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Preliminary communication

Synthesis, cytotoxicity and antiviral activity of podophyllotoxin analogues modified in the E-ring

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Abstract

Several podophyllotoxin derivatives modified in the E-ring were prepared and evaluated for their cytotoxicity on four neoplastic cell lines (P-388, A-549, HT-29 and MEL-28) and for their antiherpetic activity against Herpes simplex virus type II. The trimethoxyphenyl moiety was oxidized to *ortho*-quinone and further condensed with diamines and enamines to form different heterocycles. Most of the compounds maintained their cytotoxicity at the μM level and some of them showed antiherpetic activity.

Keywords: Podophyllotoxin; Cyclolignans; E-ring modifications; Cytotoxicity; Herpes simplex virus

1. Introduction

Cyclolignans constitute a family of natural products with very interesting antiviral and cytotoxic properties. Compounds in clinical use, such as the natural product podophyllotoxin and the semisynthetic derivatives etoposide and teniposide (Fig. 1A), belong to this family [1].

From podophyllotoxin to etoposide some chemical modifications were made that also led to a change in the mechanism of action, from the inhibition of microtubule formation by the parent compound podophyllotoxin, to DNA–topoisomerase II inhibition by etoposide and congeners. This change is related to three main chemical modifications [2]: demethylation at C-4′ of the E ring, C-7 epimerisation, and the presence of a glycosidic or related moiety at the C-7 position on the C-ring. These observations led to a great number of derivatives that were synthesized and analysed by QSAR methods by Lee and coworkers [3], while the cyclolignan skeleton was virtually untouched in every case.

In the last few years, our research group has been involved in the chemical modification of cyclolignans and has prepared a large number of derivatives with potent antiviral, cytotoxic and immunosuppressant properties [2b]. It is worth stressing the selective cytotoxicity of some derivatives [4] modified in the C- and D-rings, with the general structure shown in Fig. 1B.

In the majority of the studies related to cyclolignans, the A- and E-rings were untouched and very little research has tackled their influence on cytotoxic activity. In a previous paper [5], we reported the result of modifications affecting the A-ring. Here, we report modifications performed on the E-ring that have been related to active metabolites generated through an in vivo oxidative pathway [6]. Indeed, the main modifications in the E-ring referred to in the literature imply changes in the degree of oxidation.

It has been shown that the 3′,4′-catechol derivative of etoposide can be formed in the presence of cytochrome P-450 [7]a and that this catechol can be further oxidized to the 3′,4′-*ortho*-quinone in the presence of oxygen [7b] or under the influence of horseradish peroxidase or prostaglandin E synthetase [7c]. Both catechol and

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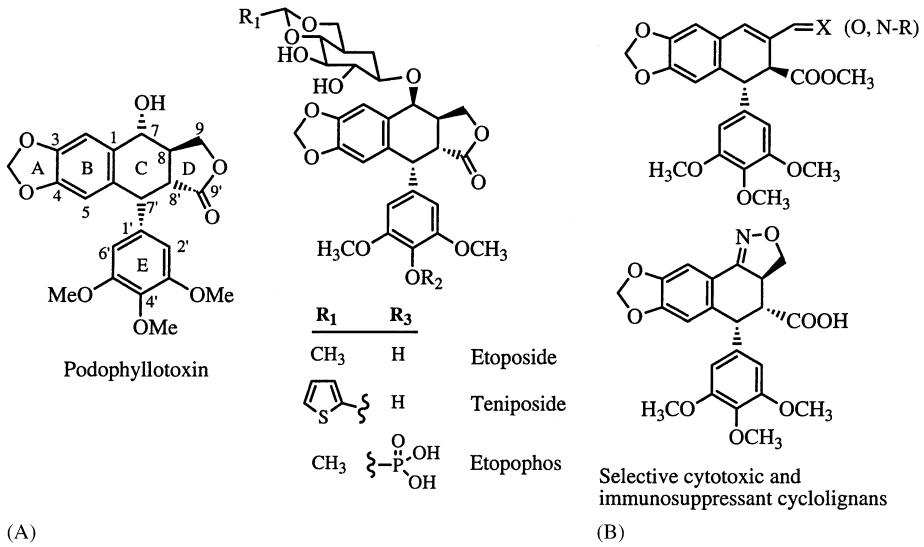

Fig. 1. Structures of podophylotoxin and related compounds. (A) Cyclolignans in clinical use. (B) Selective cytotoxic and immunosuppressant cyclolignans lacking the lactone ring.

ortho-quinone bind strongly to purified calf thymus DNA and this may contribute to the activity of these compounds, through the formation of free radicals [8] or even through the direct binding of the quinone to the DNA [9].

Based on the possibility that the catechol/quinone rings could be involved in the cytotoxicity mechanism, a series of 3′,4′-*O*-didemethylepipodophyllotoxins and 3′,4′-didemethoxy-3′,4′-dioxopodophyllotoxins, with a variety of substituents at C-7, were prepared and evaluated as antitumour agents [9]. The *ortho*-quinones were less cytotoxic than the catechols, and both were less active than the 4′-*O*-demethyl series, although some of them displayed activity comparable to the parent compound and bound to both nucleic acids and proteins.

Other modifications performed in the E-ring imply changes in the degree of oxygenation. Thus, cyclolignan analogues in which one, two or all three methoxy groups on the phenyl ring were replaced by hydrogen atoms or an alkyl group, were prepared [10–12]. The activity results showed that some of them were almost as potent as the parent compound, suggesting that the presence of the three oxygenated functions in E-ring of podophyllotoxin is not a strong determinant of cytotoxicity.

On the other hand, the possibility of transforming the *ortho*-quinone moiety into other rings has not been explored (except for a study concerning the characterization of the *ortho*-quinone as its quinoxaline derivative [13]) and nothing is reported about the effect of such changes on cytotoxicity. We therefore decided to transform the *ortho*-quinone into larger ring systems, whether aromatic or not, and to analyse their influence on cytotoxicity compared to podophyllotoxin.

Thus, a series of podophyllotoxin analogues with aza- or oxa-heterocyclic systems, instead of the trimethoxyphenyl ring, were prepared and evaluated for their cytotoxicity. Some representative compounds were also evaluated as antiviral agents.

2. Chemistry

The starting point for introducing different substituents on the cyclolignan E-ring skeleton was the transformation of the trimethoxyphenyl subunit into the quinonoid derivative. By treatment of cyclolignans **1** and **2** with nitric acid [14], the quinones **3** and **4** were obtained (Fig. 2). It is well known that during chromatography, the quinones can suffer further transformations that reduce the yields. As the reaction product was sufficiently pure as shown by the NMR spectra, it was used for the next steps without chromatographic purification. Numbering of compounds in the schemes and in the NMR tables corresponds to the usual numbering of lignans [15], for comparison purposes, although in Section 4 the systematic name is given for those derivatives with new heterocycles in ring E.

Since the hydroxyl group at C-7 of quinone **3** could interfere with later transformations, we attempted to acetylate it. It has been reported [14] that the quinone system is unaffected by acetylation; however, when **3** was treated with acetic anhydride and pyridine at room temperature, the only compound isolated was the triacetate **5a**. To obtain the quinone acetylated at C-7, nitric acid demethylation was applied to podophyllotoxin acetate **1a** yielding **3a**. Reduction of **3a** with sodium dithionite yielded the catechol **5**. Acetylation

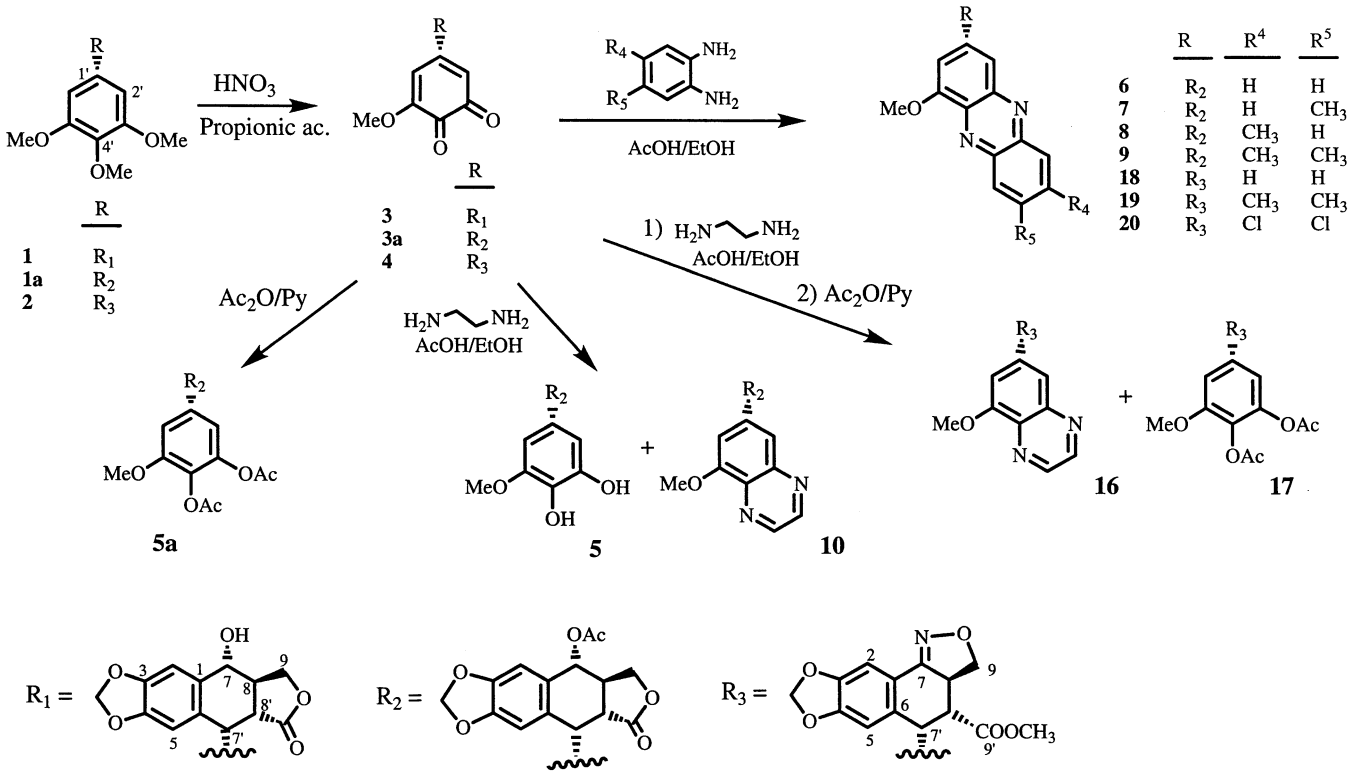

Fig. 2. Preparation of cyclolignans, nitrogenated in the E-ring, derived from podophyllotoxin and isoxazole analogues.

of this compound confirmed the structure of the triacetate **5a**.

Quinones generally react with most nucleophiles [16] and can undergo a wide range of reactions. Hence, this intermediate offers many possibilities for the modification of lignans. First, quinones served as substrates for condensation with different diamines.

When differently substituted phenylenediamines were used, the corresponding phenazines **6**–**9** and **18**–**20** were obtained (Fig. 2). When ethylenediamine was used as reagent, the quinoxalines **10** and **16** were obtained in which the new ring formed was aromatised. This result can be explained through the oxidation of the expected dihydroquinoxaline by the starting quinone, which was reduced to the catechols **5** and **17**; both were isolated as by-products from the reaction.

Catechol **5** can be transformed into different oxygenated rings in a way similar to that used for the transformation of the A-ring [5]. Thus, by reaction with dihalogenated reagents such as 1,2-dibromoethane and dibromomethane, dioxane **11** and dioxole **12** were obtained (Fig. 3).

It has been reported that α-diketones, including some *ortho*-quinones, can also form dioxane rings by reaction with 1,2-diols [17]. We tried to obtain this kind of derivative by treatment of quinone **3a** with ethylene glycol and 1,2-cyclohexanol in the presence of trimethylorthoformate, but instead of the expected dioxanes, only a small amount of the methylated derivative **13** was isolated from the complex mixture of the crude reaction product. Similar by-products were previously reported by treatment of *ortho*-quinones with methanol under weakly acidic conditions [14].

Another procedure reported for the preparation of substituted benzodioxanes from *ortho*-quinones is the reaction with enamines [18]. To apply this procedure, we used commercially available heterocyclic enamines such as pyrrolidine and morpholine enamines of cyclohexanone; these yielded a mixture of the two possible regioisomers of the corresponding tricyclic analogues **14** and **15** (Fig. 3), as deduced from the presence of several duplicated signals in their NMR spectra.

3. Biological results and discussion

3.1. Cytotoxicity

The compounds thus obtained were evaluated in vitro [19] to establish their cytotoxicity for the following cell cultures: murine leukaemia P-388, human lung carcinoma A-549, human colon carcinoma HT-29 and human melanoma MEL-28. Some general observations can be made from the results shown in Table 1.

Transformation of the trimethoxyphenyl group into the corresponding *ortho*-quinone leads to variable cytotoxicity results, depending on the substituents present in other parts of the molecule. If there is a free

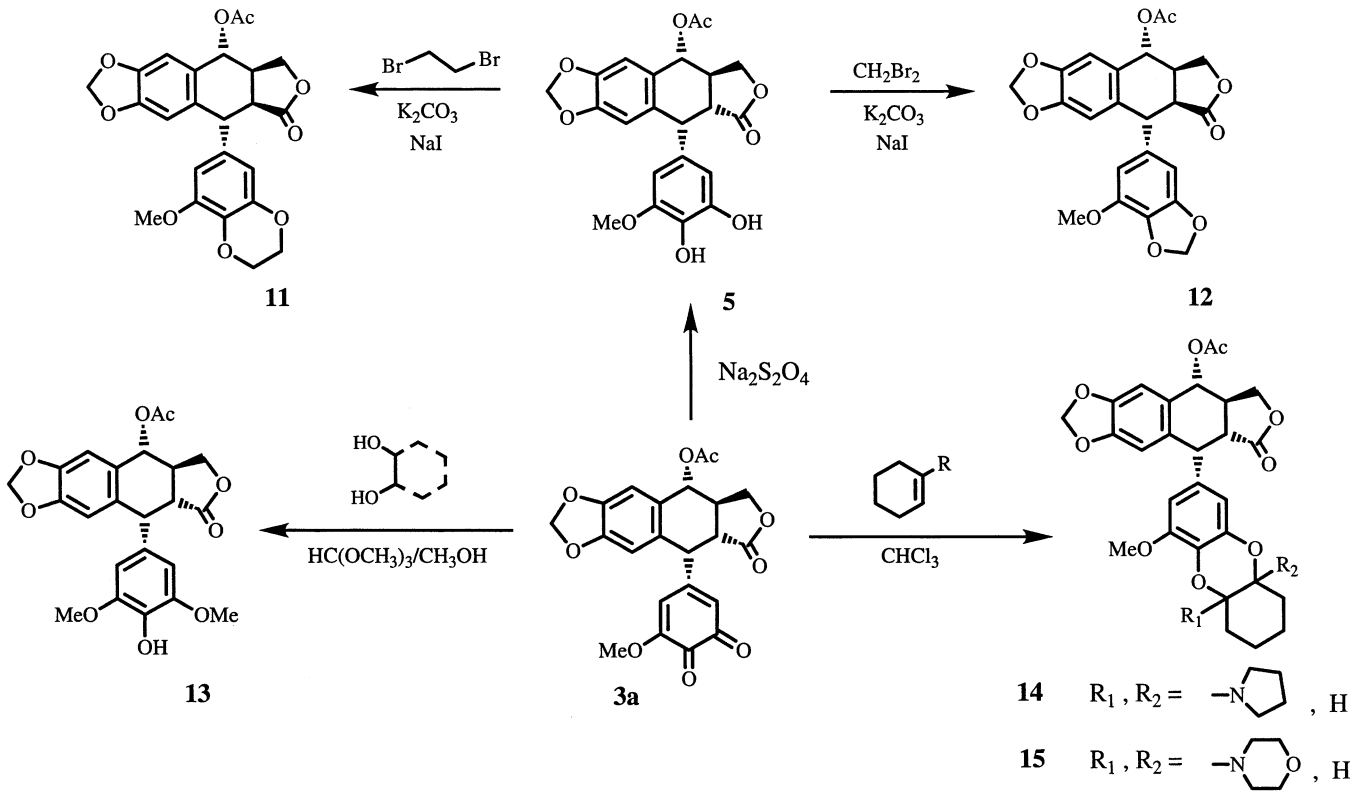

Fig. 3. Preparation of cyclolignans, oxygenated in the E-ring, derived from podophyllotoxin.

hydroxyl group at C-7, a significant decrease in potency is observed (**1** vs. **3**), while when this group is acetylated, no change in the activity is observed (**1a** vs. **3a**), **3a** being twice as potent as **3**.

Table 1
Cytotoxic activity of cyclolignans modified in the E-ring (IC_{50} μM)

Compound	P-388	A-549	HT-29	MEL-28
1	0.012	0.012	0.012	
1a	0.55	0.55	0.55	0.55
2	2.3	5.7	11	5.7
3	1.3	1.3	1.3	1.3
3a	0.59	0.59	0.59	0.59
4	12	12	12	12
5	2.8	5.84	5.84	5.84
5a	2.2	2.2	2.2	2.2
6	1.0	1.0	1.0	1.0
7+8	2.0	2.0	2.0	2.0
9	4.8	4.8	4.8	4.8
10	0.56	0.56	0.56	0.56
11	2.2	2.2	2.2	2.2
12	2.27	2.27	2.27	2.27
13	0.11	0.23	0.23	0.23
14	4.33	8.66	8.66	8.66
15	2.02	2.02	2.02	2.02
16	2.8	2.8	2.8	2.8
17	20.0	20.0	20.0	20.0
18	10.0	10.0	10.0	10.0
19	1.0	1.0	1.0	1.0
20	> 18	> 18	> 18	> 18

The catechol group in the E-ring is about four times less potent than the *ortho*-quinone (**5** vs. **3a**) and this potency is practically unchanged after acetylation (**5a** vs. **5**) but, when one of the phenol groups is methylated, the potency is partially recovered (**13** vs. **5**).

Transformation of the trimethoxyphenyl ring of the acetyl podophyllotoxin series into polyheterocyclic systems decreases the potency several times, the effect becoming more pronounced with increasing number of substituents on the phenazine system (**6** and **9** vs. **1a**). Only quinoxaline **10** retained an IC_{50} value below μM.

Those analogues in which new oxygenated rings are fused to the E-ring (dioxole and dioxanes) were also less cytotoxic than podophyllotoxin and slightly less potent than the *ortho*-quinone precursor (**11**, **12**, **14**, **15** vs. **1** and **3a**).

For the isoxazole series, the slight selectivity shown by **2** towards P-388 is lost in quinone **4**, which had values of IC_{50} in the same range as those for **2** on HT-29. Quinoxaline **16** also retained the same range of cytotoxicity as the parent compound **2** and surprisingly, phenazine **19**, which has two methyl groups as substituents, maintained the cytotoxicity, while the other derivatives (**17**, **18**, **20**) were the least potent derivatives of all the series. This is difficult to explain on the basis on the two proposed mechanisms of action of cyclolignans; therefore, a third mechanism (such as that postulated previously by Lee and coworkers [20]) could

be involved. More work will be necessary to clarify these points.

3.2. Antiviral results

The evaluation of antiherpetic activity against Herpes simplex virus type II (HSV-2) of some representative compounds was carried out using the end-point titration technique (EPTT) [21] in which the cytotoxic activity and the antiviral effect were simultaneously evaluated (Table 2). The authors stated that under defined experimental conditions only those compounds showing reduction factors (R_f) of the viral titre over 1×10^3 could be considered as having relevant antiviral activity. Following this evaluation method, phenazines **18** and **19** exhibited the highest antiviral activity against HSV-2, with a R_f of $1 \times 10^{1.5}$ and $1 \times 10^{1.0}$, respectively, when challenged with ten times the tissue culture infectious dose 50 ($TCID_{50}$) indicating a moderate activity against HSV-2. For these compounds, the nontoxic concentration needed to obtain the largest reduction of the viral titre was approximately half the cytotoxic concentration needed to detach 100% (CC_{100}) of the cell monolayer, revealing that the antiviral activity, especially for phenazine **19**, is principally due to cytotoxicity. Comparing the antiviral activity of phenazines **18** and **20**, we found that in phenazine **20** the two chloro substituents led to complete loss of activity. Furthermore, the presence at C-7′ of the 3′,4′ *ortho*-quinone moiety reduced the activity of the isoxazole derivatives (**4** vs. **18**).

Comparison of the antiviral activity of 3′,4′-catechol podophyllotoxin (**5**) and 3′,4′ *ortho*-quinone podophyllotoxin (**3a**) with that of podophyllotoxin acetate (**1a**), which is practically inactive in the systems tested, showed that the presence of catechol or quinone groups induce some HSV antiviral activity in those molecules. Compounds **1**, **1a**, **3a**, **5**, **6** and **10** were cytostatic when evaluated on confluent monolayers of Vero cells that are nonproliferative. Consequently the cytotoxic concentration needed to detach 100% (CC_{100}) of the cell monolayer was not found below 150 μg mL^{-1} (not shown). The antiherpetic activity (HSV-I) of podophyllotoxin **1** and podophyllotoxin acetate **1a** was previously reported [6,22] using the plaque elimination assay. To analyse their behaviour against HSV-2 both compounds were submitted to the EPTT assay with a lower viral challenge ($1 \times TCID_{50}$) and to the plaque elimination assay. By EPTT both compounds were shown to be slightly active against HSV-2 with a R_f value of $1 \times 10^{0.5}$ (not included). By the plaque elimination assay these compounds were tested with a low viral concentration of 100 PFU (plaque forming units). The concentrations needed for complete elimination of macroscopic plaque formation (ED_{100} effective doses of 100%) without toxicity to cell monolayers were 10 and 80 ng mL^{-1} for podophyllotoxin **1** and podophyllotoxin acetate **1a**, respectively (not shown).

4. Experimental

4.1. Chemistry

Melting points were determined by heating the compounds in an external silicone bath and were uncorrected. Optical rotations were recorded on a Perkin–Elmer 241 polarimeter in chloroform solution

Table 2
Anti-HSV-2 activity of cyclolignan on Vero cells [a] determined by the end-point titration technique (EPTT) with $10TCDI_{50}$

Cyclolignan	CC_{100} [b] (μg mL^{-1})	Viral reduction factor [c]	Antiviral activity (μg mL^{-1}) [d]
1	> 20	NA	NA
1a	> 20	NA	NA
3a	> 23	10^1	23
6	> 20	NA	NA
10	> 25	NA	NA
9	> 25	NA	NA
4	> 120	$10^{0.5}$	60
18	120	$10^{1.5}$	30
19	60	$10^{1.0}$	30
20	30	NA	NA
11	> 25	NA	NA
15	> 25	NA	NA
14	> 25	NA	NA
5	> 25	10^1	25
acyclovir	> 600	10^4	6.0

[a] VERO, *Cercopithecus aethiops* african green monkey kidney ATCC CCL 81.
[b] Minimal toxic dose that detached 100% of the cell monolayer.
[c] Ratio of the virus titre in the absence over virus titre in the presence of the tested compound.
[d] Maximal nontoxic dose that showed the highest viral reduction factor. NA, no activity.

and UV spectra on a Hitachi 100-60 spectrophotometer in ethanol. IR spectra were obtained on a Beckmann (Acculab VIII) spectrophotometer. EIMS and HRMS were run in a VG-TS-250 spectrometer working at 70 eV. NMR spectra were recorded at 200 MHz for ^{1}H and 50.3 MHz for ^{13}C in deuterochloroform using TMS as internal reference, on a Bruker WP 200 SY. Chemical shift values are expressed in ppm followed by multiplicity and coupling constants (J) in Hz. Column chromatography was performed on silica gel (Merck No 9385). TLC was carried out on silica gel 60 F_{245} (Merck, 0.25 mm thick). Solvents and reagents were purified by standard procedures as required. Elementary analyses were obtained with a LECO CHNS-932 and were within $\pm 0.4\%$ of the theoretical values.

4.1.1. Sources of precursors

Podophyllotoxin **1** was obtained from the resin of *Podophyllum emodi* by chromatographic means and was converted to acetylpodophyllotoxin (**1a**) and methyl isoxazolopodophyllate (**2**) by previously established procedures [23].

*4.1.2. Procedure for oxidative demethylation. Compounds **3**, **3a** and **4***

*4.1.2.1. 3′,4′-Didemethoxy-3′,4′-dioxopodophyllotoxin (**3**).* Nitric acid (60%, 1.2 mL) was rapidly added to a solution of podophyllotoxin (**1**) (190 mg, 0.46 mmol) in propionic acid (2 mL) at 0 °C. After exactly 4 min at 0 °C, the dark red solution was poured into water (40 mL) and extracted with EtOAc. The organic layer was washed with aq. sat. $NaHCO_3$ and brine, dried with Na_2SO_4 and the solution concentrated to a reduced volume. The residual solution was diluted with *n*-hexane and the precipitate formed after 30 min of stirring, was filtered off to yield quinone **3** (150 mg, 85%). M.p.: 179–181 °C. UV $\lambda_{max}(\varepsilon)$: 251 (3200), 257 (3400). IR (cm^{-1}): 3450, 1770, 1698, 1662, 1627, 1484, 1237, 1038. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4).

*4.1.2.2. Acetylation of **3**.* Quinone **3** (50 mg, 0.13 mmol) was acetylated with acetic anhydride in pyridine. After following the usual protocol, the reaction product was chromatographed and eluted with Cl_2CH_2–EtOAc 93:7 to yield triacetate **5a** (7,3′,4′-triacetyl-3′,4′-didemethylpodophyllotoxin) (45 mg, 68%). IR (KBr, cm^{-1}): 1776, 1735, 1609, 1505, 1486, 1236. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). Anal. $C_{26}H_{24}O_{11}$ (C, H).

The same procedure was applied to obtain the following compounds.

*4.1.2.3. 7-Acetyl-3′,4′-didemethoxy-3′,4′-dioxopodophyllotoxin (**3a**).* The acetate **3a** (78%) was obtained from **1a**. M.p.: 144–148 °C. IR (KBr, cm^{-1}): 1775, 1734, 1702, 1665, 1628, 1486, 1235, 1038. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). HRMS (FAB-POSI, M+1) Calc. for $C_{22}H_{19}O_9$ 427.1029. Found 427.1075. Anal. $C_{22}H_{18}O_9$ (C, H).

4.1.2.4. Methyl 3′,4′-didemethoxy-3′,4′-dioxoisoxazolopodophyllate 4. The isoxazole **4** (94%) was obtained from compound **2**. M.p.: 198–202 °C. UV $\lambda_{max}(\varepsilon)$: 217 (19600), 276 (10200), 314 (7100). IR (cm^{-1}): 1734, 1665, 1625, 1484, 1257, 1037. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4).

4.1.3. 7-Acetyl-3′,4′-didemethylpodophyllotoxin 5.

Quinone **3a** (214 mg, 0.5 mmol) was dissolved in ethanol (60 mL) and water (10 mL). The solution was stirred at room temperature (r.t.) and $Na_2S_2O_4$ was progressively added until the red solution became yellow. The ethanol was partially evaporated and the residual solution was extracted with EtOAc, washed with water, dried over Na_2SO_4 and the solvent evaporated. Column chromatography of the residue (eluent: Cl_2CH_2–EtOAc 9:1) yielded the catechol **5** (126 mg, 60%). M.p.: 248–252 °C (MeOH). $[\alpha]_D^{22}$ −102.4° (c, 0.5%). UV $\lambda_{max}(\varepsilon)$: 209 (31900), 285 (3600), 325 (900). IR (cm^{-1}): 3450, 3352, 1775, 1726, 1610, 1518, 1237, 1034. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). Anal. $C_{22}H_{20}O_9$ (C, H).

Acetylation of **5** with acetic anhydride and pyridine gave the triacetate **5a**.

*4.1.4. General procedure for the condensation with diamines. Compounds **6**–**10** and **16**–**20***

*4.1.4.1. (5R, 5aR, 8aS, 9R) 9-Acetoxy-5-(4-methoxyphenazin-2-yl)-5,5a,6,8,8a,9-hexahydrofuro[3′,4′:6,7]naphtho[2,3-d][1,3]dioxol-6-one (**6**).* Quinone **3a** (150 mg, 0.35 mmol) was dissolved in ethanol (7 mL) and acetic acid (2 mL) and then 1,2-phenylenediamine (76 mg, 0.70 mmol) was added. The mixture was stirred overnight at r.t. and extracted with EtOAc. The organic layer was washed with 2 N HCl, aq. Sat. $NaHCO_3$ and water. The reaction product obtained after evaporation of the solvent, was chromatographed on silica gel (Cl_2CH_2–EtOAc 9:1) to give the phenazine **6** (60 mg, 35%). $[\alpha]_D^{22}$ −35.5° (*c*, 0.11%). UV $\lambda_{max}(\varepsilon)$: 210 (35200), 259 (37700). IR (KBr, cm^{-1}): 1778, 1734, 1631, 1607, 1561, 1229, 1038. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). HRMS (FAB-POSI, M+1) Calc. for $C_{28}H_{23}N_2O_7$ 499.1505. Found 499.1487. Anal. $C_{28}H_{22}O_7N_2$ (C, H, N).

The same procedure outlined above was applied to obtain the following compounds.

*4.1.4.2. (5R, 5aR, 8aS, 9R) 9-Acetoxy-5-(4-methoxy-7(8)-methylphenazin-2-yl)-5,5a,6,8,8a,9-hexahydrofuro[3′,4′:6,7]naphtho[2,3-d][1,3]dioxol-6-one (**7**+**8**).* From quinone **3a** (183 mg, 0.43 mmol) and 3,4-

Table 3
^{1}H-NMR ($CDCl_3$–TMS, δ ppm (J Hz)) data of compounds **3**–**20**

H	**3** [a]	**3a**	**5**	**5a**	**6**	**7**+**8**	**9**	**10**
2	7.16 s	6.71 s	6.75 s	6.77 s	6.78 s	6.76 s	6.81 s	6.79 s
5	6.64 s	6.51 s	6.50 s	6.54 s	6.52 s	6.50 s	6.56 s	6.51 s
7	4.76 m	5.81 d (9.4)	5.87 d (8.7)	5.87 d (9.1)	5.91 d (9.5)	5.87 d (9.2)	5.93 d (9.1)	5.10 d (9.4)
8	2.90 m	2.83 m	2.87 m	2.80 m	2.90 m	2.87 m	2.97 m	2.86 m
9	4.63 dd (8.3; 7.3); 4.24 dd (10.4; 8.6)	4.50 dd (9.5; 7.3); 4.28 m	4.35 m; 4.20 m	4.40 dd (9.6; 6.9); 4.21 t (9.6)	4.30 m	4.30	4.35 m	4.33 dd; (9.4; 7.3); 4.23 dd (10.1; 9.4)
2′	5.36 d (1.8)	5.43 s	6.70 d (1.8)	7.02 d (1.8)	7.14 d (1.6)	7.09 bs	7.12 d (1.8)	7.49 d (1.8)
6′	6.47 d (1.8)	6.45 s	6.05 d (1.8)	6.24 d (1.8)	7.50 d (1.6)	7.47 bs	7.49 d (1.8)	7.03 d (1.8)
7′	4.41 d (5.5)	4.28 m	4.54 d (3.6)	4.64 d (4.4)	4.84 d (4.2)	4.81 d (4.7)	4.86 d (4.8)	4.82 d (4.8)
8′	3.28 dd (14.2; 5.5)	2.12 dd (14.8; 5.3)	2.87 m	2.95 dd (14.0; 4.4)	3.15 dd (14.2; 4.2)	3.12 dd (14.5; 4.8)	3.15 dd (14.3; 4.8)	3.10 dd (14.5; 4.8)
CH_3O-5′	3.75 s	3.84 s	3.86 s	3.81 s	4.21 s	4.20 s	4.21 s	4.13 s
OAc		2.17 s	2.20 s	2.28 s; 2.22 s; 2.19 s	2.20 s	2.17 s	2.21 s	2.19 s
O–CH_2–O	6.01 s	5.97 s; 5.99 s	5.97 s	5.98 s	5.96 s; 5.98 s	5.94 s; 5.96 s	5.97 s; 6.00 s	5.98 d (1.4); 5.96 d (1.4)
Others					8.12 m; 8.36 m; 7.80 (2H) m	8.11(7.97) d (8.9); 8.11(7.83) bs; 7.58(7.63) m	8.13 s; 7.85 s	8.78 d (1.0); 8.76 d (1.0)
CH_3						2.58 s	2.53 s	

H	**11**	**12**	**13**	**14**	**15**
2	6.76 s	6.75 s	6.76 s	6.74 s	6.76 s
5	6.56 s	6.54 s	6.54 s	6.50 s	6.54 s
7	5.70 d (4.9)	5.71 d (4.8)	5.87 d (8.0)	5.86 d (7.7)	5.85 d (7.7)
8	3.00 m	3.00 m	2.87 m	2.89 m	2.80 m
9	4.20–4.50 m	4.43 dd (9.6; 6.9); 4.23 dd (9.6; 3.3)	4.35 m; 4.20 m	4.35 m; 4.20 m	4.36 m; 4.25 m
2′	6.28 bs	6.33 d (1.8)	6.39 s	6.00 bs	6.05 m
6′	6.40 bs	6.40 d (1.8)	6.39 s	6.75 bs	6.74 bs
7′	4.20–4.50 m	4.36 d (3.3)	4.59 d (3.7)	4.55 d (3.3)	4.54 d (4.3)
8′	3.27 dd (9.5; 3.2)	3.25 dd (9.5; 3.7)	2.87 m	2.90 m	2.80 m
CH_3O-5′/3′	3.84 s	3.88 s	3.79 s	3.87 s	3.83 s
OAc	1.99 s	2.02 s	2.18 s	2.21	2.20 s
O–CH_2–O	5.94 bs	5.95 s	5.96 bs	5.97 bs	5.97 bs
Others	4.20–4.50 m	5.94 s		3.75 m; 2.89 m; 1.40–1.80 m	3.60 m; 2.80 m; 1.50–2.00 m

H	**4**	**16**	**17**	**18**	**19**	**20**
2	7.42 s	7.51 s	7.44 s	7.51 s	7.50 s	7.52 s
5	6.61 s	6.56 s	6.56 s	6.61 s	6.60 s	6.60 s
7						
8	3.84 m	3.94 m	3.84 m	4.00 m	4.00 m	3.96 m
9	3.84 m; 489 dt (8.0; 2.1)	3.82 dd (8.1; 13.5); 4.82 dd (8.1; 9.1)	3.84 m; 4.83 m	3.85 m; 4.83 dd (8.0; 8.4)	3.83 dd (8.3; 13.5); 4.81 dd (8.3; 9.3)	3.84 m; 4.84 dd (8.0; 7.7)
2′	5.61 d (1.6)	7.24 d (1.5)	6.43 d (1.8)	7.33 d (1.5)	7.25 d (1.5)	7.30 bs

Table 3 (*Continued*)

H	4	16	17	18	19	20
6′	5.71 d (1.6)	6.76 d (1.5)	6.36 d (1.8)	6.80 d (1.5)	6.73 d (1.5)	6.79 bs
7′	4.34 d (5.7)	4.91 d (5.3)	4.68 d (5.1)	4.94 d (5.3)	4.91 d (5.3)	4.94 d (5.5)
8′	3.28 dd (12.3; 5.7)	3.36 dd (12.4; 5.3)	3.22 dd (5.1; 12.0)	3.40 dd (5.3; 12.1)	3.37 dd (12.5; 5.3)	3.41 dd (5.5; 12.1)
CH_3O-5′	3.66 s	4.01 s	3.71 s	4.11 s	4.08 s	4.09 s
OAc			2.24 s, 2.27 s			
O–CH_2–O	6.03 bs	5.98 s; 6.01 s	5.99 s	5.98 s; 6.02 s	5.96 s; 6.00 s	6.00 s; 6.03 s
$COOCH_3$	3.76 s	3.68 s	3.65 s	3.70 s	3.67 s	3.71 s
Others		8.81 s (2H)		8.36 m, 8.15 m; 7.82 m (2H)	8.09 s, 7.86 s	8.50 s, 8.28 s
CH_3					2.53 s	

[a] NMR in acetone-d_6.

diaminotoluene (115 mg, 0.94 mmol). Column chromatography of the reaction product with Cl_2CH_2–EtOAc 85:15 yielded phenazines **7**+**8** (66 mg, 30%) and catechol **5** (25 mg, 14%). UV $\lambda_{max}(\varepsilon)$: 206 (22000), 269 (25600). IR (cm^{-1}): 1779, 1735, 1519, 1505 1229, 1038. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4).

*4.1.4.3. (5R, 5aR, 8aS, 9R) 9-Acetoxy-5-(4-methoxy-7,8-dimethylphenazin-2-yl)-5,5a,6,8,8a,9-hexahydro-furo[3′,4′:6,7]naphtho[2,3-d][1,3]dioxol-6-one (**9**).* From **3a** (300 mg, 0.54 mmol) and 4,5-dimethyl-1,2-phenylenediamine (149 mg, 1.1 mmol). Column chromatography of the reaction product with Cl_2CH_2–EtOAc 85:15 yielded phenazine **9** (60 mg, 21%). $[\alpha]_D^{22}$ +18.5°. UV $\lambda_{max}(\varepsilon)$: 207 (28600), 270 (26000). IR (cm^{-1}): 1779, 1735, 1614, 1559, 1505, 1234, 1127, 1038. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). HRMS (FAB-POSI, M+1) Calc. for $C_{30}H_{27}N_2O_7$ 527.1818. Found 527.1865. Anal. $C_{30}H_{26}N_2O_7$ (C, H, N).

*4.1.4.4. (5R, 5aR, 8aS, 9R) 9-Acetoxy-5-(8-methoxyquinoxalin-6-yl)-5,5a,6,8,8a,9-hexahydro-furo[3′,4′:6,7]naphtho[2,3-d][1,3]dioxol-6-one (**10**).* From **3a** (150 mg, 0.35 mmol) and ethylenediamine (46 mg, 0.76 mmol). Column chromatography of the acetylated reaction product with Cl_2CH_2–EtOAc 85:15 provided quinoxaline **10** (90 mg, 57%) and triacetate **5a** (27 mg, 18%). Analytical data of **10**: $[\alpha]_D^{22}$ −75.6° (*c*, 0.16%). UV $\lambda_{max}(\varepsilon)$: 208 (29300), 255 (25300). IR (KBr, cm^{-1}): 1779, 1734, 1682, 1616, 1504, 1235, 1126, 1035. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). Anal. $C_{24}H_{20}N_2O_7$ (C, H, N).

*4.1.4.5. (3aS, 4R, 5R) 5-(8-Methoxyquinoxalin-6-yl)-3,3a,4,5-tetrahydro-[1,3] dioxolo [4′,5′:6,7] naphtho [1,2-c]isoxazol-4-carboxylic acid methyl ester (**16**) and (3aS, 4R, 5R) 5-(3,4-diacetyl-5-methoxyphenyl))-3,3a,4,5-tetrahydro-[1,3]dioxolo[4′,5′:6,7]naphtho[1,2-c]isoxazol-4-carboxylic acid methyl ester (**17**).* From quinone **4** (150 mg, 0.36 mmol) and ethylenediamine (0.05 mL, 0.75 mmol). The reaction time was reduced to 2 h, the reaction product was acetylated with acetic anhydride in pyridine, and the resulting acetylated product was chromatographed on silica gel to give quinoxaline **16** (42 mg, 27%) and diacetate **17** (48 mg, 31%).

Compound **16**: m.p.: 120–125 °C. $[\alpha]_D^{22}$ −121.7° (*c*, 0.23%). UV $\lambda_{max}(\varepsilon)$: 214 (24700), 223 (25500), 256 (14100), 315 (12200). IR (cm^{-1}): 1735, 1615, 1573, 1500, 1236, 1127, 1037. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). HRMS (FAB-POSI, M+1) Calc. for $C_{23}H_{20}N_3O_6$ 434.4352. Found 434.1323. Anal. $C_{23}H_{19}N_3O_6$ (C, H, N).

Compound **17**: m.p.: 224–228 °C. $[\alpha]_D^{22}$ −117.0° (*c*, 0.54%). UV $\lambda_{max}(\varepsilon)$: 210 (18600), 217 (18500), 278

Table 4
^{13}C-NMR ($CDCl_3$–TMS, δ ppm) data of compounds **3**–**20**

C	**3** [a]	**3a**	**4**	**5**	**5a**	**6**	**7**+**8**	**9**	**10**
1	129.5	128.4	119.3	128.3	128.3	128.7	128.6	128.6	129.5
2	107.4	107.1	104.5	106.7	107.0	106.9	106.8	106.9	106.9
3	148.6	148.4	148.5	147.6	148.2	148.3	148.2	148.2	148.2
4	148.1	148.0	150.9	146.5	147.8	148.0	147.8	147.9	147.9
5	109.8	109.6	108.6	109.8	109.8	109.7	109.6	109.7	109.6
6	136.0	128.4	130.6	131.4 [b]	131.5	131.1	131.1	131.3	131.2
7	72.2	72.7	155.0	73.7	73.6	73.4	73.3	73.4	73.3
8	41.6	39.1	44.0	38.6	38.4	38.6	38.5	38.6	38.5
9	72.2	71.6	74.6	71.4	71.5	71.4	71.3	71.4	71.5
1′	152.3	151.8	155.1	131.7 [b]	142.7	143.9	143.1(142.8)	142.9	143.1
2′	124.9	124.4	109.0	110.8	116.7	122.7	122.6	122.6	122.5
3′	179.0	179.4	177.9	132.4	137.8	143.4	143.3(142.3)	142.7	143.0
4′	176.1	175.2	175.0	143.2	131.0	136.2	136.0	135.6	134.4
5′	158.2	157.1	152.7	148.2	151.8	154.3	154.2	154.3	154.4
6′	114.1	112.7	123.1	106.8	112.7	110.9	110.8	110.4	112.1
7′	44.6	44.3	48.3	43.5	43.3	44.6	44.5	44.5	44.2
8′	45.3	44.9	48.5	45.5	45.5	45.3	45.2	45.3	45.2
9′	175.1	173.4	170.8	174.0	173.5	173.7	173.3	173.8	173.6
CH_3O-5′	56.0	56.1	56.0	56.3	56.3	56.6	56.5	56.5	56.4
OAc		20.9, 171.5		21.2, 171.7	20.3, 20.6, 21.1, 167.7, 168.1, 171.4	21.1, 171.5	21.0, 171.4	21.1, 171.5	21.0, 171.5
$COOCH_3$			52.6						
O–CH_2–O	102.3	101.8	102.0	101.6	101.6	101.6	101.5	101.6	101.6
Others						128.9, 130.1, 130.2, 130.9, 142.3, 143.4	143.5(143.6), 126.9(128.3), 140.8(128.3), 129.6(141.0), 133.1(133.9), 141.7(142.1)	127.2, 128.6, 142.4, 141.6, 142.9(2C), 20.7, 20.6	145.3, 143.5

C	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1	126.1	126.1	128.2	128.2	128.2	119.3	118.9	119.2	119.3	119.3
2	104.3	102.4	106.9	106.7	106.7	104.3	103.9	104.2	104.3	104.3
3	148.4	148.4	148.0	148.0	148.0	147.9	147.8	147.9	147.9	148.0
4	147.2	147.5	147.5	147.5	147.5	150.6	150.5	150.6	150.6	150.7
5	110.0	109.8	109.6	109.9	109.8	109.1 [b]	109.3	107.9	109.1	109.0
6	131.5	131.0	132.4	132.2	131.5	133.8	134.0	133.7	134.0	133.5
7	72.3	72.1	73.6	73.5	73.7	156.0	156.1	156.0	156.1	155.9
8	39.5	39.5	38.6	38.5	38.5	43.4	43.2	43.4	43.5	43.5
9	70.7	70.6	71.3	71.3	71.3	74.4	74.4	74.3	74.3	74.4
1′	131.9	131.3	133.9	132.2	132.6	142.3	137.8	142.3 [b]	141.8 [b]	140.7
2′	109.9	108.4	107.7	107.8	107.1	121.8	116.0	122.1	122.1	121.9
3′	135.5	137.9	146.4	133.2	130.7	143.4	143.1	142.7 [b]	141.8 [b]	142.2
4′	143.8	143.5	130.3	141.3	142.0	134.4	131.2	135.9	135.4	136.2
5′	148.9	149.0	146.4	148.2	148.0	155.1	152.1	154.9	154.9	154.9

Table 4 (*Continued*)

C	11	12	13	14	15	16	17	18	19	20
6′	108.6	108.3	107.7	112.1	111.9	109.2 [b]	110.5	109.0	107.4	108.6
7′	44.0	44.1	43.5	43.3	43.4	48.2	47.5	48.4	48.4	48.4
8′	45.3	45.4	45.6	45.3	45.3	49.7	49.7	49.5	49.6	49.5
9′	177.6	177.3	173.7	173.8	173.7	171.2	171.1	171.2	171.3	171.2
CH_3O-5′	56.2	56.8	56.3	56.2	56.2	56.3	56.2	56.4	56.3	56.5
OAc	21.0, 170.6	21.0, 170.6	21.0, 171.3	21.1, 171.6	21.1, 171.5		20.2, 20.5, 167.5, 167.9			
$COOCH_3$						52.2	52.2	52.2	52.2	52.3
O–CH_2–O	101.4	101.4	101.5	101.3	101.5	101.8	101.7	101.7	101.7	101.8
Others	64.4, 64.5	101.4		22.0, 23.0, 23.6, 28.0, 34.5, 35.6, 77.0, 94.3	21.6, 26.9, 27.7, 44.5(2C), 71.3, 88.2	143.8, 145.8		129.1, 130.0, 130.4, 131.1, 143.7, 143.4	127.4, 128.4, 141.8, 142.7, 143.0, 143.1, 20.7(2C)	129.3, 130.2, 135.4, 143.8(2C)

[a] NMR in acetone-d_6.
[b] Exchangeable signals.

(8100), 314 (5300). IR (cm^{-1}): 1774, 1735, 1610, 1504, 1269, 1131, 1037. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). HRMS (FAB-POSI, M+1) Calc. for $C_{25}H_{24}NO_{10}$ 498.1400. Found 498.1421.

*4.1.4.6. (3aS, 4R, 5R) 5-(4-Methoxyphenazin-2-yl)-3,3a,4,5-tetrahydro-[1,3] dioxolo [4′,5′:6,7] naphtho[1,2-c]isoxazol-4-carboxylic acid methyl ester (**18**).* From **4** (113 mg, 0.28 mmol) and phenylenediamine (61 mg, 0.56 mmol) for 1 h. The reaction product was dissolved in methanol and the precipitated product was filtered yielding **18** (78 mg, 59%). M.p.: 124–128 °C. $[\alpha]_D^{22}$ −167.6° (*c*, 0.38%). UV $\lambda_{max}(\varepsilon)$: 212 (28700), 270 (28100), 314 (7100), 365 (3800). IR (cm^{-1}): 1735, 1610, 1520, 1504, 1257, 1129, 1038. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). HRMS (FAB-POSI, M+1) Calc. for $C_{27}H_{22}N_3O_6$ 484.1508. Found 484.1492. Anal. $C_{27}H_{21}N_3O_6$ (C, H, N).

*4.1.4.7. (3aS, 4R, 5R) 5-(4-Methoxy-7,8-dimethylphenazin-2-yl)-3,3a,4,5-tetrahydro-[1,3] dioxolo [4′,5′:6,7] naphtho[1,2-c]isoxazol-4-carboxylic acid methyl ester (**19**).* From **4** (122 mg, 0.30 mmol) and 4,5-dimethyl-1,2-phenylenediamine (81 mg, 0.60 mmol) for 1 h. Column chromatography of the reaction product provided compound **19** (71 mg, 47%). M.p.: 165–170 °C. UV $\lambda_{max}(\varepsilon)$: 265 (20300), 214 (11100), 376 (14000). IR (cm^{-1}): 1736, 1610, 1504, 1234, 1127, 1038. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). HRMS (FAB-POSI, M+1) Calc. for $C_{29}H_{26}N_3O_6$ 512.1821. Found 512.1852. Anal. $C_{29}H_{25}N_3O_6$ (C, H, N).

*4.1.4.8. (3aS, 4R, 5R) 5-(7,8-Dichloro-4-methoxyphenazin-2-yl)-3,3a,4,5-tetrahydro-[1,3] dioxolo [4′,5′:6,7] naphtho[1,2-c]isoxazol-4-carboxylic acid methyl ester (**20**).* From **4** (121 mg, 0.29 mmol) and 4,5-dichloro-1,2-phenylenediamine (105 mg, 0.59 mmol) during 4 h. The reaction product was crystallized in methanol to yield **20** (132 mg, 81%). M.p.: 192–196 °C. UV $\lambda_{max}(\varepsilon)$: 223 (26800), 270 (28800), 314 (9200), 380 (1200). IR (cm^{-1}): 1737, 1625, 1504, 1236, 1127, 1038. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). HRMS (FAB-POSI, M+) Calc. for $C_{27}H_{19}Cl_2N_3O_6$ 552.0729. Found 552.0745.

*4.1.5. Condensation of **5** with dihalogenated compounds. Compounds **11** and **12***

*4.1.5.1. (5R, 5aR, 8aS, 9R) 9-Acetoxy-5-(7-methoxybenzo[1,3]dioxol-5-yl)-5,5a,6,8,8a,9-hexahydro-furo[3′,4′:6,7]naphtho[2,3-d][1,3]dioxol-6-one (**12**).* A mixture of **5** (100 mg, 0.23 mmol), dibromomethane (101 mg, 0.58 mmol), K_2CO_3 (81 mg) and sodium iodide (2 mg) in acetone (12 mL) was refluxed for 24 h. After cooling, water was added and the product extracted with EtOAc. The organic layer

was washed with brine, dried and evaporated. Column chromatography on silica gel (eluent: CH_2Cl_2–EtOAc 95:5) of the residue produced compound **12** (21 mg, 21%). M.p.: 208–210 °C (MeOH). UV $\lambda_{max}(\varepsilon)$: 207 (36100), 290 (5800), 325 (2000). IR (cm^{-1}): 1773, 1735, 1634, 1505, 1236, 1127, 1040. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4).

*4.1.5.2. (5R, 5aR, 8aS, 9R) 9-Acetoxy-5-(8-methoxy-2,3-dihydrobenzo[1,4]dioxin-6-yl)-5,5a,6,8,8a,9-hexahydrofuro[3′,4′:6,7]naphtho[2,3-d][1,3]dioxol-6-one (**11**).* Following the same procedure, compound **11** (68%) was obtained from **5** (100 mg, 0.23 mmol) and 1,2-dibromoethane (109 mg, 0.58 mmol). M.p.: 240–242 °C (MeOH). $[\alpha]_D^{22}$ +38.5° (*c*, 0.87%). UV $\lambda_{max}(\varepsilon)$: 210 (29200), 283 (1900), 326 (800). IR (cm^{-1}): 1771, 1735, 1596, 1506, 1236, 1127, 1037. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). HRMS (FAB-POSI, M+1) Calc. for $C_{24}H_{23}O_9$ 455.1342. Found 455.1363. Anal. $C_{24}H_{22}O_9$ (C, H).

*4.1.6. Reaction of quinone **3a** with diols. 7-Acetyl-4′-demethyl podophyllotoxin (**13**)*

Quinone **3a** (150 mg, 0.35 mmol) was dissolved in methanol (10 mL). Then ethylene glycol (26 mg, 0.42 mmol), trimethylorthoformate (107 mg, 1.0 mmol) and (−) camphorsulfonic acid (10 mg, 0.05 mmol) were successively added. The mixture was refluxed under argon atmosphere for 21 h. The reaction mixture was neutralized with triethylamine (5 mg, 0.05 mmol), diluted with water and extracted with EtOAc. The organic layer was washed with brine, dried and evaporated providing a reaction product that was chromatographed on silica gel (eluent: CH_2Cl_2–EtOAc 9:1) to give derivative **13** (70 mg, 45%). IR (cm^{-1}): 3440, 1777, 1733, 1612, 1516, 1507, 1236, 1115, 1036. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4).

Column chromatography (eluent: CH_2Cl_2–EtOAc 9:1) of the reaction product obtained in the same way from **3a** (151 mg, 0.35 mmol) and 1,2-cyclohexanediol (53 mg, 0.45 mmol), provided **13** (64 mg, 42%).

*4.1.7. Condensation of **5** with enamines. Compounds **14** and **15***

*4.1.7.1. (5R, 5aR, 8aS, 9R) 9-Acetoxy-5-(4-methoxy-5a(9a)-pyrrolidin-1-yl-5a,6,7,8,9,9a-hexahydrodibenzo[1,4]dioxin-2-yl)-5,5a,6,8,8a,9-hexahydrofuro[3′,4′:6,7]naphtho[2,3-d][1,3]dioxol-6-one (**14**).* Quinone **5** (175 mg, 041 mmol) was dissolved in chloroform (15 mL) under inert atmosphere at 0 °C. Then, a solution of 1-(1-cyclohexenyl)pyrrolidine (93 mg, 0.62 mmol) in chloroform (8 mL) was added dropwise and the mixture was stirred for 3 h at 0 °C. The residue obtained after evaporation of the solvent was chromatographed on silica gel (neutralized with 1% Et_3N, eluent: C_6H_6–Et_2O 6:4) and compound **14** (124 mg, 54%) was obtained. UV $\lambda_{max}(\varepsilon)$: 207 (39900), 290 (3700), 325 (800). IR (cm^{-1}): 1779, 1733, 1597, 1506, 1237, 1125, 1038, 866, 735. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4).

*4.1.7.2. (5R, 5aR, 8aS, 9R) 9-Acetoxy-5-(4-methoxy-5a(9a)-morpholin-4-yl-5a,6,7,8,9,9a-hexahydrodibenzo[1,4]dioxin-2-yl)-5,5a,6,8,8a,9-hexahydrofuro[3′,4′:6,7]naphtho[2,3-d][1,3] dioxol-6-one (**15**).* The same procedure was applied to **5** (127 mg, 0.30 mmol) together with 4-(1-cyclohexenyl)-morpholine (101 mg, 0.60 mmol). The reaction mixture was kept 3 h at 0 °C and 21 h at r.t. Chromatography of the reaction product (eluent: hexene–EtOAc 7:3) provided compound **15** (64 mg, 36%). UV $\lambda_{max}(\varepsilon)$: 210 (43900), 289 (4100), 326 (1000). IR (cm^{-1}): 1779, 1734, 1597, 1507, 1236, 1115, 1036, 867, 735. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 4). HRMS (FAB-POSI, M+1) Calc. for $C_{32}H_{35}NO_{10}$ 593.2261. Found 593.2196. Anal. $C_{32}H_{35}O_{10}N$ (C, H, N).

4.2. Bioactivity

4.2.1. Antineoplastic assay

A screening procedure [19] was used to assess the cytotoxic activity against the following cell lines: P-388 (lymphoid neoplasms from DBA/2 mouse), A-549 (human lung carcinoma), HT-29 (human colon carcinoma) and MEL-28 (human melanoma). Cells were seeded into 16 mm wells (multidishes NUNC 42001) at concentrations of 1×10^4 (P-388) or 2×10^4 (A-549, HT-29 and MEL-28) cells/well, respectively, in 1-mL aliquots of MEM supplement with of 10 FCS medium containing the compound to be evaluated at the concentrations tested. In each case, a set of control wells was incubated in the absence of sample and counted daily to ensure the exponential growth of cells. After 3 days at 37 °C, in 10% CO_2, and 98% humidity, the P-388 cells were observed through an inverted microscopy and the degree of inhibition was determined by comparison with the controls, whereas the A-549, HT-29 and MEL-28 cells were stained with crystal violet before examination.

4.2.2. Antiviral assays

4.2.2.1. Cell culture and virus. The cell line used was: *Cercopithecus aethiops* African green monkey kidney cells (VERO cell line ATCC CCL-81). Cells were grown in MEM supplemented with 10% FBS, 100 units mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin, 2 mM L-glutamine, 0.07% $NaHCO_3$, 1% non-essential amino acids and vitamin solution. The cultures were maintained at 37 °C in humidified 5% CO_2.

HSV-2 was obtained from the Center for Disease Control (Atlanta, GA). The virus stock was prepared

from HSV-2-infected VERO cell cultures. The infected cultures were subjected to three cycles of freezing–thawing, and centrifuged at 2000 rpm for 10 min. The supernatant was collected, titrated, and stored at −170 °C in 1-mL aliquots. To titrate the virus suspension, confluent monolayer VERO cells were grown in 96-well flat-bottomed plates, infected with 0.1 mL of serial tenfold dilutions of the virus suspension in quadruplicate and incubated for 48 h.

4.2.2.2. End-point titration technique (EPTT). The virus titre was $10^{3.5}$ (the dilution of the virus required to obtained 50% lytic effect of the culture in the each well in 100 μL viral suspension, $TCDI_{50/0.1\ mL}$) using the Spearman–Käber formula [24]. The technique described by Vlietinck et al. [21] with a few modifications was used [25]. Briefly, confluent monolayer VERO cells were grown in 96-well flat-bottomed plates. Twofold dilutions of the compounds in maintenance medium (MM), identical to growth medium except for FBS which was 3%, were added 1 h before viral infection. The treated cells were infected with 0.1 mL of 10 $TCDI_{50}$ or 1.0 $TCDI_{50}$ of the previously titrated virus suspension and incubated again at 37 °C in humidified 5% CO_2 for 48 h. Controls consisted of cells with serial tenfold dilutions (from 10 to 10^{-3} $TCDI_{50}$) of HSV-2 in the absence of the compounds, treated noninfected cells and untreated noninfected cells. The antiviral activity is expressed as the maximal nontoxic dose of the test compound needed to obtain maximum reduction of virus titre. The reduction in virus titre was determined as the reduction factor (R_f) of the virus titre, i.e. the ratio of the virus titre in the absence over virus titre in the presence of the compound. Three assays were carried out in duplicate with at least five concentrations of compounds. The results are expressed as the mean obtained from three different assays.

4.2.2.3. Plaque elimination assay. Confluent monolayer VERO cells were grown in 24-well flat-bottomed plates. Twofold dilutions of 500 μL of the compounds in MM were added 1 h before viral infection. The treated cells were inoculated with 100 μL of approximately 100 PFU (plaque forming units) of virus; after 1 h 400 μL of MM with 2% carboxymethylcellulose were added and the cells well incubated again at 37 °C in humidified 5% CO_2 for 72 h. At least two assays were carried out in duplicate with four concentrations of compound and reproducible results were obtained. The ED_{100} is the dilution that completely eliminated macroscopic plaque formation without toxicity to cell monolayers.

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