The Selectivity of Beauveriolide Derivatives in Inhibition toward the Two Isozymes of Acyl-CoA : cholesterol Acyltransferase

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The selectivity of synthetic beauveriolide derivatives in inhibition toward the two isozymes of acyl-CoA : cholesterol acyltrasferase (ACAT), ACAT1 and ACAT2, was studied in cell-based assays using ACAT1- or ACAT2-expressing Chinese hamster ovary (CHO) cells. NBV274, 285 and 300 showed ACAT1 selective inhibition similar to that of natural beauveriolides I and III, NBV345 inhibited both isozymes with similar potency, but NBV281, 331 and 249 were found to selectively inhibit the ACAT2 isozyme. The structure–activity relationships indicated that a subtle structural difference in beauveriolide derivatives can affect the selectivity of inhibition of the ACAT isozymes.

Key words beauveriolide; acyl-CoA : cholesterol acyltransferase; isozyme; selective inhibition

Beauveriolides, fungal metabolites produced by *Beauveria* sp. FO-6979, were discovered as inhibitors of lipid droplet formation in mouse peritoneal macrophages.[2—4)] Study of their mechanism of action revealed that beauveriolides inhibit the synthesis of cholesteryl ester (CE), one of the main constituents of lipid droplets in macrophages by blockade of acyl-CoA : cholesterol acyltransferase (ACAT) activity. Among the seven beauveriolides we have isolated, beauveriolides I (BeauI) and III (BeauIII) (Fig. 1) were found to be the most potent ACAT inhibitors. More importantly, BeauI and BeauIII proved orally active in atherosclerogenic mouse models, reducing atherogenic lesions of the artery and the heart in apolipoprotein E knockout mice and low-density lipoprotein receptor knockout mice.[5)]

BeauI/BeauIII are 13-membered cyclodepsipeptides consisting of L-Phe, L-Ala, D-Leu/D-*allo*-Ile, and (3*S*,4*S*)3-hydroxy-4-methyloctanoic acid, respectively. Accordingly, to secure the structure–activity relationships (SAR), we have demonstrated a synthesis of beauveriolides derivatives using radiofrequency encoded combinatorial chemistry,[6,7)] and the ACAT inhibitory activity of the derivatives was evaluated using mouse macrophages.[7,8)] We first demonstrated that, in the 3-hydroxy-4-methyloctanoic acid moiety, the 3*S* configuration of the hydroxyl group is important for the inhibitory activity because 3*R* isomers lose this activity due to changes in this group, while the stereochemistry of the methyl group at C-4 did not affect the inhibition of CE synthesis in macrophages.[8)] We next demonstrated that derivatives in which L-Phe had been substituted for diphenyl alanine were 20-fold more potent than BeauIII.[6,7)] Thus, we started work on derivatization of beauveriolides in an attempt to develop a new type of antiatherosclerotic agent.[5,9,10)]

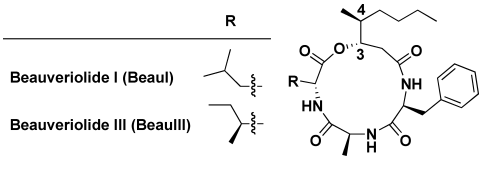

Fig. 1. The Structures of Beauveriolides I and III

Molecular biological studies have demonstrated that there are two ACAT isozymes, ACAT1 and ACAT2.[11—14)] ACAT1 is ubiquitously expressed, and high-level expression is observed in sebaceous glands, steroidogenic tissues, and macrophages, while ACAT2 is expressed predominantly in the liver and intestine.[15)] In spite of this recent knowledge about ACAT isozymes, studies on the selectivity of ACAT inhibitors, even synthetic ones, against these isozymes have not been carried out. We established a cell-based assay using ACAT1- and ACAT2-expressing Chinese hamster ovary (CHO) cells (ACAT1- and ACAT2-CHO cells, respectively) and studied the selectivity of the microbial ACAT inhibitors we have discovered.[16,17)] BeauI and BeauIII were found to inhibit ACAT1 selectively.[17)] However, the selectivity of beauveriolide derivatives toward the ACAT isozymes has not been studied.

In this study, 149 beauveriolide derivatives now available are evaluated in a cell-based assay to investigate their selectivity toward the ACAT isozymes. Regardless of the intriguing selectivity of BeauI and BeauIII, certain derivatives were found to exhibit a different selectivity. SAR are also discussed.

Experimental

Materials [1-^{14}C]Oleic acid was purchased from PerkinElmer Life and Analytical Sciences (U.S.A.). Fetal bovine serum (FBS) was bought from HyClone (U.S.A.). HAM's F12 was obtained from Sigma-Aldrich (U.S.A.). Geneticin (G-418 sulfate) and MEM vitamin solution were purchased from Wako Pure Chemical Industries (Japan). Penicillin (10000 units/ml)/streptomycin (10000 μg/ml) solution was acquired from Invitrogen (U.S.A.). Plastic microplates (48-well) were purchased from Asahi Techno Glass (Japan). Building blocks Unit I, Unit II and Unit III for combinatorial library of beauveriolides are summarized in Fig. 2. Among them, beauveriolide derivatives (149 compounds) were prepared as reported previously[6,7)] and the structures of 38 derivatives are summarized in Tables 1 to 3.

Culture of ACAT1- and ACAT2-CHO Cells Two cell lines, ACAT1- and ACAT2-CHO cells expressing African Green monkey ACAT1 and ACAT2,[16)] respectively, were maintained at 37 °C in 5% CO_2 in Ham's F-12 medium supplemented with MEM vitamins, geneticin (300 μg/ml) and 10%

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heat inactivated FBS (hereafter referred to as medium A).

Assay for ACAT1 and ACAT2 Activity Using ACAT1- and ACAT2-CHO Cells ACAT1 and ACAT2 activity were measured as the amount of [^{14}C]CE synthesized from [^{14}C]oleic acid in ACAT1- and ACAT2-CHO cells by our established method.[17] ACAT1- or ACAT2-CHO cells (1.25×10^5 cells in 250 μl of medium A) were cultured in a 48-well plastic microplate and allowed to recover overnight at 37 °C in 5% CO_2. The assays were carried out with cells that were at least 80% confluent. Following overnight recovery, a test sample (2.5 μl in methanol) and [1-^{14}C]oleic acid (1.85 kBq, 5 μl in 10% ethanol/PBS solution) were added to each culture. After a 6-h incubation at 37 °C in 5% CO_2, the medium was removed, and the cells in each well were washed twice with PBS. The cells were lysed by adding 0.25 ml of 10 mM Tris–HCl (pH 7.5) containing 0.1% (w/v) sodium dodecyl sulfate (SDS), and cellular lipids were extracted by the method of Bligh and Dyer.[18] After the organic phase had been concentrated, the total lipids were separated on a thin layer chromatography (TLC) plate (silica gel F254, 0.5 mm thick, Merck, Germany) and analyzed with a bioimaging analyzer (BAS 2000, Fuji Film, Japan). In this cell-based assay, [^{14}C]CE was produced by the reaction of ACAT1 or ACAT2. ACAT inhibitory activity (%) is defined as (1−[^{14}C]CE-drug/[^{14}C]CE-control)×100. The IC_{50} value is defined as the drug concentration that causes 50% inhibition of a biological activity. The selectivity index (SI) of a derivative is defined as $\log_{10}$(IC_{50} for ACAT1/IC_{50} for ACAT2).

Results

All combinatorial beauveriolide derivatives (149 compounds) were tested in cell-based assays using ACAT1- and ACAT2-CHO cells. Among them, 86 derivatives showed inhibitory activity against ACAT1 and/or ACAT2.

The IC_{50} values of the derivatives against ACAT1 in the assay using ACAT1-CHO cells and against CE synthesis in mouse macrophages (which express ACAT1)[6,7] are plotted in Fig. 3. A good correlation was obtained (Pearson $r=0.484$, $p<0.001$) between the two IC_{50} values. This is reasonable because mouse macrophages specifically express the ACAT1 isozyme.[11]

The IC_{50} values of these 86 derivatives against ACAT1 and ACAT2 activities are plotted in Fig. 4. Among them, 38 derivatives (Fig. 2, Tables 1 to 3), which showed potent ACAT1 and/or ACAT2 inhibition, were classified into three groups. Group 1: ACAT1 selective inhibitors (SI≦−1.0). Fifteen derivatives (NBV274, 285, 300, 280, 258, 248, 292, 293, 325, 301, 312, 348, 336, 255, 279) showed ACAT1 selective inhi-

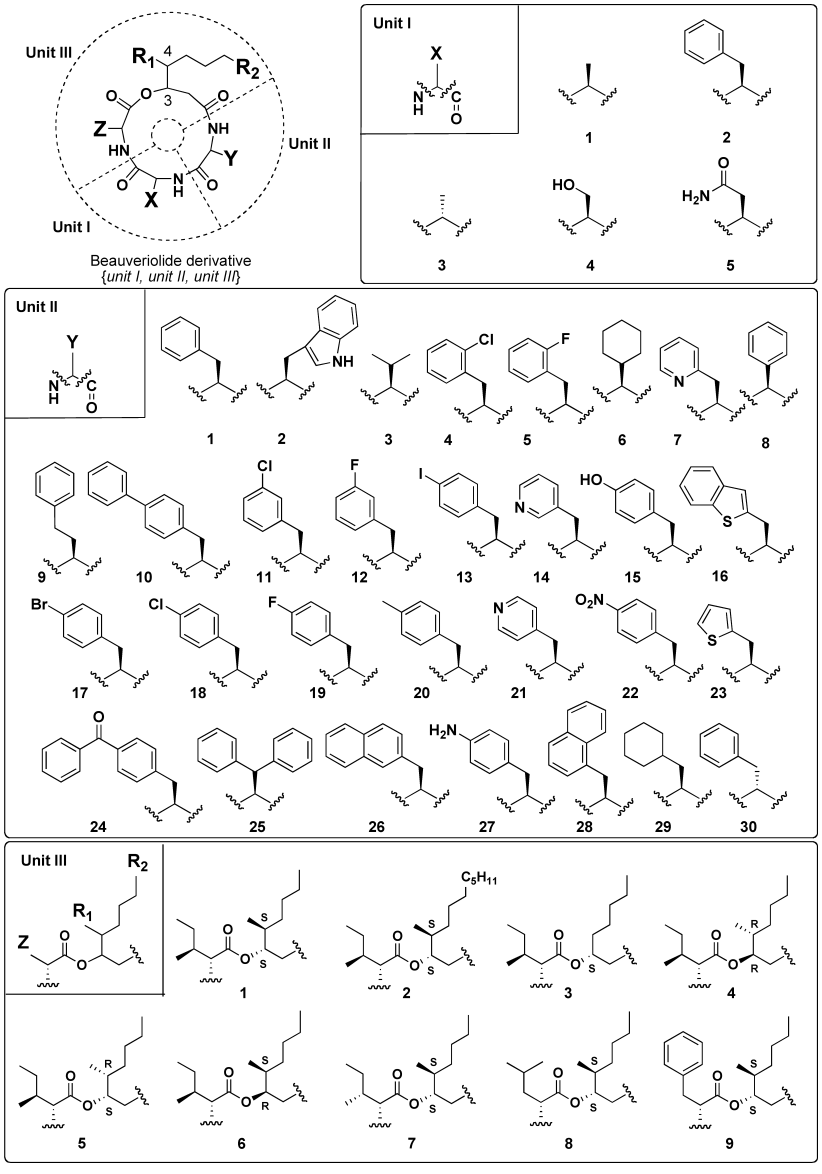


Fig. 2. The Structures of the Beauveriolide Derivatives

Beauveriolide derivatives {*unit I*, *unit II*, *unit III*} have changes in Unit I, Unit II, and Unit III, respectively.

Table 1. Beauveriolide Derivatives Showing ACAT1 Selective Inhibition

Structure[a]	{*unit I*, *unit II*, *unit III*}	IC_{50} for CE synthesis (μM)			SI[c]
		Macrophage[b]	ACAT1-CHO	ACAT2-CHO	
NBV274	{*1*, *11*, *2*}	1.2	0.15	6.5	−1.6
NBV285	{*1*, *20*, *2*}	0.40	0.10	4.0	−1.6
NBV300	{*1*, *4*, *3*}	>20	1.0	>40	<−1.6
NBV280	{*1*, *19*, *2*}	1.8	0.35	10	−1.5
NBV258	{*1*, *20*, *1*}	0.40	0.85	>20	<−1.4
NBV248	{*1*, *81*, *1*}	0.095	1.0	>20	<−1.3
NBV292	{*1*, *23*, *2*}	6.5	1.0	21	−1.3
NBV293	{*1*, *1*, *2*}	0.60	0.71	11	−1.2
NBV325	{*1*, *1*, *9*}	7.5	0.73	11	−1.2
NBV301	{*1*, *11*, *3*}	0.50	0.15	2.0	−1.1
NBV312	{*1*, *20*, *3*}	1.5	3.2	>40	<−1.1
NBV348	{*4*, *1*, *8*}	13	1.5	18	−1.1
NBV336	{*3*, *1*, *8*}	0.48	0.51	6.0	−1.1
NBV255	{*1*, *28*, *1*}	0.74	0.80	9.0	−1.1
NBV279	{*1*, *12*, *2*}	0.86	0.80	8.0	−1.0
BeauI[d]	{*1*, *1*, *8*}	0.78	0.60	20	−1.5
BeauIII[d]	{*1*, *1*, *1*}	0.41	0.90	>20	<−1.3

a) See Fig. 2. *b*) IC_{50} values in mouse macrophages are cited from refs. 5, 6. *c*) Selectivity index (SI): log[IC_{50} for ACAT1/IC_{50} for ACAT2]. *d*) Data cited from ref. 16. The data are expressed as the mean (n=4).

Table 2. Beauveriolide Derivatives Showing ACAT1 and ACAT2 Inhibition

Structure[a]	{*unit I*, *unit II*, *unit III*}	IC_{50} for CE synthesis (μM)			SI[c]
		Macrophage[b]	ACAT1-CHO	ACAT2-CHO	
NBV345	{*3*, *25*, *9*}	0.24	0.090	0.060	+0.2
NBV333	{*1*, *25*, *9*}	0.080	0.20	0.080	+0.4
NBV344	{*3*, *25*, *8*}	0.070	0.25	0.45	−0.3
NBV342	{*3*, *25*, *1*}	0.020	0.29	0.23	+0.1
NBV337	{*3*, *1*, *9*}	10	0.40	1.0	−0.4
NBV218	{*2*, *1*, *1*}	6.0	0.60	0.80	−0.1
NBV334	{*3*, *1*, *1*}	0.80	0.62	2.2	−0.6
NBV335	{*3*, *1*, *7*}	0.65	0.62	2.5	−0.6
NBV343	{*3*, *25*, *7*}	0.020	0.64	0.11	+0.8
NBV320	{*1*, *1*, *3*}	0.60	0.70	1.5	−0.3
NBV247	{*1*, *11*, *1*}	0.14	0.75	4.5	−0.8
NBV272	{*1*, *29*, *2*}	0.45	0.85	1.0	−0.1

a) See Fig. 2. *b*) IC_{50} values in mouse macrophages are cited from refs. 5, 6. *c*) Selectivity index (SI): log[IC_{50} for ACAT1/IC_{50} for ACAT2]. The data are expressed as the mean (n=4).

Table 3. Beauveriolide Derivatives Showing ACAT2 Selective Inhibition

Structure[a]	{*unit I*, *unit II*, *unit III*}	IC_{50} for CE synthesis (μM)			SI[c]
		Macrophage[b]	ACAT1-CHO	ACAT2-CHO	
NBV281	{*1*, *26*, *2*}	>20	>20	0.25	>+1.9
NBV331	{*1*, *25*, *7*}	0.020	4.0	0.10	+1.6
NBV249	{*1*, *25*, *1*}	0.040	10	0.27	+1.6
NBV332	{*1*, *25*, *8*}	0.080	>20	0.75	>+1.4
NBV224	{*6*, *2*, *1*}	9.0	9.0	0.43	+1.3
NBV269	{*1*, *10*, *1*}	>20	>20	0.90	>+1.3
NBV327	{*1*, *30*, *7*}	>20	20	1.2	+1.2
NBV276	{*1*, *25*, *2*}	0.97	7.0	0.60	+1.1
NBV223	{*2*, *2*, *4*}	11	10	0.91	+1.0
NBV341	{*3*, *30*, *9*}	0.30	15	1.4	+1.0
NBV308	{*1*, *26*, *3*}	>20	>20	2.0	>+1.0

a) See Fig. 2. *b*) IC_{50} values in mouse macrophages are cited from refs. 5, 6. *c*) Selectivity index (SI): log[IC_{50} for ACAT1/IC_{50} for ACAT2]. The data are expressed as the mean (n=4).

bition (Table 1). Among the 15 derivatives, NBV274, 285 and 300 (SI, −1.6, −1.6 and <−1.6, respectively) are more selective toward ACAT1 than BeauI and BeauIII (SI, −1.5 and <1.3, respectively).[17] Group 2: Non-selective inhibitors (−1.0<SI<+1.0). Most beauveriolide derivatives belong to this group. In fact, among the 86 derivatives described above, 60 inhibited both ACAT1 and ACAT2 isozymes. In paticular, 12 derivatives (NBV345, 333, 344, 342, 337, 218, 334, 335,

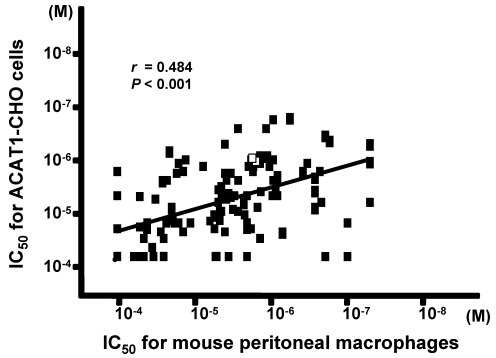


Fig. 3. The Relationship between the IC_{50} of Beauveriolide Derivatives against CE Synthesis in Mouse Peritoneal Macrophages and in ACAT1-CHO Cells

The IC_{50}s for mouse peritoneal macrophages and for ACAT1-CHO cells of each derivatives (■) were plotted on the X and Y axes, respectively. The coordinates of BeauI and BeauIII (□) were also plotted for comparison.

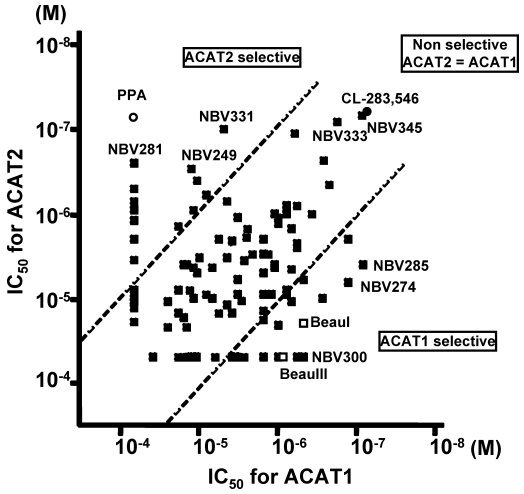


Fig. 4. The Relationship between the IC_{50} of Beauveriolide Derivatives against ACAT1 and ACAT2 Activities

The IC_{50}s for ACAT1 and ACAT2 of each derivative (■) were plotted on the X and Y axes, respectively. The coordinates of BeauI and BeauIII (□), CL-283,546 (●), and pyripyropene A (○, PPA) were also plotted for comparison.

343, 320, 247, 272) showed very strong inhibition with nano molar IC_{50} values (Table 2). NBV345 is the most potent (IC_{50}, 90 and 60 nM, respectively), followed by NBV333. Group 3: ACAT2 selective inhibitors (SI≧+1.0). Interestingly, 11 derivatives (NBV281, 331, 249, 332, 224, 269, 327, 276, 223, 341, 308) were found to selectively inhibit ACAT2 activity (Table 3). In particular, NBV281, 331 and 249 were highly selective toward the ACAT2 isozyme (SI, >+1.9, +1.6 and +1.6, respectively).

Discussion

A number of beauveriolide derivatives, prepared by combinatorial chemistry, have already been evaluated in a mouse macrophages assay.[6,7] It is known that mouse macrophages express the ACAT1 isozyme.[11] In this study, we reevaluated the derivatives in a sophisticated cell-based assay using ACAT1- and ACAT2-CHO cells[16] to confirm their selectivity toward the two isozymes.

As shown in Fig. 3, the data obtained from the macrophage assay[6,7] and from the ACAT1-CHO cell-based assay

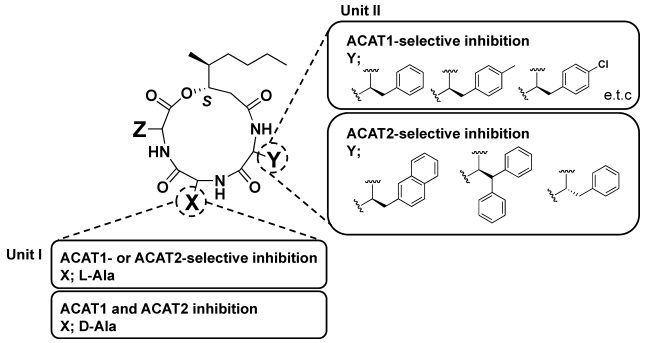
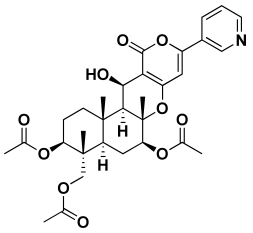


Fig. 5. The Structure–Activity Relationships of Beauveriolide Derivatives in the Inhibition toward ACAT Isozymes



Pyripyropene A

Fig. 6. The Structure of Pyripyropene A

showed a good correlation. Both assays are reliable for evaluating inhibitors of the ACAT1 isozyme.

In the ACAT1- and ACAT2-CHO cells, BeauI and BeauIII showed selective inhibition toward ACAT1 with SI values of −1.5 and <−1.3, respectively (Table 1).[17] The 15 derivatives listed in Table 1 showed the same ACAT1 selectivity (SI≦−1.0), as BeauI and BeauIII, indicating that the L-Phe at Unit II can be substituted for *m*-chlorophenyl (for example, NBV274), *p*-chlorophenyl (NBV248) or *p*-methylphenyl (NBV285) alanine, whilst maintaining the derivative's selectivity and potency (Fig. 5). Interestingly, most (60) derivatives showed non-selective inhibition (−1.0<SI<+1.0). In particular, the 12 derivatives listed in Table 2 strongly inhibited both ACAT1 and ACAT2 isozymes. Thus, derivatives having D-Ala at Unit I and diphenylalanine at Unit II (for example, NBV345, 344, 342, 343) tended to show potent non-selective inhibition (Fig. 5). To our surprise, the 11 derivatives listed in Table 3 were found to show ACAT2 selectivity, a characteristic opposite to those of BeauI and BeauIII. Combination of L-Ala at Unit I and diphenylalanine or 2-naphthylalanine at Unit II appears to be responsible for ACAT2 selectivity. The 3*S* configuration of the hydroxyl group at Unit III is important for potent inhibition of ACAT activity, while amino acid residues at Unit III have little impact on the selectivity toward the two isozymes. These results indicate that the substitution of amino acid residues at Unit I and II in beauveriolide causes a structural conformation change in the affinity of the derivatives for ACAT1 and/or ACAT2 isozymes. On the other hand, pyripyropene A is well known as a highly selective ACAT2 inhibitor (Fig. 6).[16,17] A number of pyripyropene derivatives were prepared at the three *O*-acyl positions.[19—24] Most of them showed ACAT2 selective inhibition, but none were ACAT1 selective.[25] The skeleton of pyripyropenes might be important for ensuring that they fit in

the putative binding site in the transmembrane domain of ACAT2.[26] As confirmed in this study, beauveriolide derivatives have the very unique characteristics of inhibiting ACAT1 and/or ACAT2. Therefore, they are expected to work as a new type of anti-atherosclerotic agent or as useful probes to study the function of the ACAT isozymes.

Acknowledgments This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences (to H. T.) from the National Institute of Biomedical Innovation (NIBIO), by a grant-in-aid for Scientific Research (B) 18390008 (to H. T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the Hoh-ansya Foundation (to H. T.), Japan.

References and Notes

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2) Namatame I., Tomoda H., Si S., Yamaguchi Y., Masuma R., Ōmura S., *J. Antibiot.*, **52**, 1—6 (1999).
3) Namatame I., Tomoda H., Tabata N., Si S., Ōmura S., *J. Antibiot.*, **52**, 7—12 (1999).
4) Matsuda D., Namatame I., Tomoda H., Kobayashi S., Zocher R., Kleinkauf H., Ōmura S., *J. Antibiot.*, **57**, 1—9 (2004).
5) Namatame I., Tomoda H., Ishibashi S., Ōmura S., *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 737—742 (2004).
6) Nagai K., Doi T., Sekiguchi T., Namatame I., Sunazuka T., Tomoda H., Ōmura S., Takahashi T., *J. Comb. Chem.*, **8**, 103—109 (2006).
7) Nagai K., Doi T., Ohshiro T., Sunazuka T., Tomoda H., Takahashi T., Ōmura S., *Bioorg. Med. Chem. Lett.*, **18**, 4397—4400 (2008).
8) Ohshiro T., Namatame I., Nagai K., Sekiguchi T., Doi T., Takahashi T., Akasaka K., Rudel L. L., Tomoda H., Ōmura S., *J. Org. Chem.*, **71**, 7643—7649 (2006).
9) *Chem. Eng. News*, **18**, 15 (2004).
10) Tomoda H., Doi T., *Acc. Chem. Res.*, **41**, 32—39 (2008).
11) Chang C. C., Huh H. Y., Cadigan K. M., Chang T. Y., *J. Biol. Chem.*, **268**, 20747—20755 (1993).
12) Anderson R. A., Joyce C., Davis M., Reagan J. W., Clark M., Shelness G. S., Rudel L. L., *J. Biol. Chem.*, **273**, 26747—26754 (1998).
13) Cases S., Novak S., Zheng Y. W., Myers H. M., Lear S. R., Sande E., Welch C. B., Lusis A. J., Spencer T. A., Krause B. R., Erickson S. K., Farese R. V. Jr., *J. Biol. Chem.*, **273**, 26755—26764 (1998).
14) Oelkers P., Behari A., Cromley D., Billheimer J. T., Sturley S. L., *J. Biol. Chem.*, **273**, 26765—26771 (1998).
15) Parini P., Davis M., Lada A. T., Erickson S. K., Wright T. L., Gustafsson U., Sahlin S., Einarsson C., Eriksson M., Angelin B., Tomoda H., Ōmura S., Willingham M. C., Rudel L. L., *Circulation*, **110**, 2017—2023 (2004).
16) Lada A. T., Davis M., Kent C., Chapman J., Tomoda H., Ōmura S., Rudel L. L., *J. Lipid Res.*, **45**, 378—386 (2004).
17) Ohshiro T., Rudel L. L., Ōmura S., Tomoda H., *J. Antibiot.*, **60**, 43—51 (2007).
18) Bligh E. G., Dyer W., *Can. J. Biochem. Physiol.*, **37**, 911—917 (1959).
19) Obata R., Sunazuka T., Li Z., Tomoda H., Ōmura S., *J. Antibiot.*, **48,** 749—750 (1995).
20) Obata R., Sunazuka T., Tomoda H., Harigaya Y., Ōmura S., *Bioog. Med. Chem. Lett.*, **5**, 2683—2688 (1995).
21) Obata R., Sunazuka T., Li Z., Tian Z., Harigaya Y., Tabata N., Tomoda H., Ōmura S., *J. Antibiot.*, **49**, 1133—1148 (1996).
22) Obata R., Sunazuka T., Kato Y., Tomoda H., Harigaya Y., Ōmura S., *J. Antibiot.*, **49**, 1149—1156 (1996).
23) Obata R., Sunazuka T., Tian Z., Tomoda H., Harigaya Y., Ōmura S., *J. Antibiot.*, **50**, 229—236 (1997).
24) Obata R., Sunazuka T., Tian Z., Tomoda H., Harigaya Y., Ōmura S., Smith A. B., III, *Chem. Lett.*, **26**, 935—936 (1997).
25) Ohshiro T., Ohte S., Matsuda D., Ohtawa M., Nagamitsu T., Sunazuka T., Harigaya Y., Rudel L. L., Ōmura S., Tomoda H., *J. Antibiot.*, **61**, 503—508 (2008).
26) Das A., Davis M. A., Tomoda H., Ōmura S., Rudel L. L., *J. Biol. Chem.*, **283**, 10453—10460 (2008).