Synthesis and Antiproliferative Activity of a New Compound Containing an α-Methylene-γ-Lactone Group

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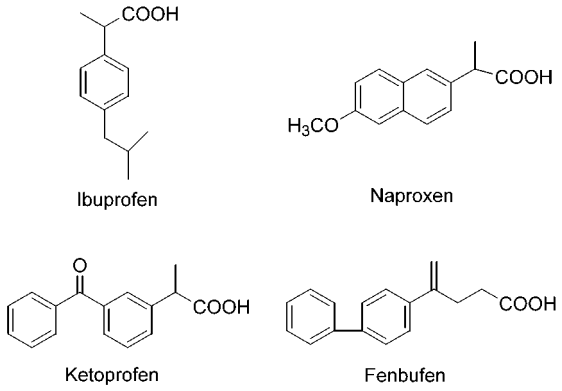
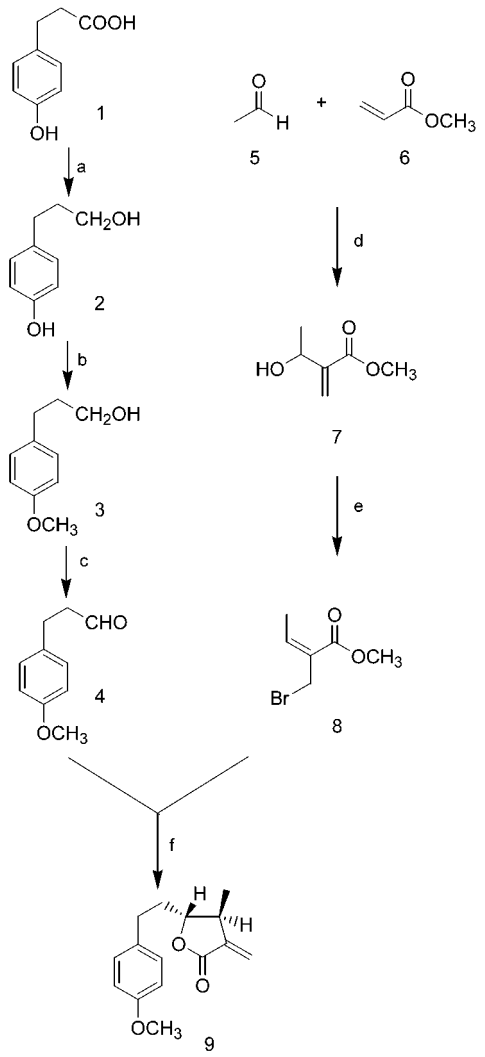
Abstract: The biological activity of compound **9** obtained by introducing an α-methylene-γ-butyrolactone group into 3-(4-hydroxyphenyl)propionic acid, **1**, was studied for possible effects on HL-60 cells, murine splenocytes, and human peripheral mononuclear cells (PBMC). **9** induced apoptosis in the HL-60 cell line and has a clear capacity to inhibit proliferation induced in murine splenocytes and PBMC by different mitogenic agents with no apparent toxic side effects. **9** was synthesized from **1**, and its structure and stereochemistry were elucidated by spectroscopic methods.

Introduction. Propionic acid derivatives[1] are very important substances, both commercially and medically speaking, within the group of nonsteroidal antiinflammatory therapeutic agents. Ibuprofen[1] was the first of a series of products within this subgroup, which now includes naproxen,[2] ketoprofen,[3] and fenbufen.[4] The



pharmacodynamic characteristics of these products are similar, with different degrees of antiinflammatory, antipyretic, analgesic, and antiplatelet activity, and all are nonselective inhibitors of cyclooxygenases.[5] The structural similarity of ibuprofen and 3-(4-hydroxyphenyl)propionic acid, **1**, originally obtained as the major product from the fern *Asplenium onopteris*[6] prompted a pharmacological study of this molecule once it had been modified as shown in Scheme 1.

Scheme 1[a]



[a] Reagents: (a) $LiAlH_4$, THF, reflux 6 h, 50%; (b) CH_3I, acetone, K_2CO_3, 95%; (c) PCC, CH_2Cl_2, 1.5 h, room temp, 52%; (d) DABCO, 7 days, room temp, 90%; (e) NBS, $(CH_3)_2S$, 0 °C to room temp, 24 h, 92%; (f) Sn, $(CH_3CH_2)_2O$, HOAc, *p*-(TsOH), C_6H_6, reflux 9 h, 70%.

The antitumoral activity of sesquiterpene lactones was known well before their allergenic function and is related to the presence of the α-methylene-γ-lactone group and its propensity for Michael-type additions with −SH or −NH nucleophiles in compounds that may play an important role in the mechanisms through which lactones carry out their biological activity.[7]

According to Hanson et al.,[8] the α-methylene-γ-lactone group in vernolepin and elephantopin with biological −NH either reacts very slowly (as in the case of lysine) or not at all (the case of guanine) while with the −SH (as occurs with cysteine) the reaction rate is similar to that of the iodoacetate, a common reagent for

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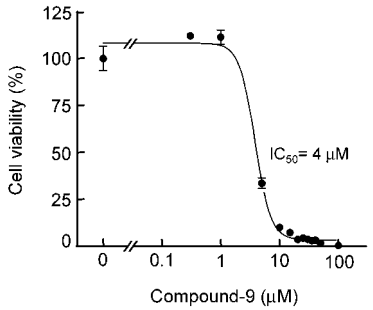

Figure 1. Representative experiment to demonstrate the effects of **9** on the growth of HL-60 cells. Cells were seeded at densities of 1×10^4 per well, and after 24 h exposure, MTT assays were performed.[17]

the sulfhydryl group.[9] It therefore seems clear that the antitumoral activity of sesquiterpene lactones is related to the ability of the α-methylene-γ-lactone group to bind to biological nucleophiles.

Apoptotic cell death plays a critical role in normal cell development, tissue homeostasis, and the regulation of the immune system.[10] Many anticancer drugs have been shown to cause the death of sensitive cells through the induction of apoptosis.

In this work we report the synthesis and biological activity of a compound containing an α-methylene-γ-butyrolactone group.

Chemistry. The 5-[2-(4-methoxyphenyl)ethyl]-3-methylen-4-methyldihydrofuran-2-one (**9**) derivative[11] was synthesized as follows. 3-(4-Hydroxyphenyl)propionic acid, **1**, isolated for the first time as a natural product from the fern *Asplenium onopteris*,[6] was used as starting material. Reduction of **1** with $LiAlH_4$ gave compound **2**, which, when methylated, afforded methyl ether **3**. This was then oxidized with pyridinium chlorochromate to give aldehyde **4**.[12]

Reaction of an acetaldehyde, **5**, with methyl acrylate, **6**, in the presence of 1,4-diazabicyclo[2,2,2]octane (DABCO) yielded **7**,[13] and treatment of **7** with *N*-bromosuccinimide (NBS) afforded **8** regio- and stereoselectively.[14] 2-Carbomethoxyallylation of **4** with 2-carbomethoxyallyl bromide **8** in the presence of metallic tin and catalytic amounts of acetic and *p*-toluenesulfonic acids yielded **9**[15] (Scheme 1). The stereochemistry of the lactone ring was determined in accordance with Löffer et al.[16]

Induction of Apoptosis by Compound 9. Compound **9** was found to inhibit the growth of HL-60 cells in culture. The inhibitory concentration IC_{50} of compound **9** was 4 μM at 24 h (Figure 1). To determine whether the extensive cell death observed in HL-60 cells after treatment with **9** was due to apoptosis or necrosis, we performed two independent assays. The first of these involved QFM (quantitative fluorescent microscopy).[18] Treatment of HL-60 cells with **9** induced apoptosis identified by characteristic morphological changes including chromatin condensation, compaction along the periphery of the nucleus, and nuclear segmentation into three or more chromatin fragments (Figure 2). When quantitative fluorescent microscopy was used, apoptosis was observed to be dose-dependent (Figure 3).

Second, DNA fragmentation was analyzed. HL-60 cells were treated with **9** for 6 and 24 h, and DNA samples were subjected to gel electrophoresis. The results showed that exposure to 5 μM of compound **9** results in endonucleolytic DNA cleavage (Figure 4) and would increase with drug concentration. This phenomenon was associated with a decrease in p32 CPP32 proenzyme level (Figure 5).

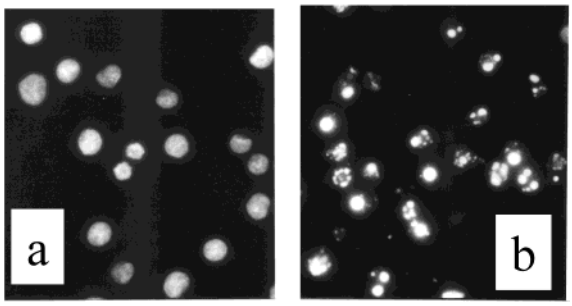

Figure 2. Photomicrographs of representative fields of HL-60 cells stained with bisbenzimide trihydrochloride to evaluate chromatin condensation after treatment for 24 h with no drug (a) or 20 μM of **9** (b).

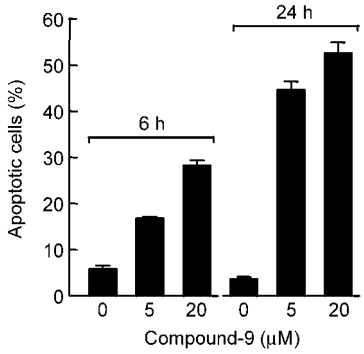

Figure 3. HL-60 cells were counted and scored for apoptotic chromatin condensation by quantitative fluorescent microscopy. Columns represent the mean number of cells counted that undergo apoptosis as a percentage of 500 total cells randomly counted in triplicate samples; bars represent the standard deviation SD.

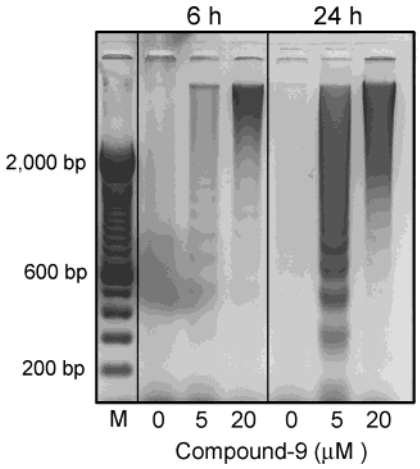

Figure 4. HL-60 cells were treated with 5 and 20 μM compound **9** and harvested at 6 and 24 h, and DNA fragmentation was monitored by electrophoresis in 2% agarose gels.

In Vitro Antiproliferative Effects. The *p*-hydroxyphenylpropionic acid derivative **9** was tested in vitro for its immunomodulator effect and its impact on the proliferative response of peripheral blood mononuclear cells (PBMC) to T lymphocyte mitogens. PBMC were purified from healthy human donors and cultured in the presence of optimal doses of the indicated mitogens in the presence or absence of different concentrations of

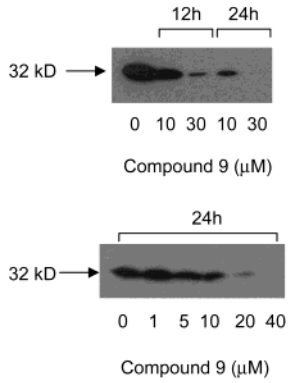

Figure 5. HL-60 cells were treated with **9** and harvested at 12 and 24 h. Total cell lysates were analyzed by immunoblotting with anti-CPP32 antibody.

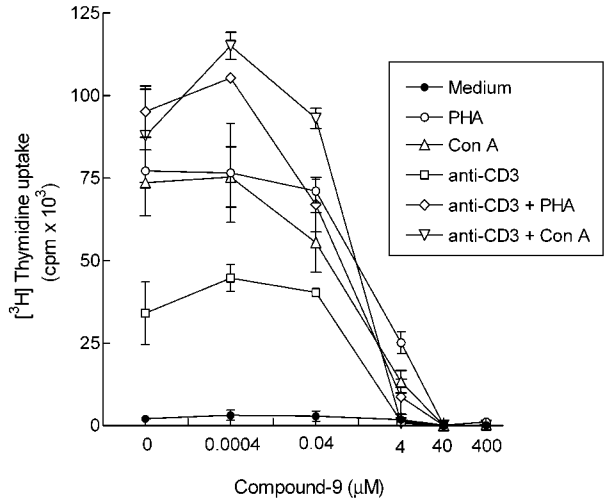

Figure 6. Compound **9** inhibits the proliferative response of PBMC to mitogens.

9, as previously described.[19] The presence of **9** in the culture medium induced a marked dose-dependent inhibitory effect on the [^{3}H]-thymidine intake of mitogenic stimulated PBMC (Figure 6).

The proliferative response of PBMC to PHA, Con A, immobilized anti-CD3, immobilized anti-CD3 plus PHA, and immobilized anti-CD3 plus Con A, and stimulation was significantly decreased by **9** at 0.04, 0.04, 4, 0.04, and 4 μM, respectively. The presence of these concentrations of **9** in the culture medium did not induce significant variations in the percentage of viable cells in the PBMC cultures measured by trypan blue exclusion and flow cytometry.[20] Taking into account the relevant role played by recombinant human interleukin 2 (IL-2) in the regulation of T lymphocyte proliferation, we investigated the effects of this cytokine on the inhibitory activity shown by **9** on the proliferative response of PBMC to T lymphocyte mitogens. Saturated amounts of IL-2 (100 UI/mL) were added to PBMC cultures in the presence of PHA and **9**. IL-2 clearly postponed the suppressor effect of **9** upon the proliferative response of PBMC to PHA stimulation (Figure 7). Similar results were obtained in the presence of the other mitogenic signals analyzed (data not shown).

The lactone showed its ability to inhibit proliferation induced in murine splenocytes by different mitogenic agents (Con A, anti-CD3, and LPS) but not that induced by PHA (Figure 8).

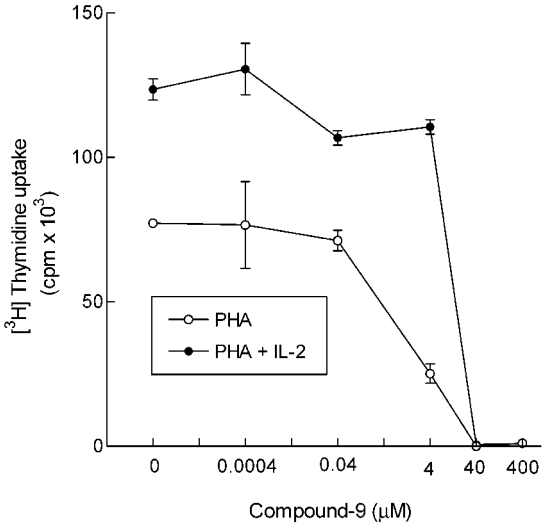

Figure 7. Interleukin 2 (IL-2) postpones the inhibitory effect of **9** on PBMC proliferation.

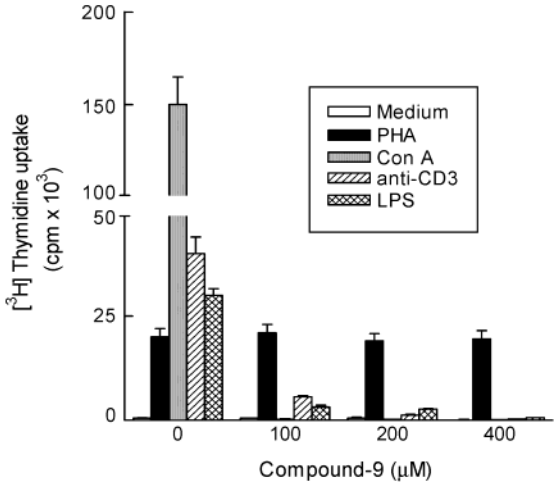

Figure 8. Compound **9** inhibits the proliferative response of splenocytes to mitogens.

We found that **9** inhibits the growth of HL-60 myeloid leukemia cells in culture (IC_{50} = 4 μM) and that the mechanism by which it achieves this cytotoxic effect is by activating apoptosis. Treatment of HL-60 cells with **9** induces morphological changes and internucleosomal DNA fragmentation characteristic of apoptotic cell death and also is associated with activation of caspase-3 by inducing cleavage of the proenzyme procaspase-3. However, **9** does not induce toxicity in T lymphocytes or in murine splenocytes. These findings are very similar to those found for the thiol alkylating agent imexon, a cyanoaziridine derivative that induces apoptosis in multiple myeloma,[21] whereas normal lymphocytes are less sensitive.[22]

In summary, we found that compound **9**, which differs in its primary cellular targeting from classical chemotherapeutic drugs, possesses extremely interesting properties with regard to its apoptosis-inducing ability in HL-60 cells.

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Supporting Information Available: Experimental procedures and spectral and analytical data for compound **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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