博士論文（要約）

論文題目　Comprehensive Structure-Activity Relationship Study of Lysocin E
（ライソシンEの網羅的構造活性相関研究）

氏　名　加治　拓哉

Introduction

Lysocin E[1] (**1**, Figure 1) is a 37-membered cyclic depsipeptide isolated from *Lysobacter* sp. Peptide **1** exhibits antimicrobial activity against methicillin-resistant *S. aureus* (MRSA) with a minimum inhibitory concentration (MIC) of 4 μg/mL. Therefore **1** is expected to be a promising seed for MRSA treatment.

The molecular target of **1** is distinct from that of any other reported antibiotics. A series of mutational analyses revealed that **1** directly binds to menaquinone (MK) within the bacterial membrane. MK is an essential factor for electron transfer in the bacterial respiratory chain. Formation of the **1**-MK complex is considered to disrupt the functional integrity of the bacterial membrane, resulting in rapid bacteriolysis. In contrast, no complexation occurs between **1** and ubiquinone (UQ), a coenzyme in the mammalian respiratory chain. The bacterial/mammalian cell selectivity of **1** is attributable to the selectivity of **1** toward MK over UQ. However, structural requirement of **1** for its potent biological activity remained to be elucidated. Herein, comprehensive structure-activity relationship (SAR) study of **1** was conducted 1) to investigate the side-chain functionalities relevant to the molecular mode of action and 2) to create more potent derivatives.

Total synthesis and functional evaluation of fourteen derivatives of lysocin E

To investigate the importance of side-chain functionalities of **1**, side-chain modified derivatives of **1** were to be designed and synthesized. Prior to the SAR study of **1**, the following three potential interactions among **1**, MK, and phospholipids were hypothesized: 1) an electrostatic interaction of the anionic carboxylate group or the cationic guanidine moieties with the polar head group of phospholipids or the carbonyl groups of MK; 2) an aromatic-aromatic interaction of the phenyl group or indole ring with the naphthoquinone ring of MK; and 3) a hydrophobic interaction of the acyl chain with the lipid chains of MK or phospholipids. To systematically investigate the significance of each of these interactions, fourteen analogues **2**-**15** were designed (Figure 1).

Syntheses of the natural **1**, amine analogues **4**, **16a**, **16b**, and **16c** were envisioned to permit rapid access to analogues **2**/**3**, **5**-**7**, **8**-**13**, **14**, and **15** respectively, by applying chemoselective single-step reactions (Figure 2). Fmoc solid-phase peptide synthesis strategies enabled efficient construction of the main chain structure without purification of intermediates (Figure 2A and 2B).[2,3] Compound **25** was used to incorporate acyl chain of **1** and **4** in SPPS (Figure 2A, **22**→**34**→**35**→**1** or **4**). In contrast, compound **30** was employed to incorporate ester linkage and Boc-protected amine for post-SPPS modification of acyl chain (Figure 2B, **22**→**37**→**38**→**16**). These synthetic strategies were applied to prepare **1**, **4**, **16a**, **16b**, and **16c** in 8.0, 6.1, 26, 12, and 6.5% overall yields, respectively. Condensation of **1** with **34** and **35** in the presence of PyBOP afforded amide analogues **2** and **3** in 48 and 44% yields, respectively. Treatment of **4** with **36**, **37**, and **38** gave rise to the dimethylguanidine (**5**), urea (**6**), and acetyl (**7**) analogues in 46, 58, and 27% yields, respectively. Treatment of **16a**-**16c** with activated carboxylic acids, which were prepared from **39**-**45** in the presence of isobutylchloroformate and *N*-methylmorpholine, afforded analogues **8**, **9**, **10**, **11**, **12**, **13**, **14**, and **15** in 34, 51, 29, 33, 41, 25, 22, and 34% yields, respectively.

compounds	R^1	R^2	R^3	R^4	R^5	membrane disruption [%] 17 2.5 μM	17 10 μM	18 2.5 μM	18 10 μM	MIC [μg/mL]
lysocin E (**1**)		Ph	3-indolyl	guanidyl	OH	62		1.0		4
2		Ph	3-indolyl	guanidyl	NHBn	64		0		4
3		Ph	3-indolyl	guanidyl		93		0		2
4		Ph	3-indolyl	NH_2	OH	62		2.2		4
5		Ph	3-indolyl		OH	55		5.1		4
6		Ph	3-indolyl		OH	7.4		0.9		8
7		Ph	3-indolyl		OH	0	9.3	0	0	16
8		Ph	3-indolyl	guanidyl	OH	51		0		16
9		Ph	3-indolyl	guanidyl	OH	48		0		4
10		Ph	3-indolyl	guanidyl	OH	65		0		2
11		Ph	3-indolyl	guanidyl	OH	62		0		4
12		Ph	3-indolyl	guanidyl	OH	42		0.7		4
13		Ph	3-indolyl	guanidyl	OH	20		0		32
14		H	3-indolyl	guanidyl	OH	0.8	65	0	0	8
15		Ph	H	guanidyl	OH	0	0	0	0	> 128

compounds	R^1	R^2	R^3	R^4	R^5
16a	H•TFA	Ph	3-indolyl	guanidyl	OH
16b	H•TFA	H	3-indolyl	guanidyl	OH
16c	H•TFA	Ph	H	guanidyl	OH

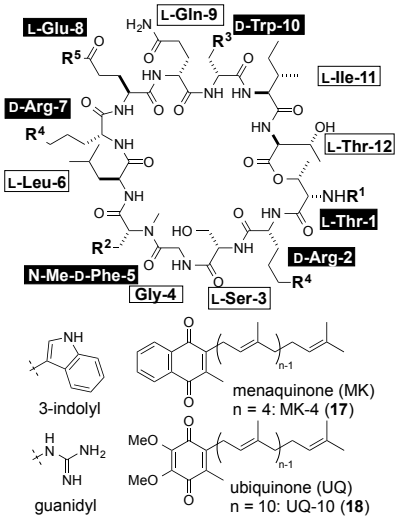

Figure 1. Structures of lysocin E (**1**), analogues **2-15**, intermediates **16a-c**, menaquinone-4 (**17**), and ubiquinone-10 (**18**). Membrane disrupting activities and antimicrobial activities of **1-15** are also displayed.

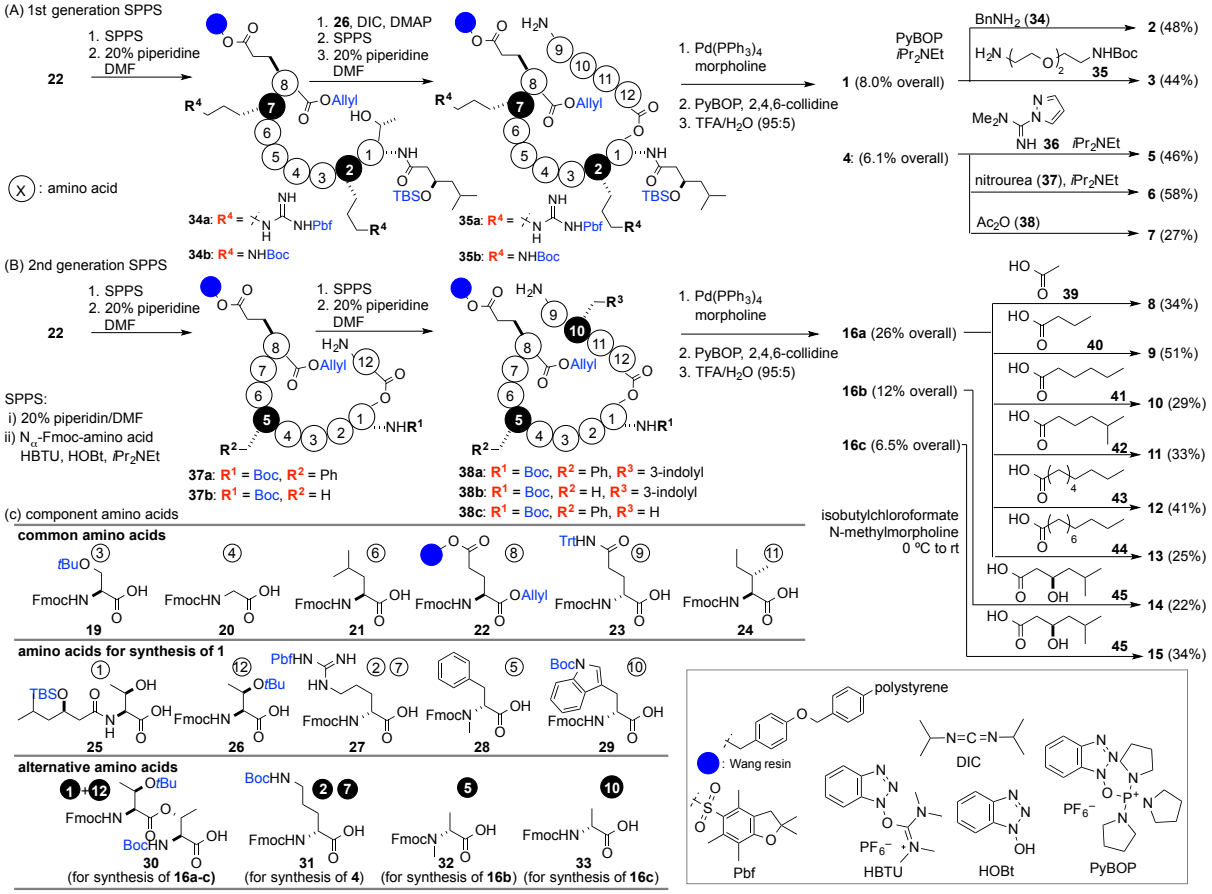

Figure 2. Solid-phase peptide syntheses of **1**, **4**, and **16a-c**, synthesis of **2**, **3**, and **5-15**, and component amino acids **19-33**.

Biological functions of **1** and its analogues were systematically evaluated based on the MK-dependent membrane lysis of liposomes and antimicrobial activity against *S. aureus*. To assess membrane lytic activity, large unilamellar vesicles (LUVs) comprising a 1:1 ratio of EYPC/EYPG were prepared in the presence of 1.25 mol% of MK-4 (**16**) or UQ-10 (**17**). Carboxyfluorescein (CF) was encapsulated as a fluorescent indicator in the LUVs. Membrane disruption caused by **1**-**15** was evaluated by fluorescence from released CF molecules.

The selective membrane lysis toward LUVs containing **17** over **18** was consistently observed for **1**-**14**. The natural **1** exhibited 62% membrane disruption at 2.5 μM and MIC of 4 μg/mL. Exchange of the anionic carboxylate with the neutral amides of **2** and **3** did not decrease membrane lytic activity (64% for **2**, 93% for **3**) and antimicrobial activity (MIC 4 μg/mL for **2** and 2 μg/mL for **3**). When the cationic guanidine moieties were exchanged to cationic amine (**4**) or dimethylguanidine (**5**), the potency of membrane disruption (62% for **4** and 55% for **5**) and antimicrobial activities (MIC 4 μg/mL for **4** and **5**) was retained. In contrast, incorporation of neutral urea (**6**) and amide (**7**) analogues decreased both membrane lytic activity (7.4% for **6** and 0% for **7**) and antimicrobial activity (MIC 8 μg/mL for **6** and 16 μg/mL for **7**), emphasizing the significance of the cationic functionalities. C2- (**8**), C4- (**9**), C6- (**10**), C7- (**11**), and C9- (**12**) acyl chain-modified analogues exhibited similar membrane lytic activities (51, 48, 65, 62, and 42%, respectively). C11-acyl chain modified analogue **13** showed lower membrane lytic activity (20%). Despite their relative unimportance in the liposome experiments, the lengths of the acyl chains influenced the MIC (2-4 μg/mL for **9**-**12**, 16 μg/mL for **8**, and 32 μg/mL for **13**), indicating the importance of the appropriate hydrophobicity of this moiety for the bioactivity. Although des-phenyl analogue **14** exhibited weak membrane lytic activity (65% at 10 μM) and antimicrobial activity (MIC 8 μg/mL), deletion of indole ring (**15**) totally abolished both membrane lytic activity (0% at 10 μM) and antimicrobial activity (MIC >128 μg/mL). The indole ring appeared to be the most essential part of **1** for the MK-selective membrane disruption and antimicrobial activity.[4]

Comprehensive Structure-Activity Relationship study of lysocin E

More comprehensive SAR experiment was conducted expanding the number of analogues to be evaluated by using one-bead-one-compound (OBOC) strategy[5]. Resin-bound 2,401 lysocin analogues were synthesized through split-pool synthesis by randomizing four amino-acid residues (L-Ser-3, L-Leu-6, D-Gln-9, and L-Ile-11) into seven amino acids (Val, Orn, Asp, Asn, Ser, Tyr, Ala). Method to evaluate MK-binding property of on-resin analogues was established by quantification of the resin-adsorbed MK. In addition, methods to evaluate the antimicrobial activity of the analogues derived from a single bead were established under carefully optimized conditions. Furthermore, structural determination of the analogues was accomplished by

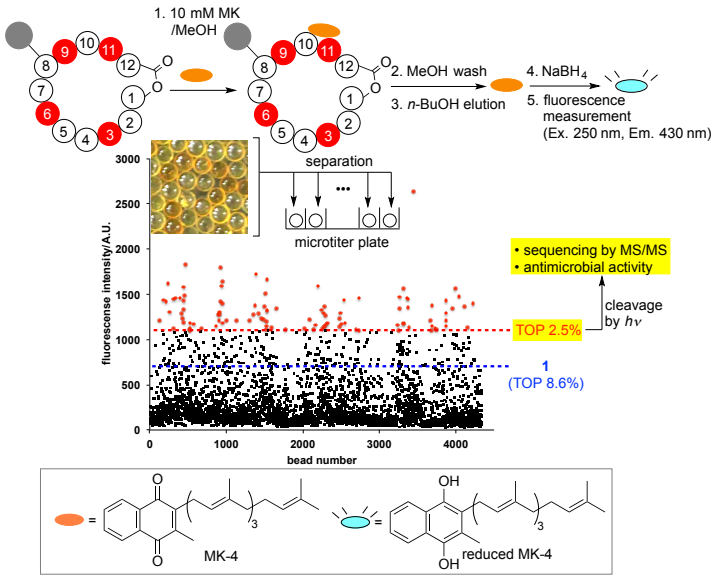

Figure 3. On-bead MK-adsorption assay of OBOC library of **1**.

tandem mass spectrometry. Screening of 2,401 analogues is currently underway.

Summary

Fourteen side-chain analogues of **1** were synthesized by using the solid-phase strategy and chemoselective single-step modification. The key functional groups for the potent activity of **1** were found to be cationic groups, hydrophobic acyl group, and the indole ring. These results offered a clearer picture of the mode of action of **1**. The cationic guanidine moieties and the hydrophobic acyl chain help **1** bind through the anionic polar heads and hydrophobic lipid tails of the bacterial membrane, respectively. On the membrane surface, the electron-rich indole and the electron-deficient naphthoquinone of MK bind as a result of the aromatic-aromatic interaction, leading to the formation of the **1**-MK complex. Finally, the complexation causes membrane damage and eventual cell death (Figure 4). Sequential analyses of on-bead and solution-phase assay were applied to the OBOC library composed of 2,401 analogues. Comprehensive structure-activity relationship study is currently underway. Furthermore, this new hypothetical mechanism of action and comprehensive structure-activity relationship study of OBOC library will provide us with valuable information for designing more active derivatives of **1**.

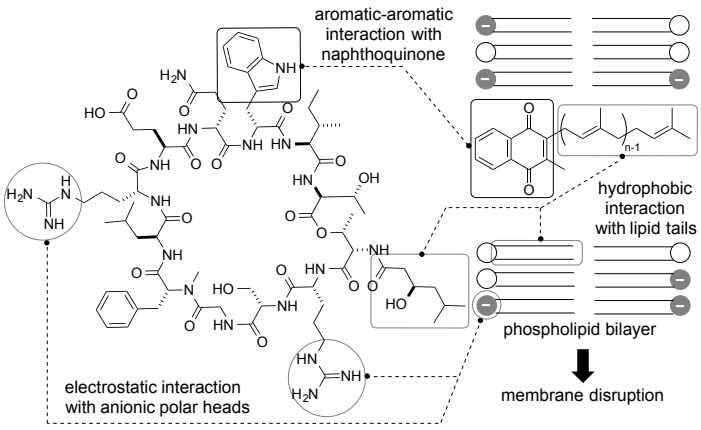

Figure 4. Hypothetical mechanism of action of **1**.

References

1) Hamamoto, H.; Urai, M.; Ishii, K.; Yasukawa, J.; Paudel, A.; Murai, M.; Kaji, T.; Kuranaga, T.; Hamase, K.; Katsu, T.; Sue, J.; Adachi, T.; Uchida, R.; Tomoda, H.; Yamada, M.; Souma, M.; Kurihara, H.; Inoue, M.; Sekimizu, K. *Nat. Chem. Biol.* **2015**, *11*, 127.

2) Murai, M.; Kaji, T.; Kuranaga, T.; Hamamoto, H.; Sekimizu, K.; Inoue, M. *Angew. Chem. Int. Ed.* **2015**, *54*, 1556.

3) (a) Murai, M. *Ph.D. thesis*, **2014**. (b) Kaji, T. *Master thesis*, **2014**.

4) Kaji, T.; Murai, M.; Itoh, H.; Yasukawa, J.; Hamamoto, H.; Sekimizu, K.; Inoue, M. *Chem. Eur. J.* **2016**, *22*, 16912.

5) Lam, K. S.; Lebl, M.; Krchňák, V. *Chem. Rev.* **1997**, *97*, 411.