Research article

Phenolic peptides as antioxidant and anti-proliferative agents

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Abstract

We report an efficient synthesis of phenolic peptides starting from 3,4-dihydroxyphenylacetic acid (DOPAC) and L-configured amino acid esters (glycine, phenylalanine, valine, serine, tryptophan, and cystine) using different coupling reagents. The combination of a phenolic scaffold with an amino acid residue might modulate the bioavailability and the therapeutic properties of title derivatives. Moreover, the incorporation of a catechol group, with inherent redox activity, can contribute to alter the redox status of the cancer cells, and therefore, provide anti-proliferative properties. Their activities as antioxidants (i.e. scavenging free radicals and H_2O_2 as well inhibition of lipid peroxidation) and as anti-proliferative agents against three human cervical carcinoma cell lines (HeLa, ViBo, and CaSki) and normal lymphocytes were evaluated. All compounds exhibited an excellent antioxidant activity; remarkably, the peptide derived from L-cystine exhibited the best antioxidant activity, displaying a 2.5-fold increase in radical-scavenging activity when compared with the natural 2-(3',4'-dihydroxyphenyl)ethanol (hydroxytyrosol, HT). Moreover, this compound was also the most potent antitumor agent against the three human tumor cell lines (IC_{50} values 108-122 μM), with a 2-7-fold increase in activity when compared with natural DOPAC and HT, used as reference compounds. Importantly, the cytotoxic activity of these phenolic peptidomimetics against normal human lymphocytes was very low, hence confirming their selectivity towards tumor cells. Moreover, a disulfide-containing peptide also exhibited negligible cell necrosis and a high selectivity against tumor cells when compared to normal lymphocytes. Such derivative incorporates two fragments characterized with redox properties, the catechol moiety, and the disulfide linker. Thus, disulfide-containing phenolic peptidomimetics emerge as good lead candidates for the development of a novel family of anti-tumor agents.

Keywords

Polyphenols, peptides, antioxidant, antiradical, anti-proliferative, cervical cancer.

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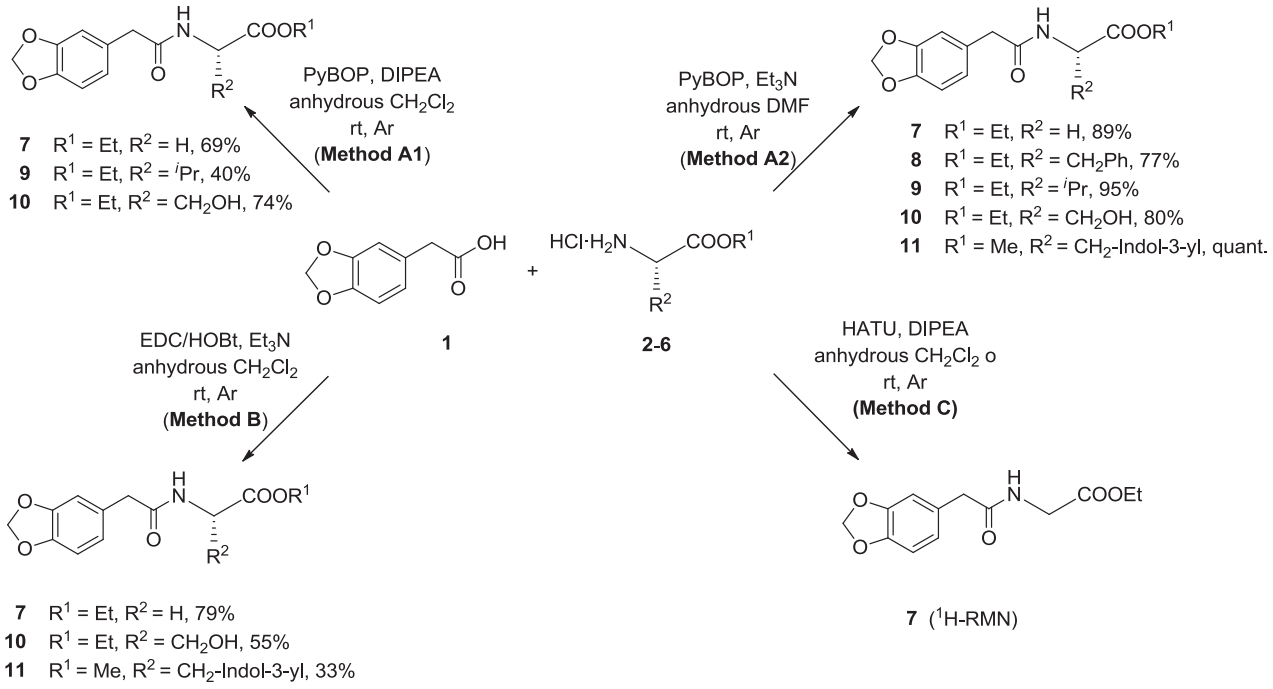
1. Introduction

Oxidative stress is a cellular state characterized by the disruption of the balance between pro-oxidants and natural antioxidant defences [1]. Pro-oxidants, including *Reactive Oxygen Species* (ROS) and *Reactive Nitrogen Species* (RNS), are comprised of a series of highly reactive compounds (free radicals, hydrogen peroxide, superoxide anion, alkyl peroxides, nitric oxide, etc.) that are produced either endogenously, mainly in mitochondrial oxidative phosphorylation and inflammatory processes, or, alternatively by exogenous agents including ionizing radiation, certain chemicals or pathogens [2]. When present at low concentrations, these pro-oxidants exhibit important biological activities including their acting as *redox messengers* in intracellular signalling pathways [3]; however, at higher concentrations, they inflict oxidative damage which can be observed in virtually all biomolecules. Oxidative stress has been demonstrated to participate in pathogenic pathways related to cell ageing [4], inflammatory processes [5], the development of cardiovascular (e.g. atherogenesis) [6] and neurodegenerative diseases (e.g. Parkinson and Alzheimer) [7], or carcinogenesis [2]. In fact, regulation of redox homeostasis has been proposed as a key point for preventing or combating such disorders [8].

In order to counteract the deleterious effect of high concentrations of pro-oxidant species, a complex machinery of detoxification has been developed in living organisms, ranging from antioxidant enzymes (e.g. glutathione peroxidase) [9], certain proteins (e.g. albumin) [10], and an heterogeneous group of low molecular weight molecules, comprised of glutathione [11], vitamins [12], carotenes [13], and polyphenols [14]. The latter compounds are ubiquitous phytochemicals especially abundant in fruits, wine, tea, cocoa and virgin olive oil [15] as secondary metabolites [14]. Numerous polyphenols, either naturally-occurring or synthetic are strong scavengers [16, 17] of pro-oxidant species. Moreover, they have also shown remarkable bioactivities as antimicrobials [18], anti-inflammatory [19], cardio- [20], and neuroprotective [21] agents or glycosidase inhibitors with hypoglycemic effects [22]. Polyphenols have also shown anti-cancer activity against a plethora of human tumor cells [23]. In this respect, hydroxytyrosol, an abundant polyphenol in olive tree and in extra-virgin olive oil is particularly remarkable; epidemiological studies have demonstrated cardio- and



Scheme 1. Peptide coupling between *O*-protected phenolic acid **1** and amino acid esters **2-6**.

neuroprotective effects of hydroxytyrosol, enhancement of immune system, and protection against inflammatory processes [24]. Moreover, its anti-proliferative effects against a series of tumor cell lines are also widely reported [25]. In this context, Fabiani and co-workers have demonstrated the pro-apoptotic effect of hydroxytyrosol against breast, prostate, colon and human leukemia tumor cells by the extracellular production of H_2O_2 [26].

For all these reasons, polyphenols have emerged as promising candidates to be used as components in the fields of cosmetics, pharmaceuticals, or as additives in functional foods.

2. Results and Discussion

2.1. Chemistry

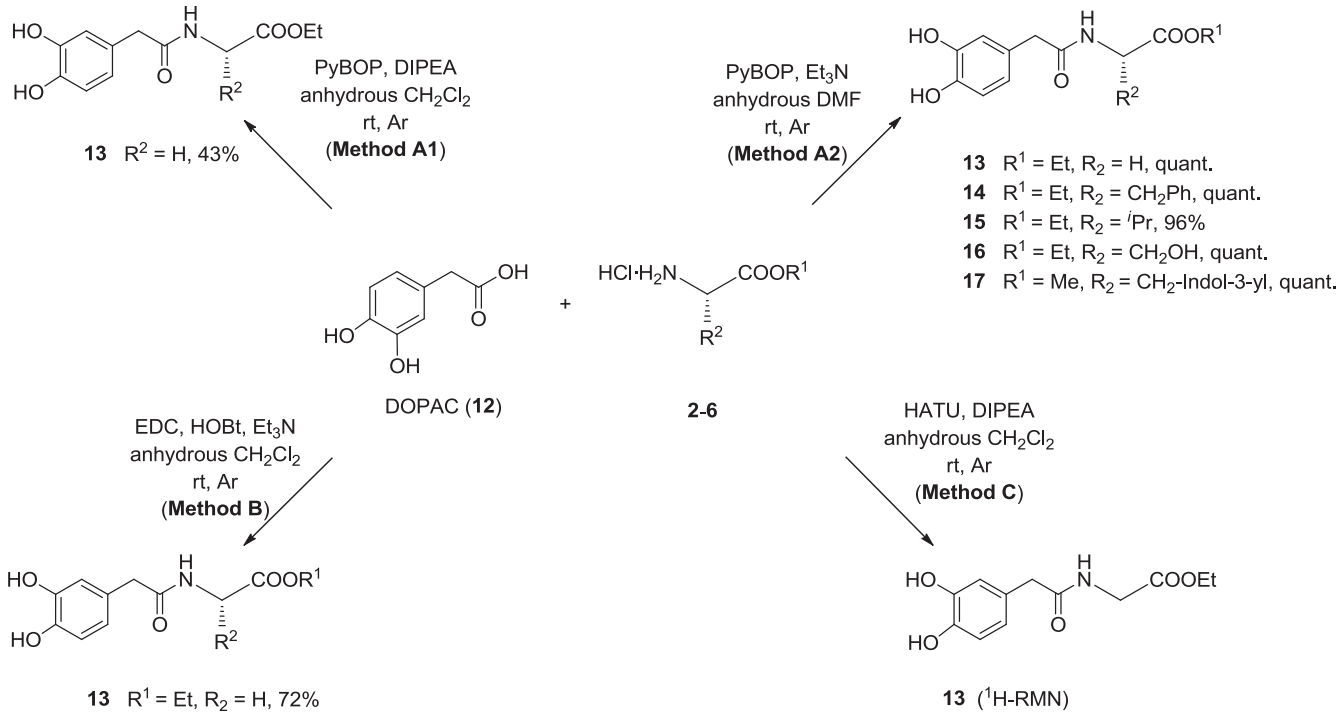
Herein we report the efficient synthesis of phenolic peptides by coupling amino acid esters and phenolic acids in the presence of a series of promoters; we then evaluated their activities as both antioxidant and anti-proliferative agents. The attachment of different amino acid residues to a phenolic structure might modulate both their bioavailability by improving uptake through the cell membrane, and their biological properties. This might therefore supply valuable information for structure-activity relationship studies.

A plethora of coupling reagents has been reported to date for the activation of the carboxylic acid moiety in peptide bond formation [27]. Among them, a carbodiimide, a phosphonium salt and a uranium salt were selected herein, giving four different synthetic pathways (Scheme 1, Methods A1, A2, B and C). Methods A1 and A2 involve the use of PyBOP (benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate) with different solvents and basic conditions (anhydrous CH_2Cl_2 and DIPEA for A1, and anhydrous DMF and Et_3N for A2), whereas EDC/HOBt 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride)/1-hydroxybenzotriazole) and HATU (1-(bis(dimethylamino)methylene)-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate) were selected in methods B and C, respectively.

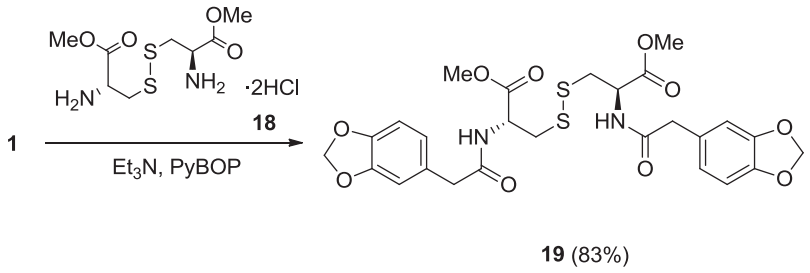
In this context, the chosen phenolic templates were 3,4-dihydroxyphenylacetic acid (DOPAC) **12** and its *O*-protected counterpart **1**. The former is a naturally-occurring phenolic compound formed by monoamine oxidase-mediated dopamine metabolism [28], exhibiting relevant biological properties; for instance, sub-stoichiometric concentrations of DOPAC have proven to inhibit the fibrillation of α-synuclein, a critical step in the etiology of Parkinson's disease [29]. A series of derivatives bearing the 1,2-methylenebenzene scaffold, present in compound **1**, have been reported to exhibit different biological properties [30]. Herein, the four methodologies indicated above were applied to compound **1** together with amino acid ester hydrochlorides **2-6**, derived from glycine, phenylalanine, valine, serine and tryptophan (Scheme 1). When the uranium salt HATU was used as the coupling reagent (Scheme 1, method C), the formation of the targeted peptide **7** [30]^b was observed by ^{1}H-NMR spectroscopy, but the reaction was not complete even after 60 h, and furthermore, a significant amount of side-products was also observed; for all these reasons, this procedure was discarded.

Replacement of HATU with the phosphonium salt PyPOP in CH_2Cl_2 (method A1) or with the combination of EDC and HOBt as an additive (method B) allowed for the isolation of **7**, **9-11** with moderate to good yields (40-74%, or 33-79%, respectively), but the reaction times were still relatively long, especially for the latter conditions (22-60 h). Remarkably, replacement of anhydrous CH_2Cl_2 by dimethylformamide (DMF) as a solvent, but retaining PyBOP as the coupling reagent (method A2), led to a significant increase in the yield of peptides **7-11** (77%-quantitative), and a general decrease in the reaction time (24-28 h), as depicted in Scheme 1.

The same conditions were employed for DOPAC **12** and amino acid ester derivatives **2-6** (Scheme 2).



Scheme 2. Peptide coupling between DOPAC **12** and amino acid esters **2-6**.



Scheme 3. Preparation of L-cystine derivative **19**.

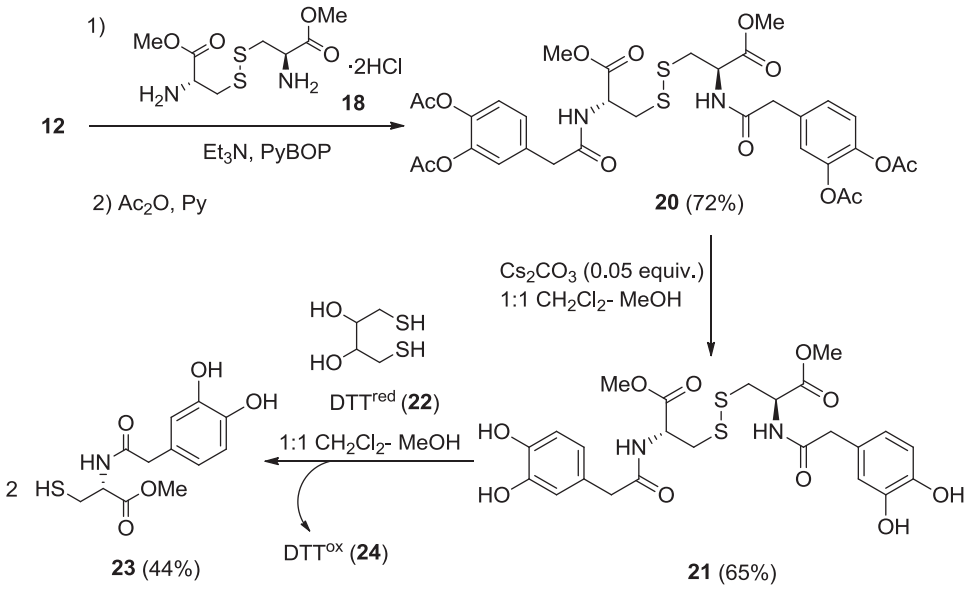
Regarding the formation of the peptidomimetic derived from glycine **13**, its synthesis was attempted using the four synthetic methodologies indicated in Scheme 2. Analogously to the preparation of its *O*-protected counterpart **7**, a high amount of side-products was observed in ^{1}H-NMR when Method C was selected; the use of HATU was therefore discarded. Methods A1, A2 and C led to the formation of **13** in 43%, quantitative, and 72% yields, respectively.

Methods A1 and B proceeded with moderate to good yields, but significant amounts of starting materials were observed even after 60 h of reaction. Remarkably, Method A2 has again proven to be the best synthetic choice, where reactions took place in shorter reaction times (15-38 h) giving rise to compounds **13**-**17** in excellent to quantitative yields (Scheme 2).

Considering the excellent pro-apoptopic properties against HL60 tumor cells observed by hydroxytyrosyl disulfide, and chalcogen-containing polyphenols, recently prepared in our research group [31], we envisioned the possibility of preparing amides derived from L-cystine, where the combination of a dimeric phenolic template, together with a disulfide tether, might reveal significant antiproliferative activities. Thus, using the optimized conditions of Method A2 (PyBOP, Et_3N, DMF), coupling between *O*-methylidene-protected DOPAC **1** and L-cystine methyl ester dihydrochloride **18** took place in a successful manner, with peptidomimetic **19** being obtained with a yield of 83% (Scheme 3).

We also attempted the preparation of unprotected disulfide **21** by direct PyBOP-mediated coupling reaction between **12** and **18**. Nevertheless, attempts to isolate compound **21** using this procedure were unsuccessful, due to the difficulty in the chromatographic separation of the target compound from side-products derived from the coupling reagent. In order to overcome this problem, the crude coupling reaction between **12** and **18** was conventionally acetylated to furnish tetra-*O*-acetyl derivative **20**, in a 72% overall yield for the two steps (Scheme 4).

Deprotection of the phenolic ester motifs was a more difficult task than previously anticipated. Firstly, strongly basic conditions (NaOMe/MeOH) should be avoided, as they enhance significantly the catechol oxidation to *o*-quinones, and subsequently to a complex mixture of compounds [32]. Nevertheless, mild conditions (K_2CO_3/MeOH or NH_4OAc/MeOH), or even the use of lipases from *C. Antarctica* and *P. cepacia* for the deprotection of **20** failed, as



Scheme 4. Preparation of L-cystine and L-cysteine derivatives **21** and **23**.

extensive decomposition or a complex mixture of compounds was observed. To our delight, treatment with $CsCO_3$ in a 1:1 mixture of CH_2Cl_2–MeOH afforded unprotected dimeric disulfide **21** in a good yield (65%). To our knowledge, this is the first example of a L-cystine-containing phenolic peptidomimetic.

The transformation of compound **21** into the L-cysteine derivative **23** was accomplished in a straightforward redox pathway by treatment of **21** with (±)-dithiothreitol (Scheme 4), an inexpensive dithiol quite useful in the mild reduction of disulfide linkages [33]; derivative **23** was isolated with a 44% yield after chromatographic purification.

2.2. *Biological evaluation*

2.2.1. Antioxidant activity

The capacity of unprotected peptidomimetics **13**-**17**, **21** and **23** to scavenge ROS (free radicals, H_2O_2 and alkyl hydroperoxides) has been evaluated; the results were compared with natural hydroxytyrosol (HT), an abundant phenolic compound in olives. [32][b] This study is an important task for the preliminary evaluation of the compounds, as their inherent antioxidant capacity is responsible for many of their biological properties.

To evaluate the antiradical activity, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used [34]. DPPH is a commercially-available free radical widely used for the quantification of the free radical scavenging activity of an antioxidant agent. DPPH solutions exhibit a deep purple color, with a λ_{max} at 515 nm; the presence of an H-donor acting as a free radical scavenger reduces DPPH, and hence a decrease in the absorbance is observed. Quantification of the potency of an antioxidant is carried out by calculating EC_{50}, that is, the concentration of the antioxidant that scavenges 50% of the original DPPH. Therefore, there is an inverse relationship between EC_{50} values and the potency of the antioxidant. Calculated EC_{50} values in the DPPH assay for various compounds, along with that found for HT, are depicted in Figure 1. It can be observed that phenolic peptides **13**-**17** and **23** exhibit an antiradical activity similar to HT ($EC_{50} = 11.3 \pm 1.3$ μM), with EC_{50} values ranging from 10.5-18.4 μM. The activity found for disulfide **21** is especially remarkable as it behaved as a potent antioxidant agent ($EC_{50} = 4.5 \pm 0.2$ μM), with a 2.5-fold increase in activity when compared with natural HT.

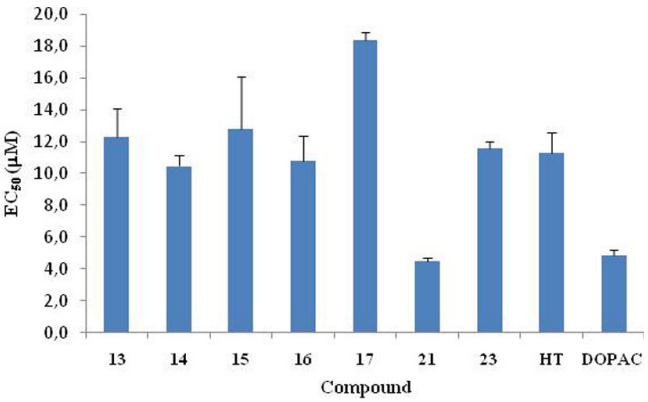


Fig. 1. Evaluation of antiradical activity using the DPPH assay.

To determine H_2O_2 scavenging activity, we used the methodology developed by Bahorun *et al*. [35]; in this assay, the peroxidase-mediated oxidation of phenol red with H_2O_2 is followed spectrophotometrically at 610 nm. The percentage of scavenging exerted by the tested compounds at 1.0 mM concentration is depicted in Figure 2. Compounds **14**-**16** exhibited virtually the same scavenging activity (60.7-65.1%) as HT (68.8 ± 5.0%); derivatives **17** and **23** showed reduced activity compared to reference compound (34.5 ± 1.0, 54.0 ± 5.3%, respectively). Remarkably, glycine and L-cystine derivatives **13** and **21** behaved as more potent H_2O_2 scavengers (74.6 ± 2.3% and 77.7 ± 10.3%, respectively) than HT.

The inhibition of the first stages of the lipid peroxidation was also evaluated. For this purpose, linoleic acid was used as the lipid model, and AAPH (2,2'-azobis(2-methylpropionamidine) dihydrochloride) was employed as a free radical initiator that affords alkyl peroxides by thermal degradation of linoleic acid [36]. The inhibition of the

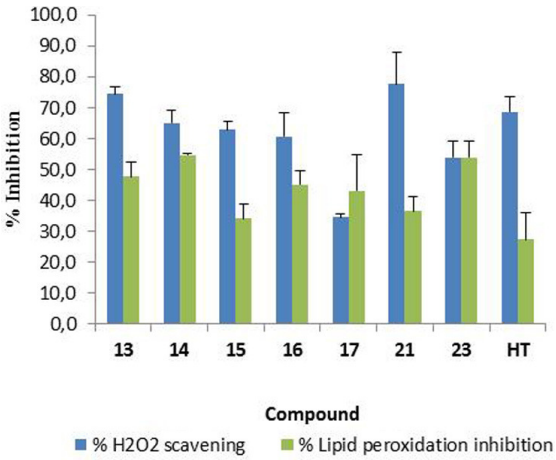


Fig. 2. H_2O_2 scavenging and inhibition of lipid peroxidation exerted by compounds **13**-**17**, **21**, **23**, DOPAC and HT.

lipid peroxidation exerted by the tested compounds at 0.74 mM concentration is depicted in Figure 2. In this assay, HT behaved as a poor antioxidant agent (27.4 ± 8.6% inhibition). Remarkably, peptides derived from glycine **13** (47.7 ± 4.9%), L-phenylalanine **14** (54.8 ± 0.4%), L-serine **16** (45.2 ± 4.6%), L-tryptophan **17** (43.1 ± 11.7%) and L-cysteine **23** (53.9 ± 5.3%) turned out to be more efficient antioxidants for lipid protection than natural HT.

2.2.2. Anti-proliferative activity assay

Some natural polyphenols, like curcumin, ferulic acid, green tea polyphenols, or resveratrol, have been reported to exhibit chemopreventive and chemotherapeutic effects against cervical cancer [37], the second most common cancer worldwide [38]. This prompted us to assess the anti-proliferative effects of the synthesized peptidomimetics and to compare their activity with natural products like DOPAC and HT; in this study, three different cervical cancer cell lines were used: HeLa and CaSki, associated with the human papillomavirus (HPV), and ViBo, which is not associated with HPV.

The degree of necrosis involved in cell death, together with the selectivity against tumor cells compared to normal healthy human lymphocytes were also determined, a key property of chemotherapeutic agents.

In vitro anti-proliferative activity

Anti-proliferative activity against human cervical cancer cell lines (HeLa, CaSki, and ViBo) was determined after 24 h of incubation of the tested compounds (**13**-**16**, **20**, **21**, **23**, HT and DOPAC) by crystal violet staining [39], and using MeOH or DMSO as solvent vehicles. Compound **17** could not be tested due to the lack of solubility under the experimental conditions. The inhibitory effect of title compounds on the proliferation of the tumor cell lines was observed to occur in a dose-dependent manner; calculated IC_{50} values are depicted in Table 1.

Natural phenolic compounds HT and DOPAC behaved as moderate antitumor agents, with EC_{50} values ranging from 227.8–765.0 μM (Table 1). In general, synthetic peptides **13**-**16** exhibited reduced activity compared to HT and DOPAC. *O*-Acetylated peptide **20**, bearing a disulfide linkage behaved as a slightly better antitumor agent (IC_{50} 199.7–229.6 μM) than HT. Similar results were obtained for unprotected L-cysteine derivative **23**. Remarkably, symmetrical

Table 1. Antiproliferative activities of tested compounds against cervical cancer (HeLa, CaSki and ViBo cell lines) (mean ± SD, 6 independent experiments)

Compound	IC_{50} (μM)		
	HeLa	CaSki	ViBo
13	419.5 ± 19.3	299.3 ± 11.0	720.3 ± 52.2
14	772.5 ± 48.2	706.5 ± 19.2	746.7 ± 50.0
15	790.0 ± 41.8	530.7 ± 40.0	779.3 ± 87.9
16	603.7 ± 72.4	734.3± 58.7	590.0 ± 88.5
20	199.7 ± 18.7	238.9 ± 14.9	260.5 ± 20.6
21	121.8 ± 22.7	108.2 ± 18.7	134.8 ± 13.2
23	373.4 ± 41.2	174.9 ± 27.6	281.6 ± 65.9
HT	239.0 ± 12.0	227.8 ± 22.8	237.5 ± 11.9
DOPAC	286.6 ± 41.9	534.1 ± 59.8	765.0 ± 86.4

fully unprotected compound **21**, derived from L-cystine, was found to be the strongest anti-proliferative agent (108.2-121.8 μM), with a 2-7-fold increase in activity in comparison with natural DOPAC and HT. This result indicates that combining a dimeric phenolic template with a disulfide linkage results in a substantial enhancement of the anti-proliferative properties. A similar behavior was found for hydroxytyrosyl disulfide against HL60 tumor cells [31].

Evaluation of cell necrosis in tumor and non-tumor cells

Cell death can occur *via* two different pathways: apoptosis (a programmed cell death) or necrosis (an unordered and accidental form of cell death) [40]. As the desired cellular death in a chemotherapy treatment is apoptosis, measurement of the degree of necrosis is an important concern when developing antitumor agents. In order to determine if a necrotic process is involved in the anti-proliferative activity, the lactate dehydrogenase (LDH) assay was carried out. The amount of LDH released to the supernatant is a measure of the loss of membrane integrity, and thus, an indication that a necrotic process takes place. Triton X-100, a non-ionic polyethoxylated surfactant detergent was used as a reference compound to induce cellular lysis [41]. The three tumor cell lines were treated with Triton X-100 in three independent experiments, and released LDH was adjusted to 100%. Cervical tumor cell cultures were also stimulated with the tested compounds at their IC_{50} concentrations, and the release of LDH was compared to the control groups (Figure 3A-C). The same experiments were also undertaken when studying human lymphocytes instead of human tumor cell lines (Figure 3D).

Regarding tumor cells, the compound provoking the highest degree of necrosis was the L-valine derivative **15** (37%, 22% and 37% for HeLa, CaSki and ViBo, respectively). The rest of the peptides exhibited an exceptionally low ratio of necrosis; HT, which exhibited a good IC_{50} value, turned out to be involved to a significant extent in necrosis when considering tumor cells death (35% and 37% for HeLa and ViBo cells, respectively). It is worth mentioning the behavior found for compounds **21** (5% and 4% of necrosis against HeLa and ViBo cells) and **23** (3%, 1% and $< 0.5\%$ of necrosis against the three cervical cultures). These data strongly suggest that when these compounds exert their cytotoxic activity against the tumor cell lines studied, they do not act via necrosis but probably *via* an apoptotic pathway.

Concerning human lymphocytes, the worst compound in terms of necrosis was L-phenylalanine derivative **14**. Remarkably, *O*-

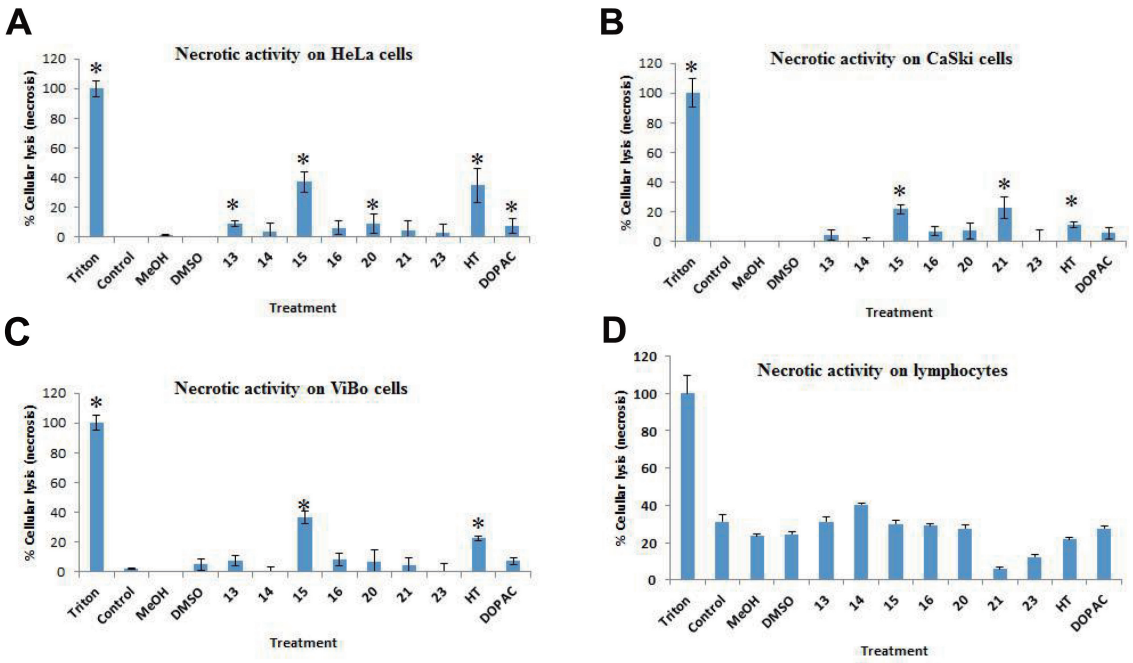


Fig. 3. Necrotic activity of tested compounds in the human cervical cell lines HeLa (A), CaSki (B), ViBo (C) as well as normal human lymphocytes (D). 7500 cells/well were seeded in 96-well tissue culture plates. After 24 h, the growth medium was discarded and cells were treated with tested compounds at their IC_{50} concentrations, or with the vehicle solvent (MeOH or DMSO), and evaluated after 24 additional hours by the amount of LDH released to the medium. Experimental data are expressed as the mean ± SD of three independent experiments performed in triplicates. $*p < 0.05$ *vs.* vehicle.

unprotected Sulphur-containing peptidomimetics **21** and **23** were found to exhibit a necrotic activity that was even lower than the corresponding solvent used as a vehicle, which could indicate that they do not provoke the death of lymphocytes by necrosis, but even a moderate activation of such cells.

Evaluation of anti-proliferative activity on non-tumor cells

One of the major problems associated with conventional chemotherapeutic treatments is the lack of selectivity of the cytotoxic agents, leading to the appearance of a series of undesirable side-effects; such side effects are predominantly due to the suppression of the host immune system by affecting normal lymphocytes. It is therefore crucial to assess the selectivity of cytotoxic agents towards non-tumor cells. Herein we carried out the study of the anti-proliferative effect of the tested compounds towards peripheral blood lymphocytes. In this assay, an enriched lymphocyte population (ELP) from a normal blood donor is labelled with carboxyfluorescein succinimidyl ester (CFSE), a fluorescent dye widely used in monitoring cell division, and stimulated with phytohemagglutinin (PHA) and/or treated with the tested compounds or with the pure vehicle. After 72 h, cells were harvested and the anti-proliferative activity was determined by flow cytometry [42]; results are depicted in Figure 4. Untreated control proliferating cells were around 87% under normal conditions, and after treatment with the vehicle, roughly 83% (MeOH) and 80% (DMSO). Whereas, peptides **15**, **21** and **23** displayed a remarkable selectivity against tumor cell lines, as normal human lymphocytes were practically unaffected, as shown in Figure 4 (i.e. only 2-9% loss of lymphocyte proliferation was observed). These results strongly contrast with the natural HT and DOPAC, which, although active against tumor cells, also elicited a significant decrease in lymphocyte proliferation (roughly 24% and 26%, respectively). Moreover, compound **20**, the tetra-*O*-acetylated derivative of **21** exhibited a decreased selectivity, as it inflicted a ∼ 25% decrease in lymphocyte proliferation. This observation suggests the importance of free phenolic OH's for exhibiting a good selectivity over normal healthy cells.

3. Materials and Methods

3.1. General procedures

General procedures concerning NMR, MS, TLC visualization and UV-Vis spectroscopy can be found in references [31, 43, 44].

3.2. Determination of the antioxidant activity

3.2.1. DPPH method

The antiradical activity of the phenolic peptides (DPPH method) was measured using the procedure reported by Prior *et al* [34].

3.2.2. H_2O_2 scavenging activity

The H_2O_2 scavenging activity of the peptides was measured using the procedure reported by Bahorun *et al.* [35].

3.2.3. Lipid peroxidation assay (ferric thiocyanate method, FTC)

Inhibition of the lipid perxoxidation was measured using the ferric thiocyanate method (FTC) [36].

3.3. General procedures for anti-proliferation assays

3.3.1. Cell culture

General procedures for the cell culture of HeLa, CaSki and ViBo cell lines can be found in references [45, 46].

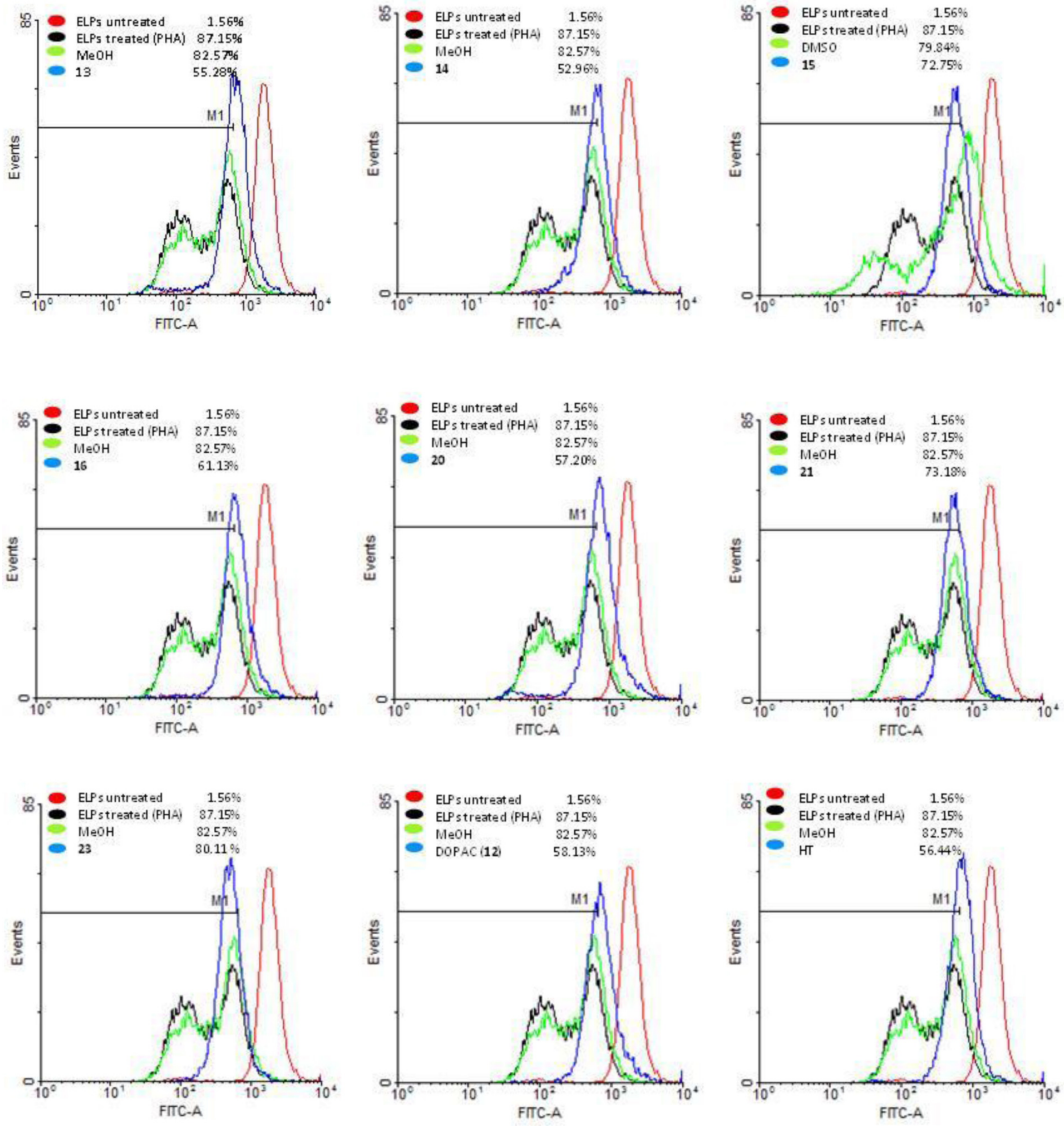


Fig. 4. Effect of compounds **13**-**16**, **20**, **21**, **23**, HT and DOPAC on the proliferation of peripheral blood lymphocytes as determined by flow cytometry. Cells were seeded in 96-well tissue culture plates, stimulated with phytohemagglutinin (PHA, 10 μg/mL) and treated with title compounds at their IC_{50} concentrations; analyses were performed after 72 h of incubation, by measuring carboxyfluorescein incorporation. M1 stands for proliferating cells.

3.3.2. Cell proliferation assay

Assays were performed as reported in reference [47]. (IC_{50}) values were determined after 24 h using crystal violet staining [39].

3.3.3. Cell necrosis study

The necrotic activity was determined as reported in reference [48].

3.3.4. Carboxyfluorescein succinimidyl ester (CSFE) labeling assay

Assays involving carboxyfluorescein were carried out as reported in reference [48].

3.3.5. Statistical analysis

For antioxidant assays, all tests were run in triplicates for each experimental condition. Values are expressed as the confidence interval, which was calculated for $n = 3$, $P = 0.95$ using the Student's t-distribution.

For anti-proliferative assays, the median and standard deviation (SD) for six measurements were calculated. Statistical analysis of differences was performed by analysis of variance (ANOVA) using SPSS 10.0 for Windows. A p-value < 0.05 (Tukey's t-test) was considered to be significant.

3.3.6. General procedures for the preparation of peptides derived from (3,4-methylenedioxy)phenylacetic acid 1

Method A1. To a solution of **1** (50 mg, 0.28 mmol) in anhydrous CH_2Cl_2 (1.5 mL), PyBOP (166 mg, 0.32 mmol, 1.15 equiv.), the corresponding amino acid ester hydrochloride **2**, **4** or **5** (0.28 mmol, 1.0 equiv.) and DIPEA (0.83 mL, 4.86 mmol, 17.4 equiv.) were added under N_2. The corresponding mixture was stirred at room temperature for 24−48 h. After that, the solvent was removed under

reduced pressure, and the residue was purified as indicated in each case.

Method A2. To a solution of **1** (50 mg, 0.28 mmol) in anhydrous DMF (4.0 mL) at 0°C, PyBOP (144 mg, 0.28 mmol, 1.0 equiv.), the corresponding amino acid ester hydrochloride **2**-**6** (0.28 mmol, 1.0 equiv.) and Et_3N (0.16 mL, 1.11 mmol, 4.0 equiv.) were added under N_2. The corresponding mixture was stirred at room temperature for 15−38 h. Thereafter, the solvent was eliminated under reduced pressure, and the residue was purified as indicated in each case.

Method B. To a solution of **1** (50 mg, 0.28 mmol) in anhydrous CH_2Cl_2 (3.0 mL), HOBt (41 mg, 0.31 mmol, 1.1 equiv.), EDC (64 mg, 0.33 mmol, 1.2 equiv.), the corresponding amino acid ester hydrochloride **2**, **5** or **6** (0.31 mmol, 1.1 equiv.) and Et_3N (0.06 mL, 0.42 mmol, 1.5 equiv.) were added under N_2. The corresponding mixture was stirred at room temperature for 60 h. Then, the solution was diluted with EtOAc (15 mL) and washed with H_2O (1 x 15 mL), and the aqueous phase was further washed with EtOAc (3 x 10 mL). The combined organic fractions were washed with sat. aq. $NaHCO_3$ (1x10 mL) and brine (1x10 mL), dried over $MgSO_4$ and filtrated; the filtrate was concentrated to dryness and the residue was purified by column chromatography using the eluent indicated in each case.

Method C. To a solution of **1** (50 mg, 0.28 mmol) in anhydrous CH_2Cl_2 (2.0 mL), HATU (116 mg, 0.31 mmol, 1.1 equiv.), glycine ethyl ester hydrochloride **2** (41 mg, 0.29 mmol, 1.05 equiv.) and DIPEA (0.10 mL, 0.58 mmol, 2.1 equiv.) were added under N_2. The corresponding mixture was stirred at room temperature for 72 h. Thereafter, the crude reaction was washed with 1M HCl (1 x 10 mL), saturated aqueous $NaHCO_3$ (1 x 10 mL) and brine (1 x 10 mL). The organic phase was dried over $MgSO_4$, filtered and the filtrate was concentrated to dryness.

Spectroscopical data of the new compounds prepared herein can be found in https://idus.us.es/xmlui/handle/11441/53604 (research repository of Seville University, Doctoral Thesis of Azucena Marset-Castro).

3.3.7. Ethyl 2-[2'-(3",4"-methylenedioxyphenyl)acetamido]acetate (7) [30][b]

Method A1. Glycine ethyl ester hydrochloride **2** was used (39 mg, 0.28 mmol), and the reaction proceeded for 48 h. Then, the solvent was removed under reduced pressure, and the residue was purified by column chromatography (hexane → 1:2 hexane–EtOAc) to give compound **7**: 51 mg, 69%.

Method A2. Glycine ethyl ester hydrochloride **2** was used (39 mg, 0.28 mmol), and the reaction proceeded for 24 h. Thereafter, the crude reaction was washed with 1M HCl (1 x 10 mL), sat. aq. $NaHCO_3$ (1 x 10 mL) and brine (1 x 10 mL). The organic layer was dried over $MgSO_4$, filtered and the filtrate was concentrated to dryness to give compound **7**: 65 mg, 89%.

Method B. Glycine ethyl ester hydrochloride **2** was used (43 mg, 0.31 mmol), and the reaction proceeded for 48 h. Column chromatography (hexane → 1:2 hexane−EtOAc) yielded compound **7**: 58 mg, 79%.

Method C. Glycine ethyl ester hydrochloride **2** was used (41 mg, 0.29 mmol), and the reaction proceeded for 72 h. After removal of the solvent, the crude product was analyzed by ^{1}H-NMR.

R_F 0.62 (1:2 hexane-EtOAc); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.79-6.76 (m, 2H, H-2", H-5"), 6.71 (dd, 1H, $J_{2'',6''} = 1.7$ Hz, $J_{5'',6''} = 7.8$ Hz, H-6"), 6.03 (brs, 1H, NH), 5.94 (s, 2H, O-CH_2-O), 4.17 (q, 2H, $J_{H,H} = 7.2$ Hz, CH_2-CH_3), 3.98 (d, 2H, $J_{2,NH} = 5.3$ Hz, H-2), 3.51 (s, 2H, H-2'), 1.25 (t, 3H, CH_2-CH_3); ^{13}C-NMR (75.5 MHz, $CDCl_3$) δ 171.3, 169.9 (C-1', C-1), 148.2 (C-3"), 147.1 (C-4"), 128.1 (C-1"), 122.8 (C-6"), 109.9 (C-2"), 108.7 (C-5"), 101.2 (O-CH_2-O), 61.6 (CH_2-CH_3), 43.1 (C-2'), 41.6 (C-2), 14.2 (CH_2-CH_3); CI-MS m/z 265 ([M]$^+$, 57%); HRCI-MS calculated for $C_{13}H_{15}NO_5$ ([M]$^+$): 265.0950, found: 265.0946.

3.3.8. Ethyl (*S*)-2-[2'-(3",4"-methylenedioxyphenyl)acetamido]-3-phenylpropanoate (8)

Method A2. L-Phenylalanine ethyl ester hydrochloride **3** was used (64 mg, 0.28 mmol), and the reaction proceeded for 38 h. Column chromatography (hexane → 1:1 hexane−EtOAc) yielded **8**: 76 mg, 77%.$[\alpha]_D^{26}$ -5 (*c* 0.94, MeOH); R_F 0.48 (1:2 hexane−EtOAc 1:2); ^{1}H-NMR (300 MHz, CD_3OD) δ 7.28−7.16 (m, 3H, Ar-H), 7.15−7.10 (m, 2H, Ar-H), 6.70 (d, 1H, $J_{5'',6''} = 7.8$ Hz, H-5"), 6.67 (d, 1H, $J_{2'',6''} = 1.3$ Hz, H-2"), 6.63 (dd, 1H, H-6"), 5.91 (s, 2H, O-CH_2-O), 4.64 (dd, 1H, $J_{2,3a} = 5.7$ Hz, $J_{2,3b} = 8.8$ Hz, H-2), 4.13 (q, 2H, $J_{H,H} = 7.2$ Hz, CH_2-CH_3), 3.39 (s, 2H, H-2'), 3.14 (dd, 1H, $J_{3a,3b} = 13.9$ Hz, H-3a), 2.96 (dd, 1H, H-3b), 1.20 (t, 3H, CH_2-CH_3); ^{13}C-NMR (75.5 MHz, CD_3OD) δ 173.9, 173.0 (C-1', C-1), 149.2 (C-3"), 148.0 (C-4"), 138.0 (Ar-C), 130.2 (x2) (2Ar-C), 130.1 (x2) (2Ar-C), 129.5 (C-1"), 127.9 (Ar-C), 123.4 (C-6"), 110.4 (C-2"), 109.1 (C-5"), 102.3 (O-CH_2-O), 62.4 (CH_2-CH_3), 55.3 (C-2), 43.1 (C-2'), 38.3 (C-3), 13.4 (CH_2-CH_3); CI-MS m/z 355 ([M]$^+$, 52%); HRCI-MS calculated for $C_{20}H_{21}NO_5$ ([M]$^+$): 355.1420, found: 355.1408.

3.3.9. Ethyl (*S*)-3-methyl-2-[2'-(3",4"-methylenedioxyphenyl) acetamido]butanoate (9)

Method A1. L-Valine ethyl ester hydrochloride **4** was used (50 mg, 0.28 mmol), and the reaction proceeded for 44 h. Column chromatography (hexane → 2:1 hexane−EtOAc) yielded **9**: 34 mg, 40%.

Method A2. L-Valine ethyl ester hydrochloride **4** was used (50 mg, 0.28 mmol), and the reaction proceeded for 17 h. Column chromatography (hexane → 1:2 hexane−EtOAc) yielded compound **9**: 81 mg, 95%. $[\alpha]_D^{24}$ +9 (*c* 0.98, CH_2Cl_2); R_F 0.54 (1:2 hexane−EtOAc); CI-MS m/z 307 ([M]$^+$, 64%); HRCI-MS m/z calcd for $C_{16}H_{21}NO_5$ ([M]$^+$): 307.1420, found: 307.1424.

3.3.10. Ethyl (*S*)-3-Hydroxy-2-[2'-(3",4"-methylenedioxyphenyl) acetamido]propanoate (10)

Method A1. L-Serine ethyl ester hydrochloride **5** was used (47 mg, 0.28 mmol), and the reaction proceeded for 30 h. Column chromatography (hexane → 1:3 hexane−EtOAc) resulted in compound **10**: 61 mg, 74%.

Method A2. L-Serine ethyl ester hydrochloride was used (47 mg, 0.28 mmol), and the reaction proceeded for 19 h. Column chromatography (hexane → 1:3 hexane−EtOAc) yielded compound **10**: 65 mg, 80%.

Method B. L-Serine ethyl ester hydrochloride was used (52 mg, 0.31 mmol), and the reaction proceeded for 60 h. Liquid-liquid extractions, as described in the general procedure, yielded pure **10**: 45 mg, 55%. $[\alpha]_D^{23}$ +3 (*c* 1.22, MeOH); R_F 0.64 (1:2 hexane-EtOAc); HRCI-MS calcd for $C_{14}H_{17}NO_6$ ([M]$^+$): 295.1056, found: 295.1049.

3.3.11. Methyl (*S*)-3-(1'''*H*-indol-3'''-yl)-2-[2'-(3",4"-methylenedioxyphenyl)acetamido]propanoate (11)

Method A2. L-tryptophan methyl ester hydrochloride **6** was used (71 mg, 0.28 mmol), and the reaction proceeded for 15 h. Column chromatography (hexane → 1:7 hexane−EtOAc) resulted in compound **11**: 106 mg, quant.

Method B. L-tryptophan methyl ester hydrochloride **6** was used (78 mg, 0.31 mmol), and the reaction proceeded for 60 h. Column chromatography (hexane → 1:2 hexane−EtOAc) yielded compound **11**: 35 mg, 33%. $[\alpha]_D^{24}$ +28 (*c* 1.10, $(CH_3)_2CO$); R_F 0.55 (1:2 hexane-EtOAc); CI-MS 381 ($[M]^+$, 21%); HRCI-MS calcd for $C_{21}H_{21}N_2O_5$ ($[M+H]^+$): 381.1450, found: 381.1436.

3.3.12. General procedures for the preparation of peptides derived from (3,4-dihydroxyphenyl)acetic acid 12 (DOPAC)

Method A1. To a solution of DOPAC (50 mg, 0.30 mmol) in anhydrous CH_2Cl_2 (1.5 mL), PyBOP (178 mg, 0.34 mmol, 1.15 equiv.), glycine ethyl ester hydrochloride **2** (42 mg, 0.30 mmol, 1.0 equiv.) and DIPEA (0.89 mL, 5.20 mmol, 17.4 equiv.) were added under N_2. The corresponding mixture was stirred at room temperature during 48 h. Then, the solvent was evaporated under reduced pressure, and the residue was purified by column chromatography (CH_2Cl_2 → 10:1 CH_2Cl_2–MeOH).

Method A2. To a solution of DOPAC (50 mg, 0.30 mmol) in anhydrous DMF (4.0 mL) at 0°C, PyBOP (155 mg, 0.30 mmol, 1.0 equiv.), the corresponding amino acid ester hydrochlorides **2**-**6** (0.30 mmol, 1.0 equiv.) and Et_3N (0.17 mL, 1.19 mmol, 4.0 equiv.) were added under N_2. The corresponding mixture was stirred at room temperature for 16-38 h. Thereafter, the solvent was eliminated under reduced pressure, and the residue was purified by column chromatography using the eluant indicated in each case.

Method B. To a solution of DOPAC (50 mg, 0.30 mmol) in anhydrous CH_2Cl_2 (3.0 mL), HOBt (44 mg, 0.33 mmol, 1.1 equiv.), EDC (68 mg, 0.36 mmol, 1.2 equiv.), glycine ethyl ester hydrochloride **2** (46mg, 0.33 mmol, 1.1 equiv.) and Et_3N (0.06 mL, 0.42 mmol, 1.5 equiv.) were added under N_2. The corresponding mixture was stirred at room temperature for 60 h. Then, the solution was diluted with EtOAc (15 mL) and washed with H_2O (1 x 15 mL), and the aqueous phase was further washed with EtOAc (3 x 10 mL). The combined organic fractions were washed with 1M HCl (1 x 10 mL), sat. aq. $NaHCO_3$ (1 x 10 mL) and brine (1x10 mL), dried over $MgSO_4$ and filtered; the filtrate was then concentrated to dryness and the residue was purified by column chromatography (CH_2Cl_2 → 10:1 CH_2Cl_2–MeOH).

Method C. To a solution of DOPAC (50 mg, 0.30 mmol) in anhydrous CH_2Cl_2 (2.0 mL), HATU (124 mg, 0.33 mmol, 1.1 equiv.), glycine ethyl ester hydrochloride **2** (44 mg, 0.31 mmol, 1.05 equiv.) and DIPEA (0.11 mL, 0.62 mmol, 2.1 equiv.) were added under N_2. The corresponding mixture was stirred at room temperature for 72 h. Thereafter, the crude reaction was washed with 1M HCl (1x10 mL), sat. aq. $NaHCO_3$ (1x10 mL) and brine (1x10 mL). The organic phase was dried over $MgSO_4$, filtered and the filtrate was concentrated to dryness.

3.3.13. Ethyl 2-[2'-(3",4"-Dihydroxyphenyl)acetamido]acetate (13)

Method A1. Glycine ethyl ester hydrochloride **2** was used (42 mg, 0.30 mmol). Chromatographic purification afforded **13**: 32 mg, 43%.

Method A2. Glycine ethyl ester hydrochloride **2** was used (42 mg, 0.30 mmol), and the reaction proceeded for 22 h. Column chromatography (CH_2Cl_2 → 10:1 CH_2Cl_2–MeOH) gave **13**: 76 mg, quant.

Method B. Glycine ethyl ester hydrochloride **2** was used (46 mg, 0.33 mmol). Column chromatography gave **13**: 54 mg, 72%.

Method C. Glycine ethyl ester hydrochloride **2** was used (44 mg, 0.31 mmol). After removal of the solvent, the crude product was analyzed by ^{1}H-NMR.

R_f 0.81 (40:1 CH_2Cl_2–MeOH); HRLSI-MS calcd for $C_{12}H_{15}NNaO_5$ ($[M + Na]^+$: 276.0848, found: 276.0842.

3.3.14. Ethyl (*S*)-2-[2'-(3",4"-dihydroxyphenyl)acetamido]-3-phenylpropanoate (14)

Method A2. L-Phenylalanine ethyl ester hydrochloride **3** was used (68 mg, 0.30 mmol), and the reaction proceeded for 38 h. Column chromatography (hexane → 1:4 hexane–EtOAc) afforded **14**: 102 mg, quant.$[\alpha]_D^{21}$ –4 (*c* 0.95, MeOH); R_F 0.40 (10:1 CH_2Cl_2–MeOH); HRCI-MS calcd for $C_{19}H_{22}NO_5$ ($[M+H]^+$): 344.1498, found: 344.1503.

3.3.15. Ethyl (*S*)-2-[2'-(3",4"-dihydroxyphenyl)acetamido]-3-methylbutanoate (15)

Method A2. L-Valine ethyl ester hydrochloride **4** was used (54 mg, 0.30 mmol), and the reaction proceeded for 16 h. Column chromatography (hexane → 1:2 hexane−EtOAc) yielded **15**. Yield: 84 mg, 96%. $[\alpha]_D^{25}$ –6 (*c* 0.71, DMSO); R_F 0.38 (10:1 CH_2Cl_2–MeOH); HRCI-MS calcd for $C_{15}H_{21}NO_5$ ($[M]^+$): 295.1420, found: 295.1428; calcd for $C_{15}H_{22}NO_5$ ($[M + H]^+$): 296.1498, found: 296.1488.

3.3.16. Ethyl (*S*)-2-[2'-(3",4"-Dihydroxyphenyl)acetamido]-3-hydroxypropanoate (16)

Method A2. L-Serine ethyl ester hydrochloride **5** was used (50 mg, 0.30 mmol), and the reaction proceeded for 38 h. Column chromatography (hexane → 1:2 hexane-EtOAc) afforded **16**: 84 mg, quant.; $[\alpha]_D^{25}$ –6 (*c*1.02, MeOH); R_F 0.39 (1:2 hexane–EtOAc); HRCI-MS calcd for $C_{13}H_{17}NO_6$ ($[M]^+$): 283.1056, found: 283.1054.

3.3.17. Methyl (*S*)-2-[2'-(3",4"-dihydroxyphenyl)acetamido]-3-(1'''*H*-indol-3'''-yl)propanoate (17)

Method A2. L-Tryptophan methyl ester hydrochloride **6** was used (76 mg, 0.30 mmol), and the reaction proceeded for 38 h. Column chromatography (hexane → 1:7 hexane–EtOAc) yielded **17**: 110 mg, quant. $[\alpha]_D^{23}$ +4 (*c* 1.15, DMF); R_F 0.33 (1:2 hexane–EtOAc); HRLSI-MS calcd for $C_{20}H_{20}N_2NaO_5$ ($[M + Na]^+$): 391.1270, found: 391.1276.

3.3.18. Methyl (*R*,*R*)-3,3'-Dithiobis{2-[2"-(3"',4"'-methylenedioxyphenyl)acetamido]propanoate} (19)

To a suspension of L-cystine dimethyl ester dihydrochloride **18** (102 mg, 0.03 mmol) in DMF (4.0 mL) at 0°C, Et_3N (0.33 mL, 2.40 mmol), (3,4- methylenedioxy)phenylacetic acid **1** (108 mg, 0.60 mmol) and PyBOP (312 mg, 0.60 mmol) were added under N_2. The corresponding mixture was kept under stirring at room temperature and in the dark for 7 h. Then, the reaction was concentrated to dryness and the residue was purified by column chromatography

(CH_2Cl_2 → 5:1 CH_2Cl_2−MeOH) to give **19** as a white solid. Yield: 147 mg, 83%; $[\alpha]_D^{28}$ +77 (*c* 1.0, CH_2Cl_2); R_F 0.44 (20:1 CH_2Cl_2–MeOH); m.p. 133−135 °C; CI-MS *m/z* 593 ([M + H]$^+$, 4%); HRCI-MS calcd for $C_{26}H_{29}N_2O_{10}S_2$ ([M + H]$^+$): 593.1264, found: 593.1245.

3.3.19. Methyl (*R*,*R*)-3,3'-Dithiobis{2-[2"-(3"',4"'-diacetoxyphenyl) acetamido]propanoate} (20)

To a suspension of L-cystine dimethyl ester dihydrochloride **18** (102 mg, 0.30 mmol) in DMF (4.0 mL), Et_3N (0.33 mL, 2.40 mmol), DOPAC (101 mg, 0.60 mmol) and PyBOP (312 mg, 0.60 mmol) were added under N_2. The corresponding mixture was stirred at room temperature in the dark for 12 h. Then, the solvent was removed under reduced pressure and the residue was conventionally acetylated with a 1:1 Ac_2O−Py mixture (2.0 mL) for 6.5 h. Then, the excess of Ac_2O was hydrolyzed with crushed ice, and concentrated to dryness; the residue was purified by column chromatography (CH_2Cl_2 → 60:1 CH_2Cl_2−MeOH) to give **20**. Yield: 158 mg, 72%; $[\alpha]_D^{25}$ +1 (*c* 1.0, CH_2Cl_2); R_F 0.64 (10:1 CH_2Cl_2−MeOH); LSI-MS *m/z* 759 ([M + Na]$^+$, 41%);HRLSI-MS calcd for $C_{32}H_{36}N_2NaO_{14}S_2$ ([M + Na]$^+$): 759.1506, found: 759.1502.

3.3.20. Methyl (*R*,*R*)-3,3'-Dithiobis{2-[2"-(3"',4"'-dihydroxyphenyl) acetamido]propanoate} (21)

To a solution of **20** (96 mg, 0.13 mmol) in a 1:1 CH_2Cl_2−MeOH mixture (2.0 mL) $CsCO_3$ was added (10 mg, 0.031 mmol), and the corresponding mixture was stirred at room temperature in the dark under inert atmosphere for 30 min. Then, the crude reaction was neutralized using Amberlite IR-120(H^+) resin, filtered, washed with MeOH, and the filtrate was concentrated to dryness. The residue was purified by column chromatography (CH_2Cl_2 → 20:1 CH_2Cl_2−MeOH) to give **21** as a yellowish syrup. Yield: 48 mg, 65%; $[\alpha]_D^{26}$ +28 (*c* 1.0, MeOH); R_F 0.38 (10:1 CH_2Cl_2−MeOH); LSI-MS *m/z* 591 ([M+Na]$^+$, 11%); HRLSI-MS calcd for $C_{24}H_{28}N_2NaO_{10}S_2$ ([M + Na]$^+$): 591.1083, found: 591.1058.

3.3.21. Methyl (*R*)-2-[2'-(3",4"-dihydroxyphenyl)acetamido]-3-mercaptopropanoate (23) [49]

To a solution of **21** (43 mg, 0.076 mmol) in an anhydrous 1:1 CH_2Cl_2−MeOH mixture (2.0 mL) (±)-dithiothreitol was added (12 mg, 0.076 mmol). The corresponding mixture was stirred at room temperature in the dark under inert atmosphere for 2 h. Then it was concentrated to dryness and the residue was purified by column chromatography (hexane → 1:10 hexane–EtOAc) to give **23** as a syrup. Yield: 19 mg, 44%; $[\alpha]_D^{25}$ −15 (*c* 1.2, MeOH); R_F 0.48 (1:5 1:10 hexane−EtOAc); HRLSI-MS calcd for $C_{12}H_{15}NNaO_5S$ ([M + Na]$^+$): 308.0569, found: 308.0569.

4. Conclusions

A one-step and almost quantitative transformation of *O*-unprotected DOPAC and L-amino acid esters (glycine, phenylalanine, valine, serine, tryptophan and cystine) into phenolic peptidomimetics has been achieved using PyBOP as the coupling reagent. A peptide derived from L-cysteine was also been prepared by dithiothreitol-mediated reduction of the disulfide linkage of the L-cystine derivative.

Title compounds have been evaluated as scavengers of ROS (anti-radical and H_2O_2 scavenging and inhibition of lipid peroxidation) as well as anti-proliferative agents against three human cervical cancer cell lines (HeLa, CaSki and ViBo); the degree of cell necrosis, and the selectivity of the various compounds against tumor cells over normal human lymphocytes has been evaluated. Remarkably, disulfide-containing peptide **21** was found to be the most active compound, with exceptional antioxidant properties, stronger than natural HT and a substantial anti-proliferative activity (IC_{50} 108.2−121.8 μM), with a 2-7-fold increase in this cytotoxic activity when compared with HT and DOPAC; moreover, its mode of action involved almost no cell necrosis, and essentially did not affect normal lymphocytes. Therefore, compound **21** might constitute a good lead candidate for the development of a novel family of anti-proliferative agents.

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Electronic Supplementary Information (ESI) available: ^{1}H- and ^{13}C-NMR spectra of compounds **7**-**11**, **13**-**17**, **20**, **21**, **23**.

Conflict of interest

We have no conflict of interest to declare.

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