Gastroprotective Effect of Carnosic Acid γ-Lactone Derivatives[§]

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Carnosic acid (**1**) has been shown to possess gastroprotective activity in vitro and in vivo. However, little is known of the gastroprotective effect or cytotoxicity of carnosic acid γ-lactone (**3**). To determine structure−activity relationships, a series of 17 esters of **3** were prepared including aliphatic, aromatic, and heterocyclic derivatives. Also, two units of **3** were coupled with succinic and phthalic acid as linkers. The compounds were assessed for their gastroprotective effect in the HCl/EtOH-induced gastric lesions model in mice and for cytotoxicity in human lung fibroblasts, human adenocarcinoma AGS cells, and Hep G2 hepatocellular carcinoma cells. At a single oral dose of 40 mg/kg, the gastroprotective effect increased moderately with the length of the alkyl chain. The best effects were observed for the butyrate (**9**) and chloroacetate (**6**) derivatives. Activity of fatty acid esters increased with chain length but decreased with unsaturation. The best gastroprotective effect, with lowest cytotoxicity, was found for the palmitate (**11**) and oleate (**12**) derivatives.

The Mediterranean medicinal and aromatic plants *Rosmarinus officinalis* L. (rosemary) and *Salvia officinalis* L. (sage) are well known for their biological effects, including chemopreventive and antioxidant activities.[1,2] The gastroprotective effect of sage and rosemary and the main active constituents from the plant extracts were reported previously.[3,4] Rosemary and sage extracts contain carnosic (**1**) and rosmarinic acids as the main bioactive constituents that have been associated with several of the reputed therapeutic actions of the crude drugs.

The abietane diterpenes from *R. officinalis* have shown cytotoxicity to P388 murine leukemia cells,[5] as well as antibacterial[6,7] and antioxidant effects.[2] Carnosic acid is the main phenolic diterpene found in *R. officinalis* leaves. It is usually isolated with variable amounts of carnosol.[8] Other phenolic diterpenes occurring in the plant are rosmanol,[9,10] epirosmanol,[2,9] carnosaldehyde,[2] and rosmadial.[11]

Carnosic acid (**1**) has been proposed to be the gastroprotective constituent of *S. officinalis* (sage). While **1** has been extensively investigated for several biological effects, much less is known on the effects and structure−activity relationship of the γ-lactone and derivatives. The lactone carnosol has been described as an anti-inflammatory,[12] hepatoprotective,[13] and antitumor[14] compound. The aim of the present study was to assess the effect of **3** and various esters toward prevention of gastric ulcers.

Most studies on *R. officinalis* have been focused on antioxidant activity of the extract and its constituents. The main compounds with higher free radical scavenging/antioxidant effect were carnosic acid and rosmarinic acid.[15] Antibacterial activity of 20 diterpenes isolated from *Salvia* species against Gram-positive and Gram-negative bacteria was previously evaluated.[16] The compounds assessed included products with a lactone group between C-20 and C-6 and between C-20 and C-7, carnosic acid derivatives, and other diterpenes with various oxidation and substitution patterns. A carnosic acid derivative and a C-20 and C-6 lactone with a quinoid system in the aromatic ring gave MICs of 20−25 μg/mL against *Staphylococcus aureus* and 8−10 μg/mL against *Bacillus subtilis*, respectively. The isolation, structural modification, and cytotoxicity of abietane diterpenes from *Perovskia abrotanoides* on murine leukemia cells P388 were reported recently.[5] The authors presented results relating to substitution at the C-20 carboxylic acid and cytotoxicity toward the P388 leukemia cell line.

Herein, we describe the preparation of various semisynthetic derivatives of carnosic acid γ-lactone (**3**) and examine the structure−activity relationships of these compounds as gastroprotective agents and their cytotoxicity. The effects were compared with those of the parent compound toward normal lung fibroblasts MRC-5 (ATCC CCL-171), human gastric adenocarcinoma AGS cells (ATCC CRL-1739), and human Hep G2 hepatocellular carcinoma cells, as well as on the HCl/EtOH-induced gastric lesions model in mice. The MRC-5 cells were used as a control of basal cytotoxicity. Epithelial gastric AGS cells were selected because we were looking for gastroprotective activity. Hepatocytes, Hep G2 (ATCC HB-8065), were included because they express the P450 cytochrome system, differing from the two previous cell lines, which are unable to metabolize the compounds.

Results and Discussion

Carnosic acid γ-lactone (**3**) was prepared by an intramolecular esterification of **1** using dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) in dry CH_2Cl_2. In the same way, carnosic acid γ-lactone 12-methyl ether (**4**) was prepared from carnosic acid 12-methyl ether (**2**). Compound **5** was obtained by acetylation of **3**. For the preparation of derivatives **6**−**20**, 100 mg (0.3 mmol) of **3** was used for each reaction. DCC (80 mg, 0.39 mmol) and a catalytic amount of DMAP were employed in all of the reactions. Esterification of **3** was performed using chloroacetic (57 mg), isobutyric (53 mg), propionic (45 mg), butyric (53 mg), lauric (120 mg), palmitic (154 mg), oleic (169 mg), or linoleic (168 mg) acid, with DCC/DMAP in CH_2Cl_2 for 2 h at room temperature, affording the corresponding chloroacetate **6**, isobutyrate **7**, propionate **8**, butyrate **9**, laurate **10**, palmitate **11**, oleate **12**, and linoleate **13** in 18−83% w/w yields. Treatment of **3** with benzoic (73 mg), nicotinic (82 mg), phenylacetic (74 mg), indoleacetic (106 mg), and indolebutyric (122 mg) acid (Sigma Chemical Co.) using the same protocol as described above afforded the benzoate **14**, nicotinate **15**, phenylacetate **16**, indoleacetate **17**, and indolebutyrate **18** in 28−85% w/w yields. Treatment of **3** with succinic (100 mg) and phthalic (71 mg) acid and DCC/DMAP in CH_2Cl_2 gave the corresponding diesters (**19** and **20**) in 41% and 32% yield, respectively. The purity of all derivatives was over 98%, as

[§] Dedicated in memory of Prof. Jaime A. Rodríguez.

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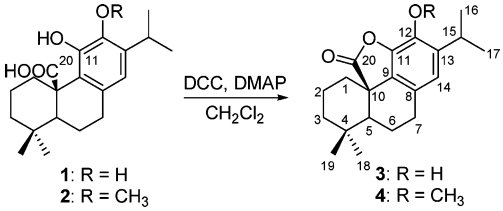
Scheme 1. Preparation of Compounds **3** and **4**

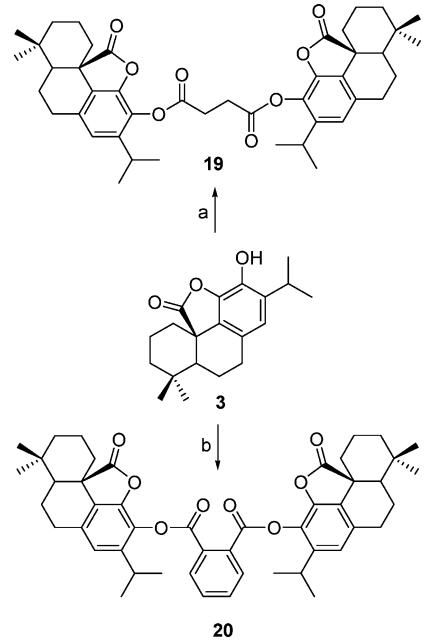


assessed by ^{1}H NMR spectroscopy. The syntheses are summarized in Schemes 1−3. All of the compounds were characterized by spectroscopic means, and compounds **6**−**20** are described for the first time. Details of the preparation of the above esters and tables of their ^{1}H and ^{13}C NMR spectra are provided in the Supporting Information. The compounds were then evaluated for gastroprotective effect and cytotoxicity. The results are summarized in Table 1.

Carnosic acid γ-lactone 12-methyl ether was previously isolated from the aerial parts of flowering *S. officinalis*.[17,18] In the present study, the parent compound (**3**) was tested for gastroprotective effect on the experimental model of HCl/EtOH-induced gastric lesions in mice (Table 1). A significant, dose-dependent gastroprotective effect was found for **3**, reducing gastric lesions by 59.5% at an oral dose of 40 mg/kg. Under the same experimental conditions the reference drug lansoprazole, at 20 mg/kg, reduced the lesions by 80%. For comparison purposes and to study structure−activity relationships, the 17 derivatives of **3** were evaluated at a single oral dose of 40 mg/kg in mice.

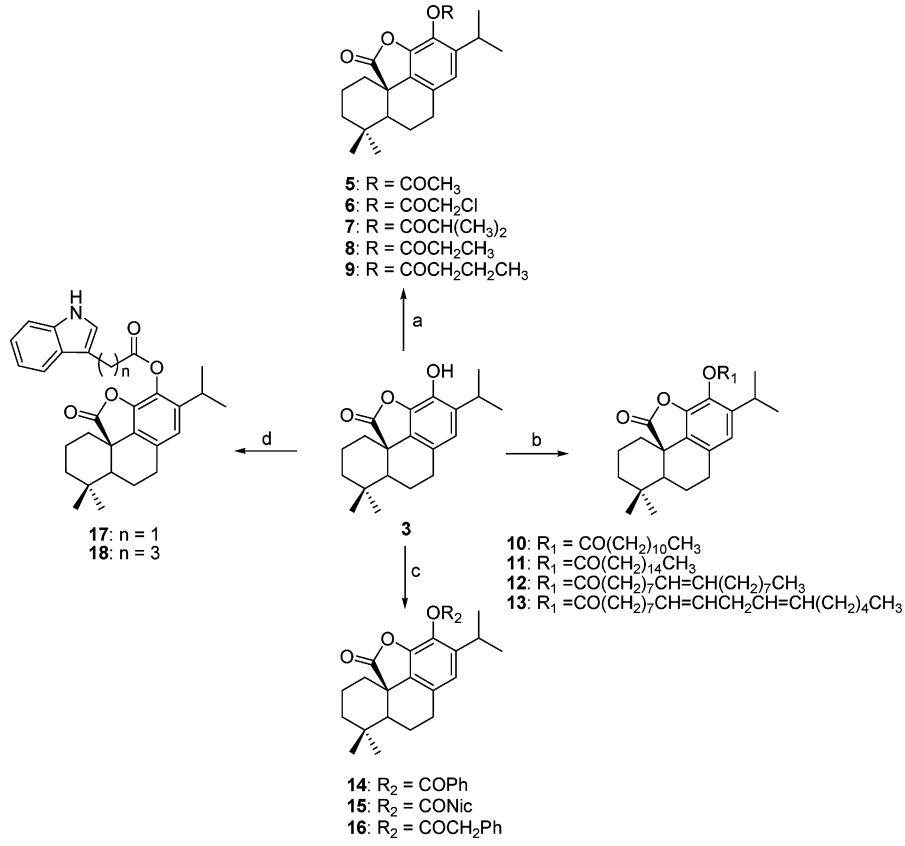
The gastroprotective effect increased moderately with the alkyl chain length, as can be observed by comparing the effects of the acetate (**5**) and propionate (**8**) with the butyrate (**9**). However, the prevention of gastric lesions was lower for the C_{12} derivative (**10**, 36% reduction) and increased in the C_{16} and C_{18} esters (**11** and **12**) to 65% and 70%, respectively. Among the fatty acid esters, activity

Scheme 3. Preparation of Compounds **19** and **20**[a]



[a] Reagents and conditions: (a) succinic acid; DCC; DMAP; CH_2Cl_2; (b) phthalic acid; DCC; DMAP; CH_2Cl_2. Reactions were carried out at room temperature.

Scheme 2. Preparation of Compounds **5**−**18**[a]



[a] Reagents and conditions: (a) acetic anhydride; pyridine or appropriate organic acid; DCC; DMAP; CH_2Cl_2; (b) appropriate fatty acid; DCC; DMAP; CH_2Cl_2; (c) appropriate aromatic acid; DCC; DMAP; CH_2Cl_2; (d) appropriate indolic acid; DCC; DMAP; CH_2Cl_2. All reactions were carried out at room temperature.

Table 1. Dose−Response Gastroprotective Effect of Carnosic Acid γ-Lactone (**3**), Gastroprotective Effect at 40 mg/kg, and Cytotoxicity (IC_{50}, μM) of Derivatives **1**−**20**[a]

compound	lesion index (mm)	% lesion reduction	cytotoxicity (IC_{50}, μM)		
carnosic acid γ-lactone (**3**)					
10 mg/kg	35.7 ± 11.3	20.8	MRC-5	AGS	Hep G2
20 mg/kg	31.3 ± 12.3	30.6	293	246	240
40 mg/kg	18.3 ± 6.1[b]	59.5			
control	45.1 ± 8.2				
1	nt		21.8 ± 3.2	89.8 ± 5.2	41.3 ± 2.6
2	nt		54.9 ± 3.9	45.2 ± 3.1	43.5 ± 4.2
4	13.3 ± 7.2[b]	71	31.9 ± 1.6	26.7 ± 1.8	58.1 ± 4.1
5	17.6 ± 9.8[b]	62	290 ± 11	275 ± 19	294 ± 17
6	8.5 ± 5.1[b]	81	130 ± 7	103 ± 8	203 ± 12
7	12.9 ± 8.0[b]	72	119 ± 7	105 ± 7	112 ± 9
8	18.7 ± 5.5[b]	59	125 ± 6	113 ± 6	196 ± 15
9	8.0 ± 3.6[b]	82	44.4 ± 2.1	99.5 ± 4.9	56.1 ± 2.8
10	29.3 ± 10.5[b]	36	>1000	413 ± 25	>1000
11	16.0 ± 7.0[b]	65	>1000	>1000	>1000
12	13.9 ± 4.4[b]	70	>1000	822 ± 49	>1000
13	25.0 ± 13.3[b]	45	293 ± 21	253 ± 18	289 ± 20
14	23.8 ± 11.4[b]	48	512 ± 41	503 ± 25	510 ± 35
15	41.7 ± 14.1	9	296 ± 22	280 ± 22	264 ± 13
16	25.6 ± 9.0[b]	44	516 ± 36	133 ± 8	522 ± 31
17	22.1 ± 8.9[b]	51	109 ± 5	85.6 ± 5.1	251 ± 21
18	23.0 ± 13.5[b]	50	54.5 ± 3.3	46.8 ± 2.8	120 ± 7
19	17.3 ± 9.2[b]	62	>1000	623 ± 43.6	>1000
20	38.6 ± 15.1	15	678 ± 42	375 ± 23	611 ± 43
control	45.7 ± 9.6				
lansoprazole (20 mg/kg)	9.1 ± 6.2[b]	80	306 ± 16	162 ± 11	221 ± 13

[a] Results are expressed as means ± SD. $n = 7$. ANOVA followed by Dunnett's multiple comparison test. nt: not tested. [b] $P < 0.01$ compared to control group. Each concentration was tested in quadruplicate together with the control and repeated three times in separate experiments.

increased with the alkyl chain length but decreased with unsaturation, as can be seen by comparing the lesion reduction by the $C_{18:1}$ and $C_{18:2}$ derivatives **12** and **13**, which was 70% and 45%, respectively.

The highest gastric lesion prevention activity was elicited by the butyrate (**9**, 82%) and chloroacetate (**6**, 81%). The relevance of the chlorine atom can be deduced by comparing the effect of the acetate **5** with that of compound **6**, bearing the halogen. The effect of the aromatic derivatives **14** and **16** as well as **17** and **18**, differing in the CH_2 units between the carbonyl ester function and the aromatic ring of the side chain, was similar. In the diesters with succinic and phthalic acid (**19** and **20**), only the succinate presented a significant gastroprotective effect (62%).

When the γ-lactone derivatives were tested against human cell lines, the highest cytotoxicity was found for the more lipophilic γ-lactone methyl ether (**4**), with low selectivity and IC_{50} values of 31.9, 26.7, and 58.1 μM for fibroblasts, AGS cells, and HepG2 cells, respectively. A C_4 side chain such as the butyrate (**9**), or a C_4 side chain linked to a heterocycle as in the indolebutyrate (**18**), increased toxicity when compared with the parent compound. While the cytotoxicity of compound **9** was higher for fibroblasts and Hep G2 cells (IC_{50} of 44.4 and 56.1 μM, respectively) than AGS cells (IC_{50}: 99.5 μM), and compound **18** was more toxic to fibroblasts and AGS cells (IC_{50} of 54.5 and 46.8 μM, respectively) than against Hep G2 hepatocytes (IC_{50}: 120 μM), the selectivity was not sufficient to be of significance.

The cytotoxicity of acetate **5** was similar to that of carnosic acid γ-lactone itself, but increased when a chlorine atom was placed in the ester chain as in the chloroacetate **6**. A remarkable change in the activity was found for the long-chain fatty acid esters **10**−**12**, being far less cytotoxic than the starting compound, with IC_{50} values >1000 μM toward fibroblasts and Hep G2 cells. Cytotoxicity increased with unsaturation, as can be seen comparing the IC_{50} values of the oleate (**12**) with that of the linoleate (**13**). For the esters with heterocycles (**17** and **18**), differing in two CH_2 units, higher cell toxicity was displayed by the indolebutyrate (**18**), with an IC_{50} value about half that of the indoleacetate (**17**). When two units of **3** were coupled with a linker to form diesters, the cytotoxicity was less pronounced for **19** than for **20**, and both compounds were less toxic than **3**.

In a report on the semisynthesis, cytotoxicity, and antimicrobial effect of aromatic abietanes, the authors presented the IC_{50} data (in μM) of the compounds prepared and the reference compound 6-mercaptopurine.[19] Several of the compounds described were more toxic than the products described in this report.

The compound with the highest gastroprotective effect (81%) was **6**; however, it was more cytotoxic than compounds **11** and **12**, which present IC_{50} values of >1000 μM against MRC-5 and HepG2 cells and >1000 and 822 μM toward AGS cells, respectively. Taking together both gastroprotective effect and cytotoxicity, the best effect with the lowest toxicity was found for the fatty acid derivatives C_{16} (**11**) and $C_{18:1}$ (**12**).

Experimental Section

General Experimental Procedures. Melting points were determined on a Koffler hot stage apparatus (Electrothermal 9100) and were uncorrected. Optical rotations were measured on a Jasco DIP 370 (Jasco Analytical Instruments, Easton, MD) polarimeter in $CHCl_3$ at 20 °C. IR spectra were recorded on a Nicolet Nexus 470 FT-IR instrument (Thermo Electron Corporation, Waltham, MA). The NMR spectra were recorded on a Bruker Avance 400 (Bruker, Rheinstetten, Germany) spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C in $CDCl_3$. Chemical shifts are given in δ (ppm) with TMS as the internal standard. Low-resolution mass spectra were run on a VG Micromass ZAB-2F and high-resolution mass spectra on a VG Micromass ZAB-2F at 70 eV (Varian Inc., Palo Alto, CA). Merck silica gel (0.063−0.2) was used for column chromatography; precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) were used for TLC analysis. TLC spots were visualized by spraying the chromatograms with *p*-anisaldehyde/ethanol/acetic acid/H_2SO_4 (2:170:20:10 v/v) and heating at 110 °C for 3 min. Dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) were from Merck (Schuchardt, Germany).

Plant Material. Carnosic acid (**1**) and carnosic acid 12-methyl ether (**2**) were isolated from the aerial parts of *R. officinalis* cultivated in Curicó, VII Región, Chile. A voucher herbarium specimen (Pertino 001/2007) has been kept at the Herbario de la Universidad de Talca. Compound **1** was obtained after extraction of the air-dried and powdered plant material with petroleum ether/ethyl acetate (EtOAc) (1:1) and

successive purification by column chromatography on silica gel, followed by crystallization. Preparation of the following compounds is described in the Supporting Information.

Carnosic acid γ-lactone (3): colorless crystals; mp 106−109 °C; $[\alpha]_D^{20}$ +79 (*c* 0.12, $CHCl_3$); IR (film) ν_{max} 3382, 2956, 1788, 1411 cm^{-1}; EIMS *m/z* 286 $[M - CO]^+$ (100), 271 (30), 243 (34), 230 (57), 218 (42), 204 (24), 69 (13); HREIMS *m/z* 286.1920 $[M - CO]^+$ (calcd for $C_{19}H_{26}O_2$, 286.1933).

Carnosic acid γ-lactone 12-methyl ether (4): colorless crystals; mp 110−113 °C; $[\alpha]_D^{20}$ +69 (*c* 0.18, $CHCl_3$); IR (film) ν_{max} 2954, 1794, 1623, 1430 cm^{-1}; HRMS *m/z* 323.1966 $[M - CO + Na]^+$ (calcd for $C_{20}H_{28}O_2Na$: 323.1987).

Carnosic acid γ-lactone 12-acetate (5): pale yellow oil; $[\alpha]_D^{20}$ +64 (*c* 0.27, $CHCl_3$); IR (film) ν_{max} 2952, 1808, 1760, 1437, 1190 cm^{-1}; EIMS *m/z* 328 $[M - CO]^+$ (40), 286 (100), 271 (15), 243 (19), 230 (36), 218 (19), 204 (14), 69 (10); HREIMS *m/z* 328.2061 $[M - CO]^+$ (calcd for $C_{21}H_{28}O_3$, 328.2049).

Carnosic acid γ-lactone 12-chloroacetate (6): pale yellow oil; $[\alpha]_D^{20}$ +70 (*c* 0.18; $CHCl_3$); IR (film) ν_{max} 2968, 1824, 1784, 1437, 1126 cm^{-1}; EIMS *m/z* 362 $[M - CO]^+$ (100), 347 (40), 319 (25), 306 (40), 286 (31), 269 (23), 230 (44), 218 (27), 149 (22), 77 (24), 69 (30); HREIMS *m/z* 413.1475 $[M + Na]^+$ (calcd for $C_{22}H_{27}ClO_4Na$, 413.1496).

Carnosic acid γ-lactone 12-isobutyrate (7): pale yellow oil; $[\alpha]_D^{20}$ +69 (*c* 0.19; $CHCl_3$); IR (film) ν_{max} 2957, 1809, 1765, 1435, 1122 cm^{-1}; EIMS *m/z* 356 $[M - CO]^+$ (32) 286 (100), 243 (15), 230 (28), 218 (12), 71 (12); HREIMS *m/z* 407.2178 $[M + Na]^+$ (calcd for $C_{24}H_{32}O_4Na$, 407.2198).

Carnosic acid γ-lactone 12-propionate (8): pale yellow oil; $[\alpha]_D^{20}$ +46 (*c* 0.08; $CHCl_3$); IR (film) ν_{max} 2957, 1804, 1770, 1439, 1130 cm^{-1}; EIMS *m/z* 342 $[M - CO]^+$ (30), 286 (100), 243 (20), 230 (38), 218 (19), 69 (8); HREIMS *m/z* 393.2013 $[M + Na]^+$ (calcd for $C_{23}H_{30}O_4Na$, 393.2042).

Carnosic acid γ-lactone 12-butyrate (9): pale yellow oil; $[\alpha]_D^{20}$ +73 (*c* 0.08; $CHCl_3$); IR (film) ν_{max} 2961, 1800, 1765, 1443, 1126 cm^{-1}; EIMS *m/z* 356 $[M - CO]^+$ (21), 286 (100), 243 (15), 230 (27), 218 (13), 71 (9); HREIMS *m/z* 407.2212 $[M + Na]^+$ (calcd for $C_{24}H_{32}O_4Na$, 407.2198).

Carnosic acid γ-lactone 12-laurate (10): colorless oil; $[\alpha]_D^{20}$ +57 (*c* 0.14; $CHCl_3$); IR (film) ν_{max} 2956, 1816, 1764, 1437, 1126 cm^{-1}; EIMS *m/z* 468 $[M - CO]^+$ (18), 450 (8), 323 (10), 286 (100), 271 (12), 230 (16), 218 (9); HREIMS *m/z* 491.3526 $[M - CO + Na]^+$ (calcd for $C_{31}H_{48}O_3Na$, 491.3501).

Carnosic acid γ-lactone 12-palmitate (11): colorless oil; $[\alpha]_D^{20}$ +37 (*c* 0.11; $CHCl_3$); IR (film) ν_{max} 2952, 1804, 1764, 1441, 1134 cm^{-1}; EIMS *m/z* 524 $[M - CO]^+$ (13), 506 (8), 286 (100), 230 (12), 98 (7); HREIMS *m/z* 575.4058 $[M + Na]^+$ (calcd for $C_{36}H_{56}O_4Na$, 575.4077).

Carnosic acid γ-lactone 12-oleate (12): colorless oil; $[\alpha]_D^{20}$ +48 (*c* 0.12; $CHCl_3$); IR (film) ν_{max} 2956, 1812, 1764, 1433, 1118 cm^{-1}; EIMS *m/z* 550 $[M - CO]^+$ (9), 286 (100), 271 (7), 230 (13), 218 (7), 69 (6); HREIMS *m/z* 601.8582 $[M + Na]^+$ (calcd for $C_{38}H_{58}O_4Na$, 601.8570).

Carnosic acid γ-lactone 12-linoleate (13): pale yellow oil; $[\alpha]_D^{20}$ +32 (*c* 0.09; $CHCl_3$); IR (film) ν_{max} 2956, 1809, 1757, 1435, 1117 cm^{-1}; EIMS *m/z* 548 $[M - CO]^+$ (8), 323 (7), 286 (100), 271 (11), 230 (14), 218 (10), 69 (7); HREIMS *m/z* 548.4251 $[M - CO]^+$ (calcd for $C_{37}H_{56}O_3$, 548.4229).

Carnosic acid γ-lactone 12-benzoate (14): colorless powder; mp 152−153 °C; $[\alpha]_D^{20}$ +48 (*c* 0.10; $CHCl_3$); IR (film) ν_{max} 2964, 1800, 1744, 1441, 1051 cm^{-1}; EIMS *m/z* 390 $[M - CO]^+$ (34), 375 (9), 285 (16), 105 (100), 77 (8); HREIMS *m/z* 441.2063 $[M + Na]^+$ (calcd for $C_{27}H_{30}O_4Na$, 441.2042).

Carnosic acid γ-lactone 12-nicotinate (15): colorless powder; mp 155−157 °C; $[\alpha]_D^{20}$ +48 (*c* 0.10; $CHCl_3$); IR (film) ν_{max} 2964, 1796, 1744, 1437, 1083 cm^{-1}; EIMS *m/z* 391 $[M - CO]^+$ (76), 376 (18), 285 (35), 269 (10), 106 (100), 78 (12); HREIMS *m/z* 391.2126 $[M - CO]^+$ (calcd for $C_{25}H_{29}NO_3$, 391.2147).

Carnosic acid γ-lactone 12-phenylacetate (16): pale yellow oil; $[\alpha]_D^{20}$ +59 (*c* 0.20; $CHCl_3$); IR (film) ν_{max} 2956, 1800, 1760, 1441, 1119 cm^{-1}; EIMS *m/z* 404 $[M - CO]^+$ (26), 286 (100), 243 (12), 230 (27), 218 (13), 91 (19), 69 (8); HREIMS *m/z* 404.2367 $[M - CO]^+$ (calcd for $C_{27}H_{32}O_3$, 404.2351).

Carnosic acid γ-lactone 12-indoleacetate (17): yellow oil; $[\alpha]_D^{20}$ +45 (*c* 0.10; $CHCl_3$); IR (film) ν_{max} 3418, 2964, 1804, 1752, 1445, 1114 cm^{-1}; EIMS *m/z* 443 $[M - CO]^+$ (15), 425 (13), 286 (42), 230 (12), 157 (36), 130 (100); HREIMS *m/z* 494.2330 $[M + Na]^+$ (calcd for $C_{30}H_{33}NO_4Na$, 494.2307).

Carnosic acid γ-lactone 12-indolebutyrate (18): yellow oil; $[\alpha]_D^{20}$ +47 (*c* 0.09; $CHCl_3$); IR (film) ν_{max} 3418, 2956, 1800, 1748, 1433, 1126 cm^{-1}; EIMS *m/z* 499 $[M]^+$ (5), 453 (22), 286 (71), 71 (12), 230 (15), 218 (10), 186 (100), 130 (19); HREIMS *m/z* 499.2741 $[M]^+$ (calcd for $C_{32}H_{37}NO_4$, 499.2723).

Carnosic acid γ-lactone 12-succinate (19): pale yellow oil; $[\alpha]_D^{20}$ +52 (*c* 0.08; $CHCl_3$); IR (film) ν_{max} 2956, 1804, 1764, 1445, 1126 cm^{-1}; HREIMS *m/z* 733.3725 $[M + Na]^+$ (calcd for $C_{44}H_{54}O_8Na$, 733.3716).

Carnosic acid γ-lactone 12-phthalate (20): colorless powder; mp 234−237 °C; $[\alpha]_D^{20}$ +62 (*c* 0.10; $CHCl_3$); IR (film) ν_{max} 2956, 1856, 1792, 1772, 1744, 1433, 1126 cm^{-1}; HREIMS *m/z* 781.3746 $[M + Na]^+$ (calcd for $C_{48}H_{54}O_8Na$, 781.3716).

The ^1H NMR and ^{13}C NMR data of compounds **3**−**9** as well as **10**−**20** are presented in Tables S1 and S2 for ^1H and Tables S3 and S4 for ^{13}C, respectively (see the Supporting Information). For preparation of the compounds, see the Supporting Information. The structures of the compounds were authenticated by comparing spectroscopic data with those published for carnosic acid γ-lactone 12-methyl ether (**4**)[17,18] and related diterpenes.[5,6,10]

HCl/EtOH-Induced Ulcer Model in Mice. The gastroprotective effect of the compounds was assessed by the HCl/EtOH-induced gastric lesions model in mice.[20] Male Swiss albino mice weighing 30 ± 3 g were used. Animals were purchased from the Instituto de Salud Pública de Chile, Santiago de Chile. Animals were fed on certified Champion diet with free access to water under standard conditions of 12 h dark−light period, 50% relative humidity, and 22 °C. Mice were randomly distributed into groups of 7 animals each and fasted for 24 h with free access to water prior to the experiment. For carnosic acid γ-lactone, three different doses were used: 40, 20, and 10 mg/kg. For dose−response studies, at least three doses should be used. Doses of 40, 20, and 10 mg/kg were selected on the basis of previous work on gastroprotective diterpenes to find a dose at which gastroprotection was about 50% for the starting compound (carnosic acid γ-lactone).

For comparison purposes, the other compounds were assessed at 40 mg/kg. Compounds were suspended in 12% Tween 80 and administered orally. Fifty minutes after treatment, all groups received orally 0.2 mL of a solution containing 0.3 M HCl/60% EtOH (HCl/EtOH) for gastric lesion induction. The antisecretory drug lansoprazole was used as the reference compound. The control group received 12% Tween 80. Animals were sacrificed by cervical dislocation 1 h after the administration of HCl/EtOH, and the stomachs were excised and inflated by injection of saline (1 mL). The ulcerated stomachs were fixed in 5% formalin for 30 min and opened along the greater curvature. The length (mm) of each lesion was measured, and the lesion index was expressed as the sum of the length of all lesions.[20,21] The protocols were approved by the Universidad de Talca Institutional Animal Care and Use Committee, which follows the recommendations of the Canadian Council on Animal Care.[22]

Cytotoxicity Assay. The human cell lines MRC-5 normal lung fibroblasts (CCL-171), AGS gastric adenocarcinoma cells (CRL-1739), and Hep G2 hepatocellular carcinoma cells (HB-8065) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were grown as monolayers in the following media: MRC-5 and Hep G2 in MEM and AGS in Ham F-12. The MEM medium contained 2 mM L-glutamine, 1 mM sodium pyruvate, and 1.5 g/L sodium bicarbonate. Ham F-12 was supplemented with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate. All media were supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 in air at 37 °C. For the experiments, cells were plated at a density of 25 000 cells/mL in 96-well plates. Confluent cultures of the different cell lines were treated with medium containing the compounds at concentrations ranging from 0 up to 1000 μM. Compounds were first dissolved in DMSO and then in medium. The final concentration of DMSO in the test medium and controls was 1%. Cells were exposed to test medium for 24 h, with or without the compound (control). Each concentration was tested in quadruplicate together with the control and repeated three times in separate experiments. Cell viability was determined at the end of the incubation by means of the neutral red uptake (NRU) assay.[23] Results were converted to percentage of controls, and the IC_{50} values were graphically obtained from the dose−response curves.

Statistical Analysis. Results were expressed as the mean ± SD. Statistical differences between treatments and their respective control

were determined by one-way analysis of variance (ANOVA), and when the F value was significant, post hoc differences were determined by the Dunnett's multiple comparison test. The level of significance was set at $P < 0.01$. All statistical analyses were performed using the software Statistica 5.1 (StatSoft, Inc.) and Statistical Package S-Plus 2000.

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Supporting Information Available: Preparation of compounds **3**−**20** and the NMR data of compounds are presented in Tables S1 and S2 for ^{1}H and S3 and S4 for ^{13}C, respectively. This information is available free of charge via the Internet at http://pubs.acs.org.

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