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Synthesis and antiproliferative activity of two diastereomeric lignan amides serving as dimeric caffeic acid-L-DOPA hybrids

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ABSTRACT

Two new diastereomeric lignan amides (**4** and **5**) serving as dimeric caffeic acid-L-DOPA hybrids were synthesized. The synthesis involved the $FeCl_3$-mediated phenol oxidative coupling of methyl caffeate to afford *trans*-diester **1a** as a mixture of enantiomers, protection of the catechol units, regioselective saponification, coupling with a suitably protected L-DOPA derivative, separation of the two diastereomers thus obtained by flash column chromatography and finally global chemoselective deprotection of the catechol units. The effect of hybrids **4** and **5** and related compounds on the proliferation of two breast cancer cell lines with different metastatic potential and estrogen receptor status (MDA-MB-231 and MCF-7) and of one epithelial lung cancer cell line, namely A-549, was evaluated for concentrations ranging from 1 to 256 μM and periods of treatment of 24, 48 and 72 h. Both hybrids showed interesting and almost equipotent antiproliferative activities (IC_{50} 64–70 μM) for the MDA-MB-231 cell line after 24–48 h of treatment, but they were more selective and much more potent (IC_{50} 4–16 μM) for the MCF-7 cells after 48 h of treatment. The highest activity for both hybrids and both breast cancer lines was observed after 72 h of treatment (IC_{50} 1–2 μM), probably as the result of slow hydrolysis of their methyl ester functions.

1. Introduction

Nature has been always considered as a huge "laboratory" providing constantly innumerous compounds with significant biological activity. Lignans comprise a large family of optically active compounds isolated from plant extracts sharing a common feature, that is, two phenylpropanoid units coupled through the central carbons of their propane side chains. In plants, the biosynthesis of lignans is probably realized through a peroxidase enzyme-catalyzed free radical dimerization of HCA derivatives [1] or of their reduced analogs. This procedure, also known as phenol oxidative coupling (POC), generates a large library of compounds with structural diversity and hence an array of different biological activities [2], with the anticancer activity being probably the most important one. Indeed, polyphenolic compounds generated in nature exhibit a wide range of biological activities including anti-inflammatory, antioxidant, antiapoptotic and cytotoxic actions [3]. The naturally occurring lignan podophyllotoxin is the starting material for the synthesis of etoposide which is used against small cell lung cancer, lymphomas, leukemia or brain tumor [4].

Abbreviations: Boc, *tert*- butoxycarbonyl; CAPE, caffeic acid phenethyl ester; L-DOPA, (*S*)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid; ER, estrogen receptor; FCC, flash column chromatography; FBS, fetal bovine serum; HCA, hydroxycinnamic acid; PBS, phosphate-buffered saline; POC, phenol oxidative coupling; PyBrOP, br omotripyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid.

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Caffeic acid is one of the most prominent members of the HCA family. It is considered one of the constituents responsible for the antiproliferative effects of honeybee propolis [5]. In addition, many studies have further established the wide range of its potential antitumor activities. Its phenethyl ester (CAPE) can arrest the growth of human leukemia HL-60 cells [6], it can act as a potential antimetastatic agent [7] or it can play an important role for the treatment and/or prevention of estrogen-related diseases, such as breast or uterine cancer [8,9]. On the other hand, 1-aryl-1, 2-dihydronaphthalene **1a** (Fig. 1), which can be derived from the ferrous chloride-mediated oxidative dimerization of methyl caffeate, has shown a very interesting biological profile as a topoisomerase inhibitor [10] and was also found to be remarkably more active than caffeic acid against breast cancer cells [11].

Furthermore, *N*-*trans*-caffeoyl-L-DOPA (**2**), also known as Clovamide, belongs to the class of *N*-phenylpropenoyl amino acids and it was identified in the antioxidant polyphenolic fraction of *Theobroma cacao* L. [12]. Clovamide or its methyl ester **3** have shown a significant binding affinity for the p56lck SH2 domain, highlighting that way interesting properties toward the treatment of a wide range of diseases such as cancer, osteoporosis or chronic inflammatory disease [13].

Over the last years, the synthesis of new compounds called hybrids has gained ground among researchers. Hybrid molecules can be derived from the covalent connection (through cleavable or non-cleavable bonds) of two or more different chemical entities with the intention to enhance the biological activity that each one of these pharmacophores exhibits [14].

Prompted by the afore mentioned significant biological profiles of both caffeic acid dimer **1a** and hybrid compound Clovamide (**2**), we thought of interest to synthesize the diastereomeric lignan amides **4** and **5** (Fig. 1), which could serve as dimeric caffeic acid-L-DOPA hybrids, and to further explore their antiproliferative activity initially on two breast cancer cell lines, namely MDA-MB-231 and MCF-7 with different metastatic potential and estrogen receptor (ER) status, and one epithelial lung cancer cell line, namely A-549.

2. Results and discussion

2.1. Chemistry

The synthesis of the projected lignan amides **4** and **5** was accomplished using a convergent methodology. The two requested, suitably protected, building blocks **9** and **12** for the assembly of the skeleton of these amides (Scheme 3) were synthesized from L-DOPA (Scheme 1) and caffeic acid (Scheme 2), respectively. Compound **9** was obtained after TFA deprotection of fully protected compound **8** which was synthesized according to a published procedure [15] (see Supplementary material). Thus, commercially available L-DOPA was converted to its methyl ester **6** in 95% yield, after treatment with $SOCl_2$ in MeOH at ambient temperature. The latter was protected at its amino function with the Boc group, using di-*tert*-butyl dicarbonate in THF in the presence of a saturated aqueous solution of $NaHCO_3$ as base. That way, compound **7** was obtained in 85% yield. The next step involved the protection of the catechol unit, which was realized upon treatment of compound **7** with benzyl bromide in refluxing acetone in the presence of K_2CO_3. Following routine purification by FCC, the fully protected compound **8** was obtained in 92% yield. Finally, TFA-mediated N-deprotection provided the trifluoroacetate salt **9** in 93% yield.

On the other hand, compound **1a** was readily synthesized according to a published procedure [16]. Thus, methyl caffeate **10** [17] was subjected to a POC reaction with an aqueous solution of $FeCl_3 \cdot 6H_2O$ in acetone (Scheme 2). After overnight stirring at 0 °C, followed by FCC purification, compound **1a** was isolated as the main product in 38% yield (75% based on recovered starting material **10**), in the form of a mixture of enantiomers (only one enantiomer is drawn in the scheme).

The relative stereochemistry (*trans*) of the stereogenic centers C1 and C2 in dimer **1a** was established by measuring the coupling constant (J = 2.8 Hz) of protons H1 and H2 in the ^{1}H NMR spectrum of the synthesized dimer **1a**. This coupling constant is consistent with J values reported for other *trans*-aryldihydronaphthalene lignans [16,17]. Interestingly, quantum mechanical calculations (see Supplementary material) of the structures of the two possible diastereomers **1a** and **1b** (again only one enantiomer is drawn in the scheme) anticipated as POC products from this experiment, revealed that the dihedral angles H1-C1-C2-H2 (H31-C7-C8-H32 in Fig. 2) are −81.3 and 56.3°, respectively. The corresponding calculated J values for the two diastereomers **1a** (*trans*) and **1b** (*cis*) are 1.43 and 5.57 Hz, thus verifying the correctness of the proposed structure **1a** for the main product of POC of methyl caffeate. The use of quantum chemical methods in this work aims at the calculation of stable molecular structures. The use of rigorous quantum mechanical methods in recent years has added new impetus to extended scope disciplines as organic synthesis [18] and medicinal chemistry [19]. Highly performant quantum chemical methods can provide rational interpretations to chemical phenomena. What is more, the determination of accurate molecular descriptors via the calculation of molecular properties enables the development of realistic Quantitative Structure-Activity (QSAR) and Structure-Property (QSPR) Relationships [20]. The choice of level of theory usually employed in large molecular architectures depends on their size. As the size of the molecules increases, the computational cost of very accurate, time-consuming methods and large, flexible basis sets becomes prohibitive [21–24].

Furthermore, treatment of **1a** with benzyl bromide in refluxing acetone in the presence of K_2CO_3 led to the fully protected dimer **11** in 74% yield. Diester **11** was then subjected to regioselective saponification with 1.0 equivalent LiOH in THF/MeOH/H_2O to give mono-acid (±)−**12** in 55% yield.

Having both key-intermediates **9** and **12** at hand, the assembly of the projected skeleton was readily realized using PyBrOP as coupling agent in the presence of Et_3N. That way, the fully protected lignan amides **13** and **14** were obtained in 63% yield as a mixture of diastereomers. Complete separation and isolation of amides **13** and **14** in almost equimolar amounts was accomplished through FCC (Scheme 3). The final step of the synthesis involved the global deprotection of all catechol units. Due to the presence of the double bond in the dihydronaphthalene moiety, the obvious use of H_2 in the presence of Pd/C was not examined as the method of choice for the chemoselective removal of the benzyl groups. Interestingly, Lamidey et al. [25] had described a very efficient silane-promoted Pd-mediated hydrogenolysis en route the synthesis of (-)-(*2R*,*3R*)-chicoric acid. By applying this particular methodology to each of the diastereomeric amides **13** and **14**, the projected compounds **4** and **5** were obtained in 68% and 72% yield, respectively, following FCC purification.

The structure of compounds **4** and **5** was established by analytical and spectroscopic methods and in particular NMR. Table S5 (in supplementary material) contains the complete proton and carbon assignment of compounds **4** and **5**. In particular, the absolute stereochemistry (configuration) of the two stereogenic centers at C1 and C2 (the third stereogenic center at C11 is of the *S* configuration due to the L-DOPA used as starting material) of the diastereomers **4** and **5** was determined on the basis of NMR studies as well as quantum mechanical calculations of the structures of the two configurational arrangements, that is 1*S*,2*R*,11*S* (diastereomer **4**) and 1*R*,2*S*,11*S* (diastereomer **5**). A noticeable difference, which seems to be of particular diagnostic interest, is

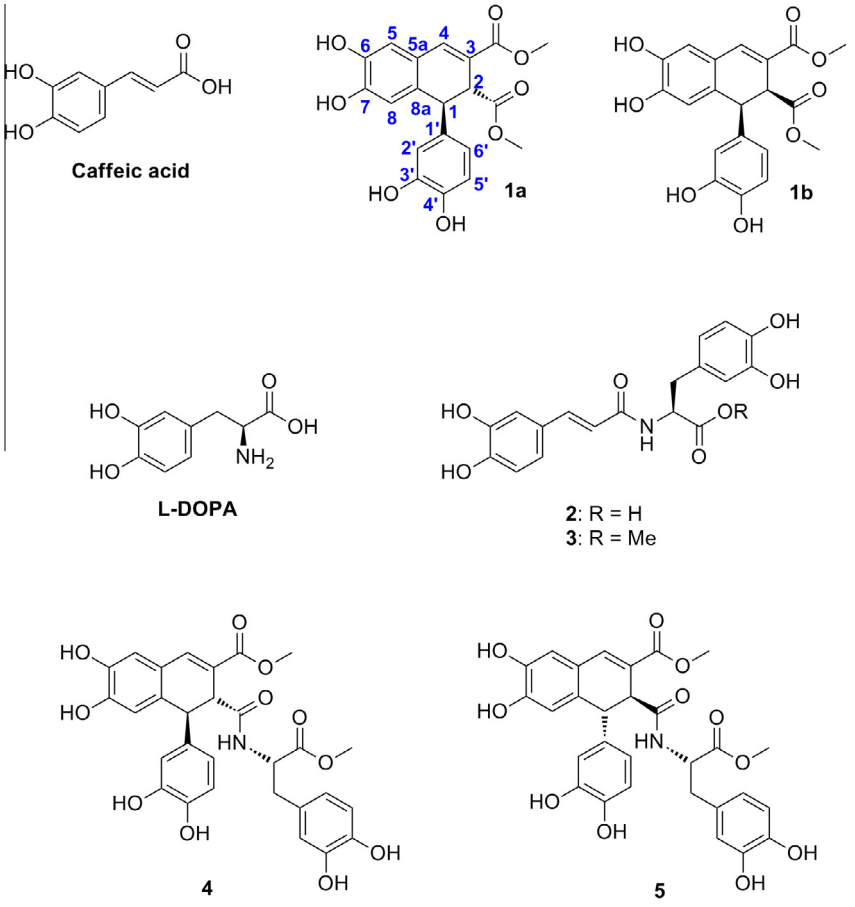
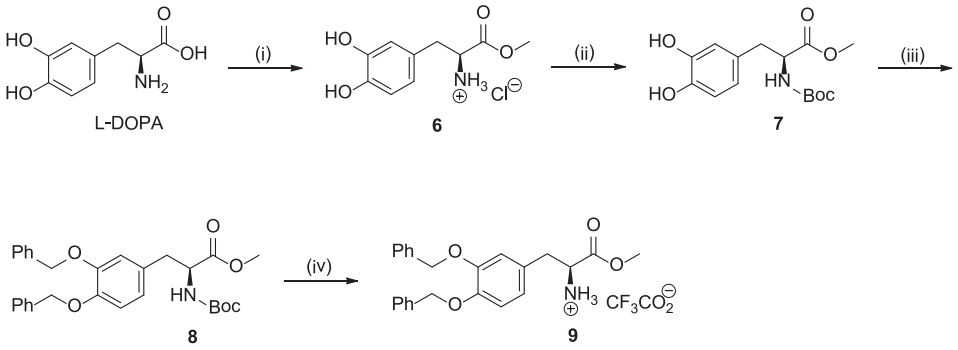


Fig. 1. Structures of compounds related to the present work.



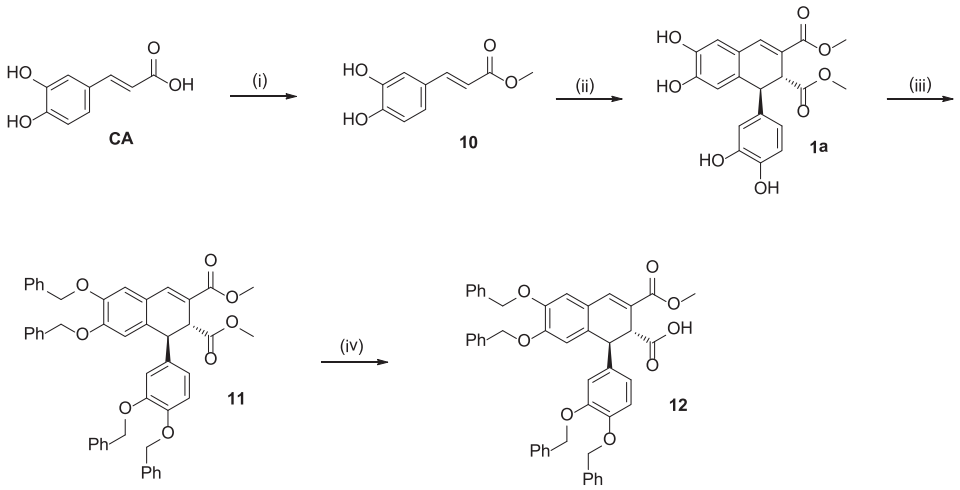
Scheme 1. Synthetic route for the preparation of suitably protected L-DOPA key-intermediate **9**. Reagents and conditions: (i) $SOCl_2$, MeOH, 16 h, RT, 95%; (ii) Boc_2O, sat. aq. $NaHCO_3$, THF, 1 h, RT, 85%; (iii) $PhCH_2Br$, K_2CO_3, Acetone, 5 h, reflux, 92%; and (iv) 50% TFA/CH_2Cl_2, 1 h, 93%.

in the chemical shifts and coupling patterns of protons H11 and $H12_a$ and $H12_b$ in the L-DOPA moiety of the two diastereomers. Thus, in the ^1H NMR spectrum of the less polar diastereomer (R_f = 0.22, see experimental section), these particular protons resonate at δ 4.499 and 2.815 ppm and appear as triplet (J = 6.4 Hz) and doublet (J = 6.4 Hz), respectively. On the other hand, in the ^1H NMR spectrum of the more polar diastereomer (R_f = 0.13, see experimental section), the H11 proton resonate at δ 4.476 as dd (J = 5.6 and 7.6 Hz) and the $H12_a$ and $H12_b$ protons resonate at 2.871 ppm as dd (J = 5.6 and 14 Hz) and at 2.746 also as dd (J = 7.6 and 14 Hz).

This is taken to mean that in the more polar diastereomer restricted rotation is imposed on the L-DOPA aliphatic chain, thus making the $H12_a$ and $H12_b$ protons diasterotopic. Quantum mechanical calculation of the two configurational arrangements, that is 1*S*, 2*R*, 11*S* (diastereomer **4**) and 1*R*, 2*S*, 11*S* (diastereomer **5**), showed (Fig. 3 and supplementary material) that restricted rotation is indeed imposed in the diastereomer **5** and thus the less polar diastereomer should be correlated to structure **4** and accordingly, the more polar diastereomer should be correlated to structure **5**.

2.2. Biology

In order to determine possible antiproliferative effects of the diastereomeric lignan amides **4** and **5** against cancer cells, the cell proliferation/viability rates in three cancer biological models, including two breast and one lung cancer cell lines, was evaluated.



Scheme 2. Synthetic route for the preparation of mono-acid (±)-**12**. Reagents and conditions: (i) H_2SO_4, MeOH, 1 h, reflux, 88%; (ii) $FeCl_3{\cdot}6H_2O$, Acetone, H_2O, overnight, 0 °C, 38% (75% based on recovered **10**); (iii) $PhCH_2Br$, K_2CO_3, Acetone, 16 h, reflux, 74%; and (iv) LiOH, THF/MeOH/H_2O (3:1:1), 16 h, RT, 55%.

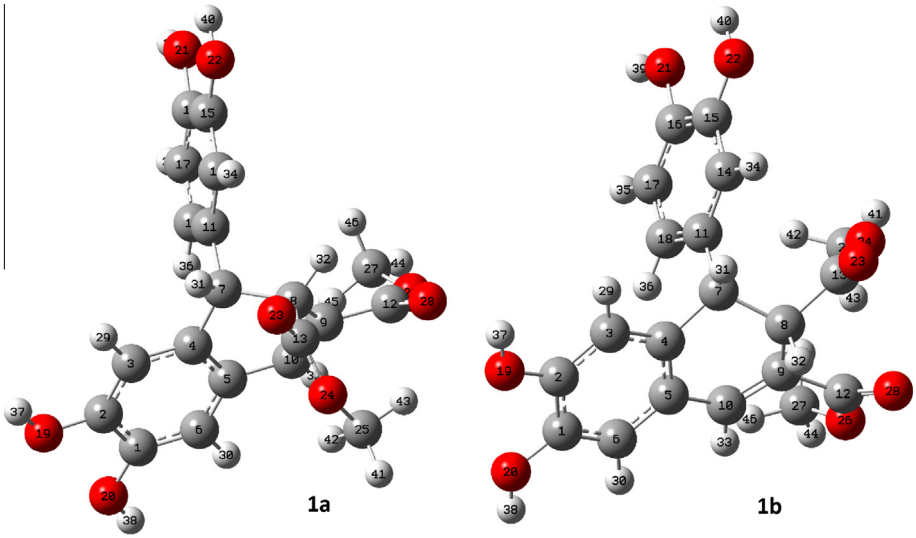
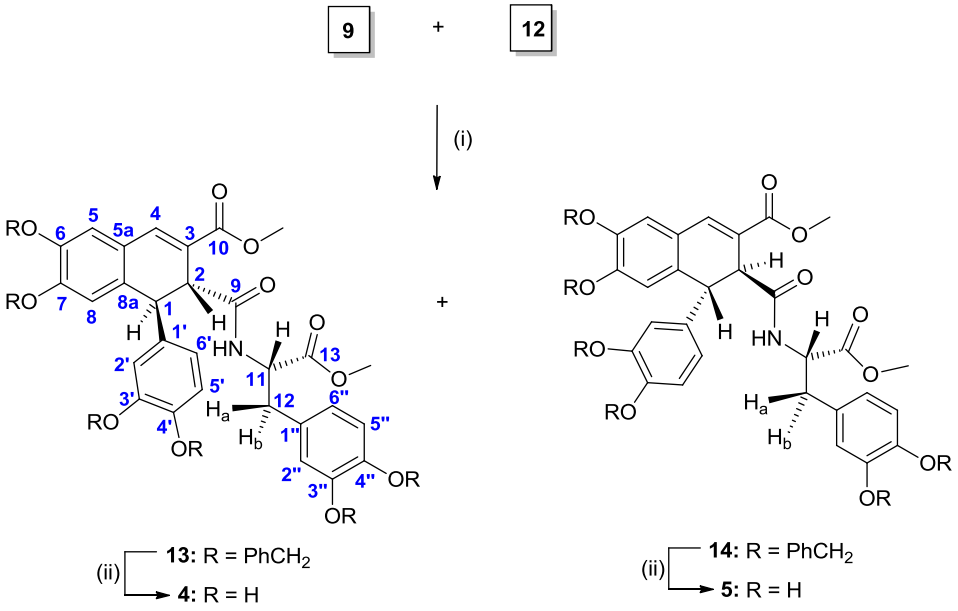


Fig. 2. Minimum energy structures of diastereomers **1a** (*trans*) and **1b** (*cis*) as obtained through quantum mechanical calculations.

We included in these studies the dimeric methyl caffeate **1a,** the methyl ester **3** of the amide hybrid caffeic acid-L-DOPA (Clovamide), and the methyl esters **6** and **10** of L-DOPA and caffeic acid, respectively, in order to draw meaningful conclusions on the effect of dimerization of caffeic acid on the potential antiproliferative activity of these compounds. The effects on cell proliferation were determined upon treatment of cancer cells with these compounds in increasing concentrations, ranging between 1 and 256 μM, and for time periods of 24, 48 and 72 h. In the present study, 0.1% DMSO in H_2O for dissolution and dilution of the sample to be tested was used. This concentration of DMSO was utilized since following pilot experiments no cytotoxic effects were found. The breast cancer cell lines used were MDA-MB-231 (highly metastatic breast cancer cell line) and MCF-7 (low metastatic potential breast cancer cell line), whereas that for lung cancer was A-549 epithelial lung cancer cell line.

The initial biological evaluation generally revealed that the majority of the compounds tested in the three cancer cell lines exhibited statistically significant antiproliferative effects in a dose- and in some cases time-dependent manner at most of the doses applied. It has been reported that the role of caffeic acid is increasingly regarded as oncoprotective [26], whereas compounds **3** and **6** exhibit anticancer activity. Indeed, it was generally observed that after 24 h of treatment with compounds **3** and **6**, cancer cells exhibited a dose-dependent response; this effect was more profound in higher concentrations. Furthermore, compounds **1a** and **10** induced a dose-dependent effect in the three cancer cell lines, after a 24 h treatment period with elevated concentrations of these compounds (1–256 μM). This effect was more intense in the more aggressive breast cancer cell line MDA-MB-231 with an IC_{50} value at low concentrations (16 μM). In addition, as for the two dimeric caffeic acid-L-DOPA hybrids, **4** and **5**, the effect on MDA-MB-231 breast cancer cell line was more intense than in MCF-7 low metastatic breast cancer cells.

In particular, the involvement of all tested compounds in the mediation of the proliferation capacity of A-549 lung cancer cells was first assessed. This assessment revealed that the compounds exhibited antiproliferative activity even at low concentrations (Fig. 4). More specifically, it was observed that compounds **3**, **5** and **6** exhibited dose-dependent effects on cell proliferation rates with IC_{50} values of 130 μM (Table 1). However, the hybrid **5** presented antiproliferative action even at the lowest concentration



Scheme 3. Synthesis of lignan amides **4** and **5**. Reagents and conditions: (i) Et_3N, PyBrOP, $CHCl_3$, 40 min, RT, 63%; and (ii) $Pd(OAc)_2$, Et_3N, Et_3SiH, CH_2Cl_2, overnight, RT, 68% (for **4**), 72% (for **5**).

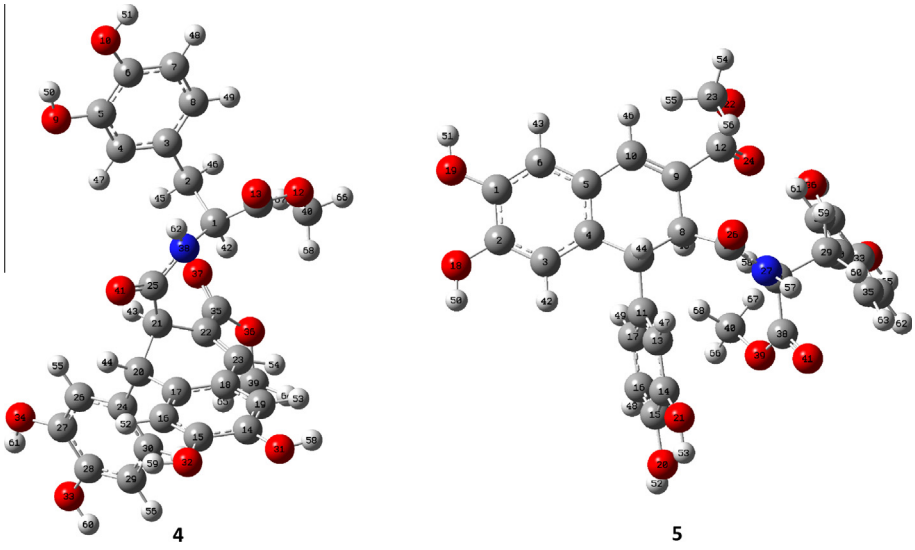


Fig. 3. Minimum energy structures of diastereomers **4** and **5** as obtained through quantum mechanical calculations.

tested (1 μM), as it reduced significantly cell viability (40%). On the other hand, hybrid **4** did not cause significant reduction of cell proliferation of lung cancer cells. Moreover, compounds **1a** and **10** exhibited stronger effects in a dose-dependent manner (IC_{50} 32 and 40 μM, respectively).

Thus, with the exception of **4**, there is a strong anticancer action of these compounds on A-549 lung cancer cells, after 24 h of treatment, a fact that can be correlated with the antiproliferative potential on our cancer cellular system, verifying the already known literature results mentioned above. Interestingly, dimerization of CA leads to a slightly better antiproliferative effect, whereas conjugation of L-DOPA to CA does not offer any particular advantage to L-DOPA but it lowers ca. 3 times the activity of CA. In addition, although conjugation of dimeric CA (**1a**) to L-DOPA, creating hybrid **5**, leads to a less active compound compared to **1a** itself. It appears that the relative stereochemistry of diastereomeric hybrids **4** and **5** plays a significant role in activity, with **4** being much less active than **5**.

As far as MDA-MB-231 breast cancer cells are concerned (Fig. 5), the obtained data revealed that, following 24 h incubation period, the hybrids **4** and **5** as well as compound **6** exhibited a dose-dependent effect in breast cancer cell proliferation, with IC_{50} values at higher concentrations. Although compound **3** exhibited a slight antiproliferative effect, it reduced significantly cell proliferation rates of the highly metastatic breast cancer cells (60%), as compared to untreated cells, at the highest concentration tested (256 μM). As for compounds **1a** and **10**, they pinpointed more intense effects among all the derivatives tested, as their IC_{50} values are 16 μM (Table 1).

Interestingly, dimerization of CA does not change the antiproliferative effect of the monomer (CA), whereas conjugation of L-DOPA to CA lowers ca. 2 times the activity of L-DOPA and 16 times the

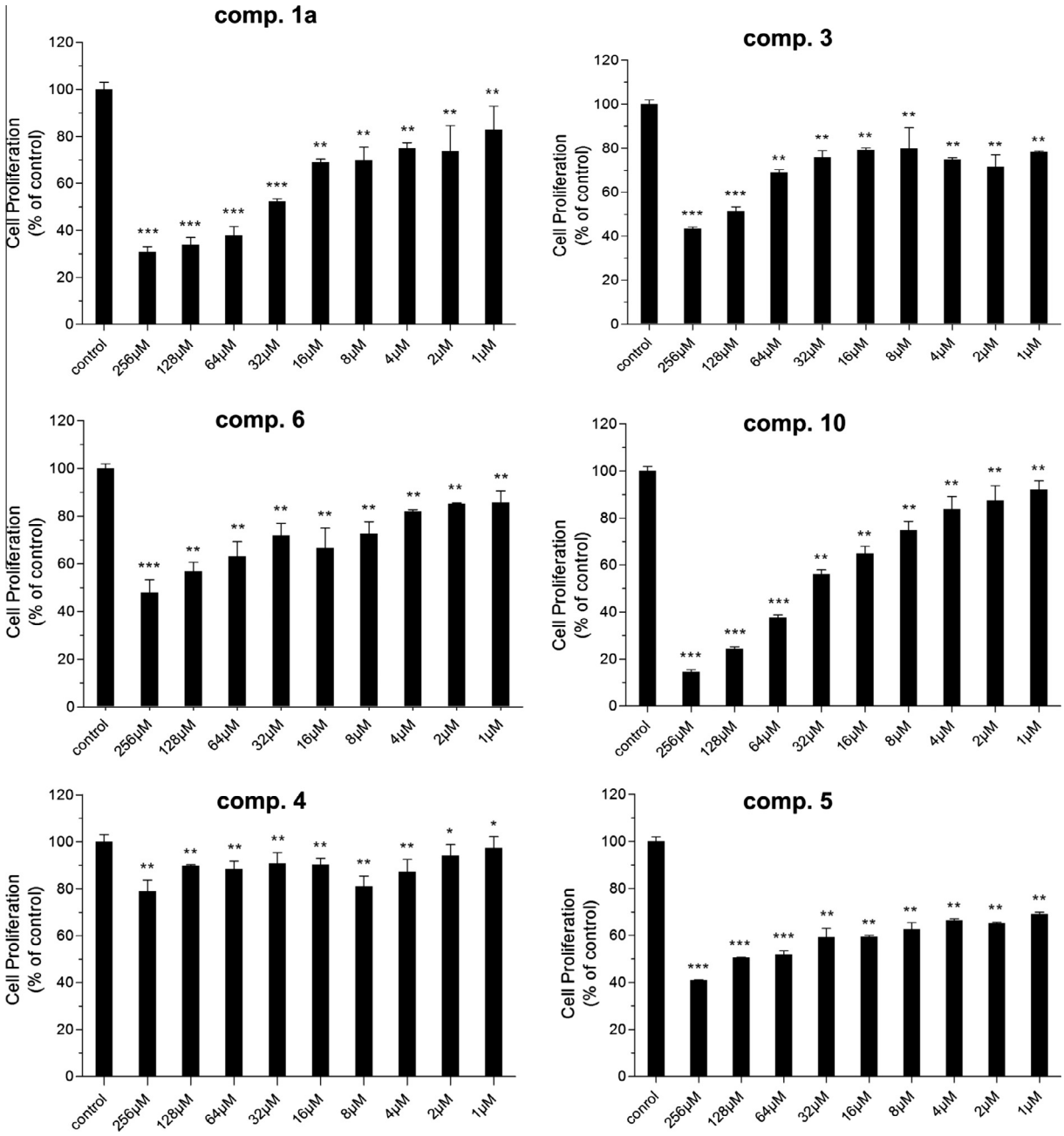


Fig. 4. Dose-dependent responses of A-549 lung cancer cells to the tested compounds. The concentration range was from 1 to 256 μM and the incubation time 24 h. The results are expressed as mean ± SD from experiments in triplicate. Symbols mark the statistically significant levels as follows: (*), (**) and (***) indicate $p < 0.05$, $p < 0.01$ and $p < 0.005$, respectively, as compared to control cells.

Table 1
IC_{50} values of three cancer cell lines upon treatment with the tested compounds for 24 h.

Compound	Cancer Cell Lines		
	A-549	MDA-MB-231	MCF-7
	IC_{50} Values (μM)		
1a	32	16	128
3	130	256	>256
6	130	130	>256
10	40	16	60
4	>256	64	>256
5	130	64	256

activity of CA. In addition, conjugation of dimeric CA (**1a**) to L-DOPA, creating hybrid **4** or **5**, leads to a 4 times less active compound compared to **1a** itself, it appears that in this cancer line the relative stereochemistry of diastereomeric hybrids **4** and **5** does not play any role in activity.

Treatment of MCF-7 breast cancer cells with these compounds for 24 h induced only slight effects in cell proliferation rates (Fig. 6). More specifically, in the presence of the hybrid **5** and also of compounds **3** and **6** a reduction in cell proliferation up to 40%, as compared to control cells, was observed. Moreover, cell proliferation reduced up to 50%, as compared to untreated cells, in the presence of both **5** and **1a** only near the highest concentration tested (256 μM). Only in the case of **10** there is a strong antiproliferative effect in the proliferation capacity of MCF-7 breast cancer cells.

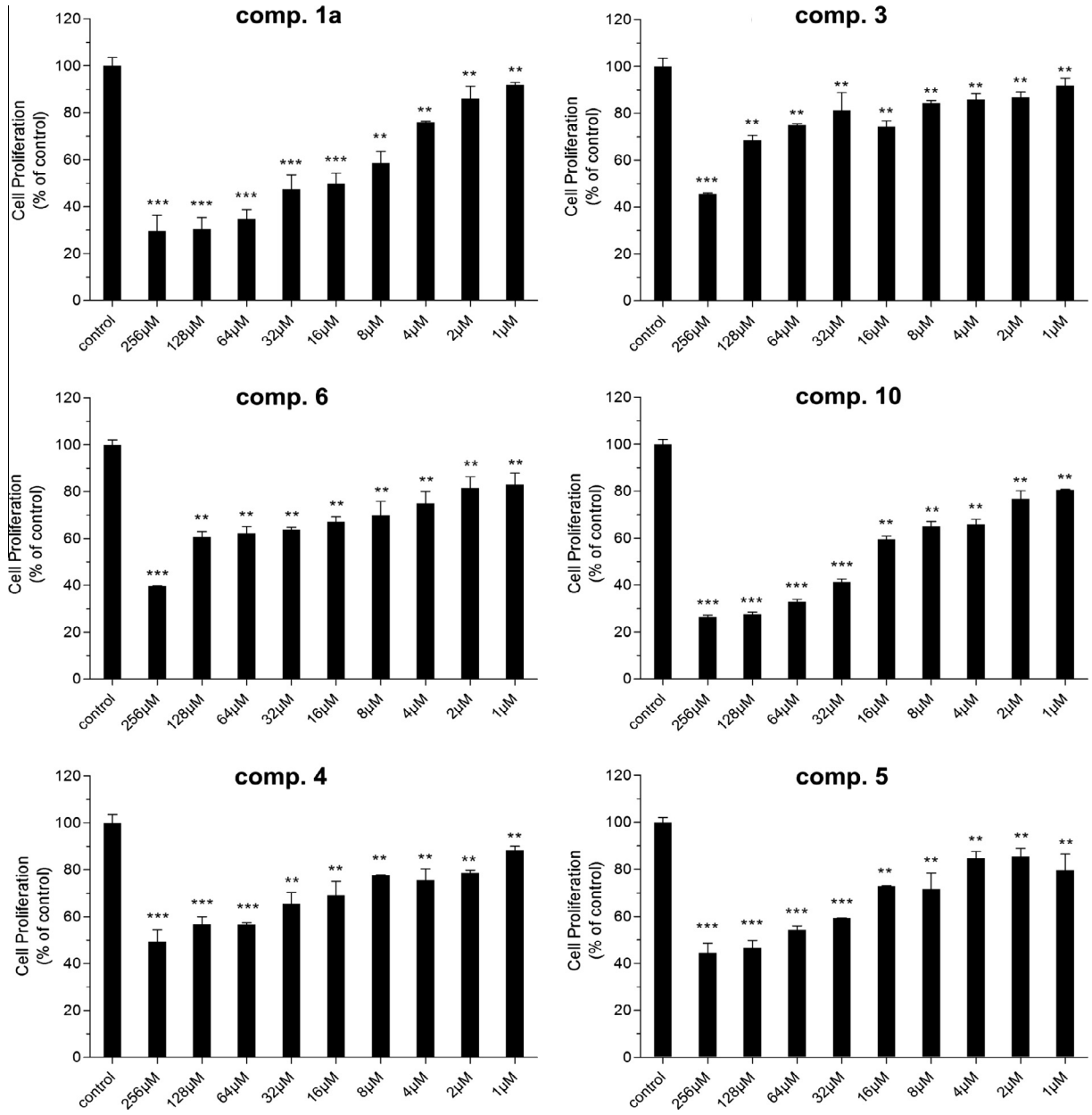


Fig. 5. Dose-dependent responses of MDA-MB-231 breast cancer cells to the tested compounds. The concentration range was from 1 to 256 μM and the incubation time 24 h. The results are expressed as mean ± SD from experiments in triplicate. Symbols mark the statistically significant levels as follows: (**) and (***) indicate $p < 0.01$ and $p < 0.005$, respectively, as compared to control cells.

Interestingly, dimerization of CA almost halves the antiproliferative effect of the monomer (CA), whereas conjugation of L-DOPA to CA does not seem to offer any particular advantage on the activity of L-DOPA. In addition, conjugation of dimeric CA (**1a**) to L-DOPA, creating hybrid **4** or **5**, almost halves the activity of **1a** itself. Also, in this cancer line the relative stereochemistry of diastereomeric hybrids **4** and **5** does not seem to play any noticeable role in activity.

Taking into consideration these data, it is plausible to suggest that the anticancer effect of the derivatives tested for a short-term treatment is related with the metastatic potential of breast cancer cells and that there is a potential for more intense effects after long-term treatment with these compounds.

It is well established that MDA-MB-231 and MCF-7 breast cancer cells are characterized by different ER status. More specifically, MCF-7 breast cancer cells are indicated as ERα positive, whereas MDA-MB-231 cells are positive for ERβ and negative for ERα [27–29]. This discrimination may be correlated with the differentiated metastatic potential of these two breast cancer cell lines. While ERα contribution in breast cancer growth and development has been thoroughly studied, the role of its isoform ERβ is less elucidated. In addition, it is documented that both ERα and ERβ present antagonistic actions, regulating the cell behavior in breast cancer initiation and differentiation [30,31].

Taking into account the fact that the methyl esters are slowly catabolized in the body, the antiproliferative effect of the two new hybrids **4** and **5** after a long-term treatment (48, 72 h) was evaluated. We focused on the breast cancer cell lines, in order to highlight the possible anticancer effect in the breast cancer cellular model. We expected that the IC_{50} of the hybrids will be lower than that observed following a 24 h treatment. Indeed, it was found that after 48 h treatment with hybrids **4** and **5**, the IC_{50} values (Table 2) were significantly lower than those of 24 h of treatment. More

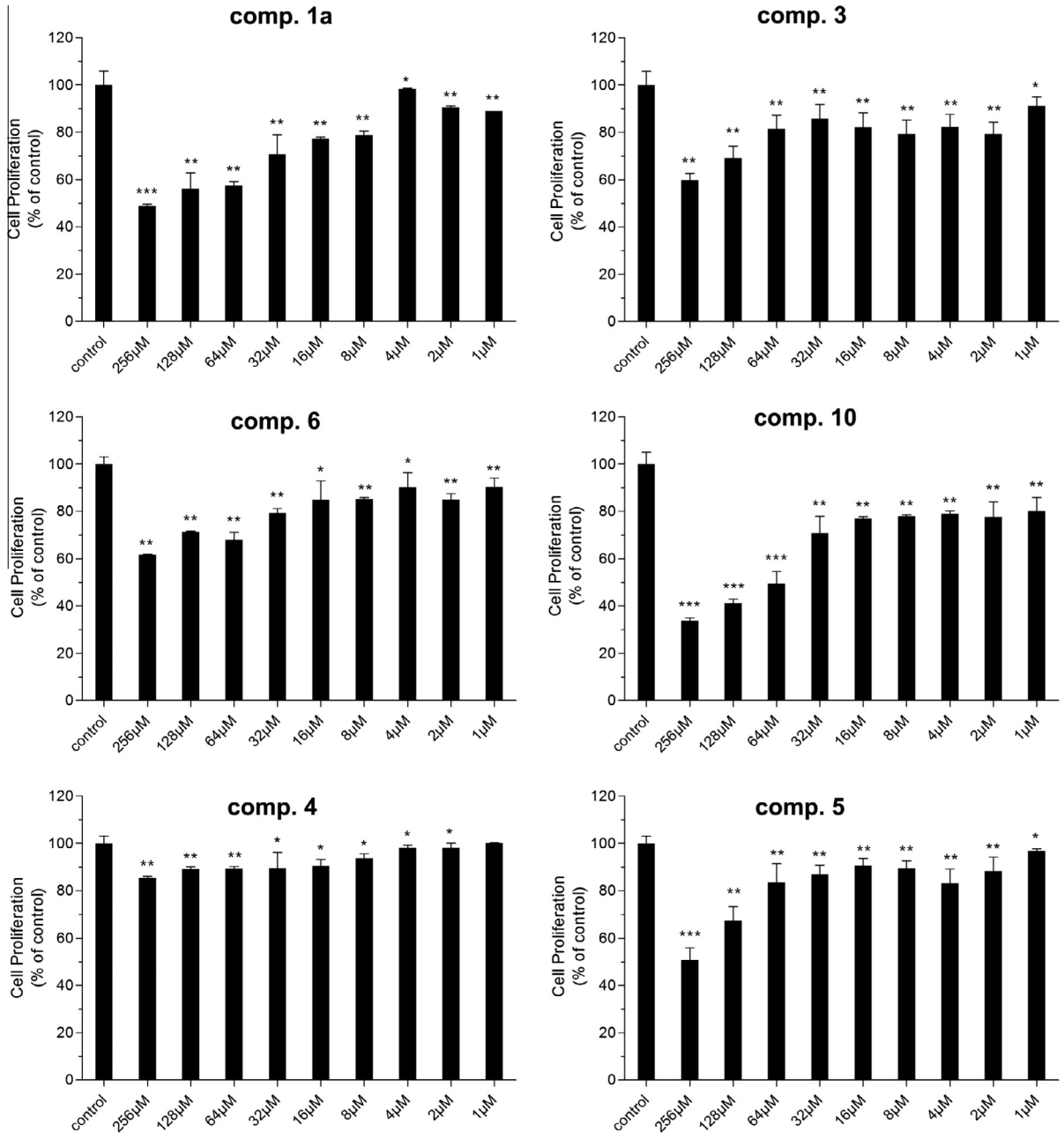


Fig. 6. Dose-dependent responses of MCF-7 breast cancer cells to the tested compounds. The concentration range was from 1 to 256 μM and the incubation time 24 h. The results are expressed as mean ± SD from experiments in triplicate. Symbols mark the statistically significant levels as follows: (*), (**) and (***) indicate $p < 0.05$, $p < 0.01$ and $p < 0.005$, respectively, as compared to control cells.

specifically, as far as MDA-MB-231 breast cancer cells are concerned, it was observed that 48 h of treatment with both **4** and **5** resulted in a dose-dependent decrease in proliferation rates with increased concentration (Fig. 7).

This effect becomes more profound when highly metastatic breast cancer cells are treated with the tested materials for 72 h (Fig. 8). In this case, a 1 μM IC_{50} value for both **4** and **5** was calculated. This is significantly lower compared to that following a 48 h treatment (IC_{50} value was 70 μM for both tested hybrids). As for MCF-7 breast cancer cell line, our data revealed that the IC_{50} values after 48 h treatment were approximately 16 μM and 4 μM regarding **4** and **5**, respectively (Fig. 7). On the other hand, when these cells were long-term-treated with the tested materials (72 h) the IC_{50} values become significantly lower (2 μM and 1 μM regarding **4** and **5**, respectively) (Fig. 6). It is therefore apparent that the relative stereochemistry of the two hybrids does not play any significant role in the highly metastatic cancer lines after long-term treatment whereas for the MCF-7 cancer lines hybrid **4** seems to

Table 2
IC_{50} values of breast cancer cell lines (MDA-MB-231 and MCF-7) upon treatment with the tested compound for 48 and 72 h.

Compound	Cancer Cell Lines			
	MDA-MB-231		MCF-7	
	48 h	72 h	48 h	72 h
	IC_{50} Values (μM)			
4	70	1	16	2
5	70	1	4	1

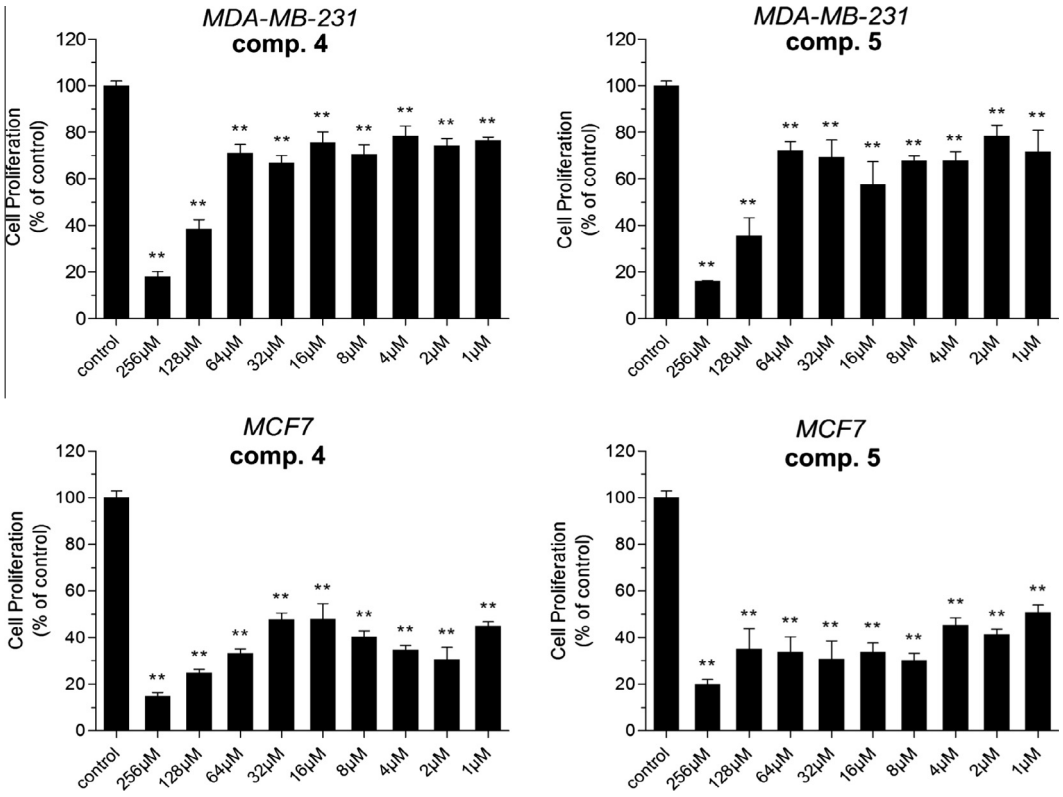


Fig. 7. Dose-dependent responses of MDA-MB-231 and MCF-7 breast cancer cells to the tested diastereomeric lignan amides **4** and **5**. The concentration range was from 1 to 256 μM and the incubation time 48 h. The results are expressed as mean ± SD from experiments in triplicate. Symbols mark the statistically significant levels as follows: (**) indicate $p < 0.01$, as compared to control cells.

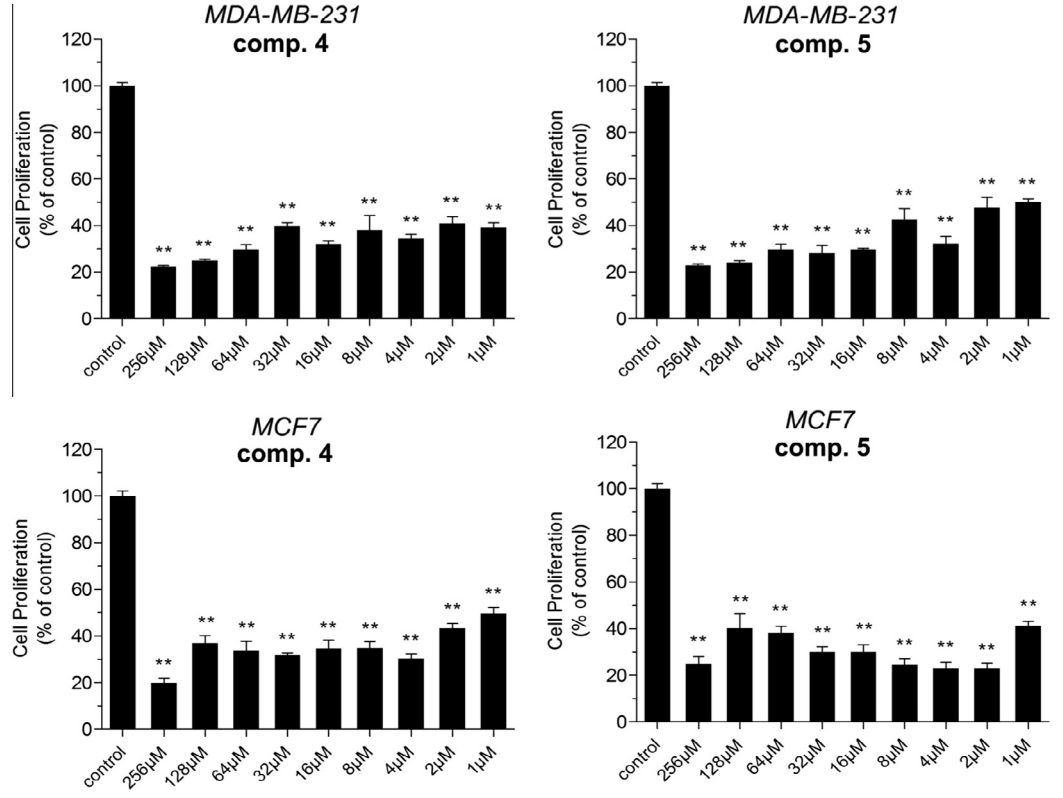


Fig. 8. Dose-dependent responses of MDA-MB-231 and MCF-7 breast cancer cells to the tested diastereomeric lignan amides **4** and **5**. The concentration range was from 1 to 256 μM and the incubation time 72 h. The results are expressed as mean ± SD from experiments in triplicate. Symbols mark the statistically significant levels as follows: (**) indicate $p < 0.01$, as compared to control cells.

be 4 and 2 times more active than hybrid **5** after 48 and 72 h treatment, respectively.

3. Conclusions

Two diastereomeric amide-type hybrids **4** and **5** of POC-derived dimeric CA with L-DOPA were efficiently synthesized using a convergent methodology. The two key-intermediates required for the assembly of the skeleton of the hybrids, namely compounds **9** and **12** were obtained in ca. 30% and 69% overall yield from the commercially available L-DOPA and caffeic acid, respectively. Coupling of these intermediates, afforded two diastereomeric compounds (**13** and **14**) which could be easily separated by FCC. Each one of these fully protected compounds was subjected to a palladium-mediated chemoselective deprotection of the catechol functionalities providing an almost equimolar mixture of hybrids **4** and **5**, in 44% yield for the two steps.

The antiproliferation effect of the hybrids **4** and **5**, the dimeric methyl caffeate **1a,** the methyl ester **3** of the CA-L-DOPA (Clovamide), and the methyl esters **6** and **10** of L-DOPA and CA, respectively, on the epithelial lung cancer cell line A-549, the highly metastatic breast cancer cell line MDA-MB-231 and the low metastatic potential breast cancer cell line MCF-7 was evaluated for concentrations ranging from 1 to 256 μM and for periods of treatment of 24, 48 and 72 h. The preliminary biological evaluation of the compounds at 24 h revealed that hybrids **4** and **5** were particularly active and equipotent on the MDA-MB-231 cell line and of very low activity on the MCF-7 cell line, whereas on the A-549 cell line hybrid **5** was substantially more active than hybrid **4**. Interestingly, most active compounds in on all three cell lines examined were the methyl esters of monomeric (**10**) and the dimeric (**1a**) CA, thus showing that their conjugation to L-DOPA does not offers any advantage on their antiproliferative potential. Also, dimerization of CA seems not to lead to a particularly more active compound.

In particular, as concerns the breast cancer cells, the antiproliferative effects of these hybrids were much stronger in MCF-7 breast cancer cells after 48 and 72 h treatment, but in both breast cancer cell lines, there was a dose-dependent effect, as it was conducted by the rudimentary biological evaluation. It has been reported that CA derivatives, such as CAPE, have estrogenic effects and it seems to have selective binding affinity to ERβ rather than ERα [9]. Indeed, we observed that our compounds exhibited higher IC_{50} values for the MDA-MB-231 than the MCF-7 breast cancer cells, but only for the 24 h treatment, whereas for longer periods of treatment, that is 48 h, both hybrids showed much higher activity for the MCF-7 cell line with hybrid **4** being 4 times less active than hybrid **5**. A powerful antiproliferative effect is however observed after 72 h of treatment for both hybrids and both cell lines with hybrid **4** being more selective for MDA-MB-231. The higher antiproliferative activity of hybrids **4** and **5** after longer periods of treatment is probably due to the slow hydrolysis of their methyl ester moieties, indicating that these compounds act as prodrugs. The observed differentiated effects of these compounds among the two breast cancer cell lines suggest that there is a correlation with the different ER status among them. This generates the suggestion that the present dimeric CA-L-DOPA hybrids serve as selective estrogen receptor modulators, which identifies them as effective tools for breast cancer focused treatment approaches.

4. Material and methods

4.1. Chemistry

Melting points were determined with a Buchi SMP-20 apparatus and are uncorrected. IR spectra were recorded as KBr pellets, unless otherwise stated, on a Perkin-Elmer 16PC FT-IR spectrophotometer. Routine ^{1}H NMR spectra were recorded at 400.13 MHz and ^{13}C NMR spectra at 100.62 MHz on a Bruker DPX spectrometer. Tetramethylsilane (TMS) was used as internal standard. Chemical shifts are reported in δ units, parts per million (ppm) downfield from TMS. 2D homonuclear and heteronuclear NMR experiments were performed on a Bruker AV-500, equipped with a cryoprobe. Electro-spray ionization (ESI) mass spectra were recorded on a Micromass-Platform LC spectrometer using MeOH as solvent. Elemental analyses were determined on a Carlo Erba EA 1108 CHNS elemental analyzer in the Instrumental Analysis Centre of the University of Patras and the results are within ±0.4% of the calculated values. Optical rotations were determined with a half-automatic Schmidt + Haensch Polatronic D MHZ-8 at the sodium D line (589 nm) using a 500 mm path-length cell and are reported as follows: $[a]_D^{25}$, concentration (g/100 mL) and solvent. FCC was performed on Merck silica gel 60 (230–400 mesh) and TLC on 60 Merck 60 F254 films (0.2 mm) precoated on aluminum foil. Spots were visualized with UV light at 254 nm and the ninhydrin or the charring agent. All solvents were dried and/or purified according to standard procedures prior to use. Anhydrous Na_2SO_4 was used for drying organic solvents and subsequently solvents were routinely removed at ca. 40 °C under reduced pressure (water aspirator). All reagents employed in the present work were purchased from commercial suppliers and used without further purification. Reactions were run in flame-dried glassware under an atmosphere of argon with the exception of those involving aqueous solutions. Clovamide methyl ester (**3**) [13] was synthesized according to published procedure.

4.1.1. Dimethyl 1-(3,4-dihydroxyphenyl)-6,7-dihydroxy-1,2-dihydronaphthalene-2,3-dicarboxylate *[(±)-1a]*

To a solution of ester **10** (3.88 g, 20 mmol) in acetone (120 mL), a solution of $FeCl_3{\cdot}H_2O$ (6.05 g, 22.4 mmol) in H_2O (5.2 mL) was added at 5 °C over 1.5 h and the mixture was kept at 0 °C overnight. Then, the reaction mixture was evaporated to a minimum volume and extracted thrice with Hex/EtOAc (1:1). The combined organic layers were washed twice with water, dried over Na_2SO_4 and evaporated to dryness. The residue was subjected to FCC using $CHCl_3$/MeOH (95:5) as eluent to collect unreacted caffeic acid (1.54 g) and then $CHCl_3$/MeOH (9:1) to obtain pure **(±)-1a**.

Yield: 1.47 g (38%); white solid, mp: 238–240 °C, lit [16]. mp: 239–242 °C; R_f ($CHCl_3$/MeOH 9:1): 0.26; IR (KBr, cm^{-1}): 3384, 1716, 1686, 1610, 1458, 1266; MS (ESI, 30 eV): *m/z* 795.19 [2 M +Na], 425.30 [M+K], 409.37 [M+Na]; ^{1}H NMR (d_6-DMSO): δ 7.56 (s, 1H), 6.88 (s, 1H), 6.56 (d, *J* = 8.0 Hz, 1H), 6.48 (s, 1H), 6.30 (d, *J* = 2.0 Hz, 1H), 6.25 (dd, *J* = 2.0 and 8.0 Hz, 1H), 4.27 (d, *J* = 2.8 Hz, 1H), 3.72 (d, *J* = 2.8 Hz, 1H), 3.65 (s, 3H), 3.53 (s, 3H); ^{13}C NMR (d_6-DMSO): δ 172.8, 167.0, 148.4, 145.3, 144.8, 144.4, 138.5, 134.6, 129.5, 123.0, 120.9, 118.4, 116.9, 116.6, 115.9, 115.0, 52.6, 52.1, 47.5, 45.0.

4.1.2. Dimethyl 6,7-bis(benzyloxy)-1-(3,4-bis(benzyloxy)phenyl)-1,2-dihydronaphthalene-2,3-dicarboxylate *[(±)-11]*

To a suspension of **(±)-1** (0.96 g, 2.48 mmol) and K_2CO_3 (2.73 g, 19.86 mmol) in acetone (10 mL), benzyl bromide (2.36 mL, 19.86 mmol) was added. The reaction mixture was refluxed overnight and then filtrated under vacuo. The filtrate was evaporated; the residue was taken up in CH_2Cl_2, washed twice with water, dried over dried over Na_2SO_4 and evaporated to dryness. The oily residue was subjected to FCC using Hex/EtOAc (9:1) to elute excess benzyl bromide and then Hex/EtOAc (7:3) to obtain compound **(±)-11**.

Yield: 1.37 g (74%); light yellow oil; R_f (Hex/EtOAc7:3): 0.25; IR (KBr, cm^{-1}): 3030, 2950, 1734, 1634, 1508, 1238, 1012, 738, 696;

MS (ESI, 30 eV): *m/z* 785.23 [M+K], 769.30 [M+Na], 747.19 [M+H]; ^{1}H NMR ($CDCl_3$): δ 7.53 (s, 1H), 7.50–7.31 (m, 20H), 6.92 (s, 1H), 6.79 (d, J = 8.0 Hz, 1H), 6.78 (s, 1H), 6.54 (d, J = 8.0 Hz, 1H), 6.52 (s, 1H), 5.20 (s, 2H), 5.13 (s, 2H), 5.06 (s, 2H), 5.02 (s, 2H), 4.55 (d, J = 2.8 Hz, 1H), 3.95 (d, J = 2.8 Hz, 1H), 3.76 (s, 3H), 3.62 (s, 3H); ^{13}C NMR ($CDCl_3$): δ 172.8, 166.9, 150.9, 148.5, 148.0, 147.8, 137.5, 137.4, 137.2, 137.1, 136.6, 135.6, 130.9, 128.6 (two C), 128.5 (four C), 128.4 (two C), 127.9 (two C), 127.7 (two C), 127.4 (two C), 127.3 (two C), 127.2 (four C), 124.6, 122.6, 120.5,115.6, 115.0, 114.9, 114.7, 71.5, 71.3, 71.1, 70.9, 52.4, 51.9, 47.2, 45.5.

*4.1.3. 6,7-bis(benzyloxy)-1-(3,4-bis(benzyloxy)phenyl)-3-(methoxycarbonyl)-1,2-dihydronaphthalene-2-carboxylic acid [**(±)-12**]*

To an ice-cold solution of **(±)-11** (1.3 g, 1.74 mmol) in THF/MeOH (3:1, 14 mL), a solution of LiOH (0.042 g, 1.74 mmol) in H_2O (3.5 mL) was added and the reaction mixture was stirred at ambient temperature overnight. Then, it was acidified with 0.5 N HCl and extracted twice with EtOAc. The combined organic layers were washed twice with water, dried over Na_2SO_4 and evaporated to dryness. The residue was subjected to FCC using $CHCl_3$/MeOH (95:5) as eluent to afford compound **(±)-12**. Unreacted starting material can be recovered from FCC and subjected once again to saponification.

Yield: 0.70 g (55%); colorless oil; R_f ($CHCl_3$/MeOH 95:5): 0.20; IR (KBr, cm^{-1}): 3300–2900, 1704, 1602, 1508, 1240, 1134, 1014, 737, 696; MS (ESI, 30 eV): *m/z* 771.25 [M+K], 755.19 [M+Na]; ^{1}H NMR ($CDCl_3$): δ 7.54 (s, 1H), 7.49–7.29 (m, 20H), 6.92 (s, 1H), 6.77 (d, J = 8.4 Hz, 1H), 6.71 (s, 1H), 6.49 (d, J = 8.4 Hz, 1H), 6.47 (s, 1H), 5.18 (s, 2H), 5.12 (s, 2H), 5.01 (s, 2H), 5.00 (s, 2H), 4.89 (d, J = 2.8 Hz, 1H), 3.92 (d, J = 2.8 Hz, 1H), 3.76 (s, 3H); ^{13}C NMR ($CDCl_3$): δ 177.0, 167.6, 151.2, 148.5, 148.1, 147.8, 138.1, 137.4, 137.1, 137.0, 136.5, 135.6, 130.8, 128.6 (two C), 128.5 (four C), 128.4 (two C), 128.0 (two C), 127.8 (two C), 127.5 (two C), 127.3 (two C), 127.2 (four C), 124.2, 121.2, 120.3, 115.7, 115.0, 114.8, 114.6, 71.6, 71.2, 71.1, 70.9, 52.2, 47.1, 44.9.

*4.1.4. Synthesis of lignan amides **13** and **14***

To an ice-cold solution of amine **9** (0.54 g, 1.05 mmol), acid **(±)-12** (0.6 g, 0.81 mmol) and Et_3N (0.45 mL, 3.24 mmol) in $CHCl_3$ (1.2 mL), PyBrOP (0.48 g, 1.05 mmol) was added. The mixture was stirred at 0 °C for 10 min and then at ambient temperature for additional 30 min. Then, the reaction mixture was diluted with $CHCl_3$, washed twice with water, dried over Na_2SO_4 and evaporated to dryness to give a mixture of diastereomers which were separated by routine FCC using Hex/EtOAc (6:4) as eluent.

*4.1.4.1. Methyl (1S,2R)-6,7-bis(benzyloxy)-1-(3,4-bis(benzyloxy)phenyl)-2-(((S)-3-(3,4-bis(benzyloxy)phenyl)-1-methoxy-1-oxopropan-2-yl)carbamoyl)-1,2-dihydronaphthalene-3-carboxylate (**13**).* Yield: 0.30 g (33%); White foam; $[a]_D^{25}$ = +132 (*c* 1.0, $CHCl_3$); R_f (Hex/EtOAc 6:4): 0.26; IR (KBr, cm^{-1}): 3033, 2924, 1740, 1686, 1508, 1240, 1136, 1018, 736, 696; MS (ESI, 30 eV): *m/z* 1144.39 [M+K], 1128.39 [M+Na], 1106.03 [M+H]; ^{1}H NMR ($CDCl_3$): δ 7.47–7.27 (m, 32H), 6.85 (s, 1H), 6.80 (d, J = 7.2 Hz, 1H), 6.77 (d, J = 5.6 Hz, 1H), 6.74 (d, J = 8.0 Hz), 6.64 (d, J = 1.6 Hz, 1H), 6.48 (s, 1H), 6.47 (d, J = 7.2 Hz, 1H), 6.31 (dd, J = 1.6 and 8.0 Hz, 1H), 5.13–4.99 (m, 12H), 4.75 (m, 1H), 4.61 (unresolved d, 1H), 3.74 (unresolved d, 1H), 3.67 (s, 3H), 3.66 (s, 3H), 2.97 (dd, J = 5.2 and 14.0 Hz, 1H), 2.86 (dd, J = 6.8 and 14.0 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ 171.8, 170.8, 167.7, 151.6, 148.9, 148.3, 147.8, 147.6, 147.5, 138.6, 137.3, 137.2, 137.1, 137.0, 136.9, 136.5, 136.2, 132.4, 129.0, 128.4–127.1 (13 signals, thirty C), 123.6, 122.0, 121.3, 120.3, 115.7, 115.6, 114.9, 114.7, 114.6, 114.5, 71.4, 71.2, 71.1, 71.0, 70.9, 70.6, 53.0, 52.1, 52.0, 48.1, 44.5, 37.0.

*4.1.4.2. Methyl (1R,2S)-6,7-bis(benzyloxy)-1-(3,4-bis(benzyloxy)phenyl)-2-(((S)-3-(3,4-bis(benzyloxy)phenyl)-1-methoxy-1-oxopropan-2-yl)carbamoyl)-1,2-dihydronaphthalene-3-carboxylate (**14**).* Yield: 0.27 g (30%); colorless oil; $[a]_D^{25}$ = −142 (*c* 0.66, $CHCl_3$); R_f (Hex/EtOAc 6:4): 0.15; IR (KBr, cm^{-1}): 3029, 2930, 1735, 1671, 1517, 1248, 1136, 1022, 741, 696; MS (ESI, 30 eV): *m/z* 1144.23 [M+K], 1128.31 [M+Na], 1106.35 [M+H]; ^{1}H NMR ($CDCl_3$): δ 7.53 (s, 1H), 7.48–7.29 (m, 30H), 6.88 (d, J = 8.0 Hz, 1H), 6.87 (s, 1H), 6.78–6.72 (m, 3H), 6.68 (d, J = 8.0 Hz, 1H), 6.59 (unresolved dd, 1H), 6.51 (unresolved dd, 1H), 6.48 (unresolved d, 1H), 5.18–4.96 (m, 12H), 4.72–4.66 (m, 1H), 4.56 (unresolved d, 1H), 3.75 (unresolved d, 1H), 3.70 (s, 3H), 3.51 (s, 3H), 3.00–2.96 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 171.4, 170.9, 167.5, 151.5, 148.9, 148.3, 148.0, 147.6, 147.5, 138.7, 137.3, 137.2, 137.1, 137.0 (two C), 136.5, 136.3, 132.1, 129.2, 128.5–127.1 (14 signals, thirty C), 123.6, 122.2, 121.5, 120.3, 115.9, 115.6, 115.0, 114.6, 114.5 (two C), 71.4, 71.3, 71.2, 71.1, 70.9, 70.6, 53.2, 52.0, 51.9, 48.3, 44.8, 37.2.

*4.1.5. Global deprotection – Synthesis of hybrids **4** and **5***

To a solution of $Pd(OAc)_2$ (0.081 g, 0.36 mmol) in CH_2Cl_2 (2.5 mL) Et_3N (50 μL, 0.36 mmol) was added and the mixture was stirred for 10 min. Then, a solution of **13** or **14** (0.17 g, 0.15 mmol) in CH_2Cl_2 (2.5 mL) was added dropwise over 30 min. The resulting mixture was stirred for 10 min followed by the dropwise addition of Et_3SiH (0.58 mL, 3.6 mmol) over 1 h. Finally, the reaction was stirred at ambient temperature overnight. Addition of MeOH followed by filtration and evaporation of the filtrate gave an oily residue which was diluted EtOAc, washed twice with an aqueous solution 5% citric acid and once with water, dried over Na_2SO_4 and evaporated to dryness. Final products were obtained after FCC purification using $CHCl_3$/MeOH (8:2) as eluent.

*4.1.5.1. Methyl (1S,2R)-1-(3,4-dihydroxyphenyl)-2-(((S)-3-(3,4-dihydroxyphenyl)-1-methoxy-1-oxopropan-2-yl)carbamoyl)-6,7-dihydroxy-1,2-dihydronaphthalene-3-carboxylate (**4**).* Yield: 0.057 g (68%); yellowish solid, mp: 234–237 °C (decomp.); $[a]_D^{25}$ = +197 (*c* 0.6, MeOH); R_f ($CHCl_3$/MeOH 8:2): 0.22; IR (KBr, cm^{-1}): 3380, 2924, 1726, 1692, 1642, 1606, 1518, 1440, 1336, 1262; MS (ESI, 30 eV): *m/z* 604.23 [M+K], 588.23 [M+Na], 566.38 [M+H]; ^{1}H NMR (d_4-MeOH): δ 7.61 (s, 1H), 6.86 (s, 1H), 6.61 (d, J = 2.0 Hz, 1H), 6.59 (d, J = 2.0 Hz, 1H), 6.52 (s, 1H), 6.47 (d, J = 8.0 Hz, 1H), 6.40 (d, J = 8.0 Hz, 1H), 6.38 (dd, J = 2.0 and 8.0 Hz, 1H), 6.15 (dd, J = 2.0 and 8.0 Hz, 1H), 4.50 (t, J = 6.4 Hz, 1H), 4.27 (d, J = 2.8 Hz, 1H), 3.71 (d, J = 2.8 Hz, 1H), 3.67 (s, 3H), 3.65 (s, 3H), 2.81 (d, J = 6.4 Hz, 2H); ^{13}C NMR (d_4-MeOH): δ 172.8, 171.8, 167.8, 148.1, 144.8, 144.6, 144.1, 143.9, 143.5, 139.7, 135.2, 130.6, 127.4, 123.2, 120.8, 120.3, 118.6, 116.1, 116.0, 115.7, 115.0, 114.8, 114.4, 53.7, 51.3, 51.1, 49.0, 45.7, 36.2.

*4.1.5.2. Methyl (1R,2S)-1-(3,4-dihydroxyphenyl)-2-(((S)-3-(3,4-dihydroxyphenyl)-1-methoxy-1-oxopropan-2-yl)carbamoyl)-6,7-dihydroxy-1,2-dihydronaphthalene-3-carboxylate (**5**).* Yield: 0.061 g (72%); yellowish solid, mp: 204–207 °C (decomp.); $[a]_D^{25}$ = −170 (*c* 0.6, MeOH); R_f ($CHCl_3$/MeOH 8:2): 0.13; IR (KBr, cm^{-1}): 3382, 2927, 1734, 1690, 1642, 1518, 1444, 1337, 1262; MS (ESI, 30 eV): *m/z* 604.26 [M+K], 588.27 [M+Na], 566.43 [M+H]; ^{1}H NMR (d_4-MeOH): δ 7.59 (s, 1H), 6.79 (s, 1H), 6.68 (d, J = 8.0 Hz, 1H), 6.61 (d, J = 8.0 Hz, 1H), 6.51 (d, J = 2.0 Hz, 1H), 6.44 (s, 1H), 6.40–6.38 (m, 2H), 6.36 (dd, J = 2.0 and 8.0 Hz, 1H), 4.48 (dd, J = 5.6 and 7.6 Hz, 1H), 4.06 (d, J = 2.8 Hz, 1H), 3.69 (s, 3H), 3.67 (d, J = 2.8 Hz, 1H), 3.56 (s, 3H), 2.87 (dd, J = 5.6 and 14 Hz, 1H), 2.75 (dd, J = 7.6 and 14 Hz, 1H); ^{13}C NMR (d_4-MeOH): δ 173.0, 171.8, 167.6, 147.9, 145.0, 144.6, 144.1, 143.9, 143.6, 139.7, 135.2, 130.4, 127.6, 123.2, 121.0, 120.4, 118.7, 116.0, 115.9, 115.7, 115.1, 114.8, 114.4, 53.5, 51.2, 51.1, 49.3, 46.2, 36.3.

4.2. Biology

4.2.1. Cell cultures

MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC). A-549 human lung carcinoma cell line was obtained from Cell Lines Service (CLS, Germany). Breast cancer cells were cultured in complete medium Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Lung cancer cells were cultured as monolayers in DMEM Ham's F12 supplemented with 5% (v/v) FBS. Cell culture mediums were supplemented with 1.0 mM sodium pyruvate, 2 mM L-glutamine and a cocktail of antimicrobial agents (100 IU/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL gentamycin sulfate and 2.5 μg/mL amphotericin B). Cells were routinely grown at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 and 95% air. Culture medium was changed every 48–72 h and the cultures were not left to become confluent. Cells were harvested by trypsinization with 0.05% (w/v) trypsin in PBS containing 0.02% (w/v) Na_2EDTA.

4.2.2. Proliferation assay

MDA-MB-231 and MCF-7 breast cancer cells and A-549 lung cancer cells were seeded in the presence of serum into 96-well plates at a density of 5000, 7500 and 4500 cells/well, respectively, and then the agents were added according to the experimental plan, in elevated concentrations (1, 2, 4, 8, 16, 32, 64, 128, 256 μM) in serum-free culture medium. After 24 h, 48 h or 72 h of incubation, Premix WST-1 (water-soluble tetrazolium salt) Cell Proliferation Assay System (Takara Bio Inc., Japan) was added at a ratio 1:10. The assay is based on the reduction of WST-1 by viable cells, producing a soluble formazan salt absorbing at 450 nm (reference wavelength at 650 nm).

4.2.3. Statistical analysis

Reported values are expressed as mean ± standard deviation (SD) of experiments in triplicate. Statistically significant differences were evaluated using the analysis of variance (ANOVA) test and were considered statistically significant at the level of at least $p \leqslant 0.05$.

4.3. Theoretical calculations

The use of quantum chemical methods in this work aims at the calculation of stable molecular structures. The basis set used in the calculations is the standard Pople basis set 6-31G [32] augmented with a rather tight d-GTF (Gaussian-type function) for the C, N and O centers [33]. The exponent of the d-GTF is $\eta_d = 0.8\ a_0^{-2}$. Six-membered d-GTF was used in all cases. In the case of lignan amide **4** we used a much larger basis set 6-31G** which contains an additional p-GTF on H centers with the tight exponent $\eta_p = 1.1\ a_0^{-2}$. The theoretical method employed in the calculations is the widely used B3LYP density functional theory (DFT) technique [34], as implemented in the GAUSSIAN 03 and GAUSSIAN 09 programs. All methods and basis sets used in this work follow the available implementation in the GAUSSIAN 03 [35] and GAUSSIAN 09 [36] programs.

4.4. NMR studies

NMR spectra, for the complete assignment of lignan amides **4** and **5**, were recorded on a Bruker AV-500 spectrometer equipped with a cryogenic probe, at 300 K, using methanol-d_4 as solvent. Routine parameters were used recording the 1H spectra. The spectral width was 6000 Hz and the chemical shifts are reported with respect to the resonance of the solvent, which was used as internal standard. TOCSY and ROESY 2D spectra were acquired using TPPI method for quadrature detection, while COSY 2D spectra were acquired using QF method. The 2D measurements were recorded using 512 increments of 2 K complex data points and 16 scans per increment for 2D ^{1}H ROESY, 8 scans for 2D ^{1}H TOCSY and 4 scans for 2D COSY experiments, respectively. The mixing time for ROESY spectra was 200 ms, and that for TOCSY was 100 ms. ^{1}H–^{13}C HSQC and HMBC 2D spectra were acquired using echo-antiecho and QF method for detection, respectively. Hetero-nuclear experiments were recorded using 1 K increments of 2 K complex data points and 4 scans per increment for ^{1}H–^{13}C HSQC and 32 scans for ^{1}H–^{13}C HMBC experiments, respectively. Data were processed using the TOPSPIN standard software. The t_1 dimension for both homonuclear and heteronuclear experiments was zero-filled to 1 K real data points, and 60° phase-shifted square sine bell window functions were applied in both dimensions, except for COSY and HMBC experiments where 0° phase-shifted sine bell window functions were applied in both dimensions.

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Appendix A. Supplementary material

Experimental procedures for the synthesis of compound **9** and full characterization of all intermediates. Quantum chemical calculations for compounds **1a**, **1b**, and diastereomeric lignan amides **4** and **5**. Selected ^{1}H and ^{13}C NMR spectra of key intermediates and final products.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2016.04.003. These data include MOL files and InChiKeys of the most important compounds described in this article.

References

[1] T. Markkanen, M.L. Makinen, E. Maunuksela, P. Himanen, Podophyllotoxin lignans under experimental antiviral research, Drugs Exp. Clin. Res. 7 (1981) 711–718.
[2] G.E. Magoulas, D. Papaioannou, Bioinspired syntheses of dimeric hydroxycinnamic acids (Lignans) and hybrids, using phenol oxidative coupling as key reaction, and medicinal significance thereof, Molecules 19 (2014) 19769–19835.
[3] F.M.F. Roleira, E.J. Tavares-da-Silva, C.L. Varela, S.C. Costa, T. Silva, J. Garrido, F. Borges, Plant derived and dietary phenolic antioxidants: anticancer properties, Food Chem. 183 (2015) 235–258.
[4] T.F. Imbert, Discovery of podophyllotoxins, Biochimie 80 (1998) 207–222.
[5] S.K. Jaganathan, M. Mandal, Antiproliferative effects of honey and of its polyphenols: a review, J. Biomed. Biotechnol. (2009). and references cited therein article ID: 830616.
[6] J. Chen, Y. Shao, M. Huang, C. Chin, C. Ho, Inhibitory effect of caffeic acid phenethyl ester on human leukemia HL-60 cells, Cancer Lett. 108 (1996) 211–214.
[7] H. Liao, Y. Chen, J. Liu, M. Hsu, H. Shieh, H. Liao, C. Shieh, M. Shiao, Y. Chen, Inhibitory effect of Caffeic Acid Phenethyl Ester on angiogenesis, tumor invasion, and metastasis, J. Agric. Food Chem. 51 (2003) 7907–7912.
[8] J. Wu, C. Omene, J. Karkoszka, M. Bosland, J. Eckard, C.B. Klein, K. Frenkel, Caffeic acid phenethyl ester (CAPE), derived from a honeybee product propolis, exhibits a diversity of anti-tumor effects in pre-clinical models of human breast cancer, Cancer Lett. 308 (2011) 43–53.
[9] B. Jung, M. Kim, H. Kim, D. Kim, J. Yang, H. Her, Y.S. Song, Caffeic acid phenethyl ester, a component of beehive propolis, is a novel selective estrogen receptor modulator, Phytother. Res. 24 (2010) 295–300.
[10] Y. Kashiwada, K.F. Bastow, K.H. Lee, Novel lignan derivatives as selective inhibitors of DNA topoisomerase II, Bioorg. Med. Chem. Lett. 5 (1995) 905–908.
[11] F. Bailly, R.-A. Toillon, O. Tomavo, N. Jouy, H. Hondermarck, P. Cotelle, Antiproliferative and apoptotic effects of the oxidative dimerization product of

methyl caffeate on human breast cancer cells, Bioorg. Med. Chem. Lett. 23 (2013) 574–578.
[12] L. Alemanno, T. Ramos, A. Gargadenec, C. Andary, N. Ferriere, Localization and identification of phenolic compounds in *Theobroma cacao* L. somatic embryogenesis, Ann. Bot. 92 (2003) 613–623.
[13] S.-H. Park, H.-S. Oh, M.-A. Kang, H. Cho, J.B. Prasad, J. Won, K.-H. Lee, The structure-activity relationship of the series of non-peptide small antagonists for p56lck SH2 domain, Bioorg. Med. Chem. 15 (2007) 3938–3950.
[14] (a) B. Meunier, Hybrid molecules with a dual mode of action: Dream or reality?, Acc Chem. Res. 41 (2008) 69–77;
(b) S. Fortin, G. Bérubé, Advances in the development of hybrid anticancer drugs, Expert Opin. Drug Discov. 8 (2013) 1029–1047.
[15] A. Gaucher, L. Dutot, O. Barbeau, W. Hamchaoui, M. Wakselman, J.-P. Mazaleyrat, Synthesis of terminally protected (S)-β3-H-DOPA by Arndt-Eistert homologation: an approach to crowned β-peptides, Tetrahedron Asymmetry 16 (2005) 857–864.
[16] I. Agata, T. Hatano, S. Nishibe, T. Okuda, A tetrameric derivative of caffeic acid from Rabdosia japonica, Phytochemistry 28 (1989) 2447–2450.
[17] C. Daquino, A. Rescifina, C. Spatafora, C. Tringali, Biomimetic synthesis of natural and "unnatural" lignans by oxidative coupling of caffeic esters, Eur. J. Org. Chem. (2009) 6289–6300. and references cited therein.
[18] F. Diederich, P.J. Stang, R.R. Tykwinski (Eds.), In Acetylene Chemistry. Chemistry, Biology And Material Science, Wiley-VCH, Weinheim, 2005.
[19] P. Carloni, F. Alber (Eds.), In Quantum Medicinal Chemistry, Wiley-VCH, Weinheim, 2003.
[20] R. Todeschini, V. Consonni, In Handbook of Molecular Descriptors, Wiley-VCH, Weinheim, 2000.
[21] G. Maroulis, C. Pouchan, Size and electric dipole (hyper)polarizability in small cadmium sulfide clusters: an ab initio study on (CdS)n, n = 1, 2, and 4, J. Phys. Chem. B 107 (2003) 10683–10686.
[22] P. Karamanis, G. Maroulis, C. Pouchan, Molecular geometry and polarizability of small cadmium selenide clusters from all-electron ab initio and Density Functional Theory calculations, J. Chem. Phys. 124 (2006) 071101.
[23] G.E. Magoulas, S.E. Bariamis, C.M. Athanassopoulos, A. Haskopoulos, P.G. Dedes, M.G. Krokidis, N.K. Karamanos, D. Kletsas, D. Papaioannou, G. Maroulis, Syntheses, antiproliferative activity and theoretical characterization of acitretin-type retinoids with changes in the lipophilic part, Eur. J. Med. Chem. 46 (2011) 721–737.
[24] S.E. Bariamis, G.E. Magoulas, K. Grafanaki, E. Pontiki, T. Tsegenidis, C.M. Athanassopoulos, G. Maroulis, D. Papaioannou, D. Hadjipavlou-Litina, Synthesis and biological evaluation of new C-10 substituted dithranol pleiotropic hybrids, Bioorg. Med. Chem. 23 (2015) 7251–7263.
[25] A.-M. Lamidey, L. Fernon, L. Pouységu, C. Delattre, S. Quideau, P. Pardon, A convenient synthesis of the Echinacea-derived immunostimulator and HIV-1 integrase inhibitor (-)-(2R,3R)-chicoric acid, Helv. Chim. Acta 85 (2002) 2328–2334.
[26] G. Ozturk, Z. Ginis, S. Akyol, G. Erden, A. Gurel, O. Akyol, The anticancer mechanism of caffeic acid phenethyl ester (CAPE): review of melanomas, lung and prostate cancers, Eur. Rev. Med. Pharmacol. Sci. 16 (2012) 2064–2068.
[27] C. DeSantis, J. Ma, L. Bryan, A. Jemal, Breast cancer statistics, 2013, CA Cancer J. Clin. 64 (2014) 52–62.
[28] P. Bouris, S.S. Skandalis, Z. Piperigkou, N. Afratis, K. Karamanou, A.J. Aletras, A. Moustakas, A.D. Theocharis, N.K. Karamanos, Estrogen receptor alpha mediates epithelial to mesenchymal transition, expression of specific matrix effectors and functional properties of breast cancer cells, Matrix Biol. 43 (2015) 42–60.
[29] A.D. Theocharis, S.S. Skandalis, T. Neill, H.A. Multhaupt, M. Hubo, H. Frey, S. Gopal, A. Gomes, N. Afratis, H.C. Lim, J.R. Couchman, J. Filmus, R.D. Sanderson, L. Schaefer, R.V. Iozzo, N.K. Karamanos, Insights into the key roles of proteoglycans in breast cancer biology and translational medicine, Biochim. Biophys. Acta 1855 (2015) 276–300.
[30] E.M. Fox, R.J. Davis, M.A. Shupnik, ERβ in breast cancer-Onlooker, passive player, or active protector?, Steroids 73 (2008) 1039–1051
[31] A.I. Tsonis, N. Afratis, C. Gialeli, M.-I. Ellina, Z. Piperigkou, S.S. Skandalis, A.D. Theocharis, G.N. Tzanakakis, N.K. Karamanos, Evaluation of the coordinated actions of estrogen receptors with epidermal growth factor receptor and insulin-like growth factor receptor in the expression of cell surface heparan sulfate proteoglycans and cell motility in breast cancer cells, FEBS J. 280 (2013) 2248–2259.
[32] R. Ditchfield, W.J. Hehre, J.A. Pople, Self-consistent molecular-orbital methods. IX. An extended gaussian-type basis for molecular-orbital studies of organic molecules, J. Chem. Phys. 54 (1971) 720–723.
[33] M.J. Frisch, J.A. Pople, J.S. Binkley, Self-consistent molecular orbital methods 25. Supplementary functions for Gaussian basis sets, J. Chem. Phys. 80 (1984) 3265–3269. and references therein.
[34] F. Jensen, Introduction to Computational Chemistry, Wiley-VCH, Weinheim, 1999. and references therein.
[35] M.J. Frisch, G.W. Trucks, H.B. Schlegel, et al., GAUSSIAN 03, Revision D.01, Gaussian Inc, Wallingford, CT, 2004.
[36] M.J. Frisch, G.W. Trucks, H.B. Schlegel, et al., GAUSSIAN 09, Revision A.01, Gaussian Inc, Wallingford, CT, 2009.