Synthesis and In Vitro Cytotoxic Activity of Semisynthetic Derivatives in the Santonin Series

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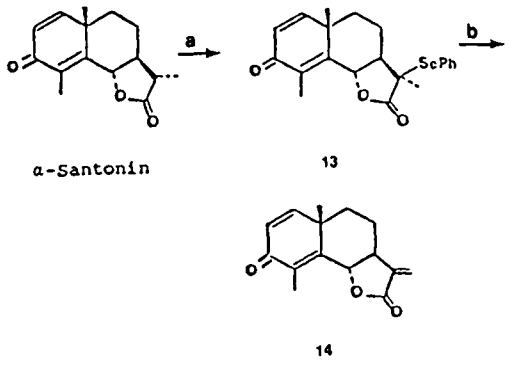
Abstract □ The synthesis of two new santonin derivatives namely 3-oxo-6β-H-11β-phenylselenoeudesm-1,4-dien-6,13-olide **(13)** and 3-oxo-6βH-eudesm-1,4,11-trien-6,13-olide **(14)** is reported along with the results of a series of santonins tested for activity against the growth of KB cells in vitro, a human epidermoid nasopharynx carcinoma. Select compounds were found to be active at concentrations lower than 5×10^{-5} M. In particular, the compound 2α-bromo-3β-hydroxy-6βH-eudesm-11-en-6,13-olide **(11)** exhibits an extremely low ID_{50} value at 0.33×10^{-6} M. Some relationships between chemical structure and cytotoxic activity are suggested, i.e. the α-methylene-γ-lactone moiety appears to be necessary for cytotoxic activity toward KB cells growth in vitro by santonin derivatives.



Scheme I—*Reagents and conditions were* (a) *Lithium diisopropylamide/Ph_2Se_2–Hexamethylphosphoramide/−78 °C,* (b) *H_2O_2/O °C.*

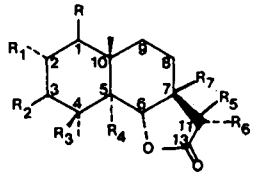
Sesquiterpene lactones are characteristic constituents of various genera of *Compositae*. During the past three decades approximately 1000 sesquiterpene lactones have been isolated, identified, and, in some cases, synthesized.[1] The interest in their bioactive properties has gradually increased since they appear to possess pronounced anti-inflammatory,[2] antibacterial,[3] and cytotoxic activity. The last property is of a particular interest since some of them are effective, because of their cell growth inhibitory activity,[4–9] against numerous tumor models.

Recently we described the synthesis of two nor-sesquiterpene-γ-lactones 1 and 2,[10,11] which occur naturally in *Crepis Pygmaea* (Compositae), and of a number of their semisynthetic derivatives 3–12.[12,13] In the present paper the synthesis of the new derivatives 13 and 14 is reported along with the results of the in vitro cytotoxic assays of compounds 1–14.

In particular it was important to evaluate the biological effect of the phenylseleno derivatives 7–9 and 13 in view of a recent report[14] indicating the interesting biological activity of a number of phenylseleno derivatives characterized as potential "pro-cytotoxic" agents. Thus our phenylseleno derivatives 7, 8, 9, and 13 were tested in order to compare their biological activity with that of the parent α-methylene-γ-lactones 10, 11, and 14. In compounds 7, 8, 9 and 13 the SePh group is part of the lactone ring and not the side chain as in the previous report.[14]

Compounds 13 and 14 were obtained following the pathway shown in Scheme I. In particular, α-santonin was converted into compound 13 (21%) using a previously described method.[15] The final product exhibited in the ^1H NMR spectrum a new group of bands at δ 7.7–7.3 ppm, typical of the aromatic protons, and a singlet at δ 1.60 ppm, due to the appearance of a tertiary methyl group. In addition, the doublet at δ 1.30 ppm, attributed to the secondary methyl group in the starting material, had disappeared. All these features are characteristic of structure 13.

Compound 14 was obtained in 81% yield by the action of hydrogen peroxide (30%) on 13. The presence in the ^1H NMR spectrum of 14 of the doublets at δ 6.67 and 6.20 ppm, characteristic of the methylene group, in addition to the disappearance of the aromatic protons at ~δ 7 ppm and of the signal at δ 1.60 ppm, attributed to the tertiary methyl group, indicated that the desired reaction had occurred.



Results and Discussion

The potential cytotoxic activity of the sesquiterpenes reported in this paper was evaluated according to the method of Geran et al.[16] by testing the compounds in vitro against the growth of human KB tumor cells (Table I). Most compounds, dissolved in Me_2SO, exhibited a significant cytotoxic effect, and, in particular, compounds 4, 10, 11, 12 and 14 were shown to be very active; lactone 11 was found to afford inhibition of growth equal to 6-mercaptopurine (6-MP), used for comparative purposes on a μg/mL basis. However, its ID_{50} value, when expressed in a molar concentration, is about three times lower than that observed for 6-MP toward KB cell growth.

From the aforementioned data some observations are evident about the relationship between the chemical structure and the cytotoxic effect. The presence of bromine in C–2 does not seem to influence particularly the cytotoxic activity, although compounds 3 and 4 were more active than 2 and 6. The stereochemistry of the hydroxyl group at C–3 markedly influences the biological activity (see i.e. compounds 4 and 7 and their respective stereoisomers 5 and 8). The difference in activity may be due to the stereospecific interaction of the

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Table I—Cytotoxic activity against KB cells

Compounds	R	R_1	R_2	R_3	R_4	R_5	R_6	R_7	ID_{50}, μg/mL[a]	
									Me_2SO[b]	EtOH
1		=	O	H	H	OH	=		9.36 (3.83×10^{-5})	—
2	H	H	O	H	H	OH	=		12.53 (4.60×10^{-5})	—
3	H	Br	O	H	H	OH	=		7.50 (2.28×10^{-5})	—
4	H	Br	α-OH	H	H	H	CH_3	H	0.56 (1.69×10^{-6})	—
5	H	Br	β-OH	H	H	H	CH_3	H	inactive	—
6	H	H	OH	H	H	H	CH_3	H	inactive	—
7	H	Br	α-OH	H	H	SePh	CH_3	H	inactive	3.66 (7.53×10^{-6})
8	H	Br	β-OH	H	H	SePh	CH_3	H	inactive	11.73 (2.41×10^{-5})
9	H	H	OH	H	H	SePh	CH_3	H	11.97 (2.94×10^{-5})	3.96 (9.73×10^{-6})
10	H	Br	α-OH	H	H		=	H	1.75 (5.32×10^{-6})	—
11	H	Br	β-OH	H	H		=	H	0.11 (0.33×10^{-6})	—
12	H	H	O	H	H		=	H	0.81 (3.27×10^{-6})	—
13		=	O		=	SePH	CH_3	H	11.84 (2.95×10^{-5})	8.62 (2.15×10^{-5})
14		=	O		=		=	H	0.48 (1.97×10^{-6})	—
6-MP[c]									0.13 (0.85×10^{-6})	—

[a] The ID_{50} values (compound concentration at which the cells showed a 50% inhibition of growth) are indicated in molar concentrations in parentheses. [b] Compounds dissolved incompletely in Me_2SO. [c] 6-Mercaptopurine.

OH with the corresponding site on the biological nucleophile or protein. However the presence itself of the hydroxyl groups and their respective space configurations has little effect on the cytotoxic effect when the α-methylene-γ-lactone moiety is present in the molecule (see compounds **10, 11** and **12**). In fact the basic structural requirement for the high activity of these sesquiterpenes appears to be associated with the presence of an exocyclic methylene group in the γ-lactone ring (see compounds **10, 11, 12** and **14**). This is in agreement with what has been reported concerning the important role played by this moiety in enzyme inhibition by Michael addition type reactions.[7,17,18] The only exception to this behavior is represented by compound **4** which displays an unexpectedly high activity in spite of the lack of α-methylene-γ-lactone moiety.

The introduction of endo conjugation into the γ-lactone ring as well as the presence of an additional hydrophilic function in C–11, on the contrary (see compounds **1, 2** and **3**), does not contribute to the biological effect. This reduction in activity may be ascribed to the fact that endocyclic α,β-unsaturated γ-lactones react slowly with cysteine to form unstable adducts as noted by Kupchan et al.[19]

The phenylselenide derivatives **7, 8, 9** and **13**, exhibited different results when tested in Me_2SO or in 95% EtOH. Enhancement of cytotoxic activity was observed when compounds **7, 8, 9,** and **13** were dissolved in EtOH. However these phenylselenide derivatives exhibited only poor activity against KB cell growth suspended in either vehicle with the exception of compounds **7** and **9** in EtOH.

In these compounds the SePh group is attached to the lactone ring and is not part of the side chain as previously reported for the effective "pro-cytotoxic" agents.[14] The low cytotoxic activity observed for these compounds may be due to the fact that the nuclear SePh group is probably quite stable thus preventing the biological activation of these "masked" compounds.

Experimental Section

Melting points were measured using a Kofler hot stage apparatus and are uncorrected. IR spectra were determined in $CHCl_3$ solution with a Perkin-Elmer 1320 spectrophotometer. The mass spectra were recorded with an AEI MS 902 spectrometer (70 eV; 150 °C). UV spectra were measured with a Perkin-Elmer 551S DB instrument in ethanol solution. ^1H NMR spectra were recorded with a Varian EM-390 (90 MHz) instrument in $CDCl_3$ solution, using Me_4Si as an internal standard. ^{13}C NMR spectra were recorded with a Varian XL-100 (25.2 MHz) instrument. $[\alpha]_D$ values were measured with a Roussel Jouan 71 in $CHCl_3$ solution. Merck DS-Alufolien Kieselgel pre-coated plates were used for TLC; for detection of spots the plates were exposed to a UV lamp and/or sprayed with phosphomolybdic acid (10% methanol solution) and with H_2SO_4 (10% aqueous solution) and heated at 120 °C for 5 min. The compounds exhibited satisfactory elemental analyses (±0.4%, C, H, Se; Carlo Erba Elemental Analyzer Mod. 1106, Institute of Organic Chemistry, University of Perugia, Italy).

3-Oxo-6βH-11β-phenylselenoeudesm-1,4-dien-6,13-olide (13)—To a solution of 24 mmol of lithium diisopropylamide (LDA, prepared from 3.5 mL of diisopropylamine and 16 mL of 1.65 M butyllithium in *n*-hexane under nitrogen at −78 °C) in 25 mL of anhydrous tetrahydrofuran (THF) was added dropwise over a period of 1 h, a solution of 2.46 g (10 mmol) of α-santonin in 15 mL of THF. After enolate formation was complete, 3.75 g (12 mmol) of diphenylselenide in 10 mL of THF containing 2.15 g (12 mmol) of hexamethylphosphoramide (HMPA) was rapidly added in a dropwise manner at −78 °C. The mixture was stirred at −78 °C for 40 min, then warmed to −40 °C for 1.5 h. The mixture was quenched by the addition of 10% HCl and diluted with ether. The organic phase was washed with water and sat. NaCl, dried over $MgSO_4$, and the solvent was removed under reduced pressure.

Column chromatography of the yellow crystalline residue on silica gel (eluant-$CHCl_3$) afforded 2.25 g of starting material and 0.850 g of the α-phenylseleno lactone **13** as white cubes (21% yield), which was recrystallized twice from ether, mp 192–194 °C, R_f = 0.60 (Et_2O); EIMS: *m/z* 401 (M^+); $[\alpha]_D$ −67° (c 1.27 $CHCl_3$); UV max: 202 nm (ε 22 000), 224 (ε 18 900); IR: 1775, 1665 cm^{-1}; ^1H NMR: δ 7.7–7.3 (m, 5, ArH), 6.63 (d, 1, *J* = 9.6 Hz, H-2), 6.20 (d, 1, *J* = 9.6 Hz, H-1), 5.17 (dd, 1, *J* = 12 and 0.5 Hz, H-6), 2.13 (d, 3, *J* = 0.5 Hz, 4-CH_3), 1.60 (s, 3, 11-CH_3) and 1.33 ppm (s, 3, 10-CH_3); ^{13}C NMR: δ 184.87 (C-3), 172.23 (C-12), 153.88 (C-1), 150.14 (C-5), 137.05 (C-3′ and C-5′), 128.87 (C-4′), 128.13 (C-2′ and C-6′), 127.63 (C-4), 124.66 (C-2), 122.92 (C-1′), 78.21 (C-6), 56.31 (C-7), 47.84 (C-11), 40.24 (C-10), and 36.27 (C-9).

3-Oxoeudesm-6βH-1,4,11-trien-6,13-olide(14)—A solution of **13** (1.2 g, 3 mmol) in THF (38 mL) containing 0.45 mL of glacial acetic acid was cooled to 0 °C and then 2.16 mL of 30% hydrogen peroxide was added. The mixture was stirred for 30 min at 0 °C; poured into cold sat. Na_2CO_3 and extracted with $CHCl_3$. The organic phase was dried over $MgSO_4$ and the solvent removed under reduced pressure. Column chromatography on silica gel (eluant $CHCl_3$), afforded 600 mg of **14** (82% yield) which crystallized from ether as white needles, mp 149–150 °C, R_f = 0.58 (Et_2O) EIMS: *m/z* 244 (M^+; $[\alpha]_D$ −88° (c 3.52 $CHCl_3$); UV max: 205 nm (ε 16 400), 234 (ε 9200); IR: 1775, 1665 cm^{-1}; ^1H NMR: δ 6.67 (d, 1, *J* = 9.6 Hz, 2-H), 6.20 (d, 1, *J* = 9.6 Hz, 1-H), 6.15 (d, 1, *J* = 3 Hz, 11-CH_2), 5.52 (d, 1, *J* = 3 Hz, 11-CH_2), 4.70 (dd, 1, *J* = 12 and 0.5 Hz, 6-H), 2.13 (d, 3, *J* = 0.5 Hz, 4-CH_3), and 1.30 ppm (s, 3, 10-CH_3); ^{13}C NMR: δ 184.93 (C-3), 169.39 (C-12), 153.96 (C-1), 150.04 (C-5), 136.40 (C-11), 127.34 (C-4), 124.56 (C-2),

118.44 (C-13), 80.25 (C-6), 49.14 (C-7), 40.25 (C-10), 36.46 (C-9), 23.96 (C-15), 20.41 (C-8), and 9.62 (C-14).

Determination of Cytotoxic Activity—An established cell line of human epidermoid carcinoma of the nasopharynx (KB cells) (Flow Laboratories Ltd)[20] was employed for the cytotoxic assay using the method of Geran et al.[16] The cells were grown in 25 cm^2 tissue culture flasks with Eagle's Minimal Essential Medium (MEM) (Difco) supplemented with 10% newborn calf serum (GIBCO, Grand Island, NY), 10% nonessential amino acids (GIBCO), and glutamine 2 mM and buffered with TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 3 mM), BES (*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 3 mM), HEPES (*N*,2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 3 mM), and TRICINE (*N*-tris(hydroxymethyl)methylglycine, 3 mM).[21] For the in vitro cytotoxic assay the cells in logarithmic growth phase which were refed 24 h before testing were used. The cells were treated for 5 min at 37 °C with 0.05% 1:250 trypsin solution and then suspended in MEM to obtain a concentration of 10^5 cells/mL. One milliliter was seeded in each Leighton tube (Bellco). The samples were allowed to incubate for 24 h at 37 °C. After this time interval, the viable cells were attached to the bottom of the tubes. The tubes were regrouped at random and the base line was evaluated in five of these by counting with a Bürker chamber the cells detached from the glass surface by trypsin solution.[22] The culture medium of the other Leighton tubes was discarded and the cells were fed with 4 mL MEM (control tubes) and with 4 mL MEM containing the compounds to be tested (treated tubes). The compounds were dissolved immediately before use in sterile Me_2SO. Nevertheless, since the SePh derivatives appeared to dissolve incompletely in this solvent, they were tested also using 95% EtOH as vehicle. Further dilutions were performed with the growth medium to the desired drug concentration. The final solvent concentration in MEM (0.5% in every tube) was previously tested by us and did not show cytotoxic effect. At least five concentration levels were used for each compound and each concentration value was tested in triplicate. The incubation was carried out at 37 °C for 72 h, the time interval in which exponential growth occurs. Cell growth was estimated by counting the viable cells as previously described.[22] The cytotoxic activity was evaluated as growth inhibition percentage of the present cells in the treated tubes with respect to the controls on the basis of the formula: $100 - [T - B)/(C - B) \times 100]$, where B was the base line and T and C were the number of viable cells, respectively, in the treated and the control tubes after 72 h incubation. The significance of these results was evaluated by use of the t test ($p < 0.01$). The inhibition values were plotted against log D, where D is the drug concentration in μg/mL of MEM. From these curves the ID_{50} values were obtained, where ID_{50} is the compound concentration at which the cells showed 50% growth inhibition in relation to the control values. The ID_{50} of 4 μg/mL was chosen as the upper limit criterion for a significant level of activity. Positive control values with 6-mercaptopurine were also determined.

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