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Laboratory note

Design and synthesis of inhibitors of inducible nitric oxide synthase. Discovery of a new chemical lead with potential for oral bioavailability

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Abstract

A series of 2-iminopiperidines fused to small-membered rings (Tables 1 and 2) were synthesised and biologically evaluated using an in vitro human nitric oxide synthase (NOS) inhibition assay. Fused bicyclic compounds **5**–**9** exhibited nearly the same potency as compound **1** in the hiNOS inhibition assay. Among these, the 1-methyl analogues **8** and **9** showed better isoform selectivity than their corresponding unsubstituted analogues **7** and **6**, respectively. Compounds **5** and **6** were also evaluated by an in vivo NO accumulation assay in a mouse model. The discovery process of new chemical leads for an orally bioavailable inhibitor of human inducible NOS (iNOS) is reported. The structure–activity relationship (SAR) study and chemistry of these compounds are also reported.

Keywords: Inhibitor of inducible nitric oxide synthase; 5-Methyl-2-azabicyclo[4.1.0]heptan-3-imines; Cyclopropanation; Dibromocarbene; Amidine

1. Introduction

In recent years, nitric oxide (NO) has been identified as one of the important and unique mediators of diverse physiological processes [1,2]. Endogenous NO is produced by nitric oxide synthase (NOS), which catalyses the oxidation of L-arginine to L-citrulline to yield NO [3–5]. The NOS family of enzymes is composed of an inducible form and two constitutive forms. Inducible NOS (iNOS) is produced by cells such as activated macrophages, smooth muscle cells, and hepatocytes. iNOS produces a large amount of NO, which is sustained over a long period of time after the enzyme induction by cytokines or endotoxins. The two constitutive isoforms of NOS (cNOS) that have been elucidated are as follows: the neuronal enzyme (nNOS), found in brain cells and peripheral nerve cells, and the endothelial enzyme (eNOS) found in vascular endothelial cells [3]. Unlike iNOS, both nNOS and eNOS are intermittently activated by transient elevations in the intracellular calcium level leading to calmodulin binding and stimulation of enzyme activity. Recent studies have shown that overproduction of NO is associated with several pathophysiological states [6–8]. Overproduction of NO by iNOS has been implicated in the tissue destruction that occurs in states such as chronic inflammation, septic shock, vascular dysfunction in diabetes, and transplant rejection. Substrate analogue inhibitors such as N^G-methyl-L-arginine (L-NMA) [9,10], N^G-nitro-L-arginine (L-NNA) [11], *N*-iminoethyl-L-ornitine (L-NIO) [12], and L-thiocitrulline [13] have been used in both animal models and clinical trials to block iNOS-mediated synthesis of NO and to treat hypotension associated with septic shock [14,15]. However, the patient's blood pressure must be monitored continuously during treatment since these com-

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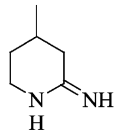
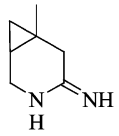
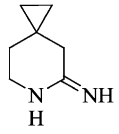
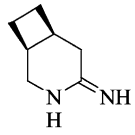
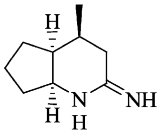
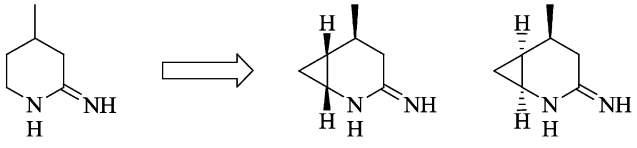
Table 1
Inhibitory activities of fused- and spiro-bicyclic 2-iminopiperidines against hiNOS, heNOS and their isoform selectivity[a]

Structure	Compound	hiNOS IC_{50} (μM)	heNOS IC_{50} (μM)	Selectivity heNOS/hiNOS
	**1** [b]	0.14	1.1	7.9
	**2**	2.2	2.0	0.9
	**3**	0.20	0.34	1.7
	**4**	0.74	4.4	5.9
	**5** [c]	0.10	0.76	7.6

[a]All compounds were prepared as a racemic mixture. [b]Reference [16]. [c]Reference [25].

pounds nonselectively inhibit both cNOS and iNOS. Thus, the development of selective iNOS inhibitors is currently of considerable interest in pharmaceutical research, because of their predicted large therapeutic potential.

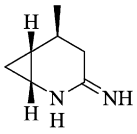
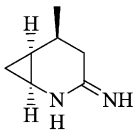
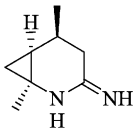
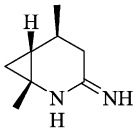
Many studies have focused on the design and synthesis of non-amino acid structures, and a number of non-amino acids have been reported as selective iNOS inhibitors [16,17]. In our previous paper [18], we reported on 5,6-dihyrdopyridin-2-imines as potent inhibitors of iNOS, with a heNOS/hiNOS selectivity of 0.6- to 11-fold. Since the discovery of 2-iminopiperidine [16] as a potent non-amino acid inhibitor of iNOS, this small molecule seems to be one of the most attractive



	1	**6**	**7**
iNOS IC_{50} (μM)	0.14	0.087	0.047
Selectivity heNOS/hiNOS	7.9	2.1	3.6

Fig. 1. Molecular design of a bicyclo[4.1.0] framework.

Table 2
Inhibitory activities of 2-aza-bicyclo[4.1.0]heptan-3-imines against hiNOS, heNOS and their isoform selectivity[a]

Structure	Compound	hiNOS IC_{50} (μM)	heNOS IC_{50} (μM)	Selectivity heNOS/hiNOS
	**6** (*anti*)	0.087	0.18	2.1
	**7** (*syn*)	0.047	0.17	3.6
	**8** (*syn*)	0.22	1.60	7.3
	**9** (*anti*)	0.13	0.61	4.7

[a]All compounds were prepared as a racemic mixture.

chemical leads because of its ability to accept a diversity of chemical modifications. As described in Fig. 1, our chemical modifications of **1** started with the preparation of 2-iminopiperidines fused to a small-membered ring and a spiro-derivative, such as the compounds listed in Table 1. Here we report on the discovery process leading to a series of bicyclic 2-iminopiperidines fused to small-membered rings as new chemical leads for selective inhibitors of iNOS.

2. Chemistry

The test compounds **2**–**4** and **6**–**9** in Tables 1 and 2 were prepared as outlined in Figs. 2–5. As shown in Fig. 2, **2** was prepared from **10**. Birch reduction of **10** provided 4-methyl-3,6-dihydropyridin-2-one (**11**) at a good yield (84%) [19]. Cyclopropanation of the double bond in **11** by a modified Simmons–Smith reaction [20] afforded **12** at a 35% yield, which was converted to **2** by the usual procedures to prepare an amidine from the corresponding amide.

As described in Fig. 3, the spiro-derivative **3** was prepared from dinitrile **13** [21]. Selective reduction of one of the nitrile functions of **13** afforded an aminonitrile **14** at a 32% yield, which was transformed into an ethyl imidate **15**. Conversion of **15** to **3** was accomplished by the usual aminolysis, followed by treatment with hydrogen chloride.

As described in Fig. 4, **4** was prepared from a cyclopentanone fused to a cyclobutane, which was synthesised from the *cis*-1,2-bis(iodomethyl)cyclobutane (**16**) [22]. Direct Beckmann rearrangement of the bicyclic cyclopentanone yielded **17**, which was transformed to **4** by the usual procedure used to prepare an amidine from the corresponding amide.

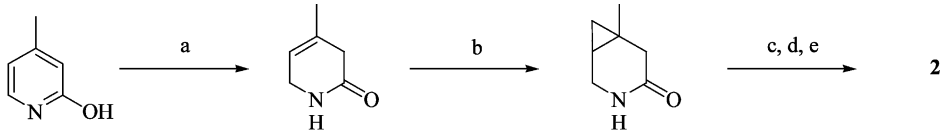


Fig. 2. Synthesis of **2**. Reagents: (a) Li, liquid NH_3, THF–EtOH, −78 °C then H_2O (84%); (b) CH_2I_2, $AlMe_3$, CH_2Cl_2, 0 °C (35%); (c) $Et_3O^+BF_4^-$, CH_2Cl_2; (d) NH_3, EtOH; (e) 4 M HCl dioxane (66% in three steps).

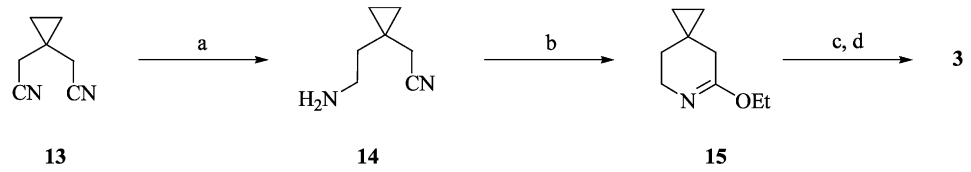


Fig. 3. Synthesis of **3**. Reagents: (a) $NaBH_4$, $CoCl_6 \cdot 6H_2O$, MeOH, −15 °C (32%); (b) HCl (gas), MeOH, room temperature; (c) NH_3, EtOH; (d) 4 M HCl dioxane (78% in three steps).

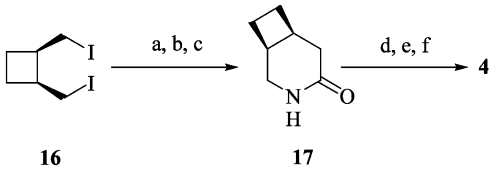


Fig. 4. Synthesis of **4**. Reagents: (a) KH, FAMSO, THF; (b) H_2SO_4 aq, acetone; (c) NH_2OSO_3H, HCO_2H, reflux (9% in three steps); (d) $Et_3O^+BF_4^-$, CH_2Cl_2; (e) NH_3, EtOH; (f) 4 M HCl dioxane (33% in three steps).

Compounds **6**–**9** were prepared from **18** as outlined in Fig. 5. Aminolysis of **18** with *p*-methoxybenzylamine, followed by formation of an imide with acetic anhydride, resulted in **19** at a 95% yield. Partial reduction of **19** with sodium borohydride afforded **20** at a 95% yield. Methylation of **19** with methyl lithium gave **21** at a 95% yield. Dehydration of **20** and **21** with *p*-toluenesulfonic acid resulted in **22** and **23** at a yield of 60 and 99%, respectively. Insertion of a dibromocarbene into the newly formed double bonds of **22** gave **24** (**24**-*anti*: 30% yield, **24**-*syn*: 12% yield). Cyclopropanation of **23** with dibromocarbene gave **25** (**25**-*anti*: 26% yield, **25**-*syn*: 7% yield). Both of these cyclopropanations led to two stereoisomers, the *syn*-isomer [23] (minor product) and the *anti*-isomer [24] (major product), respectively, which were separable by column chromatography.

Deprotection of the *p*-methoxybenzyl group of **24** and **25** resulted in **26** (**26**-*anti*: 36% yield, **26**-*syn*: 61% yield) and **28** (**28**-*anti*: 90% yield, **28**-*syn*: 76% yield), respectively. Reductive elimination of the *gem*-dibromine of **26** and **28** afforded **27** (**27**-*anti*: 90% yield, **27**-*syn*: 91% yield) and **29** (**29**-*anti*: 14% yield, **29**-*syn*: 87% yield), respectively.

The stereochemistry of **27**-*anti*, **29**-*anti* was determined based on the nuclear overhauser effects (NOEs) between 5-CH_3 and H_6, and between $H_{4'}$, H_{7endo}, and H_5 using NMR technique (Fig. 6a). The stereochemistry of **27**-*syn* and **29**-*syn* was also determined based on the NOEs between 5-CH_3, H_{7endo}, and H_{7exo}, and between H_5 and H_6 (Fig. 6b).

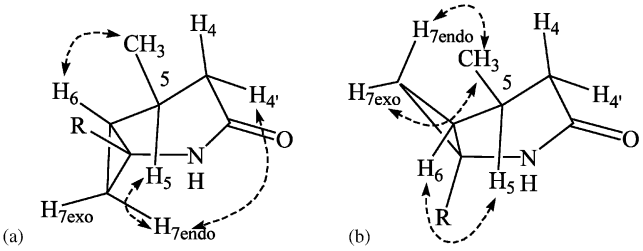


Fig. 6. (a) NOE of **27**-*anti* (R = H) and **29**-*anti* (R = Me); (b) NOE of **27**-*syn* (R = H) and **29**-*syn* (R = Me).

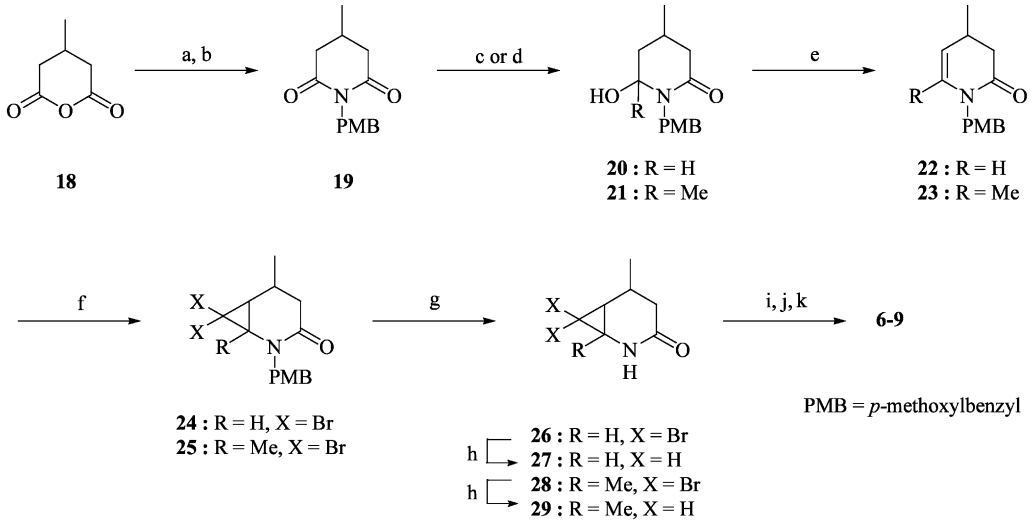


Fig. 5. Synthesis of **6**–**9**. Reagents: (a) $PMBNH_2$, toluene, room temperature; (b) Ac_2O, Et_3N, 80 °C (95% in two steps); (c) $NaBH_4$, EtOH, 0 °C (95%); (d) MeLi, THF, −78 °C (95%); (e) *p*-TsOH·H_2O, toluene, 80 °C (**22**: 60%, **23**: 99%); (f) $CHBr_3$, 50% NaOH aq, aliquat-336 (**24**-*anti*: 30%, **24**-*syn*: 12%, **25**-*anti*: 26%, **25**-*syn*: 7%); (g) $BF_3 \cdot OEt_2$, anisole, 100 °C (36–90%); (h) *n*-Bu_3SnH, AlBN, benzene, reflux (14–91%); (i) $Et_3O^+BF_4^-$, CH_2Cl_2, (j) NH_3, EtOH, (k) 4 M HCl dioxane (16–61% in three steps).

Compounds **27** and **29** were converted to their corresponding cyclic amidines **6**–**7** and **8**–**9**, respectively, by the usual procedures. The *anti*-isomers **6** and **9** were prepared from the corresponding *anti*-isomers of **27** (**27**-*anti*) and **29** (**29**-*anti*), respectively, which were obtained as major products of the dibromocyclopropanation of **22** and **23**, respectively. The *syn*-isomers **7** and **8** were prepared from the corresponding *syn*-isomers of **27** (**27**-*syn*) and **29** (**29**-*syn*), respectively, which were obtained as minor products of the cyclopropanation of **22** and **23**, respectively.

3. Results and discussion

Test compounds were biologically evaluated for their ability to inhibit the two isoforms of NOS, i.e. human iNOS (hiNOS) and human eNOS (heNOS), and their isoform selectivity was determined by the ratio of the two IC_{50} values: heNOS/hiNOS. 2-Imino-4-methylpiperidine **1** [16] was prepared as a mixture of racemates and biologically evaluated as a standard compound, which demonstrates inhibitory activities against the two isoforms of NOS, hiNOS ($IC_{50} = 0.14$ μM) and heNOS ($IC_{50} = 1.1$ μM), showing an isoform selectivity of nearly 8-fold. Cyclopropanation of 4-methyl-3,6-dihydropyridin-2-imine [18] yielded **2**, which showed a marked reduction in the potency of hiNOS and heNOS inhibition as well as less isoform selectivity relative to **1**.

The spiro analogue **3** showed a 1.4-fold reduction in inhibitory activity and less isoform selectivity compared with **1**. Compound **4**, a 2-iminopiperidine fused to a cyclobutane in the *cis*-manner, demonstrated a 5-fold reduction in the potency of hiNOS inhibition and a 4-fold reduction in heNOS inhibition, so the isoform selectivity was 6-fold. The *cis*-fused 5,6-bicyclic derivative **5** [25] was also synthesised and biologically evaluated. Compound **5** demonstrated nearly the same potency and isoform selectivity as compound **1**. Cyclopropanation of 4-methyl-3,4-dihydropyridin-2-imine yielded **6** as the main product, in which the *cis*-fused cyclopropane moiety showed *anti*-stereochemistry with respect to the 4-methyl group. This compound showed a 1.6-fold increase in hiNOS inhibition relative to **1**, with 2.1-fold isoform selectivity. The corresponding *syn*-isomer **7**, which was obtained as the minor product of the above-mentioned cyclopropanation reaction, also demonstrated more potent hiNOS inhibition than **1**, with 3.6-fold isoform selectivity.

It has been reported that introduction of an alkyl group at position-6 of 2-iminopiperidines increases the potency of iNOS inhibition [16]. On the basis of this information, a methyl group was introduced at the corresponding position of the more active *syn*-isomer **7**, yielding **8** with reduced iNOS inhibition and increased isoform selectivity. Introduction of a methyl group at the corresponding position of the *anti*-isomer **6** afforded **9**, which retained its potency for iNOS inhibition and showed increased isoform selectivity. The introduction of a methyl group at position-6 of **6** and **7** was effective in increasing the isoform selectivity without any marked loss of potency for iNOS inhibition.

In order to evaluate the newly identified chemical leads **5** and **6** for their ability to inhibit iNOS in vivo, mice were subcutaneously (sc) injected with **5** and **6** at 3 h after lipopolysaccharide (LPS) treatment [26]. Then the plasma NOx accumulation from 3 to 6 h after LPS injection was determined. As described in Table 3, test compounds **5** and **6** inhibited NOx accumulation in plasma and their ID_{50} values were 0.036 and 0.013 mg kg^{-1}, sc. To assess the acute toxicity of each compound, the maximum tolerated dose (MTD) was determined. As shown in Table 3, the MTD of **5** and **6** was 10 and 20 mg kg^{-1}, respectively, when a single intravenous (iv) dose was given to normal mice. Safety was assessed from the ratio of the MTD and ID_{50} values for NOx accumulation. According to the MTD/ID_{50} of NOx accumulation data for the compounds, **6** was thought to be tolerable enough to allow for an in vivo evaluation.

4. Conclusions

In conclusion, 2-iminopiperidines fused to small-membered rings and a spiro analogue were synthesised and biologically evaluated. Among these, compounds, *cis*-fused cyclisation of position-5 and position-6 of **1** was effective in retaining the potency of iNOS inhibition, as illustrated in **5**–**7**. Compounds **2** and **4**, in which cyclopropane and cyclobutane were fused to position-4 and -5, respectively, in a *cis*-manner, demonstrated weaker inhibitory activity. A spiro analogue **3** also retained the same potency for iNOS inhibition as **1**, but showed a loss of isoform selectivity. The *cis*-fused

Table 3
Pharmacological evaluation of **5** and **6** in mice

Compound	Mouse iNOS IC_{50} (μM)	NOx ID_{50} (mg kg^{-1}, sc)	MTD (mg kg^{-1}, iv)	MTD/NOx
5	0.030	0.036	10	280
6	0.007	0.013	20	1500
L-NMMA	3.5	26	3000	120

bicyclic analogue **5** retained both the potency and iNOS selectivity of **1**. Other *cis*-fused bicyclic analogues, **6** and **7**, also retained the potency of **1**, but showed reduced isoform selectivity. Introduction of a methyl group at position-1 of bicyclic analogues **6** and **7** was effective for increasing isoform selectivity and retaining hiNOS inhibitory activity, as illustrated in **8**–**9**. Further optimisation of 2-imino-4-methylpiperidines fused to cyclopropanes and oral bioavailability of the optimised inhibitors will be reported elsewhere.

5. Experimental

5.1. Chemistry

Melting points (m.p.) were determined by Yanaco micro m.p. apparatus MP-500D and are uncorrected. Analytical samples were homogeneous as confirmed by thin layer chromatography (TLC), and afforded spectroscopic results consistent with the assigned structures. All ^{1}H-NMR spectra were obtained on a Varian Gemini-200, VXR-200s spectrometer. The chemical shift values are reported in ppm (δ) and coupling constants (J) in Hertz (Hz). Fast atom bombardment mass spectra (FABMS) and electron ionisation (EI) were obtained on a JEOL JMS-DX303HF or PerSeptive Voyager Elite spectrometer. Atmospheric pressure chemical ionisation (APCI) was determined on a Hitachi M1200H spectrometer. Matrix assisted laser desorption ionisation-time of flight high-resolution mass spectra (MALDI-TOF HRMS) were obtained on a PerSeptive Voyager Elite spectrometer. IR spectra were measured on a Perkin–Elmer FTIR 1760X or JASCO FTIR-430 spectrometer. Column chromatography was carried out on silica gel [Merck silica gel 60 (0.063–0.200 mm) or Wako Gel C200 or Fuji Silysia FL60D]. TLC was performed on silica gel (Merck TLC plate, silica gel 60 F_{254}).

5.1.1. Starting materials

2-Hydroxy-4-methylpyridine (**10**) and 3-methylglutaric anhydride (**18**) are commercially available. Compounds **13**, **16** were synthesised according to the literature [21,22a].

5.1.2. Preparation of compound *12*

*5.1.2.1. 4-Methyl-3,6-dihydropyridin-2-one (**11**).* To a stirred solution of 2-hydroxy-4-methylpyridine (**10**) (5 g, 45.8 mmol) in THF (45 mL) and EtOH (4 mL) was added liquid NH_3 (150 mL) at −78 °C. Small pieces of lithium metal (954 mg, 137.4 mmol) were portionwisely added to the reaction mixture and the formed solution was stirred for an additional 1.5 h at −78 °C before quenched with water (50 mL). The reaction mixture was slowly warmed up to room temperature (r.t.) removing the ammonia with a stream of nitrogen and then treated with additional water (50 mL). The reaction mixture was extracted with $CHCl_3$ (50 mL × 3). The combined organic layers were washed with 1 M HCl and brine, dried over anhydrous sodium sulphate and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel ($CHCl_3$–MeOH, 5/1) to afford **11** as a white crystal (4.3 g, 84%). M.p.: 83–85 °C. IR (KBr) (cm^{-1}) 3207, 3056, 2893, 1704, 1663, 1499, 1440, 1406, 1378, 1339, 1289, 1155, 1130, 1043, 1002, 949, 838, 805, 726, 536, 492, 464. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.60–6.70 (brs, 1H), 5.43 (m, 1H), 3.90 (m, 2H), 2.80 (m, 2H), 1.72 (s, 3H). MS (APCI, Pos.) m/z 112 $[M+H]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_6H_9N_1O_1+H^+$: 112.0762; found: 112.0742. TLC R_f 0.51 ($CHCl_3$–MeOH, 10/1).

*5.1.2.2. 6-Methyl-3-azabicyclo[4.1.0]heptan-4-one (**12**).* To a stirred solution of **11** (2.0 g, 18.0 mmol) and CH_2I_2 (8.8 mL, 109 mmol) in CH_2Cl_2 (81 mL) was added Me_3Al (1 M in *n*-hexane, 72 mL, 72 mmol) at 0 °C under an argon atmosphere and stirred for 3 days at r.t.. The reaction mixture was diluted with CH_2Cl_2 (60 mL) and cooled to 0 °C. KF (12.1 g, 208 mmol) and water (3.9 mL) were added carefully. The insoluble substances were dissolved in water and extracted with $CHCl_3$. The organic layer was washed with saturated $Na_2S_2O_3$ and brine, dried over anhydrous magnesium sulphate and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (*n*-hexane–EtOAc, 1/2 to $CHCl_3$–MeOH, 30/1) to afford **12** as a pale yellow oil (785 mg, 35%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 6.00–5.65 (brs, 1H), 3.71 (dd, $J=12.6$, 2.7 Hz, 1H), 3.41 (ddd, $J=12.6$, 5.4, 1.8 Hz, 1H), 2.51 (d, $J=17.2$ Hz, 1H), 2.25 (d, $J=17.2$ Hz, 1H), 1.17 (s, 3H), 1.00 (m, 1H), 0.67 (t, $J=5.0$ Hz, 1H), 0.46–0.38 (m, 1H). MS (APCI, Pos.) m/z 126 $[M+H]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_7H_{11}N_1O_1+H^+$: 126.0919; found: 126.0923. TLC R_f 0.53 ($CHCl_3$–MeOH, 10/1).

5.1.3. Preparation of compound *3*

*5.1.3.1. [1-(2-Aminoethyl)cyclopropyl]acetonitrile (**14**).* To a stirred solution of **13** [21] (5.0 g, 41.6 mmol) in MeOH (200 mL) was added $CoCl_2 \cdot 6H_2O$ (5.0 g, 21.0 mmol) and $NaBH_4$ (4.2 g, 111 mmol) at −15 °C under an argon atmosphere. The reaction mixture was stirred for 15 min and was treated with 2 M HCl carefully. The resulting solution was warmed up to r.t., stirred for 15 min, evaporated and diluted water. The resulting mixture was washed with EtOAc and the aqueous layer was treated with 5 M NaOH to adjust the pH value to 9. After removing insoluble substance by filtration, the aqueous layer was extracted with $CHCl_3$. The organic

layer was washed with brine, dried over anhydrous magnesium sulphate and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel ($CHCl_3$–MeOH, 5/1–1/2) to afford **14** as a pale yellow oil (1.7 g, 32%). IR (neat) (cm^{-1}) 2245. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 2.85 (t, $J = 7.6$ Hz, 2H), 2.41 (s, 2H), 1.59 (t, $J = 7.6$ Hz, 2H), 0.54 (s, 4H). MS (APCI, Pos.) *m/z* 125 $[M+H]^+$. TLC R_f 0.17 ($CHCl_3$–MeOH–AcOH, 10/1/1).

*5.1.3.2. 6-Azaspiro[2.5]octan-5-imine hydrochloride salt (**3**).* To a stirred solution of **14** (830 mg, 6.7 mmol) in EtOH (13 mL) was bubbled with HCl gas at 0 °C and stirred for 3 h at r.t.. The reaction mixture was evaporated to give **15**, which was used for the subsequent reaction without further purification.

To a stirred solution of **15** in EtOH (3 mL) was added saturated ethanolic ammonia (12 mL) under an argon atmosphere at r.t. and stirring was continued for an additional 24 h. The reaction mixture was diluted with $CHCl_3$ (16 mL) and the resulting precipitates were removed by filtration. The filtrate was concentrated under reduced pressure. The reaction mixture was treated with 2 M NaOH (19 mL) and extracted with $CHCl_3$ (30 mL × 2). The combined organic layers were dried over anhydrous sodium sulphate and evaporated under reduced pressure. The residue was converted to its hydrochloride with 4 M HCl–EtOAc–EtOH at 0 °C and further purified by column chromatography on silica gel ($CHCl_3$–MeOH, 5/1) to afford **3** as a pale yellow solid (839 mg, 78%). IR (KBr) (cm^{-1}) 3292, 3122, 3002, 2946, 2877, 1683, 1537, 1444, 1422, 1379, 1340, 1017, 815. ^{1}H-NMR (200 MHz, DMSO-d_6) δ 9.75 (brs, 1H), 8.78 (brs, 1H), 8.53 (brs, 1H), 3.35 (m, 2H), 2.45 (s, 2H), 1.56 (t, $J = 5.8$ Hz, 2H), 0.45 (s, 4H). M.p.: 103–105 °C; MS and HRMS analysis showed only the ion from $C_7H_{12}N_2$ corresponding to the loss of HCl, MS (FAB, Pos.) *m/z* 125 $[M+H]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_7H_{12}N_2+H^+$: 125.1079; found: 125.1050. TLC R_f 0.33 ($CHCl_3$–MeOH–AcOH, 10/1/1).

*5.1.4. Preparation of compound **17***

*5.1.4.1. (DL)-(1S,6S)-3-azabicyclo[4.2.0]octan-4-one (**17**).* To a stirred suspension of KH (650 mg, 16.2 mmol) in THF (8 mL) was added methyl methylsulfinylmethyl sulphide (FAMSO) (589 mg, 4.7 mmol) in THF (2 mL) at 0 °C under an argon atmosphere. After 10 min, *cis*-1,2-bis(iodomethyl)cyclobutane (**16**) [22a] (1.4 g, 4.3 mmol) was added to this suspension and stirred for 4 h at r.t. The reaction mixture was diluted with CH_2Cl_2 (7 mL) and the resulting precipitates were removed by filtration. Then the filtrate was concentrated under reduced pressure.

To a stirred solution of the compound obtained above in acetone (5 mL) was added 9 M H_2SO_4 (0.3 mL) at r.t. and the reaction mixture was heated at reflux temperature for 3 h. After completing the reaction, the reaction mixture was cooled to r.t. and evaporated under reduced pressure. The residue was treated with water and extracted with Et_2O. The organic layer was washed with saturated aqueous sodium bicarbonate, brine, dried over anhydrous magnesium sulphate and evaporated to afford the bicyclo[3.2.0]heptan-3-one (480 mg), which was used for the subsequent reaction without further purification.

To a stirred solution of the bicyclo[3.2.0]heptan-3-one (480 mg) obtained above in HCO_2H (8 mL) was added NH_2OSO_3H (725 mg, 6.4 mmol) and the reaction mixture was heated at reflux temperature for 3 h. After completing the reaction, the reaction mixture was cooled to r.t. and treated with 5 M NaOH to adjust the pH value to 7. The mixture was extracted with $CHCl_3$ and washed with brine, dried over anhydrous magnesium sulphate, and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc to EtOAc–MeOH, 9/1) to afford **17** as a pale yellow oil (48 mg, 9%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 6.55 (brs, 1H), 3.39 (ddd, $J = 13.2$, 4.2, 2.0 Hz, 1H), 3.09 (ddd, $J = 13.2$, 5.6, 2.6 Hz, 1H), 2.89 (m, 1H), 2.70 (m, 1H), 2.40 (dd, $J = 15.4$, 5.6 Hz, 1H), 2.32–2.10 (m, 3H), 1.90–1.76 (m, 2H). MS (APCI, Pos.) *m/z* 126 $[M+H]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_7H_{11}N_1O_1+H^+$: 126.0919; found: 126.0893. TLC R_f 0.50 ($CHCl_3$–MeOH, 9/1).

*5.1.5. General procedure for preparation of the key intermediate (**22**), (**23**)*

*5.1.5.1. 1-(4-Methoxybenzyl)-4-methylpiperidine-2,6-dione (**19**).* To a stirred solution of 3-methylglutaric anhydride (**18**) (20 g, 156 mmol) in THF (300 mL) was added *p*-methoxybenzylamine (23 g, 168 mmol) at r.t. and stirred for 30 min. After completing the reaction, the reaction mixture was evaporated and the residue was diluted with EtOAc. The mixture was treated with 1 M HCl and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous magnesium sulphate, and evaporated to afford the residue, which was used for the subsequent reaction without further purification.

To a stirred solution of the compound obtained above in Ac_2O (100 mL) was added Et_3N (20 mL) at r.t. The reaction mixture was heated at 80 °C for 1 h. After completing the reaction, the mixture was cooled to r.t. and evaporated under reduced pressure. The residue was diluted with EtOAc and water. The organic layer was washed with 1 M HCl, saturated aqueous sodium bicarbonate, brine and dried over anhydrous magnesium sulphate and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc–*n*-hexane, 10/1–1/1) to afford **19** as a

pale yellow solid (37 g, 95%). M.p.: 50–51 °C. IR (KBr) (cm^{-1}) 3368, 2968, 2871, 1719, 1671, 1611, 1514, 1469, 1428, 1387, 1339, 1291, 1347, 1218, 1175, 1142, 1109, 1062, 1029, 970, 937, 886, 832, 806, 776, 646, 628, 595, 562, 518. ^{1}H-NMR (200 MHz, CDCl3) δ 7.32 (d, J = 9.0 Hz, 2H), 6.80 (d, J = 9.0 Hz, 2H), 4.88 (s, 2H), 3.77 (s, 3H), 2.80–2.65 (m, 2H), 2.40–2.12 (m, 3H), 1.04 (d, J = 6.2 Hz, 3H). MS (APCI, Pos.) m/z 248 $[M+H]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_{14}H_{17}N_1O_3+H^+$: 248.1287; found: 248.1259. TLC R_f 0.82 (EtOAc–*n*-hexane, 1/1).

*5.1.5.2. 6-Hydroxy-1-(4-methoxybenzyl)-4-methylpiperidin-2-one (**20**).* To a stirred solution of **19** (14 g, 56.6 mmol) in EtOH (300 mL) was added $NaBH_4$ (4.2 g, 111 mmol) at 0 °C. The reaction mixture was stirred for 2 h at r.t.. After completing the reaction, the resulting mixture was cooled to 0 °C and treated with 1 M HCl to adjust the pH value to 7. After removal of the solvent by evaporation, the resulting mixture was extracted with EtOAc and washed with brine. The combined organic layers were dried over anhydrous sodium sulphate and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc–*n*-hexane, 1/1) to afford **20** as a white crystal (13 g, 95%). M.p.: 130–131 °C. IR (KBr) (cm^{-1}) 3259, 2952, 1626, 1514, 1479, 1413, 1299, 1249, 1170, 1101, 1065, 1025, 974, 902, 838, 819, 767, 742, 619, 583, 528. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.31 (d, J = 9.0 Hz, 2H), 6.80 (d, J = 9.0 Hz, 2H), 5.10–4.70 (m, 2H), 4.40–4.25 (m, 1H), 3.75 (s, 3H), 2.80–2.05 (m, 3H), 1.95–1.60 (m, 2H), 1.47 (ddd, J = 13.6, 12.8, 3.6 Hz, 1H), 1.04 (d, J = 6.6 Hz, 3H). MS (APCI, Pos.) m/z 250 $[M+H]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_{14}H_{19}N_1O_3+H^+$: 250.1443; found: 250.1437. TLC R_f 0.24 (EtOAc–*n*-hexane, 1/1).

*5.1.5.3. 1-(4-Methoxybenzyl)-4-methyl-3,4-dihydropyridin-2-one (**22**).* A solution of **20** (3.4 g, 13.6 mmol) and *p*-toluenesulfonic acid monohydrate (150 mg, 0.8 mmol) in toluene (70 mL) was stirred for 1.5 h at 80 °C. After cooling at r.t., the reaction mixture was diluted with EtOAc and washed sequentially with saturated aqueous sodium bicarbonate and brine. The organic layer was dried over magnesium sulphate and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc–*n*-hexane, 1/5–1/3) to afford **22** as a pale yellow oil (1.9 g, 60%). IR (neat) (cm^{-1}) 3421, 2957, 2836, 1668, 1612, 1585, 1513, 1457, 1409, 1385, 1304, 1248, 1212, 1176, 1147, 1105, 1034, 946, 822, 717, 580, 518. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.22 (d, J = 8.6 Hz, 2H), 6.85 (d, J = 8.6 Hz, 2H), 5.96 (dd, J = 7.8, 1.5 Hz, 1H), 5.03 (dd, J = 7.8, 3.3 Hz, 1H), 4.70–4.50 (m, 2H), 3.79 (s, 3H), 2.80–2.05 (m, 3H), 1.05 (d, J = 6.8 Hz, 3H). MS (APCI, Pos.) m/z 232 $[M+H]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_{14}H_{17}N_1O_2+H^+$: 232.1337; found: 232.1313. TLC R_f 0.78 (EtOAc–*n*-hexane, 1/1).

*5.1.5.4. 6-Hydroxy-1-(4-methoxybenzyl)-4,6-dimethylpiperidin-2-one (**21**).* To a stirred solution of **19** (20 g, 81 mmol) in THF (300 mL) was added MeLi (1 M in Et_2O, 87 mL, 87 mmol) at −78 °C under an argon atmosphere. After completing the reaction, the reaction mixture was diluted with Et_2O and saturated aqueous ammonium chloride (100 mL) was added. The organic layer was washed with brine, dried over magnesium sulphate and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc–*n*-hexane, 1/1) to afford **21** as a white solid (20 g, 95%). M.p.: 66–68 °C. IR (KBr) (cm^{-1}) 3309, 2963, 1704, 1633, 1548, 1515, 1459, 1370, 1304, 1252, 1173, 1160, 1036, 830, 696, 589. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.20 (d, J = 8.8 Hz, 2H), 6.86 (d, J = 8.8 Hz, 2H), 5.85 (br, 1H), 4.36 (d, J = 5.6 Hz, 2H), 3.80 (s, 3H), 2.63–2.00 (m, 5H), 2.13 (s, 3H), 1.00 (d, J = 6.4 Hz, 3H). MS (APCI, Pos.) m/z 264 $[M+H]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_{15}H_{21}N_1O_3+H^+$: 264.1600; found: 264.1585. TLC R_f 0.13 (EtOAc–*n*-hexane, 1/1).

*5.1.5.5. 1-(4-Methoxybenzyl)-4,6-dimethyl-3,4-dihydropyridin-2-one (**23**).* Compound **23** was prepared from **21** in 99% yield according to the same procedure as described for the preparation of **22** from **20**. Colourless oil; IR (neat) (cm^{-1}) 2957, 2836, 1674, 1614, 1513, 1457, 1388, 1248, 1176, 1110, 1034, 959, 891, 819, 772, 659, 548. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.11 (d, J = 8.8 Hz, 2H), 6.84 (d, J = 8.8 Hz, 2H), 4.92 (d, J = 15.8 Hz, 1H), 4.89 (s, 1H), 4.70 (d, J = 15.8 Hz, 1H), 3.78 (s, 3H), 2.80–2.20 (m, 3H), 1.85 (s, 3H), 1.04 (d, J = 7.0 Hz, 3H). MS (APCI, Pos.) m/z 246 $[M+H]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_{15}H_{19}N_1O_2+H^+$: 246.1494; found: 246.1477. TLC R_f 0.58 (EtOAc–*n*-hexane, 1/1).

*5.1.6. General procedure for preparation of compounds **27** and **29***

*5.1.6.1. (DL)-(1S,5S,6R)-7,7-dibromo-2-(4-methoxybenzyl)-5-methyl-2-azabicyclo[4.1.0]heptan-3-one (**24**-anti) and (DL)-(1R,5S,6S)-7,7-dibromo-2-(4-methoxybenzyl)-5-methyl-2-azabicyclo[4.1.0]heptan-3-one (**24**-syn).* To a stirred solution of **22** (2 g, 8.7 mmol) in $CHBr_3$ (25 mL, 287 mmol) were added aliquat-336 (0.1 mL, 0.22 mmol) and 50% aqueous sodium hydroxide (6.4 g) at 0 °C under an argon atmosphere. The reaction mixture was stirred for 22 h at 0 °C. After completing the reaction, the reaction mixture was treated with saturated aqueous ammonium chloride and extracted with Et_2O. The organic layer was washed with brine and dried over magnesium sulphate, and evaporated under reduced pressure. The residue was

purified by column chromatography on silica gel (EtOAc–*n*-hexane, 1/5–1/3) to afford **24**-*anti* as a pale yellow oil (1.1 g, 30%) and **24**-*syn* as a pale yellow oil (420 mg, 12%).

*5.1.6.1.1. Compound **24**-anti.* IR (neat) (cm^{-1}) 2961, 2836, 1667, 1612, 1513, 1415, 1379, 1248, 1175, 1075, 1033, 845, 764, 721, 581, 503. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.29 (d, $J = 8.4$ Hz, 2H), 6.89 (d, $J = 8.4$ Hz, 2H), 5.47 (d, $J = 14.8$ Hz, 1H), 3.81 (d, $J = 14.8$ Hz, 1H), 3.81 (s, 3H), 2.98 (d, $J = 9.8$ Hz, 1H), 2.40–2.00 (m, 3H), 1.77 (dd, $J = 9.8$, 5.4 Hz, 1H), 1.26 (m, 3H). MS (APCI, Pos.) *m/z* 404 $[M+H, {}^{79}Br, {}^{81}Br]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_{15}H_{17}Br_2N_1O_2+H^+$: 401.9704; found: 401.9717. TLC R_f 0.38 (EtOAc–*n*-hexane, 1/2).

*5.1.6.1.2. Compound **24**-syn.* IR (neat) (cm^{-1}) 2962, 2836, 1652, 1513, 1456, 1415, 1389, 1341, 1303, 1249, 1176, 1108, 1033, 885, 849, 773, 582, 545. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.33 (d, $J = 8.6$Hz, 2H), 6.91 (d, $J = 8.6$ Hz, 2H), 4.93 (d, $J = 14.2$ Hz, 1H), 4.54 (d, $J = 14.2$ Hz, 1H), 3.82 (s, 3H), 3.27 (d, $J = 10.2$ Hz, 1H), 2.50–2.45 (m, 3H), 2.20–2.10 (m, 1H), 1.28 (d, $J = 6.2$ Hz, 3H). MS (APCI, Pos.) *m/z* 404 $[M+H, {}^{79}Br, {}^{81}Br]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_{15}H_{17}Br_2N_1O_2+H^+$: 401.9704; found: 401.9703. TLC R_f 0.18 (EtOAc–*n*-hexane, 1/2).

*5.1.6.2. (DL)-(1S,5S,6R)-7,7-dibromo-5-methyl-2-azabicyclo[4.1.0]heptan-3-one (**26**-anti).* A solution of **24**-*anti* (993 mg, 2.5 mmol), anisole (1.7 mL, 15.6 mmol) in $BF_3 \cdot OEt_2$ complex (4.0 mL) was stirred for 35 h at 100 °C under an argon atmosphere. After cooling in an ice bath, water and 5 M NaOH were slowly added to the reaction mixture. The resulting mixture was extracted with $CHCl_3$ and washed with saturated aqueous sodium bicarbonate and then brine. The organic layer was dried over magnesium sulphate and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc–*n*-hexane, 1/2–2/1) to afford **26**-*anti* as a white powder (530 mg, 36%). M.p.: 106–109 °C. IR (KBr) (cm^{-1}) 3205, 3099, 2960, 1656, 1474, 1452, 1407, 1365, 1352, 1286, 1200, 1121, 1096, 1072, 1015, 933, 884, 774, 717, 553, 497, 467. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.40–6.90 (brs, 1H), 3.20 (d, $J =$ 9.8 Hz, 1H), 2.30–1.95 (m, 3H), 1.80 (dd, $J = 9.8$, 4.4 Hz, 1H), 1.29 (d, $J = 6.0$ Hz, 3H). MS (APCI, Pos.) *m/z* 286 $[M+H, {}^{81}Br, {}^{81}Br]^+$, 284 $[M+H, {}^{79}Br, {}^{81}Br]^+$, 282 $[M+H, {}^{79}Br, {}^{79}Br]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_7H_9Br_2N_1O_1+H^+$: 281.9129; found: 281.9130. TLC R_f 0.75 ($CHCl_3$–MeOH, 10/1).

*5.1.6.3. (DL)-(1R,5S,6S)-7,7-dibromo-5-methyl-2-azabicyclo[4.1.0]heptan-3-one (**26**-syn).* Compound **26**-*syn* was prepared from **24**-*syn* in 61% yield according to the same procedure as described for the preparation of **26**-*anti* from **24**-*anti*. White powder; m.p.: 113–116 °C. IR (KBr) (cm^{-1}) 3196, 2967, 1658, 1475, 1385, 1343, 1309, 1017, 932, 882, 763, 707, 522. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 6.86 (brs, 1H), 3.38 (dd, $J = 10.0$, 4.0 Hz, 1H), 2.52–2.34 (m, 3H), 2.18 (dd, $J = 10.0$, 6.2 Hz, 1H), 1.33 (d, $J = 6.2$ Hz, 3H). MS (APCI, Pos.) *m/z* 286 $[M+H, {}^{81}Br, {}^{81}Br]^+$, 284 $[M+H, {}^{79}Br, {}^{81}Br]^+$, 282 $[M+H, {}^{79}Br, {}^{79}Br]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_7H_9Br_2N_1O_1+H^+$: 281.9129; found: 281.9117. TLC R_f 0.32 (EtOAc–*n*-hexane, 1/2).

*5.1.6.4. (DL)-(1R,5S,6R)-5-methyl-2-azabicyclo[4.1.0]heptan-3-one (**27**-anti).* To a stirred mixture of **26**-*anti* (530 mg, 1.9 mmol) in benzene (1 mL) were added *n*-Bu_3SnH (4 mL, 14.9 mmol), azobisisobutylonitrile (42 mg, 0.26 mmol) and the reaction mixture was stirred with heating at reflux temperature for 2 h under an argon atmosphere. After cooling in an ice bath, the reaction mixture was diluted with EtOAc (10 mL) and washed with 10% aqueous potassium fluoride (10 mL). Insoluble substance was removed by filtration. The organic layer was washed with brine and dried over magnesium sulphate, and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (EtOAc–*n*-hexane, 1/6–2/1) to afford **27**-*anti* as a white powder (214 mg, 90%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 6.50–6.05 (brs, 1H), 2.70–2.56 (m, 1H), 2.24–1.76 (m, 3H), 1.19 (d, $J = 6.6$ Hz, 3H), 1.05–0.84 (m, 2H), 0.60–0.40 (m, 1H). MS (APCI, Pos.) *m/z* 126 $[M+H]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_7H_{11}N_1O_1+H^+$: 126.0919; found: 126.0906. TLC R_f 0.11 (EtOAc–*n*-hexane, 4/1).

*5.1.6.5. (DL)-(1S,5S,6S)-5-methyl-2-azabicyclo[4.1.0]heptan-3-one (**27**-syn).* Compound **27**-*syn* was prepared from **26**-*syn* in 91% yield according to the same procedure as described for the preparation of **27**-*anti* from **26**-*anti*. White powder; ^{1}H-NMR (200 MHz, DMSO-d_6) δ 7.86 (brs, 1H), 2.60 (m, 1H), 2.22 (m, 1H), 1.98 (dd, $J = 16.2$, 6.0 Hz, 1H), 1.64 (dd, $J =$ 16.2, 11.8 Hz, 1H), 1.17 (m, 1H), 1.01 (d, $J = 6.4$ Hz, 3H), 0.58–0.44 (m, 2H). MS (APCI, Pos.) *m/z* 126 $[M+H]^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_7H_{11}N_1O_1+H^+$: 126.0919; found: 126.0933. TLC R_f 0.12 (EtOAc–*n*-hexane, 1/2).

*5.1.6.6. (DL)-(1S,5S,6R)-7,7-dibromo-2-(4-methoxybenzyl)-1,5-dimethyl-2-azabicyclo[4.1.0]heptan-3-one (**25**-anti).* Compounds **25**-*anti* and **25**-*syn* were prepared from **23** in 26 and 7% yields, respectively, according to the same procedure as described for the preparation of **24**-*anti* and **24**-*syn* from **22**.

Pale yellow solid; m.p.: 118–119 °C. IR (KBr) (cm^{-1}) 3435, 2965, 1655, 1512, 1438, 1305, 1243, 1182, 1033, 856, 756, 588, 512. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.24 (d, $J = 8.8$ Hz, 2H), 6.84 (d, $J = 8.8$ Hz, 2H), 5.37 (d, $J = 15.4$ Hz, 1H), 3.83 (d, $J = 15.4$ Hz, 1H), 3.79 (s, 3H), 2.40–2.00 (m, 3H), 1.61–1.40 (m, 1H), 1.50 (s, 3H), 1.31

(d, $J = 6.2$ Hz, 3H). MS (APCI, Pos.) *m/z* 420 [M+H, ^{81}Br, ^{81}Br]$^+$, 418 [M+H, ^{79}Br, ^{81}Br]$^+$, 416 [M+H, ^{79}Br, ^{79}Br]$^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_{16}H_{19}Br_2N_1O_2+H^+$: 415.9861; found: 415.9845. TLC R_f 0.40 (EtOAc–*n*-hexane, 1/2).

*5.1.6.7. (DL)-(1R,5S,6S)-7,7-dibromo-2-(4-methoxybenzyl)-1,5-dimethyl-2-azabicyclo[4.1.0]heptan-3-one (**25**-syn).* Pale yellow oil; IR (neat) (cm^{-1}) 2961, 2835, 1652, 1514, 1455, 1404, 1304, 1248, 1177, 1108, 1034, 808, 759, 648, 540. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 7.28 (d, $J = 8.8$ Hz, 2H), 6.86 (d, $J = 8.8$ Hz, 2H), 5.17 (d, $J = 15.6$ Hz, 1H), 4.29 (d, $J = 15.6$ Hz, 1H), 3.80 (s, 3H), 2.80–2.30 (m, 3H), 1.70–1.50 (m, 1H), 1.66 (s, 3H), 1.31 (d, $J = 6.4$ Hz, 3H). MS (APCI, Pos.) *m/z* 420 [M+H, ^{81}Br, ^{81}Br]$^+$, 418 [M+H, ^{79}Br, ^{81}Br]$^+$, 416 [M+H, ^{79}Br, ^{79}Br]$^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_{16}H_{19}Br_2N_1O_2+H^+$: 415.9861; found: 415.9868. TLC R_f 0.31 (EtOAc–*n*-hexane, 1/2).

*5.1.6.8. (DL)-(1S,5S,6R)-7,7-dibromo-1,5-dimethyl-2-azabicyclo[4.1.0]heptan-3-one (**28**-anti).* Compound **28**-*anti* was prepared from **25**-*anti* in 90% yield according to the same procedure as described for the preparation of **26**-*anti* from **24**-*anti*. Pale yellow powder; m.p.: 165–167 °C. IR (KBr) (cm^{-1}) 3183, 3083, 2960, 1669, 1394, 1272, 1184, 1055, 992, 804, 767, 649, 556. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 6.10 (brs, 1H), 2.27 (dd, $J =$ 13.0, 2.8 Hz, 1H), 2.08–1.95 (m, 2H), 1.72 (s, 3H), 1.54 (dd, $J = 4.5$, 1.1 Hz, 1H), 1.31 (d, $J = 6.4$ Hz, 3H); MS (APCI, Pos.) *m/z* 300 [M+H, ^{81}Br, ^{81}Br]$^+$, 298 [M+H, ^{79}Br, ^{81}Br]$^+$, 296 [M+H, ^{79}Br, ^{79}Br]$^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_8H_{11}Br_2N_1O_1+H^+$: 295.9286; found: 295.9293. TLC R_f 0.37 (EtOAc–*n*-hexane, 4/1).

*5.1.6.9. (DL)-(1R,5S,6S)-7,7-dibromo-1,5-dimethyl-2-azabicyclo[4.1.0]heptan-3-one (**28**-syn).* Compound **28**-*syn* was prepared from **25**-*syn* in 76% yield according to the same procedure as described for the preparation of **26**-*anti* from **24**-*anti*. Pale yellow powder; m.p.: 128–130 °C. IR (KBr) (cm^{-1}) 3434, 3190, 3090, 2961, 1656, 1396, 1302, 1086, 923, 801, 751, 524. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 6.50 (brs, 1H), 2.70–2.30 (m, 3H), 1.77 (s, 3H), 1.80–1.50 (m, 1H), 1.34 (d, $J = 6.6$ Hz, 3H). MS (APCI, Pos.) *m/z* 300 [M+H, ^{81}Br, ^{81}Br]$^+$, 298 [M+H, ^{79}Br, ^{81}Br]$^+$, 296 [M+H, ^{79}Br, ^{79}Br]$^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_8H_{11}Br_2N_1O_1+H^+$: 295.9286; found: 295.9298. TLC R_f 0.30 (EtOAc–*n*-hexane, 1/1).

*5.1.6.10. (DL)-(1R,5S,6R)-1,5-dimethyl-2-azabicyclo[4.1.0]heptan-3-one (**29**-anti).* Compound **29**-*anti* was prepared from **28**-*anti* in 14% yield according to the same procedure as described for the preparation of **27**-*anti* from **26**-*anti*. Pale yellow powder; ^{1}H-NMR (200 MHz, $CDCl_3$) δ 6.21 (brs, 1H), 2.17 (dd, $J =$ 6.6, 2.2 Hz, 1H), 2.08 (dd, $J = 6.6$, 3.8 Hz, 1H), 1.93 (m, 1H), 1.36 (s, 3H), 1.17 (d, $J = 6.6$ Hz, 3H), 0.86–0.79 (m, 2H), 0.66 (m, 1H). MS (FAB, Pos.) *m/z* 140 [M+H]$^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_8H_{13}N_1O_1+H^+$: 140.1075; found: 140.1049. TLC R_f 0.23 (EtOAc–*n*-hexane, 4/1).

*5.1.6.11. (DL)-(1S,5S,6S)-1,5-dimethyl-2-azabicyclo[4.1.0]heptan-3-one (**29**-syn).* Compound **29**-*syn* was prepared from **28**-*syn* in 87% yield according to the same procedure as described for the preparation of **27**-*anti* from **26**-*anti*. Pale yellow powder; ^{1}H-NMR (200 MHz, $CDCl_3$) δ 6.30 (brs, 1H), 2.36 (m, 1H), 2.26 (m, 1H), 1.66 (dd, $J = 16.6$, 11.6 Hz, 1H), 1.38 (s, 3H), 1.13 (m, 1H), 1.08 (d, $J = 6.4$ Hz, 3H), 0.71 (t, $J = 6.0$ Hz, 1H), 0.58 (dd, $J = 9.2$, 6.0 Hz, 1H). MS (APCI, Pos.) *m/z* 140 [M+H]$^+$: HRMS (MALDI-TOF, Pos.) calc. for $C_8H_{13}N_1O_1+H^+$: 140.1075; found: 140.1082. TLC R_f 0.22 (EtOAc–*n*-hexane, 4/1).

*5.1.7. Genaral procedure for preparation of compounds **2**, **4**, **6**, **7**, **8** and **9***

*5.1.7.1. 6-Methyl-3-azabicyclo[4.1.0]heptan-4-imine hydrochloride salt (**2**).* To a stirred solution of **12** (781 mg, 6.2 mmol) in CH_2Cl_2 (6 mL) was added triethyloxonium tetrafluoroborate (2 M in CH_2Cl_2, 3.5 mL, 7 mmol) under an argon atmosphere, and the reaction mixture was stirred at r.t. for 5 h. Concentration of the reaction mixture was done under reduced pressure, and it was used for the subsequent reaction without further purification.

To a stirred solution of the compound obtained above in EtOH (3 mL) was added saturated ethanolic ammonia (12 mL) under an argon atmosphere at r.t. and stirring was continued for an additional 24 h. Then the reaction mixture was diluted with $CHCl_3$ (15 mL) and the resulting precipitates were removed by filtration. The filtrate was concentrated under reduced pressure. The residue was treated with 2 M NaOH (17 mL) and extracted with $CHCl_3$ (30 mL × 2). The combined organic layers were dried over anhydrous sodium sulphate and evaporated under reduced pressure. The residue was converted to its hydrochloride with 4 M HCl–EtOAc–EtOH at 0 °C and further purified by column chromatography on silica gel ($CHCl_3$–MeOH, 5/1) to afford **2** as a pale yellow powder (657 mg, 66%). IR (KBr) (cm^{-1}) 3273, 3109, 2926, 1733, 1674, 1453, 1023, 750, 698. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 9.70–9.50 (brs, 1H), 9.10–8.90 (brs, 1H), 8.75–8.50 (brs, 1H), 3.75–3.50 (m, 2H), 3.13 (d, $J = 17.5$ Hz, 1H), 2.66 (d, $J = 17.5$ Hz, 1H), 1.21 (s, 3H), 1.15–1.02 (m, 1H), 0.67–0.47 (m, 2H). M.p.: 191–192 °C. MS and HRMS analysis showed only the ion from $C_7H_{12}N_2$ corresponding to the loss of HCl, MS (APCI, Pos.) *m/z* 125 [M+

H]$^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_7H_{12}N_2+H^+$: 125.1079; found: 125.1062. TLC R_f 0.29 ($CHCl_3$–MeOH–AcOH, 20/4/1).

*5.1.7.2. (DL)-(1S,6S)-3-azabicyclo[4.2.0]octan-4-imine hydrochloride salt (**4**).* Compound **4** was prepared from **17** in 33% yield according to the same procedure as described for the preparation of **2** from **12**. Beige powder; IR (KBr) (cm^{-1}) 3317, 3104, 2975, 2933, 1677, 1520, 1435, 1418, 1361, 1329, 1197, 1158, 960, 814, 680, 604, 548, 491. ^{1}H-NMR (200 MHz, DMSO-d_6) δ 9.74 (brs, 1H), 9.20 (brs, 1H), 8.83 (brs, 1H), 3.28–3.12 (m, 2H), 2.94–2.42 (m, 4H), 2.28–2.02 (m, 2H), 1.67 (m, 1H), 1.46 (m, 1H). M.p.: 138–140 °C. MS and HRMS analysis showed only the ion from $C_7H_{12}N_2$ corresponding to the loss of HCl, MS (APCI, Pos.) *m/z* 125 [M+H]$^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_7H_{12}N_2+H^+$: 125.1079; found: 125.1097. TLC R_f 0.16 ($CHCl_3$–MeOH–AcOH, 20/2/1).

*5.1.7.3. (DL)-(1R,5S,6R)-5-methyl-2-azabicyclo[4.1.0]heptan-3-imine hydrochloride salt (**6**).* Compound **6** was prepared from **27**-*anti* in 34% yield according to the same procedure as described for the preparation of **2** from **12**. White powder; IR (KBr) (cm^{-1}) 3108, 1670, 1626, 1418, 1351, 1227, 1030, 894, 796, 725. ^{1}H-NMR (200 MHz, DMSO-d_6) δ 9.98 (brs, 1H), 8.95 (brs, 1H), 8.54 (brs, 1H), 2.82 (m, 1H), 2.46 (dd, J = 16.0, 5.0 Hz, 1H), 2.27 (dd, J = 16.0, 7.0 Hz, 1H), 2.02 (m, 1H), 1.2–1.0 (m, 1H), 1.08 (d, J = 6.8 Hz, 3H), 0.88 (m, 1H), 0.73 (m, 1H). MS and HRMS analysis showed only the ion from $C_7H_{12}N_2$ corresponding to the loss of HCl, MS (APCI, Pos.) *m/z* 125 [M+H]$^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_7H_{12}N_2+H^+$: 125.1079; found: 125.1054; TLC R_f 0.18 ($CHCl_3$–MeOH–AcOH, 15/2/1).

*5.1.7.4. (DL)-(1S,5S,6S)-5-methyl-2-azabicyclo[4.1.0]heptan-3-imine hydrochloride salt (**7**).* Compound **7** was prepared from **27**-*syn* in 61% yield according to the same procedure as described for the preparation of **2** from **12**. Pale yellow amorphous; IR (KBr) (cm^{-1}) 2955, 1677, 1423, 1370, 1150, 1082, 1020, 973, 885, 841, 736, 457, 431. ^{1}H-NMR (200 MHz, DMSO-d_6) δ 9.98 (brs, 1H), 8.72 (brs, 1H), 8.30 (brs, 1H), 2.90 (m, 1H), 2.36 (m, 1H), 2.20–1.98 (m, 2H), 1.30 (m, 1H), 1.02 (d, J = 6.2 Hz, 3H), 0.82 (dt, J = 6.4, 3.4 Hz, 1H), 0.64 (dt, J = 9.2, 6.4 Hz, 1H). M.p.: 112–114 °C. MS and HRMS analysis showed only the ion from $C_7H_{12}N_2$ corresponding to the loss of HCl, MS (APCI, Pos.) *m/z* 125 [M+H]$^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_7H_{12}N_2+H^+$: 125.1079; found: 125.1087. TLC R_f 0.21 ($CHCl_3$–MeOH–AcOH, 20/2/1).

*5.1.7.5. (DL)-(1S,5S,6S)-1,5-dimethyl-2-azabicyclo[4.1.0]heptan-3-imine hydrochloride salt (**8**).* Compound **8** was prepared from **29**-*syn* in 16% yield according to the same procedure as described for the preparation of **2** from **12**. Orange oil; IR (neat) (cm^{-1}) 3404, 2976, 1673, 1459, 1212, 1111. ^{1}H-NMR (200 MHz, $CDCl_3$) δ 10.18 (brs, 1H), 8.75 (brs, 1H), 8.57 (brs, 1H), 2.80 (m, 1H), 2.38–2.16 (m, 1H), 2.00–1.78 (m, 1H), 1.52 (s, 3H), 1.32–1.18 (m, 1H), 1.14 (3H, d, J = 6.4 Hz, 3H), 0.82 (m, 1H), 0.71 (m, 1H). MS and HRMS analysis showed only the ion from $C_8H_{14}N_2$ corresponding to the loss of HCl, MS (APCI, Pos.) *m/z* 139 [M+H]$^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_8H_{14}N_2+H^+$: 139.1235; found: 139.1253. TLC R_f 0.43 ($CHCl_3$–MeOH–AcOH, 10/1/1).

*5.1.7.6. (DL)-(1R,5S,6R)-1,5-dimethyl-2-azabicyclo[4.1.0]heptan-3-imine hydrochloride salt (**9**).* Compound **9** was prepared from **29**-*anti* in 59% yield according to the same procedure as described for the preparation of **2** from **12**. Yellow oil; IR (neat) (cm^{-1}) 3370, 1673, 1522, 1458, 1158, 1114. ^{1}H-NMR (200 MHz, DMSO-d_6) δ 10.12 (brs, 1H), 8.92 (brs, 1H), 8.38 (brs, 1H), 2.44 (dd, J = 16.6, 5.4 Hz, 1H), 2.28 (d, J = 16.6, 7.0 Hz, 1H), 1.98 (m, 1H), 1.36 (s, 3H), 1.06 (d, J = 7.0 Hz, 3H), 1.00–0.90 (m, 1H), 0.85–0.81 (m, 2H). MS and HRMS analysis showed only the ion from $C_8H_{14}N_2$ corresponding to the loss of HCl, MS (FAB, Pos.) *m/z* 139 [M+H]$^+$; HRMS (MALDI-TOF, Pos.) calc. for $C_8H_{14}N_2+H^+$: 139.1235; found: 139.1214. TLC R_f 0.41 ($CHCl_3$–MeOH–AcOH, 10/1/1).

5.2. Biological assays

5.2.1. Preparation of partially purified enzyme and determination of K_i values

Human eNOS was overexpressed in Sf-21 cells, by infecting the cells with baculovirus carrying heNOS cDNA. The hiNOS was overexpressed in A549 by stimulation with LPS (10 μg mL^{-1}) plus cytokines (10 ng mL^{-1} TNF-α, 5 ng mL^{-1} IL-1β and 100 ng mL^{-1} interferon-γ). Human eNOS and iNOS were partially purified by chromatography on 2′, 5′-ADP-Sepharose gels. NOS activity was determined by the method for the conversion of [^{14}C]-L-arginine to L-citrulline with a minor modification. The conversion rates for various concentrations of the test compounds and L-arginine were measured. Dixon and lineweaver-Bulk plots were constructed to determine the K_i values and the mode of inhibition.

Selectivity was evaluated as the rate of the IC_{50} values for heNOS and hiNOS.

5.2.2. Enzyme assay with recombinant mouse iNOS

Recombinant mouse iNOS was purchased from Cayman Chemical (Cat. No. 60862) and the inhibitory activities of the test compounds were evaluated by measuring the conversion rate from [^{14}C]-L-arginine to

[^{14}C]-L-citrulline, and then the IC_{50} values were determined.

The ID_{50} value was determined from a log–logit transformation of the dose–response curves (**5** and **6**; 3, 10, 30 μg kg^{-1}, sc L-NMMA; 10, 30 and 100 mg kg^{-1}, sc). The ID_{50} value was defined as the concentration of test compound that produced a 50% inhibition in the NOx accumulation induced by LPS treatment alone [26]. The MTD was defined as the maximum dose at which no death was observed within 24 h after an iv injection administration. The doses used were 5, 10, 20, 30, 40 and 50 mg kg^{-1} for **5** and **6** and 1000, 2000, 3000, 4000 and 5000 mg kg^{-1} for L-NMMA.

5.2.3. Inhibition of NOx accumulation and the maximum tolerated dose (MTD) in mice

The test compounds or saline were administered subcutaneously at 3 h after the LPS (10 mg kg^{-1}, iv) injection into 7 weeks old Balb/c mice (Charles River Japan, Inc.). Blood was collected by venipuncture from the abdominal aorta under light anaesthesia at 6 h after LPS treatment. Plasma was obtained by centrifugation and the concentration of accumulated NOx over 3 h was determined by the method described in ref. [26]. To evaluate the acute toxicity, the MTD (iv maximum dose where no death was observed within 24 h after the administration) of the test compound was determined.

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