10-Oximeguanacone, the First Nitrogenated Acetogenin Derivative Found To Be a Potent Inhibitor of Mitochondrial Complex I

Teresa Gallardo,[†] Jairo Saez,[‡] Hillmer Granados,[‡] José R. Tormo,[†] Ivan D. Velez,[‡] Nestor Brun,[‡] Beatriz Torres,[‡] and Diego Cortes*[,†]

Departamento de Farmacología, Farmacognosia y Farmacodinamia, Facultad de Farmacia, Universidad de Valencia, 46100 Burjassot, Valencia, Spain, and Departamento de Química, Universidad de Antioquia, AA1226 Medellin, Colombia

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A new 10-keto bis-tetrahydrofuran acetogenin, guanacone (**1**), has been isolated from a cytotoxic extract of *Annona* aff. *spraguei* seeds. The 10-oximeguanacone derivative **1f** is the first bioactive nitrogenated acetogenin found to be a very potent inhibitor of complex I. In addition, a SAR study of guanacone analogues is reported based on the titration of the NADH oxidase and NADH:ubiquinone oxidoreductase activities.

Several species of the family Annonaceae yield a group of bioactive secondary metabolites known as acetogenins. These polyketide compounds are a class of promising anticancer, antiparasitic, and pesticidal agents.[1−3] Studies on their mode of action have established that Annonaceous acetogenins are the most powerful specific inhibitors of the mammalian electron transport chain complex I.[4,5] Some acetogenins also inhibit a new NADH oxidase located in the cytoplasmatic membrane of cancer cells, which is involved in signal recognition.[6] This second target could increase the sensitivity of the tumor cells to acetogenins.[7] We have recently studied the mechanism of the cytotoxic activity of rollimembrin[8] on mitochondrial complex I from beef heart, in comparison with the known rolliniastatin-1[5] and membranacin,[9] the only acetogenins with the rare *threo*/*cis*/*threo*/*cis*/*erythro* relative configuration.

Results and Discussion

In our present work on *Annona* aff. *spraguei* seeds from a Colombian tree known as "guanacona" or "tio-tio" an unusual 10-keto bis-tetrahydrofuran (THF) acetogenin, guanacone (**1**), was isolated from the EtOAc extract as a colorless wax. Its molecular weight was determined by peaks at *m*/*z* 643 $[M + Na]^+$ and *m*/*z* 621 $[MH]^+$ in the FABMS, corresponding to the molecular formula $C_{37}H_{64}O_7$. The existence in **1** of an α,β-unsaturated γ-lactone was first suggested by a positive Kedde reaction and by a 1751 cm^{-1} carbonyl absorption band in the IR spectrum and was confirmed by the characteristic signals in the ^1H and ^{13}C NMR spectrum, which proved the absence of an OH at the C-4 position typically found in most acetogenins (Figure 1).[1−3]

The presence of a keto group in **1** was suggested by the existence of a triplet at δ 2.39 (H-9,11) in the ^1H NMR spectrum and ^{13}C NMR resonances at δ 211.46 (C-10) and 42.70 (C-9,11) due to the keto-bearing carbon and the two flanked methylene carbons, respectively. The location of the keto group was confirmed by the fragments at *m*/*z* 223 and *m*/*z* 195 in the EIMS.

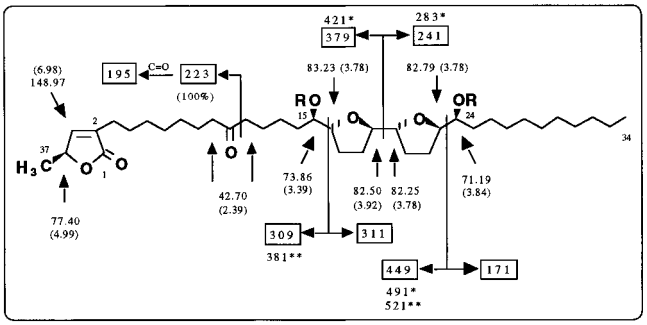
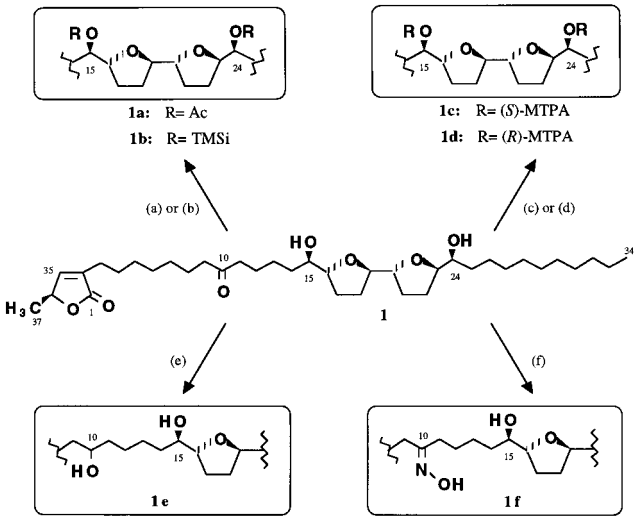


Figure 1. Diagnostic ^{13}C NMR and ^1H NMR values (in parentheses) of guanacone (**1**); and EIMS (*m*/*z*) of **1** (R = H), diacetylguanacone (**1a**)* (R = Ac), and di-TMSi-guanacone (**1b**)** (R = TMSi).



(a): Ac_2O/Pyr; (b); N,O-bis(TMSi)-acetamide/Pyr; (c) and (d): 4-(dimethylamino)pyridine, (*S*) or (*R*)-MTPA-Cl; (e): $NaBH_4$/MeOH; (f): $ClNH_4OH$/Pyr

Figure 2. Synthesis of guanacone (**1**) derivatives.

Moreover, two OH groups in **1** could be proposed from the prominent IR absorption at 3416 cm^{-1}, two successive losses of H_2O from the $[MH]^+$ in the FABMS, and the preparation of diacetate (**1a**) and di-TMSi (**1b**) derivatives. An adjacent bis-THF system in **1** could be unambiguously assigned by 1D (^1H, ^{13}C, and DEPT) and 2D (COSY and HMQC) NMR experiments, and its

* To whom correspondence should be addressed. Tel.: (34) 963-86-49-75. Fax: (34) 963-86-49-43. E-mail: dcortes@uv.es.
[†] Universidad de Valencia.
[‡] Universidad de Antioquia.

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Table 1. 1D and 2D NMR Data (400 MHz, $CDCl_3$) of Guanacone (**1**)

H	δ (*J*, Hz)	COSY 45 coupling	HMQC coupling (multiplicity DEPT ^{13}C)
1			174.90 (C)
2			134.10 (C)
3	2.24 m (7.2)	H-4 (1.55)	25.24 (CH_2)
4	1.55 m	H-3 (2.24), H-5 (1.24)	27.26 (CH_2)
5−7	1.24 m		29.57−29.02 (CH_2)
8,12	1.55 m	H-9,11 (2.39), H-7,13 (1.24)	23.69 (CH_2)
9,11	2.39 t (7.5)	H-8,12 (1.55)	42.70 (CH_2)
10			211.46 (C)
13	1.24 m	H-14 (1.39)	25.08 (CH_2)
14	1.39 m	H-13 (1.24), H-15 (3.39)	32.82 (CH)
15	3.39 m	H-16 (3.78), H-14 (1.39)	73.86 (CH)
16	3.78 m	H-15 (3.39), H-17 (1.96, 1.60)	83.23 (CH)
17,18	1.96,1.60 m	H-16 (3.78), H-19 (3.92)	28.36−24.38 (CH_2)
19	3.92 m	H-18 (1.96, 1.60), H-20 (3.78)	82.50 (CH)
20	3.78 m	H-19 (3.92), H-21 (1.96, 1.60)	82.25 (CH)
21,22	1.96, 1.60 m	H-20 (3.78), H-23 (3.78)	28.36−24.38 (CH_2)
23	3.78 m	H-22 (1.96, 1.60), H-24 (3.84)	82.79 (CH)
24	3.84 m	H-23 (3.78), H-25 (1.39)	71.19 (CH)
25	1.39 m	H-24 (3.84), H-26 (1.24)	32.30 (CH_2)
26	1.24 m	H-25 (1.39)	25.08 (CH_2)
27−31	1.24 m		29.57−29.02 (CH_2)
32	1.24 m		31.85 (CH_2)
33	1.24 m	H-34 (0.86)	22.64 (CH_2)
34	0.88 t (5.8)	H-33 (1.24)	14.08 (CH_3)
35	6.98 d (1.5)	H-36 (4.99)	148.97 (CH)
36	4.99 dq (6.7,1.5)	H-35 (6.98), H-37 (1.40)	77.40 (CH)
37	1.40 d (6.7)	H-36 (4.99)	19.16 (CH_3)

Table 2. ^1H NMR (400 MHz, $CDCl_3$) Data of Mosher Esters of **1** (**1c** and **1d**)

	H-14	H-16	H-17/18
(*S*)-MTPA-**1** (**1c**)	ca. 1.30	3.79	1.90−1.75
(*R*)-MTPA-**1** (**1d**)	ca. 1.30	3.81	2.00−1.80
$\Delta\delta_{S-R}$		−0.02	−0.10/−0.05
configuration	C-15*R*		

placement in the alkyl chain was deduced by the EIMS of **1**, **1a**, and **1b**. The relative stereochemistry across this α,α′-dihydroxylated bis-THF system, was deduced as *threo*/*trans*/*threo*/*trans*/*erythro* based on the ^1H and ^{13}C NMR data of **1** and **1a**, which were consistent with those of model compounds (Table 1 and Figure 1).[10,11]

The absolute stereochemistry of the carbinol stereogenic centers of **1** was determined by preparing the Mosher esters on the basis of the differences between the ^1H NMR chemical shifts of (*S*)- and (*R*)-MTPA (methoxy-trifluoromethyl-phenylacetate) ester derivatives,[12−14] namely (*S*)-MTPA (**1c**) and (*R*)-MTPA (**1d**). The negative $\Delta\delta_H$ ($\delta_S - \delta_R$) values of H-16 to H-18 confirmed a (15*R*) absolute configuration of this carbinol center. The relative configuration of **1**, identical to that of rolliniastatin-2 (**2**),[15] allowed us to establish the 15*R*, 16*R*, 19*R*, 20*R*, 23*R*, and 24*S* absolute configuration (Table 2).

The availability of guanacone derivatives to conduct a SAR study of these compounds prompted us to produce the 10-dihydroguanacone, (**1e**) and the 10-oximeguanacone, (**1f**) where the ketone function has been modified by selective reduction or imine preparation, respectively. In addition, three other acetogenins with identical *threo*/*trans*/*threo*/*trans*/*erythro* relative configuration also isolated from *A.* aff. *spraguei*, rolliniastatin-2 (**2**), desacetyluvaricin (**3**), and motrilin (**4**)[1−3] were selected for the biological assays.

We have studied the mode of action of these six acetogenins on the basis of their inhibition of complex I on inverted submitochondrial particles from beef heart.[16] None of the acetogenins has been proved to inhibit mammalian mitochondrial complexes other than complex I.[4] The NADH oxidase assay represents an integrated activity in which NADH is oxidized and the electrons are transferred along the respiratory chain to be finally accepted by molecular oxygen. Therefore, the inhibition of NADH oxidase activity is directly attributed to the inhibition of NADH:ubiquinone oxidoreductase and represents a good method for evaluating the different potency of related compounds such as guanacone (**1**) and its analogues (**1e**, **1f**, **2**−**4**). Figure 3 shows the titration curves of the six bis-THF acetogenins against NADH oxidase activity. We have also included in this study rollimembrin (**5**)[8] and rotenone,

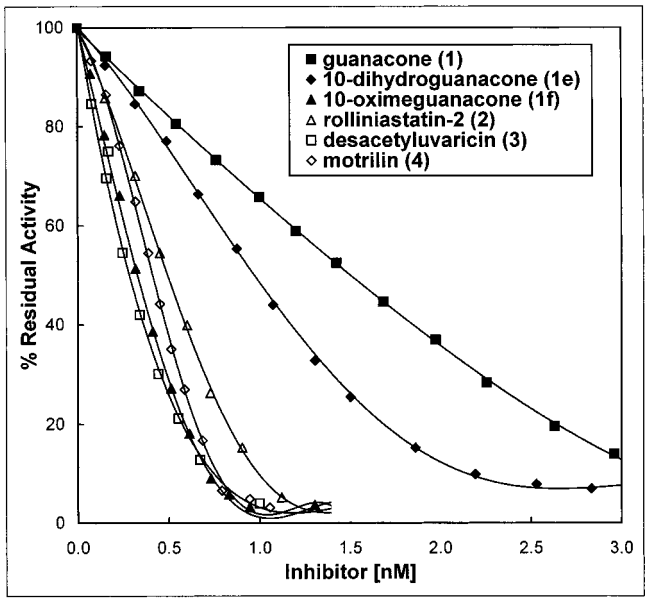


Figure 3. Titrations of acetogenins **1e**, **1f**, **2**−**4** against integrated respiratory chain, NADH oxidase activity.

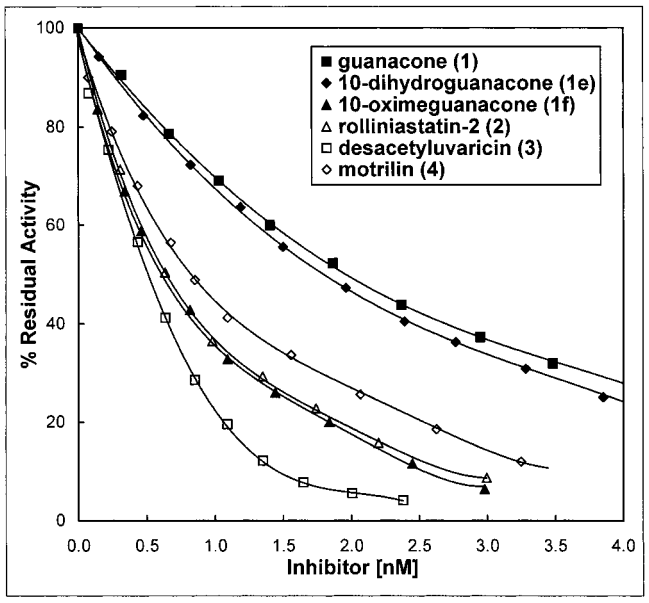
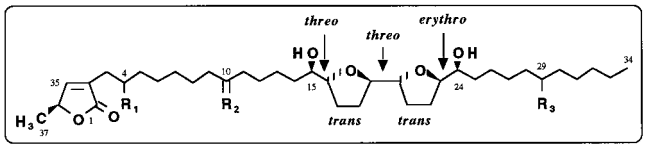
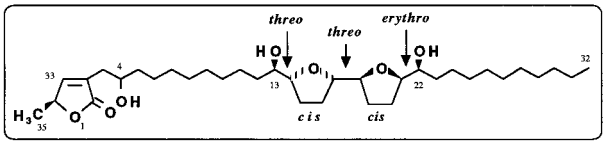


Figure 4. Titrations of acetogenins **1e**, **1f**, **2**−**4** against NADH:ubiquinone oxidoreductase activity (complex I).

the classic complex I inhibitor, for comparative purposes. All six acetogenins showed typical hyperbolic curves. 10-Oximeguanacone (**1f**) and desacetyluvaricin (**3**) were the most powerful inhibitors of NADH oxidase activity, with no significant differences. However, guanacone (**1**) and 10-dihydroguanacone (**1e**) were the weakest inhibitors of the integrated respiratory chain in this series, with a maximal inhibition not completed even at a relatively high concentration of the inhibitor. As we have previously reported,[8] rollimembrin (**5**) inhibited this activity at a similar concentration as **1f** and **3**. Nevertheless, it gave a sigmoidal titration curve. Rolliniastatin-2 (**2**) and motrilin (**4**) showed intermediate hyperbolic titration curves.



1: R_1=R_3=H; R_2=O: **guanacone** [$C_{37}H_{64}O_7$]
2: R_1=OH; R_2= R_3=H: **rolliniastatin-2** [$C_{37}H_{66}O_7$]
3: R_1=R_2= R_3=H: **desacetyluvaricin** [$C_{37}H_{66}O_6$]
4: R_1=R_2=H; R_3=OH: **motrilin** [$C_{37}H_{66}O_7$]



5: **rollimembrin** [$C_{35}H_{62}O_7$]

NADH:ubiquinone oxidoreductase activity represents a direct activity in which water-soluble NADH is oxidized by a lipid-soluble quinone through the enzymatic complex I. Figure 4 shows the titration curves of the six acetogenins against the NADH:ubiquinone oxidoreductase activity measured with decylubiquinone as an ubiquinone analogue.[17] IC_{50} values for the NADH oxidase activity were lower with respect to those of the NADH:ubiquinone oxidoreductase activity (see Figure 5). All guanacone analogues showed again typical hyperbolic curves. However, the potency order was slightly different. Desacetyluvaricin (**3**) was the most powerful inhibitor of the series, followed by 10-oxymeguanacone (**1f**) and rolliniastatin-2 (**2**). There were no significant differences between the titrations of both guanacone (**1**) and 10-dihydroguanacone (**1e**), whereas motrilin (**4**) still behaved with an intermediate potency. Maximal inhibition of NADH:ubiquinone oxidoreductase assay was not complete (15−20% residual activity) even at a relatively high concentration of the inhibitor, but it was in the same range as rotenone. As observed for other acetogenins, with the exception of rolliniastatin-1 and rollimembrin, maximal inhibition was achieved at approximately fivefold of the IC_{50} value. Also, this tendency seems to be similar to that observed for rotenone.

Hydrophobicity of the acetogenins is a key factor in their interaction with the mitochondrial complex I. It has been previously reported[18] that the presence of three hydroxyl groups results in an intermediate polarity and greater potency of the mono-THF acetogenins against cell respiration. As the family of compounds related to guanacone (**1**) has a bis-THF moiety, which includes an additional oxygenated heterocycle, the structural difference with the mono-THF series could explain that analogues of **1** showed a greater inhibitory potency for molecules with only two hydroxyl groups, such as desacetyluvaricin (**3**), being followed by **2** and **4**. This order seems to indicate that one of the THF moieties (in bis-THF acetogenins) could be equivalent to one of the hydroxyl groups needed for improving the interaction of mono-THF acetogenins with complex I.[18]

Although the general pattern shown by these compounds indicates that a medium polarity of the acetogenins is optimal for a high inhibitory potency, we cannot disregard the fact that several specific interactions with the enzyme are the key to understanding the SAR. The high potency found for 10-oximeguanacone (**1f**), potency that reaches that of desacetyluvaricin (**3**) and makes this semisythetic acetogenin one of the most potent complex I inhibitor to date, cannot be understood only on the basis of hydrophobicity. A more specific interaction with the complex I may explain this high potency. Reduction of guanacone (**1**) to 10-dihydroguanacone (**1e**) increases the inhibitory potency, and it becomes greater with the formation of the oxime moiety in the 10-oximeguanacone (**1f**). Therefore, the presence of a keto group in **1** seems to act opposite to the oxime moiety. This would suggest that the potency of this type of bis-THF acetogenins may be closely correlated to the basicity of the group at the 10 position (**1f**).

Experimental Section

General Experimental Procedures. Optical rotations were determined with a Perkin−Elmer 241 polarimeter. IR spectra (film) were run on a Perkin−Elmer 843 spectrometer. UV spectrum was obtained on a Perkin−Elmer Lambda 15 UV/vis spectrophotometer in MeOH solution. EIMS and LSIMS were determined on a VG Auto Spec Fisons spectrometer. NMR spectra were recorded on a Bruker AC-250 or a Varian Unity-300 and 400 spectrometer at 250, 300, or 400 MHz for

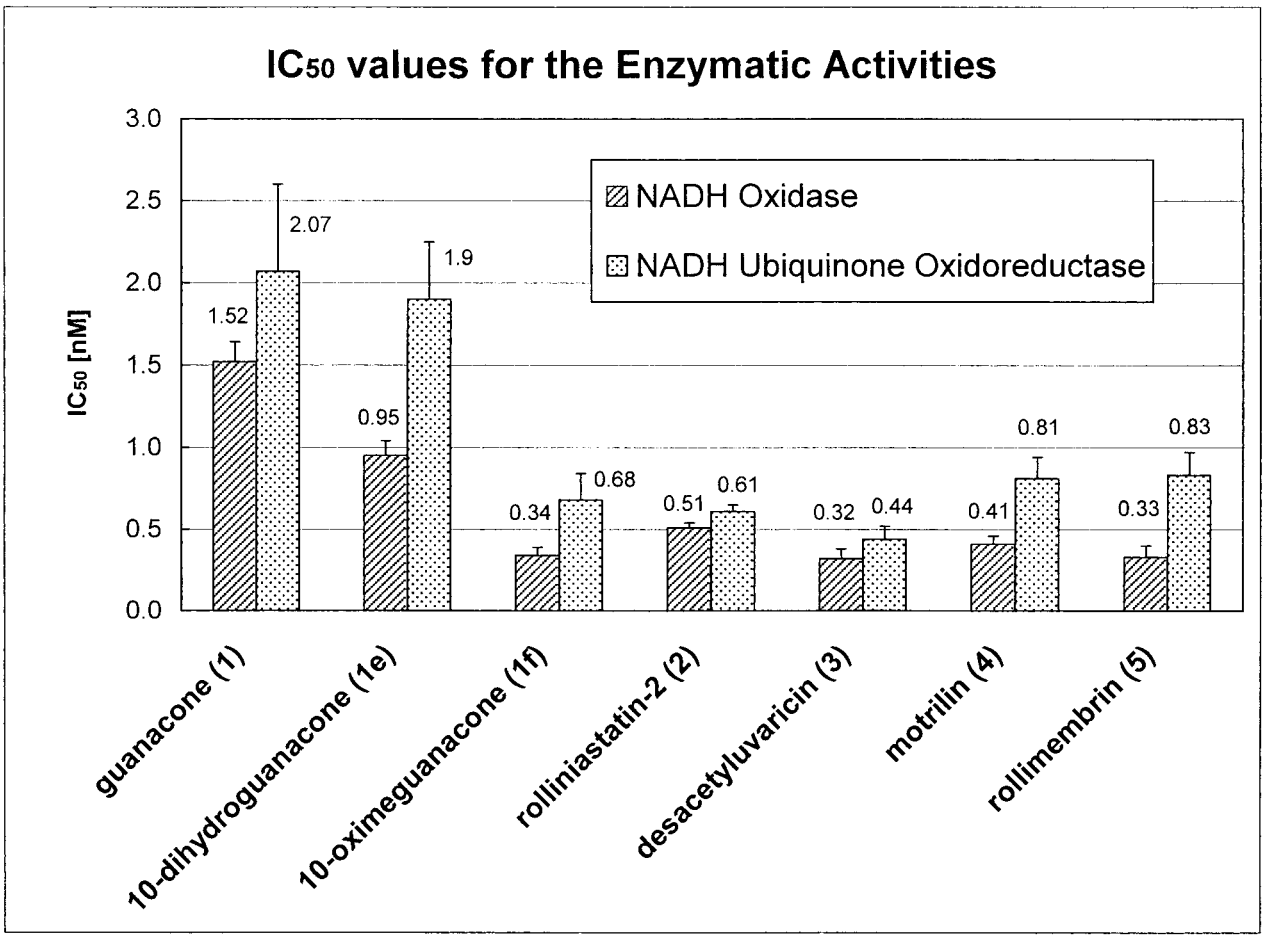


Figure 5. IC_{50} of NADH oxidase and NADH: ubiquinone oxidoreductase activities.

^1H, and 100 MHz for ^{13}C, using the solvent signal as reference ($CDCl_3$ at δ 7.26 and δ 77.0). Multiplicities of ^{13}C NMR resonances were assigned by DEPT experiments. COSY 45 and HMQC correlations were run using a Varian Unity-400 MHz instrument. Semipreparative HPLC was carried out on a Merck−Hitachi, LiChroCART 100 RP-18 column (25 × 1 cm i.d., 10-μm particle size) using MeOH−H_2O (80:20) (flow rate: 6 mL/min, detector: UV 210 nm).

Bioassays. The bioactivity of the acetogenins was assayed using inverted submitochondrial particles (SMP) from beef heart. They were obtained by extensive ultrasonic disruption of frozen−thawed mitochondria in such way to produce open membrane fragments where permeability barriers to substrates were lost. They were ultracentrifuged, resuspended in 250 mM sucrose, 10 mM Tris−HCl buffer, pH 7.4, and stored frozen at −80 °C. For the inhibitor tritrations, the test acetogenins were diluted in absolute EtOH at 2 mM. The stock solution was kept in the dark at −20 °C. Appropriate dilutions between 2 and 10 μM were made before the tritrations. Beef heart SMP were diluted to 0.5 mg·mL^{-1} in sucrose−Tris buffer and treated with 300 μM NADH to activate complex I. Increasing concentrations of the EtOH solution of inhibitor were added to this preparation, with about 5 min incubation on ice between each addition. Maximal EtOH concentration never exceeded 2% of the total volume, and control activity was not affected by this concentration. After each addition of inhibitor, NADH oxidase activity was measured. This integrated enzymatic activity was assayed at 22 °C in 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, in a double beam spectrophotometer. SMP were diluted to 6−7 μg·mL^{-1} in the cuvette. Aerobic NADH oxidation was measured in the presence of 75 μM of NADH and following the decrease in absorbance at 340 nm (ϵ = 6.22 mM^{-1}·cm^{-1}). Data from four titrations were fitted for graphics. The IC_{50} was taken as the final compound concentrations in the assay cuvette that yielded 50% inhibition of NADH oxidase activity. Data from individual titrations were used to assess the means and standard deviations. The NADH:ubiquinone oxidoreductase assay was measured with 75 μM of decylubiquinone and the presence of antimycin and cyanide. Other conditions were the same as the NADH oxidase assay.

Plant Material. Seeds of *A.* aff. *spraguei* Safford (Annonaceae) were collected at 20 m altitude, from Sahagún town in Córdoba region (Colombia). A voucher specimen was deposited, under ref 36732, at the National University of Medellin, Colombia.

Extraction and Isolation. Dried, powdered seeds (400 g) from *A.* aff. *spraguei*, were extracted by percolation with petroleum ether. The deffated seeds were extracted with EtOAc, and the concentrated extract (19.4 g) was subjected to column chromatography over Si gel and gradually eluted by mixtures CH_2Cl_2−EtOAc and EtOAc−MeOH. Guanacone (**1**) (38 mg), rolliniastatin-2 (**2**) (24 mg), desacetyluvaricin (**3**) (19 mg), and motrilin (**4**) (21 mg) were isolated.

Guanacone (1): $[\alpha]_D$ +22° (*c* 1, EtOH); IR (film) ν_{max} 3416, 2922, 1751, 1647, 1548, 1066 cm^{-1}; UV (EtOH)

λ_{max} (log ϵ) 208 (3.83); NMR data, see Table 1 and Figure 1; FABMS *m/z* 643 $[M + Na]^+$, 621 $[MH]^+$; EIMS *m/z* 449 (4), 379 (12), 361 (37), 311 (35), 309 (97), 293 (21), 291 (73), 241 (16), 223 (100), 195 (17) (Figure 1).

15,24-Diacetylguanacone (1a): prepared from **1** (9 mg) by Ac_2O and pyridine at room temperature for 8 h, to yield 10 mg of **1a**; $[\alpha]_D$ +36° (*c* 1, EtOH); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.06 (6H, s, OCOCH_3-15 and -24), 3.87−3.98 (4H, 2m, H-16, -19, -20, -23), 4.89 (2H, m, H-15, -24); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.62 (O*C*OCH$_3$), 81.63 and 81.28 (C-16, -23), 80.43 and 79.96 (C-19, -20), 75.43 (C-15), 74.97 (C-24), 21.22 (OCO*C*H$_3$); FABMS *m/z* 727 $[M + Na]^+$, 705 $[MH]^+$; EIMS, see Figure 1.

15,24-DiTMSi-guanacone (1b): prepared from **1** (1.2 mg) by *N*,*O*-bis(TMSi)-acetamide and pyridine at 70 °C for 0.5 h, to yield 2 mg of **1b**; EIMS, see Figure 1.

Preparation of the C(15,24)-(*S*)- and (*R*)-MTPA Esters of 1. To a stirred solution of **1** (2.8 mg) in CH_2Cl_2 at room temperature, was added pyridine, 4-(dimethylamino)pyridine and (*R*)-MTPA-Cl or (*S*)-MTPA-Cl.[14] The mixture was allowed to sit for 2 h at room temperature and then saturated with $NaHCO_3$ and extracted with CH_2Cl_2. By usual treatment, MTPA esters of **1** (**1c** and **1d**) were obtained.

10-Dihydroguanacone (1e): prepared from **1** (15.5 mg) by $NaBH_4$ and MeOH at room temperature for 0.5 h, to yield 15 mg of **1e**; $[\alpha]_D$ +27°(*c* 1, EtOH); ^1H NMR ($CDCl_3$, 250 MHz) δ 1.38 (4H, m, H-8, -12), 1.53 (4H, m, H-9, -11), 3.57 (1H, t, H-10); ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 71.73 (C-10), 37.36 (C-9, -11), 26.03 (C-8, -12); FABMS *m/z* 645 $[M + Na]^+$, 623 $[MH]^+$; EIMS *m/z* 381 (15), 363 (51), 345 (77), 311 (60), 293 (100), 275 (95), 241 (36), 225 (40).

10-Oximeguanacone (1f): prepared from **1** (20 mg) by $ClNH_4OH$ and pyridine at 70 °C for 1 h, to yield 13 mg of **1f**; $[\alpha]_D$ +25 ° (*c* 1, EtOH); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.11 (4H, t, H-9, -11), 2.25 (2H, t, H-3), 3.42 (1H, m, H-15), 3.83−3.90 (4H, m, H-16, -20, -23, -24), 4.02 (1H, m, H-19); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 161.36 (C-10), 72.48 (C-15), 70.66 (C-24), 33.65 and 33.24 (C-9,11); FABMS *m/z* 658 $[M + Na]^+$, 636 $[MH]^+$; EIMS *m/z* 464 (6), 394 (15), 324 (95), 311 (37), 275 (95), 241 (13), 238 (100).

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