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Guanaconetins, new antitumoral acetogenins, mitochondrial complex I and tumor cell growth inhibitors

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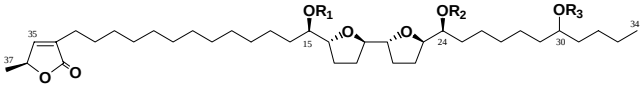
Abstract—The antitumoral activity of a series of acetylated bis-tetrahydrofuranic acetogenins with a *threo/trans/threo/trans/erythro* relative configuration was characterized by four new natural and two semisynthetic, 15,24,30-trioxygenated acetogenins that were found to inhibit mitochondrial complex I enzyme as well as growth of several tumor cell lines. Placement of acetyl groups along the alkyl chain modulated the potency of the bis-tetrahydrofuranic acetogenins and could be important for future utilization of these compounds as chemotherapeutic agents.

1. Introduction

In 1982, uvaricin, a new class of antitumor agents, was isolated from the roots of *Uvaria acuminata* (Annonaceae).[1] Chemically, uvaricin is a bis-tetrahydrofuranic (THF) acetogenin characterized by a terminal α,β-unsaturated γ-lactone, an acetoxyl group at C-24, and a *threo/trans/threo/trans/erythro* relative configuration in the α,α′-bis-THF system. Nineteen more acetylated acetogenins (linear, mono- or bis-THF) have been reported since that time.[2]

We have recently examined the structure–activity relationships (SARs) of some natural bis- and mono-THF acetogenins and semisynthetic derivatives as growth inhibitors against several human tumor cell lines. The trends in the cytotoxicity assays were compared with the potency of the acetogenins as inhibitors of the mitochondrial respiratory chain.[3–6]

The selective acetylation at different positions of a series of bis-THF acetogenins 15,24,30-trioxygenated (four of them were new natural compounds (**1**–**4**) isolated from *Annona* aff. *spraguei*, and two were semisynthetic (**5**,**6**) derivatives) was determined by comparing the SARs of these acetogenins, with a *threo/trans/threo/trans/erythro* relative configuration, with respect to their activity as complex I inhibitors and as tumor cell growth inhibitors.



1: R_1= H; R_2= R_3= $COCH_3$ (**guanaconetin-1**)
2: R_1= R_3= $COCH_3$; R_2= H (**guanaconetin-2**)
3: R_1= R_3= H; R_2= $COCH_3$ (**guanaconetin-3**)
4: R_1= R_2= H; R_3= $COCH_3$ (**guanaconetin-4**)
5: R_1= R_2= R_3= $COCH_3$
6: R_1= R_2= R_3= H

Keywords: Acetylated annonaceous acetogenins; Complex I inhibitors; Growth tumor cell inhibitors; Structure–activity relationship.
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2. Results and discussion

2.1. Isolation

Four mono- or di-acetylated bis-THF acetogenins, 15,24,30-trioxygenated (**1**–**4**), were isolated from seeds of the Colombian species *Annona* aff. *spraguei* (Annonaceae). Guanaconetin-1, guanaconetin-2, guanaconetin-3, and guanaconetin-4 (**1**–**4**) were obtained by classic chromatographic purification and semipreparative HPLC partition with methanol/water/THF 85:15:5.

The molecular weight of guanaconetin-1 (**1**) was determined by FAB-MS, *m*/*z* 729 $[M+Na]^+$, and corresponded to the molecular formula $C_{41}H_{70}O_9$. The presence of an α,β-unsaturated γ-lactone, an α,α′-dioxygenated bis-THF system, and two acetoxyl groups in **1** was deduced from ^1H–^{13}C NMR homo- and heteronuclear correlations (see Fig. 1). The existence in **1** of one hydroxyl and one acetoxyl groups flanked the adjacent bis-tetrahydrofuran moiety, and the presence of a second acetoxyl group in the terminal hydrocarbon chain, was confirmed by 2D NMR and EI-MS experiments. Location of the hydroxyl (position 15) and one of the acetoxyl (position 24) groups was deduced from the observed fragments in the EI-MS, at *m*/*z* 295, *m*/*z* 347, and *m*/*z* 399 (see Fig. 1). Location of the second acetoxyl group at C-30 was determined by an exhaustive analysis of the ^1H and ^{13}C NMR values of **1** and those of the other five C-30 oxygenated analogues (**2**–**6**), one of them corresponding to a known acetogenin, bullanin (**6**) (see Figs. 2–5).[7,8] Unambiguous differences could be observed between the ^{13}C NMR chemical shifts, concerning the terminal chain (C-29 to C-34), δ 37.2, δ 71.9, δ 37.3, δ 27.8, δ 22.7, and δ 14.1, respectively, for the C-30 hydroxylated analogues (**3** and **6**), and δ 34.0, δ 74.3, δ 34.0, δ 31.7, δ 22.5, and δ 14.0, respectively, for the C-30 acetylated analogues (**1**, **2**, **4**, and **5**) (see Figs. 1–5). In the ^1H NMR spectrum, the most important difference was observed for the proton at C-30, δ 3.60 (hydroxylated derivatives, **3** and **6**), and δ 4.86 (acetylated derivatives, **1**, **2**, **4**, and **5**).

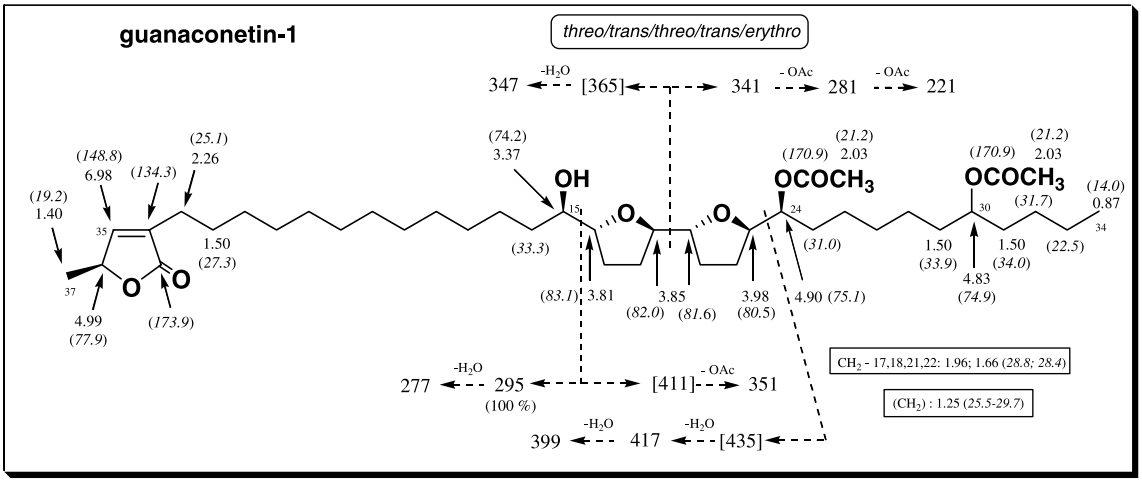

Figure 1. ^1H NMR and ^{13}C NMR (in parentheses) spectral data and significant EI-MS fragment ions of guanaconetin-1 (**1**).

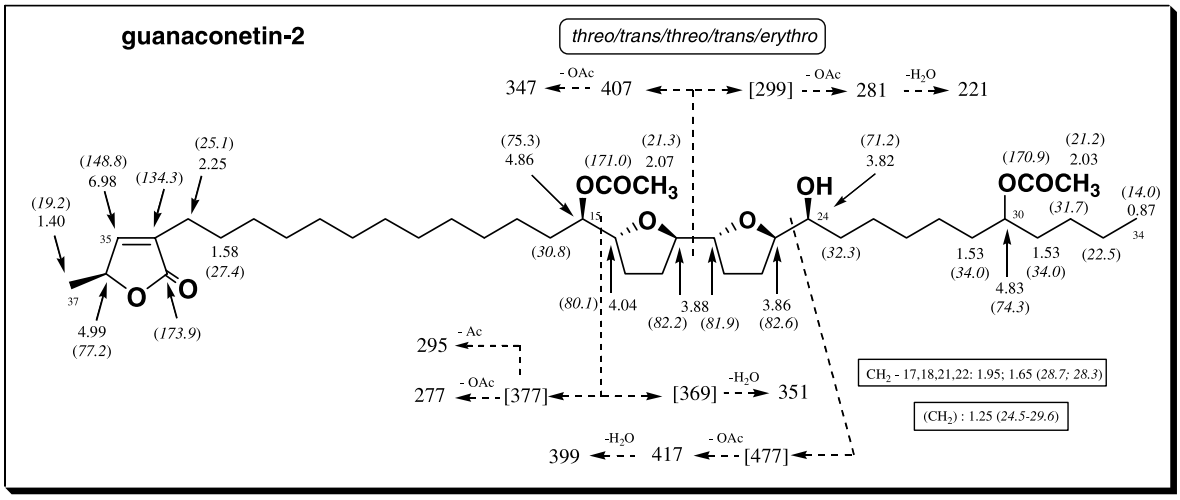

Figure 2. ^1H NMR and ^{13}C NMR (in parentheses) spectral data and significant EI-MS fragment ions of guanaconetin-2 (**2**).

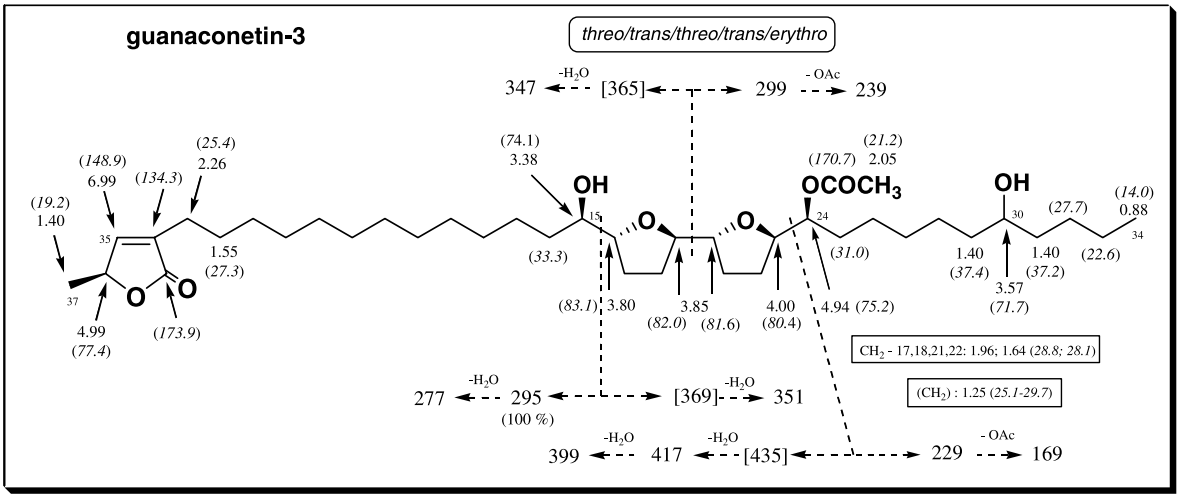

Figure 3. ^{1}H NMR and ^{13}C NMR (in parentheses) spectral data and significant EI-MS fragment ions of guanaconetin-3 (**3**).

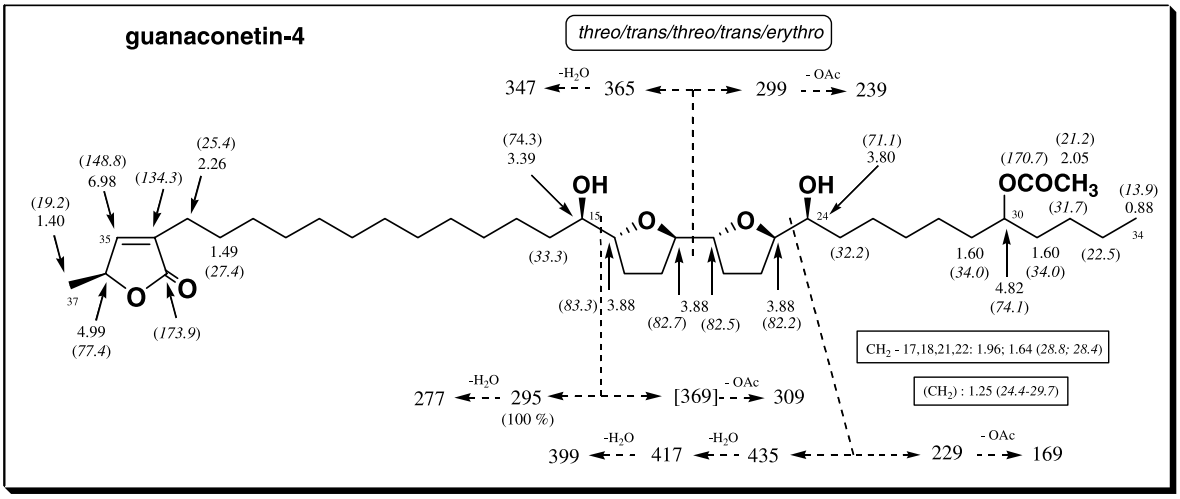

Figure 4. ^{1}H NMR and ^{13}C NMR (in parentheses) spectral data and significant EI-MS fragment ions of guanaconetin-4 (**4**).

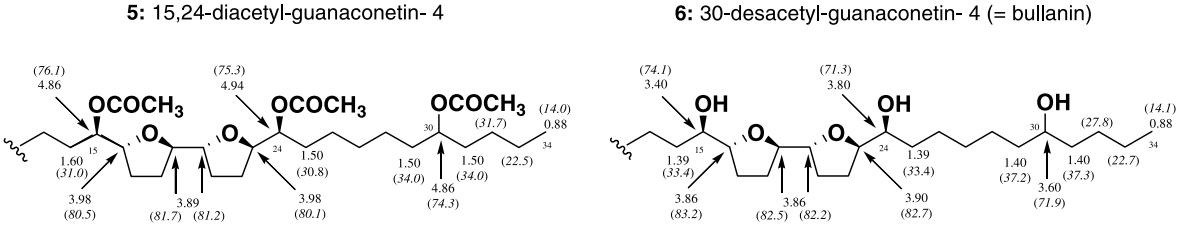

Figure 5. ^{1}H NMR and ^{13}C NMR (in parentheses) spectral data of the compounds **5** and **6**.

A chemical correlation was established by acetylation of compounds **1**–**4** to give the same triacetylated compound (**5**), and by deacetylation of guanaconetin-4 (**4**) to give compound **6**, identical to bullanin.[7,8]

The relative stereochemistry across the α,α′-dioxygenated bis-THF system of natural compounds (**1**–**4**) was deduced to be *threo*/*trans*/*threo*/*trans*/*erythro* based on the ^{1}H and ^{13}C NMR spectral data of uvaricin and other natural acetylated acetogenins.[1,2,9–11]

2.2. Biological activity

Table 1 shows the ED_{50} values against the tumor cell lines of compounds **1**–**5** and IC_{50} for inhibition of complex I of compounds **1**–**6**. The ED_{50} values were in the micromolar range, whereas IC_{50} values obtained were in the nanomolar range and were similar to what had been observed in previous sets of *threo*/*trans*/*threo*/*trans*/*erythro* acetogenins.[6] Human liver carcinoma (HepG2) was the most sensitive cell line to the set of

Table 1. Biological activity of the *threo/trans/threo/trans/erythro* bis-THF ACG studied with standard deviations

No.	Compound	[Max] (μM)	ED_{50} ± SD (μM)						IC_{50} ± SD (nM)
			MCF-7	HepG2	HT-29	A-549	MES-SA	MES-SA/DX5	NADH Ox
1	Guanaconetin-1	250	107 ± 8	40 ± 8	>250 (16%)	>250 (47%)	77 ± 17	182 ± 29	3.4 ± 1.2
2	Guanaconetin-2	250	68 ± 18	31 ± 4	>250 (25%)	213 ± 25	66 ± 3	89 ± 9	7.5 ± 1.6
3	Guanaconetin-3	250	33 ± 3	17 ± 6	129 ± 14	102 ± 21	30 ± 6	77 ± 3	2.4 ± 0.1
4	Guanaconetin-4	250	77 ± 6	27 ± 11	235 ± 14	219 ± 25	64 ± 7	102 ± 6	2.7 ± 0.2
5	15,24-Diacetyl-guanaconetin-4	250	97 ± 25	132 ± 47	>250 (16%)	>250 (44%)	>250 (32%)	>250 (45%)	4.7 ± 0.8
6	30-Deacetyl-guanaconetin-4	250	—	—	—	—	—	—	0.5 ± 0.1
7	Rotenone	500	161 ± 14	15 ± 6	202 ± 14	26 ± 7	147 ± 31	214 ± 41	5.1 ± 0.9
8	Doxorubicin	500	8 ± 3	102 ± 9	274 ± 19	152 ± 31	173 ± 54	>500 (40%)	—

Data shown are the ED_{50} (effective dose 50) observed for 50% inhibition of cell growth in the MTT cytotoxicity assay and the IC_{50} (inhibitory concentration 50) observed for NADH oxidase enzymatic inhibition.

ACG, followed by breast (MCF-7), ovary (MES-SA), and ovary doxorubicin-resistant (MES-SA/Dx5), while lung (A-549) and colon (HT-29) were the least sensitive carcinomas toward this set of acetogenins. Cytotoxicity against the MES-SA cell line was slightly higher than that observed against the ovary doxorubicin-resistant cell line (MES-SA/Dx5). Trends were similar to those previously observed for other *threo/trans/threo/trans/erythro* acetogenins,[6] although ED_{50} values were higher among this set. In addition, the selectivity of these acetogenins against the liver adenocarcinoma cell line was more apparent.

Guanaconetin-3 (**3**) showed a higher cell growth inhibition for all cell lines, whereas 15,24-diacetyl-guanaconetin-4 (**5**) was the least potent. Specifically, compound **3** was the most potent in all series, followed by **2** or **4**, both with similar potencies, and by **1** or **5**, being **5** the least potent in the MES-SA, MES-SA/Dx5, and HepG3 cell lines. As a general trend, a single acetyl moiety showed improved activity against the tumor cell lines. This potency seemed to decrease as the number of acetyl groups in the molecule increased. When compounds with the same number of acetyl groups were compared, an acetyl in position 30 of the alkyl chain decreased the potency of the acetogenin to the level of those with two acetyl groups (**4** and **2** showed similar potencies and were less potent than **3**).

On the other hand, potency toward the target enzyme (mitochondrial complex I), measured as the inhibition of the integrated NADH oxidase enzymatic activity, tended to agree with the trends for inhibition of human tumor cell lines. 30-Deacetyl-guanaconetin-4 (**6**) was the most potent, but with IC_{50} values higher than those described for other sets of ACG with *threo/trans/threo/trans/erythro* relative configuration.[6] The small quantities that were isolated prevented the determination of compound ED_{50} values against the tumor cell panel.

In the enzyme inhibition assay, acetogenins without acetyl moieties (**6**) showed IC_{50} values lower than those of acetogenins with one acetyl group (**3** and **4**), followed by the acetogenin with two acetyl groups (**1**), and by the acetogenin with three acetyl groups along the alkyl chain (**5**). Remarkably, the acetyl group in 30 position decreased the cell growth inhibition in the antitumoral panel, which is a similar trend as in the case of the guanaconetin-2 (**2**) that resulted in IC_{50} values even higher than that of the assay control rotenone.

Placement of the acetyl groups along the alkyl chain in this series has a significant effect on activity. It is unclear if the decreased cell growth inhibition of the acetogenins was due to increased lipophobicity or was caused by steric impediments that these moieties present. Nevertheless, the trends indicated that the position of these chemical groups along the alkyl chain is important for modulating the potency and that their placement must be considered if the bis-THF acetogenins are intended to be used in the future as chemotherapeutic agents.

3. Experimental section

3.1. General experimental procedure

Optical rotations were measured in EtOH using a Perkin-Elmer 241 polarimeter. IR spectra (film) were run on a Satellite FTIR-Mattson Serial 980514 spectrometer. MS (EI-MS or HREIMS) were determined on a VG Auto Spec Fisons spectrometer. ^{1}H NMR (300 or 400 MHz) and ^{13}C NMR (75 or 100 MHz) spectra were recorded on Varian Unity-300 or Varian Unity-400 instruments, using the solvent signal as reference ($CDCl_3$ at δ 7.26 and 77.0). Multiplicities of ^{13}C NMR resonances were assigned by DEPT experiments. COSY 45 HMSC and HMBC correlations were run using a Varian Unity-400 MHz instrument. Chromatographic separations were carried out by column chromatography on silica gel 60H (5–40 μm, 7336 Merck) or by flash chromatography on silica gel 60 (230–400 μm, 9385 Merck), and semipreparative HPLC on a LiChroCartR 100 RP-18 column, flow rate 4.5 ml/min, using methanol/water/THF 85:15:5.

3.2. Plant material

The seeds of *Annona* aff. *spraguei* Safford (Annonaceae), a local tree known as 'guanacona' or 'tio-tio,' were collected from Sahagún town in Cordoba region (Colombia). A voucher specimen was deposited, under ref 36732, at the National University of Medellin, Colombia.

3.3. Extraction and isolation

Dried and powdered seeds (1000 g) of *A*. aff. *spraguei* were defatted with petroleum ether. The defatted seeds were extracted with EtOAc and the concentrated extract (52 g) was partitioned between hexane and methanol. Five grams of the methanolic solution was subjected to column chromatography on silica gel 60 H (CH_2Cl_2/AcOEt 50:50) and then to semipreparative HPLC (methanol/water/THF 85:15:5). Guanaconetin (**1**, 35 mg) (t_R = 27.2 min), guanaconetin-2 (**2**, 40 mg) (t_R = 24.5 min), guanaconetin-3 (**3**, 12 mg) (t_R = 15.3 min), and guanaconetin-4 (**4**, 15 mg) (t_R = 18.2 min) were isolated.

3.3.1. Guanaconetin-1 (1). Amorphous powder; $C_{41}H_{70}O_9$; $[\alpha]_D^{25}$ +4.8° (*c* 0.62, EtOH); IR, ν_{max} (film) cm^{-1} 3380, 2928, 2856, 1750, 1738, 1256, 749; FAB-MS, *m/z* 729 $[M+Na]^+$; EIMSHR, *m/z* 351.2493 (calcd for $C_{21}H_{35}O_4$, 351.2535), 341.2313 (calcd for $C_{19}H_{33}O_5$, 341.2328), 295.2251 (calcd for $C_{18}H_{31}O_3$, 295.2273), 281.2096 (calcd for $C_{17}H_{29}O_3$, 281.2116), 277.2158 (calcd for $C_{18}H_{29}O_2$, 277.2167); EI-MS, ^{1}H NMR and ^{13}C NMR data, see Figure 1.

3.3.2. Guanaconetin-2 (2). Amorphous powder; $C_{41}H_{70}O_9$; $[\alpha]_D$ +9.3° (*c* 1.08, EtOH); IR, ν_{max} (film) cm^{-1} 3390, 2928, 2850, 1752, 1738, 1242; FAB-MS, *m/z* 729 $[M+Na]^+$; EI-MS, ^{1}H NMR and ^{13}C NMR data, see Figure 2.

3.3.3. Guanaconetin-3 (3). Amorphous powder; $C_{39}H_{68}O_8$; $[\alpha]_D$ +11.1° (*c* 1.08, EtOH); IR ν_{max} (film) cm^{-1}: 3420, 2928, 1751, 1735, 1240; FAB-MS, *m/z* 687 $[M+Na]^+$, *m/z* 664 $[M]^+$, *m/z* 646 $[M-H_2O]^+$, *m/z* 628 $[M-2H_2O]^+$, *m/z* 604 $[M-AcOH]^+$; EIMSHR, *m/z* 665.5031 (calcd for $C_{39}H_{69}O_8$, 665.4992), EI-MS, ^{1}H NMR and ^{13}C NMR data, see Figure 3.

3.3.4. Guanaconetin-4 (4). Amorphous powder; $C_{39}H_{68}O_8$; $[\alpha]_D$ +9.0° (*c* 0.88, EtOH); IR ν_{max} (film) cm^{-1}: 3410, 2926, 2854, 1757, 1735, 1244, 1070; LSIMS, *m/z* 687 $[M+Na]^+$, *m/z* 665 $[MH]^+$, *m/z* 647 $[MH-H_2O]^+$, *m/z* 604 $[MH-AcOH]^+$; EI-MS, ^{1}H NMR and ^{13}C NMR data, see Figure 4.

3.4. Preparation of 15,24-diacetylguanaconetin-4 (5)

This compound was prepared from **4** (5 mg) by Ac_2O and pyridine at room temperature for 8 h to yield 5 mg of **5** (same procedure was used from **1**, **2**, and **3**, to yield identical compound **5**). Amorphous powder; $C_{43}H_{72}O_{10}$; $[\alpha]_D$ +8.2° (*c* 1.6 EtOH); FAB-MS, *m/z* 769 $[M+Na]^+$, *m/z* 747 $[MH]^+$; ^{1}H NMR and ^{13}C NMR data, see Figure 5.

3.5. Preparation of 30-deacetylguanaconetin-4 (6)

Compound **4** (5 mg) in MeOH/HCl (2:0.1 ml) mixture was maintained at 0 °C with stirring for 8 h. The crude product of the reaction was extracted with CH_2Cl_2 and the residue was purified by flash chromatography, eluting with cyclohexane/ethylacetate 3:7 to give **6** (1 mg), identical to bullanin.[7,8] Amorphous powder; $C_{37}H_{66}O_7$; FAB-MS, *m/z* 623 $[MH]^+$; ^{1}H NMR and ^{13}C NMR data see Figure 5.

3.6. Bioassay of experimental procedures

3.6.1. Enzyme inhibition. The inhibitory potency of the compounds was assayed using submitochondrial particles (SMP) from beef heart. SMP were obtained by an extensive ultrasonic disruption of freeze-thawed mitochondria to produce open membrane fragments where permeability barriers to substrates were lost. Active complex I content in SMP preparations was estimated as previously described,[12] giving a concentration of 45.8 ± 0.3 pmol/mg. SMP were diluted to 0.5 mg/ml in 250 mM sucrose, 10 mM Tris–HCl buffer, pH 7.4, and treated with 300 μM NADH to activate complex I before starting experiments.

The enzymatic activity was assayed at 22 °C in 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA with the SMP diluted to 6 mg/ml (0.28 ± 0.01 nM complex I) in the cuvette.[13] NADH oxidase activity was measured as the aerobic oxidation of 75 μM NADH in the absence of external quinone substrates and other respiratory chain inhibitors. Reaction rates were calculated from the linear decrease of NADH concentration (λ = 340 nm, ε = 6.22 mM^{-1} cm^{-1}) in an end-window photomultiplier spectrophotometer ATI-Unicam UV4-500.

Stock solutions (2 mM in absolute ethanol) of the acetogenins were prepared and kept in the dark at −20 °C. Appropriate dilutions between 5 and 50 μM were made before the titrations. Increasing concentrations of these ethanolic solutions were then added to the diluted SMP preparations with 5 min incubation on ice between each addition. Maximum ethanol concentration never exceeded 2% of volume and control activity was unaffected at this concentration.[14] After each addition of inhibitor, the enzymatic activities involving complex I were measured as described. The inhibitory concentration 50 (IC_{50}) was taken as the final compound concentration in the assay medium that yielded 50% inhibition of NADH oxidase activity. Given values are means ± SD of four assays for each compound.

3.6.2. Tumor cell lines. General growth and incubation conditions were the same as those in earlier experiments[4–6] with six cell lines obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A-549 monolayer cells (human lung carcinoma, CCL-185) were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, HepG2 cells (human liver carcinoma, CCL-8085) were grown in MEM with 10% qualified FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 μM MEM-non-essential amino acids, MCF-7 cells (human breast HTB-22) were maintained in the previous medium supplemented with 0.01 mg/ml bovine insulin and HT-29 cells (human colon carcinoma HTB-38), MES-SA cells (human ovary carcinoma CRL-1976), and MES-SA/Dx5 monolayer cells (human ovary carcinoma doxorubi-

cin-resistant CRL-1977) were cultured routinely in McCoy's 5A medium modified with 10% FBS and 2 mM glutamine. All cell cultures were kept at 37 °C under a humidified atmosphere of 5% CO_2.

3.6.3. Antitumor assay. The general cytotoxicity activity method used was similar to the one previously used for evaluating sets of bis-THF acetogenin[4,6] and mono-THF acetogenin[5] compounds. The MTT test was applied to the six cell lines for the evaluation of cytotoxic activity. It indirectly measures the cytotoxicity and is based on the impaired ability of drug-treated cells to reduce the pale yellow MTT to a deep blue formazan. NADH was provided directly by the cells which, in turn, required a proper metabolic function. Therefore, inhibition of the MTT reduction rate was an indicator of the functional integrity of the mitochondria and, hence, of cellular viability.[15] Samples (10 μl) were incubated with 60–80% of confluent culture of each cell line for 24 h in an atmosphere of 5% CO_2 at 37 °C. The number of cells per culture well was 200,000 for MES-SA, 300,000 for MES-SA/Dx5, 150,000 for HepG2, 500,000 for MCF-7, 150,000 for HT-29, and 100,000 for A-549. The titration range started at the concentrations indicated in Table 1. Each compound was titrated in twofold serial dilutions per triplicate. Absorption at 490 nm was measured in a Victor2™ Wallac spectrofluorometer. Effective dose 50 (ED_{50}) was determined as the inhibitor concentration that killed 50% of tumor cells. Doxorubicin was used as control.

Acknowledgments

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