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Synthesis and Structure–Activity Relationships of Constrained Heterocyclic Analogues of Combretastatin A4

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A series of combretastatin A4 (CA4) analogues with a lactam or lactone ring fused to the trimethoxyphenyl or the B-phenyl moiety were synthesized in an efficient and stereoselective manner by using a domino Heck–Suzuki–Miyaura coupling reaction. The vascular-disrupting potential of these conformationally restricted CA4 analogues was assessed by various in vitro assays: inhibition of tubulin polymerization, modification of endothelial cell morphology, and disruption of endothelial cell cords. Compounds were also evaluated for their growth inhibitory effects against murine and human tumor cells. B-ring-constrained derivatives that contain an oxindole ring (in contrast to compounds with a benzofuranone ring) as well as analogues bearing a six-membered lactone core fused to the trimethoxyphenyl ring are endowed with significant biological activity. The most potent compound of this series (oxindole **9b**) is of particular interest, as it combines chemical stability and a biological activity profile characteristic of a vascular-disrupting agent.

Introduction

Tumor vasculature represents an attractive target for cancer therapy, as solid tumors require blood vessels for growth and metastasis.[1] The antivascular approach, which exploits differences between normal and immature tumor blood vessels, aims to selectively damage the newly established tumor vasculature.[2] To date, vascular-disrupting agents (VDAs) are essentially small molecules that inhibit tubulin polymerization.[3] Microtubules are dynamic polymers of α and β tubulin that play a crucial role in several cellular processes, including cell division, cell signaling, motility, transport, and maintenance of cell shape.[4] Among VDAs that perturb microtubule dynamics, combretastatin A4 (CA4) is the most promising compound.[5]

Early works showed that the natural product CA4 strongly inhibits the proliferation of a broad spectrum of human cancer cells and acts as a tubulin polymerization inhibitor by binding at the colchicine site. The water-soluble phosphate prodrug CA4P, selected for further preclinical development, has drawn significant attention due to its potent and selective effect on tumor blood vessels, causing rapid vascular shutdown, leading to necrosis of the central tumor, an area that is often resistant to conventional anticancer treatments. The prodrug CA4P (fosbretabulin) is currently being evaluated, either alone or in combination with paclitaxel or carboplatin, in clinical studies for the treatment of patients with anaplastic thyroid carcinoma[6] or advanced cancer.[7,8]

By binding to tubulin in tumor endothelial cells, VDAs such as CA4 cause rapid microtubule depolymerization. This damage triggers disruption of the cell–cell junction involving the protein vascular endothelial cadherin[9] and further cytoskeletal rearrangements (assembly of actin stress fibers) through activation of the Rho/Rho kinase pathway.[2a,10] The net result of these effects is a rounding up and membrane blebbing of endothelial cells, together with increased vessel permeability to macromolecules, leading to blood flow shutdown in vivo. The consequent decrease in nutrient supply induces necrosis in the tumor center, with a viable rim remaining at the periphery.[2,11]

The encouraging antivascular and antitumor profile of CA4 has contributed greatly to the current interest in the design and synthesis of several CA4 analogues.[12] Through SAR studies, it has been established that the *cis* orientation of both phenyl groups is an essential requirement for efficient tubulin affinity, forcing the two aromatic rings to stay within an appro-

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priate distance in the colchicine binding site. In fact, the *cis* double bond of CA4 or analogues easily undergoes isomerization, leading to *trans* isomers that display dramatically decreased inhibition of cancer cell growth and tubulin assembly.[12]

Many linkers that constrain the two phenyl rings to a similar *cis*-restricted conformation have been reported to increase the activity and stability of the designed compounds relative to CA4. This approach was mainly achieved by replacement of the double bond with other rigid linkers[13] or different cyclic moieties,[14] or by introducing another ring.[15] These considerations led us to design a new series of conformationally restricted CA4 analogues by inserting an additional ring between the *cis*-olefinic bond and one of the aromatic moieties of the CA4 structure.[16]

Herein we report the synthesis of a broad range of heterocyclic CA4 analogues with a five- or six-membered core of a different nature (lactam or lactone) fused to the trimethoxyphenyl ring or the B-phenyl moiety. We also extended the SAR of this series by examining various substitutions on the B-ring. Dienic compounds were also investigated as analogues of the diarylbutadiene **1** that we previously reported as a potent tubulin polymerization inhibitor (Figure 1).[17] The synthesized compounds were then evaluated in vitro for their capacity to act as potential VDAs. Moreover, the tubulin binding mode of selected derivatives is proposed by molecular docking investigations.

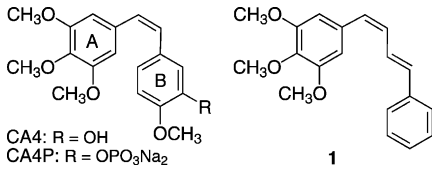


Figure 1. Structures of CA4, the prodrug CA4P, and vinylogous analogue **1**.

Results and Discussion

Chemistry

The stereoselective preparation of fused heterocyclic systems bearing an exocyclic tri- or tetrasubstituted double bond has been reported, by the use of palladium-catalyzed domino reactions (Figure 2). One of the more reliable synthetic pathways is the Heck carbocyclization/Suzuki–Miyaura coupling protocol (Path A) that uses alkyne-tethered halogenated arenes and boronic acids as coupling partners.[18] More recently, compounds with an oxindole moiety were successfully prepared by a sequence of carbopalladation, C–H activation, and C–C bond formation, from anilides and aryl iodides as the starting materials (Path B).[19] We therefore decided to investigate the preparation of heterocyclic CA4 analogues containing a stereochemically defined substituted exocyclic alkene, by using these palladium-catalyzed domino processes.[20]

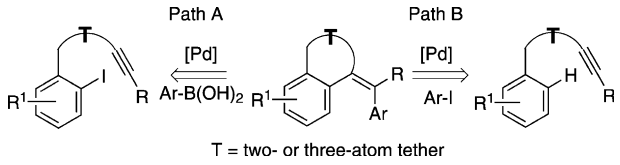
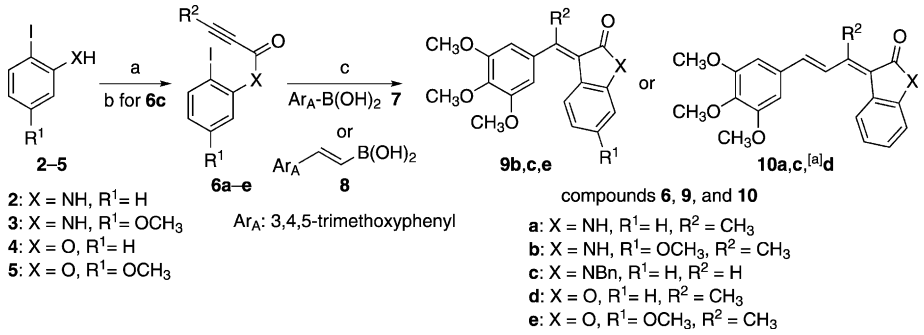


Figure 2. Retrosynthetic approach to the stereoselective preparation of fused heterocyclic systems containing an exocyclic tri- or tetrasubstituted double bond, by palladium-catalyzed domino reactions.

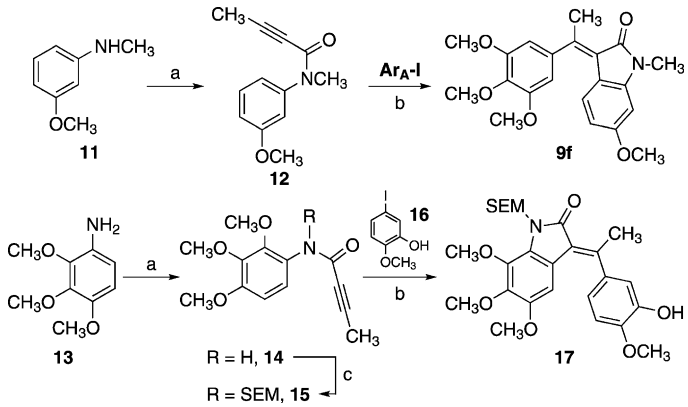
We first planned to synthesize CA4 analogues with a heterocycle fused to the B-ring by using the tandem Heck carbocyclization/Suzuki cross-coupling process (Scheme 1). As we previously reported, under the optimized reaction conditions [$Pd(OAc)_2$, PPh_3, CsF] this tandem reaction occurred with the N-protected propynamide **6c** and the N-free butynamides **6a** and **6b** as starting materials.[21] It should be pointed out that the substitution of the newly formed double bond by a methyl group (R^2), in preventing isomerization of the double bond, stabilizes the coupling products and furthermore allowed a domino reaction with secondary amide substrates (X = NH). Thus, the (*E*)-3-arylmethyleneoxindoles **9b**, **9c** and the (*E*,*E*)-3-alkylideneoxindoles **10a**, **10c** were efficiently obtained, in a stereoselective manner, from the 2-iodoanilides **6a–c** and the 3,4,5-trimethoxyphenyl boronic acid **7** or the corresponding styryl derivative **8**, respectively. The alkyne esters **6d** and **6e** were similarly treated to afford the corresponding lactones **9e** and **10d**. These domino reactions required the use of the 2-iodoaryl derivatives **6a–e**, which were derived from 2-iodoanilines **2** and **3** or 2-iodophenols **4** and **5** by a carbodiimide-mediated coupling with propynoic or butynoic acids.

To access such heterocyclic derivatives, it seemed advantageous to use a domino process involving an arene C–H activa-



Scheme 1. Synthesis of B-ring-constrained CA4 analogues via tandem Heck–Suzuki–Miyaura reaction. *Reagents and conditions:* a) **2**, **3**, **4**, or **5**, CH_3–C≡C–CO_2H, DCC, DMAP for **6d** and **6e**, CH_2Cl_2, 0 °C→RT, 3–18 h, 36–96%; b) 1. **2**, H–C≡C–CO_2H, DCC, CH_2Cl_2, 0 °C→RT, 3 h, 58%; 2. NaH, THF, 0 °C, 1 h, then BnBr, TBAB, RT, 2 h, 77%; c) Coupling conditions A: boronic acid **7** or **8** (1.1 equiv), $Pd(OAc)_2$ (5 mol%), PPh_3 (10 mol%), CsF (3 equiv) in THF, reflux, 1–3 h, 47–80%; for precursor **6e** and boronic acid **8**: RT, 2 h, 47%. [a] Presence of the *Z* isomer (*EE*/*EZ*: 97:3).

tion that requires readily available non-halogenated aromatic precursors. Zhu and co-workers reported the synthesis of 3-(diarylmethylene)oxindoles from *N*-methyl- or *N*-trimethylsilylethoxymethyl (SEM)-3-arylpropynamides (Figure 2, path B, R = phenyl) and aryl iodides as the other coupling partner.[19] Interestingly, under the reported reaction conditions [$Pd(OAc)_2$, NaOAc, DMF], the methylacetylene derivative **12** proved to be a suitable partner (Scheme 2). After heating at 80 °C for 2 h in the presence of 3,4,5-trimethoxyphenyl iodide, the *N*-methyloxindole **9 f** was obtained stereoselectively, albeit in moderate yield (46 %).[22]



Scheme 2. Synthesis of oxindoles **9 f** and **17** via hydroarylation. *Reagents and conditions*: a) CH_3–C≡C–CO_2H, DCC, CH_2Cl_2, 0 °C→RT, 3 h, 77 % for **12**, 88 % for **14**; b) Coupling conditions B: Ar–I (1.15 equiv), $Pd(OAc)_2$ (0.05 equiv), NaOAc (2 equiv), DMF; 80 °C, 2 h for **9 f** (46 %); reflux, 5 min for **17** (46 %); c) NaH, THF, 0 °C, 30 min, then SEMCl, 0 °C→RT, 65 h, 75 %.

Encouraged by these results, we envisioned extension of the same methodology to the synthesis of CA4 analogues bearing a hetero-ring annulated to the trimethoxyphenyl moiety. We first studied the oxindole series. Because this reaction proceeds well with a tertiary amide, we selected an acetylenic precursor with a removable N-protecting group, the *N*-SEM-butynamide **15** (Scheme 2).[23,24] With 5-iodo-2-methoxyphenol **16**[25] as the other coupling partner, and under the same conditions as reported above (80 °C, 2 h), the expected oxindole **17** was obtained, but in low yield (23 %). Nevertheless, running the reaction at reflux briefly (5 min) increased the yield to 46 %.[26]

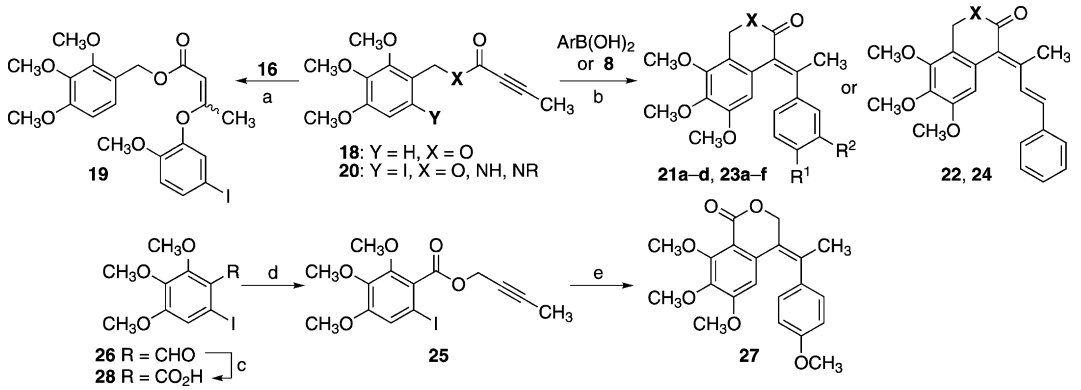
We next turned our attention to the synthesis of CA4 analogues with a six-membered heterocycle fused to the trimethoxyphenyl ring: isoquinolinone and isochromanone derivatives with a multi-substituted *exo* double bond. Unfortunately, ester **18** failed to react following the direct arylation process (Scheme 3). Instead, the Michael addition product of phenol **16** to the activated triple bond was recovered (ether **19**). However, the desired isochroman-3-ones **21 a–d** and **22** were obtained under the Heck–Suzuki coupling protocol from ester **20** (X = O) using several boronic acids as the trapping agents.[27] Isoquinolin-3-ones **23 a–f** and **24** were prepared in the same manner from the appropriate alkynamides **20** (X = NH, NR).

Access to the isomeric isochroman-1-one series was also attempted (Scheme 3). Under the same procedure, reaction of the benzoate **25**, obtained from the known benzaldehyde **26**,[28] with the 4-methoxyphenylboronic acid afforded the desired lactone **27**, but along with the corresponding direct coupling product (ratio 6:4). Despite different reaction conditions, the styryl and more electron-rich boronic acids failed to produce the expected products. The configuration of the newly formed double bond of all the synthesized compounds was unambiguously supported by NOESY experiments.[21,27]

Biological evaluation

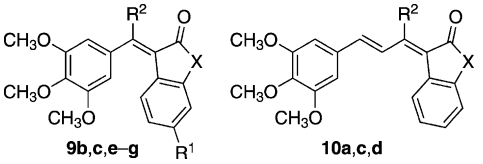
The potential antivascular effects of the synthesized compounds were assessed by their ability to inhibit tubulin polymerization (fluorimetric assay)[29] and to rapidly induce a rounding up of endothelial cells.[30] This morphological test is considered to be predictive of potential in vivo antivascular activity, and it allows determination of the lowest active concentration that causes rounding of immortalized HUVEC (EAhy 926 cells) within 2 h. Compounds were also evaluated for their growth inhibitory effects against two murine cancer cell lines: B16 melanoma cells and Lewis lung carcinoma (3LL) cells.

The biological activities of the B-ring-constrained CA4 analogues, with CA4 and diarylbutadiene **1** as reference com-



Scheme 3. Synthesis of A-ring-constrained CA4 analogues.[a] *Reagents and conditions:* a) **18**, coupling conditions B, 70 °C, 20 min; b) **20**, Ar–$B(OH)_2$ or **8**, coupling conditions A, 1–10 h, 29–92 %;[27] c) $KMnO_4$, H_2O, 75 °C, 5 h, 84 %; d) butynol, DCC, DMAP, CH_2Cl_2, RT, 65 h, 67 %; e) 4-methoxyphenylboronic acid, coupling conditions A, 30 h, 36 %. [a] See Table 2 for a complete description of X, R^1, and R^2 groups.

Table 1. Inhibition of tubulin polymerization, morphological effects on endothelial cells, and cytotoxicity of B-ring-constrained CA4 analogues.



Compd	X	R^2	R^1	ITP IC_{50} [μM][a]	Morphology HUVEC [μM][b]	Cytotoxicity IC_{50} [μM][c] 3LL	B16
9c	N-Bn	H	H	20	10	5.3	9.4
9g	NH	CH_3	H	>20	>10	19	32
9b	NH	CH_3	OCH_3	0.6	0.05	0.09	1.1
9f	N-CH_3	CH_3	OCH_3	14	0.3	0.79	16
9e	O	CH_3	OCH_3	6.7	na[d]	>50	>50
10c	N-Bn	H	–	19	na	5.7	22
10c	NH	CH_3	–	5.6	1.3	8.4	>50
***trans*-10a**	NH	CH_3	–	2.2	5	3.1	12
10d	O	CH_3	–	13	na	>50	>50
CA4				0.27	0.006	0.004	0.005
1				0.56	0.10	nd[e]	0.13

[a] Compound concentration required to inhibit tubulin polymerization (ITP) by 50%; in vitro microtubule assembly was monitored using a fluorescent probe (DAPI).[29] [b] Morphological activity on immortalized HUVEC (EAhy 926 cells) is expressed as the lowest concentration at which cell rounding was observed following incubation with the tested drug for 2 h. [c] Compound concentration required to inhibit murine tumor cell proliferation by 50% after an incubation time of 48 h; 3LL: Lewis lung carcinoma; B16: melanoma. [d] Not active at maximum solubility in cell culture medium. [e] Not determined.

pounds, are presented in Table 1. The *N*-benzyloxindole **9c** does not markedly inhibit tubulin polymerization and was also shown to gradually isomerize in solution to the (*Z*)-olefinic form. This instability was previously observed, by our research group and others, with similar oxindole derivatives bearing a methine bridge.[16c,d,21] Nevertheless, as we previously reported, substitution of the exocyclic double bond by a methyl group leads to chemically stable compounds. Therefore, only derivatives with a methyl-substituted alkene were further investigated.

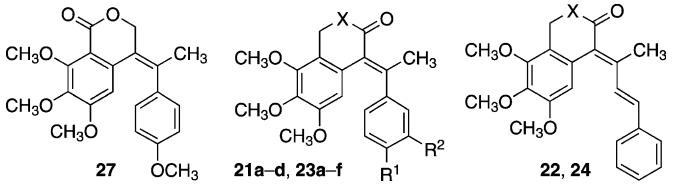
The stabilized N-free oxindole **9g**[31] was also devoid of biological activity. Interestingly, by analogy with CA4, substitution at the C6 position of the oxindole core with a methoxy group (compound **9b**) considerably enhanced biological activity. Compound **9b** showed an inhibition of tubulin polymerization (IC_{50} = 0.6 μM) within the same order of magnitude as that of CA4 (IC_{50} = 0.27 μM) and clearly induced rapid changes in endothelial cell morphology at low concentration (rounding up at 50 nM).[32] Antiproliferative activity was also increased, but did not reach the IC_{50} values of CA4 (IC_{50} = 0.09 and 1.1 μM versus 0.005 for CA4). However, methylation of the oxindole nitrogen (compound **9f**) led to significant loss of anti-tubulin activity,[33] although a morphological effect on endothelial cells was observed at 0.3 μM. Replacement of the methoxyoxindole ring by a methoxybenzofuran ring (compound **9e**) had a detrimental effect on activity. Considering the above results, it appears that the key structural factors for potent constrained derivatives bearing an olefinic linker are a methoxy group and a free nitrogen atom on the heterocyclic ring.

Compounds **10**, which did not bear the methoxy group essential for activity in the previous series, were investigated as analogues of the dienic derivative **1**.[34] The *N*-benzyloxindole **10c** (a vinylogous analogue of **9c**) presented the same biological profile as **9c;** that is, both failed to interact with tubulin and displayed a moderate effect on the proliferation of 3LL cells (IC_{50} = 5–6 μM). Unfortunately, with regard to the potent compound **9b**, such a similarity was not observed with the stabilized N-free oxindole **10a**, which showed a significant loss of potency. Surprisingly, its isomer, with a *trans*,*trans*-butadienic linker (***trans*-10a**)[35] displayed a slightly better inhibition of tubulin polymerization. In this dienic series a benzofuran core was also unsuitable for activity (compound **10d**).

Table 2 lists the biological activity values of the synthesized compounds bearing a heterocyclic moiety fused to the trimethoxyphenyl ring. The *N*-SEM-oxindole derivative **17** is devoid of any appreciable anti-tubulin and antiproliferative activities. All the other products studied presented a larger six-membered lactam or lactone ring, therefore offering the possibility to adopt a more suitable conformation to interact with tubulin.

To estimate the effect of the position of the ester function inside the ring on biological activity, the isomeric isochromanones **21a** and **27** were compared. Whereas compound **27** with the carbonyl group in the benzylic position was found inactive, the 3-isochromanone **21a** affected tubulin polymerization (3.9 μM), endothelial cell morphology, and proliferative activity. Therefore, several 3-isochromanones variously substituted on the B-ring were prepared and evaluated. Replacement of the 4-methoxy substituent with the lipophilic trifluoromethyl group (compound **21b**) proved detrimental to activity. However, the dimethylamino derivative **21c** and compound **21d**, which possesses the structural feature of CA4, displayed a substantial ability to inhibit tubulin polymerization and produced a potent morphological effect at sub-micromolar concentrations. These three interesting isochromanones displayed modest antiproliferative activity in the micromolar range. Several isoquinolinones with a free or alkylated nitrogen atom and a variously substituted B-ring were then evaluated. However, these compounds (**23a**–**f**) were essentially devoid of activity. In summary, for the A-ring-constrained CA4 analogues investigated, the dimethylaniline and methoxyphenol derivatives **21c** and **21d** proved to be the best tubulin polymerization inhibitors, with IC_{50} values (1.4 and 1.8 μM) slightly lower than that of CA4.

Table 2. Inhibition of tubulin polymerization, morphological effects on endothelial cells, and cytotoxicity of A-ring-constrained CA4 analogues.



Compd	X	R^1	R^2	ITP IC_{50} [μM][a]	Morphology HUVEC [μM][b]	Cytotoxicity IC_{50} [μM][c] 3LL	B16
17	–	–	–	>20	25		19
27	–	–	–	nd[d]	>30	55	39
21a	O	OCH_3	H	3.9	1.3	1.1	1.9
21b	O	CF_3	H	>20	12.5	13	12
21c	O	$N(CH_3)_2$	H	1.4	0.65	0.85	1.8
21d	O	OCH_3	OH	1.8	0.65	1.2	1.0
23a	NH	OCH_3	H	>20	12.5	nd	20
23b	N-Bn	OCH_3	H	>20	>30	nd	15
23c	N-CH_3	OCH_3	H	>20	>30	nd	23
23d	N-CH_3	CF_3	H	>20	>30	nd	28
23e	N-CH_3	$N(CH_3)_2$	H	>20	25	nd	23
23f	N-CH_3	OCH_3	OH	>20	25	nd	25
22	O	–	–	1.0	0.12	3.1	0.9
24	N-CH_3	–	–	12	12.5	nd	>50
CA4				0.27	0.006	0.004	0.005
1				0.56	0.10	nd	0.13

[a] Compound concentration required to inhibit tubulin polymerization (ITP) by 50%; in vitro microtubule assembly was monitored using a fluorescent probe (DAPI).[29] [b] Morphological activity on immortalized HUVEC (EAhy 926 cells) is expressed as the lowest concentration at which cell rounding was observed following incubation with the tested drug for 2 h. [c] Compound concentration required to inhibit murine tumor cell proliferation by 50% after an incubation time of 48 h; 3LL: Lewis lung carcinoma; B16: melanoma. [d] Not determined.

With dienic derivatives, the lactam ring was also detrimental to biological activity (compound **24**). However, lactone **22** potently inhibited tubulin polymerization ($IC_{50}=1.0$ μM) and caused rounding up of endothelial cells (0.12 μM) at a concentration lower than the cytotoxic IC_{50} values (micromolar range). Notably, the constrained compound **22** displayed a similar biological activity profile as that of diene **1**.

Altogether, these results show that some constrained derivatives exhibited interesting biological properties; these include oxindoles **9b** and **9f**, and the isoquinolones **21c**, **21d**, and **22** (Table 3). These five compounds are able to cause endothelial cell rounding at low micromolar concentrations, with the B-ring-constrained derivative **9b** still affecting cellular shape at 50 nM. Representative micrographs of the rounding up effects of some of these compounds are presented in Figure 3. Alteration of endothelial cell morphology is a distinctive feature of the activity of VDAs and is usually associated with microtubule network disorganization. Indeed, alteration of endothelial cell morphology by compounds **9b**, **21c**, **21d**, and **22** is well correlated with their ability to effectively inhibit tubulin polymerization. However, correlation between those assays did not hold true for derivative **9f**, for which disruption of the endothelial cell network could result from alteration of the organization of other cytoskeletal components.[9,30] It is noteworthy that the desmethyl analogue of **9f**, which does not bear a methyl group on the olefinic bond, was recently reported to display not only a vascular-disrupting effect, but also anti-angiogenic activities.[36]

The effect of these promising compounds was next evaluated on the proliferation of EAhy 926 endothelial cells. After incubation for 48 h, compounds **9b**, **21c**, **21d**, and **22** exhibited significant inhibition of endothelial cell proliferation (Table 3). However, it should be noted that these compounds were not cytotoxic ($IC_{50}>50$ μM) following an incubation time of 2 h (experimental conditions for the morphological test). These results indicate that the morphological effects observed are not caused by antiproliferative activity within the 2 h incubation period.

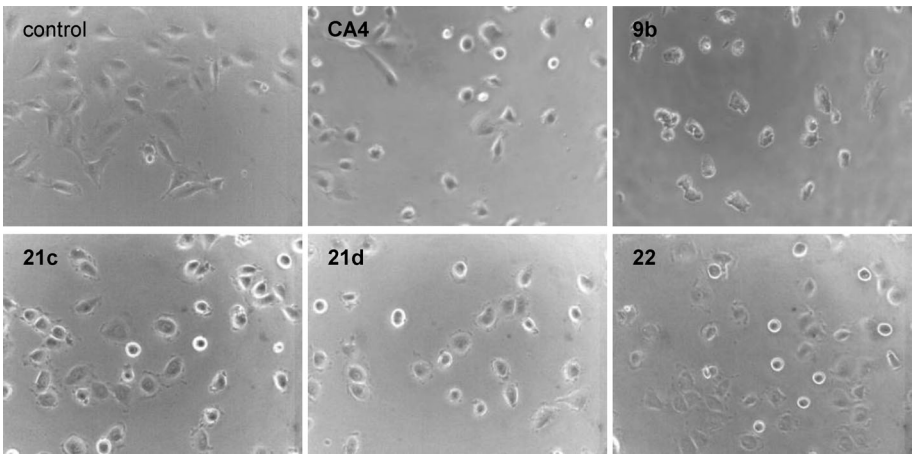
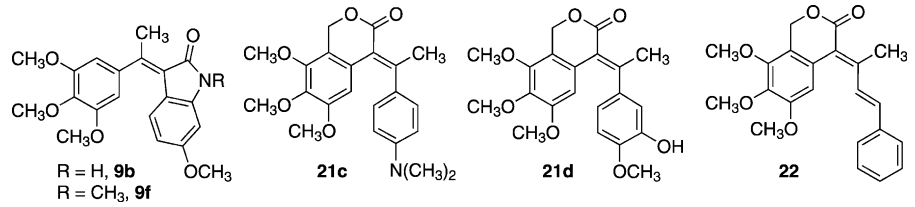


Figure 3. Morphological effects of selected CA4 analogues on EAhy 926 endothelial cells. Cells in exponential growth were exposed to the indicated compound for 2 h at the following concentration: control, 1% DMSO; CA4, 0.006 μM; **9b**, 0.10 mM; **21c**, 0.65 μM; **21d**, 0.65 μM; **22**, 0.16 μM. Representative micrographs were taken at 360× magnification.

Table 3. Biological activities of representative constrained derivatives.



Compd	ITP IC_{50} [μM][a]	Morphology HUVEC [μM][b]	Cytotoxicity IC_{50} [μM] 3LL[c]	B16[c]	HCT116[d]	MCF7 $IC_{1/2max}$ [μM][d,e]	Endothelial cells[f]
9b	0.6	0.05	0.09	1.1	0.10	0.039	0.008
9f	14	0.3	0.79	16	nd[g]	nd	nd
21c	1.4	0.65	0.85	1.8	0.21	0.51	0.9
21d	1.8	0.65	1.2	1.0	nd	nd	1.0
22	1.0	0.12	3.1	0.9	0.10	0.079	0.17
CA4	0.27	0.006	0.005	0.004	0.001	0.001	0.005
1	0.56	0.10	nd	0.13	0.50[17]	nd	0.3

[a] Compound concentration required to inhibit tubulin polymerization (ITP) by 50%; in vitro microtubule assembly was monitored using a fluorescent probe (DAPI).[29] [b] Morphological activity on immortalized HUVEC (EAhy 926 cells) is expressed as the lowest concentration at which cell rounding was observed following incubation with the tested drug for 2 h. [c] Compound concentration required to inhibit murine tumor cell proliferation by 50% after an incubation time of 48 h; 3LL: Lewis lung carcinoma; B16: melanoma. [d] Compound concentration required to inhibit human tumor cell proliferation by 50% after 72 h incubation. [e] IC_{50} values could not be determined with the MCF7 cell line, as growth inhibition did not exceed 60%; $IC_{1/2max}$ is the half-maximal inhibition concentration. [f] Compound concentration required to inhibit immortalized endothelial cell (EAhy 926) proliferation by 50% after 48 h incubation; following incubation for 2 h, $IC_{50} > 50$ μM. [g] Not determined.

We next evaluated the ability of compounds **9b**, **21c**, **21d**, and **22** to disrupt a network of capillary-like tubular structures formed by endothelial cells seeded on a Matrigel matrix.[37] Their effects on the preformed cords are depicted in Figure 4 and are compared with CA4. Over the course of 2.5 h, these constrained CA4 analogues can disrupt the tube-like structures at concentrations of 1 μM (CA4, **9b**, **9f**, and **22**) and 10 μM (**1**, **21c**, and **21d**). These effects were observed at a concentration previously shown non-cytotoxic for endothelial cells after a short exposure time.

To further assess the cytotoxic profile of oxindole **9b** and lactones **21c** and **22**, we investigated their effects against two human cancer cell lines: HCT116 colon carcinoma cells and MCF7 hormone-dependent breast carcinoma cells. Against HCT116, these three compounds displayed antiproliferative activities of 0.1–0.2 μM (Table 3). For the MCF7 cell line, IC_{50} values could not be determined, as growth inhibition did not exceed 60%, even for CA4 (Supporting Information, table 1). Whereas the reference CA4 inhibited the proliferation of the four tumor cell lines with IC_{50} values in the nanomolar range, our compounds exhibited good to moderate cytotoxicity depending on the tumor cell type (IC_{50} values ranging from 0.1 to 1–3 μM). However, these compounds displayed significantly lower cytotoxicity than CA4, while they showed a potent ability to inhibit the assembly of tubulin. In fact, this discrepancy between cytotoxic activity and anti-tubulin activity represents an interesting goal in the search for new antivascular agents.[38]

Molecular docking

To determine the possible binding modes of this series of compounds with tubulin, docking studies were carried out with the most potent compounds: oxindole **9b** and the A-ring-constrained derivatives **21c**, **21d**, and **22**, by using the reported high-resolution crystal structure of the tubulin–DAMA-colchicine complex (PDB ID: 1SA0).[39] The colchicine binding site is mostly buried in the β subunit, bordered by the S8 and S9 strands, the T7 loop, and the H7 and H8 helices. The ligand also maintains few interactions with the T5 loop of the neighboring α subunit. Several important residues for binding of colchicine-type ligands to tubulin have been identified, including Cysβ241, Metβ259, Thrα179, and Valα181.[40]

Docking simulations were performed with GOLD software version 4.1 with default parameters and without constraints on the ligand.[41] With such parameters, GOLD generates poses until it finds three with RMSD < 1.5 Å. GoldScore was used as the scoring function to rank the poses. To validate our docking process, DAMA-colchicine was first extracted from the complex then re-docked. The three top-scored conformers obtained superimposed well with the original crystallized structure. CA4 was found to adopt a very similar binding mode, the trimethoxyphenyl ring lies in a hydrophobic pocket close to Cysβ241, and the 4′-methoxy group occupies the same position as the corresponding group on ring C of colchicine. As previously reported, CA4 interacts extensively with β-tubulin and with only two residues in α-tubulin (Thr 179 and Val 181).[40,42]

Our docking process applied to the oxindole **9b** identifies two potential binding modes. In the best-scored pose, **9b** overlays correctly with CA4 (Figure 5A). The 3- and 4-methoxy oxygen atoms of the TMP ring are involved in hydrogen bonds with the thiol group of Cysβ241, and the methoxy group of the oxindole core establishes hydrophobic contacts with the side chain of Metβ259. Hydrophobic interactions are also observed between the methyl group of the bridge and residues Leu 248 and Ala 250 of the β-tubulin subunit. Furthermore, the indole NH group forms a stable hydrogen bond (1.6 Å) with the amide oxygen atom of Asnβ258.[43–45] This could explain the observed good biological results for **9b** and the loss of activity for the *N*-methyl analogue **9f**, or for the derivative **9e**, which bears an oxygen atom in place of a hydrogen bond donor atom. Furthermore, residues Leu 248 and Ala 250 of the

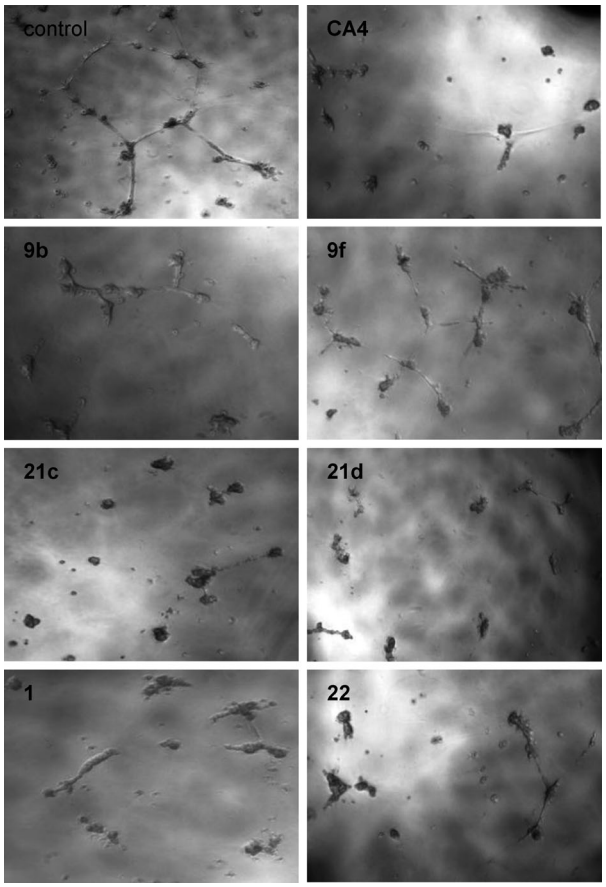


Figure 4. Effects of selected CA4 analogues on preformed endothelial cell cords (HUVEC) on Matrigel. Micrographs shown were taken 2.5 h after addition of the compounds at the following concentration: control, 1% DMSO; CA4, 1 μM; **9b**, 1 μM; **9f**, 1 μM; **21c**, 10 μM; **21d**, 10 μM; **1**, 10 μM; **22**, 1 μM. Original magnification of 40×.

β-tubulin subunit are involved in hydrophobic contacts with the methyl group of the olefinic bridge.

In contrast, in the second binding mode (score value of 43.3, regarding the score of 45.7 for the best pose), the TMP ring lies on the edge of the colchicine binding site, pointing toward the GTP bound to the α-subunit (Figure 5B). In this case, hydrogen bonds could be established between two methoxy oxygen atoms of the TMP ring, the 3-methoxy group with the side chain amino group of Asn α101 (3.0 Å) and the 5-methoxy group with the side chain hydroxy of Ser α178 (3.0 Å). Hydrogen bond interactions with Asn 101 and Ser 178 of α-tubulin are also found to be involved in the binding of potent tubulin polymerization inhibitors which show an orientation different from that of DAMA-colchicine in the tubulin binding site (heterocyclic derivatives with a trimethoxybenzoyl moiety[46] or a recently reported compound[47] discovered by virtual screening).[48] Although the indole ring is positioned differently, hydrophobic contacts of the methoxy group with Met β259 could be maintained, as well as interactions with the olefinic methyl substituent. The three A-ring-constrained compounds **21c**, **21d**, and **22** dock in a very similar—albeit quite unexpected—position, thus indicating less efficient binding to tubulin (see Supporting Information, figure 1).

Conclusions

We designed and prepared a set of conformationally restricted CA4 analogues with a lactam or lactone ring fused to the trimethoxyphenyl or the B-phenyl moieties of CA4. Another series included compounds with an (*E*,*Z*)-dienic linker. Access to these compounds was efficiently achieved in a stereoselective manner by using a domino Heck–Suzuki–Miyaura coupling reaction. Oxindole derivatives could be obtained via C–H arylation; however, the sequence could not be extended to compounds with a lactam ring.

The vascular-disrupting potential of these molecules was assessed with various in vitro assays. For the B-ring-constrained derivatives with an olefinic linker, a methoxy group and a free nitrogen atom on the heterocyclic moiety are required structural factors for potent activity. Although the oxindole **9b** is endowed with moderate antiproliferative activity, its ability to disorganize the microtubule network is similar to that of the reference CA4 and is well supported by the molecular modeling studies. In contrast, this structural modification is detrimental in the dienic series.

Results of the biological evaluation of compounds bearing a six-membered heterocyclic core fused to the trimethoxyphenyl ring indicate that a lactone moiety is well tolerated in both series. The significant anti-tubulin activity displayed by the 3-isochromanones **21c**, **21d**, and **22** indicates that structural modification carried out on this A-part of the CA4 molecule, although rarely considered so far, is a promising way to provide access to novel antivascular compounds.

The most active compound **9b** was of particular interest, as it combines chemical stability and the biological activity profile characteristic of a vascular-disrupting agent. The promising antivascular potentiality of this compound will be investigated further with in vivo murine experiments.

Experimental Section

Chemistry

Unless otherwise noted, all materials were obtained from commercial sources and were used without purification. THF was dried over sodium/benzophenone, CH_2Cl_2 over P_2O_5, DMF and MeOH over molecular sieves. Some cycloadducts have already been described by our research group and others: **6a**, **6c**,[49] **10c**,[21] and **21**–**24**.[27] Aniline **3**,[50] phenol **5**,[51] and aldehyde **26**[28] were prepared according to published procedures. ^{1}H NMR spectra were recorded with a Bruker ACP 300 spectrometer at 300 MHz for ^{1}H NMR, and at 75 MHz for ^{13}C NMR spectroscopy. Chemical shift values (δ) are given in ppm relative to the residual solvent peak ($CHCl_3$) as the internal reference, and coupling constants (*J*) are given in Hertz. All ^{13}C NMR spectra were recorded with complete proton decoupling. Peak assignment was unambiguously performed using HMQC, HMBC, and NOESY techniques. Melting points were determined by the capillary method using an Electrothermal 9200 apparatus and are uncorrected. Mass spectra were recorded on a Waters ZQ 2000 system using electrospray ionization (ESI). High-resolution ESIMS data were acquired from the "imagif" service (CNRS-ICSN, 91198 Gif-sur-Yvette, France) on a Waters LCT spectrometer. Reactions were monitored by thin-layer chromatog-

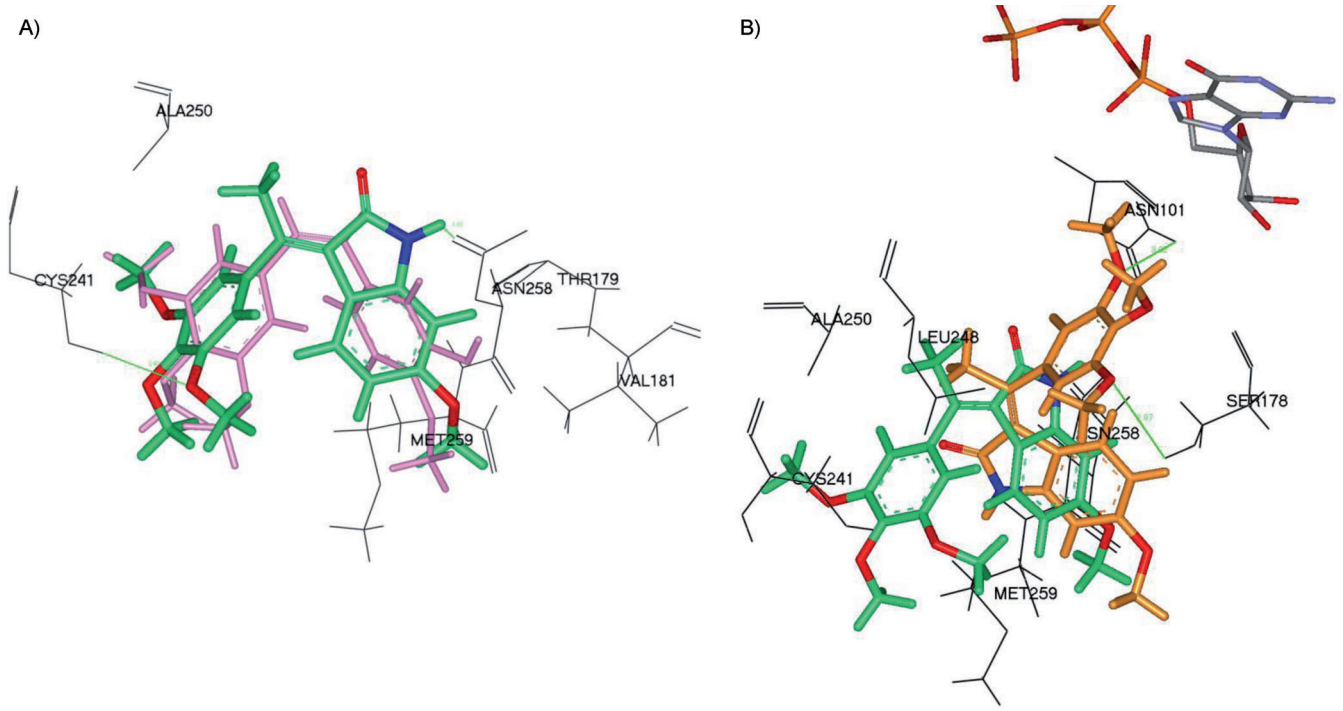
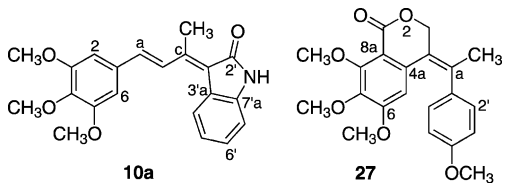


Figure 5. Proposed binding mode of CA4 and oxindole **9b** in the colchicine binding site of tubulin. A) Superimposition of CA4 (pink) and the best-scored pose of compound **9b** (green). B) Superimposition of the two best-scored poses of compound **9b**. Only relevant residues are indicated.

raphy (TLC) with Merck silica gel 60 F_{254}. Flash chromatography was carried out on Merck silica gel (320–400 mesh). Representative structures with indicative numbering for NMR assignments are shown:



6-Iodo-2,3,4-trimethoxybenzoic acid (28). A solution of $KMnO_4$ (380 mg, 2.40 mmol) in H_2O (20 mL) was added dropwise to a suspension of aldehyde **26**[28] (500 mg, 1.55 mmol) in H_2O (15 mL) while stirring at 75 °C.[52] After stirring at 75 °C for 5 h, the reaction mixture was cooled to room temperature, brought to pH 12 by addition of aqueous KOH (20%), then filtered on a pad of Celite. The filtrate was acidified to pH 2 (2 N HCl), and then extracted with Et_2O (50 mL×3). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was recrystallized from CH_2Cl_2/hexane to give acid **28** as a white solid (440 mg, 84%); mp: 100–101 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.91 (s, 1H, CO_2H), 7.11 (s, 1H, H-5), 3.90 (s, 3H, OCH_3), 3.85 ppm (s, 6H, OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.6 (CO_2H), 155.2 (C-4), 151.5 (C-2), 142.4 (C-3), 127.3 (C-1), 118.6 (CH-5), 84.3 (C-6), 62.2 (OCH_3), 61.0 (OCH_3), 56.4 ppm (OCH_3); MS (ES^+): *m/z*: 361 [*M*+Na]$^+$; Anal. calcd for $C_{10}H_{11}IO_5$: C 35.52, H 3.28, found: C 35.37, H 3.21.

Acetylenic derivatives

Preparation of amides 6b, 12, and 14: general procedure: A solution of DCC (1 equiv) in CH_2Cl_2 was added dropwise at 0 °C to a solution of the appropriate aniline and but-2-ynoic acid (1 equiv) in dry CH_2Cl_2. After stirring for 3 h at room temperature, the mixture was cooled to 0 °C and filtered. The residue was washed with CH_2Cl_2. The filtrate was washed with 2 N HCl, saturated aqueous $NaHCO_3$, and H_2O. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure.

***N*-(2-Iodo-5-methoxyphenyl)but-2-ynamide (6b):** Aniline **3**[50] (375 mg, 1.5 mmol) afforded amide **6b** as a white solid (155 mg, 36%) after chromatography (cyclohexane/EtOAc 8:2); mp: 136–138 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.93 (d, *J* = 2.9 Hz, 1H, H-6), 7.61 (d, *J* = 8.8 Hz, 1H, H-3), 7.60 (brs, 1H, NH), 6.49 (dd, *J* = 8.8, 2.9 Hz, 1H, H-4), 3.79 (s, 3H, OCH_3), 2.04 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 160.6 (C-5), 150.9 (CO), 138.7 (C-1), 138.6 (CH-3), 113.2 (CH-4), 107.5 (CH-6), 85.2 (C-d), 77.2 (C-2), 75.2 (C-c), 55.5 (OCH_3), 3.9 ppm (CH_3); MS (ES^+): *m/z*: 338 [*M*+Na]$^+$; Anal. calcd for $C_{11}H_{10}INO_2$: C 41.93, H 3.20, N 4.45, found: C 41.89, H 3.18, N 4.33.

***N*-(3-Methoxyphenyl)-*N*-methylbut-2-ynamide (12)**. *N*-Methylaniline **11** (500 mg, 3.65 mmol) afforded amide **12** as a colorless oil (574 mg, 77%) after chromatography (cyclohexane/EtOAc 7:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.29 (t, *J* = 8.1 Hz, 1H, H-5), 6.87–6.81 (m, 3H, H-2, H-4, H-6), 3.82 (s, 3H, OCH_3), 3.29 (s, 3H, N-CH_3), 1.76 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 160.0 (C-3), 154.2 (CO), 144.3 (C-1), 129.7 (C-5), 119.3 (C-6), 113.2 (C-4), 112.9 (C-2), 89.8 (C-d), 74.0 (C-c), 55.4 (OCH_3), 36.3 (CH_3), 3.9 ppm (CH_3); MS (ES^+) *m/z*: 204 [*M*+H]$^+$, 226 [*M*+Na]$^+$; HRMS (DCI/CH_4): *m/z* [*M*+H]$^+$ calcd for $C_{12}H_{14}O_2N$: 204.1025, found: 204.1021.

***N*-(2,3,4-Trimethoxyphenyl)but-2-ynamide (14):** Trimethoxyaniline **13**[23,53] (1.16 g, 6.33 mmol) afforded amide **14** as cottony white

crystals (1.40 g, 88%) after recrystallization (EtOH/hexane); mp: 130–131 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.96 (d, J = 9.0 Hz, 1H, H-6), 7.82 (s, 1H, NH), 6.62 (d, J = 9.0 Hz, 1H, H-5), 3.96 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 2.08 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 150.7 (CO), 150.1 (C-4), 142.5 (C-2), 141.6 (C-3), 124.9 (C-1), 115.2 (CH-6), 106.9 (CH-5), 84.0 (C-d), 75.6 (C-c), 61.3 (OCH_3), 60.9 (OCH_3), 56.1 (OCH_3), 3.8 ppm (CH_3); MS (ES^+) m/z: 250 $[M+H]^+$; Anal. calcd for $C_{13}H_{15}NO_4$: C 62.64, H 6.07, N 5.62, found: C 62.70, H 6.01, N 5.61.

***N*-[(2-(Trimethylsilyl)ethoxy)methyl]-*N*-(2,3,4-trimethoxyphenyl)-but-2-ynamide (15):** NaH (60% in mineral oil, 138 mg, 3.44 mmol) was added to a stirred solution of amide **14** (660 mg, 2.65 mmol) in dry THF (30 mL) at 0 °C. After 30 min at 0 °C, SEMCl (780 μL, 3.97 mmol) was added, and the mixture was stirred at room temperature for 65 h before being quenched with saturated aqueous $NaHCO_3$ and extracted with Et_2O (100 mL × 2). The combined organic layers were washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by flash chromatography (cyclohexane/EtOAc 75:25) to give amide **15** (two rotamers at a ratio of 7:3) as a colorless oil (745 mg, 75%); ^{1}H NMR (300 MHz, $CDCl_3$), *major*/minor: δ = *6.89*/6.83 (d, J = 8.8 Hz, 1H, H-6), *6.63*/6.66 (d, J = 8.8 Hz, 1H, H-5), 5.40 (d, J = 10.3 Hz, 1H, N-CH*Ha*), 4.68 (d, J = 10.3 Hz, 1H, N-CH*Hb*), 3.90 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.59–3.68 (m, 2H, O-CH_2), *1.73*/2.05 (s, 3H, CH_3), 0.95–0.88 (m, 2H, Si-CH_2), 0.01 ppm (s, 9H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$), *major*/minor: δ = *155.9*/155.1 (CO), *154.0*/153.7 (C-4), *150.5*/149.7 (C-2), *142.4*/142.8 (C-3), *127.4*/125.6 (C-1), *124.9*/123.7 (CH-6), *106.4*/106.9 (CH-5), *89.8*/90.1 (C-d), 76.1 (CH_2-N), *74.1*/73.5 (C-c), 66.2/65.6 (CH_2-O), *61.2*/61.1 (OCH_3), 60.8 (OCH_3), 55.9 (OCH_3), *18.1*/17.9 (CH_2-Si), *3.8*/4.1 (CH_3), −1.4 ppm (CH_3); MS (ES^+) m/z: 380 $[M+H]^+$, 402 $[M+Na]^+$; HRMS (DCI/CH_4) m/z $[M+H]^+$ calcd for $C_{19}H_{30}O_5NSi$: 380.1893, found: 380.1899.

2-Iodophenylbut-2-ynoate (6d): A solution of DCC (467 mg, 2.27 mmol) in dry CH_2Cl_2 (15 mL) was added dropwise to a solution of phenol **4** (500 mg, 2.27 mmol), but-2-ynoic acid (186 mg, 2.27 mmol) and DMAP (25 mg, 0.22 mmol) in dry CH_2Cl_2 (10 mL) at 0 °C. After stirring at room temperature for 3 h, the mixture was cooled to 0 °C and filtered. The filtrate was then washed with 2 N HCl, saturated aqueous $NaHCO_3$, and H_2O. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by flash chromatography (cyclohexane/EtOAc 95:5) to give ester **6d** as a colorless oil (435 mg, 67%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.83 (d, J = 7.7 Hz, 1H, H-3), 7.37 (t, J = 7.7 Hz, 1H, H-5), 7.12 (d, J = 7.7 Hz, 1H, H-6), 6.99 (t, J = 7.7 Hz, 1H, H-4), 2.08 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 151.0 (CO), 150.5 (C-1), 139.6 (CH-3), 129.5 (CH-5), 128.1 (CH-4), 122.9 (CH-6), 90.0 (C-2), 88.9 (C-d), 71.9 (C-c), 4.1 ppm (CH_3); MS (ES^+) m/z: 287 $[M+H]^+$, 309 $[M+Na]^+$; HRMS (DCI/CH_4) m/z $[M+H]^+$ calcd for $C_{10}H_8O_2I$: 286.9569, found: 286.9563.

2-Iodo-5-methoxyphenylbut-2-ynoate (6e): Ester **6e**, prepared by following the above procedure employing phenol **5** (500 mg, 2 mmol) and but-2-ynoic acid (168 mg, 2 mmol), was obtained after recrystallization (CH_2Cl_2/hexane) as white crystals (610 mg, 96%); mp: 64–66 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.66 (d, J = 8.9 Hz, 1H, H-3), 6.70 (d, J = 2.8 Hz, 1H, H-6), 6.61 (dd, J = 8.8, 2.8 Hz, 1H, H-4), 3.77 (s, 3H, OCH_3), 2.08 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 160.9 (C-5), 151.2 (C-1), 150.9 (CO), 139.3 (CH-3), 114.6 (CH-4), 109.1 (CH-6), 88.9 (C-d), 78.3 (C-2), 72.0 (C-c), 56.6 (OCH_3), 4.1 ppm (CH_3); MS (ES^+) m/z: 317 $[M+H]^+$; Anal. calcd for $C_{11}H_9IO_3$: C 41.80, H 2.87, found: C 42.36, H 3.09.

2,3,4-Trimethoxybenzylbut-2-ynoate (18): A solution of DCC (1.87 g, 9.08 mmol) in dry CH_2Cl_2 (50 mL) was added dropwise to a solution of 2,3,4-trimethoxybenzyl alcohol obtained by reduction[54] of the corresponding benzaldehyde (1.5 g, 7.57 mmol), but-2-ynoic acid (763 mg, 9.08 mmol), and DMAP (85 mg, 0.75 mmol) in dry CH_2Cl_2 (10 mL) at 0 °C. After stirring for 16 h at room temperature, the reaction mixture was cooled to 0 °C. The precipitate was filtered off and eluted with CH_2Cl_2 (50 mL × 2). The filtrate was concentrated under reduced pressure, and the residue was subjected to flash chromatography (cyclohexane/EtOAc 8:2) to give ester **18** as a colorless oil (1.4 g, 70%); ^{1}H NMR (300 MHz, CDCl3): δ = 7.04 (d, J = 8.5 Hz, 1H, H-6), 6.64 (d, J = 8.5 Hz, 1H, H-5), 5.12, (s, 2H, CH_2), 3.92 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 1.95 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 153.6 (CO), 152.4 (C-4), 152.6 (C-2), 142.1 (C-3), 125.7 (CH-6), 121.0 (C-1), 107.0 (CH-5), 85.6 (C-e), 72.4 (C-d), 63.0 (CH_2), 61.4 (OCH_3), 60.7 (OCH_3), 55.9 (OCH_3), 3.7 ppm (CH_3); MS (ES^+): m/z: 287 $[M+Na]^+$.

6-Iodo-2,3,4-trimethoxybenzylbut-2-ynoate (25): Ester **25**, prepared by following the above procedure (reaction time: 65 h) employing acid **28** (350 mg, 1.03 mmol) and butynol (115 μL, 1.54 mmol) was obtained after flash chromatography (cyclohexane/EtOAc 8:2) as a colorless oil (271 mg, 67%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.05 (s, 1H, H-5), 4.88 (q, $J_{8,11}$ = 2.4 Hz, 2H, O- CH_2), 3.90 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 1.87 ppm (t, $J_{8,11}$ = 2.4 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 166.4 (CO), 155.1 (C-4), 151.5 (C-2), 142.4 (C-3), 127.9 (C-1), 118.1 (CH-5), 84.0 (C-6), 83.8 (C-e), 72.7 (C-d), 54.2 (CH_2), 61.9 (OCH_3), 60.8 (OCH_3), 56.4 (OCH_3), 3.7 ppm (CH_3); MS (ES^+): m/z: 413 $[M+Na]^+$; HRMS (DCI/CH_4) m/z $[M+H]^+$ calcd for $C_{14}H_{16}O_5I$: 391.0043, found: 391.0045.

General procedure for the tandem Heck–Suzuki–Miyaura reaction (path A).[21,27] A flask, flame-dried under high vacuum, was charged with the appropriate alkyne (1 equiv), boronic acid (1.1 equiv), CsF (3.3 equiv, flame-dried under high vacuum prior to use) and purged with argon three times. Anhydrous THF (0.03 M) was added, and the resulting mixture was degassed (bubbling argon, 15 min) before $Pd(OAc)_2$ (5 mol%) and PPh_3 (10 mol%) were added. After heating at reflux under argon for the appropriate time, the reaction was cooled to room temperature, quenched with H_2O, and extracted twice with Et_2O. The combined organic layers were washed with H_2O, brine, dried over $MgSO_4$, concentrated under reduced pressure, and purified by flash chromatography (cyclohexane/EtOAc 8:2 or 6:4) to provide the expected cycloadducts.

(*E*)-6-Methoxy-3-(1-(3,4,5-trimethoxyphenyl)ethylidene)indolin-2-one (9b): Oxindole **9b** was obtained from alkyne **6b** (44 mg, 0.14 mmol) and boronic acid **7** as pale-yellow crystals (39 mg, 80%); mp: 192–193 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.90 (br s, 1H, NH), 6.50 (s, 2H, H-2, H-6), 6.37 (d, J = 1.9 Hz, 1H, H-7′), 6.22 (m, 2H, H-4′, H-5′), 3.92 (s, 3H, OCH_3), 3.83 (s, 6H, OCH_3), 3.74 (s, 3H, OCH_3), 2.74 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.4 (C-2′), 160.1 (C-6′), 153.8 (C-3, C-5), 151.8 (C-a), 139.1 (C-7′a), 138.5 (C-4), 137.7 (C-1), 124.3 (C-4′), 123.2 (C-3′), 116.2 (C-3′a), 106.3 (C-5′), 103.4 (CH-2, CH-6), 96.1 (CH-7′), 61.1 (OCH_3), 56.2 (OCH_3 × 2), 55.4 (OCH_3), 22.4 ppm (CH_3); MS (ES^+): m/z: 378 $[M+Na]^+$; HRMS (ESI) m/z $[M+H]^+$ calcd for $C_{20}H_{22}O_5N$: 356.1498, found: 356.1490.

(*E*)-1-Benzyl3-(3,4,5-trimethoxybenzylidene)indolin-2-one (9c): Oxindole **9c** was obtained from alkyne **6c** (200 mg, 0.55 mmol) and boronic acid **7** as an orange oil (88 mg, 50%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.85 (s, 1H, H-a), 7.80 (d, J = 7.8 Hz, 1H, H-4′), 7.31–7.34 (m, 5H, Ph-H), 7.16 (t, J = 7.8 Hz, 1H, H-6′), 6.92 (s, 2H, H-

2, H-6), 6.88 (t, $J=7.8$ Hz, 1H, H-5′), 6.74 (d, $J=7.8$ Hz, 1H, H-7′), 5.00 (s, 2H, CH_2), 3.93 (s, 3H, OCH_3), 3.87 ppm (s, 6H, OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=168.6$ (CH-2′), 153.2 (C-3, C-5), 143.4 (C-7′a), 139.2 (C-4), 137.7 (CH-a), 136.0 (C-Ph), 130.1 (C-1), 129.6 (CH-6′), 128.7 (CH-Ph×2), 127.6 (CH-Ph×2), 127.5 (CH-Ph), 126.3 (C-3′a), 122.9 (CH-4′), 121.7 (CH-5′), 121.3 (C-3′), 109.3 (CH-7′), 106.6 (CH-2, CH-6), 61.0 (OCH_3), 56.2 ($OCH_3\times2$), 43.7 ppm (CH_2); MS (ES^+): *m/z*: 402 $[M+H]^+$, 424 $[M+Na]^+$; HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{25}H_{23}O_4N$: 402.1705, found: 402.1704

(*E*)-6-Methoxy-3-(1-(3,4,5-trimethoxyphenyl)ethylidene)benzofuran-2-one (9e): Benzofuranone **9e** was obtained from alkyne **6e** (150 mg, 0.47 mmol) and boronic acid **7** as a yellow crystalline solid (73 mg, 44%); mp: 147–148 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta=6.59$ (s, 1H, H-7′), 6.51 (s, 2H, H-2, H-6), 6.37 (m, 2H, H-4′, H-5′), 3.92 (s, 3H, OCH_3), 3.83 (s, 6H, OCH_3), 3.76 (s, 3H, OCH_3), 2.70 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=167.9$ (C-2′), 160.9 (C-6′), 154.7 (C-a), 153.9 (C-3, C-5, C-7′a) 138.2 (C-4), 137.3 (C-1), 123.7 (C-4′), 118.9 (C-3′), 116.2 (C-3′a), 109.3 (C-5′), 103.4 (CH-2, CH-6), 96.7 (CH-7′), 61.1 (OCH_3), 56.6 ($OCH_3\times2$), 55.6 (OCH_3), 22.7 ppm (CH_3); MS (ES^+) *m/z*: 357 $[M+H]^+$, 379 $[M+Na]^+$; HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{20}H_{21}O_6$: 357.1338, found: 357.1337.

(*E*)-3,(*E*)-4-(3,4,5-Trimethoxyphenyl)but-3-en-2-ylidene)indolin-2-one (10a): Oxindole **10a** was obtained from alkyne **6a** (50 mg, 0.175 mmol) and styrylboronic acid **8** as a bright orange powder (43 mg, 70%); mp: 218–220 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=7.90$ (br s, 1H, NH), 7.73 (d, $J=15.9$ Hz, 1H, H-b), 7.61 (d, $J=7.8$ Hz, 1H, H-4′), 7.20 (t, $J=7.8$ Hz, 1H, H-6′), 7.13 (d, $J=15.9$ Hz, 1H, H-a), 7.03 (t, $J=7.8$ Hz, 1H, H-5′), 6.88 (d, $J=7.8$ Hz, 1H, H-7′), 6.80 (s, 2H, H-2, H-6), 3.93 (s, 6H, OCH_3), 3.90 (s, 6H, OCH_3), 2.76 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=170.0$ (C-2′), 153.6 (C-3, C-5), 149.3 (C-c), 139.6 (C-4), 139.4 (C-7′a), 137.9 (CH-a), 132.2 (C-1), 128.0 (CH-6′), 124.5 (CH-4′), 124.0 (C-3′a), 123.7 (C-3′), 121.7 (CH-5′), 109.5 (CH-7′), 104.6 (CH-2, CH-6), 61.0 (OCH_3), 56.2 ($OCH_3\times2$), 15.4 ppm (CH_3); MS (ES^+) *m/z*: 352 $[M+H]^+$, 374 $[M+Na]^+$; HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{22}O_4N$: 352.1549, found: 352.1549. The **(*E*)-3,(*Z*)-4** isomer ***trans*-10a** was obtained when the coupling reaction occurs with NaOH as additive[21]: mp: 220–221 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=9.18$ (d, $J=16$ Hz, 1H, H-b), 7.77 (br s, 1H NH), 7.62 (d, $J=7.6$ Hz, 1H, H-4′), 7.19 (t, $J=7.6$ Hz, 1H, H-6′), 7.13 (d, $J=16$ Hz, 1H, H-a), 7.03 (t, $J=7.6$ Hz, 1H, H-5′), 6.87 (s, 2H, H-2, H-6), 6.84 (d, $J=7.6$ Hz, 1H, H-7′), 3.93 (s, 6H, OCH_3), 3.88 (s, 3H, OCH_3), 2.55 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=169.3$ (C-2′), 153.4 (C-3, C-5), 148.8 (C-c), 139.7 (C-7′a), 139.1 (C-4), 136.9 (CH-a), 132.7 (C-1), 128.0 (CH-6′), 126.9 (CH-b), 124.9 (C-3′a), 124.7 CH-4′), 122.8 (C-3′), 121.7 (CH-5′), 109.3 (CH-7′), 104.8 (CH-2, CH-6), 61.0 (OCH_3), 56.1 ($OCH_3\times2$), 16.8 ppm (CH_3); MS (ES^+) *m/z*: 352 $[M+H]^+$, 374 $[M+Na]^+$.

(*E*)-3,(*E*)-4-(3,4,5-Trimethoxyphenyl)but-3-en-2-ylidene)benzofuran-2-one (10d): Benzofuranone **10d** was obtained from alkyne **6d** (64 mg, 0.22 mmol) and styrylboronic acid **8** as an orange powder (38 mg, 47%); mp: 172–173 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=7.68$ (d, $J=16.0$ Hz, 1H, H-b), 7.64 (d, $J=7.8$ Hz, 1H, H-4′), 7.30 (t, $J=7.8$ Hz, 1H, H-6′), 7.27 (d, $J=16.0$ Hz, 1H, H-a), 7.16 (t, $J=7.8$ Hz, 1H, H-5′), 7.13 (d, $J=7.8$ Hz, 1H, H-7′), 6.81 (s, 2H, H-2, H-6), 3.94 (s, 6H, OCH_3), 3.91 (s, 3H, OCH_3), 2.72 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=167.9$ (C-2′), 153.6 (C-3, C-5), 152.7 (C-7′a), 152.2 (C-c), 139.9 (C-4), 139.6 (CH-a), 131.7 (C-1), 128.9 (CH-6′), 126.8 (CH-b), 124.4 (C-3′), 123.8 (CH-4′), 123.7 (CH-5′), 118.5 (C-3′a), 110.9 (CH-7′), 105.0 (CH-2, CH-6), 61.1 (OCH_3), 56.3 ($OCH_3\times2$), 15.7 ppm (CH_3); MS (ES^+): *m/z*: 353 $[M+H]^+$, 375 $[M+Na]^+$; HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{21}O_5$: 353.1389, found: 352.1383.

(*E*)-6,7,8-Trimethoxy-4-[1-(4-methoxyphenyl)ethylidene]isochroman-1-one (27): Isochromanone **27** was obtained from alkyne **25** (50 mg, 0.128 mmol) and 4-methoxyphenylboronic acid as beige crystals (17 mg, 36%); mp: 99–101 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=7.10$ (d, $J=8.7$ Hz, 2H, H-2′, H-6′), 6.84 (d, $J=8.7$ Hz, 2H, H-3′, H-5′), 5.90 (s, 1H, H-5), 4.93 (s, 2H, CH_2-3), 3.97 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 3.27 (s, 3H, OCH_3), 2.24 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=162.1$ (C-1), 159.2 (C-4′), 156.0 (C-6), 155.0 (C-8), 142.1 (C-7), 138.7 (C-a), 136.4 (C-4a), 134.5 (C-1′), 129.9 (CH-2′, CH-6′), 123.4 (C-4), 114.1 (CH-3′, CH-5′), 112.1 (C-8a), 107.2 (CH-5), 67.2 (CH_2-3), 62.2 (OCH_3), 61.1 (OCH_3), 55.3 ($OCH_3\times2$), 21.7 ppm (CH_3); MS (ES^+): *m/z*: 393 $[M+Na]^+$; HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{23}O_6$: 371.1495, found: 371.1496

Compounds obtained through C–H activation (path B)

(*E*)-6-Methoxy-1-methyl-3-[1-(3,4,5-trimethoxyphenyl)ethylidene]indolin-2-one (9f): Propynamide **12** (28 mg, 0.137 mmol), trimethoxyiodobenzene (46 mg, 0.157 mmol), NaOAc (23 mg, 0.280 mmol), and dry DMF (2 mL) were added under argon to a flame-dried flask. The solution was purged with argon for 20 min, and $Pd(OAc)_2$ (1.5 mg, 0.0066 mmol) was added. After stirring at 80 °C for 2 h, the reaction mixture was cooled to room temperature, quenched with H_2O, and extracted twice with EtOAc (10 mL×2). The combined organic layers were washed with brine (10 mL×2), dried ($MgSO_4$), and concentrated under reduced pressure. The residue was subjected to flash chromatography (cyclohexane/EtOAc 75:25) to give **9f** as a pale-yellow solid which was recrystallized from Et_2O/hexane (23 mg, 46%); mp: 155–158 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.48$ (s, 2H, H-2, H-6), 6.33 (s, 1H, H-7′), 6.23 (m, 2H, H-4′, H-5′), 3.92 (s, 3H, OCH_3), 3.82 (s, 6H, OCH_3), 3.76 (s, 3H, OCH_3), 3.23 (s, 3H, N-CH_3), 2.75 ppm (s, 3H, C-CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=168.7$ (C-2′), 160.3 (C-6′), 153.9 (C-3, C-5), 151.3 (C-a), 143.7 (C-7′a), 138.6 (C-1), 137.7 (C-4), 124.0 (CH-4′), 123.0 (C-3′), 115.5 (C-3′a), 105.5 (CH-5′), 103.6 (CH-2, CH-6), 95.1 (CH-7′), 61.1 (OCH_3), 56.2 ($OCH_3\times2$), 55.4 (OCH_3), 25.7 (N-CH_3), 22.4 ppm (C-CH_3); MS (DCI/CH_4): *m/z*: 370 $[M+H]^+$; HRMS (DCI/CH_4) *m/z* $[M+H]^+$ calcd for $C_{21}H_{24}O_5N$: 370.1654, found: 370.1654.

(*E*)-3-[1-(3-Hydroxy-4-methoxyphenyl)ethylidene]-5,6,7-trimethoxy-1-[(2-(trimethylsilyl)ethoxy)methyl]indoline-2-one (17): Employing propynamide **15** (116 mg, 0.306 mmol) and aryliodide **16** (88 mg, 0.351 mmol) and following the same procedure as above, but after a heating at 150 °C for 5 min, the cycloadduct **17** was obtained after flash chromatography (cyclohexane/EtOAc 75:25) as a yellow oil (70 mg, 46%); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=$ 6.95 (d, $J=8.1$ Hz, 1H, H-5′), 6.88 (s, 1H, H-2′), 6.79 (d, $J=8.1$ Hz, 1H, H-6′), 5.78 (s, 1H, H-4), 5.71 (s, 1H, OH), 5.38 (s, 2H, N-CH_2), 3.93 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.38 (s, 3H, OCH_3), 3.63 (t, $J=8.1$ Hz, 2H, O-CH_2), 2.74 (s, 3H, CH_3), 0.94 (t, $J=8.1$ Hz, 2H, Si-CH_2), −0,01 ppm (s, 9H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=168.9$ (C-2), 154.1 (C-a), 148.2 (C-5), 146.6 (C-4′), 146.2 (C-3′), 143.2 (C-6), 139.3 (C-7), 136.1 (C-1′), 127.2 (C-7a), 123.3 (C-3), 118.6 (CH-6′), 118.3 (C-3a), 113.3 (CH-2′), 111.1 (CH-5′), 103.7 (CH-4), 70.2 (N-CH_2), 65.7 (O-CH_2), 61.4 (OCH_3), 60.9 (OCH_3), 56.1 (OCH_3), 55.7 (OCH_3), 22.9 (CH_3-a), 18.0 (Si-CH_2), −1,4 ppm (CH_3); MS (ES^+): *m/z*: 524 $[M+Na]^+$; HRMS (DCI/CH_4) *m/z* $[M+H]^+$ calcd for $C_{26}H_{36}O_7NSi$: 502.2261, found: 502.2243.

2,3,4-Trimethoxybenzyl 3-(5-iodo-2-methoxyphenoxy)but-2-enoate (19). Employing propynamide **18** (100 mg, 0.378 mmol) and aryliodide **16** (108 mg, 0.434 mmol) and following the same procedure as for **9f**, but after a heating at 70 °C for 20 min, ether **19** was obtained after flash chromatography (toluene/EtOAc 97:3)

as a yellow oil (191 mg, 98%); ^{1}H NMR (300 MHz, CDCl3): $\delta = 7.45$ (dd, $J = 8.6$, 2.1 Hz, 1H, H-4′), 7.27 (d, $J = 2.1$ Hz, 1H, H-6′), 7.02 (d, $J = 8.5$ Hz, 1H, H-6), 6.71 (d, $J = 8.6$ Hz, 1H, H-3′), 6.65 (d, $J = 8.5$ Hz, 1H, H-5), 5.05 (s, 2H, CH_2-a), 4.83 (s, 1H, H-d), 3.87 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 2.48 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 172.1$ (C-e), 167.4 (CO), 154.1 (C-4), 152.5 (C-2), 151.4 (C-2′), 142.4 (C1′), 142.1 (C-3), 135.5 (CH-4′), 131.7 (CH-6′), 125.2 (CH-2), 122.2 (C-1), 114.7 (CH-3′), 107.1 (CH-5), 95.3 (CH-d), 81.6 (C-5), 61.4 (OCH_3), 60.7 (CH_2-a), 60.8 (OCH_3), 55.9 (OCH_3), 55.8 (OCH_3), 18.1 ppm (CH_3); MS (ES^+): *m/z*: 536 $[M+Na]^+$.

(*E*)-3-(1-(3,4,5-Trimethoxyphenyl)ethylidene)indolin-2-one (9g). Pyrrolidine (1.8 mL, 21 mmol) was added dropwise to a well-stirred mixture of 2-indolinone (2.0 g, 15 mmol) and 3,4,5-trimethoxyacetophenone (3.15 g, 15 mmol) in MeOH (20 mL). After heating at reflux for 16 h, the mixture was cooled, and the orange solid product was filtered to give, after recrystallization in MeOH, pure **9g** (2.8 g, 57%); mp: 215–216 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.96$ (br s, 1H, NH), 7.09 (t, $J = 7.7$ Hz, 1H, H-6′), 6.80 (d, $J = 7.7$ Hz, 1H, H-7′), 6.68 (t, $J = 7.7$ Hz, 1H, H-5′), 6.50 (s, 2H, H-2, H-6), 6.33 (d, $J = 7.7$ Hz, 1H, H-4′), 3.93 (s, 3H, OCH_3), 3.83 (s, 6H, OCH_3), 2.78 ppm (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 169.6$ (C-2′), 155.2 (C-a), 153.9 (C-3, C-5), 139.3 (C-7′a), 138.3 (C-4), 137.9 (C-1), 128.2 (CH-6′), 123.6 (C-3′a), 123.4 (CH-4′), 123.3 (C-3′), 121.5 (CH-5′), 109.2 (CH-7′), 103.3 (CH-2, CH-6), 61.1 (OCH_3), 56.2 ($OCH_3 \times 2$), 22.8 ppm (CH_3); MS (ES^+): *m/z*: 326 $[M+H]^+$, 348 $[M+Na]^+$; Anal. calcd for $C_{19}H_{19}NO_4$: C 70.14, H 5.89,N 4.31, found: C 70.12, H 5.85, N 4.31.

Biological evaluations

Inhibition of tubulin polymerization: Microtubule disruption was monitored by using the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI)[55] in a 96-well plate format as described by Barron et al.[29b] and Bane et al.[56] The standard assay was performed as follows: wells were charged with tubulin (Cytoskeleton, 97% pure, final concentration 1 mg mL^{-1}) in PME buffer (100 mM 1,4-piperazinebis(ethanesulfonic acid (PIPES), 1 mM $MgSO_4$, 2 mM EGTA) with 10 μM DAPI and varying concentrations of the test compounds, using colchicine as an internal control. After pre-incubation at room temperature for 45 min, 1 mM GTP (5 μL) was added to each well to initiate tubulin polymerization, and the plate was then transferred to a thermostated Victor plate reader at 37 °C for an additional 2 h. Fluorescence was then read ($\lambda_{excitation}$: 360 nm, $\lambda_{emission}$: 450 nm. Percent inhibition was determined as follows: $1-(\Delta F_{sample}/\Delta F_{control}) \times 100$, for which $\Delta F_{control} = F$(no inhibition)$-F$(complete inhibition), and $\Delta F_{sample} = F$(sample)$-F$(complete inhibition with colchicine). The IC_{50} for compound-induced inhibition of tubulin polymerization is the concentration of compound at which the extent of inhibition of polymerization is 50% of the maximum value, as determined from the semi-logarithmic plot of percent inhibition as a function of drug concentration.

Endothelial cell morphology: Effect on the morphology of transformed HUVEC (EAhy 926) cells. To assess the effects of the compounds on the morphology of endothelial cells, we used the EAhy 926 endothelial cell line, which is derived from the fusion of human umbilical vein endothelial cells (HUVEC) with the permanent human cell line A549.[57] The EAhy 926 cell line is considered to be one of the best immortalized HUVEC lines, because these cells express most of the biochemical markers of parental HUVEC EAhy 926 cells,[58] originally obtained from Dr. Cora-Jean S. Edgell (Pathology Department, University of North Carolina, Chapel Hill, NC 27599-7525, USA); these were used with her permission, and were grown in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin (37 °C, 5% CO_2). EAhy 926 cells in exponential growth were plated onto 96-well plates at 5000 cells per 100 μL per well. Twenty-four hours after plating, the medium was aspirated, and 100 mL medium containing the test compound was added to the well containing the cells (in triplicate) in 10-fold dilutions, and incubated for 2 h. After the 2 h incubation period, digital micrographs were taken of representative center areas of each well at a magnification of 100× and 200×. CA4 was routinely included in the experiments as internal standard.

MTT cell proliferation assays: Murine B16 melanoma cells, murine 3LL Lewis lung carcinoma cells, and EAhy 926 endothelial cells were grown in DMEM medium containing 2 mM L-glutamine, 10% FBS, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (37 °C, 5% CO_2). All compounds were initially dissolved in DMSO at a stock concentration of 2.5 mg mL^{-1} and were further diluted in cell culture medium. For comparative purposes, CA4 was routinely included in the experiments as reference compound. Cells in exponential growth were plated onto 96-well plates at 5000 cells per well in 100 μL culture medium. Twenty-four hours after plating, 100 μL medium containing the compound of interest at final concentrations ranging from 0.01 to 30 μM were added to the wells (in triplicate) containing the cells, and incubated for 48 h at 37 °C and 5% CO_2. After the 48 h exposure period to the test compounds, cell viability was assayed using the MTT test,[59] and absorbance was read at λ 562 nm in a microplate reader (BioKinetics Reader, EL340). Appropriate controls with DMEM only and MTT were run to subtract background absorbance. The concentration of compound that inhibited cell viability by 50% (inhibitory concentration for 50% of cells, or IC_{50}) was determined using GraphPad Prism software. Results are presented as the mean of three independent experiments each run in triplicate.

Antiproliferative assays on human carcinoma cell lines: These assays were monitored at the laboratory "Ciblothèque Cellulaire", ICSN-CNRS, Gif-sur-Yvette, France. HCT116 (colon carcinoma) and MCF7 (hormone-dependent breast carcinoma) cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, streptomycin, and amphotericin B (Fungizone®), and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells (500–800 per well) were seeded in 96-well microplates containing 200 mL growth medium. After culturing for 24 h, cells were exposed to varying concentrations (0.5 nM–10 mM) of the test compound dissolved in DMSO (< 1% in each preparation). After 72 h of incubation, the MTS reagent (40 mL, Promega) was added 2 h before the absorbance at λ 490 nM was recorded. IC_{50} corresponds to the concentration of the test compound that elicits 50% inhibition of cell growth. Experiments were performed in triplicate.

Cord disruption assay: HUVEC were prepared and cultured in EGM-2MV microvascular endothelial cell growth medium (Lonza, Walkersville, MD, USA), as previously described.[60] In brief, HUVEC were plated in 96-well plates on a thick layer of Matrigel (Becton Dickinson, 75 μL per well) and allowed to form tube-like structures over 24 h. Test compounds were dissolved in DMSO (< 0.1% in each preparation, 100 μL per well) and were added to the formed cords and incubated for 2.5 h. The effects of the compounds on capillary tube disruption were evaluated by light microscopy (40× magnification). Experiments were done in triplicate.

Docking protocol: The reported 3D structure of tubulin was retrieved from the Protein Data Bank (http://www.rcsb.org/, PDB ID:

1SA0).[39] Subunits C, D, and E were removed. Only subunits A, B (colchicine binding site), and small molecules DAMA-colchicine (CN2), GTP, and the Mg^{2+} ion in this site were conserved. We ensured that correct ionization states were established for Asp, Glu, and His residues. The orientations of the hydroxy group hydrogen atoms from the Ser, Thr, and Tyr amino acids were not considered, as these orientations are optimized during the docking runs. Finally, no charge was added to the whole protein, because the algorithm used processes it automatically. All docking runs were performed into the colchicine binding site of tubulin, applying GOLD (Genetic Optimization for Ligand Docking), with its default parameters. This algorithm uses a stochastic genetic strategy for the conformational search and is also able to take into account conformational freedom of the residues. GoldScore is the GOLD fitness scoring function, which is made of four components (external hydrogen bond, van der Waals and external van der Waals, internal van der Waals, and internal torsion). This scoring function was optimized for the prediction of ligand binding positions. Consequently, it remains adapted in our comparison study. Ligands were built within the builder module of Sybyl[61] and next submitted to Corina,[62] a 3D structure generator. Obtained conformations were next minimized within Sybyl using the Tripos force field.

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