Dynamic Function of the Alkyl Spacer of Acetogenins in Their Inhibitory Action with Mitochondrial Complex I (NADH-Ubiquinone Oxidoreductase)†

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ABSTRACT: Studies on the inhibitory mechanism of acetogenins, the most potent inhibitors of mitochondrial complex I (NADH-ubiquinone oxidoreductase), are useful for elucidating the structural and functional features of the terminal electron transfer step of this enzyme. Previous studies of the structure−activity relationship revealed that except for the alkyl spacer linking the two toxophores (i.e., the hydroxylated THF and the γ-lactone rings), none of the multiple functional groups of these inhibitors is essential for potent inhibition. To elucidate the function of the alkyl spacer, two sets of systematically selected analogues were synthesized. First, the length of the spacer was varied widely. Second, the local flexibility of the spacer was specifically reduced by introducing multiple bond(s) into different regions of the spacer. The optimal length of the spacer for inhibition was approximately 13 carbon atoms. The decrease in the strength of the inhibitory effect caused by elongating the spacer from 13 carbons was much more drastic than that caused by shortening. Local flexibility in a specific region of the spacer was not important for the inhibition. These observations indicate that the active conformation of the spacer is not an extended form, and is not necessarily restricted to a certain rigid shape. Moreover, an analogue in which a spacer covering 10 carbon atoms was hardened into a rodlike shape still maintained a potent inhibitory effect. Our results strongly suggest that the spacer portion is free from steric congestion arising from the putative binding site probably because there is no cavity-like binding site for the spacer portion. The manner of acetogenin binding to the enzyme may not be explained by a simple "key and keyhole" analogy.

More than 400 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, and pesticidal activities (*2*, *3*). Recently, Lannuzel et al. showed that annonacin, one of natural acetogenins, promotes dopaminergic neuronal death by impairing energy production, and suggested that acetogenins in tropical plants of the Annonaceae family play a role in some forms of Parkinsonism (*4*). The inhibitory effect of acetogenins on mitochondrial NADH-ubiquinone oxidoreductase (complex I)[1] is of particular importance since their diverse biological activities are thought to be attributable to this effect. Some acetogenins, such as bullatacin (Figure 1) and rolliniastatin-1, are the most potent inhibitors of bovine heart mitochondrial complex I identified to date (*5*−*8*). Although 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 bind to the ubiquinone reduction site (*9*). Additionally, there are few structural similarities between acetogenins and ordinary complex I inhibitors such as piericidin A, rotenone, and several synthetic agrochemicals such as Fenpyroximate. Thus, if the unusual structural characteristics as well as the very strong inhibitory effect of acetogenins are taken into account, a detailed analysis of the inhibitory action of these inhibitors would provide valuable insights into the terminal electron transfer step of complex I. Toward this end, identification of the crucial structural factors responsible for the potent inhibition should be useful.

The chemical structure of most natural acetogenins is characterized by four segments, namely, an α,β-unsaturated γ-lactone ring, one to three tetrahydrofuran (THF) ring(s) with flanking OH group(s), a long alkyl tail, and an alkyl spacer linking the γ-lactone and THF moieties. On the basis of studies of the structure−activity relationship (SAR) carried out by ourselves and other groups using systematically selected natural and synthetic acetogenins, the structural factors responsible for the potent inhibition of mitochondrial complex I have been elucidated as follows. First, an α,β-unsaturated γ-lactone ring, a structural feature common to a large number of natural acetogenins (*1*−*3*), is not crucial for the activity and can be substituted with other structures

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[1] Abbreviations: complex I, mitochondrial proton-pumping NADH-ubiquinone oxidoreductase; Q_1, ubiquinone-1; rt, room temperature; SAR, structure−activity relationship; SMP, submitochondrial particles; THF, tetrahydrofuran.

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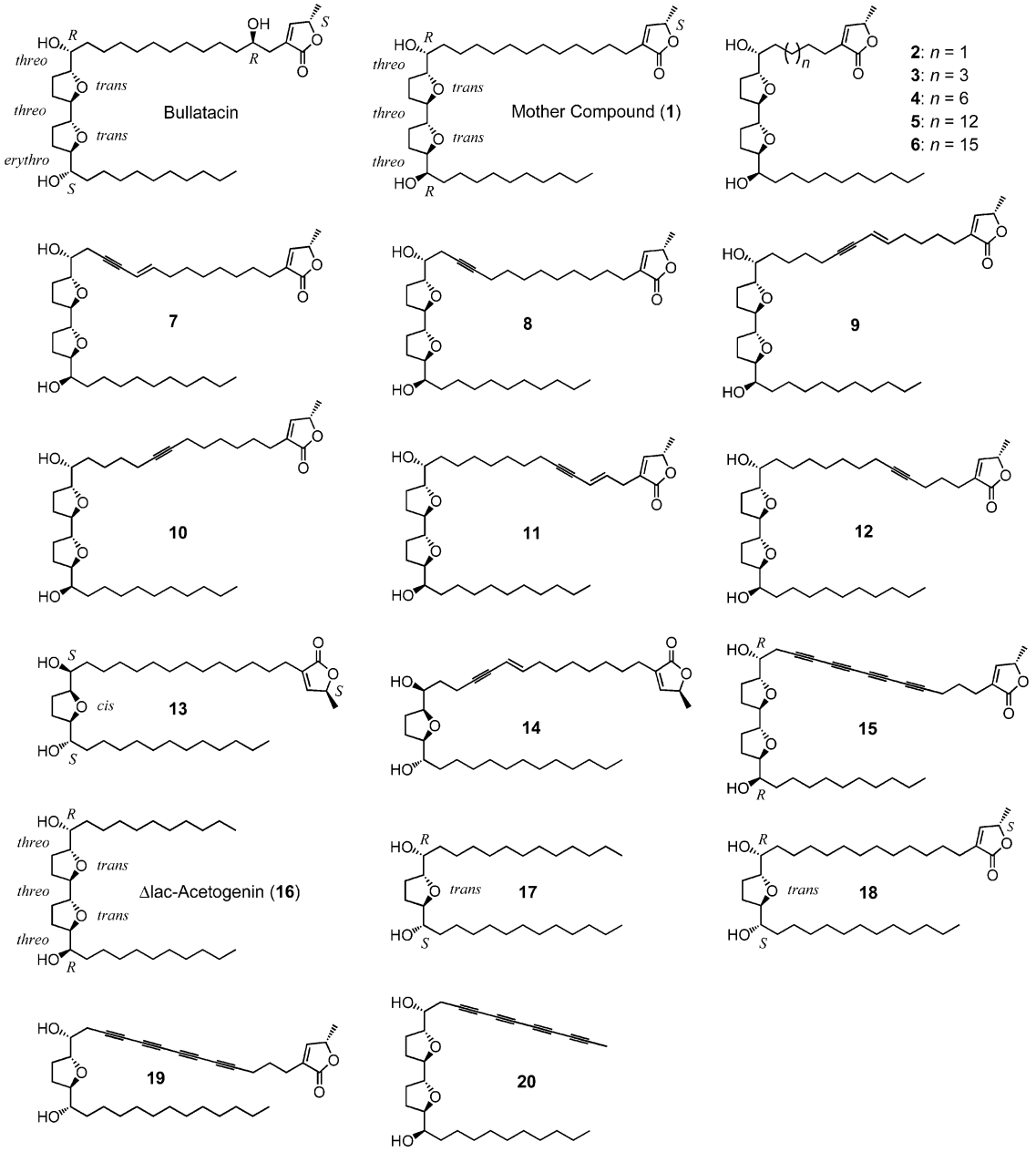
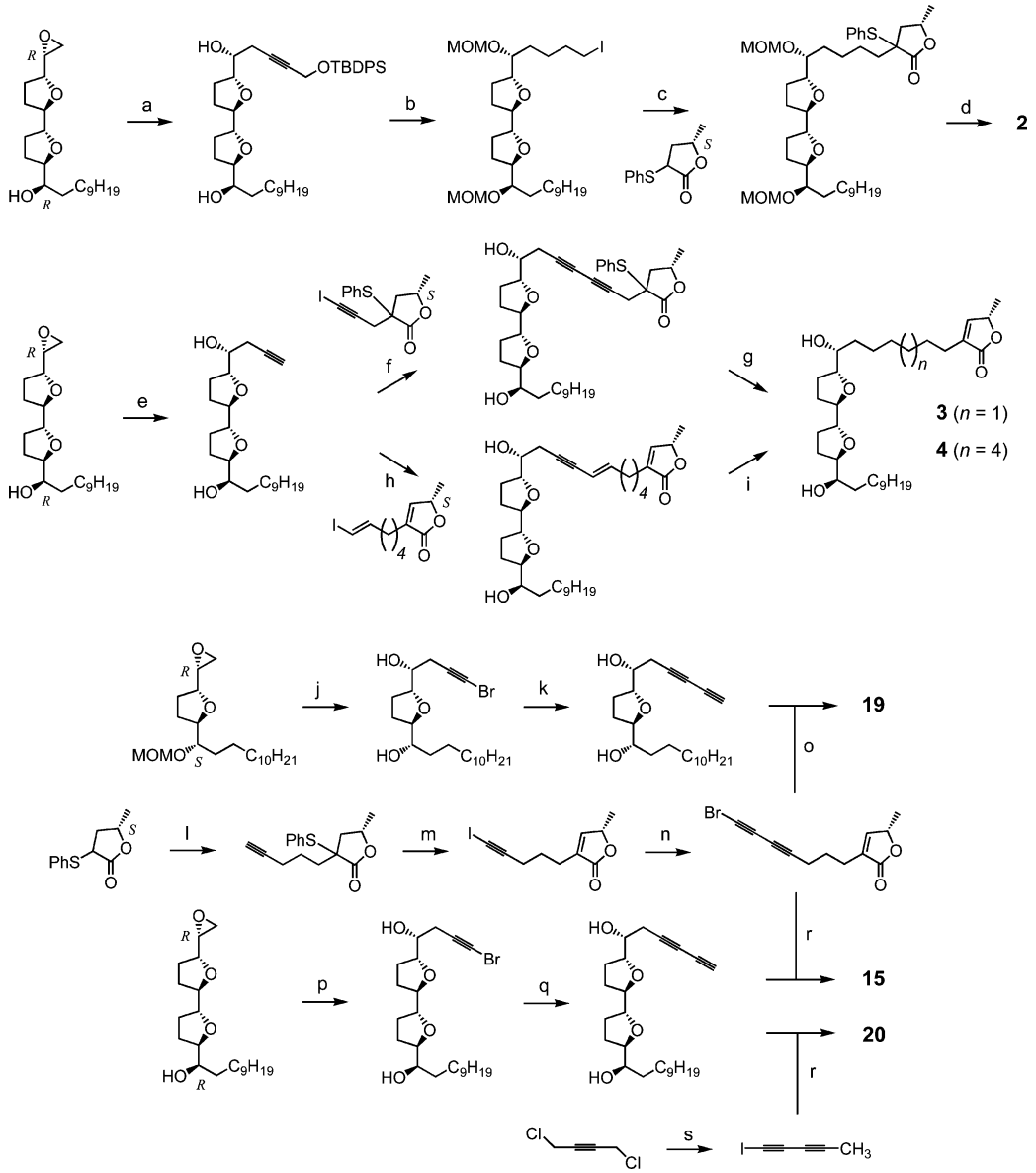


FIGURE 1: Structures of acetogenin derivatives examined in this study.

(*10*−*14*). With regard to the hydroxylated THF ring moiety, neither the number of THF rings nor the stereochemistry around this portion is an essential factor (*5*, *15*−*17*). The presence of either of two OH groups adjacent to the THF ring(s) sufficiently sustains the potent activity (*18*). With regard to the alkyl tail, a long tail is preferable, but not essential since even a methyl derivative elicited strong inhibition at the nanomolar level (*19*). With respect to the spacer moiety, the presence of polar functional group(s) like an OH group is not crucial (*5*, *15*, *20*). Importantly, neither of the two components of the inhibitor synthesized independently, i.e., the hydroxylated bis-THF ring with two alkyl chains and the γ-lactone ring with an alkyl chain, had an inhibitory effect by itself, and there was no synergistic enhancement of the inhibitory activity between the two components (*21*, *22*). These findings indicate that acetogenins work as a strong inhibitor only when the hydroxylated THF and the γ-lactone moieties are directly linked by an alkyl spacer. Thus, except for the alkyl spacer, the crucial structural factors of acetogenins are entirely ambiguous, suggesting that complex I recognizes each of the multiple functional groups of the inhibitors in a fairly loose way. When the very high affinity of some acetogenins for the enzyme is taken into account [$K_d = 2-5$ nM (*7*)], these observations seem to be confusing.

Accordingly, elucidation of the dynamic function of the spacer moiety is necessary to understand the inhibitory action of acetogenins. In this study, to elucidate the optimal length of the spacer for the inhibitory effect, we synthesized a series of acetogenin derivatives in which the spacer's length was varied while other structural factors were the same (Figure 1). Further, to obtain insights into the active conformation of acetogenin, we also synthesized a series of derivatives in which the local flexibility of the spacer was specifically reduced by introducing multiple bond(s) into different regions of the spacer. Our results demonstrated that the γ-lactone

Scheme 1[a]



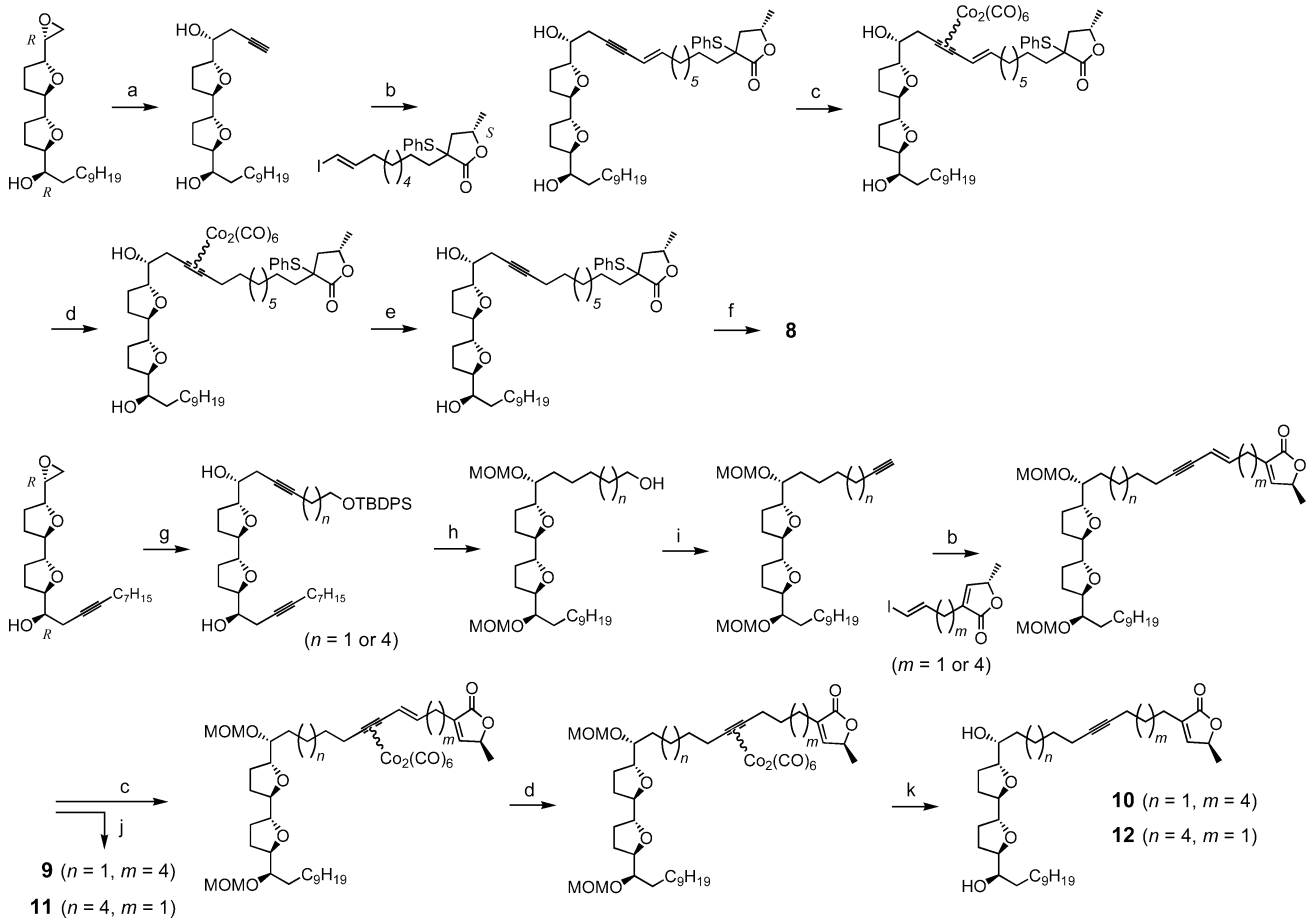
[a] Reagents and conditions: (a) 3-(*tert*-butyldiphenylsilyloxy)-1-propyne, *n*-BuLi, Et_2AlCl, dry toluene, 0 °C, 15 min, 76%; (b) (i) H_2, 10% Pd/C, EtOH, rt, overnight, 99%, (ii) chloromethyl methyl ether, $(i\text{-Pr})_2NH$, CH_2Cl_2, rt, 10 h, 97%, (iii) TBAF, THF, rt, 2 h, 92%, (iv) MsCl, Et_3N, THF, rt, 2 h, 97%, (v) NaI, acetone, rt, overnight, 85%; (c) lithium bis(trimethylsilyl)amide, THF, HMPA, −78 to −20 °C, 2 h, 40%; (d) (i) *m*CPBA, CH_2Cl_2, −40 °C, 1 h, (ii) toluene, reflux, 1 h, 67% (2 steps), (iii) 5% AcCl (in MeOH), CH_2Cl_2, rt, 88%; (e) (i) TMS-acetylene, *n*-BuLi, Et_2AlCl, toluene, 0 °C, 15 min, 93%, (ii) K_2CO_3, MeOH, 88%; (f) tri(2-furyl)phosphine, $Pd_2(dba)_3$, $(i\text{-Pr})_2NH$, dry benzene, rt, overnight, 62%; (g) (i) H_2, $(Ph_3P)_3RhCl$, dry benzene, rt, 2 days, 69%, (ii) *m*CPBA, CH_2Cl_2, −40 °C, 1 h, (iii) toluene, reflux, 1 h, 78% (2 steps); (h) $Pd(Ph_3P)_4$, CuI, Et_3N, rt, 4 h, 90%; (i) $TsNHNH_2$, NaOAc, DME/H_2O (5:3), reflux, 4 h, 78%; (j) (i) TMS-acetylene, *n*-BuLi, Et_2AlCl, toluene, 0 °C, 15 min, 93%, (ii) 5% AcCl (in MeOH), CH_2Cl_2, rt, 97%, (iii) NBS, $AgNO_3$, CH_2Cl_2, rt, 2 h, 86%; (k) (i) TMS-acetylene, $Pd(Ph_3P)_2Cl_2$, $(i\text{-Pr})_2NH$, dry benzene, rt, overnight, 64%, (ii) K_2CO_3, MeOH, 68%; (l) sodium bis(trimethylsilyl)amide, 5-iodo-1-pentyne, HMPA, rt, 2 h, 76%; (m) (i) I_2, morpholine, overnight, 82%, (ii) *m*CPBA, CH_2Cl_2, −40 °C, 1 h, (iii) toluene, reflux, 1 h, 53% (2 steps); (n) (i) TMS-acetylene, tri(2-furyl)phosphine, $Pd_2(dba)_3$, $(i\text{-Pr})_2NH$, dry benzene, rt, overnight, 49%, (ii) TBAF (1 equiv), THF/H_2O (95:5), rt, 2 h, 90%, (iii) NBS, $AgNO_3$, CH_2Cl_2, rt, 2 h, 73%; (o) $Pd(Ph_3P)_2Cl_2$, $(i\text{-Pr})_2NH$, dry benzene, rt, overnight, 78%; (p) (i) TMS-acetylene, *n*-BuLi, Et_2AlCl, toluene, 0 °C, 15 min, 93%, (ii) K_2CO_3, MeOH, 88%, (iii) NBS, $AgNO_3$, CH_2Cl_2, rt, 2 h, 71%; (q) (i) TMS-acetylene, tri(2-furyl)phosphine, $Pd_2(dba)_3$, $(i\text{-Pr})_2NH$, dry benzene, rt, overnight, 47%, (ii) K_2CO_3, MeOH, 68%; (r) tri(2-furyl)phosphine, $Pd_2(dba)_3$, $(i\text{-Pr})_2NH$, dry benzene, rt, overnight, 57% (**15**), 53% (**20**); (s) (i) *n*-BuLi, CH_2Cl_2, −78°C, 4 h, then CH_3I, −40 °C, overnight, (ii) *n*-BuLi, I_2, −78 °C, overnight, 21% (2 steps).

and the hydroxylated THF ring moieties act in a cooperative manner on the enzyme with the support of the spacer and that an extended form of the spacer is not an active conformation of the inhibitors. To take an active conformation, a certain rigid form of the spacer was not necessarily required, probably because there is no cavity-like binding domain for this portion. Our sets of compounds enabled detailed investigation of the functional role of the spacer portion.

EXPERIMENTAL PROCEDURES

Synthesis. A series of acetogenin analogues (Figure 1) was synthesized according to the procedures outlined in Schemes 1 and 2. The synthetic details and the spectral data for the

Scheme 2[a]



[a] Reagents and conditions: (a) (i) TMS-acetylene, *n*-BuLi, Et_2AlCl, dry toluene, 0 °C, 15 min, 93%, (ii) K_2CO_3, MeOH, 88%; (b) $Pd(Ph_3P)_4$, CuI, Et_3N, rt, 5 h, 85% (**8**), 79% (**10**), 69% (**12**); (c) $Co_2(CO)_8$, THF, rt, 30 min, 82% (**8**), 84% (**10**), 64% (**12**); (d) $TsNHNH_2$, NaOAc, DME/H_2O (5:3), reflux, 4 h, 77% (**8**), 58% (**10**), 75% (**12**); (e) toluene, reflux, 2 h, 11%; (f) (i) *m*CPBA, CH_2Cl_2, −40 °C, 1 h, (ii) toluene, reflux, 1 h, 60% (2 steps); (g) 4-(*tert*-butyldiphenylsilyloxy)-1-butyne (for **10**), 6-(*tert*-butyldiphenylsilyloxy)-1-hexyne (for **12**), *n*-BuLi, Et_2AlCl, dry toluene, 0 °C, 15 min, 88% (**10**), 86% (**12**); (h) (i) chloromethyl methyl ether, $(i\text{-Pr})_2NH$, CH_2Cl_2, rt, 10 h, 93% (**10**), 90% (**12**), (ii) TBAF, THF, rt, 2 h, 87% (**10**), 88% (**12**), (iii) H_2, 10% Pd/C, EtOH, rt, 5 h, 95% (**10**), 99% (**12**); (i) (i) Dess−Martin periodinane, CH_2Cl_2, 0 °C, 30 min, 81% (**10**), 73% (**12**), (ii) dimethyl(1-diazo-2-oxopropyl)phosphonate, K_2CO_3, MeOH, 98% (**10**), 80% (**12**); (j) 5% AcCl (in MeOH), CH_2Cl_2, rt, 99% (**9**), 92% (**11**); (k) (i) I_2, THF, rt, 2 h, (ii) 5% AcCl (in MeOH), CH_2Cl_2, rt, 83% (**10**), 69% (**12**) (2 steps).

compounds are described in the Supporting Information. Compounds **1**, **5**−**7**, and **16**−**18** are the same samples as those used previously (*10*, *16*, *22*).

Measurement of Complex I Activity. Bovine heart submitochondrial particles (SMP) were prepared by the method of Matsuno-Yagi and Hatefi (*23*) 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 instrument (340 nm, $\epsilon = 6.2$ 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 IC_{50} values were averaged from three independent experiments. For some test compounds, the inhibition of NADH-Q_1 oxidoreductase activity was also assessed under the same experimental conditions, except that the reaction medium contained 0.2 μM antimycin A and 2 mM KCN.

Measurement of Superoxide Production. Superoxide production was assessed by following the superoxide-dependent oxidation of epinephrine to adrenochrome (*24*) with a Shimadzu UV-3000 spectrophotometer (485−575 nm, $\epsilon = 2.96$ mM^{-1} cm^{-1}) at 25 °C. The reaction medium (2.5 mL) contained 0.25 M sucrose, 1 mM epinephrine, 1 mM EDTA, 1 μM catalase, and 10 mM Tris-HCl buffer (pH 7.4). The final protein concentration of SMP was 0.3 mg/mL. The reaction was started by adding 100 μM NADH after the equilibration of SMP with 1.2 μM inhibitor for 4 min. Superoxide dismutase was used at a final concentration of 60 units/mL to give the assay specificity.

Materials. Piericidin A and bullatacin were generous gifts from S. Yoshida (The Institute of Physical and Chemical Research, Saitama, Japan) and J. McLaughlin (Purdue University, West Lafayette, IN), respectively. Other chemicals were commercial products of analytical grade.

RESULTS

Optimal Length of the Spacer for the Inhibitory Effect. We previously examined the effect of the length of the alkyl spacer on the inhibitory potency using natural and synthetic

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

compd	IC_{50} (nM)	compd	IC_{50} (nM)
1	0.85 ± 0.05	**12**	0.85 ± 0.09
2	14 ± 2.5	**13**	5.1 ± 0.67[b]
3	1.6 ± 0.21	**14**	5.2 ± 0.83
4	1.2 ± 0.09	**15**	1.7 ± 0.14
5	13 ± 4.0	**16**	1.6 ± 0.08
6	271 ± 18	**17**	>25000
7	0.92 ± 0.11	**18**	2.3 ± 0.39
8	1.0 ± 0.07	**19**	280 ± 15
9	1.2 ± 0.08	**20**	172 ± 14
10	0.83 ± 0.09	bullatacin	0.83 ± 0.06
11	1.1 ± 0.07	piericidin A	1.3 ± 0.11

[a] The IC_{50} value is the molar concentration needed to reduce the control NADH oxidase activity [0.59−0.63 μmol of NADH min^{-1} (mg of protein)$^{-1}$] in SMP by half. Values are means ± the standard deviation of at least three independent experiments. [b] The lower inhibitory potency of this inhibitor compared to that of **18** is due to its longer spacer; 14 and 13 carbon atoms for compounds **13** and **18**, respectively.

acetogenins, and concluded that a spacer of approximately 13 carbon atoms, which corresponds in length to the spacers of the most active natural acetogenins such as bullatacin and rolliniastatin-1, is optimal (*10*). However, structural factors other than the length of the spacer were not necessarily identical in the previous compound set since we included natural acetogenins (*10*). We therefore cannot exclude the possibility that other structural factors may be also responsible for the changes in activity. To make this key point clearer, we newly synthesized compounds **2**−**4**, and reexamined the effect of the length of the alkyl spacer on the inhibitory potency using compounds **2** (C_5), **3** (C_7), **4** (C_{10}), **1** (C_{13}), **5** (C_{16}), and **6** (C_{19}) (Figure 1). All structural factors except for the length of the spacer were set to be identical in this set of compounds.

As listed in Table 1, compound **1** possessing 13 carbons was the most potent inhibitor among this set. The longer the length of the spacer from 13 carbons, the weaker the effect became (**1** vs **5** and **6**). It is noteworthy that the extent of activity loss of compound **6** is the largest class that we have observed by wide structural modification of acetogenins. For instance, even the deletion of both OH groups adjacent to the bis-THF ring of **1** resulted in an increase in the IC_{50} value to 85 ± 9 nM under the same experimental conditions (*18*). One can therefore notice the remarkable adverseness in the inhibitory effect due to the elongation up to 19 carbon atoms. A significant decrease in activity with elongation of the spacer from 13 to 15 carbons was also reported for mono-THF acetogenins (*17*). On the other hand, shortening the spacer from 13 to 7 carbons resulted in just a slight decrease in the inhibitory potency (**1** vs **3** and **4**). This result is consistent with the fact that some natural acetogenins possessing a spacer of 7 carbons such as gigantetrocin A and longimicin C exhibit fairly potent cytotoxicity against several cancer cell lines (*1*, *2*, *25*). Further shortening of the spacer length resulted in a decline in activity of ∼20-fold (**1** vs **2**). Thus, the decrease in potency caused by elongating the spacer was much more drastic than that caused by shortening (Figure 2). This result strongly suggests that an extended form of the spacer is not an active conformation for potent acetogenins such as **1**, as discussed later.

To verify whether a derivative possessing a remarkably short spacer acts in the same manner as ordinary acetogenins,

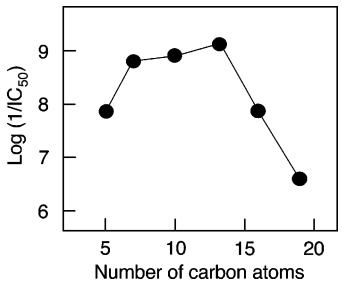


FIGURE 2: Relationship between the number of carbon atoms in the spacer and the inhibitory potency (log $1/IC_{50}$). The IC_{50} values are taken from Table 1.

we examined the additivity of the inhibitory effect between **3** and bullatacin through double-inhibitor titration of complex I activity. Double-inhibitor titration of steady-state complex I activity is useful in examining whether two inhibitors of interest bind to different sites (*26*). If the binding sites 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 the 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 the additional inhibitor.

Using bullatacin as the standard inhibitor because of its very high binding affinity for bovine complex I (*7*) and the resulting reproducible linearity of the titration curve, we previously showed that the effects of the mother compound (**1**) and bullatacin are additive, but the effects of diphenyleneiodonium, which is known to block the electron input into complex I (*27*), and bullatacin are not additive (*22*, *28*). The titration of NADH oxidase activity by bullatacin in combination with diphenyleneiodonium is shown in Figure 3A as a reference. To examine the behavior of **3**, we performed the double-inhibitor titration of bullatacin in combination with **3** (Figure 3B). In the presence of **3** giving ∼25 and ∼50% inhibition, complete inhibition by bullatacin was achieved at concentrations lower than those needed without **3**, indicating additive behavior. Thus, the binding site of **3** can be regarded as identical to that of bullatacin.

Effect of Local Flexibility of the Spacer on the Inhibition. To gain insight into the active form of the spacer and, in turn, the active conformation of the entire inhibitor molecule, we synthesized compounds **7**−**12** in which the local flexibility of the spacer was specifically reduced by introducing multiple bond(s) into different positions, but the total number of carbon atoms of the spacer was fixed at 13. The flexibility of 5 and 4 carbon atoms is almost completely reduced in the enyne (**7**, **9**, and **11**) and mono triple-bond (**8**, **10**, and **12**) derivatives, respectively. All structural factors except for the spacer portion are identical in this compound set.

Compared to that of the mother compound (**1**), the inhibitory potency was not significantly affected by the loss of local flexibility irrespective of the position of the multiple bonds (Table 1). No significant difference in inhibitory effect was observed between the enyne and mono triple-bond derivatives. Also, the inhibitory potency of mono-THF acetogenins did not decrease with the introduction of an enyne unit (**13** vs **14**). These results clearly indicate that the local flexibility in a specific region of the spacer is not crucial

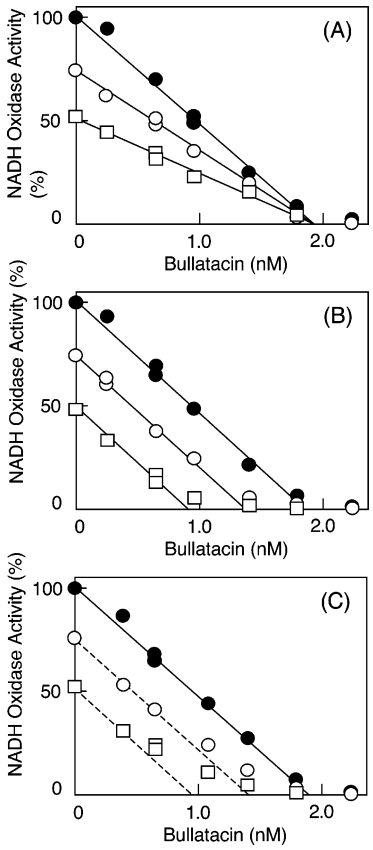


FIGURE 3: Inhibition of NADH oxidase activity in SMP by bullatacin. The titration curve for bullatacin alone is shown with filled circles. Titration was performed in the presence of diphenyleneiodonium [(A) 1.8 (○) and 2.5 μM (□)], compound **3** [(B) 0.69 (○) and 1.7 nM (□)], or compound **15** [(C) 0.78 (○) and 1.6 nM (□)]. In panel C, the pattern of ideal additive behavior is shown by the dashed lines for reference. Data shown are representative of three independent experiments.

for taking an active conformation. This means that an active conformation of the spacer may not be restricted to a certain rigid shape. It is therefore likely that any loss in the local flexibility of the spacer is compensated by taking alternative conformations. We confirmed that **9** and bullatacin exhibit additive inhibition behavior based on the double-inhibitor titration (data not shown).

Effect of Extensive Flexibility of the Spacer on the Inhibition. We next synthesized a tetrayne derivative (**15**) in which a spacer covering 10 carbon atoms was hardened to give a rodlike shape. The total number of spacer carbons was set at 13, as in the above compound sets. The molecular shape of the spacer portion of this compound resembles the extended form of compound **1**. Unexpectedly, this compound still exhibited potent inhibition at the nanomolar level (Table 1). The inhibition of NADH-Q_1 oxidoreductase activity was also observed in the same concentration range (data not shown). This result seems to be in conflict with the observation that the derivatives possessing a short spacer such as **2** and **3** exhibited potent activity, since the spatial distance between the bis-THF and γ-lactone moieties in their energetically stable conformations differs greatly between **15** and **2** (or **3**).

To know whether **15** acts in the same manner as common acetogenins, we examined the additivity of the inhibitory activity between **15** and bullatacin by the double-inhibitor titration of complex I activity. The titration pattern of bullatacin in combination with **15** reproducibly lay between the ideal additive and nonadditive behavior (Figure 3C). This result suggests that the action site of **15** is not identical to that of common acetogenins. A similar titration pattern was observed for some of the complex I inhibitors (*26*).

To elucidate the mechanism of inhibition by **15**, the unique inhibitory action of the new acetogenin mimics Δlac-acetogenins may be suggestive. We recently developed Δlac-acetogenins that possess two alkyl tails *without* an α,β-unsaturated γ-lactone ring, as shown in Figure 1 taking **16** as an example (*22*, *28*). An electron paramagnetic resonance (EPR) spectroscopic study indicated that Δlac-acetogenins act downstream of the iron−sulfur cluster N2, as is the case for other ordinary complex I inhibitors (*6*, *7*), but several lines of evidence revealed that the binding site of the inhibitors is different from that of natural acetogenins as well as ordinary complex I inhibitors (*22*, *28*). The structural factors of Δlac-acetogenins required for the potent inhibitory effect are just two: the presence of the hydroxylated adjacent bis-THF ring and two hydrophobic tails attached to the THF portion. If the γ-lactone ring of **15** does not act like that of common acetogenins because of severe steric restriction in the spacer portion, **15** would seem to have two hydrophobic tails, and hence fulfill these structural requirements. Therefore, we cannot exclude the possibility that **15** elicited the inhibition by serving as a Δlac-acetogenin-type inhibitor rather than common acetogenin.

While characterizing Δlac-acetogenins (*28*), we found two features which distinguish them from common acetogenins: (i) Δlac-acetogenins lose all inhibitory activity if the adjacent bis-THF ring is replaced by a mono-THF ring as observed for **17** (IC_{50} > 25 μM), and (ii) the rate of superoxide production from complex I induced by Δlac-acetogenins is markedly lower than that induced by common acetogenins as well as ordinary complex I inhibitors such as piericidin A and rotenone. To examine the first point, we synthesized compound **19** possessing a mono-THF ring. If **15** served as a Δlac-acetogenin rather than a common acetogenin, **19** would have absolutely no inhibitory effect, but this was not the case (Table 1). Although the inhibitory potency of **19** was much weaker than that of reference inhibitor **18**, it is reasonable to consider that in contrast to the complete loss of activity for **17**, the inhibitory effect was significantly restored for **19** by the presence of the γ-lactone ring. Additionally, we also synthesized a Δlac-acetogenin analogue possessing a tetrayne unit on one of the two tails (**20**). The inhibitory potency of **20** was markedly decreased compared to that of **16**, indicating that the rodlike tetrayne unit is quite unfavorable as the tail of Δlac-acetogenin. Thus, the synthetic experiments do not support the possibility that compound **15** served as a Δlac-acetogenin-type inhibitor.

We next examined the second point. Superoxide is produced by the single-electron reduction of oxygen by an electron carrier within the mitochondrial electron transport chain. The reductant of oxygen producing superoxide in complex I is not known, and published results are highly controversial. Recently, Lambert and Brand (*29*) suggested that the rate of superoxide production in complex I differs, reflecting a slight difference in the binding site (or manner) of inhibitors. We measured therefore the rate of superoxide production induced by compound **15** using SMP and

Table 2: Rates of Superoxide Production from Complex I Induced by Inhibitors[a]

inhibitor	rate [nmol of O_2^- min^{-1} (mg of protein)$^{-1}$]
1	0.81 ± 0.07
15	0.50 ± 0.02
16	0.33 ± 0.03
rotenone	1.06 ± 0.07

[a] The reaction was started by adding 100 μM NADH after the equilibration of SMP (0.3 mg of protein/mL) with 1.2 μM inhibitor for 4 min. Superoxide dismutase was used at a final concentration of 60 units/mL to give the assay specificity. Values are means ± the standard deviation of four independent experiments.

compared it with that induced by Δlac-acetogenin (**16**) as well as ordinary complex I inhibitors (Table 2). We preliminarily confirmed that each inhibitor caused maximal inhibition of complex I activity at the concentration used. The rate of superoxide production induced by **15** was between that induced by a Δlac-acetogenin (**16**) and by a common acetogenin (**1**). On the basis of this result along with the synthetic studies described above, we exclude the possibility that **15** served as a Δlac-acetogenin rather than a common acetogenin.

DISCUSSION

There are a variety of inhibitors of mitochondrial complex I (*30*), and except rhein (*31*) and diphenyleneiodonium (*27*), which inhibit electron input into the enzyme, all inhibitors are thought to act at the terminal electron transfer step to ubiquinone. Ordinary complex I inhibitors such as piericidin A, rotenone, and several synthetic agrochemicals like Fenpyroximate have structural features in common, notably a polar and/or heterocyclic ring possessing hydrogen bond ability and a hydrophobic "tail" structure (*32*). The former may play an important role in binding to the enzyme through specific interactions. The primary role of the tail moiety in the inhibition should be enhancement of the hydrophobicity of the entire molecule. The high degree of hydrophobicity of the inhibitor is energetically favorable for partitioning into and passage through the membrane-embedded segment of the enzyme. For acetogenins, it would not be easy to notice this feature of functional division since the two toxophores (the hydroxylated THF and the γ-lactone moieties) are separated by a long alkyl spacer. Therefore, the inhibitory action of acetogenins is thought to differ from that of ordinary inhibitors. However, radioligand and fluorescent-ligand binding (*7*) and photoaffinity labeling (*33*, *34*) studies suggested that a variety of inhibitors, including acetogenins, share a common large binding domain with partially overlapping sites. It should, however, be realized that in these studies (*7*, *33*, *34*), it was demonstrated that the binding of a certain marker ligand to complex I is completely suppressed in the presence of an excess amount of competitors, i.e., other complex I inhibitors. Under these experimental conditions, one cannot rule out the possibility that even though the binding sites of the ligand and competitors are quite different, the binding of an excess of competitor induced structural change in the ligand binding site, which resulted in the suppression of ligand binding (*9*, *26*). In fact, several studies suggested that complex I undergoes dynamic conformational change (*35*−*37*). Ino et al. (*26*) suggested that apparent competitive behavior among potent complex I inhibitors

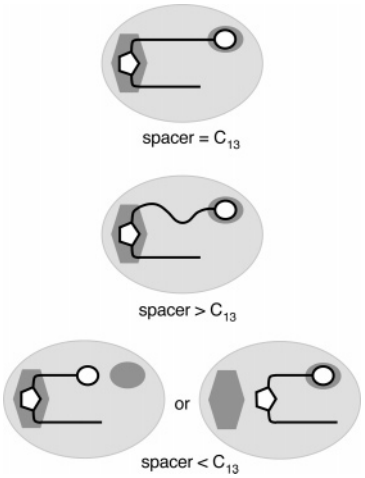


FIGURE 4: Schematic presentation of a model of binding of acetogenin to complex I. The model is drawn on the assumption that when the inhibitor elicits the inhibitory effect, the hydroxylated THF ring (⬠) and the γ-lactone ring (○) must interact with the putative two binding sites (dark areas) separated by ~13 carbons at the same time. The gray circle represents the enzyme.

cannot be explained simply on the basis of competition for the same binding region using an original fluorescent inhibitor. It remains, therefore, unclear how binding sites of complex I inhibitors relate to each other.

The aim of this study was to elucidate the inhibitory mechanism of acetogenins through investigation of the dynamic nature of the spacer. To that end, we designed and synthesized sets of compounds that enable investigation of the functional role solely of the alkyl spacer. Using a series of synthetic acetogenins in which the length of the spacer was widely varied, it was revealed that the optimal length of the spacer is approximately 13 carbon atoms and that the decrease in inhibitory potency caused by elongating the spacer further is much more drastic than that caused by shortening. If there are two binding sites for each of the two toxophores, which are separated by a distance of ~13 carbons, and the toxophores must bind to the two sites at the same time when the inhibitor elicits inhibition as illustrated in Figure 4, the derivatives having a spacer longer than 13 carbons would be able to fit their toxophores to the sites by folding the spacer properly, but the derivatives having a shorter spacer could not bind to the sites at the same time and hence the inhibitory effect would be lost. However, this was not the case; rather the result was the opposite. This therefore indicates that an extended form of the spacer is not an active conformation for potent acetogenins, and the two toxophores may be located closer together with the support of the folded spacer. On the other hand, the remarkable loss of the activity of **6** cannot be explained solely by inappropriate binding of the two toxophores to the enzyme. An excessive increase in hydrophobicity of the spacer would be rather adverse to the inhibitory effect because of some sort of trapping in the hydrophobic lipid bilayer of the membrane as observed for some natural acetogenins possessing an excessively hydrophobic alkyl tail (*15*).

Furthermore, we specifically reduced the flexibility in specific regions of the spacer to examine the effect of local flexibility on the inhibition. The results showed that the local flexibility of the spacer does not play an important part in

the adoption of an active conformation, indicating that an active conformation of the spacer is not restricted to a certain rigid shape. As it is unlikely that the binding positions of the two toxophores in the active conformation vary depending upon the local flexibility of the spacer, this observation suggests that the spacer portion is free from the steric congestion arising from the putative binding site. It is also noteworthy that the slight change in inhibitory potency due to variation of the spacer length between 13 and 7 carbon atoms (Figure 3) may not be explained assuming a rigid interaction between the spacer portion and its binding site. Taken together, our results strongly suggest that there is no cavity-like binding site for the spacer portion. Natural acetogenins have a variety of substitution patterns of polar functional group(s) in the spacer portion such as OH and C═O, whereas no specific substitution pattern is required for potent inhibition (*5*, *15*, *20*). This fact also supports the above notion. Considering that neither of the two toxophores synthesized independently has an inhibitory effect by itself and there is no synergistic enhancement between the two toxophores (*21*, *22*), we propose that acetogenins exhibit activity only when the two toxophores cooperatively act on the enzyme with the support of the spacer. In this sense, the manner in which acetogenins bind to the enzyme may not be explained by a simple "key and keyhole" analogy. While details of the binding of acetogenins remain to be elucidated, the unique structural feature of these inhibitors would be responsible for the unique inhibitory action.

It was entirely unexpected that a tetrayne derivative (**15**) elicited very potent inhibition. The synthetic study, double-inhibitor titration, and the effect on superoxide production revealed that the apparent inhibitory manner of **15** is different from that of common acetogenins, whereas this inhibitor cannot be categorized as a Δlac-acetogenin analogue which elicits potent inhibitory effect without a γ-lactone ring. It is thus obvious that the presence of the γ-lactone ring in **15** definitively differentiates this compound from Δlac-acetogenin-type inhibitors and determines its inhibitory activity. In view of the crucial role of the γ-lactone ring, we presume that this inhibitor functions such as common acetogenins, but in a somewhat different manner due to the remarkably severe steric restriction in the spacer portion. Since it is unlikely that there is a barrel-shaped cavity in complex I large enough to accommodate the rodlike spacer (with a γ-lactone ring) of **15**, the spacer portion of this inhibitor may be also free from steric congestion arising from the enzyme. On the other hand, the effect of the introduction of the tetrayne unit into the spacer differed significantly between the adjacent bis-THF and mono-THF derivatives (**15** vs **19**). It is, however, not inconceivable that the flexibility of the spacer affects the cooperativity of the two toxophores in a somewhat different way between the two derivatives since the spatial distance between the functional groups in the THF moiety [i.e., THF oxygen(s) and flanking OH groups] and the γ-lactone ring is not identical.

Shimada et al. (*38*) proposed a model for the active conformation of acetogenins on the basis of the partitioning of these inhibitors into the liposomal membrane, which was drived from ^{1}H NMR and differential scanning calorimetry studies. According to their model, the THF ring(s) with flanking hydroxy groups resides near the glycerol backbone of phosphatidylcholine irrespective of the number of THF rings and acts as a hydrophilic anchor at the membrane surface; on the other hand, the γ-lactone ring directly interacts with the target site of complex I through lateral diffusion inside the inner mitochondrial membrane. In this model, the γ-lactone ring is regarded as the only reactive species directly interacting with the enzyme. The marked decrease in the cytotoxicity of bullatacin against carcinoma cells due to saturation of the double bond in the α,β-unsaturated γ-lactone ring was one of the most important pieces of evidence for the essential role of the γ-lactone ring in the model (*38*).[2] This observation is, however, in conflict with the study by Queiroz et al. (*39*), wherein the saturation of the double bond in the α,β-unsaturated γ-lactone of squamocin was shown to have no effect on the cytotoxicity against several cancer cell lines. The SAR studies concerning the α,β-unsaturated γ-lactone ring (*10*−*14*) do not support the putative crucial role of this moiety in the inhibition of complex I. Additionally, if the diffusional movement of the γ-lactone is crucial as proposed, both the shortening and the hardening of the spacer may be significantly unfavorable to the inhibitory action, but this was not the case as revealed in the study presented here. Nevertheless, we cannot necessarily exclude the validity of their model for *partitioning* of acetogenins into the liposomal membrane. In the liposomal membrane, the average location of THF and γ-lactone ring moieties would be primarily determined by their hydrophobicity (*40*).

SUPPORTING INFORMATION AVAILABLE

Synthetic procedures and spectral data for newly synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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[2] In ref *38*, the authors emphasized a crucial role for the α,β-unsaturated γ-lactone ring in the inhibitory action by quoting an article written by the same group [Rupprecht et al. (1990) *J. Nat. Prod. 53*, 237−278], wherein assays of the cytotoxicity of bullatacin and dihydrobullatacin with several cancer cell lines had been reported. However, the cytotoxicity of bullatacin and related natural acetogenins, in terms of the ED_{50} (micrograms per milliliter) value, reported in this paper is much greater, at least by several orders of magnitude, than that reported by other research groups such as Myint et al. [(1991) *Phytochemistry 30*, 3335−3338], Naito et al. [(1995) *J. Org. Chem. 60*, 4419−4427], Queiroz et al. [(2000) *J. Med. Chem. 43*, 1604−1610], and Nakanishi et al. [(2003) *J. Med. Chem. 46*, 3185−3188].

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