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Essential structural factors of annonaceous acetogenins as potent inhibitors of mitochondrial complex I

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Abstract

The annonaceous acetogenins are the most potent of the known inhibitors of bovine heart mitochondrial complex I. These inhibitors act, at the terminal electron transfer step of the enzyme, in a similar way to the usual complex I inhibitors, such as piericidin A and rotenone; however, structural similarities are not apparent between the acetogenins and these known complex I inhibitors. A systematic set of isolated natural acetogenins was prepared and examined for their inhibitory actions with bovine heart mitochondrial complex I to identify the essential structural factors of these inhibitors for the exhibition of potent activity. Despite their very potent activity, the structural requirements of the acetogenins are not particularly rigid and remain somewhat ambiguous. The most common structural units, such as adjacent bis-tetrahydrofuran (THF) rings and hydroxyl groups in the 4- and/or 10-positions, were not essential for exhibiting potent activity. The stereochemistry surrounding the THF rings, surprisingly, seemed to be unimportant, which was corroborated by an exhaustive conformational space search analysis, indicating that the model compounds, with different stereochemical arrangements around the THF moieties, were in fairly good superimposition. Proper length and flexibility of the alkyl spacer moiety, which links the THF and the α,β-unsaturated γ-lactone ring moieties, were essential for the potent activity. This probably results from some sort of specific conformation of the spacer moiety which regulates the two ring moieties to locate into an optimal spatial position on the enzyme. It is, therefore, suggested that the structural specificity of the acetogenins, required for optimum inhibition, differs significantly from that of the common complex I inhibitors in which essential structural units are compactly arranged and conveniently defined. The structure–activity profile for complex I inhibition is discussed in comparison with those for other biological activities.

Keywords: NADH-ubiquinone oxidoreductase; Acetogenin; Structure–activity relationship

1. Introduction

A large number of natural acetogenins have been isolated from several genera of the plant family, Annonaceae [1,2]. Many of these compounds have diverse biological effects, such as cytotoxic, in vivo

Abbreviations: Complex I, NADH-ubiquinone oxidoreductase; Q, ubiquinone; Q_1, ubiquinone-1; Q_2, ubiquinone-2; Q_{10}, ubiquinone-10; diethoxy-Q_2, 2,3-diethoxy-6-geranyl-5-methyl-1,4-benzoquinone; SMP, submitochondrial particles; THF, tetrahydrofuran

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antitumor, antimalarial, pesticidal, and antifeedant activities [3–5]. In particular, the inhibitory effect of acetogenins on mitochondrial NADH-ubiquinone oxidoreductase (complex I) is worthy of notice not only because some of the compounds, such as bullatacin and rolliniastatin 1, are the most potent inhibitors of the enzyme so far known [6–8], but also because it is quite difficult to visualize structural similarities between the acetogenins and usual complex I inhibitors, such as piericidin A and rotenone; yet, the acetogenins act at the terminal electron transfer step of complex I (i.e. between Fe-S cluster 2 and the ubiquinone pool) similar to the usual complex I inhibitors [6,7,9].

The annonaceous acetogenins are fairly large molecules compared to the ordinary complex I inhibitors including the newer potent agrochemicals, such as fenpyroximate and SAN548A. The average length of the acetogenin molecules is about twice that of piericidin A when they are compared in an extended conformation. The structural specificity of these acetogenins is that the two functional units (i.e. the hydroxylated tetrahydrofuran (THF) and α,β-unsaturated γ-lactone ring moieties) are separated by a long alkyl chain, although the two moieties could each play significant roles in binding interactions to the enzyme. Thus, considering the unusual structural characteristics as well as the markedly strong inhibitory potency of acetogenins, the study of the action of these inhibitors is important to elucidate structural and functional features of the terminal electron transfer step of complex I. As the first step toward this purpose, the identification of the essential structural factors of the acetogenins for exhibiting the most potent inhibition is earnestly required.

In general, to identify important structural factors of biologically active compounds based on their structure–activity relationships, a series of derivatives is needed in which chemical structures are widely and systematically modified. Several structure–activity studies of acetogenins for complex I inhibition have been carried out [6–8,10–12]; the important structural factors have not been fully defined yet, and only limited structural variations of the acetogenins have been tested previously. Since total syntheses of a variety of acetogenins is still difficult [13–16], we have utilized structurally diverse representatives of the series of isolated natural products that we have available. In this study, we prepared a systematic set of such isolated natural acetogenins (Fig. 1) among which comparisons of activity changes of closely related derivatives is feasible, and we have examined their inhibition of bovine heart mitochondrial complex I. This study is the first detailed structure–activity study of the acetogenins for the inhibition of mitochondrial complex I, and it has revealed several interesting structural factors of the acetogenins required for the potent inhibition of this enzyme.

2. Materials and methods

2.1. Materials

Antimycin A was purchased from Sigma. MOA-stilbene was provided by Aburahi Laboratories, Shionogi (Shiga, Japan). Q_1 (ubiquinone-1) was a generous gift from Eisai (Tokyo, Japan). Piericidin A was generously provided by Dr. Shigeo Yoshida (RIKEN, Japan). Diethoxy-Q_2 (2,3-diethoxy-6-geranyl-5-methyl-1,4-benzoquinone) was from a previous sample [17]. Other chemicals were commercial products of analytical grade.

2.2. Preparation of acetogenins

The acetogenins used in this study (Fig. 1) were isolated from ethanol extracts of various annonaceous plants [1–3]. The purity of the acetogenins was chromatographically (thin-layer and HPLC) and spectroscopically (^{1}H- and ^{13}C-NMR spectroscopy) confirmed. NMR spectra were recorded on a Bruker ARX-300 and a Varian VXR-500.

2.3. Methods

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 −78°C.

The NADH oxidase activity was followed spectrometrically with a Shimadzu UV-3000 at 340 nm ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$) at 30°C. The reaction medium

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 SMP were equilibrated with inhibitor for 5 min. The NADH-Q oxidoreductase activity was determined following NADH oxidation at 30°C in the same reaction medium in the presence of indicated concentrations of exogenous Q, 0.2 μM antimycin A, 0.2 μM MOA-stilbene and 2 mM KCN.

3. Results

Acetogenin molecules investigated in this study can be dissected into four chemical portions, for simplicity, as follows: the hydroxylated THF ring moiety, the α,β-unsaturated γ-lactone ring moiety, the spacer moiety linking the two rings, and the hydrophobic side chain attached to the THF ring(s) which ends with the terminal methyl. We examined the roles of each portion in exhibiting potent inhibitory action with bovine complex I. The inhibitory potencies of all compounds in terms of the I_{50} value, which is the molar concentration in the reaction medium needed to inhibit one-half of the control enzyme (NADH oxidase) activity, are listed in Table 1.

Table 1
Summary of inhibitory potencies of acetogenins

Compound no.	I_{50} (nM)	Compound no.	I_{50} (nM)
1	1.2	12	330
2	1.4	13	3.8
3	1.9	14	4.1
4	14	15	6.1
5	5.4	16	21
6	1.8	17	2.4
7	1.6	18	1.6
8	1.4	19	9.4
9	1.6	20	17
10	3.3	21	2.9
11	1.5	22	26
		(Piericidin A)	2.1

The I_{50} value is the molar concentration in the reaction medium needed to halve the control NADH oxidase activity. The control enzyme activity was 0.55–0.60 μmol NADH/min/mg of protein. The values were averaged from two independent measurements.

3.1. The role of the stereochemistry and number of the THF rings

Compound 1 (bullatacin) was revealed to be more potent than piericidin A, and, as noted previously, it was also one of the most potent acetogenins with bovine complex I [6,7]. We confirmed that compound 1 is about two-times more potent than piericidin A when the I_{50} values were compared under the present experimental conditions. A different structural factor between compounds 1 and 2 (trilobacin) is solely the configuration of the adjacent bis-THF ring moiety. The inhibitory potencies of the two compounds were almost identical, taking into account experimental errors. This indicated that the stereochemical arrangement of the bis-THF ring was not an essential structural factor for potent activity. This notion would be supported by the earlier observation that the potencies of rolliniastatin 1 and bullatacin, which also differ from each other only in the stereochemical arrangement of the THF rings, are almost identical [6]. In [6], the authors emphasized a slight difference in the inhibitory potencies of rolliniastatin 1 and bullatacin, whereas the I_{50} and K_i values of the two compounds would be taken as almost identical, considering experimental variations in their work (see Figs. 2 and 3 in [6]). Three-dimensional structural features of the hydroxylated adjacent bis-THF moieties are not so different between the two stereoisomers, as discussed later.

Compounds 8 (sylvaticin) and 9 (bullatalicin) retained the potent activity despite bearing different configurations in one of the two non-adjacent THF rings. This finding indicates not only that adjacent bis-THF rings are not essential for the activity, but also that the configurations of the THF ring moieties are not so important, as mentioned above (compounds 1 vs. 2). In addition, the fact that the I_{50} value of compound 11 (muricatetrocin B) was almost identical to that of the most potent inhibitors like compound 1 indicates that the presence of a mono-THF ring can be sufficient enough to retain the potent activity.

To compare the three-dimensional structures of the hydroxylated adjacent bis-THF rings between stereoisomers, the method of exhaustive conformational space search [19] was employed for model compounds A and B (Fig. 2) which are component

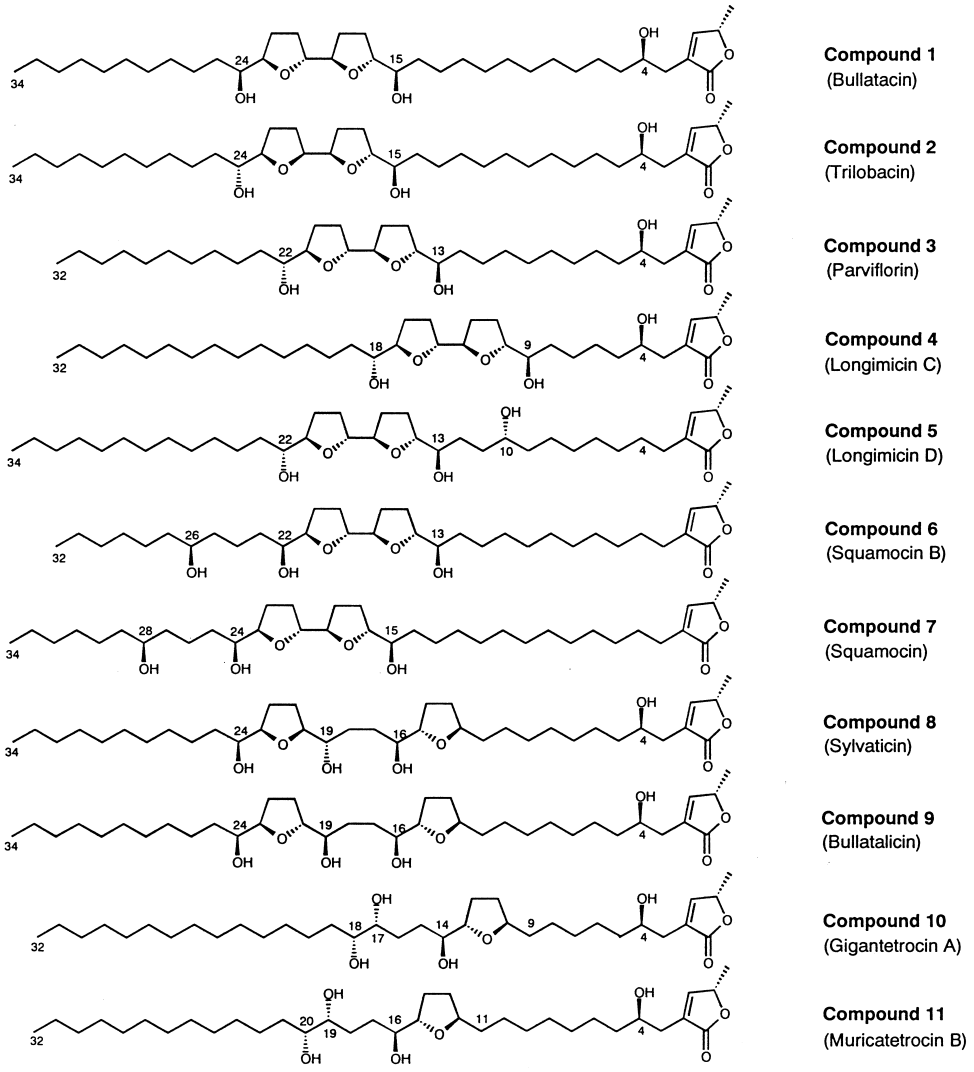

Fig. 1. Structures of acetogenins used in this study.

parts of compounds 1 and 2, respectively. In each model compound, single bonds were rotated by the step of 60°, and 7776 initial conformations were optimized by quantum chemical calculations (MNDO-AM1). A total of 185 and 169 stable conformations were obtained for models A and B, respectively. COMPASS (common geometrical pattern search system) [20] followed by molecular field fitting was implemented for all combinations of the stable conformations of each molecule. Although stereochemical arrangement of the left side moiety of the model compounds is different, molecular field fitting resulted in fairly good superimposition. In 1786 of 31 265 (=185×169) cases, the similarity index was greater than 90%. Some of the best fittings (i.e. similarity index greater than 95%) are shown in Fig. 2. These results strongly suggested that stereochemical differences of the bis-THF rings make little difference in the three-dimensional structure of this moiety.

Recently, Shimada et al. [21] reported that the THF rings of the annonaceous acetogenins strongly interact with the glycerol backbone region of lipids in liposomal membranes, and it was proposed that the THF rings may serve a role as an anchor in the membrane to optimize the location and the conformation of the functional group(s) of the acetogenins. If the role of the THF rings is, indeed, as an anchor at the interface of the membrane, the stereochemical

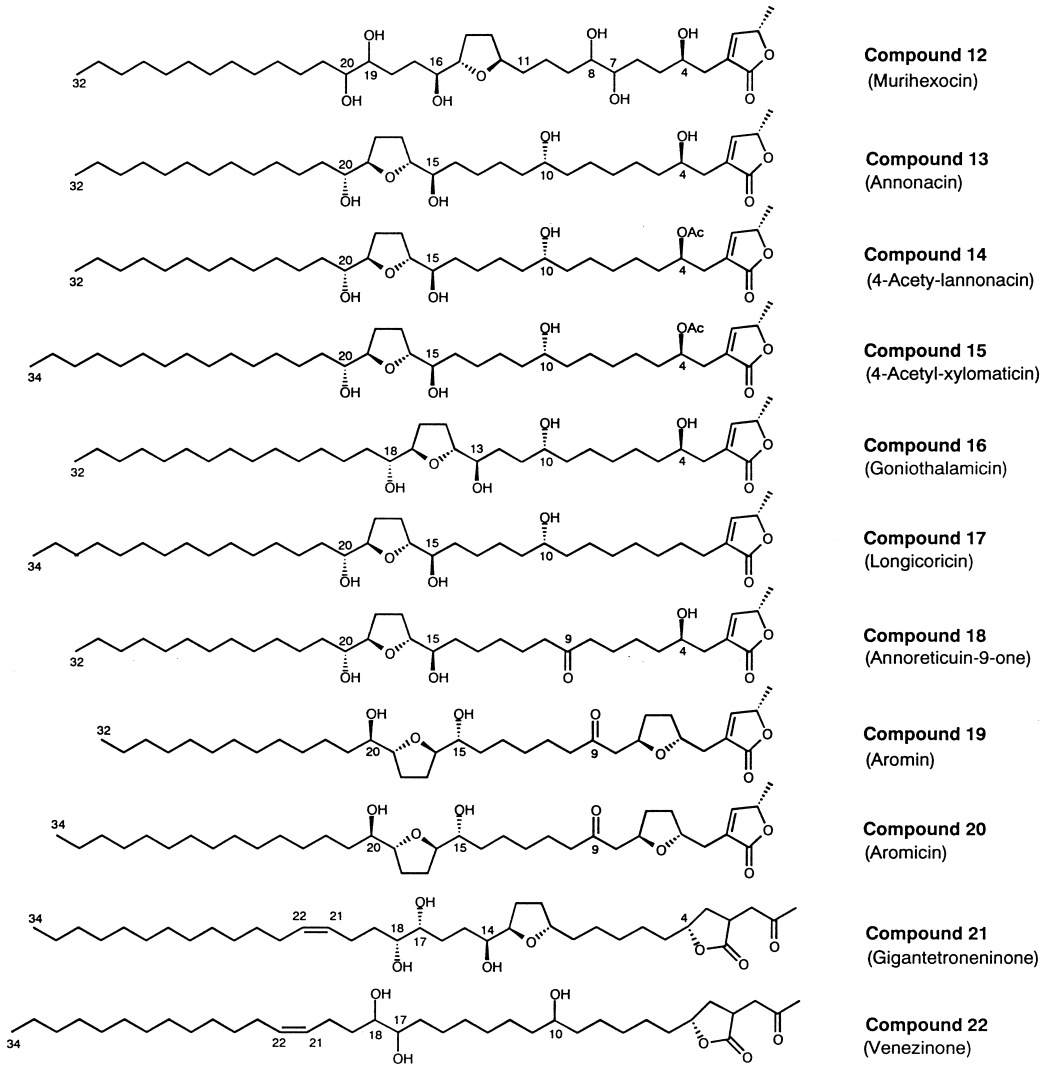

Fig. 1 (continued).

differences within the THF rings of the acetogenins should not make much difference in their bioactivity profiles which agrees with the results of the COMPASS analysis.

3.2. The role of the length of the spacer moiety

Comparing the I_{50} values of compounds 1, 3 (parviflorin) and 4 (longimicin C), clearly revealed that the shorter the length of the spacer moiety, the weaker the potency becomes. This notion was also supported from comparisons of compounds 10 (gigantetrocin A) vs. 11 (muricatetrocin B) and compounds 13 (annonacin) vs. 16 (goniothalamicin), wherein structural features, such as total number of carbon atoms, substituted positions of OH groups, and configuration of the THF moiety, are identical except for the length of the spacer. Therefore, the spacer moiety seems to regulate some sort of significant spatial arrangement between the γ-lactone and THF ring moieties irrespective of the number of the THF rings.

3.3. The role of hydroxy groups in the spacer moiety

Compound 5 (longimicin D), in which the length of the spacer is the same as that of compound 3 but with one OH group moved from the 4-position to the

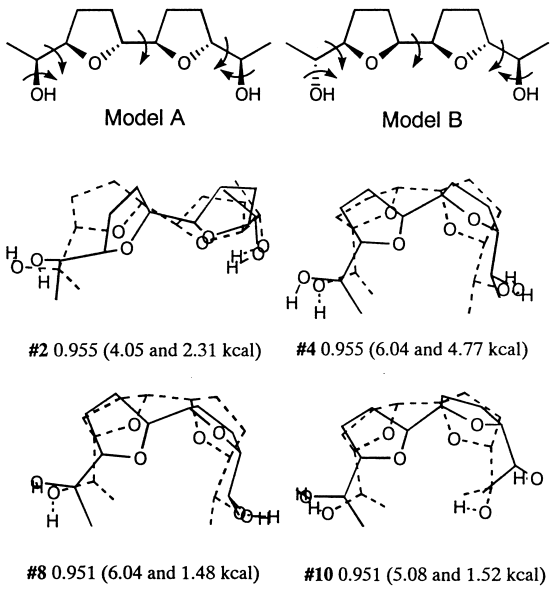

Fig. 2. Superimposition of model compounds A (solid line) and B (dotted line). Model compounds A and B are the model of the bis-THF rings moiety of compounds 1 (bullatacin) and 2 (trilobacin), respectively. The single bonds indicated by arrows were rotated by the step of 60°. Similarity index and conformation energy for each superimposition are shown below the view.

10-position, was slightly less potent than compound 3. However, since the I_{50} value of compound 5 was retained at the nM level, the 4-OH group itself seems not to be essential for the activity. In support of this, compounds 6 (squamocin B) and 7 (squamocin), in which the positions of the third OH groups are in the terminal alkyl chain at the 26- and 28-positions, respectively, instead of between the two ring moieties, maintained the potent activities. This conclusion was valid irrespective of the number of the THF rings (one or two) in the middle of the molecule, as seen in compounds 13–15 and 17. In addition, compound 18 (annoreticuin-9-one) was rather more potent than compound 13, though the only structural difference between them is that the 10-OH group of the latter is transformed to 9-oxo in the former. This also indicates that the common 4,10-dihydroxy substitution pattern in the spacer moiety is not essential for the potent activity.

Compound 12 (murihexocin), in which two additional OH groups are attached in the spacer moiety of compound 11, appeared not only to be a much poorer inhibitor than compound 11, but also to be the poorest inhibitor among all of the compounds evaluated in this study. This is probably because the presence of the two vicinal OH groups in this spacer region prevents the γ-lactone and THF ring moieties from positioning into the optimal spatial location suggested above. Considering that both compounds 11 and 12 possess the same total number of carbon atoms between the THF and the γ-lactone rings, the alkyl chain length between the two ring systems cannot be the reason for their different activity profiles, but the difference in their polarities in their spacer regions would seem to be quite important. Considering this and the above results, it seems likely that no polar functional group is required within the spacer moiety to retain the potent activity and too many polar functional groups therein hinder the activity.

The structural profile of compounds 19 (aromin) and 20 (aromicin) resemble compound 18, but these compounds possess one THF ring within the spacer moiety. The activities of compounds 19 and 20 were decreased compared to that of compound 18. It can be argued that the THF ring (located in the middle of the molecule) and the γ-lactone ring are not correctly positioned for optimal spatial location due to the presence of the rigid THF ring at positions 4–7. This result also suggested that, when similar acetogenins possess the same number of carbons between the THF and the γ-lactone ring systems, the different chemical groups (a hydroxyl vs. a THF ring in this

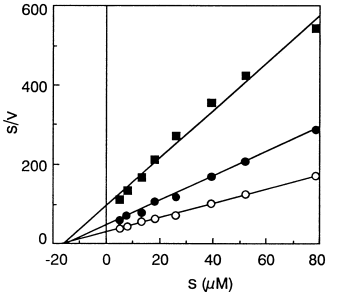

Fig. 3. Hanes–Woolf plot of kinetics data of NADH-Q_1 oxidoreductase activity in the presence of compound 1 (bullatacin). The reaction medium, in a final volume of 2.5 ml, contained 50 mM phosphate buffer (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 50 μM Q_1, 0.2 μM antimycin A, 0.2 μM MOA-stilbene and 2 mM KCN. The final mitochondrial protein concentration was 30 μg of protein/ml. The reaction was started by adding 50 μM NADH. The concentration of compound 1 was 0 nM (○), 0.9 nM (●) or 1.5 nM (■).

case) in their spacer regions are responsible for their different activity profiles.

3.4. The role of the length of the alkyl side chain

Compound 20 was 50% less active than compound 19, although the tail moiety of the former is longer than that of the latter by two carbon atoms. This finding indicates that if other structural factors are identical, a less hydrophobic tail is favorable for the activity. This tendency is also observed with the pair of compounds 14 and 15. It can be suggested that 13 carbons in the tail moiety of compounds 19 and 14 are sufficiently hydrophobic, and a further increase in hydrophobicity of the tail is rather adverse to the activity due to some sort of trapping in the hydrophobic lipid bilayer of the membrane. Similar behavior has been observed for very hydrophobic inhibitors of respiratory enzymes, such as synthetic antimycins [22] and piericidins [23]. It should be mentioned, however, that this consideration does not necessarily mean that a shorter tail structure would be better for the activity.

3.5. Characterization of inhibitory action of acetogenins

The inhibition mechanism of compound 1 (bullatacin), the most potent acetogenin studied here, was examined in the NADH-Q_1 oxidoreductase assay

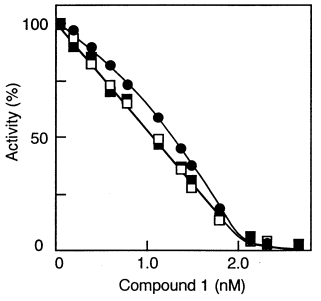

Fig. 4. The inhibition of electron transfer by compound 1 (bullatacin) with various ubiquinones. For NADH-Q oxidoreductase activity, the reaction conditions were the same as in the legend to Fig. 3. As an electron acceptor, 50 μM Q_1 (□) or 100 μM 2,3-$(EtO)_2$-Q_2 (■) was used. The inhibition of NADH oxidase activity (●) was also examined under the same reaction conditions, except that complexes III and IV inhibitors were omitted from the reaction medium. The extent of inhibition attained by 0.1 μM piericidin A was taken as 100% inhibition.

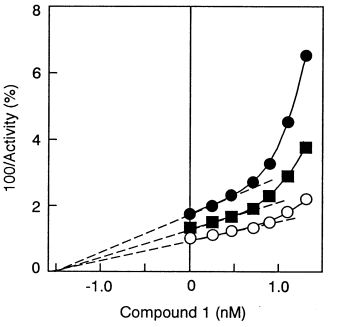

Fig. 5. Dixon plots of compound 1 (bullatacin) in the presence of rotenone. NADH oxidase activity was determined under the experimental conditions given in Fig. 4. Rotenone concentrations were 0 nM (○), 1.4 nM (■) and 2.7 nM (●).

with a series of concentrations of Q_1 added to the system. As shown in Fig. 3, the inhibition mechanism was non-competitive against Q_1. Non-competitive inhibition was also suggested by other plots of the experimental data, such as 1/[S] vs. 1/rate and rate/[S] vs. rate. In support of this, the inhibitory potency of this compound was not affected irrespective of which Q structure was used as an electron acceptor, as shown in Fig. 4. Complete inhibition was attained in the presence of about 2 nM of compound 1 in all cases. Although a titration curve was almost linear when exogenous Q (Q_1 or diethoxy-Q_2) was used as an electron acceptor, that for the NADH oxidase activity (closed circles) was slightly sigmoidal. A similar sigmoidal titration curve was observed with rolliniastatin 1 in the earlier study [8], whereas the reason for this remains to be elucidated. The fact that the sensitivity of bulky diethoxy-Q_2 to the inhibition by compound 1 was similar, but not identical, to that of endogenous Q (Q_{10}) supports the previous finding that this bulky substrate accepts electrons from the physiological site of the enzyme [17].

We also performed the same experiments for compounds 12 and 18. Similarly to compound 1, the inhibition mechanism of these two compounds was non-competitive against Q_1 despite a large difference in the inhibitory potencies, and their inhibitory potencies were not affected irrespective of which Q structure was used as an electron acceptor (data not shown).

Earlier studies [6,11] investigated the Dixon plot analysis to determine the mutual exclusivity between acetogenins as well as between acetogenins and rote-

none. We carried out the same analysis by titrating compound 1 in the presence of different fixed concentrations of rotenone which exhibited inhibition less than 50%. As shown in Fig. 5, the plots were curvilinear especially in the region of high concentrations of compound 1. Extrapolation of the linear part of the plots at the low concentration range gave the common intercept at the *x*-axis (dotted lines), suggesting that compound 1 and rotenone are mutually non-exclusive inhibitors. Although this result seems to be consistent with the earlier observations [6,11], the Dixon plot analysis should be interpreted with care since the extrapolated straight line is significantly affected depending upon the region of inhibitor concentrations of interest, as readily realized from Fig. 5. The earlier studies did not mention this, and the Dixon plots therein were linearly extrapolated throughout all inhibitor concentrations. To draw precise conclusions about the mutual exclusivity between acetogenins as well as between acetogenins and rotenone, radioligand assay is required.

4. Discussion

The annonaceous acetogenins, which are previously reported to be the most potent complex I inhibitors, are those with adjacent bis-THF rings in the middle of the molecules [6–8]. The present structure–activity study clearly indicates that the adjacent bis-THF ring moiety is not an essential structural factor for exhibiting potent inhibition, and the mono-THF ring compounds can maintain the potent activity. The natural acetogenins have various stereochemical configurations around the hydroxylated THF ring moiety. This stereochemical factor was also not essential for potent activity irrespective of the number (one or two) of THF rings. This conclusion seemed to be supported by the exhaustive conformational space research study, which revealed that some stable conformations of the adjacent bis-THF rings of stereoisomers overlapped well. This conclusion also agrees with the observation by Shimada et al. [21] that the THF rings of the acetogenins had strong interactions with the interface of lipid bilayers irrelevant with the stereochemistry of the THF region. The presence of the 4- and/or 10-OH groups in the spacer region was also not essential for the activity. It seems that no polar substituent is required in this moiety.

On the other hand, it was demonstrated that the spacer moiety is very important for the potent activity. This finding, however, does not necessarily indicate that the distance, itself, between the THF ring(s) and the α,β-unsaturated γ-lactone ring is important for the activity, since the activities of the compounds which have the same or similar number of carbon atoms in the spacer often differed markedly (compounds 11 vs. 12 and compounds 18 vs. 19). If an extended conformation of the spacer moiety would be an active conformation, activities of the compounds which possess alkyl spacers of less than 13 carbon atoms, such as compounds 3, 4 and 16, would be reduced more drastically than those possessing alkyl chains of 13 carbon atoms, but the results were not so. Therefore, some sort of specific conformation of the spacer might regulate the two ring moieties to locate into an optimal spatial position which is essential to elicit the most potent inhibition. The design syntheses of acetogenins, in which the spatial position of the two ring moieties could be fixed to each other in different manners by specific structures of the spacer, would be helpful to elucidate the optimal conformation of the acetogenin molecule as well as the function of the spacer.

There are several structure–activity studies of very potent inhibitors of respiratory enzymes, such as antimycin A, stigmatellin, and piericidin A, which completely inhibit the enzyme activity at stoichiometric amounts with respect to their target enzymes (i.e. complex I or III). For these inhibitors, essential structural factors which affect the inhibitory potency by several orders of magnitude have been identified. For instance, a phenolic hydroxyl group of the salicylic acid moiety of antimycin A is essential for the potent activity since methylation of this functional group resulted in marked reduction of the potency by about four orders of magnitude [24,25]. Methylation of the pyridinol hydroxyl group of piericidin A reduced the potency by about 500-fold [26]. Modification of either the 8-hydroxy or the 4-oxo groups of the chromone ring system of stigmatellin affect the potency by three orders of magnitude [27]. Considering these facts, the structure–activity profile of acetogenins revealed in this study seems to be unusual since marked changes in the inhibitory potency (say,

by several orders of magnitudes) due to single structural modifications were not observed except for compound 12. Although an important role of the spacer moiety was revealed, other derivatives wherein the structures of the THF ring and/or the α,β-unsaturated γ-lactone moieties are widely modified, are required to know whether these common structural units are essential for the potent activity.

Concerning this respect, it should be noted that the number of isolated natural acetogenins in which the THF and/or the α,β-unsaturated γ-lactone moieties are deleted or modified is highly limited. However, we isolated two unusual compounds (compounds 21 (gigantetroneninone) and 22 (venezinone)) and investigated their activity as a preliminary examination to find a clue to the above issue. Both compounds have ketolactones in place of the α,β-unsaturated γ-lactone, and compound 22 has no THF ring in the middle of the molecule. The fact that the I_{50} value of compound 21 was retained at the nM level suggested that the common α,β-unsaturated γ-lactone is not really essential for potent activity. Although the length between the THF ring and the ketolactone of compound 21 is rather shorter than that of compounds 4 and 10, the former was more potent than the latter two. This suggests that the role of the spacer moiety might differ somewhat between the α,β-unsaturated γ-lactone and the ketolactone compounds. The potency of compound 22 decreased to about 1/10th that of compound 21, suggesting that the THF ring is important to maintain the most potent activity, but it is not essential for eliciting the inhibition. Further derivatives are needed to draw conclusions on the functions of the THF ring and the α,β-unsaturated γ-lactone.

An earlier study [6] reported that the acetogenins inhibit complex I activity in non-competitive or uncompetitive manners against exogenous Q depending upon inhibitor structures. The inhibition by bullatacin (compound 1) was uncompetitive against exogenous Q [6,11]. In contrast to this, the present study showed that the inhibition manner of acetogenins is non-competitive against Q_1 irrespective of the inhibitor structures. Although we have no proper explanation for this discrepancy, it could be mentioned that the inhibition mechanism was investigated in the earlier studies at a certain concentration of the inhibitor which elicits a fairly large extent of inhibition (i.e. at low residual enzyme activity). In addition, although the previous studies [6,11] discussed the binding manners (or sites) of acetogenins in relation to the proposed multiple Q binding sites model [28,29], we think that this model should be inferred by further experimental evidence[1] and such classification is impractical at present.

The structure–activity profile of acetogenins revealed in this study seems to be comparable to that observed for the inhibition of the growth of adriamycin resistant human mammary adenocarcinoma (MCF-7/Adr) cells [5] and the yellow fever mosquito larvae microtiter plate assay [30]. For example, conclusions about the structure–activity relationships with MCF-7/Adr cells can be drawn as follows: (1) a hydroxyl group at the 4-position is not required as long as there are a total of three hydroxyl groups in the bis-adjacent THF series; (2) distance of not less than 13 carbon atoms in the spacer moiety is optimum; and (3) the non-adjacent bis-THF ring and mono-THF compounds have similar potencies to the bis-adjacent THF compounds. The similar structure–activity profiles between complex I and MCF-7/Adr cells would be explained by the fact that the ability of the acetogenins to lower ATP levels, via inhibition of complex I in mitochondria, and, possibly, inhibition of the NADH oxidase of the plasma membranes of cancerous cells [31], would seem to inhibit the multidrug resistance (MDR) transporter proteins which require ATP for energizing the P-gp efflux pump [32,33]. Although our present study showed little difference in activity among acetogenins with different stereochemical arrangements of the THF rings, it was reported that the stereochemical arrangement of the THF rings has slight effect on the activity with MCF-7/Adr cells [5], and, thus, this assay has to take into consideration factors, such

[1] The proposal for a model with multiple binding sites for complex I inhibitors is primarily based on the earlier studies using ^{14}C-labeled rotenone and piericidin A (Horgan et al., J. Biol. Chem. 243 (1968) 834-843 and Gutman et al., J. Biol. Chem. 245 (1970) 1992–1997). However, these earlier studies were questioned by the recent radioligand assay using [^{3}H]dihydrorotenone (D.S. Higgins, J.T. Greenamyre, J., Neurosci. 16 (1996) 3807–3816), wherein the signal-to-noise ratio was markedly improved due to high specific activity of the ligand.

as membrane transport, intracellular transport and metabolic inactivation.

Acknowledgements

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References

[1] L. Zeng, Q. Ye, N.H. Oberlies, G. Shi, Z.-M. Gu, K. He, J.L. McLaughlin, Nat. Prod. Rep. 13 (1996) 275–306.
[2] Z.-M. Gu, G.-X. Zhao, N.H. Oberlies, J.L. McLaughlin in: J.T. Arnason, R. Mata, J.T. Romeo (Eds.), Recent Advances in Phytochemistry, Vol. 29, Plenum Press, New York, 1995, pp. 249–310.
[3] X.-P. Fang, M.J. Rieser, Z.-M. Gu, G.-X. Zhao, J.L. McLaughlin, Phytochem. Anal. 4 (1993) 27–67.
[4] J.L. McLaughlin, L. Zeng, N.H. Oberlies, D. Alfonso, H.A. Johnson, B.A. Cummings, in: P.A. Hedin, R.M. Hollingworth, E.P. Masler, J. Miyamoto, D.G. Thompson (Eds.), ACS Symposium Series, Phytochemicals for Pest Control, 658, American Chemical Society, Washington, DC, 1997, pp. 117–133.
[5] N.H. Oberlies, C.-J. Chang, J.L. McLaughlin, J. Med. Chem. 40 (1997) 2102–2106.
[6] M. Degli Esposti, A. Ghelli, M. Ratta, D. Cortes, E. Estornell, Biochem. J. 301 (1994) 161–167.
[7] R.M. Hollingworth, K.I. Ahammadsahib, G. Gadelhak, J.L. McLaughlin, Biochem. Soc. Trans. 22 (1994) 230–233.
[8] M.C. Gonzalez, J.R. Tormo, A. Bermejo, M.C. Zafra-Polo, E. Estornell, D. Cortes, Bioorg. Med. Chem. Lett. 7 (1997) 1113–1118.
[9] T. Friedrich, P. Van Heek, H. Leif, T. Ohnishi, E. Forche, B. Kunze, R. Jansen, W. Trowitzsch-Kienast, G. Höfle, H. Reichenbach, H. Weiss, Eur. J. Biochem. 219 (1994) 691–698.
[10] J.L. Landolt, K.I. Ahammadsahib, R.M. Hollingworth, F.L. Barr, N.L. Crane, N.L. Buerck, G.P. McCabe, J.L. McLaughlin, Chemico-Biol. Interact. 98 (1995) 1–13.
[11] E. Estornell, J.R. Tormo, D. Cortes, Biochem. Biophys. Res. Commun. 240 (1997) 234–238.
[12] D. Alfonso, H.A. Johnson, T. Colman-Saizarbitoria, C.P. Presley, G.P. McCabe, J.L. McLaughlin, Nat. Toxins 4 (1996) 181–188.
[13] H. Naito, E. Kawahara, K. Maruta, M. Maeda, S. Sasaki, J. Org. Chem. 60 (1995) 4419–4427.
[14] S.C. Sinha, A. Sinha, A. Yazbak, Z. Keinan, J. Org. Chem. 61 (1996) 7640–7641.
[15] J.A. Marshall, K.W. Hinkle, J. Org. Chem. 62 (1997) 5989–5995.
[16] J.A. Marshall, M. Chen, J. Org. Chem. 62 (1997) 5996–6000.
[17] H. Miyoshi, M. Inoue, S. Okamoto, M. Ohshima, K. Sakamoto, H. Iwamura, J. Biol. Chem. 272 (1997) 16176–16183.
[18] A. Matsuno-Yagi, Y. Hatefi, J. Biol. Chem. 260 (1985) 14424–14427.
[19] T. Akagi, Y. Takahashi, S. Sasaki, Quant. Struct.-Act. Relat. 15 (1996) 290–295.
[20] Y. Takahashi, T. Akagi, S. Sasaki, Tetrahedron Computer Methodology 3 (1990) 27–35.
[21] H. Shimada, J.B. Grutzner, J.F. Kozlowski, J.L. McLaughlin, Biochemistry 37 (1998) 854–866.
[22] N. Tokutake, H. Miyoshi, H. Nakazato, H. Iwamura, Biochim. Biophys. Acta 1142 (1993) 262–268.
[23] K.H. Chung, K.Y. Cho, Y. Asami, N. Takahashi, S. Yoshida, Z. Naturforsch. 44c (1989) 609–616.
[24] N. Tokutake, H. Miyoshi, T. Satoh, T. Hatano, H. Iwamura, Biochim. Biophys. Acta 1185 (1994) 271–278.
[25] H. Miyoshi, N. Tokutake, Y. Imaeda, T. Akagi, H. Iwamura, Biochim. Biophys. Acta 1229 (1995) 149–154.
[26] D.J. Horgan, H. Ohno, T.P. Singer, J.E. Casida, J. Biol. Chem. 243 (1968) 5967–5976.
[27] G. Thierbach, B. Kunze, H. Reichenbach, G. Höfle, Biochim. Biophys. Acta 765 (1984) 227–235.
[28] M. Degli Esposti, A. Ghelli, Biochim. Biophys. Acta 1187 (1994) 116–120.
[29] U. Brandt, Biochim. Biophys. Acta 1318 (1997) 79–81.
[30] K. He, L. Zeng, Q. Ye, G. Shi, N.H. Oberlies, G.-X. Zhao, J. Njoku, J.L. McLaughlin, Pestic. Sci. 49 (1997) 372–378.
[31] J. Morré, R. DeCabo, C. Farley, N.H. Oberlies, J.L. McLaughlin, Life Sci. 56 (1995) 343–348.
[32] S.M. Simon, M. Schindler, Proc. Natl. Acad. Sci. USA 91 (1994) 3497–3504.
[33] V. Ling, Cancer Res. 69 (1992) 2603–2609.