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Methyltrioxorhenium catalysed synthesis of highly oxidised aryltetralin lignans with anti-topoisomerase II and apoptogenic activities

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Abstract—A novel and efficient procedure to prepare highly oxidised aryltetralin lignans, such as isopodophyllotoxone and (−)-aristologone derivatives, by oxidation of podophyllotoxin and galbulin with methylrhenium trioxide (MTO) and novel MTO heterogeneous catalysts is reported. It is noteworthy that in the case of isopodophyllotoxone derivatives the functionalisation of the C-4 position of the C-ring and the ring-opening of the D-lactone moiety increased the activity against topoisomerase II while causing the undesired inhibition of tubulin polymerisation to disappear. The novel (−)-aristologone derivatives showed apoptogenic activity against resistant human lymphoma cell lines.

1. Introduction

Lignans are a family of natural products with a broad variety of biological and pharmacological activities.[1] Among them, aryltetralin derivatives are of special interest owing to their powerful antitumoral, antimitotic, antiviral, cardiovascular and immunosuppressive activity.[2] Aryltetralin derivatives show also a selective nonredox inhibition of 5-lipoxygenase by interaction with the arachidonic acid binding site and could form a new class of therapeutic agents for the treatment of asthma and rheumatoid arthritis.[3] Podophyllotoxin **1**, isolated from different plants of the genus *Podophyllum*, is the most investigated aryltetralin derivative. It is a well-established inhibitor of cell division at the level of the microtubule assembly by freezing polymerisation of tubulin at the colchicine site.[4] This activity has led to the design of semi-synthetic derivatives, such as etoposide (4′-demethyl-7-[4,6-*O*-ethylidene-β-D-gluco-pyranosyl epipodophyllotoxin) and teniposide (4′-demethyl-7-[4,6-*O*-thenilidene-β-D-glucopyranosyl epipodophyllotoxin), which have been widely used for the treatment of small-cell lung cancer, testicular cancer, lymphoma and acute lymphocytic leukaemia.[5] Etoposide and teniposide show different side effect profiles compared to podophillotoxin due to their action as selective inhibitors of DNA topoisomerase II, a key enzyme involved in DNA transcription, replication, recombination and possibly DNA repair.[6] In recent years, several syntheses of podophyllotoxin and aryltetralin lignan derivatives have been reported in the literature which mainly focused on structural modifications of leader molecules to obtain less toxic analogues with high biological activities. On the other hand, a few data are available on oxidative functionalisation,[7] a process that plays a relevant role in their biological mechanism of action. For example, it is known that etoposide undergoes oxidative *ortho*-demethylation by a cytochrome P450-dependent metabolic process to a 3′,4′-dihydroxy derivative.[8] This derivative is further oxidised to the corresponding *ortho*-benzoquinone, a highly reactive intermediate responsible for the irreversible binding to proteins and DNA.[9] In a similar way, a semibenzoquinone free radical intermediate of etoposide is responsible for DNA strand breakage.[10] Since general and selective methods for the oxidation of aryltetralin lignans are still lacking,[11] novel synthetic strategies are needed to

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prepare highly oxidised derivatives for the evaluation of their biological activity. A novel catalyst useful for this purpose is methyltrioxorhenium ($MeReO_3$, MTO).[12] In the last decade MTO, in combination with H_2O_2, has been used in several organic trasformations.[13] The reactive intermediates for these oxidations are monoperoxo [$MeRe(O)_2O_2$] and bis-peroxo [$MeReO(O)_2$]η^2-rhenium complexes.[14] Accordingly, with this high reactivity, MTO has been used for the oxidation of phenols and methoxy benzene derivatives.[15] In this latter case, the reaction proceeds through the formation of epoxide intermediates that are further rearranged and oxidised to corresponding benzoquinones.[16] The efficient oxidation of several aromatic derivatives to *ortho*- and *para*-benzoquinones has also been reported by use of novel heterogeneous rhenium compounds based on the heterogenation of MTO on easily available and low-cost poly(4-vinylpyridine) 2% cross-linked with divinylbenzene (PVP-2/MTO, **I**) and microencapsulation on polystyrene (PS-2/MTO, **II**).[13,17]

Here, we report that MTO and MTO heterogeneous catalysts **I** and **II**, whose structures are reported in Figure 1, can be used for the unprecedented one-pot preparation of highly oxidised isopodophyllotoxone derivatives from podophyllotoxin using H_2O_2 as primary oxidant. Notably, the oxidation of the C-4 position of the C-ring and the concomitant ring-opening of the D-lactone moiety enhances the activity against topoisomerase II, while causing the undesired inhibition of tubulin polymerisation to disappear. The generality of this procedure was further shown by the unprecedented synthesis of benzoquinone derivatives of galbulin, an aryltetralin lignan isolated from *Galbulimima belgraveana*.[18] Benzoquinone derivatives of galbulin were evaluated for apoptogenic activity against human lymphoma cell lines BL41 (EBV−) prone to apoptosis and E2R (EBV+) which is strongly resistant to chemical treatment.

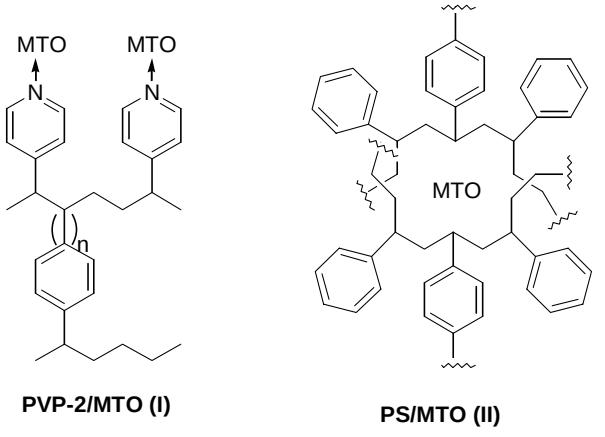


Figure 1.

2. Chemistry

Initially, the oxidation of aryltetralin derivatives was investigated using MTO under homogeneous conditions. As a general procedure, podophyllotoxin **1** (1.0 mmol) was added to a solution of MTO (0.05 mmol) in the appropriate solvent (vide infra). Hydrogen peroxide (H_2O_2, 4.0 mmol, 30% aqueous solution) was added to the reaction mixture repeatedly over 24 h period. The results of the oxidations are summarised in Table 1 and Scheme 1.

In the absence of the catalyst, less than 2% conversion of substrate took place under otherwise identical conditions. The reaction performed in acetic acid (AcOH, 5 mL) at 25 °C gave the *ortho*-benzoquinone of isopodophyllotoxone, compound **2**, as the only recovered product in 35% yield and quantitative conversion of substrate (Table 1, entry 1).

In accordance with the high reactivity reported for MTO in the presence of AcOH, the low-mass balance with respect to isolated product might be due to the formation of polar hydrophilic over-oxidation products not recovered by usual work-up procedures.[19] Probably, the reaction involves the formation of highly reactive arene oxide intermediates that can be further rearranged and oxidised at both the C- and E-rings.[15a] *ortho*-Benzoquinone analogues of podophyllotoxin and 4′-de-*O*-methylpodophyllotoxin (not shown) bearing the quinone

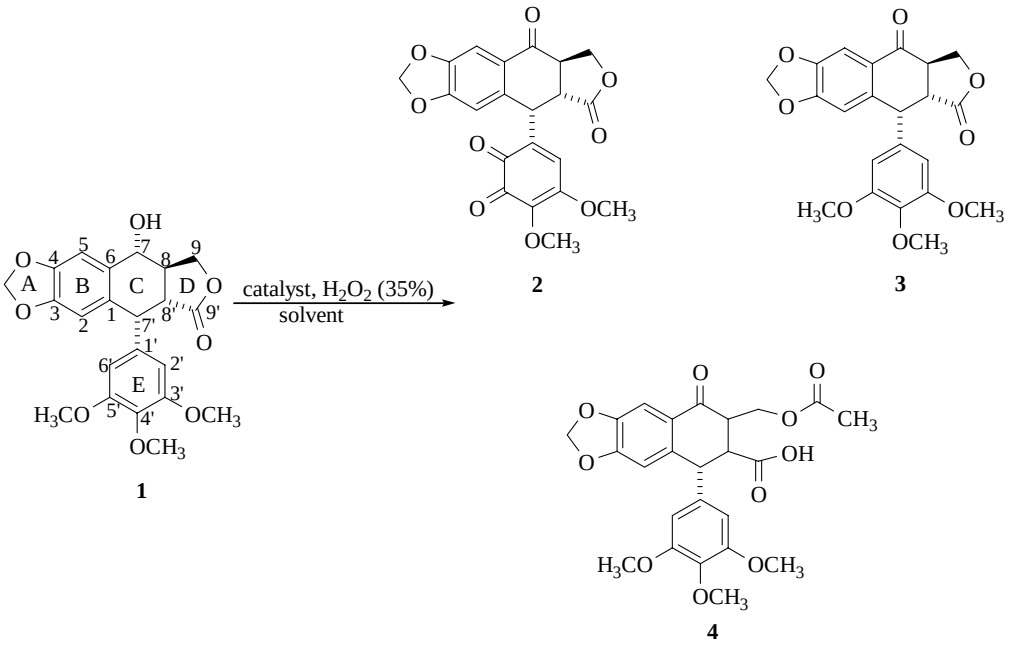
Table 1. Oxidative functionalisation of podophyllotoxin **1** and galbulin **5** with MTO and MTO heterogeneous catalysts **I** and **II**

Entry	Solvent	Substrate	*T* (°C)	*t* (h)	Catalyst	Conversion (%)	Product(s)	Yield (%)
1	CH_3COOH	**1**	20	24	MTO[a]	>96	2	35
2	CH_2Cl_2/CH_3CN	**1**	50	24	MTO[a]	80	2	60
3	CH_2Cl_2/EtOH	**1**	20	24	MTO[a]	>99	2	15
4	CH_2Cl_2/EtOH	**1**	−10	24	MTO[a]	>99	2	71
5	CH_3COOH	**1**	40	66	PVP-2/MTO(**I**)[b,c]	>99	2	69
6	CH_3COOH	**1**	20	66	PS/MTO(**II**)[b,c]	>99	2(3)[4]	13(4)[62]
7	CH_2Cl_2/EtOH	**1**	20	66	PVP-2/MTO(**I**)[b,c]	>99	2	65
8	CH_2Cl_2/CH_3CN	**5**	20	24	MTO	44	6(7)[8]{9}	25(17)[21]{28}
9	CH_2Cl_2/CH_3CN	**5**	20	66	PVP-2/MTO(**I**)[b,c]	40	6(7)[8]{9}	36(6)[10]{9}
10	CH_2Cl_2/CH_3CN	**5**	20	7	PS/MTO(**II**)[b,c]	65	7(8)[9]	9(12)[40]

[a] All the reactions were performed with H_2O_2 (35% aqueous solution) using 0.05 mmol catalyst and 1.0 mmol substrate.

[b] Catalysts **I** and **II** were prepared adding MTO (1.0 mmol) to a suspension of the resin [poly(4-vinylpyridine) or polystyrene 2% cross-linked with divinyl benzene; 1.0 g] in ethanol (5 mL) at room temperature.

[c] All the reactions were performed treating podophyllotoxin **1** and galbulin **5** (1.0 mmol), dissolved in the appropriate solvent, with catalysts **I** and **II** (100 mg, loading factor 1.0) and H_2O_2 (4.0 mmol).



Scheme 1.

moiety on the 3′- and 4′-positions of the E-ring have been previously synthesized by oxidative demethylation with sodium metaperiodate and nitric acid.[20] In this latter case, the presence of a substituent on the C-2′ position of the E-ring was found to stabilise the stereochemistry of products against epimerisation.[21] To the best of our knowledge, this is the first example of synthesis of *ortho*-benzoquinone derivative of podophyllotoxin showing the quinone moiety on the 2′-, 3′- positions of the E-ring. When the reaction was performed in CH_2Cl_2/CH_3CN (5 mL, 1:1 v/v) at 50 °C compound **2** was isolated as the only recovered product in 60% yield and 80% conversion of substrate (Table 1, entry 2). A better result was observed performing the reaction in CH_2Cl_2/EtOH (5 mL, 1:1 v/v) at −10 °C in which case compound **2** was recovered in 71% yield (Table 1, entry 4). In contrast, a low selectivity was obtained in CH_2Cl_2/EtOH (5 mL, 1:1 v/v) at 25 °C (Table 1, entry 3). Next, we studied the oxidation of podophyllotoxin **1** with heterogeneous catalysts **I** and **II**. The reactivity and selectivity of MTO in these catalysts can be tuned by the chemical–physical properties of the resin used as a support of the active species.[22] Briefly, MTO (1.0 mmol) was added to the suspension of the appropriate resin poly(4-vinylpyridine) (PVP-2) or polystyrene (PS) 2% cross-linked with divinylbenzene, in ethanol (5 mL) at 25 °C. After 1 h, the solvent was removed by filtration and the catalysts were used without further purification. Podophyllotoxin **1** dissolved in the appropriate solvent (vide infra) was added portion wise to catalysts **I** and **II** (100 mg, loading factor 1. The loading factor is referred as mmol of active species for gram of resin) and H_2O_2 (4.0 mmol) at 20–40 °C. At the end of the reaction catalysts were recovered by filtration. As reported in Scheme 1, the performance of poly(4-vinylpyridine)/MTO catalyst **I** in AcOH at 40 °C was similar to that observed for MTO in mixed solvents affording compound **2** as the only recovered product in quantitative conversion of substrate and 69% yield (Table, entry 5 vs entries 2 and 4). Similar results were obtained in CH_2Cl_2/EtOH mixture (1:1, v/v) (Table 1, entry 6). Notably, a different reaction pathway was observed with microencapsulated MTO catalyst **II**. In this latter case, the oxidation in AcOH at 25 °C gave the novel acetylated D-ring opened isopodophyllotoxone derivative **4** as the main product (62%) beside compound **2** (13%) and low amount of isopodophyllotoxone **3** (4%) (Table 1, entry 7).[23] Modification of the D-ring of podophyllotoxin including the ring-opening of the lactone moiety has been reported in the literature to give products with different degrees of oxidation at positions C-9 and C-9′.[24] These modifications include the synthesis of the hydrazine derivative, GP-11, which is almost equipotent than etoposide.[25] A different selectivity depending on the nature of the support used for the heterogenisation process of MTO has been previously observed in the oxidation of *N*,*N*′-disubstituted hydroxylamines to nitrones, and during the preparation of benzoquinone and γ-lactone derivatives from natural phenols. For example, higher conversions and yields of benzoquinones were obtained during the oxidation of substituted hydrogenated cardanols (3-*n*-pentadecylphenols) with catalyst **I** with respect to MTO and catalyst **II** because of a support mediated molecular recognition process based on hydrogen bonding interactions between the pyridinyl moiety and the phenolic group of substrate.[26] Irrespective of the catalyst used for the oxidation, the lower reactivity of heterogeneous catalysts **I** and **II** with respect to MTO (see for example reaction times in Table 1: entries 5 and 6 vs 1) was probably due to the presence of a kinetic barrier to the approach of compound **1** to rhenium polymeric compounds.

To evaluate the generality of this procedure, galbulin **5** was oxidised with MTO under similar experimental conditions. Treatment of **5** (1.0 mmol) with MTO

(0.05 mmol) and H_2O_2 (4.0 mmol, 30% aqueous solution) in CH_2Cl_2/CH_3CN (1:1 v/v, 5 mL) at 25 °C gave the *para*-benzoquinone derivatives of (−)-aristoligone [(7′*R*,8*S*,8′*R*)-8,8′-dimethyl-3′,4′,4,5-tetramethoxy-2,7′-cyclolignan-7-one], compounds **6**–**9**, in similar yields and acceptable conversion of substrate (Table 1, entry 8, Scheme 2). A better result was obtained performing the reaction with catalyst **I** in which case *para*-benzoquinone **6** was recovered as the main reaction product in 36% yield (Table 1, entry 9, Scheme 2). Again, a different selectivity was observed with catalyst **II**. In this latter case, the reaction performed under similar experimental conditions afforded *para*-benzoquinone **9** as the main reaction product in 40% yield beside low amount of compounds **7** and **8** (Scheme 2, Table 1, entry 10).

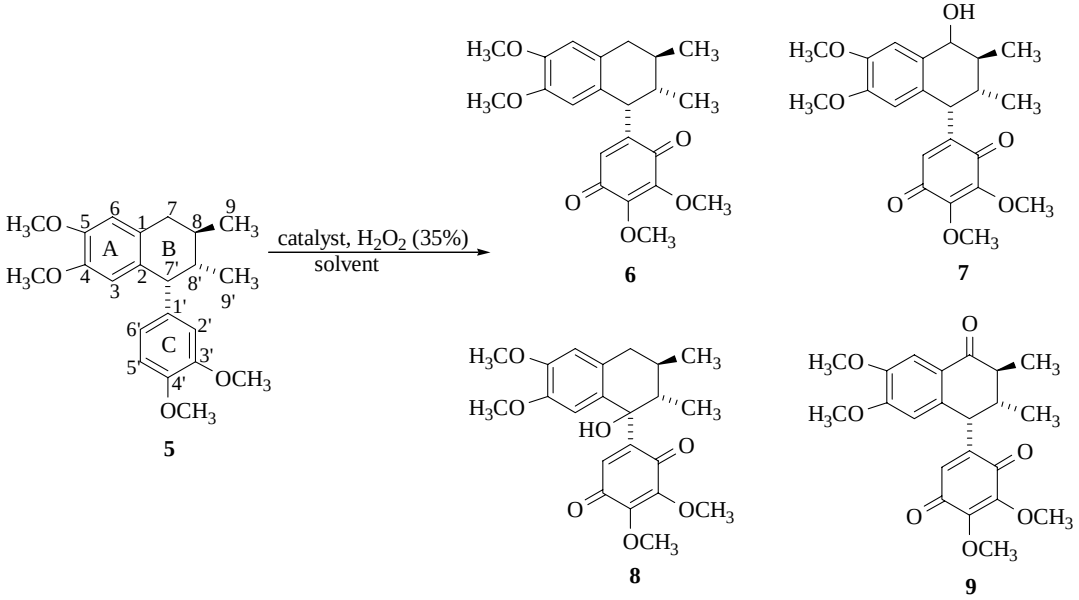
Low amounts of (−)-aristologone have been detected in the roots of *Holostylis reniformis* and in *Aristolochia* species used in Brazilian traditional medicine.[27] To the best of our knowledge there are no data available in the literature on the synthesis and biological activity of benzoquinone derivatives of (−)-aristoligone.

3. Biology

3.1. Activity of isopodophyllotoxone derivatives 2–4 against topoisomerase II

To evaluate the activity and mechanism of action of isopodophyllotoxone derivatives **2**–**4**, we analysed the mitotic indices (MI) and the frequencies of chromosomal aberrations following a 3 h treatment with four different dose levels of each compound (selected in preliminary dose-range finder experiments) in the G_2 phase of cell cycle in a Chinese hamster ovary (CHO) cell line. This cell line is routinely used in mutagenicity testing as indicated in the guidelines of EEC Council 79/831 and OECD for the test of chemical No. 471. The positive control Colcemid® which causes accumulation of cells in metaphase by inhibiting the depolymerisation of tubulin was also included at 0.2 μg/mL, a very low concentration routinely employed in cytogenetic assays to accumulate cells in a metaphase-like stage. Evaluation of mitotic indices (number of metaphases out of 1000 cells scored expressed as percentage) was used to assess the interference of compounds **2**–**4** with polymerisation of tubulin during assembly of mitotic spindle apparatus while analyses of chromosomal aberrations in the G_2 phase of cell cycle were used to assess the inhibition of DNA topoisomerase II. This indirect evaluation of activity against topoisomerase II is based on the fact that non catalytic inhibitors of both DNA topoisomerases I and II act by stabilising a ternary complex known as the 'cleavable complex' in which the enzyme is covalently linked to DNA. In the topoisomerase II reaction both single (SSBs) and double DNA strand breaks (DSBs) are formed while in the topoisomerase I reaction only DNA SSBs are generated. These DNA breaks are therefore produced by an enzyme-mediated process and being 'protein concealed,' they can be detected by DNA filter elution methodology only if the cell lysate is digested with a proteinase before elution.[28] Inhibitors of DNA topoisomerase II, which give rise to DSBs, the ultimate lesion responsible for the production of chromosomal aberrations, are able to induce chromosomal aberrations in all phases of the cell cycle, acting similar to ionising radiation by a typical 'S-independent' mechanism. Furthermore, unlike the majority of 'S-independent' agents, they are also able to induce sister chromatid exchanges (SCE's) when the treatment is performed in the S phase of the cell cycle.[28b]

MI for the podophyllotoxin and isopodophyllotoxone derivatives **2**–**4** are displayed in Figure 2, A–D, respectively. Tables 2–5 show the incidence of chromosomal aberrations induced by phodophillotoxin and compounds **2**–**4**, respectively. In these latter tables, at each dose level, the total number of cell scored, the frequency of chromatid and chromosome deletions,



Scheme 2.

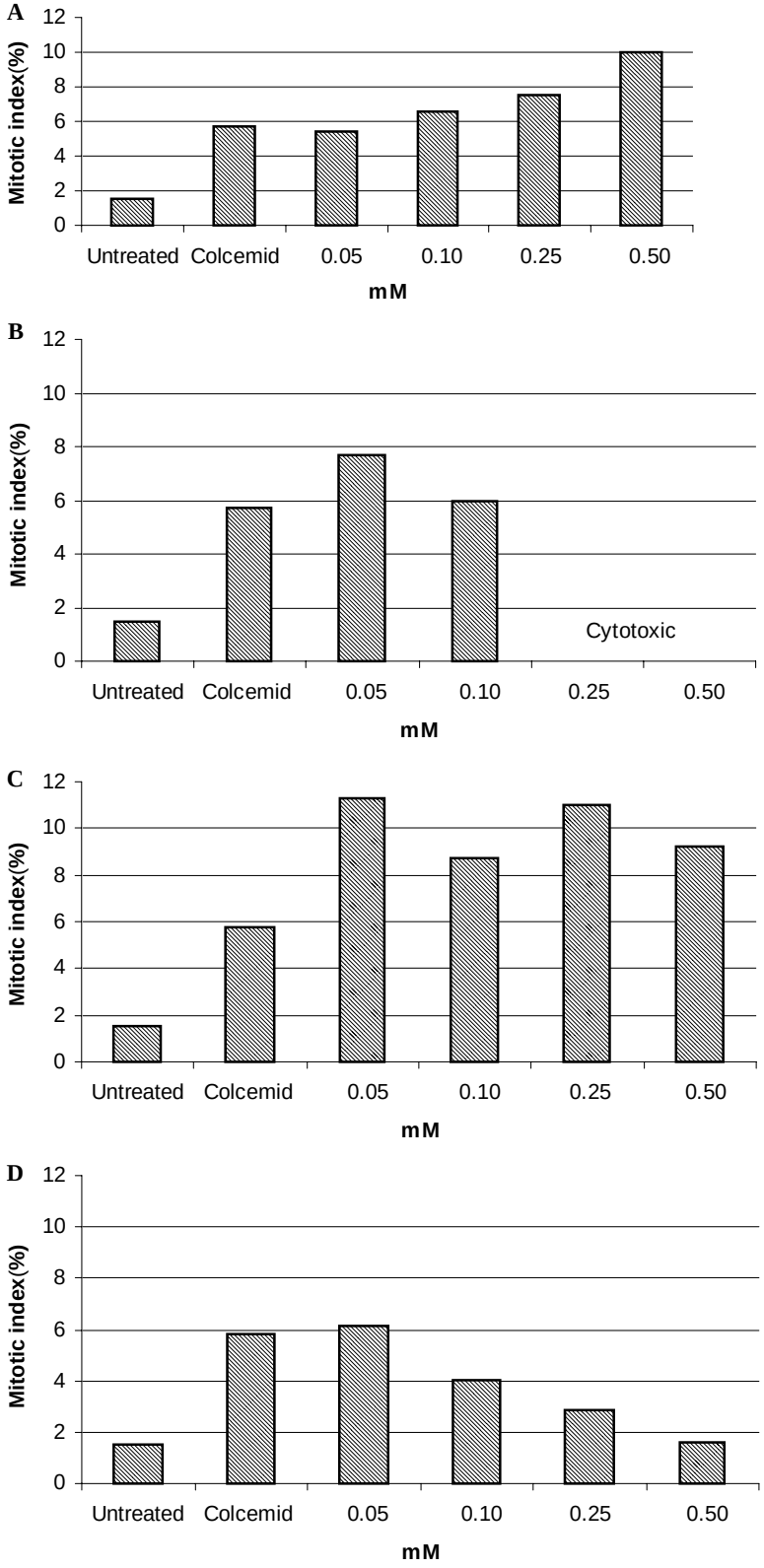


Figure 2. Mitotic index values (MI) of podophyllotoxin **1** (A), benzoquinone derivative **2** (B), isopodophyllotoxone **3** (C), and isopodophyllotoxone derivative **4** (D) in a Chinese hamster ovary (CHO) cell line.

exchanges and the percentage of cells bearing aberrations excluding gaps are reported. Statistical significance calculated on the number of cells bearing aberrations is also shown.

Podophyllotoxin **1** (Fig. 2A, Table 2) induced marked and dose-related increases in the MI, compared to the untreated and positive control (colcemid)-treated cultures. At the high-dose level this increase was 1.74 times

Table 2. Incidence of chromosomal aberrations in CHO cells by podophyllotoxin **1**

Treatment	Dose level (mM)	*N*	Total number of aberration (%)				*F*	Aberrant cells (%)	Stat. sig.	Polyploid cells (%)	Relative MI (%)
			Chromatid		Chromosome						
			Breaks	Exch.	Breaks	Exch.					
Untreaded positive control	0.00	100	2	0	0	0	0	2.0	NS	0	26
Colcemid	0.20	100	2	0	0	0	0	0.0	—	2.0	100
Compound **1**	0.05	100	1	0	1	0	0	2.0	NS	1.0	105
Compound **1**	0.10	100	4	0	0	0	0	4.0	NS	2.0	115
Compound **1**	0.25	100	2	0	0	0	0	2.0	NS	4.0	130
Compound **1**	0.50	100	5	0	0	0	0	6.0	NS	3.0	173

N: total number of metaphases scored (100 metaphases/culture). *F*: isolocus events which include isochromatid and isolocus breaks when these cannot be distinguished.
Aberrant cells (%): percentage of cells bearing aberrations (excluding gaps).
Relative MI: mitotic index relative to colcemid-treated culture (percent).
Stat. sig.: statistical significance (test compound-treated culture vs colcemid treated culture). NS: not significant.

Table 3. Incidence of chromosomal aberrations in CHO cells by benzoquinone **2**

Treatment	Dose level (mM)	*N*	Total number of aberration (%)				*F*	Aberrant cells (%)	Stat. sig.	Polyploid cells (%)	Relative MI (%)
			Chromatid		Chromosome						
			Breaks	Exch.	Breaks	Exch.					
Untreaded positive control	0.00	100	2	0	0	0	0	2.0	NS	0	26
Colcemid	0.20	100	2	0	0	0	0	2.0	NS	2.0	100
Compound **2**	0.05	100	0	0	0	0	0	0.0	NS	1.0	134
Compound **2**	0.10	100	5	0	1	0	0	6.0	NS	10.0	104
Compound **2**	0.25	No metaphases found (cytotoxic)									
Compound **2**	0.50	No metaphases found (cytotoxic)									

N: total number of metaphases scored (100 metaphases/culture). *F*: isolocus events which include isochromatid and isolocus breaks when these cannot be distinguished.
Aberrant cells (%): percentage of cells bearing aberrations (excluding gaps).
Relative MI: mitotic index relative to colcemid-treated culture (percent).
Stat. sig.: statistical significance (test compound-treated culture vs colcemid treated culture). NS: not significant.

Table 4. Incidence of chromosomal aberrations in CHO cells by isopodophyllotoxone **3**

Treatment	Dose level (mM)	*N*	Total number of aberration (%)				*F*	Aberrant cells (%)	Stat. sig.	Polyploid cells (%)	Relative MI (%)
			Chromatid		Chromosome						
			Breaks	Exch.	Breaks	Exch.					
Untreaded positive control	0.00	100	2	0	0	0	0	2.0	NS	0	26
Colcemid	0.20	100	2	0	0	0	0	0.0	—	2.0	100
Compound **3**	0.05	100	0	0	0	0	0	0.0	NS	1.0	196
Compound **3**	0.10	100	5	0	0	1	3	9.0	**	0.0	151
Compound **3**	0.25	100	1	0	0	1	0	2.0	NS	4.0	191
Compound **3**	0.50	100	10	0	0	0	0	10.0	***	1.0	160

N: total number of metaphases scored (100 metaphases/culture). *F*: isolocus events which include isochromatid and isolocus breaks when these cannot be distinguished.
Aberrant cells (%): percentage of cells bearing aberrations (excluding gaps).
Relative MI: mitotic index relative to colcemid-treated culture (percent).
Stat. sig.: statistically significance (test compound-treated culture vs colcemid treated culture). NS: not significant.
** Statistically at $P < 0.05$ with Fisher's exact test.
*** Statistically at $P < 0.01$ with Fisher's exact test.

Table 5. Incidence of chromosomal aberrations in CHO cells by isopodophyllotoxone derivative **4**

Treatment	Dose level (mM)	*N*	Total number of aberration (%)				*F*	Aberrant cells (%)	Stat. sig.	Polyploid cells (%)	Relative MI (%)
			Chromatid		Chromosome						
			Breaks	Exch.	Breaks	Exch.					
Untreaded positive control	0.00	100	2	0	0	0	0	2.0	NS	0	26
Colcemid	0.20	100	2	0	0	0	0	0.0	—	2.0	100
Compound **4**	0.05	100	3	0	0	3	0	6.0	NS	3.0	106
Compound **4**	0.10	100	20	0	0	0	0	20.0	***	4.0	70
Compound **4**	0.25	Very few metaphases (cytotoxic)									25
Compound **4**	0.50	Very few metaphases (cytotoxic)									15

N: total number of metaphases scored (100 metaphases/culture). *F*: isolocus events which include isochromatid and isolocus breaks when these cannot be distinguished.
Aberrant cells (%): percentage of cells bearing aberrations (excluding gaps).
Relative MI: mitotic index relative to colcemid-treated culture (percent).
Stat. sig.: statistical significance (test compound-treated culture vs colcemid treated culture). NS: not significant.
*** Statistically at $P < 0.01$ with Fisher's exact test.

the value of colcemid-treated cultures. Alternatively, podophyllotoxin did not induce significant increases in the frequency of chromosomal aberrations compared to control values. Polyploid cells were also observed.

Compound **2** showed a cytotoxic effect at the two higher-dose levels as indicated by the absence of mitotic activity. At lower-dose levels an increase in the mitotic index values similar to podophyllotoxin was observed (Fig. 2B, Table 3).

On the contrary, treatments with compound **3** induced significant increases in the frequency of chromosomal aberrations compared to control cultures and elevated values of mitotic indices not related to the dose levels which in some cases were even higher than podophyllotoxin ones (Fig. 2C, Table 4).

Notably, compound **4** induced marked and dose-related decreases of mitotic indices compared to control values. At the two higher-dose levels employed, mitotic indices reached 25% and 47% of control values indicating a clear cytotoxic effect. At immediate lower-dose level, mitotic index value reached 68% of control one and significant increases in chromosomal aberrations ($p < 0.01$) were observed at this dose level. Such a result indicates that the mechanism of action of compound **4** is different compared to the podophyllotoxin . In fact, in this latter case the polymerisation of tubulin into microtubule appears not be influenced as evinced by decreased mitotic index values. Alternatively, a new plausible antitopoismerase II activity appears to be generated as evinced by induction of chromosomal aberrations (Fig. 2D, Table 5). The profile of results on MI and chromosomal aberrations for compound **4** is influenced by general cytotoxic effects. A possible buffering effect on tubulin can be excluded by the observation of very few metaphases (cytotoxic effects) at dose levels higher than 0.1 mM (Table 5).

On the basis of these results it is possible to define the following qualitative structure–activity relationships: (i) compound **2**, characterised by the presence of the 2′,3′-*ortho*-benzoquinone moiety on the E-ring and by the contemporary oxidation of the C-7 benzylic position on the C-ring, shows a decreased inhibitory activity polymerisation of tubulin accompanied by a global increase of cytotoxicity. (ii) Isopodophyllotoxone **3**, in which the oxidation of the C-7 benzylic position on the C-ring is the only oxidative functionalisation, shows an enhanced inhibitory activity of polymerisation of tubulin and concurrent generation of anti-topoisomerase II activity. (iii) Finally, the oxidation of the C-7 benzylic position, the concomitant ring-opening of the D-lactone moiety and the acetylation of the newly formed hydroxyl group further enhance the activity of compound **4** against topoisomerase II causing at the same time a significant decrease in the inhibitory activity against tubulin polymerisation. To the best of our knowledge, this is the first example reported in the literature of a isopodophyllotoxone derivative characterised by a significant anti-topoisomerase II activity.

4. Apoptogenic activity of (−)-aristologone derivatives 6–9 against human lymphoma cells lines, BL41 (EBV−) and E2R (EBV+)

Lignans are a promising class of apoptogenic compounds. For example, NDGA, epiashantin and arctigenin causes mitochondrial dependent apoptosis in colorectal tumor cells.[29] Lignans are inhibitors of 5′-lipooxygenase LOX (e.g., NDGA) and generally of arachidonic acid metabolism whose modulation or inhibition has dramatic effect on survival/apoptosis of cancer cells. The choice of the cell line utilised to assay the toxicity of these compounds seems to be of great relevance. In fact, the extent of apoptosis resistance, which largely differs among the cell lines, may lead to mis-interpretation of the results. So the utilisation of a couple of isogenic cell lines, which differ for apoptosis resistance, may give a more complete information about toxicity and may be of great interest to identify new compounds capable of inducing apoptosis in resistant

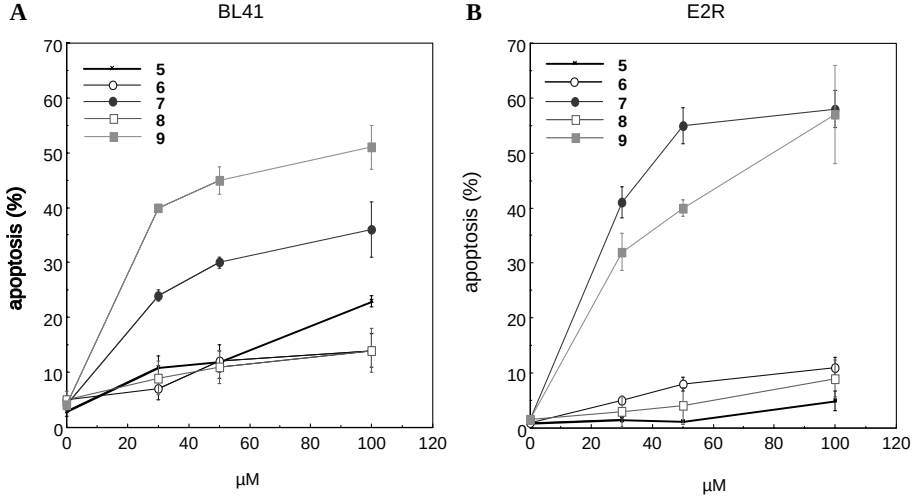


Figure 3. Apoptogenic activity of galbulin **5** and (−)-aristologone derivatives **6**–**9** against human lymphoma cell line BL41(EBV−) (A) prone to apoptosis and strongly resistant cells E2R (EBV+) (B).

cell lines. In this light,[30] galbulin **5**, and (−)-aristologone derivatives **6**–**9** were tested on human lymphoma cell line BL41(EBV−) prone to apoptosis and on the converted ones E2R (EBV+) which is strongly resistant.[31] Galbulin **5**, and compounds **6** and **8** resulted to be weak inducers of apoptosis only in the sensitive cell line (Fig. 3A and B). Notably, compounds **9** and **7** resulted to be strongly apoptogenic both on sensitive and apoptosis resistant cell lines, being **7** more effective on E2R. Thus, the highest apoptogenic activity was observed in (−)-aristologone derivatives **9** and **7** characterised by both the *para*-benzoquinone moieties on the C-ring and by the oxidation of the benzylic C-7 position of the B-ring to ketone or hydroxyl moiety. On the contrary, the oxidation of the benzylic C-1 position of the B-ring was not important for the apoptogenic activity.

The compounds, at the tested doses, did not show the capability to producing necrosis, using the trypan blue test (data not shown) hence stressing the notion that the strong apoptogenic activities on human lymphoma cell lines are specific.

5. Conclusions

MTO, poly(4-vinylpyridine)/MTO **I** and microencapsulated polystyrene/MTO **II** were efficient catalysts for the functionalisation of podophyllotoxin **1** and galbulin **5** with H_2O_2 as primary oxidant. Irrespective of experimental conditions used for the transformations, both aryltetralin derivatives were oxidised at the corresponding benzylic positions and on the aromatic moieties. In the oxidation of **1**, the *ortho*-benzoquinone **2** produced by de-alkylation of one methoxy substituent and contemporary oxygen atom insertion on the benzylic C-7 position was obtained as the only recovered product with MTO and catalyst **I**. The products of C-7 benzylic oxidation and ring-opening of the lactone on the D-ring, compounds **3** and **4**, were isolated in the presence of catalyst **II**. *para*-Benzoquinones **6**–**9**, with a different degree of oxidation at the benzylic positions, were synthesized by oxidation of **5** both with MTO and **I**.

From the biological point of view, the presence of the *ortho*-benzoquinone moiety in **2** does not increase the activity against topoisomerase II. On the contrary, the D-ring opened derivative **4** showed the highest biological activity as evaluated by mitotic index and aberration studies, with isopodophyllotoxone **3** possessing an intermediate behaviour. The anti-topoisomerase II activity of **4** is noteworthy. It is known from the literature that some cyclolignan derivatives lacking the lactone moiety are potent inhibitors of topoisomerase II.[24c] In accordance with the hypotheses suggested to rationalise this behaviour,[32] the significant activity of **4** could be accounted for in terms of a spontaneous or enzyme-mediated transformation of the acetylated alcoholic moiety into a more reactive electrophilic intermediate that was able to react with nucleophiles of biomolecules. In the case of (−)-aristologone derivatives **6**–**9**, the presence of the *para*-benzoquinone moiety on the C-ring was an important structural property to increase the apoptogenic activity. The highest activity was observed when the benzylic C-7 position on the B-ring was also oxidised as, for example, in the case of compound **7**. Noteably, the oxidation of the benzylic C-7′ position in 8 does not enhance the biological activity, showing that the site of the oxy-functionalisation on the B-ring is of relevant importance for the apoptogenic properties of these derivatives.

6. Experimental

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AM 400, or Bruker 200 spectrometers. Mass spectroscopy (MS) was performed with a GC Shimadzu GC-17A and a mass-selective detector QP 6000. All solvents were of ACS reagent grade, and were redistilled and dried according to the standard procedures. Chromatographic purifications were performed on columns packed with Merck silica gel 60, 230–400 mesh for flash technique. Thin layer chromatography was carried out using Merck platten Kieselgel 60 F254.

6.1. Starting materials

Podophyllotoxin **1** and MTO were commercial availables (Aldrich). Galbulin **5** was prepared as previously reported.[33] Dimethylsulfoxide (DMSO) of spectroscopic grade was obtained from Fluka AG (Switzerland). Colcemid (Gibco BRL) was used at a final concentration of 0.2 μg/mL. Poly(4-vinyl pyridine)/MTO **I** and polystyrene/MTO **II** catalysts were prepared as previously reported.[17] In summary, to a suspension of 600 mg of the appropriate resin in 4 mL of ethanol (tetrahydrofuran in the case of polystyrene) 77 mg (0.3 mmol) MTO, was added and the mixture was stirred for 1 h using a magnetic stirrer. Coocervates were found to envelop the solid core dispersed in the medium and 5.0 mL hexane added to harden the capsule walls. The solvent was removed by filtration, and the solid residue was washed with ethyl acetate and finally dried under high vacuum. In each case, MTO had completely become bound to the polymer. This result was confirmed by spectroscopic analysis of the residue obtained after evaporation of the organic layers. Catalysts were used without any further purification.

6.2. Oxidation of podophyllotoxin 1 and galbulin 5

6.2.1. Homogeneous oxidation. General procedure. A 10 mL reaction flask was charged sequentially with substrate (1.0 mmol), the appropriate solvent (5 mL; AcOH, CH_2Cl_2/CH_3CN 1:1 v/v, CH_2Cl_2/EtOH 1:1 v/v), MTO (0.05 mmol) and H_2O_2 (35% aqueous solution, 4.0 mmol). The stirred solution became yellow due to formation of peroxo species. The mixture was stirred at the appropriate temperature (see Table 1) until no more starting material could be detected on TLC. The mixture was extracted with CH_2Cl_2 (3× 50 mL), dried over Na_2SO_4, and then concentrated. The crude was purified by preparative TLC (CH_2Cl_2/MeOH 9.7:0.3) and by flash chromatography. Products were identified

by their ^{1}H NMR, ^{13}C NMR, MS and elemental analyses, and by comparison with authentic sample (in the case of isopodophyllotoxone).

6.2.2. Heterogeneous oxidation. General procedure. To the solution of the substrate (1.0 mmol) in AcOH or CH_2Cl_2 /EtOH 1:1 v/v (5.0 mL) were added catalysts I and II (100 mg, loading factor 1) and H_2O_2 (4.0 mmol) at 25–50 °C. The mixture was stirred until no more starting material could be detected on TLC. The suspension was filtered off, and the recovered catalyst was washed with CH_2Cl_2. The crude was purified by preparative TLC (CH_2Cl_2/MeOH 9.7:0.3) and by flash chromatography. Products were identified by ^{1}H NMR, ^{13}C NMR, MS and elemental analyses, and by comparison with authentic sample (in the case of isopodophyllotoxone).

6.2.3. Benzoquinone 2. δ_H ($CDCl_3$): 7 (1H, s, PhH), 6.25 (1H, s, PhH), 6.13 (1H, s, PhH), 6 (2H, m, CH_2), 4.9 (1H, d, $J = 6.4$ Hz, CH), 4.8–4.5 (2H, m, CH_2), 4.0 (6H, s, OCH_3), 3–2.6 (2H, m, CH). δ_C ($CDCl_3$): 207 (C=O), 183.95 (C=O), 182.61 (C=O), 174.24 (C), 149.44 (C), 147.47 (C), 145.37 (C), 144.07 (C), 134.44 (C), 132.65 (C), 128.58 (PhH), 128.25 (C), 108.77 (PhH), 106.04 (PhH), 101.62 (CH_2), 71.63 (CH_2), 61.28 (CH_2), 60.97 (OCH_3), 43.81 (CH), 41.70 (CH), 33.57 (CH); *m/z* (EI) 412 (M^+). Anal. Calcd for $C_{21}H_{16}O_9$: C, 61.17; H, 3.91. Found: C, 61; H, 3.89.

6.2.4. Isopodophyllotoxone 3. δ_H ($CDCl_3$): 7.095 (1H, s, PhH), 6.49 (1H, s, PhH), 6.35 (2H, s, PhH), 5.95 (2H, m, CH_2), 4.59 (2H, m, CH_2), 4.08 (1H, m, CH), 3.79 (9H, s, OCH_3), 2.81–2.75 (2H, m, CH). δ_C ($CDCl_3$): 188 (C=O), 173 (C=O), 153 (C), 152.7 (C), 152.2(C), 147.8 (C), 140.36 (C), 137.55 (C), 133.9 (C), 128 (C), 110.2 (PhH), 107.5 (PhH), 105.5 (PhH), 102.2 (CH_2), 66.8 (CH_2), 60.6 (CH_3O), 56.1 (CH_3O), 46.5 (CH), 44.5 (CH), 43.3 (CH); *m/z* (EI) 412 (M^+). Anal. Calcd for $C_{22}H_{20}O_8$: C, 64.07; H, 4.89. Found: C, 63.98; H, 4.79.

6.2.5. Isopodophyllotoxone derivative 4. δ_H ($CDCl_3$): 6.85 (1H, s, PhH), 6.53 (1H, s, PhH), 6.24 (2H, s, PhH), 5.96 (2 H, m, CH_2), 4.65 (1 H, m, CH_2), 4.33 (1H, m, CH_2), 4 (1H, m, CH), 3.83 (9H, m, OCH_3), 3–2.80 (2H, m, CH), 2.10 (3H, s, $COOCH_3$). δ_C ($CDCl_3$): 197 (C=O), 174.12 (COOH), 170.55 (C), 152.67 (OCH_3), 147.43 (C), 134.51 (C), 132.88 (C), 127.79 (C), 110.19 (PhH), 109.05 (PhH), 108.12 (PhH), 101.65 (PhH), 68.08 (C=O), 67.42 (CH_2), 60.71 (CH_3O), 60.35 (CH_3O), 56.23 (CH_3O), 43.79 (CH), 41.47 (CH), 36.71 (CH); *m/z* (EI) 472 (M^+). Anal. Calcd for $C_{24}H_{24}O_{10}$: C, 61.01; H, 5.12. Found: C, 59.98; H, 5.12.

6.2.6. Benzoquinone 6. δ_H ($CDCl_3$): 6.95 (1H, s, CH), 6.7 (1H, s, CH), 5.24 (1H, s, CH), 3.87 (3H, s, CH_3), 3.83 (3H, s, CH_3), 3.62 (3H, s, CH_3), 3.52 (3H, s, CH_3), 3.15 (1H, m, CH), 2.65–2.1 (2H, m, CH_2), 1.8–1.5 (2H, m, CH), 1.1 (3H, d, $J = 7.6$ Hz, CH_3), 0.85 (3H, d, $J = 6.2$ Hz, CH_3). δ_C ($CDCl_3$): 183.46 (C=O), 177.41 (C=O), 149.94 (C), 147.36 (C), 147 (C), 145.23 (C), 144.21 (C), 130.59 (C), 129.18 (PhH), 129.12 (C), 113.39 (C), 112.23 (PhH), 61.01 (CH_3O), 60.82 (CH_3O), 55.89 (CH_3O), 55.7 (CH_3O), 44.97 (CH), 37.16 (CH), 35.41 (CH), 18.57(CH_3), 14.42(CH_3); *m/z* (EI) 386. (M^+). Anal. Calcd for $C_{22}H_{26}O_6$: C, 68.38; H, 6.78. Found: C, 68.28; H, 6.75.

6.2.7. Benzoquinone 7. δ_H ($CDCl_3$): 6.98 (1H, s, PhH), 6.92 (1H, s, PhH), 5.18 (1H, s, PhH), 4.51 (1H, br s, OH), 3.84 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 3.66 (3H, s, OCH_3), 3.62 (1H, m, CH), 3.39 (3H, s, OCH_3), 2.8 (1H, m, CH), 1.84–1.4 (2H, m, CH_2), 1.2 (3H, d, $J = 6.55$ Hz, CH_3), 0.85 (3H, d, $J = 6.42$ Hz, CH_3). δ_C ($CDCl_3$): 183.46 (C=O), 177.41 (C=O), 149.94 (C), 147.98 (C), 147.78 (C), 145.23 (C), 144.21 (C), 129.88 (C), 129.36 (C), 129.18 (PhH), 113.66 (PhH), 112.01 (PhH), 71.58 (CH), 61.01 (CH_3O), 60.82 (CH_3O), 55.9 (CH_3O), 44.84 (CH), 43.67 (CH), 34.99 (CH), 14.82 (CH_3), 14.29 (CH_3); *m/z* (EI) 402. (M^+). Anal. Calcd for $C_{22}H_{26}O_7$: C, 65.66; H, 6.51. Found: C, 65.59; H, 6.50.

6.2.8. Benzoquinone 8. δ_H ($CDCl_3$): 6.96 (1H, s, PhH), 6.68 (1H, s, PhH), 5.24 (1H, s, PhH), 4.38 (1H, br s, OH), 3.87 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 3.64 (3H, s, OCH_3), 3.39 (3H, s, OCH_3), 2.92–2.5 (2H, m, CH_2), 1.5 (2H, m, CH), 1.23–1.20 (3H, d, $J = 6.2$ Hz, CH_3), 1.09–1.05 (3H, d, $J = 6.0$ Hz, CH_3). δ_C ($CDCl_3$): 193.52 (C=O), 192.9 (C=O), 155.35 (C), 147.9 (C), 147.62 (C), 138 (C), 134.15 (PhH), 127.95 (C), 113.68 (PhH), 109 (PhH), 79.92 (C), 73.78 (CH), 73.56 (CH), 57.41 (CH_3O), 55.98 (CH_3O), 55.73 (CH_3O), 45.27 (CH), 36.75 (CH_2), 34.38 (CH), 19 (CH_3), 12.46 (CH_3); *m/z* (EI) 404. (M^+). Anal. Calcd for $C_{22}H_{28}O_7$: C, 65.33; H, 6.98. Found: C, 65.28; H, 6.95.

6.2.9. Benzoquinone 9. δ_H ($CDCl_3$): 6.71 (1H, s, PhH), 6.62 (1H, s, PhH), 5.63 (1H, s, PhH), 3.86 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 3.67 (3H, s, OCH_3), 3.55 (1H, m, CH), 2–1.9 (2H, m, CH), 1.0 (3H, d, $J = 6.45$ Hz, CH_3), 0.89 (3H, d, $J = 6.33$ HZ, CH_3). δ_C ($CDCl_3$): 201.49 (C=O), 183.46 (C=O), 177.41 (C=O), 149.94 (C), 149.02 (C), 145.23 (C), 144.21 (C), 133.6(C), 129.8 (PhH), 129.26 (C), 113.9 (CH), 110.77 (PhH), 61.01 (CH_3O), 60.82 (CH_3O), 55.89 (CH_3O), 55.65 (CH_3O), 44.2 (CH), 42.67 (CH), 35.55 (CH), 14.68 (CH_3), 14.36 (CH_3); *m/z* (EI) 400. (M^+). Anal. Calcd for $C_{22}H_{24}O_7$: C, 65.99, H, 6.04. Found: C, 65.87; H, 6.0.

6.3. Biological assay

6.3.1. Evaluation of mitotic index and anti-topoisomerase II activity. The parent compound podophyllotoxin **1** and isopodophyllotoxone derivatives **2**–**4** were dissolved in dimethylsulfoxide (DMSO). Stock solutions were prepared at 5.0 mM and kept frozen at −20 °C. For treatment of cultures, 50 μL of appropriately diluted solutions were added to 5 mL culture such that the final concentration of solvent did not exceed 1%.

6.3.1.1. Cell cultures and media. Chinese hamster ovary (CHO) cells used in this work were obtained from Prof. A.T. Natarajan (State University of Leiden, The

Netherland). This cell line derives from the CHO isolated from an explant of the ovary of the Chinese hamster (*Cricetulus griseus*, $2n = 22$) originally described by Kao and Puck.[34] The Chinese hamster ovary (CHO) cell line is particularly useful for this kind of studies because of its stable karyotype (modal number is 21 chromosomes), short cell cycle (12–14 h) and its high plating efficiency.

Permanent stocks of CHO cells are stored at −163 °C under liquid nitrogen and subcultures are prepared from these stocks for experimental use. Cultures are grown as monolayer cultures in Ham's F-10 medium (Gibco BRL) supplemented with 15% foetal bovine serum, 4mM L-glutamine, and the antibiotics penicillin (50 IU/mL) and streptomycin (50 μg/mL). All incubations are at 37 °C in a 5% carbon dioxide (CO_2) atmosphere (100% humidity nominal).

6.3.1.2. G_2 treatment of cells. Approximately, 24–30 h before treatment exponentially growing cells were detached by trypsin action and an appropriate number of 25 sq cm plastic cell culture flasks containing 5 mL complete culture medium was individually inoculated with 3.0×10^5 cells.

Cultures were treated according to the following experimental schedule: (i) Three hours incubation with podophyllotoxin **1** or individually isopodophyllotoxone derivatives **2**–**4** at 0.05, 0.1, 0.25 and 0.50 mM. (ii) Three hours pre-treatment with colcemid at 0.2 μg/mL which served as positive control for inhibition of cell division interfering with the microtubule assembly by freezing polymerisation of tubulin. Untreated and solvent treated controls were also included. At the end of treatment cultures were trypsinised, and hypotonic treatment (KCl 0.075 M) and fixation were carried out according to standard procedures. Air-dried preparations were stained with Giemsa (3%) for 5 min. For each experimental point 100 metaphases were scored for chromosomal aberrations and were classified according to the description of Savage.[35] The mitotic index was expressed in percentage based on the number of metaphases present after a total of 1000 cells scored (interphases and metaphases).

6.3.1.3. Evaluation of results. For the chromosome aberration assay the number of aberration-bearing cells (excluding gaps) was utilised for statistical analyses. To determine the statistical significance Fisher's exact test was used. The test compounds were considered positive when statistically significant increases in aberration-bearing cells compared with the concurrent solvent controls were observed at two consecutive dose levels or the higher-dose level end exceeded the historical control mean values.

6.3.2. Evaluation of apoptogenic activity of galbulin 5 and (−)-aristologone derivatives 6–9. Human lymphoma cell lines BL41(EBV−) and E2R (EBV+) were seeded at conc 3×10^5cells/mL and the compounds **5**–**9** were added during 18 h. Dimethylsulphoxide (DMSO) used to solubilise the compounds never excedeed 0.5% v/v. For morphological analysis of apoptogenic activity, $2–6 \times 10^5$ cells were fixed in 4 % (v/v) paraformeldehyde and stained with a solution (0.2 μg/mL) of 4,6-diamino-2-phenyl-indole (DAPI). Apoptosis was quantified by scoring cells with condensed and fragmented nuclei, according to Ghibelli et al.[36] major than 500 cells in random fields were scored using fluorescent microscopy.

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