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New functionalized aminofurazans as potential antimitotic agents in the sea urchin embryo assay

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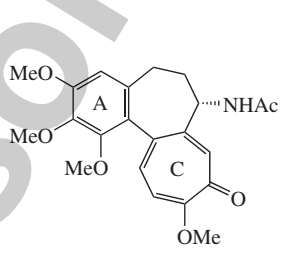
A series of new furazan (1,2,5-oxadiazole) derivatives based on structural overlap with combretastatin have been synthesized. Targeted molecules were evaluated using the sea urchin embryo assay; several agents demonstrated 1–4 μmol dm^{-3} antiproliferative activity in this *in vivo* model.

Targeting tubulin in rapidly dividing tumor cells has been a well validated strategy for cancer therapy.[1,2] Screening of natural products for cytotoxic activity yielded structurally diverse classes of mitotic spindle poisons. Most of the known anti-mitotic agents, including colchicine and *Vinca* alkaloids, inhibit tubulin polymerization. In contrast, Taxol™ has an opposite action: it stimulates polymerization *in vitro* and stabilizes spindle MT's.[3] High toxicity found for the mitotic poisons[4–9] prompted scientists to expand their search for the synthetic modulators of tubulin that (a) mimic natural products, (b) result in the same anti-mitotic effect and (c) display better efficacy/safety window. In general, colchicine derivatives are structurally simpler than *Vinca* alkaloids or Taxol™ derivatives, as exemplified by combretastatin A-4 (**CA-4**). This agent features a tilted 'biaryl' structural motif (A and B rings) connected by a hydrocarbon bridge of variable length. The linker provides for *cis*-configuration of the biaryl template necessary for the efficient interaction of a molecule with the colchicine binding site of tubulin.[10]

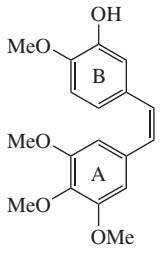
Note that five-membered heterocycle templates were reported to provide both non-isomerizable and metabolically stable isosteric replacement for the *cis*-styrene featured in combretastatins.[11] For example, combretafurazans were reported to be more potent in anti-mitotic agents compared to **CA-4** in neuroblastoma cells.[12]

In our search for new synthetic anti-mitotic agents, we focused on heterocyclic molecules with pharmacophore arrangement that mimics combretastatin and related compounds. Initially, we studied derivatives of both 1,3,4-oxadiazoles[13,14] and 1,3,4-triazoles.[15] The biaryl (1,2,4-oxadiazol-3-yl)furazan template was expected to provide for the (i) isosteric replacement of the *cis*-styrene bond while maintaining proper alignment of the aryl substituents for the tubulin activity and (ii) yield better cell permeability compared to the substituted phenyl group.[13–15] Considering complexity of both *in vitro* and *ex vivo* assays measuring tubulin activity of an agent, we decided to profile newly synthesized compounds in the sea urchin embryo model instead.[16] This phenotypic *in vivo* assay includes (i) a fertilized egg test for antimitotic activity displayed by the cleavage alteration/arrest, and (ii) the behavioral monitoring of a free-swimming blastulae treated immediately after hatching.[16,17]

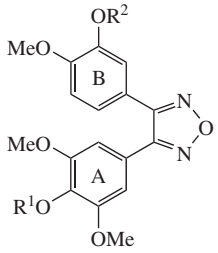
Our chemistry efforts were centered around structural modifications of a 4-amino-3-(1,2,4-oxadiazol-3-yl)furazan template. An overview of this approach is summarized in Scheme 1.



Colchicine



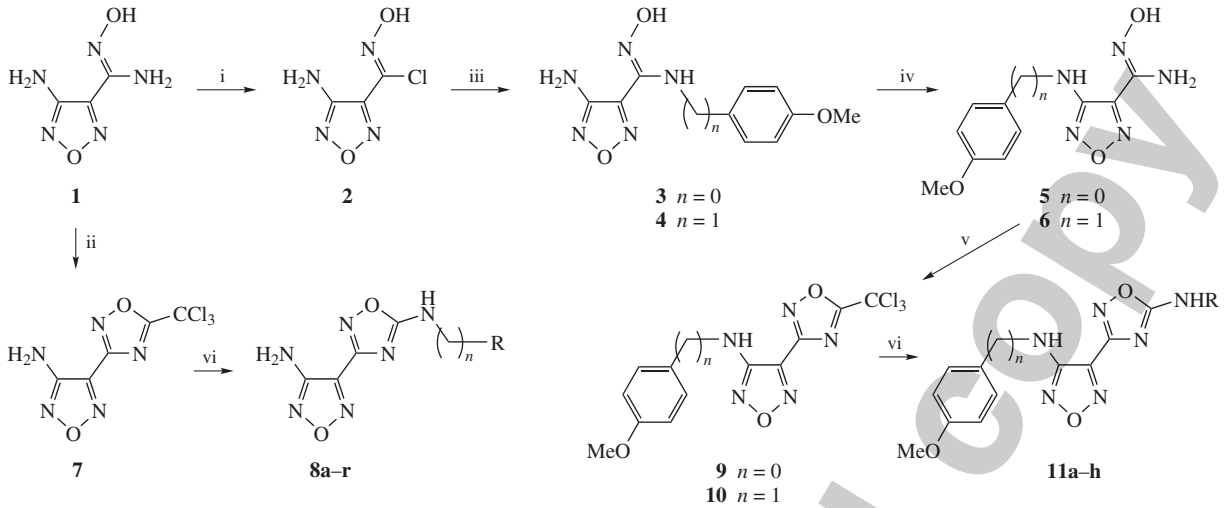
Combretastatin A-4
CA-4



'Combretafurazans'

Commercially available furazan derivative **1** provided for a good starting point in the synthesis. Key intermediate **2**[18,19] was prepared by diazotization of amidoxime **1** as described previously. Treatment of **2** with substituted amines in the presence of Et_3N yielded corresponding amidoximes **3** and **4**. The subsequent ring-to-ring interconversion reaction[20–22] took place with KOH in ethylene glycol at reflux to furnish **5** and **6**. Notably, this is the first example of the rearrangement applied to the synthesis of a bicyclic (1,2,4-oxadiazol-3-yl)furazan ring system. This step is both robust and high-yielding (~80% for both **5** and **6**).

In order to introduce a 1,2,4-oxadiazolyl-*N*-substituent into the targeted molecules, we first prepared trichloromethyl derivative **7**[23] from furazan derivative **1**[24,25] using a modification of a published procedure. Specifically, the nucleophilic displacement of the CCl_3 group[26,27] in compound **7** was achieved with amines in THF to afford desired molecules **8a–r**.† A wide variety of amine reagents were tolerated under the reaction conditions (Table 1). The yields of **8a–r** were 76–96%. Unfortunately, our attempts to further modify the unsubstituted amino group in these compounds *via* the reductive amination were unsuccessful



Scheme 1 *Reagents and conditions*: i, see refs. 18, 19; ii, see ref. 23; iii, *p*-MeOC$_6$H$_4$(CH$_2$)$_n$NH$_2$/NEt$_3$, EtOH or Pr^{i}OH; iv, KOH, ethylene glycol, reflux; v, CCl$_3$COCl, BuOAc, reflux; vi, RNH$_2$, THF.

under a variety of experimental conditions. Easy access to intermediates **5** and **6** (*vide supra*) allowed us to address this issue. Namely, ring-closing reactions of **5** and **6** to form targeted 1,2,4-oxadiazole derivatives **9** and **10** were accomplished with CCl$_3$COCl in butyl acetate at reflux. A final step towards desired molecules **11a–h** was accomplished *via* the nucleophilic displacement of a CCl$_3$ moiety with the respective amines in THF at room temperature. The yields ranged from 72 to 86% (Table 1).

We further elaborated the stepwise nucleophilic displacement of both NO$_2$ groups in 3,4-dinitrofurazan **12**[28] with N- and O-nucleophiles to access compound **14** as suggested by the earlier experimental data on the tubulin activity within the furazan series (Scheme 2).[12,27,29] Synthetic protocols describing similar transformations in the furazan series are scarce, primarily due to the hazard of starting compound **12**. In our hands, this safety concern was successfully addressed by working with dilute (< 0.35 mol dm^{-3}) solutions of **12** in CH$_2$Cl$_2$ at room temperature. While studying this conversion, we found that the initial introduction of the N-substituent was critical to the nucleophilic displacement of the remaining NO$_2$ group with the O-nucleophile. Following this protocol, we isolated compound **14** in 70.5% overall yield. Reversing the order of reactions, namely reacting **12** with the O-nucleophile followed by the N-nucleophile led to a complex mixture of products, none of them major (Scheme 2).

We further tested the molecules of **8a–r**, **11a–h** and **12** in the phenotypic sea urchin embryo assay in order to identify compounds targeting tubulin.[16,17] Combretastatin A-4 disodium phosphate (CA-4P, OxiGene) served as a benchmark reference compound.[16] We monitored the effect of furazans **8**, **11** and **14** on two specific developmental stages of the sea urchin embryo, namely: (i) fertilized egg to assess antimitotic activity and (ii) behavioral monitoring of a free-swimming blastulae to detect changes in the embryo swimming pattern. In this assay,

† Both ^{1}H and ^{13}C NMR data confirmed structures of the targeted furazans. For example, ^{13}C NMR spectra of compounds **8a–r** featured four signals specific to quaternary carbons of the furazan-1,2,4-oxadiazole core. The IR spectrum of these molecules displayed intense aminofurazan-NH$_2$ vibrational bands at *ca.* 3440 and *ca.* 3320 cm^{-1}. Typical ranges for the ^{1}H and ^{13}C NMR shifts of compounds **8a–r** are shown.

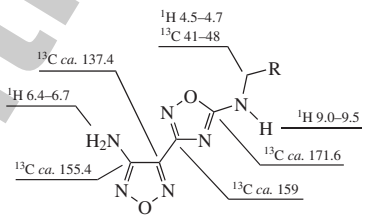
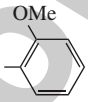
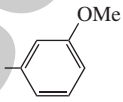
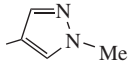
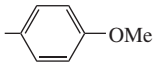
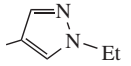
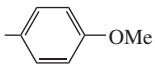
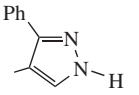
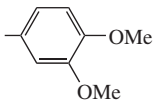
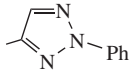
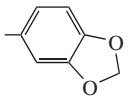
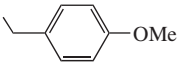
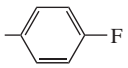
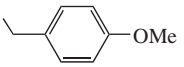
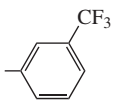
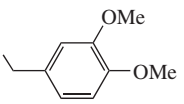
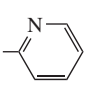
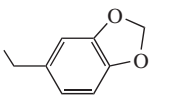
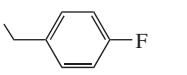
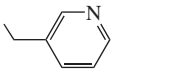
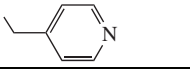
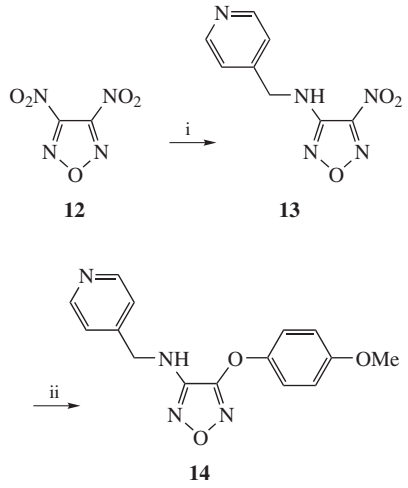


Table 1 Synthesis of 4-amino-3-(1,2,4-oxadiazol-3-yl)furazans **8a–r** and **11a–h**.

Product	*n*	R	Yield (%)[a]	Product	*n*	R	Yield (%)[a]
8a	1		94	**8n**	1		79
8b	1		89	**8o**	1		93
8c	1		90	**8p**	1		92
8d	2		96	**8q**	1		79
8e	1		88	**8r**	1		78
8f	1		93	**11a**	1	H	73
8g	1		85	**11b**	1		76
8h	1		76	**11c**	0		84
8i	1		91	**11d**	0		86
8j	1		87	**11e**	0		80
8k	1		92	**11f**	0		81
8l	1		85	**11g**	0		83
8m	1		79	**11h**	0		76

[a]Yields refer to the isolated analytically pure materials.†



Scheme 2 *Reagents and conditions*: i, *p*-$PyCH_2NH_2$, CH_2Cl_2, Et_3N, room temperature; ii, *p*-$MeOC_6H_4OH$, K_2CO_3, DMSO, 80–100 °C.

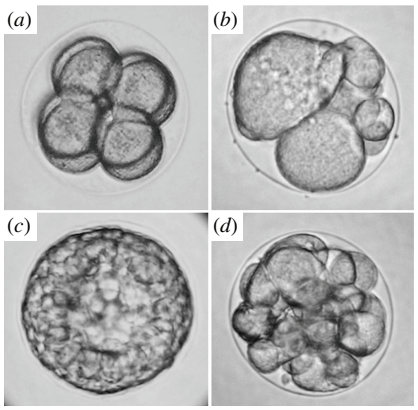


Figure 1 Effect of aminofurazans on the sea urchin embryo development. (*a*), (*c*) Intact embryos: (*a*) 8-cell stage; (*c*) early blastula. (*b*), (*d*) Typical cleavage abnormalities caused by aminofurazans. Fertilized eggs were exposed continuously to (*b*) 4 μmol dm^{-3} of **8c** or (*d*) 2 μmol dm^{-3} of **11g**. Time after fertilization: (*a*), (*b*) 3 h; (*c*), (*d*) 6 h. Average embryo diameter is 115 mm.

the molecules of **8b**,**c** and **11d**,**g** displayed EC_{50} values of about 1–4 μmol dm^{-3} suggesting an antimitotic tubulin destabilizing effect (Figure 1).

In summary, a series of furazan derivatives based on structural overlap with combretastatin have been designed, synthesized and tested in the phenotypic sea urchin embryo *in vivo* assay. The targeted molecules were prepared *via* a synthetic sequence involving the formation of key chloroamidoxime **2**. Compound **2** was converted to trichloromethyl 1,2,4-oxadiazoles **7**, **9** and **10** *via* a two-step protocol, namely, the ring-to-ring interconversion reaction of amidoximes **3** and **4** to yield compounds **5** and **6** with their subsequent cyclization. A nucleophilic displacement of the CCl_3 group in **7**, **9** and **10** with a series of amines furnished targeted furazans **8a**–**r** and **11a**–**h** in high yields. A furazan analogue of combretastatin **14** was prepared from 3,4-dinitrofurazan **12** *via* stepwise nucleophilic displacement of nitro groups with N- and O-nucleophiles. The order of reactions for **12** was discovered to be critical to the outcome of this conversion. All targeted furazan derivatives were conveniently purified *via* a straightforward recrystallization to furnish analytically pure materials immediately suitable for biological assays.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mencom.2010.05.002.

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