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Catechols from Abietic Acid: Synthesis and Evaluation as Bioactive Compounds

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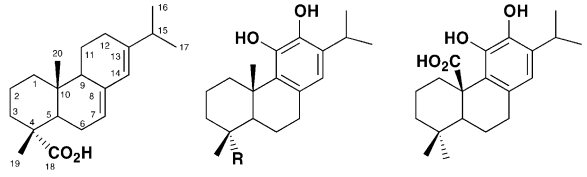
Abstract—Catechols from abietic acid were prepared by a short and good yielding chemical process and further evaluated for several biological activities namely, antifungal, antitumoral, antimutagenic, antiviral, antiproliferative and inhibition of nitric oxide. Their properties were compared with those of carnosic acid (**6**), a naturally occurring catechol with an abietane skeleton and known to possess potent antioxidant activity, as well as anticancer and antiviral properties. From all the synthetic catechols tested compound **2** showed the best activities, stronger than carnosic acid.

Introduction

Phenols are an important class of compounds widely used to inhibit the oxidation of materials of both commercial and biological importance. In general, antioxidants can intercept and react with free radicals at a rate faster than the substrate, and since free radicals are able to attack a variety of targets including lipids and proteins, it is believed that they are implicated in a number of important degenerative diseases.[1] Naturally occurring phenols with a tricyclic diterpenic structure and a catechol moiety are amongst the most used and studied compounds because of their antioxidant and biological activities.[2–10]

The search for abietic acid (**1**) derivatives with potentially interesting properties led to methyl 11,12-dihydroxyabietate-8,11,13-trien-15-oate (**2**), a catechol possessing an dehydroabietane skeleton, previously described as an intermediate in the hemisynthesis of sesquiterpene[11] or taxodione-type diterpene derivatives.[12]



1 **2 - R = CO_2CH_3** **3 - R = CO_2H** **4 - R = CH_2OH** **5 - R = CH_3** **6**

Compounds **2–5** possess a skeleton and an aromatic moiety similar to carnosic acid (**6**), one of the constituents of extracts from rosemary and sage leaves,[2,3] responsible for the application as antioxidant.[4–6,10] Anticancer[4,5,7] and antiviral properties[5,6,8] of **6** were also documented.

In previously described procedures,[11,12] **2** and **5** were obtained by a four-step procedure using, respectively, methyl 12-hydroxyabieta-8,11,13-trien-18-oate (**7**)[11] or ferruginol[12] (12-hydroxyabieta-8,11,13-triene) (**9**) as starting materials, followed by nitration, reduction, oxidation and hydrogenation.

Here, a shorter and high yielding procedure (Schemes 1 and 2) to obtain **2–5** is described. The catechols **2–5**

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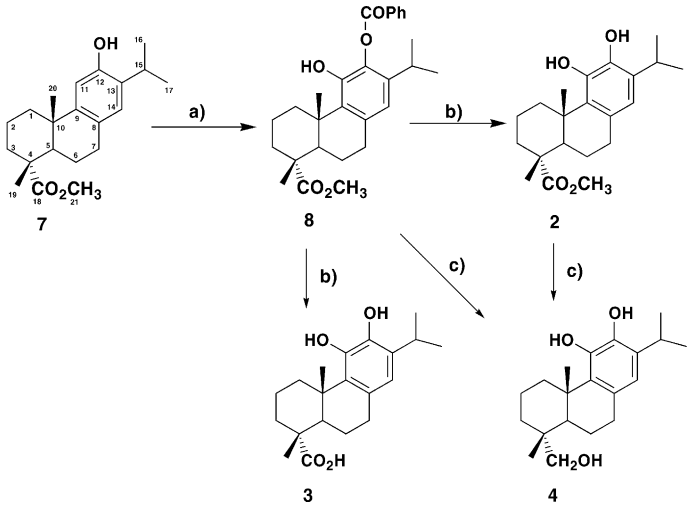
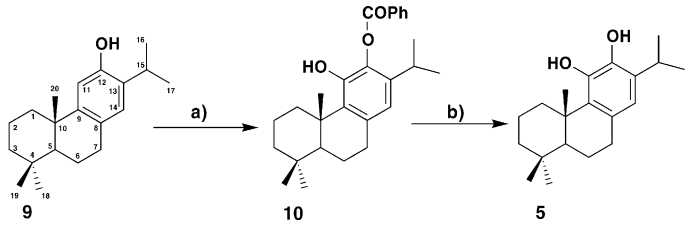


Scheme 1. (a) $(PhCO)_2O_2$, CH_3Cl, Δ, 2 h; (b) $AlCl_3$, EtSH, CH_2Cl_2, 3- 5 °C, 4 h for **2** or 48 h for **3**; (c) Dibal-H, Et_2O, −78 °C, 10 min.



Scheme 2. (a) $(PhCO)_2O_2$, CH_3Cl, Δ, 2 h; (b) Dibal-H, Et_2O, −78 °C, 10 min.

were evaluated for antifungal activity, as well as for their ability to interfere with the proliferation of human lymphocytes, production of nitric oxide and growth of human cancer lines. The antimutagenic and antiviral activities of compound **2** were also investigated. All the activities tested were compared with those of carnosic acid (**6**).[2,3]

Results and Discussion

Synthesis of compounds

Methyl 12-hydroxyabieta-8,11,13-trien-18-oate (**7**)[13] was converted into methyl 11,12-dihydroxyabieta-8,11,13-trien-18-oate (**2**) by a two-step procedure. First, **7** was oxidized with benzoyl peroxide in refluxing chloroform to give methyl 12-benzoyloxy-11-hydroxyabieta-8,11,13-trien-18-oate (**8**) as a major product.[14] The benzoyloxy-phenol **8** is the key intermediate in the synthesis of catechols **2**–**4** (Scheme 1).

Catechol **2** was obtained from **8** by cleavage of the aromatic ester group at C-12 with an aluminium chloride-ethanethiol system and dichloromethane as the co-solvent, previously reported for the cleavage of benzyl and methyl esters of aliphatic and aromatic carboxylic acids[15] and ethers.[16] By controlling the reaction time, it is possible to cleave selectively the aromatic ester group leaving the methyl ester group at C-18 intact (4 h) to give **2**, or allowing the reaction to proceed for a longer time (48 h) in order to cleave both groups and to afford **3**, as detailed in the Experimental.

The catechol **4** can be obtained from the benzoyloxyphenol **8** or the catechol **2**, using diisobutylaluminiumhydride (DIBAL-H).[17] Other procedures for the cleavage of the ester groups had less success in terms of selectivity or yield.[18,19]

The catechols **3** and **4** were disclosed by Ohtsuka and Tahara[11] but not characterized. As for all the catechols, the *ortho*-diphenol moiety of **3** and **4** were first assigned by the green coloured spots on the TLC plates used to monitor the reaction, after spraying with a solution of ferric chloride.[20] These structures were established using FTIR, ^1H and ^{13}C NMR and mass spectrometry.

The above benzoyl peroxide oxidation was applied to ferruginol (**9**),[12] to give 12-benzoyloxyabieta-8,11,13-trien-11-ol (**10**), which was then converted into 11,12-dihydroxyabieta-8,11,13-triene (**5**) (Scheme 2). Both cleavage methods previously mentioned could be used, although the reductive cleavage with DIBAL-H provides a quantitative yield of **5**, with spectral data in agreement with the literature data.[12]

The structural characterization of the starting materials **6**, **8**, the intermediates **7**, **9** and the target products **2**–**5** were made on the basis of the spectroscopic properties and in the case of known compounds **2**, **5**–**10** by comparison with previous data. The new compounds **3** and **4** were fully characterized.

Carnosic acid (**6**), used as reference, was extracted from rosemary according to the literature.[2,3,5]

Biological Activity

Antifungal activity. Catechols **2**–**5** and carnosic acid (**6**) were screened for antifungal activity against the yeasts *Candida albicans*, *Candida glabrata*, *Cryptococcus neoformans*, the mould *Aspergillus fumigatus* and the dermatophytes *Microsporum canis, Trichophyton mentagrophytes* and *Epidermophyton floccosum*. No activity was detected against *C. albicans*, *C. glabrata*, *C. neoformans* and *A. fumigatus* even when the compounds were tested at 750 μM. However, an inhibition of the dermatophytes' growth was observed with all the compounds. The activities, expressed in minimum lethal concentration (MLC) and defined as the lowest concentration showing no visible fungal growth after the incubation time, are shown in Table 1.

M. canis showed to be less sensitive to the tested catechols than *T. mentagrophytes* and *E. floccosum*. Catechols **2** and **5** were the most active compounds, exhibiting a potent antifungal activity, greater than that presented by the well-known antifungal agent fluconazole. Carnosic acid (**6**) showed less intense activity when compared with these two catechols for the strains tested.

Effect on lymphocyte proliferation and NO production. Compounds **2**–**6** were evaluated for their effect on the mitogenic response of human lymphocytes to PHA as well as on the production of nitric oxide (NO) by the murine macrophage cell line J774 stimulated with a combination of lipopolysaccharide (LPS) and gamma-interferon (IFN-γ). The results, given in concentrations that were able to produce 50% inhibition of lymphocyte proliferation or inhibition of NO formation (IC_{50}), are summarized in Table 2.

All the catechols studied exhibited a dose-dependent suppressor effect on the mitogenic response of human lymphocytes to PHA. Catechols **2**–**5** showed an interesting antiproliferative activity, stronger than carnosic acid (**6**), inhibiting lymphocytes proliferation at concentrations below 10 μM. Compounds **2** and **5** were the most potent compounds, exhibiting IC_{50} of 2.8 and 2.9 μM, respectively. It is noteworthy that their antiproliferative activity was only ten fold lower than that of the well-known imunosuppressor cyclosporin A ($IC_{50} = 0.34$ μM) which was used as positive control.

Catechols **2**–**5** were also potent inhibitors of NO production. Their effects were stronger than those of carnosic acid (**6**) and the positive control drug L-NAME (*N*-nitro-L-arginine methyl ester). Compounds **2** and **5** displayed similar potency and were the most potent inhibitors.

Effect on the growth of human cancer cell lines. The effects of compounds **2**–**5** and of carnosic acid (**6**) on the in vitro growth of five human cancer cell lines MCF-7 (breast), NCI-H460 (lung), SF-268 (CNS), TK-10 (renal) and UACC-62 (melanoma) were evaluated and the results, expressed as concentrations that were able to cause 50% cell growth inhibition (GI_{50}), are summarized in Table 3.

All these compounds (**2**–**6**) produce a dose-dependent inhibition of the five human cancer cell lines. The inhibitory effect presented by these compounds can not be attributed to a toxic effect, as inferred from the sulforhodamine B (SRB) assay (data not shown).

At concentrations above 20 μM, **3** and **4** as well as carnosic acid (**6**) showed a moderate effect on the inhibition of cell growth. Compounds **2** and **5** exhibited the strongest growth inhibitory effects, which were always higher than those presented by carnosic acid for all the cancer cell lines tested. Nevertheless compounds **2**–**6** are far to be as active as doxorubicin.

Antimutagenic activity. The antimutagenic activity of catechol **2** and carnosic acid (**6**) was also evaluated in the Ames test using the strain *Salmonella typhimurium* TA102, which is known to respond readily to active oxygen-containing species. Based on the dose–response curve of *tert*-butyl-hydroperoxide (*t*-BHP) (data not shown) a dose of 0.44 μmol was used for the antimutagenicity studies with **2** and carnosic acid (**6**). Compound **2** dose-dependently reduced the *t*-BHP-induced mutagenicity (Fig. 1a). The same was observed with carnosic acid (**6**), which strongly inhibited the mutagenicity of *t*-BHP (Fig. 1b). Carnosic acid (**6**) seems to act as a powerful scavenger of hydroxyl radicals generated by the *t*-BHP.[4] The chemical structure of **2** can explain the similar inhibitory effect on the mutagenicity of *t*-BHP when compared with **6**.

The present results on the inhibitory effects of compound **2** on the mutagenicity of *t*-BHP in strain TA102 are similar to those obtained with carnosic acid (**6**) (Fig. 2), and better than those with carnosol, which showed a weak inhibitory effect at the same concentrations.[4] Additionally, the present results indicate that compounds **2** and **6** both exert strong antimutagenic effect, possibly by

Table 1. Effect of catechols **2**–**5** and carnosic acid (**6**) on the growth of dermatophytes

Compd	MLC (μM)		
	Microsporum canis	*Trichophyton mentagrophytes*	*Epidermophyton floccosum*
2	90.3±15.1	22.6±2.4	22.6±2.4
3	376.5±62.7	94.1±9.9	94.1±15.7
4	393.1±78.9	196.5±39.4	98.3±16.4
5	210.0±22.1	26.2±5.5	13.1±2.2
6	376.5±62.7	94.1±7.8	188.3±15.7
Fluconazole	>208.9	104.5±17.4	52.2±8.7

Fluconazole was used as positive control. Results show means±SEM of 4–6 independent experiments performed in duplicate.

Table 2. Effect of catechols **2**–**5** and of carnosic acid (**6**) on lymphocyte proliferation and NO production

Compd	IC_{50} (μM)	
	Lymphocyte proliferation	NO production
2	2.8±0.9	5.2±1.2
3	8.3±0.2	7.9±0.9
4	10.6±1.0	12.7±1.3
5	2.9±0.7	5.3±0.4
6	35.4±3.1	18.5±2.1
Cyclosporin A	0.34±0.04	—
L-NAME	—	101.4±4.5

Cyclosporin A and L-NAME were used as positive controls; results show means±SEM of 3–4 independent observations performed in duplicate.

scavenging oxygen species. Since these active species are involved in the process of carcinogenicity it is possible that compound **2** could exhibit anticarcinogenic properties.

Antiviral activity. Activity against HSV-1/2, vaccinia virus and ASFV. Compounds **2** and **6** were also evaluated for their antiviral activity against herpes simplex viruses 1 (HSV-1), herpes simplex viruses 2 (HSV-2), vaccinia virus (VAC) and African swine fever virus (ASFV).

The results obtained (Table 4) show that the synthetic catechol **2** has a stronger effect on the viruses studied when compared with carnosic acid (**6**), being HSV-1 and HSV-2 the viruses most inhibited by **2**.

The effect of carnosic acid (**6**) on HSV was previously reported.[5] These authors[5] demonstrated that both, HSV-1 and HSV-2, were more inhibited in presence of the same concentration of **6**. However, their methodology was different. They studied the direct effect of **6** on the virus, whereas in our work it was studied the effect of **6** on viral infectivity (viral plaque formation) and on viral yield (number of infectious extracellular virions produced).

Activity against HIV. Compounds **2** and **6** were also evaluated against Human Immunodeficiency Virus type 1 (HIV-1, NL4-3 strain). The results obtained indicate

Table 3. Effects of catechols **2**–**5** and of carnosic acid (**6**) on the growth of human cancer cell lines

Compd	GI_{50} (μM)				
	MCF-7 (breast)	NCI-H460 (lung)	SF-268 (CNS)	TK-10 (renal)	UACC-62 (melanoma)
2	9.8±0.1	20.9±0.1	9.5±0.5	12.3±0.4	17.7±0.7
3	21.9±0.4	88.4±3.0	65.3±1.0	22.9±0.4	59.5±1.4
4	20.5±2.2	62.9±3.1	63.9±1.0	23.6[a]	44.8±2.0
5	8.9±0.1	25.6±2.3	22.1±0.5	12.9±1.1	19.9±0.4
6	24.2±0.3	41.2±6.3	24.3±1.7	47.2±9.9	21.6±1.1
Doxorubicin	0.04±0.01	0.09±0.01	0.09±0.01	0.55±0.06	0.09±0.01

Doxorubicin was used as positive control. Results show means±SEM of 3–6 independent experiments performed in duplicate.
[a]Result shows mean from one experiment performed in duplicate.

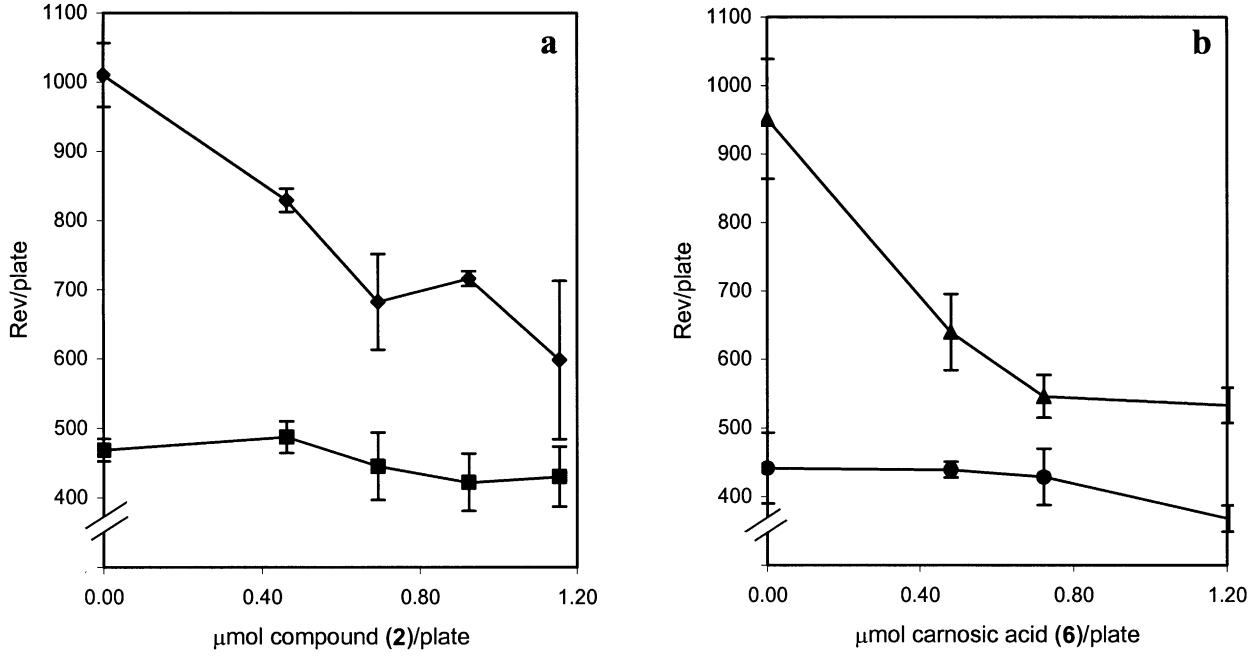


Figure 1. Antimutagenic effect of compound (**2**) and carnosic acid (**6**) on *S. typhimurium* TA 102 treated with *t*-BHP (0.44 μmol/plate). (a) ν compound (**2**), υ *t*-BHP + compound (**2**); (b) λ carnosic acid (**6**), σ *t*-BHP + carnosic acid (**6**). The error bars represent the standard deviation of the mean (SEM).

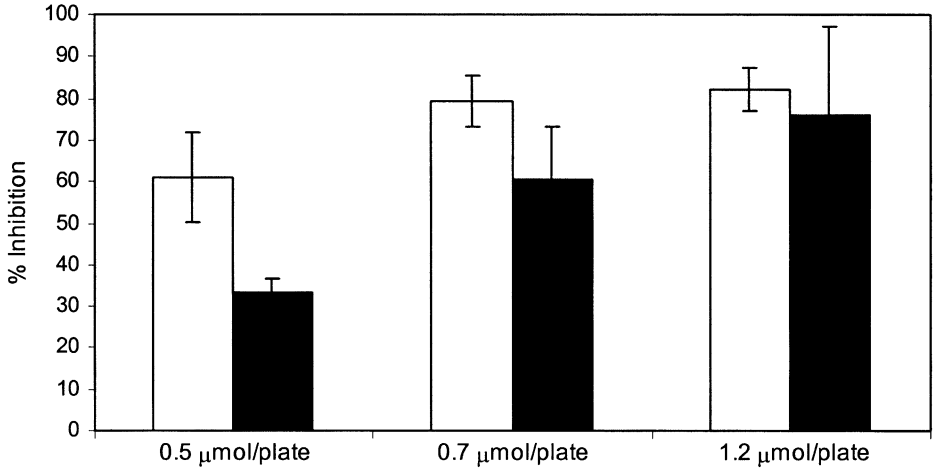


Figure 2. Inhibitory comparison of carnosic acid (**6**) (white bars) with compound (**2**) (black bars) on the mutagenicity of *t*-BHP, using *S. typhimurium* TA 102. The results are the average of three independent experiments for each inhibitory dose tested. The error bars represent the standard deviation of the mean.

Table 4. Effect of catechols **2**–**5** and of carnosic acid (**6**) on viruses infectivity

Compd	Concentration (μM)	Virus tested	Virus yield inhibition (% control)	Plaque formation inhibition (% control)
2	15	HSV-1	80.01±1.72	59.97±4.01
		HSV-2	79.21±3.00	65.00±3.36
		VAC	29.98±0.92	0.02±0.39
		ASFV	0.01±0.05	49.87±0.80
6	15	HSV-1	29.89±4.30	50.02±6.96
		HSV-2	35.00±3.74	nd
		ASFV	nd	40.11±2.51

For the effect on viral yield, the drug was present all the time after infection (1 pfu/cell), and the titres were determined by plaque assay as referred in the Experimental. For the study of plaque formation the viruses were titrated in presence of the drug as referred in the Experimental. Each value represents the average±SEM of three separate experiments performed in duplicate. All drugs were used at maximum concentration non cytotoxic. nd, not done.

that the effective concentration to achieve 50% protection for carnosic acid (**6**) (EC_{50} = 458 μM) is higher than for catechol **2** (EC_{50} = 275 μM) and AZT (EC_{50} = 50 μM). Although, **2** was more potent than **6**, it is 7-fold less potent than AZT.

When viral particles were exposed to catechols **2** and **6** before infection of cells no inhibition was detected in viral replication. An effect in viral infectivity was detected only when the cells were previously incubated with these compounds. This leads to the conclusion that the effect of these compounds is intracellular.

Furthermore, a quantification of proliferation and viability was performed on cells exposed to the inhibitory concentrations of compounds **2** and **6**, by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases of viable cells. Cells in the presence of compounds have similar growth curves when compared with non-exposed cells (data not shown).

Conclusions

From this work, we have shown that the catechols (**2**–**5**), analogues to carnosic acid (**6**), can be easily obtained from abietic acid (**1**), an abundant natural product. They show promising biological activities namely, antifungal, antitumoral, antimutagenic, antiviral, antiproliferative and nitric oxide inhibition, in general better than **6**, used as the reference. Among the tested catechols, compound **2** showed the best activities for all the tests that are, nevertheless, lower than that of standards. Further tests and development of new compounds within that class of molecules are under investigation.

Experimental

FTIR spectra were recorded on Perkin-Elmer 1725 and UV–VIS spectra on Hitachi 150-20 spectrophotometers. NMR spectra were run on a General Electric QE-300 spectrometer with resonance frequence of 300.65 MHz for ^{1}H and 75.6 MHz for ^{13}C, with appropriate solvent. The chemical shifts (δ) are reported in ppm, and coupling constants (*J*) in Hz. EI mass spectra were determined on a Kratos MS 25RF instrument at 70 eV. Microanalyses were performed on a Carlo Erba 1106R microanalyser. Silica gel for TLC refers to Merck silica gel GF254 and for flash chromatography to Merck silica gel 60, 230–400 mesh. Organic extracts were dried over anhydrous sodium sulfate.

All the reagents were of the highest quality available. Solvents for the reactions were reagent grade, dried and

distilled prior to use following standard procedures. The solvents for extraction and chromatography were of technical grade, freshly distilled before use.

Methyl 12-benzoyloxy-11-hydroxyabieta-8,11,13-trien-18-oate (8). Methyl 12-hydroxyabieta-8,11,13-trien-18-oate (**7**) (5.0 g) (mp 160–162 °C) obtained as described in the literature,[13] was oxidized with benzoyl peroxide (3.7 g) in chloroform (60 mL) at reflux for 2 h under nitrogen. After the workup the residue was chromatographed to obtain the major of four products, methyl 12-benzoyloxy-11-hydroxyabieta-8,11,13-trien-18-oate (**8**), 60% yield, mp 155–156 °C (methanol) (lit.:[14] 155–157 °C; from methanol).

Methyl 11,12-dihydroxyabieta-8,11,13-trien-18-oate (2). Anhydrous aluminum chloride (650 mg) was added slowly, under nitrogen, to a stirred solution of methyl 12-benzoyloxy-11-hydroxyabieta-8,11,13-trien-18-oate (**8**) (300 mg) and ethanethiol (0.51 mL) in dry dichloromethane (10 mL) at 0 °C in a ice-water bath during a 15 min period. After stirring at this temperature for 15 min and at room temperature for 4 h, the mixture was poured into ice-diluted hydrochloric acid and extracted with ether. The extract was washed with brine, dried over sodium sulfate and evaporated in vacuum. The residue (230 mg, 90%) was chromatographed on silica gel, using dichloromethane as eluent, and the product recrystallized as white crystals, mp 155–157 °C from chloroform/hexane (lit.:[11] 145–146.5 °C from ether/petroleum ether), FTIR (KBr) ν_{max} 3500, 3392, 1700 cm^{-1}; ^1H NMR ($CDCl_3$/TMS) δ 1.22 (3H, d, $J=6$, 16-H or 17-H), 1.24 (3H, d, $J=6$, 16-H or 17-H), 1.28 (3H, s, 19-H), 1.35 (3H, s, 20-H), 1.42 (1H, dd, $J_{1a,1e}=13.2$ and $J_{1a,2e}=3.6$, 1-H_a), 1.5–1.9 (2H, m, 3-H), 1.62 (1H, dt, $J_{2e,2a}=9.9$ and $J_{2e,1a}=3.9$, 2-He), 1.65–1.75 (1H, m, 6-Ha), 1.69 (1H, dd, $J_{6e,6a}=12.6$ and $J_{6e,7a}=6$, 6-He), 1.78 (1H, d, $J_{2a,2e}=10.2$, 2-Ha), 2.21 (1H, d, $J_{5a,6a}=12$, 5-H), 2.72 (1H, dd, $J_{7e,7a}=16.2$ and $J_{7e,6a}=4.8$, 7-He), 2.85 (1H, ddd, $J_{7a,7e}=16.2$, $J_{7a,6a}=12.3$ and $J_{7a,6e}=6.3$, 7-Ha), 2.99 (1H, sept, $J=6$, 15-H), 3.15 (1H, dt, $J_{1e,1a}=12.6$ and $J_{1e,2e}=2.1$, 1-He), 3.67 (3H, *s*, 18-H), 4.85 (1H, *brs*, 12-OH, D_2O exchange), 5.81 (1H, *s*, 11-OH, D_2O exchange), 6.41 (1H, *s*, 14-H) ppm; ^{13}C NMR ($CDCl_3$/TMS) δ 16.82 (C-19), 18.62 (C-2), 20.26 (C-20), 22.29 (C-6), 22.51 (C-16 or C-17), 22.77 (C-16 or C-17), 27.17 (C-15), 31.82 (C-7), 35.82 (C-1), 36.47 (C-3), 38.52 (C-10), 46.96 (C-5), 48.39 (C-4), 51.97 (C-21), 117.13 (C-14), 129.55 (C–Ar), 131.93 (C–Ar), 132.38 (C–Ar), 137.93 (C–Ar), 143.25 (C–Ar), 179.64 (C-18) ppm; FABMS *m/z* 346 [M^+,100], 331 (11), 285 (28), 271 (66), 229 (16), 205 (27), 165 (82). Elemental analysis (C 72.80, H 8.73%, calcd for $C_{21}H_{30}O_4$): C 72.65, H 9.08%.

11,12-Dihydroxyabieta-8,11,13-trien-18-oic acid (3). This was obtained from **8** using the same procedure as for **2** with stirring at room temperature for 48 h. After flash chromatography on silica gel, using hexane/diethyl ether (7:3) as an eluent, **3** was obtained as a yellowish powder (40%). Recrystallization from a mixture of chloroform and hexane gave pale yellow crystals of **3**: mp 180–182 °C; FTIR (KBr) ν_{max} 3450, 1691 cm^{-1}; ^1H NMR ($CDCl_3$/TMS) δ 1.23 (3H, d, $J=6.3$, 16-H or 17-H), 1.25 (3H, d, $J=6.3$, 16-H or 17-H), 1.30 (3H, s, 19-H), 1.37 (3H, s, 20-H), 1.42–1.49 (1H, m, 1-H_a), 1.6–1.9 (6H, m, 2-H, 3-H and 6-H), 2.23 (1H, d, $J_{5a,6a}=12$, 5-H), 2.75 (1H, dd, $J_{7e,7a}=16.5$ and $J_{7e,6a}=4.8$, 7-He), 2.91 (1H, ddd, $J_{7a,7e}=16.5$, $J_{7a,6a}=12.3$ and $J_{7a,6e}=6.3$, 7-Ha), 2.93 (1H, sept, $J=6.9$, 15-H), 3.16 (1H, dt, $J_{1e,1a}=13.5$, 1-He), 4.49 (1H, brs, 12-OH, D_2O exchange), 5.73 (1H, *s*, 11-OH, D_2O exchange), 6.43 (1H, *s*, 14-H) ppm; ^{13}C NMR ($CDCl_3$/TMS) δ 16.65 (C-19), 18.60 (C-2), 20.28 (C-20), 22.35 (C-6), 22.49 (C-16 or C-17), 22.76 (C-16 or C-17), 27.31 (C-15), 31.81 (C-7), 35.80 (C-1), 36.55 (C-3), 38.47 (C-10), 46.71 (C-5), 48.08 (C-4), 117.23 (C-14), 129.66 (C–Ar), 131.66 (C–Ar), 132.35 (C–Ar), 137.87 (C–Ar), 143.10 (C–Ar), 183.31 (C-18) ppm; FABMS *m/z* 332(M^+,100), 317(21), 271(36), 229(20), 165(93). Elemental analysis (C 72.26, H 8.49%, calcd for $C_{20}H_{28}O_4$): C 72.20, H 8.83%.

11,12-Dihydroxyabieta-8,11,13-trien-18-ol (4). To a solution of methyl 11,12-dihydroxyabieta-8,11,13-trien-18-oate (**2**) or methyl 12-benzoyloxy-11-hydroxyabieta-8,11,13-trien-18-oate (**8**) (100 mg) in dry ether (20 mL), at −78 °C and under N_2, was slowly added diisobutylaluminum hydride (Dibal-H) (1 mL). After stirring at this temperature for 10 min, the mixture was worked up with a saturated solution of sodium bisulfide and allowed to warm up to room temperature. Then the product was extracted with ether, dried over anhydrous sodium sulfate and the solvent was evaporated in vacuum. The product (90 mg, 98%) was recrystallized from a mixture of chloroform and hexane as white crystals of **4**: mp 161–163 °C; FTIR (KBr) ν_{max} 3526, 3505, 3289 cm^{-1}; ^1H NMR ($CDCl_3$/TMS) δ 0.88 (3H, *s*, 19-H), 1.22 (3H, d, $J=6.3$, 16-H or 17-H), 1.24 (3H, d, $J=6.3$, 16-H or 17-H), 1.27–1.35 (1H, m, 5-H), 1.37 (3H, s, 20-H), 1.45 (1H, dd, $J_{1a,1e}=12.9$ and $J_{1a,2e}=4.2$, 1-H_a), 1.5–1.9 (6H, m, 2-H, 3-H and 6-H), 2.7–2.9 (2H, *m*, 7-H), 2.99 (1H, sept, $J=6.9$, 15-H), 3.09 (1H, dt, $J_{1e,1a}=13.2$ and $J_{1e,2e}=3.3$, 1-He), 3.21 and 3.49 (2H, 2d, $J=10.9$, 18-H), 3.48 (1H, sh, 18-OH, D_2O exchange), 4.50 (1H, brs, 12-OH, D_2O exchange), 5.70 (1H, s, 11-OH, D_2O exchange), 6.43 (1H, s, 14-H) ppm; ^{13}C NMR ($CDCl_3$/TMS) δ 17.93 (C-19), 18.72 (C-2), 19.04 (C-6), 20.62 (C-20), 22.51 (C-16 or C-17), 22.77 (C-16 or C-17), 27.29 (C-15), 32.10 (C-7), 34.76 (C-1), 36.22 (C-3), 38.01 (C-10), 39.07 (C-4), 46.48 (C-5), 72.43 (C-18), 117.24 (C-14), 129.65 (C–Ar), 131.61 (C–Ar), 132.89 (C–Ar), 138.10 (C–Ar), 143.05 (C–Ar) ppm; FABMS *m/z* 318 (M^+, 100), 285(22), 217(27), 205(62), 191(46), 165(63). Elemental analysis (C 75.43, H 9.50%, calcd for $C_{20}H_{30}O_3$): C 75.45, H 9.28%.

12-Benzoyloxy-11-hydroxyabieta-8,11,13-triene (10). Ferruginol [12-hydroxyabieta-8,11,13-triene (**9**)] (400 mg) obtained as described in the literature,[12] was oxidized with benzoyl peroxide (400 mg) as described above. After the workup and column chromatography (Et_2O/hexane, 5–40%), the major of four products was isolated, 12-benzoyloxy-11-hydroxyabieta-8,11,13-triene (**10**), 347 mg, 61% yield, mp 130–132 °C from petroleum ether (lit.:[14] 132.5–133 °C; from petroleum ether).

11,12-Dihydroxyabieta-8,11,13-triene (5). This was obtained from 10 (100 mg) using either the cleavage system $AlCl_3$/EtSH or Dibal-H. With Dibal-H as described above for **4**, after the workup and column chromatography (Et_2O/hexane) a white gum (63.5 mg; 86%) was obtained. The IR and ^1H NMR spectra were identical to literature.[12]

Carnosic acid (6). This was extracted from dry rosemary leaves (22 g).[5] Column chromatography of the extract (1.19 g) on polyamide with CH_2Cl_2/hexane gave a fraction from which, after repeated recrystallization from $CHCl_3$/hexane, pale crystals of **6** (46 mg, 3.9%) mp 192–197 °C (lit.:[6] 189–192 °C, from hexane; lit.:[2] 192–197 °C, from hexane/C_6H_6) were obtained. ^1H and ^{13}C NMR spectra of **6** were identical to data reported for carnosic acid.[3]

Biological activity

Stocks solutions of catechols were prepared in DMSO and stored at −20 °C, providing uniform samples for re-tests. These frozen concentrates were then diluted to the desired final concentrations with the appropriate solvents just prior to the different assays. Final concentrations of DMSO showed no interference with the biological activities tested.

Antifungal activity. The antifungal activity of compounds was evaluated as described elsewhere.[21] Seven clinical fungi strains were used, the yeasts *C. albicans*, *C. glabrata*, *C. neoformans*, the mould *A. fumigatus* and the dermatophytes *M. canis*, *T. mentagrophytes* and *E. floccosum*. They were grown on Sabouraud chloramphenicol agar at 30 °C for 48–72 h in the case of yeasts and moulds and on Sabouraud Chloramphenicol cycloheximide at 30 °C for 7 days just up to 15 days for dermatophytes. Fluconazole was used as the positive control.

Lymphocyte proliferation assay. The effects of compounds on the mitogenic response of human lymphocytes to PHA (10 μg/mL) were evaluated using a modified colorimetric MTT assay.[22] This assay was previously described by us.[23] Cyclosporin A was used as positive control.

NO production assay. The effects of catechols on the production of nitric oxide (NO) were evaluated quantifying nitrite accumulation in cell culture supernatant by the Griess reaction.[24] The murine macrophage cell line J774 was cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 50 μg/mL gentamicine and 10% foetal bovine serum. Cells were plated in 96-well culture plates at a density of 0.5×10^6 cells/well and allowed to adhere for 2 h at 37 °C in a 5% CO_2 humidified atmosphere. To induce nitric oxide synthase, culture medium was replaced by fresh media containing the LPS (1 μg/mL) and IFN-γ (100 U/mL).

Cancer cell growth assay. The effects of catechols on the growth of cancer cell lines were evaluated according to the procedure adopted in the NCI's in vitro anticancer drug screening that use the sulforhodamine B (SRB) assay to assess growth inhibition.[25] Five human tumor cell lines were used, MCF-7 (breast cancer), TK-10 (renal cancer), UACC-62 (melanoma), NCI-H460 (non-small cell lung cancer) and SF-268 (CNS cancer). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 5% heat-inactivated fetal bovine serum, 2 mM glutamine and 50 μg/mL gentamicin at 37 °C in an humidified air incubator containing 5% CO_2. Doxorubicin was used as the positive control. Growth inhibition of 50% (GI_{50}) as well as toxicity of compounds was calculated as described elsewhere.[26]

Mutagenicity experiments. The antimutagenicity of compound **2** and carnosic acid (**6**) was evaluated using the Ames test. *Salmonella typhimurium* strain TA 102 was used as tester strain in the pre-incubation assay.[27] This strain has been shown to be highly sensitive to reactive oxygen species, including those generated by *t*-BHP.[27] Mutagenic activity was expressed as His^+ revertants per plate (Rev/plate). Dose–response curves were generated and three independent experiments were performed for *t*-BHP. A concentration of 0.44 μmol *t*-BHP was used for the inhibition experiments with the compound **2** and the carnosic acid (**6**). This dose of 0.44 μmol *t*-BHP was chosen from the linear portion of the dose–response curve of this compound using strain TA 102 (data not shown).

Anti-HSV, VA, ASFV assays. Vero cells were grown as monolayers in Dulbecco's modified Eagle's medium (DME), supplemented with 10% newborn calf serum (NCS) and 50 μg/mL gentamycin. HSV-1 strain SC 16, HSV-2 strain HD, VAC strain WR from ATCC (USA) and the Lisbon 60 strain of African swine fever virus (ASFV)[28] were used. All viruses were grown in Vero cells, as described.[29] The cytotoxicity of catechols **2** and **6** was previously evaluated in order to determine their maximum concentration non cytotoxic (MCNC) to Vero cells by dye uptake assay.[30]

The effect of **2** and **6** on viral plaque formation and on viral replication were done as described previously.[31,32]

Anti-HIV-1 assays. Suspension culture of Jurkat cells was incubated for 30 min at 37 °C with the compounds in predetermined concentrations. The cells were washed with RPMI supplemented with 10% FBS at the same temperature. Jurkat cells were then infected with HIV-1 at a multiplicity of infection (m.o.i.) of 1 (1000 $TCID_{50}/1\times10^6$) and in the presence of compounds **2** and **6**. After adsorption of the viruses for 2 h at 37 °C the cells were again washed and cultured in same media. As a control, cells with no HIV-1 infection were also incubated with the same concentrations of those compounds. Cells with or without infection were maintained in culture for 7 days at 37 °C, with media change at day 4. At day 9 cells were harvested and HIV p24 was quantified. The 50% inhibitory concentration of the drug was determined using the median effect equation.

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