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# Synthesis and biological evaluation of phenstatin metabolites

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ABSTRACT

Previous investigations on the incubation of phenstatin with rat and human microsomal fractions revealed the formation of nine main metabolites. The structures of eight of these metabolites have been now confirmed by synthesis and their biological properties have been reported. Eaton's reagent was utilized as a convenient condensing agent, allowing, among others, a simple multigram scale preparation of phenstatin. Synthesized metabolites and related compounds were evaluated for their antiproliferative activity in the NCI-60 cancer cell line panel, and for their effect on microtubule assembly. Metabolite **23** (2′-methoxyphenstatin) exhibited the most potent in vitro cytotoxic activity: inhibition of the growth of K-562, NCI-H322M, NCI-H522, KM12, M14, MDA-MB-435, NCI/ADR-RES, and HS 578T cell lines with $GI_{50}$ values <10 nM. It also showed more significant tubulin polymerization inhibitory activity than parent phenstatin (**3**) ($IC_{50}$ = 3.2 μM vs 15.0 μM) and induced G2/M arrest in murine leukemia DA1-3b cells. The identification of this active metabolite led to the design and synthesis of analogs with potent in vitro cytotoxicity and inhibition of microtubule assembly.



## 1. Introduction

Tubulin is a heterodimer of closely related and tightly linked globular α- and β-tubulin proteins, capable to polymerize in hollow tubes called microtubules. These cytoskeletal structures are involved in many cellular functions.[1] One of their key roles is in the mitotic spindle, intimately involved in cell division, which bridges between the chromosomes in the center and the centrosomes at opposite poles of the cell, and leading to, at the metaphase/anaphase transition, the separation of the sister chromatids and then their segregation into the daughter cells. Their importance in cell division makes microtubules an important target for anticancer drugs.[2] Tubulin-binding agents interfere with the dynamic instability of microtubules and thereby arrest mitotic cells in the G2/M phases of the cell division cycle, causing mitotic catastrophy and finally apoptotic cell death.[3] Colchicine and its analogs predominantly bind to a high affinity site, called colchicine binding site, located at the interface between α- and β-tubulin, facing the lumen of the microtubule.

Combretastatin A-4 (CA-4) (**1**) (Fig. 1), extracted from a South African tree *Combretum caffrum* by Pettit et al.,[4] depicts high affinity for the colchicine binding site, and is one of the most potent antimitotic agents,[5] also active towards multi-drug-resistant (MDR) cell lines.[6] Research on combretastatin A-4 in order to improve its water solubility and in vivo activity[7] led to its water-soluble prodrug combretastatin A-4 phosphate (CA-4P, Zybrestat) (**2**) (Fig. 1), presently in phase II human cancer clinical trials[8] with phase III trials under way. CA-4P acts as a vascular disrupting agent (VDA)[9]: its mechanism of action is believed to be related to tubulin-binding properties that result in rapid tumor endothelial cell damage, neovascular shutdown and subsequent tumor cell death.[10] In contrast to colchicine, the anti-vascular effects of CA-4 in vivo are apparent well below the maximum tolerated dose, offering a wide therapeutic window. CA-4P does not induce the common side effects of existing chemotherapies such as alopecia and bone marrow toxicity. However,


**1** R = H Combretastatin A-4 (CA-4)
**2** R = $PO_3Na_2$ Combretastatin A-4P (CA-4P)
**3** R = H Phenstatin
**4** R = $PO_3Na_2$ Phenstatin-P

**Figure 1.** Structure of combretastatin and phenstatin derivatives.

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cardiovascular toxicity and neurotoxicity are dose limiting for this compound.[11] These significant side effects currently represent the main obstacles to broad clinical application of CA-4P. For this reason, it is necessary to develop other CA-4 structurally related compounds with more specificity for tumor endothelial cells than normal endothelial cells to avoid cardiac toxicity from endothelial damage.[12]

A closely related compound is phenstatin (**3**) (Fig. 1), which resulted from a SAR-study on combretastatin A-4 and exhibited potent inhibition of cancer cell growth.[13,14] The water-soluble phenstatin phosphate prodrug (**4**) and the parent phenstatin (**3**) (Fig. 1) were essentially indistinguishable and very similar to the combretastatin A-4 phosphate prodrug (**2**) in terms of both potency and differential cytotoxicity.[14] The biotransformation pathways of CA-4 (Fig. 2),[15] and the ones of an indole analog of phenstatin (BPR0L075, **13**) (Fig. 3)[16] have been published but, curiously, nothing was known about phenstatin bioconversion before our study, and this compound has not been advancing in human clinical trials. These considerations, linked to our interest in the medicinal chemistry of CA-4 and phenstatin derivatives, led us to study the metabolic fate of **3** in rat and in human microsomal preparations.[17]

It was observed that combretastatin A-4 (CA-4, **1**), in the presence of rat or human microsomes, was metabolized into compounds **5**–**12**: one of the main transformations concerned the isomerization of the double bound leading to the *trans*-stilbenes **5**, **7**, **9** and **11**. However, *O-demethylases* yield phenols **5**, **6**, **7** and **8**, while *aromatic hydroxylases* give *para* diphenols **9** and **10**, probably transformed by *oxidases* in quinones **11** and **12** (Fig. 2).[15] In the case of ketone **13**, *O-demethylases* yield phenols **15**, **17** and **19** and *aromatic hydroxylases* generate compounds **14**, **16** and **18** (Fig. 3).[16]

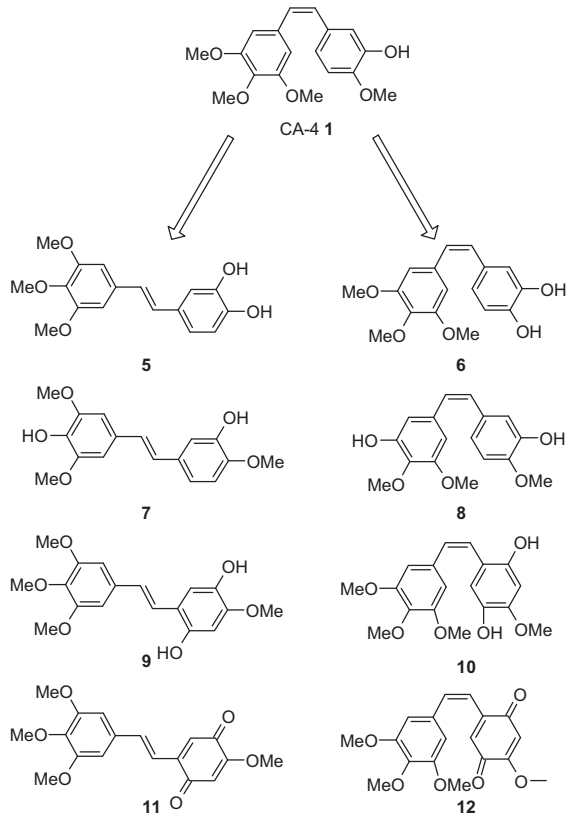



**Figure 2.** Structure of combretastatin A-4 (**1**) and its main metabolites described in the literature; the metabolite **8** observed by MS–MS was not synthesized.[15]

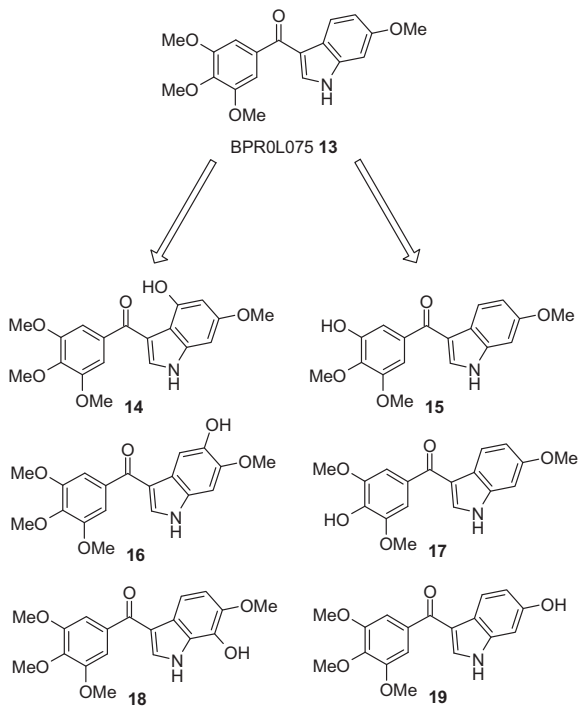



**Figure 3.** Structure of 6-methoxy-3-(3′,4′,5′-trimethoxybenzoyl)-1*H*-indole (**13**, BPR0L075) and its main metabolites described in the literature.[16]

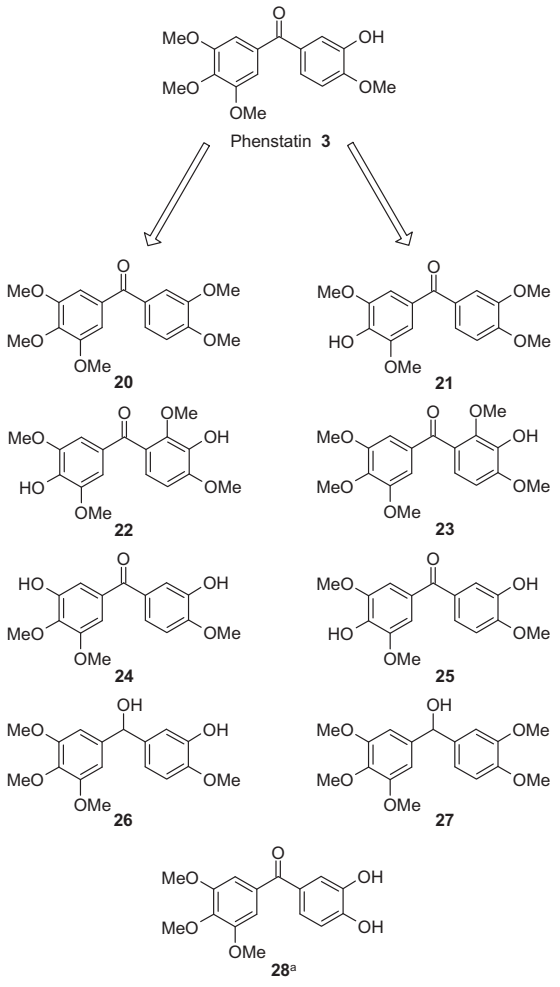



**Figure 4.** Structure of phenstatin (**3**) and its main metabolites **20**–**27**. [a]The predictable metabolite **28** was found not to be a product of phenstatin biotransformation.[17]

We have examined the metabolic fate of CA-4 (**1**) (Fig. 2),[15] the one of the aromatic ketone BPR0L075 (**13**) (Fig. 3)[16] and the molecular weight of major metabolites observed during the MS-MS study of phenstatin (**3**) biotransformation in the same conditions. This study led us to consider *O*-methylations (**20**, **21** and **27**), aromatic hydroxylations followed by *O*-methylations (**22**, **23**), ether demethylations (**21**, **22**, **24**, **25**, **28**), and ketone reductions (**26**, **27**) as possible metabolic processes for phenstatin (**3**) (Fig. 4). However, MS–MS study excluded the formation of quinones as for CA-4. It was later observed that compounds **20**–**27** were indeed metabolites of phenstatin, and that catechol **28**, which was included by comparison with metabolization of CA-4, was not a product of this biotransformation.

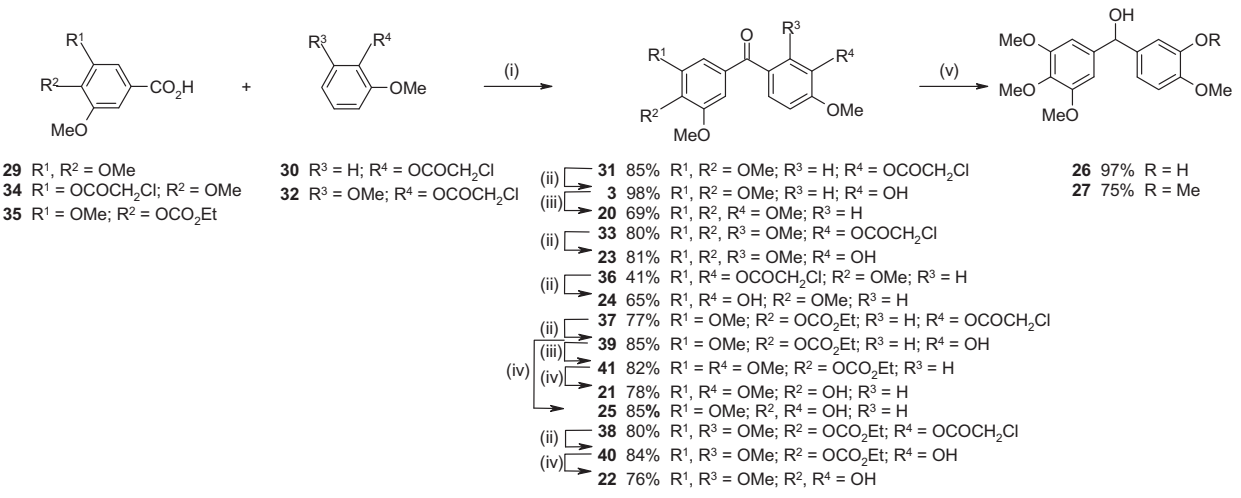
We report here the synthesis of several metabolites of phenstatin (**3**) and their biological evaluation.[17] Only three of these compounds had been already described. Using Eaton's reagent as a condensing medium, allowed the preparation of this metabolite in an easier and a more convenient manner. Inter alia, a multigram synthesis of phenstatin was described. In addition, on the basis of the insight gained about the major metabolic pathways of phenstatin,[17] we have determined metabolite **23**, which has globally an improved in vitro efficacy on tubulin polymerization and on cancer cell lines than that of parent compound **3**. Identification of this active metabolite of **3** led us to design and synthesize analogs which exhibit potent in vitro cytotoxicity.

## 2. Chemistry

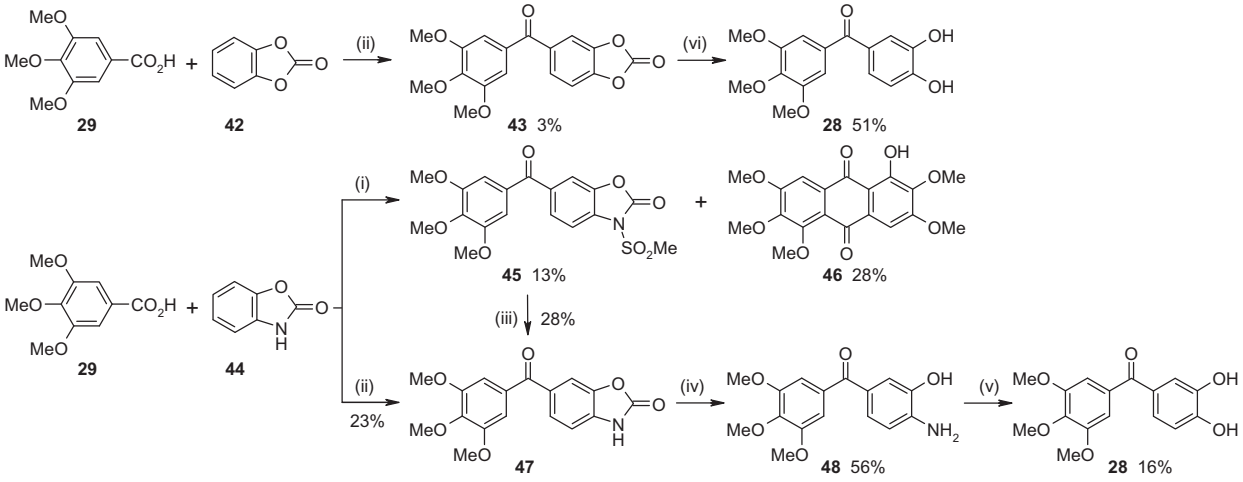
The general synthetic procedure for the target compound synthesis is illustrated in Scheme 1. Literature described that Jacobsen oxidation of CA-4 led to phenstatin (**3**).[14] This compound can also be obtained by Friedel–Crafts reaction from a protected methoxyphenol and trimethoxybenzoic acid in PPA.[18] However, this is a highly viscous medium which is difficult to stir and hydrolyze at the end of the reaction; then, we decided to use Eaton's reagent ($MeSO_3H/P_2O_5$).[19] Thus, phenstatin (**3**) was obtained by reacting trimethoxybenzoic acid **29** with protected phenol **30** in Eaton's reagent at 60 °C to give ketone **31** (85%), followed by removal of the chloroacetyl protection group by using sodium acetate in methanol (98%) (litt. 91%[18]). The same sequence applied to the protected phenol **32** led to ketone **33** (80%), and then to 2′-*O*-methylphenstatin **23** (81%). The reduced phenstatin **26** was previously obtained from Grignard reaction of the corresponding aldehyde,[20] however $NaBH_4$ reduction of ketones **3** and **20** provided a simpler route to benzhydrols **26** (97%) and **27**[21] (75%). Friedel–Crafts reaction, promoted by Eaton's reagent, of the protected dimethoxyacids **34** or **35** with the protected phenols **30** or **32** led, respectively, to compounds **36** (41%), **37** (77%) and **38** (80%). The sodium acetate removing of the chloroacetyl protective group of these ketophenols yielded metabolite **24** (65%), and compounds **39** (85%) and **40** (84%). Dimethylsulfate methylation of phenstatin (**3**) gave known pentamethoxylated metabolite **20** (69%). Synthesis of this ketone was also described from tin tetrachloride mediated reaction of trimethoxybenzoyl chloride with veratrole (84%).[22] The same methylation reaction applied to phenol **39** yielded ketone **41** (82%), and then ethanolamine cleavage[23] of the remaining carbonate protecting group of intermediates **39**, **40** and **41** furnished metabolites **25** (85%), **22**[24] (76%) and **21** (78%), respectively. Alternatively, cleavage of the central methoxy group of phenstatin (**3**) in order to give phenol **25** was realized in 52% by refluxing **3** with 3 equiv of aluminum chloride in dichloromethane.[25] Noteworthy, no such transformation was obtained by using $BCl_3$.

During the syntheses described in Scheme 1, we observed that chloroacetyl and ethyl carbonate protections of phenol groups were rather stable when heated in Eaton's reagent. However, when the acid **29** was opposed to catechol protected in these ways, many products were observed by TLC of crude reaction media, and only monoesterified catechol[26] (see Supplementary information section) was isolated in 36–63% yield. In a similar procedure, cyclic carbonate **42** was reacted with trimethoxybenzoic acid **29** in Eaton's reagent. Thus, the benzophenone **43** was obtained in very low (3%) yield; deprotection of which gave 51% of potential metabolite **28** (Scheme 2).

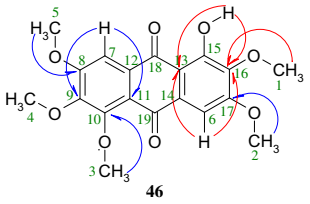
Due to disappointing results obtained in these attempts to prepare ketone **28**, another approach was considered: an amino group was used as a latent phenol function. The Friedel–Crafts acylation of benzoxazolones with aromatic carboxylic acids in PPA is known.[27] However, by heating at 60 °C a homogeneous mixture of acid **29** and benzoxazolone **44** in Eaton's reagent, the sulfonated benzoxazolone **45** was isolated in low (13%) yield, accompanied by 28% of the pentamethyl rufigallic acid **46**. Obtention of the benzoquinone **46** with milder condition reactions is of interest since the formation of such quinones has only been described with sulfuric acid at very high temperatures.[28] In this compound, the position of the hydroxyl group was deduced from $^1H$ and $^{13}C$ spectra, and from HMBC correlations shown in Figure 5. Removing of the methanesulfonyl group in **45** was performed in presence of *n*-tetrabutylammonium fluoride in THF at room temperature for 24 h,[29] leading




**Scheme 1.** *Reagents and conditions:* (i) Eaton's reagent, 60 °C; (ii) $AcONa{\cdot}3H_2O$, MeOH, reflux; (iii) $Me_2SO_4$, $K_2CO_3$, acetone, reflux; (iv) ethanolamine, EtOH 95%, rt; (v) $NaBH_4$ 2.2 equiv, $EtOH/H_2O$, rt.




**Scheme 2.** *Reagents and conditions:* (i) Eaton's reagent 4 equiv, 60 °C, 30 h; (ii) Eaton's reagent 4 equiv (added in small portions), 50 °C, 24 h; (iii) *n*-$(C_4H_9)_4N^+F^-\cdot 3H_2O$ 3 equiv, THF, rt, 24 h; (iv) NaOH 0.38 N 5 equiv, 80 °C, 10 h; (v) $NaIO_4$, HCl, AcOH, rt, 14 h; (vi) ethanolamine, EtOH 95%, rt, 5 h.




| C δ (ppm) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 61.4 | 56.5 | 60.9 | 61.6 | 56.4 | 106.1 | 103.9 | **157.5** | **148.5** | **155.0** | **121.1** | 130.7 | **111.5** | 130.6 | 156.0 | **140.6** | **158.4** | 186.7 | 180.3 |
| **H δ (ppm)** | 4.03 | 4.00 | 4.01 | 4.05 | 3.99 | 7.68 | 7.42 | **7.68** | **7.68** | **7.68** | **7.68** | 7.68 | **12.64** | 7.42 | 12.64 | **12.64** | **7.42** | 7.68 | 7.42 |

**Figure 5.** Main $^1$H HMBC NMR correlations for the anthraquinone **46** (selected correlations in blue, informative correlations in red).

to 28% of ketone **47** (Scheme 2). It was thought that, because of the low reactivity of benzoxazolones, self-condensation of trimethoxybenzoic acid firstly occurred. Consequently, reaction conditions were modified, and acid **29** was slowly added by parts to a solution of benzoxazolone **44** in Eaton's reagent at 50 °C: benzophenone **47** was then isolated in 23% yield, accompanied by a low amount of an unknown red by-product.[30] Opening of the oxazolone ring was obtained by refluxing compound **47** in sodium hydroxide, and benzophenone **48** was then isolated in 56% yield. Sodium periodate oxidation[31] of **48** was performed, leading to potential metabolite **28** in low 16% yield. A sufficient quantity of this compound **28** was thus obtained allowing the comparison of its structure to those of known metabolites of phenstatin, confirming that this compound is not such a metabolite. So, no more attempts were realized in order to improve the yield of these syntheses.

The in vitro anticancer properties obtained for metabolite **23** prompted us to synthesize some analogs to be evaluated in tubulin polymerization or cytotoxicity. Friedel–Crafts reaction promoted by Eaton's reagent of trimethoxybenzoic acid **29** with the protected aniline **52** led to ketone **53** (41%), and then hydrochloric acid cleavage of the acetyl protecting group of intermediate **53** yielded amino analog **54** of phenol **23** (68%). The same sequence of Friedel–Crafts condensation between anisic acid **55** and aromatic ester **32** led to 53% of protected compound **56**. Then, the sodium acetate removing of the chloroacetyl protective group of **56** yielded ketone **57** (87%), an analog of **23**, with only one methoxy group on cycle A.

## 3. Biological evaluation

The three metabolites **21**, **22**, and **23**, the three intermediate compounds **45**, **46**, and **47**, and the three 2′-methoxybenzophenones **53**, **54**, and **57** were selected by NCI for screening against 60 human tumor cell lines. The representative results are summarized in Tables 1 and 2. Anthraquinone **46** and benzoxazolone **47** generally showed modest cell growth inhibitory activity at $10^{-5}$ M concentration (only 60% inhibition on OVCAR-4 for compound **46** and 34% inhibition on K-562 for **47**) (Table 1). The *O*-demethylated metabolites **21** and **22** showed decreased cytotoxicity compared to parent phenstatin (**3**). In preliminary screening, **23** showed significant cytotoxicity against melanoma cell lines (Fig. 6). In addition, it also exhibited more potent anti-proliferative activity against several types of cancer cells, such as DU145, SF-295, and MCF7 cell lines, than phenstatin (Table 1). Interestingly, the results obtained from NCI revealed that intermediate **45** had also a moderate cytotoxic effect with $IC_{50}$ values between 0.29 and 22.3 μM (Table 1).

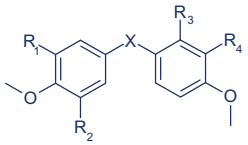
In order to examine whether these metabolite and intermediate compounds interact with tubulin and inhibit in vitro tubulin polymerization, we performed a turbidimetric tubulin polymerization assay. The results are shown in Table 1. Metabolite **23** exhibited the most potent inhibitory effect and its 50% tubulin polymerization inhibition was as low as 3.2 μM, being more active than phenstatin itself (15.0 μM) in our testing conditions.[32]

**Table 1**
Results of the in vitro human cancer cell growth inhibition[a,b] and tubulin polymerization inhibition[c] for metabolites **21**, **22** and **23** and intermediates **45**, **46** and **47**

| Cell type | Compound | Phenstatin (**3**) | **23** | **22** | **21** | **45** | **46** | **47** |
|---|---|---|---|---|---|---|---|---|
| | $IC_{50}$ (μM) tubulin[c] | 15.0 ± 0.2[d] | 3.2 ± 0.2[d] | 14.9 ± 0.9[d] | >100[d] | >100[d] | >100[d] | >100[d] |
| | Cell line | $GI_{50}$ ($10^{-a}$ M)[a,b] | | | | | Cell growth inhibition, % ($10^{-5}$ M) | |
| Leukemia | CCRF-CEM | – | 2.88E−8 | 8.96E−7 | 4.05E−6 | 3.35E−6 | 0 | 0 |
| | HL-60(TB) | – | 2.35E−8 | 3.64E−7 | 1.25E−6 | 2.52E−6 | 6.14 | 0 |
| | K-562 | – | <1.00E−8 | 1.53E−7 | 6.66E−7 | 5.88E−7 | 0 | 33.5 |
| Non-small cell lung cancer | A549/ATCC | – | 4.13E−8 | 3.47E−6 | 5.91E−5 | 4.68E−6 | 26.12 | 0 |
| | EKVX | – | 6.11E−7 | 4.76E−6 | >1.00E−4 | 4.58E−6 | 13.48 | 13.74 |
| | NCI-H322M | – | <1.00E−8 | 4.99E−6 | 3.44E−6 | 5.38E−6 | 0 | 0 |
| | NCI-H460 | 5.70E−9[e] | 5.07E−8 | 1.97E−6 | 3.44E−6 | 2.64E−6 | 9.33 | 0 |
| | NCI-H522 | – | <1.00E−8 | 2.90E−7 | 3.64E−6 | 2.34E−6 | 29.5 | 0 |
| Colon cancer | COLO 205 | 4.86E−6[f] | 6.20E−6 | 4.83E−6 | 1.42E−5 | 2.50E−6 | 0 | 0 |
| | HCC-2998 | – | 2.11E−7 | 2.89E−6 | N.D.[g] | 6.75E−6 | 0 | 0 |
| | HCT-116 | – | 3.20E−8 | 1.41E−6 | 2.50E−6 | 2.31E−6 | 11.78 | 0 |
| | HCT-15 | – | 1.06E−8 | 6.11E−7 | 2.07E−6 | 2.00E−6 | 0 | 0 |
| | KM12 | – | <1.00E−8 | 4.09E−7 | 1.09E−6 | 1.15E−6 | 0 | 0 |
| Melanoma | M14 | – | <1.00E−8 | 4.55E−7 | 9.02E−7 | 1.36E−6 | 0 | 0 |
| | MDA-MB-435 | – | <1.00E−8 | 2.82E−7 | 2.73E−7 | 2.89E−7 | 16.85 | 11.24 |
| | SK-MEL-2 | – | 2.66E−8 | >1.00E−4 | 5.29E−6 | 5.11E−6 | 0 | 0 |
| | SK-MEL-28 | – | 9.46E−6 | >1.00E−4 | 9.46E−6 | 1.13E−5 | 14.98 | 0 |
| | UACC-62 | – | 2.73E−8 | 5.01E−6 | 8.41E−6 | 5.30E−6 | 6.13 | 0 |
| Ovarian cancer | OVCAR-3 | 2.30E−9[e] | 2.22E−8 | 3.63E−7 | 2.33E−6 | 1.56E−6 | 0 | 0 |
| | OVCAR-4 | – | 7.30E−8 | >1.00E−4 | >1.00E−4 | 2.23E−5 | 60.25 | 0 |
| | OVCAR-5 | – | 6.51E−7 | 4.92E−6 | 1.29E−5 | 5.77E−6 | 7.52 | 0 |
| | NCI/ADR-RES | – | <1.00E−8 | 5.15E−7 | 8.54E−7 | 2.03E−6 | 20.26 | 0 |
| | SK-OV-3 | – | 8.96E−8 | 9.12E−7 | 2.20E−6 | 2.84E−6 | 9.78 | 0 |
| Renal cancer | A498 | 3.80E−7[e] | 2.72E−6 | 1.70E−6 | 3.36E−6 | 3.11E−6 | N.D.[g] | 17.01 |
| | CAKI-1 | – | 1.20E−7 | 1.90E−6 | 3.01E−6 | 2.64E−6 | 7.07 | 0 |
| | RXF 393 | – | 1.59E−8 | 7.55E−7 | 1.88E−6 | 4.63E−6 | 2.66 | 0 |
| | SN12C | – | 4.69E−8 | 1.37E−5 | 6.68E−6 | 7.79E−6 | 8.73 | 0 |
| | UO-31 | – | 4.89E−8 | 1.66E−5 | 2.16E−6 | 6.81E−6 | 0 | 0 |
| Prostate cancer | DU145 | 3.40E−8[f] | 2.09E−8 | 8.18E−7 | 4.49E−6 | 3.04E−6 | 7.83 | 0 |
| Central nervous system cancer | SF-268 | – | 2.11E−8 | 4.25E−6 | | 7.95E−6 | 22.19 | 0 |
| | SF-295 | 5.20E−8[e] | 1.99E−8 | 1.40E−6 | 2.08E−6 | 1.61E−6 | 37.85 | 0 |
| | SF-539 | – | 1.51E−8 | 6.11E−7 | 2.96E−6 | 2.36E−6 | 16.23 | 0 |
| Breast cancer | MCF7 | 4.37E−7[f]/3.40E−8[h] | 2.64E−8 | 1.01E−6 | 4.33E−7 | 4.43E−6 | 26.69 | 0 |
| | MDA-MB-231/ATCC | – | 2.46E−8 | 2.16E−6 | 4.79E−6 | 4.85E−6 | 21.33 | 0 |
| | HS 578T | – | <1.00E−8 | 3.58E−7 | 4.92E−6 | 3.16E−6 | 16.89 | 0 |
| | MDA-MB-468 | – | 2.49E−7 | 8.64E−7 | 1.97E−6 | 2.82E−6 | 22.4 | 0 |

[a] Data obtained from NCI's in vitro 60 cell 5-dose screen.
[b] $GI_{50}$ is the molar concentration of synthetic compound causing 50% growth inhibition of tumor cells.
[c] See Ref.[32] and Supplementary information.
[d] Data obtained from Ref.[16]
[e] Data obtained from Ref.[14]
[f] Data obtained from Ref.[13]
[g] Not determined.
[h] Data obtained from Ref.[33]

**Table 2**
Tubulin polymerization inhibitory activity and cytotoxicity for compounds **53**, **54**, **57** references **3** (phenstatin) and **1** (CA-4), metabolite **23**, and known antitubulin agents **58**–**61**

| Compound |  | | | | | TPI $IC_{50}$ (μM) | In vitro cell growth inhibition, $GI_{50}$ (nM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $R_1$ | $R_2$ | X | $R_3$ | $R_4$ | | HL-60 | A-549 | HT-29 | MDA-MB-435 | MCF7 | SF-295 | OVCAR-3 | A498 | DU-145 |
| **23** | OMe | OMe | C=O | OMe | OH | 3.2[a] | 23.5 | 41.3 | 3460 | <10 | 26.4 | 19.9 | 22.2 | 2720 | 20.9 |
| **53** | OMe | OMe | C=O | OMe | $NHCOCH_3$ | >50[a] | 3530 | 4620 | 4330 | 344 | 9600 | 293 | 3300 | 85.5 | 292 |
| **54** | OMe | OMe | C=O | OMe | $NH_2$ | 10[a] | 33.1 | 39.0 | 32.3 | <10 | 10.6 | 19.9 | 20.2 | 22.1 | 27.4 |
| **57** | H | H | C=O | OMe | OH | >50[a] | N.D.[b] | N.D.[b] | N.D.[b] | N.D.[b] | N.D.[b] | N.D.[b] | N.D.[b] | N.D.[b] | N.D.[b] |
| Phenstatin (**3**) | OMe | OMe | C=O | H | OH | 15.0[a] | 31.0[c] | 290[c] | 1800[c] | | 437[d] | 163.3[e] | 7.2[e] | 1194[e] | 34[d] |
| CA-4 (**1**) | OMe | OMe | CH=CH | H | OH | 12.7[a] | 31.6[f] | 1200[f] | 631[f] | 31.6[f] | 39.8[f] | 63.1[f] | 39.8[f] | 50.1[f] | 2.5[g] |
| **58** | OMe | OMe | CH=CH | OH | OH | 1.9[h] | | | | | | | | | 511.5[g] |
| **59** | OMe | OMe | C=O | $NH_2$ | H | 0.4[i] | | | 42[j] | | 23[d] | | | | 36[d] |
| **60** | OMe | OMe | C=O | H | $NH_2$ | 1.5[f] | 30[f] | 57[f] | 33[i] | | | | | | |
| **61** | OMe | OMe | C=O | OH | OH | 0.8[g] | | | | | | 119.6[g] | | | 143.6[g] |

Compound **57** showed very modest cell growth inhibition at $10^{-5}$ M concentration (only 26.5% inhibition of renal cancer cell line UO-31 and 23% of HOP-92 (non-small cell lung cancer)).

[a] See Ref.[40] and Supplementary information.
[b] Not determined.
[c] Data obtained from Ref.[35]
[d] Data obtained from Ref.[36]
[e] Data obtained from Ref.[14]
[f] Data obtained from Ref.[37]
[g] Data obtained from Ref.[13]
[h] Data obtained from Ref.[38]
[i] Data obtained from Ref.[39]
[j] Data obtained from Ref.[40]

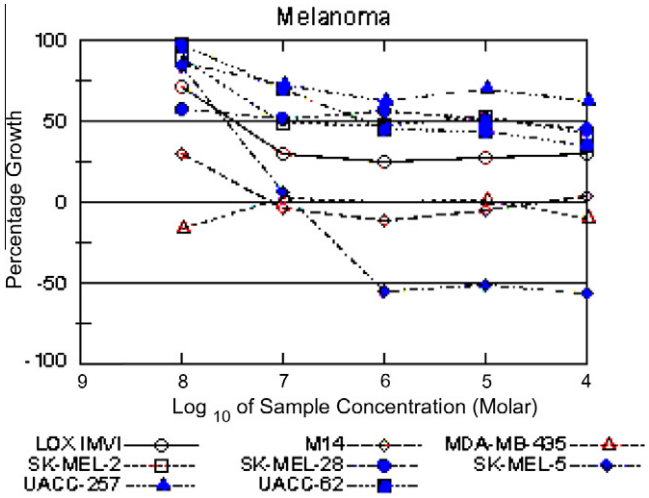



**Figure 6.** Dose–response curve of **23** against different melanoma cell lines.

Furthermore, cell cycle analysis of metabolite **23** was performed using flow cytometry (Fig. 7). We observed that compound **23** strongly induced G2/M arrest in murine leukemia DA1-3b[34] cells, and the effect was observed in a dose-independent manner after treatment for 24 h with increased amounts of this compound. About 25.3% of the cells were arrested in the G2/M phase while 47.2% of the cells were found to be in the G2/M phase in presence of 100 and 1 μM of metabolite **23**, respectively. These results confirmed that metabolite **23** is a potent antitubulin agent.

Because of the interesting biological activities of 2′-methoxyphenstatin (**23**), and the fact that 2′-hydroxyphenstatin (**61**; Table 2) is more active than phenstatin (**3**),[13] it was interesting to reexamine structure-activity relationships concerning the C2′ position of the phenstatin scaffold. Hence, we investigated the effect of introducing a different substituent at the C3′ position of benzophenone ring B on tubulin polymerization. Methylation of the 2′-hydroxyl group of **61** led to **23**, with an increase of six times of its cytotoxicity against SF-295 and DU-145; substitution of the 3′-hydroxyl group of **23** by an amino moiety (**54**) resulted in a slight decrease of the inhibitory activity on tubulin, and in a two-digit increasing of the cellular growth inhibition for HT-29 and for A498 cell lines. Except for MDA-MB-435 and DU-145, compound **54** presents an equal or better cytotoxicity as phenstatin or CA-4. As it could be predicted, acetylation of the 3-amino group of **54** lead to amide **53** resulting in lower antiproliferative potential, and abolishes the efficacy on microtubule assembly. The removal of methoxy groups at the C3 and C5 positions of metabolite **23** ring A (compound **57**) resulted in a complete loss of

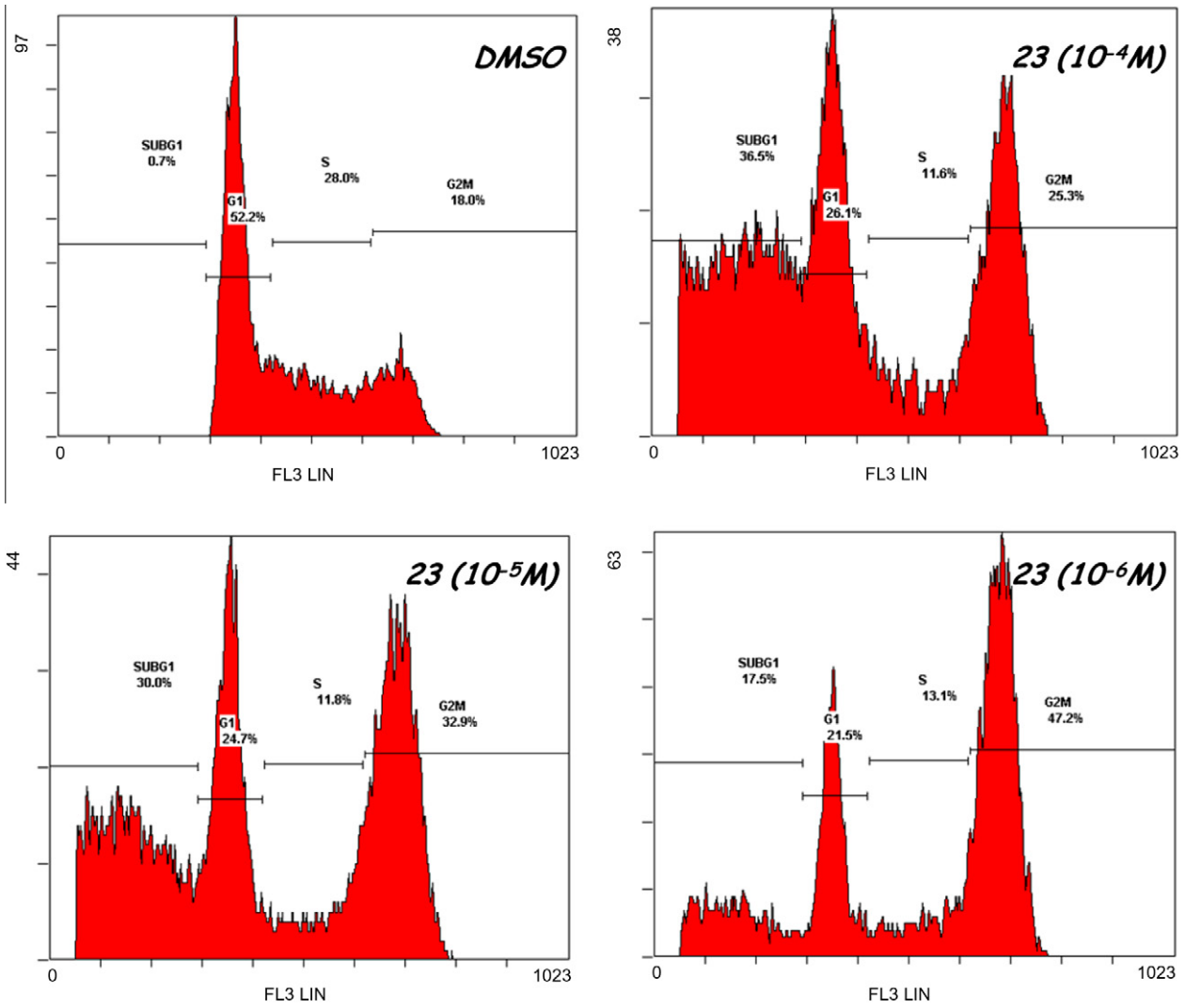



**Figure 7.** Effects of metabolite **23** on cell cycle progression of DA1-3b cells were determined by flow cytometry analysis. DA1-3b cells were treated with different concentrations of metabolite **23**. The percentage of cells in each cycle phase was indicated.

biological activity (Table 2). Thus, this study shows that introduction of a methoxy group in position 2 of the B-ring leads to compounds **23** and **54** with improved or similar growth inhibitory properties compared to known antitubulin agents as CA-4 (**1**), phenstatin (**3**), CA-1 (**58**), aminobenzophenones **59**–**60**, and hydroxyphenstatin **61** (Table 2).

## 4. Conclusions

Through Friedel–Crafts reaction using Eaton's reagent, we have developed a new synthesis of phenstatin which is currently the easiest method cited in the literature. Syntheses of eight metabolites of phenstatin, and some related compounds were described, and we showed that phenstatin exhibits a metabolic profile with significant differences compared to CA-4. It is noteworthy that metabolite **23** leads tumoral cells to arrest in G2/M, and has potent cytotoxicity against K-562, NCI-H322M, NCI-H522, HCT-15, KM-12, M14, MDA-MB-435, NCI/ADR-RES, HS 578T, and DU-145 cells in the nanomolar concentration range. The same compound showed a better inhibition of tubulin polymerization than the parent phenstatin (**3**). Further structure-activity relationship studies at the 2′ position on B ring of phenstatin provided analogs **53**, **54**, and **57**. When acetamido moiety was introduced in place of the hydroxy group onto the 3′ position of metabolite **23**, the resulting compound showed decreased potency relative to parent compound, while the amino derivative **54** proved to be the most potent derivative synthesized. Overall, the 2′-methoxybenzophenones exhibited interesting biological properties and have potential for further investigation as anticancer agents.

## 5. Experimental

### 5.1. Chemistry

#### 5.1.1. Materials and methods

Starting materials were commercially available. Melting points were measured on a Electrothermal® apparatus and are uncorrected. NMR spectra were acquired at 200 MHz for $^1$H NMR and 50 MHz for $^{13}$C NMR on a Varian Gemini 2000® spectrometer, or at 400 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR on a Varian 400 MHz Premium Shielded® spectrometer. Chemical shifts ($\delta$) are expressed in ppm relative to TMS as internal standard. Thin layer chromatographies were performed on Macherey Nagel silica gel plates with a fluorescent indicator and were visualized with UV-lamp at 254 and 366 nm. Column chromatographies were performed on silica gel (40–60 μm; Macherey-Nagel). Elemental analyses (C, H, N, S) of new compounds were determined by 'Service de Microanalyses', Faculté de Sciences Mirande, Université de Bourgogne, Dijon, France.

#### 5.1.2. General procedure A for Friedel–Crafts reactions in the presence of Eaton's reagent

Eaton's reagent was prepared from phosphorus pentoxide ($P_2O_5$) and methanesulfonic acid ($CH_3SO_3H$) (weight ratio $P_2O_5$:$CH_3SO_3H$ 1:10). The mixture was heated at 40 °C under nitrogen atmosphere until complete homogeneity. Carboxylic acid (1.15–1.5 equiv) and aromatic derivative (1.0 equiv) were then added to Eaton's reagent. The mixture was heated at 40–80 °C under inert atmosphere for 3–30 h. After cooling to room temperature, the reaction medium was diluted with dichloromethane and carefully poured into a separatory funnel containing sodium bicarbonate aqueous solution (50% $NaHCO_3$). The aqueous solution was extracted with dichloromethane, and the combined organic layers were dried ($MgSO_4$). Solvent was removed under reduced pressure to produce a brownish oil. The crude product was purified by column chromatography on silica gel to afford pure benzophenones (**31**, **33**, **37**, **38**, **43**, **45**, **47**, **53**, and **56**).

##### 5.1.2.1. 2-Methoxy-5-(3,4,5-trimethoxybenzoyl)phenyl chloroacetate (31) (Scheme 1).

The general procedure A was followed using 3,4,5-trimethoxybenzoic acid **29** (95.19 g, 448.6 mmol), 2-methoxyphenyl chloroacetate **30** (60.00 g, 299.1 mmol) and Eaton's reagent (43.27 g $P_2O_5$ in 292.2 mL $CH_3SO_3H$). The mixture was heated at 60 °C for 4 h. The final brown oil was purified by column chromatography on silica gel with EtOAc/*n*-heptane 3/7 to give pure chloroacetate **31** (100.4 g, 85%) (lit. (with PPA)[18] 80%) as an off-white solid; mp (EtOH/$Et_2O$) 150–152 °C; TLC $R_f$ (EtOAc/*n*-heptane 7/3) = 0.62; $^1$H ($CDCl_3$, 200 MHz) $\delta$ (ppm) 3.89 (s, 6H, 2OC*H*$_3$), 3.94 (s, 6H, 2OC*H*$_3$), 4.36 (s, 2H, OCOC*H*$_2$Cl), 7.03 (s, 2H, Ar*H*), 7.07 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.62 (d, *J* = 2.2 Hz, 1H, Ar*H*), 7.79 (dd, *J* = 8.6, 2.2 Hz, 1H, Ar*H*). Calcd for $C_{19}H_{19}O_7Cl$: C, 57.80; H, 4.85. Found: C, 57.74; H, 4.78.

##### 5.1.2.2. 2,6-Dimethoxy-3-(3,4,5-trimethoxybenzoyl)phenyl chloroacetate (33) (Scheme 1).

The general procedure A was followed using 3,4,5-trimethoxybenzoic acid **29** (2.50 g, 11.8 mmol), 2,6-dimethoxyphenyl chloroacetate **32** (1.81 g, 7.8 mmol) and Eaton's reagent (1.14 g $P_2O_5$ in 7.67 mL $CH_3SO_3H$). The mixture was heated at 60 °C for 5 h. The final brown oil was purified on silica gel with EtOAc/*n*-heptane 4/6 to give pure chloroacetate **33** (2.70 g, 80%) as an off-white solid; mp (EtOH) 118–120 °C; TLC $R_f$ (EtOAc/*n*-heptane 4/6) = 0.15; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ (ppm) 3.68 (s, 3H, OC*H*$_3$), 3.86 (s, 6H, 2OC*H*$_3$), 3.91 (s, 3H, OC*H*$_3$), 3.93 (s, 3H, OC*H*$_3$), 4.38 (s, 2H, OCOC*H*$_2$Cl), 6.81 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.10 (s, 2H, Ar*H*), 7.33 (d, *J* = 8.6 Hz, 1H, Ar*H*). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 40.4 ($CH_2$), 56.2 (2$CH_3$), 56.3 ($CH_3$), 60.9 ($CH_3$), 62.6 ($CH_3$), 106.9 (CH), 107.4 (2CH), 125.7 (C), 128.3 (CH), 132.3 (C), 132.5 (C), 142.6 (C), 151.8 (C), 152.9 (2C), 154.3 (C), 165.0 (C), 193.5 (C). IR $\nu$ cm$^{-1}$: 522, 758, 814, 1093, 1120, 1293, 1290, 1414, 1489, 1667, 1754, 1781. Calcd for $C_{20}H_{21}O_8Cl$: C, 56.54; H, 4.98. Found: C, 56.37; H, 4.81.

##### 5.1.2.3. 5-{4-[(Ethoxycarbonyl)oxy]-3,5-dimethoxybenzoyl}-2-methoxyphenyl chloroacetate (37) (Scheme 1).

The general procedure A was followed using 4-[(ethoxycarbonyl)oxy]-3,5-dimethoxybenzoic acid (see Supplementary information) (10.10 g, 37.38 mmol), 2-methoxyphenyl chloroacetate **30** (5.00 g, 24.92 mmol) and Eaton's reagent (3.67 g $P_2O_5$ in 24.80 mL $CH_3SO_3H$). The mixture was heated at 50 °C for 3 h. The resulting brown oil was crystallized in 95% EtOH to give pure product **37** (8.70 g, 77%) as a white solid; mp (EtOH) 171–172 °C; $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ (ppm) 1.41 (t, *J* = 7.1 Hz, 3H, $OCO_2CH_2CH_3$), 3.87 (s, 6H, 2OC*H*$_3$), 3.94 (s, 3H, OC*H*$_3$), 4.35 (q, *J* = 7.1 Hz, 2H, OCOC*H*$_2$CH$_3$), 4.37 (s, 2H, OCOC*H*$_2$Cl), 7.02 (s, 2H, Ar*H*), 7.07 (d, *J* = 8.5 Hz, 1H, Ar*H*), 7.63 (d, *J* = 2.2 Hz, 1H, Ar*H*), 7.81 (dd, *J* = 8.5, 2.2 Hz, 1H, Ar*H*). $^{13}$C NMR ($CDCl_3$, 50 MHz) $\delta$ 14.0 ($CH_3$), 40.4 ($CH_2$), 56.1 ($CH_3$), 56.3 ($CH_3$), 65.2 ($CH_2$), 106.3 (CH), 106.8 (CH), 111.4 (CH), 111.9 (CH), 124.6 (C), 125.1 (C), 129.8 (CH), 130.3 (C), 135.6 (C), 138.6 (C), 152.0 (2C), 152.5 (C), 154.6 (C), 165.3 (C), 193.3 (C). IR $\nu$ cm$^{-1}$: 760, 1105, 1127, 1209, 1253, 1336, 1409, 1597, 1651, 1770, 1792. Calcd for $C_{21}H_{21}O_9Cl$: C, 55.70; H, 4.67. Found: C, 55.64; H, 4.79.

##### 5.1.2.4. 3-{4-[(Ethoxycarbonyl)oxy]-3,5-dimethoxybenzoyl}-2,6-dimethoxyphenyl chloroacetate (38) (Scheme 1).

The general procedure A was followed using 4-[(ethoxycarbonyl)oxy]-3,5-dimethoxybenzoic acid (see Supplementary information) (1.32 g, 4.88 mmol), 2,6-dimethoxyphenyl chloroacetate **32** (0.75 g, 3.25 mmol) and Eaton's reagent (0.48 g $P_2O_5$ in 3.24 mL $CH_3SO_3H$). The resulting mixture was heated at 50 °C for 3 h. The final brown oil was purified by column chromatography

(EtOAc/*n*-heptane 3/7) to give pure product **38** (1.18 g, 80%) as white solid; TLC $R_f$ (EtOAc/*n*-heptane 5/5) = 0.39; $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ (ppm) 1.40 (t, $J$ = 7.1 Hz, 3H, $OCO_2CH_2CH_3$), 3.68 (s, 3H, $OCH_3$), 3.85 (s, 6H, $2OCH_3$), 3.91 (s, 3H, $OCH_3$), 4.33 (q, $J$ = 7.1 Hz, 2H, $OCOCH_2CH_3$), 4.38 (s, 2H, $OCOCH_2Cl$), 6.81 (d, $J$ = 8.8 Hz, 1H, Ar*H*), 7.10 (s, 2H, Ar*H*), 7.36 (d, $J$ = 8.8 Hz, 1H, Ar*H*). IR $\nu$ cm$^{-1}$: 760, 1105, 1127, 1209, 1253, 1336, 1409, 1597, 1651, 1770, 1792. Calcd for $C_{22}H_{23}O_{10}Cl$: C, 54.72; H, 4.80. Found: C, 55.08; H, 4.74.

#### 5.1.2.5. 5-(3,4,5-Trimethoxybenzoyl)-1,3-benzodioxol-2-one (43) (Scheme 2).

The general procedure A was followed using 3,4,5-trimethoxybenzoic acid **29** (0.30 g, 2.20 mmol), carbonate **42** (0.70 g, 3.31 mmol) and Eaton's reagent (0.25 g $P_2O_5$ in 1.72 mL $CH_3SO_3H$). The mixture was heated at 50 °C for 26 h and turned purple very quickly. The final brown oil was purified by column chromatography on silica gel with EtOAc/*n*-heptane 2/8 to afford pure 5-(3,4,5-trimethoxybenzoyl)-1,3-benzodioxol-2-one **43** (21 mg, 3%). This compound was not characterized and utilized directly in the next step.

#### 5.1.2.6. 3-(Methylsulfonyl)-6-(3,4,5-trimethoxybenzoyl)-1,3-benzoxazol-2(3*H*)-one (45) and 1-hydroxy-2,3,5,6,7-pentamethoxyanthra-9,10-quinone (46) (Scheme 2).

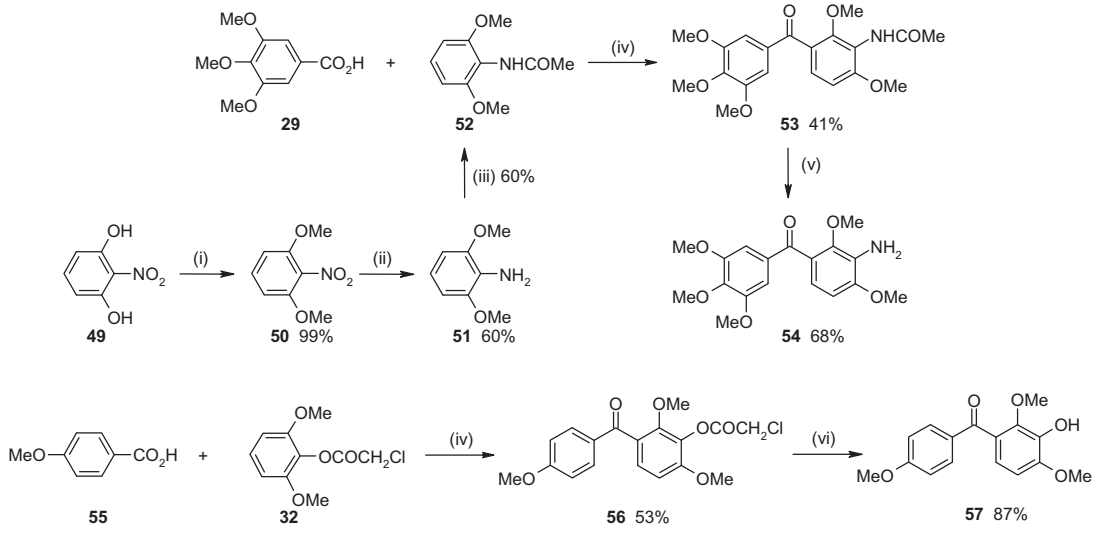
The general procedure A was followed using 3,4,5-trimethoxybenzoic acid **29** (47.11 g, 222.0 mmol), 1,3-benzoxazol-2(3*H*)-one **44** (20.00 g, 148.0 mmol) and Eaton's reagent (17.13 g $P_2O_5$ in 115.7 mL $CH_3SO_3H$). The mixture was heated at 60 °C for 30 h. The final brown oil was purified by column chromatography on silica gel with EtOAc/*n*-heptane 1/9 to give pure compounds **45** (7.84 g, 13%) and **46** (15.51 g, 28%) as yellow solids; Physico-chemical caracteristics of compound **45**: $^1$H ($CDCl_3$, 400 MHz) $\delta$ (ppm) 3.48 (s, 3H, $SO_2CH_3$), 3.88 (s, 6H, $2OCH_3$), 3.95 (s, 3H, $OCH_3$), 7.02 (s, 2H, Ar*H*), 7.06 (d, $J$ = 8.2 Hz, 1H, Ar*H*), 7.71 (d, $J$ = 1.6 Hz, 1H, Ar*H*), 7.75 (dd, $J$ = 8.2, 1.6 Hz, 1H, Ar*H*). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 28.4 ($CH_3$), 56.3 ($2CH_3$), 61.0 ($CH_3$), 107.3 (CH), 107.6 (2CH), 111.6 (CH), 127.2 (CH), 132.5 (C), 132.6 (C), 135.4 (C), 142.1 (C), 142.3 (C), 153.0 (2C), 154.6 (C), 194.1 (C); mp (EtOAc/*n*-heptane) 165–168 °C. Calcd for $C_{18}H_{17}O_8NS$: C, 53.07; H, 4.21; N, 3.44; S, 7.87. Found: C, 53.29; H, 4.60; N, 3.68; S, 7.53. Physico-chemical caracteristics of compound **46**: $^1$H ($CDCl_3$, 400 MHz) $\delta$ (ppm) 3.99 (s, 3H, $OCH_3$), 4.00 (s, 3H, $OCH_3$), 4.01 (s, 3H, $OCH_3$), 4.03 (s, 3H, $OCH_3$), 4.05 (s, 3H, $OCH_3$), 7.42 (s, 1H, Ar*H*), 7.68 (s, 1H, Ar*H*), 12.64 (s, 1H, ArO*H*). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 56.4 ($CH_3$), 56.5 ($CH_3$), 60.9 ($CH_3$), 61.4 ($CH_3$), 61.6 ($CH_3$), 103.9 (CH), 106.1 (CH), 111.5 (C), 121.1 (C), 130.6 (C), 130.7 (C), 140.6 (C), 148.5 (C), 155.0 (C), 156.0 (C), 157.5 (C), 158.4 (C), 180.3 (C), 186.7 (C); mp (EtOAc/*n*-heptane) >250 °C. Calcd for $C_{19}H_{18}O_8$: C, 60.96; H, 4.85. Found: C, 60.83; H, 4.78.

#### 5.1.2.7. 6-(3,4,5-Trimethoxybenzoyl)-1,3-benzoxazol-2(3*H*)-one (47) (Scheme 2).

The general procedure A was followed using 1,3-benzoxazol-2(3*H*)-one **44** (4.00 g, 29.6 mmol) and Eaton's reagent (4.00 g $P_2O_5$ in 27.00 mL $CH_3SO_3H$). 3,4,5-Trimethoxybenzoic acid **29** (10.99 g, 51.8 mmol) was added to the reaction mixture in small portions (1.0 g every 30 min) and the resulting viscous solution was heated at 60 °C for 30 h. The final brown oil was purified by column chromatography on silica gel with EtOAc/*n*-heptane 1/9 to give pure compound **47** (2.24 g, 23%); mp (EtOAc/*n*-heptane) 220–221 °C; $^1$H ($CDCl_3$, 400 MHz) $\delta$ (ppm) 3.89 (s, 6H, $2OCH_3$), 3.95 (s, 3H, $OCH_3$), 7.02 (s, 2H, Ar*H*), 7.16 (d, $J$ = 8.6 Hz, 1H, Ar*H*), 7.71 (dd, $J$ = 8.6, 1.7 Hz, 1H, Ar*H*), 7.73 (d, $J$ = 1.7 Hz, 1H, Ar*H*), 8.32 (large s, 1H, N*H*). $^{13}$C NMR ($CDCl_3$ + DMSO-$d_6$, 100 MHz) $\delta$ 56.9 ($2CH_3$), 61.4 ($CH_3$), 108.1 (2CH), 109.8 (CH), 111.5 (CH), 127.8 (CH), 132.3 (C), 133.5 (C), 135.4 (C), 142.3 (C), 144.3 (C), 153.5 (2C), 155.7 (C), 194.7 (C). IR $\nu$ cm$^{-1}$: 583, 704, 815, 922, 1104, 1119, 1290, 1413, 1493, 1574, 1643, 1773, 3021. Calcd for $C_{17}H_{15}O_6N$: C, 62.00; H, 4.59; N, 4.25. Found: C, 61.89; H, 4.40; N, 4.19.

#### 5.1.2.8. *N*-[2,6-Dimethoxy-3-(3,4,5-trimethoxybenzoyl)phenyl] acetamide (53) (Scheme 3).

The general procedure A was followed using 3,4,5-trimethoxybenzoic acid **29** (1.30 g, 6.1 mmol), *N*-(2,6-dimethoxyphenyl)acetamide **52** (1.00 g, 5.1 mmol) and Eaton's reagent (0.47 g $P_2O_5$ in 3.2 mL $CH_3SO_3H$). The mixture was heated at 60 °C for 24 h. The final beige oil was purified on silica gel with EtOAc/*n*-heptane 4/6 to give pure compound **53** (0.82 g, 41%) as a white solid; mp (EtOAc/*n*-heptane) 176–177 °C; TLC $R_f$ (EtOAc) = 0.31; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ (ppm) 2.23 (large s, 3H, $NHCOCH_3$), 3.66 (s, 3H, $OCH_3$), 3.88 (s, 6H, $2OCH_3$), 3.92 (s, 3H, $OCH_3$), 3.93 (s, 3H, $OCH_3$), 6.70 (large s, 1H, ArN*H*), 6.77 (d, $J$ = 8.6 Hz, 1H, Ar*H*), 7.13 (s, 2H,




**Scheme 3.** *Reagents and conditions:* (i) $Me_2SO_4$ 3 equiv, $K_2CO_3$ 5 equiv, acetone, reflux, 1 h; (ii) $SnCl_2{\cdot}2H_2O$ 5 equiv, conc HCl, EtOH, rt, 30 h; (iii) $Ac_2O$ 2 equiv, $CH_2Cl_2$, rt, 24 h; (iv) Eaton's reagent 4 equiv, 60 °C; (v) HCl 10%, MeOH, reflux, 24 h; (vi) $CH_3COONa{\cdot}3H_2O$ 4.5 equiv, MeOH, reflux, 3 h.

Ar*H*), 7.35 (d, *J* = 8.6 Hz, 1H, Ar*H*). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 23.5 ($CH_3$), 56.1 ($CH_3$), 56.3 (2$CH_3$), 60.9 ($CH_3$), 62.1 ($CH_3$), 106.1 (CH), 107.4 (2CH), 123.6 (CH), 125.1 (CH), 132.8 (C), 142.5 (C), 152.9 (2C), 155.8 (C), 157.5 (C), 169.2 (C), 194.0 (C), 217.4 (C). IR $\nu$ cm$^{-1}$: 1098, 1126, 1464, 1502, 1537, 1576, 1596, 1658. Calcd for $C_{20}H_{23}O_7N$: C, 61.69; H, 5.95; N, 3.60. Found: C, 61.42; H, 5.76; N, 3.45.

#### 5.1.2.9. 2,6-Dimethoxy-3-(4-methoxybenzoyl)phenyl chloroacetate (56) (Scheme 3).

The general procedure A was followed using 4-methoxybenzoic acid **55** (7.05 g, 46.3 mmol), 2,6-dimethoxyphenyl chloroacetate **32** (5.34 g, 23.2 mmol) and Eaton's reagent (2.43 g $P_2O_5$ in 16.4 mL $CH_3SO_3H$). The mixture was heated at 60 °C for 3 h. The final beige oil was purified on silica gel with EtOAc/*n*-heptane 3/7 to give chloroacetate **56** (4.48 g, 53%) as a white solid; mp (EtOAc) 48–50 °C; TLC $R_f$ (EtOAc/*n*-heptane 3/7) = 0.12; $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ (ppm) 3.65 (s, 3H, OC*H*$_3$), 3.88 (s, 3H, OC*H*$_3$), 3.89 (s, 3H, OC*H*$_3$), 4.39 (s, 2H, OCOC*H*$_2$Cl), 6.88 (d, *J* = 8.8 Hz, 1H, Ar*H*), 6.93 (d, *J* = 9.3 Hz, 2H, Ar*H*), 7.32 (d, *J* = 8.8 Hz, 1H, Ar*H*), 7.82 (d, *J* = 9.3 Hz, 2H, Ar*H*). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 40.4 ($CH_2$), 55.5 ($CH_3$), 56.3 ($CH_3$), 62.5 ($CH_3$), 106.9 (CH), 113.6 (2CH), 126.2 (C), 128.2 (CH), 130.4 (C), 132.2 (C), 132.4 (2CH), 151.5 (C), 154.1 (C), 163.7 (C), 165.1 (C), 193.2 (C). Calcd for $C_{18}H_{17}O_6Cl$: C, 59.27; H, 4.70. Found: C, 59.48; H, 4.77.

### 5.1.3. General procedure B for the synthesis of phenols from chloroacetic esters

(Mono or di)chloroacetic ester (1 equiv) and sodium acetate (4.5 equiv or 6 equiv) were dissolved in methanol. The solution was refluxed for 1–3 h. After cooling at rt, the mixture was concentrated under reduced pressure. The residue was taken into distilled water. The resulting precipitate was filtered, washed with water several times to remove remaining sodium acetate. The solid was recrystallized from ethanol.

#### 5.1.3.1. (3-Hydroxy-4-methoxyphenyl)-(3,4,5-trimethoxyphenyl) methanone (Phenstatin, 3) (Scheme 1).

The general procedure B was followed using chloroacetate **31** (150.0 g, 0.38 mol) and sodium acetate (AcONa·3$H_2O$) (232.65 g, 1.71 mol) in MeOH (1 L). The reaction mixture was refluxed for 2 h. The formed solid was collected by filtration and recrystallized from EtOH to obtain phenstatin (**3**) (118.52 g, 98%) as a white solid with the same physico-chemical properties as described in the literature[18]; TLC $R_f$ (EtOAc/*n*-heptane 5/5) = 0.32; mp (EtOH) 150–152 °C; $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ (ppm) 3.89 (s, 6H, 2OC*H*$_3$), 3.93 (s, 3H, OC*H*$_3$), 3.99 (s, 3H, OC*H*$_3$), 5.70 (s, 1H, ArO*H*), 6.92 (d, *J* = 8.2 Hz, 1H, Ar*H*), 7.03 (s, 2H, Ar*H*), 7.39 (dd, *J* = 8.2, 2.1 Hz, 1H, Ar*H*), 7.44 (d, *J* = 2.1 Hz, 1H, Ar*H*). Calcd for $C_{17}H_{18}O_6$: C, 64.14; H, 5.70. Found: C, 64.07; H, 5.76.

#### 5.1.3.2. (3-Hydroxy-2,4-dimethoxyphenyl)-(3,4,5-trimethoxyphenyl)methanone (23) (Scheme 1).

The general procedure B was followed using chloroacetate **33** (1.2 g, 2.8 mmol) and sodium acetate (AcONa·3$H_2O$) (1.73 g, 12.7 mmol) in MeOH (10 mL). The reaction mixture was refluxed for 2 h. The formed solid was collected by filtration and recrystallized from EtOH to obtain **23** (0.80 g, 81%) as white needles; TLC $R_f$ (EtOAc/*n*-heptane 5/5) = 0.30; mp (EtOH) 156–157 °C; $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ (ppm) 3.81 (s, 3H, OC*H*$_3$), 3.86 (s, 6H, 2OC*H*$_3$), 3.93 (s, 3H, OC*H*$_3$), 3.97 (s, 3H, OC*H*$_3$), 5.74 (s, 1H, ArO*H*), 6.71 (d, *J* = 8.5 Hz, 1H, Ar*H*), 6.93 (d, *J* = 8.5 Hz, 1H, Ar*H*), 7.10 (s, 2H, Ar*H*). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 56.2 (2$CH_3$), 56.3 ($CH_3$), 60.9 ($CH_3$), 61.9 ($CH_3$), 105.7 (CH), 107.6 (2CH), 120.9 (C), 125.7 (C), 133.1 (C), 138.4 (C), 142.5 (C), 145.9 (C), 149.8 (C), 152.8 (2C), 194.2 (C). IR $\nu$ cm$^{-1}$: 611, 1004, 1091, 1131, 1283, 1332, 1409, 1456, 1577, 1607, 1654, 3325. Calcd for $C_{18}H_{20}O_7$: C, 62.06; H, 5.79. Found: C, 62.00; H, 5.88.

#### 5.1.3.3. (3-Hydroxy-4,5-dimethoxyphenyl)(3-hydroxy-4-methoxyphenyl) methanone (24) (Scheme 1).

The general procedure B was followed using **36** (1.52 g, 3.32 mmol) and sodium acetate (AcONa·3$H_2O$) (2.04 g, 14.95 mmol) in MeOH (25 mL). The resulting reaction mixture was stirred at reflux for 3 h. The crude solid was poured into water and the precipitate was filtered, dried and recrystallized from EtOH to give pure metabolite **24** (0.66 g, 65%) as an off-white solid; mp (EtOH) 166–167 °C; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ (ppm) 3.91 (s, 3H, OC*H*$_3$), 3.98 (s, 3H, OC*H*$_3$), 3.99 (s, 3H, OC*H*$_3$), 5.64 (s, 1H, ArO*H*), 5.82 (s, 1H, ArO*H*), 6.91 (d, *J* = 8.3 Hz, 1H, Ar*H*), 6.99 (d, *J* = 2.0 Hz, 1H, Ar*H*), 7.00 (d, *J* = 2.0 Hz, 1H, Ar*H*), 7.39 (dd, *J* = 8.3, 2.0 Hz, 1H, Ar*H*), 7.43 (d, *J* = 2.0 Hz, 1H, Ar*H*). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 56.1 ($CH_3$), 56.2 ($CH_3$), 61.0 ($CH_3$), 105.7 (CH), 109.7 (CH), 110.6 (CH), 116.2 (CH), 123.7 (CH), 130.9 (C), 133.5 (C), 138.8 (C), 145.2 (C), 148.5 (C), 150.2 (C), 152.1 (C), 194.6 (C). IR $\nu$ cm$^{-1}$: 759, 1113, 1128, 1238, 1253, 1267, 1348, 1580, 1629, 1717, 1755, 3421. Calcd for $C_{16}H_{16}O_6$: C, 63.15; H, 5.30. Found: C, 63.32; H, 5.28.

#### 5.1.3.4. Ethyl 4-(3-hydroxy-4-methoxybenzoyl)-2,6-dimethoxyphenyl carbonate (39) (Scheme 1).

The general procedure B was followed using **37** (3.00 g, 6.62 mmol) and sodium acetate (AcONa·3$H_2O$) (4.06 g, 29.81 mmol) in MeOH (35 mL). The resulting reaction mixture was stirred at reflux for 1 h. The crude solid was poured into water and the precipitate was filtered, dried and recrystallized from EtOH to give pure product **39** (2.12 g, 85%) as an off-white solid; mp (EtOH) 114–116 °C; $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ (ppm) 1.41 (t, *J* = 7.1 Hz, 3H, OCO$_2$CH$_2$C*H*$_3$), 3.87 (s, 6H, 2OC*H*$_3$), 3.99 (s, 3H, OC*H*$_3$), 4.35 (q, *J* = 7.1 Hz, 2H, OCO$_2$C*H*$_2$CH$_3$), 5.71 (s, 1H, ArO*H*), 6.92 (d, *J* = 8.3 Hz, 1H, Ar*H*), 7.03 (s, 2H, Ar*H*), 7.41 (dd, *J* = 8.3, 1.8 Hz, 1H, Ar*H*), 7.47 (d, *J* = 1.8 Hz, 1H, Ar*H*). IR $\nu$ cm$^{-1}$: 758, 1052, 1126, 1175, 1202, 1254, 1335, 1414, 1456, 1596, 1649, 1762, 3410. Calcd for $C_{19}H_{20}O_8$: C, 60.64; H, 5.36. Found: C, 61.01; H, 5.42.

#### 5.1.3.5. Ethyl 4-(3-hydroxy-2,4-dimethoxybenzoyl)-2,6-dimethoxyphenyl carbonate (40) (Scheme 1).

The general procedure B was followed using **38** (1.07 g, 2.36 mmol) and sodium acetate (AcONa·3$H_2O$) (1.93 g, 14.17 mmol) in MeOH (20 mL). The resulting reaction mixture was stirred at reflux for 1 h. The crude solid was poured into water and the precipitate was filtered, dried and recrystallized from EtOH to give pure product **40** (0.81 g, 84%) as an off-white solid; mp (EtOH) 140–143 °C; $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ (ppm) 1.40 (t, *J* = 7.1 Hz, 3H, OCO$_2$CH$_2$C*H*$_3$), 3.81 (s, 3H, OC*H*$_3$), 3.84 (s, 6H, 2OC*H*$_3$), 3.97 (s, 3H, OC*H*$_3$), 4.33 (q, *J* = 7.1 Hz, 2H, OCO$_2$C*H*$_2$CH$_3$), 6.70 (d, *J* = 8.6 Hz, 1H, Ar*H*), 6.96 (d, *J* = 1.9 Hz, 1H, Ar*H*), 7.10 (s, 2H, Ar*H*). IR $\nu$ cm$^{-1}$: 776, 1090, 1129, 1268, 1342, 1416, 1468, 1600, 1660, 1746, 3447. Calcd for $C_{20}H_{22}O_9$: C, 59.11; H, 5.46. Found: C, 59.57; H, 5.38.

#### 5.1.3.6. (3-Hydroxy-2,4-dimethoxyphenyl)(4-methoxyphenyl) methanone (57) (Scheme 3).

The general procedure B was followed using **56** (2.51 g, 6.88 mmol) and sodium acetate (AcONa·3$H_2O$) (4.68 g, 34.4 mmol) in MeOH (35 mL). The resulting reaction mixture was stirred at reflux for 3 h. The crude solid was poured into water and the precipitate was filtered, dried over magnesium sulfate and recrystallized from EtOH to give pure phenol **57** (1.72 g, 87%) as a white solid; mp (EtOH) 115–117 °C; TLC $R_f$ (EtOAc/*n*-heptane 3/7) = 0.66; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ (ppm) 3.75 (s, 3H, OC*H*$_3$), 3.88 (s, 3H, OC*H*$_3$),

3.96 (s, 3H, OC$H_3$), 5.72 (s, 1H, ArO$H$), 6.71 (d, $J$ = 8.6 Hz, 1H, Ar$H$), 6.92 (d, $J$ = 8.6 Hz, 1H, Ar$H$), 6.93 (d, $J$ = 8.9 Hz, 2H, Ar$H$), 7.83 (d, $J$ = 8.9 Hz, 2H, Ar$H$). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 55.5 ($CH_3$), 56.3 ($CH_3$), 61.8 ($CH_3$), 106.0 (CH), 113.5 (2CH), 120.7 (CH), 126.2 (C), 130.8 (C), 132.4 (2CH), 138.3 (C), 145.6 (C), 149.6 (C), 163.5 (C), 194.0 (C). Calcd for $C_{16}H_{16}O_5$: C, 66.66; H, 5.59. Found: C, 66.56; H, 5.62.

### 5.1.4. General procedure C for the synthesis of benzhydrols by reduction of the corresponding ketones

An aqueous solution of sodium borohydride (1.1–2.2 equiv) was added dropwise to a solution of benzophenone (1 equiv) in absolute ethanol. After stirring for 5–8 h at room temperature, the reaction media is neutralized with a hydrochloric acid solution (1.5 M) and extracted with ethyl acetate (3 × 25 mL). Combined organic layers were dried over magnesium sulfate and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel or recrystallized from $H_2O$ to afford the required benzhydrols **26** or **27**.

#### 5.1.4.1. 5-[Hydroxy(3,4,5-trimethoxyphenyl)methyl]-2-methoxyphenol (26) (Scheme 1).

The general procedure C was followed using $NaBH_4$ (0.26 g, 6.9 mmol) in water (10 mL) and phenstatin (**3**) (1.0 g, 3.1 mmol) in EtOH (30 mL). The mixture was stirred at rt for 5 h. The residue was recrystallized from $H_2O$ to give benzhydrol **26** (0.98 g, 97%) as a brown solid with the same physical properties as described in the literature[20]; mp ($H_2O$) 145–147 °C; $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ (ppm) 3.84 (s, 9H, 3OC$H_3$), 3.89 (s, 3H, OC$H_3$), 5.20 (s, 1H, ArO$H$), 5.69 (s, 1H, ArO$H$), 6.60 (s, 1H, ArC$H$Ar), 6.61 (s, 2H, Ar$H$), 6.80–6.95 (m, 3H, Ar$H$). IR $\nu$ cm$^{-1}$: 1121, 1422, 1465, 1507, 1591, 3249, 3480. Calcd for $C_{17}H_{20}O_6$: C, 63.74; H, 6.29. Found: C, 63.50; H, 6.11.

#### 5.1.4.2. (3,4-Dimethoxyphenyl)(3,4,5-trimethoxyphenyl)methanol (27) (Scheme 1).

The general procedure C was followed using $NaBH_4$ (0.05 g, 0.6 mmol) in water (3 mL) and benzophenone **20** (0.20 g, 1.30 mmol) in EtOH (10 mL). The mixture was stirred at rt for 8 h. The residue was purified by chromatography on silica gel, eluting EtOAc/*n*-heptane 4/6 to deliver pure benzhydrol **27** (0.15 g, 75%) as a viscous beige oil; TLC $R_f$ (EtOAc/*n*-heptane 4/6) = 0.32; $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ (ppm) 3.82 (s, 3H, OC$H_3$), 3.83 (s, 6H, 2OC$H_3$), 3.87 (s, 6H, 2OC$H_3$), 5.24 (s, 1H, ArO$H$), 6.57 (s, 2H, Ar$H$), 6.60 (s, 1H, ArC$H$Ar), 6.81 (dd, $J$ = 7.8, 1.6 Hz, 1H, Ar$H$), 6.86 (d, $J$ = 7.8 Hz, 1H, Ar$H$), 6.91 (d, $J$ = 1.6 Hz, 1H, Ar$H$). Calcd for $C_{18}H_{22}O_6$: C, 64.66; H, 6.63. Found: C, 64.58; H, 7.19.

### 5.1.5. General procedure D for the synthesis of phenols by cleavage of ethyl carbonates

Ethylcarbonate (1 equiv) and ethanolamine (5 equiv) were dissolved in 95% ethanol. The resulting yellow solution was stirred at rt for 5 h. After reaction completion, the final suspension was concentrated in vacuo, treated with distilled water, and then neutralized to pH 7 by adding slowly concentrated HCl. The aqueous solution was extracted with dichloromethane. The organic phase was dried ($MgSO_4$), filtered and concentrated in vacuo. The residue was purified by chromatography on silica gel or recrystallized from acetone to afford phenol.

#### 5.1.5.1. (3-Hydroxy-2,4-dimethoxyphenyl)(4-hydroxy-3,5-dimethoxyphenyl)methanone (22) (Scheme 1).

The general procedure D was followed using ethyl 4-(3-hydroxy-2,4-dimethoxybenzoyl)-2,6-dimethoxyphenyl carbonate **40** (0.4 g, 1.0 mmol) and ethanolamine (0.3 mL, 4.9 mmol) in EtOH 95% (25 mL). The final residue was purified by chromatography on silica gel eluting EtOAc/*n*-heptane 6/4 to isolate phenol **22** (0.25 g, 76%) as a white cotton; mp (EtOAc/*n*-heptane) 162–164 °C; TLC $R_f$ (EtOAc/*n*-heptane 6/4) = 0.27; $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ (ppm) 3.80 (s, 3H, OC$H_3$), 3.90 (s, 6H, 2OC$H_3$), 3.97 (s, 3H, OC$H_3$), 5.74 (s, 1H, ArO$H$), 5.98 (s, 1H, ArO$H$), 6.71 (d, $J$ = 8.6 Hz, 1H, Ar$H$), 6.92 (d, $J$ = 8.6 Hz, 1H, Ar$H$), 7.15 (s, 2H, Ar$H$). $^{13}$C NMR ($CDCl_3$, 50 MHz) $\delta$ 56.0 ($CH_3$), 56.1 ($CH_3$), 56.3 (2$CH_3$), 107.0 (CH), 107.5 (2CH), 120.1 (C), 120.7 (C), 125.6 (C), 128.9 (CH), 138.1 (C), 139.4 (C), 146.3 (2C), 149.4 (C), 193.7 (C). IR $\nu$ cm$^{-1}$: 602, 751, 1089, 1121, 1203, 1288, 1336, 1450, 1609, 1651, 3468. Calcd for $C_{17}H_{18}O_7$: C, 61.07; H, 5.43. Found: C, 60.66; H, 5.39.

#### 5.1.5.2. (4-Hydroxy-3,5-dimethoxyphenyl)(3-hydroxy-4-methoxyphenyl)methanone (25) (Scheme 1).

The general procedure D was followed using ethyl 4-(3-hydroxy-4-methoxybenzoyl)-2,6-dimethoxyphenyl carbonate **39** (2.0 g, 5.3 mmol) and ethanolamine (1.6 mL, 26.6 mmol) in EtOH 95% (30 mL). The residue was recrystallized from acetone to give benzophenone **25** (1.37 g, 85%) as an off-white compact solid; mp (acetone) 162–164 °C; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ (ppm) 3.92 (s, 6H, 2OC$H_3$), 3.99 (s, 3H, OC$H_3$), 5.68 (s, 1H, ArO$H$), 5.91 (s, 1H, ArO$H$), 6.93 (d, $J$ = 8.5 Hz, 1H, Ar$H$), 7.10 (s, 2H, Ar$H$), 7.37 (dd, $J$ = 8.5, 2.0 Hz, 1H, Ar$H$), 7.42 (d, $J$ = 2.0 Hz, 1H, Ar$H$). IR $\nu$ cm$^{-1}$: 752, 1026, 1112, 1274, 1291, 1319, 1415, 1462, 1488, 1552, 1619, 3101. Calcd for $C_{16}H_{16}O_6$, 5/3 $H_2O$: C, 57.48; H, 5.83. Found: C, 57.10; H, 6.22.

#### 5.1.5.3. (3,4-Dimethoxyphenyl)(4-hydroxy-3,5-dimethoxyphenyl)methanone (21) (Scheme 1).

The general procedure D was followed using ethyl 4-(3,4-dimethoxybenzoyl)-2,6-dimethoxyphenyl carbonate **41** (0.4 g, 1.0 mmol) and ethanolamine (0.31 mL, 5.1 mmol) in EtOH 95% (15 mL). The residue was purified by chromatography on silica gel, eluting with EtOAc/*n*-heptane 6/4 to give pure benzophenone **21** (0.25 g, 78%) as an off-white solid; mp (EtOAc/*n*-heptane) 150–153 °C; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ (ppm) 3.93 (s, 6H, 2OC$H_3$), 3.95 (s, 3H, OC$H_3$), 3.97 (s, 3H, OC$H_3$), 5.91 (s, 1H, ArO$H$), 6.92 (d, $J$ = 8.6 Hz, 1H, Ar$H$), 7.10 (s, 2H, Ar$H$), 7.39 (dd, $J$ = 8.5, 2.0 Hz, 1H, Ar$H$), 7.43 (d, $J$ = 2.0 Hz, 1H, Ar$H$). $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 56.0 ($CH_3$), 56.1 ($CH_3$), 56.5 (2$CH_3$), 107.6 (2CH), 109.8 (CH), 112.4 (CH), 124.7 (CH), 129.2 (C), 130.7 (C), 138.9 (C), 146.5 (2C), 148.9 (C), 152.6 (C), 194.5 (C). IR $\nu$ cm$^{-1}$: 739, 754, 1023, 1117, 1216, 1264, 1330, 1414, 1465, 1511, 1578, 1641, 3432. Calcd for $C_{17}H_{18}O_6$: C, 64.14; H, 5.70. Found: C, 64.01; H, 5.38.

#### 5.1.5.4. (3,4-Dihydroxyphenyl)(3,4,5-trimethoxyphenyl)methanone (28) (Scheme 2).

*From benzophenone **43**:* A mixture of benzophenone **43** (0.021 g, 0.063 mmol) and ethanolamine (0.1 mL, 1.656 mmol) in EtOH 95% was stirred at room temperature for 5 h. The final suspension was concentrated in vacuo, treated with distilled water, and then neutralized to pH 7 by adding slowly concentrated HCl. The aqueous solution was extracted with dichloromethane. The organic phase was dried ($MgSO_4$), filtered and concentrated in vacuo. The residue was purified by column chromatography on silica gel (EtOAc/*n*-heptane 5/5) affording pure compound **28** (9.9 mg, 51%) as a red solid.

*From aminophenol **48**:* A solution of **48** (0.25 g, 0.82 mmol) in AcOH (100 mL) was added to a vigorously stirred solution of sodium metaperiodate (3.84 g, 17.95 mmol) in 0.1 N HCl (269 mL) at rt for 4 h. The reaction mixture was extracted with $CHCl_3$. The organic layer was washed with water and, after addition of AcOH (10 mL) and KI (1.25 g, 7.53 mmol), was stirred at rt for 10 h. The iodine formed was reduced with aqueous $NaHSO_3$. The organic

layer was washed with water, acidified with AcOH (5 mL) and dried ($MgSO_4$). After evaporation of the solvent, the residue was purified by column chromatography on silica gel with EtOAc/*n*-heptane 3/7 to give pure compound **28** (40.1 mg, 16%); $^1H$ ($CDCl_3$, 400 MHz) $\delta$ (ppm) 3.84 (s, 6H, 2OC*H*$_3$), 3.96 (s, 3H, OC*H*$_3$), 5.64 (large s, 2H, ArO*H*), 6.53 (s, 1H, Ar*H*), 7.07 (s, 2H, Ar*H*), 7.40 (d, *J* = 8.2 Hz, 1H, Ar*H*), 7.91 (d, *J* = 8.2 Hz, 1H, Ar*H*). This compound was not further analyzed, but directly utilized in the studies on metabolization of phenstatin.

### 5.1.6. General procedure E for methylation of phenolic functions

Benzophenones (1 equiv) were dissolved in anhydrous acetone, under inert atmosphere. Potassium carbonate (3–5 equiv) was then added to the mixture. Dimethyl sulfate (1–2.5 equiv) was added dropwise to the resulting suspension. The reaction mixture was refluxed for 2 h, then cooled at rt, and finally concentrated under reduced pressure. The finally residue was recrystallized from water to afford *O*-methylated compounds.

#### 5.1.6.1. (3,4-Dimethoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (20) (Scheme 1).

The general procedure E was followed using phenstatin (**3**) (0.25 g, 0.8 mmol), $K_2CO_3$ (0.33 g, 2.4 mmol), dimethyl sulfate (0.11 mL, 1.2 mmol) and acetone (20 mL). The mixture was refluxed for 2 h. The final precipitate was recrystallized from water to give the corresponding *O*-methylated ketone **20** (0.18 g, 69%) as an off-white powder; mp ($H_2O$) 121–122 °C (lit. 122–123 °C,[22a] 119–120 °C[22b]); $^1H$ NMR ($CDCl_3$, 200 MHz) $\delta$ (ppm) 3.89 (s, 6H, 2OC*H*$_3$), 3.94 (s, 3H, OC*H*$_3$), 3.96 (s, 3H, OC*H*$_3$), 3.98 (s, 3H, OC*H*$_3$), 6.92 (d, *J* = 8.3 Hz, 1H, Ar*H*), 7.04 (s, 2H, Ar*H*), 7.41 (dd, *J* = 8.3, 1.8 Hz, 1H, Ar*H*), 7.47 (d, *J* = 1.8 Hz, 1H, Ar*H*). $^{13}C$ NMR ($CDCl_3$, 100 MHz) $\delta$ 56.1 ($2CH_3$), 56.3 ($2CH_3$), 61.0 ($CH_3$), 107.5 (2CH), 109.8 (CH), 112.3 (CH), 125.1 (CH), 130.3 (C), 133.3 (C), 141.6 (C), 149.0 (C), 152.8 (2C), 152.9 (C), 194.6 (C). IR $\nu$ $cm^{-1}$: 763, 998, 1118, 1242, 1265, 1329, 1412, 1578, 1632, 1732. Calcd for $C_{18}H_{20}O_6$: C, 65.05; H, 6.07. Found: C, 65.17; H, 6.38.

#### 5.1.6.2. 4-(3,4-Dimethoxybenzoyl)-2,6-dimethoxyphenyl ethyl carbonate (41) (Scheme 1).

The general procedure E was followed using ethyl 4-(3-hydroxy-4-methoxybenzoyl)-2,6-dimethoxyphenyl carbonate **39** (0.50 g, 1.3 mmol), $K_2CO_3$ (0.55 g, 4.0 mmol), dimethyl sulfate (0.19 mL, 2.0 mmol) and acetone (20 mL). The reaction mixture was stirred and refluxed for 2 h. The final precipitate was recrystallized from water to give the corresponding *O*-methylated ketone **41** (0.43 g, 82%) as an off-white solid; mp ($H_2O$) 142–144 °C; $^1H$ NMR ($CDCl_3$, 400 MHz) $\delta$ (ppm) 1.41 (t, *J* = 7.1 Hz, 3H, $OCO_2CH_2CH_3$), 3.87 (s, 6H, 2OC*H*$_3$), 3.96 (s, 3H, OC*H*$_3$), 3.98 (s, 3H, OC*H*$_3$), 4.35 (q, *J* = 7.1 Hz, 2H, $OCO_2CH_2CH_3$), 6.91 (d, *J* = 8.4 Hz, 1H, Ar*H*), 7.03 (s, 2H, Ar*H*), 7.42 (dd, *J* = 8.3, 1.9 Hz, 1H, Ar*H*), 7.49 (d, *J* = 1.9 Hz, 1H, Ar*H*). $^{13}C$ NMR ($CDCl_3$, 100 MHz) $\delta$ 14.1 ($CH_3$), 56.1 ($2CH_3$), 56.4 ($2CH_3$), 65.2 ($CH_2$), 106.7 (2CH), 109.8 (CH), 112.1 (CH), 125.3 (CH), 129.9 (CH), 132.1 (C), 136.3 (C), 149.1 (C), 152.0 (2C), 152.6 (C), 153.1 (C), 194.4 (C). Calcd for $C_{20}H_{22}O_8$: C, 61.53; H, 5.68. Found: C, 61.14; H, 5.73.

#### 5.1.6.3. (4-Amino-3-hydroxyphenyl)(3,4,5-trimethoxyphenyl) methanone (48) (Scheme 2).

A mixture of benzoxazolone **47** (0.50 g, 1.52 mmol) and sodium hydroxide (0.30 g, 7.50 mmol) in water (20 mL) was stirred at 80 °C for 10 h. The final suspension was neutralized to pH 7 by adding slowly concentrated HCl. The aqueous solution was extracted with dichloromethane. The organic phase was dried ($MgSO_4$), filtered and concentrated in vacuo. The residue was purified by column chromatography on silica gel (EtOAc/*n*-heptane 5/5) affording desired *O*-methylated product **48** (0.26 g, 56%) as a beige solid; $^1H$ ($CDCl_3$, 400 MHz) $\delta$ (ppm) 3.88 (s, 6H, 2OC*H*$_3$), 3.93 (s, 3H, OC*H*$_3$), 6.44 (s large, 1H, ArO*H*), 6.69 (d, *J* = 8.2 Hz, 1H, Ar*H*), 7.00 (s, 2H, Ar*H*), 7.31 (dd, *J* = 8.2, 1.8 Hz, 1H, Ar*H*), 7.45 (d, *J* = 1.8 Hz, 1H, Ar*H*). Calcd for $C_{16}H_{17}O_5N$: C, 63.36; H, 5.65; N, 4.62. Found: C, 63.61; H, 5.77; N, 4.80. This compound was not further analyzed, but utilized directly in the next step.

#### 5.1.6.4. (3-Amino-2,4-dimethoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (54) (Scheme 3).

A mixture of acetamide **53** (0.50 g, 1.28 mmol) and a 10% hydrochloric acid solution (15 mL) in methanol (20 mL) was stirred at 80 °C for 24 h. The final suspension was neutralized to pH 7 by slow addition of an ammoniacal solution. The aqueous solution was extracted with dichloromethane. The organic phase was dried ($MgSO_4$), filtered and concentrated in vacuo. The residue was recrystallized from $Et_2O$ to afford pure aniline **54** (0.30 g, 68%) as a beige solid; mp ($Et_2O$) 145–147 °C; TLC $R_f$ (EtOAc/*n*-heptane 4/6) = 0.26; $^1H$ ($CDCl_3$, 400 MHz) $\delta$ (ppm) 3.72 (s, 3H, OC*H*$_3$), 3.86 (s, 6H, 2OC*H*$_3$), 3.93 (s, 6H, 2OC*H*$_3$), 4.05 (large s, 2H, ArN*H*$_2$), 6.63 (d, *J* = 8.6 Hz, 1H, Ar*H*), 6.78 (d, *J* = 8.6 Hz, 1H, Ar*H*), 7.11 (s, 2H, Ar*H*). $^{13}C$ NMR ($CDCl_3$, 100 MHz) $\delta$ 55.8 ($CH_3$), 56.2 ($2CH_3$), 60.9 ($CH_3$), 61.3 ($CH_3$), 105.1 (CH), 107.7 (2CH), 119.1 (CH), 125.1 (C), 129.8 (C), 133.2 (C), 142.3 (C), 145.8 (C), 150.1 (C), 152.7 (2C), 197.1 (C). IR $\nu$ $cm^{-1}$: 1123, 1459, 1500, 1580, 1596, 1657. Calcd for $C_{18}H_{21}O_6N$: C, 62.24; H, 6.09; N, 4.03. Found: C, 62.14; H, 6.11; N, 4.34.

### 5.2. Tubulin studies

All our compounds and the two reference compounds (phenstatin and CA-4) have been tested in identical operating conditions.[32] Turbidimetric assays of microtubules were performed as described,[41] except when 50–150 μL quartz microcuvettes were utilized instead of 96-well plates. Lyophilized bovine brain tubulin (97%) was purchased from Cytoskeleton Inc. and was reconstituted to 5 mg/mL with the following buffer: 80 mM PIPES, pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA, 1.0 mM GTP, 5% glycerol. To 63 μL of this solution in ultra micro quartz cuvette at 0 °C was added 7 μL of test compound in dimethyl sulfoxide. The increase in absorbance was monitored at 340 nm and 37 °C on a HeλIOS Gamma&Delta. The $IC_{50}$ was defined as the compound molar concentration that inhibited the extent of assembly by 50% after 30 min incubation.

### 5.3. Cell proliferation assay

Six metabolites and intermediates compounds were tested against a panel of 60 human cancer cell lines at the National Cancer Institute, Bethesda, MD.[42a] The cytotoxicity studies were conducted using a 48 h exposure protocol using the sulforhodamine B assay.[42b]

### 5.4. Cell cycle analysis for metabolite 23

The frequency of G2/M cells was assessed by studying the cell cycle after fixation overnight at 4 °C with 70% ice-cold ethanol/phosphate-buffered saline (PBS) followed by staining with a solution containing PI (50 μg/mL) and RNase A (0.5 mg/mL), and analyzed with a Becton Dickinson FACScan cytofluorometer. DA1-3b cells were incubated for 24 h in presence of metabolite **23** and then analyzed.

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## Supplementary data

Supplementary data (Syntheses and physico-chemical characterization of all intermediates and by-products are related in this section) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.08.047.

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