Epoxy-Acetogenins and other Polyketide Epoxy Derivatives as Inhibitors of the Mitochondrial Respiratory Chain Complex I

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Abstract: **Annonaceous acetogenins (ACG), an extensive group of cytotoxic natural products, are antitumor agents whose main mode of action is inhibition of the mammalian mitochondrial complex I. Herein we describe the importance of the different chemical groups along the alkyl chain for optimal inhibitory potency, discussing the structurally relevant factors present in these compounds. For this purpose, a series of epoxide derivatives from α-linolenic acid were prepared and their activity compared with that of epoxy-acetogenins and tetrahydrofuranic (THF) acetogenins isolated from *Rollinia membranacea*.**

Key words: **Annonaceae, *Rollinia membranacea*, epoxy-acetogenins, tripoxyrollin, diepomuricanin A, membrarollin, α-linolenic acid derivatives, inhibitors of mitochondrial complex I.**

Introduction

Annonaceous acetogenins (ACG) have attracted much interest because of their cytotoxic, antitumor, parasitic and insecticide activities (1 – 3). These biological effects have been related with the ability to inhibit the NADH:ubiquinone oxidoreductase (complex I) of the mitochondrial electron transport chain (4 – 6). To study the structurally relevant factors for the inhibitory potency of these compounds we decided to select a series of epoxy ACG, potential precursors of the THF-ACG (7), and a series of alkyl chain epoxide derivatives without the methyl-γ-lactone characteristic of the ACG. This is the first study that describes the inhibitory action of epoxy-ACG: tripoxyrollin (**1**) and diepomuricanin A (**2**) (8, 9), and compares their potency with the ones observed for THF-acetogenins (**4**), and alkyl chain epoxide derivatives (**3b – 3d**) semisynthesized from the unsaturated fatty acid, α-linolenic acid, (**3**) (Fig. **1**). The relevance of the number of epoxy groups and the presence or the absence of the lactone ring is discussed in an attempt to establish a structure-activity relationship (SAR).

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Materials and Methods

Chemical experimental procedures

Optical rotations were measured with a Perkin-Elmer 241 polarimeter. IR spectra (film) were recorded on a Perkin-Elmer 843 infrared spectrophotometer. CIMS and LSIMS were determined on a VG Auto Spec Fisons spectrometer. NMR spectra were taken on a Varian Unity-400 spectrometer at 400 MHz for ^{1}H, and 100 MHz for ^{13}C, using $CDCl_3$ (δ 7.26 ppm and δ 77.0 ppm) as reference standard. Multiplicities of ^{13}C-NMR resonances were assigned by DEPT experiments. COSY 45 and HMQC correlations were performed using a Varian Unity-400 MHz instrument. Column chromatography was carried out over silica gel 60H (Merck 7736). Analytical TLC was performed on Merck precoated silica gel 60 F_{254} plates and spots were detected by spraying with phosphomolybdic acid.

Extraction and isolation from plant material

Tripoxyrollin (**1**), diepomuricanin-A (**2**), and membrarollin (**4**) were isolated by our group from *Rollinia membranacea* (Annonaceae) seeds, as previously described (8 – 10).

Other products

Pure α-linolenic acid (**3**) and rotenone (**5**) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Salts and solvents were from Merck (Darmstadt, Germany).

*Chemical transformations of α-linolenic acid (**3**)*

Methylation of **3**: Concentrated H_2SO_4 (5 drops) was added to a solution of **3** (117 mg, 0.42 mmol) in MeOH (20 ml) and the mixture stirred and refluxed for 2 h. After concentration of the solvent, 5% $NaHCO_3$ (aq.) up to pH 7 was added to the mixture which was extracted with CH_2Cl_2 (3 × 25 ml). Then the organic layer was washed with H_2O, dried over anhydrous Na_2SO_4 and evaporated to yield **3a** (115 mg, 95%).

Epoxidation of **3a**: *m*-CPBA (306 mg) in dry CH_2Cl_2 (20 ml) was added dropwise during 1 h to a stirred solution of **3a** (107 mg, 0.36 mmol) in dry CH_2Cl_2 (20 ml) at 0 °C. The reaction mixture was then maintained at room temperature for 2 h, washed with 10% Na_2CO_3 (2 × 30 ml), saturated NaCl (20 ml), dried over anhydrous NaO_4 and evaporated. The residue (125 mg)

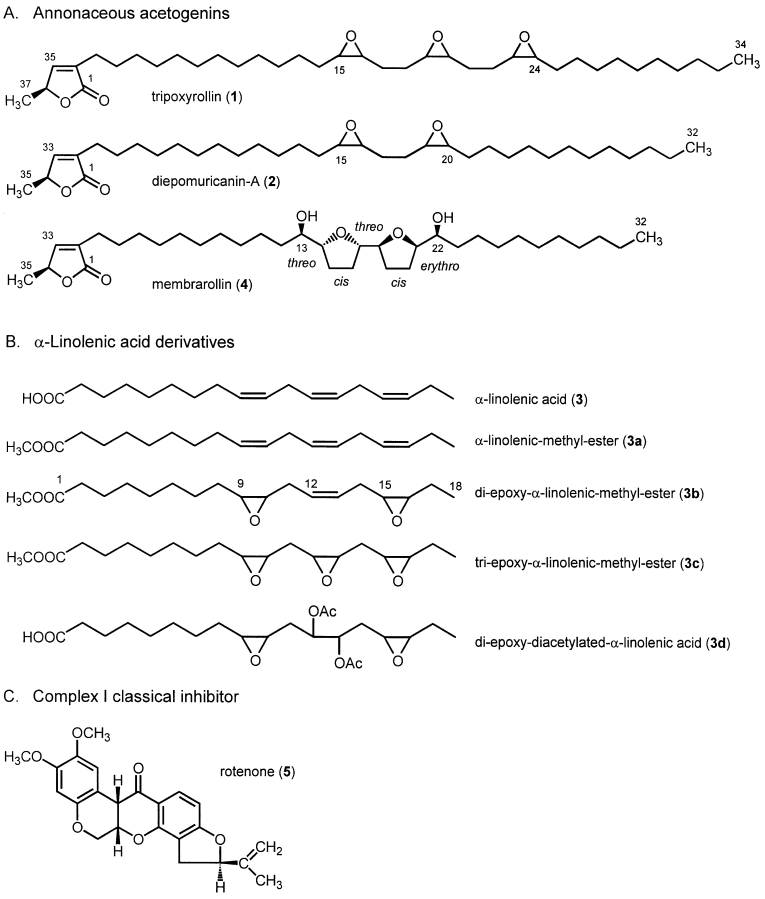

Fig. 1 Structures of compounds assayed as complex I inhibitors.

was purified by CC on silica gel with *n*-hexane-EtOAc (8 : 2) to yield **3b** (7 mg, 5.8 %) and **3c** (77.7 mg, 63.9 %).

Opening of the 12,13-oxirane ring in **3c**: The triepoxide **3c** (40 mg, 0.117 mmol) in MeOH/H_2O (8 : 2; 10 ml) and 1 N NaOH (2 ml) was stirred at 50 °C for 3 h. The mixture was cooled to room temperature, and neutralized with 5 % HCl. Then it was saturated with NaCl and extracted with CH_2Cl_2 (3 × 25 ml). The extracts were dried over anhydrous Na_2SO_4 and concentrated to dryness. Pyridine (1.5 ml) and Ac_2O (3 ml) were added and the mixture was allowed to stand for 12 h. A usual work-up gave a residue (50 mg) which was purified by flash chromatography with CH_2Cl_2 : MeOH (98 : 2) to provide **3d** (42 mg, 81.2 %).

Physical properties

Methyl Z,Z,Z-octadeca-9,12,15-trienoate (α-linolenic methyl ester, **3a**): Colorless viscous oil; IR (CH_2Cl_2): ν_{max} = 1733 (CO ester) cm^{-1}; LSI-MS: *m/z* = 293 $[MH]^+$; ^{1}H-NMR and ^{13}C-NMR, Tables **1** and **2**.

Methyl 9,10,15,16-diepoxy-octadec-12(Z)-enoate (diepoxy-α-linolenic methyl ester, **3b**): $C_{19}H_{32}O_4$; Optically inactive colorless oil; IR (CH_2Cl_2): ν_{max} = 1735 cm^{-1}; LSI-MS: *m/z* = 325 $[MH]^+$; CIMS: *m/z* = 325 $[MH]^+$, 309, 307, 293, 275, 253, 239, 221, 199, 185, 167, 155, 137, 125, 109, 97; ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz), Tables **1** and **2**.

Methyl 9,10,12,13,15,16-triepoxyoctadecanoate (triepoxy-α-linolenic methyl ester, **3c**): $C_{19}H_{32}O_5$; Optically inactive colorless oil; IR (CH_2Cl_2): ν_{max} = 1735 cm^{-1}; LSI-MS: *m/z* = 341 $[MH]^+$; CIMS: *m/z* = 341 $[MH]^+$, 323, 305, 291, 275, 273, 268, 255, 243, 237, 225, 221, 213, 199, 185, 167, 155, 141, 137, 127, 109, 97, 85, 71; ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz), Tables **1** and **2**.

12,13-Diacetoxy-9,10,15,16-diepoxyoctadecanoic acid (diepoxy-diacetylated α-linolenic acid, **3d**): $C_{22}H_{36}O_8$; Optically inactive colorless oil; IR (CH_2Cl_2): ν_{max} = 1734 cm^{-1}; LSI-MS: *m/z* = 429 $[MH]^+$; CIMS: *m/z* = 429 $[MH]^+$, 411 $[MH - H_2O]^+$, 401 $[MH - CO]^+$, 369 $[MH - AcOH]^+$, 341 $[401 - AcOH]^+$, 309 $[MH - 2AcOH]^+$, 291, 281, 249, 211, 185, 155, 109; ^{1}H-NMR (400 MHz), Table **1**; ^{13}C-NMR (100 MHz): δ 179.4 (COOH), 170.7 and 170.6 (OCOMe), 21.1 and 21.0 (OCOMe).

Bioassay experimental procedures

The bioactivity of the compounds was assayed using submitochondrial particles (SMP) from beef heart. These SMP were obtained by extensive ultrasonic disruption of frozen-thawed mitochondria in such a way to produce open membrane fragments where permeability barriers to substrates were lost. Finally, they were ultracentrifugated, resuspended in 250 mM sucrose, 10 mM Tris-HCl pH 7.4 buffer, and stored frozen at – 80 °C (11). For the inhibitor titrations, compounds were initially diluted in absolute ethanol at 15 mM. These stock solutions were kept in the dark at – 20 °C. Appropriate dilutions

between 2 and 10 mM were made before the titrations. 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 ethanolic solution of inhibitor were added to this preparation with 5 min incubation on ice between each addition. Maximal ethanol concentration never exceeded 2% of volume and control activity was not affected by this concentration (5).

After each addition of inhibitor the enzymatic activity was measured at 22 °C in SMP diluted to 6 – 7 mg·ml^{-1} in the cuvette with 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, as both, NADH oxidase and NADH : ubiquinone (NADH : DB) oxidoreductase activities. NADH oxidase activity was measured as the aerobic oxidation of 75 mM NADH following the decrease in absorbance at 340 nm (ε = 6.22 mM^{-1}·cm^{-1}) in an end-window photo-multiplier double-beam spectrophotometer ATI-Unicam UV4-500. NADH : ubiquinone oxidoreductase was measured in the same manner but with 30 μM decylubiquinone (DB) as ubiquinone analogue, in presence of 2 mM KCN and 2 μM antimycin A (12). Rotenone sensitivity was routinely assessed in all samples for both assays (11, 12). Data from four titrations were pooled and fitted for graphics. Individual IC_{50} for each titration were used for the statistic means and the standard deviations.

Results and Discussion

Chemistry

α-Linolenic acid (**3**) was taken as an appropriate substrate for epoxidation. So, after methylation with MeOH and concentrated sulfuric acid the methyl ester derivative (**3a**) was epoxidized with *m*-CPBA. Two compounds, **3b** and **3c**, were found in the reaction mixture and further purified by column chromatography.

Compound **3b** was obtained as a colorless oil. The LSI-MS showed ion peaks at *m/z* 347 $[M + Na]^+$ and *m/z* 325 $[MH]^+$, indicating a molecular formula of $C_{19}H_{32}O_4$. Extensive analysis of the ^{1}H- and ^{13}C-NMR spectra (see Tables **1** and **2**) together with DEPT, ^{1}H-^{1}H COSY and HMQC data indicated the presence of a diepoxide derivative. Two olefinic protons at δ 5.60 in the ^{1}H-NMR spectra and a multiplet resonance at δ 2.93, integrating for four protons, also indicated the presence of two epoxy rings. A unique carbon signal resonance at δ 126.8 established the double bond between C-12 and C-13 and the placement of the two epoxy groups at C-9, C-10 and C-15, C-16. This was also supported by the presence of two pairs of fragment ions at *m/z* 167/155 and 137/125 towards the terminal methyl group, and a peak at *m/z* 253 (cleavage at C-14/C-15 towards the ester group).

Compound **3c**, observed as only one spot in TLC, was obtained as an oil. The LSI-MS exhibited peaks at *m/z* = 363 $[M + Na]^+$ and *m/z* 341 $[MH]^+$ which agreed with a molecular formula $C_{19}H_{32}O_5$ and with the formation of a tri-epoxy derivative. Examination of both ^{1}H- and ^{13}C-NMR evidenced a series of double signal resonances (see Tables **1** and **2**), suggesting that **3c** could be a mixture of *meso*-triepoxides obtained from the diepoxide **3b** during the epoxidation reaction of **3a**. As described by Hoye and Suhadolnik (13), if **3b** is the all-*S*- or the all-*R*-diastereomer only one compound will arise from epoxidation because the two faces of the olefin are interconverted by rotation. Therefore **3b** presumably was the *meso*-diastereomer. We found no way to separate these *meso*-derivatives and so they were assayed as the mixture for their biological activity.

The triepoxide **3c** treated first with NaOH and then acetylated by pyridine/Ac_2O, yielded **3d**. The CIMS of **3d** showed a peak at *m/z* 429 $[MH]^+$ for a molecular formula of $C_{22}H_{36}O_8$. Se-

Table 1 ^{1}H-NMR spectral data of compounds **3a – 3d** ($CDCl_3$, *J* in Hz, δ in ppm).

Proton Nº	Compounds **3a**	**3b**	**3c**	**3d**
2	2.29 t (7.6)	2.30 t (7.6)	2.25 t (7.5)	2.31 t (7.6)
3	1.61 m	1.61 m	1.60 m	1.60 m
4 – 7	1.30 m	1.32 m	1.28 m	1.31 m
8	2.06 m	1.56 m	1.60 m	1.60 m
9	5.35 m	2.93 m	2.92 m	2.96 m
10	5.35 m	2.93 m	3.07 m	3.09 m
11	2.80 brt (5.6)	2.23 m	1.75 m	1.72 m
12	5.35 m	5.60 m	3.12 m	4.21 m
13	5.35 m	5.60 m	3.12 m	4.21 m
14	2.80 brt (5.6)	2.23 m	1.75 m	1.72 m
15	5.35 m	2.93 m	3.07 m	3.09 m
16	5.35 m	2.93 m	2.92 m	2.96 m
17	2.06 m	1.56 m	1.49 m	1.49 m
18	0.97 t (7.6)	1.05 t (7.6)	1.03/1.00 t (7.6)	1.03/0.87 t (7.6)
OMe	3.66 s	3.66 s	3.61 s	
OCOMe				2.03 s
OCOMe				2.06 s

Table 2 ^{13}C-NMR chemical shifts of compounds **3a – 3c** ($CDCl_3$; δ in ppm)*.

Carbon Nº	Compounds **3a**	**3b**	**3c**
1	174.2	174.2	174.2
2	34.1	34.0	34.0
3	24.9	24.8	24.8
4 – 7	29.1 – 29.5	29.0 – 29.3	28.9 – 29.2
8	27.1	27.4[b]	27.5
9	127.1	56.3[a]	56.9/56.6[a]
10	131.9	57.1[a]	53.7/54.1[a]
11	25.5	26.4	27.3[b]
12	128.2	126.8	53.7/54.1[a]
13	128.2	126.8	53.7/54.1[a]
14	25.5	26.4	27.0[b]
15	127.7	58.3[a]	53.7/54.1[a]
16	130.2	56.3[a]	58.1/57.8[a]
17	20.5	27.7[b]	27.8
18	14.2	10.6	10.4/10.3
OMe	51.4	51.4	51.4

* Assignments from DEPT and HMQC data.

[a, b] Assignments may be interchanged within the column.

quential losses of two 60 mass units from the MH^+ in the CIMS, as well as the ^1H- and ^{13}C-chemical shifts at δ 2.03/2.06 and δ 170.7/170.6, respectively, suggested the presence of a diacetoxy derivative. Careful examination of fragment ions in the CIMS and the ^1H-^1H COSY spectral data confirmed the opening of the oxirane ring placed at C-12/C-13.

Bioassays of α-linolenic acid derivatives

It has been well established that the ACG are potent specific inhibitors of the mitochondrial complex I (NADH : ubiquinone oxidoreductase (4 – 6). Complex I is a limiting step in the production of cell energy by the mitochondria. Therefore, inhibition of this enzyme compromises the tumor cell metabolism because of the high demand of energy production needed for tumor growth (1 – 3).

The study of the different epoxy moieties along the alkyl chain of ACG was the main purpose of this research. In that way, the α-linolenic acid derivatives obtained (**3b – 3d**), two natural epoxy-acetogenins, tripoxyrollin (**1**) and diepomuricanin A (**2**), and a bis-THF ACG, membrarollin (**4**), were biologically tested as inhibitors of mitochondrial respiratory chain. A classical inhibitor of the mitochondrial complex I, rotenone (**5**), was also used for reference.

The assays included not only the whole respiratory chain, but also the complex I specific activity. In the first case, an NADH oxidase assay was performed. It 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. As acetogenins only inhibit mitochondrial complex I (4 – 6), the inhibition of this activity is directly attributed to the inhibition of complex I. Measurement of complex I specific activity was performed as the NADH : ubiquinone oxidoreductase assay with decylubiquinone (DB) as an ubiquinone analogue. This activity, referred to as the NADH : DB assay, is a less physiological assay because a high quantity of the quinone is added. This measurement presents lesser-observed enzymatic activities compared with those of the NADH oxidase assay, probably due to a limited solubility of the DB in the assay media (14). Moreover, inhibitors are partially displaced by DB (15, 16) showing greater IC_{50} values compared with those obtained in the NADH oxidase assay. Nevertheless, the NADH : DB assay is a good measurement of the complex I specific activity (12, 14) and it is needed in the studies of complex I inhibitors for adequate comparisons (16) despite its limitations.

Figure **2A** shows the titration curves of α-linolenic acid derivatives (**3a – 3d**) against the NADH oxidase activity. All the four compounds showed typical hyperbolic curves with a micromolar (μM) level potency. The triepoxide (**3c**) was the most potent of the series. However the opening of the central epoxy ring in addition to the loss of the methyl ester group decreased the potency of the compound with the result that **3d** was the weakest inhibitor of the study (Table **3**). Compounds **3a** and **3b** exhibited an intermediate potency. The titration curves for compounds **3a – 3d** against the specific complex I activity are represented in Fig. **2B**. All the α-linolenic acid derivatives had similar tendencies except **3b** and **3a** that showed crossing curves. This little difference could be due to interference with the externally added quinone (16).

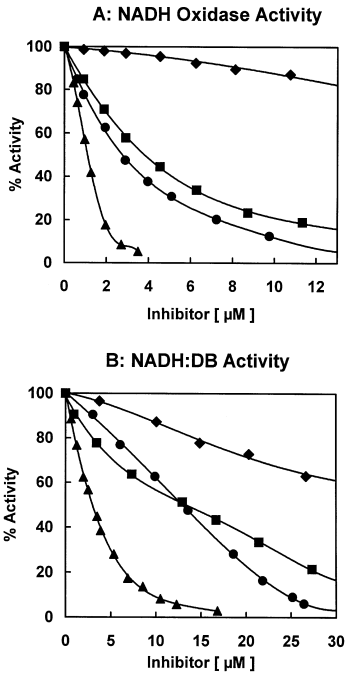

Fig. 2 Titrations of α-linolenic acid derivatives as respiratory chain inhibitors. (■) α-linolenic methyl ester (**3a**); (●) di-epoxy-α-linolenic methyl ester (**3b**); (▲) tri-epoxy-α-linolenic methyl ester (**3c**); (◆) di-epoxy-diacetylated-α-linolenic acid (**3d**). Control acitivities ($\mu mol \cdot min^{-1} \cdot mg^{-1}$) were 0.96 ± 0.09 for NADH oxidase and 0.57 ± 0.11 for NADH : DB oxidoreductase.

It has been suggested that hydrophobicity is a key factor to understand the SAR of a complex I inhibitor (15). Differential solubility in the lipophilic membrane bilayer could affect the access of the inhibitor to the enzyme. A too hydrophilic compound will not be able to get dissolved in the membrane in sufficient amounts to be active. A too hydrophobic compound will be retained by the membrane and will not reach the enzyme core. We found by TLC that all **3a – 3d** compounds have a similar hydrophobicity. This indicates that the differences observed in the inhibitory potencies were produced by the presence or the absence of the epoxy groups along the alkyl chain. Therefore it was not merely an effect of a hydrophobicity factor.

The IC_{50} values indicated that the presence of two distanced epoxy moieties along the alkyl chain (**3b**) increases significantly the potency of the inhibitor in the NADH oxidase assay with regard to **3a**. This pattern was also observed in the NADH : DB assay but with less significant differences. Moreover, if an additional epoxy ring is present (**3c**), the inhibitory potency is clearly increased in both assays (Table **3**). It is important to note that this structural change increased the NADH : DB oxidoreductase inhibition by five fold for **3c** with respect to **3b**, avoiding the decylubiquinone displacement by one half (NADH : DB oxidoreductase/NADH oxidase ratio decreased from 4.7 to 2.4) which is indicative of a more tight binding to the enzyme (16). The cleavage of the central epoxy ring (**3d**) effected not only loss of potency but also a null and void inhibition of the enzyme at the experimental range, probably due to an esteric effect along the other epoxy groups produced by the acetoxy moieties (see Table **3** for tentative IC_{50} values for compound **3d**).

Bioassays of epoxy-acetogenins

Isolated linear acetogenins are claimed as the biogenetic precursors of the epoxy-acetogenins (7, 8), and differ in the de-

Table 3 IC_{50} values for compounds assayed as complex I inhibitors.

Inhibitor	NADH oxidase	NADH : DB oxidoreductase	Ratio
A. Annonaceous acetogenins	IC_{50} (nM)		
tripoxyrollin (**1**)	0.83 ± 0.16	19.3 ± 5.4	23
diepomuricanin-A (**2**)	17.0 ± 1.4	>250	>15
membrarollin (**4**)	0.29 ± 0.02	0.83 ± 0.18	2.9
B. α-Linolenic acid derivatives	IC_{50} (μM)		
α-linolenic methyl ester (**3a**)	3.69 ± 0.11	15.0 ± 3.6	4.1
di-epoxy-α-linolenic methyl ester (**3b**)	2.85 ± 0.37	13.5 ± 0.6	4.7
tri-epoxy-α-linolenic methyl ester (**3c**)	1.17 ± 0.16	2.82 ± 0.91	2.4
di-epoxy-diacetylated-α-linolenic (**3d**)	>30	>60	–
C. Other complex I inhibitors	IC_{50} (nM)		
rotenone (**5**)	5.1 ± 0.9	28.8 ± 1.5	5.6

gree of unsaturation and hydroxylation of their alkyl chains (1–3). They are similar to the α-linolenic derivatives of our study. Natural epoxy-acetogenins, originated by oxidation of linear olefinic acetogenins, are also key metabolites in the biosynthesis pathway of mono-, bis- or tri-THF acetogenins. The existence of three epoxy groups at the proper carbon distance affords in biogenetic conditions an α,α′-dihydroxylated-bis-THF acetogenin (7). For that reason we also compared the inhibitory potency values found for tripoxyrollin (**1**) with those of membrarollin (**4**), an α,α′-dihydroxylated-bis-THF acetogenin with a *threo/cis/threo/cis/erythro* relative configuration (10). From IC_{50} values (Table **3**) we can deduce that the tetrahydrofuran formation increases the potency against both respiratory-chain activities.

Figure **3** shows titrations of both NADH oxidase (**3A**), and NADH : DB oxidoreductase (**3B**) activities. All natural ACG showed inhibitory potency at the nano-molar (nM) level. The presence of the terminal methyl-γ-lactone moiety (as in **1**, **2**, and **4**) increased the potency of the compounds by three orders of magnitude with regard to **3a–3d**. This demonstrated that the lactone moiety is a key factor in the interaction of the acetogenin with the enzyme, in agreement with recent studies on the membrane conformations of several ACG and their cytotoxic effects (17). The same SAR found for the α-linolenic acid derivatives was present in the epoxy-acetogenins because the presence of a third epoxy ring increased inhibition of both NADH oxidase and NADH : DB oxidoreductase activities. Moreover, it is important to note that the presence of the lactone moiety gives the compound the ability to inhibit the mammalian complex I at a range similar to that found for other classical potent complex I inhibitors like rotenone (**5**) (Table **3**).

All these data can be summarized in the following structure-activity relationship conclusions: (i) An increasing number of epoxy rings improves the inhibitory potency of both, acetogenins and alkyl chain derivatives without a terminal methyl-γ-lactone, (ii) tetrahydrofuranic acetogenins are more potent than epoxy-acetogenins, being the last ones followed in potency by alkyl chain epoxides without the lactone moiety, and (iii) the terminal methyl-γ-lactone of the ACG is a relevant factor for the inhibitory potency because its presence in the molecule decreases the IC_{50} values of the compound by three orders of magnitude.

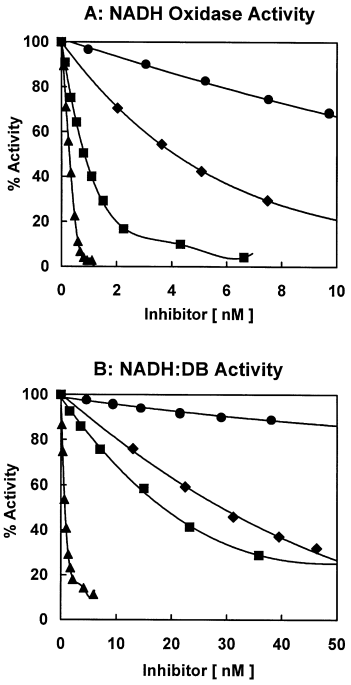

Fig. 3 Titrations of acetogenins and rotenone as respiratory chain inhibitors. (■) tripoxyrollin (**1**); (●) diepomuricanin-A (**2**); (▲) membrarollin (**4**); (◆) rotenone (**5**). Control activities ($\mu mol \cdot min^{-1} \cdot mg^{-1}$) were 0.94 ± 0.09 for NADH oxidase and 0.56 ± 0.09 for NADH : DB oxidoreductase.

This study contributes to our knowledge of the natural Annonaceous acetogenins. SAR conclusions open a new perspective in the study of these compounds and focus the research efforts on the study of the rules that make the methyl-γ-lactone, tetrahydrofuran, and epoxy rings along the alkyl chain so important, and in that order, for improving the drug design of this family of future antitumor drugs.

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