# Cytotoxic Sesquiterpenoids from *Ratibida columnifera*

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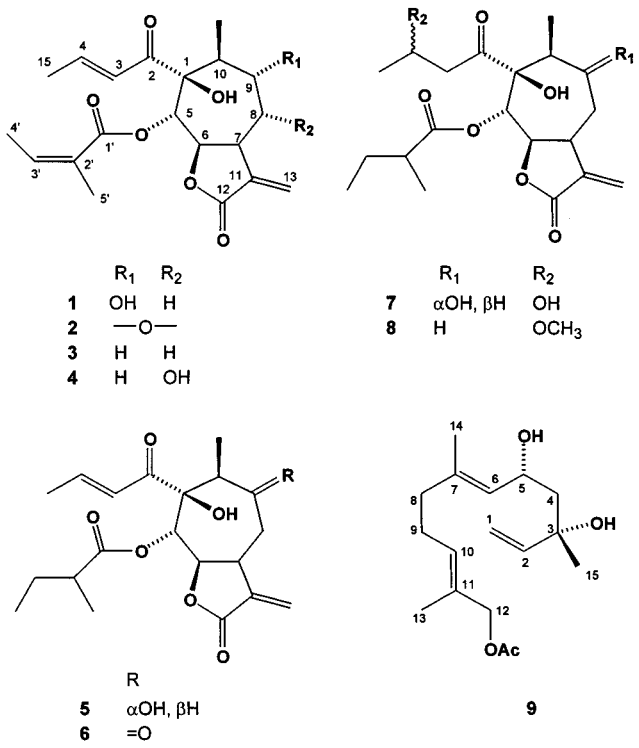
Bioassay-directed fractionation of the flowers and leaves of *Ratibida columnifera* using a hormone-dependent human prostate (LNCaP) cancer cell line led to the isolation of 10 cytotoxic substances, composed of five novel xanthanolide derivatives (**2**−**4**, **7**, and **8**), a novel nerolidol derivative (**9**), and three known sesquiterpene lactones, 9α-hydroxy-*seco*-ratiferolide-5α-*O*-angelate (**1**), 9α-hydroxy-*seco*-ratiferolide-5α-*O*-(2-methylbutyrate) (**5**), 9-oxo-*seco*-ratiferolide-5α-*O*-(2-methylbutyrate) (**6**), as well as a known flavonoid, hispidulin (**10**). On the basis of its cytotoxicity profile, compound **5** was selected for further biological evaluation, and was found to induce G1 arrest and slow S traverse time in parental wild type p53 A2780S cells, but only G2/M arrest in p53 mutant A2780R cells, with strong apoptosis shown for both cell lines. The activity of **5** was not mediated by the multidrug resistance (MDR) pump, and it was not active against several anticancer molecular targets (i.e., tubulin polymerization/depolymerization, topoisomerases, and DNA intercalation). While these results indicate that compound **5** acts as a cytotoxic agent via a novel mechanism, this substance was inactive in in vivo evaluations using the murine lung carcinoma (M109) and human colon carcinoma (HCT116) models.

As part of a search for novel naturally occurring plant-derived anticancer agents, we have investigated separate batches of the flowers and leaves of *Ratibida columnifera* (Nutt.) Wood & Standl. (Compositae). The chloroform-soluble extracts from these plant parts were significantly cytotoxic when evaluated against a hormone-dependent human prostate (LNCaP) cancer cell line. Four species of the genus *Ratibida* have been investigated previously, namely, *R. columnifera*, *R. latipalearis*, *R. mexicana*, and *R. peduncularis*. The isolation of hispidulin (6-methoxy-5,7,4′-trihydroxyflavone) from *R. columnifera* was first reported in 1972 by Carman et al.[1] The aerial parts of *R. columnifera* were studied chemically in 1985, and a number of sesquiterpene lactones were obtained, along with several other compounds.[2] In 1987, 10 xanthanolides, a nerolidol derivative, a phenylpropanoid, and a tetrayne thioenol analogue were reported from the aerial parts of *R. columnifera* and *R. peduncularis*.[3] Mata et al. reported the isolation of the sesquiterpene lactones, ratibinolide I,[4] ratibinolide II, and ratibinolide III,[6] from the aerial parts of *R. latipalearis*. Ratibinolides I and II showed cytotoxic activity with the P388 murine cell-culture system ($ED_{50}$ values of 2.3 and 0.2 μg/mL, respectively).[6] The roots of *R. mexicana* have afforded two sesquiterpene lactones which showed inhibition of radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crus-galli*, and demonstrated moderate cytotoxic activity with three human solid tumor cell lines.[7]

The cytotoxic constituents of *R. columnifera* have not been studied previously. In this communication, we report the isolation and structure characterization of six new constituents, five xanthanolides (**2**−**4**, **7**, and **8**) and a nerolidol derivative (**9**), along with three known xanthanolides (**1**, **5**, and **6**) and hispidulin (**10**) (Chart 1). Compounds **1**−**4**, **9**, and **10**, and compounds **5**−**8** were isolated from the flowers and leaves of *R. columnifera*, respectively. All 10 compounds from *R. columnifera* were evaluated for in vitro cytotoxic activity against a panel of human cancer cell lines, to determine if any selective cytotoxicity was evident.

**Chart 1**




Of the cytotoxic isolates obtained in this study, compound **5** [9α-hydroxy-*seco*-ratiferolide-5α-*O*-(2-methylbutyrate)]

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was selected for additional biological and mechanism of action evaluations, comprising cytotoxicity testing against a panel of 25 cell lines, and effects on the cell cycle, apoptosis, tubulin proliferation, DNA intercalation, and inhibitory activity against topoisomerases. In addition, compound **5** was subjected to in vivo evaluations using murine lung carcinoma (M109) model and human colon carcinoma (HCT116) models.

## Results and Discussion

In preliminary experiments, separate $CHCl_3$-soluble extracts of the flowers and leaves of *R. columnifera* were found to be significantly cytotoxic against the LNCaP (hormone-dependent human prostate) cell line ($ED_{50}$ 0.5 and 0.8 $\mu$g/mL, respectively). Using this cell line to direct the fractionation of the $CHCl_3$-soluble extract of *R. columnifera* flowers, compound **1** was isolated as a major active constituent and identified as the known compound, 9α-hydroxy-*seco*-ratiferolide-5α-*O*-angelate,[3] accompanied by the known flavonoid, hispidulin (**10**).[10−12] Compounds **5** and **6**, isolated as active compounds from the leaves of *R. columnifera*, were identified as 9α-hydroxy-*seco*-ratiferolide-5α-*O*-(2-methylbutyrate) and 9-oxo-*seco*-ratiferolide-5α-*O*-(2-methylbutyrate),[3] respectively. All four of these known compounds were obtained previously from the aerial parts of this plant and their structural identifications were carried out by comparison with published physical and spectral data.[3,8,9]

The novel compound **2**, isolated from the flowers, was assigned a molecular formula of $C_{20}H_{24}O_7$ (376.1517 Da) on the basis of HREIMS. Its IR spectrum showed three carbonyl group absorbances at 1772, 1729, and 1692 $cm^{-1}$, indicating the presence of two ester or lactone groups and one conjugated carbonyl group. The $^1H$ and $^{13}C$ NMR spectra of **2** were closely comparable to those of compound **1**, suggesting that the two compounds are based on the same carbon skeleton. In the $^1H$ NMR spectrum of **2**, signals assigned to H-8 and H-9 in **1** were replaced by a multiplet at $\delta$ 3.16 (2H) which was correlated to the $^{13}C$ NMR signals at $\delta$ 56.3 (d) and 56.8 (d), according to the $^1H-^1H$ COSY, homo-decoupling, and $^1H-^{13}C$ HETCOR NMR experiments. These observations indicated the presence of an 8,9-epoxy function in **2**, which was supported by a molecular ion peak at *m/z* 376 in its EIMS, 2 Da less than that of **1**. The $^1H$ NMR signals at $\delta$ 5.47 (1H, d, $J$ = 10.7 Hz), 5.09 (1H, dd, $J$ = 8.6, 10.7 Hz), and 3.51 (1H, m) assigned to H-5, H-6, and H-7, respectively, were very similar to those of **1**, indicating the presence of the same 5α,6$\beta$,7$\beta$-configuration. In a NOE difference NMR experiment on **2**, irradiation of H-5 ($\delta$ 5.47) led to enhancement of the H-8, H-9 signal ($\delta$ 3.16, 8.4%), as well as H-13b ($\delta$ 6.04, 4.6%), while irradiation of H-6 ($\delta$ 5.09) did not lead to the enhancement of H-8 and H-9, indicating that the epoxy group has a 8α,9α-*cis*-stereochemistry, which was also supported by the magnitude of the coupling constant between H-9 and H-10 ($J_{9,10}$ = 8.5 Hz). By comparing the $^1H$ and $^{13}C$ NMR spectra of **2** with those of (1*S*,5*S*,6*R*,7*R*,8*R*,9*S*,10*S*,11*R*)-5-angeloyloxy-8,9-epoxy-1-hydroxy-2-oxoxanth-3-en-6,12-olide, a related sesquiterpene lactone previously isolated from the aerial parts of this plant,[3] similar signals were apparent except for the lactone moiety. The partial structure of the latter in **2** was established as an 11-en-6,12-olide based on the $^1H$ NMR signals at $\delta$ 6.51 (1H, d, $J$ = 2.7 Hz) and 6.04 (1H, d, $J$ = 2.5 Hz), and the $^{13}C$ NMR signals at $\delta$ 136.4 (s, C-11), 168.0 (s, C-12), and 125.9 (t, C-13), in direct comparison with the same signals for **1**. All $^1H$ and $^{13}C$ NMR assignments for **2** were made by performing appropriate $^1H-^1H$ COSY, homo-decoupling, $^1H-^{13}C$ HETCOR, APT, SFORD, and selective INEPT NMR experiments. Hence, the structure of **2** was established as (1*S*,5*S*,6*R*,7*R*,8*R*,9*S*,10*S*)-5-angeloyloxy-8,9-epoxy-1-hydroxy-2-oxoxantha-3,11-dien-6,12-olide.

The elemental formula of compound **3** was suggested as $C_{20}H_{26}O_6$ by its HREIMS at *m/z* 362.1729 ($[M]^+$, calcd 362.1728), one oxygen atom less than that of **1**. The $^1H$ NMR signals of **3** were very similar to those of **1** except that only two methine protons bearing oxygen atoms were observed in **3**, and were assignable to H-5 ($\delta$ 5.31, 1H, br d, $J$ = 10.5 Hz) and H-6 ($\delta$ 5.01, 1H, br dd, $J$ = 8.3, 10.5 Hz), respectively. The $^{13}C$ NMR spectrum of **3** exhibited two methylene carbon signals at $\delta$ 28.7 (t) and 27.6 (t) due to C-8 and C-9, respectively, according to APT and SFORD NMR experiments, which indicated the absence of a hydroxyl substituent at either the C-8 or the C-9 position. Comparison of the $^1H$ and $^{13}C$ NMR spectra of **3** with analogous data for the related compound, (1*S*,5*S*,6*R*,7*S*,-10*S*,11*R*)-5-angeloyloxy-1-hydroxy-2-oxoxanth-3-en-6,12-olide,[3] isolated previously from the aerial parts of *R. columnifera*, indicated a close correlation, except for those resonances due to the lactone moieties. The $^1H$ NMR signals observed for **3** at $\delta$ 6.34 (1H, d, $J$ = 3.0 Hz, H-13a) and 5.61 (1H, brs, H-13b), and the $^{13}C$ NMR signals at $\delta$ 137.9 (s, C-11), 169.3 (s, C-12), and 123.1 (t, C-13), were attributable to an 11-en-6,12-olide system, as in the case of **2**. The molecular ion peak at *m/z* 362, and major ion fragments at *m/z* 293 $[M - C_4H_5O]^+$ and 193 $[M - C_4H_5O$ - ang$]^+$ in the EIMS of **3**, were consistent with the structure proposed for this compound. Consequently, compound **3** was assigned as (1*S*,5*S*,6*R*,7*S*,10*S*)-5-angeloyloxy-1-hydroxy-2-oxoxantha-3,11-dien-6,12-olide.

Compound **4**, having a molecular formula of $C_{20}H_{26}O_7$ as determined by HREIMS, exhibited the same molecular ion peak at *m/z* 378, and characteristic ion peaks at *m/z* 309 and 209 in the low-resolution EIMS, as observed for compound **1**. The $^1H$ NMR spectrum of **4** displayed very similar signals and coupling constants to those of **1**, with slight differences of the signals due to H-3 (+0.25 ppm), H-4 (−0.11 ppm), H-5 (+0.20 ppm), H-7 (−0.32 ppm), H-13b (+0.23 ppm), and H-14 (−0.10 ppm). The presence of a hydroxyl group at the C-8 position was indicated on the basis of a $^1H-^1H$ COSY correlation between H-7 ($\delta$ 3.39) and H-8 ($\delta$ 3.83), and supported by the observation of another correlation between H-10 ($\delta$ 2.45) and a methylene (APT, HETCOR) proton ($\delta$ 2.38, H-9a). In addition, irradiation ($^3J_{CH}$ = 6 Hz) of H-14 ($\delta$ 0.82, 3H, d, $J$ = 6.0 Hz) in a selective INEPT NMR experiment on **4** led to the enhancement of the C-1 and C-9 resonances at $\delta$ 83.6 (s) and 38.3 (t), respectively, giving further evidence for the location of a hydroxyl group at C-8. The stereochemistry of the C-8 hydroxyl group was established as having an α-configuration on the basis of observed $^1H-^1H$ NOE difference correlations between H-5, H-8, HO-1, and H-13b. Irradiation of H-5 ($\delta$ 5.55) led to the enhancement of H-8 ($\delta$ 3.83, 5.4%), OH-1 ($\delta$ 4.29, 3.8%), and H-13a ($\delta$ 6.37, 3.8%). The $^1H$ and $^{13}C$ NMR assignments for this isolate were achieved using appropriate $^1H-^1H$ COSY,$^1H-^{13}C$ HETCOR, and selective INEPT NMR methods. Thus, the structure of compound **4** was assigned as (1*S*,5*S*,6*R*,7*R*,8*S*,10*S*)-5-angeloyloxy-1,8-dihydroxy-2-oxoxantha-3,11-dien-6,12-olide.

Compounds **7** and **8** were found to have the molecular formulas $C_{20}H_{30}O_8$ and $C_{21}H_{30}O_8$, respectively, as determined by their HREIMS data. The $^1H$ NMR spectra of **7** and **8** showed that their structures were, in turn, very close

**Table 1.** $^{13}C$ NMR Spectral Data for Compounds **1**−**5**, **7**, and **8**[a]

| | compound | | | | | | |
|---|---|---|---|---|---|---|---|
| carbon | **1** | **2** | **3** | **4** | **5**[b] | **7** | **8** |
| 1 | 82.5 s | 83.9 s | 83.4 s | 83.6 s | 82.2 s | 83.1 s | 81.8 s |
| 2 | 197.4 s | 199.2 s | 199.3 s | 200.9 s | 196.8 s | 211.8 s | 211.9 s |
| 3 | 125.2 d | 125.1 d | 125.2 d | 126.2 d | 125.3 d | 47.7 t | 47.7 t |
| 4 | 145.9 d | 146.8 d | 145.8 d | 144.9 d | 145.6 d | 63.9 d | 73.0 d |
| 5 | 78.4 d | 78.6 d | 78.7 d | 78.8 d | 78.6 d | 77.9 d | 75.5 d |
| 6 | 78.4 d | 76.0 d | 77.9 d | 78.8 d | 77.0 d | 76.2 d | 75.8 d |
| 7 | 41.4 d | 41.8 d | 38.9 d | 45.2 d | 34.1 d | 34.2 d | 36.1 d |
| 8 | 37.1 t | 56.8 d | 28.7 t | 64.9 d | 37.1 t | 37.8 t | 44.0 t |
| 9 | 71.1 d | 56.3 d | 27.6 t | 38.3 t | 70.9 d | 70.4 d | 207.1 s |
| 10 | 34.2 d | 39.9 d | 35.0 d | 33.9 d | 41.4 d | 42.4 d | 48.8 d |
| 11 | 137.6 s | 136.4 s | 137.9 s | 134.8 s | 137.6 s | 137.8 s | 137.0 s |
| 12 | 168.9 s | 168.0 s | 169.3 s | 168.2 s | 168.8 s | 176.6 s | 167.8 s |
| 13 | 123.9 t | 125.7 t | 123.1 t | 126.5 t | 123.8 t | 124.1 t | 124.2 t |
| 14 | 12.8 q | 11.9 q | 15.3 q | 15.5 q | 12.7 q | 12.6 q | 11.4 q |
| 15 | 18.6 q | 18.7 q | 18.5 q | 18.5 q | 18.4 q | 22.6 q | 18.9 q |
| 1′ | 166.9 s | 168.0 s | 167.7 s | 169.9 s | 175.6 s | 168.6 s | 175.5 s |
| 2′ | 126.3 s | 126.3 s | 126.4 s | 127.9 s | 40.9 d | 40.9 d | 41.0 d |
| 3′ | 141.0 d | 141.7 d | 141.0 d | 141.1 d | 26.2 t | 26.5 t | 26.7 t |
| 4′ | 15.8 q | 15.9 q | 15.8 q | 16.1 q | 11.4 q | 11.3 q | 9.0 q |
| 5′ | 19.9 q | 20.0 q | 20.2 q | 20.5 q | 16.1 q | 16.2 q | 16.1 q |
| $OCH_3$ | | | | | | | 56.0 q |

[a] Chemical shifts given in ppm using TMS as internal reference in $CDCl_3$ (90.8 MHz). [b] $^{13}C$ NMR data for this known compound were not reported previously.[3]

to those of **5** and **6**, and that only signals due to the C-1 side chain were different. The C-1 side chains in **7** and **8** appeared to be saturated due to the absence of any olefinic carbon signals at the C-3 and C-4 positions when compared with the $^{13}C$ NMR data of **5** and **6**. Moreover, a signal at $\delta$ 4.26 (1H, m) in **7** and $\delta$ 3.80 (1H, m) in **8**, respectively, indicated the presence of a methine proton bearing an oxygen atom in the C-1 side chain of each molecule, which was supported by the corresponding carbon signals at $\delta$ 63.9 (d, C-4) and 73.0 (d, C-4) in the $^{13}C$ NMR spectra of **7** and **8**. The presence of an additional methoxy group in the C-1 side chain of **8** was suggested by a proton signal at $\delta$ 3.29 (3H, s) and a carbon signal at $\delta$ 56.0 (q) based on the analysis of the $^1H$ and $^{13}C$ NMR spectra of **6** and **8**. The C-15 methyl groups in **7** and **8** were observed as doublets at $\delta$ 1.25 ($J$ = 6.2 Hz) and 1.24 ($J$ = 6.1 Hz) in their $^1H$ NMR spectra, and were shifted upfield 0.69 and 0.70 ppm when compared with the analogous data of **5** and **6**. This suggested that **7** and **8** were a $\beta$-hydroxy and a $\beta$-methoxy ketone derivative, respectively, observations supported by comparison of the $^{13}C$ NMR spectra of **7** and **8** with those of the C-4 epimeric $\beta$-hydroxyketones (1*S*,5*S*,6*R*,7*S*,10*S*,11*R*)-5-angeloyloxy-1,4-dihydroxy-2-oxoxanthan-6,12-olide, previously isolated from the aerial parts of the same plant.[2] An attempt to use Horeau's method to distinguish between these two C-4 hydroxyketone epimers resulted in dehydration, and the absolute configurations at C-4 could not be determined.[2] Accordingly, compounds **7** and **8** also were not derivatized in this manner in the present investigation, and their structures were elucidated as (1*S*,5*S*,6*R*,7*S*,9*R*,10*S*)-5-methylbutanoyloxy-1,4,9-trihydroxy-2-oxoxanth-11-en-6,12-olide and (1*S*,5*S*,6*R*,7*S*,10*R*)-1-hydroxy-4-methoxy-5-methylbutanoyloxy-2,9-dioxoxanth-11-en-6,12-olide, respectively.

The elemental formula of compound **9** was suggested as $C_{17}H_{28}O_4$ by positive-ion HRFABMS of the apparent molecular ion peak at *m*/*z* 297.2081 ($[M + H]^+$, calcd 297.2066). Analysis of its $^1H$ and $^{13}C$ NMR spectra indicated that **9** was a nerolidol derivative.[2,3,10] Compound **9** showed a methyl group at $\delta$ 2.07 (3H, s) in the $^1H$ NMR spectrum, and two signals at $\delta$ 21.0 (q) and 171.4 (s) in the $^{13}C$ NMR spectrum, respectively, indicating the presence of an acetyl group in the molecule. NMR spin-decoupling and a selective INEPT experiment demonstrated that the oxygen functions were located at C-3, C-5, and C-12. The position of the acetoxyl group was inferred as being at C-12 from the NMR data of **9**, and in particular, the chemical shifts of H-10 and C-10 excluded a 13-acetoxyl group.[3] The stereochemistry was deduced by comparison of the $^1H$ and $^{13}C$ NMR spectral data with those of similar nerolidol derivatives.[2,3,10]

As summarized in Table 2, the xanthanolides **1**−**6** demonstrated broad cytotoxic activity against a panel of human cancer cell lines, of which **2** showed greatest activity ($ED_{50}$ 0.16 $\mu$g/mL) against the HT cell line. Compound **4** was in general less active than **1**−**3** and **5**−**6** in the cell line panel. The xanthanolide $\beta$-hydroxyketone derivative **7** was moderately active against most cell lines tested in the panel, except for the multidrug-resistant KB-V1 cell lines. The greatest activity for the xanthanolide methyl ether **8** was obtained when tested against the LNCaP and BC1 cell lines ($ED_{50}$ 0.5 and 1.2 $\mu$g/mL, respectively). The nerolidol derivative **9** showed weak general cytotoxic activity, with an $ED_{50}$ value of $<4$ $\mu$g/mL being observed only with the KB cell line ($ED_{50}$ 3.5 $\mu$g/mL). The flavone, hispidulin (**10**), is well-known for its cytotoxic properties,[11] and was first identified from the leaves of *Ambrosia hispida*.[12] In the present investigation, **10** was found to be active when evaluated against the KB-V1 cell line (in the presence of vinblastine) and ZR-75-1 cell line ($ED_{50}$ 1.3 and 1.2 $\mu$g/mL, respectively). None of the test materials mediated an antimitotic response, as evaluated in the ASK test system.

Several of these pure constituents of *R. columnifera* were evaluated for cytotoxicity in a 25 cell line tumor panel, representing a diverse group of mouse and human tumors, fibroblasts, and normal bovine endothelial cells. Of the compounds tested, **5** [9α-hydroxy-*seco*-ratiferolide-5α-*O*-(2-methylbutyrate)] proved to be the most interesting, and exhibited a mean $IC_{50}$ of 1.46 $\mu$M and demonstrated a novel selectivity pattern. Thus, for **5**, p53 mutant A2780R cells had a similar sensitivity to parental wild-type A2780S cells (both ovarian cancer), $MDR^+$ HCT116/VVM46 cells had a similar sensitivity to $MDR^-$ HCT116 cells (both colon cancer), and leukemic cells (HL-60, CCRF-CEM) had intermediate sensitivity. Relative to the tumor cells in the

**Table 2.** Cytotoxic Activity of Isolates Obtained from *R. columnifera*[a]

| | cell line[b] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| compound | BC1 | HT | Lu1 | Mel2 | Col2 | KB | KB-V1$^+$ | KB-Vl$^-$ | A431 | LNCaP | ZR-75-1 | U373 | ASK[c] |
| **1** | 0.6 | 0.3 | 1.1 | 0.5 | 0.5 | 0.3 | 1.3 | 1.2 | 0.2 | 0.16 | 0.2 | 0.2 | − |
| **2** | 0.3 | 0.16 | 0.5 | ND | 0.8 | 0.3 | 0.9 | 0.2 | ND | ND | ND | 0.2 | − |
| **3** | 0.8 | 0.4 | 0.4 | ND | 0.9 | 0.4 | 0.3 | 1.6 | ND | 1.0 | 1.4 | 0.2 | − |
| **4** | 4.2 | ND | 2.3 | ND | 5.7 | 1.4 | 1.7 | 3.0 | ND | 0.4 | 1.4 | ND | − |
| **5** | 0.8 | ND | 0.9 | ND | 1.1 | 1.0 | 1.6 | 0.9 | ND | 0.5 | ND | ND | − |
| **6** | 0.5 | ND | 2.3 | ND | 1.2 | 1.1 | 1.1 | 2.0 | ND | 0.4 | ND | ND | − |
| **7** | 9.0 | ND | 16.8 | ND | 8.7 | 12.7 | >20 | >20 | ND | 8.1 | ND | ND | − |
| **8** | 1.2 | ND | 4.4 | ND | 3.1 | 6.6 | 5.0 | 4.0 | ND | 0.5 | ND | ND | − |
| **9** | 8.7 | 9.5 | >20 | ND | 6.2 | 3.5 | >20 | 10.9 | ND | >20 | ND | 6.3 | − |
| **10** | >20 | 10.1 | >20 | 14.6 | >20 | 18.8 | 1.3 | 8.7 | 12.8 | 7.1 | 1.2 | 8.8 | − |

[a] Results are expressed as $ED_{50}$ values ($\mu$g/mL). [b] Key: BC1 = human breast cancer; HT = human fibrosarcoma; Lu1 = human lung cancer; Mel2 = human melanoma; KB = human epidermoid carcinoma; KB-V1$^+$ = multidrug-resistant KB assessed in the presence of vinblastine (1 $\mu$g/mL); KB-V1$^-$ = multidrug-resistant KB assessed in the absence of vinblastine; A431 = human epidermoid carcinoma; LNCaP = hormone-dependent human prostate cancer; ZR-75-1 = hormone-dependent human breast cancer; U373 = human glioblastoma; ASK = human astrocytoma; ND = not determined. [c] − = not active when tested at a concentration of 20 $\mu$g/mL for the ASK cell line.

**Table 3.** Induction of Apoptosis by Compound **5** in Human Ovarian Carcinoma Cells after a 24-Hour Exposure[a]

| | % apoptotic cells | |
|---|---|---|
| concentration ($\mu$M) | A2780S (p53 wt) | A2780R (p53 mut) |
| 0 | 1.49 | 0.85 |
| 0.15 | 1.17 | 0.77 |
| 1.5 | 1.27 | 1.66 |
| 7.5 | 61.31 | 2.63 |
| 15.0 | 51.67 | 31.87 |
| 30.0 | 78.14 | 49.25 |

[a] For protocols used, see Experimental Section.

panel, "normal" bovine aortic endothelial (ABAE) cells seemed resistant.

Compound **5** was then investigated for its effects on the cell cycle and apoptosis, and was found to induce G1 arrest at the $IC_{50}$ concentration level (1.16 $\mu$g/mL) in wild-type p53 A2780S cells. At 5−10 times the $IC_{50}$ value, in addition to G1 arrest, S traverse time was reduced. The only effect on p53 mutant A2780R cells was G2/M arrest at 10 times the $IC_{50}$ value. Both the p53 wild-type and p53 mutant ovarian cancer cell lines underwent apoptosis at higher concentrations of **5**, although the p53 wild-type cells were more sensitive, requiring only half the concentration necessary for p53 mutant cells (Table 3). This cell-cycle profile was suggestive of a mechanism which was only qualitatively modified by p53, but does not significantly influence the overall potential of the two cell lines to undergo apoptosis. The unusual manner by which compound **5** altered the cell cycle prompted an evaluation of this sesquiterpene lactone in various additional assays in an attempt to identify its molecular target. However, in the concentration range 10−100 $\mu$M, **5** did not inhibit or induce tubulin polymerization, nor inhibit the catalytic activity of either topoisomerase I or topoisomerase II, nor intercalate with DNA (data not shown). Consequently, the novel cytotoxic mechanism of **5** remains unknown.

Compound **5** was evaluated in two in vivo murine xenograft models, represented by the mouse 109 murine lung carcinoma and the HCT116 human colon tumor systems. When tested in the M109 system at 8 and also at 24 mg/kg/injection (the maximum tolerated dose, MTD), compound **5** was inactive, demonstrating T/C values of 103 and 113%, respectively. In the HCT116 model planted subcutaneously in athymic mice, **5** was not found to reach a MTD and was inactive at all doses tested, with 30 mg/kg/injection being the highest dose used.

## Experimental Section

**General Experimental Procedures.** Melting points are uncorrected. Optical rotations were measured with a Perkin-Elmer model 241 polarimeter. The UV spectra were obtained using a Beckman DU-7 spectrometer and IR spectra were recorded on a Midac Collegian FT-IR spectrometer. Unless stated otherwise, $^1H$ and $^{13}C$ NMR spectra were recorded with TMS as internal standard, employing either a Nicolet-360 or a Varian XL-300 instrument (360 or 300 MHz, respectively). Low- and high-resolution mass spectra were obtained on a Finnigan MAT-90 instrument. Preparative TLC was performed on Merck silica gel G and Whatman reversed-phase $C_{18}$ plates [0.5 and 0.25 (mm) layer thickness, respectively].

**Plant Material.** Separate collections of the flowers and leaves of *R. columnifera* were made near Maple, TX, in June, 1992, and identified by one of us (J.C.T.). Voucher specimens for the flowers (A01517) and the leaves (A01515) of *R. columnifera* have been deposited at the Field Museum of Natural History, Chicago, IL.

**Extraction and Isolation.** The air-dried ground flowers (540 g) of *R. columnifera* were extracted with MeOH three times (1 × 2 L; 2 × 1.5 L) at room temperature. The resultant extracts were combined, concentrated, and diluted with $H_2O$ to afford an 80% aqueous MeOH solution, which was washed with hexanes (3 × 200 mL). The MeOH layer was concentrated, diluted with $H_2O$, and partitioned between 10% MeOH solution and $CHCl_3$ (3 × 200 mL). The $CHCl_3$-soluble extract exhibited strong cytotoxic activity in several cell lines (most potent activity, $ED_{50}$ 0.5 $\mu$g/mL against the LNCaP cell line). A portion of the $CHCl_3$-soluble extract (22 g) was chromatographed over a silica gel column eluted with hexanes−acetone mixtures (9:1 → 1:1). Seven fractions were collected and tested against the LNCaP cell line to afford two active fractions, F005 (1.2 g) and F006 (12.5 g) ($ED_{50}$ 1.1 and 1.0 $\mu$g/mL, respectively). F005 was subjected to silica gel chromatography eluted with hexanes−acetone−MeOH mixtures of increasing polarity. Fractions 11−12 (465 mg) were fractionated by RP-$C_{18}$ silica gel column chromatography and eluted with 50−60% MeOH. Subfractions 15−23 (128 mg) were purified by column chromatography over silica gel [hexanes−acetone−MeOH (5:1:0.05 → 4:1:0.05)] and preparative silica gel G TLC [benzene−EtOAc (5:1)] to yield compounds **2** (26.5 mg) and **3** (9.6 mg), respectively. F006 was absorbed on silica gel and subfractionated by silica gel column chromatography using hexanes−EtOAc−MeOH mixtures of increasing polarity as solvent system, and washed with MeOH. Subfractions 14−21 (4.6 g) were combined and purified over a reversed-phase $C_{18}$ column [MeOH−$H_2O$−$CH_3CN$ (60:35:5)] to afford **1** (2.5 g). Subfraction 12 (480 mg) was chromatographed over a RP-$C_{18}$ silica gel column eluted with 40, 45, 50, and 60% MeOH to yield fractions 19−23 which were purified by preparative TLC over silica gel G [hexanes−acetone−MeOH (11:9:1)], and finally over RP-$C_{18}$ silica gel [MeOH−$H_2O$−$CH_3CN$ (65:35:5)] to afford **4** (14.5 mg). Subfractions 7−11 (156 mg) were chromatographed over a RP-$C_{18}$ silica gel column using 45−60% MeOH as solvent system, and fractions 52−82 were purified by silica gel column chromatography [hexanes−EtOAc−MeOH (4:1:1)] and by prepara-

tive RP-$C_{18}$ silica gel TLC (70% MeOH + 1% $CH_3CN$) to yield **9** (8.6 mg). Subfractions 5−9 (120 mg) of F006 were purified by column chromatography over silica gel eluted with hexanes−EtOAc (2:1) to give **10** (5.6 mg).

The air-dried leaves (2.2 kg) of *R. columnifera* were extracted with MeOH (1 × 6 L; 2 × 4 L) at room temperature. Removal of MeOH under vacuum afforded a greenish residue which was extracted successively with hexanes (3 × 300 mL) and $CHCl_3$ (3 × 300 mL). The $CHCl_3$-soluble extract (50 g) showed significant cytotoxic activity when tested against the LNCaP cell line ($ED_{50}$ 0.8 $\mu$g/mL). The $CHCl_3$-soluble extract was chromatographed over a silica gel (1.5 kg) column eluted with hexane−acetone mixtures (8:1 → 1:1) in a gradient and afforded seven fractions which were evaluated against the same cell line. The second fraction (4.6 g) was fractionated by silica gel (150 g) column chromatography eluted with hexane−acetone mixtures (75:25 → 65:35). A subfraction eluted with 30% hexane−acetone was purified by column chromatography over silica gel [petroleum ether−EtOAc (80:20)] and Sephadex LH-20 (60 × 1.2 cm) [MeOH−$CHCl_3$ (80:20)] to yield compounds **6** (58.5 mg) and **8** (18.3 mg). The sixth fraction, derived from the initial column chromatography of the $CHCl_3$-soluble extract (6.3 g), was subjected to passage over silica gel (180 g), eluted with $CHCl_3$−MeOH mixtures (98:2 → 80:20). Subfraction 6 (5% $CHCl_3$−MeOH) was purified by repeated silica gel column chromatography using $CHCl_3$−MeOH (95:5) and petroleum ether−acetone (80:20) for elution. The final purification was effected by passage over a Sephadex LH-20 (60 × 12 cm) column eluted with MeOH−$CHCl_3$ (80:20), to afford compound **7** (11.7 mg). Subfraction 7 (6% $CHCl_3$−MeOH) was purified by silica gel [$CHCl_3$−MeOH (94:6)] and Sephadex LH-20 [MeOH−$CHCl_3$ (80:20)] column chromatogaphy to yield compound **5** (17.2 mg).

A MeOH solution of compound **6** (2 mg/mL) was incubated in a water bath at 40° C for 2 days. The resultant product was analyzed by silica gel TLC developed in hexanes−acetone (4:1), using compound **8** as a standard, and by $^1$H NMR after purification by preparative TLC. This procedure demonstrated that compound **8** was not an artifact derived from **6** during the procedure of extraction and separation.

**9α-Hydroxy-*seco*-ratiferolide-5α-*O*-angelate (1):** colorless oil; $[\alpha]_D^{20}$ −105.8° (*c* 0.35, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 220 (4.08) nm; exhibited comparable spectral (IR, $^1$H NMR, $^{13}$C NMR, EIMS) data to published values.[3]

**(1*S*,5*S*,6*R*,7*R*,8*R*,9*S*,10*S*)-5-Angeloyloxy-8,9-epoxy-1-hydroxy-2-oxoxantha-3,11-dien-6,12-olide (2):** colorless oil; $[\alpha]_D^{20}$ −107.3° (*c* 0.11, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 220 (4.26) nm; IR (dry film) $\nu_{max}$ 3421 (OH), 2925 (C−H), 1772 ($\gamma$-lactone), 1729 ($CO_2R$), 1692 (C=C=O), 1225, 1132 cm$^{-1}$; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 7.10 (1H, dq, *J* = 7.0, 15.3 Hz, H-4), 6.68 (1H, dd, *J* = 1.5, 15.3 Hz, H-3), 6.51 (1H, d, *J* = 2.7 Hz, H-13a), 6.18 (1H, dq, *J* = 1.5, 7.3 Hz, H-3′), 6.04 (1H, d, *J* = 2.5 Hz, H-13b), 5.47 (1H, d, *J* = 10.7 Hz, H-5), 5.09 (1H, dd, *J* = 8.6, 10.7 Hz, H-6), 4.55 (1H, s, OH), 3.51 (1H, m, H-7), 3.16 (2H, m, H-8, H-9), 1.96 (3H, dd, *J* = 1.5, 7.3 Hz, $H_3$-4′), 1.94 (3H, dd, *J* = 1.5, 7.0 Hz, $H_3$-15), 1.86 (1H, qd, *J* = 6.8, 8.5 Hz, H-10), 1.79 (3H, quint., *J* = 1.5 Hz, $H_3$-5′), 1.08 (3H, d, *J* = 6.8 Hz, $H_3$-14); $^{13}$C NMR data, see Table 1; EIMS *m*/*z* $[M]^+$ 376 (1) 348 (1), 307 (100), 207 (2), 151 (3), 111 (22), 109 (3); HREIMS *m*/*z* $[M]^+$ 376.1517 (calcd for $C_{20}H_{24}O_7$, 376.1522).

**(1*S*,5*S*,6*R*,7*S*,10*S*)-5-Angeloyloxy-1-hydroxy-2-oxoxantha-3,11-dien-6,12-olide (3):** colorless oil; $[\alpha]_D^{20}$ −32.2° (*c* 0.09, MeOH); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 219 (4.21) nm; IR (dry film) $\nu_{max}$ 3420 (OH), 2921 (C−H), 1768 ($\gamma$-lactone), 1734 ($CO_2R$), 1692 (C=C=O), 1228, 1134 cm$^{-1}$; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 7.08 (1H, dq, *J* = 6.9, 15.2 Hz, H-4), 6.56 (1H, br d, *J* = 15.2 Hz, H-3), 6.34 (1H, d, *J* = 3.0 Hz, H-13a), 6.16 (1H, br q, *J* = 7.2 Hz, H-3′), 5.61 (1H, brs, H-13b), 5.31 (1H, br d, *J* = 10.5 Hz, H-5), 5.01 (1H, br dd, *J* = 8.3, 10.5 Hz, H-6), 4.36 (1H, brs, OH), 3.45 (1H, m, H-7), 2.21 (1H, m, H-10), 1.98 (3H, br d, *J* = 6.2 Hz, $H_3$-4′), 1.91 (3H, br d, *J* = 6.9 Hz, $H_3$-15), 1.87−1.64 (4H, m, $H_2$-8, $H_2$-9), 1.84 (3H, brs, $H_3$-5′), 0.74 (3H, d, *J* = 6.3 Hz, $H_3$-14); $^{13}$C NMR data, see Table 1; EIMS *m*/*z* $[M]^+$ 362 (10), 293 (100), 193 (8), 149 (2), 113 (2), 111 (2), 109 (2); HREIMS *m*/*z* $[M]^+$ 362.1738 (calcd for $C_{20}H_{26}O_6$, 362.1729).

**(1*S*,5*S*,6*R*,7*R*,8*S*,10*S*)-5-Angeloyloxy-1,8-dihydroxy-2-oxoxantha-3,11-dien-6,12-olide (4):** colorless oil; $[\alpha]_D^{20}$ −42.5° (*c* 0.08, MeOH); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 220 (4.37) nm; IR (dry film) $\nu_{max}$ 3439 (OH), 2922 (C−H), 1769 ($\gamma$-lactone), 1729 ($CO_2R$), 1694 (C=C=O), 1229, 1136 cm$^{-1}$; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 7.01 (1H, dq, *J* = 7.0, 15.3 Hz, H-4), 6.78 (1H, br d, *J* = 15.3 Hz, H-3), 6.37 (1H, d, *J* = 1.8 Hz, H-13a), 6.18 (1H, dq, *J* = 1.2, 7.3 Hz, H-3′), 5.93 (1H, brs, H-13b), 5.55 (1H, d, *J* = 10.1 Hz, H-5), 5.07 (1H, dd, *J* = 8.0, 10.1 Hz, H-6), 4.29 (1H, s, OH), 3.83 (1H, brs, H-8), 3.39 (1H, m, H-7), 2.45 (1H, m, H-10), 2.38 (1H, m, H-9a), 1.94 (3H, dd, *J* = 1.3, 7.3 Hz, $H_3$-4′), 1.89 (3H, dd, *J* = 1.3, 7.0 Hz, $H_3$-15), 1.84 (1H, brs, $H_3$-5′), 1.60 (1H, m, H-9b), 0.82 (3H, d, *J* = 6.0 Hz, $H_3$-14); $^{13}$C NMR data, see Table 1; EIMS *m*/*z* $[M]^+$ 378 (15), 360 (1), 309 (100), 278 (4), 264 (5), 209 (7), 191 (3), 165 (8), 149 (2), 111 (4), 109 (2); HREIMS *m*/*z* $[M]^+$ 378.1683 (calcd for $C_{20}H_{26}O_7$, 378.1679).

**9α-Hydroxy-*seco*-ratiferolide-5α-*O*-(2-methylbutyrate) (5):** colorless oil; $[\alpha]_D^{20}$ −142.0° (*c* 0.41, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 237 (3.94) nm; $^{13}$C NMR data, see Table 1; exhibited comparable spectral (IR, $^1$H NMR, EIMS) data to published values.[3]

**9-Oxo-*seco*-ratiferolide-5α-*O*-(2-methylbutyrate) (6):** white needles; mp 131−132 °C (MeOH); $[\alpha]_D^{20}$ −242.0° (*c* 0.52, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 238 (4.05) nm; exhibited comparable spectral (IR, $^1$H NMR, $^{13}$C NMR, EIMS) data to published values.[3]

**(1*S*,5*S*,6*R*,7*S*,9*R*,10*S*)-5-Methylbutanoyloxy-1,4,9-trihydroxy-2-oxoxanth-11-en-6,12-olide (7):** colorless gum; $[\alpha]_D^{20}$ −105.6° (*c* 0.54, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 223 (3.02) nm; IR (dry film) $\nu_{max}$ 3480 (OH), 2982 (C−H), 1783 ($\gamma$-lactone), 1730 ($CO_2R$), 1610 (C=C=O), 1140 cm$^{-1}$; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 6.35 (1H, d, *J* = 2.9 Hz, H-13a), 5.71 (1H, d, *J* = 2.9 Hz, H-13b), 5.28 (1H, br d, *J* = 11.0 Hz, H-5), 4.98 (1H, br dd, *J* = 8.9, 11.0 Hz, H-6), 4.39 (1H, brs, OH), 4.26 (1H, m, H-4), 3.84 (1H, m, H-9), 3.65 (1H, m, H-7), 3.15 (1H, s, OH), 2.83 (1H, dd, *J* = 8.2, 17.9 Hz, H-3a), 2.68 (1H, dd, *J* = 2.7, 17.9 Hz, H-3b), 2.34 (1H, m, H-2′), 2.25−2.30 (2H, m, $H_2$-8), 1.99 (1H, m, H-10), 1.65 (1H, m, H-3′a), 1.48 (1H, m, H-3′b), 1.25 (3H, d, *J* = 6.2 Hz, $H_3$-15), 1.16 (3H, d, *J* = 6.7 Hz, $H_3$-5′), 0.95 (3H, d, *J* = 4.4 Hz, $H_3$-14), 0.91 (3H, t, $H_3$-4′); $^{13}$C NMR data, see Table 1; EIMS *m*/*z* $[M - OH]^+$ 381 (8), 311 (100), 297 (9), 227 (7), 209 (56), 191 (18), 163 (8), 111 (14); HREIMS *m*/*z* $[M + H]^+$ 381.1903 (calcd for $C_{20}H_{29}O_7$, 381.1913).

**(1*S*,5*S*,6*R*,7*S*,10*R*)-1-Hydroxy-4-methoxy-5-methylbutanoyloxy-2,9-dioxoxanth-11-en-6,12-olide (8):** colorless gum; $[\alpha]_D^{20}$ −189.1° (*c* 0.41, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 228 (3.06) nm; IR (dry film) $\nu_{max}$ 3420 (OH), 1770 ($\gamma$-lactone), 1740 ($CO_2R$), 1700 (C=C=O), 1140 cm$^{-1}$; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 6.34 (1H, d, *J* = 2.2 Hz, H-13a), 5.68 (1H, d, *J* = 2.2 Hz, H-13b), 5.40 (1H, d, *J* = 10.2 Hz, H-5), 4.87 (1H, dd, *J* = 8.0, 10.2 Hz, H-6), 3.80 (1H, m, H-4), 3.70 (1H, m, H-7), 3.47 (1H, q, H-10), 3.29 (3H, s, $-OCH_3$), 3.22 (1H, dd, *J* = 9.2, 14.6 Hz, H-3a), 2.75 (2H, m, H-8), 2.44 (1H, dd, *J* = 3.7, 14.6 Hz, H-3b), 2.40 (1H, m, H-2′), 1.70 (1H, m, H-3′a), 1.50 (1H, m, H-3′b), 1.24 (3H, d, *J* = 6.1 Hz, $H_3$-15), 1.17 (3H, d, *J* = 7.0 Hz, $H_3$-5′), 0.97 (3H, d, *J* = 7.4 Hz, $H_3$-14), 0.91 (3H, t, $H_3$-4′); $^{13}$C NMR data, see Table 1; EIMS *m*/*z* $[M + H]^+$ 410 (4), 309 (100), 239 (23), 207 (33), 191 (29); HREIMS *m*/*z* $[M]^+$ 410.1945 (calcd for $C_{21}H_{30}O_8$, 410.1940).

**12-Acetoxy-5-hydroxynerolidol (9):** colorless oil; $[\alpha]_D^{20}$ −62.5° (*c* 0.10, MeOH); IR (dry film) $\nu_{max}$ 3412 (OH), 2926 (C−H), 1736 (OAc), 1450, 1377, 1238, 1026 cm$^{-1}$; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 5.95 (1H, dd, *J* = 10.7, 17.3 Hz, H-2), 5.41 (1H, dt, *J* = 1.2, 6.0 Hz, H-10), 5.25 (1H, dd, *J* = 1.2, 17.3 Hz, H-1a), 5.21 (1H, dd, *J* = 1.2, 8.4 Hz, H-6), 5.03 (1H, dd, *J* = 1.2, 10.7 Hz, H-1b), 4.77 (1H, ddd, *J* = 3.2, 8.4, 8.6 Hz, H-5), 4.44 (2H, s, $H_2$-12), 2.18 (2H, m, H-8a, H-9a), 2.07 (3H, s, $OCOCH_3$), 1.80 (1H, dd, *J* = 8.6, 14.8 Hz, H-4a), 1.68 (3H, d, *J* = 1.2 Hz, $H_3$-15), 1.65 (3H, d, *J* = 1.2 Hz, $H_3$-13), 1.56 (1H, dd, *J* = 3.2, 14.8 Hz, H-4b), 1.40 (3H, s, $H_3$-14), 1.18−1.32 (2H, m, H-8b, H-9b); $^{13}$C NMR (90.8 MHz, $CDCl_3$) $\delta$ 171.4 (s, $CH_3CO$), 145.8

(d, C-2), 137.2 (s, C-7), 130.3 (s, C-11), 128.8 (d, C-10), 128.1 (d, C-6), 111.3 (t, C-1), 73.2 (s, C-3), 70.2 (t, C-12), 66.2 (d, C-5), 47.1 (t, C-4), 38.7 (t, C-8), 26.7 (q, C-13), 25.7 (t, C-9), 21.0 (q, *C*$H_3$CO), 16.4 (q, C-14), 14.0 (q, C-15); positive FABMS *m/z* $[M + H]^+$ 297 (13), 279 (28), 219 (5), 201 (5), 149 (100), 131 (17), 121 (16), 107 (27), 44 (43); positive HRFABMS *m/z* $[M]^+$ 297.2081 (calcd for $C_{17}H_{29}O_4$, 297.2066).

**Hispidulin (10):** yellow powder; $[\alpha]_D^{20}$ −58.0° (*c* 0.20, $CHCl_3$); exhibited comparable spectral (UV, IR, $^1$H NMR) data to published values.[1,12]

**Preliminary Biological Evaluation of Compounds 1−10.** Compounds **1**−**10** were evaluated for cytotoxicity against a panel of human cancer cell lines (Table 2) according to established protocols,[13] and antimitotic activity was assessed using cultured ASK cells (Table 2).[14]

**Oncology Diverse Cell Assays.** Compound **5** was assessed in more detail for cytotoxicity in a panel of 25 tumor cell lines, using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-2*H*-tetrazolium, inner salt] assay.[15] Cells were plated at 4000 per well in 96-well microtiter plants, and 24 h later, the test compound was added and serially diluted. The cells were incubated at 37 °C for 72 h, at which time the tetrazolium dye (MTS) at 333 (g/mL final concentration, in combination with the electron-coupling agent, phenazine methosulfate (25 $\mu$M final concentration), was added. A dehydrogenase enzyme in live cells reduces MTS to a form that absorbs light at 492 nm, which can be quantitated spectrophotometrically, with absorbance being proportional to the number of live cells. The results are expressed for each cell line as an $IC_{50}$ value, which is the concentration of drug required to inhibit cell proliferation (i.e., absorbance at 492 nm) to 50% of that of untreated control cells. The mean $IC_{50}$ values of compound **5** in all 25 cell lines was determined from a mean bar-graph.

**Cell-Cycle Arrest Studies.** Compound **5** was evaluated against A2780S (parental wild-type p53, $bcl2^-$) and A2780R (mutant p53, $bcl2^{2+}$) ovarian carcinoma cell lines. Briefly, $5 \times 10^5$ cells/2 mL were incubated overnight and then treated with the test compound for 24 h. Cells were trypsinized, fixed overnight in 70% EtOH at −20 °C, and stained with propidium iodide (50 $\mu$g/mL) and 1 mg/mL RNase (1 mg/mL) for at least 1 h before DNA evaluation. A FACScalibur with Cellquest software (Becton-Dickinson Immunocytometry Systems) instrument was used to evaluate the cell-cycle profiles.

**Evaluation of Apoptosis.** Compound **5** was evaluated for induction of apoptosis with A2780S and A2780R cells. Cells ($10^6$) were trypsinized as described above, and processed following the steps described in the APO-Direct Kit (Cat. No. 6536KK, Pharmingen, San Diego, CA), with minor modifications. In short, cells harvested from cultures were pelleted and resuspended in 1 mL of 1% formaldehyde solution in PBS [Polysciences Formaldehyde (MeOH free), 10% ultrapure EM grade] and incubated for 15 min on ice. Cells were washed twice with 1 mL PBS and the final pellet fixed in 1 mL 80% EtOH at −20 °C while mantaining continuous vortexing for 5 s. Samples were stored at −20 °C for 16 h before proceeding with the TdT assay as described by the manufacturer.

**In Vivo Evaluations of Compound 5.** Compound **5** (produced in sufficient quantity by reisolation from *R. columnifera* leaves) was evaluated in two in vivo test systems, namely, the M109 murine lung carcinoma and HCT 116 human colon tumor models. The M109 murine lung carcinoma grows both as a solid subcutaneous (sc) tumor and an intraperitoneal (ip) tumor. Solid tumors were harvested, and a 2% w/v brei (homogenate) was prepared in RPMI medium. Female CDF1 mice (six mice per group) were implanted intraperitoneally with 0.5 mL of the brei. Compound **5** was administered by the ip route (24 and 8 mg/kg/injection), starting 1 day after the ip implantation every day for 5 days. Since the ip tumors are lethal, the endpoint monitored was lifespan determination. Data were expressed as a %T/C value (lifespan of test mice over control mice × 100%; threshold activity is deemed significant at T/C 125%).

The HCT116 human colon carcinoma grows as a solid sc tumor in athymic nude mice. Tumors were harvested, and fragments (2−3 $mm^2$) were prepared and implanted sc with sterile trochars. After a period of time the tumors were measured and sorted into a tumor weight range of 60−160 mg [using the formula $W$ (mm) × $L$ $(mm)^2/2$ = tumor weight (mg)] for the commencement of the study. Compound **5** was administered by the ip route (30 mg/kg/injection), starting on the day the mice were sorted into the specific tumor weight range. Mice were treated on alternate days for a total of five doses. Thereafter, the tumors were measured twice weekly until they reached a target weight of 500 mg. The time (number of days) to reach the target for the untreated control (C) mice was subtracted from the time to reach the target for the treated (T) mice, to give a T − C value. This figure was divided by the tumor volume doubling time (TVDT), and multiplied by 3.32 to yield a gross log cell kill (LCK) value, i.e., (T − C)/TVDT × 3.32 = 1 LCK (days). An active compound has a LCK of 1.0 or greater.

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