ChemPubSoc Europe

DOI: 10.1002/chem.201604022

CHEMISTRY
A European Journal
Full Paper

Natural Products

Total Synthesis and Functional Evaluation of Fourteen Derivatives of Lysocin E: Importance of Cationic, Hydrophobic, and Aromatic Moieties for Antibacterial Activity

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Abstract: Lysocin E (**1**) is a structurally complex 37-membered depsipeptide comprising 12 amino-acid residues with an N-methylated amide and an ester linkage. Compound **1** binds to menaquinone (MK) in the bacterial membrane to exert its potent bactericidal activity. To decipher the biologically important functionalities within this unique antibiotic, we performed a comprehensive structure-activity relationship (SAR) study by systematically changing the side-chain structures of L-Thr-1, D-Arg-2, N-Me-D-Phe-5, D-Arg-7, L-Glu-8, and D-Trp-10. First, we achieved total synthesis of the 14 new side-chain analogues of **1** by employing a solid-phase strategy. We then evaluated the MK-dependent liposomal disruption and antimicrobial activity against *Staphylococcus aureus* by **1** and its analogues. Correlating data between the liposome and bacteria experiments revealed that membrane lysis was mainly responsible for the antibacterial functions. Altering the cationic guanidine moiety of D-Arg-2/7 to a neutral amide, and the C7-acyl group of L-Thr-1 to the C2 or C11 counterpart decreased the antimicrobial activities four- or eight-fold. More drastically, chemical mutation of D-Trp-10 to D-Ala-10 totally abolished the bioactivities. These important findings led us to propose the biological roles of the side-chain functionalities.

Introduction

Bacterial infections increasingly evade standard treatments with growing resistance to multiple antibiotics.[1] Antibiotic-resistant infectious diseases thus pose a threat to public health around the world.[2] In particular, nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals are a serious clinical problem. The emergence of multidrug resistance has created an urgent need for the development of effective antibiotics with new modes of action against such resistant strains.[3,4] Lysocin E (**1**, Figure 1) was isolated from *Lysobacter* sp. RH2180-5 strain as a novel antibiotic, and structurally determined to be a 37-membered depsipeptide (molecular weight = 1618 Da).[5] Compound **1** exhibits potent antimicrobial activity against MRSA with a minimum inhibitory concentration (MIC) of 4 $\mu g\,mL^{-1}$ (2.5 μM). Moreover, treatment of *S. aureus* infected mice with **1** markedly increased their survival rate compared with that of untreated mice ($ED_{50} = 0.5\ mg\,kg^{-1}$ body weight). Remarkably, this therapeutic effect of **1** was even stronger than that of vancomycin ($ED_{50} = 5.8\ mg\,kg^{-1}$), which is widely used to treat MRSA infections.

The molecular target of **1** is distinct from that of any other reported antibiotic. 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.[6] Formation of the **1**-MK complex is considered to disrupt the functional integrity of the bacterial membrane, resulting in rapid bacteriolysis.[5] Isothermal titration calorimetry experiments further demonstrated that **1** forms a 1:1 complex with MK, with a dissociation constant of 4.5 μM. In sharp contrast, no complexation occurs between **1** and ubiquinone (UQ), a coenzyme in the mammalian respiratory chain. The selectivity of **1** toward MK over UQ is attributable to the bacterial/mammalian cell selectivity of **1**. Generally, antibiotics that disrupt membranes are less likely to induce drug resistance than antibiotics that target other bacterial systems due to the relatively conserved molecular composition of lipids.[7] The potent bactericidal activity and advantageous mode of action make naturally occurring **1** a useful lead compound for developing new antibiotics.[8]

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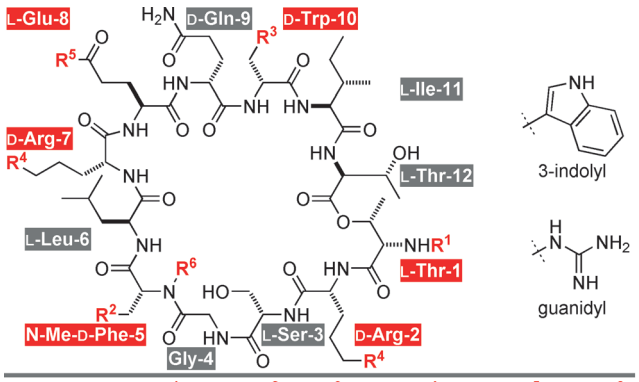
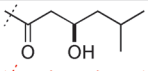
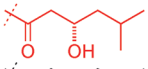
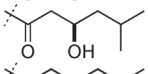
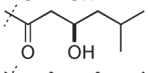
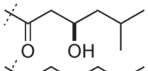
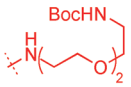
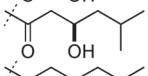
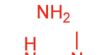
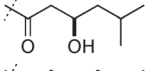
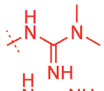
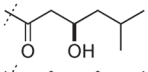
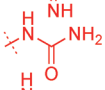
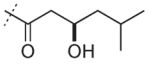
Supporting information containing experimental procedures, characterization of the compounds, assay data, and spectroscopic data for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201604022.



compounds	R^1	R^2	R^3	R^4	R^5	R^6
lysocin E (**1**)		Ph	3-indolyl	guanidyl	OH	Me
2		Ph	3-indolyl	guanidyl	OH	Me
3		Ph	3-indolyl	guanidyl	OH	H
4		Ph	3-indolyl	guanidyl	NHBn	Me
5		Ph	3-indolyl	guanidyl		Me
6		Ph	3-indolyl		OH	Me
7		Ph	3-indolyl		OH	Me
8		Ph	3-indolyl		OH	Me
9		Ph	3-indolyl		OH	Me
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11		Ph	3-indolyl	guanidyl	OH	Me
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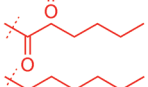
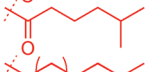
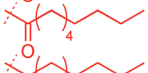
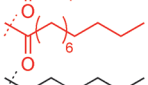
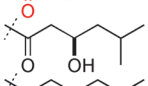
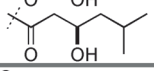
13		Ph	3-indolyl	guanidyl	OH	Me
14		Ph	3-indolyl	guanidyl	OH	Me
15		Ph	3-indolyl	guanidyl	OH	Me
16		Ph	3-indolyl	guanidyl	OH	Me
17		H	3-indolyl	guanidyl	OH	Me
18		Ph	H	guanidyl	OH	Me

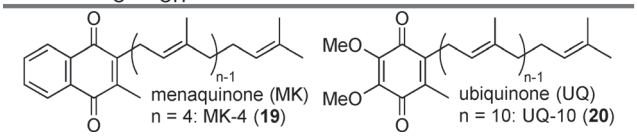


Figure 1. Structures of lysocin E (**1**), analogues **2**-**18**, menaquinone-4 (MK-4, **19**), and ubiquinone-10 (UQ-10, **20**). The names of the amino acid residues of **1** are written in the structure. Substituted moieties of the analogues are displayed in red. Boc = *tert*-butoxycarbonyl; TFA = trifluoroacetic acid.

The 37-membered macrocycle of **1** comprises 12 amino acid residues with an N-methylated amide at N-Me-D-Phe-5 and an ester linkage between L-Thr-1 and L-Thr-12 (Figure 1). The sequence contains one Gly-4, five types of L-configured amino acids (L-Thr-1/12, L-Ser-3, L-Leu-6, L-Glu-8, L-Ile-11), and four types of D-configured amino acids (D-Arg-2/7, N-Me-D-Phe-5, D-Gln-9, D-Trp-10), and (*R*)-3-hydroxy-5-methylhexanamide is appended to L-Thr-1 of the macrocyclic core. We recently reported the first total synthesis of this structurally complex peptidic natural product **1** by applying a full solid-phase strategy.[9,10] The approach we developed allowed for synthetic access to the enantiomeric (*ent*-**1**), epimeric (**2**), and N-demethylated (**3**) analogues of **1**. Synthesized **1** and its three analogues were biologically evaluated in an antimicrobial assay against *S. aureus*. The enantiomer *ent*-**1** exhibited activity (4 μg mL^{-1}) comparable to that of **1**, and a single stereocenter change in the acyl chain of Thr-1 (**1**→**2**) did not largely affect the potency (8 μg mL^{-1}). In contrast, the antibacterial property of N-demethylated analogue **3** (32 μg mL^{-1}), which has a different macrocycle conformation from that of **1** based on NMR analysis, was decreased eight-fold.[11] Indifference of the absolute configuration of **1** corroborated the importance of the recognition of achiral MK, and not that of chiral biomolecules such as proteins for the activity, and the decreased activity of **3** suggested the significance of the main-chain structure for organizing the bioactive three-dimensional shape.

The preliminary structure-activity relationship (SAR) study prompted us to perform another set of SAR studies on the side-chains of **1** (Figure 1).[12–14] Specifically, the 15 newly designed analogues **4**–**18** all have the same main-chain structure but the side-chain structures are diversified. Herein we report the highly efficient total syntheses of the 15 lysocin analogues **4**–**18** by utilizing two solid-phase strategies. The functions of **1** and its analogues were systematically evaluated based on the MK-dependent membrane lysis of liposomes and antibacterial activity against *S. aureus*. Comprehensive SAR studies of the analogues shed light on the side-chain functionalities relevant to the molecular mode of action of **1**, thereby providing valuable information for the search of optimized antibiotics.

Results and Discussion

Before planning the sites for the chemical modification of **1**, we hypothesized the following three potentially attractive interactions among **1**, MK, and phospholipids (Figure 1): 1) an electrostatic interaction of the anionic carboxylate group of L-Glu-8 or the cationic guanidine moieties of D-Arg-2/7 with the polar head group of phospholipids or the carbonyl groups of MK; 2) a hydrophobic interaction of the acyl chain of L-Thr-1 with the lipid chains of MK or phospholipids; and 3) an aromatic–aromatic interaction of the phenyl group of N-Me-D-Phe-5 or the indole ring of D-Trp-10 with the naphthoquinone structure of MK. To systematically investigate the significance of each of these interactions, the functional groups of L-Thr-1, D-Arg-2, N-Me-D-Phe-5, D-Arg-7, L-Glu-8, and D-Trp-10 were planned to be replaced in analogues **4**–**18**. In **4** and **5**, we substituted a neutral amide for the carboxylic acid of **1**, and in **6**–**9** we altered the guanidine groups of D-Arg-2/7. In **10**, we removed the acyl chain of **1**; in **11**–**16**, we exchanged the C7-

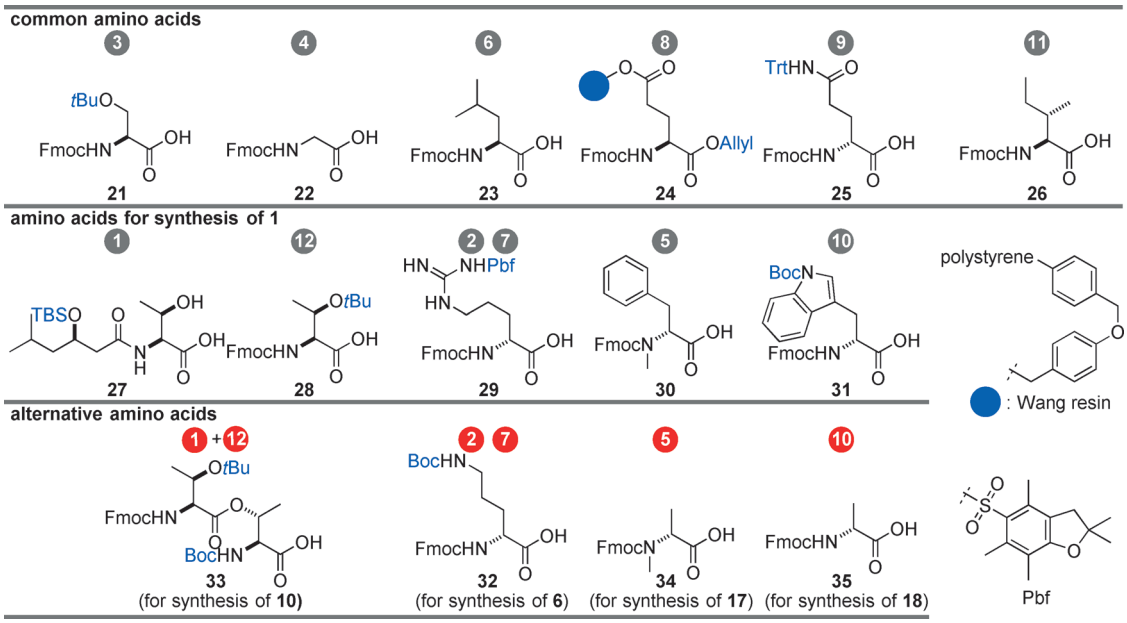
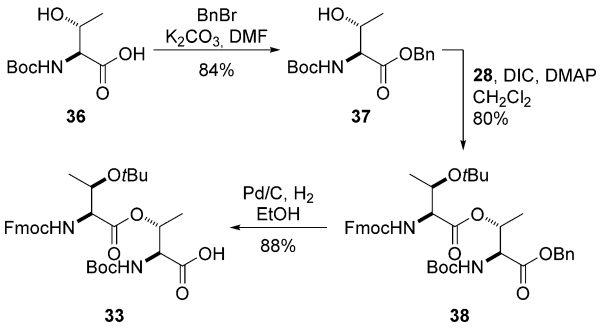


Figure 2. Building blocks for total syntheses of **1** and analogues **4**–**18**. Circled numbers are residue numbers (gray: common amino acids, red: alternative amino acids for the synthetic analogues). TBS = *tert*-butyldimethylsilyl; Fmoc = 9-fluorenylmethoxycarbonyl; Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; *t*Bu = *tert*-butyl; Trt = triphenylmethyl.

acyl chain of **1** into the C2- to C11-counterparts; and in **17** and **18**, we deleted the phenyl and indole rings, respectively.

The total synthesis of **1** was previously achieved by using the full solid-phase strategy without tedious purification of the intermediates.[9] The route to **1** employed **21**–**31** as monomers (Figure 2) and involved the following operations: 1) stepwise solid-phase assembly of the linear 12-mer sequence using Fmoc-based solid-phase peptide synthesis (SPPS)[15,16] from L-Glu-8 (**24**), the side-chain of which was anchored to Wang resin;[17] 2) orthogonal deprotection of the allyl group of L-Glu-8 to selectively liberate a C_α-carboxyl group; 3) intramolecular amidation between the free C_α-carboxyl and N_α-amino groups, taking advantage of the pseudo high dilution phenomenon of the resin-bound molecule;[18] and 4) simultaneous acid-promoted cleavage (Wang resin) and global deprotection of the side-chain protective groups (Pbf,[19] *t*Bu, TBS, Boc, and Trt) to release **1**.[20] This first-generation strategy was utilized to synthesize **4**–**9** with the same acyl group, and was modified to the second-generation strategy to prepare another series of analogues, **10**–**18**. Specifically, the macrocyclic amine **10** was designed as a common intermediate for the alternative strategy to enable efficient synthetic access to **11**–**16** by a single-step acylation. To construct **10**, the ester linkage within the macrocycle was envisioned to be preformed by solution-phase chemistry to avoid ester condensation, which is less efficient than the amide counterpart on the solid matrices. Therefore, the second-generation strategy was planned to utilize N-Boc-protected ester **33** instead of the acyl chain-linked **27** and **28**.

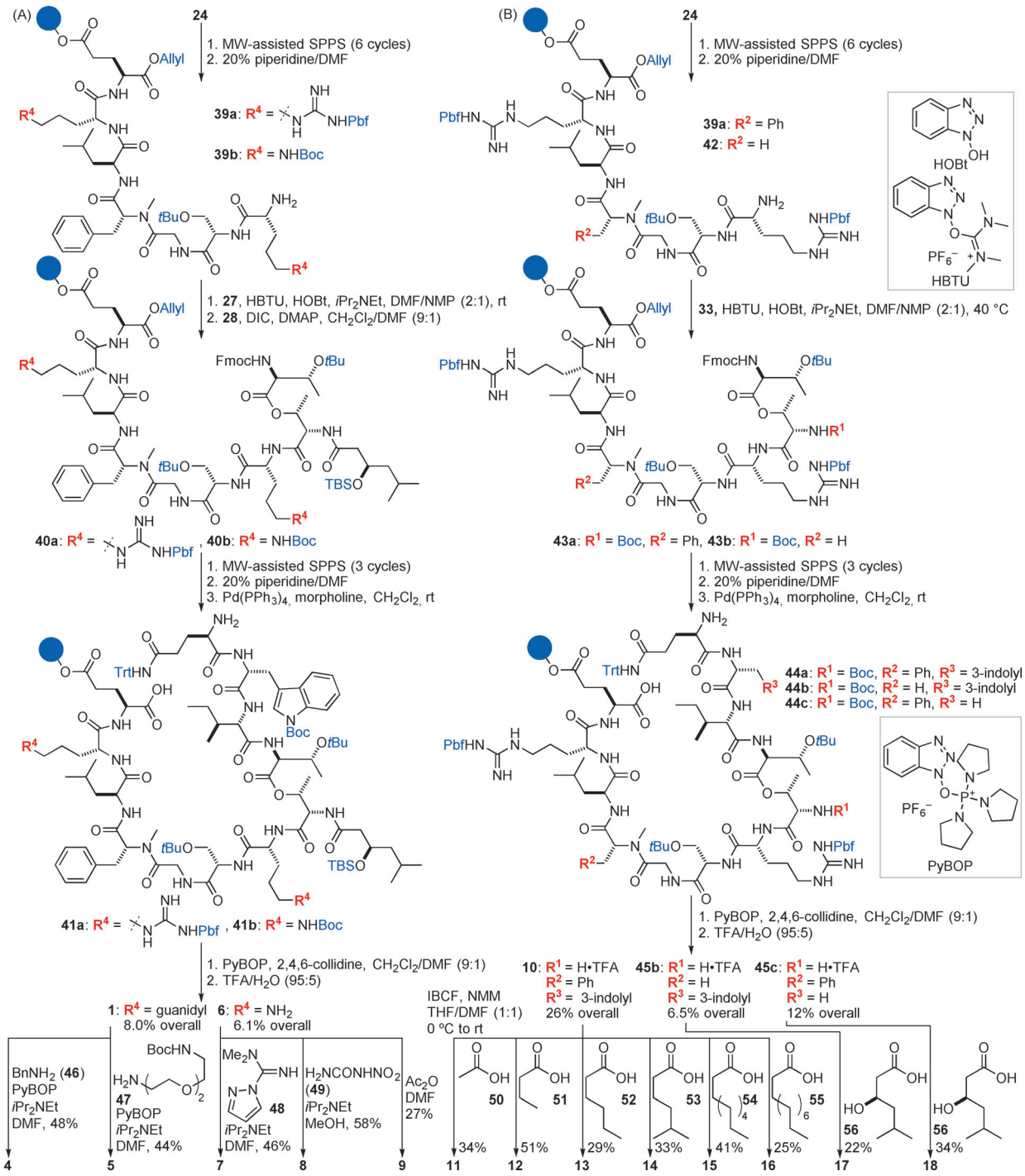
Among all the components **21**–**35** necessary for the assembly of **4**–**18**, **27** and **33** required synthetic preparation (Figure 2). The route to **27** was described previously.[9] Scheme 1 depicts the three-step synthesis of the building



Scheme 1. Synthesis of ester **33**. DIC=*N*,*N*′-diisopropylcarbodiimide; DMAP = *N*,*N*-dimethyl-4-aminopyridine; DMF = *N*,*N*-dimethylformamide.

block **33** from Boc-L-Thr-OH (**36**) for the second-generation synthesis. Treatment of carboxylic acid **36** with BnBr and K_2CO_3 afforded benzyl ester **37**. The hydroxy group of **37** was in turn condensed with the carboxylic acid of Fmoc-L-Thr(*t*Bu)-OH (**28**) in the presence of DIC and DMAP to produce **38**. Hydrogenolysis of the benzyl group of **38** with Pd/C gave rise to the requisite Boc-protected ester **33** in 88% yield.

With all the building blocks in hand, we began the first-generation solid-phase synthesis of lysocin E (**1**) and **6** from allyl glutamate-loaded resin **24** (Scheme 2 A). Natural **1** and its analogue **6** were assembled from components **21**-**31**, and **21**-**28**, **30**, **31**, and **32**, respectively. Based on our previous report,[9] the peptide chain of **1** or **6** was elaborated at 40 °C under microwave (MW)-assisted conditions[21] to facilitate the reactions. Cycles of piperidine-promoted N_α-deprotection and HOBt/HBTU[22]-mediated amide coupling were applied to **24** using **29**, **23**, **30**, **22**, **21**, and **29** for **1**; or **32**, **23**, **30**, **22**, **21**, and **32**



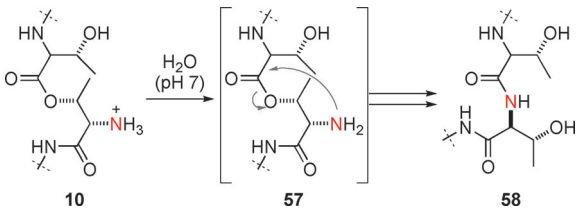
Scheme 2. A) First-generation solid-phase synthesis of **1** and **6**, and preparation of **4**–**9**. B) Second-generation solid-phase synthesis of **10** and **45 b/c**, and preparation of **11**–**18**. MW-assisted SPPS: i) 20% piperidine/DMF; ii) N_α-Fmoc-protected amino acid, HBTU, HOBt, *i*Pr$_2$NEt, DMF, NMP, 40 °C. HBTU = *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate; HOBt = 1-hydroxybenzotriazole; IBCF = isobutylchloroformate; NMM = *N*-methylmorpholine; NMP = *N*-methyl-2-pyrrolidone; PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate.

for **6**. This sequence resulted in the formation of the resin-bound heptapeptide **39 a** or **39 b** after removing the Fmoc group at the N-terminus. To prevent epimerization at the C_α-position of amide **27**, condensation of amine **39 a/b** with (*R*)-3-hydroxy-5-methylhexanamide-substituted **27** was preemptively conducted at room temperature without MW irradiation to produce the octapeptide. The subsequent DIC/DMAP-promoted esterification of the obtained peptide with **28** gave rise to nonapeptide **40 a/b**. The MW-assisted N_α-deprotection/condensation protocol was re-applied to **40 a/b** for stepwise elongation of the three residues (**26**, **31**, and **25**) to deliver the linear dodecapeptide. The Fmoc group of the N-terminus and the allyl group of the C-terminus of the product were then removed by treatment with piperidine, and subsequently with

catalytic $Pd(PPh_3)_4$[23] and an excess of morpholine to provide the macrolactam precursor **41a**/**b**. Facile on-resin cyclization of **41a**/**b** was then effected using PyBOP[24]/2,4,6-collidine, leading to the 37-membered macrolactam. Finally, treatment of the macrolactam with 95% aqueous TFA simultaneously realized cleavage from the Wang resin and global deprotection of the acid-labile protective groups (Pbf, *t*Bu, Boc, Trt, and TBS groups), releasing **1**/**6** into solution. After purification by reversed-phase HPLC, lysocin E (**1**) and its derivative **6** were obtained in 8.0 and 6.1% yields, respectively, over 25 steps from **24**.

The thus-obtained fully deprotected **1** and **6** were further transformed into the five analogues **4**, **5**, and **7**–**9**, by applying chemoselective single-step reactions. The carboxylic acid of L-Glu-8 of **1** was condensed with benzylamine **46** or mono-Boc-protected diamine **47** by using PyBOP to furnish the amide analogues **4** and **5** in 48 and 44% yields, respectively. Alternatively, the two primary amines of **6** were functionalized to generate **7**–**9**. Treatment of **6** with 1*H*-pyrazole-*N*,*N*-dimethyl-1-carboxamidine (**48**),[25] nitrourea (**49**), and Ac_2O resulted in the formation of dimethylguanidine (**7**, 46%), urea (**8**, 58%), and acetyl (**9**, 27%) analogues, respectively.

Next, the second-generation strategy was utilized to construct the common macrocyclic amine **10** (Scheme 2B). Heptapeptide **39a**, which was also used in the first-generation synthesis, was prepared from the Wang-resin tethered **24** by repeated cycles of piperidine-promoted Fmoc removal and MW-assisted condensation with the six Fmoc-amino acids. N-Boc-protected ester **33** was in turn attached using HBTU/HOBt, giving rise to nonapeptide **43a**. Three additional cycles of N_α-deprotection and MW-promoted chain-elongation (**26**, **31**, and **25**), followed by detachment of the Fmoc and allyl groups, provided the macrolactamization precursor **44a**. Finally, the on-resin macrolactamization by the action of PyBOP, and the TFA-promoted global deprotection and resin cleavage furnished amine **10** as its TFA salt (26% yield over 24 steps from **24**). The excellent overall yield of **10** compared with that of **1** (8.0%) verified the advantage of direct attachment of the pre-esterified **33** over separate application of the two monomers **27** and **28**. Despite its stability under acidic conditions, the 37-membered lactone of **10** readily converted into 36-membered lactam **58** via an O to N acyl transfer in solution at pH 7 or above (Scheme 3, see also Supporting Information).



10 57 58

Scheme 3. Undesired O to N acyl transfer of **10** in pH 7 buffer.

Based on this specific physicochemical feature of **10**, we decided not to use **10** for the functional evaluation, and it was instead directly subjected to acylation reactions.

Remarkably, the post-SPPS acylation of non-protected **10** chemoselectively occurred at the N_α-amino group of L-Thr-1, permitting rapid access to the six acyl-chain analogues. Namely, TFA salt **10** was treated with activated carboxylic acids, which were prepared from carboxylic acids **50**, **51**, **52**, **53**, **54**, and **55** in the presence of IBCF and NMM, leading to analogues **11**, **12**, **13**, **14**, **15**, and **16** in 34, 51, 29, 33, 41, and 25% yields, respectively.

The second-generation strategy was applicable for the total syntheses of N-Me-D-Phe-5-substituted **17** and D-Trp-10-substituted **18**. By use of monomers **34** and **35** in place of the original **30** and **31**, the linear dodecapeptides **44b** and **44c** were obtained by the Fmoc-based SPPS (**24**→**42**→**43b**→**44b**, and **24**→**39a**→**43a**→**44c**, respectively). Macrolactamization and subsequent TFA treatment transformed **44b** and **44c** into **45b** in 6.5% overall yield, and **45c** in 12% overall yield, respectively. Lastly, the targeted aromatic ring analogues **17** and **18** were synthesized from amine-TFA salts **45b** and **45c** using the IBCF-activated ester of **56** and NMM in 22 and 34% yields, respectively.[26] The total syntheses of the 15 structurally complex and diverse analogues **4**–**18** clearly demonstrated the robustness and generality of the present synthetic strategies.

Having successfully prepared all of the analogues, the MK-dependent membrane disruption and antimicrobial activities of the 14 analogues (**4**–**9** and **11**–**18**) were assessed along with the parent compound **1**. To estimate the MK-dependent membrane lytic activity without the interference of other biological molecules, the first assay employed liposomes containing only MK or UQ and lipids.[5] In general, bacterial membranes are negatively charged with lipids bearing phospholipid head groups such as phosphatidylglycerol (PG) and cardiolipin, whereas mammalian membranes are enriched in zwitterionic phospholipids (neutral in net charge) such as phosphatidylcholine (PC) sphingomyelin.[27] To mimic the bacterial membranes, large unilamellar vesicles (LUVs) containing PGs were used as liposomes. Specifically, LUVs comprising a 1:1 ratio of egg yolk PC (EYPC)/egg yolk PG (EYPG) were prepared in the presence of 1.25 mol% of MK-4 (**19**)[28] or UQ-10 (**20**). Carboxyfluorescein (CF) was encapsulated as a fluorescent indicator in the LUVs. Although fluorescence of the CF molecules within the LUVs is self-quenched due to the high concentration, membrane disruption by peptides causes the CF to leak from LUVs, resulting in dilution of the CF molecules and increase of fluorescence intensity. Therefore, fluorescence was measured as an indicator of the LUV membrane disruption. The fluorescence change caused by lysis in the presence of each peptide was standardized according to the maximum intensity induced by adding Triton X-100.

When 2.5 μM of lysocin E (**1**) was added to the LUVs with either 1.25 mol% of MK (**19**) or UQ (**20**) (Figure 3), only the MK-containing LUVs showed increased fluorescence, corroborating the MK-dependency of the membrane rupture by **1**. The non-activity toward the UQ-containing LUVs was consistent for all of the analogues, confirming their non-disruption activities without MK (red lines). Interestingly, the ability to disrupt the MK-doped membrane varied significantly. The fluorescence changes (%) of all the analogues after reaching their plateaus

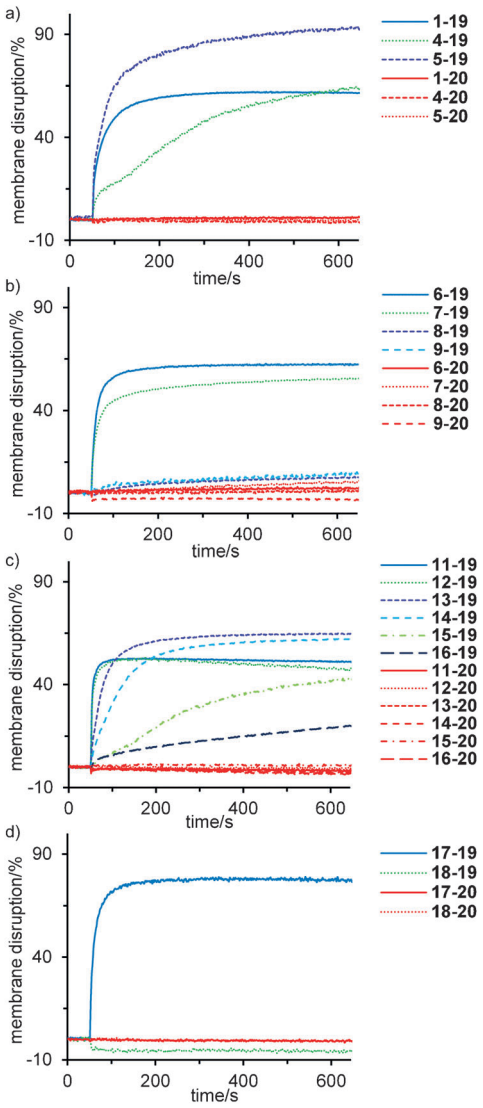


Figure 3. Time-course of CF leakage from LUVs [EYPC/EYPG (1:1) containing 1.25 mol% **19** or **20**] caused by a) 2.5 μM of **1**, **4**, and **5**, b) 2.5 μM of **6**–**8** and 10 μM of **9**, c) 2.5 μM of **11**–**16**, and d) 10 μM of **17** and **18**. Each analogue was added at 50 s. CF leakage caused by each analogue was determined by the fluorescence intensity of leaked CF standardized against the maximum leakage by Triton X-100. CF = carboxyfluorescein; EYPC = egg yolk phosphatidylcholine; EYPG = egg yolk phosphatidylglycerol; LUV = large unilamellar vesicle.

(600–650 s) were quantified for comparison of the activities (Table 1). In these experiments, the natural **1** exhibited 62% disruption at 2.5 μM. Exchange of the anionic carboxylate of L-Glu-8 of **1** with the neutral amides of **4** and **5** did not decrease the membrane-disrupting activity compared to **1** (62% for **1**, 64% for **4**, and 93% for **5**). When the cationic guanidine moieties of D-Arg-2/7 of **1** were substituted with the other cationic amine (**6**) and dimethyl guanidine (**7**) functionalities, the potency was retained (62% for **6** and 55% for **7**) despite the difference in steric sizes of the functional groups. In sharp contrast, applying the neutral urea (**8**) and amide (**9**) analogues diminished the fluorescence change (7.4% for **8** and 0% for **9**). A higher concentration of **9** (10 μM) was required to observe

Table 1. Membrane-disrupting and antimicrobial activities of the natural **1**, analogues **4**–**9** and **11**–**18**.

Compounds	Membrane disruption [%][a]				MIC[e] [μg mL^{-1}]
	1.25 mol% **19**		1.25 mol% **20**		
	2.5 μM[b,d]	10 μM[c,d]	2.5 μM[c,d]	10 μM[c,d]	
1	62		1.0		4
4	64		0		4
5	93		0		2
6	62		2.2		4
7	55		5.1		4
8	7.4		0.9		8
9	0	9.3	0	0	16
11	51		0		16
12	48		0		4
13	65		0		2
14	62		0		4
15	42		0.7		4
16	20		0		32
17	0.8	65	0	0	8
18	0	0	0	0	> 128

[a] The membrane disruption activities (%) were determined from fluorescence intensity between 600–650 s. The LUVs consisted of EYPC/EYPG (1:1) containing 1.25 mol% **19** or **20**. [b] The mean values of the duplicate experiments. [c] The values of the single experiment. [d] Final concentration of peptides. [e] Minimal concentration required to inhibit cell growth of methicillin-susceptible *S. aureus*. 4 μg mL^{-1} of lysocin E (**1**) corresponds to 2.5 μM.

the MK-dependent membrane rupture (9.3%). The assay data of **4**–**9** revealed the lack of impact of the anionic character of L-Glu-8 and the importance of the cationic character of D-Arg-2/7 for the activity. Accordingly, it is likely that the guanidine (**1**), amine (**6**), and dimethylguanidine (**7**) structures play an important role in binding to the anionic lipid head groups of PG through attractive electrostatic interactions to induce membrane rupture.

The C2- (**11**), C4- (**12**) C6- (**13**), C7- (**14**), and C9- (**15**) acyl chain-modified analogues exhibited similar membrane-disrupting activities to that of the parent **1** with the hydroxylated C7-acyl group at L-Thr-1 (51, 48, 65, 62, and 42% for **11**, **12**, **13**, **14**, and **15**, respectively). Although the more hydrophobic molecule **16** with the C11-acyl chain was expected to more favorably insert into the hydrophobic membrane, **16** turned out to have lower activity (20%). The lower water solubility of **16** likely reduced the effective concentration of **16**, thereby decreasing its activity.

Drastic changes in the membrane-disrupting activities were observed upon assay of the aromatic ring-modified analogues **17** and **18**. Removing the phenyl group of N-Me-D-Phe-5 (**17**) or the indole ring of D-Trp-10 (**18**) abolished the activities (0.8% for **17** and 0% for **18**). When the concentrations of the peptides were increased from 2.5 μM to 10 μM, the des-phenyl analogue **17** regained its activity (65%), but the des-indole analogue **18** was inert. Because all 14 analogues except for **18** possessed the activity, the indole ring appeared to be the most essential structural unit of **1** for the MK-dependent membrane-disruption.

Finally, an antibacterial assay for the 14 lysocin derivatives **4**–**9** and **11**–**18** was carried out using *S. aureus* (Table 1). All 14 analogues, except for **18**, were found to possess antimicrobial activitity. The MIC values of the parent **1** and 10 analogues (**4**, **5**, **6**, **7**, **8**, **12**, **13**, **14**, **15**, and **17**) were within single-digit micromolar range (2-8 $\mu g\,mL^{-1}$), and those of the other three analogues (**9**, **11**, and **16**) were double-digit micromolar (16-32 $\mu g\,mL^{-1}$). The des-indole analogue **18** showed no antimicrobial activity up to 128 $\mu g\,mL^{-1}$. The effect of the polar functional groups of **1** on the antibacterial activities was well-correlated with the membrane-disrupting activities. The low MIC values (2 or 4 $\mu g\,mL^{-1}$) were preserved upon alteration of the anionic carboxylate group of L-Glu-8 of **1** to the neutral amides (**4**, **5**), or exchange of the cationic guanidines of D-Arg-2/7 to the cationic amines (**6**) and dimethyl guanidines (**7**). On the other hand, deletion of the cationic character increased the MIC values (8 $\mu g\,mL^{-1}$ for **8**, and 16 $\mu g\,mL^{-1}$ for **9**), again emphasizing the significance of the cationic functions of **1** for the potent activities.

Despite their relative unimportance in the liposome experiments, the lengths of the acyl chains at L-Thr-1 influenced the MIC values of **11**–**16**. The antimicrobial activity of analogues **12**–**15** possessing C4- to C9-acyl chains was comparable (2-4 $\mu g\,mL^{-1}$) to that of **1**, whereas that of C2-acyl analogue **11** and C11-acyl analogue **16** was weaker (16 and 32 $\mu g\,mL^{-1}$, respectively). The discrepancies between the liposome and bacteria assays could originate from the differences in the surface structures between the simple model membranes and the bacterial plasma membrane/peptidoglycan layer containing many other components. Nevertheless, these antimicrobial data indicated the importance of the appropriate hydrophobicity of this moiety for the bioactivity. Due to the likelihood that most hydrophilic **11** does not partition into hydrophobic lipids, and most hydrophobic **16** does not dissolve in aqueous media in effective fashions, the lower activities of **11** and **16** are attributable to the changed physicochemical properties.

As expected from the membrane-disrupting activities of **17** and **18**, deletion of the phenyl ring or the indole ring reduced the antimicrobial activity. The MIC value of the N-Me-D-Phe-5-substituted analogue **17** was 2-fold higher (8 $\mu g\,mL^{-1}$) than that of **1**. On the other hand, the D-Trp-10-substituted analogue **18** totally lost its activity ($>128\ \mu g\,mL^{-1}$). The activities of **17** and **18** proved that the indole structure of **1** is more biologically essential than the phenyl ring. As all the lysocin-based structures with the indole ring (**1**, **4**–**9** and **11**–**17**) retained both the MK-dependent membrane-disrupting and antimicrobial activities, the indole ring could serve as the requisite recognition unit of the MK structure.

Overall, the key structural features that are crucial for the two types of activities were established to be 1) the cationic functionalities of D-Arg-2 and -7, 2) the hydrophobic acyl group of four to nine carbons at L-Thr-1, and 3) the indole ring of D-Trp-10. These results offered a clearer picture of the mode of action of the original **1**. The cationic guanidine moieties of D-Arg-2/7 and the hydrophobic (*R*)-3-hydroxy-5-methylhexanamide of L-Thr-1 help **1** bind through the anionic polar heads[29] and hydrophobic lipid tails of the bacterial membrane, respectively. On the membrane surface, the electron-rich indole of D-Trp-10 and the electron-deficient naphthoquinone of MK bind as a result of the aromatic-aromatic interaction,[30] leading to the formation of the equimolar **1**-MK complex. Finally, the complexation causes membrane damage and eventual cell death. This new hypothetical mechanism of action provides us with valuable information for designing more active and selective antibiotics based on the lysocin structure.

Conclusion

In summary, we performed comprehensive SAR studies of the 14 new analogues of a structurally complex 37-membered depsipeptide, lysocin E (**1**). The 14 analogues **4**–**9** and **11**–**18** were designed to decipher the biological roles of the anionic residue L-Glu-8, the cationic residues D-Arg-2/7, the hydrophobic N-acyl group of L-Thr-1, and the aromatic residues D-Trp-10/N-Me-D-Phe-5. The two different full solid-phase synthetic routes allowed for the efficient access to **1** and the 14 structurally diverse analogues **4**–**9** and **11**–**18**. The first- and second-generation SPPS strategies assembled **1**, **6**, **10**, **45b**, and **45c**, which were further derivatized into **4**/**5**, **7**–**9**, **11**–**16**, **17**, and **18**, respectively, by chemoselective post-SPPS modifications. MK-dependent membrane-disrupting activity using LUVs and antibacterial activity using *S. aureus* were evaluated for the 14 synthesized analogues, **4**–**9** and **11**–**18**. The prominent functionalities that were necessary for the potent liposomal and antimicrobial activities were established to be the cationic guanidines of D-Arg-2/7, the hydrophobic acyl group of four to nine carbons at L-Thr-1, and the indole ring at D-Trp-10. These novel findings provide the first evidence for the hypothetical roles of these functionalities. After the cationic guanidine moieties and the hydrophobic acyl chain effectively bind to the negatively charged bacterial membrane, the aromatic-aromatic interaction between the indole ring and the naphthoquinone of MK within the membrane leads to critical membrane damage. The present achievements highlight the power of robust and divergent solid-phase strategies for systematic SAR studies, and chart a rational path forward for development of lysocin-based next-generation antibiotics for treating various infectious diseases.

Acknowledgements

This research was financially supported by Grant-in-Aids for Scientific Research (A) (JSPS KAKENHI Grant Number JP26253003) and Scientific Research on Innovative Areas (JSPS KAKENHI Grant Number JP16H01130) to M.I., for Research Activity Start-up (JSPS KAKENHI Grant Number JP15H06156) to H.I., and for Scientific Research (S) (JSPS KAKENHI Grant Number JP15H05783) and JST A-STEP to K.S. A JSPS fellowship to T.K. is gratefully acknowledged. We thank Drs. T. Kuranaga (Hokkaido University) and M. Urai (National Institute of Infectious Diseases) for valuable discussion.

Keywords: antibiotics · peptides · solid-phase synthesis · structure-activity relationships · total synthesis

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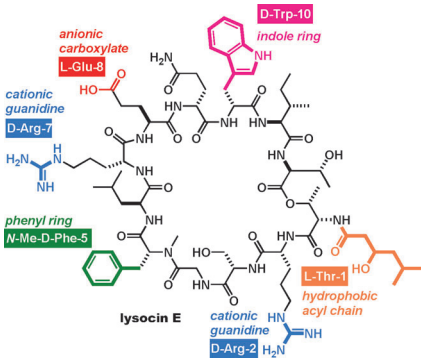
Received: August 24, 2016
Published online on ■■ ■■, 0000

FULL PAPER

What parts work best? Antibiotic lysocin E (**1**), a 37-membered depsipeptide, binds to menaquinone in the bacterial membrane to exert its potent bactericidal activity (see figure). Comprehensive SAR studies of **1** were performed by systematically changing the side-chain structures. The key structural features for the antimicrobial activities were established to be the cationic functionalities at D-Arg-2/7, the hydrophobic acyl group at L-Thr-1, and the indole ring at D-Trp-10.



Natural Products

*T. Kaji, M. Murai, H. Itoh, J. Yasukawa, H. Hamamoto, K. Sekimizu, M. Inoue**

■■ – ■■

Total Synthesis and Functional Evaluation of Fourteen Derivatives of Lysocin E: Importance of Cationic, Hydrophobic, and Aromatic Moieties for Antibacterial Activity