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A novel and efficient synthesis of highly oxidized lignans by a methyltrioxorhenium/hydrogen peroxide catalytic system. Studies on their apoptogenic and antioxidant activity

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ABSTRACT

Highly oxidized lignans produced during the cytochrome P-450 metabolism in the cells show biological activities significantly different from those of their parent natural compounds. Lignans precursors of mammalian enterolignans were treated with a methyltrioxorhenium/hydrogen peroxide catalytic system to afford new compounds oxidized at benzylic as well as in arylic positions. The evaluation of the antioxidant and apoptogenic activity by in vivo protocols of these compounds showed some interesting structure–activity relationships related to the oxidation degree of the molecules.

1. Introduction

Lignans are a large group of naturally occurring phenols widely spread within the plant kingdom, that are derived from the shikimic acid biosynthetic pathway. These compounds show dimeric structures formed by a β,β′-linkage between two phenyl propane units with a different degree of oxidation in the side-chain and by a different substitution pattern in the aromatic moieties.[1] Because of their biological activities, such as antioxidant,[2] antimicrobial,[3] antitumour,[4] anti-inflammatory[5] and antiviral properties,[6] lignans have been used for a long time both in the ethnical and conventional medicine.[7] To date, most attention has been devoted to the antioxidant activity, mainly because due to the radical scavenging properties of these compounds. Some activity–structure relationship studies have been made to correlate the antioxidant activity to the degree of oxidation of the molecule.[2a] As a general reaction pattern, the presence of a catechol moiety increases the antioxidant activity, while oxidation at the benzylic position (the C-1 position in the side chain of the phenyl propane unit) decreases the activity, the presence of a keto group in this position being more inhibitory than that of the hydroxyl moiety. It is worth to note that these studies are usually performed under in vitro conditions by monitoring the conversion of a sacrificial substrate in the presence of active oxygen species and lignans. On the other hand, lignans that are characterized by a high value of antioxidant activity under in vitro conditions can modify their pharmacological profile in vivo, due to the metabolism in the cell. In this general context, lignans are subjected to oxidative metabolic transformations by enzymes of the cytochrome P-450 family.[8] During these transformations, lignans are oxidized both in the benzylic position by oxygen atom insertion on the activated sigma C–H bond, and on the arene moieties. In the latter case, the reaction proceeds through an initial epoxidation process, with or without de-O-methylation of the guaiacyl group, and successive oxidation steps to afford the corresponding hydroquinone and catechol or quinone derivatives. It is well known from the literature that these metabolites can tune the redox potential of the cell and, in particular quinones, can act as electrophilic species in alkylation of nucleophilic sites in proteins and nucleic acids.[9] Thus, the biological activity of lignans should be compared to that of the corresponding highly oxidized metabolites, to account for the potential role of metabolism in their biological activity. Unfortunately, only few data about the biological activity of oxidized lignans are available.[10] This is

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mainly due to the small amount of lignan metabolites recoverable from physiological fluids during in vivo treatments (less than ppm amounts of lignans metabolites are detectable by GC–MS and LC–MS analyses of organic fluid samples), associated with the difficulty to design efficient chemical strategies for the selective transformation of lignans stable, and some time recalcitrant, to oxidation.

In the last few years we have been involved in the design and development of novel stoichiometric and catalytic procedures for the oxidation of biologically active natural substances, including products from the primary (nucleic acids components, amino acids and peptides) and secondary (phenols and terpenes) metabolic pathways.[11] During these studies we observed that methyltrioxorhenium ($MeReO_3$, MTO), in combination with hydrogen peroxide (H_2O_2) as the primary oxidant, was a selective and efficient organometallic catalyst for the oxidation of several phenols,[12] flavonoids,[13] lignans, and lignin derivatives.[14] These oxidations, which proceed through two reactive intermediates, the monoperoxo $[MeRe(O)_2(O_2)]$ and bis-peroxo $[MeRe(O)(O_2)_2]$ η^2-rhenium complexes,[15] showed a selectivity that strictly depends on the stereoelectronic properties and substituents pattern of the substrates. As a general pathway, the oxidation of arene derivatives proceeds by a concerted oxygen atom transfer by a butterfly-like transition state, with formation of epoxide intermediates that are further rearranged and oxidized to corresponding quinones and hydroquinone or catechol derivatives. No radical pathways have been observed suggesting that the homolytic activation of H_2O_2 is not an operative process under these experimental conditions.

In the case of the oxidation of lignans of the aryltetralin family, we described that the oxidation of podophyllotoxin, isolated from different plants of the genus *Podophyllum*, with the MTO/H_2O_2 system, afforded quinone and isopodophyllotoxone derivatives, which have never been previously reported.[14a] The evaluation of the toxicity of these compounds showed an unprecedented shift of the biological activity from the inhibition of cell division at the level of the microtubule assembly by freezing the polymerization of tubulin at the colchicine site, which is characteristic of the parent podophyllotoxin, to the inhibition of topoisomerase II, a more interesting goal from a therapeutic point of view. More exciting was that the biological activity changed gradually so that some oxidation products showed both properties, inhibition of tubulin and activity against topoisomerase II. In particular, we observed a decreased inhibitory activity of polymerization of tubulin in the case of 2′,3′-*ortho*-benzoquinone derivatives versus a selective activity against topoisomerase II, when the oxidation of the benzylic C-7 position, affording isopodophyllotoxone, was accompanied by the ring-opening of the D-lactone moiety.[14a]

A similar structure activity relationships was also observed in the evaluation of the apoptogenic activity of products of the MTO/H_2O_2 oxidation of galbulin, an aryltetraline lignan isolated from *Galbulimima belgraveana*, against both human lymphoma cells lines BL41 (EBV-) prone to apoptosis and E2R (EBV+), which is strongly resistant to chemical treatment.[14a] Moreover, an unprecedented diastereoselective dearylation process, and production of rare and enantiomerically pure acuminatolide, was observed by treatment of the furfuran lignan asarinin with the MTO/H_2O_2 system.[14e]

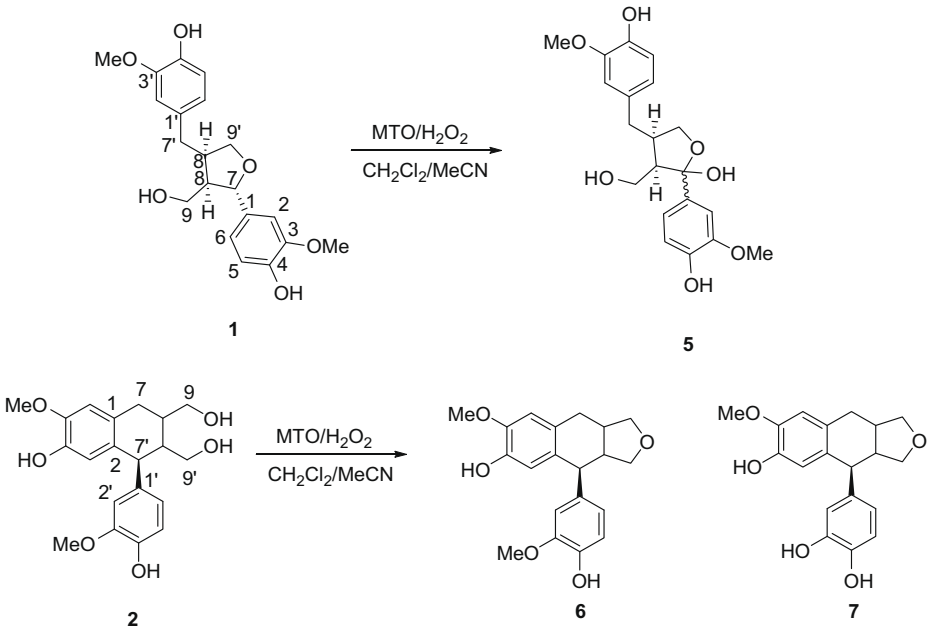
As a continuation of this study and with the aim to further evaluate the biological activity of highly oxidized lignans, we focused here the attention on the oxidation of lariciresinol **1**, isolariciresinol **2**, matairesinol **3** and 7-hydroxymatairesinol **4**, which are all precursors of mammalian enterolignans, with the MTO/H_2O_2 system. Enterolignans, that are enterolactones and enterodiols, are phytoestrogens that are formed by the human colonic microflora from plant lignans. Enterolactones have been suggested to inhibit the growth and development of breast and prostate cancer.[16,17] However, little is known about the mechanistic basis for its anticancer activity. In fact, these compounds can have strong apoptogenic activity on tumour cells by various mechanisms of action. In this paper, novel lignans have been evaluated for their cytotoxicity by an apoptosis assay and in vivo antioxidant activity to define qualitative structure–activity relationships with respect to parent compounds.

2. Chemical part

2.1. Catalytic oxidation with H_2O_2/MTO

For the oxidation of lignans **1–4** with MTO, the appropriate substrate (1.0 mmol) was added to a solution of MTO (5.0% w/w of the substrate) in CH_2Cl_2/CH_3CN (1:1 ratio; 12.0 mL), and a small excess of the primary oxidant H_2O_2 (1.5 equiv; 35% water solution) was added to the reaction mixture in several batches at 0 °C. In the absence of MTO, less than 2% of conversion of the substrate was observed. Treatment of **1** with the MTO/ H_2O_2 system gave 3,3′-dimethoxy-4,4′,7,9-tetrahydroxy-7,9′-epoxylignan **5,** a product of oxygen atom insertion at the C-7 benzylic ethereal position (Scheme 1) as the only recovered product at an acceptable conversion of substrate and high yield, besides the unreacted substrate (Table 1, entry 1). The absence of oxidation products characteristic of benzylic C-7′ position suggests that the electronic effect of the oxygen atom on the furan scaffold was a crucial factor for the regioselectivity of the reaction. Moreover, other possible products of the oxidation at the C-9 position (primary alcohol) and of the aromatic moieties were not detected in the reaction mixture by gas chromatography–mass spectrometry (GC–MS). These results are in accordance with previously reported data on the oxidation of the tetrahydrofuran lignan asarinin (not shown) with MTO, in which case the benzylic ethereal position was selectively oxidized. The configuration of the C-7 carbon in **5** was confirmed by NOE between H-8 and H-2(H-6). Unfortunately, compound **5** showed a low stability in solution even when stored at low temperature probably due to the ring-opening of the hemiacetalic moiety. The oxidation of isolariciresinol **2** afforded isoshonanin **6** and the catechol derivative **7** as the only recovered products in acceptable yields besides the presence of unreacted substrate (Scheme 1, Table 1, entry 2). No products of the benzylic oxidation of the C-7 and C-7′ positions were observed. In this latter case, MTO showed some multifunctional catalytic properties, acting contemporary as an acidic catalyst during the ring-closure of the new furan ring by dehydration of the C-9 and C-9′ primary alcohols, and as oxidative catalyst in the de-O-methylation of the guaiacyl moiety. The de-O-methylation process was regioselective at the guaiacyl moiety on the C-ring as suggested by the disappearance of the down-shielded C(3′)-OMe (δ_H 3.80 ppm) characteristic of the C-ring in both compounds **2** and **6** in ^1H NMR, and by the presence of the characteristic fragment *m/z* 266 which indicated the presence of a catechol moiety on the C-ring in GC–MS analysis.[2a] Probably, the reaction proceeded by initial formation of **6** and successive de-O-methylation to **7**. This hypothesis was confirmed by the quantitative oxidation of isolated **6** to **7** under identical experimental conditions.

Next we studied the oxidation of the butyrolactone lignans, matairesinol **3** and 7-hydroxymatairesinol **4**. The oxidation of **3** afforded a mixture of three reaction products in similar yield, 3-methoxy-3′,4,4′-trihydroxylignano-9,9′-butyrolactone **8**, 7′-hydroxymatairesinol **9** and the γ-hydroxyacid derivative **10** (Scheme 2, Table 1, entry 3). It is interesting to note that, in accordance with data previously reported for the oxidation of **2**, MTO again catalyzed the regioselective de-O-methylation of only one of the guaiacyl moieties in **3**, to give **8** that was identical to an



Scheme 1. Oxidation of lariciresinol **1** and isolariciresinol **2** with the hydrogen peroxide/methyltrioxorhenium catalytic system.

Table 1
Experimental data of oxidation as depicted in Schemes 1 and 2[a]

Entry	Substrate	Conv. (%)	Yield[b] (%)
1	**1**	60	**5**: 98
2	**2**	70	**6**: 30 **7**: 57
3	**3**	55	**8**: 30 **9**: 30 **10**: 40
4	**4**	82	**11**: 35 **12**: 37 **13**: 16 **14**: 8

[a] In a typical experiment, hydrogen peroxide (1.5 equiv, 35% water solution) and the substrate (1.0 mmol) to be oxidized were added to a solution of MTO (5% w/w) in CH_2Cl_2/CH_3CN at $T = 0$ °C.
[b] Calculated on converted substrate.

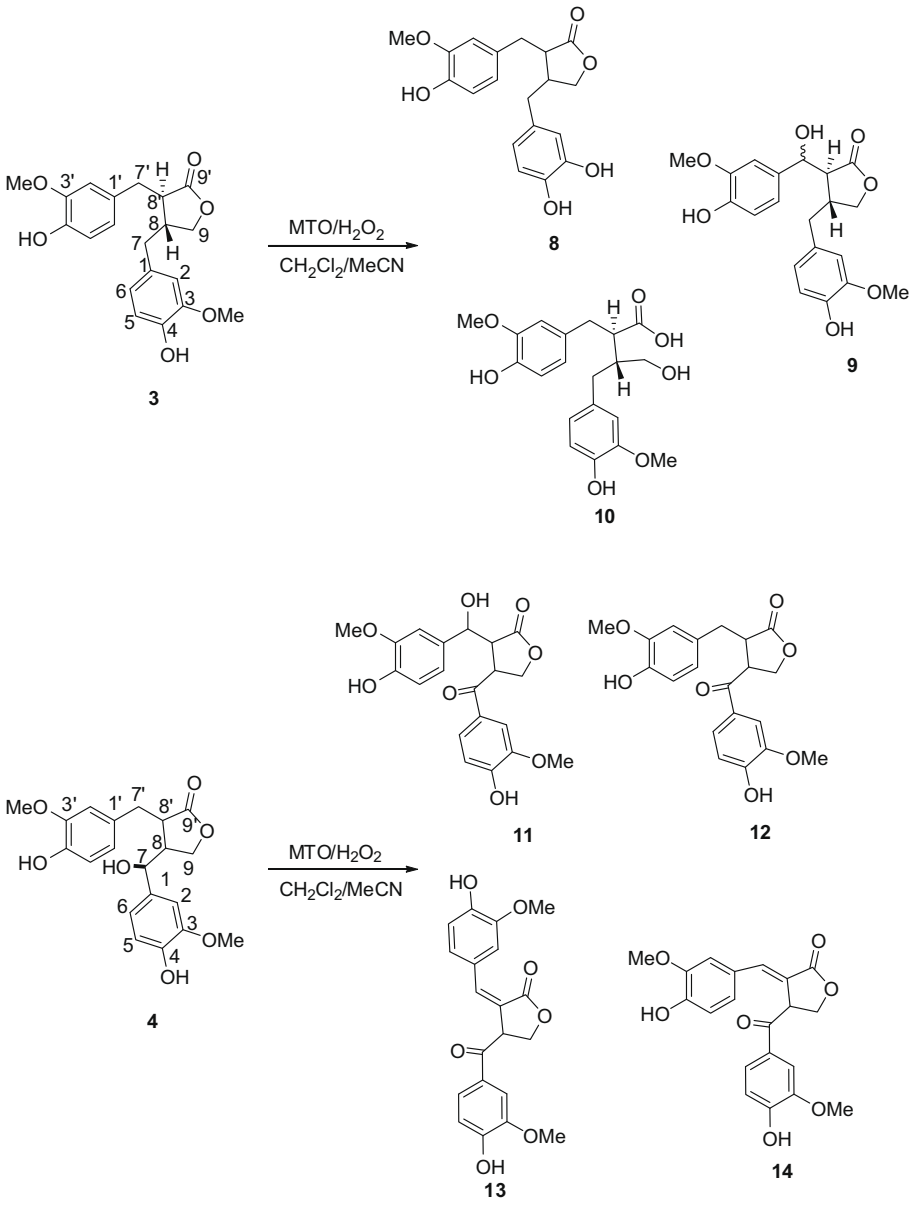
authentic sample (see for example $[\alpha]_D$ of **8** −21.3 *c* 0.114 MeOH versus $[\alpha]_D$ −20° *c* 0.114 MeOH or $[\alpha]_D$ −22.8 *c* 0.114 MeOH).[18] These data suggest that the reactivity of the guaiacyl moiety towards electrophilic oxidants like activated MTO complexes strictly depends on its position on the lignan scaffold probably due to stereoelectronic effects exerted by the other substituents on the arene moieties. A significative regioselectivity was also observed in the formation of **9** in which case the oxygen atom insertion at the C-7′ benzylic position was the only oxidative process operative in the side-chain of the molecule. In this latter case, stereoelectronic effects occurred even if a possible orientating effect of the carbonyl group on the approach of the catalytic species of the substrate can not be completely ruled out. Finally, compound **10** was simply obtained by hydrolytic ring-opening of the lactone moiety in **3**.

During the oxidation of **4**, a more complex mixture of reaction products was observed, including 7′-hydroxy-7-oxomatairesinol **11**, 7-oxo-matairesinol **12** and isomeric 7′,8′-dehydro-7-hydroxymatairesinol (*Z*) **13** and 7′,8′-dehydro-7-hydroxymatairesinol (*E*) **14,** compounds **11** and **12** being synthesized in highest yield (Table 1, entry 4). The structures of all products were confirmed by comparison with authentic samples.[19] The products formed in the reaction above support the presence of an initial oxidation of the benzylic C-7 position to afford **12**, followed by successive oxygen atom insertion into the C-7 benzylic position (as in the case of **11**) or a dehydration process to give isomers **13** and **14**. This reaction pattern is similar to that previously reported for the oxidation of **4** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in dioxane.[19] In this latter case, the initial formation of benzylic cations by abstraction of hydride ion was a crucial step. Instead, on the basis of the known property of activated MTO complexes, the transfer of the oxygen atom to the substrate in our case required a concerted mechanism.[15]

3. Biological part

3.1. Apoptosis evaluation

The cytotoxicity of selected lignans **6–7**, **8–9** and **11–13** and their parent compounds **2, 3**, and **4** have been evaluated by apoptosis induction (Figs. 1 and 2). Lignan **1** and α-conidendrin **15** (not shown) were also evaluated as references. Lignan **5** has not been included in our study since it was not stable enough in solution to perform biological tests. Apoptosis induction has been tested on prone human B cell lymphoma line (BL41) and on the resistant isogenic derivative (E2R).[20] The apoptosis assay was done in parallel with both cell lines. In addition, E2R cell line (apoptosis resistant) was employed in an acute in vivo test to evaluate the ability of the lignans to modulate the oxidative status of the cells. The parent lignans lariciresinol **1**, isolariciresinol **2**, hydroxymatairesinol **4**, and α-conidendrin were scarcely apoptogenic in both cell lines BL41 and E2R, with the exception of matairesinol **3** which showed an appreciable activity. In the case of isolariciresinol-type lignans **6** and **7**, compound **6** resulting from the ring-closure of the dihydroxy moiety present in parent lignan, and resembling to a deoxo-α-conidendrin derivative, showed the highest apoptogenic activity for both cell lines. Thus, the process of ring-closure of the dihydroxy moiety increases the cytotoxicity. On the contrary, the de-O-methylation of the guaiacyl moiety in parent compound, decreases the cytotoxicity as shown by the comparison between derivatives **6** and **7**, the apoptogenic activity of **7** being comparable to that of the low-toxic lignan **2** (Fig. 1, panels A and B).



Scheme 2. Catalytic oxidation of matairesinol **3** and 7-hydroxymatairesinol **4**.

In the family of butyrolactone lignans, the oxidation of the C-7 position (compound **4**) or C-7′ position (compound **9**) decreases the apoptogenic activity against both cell lines with respect to **3**, in which case there are not hydroxyl moieties in the side-chain (Fig. 2, panels A and B). On the other hand, when both benzylic C-7 and C-7′ positions are contemporary oxidized, one of which to carbonyl moiety (C-7 position), as in the case of **11**, the most intensive apoptogenic activity was obtained for the resistant E2R cell line. A similar behaviour was observed in the case of side-chain dehydrated **13**. In accordance with data previously shown for compound **7**, the presence of the cathecol moiety in **8** slightly decreases the apoptogenic activity with respect to parent matairesinol **3**.

3.2. Measuring the oxidative intracellular status

Next, we examined the antioxidant or pro-oxidant effect of the lignans on the intracellular redox level. This in vivo assay was performed by using the resistant E2R cell line in the presence of the probe dichlorodehydrofluoresceine diacetate (DCFH-DA) that is a selective probe versus oxygen and nitrogen peroxides,[20] in flow cytometric analysis. Cells were exposed to the same doses of lignan samples employed for the apoptosis evaluation, but for a short exposition time to avoid interference of possible degenerative processes (apoptosis, necrosis, etc.) on the measure of oxidative status of the cells. The flow cytometric analysis was both qualitative, allowing to evaluate the oxidation at single cell level and providing information on the overall status of cell population in the culture, and quantitative in that the results can be treated by integrating the values of population/subpopulations from the pool of single measurements. As shown in Figure 3 none of the parent lignans showed a pro-oxidant activity. Matairesinol **3** and α-conidendrin **15** were the only compounds among the parent lignans that displayed a significant antioxidant activity. Since compound **3** showed a significant high toxicity compared to α-conidendrin (see above), the toxicity of these compounds can not be directly correlated to their in vivo antioxidant activity. Lignans **6** and **7** did not show a significant antioxidant activity (Fig. 3, panel A). In the family of butyrolactone lignans, **3** and **8** were more active than

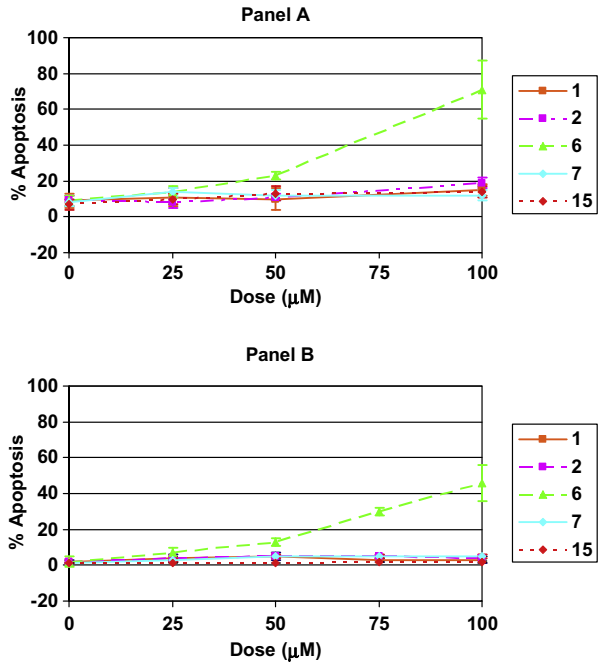


Figure 1. Apoptosis evaluation of compounds **1**, **2**, **6**, **7** and **15**. Panel A: prone human B cell lymphoma line (BL41). Panel B: resistant isogenic derivative (E2R).

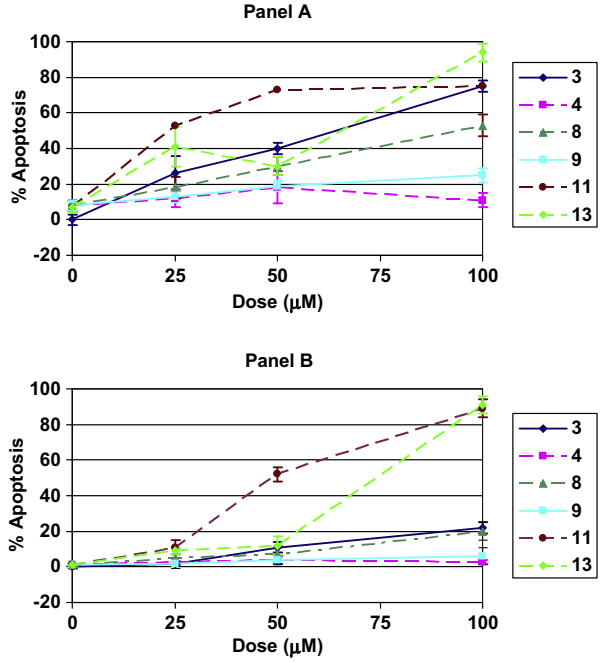


Figure 2. Apoptosis evaluation of compounds **3**, **4**, **8**, **9**, **11** and **13**. Panel A: prone human B cell lymphoma line (BL41). Panel B: resistant isogenic derivative (E2R).

4, **9**, **11** and **13** (Fig. 3, panel B). It is worth to note that the catechol moiety has a significant role in the antioxidant activity while the oxidation of the benzylic C-7 and C-7′ positions did not influence or even decreases the antioxidant activity (compare activity of compounds **8** and **9**).

4. Conclusions

On the basis of the data previously reported on the apoptogenic activity of lignan derivatives it is reasonable to suggest that the

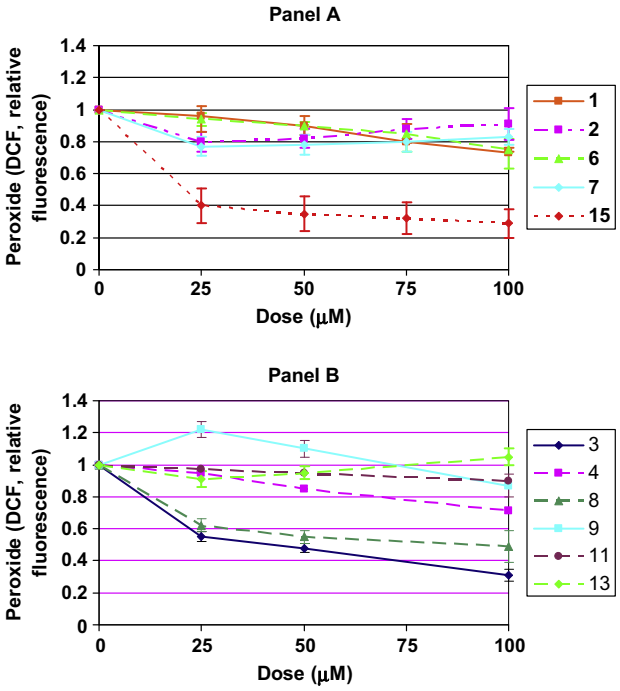


Figure 3. Antioxidant or pro-oxidant effect of the lignans on the intracellular redox level.

presence of a cathecol moiety or the mono-oxidation of the benzylic C-7 and C-7′positions decreases the cytotoxicity, while the ring-closure process to a novel tetrahydro furane ring, as well as, the contemporary oxidation of both benzylic positions increases the toxicity. Finally, the presence of the lactone moiety do not seems to be relevant per se for the apoptogenic activity. About the antioxidant activity, all lignans that are characterized by a relevant antioxidant activity contain the lactone moiety. In this latter case, the oxidation of the benzylic positions always decreases the antioxidant activity.

5. Experimental

All solvents and reagents used were of analytical grade and were purchased from Aldrich Chemical Co. Silica Gel 60 F254 plates and Silica Gel 60 were furnished from Merck. Lignans **1–4** were purchased from Oy ArboNova Ab. The nomenclature of lignans and neolignans has been attributed according to IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN), Recommendations 2000.

5.1. Oxidation with MTO/H_2O_2. General procedure

In a typical experiment, hydrogen peroxide (1.5 equiv, 35% water solution) and the substrate (1.0 mmol) to be oxidized were added to a solution of the methyltrioxorhenium (5% w/w) in CH_2Cl_2/CH_3CN, 1:1 (12 mL) and the mixture was stirred at $T = 0$ °C. Oxidations were monitored by TLC using the mixture CH_2Cl_2/CH_3OH (9.2/0.8 v/v) as eluent. At the end of the reaction, MnO_2 was added to the solution to degrade the excess of hydrogen peroxide and the reaction mixture was vigorously stirred for 15 min. After filtration, the solvent mixture was evaporated under vacuum. The reaction products were extracted with ethyl acetate (3×20 mL) and washed with a NaCl saturated solution (2×20 mL). The organic layers were dried over anhydrous sodium sulfate. The crude product was purified by preparative TLC using dichloromethane/methanol (9.5/0.5 v/v) mixtures as eluents. All

the products were identified by ^{1}H NMR, ^{13}C NMR and GC–MS spectra. ^{1}H NMR and ^{13}C NMR were recorded on a Bruker 200 MHz spectrometer using $CDCl_3$ (99.8% in deuterium), CD_3OD (99.8% in deuterium) or CD_3COCD_3 (99.8% in deuterium) as solvents. All chemical shift are expressed in parts per million (δ scale) and coupling constants in Hertz (Hz).

5.1.1. 3,3′-Dimethoxy-4,4′,7,9-tetrahydroxy-7,9′-epoxylignan (5)

Oil. δ_H (200 MHz, $CDCl_3$, $(CD_3)_2CO$): 2.65 (1H, m, H-8′), 2.7 (1H, m, H-8), 3.0 (2H, m, H-6), 4.04–4.14 (4H, m, H-9 and H-9′), 4.22 (3H, s, OMe), 4.26 (3H, s, OMe), 7.02–7.20 (6H, m, Ar-H); δ_C (200 MHz, $CDCl_3$, $(CD_3)_2CO$): 36.3 (C-7′), 40.2 (C-8′), 55.4 (2 × OMe), 59.8 (C-9), 61.5 (C-8), 72.4 (C-9′), 109.5 (C-7), 112.1 (C-2′and C-2), 114.4 (C-5′), 114.5 (C-5), 119.7 (C-6′), 121.8 (C-6), 132.5 (C-6′), 132.5 (C-1′), 136.1 (C-1), 144.5 (C-4′), 145.0 (C-4), 147.1 (C-3′),147.2 (C-3). MS (EI): *m/z* 376 (M^+).

5.1.2. Isoshonanin (6)

Oil.[21] δ_H (200 MHz, $CDCl_3$): 2.24 (2H, m, H-8 and H-8′), 2.70 (1H, m, H-7), 2.96 (1H, m, H-7), 3.50–3.82 (4H, m, H-9 and H-7′ and H-9′), 3.80 (3H, s, OMe), 3.85 (3H, s, OMe), 4.2 (1H, m, H-9), 6.37 (1H, s, H-3), 6.54–6.82 (4H, m, H-2′, H-6, H-6′ and H-5′); δ_C (200 MHz, $CDCl_3$): 32.5 (C-7), 42.4 (C-8′), 49.6 (C-7′), 50.6 (C-8), 55.8 (OMe), 55.9 (OMe), 72.4 (C-9′), 73.2 (C-9), 110.4 (C-6), 111.0 (C-2′), 114.3 (C-5′), 115.2 (C-3), 121.5 (C-6′), 127.6 (C-1), 133.0 (C-2), 136.5 (C-1′), 143.8 (C-4′), 144.4 (C-4), 145.0 (C-3′), 146.7 (C-5). MS (EI): *m/z* 342 (M^+).

5.1.3. 3′,4′-Dihydroxy-5-methoxy-2,7′-cyclolignan-9,9′-furan (7)

Oil. δ_H (200 MHz, $CDCl_3$): 1.94–2.22 (2H, m, H-8′ and H-8), 2.51–2.92 (2H, m, H-7), 3.34–3.82 (3H, m, H-7′ and H-9), 3.84 (3H, s, OMe), 4.27–4.44 (2H, m, H-9′), 6.48 (1H, s, H-6), 6.59 (1H, s, H-6′),6.67–7.12 (3H, m, H-3, H-2′and H-5′); δ_C (200 MHz, $CDCl_3$): 37.7 (C-7), 38.3 (C-8′), 49.6 (C-8), 53.6 (C-7′), 55.8 (OMe), 72.2 (C-9), 78.7 (C-9′), 112.9 (C-6), 114.2 (C-2′), 114.3 (C-5′), 117.9 (C-3), 121.2 (C-6′), 128.8 (C-1), 133.6 (C-1′), 138.4 (C-2), 143.4 (C-4′), 144.2 (C-3′), 144.9 (C-4), 145.5 (C-5). MS (EI): *m/z* 328 (M^+).

5.1.4. 3-Methoxy-3′,4,4′-hydroxylignan-9,9′-butyrolactone (8)

Amorphorus powder.[18b] δ_H (200 MHz, $CDCl_3$, $(CD_3)_2CO$): 2.45–2.64 (4H, m, H-7, H-8 and H-8′), 2.85 (2H, m, H-7′), 3.83 (3H, s, OMe), 3.88 (1H, m, H-9), 4.12 (1H, m, H-9), 6.45–6.80 (6H, m, Ar-H); δ_C (200 MHz, $CDCl_3$, $(CD_3)_2CO$): 35.2 (C-7′), 38.7 (C-7), 42.4 (C-8), 47.9 (C-8′), 56.3 (OMe), 72.8 (C-9), 113.8 (C-2′), 116.0 (C-5′), 116.3 (C-5), 116.7 (C-2), 120.9 (C-6), 130.5 (C-1′), 131.3 (C-1), 122.9 (C-6′), 144.8 (C-4), 146.2 (2×, C-3 and C-4′), 148.1 (C-3′), 181.4 (C-9′). MS (EI): *m/z* 344 (M^+).

5.1.5. 7′-Hydroxymatairesinol (9)

Oil. δ_H (200 MHz, $CDCl_3$): 2.15–2.55 (3H, m, H-8′ and H-7′), 2.93 (1H, m, H-8), 3.62 (3H, s, OMe), 3.79 (3H, s, OMe), 3.92 (2H, m, H-9′), 5.35 (1H, s, H-7), 6.41–6.82 (6H, m, H-2, H-2′, H-6, H-6′, H-5 and H-5′); δ_C (200 MHz, $CDCl_3$): 35.6 (C-7′), 42.8 (C-8′), 55.5 (C-8), 55.8 (OMe), 55.9 (OMe), 72.0 (C-9′), 74.5 (C-7), 110.9 (C-2), 111.2 (C-2′), 114.3 (C-5′), 115.9 (C-5), 120.2 (C-6), 120.8 (C-6′), 132.6 (C-1′), 133.2 (C-1), 145.2 (C-4′), 146.3 (C-3′), 147.0 (C-4), 148.7 (C-3), 178.1 (C-9). MS (EI): *m/z* 374 (M^+).

5.1.6. γ-Hydroxyacid derivative (10)

Oil. δ_H (200 MHz, $CDCl_3$): 2.08–2.61 (2H, m, H-8′ and H-8), 3.46 (2H, m, H-9′), 3.76 (3H, s, OMe), 3.79 (3H, s, OMe), 5.13 (1H, s, H-7), 6.31–6.57 (6H, m, H-2, H-2′, H-6, H-6′, H-5 and H-5′); δ_C (200 MHz, $CDCl_3$): 36.6 (C-7′), 39.1 (C-8′), 56.5 (C-8), 55.7 (OMe), 55.8 (OMe), 65.3 (C-9′), 76.4 (C-7), 110.7 (C-2), 112.5 (C-2′), 115.7 (C-5′), 116.1 (C-5), 119.9 (C-6), 122.8 (C-6′), 131.8 (C-1), 132.2 (C-1′), 145.6 (C-4′), 147.0 (C-3′), 147.3 (C-4), 148.9 (C-3), 177.7 (C-9). MS (EI): *m/z* 392 (M^+).

5.1.7. 7′-Hydroxy-7-oxomatairesinol (11)

Oil.[19] δ_H (200 MHz, $CDCl_3$, $(CD_3)_2CO$): 3.38 (1H, m, H-8′), 3.47 (3H, s, OMe), 3.73 (3H, s, OMe), 3.90–4.10 (3H, m, H-9 and H-8), 5.11–5.15 (1H, m, H-7′), 6.57–6.77 (6H, m, Ar-H); δ_C (200 MHz, $CDCl_3$, $(CD_3)_2CO$): 45.6 (C-8), 50.0 (C-8′), 56.0 (2 × OMe), 67.2 (C-9), 73.6 (C-7′), 109.2 (C-2), 110.9 (C-2′), 114.3 (C-5), 117.0 (C-5′), 120.2 (C-6), 124.6 (C-6′), 129.7 (C-1), 133.2 (C-1), 147.0 (C-3), 149.7 (C-3′), 151.6 (C-4), 176.3 (C-9′),194.7 (C-7). MS (EI): *m/z* 388 (M^+).

5.1.8. 7-Oxo-matairesinol (12)

Oil.[19] δ_H (200 MHz, $CDCl_3$, $(CD_3)_2CO$) 2.90–2.96 (2H, m, H-7′), 3.44 (1H, m, H-8′), 3.67 (3H, s, OMe), 3.86 (3H, s, O-Me), 4.0–4.30 (3H, m, H-8 and H-9), 6.48 (1H, m, H-6′), 6.54 (1H, m, H-2′), 6.55 (1H, m, H-5′), 6.81 (1H, m, H-5), 7.13 (1H, m, H-6), 7.27 (1H, m, H-2); δ_C (200 MHz, $CDCl_3$, $(CD_3)_2CO$): 34.5 (C-7′), 44.9 (C-8′), 46.5 (C-8), 55.8 (OMe), 56.2 (OMe), 69.4 (C-9), 110.1 (C-2), 111.8 (C-2′), 113.9 (C-5), 114.4 (C-5′), 122.37 (C-6′), 123.6 (C-6), 128.7 (C-1), 128.9 (C-1′), 144.7 (C-4′), 146.6 (C-3′), 147.1 (C-3), 151.4 (C-4),177.3 (C-9′), 195.0 (C-7). MS (EI): *m/z* 372 (M^+).

5.1.9. 7′,8′-Dehydro-7-hydroxymatairesinol (*Z*) (13)

Oil.[19] δ_H (200 MHz, $CDCl_3$, $(CD_3)_2CO$): 3.36 (1H, m, H-8), 3.81 (3H, s, OMe), 3.85 (3H, s, OMe), 4.29 (1H, m, H-9), 4.52 (1H, m, H-9), 4.80 (1H, m, H-7), 6.50 (1H, m, H-7′), 6.78-7.10 (5H, m, H-2, H-5, H-5′, H-6, H-6′), 8.11 (1H, d, J = 2.0 Hz, H-2′); δ_C (200 MHz, $CDCl_3$, $(CD_3)_2CO$): 50.9 (C-8), 56.0 (OMe), 56.4 (OMe), 67.3 (C-9), 75.9 (C-7), 111.0 (C-2), 115.0 (C-2′), 115.1 (C-5′), 115.3 (C-5), 120.0 (C-6), 123.9 (C-8′), 126.9 (C-6′), 127.2 (C-1′), 135.5 (C-1), 142.3 (C-7′), 146.6 (C-4), 147.5 (C-3), 149.0 (C-4′), 170.8 (C-9′). MS (EI): *m/z* 372 (M^+).

5.1.10. 7′,8′-Dehydro-7-hydroxymatairesinol (*E*) (14)

Oil.[19] δ_H (200 MHz, $CDCl_3$, $(CD_3)_2CO$) 3.86 (3H, s, OMe), 3.92 (3H, s, OMe), 4.10 (1H, m, H-8), 4.14 (1H, m, H-9), 4.51 (1H, m, H-9), 5.11 (1H, m, H-7), 6.80(1H, m, H-5), 6.80–7.29(5H, m, H-2, H-2′, H-5′, H-6, H-6′), 7.45 (1H, d, J = 2.1 Hz, H-7′); δ_C (200 MHz, $CDCl_3$, $(CD_3)_2CO$): 45.7 (C-8), 56.1 (OMe), 56.4 (OMe), 66.2 (C-9), 71.6 (C-7), 110.0 (C-2), 114.2 (C-2′), 114.9 (C-5′), 116.0 (C-5), 118.9 (C-6), 121.7 (C-8′), 124.8 (C-6′), 127.1 (C-1′), 134.6 (C-1), 137.3 (C-7′), 146.3 (C-4), 148.2 (C-3),149.1 (C-4′), 173.0 (C-9′). MS (EI): *m/z* 372 (M^+).

5.2. Cell culture

BL 41 is an EBV negative cell line derived from a Burkitt Lymphoma while E2R is the isogenic counterpart, converted to EBV positive, and apoptosis resistant.[20] Both cell lines were grown in RPMI medium with 4 mM L-glutamine and 10% FCS. All incubations were at 37 °C in a 5% CO_2 atmosphere.

5.3. Apoptosis evaluation

Both BL41 and E2R cell lines were seeded at concentration of 3 × 105 cells/mL and the compounds were added for 18 h of incubation. Compounds were dissolved in DMSO which never exceeded 0.5% v/v. For morphological analysis of apoptogenic activity cell were fixed in 4% (v/v) *p*-formaldehyde and stained with a solution (0.2 μg/mL) of DAPI. Apoptosis was quantified by scoring cells condensed and fragmented nuclei according to the reported procedure.[22] At least 500 cells, in random fields, were scored using an epifluorescent microscopy.

5.4. Flow cytometric analysis of ROS

Intracellular peroxide levels were assessed using an oxidation-sensitive fluorescent probe DCFH-DA (Sigma–Aldrich). In presence of a variety of intracellular peroxides, DCFH-DA is oxidized to 2′,7′-dichlorofluorescein. Cells after treatments of 1 h with the tested compounds (dissolved in DMSO 0.2%.) in RPMI medium were incubated in the same medium with 10 μM DCFH-DA for 20′ and at 37 °C in CO_2 incubator. The analysis was performed using FACScalibur cytometer (Becton & Dickinson) with excitation at $\lambda = 488$ nm, emission at $\lambda = 533$ nm recorded in FL-1 and 10,000 events were recorded for each sample.

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