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Synthesis and biological activity of optically active NCL-1, a lysine-specific demethylase 1 selective inhibitor

Daisuke Ogasawara [a], Takayoshi Suzuki [a,b,*], Koshiki Mino [c], Rie Ueda [a], Mohammed Naseer Ahmed Khan [a], Takuya Matsubara [a], Koichi Koseki [c], Makoto Hasegawa [c], Ryuzo Sasaki [d], Hidehiko Nakagawa [a], Tamio Mizukami [c,*], Naoki Miyata [a,*]

[a] Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan
[b] PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan
[c] Graduate School of Bio-Science, Nagahama Institute of Bio-Science and Technology, 1266 Tamura-cho, Nagahama, Shiga 526-0829, Japan
[d] Frontier Pharma Inc., 1281-8 Tamura-cho, Nagahama, Shiga 526-0829, Japan

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ABSTRACT

Optically active (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 were synthesized and evaluated for their lysine-specific demethylase 1 inhibitory activity and cell growth inhibitory activity. In enzyme assays, the (1*S*,2*R*)-isomer was approximately four times more potent than the (1*R*,2*S*)-isomer. In cell growth inhibition assays, the two isomers showed similar activity in HEK293 cells and SH-SY5Y cells, whereas the (1*S*,2*R*)-isomer showed approximately four times more potent activity than the (1*R*,2*S*)-isomer in HeLa cells.

1. Introduction

Reversible histone methylation, a process strictly controlled by histone methyltransferases and histone demethylases, plays a pivotal role in the regulation of epigenetic gene expression.[1] Lysine-specific demethylase 1 (LSD1) removes methyl groups from mono- and dimethylated Lys4 of histone H3 (H3K4me1/2) through flavin adenine dinucleotide (FAD)-dependent enzymatic oxidation.[2] In prostate cell lines, LSD1 also demethylates H3K9me1/2 and regulates androgen-receptor-mediated transcription.[3] It has recently been reported that H3K9me1/2 is demethylated by LSD1 in cells infected by herpes virus.[4] The targets of LSD1 regulatory demethylation are not limited to histone H3; LSD1 also demethylates p53, DNA methyltransferase 1, and E2F1, and regulates their cellular functions.[5]

Histone demethylation by LSD1 is suggested to be associated with certain disease states, such as cancer and herpes simplex.[1a,4,6] Therefore, LSD1 inhibitors are of interest not only as tools for elucidating in detail the biological functions of the enzyme, but also as potential therapeutic agents. Several LSD1 inhibitors, such as *trans*-2-phenylcyclopropylamine (PCPA) (Fig. 1), have been identified so far.[7] We have recently reported NCL-1 (Fig. 1) as the first cell-active LSD1 selective inhibitor.[8] We showed that NCL-1 potently inhibits LSD1 in preference to monoamine oxidase-A (MAO-A) and MAO-B, which are FAD-dependent oxidases, and represses cancer cell growth with the accumulation of H3K4me2. However, the NCL-1 we reported[8] is a mixture of (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 (Fig. 1). Recently, Mai and co-workers investigated the LSD1 and MAO inhibitory activities of chiral PCPAs and their derivatives.[7k] That report prompted us to examine the biological activity of optically active NCL-1.

Here we report the synthesis, absolute structure determination, LSD1 inhibition, and cell growth inhibition of (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1.

2. Chemistry

The routes used for the synthesis of (1*S*,2*R*)-NCL-1 are shown in Schemes 1–3. Scheme 1 shows the preparation of intermediate **4**. *N*-Boc homoserine **1** was treated with benzylamine in the presence of PyBOP to give amide **2**.

Deprotection of the Boc group of **2** afforded amine **3**. Amine **3** was treated with benzoic acid in the presence of PyBOP to give amide **4**.

Scheme 2 shows the preparation of optically active cyclopropanecarboxamides (1*S*,2*S*)-**10** and (1*R*,2*R*)-**10**. Carboxylic acid **5** was converted into methyl ester **6** under acidic conditions.

* Corresponding authors.
E-mail address: miyata-n@phar.nagoya-cu.ac.jp (N. Miyata).

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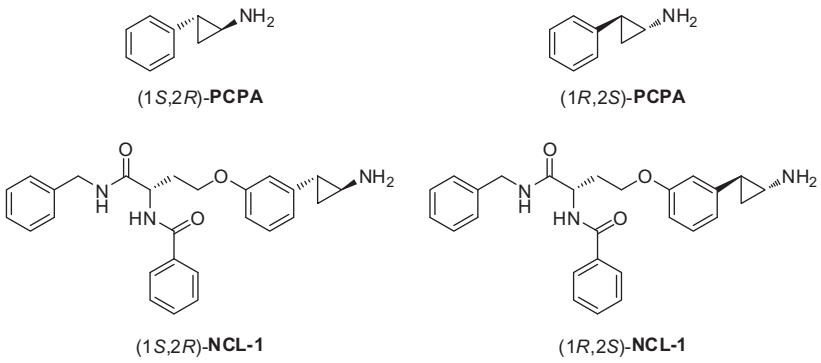
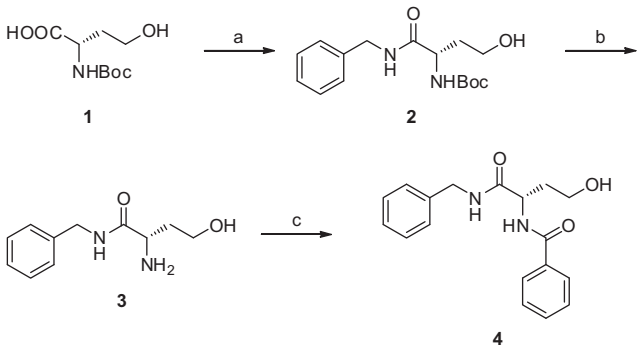


Figure 1. Structures of PCPA and NCL-1.



Scheme 1. Reagents and conditions: (a) benzylamine, PyBOP, Et_3N, DMF, rt; (b) HCl, 1,4-dioxane, CH_2Cl_2, rt; (c) benzoic acid, PyBOP, Et_3N, DMF, rt.

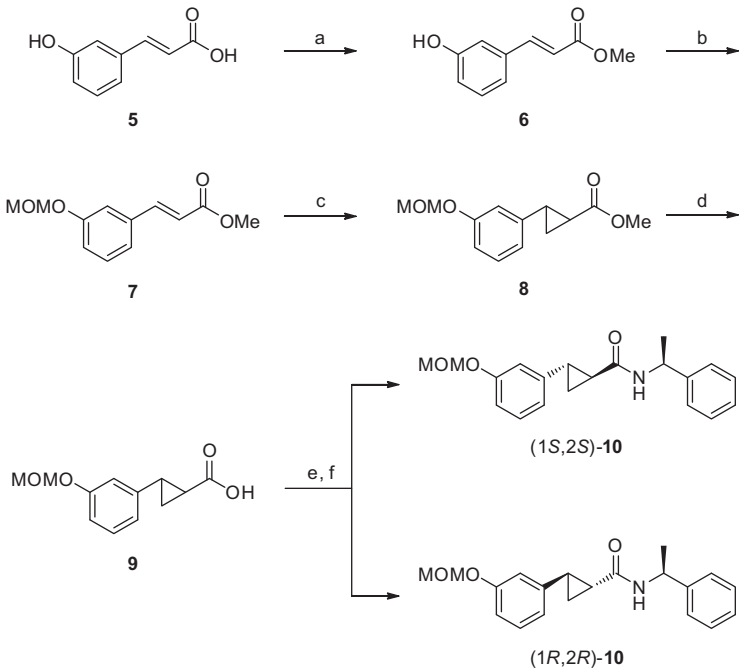
MOM protection of the hydroxyl group of **6** afforded compound **7**. Cyclopropanation of olefin **7** was achieved through the Corey–Chaykovsky reaction to give cyclopropane compound **8**. Methyl ester **8** was hydrolyzed under alkaline conditions to give carboxylic acid **9**. Carboxylic acid **9** was treated with (*S*)-phenylethylamine in the presence of EDCI and HOBt to give a mixture of (1*S*,2*S*)-**10** and (1*R*,2*R*)-**10**. The mixture was separated by column chromatography to give optically active (1*S*,2*S*)-**10** and (1*R*,2*R*)-**10**.

Scheme 3 shows the synthesis of (1*S*,2*R*)-NCL-1 from (1*S*,2*S*)-**10**. Amide (1*S*,2*S*)-**10** was hydrolyzed under alkaline conditions to give carboxylic acid (1*S*,2*S*)-**9**, which, in turn, was converted into amine (1*S*,2*R*)-**11** by the Curtius rearrangement. Deprotection of the Boc and MOM groups of compound (1*S*,2*R*)-**11** under acidic conditions and subsequent Boc protection afforded (1*S*,2*R*)-**12**. Compound (1*S*,2*R*)-**12** and compound **4** were subjected to the Mitsunobu reaction to give (1*S*,2*R*)-**13**. Deprotection of the Boc group of (1*S*,2*R*)-**13** under acidic conditions afforded (1*S*,2*R*)-NCL-1. (1*R*,2*S*)-NCL-1 was synthesized from (1*R*,2*R*)-**10** using a similar procedure to that described above. The absolute structure of optically active NCL-1 was determined as described below.

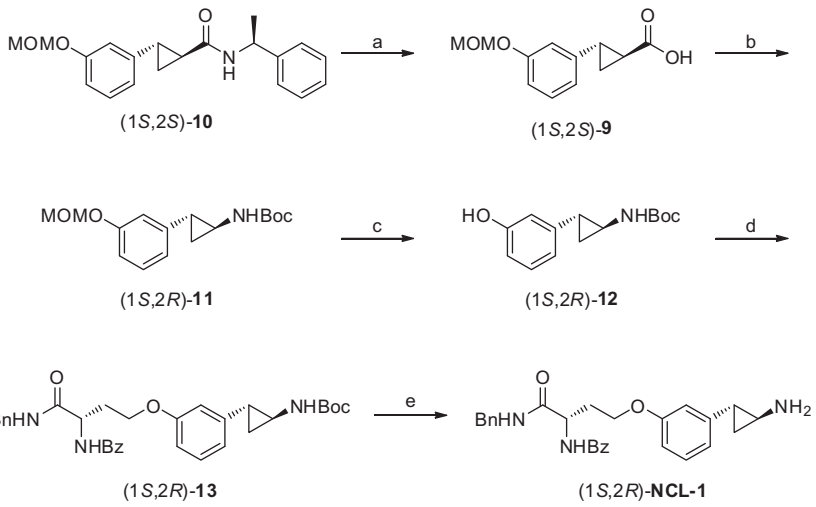
3. Results and discussion

3.1. Absolute structure determination

We initially tried to determine the absolute structure of prepared NCL-1 by X-ray crystal structure analysis. Although we



Scheme 2. Reagents and conditions: (a) concd H_2SO_4, MeOH, reflux; (b) MOMCl, K_2CO_3, acetone, rt; (c) $Me_3S(O)I$, NaH, DMSO, rt; (d) KOH, MeOH, rt; (e) (*S*)-phenylethylamine, EDCI, HOBt, DMF, rt; (f) column chromatography.



Scheme 3. Reagents and conditions: (a) KOH, hydrazine, ethylene glycol, 120 °C; (b) DPPA, Et_3N, *t*-BuOH, cyclohexane, reflux; (c) (i) HCl, AcOEt, CH_2Cl_2, rt; (ii) Boc_2O, Et_3N, 1,4-dioxane, H_2O, rt; (d) **4**, PPh_3, DIAD, THF, rt; (e) HCl, AcOEt, CH_2Cl_2, rt.

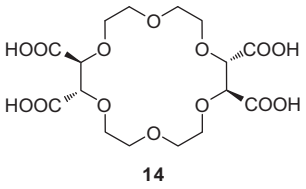


Figure 2. Structure of chiral shift reagent **14**.

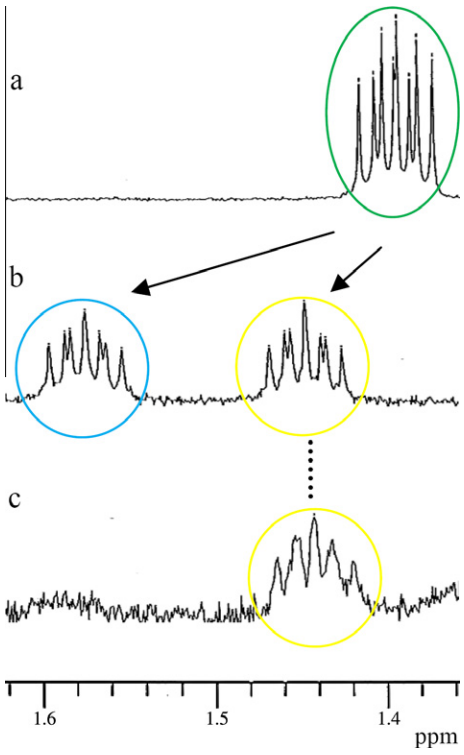


Figure 3. ^{1}H NMR spectra of PCPA. (a) PCPA (racemate), (b) PCPA (racemate) in the presence of **14**, (c) (1*S*,2*R*)-PCPA in the presence of **14**.

tried to crystallize optically active NCL-1 and several intermediates, we could not obtain crystals suitable for the X-ray crystal

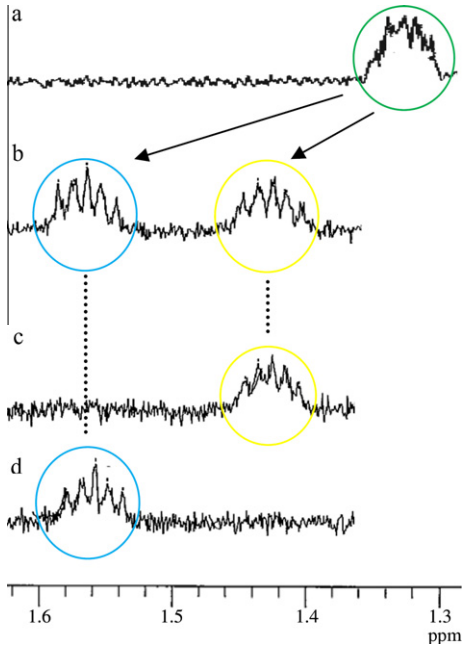


Figure 4. ^{1}H NMR spectra of NCL-1. (a) A mixture of (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1, (b) a mixture of (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 in the presence of **14**, (c) (1*S*,2*R*)-NCL-1 (prepared as shown in Scheme 3) in the presence of **14**, (d) (1*R*,2*S*)-NCL-1 (the other isomer) in the presence of **14**.

structure analysis. We then decided to determine its absolute structure by ^{1}H NMR analysis using a chiral shift reagent.

As NCL-1 has a PCPA moiety (Fig. 1), the absolute structure of the prepared NCL-1 can be determined by comparing its ^{1}H NMR spectrum with that of an enantiopure PCPA in the presence of a chiral shift reagent. To enantiodiscriminate the PCPA part of NCL-1 by ^{1}H NMR analysis, we used (18-crown-6)-2,3,11,12-tetracarboxylic acid **14** (Fig. 2) as the chiral shift reagent, because it has been used to discriminate the chirality of various amines.[9] We initially analyzed the ^{1}H NMR spectrum of PCPA (Fig. 3). Peaks assignable to the C2 methine proton of the cyclopropane of racemic PCPA were observed at 1.43–1.37 ppm (Fig. 3a). Multiple peaks of racemic PCPA were split into two sets of peaks by the addition of chiral shift reagent **14** (Fig. 3b). One of the two sets of peaks was identical to that of (1*S*,2*R*)-PCPA[7k] in the presence of compound **14** (Fig. 3c).

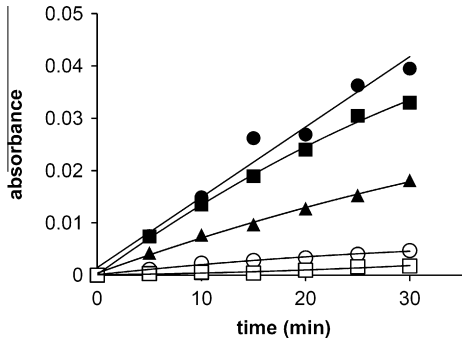


Figure 5. Steady-state progress curves obtained for the inactivation of LSD1 with 0 (●), 0.8 (■), 2.3 (▲), 6.9 (○), and 21 (□) μM (1*S*,2*R*)-NCL-1.

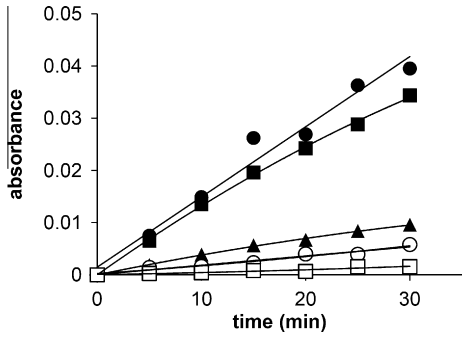


Figure 6. Steady-state progress curves obtained for the inactivation of LSD1 with 0 (●), 2.3 (■), 6.9 (▲), 20.6 (○), and 62 (□) μM (1*R*,2*S*)-NCL-1.

These results showed that the peaks at higher magnetic fields (1.47–1.43 ppm, yellow circle) are derived from (1*S*,2*R*)-PCPA and those at lower magnetic fields (1.60–1.55 ppm, blue circle) are derived from (1*R*,2*S*)-PCPA. Next, we analyzed the ^{1}H NMR spectrum of NCL-1 (Fig. 4). As is the case in PCPA (Fig. 3), the addition of chiral shift reagent **14** split the multiple peaks at 1.32–1.29 ppm of the C2 methine proton of a mixture of (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 (Fig. 4a) into two sets of peaks at 1.58–1.53 and 1.44–1.40 (Fig. 4b). One of the two sets of peaks was identical to that of one of the two optically active NCL-1s we prepared, as shown in Scheme 3 (Fig. 4c), and the other set of peaks corresponded to that of the other optically active NCL-1 (Fig. 4d). Comparing the peak shift pattern of NCL-1 (Fig. 4) with that of PCPA (Fig. 3), the optically active NCL-1 prepared as shown in Scheme 3 was determined to be (1*S*,2*R*)-NCL-1, and the other compound was determined to be (1*R*,2*S*)-NCL-1.

3.2. Enzyme assays

(1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 were assayed against LSD1 using a peroxidase-coupled assay as described before.[8] Because a mixture of (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 has been reported to be an irreversible inhibitor that covalently binds to FAD,[8] we initially determined whether the inhibition by (1*S*,2*R*)-NCL-1 or (1*R*,2*S*)-NCL-1 was time-dependent or not. The time course of product formation was monitored in the absence and presence of (1*S*,2*R*)-NCL-1 or (1*R*,2*S*)-NCL-1. As shown in Figures 5 and 6, both (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 were time-dependent inhibitors of LSD1, exhibiting nonlinear progress curves and reaching a plateau. These data suggest that both (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 are irreversible inhibitors.

Table 1
In vitro LSD1 inhibitory activities (IC_{50} values) of PCPA, (1*S*,2*R*)-NCL-1, and (1*R*,2*S*)-NCL-1[a]

Compound	PCPA	(1*S*,2*R*)-NCL-1	(1*R*,2*S*)-NCL-1
IC_{50} (μM)	21	1.6	6.7

[a] Values are means of at least three separate experiments.

Table 2
Growth inhibition of HeLa cells, HEK 293 cells, and SH-SY5Y cells by PCPA, (1*S*,2*R*)-NCL-1, and (1*R*,2*S*)-NCL-1[a]

Cell	PCPA GI_{50} (μM)	(1*S*,2*R*)-NCL-1 GI_{50} (μM)	(1*R*,2*S*)-NCL1 GI_{50} (μM)
HeLa	>500	20	74
HEK293	>500	17	25
SH-SY5Y	500	9.0	9.0

[a] Values are means of at least three separate experiments.

The IC_{50} values of (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 were also determined. We used the data after the product concentration reached a plateau for the determination of the IC_{50} values (Figs. 5 and 6). As expected from previous data, both (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 showed more potent activity than PCPA. (1*S*,2*R*)-NCL-1 was approximately four times more potent than (1*R*,2*S*)-NCL-1 (IC_{50} of (1*S*,2*R*)-NCL-1 = 1.6 μM; IC_{50} of (1*R*,2*S*)-NCL-1 = 6.7 μM) (Table 1).

3.3. Cell growth inhibition assays

We reported that a mixture of (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 exerts cell growth inhibitory activity with the accumulation of H3K4me2.[8] Therefore, (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 were evaluated for their ability to inhibit the growth of three cell lines (Table 2). Interestingly, (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 displayed similar activities against HEK293 cells and SH-SY5Y cells, whereas (1*S*,2*R*)-NCL-1 showed approximately four times more potent inhibitory activity against HeLa cells than (1*R*,2*S*)-NCL-1. The reasons for the differences in results between enzyme assays and cell-based assays and between HeLa cell line and the other two cell lines are not clear. Nevertheless, we surmise that the differences may be due to the sensitivity to LSD1 that complexed with proteins in the cells. LSD1 has been reported to bind to such proteins as HDAC1, CoREST, and TLX.[10] The activity of NCL-1 may depend on which protein LSD1 binds to in cells. The binding of LSD1 to the proteins may alter the structure of the active site and affect the binding of NCL-1 to LSD1.

4. Conclusion

We have synthesized optically active (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 and evaluated their LSD1 inhibitory activity and cell growth inhibitory activity. In the enzyme assays, (1*S*,2*R*)-NCL-1 was more potent than (1*R*,2*S*)-NCL-1. In the cell growth inhibition assays, (1*S*,2*R*)-NCL-1 and (1*R*,2*S*)-NCL-1 showed similar activity in HEK293 cells and SH-SY5Y cells, whereas (1*S*,2*R*)-NCL-1 showed approximately four times more potent activity in HeLa cells than (1*R*,2*S*)-NCL-1. These findings are expected to contribute to the development of PCPA-based LSD1 inhibitors.

5. Experimental section

5.1. Chemistry

Melting points were determined with a Yanagimoto micro melting point apparatus or a Büchi 545 melting point apparatus and were left uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JEOL JNM-LA500 spectrome-

ter in solvent as indicated. Chemical shifts (δ) were reported in parts per million relative to the internal standard, tetramethylsilane. Elemental analysis was performed with a Yanaco CHN CORDER NT-5 analyzer, and all values were within ±0.4% of the calculated values. Fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMS-SX102A mass spectrometer. Reagents and solvents were purchased from Aldrich, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, and Kanto Kagaku and used without purification. Flash column chromatography was performed using Silica Gel 60 (particle size 0.046–0.063 mm) supplied by Merck.

5.1.1. (*S*)-1-Benzylcarbamoyl-3-hydroxypropyl *tert*-butylcarbamate (2)

A solution of **1** (1.42 g, 6.50 mmol), PyBOP (3.38 g, 6.50 mmol), Et_3N (1.8 mL, 12.9 mmol), and benzylamine (0.85 mL, 7.77 mmol) in DMF (13.5 mL) was stirred for 14 h at room temperature. The reaction mixture was poured into water and extracted with $CHCl_3$. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, concentration in vacuo, and purification by silica gel flash column chromatography (AcOEt/*n*-hexane = 2:1) gave 1.24 g (62%) of **2** as a white solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.35–7.32 (2H, m), 7.29–7.25 (3H, m), 6.71 (1H, br s), 5.58 (1H, d, J = 7.3 Hz), 4.45 (2H, s), 4.35 (1H, s), 3.71 (2H, s), 3.27 (1H, br s), 2.04–1.98 (1H, m), 1.72–1.85 (1H, m), 1.43 (9H, s).

5.1.2. (*S*)-2-Amino-*N*-benzyl-4-hydroxybutanamide hydro- chloride (3·HCl)

To a solution of **2** (1.24 g, 3.89 mmol) obtained above in CH_2Cl_2 (13 mL) was added 4 N HCl in 1,4-dioxane (20 mL) and the mixture was stirred for 3 h at room temperature. Concentration in vacuo gave 951 mg (q. y.) of **3** as a white solid; ^{1}H NMR (CD_3OD, 500 MHz, δ; ppm) 7.44–7.41 (2H, m), 7.34–7.30 (3H, m), 7.27 (1H, br s), 4.43 (2H, s), 3.94 (1H, d, J = 7.0 Hz), 2.03 (2H, s).

5.1.3. (*S*)-*N*-(1-Benzylcarbamoyl-3-hydroxypropyl)benzamide (4)

A solution of **3** (951 mg, 3.89 mmol) obtained above, PyBOP (3.06 g, 5.88 mmol), benzoic acid (598 mg, 4.90 mmol), and Et_3N (1.3 mL, 9.80 mmol) in DMF (10 mL) was stirred for 12 h at room temperature. The reaction mixture was poured into water and extracted with CH_2Cl_2. The organic layer was washed with water and brine and dried over Na_2SO_4. Filtration, concentration in vacuo, and purification by silica gel flash column chromatography (AcOEt/*n*-hexane = 2:1) gave 1.00 g (65%) of **4** as a white solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.83 (2H, d, J = 7.0 Hz), 7.56–7.53 (1H, m), 7.47–7.44 (2H, m), 7.36–7.33 (2H, m), 7.30–7.26 (3H, m), 6.62 (1H, s), 4.86–4.81 (1H, m), 4.54–4.45 (2H, m), 3.78–3.75 (2H, m), 3.63–3.61 (1H, m), 2.17–2.09 (1H, m), 1.94–1.89 (1H, m).

5.1.4. Methyl *trans*-3-(3-hydroxylphenyl)propenoate (6)

To a solution of **5** (25.0 g, 0.152 mol) in MeOH (82 mL) was added concentrated H_2SO_4 (2.5 mL) at 0 °C and the mixture was stirred at reflux temperature for 6 h. The reaction mixture was concentrated in vacuo and the residue was dissolved in AcOEt. The organic layer was washed with water, saturated $NaHCO_3$, and brine, and dried over Na_2SO_4. Filtration and concentration in vacuo gave 26.3 g (97%) of **6** as a white solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.60 (1H, d, J = 15.8 Hz), 7.21 (1H, t, J = 8.0 Hz), 7.04 (1H, d, J = 8.0 Hz), 6.98 (1H, t, J = 1.8 Hz), 6.82 (1H, dd, J = 8.0, 1.8 Hz), 6.44 (1H, d, J = 15.8 Hz), 3.77 (3H, s).

5.1.5. Methyl *trans*-3-(3-methoxymethoxyphenyl)propenoate (7)

To a solution of **6** (26.3 g, 0.148 mmol) obtained above in acetone (220 mL) was added dry K_2CO_3 (40.8 g, 0.295 mmol), and the solution was stirred at room temperature for 15 min. Then, MOMCl (11.2 mL, 0.1476 mmol) was added slowly with a syringe through a septum. After complete addition, the reaction mixture was stirred at room temperature for 12 h. Filtration, concentration in vacuo, and purification by silica gel flash column chromatography (AcOEt/*n*-hexane = 1:10) gave 27.5 g (84%) of **7** as a colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.66 (1H, d, J = 16.2 Hz), 7.30 (1H, t, J = 8.0 Hz), 7.20 (1H, s), 7.17 (1H, d, J = 8.0 Hz), 7.07 (1H, dd, J = 8.0, 2.4 Hz), 6.43 (1H, d, J = 16.0 Hz), 5.19 (2H, s), 3.81 (3H, s), 3.49 (3H, s).

5.1.6. Methyl *trans*-2-(3-methoxymethoxyphenyl)cyclopropanecarboxylate (8)

To a mixture of NaH (6.43 g, 0.161 mol, 60 wt % in mineral oil) and trimethylsulfoxonium iodide (35.4 g, 0.161 mol) was added dropwise dry DMSO (140 mL) with stirring at room temperature. The reaction mixture was stirred at room temperature for 1 h. To the mixture was added dropwise a solution of **7** (27.5 g, 0.124 mol) obtained above in dry DMSO (140 mL). The mixture was stirred at room temperature for 6 h, acidified to pH 4 by adding 10% citric acid, and extracted with AcOEt. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, concentration in vacuo, and purification by silica gel flash column chromatography (AcOEt/*n*-hexane = 1:10) gave 4.63 g (16%) of **8** as a colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.19 (1H, t, J = 8.0 Hz), 6.89 (1H, dd, J = 8.0, 2.5 Hz), 6.78 (1H, t, J = 2.5 Hz), 6.74 (1H, d, J = 8.0 Hz), 5.16 (2H, s), 3.71 (3H, s), 3.47 (3H, s), 2.51–2.49 (1H, m), 1.92–1.89 (1H, m), 1.61–1.57 (1H, m), 1.34–1.31 (1H, m).

5.1.7. 2-(3-Methoxymethoxyphenyl)cyclopropanecarboxylic acid (9)

To a solution of **8** (4.60 g, 19.5 mmol) obtained above in MeOH (38 mL) was added a solution of KOH (10.9 g, 194 mmol) in MeOH (89 mL) at 0 °C. The reaction mixture was stirred for 5 h at room temperature. The solution was concentrated in vacuo and the residue was suspended in water and extracted with CH_2Cl_2. The organic layer was discarded and the aqueous phase was acidified with 2 N aqueous HCl to pH 1 and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4. Filtration and concentration in vacuo gave 3.70 g (86%) of **9** as a colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.20 (1H, t, J = 8.0 Hz), 6.90 (1H, dd, J = 8.0, 1.9 Hz), 6.79 (1H, t, J = 1.9 Hz), 6.75 (1H, d, J = 8.0 Hz), 5.16 (2H, s), 3.48 (3H, s), 2.60–2.56 (1H, m), 1.92–1.89 (1H, m), 1.65 (1H, quintet, J = 5.0 Hz), 1.42–1.38 (1H, m).

5.1.8. (1*S*,2*S*)-2-(3-(Methoxymethoxy)phenyl)-*N*-((*S*)-1-phenylethyl) cyclopropanecarboxamide ((1*S*,2*S*)-10) and (1*R*,2*R*)-2-(3-(methoxymethoxy)phenyl)-*N*-((*S*)-1-phenylethyl) cyclopropanecarboxamide ((1*R*,2*R*)-10)

To a solution of **9** (3.6 g, 16.2 mmol) obtained above in DMF (53 mL) were added EDCI (4.66 g, 24.3 mmol), HOBt·H_2O (3.72 g, 24.3 mmol), and (*S*)-1-phenylethylamine (3.1 mL, 24.3 mmol). The reaction mixture was stirred for 13 h at room temperature. The mixture was poured into water and extracted with $CHCl_3$. The organic layer was washed with water and brine, and dried over Na_2SO_4. Filtration, concentration in vacuo, and purification by silica gel flash column chromatography (AcOEt/*n*-hexane = 1:4) gave 1.35 g (51%) of (1*S*,2*S*)-**10** as a white solid and 1.33 g (50%) of (1*R*,2*R*)-**10** as a white solid. (1*S*,2*S*)-**10**: ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.34–7.33 (4H, m), 7.28–7.25 (1H, m), 7.18 (1H, t, J = 8.0 Hz), 6.87 (1H, dd, J = 8.2, 2.4 Hz), 6.76–6.73 (2H, m), 5.85 (1H, br s), 5.15 (2H, s), 3.47 (3H, s), 2.50–2.45 (1H, m), 1.61–1.57 (1H, m), 1.59 (3H, s), 1.50–1.49 (2H, m), 1.21–1.18 (1H, m). (1*R*,2*R*)-**10**: ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.35–7.31 (4H, m), 7.28–7.24 (1H, m), 7.17 (1H, t, J = 8.0 Hz), 6.86 (1H, dd, J = 8.2, 2.4 Hz), 6.73–6.72 (1H, m), 6.71 (1H, s), 5.84

(1H, br s), 5.14 (2H, s), 3.47 (3H, s), 2.47–2.44 (1H, m), 1.63–1.57 (1H, m), 1.58 (3H, s), 1.52–1.50 (2H, m), 1.23–1.22 (1H, m).

5.1.9. (1*S*,2*S*)-2-(3-(Methoxymethoxy)phenyl)cyclopropanecarboxylic acid ((1*S*,2*S*)-9)

To a suspension of (1*S*,2*S*)-**10** (1.35 g, 4.15 mmol) obtained above in ethylene glycol (21 mL) were added hydrazine monohydrate (1.0 mL, 16.60 mmol) and KOH (1.40 g, 25.0 mmol), and the mixture was stirred for 7 days at 120 °C. The reaction mixture was poured into 2 N aqueous HCl and extracted with $CHCl_3$. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, concentration in vacuo, and purification by silica gel flash column chromatography (AcOEt/*n*-hexane = 1:3) gave 531 mg (58%) of (1*S*,2*S*)-**9** as a yellow solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.20 (1H, t, J = 8.0 Hz), 6.90 (1H, dd, J = 8.0, 2.0 Hz), 6.79 (1H, s), 6.75 (1H, d, J = 8.0 Hz), 5.16 (2H, s), 3.48 (3H, s), 2.60–2.55 (1H, m), 1.93–1.90 (1H, m), 1.66–1.63 (1H, m), 1.42–1.38 (1H, m).

5.1.10. *tert*-Butyl ((1*S*,2*R*)-2-(3-(methoxymethoxy)phenyl)cyclopropyl)carbamate ((1*S*,2*R*)-11)

To a solution of (1*S*,2*S*)-**9** (531 mg, 2.39 mmol) obtained above in dry cyclohexane (22 mL) were added DPPA (0.6 mL, 2.88 mmol), Et_3N (0.4 mL, 2.87 mmol), and dry *tert*-butanol (9.1 mL, 95.1 mmol) at 0 °C under nitrogen atmosphere. The reaction mixture was refluxed for 12 h and then cooled to room temperature. Concentration in vacuo and purification by silica gel flash column chromatography (AcOEt/*n*-hexane = 1:7) gave 346 mg (49%) of (1*S*,2*R*)-**11** as a yellow solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.17 (1H, t, J = 8.0 Hz), 6.85 (1H, dd, J = 8.0, 1.5 Hz), 6.77 (1H, s), 6.76 (1H, s), 5.15 (2H, s), 3.47 (3H, s), 2.75–2.72 (1H, m), 2.05–1.99 (1H, m), 1.45 (9H, s), 1.17–1.15 (2H, m).

5.1.11. *tert*-Butyl ((1*S*,2*R*)-2-(3-hydroxyphenyl)cyclopropyl)carbamate ((1*S*,2*R*)-12)

To a solution of (1*S*,2*R*)-**11** (346 mg, 1.18 mmol) obtained above in CH_2Cl_2 (4.0 mL) was added 4 N HCl in AcOEt (4.5 mL, 18.0 mmol), and the solution was stirred for 2.5 h at room temperature. The solvent was removed by evaporation and the residue was dissolved in 1,4-dioxane (2.2 mL) and H_2O (2.2 mL). To the solution were added Et_3N (1.3 mL, 9.35 mmol) and Boc_2O (0.3 mL, 1.31 mmol), and the reaction mixture was stirred at room temperature. After 9 h, the reaction mixture was poured into aqueous 10% citric acid and extracted with AcOEt. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, concentration in vacuo, and purification by silica gel flash column chromatography (AcOEt/*n*-hexane = 1:5) gave 214 mg (73%) of (1*S*,2*R*)-**12** as a colorless oil; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.12 (1H, d, J = 8.0 Hz), 6.70 (1H, d, J = 8.0 Hz), 6.64 (1H, d, J = 8.0 Hz), 6.61 (1H, s), 4.82 (1H, br s), 4.73 (1H, br s), 2.74–2.69 (1H, m), 2.01–1.97 (1H, m), 1.45 (9H, s), 1.16–1.12 (2H, m).

5.1.12. *tert*-Butyl ((1*S*,2*R*)-2-(3-((*S*)-3-benzamido-4-(benzylamino)-4-oxobutoxy)phenyl)cyclopropyl)carbamate ((1*S*,2*R*)-13)

To a solution of (1*S*,2*R*)-**12** (214 mg, 0.858 mmol), **4** (536 mg, 1.72 mmol), and PPh_3 (675 mg, 2.57 mmol) in dry THF (2.0 mL) was added DIAD (1.3 mL, 2.57 mmol, 40% in toluene) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then at room temperature for 9 h. Concentration in vacuo and purification by silica gel flash column chromatography (AcOEt/*n*-hexane = 1:2) gave 131 mg (28%) of (1*S*,2*R*)-**13** as a white solid; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.82 (2H, d, J = 8.5 Hz), 7.52 (1H, t, J = 8.5 Hz), 7.46 (2H, t, J = 8.5 Hz), 7.31–7.21 (5H, m), 7.16 (1H, t, J = 8.0 Hz), 6.92 (1H, s), 6.75 (1H, d, J = 8.0 Hz), 6.63 (1H, d, J = 8.0 Hz), 4.88 (1H, q, J = 6.0 Hz), 4.47 (2H, d, J = 6.0 Hz), 4.32–4.29 (1H, m), 4.13–4.09 (1H, m), 2.69–2.68 (1H, m), 2.43–2.37 (2H, m), 1.99–1.97 (1H, m), 1.45 (9H, s), 1.14–1.10 (2H, m).

5.1.13. (1*S*,2*R*)-2-(3-((*S*)-3-Benzamido-4-(benzylamino)-4-oxobutoxy)phenyl)cyclopropylamine hydrochloride ((1*S*,2*R*)-NCL-1·HCl)

To a solution of (1*S*,2*R*)-**13** (131 mg, 0.241 mmol) obtained above in CH_2Cl_2 (1.6 mL) was added 4 N HCl in AcOEt (2.8 mL, 11.2 mmol), and the mixture was stirred for 7 h at room temperature. The solvent was removed by evaporation and the residue was recrystallized from $CHCl_3$ and MeOH to give 70 mg (61%) of (1*S*,2*R*)-NCL-1·HCl as colorless crystals: >99% ee (^{1}H NMR), mp 208–209 °C; ^{1}H NMR (CD_3OD, 500 MHz, δ; ppm) 7.84 (2H, d, J = 8.5 Hz), 7.54 (1H, t, J = 8.5 Hz), 7.46 (2H, t, J = 8.5 Hz), 7.26–7.17 (6H, m), 6.77 (1H, d, J = 8.0 Hz), 6.72 (1H, d, J = 8.0 Hz), 6.67 (1H, d, J = 2.0 Hz), 4.40 (2H, m), 4.08 (2H, t, J = 6.0 Hz), 2.78–2.76 (1H, m), 2.42–2.40 (1H, m), 2.29–2.24 (2H, m), 1.37–1.31 (1H, m), 1.28–1.24 (1H, m); ^{13}C NMR (CD_3OD, 125 MHz, δ; ppm) 173.94, 170.34, 160.52, 141.32, 139.79, 135.21, 132.96, 130.81, 129.57, 129.53, 128.54, 128.45, 128.19, 120.00, 114.14, 113.80, 65.91, 53.13, 44.15, 32.66, 31.95, 22.57, 13.83; MS (FAB) m/z 444 (M^+). Anal. Calcd for $C_{27}H_{30}ClN_3O_3 \cdot 1/2H_2O$: C, 66.32; H, 6.39; N, 8.59. Found: C, 66.33; H, 6.16; N, 8.58.

5.1.14. (1*R*,2*S*)-2-(3-((*S*)-3-Benzamido-4-(benzylamino)-4-oxobutoxy)phenyl)cyclopropylamine hydrochloride ((1*R*,2*S*)-NCL-1·HCl)

(1*R*,2*S*)-NCL-1·HCl was synthesized from (1*R*,2*R*)-**10** using the procedure described for (1*S*,2*R*)-NCL-1·HCl as colorless crystals: >99% ee (^{1}H NMR), mp 138–142 °C; ^{1}H NMR (CD_3OD, 500 MHz, δ; ppm) 7.84 (2H, d, J = 8.5 Hz), 7.54 (1H, t, J = 8.5 Hz), 7.46 (2H, t, J = 8.5 Hz), 7.26–7.18 (6H, m), 6.77 (1H, d, J = 8.0 Hz), 6.72 (1H, d, J = 8.0 Hz), 6.67 (1H, s), 4.40 (2H, m), 4.08 (2H, t, J = 6.0 Hz), 2.80–2.76 (1H, m), 2.44–2.39 (1H, m), 2.29–2.24 (1H, m), 1.36–1.35 (1H, m), 1.28–1.25 (1H, m); ^{13}C NMR (CD_3OD, 125 MHz, δ; ppm) 173.93, 170.34, 160.52, 141.32, 139.79, 135.21, 132.96, 130.80, 129.57, 129.53, 128.54, 128.45, 128.19, 120.01, 114.04, 113.80, 65.87, 53.11, 44.15, 32.67, 31.95, 22.57, 13.85; MS (FAB) m/z 444 (M^+). Anal. Calcd for $C_{27}H_{30}ClN_3O_3 \cdot 7/6H_2O$: C, 64.73; H, 6.50; N, 8.39. Found: C, 64.52; H, 6.13; N, 8.73.

5.2. Biology

5.2.1. Assay for LSD1 inhibitory activity

The assay for LSD1 inhibitory activity was conducted at 25 °C using the peroxidase-coupled method as described previously.[8] The chemically synthesized peptide consisted of the first 21 amino acid residues of histone H3, incorporating dimethylated lysine at position 4 (H3K4me2 peptide) (Sigma–Aldrich), and was used as the substrate of LSD1. The reaction mixture contained 50 mM HEPES–NaOH, pH 7.5, 0.1 mM 4-aminoantipyrine, 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 5.5 units/mL horseradish peroxidase, 20 μM H3K4me2 peptide, and an appropriate amount of LSD1. To assess the inhibitory effect of the test compounds on LSD1 activity in comparison with that of PCPA, partially purified LSD1 obtained at the purification step by HisTrap HP chromatography was dissolved in buffer C and used. The test compounds were dissolved in DMSO. The final concentration of DMSO in the reaction mixture was adjusted to 5%, and it was confirmed that 5% DMSO did not affect LSD1 activity. Reaction without the inhibitors was also conducted as control. Reaction mixtures (18 μL) containing all the materials except H3K4me2 peptide were incubated for 5 min. Then, the reactions were started by adding 2 μL of 0.2 mM peptide solution into the reaction mixtures. The absorbance at 515 nm was monitored for 30 min in a 384-well plate (Nunc) by using a microplate reader (SpectraMax M2e; Molecular Devices).

For the determination of IC_{50} values, enzyme activity was determined from the linear part of the reaction curve. The ratio of the enzyme activity measured in the presence of the inhibitor to the activity of the control was plotted against log[Inh].

5.2.2. Assay for cell growth inhibitory activity

Cells were plated at an initial density of 5000 cells/well (50 μL/well) in 96-well plates in RPMI 1640 with 10% fetal bovine serum and allowed to attach overnight. The cells were exposed to inhibitors for 48 h at 37 °C in a 5% CO_2 incubator. A solution (5 mg/mL) of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added (10 μL/well) and incubation was carried out for 3 h before solubilization buffer (0.04 mol/L HCl–isopropanol) was added (100 μL/well) onto the cultured cells. The solubilized dye was quantified by colorimetric measurement at 560 nm using a reference wavelength of 750 nm. The absorbance values of control wells (C) and test wells (T) were measured. Absorbance of the test wells (T_0) was also measured at time 0 (addition of compounds). Using these measurements, cell growth inhibition (percentage of growth) by a test inhibitor at each concentration was calculated as follows: % growth = $100 \times [(T - T_0)/(C - T_0)]$, when $T > T_0$ and % growth = $100 \times [(T - T_0)/T]$, when $T < T_0$. Computer analysis of the % growth values afforded the 50% growth inhibition parameter (GI_{50}). GI_{50} was calculated as $100 \times [(T - T_0)/(C - T_0)] = 50$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.12.024.

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