Synthesis and Inhibitory Action of Novel Acetogenin Mimics with Bovine Heart Mitochondrial Complex I†

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Received November 24, 2003; Revised Manuscript Received January 23, 2004

ABSTRACT: Studies on the inhibition mechanism of acetogenins, the most potent inhibitors of complex I, are useful to elucidate the structural and functional features of the terminal electron-transfer step of this enzyme. We synthesized acetogenin mimics that possess two alkyl tails without a γ-lactone ring, named Δlac-acetogenin, and examined their inhibitory action on bovine heart mitochondrial complex I. Unexpectedly, the Δlac-acetogenin carrying two *n*-undecanyl groups (compound **3**) elicited very potent inhibition comparable to that of bullatacin. The inhibitory potency of compound **3** markedly decreased with shortening the length of either or both alkyl tails, indicating that symmetric as well as hydrophobic properties of the inhibitor are important for the inhibition. Both acetylation and deoxygenation of either or both of two OH groups adjacent to the tetrahydrofuran (THF) rings resulted in a significant decrease in inhibitory potency. These structural dependencies of the inhibitory action of Δlac-acetogenins are in marked contrast to those of ordinary acetogenins. Double-inhibitor titration of steady-state complex I activity showed that inhibition of compound **3** and bullatacin are not additive, though the inhibition site of both inhibitors is downstream of iron−sulfur cluster N2. Our results indicate that the mode of inhibitory action of Δlac-acetogenins differs from that of ordinary acetogenins. Therefore, Δlac-acetogenins can be regarded as a novel type of inhibitor acting on the terminal electron-transfer step of complex I.

More than 350 annonaceous acetogenins have been isolated from the plant family *Uvaria accuminata* (Annonaceae) in the past two decades (*1*−*3*). Acetogenins have very potent and diverse biological effects such as antitumor, antimalarial, pesticidal, and antifeedant activities (*3*). The inhibitory effects of acetogenins on mitochondrial NADH-ubiquinone oxidoreductase (complex I) are of particular importance because their diverse biological activities are thought to be attributable to this effect (*3*). Actually some acetogenins, such as bullatacin (rolliniastatin-2, Figure 1) and rolliniastatin-1, are the most potent inhibitors of this enzyme identified to date (*4*−*7*). Although the acetogenins are thought to act at the terminal electron-transfer step of complex I (*6*, *7*), there is still no hard experimental evidence to verify whether the inhibitors in fact bind to the ubiquinone reduction site. Additionally, there are few structural similarities between acetogenins and ordinary complex I inhibitors such as piericidin A and rotenone. Thus, considering the unusual structural characteristics, as well as the very strong inhibitory effect, of acetogenins, a detailed analysis of the inhibitory actions of these inhibitors is important to elucidate the structural and functional features of the terminal electron-transfer step of complex I. As the first step toward this end, identification of the crucial structural factors of acetogenins responsible for potent inhibition would be useful.

In previous structure−activity studies using a series of natural and synthetic acetogenins (*8*−*12*), we have shown that (i) the presence of polar functional groups like an OH group in the spacer, the number of THF rings, and the stereochemistry around the THF ring(s) with flanking OH groups are not essential structural factors for potent activity, (ii) the natural γ-lactone ring itself is not crucial for activity and can be substituted with a ubiquinone ring, and (iii) acetogenin acts as a strong inhibitor only when the γ-lactone and the THF ring moieties are directly linked by an alkyl spacer, the optimal length of which is about 13 carbon atoms. Thus, except for the important role of the alkyl spacer, crucial structural factors including the active conformation of acetogenins remain to be elucidated. Nevertheless, on the basis of these results, we proposed that the γ-lactone and THF ring moieties act in a cooperative manner on the enzyme with the support of some specific conformation of the spacer (*8*−*10*).

The presence of an α,β-unsaturated γ-lactone ring is one of the common structural features of a large number of natural acetogenins. Recent synthetic studies from different laboratories demonstrated that the ubiquinone ring, a physiological substrate of complex I, is completely substitutable for the γ-lactone ring of natural acetogenins (*12*, *13*). Although this finding does not necessarily mean that acetogenins occupy the ubiquinone reduction site of the enzyme, as discussed in ref *12*, it prompted us to thoroughly

† This work was supported in part by Grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science (Grant 15380083 to H.M.).

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[1] Abbreviations: Complex I, mitochondrial proton-pumping NADH-ubiquinone oxidoreductase; EPR, electron paramagnetic resonance; Q_1, ubiquinone-1; SMP, submitochondrial particles; THF, tetrahydrofuran.

10.1021/bi030242m CCC: $27.50
Published on Web 03/06/2004

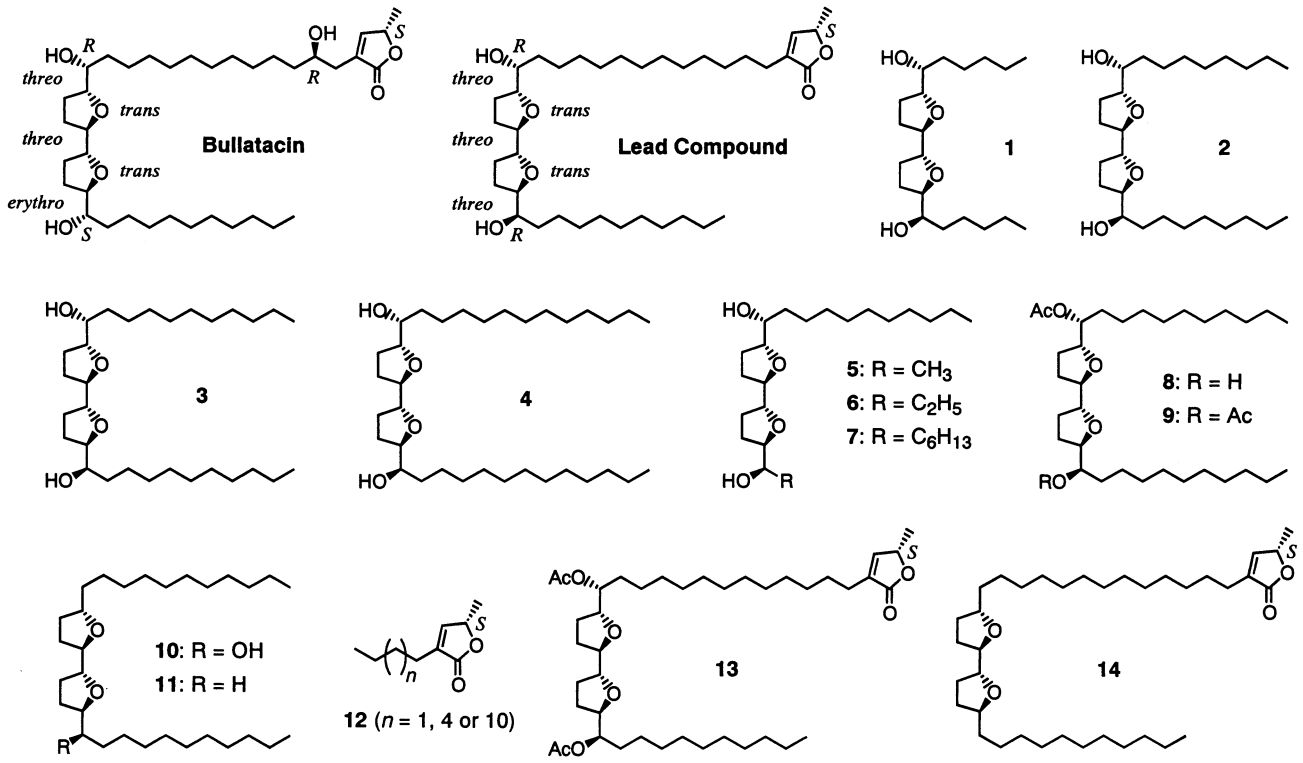


FIGURE 1: Structures of acetogenins studied in these experiments.

reconsider the role of the terminal γ-lactone ring moiety. In this study, we synthesized acetogenin mimics that possess no terminal heterocyclic system (named Δlac-acetogenin) and investigated their inhibitory effect on bovine heart mitochondrial complex I. Unexpectedly, some Δlac-acetogenins maintained very potent activities. The inhibitory action of this novel class of inhibitors is discussed in comparison with common acetogenins.

EXPERIMENTAL PROCEDURES

Materials. Bullatacin was a generous gift from Prof. J. L. McLaughlin (Purdue University, West Lafayette, IN). Lead compound, compounds **1**, **12**, **13**, and **14**, were the same samples as those used previously (*8*, *14*). Other chemicals were commercial products of analytical grade.

*Synthetic Procedures: Compounds **2**, **3**, and **4**.* The synthetic procedures are outlined in Scheme 1. To a mixture of **15** (0.80 g, 1.63 mmol), which was synthesized as described previously (*11*), DMAP (0.12 g, 0.98 mmol), and Et_3N (2.3 mL) in CH_2Cl_2 (3 mL) was added TsCl (1.4 g, 7.34 mmol) at room temperature (rt). The reaction mixture was stirred at 30 °C for 24 h and then quenched and washed with brine, dried, and concentrated. The residue was purified by silica gel column chromatography (hexanes−EtOAc, 7:3) to give **16** (1.19 g, 91%). To a solution of **16** (0.5 g, 0.63 mmol) in dry THF (15 mL) was added TBAF (1.0 M in THF, 1.5 mL, 2.63 mmol) at rt, and the mixture was stirred at 40 °C for 2 h. The reaction mixture was quenched with saturated aqueous NH_4Cl, washed with brine, dried, and concentrated. The crude product was purified by silica gel column chromatography (hexanes−EtOAc, 1:1) to afford diepoxide **17** (0.11 g, 77%). The ^1H and ^{13}C NMR data and an optical rotation of **17** were completely identical to those reported (*11*, *15*).

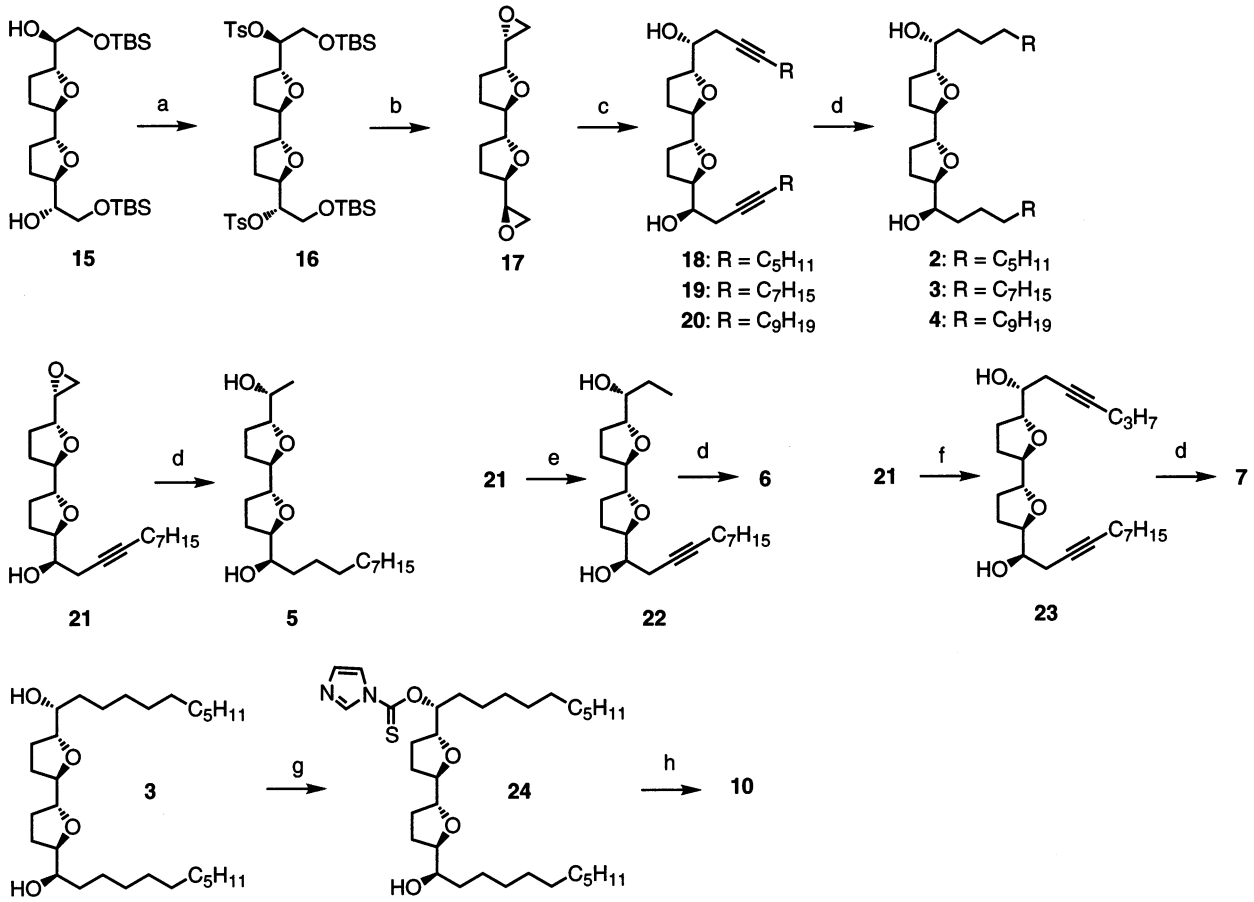
To a solution of 1-heptyne (85 mg, 0.88 mmol) in dry THF (1.5 mL) at −78 °C was added a solution of *n*-BuLi (1.6 M in hexane, 0.6 mL, 0.85 mmol). After 30 min, BF_3·Et_2O (0.11 mL, 0.85 mmol) was added, and the mixture was stirred for 15 min (*16*). To the mixture was added a solution of **17** (40 mg, 0.18 mmol) in THF (1.8 mL), and the mixture was stirred at −78 °C for 30 min. The reaction mixture was worked up with saturated aqueous NH_4Cl, washed with brine, dried, and concentrated. The residue was purified by silica gel column chromatography (hexanes−EtOAc, 1:1) to give **18** (56 mg, 75%). A mixture of **18** (39 mg, 0.093 mmol) and 10% Pd/C (8 mg) in ethanol (2 mL) was stirred under H_2 atmosphere at rt for 8 h. The crude product was purified by silica gel column chromatography ($CHCl_3$−MeOH, 98:2) to afford **2** (39 mg, 98%) as a colorless oil. ^1H NMR (300 MHz, $CDCl_3$) δ 3.89−3.80 (m, 4H), 3.40−3.38 (m, 2H), 2.48 (d, J = 4.0 Hz, 2H), 2.01−1.95 (m, 4H), 1.71−1.60 (m, 4H), 1.60−1.25 (m, 28H), 0.88 (t, J = 6.6 Hz, 6H). ESI-MS (*m*/*z*) 450 $[M + Na]^+$.

Compounds **3** and **4** were synthesized by the same procedures, except that 1-nonyne and 1-undecyne were used in step c, respectively, in place of 1-heptyne.

Compound **3**: ^1H NMR (300 MHz, $CDCl_3$) δ 3.89−3.80 (m, 4H), 3.40−3.38 (m, 2H), 2.48 (d, J = 4.0 Hz, 2H), 2.01−1.95 (m, 4H), 1.71−1.60 (m, 4H), 1.60−1.25 (m, 36H), 0.88 (t, J = 6.6 Hz, 6H). ESI-MS (*m*/*z*) 506 $[M + Na]^+$. Compound **4**: ^1H NMR (300 MHz, $CDCl_3$) δ 3.89−3.80 (m, 4H), 3.40−3.38 (m, 2H), 2.48 (d, J = 4.0 Hz, 2H), 2.01−1.95 (m, 4H), 1.71−1.60 (m, 4H), 1.60−1.25 (m, 44H), 0.88 (t, J = 6.6 Hz, 6H). ESI-MS (*m*/*z*) 562 $[M + Na]^+$.

*Synthetic Procedures: Compound **5**.* A mixture of epoxide **21** (50 mg, 0.14 mmol), which was synthesized as described previously (*12*), and 10% Pd/C (10 mg) in ethanol (2 mL) was stirred under H_2 atmosphere at rt for 24 h. The crude

Scheme 1 [a]



[a] Reaction conditions: (a) TsCl, 4-(dimethylamino)pyridine, Et_3N, CH_2Cl_2, 30 °C, 24 h, 91%; (b) TBAF (4 equiv), THF, 45 °C, 2 h, 77%; (c) 1-heptyne, 1-nonyne or 1-undecyne, *n*-BuLi, $BF_3{\cdot}Et_2O$, −78 °C, 30 min, 70−80%; (d) H_2, Pd/C, EtOH, 8 h, 98%; (e) MeMgBr, THF, CuI, 0 °C, 1 h, 85%; (f) 1-pentyne, *n*-BuLi, $BF_3{\cdot}Et_2O$, −78 °C, 30 min, 74%; (g) thiocarbonyldiimidazole (1.0 equiv), 4-(dimethylamino)pyridine, CH_2Cl_2, rt, 16 h, 90%; (h) *n*-Bu_3SnH, AIBN, dry toluene, 50 °C, 1.5 h, 66%.

product was purified by silica gel column chromatography (hexanes−EtOAc, 3:7) to afford the secondary alcohol **5** (49 mg, 98%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.89−3.75 (m, 4H), 3.58 (m, 1H), 3.39 (m, 1H), 2.94 (br s, 1H), 2.73 (br s, 1H), 2.01−1.95 (m, 4H), 1.73−1.58 (m, 4H), 1.58−1.25 (m, 18H), 1.12 (d, J = 6.3 Hz, 3H), 0.88 (t, J = 6.6 Hz, 3H). ESI-MS (*m*/*z*) 379 $[M + Na]^+$.

*Synthetic Procedures: Compound **6**.* To a mixture of MeMgBr (3.0 M in hexane, 0.16 mL, 0.46 mmol) and CuI (2 mg) in dry THF (5 mL) at 0 °C was added a solution of **21** (54 mg, 0.15 mmol) in dry THF (1 mL), and the mixture was stirred for 1 h. The reaction mixture was quenched with saturated aqueous NH_4Cl, washed with brine, dried, and concentrated. The residue was purified by silica gel column chromatography (hexanes−EtOAc, 1:1) to afford **22** (44 mg, 85%). A mixture of **22** (44 mg, 0.12 mmol) and 10% Pd/C (9 mg) in ethanol (2 mL) was stirred under H_2 atmosphere at rt for 2.5 h. The crude product was purified by silica gel column chromatography (hexanes−EtOAc, 7:3) to afford **6** (43 mg, 98%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.95−3.83 (m, 4H), 3.41−3.30 (m, 2H), 2.54 (br s, 2H), 2.01−1.95 (m, 4H), 1.73−1.60 (m, 4H), 1.60−1.25 (m, 20H), 0.99 (t, J = 6.7 Hz, 3H), 0.88 (t, J = 6.6 Hz, 3H). ESI-MS (*m*/*z*) 393 $[M + Na]^+$.

*Synthetic Procedures: Compound **7**.* To a solution of 1-pentyne (19.5 mg, 0.28 mmol) in dry THF (5 mL) at −78 °C was added a solution of *n*-BuLi (1.56 M in hexane, 0.18 mL, 0.28 mmol). After 20 min, $BF_3{\cdot}Et_2O$ (0.036 mL, 0.28 mmol) was added, and the mixture was stirred for 30 min. To the mixture was added a solution of **21** (50 mg, 0.14 mmol) in THF (1.5 mL), and the mixture was stirred at −78 °C for 15 min. The reaction mixture was worked up with saturated aqueous NH_4Cl, washed with brine, dried, and concentrated. The residue was purified by silica gel column chromatography (hexanes−EtOAc, 3:2) to afford **23** (44 mg, 74%). A mixture of **23** (44 mg, 0.10 mmol) and 10% Pd/C (8 mg) in ethanol (2 mL) was stirred under H_2 atmosphere at rt for 8 h. The crude product was purified by silica gel column chromatography (hexanes−EtOAc, 1:1) to afford **7** (43 mg, 98%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.89−3.80 (m, 4H), 3.40−3.38 (m, 2H), 2.56 (br s, 2H), 2.01−1.95 (m, 4H), 1.71−1.60 (m, 4H), 1.60−1.25 (m, 28H), 0.88 (t, J = 6.6 Hz, 6H). ESI-MS (*m*/*z*) 450 $[M + Na]^+$.

*Synthetic Procedures: Compounds **8** and **9**.* To a mixture of **3** (51 mg, 0.11 mmol) and DMAP in CH_2Cl_2 (6 mL) at 0 °C was added acetyl chloride (16 mg, 0.21 mmol), and the mixture was stirred for 3 h. The reaction mixture was quenched with saturated aqueous NH_4Cl, washed with brine, dried, and concentrated. The mixture of **8**, **9**, and recovered **3** was separated by silica gel column chromatography (hexanes−EtOAc, 7:3) to afford **8** (26 mg, 47%) and **9** (12

mg, 21%) as a colorless oil. Compound **8**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.86 (m, 1H), 4.03 (m, 1H), 3.94−3.78 (m, 3H), 3.37 (m, 1H), 2.42 (br s, 1H), 2.08 (s, 3H), 2.01−1.95 (m, 4H), 1.80−1.25 (m, 40H), 0.88 (t, J = 6.6 Hz, 6H). ESI-MS (*m*/*z*) 548 [M + Na]$^+$. Compound **9**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.84 (m, 2H), 3.98 (m, 2H), 3.91 (m, 2H), 2.05 (s, 6H), 2.03−1.75 (m, 8H), 1.58 (m, 4H), 1.38−1.23 (m, 32H), 0.88 (t, J = 6.6 Hz, 6H). ESI-MS (*m*/*z*) 590 [M + Na]$^+$.

*Synthetic Procedures: Compounds **10** and **11***. A solution of **3** (61 mg, 0.13 mmol), thiocarbonyldiimidazole (22 mg, 0.13 mmol) and DMAP (2 mg) in CH_2Cl_2 (3 mL) was stirred at rt for 16 h (*17*). The crude product was chromatographed over silica gel (hexanes−EtOAc, 1:1) to afford **24** (67 mg, 85%). Tributyltinhydride (0.18 mL, 0.68 mmol) was added dropwise to a solution of **24** (67 mg, 0.11 mmol) and 2,2′-azobisisobutyronitrile (AIBN, 3 mg) in dry toluene (6 mL) at 50 °C, and the mixture was stirred at that temperature for 1.5 h. The reaction mixture was concentrated in vacuo, and the residue was purified by silica gel column chromatography (hexanes−EtOAc, 7:3) to afford **10** (35 mg, 66%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.93−3.75 (m, 4H), 3.38 (m, 1H), 2.52 (br s, 1H), 2.01−1.95 (m, 4H), 1.73−1.58 (m, 4H), 1.58−1.22 (m, 38H), 0.88 (t, J = 6.6 Hz, 6H). ESI-MS (*m*/*z*) 490 [M + Na]$^+$. Compound **11** was obtained by deoxygenation of **10** by the same procedures. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.95−3.75 (m, 4H), 2.05−1.90 (m, 4H), 1.66−1.50 (m, 4H), 1.50−1.25 (m, 40H), 0.88 (t, J = 6.6 Hz, 6H). ESI-MS (*m*/*z*) 474 [M + Na]$^+$.

Complex I Assays. Bovine heart submitochondrial particles (SMP) were prepared by the method of Matsuno-Yagi and Hatefi (*18*) using a sonication medium containing 0.25 M sucrose, 1 mM succinate, 1.5 mM ATP, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 10 mM Tris-HCl (pH 7.4) and stored in a buffer containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4) at −84 °C.

The NADH oxidase activity in SMP was followed spectrometrically with a Shimadzu UV-3000 (340 nm, ϵ = 6.2 mM^{-1} cm^{-1}) at 25 °C. The reaction medium (2.5 mL) contained 0.25 M sucrose, 1 mM $MgCl_2$, and 50 mM phosphate buffer (pH 7.4). The final mitochondrial protein concentration was 30 μg of protein/mL. The reaction was started by adding 50 μM NADH after the equilibration of SMP with inhibitor(s) for 5 min. The NADH-Q_1 oxidoreductase activity was also determined under the same experimental conditions, except that the reaction medium contained 0.2 μM antimycin A and 2 mM KCN. The 50% inhibitory concentration (IC_{50}) values were averaged from three independent experiments.

EPR Spectroscopy. To test for possible effects of compound **3** on the redox states of iron sulfur clusters in SMP, as well as in isolated bovine complex I samples containing 3 mg/mL protein, were incubated with varying inhibitor concentrations for 5 min at ambient temperature. After addition of 3 mM NADH and 1 min reaction time, samples were quick-frozen in cold isopentane/methylcyclohexane (5:1) and stored in liquid nitrogen. X-band EPR spectra were recorded with a Bruker ESP 300E spectrometer equipped with a frequency counter (HP 53159 A, Hewlett-Packard), an ER 035 M NMR gaussmeter (Bruker), and a liquid helium continuous flow cryostat (Oxford Instruments). Accuracy of field positions was checked by using BDPA (α,γ-bisphenylene-β-phenylallyl-benzolate, g = 2.0025) in polystyrol as a g-standard. Complex I was purified as described in ref *19*.

Table 1: Summary of the Inhibitory Potencies (IC_{50}) of the Test Compounds[a]

compd no.	IC_{50} (nM)	compd no.	IC_{50} (nM)
1	4500 ± 300	9	330 ± 30
2	45 ± 4	10	14 ± 1
3	1.6 ± 0.2	11	620 ± 70
4	9.0 ± 0.7	13	7.2 ± 0.6
5	280 ± 30	14	85 ± 9
6	85 ± 8	lead compd	0.8 ± 0.04
7	3.2 ± 0.3	bullatacin	0.9 ± 0.04
8	5.5 ± 0.4		

[a] The IC_{50} value is the molar concentration needed to reduce the control NADH oxidase activity in SMP by half.

RESULTS

Structure−Activity Relationship of Δ*lac-Acetogenins.* We have shown previously that compounds **1** and **12**, which are substructures of the lead compound corresponding to the THF and γ-lactone ring moieties, respectively, elicit negligibly weak inhibition (IC_{50} > 4.5 μM) with bovine complex I and that combined use of both compounds at various molar ratios does not result in synergistic enhancement of inhibition (*8*). On the basis of these results, we concluded that acetogenins show potent activity only when the THF and the γ-lactone ring moieties are directly linked by the alkyl spacer. Although this conclusion itself is valid as discussed later, we cannot exclude the possibility that the very weak activity of compound **1** is attributable to its poor hydrophobicity, that is, short alkyl tail.

We therefore synthesized three compounds possessing two alkyl tails with different length (compounds **2**−**4**). Compounds **2**, **3**, and **4** have tails of 9, 11, and 13 carbon atoms, respectively. The stereochemistry around the hydroxylated bis-THF ring moiety of these and the following derivatives studied in this work was fixed as identical to that of the lead compound, that is, *threo*/*trans*/*threo*/*trans*/*threo*. Compared to compound **1**, the inhibitory potency markedly increased with lengthening the alkyl tails (Table 1). Compound **3** carrying two *n*-undecanyl groups elicited very potent activity comparable to that of bullatacin, one of the most potent inhibitors of bovine complex I (*4*−*6*). This compound also inhibited NADH-Q_1 oxidoreductase activity very strongly (data not shown). Further lengthening of the tail beyond an *n*-undecanyl group resulted in a slight loss of the activity (compounds **3** vs **4**). Although this result seems to be somewhat peculiar, a similar tendency was reported for natural acetogenins, which have sufficiently long alkyl tails (*9*). An excessive increase in hydrophobicity of the tail may be rather adverse to the activity probably because of some sort of trapping in the hydrophobic lipid bilayer of the membrane. It should be noted that the inhibitory potencies of compounds **3** and **4** were not affected in the presence of various concentrations of compound **12**, that is, no synergistic effect was observed between them. This result supports our previous conclusion that ordinary acetogenins show potent activity only when the THF and the γ-lactone ring moieties are directly linked by the alkyl spacer (*8*, *10*).

Because the above results provided significant insights into the role of the γ-lactone ring of acetogenins, the structure−

activity profile of Δlac-acetogenins was further investigated. We next shortened one of the two *n*-undecanyl tails of compound **3** (compounds **5**−**7**). The inhibitory potency decreased markedly with shortening of either tail, indicating that symmetric as well as hydrophobic properties of compound **3** are important for inhibitory action. This structural dependency is in striking contrast to natural acetogenins, where inhibitory potency was almost completely retained even after shortening the tail drastically from *n*-undecanyl to methyl (*11*).

Role of Hydroxy Groups of Δlac-Acetogenins. The mechanistic significance of the OH group(s) adjacent to the bis-THF rings for inhibitory action is still unknown for common acetogenins. It was previously shown that acetylation of both OH groups of the lead compound (i.e., compound **13**) resulted in a rather slight decrease in inhibitory potency (Table 1). Similar observations were reported for natural acetogenins (*20*). To examine the role of the OH group(s) in Δlac-acetogenin, we prepared compounds **8** and **9** by acetylation of either or both OH groups of compound **3**. Acetylation of one of the two OH groups resulted in a slight decrease in the inhibitory potency (compounds **3** vs **8**). Contrary to common acetogenins, acetylation of both OH groups diminished the inhibitory potency by more than 2 orders of magnitude (compounds **9** vs **13**). These results indicate that the presence of both free OH groups is important to elicit potent activity, though the presence of one free OH group can sustain fairly potent inhibition.

Looking at the above results from another point of view, one could state that a submicromolar IC_{50} even after masking both free OH groups in compound **3** still represents a significant inhibitory effect. This remaining activity may have been supported by the presence of two rather polar acetyl groups replacing the OH groups. To further examine the role of these OH group(s), we removed one or both OH groups of compound **3** by deoxygenation to obtain compounds **10** and **11**, respectively.

Removal of one of the two OH groups resulted in a slight, but significant, loss of activity. Again one free OH group was sufficient to sustain most of the inhibitory potency. However, the increase of the IC_{50} value was greater than in the case of the monoacetyl derivative (compounds **10** vs **8**). In contrast to common acetogenins (*14*), removal of both OH groups drastically diminished inhibitory potency (compounds **11** vs **14**). Compound **11** did not show 100% inhibition even at 5 μM, demonstrating that loss of the inhibitory potency was much more drastic than for the diacetyl derivative (compounds **9** vs **11**). Thus, effects of removal, as well as acetylation, of both OH groups on the activity differed significantly between common acetogenins and Δlac-acetogenins. Taking into consideration that hydrophilic indices of −OH and $-OCOCH_3$ groups, in terms of the π value in *n*-octanol/water system, are rather comparable (i.e., −0.67 and −0.55, respectively) (*21*), the capacity to act as hydrogen bond donor in the vicinity of the THF ring moiety seems critical for the inhibitory potency of Δlac-acetogenins.

Double-Inhibitor Titration of Complex I Activity. To find a clue as to whether natural acetogenins and Δlac-acetogenins bind to different sites, we examined whether in double-inhibitor titration of steady-state complex I activity the effect of natural and Δlac-acetogenins is additive. If the binding sites of the two inhibitors are identical, the extent of inhibition by the two will be additive and the maximum inhibition by one inhibitor will be attained at a lower concentration than that without an additional inhibitor. However, if the binding sites are not identical and there is no cooperativity between the two sites, the inhibition will not be additive and the concentration giving maximum inhibition by one inhibitor will not be affected irrespective of the presence of an additional inhibitor. A typical example of double-inhibitor titration is seen in the case of a combination of Q_i and Q_o inhibitors of cytochrome bc_1 complex (*22*), wherein the two sites are located at different positions of the enzyme and work sequentially in enzyme turn over.

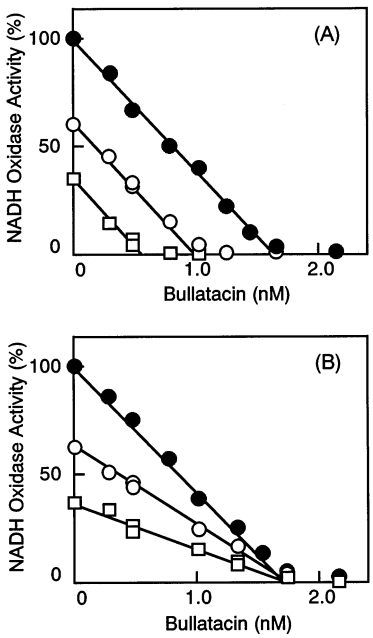


FIGURE 2: Inhibition of NADH oxidase activity by bullatacin. Titration by bullatacin alone is shown by closed circles. Titration was performed in the presence of the lead compound (panel A, 0.5 nM, open circles; 0.9 nM, open squares) or compound **3** (panel B, 1.2 nM, open circles; 3.5 nM, open squares). Data shown are representative of two independent experiments.

Figure 2 shows a double-inhibitor titration of NADH oxidase activity with different pairs of inhibitors. We used bullatacin as the standard inhibitor because of its very high binding affinity to complex I ($K_d = 2-5$ nM, ref *6*) and the resulting reproducible linearity of the titration curve. In the presence of the lead compound giving about 30% and 60% inhibition (Figure 2A), complete inhibition by bullatacin was achieved at significantly lower concentrations than those obtained without the lead compound, indicating additivity of inhibition for the two inhibitors. By contrast, in the presence of compound **3** (Figure 2B), bullatacin attained complete inhibition at an identical concentration to that obtained without compound **3**. As a control, a titration of bullatacin in combination with diphenyleneiodonium, which is known to inhibit the electron input into complex I (*23*), was carried out, and as expected indicated nonadditive behavior comparable to that observed for compound **3** (data not shown). Taken together, no additivity of inhibition was observed between common acetogenins and Δlac-acetogenins.

We also examined the inhibition mechanism of compound **3** by Lineweaver−Burk plots of the kinetic data of NADH-

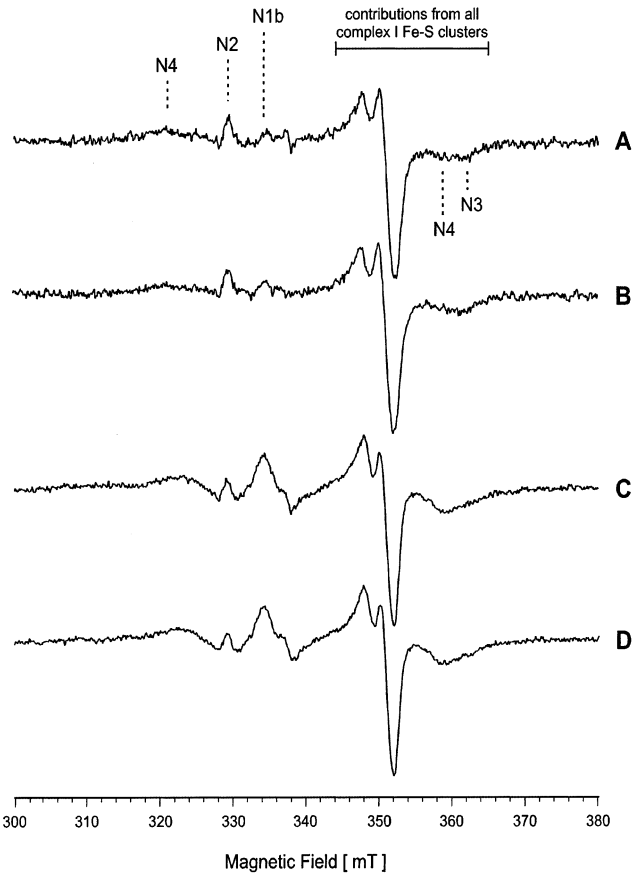


FIGURE 3: EPR spectra of isolated complex I and SMP reduced with NADH. Spectra were recorded with the following instrument settings: microwave frequency, 9.468 GHz; modulation amplitude, 0.64 mT; microwave power, 1 mW; temperature, 16 K. Some field positions, where characteristic signals from iron−sulfur clusters of complex I can be identified, are indicated. Trace A is purified complex I (3 mg/mL) in the presence of compound **3** (26 μM); trace B is purified complex I without inhibitor; trace C is SMP (20 mg/mL) in the presence of compound **3** (26 μM); trace D is SMP in the absence of inhibitor.

Q_1 oxidoreductase activity. The inhibition mechanism was competitive against Q_1 (data not shown), though noncompetitive inhibition was reported for common acetogenins (*9*, *12*).

Effect of Δlac-Acetogenin on Redox State of Fe−S Clusters. The results of double-inhibitor titration along with the unusual structural nature of Δlac-acetogenins raised the possibility that the inhibition site of these compounds may differ from that of other known complex I inhibitors, which act downstream of iron−sulfur cluster N2. We therefore examined the effect of compound **3** on the redox state and the spectral properties of the iron−sulfur clusters in complex I by EPR spectroscopy of isolated enzyme and SMP (Figure 3). In SMP as well as in purified enzyme, inhibitor concentrations that led to full inhibition of catalytic activity had no effect on the shape of the EPR spectra of any of the visible iron−sulfur clusters. Even when the inhibitor concentration was increased to 160 μM, no change in the EPR spectra was detectable. This suggests that the inhibition site of compound **3** is also downstream of iron−sulfur cluster N2.

DISCUSSION

Some of the natural acetogenins such as bullatacin are the most potent inhibitors of complex I identified today (*4*−*6*). There are few structural similarities between acetogenins and ordinary complex I inhibitors such as piericidin A, fenpyroximate, and pyridaben. Ordinary complex I inhibitors have some broad structural features in common, namely, a heterocyclic ring terminated by a hydrophobic side chain moiety (*24*). Compared to natural acetogenins as well as ordinary inhibitors, the structural nature of Δlac-acetogenins, especially compound **3**, is highly unique and resembles that of phospholipid or detergent molecules. Okun et al. have shown that polyoxyethylene ether detergents such as Triton-X and Thesit act as specific inhibitors of bovine complex I (*25*). The IC_{50}'s of Triton-X and Thesit are 5.8 and 150 μM, respectively (*25*). Similar effect of Triton-X on complex I activity has also described by Ushakova et al. (*26*). Compared to these detergents, compound **3** is a much more potent inhibitor with an IC_{50} value that is more than 3 orders of magnitude lower. Therefore its mode of inhibition may be different from that of the detergent-type inhibitors.

It has been shown that a terminal γ-lactone ring itself is not essential for common acetogenins to elicit potent inhibition but that it can be substituted with a ubiquinone ring (*12*, *13*). At first sight, the present study seems to support this idea, but careful interpretation of the results strongly suggests that the mode of inhibition for the Δlac-acetogenins in fact differs from that of common acetogenins: first, the structure−activity profiles were entirely different; second, the double-inhibitor titrations indicated that the inhibition by both types of inhibitors is not additive. Therefore, one has to consider that deletion of a terminal γ-lactone ring makes Δlac-acetogenins a different type of complex I inhibitors. Still the site of action for these inhibitors remains downstream of iron−sulfur cluster N2, similar to common acetogenins (*5*).

On the basis of the ^{1}H NMR spectroscopic studies, Shimada et al. showed that when natural acetogenins partition into liposomal membrane made from dimyristoylphosphatidylchholine, the hydroxylated THF ring moiety resides near the polar interfacial headgroup region of the membrane, while the tail and spacer (plus γ-lactone ring) is found in the hydrophobic membrane interior (*27*). This partitioning model in the liposomal system is thermodynamically reasonable because the average location of portions of the molecule is primarily determined by hydrophobicity (*28*). Based on this model, it is likely that the polar hydroxylated THF ring moiety of Δlac-acetogenins resides in the hydrophilic region of the enzyme with support of the long alkyl tails bound to hydrophobic regions of the enzyme or membrane environment. The precise location of the THF ring moiety in the enzyme seems critical for inhibition since the balance of the hydrophobicities of the two alkyl tails was found to be a very important structural feature of Δlac-acetogenins. Additionally, the function of both OH groups as hydrogen bond donors was shown to be crucial for activity, suggesting that they form critical bonds to some residue(s) of the enzyme.

Mutagenesis (*29*, *30*) and ligand binding (*6*, *31*, *32*) studies suggested that a wide variety of complex I inhibitors share a common large binding domain with partially overlapping sites and that PSST, ND5, ND1, and 49 kDa subunits contribute to the domain. However, the possibility that these inhibitors prevent binding of other inhibitors by inducing a conformational change of the enzyme, rather than by occupying the same site, cannot be fully excluded at this

point (*33*). Actually, using a fluorescent inhibitor, Ino et al. suggested that apparent competitive behavior among potent complex I inhibitors cannot be explained simply on the basis of competition for the same binding region (*34*). On the other hand, it has been commonly assumed that the inhibitor binding site(s) locate in the hydrophobic membrane part of the enzyme and may be identical to the ubiquinone reduction site. However this idea was challenged by recent studies. Zickermann et al. demonstrated that the position of the 49 kDa subunit, which was shown to be part of the "catalytic core" conferring ubiquinone reduction (*29*), is located on the peripheral arm in complex I and separated from the membrane part of the enzyme (*35*). Gong et al. have shown that the binding of [^{3}H]azido-Q to subunit NuoM (ND4) is not suppressed by various complex I inhibitors, suggesting that the ubiquinone binding site may not be identical to the inhibitor binding site (*36*). Thus the location and nature of the inhibitor (or ubiquinone) binding site(s) in complex I is still under debate. To reconcile the peripheral location of 49 kDa subunit, Zickermann et al. proposed that a hydrophobic ramp or crevice connects the membrane part and the catalytic site in the peripheral arm, providing a route for ubiquinone, and that complex I inhibitors may act on this route (*35*). Taking into account this model, the structural nature of Δlac-acetogenins is very suggestive since the polar hydroxylated THF moiety may locate at the interface of the hydrophilic and hydrophobic part of the enzyme due to the typical amphiphilic nature of the inhibitor. Further studies on the inhibitory action of these unique inhibitors will be helpful to elucidate the structural and functional features of complex I.

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BI030242M