Dynamic Function of the Spacer Region of Acetogenins in the Inhibition of Bovine Mitochondrial NADH-Ubiquinone Oxidoreductase (Complex I)[†]

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ABSTRACT: Studies of the action mechanism of acetogenins, the most potent and structurally unique inhibitors of bovine heart mitochondrial complex I (NADH-ubiquinone oxidoreductase), are valuable in characterizing the inhibitor binding site in this enzyme. Our previous study deepened our understanding of the dynamic function of the spacer region of bis-THF acetogenins [Abe, M., et al. (2005) *Biochemistry 44*, 14898−14906] but, at the same time, posed new important questions. First, while the two toxophores (i.e., the hydroxylated THF and the γ-lactone rings) span a distance shorter than that of the extended 13 carbon atoms [-$(CH_2)_{13}$-], what is the apparent optimal length of the spacer for the inhibition of 13 carbon atoms? In other words, what is the functional role of the additional methylene groups? Second, why was the inhibitory potency of the mono-THF derivative, but not the bis-THF derivative, drastically reduced by hardening the spacer covering 10 carbon atoms into a rodlike shape [-CH_2-$(C\equiv C)_4$-CH_2-]? This study was designed not only to answer these questions but also to further disclose the dynamic functions of the spacer. We here synthesized systematically designed acetogenins, including mono- and bis-THF derivatives, and evaluated their inhibitory effects on bovine complex I. With regard to the first question, we demonstrated that the additional methylenes enhance the hydrophobicity of the spacer region, which may be thermodynamically advantageous for bringing the polar γ-lactone ring into the membrane-embedded segment of complex I. With regard to the second question, we observed that a decrease in the flexibility of the spacer region is more adverse to the action of the mono-THF series than that of the bis-THF series. As a cause of this difference, we suggest that for bis-THF derivatives, one of the two THF rings, being adjacent to the spacer, is capable of working as a pseudospacer to overcome the remarkable decrease in the conformational freedom and/or the length of the spacer. Moreover, using photoresponsive acetogenins that undergo drastic and reversible conformational changes with alternating UV−vis irradiation, we provided further evidence that the spacer region is free from steric congestion arising from the putative binding site probably because there is no receptor wall for the spacer region.

More than 400 annonaceous acetogenins have been isolated from the plant family *Annonaceae* since the discovery of uvaricin in 1982 (*1*–*3*). Acetogenins have very potent and diverse biological effects such as antitumor, antimalarial, and pesticidal activities (*2, 3*). Annonacin, the major acetogenin in the tropical plant *Annona muricaca*, is highly toxic to cultured neurons and may play a role in some neurodegeneration in humans (*4, 5*). 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. Acetogenins such as bullatacin (Figure 1) are among the most potent inhibitors of bovine heart mitochondrial complex I (*6*–*9*). Although there is little structural similarity between acetogenins and ordinary complex I inhibitors such as piericidin A, rotenone, and several synthetic agrochemicals like fenpyroximate, acetogenins have been thought to act at the terminal electron transfer step of complex I (*7, 8*). We recently revealed that an acetogenin derivative synthesized as a photoaffinity labeling probe binds to the ND1 subunit with high specificity (*10*). Considering the unusual structural characteristics as well as the very strong inhibitory effect of acetogenins, a detailed analysis of the inhibitory action of these inhibitors would provide

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[1] Abbreviations: complex I, mitochondrial proton-pumping NADH-ubiquinone oxidoreductase; IC_{50}, molar concentration (nanomolar) needed to reduce the control NADH oxidase activity in SMP by half; SAR, structure−activity relationship; SMP, submitochondrial particles; THF, tetrahydrofuran.

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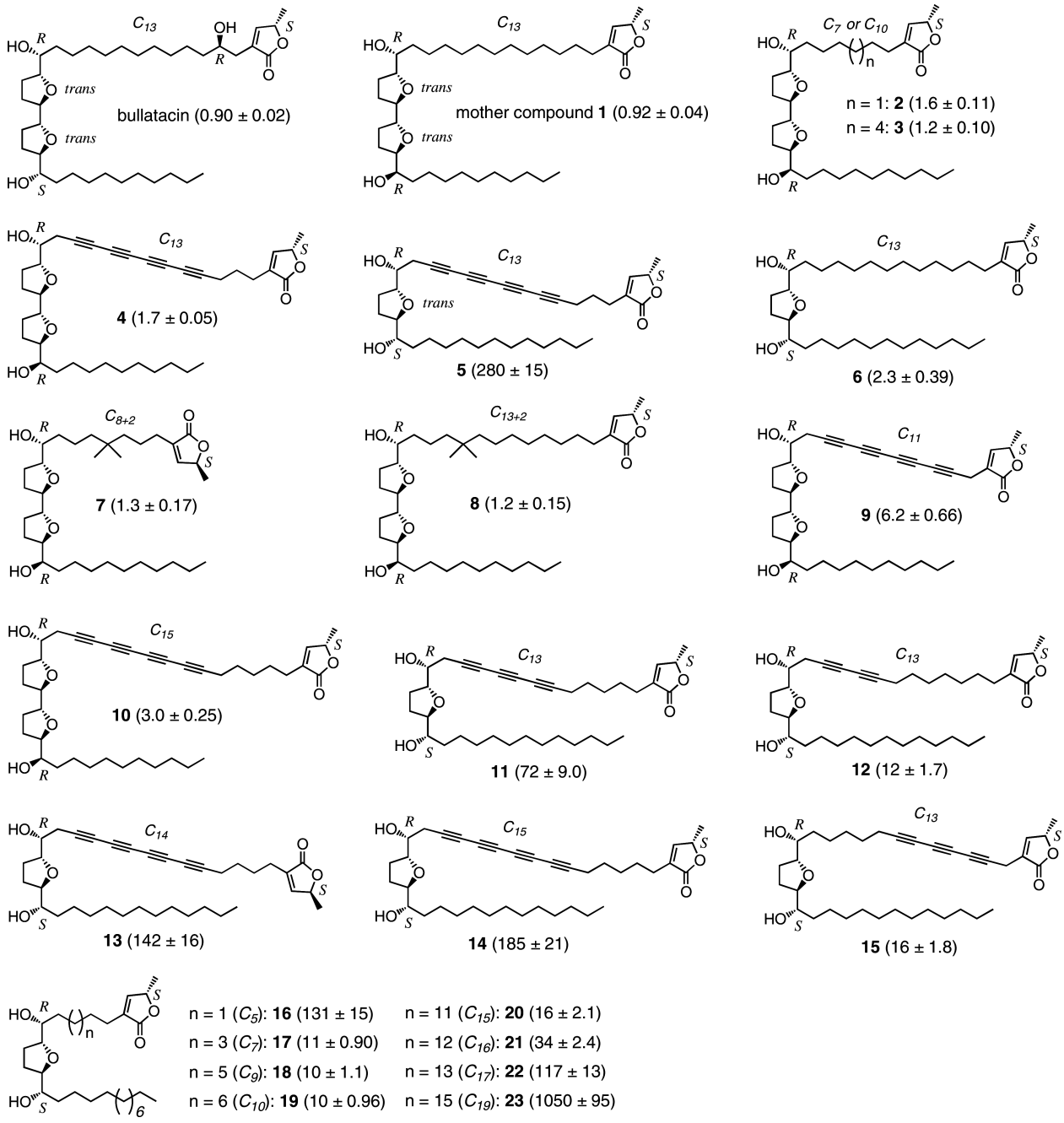

FIGURE 1: Structure of bullatacin and synthetic acetogenins studied herein. Compounds **7**−**23** were newly synthesized. The total number of carbon atoms of the spacer portion is shown for each compound. The numbers in parentheses are IC_{50} values, which is the molar concentration (nanomolar) needed to reduce the control NADH oxidase activity [0.65−0.78 μmol of NADH min^{-1} (mg of protein)$^{-1}$] in SMP by half. Values are means ± the standard deviation of three independent experiments.

valuable insights into the terminal electron transfer step of complex I (*11*).

The chemical structure of most natural acetogenins is characterized by four segments: (i) an α,β-unsaturated γ-lactone ring, (ii) one to three tetrahydrofuran (THF) rings with flanking OH groups, (iii) a long alkyl tail, and (iv) an alkyl spacer linking the two toxophores (i.e., 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 roles of each segment in the inhibitory action with bovine complex I have been elucidated as follows. (i) A natural α,β-unsaturated γ-lactone ring itself is not crucial for the activity and can be substituted with other structures (*12–16*). (ii) Neither the number of THF rings nor the stereochemistry around the hydroxylated THF ring moiety is an essential factor (*6, 17–19*), and the presence of either of two OH groups adjacent to the THF ring(s) sufficiently sustains the potent activity (*20*). (iii) A long alkyl tail is preferable, but not essential since even a methyl derivative elicited strong inhibition at the nanomolar level (*21*). (iv) The presence of polar functional group(s) such as hydroxy and carbonyl groups is not crucial for the activity (*6, 17, 22*). Thus, 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. However, it is noteworthy that neither the hydroxylated THF nor the γ-lactone ring moiety synthesized

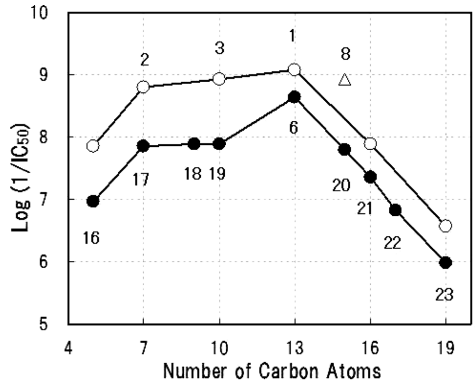

FIGURE 2: Relationship between the number of carbon atoms of the spacer and the inhibitory potency (log $1/IC_{50}$). The figures refer to the compound numbers in Figure 1: bis-THF series (○), mono-THF series (●), and compound **8** (△).

separately has an inhibitory effect by itself and that there is no synergistic enhancement of the inhibitory effect between the two moieties (*23, 24*). Thus, acetogenins act as strong inhibitors only when the THF and γ-lactone ring moieties are directly linked by an alkyl spacer.

Accordingly, elucidation of the dynamic function of the spacer moiety is crucial in determining the inhibitory action of acetogenins in detail. We previously carried out the SAR study of a series of synthetic bis-THF acetogenins in which the spacer region was systematically modified and made the following findings (*25*). First, the optimal length of the spacer required for strong inhibition is 13 carbon atoms (compound **1** in Figure 1; also see the white circles in Figure 2), which corresponds in length to the spacers of the most potent natural acetogenins such as bullatacin. Second, the decrease in inhibitory potency caused by elongating the spacer from 13 carbons is much more drastic than that caused by shortening it (Figure 2). With regard to this point, it should be noted that even the derivatives possessing a short spacer of 7 (**2**) or 10 (**3**) carbon atoms elicited a potent inhibitory effect at the nanomolar level. Third, local flexibility in a specific region of the spacer is not important for the inhibition. Together, these findings strongly suggest that an active conformation of the spacer of strong acetogenins is not an extended form, and hence, the THF and γ-lactone moieties are brought close together by a properly folded spacer. An active conformation of the spacer may not necessarily be restricted to a certain rigid shape probably because there is no cavity-like binding site for the spacer region. Additionally, as the presence of multiple hydroxy groups in the spacer region is markedly adverse to the activity (*17*), the spacer may reside in a hydrophobic environment.

It is worth noting that a bis-THF derivative (**4**) in which the spacer covering 10 carbon atoms was hardened into a rodlike shape maintained a potent inhibitory effect, whereas a mono-THF derivative (**5**) drastically lost the activity with this structural modification (**5** vs **6**) (*25*). Considering that the profiles of SARs concerning other parts of the inhibitor's structure are comparable between the bis- and mono-THF series (e.g., refs *17–19*), this result seems confusing at first. From another angle, however, this observation may provide new insights into the dynamic functions of the spacer.

Thus, the previous study (*25*) deepened our knowledge with regard to the dynamic function of the spacer but, at the same time, posed other important questions. First, while the two ring moieties are located within a distance fairly shorter than that of 13 linear carbon atoms, why is the apparent optimal length of the spacer 13 carbon atoms? In other words, what is the functional role of the additional methylene ($-CH_2-$) groups? Second, why did the introduction of a tetrayne structure into the spacer drastically reduce the inhibitory potency of a mono-THF derivative (**5**), but not a bis-THF derivative (**4**)? To answer these questions and further disclose the function of the spacer region, we synthesized systematically designed compounds and evaluated their inhibitory effects on bovine mitochondrial complex I.

EXPERIMENTAL PROCEDURES

Materials. Compounds **7**−**19** and **21**−**23** and photoresponsive derivatives (**24** and **25**) were newly synthesized by the procedures described in the Supporting Information. Compounds **1**−**6** and **20** are the same samples that were used previously (*19, 25*). Bullatacin was a generous gift from J. L. McLaughlin (Purdue University, West Lafayette, IN). Other chemicals were commercial products of analytical grade.

Measurement of Complex I Activity. Bovine heart submitochondrial particles (SMP) were prepared by the method of Matsuno-Yagi and Hatefi (*26*) 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 7.5). 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 an inhibitor 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 determined under the same experimental conditions, except that the reaction medium contained 0.2 μM antimycin A and 2 mM KCN.

Assay with Photoresponsive Acetogenins. The photoirradiation of **24** or **25** in SMP was carried out using a LAX-102 illuminator containing a 100 W xenon arc lamp (Asahi Spectra, Tokyo, Japan) equipped with a mirror module and narrow bandpass UV−vis filters (±10 nm bandwidth) to select the required wavelength. When a change in complex I activity due to photoirradiation was monitored, UV or visible light was focused on a quartz reaction cuvette containing a reaction mixture (2.5 mL) using an appurtenant fiber cable at a distance of 3 cm. The NADH oxidase activity (30 μg of protein/mL) was monitored for 1 min immediately after a 1 min exposure to UV light in a quartz reaction cuvette containing SMP and inhibitor, and consequently, the reaction cuvette was exposed to visible light for 1 min and then the enzyme activity monitored again. We repeated these operations continuously without additional NADH and/or inhibitor. Under the experimental conditions, 250 μM NADH added initially was enough for the continuous monitoring of the enzyme activity throughout the repeated operation.

RESULTS

Role of the Spacer in the Inhibition by the Bis-THF Series. To address the first question noted in the introductory section, we established a working hypothesis, that the additional methylene groups enhance the hydrophobicity of the spacer, which may be thermodynamically advantageous for bringing the polar γ-lactone ring into the membrane-embedded segment of complex I. To verify this idea, we synthesized compound **7**, in which the spacer is fixed at a length of eight carbon atoms but has additional geminal methyl groups (a total of 10 carbon atoms) to enhance its hydrophobicity. Irrespective of the significant steric hindrance arising from the geminal methyl groups, the inhibitory potency of **7** (IC_{50} = 1.3 ± 0.17 nM) was almost identical to that of the derivative (**3**) possessing 10 carbon atoms without the branched structure (IC_{50} = 1.2 ± 0.10 nM). A compound possessing a spacer of eight carbon atoms without a branched methyl group would be ideal as the other reference but had not been in the previous work (*25*). As the next best reference, compound **2** possessing a spacer of seven carbon atoms had an IC_{50} of 1.6 ± 0.11 nM, indicating that **7** is slightly more active than **2**. Thus, while the extent of the increase in inhibitory activity from **2** (C_7) to **1** (C_{13}) is essentially slight (white circles in Figure 2), it is likely that an increase in the hydrophobicity of the spacer region is favorable for the inhibition.

We previously showed that elongation of the spacer from 13 to 16 or 19 carbon atoms results in a drastic decrease in activity (white circles in Figure 2). To explain this phenomenon, we suspected that an excessive increase in the hydrophobicity of the spacer causes some sort of trapping of the inhibitor in the hydrophobic lipid bilayer of the membrane (*25*). However, we cannot exclude the possibility that the drastic decrease in activity can be attributed to an entropic disadvantage inherent in the folding of the markedly long alkyl spacer for enabling cooperative binding of the two toxophores to the putative binding sites. To address this point, we synthesized the derivative **8** in which the length of the spacer is fixed at 13 carbon atoms with additional geminal methyl groups, giving a total of 15 carbon atoms. To simplify the comparison, the geminal methyl groups were introduced at the same position as those in **7**. Regardless of an increase in hydrophobicity, compound **8** retained potent activity (IC_{50} = 1.2 ± 0.15 nM; a triangle in Figure 2), indicating that an excessive increase in the hydrophobicity of the spacer is not significantly adverse to the activity. It is therefore likely that the drastic decrease in activity due to the elongation beyond 13 carbon atoms is primarily due to the entropic disadvantage mentioned above.

Effect of the Multiple-Yne Structure on the Inhibition. We next addressed the second question: why the inhibitory potency of a mono-THF derivative (**5**), but not a bis-THF derivative (**4**), was drastically reduced via introduction of the tetrayne structure [-$(C{\equiv}C)_4$-] into the spacer. Answering this question would be equivalent to elucidating how both toxophoric rings (i.e., the γ-lactone and the THF rings) in **4** reach the two putative binding sites by overcoming such a remarkable disadvantage in conformational freedom.

To clarify the effect of the introduction of the tetrayne structure into the spacer, we newly synthesized compounds **9** and **10** which possess spacers of 11 and 15 carbon atoms, respectively. Interestingly, **9** still maintained strong activity at the nanomolar level, though the activity was slightly decreased compared to that of **4**. Although elongation of the spacer beyond 13 carbons is significantly unfavorable for the activity for the simple alkane derivatives (Figure 2), compound **10** elicited strong activity similar to that of **4**. This is probably because the rigid tetrayne structure lightens the entropic disadvantage mentioned above compared with the corresponding saturated alkane. Anyhow, these results mean that the bis-THF series are able to overcome the remarkable loss in conformational freedom of the spacer region. On the other hand, using newly synthesized triyne (**11**) and diyne (**12**) derivatives, we confirmed that the inhibitory potency of **5** significantly recovered with an increase in the conformational freedom of the spacer [**5** (tetrayne) < **11** (triyne) < **12** (diyne)].

In anticipation of a recovery of inhibitory potency, we extended the spacer of **5** by one and two methylene units to give **13** (C_{14}) and **14** (C_{15}), respectively. However, the recovery was only 1.5−2-fold (**5** vs **13** and **14**). This result suggests that an increase in conformational freedom in the spacer region close to the γ-lactone ring is not effective at recovering the activity. On the basis of this result, the conformational flexibility of the spacer region close to the THF ring may be more significant for the inhibitory action than the region close to the γ-lactone ring. To verify this idea, we synthesized **15** in which a triyne unit in **11** was shifted to the γ-lactone ring by four methylene units. The inhibitory potency of **15** turned out to be much improved compared to that of **11**.

Effect of the Length of the Spacer on Inhibition. To elucidate the remarkable difference in the spacer's effect between the mono- and bis-THF series, we decided to examine the relationship between the length of the alkyl spacer and the inhibitory potency for the mono-THF series and compare it with the relationship for the bis-THF series studied previously (*25*). We newly synthesized a series of mono-THF derivatives possessing spacers of different lengths, compounds **16**−**23** (C_5−C_{19}). As seen in Figure 2 (●), compound **6** possessing 13 carbons was the most potent inhibitor among the derivatives tested, though **6** was somewhat weaker than the corresponding bis-THF derivative (**1**). The longer the length of the spacer from 13 carbons, the weaker the effect became. Shortening the spacer from 13 to seven carbons resulted in a decrease in the inhibitory potency of 1 order of magnitude. For both series, the decrease in activity caused by shortening the spacer from seven to five carbons was striking. Thus, it is obvious that the decrease in activity due to the shortening is more pronounced for the mono-THF series than the bis-THF series. This finding means that adaptation to shorten the spacer is less effective in the mono-THF series than in the bis-THF series.

The relationship between the length of the spacer and the activity for the mono-THF series did not completely match that for the bis-THF series. Considering that the hydrophobicity of alkyl groups increases linearly with their length, the variation in the length of the spacer affects not only the distance between the two toxophores but also the position of the polar THF portion in the membrane environment by altering the hydrophobicity−hydrophilicity balance. Therefore, this inconsistency probably exists because contributions of the two effects to the inhibitory action are not identical

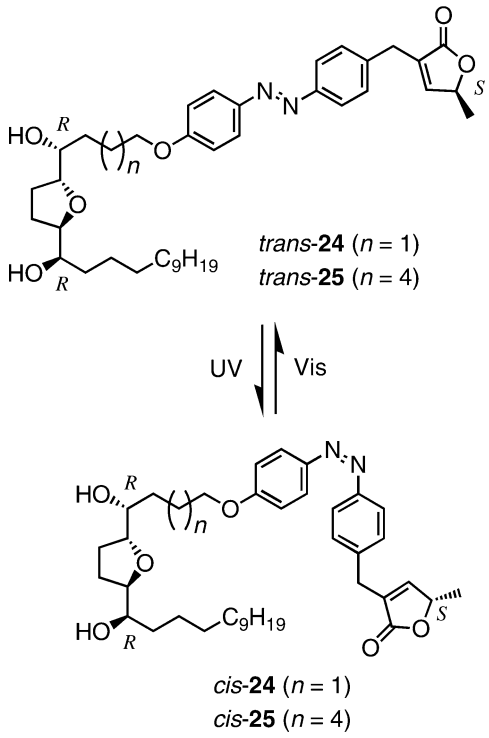

FIGURE 3: Structure of *trans* and *cis* isomers of compounds **24** and **25**. The azobenzene is *trans*−*cis* isomerized by alternating UV−visible irradiation.

between the bis- and mono-THF series because of a difference in hydrophilicity around the THF moiety.

Effect of Light-Induced Conformational Changes of the Spacer on Inhibition. To gain further insight into the dynamic function of the spacer, we synthesized azobenzene derivatives of acetogenin (Figure 3). Azobenzene has been widely used as a photoresponsive switch which can reversibly *trans*−*cis* isomerize in response to photoirradiation. We previously showed that the inhibitory effect of a photoresponsive complex I inhibitor, which was synthesized using Δlac-acetogenin as a template (*24, 27*), can be reversibly changed by alternating UV−visible irradiation (*28*). As the relative spatial distance (or orientation) of the γ-lactone and the THF moieties drastically differs between the *trans* and *cis* isomers, we suspected a remarkable difference in the inhibitory potencies of the isomers. Before examining the inhibition of complex I in SMP, we characterized *trans*−*cis* isomerization by UV−visible irradiation according to the previously described method (*28*).

The *trans*−*cis* photoisomerization was confirmed from the UV−visible absorption spectra of **24**. When **24** was irradiated at 370 ± 10 nm in ethanol, the intensity of the absorption band centered at 350 nm decreased, while that of the absorption band centered at 445 nm associated with an increase in the *cis* form increased [Figure S1 (A)]. These spectral changes are typical for the *trans*−*cis* isomerization of azobenzene derivatives (*29*). On the other hand, the reverse isomerization took place upon irradiation at 470 ± 10 nm [Figure S1 (B)]. For both processes, a 1 min irradiation was enough to attain the maximal photoisomerization under the experimental conditions. We confirmed that by alternating irradiation at 370 and 470 nm for 1 min, maximal photoisomerization is reproducible (Figure S2). Thus, the azobenzene unit can reversibly, but not completely (*29*), *trans*−*cis* isomerize in response to photoirradiation.

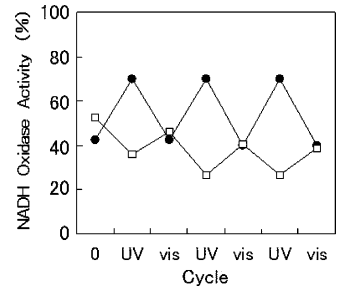

FIGURE 4: Changes in NADH oxidase activity after alternating photoirradiation with UV and visible light. The reaction mixture was irradiated at 370 and 470 (±10) nm for 1 min at 25 °C. The concentrations of **24** (●) and **25** (□) were 3.8 and 8.2 nM, respectively. The final mitochondrial protein concentration is 30 μg of protein/mL. The control NADH oxidase activity was 0.68 ± 0.04 μmol of NADH min^{-1} (mg of protein)$^{-1}$. Data are representative of three independent experiments.

We examined the changes in the inhibitory effects of **24** due to photoisomerization in SMP. SMP were preincubated with **24** in the dark for 5 min before the enzyme reaction started. The IC_{50} value of *trans*-**24**, which was prepared by keeping the sample in the dark at room temperature for 4 days, was 2.6 ± 0.32 nM. After a 1 min exposure to UV (370 ± 10 nm) or visible (470 ± 10 nm) light in a quartz reaction cuvette containing SMP, the enzyme activity (NADH oxidase activity) was measured. As shown in Figure 4 (●), alternating irradiation in the presence of 3.8 nM **24** reproducibly enhanced and reduced the enzyme activity between ∼40 and ∼70%. This result means that the inhibitory potency of the *trans* form (extended form) is stronger than that of the *cis* form (short form). Since it was impractical to determine the actual *trans*:*cis* ratio of **24** in an equilibrium state in SMP (*28*), we cannot conclude whether the *cis* isomer completely lost the inhibitory effect solely from this result. However, as we will describe below, we interpreted this result to mean that the *cis* isomer does not completely lose the inhibitory effect.

Next, we examined the inhibitory effects of **25** possessing a longer spacer than **24** (Figure 3). The distance between the two toxophores of the extended *trans*-**25** is considerably longer than the extended 13 methylenes, presumably corresponding to the extended 18−19 methylenes. The IC_{50} value of *trans*-**25** was 8.5 ± 0.31 nM. Interestingly, the relative inhibitory effects of the *trans* and *cis* isomers were reversed compared with those of **24** [Figure 4 (□)]. Considering the case of **24**, it is likely that the spatial distance between the two toxophores in *trans*-**25** is too long for optimal occupation of the putative binding sites due to elongation of the residual alkyl spacer. Taken together, the experiments using the azobenzene derivatives indicate that both isomers of **24** and **25** retain a strong inhibitory effect. As the conformation of the rigid azobenzene core differs greatly between the *trans* and *cis* isomers (*30*), the residual spacer moiety has to adopt a markedly different conformation to allow the toxophores to occupy the putative binding sites.

DISCUSSION

In this study, we further explored the dynamic function of the alkyl spacer of acetogenins by synthesizing a series of mono-THF analogues and comparing their inhibitory action with that of the bis-THF series. The optimal length

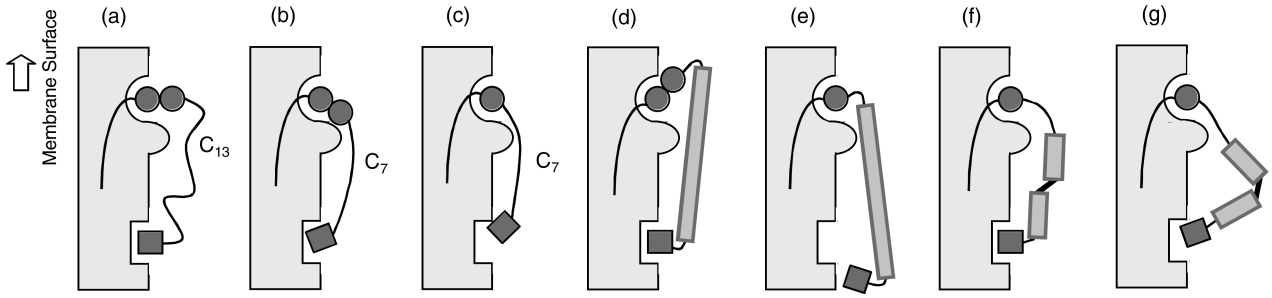

FIGURE 5: Schematic presentation of a model of the binding of acetogenin to complex I. When the inhibitor exhibits its effect, the hydroxylated THF ring (●) and the γ-lactone ring (■) have to bind simultaneously to the two putative binding sites, which are located within a distance shorter than that of the extended 13-carbon methylene chain. We propose some steric congestion in the vicinity of the THF ring moiety, as illustrated by a protuberance adjacent to the binding pocket: (a) bis-THF derivative possessing 13 carbon atoms, (b) bis-THF derivative possessing seven carbon atoms, (c) mono-THF derivative possessing seven carbon atoms, (d) bis-THF derivative possessing a tetrayne structure, (e) mono-THF derivative possessing a tetrayne structure, (f) *trans*-**24**, and (g) *cis*-**24**.

of the spacer for both series was shown to be 13 carbon atoms. This finding is consistent with the fact that regardless of the number of THF rings, natural acetogenins possessing 13 carbon atoms elicit the most potent activity among the related analogues (*6, 17–19, 22*). Given that the two toxophores are located within a distance shorter than that of the 13 extended carbon atoms (methylenes), the additional methylenes enhance the hydrophobicity of the spacer region, which may be thermodynamically advantageous for bringing the polar γ-lactone ring into the membrane-embedded segment of complex I. A photoaffinity labeling study revealed that the binding site of the γ-lactone portion is in the ND1 subunit that resides in the membrane arm (*10*). Elongation beyond 13 carbon atoms is adverse to the activity primarily because of the entropic disadvantage inherent in folding the long alkyl spacer for enabling cooperative binding of the two toxophores. Furthermore, we observed that a decrease in the flexibility of the spacer region is more adverse to the action of the mono-THF series than that of the bis-THF series. One of the primary aims of this study is to clarify the cause of this difference.

On the basis of the SAR studies performed in this work and previously (*25*), we propose a model for the binding of acetogenins to complex I, including both series (Figure 5). The polar hydroxylated THF ring moiety (●) may reside at the membrane surface area in the enzyme. When acetogenins exhibit inhibition, both the THF and the γ-lactone (■) rings have to occupy simultaneously the two putative binding sites (model a), which must be located within a distance shorter than that of the 13-carbon extended methylene chain, as mentioned above.

All of our observations with respect to the SAR of acetogenins can be explained assuming that (i) both THF rings of the bis-THF series are not necessarily crucial for the inhibitory action and the THF ring adjacent to the spacer, termed the "additional THF", is capable of working as a pseudospacer and (ii) there is some steric congestion in the vicinity of the THF ring moiety, as illustrated by a protuberance adjacent to the binding pocket (Figure 5). The first assumption is supported by numerous SAR studies indicating that the recognition of the hydroxylated THF moiety by the enzyme is not rigorous since neither the number of THF rings nor the stereochemistry around the hydroxylated THF ring moiety is a crucial structural factor for the inhibition (*6, 17–19, 22, 31*). That the hydroxy group flanking the additional THF is not essential for the activity (*20*) also seems to support the idea. The second assumption may be supported by the fact that the hardening of the spacer close to the THF ring is more adverse to the activity than the hardening of the region close to the γ-lactone ring (**11** vs **15**). No such positional specificity in the hardening was found in the bis-THF series (*25*). On the basis of this model, we will describe three observations that seem to be complicated to reconcile.

First is the observation that the bis-THF derivatives (**2** and **3**) possessing a short spacer are more active (by ~10-fold) than the corresponding mono-THF derivatives (**17** and **19**). This result can be explained as follows: the bis-THF derivatives are able to overcome the shortage of the spacer's length more efficiently than the mono-THF derivatives with the help of the additional THF which acts as a pseudospacer (model b vs model c). Second, in contrast to the mono-THF series, the tetrayne derivatives of the bis-THF series retain strong activity (e.g., **4** vs **5**). Similarly, this result can be explained by the notion that the additional THF serves as a pseudospacer and manipulates the rodlike tetrayne structure to properly adjust the binding of the γ-lactone ring to the enzyme, which mono-THF derivatives are less capable of doing (model d vs model e). Third, we will consider two findings concerning azobenzene derivatives: (i) regardless of the drastic difference in conformational properties, both the *trans* and *cis* isomers of the azobenzene derivatives are able to exhibit potent activity, and (ii) the relative inhibitory potencies of the *trans* and *cis* isomers were reversed between **24** and **25**. These findings indeed cannot be explained supposing the presence of a receptor wall for the spacer region. The flexible methylene chain in the spacer should be capable of manipulating the position of the γ-lactone ring attached to the rigid azobenzene by properly folding (model f vs model g). Given that the spatial distance between the two para positions in the azobenzene is longer for the *trans* isomer (extended form) than for the *cis* isomer (short form) (*30*), it is reasonable that the *trans* and *cis* isomers are favorable for the activity of the derivatives possessing a shorter (**24**) and a longer (**25**) alkyl spacer, respectively. Presumably, as a common phenomenon supporting the intensive inhibitor−enzyme interaction described above, some sort of ligand-induced conformational change in the binding domain might occur, which enables the cooperative binding of the two toxophores.

In conclusion, acetogenins exhibit potent inhibition of bovine complex I only when the two toxophoric moieties are directly linked by the alkyl spacer and cooperatively bind

to the two putative binding sites. Regardless of marked changes in the conformation and/or length of the spacer region, the spacer of the bis-THF series dynamically regulates the cooperative binding of the two toxophores to the sites. One of the two THF rings, being adjacent to the spacer, in bis-THF acetogenins may serve as a pseudospacer to overcome the significant structural disadvantage that occurrs in the spacer portion, whereas mono-THF acetogenins cannot efficiently adapt to such structural changes. This study along with the previous work (*25*) strongly suggests that there should be no receptor wall for the spacer region of acetogenins bound to the enzyme.

SUPPORTING INFORMATION AVAILABLE

Syntheses of compounds **7**−**19** and **21**−**25** and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Zeng, L., Ye, Q., Oberlies, N. H., Shi, G., Gu, Z. M., He, K., and McLaughlin, J. L. (1996) Recent advances in annonaceous acetogenins. *Nat. Prod. Rep. 13*, 275–306.
2. Alali, F. Q., Liu, X. X., and McLaughlin, J. L. (1999) Annonaceous acetogenins: Recent progress. *J. Nat. Prod. 62*, 504–540.
3. Bermejo, A., Figadere, B., Zafra-Polo, M.-C., Barrachina, I., Estornell, E., and Cortes, D. (2005) Acetogenins from Annonaceae: Recent progress in isolation, synthesis and mechanisms of action. *Nat. Prod. Rep. 22*, 269–303.
4. Lannuzel, A., Michel, P. P., Höglinger, G. U., Champy, P., Jousset, A., Medja, F., Lombes, A., Darios, F., Gleye, C., Laurens, A., Hocquemiller, R., Hirsch, E. C., and Ruberg, M. (2003) The mitochondrial complex I inhibitor annonacin is toxic to mesencephalic dopaminergic neurons by impairment of energy metabolism. *Neuroscience 121*, 287–296.
5. Escobar-Khondiker, M., Höllerhage, M., Muriel, M. P., Champy, P., Bach, A., Depienne, C., Respondek, G., Yamada, E., Lannuzel, A., Yagi, T., Hirsch, E. C., Oertel, W. H., Jacob, R., Michel, P. P., Ruberg, M., and Höglinger, G. U. (2007) Annonacin, a natural mitochondrial complex I inhibitor, causes tau pathology in cultured neurons. *J. Neurosci. 27*, 7827–7837.
6. Degli Esposti, M., Ghelli, A., Ratta, M., Cortes, D., and Estornell, E. (1994) Natural substances (acetogenins) from the family *Annonaceae* are powerful inhibitors of mitochondrial NADH dehydrogenase (complex I). *Biochem. J. 301*, 161–167.
7. Friedrich, T., Van Heek, P., Leif, H., Ohnishi, T., Forche, E., Kunze, B., Jansen, R., Trowitzsch-Kienast, W., Höfle, G., Reichenbach, H., and Weiss, H. (1994) Two binding sites of inhibitors in NADH: ubiquinone oxidoreductase (complex I). *Eur. J. Biochem. 219*, 691–698.
8. Okun, J. G., Lümmen, P., and Brandt, U. (1999) Three classes of inhibitor share a common binding domain in mitochondrial complex I (NADH-ubiquinone oxidoreductase). *J. Biol. Chem. 274*, 2625–2630.
9. Miyoshi, H. (2001) Probing the ubiquinone reduction site in bovine mitochondrial complex I using a series of synthetic ubiquinones and inhibitors. *J. Bioenerg. Biomembr. 33*, 223–231.
10. Murai, M., Ishihara, A., Nishioka, T., Yagi, T., and Miyoshi, H. (2007) The ND1 subunit constructs the inhibitor binding domain in bovine heart mitochondrial complex I. *Biochemistry 46*, 6409–6416.
11. Yagi, T., and Matsuno-Yagi, A. (2003) The proton-translocating NADH-quinone oxidoreductase in the respiratory chain: The secret unlocked. *Biochemistry 42*, 2266–2274.
12. Takada, M., Kuwabara, K., Nakato, H., Tanaka, A., Iwamura, H., and Miyoshi, H. (2000) Definition of crucial structural factors of acetogenins, potent inhibitors of mitochondrial complex I. *Biochim. Biophys. Acta 1460*, 302–310.
13. Hoppen, S., Emde, U., Friedrich, T., Grubert, L., and Koert, U. (2000) Natural-product hybrid: Design, synthesis, and biological evaluation of quinone-annonaceous acetogenins. *Angew. Chem., Int. Ed. 39*, 2099–2102.
14. Torm, J. R., Estornell, E., Gallardo, T., Gonzalez, M. C., Cave, A., Granell, S., Cortes, D., and Zafra-Polo, M. C. (2001) γ-Lactone-functionalized antitumal acetogenins are the most potent inhibitors of mitochondrial complex I. *Bioorg. Med. Chem. Lett. 11*, 681–684.
15. Torm, J. R., Gallardo, T., Peris, E., Bermejo, A., Cabedo, N., Estornell, E., Zafra-Polo, M. C., and Cortes, D. (2003) Inhibitory effects on mitochondrial complex I of semisynthetic mono-tetrahydrofuran acetogenin derivatives. *Bioorg. Med. Chem. Lett. 13*, 4101–4105.
16. Yabunaka, H., Abe, M., Kenmochi, A., Hamada, T., Nishioka, T., and Miyoshi, H. (2003) Synthesis and Inhibitory Activity of Ubiquinone-Acetogenin Hybrid Inhibitor with Bovine Mitochondrial Complex I. *Bioorg. Med. Chem. Lett. 13*, 2385–2388.
17. Miyoshi, H., Ohshima, M., Shimada, H., Akagi, T., Iwamura, H., and McLaughlin, J. L. (1998) Essential structural factors of Annonaceous acetogenins as potent inhibitors of mitochondrial complex I. *Biochim. Biophys. Acta 1365*, 443–452.
18. Makabe, H., Miyawaki, A., Takahashi, R., Hattori, Y., Konno, H., Abe, M., and Miyoshi, H. (2004) Synthesis of two possible diastereomers of reticulatain-1. *Tetrahedron Lett. 45*, 973–977.
19. Makabe, H., Hattori, Y., Kimura, Y., Konno, H., Abe, M., Miyoshi, H., Tanaka, A., and Oritani, T. (2004) Total synthesis of *cis*-solamin and its inhibitory action with bovine heart mitochondrial complex I. *Tetrahedron 60*, 10651–10657.
20. Abe, M., Kenmochi, A., Ichimaru, N., Hamada, T., Nishioka, T., and Miyoshi, H. (2004) Essential structural features of acetogenins: Role of hydroxy groups adjacent to the bis-THF rings. *Bioorg. Med. Chem. Lett. 14*, 779–782.
21. Motoyama, T., Yabunaka, H., and Miyoshi, H. (2002) Essential structural factors of acetogenins, potent inhibitors of mitochondrial complex I. *Bioorg. Med. Chem. Lett. 12*, 2089–2092.
22. Tormo, J. R., Gallardo, T., Aragon, R., Cortes, D., and Estornell, E. (1999) Specific interactions of mono-tetrahydrofuranic annonaceous acetogenins as inhibitors of mitochondrial complex I. *Chem.-Biol. Interact. 122*, 171–183.
23. Kuwabara, K., Takada, M., Iwata, J., Tatsumoto, K., Sakamoto, K., Iwamura, H., and Miyoshi, H. (2000) Design syntheses and mitochondrial complex I inhibitory activity of novel acetogenin mimics. *Eur. J. Biochem. 267*, 2538–2546.
24. Hamada, T., Ichimaru, N., Abe, M., Fujita, D., Kenmochi, A., Nishioka, T., Zwicker, K., Brandt, U., and Miyoshi, H. (2004) Synthesis and inhibitory action of novel acetogenin mimics with bovine heart mitochondrial complex I. *Biochemistry 43*, 3651–3658.
25. Abe, M., Murai, M., Ichimaru, N., Kenmochi, A., Yoshida, T., Kubo, A., Kimura, Y., Moroda, A., Makabe, H., Nishioka, T., and Miyoshi, H. (2005) Dynamic function of the alkyl spacer of acetogenins in their inhibitory action with mitochondrial complex I (NADH-ubiquinone oxidoreductase). *Biochemistry 44*, 14898–14906.
26. Matsuno-Yagi, A., and Hatefi, Y. (1985) Studies on the mechanism of oxidative phosphorylation. *J. Biol. Chem. 260*, 14424–14427.
27. Ichimaru, N., Murai, M., Abe, M., Hamada, T., Yamada, Y., Makino, S., Nishioka, T., Makabe, H., Makino, A., Kobayashi, T., and Miyoshi, H. (2005) Synthesis and inhibition mechanism of Δlac-acetogenins: A novel type of inhibitor of bovine heart mitochondrial complex I. *Biochemistry 44*, 816–825.
28. Fujita, D., Murai, M., Nishioka, T., and Miyoshi, H. (2006) Light control of mitochondrial complex I activity by a photoresponsive inhibitor. *Biochemistry 45*, 6581–6586.
29. Yang, L., Takisawa, N., Hayashita, T., and Shirahama, K. (1995) Colloid chemical characterization of the photosurfactant 4-ethylazobenzene 4′-(oxyethyl)trimethylammonium bromide. *J. Phys. Chem. 99*, 8799–8803.
30. Henzl, J., Mehlhorn, M., Grawronski, H., Rieder, K. H., and Morgenstern, K. (2006) Reversible *cis-trans* isomerization of a single azobenzene molecule. *Angew. Chem., Int. Ed. 45*, 603–606.
31. Fujita, D., Ichimaru, N., Abe, M., Murai, M., Hamada, T., Nishioka, T., and Miyoshi, H. (2005) Synthesis of non-THF analogs of acetogenin toward simplified mimics. *Tetrahedron Lett. 46*, 5775–5779.

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