



Combretastatin A-4 sulfur-containing heterocyclic derivatives: synthesis, antiproliferative activities and molecular docking studies

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ABSTRACT

Combretastatin A-4 inspired heterocyclic derivatives were synthesized and evaluated for their biological activities on tubulin polymerization and cell proliferation. Among the 19 described sulfur-containing compounds, derivatives (Z)-**4h** and (Z)-**4j** exhibited interesting *in cellulo* tubulin polymerization inhibition and antiproliferative activities with IC₅₀ values for six different cell lines between 8 and 27 nM. Furthermore, *in silico* docking studies within the colchicine/CA-4 binding site of tubulin were carried out to understand the interactions of our products with the protein target. The effects on the cell cycle of follicular lymphoma cells were also investigated at 1-10 nM concentrations showing that apoptotic processes occurred.

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1. Introduction

With an estimated 18 million cases in the world in 2018 [1], cancer still remains one of the most challenging public health issue. Beside surgery and radiotherapy, the use of selective and efficient chemotherapeutic treatments, which would completely eradicate tumor cells with minimal side effects, is the main goal of current anticancer therapeutic strategies.

Historically, nature has been the source of many active ingredients of medicines and has inspired the design of numerous active pharmaceutical ingredients: the treatment of cancer has followed this tendency [2]. In our modern times, a striking example is the tubulin-targeting agent combretastatin A-4 (Fig. 1), also known as CA-4, which was originally isolated from the bark of the South African willow tree *Combretum caffrum* by G.R. Pettit in 1989 [3]. It appeared that the dynamic polymerization of α and β tubulin heterodimers [4], which would generally lead to the formation of microtubules, is efficiently inhibited by CA-4, then disrupting the cytoskeleton. This (*Z*)-stilbenoid proved to bind specifically to a region known as the colchicine binding site [5] of tubulin, a compound acting as a poison of the mitotic spindle [6]. Consequently, CA-4 triggers a G2/M cell cycle phase arrest, initiating an apoptotic program and, remarkably, its water-soluble phosphate and serinol derivatives fosbretabulin and ombrabulin are responsible of vascular targeting and disrupting abilities [7].

Many CA-4 analogues have been described [8], mainly focusing on B ring modifications (e.g. heterocyclic derivative **1** [9], Fig. 1) and central (*Z*) double bond variations (e.g. compound **2** [10], Fig. 1). Indeed, it is also established that CA-4 tends to be unstable *in vivo* [11] and an impeding (*Z*) to (*E*) isomerization phenomenon was observed, (*E*)-CA-4 being thermodynamically more stable but less active than its diastereoisomer [12]. Though this stereoconversion is not deleterious for clinical studies, there is definitely an interest to develop CA-4 derivatives overcoming this obstacle. Numerous strategies were imagined to this purpose, as for instance the addition of substituents onto the central double bond [13], its replacement with heterocyclic rings [14], a ketone [15] (phenstatin derivatives) or with an *iso*-methylene bond, leading to the so called *iso* CA-4 analogues (Fig. 1) [16].

In the work described herein, we report the synthesis and biological evaluations of a series of microtubules targeting derivatives containing either substituted thiophenes / benzo[*b*]thiophenes [17] or benzo[*d*]thiazoles instead of the CA-4 non-essential B ring (Fig. 2). In addition to the structure-activity relationship (SAR) exploration of these chalcogenic heterocycles for their tubulin polymerization inhibitory properties, a second modification was also investigated with the replacement of the (*Z*) central olefin by various structural units. Studying these different analogs and bioisosteric equivalents enables not only to assess the SARs of these tubulin polymerization inhibitors but also to expand our knowledge in designing future potentially active drugs.

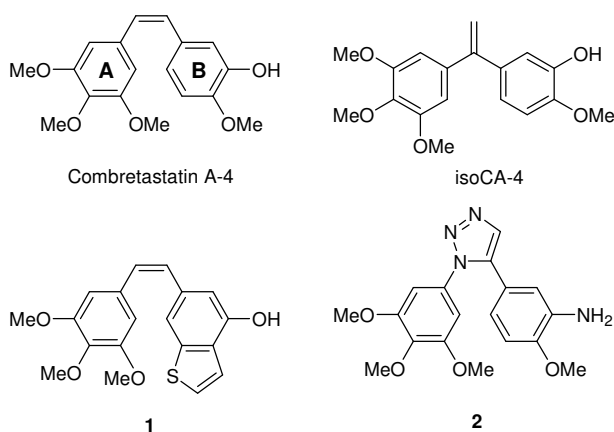


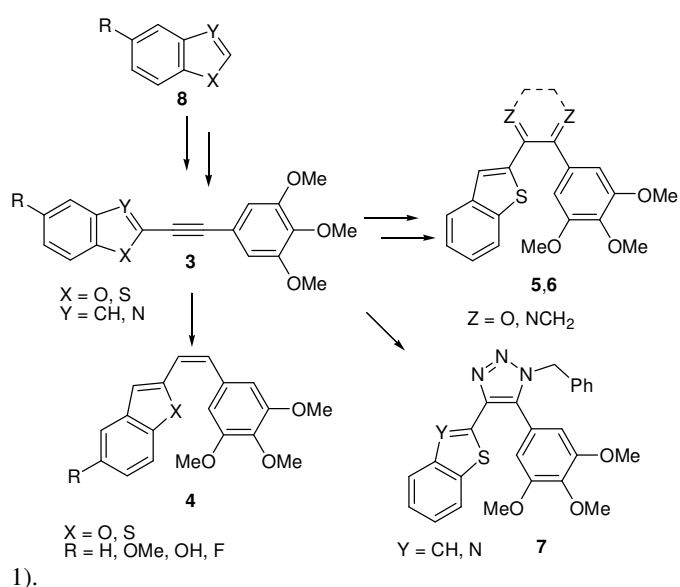
Fig. 1. Structures of CA-4, *iso*CA-4 and other reported heterocyclic analogs **1** and **2**.

2. Results and discussion

2.1. Chemistry

The syntheses of the target products depicted in this full paper required the use of different synthetic pathways, with the central position of acetylenic derivatives **3**. These compounds were previously proved to be crucial building blocks for the stereoselective synthesis of (*Z*) isomers of compounds **4** [18] and they also appeared to be helpful for the preparation of other targets such as 1,2-ethanediones **5** ($Z = O$) dihydropyrazines **6** ($Z = NCH_2$) and benzyltriazoles **7** described herein (Fig. 2).

Though some starting materials **8** such as benzo[*b*]thiophene and benzo[*d*]thiazole were commercially available, some derivatives with different substituents in the benzo[*b*]thiophene series were first prepared, following literature procedures (R groups, Scheme



1).

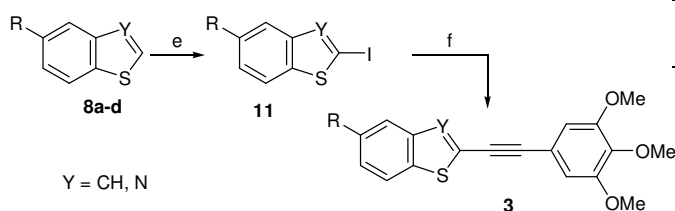
Fig. 2. General synthetic strategy for the preparation of (*Z*)-3,4,5-trimethoxystyrenes **4**, diketo **5** and heterocyclic derivatives **6** and **7**.

After alkylation of *para*-substituted thiophenols **9** to obtain the functionalized thioethers **10**, an acidic ring closure step allowed us to reach benzo[*b*]thiophenes **8a,b** [19]. 5-Methoxy derivative **8b** was then subjected to BBr_3 -mediated demethylation [19e] and the resulting hydroxyl group was protected as a *tert*-butyldimethylsilyl (TBDMS) ether **8c** (Scheme 1).

Scheme 1. Reagents and conditions: (a) bromoacetaldehyde diethyl acetal, K_2CO_3 , acetone, rt, 18 h, 69% for **10a** (R = F), 96% for **10b** (R = OMe). (b) Polyphosphoric acid, chlorobenzene, 130 °C, 18 h, 60% for **8a** (R = F), 85% for **8b** (R = OMe). (c) $BBr_3 \cdot Me_2S$, chlorobenzene, 130 °C, 5 h, 78%. (d) TBDMSO, imidazole, DMF, 90°C, 3 h, 84%.

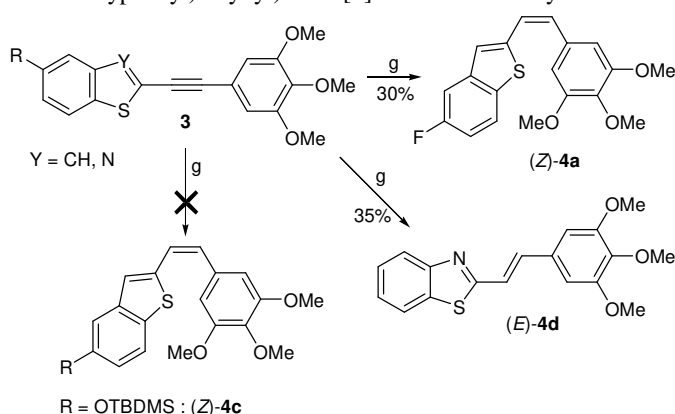
According to our synthetic strategy (Fig. 2), the preparation of diarylacetylenes **3** was crucial and involved a Sonogashira coupling reaction [20] between 5-ethynyl-1,2,3-trimethoxybenzene and heterocyclic iodo derivatives. These last substrates were prepared using a selective deprotonation of heterocyclic rings **8a-d** in the presence of the mixed aggregate reagent lithium 2,2,6,6-tetramethylpiperidide (TMPLi)/ $ZnCl_2$ (*in situ* generated by the mixture of $ZnCl_2 \cdot TMEDA$ and TMPLi) [21] followed by iodine quenching, giving compounds **11** (2-iodobenzo[*d*]thiazole or substituted 2-iodobenzo[*b*]thiophenes) in very good to quantitative yields (Scheme 2).

Then, the palladium-catalyzed cross-coupling was carried out between the aryl iodides **11** and 5-ethynyl-1,2,3-trimethoxybenzene which resulted in the formation of a variety of alkynes **3** in moderate to excellent yields (Scheme 2 and Table 1).

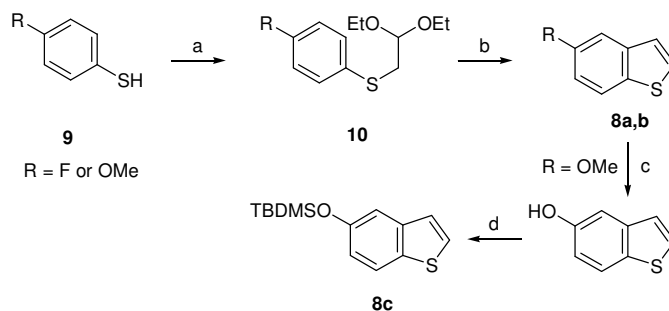


Scheme 2. Reagents and conditions: (e) 1) 2,2,6,6-tetramethylpiperidine, *n*-BuLi / $ZnCl_2 \cdot TMEDA$, THF, 15 min at 0°C then 2 h at rt. 2) I_2 in THF, rt, 18 h, 99% for **11a** (Y = CH, R = F), 83% for **11b** (Y = CH, R = OMe), 82% for **11c** (Y = CH, R = OTBDMS), 85% for **11d** (Y = N, R = H). (f) $PdCl_2(PPh_3)_2$ 2 mol%, CuI 4 mol%, Et_3N , THF, rt, 19 h, 67% for **3a** (Y = CH, R = F), 88% for **3b** (Y = CH, R = OMe), 95% for **3c** (Y = CH, R = OTBDMS), 66% for **3d** (Y = N, R = H).

To achieve the synthesis of (*Z*) derivatives **4** in a stereoselective manner, we used the semi-reduction of acetylenic derivatives **3**, as previously studied [18]. The use of *N,N*-dimethylformamide as the molecular hydrogen source (after its *in situ* basic hydrolysis) in the presence of a catalytic amount of palladium acetate [22] was previously found to be particularly efficient in selectively obtaining (*Z*)-isomers of **4** with benzofurane, benzo[*b*]thiophene and 5-methoxybenzo[*b*]thiophene rings (Fig. 2) [18]. As such, we applied this chemo- and stereoselective method to new substrates **3a-d** and noticed heterogeneous results. Whereas the partial hydrogenation of compound **3a** resulted in the stereoselective formation of (*Z*)-5-fluorobenzo[*b*]thiophene derivative **4a**, albeit in low yield (Scheme 3) [23], the reduction of the 2-((3,4,5-trimethoxyphenyl)ethynyl)benzo[*d*]thiazole **3d** solely resulted in



the formation of (*E*)-isomer [24] of **4d** with a moderate 35%



yield (Scheme 3). Moreover, though a perfect selectivity for the (*Z*) stereoisomer was already observed with the 5-methoxybenzo[*b*]thiophene substrate **3b** [18], we were surprised to see that the reaction was not complete (39% of desilylated starting material remained after a 6 hours reaction time, based on LC/MS analysis of the crude product) when carried out on silyl ether **3c** and gave a mixture among which the deprotected compound **4e** (R = OH) was detected (with a 92/8 selectivity in the favor of the (*Z*) isomer, according to LC/MS data analysis, based on MH^+ molecular ions).

Scheme 3. Reagents and conditions: (g) $Pd(OAc)_2$ 2 mol%, KOH 1.5 eq., DMF, sealed tube, 145°C, 6 h.

Table 1. Preparation of iodo derivatives **11** and diarylacetylenes **3**.

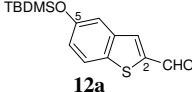
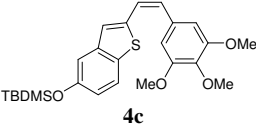
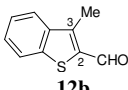
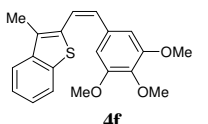

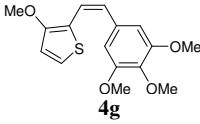
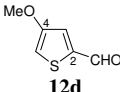
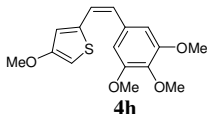
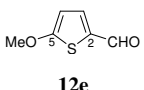
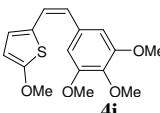
Entry	Iodination products 11	Yield %	Sonogashira coupling products 3	Yield %
1		99		67
2		83		88 ^a
3		82		95
4		85		66

^a The Sonogashira coupling between 5-ethynyl-1,2,3-trimethoxybenzene and 5-methoxy-2-bromobenzo[*b*]thiophene gave compound **3b** in a lower 30% yield [18].

This last result prompted us to investigate other synthetic approaches to reach the target compound **4c** and, despite of its reported lower selectivity, the Wittig reaction [25,26] was chosen for a straightforward access to this compound and other derivatives, necessary to complete the SAR study (Scheme 4 and Table 2). After the preparation of the appropriate aldehydes **12** in different series, using a deprotonation at position 2 of thiophenes or benzo[*b*]thiophenes **13** followed by electrophilic quenching with DMF, these building blocks were engaged in a Wittig reaction with 3,4,5-trimethoxybenzyl triphenylphosphonium salt, using *n*-BuLi as a base. These Wittig conditions led to the formation of a (*Z*)/(*E*) stereoisomers mixture of the expected 3,4,5 trimethoxystyrene derivatives **4c,f-i** (Scheme 4 and Table 2).

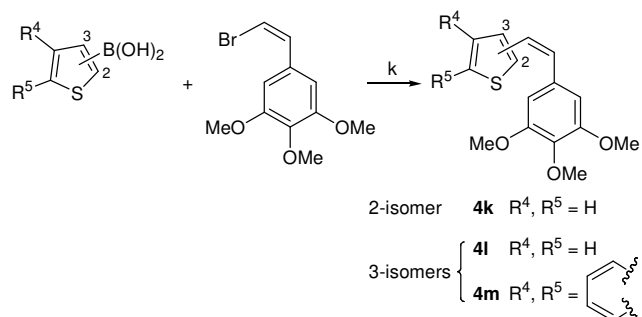
Scheme 4. Reagents and conditions: (h) *n*-BuLi 1.1 equiv, THF, -78°C, 1h then addition of DMF (1.2 equiv), -78°C, 20 min then rt, 2h, 37-83%. (i) (1) 3,4,5-Trimethoxybenzyltriphenylphosphonium bromide 1.0 equiv, THF, *n*-BuLi, -78°C, 30 min, then rt, 1h. (2) Aldehyde **12** addition, THF, -78°C, 1h then rt, 24h, 14-47%. (j) *n*-Bu₄NF, THF, rt, 1h, 63%.

Table 2. Preparation of aldehydes **12** and (*Z*)-ethylene derivatives **4**.

Entry	Aldehydes 12	Yield %	Wittig products 4 (isolated yield for (<i>Z</i>))	Yield % ^a
1		81		33
2		73		21
3		47		47
4		37		23
5		83		14

^a Isolated yield for (*Z*) product.

Depending on the substrates **12a-e**, the regioselectivity of the olefin formation was variable: we were strictly focused on the development of (*Z*)-stereoisomers, isolated after tedious purifications by flash chromatography on silica gel (Table 2). As a result, a small library of original compounds, including the hydroxyl derivative **4e**, obtained after fluoride-mediated

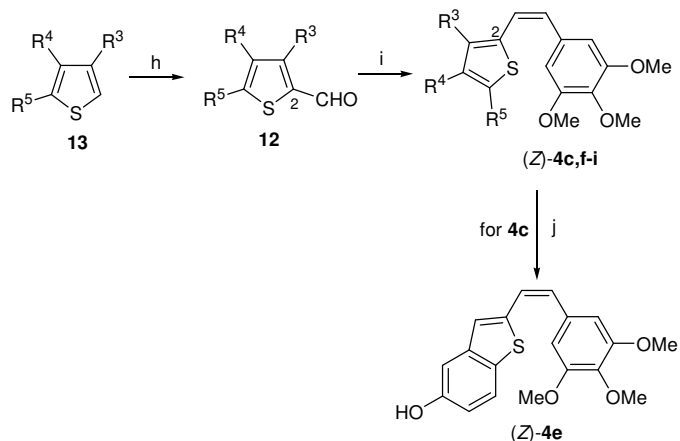


desilylation on **4c** (Scheme 4) was prepared using this approach, involving a Wittig reaction.

The preparation of the other (*Z*)-ethylenic derivatives **4k-m** that are described in this paper relies on a Suzuki coupling reaction between 2- or 3-(benzo)thiophene boronic acids and stereodefined (*Z*)-1-(2-bromovinyl)-3,4,5-trimethoxybenzene (Scheme 5) [18,27].

Scheme 5. Reagents and conditions: (k) Pd(PPh₃)₄ 5 mol%, Na₂CO₃ 1.0 equiv, DME/water, 90 °C, 20 h, 38% for **4k**, 59% for **4l**, 28% for **4m**.

To complete the SARs for these (benzo)thiophene derivatives,



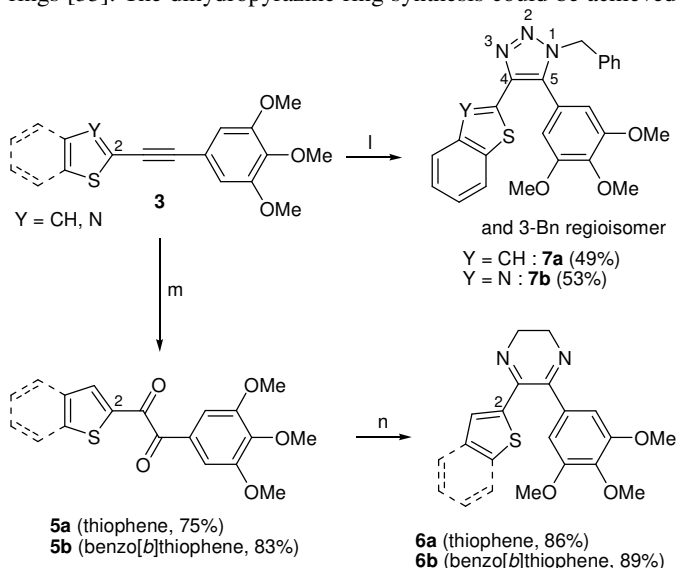
some compounds aiming to modify the central (*Z*) double bond were prepared and the acetylenic derivatives **3** proved to be of real interest to this purpose. Indeed, when engaged in different reactions, the triple bond is an interesting platform to enhance the molecular diversity.

The design of 1,2,3-triazole-containing combretastatin analogues [28] have been reported to exhibit interesting antiproliferative activities, sometimes almost equal to that of CA-4 (e.g. compound **2** [10] in Fig. 1). This modification on the central part of **2** is known to evade the isomerization issue. To achieve the synthesis of such analogues in our sulfur-containing heterocycles series, we performed a 1,3-dipolar Huisgen cycloaddition, which is a well-described high yielding method to access triazoles [29]. After an unsuccessful attempt to add benzyl azide on substrates **3** with a copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) [29b], maybe due to the use of an internal alkyne, this “click” reaction was carried out under thermal conditions [30]. Consequently, we attempted this procedure on substrates **3d** (benzo[*d*]thiazole ring) and **3e** (benzo[*b*]thiophene ring), which respectively led to triazole derivatives **7a** and **7b** with moderate yields, as regioisomers' mixture (Scheme 6). In order to obtain stereopure triazoles, mixture **7a** was further purified by flash column chromatography to give stereopure fractions **7aa** and **7ab** which were characterized using NOESY experiments (Supplementary Figure S1).

Additionally, Alami and co-workers [31] introduced the benzil-derived CA-4 derivatives as a replacement of the ethylenic bridge. The study of such compounds suggested that the replacement by two carbonyl functions would prevent the isomerization issue while maintaining the sp² hybridization and the appropriate carbon-carbon distance between the two aromatic rings. They also presented a synthetic approach consisting in the oxidation of diarylalkynes with DMSO, in the presence of a FeBr₃ promoter [32]. We performed our experiment by gradually heating the reaction mixture with an oil bath. As a result, we

efficiently synthesized compounds **5a,b** with very good 75% and 83% yields, respectively (Scheme 6).

Next, as an extension of our pharmacomodulation studies of these sulfur heterocyclic analogues, starting from the 1,2-diarylketones analogs, we decided to investigate the effect of the dihydropyrazine moiety on the biological activities of CA-4 analogs. Indeed, whilst numerous 5-membered nitrogen (poly)cyclic ring containing derivatives are described in the literature [33,34], little is known about the impact of 6-membered rings [35]. The dihydropyrazine ring synthesis could be achieved



through different chemical processes [36,37]: thus, we submitted compounds **5a,b** to a solution of ethylenediamine in anhydrous ether during 3 hours, the mixture being warmed from 0 to 30°C. The reactive mechanism relies on cyclization of both ketones with ethylenediamine and water elimination, leading to the formation of the dihydropyrazine moiety with excellent yields (86% for **6a** and 89% for **6b**, Scheme 6).

Scheme 6. Reagents and conditions: (l) Benzyl azide, 100°C, sealed tube, 17 h. (m) FeBr₃ 10 mol%, DMSO, 140°C, 23 h. (n) Ethylenediamine, Et₂O, 0°C, then 30°C, 6 h.

In parallel to these modulations needed to understand more deeply the SARs, we have also proceeded to the replacement of the ethylenic (*Z*) double bond by an *isomethylene* one. This structural modification has been described in the literature for CA-4, simultaneously by Alami [16a] and Pelaez [16b] groups who named these derivatives *isoCA-4*.

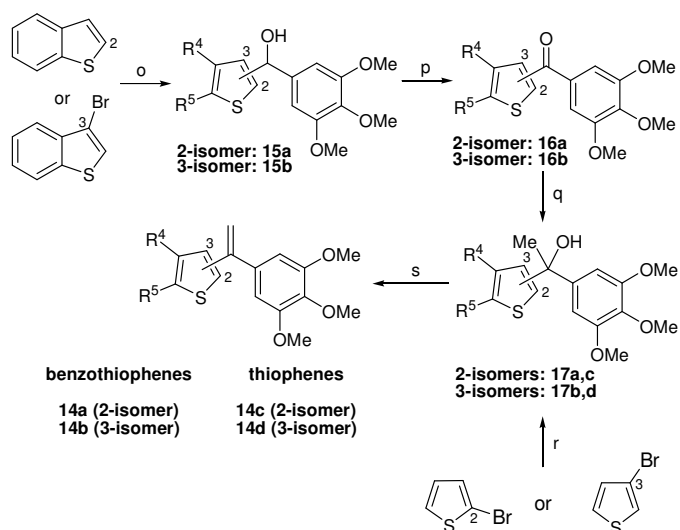
To do so for the benzothiophene series, we based our synthetic strategy on a 4 steps sequence leading to the desired *isomethylene* derivatives **14a,b** (Scheme 7 and Fig. 3), that also yielded the ketones **16a,b** for biological evaluations. This

approach, which was originally described by our group in a former preliminary communication [38], starts with the electrophilic trapping of 2- or 3-aryllithium species (generated after deprotonation at position 2 or bromine/lithium exchange at position 3 with *n*-BuLi) by 3,4,5-trimethoxybenzaldehyde (Scheme 7). This process led to secondary alcohols **15a,b**, which were then oxidized in the corresponding ketones **16a,b** [39] using an excess of manganese oxide in acetonitrile (Scheme 7).

The subsequent addition of the methyl magnesium iodide Grignard reagent in THF resulted in the formation of methylcarbinols **17a,b**.

Alternatively, tertiary alcohols **17c,d** (being grafted at positions 2 and 3 of the thiophene ring, respectively) were prepared in one step by trapping the 2- or 3-thiophenyl lithium species by the methyl ketone 1-(3,4,5-trimethoxy-phenyl)-ethanone in good 67 and 76% yields, respectively.

These tertiary alcohols **17a-d** were finally dehydrated to give the desired *isoCA-4* compounds **14a-d** (Fig. 3) with moderate to good overall yields, over 2 or 4 steps, depending on the heterocyclic scaffold.



Scheme 7. Reagents and conditions: (o) For **15a**: *n*-BuLi 1.3 equiv, Et₂O, 0°C, 1.5 h then addition of 3,4,5-trimethoxybenzaldehyde (1.3 equiv), 0°C, 3 h, 85%. For **15b**: *n*-BuLi 1.2 equiv, Et₂O, -70°C, 1 h then addition of 3,4,5-trimethoxybenzaldehyde (1.3 equiv), -70°C, 1 h then rt, 4 h, 82%. (p) MnO₂ 6 equiv, CH₃CN, rt, 4 h, 99% for **16a** and 80% for **16b**. (q) MeMgI 3 equiv, THF, 0°C, 30 min then rt, 15 min then 75°C, 2 h, 95% for **17a** and 92% for **17b**. (r) *n*-BuLi 1.2 equiv, Et₂O, -70°C, 1 h then addition of 3,4,5-trimethoxyacetophenone (1.3 equiv), -70°C, 1 h then rt, 4 h, 67% for **17c**, 76% for **17d**. (s) *p*-TsOH 5 mol%, toluene, reflux, 2 h for **14a**, 93%, 1 h for **14b**, 89%, 2 h for **14c**, 98%, 2 h for **14d**, 94%.

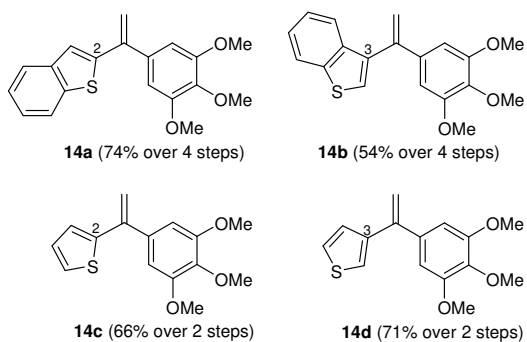


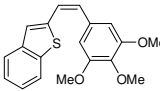
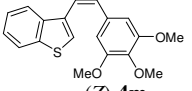
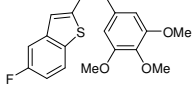
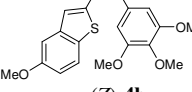
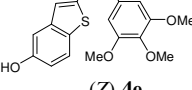
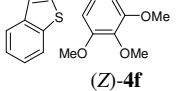
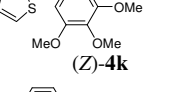
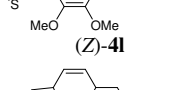
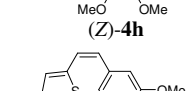
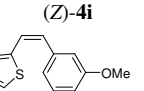
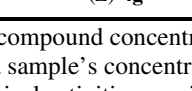
Fig. 3. 1,1-diarylethylene derivatives **14a-d** (overall yield for the whole sequences under brackets).

2.2. Biological evaluations

The purpose of this article was to complete our knowledge in the biological effects of thiophene/benzothiophene CA-4 analogues and, consequently, the majority of the prepared compounds were evaluated *in vitro*.

First, the heteroaromatic products were tested for their ability to inhibit tubulin assembly into microtubule, using the *in cellulo* assay described by Vassal *et al.* [40]. This test is based on double-immunofluorescence staining using antibodies specific to tyrosinated and detyrosinated tubulin and allows the detection of stabilizing agents such as paclitaxel or docetaxel and destabilizing drugs like the well-known colchicine or CA-4.

Table 3. Biological evaluations of (Z)-ethylenic derivatives **4**.

Entry	compound	Tubulin polymerization inhibition EC ₅₀ ^a	Antiproliferative effects					
			IC ₅₀ ^b					
			IC8 Melanoma	MES-SA Uterine sarcoma	MES-SA Dx5 Uterine sarcoma	RL Lymphoma	A2780 Ovarian carcinoma	MIA PaCa-2 Pancreatic carcinoma
1	colchicine	200 nM ^{d0}	15 nM ^c	15 nM ^c	757 nM	4.4 nM	14 nM	6.1 nM
2	CA-4	9 nM ^c	4 nM ^c	3 nM ^c	3 nM	1.4 nM	2.5 nM	2.1 nM
3	 (Z)-4j	80 nM ^c	17 nM ^c	13 nM ^c	19 nM	7.6 nM	7.9 nM	7.8 nM
4	 (Z)-4m	2430 nM ^c	288 nM ^c	191 nM ^c	288 nM	n.d.	n.d.	n.d.
5	 (Z)-4a	523 nM	n.d.	n.d.	n.d.	38 nM	200 nM	200 nM
6	 (Z)-4b	62% at 2 μM ^d 14% at 1 μM ^d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	 (Z)-4e	717 nM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	 (Z)-4f	555 nM	n.d.	n.d.	n.d.	61 nM	360 nM	120 nM
9	 (Z)-4k	> 10 μM ^c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	 (Z)-4l	> 10 μM ^c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	 (Z)-4h	81 nM ^c	27 nM ^c	25 nM ^c	27 nM	11 nM	13 nM	14 nM
12	 (Z)-4i	1558 nM ^c	76 nM ^c	53 nM ^c	75 nM	n.d.	n.d.	n.d.
13	 (Z)-4g	> 10 μM	n.d.	n.d.	n.d.	> 1 μM	> 1 μM	> 1 μM

^a EC₅₀: compound concentration required to decrease dynamic, tyrosinated microtubules content by 50%.

^b IC₅₀: a sample's concentration which produces a 50% reduction in cell activity.

^c Biological activities previously described by our group [38].

^d Percentage of Tubulin Polymerization Inhibition (TPI) at the given concentration, determined on isolated protein, according to ref. [40]. For comparison, TPI on isolated tubulin for benzo[*b*]thiophene (Z)-4j: IC₅₀ = 2.6 μM [17b].

n.d.: not determined.

Beside tubulin polymerization inhibition (TPI) assays carried out on isolated protein, which present the major drawback that they do not give any insight about the cellular response, this assay gives directly information on both the efficient penetration of compounds within cells and intracellular microtubules' formation [42].

We used colchicine, CA-4 and our previously described derivative **4j** as reference compounds (Table 3, entries 1-3). In previous communications, we have indeed shown that the more potent analogs were obtained in the benzo[*b*]thiophene, rather than benzofuran and indole series [17b] but also indicated that the grafting on position 2 of the sulfur-containing rings was essential for the TPI activity (Table 3, entries 3 *versus* 4) [38]. In this full paper, we wish to report on the effect of the substitution of this benzo[*b*]thiophene ring and some other structural modifications such as the replacement of the central double bond, by e.g. nitrogen heterocycles (dihydropyrazine and benzyl triazole units).

The substitution of the benzo[*b*]thiophene at position 5 was first studied by adding various groups. The introduction of the electro-donating methoxy group resulted in a dramatic loss of activity (Table 3, entry 6) whereas the hydroxy derivative (*Z*)-**4e** have shown a TPI EC₅₀ equal to 717 nM (Table 3, entry 7), albeit lower than the unsubstituted compound (*Z*)-**4j** (TPI IC₅₀ = 80 nM). A slight improvement was noticed with the isosteric fluorine atom as TPI potency for (*Z*)-**4a** (EC₅₀ = 523 nM, Table 3, entry 5), compared to (*Z*)-**4e**.

The introduction of a methyl group at position 3 of the benzo[*b*]thiophene ring, that could modify the dihedral angle between the two aromatic ring planes A and B [43], was also envisaged in (*Z*)-**4f**, but was found to be disappointing as TPI activity (EC₅₀ = 555 nM, Table 3, entry 8) was lower than the parent compound (*Z*)-**4j** (EC₅₀ = 80 nM, Table 3, entry 3).

The SARs show that the fused aromatic ring was essential as thiophene analogue (*Z*)-**4k** completely lacks TPI properties (Table 3, entry 9). However, in this thiophene series, we have demonstrated the crucial effect of the methoxy substitution at an adequate 4 position as (*Z*)-**4h** displayed a much better TPI potency than its 5-regioisomer (*Z*)-**4i** (Table 3, entries 11 and 12, respectively), equal to that of benzo[*b*]thiophene derivative (*Z*)-**4j**. To complete this study, we describe herein the 2-thiophene analogue, with a methoxy group at position 3 (*Z*)-**4g**, which exhibited no TPI properties (Table 3, entry 13).

The anti-proliferative effects were then evaluated for the most active tubulin polymerization inhibitors **4** on a panel of six different cancer cell lines: IC8 (melanoma) [44] MES-SA (uterine sarcoma) and its Multi-Drug Resistant version MES-SA Dx5 [45], RL (follicular lymphoma) [46], A2780 (ovarian carcinoma) [47] and MIA PaCa-2 (pancreatic carcinoma) [48]. The designed CA-4 analogs and references were incubated for 72 hours with these cell lines and the remaining living cells were quantified using MTT [49] or resazurin [50] dying systems.

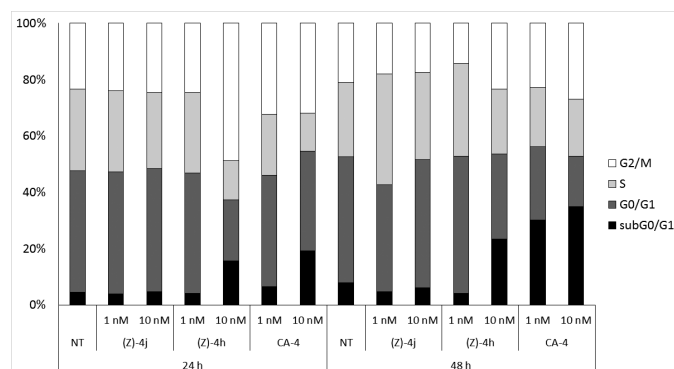
Interestingly, we have noticed a correlation between high TPI activities and low IC₅₀ values for anti-proliferative effects and, in case of the negative control (*Z*)-**4g** (TPI EC₅₀ > 10 μM), no anti-proliferative effects (IC₅₀ > 1 μM for three different cell lines) were noticed (Table 3, entry 11).

Analog (*Z*)-**4a,f** did not exhibit striking anti-proliferative properties (Table 3, entries 4 and 7), except for the more sensitive RL cell lines. However, for some other compounds, a high anti-proliferative effect was observed as IC₅₀ were below 30 nM ((*Z*)-**4h**, Table 3, entry 9) and even in the nanomolar range for our

most active compound (*Z*)-**4j** (IC₅₀ = 10 nM, Table 3, entry 3), after a 72h treatment. In this last case, activities were lower to that of CA-4 (Table 3, entry 2) but, to a lesser extent for (*Z*)-**4h**, similar to that of colchicine (Table 3, entry 1). In addition, whereas a MDR phenomenon was observed with this natural product (IC₅₀ for MES-SA Dx5 dropped to 757 nM, compared to 15 nM for the sensitive wild type cell line MES-SA), this was not observed with compounds (*Z*)-**4h,j**, as anti-proliferative activities were indeed conserved for these two derivatives in MES-SA cells expressing or not P-gp (IC₅₀ = 19 nM *versus* 13 nM and IC₅₀ = 27 nM *versus* 25 nM, respectively for MDR *versus* sensitive cells).

Selected active compounds were evaluated for their ability to induce cell cycle arrest in RL cells. Exposure to 10 nM (*Z*)-**4h** was associated with important arrest in G2/M phase (48.8% vs 23.3%) and induction of cell death (15.6% vs 4.5% in subG0/G1 phase) after 24 hours and with cell death (23.4% vs 8.0%) after 48 hours, whereas 1 nM of the same compound did not modify the cell cycle distribution (Fig. 4 and supplementary figure S3). CA-4 was used as a positive control, inducing similar cell death (19.3% vs 4.5%) at 24 hours with 10 nM, and even higher at 48 hours both with 1 nM (30.1% vs 8.0%) and 10 nM (34.9% vs 8.0%). The toxicity of (*Z*)-**4h** and CA-4 was confirmed by the low number of cells that were gated in the cell cycle at 48 hours (45-50%) as compared with unexposed cells (80-85%). We did not observe any notable activity with (*Z*)-**4j**. These results confirm that the effects of compounds observed in the MTT assay correlate both to disturbance of cell cycle and induction of cell death, as it is expected for antimicrotubular agents.

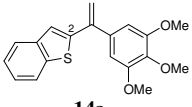
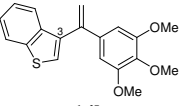
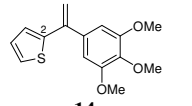
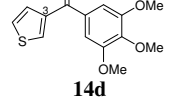
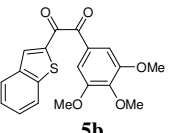
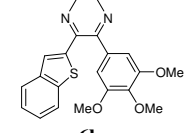
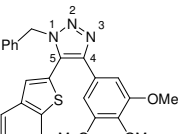
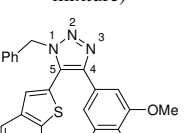
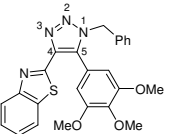
Fig. 4. Cell cycle distribution of RL cells incubated 24 hours and 48 hours in



indicated conditions. The results are mean values from two independent experiments. SubG0/G1 corresponds to apoptotic cells. NT: non treated, CA-4: combretastatin A-4.

At the same time, we also explored the biological effects of some thiophene and benzo[*b*]thiophene derivatives aiming to block the *in vivo* (*Z*) to (*E*) isomerization issue that is described for CA-4 [11] and to improve physicochemical properties such as water solubility. Among the different strategies described in the literature, this was envisaged through the replacement of the central double bond by an isomethylene bond (compounds **14a-f**), a diketone (compound **5b**) or by nitrogen heterocycles such as dihydropyrazine (compound **6b**) or benzyltriazole (compounds **7a,b**), (in which N heteroatom could be converted into hydrochloride salts to improve water solubility).

Table 4. Biological evaluations of isomethylene derivatives **14a-d**, 1,2-dione derivative **5b**, dihydropyrazine **6b** and triazoles

Entry	compound	TPI	Antiproliferative effects
		EC ₅₀ ^a	IC ₅₀ ^b
1	 14a	321 nM ^c	IC8: 101 nM ^c MES-SA: 95 nM ^c MES-SA Dx5: 150 nM
2	 14b	3897 nM ^c	n.d.
3	 14c	> 10 μM ^c	n.d.
4	 14d	> 10 μM ^c	n.d.
5	 5b	35% inhibition at 14 μM ^d	n.d.
6	 6b	14 μM ^d	n.d.
7	 7a (72/28 regioisomer mixture)	> 10 μM	RL: 560 nM A2780: > 1 μM MIA PaCa-2: 520 nM
8	 7aa (major regioisomer)	> 10 μM	RL: > 1 μM A2780: > 1 μM MIA PaCa-2: > 1 μM
9	 7b (59/41 regioisomer mixture)	2120 nM	RL: 500 nM A2780: 470 nM MIA PaCa-2: 490 nM

7a,b.

^a EC₅₀: compound concentration required to decrease dynamic, tyrosinated microtubules content by 50%.

^b IC₅₀: a sample's concentration which produces a 50% reduction in cell proliferation.

^c Biological activities previously described by our group [38].

^d Percentage of Tubulin Polymerization Inhibition (TPI) at the given concentration, determined on isolated protein, according to ref. [41].

n.d.: not determined.

In the isomethylene series, our previous study [38] has shown that both the nature of the sulfur heterocycle and its grafting to the *iso* double bond were critical, the benzo[*b*]thiophene ring substituted at the 2-position **14a** being the most interesting derivative (Table 4, entries 1 to 4), for its TPI and anti-proliferative activities. In the present study, we also demonstrated that this compound is still active on MES-SA Dx5 resistant cells (Table 4, entry 1), similarly to what was observed with (*Z*)-ethylenic compounds **4h,i,j,m**.

Other structural modifications such as a central diketone or dihydropyrazine introduction resulted in less active compounds (Table 4, entries 5 and 6).

Finally, benzyltriazoles **7a,b** were evaluated as a mixture of regioisomers and did not show high TPI or anti-proliferative activities (Table 4, entries 7 to 9). It is noteworthy that benzylated derivative **7aa**, evaluated as a sole product, was the less active regioisomer of the benzo[*b*]thiophene analog **7a**. Notably, Crooks and co-workers have reported the TPI and anti-proliferative activities of the debenzylated analog of compound **7a** and the activity range was similar to that of CA-4 [51]: the presence of a benzyl substituent on the triazole ring proved consequently to be deleterious for the TPI and antiproliferative activities.

3. Docking studies

To gain insight into the binding mode of the most active CA-4 derivatives, molecular docking was performed onto two X-ray structures of the bovine tubulin dimer, namely, tubulin bound to CA-4 (PDB ID: 5LYJ) and tubulin bound to the thiol derivative of colchicine 2-mercapto-*n*-[1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydro-benzo[*a*]heptalen-7-yl]acetamide (CN2) (PDB ID: 1SA0), respectively. Prior to docking, all ligands, cofactors and additional interacting proteins were removed from both structures. Tested compounds include CA-4, colchicine, all (*Z*)-ethylenic derivatives **4** as well as compounds **14a** and **14b**. Docking was run using multiple programs including AutoDock4 [52], AutoDock Vina [53] and DOCK6 [54], in order to increase the probability of sampling the correct pose. Selection of the top docking pose was carried out by rescoring all the generated poses with DSX scoring function [55] and extract the one with the best score. As a validation of our protocol, CA-4 and CN2 top poses were found to match perfectly their corresponding crystal pose (Fig. 5). As expected, docking scores for CA-4 and its derivative were all much higher (in absolute value) for the 5LYJ structure, where the binding pocket is suitable for the binding of CA-4, than for 1SA0, while this trend is inverted in the case of colchicine (see Table S2). Overall, we found that the binding

modes generated for all derivatives were similar to CA-4 pose, especially along their shared motif also present in CA-4 (Fig. 6).

Compared to CA-4, other interactions were identified for the evaluated compounds. Interestingly, one of the most active derivative (*Z*)-**4h** was characterized by a cation- π interaction occurring between its thiophene motif and K352 residue of the β tubulin monomer (Fig. 7). This interaction may explain the differences between the strong inhibitory activity of this compound, compared to those of (*Z*)-**4g** (inactive) and (*Z*)-**4i** (less active), in which this interaction was not noticed.

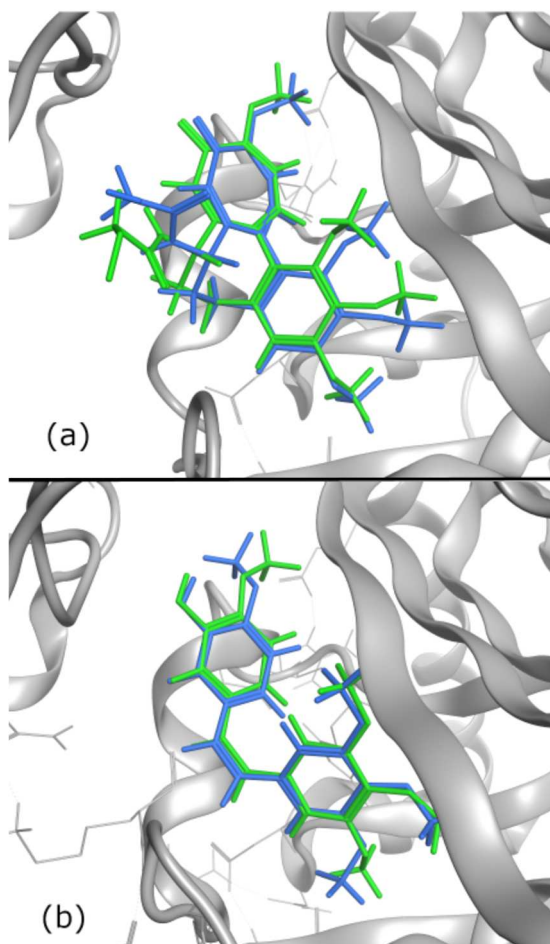


Fig. 5. (a) Comparison of top docking pose (green) and crystal pose (blue) of the colchicine analog CN2 (2-mercapto-n-[1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydro-benzof[a]heptalen-7-yl]acetamide). (b) Comparison of top docking pose (green) and crystal pose (blue) of CA-4

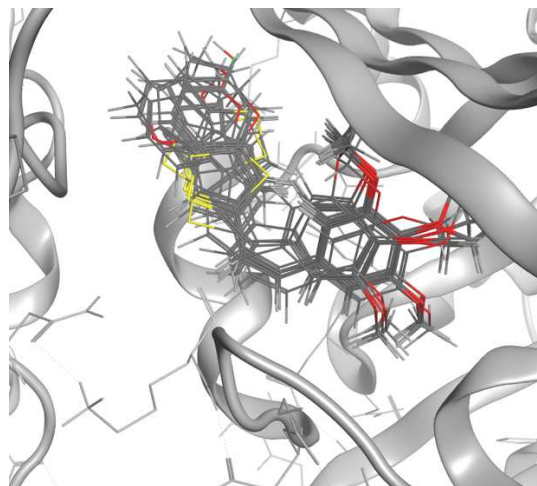


Fig. 6. Superimposition of the top docking poses of all tested derivatives. Docking revealed similar binding modes, especially around their shared motif common to CA-4.

To evaluate whether the docking scores of the tested compounds are representative of their inhibitory activity, multiple linear regression was performed using DSX and AutoDock4 scores calculated for the top docking pose of each compound. An estimate of the log of the partition coefficient ($\log P$) was also included as a variable in the regression model. A satisfactory R^2 -value of 0.77 was obtained when fitting our model with the pEC50 values reported in Table 3 and Table 4 (Fig. 8).

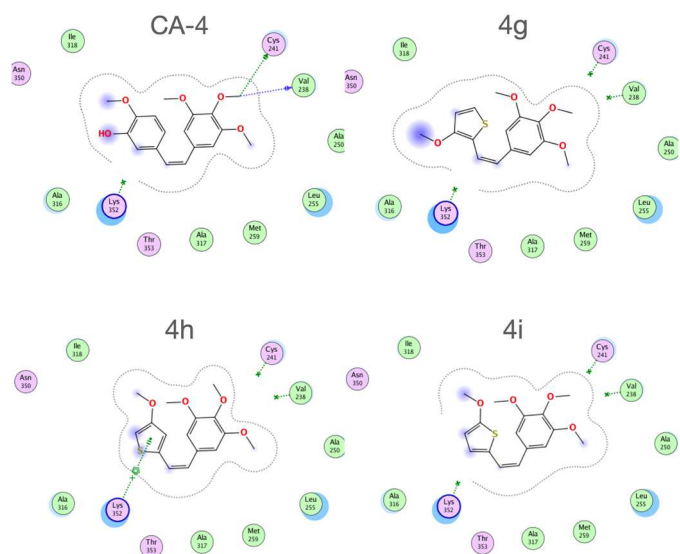


Fig. 7. Top docking pose obtained for CA-4, (*Z*)-**4g**, (*Z*)-**4h** and (*Z*)-**4i**. Docking pose with (*Z*)-**4h** shows a cation- π interaction between its thiophene motif and K352 of the β monomer. Hydrophobic interactions are not represented.

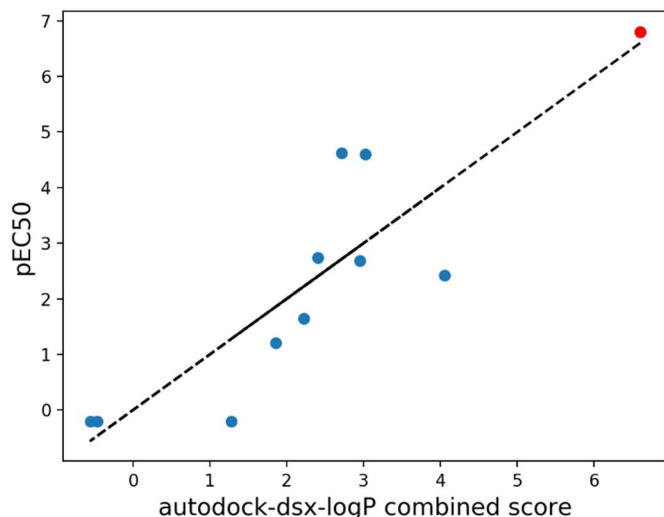


Fig. 8. Multiple linear regression showing the relationship between pEC₅₀s (dependent variable) and Autodock4 and DSX docking scores as well as log P values (independent variable). The red dot corresponds to CA-4 while blue dots stand for compounds **14a** and **14b** and all (Z)-ethylenic derivatives **4** excluding (Z)-**4b**. An R²-value of 0.77 was reported. The combined score on the x axis was computed as $\alpha S_{Autodock4} + \beta S_{DSX} + \gamma \log P$ where $\alpha = -2.63$, $\beta = -0.02$ and $\gamma = -4.19$ are coefficients calculated from the regression model.

4. Conclusion

In conclusion, our efforts in expanding the SAR knowledge of the Tubulin Polymerization Inhibition properties of this family of compounds resulted in the synthesis of 19 CA-4 analogs, including 9 compounds newly reported in this full paper. We mainly focused our work on the modification of the B ring of CA-4 but also of the olefinic central double bond.

In this last mentioned case, results were quite disappointing as none of the designed diketo, benzyltriazole and dihydropyrazine derivatives, aiming to limit the (Z) to (E) isomerization, were as/more active than the (Z) alkene.

The pharmacomodulation of the benzothiophene ring was carried out by introducing methoxy/hydroxyl/fluorine/methyl groups at different positions of this heterocyclic ring, thus resulting in less active compounds than the parent derivative **4j**. In the thiophene series, the most potent compound remains the previously described 4-methoxythiophene **4h** having a (Z)-3,4,5-trimethoxystyrene motif at its 2 position.

The biological evaluations were investigated on 19 final compounds, combining TPI properties and, for the most active ones, the determination of anti-proliferative effects on six different cell lines, including one having a MDR phenotype. We were pleased to see that compounds **4a,f,h,j** and **14a** exhibited promising anti-proliferative activities on these six cell lines, with EC₅₀ below 200 nM (**4a,f** and **14a**) or in the nanomolar range (**4h,j**) and that they were still active on a MDR resistant cell line, in contrast to the reference compound colchicine. After selection of the two most potent analogs **4h** and **4j**, cell cycle analyses were carried out on follicular lymphoma RL cells. Treatment with low 1 nM and 10 nM concentrations of these two derivatives revealed a G2/M transition blockade, similarly to other compounds of the CA-4 family.

Finally, our docking study has provided a very interesting insight into the docking modes of the prepared compounds, compared to

both CA-4 and the sulfur analogue of colchicine CN2, within the two described X-ray crystallographic structures 1SA0 and 5LYJ of tubulin. Furthermore, a correlation between pEC₅₀ values and a score built out of LogP values combined with Autodock4 and DSX docking scores was established. We expect that our model could provide strong support for the prediction and the design of other inhibitors of tubulin polymerization targeting the same binding site.

Acknowledgements

Authors would like to thank Dr. Sylviane Thoret and Ms. Joelle Dubois for preliminary tubulin polymerization studies of compounds **4b**, **5b** and **6b**.

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Supplementary data

Supporting information associated to this article contains ¹H and ¹³C NMR spectra of chemical intermediates **11a-c**, **3a,c,d**, **12a**, **4c**, **15a,b**, **16a,b**, **17a-d**, final compounds **4a,d-i,j,l,m**, **14c,d**, **5a,b**, **6a,b**, **7aa**, **7ab**, **7b**, **14a-d**, NOESY spectra of **7aa** and **7ab** and DSX/Autodock4 scores of colchicine derivative CN2 and CA-4.

This supporting information also include effect/dose curves for tubulin polymerization inhibition, anti-proliferative effects (MES-SA, MES-SA/Dx5, RL, A2780, MiaPaCa-2 cells) for synthesized compounds and cell cycle distribution diagrams for RL cells upon treatment with (Z)-**4h**, (Z)-**4j** and CA-4.

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5. Experimental section

5.1. Chemistry - General

All moisture/air-sensitive reactions were carried out under a positive pressure of argon and with oven-dried glassware. Melting points were measured on a Büchi B-540 melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on Bruker ALS300, DRX300 and DRX400 Fourier transform spectrometers, using an internal deuterium lock, operating at 300 or 400 MHz and carbon nuclear magnetic resonance (^{13}C NMR) on a Bruker AV400 spectrometer using an internal deuterium lock, operating at 100 MHz. Chemical shifts are reported in parts per million (ppm) relative to internal standards (tetramethylsilane, $\delta_{\text{H}} = \delta_{\text{C}} = 0.00$; CDCl_3 , $\delta_{\text{H}} = 7.26$ and $\delta_{\text{C}} = 77.16$; $\text{DMSO}-d_6$, $\delta_{\text{H}} = 2.50$ and $\delta_{\text{C}} = 39.52$; acetone- d_6 , $\delta_{\text{H}} = 2.05$ and $\delta_{\text{C}} = 29.84$ [56]. Data are presented as follows: chemical shift (δ , ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quadruplet, dd = doublet of doublet, m = multiplet, br = broad), coupling constant, integration. Atom numbering refers to thiophene and benzo[*b*]thiophene nomenclature.

Agilent uHPLC/MS consists of a 1290 Infinity system with a binary pump, degasser, autosampler, thermostated column compartment, 1260 Diode Array Detector and 6120 single quadrupole mass spectrometer. The entire system was controlled by Chemstation software (Agilent technologies). The column was an Agilent Poroshell 120 SB-C18, 2.7 μm , 2.1x50 mm. The samples were analysed in the positive ion mode of the Electro Spray Ionisation (ESI) source, whose conditions were as follow: gas temperature, 350°C, drying gas at 12.0 l/min, nebulizer gas at 35 psig, Vcap at 3000 V, fragmentor at 60 V.

High-Resolution Mass Spectra were recorded on a Bruker MicroTOF Q (ESI mode), QTOF Impact II (ESI mode), Thermoquest Finnigan MAT 95 XL spectrometer (chemical ionization CI mode with isobutane) or on Agilent 7200 GC-MS QTOF (electronic impact EI mode).

Reactions were monitored with analytical Thin Layer Chromatography (TLC), which was carried out using Merck commercial aluminium sheets coated (0.2 mm layer thickness) with Kieselgel 60 F₂₅₄, with visualization by ultraviolet and anisaldehyde stain solution. Proportions of solvents used for TLC are by volume. Product purification by flash column chromatography was performed using Merck Kieselgel 60 Å (40-63 mm). Proportions of solvents used for column chromatography are by volume. Tetrahydrofuran (THF), *N,N*-Dimethylformamide (DMF), dimethylsulfoxide (DMSO) (Acroseal[®], over molecular sieves) are purchased by Acros Organics. For extraction/purification, diethyl ether, dichloromethane, cyclohexane and ethyl acetate (AcOEt) were of reagent grade. All other chemical reagents were used as received. Commercial *n*-BuLi solutions in hexanes were titrated using *N*-benzylbenzamide [57].

Final compounds (*Z*)-**4b**, (*Z*)-**4j** and (*Z*)-**4k** were prepared according to literature procedures from our group [18]. 5-Fluorobenzo[*b*]thiophene **8a** [19a,b], 5-methoxybenzo[*b*]thiophene **8b** [19c,d], benzo[*b*]thiophen-5-ol [19e], (benzo[*b*]thiophen-5-yloxy)-*tert*-butyl-dimethyl-silane **8c** [58], $\text{ZnCl}_2 \cdot \text{TMEDA}$ [59], 3,4,5-trimethoxybenzyltriphenylphosphonium bromide [60], 2-(3,4,5-trimethoxyphenylethynyl)-thiophene [18], 2-((3,4,5-trimethoxyphenyl)ethynyl)benzo[*b*]thiophene **3e** [18], were prepared according to literature procedures.

5.2. General procedure A for the preparation of heteroaryl iodides **11** using a zinc/lithium mixed aggregate 2-deprotonation and subsequent iodination

To a stirred, cooled (0°C) solution of 2,2,6,6-tetramethylpiperidine (TMP) (0.55 ml, 3.0 mmol) in THF (2.5 ml) were successively added *n*-BuLi (1.2 ml of a 2.5 M hexane solution, 3.0 mmol) and, 5 min later, $\text{ZnCl}_2 \cdot \text{TMEDA}$ (0.250 g, 1.0 mmol). The mixture was stirred for 15 min at 0 °C before introduction of the benzo[*b*]thiophene derivative (2.0 mmol) at 10°C. After 2 h at room temperature, a solution of I_2 (0.76 g, 3.0 mmol) in THF (5 ml) was added. The mixture was stirred overnight before addition of an aqueous saturated solution of $\text{Na}_2\text{S}_2\text{O}_3$ (2 mL) and extraction with EtOAc (3 × 10 ml). The combined organic layers were dried over Na_2SO_4 , filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (EtOAc / cyclohexane) to give the desired product.

5.2.1. Preparation of 2-iodo 5-fluoro benzo[*b*]thiophene **11a**

Following general procedure A, scale: 5-fluorobenzo[*b*]thiophene (0.350g, 2.0 mmol), TMP (0.55 ml, 3.0 mmol) in THF (2.5 ml) were successively added *n*-BuLi (1.2 ml, 3.0 mmol) and, 5 min later, $\text{ZnCl}_2 \cdot \text{TMEDA}$ (0.253 g, 1.0 mmol), I_2 (0.761 g, 3.0 mmol) in THF (5 ml). The crude product was purified by flash chromatography (EtOAc / cyclohexane 5 / 95) to give the desired product (0.550 g, 99 %) as an orange solid, mp 98-99°C. ^1H NMR ($\text{DMSO}-d_6$, 400 MHz): $\delta = 7.21$ (td, $J = 8.9 - 2.6$, 1H), 7.61 (dd, $J = 9.8 - 2.5$, 1H), 7.68 (m, 1H), 7.98 (dd, $J = 8.8 - 5.0$, 1H). ^{13}C NMR ($\text{DMSO}-d_6$, 100 MHz): $\delta = 84.25$ (C), 107.77 (d, $J = 23.4$, CH), 112.84 (d, $J = 25.1$ Hz, CH), 123.18 (d, $J = 9.4$ Hz, CH), 133.23 (CH), 139.46 (C), 141.77 (d, $J_{\text{C-F}} = 9.7$ Hz, C), 161.11 (d, $J_{\text{C-F}} = 240.4$ Hz, C). HRMS (EI): m/z calcd for $\text{C}_8\text{H}_4\text{FIS} [\text{M}^+]$: 277.9057; found: 277.9060 (done with GCMS).

5.2.2. Preparation of 2-iodo-5-methoxybenzo[*b*]thiophene **11b**

Following general procedure A, scale: 5-methoxybenzo[*b*]thiophene (0.310 g, 1.90 mmol), TMP (0.52 ml, 2.83 mmol), THF (7 ml), *n*-BuLi (1.14 ml, 2.83 mmol), $\text{ZnCl}_2 \cdot \text{TMEDA}$ (0.238 g, 0.95 mmol), I_2 (0.718 g, 2.83 mmol). The crude product was purified by flash chromatography (EtOAc / cyclohexane 10 / 90) to give the desired product (0.452 g, 83 %) as an orange solid, mp 94-95°C. ^1H NMR (400 MHz, acetone- d_6): $\delta = 3.84$ (s, 3H), 6.98 (dd, $J = 8.9 - 2.5$, 1H), 7.35 (d, $J = 2.5$, 1H), 7.60 (s, 1H), 7.77 (d, $J = 8.9$, 1H). ^{13}C NMR (acetone- d_6 , 100 MHz): $\delta = 55.79$ (CH_3), 79.94 (C), 105.50 (CH), 115.50 (CH), 122.78 (CH), 134.79 (CH), 137.43 (C), 142.98 (C), 158.73 (C). HRMS (EI): m/z calcd for $\text{C}_9\text{H}_7\text{IOS} [\text{M}^+]$: 289.9257; found: 289.9262 (done with GCMS).

5.2.3. Preparation of *tert*-butyl-(2-iodobenzo[*b*]thiophen-5-yloxy)-dimethylsilane **11c**

Following general procedure A, scale: benzo[*b*]thiophen-5-yloxy(*tert*-butyl)dimethylsilane (0.313 g, 1.18 mmol), TMP (0.33 ml, 1.77 mmol), THF (3.5 mL), *n*-BuLi (0.72 mL, 1.77 mmol), $\text{ZnCl}_2 \cdot \text{TMEDA}$ (0.150 g, 0.59 mmol), I_2 (0.450 g, 1.77 mmol). The crude product was purified by flash chromatography (EtOAc / cyclohexane 5 / 95) to give the desired product (0.378 g, 82 %) as a yellow solid, mp 62-63°C. ^1H NMR (CDCl_3 , 400MHz): $\delta = 0.27$ (s, 6H), 1.06 (s, 9H), 6.69 (dd, $J = 7.8 - 0.9$, 1H), 7.18 (t, $J = 7.8$, 1H), 7.33 (dd, $J = 7.8 - 0.9$, 1H), 7.50 (s, 1H). ^{13}C NMR (CDCl_3 , 100 MHz): $\delta = -4.12$ (CH_3), 18.34 (C), 25.80

(CH₃), 78.77 (C), 112.85 (CH), 115.53 (CH), 125.89 (CH), 134.41 (CH), 136.83 (C), 142.85 (C), 149.32 (C). HRMS (ESI⁺): m/z calcd for C₁₄H₂₀IOSSi [MH⁺]: 391.0043; found: 391.0049.

5.2.4. Preparation of 2-iodobenzo[d]thiazole **11d**

Following general procedure A, scale: benzo[d]thiazole (0.541 g, 4.0 mmol), TMP (1.1 ml, 6.0 mmol), THF (15 ml), *n*-BuLi (2.4 ml, 6.0 mmol), ZnCl₂·TMEDA (0.505 g, 2.0 mmol), I₂ (1.523 g, 6.0 mmol). The crude product was purified by flash chromatography (EtOAc / cyclohexane 10 / 90) to give the desired product (0.882 g, 85 %) as an orange solid. ¹H NMR (CDCl₃, 300 MHz): δ = 7.29–7.39 (m, 2H), 7.77 (d, J = 8.0, 1H), 7.96 (d, J = 7.7, 1H). Spectral data were in agreement to those reported in the literature [21].

5.3. General procedure B for the preparation of the acetylenic derivatives **3** using a Sonogashira coupling reaction

To a solution of 2-iodo-heteroaryl derivative (2.0 mmol) and PdCl₂(PPh₃)₂ (0.028 g, 0.04 mmol) in THF (12 ml) under an argon atmosphere were added CuI (0.015 g, 0.08 mmol), triethylamine (0.36 ml, 2.6 mmol) and then 5-ethynyl-1,2,3-trimethoxybenzene (0.461 g, 2.4 mmol). After stirring for 19 h at room temperature, the reaction mixture was filtered over a sintered-glass filter and washed with THF (2 x 20 mL). The combined organic layers were concentrated under vacuum. The crude brown oil was purified by column chromatography (ethyl acetate / cyclohexane).

5.3.1. Preparation of 5-fluoro-2-((3,4,5-trimethoxyphenyl)ethynyl)benzo[b]thiophene **3a**

Following general procedure B, scale: 2-iodo-5-fluorobenzo[b]thiophene (0.500 g, 1.8 mmol), PdCl₂(PPh₃)₂ (0.025 g, 0.04 mmol), CuI (0.014 g, 0.07 mmol), THF (11 ml), Et₃N (0.32 ml, 2.34 mmol), 5-ethynyl-1,2,3-trimethoxybenzene (0.414 g, 2.16 mmol). The crude product was purified by silica gel flash column chromatography (AcOEt / cyclohexane 10 / 90) to afford the desired product as a yellow powder (0.411 g, 67 %), mp 102–103°C. ¹H NMR (400 MHz, DMSO-*d*₆): δ = 3.72 (s, 3H) 3.83 (s, 6H), 6.93 (s, 2H), 7.29 - 7.37 (m, 1H), 7.63 - 7.70 (m, 2H), 8.01 (dd, J = 8.8 - 5.0, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ = 56.07 (CH₃), 60.16 (CH₃), 81.14 (C), 95.88 (C), 108.93 (CH), 109.32 (d, J_{C-F} = 23.3, CH), 114.46 (d, J = 25.2, CH), 116.23 (C), 124.08 (d, J = 9.3, CH), 124.63 (C), 128.80 (d, J = 4.4, CH), 135.13 (C), 139.11 (C), 139.98 (d, J = 10.0, C) 153.02 (C), 160.51 (d, J_{C-F} = 240.2, C). HRMS (ESI⁺): m/z calcd for C₁₉H₁₆FO₃S [MNa⁺]: 343.0799; found: 343.0785.

5.3.2. Preparation of 5-methoxy-2-((3,4,5-trimethoxyphenylethynyl)-benzo[b]thiophene **3b**

Following general procedure B, scale: 2-iodo-5-methoxybenzo[b]thiophene (0.400 g, 1.38 mmol), PdCl₂(PPh₃)₂ (0.020 g, 0.028 mmol), CuI (0.011 g, 0.055 mmol), THF (9 ml), Et₃N (0.25 ml, 1.9 mmol), 5-ethynyl-1,2,3-trimethoxybenzene (0.318 g, 1.65 mmol). The crude product was purified by silica gel flash column chromatography (AcOEt / cyclohexane 20 / 80) to afford the desired product as an orange powder (0.428 g, 88 %). ¹H NMR (400 MHz, CDCl₃): δ = 3.86 (s, 3H), 3.88 (s, 9H), 6.79 (s, 2H), 7.02 (dd, J = 6.6 - 1.8, 1H),

7.20 (d, J = 1.8, 1H), 7.41 (s, 1H), 7.63 (d, J = 6.6, 1H). The spectral data were in accordance to those published in the literature [18].

5.3.3. Preparation of tert-butyl(dimethyl)((2-((3,4,5-trimethoxyphenyl)ethynyl)benzo[b]thiophen-5-yl)oxy)silane **3c**

Following general procedure B, scale: tert-butyl((2-iodobenzo[b]thiophen-5-yl)oxy)dimethylsilane (0.115 g, 0.30 mmol), PdCl₂(PPh₃)₂ (0.005 g, 0.06 mmol), CuI (0.003 g, 0.12 mmol), THF (2 ml), Et₃N (0.06 ml, 0.43 mmol), 5-ethynyl-1,2,3-trimethoxybenzene (0.068 g, 0.35 mmol). The crude product was purified by silica gel flash column chromatography (AcOEt / cyclohexane 10 / 90) to afford the desired product as an orange oil (0.128 g, 95 %). ¹H NMR (400 MHz, acetone-*d*₆): δ = 0.25 (s, 6H), 1.02 (s, 9H), 3.77 (s, 3H), 3.88 (s, 6H), 6.87 (s, 2H), 7.04 (dd, J = 8.7 - 2.4, 1H), 7.35 (d, J = 2.4, 1H), 7.53 (s, 1H), 7.78 (d, J = 8.7, 1H). ¹³C NMR (100 MHz, acetone-*d*₆): δ = -4.31 (CH₃), 18.81 (C), 26.06 (CH₃), 56.59 (CH₃), 60.71 (CH₃), 82.35 (C), 95.86 (C), 109.88 (CH), 114.53 (CH), 117.99 (C), 121.00 (CH), 123.64 (CH), 124.82 (C), 129.39 (CH), 134.05 (C), 140.77 (C), 141.59 (C), 154.49 (C), 154.56 (C). HRMS (ESI⁺): m/z calcd for C₂₅H₃₁O₄SSi [MH⁺]: 455.1707; found: 455.1700.

5.3.4. Preparation of 2-((3,4,5-trimethoxyphenyl)ethynyl)benzo[d]thiazole **3d**

Following general procedure B scale: 2-iodobenzo[d]thiazole (0.500 g, 1.92 mmol), PdCl₂(PPh₃)₂ (0.027 g, 0.04 mmol), CuI (0.015 g, 0.08 mmol) in THF (14 ml), Et₃N (0.41 ml, 2.9 mmol), 5-ethynyl-1,2,3-trimethoxybenzene (0.420 g, 2.2 mmol). The crude product was purified by silica gel flash column chromatography (AcOEt / cyclohexane: 10 / 90) to afford the desired product as a yellow powder (0.410 g, 66 %), mp 111–112°C. ¹H NMR (400 MHz, acetone-*d*₆): δ = 3.80 (s, 3H), 3.91 (s, 6H), 7.01 (s, 2H), 7.54 (td, J = 7.5 - 1.3, 1H), 7.60 (td, J = 7.7 - 1.2, 1H), 8.08 (dd, J = 12.0 - 7.8, 1H). ¹³C NMR (100 MHz, acetone-*d*₆) δ = 56.66 (CH₃), 60.75 (CH₃), 82.41 (C), 96.66 (C), 110.46 (CH), 116.28 (C), 122.67 (CH), 124.23 (CH), 127.30 (CH), 127.78 (CH), 136.16 (C), 141.63 (C), 148.87 (C), 154.14 (C), 154.55 (C). HRMS (ESI⁺): m/z calcd for C₁₈H₁₆NO₃S [MH⁺]: 326.0845; found: 326.0840.

5.4. General procedure C for the semi-hydrogenation of the acetylenic derivatives **3**

The acetylenic derivative **3** (1 eq.), KOH (1.5 eq), Pd(OAc)₂ (0.02 eq.), and DMF (2.5 ml) were placed in a 25 ml screw-cap tube under an argon atmosphere. The tube was sealed and the mixture heated in an oil bath at 145°C with stirring for 6 h. After the reaction mixture was cooled to room temperature, the crude reaction mixture was diluted with H₂O (10 ml), extracted three times with EtOAc (3 x 30 ml), dried over Na₂SO₄ and concentrated under reduced pressure to give a thick brown oil. The residue was then subjected to silica gel column chromatography (AcOEt / cyclohexane) to afford the desired product.

5.4.1. Preparation of (Z)-5-fluoro-2-((3,4,5-trimethoxystyryl)benzo[b]thiophene **4a**

Following general procedure C, scale: 5-fluoro-2-((3,4,5-trimethoxyphenyl)ethynyl)benzo[b]thiophene (0.342 mg, 1

mmol), KOH (84 mg, 1.5 mmol), Pd(OAc)₂ (5 mg, 0.02 mmol), and DMF (2.5 ml). The crude product was purified by silica gel flash column chromatography (AcOEt / Cyclohexane 5 / 95) to afford the desired product as a yellow oil (0.100 g, 30 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ = 3.71 (s, 9H), 6.70 (d, J = 0.7 Hz, 2H), 6.73 (d, J = 12.0 Hz, 1H), 6.88 (d, J = 12.0 Hz, 1H), 7.16 (td, J = 9.1, 2.6 Hz, 1H), 7.46 (s, 1H), 7.59 (dd, J = 9.8 - 2.5 Hz, 1H), 7.86 (dd, J = 8.8 - 5.1 Hz, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ = 55.81 (CH₃), 60.16 (CH₃), 106.01 (CH), 108.63 (CH, d, J_{C-F} = 23.0 Hz), 113.34 (CH, d, J_{C-F} = 24.9 Hz), 123.26 (CH), 123.94 (CH, d, J_{C-F} = 9.7 Hz), 125.85 (CH, d, J_{C-F} = 4.4 Hz), 131.41 (C), 131.62 (CH), 134.98 (C), 137.39 (C), 139.65 (C, d, J_{C-F} = 9.7 Hz), 142.03 (C), 152.86 (C), 160.22 (C, d, J_{C-F} = 239.1 Hz). HRMS (ESI⁺): m/z calcd for C₁₉H₁₇FNaO₃S [MNa⁺]: 367.0775; found: 367.0761.

5.4.2. Preparation of (*E*)-2-(3,4,5-trimethoxystyryl)benzo[*d*]thiazole **4d**

Following general procedure C, scale: 2-((3,4,5-trimethoxyphenyl)ethynyl)benzo[*d*]thiazole (0.325 g, 1 mmol), KOH (84 mg, 1.5 mmol), Pd(OAc)₂ (5 mg, 0.02 mmol), and DMF (2.5 ml) were placed in a screw-cap tube (25 ml). The crude product was purified by silica gel flash column chromatography (AcOEt / cyclohexane 20 / 80) to afford the desired product as a yellow viscous oil (111 mg, 34 %). ¹H NMR (400 MHz, DMSO-*d*₆) δ = 3.70 (s, 3H), 3.85 (s, 6H), 7.15 (s, 2H), 7.43 (td, J = 7.6 - 1.2, 1H), 7.51 (td, J = 7.6 - 1.4, 1H), 7.60 (d, J = 16.3, 1H), 7.66 (d, J = 16.3, 1H), 7.96 (d, J = 8.1, 1H), 8.09 (d, J = 8.0, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ = 56.03 (CH₃), 60.13 (CH₃), 105.24 (CH), 121.24 (CH), 122.20 (CH), 122.45 (CH), 125.38 (CH), 126.53 (CH), 130.84 (C), 133.98 (C), 137.67 (CH), 138.73 (C), 153.14 (C), 153.51 (C), 166.60 (C). HRMS (ESI⁺): m/z calcd for C₁₈H₁₈NO₃S [MH⁺]: 328.1002; found: 328.0996.

5.5. General procedure D for the aldehyde preparation according to the *n*-BuLi 2-deprotonation / DMF electrophilic trapping

To a solution of the (benzo)thiophene derivative (2.30 mmol) in anhydrous THF (4 ml) under argon at -78°C was added dropwise a 2.5 M solution of *n*-BuLi in hexanes (1.00 ml, 2.50 mmol). The reaction mixture was stirred for 1 h at -78°C and DMF (0.25 ml, 3.20 mmol) was then added. The resulting mixture was allowed to stand 2 hours at room temperature and then poured into water (10 ml) and extracted three times with ethyl acetate (3 × 20 ml). The organic phase was washed with H₂O (20 ml), dried with Na₂SO₄ and the volatiles were removed under reduced pressure. The crude product was then purified by flash chromatography (AcOEt / cyclohexane) on silica gel.

5.5.1. Preparation of 5-((*tert*-butyldimethylsilyloxy)benzo[*b*]thiophene-2-carbaldehyde **12a**

Following general procedure D, scale: (benzo[*b*]thiophen-5-yloxy)(*tert*-butyl)dimethylsilane (0.600 g, 2.3 mmol), THF (4 ml), *n*-BuLi (1.0 mL, 2.5 mmol), DMF (0.25 ml, 3.2 mmol). The crude product was purified by flash chromatography on silica gel (AcOEt / cyclohexane 10 / 90) and the *title* product was obtained as a yellow solid (0.540 g, 81 %), mp 54-55°C. ¹H NMR (400 MHz, acetone-*d*₆): δ = 0.27 (s, 6H), 1.02 (s, 9H), 7.19 (dd, J = 8.8, 2.4 Hz, 1H), 7.56 (d, J = 2.4 Hz, 1H), 7.93 (d, J = 8.8 Hz, 1H),

8.23 (s, 1H), 10.14 (s, 1H). ¹³C NMR (100 MHz, acetone-*d*₆): δ = -4.34 (CH₃), 18.82 (C), 26.04 (CH₃), 116.46 (CH), 123.90 (CH), 125.00 (CH), 135.55 (CH), 136.41 (C), 141.22 (C), 145.53 (C), 154.72 (C), 185.91 (CH). HRMS (ESI⁺): m/z calcd for C₁₅H₂₁O₂Si [MH⁺]: 293.1026; found: 293.1023.

5.5.2. Preparation of 3-methylbenzo[*b*]thiophene-2-carbaldehyde **12b**

Following general procedure D, scale: 3-methylbenzo[*b*]thiophene (0.5 g, 3.4 mmol), THF (6 ml), *n*-BuLi (1.62 ml, 4.0 mmol), DMF (0.3 ml, 3.87 mmol). Purification by flash chromatography on silica (AcOEt / cyclohexane 10 / 90) afforded the desired product as a yellow solid (0.435 g, 73 %). ¹H NMR (300 MHz, DMSO-*d*₆): δ = 2.79 (s, 3H), 7.42 - 7.53 (m, 2H), 7.85 - 7.90 (m, 2H), 10.34 (s, 1H). Spectral data were in agreement to those reported in the literature [61].

5.5.3. Preparation of 3-methoxythiophene-2-carbaldehyde **12c** and 4-methoxythiophene-2-carbaldehyde **12d**

Following general procedure D, scale: 3-methoxythiophene (2 g, 17.5 mmol), THF (20 ml), *n*-BuLi (7.75 ml, 19.4 mmol), DMF (1.64 ml, 21.2 mmol). The extraction was carried out with CH₂Cl₂ (3 × 75 ml). Purification by flash chromatography on silica (AcOEt / cyclohexane 20 / 80) afforded 4-methoxythiophene-2-carbaldehyde **12d** (less polar fraction) as a yellow solid (1.1717 g, 47 %) and 3-methoxythiophene-2-carbaldehyde **12c** (more polar fraction) as an orange solid (0.917 g, 37 %).

3-methoxythiophene-2-carbaldehyde **12c**: ¹H NMR (CDCl₃) δ = 3.99 (s, 3H), 6.87 (d, J = 5.5, 1H), 7.64 (dd, 5.5 - 1.3, 1H), 9.99 (d, J = 5.5, 1.3, 1H). Spectral data were in agreement to those reported in the literature [62].

4-methoxythiophene-2-carbaldehyde **12d**: ¹H NMR (CDCl₃) δ = 3.85 (s, 3H), 6.75 (dd, J = 1.7-1.1, 1H), 7.40 (d, 1.9, 1H), 9.82 (d, J = 1.1, 1H). Spectral data were in agreement to those reported in the literature [63].

5.6. Preparation of 5-methoxythiophene-2-carbaldehyde **12e**

A 2.5 M solution of *n*-BuLi in hexanes (1.53 ml, 3.81 mmol) was added dropwise under argon at 0°C to a stirred solution of 2-methoxythiophene (0.617 g, 5.4 mmol) in anhydrous diethyl ether (8 ml). The reaction mixture was then stirred for 1 h at 0°C and was allowed to stand 15 min at room temperature. DMF (0.254 g, 3.47 mmol) dissolved in anhydrous diethyl ether (7 ml) was added dropwise at room temperature. The mixture was heated at 40°C for 1 h. The mixture was then poured into water (50 ml) and extracted with ethyl acetate (3 × 75 ml). The combined organic extracts were washed with H₂O (100 ml), dried with MgSO₄ and the solvent was evaporated under reduced pressure. Purification by flash chromatography on silica (AcOEt / cyclohexane 20 / 80) afforded the desired product as an orange oil (0.410 g, 83 %).

¹H NMR (CDCl₃): δ = 3.97 (s, 3H), 6.32 (d, J = 7.5, 1H), 7.49 (d, J = 7.5, 1H), 9.63 (s, 1H). Spectral data were in agreement to those reported in the literature [64].

5.7. General synthetic procedure E for the Wittig synthesis: preparation of (Z)-3,4,5 trimethoxystyrene derivatives **4c,f,i**

To a stirred suspension of 3,4,5-trimethoxybenzyltriphenylphosphonium bromide (1 eq.) in THF at -78°C was added dropwise (10 min) *n*-BuLi 2.5 M in hexane (2 eq.). The stirring was continued at -78°C for 30 min and at room temperature for 1 h. The dark red reaction mixture was then cooled to -78°C and a solution of (benzo)thiophene aldehyde (1 eq.) in THF was added dropwise (15 min). The stirring was continued at -78°C for 1 h and at room temperature for 18 h. Subsequently, the mixture was quenched with ice-cold water and ethyl acetate was then added. After decantation, the aqueous layer was extracted three times with ethyl acetate and three times with dichloromethane. The combined organic layers were dried over Na_2SO_4 , filtered, and concentrated under reduced pressure to give a yellow residue. We have chosen to only isolate the most active (Z) isomer after purification of the crude product by silica gel (treated with triethylamine) flash column chromatography, (AcOEt / cyclohexane).

5.7.1. Preparation of (Z)-*tert*-butyldimethyl((2-(3,4,5-trimethoxystyryl)benzo[*b*]thiophen-5-yl)oxy)silane **4c**

Following general procedure E, scale: 3,4,5-trimethoxybenzyltriphenylphosphonium bromide (0.523 g, 1.0 mmol) solution in THF (11 ml), *n*-BuLi 2.5 M in hexane (0.8 ml, 2 mmol), 5-((*tert*-butyldimethylsilyl)oxy)benzo[*b*]thiophene-2-carbaldehyde (0.292 g, 1.0 mmol) in THF (4 ml). The crude product was purified by silica gel flash column chromatography (AcOEt / cyclohexane 10 / 90) to afford the (Z) isomer as a yellow oil (0.152 g, 33 %).

^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ = 0.18 (s, 6H), 0.95 (s, 9H), 3.70 (s, 9H), 6.67 (d, J = 12.1, 1H), 6.70 (s, 2H), 6.83 (m, 2H), 7.21 (d, J = 2.5, 1H), 7.37 (s, 1H), 7.67 (d, J = 8.6, 1H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ = -4.53 (CH_3), 17.99 (C), 25.61 (CH_3), 55.79 (CH_3), 60.16 (CH_3), 106.06 (CH), 113.04 (CH), 118.96 (CH), 123.00 (CH), 123.46 (CH), 130.88 (CH), 131.55 (C), 132.53 (C), 137.31 (C), 139.86 (C), 140.54 (C), 152.79 (2C). HRMS (ESI $^+$): m/z calcd for $\text{C}_{25}\text{H}_{33}\text{NaO}_4\text{SSi}$ [MNa^+]: 479.1683; found: 479.1691.

5.7.2. Preparation of (Z)-3-methyl-2-(3,4,5-trimethoxystyryl)benzo[*b*]thiophene **4f**

Following general procedure E, scale: 3,4,5-trimethoxybenzyltriphenylphosphonium bromide (1.26 g, 2.4 mmol) solution in THF (25 mL), *n*-BuLi 2.5 M in hexane (1.92 ml, 4.8 mmol), 3-methylbenzo[*b*]thiophene-2-carbaldehyde (0.424 g, 2.4 mmol) in THF (8 mL). The crude product was purified by silica gel flash column chromatography treated with triethylamine (AcOEt / Cyclohexane 15 / 85) to afford the (Z) isomer as a yellow oil (0.165 g, 21 %). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ = 2.30 (s, 3H), 3.57 (s, 6H), 3.66 (s, 3H), 6.62 (s, 2H), 6.74 (d, J = 12.0, 1H), 6.83 (d, J = 12.0, 1H), 7.31 (td, J = 7.1 - 1.2, 1H), 7.36 (td, J = 7.7 - 1.5, 1H), 7.71 (dd, J = 7.6 - 1.0, 1H), 7.83 (dd, J = 7.6 - 1.0, 1H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ = 11.98 (CH_3), 55.54 (CH_3), 60.06 (CH_3), 106.14 (CH), 121.21 (CH), 121.81 (CH), 122.13 (CH), 124.13 (CH), 124.86 (CH), 130.65 (C), 131.60 (C), 132.04 (CH), 133.38 (C), 137.22 (C), 138.30 (C), 139.39 (C), 152.58 (C). HRMS (ESI $^+$): m/z calcd for $\text{C}_{20}\text{H}_{20}\text{NaO}_3\text{S}$ [MNa^+]: 363.1025; found: 363.1011.

5.7.3. Preparation of (Z)-3-methoxy-2-(3,4,5-trimethoxystyryl)thiophene **4g**

Following general procedure E, scale: 3,4,5-trimethoxybenzyltriphenylphosphonium bromide (1.57 g, 3 mmol) solution in THF (31 ml), *n*-BuLi 2.5 M in hexane (2.4 ml, 6 mmol), 3-methoxythiophene-2-carbaldehyde (0.426 g, 3 mmol) in THF (10 ml). The crude product was purified by silica gel flash column chromatography treated with triethylamine (AcOEt / cyclohexane 15 / 85) to afford the (Z) isomer as a yellow oil (0.434 g, 47 %). ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ = 3.67 (s, 3H) 3.72 (s, 6H), 3.83 (s, 3H), 6.36 (d, J = 12.2, 1H), 6.63 (d, J = 12.2, 1H), 6.65 (s, 2H), 6.95 (d, J = 5.6, 1H), 7.29 (d, J = 5.6, 1H). ^{13}C (100 MHz, $\text{DMSO}-d_6$): δ = 55.75 (CH), 58.60 (CH), 60.11 (CH), 105.78 (CH), 115.16 (C), 115.75 (CH), 119.08 (CH), 124.45 (CH), 125.47 (CH), 132.45 (C), 136.88 (C), 152.77 (C), 156.99 (C). HRMS (ESI $^+$): m/z calcd for $\text{C}_{16}\text{H}_{18}\text{NaO}_4\text{S}$ [MNa^+]: 329.0818; found: 329.0814.

5.7.4. Preparation of (Z)-4-methoxy-2-(3,4,5-trimethoxystyryl)thiophene **4h**

Following general procedure E, scale: 3,4,5-trimethoxybenzyltriphenylphosphonium bromide (1.57 g, 3 mmol) solution in THF (31 ml), *n*-BuLi 2.5 M in hexane (2.4 ml, 6 mmol), 4-methoxythiophene-2-carbaldehyde (0.426 g, 3 mmol) in THF (12 ml). The crude product was purified by silica gel flash column chromatography treated with triethylamine (AcOEt / cyclohexane 15 / 85) to afford the (Z) isomer as a light yellow solid (0.213 g, 23 %), mp 54-55 $^{\circ}\text{C}$. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ = 3.68 (s, 6H), 3.73 (s, 6H), 6.43 (d, J = 1.9, 1H), 6.51 (d, J = 12.1, 1H), 6.61 (d, J = 12.1, 1H), 6.66 (s, 2H), 6.76 (d, J = 1.5, 1H). ^{13}C (100 MHz, $\text{DMSO}-d_6$): δ = 55.78 (CH_3), 57.02 (CH_3), 60.13 (CH_3), 98.16 (CH), 105.80 (CH), 120.52 (CH), 122.97 (CH), 129.06 (CH), 131.83 (C), 137.09 (C), 137.68 (C), 152.86 (C), 157.09 (C). HRMS (ESI $^+$): m/z calcd for $\text{C}_{16}\text{H}_{18}\text{NaO}_4\text{S}$ [MNa^+]: 329.0818; found: 329.0821.

5.7.5. Preparation of (Z)-5-methoxy-2-(3,4,5-trimethoxystyryl)thiophene **4i**

Following general procedure E, scale: 3,4,5-trimethoxybenzyltriphenylphosphonium bromide (1.55 g, 2.96 mmol) solution in THF (31 ml), *n*-BuLi 2.5 M in hexane (2.36 ml, 5.90 mmol), 4-methoxythiophene-2-carbaldehyde (0.420 g, 2.96 mmol) in THF (12 ml). The crude product was purified by silica gel flash column chromatography treated with triethylamine (AcOEt / cyclohexane 15 / 85) to afford the (Z) isomer as a pale yellow oil (0.165 g, 14 %). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ = 3.68 (s, 3H) 3.74 (s, 6H), 3.77 (s, 3H), 6.17 (d, J = 4.0, 1H), 6.31 (d, J = 12.0, 1H), 6.57 (d, J = 12.5, 1H), 6.63 (s, 2H), 6.81 (d, J = 4.0, 1H). ^{13}C (100 MHz, $\text{DMSO}-d_6$): δ = 55.82 (CH_3), 59.86 (CH_3), 60.16 (CH_3), 102.86 (CH), 105.84 (CH), 124.05 (CH), 124.81 (CH), 125.16 (C), 128.49 (CH), 132.25 (C), 136.94 (C), 152.89 (C), 165.59 (C). HRMS (ESI $^+$): m/z calcd for $\text{C}_{16}\text{H}_{18}\text{NaO}_4\text{S}$ [MNa^+]: 329.0818; found: 329.0805.

5.8. Preparation of (Z)-2-(3,4,5-trimethoxystyryl)benzo[*b*]thiophen-5-ol **4e** by fluoride-mediated deprotection of silyl ether

To a stirred solution of (Z)-*tert*-butyldimethyl((2-(3,4,5-trimethoxystyryl)benzo[*b*]thiophen-5-yl)oxy)silane **4c** (0.150 g, 0.33 mmol), in THF (2 mL) at room temperature, was added a 1M TBAF solution in THF (1.0 ml, 1 mmol). The stirring was

continued at room temperature for 1 h. Subsequently, the mixture was diluted with EtOAc (10 mL) and washed with a saturated NaHCO₃ aqueous solution (2 × 5 ml), dried over Na₂SO₄ and concentrated under reduced pressure. The crude product was purified by silica gel flash column chromatography (AcOEt / cyclohexane 30 / 70) to afford the desired product as a yellow oil (71 mg, 63 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ= 3.70 (d, 3H), 3.71 (d, 6H), 6.64 (d, *J* = 12.0, 1H), 6.70 (s, 2H), 6.80 (m, 2H), 7.07 (d, *J* = 2.3, 1H), 7.30 (s, 1H), 7.57 (d, *J* = 8.7, 1H), 9.39 (s, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ= 55.79 (CH₃), 60.16 (CH₃), 106.06 (CH), 107.85 (CH), 115.28 (CH), 122.77 (CH), 123.63 (CH), 125.91 (CH), 130.18 (C), 130.47 (CH), 131.64 (C), 137.26 (C), 139.83 (C), 140.06 (C), 152.76 (C), 154.94 (C). HRMS (ESI⁺): *m/z* calcd for C₁₉H₁₈NaO₄S [MNa⁺]: 365.0818; found: 365.0806.

5.9. Preparation of (Z)-ethylenic derivatives **4l,m** using a Suzuki coupling

5.9.1. Preparation of **4l**

1-(2-Bromovinyl)-3,4,5-trimethoxybenzene (*Z/E* ratio 91/9, determined by ¹H NMR) (0.273 g, 1.0 mmol) and tetrakis(triphenylphosphine)palladium (0) (0.060 g, 0.05 mmol) were stirred in 1,2-dimethoxyethane (15 ml) under argon for 20 min. 3-thiopheneboronic acid (0.172 g, 1.34 mmol) and sodium carbonate (0.100 g, 0.95 mmol) in water (10 ml) were added, the mixture was heated under reflux during 20 h. After cooling and decantation, the aqueous layer was separated and extracted with ethyl acetate (2 × 7 ml). The combined organic phases were washed with brine (7 ml), dried over Na₂SO₄, filtered and concentrated under reduced pressure. This crude product was purified by column chromatography (AcOEt / cyclohexane 10 / 90) to give the (*Z*) isomer of compound **4l** as a yellow oil (0.160 g, 59 %). ¹H NMR (300 MHz, DMSO-*d*₆): δ= 3.64 (s, 6H) 3.66 (s, 3H), 6.49 (d, *J* = 12.4, 1H), 6.55 (d, *J* = 12.4, 1H), 6.57 (s, 2H), 6.93 (dd, *J* = 1.2 - 4.8, 1H), 7.41-7.42 (m, 1H), 7.46 (dd, *J* = 2.8 - 4.8, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ= 55.60 (CH₃), 60.08 (CH₃), 105.84 (CH), 123.99 (CH), 124.67 (CH), 125.95 (CH), 127.70 (CH), 129.30 (CH), 132.49 (C), 136.77 (C), 137.87 (C), 152.61 (C). HRMS (ESI⁺): *m/z* calcd for C₁₅H₁₆NaO₃S [MNa⁺]: 299.0712; found: 299.0701.

5.9.2. Preparation of **4m**

1-(2-Bromovinyl)-3,4,5-trimethoxybenzene (*Z/E* ratio 91/9, determined by ¹H NMR) (0.546 g, 2.00 mmol) and tetrakis(triphenylphosphine)palladium (0) (0.100 g, 0.10 mmol) were stirred in 1,2-dimethoxyethane (30 ml) under argon at room temperature for 20 min. 3-benzo[*b*]thiopheneboronic acid (0.41 g, 2.30 mmol) and sodium carbonate (0.20 g, 1.90 mmol) in water (20 ml) were added, the mixture was heated under reflux during 20 h. After cooling and decantation, the aqueous layer was separated and extracted with ethyl acetate (2 × 30 ml). The combined organic phases were washed with brine (20 ml), dried over Na₂SO₄, filtered and concentrated under reduced pressure. This crude product was purified twice by column chromatography on silica gel deactivated by Et₃N (AcOEt / cyclohexane 85 / 15) to give the (*Z*) isomer as a yellow oil (0.179 g, 28 %). ¹H NMR (400 MHz, CDCl₃): δ= 3.53 (s, 6H), 3.79 (s, 3H), 6.45 (s, 2H), 6.67 (d, *J* = 12.0, 1H), 6.71 (d, *J* = 12.0, 1H), 7.31 - 7.37 (m, 3H), 7.66 - 7.69 (m, 1H), 7.84 - 7.87 (m, 1H). ¹³C NMR (100 MHz, CDCl₃): δ= 55.78 (CH₃), 60.94 (CH₃),

106.00 (CH), 121.98 (CH), 122.69 (CH), 122.84 (CH), 123.89 (CH), 124.27 (CH), 124.56 (CH), 132.37 (C), 132.5 (CH), 133.07 (C), 137.40 (C), 138.13 (C), 139.82 (C), 152.88 (C). HRMS (ESI⁺): *m/z* calcd for C₁₉H₁₈NaO₃S [MNa⁺]: 349.0869; found: 349.0861.

5.10. Preparation of 1,2,3-triazole CA-4 derivatives **7**

5.10.1 Preparation of 5-(benzo[*b*]thiophen-2-yl)-1-benzyl-4-(3,4,5-trimethoxyphenyl)-1*H*-1,2,3-triazole **7aa** and 4-(benzo[*b*]thiophen-2-yl)-1-benzyl-5-(3,4,5-trimethoxyphenyl)-1*H*-1,2,3-triazole **7ab**

2-((3,4,5-trimethoxyphenyl)ethynyl)benzo[*b*]thiophene (1.0349 g, 3.19 mmol), and benzyl azide (1000 μl, 7.80 mmol) were placed in a screw-cap tube under a nitrogen atmosphere. The tube was capped and the mixture heated in an oil bath at 100°C with stirring for 17 h. After the reaction mixture was cooled to room temperature, the crude reaction mixture was diluted with EtOAc and concentrated under reduced pressure. Then, the thick brown residue was diluted with diisopropyl ether (5 ml), heated and cooled down to room temperature, then to -30°C, thus allowing the crystallization of a solid. The supernatant was carefully removed. This operation was repeated 3 times on the solid with diisopropyl ether (2 ml) and compound **7a** was obtained (720.1 mg, 49% yield) as a grey solid and a 72/28 regioisomers' ratio.

This grey solid was finally subjected to two silica gel column chromatographies (AcOEt / cyclohexane 20 / 80 then dichloromethane / methanol 99 / 1) to afford the desired products **7aa** (less polar, 518.5 mg, 35 %) as a white powder, mp 102.0-104.8°C and **7ab** (more polar, 201.6 mg, 14 %) as a white powder, mp 160.0-162.8°C.

Less polar compound **7aa** (major regioisomer),

¹H NMR (400 MHz, CDCl₃): δ= 3.68 (s, 6H) 3.98 (s, 3H), 5.44 (s, 2H), 6.38 (s, 2H), 7.09-7.11 (m, 2H), 7.25-7.34 (m, 5H), 7.35 (d, *J* = 0.6, 1H), 7.64-7.68 (m, 1H), 7.76-7.80 (m, 1H). ¹³C NMR (100 MHz, CDCl₃) δ= 52.38 (CH₂), 56.20 (CH₃), 61.18 (CH₃), 107.29 (CH), 120.51 (CH), 121.68 (C), 122.23 (CH), 123.64 (CH), 124.46 (CH), 124.50 (CH), 127.51 (CH), 128.32 (CH), 128.87 (CH), 133.41 (C), 133.90 (C), 135.69 (C), 139.36 (C), 139.39 (C), 139.90 (C), 140.46 (C), 153.77 (C). HRMS (ESI⁺): *m/z* calcd for C₂₆H₂₃N₃NaO₃S [MNa⁺]: 480.1352; found: 480.1353.

More polar compound **7ab** (minor regioisomer),

¹H NMR (400 MHz, CDCl₃): δ= 3.59 (s, 6H) 3.81 (s, 3H), 5.54 (s, 2H), 6.93 (s, 2H), 7.09-7.12 (m, 2H), 7.17 (s, 1H) 7.25-7.30 (m, 3H), 7.40-7.44 (m, 2H), 7.74-7.79 (m, 1H), 7.84-7.88 (m, 1H).

¹³C NMR (100 MHz, CDCl₃) δ= 52.42 (CH₂), 55.79 (CH₃), 60.88 (CH₃), 103.91 (CH), 122.32 (CH), 124.23 (CH), 125.06 (CH), 125.67 (CH), 125.69 (C), 126.84 (C), 127.43 (C), 127.48 (CH), 128.11 (CH), 128.34 (CH), 128.80 (CH), 135.18 (C), 137.94 (C), 139.08 (C), 141.15 (C), 146.16 (C), 153.21 (C).

5.10.2 Preparation of 2-[1-Benzyl-5-(3,4,5-trimethoxy-phenyl)-1*H*-[1,2,3]triazol-4-yl]-benzothiazole and 2-[3-Benzyl-5-(3,4,5-trimethoxy-phenyl)-3*H*-[1,2,3]triazol-4-yl]-benzothiazole as a regioisomer mixture **7b**

Following the synthesis of triazole derivative **7a**, scale: 2-((3,4,5-trimethoxyphenyl)ethynyl)benzo[d]thiazole **3d** (366.4 mg, 1.13 mmol), benzyl azide (350 μ l, 2.80 mmol). The crude product did not crystallize upon treatment with diisopropyl ether and was purified by silica gel flash chromatography (AcOEt/cyclohexane 30 /70 then dichloromethane / methanol 99 / 1) to afford compound **7b** (272.9 mg, 53 %) as a yellow oil and a non-separable 59/41 regioisomers' ratio.

Regioisomer 1 (major compound): ^1H NMR (400 MHz, acetone- d_6): δ = 3.75 (s, 6H), 3.78 (s, 3H), 6.11 (s, 2H), 6.97 (s, 2H), 7.24-7.37 (m, 5H), 7.51 (td, J = 7.6 – 1.2, 1H), 7.60 (td, J = 7.7 – 1.2, 1H), 8.07 (d, J = 8.8, 1H), 8.19 (d, J = 8.2, 1H). ^{13}C NMR (100 MHz, acetone- d_6): 53.98 (CH₂), 56.43 (CH₃), 60.66 (CH₃), 107.29 (CH), 124.31 (CH), 126.28 (C), 127.25 (CH), 127.70 (CH), 128.80 (CH), 128.85 (CH), 128.87 (CH), 129.42 (CH), 136.83 (C), 136.92 (C), 137.43 (C), 140.04 (C), 148.60 (C), 153.64 (C), 154.52 (C), 154.73 (C).

Regioisomer 2 (minor compound): ^1H NMR (400 MHz, CDCl₃): δ = 3.74 (s, 6H), 3.82 (s, 3H), 5.70 (s, 2H), 6.88 (s, 2H), 7.16 (br d, J = 6.7, 2H), 7.24-7.37 (m, 3H) 7.40 (td, J = 7.6 – 1.2, 1H), 7.46 (td, J = 7.6 – 1.3, 1H), 7.86 (d, J = 7.5, 1H), 8.04 (d, J = 8.2, 1H). ^{13}C NMR (100 MHz, acetone- d_6): 52.93 (CH₂), 56.50 (CH₃), 60.71 (CH₃), 109.09 (CH), 121.69 (C), 122.58 (CH), 122.93 (CH), 123.76 (CH), 126.21 (CH), 127.05 (CH), 127.91 (C), 128.11 (CH), 129.61 (CH), 135.50 (C), 136.07 (C), 140.15 (C), 140.48 (C), 154.28 (C), 155.19 (C), 160.35 (C).

HRMS (ESI⁺): m/z calcd for C₂₅H₂₃N₄O₃S [MH⁺]: 459.1485; found: 459.1477.

5.11. General synthetic procedure E for the synthesis of 1,2-diaryl-1,2-dione 5

The acetylenic derivative (1 mmol) and FeBr₃ (0.1 mmol) were stirred overnight in DMSO (5.0 ml) at 140°C under argon. After cooling to room temperature, H₂O (6 ml) was added to the crude product and the mixture was extracted 3 times with ethyl acetate (3 x 6 ml). Organic layers were then washed with an aqueous saturated NH₄Cl solution (16 ml), dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude mixture was then purified by column chromatography on silica gel (cyclohexane/ethyl acetate, 80/20) followed by washing with Et₂O to give the desired product as a colored solid.

5.11.1. Preparation of 1-(thiophen-2-yl)-2-(3,4,5-trimethoxyphenyl)ethane-1,2-dione 5a

Following the experimental procedure E. Scale: 2-((3,4,5-trimethoxyphenyl)ethynyl)thiophene (0.136 g, 0.5 mmol) and FeBr₃ (0.015 g, 0.05 mmol) were stirred in DMSO (4 ml). Reaction time at 140°C: 23h. The crude product was then purified by column chromatography on silica gel (cyclohexane/ethyl acetate, 80/20 then 70/30). 1-(thiophene-2-yl)-2-(3,4,5-trimethoxyphenyl)ethane-1,2-dione was obtained as a yellow solid (0.115 g, 75%), mp 183-184°C. ^1H NMR (400 MHz, CDCl₃): δ = 3.89 (s, 6H), 3.94 (s, 3H), 7.18 – 7.20 (m, 1H), 7.30 (s, 2H), 7.80 (d, J = 3.8 Hz, 1H), 7.84 (d, J = 5.3 Hz, 1H). ^{13}C NMR (100 MHz, CDCl₃): δ = 56.46 (CH₃), 61.17 (CH₃), 107.67 (CH), 127.58 (C), 128.94 (CH), 136.89 (CH), 136.99 (CH), 140.03 (C), 144.40 (C), 153.39 (C), 185.52 (C), 190.85 (C). HRMS (ESI⁺): m/z calcd for C₁₅H₁₄O₅SNa [MNa⁺]: 329.0454; found: 329.0447.

5.11.2. Preparation of 1-(Benzo[b]thiophen-2-yl)-2-(3,4,5-trimethoxyphenyl)ethane-1,2-dione 5b

Following the experimental procedure E: Scale: 2-((3,4,5-trimethoxyphenyl)ethynyl)-benzo[b]thiophene (0.162 g, 0.5 mmol) and FeBr₃ (0.015 g, 0.05 mmol) were stirred in DMSO (4 ml). Reaction time at 140°C: 23h. The crude product was then purified by column chromatography on silica gel (cyclohexane/ethyl acetate, 80:20). 1-(Benzo[b]thiophene-2-yl)-2-(3,4,5-trimethoxyphenyl)ethane-1,2-dione was obtained as a yellow solid (0.149 g, 83%), mp 140-141°C. ^1H NMR (400 MHz, CDCl₃): δ = 3.90 (s, 6H), 3.95 (s, 3H), 7.32 (s, 2H), 7.42 (td, J = 7.6 – 0.8, 1H), 7.52 (td, J = 7.6 – 1.1, 1H), 7.88 (d, J = 8.0, 1H), 7.92 (d, J = 8.2, 1H), 8.04 (s, 1H). ^{13}C NMR (100 MHz, CDCl₃): δ = 56.47 (CH₃), 61.19 (CH₃), 107.65 (CH), 123.19 (CH), 125.54 (CH), 126.83 (CH), 127.54 (C), 128.59 (CH), 134.87 (CH), 139.06 (C), 139.59 (C), 143.56 (C), 144.52 (C), 153.44 (C), 187.49 (C), 190.68 (C). HRMS (CI): m/z calcd for C₁₉H₁₇O₅S [MH⁺]: 357.0797; found: 357.0797.

5.12. General synthetic procedure F for the synthesis of 5,6-diaryl-1,2-dihydropyrazines 6

Ethylenediamine (1.2 mmol) in freshly distilled Et₂O (2.5 ml) was added to a solution of 1,2-diaryl-1,2-dione derivative (1 mmol) in freshly distilled Et₂O at 0°C under an argon atmosphere. The mixture was stirred at 30°C for 6h, quenched with water (50 ml), and extracted three times with ethyl acetate (3 x 150 ml). The organic layers were then combined, dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude mixture was purified by column chromatography on silica gel to give the final product.

5.12.1. Preparation of 5-(thiophen-2-yl)-6-(3,4,5-trimethoxyphenyl)-2,3-dihydropyrazine 6a

Following the experimental procedure F. Scale: ethylenediamine (20 μ l, 0.3 mmol) in diethyl ether (240 μ l), 1-(thiophen-2-yl)-2-(3,4,5-trimethoxyphenyl)ethane-1,2-dione (0.027 g, 0.1 mmol) in diethyl ether (240 μ l). Reaction time at 30°C: 6h. The crude product was purified by column chromatography on silica gel (cyclohexane/ethyl acetate, 50/50) to give the title compound as a light yellow solid (0.028 g, 86%), mp 122.3-124.7°C. ^1H NMR (400 MHz, CDCl₃): δ = 3.65 (s, 4H), 3.76 (s, 6H), 3.87 (s, 3H), 6.64 (d, J = 3.3 Hz, 1H), 6.74 (s, 2H), 6.85 (dd, J = 5.0 – 3.8, 1H), 7.34 (d, J = 5.0 Hz, 1H). ^{13}C NMR (100 MHz, CDCl₃): δ = 45.64 (CH₂), 46.02 (CH₂), 56.26 (CH₃), 61.13 (CH₃), 105.60 (CH), 127.56 (CH), 129.06 (CH), 130.88 (CH), 133.31 (C), 139.83 (C), 142.43 (C), 153.02 (C), 154.59 (C), 159.61 (C). LC/MS (retention time: 1.920 min) m/z (ESI⁺) 175.1 (74.8%), 349.1 (100, (M+H₂O)H⁺), 350 (20.7), 351 (6.5).

5.12.2. Preparation of 5-(benzo[b]thiophen-2-yl)-6-(3,4,5-trimethoxyphenyl)-2,3-dihydropyrazine 6b

Following the experimental procedure F: Scale: ethylenediamine (8 μ l, 0.12 mmol), in diethyl ether (240 μ l), 1-(benzo[b]thiophen-2-yl)-2-(3,4,5-trimethoxyphenyl)ethane-1,2-dione (0.032 g, 0.1 mmol) in diethyl ether (240 μ l). Reaction time at 30°C: 6h. The crude product was purified by column chromatography on silica gel (cyclohexane/ethyl acetate, 50/50) to give the title compound (0.034 g, 89%) as a light yellow solid, mp 149.5-151.4°C. ^1H

NMR (400 MHz, CDCl₃): δ = 3.65-3.68 (m, 2H), 3.71-3.75 (m, 2H), 3.76 (s, 6H), 3.89 (s, 3H), 6.82 (s, 2H), 6.85 (s, 1H), 7.28 (t, J = 7.0, 1H), 7.35 (t, J = 7.6, 1H), 7.57 (d, J = 8.1 Hz, 1H), 7.81 (d, J = 8.1 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃): δ = 46.01 (CH₂), 46.08 (CH₂), 56.31 (CH₃), 61.12 (CH₃), 105.54 (CH), 122.38 (CH), 124.63 (CH), 125.07 (CH), 126.32 (CH), 127.96 (CH), 133.30 (C), 139.51 (C), 139.97 (C), 140.29 (C), 142.58 (C), 153.11 (2 x C), 155.24 (C), 159.35 (C). LC/MS (retention time: 2.66 min) m/z (ESI⁺) 200.1 (59.1%), 399.2 (100, (M+H₂O)H⁺), 400 (23.2), 401 (7.3).

5.13. Preparation of 1,1-diarylethylene derivatives **14a-d**

5.13.1. Preparation of the secondary alcohol benzo[*b*]thiophen-2-yl(3,4,5-trimethoxyphenyl)methanol **15a**

To a solution of benzo[*b*]thiophene (1.38 g, 10.0 mmol) in Et₂O (10 mL) in a 100 mL round bottom flask under argon at 0°C, was added dropwise *n*-BuLi 2.5 M in hexane (5.2 ml, 13.0 mmol) was added dropwise and stirred for 1h00. To the reaction mixture was then added a solution of 3,4,5-trimethoxybenzaldehyde (2.57 g, 13 mmol) in Et₂O (60 mL), which was stirred 1h00 at -70°C and 4h00 at room temperature. The solution was quenched with brine (60 mL) and extracted three times with ethyl acetate (3 x 100 mL). The combined organic phases were finally dried with Na₂SO₄, filtered, evaporated and the resulting residue was then purified by flash chromatography (EtOAc / Cyclohexane 30 / 70) to give compound **15a** as a white solid (2.81 g, 85 %), mp 141-142°C. ¹H NMR (400 MHz, CDCl₃): δ = 3.85 (s, 9H), 6.03 (s, 1H), 6.72 (s, 2H), 7.13 (s, 1H), 7.29 - 7.33 (m, 2H), 7.70 (dd, J = 7.2 - 1.9, 1H), 7.79 (d, J = 7.6, 1H). ¹³C NMR (100 MHz, CDCl₃): δ = 56.24 (CH₃), 60.99 (CH₃), 73.18 (CH), 103.44 (CH), 121.38 (CH), 122.55 (CH), 123.74 (CH), 124.42 (CH), 137.72 (C), 138.36 (C), 139.45 (C), 139.97 (C), 148.54 (C), 153.44 (C). LC/MS (retention time 3.50 min) m/z (ESI⁺) 313.1 (MH⁺ - H₂O, 100%), 314.1 (22), 315.1 (6.5), 353.1 (MNa⁺, 6.5), 683.2 (2MNa⁺, 14.5).

5.13.2. Preparation of the secondary alcohol benzo[*b*]thiophen-3-yl(3,4,5-trimethoxyphenyl)methanol **15b**

Following the synthesis of benzo[*b*]thiophen-2-yl(3,4,5-trimethoxyphenyl)methanol, scale: 3-bromobenzo[*b*]thiophene (0.85 g, 4.0 mmol), *n*-BuLi 2.5 M (2 ml, 4.8 mmol), Et₂O (35 ml), 3,4,5-trimethoxybenzaldehyde (1.02 g, 5.2 mmol). The crude product was purified by flash chromatography (EtOAc / cyclohexane 30/70) to give compound **15b** (1.08 g, 82 %) as a yellow solid, mp 98-99°C. ¹H NMR (400 MHz, CDCl₃): δ = 3.81 (s, 6H), 3.84 (s, 3H), 6.08 (s, 1H), 6.68 (s, 2H), 7.31 (s, 1H), 7.34 (m, 2H), 7.78 (m, 1H), 7.85 (m, 1H). ¹³C NMR (100 MHz, CDCl₃): δ = 56.22 (CH₃), 60.96 (CH₃), 72.23 (CH), 103.92 (CH), 122.68 (CH), 122.98 (CH), 124.20 (CH), 124.25 (CH), 124.61 (CH), 137.39 (C), 137.62 (C), 138.05 (C), 138.58 (C), 141.06 (C), 153.42 (C). LC/MS (retention time 3.40 min) m/z (ESI⁺) 313.1 (MH⁺ - H₂O, 100%), 314.1 (22), 315.1 (6.5), 353.1 (MNa⁺, 9), 683.2 (2MNa⁺, 12.5).

5.13.3. Preparation of ketone benzo[*b*]thiophen-2-yl(3,4,5-trimethoxyphenyl)methanone **16a**

To a solution of benzo[*b*]thiophen-2-yl(3,4,5-trimethoxyphenyl)methanol (0.99 g, 3 mmol) in acetonitrile (150 ml) in a 250 ml round bottom flask at room temperature, MnO₂ (0.7824 g,

9 mmol) was added and the heterogenous mixture was stirred during 2h. Then, a second equal portion of MnO₂ (0.7824 g, 9 mmol) was added and the mixture was stirred during 2h. The suspension was then filtered through a thin layer of silica gel to remove the black solid, washing 3 times with CH₃CN. After removal of the volatiles under reduced pressure, the crude product was purified *via* flash chromatography (EtOAc / Cyclohexane 30 / 70) to give compound **16a** (0.95 g, 97 %) as a yellow solid, mp 113-115°C. ¹H NMR (300 MHz, CDCl₃): δ = 3.92 (s, 6H), 3.95 (s, 3H), 7.19 (s, 2H), 7.43 (m, 1H), 7.50 (m, 1H), 7.91 (m, 3H). ¹³C NMR (100 MHz, CDCl₃): δ = 56.47 (CH₃), 61.11 (CH₃), 107.04 (CH), 122.96 (CH), 125.17 (CH), 126.11 (CH), 127.50 (CH), 131.79 (CH), 133.00 (C), 139.01 (C), 142.17 (C), 142.65 (C), 142.90 (C), 153.13 (C), 188.59 (C). HRMS (ESI⁺): m/z calcd for C₁₈H₁₆NaO₄S [MNa⁺]: 351.0662; found: 351.0650. Spectral data were in agreement to those reported in the literature [15a].

5.13.4. Preparation of ketone benzo[*b*]thiophen-3-yl(3,4,5-trimethoxyphenyl)methanone **16b**

Following to the same protocol than for the synthesis of benzo[*b*]thiophen-2-yl(3,4,5-trimethoxyphenyl)methanone, scale: benzo[*b*]thiophen-3-yl(3,4,5-trimethoxyphenyl)methanol (0.95 g, 2.87 mmol), first addition of MnO₂ (0.75 g, 8.61 mmol), second addition of MnO₂ (0.75 g, 8.61 mmol), CH₃CN (100 ml). The crude product was purified by flash chromatography (EtOAc / cyclohexane: 30 / 70) to give compound the title compound (0.756 g, 80 %) as a white solid, mp 123-124°C. ¹H NMR (400MHz, CDCl₃): δ = 3.89 (s, 6H), 3.95 (s, 3H), 7.14 (s, 2H), 7.44 (m, 1H), 7.51 (m, 1H), 7.91 (d, J = 8.0, 1H), 8.02 (s, 1H), 8.48 (d, J = 8.0, 1H). ¹³C NMR (100 MHz, CDCl₃): δ = 56.41 (CH₃), 61.09 (CH₃), 107.28 (CH), 122.47 (CH), 125.11 (CH), 125.69 (CH), 125.71 (CH), 134.34 (C), 134.89 (C), 137.23 (CH), 137.58 (C), 140.12 (C), 142.07 (C), 153.09 (C), 189.90 (C). HRMS (ESI⁺): m/z calcd for C₁₈H₁₆NaO₄S [MNa⁺]: 351,0662; found: 351,0657.

5.13.5. Preparation of tertiary alcohol 1-(benzo[*b*]thiophen-2-yl)-1-(3,4,5-trimethoxyphenyl)ethanol **17a** by Grignard reagent addition on ketone **16a**

To a solution of benzo[*b*]thiophen-2-yl(3,4,5-trimethoxyphenyl)methanone **16a** (0.328 g, 1.00 mmol) in THF (20 mL) in a 100 mL round bottom flask at 0°C was slowly added a 3M CH₃MgI solution in Et₂O (1 mL, 3.00 mmol) during 15 minutes. The mixture was stirred at room temperature during 30 minutes, and then the reaction was heated to reflux at 75°C for 2h. After cooling to room temperature, a saturated aqueous solution of NH₄Cl (10 mL) was then added, followed by water (10 mL): the solution was extracted 3 times with EtOAc (3 x 20 mL). After drying of the combined organic phases with Na₂SO₄ and filtration, the volatiles were removed under pressure. The crude product was purified by flash chromatography (AcOEt/cyclohexane 30/70) to give compound **17a** (0.327 g, 95%) as a white solid, mp 133-134°C. ¹H NMR (400 MHz, acetone-*d*₆): δ = 1.25 (s, 3H), 2.89 (s, 3H), 2.98 (s, 6H), 4.41 (s, 1H), 6.13 (s, 2H), 6.48 (m, 3H), 6.93 (d, J = 6.8, 1H), 7.00 (d, J = 7.7, 1H). ¹³C NMR (100 MHz, acetone-*d*₆): δ = 31.93 (CH₃), 56.40 (CH₃), 60.44 (CH₃), 75.07 (C), 104.10 (CH), 120.46 (CH), 122.95 (CH), 124.29 (CH), 124.75 (CH), 124.88 (CH), 137.98 (C), 140.58 (C), 140.74 (C), 144.37 (C), 153.85 (C), 156.63 (C). LC/MS (retention time 3.75 min) m/z (ESI⁺) 327.2 (MH⁺ - H₂O, 100%), 328.1 (21.5), 329.1 (7), 367.1 (MNa⁺, 11.5), 711.2 (2MNa⁺, 19.5).

5.13.6. Preparation of tertiary alcohol 1-(benzo[b]thiophen-3-yl)-1-(3,4,5-trimethoxyphenyl)ethanol 17b by Grignard reagent addition on ketone 16b

Following the same protocol than for the synthesis of 1-(benzo[b]thiophen-2-yl)-1-(3,4,5-trimethoxyphenyl)ethanol, scale: benzo[b]thiophen-3-yl-(3,4,5-trimethoxy-phenyl)-methanone **16b** (0.328 g, 1.00 mmol), 3M CH₃MgI solution in Et₂O (1 mL, 3.00 mmol), THF (30 mL). The crude product was purified by flash chromatography (AcOEt/cyclohexane 30/70) to give compound **17b** (0.316 g, 92%) as a slightly red solid. ¹H NMR (300 MHz, CDCl₃): δ = 2.03 (s, 3H), 3.76 (s, 6H), 3.82 (s, 3H), 6.67 (s, 2H), 7.20 (m, 1H), 7.28 (m, 1H), 7.48 (s, 1H), 7.62 (d, J = 7.6, 1H), 7.82 (d, J = 7.6, 1H). ¹³C NMR (100 MHz, acetone-*d*₆): δ = 32.26 (CH₃), 56.32 (CH₃), 60.42 (CH₃), 75.11 (C), 104.05 (CH), 122.95 (CH), 123.18 (CH), 124.12 (CH), 124.70 (CH), 126.32 (CH), 137.79 (C), 138.68 (C), 141.72 (C), 144.17 (C), 144.36 (C), 153.85 (C).

5.13.7. Preparation of tertiary alcohol 1-thiophen-2-yl-1-(3,4,5-trimethoxy-phenyl)-ethanol 17c by addition of 2-thiophenyl lithium reagent onto 3,4,5 trimethoxyacetophenone

To a solution of 2-bromothiophene (0.98 g, 6.0 mmol) in THF (30 mL) in a 100 ml round bottom flask under argon at -70°C, *n*-BuLi 2.5 M in hexane (3.60 ml, 9.0 mmol) was added dropwise and stirred for 1h. To the reaction mixture was then added a solution of 3,4,5 trimethoxyacetophenone (1.50 g, 7.2 mmol) which was stirred 1h at -70°C and 4h at room temperature. The solution was quenched with saturated NaCl aqueous solution (60 ml) and extracted with ethyl acetate (3 × 100 ml). The combined organic phases were dried with Na₂SO₄, filtered, evaporated and the crude product was purified by flash chromatography (EtOAc/Cyclohexane 30/70) to give compound **17c** (1.19 g, 67%) as a white solid, mp 70-71°C. ¹H NMR (400 MHz, acetone-*d*₆): δ = 1.45 (s, 3H), 3.18 (s, 3H), 3.26 (s, 6H), 4.50 (s, 1H), 6.35 (s, 2H), 6.39 (dd, J = 4.8 - 3.6, 1H), 6.46 (dd, J = 4.0 - 1.2, 1H), 6.76 (dd, J = 5.2 - 1.2, 1H). ¹³C NMR (100 MHz, acetone-*d*₆): δ = 32.50 (CH₃), 56.34 (CH₃), 60.43 (CH₃), 74.81 (C), 104.04 (CH), 124.11 (CH), 125.11 (CH), 126.97 (CH), 137.92 (C), 145.12 (C), 153.72 (C), 155.72 (C). LC/MS (retention time 2,98 min) m/z (ESI⁺) 277.1 (MH⁺ - H₂O, 100%), 278.1 (17.4), 279.0 (5.8), 317.1 (MNa⁺, 5.0), 611.2 (2MNa⁺, 7.0).

5.13.8. Preparation of tertiary alcohol 1-thiophen-3-yl-1-(3,4,5-trimethoxy-phenyl)-ethanol 17d by addition of 3-thiophenyl lithium reagent onto 3,4,5 trimethoxyacetophenone

Following the same protocol than for the synthesis of 1-thiophen-2-yl-1-(3,4,5-trimethoxy-phenyl)ethanol, scale: 3-bromothiophene (0.98 g, 6 mmol), Et₂O (80 ml), *n*-BuLi (3.6 ml, 9 mmol), 3,4,5-trimethoxyacetophenone (1.50 g, 7.2 mmol). The crude product was purified by flash chromatography (EtOAc / Cyclohexane 30:70) to give compound **17d** (1.40 g, 76%) as a white solid, mp 75-76°C. ¹H NMR (400 MHz, acetone-*d*₆): δ = 1.89 (s, 3H), 3.69 (s, 3H), 3.76 (s, 6H), 4.66 (s, 1H), 6.80 (s, 2H), 7.08 (dd, J = 4.4 - 1.2, 1H), 7.32 (m, 2H). ¹³C NMR (100 MHz, acetone-*d*₆): δ = 31.49 (CH₃), 56.32 (CH₃), 60.41 (CH₃), 74.55 (C), 104.11 (CH), 120.63 (CH), 125.97 (CH), 127.95 (CH), 137.73 (C), 145.42 (C), 151.93 (C), 153.73 (C). LC/MS (retention time 3.40 min) m/z (ESI⁺) 313.1 (MH⁺ - H₂O, 100%), 314.1 (22), 315,1 (6.5), 353,1 (MNa⁺, 9.0), 683.2 (2MNa⁺, 12.5).

5.13.9. Preparation of 2-[1-(3,4,5-trimethoxy-phenyl)-vinyl]-benzo[b]thiophene 14a

To a solution of 1-(benzo[b]thiophen-2-yl)-1-(3,4,5-trimethoxy-phenyl)-ethanol (0.172 g, 0.50 mmol) in toluene (50 mL), *p*-TsOH (0.004 g, 0.025 mmol) was added and the reaction mixture was stirred at 130°C for 2h. The mixture was then dried with Na₂SO₄, filtered and evaporated under reduced pressure until a solution volume ≈ 10 mL. Finally, this solution was directly purified by flash chromatography (EtOAc/cyclohexane 30/70) to give compound **14a** (0.151 g, 93%) as a yellow oil. ¹H NMR (400 MHz, CDCl₃): δ = 3,86 (s, 3H), 3,92 (s, 6H), 5,38 (s, 1H), 5,69 (s, 1H), 6,70 (s, 2H), 7,16 (s, 1H), 7,32 (m, 2H), 7,68 (m, 1H), 7,80 (m, 1H). ¹³C NMR (100 MHz, CDCl₃): δ= 56.30 (CH₃), 61.07 (CH₃), 105.82 (CH), 115.67 (CH₂), 122.24 (CH), 123.71 (CH), 123.89 (CH), 124.55 (CH), 124.92 (CH), 136.25 (C), 138.11 (C), 139.53 (C), 140.16 (C), 143.93 (C), 144.66 (C), 153.07 (C). HRMS (ESI⁺): m/z calcd for C₁₉H₁₈NaO₃S [MNa⁺]: 349.0869; found: 349.0864.

5.13.10. Preparation of 3-[1-(3,4,5-trimethoxy-phenyl)-vinyl]-benzo[b]thiophene 14b

Following the same protocol than for the synthesis of 2-[1-(3,4,5-trimethoxy-phenyl)-vinyl]-benzo[b]thiophene, scale: 1-(benzo[b]thiophen-3-yl)-1-(3,4,5-trimethoxyphenyl)ethanol (0.172 g, 0.50 mmol), toluene (50 ml), *p*-TsOH (0.004 g, 0.025 mmol), reaction time 1h. The crude product was purified by flash chromatography (AcOEt / cyclohexane : 30 / 70) to give the desired compound (0.145 g, 89 %) as a yellow oil. ¹H NMR (400 MHz, CDCl₃) δ= 3.77 (s, 6H), 3.87 (s, 3H), 5.50 (d, J = 1.4, 1H), 5.69 (d, J = 1.4, 1H), 6.60 (s, 2H) 7.28 - 7.36 (m, 2H), 7.39 (s, 1H), 7.54 (d, J = 8.1, 1H), 7.88 (d, J=8.1, 1H). ¹³C NMR (100 MHz, CDCl₃): δ= 56.27 (CH₃), 61.09 (CH₃), 104.84 (CH), 115.53 (CH₂), 122.85 (CH), 123.81 (CH), 124.22 (CH), 124.43 (CH), 125.43 (CH), 136.65 (C), 137.62 (C), 138.20 (C), 138.30 (C), 140.44 (C), 143.98 (C), 153.15 (C). HRMS (ESI⁺): m/z calcd for C₁₉H₁₈NaO₃S [MNa⁺]: 349.0869; found: 349.0851

5.13.11. Preparation of 2-[1-(3,4,5-Trimethoxy-phenyl)-vinyl]-thiophene 14c

Following the same protocol than for the synthesis of 2-[1-(3,4,5-trimethoxy-phenyl)-vinyl]-benzo[b]thiophene, scale: 1-thiophen-2-yl-1-(3,4,5-trimethoxy-phenyl)-ethanol (0.294 g, 1.00 mmol), toluene (50 mL), *p*-TsOH (0.009 g, 0.05 mmol), reaction time at 130°C: 2h. This crude product was purified by flash chromatography (EtOAc/cyclohexane 20/80) to give compound **14c** (0.270 g, 98%) as a yellow oil. ¹H NMR (400 MHz, CDCl₃): δ= 3.85 (s, 6H), 3.89 (s, 3H), 5.23 (s, 1H), 5.56 (s, 1H), 6.67 (s, 2H), 6.98 (m, 2H), 7.25 (dd, J = 4.5 - 1.6, 1H). ¹³C NMR (100 MHz, CDCl₃): δ= 56.26 (CH₃), 61.04 (CH₃), 105.68 (CH), 113.29 (CH₂), 125.33 (CH), 126.62 (CH), 127.44 (CH), 136.75 (C), 138.05 (C), 143.41 (C), 144.70 (C), 152.97 (C). HRMS (ESI⁺): m/z calcd for C₁₅H₁₆NaO₃S [MNa⁺]: 299.0712; found: 299.0708.

5.13.12. Preparation of 3-[1-(3,4,5-Trimethoxy-phenyl)-vinyl]-thiophene 14d

Following the same protocol than for the synthesis of 2-[1-(3,4,5-trimethoxy-phenyl)-vinyl]-benzo[b]thiophene, scale: 1-thiophen-3-yl-1-(3,4,5-trimethoxy-phenyl)-ethanol (0.294 g, 1.00 mmol), toluene (50 mL), *p*-TsOH (0.009 g, 0.05 mmol), reaction time 2h. The crude product was purified by flash chromatography

(EtOAc/cyclohexane 20/80) to give compound **14d** (0.260 g, 94%) as a yellow oil. ¹H NMR (400 MHz, CDCl₃): δ= 3.84 (s, 6H), 3.88 (s, 3H), 5.31 (d, J= 1.2, 1H), 5.50 (J= 1.2, 1H), 6.61 (s, 2H), 7.19 (m, 2H), 7.31 (dd, J = 5.1 - 3.2, 1H). ¹³C NMR (100 MHz, CDCl₃) δ= 56.24 (CH₃), 61.01 (CH₃), 105.46 (CH) 113.12 (CH₂), 123.51 (CH), 125.61 (CH), 127.24 (CH), 137.32 (C), 137.85 (C), 142.41 (C), 144.63 (C), 152.98 (C). HRMS (ESI+): m/z calcd for C₁₅H₁₆NaO₃S [MNa⁺]: 299.0712; found: 299.0719.

5.14 Biological evaluations

5.14.1 In cellulo ITP assay

This assay was realized according to our previously established protocol [40].

5.14.2 Antiproliferative activities – Alizarine protocol

The human uterine sarcoma MES-SA cell line and its multidrug resistant derivative, MES-SA/DX5 cell line were cultured in McCoy's 5A medium (Life Technologies, Paisley, UK) supplemented with 10 % fetal calf serum (Hyclone, Cramlington, UK) and 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Carlstad, CA), at 37°C, 5% CO₂, in a humidified atmosphere. For PrestoBlue® anti-proliferative assay, the cells were seeded in 96-well culture plates (Greiner Bio-One, Kremsmuenster, Austria) at 2 500 cells/well. The next day, cells were treated with the different drugs prepared in DMSO (Sigma Aldrich, St Louis, MO), in triplicate. Final concentration of DMSO in the culture medium was 0.05%. After 72 hours of treatment, the PrestoBlue® Cell Viability Reagent (Invitrogen, Carlsbad, CA) was added to the wells and the anti-proliferative assay was performed according to the manufacturer's recommendations. Fluorescence was measured at 590 nm after excitation at 560nm, with an Infinite® M1000 microplate reader (Tecan, Männedorf, Switzerland). Each concentration point was done at least in triplicate for each independent experiment. The results are expressed as means. The results were then analyzed by Sigmaplot software to determine IC₅₀ values. The IC₅₀ values in tables are a mean of three independent experiments.

5.14.3 Antiproliferative activities – MTT protocol

Human follicular lymphoma cells (RL) were grown in complete RPMI media and seeded in 96 well plates (10000 cells per well, 100 µl) containing 100 µl of media with various concentrations of compounds. Human ovarian cancer (A2780) and pancreatic adenocarcinoma (MIA-PaCa-2) cells were grown in complete DMEM media seeded in 96 well plates (4000 and 3000 cells per well respectively, 100 µl) and adhered for 24 hours. Then, compounds were added at various concentrations. After 72 hours of exposure to compounds, metabolically active cells were quantified by the MTT assay. IC₅₀ values were determined using the CompuSyn software. Results are expressed as mean values of IC₅₀ of three independent experiments.

5.14.4 Cell cycle analyses

The distribution of cells in the cell cycle was determined after 24 and 48 hours of incubation with compounds, as described previously [65]. RL cells (5.10⁵ cells per well) were incubated for 24 h in the absence or presence of drugs in a final volume of 500

µL, washed with PBS, and incubated 30 minutes with 300 µL containing propidium iodide (50 µg/mL), Nonidet P-40 (0.05 %), and sodium citrate (3.6 µM). Twenty thousand cells were analyzed on LSR II flow cytometer (BD Biosciences, Le Pont-de-Claix, France) and cell cycle distribution was determined on gated cells excluding doublets and cell debris. Results were expressed as percentage of cells in each phase of the cell cycle. Cisplatin and vincristine were used at high concentrations as internal controls for each experiment.

5.15 In silico study

Prediction of the binding modes and activities of CA-4 derivatives was performed using a structure-based approach that includes structure preparation of both targets and compounds, docking and regression modeling. Two different structures of the bovine tubulin dimer were considered as docking targets, namely, tubulin in complex with CA-4 (PDB ID: 5LYJ) and tubulin in complex with CN2, a colchicine analog (PDB ID: 1SA0). Both structures were downloaded from the Protein Data Bank. Docked compounds include CA-4, colchicine, all (Z)-ethylenic derivatives **4** as well as compounds **14a** and **14b**.

5.15.1 Structure preparation

Tubulin structures were prepared using the MOE 2018's Structure Preparation tool [66]. All ligands, cofactors and additional interacting proteins were manually removed. Preparation was carried out in two steps: (1) structure correction which comprises the capping of C- and N-termini, addition of missing atoms, if any, modification of residues consistently with protein sequence, building of missing loops by homology modeling; (2) structure protonation using the Protonate3D wizard at pH=7.4, T=298K and 0.15M of salt concentration. Compounds were prepared via the MOE's Wash utility which enables to predict all the 3D protonation states of input molecules at a given pH. In the present case, only the most probable protonation state of each compound at pH=7.4 was docked.

5.15.2 Docking

Docking was conducted using the DockBox package [67], a python wrapper library which provides an automated way of docking and rescoring docking poses with multiple programs. Sampling of the docking poses was performed using three programs: AutoDock4 [52], AutoDock Vina [53] and DOCK6 [54]. For each program, 10 docking poses were generated resulting in a total of 30 poses being produced for every compound. A 30-Å³ docking box centered on the center of mass of the co-crystal ligand, i.e. CA-4 for 5LYJ and CN2 for 1SA0, was considered. Subsequently to docking, poses were energy-minimized with Amber16 and rescored using DSX-CSD and AutoDock4 scoring functions. For each compound, the pose with the highest DSX score over both tubulin targets was considered as the top pose, i.e., was used for future investigation of the binding mode and binding affinity. Visualization of the binding poses was done using MOE 2018 [66].

5.15.3 Linear regression

Multiple linear regression was built to fit experimentally-measured EC₅₀s of the docked compounds. Both DSX and

Autodock4 scores of the top docking poses, as well as an estimate of the logarithm of the partition coefficient LogP from Wildman-Crippen algorithm [68], were used as input variables for the model. Estimation of the LogP values and construction of the linear regression model were carried out via the python scikit-learn module [69]. Colchicine and compound (Z)-4b for which no accurate EC₅₀ value was measured were excluded from the fit. EC₅₀s of compounds (Z)-4k, (Z)-4l and (Z)-4g were set to 10000 nM.

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