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Article

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Design, Synthesis and Biological Evaluation of Combretabenzodiazepines: a Novel Class of Antitubulin Agents

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ABSTRACT: In the present manuscript, starting from the 1,4-benzodiazepin-2-one nucleus, a privileged structure in medicinal chemistry, we have synthesized a novel class of *cis*-locked combretastatins named combreatabenzodiazepines. They show similar cytotoxic and antitubulin activity compared to combretastatin A-4 in neuroblastoma cells, showing a better pharmacokinetic profile. This class of compounds has therefore the potential for further development as antitubulin agents.

INTRODUCTION

The naturally-occurring combretastatin A-4[1] (CA-4, **1**) and its analogue phenstatin (**2**; Figure 1), serendipitously discovered in 1998,[2] block the polymerization of tubulin, strongly interacting with the colchicine binding site.[3] These have displayed a high degree of cytotoxicity on several cancer cell lines, including those that exhibit multidrug resistance,[4] and have anti-tumoural properties in vivo models.[5] Furthermore, both agents induce a rapid vascular shutdown in tumors, occluding the blood flow, with the net result of starving the proliferating cancer cells.[6]

CA-4, in its water soluble phosphate pro-drug,[7] both as a single agent and in combination therapy, has entered clinical trials for the treatment of anaplastic thyroid cancer and age-related macular degeneration.[8]

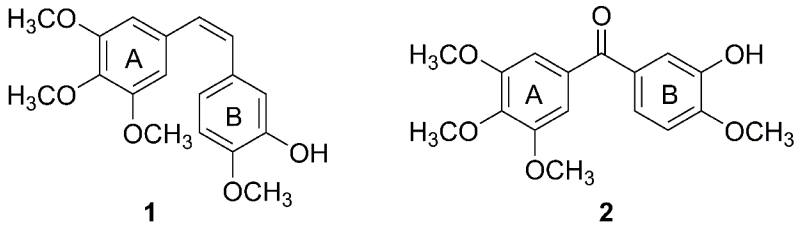


Figure 1. CA-4 (**1**) and phenstatin (**2**).

Over the last decades, these two relative simple molecules have been the starting point of many medicinal chemistry programs directed at the rational design of novel antitubulin agents. For CA-4, the replacement of the unstable olefinic bond[9] with several five-membered heterocyclic rings[10] has been the main strategy pursued. In the case of phenstatin, the replacement of the ketone with an ethylene bridge (**3**),[11] a methyl (**4**),[12] a *N*-methyl group (**5**)[13] or the formation of rigid analogues between the ethylene group and the B-ring[14] (**6**,[15] **7**,[16] **8**,[17] **9**,[18] **10**[19]) or the A-ring (**11**)[20] has unveiled novel agents endowed with cytotoxic activity (Figure 2).

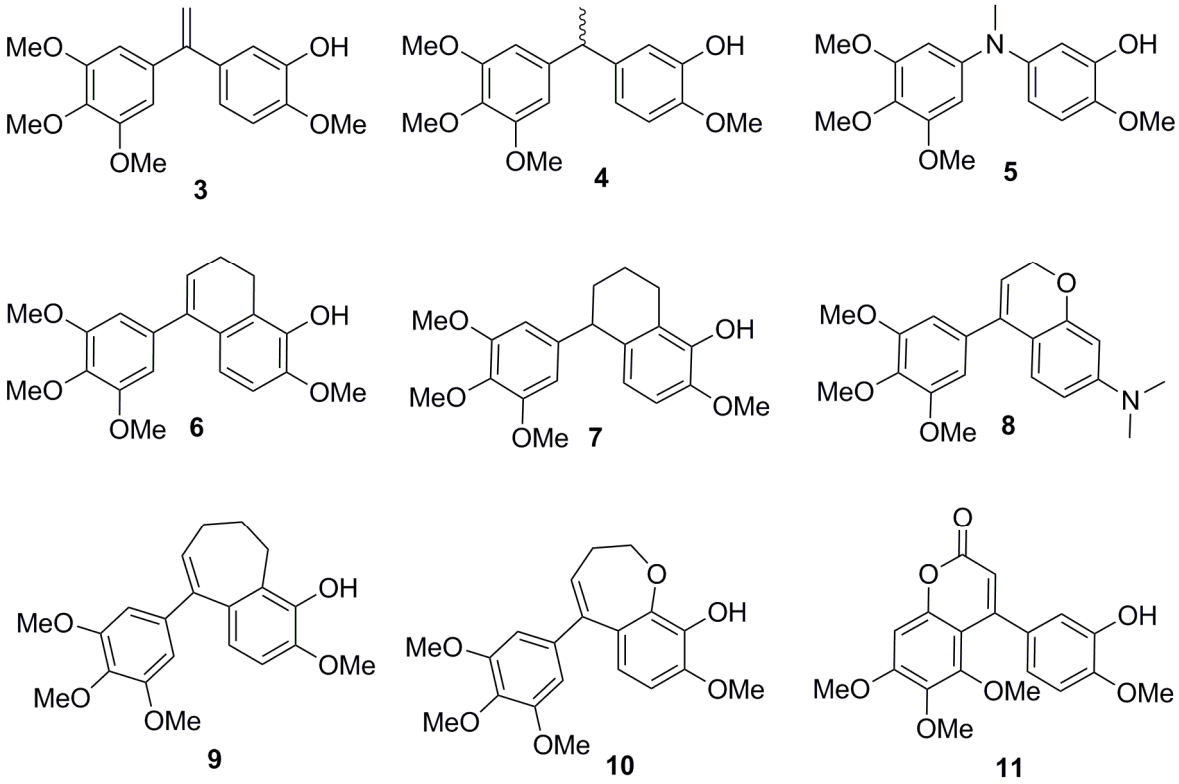
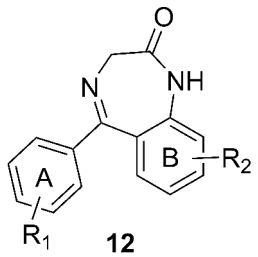


Figure 2. Analogues of phenstatin endowed with strong cytotoxic activity.

The important features that maintain the tubulin interaction for CA-4,[21] phenstatin and all the related compounds shown in Figure 2 are: *i*) the presence of the trimethoxyphenyl A-ring and *ii*) the lack of planarity for the two phenyl rings which must be inclined towards each other with a dihedral angle between 50° and 80°.[14]

In search of novel potential rigid analogues of phenstatin, we hypothesised that the replacement of the benzosuberene ring of **9** with a 1,4-benzodiazepine-2-one nucleus (**12**) (Figure 3) could be a successful strategy to unravel a novel class of antitubulin agents. Indeed, the 1,4-benzodiazepine-2-one nucleus is one of the most widely used molecular scaffold in medicinal chemistry due to its favourable pharmacokinetic features and its ability to act as a

privileged structure when properly decorated. In this manuscript, we report the design, synthesis, and biological evaluation of a novel class of antitubulin agents named combretabenzodiazepines.

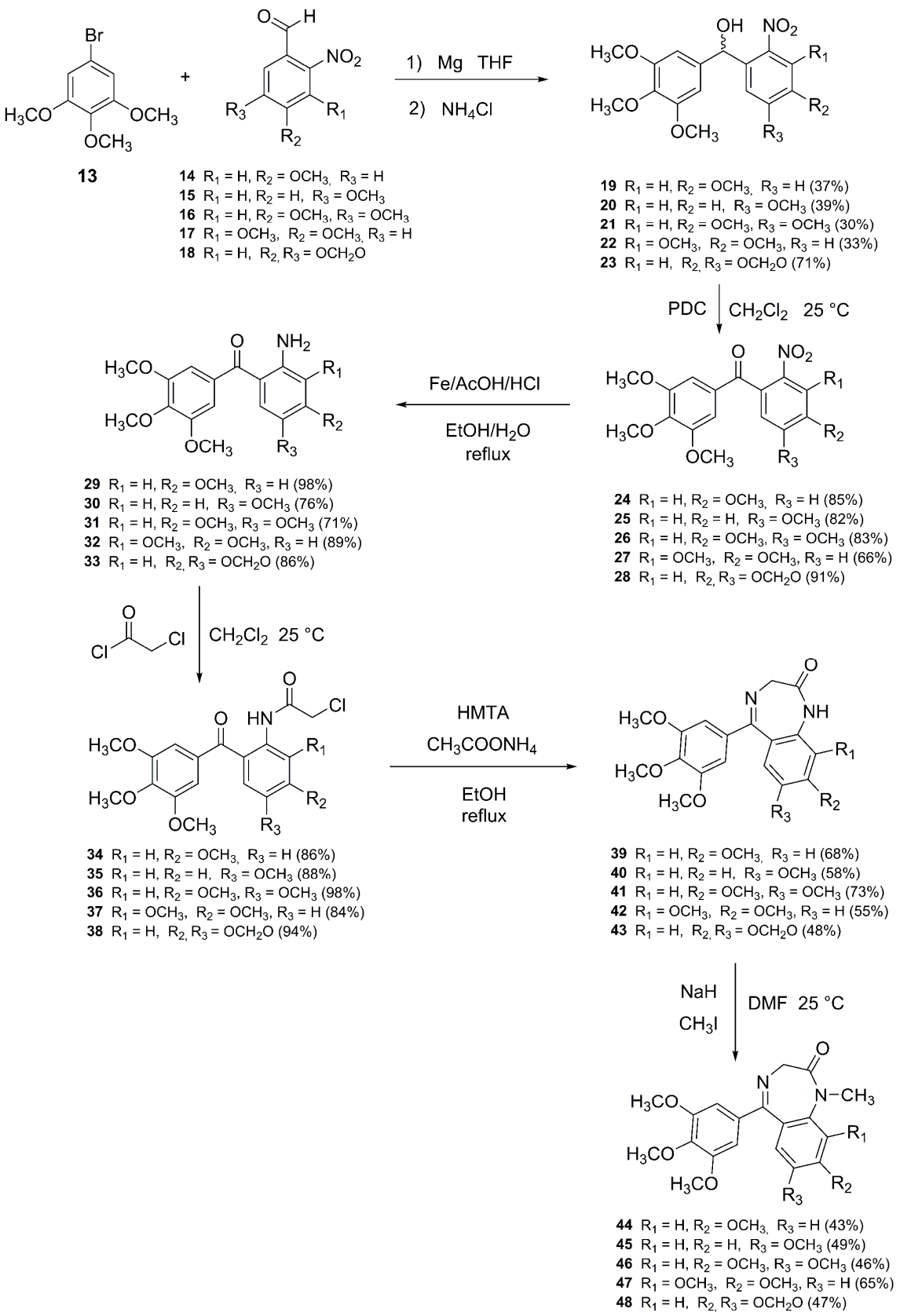


12

Figure 3. The combretabenzodiazepine scaffold.

RESULTS AND DISCUSSION

The synthesis of the combretabenzodiazepines starts with a Grignard reaction between the 5-bromo-1,2,3-trimethoxybenzene (**13**) and the appropriate aldehydes (**14**-**18**) to afford the corresponding 2-nitrobenzydrols (**19**-**23**). The latter were oxidized with pyridinium dichromate (PDC) to benzophenones (**24**-**28**). The nitro group was then reduced using iron/acetic acid/hydrochloric acid under reflux conditions to give the 2-aminobenzophenones (**29**-**33**).[22] The amino group was acetylated in the presence of chloroacetyl chloride to form the chloroacetamidobenzophenones (**34**-**38**). Finally, the 1,4-benzodiazepine-2-one nucleus was obtained using an improved Blažević-Kajfež procedure[23] via ammonium acetate and hexamethylenetetramine (HMTA)[24] to afford final compounds **39**-**43**. These combretabenzodiazepines were then *N*1-methylated to give compounds **44**-**48** using sodium hydride and methyl iodide in DMF (Scheme 1).


Br
H3CO
OCH3
OCH3
13
O
H
NO2
R3
R1
R2
14 R1 = H, R2 = OCH3, R3 = H
15 R1 = H, R2 = H, R3 = OCH3
16 R1 = H, R2 = OCH3, R3 = OCH3
17 R1 = OCH3, R2 = OCH3, R3 = H
18 R1 = H, R2, R3 = OCH2O
1) Mg THF
2) NH4Cl
OH
NO2
19 R1 = H, R2 = OCH3, R3 = H (37%)
20 R1 = H, R2 = H, R3 = OCH3 (39%)
21 R1 = H, R2 = OCH3, R3 = OCH3 (30%)
22 R1 = OCH3, R2 = OCH3, R3 = H (33%)
23 R1 = H, R2, R3 = OCH2O (71%)
PDC
CH2Cl2 25 °C
24 R1 = H, R2 = OCH3, R3 = H (85%)
25 R1 = H, R2 = H, R3 = OCH3 (82%)
26 R1 = H, R2 = OCH3, R3 = OCH3 (83%)
27 R1 = OCH3, R2 = OCH3, R3 = H (66%)
28 R1 = H, R2, R3 = OCH2O (91%)
Fe/AcOH/HCl
EtOH/H2O
reflux
NH2
29 R1 = H, R2 = OCH3, R3 = H (98%)
30 R1 = H, R2 = H, R3 = OCH3 (76%)
31 R1 = H, R2 = OCH3, R3 = OCH3 (71%)
32 R1 = OCH3, R2 = OCH3, R3 = H (89%)
33 R1 = H, R2, R3 = OCH2O (86%)
Cl
Cl
CH2Cl2 25 °C
HN
34 R1 = H, R2 = OCH3, R3 = H (86%)
35 R1 = H, R2 = H, R3 = OCH3 (88%)
36 R1 = H, R2 = OCH3, R3 = OCH3 (98%)
37 R1 = OCH3, R2 = OCH3, R3 = H (84%)
38 R1 = H, R2, R3 = OCH2O (94%)
HMTA
CH3COONH4
EtOH
reflux
N
NH
39 R1 = H, R2 = OCH3, R3 = H (68%)
40 R1 = H, R2 = H, R3 = OCH3 (58%)
41 R1 = H, R2 = OCH3, R3 = OCH3 (73%)
42 R1 = OCH3, R2 = OCH3, R3 = H (55%)
43 R1 = H, R2, R3 = OCH2O (48%)
NaH
CH3I
DMF 25 °C
N-CH3
44 R1 = H, R2 = OCH3, R3 = H (43%)
45 R1 = H, R2 = H, R3 = OCH3 (49%)
46 R1 = H, R2 = OCH3, R3 = OCH3 (46%)
47 R1 = OCH3, R2 = OCH3, R3 = H (65%)
48 R1 = H, R2, R3 = OCH2O (47%)

Scheme 1. Scheme for the synthesis of combretabenzodiazepines **39-48**.

In order to investigate the cytotoxicity and tubulin-polymerization aspects of these compounds, we first performed an MTT assay on the SH-SY5Y neuroblastoma cell line, as we have evidence that this model is highly responsive to CA-4 and phenstatin.[25] The initial screening at 1 μM showed that most compounds (except **41**, **46**) were active at this concentration.

To investigate the activity further, we chose the compounds that induced at least 50% cell death and performed complete concentration-response curves (Figure 4).

A

Comp.	% of viability SH-SY5Y (1 μM)	IC_{50} (nM) MTT assay	Inhibition of tubulin polymerization IC_{50} (μM)
1	33.4 ± 3.0	2.7 ± 2.1	1.3 ± 0.1
39	24.7 ± 3.0	25.1 ± 14.7	9.0 ± 1.3
40	33.9 ± 4.3	255.6 ± 60.4	n.d.
41	102 ± 6.7	n.d.	n.d.
42	46.8 ± 3.0	90.3 ± 14.3	n.d.
43	35.1 ± 6.5	71.0 ± 14.1	13.6 ± 1.1
44	32.9 ± 7.1	279.5 ± 115.9	n.d.
45	44.4 ± 2.9	862.0 ± 338.7	n.d.
46	105.2 ± 5.4	n.d.	n.d.
47	76.4 ± 6.5	n.d	n.d.
48	46.4 ± 5.8	n.d.	n.d.
61	27.5 ± 2.9	3.9 ± 2.6	1.3 ± 0.1
62	95.2 ± 2.9	n.d.	n.d.
64	64.8 ± 4.6	414.8 ± 345.0	n.d.

B

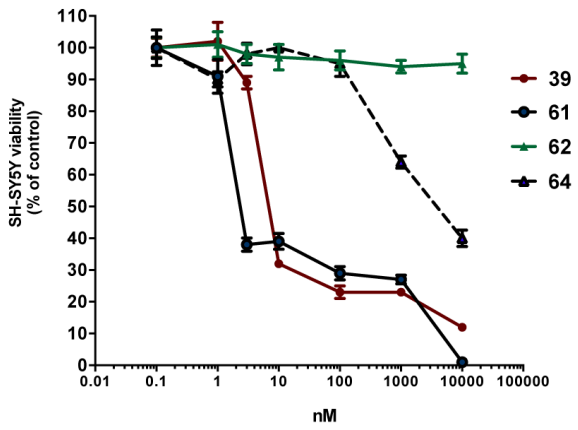


Figure 4. Cytotoxicity in SH-SY5Y cells of the synthesized compounds. (**A**) Values in the second column represent the % of viable cells (determined by MTT assay) after 48 hours of treatment with 1 μM of the indicated compounds. Values in the third column represent the calculated IC_{50} values (using Kaleidagraph software). Values in last column represent the IC_{50} value calculated for inhibition of tubulin polymerization *in vitro*. n.d.: not determined since full

cytotoxicity was not reached at concentration at 1 μM and up to 1 μM. Values are the mean ± SD of 8-12 determinations from 3 different experiments. (**B**) Concentration response curves for cytotoxicity (MTT assay) in SH-SY5Y treated for 48 hours with compounds **39**, **61**, **62** and **64**. Values are the mean ± SD of 8 determinations from 2 different experiments. n.d.: not determined.

Remarkably, the *N*-methylated combretabenzodiazepines **44-48** were far less active compared to to the corresponding combretabenzodiazepines **39-43.**

Compounds **42** and **43** displayed IC_{50}s of 90.3 ± 14.3 nM and 71.0 ± 14.1 nM, respectively. The most potent compound of this series was compound **39**, displaying an apparent IC_{50} of 25.1 ± 14.7 nM. To investigate whether the cytotoxic effect of the two most potent compounds could be re-conducted to tubulin polymerization, we performed flow cytometry analysis of the cell cycle of cells treated with **39** or **43**. The typical signature of tubulin depolymerizing agents, *i.e.* a G2/M arrest, was evident and quantitatively similar to CA-4, for both compounds (Figure 5).

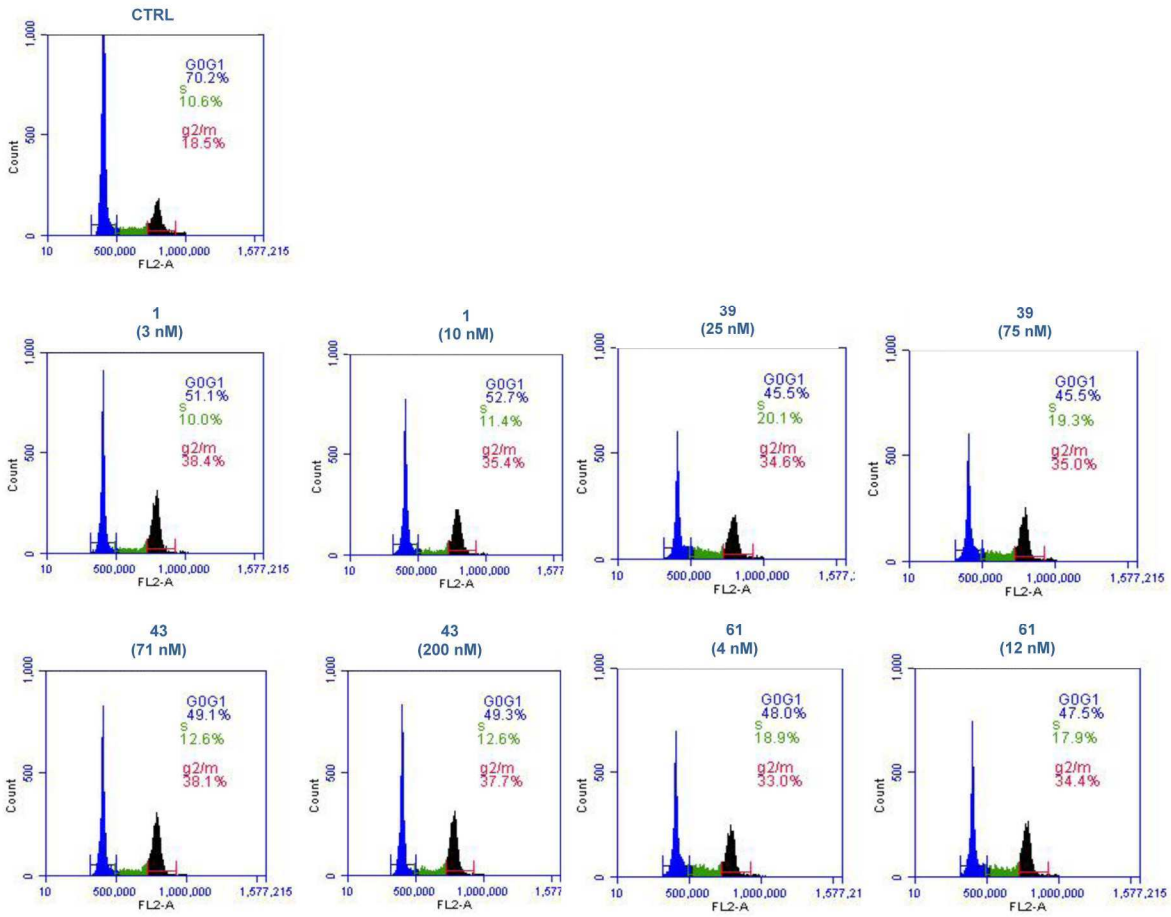


Figure 5. CA-4 and the most potent compounds synthesized induce G2/M arrest. Cell cycle analysis (using BD Accuri C6 flow cytometry) of SH-SY5Y cells treated for 16 hours with vehicle (CTRL) or with the indicated compounds at the IC_{50} or 3 x IC_{50} values. The Y-axis represents the cell number, and the X-axis represents propidium iodide (PI) fluorescence on a linear scale. FL2-A: PI staining. Data are representative of 3 different experiments.

To confirm this finding, we also performed an in vivo tubulin polymerization assay in SH-SY5Y cells. Briefly, in this assay, cells are grown for 16 h in the presence of pharmacological agents after which cells are lysed in the presence of paclitaxel to maintain tubulin status. Polymerized tubulin is then pelleted by centrifugation while monomers remain in the supernatant. As it can be observed in Figure 6, **1**, **39**, and **43** significantly reduced the amount of

pelletable (polymerized) tubulin, strongly suggesting that these agents act on tubulin by inhibiting polymerization.

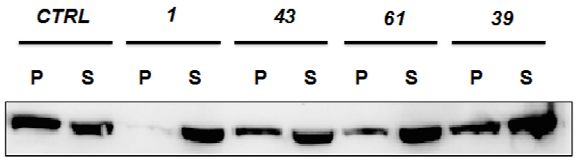


Figure 6. CA-4 and compounds 43, 61 and 39 synthesized affect tubulin polymerization. Western blot of α-tubulin extracted in the presence of paclitaxel from SH-SY5Y cells treated with vehicle (CTRL) or the indicated compounds at 3 X IC_{50} value for 16 hours. Results are representative of 3 separate experiments. P = pelletable fraction, S = soluble fraction.

Given that the two methods above may be seen as indirect evidences of an effect on tubulin polymerization, we also visualized tubulin in intact cells via immunocytochemistry and investigated directly the effect of compounds **43** and **39** on tubulin polymerization *in vitro*. As predicted, the ordered tubulin network seen in control cells by immunocytochemistry was deranged when cells were treated with combretastatin, **39** or **43** at the IC_{50} value (see supporting information). Furthermore, tubulin polymerization was inhibited by both **39** and **43** in a dose-dependent manner (Figure 7) with IC_{50s} of 9.0 ± 1.3 μM and 13.6 ± 1.1 μM, respectively (compared to 1.3 ± 0.1 μM of **1**).

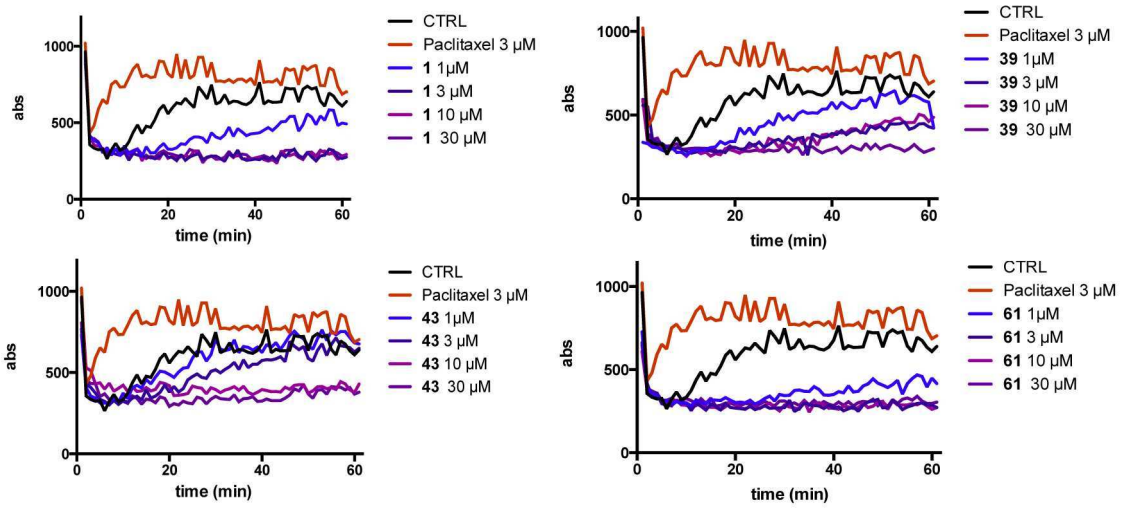
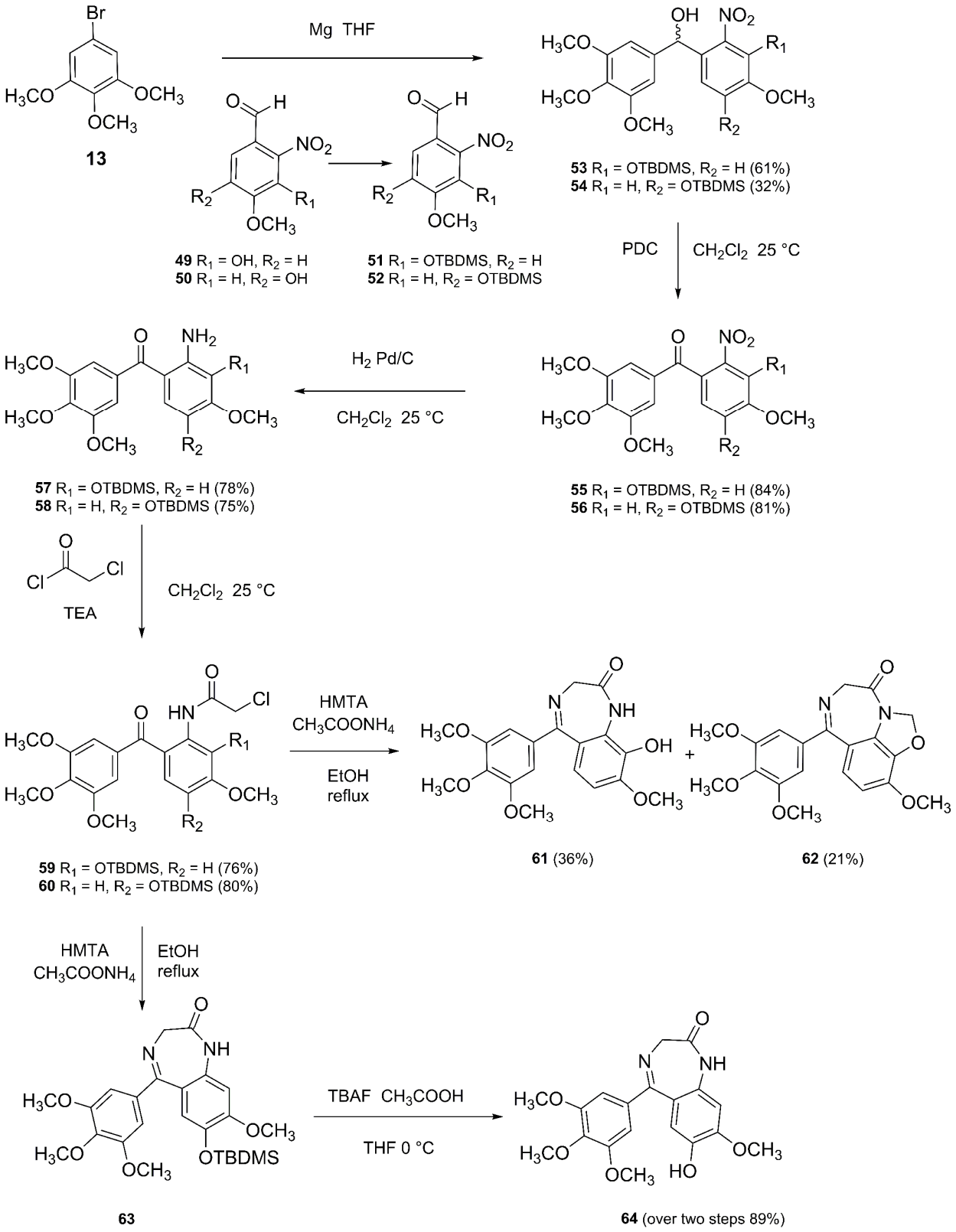


Figure 7. Representative traces of the tubulin in vitro polymerization assay using a commercial kit (see materials and methods). Experiments depicted are from a single 96-well plate and therefore the control and paclitaxel traces are identical for all four panels are presented each time for comparison.

From previous SAR studies of **1** and **2,** an isovanillin moiety in the B-ring significantly increases potency,[10] but due to the rigidity of the 1,4-benzodiazepine-2-one scaffold two positions were available to graft the phenyl. To understand the spatial orientation of combretabenzodiazepines in the tubulin pocket we pursued two strategies: (i) we synthesized the two isovanillin-containing combretabenzodiazepines; and (ii) we performed docking studies.

For the synthesis, starting from the aldehydes **49** and **50**, the same procedure developed for the combretabenzodiazepines **39**-**43** was used. In order to successfully carry out the Grignard reaction, the hydroxyl groups of the aldehydes were protected with *tert*-butyldimethylsilyl chloride to afford the protected aldehydes **51** and **52**. The reduction of the nitro group to amino was not carried out with iron/acetic acid/hydrochloric acid as in the presence of these strong acid conditions the silyl protecting group was cleaved (the silyl deprotection was even observed when

the nitro reduction was attempted using sodium dithionite in acetone at 50 °C). In this case hydrogenation in presence of Pd/C 10% successfully afforded the silyl protected 2-aminobenzophenones **57** and **58**. We point out that during the cyclization procedure, besides the desired combretabenzodiazepine **61**, obtained in 36% yield, we isolated a second combretabenzodiazepine **62** in 21% yield. The extra ring was formed by the reaction between the formaldehyde deriving from thermal decomposition of HMTA with the NH and OH group of the B-ring. Remarkably the silyl group was lost during this stage, while to obtain **64** it was necessary performed a deprotection TBAF mediated (Scheme 2).



Scheme 2. Scheme for the synthesis of combretabenzodiazepines **61** and **64**.

When testing the three new compounds, **62** was devoid of activity up to 1 μM, compound **64** was poorly active while **61** gained a 10-fold potency over the parent compound **39**, and showed similar potency in respect to CA-4 displaying an IC_{50} of 3.9 ± 2.6 nM (Figure 4).

As expected, compound **61** induced a G2/M cell cycle arrest (Figure 5), inhibited tubulin polymerization in the in vivo (Figure 6) and in the in vitro assays (Figure 7), and deranged tubulin organization in intact cells as visualized by immunocytochemistry (see Supporting Information Figure S5). This confirmed that the mechanism of action of **61** is to inhibit tubulin polymerization.

Given that we wanted to explore the docking mode of **61** to tubulin, we first needed to provide formal proof that, similarly to **1** and **2**, it bound to the colchicine binding site. To investigate this, we performed [^{3}H]colchicine radioligand binding assays. As shown in Table 1, **61** inhibited [^{3}H]colchicine binding at similar concentrations compared to non-radioactive colchicine, which was used as control.

Table 1. Compound **61** competes with [^{3}H]colchicine for the same binding site on tubulin. Values represent mean ± SEM of at least 7 replicates from three separate experiments.

Compound	**Inhibition of colchicine binding**	
	% inhibition	
	1 μM	10 μM
1	65 ± 23	89 ± 8

61	48 ± 6	80 ± 4

Molecular modeling studies were then performed to investigate the potential pose of **61** to the colchicine binding site of α,β-tubulin. Docking studies showed that **61**, the most active compound, occupies the colchicine binding site of tubulin in agreement with the X-ray structure complex of DAMA-colchicine-α,β-tubulin (PDB id: 1SA0).[26] The trimethoxyphenyl moiety in ring A of **61** was positioned in the binding cavity buried in the β-subunit. The thiol group of Cysβ241 formed a hydrogen bond with the oxygen atom of one of the methoxy groups, and several amino acids of β-tubulin formed hydrophobic interactions with the trimethoxyphenyl moiety. The hydroxyl group of ring B formed a hydrogen bond with the main chain nitrogen atom of Valα181 (Figure 8). In the case of compound **64** the docked pose is slight shifted compared to the colchicine binding mode, the trimethoxyphenyl group occupies the same cavity around Cysβ241, without forming an hydrogen bond. The different position of the hydroxyl group on ring B implies a missing hydrogen bonding interaction with Valα181 and an unfavorable interaction with the hydrophobic region around Metβ259.

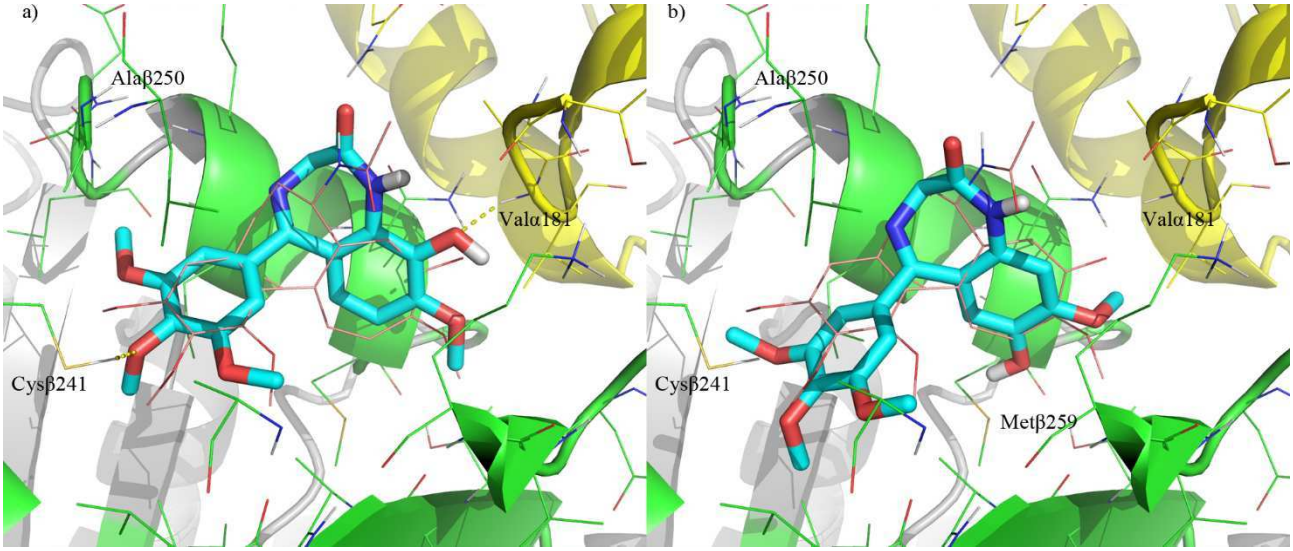


Figure 8. Superimposition of the docked conformation of compounds **61** (a) and **64** (b) as cyan stick models on top of the X-ray structure of DAMA-colchicine (pink wire model). The backbone of tubulin is shown as ribbon representation (α-tubulin: yellow, β-tubulin: green). The amino acids of tubulin within 4.0 Å from colchicine are shown as wire models. Hydrogen bonds (distance < 3 Å) are shown as dotted yellow lines.

CONCLUSIONS

In conclusion, we now report a novel class of compounds named combretabenzodiazepines with similar efficacy and potency compared to CA-4. Although CA-4 has entered clinical trials, double-bond isomerisation, a short half-life and metabolic stability could represent an Achille's heel and therefore a point of improvement. We therefore also compared the metabolic profile (phase I/II) of CA-4 and **61**. When incubated in the presence of human liver microsomes, CA-4 underwent a relevant phase I oxidative transformation, with a residual substrate after 1 h ranging between 75% to 67% depending on the presence or absence of GSH respectively (Table 2); in

the phase II incubations the residual substrate was about 39%. Conversely, a greater metabolic stability was observed for **61**, undergoing poor substrate depletion compared to CA-4, being the residual substrate >95% and 92% for phase I/II respectively (Table 2). The structural characterization of the metabolites and the corresponding metabolic pathways involved in the metabolism of **61** were achieved by tandem mass spectrometry (LC-ESI-MS^2) analysis and are present in the supporting information.

Table 2. Metabolic profile for **1** and **61**. Data are expressed as the peak area percent relative to controls (without microsomes) obtained from LC-UV analyses of incubation extracts (t = 60 min, phase I or 30 min, phase II incubations).

Compound	**Phase I**			**Phase II**
	HLM	*HLM+NADPH*	*HLM+NADPH+GSH*	*HLM+UDPGA*
1	100%	75%	67%	39%
61	95%	96%	97%	92%

Overall, these data show that **61** is characterized by a greater in vitro phase I/II metabolic stability with respect to the reference compound CA-4. The 1,4-benzodiazepin-2-one nucleus appears, alongside avoiding the isomerization problem associated with the double bond, to mitigate the occurrence of both the *O*-demethylation and aromatic hydroxylation pathways[9] and the extensive glucuronidation of the phenolic function.[27]

Table 3. Summary of metabolic profile for **1** and **61**.

Metabolic pathways	**1**	**61**
Aromatic hydroxylation	+	-

O-demethylation	+	+
Reactive metabolites	+	-
Isomerization	+	-
Glucuronidation	+++	+
Hydrolysis (*in vitro* 1h)	-	-

Finally, as a consequence of the lack of aromatic hydroxylation, the formation of glutathione adducts was not observed indicating the absence of reactive metabolites in the metabolism of **61** (Table 3). On the contrary the metabolic fate of CA-4 is characterized by the formation of electrophilic quinone species that reacted with nucleophilic thiols.

These compounds, in our opinion deserve consideration for the development of novel antitubulin agents.

EXPERIMENTAL SECTION

Chemistry General Procedures. Commercially available reagents and solvents were purchased from Aldrich or Alfa Aesar and were used without further purification. Tetrahydrofuran (THF) was distilled immediately before use from Na/benzophenone under a slight positive atmosphere of dry nitrogen. *N,N'*-Dimethylformamide (DMF) was distilled under vacuum from KOH and stored on activated molecular sieves (4 Å). Dichloromethane was dried by distillation from P_2O_5 and stored on activated molecular sieves (4 Å). When needed the reactions were performed in flame- or oven-dried glassware under a positive pressure of dry

nitrogen. Melting points were determined in open glass capillary with a Stuart scientific SMP3 apparatus and are uncorrected. All compounds were checked by IR (FT-IR THERMO-NICOLET AVATAR), ^{1}H and ^{13}C APT (JEOL ECP 300 MHz spectrometer), and mass spectrometry (Thermo Finningan LCQ-deca XP-plus) equipped with an ESI source and an ion trap detector. Chemical shifts are reported in parts per million (ppm). Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230–400 mesh ASTM). Thin layer chromatography (TLC) was carried out on 5x20 cm plates with a layer thickness of 0.25 mm (Merck Silica gel 60 F_{254}). When necessary they were developed with $KMnO_4$ reagent. Purity of tested compounds was established by elemental analysis. Elemental analysis (C, H, N) of the target compounds are within ±0.4% of the calculated values.

*Preparation of aldehydes **14-18, 49** and **50**.* The 4-methoxy-2-nitrobenzaldehyde (**14**) was synthesized according to the literature.[28] The 5-methoxy-2-nitrobenzaldehyde (**15**) was synthesized according to the literature.[29] The 4,5-dimethoxy-2-nitrobenzaldehyde (**16**) and 6-nitrobenzo[1,3]dioxole-5-carbaldehyde (**18)** were commercially available. The 3,4-dimethoxy-2-nitrobenzaldehyde (**17**) was synthesized according to the literature.[30] The 3-hydroxy-4-methoxy-2-nitrobenzaldehyde (**49**) and 5-hydroxy-4-methoxy-2-nitrobenzaldehyde (**50**) were synthesized according to the literature.[31]

*Preparation of OTBDMS-protected aldeydes **51** and **52**.* The 3-(*tert*-butyldimethylsilyloxy)-4-methoxy-2-nitrobenzaldehyde (**51)** and the 5-(*tert*-butyldimethylsilyloxy)-4-methoxy-2-nitrobenzaldehyde (**52)** were synthesized starting from aldehydes **49** and **50** respectively, according to the literature.[32]

*General procedure for the preparation of 2-nitrobenzhydrols **19-23, 53-54**.* To a dried three-necked flask equipped with a dropping funnel, reflux condenser, and magnetic stirrer was added magnesium turnings (10 mmol, 1.4 eq), a small piece of iodine and 3.0 mL of dry THF. The mixture was gentle heated while approximately 1/4 of 3,4,5-trimethoxybromobenzene solution (**13**) (10 mmol, 1.4 eq) in dry THF (10.0 mL) was added dropwise. As soon as the solution became colorless the remaining 3,4,5-trimethoxylbromobenzene solution was added dropwise under mild reflux and stirring was continued for 1 h at room temperature. Afterward the 3,4,5-trimethoxyphenylmagnesium bromide solution prepared was slowly added to the corresponding substituted 2-nitrobenzaldehyde (7 mmol, 1 eq) in 10.0 mL of dry THF at 0 °C. After complete addition, the solution was allowed to stir at room temperature for 12 h. A saturated aqueous NH_4Cl solution was slowly added to hydrolyze the adduct at 0 °C, and the mixture was stirred for 10 min. The phases were separated, and the aqueous layer was extracted with EtOAc (x3). The combined organic layers were washed with brine (x1), dried over sodium sulfate, and filtered. The filtrate was concentrated *in vacuo*, and the residue was purified by column chromatography to afford the desired 2-nitrobenzhydrol.

(4-methoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanol (19). The title compound was prepared from **14**. The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 7:3 as eluants and was crystallized with MeOH to give a pale yellow solid, yield 37%, m.p. 130-132 °C. IR (KBr) 3471, 2956, 2828, 1595, 1537, 1324, 1128, 1003, 851 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.49 (d, J = 8.5 Hz, 1-H), 7.39 (d, J = 1.5 Hz, 1-H), 7.01 (dd, J = 8.5/1.8 Hz, 1-H), 6.55 (s, 2-H), 6.24 (s, 1-H), 3.84 (s, 3-H), 3.80-3.78 (m, 9-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 159.3, 153.3, 149.1, 137.7, 137.4, 119.7, 109.4, 103.9, 70.9, 60.9, 56.2, 56.0 ppm; MS (ESI) m/z 332 $[M+H-H_2O]^+$.

(5-methoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanol (20). The title compound was prepared from **15**. The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 7:3 as eluants and was crystallized with MeOH to give a brown solid, yield 39%, m.p. 110-112 °C. IR (KBr) 3505, 2941, 2841, 1592, 1506, 1237, 1126, 835 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.08 (d, J = 9.1 Hz, 1-H), 7.19 (d, J = 2.7 Hz, 1-H), 6.90 (dd, J = 9.1/2.7 Hz, 1-H), 6.57 (s, 2-H), 6.44 (s, 1-H), 3.88 (s, 3-H), 3.82 (s, 3-H), 3.81 (s, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 163.8, 153.3, 142.0, 141.2, 137.6, 137.3, 128.0, 114.5, 113.0, 104.2, 71.6, 60.9, 56.2, 56.0 ppm; MS (ESI) m/z 332 $[M+H-H_2O]^+$.

(4,5-dimethoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanol (21). The title compound was prepared from **16**. The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 5:5 as eluants and was crystallized with MeOH to give a pale yellow solid, yield 30%, m.p. 148-149 °C. IR (KBr) 3438, 2935, 2836, 1586, 1526, 1336, 1262, 1211, 1126, 1067, 742 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.60 (s, 1-H), 7.16 (s, 1-H), 6.58 (s, 2-H), 6.44 (s, 1-H), 3.95 (s, 3-H), 3.92 (s, 3-H), 3.82 (s, 3-H), 3.81 (s, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 153.4, 153.1, 148.0, 140.3, 137.8, 137.4, 134.0, 110.5, 108.0, 104.1, 71.2, 60.8, 56.4, 56.3, 56.1 ppm; MS (ESI) m/z 362 $[M+H-H_2O]^+$.

(3,4-dimethoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanol (22). The title compound was prepared from **17**. The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 7:3 as eluants and was crystallized with MeOH to give a yellow-orange solid, yield 33%, m.p. 126-127 °C. IR (KBr) 3420, 2941, 2831, 1650, 1596, 1539, 1504, 1282, 1131, 1054 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.03 (d, J = 8.7, Hz, 1-H), 6.96 (d, J = 8.7 Hz, 1-H), 6.59 (s, 2-H), 5.79 (s, 1-H), 3.92 (s, 3-H), 3.88 (s, 3-H), 3.82 (brs, 9-H) ppm; ^{13}C-NMR

(75 MHz, $CDCl_3$) δ 153.1, 152.5, 145.0, 140.4, 137.8, 137.1, 127.9, 123.2, 114.0, 103.3, 70.6, 62.1, 60.8, 56.2, 56.0 ppm; MS (ESI) m/z 362 $[M+H-H_2O]^+$.

(6-nitrobenzo[d][1,3]dioxol-5-yl)(3,4,5-trimethoxyphenyl)methanol (23). The title compound was prepared from **18**. The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 7:3 as eluants and was crystallized with MeOH to give a yellow solid, yield 71%, m.p. 195-197 °C. IR (KBr) 3064, 2950, 2842, 1591, 1520, 1501, 1330, 1263, 1132, 1029, 920 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.47 (s, 1-H), 7.04 (s, 1-H), 6.59 (s, 2-H), 6.35 (s, 1-H), 6.11 (s, 1-H), 3.82 (brs, 9-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 153.3, 152.2, 147.3, 142.3, 137.6, 137.5, 136.4, 108.3, 105.5, 103.9, 103.2, 70.9, 60.9, 56.2 ppm; MS (ESI) m/z 346 $[M+H-H_2O]^+$.

(3-(tert-butyldimethylsilyloxy)-4-methoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanol (53). The title compound was prepared from **51**. The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 7:3 as eluants and was crystallized with MeOH to give a yellow solid, yield 61%, m.p. 99-101 °C. IR (KBr) 3291, 2937, 2858, 1533, 1506, 1292, 1129, 830 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.89 (d, J = 8.6 Hz, 1-H), 6.84 (d, J = 8.6 Hz, 1-H), 6.56 (s, 2-H), 5.75 (s, 1-H), 3.80–3.76 (m, 12-H), 0.94–0.91 (m, 9-H), 0.18–0.16 (m, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 153.3, 150.3, 143.5, 137.5, 137.3, 137.2, 128.0, 120.0, 112.8, 103.4, 71.2, 60.9, 56.2, 55.6, 25.7, 18.6, -3.96 ppm; MS (ESI) m/z 462 $[M+H-H_2O]^+$.

(5-(tert-butyldimethylsilyloxy)-4-methoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanol (54). The title compound was prepared from **52**. The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 7:3 as

eluants and was crystallized with MeOH to give a yellow solid, yield 32%, m.p. 118-119 °C. IR (KBr) 3448, 2937, 1595, 1516, 1459, 1330, 1282, 1131, 1060, 836, 796 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.55 (s, 1-H), 7.00 (s, 1-H), 6.54 (s, 2-H), 6.32 (s, 1-H), 3.85–3.77 (m, 12-H), 335 (brs, 1-H), 0.92 (brs, 9-H), 0.11 (brs, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 153.2, 150.3, 150.1, 141.1, 137.7, 137.2, 133.7, 120.7, 108.9, 103.9, 71.1, 60.8, 56.0, 56.0, 25.6, 18.5, -4.4 ppm; MS (ESI) m/z 462 $[M+H-H_2O]^+$.

*General procedure for the preparation of 2-nitrobenzophenones **24-28, 55-56**.* To a stirred solution of the corresponding 2-nitrobenzhydrol (4 mmol, 1 eq) and 4 Å molecular sieves (0.40 g) in dry dichloromethane (30.0 mL) was added pyridinium dichromate (PDC, 6 mmol, 1,5 eq) portion-wise at 0 °C. The reaction mixture was stirred under nitrogen atmosphere at room temperature for 12 h. Afterward the mixture was diluted with dichloromethane (30.0 mL) and filtered through a pad of Celite. The filtrate was concentrated *in vacuo* and the residue was purified by column chromatography to obtain the desired 2-nitrobenzophenone.

(4-methoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanone (24). The title compound was prepared from **19**. The crude material was purified by column chromatography using PE/EtOAc 7:3 and PE/EtOAc 5:5 as eluants and was crystallized with MeOH to give an off white solid, yield 82%, m.p. 164-165 °C. IR (KBr) 3103, 2945, 2843, 1678, 1584, 1531, 1332, 1233, 1129, 995, 864 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.64 (d, J = 1.8 Hz, 1-H), 7.44 (d, J = 8.5 Hz, 1-H), 7.26 (dd, J = 8.2/3.4 Hz, 1-H), 6.98 (s, 1-H), 3.96 (s, 3-H), 3.91 (s, 3-H), 3.82 (s, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 192.1, 161.2, 153.3, 148.8, 143.3, 131.6, 130.6, 127.9, 119.8, 109.4, 107.0, 61.0, 56.4, 56.3 ppm; MS (ESI) m/z 348 $[M+H]^+$.

(5-methoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanone (25). The title compound was prepared from **20**. The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 5:5 as eluants to give a pale yellow solid, yield 82%, m.p. 156-157 °C. IR (KBr) 3101, 2972, 2943, 2845, 1671, 1579, 1333, 1233, 1124, 755 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.22 (d, J = 9.2 Hz, 1-H), 7.07 (dd, J = 9.2/2.8 Hz, 1-H), 6.99 (s, 2-H), 6.88 (d, J = 2.8 Hz, 1-H), 3.92 (s, 3-H), 3.90 (s, 3-H), 3.81 (s, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 192.1, 164.1, 153.3, 143.4, 139.6, 138.7, 131.0, 127.1, 115.4, 113.6, 106.8, 61.0, 56.4, 56.4 ppm; MS (ESI) m/z 348 $[M+H]^+$.

(4,5-dimethoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanone (26). The title compound was prepared from **21**. The crude material was purified by column chromatography using PE/EtOAc 7:3 and PE/EtOAc 4:6 as eluants to give an off white solid, yield 82%, m.p. 151 °C. IR (KBr) 3101, 2937, 2832, 1661, 1582, 1524, 1415, 1334, 1284, 1219, 1130, 757 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.71 (s, 1-H), 6.99 (s, 2-H), 6.86 (s, 1-H), 4.03 (s, 3-H), 3.98 (s, 3-H), 3.92 (s, 3-H), 3.82 (s, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 192.3, 153.9, 153.4, 149.8, 143.4, 139.8, 131.4, 130.2, 110.2, 106.9, 106.8, 61.1, 56.8, 56.7, 56.4 ppm; MS (ESI) m/z 378 $[M+H]^+$.

(3,4-dimethoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanone (27). The title compound was prepared from **22**. The crude material was purified by column chromatography using PE/EtOAc 6:4 and PE/EtOAc 5:5 as eluants to give a brown solid, yield 66%, m.p. 161-163 °C. IR (KBr) 3002, 2944, 2141, 1659, 1536, 1335, 1280, 1132, 990, 754 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.03 (d, J = 8.7 Hz, 1-H), 6.96 (d, J = 8.7 Hz, 1-H), 6.59 (s, 2-H), 5.79 (s, 1-H), 3.92 (s, 3-H), 3.88 (s, 3-H), 3.82 (brs, 9-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 153.1, 152.5,

145.0, 140.4, 137.8, 137.1, 127.9, 123.2, 114.0, 103.3, 70.6, 62.1, 60.8, 56.2 ppm; MS (ESI) m/z 378 $[M+H]^+$.

(6-nitrobenzo[d][1,3]dioxol-5-yl)(3,4,5-trimethoxyphenyl)methanone (28). The title compound was prepared from **23**. The crude material was purified by column chromatography using PE/EtOAc 5:5 and PE/EtOAc 2:8 as eluants to give a pale orange solid, yield 91%, m.p. 195-197 °C. IR (KBr) 3051, 2939, 2 l'842, 1661, 1584, 1536, 1504, 1421, 1333, 1128, 1030 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.62 (s, 1-H), 6.97 (s, 2-H), 6.81 (s, 1-H), 6.21 (s, 2-H), 3.89 (s, 3-H), 3.81 (brs, 6-H), ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 191.6, 153.3, 152.7, 149.1, 143.3, 141.4, 132.6, 131.0, 107.8, 106.7, 105.0, 103.8, 61.0, 56.4 ppm; MS (ESI) m/z 360 $[M-H]^-$.

(3-(tert-butyldimethylsilyloxy)-4-methoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanone (55). The title compound was prepared from **53**. The crude material was purified by column chromatography using PE/EtOAc 6:4 as eluant to give a white solid, yield 84%, m.p. 138-139 °C. IR (KBr) 3009, 2937, 2358, 1652, 1543, 1500, 1416, 1331, 1127, 840 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.14 (d, J = 8.5 Hz, 1-H), 7.04 (s, 2-H), 7.98 (d, J = 8.5 Hz, 1-H), 3.92–3.85 (m, 12-H), 0,94 (brs, 9-H), 0.21 (brs, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 191.3, 153.8, 153.0, 143.5, 142.8, 138.6, 131.6, 125.0, 123.3, 111.5, 107.5, 61.1, 56.4, 55.9, 25.6, 18.6, -4.0 ppm; MS (ESI) m/z 478 $[M+H]^+$.

(5-(tert-butyldimethylsilyloxy)-4-methoxy-2-nitrophenyl)(3,4,5-trimethoxyphenyl)methanone (56). The title compound was prepared from **54**. The crude material was purified by column chromatography using PE/EtOAc 8:2 as eluant to give a pale yellow solid, yield 81%, m.p. 150-152 °C. IR (KBr) 3040, 2941, 2856, 1675, 1567, 1518, 1415, 1331, 1294, 1134, 838 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) 7.70 (s, 1-H), 6.97 (s, 2-H), 6.83 (s, 1-

H), 3.94 (s, 3-H), 3.89 (s, 3-H), 3.79 (s, 6-H), 0.96 (brs, 9-H), 0.18 (brs, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 192.1, 153.3, 151.7, 150.8, 143.3, 140.3, 131.5, 130.2, 120.0, 107.9, 106.8, 61.0, 56.4, 56.1, 25.6, 18.6, -4.4 ppm; MS (ESI) m/z 478 $[M+H]^+$.

*General procedure for the preparation of 2-aminobenzophenones **29-33**.* A mixture of the corresponding 2-nitrobenzophenone (2.8 mmol, 1 eq) and iron powder (7.7 eq), in ethanol (10.0 mL), acetic acid (10 mL), water (5.0 mL), and 35% hydrochloric acid (2 drops) was vigorously stirred and refluxed for 2 h. The suspension was cooled and filtered through a pad of Celite. The filtrate was diluted with water and extracted with dichloromethane (x3). The combined organic layers were washed with saturated aqueous $NaHCO_3$ solution (x1), water (x1), dried over sodium sulfate and concentrated *in vacuo*. Finally the residue was purified by column chromatography to afford the desired 2-aminobenzophenone.

(2-amino-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (29). The title compound was prepared from **24**. The crude material was purified by column chromatography using PE/EtOAc 7:3 as eluant to give a yellow solid, yield 98%, m.p. 94-95 °C. IR (KBr) 3441, 3338, 2935, 2832, 1616, 1582, 1343, 1228, 1120, 770 cm^{-1}. ^{1}H-NMR (300 MHz, CD_3OD) δ 7.34 (d, J = 9.2 Hz, 1-H), 6.82 (s, 2-H), 6.31 (d, J = 2.5 Hz, 1-H), 6.15 (dd, J = 9.2/2.4 Hz, 1-H), 3.84 (s, 6-H), 3.83 (s, 3-H), 3.80 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, CD_3OD) δ 198.3, 166.2, 156.0, 154.1, 141.3, 137.6, 137.5, 112.6, 107.6, 105.0, 99.9, 61.2, 56.7, 55.7 ppm; MS (ESI) m/z 318 $[M+H]^+$.

(2-amino-5-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (30). The title compound was prepared from **25**. The crude material was purified by column chromatography using PE/EtOAc 7:3 as eluant to give a yellow solid, yield 76%, m.p. 130-132 °C. IR (KBr) 3420, 3308, 2944, 2832, 1639, 1583, 1411, 1333, 1213, 1134, 777 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ

7.01-6.94 (m, 4-H), 6.77 (d, J = 8.6 Hz, 1-H), 3.92 (s, 3-H), 3.87 (br s, 6-H), 3.68 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 197.5, 152.9, 149.9, 145.3, 141.1, 134.9, 122.8, 118.6, 116.7, 107.0, 61.0, 56.4, 56.1 ppm; MS (ESI) m/z 318 $[M+H]^+$.

(2-amino-4,5-dimethoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (31). The title compound was prepared from **26**. The crude material was purified by column chromatography using PE/EtOAc 7:3 as eluant to give a yellow solid, yield 71%, m.p. 129-131 °C. IR (KBr) 3452, 3323, 2944, 2833, 1629, 1582, 1414, 1337, 1207, 1134, 775 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.00 (s, 1-H), 6.88 (s, 2-H), 6.35 (s, 1-H), 3.91 (s, 6-H), 3.86 (s, 6-H), 3.69 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 196.1, 155.4, 152.8, 148.2, 140.4, 139.8, 135.8, 116.3, 110.0, 106.5, 99.6, 61.0, 56.6, 56.3, 55.9 ppm; MS (ESI) m/z 348 $[M+H]^+$.

(2-amino-3,4-dimethoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (32). The title compound was prepared from **27**. The crude material was purified by column chromatography using PE/EtOAc 7:3 as eluant to give a yellow solid, yield 89%, m.p. 140-141 °C. IR (KBr) 3481, 3346, 2968, 2942, 2833, 1617, 1578, 1529, 1241, 1129, 997, 743 cm^{-1}. ^{1}H-NMR (300 MHz, CD_3OD) δ 7.27 (d, J = 9.3 Hz, 1-H), 6.84 (s, 2-H), 6.23 (d, J = 9.3 Hz, 1-H), 3.90–3.85 (m, 15-H) ppm; ^{13}C-NMR (75 MHz, CD_3OD) δ 197.1, 156.0, 152.8, 146.2, 140.5, 135.8, 134.7, 131.3, 113.6, 106.6, 99.8, 61.0, 59.8, 56.3, 55.9 ppm; MS (ESI) m/z 348 $[M+H]^+$.

(6-aminobenzo[d][1,3]dioxol-5-yl)(3,4,5-trimethoxyphenyl)methanone (33). The title compound was prepared from **28**. The crude material was purified by column chromatography using PE/EtOAc 7:3 and PE/EtOAc 5:5 as eluants to give a yellow solid, yield 86%, m.p. 150-152 °C. IR (KBr) 3450, 3332, 3003, 2904, 1583, 1560, 1496, 1413, 1333, 1233, 1133, 783 cm^{-1}.

^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.85 (s, 1-H), 6.77 (s, 2-H), 6.42 (brs, 2-H), 6.18 (s, 1-H), 5.84 (s, 2-H), 3.85–3.82 (brs, 9-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 196.1, 153.2, 152.8, 150.5, 140.0 138.3, 136.1, 111.3, 110.0, 106.1, 101.3, 96.8, 60.9, 56.2 ppm; MS (ESI) m/z 332 $[M+H]^+$.

*General procedure for the preparation of OTBDMS-protected 2-aminobenzophenones **57-58**.* To a solution of the corresponding OTBDMS protected 2-nitrobenzophenone (0.122 g, 0.25 mmol, 1 eq) in dichloromethane (7.0 mL), was added Pd/C 10% (0.036 g). The resulting mixture was vigorously stirred under a H_2 atmosphere (1 atm) at room temperature. After 10 h the suspension was filtered through a pad of Celite and the solvent was removed *in vacuo* The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 7:3 as eluants.

(2-amino-3-(tert-butyldimethylsilyloxy)-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (57). The title compound was prepared from **55**. Yellow oil, yield 78%. IR (KBr) 3498, 3359, 2934, 2857, 1609, 1579, 1502, 1275, 1127, 834 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.15 (d, J = 9.1 Hz, 1-H), 6.86 (s, 2-H), 6.22 (d, J = 9.1 Hz, 1-H), 3.90–3.86 (m, 12-H), 1.04 (brs, 9-H), 0.22 (brs, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 197.2, 152.8, 144.9, 140.3, 136.0, 130.7, 128.5, 113.5, 106.6, 99.5, 61.0, 56.3, 55.0, 26.2, 18.8, -3.9 ppm; MS (ESI) m/z 448 $[M+H]^+$.

(2-amino-5-(tert-butyldimethylsilyloxy)-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (58). The title compound was prepared from **56**. Yellow solid, yield 75%,%, m.p. 124-127 °C. IR (KBr) 3470, 3359, 2934, 2856, 1623, 1578, 1533, 1251, 1116, 832, 783 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.94 (s, 1-H), 6.82 (s, 2-H), 6.17 (s, 1-H), 3.90–3.83 (m, 12-H), 0.91 (brs, 9-H), 0.07 (brs, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 196.6, 157.2,

152.9, 148.4, 140.2, 136.1, 135.2, 124.7, 110.8, 106.3, 99.3, 61.1, 56.3, 55.5, 25.8, 18.4,-4.5 ppm; MS (ESI) m/z 448 $[M+H]^+$.

*General procedure for the preparation of 2-chloroacetamides **34-38, 59-60**.* To a cooled (0 °C) solution of the corresponding 2-aminobenzophenone (1 eq) in dry dichloromethane (0.2 M), was added chloroacetyl chloride (1.1 eq) dropwise, and the reaction mixture was stirred at room temperature for 1h. Afterward a saturated aqueous $NaHCO_3$ solution was added at 0 °C and the mixture was vigorously stirred for 10 min. The organic layer was separated, washed with aqueous saturated $NaHCO_3$ solution (x1), dried over sodium sulfate and concentrated under reduced pressure. Finally the crude material was purified by column chromatography to afford the desired 2-chloroacetamide.

2-chloro-*N*-(5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl)acetamide (34). The title compound was prepared from **29**. The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 4:6 as eluants to give a white solid, yield 86%, m.p. 183-185 °C. IR (KBr) 3140, 2969, 2839, 1669, 1579, 1408, 1341, 1248, 1127, 617 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 12.05 (br s, 1-H), 8.32 (s, 1-H), 7.59 (d, J = 8.8 Hz, 1-H), 6.91 (s, 2-H), 6.65 (d, J = 8.8 Hz, 1-H), 4.20 (s, 2-H), 3.92 (s, 3-H), 3.91 (s, 3-H), 3.87 (s, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 197.5, 165.8, 164.3, 152.9, 142.2, 141.7, 135.8, 134.2, 116.8, 109.5, 107.4, 105.8, 61.0, 56.4, 55.8, 43.3 ppm; MS (ESI) m/z 394 $[M+H]^+$.

2-chloro-*N*-(4-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl)acetamide (35). The title compound was prepared from **30**. The crude material was purified by column chromatography using PE/EtOAc 7:3 and PE/EtOAc 6:4 as eluants to give a pale yellow solid, yield 88%, m.p. 139-140 °C. IR (KBr) 3208, 2954, 2835, 1674, 1585, 1540, 1337, 1137, 775 cm^{-1}. ^{1}H-NMR (300

MHz, $CDCl_3$) δ 10.90 (br s, 1-H), 8.39 (d, J = 8.9 Hz, 1-H), 7.16-7.10 (m, 2-H), 7.02 (s, 2-H), 4.16 (s, 2.H), 3.94 (s, 3-H), 3.87 (br s, 6-H), 3.78 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 197.1, 165.0, 155.1, 153.0, 142.6, 132.8, 131.6, 126.8, 123.9, 118.8, 117.7, 107.9, 61.1, 56.4, 55.8, 43.1 ppm; MS (ESI) m/z 394 $[M+H]^+$

2-chloro-*N*-(4,5-dimethoxy-2-(3,4,5-trimethoxybenzoyl)phenyl)acetamide (36). The title compound was prepared from **31**. The crude material was purified by column chromatography using PE/EtOAc 5:5 and EtOAc as eluants to give an off white solid, yield 98%, m.p. 185-186 °C dec. IR (KBr) 3119, 2958, 2832, 1671, 1583, 1524, 1328, 1208, 1136, 988, 776 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 11.9 (br s, 1-H), 8.37 (s, 1-H), 7.13 (s, 1-H), 6.96 (s, 2-H), 4.19 (s, 2-H), 4.00 (s, 3-H), 3.94 (s, 3-H), 3.87 (br s, 6-H), 3.77 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 197.0, 165.5, 153.8, 153.0, 144.0, 141.8, 135.8, 134.0, 116.2, 115.7, 107.4, 104.7, 61.1, 56.4, 56.3, 43.3 ppm; MS (ESI) m/z in 424 $[M+H]^+$.

2-chloro-*N*-(2,3-dimethoxy-6-(3,4,5-trimethoxybenzoyl)phenyl)acetamide (37). The title compound was prepared from **32**. The crude material was purified by column chromatography using PE/EtOAc 7:3 and PE/EtOAc 6:4 as eluants to give a pale yellow solid, yield 84%, m.p. 137-139 °C. IR (KBr) 3389, 3023, 2944, 2840, 1698, 1654, 1413, 1323, 1130, 999, 765 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.13 (brs, 1-H), 7.19 (d, J = 8.4 Hz, 1-H), 7.09 (s, 2-H), 6.80 (d, J = 8.7 Hz, 1-H), 4.08 (s, 2-H), 3.93–3.85 (m, 15-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 194.1, 164.4, 155.5, 152.9, 142.9, 142.3, 132.6, 129.3, 126.7, 126.0, 108.5, 107.9, 61.0, 60.8, 56.4, 56.1, 43.0 ppm; MS (ESI) m/z 446 $[M+Na]^+$.

2-chloro-*N*-(6-(3,4,5-trimethoxybenzoyl)benzo[d][1,3]dioxol-5-yl)acetamide (38). The title compound was prepared from **33**. The crude material was purified by column chromatography

using PE/EtOAc 7:3, PE/EtOAc 5:5 and a EtOAc as eluants to give a pale yellow solid, yield 94%, m.p. 188-190 °C. IR (KBr) 3119, 2041, 2836, 1681, 1522, 1327, 1231, 1134, 779, 703, 452 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 11.85 (brs, 1-H), 8.18 (s, 1-H), 7.04 (s, 1-H), 6.90 (s, 2-H), 6.05 (s, 2-H), 4.17 (s, 2-H), 3.91–3.80 (m, 9-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 197.0, 165.6, 153.0, 152.2, 143.1, 141.9, 136.8, 133.9, 117.9, 112.0, 107.4, 103.1, 102.4, 61.1, 56.5, 43.2 ppm; MS (ESI) m/z 430 $[M+Na]^+$.

***N*-(2-(tert-butyldimethylsilyloxy)-3-methoxy-6-(3,4,5-trimethoxybenzoyl)phenyl)-2-chloroacetamide (59).** The title compound was prepared from **57** according to the general procedure with addition of TEA (1.2 eq). The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 7:3 as eluants to give an off white solid, yield 76%, m.p. 115-116 °C. IR (KBr) 3275, 2937, 2857, 1697, 1582, 1454, 1333, 1127, 839, 783 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.90 (brs, 1-H), 7.11 (s, 2-H), 7.05 (d, J = 8.6 Hz, 1-H), 6.75 (d, J = 8.6 Hz, 1-H), 4.04 (s, 2-H), 3.92–3.86 (m, 12-H), 1.00 (brs, 9-H), 0.21 (brs, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 194.4, 164.0, 153.0, 152.8, 142.1, 139.4, 132.8, 126.8, 126.7, 123.9, 107.8, 107.7, 61.0, 56.4, 55.3, 42.9, 25.9, 18.8, -3.8 ppm; MS (ESI) m/z 546 $[M+Na]^+$.

***N*-(4-(tert-butyldimethylsilyloxy)-5-methoxy-2-(3,4,5-trimethoxybenzoyl)phenyl)-2-chloroacetamide (60).** The title compound was prepared from **58** according to the general procedure with addition of TEA (1.2 eq). The crude material was purified by column chromatography using PE/EtOAc 8:2 and PE/EtOAc 7:3 as eluants to give an off white solid, yield 80%, m.p. 158-160 °C. IR (KBr) 3002, 2955, 2858, 1686, 1579, 1523, 1346, 1126, 939, 838, 785 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 11.88 (brs, 1-H), 8.33 (s, 1-H), 7.11 (s, 1-H), 6.93 (s, 2-H), 4.21 (s, 2-H), 3.96–3.83 (m, 12-H), 0.95 (brs, 9-H), 0.13 (brs, 6-H) ppm; ^{13}C-NMR (75

MHz, $CDCl_3$) δ 197.4, 165.6, 155.8, 153.0, 141.8, 140.0, 135.9, 134.2, 125.0, 116.8, 107.4, 105.0, 61.2, 56.4, 55.9, 43.3, 25.7, 18.5, -4.4 ppm; MS (ESI) m/z 546 $[M+Na]^+$.

*General procedure for the preparation of combretabenzodiazepines **39-43, 61-62, 64**.* To a solution of the corresponding 2-chloroacetamide (1 eq) in 96% ethanol (0.16 M), hexamethylenetetramine (HMTA) (2.5 eq) and ammonium acetate (2.5 eq) were added. The reaction mixture was vigorously stirred at reflux temperature for 6h. Then water was added and the resulting suspension was extracted with dichloromethane (x2). The combined organic layers were washed with brine (x1), dried over sodium sulfate and concentrated *in vacuo*. Finally the crude material was purified by column chromatography to afford the desired combretabenzodiazepine.

8-methoxy-5-(3,4,5-trimethoxyphenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (39). The title compound was prepared from **34**. The crude material was purified by column chromatography using PE/EtOAc 5:5 and EtOAc as eluants and was crystallized with EtOAc to give an off white solid, yield 68%, m.p. 213-215 °C. IR (KBr) 3064, 2953, 2844, 1677, 1616, 1414, 1346, 1128, 857 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.85 (br s, 1-H), 7.27 (d, J = 8.0 Hz, 1-H), 6.77 (s, 2-H), 6.71-6.68 (m, 2-H), 4.27 (s, 2-H), 3.87 (s, 3-H), 3.86 (s, 3-H), 3.81 (s, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 172.0, 170.8, 162.3, 152.9, 140.8, 140.2, 135.1, 133.2, 120.0, 110.8, 107.4, 105.0, 61.0, 56.6, 56.3, 55.7 ppm; MS (ESI) m/z 357 $[M+H]^+$. Anal. Calcd for $C_{19}H_{20}N_2O_5$: C 64.04, H 5.66, N 7.86, found C, 64.05; H, 5.72, N, 7.70.

7-methoxy-5-(3,4,5-trimethoxyphenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (40). The title compound was prepared from **35**. The crude material was purified by column chromatography using PE/EtOAc 7:3 and PE/EtOAc 3:7 as eluants and was crystallized with

EtOAc to give a white solid, yield 58%, m.p. 222-224 °C. IR (KBr) 3063, 2944, 1681, 1576, 1503, 1351, 1223, 1134, 838 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.98 (br s, 1-H), 7.12 (br s, 2-H), 6.84 (br s, 3-H), 4.27 (br s, 1-H), 3.90-3.84 (m, 10-H), 3.74 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 171.1, 169.0, 153.8, 151.7, 139.0, 133.4, 131.5, 126.8, 121.7, 117.9, 113.4, 106.1, 59.8, 55.4, 55.1, 54.6 ppm; MS (ESI) m/z 357 $[M+H]^+$. Anal. Calcd for $C_{19}H_{20}N_2O_5$: C 64.04, H 5.66, N 7.86, found C, 63.83; H, 5.43; N, 7.95.

7,8-dimethoxy-5-(3,4,5-trimethoxyphenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (41). The title compound was prepared from **36**. The crude material was purified by column chromatography using PE/EtOAc 5:5 and EtOAc as eluants and was crystallized with EtOAc to give a white solid, yield 73%, m.p. 257-258 °C dec. IR (KBr) 2994, 2937, 2829, 1677, 1507, 1349, 1259, 1133, 1028, 837 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.72 (br s, 1-H), 6.84 (s, 2-H), 6.76 (s, 1-H), 6.69 (s, 1-H), 4.26 (br s, 2-H), 3.96 (s, 3-H), 3.89 (s, 3-H), 3.83 (br s, 6-H), 3.75 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 171.8, 170.2, 153.0, 144.8, 140.3, 134.7, 134.1, 119.3, 112.8, 107.4, 104.0, 61.0, 56.4, 56.3 ppm; MS (ESI) m/z 387 $[M+H]^+$. Anal. Calcd for $C_{20}H_{22}N_2O_6$: C 62.17, H 5.74, N 7.25, found C, 62.45; H, 5.89; N, 7.45.

8,9-dimethoxy-5-(3,4,5-trimethoxyphenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (42). The title compound was prepared from **37**. The crude material was purified by column chromatography using PE/EtOAc 5:5 and PE/EtOAc 3:7 as eluants to give a white solid, yield 55%, m.p. 240-242 °C dec. IR (KBr) 3170, 3069, 2941, 2839, 1683, 1578, 1506, 1341, 1295, 1123, 799 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.12 (brs, 1-H), 7.07 (d, J = 9.0 Hz, 1-H), 6.74 (s, 2-H), 6.72 (d, J = 9.0 Hz, 1-H), 4.28 (s, 2-H), 3.94–3.81 (m, 15-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.8, 170.3, 153.9, 152.8, 139,9, 137.5, 135.2, 133.3, 127.2, 120.3, 107.2, 107.0, 61.0,

60.9, 56.8, 56.2, 56.1 ppm; MS (ESI) m/z 387 $[M+H]^+$. Anal. Calcd for $C_{20}H_{22}N_2O_6$: C 62.17, H 5.74, N 7.25, found C, 62.45; H, 5.92, N, 7.23.

9-(3,4,5-trimethoxyphenyl)-5,7-dihydro-6H-[1,3]dioxolo[4',5':4,5]benzo[1,2-e][1,4]diazepin-6-one (43). The title compound was prepared from **38**. The crude material was purified by column chromatography using PE/EtOAc 3:7, EtOAc and EtOAc/MeOH 98:2 as eluants and to give a pale yellow solid, yield 48%, m.p. 250-251 °C dec. IR (KBr) 3073, 2964, 2834, 1681, 1505, 1487, 1247, 1132, 1037, 843 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.56 (brs, 1-H), 6.77 (s, 2-H), 6.72 (s, 1-H), 6.66 (s, 1-H), 6.03 (s, 2-H), 4.26 (brs, 1-H), 3.87–3.83 (m, 10-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 171.8, 170.1, 153.0, 150.8, 143.9, 140.2, 134.9, 127.1, 121.0, 109.5, 107.4, 102.4, 101.9, 61.0, 56.7, 56.4 ppm; MS (ESI) m/z 371 $[M+H]^+$. Anal. Calcd for $C_{19}H_{18}N_2O_6$: C 61.62, H 4.90, N 7.56, found C, 61. 65; H, 5.00; N, 7.75.

9-hydroxy-8-methoxy-5-(3,4,5-trimethoxyphenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (61). The title compound was prepared from **59**. The crude material was purified by column chromatography using PE/EtOAc 5:5 and EtOAc as eluants to give a white solid, yield 36%, m.p. 251-252 °C dec. IR (KBr) 3274, 2940, 2837, 2504, 1694, 1579, 1507, 1348,1293, 1122 cm^{-1}. ^{1}H-NMR (300 MHz, DMSO) δ 9.48 (s, 1-H), 9.39 (s, 1-H), 6.90 (d, J = 7.9 Hz, 1-H), 6.80 (s, 2-H), 6.78 (d, J = 10.7 Hz, 1-H), 4.05 (brs, 1-H), 3.89 (s, 3-H), 3.74–3.71 (m, 10-H) ppm; ^{13}C-NMR (75 MHz, DMSO) δ 169.9, 169.1, 152.4, 149.1, 139.4, 136.4, 134.8, 128.4, 121.4, 120.8, 107.1, 60.2, 56.8, 56.2, 56.0 ppm; MS (ESI) m/z 373 $[M+H]^+$. Anal. Calcd for $C_{19}H_{20}N_2O_6$: C 61.28, H 5.41, N 7.52, found C, 61.25; H, 5.55; N, 7.43.

3-methoxy-6-(3,4,5-trimethoxyphenyl)-1H-2-oxa-7,9a-diazabenzo[cd]azulen-9(8H)-one (62). The title compound was prepared from **59**. The crude material was purified by column

chromatography using PE/EtOAc 5:5 and EtOAc as eluants to give a white solid, yield 21%, m.p. 242-244 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.03 (d, J = 8.6 Hz, 1-H), 6.76 (s, 2-H), 6.60 (d, J = 8.6 Hz,1-H), 5.72 (s, 2-H), 4.70 (s, 2-H), 3.94 (s, 3-H), 3.89–3.84 (m, 9-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 163.8, 161.9, 153.3, 151.4, 139.8, 132.8 (2-C), 127.0, 123.4, 110.7, 106.5, 106.2, 68.1, 61.1, 58.2, 56.4 ppm; MS (ESI) m/z 385 $[M+H]^+$. Anal. Calcd for $C_{20}H_{20}N_2O_6$: C 62.49, H 5.24, N 7.29, found C, 62.72; H, 5.47; N, 7.21.

7-hydroxy-8-methoxy-5-(3,4,5-trimethoxyphenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (64). The title compound was prepared from **60**. After concentration *in vacuo* the residue was dissolved in 4.0 mL of dry THF. To the solution, glacial acetic acid (1 eq) and TBAF 1 M in THF (1 eq) were added dropwise at 0 °C. After 15 min. water was added and the resulting mixture was extracted with dichloromethane (x3). The combined organic layers were washed with brine (x1), dried over sodium sulfate and concentrated *in vacuo*. The crude material was purified by column chromatography using PE/EtOAc 3:7 and EtOAc as eluants to give a white solid, yield 89%, m.p. 208-211 °C dec. IR (KBr) 3551, 3414, 2939, 2840, 1663, 1509, 1347, 1276, 1127, 1004, 789 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.64 (s, 1-H), 6.84 (s, 1-H), 6.76 (s, 2-H), 6.62 (s, 1-H), 5.84 (brs, 1-H), 4.25 (brs, 2-H), 3.94 (s, 3-H), 3.86 (s, 3-H), 3.80 (s, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 171.9, 170.4, 152.9, 149.7, 141.6, 140.2, 134.9, 133.0, 120.5, 116.0, 107.3, 103.4, 61.0, 56.6, 56.4, 56.3 ppm; MS (ESI) m/z 373 $[M+H]^+$. Anal. Calcd for $C_{19}H_{20}N_2O_6$: C 61.28, H 5.41, N 7.52, found C, 61.03; H, 5.21; N; 7.61.

*General procedure for the preparation of N1-methyl combretabenzodiazepines **44-48**.* Under nitrogen atmosphere the corresponding combretabenzodiazepine (**39-43**) (1 eq) was dissolved in dry DMF (0.17 M). To the cooled (0 °C) solution NaH 60% (1.2 eq) was added portionwise and the reaction mixture was stirred for 1h. Then methyl iodide (1.5 eq) was added and the mixture

was stirred for 12h at room temperature. Afterward the reaction mixture was diluted with EtOAc and washed with brine (x3), dried over sodium sulfate and concentrated *in vacuo*. Finally the crude material was purified by column chromatography to afford the desired *N*1-methyl-combretabenzodiazepine.

8-methoxy-1-methyl-5-(3,4,5-trimethoxyphenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (44). The title compound was prepared from **39**. The crude material was purified by column chromatography using PE/EtOAc 5:5 and PE/EtOAc 3:7 as eluants to give a white solid, yield 43%, m.p. 137-139 °C dec. IR (KBr) 2987, 2937, 2839, 1672, 1606, 1413, 1352, 1232, 1126, 755 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.25 (d, J = 8.6 Hz, 1-H), 6.82 (s, 2-H), 6.78 (d, J = 2.4 Hz, 1-H), 6.78 (d, J = 2.4 Hz, 1-H), 6.71 (dd, J = 8.6/2.4 Hz, 1-H), 4.70 (d, J = 10.7 Hz, 1-H), 3.87 (s, 3-H), 3.84 (s, 3-H), 3.80 (s, 6-H), 3.72 (d, J = 10.7 Hz, 1-H), 3.36 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.1, 169.7, 161.9, 152.9, 145.8, 140.4, 134.3, 132.3, 121.5, 109.8, 107.4, 106.3, 60.9, 56.8, 56.3, 55.7, 35.0 ppm; MS (ESI) m/z 371 $[M+H]^+$. Anal. Calcd for $C_{20}H_{22}N_2O_5$: C 64.85, H 5.99, N 7.56, found C, 64.93; H, 6.12; N, 7.50.

7-methoxy-1-methyl-5-(3,4,5-trimethoxyphenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (45). The title compound was prepared from **40**. The crude material was purified by column chromatography using PE/EtOAc 7:3 and PE/EtOAc 3:7 as eluants to give a pale yellow solid, yield 49%, m.p. 145-147 °C. IR (KBr) 2940, 2837, 1670, 1573, 1498, 1348, 1287, 1131, 816 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.27 (d, J = 8.9 Hz, 1-H), 7.11 (d, J = 9.1 Hz, 1-H), 6.90 (s, 2-H), 6.82 (s, 1-H), 4.75 (d, J = 10.4 Hz, 1-H), 3.88 (br s, 3-H), 3.84 (br s, 6-H), 3.74 (br s, 4-H), 3.36 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.4, 169.1, 155.3, 153.1, 140.6, 137.9, 133.9, 129.5, 122.6, 118.5, 114.1, 107.4, 61.1, 57.0, 56.5, 55.9, 35.0; MS (ESI) m/z 371 $[M+H]^+$. Anal. Calcd for $C_{20}H_{22}N_2O_5$: C 64.85, H 5.99, N 7.56, found C, 64.72; H, 5.82; N, 7.81.

7,8-dimethoxy-1-methyl-5-(3,4,5-trimethoxyphenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (46). The title compound was prepared from **41**. The crude material was purified by column chromatography using PE/EtOAc 4:6 and EtOAc as eluants and was crystallized with EtOAc to give a pale yellow solid, yield 46%, m.p. 224-225 °C dec. IR (KBr) 2991, 2952, 2833, 1666, 1519, 1507, 1341, 1130, 1005, 865 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.89 (s, 2-H), 6.77 (s, 1-H), 6.74 (s, 1-H), 4.74 (d, J = 10.4 Hz, 1-H), 3.96 (s, 3-H), 3.86 (s, 3-H), 3.82 (br s, 6-H), 3.75 (br s, 4-H), 3.37 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 169.9, 169.3, 153.0, 151.9, 145.1, 140.6, 139.0, 133.5, 120.7, 112.2, 107.5, 104.2, 61.0, 56.5, 56.4, 56.3, 35.2 ppm; MS (ESI) m/z 401 $[M+H]^+$. Anal. Calcd for $C_{21}H_{24}N_2O_6$: C 62.99, H 6.04, N 7.00, found C, 63.23; H, 6.32; N, 7.00.

8,9-dimethoxy-1-methyl-5-(3,4,5-trimethoxyphenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one (47). The title compound was prepared from **42**. The crude material was purified by column chromatography using PE/EtOAc 3:7 as eluant to give an off white solid, yield 65%, m.p. 157-159 °C dec. IR (KBr) 3416, 2940, 2837, 1677, 1592, 1456, 1346, 1278, 1126, 1004 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.08 (d, J = 8.9 Hz, 1-H), 6.91 (s, 2-H), 6.84 (d, J = 8.9 Hz, 1-H), 4.73 (d, J = 10.4 Hz, 1-H), 3.96 (s, 3-H), 3.88–3.83 (m, 12-H), 3.75 (d, J = 10.6 Hz, 1-H), 3.30 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 169.8, 169.1, 155.0, 153.0, 142.2, 140.4, 138.4, 134.2, 125.8, 123.6, 109.0, 107.3, 61.0, 60.6, 56.8, 56.4, 56.3, 36.2 ppm; MS (ESI) m/z 401 $[M+H]^+$. Anal. Calcd for $C_{21}H_{24}N_2O_6$: C 62.99, H 6.04, N 7.00, found C, 63.32; H, 6.31; N, 7.12.

5-methyl-9-(3,4,5-trimethoxyphenyl)-5,7-dihydro-6H-[1,3]dioxolo[4',5':4,5]benzo[1,2-e][1,4]diazepin-6-one (48). The title compound was prepared from **43**. The crude material was purified by column chromatography using PE/EtOAc 4:6 and PE/EtOAc 3:7 as eluants and was

crystallized with MeOH to give an off white solid, yield 47%, m.p. 202-205 °C dec. IR (KBr) 2992, 2900, 1677, 1577, 1505, 1487, 1413, 1344, 1233, 1128, 1035 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.85 (s, 2-H), 6.81 (s, 1-H), 6.73 (s, 1-H), 6.10 (brs, 1-H), 6.03 (brs, 1-H), 4.75 (d, J = 10.7Hz, 1-H), 3.88–3.85 (m, 9-H), 3.86 (d, J = 10.7 Hz, 1-H), 3.34 (s, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.2, 169.1, 153.1, 150.4, 143.9, 140.0, 134.8, 134.2, 122.5, 108.8, 107.4, 102.4, 102.0, 61.1, 57.0, 56.5, 35.3 ppm; MS (ESI) m/z 385 $[M+H]^+$. Anal. Calcd for $C_{20}H_{20}N_2O_6$: C 62.49, H 5.24, N 7.29, found C, 62.76; H, 5.45; N 6.91.

Molecular modeling. All molecular modeling studies were performed on a Tesla workstation. Different crystal structures of colchicine domain inhibitors have been reported; in our study, the X-ray structure of the DAMA-colchicine-α,β-tubulin complex was used (PDB ID: 1SA0).[26] The stathmin-like domain, subunits C and D, colchicine, and water molecules were removed; the binding site was detected using the original ligand coordinates. Ligand structures were built from a SMILES string and were minimized using Omega2.[33] The docking simulations were performed using FRED, and default settings were used.[34] To validate the use of the FRED program, the docking studies were performed on the reference compound colchicine. FRED successfully reproduced the binding conformations reported in the X-ray structure with acceptable root-mean-square deviation (rmsd < 1 Å) of atom coordinates. All structural images were prepared using PyMOL.[35]

Cell Culture. The SH-SY5Y human neuroblastoma cell line was obtained from ATCC and cultured in 50% DMEM and 50% F-12 supplemented with 10% foetal bovine serum, 2 mM L-glutamine, penicillin (100 μg/mL), and streptomycin (100 μg/ mL).

Cytotoxicity assay. For cytotoxicity assay, cells were plated on 24-well plates and grown for 48 hours in the presence or absence of CA-4 or the combretabenzodiazepines. After the period of treatment cells were washed with Lock solution (134 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 10 mM HEPES [7.6], 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM sucrose) and incubated for 1 hour with MTT (250 µg/mL in Locke's solution) at 37 °C. After 1 hour, the reaction was stopped and formazan crystals solubilized with isopropanol and 0.1 M of HCl and kept at RT for 15', before being read at 570 nm using a spectrophotometer (Victor). IC_{50} values were calculated using Kaleidagraph software.

Flow-cytometric analysis. SH-SY5Y grown in the presence or absence of compounds for 16 or 24 hours were washed once in PBS and resuspended in 1 mL of 70% EtOH and stored at -20 °C. Cells were then washed twice in PBS and resuspended in PBS containing RNAse (100 μg/mL) for 1 h at 37 °C. DNA was then stained with a PBS solution containing 5 mM EDTA and 25 μg/mL propidium iodide for 30 min at 4 °C in the dark. Cell cycle analysis was determined with a BD Accuri FACS.

In vivo and in vivo tubulin polymerization assay. 1 x 10^^{6} Cells were grown in 10 mm^3 dish in the presence of absence of drug for 16 hours. Cells were then tripsinised and centrifuged twice at 600 g for 5'. Cells were then resuspended in 70 µL of Hypotonic buffer (20 mM Tris-HCl pH 6.8, 2 mM EGTA, 1 mM $MgCl_2$, 0.5% of Igepal and protease inhibitors) containing 4 µg/mL paclitaxel. Lysates are incubated for 10' at room temperature in the dark, then vortexed. Protein amounts were determined using Bradford assay (Sigma-Aldrich) and 50 µg were centrifuged at 13000 rpm for 15' at room temperature. Supernatant and pellet were then resuspended in equal volume of SDS-loading buffer and run on 12% SDS-page polyacrylamide gel. Tubulin was identified using anti-tubulin antibody (Sigma-Aldrich).

For *in vitro* characterization, the effects of compounds on the polymerization of tubulin were determined by employing a fluorescence-based tubulin polymerisation assay kit (Cytoskeleton-Cat. #BK011P) according to the manufacturer's protocol. Tubulin was resuspended in ice-cold tubulin buffer (80 mM PIPES, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP, 20% (*v*/*v*) glycerol) and added to wells on a 96-well plate containing the designated concentration of the drug or vehicle. The samples were mixed well, and tubulin assembly was monitored at 1 min intervals for 90 min at 37 °C using a plate reader (Victor3V, PerkinElmer Life Sciences). The IC_{50} values were calculated with a non-linear regression curves in *Prism* and using Cytoskeleton template (http://www.cytoskeleton.com/bk011p).

Binding of [^{3}H]colchicine to tubulin. The binding of [^{3}H]colchicine to tubulin was measured by an adaptation of the method described by Kang et al.[36] Briefly, reaction mixtures contained 0.2 mg/mL tubulin (T238P-A, Cytoskeleton, Inc), 1.0 M monosodium glutamate, 0.1 M glucose-1 phosphate, 1.0 mM GTP, 1.0 $MgCl_2$, 0.5 mg/mL bovine serum albumin, 1% dimethyl sulfoxide and vehicle or inhibitors at different concentrations as indicated. These reaction conditions strongly stabilize binding activity of tubulin. Samples were pre-incubated with different concentrations of non-radioactive colchicine (Sigma-Aldrich) or compound **61** for 30 min at 37 °C. After this period 0.2 μCi [^{3}H]colchicine (colchicine, [ring C, Methoxy-^{3}H]-, 250 μCi, 9.25 MBq; PerkinElmer) was added, and the samples were incubated for 1 h at 37 °C. The reactions were stopped with ice-cold water and filtered using 0.45 μm nitrocellulose-Millipore filters. Filters were washed three times and 3 mL of scintillation were added to each sample. Samples were vortexed and the read on a β-counter.

ASSOCIATED CONTENT

Supporting Information. Images of immunocytochemistry using an anti-tubulin antibody and metabolism procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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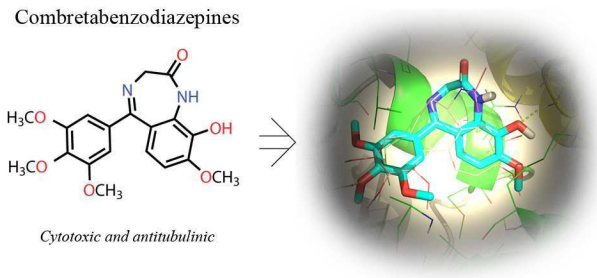
Table of Contents Graphic



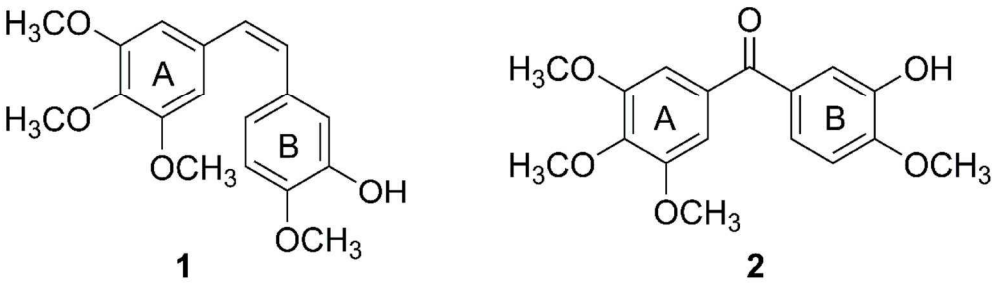


Figure 1. Combretastatin A-4 (1) and phenstatin (2).
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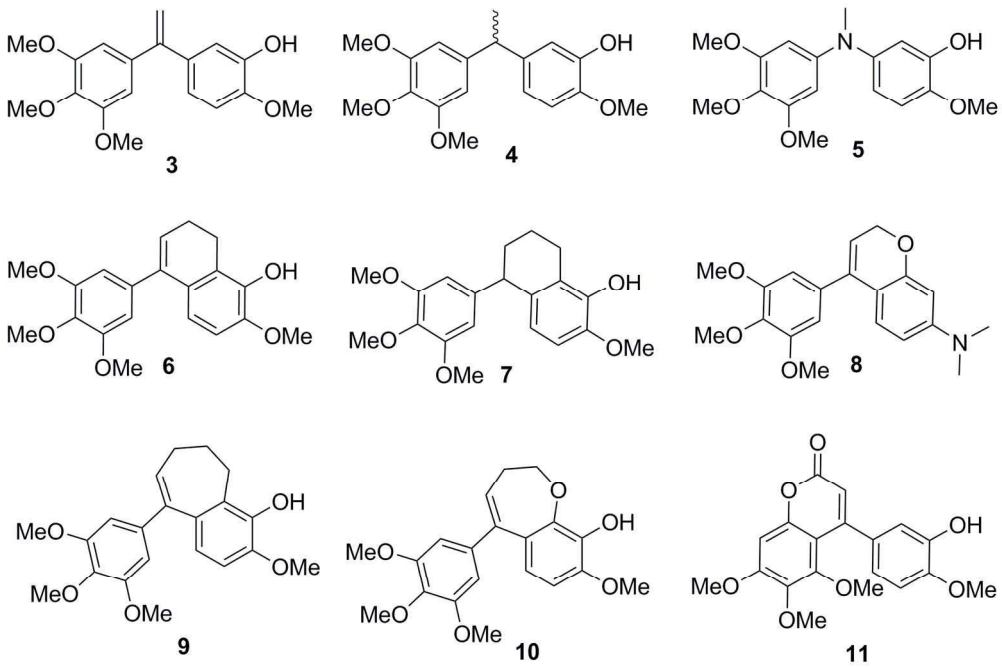

Figure 2. Analogues of phenstatin endowed with strong cytotoxic activity.
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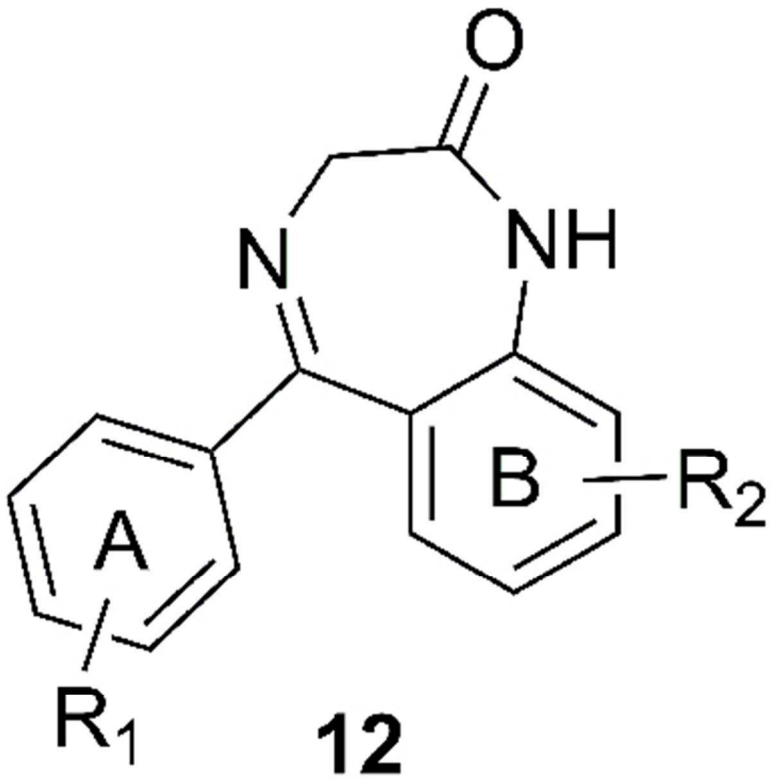

Figure 3. The combretabenzodiazepine scaffold.
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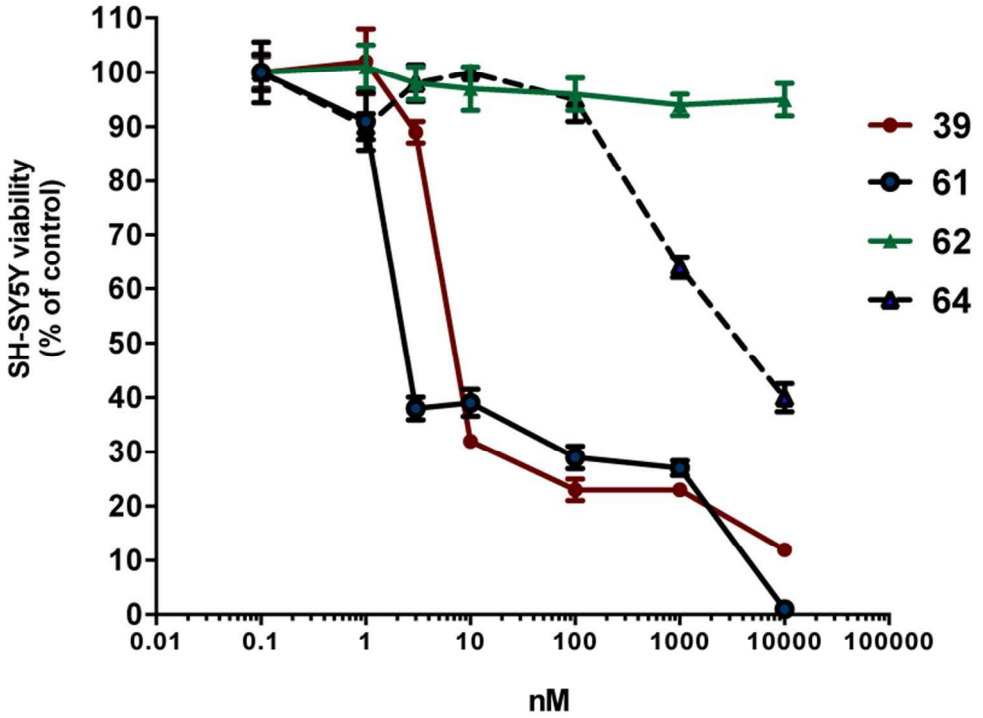

Figure 4. Cytotoxicity in SH-SY5Y cells of the synthesized compounds. (A) Values in the second column represent the % of viable cells (determined by MTT assay) after 48 hours of treatment with 1 μM of the indicated compounds. Values in the third column represent the calculated IC50 values (using Kaleidagraph software). Values in last column represent the IC50 value calculated for inhibition of tubulin polymerization in vitro. n.d.: not determined since full cytotoxicity was not reached at concentration at 1 μM and up to 1 μM. Values are the mean ± SD of 8-12 determinations from 3 different experiments. (B) Concentration response curves for cytotoxicity (MTT assay) in SH-SY5Y treated for 48 hours with compounds 39, 61, 62 and 64. Values are the mean ± SD of 8 determinations from 2 different experiments. n.d.: not determined.
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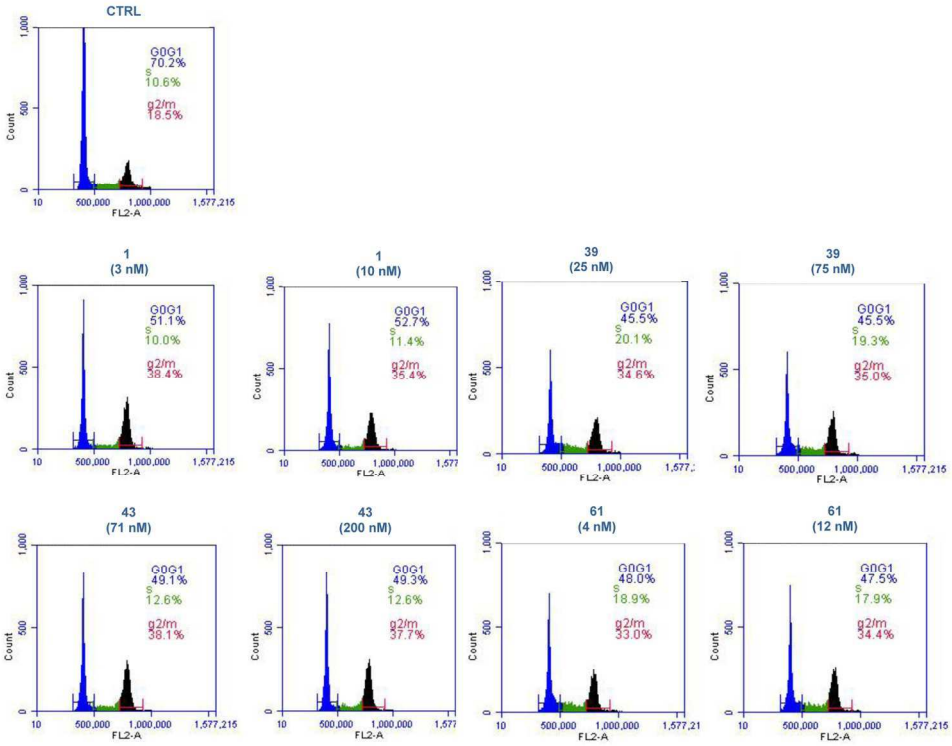


Figure 5. Combretastatin A-4 and the most potent compounds synthesized induce G2/M arrest. Cell cycle analysis (using BD Accuri C6 flow cytometry) of SH-SY5Y cells treated for 16 hours with vehicle (CTRL) or with the indicated compounds at the IC50 or 3 x IC50 values. The Y-axis represents the cell number, and the X-axis represents propidium iodide (PI) fluorescence on a linear scale. FL2-A: PI staining. Data are representative of 3 different experiments.
162x125mm (300 x 300 DPI)

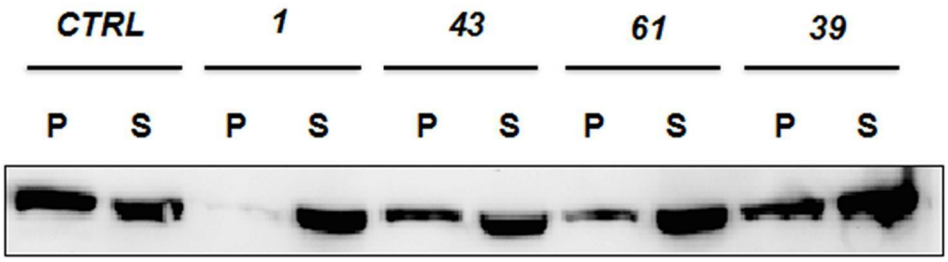


Figure 6. Combretastatin A-4 and compounds 43, 61 and 39 synthesized affect tubulin polymerization. Western blot of α-tubulin extracted in the presence of paclitaxel from SH-SY5Y cells treated with vehicle (CTRL), combretastatin A-4 (Comb) or the indicated compounds at 3 X IC50 value for 16 hours. Results are representative of 3 separate experiments. P = pelletable fraction, S = soluble fraction.
80x25mm (300 x 300 DPI)

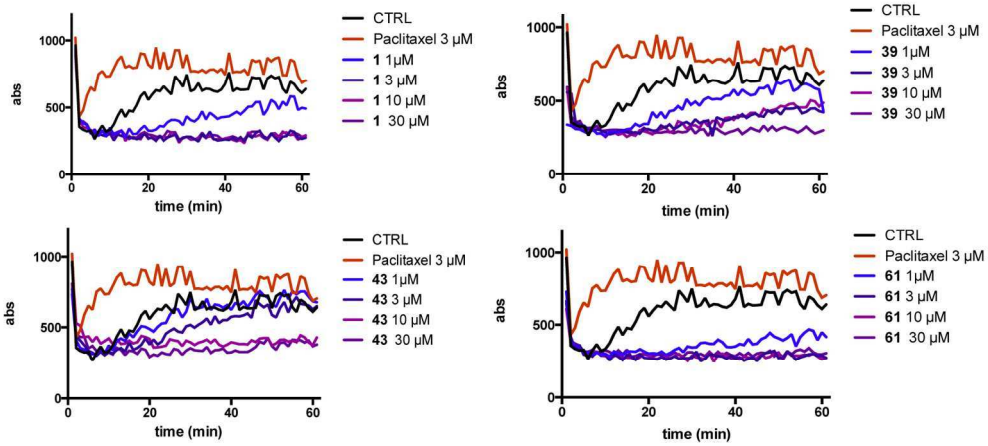


Figure 7. Representative traces of the tubulin in vitro polymerization assay using a commercial kit (see materials and methods). Experiments depicted are from a single 96-well plate and therefore the control and paclitaxel traces are identical for all four panels are presented each time for comparison.
150x70mm (300 x 300 DPI)

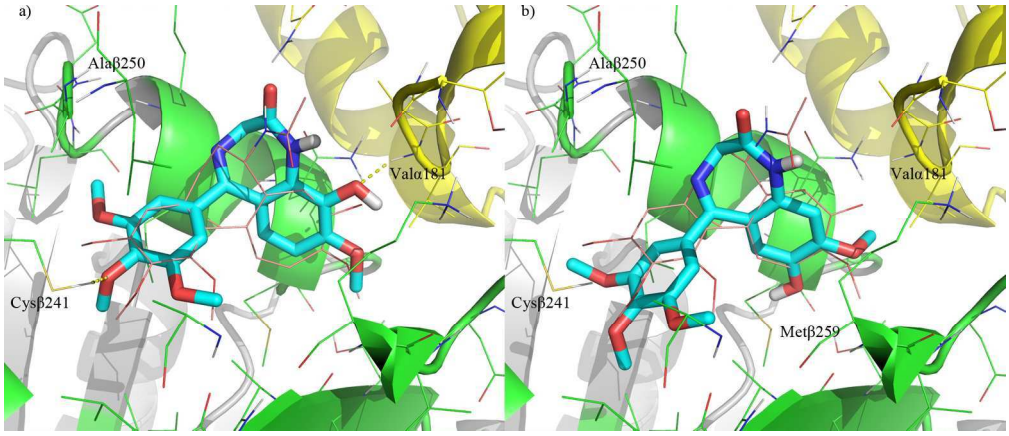
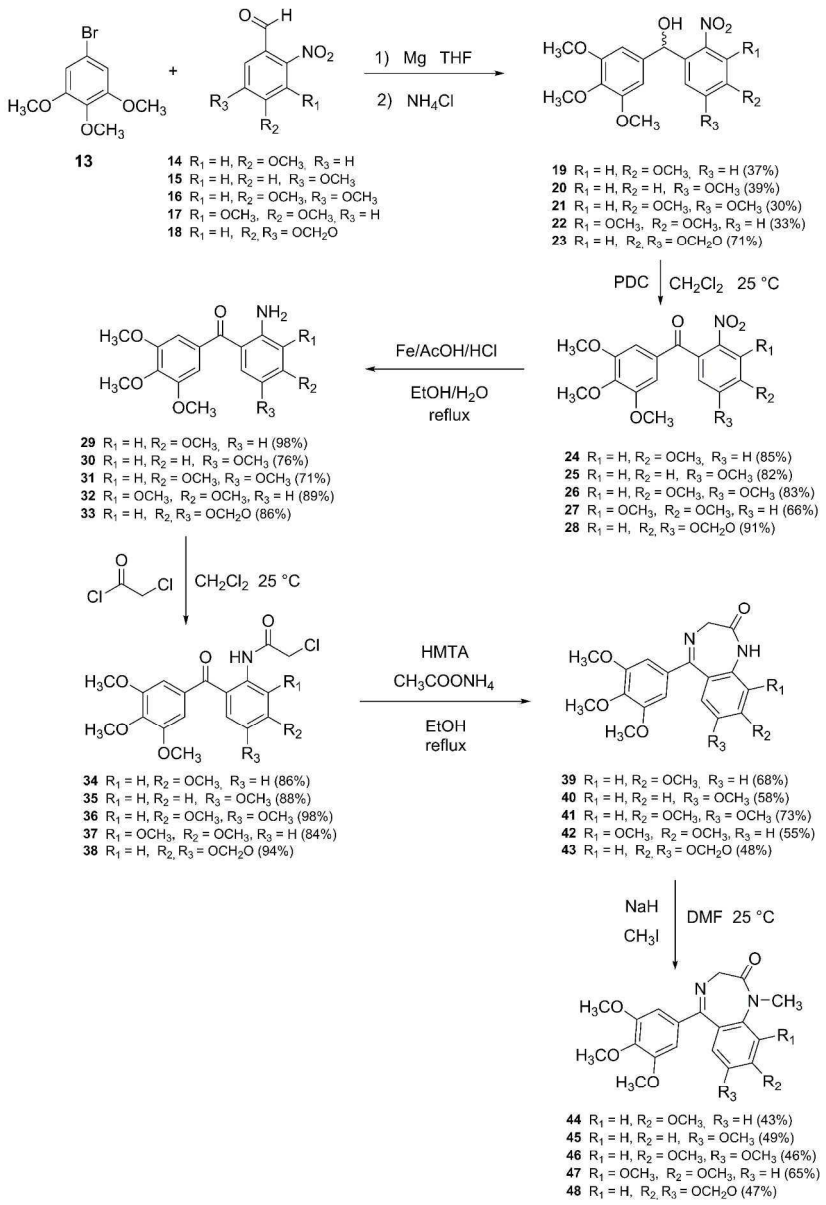
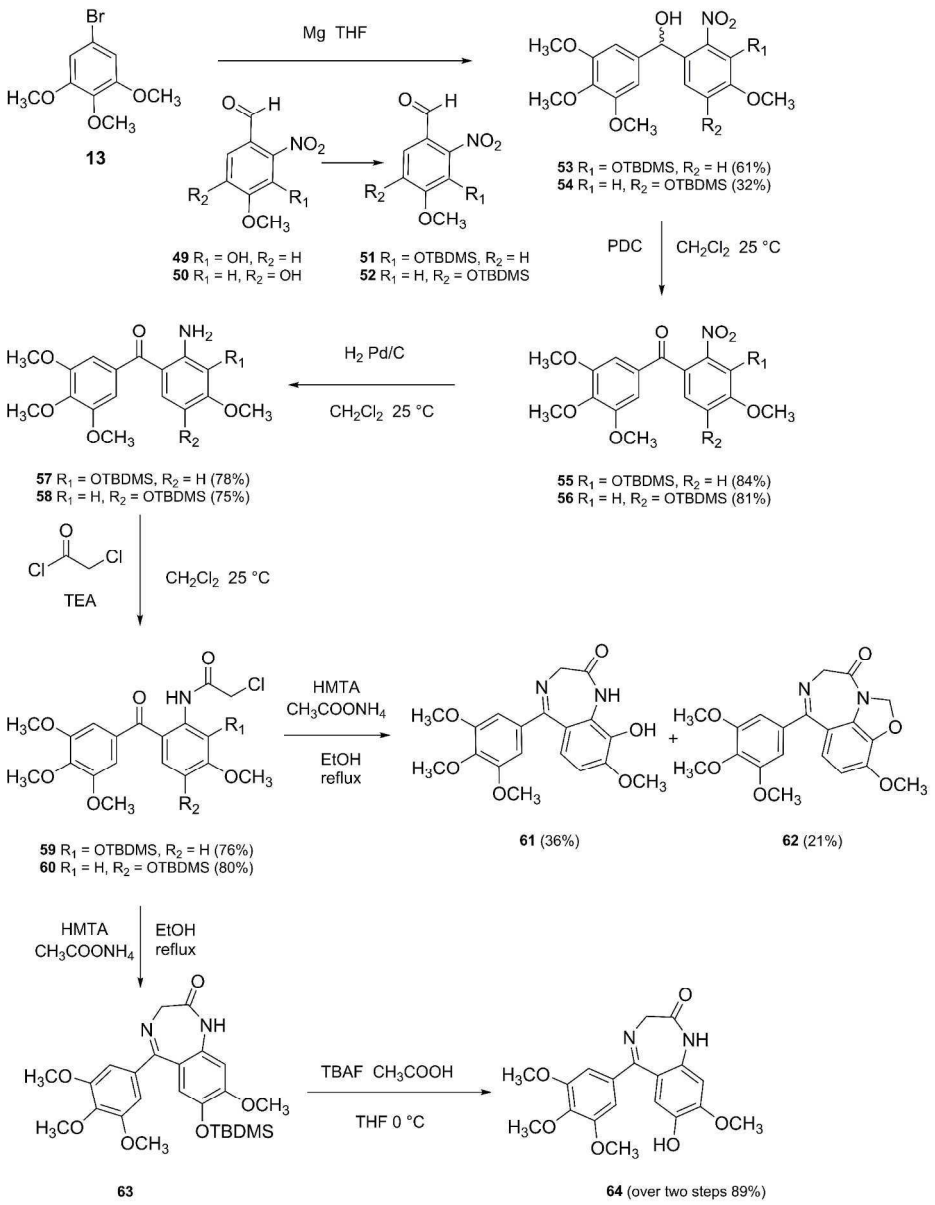


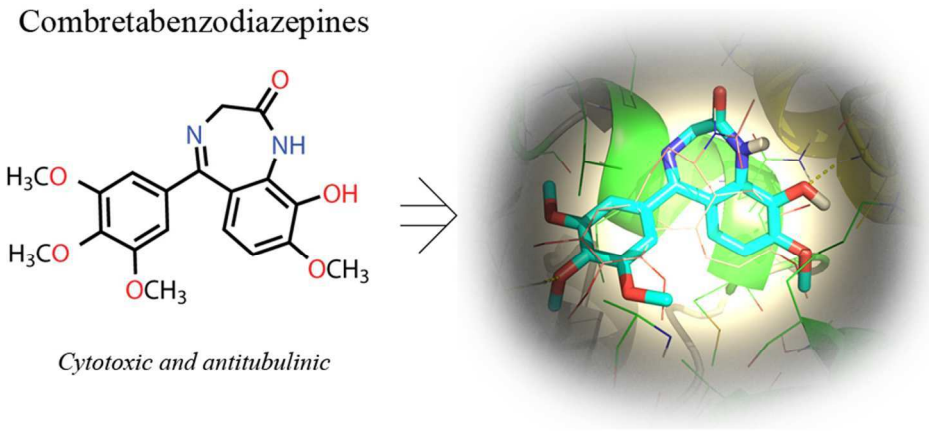
Figure 8. Superimposition of the docked conformation of compounds 61 (a) and 64 (b) as cyan stick models on top of the X-ray structure of DAMA-colchicine (pink wire model). The backbone of tubulin is shown as ribbon representation (α-tubulin: yellow, β-tubulin: green). The amino acids of tubulin within 4.0 Å from colchicine are shown as wire models. Hydrogen bonds (distance < 3 Å) are shown as dotted yellow lines.
170x72mm (300 x 300 DPI)



Scheme 1. Scheme for the synthesis of combretabenzodiazepines 39-48.
172x251mm (300 x 300 DPI)



Scheme 2. Scheme for the synthesis of combretabenzodiazepines 61 and 64.
177x229mm (300 x 300 DPI)



Graphical abstract
85x39mm (300 x 300 DPI)