Articles

Enantiospecific Semisynthesis of (+)-Almuheptolide-A, a Novel Natural Heptolide Inhibitor of the Mammalian Mitochondrial Respiratory Chain

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The development of novel styryl lactone derivatives as bioactive compounds and the semisynthesis of both 4,5-dialkoxylated eight-membered-ring lactones with a heptolide skeleton (almuheptolide-A (**1**) type) and 7-alkoxylated δ-lactones with a saturated furanopyrone skeleton (etharvensin (**8**) type) have been successfully achieved from the chiral unsaturated α-pyrone altholactone (**7**). This new method is a direct and one-step enantiospecific alkoxylation of altholactone (**7**) in concentrated acid medium, followed by formation of the eight-membered-ring ζ-lactone. The reaction mechanism operating in the synthesis of the heptolide skeleton is postulated to be a direct Michael-type addition. Concerted opening of both the α-pyrone and tetrahydrofuran rings and subsequent intramolecular rearrangement with the ring closure lead to almuheptolide-A (**1**). This compound (**1**) and its diacetated derivative (**1a**) showed potent and selective inhibitory activity toward mammalian mitochondrial respiratory chain complex I. This mechanism of action, reported here for the first time, provides a possible explanation for the cytotoxic and antitumor activities previously described for related natural compounds.

Introduction

Several plants of the Annonaceae family are known as a source of natural, potent, biologically active compounds. As a part of our search for new cytotoxic compounds in Annonaceous plants,[1−4] series of styryl lactones were isolated from the methanolic extract of *Goniothalamus arvensis* stem barks.[5−7] In this paper we report the isolation and structure elucidation of (+)-almuheptolides-A (**1**) and -B (**2**), new natural heptolides from *G. arvensis*, and the first method for the preparation of pentasubstituted heptolides from furano-α-pyrone structures (Figure 1). (+)-Almuheptolide-A (**1**) possesses an unusual skeleton similar to that of the known (+)-gonioheptolides-A (**3**) and -B (**4**)[8] and related to that described for the natural marine products octalactins-A (**5**) and -B (**6**) (Figure 1).[9]

The cytotoxic activities previously reported for eight-membered-ring ζ-lactones[8,9] and the structural resemblance of these compounds to antimycin A (a nine-membered dilactone with a salicylamide moiety and a well-known inhibitor of the respiratory chain)[10] prompted us to assay the (+)-almuheptolide-A (**1**) and its diacetate (**1a**) against the mammalian mitochondrial respiratory chain activities. The current research on respiratory chain inhibitors has provided very interesting compounds with high potential for biomedical basic research,[11] antitumor therapy,[1−3,12,13] and agrochemical pest control.[14] We describe herein the selective inhibitory action against the mitochondrial complex I (NADH: ubiquinone oxidoreductase) of the (+)-almuheptolide-A (**1**) and its diacetate derivative (**1a**). Therefore, in addition to the new rapid and convenient method for semisynthesis, the mechanism of action of this type of

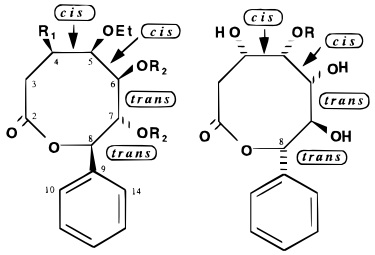

Figure 1. Natural eight-membered-ring lactones.

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Table 1. 1D (^{1}H and ^{13}C NMR) and 2D (^{1}H−^{1}H COSY 45, ^{1}H−^{13}C HMQC, and HMBC) Experiments of **1** ($CDCl_3$, 400 MHz)

	^{1}H NMR and COSY 45		^{13}C NMR, HMQC, and HMBC		
position	δ_H (J, Hz)	^{1}H−^{1}H COSY 45	δ_C (multiplicity)	HMQC (^1J)	HMBC (^2J and ^3J)
2			171.80 (C)		H-3a, H-3b, H-4
3a	2.84 dd (16.0, 6.0)	H-3b, H-4	36.38 (CH_2)	H-3a, H-3b	H-4, H-5
3b	2.75 dd (16.0, 6.4)	H-3a, H-4			
4	4.24 ddd (6.4, 6.0, 4.4)	H-3a, H-3b, H-5	75.39 (CH)	H-4	H-3a, H-3b
5	4.17 dd (4.9, 4.4)	H-4, H-6	80.62 (CH)	H-5	H-3a, H-3b, H-7
6	4.22 dd (4.9, 3.9)	H-5, H-7	79.58 (CH)	H-6	
7	4.08 dd (5.1, 3.9)	H-6, H-8	84.72 (CH)	H-7	H-8
8	4.62 d (5.1)	H-7	85.41 (CH)	H-8	H-10, H-14
9			140.00 (C)		H-7, H-8, H-11, H-13
10,14	7.45 dd (7.6, 1.9)	H-11, H-13, H-12	126.26 (2 CH)	H-10, H-14	H-8, H-12 (C-10→H-14 and C-14→H-10)
11,13	7.35 dt (7.6, 1.9)	H-10, H-14, H-12	128.36 (2 CH)	H-11, H-13	C-11→H-13 and C-13→H-11
12	7.23 dd (7.6, 1.9)	H-10, H-14, H-11, H-13	127.76 (CH)	H-12	H-10, H-14
15	4.14 q (7.0)	16-CH_3	60.78 (CH_2)	15-CH_2	16-CH_3
16	1.26 t (7.0)	15-CH_2	14.14 (CH_3)	16-CH_3	15-CH_2
17	3.68 dq (6.8)	18-CH_3	65.66 (CH_2)	17-CH_2	18-CH_3
18	1.20 t (6.8)	17-CH_2	15.55 (CH_3)	18-CH_3	17-CH_2

Table 2. 1D (^{1}H NMR) and 2D (^{1}H−^{1}H COSY 45) Experiments in $CDCl_3$ of **1a** (400 MHz), **2**, and **2a** (250 MHz)

	diacetylalmuheptolide-A (**1a**)		almuheptolide-B (**2**)		diacetylalmuheptolide-B (**2a**)	
proton	δ_H (J, Hz)	^{1}H−^{1}H COSY 45	δ_H (J, Hz)	^{1}H−^{1}H COSY 45	δ_H (J, Hz)	^{1}H−^{1}H COSY 45
2						
3a	2.53 m	H-3b, H-4	2.64 dd (16.7, 6.6)	H-3b, H-4a, H-4b	2.52 m	H-4a, H-4b
3b	2.51 m	H-3a, H-4	2.47 dd (16.7, 7.2)	H-3a, H-4a, H-4b		
4a	4.19 m	H-3a, H-3b, H-5	2.10 ddd, 2H (15.1, 7.2, 6.6)	H-3a, H-3b, H-5	2.15 m	H-3a, H-3b, H-4b
4b					1.93 m	H-3a, H-3b, H-4a, H-5
5	4.31 brd (3.9)	H-4, H-6	4.23 m	H-4a, H-4b, H-6	4.23 brd (4.0)	H-4a, H-4b, H-6
6	5.27 dd (3.9, 2.0)	H-5, H-7	4.20 m	H-5, H-7	5.24 dd (4.0, 1.1)	H-5, H-7
7	5.09 dd (3.5, 2.0)	H-6, H-8	4.07 m	H-6, H-8	5.03 dd (3.5, 1.1)	H-6, H-8
8	4.92 d (3.5)	H-7	4.63 m	H-7	4.81 d (3.5)	H-7
9						
10,14	7.48 dd (7.3, 1.7)	H-11,13, H-12	7.43 dd (7.4, 1.2)	H-11,13, H-12	7.44 dd (7.1, 1.8)	H-11,13, H-12
11,13	7.35 dt (7.3, 1.7)	H-10,14, H-12	7.36 dt (7.4, 1.2)	H-10,14, H-12	7.31 dt (7.1, 1.8)	H-10,14, H-12
12	7.30 dd (7.3, 1.7)	H-10,14, H-11,13	7.32 dd (7.4, 1.2)	H-10,14 H-11,13	7.34 dd (7.1, 1.8)	H-10,14, H-11,13
15	4.16 q (7.0)	16-CH_3	4.15 q (7.0)	16-CH_3	4.14 q (7.0)	16-CH_3
16	1.27 t (7.0)	15-CH_2	1.25 t (7.0)	15-CH_2	1.26 t (7.0)	15-CH_2
17	3.79 dq (6.8)	18-CH_3				
18	1.19 t (6.8)	17-CH_2				
6-$OCOCH_3$	2.12 s				2.13 s	
7-$OCOCH_3$	1.94 s				2.00s	

cytotoxic compounds is established for the first time, which opens a guideline for potential applications.

Results and Discussion

Isolation and Chemistry. (+)-Almuheptolides-A (**1**) and -B (**2**) are members of a small class of natural products which contain a saturated eight-membered-ring lactone (ζ-lactone). Both **1** and **2** have the same heptolide skeleton as the bioactive natural compounds **3** and **4** previously isolated from *G. giganteus*[8] and a lactone system similar to **5** and **6**, cytotoxic metabolites isolated from marine actinomycetes belonging to the *Streptomyces* genus[9] (Figure 1).

(+)-Almuheptolide-A (**1**) was first isolated from *G. arvensis* stem bark. Inspection of the 1D (^{1}H and ^{13}C NMR spectra), 2D homonuclear (^{1}H−^{1}H COSY 45), and 2D heteronuclear correlation spectroscopy (HMQC, proton-detected heteronuclear multiple-quantum coherence, and HMBC, proton-detected multiple-bond heteronuclear multiple-quantum coherence) (Table 1) revealed that **1** has a heptolide skeleton (8-phenyl-2-oxocanone) similar to that of (+)-gonioheptolides-A (**3**) and -B (**4**).[8] Compound **1** is an optically active, yellowish oil, $[\alpha]_D$ +11.7° (EtOH, *c* 1.8), with a molecular formula $C_{17}H_{24}O_6$, as was indicated by an EIMS peak at *m*/*z* 324 $[M]^+$. The presence of two hydroxyl groups was indicated by an IR band at 3422 cm^{-1} and corroborated by the preparation of a diacetate derivative (**1a**). Two ethoxyl groups in **1** were evidenced by the EIMS peaks *m*/*z* 278 $[M - HOCH_2CH_3]^+$ and 232 $[M - 2\ HOCH_2CH_3]^+$. The ^{1}H NMR spectrum resonances at δ 4.14 (q, 2H), 1.26 (t, 3H) and δ 3.68 (dq, 2H), 1.20 (t, 3H) and the ^{13}C NMR spectrum resonances at δ 60.78 (CH_2), 14.14 (CH_3) and δ 65.66 (CH_2), 15.55 (CH_3) confirmed the presence of these ethoxyl groups (Table 1). A saturated ζ-lactone moiety in **1** was suggested by both an IR carbonyl absorption band at 1716 cm^{-1} and a weak downfield quaternary carbon signal at δ 171.80 (C-2) in the ^{13}C NMR spectrum. The presence of a methylene in the ring of **1** was indicated in the ^{1}H NMR spectrum by two resonances at δ 2.84 (dd, H-3a) and 2.75 (dd, H-3b) with $J_{3a,3b} = 16.0$ Hz and by a signal at δ 36.38 (C-3) in the ^{13}C NMR spectrum.

^{1}H−^{1}H COSY 45 spectra of **1** and **1a** (Tables 1 and 2) revealed the correlation between the methylene at C-3 and a methine at C-4 and the successive correlations of five oxygenated methine protons (CH-4 to CH-8), as well as with their corresponding carbons in the HMQC spectrum (Table 1). Indeed, NOEs observed in **1a** between H-8 and the aromatic ring protons H-10 and H-14 (Figure 2) are consistent with a phenyl group placed at position 8, excluding the location of the

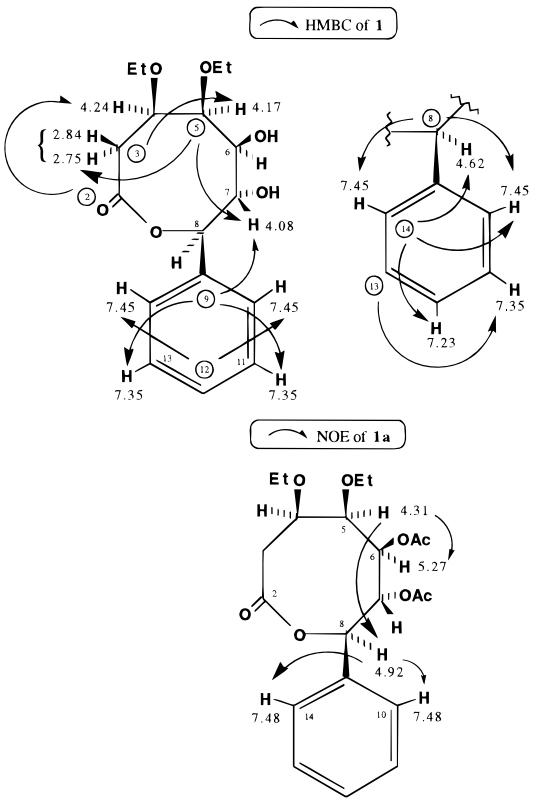


Figure 2. $^1H-^{13}C$ long-range correlations (^3J) established from HMBC of **1** and NOE of **1a**.

aromatic ring at other positions. Moreover, 2D long-range correlations HMBC observed between the aromatic ring protons, H-10 and H-14, and the carbon nucleus at C-8 and between H-7 and C-9 supported the placement of the aromatic moiety at C-8 of the saturated ζ-lactone system. The combined analysis of HMBC and HMQC data (Table 1 and Figure 2) indicated the linkages between the constitutive parts of **1** and the allowed resonance assignments. Figure 2 shows the cross-peaks observed due to the three-bond (^3J) heteronuclear coupling.

The relative stereochemistry of the chiral centers in **1** was deduced from both the ^1H-coupling constant data and the NOEDIFF experiments for **1a**. NOEs between H-5/H-6 and H-5/H-8 (Figure 2) were in agreement with a *cis*-relationship for these three protons. The relative configuration of **1** is therefore *cis*-4,5, *cis*-5,6, *trans*-6,7, and *trans*-7,8, and thus, it is similar to that of the known compounds **3** and **4**.[8] To observe this stereochemistry of **1**, a 3D representation of the fully energy-minimized structure was built using a molecular modeling program with the MM2 derived force field (Figure 3). In conclusion, the novel (+)-almuheptolide-A (**1**) is the 4,5-diethoxy-6,7-dihydroxy-8-phenyl-2-oxocanone (Figure 1), where its stereochemistry (4*R*,5*R*,6*R*,7*R*,8*R*) is postulated on the basis of the known absolute configuration of the starting semisynthetic furanopyrones altholactone (**7**) and etharvensin (**8**).[6]

Almuheptolide-B (**2**) was also isolated from *G. arvensis* as a yellowish oil, $[\alpha]_D$ +5.6° (EtOH, *c* 1.6). Its molecular weight was indicated by a peak in the EIMS at *m*/*z* 280 $[M]^+$ corresponding to the molecular formula $C_{15}H_{20}O_5$. The IR, UV, MS, and NMR spectra of **2** were very similar to those of **1**. The assignments and

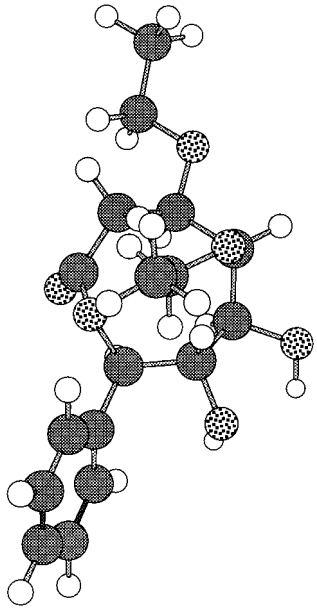


Figure 3. Perspective of the fully energy-minimized structure of (+)-almuheptolide-A (**1**) by MM2 calculations.

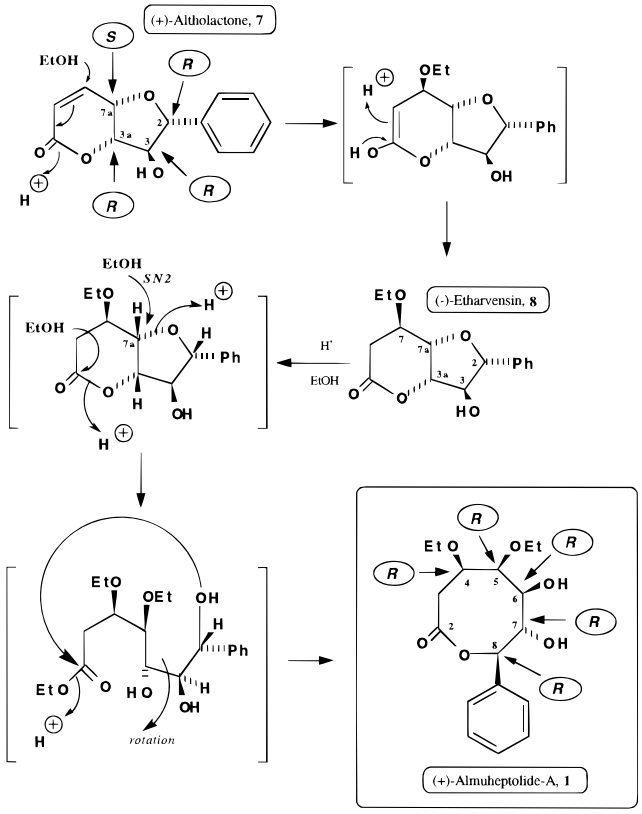
Table 3. ^{13}C NMR Data in $CDCl_3$ of **1a** (100 MHz), **2**, and **2a** (62.5 MHz)

	δ_C (DEPT)		
carbon	**1a**	**2**	**2a**
2	172.00 (C)	174.50 (C)	173.08 (C)
3	37.34 (CH_2)	30.80 (CH_2)	30.88 (CH_2)
4	74.95 (CH)	23.50 (CH_2)	23.93 (CH_2)
5	82.60 (CH)[a]	81.00 (CH)	79.78 (CH)
6	77.10 (CH)	79.00 (CH)	77.52 (CH)
7	82.61 (CH)[a]	85.00 (CH)	83.73 (CH)
8	84.85 (CH)	86.00 (CH)	84.83 (CH)
9	139.00 (C)	140.00 (C)	138.87 (C)
10,14	126.15 (2 CH)	126.00 (2 CH)	126.24 (2 CH)
11,13	128.20 (2 CH)	128.50 (2 CH)	128.28 (2 CH)
12	127.83 (CH)	127.80 (CH)	127.91 (CH)
15	60.70 (CH_2)	61.40 (CH_2)	60.51 (CH_2)
16	14.18 (CH_3)	14.00 (CH_3)	14.20 (CH_3)
17	67.14 (CH_2)		
18	15.58 (CH_3)		
6-$OCOCH_3$	169.00 (C)[b]		169.70 (C)[d]
	20.89 (CH_3)[c]		20.93 (CH_3)[e]
7-$OCOCH_3$	168.50 (C)[b]		169.69 (C)[d]
	20.90 (CH_3)[c]		20.67 (CH_3)[e]

[a–e] Exchangeable values.

comparisons of the ^1H NMR, ^{13}C NMR, and COSY spectra of **2** and its diacetate derivative (**2a**) with those of **1** and **1a** suggested that these compounds had the same plain heptolide skeleton and relative stereochemistry, but **2** had only one ethoxyl group at C-5 and therefore two adjacent methylene carbons placed at 3 and 4 (Tables 2 and 3). The $^1H-^1H$ COSY 45 spectrum of **2** revealed the correlations between the spin resonance at δ 2.10 (ddd, 2H, H-4a,4b) and the proton signals at δ 2.64 (dd, H-3a), 2.47 (dd, H-3b), and 4.23 (m, H-5), as well as the consecutive proton correlations between four methines (H-5 to H-8). Indeed, the ^1H NMR spectrum of **2a** showed that, upon acetylation, the chemical shifts of H-6 and H-7 moved downfield in agreement with the location of the two hydroxyl groups at these positions. In conclusion, (+)-almuheptolide-B (**2**) is the 6,7-dihydroxy-5-ethoxy-8-phenyl-2-oxocanone, with a similar stereochemistry to that of **1** (Figure 1).

Scheme 1. Preparation of **1** and **8** from **7**



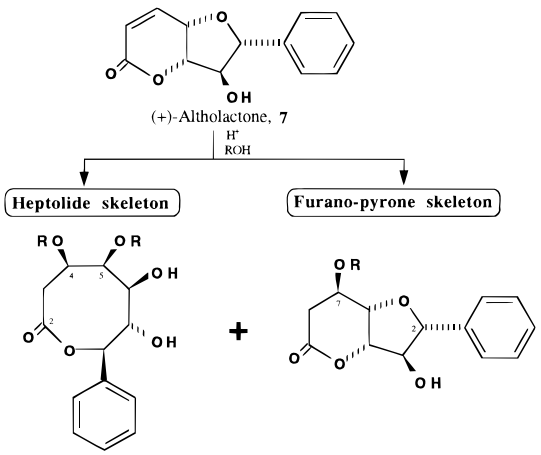
Semisynthesis. Because octalactin-A (**5**) has an attractive structure and a potent biological activity, several research groups have attempted its total synthesis.[15,16]

Therefore, in view of the original and unusual eight-membered-ring lactone of **1**, similar to that of **5**, we decided to carry out an enantiospecific semisynthesis of **1** starting from (+)-altholactone (**7**), an unsaturated furano-α-pyrone, using ethanol and concentrated H_2SO_4 (Scheme 1). Almuheptolide-A (**1**) was obtained as a single diastereoisomer (yield 40%) when the mixture was refluxed for 3 h, via the intermediate furanopyrone skeleton etharvensin (**8**). This reaction was carried out under a variety of conditions, such that only **8** was obtained (yield 90%) when **7** was refluxed for 1.5 h,[6] while **1** was prepared in a yield of 62% when **8** was refluxed for 5 h (Scheme 2).

In our semisynthetic plan, depicted in Scheme 1, we envisaged that a saturated ζ-lactone unit (2-oxocanone ring) could be easily obtained from the unsaturated δ-lactone **7**. Formation of intermediate etharvensin (**8**)[6] suggested that the mechanism operating in this transformation, and the key connection between **7** and **1** reported in this paper, involved alkoxylation of **7** by Michael-type addition of an EtOH molecule across the α,β-unsaturated δ-lactone double bond from the less electron dense position (β of the C=O group). Such addition took place under acidic conditions at the less hindered face to afford intermediate **8**. Subsequent nucleophilic attack with a second EtOH molecule by a S_N2 mechanism would lead to an inversion of configuartion of C-7a of **7** (C-5 in **1**), followed by a concerted opening of the α-pyrone and tetrahydrofuran rings. This allowed the rotation, rearrangement, and subsequent intramolecular eight-ring closure.

Scheme 2. General Procedure for Alkoxylation: Semisynthesis of 4,5-Dialkoxylated Heptolides and 7-Alkoxyfuranopyrones from **7**

Starting material	Conditions	Products
	Reagents (reaction time)	Styryl-lactone derivatives (yield)
7	EtOH, H_2SO_4 (3 h)	**1** (40 %) + **8** (28 %)
7	EtOH, H_2SO_4 (1.5 h)	**8** (90 %)
8	EtOH, H_2SO_4 (5 h)	**1** (62 %)
7	MeOH, H_2SO_4 (6 h)	**7a** (32 %) + **7b** (36 %)
7	*i*-PrOH, H_2SO_4 (9 h)	**7c** (16.5 %) + **7d** (40 %)



1: R= Et: (+)-almuheptolide-A
7a: R= Met
7c: R= *i*Pr
8: R= Et: (-)-etharvensin
7b: R= Met
7d: R= *i*Pr

To corroborate this mechanistic hypothesis, we have carried out the solvolysis using other alcohols as nucleophilic agents in the acid medium, MeOH/H_2SO_4 and *i*-PrOH/H_2SO_4. Thus, with compound **7**, under the same conditions, mixtures of **7a**,**b** or **7c**,**d** were obtained, respectively. Scheme 2 illustrates the general semisynthetic route used for the preparation of compounds **1**, **8**, and **7a**−**d**.

This protocol constitutes a new enantiospecific method for the preparation of this class of compounds. In addition, we have shown that the substituted eight-membered-ring lactone can be achieved using a simple alkoxylation procedure involving a concerted opening of the δ-lactone and tetrahydrofuran rings to yield a ζ-lactone by direct translactonization. On the other hand, the natural starting material for this semisynthesis, altholactone (**7**), is a compound isolated in large quantity from *G. arvensis*,[5] and therefore, it is a good material for the preparation of other related compounds. Although several total syntheses of macrocyclic lactones have been previously reported,[15−18] none of them are easily available. This is the first report of the semisynthesis of pentasubstituted eight-membered-ring lactones, constituting a novel, efficient, and direct method for the preparation of 8-phenyl-2-oxocanone compounds from a readily available substrate.

Bioactivities. Almuheptolide-A (**1**) and its diacetate (**1a**) were initially found to be inhibitors of the integrated mitochondrial electron-transport chain, measured as aerobic NADH oxidation, with similar potency,

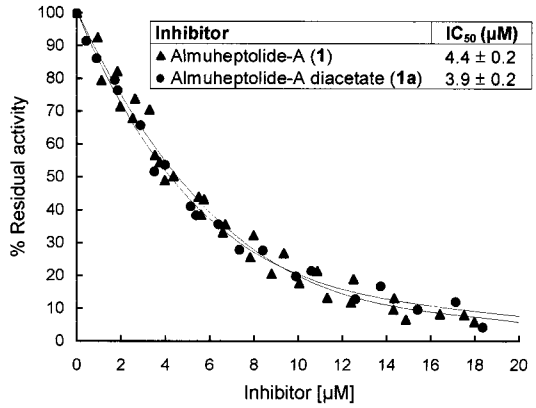


Figure 4. Titration of (+)-almuheptolide-A (**1**) and the diacetate derivative (**1a**) against NADH oxidase activity in bovine heart submitochondrial particles. Control activity was 0.95 μmol·min^{-1}·mg^{-1}.

as shown in Figure 4. Almuheptolide-A (**1**) showed an IC_{50} of 4.4 ± 0.2 μM, whereas its diacetate (**1a**) gave an IC_{50} of 3.9 ± 0.2 μM. Taking into account that most of the respiratory chain inhibitors with potential biomedical interest act within this order of magnitude on mammalian submitochondrial particles,[1,19−21] both compounds were effective inhibitors of the whole respiratory chain. However, the NADH oxidase activity involves all three energy-conserving enzymatic complexes of the respiratory chain, and thus the inhibitory action of the compounds might be affecting one or more of these electron-transfer chain components.

To define the target enzyme within the respiratory chain, the time course of the reduction of ferricytochrome *c* by both NADH and succinate was followed after addition of almuheptolide-A (**1**) as shown in Figure 5. The inhibitor did not affect the succinate:cytochrome *c* reductase activity (Figure 5A) at a concentration that fully inhibited the NADH oxidase activity, whereas the reduction of cytochrome *c* was abolished when antimycin A (specific inhibitor of the ubiquinol:cytochrome *c* reductase or complex III) was added. In turn, almuheptolide-A (**1**) inhibited the reduction of ferricytochrome *c* by NADH (Figure 5B), but when succinate was added, the cytochrome *c* was further reduced. Finally, the aerobic oxidation of ferrocytochrome *c* was not affected by the compound (Figure 5C), but it was fully inhibited by cyanide, a common inhibitor of the cytochrome *c* oxidase or complex IV. Similar results were obtained with the diacetate derivative (**1a**).

These results indicate that the heptolides act primarily by inhibiting the mitochondrial complex I. To confirm the target enzyme of these compounds, individual activity of respiratory chain enzymes was measured in the presence of almuheptolide-A (**1**). Neither succinate:ubiquinone reductase (complex II) nor ubiquinol:cytochrome *c* reductase (complex III) were affected by the compound, even at relatively high concentration (more than 97% activity with more than 100 μM). Indeed, almuheptolide-A showed a selective inhibition of the NADH:ubiquinone reductase (complex I) with an IC_{50} of 32 ± 3 μM. This loss of potency or weakened affinity with respect to the integrated NADH oxidase assay is commonly observed with many complex I inhibitors,[20,22,23] and it is probably due to a partial competition with the ubiquinone analogue used for the

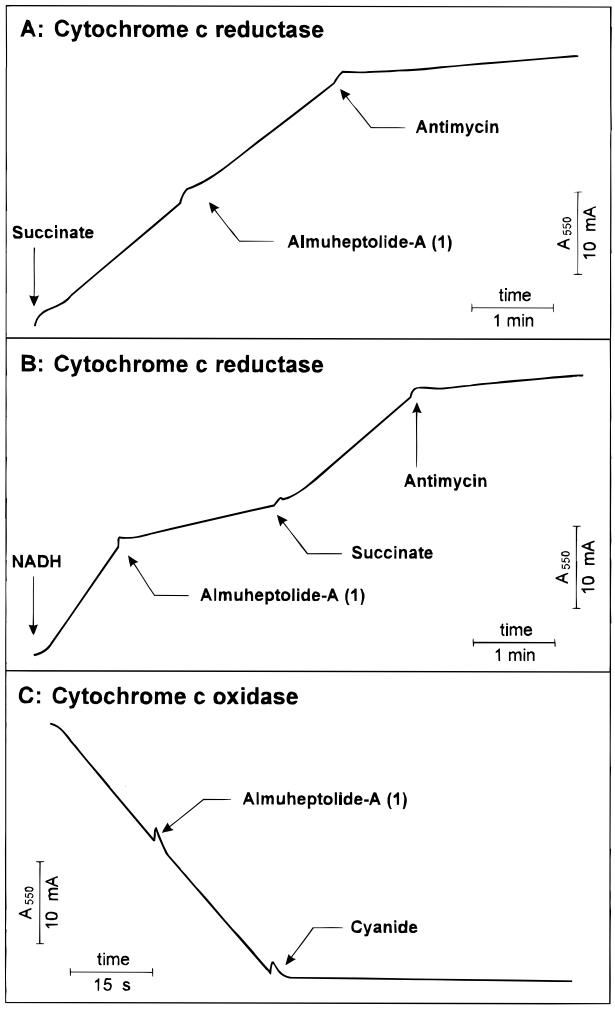


Figure 5. Effect of (+)-almuheptolide-A (**1**) on the time-course reduction and oxidation of cytochrome *c* in bovine heart submitochondrial particles. Succinate- and NADH-induced cytochrome *c* reductase activities were 0.12 and 0.15 μmol·min^{-1}·mg^{-1}, respectively. Cytochrome *c* oxidase activity was 0.42 μmol·min^{-1}·mg^{-1}. (+)-Almuheptolide-A was added at 15 μM, antimicyn A at 5 μM, and cyanide at 2 mM.

activity assay which is added at high concentration with respect to the natural content of the endogenous ubiquinone.[24]

The most recent mechanistic hypotheses on mitochondrial complex I function have established three ubiquinone binding sites within the transmembrane segment of the complex.[25−27] Although not completely proved, inhibitors of the enzyme could act by binding independently to one of these sites. Inhibition kinetic pattern is useful to classify the complex I inhibitors.[12,21,28] Figure 6A shows that almuheptolide-A behaved as a noncompetitive inhibitor with regard to the ubiquinone analogue substrate, and thus, it mimicked the rotenone behavior. Although most of the noncompetitive inhibitors of the complex I act by overlapping the rotenone binding site, some inhibitors could act at a different site with the same inhibitor kinetics.[25] To determine the mutual exclusivity between both inhibitors, we have done the titration of one of them in the presence of the other to reveal the kinetic displacement.[12,28] Figure 6B shows Dixon plots obtained by titration of almuheptolide-A in the presence of rotenone. As observed in the graphic, rotenone

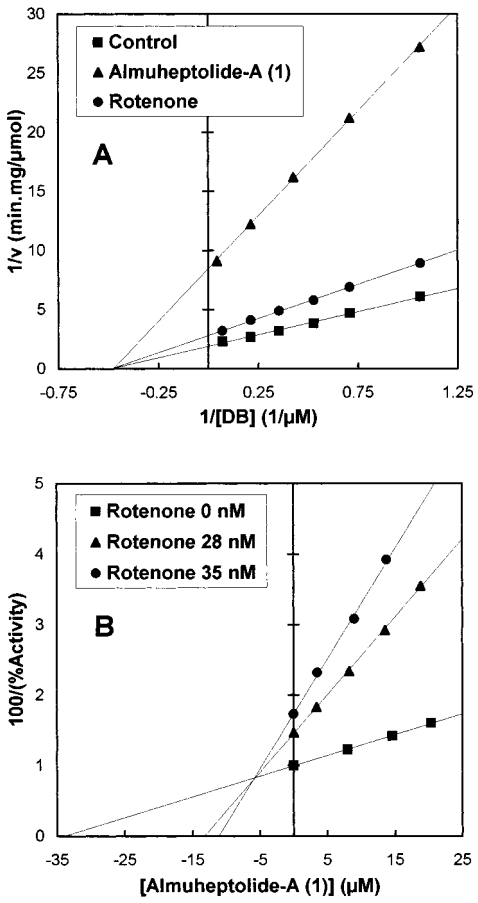


Figure 6. Inhibition kinetics and kinetic displacement of (+)-almuheptolide-A (**1**) and rotenone. (A) Reciprocal plots of the NADH:ubiquinone oxidoreductase activity in the presence of (+)-almuheptolide-A (45 μM) and rotenone (18.5 nM). (B) Dixon plots of (+)-almuheptolide-A titrated at different fixed concentrations of rotenone (only the lineal part of the plot is shown).

modified the K_i of the new inhibitor (*x*-axis intercept), and thus, both compounds compete by the same binding site. Therefore, we can assess that almuheptolide-A selectively binds the energy-coupling site of the complex I, namely, the "rotenone site",[22] site B,[27] or site P_I.[30]

The initial hypothesis of the present work was that the newly synthesized compound almuheptolide-A might be a complex III inhibitor due to its resemblance with the antimycin A molecule. Surprisingly, it did not inhibit this segment of the respiratory chain. Close scrutiny of the chemical structure of almuheptolide-A shows that it lacks the functional groups that bear the antimycin A and its derivatives that have been recently characterized as involved in the binding to the Qn pocket of the complex III.[10] Therefore, this is additional evidence of the relevance of the colateral chemical groups to drive selective inhibition of respiratory complexes.

Instead, we have found that almuheptolide-A is a very specific inhibitor of the site B of the complex I. According to previous studies, this ubiquinone binding site of the complex should be wide enough to accommodate a great variety of chemical structures acting as inhibitors.[19,22] Note that neither eight-membered-ring lactones nor related structures have ever been described as complex I inhibitors up to date, and thus, inhibitors of complex I are expanded with a new interesting type of molecular structures as the heptolides described herein.

Conclusion

In this paper we have described for the first time a fast, easy, and stereospecific semisynthesis method for a high-yield obtention of pentasubstituted eight-membered-ring lactones (**1**,**7a**,**c**). One of them, a new 8-phenyl-2-oxocanone (almuheptolide-A, **1**) isolated from *G. arvensis*, was found to be a noncompetitive inhibitor of the mammalian respiratory chain complex I. Inhibition of the transmembrane segment (ubiquinone region) of the complex I has been correlated with oxidative damage of the cell. Electrons trapped in the peripheral part of the complex are shunted to radical oxygen species formation,[29−32] and thus, cellular damage is produced at two levels: ATP depletion by inhibition of the oxidative phosphorylation and free-radical attack to mitochondrial components. The high metabolic rate of tumoral cells might explain their sensitivity to mitochondrial electron-transfer chain inhibitors. Although some complex I inhibitors such as rotenoids, piericidins, and Annonaceous acetogenins[3,12,28] are highly potent (with IC_{50} 1 order of magnitude below), the antitumor trials have not been successful due to the extreme toxicity of these compounds. Instead, the current tendency for searching for new antitumoral agents targeting complex I is moving toward less potent inhibitors[33] which act at the micromolar range in in vitro assays, an intermediate potency matched by almuheptolide-A.

This new 8-phenyl-2-oxocanone, isolated first from the natural source *G. arvensis*, is easily obtained by the fast, stereospecific, and efficient method for the semisynthesis of pentasubstituted eight-membered-ring lactones described herein. Therefore, almuheptolide-A is a very interesting molecule that might be exploited in the future for antitumor chemotherapy, biomedical research, and insect control.

Experimental Section

General Instrumentation. Optical rotations were determined on a Perkin-Elmer 241 polarimeter. IR spectra were run in film using NaCl plates on a Perkin-Elmer 843 spectrometer. UV spectra were obtained on a Perkin-Elmer lambda computer 15 UV/vis spectrophotometer. Mass spectra were recorded with a VG Auto Spec Fisons spectrometer, and the liquid secondary ion mass spectrum (LSIMS) was obtained by fast ion bombardment. ^{1}H NMR (250 and 400 MHz), ^{13}C NMR, and DEPT (62.5 and 100 MHz) spectra were recorded on Bruker AC-250, Varian Unity-300, and Varian Unity-400 instruments. Silica gel TLC plates were observed under UV light (254 nm) and after spraying with anisaldehyde−sulfuric acid. Preparative TLC was performed using silica gel (0.5 mm plates).

Plant Material. *G. arvensis* Scheff. (Annonaceae) was collected in the National Park of Varirata, located in the Central Province of Papua New Guinea. A voucher specimen was deposited in the herbarium of the University of Papua New Guinea.

Extraction and Isolation. Dried and powdered stem bark of *G. arvensis* (368 g) was macerated with MeOH at room temperature. The concentrated MeOH extract was partitioned between hexane (extract A) and 50% aqueous MeOH. The aqueous extract was fractionated successively into CH_2Cl_2 and EtOAc (extracts B and C, respectively). Extract B (7 g) was applied to a silica gel 60H column (Merck 7736) and eluted

with CH_2Cl_2−EtOAc (60:40). Repeated column chromatography of some fractions afforded **1** (5 mg) and **2** (8 mg).

(+)-Almuheptolide-A (1): $C_{17}H_{24}O_6$; $[\alpha]_D$ +11.7° (EtOH, *c* 1.8); UV λ_{max} EtOH nm (log ϵ) 229 (2.82) and 257 (2.35); IR ν_{max} (film) cm^{-1} 3422, 3059, 3026, 2974, 2926, 1716, 1602, 1492, 1449, 1370, 1282, 1182, 1085, 1042, 857, 757, 699; EIMS *m/z* (%) 324 $[M]^+$, 306 $[M - H_2O]^+$ (8), 288 $[M - 2H_2O]^+$ (3), 279 (22), 278 $[M - HOCH_2CH_3]^+$ (64), 262 (30), 260 $[M - HOCH_2CH_3 - H_2O]^+$ (20), 232 $[M - 2HOCH_2CH_3]^+$ (30), 173 (34), 162 (88), 145 (85), 133 (100), 117 (85), 103 (89), 97 $[C_5H_5O_2]^+$ (72), 91 (92), 77 (66); ^1H and ^{13}C NMR ($CDCl_3$, 400 and 100 MHz) Table 1.

(+)-6,7-Diacetylalmuheptolide-A (1a). **1** (3.9 mg) was treated with pyridine (1 mL) and Ac_2O (1.5 mL). The reaction mixture was stirred for 12 h at room temperature. After the usual workup the diacetate **1a** (4.0 mg, 82%) was obtained: $C_{21}H_{28}O_8$; $[\alpha]_D$ +22.31° (EtOH, *c* 1.3); UV λ_{max} EtOH nm (log ϵ) 218 (3.2) and 260 (2.4); IR ν_{max} (film) cm^{-1} 2975, 2925, 1742, 1448, 1368, 1232, 1093, 1038, 940, 758, 698; LSIMS *m/z* (%) $[MH]^+$ 409 (100); CIMS *m/z* (%) $[MH]^+$ 409 (50), 408 $[M]^+$ (20), 349 $[MH - HOCOCH_3]^+$ (36), 289 $[MH - 2HOCOCH_3]^+$ (12), 242 $[M - 2HOCOCH_3 - HOCH_2CH_3]^+$ (65), 107 (69); ^1H and ^{13}C NMR ($CDCl_3$, 400 and 100 MHz) Tables 2 and 3.

(+)-Almuheptolide-B (2): $C_{15}H_{20}O_5$; $[\alpha]_D$ +5.6° (EtOH, *c* 1.6); UV λ_{max} EtOH nm (log ϵ) 227 (2.65) and 260 (2.0); IR ν_{max} (film) cm^{-1} 3422, 2921, 1708, 1451, 1370, 1262, 1035, 758, 699; EIMS *m/z* (%) 280 $[M]^+$ (1), 279 $[M - 1]^+$ (2), 262 $[M - H_2O]^+$ (4), 244 $[M - 2H_2O]^+$ (19), 234 $[M - HOCH_2CH_3]^+$ (16), 217 $[M - OCH_2CH_3 - H_2O]^+$ (11), 198 $[M - 2H_2O - HOCH_2CH_3]^+$ (2), 167 $[M - 2H_2O - Ph]^+$ (5), 107 (73), 97 (16), 91 (100), 77 (55); ^1H and ^{13}C NMR ($CDCl_3$, 250 and 62.5 MHz) Tables 2 and 3.

(+)-6,7-Diacetylalmuheptolide-B (2a). **2** (7.9 mg) was treated with pyridine (2 mL) and Ac_2O (2.5 mL). The reaction mixture was stirred for 12 h at room temperature. After the usual workup the diacetate **2a** (7.1 mg, 69%) was obtained: $C_{19}H_{24}O_7$; $[\alpha]_D$ +15.0° (EtOH, *c* 2.0); UV λ_{max} EtOH nm (log ϵ) 226 (3.0) and 256 (3.0); IR ν_{max} (film) cm^{-1} 2918, 2341, 2327, 1740, 1720, 1541, 1449, 1369, 1224, 1117, 1037, 698; LSIMS *m/z* (%) 365 $[MH]^+$ (100); EIMS *m/z* (%) 365 $[MH]^+$ (2), 319 $[MH - HOCH_2CH_3]^+$ (54), 260 $[M - HOCH_2CH_3 - OCOCH_3]^+$ (50), 245 $[MH - 2HOCOCH_3]^+$ (98), 244 (85), 243 (76), 231 (30), 217 (56), 215 (72), 199 (64), 128 (60), 125 (54), 115 (51), 105 (83), 99 (47), 91 (70), 77 (47); ^1H and ^{13}C NMR ($CDCl_3$, 250 and 62.5 MHz) Tables 2 and 3.

General Procedure for Heptolide Skeleton Preparation: 1. Semisynthesis of (+)-Almuheptolide-A (1). To an ethanol solution (10 mL) of altholactone (**7**; 60 mg, 0.258 mmol) was added dropwise at 0 °C concentrated H_2SO_4 (96%, 1.5 mL). After the mixture was stirred and refluxed for 3 h, water was added and the reaction mixture was extracted into CH_2Cl_2. The organic solution after usual workup was purified by silica gel 60H column chromatography (eluting with $CHCl_3$−EtOAc (70:30)), affording **1** (34 mg, 0.105 mmol, 40%) and **8** as synthetic intermediate (20 mg, 0.072 mmol, 28%). Compound **1** was obtained in a yield of 62% when **8** was refluxed for 5 h and was found to be identical in all respects to the previously isolated (+)-almuheptolide-A ($[\alpha]_D$, NMR, MS data).

Compound 1 ((+)-Almuheptolide-A): see extraction and isolation.

Compound 8 ((−)-Etharvensin): $C_{15}H_{18}O_5$; $[\alpha]_D$ −6.5° (EtOH, *c* 2.0); IR (film) ν_{max} 3416 (OH), 1740 (C=O); HREIMS *m/z* (%) $[M]^+$ 278.1148 (calcd 278.1154 for $C_{15}H_{18}O_5$) (8), $[M - HOEt]^+$ 232.0740 (calcd 232.0735 for $C_{13}H_{12}O_4$) (12), 133.0653 (calcd 133.0653 for C_9H_9O) (100); ^1H NMR ($CDCl_3$, 400 MHz), ^{13}C NMR ($CDCl_3$, 100 MHz), COSY 45, HMQC, and other physical and chemical data, see ref 6.

2. Semisynthesis of Compound 7a. A similar procedure ($MeOH/H_2SO_4$, 6 h) and workup afforded **7a** (24 mg, 0.080 mmol, 32%) and **7b** as synthetic intermediate (24 mg, 0.09 mmol, 36%).

Compound 7a: $C_{15}H_{20}O_6$; $[\alpha]_D$ +15° (EtOH, *c* 0.8); UV λ_{max} EtOH nm (log ϵ) 217 (3.2) and 257 (2.02); IR ν_{max} (film) cm^{-1} 3422, 2932, 2103, 1735, 1635, 1493, 1452, 1381, 1239, 1198, 1156, 1096, 1053, 919, 760, 699; LSIMS *m/z* (%) $[MH]^+$ 297 (100), 265 $[MH - HOCH_3]^+$ (23); EIMS *m/z* (%) 264 $[M - HOCH_3]^+$ (2), 230 $[MH - OCH_3 - 2H_2O]^+$; ^1H NMR ($CDCl_3$, 250 MHz) δ 7.45 (d, H-10,14), 7.35 (t, H-11,13), 7.29 (d, H-12), 4.63 (d, H-8), 4.23 (dd, H-4), 4.20 (dd, H-6), 4.14 (brd, H-5), 4.09 (dd, H-7), 3.71 (s, 3H, 15-CH_3), 3.49 (s, 3H, 16-CH_3), 2.77 (dd, H-3b), 2.86 (dd, H-3a); $J_{3a,3b}$ = 16.0 Hz, $J_{3a/3b,4}$ = 6.3 Hz, $J_{4,5}$ = 4.5 Hz, $J_{5,6}$ = 5.0 Hz, $J_{6,7}$ = 4.0 Hz, $J_{7,8}$ = 5.1 Hz, $J_{10,11}$ = $J_{11,12}$ = $J_{12,13}$ = $J_{13,14}$ = 6.9 Hz, $J_{10,12}$ = $J_{11,13}$ = $J_{12,14}$ = 1.5 Hz; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.05 (C-2), 139.76 (C-9), 128.39 (C-11,13), 127.80 (C-12), 126.20 (C-10,14), 85.39 (C-8), 84.67 (C-7), 80.59 (C-5), 79.49 (C-6), 76.00 (C-4), 57.75 (15-CH_3), 51.88 (16-CH_3), 35.50 (3-CH_2).

Compound 7b: $C_{14}H_{16}O_5$; $[\alpha]_D$ −69.2° (EtOH, *c* 1.3); UV λ_{max} EtOH nm (log ϵ) 217 (2.7) and 257 (1.9); IR ν_{max} (film) cm^{-1} 3429, 2928, 2108, 1724, 1639, 1492, 1437, 1368, 1282, 1200, 1171, 1084, 1044, 759, 700; EIMS *m/z* (%) $[M]^+$ 264 (7), 232 $[M - HOCH_3]^+$ (3), 215 $[M - OCH_3 - H_2O]^+$ (3), 177 (3), 162 (20), 144 (34), 133 $[C_9H_9O]^+$ (100), 107 (29), 105 (40), 97 (20), 91 (89), 77 (42); ^1H NMR ($CDCl_3$, 250 MHz) δ 7.38−7.32 (m, H-9 to H-13), 4.88 (dd, H-3a), 4.70 (d, H-2), 4.39 (brt, H-7a), 4.28 (brt, H-3), 3.93 (dd, H-7), 3.46 (s, 3H, CH_3), 2.86 (dd, H-6b), 2.73 (dd, H-6a); $J_{2,3}$ = 6.0 Hz, $J_{3,3a}$ = 2.1 Hz, $J_{3a,7a}$ = 4.5 Hz, $J_{7a,7}$ = 4.0 Hz, $J_{7,6b}$ = 3.8 Hz, $J_{7,6a}$ = 5.7 Hz, $J_{6a,6b}$ = 16.7 Hz; ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 169.13 (C-5), 138.07 (C-8), 128.67 (2 CH), 128.36 (CH), 126.04 (2 CH), 86.91 (C-3a), 86.05 (C-2), 83.56 (C-3), 75.25 (C-7a), 74.53 (C-7), 57.32 (14-CH_3), 32.56 (6-CH_2).

3. Semisynthesis of Compound 7c. A similar procedure (*i*-PrOH/H_2SO_4, 9 h) and workup afforded **7c** (20 mg, 0.057 mmol, 16.5%) and **7d** as synthetic intermediate (40 mg, 0.137 mmol, 40.0%).

Compound 7c: $C_{19}H_{28}O_6$; $[\alpha]_D$ +17.5° (EtOH, *c* 4.0); UV λ_{max} EtOH nm (log ϵ) 225 (2.7) and 257 (2.5); IR ν_{max} (film) cm^{-1} 3423, 3405, 2975, 2927, 2084, 1708, 1640, 1492, 1450, 1372, 1243, 1196, 1104, 1042, 824, 757, 699; LSIMS *m/z* (%) $[MH]^+$ 353 (100), 233 $[MH - 2HOCH(CH_3)_2]^+$ (15); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.46 (d, H-10,14), 7.34 (t, H-11,13), 7.28 (d, H-12), 4.63 (d, H-8), 4.36 (dd, H-4), 4.26 (dd, H-6), 4.21 (brd, H-5), 4.09 (dd, H-7), 3.87 (sept, 15-CH), 5.02 (sept, 18-CH), 2.88 (dd, H-3a), 2.71 (dd, H-3b), 1.29−1.23 (ddd, 19,20-CH_3), 1.22−1.19 (ddd, 16,17-CH_3); $J_{3a,3b}$ = 16.3 Hz, $J_{3a/3b,4}$ = 6.1 Hz, $J_{4,5}$ = 10.1 Hz, $J_{5,6}$ = 4.9 Hz, $J_{6,7}$ = 3.3 Hz, $J_{7,8}$ = 5.3 Hz, $J_{10,11}$ = $J_{11,12}$ = $J_{12,13}$ = $J_{13,14}$ = 7.1 Hz, $J_{10,12}$ = $J_{11,13}$ = $J_{12,14}$ = 1.7 Hz; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.81 (C-2), 140.00 (C-9), 128.38 (C-11,13), 127.90 (C-12), 126.20 (C-10,14), 84.87 (C-8), 84.80 (C-7), 80.43 (C-5), 79.75 (C-6), 77.80 (C-4), 68.21 (18-CH), 68.20 (15-CH), 23.23 (16,17-CH_3), 21.78 (19,20-CH_3), 37.17 (3-CH_2).

Compound 7d: $C_{16}H_{20}O_5$; $[\alpha]_D$ −3.1° (EtOH, *c* 1.9); UV λ_{max} EtOH nm (log ϵ) 219 (2.9) and 257 (2.3); IR ν_{max} (film) cm^{-1} 3417, 3031, 2970, 2923, 2124, 1737, 1635, 1493, 1451, 1380, 1236, 1174, 1142, 1079, 1054, 973, 924, 855, 759, 700; EIMS *m/z* (%) $[M]^+$ 292 (3), 232 $[M - HOCH(CH_3)_2]^+$ (3), 215 $[M - OCH(CH_3)_2 - H_2O]^+$ (1), 162 (53), 133 $[C_9H_9O]^+$ (100), 120 (16), 115 (9.5), 107 (15), 105 (15), 91 (45), 77 (12); ^1H NMR ($CDCl_3$, 250 MHz) δ 7.33−7.30 (m, H-9 to 13), 4.87 (dd, H-3a), 4.68 (d, H-2), 4.30 (brt, H-7a), 4.25 (td, H-3), 4.19 (dd, H-7), 3.80 (sept, 14-CH), 2.81 (dd, H-6b), 2.63 (dd, H-6a), 1.18 (d, 15-CH_3), 1.16 (d, 16-CH_3); $J_{2,3}$ = 5.7 Hz, $J_{3,3a}$ = 1.6 Hz, $J_{3a,7a}$ = 4.4 Hz, $J_{7a,7}$ = 4.0 Hz, $J_{7,6b}$ = 3.7 Hz, $J_{7,6a}$ = 5.2 Hz, $J_{6a,6b}$ = 16.6 Hz; ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ 169.28 (C-5), 138.32 (C-8), 128.55 (2 CH), 128.15 (2 CH), 125.95 (CH), 87.29 (C-3a), 86.10 (C-2), 83.33 (C-3), 75.87 (C-7a), 70.99 (C-7), 70.09 (14-CH), 33.63 (6-CH_2), 22.41 (15-CH_3), 22.41 (16-CH_3).

Biochemical Methods. Antimycin A, decylubiquinone, and other biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions (15 mM in absolute ethanol) of the compounds were prepared and kept in the dark at −20 °C. Appropriate dilutions (3−6 mM) were made before the experiments. Inverted submitochondrial particles (SMP) from beef heart were obtained by extensive ultrasonic disruption of frozen−thawed mitochondria to produce open membrane fragments where permeability barriers

to substrates were lost.[24] Beef heart SMP were diluted to 0.5 mg/mL in 250 mM sucrose, 10 mM Tris-HCl buffer, pH 7.4, and treated with 0.3 mM NADH to activate complex I before starting experiments.[12] The enzymatic activities were assayed at 22 °C in 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA with the SMP diluted to 6 μg/mL in the cuvette.

NADH oxidase was measured as the aerobic oxidation of 75 μM NADH in the absence of the quinone substrate and other inhibitors of the respiratory chain. NADH:ubiquinone oxidoreductase was measured with 75 μM NADH and 30 μM decylubiquinone (DB) as soluble short-chain ubiquinone analogue in the presence of 2 μM antimycin and 2 mM potassium cyanide to block any reaction downstream of the complex I.[34] Cytochrome *c* reduction by both NADH and succinate and the cytochrome *c* oxidase activity were measured as previously described.[4,35] Ubiquinol:cytochrome *c* reductase (complex III) and succinate:ubiquinone reductase (complex II) activities were assayed in the conditions previously repored.[36]

Inhibitor titrations were made as previously described in detail.[3,12] Data from four titrations in the same conditions were pulled and fitted for graphics. The inhibitory concentration 50 (IC_{50}) was taken as the final compound concentration in the assay cuvette that yielded 50% of the initial activity. Data from individual titrations were used to assess the means and standard deviations. Inhibition kinetics were evaluated against the NADH:ubiquinone oxidoreductase activity by varying the concentration of the ubiquinone analogue at a fixed concentration of the compound.[12,28] Kinetic displacement between inhibitors was evaluated by the Dixon plot using decylbenzoquinone as the complex I substrate.[28]

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