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Synthesis and X-ray structure of potent anticancer 4-(4-methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)isoxazole

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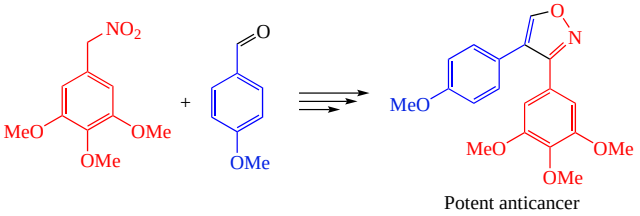
The three-step synthesis of potent anticancer 4-(4-methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)isoxazole starting from 1,2,3-trimethoxy-5-(nitromethyl)benzene and anisaldehyde was developed. In phenotypic sea urchin embryo assay, this compound exhibited antimitotic antitubulin activity comparable to that of the natural cytostatic combretastatin A4 (CA4). The title isoxazole inhibited *in vitro* growth of A549 human lung cancer cells and PC-3 human prostate cancer cells with IC_{50} values of 8 and 6 nM, respectively, exceeding the effect of CA4.

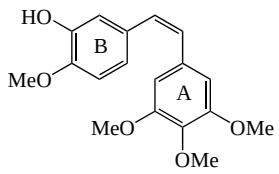


Potent anticancer

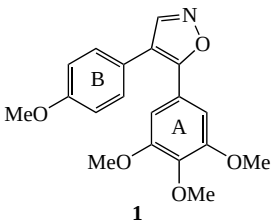
Keywords: *o*-diarylisoxazoles, nitromethylarenes, sea urchin embryo, cancer cells, X-ray.

ortho-Diaryl substituted five-membered *N*-heterocycles, referred to as combretazoles, feature *cis*-biaryl topology of the A and B rings similar to that of the natural antimitotic combretastatin A4 (CA4), and exhibit strong anticancer activity both *in vitro* and *in vivo*.[1–13] Within combretazoles, *o*-diarylisoxazoles have been identified as the most potent antimitotic compounds compared to their analogues of the pyrrole, pyrazole and 1,2,3-triazole series, both in sea urchin embryo tests and in NCI60 anticancer drug screen.[4,14–19] Some of these compounds exhibited cytotoxicity against human cancer cell lines in low nanomolar concentration (GI_{50} of 1–10 nM).

In the diarylisoxazole series, the most active antimitotic compound **1** (Figure 1) contained 3,4,5-trimethoxyphenyl pharmacophore as the ring A connected to position 5 of the isoxazole ring and 4-methoxyphenyl group as the ring B. It showed consistently high activity in NCI60 screen with



Combretastatin A4



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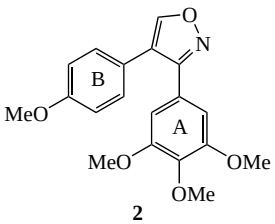

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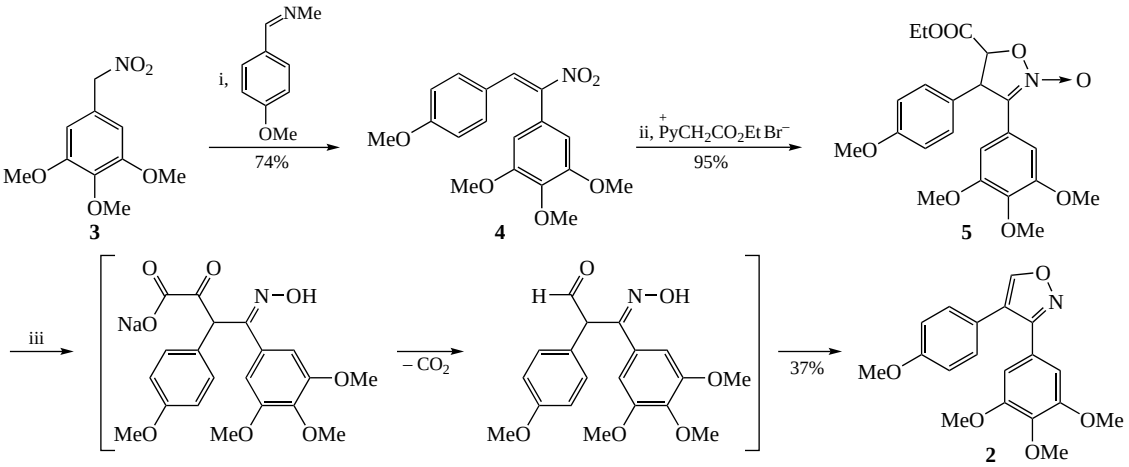
Figure 1 Structures of combretastatin A4 (CA4) and its diarylisoxazole analogues.

GI_{50} < 10 nM.[1] Particularly, GI_{50} values for HeLa human cervical carcinoma cells, HepG2 human liver carcinoma cells, and OVCAR-3 human ovarian carcinoma cells were 4.7, 4.4 and 10 nM, respectively.[4] Compound **1** was included into the Tubulin list of standards at The NCI Development Therapeutics Program Database.[20] *In vitro* studies at the P. A. Herzen Moscow Oncology Research Institute demonstrated extremely high cytotoxicity of isoxazole **1** against PC-3 human prostate cancer cell line with IC_{50} < 1 nM.[21] This compound with improved formulation was studied *in vivo* on different human and mouse xenografts using the nude mouse model before publication of synthetic route.[22] It was reported previously[1,21] that in the series of 4,5- and 3,4-diarylisoxazole isomers cytotoxicity and antitubulin activity could differ by 1–2 orders. The position of the 3,4,5-trimethoxyphenyl substituent close to the heteroatom of isoxazole linker, as in **1** (see Figure 1), was identified as favourable for the effect.[1] Therefore, it was important to develop the synthetic procedure to obtain the corresponding isomer **2** that was previously unaccessible due to the lack of starting 1,2,3-trimethoxy-5-(nitromethyl)benzene **3** (Scheme 1).

Recently,[23] the nitromethylarene **3** was synthesized by the Victor Meyer reaction with 35% yield. In the present work, the corresponding nitrostilbene **4**, and isoxazoline oxide **5** were obtained using modified methods[18] in good yields (see Scheme 1).† The intermediate isoxazoline *N*-oxide **5** was

† *5-Ethoxycarbonyl-4-(4-methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-4,5-dihydroisoxazole* N-*oxide* **5**. Pyridinium salt $Py^+CH_2CO_2Et\ Br^-$ (0.738 g, 3 mmol) was added to a solution of nitrostilbene **4** (0.518 g, 1.5 mmol) in MeOH (10 ml) at room temperature and stirring, then Et_3N (1.075 g, 4.35 mmol) was added, and the mixture was stirred for 1 h (TLC control). The solvent was evaporated *in vacuo*, the residue was washed with water and extracted with CH_2Cl_2. The extract was dried over $MgSO_4$ and evaporated *in vacuo* to give 4,5-dihydroisoxazole *N*-oxide **5**. Yellow oil (95%); ^1H NMR (500 MHz, $CDCl_3$) δ: 7.28 (d, J = 8.2 Hz, 2 H,



Scheme 1 *Reagents and conditions*: i, AcOH–MeCN, 20 °C, overnight; ii, Et_3N–MeCN, 40 °C, 5 h; iii, 2% NaOH, EtOH–H_2O, 60 °C, 6 h.

isomerized with simultaneous hydrolysis and decarboxylation to afford the target diarylisoxazole **2**.

The structure of new diarylisoxazole **2** was proved by X-ray diffraction analysis (Figure 2).‡ The compound crystallizes in the $P\bar{1}$ space group (no. 2) with one molecule in the asymmetric part of unit cell without solvent in the crystal structure. Conformation of the molecule is characterized by dihedral angles showing the turning of aromatic fragments relative to the central isoxazole ring. The values of these dihedral angles are 45.83 and 27.51° for methoxyphenyl and trimethoxyphenyl fragments, respectively.

The phenotypic sea urchin embryo assay[1] was used to confirm antimitotic microtubule destabilizing mechanism of action of the target isoxazole **2**. Compound CA4 and previously synthesized isomer **1** were selected as positive controls. This *in vivo* assay allows for rapid identification of antimitotic activity by cleavage alteration/arrest after the application of a test molecule to fertilized eggs. A specific modification of swimming pattern observed after hatching, *i.e.* spinning at the bottom of the culture vessel instead of forward swimming near the surface of the seawater, is an evidence of the microtubule destabilizing mode of action (Table 1). Compound **2** was more potent antimiotic agent than CA4, and its effect was related to microtubule destabilization as followed from its ability to induce embryo spinning. Both compounds **1** and **2** strongly inhibited growth of A549 and PC-3 cancer cells with the IC_{50} values from 0.08 to 10 nmol dm^{-3} depending on the cell line, which significantly exceeded the antiproliferative effect of CA4.[21]

In conclusion, the synthesis of potent anticancer 4-(4-methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)isoxazole **2** was accomplished. Its molecular structure was confirmed by X-ray crystallography. The study using *in vivo* sea urchin embryo assay and *in vitro* cytotoxicity test demonstrated that isoxazole **2** is strong tubuilin-targeting antimitotic agent even more potent than CA4.

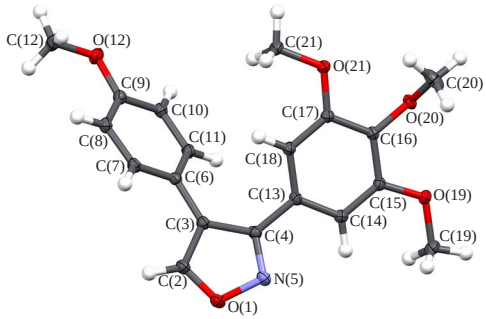


Figure 2 Structure of 4-(4-methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-isoxazole **2** in a crystal. The thermal vibrations of atoms are represented by ellipsoids in anisotropic approximation (p = 50%).

Table 1 Antimitotic microtubule destabilizing activity and cytotoxicity of compounds **1**, **2** and CA4.

Compound	Sea urchin embryo effects,[a] EC/nM			IC_{50}[b]/nM	
	Cleavage alteration	Cleavage arrest	Embryo spinning	A549[c]	PC-3[d]
CA4	2	10	50	86 ± 2[21]	40 ± 8[21]
1[e]	2	10	100	7 ± 2[21]	0.08 ± 0.01[21]
2	1	5	50	8 ± 1[21]	6 ± 1[21]

[a] The sea urchin embryo assay was conducted as described previously.[1] Fertilized eggs and hatched blastulae were exposed to 2-fold decreasing concentrations of compounds. Duplicate measurements showed no differences in effective threshold concentration (EC) values. [b] IC_{50}: concentration required for 50% cell growth inhibition (data from ref. 21). [c] A549: human lung cancer cell line. [d] PC-3: human prostate cancer cell line. [e] EC values from ref. 1.

H-2′6′), 7.14 (s, 2H, H-2,6), 6.91 (d, J = 8.7 Hz, 2H, H-3′,5′), 4.99 (d, J = 2.8 Hz, 1H, H-4 iso), 4.81 (d, J = 2.6 Hz, 1H, H-5 iso), 4.38–4.28 (m, 2H, OCH_2), 3.82 (s, 3H, OMe), 3.79 (s, 3H, OMe), 3.76 (s, 6H, 2OMe), 1.35 (t, J = 7.2 Hz, 3H, Me); ^{13}C NMR (126 MHz, $CDCl_3$) δ: 169.1, 159.8, 153.1 (2C), 139.2, 130.4, 128.3 (2C), 128.2, 120.7, 115.0 (2C), 104.4 (2C), 78.7, 62.5, 60.8, 56.1, 55.3 (2C), 54.2, 14.1.

4-(4-Methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)isoxazole **2**. 4,5-Dihydroisoxazole *N*-oxide **5** was added to a 2% solution of NaOH in EtOH (10 ml) and water (2 ml), and the mixture was stirred at 60 °C for 5–6 h. Ethanol was evaporated *in vacuo*, the residue was suspended in dichloromethane (60 ml) and washed with water. The organic layer was dried with $MgSO_4$ and evaporated. Isoxazole **2** was obtained as a yellowish oil (37%) after purification by column chromatography (benzene–benzene/ethyl acetate gradient, 1 → 10). Analytical sample was obtained by crystallization from MeOH. Yellow crystals; mp 93–94 °C (MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.45 (s, 1H, H-5 iso), 7.23 (d, J = 7.2 Hz, 2H, H-2′,6′), 6.91 (d, J = 6.9 Hz, 2H, H-3′,5′), 6.76 (s, 2H, H-2,6), 3.87 (s, 3H, OMe), 3.82 (s, 3H, OMe), 3.70 (s, 6H, 2OMe); ^{13}C NMR (126 MHz, $CDCl_3$) δ: 159.8, 159.6, 156.0 (2C), 153.2, 139.1, 130.5 (2C), 123.9, 121.2, 119.8, 114.2 (2C), 105.8 (2C), 60.9, 56.0 (2C), 55.4. HRMS (ESI/QTOF) m/z: $[M+H]^+$ calc. for $C_{19}H_{20}NO_5$ 342.1338; found 342.1336.

‡ *Crystal data for* **2**. $C_{19}H_{19}NO_5$, M_r = 341.35, triclinic, $P\bar{1}$ at 100.0(1) K, a = 7.60340(10), b = 10.6503(2) and c = 10.8878(2) Å, α = 74.5640(10), β = 89.5210(10) and γ = 75.7160(10)°, V = 822.05(2) Å^3, Z = 2, d_{calc} = 1.379 g cm^{-3}, μ(MoK$_a$) = 0.100 mm^{-1}, F(000) = 360. Total of 40048 reflections were measured and 6112 independent reflections (R_{int} = 0.0485) were used. The refinement converged to wR_2 = 0.1089 and GOF = 1.014 for all independent reflections [R_1 = 0.0447 was calculated for 4657 observed reflections with $I > 2\sigma(I)$].

CCDC 2335715 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre *via* http://www.ccdc.cam.ac.uk.

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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.2024.06.013.

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