Natural Product Synthesis

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Total Synthesis and Biological Evaluation of the Antibiotic Lysocin E and Its Enantiomeric, Epimeric, and N-Demethylated Analogues**

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Abstract: *Lysocin E, a macrocyclic peptide, exhibits potent antibacterial activity against methicillin-resistant Staphylococcus aureus (MRSA) through a novel mechanism. The first total synthesis of lysocin E was achieved by applying a full solid-phase strategy. The developed approach also provides rapid access to the enantiomeric, epimeric, and N-demethylated analogues of lysocin E. Significantly, the antibacterial activity of the unnatural enantiomer was comparable to that of the natural isomer, suggesting the absence of chiral recognition in its mode of action.*

Infectious diseases caused by antibiotic-resistant bacteria pose a threat to public health around the world.[1,2] Nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals have become an especially serious clinical problem. Recently, Sekimizu and co-workers isolated a novel peptidic natural product, lysocin E (**1a**, Scheme 1), from a culture supernatant of a *Lysobacter* species and showed that **1a** was a potent growth inhibitor of MRSA with a minimum inhibitory concentration (MIC) of 4 $\mu g\,mL^{-1}$.[3] Furthermore, **1a** was found to possess a potent therapeutic effect in mice infected with *S. aureus*. The ED_{50} value of **1a** (0.5 $mg\,kg^{-1}$) was even smaller than that of vancomycin (5.8 $mg\,kg^{-1}$), which is widely used to treat MRSA infections.

Menaquinone, a co-enzyme in the bacterial respiratory chain,[4] has been assumed to be the main target molecule of **1a** (Scheme 1).[3] Peptide **1a** selectively forms an equimolar complex with menaquinone present in the cytoplasmic membrane of *S. aureus*, effecting cell death through membrane disruption. Although the involvement of other molecules in the mechanism has not been ruled out, the mode of action of **1a** is apparently distinct from those of previously known antibiotics. As an antibiotic that functions by a novel mechanism is less likely to show cross-resistance with existing antibiotics, **1a** is viewed as a useful lead compound for the development of a suitable drug candidate.[5]

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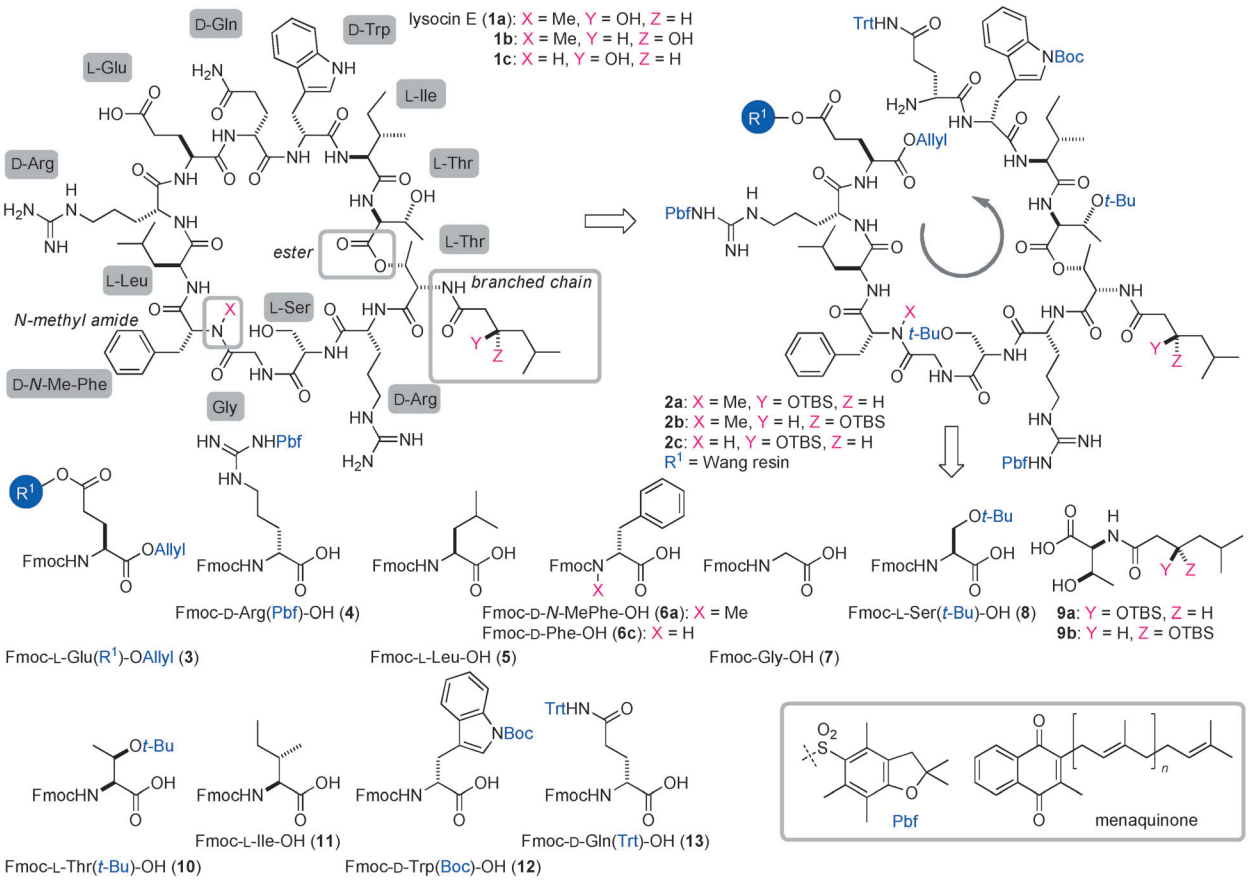
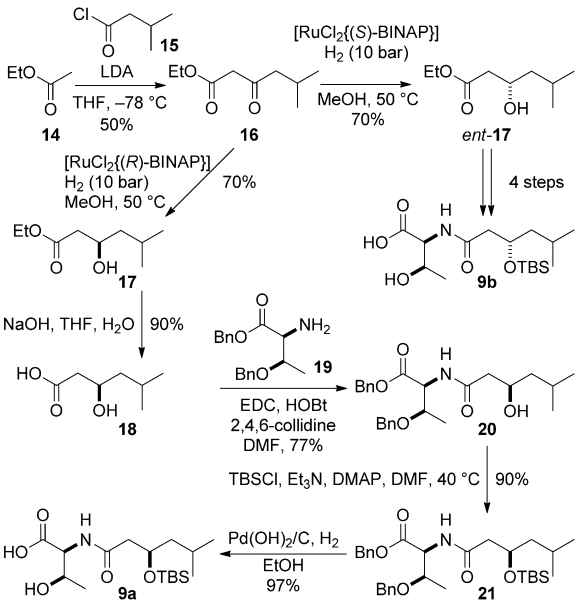
A solid-phase synthetic route to **1a** would make it possible to prepare a wider variety of lysocin E analogues than with a solution-phase route, thereby enabling a comprehensive structure–activity relationship (SAR) study of **1a** in search of more potent analogues.[6–8] Herein, the first total synthesis of lysocin **1a** by a solid-phase approach is disclosed. The developed route enabled the preparation of not only the enantiomer of **1a** (*ent*-**1a**), but also of epimeric (**1b**) and N-demethylated analogues (**1c**). A preliminary comparative study of the antibacterial properties of **1a**, *ent*-**1a**, **1b**, and **1c** sheds light on the structural factors that are relevant to the lysocin E molecular mode of action.

The core structure of **1a** is a 37-membered macrocycle comprising twelve amino acid residues with an N-methylated amide and an ester linkage (Scheme 1). There are four types of D-configured amino acids (D-Gln, D-Trp, D-Arg, D-*N*-Me-Phe) present within the sequence, and (*R*)-3-hydroxy-5-methylhexanamide is appended to the macrocyclic core.[9] To enable a full solid-phase synthesis, this complex structure was retrosynthetically disconnected into the eleven building blocks **3**–**5**, **6a**, **7**, **8**, **9a**, and **10**–**13**, the protective groups of which were designed to possess three dimensions of orthogonal reactivity. Acid-labile groups (Wang resin[10] of **3**, Pbf[11] of **4**, *t*Bu of **8** and **10**, TBS of **9a**, Boc of **12**, and Trt of **13**) were chosen for protection of the side chains, whereas the base-labile Fmoc group was used for temporary N_α protection.[12] The allyl group, cleavable under neutral conditions, was selected for the carboxylic acid moiety of **3**. Therefore, our strategy involved the following operations:[13] 1) stepwise solid-phase assembly of the linear sequence using Fmoc chemistry from side-chain-anchored **3**, 2) orthogonal deprotection of the allyl group of **2a** to selectively liberate the C_α carboxylic acid, 3) intramolecular amidation between the free C_α carboxylic acid and the N_α amine, taking advantage of the pseudo-high dilution of the resin-bound molecule,[14] and 4) simultaneous acid-promoted global deprotection and cleavage to release the targeted peptide **1a**. This strategy would allow for the unified and efficient preparation of the lysocin E derivatives *ent*-**1a**, **1b**, and **1c** by simply replacing the building blocks of **1a** for their enantiomeric counterparts, **9b**, and **6c**, respectively.

As a prelude to the solid-phase synthesis of **1a**, the branched fragment **9a** was prepared (Scheme 2). LDA-promoted addition of ethyl acetate (**14**) to isovaleryl chloride (**15**) afforded β-ketoester **16**. Asymmetric Noyori reduction of the ketone using $[RuCl_2\{(R)\text{-BINAP}\}]$[15] converted **16** into enantiomerically pure (*R*)-ethyl-3-hydroxy-5-methylhexanoate (**17**).[16] Saponification of **17** provided carboxylic acid **18**, which was condensed with L-Thr(Bn)-OBn (**19**) to give



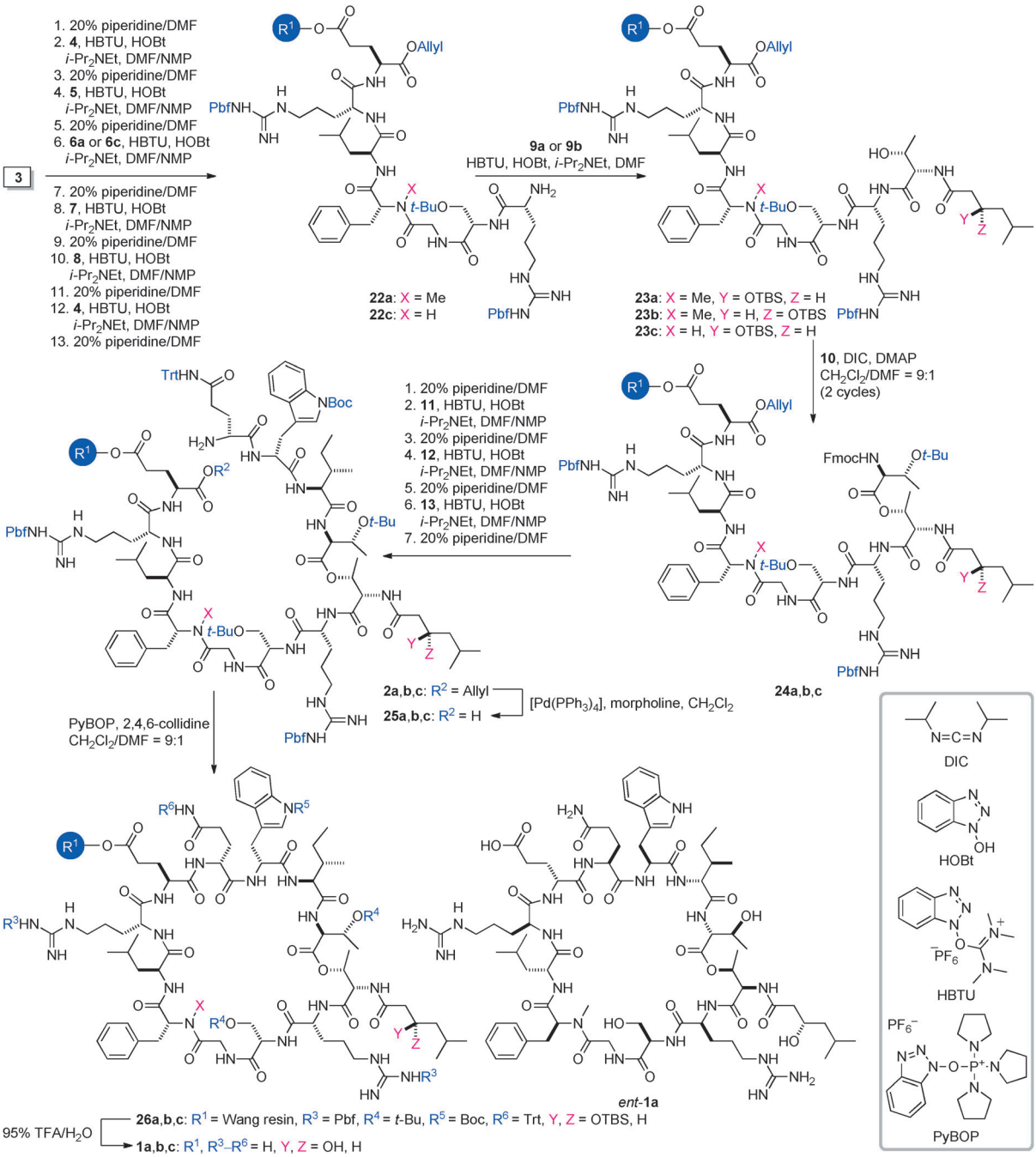
Scheme 1. Structures of lysocin E (**1a**), synthesized analogues **1b** and **1c**, and menaquinone and retrosynthesis of **1a**–**1c**. Boc = *tert*-butoxycarbonyl, Fmoc = 9-fluorenylmethoxycarbonyl, Trt = triphenylmethyl, TBS = *tert*-butyldimethylsilyl, Wang resin = 4-benzyloxybenzyl alcohol resin.



Scheme 2. Synthesis of **9a** and **9b**. BINAP = 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl, DMAP = 4-(*N*,*N*-dimethylamino)pyridine, EDC = 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, HOBt = 1-hydroxybenzotriazole, LDA = lithium diisopropylamide.

amide **20**. Then, TBS protection of the secondary alcohol of **20** and subsequent hydrogenolysis of the two benzyl groups of **21** furnished fragment **9a**. Alternatively, the diastereomeric fragment **9b** was synthesized for the total synthesis of epimeric analogue **1b** by using *ent*-**17** and **19**.

With all of the building blocks in hand, we commenced the solid-phase synthesis of lysocin E (**1a**) from the allyl glutamate loaded resin **3** (Scheme 3). The peptide chain of **1a** was elaborated at 40 °C under microwave-assisted conditions[17] to facilitate the reactions. Cycles of piperidine-promoted N_α deprotection and HOBt/HBTU[18]-mediated amide coupling were applied to **3** using **4**, **5**, **6a**, **7**, **8**, and **4**. This sequence resulted in the formation of the resin-bound heptapeptide **22a** after removal of the Fmoc group at the N terminus. As epimerization at the C_α position of amide **9a** would likely occur under forcing conditions, the condensation of amine **22a** with (*R*)-3-hydroxy-5-methylhexanamide-substituted **9a** was preemptively conducted at room temperature without microwave irradiation to produce **23a**. Next, DIC/DMAP-promoted esterification between the hindered secondary alcohol of **23a** and the carboxylic acid of **10** was performed twice to realize complete conversion of the starting material into nonapeptide **24a**. The microwave-assisted N_α deprotection/condensation procedure was re-applied to **24a** for the stepwise elongation of the three



Scheme 3. Total synthesis of lysocin E (**1a**) and its enantiomeric (*ent*-**1a**), epimeric (**1b**), and N-demethylated (**1c**) analogues. DMF = dimethylformamide, NMP = *N*-methyl pyrrolidinone, TFA = trifluoroacetic acid.

residues (**11**, **12**, and **13**); then, the Fmoc group of the product was removed, delivering linear dodecapeptide **2a**.

The last three steps completed the total synthesis of **1a** (Scheme 3). First, the allyl group of the resin-bound glutamic acid of **2a** was removed with catalytic amounts of $[Pd(PPh_3)_4]$ and an excess of morpholine to provide macrolactam precursor **25a**.[19] Second, the facile on-resin cyclization of **25a** was effected using PyBOP[20]/2,4,6-collidine, leading to 37-membered macrolactam **26a**. Third, treatment of **26a** with aqueous TFA (95%) simultaneously realized cleavage from the Wang resin and global deprotection of the acid-labile protective groups (Pbf, *t*Bu, Boc, Trt, and TBS), releasing **1a** into solution. After purification by reverse-phase HPLC, lysocin E (**1a**) was obtained in 8.0% yield over 24 steps from **3**. The ^{1}H and ^{13}C NMR data as well as the HPLC retention time of synthetic **1a** are identical to those of natural **1a**.

The presented optimized solid-phase strategy provided rapid access to three synthetic analogues. Whereas the enantiomer of lysocin E (*ent*-**1a**) was prepared from the enantiomeric eleven building blocks, epimeric **1b** and N-demethylated **1c** were synthesized from **3** by replacing the monomers **9a** and **6a** with **9b** and **6c**, respectively

(Scheme 3). The consistently high overall yields for **1b** (6.8% from **3**) and **1c** (8.7% from **3**) clearly demonstrate the robustness of our approach to obtain structurally modified lysocin E analogues.

A preliminary antibacterial study of the synthetic compounds was carried out using *S. aureus* (Table 1). The same MIC values of natural and synthetic **1a** again confirmed their chemical equivalence (4 $\mu g\,mL^{-1}$). Most importantly, enantiomeric *ent*-**1a** exhibited comparable activity (4 $\mu g\,mL^{-1}$) to **1a**. The insignificance of the absolute configuration of the molecule suggests that the mode of action mainly involves the recognition of achiral menaquinone and not that of chiral biomolecules, such as proteins. Similarly, a single inversion of a stereocenter in the branched side chain (**1a**→**1b**) did not largely affect the potency (8 $\mu g\,mL^{-1}$). In contrast to these data, an eightfold decrease in the antibacterial activity was observed with N-demethylated analogue **1c** (32 $\mu g\,mL^{-1}$), demonstrating the importance of the *N*-methyl group within the macrolactam.

Table 1: In vitro antibacterial activity.

Compound	MIC[a] [$\mu g\,mL^{-1}$]
1a (natural)	4
1a (synthetic)	4
ent-**1a**	4
1b	8
1c	32
vancomycin	1

[a] Minimal concentration required to inhibit cell growth of methicillin-susceptible *S. aureus*.

To gain insight into the relationship between the assay data and the specific structure of the macrolactam region, **1a**, **1b**, and **1c** were analyzed by NMR spectroscopy in [D_6]DMSO (Supporting Information, Figure S1 and Table S1). As expected, the ^1H and ^{13}C NMR chemical shifts for the macrocyclic core of epimeric **1b** are in excellent agreement with those of **1a**, and thus the single stereochemical inversion has negligible influence on the three-dimensional structure of the lactam. On the other hand, the chemical shifts of the macrocycle of **1c** significantly deviate from those of **1a**, reflecting the large impact of N methylation on the lactam conformation.[21] Although more structural and biological studies remain to be performed, these initial experiments indicate that the *N*-methyl moiety is an essential structural factor in organizing the core of **1a** into a bioactive conformation.

In conclusion, the total synthesis of antibiotic peptide lysocin E (**1a**) was accomplished for the first time. The full solid-phase approach is efficient and robust in the assembly of the complex 37-membered macrolactam and provided rapid access to enantiomeric (*ent*-**1a**), epimeric (**1b**), and N-demethylated (**1c**) analogues of lysocin E. Comparative evaluation of the antibacterial activities of these synthetic materials revealed crucial structure–activity relationships: The equally high activity of *ent*-**1a** suggests the absence of chiral recognition in the mode of action of **1a**, and the decreased potency of **1c** indicated the biological significance of the *N*-methyl group. More detailed SAR studies will lead to the elucidation of the mode of action, optimization of the antibacterial activity, and determination of the structure of lysocin E in complex with menaquinone. These studies will provide the foundation for the development of new antibacterial agents against MRSA with unique mechanisms.

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[1] a) F. D. Lowy, *J. Clin. Invest.* **2003**, *111*, 1265; b) The Antibiotic Alarm, *Nature* **2013**, *495*, 141.
[2] a) A. Coates, Y. Hu, R. Bax, C. Page, *Nat. Rev. Drug Discovery* **2002**, *1*, 895; b) C. Walsh, *Nat. Rev. Microbiol.* **2003**, *1*, 65; c) M. S. Butler, M. A. Cooper, *J. Antibiot.* **2011**, *64*, 413; d) K. M. G. O'Connell, J. T. Hodgkinson, H. F. Sore, M. Welch, G. P. C. Salmond, D. R. Spring, *Angew. Chem. Int. Ed.* **2013**, *52*, 10706; *Angew. Chem.* **2013**, *125*, 10904.
[3] H. Hamamoto, M. Urai, K. Ishii, J. Yasukawa, A. Paudell, M. Murai, T. Kaji, T. Kuranaga, K. Hamase, T. Katsu, J. Su, T. Adachi, R. Uchida, H. Tomoda, M. Yamada, M. Souma, H. Kurihara, M. Inoue, K. Sekimizu, *Nat. Chem. Biol.*, DOI: 10.1038/NCHEMBIO.1710.
[4] Z. Tynecka, Z. Szcześniak, A. Malm, R. Los, *Res. Microbiol.* **1999**, *150*, 555.
[5] For reviews, see: a) D. J. Newman, G. M. Cragg, *J. Nat. Prod.* **2007**, *70*, 461; b) J. W.-H. Li, J. C. Vederas, *Science* **2009**, *325*, 161; c) P. A. Wender, V. A. Verma, T. J. Paxton, T. H. Pillow, *Acc. Chem. Res.* **2008**, *41*, 40; d) A. M. Szpilman, E. M. Carreira, *Angew. Chem. Int. Ed.* **2010**, *49*, 9592; *Angew. Chem.* **2010**, *122*, 9786; e) R. M. Wilson, S. J. Danishefsky, *Angew. Chem. Int. Ed.* **2010**, *49*, 6032; *Angew. Chem.* **2010**, *122*, 6168.
[6] R. B. Merrifield, *J. Am. Chem. Soc.* **1963**, *85*, 2149.
[7] For reviews, see: a) J. M. Humphrey, A. R. Chamberlin, *Chem. Rev.* **1997**, *97*, 2243; b) S. C. Stolze, M. Kaiser, *Synthesis* **2012**, *44*, 1755.
[8] For recent accounts on SAR studies on peptidic natural products from our laboratory, see: a) H. Itoh, M. Inoue, *Acc. Chem. Res.* **2013**, *46*, 1567; b) M. Inoue, *Proc. Jpn. Acad. Ser. B* **2014**, *90*, 56.
[9] For recent reviews on non-ribosomal peptides, see: a) S. A. Sieber, M. A. Marahiel, *Chem. Rev.* **2005**, *105*, 715; b) C. T. Walsh, R. V. O'Brien, C. Khosla, *Angew. Chem. Int. Ed.* **2013**, *52*, 7098; *Angew. Chem.* **2013**, *125*, 7238.
[10] S. Wang, *J. Am. Chem. Soc.* **1973**, *95*, 1328.
[11] L. A. Carpino, H. Shroff, S. A. Triolo, E. M. E. Mansour, H. Wenschuh, F. Albericio, *Tetrahedron Lett.* **1993**, *34*, 7829.
[12] a) L. A. Carpino, G. Y. Han, *J. Am. Chem. Soc.* **1970**, *92*, 5748; b) *Fmoc Solid Phase Peptide Synthesis—A Practical Approach* (Eds.: W. C. Chan, P. D. White), Oxford University Press, Oxford, **2000**.
[13] For related approaches to cyclic peptides, see: a) A. Trzeciak, W. Bannwarth, *Tetrahedron Lett.* **1992**, *33*, 4557; b) S. A. Kates, N. A. Solé, C. R. Johnson, D. Hudson, G. Barany, F. Albericio, *Tetrahedron Lett.* **1993**, *34*, 1549; c) S. C. Stolze, M. Meltze, M. Ehrmann, M. Kaiser, *Chem. Commun.* **2010**, *46*, 8857; d) J. Lee, J. H. Griffin, T. I. Nicas, *J. Org. Chem.* **1996**, *61*, 3983; e) R. Krishnamoorthy, L. D. Vazquez-Serrano, J. A. Turk, J. A. Kowalski, A. G. Benson, N. T. Breaux, M. A. Lipton, *J. Am. Chem. Soc.* **2006**, *128*, 15392; f) M. J. Dixon, A. Nathubhai, O. A. Andersen, D. M. F. van Aalten, I. M. Eggleston, *Org. Biomol. Chem.* **2009**, *7*, 259; g) T. Walther, H.-D. Arndt, H. Waldmann, *Org. Lett.*

2008, *10*, 3199; h) M. Pelay-Gimeno, Y. García-Ramos, M. J. Martin, J. Spengler, J. M. Molina-Guijarro, S. Munt, A. M. Francesch, C. Cuevas, J. Tulla-Puche, F. Albericio, *Nat. Commun.* **2013**, *4*, 2352.

[14] a) S. Mazur, P. Jayelekshmy, *J. Am. Chem. Soc.* **1979**, *101*, 677; for a review, see: b) C. J. White, A. K. Yudin, *Nat. Chem.* **2011**, *3*, 509.

[15] R. Noyori, T. Ohkuma, M. Kitamura, *J. Am. Chem. Soc.* **1987**, *109*, 5856.

[16] L. Coutable, K. Adil, C. Saluzzo, *Tetrahedron* **2008**, *64*, 11296.

[17] a) H.-M. Yu, S.-T. Chen, K.-T. Wang, *J. Org. Chem.* **1992**, *57*, 4781; b) S. A. Palasek, Z. J. Cox, J. M. Collins, *J. Pept. Sci.* **2007**, *13*, 143; c) B. Bacsa, K. Horváti, S. Bõsze, F. Andreae, C. O. Kappe, *J. Org. Chem.* **2008**, *73*, 7532.

[18] V. Dourtoglou, B. Gross, V. Lambropoulou, C. Zioudrou, *Synthesis* **1984**, 572.

[19] S. Friedrich-Bochnitschek, H. Waldmann, H. Kunz, *J. Org. Chem.* **1989**, *54*, 751.

[20] J. Coste, D. Le-Nguyen, B. Castro, *Tetrahedron Lett.* **1990**, *31*, 205.

[21] a) J. Chatterjee, C. Gilon, A. Hoffman, H. Kessler, *Acc. Chem. Res.* **2008**, *41*, 1331; b) J. Chatterjee, F. Rechenmacher, H. Kessler, *Angew. Chem. Int. Ed.* **2013**, *52*, 254; *Angew. Chem.* **2013**, *125*, 268.

