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Original article

Synthesis and cytotoxicity of podophyllotoxin analogues modified in the A ring

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In memory of Dr. Benedicto del Rey

Abstract

Several podophyllotoxin derivatives lacking the methylenedioxy group or with different functionalization of the A-ring of the cyclolignan skeleton have been prepared and evaluated for their cytotoxic activities on four neoplastic cell lines (P-388, A-549, HT-29 and MEL-28). Most of them maintained their cytotoxicity at the μM level.

Keywords: Podophyllotoxin; Cyclolignans; A-ring modifications; Cytotoxicity

1. Introduction

Podophyllotoxin belongs to the natural product family of lignans, well known for their antineoplastic and antiviral properties [1]. These properties prompted the scientific community to perform numerous modifications on the podophyllotoxin skeleton, in order to avoid several side effects, which have resulted in the clinical introduction of etoposide, teniposide and etopophos [1,2] (Fig. 1).

The chemical modifications that led to etoposide and derivatives, led also to a change in the mechanism of action: from antimicrotubule in the parent compound podophyllotoxin to antitopoisomerase II in etoposide and congeners. This change is related to three main chemical modifications: demethylation at C-4′ of the E ring, a C-7 epimerization and the presence of a glicosidic moiety or similar at the C-7 position on the C ring. Those facts were the origin of a great number of derivatives that have been synthesized and analyzed by QSAR methods by Lee and coworkers [3] leaving the cyclolignan skeleton nearly untouched.

Over the years, we have been involved in the chemical transformation of podophyllotoxin and we have prepared a large number of cyclolignans, some of which displayed potent antiviral and cytotoxic activities [2b]. Among these, those analogues modified at the C- and D-rings with the general structure showed at Fig. 1 are particularly worthy of note [4]. These derivatives have an interesting selectivity against the human colon tumoural cell line HT-29, in spite of the absence of the lactone moiety which has always been considered an important factor for high cytotoxic activity [5].

In our previous studies, the A and E rings were untouched, and also there are few references about modifications to these and the influence of such changes on the cytotoxic activity of cyclolignans.

Regarding the A ring, several N-heterocyclic A-ring podophyllotoxin derivatives have been prepared [6] and the biological evaluation indicates no antimitotic activity, either weak inhibition of topoisomerase II in vitro. Nonetheless, one of them exhibited an improved cytotoxic profile compared to that of etoposide, so a diferent mechanism of action was postulated.

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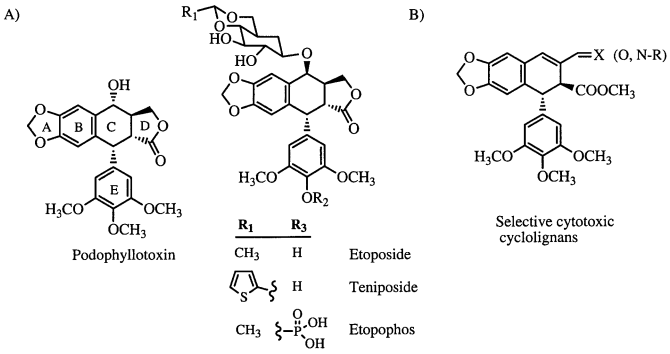

Fig. 1. Structures of podophylotoxin and related compounds. (A) Cyclolignans in clinical use. (B) Selective cytotoxic cyclolignans lacking the lactone ring.

The ring A-opened compounds have been found in naturally occurring podophyllotoxin congeners such as diphyllin and sikkimotoxin which have been shown to be less cytotoxic than podophyllotoxin [7]. Also a series of 3,4-*O*,*O*-demethylene-7β-substituted podophyllotoxins and their 4′-O-demethyl analogues have been synthesized and evaluated for their cytotoxicity and their ability to inhibit human DNA topoisomerase II. All the tested compounds were found to be less potent than the methylenedioxy-bearing analogues [8].

However we have shown in previous work [9] that analogues without the methylenedioxy group have a very interesting immunosuppressive activity. All these facts made us to think that more work is necessary to understand the influence of that group on the cytotoxicity. Thus, in this paper we present our studies directed towards the preparation of podophyllotoxin analogues lacking the methylenedioxy group and/or with different functionalization on the A ring.

2. Chemistry

The preparation of the analogues **3**–**7** and **9**–**10**, which lacked the methylene group on the dioxole ring is shown in Fig. 2 and summarized in Table 1.

The starting materials for the transformation presented here were the natural products deoxypodophyllotoxin and podophyllotoxin isolated from podophyllum resin [10]. Podophyllotoxin was also transformed into the isoxazole **8** as previously described by us [11].

Boron trichloride was used to attain the cleavage of the methylenedioxy group selectively, in the presence of the aromatic methoxyl groups of the trimethoxyphenyl moiety. The best yields of demethylenated compounds were obtained when the reaction mixture was kept one hour at low temperature and one hour under reflux in the presence of calcium carbonate. After longer reaction periods, 3,4-demethylenepodophyllotoxin (**4**) was accompanied by two other compounds, its 7-epimer **5** and the dehydration product **6**, likely formed by dehydratation followed by double bond isomerization to the more stable α,β-unsaturated enone system.

Compound **6** was also the major product of the acid-catalyzed cleavage of the methylenedioxy ring of **2** according to Schreier's method [12].

With the aim of analyzing the influence on cytotoxicity, we have considered simultaneous modifications in

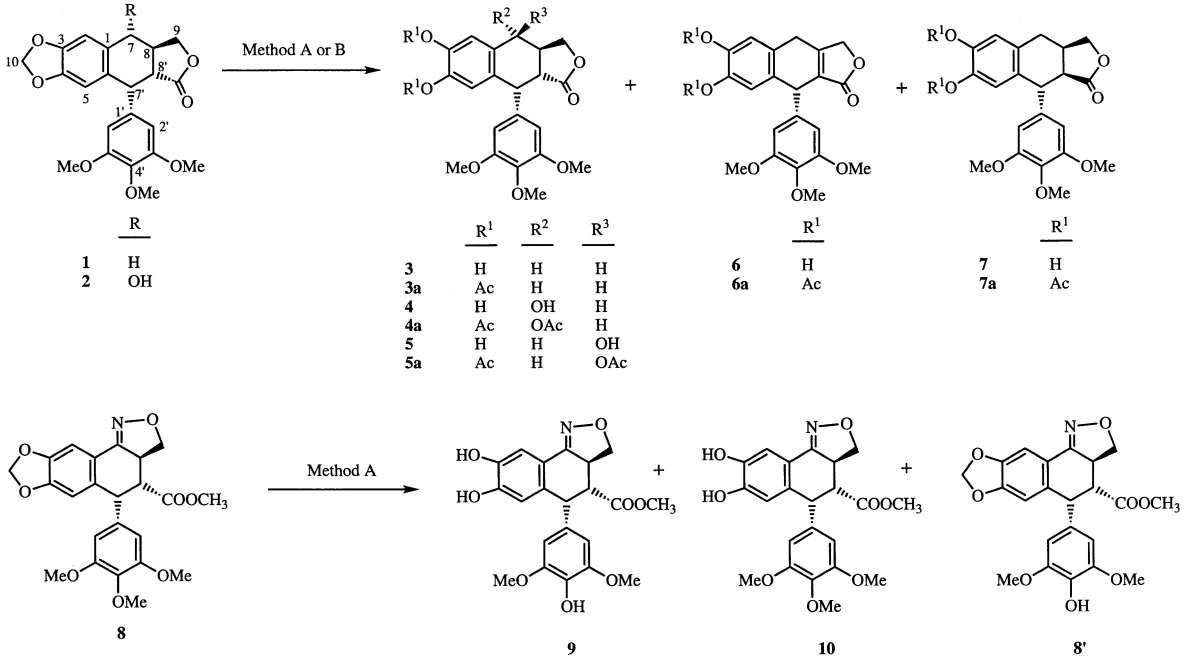

Fig. 2. Preparation of the 3,4-*O*,*O*-demethylenated cyclolignans.

Table 1
Preparation of compounds **3**–**7**

Substrate	Method [a]	Temperature (°C)	Time (i+ii) (h)	Products
1	A	−78	1+1	**3** (67%)
1	B	150	3	**3** (41%)+**7** (10%)
2	A	−65 to −70	1+1	**4** (80%)
2	A	−78	2.5+4	**4** (11%)+**5** (16%)+**6** (14%)
2	B	150	3	**6** (43%)
8	A	0	1+1.5	**9**+**10** (70%)+**8′** (6%)

[a] Method A: (i) $BCl_3-CH_2Cl_2$, low temperature; (ii) H_2O, acetone, $CaCO_3$, reflux. Method B: H_3PO_4, phenol, AcOH.

A ring and other parts of the cyclolignan skeketon that also shown immunosuppressive activity[10]. Thus, the isoxazole **8** was treated with boron trichloride to afford a 9:1 mixture of **9** and **10** as it can be deduced from the ^{1}H-NMR spectra. At this point, it was not possible the purification of **10**; this was achieved later on, after reaction with dihalogenated derivatives. It has been reported that cleavage of the methoxy groups usually requires either higher temperatures or longer reaction times[13]. However, when **8** was demethylenated at low temperature, there was a large proportion of starting material, and when the reaction was performed at 0 °C, the major product was **9** together with a small proportion of **10**. Even the compound **8′**, which had the methylenedioxy but demethylated at C-4′, was also isolated.

Once the two phenolic groups were obtained, they were transformed into other five- or six-membered rings with different substituents by condensation with dihalogenated substrates under basic conditions (Figs. 3 and 4). Thus, condensation of **3** with methyl dichloroacetate and dichloromethyl methyl ether yielded the analogous compounds **11** and **12** as a mixture of epimers at C-10 position, as it can be deduced from several duplicated signals in their espectra. During the recycling of the dioxole, epimerization of the lactone ring occurred due to the basic media used in the reaction. The purification of carboxylic acid was difficult and it was isolated as its methyl ester **11a**, obtained after treatment of the reaction product with diazomethane. The same anologue **11a** was obtained from demethylenedeoxypicropodophyllin **7** under the same conditions. When **4** was treated with methyl dichloroacetate, the only isolated product was **13**, as a result of the dehydratation and aromatization under the reflux conditions of the reaction.

Treatment of **3** with 1,2-dibromoethane gave a reaction product from which the dioxane derivatives **14** and **15** were isolated. In this case only a partial isomerization of the lactone ring occurred.

The mixture of **9** and **10** was also treated with 1,2-dibromoethane under the same conditions and the corresponding dioxanes **20** and **21** were obtained. In order to obtain the corresponding substituted dioxole analogues, the mixture of **9** and **10** was treated with the corresponding dihalogenated derivatives, giving **16** and **17** when methyl dichloroacetate was used; the mixture of epimers is clearly observed in the NMR ^{13}C spectrum of

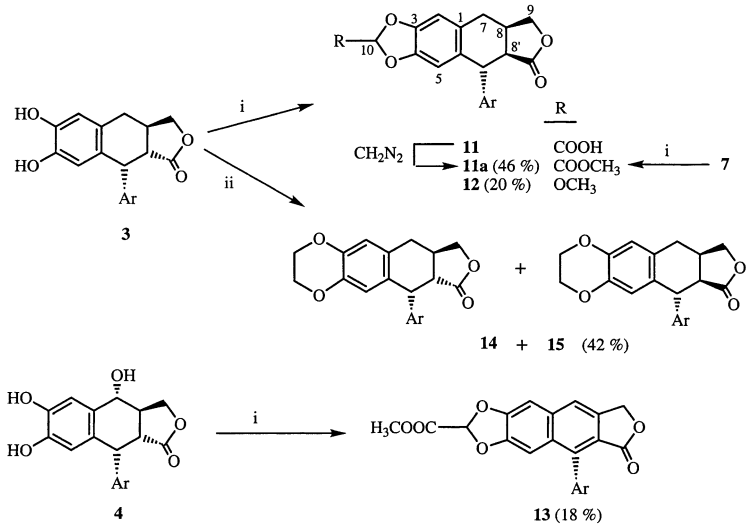

Fig. 3. Formation of new dioxole/dioxane rings from **3** and **4**. (i) $Cl_2CH-COOCH_3$ or $Cl_2CH-O-CH_3$, K_2CO_3, DMF, 90–100 °C. (ii) $Br-CH_2-CH_2-Br$, K_2CO_3, NaI, acetone, reflux.

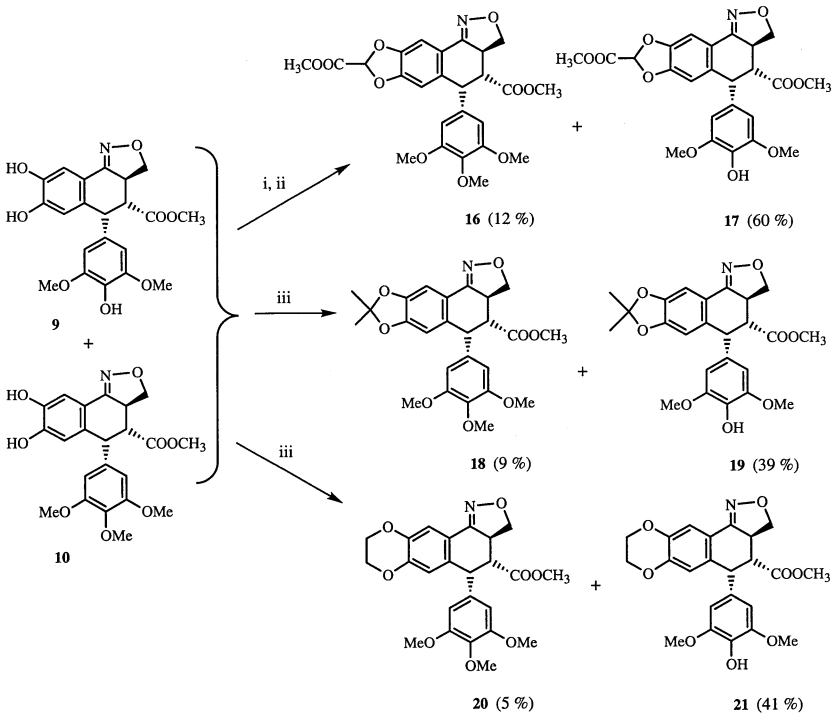

Fig. 4. Formation of new dioxole/dioxane rings from **9** and **10**. (i) $Cl_2CH-COOCH_3$, K_2CO_3, DMF, 90–100 °C. (ii) CH_2N_2, ether. (iii) $Cl_2CH-O-CH_3$ or $Br-CH_2-CH_2-Br$, K_2CO_3, NaI, acetone, reflux.

derivative **17**, in which several signals are duplicated (Table 6). In the case of dichloromethyl methyl ether, instead of the corresponding dioxole analogues, the acetonides **18** and **19** ware obtained as the result from the reaction between the phenolic groups and the acetone used as solvent.

3. Biological results and discussion

The compounds thus prepared were evaluated in vitro to establish their cytotoxicity[14] against cell cultures of P-388 murine leukaemia, A-549 human lung carcinoma, HT-29 human colon carcinoma and MEL-28 malignant human melanoma. The results obtained are shown in Table 2.

The derivatives tested showed cytotoxicity levels two or three orders of magnitude lower than those of the parent compounds podophyllotoxin and deoxypodophyllotoxin. However, the cytotoxicity remained at the micromolar level and some general observations can be made.

For the most part, there are no differences among the IC_{50} values for the four cell lines; only compounds **4**, **4a**, **5** and **8** are about twice as potent against P-388, A-549 and MEL-28 as against HT-29.

Elimination of the methylenedioxy group led, in general, to less cytotoxic compounds, the decrease in potency being larger in the case of deoxypodophyllotoxin (**3** vs. **1**) than in the podophyllotoxin derivatives (**4** vs **2**). Epimerization of the hydroxyl group at C-7 decreased the cytotoxic potency (**5** vs. **4**) four times. Acetylation of the catechol groups also led to derivatives about one order of magnitude less cytotoxic.

The derivatives with the isoxazole ring were among the least cytotoxic compounds prepared, although it is worth noticing that derivative **19** was ten times more potent against P-388 than the other tested lines.

Table 2
Cytotoxic activity of cyclolignans modified at ring A (IC_{50} μM)

Compound	P-388	A-549	HT-29	MEL-28
1	0.003	0.005	0.005	–
2	0.012	0.012	0.012	–
2a	0.55	0.55	0.55	0.55
3	1.3	1.3	1.3	1.3
4	0.62	0.62	1.2	0.62
4a	2.0	2.0	5.1	2.0
5	2.5	2.5	12.4	2.5
5a	20.6	20.6	20.6	20.6
6	5.2	5.2	5.2	–
6a	21.4	21.4	21.4	21.4
7	25.9	51.8	> 51.8	–
8	2.3	5.7	11	5.7
8′	23.4	> 23.4	> 23.4	23.4
10	> 23.3	> 23.3	> 23.3	> 23.3
11a	2.2	5.5	5.5	5.5
12	0.23	0.23	0.23	0.23
17	> 23	> 23	> 23	> 23
19	2.2	22.0	22.0	22.0

Also, the epimerization of the lactone ring (**7** vs. **3**) led to much less cytotoxic derivatives, although the cytotoxicity was partially recovered when a new dioxole ring was formed (**11a** and **12** vs. **7**). This result clearly indicates the importance of the methylenedioxy ring, which is a common moiety in several antimitotic natural products.

4. Experimental

4.1. Chemistry

Melting points were determined by heating in an external silicone bath and were uncorrected. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter in chloroform solution and UV spectra on a Hitachi 100-60 spectrophotometer in ethanol solution. IR spectra were obtained on a Beckmann (Acculab VIII) spectrophotometer in chloroform solution. 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 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. Flash chromatography was performed on silica gel (Merck no. 9385). Elemental analysis were obtained with a LECO CHNS-932 and were within $\pm 0.4\%$ of the theoretical values.

4.1.1. Isolation of lignans *1* and *2*

The resin (50 g) of Podophyllum emodi was extracted with hot $CHCl_3$. The soluble fraction was chromatographed on neutral alumina (activity II), and the following cyclolignans were eluted with $CHCl_3$: deoxypodophyllotoxin (**1**, 3.7%), podophyllotoxin (**2**, 36%) and 4′-demethylpodophyllotoxin (1.3%).

4.1.2. 3,4-O,O-Demethylenedeoxypodophyllotoxin (*3*)

4.1.2.1. Method A. To a solution of boron trichloride in dichloromethane (1 M, 1 mL) precooled at −70 to −65 °C was added dropwise **1** (100 mg, 0.25 mmol) in dichloromethane (5 mL) over 15 min. After the mixture was stirred at the same temperature for an additional 1 h, the mixture was poured into ice-water and extracted with ethyl acetate. The combined organic layers were washed with brine until neutral pH, dried over anhydrous sodium sulfate and filtered. The filtrate was evaporated to give a white solid which was put into a mixture of acetone–water–calcium carbonate (1 mL–1 mL–0.3 g) and refluxed for 1 h. The white suspension was diluted with water, neutralized with 2N HCl until pH 2–3 and extracted with ethyl acetate. Evaporation of the solvent afforded a reaction product from which **3** (65 mg, 67%) was separated after crystalization from dichloromethane: m.p. 250–254 °C. Physical data is according with those described previously [15]. $[\alpha]_D^{22}$ −100.2° (c, 0.5%). UV $\lambda_{max}(\varepsilon)$: 216 (20800), 288 (3500). IR (cm^{-1}): 3550, 3020, 1780, 1600, 1510, 1250, 1140, 1010. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5). HRMS (FAB+) Calc. for $C_{21}H_{23}O_7$ $[M+H]^+$ 387.1443, Found 387.1417. Anal. $C_{21}H_{22}O_7$ (C, H).

Acetylation of **3** with acetic anhydride in pyridine afforded **3a**. IR (cm^{-1}): 3010, 1775, 1600, 1500, 1200, 1130, 1010. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5).

4.1.2.2. Method B. A solution of **1** (500 mg, 1.25 mmol) and phenol (400 mg) in 85% H_3PO_4 (15 mL) and glacial acetic acid (5 mL) was heated for 3 h at 150 °C. The reaction mixture was diluted with water and extracted with EtOAc. The organic layer was washed with 5% aqueous $NaHCO_3$ and water and then evaporated in vacuo and fractionated on a silica gel column (Cl_2CH_2/EtOAc 1:1) to afford **3** (183 mg, 38%) and a mixture of **3**+**7** (120 mg). That mixture was acetylated and chromatographed on silica gel (Cl_2CH_2/EtOAc 9:1) to yield **3a** (15 mg, 3%) and **7a** (60 mg, 10%). Compound **7a**. IR (cm^{-1}): 2950, 1780, 1600, 1510, 1230, 1140, 1020. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5).

4.1.3. 3,4-O,O-Demethylenepodophyllotoxin (*4*)

By use of the same procedure A outline for the preparation of **3**, compound **4** (80%) was prepared from **2**. Physical data is in accord with those described previously [15]. EM (m/z): 402 [M^+]. $[\alpha]_D^{22}$ −92.9° (c, 0.5%). UV $\lambda_{max}(\varepsilon)$: 212 (29100), 285 (2900). IR (cm^{-1}): 3420, 1780, 1590, 1510, 1250, 1130, 1010. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5).

Acetylation of **4** yielded **4a**. $[\alpha]_D^{22}$ −70.4° (c, 0.9%). UV $\lambda_{max}(\varepsilon)$: 222 (15700), 268 (1400). IR (cm^{-1}): 3000, 1780, 1745, 1600, 1510, 1250, 1130, 1010. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5).

4.1.4. 3,4-O,O-Demethyleneepipodophyllotoxin (*5*)

Procedure A was applied to **2** (500 mg) with the followed modifications. The reaction mixture was keeped at −78 °C for 2.5 h, followed by 4 h reflux with calcium carbonate. The mixture was worked up as described before for **3**. The reaction product was a mixture of **4**, **5** and **6**. Purification of this product by flash column chromatography with Cl_2CH_2/EtOAc (1:1) gave **6** (65 mg, 14%), **4** (50 mg, 11%) and **5** (75 mg, 16%).

Compound **5**: EM (m/z): 402 (M^+). $[\alpha]_D^{22}$ −77.8° (c, 0.5%). UV $\lambda_{max}(\varepsilon)$: 220 (17500), 285 (3100). IR (cm^{-1}): 3490, 1780, 1750, 1600, 1510, 1240, 1125, 1010. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5).

Acetylation of **5** afforded **5a**. $[\alpha]_D^{22}$ −71.0° (c, 0.63%). UV $\lambda_{max}(\varepsilon)$: 209 (25000), 267 (1900). IR (cm^{-1}): 1780, 1745, 1600, 1510, 1230, 1135, 1015. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5).

Table 3
^{1}H-NMR data of compounds **3**–**15**

H	**3** [a]	**3a**	**4** [a]	**4a**	**5** [a]	**5a**	**6** [a]	**6a**
2	6.72 s	7.09 s	7.19 s	7.21 s	6.94 s	7.34 s	6.76 s	7.16 s
5	6.49 s	6.94 s	6.47 s	6.98 s	6.49 s	6.98 s	6.70 s	7.03 s
7	2.70–3.10 m	2.60–3.20 m	4.75 d (9.0)	5.93 d (8.9)	4.85 d (3.3)	6.20 d (3.8)	3.10 m	3.80 m
8	2.70–3.10 m	2.60–3.20 m	3.00 m	2.88 m	2.92 m	3.00 m	–	–
9	4.41 dd (8.3;6.1)	4.48 m	4.50 m	4.39 dd (9.4;6.7)	4.31 d (9.3)	4.38 dd (8.5;8.0)	4.93 m	4.90 m
	3.95 dd (9.4;8.3)	3.90 m	4.12 dd (10.1;8.8)	4.23 t (9.4)		3.97 dd (10.6;8.5)	–	–
2′,6′	6.40 s	6.30 s	6.45 s	6.33 s	6.35 s	6.21 s	6.48 s	6.37 s
7′	4.50 d (4.5)	4.71 d (3.5)	4.50 m	4.72 d (4.2)	4.51 d (5.3)	4.78 d (5.0)	4.69 bs	4.90 m
8′	2.70–3.10 m	2.60–3.20 m	3.00 m	2.98 dd (14.4;4.2)	3.32 dd (14.1;5.3)	3.30 dd (14.0;5.0)	–	–
CH_3O-3′,5′	3.67 s	3.72 s	3.68 s	3.73 s	3.66 s	3.70 s	3.72 s	3.77 s
CH_3O-4′	3.66 s	3.79 s	3.67 s	3.80 s	3.66 s	3.78 s	3.64 s	3.79 s
OAc	–	2.23 s	–	2.30	–	2.28	–	2.25
	–	2.29 s	–	2.24	–	2.23	–	2.30
		–	–	2.19	–	2.10	–	–

H	**7**	**7a**	**11a**	**12**	**13**	**14**	**15**
2	6.67 s	7.02 s	6.71 s	6.73 s	7.29 s	6.72 s	6.68 s
5	6.57 s	6.72 s	6.58(6.60) s	6.64 s	7.20 s	6.59 s	6.59 s
7	2.75 dd (15.2;6.1)	2.90 m	2.82 m	2.95 m	7.75 s	3.05 m	2.82 dd (15.1;6.0)
	2.35 dd (15.2;4.7)	2.51 dd (15.4;5.0)	2.48 dd (15.2;5.6)	2.52 dd (15.0;5.5)	–	2.73 m	2.43 dd (15.1;6.0)
8	2.99 m	2.90 s	3.00 m	3.04 m	–	2.73 m	3.00 m
9	4.40 dd (9.5;7.8)	4.42 dd (9.4;6.2)	4.43 dd (9.0;7.4)	4.47 dd (9.2;7.1)	5.40 s	4.45 dd (8.8;6.2)	4.42 dd (8.7;7.3)
	3.89 dd (9.5;3.3)	4.04 dd (11.4;9.4)	3.96 dd (9.0;2.8)	4.00 m	–	3.90 m	3.95 m
2′,6′	6.29 s	6.38 s	6.31 s	6.33(6.35) s	6.53 d (2.1)	6.32 s	6.35 s
	–	–	–	–	6.55 d (2.1)	–	–
7′	4.28 d (2.8)	4.18 d (5.0)	4.31 bs	4.38 bs	–	4.61 d (3.3)	4.28 d (3.4)
8′	3.36 dd (9.7;2.8)	3.23 dd (8.8;5.0)	3.30 m	3.35 m	–	2.73 m	3.32 dd (9.5;3.4)
CH_3O-3′,5′	3.68 s	3.77 s	3.76 s	3.78 s	3.87 s, 3.85 s	3.74 s	3.77 s
CH_3O-4′	3.78 s	3.82 s	3.80 s	3.84 s	3.99 s	3.80 s	3.81 s
OAc	–	2.24 s	–	–	–	–	–
	–	2.20 s	–	–	–	–	–
$COOCH_3$	–	–	3.83 s	–	3.84 s	–	–
O–CH–O	–	–	6.28 s	6.80(6.84) s	6.45 s	–	–
OCH_3	–	–	–	3.41(3.44) s	–	–	–
$–CH_2–CH_2–$	–	–	–	–	–	4.26 bs	4.26 bs

[a] NMR in acetone-d_6.

*4.1.5. 3,4-O,O-Demethylene-β-apopicropodophyllin (**6**)*

It was eluted from the aforementioned chromatography and also by treatment of **2** with phenol and H_3PO_4 following method B: a solution of **2** (500 mg) and phenol (200 mg) in 15 mL of H_3PO_4 and 5 mL of glacial AcOH was heated for 3 h at 150 °C. The reaction mixture was diluted with water and extracted with EtOAc. The organic layer was washed with 5% aqueous $NaHCO_3$ and water and the evaporated in vacuo and fractionated on a silica gel column (Cl_2CH_2/EtOAc 1:1) to afford 200 mg (43%) of **6**. EM (*m/z*): 384 [M^+]. $[\alpha]_D^{22}$ +67.2° (*c*, 0.25%). UV $\lambda_{max}(\varepsilon)$: 222 (23900), 286 (4800). IR (cm^{-1}): 3300, 1730, 1680, 1590, 1500, 1230, 1110, 1010. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5).

Acetylation of **6** afforded **6a**. $[\alpha]_D^{22}$ +52.2° (*c*, 0.64%). UV $\lambda_{max}(\varepsilon)$: 207 (28500), 272 (1700). IR (cm^{-1}): 3010, 1765, 1690, 1600, 1500, 1200, 1130, 1010. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5).

*4.1.6. 3,4-O,O-Demethylenedeoxypicropodophyllin (**7**)*

Procedure A was applied to deoxypicropodophyllin (100 mg obtained by basic epimerization of **1** [16]) to afford 75 mg (75%) of **7**. EM (*m/z*): 386 (M^+). $[\alpha]_D^{22}$ +46.8° (*c*, 0.96%). UV $\lambda_{max}(\varepsilon)$: 222 (23300), 288 (7200).

Table 4
^{1}H RMN data of compounds **8′**, **9**, **16**–**21**

H	**8′**	**9** [a]	**16**	**17**	**18**	**19**	**20**	**21**
2	7.45 s	7.38 s	7.54 s	7.50 s	7.37 s	7.37 s	7.56 s	7.55 s
5	6.55 s	6.61 s	6.64 s	6.61 s	6.47 s	6.46 s	6.64 s	6.63 s
8	3.80 m	3.75 m	3.80 m	3.80 m	3.90 m	3.90 m	3.90 m	3.77 m
9	4.83 t (7.3)	4.60 m	4.86 t (6.2)	4.83 t (7.7)	4.83 dd (9.0;8.0)	4.82 dd (9.1;8.0)	4.84 dd (9.1;8.1)	4.83 t (7.5)
	3.80 m	3.80 m	3.80 m	3.80 m	3.90 m	3.90 m	3.90 m	3.77 m
2′,6′	6.14 s	6.22 s	6.13 s	6.12 s	6.15 s	6.15 s	6.12 s	6.14 s
7′	4.63 d (5.1)	4.60 m	4.66 d (5.1)	4.63 d (5.1)	4.62 d (5.1)	4.60 d (5.1)	4.64 d (5.1)	4.63 d (5.1)
8′	3.20 dd (12.1;5.1)	3.27 m	3.20 m	3.20 m	3.20 dd (12.3;5.1)	3.18 dd (12.3;5.1)	3.20 dd (12.3;5.1)	3.19 dd (11.3;5.1)
CH_3O–3′,5′	3.77 s	3.69 s	3.74 s	3.75 s	3.74 s	3.77 s	3.73 s	3.77 s
CH_3O-4′	–	–	3.87 s	–	3.80 s	–	3.80 s	–
$COOCH_3$	3.66 s	3.67 s	3.69 s	3.65 s	3.67 s	3.66 s	3.68 s	3.67 s
O–CH_2–O	6.00 s	–	–	–	–	–	–	–
O–CH–O	–	–	6.34(6.36) s	6.34(6.35) s	–	–	–	–
OCH_3	–	–	3.86(3.88) s	3.83(3.85) s	–	–	–	–
–CH_2–CH_2–	–	–	–	–	–	–	4.27 s	4.27 s
CH_3	–	–	–	–	1.67 s	1.66 s	–	–
	–	–	–	–	1.68 s	1.67 s	–	–

[a] NMR in acetone-d_6.

IR (cm^{-1}): 3400, 1740, 1600, 1510, 1240, 1110, 1010. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5).

*4.1.7. Isoxazoles **9** and **8′***

Isoxazole **8** (456 mg, 1.03 mmol, obtained by described procedures [11]) was demethylenated using the method A with the following modifications: the temperature of the first step was 0 °C for 1 h and the reflux time was extended to 90 min. The reaction product was precipitated in dichloromethane to obtain 300 mg (70%) of a 9:1 mixture of **9** and **10** and 100 mg of residue which was chromatographed on silica gel (Cl_2CH_2/EtOAc 75:25) to afford 28 mg (6%) of **8′**.

9: IR (cm^{-1}): 3430, 1730, 1709, 1600, 1510, 1260, 1110. ^{1}H-NMR (Table 4). ^{13}C-NMR (Table 6). HRMS (FAB +) Calc. for $C_{21}H_{22}NO_8$ $[M+H]^+$ 416.1345, found 416.1301.

8′: $[\alpha]_D^{22}$ −145.3° (*c*, 0.5%). UV $\lambda_{max}(\varepsilon)$: 215 (26200), 274 (11600), 313 (6900). IR(cm^{-1}): 3540, 1740, 1630, 1510, 1220, 1130, 1050. ^{1}H-NMR (Table 4). ^{13}C-NMR (Table 6).

*4.1.8. Formation of the new substituted dioxole ring: compounds **11a**–**13**, **16**–**19***

*4.1.8.1. Compound **11a**.* A mixture of **3** (120 mg, 0.31 mmol), methyl dichloroacetate (0.1 mL, 0.96 mmol) and anhyd. K_2CO_3 (300 mg) in DMF (2 mL) was stirred at 90–100 °C under nitrogen for 2 h. After cooling the mixture, water (5 mL) was added and it was stirred at 90–100 °C for 40 min. The mixture was cooled, weakly acidified with 2N HCl and then weakly basified with sat. $NaHCO_3$ and washed with ethyl acetate. The aqueous layer was acidified with 2N HCl and extracted with ethyl acetate. The extract was washed with brine, dried over Na_2SO_4 and evaporated. The resulting residue was treated with an ethereal solution of diazomethane and then chromatographed on silica gel (Cl_2CH_2/EtOAc 92:8) to give **11a** (65 mg, 46%). **11a** (25%) was also obtained by reaction of **7** with methyl dichloroacetate. M.p. 95–98 °C ($MeOH–Cl_2CH_2$). UV $\lambda_{max}(\varepsilon)$: 214 (20500), 290 (3600). IR (cm^{-1}): 1770, 1600, 1500, 1240, 1135, 1010. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5). Anal. $C_{24}H_{24}O_9$ (C, H).

*4.1.8.2. Compound **12**.* From **3** (150 mg, 0.39 mmol), dichloromethyl methyl ether (0.1 mL, 1,12 mmol), anhyd. K_2CO_3 (1 g) in DMF (3 mL). The mixture was stirred at 90–100 °C under nitrogen for 2 h. After cooling, water (5 mL) was added and it was stirred at 90–100 °C for 40 min. A reaction product was obtained after the same work up described above for **11a**. After chromatography of the reaction product with Cl_2CH_2/EtOAc 9:1, **12** (32 mg, 20%) was isolated. UV $\lambda_{max}(\varepsilon)$: 214 (20500), 290 (3600). IR (cm^{-1}): 1760, 1590, 1510, 1240, 1125, 1010. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5). HRMS (FAB +) calcd for $C_{23}H_{25}O_8$ $[M+H]^+$ 429.1549, found 429.1502. Anal. $C_{23}H_{24}O_8$ (C, H).

*4.1.8.3. Compound **13**.* From **4** (100 mg, 0.25 mmol), methyl dichloroacetate (0.1 mL, 0.96 mmol), anhyd. K_2CO_3 (300 mg) in DMF (2 mL). The mixture was stirred at 90–100 °C under nitrogen for 2 h. After cooling, water (5 mL) was added and it was stirred at 90–100 °C for 40 min. A reaction product was obtained after the same work up described above for **11a**. After esterification and chromatography (Cl_2CH_2/EtOAc 92:8) of the reaction product, **13** (20 mg, 18%) was isolated. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5).

Table 5
^{13}C-NMR data of compounds **3**–**15**

C	**3** [a]	**3a**	**4** [a]	**4a**	**5** [a]	**5a**	**6** [a]	**6a**	**7**	**7a**	**11a**	**12**	**13**	**14**	**15**
1	128.1	130.6	133.7	133.7	132.3	133.0	127.6	128.0	127.4	134.0	129.1	128.5	130.6 [c]	127.9	127.8
2	116.2	123.8	114.3	122.6	117.4	125.6	115.6	123.1	115.6	122.7	108.9	108.4	104.2	116.9	116.8
3	144.7	141.1	145.5	141.8	145.4	141.7	145.0	141.4	142.7	140.7	146.0	145.2	147.8	142.5	142.3
4	145.3	141.3	145.5	142.4	146.3	143.2	145.2	141.4	143.7	140.8	146.0	145.2	149.0	142.7	142.5
5	118.0	125.6	117.0	125.5	117.6	126.0	116.6	125.0	116.4	124.0	110.0	109.4	104.4	119.0	117.8
6	130.1	133.7	130.1	134.6	130.5	134.3	129.1	129.5	129.0	134.0	131.5	130.6	130.0 [c]	130.5	130.3
7	32.8	32.8	72.6	73.1	66.5	67.1	29.4	28.8	31.3	31.6	32.0	32.1	119.3	32.3	32.1
8	34.1	32.7	41.7	38.6	39.7	36.7	123.4	137.4	33.0	33.7	33.1	33.1	140.3	32.8	33.1
9	72.5	71.8	72.0	71.2	68.4	67.3	71.7	70.9	73.4	72.1	72.6	72.7	67.9	72.1	71.9
1′	138.3	135.8	137.9	137.3	137.5	137.6	140.7	135.0	138.5	138.0	138.0	138.0	134.7 [c]	136.6	138.2
2′	110.2	108.8	109.9	108.3	109.7	108.4	106.8	106.3	105.2	105.7	105.8	105.0	108.0	108.2	104.9
3′	153.5	152.8	153.5	152.9	153.5	152.9	154.2	153.5	153.3	153.6	153.6	153.3	153.2	152.4	153.1
4′	138.4	136.3	– [b]	137.5	138.3	137.8	138.0	137.5	136.6	136.5	138.2	138.2	138.6	136.9	136.6
5′	153.5	152.8	153.5	152.9	153.5	152.9	154.2	153.5	153.3	153.6	153.6	153.3	153.2	152.4	153.1
6′	110.2	108.8	109.9	108.3	109.7	108.4	106.8	106.3	105.2	105.7	105.8	105.0	108.0	108.2	104.9
7′	44.1	43.4	44.5	43.3	41.2	41.4	42.9	42.3	45.0	44.5	45.3	45.2	140.3	43.1	44.6
8′	47.9	47.4	45.8	45.4	44.2	43.4	160.5	156.8	46.7	46.2	46.2	46.3	128.8 [c]	47.5	46.0
9′	175.5	174.4	175.4	173.4	175.8	173.8	172.8	171.9	179.9	177.6	178.0	178.3	169.3	175.1	178.4
CH_3O-3′,5′	56.5	56.4	56.5	56.2	56.4	56.3	56.4	56.4	56.2	56.3	56.4	56.1	56.4	56.2	56.0
CH_3O-4′	60.4	60.7	60.4	60.7	60.4	60.7	60.4	60.8	60.9	60.8	60.7	60.8	61.0	60.7	60.5
OAc	–	20.5	–	20.5(2C), 21.0	–	20.5(2C), 20.9	–	20.8	–	20.5	–	–	–	–	–
	–	168.0	–	168.1(2C) 171.2	–	167.8(2C) 170.3	–	168.1	–	166.1	–	–	–	–	–
$COOCH_3$	–	–	–	–		–	–	–	–	–	52.8	–	53.1	–	–
	–	–	–	–		–	–	–	–	–	165.6	–	165.0	–	–
O–CH–O	–	–	–	–		–	–	–	–	–	103.0	119.8	103.5	–	–
OCH_3	–	–	–	–		–	–	–	–	–	–	50.0	–	–	–
$-CH_2-CH_2-$	–	–	–	–		–	–	–	–	–	–	–	–	64.3	64.1(2C)
	–	–	–	–		–	–	–	–	–	–	–	–	64.4	

[a] NMR in acetone-d_6.
[b] Signal not observed.
[c] Exchangeable signals.

Table 6
^{13}C-NMR data of compounds **8′**, **9**, **16**–**21**

C	**8′**	**9** [a]	**16**	**17**	**18**	**19**	**20**	**21**
1	118.8	117.4	119.6	119.5	119.2	119.1	118.4	118.3
2	103.9	110.3	103.4	103.3	103.8	103.8	113.1	113.0
3	147.5	145.4	146.7	146.8(146.6)	150.4	147.4	143.5	143.3
4	150.5	149.0	149.4	149.3	– [b]	150.4	146.5	146.5
5	109.2	116.1	109.6	109.5(109.2)	108.9	108.9	117.9	117.8
6	130.5	131.5	134.8	134.2	134.3	134.1	133.6	133.8
7	156.4	156.5	156.2	156.1	156.5	156.5	156.2	156.1
8	43.4	43.9	43.4	43.3	43.5	43.5	43.6	43.4
9	74.4	73.9	74.6	74.5	74.4	74.3	74.4	74.3
1′	135.0	135.6	135.3	130.3	135.1	130.6	135.5	131.0
2′	106.1	107.4	106.5	106.1(106.5)	106.5	106.1	106.5	106.0
3′	146.8	147.9	153.1	153.0(152.7)	153.0	146.8	153.0	146.7
4′	134.2	133.7	137.4	135.5(135.3)	– [b]	134.5	137.3	134.1
5′	146.8	147.9	153.1	153.0(152.7)	153.0	146.8	153.0	146.7
6′	106.1	107.4	106.5	106.1(106.5)	106.5	106.1	106.5	134.1
7′	47.8	47.2	48.0	47.8	47.9	47.8	47.2	47.0
8′	50.0	50.3	49.9	49.8	50.0	50.3	50.1	50.1
9′	171.4	172.0	171.3	171.3	171.5	171.5	171.5	171.5
CH_3O-3′,5′	56.3	56.2	56.1	56.3(56.0)	56.1	56.3	56.1	56.2
CH_3O-4′	52.0	–	60.8	–	60.8	–	60.8	–
$COOCH_3$	–	51.6	53.2	53.1	52.0	52.0	52.0	51.9
O–C–O	101.6	–	104.3	104.3	– [b]	118.2	–	–
$COOCH_3$	–	–	52.1	52.0	–	–	–	–
	–	–	165.1	165.1	–	–	–	–
CH_3	–	–	–	–	25.9	25.8	–	–
$–CH_2–CH_2–$	–	–	–	–	–	–	64.2, 64.5	64.1, 64.5

[a] NMR in acetone-d_6.
[b] Signal not observed.

*4.1.8.4. Compounds **16** and **17**.* From a 9:1 mixture of **9** and **10** (70 mg), methyl dichloroacetate (0.05 mL, 0.48 mmol), anhyd. K_2CO_3 (140 mg) in DMF (2 mL). The mixture was stirred at 90–100 °C under nitrogen for 1.5 h. After cooling, water (2 mL) was added and it was stirred at 90–100 °C for 30 min. A reaction product was obtained after the same work up described above for **11a**. Column chromatography of the reaction product with Cl_2CH_2/EtOAc 6:4 yielded 11 mg of **16** (12%) and 50 mg (60%) of **17**.

16: UV $\lambda_{max}(\varepsilon)$: 224 (27800), 274 (16100), 311 (10500). IR (cm^{-1}): 1760, 1735, 1590, 1510, 1210, 1127, 1010. ^{1}H-NMR (Table 4). ^{13}C-NMR (Table 6). HRMS (FAB+) Calc. for $C_{25}H_{26}O_{10}N$ $[M+H]^+$ 500.1557. Found 500.1532. Anal. $C_{25}H_{25}O_{10}N$ (C, H, N).

17: EM (*m*/*z*): 486 (M+1). IR (cm^{-1}): 3445, 1760, 1730, 1610, 1510, 1214, 1116, 1010. ^{1}H-NMR (Table 4). ^{13}C-NMR (Table 6).

*4.1.8.5. Compounds **18** and **19**.* A 9:1 mixture of **9**+**10** (100 mg), dichloromethyl methyl ether (0.33 mL, 3.7 mmol), K_2CO_3 (83 mg) and sodium iodide (2 mg) in acetone (5 mL) was refluxed for 48 h. After cooling, water was added and extracted with ethyl acetate. The organic layer was washed with brine until pH 7, dried and evaporated off. The reaction product was chromatographed (Cl_2CH_2/EtOAc 93:7) to yield 10 mg (9%) of **18** and 45 mg of **19** (39%).

18: ^{1}H-NMR (Table 4). ^{13}C-NMR (Table 6).

19: M.p. 78–83 °C. UV $\lambda_{max}(\varepsilon)$: 222 (15900), 276 (11100), 315 (7500). IR (cm^{-1}): 3447, 1740, 1610, 1510, 1240, 1110, 1010. ^{1}H-NMR (Table 4). ^{13}C-NMR (Table 6). HRMS (FAB+) Calc. for $C_{24}H_{26}O_8N$ $[M+H]^+$ 456.1658. Found 456.1649. Anal. $C_{24}H_{25}O_8N$ (C, H, N).

*4.1.9. Formation of the new dioxane ring: compounds **14**, **15**, **20**, **21***

*4.1.9.1. Compounds **14** and **15**.* A mixture of **3** (205 mg, 0.53 mmol), 1,2-dibromoethane (0.1 mL, 1.16 mmol), K_2CO_3 (430 mg) and sodium iodide (4 mg) in acetone (5 mL) was refluxed for 40 min. After cooling, water was added and extracted with ethyl acetate. The organic layer was washed with brine until pH 7, dried and evaporated off. Flash chromatography (Cl_2CH_2/EtOAc 95:5) of the residue afforded: 43 mg (20%) of **14** and 48 mg (22%) of a mixture of **14** and **15**.

14: UV $\lambda_{max}(\varepsilon)$: 213 (98800). IR ($cm^{-1}$): 1775, 1590, 1505, 1247, 1126, 1020. ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5). HRMS (FAB+) Calc. for $C_{23}H_{25}O_7$ $[M+H]^+$ 413.1600. Found 413.1578. Anal. $C_{23}H_{24}O_7$ (C, H).

15: ^{1}H-NMR (Table 3). ^{13}C-NMR (Table 5).

*4.1.9.2. Compounds **20** and **21**.* The mixture of **9**+**10** (100 mg) and 1,2-dibromethane (0.05 mL, 0.58 mmol) in acetone (5 mL) in the presence of NaI (2 mg) and K_2CO_3 (81 mg) was refluxed for 24 h. After cooling, water was added and extracted with ethyl acetate. The organic layer was washed with brine until pH 7, dried and evaporated off. Column chromatography (Cl_2CH_2/EtOAc 9:1) of the reaction product afforded 5 mg (5%) of **20** and 43 mg (41%) of **21**.

20: ^{1}H-NMR (Table 4). ^{13}C-NMR (Table 6).

21: M.p. 135–140 °C. $[\alpha]_D^{22}$ −85.7° (*c*, 0.79%). UV $\lambda_{max}(\varepsilon)$: 222 (15600), 275 (10100), 312 (4500). IR (cm^{-1}): 3500, 1734, 1613, 1519, 1250, 1115, 1067. ^{1}H-NMR (Table 4). ^{13}C-NMR (Table 6). HRMS (FAB+) Calc. for $C_{23}H_{24}NO_8$ $[M+H]^+$ 442.1502. Found 442.1532. Anal. $C_{23}H_{23}O_8N$ (C, H, N).

4.2. Antineoplastic assays

A screening procedure [14] was used to assess the cytotoxic activity against the following cell lines: P-388 (lymphoid neoplasmas 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), 2×10^4 (A-549, HT-29 and MEL-28) cells/well, respectively, in 1 mL aliquots of MEM10FCS 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, under a 10% CO_2, 98% humid atmosphere, P-388 cells were observed through an inverted microscopy and the degree of inhibition was determined by comparison with the controls, whereas A-549, HT-29 and MEL-28 were stained with crystal violet before examination.

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References

[1] D.C. Ayres, J.D. Loike, Lignans. Chemical, Biological and Clinical Properties, Chs. 3 and 4, Cambridge University Press, Cambridge, 1990.
[2] (a) Y. Damayanthi, J.W. Lown, Curr. Med. Chem. 5 (1998) 205–252;
(b) M. Gordaliza, M.A. Castro, J.M. Miguel del Corral, A. San Feliciano, Curr. Pharmaceut. Des. 6 (2000) 1811–1839.
[3] S.J. Cho, A. Tropsha, M. Suffness, Y.C. Cheng, K.H. Lee, J. Med. Chem. 39 (1996) 1383–1395.
[4] (a) M. Gordaliza, J.M. Miguel del Corral, M.A. Castro, M.L. López-Vázquez, P.A. García, A. San Feliciano, M.D. García-Grávalos, Bioorg. Med. Chem. Lett. 5 (1995) 2465–2468;
(b) M. Gordaliza, M.A. Castro, J.M. Miguel del Corral, M.L. López-Vázquez, P.A. García, A. San Feliciano, M.D. García-Grávalos, H.B. Broughton, Tetrahedron 53 (1997) 15743–15760.
[5] (a) C.F. Brewer, J.D. Loike, S.B. Horwitz, H. Sternlicht, W.J. Gensler, J. Med. Chem. 22 (1979) 215–221;
(b) A.R. Beard, M.G.B. Drew, P. Hilgard, B.D. Hudson, J. Mann, S. Niedle, L.F.T. Wong, Anti-Cancer Drug Des. 2 (1987) 247–252.
[6] (a) S.J. Cho, Y. Kashiwada, K.F. Bastow, Y.C. Cheng, K.H. Lee, J. Med. Chem. 39 (1996) 1396–1402;
(b) E. Bertounesque, T. Imbert, C. Monneret, Tetrahedron 52 (1996) 14235–14246.
[7] H. Stahelin, A. von Wartburg, in: E. Jucker (Ed.), Prog. Drug Res. 33 (1989) 206.
[8] (a) Z.Q. Wang, H. Hu, H.X. Chen, Y.C. Cheng, K.H. Lee, J. Med. Chem. 35 (1992) 871–877;
(b) J.F. Kadow, M.M. Tun, A.R. Crosswell, W.C. Rose, D.M. Vyas, T.W. Doyle, Bioorg. Med. Chem. Let. 2 (1992) 17–22.
[9] M. Gordaliza, M.A. Castro, J.M. Miguel del Corral, M.L. López-Vázquez, A. San Feliciano, G.T. Faircloth, Bioorg. Med. Chem. Let. 7 (1997) 2781–2786.
[10] M. Gordaliza, G.T. Faircloth, M.A. Castro, J.M. Miguel del Corral, M.L. López-Vázquez, A. San Feliciano, J. Med. Chem. 39 (1996) 2865–2868.
[11] J.M. Miguel del Corral, M. Gordaliza, M.A. Castro, M.L. López-Vázquez, M.D. García-Grávalos, H.B. Broughton, A. San Feliciano, Tetrahedron 53 (1997) 6555–6564.
[12] E. Schreier, Helv. Chem. Acta 47 (1964) 1529–1555.
[13] S. Teitel, J. O'Brien, A. Brossi, J. Org. Chem. 37 (1972) 3368–3369.
[14] (a) G.T. Faircloth, D. Stewart, J.J. Clement, J. Tissue Culture Methods 11 (1988) 201–205;
(b) R.J. Bergeron, P.F. Cavaragh, Jr, S.J. Kline, R.G. Hughes, G.T. Elliot, C.W. Porter, Biochem. Biophys. Res. Commun. 121 (1984) 848–854.
[15] T. Terada, K. Fujimoto, M. Nomura, J.-I. Yamashita, T. Kobunai, S. Takeda, K. Wierzba, Y. Yamada, H. Yamaguchi, Chem. Pharm. Bull. 40 (1992) 2720–2727.
[16] J.M. Miguel del Corral, M. Gordaliza, J.L. López, E. Olmo, M.A. Castro, M.L. López, Helv. Chim. Acta 78 (1995) 1793–1796.