyl group*. A solution of H_2SO_4 (50%, 5 ml) in methanol was added dropwise to a vigorously stirred suspension of chalcone **5′** (20 mmol) and $PhI(OAc)_2$ (30 mmol) in methanol (100 ml). The mixture was stirred at room temperature for 8 h and kept at 2–6 °C overnight. Then the reaction mixture was diluted with water (300 ml) and extracted with CH_2Cl_2 (3×100 ml). The organic layer was washed with brine (2×80 ml), water (50 ml) and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* and the resulting oil was purified using flash-chromatography (ethyl acetate–hexane, 1 : 4) to afford product **6** that was used without further purification.

General procedure for the synthesis of 4,5-diarylpyrazoles **1**. $N_2H_4 \cdot 2HCl$ (0.410 g, 3.9 mmol) was added to a solution of dimethylketal **6** (3.6 mmol) in ethanol (20 ml), the mixture was refluxed for 1 h, concentrated *in vacuo* and the resulting residue was dissolved in ethyl acetate (50 ml). The organic layer was washed with 5% aq. $NaHCO_3$ (50 ml), distilled water (50 ml), dried over $MgSO_4$ and concentrated *in vacuo* to furnish crude pyrazole **1**. Meting points were measured after recrystallization from ethyl acetate.

General procedure for the synthesis of 4,5-diarylisoxazoles **2**. 4,5-Diarylisoxazoles **2** were prepared according to a modified procedure.[17] $NH_2OH \cdot HCl$ (3.9 mmol) was added to a solution of dimethylketal **6** (3.6 mmol) in ethanol (20 ml). The resulting mixture was refluxed for 2 h, concentrated *in vacuo*, the residue was treated with ethyl acetate (50 ml), and the organic phase was washed with distilled water (2×25 ml), dried over $MgSO_4$ and concentrated to afford crude isoxazole **2**. Melting points were measured after recrystallization from ethyl acetate.



Scheme 2 *Reagents and conditions:* i, NaOH, EtOH, room temperature, 24 h;[3,16] ii, AcCl, pyridine, CH_2Cl_2, room temperature, 3 h;[3,16] iii, $PhI(OAc)_2$, MeOH, H_2SO_4, room temperature, 8 h; iv, EtOH, $N_2H_4 \cdot 2HCl$, reflux, 1 h; v, EtOH, $NH_2OH \cdot HCl$, reflux, 2 h.

Table 1 Effect of diarylpyrazoles **1** and diarylisoxazoles **2** on sea urchin embryos.[a]

Compound	LOEC/μmol dm^{-3}		
	Cleavage alteration	Cleavage arrest	Embryo spinning
CA2	0.002	0.01	0.5
CA4	0.002	0.01	0.5
1a	0.01	0.02	0.5
1b	0.05	0.2	2
1c	0.1	2	>10
1d	0.1	2 TE[b]	>10
1e	0.05	0.2	4
2a	0.001	0.005	0.02
2b	0.002	0.005	0.05
2d	0.002	0.01	0.5
2e	0.05	0.2	4
2f	0.002	0.02	0.05

[a] The sea urchin embryo assay was carried out as described.[3] Fertilized eggs and hatched blastulae were exposed to twofold decreasing concentrations of test compounds. Duplicate measurements showed no difference in LOEC (lowest observed effect concentration) values. [b] TE: tuberculate eggs typical of microtubule destabilizing agents action.

of diarylpyrazole **1c** was considered unrelated to tubulin or microtubules, because this compound failed to induce embryo spinning or formation of tuberculate arrested eggs typical of microtubule destabilizing agents.

In summary, we have synthesized a series of *cis*-restricted combretastatin analogues, diarylpyrazoles and diarylisoxazoles, and demonstrated that in the sea urchin embryo model, diarylisoxazoles were more potent than the respective 4,5-diarylpyrazoles. Isoxazoles **2a**,**b**,**d**,**f** exhibited antimitotic effect comparable to that for combretastatins A-2 and A-4.

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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.2019.03.015.

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