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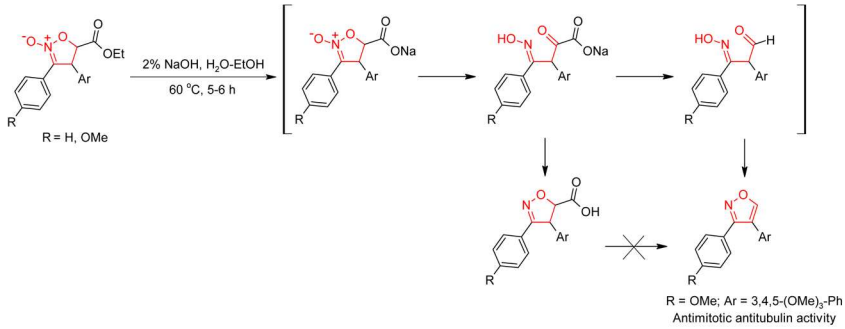

Regioselective synthesis of 3,4-diaryl-5-unsubstituted isoxazoles, analogues of natural cytostatic combretastatin A4

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ABSTRACT

4,5-Diarylisoxazoles are potent antiproliferative tubulin-targeting agents. Their isomeric 3,4-diaryl-5-unsubstituted isoxazoles are hardly accessible. The synthesis of 3,4-diaryl-5-unsubstituted isoxazoles **13** was designed based on a condensation of arylbenzaldehydes, arylnitromethanes, and ethoxycarbonylmethylpyridinium bromide followed by a selective one-step transformation of intermediate 3,4-diaryl-5-ethoxycarbonyl-4,5-dihydroisoxazole 2-oxides **8**. The orientation of aryl rings in relation to isoxazole heterocycle was confirmed by X-ray crystallography. Targeted compounds were evaluated for antimitotic microtubule destabilizing activity using a phenotypic sea urchin embryo assay. 3-(4-Methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazole **13e** and **13h** with a single methoxy substituent were the most potent. Compound **13e** showed strong cytotoxicity in NCI60 screen with GI_{50} for NCI-H522 human lung cancer cell line of 0.023 μM.

Keywords:
3,4-Diarylisoxazoles
Nitrostilbenes
Microtubule destabilization
Sea urchin embryo

Abbreviations:
CA4, combretastatin A-4
SAR, structure-activity relationship

1. Introduction

o-Diarylazoles can be considered as *cis*-restricted analogues of combretastatin A-4 (**CA4**, Fig. 1), a highly potent natural cytostatic originally isolated from the bark of African willow tree *Combretum caffrum* [1]. Numerous structure-activity relationship (SAR) studies identified that 3,4,5-trimethoxyphenyl ring A and 4-methoxyphenyl ring B bound by *cis*-double bond are essential for antimitotic microtubule destabilizing effect of **CA4**, whereas the presence of 3-hydroxy group in the ring B is irrelevant [2–4]. To avoid *cis-trans* isomerization that causes activity decrease, diverse N-containing rings have been introduced to replace the ethene linker, resulting in compounds with antiproliferative antitubulin effects comparable with those of **CA4** [4]. Among them, a number of diaryl-*o*-substituted isoxazoles were identified as potent antimitotic microtubule destabilizing agents [5–10]. Particularly, diaryl-*o*-isoxazole **1** (Fig. 1) effectively inhibited both cancer cell growth and *in vitro* tubulin polymerization [5,7].

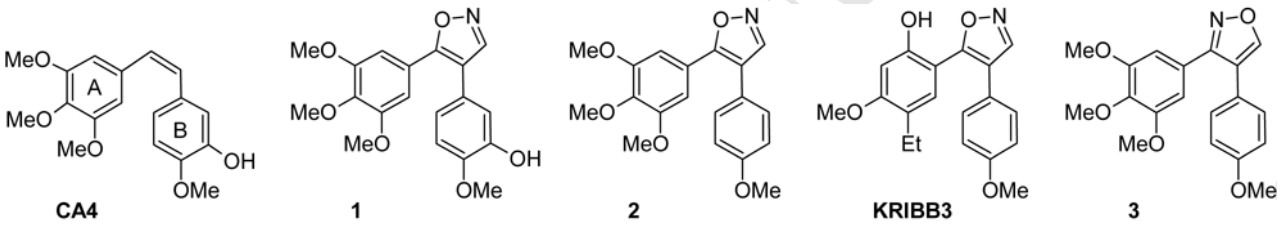


Fig. 1. Structures of combretastatin A-4 and their diaryl-*o*-substituted isoxazole analogues.

The removal of hydroxy group from the ring B also resulted in potent cytotoxic compounds **2**, KRIBB3, and **3** (Fig. 1) that strongly suppressed cancer cell growth both *in vitro* and *in vivo* in mouse xenograft model, disrupted cellular microtubules, induced mitotic arrest followed by apoptosis, and displayed antiangiogenic effect [6–8]. On the other hand, the position and number of substituents in benzene rings as well as the orientation of isoxazole heterocycle relating to aryl rings could significantly affect biological activity [5–8,10].

Up to date, no reliable and universal synthetic strategy for 3,4-diaryl-5-unsubstituted isoxazoles **4b** (Fig. 2) has been published, particularly due to the unpredictable regioselectivity. Generally, the reported reaction routes required expensive catalysts and scarcely available reagents, and could not be reproduced on a multigram scale [11]. According to literature, a conventional procedure for the synthesis of polymethoxy-4,5-diarylisoxazoles **4a** includes cyclization of easily accessible diarylketoaldehydes with hydroxylamine (Fig. 2) [5–7,10].

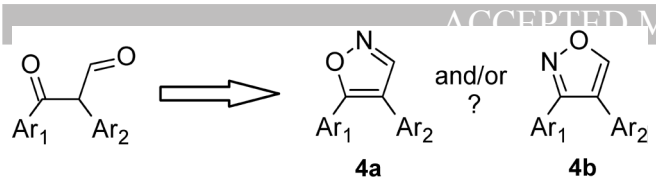


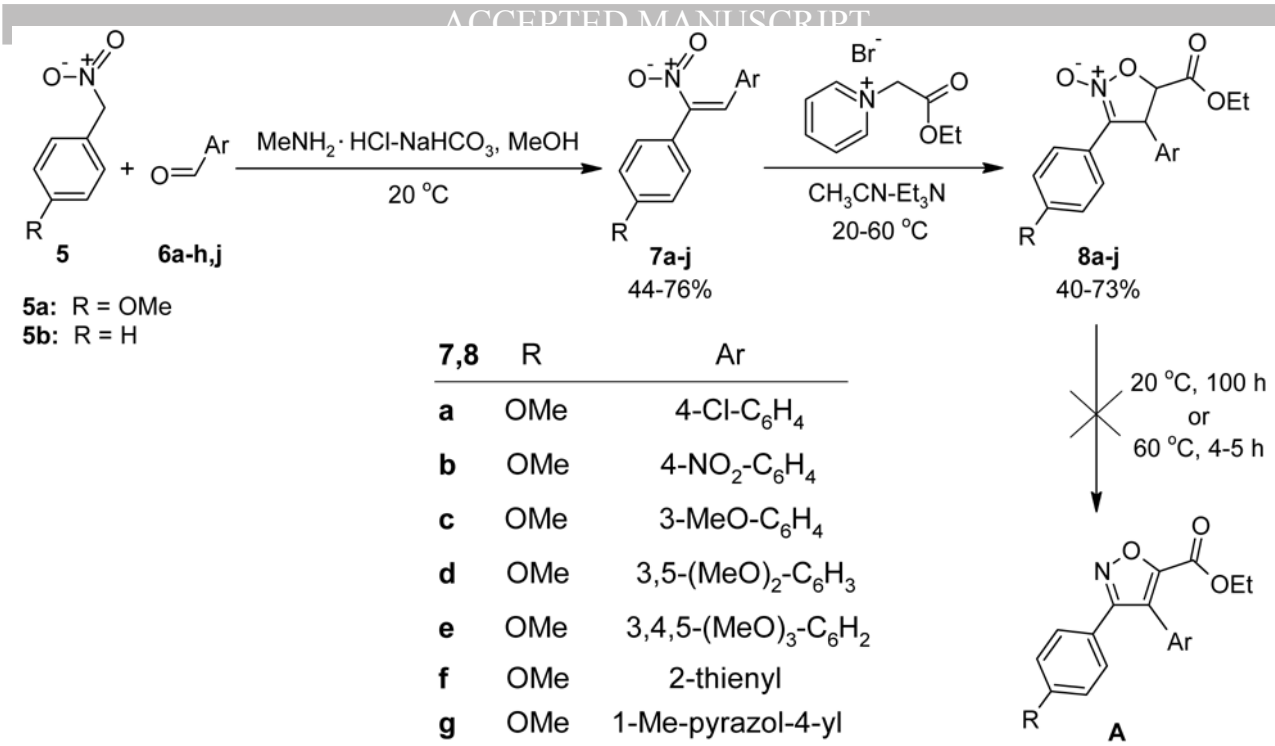
Fig. 2. General route for the preparation of diarylisoxazoles.

It was reported that the same chemical route afforded both polymethoxy-4,5-diarylisoxazoles **4a** and their 3,4-diaryl-substituted analogues **4b**, including compound **3** (Fig. 1 and 2) [6]. However, the identification of the isomers had come into question, since their published NMR spectra were identical. Similarly, the structure of 3,4-diarylisoxazoles **4b** (Fig. 2) obtained using the same reaction route by another research group [12] required further clarification by proper analytic procedures. Notably, all other literature data confirmed the formation of isomers **4a** by the above cyclization pathway (Fig. 2) [5,7,10,13]. In the present study a facile synthetic procedure for 5-unsubstituted 3,4-diarylisoxazoles using available materials was developed. The targeted molecules were evaluated for their antimitotic microtubule destabilizing activity in a sea urchin embryo model.

2. Results and discussion

2.1. Chemistry

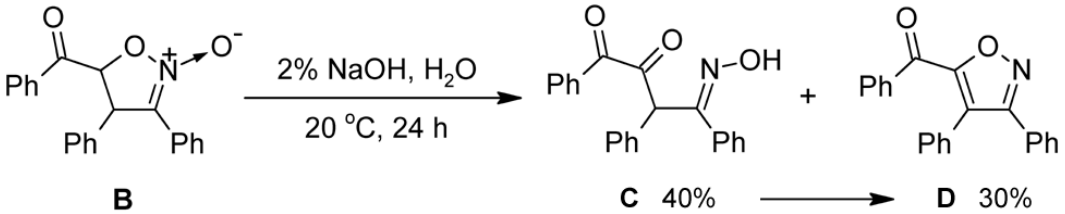
Starting diarylnitrostilbenes **7a**–**j** were easily synthesized by condensation of phenylnitromethanes **5** with aldehydes **6** [14–16]. At the next step compounds **7** reacted with ethoxycarbonyl methylpyridinium bromide in the presence of Et_3N to afford the respective 3,4-diarylisoxazoline N-oxide-5-carboxylates **8** with good yields (Scheme 1) [16].



7,8	R	Ar
a	OMe	4-Cl-C_6H_4
b	OMe	4-NO_2-C_6H_4
c	OMe	3-MeO-C_6H_4
d	OMe	3,5-$(MeO)_2$-C_6H_3
e	OMe	3,4,5-$(MeO)_3$-C_6H_2
f	OMe	2-thienyl
g	OMe	1-Me-pyrazol-4-yl
h	H	4-MeO-C_6H_4
i	H	3,4,5-$(MeO)_3$-C_6H_2
j	H	2,5-$(MeO)_2$-3,4-OCH_2O-C_6H

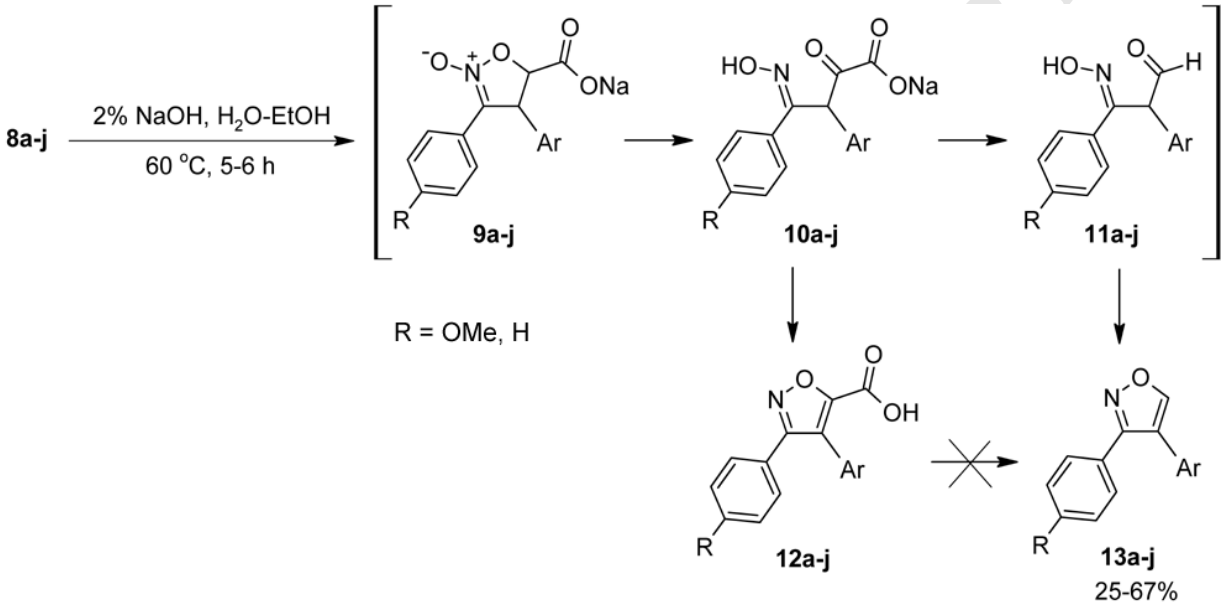
Scheme 1. Synthesis of diarylnitrostilbenes **7** and 3,4-diaryl-5-ethoxycarbonyl-4,5-dihydroisoxazole 2-oxides **8**.

The reported straightforward rearrangement of isoxazolines to the respective isoxazoles [17] implied possible application of this reaction to afford 3,4-diaryl-5-ethoxycarbonylisoxazoles **A**. It was found, however, that only isoxazolines **8a**–**j**, but not isoxazoles **A**, were formed at different conditions: 20 °C, 100–200 h, or 60 °C, 4–5 h (Scheme 1) [16]. Nitro derivative **8b** was the only exception capable to convert partially into the respective 3,4-diaryl-5-ethoxycarbonylisoxazole. Therefore, the next step of the study was to develop a chemical strategy for the transformation of **8a-j** to 3,4-diaryl-5-ethoxycarbonylisoxazoles **A.** According to literature, the rearrangement of 3,4-diphenylisoxazoline oxide **B** proceeded in water solution of 2% NaOH at room temperature during 24 h, yielding monoxime **C** (40%) and isoxazole **D** (30%) (Scheme 2). The plausible mechanism of the reaction included an opening of 3,4-diphenylisoxazoline N-oxide ring with the cleavage of N–O bond affording tricarbonylmonoxime **C**, followed by a cyclization and dehydration resulting in 3,4-diphenylbenzoylisoxazole **D** [17a,b].



Scheme 2. Rearrangement of 3,4-diphenylisoxazoline oxide **B**.

We intended to use these reaction conditions for the rearrangement of isoxazoline N-oxides **8** to 3,4-diaryl-5-ethoxycarbonylisoxazoles **A**. However, instead of anticipated compounds **A** or 5-carboxyisoxazoles **12a**–**j**, 5-unsubstituted isoxazoles **13a**–**j** with minor impurities of **12a**–**j** were formed at 20 °C and reaction time of 100–200 h. It was further found that the yields as well as relative content of minor **12** and major **13** components were similar at different temperatures. Targeted unsubstituted 3,4-diarylisoxazoles **13** were easily separated in alkaline media from byproduct acids **12**. Finally, optimal reaction conditions were found to be 60 °C (5–6 h) with the ratio of **12**:**13** = 1:2 – 1:7. A putative mechanism of this reaction is presented in Scheme 3.

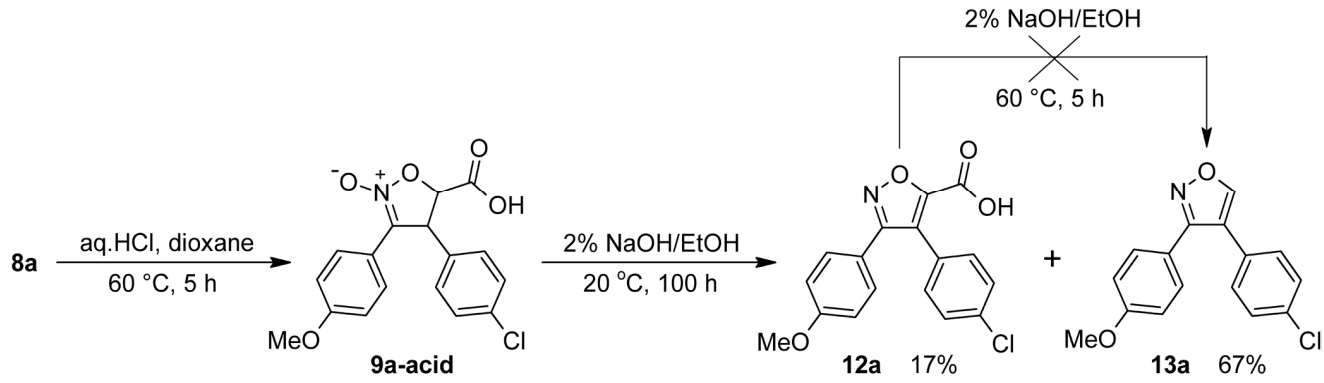


Scheme 3. Proposed mechanism of transformation of 3,4-diaryl-5-ethoxycarbonyl-4,5-dihydroisoxazole 2-oxides **8** to 5-unsubstituted 3,4-diarylisoxazoles **13**.

Starting compounds **8** were not detected by TLC in the reaction mixtures after ~ 2 h. Initially the ester group of **8** was hydrolyzed to afford isoxazoline acid Na-salts **9**. Then isoxazoline ring of **9** was recyclized with cleavage of N-O bond to afford 2-oxo-carboxylic acid salts **10.** It is well known that such acids are easily decarboxylated in acidic and alkaline media even at room temperature resulting in oxyimino aldehydes **11** [18]. Then intermediate ketocarboxylic salts **10** and oxyimino aldehydes **11** were cyclized to corresponding 5-carboxyisoxazoles **12** and unsubstituted isoxazoles **13**, respectively, like the cyclization of oxyimino ketone **C** (Scheme 2) [17a,b].

During the rearrangement of **9a** and **9c** intermediate aldehydes **11a** and **11c** were found in the reaction mixture. Aromatic protons and doublets of CHO and CH-Ar were observed in ^{1}H NMR spectra of products separated at the reaction half-time (**11a**: δ8.8, d, J = 7.5 Hz; 5.61, d, J = 7.5 Hz; **11c**: δ8.9, d, J =7.65 Hz; 5.52, d, J =7.6 Hz). Besides, there was proton signal of C=NOH in **11a** at

δ12.8 ppm. Integral intensities were corresponded to single protons in CHO, CH-Ar, and C=NOH groups. To confirm this reaction route, isoxazoline oxide acid of **9a** was synthesized by acidic hydrolysis of ester **8a** with high yield (98%). The solution of this acid was kept in EtOH–H_2O–NaOH (2%) at room temperature during 100 h, finally yielding 5-carboxyisoxazole **12a** (17%) and isoxazole **13a** (67%) (Scheme 4). Additionally, 5-carboxyisoxazole **12a** failed to transform to **13a** under the same conditions (2% NaOH/EtOH/H_2O, 60 °C, 5 h), and 90% of unconverted **12a** was separated from the reaction mixture. This observation was in a good agreement with literature data that decarboxylation of 3,4-diarylisoxazole-5-carboxylic acids proceeded at high temperature (200 °C, 3 h) and with low yield (5%) [11b].



Scheme 4. Synthesis and transformation of isoxazoline 2-oxide **9a-acid** to corresponding 5-unsubstituted 3,4-diarylisoxazole **13a**.

Finally the study of reaction mechanism resulted in the development of a simple and reliable procedure for the selective synthesis of hardly accessible 5-unsubstituted 3,4-diarylisoxazoles. The structures of **12** and **13** were proved by ^{1}H-, ^{13}C-NMR, and MS analyses. To confirm the structure of regioisomers (**4a** or **4b**, Fig. 2), 5-(4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazole **14** (Fig. 3) was synthesized according to procedure [7] and compared with the respective 3,4-diarylisoxazole **13e** by ^{1}H NMR.

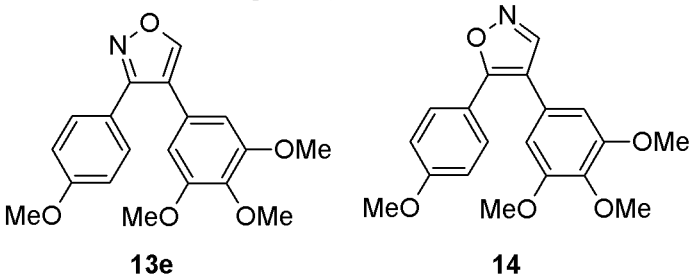


Fig. 3. Structure of 3,4-diarylisoxazole **13e** and its 4,5-diarylisoxazole regioisomer **14**.

The difference of 0.27 ppm in the chemical shift of the isoxazole proton in **13e** (8.48) and in **14** (8.31) could be considered as a reliable basis for distinguishing regioisomers **4a** and **4b** (Fig. 2). In addition,

the chemical shifts of aromatic protons in **13e** and **14** were different as well (Experimental section, *4.1.5.7* and *4.1.5.8*).

The structure of isoxazole **13e** was characterized by single-crystal X-ray diffraction study (Fig. 4). The geometrical parameters for **13e** are available in Supplementary data (Table S1). Unsubstituted at C-5 position 3,4-diarylisoxazoles were not published previously [19]. Specifically, the isoxazole ring in **13e** was planar with all bond lengths matching conventional O─N, O─C, C─C, N═C and C═C bonds reported for the related 4,5-diarylisoxazoles unsubstituted at C-3 position [20,21]. The 3- and 4-aryl substituents were not coplanar to the central isoxazole ring (the dihedral angles are 24.38(6) and 61.56(6)°, respectively). The twist angles of aryl groups at C-4 position were usually larger than those of aryl groups at C-3 and C-5 positions in the corresponding 3,4- and 4,5-diarylisoxazoles [20,21,22]. Notably, the 4-methoxy group in 3-(4-methoxyphenyl) substituent as well as 3- and 5-methoxy groups in 4-(3,4,5-trimethoxyphenyl) substituent were found to be within the planes of the corresponding benzene rings (the C10─C9─O2─C12, C14─C15─O3─C19 and C18─C17─O5─C21 torsion angles are -5.9(2), -6.9(3) and -8.4(3)°, respectively), whereas the 4-methoxy group in 4-(3,4,5-trimethoxyphenyl) substituent was rotated relative to the benzene plane (the C15─C16─O4─C20 torsion angle is -100.5(2)°).

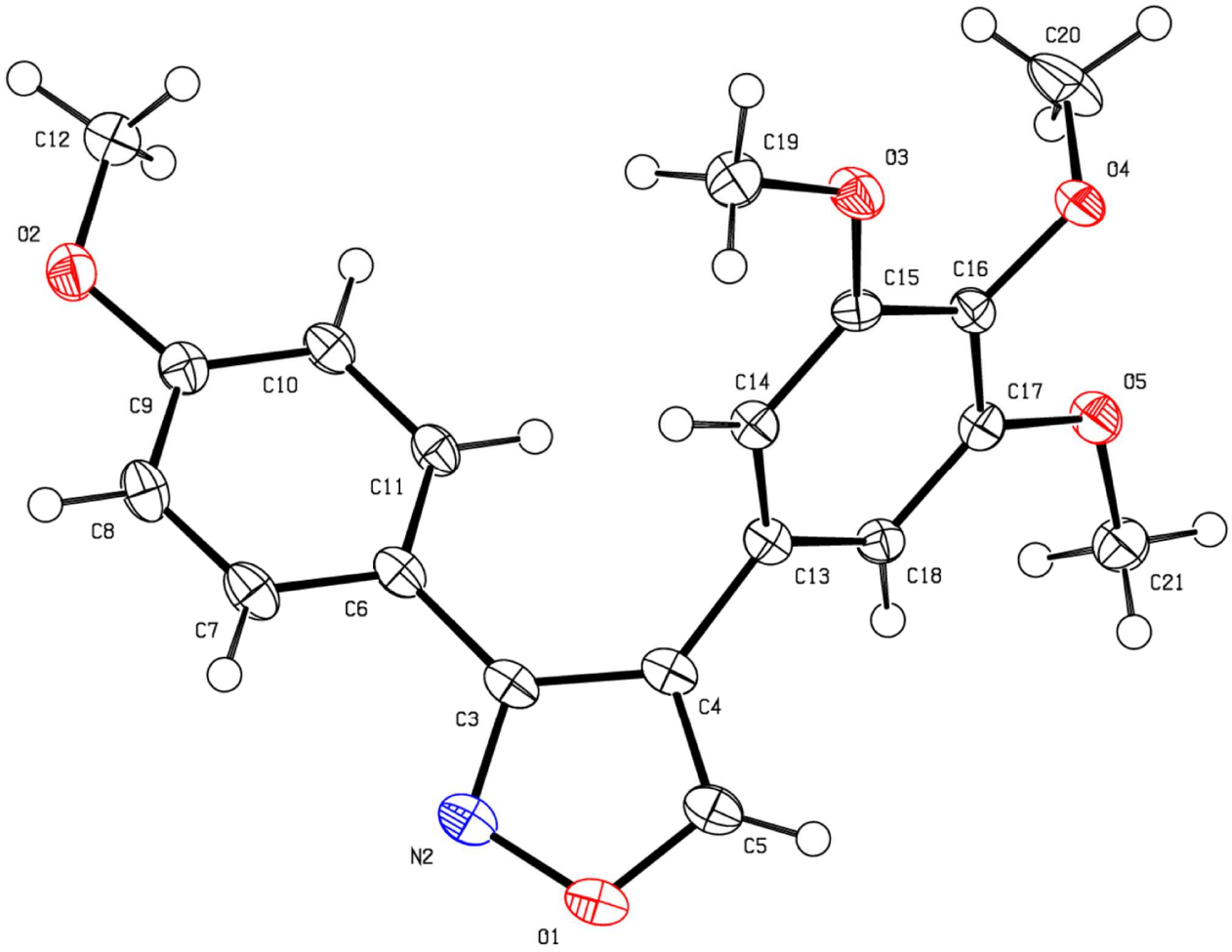


Fig. 4. Molecular structure of 3-(4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazole **13e** (50% ellipsoids).

2.2. Biological evaluation

Compounds **7**, **8**, **12** and **13** were screened for antimitotic microtubule destabilizing activity using a phenotypic sea urchin embryo assay [23]. The two-step assay includes the treatment of fertilized eggs to assess cell division (cleavage) alteration/arrest. Specific swimming changes of hatched blastulae exposed to a compound, namely, rapid spinning at the bottom of the culture well instead of forward swimming near the surface, indicate microtubule destabilizing mechanism of action. The results are presented in Table 1.

Table 1.

Effects of diarylethylenes **7** and diarylisoxazoles **8**, **12**, and **13** on sea urchin embryos

Compd	Sea urchin embryo effects, EC (μM)[a]		
	Cleavage alteration	Cleavage arrest	Embryo spinning
7e	1	4	>4
7f	0.2	2	2
7g	2	>4	>4
7h	2	4	4
7j	2	4	1
8a	>4	>4	>4
8b	>4	>4	>4
8c	4	>4	>4
8d	>4	>4	>4
8e	>4	>4	>4
8j	>4	>4	>4
12a	>4	>4	>4
12c	>4	>4	>4
12d	20	>40	>40
12e	0.2	2 (TE)[b]	>5
12j	>4	>4	>4
13a	>4	>4	>4
13b	>4	>4	>4
13c	0.52	4 (TE)[b]	>10
13d	0.2	2 (TE)[b]	>10
13e	0.01	0.1	1
14	0.005	0.1	0.5
13f	0.5	>4	>4
13g	>4	>4	>4
13h	0.005	0.05	0.1
13i	4	>4	>4
13j	2	>4	>4
CA4	0.002	0.01	0.05

[a] The sea urchin embryo assay was conducted as described previously [23]. 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] TE: tuberculate eggs typical of microtubule destabilizing agents.

It was reported previously that compound **7e** inhibited growth of chick embryonic cardiac fibroblasts [14]. Our data confirmed moderate antiproliferative effects of diarylnitroethylenes **7** associated with both microtubule destabilization and tubulin unrelated toxicity. Specifically, compounds **7e**, **7f**, and **7j** caused embryo destruction and death at 2–4 μM concentration. According to literature, introduction of NO_2 group into the double bond of benzyl **CA4** analogues dramatically decreased cytotoxicity, although did not affect antitubulin activity [24]. Similar to our results for series **7**, 1-(3′,4′,5′-trimethoxyphenyl)-2-nitroethylene, together with inhibition of tubulin polymerization, was found to exhibit tubulin-unrelated cytotoxicity [25]. Transformation of **7** to dihydroisoxazole 2-oxides **8** resulted in activity loss. Further transformation to diarylisoxazole-5-carbonic acids **12** also afforded inactive compounds with the only one exception for 3,4,5-trimethoxyphenyl derivative **12e** that produced weak microtubule destabilizing effect. Final 3,4-diarylisoxazoles **13c**–**e**, **h** with polymethoxy-substituted rings A and B displayed antimitotic antitubulin activity. Among them, surprisingly, **13h** with only one methoxy group was the most potent in the sea urchin embryo assay, whereas trimethoxy-substituted **13i** showed negligible activity. A close analogue of **CA4**, 3-(4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazole **13e**, inhibited cleavage at low nanomolar concentration. This compound exhibited pronounced cytotoxicity in NCI60 screen with average GI_{50} of 0.17 μM for 60 human cancer cell lines. NCI-H522 non-small cell lung cancer cell line was the most sensitive with GI_{50} = 0.023 μM and TGI (total growth inhibition concentration) = 0.075 μM. 4,5-Diarylisoxazole **14**, the corresponding regioisomer of **13e**, showed similar but slightly higher antimitotic antitubulin activity. Thiophene derivative **13f** could be considered as antiproliferative agent with non-tubulin mode of action.

3. Conclusions

The synthesis of 3,4-diaryl-5-unsubstituted isoxazoles **13** was developed using readily available starting phenylnitromethanes **5** and arylbenzaldehydes **6** that condensed easily with formation of 1,2-diaryl-1-nitroethylenes **7**. Further reaction with ethoxycarbonylmethylpyridinium bromide at 60 °C for 4–6 h yielded intermediate 3,4-diaryl-5-ethoxycarbonyl-4,5-dihydroisoxazole 2-oxides **8**. Subsequent one-step isomerization with decarboxylation of **8** afforded the targeted 3,4-diaryl-5-unsubstituted isoxazoles **13**. The synthesized compounds were evaluated for antiproliferative activity in a sea urchin embryo model. 4-(4-Methoxyphenyl)-3-phenylisoxazole **13h** and 3-(4-methoxyphenyl)-4-(3,4,5-

trimethoxyphenyl)isoxazole **13e** were identified as the most potent antimitotic microtubule destabilizing agents. **13e** exhibited high cytotoxicity against a panel of human cancer cell lines in NCI60 screen.

4. Experimental section

4.1. Chemistry. Materials and methods

Melting points were measured on a Boetius melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX-500 instrument [working frequencies of 500.13 MHz (^{1}H) and 125.76 MHz (^{13}C)]. Chemical shifts were stated in parts per million (ppm) and referenced to the appropriate NMR solvent peaks. Spin-spin coupling constants (*J*) were reported in Hertz (Hz). NMR spectra (Supplementary data) were prepared using an original software designed at N. D. Zelinsky Institute of Organic Chemistry RAS (Moscow, Russian Federation) (http://nmr.ioc.ac.ru:8080/SDF2PDF.kl1). Low resolution mass spectra (*m*/*z*) were recorded on a Finnigan MAT/INCOS 50 mass spectrometer at 70 eV using direct probe injection. High resolution mass spectra (HRMS) were measured on a Bruker maXis and micrOTOF II instruments using electrospray ionization (ESI). Elemental analysis was performed on the automated Perkin-Elmer 2400 CHN microanalyzer. Flash chromatography was carried out on silica gel (Acros, 0.035–0.070 mm, 60 Å). TLC was performed on Merck 60 F_{254} plates. Solvents, benzaldehydes, and ethyl bromoacetate were purchased from Acros Organics (Belgium) at the highest commercial quality and used as received. 4,7-Dimethoxy-2*H*-1,3-benzodioxole-5-carbaldehyde was synthesized from apiol, isolated from parsley seed extract [26]. Phenylnitromethanes **5** were synthesized by modified procedure [16,27].

*4.1.1. General procedure for the preparation of 1,2-diaryl-1-nitroethylenes **7** [14,15,16]*

A mixture of phenylnitromethane **5** (12 mmol), aldehyde **6** (13 mmol), MeOH (4 mL), $MeNH_2$·HCl (0.1 g, 1.46 mmol), and $NaHCO_3$ (0.04 g, 0.48 mmol) was stirred at room temperature for 120–200 h (TLC control) or at 60 °C for 4–6 h. Methanol was evaporated and methylene chloride (70 mL) was added to the mixture. The solution was washed with water (3 × 20 mL), dried by filtration through cotton wool, then the solvent was removed and the residue was triturated with minimal amount of MeOH. Nitroethylene crystals were filtered, washed with methanol and dried *in vacuo*.

*4.1.2. General procedure for the preparation of 3,4-diaryl-5-ethoxycarbonyl-4,5-dihydroisoxazole 2-oxides **8** [16].*

A mixture of the corresponding nitroethylene (5 mmol), ethoxycarbonylmethylpyridinium bromide [28] (7.5 mmol), and triethylamine (15 mmol) in dry acetonitrile (40 mL) was stirred at 60 °C for 4–5 h (TLC control). Acetonitrile was removed *in vacuo*, and then methylene chloride (150 mL) was added. The methylene chloride extract was washed with water, 5% aqueous HCl, water until pH 7, and dried by filtration through cotton wool. Methylene chloride was removed *in vacuo* and the residue was triturated in ethanol. The collected crystals were washed with ethanol and dried in air to obtain a 4,5-dihydroisoxazole 2-oxides **8**. When crystallization was unavailable, 4,5-dihydroisoxazole 2-oxides **8** were isolated by column chromatography. For each compound, the appropriate conditions are specified.

*4.1.3. Synthesis of 5-carboxy-4-(4-chlorophenyl)-3-(4-methoxyphenyl)-4,5-dihydroisoxazole 2-oxide (**9a**).*

A solution of compound **8a** (0.546 g, 1.5 mmol) in dioxane (15 mL) and aqueous HCl (9%, 3 mL) was heated at ~ 60 °C for 5 h, then kept overnight at room temperature. Dioxane was evaporated *in vacuo* and the residue was extracted with ethyl acetate (3 × 25 mL). The extracts were washed with water, brine, dried with anhydrous sodium sulfate. The solvent was removed *in vacuo* to afford **9a** as white solid (0.512 g, 98% yield); mp 116–119 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ13.75 (1H, br s, COOH), 7.85 (2H, d, J = 9.0 Hz, H-2′,6′), 7.45 (2H, d, J = 8.4 Hz, H-3″,5″), 7.39 (2H, d, J = 8.4 Hz, H-2″,6″), 6.97 (2H, d, J = 9.0 Hz, H-3′,5′), 5.39 (1H, d, J = 2,4 Hz, H-4), 4.99 (1H, d, J = 2.4 Hz, H-5), 3.75 (3H, s, OMe); ^{13}C NMR (500 MHz, DMSO-d_6) δ173.3, 1159.9, 138.0, 132.7, 129.2 (2C), 129.1 (2C), 128.2 (2C), 117.4, 114.3 (2C), 114.0, 77.4, 55.2, 51.9; EIMS m/z 347 $[M]^+$(17), 316 (17), 303 (16), 135 (100). Anal. Calcd for $C_{17}H_{14}ClNO_5$: C, 58.72; H, 4.06; N, 4.03. Found: C, 58.65; H, 4.16; N, 4.25.

*4.1.4. Synthesis of 4-(4-chlorophenyl)-3-(4-methoxyphenyl)isoxazole-5-carboxylic acid (**12a**) and 4-(4-chlorophenyl)-3-(4-methoxyphenyl)isoxazole (**13a**).*

A mixture of acid **9a** (1 mmol, 0.348 g), 2% ethanolic NaOH solution (7 mL), and water (3 mL) was stirred at 60 °C for 6 h, then ethanol was removed *in vacuo*. The residue was suspended in methylene chloride (100 mL), aqueous alkaline layer was separated, and the methylene chloride extract was washed with water (2 × 20 mL). The methylene chloride solution was dried by filtration through cotton wool and evaporated *in vacuo* to afford 0.19 g (67%) of 4-(4-chlorophenyl)-3-(4-methoxyphenyl)isoxazole **13a** as white crystals; mp 89–91 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ8.48 (1H, s, H-5), 7.41 (2H, d, J = 8.8 Hz, H-2′,6′), 7.33 (2H, d, J = 8.5 Hz, H-2″,6″), 7.19 (2H, d, J = 8.5 Hz, H-3″,5″), 6.90 (2H, d, J = 8.8 Hz, H-3′,5′), 3.83 (3H, s, OMe); ^{13}C NMR (500 MHz, $CDCl_3$) δ

160.8, 159.7, 156.2, 134.0, 130.1 (2C), 130.0 (2C), 129.0 (2C), 127.7, 120.4, 119.6, 114.2 (2C), 55.3; EIMS *m*/*z* 285 [M]+(100), 257 (29), 242 (46). Anal. Calcd for $C_{16}H_{12}ClNO_2$: C, 67.26; H, 4.23; N, 4.90. Found: C, 67.42; H, 4.31; N, 4.72.

The washings were combined with the aqueous alkaline layer. The combined alkaline solution was acidified using 10% aqueous HCl (pH 1-2) and extracted with ethyl acetate (2 × 35 mL). The combined extracts were washed with water (2 × 20 mL), brine, and dried with anhydrous Na_2SO_4. The solvent was removed *in vacuo*, and the residue was triturated in benzene. Separated crystals were filtered, washed with benzene, and dried to afford 0.056 g (17%) of acid **12a** as a white solid; mp 183–185 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ14.25 (1H, br s, COOH), 7.47 (2H, d, *J* = 8.5 Hz, H-3″,5″), 7.34 (2H, d, *J* = 8.5 Hz, H-2″,6″), 7.26 (2H, d, *J* = 8.8 Hz, H-2′,6′), 6.96 (2H, d, *J* = 8.9 Hz, H-3′,5′), 3.76 (3H, s, OMe); EIMS *m*/*z* 329 [M]+(3), 284 (4), 256 (13), 123 (100). Anal. Calcd for $C_{17}H_{12}ClNO_4$: C, 61.92; H, 3.67; N, 4.25. Found: C, 61.80; H, 3.55; N, 4.33.

*4.1.5. General procedure for the preparation of 3,4-diarylisoxazoles **13** and 3,4-diarylisoxazole-5-carboxylic acids **12**.*

A mixture of the corresponding 5-(ethoxycarbonyl)-4,5-dihydroisoxazole N-oxides- **8** (1.5 mmol), 2% ethanolic NaOH solution (10 mL) and water (2 mL) was stirred at 60 °C for 5–6 h, then ethanol was removed *in vacuo*. The residue was suspended in methylene chloride (100 mL) and the aqueous alkaline layer was separated. The methylene chloride extract was washed with water (2 × 20 mL). The methylene chloride solution was dried by filtration through cotton wool and evaporated *in vacuo*. The residue was crystallized from methanol to afford corresponding 3,4-diarylisoxazole **13**. **13f** was purified by column chromatography (silica gel, benzene/ethyl acetate 19:1). Water washings were combined with the aqueous alkaline layer, acidified with 10% aqueous HCl (pH 1–2), and extracted with ethyl acetate (100 mL). The extract was washed with water (2 × 20 mL) and brine and then dried with anhydrous Na_2SO_4. The solvent was removed, the residue was mixed with benzene, and the crystals of the corresponding 3,4-diarylisoxazole-5-carboxylic acid **12** were filtered and dried *in vacuo*. Acids **12b**,**f**–**i** were not specifically separated.

*4.1.5.1. 4-(4-Chlorophenyl)-3-(4-methoxyphenyl)isoxazole (**13a**).* 20 °C, 200 h; 0.15 g (53%).

*4.1.5.2. 3-(4-Methoxyphenyl)-4-(4-nitrophenyl)isoxazole (**13b**).* 20 °C, 190 h; cream crystals; 0.05 g (15%); mp 111–113 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ8.64 (1H, s, H-5), 8.21 (2H, d, *J* = 8.6 Hz, H-3″,5″), 7.44 (2H, d, *J* = 8.6 Hz, H-2′,6′), 7.38 (2H, d, *J* = 8.6 Hz, H-2″,6″), 6.92 (2H, d, *J* = 8.6 Hz, H-3′,5′), 3.85 (3H, s, OMe); ^{13}C NMR (500 MHz, $CDCl_3$) δ161.0, 159.7, 157.0, 147.3, 136.0, 130.0

(2C), 129.3 (2C), 124.0 (2C), 119.8, 118.4, 114.3 (2C), 55.3; EIMS *m*/*z* 296 [M]+(100), 268 (23), 253 (20), 207 (18). Anal. Calcd for $C_{16}H_{12}N_2O_4$: C, 64.86; H, 4.08; N, 9.45. Found: C, 64.72; H, 4.18; N, 9.36.

*4.1.7.3. 4-(3-Methoxyphenyl)-3-(4-methoxyphenyl)isoxazole (**13c**).* Yellowish oil; 0.16 g (57%); ^{1}H NMR (500 MHz, DMSO-d_6) δ9.21 (1H, s, H-5), 7.39 (2H, d, *J* = 8.7 Hz, H-2′,6′), 7.29 (1H, t, *J* = 7.9 Hz, H-5″), 7.01 (2H, d, *J* = 8.7 Hz, H-3′,5′), 6.93 (1H, d, *J* = 7.3 Hz, H-6″), 6.87 (1H, br s, H-2″), 6.84 (1H, d, *J* = 7.7 Hz, H-4″), 3.79 (3H, s, O′Me), 3.70 (3H, s, O″Me); ^{13}C NMR (500 MHz, DMSO-d_6) δ160.3, 159.3, 159.2, 158.0, 130.0, 129.8, 129.7 (2C), 120.7, 120.3, 119.1, 114.12 (2C), 114.08, 113.3, 55.1, 55.0; EIMS *m*/*z* 281 [M]+(100), 253 (53), 238 (41), 210 (38). Anal. Calcd for $C_{17}H_{15}NO_3$: C, 72.58; H, 5.37; N, 4.98. Found: C, 72.40; H, 5.48; N, 4.70.

*4.1.5.4. 4-(3-Methoxyphenyl)-3-(4-methoxyphenyl)isoxazole-5-carboxylic acid (**12c**).* White solid; 0.044 g (14%); mp 145–147 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ7.27–7.32 (3H, m, H-2′,6′, H-6″), 6.91 (4H, m, H-3′,5′, H-2″,5″), 6.82 (1H, br d, *J* = 7.5 Hz, H-4″), 3.75 (3H, s, O′Me), 3.71 (3H, s, O″Me); EIMS *m*/*z* 325 [M]+ (33), 298 (13), 280 (33), 252 (100). Anal. Calcd for $C_{18}H_{15}NO_5$: C, 66.46; H, 4.65; N, 4.31. Found: C, 66.70; H, 4.60; N, 4.43.

*4.1.5.5. 4-(3,5-Dimethoxyphenyl)-3-(4-methoxyphenyl)isoxazole (**13d**).* White solid; 0.296 g (64%); mp 82–84 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ8.49 (1H, s, H-5), 7.48 (2H, d, *J* = 8.8 Hz, H-2′,6′), 6.89 (2H, d, *J* = 8.8 Hz, H-3′,5′), 6.44 (1H, t, *J* = 2.3 Hz, H-4″), 6.41 (2H, d, *J* = 2.3 Hz, H-2″,6″), 3.82 (3H, s, OMe′), 3.71 (6H, s, OMe″); ^{13}C NMR (500 MHz, $CDCl_3$) δ161.3, 161.1, 160.2, 156.7 (2C), 131.4, 130.5 (2C), 121.2, 120.5, 114.4 (2C), 107.4 (2C), 100.5, 55.8 (2C), 55.7; EIMS *m*/*z* 311 [M]+ (20), 283 (29), 268 (16), 92 (100). Anal. Calcd for $C_{18}H_{17}NO_4$: C, 69.44; H, 5.50; N, 4.50. Found: C, 69.51; H, 5.61; N, 4.65.

*4.1.5.6. 4-(3,5-Dimethoxyphenyl)-3-(4-methoxyphenyl)isoxazole-5-carboxylic acid (**12d**).* White solid; 0.05 g (9%); mp 187–189 °C; ^{1}H NMR (500 MHz, D_2O, Na-salt) δ7.00 (2H, br s, H-2′,6′), 6.4 (2H, br s, H-3′,5′), 6.29–6.21 (3H, m, H-4″,2″,6″), 3.39 (6H, s, OMe″), 3.35 (3H, s, OMe′); ^{13}C NMR (500 MHz, D_2O, Na-salt) δ 162.4, 161.4, 161.1, 159.7, 159.5 (2C), 131.3, 129.1 (2C), 119.8, 119.1, 113.3 (2C), 108.2 (2C), 99.6, 54.6 (2C), 54.4; EIMS *m*/*z* 355 [M]+(100), 327 (38), 310 (28), 282 (25). Anal. Calcd for $C_{19}H_{17}NO_6$: C, 64.22; H, 4.82; N, 3.94. Found: C, 64.42; H, 4.70; N, 3.73.

*4.1.5.7. 3-(4-Methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazole (**13e**).* White solid; 0.235 g (46%); mp 143–145 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ8.48 (1H, s, H-5), 7.49 (2H, d, *J* = 8.9 Hz, H-2′,6′), 6.90 (2H, d, *J* = 8.9 Hz, H-3′,5′), 6.46 (2H, s, H-2″,6″), 3.88 (3H, s, OMe′), 3.83 (3H, s, OMe″), 3.74 (6H, s, OMe″); ^{13}C NMR (500 MHz, $CDCl_3$) δ160.7, 159.7, 155.9 (2C), 153.4, 137.8, 130.1 (2C), 124.6, 120.8, 120.0, 114.0 (2C), 106.1 (2C), 61.0, 56.1 (2C), 55.3; EIMS *m*/*z* 341 [M]+(38), 326 (4), 92 (71), 77 (100). Anal. Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.77; H, 5.52; N, 4.38.

*4.1.5.8. 5-(4-Methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazole (**14**).* White solid; 0.32 g (69%); mp 148–150 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ8.30 (1H, s, H-3), 7.62 (2H, d, *J* = 8.7 Hz, H-2′,6′), 6.91 (2H, d, *J* = 8.7 Hz, H-3′,5′), 6.58 (2H, s, H-2″,6″), 3.90 (3H, s, OMe′), 3.84 (3H, s, OMe″), 3.79 (6H, s, OMe″); ^{13}C NMR (500 MHz, $CDCl_3$) δ163.9, 160.9, 153.6 (2C), 151.7, 137.8, 128.8 (2C), 125.8, 120.1, 114.9, 114.1 (2C), 105.9 (2C), 61.0, 56.2 (2C), 55.3; EIMS *m*/*z* 341 $[M]^+$(60), 326 (14), 136 (21), 135 (100), 107 (11), 92 (15), 77 (26). Anal. Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.80; H, 5.65; N, 4.22.

*4.1.5.9. 3-(4-Methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazole-5-carboxylic acid (**12e**).* White solid; 0.15 g (25%); mp 203–205 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ14.1 (1H, br s, COOH), 8.33 (2H, d, *J* = 8.8 Hz, H-2′,6′), 7.97 (2H, d, *J* = 8.9 Hz, H-3′,5′), 6.64 (2H, s, H-2″,6″), 3.76 (3H, s, OMe′), 3.70 (3H, s, OMe″), 3.64 (6H, s, OMe″); EIMS *m*/*z* 385 [M]+(33), 342 (38), 312 (34), 298 (63), 77 (100). Anal. Calcd for $C_{20}H_{19}NO_7$: C, 62.33; H, 4.97; N, 3.63. Found: C, 62.52; H, 4.80; N, 3.81.

*4.1.5.10. 3-(4-Methoxyphenyl)-4-(2-thienyl)isoxazole (**13f**).* Gray solid; 0.169 g (33%); mp 75–77 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ8.51 (1H, s, H-5), 7.50 (2H, d, *J* = 8.8 Hz, H-2′,6′), 7.29 (1H, dd, *J* = 5.2 Hz, *J* = 1.1 Hz, H-5″), 7.01 (1H, dd, *J* = 5.2 Hz, *J* = 3.6 Hz, H-4″), 6.93 (1H, dd, *J* = 3.6 Hz, *J* = 1.1 Hz, H-3″), 6.91 (2H, d, *J* = 8.8 Hz, H-3′,5′), 3.83 (3H, s, OMe); ^{13}C NMR (500 MHz, $CDCl_3$) δ 160.8, 159.8, 156.3 (2C), 130.1, 129.4 (2C), 128.3, 127.3, 125.9, 120.4, 114.3 (2C), 55.2; EIMS *m*/*z* 257 [M]+(100), 229 (18), 214 (35), 186 (13). Anal. Calcd for $C_{14}H_{11}NO_2S$: C, 65.35; H, 4.31; N, 5.44. Found: C, 65.58; H, 4.42; N, 5.64.

*4.1.5.11. 3-(4-Methoxyphenyl)-4-(1-methylpyrazol-4-yl)isoxazole (**13g**).* White solid; 0.339 g (67%); mp 86–88 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ8.47 (1H, s, H-5), 7.52 (2H, d, *J* = 8.6 Hz, H-2′,6′), 7.43 (1H, s, H-3″), 7.26 (1H, s, H-5″), 6.93 (2H, d, *J* = 8.6 Hz, H-3′,5′), 3.88 (3H, s, OMe), 3.85 (3H, s,

NMe); ^{13}C NMR (500 MHz, $CDCl_3$) δ160.7, 159.9, 155.3, 138.6, 129.8 (2**C**), 128.7, 120.9, 114.0 (2**C**), 111.3, 109.4, 55.2, 39.1; EIMS *m/z* 255 [M]+(100), 227 (10), 212 (21), 184 (8). Anal. Calcd for $C_{14}H_{13}N_3O_2$: C, 65.87; H, 5.13; N, 16.46. Found: C, 65.68; H, 5.23; N, 16.64.

*4.1.5.12. 4-(4-Methoxyphenyl)-3-phenylisoxazole (**13h**).* Yellowish oil; 0.05 g (25%); ^{1}H NMR (500 MHz, $CDCl_3$) δ8.43 (1H, s, H-5), 7.50 (2H, d, *J* = 7.3 Hz, H-2′,6′), 7.40–7.33(3H, m, H-3′,4′,5′), 7.15 (2H, d, *J* = 8.2 Hz, H-2″,6″), 6.86 (2H, d, *J* = 8.2 Hz, H-3″,5″), 3.80 (3H, s, OMe); ^{13}C NMR (500 MHz, $CDCl_3$) δ160.1, 159.4, 155.8, 130.0 (2C), 129.5, 128.7, 128.6 (2C), 128.6 (2C), 121.0, 119.9, 114.2 (2C), 55.2; HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for $C_{16}H_{14}NO_2$ 252.1019; Found 252.1023. Anal. Calcd for $C_{16}H_{13}NO_2$: C, 76.48; H, 5.21; N, 5.57. Found: C, 76.65; H, 5.35; N, 5.77.

*4.1.5.13. 3-Phenyl-4-(3,4,5-trimethoxyphenyl)isoxazole (**13i**).* White solid; 0.13 g (54%); mp 72–77 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ8.55 (1H, s, H-5), 7.55 (2H, d, *J* = 7.5 Hz, H-2′,6′), 7.43–7.37 (3H, m, H-3′,4′,5′), 6.44 (2H, s, H-2″,6″), 3.87 (3H, s, OMe), 3.71 (6H, s, OMe); ^{13}C NMR (500 MHz, DMSO-d_6) δ160.2, 155.9, 153.4 (2C), 137.8, 129.7, 128.8 (2C), 128.64, 128.56, 124.3, 120.3, 106.0 (2C), 60.9, 56.0 (2C); HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for $C_{18}H_{18}NO_4$ 312.1230; Found 312.1231; [M + Na]+ Calcd for $C_{18}H_{17}NO_4Na$ 334.1050; Found 334.1046. Anal. Calcd for $C_{18}H_{17}NO_4$: C, 69.44; H, 5.50; N, 4.50. Found: C, 69.64; H, 5.55; N, 4.80.

*4.1.5.14. 4-(2,5-Dimethoxy-3,4-methylenedioxyphenyl)-3-phenylisoxazole (**13j**).* White solid; 0.22 g (51%); mp 166–168 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ9.04 (1H, s, H-5), 7.46–7.40 (5H, m, Ph), 6.51(1H, s, H-6″), 6.06 (2H, s, OCH_2O), 3.69 (3H, s, OMe), 3.40 (3H, s, OMe); ^{13}C NMR (500 MHz, DMSO-d_6) δ(+ or – is a sign in the APT procedure) 160.7 (–), 158.9 (+), 139.1 (–), 138.0 (–), 137.2 (–), 136.0 (–), 130.0 (+), 129.5 (+2C), 129.2 (–), 128.0 (+2C), 115.9 (–), 114.6 (–), 110.2 (+), 102.3 (–), 59.5 (+), 56.9 (+); HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for $C_{18}H_{16}NO_5$ 326.1023; Found 326.1019; [M + K]+ Calcd for $C_{18}H_{15}NO_5K$ 364.0582; Found 364.0582. Anal. Calcd for $C_{18}H_{15}NO_5$: C, 66.46; H, 4.65; N, 4.31. Found: C, 66.61; H, 4.54; N, 4.52.

*4.1.5.15. 4-(2,5-Dimethoxy-3,4-methylenedioxyphenyl)-3-phenylisoxazole-5-carboxylic acid (**12j**).* White solid; 89 mg (18%); mp 198–200 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.48–7.40 (5H, m, Ph), 6.62 (1H, s, H-6″), 6.09 (2H, s, OCH_2O), 3.72 (3H, s, OMe), 3.42 (3H, s, OMe); ^{13}C NMR (500 MHz, DMSO-d_6) δ62.6, 157.9, 157.5, 138.6, 138.0, 137.2, 135.6, 130.1, 128.8 (2C), 128.2, 127.6 (2C), 119.2, 114.0, 110.4, 102.0, 59.1, 56.6; HRMS (ESI/QTOF) m/z: [M + H]+ Calcd for $C_{19}H_{16}NO_7$

370.0921; Found 370.0913; [M + K]+ Calcd for $C_{19}H_{15}NO_7K$ 408.0480; Found 408.0484. Anal. Calcd for $C_{19}H_{15}NO_7$: C, 61.79; H, 4.09; N, 3.79. Found: C, 61.63; H, 4.22; N, 3.54.

4.1.6. X-ray crystal structure determination.

Data were collected using a Bruker APEX-II CCD diffractometer (λ(MoK$_\alpha$)-radiation, graphite monochromator, ω and φ scanning mode) and corrected for absorption using the *SADABS* program [29] (For the details, see Table S1, Supplementary data). The crystal structures of **13e** were determined by direct methods and refined by a full-matrix least squares technique on F^2 with anisotropic displacement parameters for non-hydrogen atoms. The hydrogen atoms in both compounds were placed in calculated positions and refined within the riding model with fixed isotropic displacement parameters (U_{iso}(H) = 1.5U_{eq}(C) for the CH_3-groups and 1.2U_{eq}(C) for the other groups). All calculations were carried out using the *SHELXTL* program [30]. Crystallographic data for **13e** have been deposited with the Cambridge Crystallographic Data Center. CCDC 1535084 (**13e**) contain supplementary crystallographic data for this paper. These data can be obtained free of charge from the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk or www.ccdc.cam.ac.uk).

4.2. Biology. Phenotypic sea urchin embryo assay [23].

Adult sea urchins, *Paracentrotus lividus* L. (Echinidae), were collected from the Mediterranean Sea on the Cyprus coast and kept in an aerated seawater tank. Gametes were obtained by intracoelomic injection of 0.5 M KCl. Eggs were washed with filtered seawater and fertilized by adding drops of diluted sperm. Embryos were cultured at room temperature under gentle agitation with a motor-driven plastic paddle (60 rpm) in filtered seawater. The embryos were observed with a Biolam light microscope (LOMO, St. Petersburg, Russia). For treatment with the test compounds, 5 mL aliquots of embryo suspension were transferred to six-well plates and incubated as a monolayer at a concentration up to 2000 embryos/mL. Stock solution of **12d** was prepared in distilled water at 10 mM concentration. Stock solutions of other compounds were prepared in DMSO at 10–20 mM concentration followed by a 10-fold dilution with 96% EtOH. This procedure enhanced the solubility of the test compounds in the salt-containing medium (seawater), as evidenced by microscopic examination of the samples. The maximal tolerated concentrations of DMSO and EtOH in the *in vivo* assay were determined to be 0.05% and 1%, respectively. Higher concentrations of either DMSO (≥0.1%) or EtOH (>1%) caused nonspecific alteration and retardation of the sea urchin embryo development independent of the treatment stage. Combretastatin A-4 (synthesized as reported previously [26] served as a positive control. The antiproliferative activity was assessed by exposing fertilized eggs (8–15 min after fertilization, 45–55 min before the first mitotic cycle completion) to 2-

fold decreasing concentrations of the compound. Cleavage alteration and arrest were clearly detected at 2.5 h and 5.5 h after fertilization, when control embryos reached 8-cell and early blastula stages, respectively. The effects were estimated quantitatively as an effective threshold concentration, resulting in cleavage alteration and embryo death before hatching or full mitotic arrest. At these concentrations all tested microtubule destabilizers caused 100% cleavage alteration and embryo death before hatching, whereas at 2-fold lower concentrations the compounds failed to produce any effect. For microtubule-destabilizing activity, the compounds were tested on free-swimming blastulae just after hatching (8–10 h after fertilization), which originated from the same embryo culture. Embryo spinning was observed after 15 min to 20 h of treatment, depending on the structure and concentration of the compound. Both spinning and lack of forward movement were interpreted to be the result of the microtubule-destabilizing activity of a molecule. Video illustrations are available at http://www.chemblock.com. Sea urchin embryo assay data are available at http://www.zelinsky.ru. Experiments with the sea urchin embryos fulfill the requirements of biological ethics. The artificial spawning does not cause animal death, embryos develop outside the female organism, and both post spawned adult sea urchins and the excess of intact embryos are returned to the sea, their natural habitat.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at ..... These include X-ray data for **13e**, analytical data, ^{1}H and ^{13}C NMR spectra of compounds **5a**,**b**, **7a**–**j**, **8a**–**j**, **9a**, **12a**,**c**–**e**,**j**, and **13a**–**j** (PDF).

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Highlights

- A facile synthesis of 3,4-diaryl-5-unsubstituted isoxazoles is developed.
- Rearrangement of diaryl-5-ethoxycarbonylisoxazoline 2-oxides to diarylisoxazoles.
- High antitubulin activity of 4-(4-methoxyphenyl)-3-phenyl isoxazole.