Received: 7 December 2019 | Revised: 23 December 2019 | Accepted: 26 December 2019

DOI: 10.1002/chir.23179

WILEY

SPECIAL ISSUE ARTICLE

Chiral combinatorial preparation and biological evaluation of unique ceramides for inhibition of sphingomyelin synthase

Sajeer Koolath[1] | Yuta Murai[1,2] | Yoshiko Suga[2] | Kenji Monde[1,2]

[1]Graduate School of Life Science, Hokkaido University, Sapporo, Japan

[2]Faculty of Advanced Life Science, Hokkaido University, Sapporo, Japan

Correspondence
Yuta Murai and Kenji Monde, Graduate School of Life Science, Hokkaido University, Kita 21 Nishi 11, Sapporo 001-0021, Japan.
Email: ymurai@sci.hokudai.ac.jp; kmonde@sci.hokudai.ac.jp

Funding information
Hokkaido University; MEXT, Grant/Award Numbers: 17K19188, 18K14350, 19H02836; MEXT, Grant/Award Number: Doctoral program for Data Related Innovation Exper

Abstract

Enantiomers or diastereomers of chiral bioactive compounds often exhibit different biological and toxicological properties. Here, we report the efficient synthesis of four stereoisomers of sphingosine and derivatization of unique chiral ceramides through a combinatorial chemistry by solid-phase activated resin ester. In addition, to test the effectivity of stereochemistry of ceramide, we demonstrated a cell-based assay of sphingomyelin synthase inhibition in the presence ofchiral unique ceramides, which suggested that libraries of this sort will be a rich source of biologically active synthetic molecules.

KEYWORDS

chiral ceramide, chiral combinatorial chemistry, functional resin, inhibitor, sphingomyelin synthase

1 | INTRODUCTION

Chirality is a property of matter found throughout biological systems, from basic building blocks of biomolecules such as amino acids, carbohydrates, and lipids. In the field of new drug discovery and phramacotherapeutics, information on the stereoisomerism of a bioactive compound is very important because living systems are themselves chiral and each of the stereoisomers of chiral drugs show very different effects in vivo. In fact, over 50% of commercially available drugs are chiral substances,[1] and it is therefore also important to realize the safety and efficacy of chiral drugs in order to avoid drug-induced incidents such as the thalidomide disaster in Japan in the late 1950s. Over the past several decades, protein structure-based drug design in silico using structural information from solution-state NMR[2] and X-ray crystallography[3] have often provided detailed structural information on target biomolecules and novel chiral drug candidates. In the case of membrane proteins, such as a membrane receptor and enzyme, it is often more efficient to design drug candidates from original substrates due to difficulty in using NMR and X-ray methods.

In this study, we established new chiral inhibitors based on the ceramide backbone for the membrane protein sphingomyelin synthase (SMS) and evaluated their inhibitory efficacy. SMS catalyzes ceramide and phosphatidylcholine as substrates to produce sphingomyelin (SM) and diacylglycerol.[4] It has been shown that SMS modulates the levels of SM and other sphingolipids levels, thereby regulating membrane fluidity, ceramide-dependent apoptosis, lipid metabolism, and signal transduction.[5] In addition, it was reported that mice with SMS knockout or knockdown showed a decrease in plasma inflammatory cytokines[6] and showed resistance to the development of high-fat diet-induced obesity,[7] insulin resistance,[8] Alzheimer's disease,[9] and tumorigenesis.[10]

This work is dedicated to the memory of Prof Koji Nakanishi, outstanding leader in natural products chemistry and chemical biology.

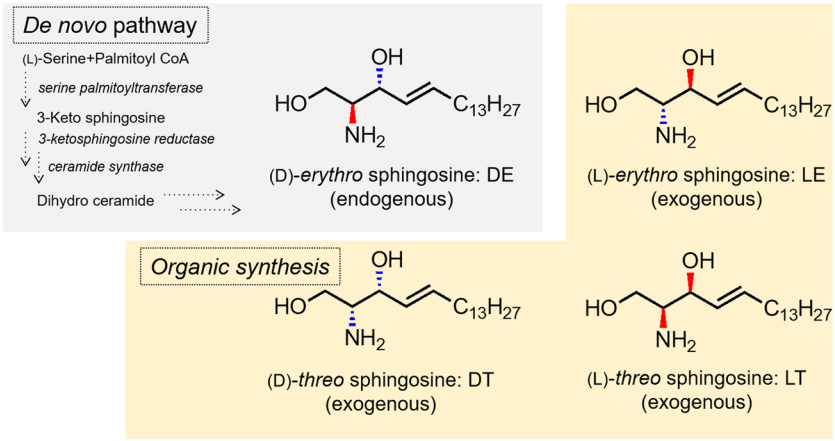

FIGURE 1 De novo pathway of endogenous (D)-*erythro* sphingosine and unnatural stereoisomers of sphingosine, (L)-*erythro*, (D)-*threo*, and (L)-*threo* sphingosines

Therefore, SMS inhibition is a novel therapeutic approach for these diseases.

Our strategy for creating SMS inhibitors is to design the structure of SMS inhibitors from ceramide as an original substrate. The most common ceramide in mammals consists of the amino alcohol sphingosine as a backbone moiety, which has two chiral centers at the C-2 and C-3 positions. Theoretically, there are four stereoisomers,

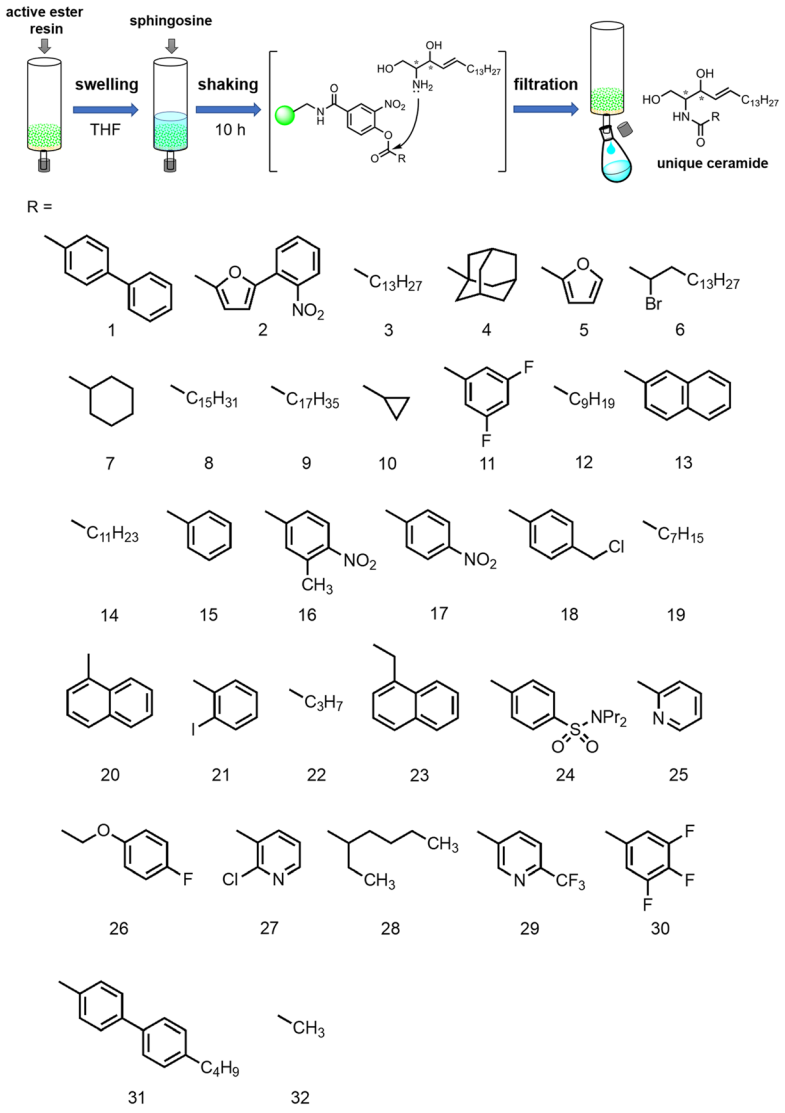

FIGURE 2 Efficient preparation of unique chiral ceramides by active ester resin

(D-*erythro*: DE), (L-*erythro*: LE), (D-*threo*: DT), and (L-*threo*: LT), in sphingosine, but only D-*erythro* type [(2*S*, 3*R*, 4*E*)-2-aminooctadec-4-ene-1,3-diol] has actually been found in mammals (Figure 1). Nakanishi and Berova et al determined their absolute configurations by sensitive and reliable stereochemical analysis utilizing high-performance liquid chromatography[11] and circular dichroism[12] techniques. We have also established an efficient methodology for those of sphingosine by using the vibrational circular dichroism technique,[13] which measures the differential absorption of left versus right circularly polarized infrared radiation. For investigating the relationship between stereochemistry and biological activity, the four isomers were synthesized in some studies and their biological activities were evaluated.[14] In addition to this, Inokuchi and Norman reported

TABLE 1 IC_{50} values are the means of each of the four unique chiral ceramides in separate determinations for SMS1 or SMS2 expressed in SMS1/2 double knockout mouse fibroblast cell lysate and were determined by using more than four concentrations of each inhibitor

	SMS1 (IC_{50}) mM				SMS2 (IC_{50}) mM			
entry (# of R)	DE	LE	LT	DT	DE	LE	LT	DT
1	20	>100	70	>100	7	15	20	30
2	3	15	7	30	0.2	1	0.3	0.4
3	50	>100	>100	>100	>100	>100	80	>100
4	4	50	25	>100	10	9	9	4
5	5	20	20	20	3	6	3	4
6	30	100	70	70	30	60	60	40
7	3	30	25	70	8	7	15	20
8	>100	>100	>100	>100	70	100	40	>100
9	>100	>100	>100	>100	70	80	90	>100
10	0.8	10	3.5	20	20	10	5	3
11	100	90	30	>100	30	25	10	10
12	50	60	25	>100	30	30	7	40
13	20	20	6	50	9	10	2	6
14	>100	>100	60	>100	>100	>100	50	>100
15	15	20	9	30	15	10	3	5
16	30	40	20	70	10	20	4	7
17	20	50	15	20	4	10	2	5
18	4	25	15	20	1.5	6	1.5	3
19	1	15	7	30	1	5	2	7
20	15	50	7	20	9	10	4	5
21	30	40	8	15	10	15	3	5
22	1	9	6	20	3	7	3	5
23	15	20	15	25	10	7	4	7
24	8	30	15	30	2	5	1.5	4
25	5	8	10	10	3	5	4	6
26	50	50	30	50	30	15	9	7
27	20	20	20	20	10	15	7	7
28	2	10	3	15	2	5	2	4
29	10	>100	40	80	3	50	15	40
30	50	100	50	100	30	30	20	30
31	30	100	40	100	30	50	40	70
32	16	30	20	30	12	20	10	20

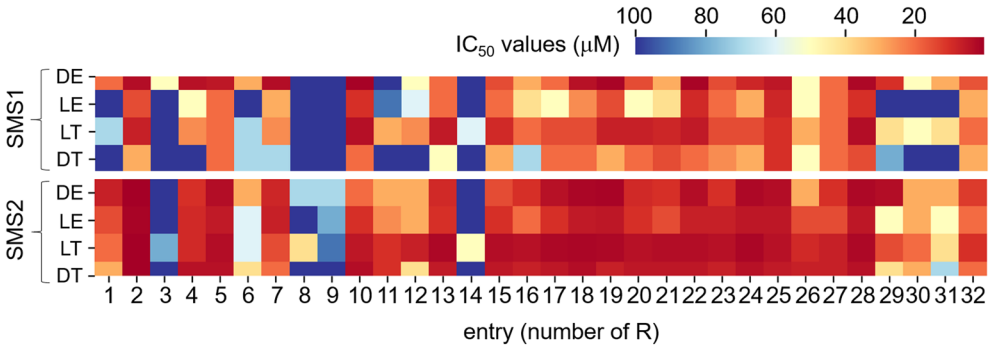

FIGURE 3 Heatmap analysis of IC_{50} values of each of the four unique chiral ceramides for SMS1 and SMS2

that an inhibitor, (D)-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), of glucosylceramide synthase suppressing GM3 in adipocytes showed a TNF-α-induced defect in insulin-dependent tyrosine phosphorylation of insulin receptor substrate-1.[15] Notably, the stereochemistry of active D-PDMP is different from that of endogenous ceramide. Stereospecific activity is a common feature for sphingolipids, suggesting that their targets also have specific spatial configurations; therefore, focusing on the stereochemistry of sphingolipids is a much attractive research point for revealing the structure-activity relationships of these pharmacologically active derivatives. However, a deeper understanding of the biological role of ceramide stereochemistry in human pathophysiology and the potential therapeutic use of SMS has still been veiled. In this study, to address these unclear points, we synthesized several unique ceramides of all of four stereoisomers by solid-phase activated resin ester and evaluated the importance of their stereochemistry efficacy towards lipid-metabolizing enzymes.

2 | RESULTS AND DISCUSSION

First, we started to synthesize all four stereoisomers of sphingosine according to established methods[16] from (L)- or (D)-serine with slight modification (Scheme S1). The specific rotations of the stereoisomers were measured, DE: $[\alpha]_D$ −4.4, LE: +4.6, DT: −2.9, and LT: +2.2 (c 1.0; $CHCl_3$). Next, to prepare unique ceramides efficiently through a combinatorial chemistry, we prepared several activated esters coupling with 32 commercially available carboxylic acids or acid chlorides and nitrophenol on polystyrene as reported by Chang et al[17] (Scheme S2). Subsequently, each of the four chiral shingosines was subjected to acylation with 32 kinds of activated ester resin, followed by filtration to generate 128 unique ceramides without starting material sphingosine and byproducts (Figure 2). The products and their purity were characterized by measuring electrospray ionization-mass spectrometry (ESI-MS) and ^{1}H-NMR of 40 randomly selected ceramides among the 128 ceramides (see the ESI).

Finally, to evaluate the inhibitory efficacy of the stereochemistry of each of the chiral four unique ceramides, we carried out cell-based assays for both of the isozymes SMS1 and SMS2[18] in the presence of the 128 unique ceramides. This assay uses a fluorescent (D)-*erythro* C6-NBD (4-nitrobenzo-2-oxa-1,3-diazole)-Cer analog as a substrate into SM moiety. As shown in Table 1, several ceramides showed relatively moderate inhibitory activities (IC_{50} 0.2-1 μM; Table 1) compared with those of our previously reported natural compounds.[19] Furthermore, to determine the differences in the inhibitory activities of the four stereoisomers, we represented the IC_{50} values as a heatmap graphical representation in which individual values contained in a matrix are displayed as colors. Although the natural stereoisomer is (D)-*erythro* type, the unnatural (L)-*threo* stereoisomer indicated that the ratio of ceramides showing strong inhibitory activities towards SMS1 and SMS2 is high (Figure 3). In addition to this, there might be a possibility that (D)-*erythro* ceramide analogue also become a substrate for SMS. Therefore, (L)-*threo* sphingolipid is the promising scaffold for the development of ceramide-based SMS inhibitors. In the present study, we demonstrated that the chiral chemistry of ceramide affected its inhibitory activity towards the lipid-metabolizing enzymes.

3 | CONCLUSION

We successfully synthesized 128 unique chiral ceramides through combinatorial chemistry by solid-phase activated ester without starting material sphingosine and byproducts. Additionally, we proved that these ceramides have good inhibitory activities (IC_{50} = 0.2-1 μM) for SMSs 1 and 2. Furthermore, according to heatmap analysis of IC_{50} values, we confirmed that most of the unnatural (L)-*threo* stereoisomer ceramide derivatives showed strong inhibitory activities towards SMS1 and SMS2, respectively, compared with the inhibitory activities of other stereoisomers. Therefore, to create competent bioactive

compounds based on the sphingolipid moiety, it is important to thoroughly consider the information on the stereoisomerism of them.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Shinji Nakaoka for helping with the heatmap analysis. This work was supported by a grant-in-aid for scientific research KAKENHI (grants 19H02836, 17K19188, and 18K14350) from the MEXT of Japan, the MEXT Doctoral program for Data Related Innovation Expert Hokkaido University (D-DRIVE-HU) program, and the Photo-excitonix Project in Hokkaido University. This work was inspired by JSPS Asian CORE Program "Asian Chemical Biology Initiative" and JSPS A3 Foresight Program. A. A. S. thanks the International Graduate Program (IGP), Hokkaido University, for the scholarship given.

ORCID

Sajeer Koolath https://orcid.org/0000-0002-3789-6547
Yuta Murai https://orcid.org/0000-0002-9115-6478
Kenji Monde https://orcid.org/0000-0002-1424-1054

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Koolath S, Murai Y, Suga Y, Monde K. Chiral combinatorial preparation and biological evaluation of unique ceramides for inhibition of sphingomyelin synthase. *Chirality*. 2020;1–6. https://doi.org/10.1002/chir.23179