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Synthesis and characterization of Δlac-acetogenins that potently inhibit mitochondrial complex I

Jean-Charles Chapuis, Omar Khdour, Xiaoqing Cai, Jun Lu, Sidney M. Hecht *

Center for BioEnergetics, The Biodesign Institute, Arizona State University, Tempe, AZ 85287, USA

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ABSTRACT

Four Δlac-acetogenins have been prepared and characterized as inhibitors of the mitochondrial respiratory chain, to define the effects of unsaturation within the alkyl substituents. In keeping with earlier reports, the presence of acetylenic functionalities within the alkyl substituents slightly diminished their potency of inhibition of NADH oxidase activity, which measures the overall transfer of electrons from NADH to oxygen through mitochondrial complexes I, III, and IV. In contrast, both of the acetylenic Δlac-acetogenins were far more active in a NADH–ubiquinone Q_1 oxidoreductase assay that measures complex I function per se.

1. Introduction

Members of the family Annonaceae produce a very interesting class of natural product known as acetogenins.[1] Many compounds in this structural class exhibit potent biological activities such as anti-tumor, anti-malarial, and pesticidal activities.[2] These diverse biological activities have been attributed to the compelling inhibitory effects of these compounds on mitochondrial complex I (NADH–ubiquinone oxidoreductase) in mammalian and insect mitochondrial electron transport systems.[3] They have also been reported as potent inhibitors of NADH oxidase of the plasma membrane of cancer cells.[4]

Many of the naturally occurring acetogenins contain α,β-unsaturated γ-methylbutyrolactone substituents at the terminus of a linear alkyl substituent, as exemplified by bullatacin, trilobin, and trilobacin (Fig. 1). The intrinsic electrophilic nature of such functionalities may be capable of mediating enhanced potency through alkylation of their target proteins, albeit with the potential for loss of selectivity of action. Bullatacin is one of the most potent inhibitors of bovine heart complex I NADH–ubiquinone oxidoreductase, having activity reported at low nanomolar concentration.

Recently, structurally related compounds lacking the α,β-unsaturated γ-methylbutyrolactone ring have been described. These Δlac-acetogenins appear to act at a site on complex I different than the prototype acetogenins, or the more traditional mitochondrial complex I inhibitors such as rotenone and piericidin.[5]

In recent studies of the structural requirements for complex I inhibition, the alkyl substituents of acetogenins and Δlac-acetogenins were altered in length and by introduction of single and multiple double and triple bonds within the alkyl substituent. While dramatic effects were observed with varying chain length, the effects of unsaturation were less clear, producing little change in some cases and a pronounced diminution of activity in others.[6,7]

As part of an effort to characterize the function of the mitochondrial respiratory chain, we have prepared four Δlac-acetogenins (**1**–**4**) (Fig. 1), two of which contain alkynes within the alkyl substituents. We have characterized these compounds in a series of biological assays including (i) complex I assay measured by NADH–ubiquinone oxidoreductase, (ii) complexes I, III, and IV assay measured by NADH oxidase, (iii) evaluation of the consequences of the compounds in inducing a shift to glycolysis, and (iv) evaluation of the cytotoxicities of all four compounds in several mammalian cell lines.

We found that acetylenic compounds **1** and **3**, not previously studied as complex I inhibitors, strongly inhibit complex I and actually exhibit significantly stronger activities than the reported inhibitors **2**[8] and **4**.[9] Presently, we detail the preparation, biochemical, and biological characterization of these interesting new Δlac-acetogenin complex I inhibitors.

2. Results and discussion

Inhibitors **1** and **2** were prepared as described previously.[9] Known[9] compounds **3** and **4** were prepared analogously but using a new route for a key synthetic intermediate (**8**), as outlined in

* Corresponding author. Tel.: +1 480 965 6625; fax: +1 480 965 0038.
E-mail address: sidney.hecht@asu.edu (S.M. Hecht).

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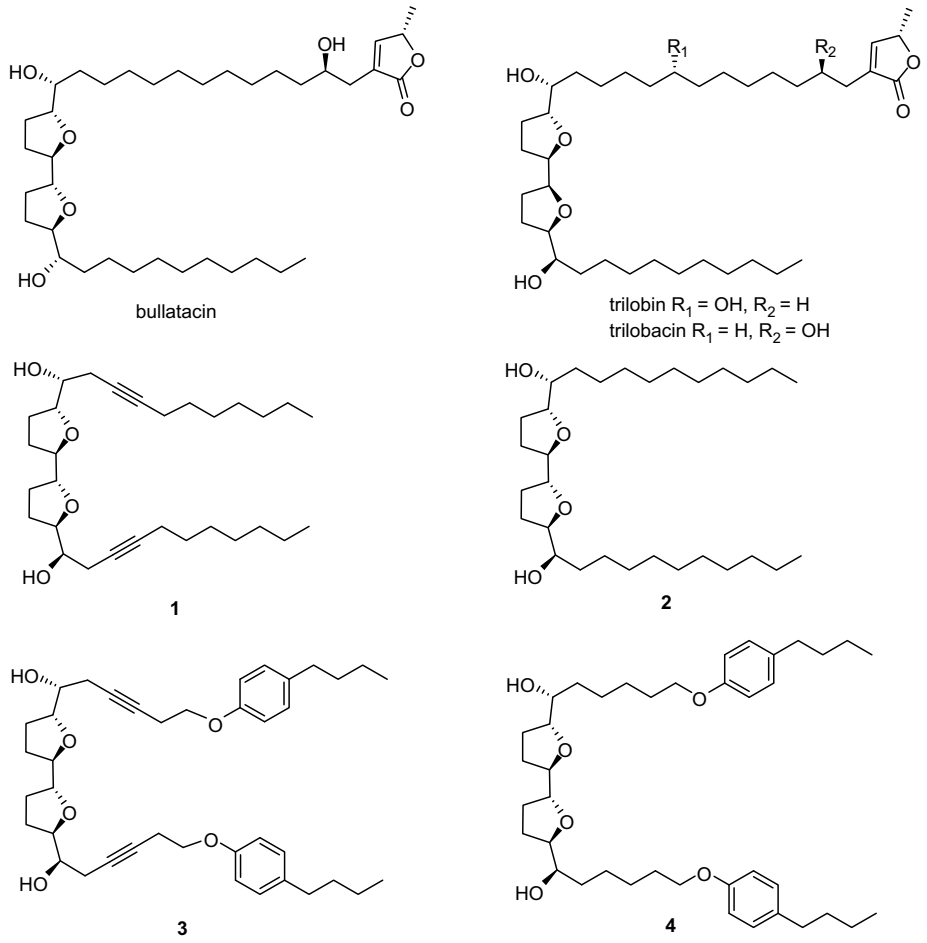


Figure 1. Δlac-acetogenins **1**–**4** and structurally related natural compounds.

Scheme 1. *n*-Butylphenoxypropionaldehyde **6** was prepared readily in two steps as shown in the Scheme, and then converted to **8** via dibromobutenyl ether **7**. Nucleophilic opening of the epoxide moieties in **9** then afforded inhibitor **3** in 99% yield as a colorless oil. Catalytic hydrogenation of **3** afforded **4** in 87% yield.

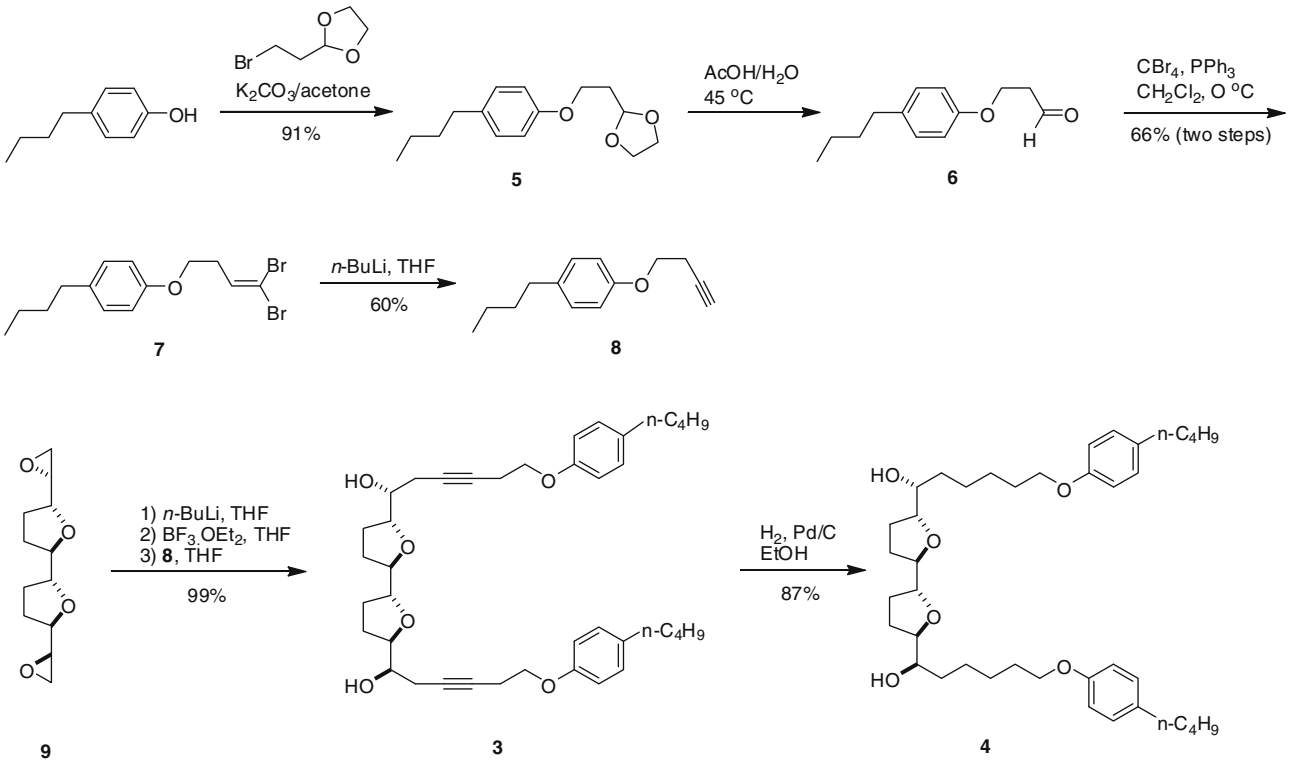
Both the NADH oxidase and NADH–ubiquinone oxidoreductase activities of compounds **1**–**4** were measured. NADH oxidase activity reflects forward electron transfer in the respiratory chain, wherein NADH is oxidized by endogenous coenzyme Q_{10} with transfer of electrons through complexes III and IV to O_2. It was found that the bovine heart submitochondrial particles (SMPs) functioned significantly more robustly in Hepes buffer,[10] than the reported[5,6,8,9] phosphate buffer. Accordingly, the assay was run in Hepes buffer. As shown in Table 1, acetylenic derivatives **1** and **3** had IC_{50} values of 30.1 and 38.8 nM in this assay, while the corresponding saturated derivatives **2** and **4** gave values of 12.3 and 19.6 nM, respectively. Thus, the presence of the triple bonds in **1** and **3** diminished the NADH oxidase activity of these compounds to some extent.[11]

Inhibition of complex I formation was also measured by measuring NADH–ubiquinone oxidoreductase activity in the presence of exogenous ubiquinone Q_1, as well as complex III inhibitor antimycin A and complex IV inhibitor cyanide. As shown in Table 1, acetylenic compound **1** was significantly more active in this assay than acetylenic compound **3** (48 vs 1700 nM IC_{50} values, respectively). Compounds **2** and **4** exhibited little inhibitory activity in this assay system.[12]

Previous studies by Miyoshi and co-workers have focused on the alkyl substituents of Δlac-acetogenins and indicated that an optimal chain length and balance of hydrophobicity of the two alkyl substituents, as well as the steric nature of the alkyl substituents, were important factors for respiratory chain inhibition.[8,14] In another study, these workers found that local flexibility in a specific region of one of the alkyl chains (studied by introducing double and triple bonds) was not obviously important for the inhibitory activity of the acetogenins.[6]

According to the Warburg effect, which is defined by the observation that cancer cells are intrinsically more glycolytic than normal cells, tumor cells depend less on mitochondrial function for ATP production and more on glycolysis than their normal counterparts.[15] Further, it has been reported that more generally when cells lack oxygen, they have a propensity to switch to anaerobic metabolism, resulting in an increase in glycolysis.[16] The Pasteur effect, defined as the inhibition of glycolysis by respiration, is based on such a principle.[17] When cells are subjected to decreased mitochondrial function due to metabolic stress, glycolysis is stimulated in order to maintain relatively constant ATP synthesis. Under such conditions, pyruvate is reduced to lactate to regenerate oxidized pyridine nucleotides. Although not as efficient as aerobic respiration, two ATP and two lactate molecules are formed per glucose metabolized. The outcome is increased lactate production sufficient to compensate for inhibition of respiration. We have used this principle to evaluate known anti-mitochondrial toxins, such as antimycin A, as well as the Δlac-acetogenins that are the focus of this study.

Figure 2 shows the titration of semi-confluent MCF-7 cells exposed to varying concentrations of compounds **1**–**4** for 3 h. Acetylenic compounds **1** and **3** stimulated significantly more lactate production than **2** and **4**. In order to compare these compounds,



Scheme 1.

Table 1
Inhibitory activities toward mitochondrial complex I[a] of the described Δlac-acetogenins

Compound	IC_{50}[b]	
	NADH oxidase (nM)	NADH–ubiquinone (Q_1) oxidoreductase (μM)
1	30.1 ± 0.8	0.048 ± 0.002
2	12.3 ± 0.3	10.4 ± 0.3
3	38.8 ± 1.3	1.7 ± 0.2
4	19.6 ± 1.9	>15

[a] Test performed on bovine heart submitochondrial particles (SMP).
[b] The molar concentration required for half-maximal inhibition of the control. Values are means ± SD of three independent experiments. NADH–ubiquinone oxidoreductase was determined with ubiquinone 1 (Q_1) as the ubiquinone analogue. Control activity was approximately 0.92 μmol min^{-1} mg^{-1} for NADH oxidase and 0.75 μmol min^{-1} mg^{-1} for NADH–ubiquinone oxidoreductase.

the concentration required to obtain half of the maximal lactate production was calculated. Table 2 presents that value for each of compounds **1**–**4** compared to antimycin A under identical conditions. Compounds **2** and **4** were found to be less able to induce a shift to glycolysis than the two acetylenic compounds **1** and **3**, the latter of which had activity in the nanmolar range. Increasing the incubation time from 3 to 6 h did not significantly change the values measured (0.44, 4.4, 0.32, and 18.8 μM for **1**–**4**, respectively).

Also determined were the cytotoxicities of **1**–**4** toward cultured mammalian cells. Compounds **1**–**4** were not strongly cytotoxic. The results were confirmed by SRB assay[18] which gave similar values (data not shown). In both assays, compound **1** was found to be the most active. 'Normal' human breast cells (MCF-10A) were found slightly more sensitive to compound **1**, although the difference was not large. Natural acetogenins, such as bullatacin and derivatives have been previously reported[19] not to be as cytotoxic toward MCF-7 cells as compared with A549 (lung) or HT-29 (colon) cells. Other workers have recently reported a lack of significant cytotoxicity of synthetic hybrid acetogenins toward a panel of 39 human cancer cell lines.[20] In the present study, additional cell lines, such as two human glioblastoma lines (Table 3), were found to be largely unaffected by these compounds as well. For the cell lines tested, **1** was generally more potently cytotoxic than **2**–**4**. One exception was Lewis lung carcinoma which was inhibited strongly by all of the compounds, especially by compounds **2**–**4**. Liu et al.[21] recently reported micromolar inhibition of HT-29 cells by a modified bullatacin derivative, but obtained nanomolar activities against cancerous human liver cells and a lack of response in normal hepatocytes. Whether these effects are all due to action at the locus of mitochondrial complex I is unclear at present.

Differences in the mode of ATP production between normal and cancer cells, that is, the extent of use of glycolysis versus the mitochondrial respiratory chain, have been suggested as a basis for selective cancer chemotherapy.[22,23] In addition to their lack of an electrophilic α,β-unsaturated γ-methylbutyrolactone constituent, the Δlac-acetogenins are of interest as mitochondrial inhibitors due to the ability of at least some of these compounds (e.g., **4**) to inhibit mitochondrial complex I function without strongly inducing superoxide production.[24] In the present study, we have demonstrated that introduction of an alkyne moiety within the aliphatic substituent of such compounds can result in more potent inhibition of NADH–ubiquinone oxidoreductase activity, and a significantly enhanced ability to induce a shift to glycolysis. This design feature may find utility in implementing therapeutic strategies that exploit differences in ATP production between normal and cancer cells.

3. Experimental

3.1. Chemistry

Chemicals and solvents were of reagent grade and were used without further purification. Anhydrous grade solvents were purchased from VWR. All reactions involving air or moisture sensitive

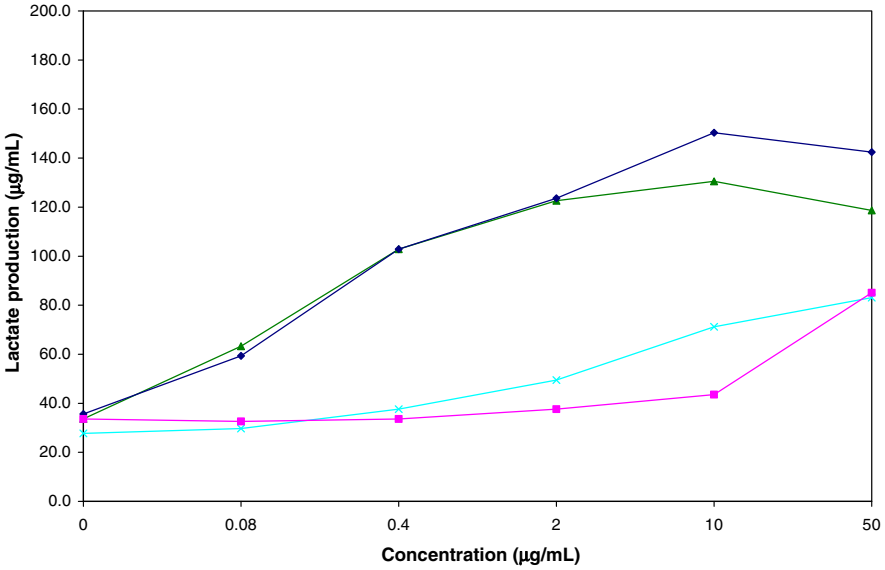


Figure 2. Lactate production by MCF-7 cells exposed to compound **1** (▲), **2** (x), **3** (♦), or **4** (■) for 3 h.

Table 2
Lactate production by MCF-7 human breast cancer cells incubated for 3 h in the presence of compounds **1**, **2**, **3**, or **4**

Compound	Concentration (μM)	Incremental lactate production (μM)
1	0.38	1080
2	6.6	616
3	0.44	1280
4	18.2	572
Antimycin A	<0.15	1520

reagents or intermediates were performed under an argon atmosphere. Flash chromatography was carried out using Silicycle 200–400 mesh silica gel. Analytical TLC was carried out using 0.25 mm EM silica gel 60 F_{250} plates that were visualized by irradiation (254 nm) or by staining with *p*-anisaldehyde stain. ^{1}H and ^{13}C NMR spectra were obtained using Gemini 300, Inova 400, Inova 500 and VNMRS 800 MHz Varian instruments. Chemical shifts were reported in parts per million (ppm, δ) referenced to the residual ^{1}H resonance of the solvent ($CDCl_3$, 7.26 ppm). ^{13}C spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, 77.0 ppm). Splitting patterns were designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass spectra of **4**, **5**, **7**, and **8** were obtained at the Michigan State University-NIH Mass Spectrometry Facility. For compound **3**, the high resolution mass spectrum was obtained at the Ohio State University Mass Spectrometry Facility.

3.1.1. 2-(2-(4-*n*-Butylphenoxy)ethyl)-1,3-dioxolane (5)

To a suspension of 3.31 g (4.00 mmol) of K_2CO_3 in 20 mL of acetone was added 900 mg (6.00 mmol) of 4-*n*-butylphenol at 70 °C. After stirring for 1 h, 2.17 g (12.0 mmol) of 2-(2-bromoethyl)-1,3-dioxolane was added to the mixture. The reaction mixture was heated at reflux overnight. The cooled reaction mixture was filtered and concentrated to dryness. The residue was dissolved in 20 mL of water and the aqueous mixture was adjusted to a pH between 2 and 3 using 1.2 N HCl. The solution was extracted with ethyl acetate and the combined organic phase was washed with brine, dried over anhydrous Na_2SO_4, and concentrated under diminished pressure to yield a crude oil. The residue was purified by flash chromatography on a silica gel column (25.4 × 2.6 cm). Elution with 1:20 ethyl ether/hexanes gave **5** as a colorless oil: yield 1.4 g (91%); silica gel TLC R_f 0.42 (1:5 ethyl ether/hexanes); ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 3H, J = 7.2 Hz), 1.32 (m, 2H), 1.53 (m, 2H), 2.12 (m, 2H), 2.53 (t, 2H, J = 8.1 Hz), 3.86 (t, 2H, J = 2.1 Hz), 3.97 (t, 2H, J = 2.7 Hz), 4.08 (t, 2H, J = 4.8 Hz), 5.01 (t, 1H, J = 4.8 Hz), 6.80 (d, 2H, J = 8.4 Hz), and 7.06 (d, 2H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$) δ 13.95, 22.28, 33.89, 33.91, 34.72, 63.62, 64.90, 102.13, 114.31, 129.21, 135.07, and 156.84; mass spectrum (EI), m/z 250.1568 (M^+) ($C_{15}H_{22}O_3$ requires m/z 250.1569).

3.1.2. 3-(4-*n*-Butylphenoxy)propionaldehyde (6)

A solution containing 200 mg (1.60 mmol) of **5** in 5 mL of 4:1 acetic acid/water was stirred at 45 °C for 5 h. The cooled reaction mixture was adjusted to a pH between 6 and 7 with satd aq $NaHCO_3$. The aqueous mixture was then extracted with ethyl acetate and the combined organic phase was washed with brine and dried over anhydrous $MgSO_4$. Concentration under diminished pressure gave a crude oil. Crude **6** was used for the next step without further purification; silica gel TLC R_f 0.38 (1:5 ethyl ether/hexanes).

3.1.3. 1-Butyl-4-(4,4-dibromobut-3-enyloxy)benzene (7)

To a dried flask were added 419 mg (1.26 mmol) of CBr_4 and 2 mL of anhydrous CH_2Cl_2 at 0 °C. A solution containing 662 mg (2.52 mmol) of PPh_3 in 2 mL of anhydrous CH_2Cl_2 was then added dropwise. The reaction mixture was stirred at 0 °C for 10 min, and

Table 3
Human and mouse cell line inhibition values (IC_{50}) in μM for compounds **1**–**4** determined by MTT assay[a]

Compound	MCF-7	MCF-10A	CRL-2365	CRL-2366	A549	3LL
1	7.6 ± 2.2	2.0 ± 0.2	20.3 ± 2.5	>20.9	13.2 ± 1.0	5.2 ± 0.6
2	>20	>20	>20	>20	>20	0.99 ± 0.06
3	>16	>16	14.6 ± 1.4	>16	>16	0.48 ± 0.14
4	>16	>16	>16	>16	>16	0.64 ± 0.06

[a] Cell type: MCF-7 (breast carcinoma); MCF-10A ('normal' human breast); CRL-2365 (brain glioblastoma); CRL-2366 (DNA-repair deficient brain glioblastoma); A549 (lung carcinoma); 3LL (mouse Lewis lung carcinoma).

a solution containing 130 mg (0.63 mmol) of **6** in 3 mL of anhydrous CH_2Cl_2 was added slowly. The reaction mixture was stirred for 1.5 h and then 3 mL of water was added. The mixture was extracted with portions of CH_2Cl_2 and the combined organic phase was washed with brine and dried over anhydrous $MgSO_4$, then concentrated under diminished pressure. Twenty milliliters of ether was added to the residue and the resulting suspension was filtered to remove triphenylphosphine oxide. The solid was washed again with ether. The combined filtrate was concentrated and the residue was purified by flash chromatography on a silica gel column (25 × 2.6 cm). Elution with 1:25 ether/hexanes gave **7** as a colorless oil: yield 130 mg (66% over two steps); silica gel TLC R_f 0.85 (1:5 ethyl ether/hexanes); ^{1}H NMR ($CDCl_3$) δ 0.92 (t, 3H, J = 7.2 Hz), 1.33 (m, 2H), 1.53 (m, 2H), 2.55 (m, 4H), 3.99 (t, 2H, J = 6.6 Hz), 6.58 (t, 1H, J = 7.2 Hz), 6.80 (d, 2H, J = 8.7 Hz), and 7.09 (d, 2H, J = 8.1 Hz); ^{13}C NMR ($CDCl_3$) δ 14.11, 22.44, 33.39, 34.03, 34.88, 65.46, 90.93, 114.56, 129.46, 135.03, 135.60 and 156.69; mass spectrum (EI), m/z 359.9715 (M^+) ($C_{14}H_{18}O^{79}Br_2$ requires m/z 359.9724).

3.1.4. 1-(But-3-ynyloxy)-4-*n*-butylbenzene (8)

A solution of 0.46 mL of *n*-BuLi in hexane (2.5 M in hexane, 1.15 mmol) was added dropwise to a stirred solution containing 190 mg (0.53 mmol) of **7** in 20 mL of anhydrous THF at −78 °C. After 45 min, the reaction mixture was slowly warmed to 0 °C. The reaction was quenched with satd NH_4Cl after 1 h. The reaction mixture was extracted with ethyl acetate and the organic phase was washed with brine, and dried over anhydrous $MgSO_4$, then concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25 × 2.6 cm). Elution with 1:60 ethyl ether/hexanes gave **8** as a colorless oil: yield 60 mg (60%); silica gel TLC R_f 0.77 (1:20 ethyl ether/hexanes); ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3H, J = 5.4 Hz), 1.36 (m, 2H), 1.57 (m, 2H), 2.06 (d, 1H, J = 2.7 Hz), 2.58 (t, 2H, J = 5.7 Hz), 2.68 (m, 2H), 4.11 (t, 2H, J = 5.4 Hz), 6.86 (d, 2H, J = 6.3 Hz), and 7.12 (d, 2H, J = 6.6 Hz); ^{13}C NMR ($CDCl_3$) δ 13.98, 19.59, 22.32, 33.90, 34.76, 66.08, 69.83, 80.57, 114.51, 129.32, 135.53, and 156.45; mass spectrum (EI), m/z 202.1360 (M^+) ($C_{14}H_{18}O$ requires m/z 202.1358).

3.1.5. (1*R*,1′*R*)-1,1′-((2*R*,2′*R*,5*R*,5′*R*)-Octahydro-2,2′-bifuran-5,5′-diyl)-bis-(6-(4-*n*-butylphenoxy)hex-3-yn-1-ol) (3)

Compound **3** was prepared according to the method of Ichimaru et al.[7] To a solution of 143 mg (0.71 mmol) of **8** in 2 mL of anhydrous THF was added 1.28 mL of a solution of *n*-BuLi in hexane (2.5 M in hexane, 0.71 mmol) at −78 °C. After 30 min, 101 mg (0.71 mmol) of $BF_3{\cdot}Et_2O$ was added and the reaction mixture was stirred for another 30 min. A solution of 7 mg (0.03 mmol) of **9**[25] in 1.0 mL of THF was then added and the reaction mixture was stirred for 1.5 h. The reaction was quenched by the addition of sat NH_4Cl. The reaction mixture was extracted with ether. The combined organic phase was washed with brine and dried over anhydrous $MgSO_4$, then concentrated under diminished pressure to give a crude oil. The residue was purified by flash chromatography on a silica gel column (20 × 1.7 cm). Elution with 1:2 ethyl ether/hexanes gave **3** as a colorless oil: yield 19.5 mg (99%); silica gel TLC R_f 0.40 (3:1 ethyl acetate/hexanes); ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 6H, J = 7.2 Hz), 1.32 (m, 4H), 1.55 (m, 4H), 1.85 (m, 4H), 1.91 (m, 4H), 2.40 (t, 4H, J = 3.9 Hz), 2.51 (t, 4H), 2.59–2.64 (m, 6H), 3.58 (br, 2H), 3.86 (m, 2H), 4.01 (m, 6H), 6.79 (d, 4H, J = 8.4 Hz) and 7.06 (d, 4H, J = 8.1 Hz); mass spectrum (ESI), m/z 653.3821 $(M+Na)^+$ ($C_{40}H_{54}O_6$ requires m/z 653.3818).

3.1.6. (1*R*,1′*R*)-1,1′-((2*R*,2′*R*,5*R*,5′*R*)-Octahydro-2,2′-bifuran-5,5′-diyl)-bis-(6-(4-*n*-butylphenoxy)hexan-1-ol) (4)

Compound **4** was prepared according to the method of Ichimaru et al.[9] A mixture of 13.8 mg (0.02 mmol) of **3** and 3 mg of 10% Pd/C in 1 mL of ethanol was stirred overnight under a H_2 atm at room temperature. The catalyst was removed by filtration and the resulting mixture was concentrated and purified by flash chromatography on a silica gel column (20 × 1.7 cm). Elution with 1:2 ethyl ether/hexanes gave **4** as a colorless oil: yield 12.2 mg (87%); silica gel TLC R_f 0.45 (1:3 hexane/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.91 (t, 6H, J = 7.5 Hz), 1.34–1.69 (m, 24H), 1.76–1.79 (m, 4H), 1.97–1.99 (m, 4H), 2.48 (br, 2H), 2.54 (t, 4H), 3.40 (m, 2H), 3.83–3.87 (m, 4H), 3.92 (t, 4H, J = 6.5 Hz), 6. 80 (d, 4H, J = 8.5 Hz), and 7.07 (d, 4H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 13.94, 22.29, 25.42, 26.15, 28.35, 28.94, 29.31, 33.37, 33.89, 34.72, 67.87, 73.94, 81.76, 83.12, 114.27, 129.18, 134.82 and 157.11; mass spectrum (ESI), m/z 639.4628 $(M+H)^+$ ($C_{40}H_{63}O_6$ requires m/z 639.4625); $[\alpha]_{25}^{D}$ +0.025° (c 0.75, CH_2Cl_2).

3.2. Biological evaluation

3.2.1. Lactate assay

Using an adaptation of the method of Everse,[26] mitochondrial toxins were assessed in MCF-7 human breast carcinoma cells. Cells were plated at 2×10^5 cells per well in tissue culture treated 12-well plates and incubated for 24 h (resulting in about 50% cell confluence). The wells were rinsed with a pre-warmed solution of Krebs–Ringer buffer (Sigma) and the compounds were then added at the appropriate concentrations in 0.5 mL of buffer and incubated for 6 h in comparison with controls containing the same amount of buffer. The supernatant was recovered and the variation in absorbance (ΔOD) was measured at 363 nm. Lactate concentration (μg/mL) was calculated from the following formula: [lactate] = [(ΔOD_{363}) (10f)(90)]/9.1 where f is the dilution factor used to bring the lactate concentration within the measurable range of the lactate titration curve, 90 represents the molecular weight of lactic acid and 9.1 is the mM extinction coefficient of the reaction at 363 nm. IC_{50} values were calculated as the dose which produced a 50% inhibition of the maximum lactate production measured.

3.2.2. MTT assay

In vitro inhibition of human cancer cell growth was assessed using the standard MTT assay as previously described.[27]

3.2.3. Mitochondrial complex I activity

Beef heart mitochondria were obtained by a large-scale procedure.[28] Inverted submitochondrial particles (SMP) were prepared by the method of Matsuno-Yagi and Hatefi,[29] and stored in a buffer containing 0.25 M sucrose and 10 mM Tris–HCl (pH 7.4) at −80 °C. Inhibitory effects of compounds **1** and **3** on bovine heart mitochondrial complex I (NADH oxidase and NADH:ubiquinone oxidoreductase) were evaluated by modification of a method described previously.[8] Stock solutions (2 mg/mL in dimethylsulfoxide) of compounds **1** and **3** (Fig. 1) were prepared and kept in the dark at −80 °C. Maximal dimethyl sulfoxide concentration never exceeded 2% and had no influence on the control enzymatic activity. Beef heart SMP were diluted to 0.5 mg/mL, and treated with 300 μM NADH to activate complex I.[30] The enzymatic activities were assayed at 30 °C and monitored spectrophotometrically with a Molecular Devices SPECTRA Max-M5 (340 nm, ε = 6.22 mM^{-1} cm^{-1}). NADH oxidase activity was determined in a reaction medium (2.5 mL) containing 50 mM Hepes, pH 7.5, containing 5 mM $MgCl_2$. The final mitochondrial protein concentration was 30 μg. The reaction was initiated by adding 50 μM NADH after the pre-equilibration of SMP with inhibitor for 5 min. The initial rates were calculated from the linear portion of the traces. The inhibition of NADH-Q_1 oxidoreductase activity was also determined under the same experimental conditions except that the reaction medium (2.5 mL) contained 0.25 M sucrose, 1 mM $MgCl_2$,

2 μM antimycin A, 2 mM KCN, 50 μM ubiquinone Q_1, and 50 mM phosphate buffer, pH 7.4.

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11. When tested for NADH oxidase activity in phosphate buffer and 1 mM Mg^{2+}, compounds **2** and **4** gave values of 7.5 ± 0.3 nM and 0.83 ± 0.04 nM, respectively.[9]
12. Residual enzymatic activity,[13] defined as NADH oxidase or NADH-Q_1 oxidoreductase activity not inhibited by a high (1 μM) concentration of inhibitor, was subtracted when calculating the IC_{50} values. Residual NADH oxidase activities were 12% and 18% for compounds **1** and **3**, respectively. Residual NADH-Q_1 oxidoreductase activities were 18% and 25% for compounds **1** and **3**, respectively.
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