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ACETOGENINS FROM *ANNONA GLABRA* SEEDS

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IN HONOUR OF THE RETIREMENT OF PROFESSOR ANDRÉ CAVÉ

Key Word Index—*Annona glabra*; Annonaceae; seeds; mono- and bis-tetrahydrofuranic; acetogenins; mitochondrial respiratory chain.

Abstract—From the cytotoxic ethanol extract of *Annona glabra* seeds, a new mono-tetrahydrofuranic (mono-THF) acetogenin, glabranin, as well as pair of 22-epimer *bis*-THF acetogenins, were isolated by semipreparative HPLC. Four known mono-THF acetogenins with an identical *threo/trans/threo* relative configuration, annonacin, annonacinone, corossolin and corossolone, were found to be potent inhibitors of complex I of the mitochondrial respiratory chain.

INTRODUCTION

Annona glabra, a tree distributed mainly in South America and in the south east of Asia, is used in traditional medicine as an insecticide and a parasiticide [1]. In a previous study, three known *bis*-(THF) acetogenins, desacetyluvaricin, asimicin and squamocin, were isolated from seeds of this species [1, 2], and shown to have insecticidial activity.

We report, herein, the isolation of eight mono-THF γ-methyl-γ-lactone acetogenins (**1**–**8**), where glabranin (**1**) is a new olefinic tetrahydroxylated acetogenin. Moreover, a pair of 22-epimer adjacent *bis*-THF acetogenins, molvizarin and parviflorin (**9** and **10**), were obtained by preparative HPLC. We also describe the presence of laherradurin (**11**) and itrabin (**12**), belonging to a very rare class of acetogenins, β-hydroxy-γ-methyl-γ-lactones, which only have been reported previously in *A. cherimolia* seeds [3, 4]. The mode of action of the cytotoxic acetogenins targets on the mitochondrial NADH: ubiquinone oxidoreductase, also known as the respiratory complex I [5, 6]. Titration against NADH: decylubiquinone oxidoreductase activity on submitochondrial particles from beef heart indicated that the mono-THF acetogenins, annonacin (**5**), annonacinone (**6**), corossolin (**7**) and corossolone (**8**) [2], are potent and specific inhibitors of the complex I activity of mammalian mitochondria.

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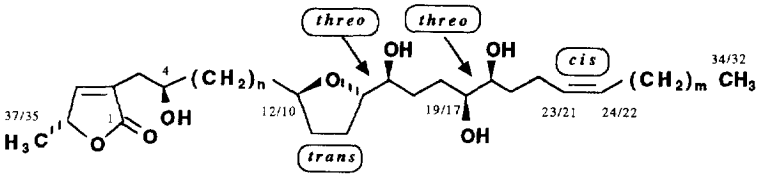
RESULTS AND DISCUSSION

Fractionation by silica gel column chromatography and semipreparative HPLC of hexane and ethanol extracts from *A. glabra* seeds, collected in Cali (Colombia), gave 12 acetogenins belonging to four different types: (a) mono-THF α-monohydrylated acetogenins, glabranin (**1**), muricatetrocin-B (**2**), gigantetronenin (**3**) and gigantetrocin-A (**4**); (b) mono-THF α,α′-dihydroxylated acetogenins, annonacin (**5**), annonacinone (**6**), corossolin (**7**) and corossolone (**8**); (c) adjacent *bis*-THF acetogenins, molvizarin (**9**) and parvilflorin (**10**); and (d) β-hydroxy-γ-methyl-γ-lactone acetogenins, laherradurin (**11**) and itrabin (**12**).

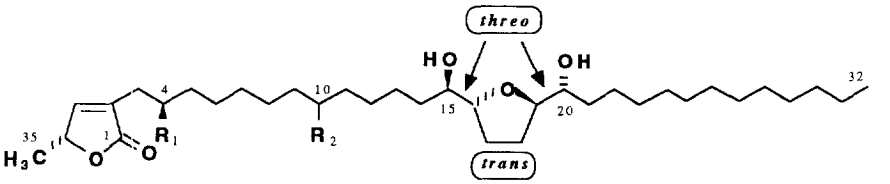
The four mono-THF α-monohydroxylated acetogenins (**1**–**4**), were obtained as two pairs of mixtures (**1**+**2** and **3**+**4**). These mixtures were resolved by semipreparative reverse-phase HPLC. Compounds **1** and **2** showed a strong IR absorption band at 1740 cm^{-1} and a positive Kedde reaction, characteristic of an α,β-unsaturated γ-lactone. The molecular formula of compounds **1** and **2** {$C_{37}H_{66}O_7$ and $C_{35}H_{64}O_7$} were indicated by FAB-mass spectrometry by peaks at *m/z* 645 $[M+Na]^+$/623 $[MH]^+$ and peaks at *m/z* 619 $[M+Na]^+$/597 $[MH]^+$, respectively. Examination of the 1D-NMR (^1H, ^{13}C and DEPT) spectra of **1** and **2** suggested the presence of acetogenins with an 4-hydroxylated α,β-unsaturated γ-methyl-γ-lactone system (Fig. 1), as well as a α-monohydroxylated mono-THF system [4]. The presence of the latter was deduced by two ^{13}C signals at δ 79.3 **1** and δ 81.7 **2** due to oxygen-bearing carbons characteristic of this

α-Monohydroxylated mono- THF acetogenins:



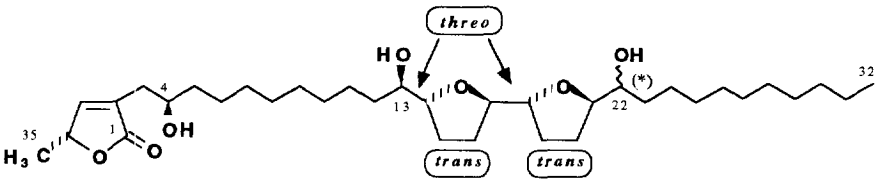
1: n= 7; m= 9; C_{23}=C_{24}: glabranin {$C_{37}H_{66}O_7$}
2: n= 7; m= 7; ----------: muricatetrocin-B {$C_{35}H_{64}O_7$}
3: n= 5; m= 11; C_{21}=C_{22}: gigantetronenin {$C_{37}H_{66}O_7$}
4: n= 5; m= 9; ----------: gigantetrocin-A {$C_{35}H_{64}O_7$}

α,α'-Dihydroxylated mono- THF acetogenins:



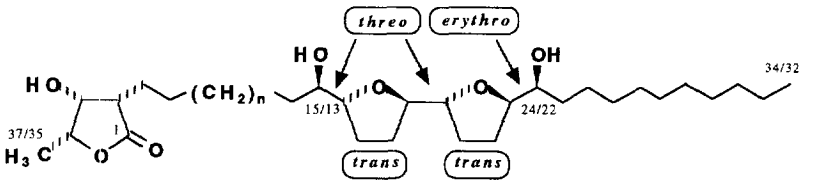
5: R_1= R_2= OH: annonacin
6: R_1= OH; R_2= O: annonacinone
7: R_1= H; R_2= OH: corossolin
8: R_1= H; R_2= O: corossolone

Adjacent *bis*-THF acetogenins:



9: (*) *erythro*: molvizarin
10: (*) *threo*: parviflorin

β-Hydroxy-γ-methyl-γ-lactone acetogenins:



11: n= 9: laherradurin
12: n= 7: itrabin

Scheme 1. Acetogenins from *Annona glabra* seeds.

group of acetogenins [7, 8]. In addition, two resonances in the ^{13}C NMR at δ 74.59 and δ 74.23, also due to methine oxygen carbons, and their corresponding resonances in the ^1H NMR at δ 3.40, confirmed the existence of a vicinal diol group in **1**, similar to that observed for muricatetrocin-B (**2**) [9] and other acetogenins isolated from *A. glabra* (**3** and **4**).

The remaining NMR signals for **1** were characteristic of olefinic mono-THF acetogenins, like senegalene [10] and annogalene [11]. Thus, two sp^2 methine resonances in the ^{13}C NMR spectrum, at δ 128.60 and δ 130.18, and the corresponding signals in the ^1H NMR spectrum at δ 5.35 and δ 5.38, as well as the chemical shifts of the neighbour methylenes, are in agreement with those of venezenin, an unusual linear acetogenin possessing a double bond located two methylenes away from a vicinal diol [2, 12] (Fig. 1). The presence of four hydroxyl groups in **1** was confirmed by the preparation of the tetraacetyl derivative (**1a**), which showed four singlets in the ^1H NMR at δ 2.02, δ 2.04, δ 2.06 and δ 2.07 (3H each).

To establish the placement of the monohydroxylated mono-THF system, the double bond and the diol group along the hydrocarbon chain, mass spectrometry studies were undertaken (Fig. 2). Three important cleavages in the structure of **1** could be considered as basic for its identification: (a) cleavage between C-3 and C-4 containing the terminal methyl (fragment ions at *m/z* 493 and 475), characteristic of 4-hydroxylated acetogenins, as for annosenegalin

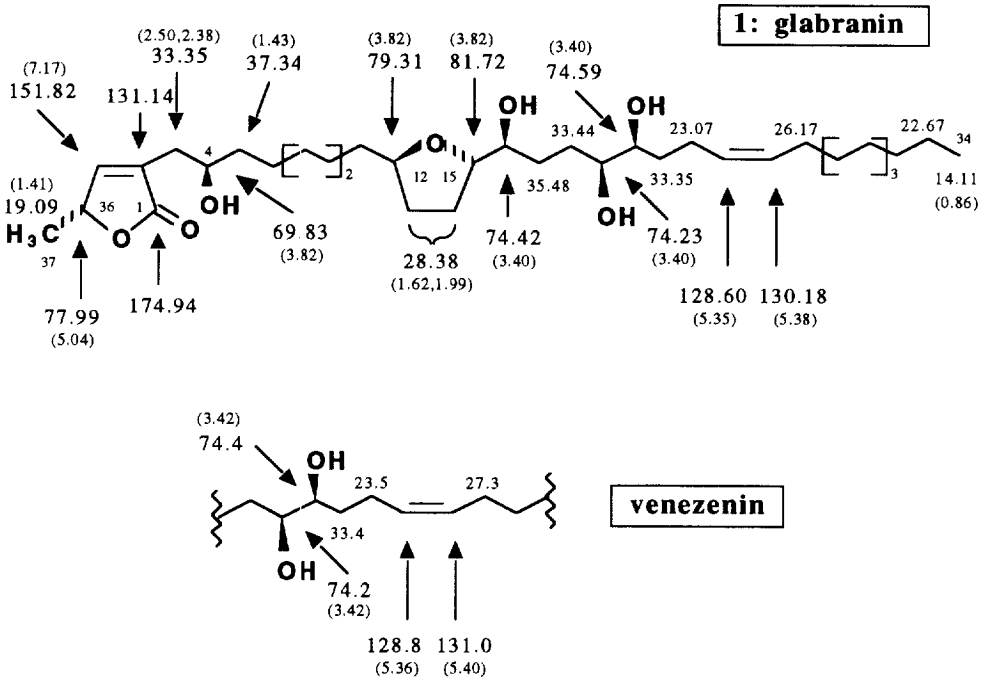


Fig. 1. ^{13}C and ^{1}H NMR (in parentheses)[#,##] of glabranin (1) and venezenin [12]. [#]^{1}H and ^{13}C NMR (and DEPT) were recorded at 400 MHz and 100 MHz, respectively, in $CDCl_3$. [##]$J_{3a,3b} = 15$ Hz; $J_{3a,4} = 8$ Hz; $J_{4,3b} = 3$ Hz; $J_{23,24} = 9$ Hz; $J_{33,34} = 7$ Hz; $J_{35,36} = 1.5$ Hz; $J_{36,37} = 7$ Hz.

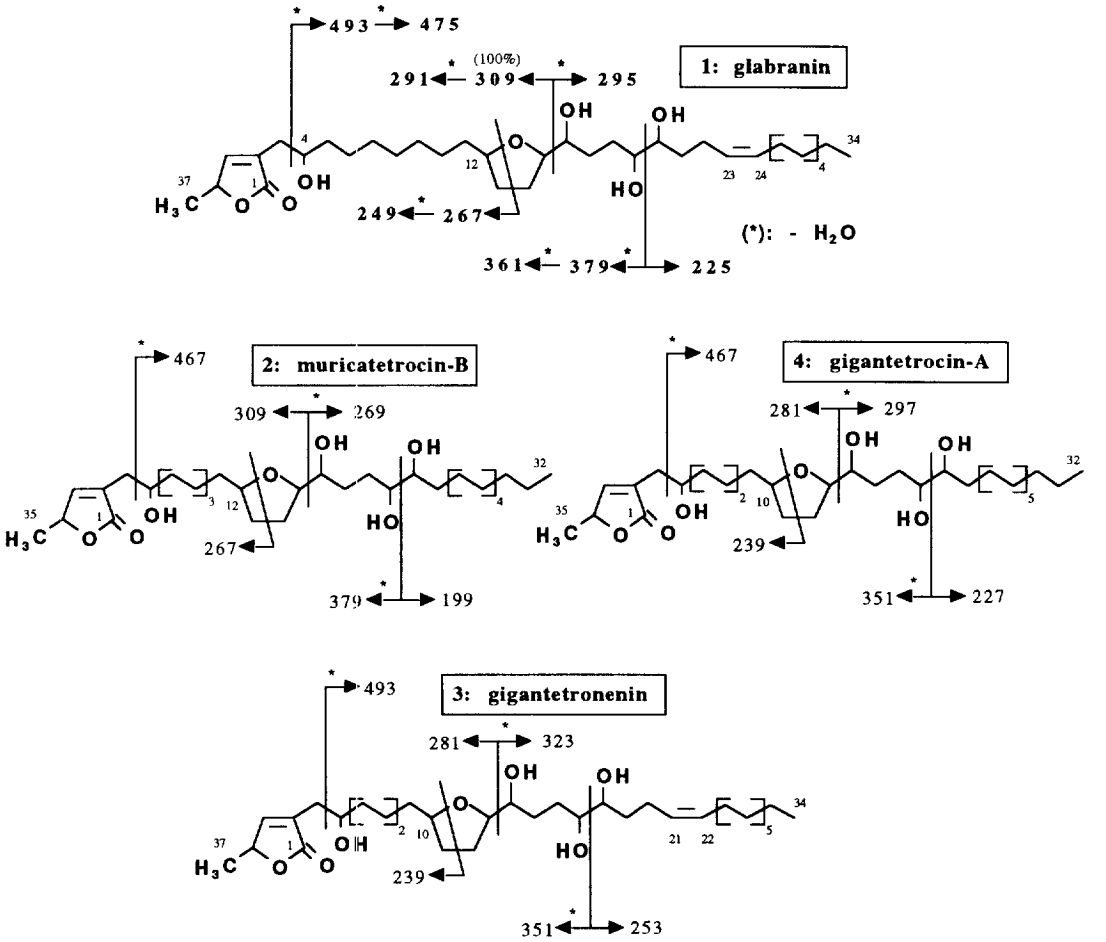


Fig. 2. Fragment ions in EI mass spectrum of glabranin (1), muricatetrocin-B (2), gigantetronenin (3) and gigantetrocin-B (4).

(mono-THF) [11] and molvizarin (**9**) (adjacent *bis*-THF) [13]; (b) cleavage over the THF ring (fragment ions at *m/z* 267 and 249), typical of acetogenins with a THF ring α-monohydroxylated, as for senegalene (mono-THF) [10] and cherimolin-1 (non-adjacent *bis*-THF) [4]; (c) cleavage between C-19 and C-20, indicating the position of the two vicinal hydroxyl moieties, as for three other mono-THF acetogenins isolated from *A. glabra*, muricatetrocin-B (**2**) [9], gigantetronenin (**3**) [14] and gigantetrocin-A (**4**) [7] (Fig. 2).

The relative configuration of glabranin (**1**) should be considered to be identical to that of gigantetronenin (**3**), *trans/threo* (mono-THF system), *threo* (vicinal diol) and *cis* (double bond), muricatetrocin-B (**2**), and gigantetrocin-A (**4**) (*trans/threo-threo*), based on similar ^{1}H and ^{13}C NMR chemical shifts from the groups concerned [7, 9, 14, 15].

A couple of 22-epimer *bis*-THF acetogenins (**9**, **10**) were separated by reverse-phase HPLC. Compounds **9** and **10** are two 4,13,22-trihydroxylated adjacent *bis*-THF acetogenins [2, 15]. Molvizarin (**9**), previously isolated from *A. cherimolia* seeds [13] possesses a classical *threo/trans/threo/trans/erythro* relative configuration [15]. However parviflorin (**10**) belongs to the less frequent type of adjacent *bis*-THF acetogenins with a *threo/trans/threo/trans/threo* relative configuration (asimicin-type) [2, 15]. A similar iso-acetogenins pair, isomolvizarins-1 and -2, isolated from *A. cherimolia* roots, were also separated by HPLC [16]. Parviflorin (**10**), was previously isolated from *Asimina parviflora* [2, 17].

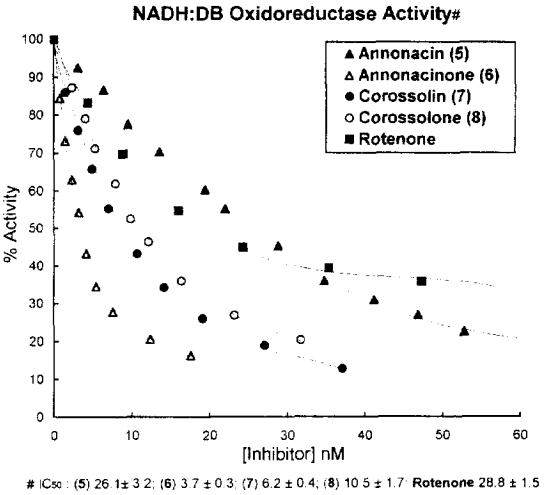
Laherradurin (**11**) and itrabin (**12**) belong to the very rare class of acetogenins characterized by a saturated β-hydroxy-γ-methyl-γ-lactone moiety [3, 4], and a classical *threo/trans/threo/trans/erythro* relative configuration concerning the α,α′-dihydroxylated *bis*-THF system, have been also isolated from *A. glabra*.

In our study of acetogenins from *A. glabra*, we found four C_{35} α,α′-dihydroxylated mono-THF acetogenins (**5**–**8**), characterized by the presence of an identical relative configuration *threo/trans/threo*. The structural differences between those compounds concern the presence and the nature of an oxygenated group at the C-10 position and at the C-4 position. As acetogenins are specific inhibitors of complex I of mammalian mitochondrial respiratory chain [2, 5, 6], we focused on the inhibition of this complex I (NADH:ubiquinone oxidoreductase) [18].

Titration against NADH-decylubiquinone oxidoreductase activity in beef-heart submitochondrial particles seems to indicate that these mono-THF acetogenins (**5**–**8**) are specific and potent inhibitors of complex I activity. From IC_{50} values of annonacin (**5**), annonacinone (**6**), corossolin (**7**) and corossolone (**8**) [corossolin (**7**) was also prepared from corossolone (**8**) by reduction with $NaBH_4$], and rotenone as a classic inhibitor reference, we can establish some requirements for their activity.

As it has been previously reported by González *et al.* [18], the different substitution of the carbon in the 4-position classifies the acetogenins in two groups. If the acetogenin possesses an 4-hydroxyl group, substitution of an hydroxyl group in the 10-position by a ketone function increases the potency of mono-THF acetogenins up to seven times on the mammalian respiratory chain complex I. However, this modification does not affect the acetogenins without an hydroxyl in the 4-position (Fig. 3).

In conclusion, in addition to the α,α′-dihydroxylated mono-THF system and the α,β unsaturated-γ-lactone moiety, the hydroxyl and keto groups in the C-4 or C-10 positions are important for the inhibitory potency of the acetogenins against mammalian mitochondrial complex I.



IC_{50} : (5) 26.1 ± 3.2; (6) 3.7 ± 0.3; (7) 6.2 ± 0.4; (8) 10.5 ± 1.7; Rotenone 28.8 ± 1.5

Fig. 3. NADH decylubiquinone oxidoreductase activity of α,α′-dihydroxylated mono-THF acetogenins from *A. glabra* (5–8).

EXPERIMENTAL

^{1}H NMR: 250 and 400 MHz, $CDCl_3$. ^{13}C NMR: 62.5 and 100 MHz, $CDCl_3$.

Plant material

Seeds of *Annona glabra* L. were collected in Cali, Colombia. A voucher specimen is deposited in the herbarium of the Department of Botany, University of Valle, Colombia, under Ref. Cabrera 4107.

Extraction and isolation

Extraction and fractionation were monitored by the Kedde reagent. Dried and powdered seeds (120 g) were extracted by Soxhlet with petroleum 30–60°. The organic extract was evapd and the residue maintained at 4° for 24 h to give a ppt. (A). This A (0.96 g) was fractionated by 60 H silica gel CC with CH_2Cl_2–EtOAc–MeOH (5.5:4:0.5), followed by semiprep. reverse-phase HPLC (Merck–Hitachi, LiChroCART 100 RP-18, 10 μm, flow-rate 6 ml min^{-1}). Deffated seeds were extracted with EtOH (B). The concd B extract (7.68 g) was partitioned between CH_2Cl_2 and H_2O. The concd CH_2Cl_2 extract (C) (2.14 g) was fractionated by 60 H silica gel CC with CH_2Cl_2–EtOAc–Me_2CO (6:3:1), followed by semiprep. reverse-phase HPLC. From ppt. A and CH_2Cl_2 extract C, 12 acetogenins were isolated, glabranin (**1**) (5 mg), muricatetrocin-B (**2**) (5 mg), gigantetronenin (**3**) (5 mg), gigantetrocin-A (**4**) (5 mg), annonacin (**5**) (130 mg), annonacinone (**6**) (20 mg), corossolin (**7**) (10 mg), corossolone (**8**) (25 mg), molvizarin and parviflorin (**9** and **10**) (12 mg), laherradurin (**11**) (10 mg) and itrabin (**12**) (10 mg).

Glabranin (**1**)

Amorphous. HPLC, MeOH–H_2O (4:1) (R_t 28.4 min); MeCN–H_2O (3:2) (R_t 48.6 min). $C_{37}H_{67}O_7$. FABMS (NMBA + NaCl), *m/z*: 645 $[M+Na]^-$, 623 $[MH]^+$. EIMS *m/z* (rel. int.): 493 (24), 475 (3), 379 (58), 361 (21), 309 (100), 295 (5), 293 (45), 291 (16), 269 (17), 267 (83), 251 (9), 249 (25), 225 (5). ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): Fig. 1.

Glabranin tetraacetate (**1a**)

Treatment of **1** (1.5 mg) with Ac_2O-pyridine and subsequent work-up gave **1a** in quantitative yield. $C_{45}H_{75}O_{11}$. FABMS (NMBA + NaCl), *m/z*: 813 $[M+Na]^+$, 791 $[MH]^+$. EIMS *m/z* (rel. int.): 421 (12), 351 (100), 309 (72), 267 (7), 261 (74), 239 (9). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.07, 2.06, 2.04, 2.02 ($OCOCH_3$-4, 16, 19 and 20).

10-*Dihydrocorossolone* (= *corossolin*, **7**).

Compound **8** (15.4 mg) dissolved in MeOH (2 ml) was treated with $NaBH_4$ (20 mg). The reaction was stirred under reflux for 30 min and, after extracting with CH_2Cl_2 and subsequent work-up, gave corossolin, **7** (11.6 mg). $C_{35}H_{64}O_6$. FABMS (NMBA + NaCl), *m/z*: 603 $[M+Na]^+$, 581 $[MH]^-$. EIMS, ^{1}H NMR and ^{13}C NMR: see Ref. [19].

Molvizarin and parviflorin (**9** and **10**)

Amorphous. HPLC, MeCN–H_2O–THF (3:2:1) (**9**: R_t 7.16 min; **10**: R_t 7.97 min). $C_{35}H_{62}O_7$. FABMS (NMBA + NaCl), *m/z*: 617 $[M+Na]^+$, 595 $[MH]^-$. EIMS *m/z* (rel. int.): 576 (16), 465 (11), 423 (8), 353 (12), 311 (20), 283 (100), 241 (12), 171 (14), 141 (12), 123 (27). ^{1}H NMR, 400 MHz ($CDCl_3$): δ 2.50, 2.35 (CH_2-3), δ 3.84 (CH-4), δ 1.46 (CH_2-5), δ 1.36 (CH_2-6), δ 1.25 (CH_2-7-10), δ 1.36 (CH_2-11, 12), δ 3.37 (CH-13), δ 3.84 (CH-14), δ 1.96, 1.62 (CH_2-15, 16), δ 3.84 (CH-17, 18), δ 1.96, 1.62 (CH_2-19, 20), δ 3.84 (CH-21), δ 3.84 (**9**), 3.37 (**10**) (CH-22), δ 1.36 (CH_2-23, 24), δ 1.25 (CH_2-25–31), δ 0.87 (CH_3-32), δ 7.16 (CH-33), δ 5.04 (CH-34), δ 1.42 (CH_3-35). ^{13}C NMR, 100 MHz ($CDCl_3$), δ: 174.5 (C-1), δ 131.0 (C-2), δ 33.3 (CH_2-3), δ 70.0 (CH-4), δ 37.4 (CH_2-5), δ 33.3 (CH-12), δ 74.0 (CH_2-13), δ 83.19 (**9**), 83.21 (**10**) (CH-14), δ 82.5 (**9**), 81.8 (**10**) (CH-17), δ 82.3 (**9**), 81.8 (**10**) (CH-18), δ 82.8 (**9**), 83.2 (**10**) (CH-21), δ 71.3 (**9**), 74.0 (**10**) (CH-22), δ 32.4 (CH_2-23), δ 22.7 (CH_2-31), δ 14.1 (CH_3-32), δ 151.8 (CH-33), δ 78.0 (CH-34), δ 19.1 (CH_3-35).

Inhibitor titrations

Titrations of different inhibitors were performed as described in [6]. Submitochondrial particles (SMP) were dild in 250 mM sucrose, 10 mM *tris*-HCl buffer, pH 7.6 to a final concn of 0.5 mg ml^{-1} and were kept in glass test tubes on ice. A quantity of 10–14 μg of SMP was assayed in KPi buffer each time with the addition of NADH (75 μM) and decylubiquinone (DB) (30 μM), and was treated with antimicin A at 2 μM in order to block the activity of the ubiquinone: cytochrome c oxidoreductase (complex III), and KCN 2 mM to block the activity of the respiratory chain complex IV (cytochrome c oxidase). Controls were performed at the beginning of the titration. The titration was performed with the addition of a small quantity of the inhibitor (0.005–0.01 mM in EtOH) sequentially to the mixt. medium. EtOH never exceeded 2% of the total vol. Time for incubation was 5 min. IC_{50} values were the final inhibitor concns in the assay cuvette that inhibit enzymatic NADH: ubiquinone activities by 50%.

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