Synthesis of PIP-LSD1 Inhibitor Coupled Molecules and Evaluation of Their Bioactivities

PIP-LSD1 阻害剤結合分子の合成と生物活性評価

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Abbreviations

A	adenine
Ac	acetyl
Ar	aryl group
Bn	benzyl
Boc	*tert*-butoxycarbonyl group
bp	base pair
C	cytosine
cat.	Catalyst
Cbz	benzyloxycarbonyl group
DMAP	4-(dimethylamino)pyridine
DMF	*N*,*N*-dimethylformamide
DNA	deoxyribonucleic acid
Dp	Dimethyl amino pripyl amide
DPPA	di-phenylphosphoryl azide
EDCI	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
eq	equivalent
Et	ethyl
Et_2O	diethyl ether
EtOAc	ethyl acetate
FAD	flavine adenine dinucleotide
G	guanine
h	hour(s)
HATU	1-[Bis(dimethylamino) methylene] -1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxide hexafluorophosphate
HDMs	histone demethylases
HMTs	histone methytransferases
HOBT	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrum
Hz	hertz
^{i}Pr	isopropyl

IR	infrared
J	coupling constant (in NMR)
LSD1	lysine-specific demethylase-1
M	mol/L
Me	methyl
min	minute(s)
mp	melting point
NMR	nuclear magnetic resonance
Ns	nosyl group
Nu	nucleophile
PCPA	trans-2-phenyl cyclopropylamine
Ph	phenyl
PIP	pyrrole imidazole polyamide
PTMs	post-translational modification
quant.	quantitative yield
rt	room temperature
T	thymine
^{t}Bu	*tert*-butyl
δ	chemical shift in parts per million downfield from tetramethylsilane

Introduction

a. Introduction of pyrrole-imidazole polyamides (PIPs)

It has been developed decades of years on drugs and therapeutics which targeted DNA, especially in oncology, and became a mainstay in recent days for their broad utilizations and remarkable efficacy. [1-2] As we know, deoxyribose nucleic acid (DNA) is a class of molecule playing initial roles in genetic activities, which performing as the essential storage media of genetic code and also conducting characterized chemically including encoding, transcription and many other functions in organisms. There are two fundamental motifs carrying the genetic information, one is four kinds of nucleobases within the helical structure, adenine (A), guanine (G), cytosine (C), and thymine (T), which tell apart the single nucleotide building blocks from tiny variant; the other is the deoxyribose sugar motifs which contain 5' and 3' hydroxyl groups oriented the DNA strand.

The specific recognitions of the DNA by certain natural or artificial molecules to acquire genetic information that storage in the double helix structure have become an ultimate challenge for worldwide researchers. From the ancient days, it has natural proteins evolved with minor changes that were occurred in their surface to enable the recognition of DNA. The changed surface of evolved protein cooperated or combined with DNA perfectly following the stringent rules. In the 1960s, DNA-recognition by some small molecules was discovered.[3] Such as dye molecules, which were applied for staining, then cell biologists and histologists found that cell nuclei could be bound with different dyes specifically. Based on the primeval finding, the "intercalation hypothesis" was established on 1961. It was launched by Leonard Lerman who was working at the Cambridge MRC laboratory. The discovery of "intercalation hypothesis" engendered a myriad of a breakthrough on DNA-targeted drugs through the biophysical, biostructural and biochemical evolvements.[4] One of them is actinomycin D which is a natural product brought profound impacts on the DNA-drug recognition field. [3,5-6]

Later on, in the 1980s, several natural products were found as the DNA minor groove binders. [7] In 1985, Dickerson reported netropsin (Fig. 1.1) which combined with

DNA on 1:1 stoichiometry. Two years later in 1987, Rich's group also reported distamycin (Fig. 1.1) which combined with DNA on 1:1 stoichiometry.[8] Then in 1995, another significant study was reported by Ramakrishnan of distamycin A which was proved could form a 2:1 complex with DNA through the binding with minor groove.[9] The unveiling of the functional molecule of distamycin A prompted researchers to achieve more progress on DNA minor groove binders and seek their therapeutic application. Besides, many other DNA-binding natural products and synthetic DNA minor groove binders were reported (Fig. a.1), for example, tallimustine, PNU 160666, brostacillin etc.[10-12]

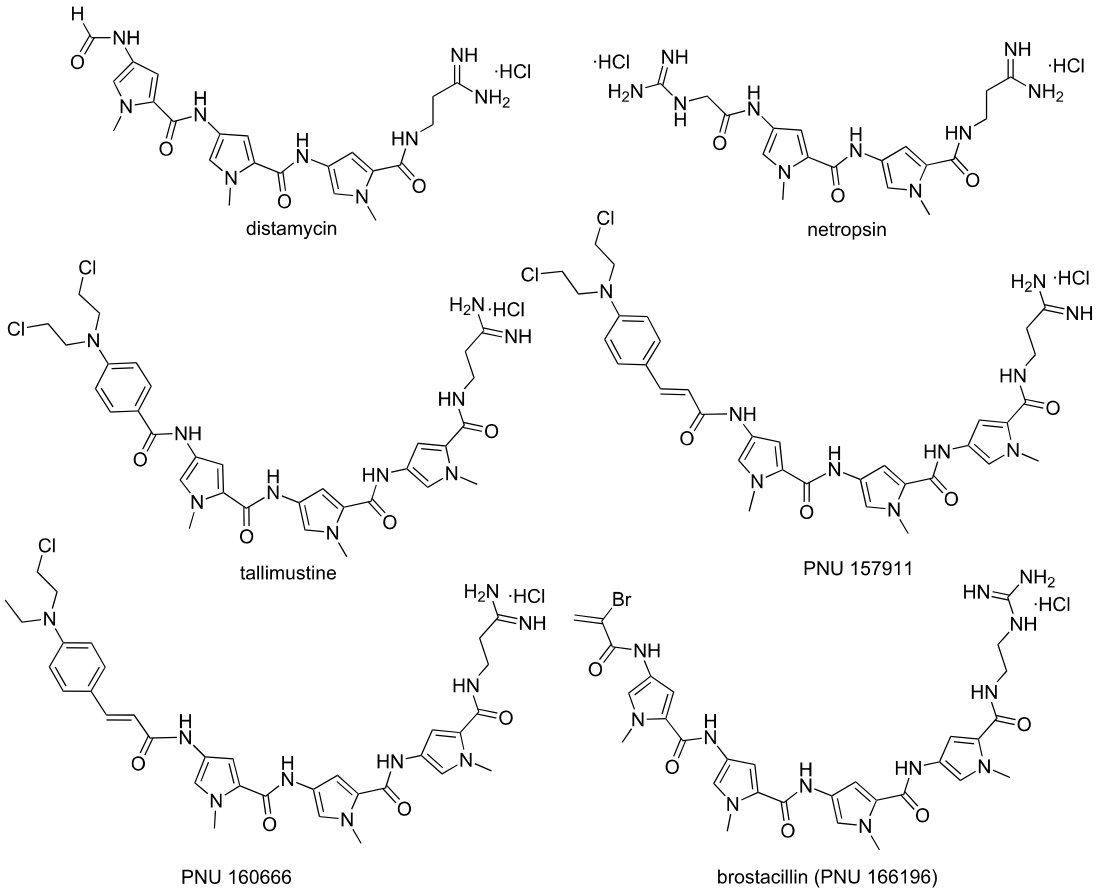


Fig. a.1: Chemical structures of naturally occurring and synthetic hybrid minor groove binders.

Since it was verified with NMR and X-ray dates that the pivotal target on recognizing DNA specifically was not the major groove but the minor groove, increasing efforts were made on this field. The development of DNA minor groove conjugates has undergone tremendous changes over the past decades. From the natural product dystamycin that was found in the very early time, to a class of heterocyclic oligomers that can be programmed chemically or biologically, which demonstrated sequence specificity and high affinity.

One of the most striking class of compounds among them is pyrrole-imidazole polyamides (PIPs). PIPs is a class of programmable DNA binder, which has a preferential affinity for minor groove. Researchers were inspired by two natural products netropsin and distamycin A which were reported as the DNA minor groove binder in 1985 and 1995 respectively. Both of them have the similarity on structure, pyrrole constitutions enable netropsin and distamycin A to interact with A/T-rich region, constituted 1:1 DNA-ligand complex. Additionally, distamycin A also could form the DNA-binding complex in 2:1 stoichiometry.[7-9] Meanwhile, the molecular architectures were demonstrated. Hydrogen bonds and van der Waals (VDW) interactions played pivotal roles. The amide nitrogen of pyrrole reactive with the third nitrogen of adenine or the second oxygen of thymine via hydrogen bonds, in addition, the second carbon-hydrogen of adenine could interact with the third carbon-hydrogen of pyrrole through VDW. Based on the clarification of structure, a new hypothesis was established by Dickerson's group. They illustrated a model that substituted the nitrogen of *N*-methylpyrrole for third carbon, for example changing the pyrrole moiety to imidazole. As indicated previously, the replaced nitrogen on imidazole would proceed to interact with the amino moiety of guanine via hydrogen bond in 1:1 stoichiometry. They assumed that the generated complex would engage to recognize specifically on G/C Watson–Crick base pairs. They also described details on this model, which permitted to recognize G/C pairs from A/T pairs, nevertheless, recognize G/C pairs from C/G pairs (Fig. a.2).[11]

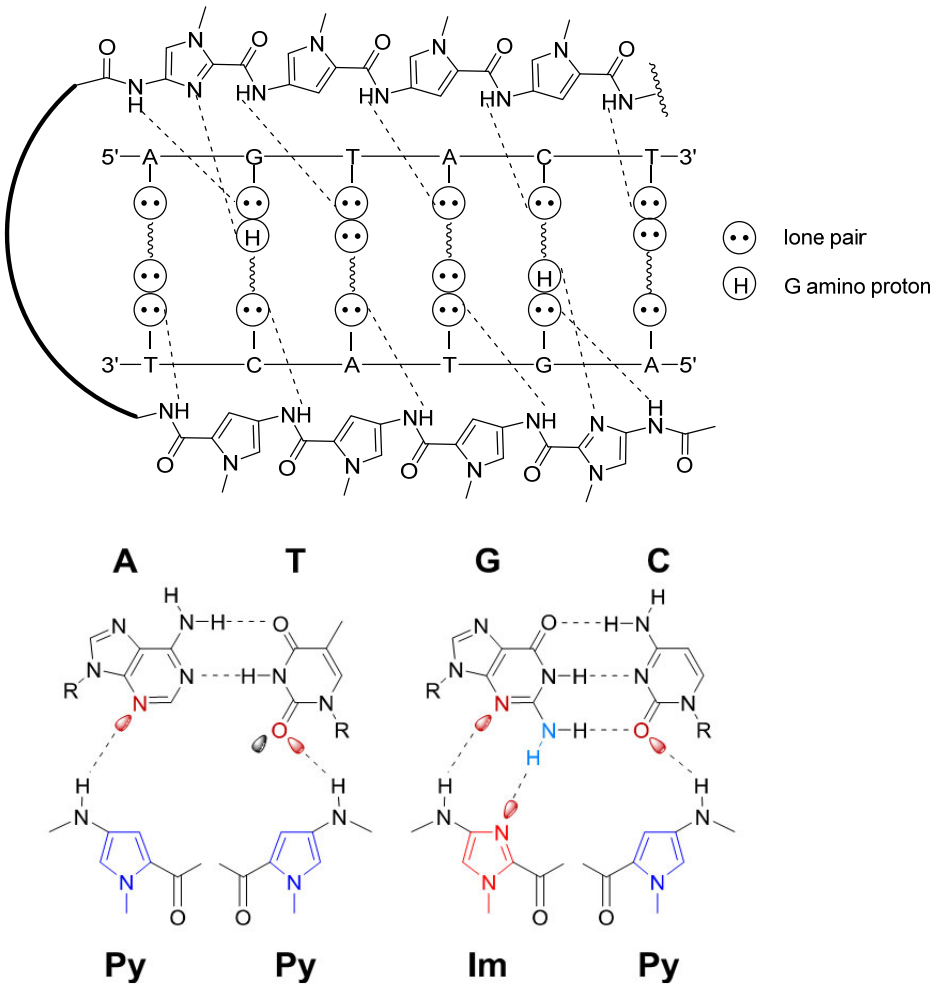


Fig. a.2 Pairing rules for DNA-binding polyamides indicating the hydrogen bonds between bases and Py, Im units.

Later on, Dervan's group pushed the boundaries. They developed a class of Distamycin A analogue ImPyPy-dimethylaminopropylamide (ImPyPy-Dp) which could bind with minor groove of DNA in 2:1 stoichiometry as a homodimer. [13] The ligand was demonstrated that had a high affinity for 5'-TGTTA-3' in an antiparallel way. Strikingly, they exposited the building block of pyrrole and imidazole as the orientated moiety which permitted the Py/Py structure recognized A/T and T/A pairs, meanwhile the Im/Py combination targeted in G/C pairs, as well as the Py/Im combination targeted in C/G pairs. [14] The demonstrated regulation by Dervan's group rendered a tremendous development of recognition on DNA minor groove. Numerous relevant works have been done base on the regulation including this work.

b. Introduction of lysine-specific demethylase (LSD1)

Along with the development of epigenetics and oncology, details of tumorous mechanism were revealed. It was indicated relations between tumorigenesis and epigenetic mutations. Histone abnormalities such as histone lysine methylations served as a class of key mutation coupled with tumorigenesis. Therefore, increasing cancer research has focused on this process.[15] It was also indicated that the methylation of histone played a pivotal role in gene-expression as well as gene stabilization. Numerous of cancers have been detected procced the mutation.[16]

Histones are a class of conserved proteins served as the moiety of chromatin of eukaryotic cells and prokaryotic cells. In eukaryotic nucleus, histones perform as the significant protein component that exists as the unit frameworks which was wrapped around by 146 bp of DNA to form the nucleosomes. 8 histone proteins are divided into pairs, H4, H3, H2B and H2A. Each of the pairs are surrounded by DNA to form globular cores known as nucleosomes and N-terminal tail that protuberant out of the histone/DNA region and are accordant with a various range of post-translational modifications, such as methylation, acetylation, ubiquitination and SUMOylation (SUMO: .Small Ubiquitin-like Modifier) [15-16].

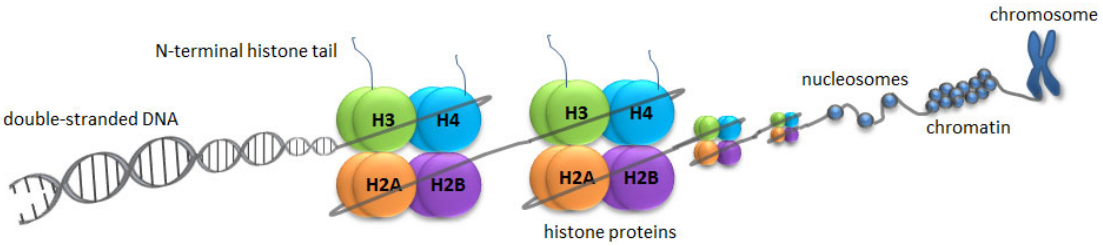


Fig. b.1 Schematic representation shows the organization and packaging of genetic material. Nucleosome are represented by DNA (grey) wrapped around eight histone proteins.

According Vincent Allfrey's studies in the early 1960s, histone modification is also known as post-translational modifications (PTMs), which are arrays of modifications influence regulatory biological processes included transcriptional activation/ inactivation, replication, chromosome packaging, repression and DNA damage/repair etc.[17] The PTMs affects gene expression by altering chromatin structure or recombinant histone modifiers. Structural studies by X-ray revealed that histone amino (N)-terminal tails can protrude from nucleosome region and interact with neighbor nucleosomes.[18-19] Thus the modified tails would alter the action modes between nucleosomes consequent on affecting the overall chromatin structure. Recent decades years, some PTMs have been well defined and understood. Here I will mainly describe methylation and demethylation.

The major site methylation occurs on the side chains of lysines and arginines that considered not changing the histone protein, which were just contrary to acetylation and phosphorylation. Histone methylation could be mono-, di- or tri-methylation manipulated by diverse histone methyltransferases (HMTs), mostly was consequent on gene activation or silence.[20] For instance, di/ trimethylation at H3K4 is involved in upregulated gene expression whereas methylation at H3K9, H3K27 and H4K20 is involved in downregulated gene expression [21]. Besides the methylation at lysine, arginine methylation is also playing a vital role in tumorigenesis. For example, PRMT5 which is known as a Type II protein arginine methyltransferases (PRMTs) was found implicated in the repression of certain tumor suppressor genes such as retinoblastoma (RB) tumor suppressors gene, analogously PRMT7 was observed upregulated in breast cancer.

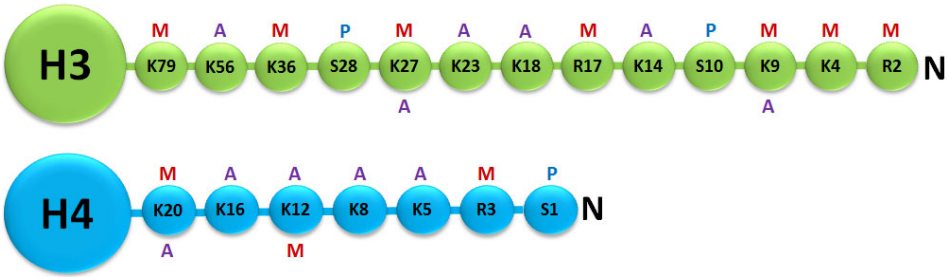


Fig. b.2 N-terminal tail modifications of H3 and H4. M = methylated, A = acetylated, P = phosphorylated.

Initially, it was thought that methylation was irreversible and dynamic not like other histone modifications which are reversible. Whereas a study in 2002 indicated a new

pathway of methylation as a thermodynamic and reversible process.[19] The revers process was conducted by histone demethylases (HDMs). In 2004, the first lysine-specific demethylase (LSD1) was reported by Shi's group in 2004.[22]

LSD1 targets lysine 4 histone H3 (H3K4) or lysine 9 of histone H3 (H3K9), catalyzes the mono- and di-demethylation but not tri-demethylation through flavin adenine dinucleotide (FAD) dependent enzymatic oxidation. In terms of regulation of epigenetic gene expression LSD1 plays pivotal roles in various biological process such as embryonic development and homeostasis without carry out any alternation in DNA sequence.[15] In addition, it has been reported that LSD1 involved the various cancers, for example colon cancer, leukemia etc.[15, 25-27] Besides, LSD1 also indicated that has potential to implicate in the infection of α- herpesvirus and globin disorder.[28-29] Therefore, compounds that inhibit the catalytic activity of LSD1 are interesting as chemical tools for studying the functions of LSD1 and as candidate therapeutic agents targeting LSD1 .One of the component of LSD1 inhibitor is tranylcypromine (PCPA) which is one of the best-studied LSD1 inhibitors and has contributed immensely to the understanding of the biology of LSD1. [26,30-33] In recent decades，PCPA has been studied by numerous scientists, the biological studies using PCPA have contributed immensely to the understanding of the enzyme. Many of these studies suggested that LSD1 inhibitor composed by PCPA derivatives could be new approaches to pharmacological designs of promising lead drugs for cancer treatment. As elucidated regulation of LSD1 inhibitor, an FDA adduct played vital role as an electron mediate to permit the compound which contained PCPA moiety inhibitory activity. On the other hand, there are improvable properties of developed PCPA-type inhibitor including inadequate inhibitory activity and selectivity.[15] Increasing researchers have concentrated on improving PCPA-type LSD1 inhibitors. Likewise, this work has made several efforts on expend the utility of NCD38 which is one of the LSD1 inhibitor, as well as overcome drawbacks in some extent.

Chapter I Synthesis of NCD38-PIP hybrid molecules and evaluation of their bioactivities

1-1 Introduction

Base on the clarification of pairing rules between PIPs and DNA minor grooves, increasing researchers has focused on the preparation of PIPs efficiently. Initially Dervan's group applied solid-phase synthesis on the synthesis of PIPs for they claimed that reaction would take more time and go through in harsh conditions. It was considered that a splendid advance for the obvious time reducing, hence a myriad of work was realized based on this strategy for preparing PIPs.[34-35] In addition, the development of a synthetic method using hydrazine resin and nucleophilic cleavage reagent was attributed to the produce of multifunctional polyamides.[36] Nevertheless, drawbacks of solid-phase synthesis such as unsatisfactory yields and long reaction time limited the utilization on synthesis of PIPs.[37] Thus they turned direction to a convergent synthesis performed in solution phase which increased the productivity up to gram-scale with minimal chromatography. The efficient synthetic methodology inspired us to devise our work in solution phase.

In our laboratory, several studies on synthesis of PIPs have been done. Previous works provided an efficient platform to this project. Base on Tor's work, the developed synthetic routes of PIPs units were applied for preparing the PIPs motifs.[38]

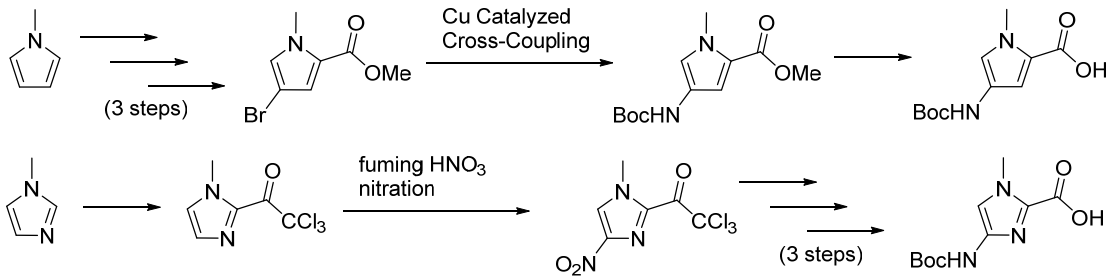


Fig. 1.1 Developed synthesis routes of PIPs units

1-2 Molecular design of NCD38-PIP hybrid molecules

Specificity is permitted by recognizing the regulations of WW(=A or T) base pairs by antiparallel pyrrole-pyrrole units. The imidazole-pyrrole units and pyrrole-imidazole units recognize the SS(S=G or C) base pairs, respectively. These outstanding properties empowered PIPs as a good delivery material for various regions of genome. Besides, it was proved that LSD1 regulates the expression of many important gene progression and cell proliferation, aberrant expression of LSD1 has been shown in many types of cancers. Therefore, LSD1 inhibitors were considered to be anticancer agents.

In 2013, Suzuki's group reported effective LSD1 inhibitors such as **1** and **2** (NCD38).[39] The inhibitor consists of 2 parts, one is a lysine part for enzyme selectivity, the other part is PCPA. Especially, the PCPA is the best-studied LSD1 inhibitor so far. Many studies suggested that LSD1 inhibitor composed by PCPA derivatives could be new approaches of leading drugs for cancer treatment.

Based on these backgrounds, coupled molecules **3a** and **3b** were designed to expend the utility of both NCD38 and PIPs. As shown in Fig. 1.2, we introduced 2 different PIPs to NCD38 moiety expected the genomic region-specific recognition could be altered. Because NCD 38 was developed as a diastereomeric mixture regarding the stereochemistry of cyclopropane unit, we didn't have enough information on the difference of bioactivity between the diastereomers. Therefore, I first planed the synthesis of both diastereomers of the inhibitor parts and determined the preferred isomer for this study.

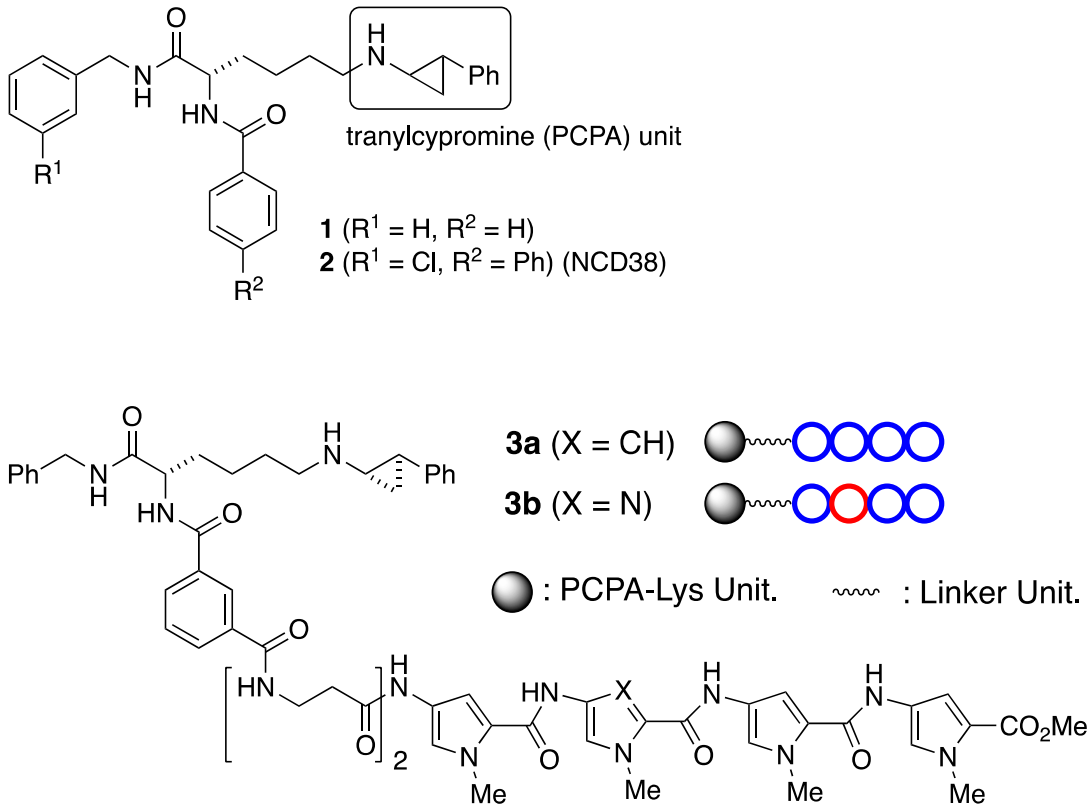
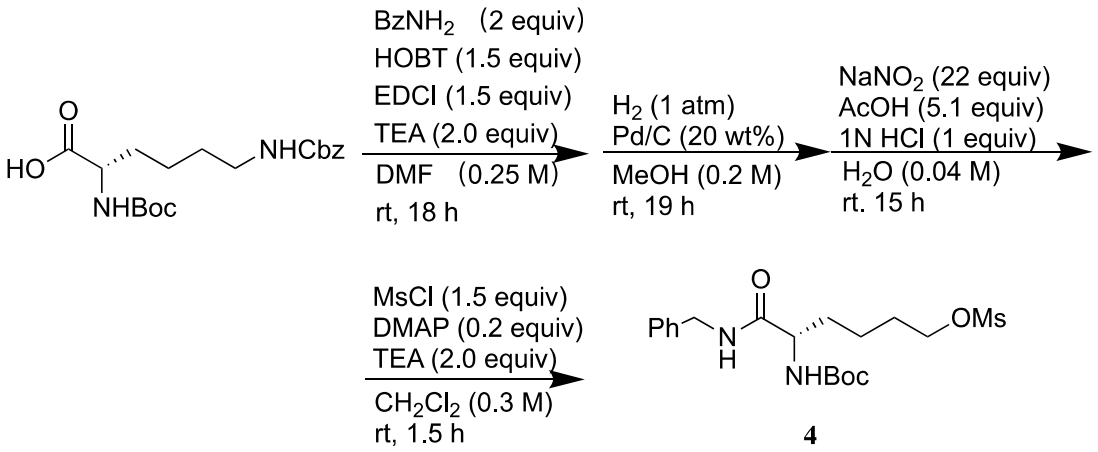


Fig. 1.2 LSD1 Inhibitors and Designed NCD38–PIP Hybrid Molecules. Chemical structure of compounds. Blue rings, red rings, gray balls and hexagons represent pyrrole, imidazole, PCPA-Lys unit and linker unit respectively.

1-3 Synthesis of NCD38-PIP hybrid molecules

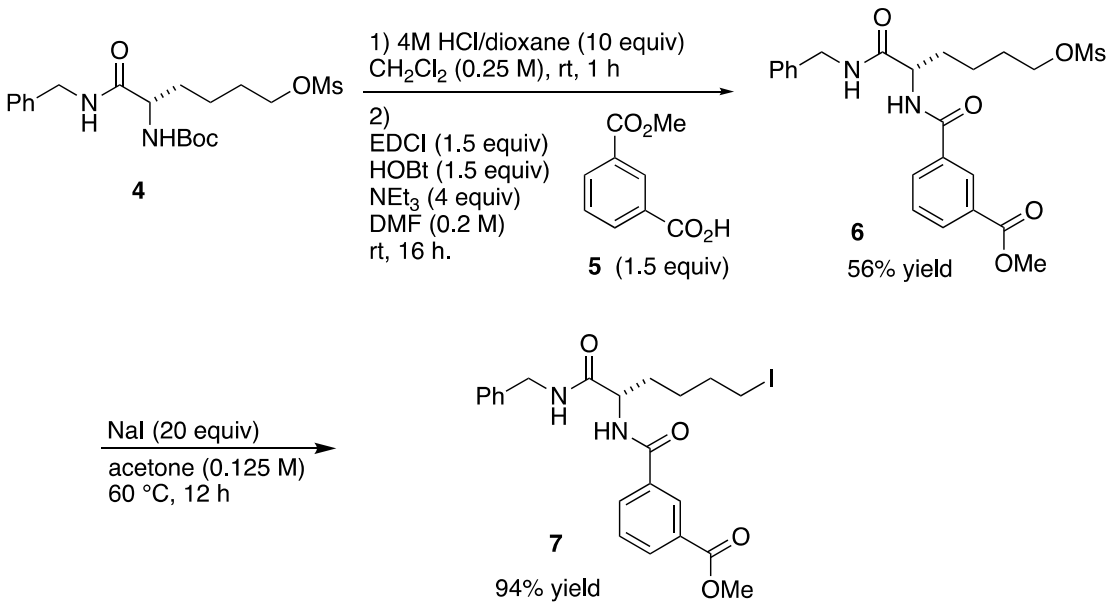
1-3.1 Synthesis of the LSD1 inhibitor Part

In reference to the reported work, we set out our synthesis of LSD1 inhibitor from the preparation of a mesylate derivative **4**, which was obtained from L-lysine applying the known synthetic route (Scheme 1). [39] A commercial reagent of protected lysine was applied as the starting material; the carboxylic acid part then was protected by benzylamine in the presence of dual condensation reagents of 1-hydroxybenzotriazole (HOBT) and *N*-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI) under basic conditions. Followed by deprotection of benzyloxycarbonyl group (Cbz) under 1 atm hydrogen and 10% palladium on carbon, the obtained amine was then converted to alcohol using diazo-reaction which was performed in the presence of 22 equivalent sodium nitrite, 1 equivalent hydrogen chloride, 5.1 equivalent acetic acid and 0.04 M water as solvent. The diazo-reaction did not yield the product efficiently as we expected especially in scale-up reaction, while still concurs with the data reported in Suzuki's paper. Consequent on coupling the hydroxy group with methanesulfonyl chloride under basic conditions, the preparation of mesylate derivative **4** was come to the conclusion.



Scheme 1.1 Preparation of mesylate derivative **4**

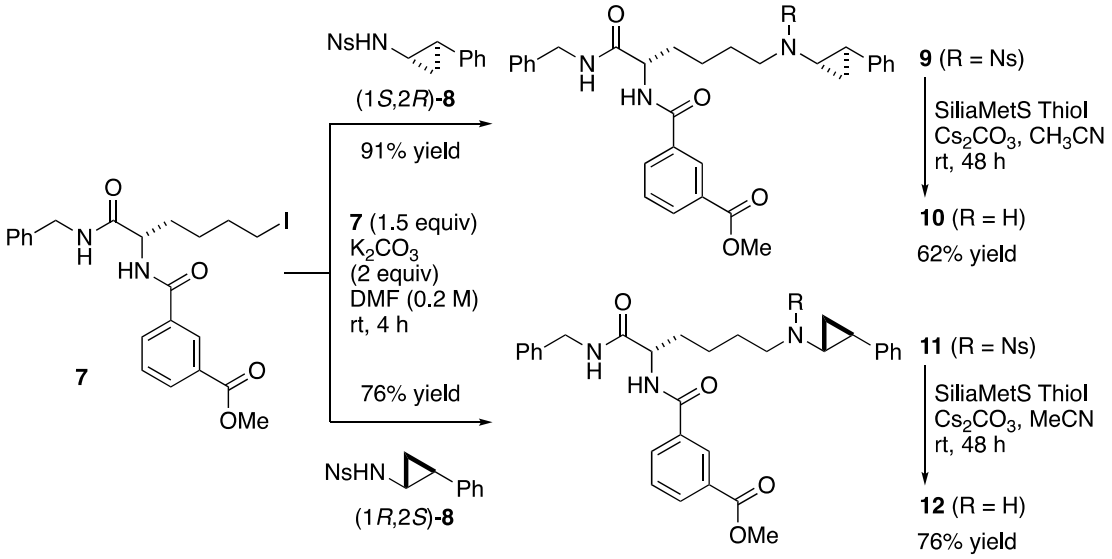
Then, the mesylate derivative **4** was dissolved in dichloromethane and deprotected the *t*-butyloxycarbonyl group (Boc) in the presence of 10 equivalent 4M HCl/dioxane, the resulting amine was coupled with the commercial reagent of 3-(methoxycarbonyl)benzoic acid **5** using dual condensation reagent of 1-hydroxybenzotriazole (HOBT) and *N*-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI), both of them were applied 1.5 equivalent in DMF generating compound **6** in 56% yield. The suspension of mesylate derivative **6** was dissolved in acetone with sodium iodide undergoing iodination reaction refluxed for 12 hours produced iodide derivative **7** in 94% yield.



Scheme 1.2 Synthesis of LSD1 inhibitor part (A)

Then we planned to coupled iodide derivative **7** with *N*-2-nosyl (Ns) phenylcyclopropylamine **8**. There is 2 different stereometric structure of **8**. To determine the more bioactive structure, the pure enantiomers of **8** was prepared. As described above, NCD38 was developed using racemic *trans*-2-Phenylcyclopropylamine.[39] Later in the 2014, Suzuki's group reported another work of synthesis of optically pure NCD serious inhibitors and evaluated their LSD1 inhibitory activities.[40] The data reported in this paper suggested that the compounds with 1*S*, 2*R* stereochemistry regarding the PCPA unit would be more bioactive compounds towards LSD1 inhibition activities.

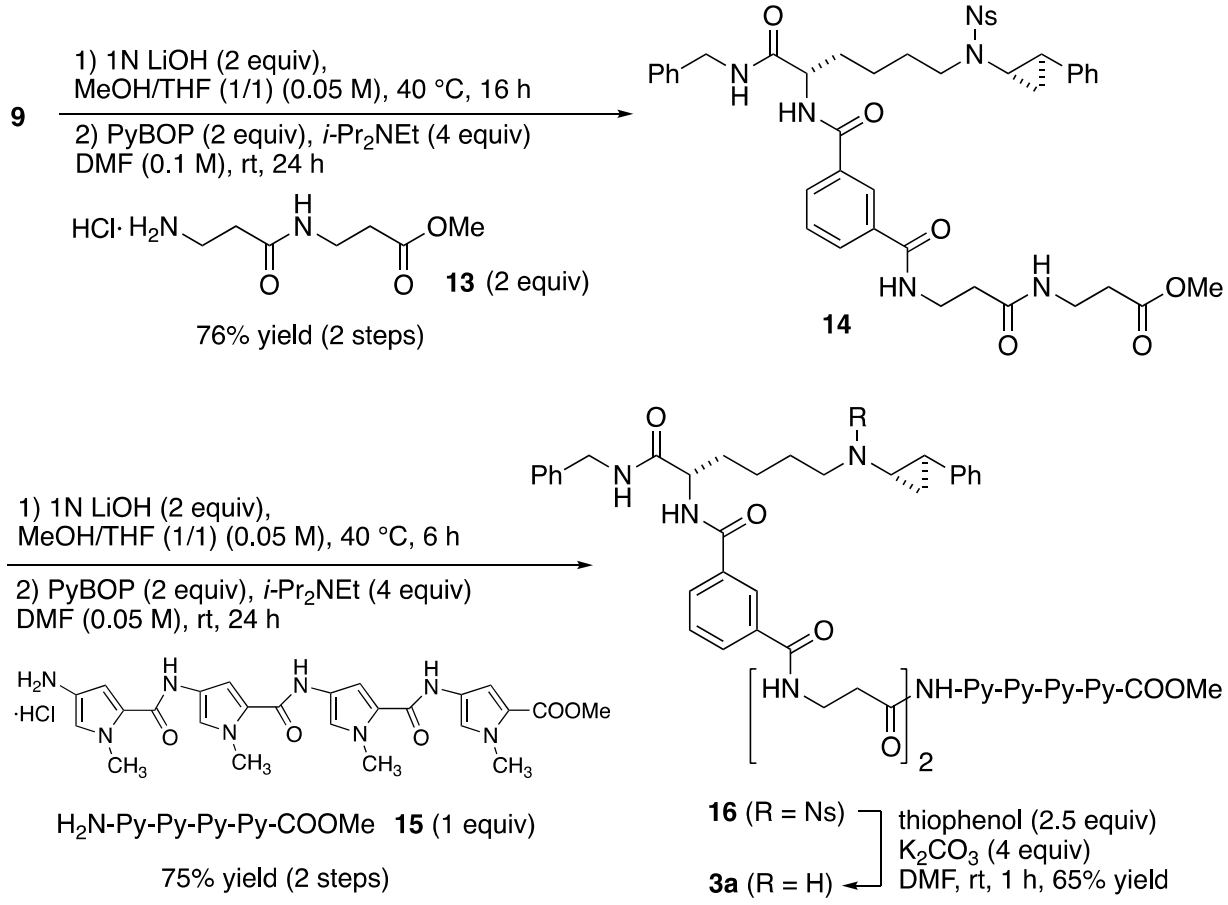
According to the 2 works as references, our work was also conducted the optical resolution of racemic **8**, then coupled the 2 enantiomers which was (1*S*, 2*R*)-**8** and (1*R*, 2*S*)-**8** with lysine moiety of compound **7**, generating diastereomers **9** and **11**.[41] The following study of LSD1 inhibitory activity revealed that the 2 diastereomer **9** and **11** had different LSD1 inhibitory activity (See Fig 1.3). The 2 enantiomers were coupled with 1.5 equivalent of iodide derivative **7** and 2 equivalent of K_2CO_3 in DMF to result compounds **9** and **11** in 91% yield and 76% yield, respectively. Another synthesis trail of compound **9** which started from mesylate derivative **6** could also had possibility be utilized. However, a pre-experiment result indicated that efficiency of this trail was less satisfactory. Then compound **9** and **11** were followed a deprotection reaction of Ns group using a silica-supported thiol (SiliaMetS® Thiol), the resulting compounds **10** and **12** were generated in 62% yield and 76% yield, respectively.



Scheme 1.3 Synthesis of LSD1 inhibitor part (B)

1-3.2 Synthesis of NCD38-PyPyPyPy hybrid molecules

Compound **9** was hydrolyzed under basic conditions, turning the methyl ester moiety to the carboxylic acid. Then it was condensed with a β-alanine dimer **13** to inaugurate a spacer unit between the LSD1 inhibitor unit and PIP unit. The reaction was conducted in the presence of 2 equivalent of PyBOP, 4 equivalents of *N, N*-diisopropylethylamine (DIPEA) and 2 equivalents of β-alanine dimer **13** in DMF, adduct **14** was afforded in 76% in 2 steps from **9**. Then compound **14** was hydrolyzed under basic conditions, turning the methyl ester moiety to the carboxylic acid. The tetramer consisted by pyrrole unit ($HCl{\cdot}NH_2$-PyPyPyPy-COOMe) **15** was coupled with the obtained carboxylic acid in the presence of 2 equivalents of PyBOP in DMF to obtain the adduct **16** in overall 75% yield in 2 steps. The nosyl group of compound **16** was removed to obtain NCD38-PyPyPyPy hybrid molecule **3a** in 65% yield.

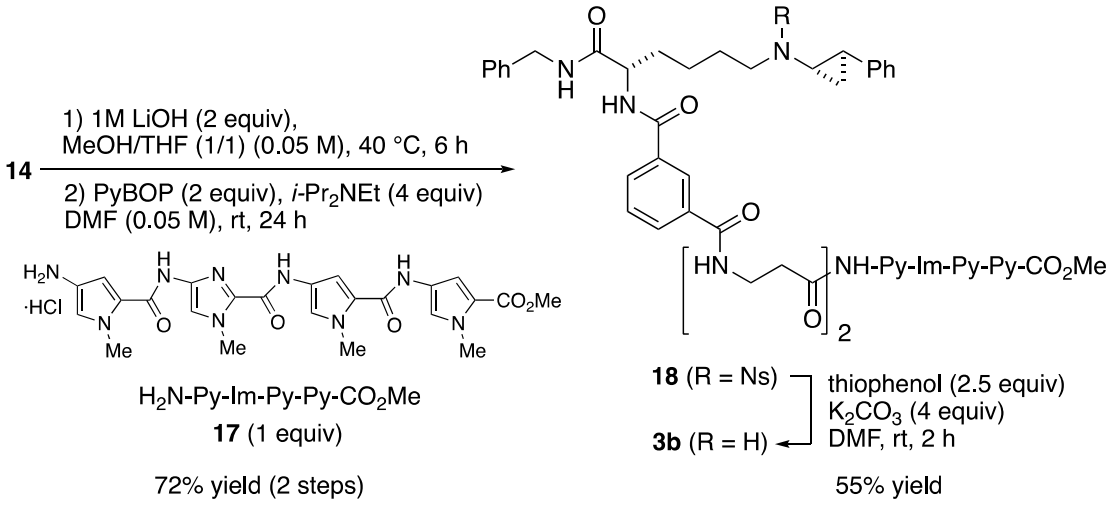


Scheme 1.4 Synthesis of NCD38-PyPyPyPy hybrid complex

1-3.3 Synthesis of NCD38-PyImPyPy hybrid molecules

Likewise, another hybrid molecules **3b** was prepared in the same way as **3a**.

The compound **14** was hydrolyzed under basic conditions, turning the methyl ester moiety to the carboxylic acid. Then the tetramer consisted by 3 pyrrole units and 1 imidazole unit ($HCl \cdot NH_2$-PyImPyPy-COOMe) **17** was coupled with the obtained carboxylic acid in the presence of 2 equivalents of PyBOP in DMF to obtain the adduct **18** in overall 72% yield in 2 steps. The nosyl group of compound **18** was removed to obtain NCD38-PyImPyPy hybrid molecule **3b** in 55% yield.

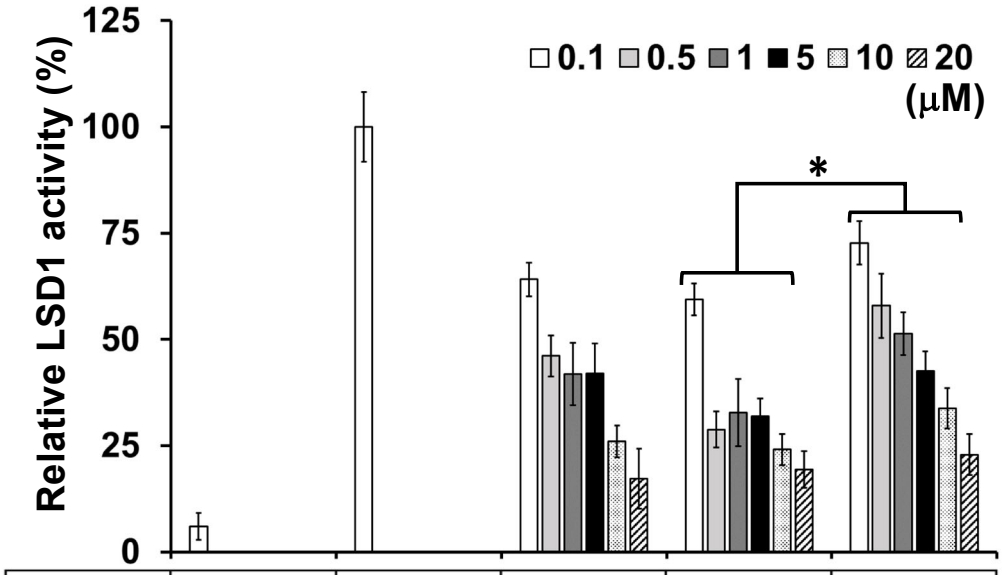


Scheme 1.5 Synthesis of NCD38-PyImPyPy hybrid complex

1-4 Bioactivities of NCD38-PIP hybrid molecules

1-4.1 Inhibition activity of LSD1

Inhibitory activities of LSD1 were assessed in gradient concentration as 9.6%, 19.4% and 22.9% using 20 μM of compound **2** (developed NCD38 molecule),[39] **10**, and **12**, respectively. (Fig. 1.3) When using 1 μM of compound **2**, **10**, and **12**, the inhibitory activities were detected as 26.2%, 32.7% and 51.4%, respectively. The consequence suggested obviously that both of the 2 diastereomeric compounds of **10** and **12** exhibited LSD1 inhibitory activities, in addition, the 2 diastereomeric compounds inhibited LSD1 as the similar level of the parental compound **2**. Furthermore, it was detected that when applied in concentration as 1 μM ($P = 0.029$), 5 μM ($P = 0.036$) and 10 μM ($P = 0.046$), compared with both parental compound **2** and compound **12**, diastereomeric compound **10** revealed apparently stronger LSD1 inhibitory activity. On the contrary, no obvious discrepancy at 20 μM ($P = 0.38$) was detected. The consequences implied that the LSD1 inhibitory activity of NCD38 could be enhanced by merged the optical resolution into synthesis route. Moreover, the bioactivity results guided us explicitly that the compound with 1*S*, 2*R* stereochemistry regarding the PCPA unit: compound **10** was a better candidate for further studies. Therefore, compound **9** was mainly used as a key synthetic intermediate for this study.



LSD1	–	+	+	+	+
DMSO	1.0%	1.0%	1.0%	1.0%	1.0%
Compound	–	–	**2**	**10**	**12**
Buffer	+	+	+	+	+

Fig. 1.3 Inhibition of LSD1 activity by the compounds in vitro. LSD1 activities were assessed using an LSD1 activity assay kit. Relative LSD1 activities are shown as bars filled with white, light grey, grey, black, dots and slashed lines in presence 0.1, 0.5, 1, 5, 10 and 20 μM of the compounds, respectively. LSD1 activities were estimated by comparison with 1% DMSO control as 100%. Data represent the mean of three independent replicates ± SD. * $P < 0.05$.

1-4.2 Inhibition activity of NCD38-PIP hybrid molecules

In the same way, LSD1 activities of **3a** and **3b** were estimated. The LSD1 activity was assessed with LSD1 activity assay kit in 6 different concentrations of the compounds **3a** and **3b**, respectively. DMSO was used as negative control for LSD1 inhibitor. The inhibitory activities of LSD1 was tested using 1% DMSO as negative control. Error bars, standard deviation of the means of triplicate samples. As the bar chart indicated clearly that both **3a** and **3b** inhibited LSD1 activities were levels similar to the parental NCD38.

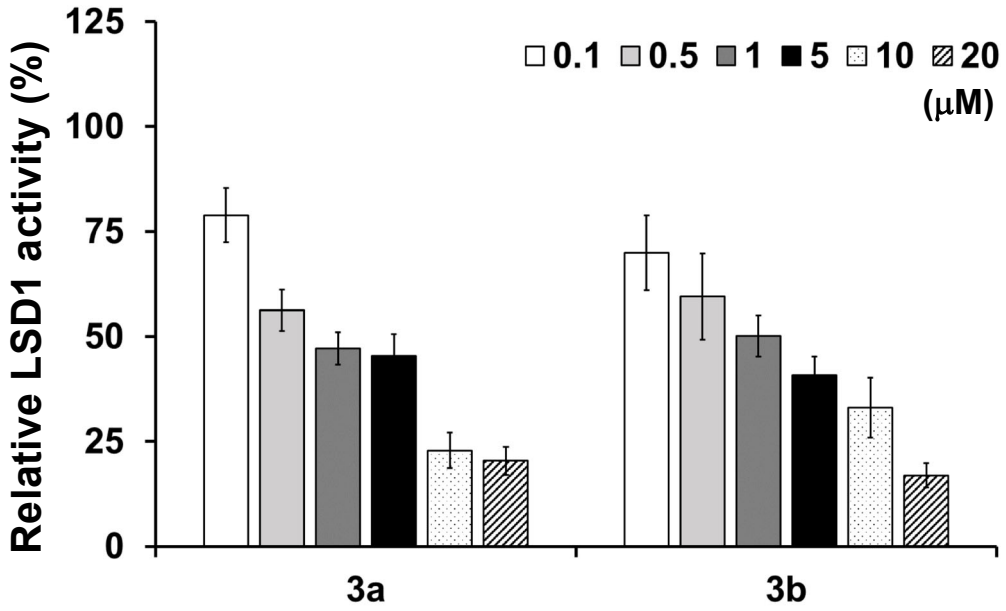


Fig. 1.4 Inhibition of LSD1 activity by conjugated **3a** and **3b** in vitro. LSD1 activities were assessed using an LSD1 fluorometric drug discovery kit. Relative LSD1 activities are shown as bars filled with white, light grey, grey, black, dots and slashed lines in presence 0.1, 0.5, 1, 5, 10 and 20 μM of the compounds, respectively. LSD1 activities were estimated by comparison with 1% DMSO control as 100%. Data represent the mean of three independent replicates ± SD.

1-4.3 ChIP-seq analyses and RNA-seq analyses of NCD38

Initially, we performed the genome-wide analysis for histone modification alterations induced by the NCD38 (Fig. 1.5. A & B). We carried out ChIP-seq analysis for H3K4me2 H3K27ac, and RNA-seq analysis for colon adenocarcinoma cells incubated in the presence of NCD38 as the inhibitor for 1 month. As the figure showed, 103 and 458 regions exhibited higher than 3 times upregulation in the 2 antibodies levels, respectively, in NCD38 processed cells compared with negative control processed cells. RNA-seq analysis implied that the expression of regulation as well as the ChIP-seq peaks of the 2 antibodies which were immensely upregulated in NCD38 processed cells compared with negative control processed cells. The consequences implied that cells processed with NCD38 was upregulated the level of the 2 antibodies, consequent in upregulated of DNA near the activation regions.

In addition, 6 base pairs DNA sequences within 250 base pairs were analyzed. (Fig. 1.5. C) As a result of investigating in 6 base unit and listing up in order of frequency, it is suggested that CG-rich sequence is high in NCD38 treatment system.

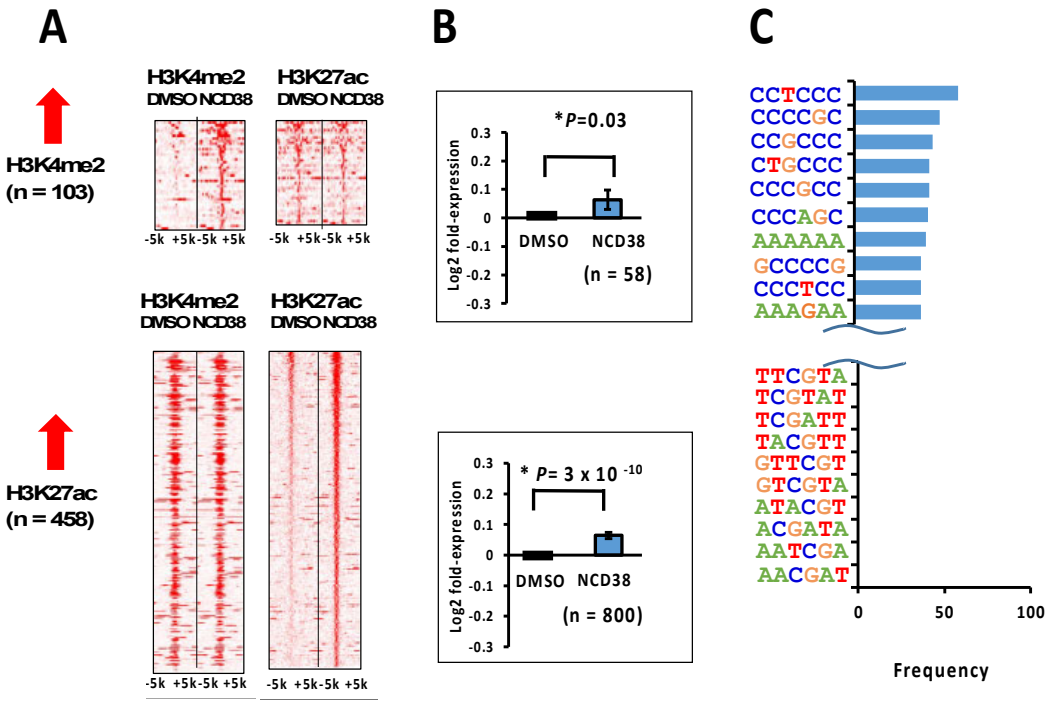


Fig. 1.5 (A) Heat maps showing the read densities of ChIP-seq within ± 5 kb around the center position of ChIP-seq peaks. Whereas increase of H3K4me3 level was hardly observed, 103 and 458 regions showed >3-fold increase of H3K4me2 and H3K27ac levels, respectively. Expression of genes nearest to the H3K4me2 and H3K27ac peaks were significantly upregulated (P = 0.03 and P = 3 × 10^{-10}, respectively, t-test). (B) RNA-seq analysis in each activation region. Each activation region had a dominant rise in gene expression levels up to. (C) 6-bp DNA sequences within 250 bp from the center of the increased H3K27ac peaks are shown. GC-content of top 10 6-bp sequences was as high as 80% ± 9% .

1-4.4 ChIP-seq analyses and RNA-seq analyses of NCD38-PyPyPyPy

Then, the compound **3a** was analyzed in the say way. (Fig. 1.6 A & B). We carried out ChIP-seq analysis for the same antibodies. The RNA-seq analysis was performed using colon adenocarcinoma cells incubated in the presence of compound **3a** for 1 month. The ChIP-seq analyses indicated that compared with the parental inhibitor, compound **3a** was showed less regions of more than 3 times upregulation in H3K27ac levels.

RNA-seq analysis implied that the expression of regulation as well as the ChIP-seq peaks of the H3K27ac antibodies which were immensely upregulated in the compound **3a** processed cells compared with negative control processed cells.

Interestingly, all of the 234 regions had no overlap of activated regions between NCD38 and compound **3a** treated cells. The sequences analysis implied that all the top 10 sequences were AT-rich region, Compared with NCD38 treated cells, the appearance of GC-rich sequences significantly downregulated in the compound **3a** treated cells.

Then we detected whether the compound **3a** had priority of targeting AT-rich regions. 6 base pairs DNA sequences within 250 base pair were analyzed.

Surprisingly, the top 10 sequences were AT-rich regions, meanwhile, expression of GC-rich regions was downregulated compared with the performance of parental inhibitor treated cells. The performance of 6 base pairs sequences of G or C indicated that there was a clearly downregulated in the compound **3a** treated cells ($P < 1 \times 10^{-15}$). On the other hand, performance of 6 base pairs sequences of A or T indicated that there was a clearly upregulated in the compound **3a** treated cells ($P < 1 \times 10^{-15}$).

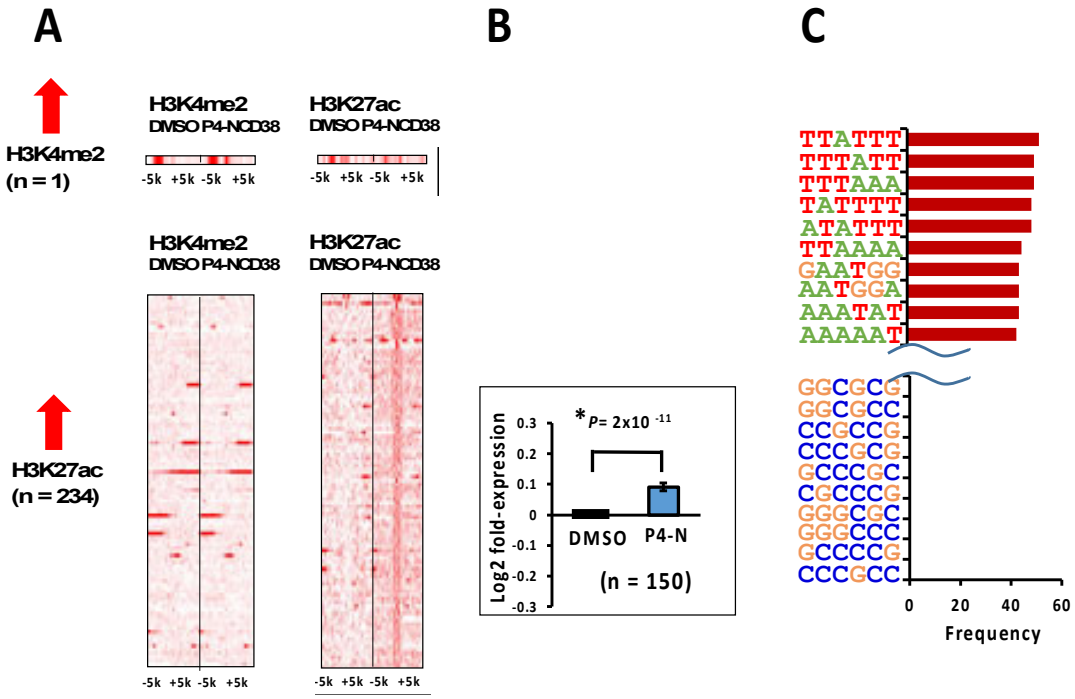


Fig. 1.6 (A) Heat maps showing read densities of H3K4me2, H3K4me3, and H3K27ac within ± 5 kb around H3K27ac-increased regions in enhancers and other regions. Genes nearest to the H3K27ac peaks in other regions were significantly upregulated ($P = 9 \times 10^{-10}$), while those in enhancer regions tended to be upregulated (P = 0.053). (B) RNA-seq analysis in each activation region. Each activation region had a dominant rise in gene expression levels up to. (C) Frequencies of appearance of 6-bp sequences within 250 bp from the center of the increased H3K27ac peaks. All the top 10 sequences were WWWWWW.

1-4.5 ChIP-seq analyses and RNA-seq analyses of NCD38-PyImPyPy

Also, we analyzed the compound **3b** in the same way (Fig. 1.7 A & B). We carried out ChIP-seq analysis for the same antibodies. The RNA-seq analysis was performed using colon adenocarcinoma cells incubated in the presence of compound **3a** for 1 month. The ChIP-seq analyses indicated that compared with the parental inhibitor, compound **3b** was showed less regions of more than 3 times upregulation in the 2 antibodies' levels. The majority (89%) of 82 upregulated regions of H3K27ac were not located in promoter regions. Besides, overlap of activation regions between parental inhibitor and the compound **3b** treatment cells was not detected. The consequences implied that cells processed with the compound **3b** could not upregulated significantly the level of DNA regulation close to H3K27ac peaks ($P = 0.1$), compared with DMSO treated cells. Then we detected whether the compound **3b** had priority of targeting WWCGWW regions. 6 base pairs DNA sequences within 250 base pair were analyzed. The consequences implied that top 5 to 10 sequences contain GC were detected as 40% ± 4% and 55% ± 6%, respectively, however, it was lower than the performance that cells treated by NCD38. Meanwhile, WWCGWW sequence was not detected in top 10 sequences, probably due to the less frequent appearance of GC sequence then the expectation.

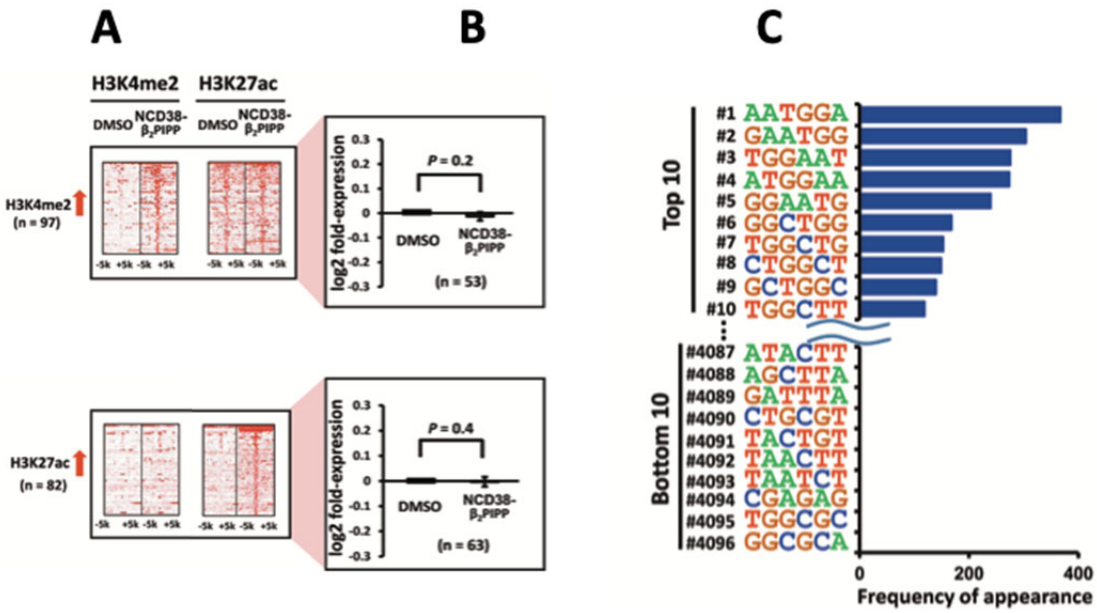


Fig. 1.7 (A) Heat maps showing the read densities of ChIP-seq within ± 5 kb around the center position of ChIP-seq peaks. (B) RNA-seq analysis in within ± 5 kb around the center position of H3K4me2 and H3K27ac. Less number of regions (97 and 82) showed more than 3-fold increases in H3K4me2 and H3K27ac levels, respectively, compared with treatment by parental NCD38. Expression of genes nearest to these H3K4me2-increased and H3K27ac-increased peaks were not significantly up-regulated. (C) Frequencies of appearance of 6-bp sequences within 250 bp from the center of the increased H3K27ac peaks. GC-contents of top 5 and top 10 sequences decreased to 40% ± 4% and 55% ± 6%, respectively

1-5 Discussion

We successfully synthesized LSD1 inhibitor–pyrrole-imidazole polyamide conjugates for region-specific alterations of histone modification. Based on the synthestic route of NCD38 which developed by Suzuki's group, we prepared the NCD38-like LSD1 inhibitor part efficiently. A (1*S*,2*R*) - trans - 2 - phenyl cyclopropylamine unit was conjugated with an L-lysine derivative using a nosyl group as a functional protecting group (Ns strategy). Coupling of LSD1 inhibitor moiety with 2 different pyrrole-imidazole polyamide tetramers was executed through amide bond formation. Additionally, LSD1 activities of **3a** and **3b** were estimated. The LSD1 activity was assessed with LSD1 activity assay kit in 6 different concentrations of the compounds **3a** and **3b**, respectively. As the results indicated clearly that both **3a** and **3b** inhibited LSD1 activities. The levels of inhibition were similar to that of the parental NCD38.

As applied the conjugation of NCD38-like LSD1 inhibitor with two different PIP tetramer units, we detected and analyzed the alternation of epigenetic regions by NCD38 and two different hybrid molecules, compound **3a** which is NCD38-β2PPPP and compound **3b** which is NCD38-β2PIPP. The recognition of specific regions had a significant alternation from the GC-rich regions to the AT-rich regions. The consequences indicated that the two different hybrid molecules targeted various sequences, indicating that the sequence-specific recognition of NCD38-PIP conjugates alternated from GC-rich regions to AT-rich regions.

Chapter II Synthesis of Simplified -PIP Hybrid Molecules and evaluation of their bioactivities

2-1 Introduction

LSD1 was found to be a highly conserved flavin-containing amino oxidase homolog that specifically removes the mono- and di-methylated lysine at lysine 4 or lysine 9 of H3. Speaking of histone methylation, which was verified as a reversible process. Modern science clarified that the major site of this process occurs on the side chains of lysines and arginines. Generally, arginine is involved in gene activation and histone methyltransferases. By comparison, lysine sites can have multiple effects on chromatin function. Therefore, increasing effort has been done on this field.

As “methyl eraser”, LSD1 regulates the expression of many important genes’ progression and cell proliferation. Aberrant expression of LSD1 has been shown in many types of cancers. In particular, LSD1 expression is upregulated in bladder, small cell lung, and colorectal clinical cancer tissues when compared with the corresponding nonneoplastic tissues. LSD1 has also been shown to be overexpressed in some breast cancers and may function as a biomarker of the aggressiveness of the disease.

2-2 Molecular Design of Simplified -PIP Hybrid Molecules

It has been proved by previous studies that PCPA derivatives exhibit LSD1 inhibitory activity. It also suggested that the PCPA itself has several shortages such as low affinity and selectivity against LSD1. Compared with LSD1, PCPA inhibited MAOs (monoamine oxidases) which are also FAD-dependent enzymes preferentially. Thus, Suzuki and coworkers developed NCD38 type LSD1 inhibitor which was designed with a lysine moiety as the PCPA carrier. The modified lysine moiety was initially designed to serve a selective inhibitor for LSD1. They examined the IC50 values of PCPA and NCD38 against LSD1, MAO A and MAO B. The IC50 value of PCPA against LSD1 was higher than MAOs, while NCD38 inhibited LSD1 with far more efficiency as expected.[39]

	IC50 (μM)		
	LSD1	MAO A	MAO B
PCPA	31	2.5	2.4
NCD38	0.59	>100	>100

Table 2.1 IC 50 values of compounds against LSD1, MAO A and MAO B

As mentioned previously, PIPs have been used to drive self-assembly on nano-platform based on the specific recognition of DNA via Watson-Crick base-pairing regimes. It is also proved that MAOs mainly localized in mitochondrial outer membranes.[42] Therefore the complex combined with PIP would deliver PCPA to nucleus over mitochondrial outer membranes to serve selectivity of LSD1 naturally. This mean the lysine moiety in NCD38-type inhibitor would not be necessarily important when using PIP unit-coupled molecules. Thus, we hypnotized a simplified LSD1 inhibitor, with both of PCPA and a simple alkyl side chain as linker moiety. It is worth to mention that we designed the linker part without cumbersome substituents, such as benzyl amino group and chloro-benzyl amino group.

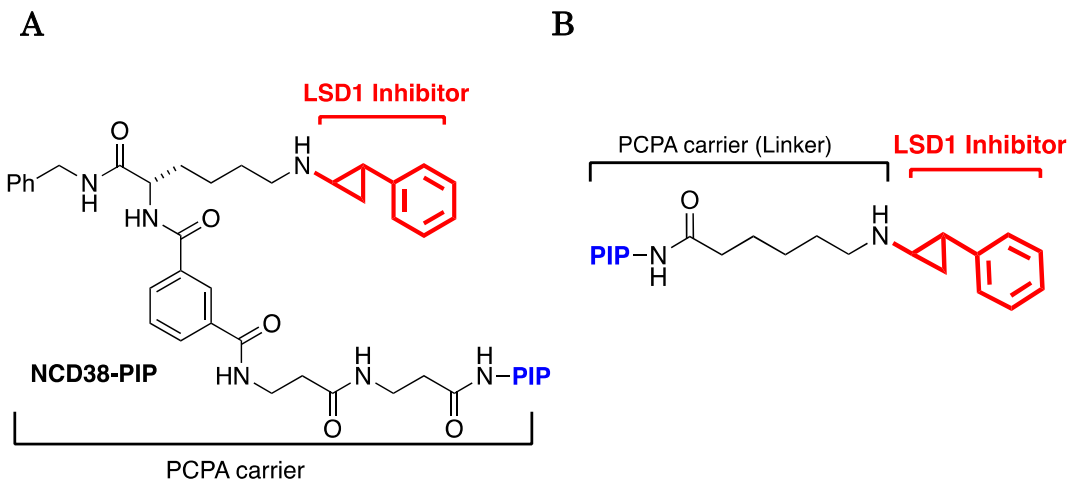


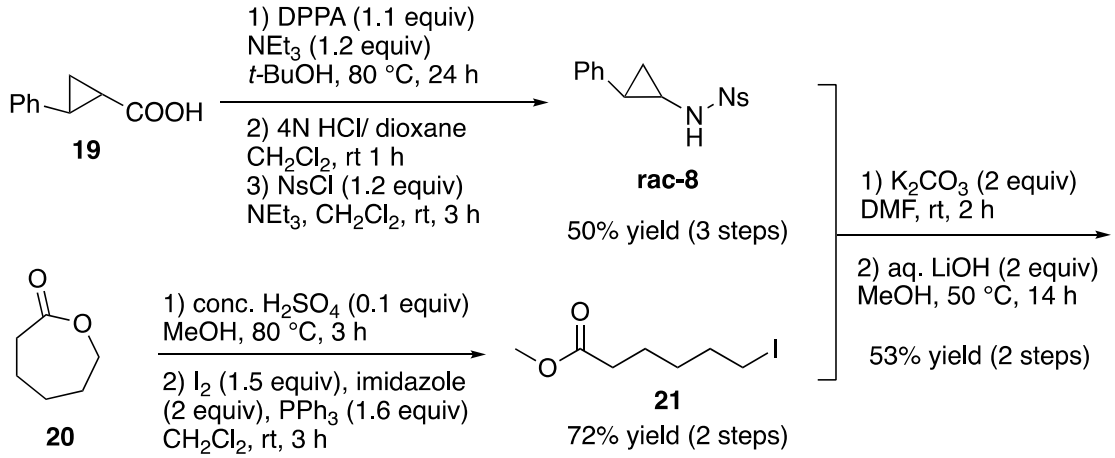
Fig. 2.1 (A) Molecular structure of NCD38-PIP complex; (B) Molecular structure of simplified LSD1 inhibitor-PIP complex

2-3 Synthesis of Simplified -PIP Hybrid Molecules

2-3.1 Synthesis of Simplified LSD1 Inhibitor

According to our design of simplified LSD1 inhibitor, it is constituted by PCPA and linker moieties. Therefore, we set out our synthesis of simplified LSD1 inhibitor from the preparation of these two parts respectively. Firstly, a commercial reagent of 2-phenylcyclopropane-1-carboxylic acid **19** was applied as the starting material, in the presence of diphenylphosphoryl azide (DPPA) as the azidation reagent, triethylamine and *tert*-butanol, an azidation reaction was conducted undergoing the Curtius rearrangement, directed the consequence of Boc protected 2-phenylcyclopropan-1-amine. The resultant was solved in dichloromethane and deprotected the *t*-butyloxycarbonyl (Boc) group in the presence of 10 equivalent 4M HCl/dioxane, afterwards, a 2-Ns group was applied as the protecting group of the amine using 1.2 equivalent 2-nitrobenzenesulfonyl chloride under the basic conditions to obtain the *rac*-**8** in 50% yield in total 3 steps. Furthermore, the linker part was also prepared according to the known synthetic methods.

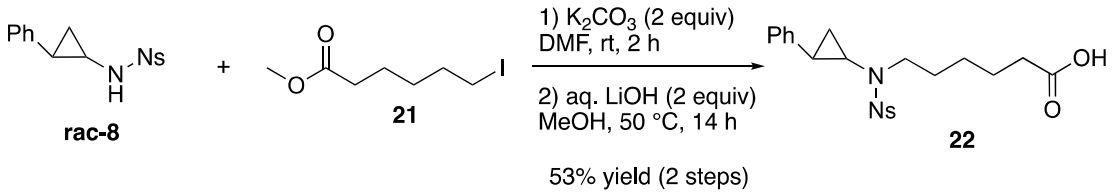
Commercial reagent of oxepan-2-one was used as the starting material, a ring-opening reaction was executed in the presence of concentrated sulfuric acid, refluxed at 80 °C for 3 hours. The residue then followed an iodination reaction to give the compound **21** in 72% yield in 2 steps.



Scheme 2.1 Synthesis of simplified LSD1 inhibitor (A)

All the reactions and compounds elucidated above is reported in previous of other researchers. We compared known analysis information with our detected ones to identify these compounds.

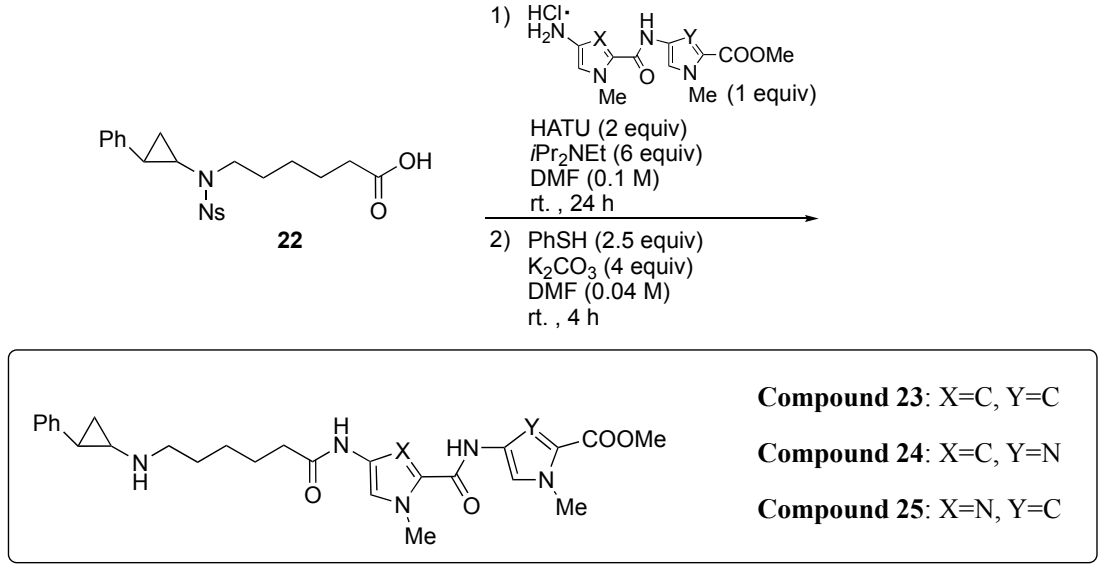
Since the preparation of PCPA and linker substrates were achieved, a nucleophilic reaction was performed between the nitrogen of *rac*-**8** and α-carbon of **21** to couple the 2 moieties together under basic conditions. The ester part of the obtained adduct was hydrolysis using the solvent of lithium hydroxide and refluxed in 50 °C for 14 hours to generate the target compound **22** in 53% yields in 2 steps.



Scheme 2.2 Synthesis of simplified LSD1 inhibitor (B)

2-3.2 Synthesis of Simplified LSD1-PIP Dimer Hybrid Molecules

The prepared carboxylic acid derivative **22** was coupled with hydrochloride of PIP dimer to introduce the linker part and PCPA part of LSD1 inhibitor. The coupling reaction was conducted in the presence of 2 equivalent HATU, 6 equiv of *i*-Pr_2NEt and 1 equivalent of hydrochloride of PIP dimer in DMF, at room temperature for 24 hours. The precursor molecule of the target hybrid compound was obtained in 64% yield. Then the precursor molecule followed a deprotection reaction of Ns group using a thiophenol and K_2CO_3 in DMF, the resulting compounds **23** was generated in 60% yield. Compound **24** and **25** was prepared using the same method as that for compound **23**. See more information at experimental section.

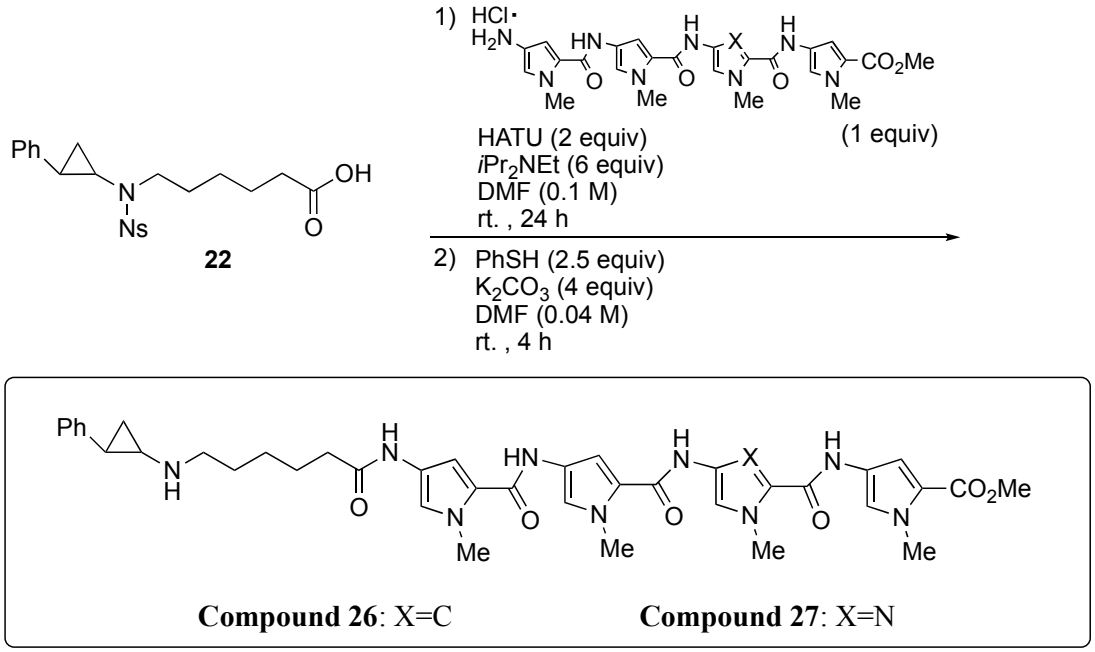


* Compound 24 did not use PIP amine hydrocloride but PIP amine.

Scheme 2.3 Synthesis of simplified LSD1-PIP dimer hybrid molecules

2-3.3 Synthesis of Simplified LSD1-PIP Teramer Hybrid Molecules

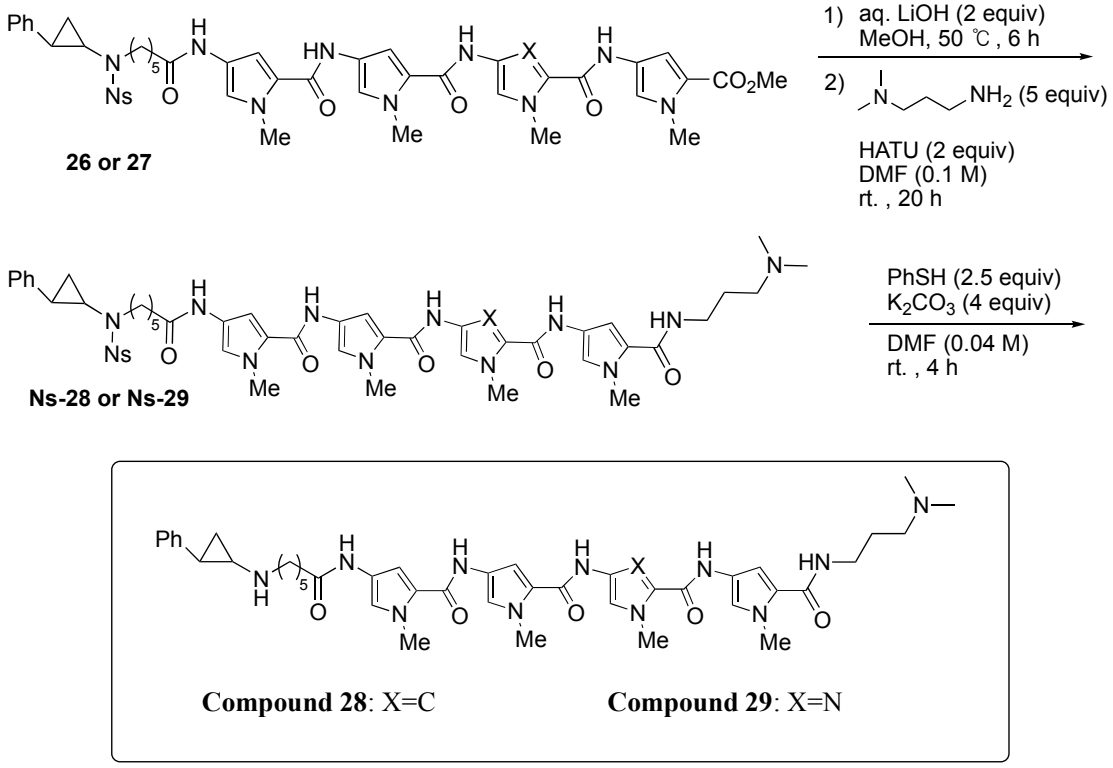
Likewise, we synthesized another 2 different of LSD1-PIP hybrid complexes. The prepared carboxylic acid derivative **22** was coupled with hydrochloride of PIP tetramer to introduce the linker part and PCPA part of LSD1 inhibitor. The coupling reaction was conducted in the presence of 2 equivalent HATU, 6 equivalents of *i*-Pr_2NEt and 1 equivalent of hydrochloride of PIP dimer in DMF, at room temperature for 24 hours. The precursor molecule of the target hybrid compound was obtained in 63% yield. Then the precursor molecule followed a deprotection reaction of Ns group using a thiophenol and K_2CO_3 in DMF, the resulting compounds **23** was generated in 61% yield. Compound **27** was prepared using the same method as that for compound **26**. See more information at experimental section.



Scheme 2.4 Synthesis of simplified LSD1-PIP tetramer hybrid molecules

2-3.4 Synthesis of simplified LSD1-PIP teramer (Dp) hybrid molecules

In addition, a modification of C-terminal of the simplified LSD1-PIP tetramer hybrid molecules was performed. After hydrolysis of the ester part in compound **26** under basic conditions, the obtained carboxylic acid derivative was coupled with *N*, *N*-dimethylpropane 1,3-diamine which may enhance the ability of cell permeability.[43] When the reaction was performed using 2 equivalent of HATU, 5 equivalent of *N*, *N*-dimethylpropane 1,3-diamine in DMF at room temperature, compound **Ns-28** was obtained in 65% yield. Then compound **Ns-28** was followed a deprotection reaction of Ns group using a thiophenol and K_2CO_3 in DMF, the resulting compound **28** was generated in 61% yield. Compound **29** was prepared using the same method as that for compound **28**. See more information at experimental section.



Scheme 2.5 Synthesis of simplified LSD1-PIP Teramer (Dp) hybrid molecules

2-4 Bioactivities of simplified LSD1-PIP hybrid molecules

2-4.1 Inhibitory activity of aimplified LSD1-PIP hybrid molecules

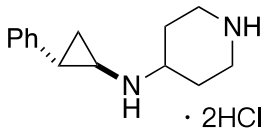
Inhibition of LSD1 activities were determined in vitro. The commercial LSD1

inhibitor of GSK-LSD1 (Fig. 2.2) was applied as the positive control. GSK-LSD1 is an irreversible, mechanism-based inhibitor of LSD1 which induces gene expression changes in cancer cell lines and inhibits cancer cell line growth. It is also implied that GSK-LSD1 has permitted itself as a chemical probe as part of the SGC epigenetics initiative.[44]

In our vitro test, GSK-LSD1 inhibits LSD1 with an IC50 of 64.794 nM with 6.907 standard error. 3 different simplified LSD1-PIP dimer complexes exhibited IC 50 various from 165.114 to 214.198 with standard error below 50. Whereas the IC 50 values of 4 different simplified LSD1-PIP tetramer complexes no matter ester or Dp C-terminal revealed slight upgraded various from 225.76 to 336.202 with standard error below 50. It was implied that LSD1-inhibiting activities of 7 types of simplified LSD1-PIP hybrid molecules were marginally down down-regulated compared with the positive control. Nevertheless, these compounds still presented considerable inhibitory activity of LSD1.

Compounds	**IC 50**	**Std. Error**
GSK-LSD1	64.794	6.907
Simplified LSD1-PyPy-Me	173.755	37.099
Simplified LSD1-PyIm-Me	165.114	25.391
Simplified LSD1-ImPy-Me	214.198	48.713
Simplified LSD1-PyPyPyPy-Me	225.76	44.659
Simplified LSD1-PyPyImPy-Me	336.202	32.678
Simplified LSD1-PyPyPyPy-Dp	375.14	49.61
Simplified LSD1-PyPyImPy-Dp	336.202	32.678
		[nM]

Table 2.2 IC 50 values of compounds against LSD1



GSK-LSD1

Fig. 2.2 Chemical structure of GSK-LSD1 inhibitor

2-4.2 EMSA analyses of simplified LSD1-PyPyPyPy (Dp) Hybrid Molecules

Furthermore ,electrophoretic mobility shift assays (EMSA) analyses was conducted to test the specific recognition of WWWWWW or WWCGWW sequence by the simplified LSD1-PyPyPyPy (Dp) hybrid molecule.

It was performed for double strand DNA (dsDNA) in presence of compound **28**. It was demonstrated that compound **28** bound to dsDNA containing WWWWWW. Whereas the recognition of dsDNA containing WWCGWW sequences could not been detected.

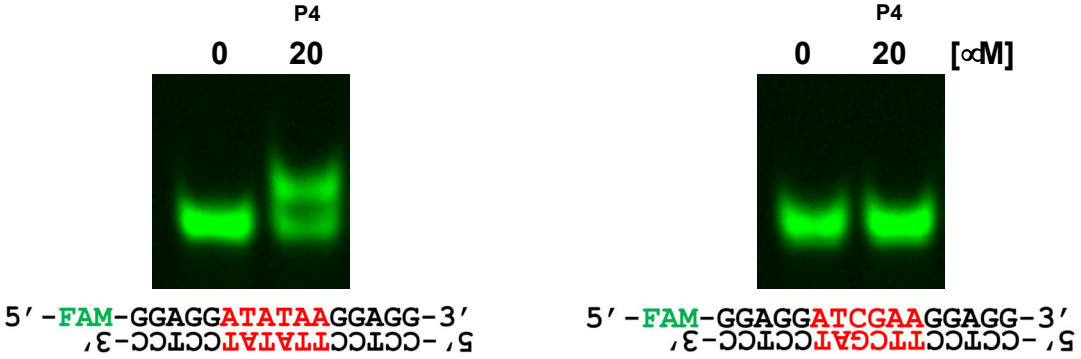


Fig. 2.4 EMSA analysis for double strand DNA (dsDNA) in presence compound **28**, vehicle control (lane 5: 1% DMSO).

2-5 Discussion

In this section, we designed a simplified LSD1 inhibitor based on the previous researches. Since numerous works implied that PCPA derivatives exhibited LSD1 inhibitory activity, albeit possessed some shortages such as low affinity and selectivity against LSD1. To improve these shortcomings, various of complexes combined PCPA with PIPs was prepared.

As the design of simplified LSD1 inhibitor, the synthetic route was shortened strikingly compared with the NCD38-type inhibitor. In terms of the synthesis of NCD38-type inhibitor, included the preparation of substrate of compound **4** and optical resolution of compound **8**, total 14 steps were necessary. On the contrary, synthesis of the simplified LSD1 inhibitor started from a commercial reagent processed 7 steps to obtain the target inhibitor carboxylic acid **22**. The synthetic efficiency was remarkably increased. I established an applicable platform for the future works.

In addition, the IC 50 values of 7 types of simplified LSD1-PIP hybrid molecules were measured, implying that LSD1-inhibiting activities were marginally down compared with the positive control. Nevertheless, these compounds still presented considerable inhibitory activity of LSD1. Besides, electrophoretic mobility shift assays (EMSA) analyses indicated that simplified LSD1-PyPyPyPy (Dp) hybrid molecule bound to dsDNA containing WWWWWW, whereas the recognition of dsDNA containing WWCGWW sequences could not been detected.

Conclusions

In this study, we conducted two parts of studies of LSD1 inhibitors conjugated PIPs molecules. In the first part, we prepared NCD38-type inhibitor moiety based on the synthetic route of NCD38 which developed by Suzuki's group. The PCPA unit that permitted the whole coupled molecules inhibitory activity was conjugated with an L-lysine derivative using a nosyl group as a functional protecting group (Ns strategy). Coupling of NCD38-type inhibitor moiety with 2 different pyrrole-imidazole polyamide tetramers was executed through amide bond formation. Additionally, LSD1 inhibitory activity was assessed using these 2 coupled molecules which are **3a** and **3b**, respectively. As the results indicated clearly that both **3a** which is NCD38-β2PPPP and **3b** which is NCD38-β2PIPP inhibited LSD1 activities. The levels of inhibition were similar to that of parental NCD38. Meanwhile, ChIP-seq and RNA-seq data of the regions epigenetically altered by parental NCD38 and the two different hybrid molecules were analyzed. It was implied that the recognition of specific regions had a significant alternation from the GC-rich regions to the AT-rich regions. The consequences indicated that the two different hybrid molecules targeted various sequences, indicating that the sequence-specific recognition of NCD38-PIP conjugates alternated from GC-rich regions to AT-rich regions.

Furthermore, we hypothesized a simplified LSD1 inhibitor for the following work for 2 reasons. Firstly, it was reported numerously that PCPA derivatives were LSD1 inhibitory activity. Besides, the PIPs could enable the hybrid molecules region-specific recognitive function whereas the complicated PCPA carrier moiety which serve the inhibitor selectivity would be simplified. Thus, LSD1 inhibitor composed with PCPA moiety as well as a simpler linker expected a practicable inhibitor structure for this work. The alternation of structure significantly shortened the synthetic route of LSD1 inhibitor. Compared with the NCD38-type inhibitor coupled molecule which was synthesized in total 14 steps. On the contrary, we prepared the simplified LSD1 inhibitor started from a commercial reagent processed 7 steps. The efficiency of preparing inhibitor was remarkably increased. We established an applicable platform for the future works.

Likewise, the IC 50 values of 7 types of simplified LSD1-PIP hybrid molecules were implied that LSD1-inhibiting activities were marginally down compared with the

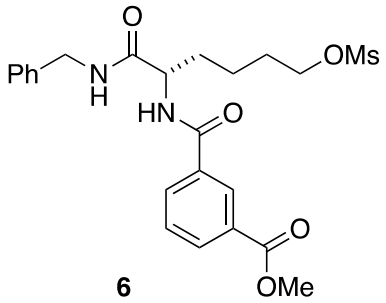
positive control. Nevertheless, these compounds still presented considerable inhibitory activity of LSD1. Besides, electrophoretic mobility shift assays (EMSA) analyses indicated that simplified LSD1-PyPyPyPy (Dp) hybrid molecule bound to dsDNA containing WWWWWW, whereas the recognition of dsDNA containing WWCGWW sequences could not been detected.

In conclusion, we established an applicable platform of synthesizing LSD1 inhibitor, which contains PCPA moiety, coupled PIPs to generated LSD1-PIPs hybrid molecules. Applying both developed NCD38-type LSD1 inhibitor and the designed new LSD1 inhibitor, various LSD1-PIPs hybrid molecules were prepared successfully. Especially, the simplified LSD1 inhibitor-PIPs hybrid molecules could be prepared efficiently which facilitated the following works such as study of bioactivities significantly. Bioactivity assessments of NCD38-PIPs were completed including inhibitory activities, ChIP-seq analysis and RNA-seq analysis. Likewise, the simplified LSD1 inhibitor-PIPs hybrid molecules would be assessed of their bioactivities. LSD1 inhibitory activities and ChIP-seq analysis of simplified LSD1-PyPyPyPy (Dp) hybrid molecule have been done, estimating of the other compounds are ongoing.

Experimental Section

General: Infrared (IR) spectra were recorded on a Fourier transform infrared spectrophotometer, equipped with ATR. NMR spectra were recorded with a 400 MHz spectrometer. Chemical shifts in $CDCl_3$ were reported downfield from TMS (= 0 ppm) for ^1H NMR. For ^{13}C NMR, chemical shifts were reported in the scale relative to the solvent signal [$CHCl_3$ (77.0 ppm)] as an internal reference. Positive-ion mass spectra were recorded by electrospray ionization (ESI-TOF). Column chromatography was performed with silica gel 60N (spherical, neutral, 40–60 μm mesh) (Kanto chemical) or CHROMATOREX NH-DM1020 (Fuji silysia chemical). Reactions were carried out in dry solvent. Other reagents were purified by the usual methods.

Synthesis of **6**

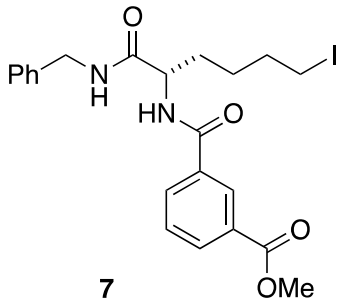


6

To a stirred solution of **4** (809 mg, 1.95 mmol) in CH_2Cl_2 (67.8 mL) at 0 °C was added 4M HCl in dioxane (4.9 mL) and the resulting mixture was stirred at room temperature. After 1 h, organic solvent was removed to give the corresponding amine hydrochloride in a quantitative yield.

To a stirred solution of 3-(methoxycarbonyl)benzoic acid **5** (135.1 mg, 0.75 mmol), 1-hydroxybenzotriazole (HOBt) (101.3 mg, 0.75 mmol), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI) (143.8 mg, 0.75 mmol), and triethylamine (0.28 mL, 2 mmol) in DMF (2.5 mL) at 0 °C was added the obtained amine hydrochloride (175.4 mg, 0.5 mmol). After 17 h, the reaction was quenched with sat. aq. NH4Cl solution and diluted with AcOEt. The resulting mixture was washed with 1M aq. HCl solution, sat. aq. $NaHCO_3$ solution, and brine, and then dried over Na_2SO_4. After concentration under reduced pressure, the residue was recrystallized from AcOEt-hexane to give compound 6 as white solid (135 mg, 56% yield). $[\alpha]^{20}_D$ = –9.8 (*c* = 0.23, $CHCl_3$); Mp. 142–143°C; ^{1}H NMR (400 MHz, CDCl3) δ 1.47–1.60 (m, 2H), 1.70– 2.08 (m, 4H), 2.95 (s, 3H), 3.91 (s, 3H), 4.13–4.25 (m, 2H), 4.36 (dd, *J* = 5.6 Hz, 14.8 Hz, 1H), 4.46 (dd, *J* = 5.6 Hz, 14.8 Hz, 1H), 4.70–4.82 (m, 1H), 7.10–7.37 (m, 7H), 7.43–7.48 (m, 1H), 7.93 (d, *J* = 8.0 Hz, 1H), 8.14 (d, *J* = 8.0 Hz, 1H), 8.38 (d, *J* = 1.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.5, 28.6, 31.9,37.3, 43.5, 52.3, 53.4, 69.6, 127.5, 127.6 (2C), 127.9, 128.6 (2C), 128.8, 130.5, 131.7, 132.7, 133.9, 137.8, 166.1, 166.5, 171.3; IR (ATR) ν 1721, 1631, 1532, 1347, 1252, 1163, 956, 821, 694 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{23}H_{28}N_2NaO_7S^+$ ($M+Na^+$): 499.1509. Found: 499.1529.

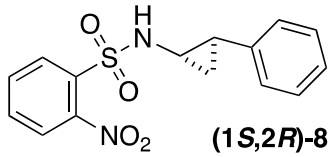
Synthesis of **7**



7

A suspension of compound **6** (95.3 mg, 0.2 mmol) and NaI (600 mg, 4.00 mmol) in acetone (4 mL) was refluxed for 12 h. After cooling down to room temperature, the reaction was diluted with $CHCl_3$. The resulting mixture was washed with brine and then dried over Na_2SO_4. After concentration under reduced pressure, the residue was recrystallized from AcOEt-hexane to give compound 7 as white solid (95 mg, 94% yield). $[\alpha]^{20}_D$ = –9.5 (*c* = 0.14, CHCl3); Mp. 153–154°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.49–1.57 (m, 2H), 1.77–1.91 (m, 3H), 1.97–2.06 (m, 1H), 3.17 (t, *J* = 6.8 Hz, 2H), 3.94 (s, 3H), 4.41 (dd, *J* = 5.6 Hz, 14.4 Hz, 1H), 4.51 (dd, *J* = 6.0 Hz, 14.4 Hz, 1H), 4.68–4.74 (m, 1H), 6.71 (dd, *J* = 5.6 Hz, 6.0 Hz, 1H), 7.01 (d, *J* = 8.0 Hz, 1H), 7.20–7.33 (m, 5H), 7.51 (dd, *J* = 8.0 Hz, 8.0 Hz, 1H), 7.97 (dd, *J* = 1.6 Hz, 8.0 Hz, 1H), 8.18 (d, *J* = 8.0 Hz, 1H), 8.40 (d, *J* = 1.6 Hz, 1H); 13C NMR (100 MHz, $CDCl_3$) δ 6.4, 26.5, 31.6, 32.9, 43.7, 52.4, 53.5, 127.6, 127.7 (2C), 127.9, 128.7 (2C), 128.9, 130.6, 131.7, 132.8, 134.0, 137.7, 166.2, 166.5, 171.3; IR (ATR) ν 3292, 2927, 1722, 1634, 1538, 1438, 1299, 1260, 729, 697 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{22}H_{25}IN_2NaO_4^+$ ($M+Na^+$): 531.0751. Found: 531.0756.

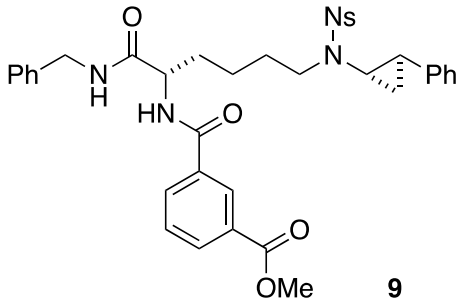
Synthesis of **(1*S*,2*R*)-8**



(1*S*,2*R*)-8

(1*S*,2*R*)-2-phenylcyclopropane-1-amine hydrochloride was prepared using the reported procedure. To a stirred solution of (1*S*,2*R*)-2-phenylcyclopropane-1-amine hydrochloride (225 mg, 1.33 mmol) and NEt_3 (0.56 mL, 4.00 mmol) in CH_2Cl_2 (13.3 mL) at room temperature was added o-nosyl chloride (354.6 mg, 1.60 mmol). The reaction mixture was kept stirring for 4 h at room temperature. To consume the unreacted 2-nosyl chloride, N,N-dimethylpropane-1,3-diamine (0.165 mL, 1.33 mmol) was added and the reaction was stirred for 1 h. After dilution with AcOEt, the resulting mixture was washed with 1M aq. HCl, sat. aq. $NaHCO_3$ and brine, and then dried over Na_2SO_4. After concentration under reduced pressure, the residue was purified by silica gel column chromatography (SiO_2, n-hexane/EtOAc = 3/1) to give (1*S*,2*R*)-**8** (309 mg, 73% yield). Stereoscopic data was identical to the reported data.

Synthesis of **9**

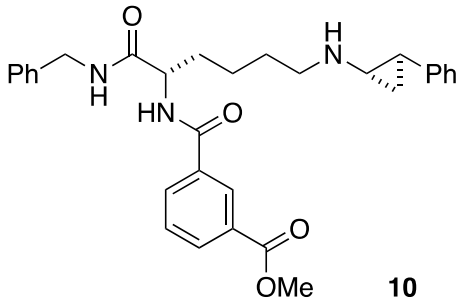


9

To a stirred suspension of compound (1*S*,2*R*)-**8** (64 mg, 0.2 mmol) and K_2CO_3 (55.3 mg, 0.4 mmol) in DMF (1 mL) at room temperature was added **7** (152.5 mg, 0.3 mmol), and the resulting mixture was stirred for 24 h at the same temperature. The reaction was diluted with AcOEt/n-hexane (4/1) and the mixture was washed with water (x2) and brine, and then dried over Na_2SO_4. After concentration in vacuo, the obtained residue was purified by silica gel column chromatography (SiO_2, n-hexane/EtOAc = 1/1 to 1/2) to give compound **9** (190.3 mg, 91% yield) as white amorphous. $[\alpha]^{20}_D$ = 13.9 (*c* = 2.64, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.10–2.15 (m, 9H), 2.60–2.66 (m, 1H), 3.30– 3.40 (m, 1H), 3.43–3.52 (m, 1H), 3.91 (s, 3H), 4.47 (d, *J* = 5.6 Hz, 1H), 4.60–4.68 (m, 1H), 6.56 (broad peak, 1H), 6.92–6.97 (m, 2H), 7.00 (d, *J* = 8.0 Hz, 1H), 7.16–7.33 (m, 8H), 7.50–7.72 (m, 4H), 7.97 (dd, *J* = 1.6 Hz, 8.0 Hz, 1H), 8.04 (d, *J* = 8.0 Hz, 1H), 8.16–8.20 (m, 1H), 8.42–8.45 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.0, 22.4, 24.9, 27.8, 31.9, 38.4, 43.5, 49.7, 52.2, 53.6, 124.0, 126.0 (2C), 126.4, 127.4, 127.6 (2C), 128.0, 128.4 (2C), 128.6 (2C), 128.7, 130.5, 131.4, 131.6, 131.7, 132.6, 132.9, 133.7, 134.0, 137.9, 139.2, 147.9, 166.2, 166.5, 171.4; IR (ATR) ν 3285, 1722, 1636, 1542, 1439, 1370, 1259, 1159, 747, 698

cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{37}H_{38}N_4NaO_8S^+$ ($M+Na^+$): 721.2303. Found: 721.2325.

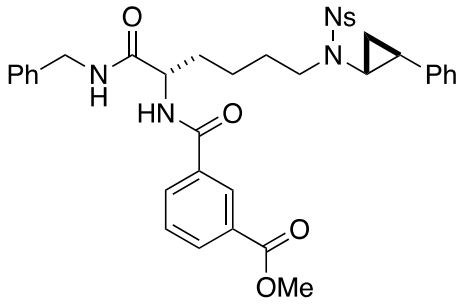
Synthesis of **10**



10

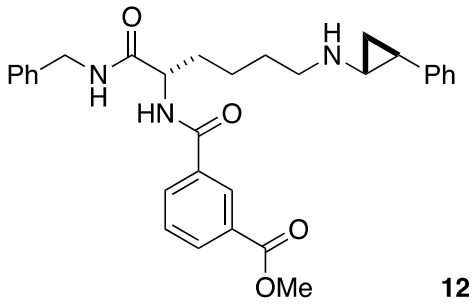
A suspension of compound **9** (33.0 mg, 0.0472 mmol), Cs_2CO_3 (61.6 mg, 0.189 mmol), and SiliaMetS Thiol (1.41 mmol/g) (134 mg, 0.189 mmol) in CH_3CN (1.2 mL) were stirred for 48 h at room temperature. The yellow suspension was directly purified by silica gel column chromatography (SiO_2, $CHCl_3$/MeOH = 20/1 to 10/1) to give compound **10** (14.9 mg, 62% yield) as colorless oil. $[\alpha]^{20}_D$ = 16.2 (*c* = 2.4, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 0.90–1.06 (m, 2H), 1.40–1.60 (m, 4H), 1.80–2.03 (m, 4H), 2.28–2.32 (m, 1H), 2.69–2.76 (m, 2H), 3.94 (s, 3H), 4.41 (dd, *J* = 5.6 Hz, 14.8 Hz, 1H), 4.50 (dd, *J* = 6.0 Hz, 15.2 Hz, 1H), 4.66–4.72 (m, 1H), 6.87 (dd, *J* = 5.6 Hz, 6.0 Hz, 1H), 7.00–7.03 (m, 2H), 7.08 (d, *J* = 7.2 Hz, 1H), 7.11–7.15 (m, 1H), 7.21–7.32 (m, 6H), 7.48–7.54 (m, 2H), 7.98–8.00 (m, 1H), 8.16–8.19 (m, 1H), 8.40–8.42 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.8, 23.2, 24.8, 29.4, 32.7, 41.4, 43.6, 49.0, 52.4, 53.6, 125.5, 125.8 (2C), 127.6, 127.7 (2C), 127.9, 128.2 (2C), 128.7 (2C), 128.9, 130.6, 131.7, 132.7, 134.1, 137.8, 142.1, 166.2, 166.3, 171.4; IR (ATR) ν 3282, 2925, 1725, 1635, 1538, 1438, 1258, 729, 696 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{31}H_{36}N_3O_4^+$ ($M+H^+$): 514.2700. Found: 514.2703.

Synthesis of **11**



This compound was prepared using the synthetic procedure for compound **9** (76% yield). White amorphous; $[\alpha]^{20}_{D}$ = –18.6 (*c* = 4.04, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.15–2.10 (m, 9H), 2.60–2.65 (m, 1H), 3.30–3.38 (m, 1H), 3.46–3.54 (m, 1H), 3.90 (s, 3H), 4.47 (d, *J* = 6.0 Hz, 1H), 4.60–4.66 (m, 1H), 6.52 (t, *J* = 6.0 Hz, 1H), 6.93 (d, *J* = 7.2 Hz, 2H), 7.00 (d, *J* = 8.0 Hz, 1H), 7.16–7.30 (m, 8H), 7.50– 7.72 (m, 4H), 7.97 (d, *J* = 8.4 Hz, 1H), 8.04 (d, *J* = 7.6 Hz, 1H), 8.18 (d, *J* = 7.6 Hz, 1H), 8.45 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.0, 22.3, 24.9, 27.7, 31.7, 38.3, 43.6, 49.5, 52.3, 53.6, 124.1, 126.0 (2C), 126.5, 127.4, 127.6 (2C), 128.0, 128.5 (2C), 128.7 (2C), 128.8, 130.5, 131.5, 131.6, 131.7, 132.7, 133.0, 133.7, 134.0, 137.9, 139.2, 147.9, 166.2, 166.5, 171.3; IR (ATR) ν 1724, 1637, 1543, 1261, 1160, 730, 696, 620, 608 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{37}H_{38}N_4NaO_8S^+$ ($M+Na^+$): 721.2303. Found: 721.2308.

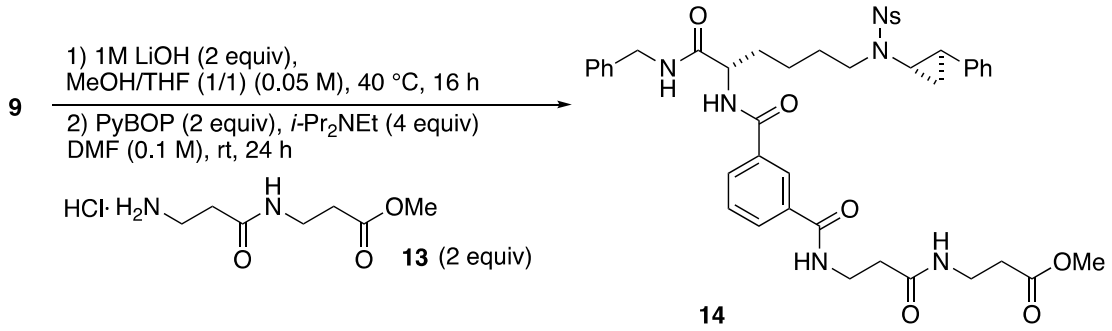
Synthesis of **12**



12

This compound was prepared using the synthetic procedure for compound **10** (76% yield). Colorless oil; $[\alpha]^{20}_{D} = -18.9$ (*c* = 3.6, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.97–1.01 (m, 1H), 1.12–1.17 (m, 1H), 1.40–1.49 (m, 2H), 1.60–1.68 (m, 2H), 1.80–2.03 (m, 3H), 2.30–2.36 (m, 1H), 2.75–2.84 (m, 2H), 3.90 (s, 3H), 4.39 (dd, *J* = 6.8 Hz, 14.8 Hz, 1H), 4.48 (dd, *J* = 6.8 Hz, 14.8 Hz, 1H), 4.70–4.76 (m, 1H), 7.00–7.03 (m, 2H), 7.15 (dd, *J* = 6.8 Hz, 6.8 Hz, 1H), 7.20–7.36 (m, 8H), 7.46–7.50 (m, 2H), 7.96–7.99 (m, 1H), 8.16 (d, *J* = 8.0 Hz, 1H), 8.42 (d, *J* = 2.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.0, 23.0, 24.1, 28.4, 32.5, 40.8, 43.6, 48.7, 52.4, 53.4, 125.9 (2C), 127.5, 127.7 (2C), 128.0, 128.3 (2C), 128.7 (2C), 128.8, 130.5, 131.7, 132.7, 134.0, 137.8, 141.4, 166.2, 166.5, 171.6; IR (ATR) ν 3297, 2931, 1725, 1637, 1541, 1439, 1264, 732, 697 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{31}H_{36}N_3O$ + ($M+H^+$): 514.2700. Found: 514.2721.

Synthesis of **14**

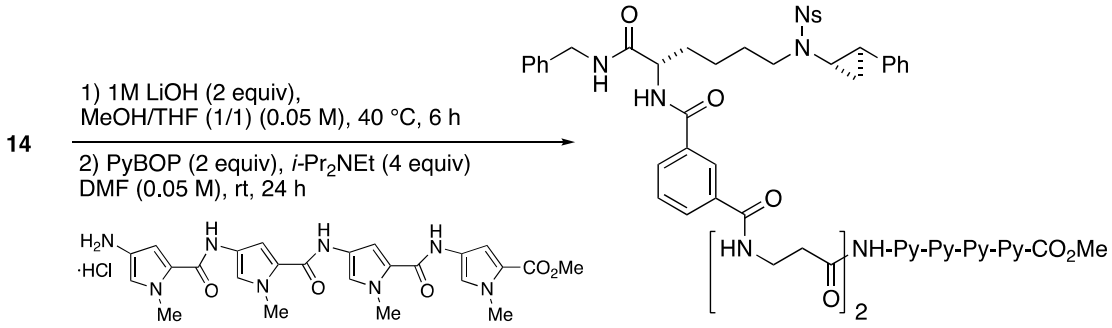


A solution of **9** (49.0 mg, 0.07 mmol) and 1M LiOH (0.14 mL, 0.14 mmol) in MeOH/THF (1/1) (1.4 mL) was stirred at 40 °C. After 16 h, the resulting mixture was acidified with 1M aq. HCl and the mixture was extracted with $CHCl_3$ and then organic layer was dried over Na_2SO_4. After concentration in vacuo, almost pure carboxylic acid was obtained in quantitative yield. The obtained residue was used for the next reaction without purification.

To a stirred solution of the crude carboxylic acid derivative, benzotriazol-1-yl-oxytripyrrolidinophosphonium-hexafluorophosphat (PyBOP) (72.8 mg, 0.14 mmol), and *i*-Pr_2NEt (49 μL mL, 0.28 mmol) in DMF (0.7 mL) at 0 °C was added b-alanine dimer **13** (29.5 mg, 0.14 mmol), and the resulting mixture was stirred for 40 h at room temperature. The reaction was diluted with AcOEt/n-hexane (4/1) and the resulting mixture was washed with 1M aq. HCl solution, sat. aq. $NaHCO_3$ solution, water, and brine, and then dried over Na_2SO_4. After concentration under reduced pressure, the obtained residue was purified by silica gel column chromatography (CHROMATOREX NH-DM1020, $CHCl_3$/MeOH = 80/1) to give compound **14** (44.7 mg, 76% yield) as colorless oil. $[\alpha]^{20}_D$ = –49.2 (*c* = 1.6, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.10–2.11 (m, 9H), 2.43–2.55 (m, 4H), 2.59–2.65 (m, 1H), 3.24–3.38 (m, 1H), 3.40–3.52 (m, 3H), 3.61 (s, 3H), 3.60–3.70 (m, 2H), 4.40 (dd, *J* = 5.6 Hz, 14.8

Hz, 1H), 4.46 (dd, J = 6.0 Hz, 14.8 Hz, 1H), 4.63–4.70 (m, 1H), 6.46 (dd, J = 5.6 Hz, 6.0 Hz, 1H), 6.88–6.95 (m, 3H), 7.14–7.70 (m, 14H), 7.87–8.00 (m, 3H), 8.22 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.0, 22.6, 24.9, 27.9, 31.7, 33.8, 35.0, 35.6, 36.3, 38.3, 43.4, 49.8, 51.8, 53.8, 124.1, 125.4, 126.0 (2C), 126.5, 127.3, 127.5 (2C), 128.4 (2C), 128.6 (2C), 128.8, 130.4, 130.6, 131.5, 131.5, 132.8, 133.7, 133.8, 134.6, 138.0, 139.2, 147.8, 166.9, 166.9, 171.7, 171.9, 172.9; IR (ATR) ν 3316, 1737, 1644, 1542, 1438, 1371, 770, 699, 671, 633 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{43}H_{48}N_6NaO_{10}S^+$ ($M+Na^+$): 863.3050. Found: 863.3048.

Synthesis of **16**

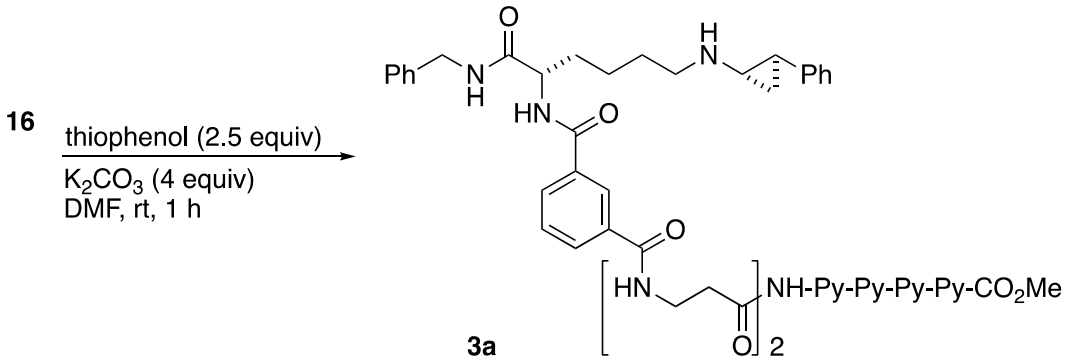


A solution of **14** (44.7 mg, 0.053 mmol) and 1M LiOH (0.106 mL, 0.106 mmol) in MeOH/THF (1/1) (1.06 mL) was stirred at 40 °C. After 16 h, the resulting mixture was acidified with 1M aq. HCl and the mixture was extracted with $CHCl_3$ and then organic layer was dried over Na_2SO_4. After concentration in vacuo, almost pure carboxylic acid was obtained in quantitative yield. The obtained residue was used for the next reaction without purification.

A solution of obtained carboxylic acid derivative (22.1 mg, 0.0267 mmol), **15** (14.9 mg, 0.0267 mmol),

benzotriazol-1-yl-oxytripyrrolidinophosphonium-hexafluorophosphate (PyBOP) (27.8 mg, 0.0534 mmol), and *i*-Pr_2NEt (0.019 mL, 0.107 mmol) in DMF (0.54 mL) was stirred for 24 h at room temperature. The reaction was diluted with $CHCl_3$/*i*-PrOH (1/1) and the resulting mixture was washed with 1M aq. HCl solution, sat. aq. $NaHCO_3$ solution, and water, and then dried over Na_2SO_4. After concentration under reduced pressure, the obtained residue was purified by silica gel column chromatography (CHROMATOREX NH-DM1020, $CHCl_3$/MeOH = 60/1 to 30/1) to give compound **16** (26.6 mg, 75% yield) as pale brown solid. Mp. 141–142°C; $[\alpha]^{20}_D$ = –29.8 (*c* = 0.88, $CHCl_3$); IR (ATR) ν 3292, 2924, 1645, 1543, 1437, 1404, 1252, 1108, 748, 608 cm-1; (+)-ESI-HRMS. Calcd for C67H72N14NaO14S+ (M+Na+): 1351.4971. Found: 1351.4917. Although all signals were detected as broad peaks in the 1H NMR analysis ($CDCl_3$) probably due to the high molecular weight, an analyzable chart was obtained. See supporting information for the 1H NMR chart.

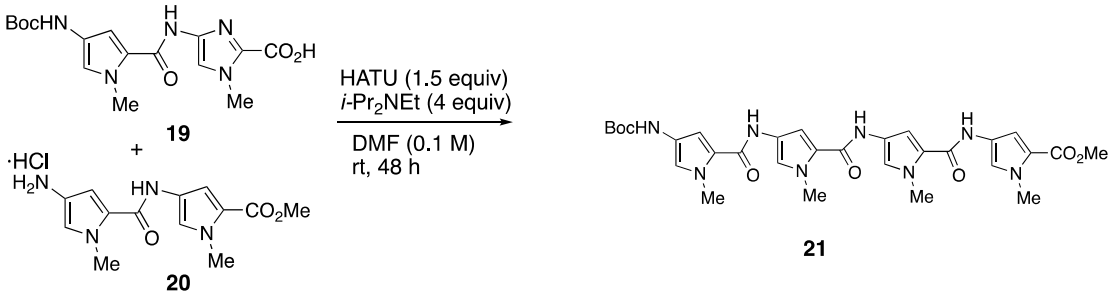
Synthesis of **3a**



To a stirred solution of **16** (11.0 mg, 0.0083 mmol), K_2CO_3 (4.6 mg, 0.033 μmol) in DMF (0.3 mL) was added thiophenol (2.1 μL, 0.021 mmol) and the resulting solution

was stirred for 1 h at room temperature. The yellow solution was directly purified by silica gel column chromatography (SiO_2, $CHCl_3$/MeOH = 20/1 to 10/1) to give compound **3a** (6.5 mg, 65% yield) as colorless solid. $[\alpha]^{20}_D$ = –41.4 (*c* = 0.48, $CHCl_3$); IR (ATR) ν 3295, 2924, 2853, 1644, 1540, 1436, 1404, 1254, 1205, 1108, 754, 699, 606 cm-1; (+)-ESI-HRMS. Calcd for $C_{61}H_{70}N_{13}O^+$ ($M+H^+$): 1144.5363. Found: 1144.5350. Although all signals 10 were detected as broad peaks in the ^1H NMR analysis ($CDCl_3$) probably due to the high molecular weight, 3 an analyzable chart was obtained.

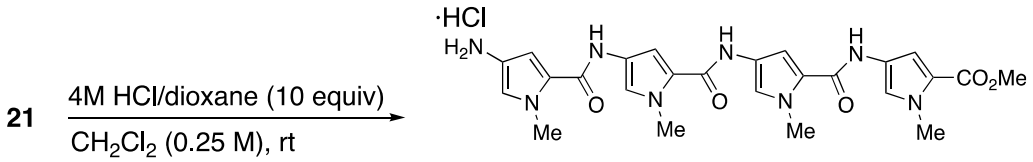
Synthesis of **21**



To a stirred solution of **19** (181.7 mg, 0.5 mmol), **20** (187.7 mg, 0.6 mmol), and HATU (380.2 mg, 1 mmol) in DMF (5 mL) at room temperature was added *i*-Pr_2NEt (0.35 mL, 2 mmol), and the resulting mixture was stirred for 48 h at the same temperature. The reaction was quenched by the addition of 1M aq. $KHSO_4$ solution and the mixture was extracted with CH_2Cl_2. The organic layer was washed with brine and dried over Na_2SO_4. After concentration under reduced pressure, the obtained residue was purified by silica gel column chromatography (SiO_2, hexane/AcOEt = 1/3) to give compound **21** (111.9 mg, 36% yield) as yellow amorphous. Mp. 155–

156°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26 (s, 9H), 3.82 (s, 3H), 3.91 (s, 3H), 3.93 (s, 3H), 3.97 (s, 3H), 4.07 (s, 3H), 6.35 (s, 1H), 6.72 (s, 1H), 6.78 (d, *J* = 2.0 Hz, 1H), 6.84 (s, 1H), 7.00 (s, 1H), 7.43 (d, *J* = 2.0 Hz, 1H), 7.48 (s, 1H), 7.67 (s, 1H), 8.41 (s, 1H), 8.61 (d, *J* = 0.8 Hz, 1H), 8.98 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.4 (3C), 29.7, 35.9, 36.7, 36.8, 51.1, 80.3, 103.6, quant. 104.3, 108.4, 114.2, 119.3, 119.5, 119.8, 120.8, 121.0, 121.9, 122.1, 123.4, 133.5, 135.7, 150.1, 153.5, 155.5, 158.6, 158.9, 161.6; IR (ATR) ν 3318, 2926, 1698, 1658, 1587, 1539, 1440, 1404, 1367, 1250, 1205, 1162, 1110, 772, 733 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{29}H_{35}N_9NaO_7^+$ ($M+Na^+$): 644.2557. Found: 644.2553.

Synthesis of **17**

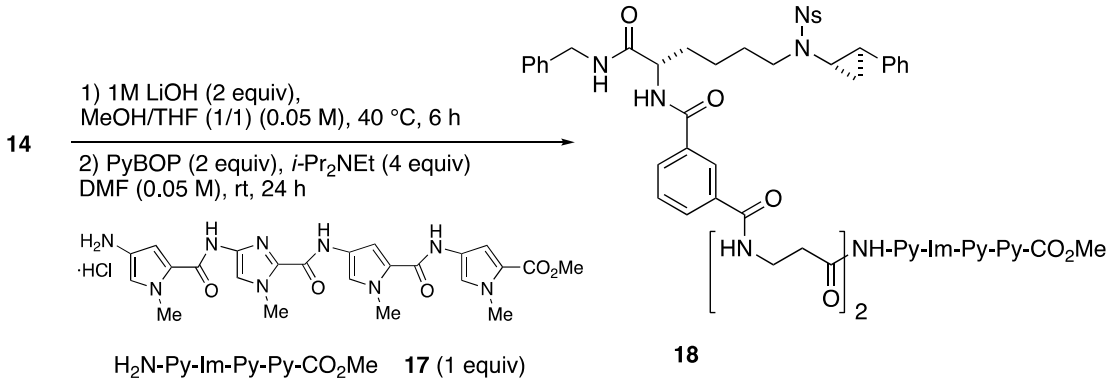


17

To a stirred solution of **21** (186.5 mg, 0.3 mmol) in CH_2Cl_2 (6.2 mL) at 0 °C was added 4M HCl in dioxane (0.75 mL) and the resulting mixture was stirred at room temperature. After 19 h, organic solvent was removed and the residue was washed with ether to give **17** (168 mg, quant.) as yellow solid, which was used for the next reaction without further purification. Mp. 188–189°C; 1H NMR (400 MHz, CD_3OD) δ 3.79 (s, 3H), 3.89 (s, 3H), 3.92 (s, 3H), 3.98 (s, 3H), 4.09 (s, 3H), 6.93 (d, *J* = 2.4 Hz, 1H), 6.98 (s, 1H), 7.02 (d, *J* = 2.4 Hz, 1H), 7.13 (d, *J* = 2.4 Hz, 1H), 7.36 (d, *J* = 2 Hz, 1H), 7.49–7.53 (m, 2H), 8.43 (dd, *J* = 1.2 Hz, 8.0 Hz, 1H), 8.73 (dd, *J* = 1.2 Hz,

4.0 Hz, 1H). (Protons in NH_2 and one amide NH proton could not be detected in CD_3OD); IR (ATR) ν 3364, 1671, 1633, 1554, 1435, 1404, 1258, 1206, 1110, 769, 638, 611 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{24}H_{27}N_9O_5^+$ ($M+H^+$): 522.2213. Found: 522.2238.

Synthesis of **18**

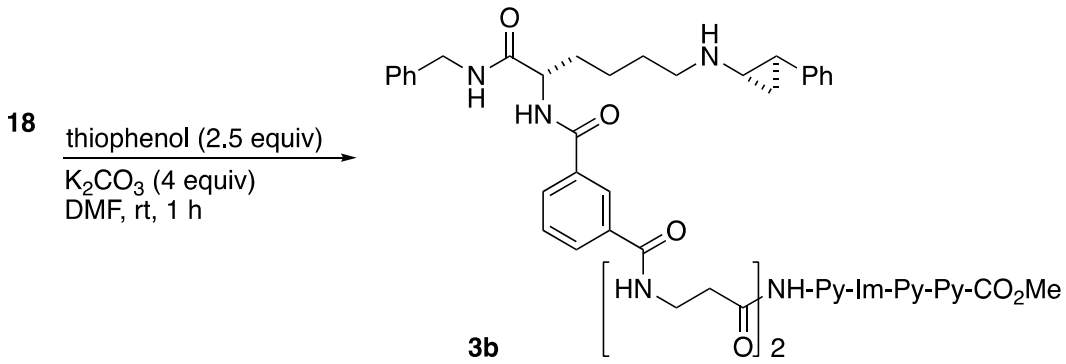


A solution of **14** (44.7 mg, 0.053 mmol) and 1M LiOH (0.106 mL, 0.106 mmol) in MeOH/THF (1/1) (1.06 mL) was stirred at 40 °C. After 16 h, the resulting mixture was acidified with 1M aq. HCl and the mixture was extracted with $CHCl_3$ and then organic layer was dried over Na_2SO_4. After concentration in vacuo, almost pure carboxylic acid was obtained in quantitative yield. The obtained residue was used for the next reaction without purification.

A solution of obtained carboxylic acid derivative (22.1 mg, 0.0267 mmol), **17** (14.9 mg, 0.0267 mmol), benzotriazol-1-yl-oxytripyrrolidinophosphonium-hexafluorophosphate (PyBOP) (27.8 mg, 0.0534 mmol), and *i*-Pr_2NEt (0.019 mL, 0.107 mmol) in DMF (0.54 mL) was

stirred for 24 h at room temperature. The reaction was diluted with $CHCl_3$/*i*-PrOH (1/1) and the resulting mixture was washed with 1M aq. HCl solution, sat. aq. $NaHCO_3$ solution, and water, and then dried over Na_2SO_4. After concentration under reduced pressure, the obtained residue was purified by silica gel column chromatography (CHROMATOREX NH-DM1020, $CHCl_3$/MeOH = 60/1 to 30/1) to give compound **18** (25.7 mg, 72% yield) as colorless oil. $[\alpha]^{20}_{D}$ = –35.7 (*c* = 0.54, $CHCl_3$); IR (ATR) ν 3296, 2925, 1647, 1542, 1441, 1405, 1255, 1117, 670, 626 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{67}H_{72}N_{14}NaO_{14}S^+$ ($M+Na^+$): 1352.4923. Found: 1352.4918. Although all signals were detected as broad peaks in the 1H NMR analysis ($CDCl_3$) probably due to the high molecular weight, an analyzable chart was obtained.

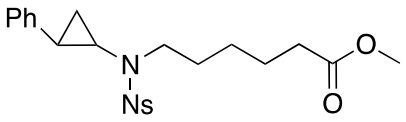
Synthesis of **3b**



To a stirred solution of **18** (12.0 mg, 0.00903 mmol), K_2CO_3 (5.0 mg, 0.0361 mmol) in DMF (0.3 mL) was added thiophenol (2.1 μL, 0.0226 mmol) and the resulting solution was stirred for 2 h at room temperature. The yellow solution was directly

purified by silica gel column chromatography (SiO_2, $CHCl_3$/MeOH = 20/1 to 10/1) to give compound **3b** (5.7 mg, 55% yield) as colorless solid. $[\alpha]^{20}_D$ = 32.1 (*c* = 0.57, $CHCl_3$); IR (ATR) ν 3284, 2924, 1643, 1531, 1440, 1404, 1251, 1204, 1107, 750 cm^{-1}; (+)-ESI- HRMS. Calcd for $C_{60}H_{69}N_{14}O_{10}^+$ ($M+H^+$): 1145.5316. Found: 1145.5296. Although all signals were detected as broad peaks in the 1H NMR analysis ($CDCl_3$) probably due to the high molecular weight, an analyzable chart was obtained.

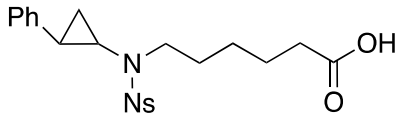
Synthesis of **22-COOMe**



22-COOMe

To a stirred solution of **rac-8** (191 mg, 0.6 mmol) in DMF (3 mL) at room temperature was added K_2CO_3 (165.8 mg, 1.2 mmol) and methyl 2-iodoacetate **21** (240.6 mg, 1.2 mmol). The resulting mixture was stirred at room temperature for 2 h. The reaction was diluted with ether and the mixture was washed with brine, and then dried over Na_2SO_4. After concentration in vacuo, the obtained residue was purified by silica gel column chromatography (SiO_2, *n*-hexane/EtOAc = 2/1) to give **22-COOMe** (254 mg, 95% yield) as white amorphous. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.14–1.43 (m, 4H), 1.60–1.72 (m, 4H), 2.09–2.14 (m, 1H),2.30 (t, *J* = 7.6 Hz, 2H), 2.63–2.67 (m, 1H), 3.29–3.50 (m, 2H), 3.66 (s, 3H), 6.96–6.98 (m, 2H), 7.17–7.28 (m, 3H), 7.75–7.71 (m, 3H), 8.00 (dd, *J* = 1.2 Hz, 8.4Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.5, 24.9, 26.2, 28.1, 33.8, 38.4, 124.0, 126.1 (2 C), 126.5, 128.5 (2 C), 131.4, 131.7, 133.1, 133.6, 139.3, 147.9, 173.9; IR (ATR) ν 2946, 1731, 1541, 1437, 1345, 1159, 1126, 1060, 851, 743, 698 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{22}H_{26}N_2O_7S^+$ ($M+Na^+$): 469.1404. Found: 469.1409.

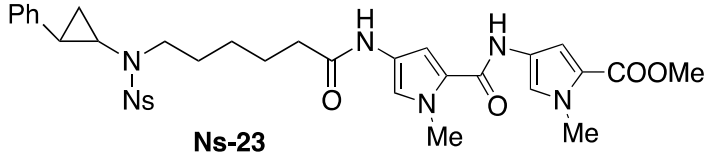
Synthesis of **22**



22

A solution of **22-COOMe** (226.8 mg, 0.5 mmol) and 1M LiOH (1 mL, 1 mmol) in MeOH (5 mL) was stirred at 50 °C. After 16 h, the resulting mixture was acidified with 1M aq. HCl and the mixture was extracted with $CHCl_3$ and then organic layer was dried over Na_2SO_4. After concentration in vacuo, The obtained residue was purified by silica gel column chromatography (SiO_2, *n*-hexane/EtOAc = 1/2) to give compound **22** (215 mg, 99% yield) as off-white amorphous. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.19–1.42 (m, 4H), 1.61–1.70 (m, 4H), 2.09–2.13 (m, 1H), 2.34 (t, *J* = 7.6 Hz, 2H), 2.63–2.67 (m, 1H), 3.33–3.49 (m, 2H), 6.96–6.98 (d, *J* = 7.2 Hz, 2H), 7.19–7.27 (m, 3H), 7.56–7.71 (m, 3H), 7.98–8.00 (dd, *J* = 0.8 Hz, 7.6Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.1, 24.1, 24.9, 26.0, 28.0, 33.7, 38.4, 50.1, 124.0, 126.0 (2 C), 126.4, 128.4 (2 C), 131.4, 131.6, 132.9, 133.7, 139.3, 147.9, 179.7; IR (ATR) ν 2929, 1705, 1541, 1439, 1345, 1158, 1125, 1079, 1060, 851, 743, 698 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{21}H_{24}N_2O_7S^+$ ($M+Na^+$): 455.1247. Found: 455.1263.

Synthesis of **Ns-23**

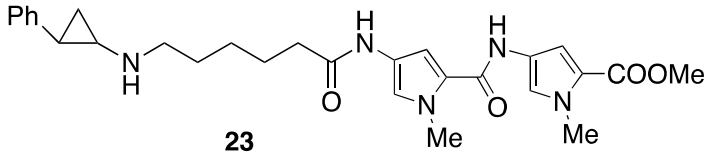


Ns-23

To a stirred solution of 6-((2-nitro-N-(2-phenylcyclopropyl) phenyl) sulfonamido) hexanoic acid **22** (129.7 mg, 0.3 mmol), 1-[Bis(dimethylamino) methylene] -1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxide hexafluorophosphate (HATU) (288.1 mg, 0.6 mmol) and N,N-diisopropylethylamine (0.31 mL, 1.8 mmol) in DMF (3 mL) at 0 °C was added the obtained amine hydrochloride $HCl \cdot NH_2$-PyPy-COOme (93.8 mg, 0.3 mmol). After 24 h, the reaction was quenched with sat. aq. $KHSO_4$ solution and diluted with AcOEt. The resulting mixture was washed with brine, and then dried over Na_2SO_4. After concentration under reduced pressure, the residue was recrystallized by NH-silica gel column chromatography from AcOEt-hexane (2/1) to give compound **Ns-23** as yellow amorphous (132 mg, 64% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.18 (dd, 6.4 Hz, 13.2 Hz, 1H), 1.26–1.35 (m, 3H), 1.66 (dd, J = 8 Hz, 15.6 Hz, 4H), 2.07–2.12(m, 1H), 2.27–2.31 (t, J = 7.2 Hz, 2H), 2.62 (t, J = 3.2 Hz, 1H), 3.28–3.47 (m, 2H), 3.76 (s, 3H), 3.83 (s, 3H), 3.86 (s, 3H), 6.72 (d, J = 1.2 Hz, 1H), 6.82 (d, J = 2 Hz, 1H), 7.13–7.27 (m, 3H), 7.38 (s, 1H), 7.54–7.71 (m, 4H), 7.94 (d, J = 7.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.9, 24.9, 25.1, 26.0, 28.1, 36.4, 36.5, 36.6, 38.4, 50.0, 50.9, 104.2, 108.5, 119.5, 120.8, 121.3, 122.0, 124.1, 126.0 (2 C), 126.4, 128.4 (2 C), 131.4, 131.5, 132.6, 133.8, 139.0, 147.7, 159.0, 161.5, 170.8;

IR (ATR) ν 2947, 1705, 1655, 1543, 1439, 1405, 1366, 1251, 1158, 1108, 1060 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{34}H_{38}N_6O_8S^+$ ($M+H^+$): 691.2545. Found: 691.2530.

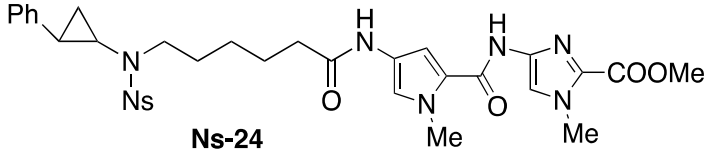
Synthesis of **23**



23

To a stirred solution of **Ns-23** (55.3 mg, 0.08 mmol), K_2CO_3 (44.2 mg, 0.32 mmol) in DMF (2 mL) was added thiophenol (20.5 μL, 0.2 mmol) and the resulting solution was stirred for 4 h at room temperature. The yellow solution was washed with brine, $NaHCO_3$ successively, then dried over Na_2SO_4. The condensed residue was purified by silica gel column chromatography (SiO_2, $CHCl_3$/MeOH = 10/1) to give compound **23** (24 mg, 60% yield) as yellow amorphous. IR (ATR) ν 2929, 1704, 1650, 1552, 1437, 1403, 1361, 1248, 1195, 1151, 1105, 1059, 822 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{28}H_{35}N_5O_4^+$ ($M+H^+$): 507.2846. Found: 507.2856. Although all signals were detected as broad peaks in the ^1H NMR analysis ($CDCl_3$) probably due to the high molecular weight, an analyzable chart was obtained.

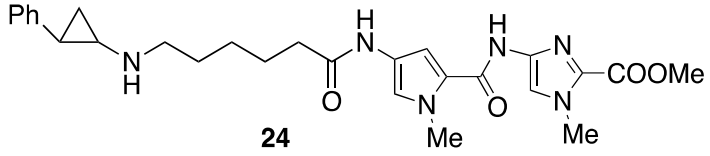
Synthesis of **Ns-24**.



Ns-24

To a stirred solution of 6-((2-nitro-N-(2-phenylcyclopropyl) phenyl) sulfonamido) hexanoic acid **22** (43.3 mg, 0.1 mmol), 1-[Bis(dimethylamino) methylene] -1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxide hexafluorophosphate (HATU) (76 mg, 0.2 mmol) and N,N-diisopropylethylamine (0.1 mL, 0.6 mmol) in DMF (1 mL) at 0 °C was added the obtained amine hydrochloride $HCl{\cdot}NH_2$-PyIm-COOme (31.4 mg, 0.1 mmol). After 24 h, the reaction was quenched with sat. aq. $KHSO_4$ solution and diluted with AcOEt. The resulting mixture was washed with brine, and then dried over Na_2SO_4. After concentration under reduced pressure, the residue was recrystallized by silica gel column chromatography (SiO_2, $CHCl_3$/MeOH = 20/1) twice to give compound **Ns-24** as yellow amorphous (104 mg, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.18–1.40 (m, 4H), 1.66–1.73 (m, 4H), 2.09–2.12(m, 1H), 2.27–2.31 (t, *J* = 7.2 Hz, 2H), 2.63–2.65 (t, *J* = 3.2 Hz, 1H), 3.32–3.45 (m, 2H), 3.90 (s, 3H), 3.91 (s, 3H), 3.99 (s, 3H), 6.55 (d, *J* = 2 Hz, 1H), 6.90 (d, *J* = 6.8 Hz, 2H), 7.18–7.28 (m, 3H), 7.36 (s, 1H), 7.55–7.67 (m, 4H), 7.98 (d, *J* = 5.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.9, 24.9, 25.0, 26.0, 28.0, 35.8, 36.3, 36.6, 38.4, 50.0, 52.1, 103.6, 114.7, 120.4, 121.6, 121.8, 124.0, 125.9 (2 C), 126.3, 128.3 (2 C), 131.0, 131.4, 132.7, 133.6, 137.2, 139.2, 147.8, 158.4, 158.9, 170.4; IR (ATR) ν 2949, 1713, 1656, 1540, 1461, 1436, 1403, 1370, 1277, 1202, 1158, 1125, 1060, 909 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{33}H_{37}N_7O_8S^+$ ($M+H^+$): 692.2497. Found: 692.2493.

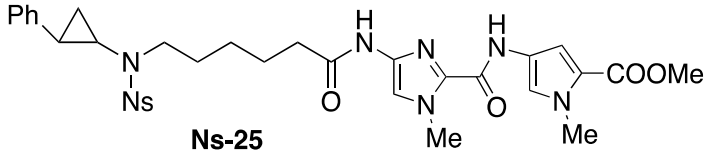
Synthesis of **24**.



24

To a stirred solution of **Ns-24** (55.3 mg, 0.08 mmol), K_2CO_3 (44.2 mg, 0.32 mmol) in DMF (2 mL) was added thiophenol (20.5 μL, 0.2 mmol) and the resulting solution was stirred for 4 h at room temperature. The yellow solution was washed with brine, $NaHCO_3$ successively, then dried over Na_2SO_4. The condensed residue was purified by silica gel column chromatography (SiO_2, $CHCl_3$/MeOH = 10/1) to give compound **24** (60 mg, quant.) as yellow amorphous. IR (ATR) ν 2925, 1715, 1658, 1542, 1461, 1438, 1404, 1372, 1278, 1127, 741, 698, 629 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{27}H_{34}N_6O_4^+$ ($M+H^+$): 507.2714. Found: 507.2709. Although all signals were detected as broad peaks in the ^1H NMR analysis ($CDCl_3$) probably due to the high molecular weight, an analyzable chart was obtained.

Synthesis of **Ns-25**.

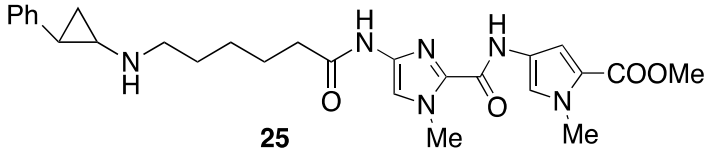


Ns-25

To a stirred solution of 6-((2-nitro-N-(2-phenylcyclopropyl) phenyl) sulfonamido) hexanoic acid **22** (51.9 mg, 0.12 mmol), 1-[Bis(dimethylamino) methylene] -1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxide hexafluorophosphate (HATU) (76 mg, 0.2 mmol) and N,N-diisopropylethylamine (0.034 mL, 0.2 mmol) in DMF (0.5 mL) at 0 °C was added the obtained amine NH_2-ImPy-COOMe (27.7 mg, 0.1 mmol). After 24 h, the reaction was diluted with AcOEt. The resulting mixture was washed with sat. aq. Na_2CO_3 aq, sat. aq. $KHSO_4$ solution, water, and brine, and then dried over Na_2SO_4. After concentration under reduced pressure, the residue was recrystallized by silica gel column chromatography ($CHCl_3$/MeOH, 50/1, NH-silica, then SiO_2, $CHCl_3$/MeOH = 40/1) to give compound **Ns-25** as yellow amorphous (28.9 mg, 42% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.16–1.45 (m, 4H), 1.66–1.77 (m, 4H), 2.09–2.13(m, 1H), 2.33–2.37 (t, *J* = 7.6 Hz, 2H), 2.64–2.67 (m, 1H), 3.31–3.52 (m, 2H), 3.81 (s, 3H), 3.90 (s, 3H), 4.04 (s, 3H), 6.82 (d, *J* = 2 Hz, 1H), 6.96 (d, *J* = 8 Hz, 2H), 7.17–7.25 (m, 3H), 7.40 (s, 1H), 7.53–7.71 (m, 4H), 8.00 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.1, 24.9, 24.9, 26.1, 28.1, 29.7, 35.6, 36.3, 36.8, 50.0, 51.1, 108.2, 114.2, 120.0, 121.6, 121.1, 124.0, 126.0 (2 C), 126.5, 128.5 (2 C), 131.5, 131.6, 133.0, 133.6, 135.8, 139.3, 147.9, 155.9, 161.5, 170.1; IR (ATR) ν 2926, 1672,

1540, 1447, 1371, 1256, 1197, 1160, 1118, 1061, 911, 733 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{33}H_{37}N_7O_8SNa^+$ ($M+Na^+$): 714.2317. Found: 714.2306.

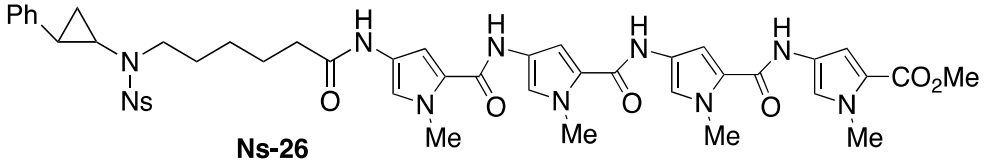
Synthesis of **25**.



25

To a stirred solution of **Ns-25** (12.4 mg, 0.018 mmol), K_2CO_3 (10 mg, 0.045 mmol) in DMF (0.45 mL) was added thiophenol (4.6 μL, 0.045 mmol) and the resulting solution was stirred for 4 h at room temperature. The yellow solution was washed with brine, extracted with AcOEt, then dried over Na_2SO_4. The condensed residue was purified by silica gel column chromatography (NH-silica, $CHCl_3$; then SiO_2, $CHCl_3$/MeOH = 20/1 to 10/1) to give compound **25** (2.9 mg, 32 %) as yellow amorphous. ^1H NMR (400 MHz, $CDCl_3$) δ 0.83–0.88 (m, 1H), 0.96–1.01 (m, 1H), 1.10–1.14 (m, 1H), 1.37–1.45 (m, 2H), 1.53–1.59 (m, 2H), 1.70–1.77 (m, 2H), 2.34–2.38 (m, 3H), 2.75–2.78 (m, 2H), 3.81 (s, 3H), 3.91 (s, 3H), 4.05 (s, 3H), 6.77 (d, J = 2.4 Hz, 1H), 7.043 (d, J = 8 Hz, 2H), 7.13–7.17 (m, 1H), 7.23–7.26 (m, 3H), 7.41 (s, 2H), 7.82 (s, 1H), 8.78 (s, 1H); IR (ATR) ν 2925, 2363, 1703, 1670, 1541, 1446, 1404, 1255, 1196, 1117, 913, 749, 698 cm^{-1}; (+)-ESI-HRMS. Calcd for $C_{27}H_{34}N_6O_4^+$ ($M+H^+$): 507.2714. Found: 507.2711.

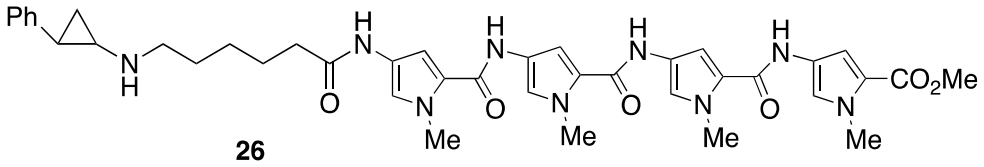
Synthesis of **Ns-26**



Ns-26

To a stirred solution of amine hydrochloride HCl·NH_2-PyIm-COOme (80.0 mg, 0.144 mmol), hexanoic acid **22** (56.2 mg, 0.13 mmol), and *i*-Pr_2NEt (45 μL, 0.26 mmol) in DMF (0.87 mL) at room temperature was added HATU (98.9 mg, 0.26 mmol) and the resulting mixture was stirred for 12 h at room temperature. After dilution with $CHCl_3$/*i*-PrOH (1/1), the resulting mixture was washed with sat. aq. Na_2CO_3, 1N HCl, H_2O, and brine and then dried over Na_2SO_4. After concentration in vacuo, the obtained residue was purified by silica gel column chromatography ($CHCl_3$/MeOH, 60/1, NH-silica) to give the target compound (76.8 mg, 63% yield) as yellow amorphous. Formation of the target molecule was confirmed by ESI Mass analysis: m/z: $[M+Na]^+$ 957.

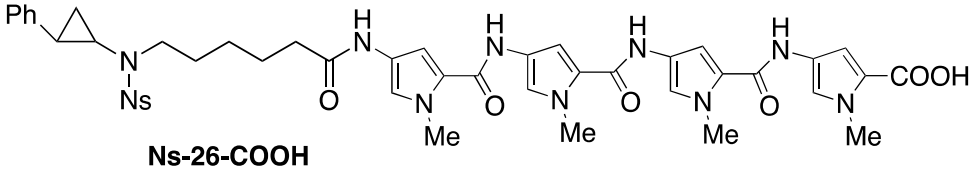
Synthesis of **26**



26

To a stirred suspension of Ns adduct **Ns-26** (12 mg, 0.0126 mmol) and K_2CO_3 (6.6 mg, 0.048 mmol) in DMF (0.3 mL) at room temperature was added thiophenol (3 mL, 0.03 mmol). The resulting mixture was stirred for 2 h at the same temperature. The reaction was directly purified by silica gel column chromatography ($CHCl_3$/MeOH, 50/1, NH-silica) to give the target compound **26**(5.8 mg, 61% yield) as yellow amorphous. IR (ATR) ν 2923, 2361, 1647, 1578, 1437, 1403, 1258, 1107, 736 cm^{-1}; (+)-ESI-TOF Mass. m/z: $[M+H]^+$ Calcd for $C_{40}H_{48}N_9O_6^+$ 750.3722; Found. 750.3737.

Synthesis of **Ns-26-COOH**

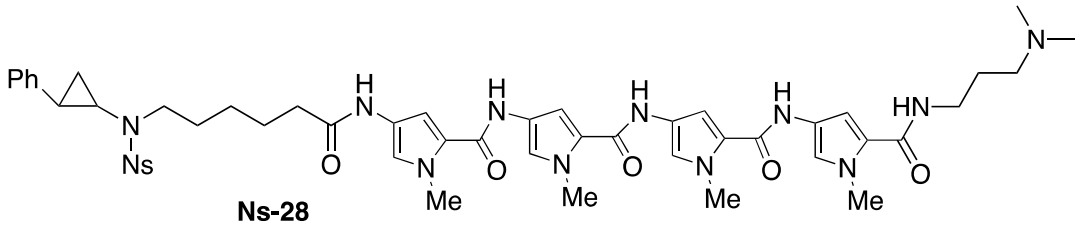


Ns-26-COOH

To a stirred solution of **Ns-26** (100 mg, 0.107 mmol) in MeOH/THF (1 mL/1 mL) at room temperature was added 1M aq. LiOH (0.2 mL, 0.2 mmol) and the resulting mixture was stirred at 40 °C. After 6 h, the reaction was cooled down to room

temperature and 1N HCl was added to the reaction. The mixture was extracted with $CHCl_3$ and the combined organic layers were dried over Na_2SO_4. After concentration in vacuo, the obtained residue was purified by silica gel column chromatography ($CHCl_3$/MeOH, 30/1 to 20/1, SiO_2, 2 times) to give the target compound **Ns-26-COOH** (28.9 mg, 31% yield). Starting material **Ns-26** was also recovered (40.8 mg, 41% yield). Formation of the target molecule was confirmed by ESI Mass analysis: m/z: $[M+Na]^+$ 943.

Synthesis of **Ns-28**

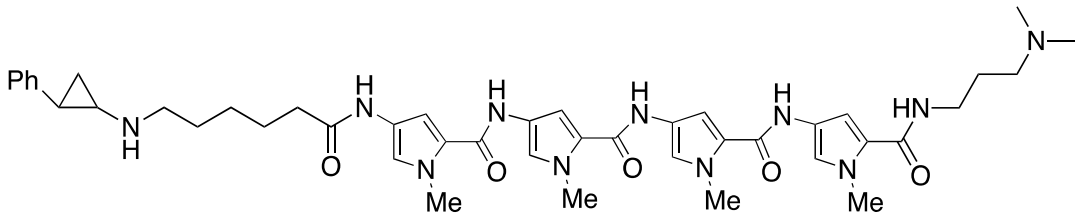


Ns-28

To a stirred solution of **Ns-26-COOH** (25.5 mg, 0.028 mmol) and HATU (21.3 mg, 0.056 mmol) in DMF (0.56 mL) at room temperature was added N,N-dimethylpropane 1,3-diamine (18 μL, 0.14 mmol), and the resulting mixture was stirred for 20 h at the same temperature. After dilution with AcOEt, the mixture was washed with sat. aq. Na_2CO_3, water, brine, and then dried over Na_2SO_4. After concentration in vacuo, the obtained residue was purified by silica gel column chromatography ($CHCl_3$/MeOH, 30/1 to 20/1, NH-silica) to give the target compound

Ns-28 (18.4 mg, 65% yield). Formation of the target molecule was confirmed by ESI Mass analysis: m/z: $[M+H]^+$ 1005.

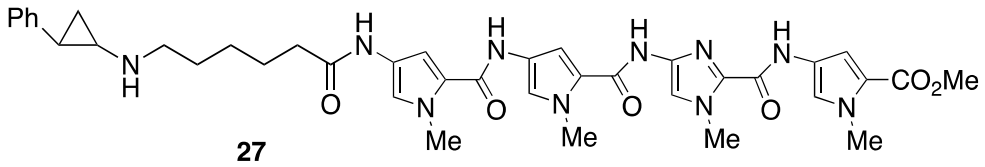
Synthesis of **28**



28

To a stirred suspension of Ns adduct **Ns-28** (11.8 mg, 0.0117 mmol) and K_2CO_3 (6.5 mg, 0.047 mmol) in DMF (0.3 mL) at room temperature was added thiophenol (3 mL, 0.035 mmol). The resulting mixture was stirred for 2 h at the same temperature. The reaction was directly purified by silica gel column chromatography ($CHCl_3$/MeOH, 50/1, NH-silica) to give the target compound **28**(2.6 mg, 27% yield) as yellow amorphous. Formation of the target molecule was confirmed by ESI Mass analysis: m/z: $[M+H]^+$ 820.

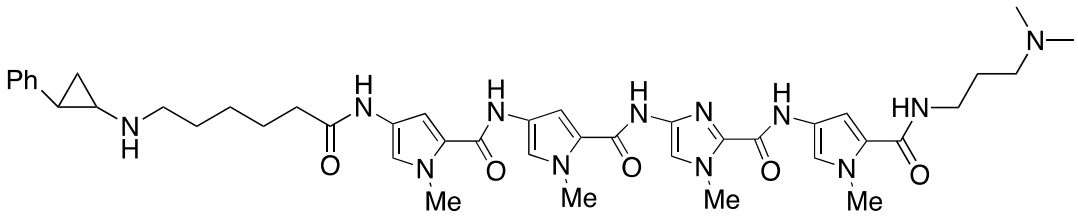
Synthesis of **27**



27

Compound **27** was prepared using the same method as that for compound **26**. Formation of the target molecule was confirmed by ESI Mass analysis: m/z: $[M+H]^+$ 1006.

Synthesis of **29**



29

Compound **29** was prepared using the same method as that for compound **28**. Formation of the target molecule was confirmed by ESI Mass analysis: m/z: $[M+H]^+$ 821.

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