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Structure−Activity Relationship Analysis of Bufadienolide-Induced in Vitro Growth Inhibitory Effects on Mouse and Human Cancer Cells

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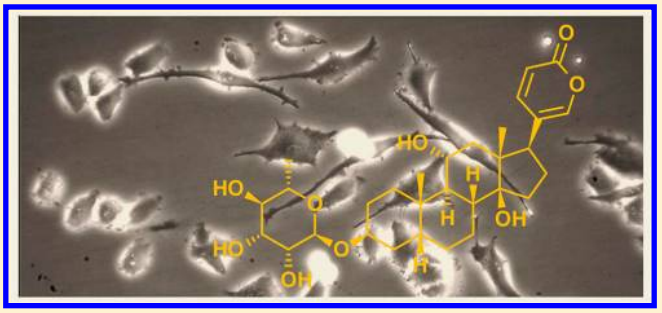
Supporting Information

ABSTRACT: The in vitro growth inhibitory effects of 27 bufadienolides and eight degradation products, with two cardenolides (ouabain and digoxin) chosen as reference compounds, were analyzed by means of an MTT colorimetric assay in six human and two mouse cancer cell lines. A structure−activity analysis was then performed to highlight the most important substituents relating to the in vitro growth inhibitory activity of bufadienolides in cancer cells. Thus, the current study revealed that various bufadienolides, including gamabufotalin rhamnoside (**1a**), bufotalin (**2a**), and hellebrin (**3a**), displayed higher growth inhibitory activities for various human cancer cell lines when compared to ouabain and digoxin. Gamabufotalin rhamnoside (**1a**) was the only compound that displayed growth inhibitory effects of <1 μM in mouse cancer cells that expressed mutated forms of the Na^+,K^+-ATPase α-1 subunit. In addition, all genins and degradation products displayed weaker (if any) in vitro growth inhibitory effects on cancer cells when compared to their respective glycosylated homologue, with the exception of hellebrigenin (**3b**), which was as active as hellebrin (**3a**).



Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries.[1] As emphasized by Jemal et al.,[2] based on the GLOBOCAN project, approximately 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008. Of these, 56% of the cases and 64% of the deaths occurred in the economically developing world. Various types of cancers, including gliomas, melanomas, non-small-cell lung cancers (NSCLC), esophageal cancers, and pancreatic cancers, among others, remain associated with dismal prognoses because they resist current radio- and chemotherapeutic treatments.[3] Indeed, these cancer types display various levels of resistance to pro-apoptotic stimuli and/or the multidrug resistance (MDR) phenotype,[3] and most of the cytotoxic agents used in oncology as well as radiotherapy induce pro-apoptotic stimuli. Novel types of anticancer drugs are thus needed to overcome apoptosis resistance and/or MDR phenotypes in those cancer types associated with dismal prognoses. Certain cardiotonic glycosides could be potential candidates; for example, 19-hydroxy-2″-oxovoruscharine displays similar and marked anticancer activity in chemosensitive versus apoptosis-resistant[4,5] and/or MDR[6] cancer cell lines. In addition, various cardiotonic steroids are also able to sensitize apoptosis-resistant cancer cells to pro-apoptotic stimuli.[7−9] At the clinical level, digoxin has already been suspected to provide therapeutic benefits to breast cancer,[10] leukemia/lymphoma,[11] and prostate cancer[12] patients. For example, Platz and colleagues[12] have conducted an in vitro prostate cancer cell cytotoxicity screen of 3187 compounds, and digoxin emerged as the leading candidate. These authors then evaluated the association between digoxin treatment (in heart failure patients) and prostate cancer risk in 47 884 men including regular digoxin users versus nonusers. Users of ≥10 years showed a 25% lower prostate cancer risk compared to nonusers.[12]

Cardiotonic steroids include two chemical classes, cardenolides (e.g., ouabain and digoxin) and bufadienolides, which both bind to the α subunits of the sodium/potassium pump (Na^+/K^+-ATPase; NaK hereafter) as specific ligands.[13,14] Bufadienolides are characterized by the presence of a six-membered lactone (α-pyrone) ring located at position C-17β.[14] While the PubMed database contained ∼37 000 references concerning cardenolides in January 2013, it included only ∼800 references concerning bufadienolides. When the PubMed search is restricted to the oncology area, i.e., using "cardenolides versus tumor" and "bufadienolides versus tumor" as keywords, the PubMed database displayed ∼1400 and ∼130 references, respectively. Thus, it seems that the potential anticancer effects

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Table 1. Determination by Means of the MTT Colorimetric Assay of the Concentration that Reduces in Vitro by 50% (after Three Days of Culture with the Test Compound) the Growth of Various Mouse and Human Cancer Cell Lines

compound[a]		IC_{50} in vitro growth inhibitory concentration (nM) after 3 days of culture								
		human cancer cell lines[b]							mouse cancer cell lines[b]	
name	code	Hs683	MCF-7	PC-3	A549	U373	SKMEL-28	mean ± SEM	CT26.WT	B16F10
gamabufotalin rhamnoside	**1a**	2	8	<1	<1	3	5	<3	877	659
bufotalin	**2a**	9	37	34	14	74	65	39 ± 11	>10 000	>10 000
hellebrin	**3a**	7	44	11	6	36	68	29 ± 10	>10 000	>10 000
5α-bufalin	**4a**	21	428	67	22	362	463	227 ± 86	>10 000	>10 000
argentinogenin	**5a**	9	36	43	53	86	70	50 ± 11	>10 000	>10 000
bovogenin A	**6a**	40	278	2431	51	62	336	533 ± 382	>10 000	>10 000
scillarenone	**7a**	73	341	284	333	399	1607	506 ± 224	>10 000	>10 000
3α-acetylamino-16-acetoxy-14,15-epoxybufadienolide	**8a**	312	726	426	302	384	3506	943 ± 517	>10 000	>10 000
arenobufagin diacetate	**9a**	2617	7264	>10 000	5910	8809	7985	>7098	>10 000	>10 000
bufarenogin	**10a**	2684	9301	7417	3843	>10 000	>10 000	>7208	>10 000	>10 000
3-*O*-acetyl-14,15-epoxy-19-oxobufadienolide	**11a**	3656	>10 000	5343	7135	7046	>10 000	>7196	>10 000	>10 000
ouabain[c]		28	133	43	27	77	211	86 ± 30	>10 000	>10 000
digoxin[c]		40	426	75	50	146	266	167 ± 62	>10 000	>10 000

[a]The chemical structure of each compound is depicted in Table 2. [b]The origin and histological type of each cell line analyzed are as follows. Human glioma model lines included the Hs683 oligodendroglioma (ATCC code HTB-138) and the U373 glioblastoma (ECACC code 89081403) cell lines. Melanoma models included the human SKMEL-28 (ATCC code HTB-72) and the mouse B16F10 (ATCC code CRL-6475) cell lines. Carcinoma models included the A549 NSCLC (DSMZ code ACC107), the MCF-7 breast (DSMZ code ACC115), and the PC-3 prostate (DSMZ code ACC465) human cancer cell lines, as well as the mouse CT26.WT colon (ATCC code CRL-2638) cancer cell line. [c]Positive control substance.

of bufadienolides have been investigated far less than those relating to cardenolides.

The aim of the present study, therefore, is the characterization of the in vitro growth inhibitory effects of 27 bufadienolides and eight degradation products thereof, with two cardenolides (ouabain and digoxin) used as reference compounds. The current study then attempted a structure–activity (SAR) analysis to determine the most important substituents for the growth inhibitory activity of bufadienolides and derivatives thereof in six human and two mouse cancer cell lines.

RESULTS AND DISCUSSION

One of the current coauthors recently contributed to an in-depth review concerning bufadienolides and their antitumor activity,[14] and it appears that several studies have already highlighted the in vivo antitumor efficacy of various bufadienolides. Bufalin exhibits significant antitumor effects in human xenografts of hepatocellular carcinoma,[15] and resibufogenin showed similar effects on HeLa human tumor growth in nude mice.[16]

In the present study, the growth inhibitory activity of selected bufadienolides and bufadienolide degradation products on six human and two mouse cancer cell lines was determined by an MTT colorimetric assay, and the results were compared to the two cardenolides ouabain and digoxin (Table 1). These two reference compounds are very well studied with respect to their in vitro activities on various cancer cell lines. Furthermore, digoxin is a marketed drug for treating congestive heart failure and atrial fibrillation (Lanoxin), so that plenty of in vivo and pharmacokinetic data are available, and ouabain is of particular interest since it is assumed to be an endogenous hormone in humans.[10−13,17]

The data in Table 1 indicate that several bufadienolides display higher in vitro growth inhibitory activities than ouabain and digoxin. For example, gamabufotalin rhamnoside (**1a**) was approximately 100 times more potent in terms of cancer cell growth inhibition than ouabain or digoxin (Table 1). Bufotalin (**2a**) and hellebrin (**3a**), while more active than ouabain and digoxin, were about 10 times less active than gamabufotalin rhamnoside (Table 1). Cardenolides and bufadienolides target the NaK α subunits. NaK is an integral membrane protein found in the cells of all higher eukaryotes and is responsible for translocating sodium and potassium ions across the cell membrane, utilizing ATP as the driving force.[3,18] It is also a versatile signal transducer and a key player in cell adhesion, and its aberrant expression and activity are implicated in the development and progression of different types of cancers.[3] NaK is composed of two subunits. The catalytic α subunit (of which four different isoforms have been characterized to date) is a multipass transmembrane protein containing the binding sites for Na^+, K^+, ATP, and cardiotonic steroids. The β regulatory subunit (of which three different isoforms have been characterized) is a transmembrane protein with several glycosylation sites that is required for the biogenesis and activity of the enzyme complex.[3,17]

Gamabufotalin rhamnoside (**1a**) was the only compound that displayed growth inhibitory activity of <1 μM in the mouse cancer cell lines studied, while the observed effects were markedly less pronounced than in human cancer cells (Table 1). In fact, rodent NaK shows a roughly 1000-fold lower affinity for ouabain compared to human NaK.[19] This feature is caused by two mutations (human-rat Q117R and N128D) in the extracellular loop between TM1 and TM2 in murine α1 compared to human α1. These mutations also account for the approximately 1000-fold weaker sensitivity of murine cancer cells to the growth inhibitory effects of cardiotonic steroids.[17] Yang et al.[20] demonstrated that the relative lack of the NaK α3 subunits in rodent cancer cells may also account for the unresponsiveness to cardiotonic steroids, but Lin et al.[21]

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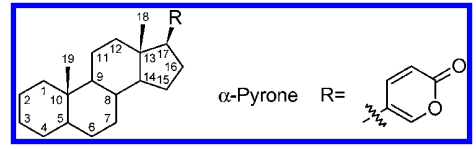
Table 2. Growth Inhibitory Effects of Various Bufadienolides and Their Degradation Products on Six Human Cancer Cell Lines Shown in Table 1



	chemical substituents[a]												
compound	3	4	5	9	10	11	12	14	15	16	17 (R =)	ring configuration	mean IC_{50} ± SEM (nM)
1a	O-Rha β	H	H β	H α	CH_3 β	OH α	H	OH β	H	H	α-pyrone	cis/trans/cis	<3 ± 1
1b	OH β	H	H β	H α	CH_3 β	OH α	H	OH β	H	H	α-pyrone	cis/trans/cis	21 ± 4
1c	O-Ac β	H	H β	H α	CH_3 β	O-Ac α	H	OH β	H	H	$COOCH_3$	cis/trans/cis	>10 000
2a	OH β	H	H β	H α	CH_3 β	H	H	OH β	H	O-Ac β	α-pyrone	cis/trans/cis	39 ± 11
2b	O-Ac β	H	H β	H α	CH_3 β	H	H	OH β	H	O-Ac β	α-pyrone	cis/trans/cis	159 ± 63
2c	=O	H	H β	H α	CH_3 β	H	H	OH β	H	O-Ac β	α-pyrone	cis/trans/cis	498 ± 210
2d	O-$CH(Ph)_2$ β	H	OH β	H α	CH_3 β	H	H	OH $\beta\beta$	H	H	$COOCH(Ph)_2$	cis/trans/cis	>10 000
3a	O-(Glc-Rha) β	H	OH β	H α	CHO β	H	H	OH β	H	H	α-pyrone	cis/trans/cis	29 ± 10
3b	OH β	H	OH β	H α	CHO β	H	H	OH β	H	H	α-pyrone	cis/trans/cis	8 ± 2
4a	OH β	H	H α	H α	CH_3 β	H	H	OH β	H	H	α-pyrone	trans/trans/cis	227 ± 86
4b	O-Ac β	H	H β	H α	CH_3 β	H	H	OH β	H	H	α-pyrone	cis/trans/cis	48 ± 21
4c	OH α	H	H β	H α	CH_3 β	H	H	OH β	H	H	α-pyrone	cis/trans/cis	2036 ± 1474
4d	O-Ac β	H	H β	H α	CH_3 β	H	H	OH β	OH α	H	α-pyrone	cis/trans/cis	>6800
4e	O-Ac β	H	H β	H α	CH_3 β	H	H	OH α	=O	H	α-pyrone	cis/trans/trans	>9600
4f	O-CHO β	H	H β	H α	CH_3 β	H	H	-O-(15) α	-O-(14) α	H	α-pyrone	cis/trans/trans	>10,000
4g	OH β	H	H β	H α	CH_3 β	H	H	-O-(15) α	-O-(14) α	H	α-pyrone	cis/trans/trans	>10,000
5a	OH β	H	H β	=	CH_3 β	=, OH	=O	OH β	H	H	α-pyrone	cis///cis	50 ± 11
6a	OH β	H	H α	H α	CHO β	H	H	OH β	H	H	α-pyrone	trans/trans/cis	533 ± 382
6b	O-Ac β	H	H α	H α	CHO β	H	H	OH β	H	H	α-pyrone	trans/trans/cis	103 ± 57
7a	=O	=	=	H α	CH_3 β	H	H	OH β	H	H	α-pyrone	///trans/cis	506 ± 224
8a	NH-Ac α	H	H β	H α	CH_3 β	H	H	-O-(15) β	-O-(14) β	O-Ac β	α-pyrone	cis/trans/cis	943 ± 517
8b	O-Ac β	H	H β	H α	CH_3 β	H	H	-O-(15) β	-O-(14) β	OH β	α-pyrone	cis/trans/cis	>4900
8c	O-Ac β	H	H β	H α	CH_3 β	H	H	-O-(15) β	-O-(14) β	OH β	$COOCH_3$	cis/trans/cis	>10 000
9a	O-Ac β	H	H β	H α	CH_3 β	O-Ac α	=O	OH β	H	H	α-pyrone	cis/trans/cis	7098 ± 1058
9b	O-Ac β	H	H β	H α	CH_3 β	O-Ac α	=O	OH β	H	H	$COOCH_3$	cis/trans/cis	>10 000
9c	O-Ac β	H	H β	H α	CH_3 β	O-Ac α	=O	O-CHO β	H	H	$COOCH_3$	cis/trans/cis	>10 000
10a	OH β	H	H β	H α	CH_3 β	=O	OH β	OH β	H	H	α-pyrone	cis/trans/cis	>5800
10b	OH β	H	H β	H α	CH_3 β	OH α	OH β	OH β	H	H	α-pyrone	cis/trans/cis	306 ± 128
10c	O-Ac β	H	H β	H α	CH_3 β	=O	O-Ac β	OH β	H	H	α-pyrone	cis/trans/cis	>5100
10d	O-Ac β	H	H β	H α	CH_3 β	=O	O-Ac α-	OH β	H	H	α-pyrone	cis/trans/cis	>10 000
10e	O-Ac β	H	H β	H α	CH_3 β	=O	O-Ac β	H α	H	H	CH_2OCOCH_3	cis/trans/tra ns	>10 000
10f	O-Ac β	H	H β	H α	CH_3 β	O-Ac α	=O	O-CO-(17) β	H	H	CO-O-(14)	cis/trans/cis	>10 000
10g	O-Ac β	H	H β	H α	CH_3 β	O-Ac α	O-Ac β	OH β	H	H	$COOCH_3$	cis/trans/cis	>10 000
11a	O-Ac β	H	H β	H α	CHO β	H	H	-O-(15) β	-O-(14) β	H	α-pyrone	cis/trans/cis	7431 ± 960
11b	N_3 β	H	H β	H α	CH_3 β	H	H	-O-(15) β	-O-(14) β	H	α-pyrone	cis/trans/cis	>10 000

[a]Positions 1, 2, 6, 7, and 8 = H; position 13 = CH_3.

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recently emphasized the importance of the NaK α1 subunit in tumor growth and cancer cell survival. According to the data illustrated in the present study, gamabufotalin rhamnoside (**1a**) would mainly target the NaK α1 subunit because it is 100 to 1000 times less active in rodent than in human cancer cells. While the α1 subunit is upregulated in non-small-cell lung cancers, gliomas, melanomas, and kidney cancers, the α3 subunit is upregulated in colon cancers.[3] Impairing α1 NaK subunit activity is much more detrimental to cancer than to normal cells.[3,5,17] We are currently accumulating the necessary amount of gamabufotalin rhamnoside (**1a**) to characterize its in vivo antitumor activity in various cancer models.

Precise SAR analysis appeared difficult to perform due to the large variety of chemical groups present in bufadienolides and their degradation products. For instance, no conclusion can be drawn regarding the effects of substituents on positions 10, 11, and 12. The only elements worth mentioning are the potent activities of compounds possessing an aldehyde moiety and an α-OH on C-11, as already observed for numerous other bufadienolides.[22,23] Other positions show trends only because too few compounds have been analyzed. This is the case for the sugar moiety at position 3, the double bond at position 9, and the −OH group at position 5 (only two or three compounds were available for comparison in each case; for the double bond between C-9 and C-11, only one compound was available). In contrast, more relevant observations can be made about the importance of some chemical functions, as detailed below. The double bond between C-4 and C-5 has no effect on the biological activity of the compounds (Table 2, compare compound **2c** vs **7a**). The β-configured alcohol on position 14 is of critical importance, as it is the case for structurally related steroids.[22,23] None of the 14 most active bufadienolides and only three of the 22 most active compounds lack this substituent. This hydroxy group is responsible for donating a hydrogen bond to an asparagine in the binding pocket.[3,22] When this donating effect is prevented by inclusion of the oxygen atom in an epoxide, the activity is lower (see compounds **6b** and **11a**).[24] As already observed by Kamano et al.,[22,23] polar or sterically hindered substituents at position 15 are rather unfavorable in terms of the growth inhibition of cancer cells (see compound **4b** or **4d**). Substituents on position 16 also have a deleterious effect on the growth inhibition of cancer cells (see compounds **2a** vs **1b** and **2b** vs **4b**), although some discrepancies were observed (e.g., compare compounds **8b** and **11a**). However, when substitution does take place, an acetoxy group is preferred to a hydroxy group, a feature previously observed in cinobufagin derivatives.[24] The reason for these effects is that this face of the steroidal backbone is directed toward a rather lipophilic area of the binding pocket.[3] The α-pyrone is essential for the growth inhibition of cancer cells, because the 23 active compounds (i.e., with a mean IC_{50} value of <10 μM on the six human cancer cell lines) possess this heterocyclic moiety at position 17. Only chemical groups efficiently mimicking the unsaturated lactone group in terms of interactive chemical group and size can be used in this position, as already pointed out by Cerri et al.[25] and Gupta.[26] Within this framework, alkylcarboxylate moieties (the alternative chemical groups for the α-pyrone in the test compound group) are not relevant for this purpose. Indeed, these groups fail in having the optimal size requested to fill the pocket (in being either too small or too large), while the α-pyrone matches it very well. Additionally, they are unable to make the right interactions, while unsaturated esters are more suitable.[22,26] Regarding the relative configuration of rings A, B, C, and D, only the cis configuration between rings C and D is crucial for the growth inhibition of cancer cells; rings C and D cis-configured compounds are the most potent growth inhibitors.

In conclusion, the current study reveals that various bufadienolides, including gamabufotalin rhamnoside (**1a**), bufotalin (**2a**), and hellebrin (**3a**), display higher growth inhibitory activities in various human cancer cell lines when compared to two cardenolides chosen as references, ouabain and digoxin. While genins of cardiac glycosides typically display weaker growth inhibitory effects on cancer cells when compared to their glycosylated homologues, the exception was hellebrigenin (**3b**), which was as active as hellebrin (**3a**). None of the tested bufadienolide degradation products lacking the α-pyrone showed activity at 10 μM or below. In agreement with previous studies, our SAR analysis showed that the 14β-OH group is very important for the activity and that a 10β-CHO moiety rather increases while a 16β-*O*-acetyl or a 15α-OH group rather decreases growth inhibitory activities in various human cancer cell lines.

EXPERIMENTAL SECTION

General Experimental Procedures. CD spectra in the range 230−650 nm were obtained from solutions in $CHCl_3$ (1 mg/mL) with a cell length of 0.1 cm on a JASCO J-810 spectropolarimeter. NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer (UltraShield) using a 5 mm switchable probe (PA BBO 500SB BBF-H-D-05-Z, ^{1}H, BB = ^{19}F and ^{31}P−^{15}N) with *z* axis gradients and an automatic tuning and matching accessory (BrukerBioSpin, Rheinstetten, Germany). The resonance frequencies were 500.13 MHz for ^{1}H NMR and 125.75 MHz for ^{13}C NMR. All measurements were performed in solutions of fully deuterated chloroform or methanol at 298 K. Standard 1D and gradient-enhanced (ge) 2D experiments, such as double-quantum filtered (DQF) COSY, NOESY, HSQC, and HMBC, were performed according to the manufacturer's instructions. The chemical shifts are referenced internally to the residual, nondeuterated solvent signal for chloroform ^{1}H (δ 7.26 ppm) or methanol ^{1}H (δ 3.31 ppm) and to the carbon signal of the solvent for chloroform ^{13}C (δ 77.00 ppm) or methanol ^{13}C (δ 49.00 ppm). ESIMS^n spectra were obtained on a 3D-ion trap mass spectrometer (HCT, Bruker Daltonics, Bremen, Germany), and HRESIMS spectra were recorded on an ESI-Qq-TOF mass spectrometer (micrOTOF-Q II, Bruker Daltonics) in the positive-ion mode by direct infusion. The HPLC analyses were performed as previously described[14,27] using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with an LC-10AD VP binary pump, an SPD-M10A VP diode array detector, an SCL-10A VP system controller, an autosampler, and a DGU-14A degasser. The samples were separated on a Nucleosil C_{18} column (4.0 × 250 mm, 5 μm, Macherey-Nagel, Düren, Germany). The mobile phase consisted of MeCN and water (adjusted to pH 3.3 with acetic acid). A multistep gradient program was used as follows: 8% MeCN (0 min), 54% MeCN (45 min), 54% MeCN (55 min), and 95% MeCN (70 min). The flow rate was 0.8 mL/min. Online UV spectra were recorded in the range 190−400 nm.

Isolation and Derivatization of the Bufadienolides. All investigated compounds (**1a**−**11b**) were obtained from the "bufadienolide collection" of the Department of Pharmacognosy, University of Vienna, and were in part gifts from Prof. Dr. Tadeus Reichstein (University of Basel, Switzerland). The purities of the above listed compounds were >98%, as determined by HPLC analysis. The acetylation of bufadienolides[28] and the degradation of bufadienolides[28−33] were described previously. Gamabufotalin 3-*O*-α-L-rhamnoside (**1a**) was isolated from red-colored bulbs of plants of the *Urginea maritima* aggregate[34] and bulbs of tetraploid *Urginea aphylla*,[35] *Urginea maritima* sensu strictu,[36] and *Urginea hesperia*.[37] Hellebrigenin (**3b**) was in part isolated from the venom of *Bufo melanosticus*[38] and in part obtained by deacetylation of 3-acetylhellebrigenin. For the deacetyla-

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tion, 36 mg of 3-acetylhellebrigenin was dissolved in 20 mL of dry methanol. Then, 5 mL of sodium methanolate solution (500 mg of sodium was dissolved in 50 mL of dry methanol) was added, and the mixture was stirred for 1 h at 20 °C. Next, 50 mL of NH_4Cl solution (5% in water) was added, and the mixture was extracted with dichloromethane (50 mL). The organic layer was separated, dried with sodium sulfate, and evaporated to dryness to give 17 mg of hellebrigenin (**3b**). The structures of all compounds were verified or determined by extensive NMR and mass spectrometry experiments. The compounds gamabufotalin (**1b**),[39] (3*β*,5*β*,11*α*,14*β*,17*β*)-3,11-bis(acetyloxy)-14-hydroxyandrostane-17-carboxylic acid methyl ester (**1c**),[40,41] bufotalin (**2a**),[39] 3-*O*-acetylbufotalin (**2b**),[42] bufotalone (**2c**),[43] (5*β*,14*β*,17*β*)-5,14-dihydroxy-3-diphenylmethoxyandrostane-17*β*-carboxylic acid diphenylmethyl ester (**2d**),[31] hellebrin (**3a**),[44] hellebrigenin (**3b**),[45] 5*α*-bufalin (**4a**),[46] 3-*O*-acetylbufalin (**4b**),[45] 3-epi-bufalin (**4c**),[47] 15*α*-hydroxybufalin 3-acetate (**4d**),[22] (3*β*,5*β*,14*α*,15*α*)-14,15-epoxy-3-hydroxybufa-20,22-dienolide (**4g**),[22] argentinogenin (**5a**),[28] bovogenin A (**6a**),[42] bovogenin A 3-acetate (**6b**),[42] scillarenone (**7a**),[42,48] desacetylcinobufagin 3-acetate (**8b**),[22] (3*β*,5*β*,14*β*,15*β*,16*β*,17*β*)-3-(acetyloxy)-14,15-epoxy-16-hydroxyandrostane-17-carboxylic acid methyl ester (**8c**),[32] arenobufagin 3,11-diacetate (**9a**),[32,33,42] (3*β*,5*β*,11*α*,14*β*,17*β*)-3,11-bis(acetyloxy)-14-hydroxy-12-oxoandrostane-17-carboxylic acid methyl ester (**9b**),[32,33] bufarenogin (**10a**),[33,49] 11*α*,12*β*-dihydroxybufalin (**10b**),[28,50] bufarenogin 3,12-diacetate (**10c**),[28,33,42] (3*β*,5*β*,12*α*)-3,12-bis(acetyloxy)-14-hydroxy-11-oxobufa-20,22-dienolide (**10d**),[33,42] (3*β*,5*β*,12*β*,17*β*)-3,12-bis(acetyloxy)-11-oxoandrostane-17-hydroxymethyl-*α*-acetate (**10e**),[33] resibufagin acetate (**11a**),[51] and 3*β*-azidoresibufogenin (**11b**)[41] were previously described.

(3*β*,5*β*,14*α*)-3-(Acetyloxy)-14-hydroxy-15-oxo-bufa-20,22-dienolide (4e): colorless solid (acetone/diethyl ether); mp 256−262 °C; CD (c 2.3 × 10^{-3} M, $CHCl_3$) λ_{max} ($\Delta\varepsilon$) 231 (+0.53), 299 (+1.21) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.28 (1H, d, J = 2.6 Hz, H-21), 7.22 (1H, dd, J = 9.3, 2.6 Hz, H-22), 6.33 (1H, d, J = 9.3 Hz, H-23), 5.09 (1H, s, H-3), 3.46 (1H, t, J = 9.5 Hz, H-17), 2.72 (1H, dd, J = 18.9, 9.5 Hz, H-16a), 2.23 (1H, m, H-7a), 2.19 (1H, dd, J = 18.9, 9.5 Hz, H-16b), 2.05 (3H, s, OCOCH_3), 2.01 (1H, m, H-8), 1.98 (1H, m, H-6a), 1.95 (1H, m, H-9), 1.93 (1H, m, H-4a), 1.91 (1H, m, H-12a), 1.69 (1H, m, H-5), 1.62 (1H, m, H-2a), 1.55 (1H, m, H-2b), 1.53 (1H, m, H-1a), 1.46 (1H, m, H-11a), 1.46 (1H, m, H-4b), 1.38 (1H, m, H-12b), 1.35 (1H, m, H-1b), 1.27 (1H, m, H-6b), 1.23 (1H, m, H-11b), 1.20 (1H, m, H-7b), 0.99 (3H, s, H-19), 0.65 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 211.53 (C, C-15), 170.73 (OCOCH$_3$), 161.54 (C, C-24), 149.20 (CH, C-21), 144.75 (CH, C-22), 116.34 (C, C-20), 115.88 (CH, C-23), 81.30 (C, C-14), 70.31 (CH, C-3), 45.73 (C, C-13), 40.97 (CH, C-17), 36.86 (CH, C-5), 36.52 (CH_2, C-16), 35.34 (C, C-10), 35.17 (CH, C-8), 32.62 (CH, C-9), 30.84 (CH_2, C-1), 30.46 (CH_2, C-4), 28.14 (CH_2, C-12), 25.92 (CH_2, C-6), 24.86 (CH_2, C-2), 23.38 (CH_3, C-19), 21.49 (OCOCH$_3$), 19.73 (CH_2, C-7), 19.56 (CH_2, C-11), 14.98 (CH_3, C-18); ESIMS m/z 443.2 [M + H]$^+$; ESIMS^2 (443.2 →) m/z 443.2 (15), 383.1 (100); ESIMS^3 (443.2 → 383.1 →) m/z 329.1 (12), 231.0 (11), 221.0 (11), 213.1 (61), 211.0 (17), 209.0 (13), 203.1 (20), 199.0 (11), 197.0 (15), 195.0 (16), 193.0 (12), 187.0 (20), 185.0 (32), 183.0 (19), 181.0 (14), 175.0 (12), 173.0 (19), 171.0 (58), 169.0 (20), 167.0 (11), 165.0 (11), 161.0 (20), 159.0 (44), 157.0 (100), 155.0 (20), 153.0 (10), 151.0 (11), 149.0 (13), 147.1 (39), 145.0 (61), 143.0 (70), 142.0 (29), 141.0 (22), 135.1 (18), 133.1 (49), 131.1 (84), 129.1 (54), 128.1 (51), 123.1 (17), 121.1 (25), 119.1 (22), 117.1 (35), 115.1 (23), 109.1 (23), 107.1 (18); HRESIMS m/z 443.2440 [M + H]$^+$ (calcd for $C_{26}H_{35}O_6^+$, 443.2428, Δ = 2.7 ppm).

(3*α*,5*β*,14*α*,15*α*)-14,15-Epoxy-3-(formyloxy)-bufa-20,22-dienolide (4f): colorless solid ($CHCl_3$); mp 240−243 °C; CD (c 2.4 × 10^{-3} M, $CHCl_3$) λ_{max} ($\Delta\varepsilon$) 230 (+1.75), 293 (+0.88) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.06 (1H, s, OC*H*O), 7.17 (1H, m, H-21), 7.16 (1H, m, H-22), 6.29 (1H, d, J = 11.1 Hz, H-23), 5.21 (1H, s, H-3), 3.53 (1H, s, H-15), 2.46 (1H, dd, J = 11.1, 6.6 Hz, H-17), 2.17 (1H, td, J = 11.8, 4.4 Hz, H-8), 2.09 (1H, dd, J = 13.6, 6.6 Hz, H-16a), 1.99 (1H, td, J = 14.2, 2.6 Hz, H-4a), 1.90 (1H, tt, J = 14.0, 4.6 Hz, H-6a), 1.78 (1H, dd, J = 11.8, 3.5 Hz, H-9), 1.72 (1H, m, H-16b), 1.68 (1H, m, H-5), 1.65 (1H, m, H-2a), 1.65 (1H, m, H-2b), 1.65 (1H, m, H-12a), 1.60 (1H, m, H-12b), 1.61 (1H, m, H-1a), 1.58 (1H, m, H-7a), 1.44 (1H, d, J = 14.2 Hz, H-4b), 1.38 (1H, m, H-1b), 1.37 (1H, m, H-11a), 1.34 (1H, m, H-7b), 1.19 (1H, m, H-6b), 1.10 (1H, m, H-11b), 0.99 (3H, s, H-19), 0.67 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 161.71 (C, C-24), 160.72 (HCOO), 148.64 (CH, C-21), 145.22 (CH, C-22), 117.19 (C, C-20), 115.77 (CH, C-23), 72.75 (C, C-14), 70.72 (CH, C-3), 57.66 (CH, C-15), 43.39 (CH, C-17), 42.11 (C, C-13), 36.95 (CH, C-5), 36.53 (CH, C-9), 34.93 (C, C-10), 34.16 (CH_2, C-12), 32.31 (CH, C-8), 30.37 (CH_2, C-1), 30.23 (CH_2, C-4), 29.26 (CH_2, C-16), 25.30 (CH_2, C-6), 24.99 (CH_2, C-2), 23.44 (CH_3, C-19), 20.71 (CH_2, C-7), 19.52 (CH_2, C-11), 16.04 (CH_3, C-18); ESIMS m/z 413.2 [M + H]$^+$; ESIMS^2 (413.2 →) m/z 413.1 (11), 367.1 (100), 349.1 (13); ESIMS^3 (413.2 → 367.1 →) m/z 367.1 (40), 349.1 (11), 225.0 (10), 223.0 (14), 221.0 (14), 215.1 (40), 213.0 (11), 211.0 (14), 209.0 (20), 207.0 (17), 205.0 (12), 199.0 (17), 197.0 (22), 195.0 (35), 193.0 (18), 187.0 (42), 185.0 (23), 183.0 (36), 181.0 (35), 180.0 (11), 179.0 (22), 175.0 (12), 173.0 (23), 171.0 (31), 169.0 (65), 167.0 (28), 166.0 (11), 165.0 (29), 161.0 (22), 159.0 (73), 157.0 (53), 155.0 (41), 154.0 (11), 153.0 (33), 149.0 (12), 147.0 (33), 145.0 (73), 144.0 (12), 143.0 (62), 142.0 (25), 141.0 (62), 135.0 (21), 133.1 (59), 131.1 (100), 129.0 (52), 128.1 (61), 121.1 (23), 119.1 (53), 117.1 (51), 115.1 (36), 109.1 (25), 107.1 (36), 105.1 (71); HRESIMS m/z 413.2355 [M + H]$^+$ (calcd for $C_{25}H_{33}O_5^+$, 413.2323, Δ = 7.9 ppm).

(3*α*,5*β*,15*β*,16*β*)-3-(Acetylamino)-16-(acetyloxy)-14,15-epoxybufa-20,22-dienolide (8a): colorless powder (acetone); mp 294−306 °C; CD (c 2.1 × 10^{-3} M, $CHCl_3$) λ_{max} ($\Delta\varepsilon$) 230 (−1.54) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.92 (1H, brd, H-22), 7.15 (1H, brd, H-21), 6.21 (1H, d, J = 9.8 Hz, H-23), 5.46 (1H, d, J = 9.5 Hz, H-16), 3.79 (1H, m, H-3), 3.64 (1H, s, H-15), 2.77 (1H, d, J = 9.5 Hz, H-17), 2.02 (1H, td, J = 12.0, 3.8 Hz, H-8), 1.96 (3H, s, NCOCH_3), 1.90 (3H, s, OCOCH_3), 1.81 (1H, m, H-1a), 1.79 (1H, m, H-6a), 1.76 (1H, m, H-12a), 1.72 (1H, m, H-2a), 1.60 (1H, td, J = 12.0, 3.2 Hz, H-9), 1.55 (1H, m, H-4a), 1.51 (1H, m, H-7a), 1.51 (1H, m, H-11a), 1.50 (1H, m, H-4b), 1.49 (1H, m, H-5), 1.38 (1H, m, H-12b), 1.29 (1H, m, H-11b), 1.28 (1H, m, H-6b), 1.16 (1H, m, H-1b), 1.16 (1H, m, H-2b), 0.95 (3H, s, H-19), 0.90 (1H, m, H-7b), 0.80 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.29 (OCOCH$_3$), 169.35 (NCOCH$_3$), 161.70 (C, C-24), 151.50 (CH, C-21), 148.23 (CH, C-22), 116.19 (C, C-20), 113.87 (CH, C-23), 74.60 (CH_2, C-16), 72.44 (C, C-14), 59.35 (CH, C-15), 50.25 (CH, C-17), 49.28 (CH, C-3), 45.11 (C, C-13), 41.62 (CH, C-5), 39.97 (CH, C-9), 39.95 (CH_2, C-12), 35.28 (CH_2, C-1), 34.84 (C, C-10), 33.27 (CH_2, C-4), 33.14 (CH, C-8), 27.88 (CH_2, C-2), 25.87 (CH_2, C-6), 23.46 (NCOCH$_3$), 23.33 (CH_3, C-19), 20.71 (CH_2, C-11), 20.63 (CH_2, C-7), 20.56 (OCOCH$_3$), 17.19 (CH_3, C-18); ESIMS m/z 484.2 [M + H]$^+$; ESIMS^2 (484.2 →) m/z 424.2 (100); ESIMS^3 (484.2 → 424.2 →) m/z 424.2 (11), 382.1 (56), 365.1 (100), 347.1 (19), 215.1 (11); ESIMS^4 (484.2 → 424.2 → 365.1 →) m/z 365.1 (14), 347.1 (100), 319.1 (12), 305.1 (16), 283.0 (18), 265.0 (11), 251.0 (11), 241.1 (47), 237.0 (12), 225.1 (14), 223.0 (11), 215.1 (16), 213.1 (32), 211.0 (16), 195.0 (12), 187.0 (15), 185.0 (11), 181.0 (12), 175.0 (13), 173.0 (14), 171.0 (18), 159.1 (54), 157.1 (59), 155.0 (14), 147.1 (15), 145.1 (36), 143.1 (23), 142.0 (12), 133.1 (11), 131.1 (91), 129.1 (15), 128.1 (11), 119.1 (13), 105.1 (13); HRESIMS m/z 484.2682 [M + H]$^+$ (calcd for $C_{28}H_{38}NO_6^+$, 484.2694, Δ = −2.4 ppm).

(3*β*,5*β*,11*α*,14*β*,17*β*)-3,11-Bis(acetyloxy)-14-(formyloxy)-12-oxo-androstane-17-carboxylic acid methyl ester (9c): colorless solid (diethyl ether); mp 131−134 °C; $[\alpha]^{25}_D$ +66 ($CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.08 (1H, s, OC*H*O), 5.46 (1H, d, J = 11.7 Hz, H-11), 5.08 (1H, s, H-3), 4.00 (1H, dd, J = 9.5, 6.0 Hz, H-17), 3.66 (3H, s, COOCH_3), 2.84 (1H, dd, J = 14.8, 7.6 Hz, H-15a), 2.76 (1H, td, J = 12.1, 3.8 Hz, H-8), 2.21 (1H, m, H-16a), 2.16 (1H, m, H-9), 2.12 (3H, s, OCOCH_3 at 11), 2.05 (3H, s, OCOCH_3 at 3), 1.95 (1H, m, H-6a), 1.89 (1H, m, H-16b), 1.81 (1H, m, H-4a), 1.77 (1H, m, H-5), 1.71 (1H, m, H-1a), 1.70 (1H, m, H-7a), 1.69 (2H, m, H-2),1.51 (1H, m, H-4b), 1.48 (1H, m, H-7b), 1.46 (1H, m, H-1b), 1.39 (1H, m, H-15b), 1.34 (1H, m, H-6b), 1.33 (3H, s, H-18), 1.16 (3H, s, H-19); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 202.73 (C, C-12), 174.68 (C, C-20), 170.55 (C, OCOCH$_3$ at 3), 169.66 (C, OCOCH$_3$ at 11), 97.31 (C, C-

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14), 74.24 (CH, C-11), 69.92 (CH, C-3), 64.39 (C, C-13), 51.63 (CH_3, OCH_3), 45.58 (CH, C-17), 38.09 (CH, C-5), 37.34 (CH, C-8), 37.02 (CH, C-9), 36.95 (C, C-10), 33.22 (CH_2, C-1), 30.72 (CH_2, C-4), 29.86 (CH_2, C-15), 25.97 (CH_2, C-6), 25.30 (CH_2, C-2), 23.40 (CH_3, C-19), 22.57 (CH_2, C-7), 22.53 (CH_2, C-16), 21.44 (CH_3, $OCOCH_3$ at 3), 20.76 (CH_3, $OCOCH_3$ at 11), 16.33 (CH_3,C-18); ESIMS m/z 515.1 $[M + Na]^+$, 447.1 $[M - HCOOH + H]^+$; $ESIMS^2$ (447.1 →) m/z 447.1 (15), 415.1 (100); $ESIMS^3$ (447.1 → 415.1 →) m/z 345.0 (23), 327.0 (12), 285.0 (100), 267.0 (42), 239.0 (16), 185.0 (14), 159.0 (34); HRESIMS m/z 515.2210 $[M + Na]^+$ (calcd for $C_{26}H_{36}O_9Na^+$, 515.2252, Δ = −8.1 ppm).

(3β,5β,11α,14β,17β)-3,11-Bis(acetyloxy)-14-hydroxy-12-oxo-androstane-17-carboxylic acid γ-lactone (10f): colorless solid (diethyl ether/petroleum ether); mp 240−258 °C; CD (c 2.3 × 10^{-3} M, $CHCl_3$) λ_{max} (Δε) 230 (+0.86), 291 (+1.81) nm; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.46 (1H, d, J = 10.1 Hz, H-11), 5.09 (1H, s, H-3), 3.01 (1H, d, J = 4.1 Hz, H-17), 2.53 (1H, m, H-8), 2.32 (1H, t, J = 10.9 Hz, H-9), 2.16 (1H, m, H-16a), 2.11 (3H, s, $OCOCH_3$ at 11), 2.06 (3H, s, $OCOCH_3$ at 3), 2.01 (1H, m, H-6a), 1.96 (1H, m, H-15a), 1.88 (1H, m, H-7a), 1.86 (1H, m, H-4a), 1.86 (1H, m, H-5), 1.78 (1H, m, H-2a), 1.68 (1H, m, H-2b), 1.75 (1H, m, H-15b), 1.62 (1H, m, H-16b), 1.55 (1H, m, H-4b), 1.49 (1H, m, H-1a), 1.43 (1H, m, H-7b), 1.37 (1H, m, H-6b), 1.33 (1H, m, H-1b), 1.29 (3H, s, H-18), 1.23 (3H, s, H-19); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 200.44 (C, C-12), 175.98 (C, C-20), 170.57 ($OCOCH_3$ at 3), 169.55 ($OCOCH_3$ at 11), 91.47 (C, C-14), 72.81 (CH, C-11), 69.84 (CH, C-3), 61.26 (C, C-13), 50.53 (CH, C-17), 40.58 (CH, C-9), 37.78 (CH, C-5), 37.19 (C, C-10), 33.98 (CH, C-8), 32.93 (CH_2, C-1), 31.00 (CH_2, C-4), 27.45 (CH_2, C-15), 26.19 (CH_2, C-6), 25.30 (CH_2, C-2), 23.76 (CH_3, C-19), 22.45 (CH_2, C-7), 21.44 ($OCOCH_3$ at 3), 20.54 ($OCOCH_3$ at 11), 18.73 (CH_2, C-16), 14.86 (CH_3, C-18); ESIMS m/z 433.1 $[M + H]^+$; $ESIMS^2$ (433.1 →) m/z 433.1 (15), 415.1 (24), 387.1 (13), 373.0 (100), 355.0 (60), 313.0 (31), 295.0 (97), 267.0 (18), 249.0 (11), 213.0 (33), 187.0 (21); $ESIMS^3$ (433.1 → 295.0 →) m/z 295.0 (12), 267.0 (21), 249.0 (23), 239.0 (38), 213.0 (100), 187.0 (54); HRESIMS m/z 433.2214 $[M + H]^+$ (calcd for $C_{24}H_{33}O_7^+$, 433.2221, Δ = −1.5 ppm).

(3β,5β,11α,12β,14β,17β)-3,11,12-Tris(acetyloxy)-14-hydroxy-androstane-17-carboxylic acid methyl ester (10g): colorless solid (acetone/diethyl ether); mp 186−190 °C; CD (c 2.0 × 10^{-3} M, $CHCl_3$) λ_{max} (Δε) 231 (+0.62) nm; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.11 (1H, m, H-12), 5.10 (1H, m, H-3), 5.10 (1H, m, H-11), 3.70 (3H, s, $COOCH_3$), 2.65 (1H, m, H-17), 2.28 (1H, m, H-9), 2.26 (1H, m, H-15a), 2.12 (3H, s, $OCOCH_3$ at 12), 2.06 (1H, m, H-16a), 2.05 (3H, s, $OCOCH_3$ at 3), 1.97 (1H, m, H-7a), 1.94 (1H, m, H-4a), 1.92 (1H, m, H-16b), 1.92 (1H, m, H-6a), 1.90 (3H, s, $OCOCH_3$ at 11), 1.89 (1H, m, H-15b), 1.78 (1H, td, J = 12.1, 3.8 Hz, H-8), 1.68 (1H, m, H-1a), 1.66 (2H, m, H-2), 1.65 (1H, m, H-5), 1.47 (1H, m, H-4b), 1.35 (1H, m, H-1b), 1.30 (1H, m, H-7b), 1.25 (1H, m, H-6b), 1.13 (3H, s, H-18), 1.01 (3H, s, H-19); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 179.36 (C, C-20), 170.73 ($OCOCH_3$ at 3), 170.13 ($OCOCH_3$ at 12), 169.86 ($OCOCH_3$ at 11), 84.07 (C, C-14), 76.78 (C, C-12), 70.97 (CH, C-11), 70.32 (CH, C-3), 52.28 (CH_3, OCH_3), 52.03 (CH, C-17), 51.69 (C, C-13), 39.45 (CH, C-8), 38.59 (CH, C-5), 35.64 (CH_2, C-15), 35.59 (C, C-10), 33.40 (CH_2, C-1), 32.46 (CH, C-9), 30.87 (CH_2, C-4), 26.57 (CH_2, C-16), 26.15 (CH_2, C-6), 25.44 (CH_2, C-2), 23.36 (CH_3, C-19), 21.49 ($OCOCH_3$ at 3), 21.20 ($OCOCH_3$ at 12), 21.17 ($OCOCH_3$ at 11), 21.04 (CH_2, C-7), 15.73 (CH_3, C-18); ESIMS m/z 531.2 $[M + Na]^+$, 491.1 $[M - H_2O + H]^+$; $ESIMS^2$ (491.1 →) m/z 459.1 (15), 431.1 (100), 389.1 (29), 371.1 (36), 311.1 (25); $ESIMS^3$ (491.1 → 431.1 →) m/z 371.1 (62), 329.1 (12), 311.1 (100), 279.0 (14), 251.0 (83), 229.0 (15); $ESIMS^3$ (491.1 → 251.0 →) m/z 251.0 (16), 223.0 (17), 209.0 (25), 195.0 (60), 183.0 (25), 181.0 (29), 171.0 (17), 169.0 (87), 159.0 (13), 157.0 (45), 155.0 (13), 143.0 (100), 119.0 (20); HRESIMS m/z 531.2566 $[M + Na]^+$ (calcd for $C_{27}H_{40}O_9Na^+$, 531.2565, Δ = 0.2 ppm).

Cancer Cell Lines. Human and murine normal and cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), the European Collection of Cell Culture (ECACC, Salisbury, UK), and the Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ, Braunschweig, Germany). The code number and histological type of each of the cell lines used in the current study are detailed in Table 1.

The U373 cell line was cultured in MEM culture medium (Lonza; code 12-136F), while the Hs683, SKMEL-28, B16F10, A549, PC-3, MCF-7, and CT26 cell lines were cultured in RPMI culture medium (Lonza; code 12-115F) supplemented with 10% heat-inactivated fetal bovine serum (Lonza; code DE14-801F). Cell culture media were supplemented with 4 mM glutamine (Lonza; code BE17-605E), 100 μg/mL gentamicin (Lonza; code 17-5182), and penicillin−streptomycin (200 units/mL and 200 μg/mL) (Lonza; code 17-602E).

Determination of the IC_{50} Growth Inhibitory Concentrations in Vitro. The MTT colorimetric assay was used as detailed previously.[52,53] Briefly, this test measures the number of metabolically active (living) cells that are able to transform the yellow substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into the blue formazan dye via a mitochondrial reduction involving succinate dehydrogenase. The amount of formazan obtained at the end of the experiment (measured by spectrophotometry) is directly proportional to the number of living cells. The determination of the optical density in the control compared to the treated cells therefore enables quantitative measurements of the effects of compounds on the growth of normal as well as cancer cells in vitro. Each experimental condition was assessed in six replicates.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H and ^{13}C NMR data of compounds **1a**−**11b**, as well as ^1H and ^{13}C NMR spectra of compounds **4e**, **4f**, **8a**, **9c**, **10f**, and **10g**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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