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Synthesis and cytotoxic evaluation of C-9 oxidized podophyllotoxin derivatives

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Abstract—A series of podophyllotoxin and podophyllic aldehyde derivatives, lacking the lactone ring and oxidized at C-9 position, has been prepared. The functionalities considered at C-9 were carboxylic acids and several derivatives such as esters, amides, nitriles or anhydrides. The synthesized compounds were cytotoxic at the micromolar level, though less potent and selective than the parent compounds, revealing the influence of the C-9 electrophilic character on the potency and selectivity of these cyclolignans.

1. Introduction

Podophyllotoxin **1** and its semisynthetic derivatives, etoposide, teniposide and etopophos, are antiviral and cytotoxic compounds in clinical use that belong to the cyclolignan family of natural products (Fig. 1).[1] Podophyllotoxin derivatives are widely used as anticancer agents, but they still have several secondary effects. Because of that, the aryltetralin lignans are the subject of extensive research and nearly every ring of the cyclolignan skeleton (A–E) has been modified[2,3] and in those cases in which the lactone ring remained, it used to be like in podophyllotoxin, that is a 9′,9-lactone.

Furthermore, there have been described several natural cyclolignans,[4] such as justicidin E, retrojusticidin B or formosolactone[5] among others (Fig. 1), that possess a 9,9′-lactone instead of a 9′,9-lactone present in podophyllotoxin-like lignans. In general, the retrolactones have been less studied, with only a few examples as that of the synthesis and antiproliferative effects of retroetoposide.[6] In those cases, the compounds were less cytotoxic although the retrolactone seemed important for the anti-HIV activity described for those derivatives.[7] Also, there are natural cyclolignans that present a free carboxylic group at C-9[4] for which, as far as the authors knows, no biological activities are described.

Over the years, our group has been involved in the chemical transformation of podophyllotoxin and has prepared a large number of cyclolignans by modifications of nearly all the rings of the skeleton looking for more potent, less toxic and more selective analogues.[8,9] In this sense, we prepared a potent cytotoxic and selective cyclolignan,[10,11] the podophyllic aldehyde **2**, that lacked the γ-lactone ring, generally considered an important feature for the bioactivity of podophyllotoxin analogues.[1–3] Thus, the podophyllic aldehyde became our lead compound for further modifications from which the imine derivatives are worth to point out because they not only maintained the level of cytotoxicity of the parent aldehyde, but have also considerably improved the selectivity against the HT-29 colon carcinoma.[12]

Those facts prompted us to prepare new analogues of podophyllic aldehyde with higher oxidation degree at C-9, in order to analyse how the electrophilic character at that position could influence the antineoplastic selectivity previously observed for the aldehyde derivatives. Thus, we now report the synthesis of new cyclolignans with several carboxyl-related derivatives (esters, amides, nitriles, etc.) at C-9 position and their cytotoxicity.

Keywords: Podophyllotoxin; Podophyllic aldehyde; C-9 oxidized cyclolignans; Cytotoxicity.
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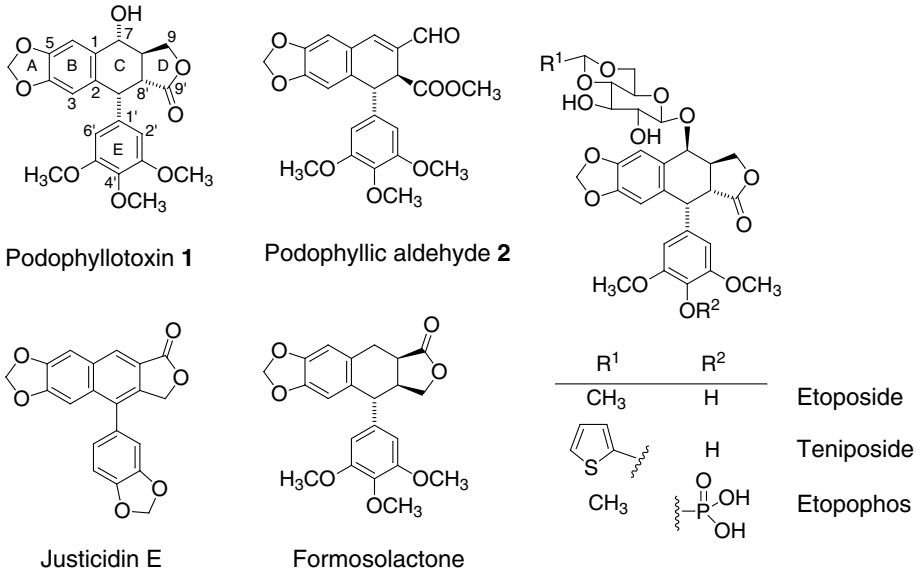


Figure 1. Structures of podophyllotoxin and related compounds.

2. Results and discussion

2.1. Chemistry

Podophyllic aldehyde **2** was the starting material for the preparation of C-9 carboxylic acid derivatives. It was obtained from podophyllotoxin **1** as previously described.[10–12]

The aldehyde group was oxidized[13] to the carboxylic acid **3** with $NaClO_2$ using 2-methyl-2-butene as scavenger, then transformed into its methyl ester **4** by treatment with an ethereal solution of diazomethane. The ^1H NMR data of diester **4** were in agreement with those published by Jones[14] and now, its ^{13}C NMR data are reported. The acid **3** was also transformed into the mixed anhydride **5** by reaction with acetic anhydride (Scheme 1).

In previous works[10,15] we proposed that the podophyllic aldehyde **2** could be cytotoxic by transformation into its lactol-lactone analogue, giving rise to a more rigid molecule, similar to the *trans*-lactones, with the four rings almost coplanar and thus more reactive and more susceptible to nucleophilic attack by several biomolecules. Under these premises, we tried to prepare several derivatives with a lactol function at C-9.

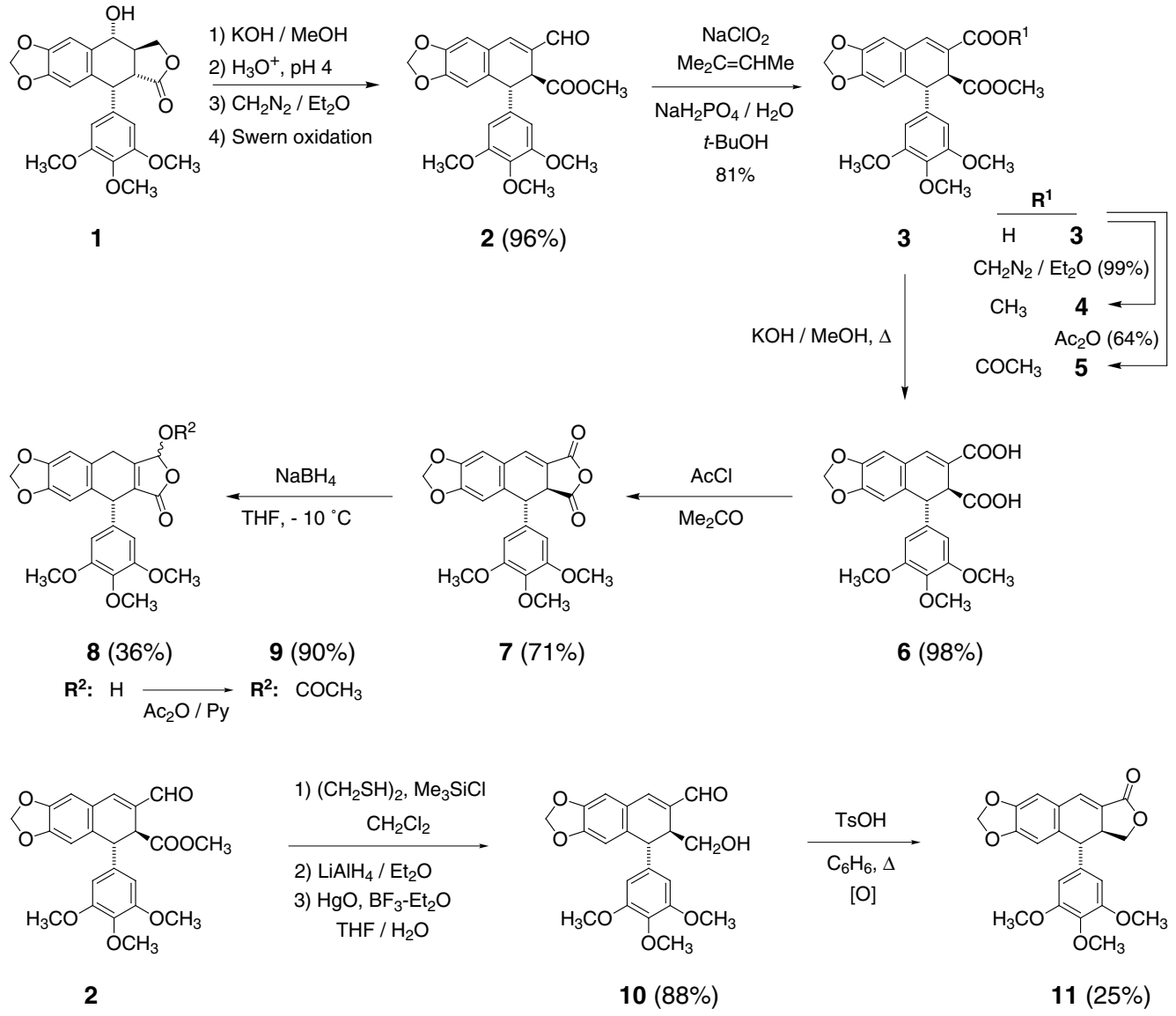
Attempts to transform directly the aldehyde **2** into the lactol by treatment with TsOH under different reaction conditions were unsuccessful, recovering either the starting material or products derived from the C-ring aromatisation. Consequently we decided to modify the functionality at C-9′ and the acid **3** was saponified to the diacid **6** and further treatment with acetyl chloride afforded the anhydride **7** which was reduced with $NaBH_4$ to yield the lactol-lactones **8**, in which a migration of the double bond from C-7 to C-8(8′) took place. The presence of the lactol at C-9 was confirmed by two-dimensional NMR experiments and by preparation of their acetyl derivatives **9**.

On the other hand, the reduction of the methyl ester **2**, after transient protection of the aldehyde group as its dithiolane, yielded the hydroxy-aldehyde **10**[11] which was treated with TsOH to give a very complex reaction mixture, from which only the 9,9′-lactone **11** was isolated after column chromatography, probably due to a further oxidation, under atmospheric oxygen, of the desired lactol (Scheme 1).

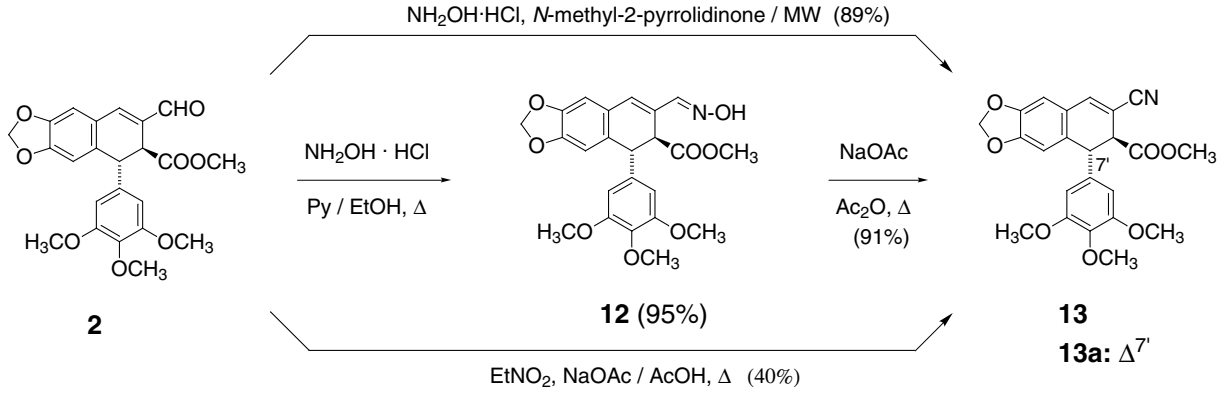
Other functions considered for being introduced at C-9 were nitriles and amides. The nitrile **13** was obtained from the aldehyde **2**, in good yield through its intermediate oxime **12** (Scheme 2), either by isolation of **12**[11a] followed by its dehydratation with acetic anhydride/sodium acetate,[16] or directly in a one-pot reaction under microwave irradiation[17] which afforded the nitrile **13** with a considerable reduction on the reaction time. Another procedure described in the literature[18,19] to obtain nitriles from aldehydes with good yields is the treatment with nitroethane and sodium acetate, however, when it was applied to **2**, the nitrile **13** was obtained in lower yields and accompanied by the corresponding aromatised nitrile **13a** in a 55:45 ratio.

Amides **14**–**20** were obtained from the acid **3** using CDI[20] or DCC/HOBT[21] as activating agents (Scheme 3) and their structures confirmed by two-dimensional NMR experiments. Thus, reaction of **3** with ethyl-, phenyl-, 4-methylphenyl- and 3,4,5-trimethoxyphenyl-amines in THF and CDI yielded the amides **14**, **16**–**18** with low yields (9–30%). When ethanolamine was used, the only isolated product was the phthalimide **19**, in which again the aromatisation of the C ring took place, together with the aminolysis of the C-9′ ester group.

Treatment of **3** with hexylamine under reflux, without any activating agent, afforded the diamide **20**; however

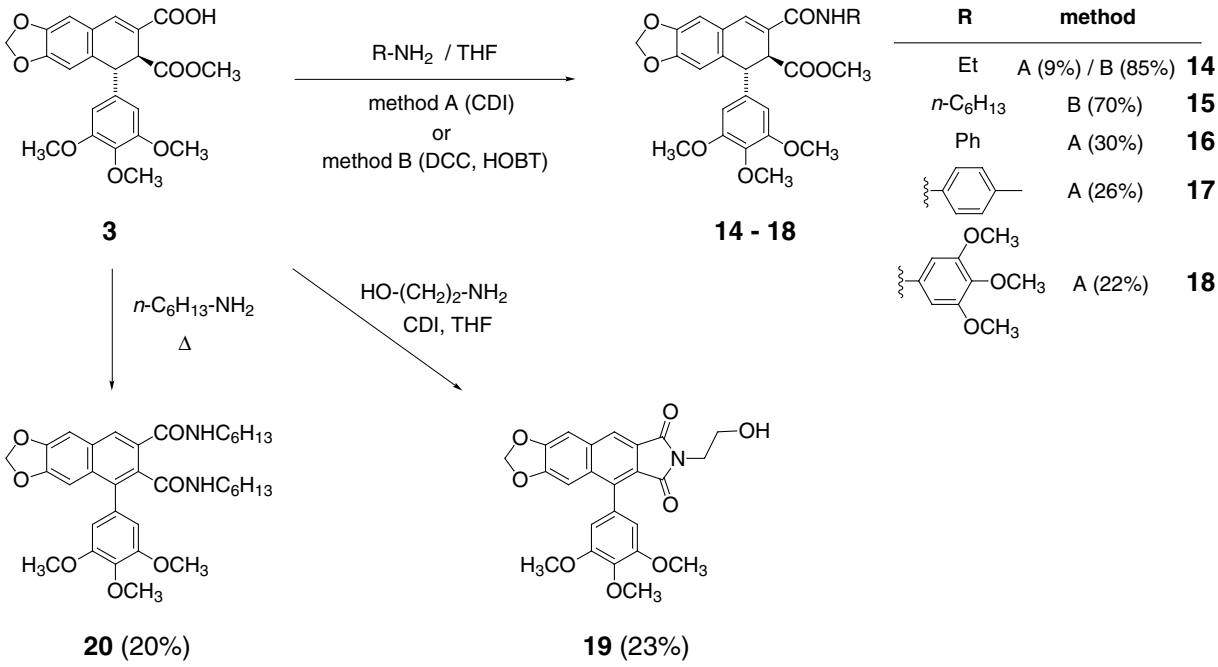


Scheme 1. Synthesis of cyclolignans **3**–**11**.



Scheme 2. Preparation of nitriles **13** and **13a**.

when the reaction was performed in the presence of DCC and HOBT, the corresponding amide **15** was obtained in good yield (70%). When the latter conditions were applied to prepare the ethylamide **14**, an important increase in the yield (from 9% with CDI to 87% with DCC) was also observed.



Scheme 3. Preparation of the amides **14**–**20**.

2.2. Cytotoxicity

Most of the compounds prepared were evaluated in vitro to establish their antineoplastic cytotoxicity[22,23] against cell cultures of P-388 murine leukaemia, A-549 human lung carcinoma, HT-29 human colon carcinoma and MEL-28 human melanoma. The results obtained are shown in Table 1, expressed in μM.

The first general observation that can be made is that no differences are observed among the IC_{50} values for the four cell lines, which means that the selectivity observed for **2** has been lost. On the other hand, although all the derivatives tested were cytotoxic, a decrease in the potency of one or two orders of magnitude was observed with respect to the parent compounds podophyllotoxin **1** and podophyllic aldehyde **2**.

Table 1. Cytotoxic activity of cyclolignan derivatives (IC_{50}, μM)

Compound	P-388	A-549	HT-29	MEL-28
1	0.012	0.012	0.012	n.d.
2	0.23	0.12	0.012	0.23
3	>20	>20	>20	>20
4	5.1	5.1	5.1	5.1
5	10	10	10	10
6	>20	>20	>20	>20
7	12	12	12	12
8	1.2	1.2	1.2	n.d.
10	0.25	0.25	0.25	0.25
11	>20	>20	>20	>20
12	2.3	2.3	2.3	2.3
13	n.d.	2.4	2.4	2.4
15	1.9	1.9	1.9	n.d.
16	1.9	1.9	1.9	n.d.
17	1.9	1.9	1.9	1.9
18	1.7	1.7	1.7	n.d.
19	2.2	2.2	2.2	n.d.
20	2.0	2.0	2.0	n.d.

n.d., not determined.

Among the carboxylic acid derivatives, there were no significant differences in the cytotoxicity measured for amides, nitriles or anhydrides. The less potent compounds of the series are those having a free carboxylic group at C-9 position (**3** and **6**) and the reverse lactone **11** resembling justicidin E. Those results indicate that the electrophilic character at that position clearly influences the cytotoxic potency and the selectivity of these cyclolignans.

The lactol-lactone **8** displayed the best cytotoxicity of the series but was less potent than we expected based on the postulation made before, although we have to take in account that **8** is not exactly the lactol expected from **2**, because the double bond in C ring is located at a different position, making the molecule less rigid. This may be compared to the *cis*-lactones, all of them being less potent than the *trans*-lactone analogues.[1–3,8,9]

In summary, we have prepared a series of podophyllotoxin and podophyllic aldehyde derivatives, lacking the lactone ring and oxidized at C-9 position to carboxylic acid derivatives such as esters, amides, nitriles or anhydrides. The compounds synthesized were cytotoxic at the micromolar range, although less potent and selective than the parent compounds.

3. Experimental

3.1. Chemistry

NMR spectra were recorded on a Bruker AC 200 at 200 MHz for ^{1}H and 50.3 MHz for ^{13}C in deuterio-

chloroform with TMS as an internal standard. 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 Nicolet Impact 410 spectrophotometer. HRMS were run in a VG-TS-250 spectrometer working at EI (70 eV) or FAB and in a hybrid quadrupole time of flight spectrometer QSTAR XL (Applied Biosystems) by ESI in positive mode (the ionization voltage was 5.0 kV). Column chromatography (CC) was performed on silica gel (Merck No. 9385). TLC was carried out on silica gel $60F_{254}$ (SDS 0.20 mm thick). Solvents and reagents were purified by standard procedures as necessary.

3.1.1. Podophyllotoxin (1). It was isolated from *Podophyllum emodi* in the same way as we previously published.[24]

3.1.2. 9′-Methyl (7′*R*,8′*S*)-3′,4′,5′-trimethoxy-4,5-methylenedioxy-9-oxo-2,7′-cyclolign-7-en-9′-oate (2). Podophyllotoxin **1** was transformed into podophyllic aldehyde **2** after saponification, esterification and Swern oxidation by a previously described method (96% overall yield).[11]

3.1.3. (7′*R*,8′*S*)-3′,4′,5′,9′-Tetramethoxy-4,5-methylenedioxy-9′-oxo-2,7′-cyclolign-7-en-9-oic acid (3). To a solution of **2** (500 mg, 1.17 mmol) and 2-methyl-2-butene (1.5 mL, 12 mmol) in *t*-butanol (25 mL), a solution of sodium chlorite (0.44 mL, 1.5 mmol) in aq 5% NaH_2PO_4 (10 mL) was slowly added. After the mixture was stirred at room temperature for 4 d, it was concentrated under reduced pressure, dissolved in EtOAc and extracted with aq satd Na_2CO_3. The aqueous phase was acidified with 2 N HCl and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure to give **3** (420 mg, 81%). $[\alpha]_D^{20}$ −129 (*c* 0.96). UV λ_{max}(lg ε): 213 (4.4), 241 (4.3), 336 (4.0) nm. IR (KBr) ν_{max}: 3600–2500 (COOH), 1732 ($COOCH_3$), 1683 (C=C–COOH) cm^{-1}. ESI-HRMS: calcd for $C_{23}H_{22}O_9$ + Na, 465.1156; found, 465.1145. ^{1}H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.4. Dimethyl (7′*R*,8′*S*)-3′,4′,5′-trimethoxy-4,5-methylenedioxy-2,7′-cyclolign-7-ene-9,9′-dioate (4). The compound **3** (30 mg, 0.068 mmol) was treated with an ethereal solution of diazomethane for 1 h. After removing the solvent, the dimethyl diester **4** (31 mg, 100%) was obtained. $[\alpha]_D^{20}$ −115 (*c* 0.88). UV λ_{max} (lg ε): 210 (3.4), 243 (4.3), 312 (3.7), 342 (3.9) nm. IR (KBr) ν_{max}: 1734 ($COOCH_3$), 1708 (C=C–$COOCH_3$) cm^{-1}. ESI-HRMS: calcd for $C_{24}H_{24}O_9$ + Na, 479.1312; found, 479.1301. ^{1}H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.5. Acetic (7′*R*,8′*S*)-3′,4′,5′,9′-tetramethoxy-4,5-methylenedioxy-9′-oxo-2,7′-cyclolign-7-en-9-oic anhydride (5). The acid **3** (50 mg, 0.11 mmol) was dissolved in acetic anhydride (5.0 mL) and stirred at room temperature for 1 d. Then, ice was added and after it melted, the mixture was diluted with EtOAc. The organic layer was washed with aq satd $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and the solvent evaporated off to give **5** (35 mg, 64%). $[\alpha]_D^{20}$ −110 (*c* 1.14). UV λ_{max} (lg ε): 211 (4.5), 242 (4.0), 336 (4.0) nm. IR (film) ν_{max}: 1732 ($COOCH_3$, C=C–COOCO), 1677 (C=C–COOCO) cm^{-1}. ESI-HRMS: calcd for $C_{25}H_{24}O_{10}$ + Na, 507.1261; found, 507.1250. ^{1}H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.6. (7′*R*,8′*S*)-3′,4′,5′-Trimethoxy-4,5-methylenedioxy-2,7′-cyclolign-7-ene-9,9′-dioic acid (6). Acid **3** (105 mg, 0.238 mmol) was dissolved in a methanolic solution of 5% KOH (5.0 mL), stirred and heated under reflux for 6 h. After cooling to room temperature, concentration under reduced pressure and dilution with EtOAc, it was extracted with aq satd Na_2CO_3. The aqueous phase was acidified to pH 2 with 2 N HCl and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure to yield the diacid **6** (100 mg, 98%). $[\alpha]_D^{20}$ −145 (*c* 0.11). UV λ_{max} (lg ε): 212 (4.5), 241 (4.4), 336 (4.1) nm. IR (KBr) ν_{max}: 3600–2500 (COOH), 1703 (COOH) cm^{-1}. ESI-HRMS: calcd for $C_{22}H_{20}O_9$ + Na, 451.0999; found, 451.1004. ^{1}H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.7. (7′*R*,8′*S*)-3′,4′,5′-Trimethoxy-4,5-methylenedioxy-2,7′-cyclolign-7-ene-9,9′-dioic anhydride (7). Compound **5** (124 mg, 0.290 mmol) was treated with acetyl chloride (0.50 mL, 7.0 mmol) in dry acetone (2.0 mL) and stirred at room temperature for 4 d. After concentration of the reaction mixture under reduced pressure, methanol was added and a yellow precipitate was formed and separated by filtration to yield the anhydride **7** (85 mg, 71%). $[\alpha]_D^{20}$ −230 (*c* 0.65). UV λ_{max} (lg ε): 214 (4.4), 246 (4.3), 283 (4.2), 328 (3.9) nm. IR (KBr) ν_{max}: 1848 (COOCO), 1770 (COOCO), 1668 (C=C–COOCO) cm^{-1}. EI-HRMS: calcd for $C_{22}H_{18}O_8$, 410.0992; found, 410.0992. ^{1}H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.8. (7′*R*)-9-Hydroxy-3′,4′,5′-trimethoxy-4,5-methylenedioxy-2,7′-cyclolign-8(8′)-eno-9′,9-lactone (8). To a solution of **7** (70 mg, 0.17 mmol) in dry THF (12 mL), sodium borohydride (1.7 mg, 0.043 mmol) was added and stirred at −10 °C for 6 h. The reaction was quenched with aq satd NH_4Cl and extracted with CH_2Cl_2. The organic phase was washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The reaction product was purified by CC on silica gel (CH_2Cl_2–EtOAc, 8:2) to yield the hydroxylactones **8** (25 mg, 36%). IR (film) ν_{max}: 3392 (OH), 1762 and 1714 (γ-hydroxy-α,β-unsaturated-γ-lactone) cm^{-1}. ^{1}H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.9. (7′*R*)-9-Acetoxy-3′,4′,5′-trimethoxy-4,5-methylenedioxy-2,7′-cyclolign-8(8′)-eno-9′,9-lactone (9). The hydroxylactones **8** (15 mg, 0.037 mmol) were treated with acetic anhydride (1.0 mL) in pyridine (1.0 mL) and stirred at room temperature for 1 h. Then, ice was added and after it melted, the solution was diluted with EtOAc. The organic layer was washed with 2 N HCl, aq satd $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give the

Table 2. ^{1}H NMR data of compounds **3**–**9** and **11**–**20** (δ ppm (J Hz))

H	3	4	5	6	7	8	9	11	12
3	6.65 s	6.65 s	6.69 s	6.66 s	6.31 s	6.61/6.59 s	6.62/6.61 s	6.30 s	6.63 s
6	6.84 s	6.83 s	6.86 s	6.84 s	6.87 s	6.73 s	6.72/6.70 s	6.86 s	6.73 s
7	7.72 s	7.62 s	7.69 s	7.72 s	7.53 d (2.6)	3.93 dd (23, 4.6)	3.65–3.85 m	7.40 d (3.2)	6.71 s
						3.62 dd (23, 4.6)/ 3.65–3.90 m			
9						6.09 br s	6.94/6.93 s		7.86 s
2′	6.24 s	6.24 s	6.22 s	6.20 s	6.40–6.60 m	6.34 s	6.37/6.35 s	6.48 s	6.25 s
6′	6.24 s	6.24 s	6.22 s	6.20 s	6.40–6.60 m	6.34 s	6.37/6.35 s	6.49 s	6.25 s
7′	4.63 d (2.6)	4.60 d (2.7)	4.66 d (2.6)	4.62 s	4.18 s	4.78/4.71 t (4.6)	4.70–4.90 m	3.78 d (2.2)	4.47 d (2.2)
8′	3.98 d (2.6)	4.01 d (2.7)	4.01 d (2.6)	3.96 s	4.13 d (2.6)			3.39–3.59 m	3.98 d (2.2)
9′								4.44 t (8.9)	
								3.98 t (8.9)	
–OCH_2O–	6.00 d (1.7)	5.98 d (1.1)	6.02 d (1.3)	5.99 s	6.01 d (1.1)	5.95 s	5.96 s	5.98 d (1.3)	5.94 s
	5.98 d (1.7)	5.97 d (1.1)	6.01 d (1.3)	5.98 s	5.99 d (1.1)	5.94 s	5.94 s	5.96 d (1.3)	5.93 s
CH_3O-3′	3.74 s	3.74 s	3.75 s	3.72 s	3.70–3.90 m	3.78 s	3.80 s	3.89 s	3.71 s
CH_3O-4′	3.78 s	3.79 s	3.80 s	3.77 s	3.91 s	3.77 s	3.80 s	3.90 s	3.76 s
CH_3O-5′	3.74 s	3.74 s	3.75 s	3.72 s	3.70–3.90 m	3.73 s	3.80 s	3.82 s	3.71 s
CH_3O-9′	3.65 s	3.65 s	3.67 s						3.62 s
OH	9.07 br s			9.71 br s		4.45 br s			8.24 br s
Others		3.77 s	2.31 s				2.19/2.20 s		

H	13	14	15	16	17	18	19	20
3	6.59 s	6.64 s	6.64 s	6.65 s	6.67 s	6.58 s	7.11 s	6.90 s
6	6.74 s	6.75 s	6.75 s	6.80 s	6.80 s	6.78 s	7.34 s	7.10 s
7	7.23 s	7.11 s	7.11 s	7.30 s	7.29 s	7.30 s	8.17 s	8.00 s
2′	6.24 s	6.30 s	6.31 s	6.31 s	6.32 s	6.30 s	6.55 s	6.60 s
6′	6.24 s	6.30 s	6.31 s	6.31 s	6.32 s	6.30 s	6.55 s	6.60 s
7′	4.60 d (4.2)	4.51 d (3.5)	4.51 d (3.4)	4.55 d (3.3)	4.56 d (3.3)	4.49 d (4.4)		
8′	3.61 d (4.2)	4.04 d (3.5)	4.03 d (3.4)	4.13 d (3.3)	4.11 d (3.3)	4.12 d (4.4)		
–OCH_2O–	5.99 d (1.3)	5.97 s	5.97 s	5.98 s	5.99 s	5.99 s	6.13 s	6.05 s
	5.98 d (1.3)		5.96 s			5.98 s		
CH_3O-3′	3.76 s	3.75 s	3.75 s	3.76 s	3.76 s	3.77 s	3.85 s	3.84 s
CH_3O-4′	3.81 s	3.79 s	3.79 s	3.79 s	3.79 s	3.80 s	3.97 s	3.92 s
CH_3O-5′	3.76 s	3.75 s	3.75 s	3.76 s	3.76 s	3.77 s	3.85 s	3.84 s
CH_3O-9′	3.73 s	3.63 s	3.63 s	3.66 s	3.66 s	3.66 s		
NH		6.05 br s	6.09 br s	8.15 br s	8.02 br s	8.21 br s		6.09 br s
Others		3.35 q (6.5)	3.32 dd (13.1, 5.7)	7.55 d (8.0)	7.45 d (8.4)	6.86 s (2H)	3.60–4.05 m	3.27–3.37 m (4 H)
		1.21 t (6.5)	1.05–1.98 m (8 H)	7.31 dd (8.0, 7.1)	7.13 d (8.4)	3.83 s (6 H)	(4 H)	1.00–1.80 m (16 H)
			0.87 t (6.6)	7.09 t (7.1)	2.31 s	3.79 s (3 H)		0.75–0.90 m (6 H)

acetoxylactones **9** (15 mg, 90%). ^{1}H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.10. (7′*R*,8′*S*)-9′-Hydroxy-3′,4′,5′-trimethoxy-4,5-methylenedioxy-2,7′-cyclolign-7-en-9-al (10). It was prepared as previously described from **2** by reduction with $LiAlH_4$ of its dithiolane protected derivative and final deprotection.[11]

3.1.11. (7′*R*,8′*S*)-3′,4′,5′-Trimethoxy-4,5-methylenedioxy-2,7′-cyclolign-7-eno-9,9′-lactone (11). A mixture of **10** (170 mg, 0.428 mmol) and TsOH (10 mg, 0.058 mmol) was stirred in benzene (15 mL) under reflux for 5 h. Then, solvent was removed under reduced pressure and the product was redissolved in EtOAc. The organic layer was washed with aq satd $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered and evaporated until dryness. CC on silica gel (CH_2Cl_2–EtOAc, 96:4) of the residue afforded **11** (43 mg, 25%). IR (film) ν_{max}: 1752 (C=C–COO) cm^{-1}. ESI-HRMS: calcd for $C_{22}H_{20}O_7$ + Na, 419.1101; found, 419.1131. ^{1}H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.12. Methyl (7′*R*,8′*S*)-9-hydroxyimino-3′,4′,5′-trimethoxy-4,5-methylenedioxy-2,7′-cyclolign-7-en-9′-oate (12). Over a solution of **2** (156 mg, 0.368 mmol) in dry EtOH (3.0 mL), dry pyridine (28 μL, 0.35 mmol) and hydroxylamine hydrochloride (28 mg, 0.40 mmol) were added. The mixture was stirred under reflux for 2 h and then allowed to cool to room temperature and the solvent removed under reduced pressure. Water was added and the product was extracted with EtOAc, washed with 2 N HCl and brine, dried over anhydrous Na_2SO_4 and filtered off. The evaporation of the solvent afforded the oxime **12** (153 mg, 95%).[11a]

3.1.13. Methyl (7′*R*,8′*S*)-8-cyano-3′,4′,5′-trimethoxy-4,5-methylenedioxy-9-nor-2,7′-cyclolign-7-en-9′-oate (13). *(a) From 12*. The oxime **12** (153 mg, 0.349 mmol) was refluxed in acetic anhydride (2.0 mL) with sodium acetate (2 mg, 0.02 mmol) for 21 h. Then, it was extracted with EtOAc and the organic phase was washed with brine and dried over anhydrous Na_2SO_4 to obtain, after evaporation of the solvent, the nitrile **13** (134 mg, 91%). IR (film) ν_{max}: 2212 (C=C–CN), 1735 ($COOCH_3$) cm^{-1}.

Table 3. ^{13}C NMR data of compounds **3**–**9** and **11**–**20** (δ ppm)

C	3	4	5	6	7	8	9	11	12	13	14	15	16	17	18	19	20
1	125.1	125.1	124.6	125.1	125.7	124.0	123.2	125.7	131.1	124.0	125.2	125.0	125.0	125.1	125.0	126.7	129.7
2	132.1	131.8	132.8	132.1	134.6[a]	129.2	129.0	134.3[a]	126.8	131.2	131.1	131.3	131.5	131.6	131.5	130.3[a]	130.4
3	109.8	109.9	109.9	110.0	103.2[b]	109.3	109.3	108.7	109.6	109.9	109.5	109.6	109.6	109.7	109.6	106.2	104.3
4	149.8	149.5	153.1	150.0	150.8	147.0	147.3	149.5	148.3	150.1	148.7	148.7	149.1	149.2	149.2	150.5	149.4
5	147.1	147.2	150.4	147.3	147.3	147.0	147.2	146.8	147.1	147.3	146.9	146.9	147.0	147.1	147.1	150.1	148.5
6	108.9	108.8	109.0	109.1	109.2[b]	108.0	107.9	109.2	107.8	108.2	108.1	108.2	108.4	108.4	108.3	105.1	103.1
7	139.3	137.3	140.6	139.8	135.7	28.3/27.9	28.2/27.8	132.3	133.2	142.2	130.9	131.0	132.3	132.2	132.3	123.0	128.1
8	122.0	122.9	121.6	121.4	120.5	156.8	154.5	127.0	126.4	103.6	127.7	127.7	127.7	127.9	127.8	123.6	130.1
9	171.8	167.0	162.0	172.0	162.5	97.6/97.4	92.9/92.5	169.5	151.4	119.0	167.1	167.1	165.2	165.2	165.4	168.0[b]	168.0
1′	137.6	137.8	137.2	137.8	135.0[a]	138.3	137.6	135.0[a]	138.2	136.6	137.7	137.7	137.5	137.6	137.4	139.0	136.4
2′	104.5	104.7	104.5	104.5	109.8	105.4[a]	105.6	108.0	105.3	104.8	104.9	104.8	104.8	104.8	104.9	107.0	107.6
3′	153.0	153.1	153.1	153.2	153.5	153.2	153.3	154.2	153.3	153.4	153.1	153.1	153.2	153.3	153.2	153.2	153.1
4′	136.8	136.9	137.2	136.9	137.6	137.3	137.2	137.6	137.6	137.3	137.0	137.0	138.0	137.1	137.1	138.0	137.6
5′	153.0	153.1	153.1	153.2	153.5	153.2	153.2	153.3	153.3	153.4	153.1	153.1	153.2	153.3	153.2	153.2	153.1
6′	104.5	104.7	104.5	104.5	109.8	105.6[a]	105.6	103.1	105.3	104.8	104.9	104.8	104.8	104.8	104.9	107.0	107.6
7′	46.5[a]	47.0[a]	46.2[a]	46.4[a]	46.9[c]	42.8/42.6	42.8	51.3	46.5	45.5	46.3	46.2	46.2	46.3	46.5	133.4[a]	133.1
8′	46.3[a]	46.5[a]	46.1[a]	46.1[a]	45.2[c]	130.6	131.6	40.8	46.5	48.8	47.1	47.1	47.2	47.3	47.7	133.4[a]	130.9
9′	172.5	172.7	171.9	177.7	168.8	169.9	169.3[a]	71.9	172.5	170.8	172.9	172.8	173.0	173.0	173.5	168.8[b]	170.1
–OCH_2O–	101.5	101.6	101.7	101.7	102.1	101.3	101.4	101.6	101.3	101.8	101.3	101.4	101.5	101.6	101.6	102.3	101.6
CH_3O-3′	55.9	56.1	56.0	56.1	56.2	56.2	56.2	56.2	56.3	56.1	56.1	56.1	56.1	56.2	56.1	56.3	56.2
CH_3O-4′	60.6	60.8	60.6	60.8	60.9	60.8	60.8	60.9	60.7	60.8	60.7	60.7	60.8	60.9	61.0	61.1	61.0
CH_3O-5′	55.9	56.1	56.0	56.1	56.2	56.2	56.2	56.2	56.3	56.1	56.1	56.1	56.1	56.2	56.1	56.3	56.2
CH_3O-9′	52.5	52.6	52.6						52.3	52.9	52.4	52.4	52.7	52.8	52.8		
Others		52.0	166.2				168.6[a]				34.7	39.9	129.0	135.5	134.2	41.1	40.3, 40.0
			22.2				20.8				14.7	31.4	119.9	120.0	97.6	61.5	31.5, 31.4
												29.3	129.0	129.5	153.2		29.4, 29.0
												26.5	124.2	134.0	134.6		26.7, 26.5
												22.5		21.0	56.1		22.6
												14.0			61.0		14.0

[a–c]Exchangeable assignments.

FAB-HRMS: calcd for $C_{23}H_{21}NO_7 + H$, 424.1396; found, 424.1379. ^1H NMR: Table 2. ^{13}C NMR: Table 3.

(b) From ***2*** *and* NH_2OH. A mixture of **2** (42 mg, 0.10 mmol), hydroxylamine hydrochloride (10 mg, 0.14 mmol) and *N*-methyl-2-pyrrolidinone (100 μL, 1.04 mmol) was irradiated at 750 W in a domestic microwave oven for 10 min (5 × 2 min). Then, it was diluted with water and extracted with EtOAc. The organic layer was washed with brine, dried, filtered and concentrated in vacuum to yield the nitrile **13** (37 mg, 89%).

(c) From ***2*** *and* $EtNO_2$. Aldehyde **2** (94 mg, 0.22 mmol), nitroethane (82 μL, 1.1 mmol) and sodium acetate (71 mg, 0.87 mmol) were refluxed in glacial acetic acid (2.0 mL) for 22 h. The mixture was allowed to cool to room temperature and then diluted with water, washed with aq satd $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered and the solvent evaporated off. Purification by silica gel CC (CH_2Cl_2–EtOAc, 95:5) afforded the mixture **13** + **13a** in a 55:45 ratio (37 mg, 40%).

3.1.14. General procedures for the synthesis of amides 14–18. *Method A*. To a solution of **3** (0.07–0.15 mmol) in dry THF (4.0 mL), *N*,*N*′-carbonyldiimidazol (CDI, 1.5 equiv) was added in small portions and the mixture stirred at room temperature for 1 h. The corresponding amine (1.5 equiv) was then added and the mixture stirred at room temperature for 24 h more. The solvent was evaporated under reduced pressure and the residue was redissolved in CH_2Cl_2. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to obtain a mixture that was purified by silica gel CC to obtain the amides in 9–30% yield.

Method B. To a solution of **3** (0.10–0.25 mmol) in dry THF (3.0 mL), *N*,*N*′-dicyclohexylcarbodiimide (DCC, 1.0 equiv), 1-hydroxybenzotriazole (HOBT, 1.0 equiv) and the corresponding amine (2.0 equiv) were added and the mixture stirred at room temperature for 24 h. The mixture was then filtered and the solvent evaporated. The residue was redissolved in CH_2Cl_2 and this organic phase was washed with aq satd $NaHCO_3$, brine, dried over anhydrous Na_2SO_4, filtered and evaporated. Purification by silica gel CC afforded the amides in 70-85% yield.

3.1.15. Methyl (7′*R*,8′*S*)-9-ethylamino-3′,4′,5′-trimethoxy-4,5-methylenedioxy-9-oxo-2,7′-cyclolign-7-en-9′-oate (14). Following the method A, the reaction between **3** (51 mg, 0.12 mmol) and ethylamine 2.0 M in THF (87 μL, 0.17 mmol) yielded, after CC purification (*n*-hexane–EtOAc, 6:4), the ethylamide **14** (5 mg, 9%).

Application of the method B to **3** (95 mg, 0.21 mmol) afforded **14** (87 mg, 85%). IR (film) ν_{max}: 3370 (NH), 1731 ($COOCH_3$), 1651 (CONH), 1532 (CONH) cm^{-1}. ESI-HRMS: calcd for $C_{25}H_{27}NO_8 + Na$, 492.1634; found, 492.1641. ^1H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.16. Methyl (7′*R*,8′*S*)-9-hexylamino-3′,4′,5′-trimethoxy-4,5-methylenedioxy-9-oxo-2,7′-cyclolign-7-en-9′-oate (15). Following method B, **3** (48 mg, 0.11 mmol) and *n*-hexylamine (29 μL, 0.22 mol) yielded **15** (40 mg, 70%) after CC (CH_2Cl_2–EtOAc, 85:15). IR (film) ν_{max}: 3319 (NH), 1731 ($COOCH_3$), 1622 (CONH), 1537 (CONH) cm^{-1}. FAB-HRMS: calcd for $C_{29}H_{35}NO_8$ + H, 526.2441; found, 526.2487. ^1H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.17. Methyl (7′*R*,8′*S*)-3′,4′,5′-trimethoxy-4,5-methylenedioxy-9-oxo-9-phenylamino-2,7′-cyclolign-7-en-9′-oate (16). From **3** (40 mg, 0.090 mmol) and aniline (13 μL, 0.14 mmol) using method A, phenylamide **16** was obtained (14 mg, 30%) after CC (*n*-hexane–EtOAc, 7:3). $[\alpha]_D^{20}$ −90 (*c* 0.20). IR (film) ν_{max}: 3353 (NH), 1731 ($COOCH_3$), 1662 (CONH), 1537 (CONH) cm^{-1}. FAB-HRMS: calcd for $C_{29}H_{27}NO_8$ + H, 518.1815; found, 518.1863. ^1H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.18. Methyl (7′*R*,8′*S*)-3′,4′,5′-trimethoxy-4,5-methylenedioxy-9-oxo-9-(*p*-tolylamino)-2,7′-cyclolign-7-en-9′-oate (17). Chromatographic purification of the reaction product (CH_2Cl_2–EtOAc, 92:8) between **3** (44 mg, 0.10 mmol) and *p*-toluidine (16 mg, 0.15 mmol) following method A gave **17** (14 mg, 26%). $[\alpha]_D^{20}$ −126 (*c* 0.26). IR (film) ν_{max}: 3343 (NH), 1731 ($COOCH_3$), 1659 (CONH), 1525 (CONH) cm^{-1}. FAB-HRMS: calcd for $C_{30}H_{29}NO_8$ + H, 532.1971; found, 532.1953. ^1H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.19. Methyl (7′*R*,8′*S*)-3′,4′,5′-trimethoxy-4,5-methylenedioxy-9-oxo-9-(3,4,5-trimethoxyphenylamino)-2,7′-cyclolign-7-en-9′-oate (18). Using method A, **3** (65 mg, 0.15 mmol) and 3,4,5-trimethoxyaniline (41 mg, 0.22 mmol) afforded **18** (20 mg, 22%) after CC (CH_2Cl_2–EtOAc, 8:2). IR (film) ν_{max}: 3341 (NH), 1732 ($COOCH_3$), 1659 (CONH), 1539 (CONH) cm^{-1}. FAB-HRMS: calcd for $C_{32}H_{33}NO_{11}$ + H, 608.2131; found, 608.2192. ^1H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.20. *N*-(2-Hydroxyethyl)-3′,4′,5′-trimethoxy-4,5-methylenedioxy-2,7′-cycloligna-7,7′-dien-9,9′-imide (19). To a solution of **3** (56 mg, 0.13 mmol) in dry THF (4.0 mL), CDI (29 mg, 0.18 mmol) was added in small portions and the mixture stirred at room temperature for 1 h. Then ethanolamine (12 μL, 0.20 mmol) was added maintaining the mixture in the same conditions for 25 h more. The solvent was evaporated under reduced pressure. Purification of the crude by silica gel CC (CH_2Cl_2–MeOH, 98:2) provided **19** (13 mg, 23%). IR (film) ν_{max}: 3444 (OH), 1760 (CO–NR–CO), 1705 (CO–NR–CO) cm^{-1}. FAB-HRMS: calcd for $C_{24}H_{21}NO_8$ + H, 452.1345; found, 452.1393. ^1H NMR: Table 2. ^{13}C NMR: Table 3.

3.1.21. *N*,*N*′-Dihexyl-3′,4′,5′-trimethoxy-4,5-methylenedioxy-2,7′-cycloligna-7,7′-diene-9,9′-diamide (20). A mixture of **3** (33 mg, 0.075 mmol) and *n*-hexylamine (1.0 mL) was refluxed for 24 h. After it was allowed to cool to room temperature, the reaction mixture was filtered through a silica gel CC (CH_2Cl_2–MeOH, 95:5) to afford **20** (9 mg, 20%). IR (film) ν_{max}: 3275 (NH), 1644 (CONH), 1552 (CONH) cm^{-1}.ESI-HRMS: calcd for $C_{34}H_{44}N_2O_7$ + Na, 615.3046; found, 615.3047. ^1H NMR: Table 2. ^{13}C NMR: Table 3.

3.2. Cytotoxic assays

A screening method previously described was used to assess the antitumoral activity against the following cell lines: P-388 (lymphoid neoplasma 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 MEM-10% FCS medium containing the compound to be evaluated at different concentrations. In each case, a set of control wells was incubated in the absence of drug and counted daily to ensure exponential cell growth. After 4 days at 37 °C, under a 10% CO_2, 98% humid atmosphere, P-388 cells were observed by inverted microscopy and the degree of inhibition was determined by comparison with the controls, while A-549, HT-29 and MEL-28 were stained with crystal violet before examination. All calculations represent the average of duplicate wells.

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References and notes

1. Ayres, D. C.; Loike, J. D. *Lignans: Chemical, Biological and Clinical Properties*; Cambridge University Press: London, 1990.
2. Damayanthi, Y.; Lown, J. W. *Curr. Med. Chem.* **1998**, *5*, 205–252.
3. Gordaliza, M.; Castro, M. A.; Miguel del Corral, J. M.; San Feliciano, A. *Curr. Pharm. Des.* **2000**, *6*, 1811–1839.
4. Ward, R. S. *Nat. Prod. Rep.* **1999**, *16*, 75–96.
5. Kuo, Y. H.; Yu, M. T. *Heterocycles* **1993**, *36*, 529–535.
6. Meresse, P.; Magiatis, P.; Bertounesque, E.; Monneret, C. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4107–4109.
7. Sagar, K. S.; Chang, C. C.; Wang, W. K.; Lin, J. Y.; Lee, S. S. *Bioorg. Med. Chem.* **2004**, *12*, 4045–4054.
8. Castro, M. A.; Miguel del Corral, J. M.; Gordaliza, M.; Gómez-Zurita, M. A.; García, P. A.; San Feliciano, A. *Phytochem. Rev.* **2003**, *2*, 219–233.
9. Gordaliza, M.; García, P. A.; Miguel del Corral, J. M.; Castro, M. A.; Gómez-Zurita, M. A. *Toxicon* **2004**, *44*, 441–459.
10. Gordaliza, M.; Miguel del Corral, J. M.; Castro, M. A.; López-Vázquez, M. L.; García, P. A.; San Feliciano, A.; García-Grávalos, M. D. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2465–2468.
11. (a) Gordaliza, M.; Castro, M. A.; Miguel del Corral, J. M.; López-Vázquez, M. L.; García, P. A.; San Feliciano, A.; García-Grávalos, M. D.; Broughton, H. B. *Tetrahe-*

dron **1997**, *53*, 15743–15760; (b) Gordaliza, M.; Castro, M. A.; Miguel del Corral, J. M.; López-Vázquez, M. L.; García, P. A.; García-Grávalos, M. D.; San Feliciano, A. *Eur. J. Med. Chem.* **2000**, *35*, 691–698.
12. Castro, M. A.; Miguel del Corral, J. M.; Gordaliza, M.; García, P. A.; Gómez-Zurita, M. A.; García-Grávalos, M. D.; Iglesia-Vicente, J.; Gajate, C.; An, F.; Mollinedo, F.; San Feliciano, A. *J. Med. Chem.* **2004**, *47*, 1214–1222.
13. Bal, B. S.; Childers, W. E.; Pinnick, H. W. *Tetrahedron* **1981**, *37*, 2091–2096.
14. Jones, D. W.; Thompson, A. M. *J. Chem. Soc., Perkin Trans. 1* **1993**, 2541–2548.
15. Gordaliza, M.; Miguel del Corral, J. M.; Castro, M. A.; López-Vázquez, M. L.; San Feliciano, A.; Carpy, A. *Bioorg. Med. Chem.* **1995**, *3*, 1203–1210.
16. Astles, P. C.; Brown, T. J.; Halley, F.; Handscombe, C. M.; Harris, N. V.; Majid, T. N.; McCarthy, C.; McLay, I. M.; Morley, A.; Porter, B.; Roach, A. G.; Sargent, C.; Smith, C.; Walsh, R. J. A. *J. Med. Chem.* **2000**, *43*, 900–910.
17. Chakraborti, A. K.; Kaur, G. *Tetrahedron* **1999**, *55*, 13265–13268.
18. Karmarkar, N.; Kelkar, S. L.; Wadia, M. S. *Synthesis* **1985**, 510–512.
19. Bergeron, R. J.; Wiegand, J.; Weimar, W. R.; Vinson, J. R. T.; Bussenius, J.; Yao, G. W.; McManis, J. S. *J. Med. Chem.* **1999**, *42*, 95–108.
20. Moulin, C.; Duflos, M.; Le Baut, G.; Grimaud, N.; Renard, P.; Caignard, D. H. *Eur. J. Med. Chem.* **1998**, *33*, 321–329.
21. Suzuki, T.; Nagaoka, H.; Kondo, Y.; Takahashi, T.; Takeuchi, M.; Hara, H.; Saito, M.; Yamada, T.; Tomioka, K.; Hamada, M.; Mase, T. *Chem. Pharm. Bull.* **1998**, *46*, 1468–1473.
22. Faircloth, G. T.; Stewart, D.; Clement, J. J. *J. Tissue Cult. Methods* **1988**, *11*, 201–205.
23. Bergeron, R. J.; Cavanaugh, P. F., Jr.; Kline, S. J.; Hughes, R. G.; Elliot, G. T.; Porter, C. W. *Biochem. Biophys. Res. Commun.* **1984**, *121*, 848–854.
24. Castro, M. A.; Miguel del Corral, J. M.; Gordaliza, M.; Grande, C.; Gómez-Zurita, M. A.; García-Grávalos, M. D.; San Feliciano, A. *Eur. J. Med. Chem.* **2003**, *38*, 65–74.