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Original article

New synthesis and promising neuroprotective role in experimental ischemic stroke of ONO-1714

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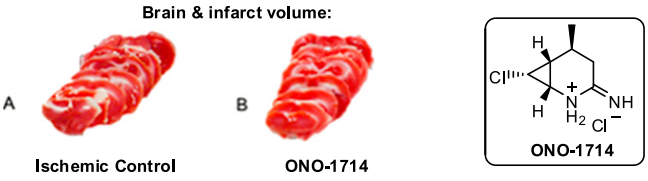
HIGHLIGHTS

- New synthesis of selective iNOS inhibitor ONO-1714.
- ONO-1714 is potentially effective as therapeutic intervention in stroke.
- Results suggest ONO-1714 is a neuroprotective molecule in the context of brain ischemia.

GRAPHICAL ABSTRACT



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ABSTRACT

In an experimental permanent stroke model, we report here the contribution of ONO-1714 to brain damage prevention. Daily drug administration, twenty-one days prior to and two days after an experimental infarct, was performed by using mini-osmotic pumps (ALZET). Infarct volumes were assessed by image analysis of sequential coronal brain 1 mm^3 sections stained following the 2,3,5-triphenyltetrazolium chloride histological staining technique. Results of this study provide evidence of a significant reduction of the brain lesion size, suggesting ONO-1714 as a potential neuroprotective agent in stroke patients. ONO-1714 was prepared in our laboratory following a procedure which resulted in the supply of the desired compound in an easy and excellent yield.

1. Introduction

Stroke is one of the main causes of death and a major cause of long-term disability in the western world. More than 80% of all strokes are caused by cerebral ischemia [1], resulting in devastating neurological sequelae accompanied by severe morphological and molecular alterations [2]. Many advances in the understanding of the mechanisms of ischemic brain injury have been done and many pharmacological approaches to protecting the brain have been considered [3,4]. There are many evidences that inflammation, among other contributing factors, plays an important role in the outcome of ischemic stroke (IS). It is accepted that inflammatory mediators, such as nitric oxide (NO), contribute to brain damage [4]. Since stroke is common and current drug therapies for the management of stroke patients are limited, the progress toward the identification of new targets and the development of more effective new drugs has become a challenging task for prevention and early management of patients [4,5]. Actually, more than 50 neuroprotective agents have been evaluated in promising Phase III clinical trials with disappointing results. Restoration of blood flow needs to be achieved as quickly as possible. Yet, only intravenous administration of the tissue plaminogen activator (tPA), a clot-

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dissolving agent, has been proven to be effective. However, to reap the full benefits of tPA, there is a very short window of opportunity for drug administration [4].

Studies with animals and humans provided increasing knowledge on the mechanisms of cell death following IS, including excitotoxicity, calcium ion [Ca^{2+}] overload, free-radicals, inflammation, and apoptosis [4]. In the past decade several studies have examined the role of the vasodilator mediator NO and its synthesizing enzyme system Nitric Oxide Synthase (NOS) in cerebrovascular diseases, including stroke [6]. NO is an intercellular messenger present in all vertebrates, modulating blood flow, thrombosis, and neural activity. Basically, there are three isoforms of the NOS: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS), each one involved in specific events in the brain. The vascular endothelium synthesizes NO through the action of eNOS, constitutively active, calcium dependent, that generates NO in response to shear stress and other physiological stimuli, and iNOS, transcriptionally regulated, calcium independent, which is produced in response to cytokines and endotoxin signals. Acute expression of highly reactive mediators, such as iNOS, and matrix metalloproteinase-9 (MMP9), participates in brain damage after stroke [6,7]. The reaction of NO with superoxide (O_2^-) to form the much more powerful oxidant peroxynitrite ($ONOO^-$) is a key element in resolving the contrasting roles of NO in physiology and pathology [8]. When ischemia has been developed, overproduction of NO generated by the neuronal or inducible isoforms (nNOS, iNOS) is neurotoxic, thus contributing to brain injury and responsible for the progression of brain damage [9]. This probably occurs through NO-induced formation of peroxynitrite and toxic free radicals leading to damage by lipid peroxidation [10]. NO over-expression has been also reported to stimulate the release of the neurotransmitter glutamate, thus contributing to excitotoxicity [11]. In striking contrast, NO derived from the eNOS isoform is beneficial because plays a prominent role in preventing neuronal injury by maintaining cerebral blood flow [12].

Taking together, available data define iNOS as an important player in IS, and also that pharmacological manipulations of the NO levels in the area of infarct by regulating iNOS activity might prevent neuronal injury and neurological deficits [5]. It has been found that selective iNOS inhibitors significantly reduced infarct volume, which is in striking contrast with non-selective inhibitors that were ineffective. ONO-1714, a cyclic amidine derivative and selective and highly potent inhibitor of rat and human iNOS ($Ki = 1.8$ nM) with a 10-fold selectivity over eNOS, has appeared as a newly developed competitive NOS inhibitor that has selective potency for iNOS [13], but few data has been reported regarding the neuroprotective effect of this compound [14].

ONO-1714 was prepared in our laboratory following a procedure which resulted in the supply of the desired compound in an easy an excellent overall yield. *In vitro* experiments carried out in this study assessed the efficiency of ONO-1714 on the inhibition of NO production in lipopolysaccharide (LPS) and interferon-gamma (INF-γ) stimulated mouse peritoneal macrophages, and on iNOS activity assay. In addition, the effect of ONO-1714 on the pathophysiology of stroke was explored in a model of permanent (p) middle cerebral artery (MCA) occlusion (pMCAO) in mouse. The pMCAO model is accepted as a pre-clinical experimental ischemic model for the evaluation of potential neuro-protective agents in preclinical assays [15,16]. Following this procedure, the results of this study suggest ONO-1714 as a potential neuroprotective agent in stroke patients.

The structure of ONO-1714 is shown in Scheme 1 and it was obtained following a procedure which involved an adaptation of the procedure described by Kawanaka [17], which was thwarted by our initial inability to cleavage satisfactoriously the remaining protecting group, *N*-PMB (*N*-*p*-methoxybenzil), that they proposed. All efforts for the deprotection step proved to be unsuccessful, so that we initiated a similar route shown in Scheme 1, with certain modifications with regard to Kawanaka's one, which resulted in the supply of the desired compound in an easier and better overall yield.

2. Results and discussion

2.1. Chemistry

The new synthesis proposed in this paper required the preparation of the optically active enamide (−)-**1** which synthesis is already described in the literature [18,19]. Thus, the cyclopropanation of (−)-**1** with $CHCl_3$ under alkaline conditions resulted in (−)-*anti*-**2** (45%) (the newly introduced cyclopropane moiety and the methyl moiety showed *anti*-stereochemistry) and (−)-*syn*-**2** (12%) (the newly introduced cyclopropane moiety and the methyl moiety showed *syn*-stereochemistry) respectively, as a separable mixture by column chromatography on silica gel [20].

The mayor isomer (−)-*anti*-**2** was reduced, to remove one chlorine, with tin hydride resulting in an inseparable mixture consisting of an endo-isomer, (−)-*anti-cis*-**3** and an exo-isomer, (−)-*anti-trans*-**3** (2:1) (74%). The mixture obtained was then transformed into the corresponding thioamido derivatives by treatment with Lawesson's reagent. We were pleased to obtain the corresponding mixture of isomeric thiolactams, which could be efficiently separated, by column chromatography on silica gel, to give (−)-*anti-cis*-**4** (48%) and (−)-*anti-trans*-**4** (18%) [21]. In addition, easily removal of the Dmob group from (−)-*anti-cis*-**4** with TFA provided (−)-*anti-cis*-**5** in 70% yield. The deprotected thioamide was then stirred with a saturated solution of NH_3 in methanol to yield the corresponding amidine **6** which was converted into its corresponding hydrochloride salt after acidification of the crude product with HCl—CH_3OH.

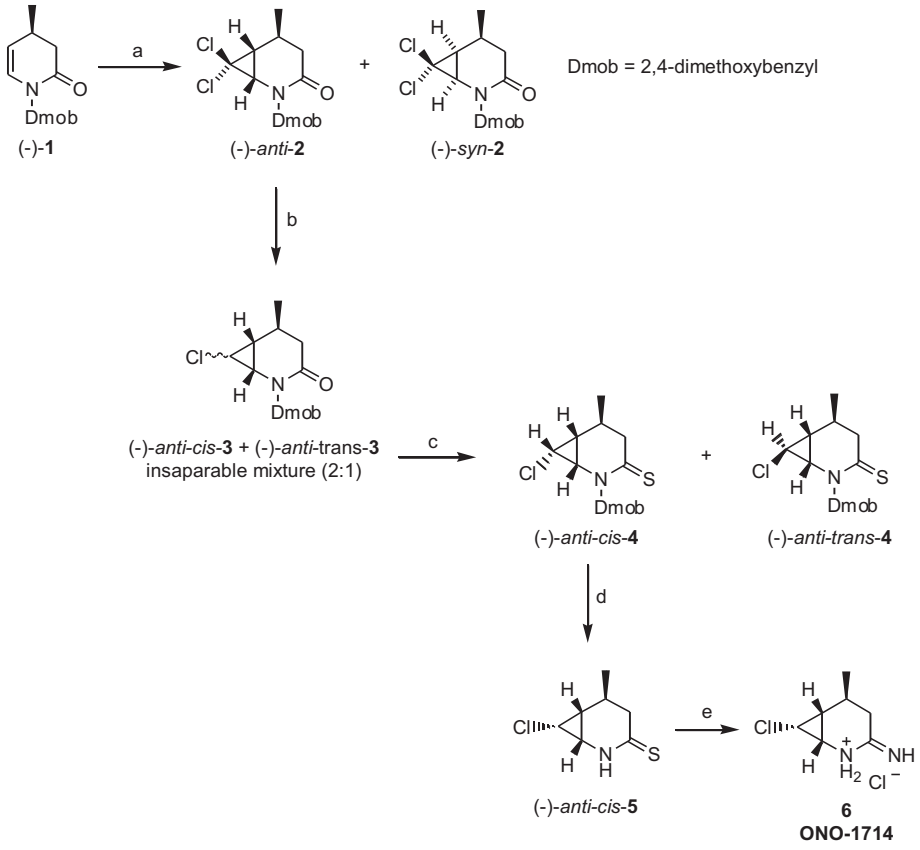
The transformation of the lactam group into the thiolactam system has revealed a convenient way to improve isomer separation and deprotection efficiency. The synthesis of amidine **6** by deprotection of (−)-**3** and afterward transformation of the amide group into thioamide was discarded as the deprotection of a mixture of 3 isomers only provided 35% yield of the corresponding amide.

In view of this efficient optimization we have extended this methodology to the synthesis of several compounds structurally related to **6** with replacement for the chloro group on the cyclopropane ring with different substituents such as $CONH_2$ [18], COOEt [19], CF_3 [18], dichloro, all in racemic and optically active forms. After being transformed into their corresponding amidine hydrochloride salts they were biologically evaluated for their ability to inhibit the iNOS. Replacement of the chloro group showed always a marked reduction of iNOS inhibition [18,19].

2.2. Biological results

2.2.1. Biological in vitro results

Endogenous iNOS inhibition was spectrophotometrically analyzed by using the Griess assay [22]. To confirm the effect of ONO-1714 on NO production, *in vitro* experiments were conducted using a LPS and INF-γ stimulated mouse peritoneal macrophage model [22]. As shown in Fig. 1A, the treatment with LPS (100 ng/mL) and INF-γ (100 ng/mL) markedly increased the production of NO from the basal level following 48 h incubation. When cells were simultaneously treated with various concentrations of ONO-1714, 100 μM, 10 μM, 1 μM and 0.1 μM, and LPS/INF-γ, NO production was significantly down-regulated in a dose-dependent manner. Characteristically, progressive reduction of nitrite-containing in



Scheme 1. Synthesis of ONO-1714. Reagents: (a) aliquat-336, $CHCl_3$, NaOH, rt. Stereochemistry correlations determined by the NOE correlation are described in Ref. 3; (b) Ph_3SnH, AIBN, benzene, 80 °C, (−)-*anti-trans*-**3** and (−)-*anti-cis*-**3** were obtained as an inseparable mixture (1:2); (c) (i) Lawesson's reagent, benzene, 80 °C, Stereochemistry correlations determined by the NOE correlation are described in Ref. 4; (ii) chromatographic separation; (d) TFA, 80 °C; (e) (i) MeOH sat. NH_3, (ii) HCl, MeOH.

macrophage samples correlated well with ONO-1714 concentrations (0.1, 1, 10, 100 μM) (Fig. 1A). The IC_{50} of the product was calculated at 0.82 μM.

The survival of mouse activated macrophages has been determined by the colorimetric sulforhodamine B (SRB) assay, a suitable survival test procedure for the screening of compounds for potential in therapy because it is a fast and efficient method that is reproducible and technically advantageous. The SRB assay is an alternative to the very laborious clonogenic assay that yields comparable results [23]. It is noteworthy that the different concentrations of the iNOS inhibitor employed showed prominent survival rates (means ± S.E.; 98.368 ± 1.52%; 93.588 ± 1.72%; 99.104 ± 0.90%; 97.242 ± 1.61%, respectively) (Fig. 1B). This ruled out the possibility that ONO-1714 might act as a cytotoxic agent. The results in this study are consistent with previous research [13].

Finally, IC_{50} value for inhibition of recombinant murine iNOS was examined using the [^{14}C]-citrulline assay in which the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline was measured. ONO-1714 potently inhibited iNOS with IC_{50} value (0.011 μM) in accordance with data reported previously [17].

2.2.2. Biological in vivo results

Animals were subjected to focal ischemia by pMCAO. After 48 h, the mice were euthanized, the brain removed, cut in coronal slices and stained with TTC to recognize and determine infarct volume. The infarct volume was larger in vehicle-treated mice compared with chronically ONO-1714 treated subjects (Fig. 2). The size of the affected brain area was 4.17 mm^3 + 0.2 mm^3 in untreated animals whereas it was faint, under the limit of detection by the TTC procedure, for the treated group ($p < 0.05$). The model of pMCAO carried out in this work involved the frontal branch of the MCA, whereas the stem of this artery remained untie. This procedure yielded a smaller infarct size than that determined by ligature of the arterial stem, allowing a better assessment of final infarct volume among control and treated groups and also reducing the sample size.

NO generated oxidatively from L-arginine by NOS isoforms, which are dependent on cofactor binding and dimerization to become active [24], mediates many physiological actions in the nervous system, such as neuronal signaling and vasodilation [25]. Under pathological conditions, accumulation of this highly reactive mediator has conversely damaging effects, leading to neurotoxicity in neurodegenerative diseases and after stroke [6,25]. In IS studies of NO functions has generated considerable debate as considered to be either a damaging or protective molecule, depending on the enzyme/cell source [26,27]. All the NOS isoforms present in the brain have influence in stroke regulating brain damage. NO generated primarily by nNOS and iNOS isoforms promotes neuronal devastation after ischemia. Particularly, uncontrolled up-regulation of iNOS is responsible for extensive brain damage [11]. In striking contrast, NO produced by the eNOS isoform in endothelial cells exert a neuroprotective role [28]. Therefore, the design of selective pharmacological regulators of the activity of NOS isozymes is of considerable interest [5,9]. In the field of regulation of NOS activity, it should be aware that NO is an important mediator of the brain physiology [9,29], therefore widespread and excessive down-regulation of NO by non-specific NOS inhibitors, such as substrate analogs of L-arginine, may be detrimental. In fact, inhibition of eNOS worsens injury in experimental stroke, but the use of the selective nNOS inhibitor 7-nitroindazole significantly decreases

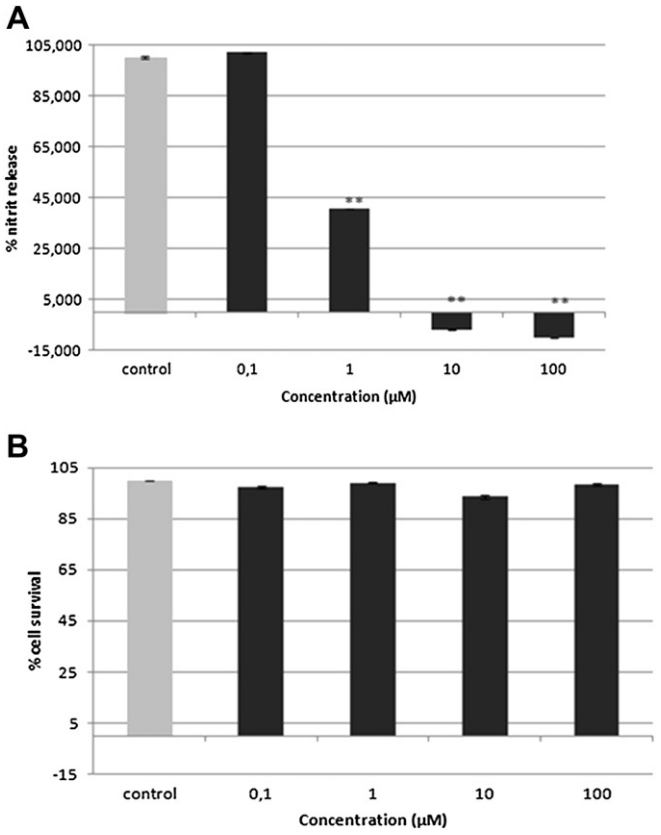


Fig. 1. A ONO-1714 inhibits mouse iNOS activity in culture medium: Spectrophotometer assay showing the percentage of nitrite release from activated mouse macrophages in comparison with non-treated cells (control). Macrophages were incubated, as described in the material and methods section, in the presence of increasing concentrations of ONO-1714. Griess assay shows progressive reduction of nitrite-containing in macrophage samples which correlated well with ONO-1714 concentrations (0.1, 1, 10, 100 μM). IC_{50} is calculated at 0.82 μM. Below 0.1 μM of the inhibitor concentration, nitrite release was unaffected. Data are expressed as percentages of the control. Values represent mean ± SEM, $n = 4$. Asterisks show significant differences as compared to control, $p < 0.05$ was considered as statistically significant as compared to control. B ONO-1714 does not affect macrophage cell survival: Spectrophotometer data obtained with the SRB assay quantitation, showing dose response curves of activated macrophages treated with different ONO-1714 concentrations: 0.1, 1, 10 and 100 μM. Experiments were performed at least four times, and the results are expressed as mean ± standard.

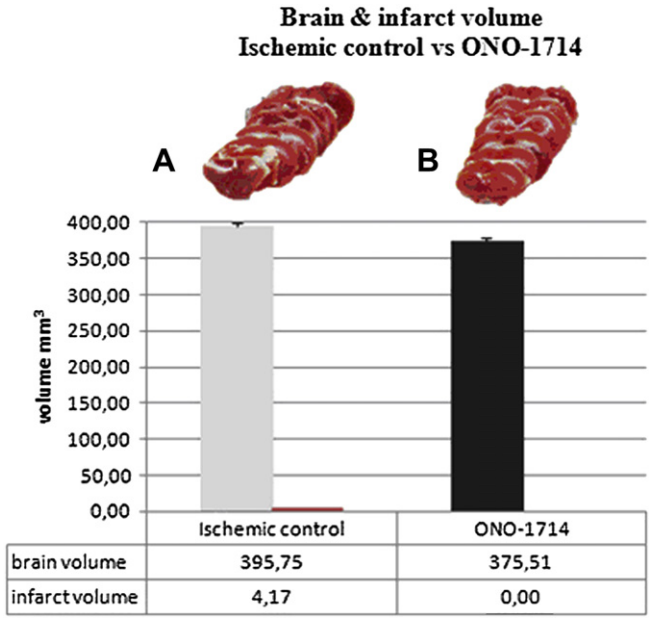


Fig. 2. Chronic treatment with ONO-1714 reduces the infarct volume after pMCAO, frontal branch, in mice. Mice were subjected to 48 h of pMCAO, and volumes of both the whole brain and the infarcted region were measured from TTC-stained serial coronal sections. Representative stacks of seven TTC-stained sections are shown for each group: vehicle treated (A) and ONO-1714 treated (B) mice. No differences between brain volumes were detected between both groups. However, mice treated with vehicle (A) reveal a larger unstained area of ischemic tissue in the infarcted neocortex when compared to their ONO-1714 treated littermates that lack detectable cortical infarct (B). Data are mean ± SEM, $n = 3$; (*; $p < 0.05$).

the infarct volume [30]. When assessed by type of inhibitor, total lesion volume was reduced in permanent models by nNOS and iNOS inhibitors, but not by nonselective inhibitors [5,17]. Inhibition of iNOS, for example, has been largely proposed as a neuroprotective strategy in stroke [31–33]. Therefore, preclinical studies are concerned to a specific NOS isozyme regulation, means of increase NO availability to the vasculature or to restore eNOS activity during IS. Therefore, the discovery and preclinical trial of new nNOS and iNOS inhibitors exhibiting higher affinity against one or both isoforms should be promoted. Herein, we report the preparation of a potent and selective iNOS inhibitor, ONO-1714 [13], that also exert a potent inhibitory action on nNOS [14,34]. *In vitro* results in this study depict ONO-1714 as a potent iNOS inhibitor with excellent pharmacokinetics which is in good agreement with previous studies that might potentially be effective as a novel and potent therapeutic intervention in stroke [13,14].

We proceed to analyze the *in vivo* effect of this molecule in a permanent occlusion model of the frontal branch of the MCA. This procedure produced a very limited cortical infarct, which made easy to statistically compare ischemic lesion volumes between animal groups. All control animals subjected to pMCAO showed infarct. In striking contrast, ONO-1714 treated mice consistently showed no infarct or under the limit of detection using the TTC procedure, suggesting that ONO-1714 is a neuroprotective molecule in the context of brain ischemia. It is well known that animals that underwent focal ischemia induced by pMCAO show significant changes in iNOS expression [7]. Following this stroke model, we have recently found no statistically significant changes among genotypes for nNOS and eNOS isoforms, but a very significant increase for iNOS when compared with control animals [7]. Now, we are showing that chronic administration of the iNOS inhibitor ONO-1714 results in an improvement of the symptoms associated with focal ischemia, particularly a significant reduction of infarct volume. Data concerning the effect of ONO-1714 in stroke in this report are consistent with previous assays that iNOS inhibitors are candidate treatments for acute IS [5,35].

Acute expression of highly reactive mediators, such as iNOS and MMP9, participates in brain damage after stroke [6]. Uncontrolled astrocytic production of NO by iNOS has been found to up-regulate MMP9 [36], proposed as a marker of brain ischemia [37] and predictor of poor outcome and mortality in stroke [38]. Also, it has been proved that chemical or genetic inhibition of MMP9 protects the brain from ischemic injury [38,39]. Therefore, a mechanism of action for ONO-1714 in this scenario might be through down-regulation of MMP9. On the other hand, it has been reported a neuroprotective effect of ONO-1714 on cytotoxicity induced by *N*-methyl-D-aspartate (NMDA) receptor activation, through inhibition of the nNOS isoform [17]. Following acute ischemic or hypoxic injury, release of glutamate into the extracellular space triggers the opening of cation-permeable channels at NMDA receptors contributing to excytotoxic cell death. By regulating NO levels, ONO-1714 might exert neuroprotection through these mechanisms, but further studies are required to confirm this.

3. Experimental protocols

3.1. Chemistry

Melting points (uncorrected) were determined on a Stuart Scientific SMP3 apparatus. Infrared (IR) spectra were recorded with a Perkin–Elmer 1330 infrared spectrophotometer. ^{1}H and ^{13}C NMR values were recorded on a Bruker 300-AC instrument. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (J) are in hertz. Mass spectra were run on a HP 5989A spectrometer. Elemental analyses (C, H, N) were performed on a Perkin–Elmer 2400 CHN apparatus at the Microanalyses Service of the University Complutense of Madrid; all the values are within ±0.4% of the theoretical compositions. Thin-layer chromatography (TLC) was run on Merck silica gel 60 F-254 plates. Unless stated otherwise, starting materials used were high-grade commercial products.

3.1.1. (1S,5S,6R)-7,7-Dichloro-2-(2,4-dimethoxybenzyl)-5-methyl-2-azabicyclo[4.1.0]heptan-3-one (−)-anti-**2** and (1S,5S,6R)-7,7-dichloro-2-(2,4-dimethoxybenzyl)-5-methyl-2-azabicyclo[4.1.0] heptan-3-one (−)-syn-**2**

To a stirred solution of the enamide (−)-**1** (0.48 g, 1.84 mmol) in $CHCl_3$ (4.42 mL) were added aliquat-336 (0.05 mL) and 50% aqueous sodium hydroxide (0.97 g) under an argon atmosphere. The reaction mixture was stirred for 22 h at room temperature. After completing the reaction, the mixture was treated with saturated aqueous ammonium chloride and extracted with Et_2O. The organic layer was washed with brine, dried over magnesium sulfate and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc 4:1–2:1) to afford 0.28 g, (45%) of (−)-*anti*-**2** as a pale yellow solid and 0.09 g (15%) of (−)-*syn*-**2** as a pale yellow oil.

(−)-*anti*-**2**: FW = 344.232. Mp = 89–91 °C. IR (KBr) 1665 (C=O) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.24 (d, 3H, CH_3, J = 6.3 Hz), 1.74 (dd, 1H, H_6, J_1 = 9.8 Hz, J_2 = 5.4 Hz), 2.11–2.20 (d, 1H, CH_2, J_1 = 14.2 Hz), 2.07–2.12 (m, 1H, CH), 2.32 (dd, 1H, CH_2, J_1 = 13.7 Hz, J_2 = 2.9 Hz), 3.08 (d, 1H, H_1, J = 9.8 Hz), 3.82 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 4.17 (d, 1H, CH_2, J = 14.2 Hz), 5.14 (d, 1H, CH_2, J = 13.7 Hz), 6.42–6.45 (m, 2H, ArH), 7.25 (d, 1H, ArH, J = 9.1 Hz). ^{13}C NMR ($CDCl_3$) δ 21.8 (CH_3), 27.7 (C5), 35.3 (C4), 39.2 (CH_2), 42.5 (C6), 44.3 (C1), 55.3 (OCH_3), 55.4 (OCH_3), 64, 52 (C7), 98.1 (Ar*C*H), 104.1 (Ar*C*H), 116.6 (ArC_{ipso}), 132.2 (Ar*C*H), 158.8 (Ar*C*–OCH_3), 160.6 (Ar*C*–OCH_3), 170.6 (C=O). MS (ESI) *m*/*z* (rel%): 367.13 (4), 366.17 $[M + Na]^+$ (37), 151 (100), 121.28 (16). Anal. calcd for $C_{16}H_{19}Cl_2NO_3$: C, 55.83; H, 5.56; N, 4.07. Found: C, 55.99; H, 5.43, N, 3.96.

(−)-*syn*-**2**: FW = 344.232. IR (KBr) 1668 (C=O) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.18 (d, 3H, CH_3, J = 6.6 Hz), 1.92 (dd, 1H, H_6, J_1 = 10.4 Hz, J_2 = 5.5 Hz), 2.15–2.23 (m, 1H, CH_2), 2.36–2.42 (m, 1H, CH), 2.45–2.50 (m, 1H, CH_2), 3.38 (d, 1H, H_1, J = 10.4 Hz), 3.79 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 4.62 (d, 1H, CH_2, J = 14.3 Hz), 4.77 (d, 1H, CH_2, J = 14.3 Hz), 6.40–6.45 (m, 2H, ArH), 7.29 (d, 1H, ArH, J = 8.8 Hz). ^{13}C NMR ($CDCl_3$) δ 21.3 (CH_3), 22.6 (C5), 36.2 (C4), 40.5 (CH_2), 41.6 (C6), 45.1 (C1), 55.2 (OCH_3), 55.3 (OCH_3), 63.8 (C7), 98.3 (Ar*C*H), 104.1 (Ar*C*H), 116.2 (ArC_{ipso}), 130.8 (Ar*C*H), 158.4 (Ar*C*–OCH_3), 160.0 (Ar*C*–OCH_3), 170.1 (C=O). MS (ESI) *m*/*z* (rel%): 346.10 (34), 344.21 $[M + H]^+$ (54), 151.10 (100), 121.27 (10). Anal. calcd for $C_{16}H_{19}Cl_2NO_3$: C, 55.83; H, 5.56; N, 4.07. Found: C, 54.97; H, 5.65, N, 4.13.

3.1.2. (1S,5S,6R,7S)-7-Chloro-2-(2,4-dimethoxybenzyl)-5-methyl-2-azabicyclo[4.1.0]-heptan-3-one (−)-anti-cis-**3** and (1S,5S,6R,7S)-7-chloro-2-(2,4-dimethoxybenzyl)-5-methyl-2-azabicyclo[4.1.0]-heptan-3-one (−)-anti-trans-**3**

To a stirred mixture of (−)-*anti*-**2** (1.00 g, 5.20 mmol) in benzene (6.60 mL) were added Ph_3SnH (2.0 g, 5.7 mmol) and azobisisobutylonitrile (0.04 g, 0.26 mmol). The reaction mixture was stirred with heating at 80 °C for 4 h under an argon atmosphere. After cooling in an ice bath, the reaction mixture was diluted with EtOAc and then washed with 10% aqueous potassium fluoride. The resulting insoluble substances were removed by filtration. The organic layer was washed with brine (10 mL), dried over magnesium sulfate and the solvents were evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc 5:1–2:1) to afford (−)-*anti-cis*-**3** and (−)-*anti-trans*-**3** as an inseparable mixture (2:1), (74%). ^{1}H NMR ($CDCl_3$): δ. Data for major isomer assigned as (−)-*anti-cis*-**3** following assignment of compounds **4**: 1.20 (d, 3H, CH_3, J = 6.3 Hz), 2.19–2.22 (m, 3H, H_4 H_6), 2.25–2.30 (m, 1H, H_5), 2.75–2.81 (m, 1H, H_7), 3.24 (dd, 1H, H_1, J_1 = 5.4 Hz, J_2 = 7.8 Hz), 3.80 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 4.00 (d, 1H, CH_2, J = 14.6 Hz), 5.20 (d, 1H, CH_2, J = 14.6 Hz), 6.44–6.47 (m, 2H, ArH), 7.22–7.25 (m, 1H, ArH). Data for minor isomer assigned as (−)-*anti-trans*-**3** following assignment of compounds **4**:1.20 (d, 3H, CH_3, J = 6.3 Hz), 1.74–1.78 (m, 1H, H_6), 2.12 (d, 1H, H_4, J = 11.1 Hz), 2.28–2.30 (m, 1H, H_5), 2.32 (d, 1H, $H_{4'}$, J = 11.1 Hz), 2.59–2.61 (m, 1H, H_7), 2.78–2.81 (m, 1H, H_1), 3.82 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 4.56 (d, 1H, CH_2, J = 14.2 Hz), 4.70 (d, 1H, CH_2, J = 14.2 Hz), 6.44–6.47 (m, 2H, ArH), 7.27–7.28 (m, 1H, ArH). ^{13}C NMR ($CDCl_3$) δ: Mixture: 21.4 (CH_3), 21.7 (CH_3), 22.6, 24.6, 29.4, 31.1, 34.4, 38.8, 39.2, 39.6, 40.0, 40.2, 42.4 (CH_2), 42.8 (CH_2), 55.3 (4 OCH_3), 98.1 (Ar*C*H), 98.2 (Ar*C*H), 103.9 (Ar*C*H), 104.0 (Ar*C*H), 117.1 (ArC_{ipso}), 117.2 (ArC_{ipso}), 130.9 (Ar*C*H), 131.5 (Ar*C*H), 158.6 (2 Ar*C*–OCH_3), 160.2 (2 Ar*C*–OCH_3), 170.7 (*C* = 0), 172.0 (*C* = 0).

3.1.3. (1S,5S,6R,7S)-7-Chloro-2-(2,4-dimethoxybenzyl)-5-methyl-2-azabicyclo[4.1.0]-heptan-3-thione (−)-anti-cis-**4** and (1S,5S,6R,7S)-7-chloro-2-(2,4-dimethoxybenzyl)-5-methyl-2-azabicyclo[4.1.0]-heptan-3-thione (−)-anti-trans-**4**

To a stirred solution of the lactams mixture of (−)-*anti-cis*-**3** and (−)-*anti-trans*-**3** (0.38 g, 1.27 mmol) in benzene (10 mL) was added Lawesson's reagent (0.80 mmol). The reaction mixture was stirred at 80 °C for 2 h. The solvent was evaporated under reduced pressure. The crude residue was separated by flash column chromatography on silica gel (hexane/EtOAc 4:1) to afford 0.20 g (48%) of (−)-*anti-cis*-**4** as a white solid and 0.07 g (18%) of (−)-*anti-trans*-**4** as a pale yellow oil

(−)-*anti-cis*-**4**: FW = 325.854. Mp = 126–129 °C. IR (KBr) 1605 (C=S) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.15 (d, 3H, CH_3, J = 6.7 Hz), 1.35–1.43 (m, 1H, H_6), 2.08–2.13 (m, 1H, CH), 2.53 (t, 1H, CH_2, J_1 = 12.6 Hz), 2.84 (m, 1H, H_7), 3.12 (dd, 1H, CH_2, J_1 = 15.2 Hz, J_2 = 3.6 Hz), 3.22 (dd, 1H, H_1, J_1 = 7.9 Hz, J_2 = 5.5 Hz), 3.78 (s, 3H, OCH_3); 3.79 (s, 3H, OCH_3), 4.34 (d, 1H, CH_2, J = 14.6 Hz), 6.02 (d, 1H, CH_2, J = 14.6 Hz), 6.42–6.44 (m, 2H, ArH), 7.31 (d, 1H, ArH, J = 9.1 Hz). ^{13}C NMR ($CDCl_3$) δ 14.0 (CH_3), 21.2 (C7), 22.6 (C6), 24.0 (C1), 31.5 (C5), 36.4 (C4), 50.1 (CH_2), 55.3 (OCH_3), 55.4 (OCH_3), 98.3 (Ar*C*H), 104.0 (Ar*C*H), 115.4 (ArC_{ipso}), 131.1 (Ar*C*H), 158.7 (Ar*C*–OCH_3), 160.6 (Ar*C*–OCH_3), 203.5 (C=S). Anal. calcd for $C_{16}H_{20}ClNO_2S$: C, 58.97; H, 6.19; N, 4.30; S, 9.84. Found: C, 58.70; H, 6.02, N, 4.37; S. 9.93.

(−)-*anti-trans*-**4**: FW = 325.854. IR (KBr) 1605 (C=S) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.16 (d, 3H, CH_3, J = 6.7 Hz), 1.48–1.51 (m, 1H, H_6), 1.71–1.77 (m, 1H, CH), 2.49 (dd, 1H, CH_2, J_1 = 15.2 Hz, J_2 = 10.9 Hz), 2.63–2.65 (m, 1H, H_7), 2.84 (dd, 1H, H_1, J_1 = 9.1 Hz, J_2 = 1.8 Hz), 2.98 (dd, 1H, CH_2, J_1 = 12.2 Hz, J_2 = 3.6 Hz), 3.79 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 5.14 (d, 1H, CH_2, J = 14.0 Hz), 5.40 (d, 1H, CH_2, J = 14.0 Hz), 6.42–6.44 (m, 2H, ArH), 7.33 (d, 1H, ArH, J = 7.9 Hz). ^{13}C NMR ($CDCl_3$) δ 20.8 (CH_3), 30.4 (C7), 30.7 (C6), 38.5 (C5), 38.6 (C1), 41.8 (C4), 49.8 (CH_2), 55.4 (OCH_3), 55.5 (OCH_3), 98.3 (Ar*C*H), 104.3 (Ar*C*H), 115.8 (ArC_{ipso}), 132.1 (Ar*C*H), 158.8 (Ar*C*–OCH_3), 160.9 (Ar*C*–OCH_3), 201.2 (C=S). Anal. calcd for $C_{16}H_{20}ClNO_2S$: C, 58.97; H, 6.19; N, 4.30; S, 9.84. Found: C, 58.90; H, 6.23, N, 4.42; S. 9.78.

3.1.4. (1S,5S,6R,7R)-7-Chloro-5-methyl-2-azabicyclo[4.1.0]heptan-3-thione (−)-anti-cis-**5**

A solution of (−)-*anti-cis*-**4** (0.12 g, 0.33 mmol) in trifluoroacetic acid (0.60 mmol) was refluxed for 16 h. The solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc 4:1) to afford 0.04 g (70%) of (−)-*anti-cis*-**5** as a white solid. FW = 175.680. IR (KBr) 1655 (C=S) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.21 (d, 3H, CH_3, J = 6.7 Hz), 1.38–1.44 (m, 1H, H_6), 1.95–2.00 (m, 1H, CH), 2.35 (dd, 1H, CH_2, J_1 = 15.6 Hz, J_2 = 12.2 Hz), 2.92–2.96 (m, 1H, H_7), 2.97–3.01 (m, 1H, CH_2), 2.95 (dd, 1H, H_1, J_1 = 15.9 Hz, J_2 = 3.8 Hz), 8.43 (s(a), 1H). ^{13}C NMR ($CDCl_3$) δ 21.2 (CH_3), 21.4 (C5), 26.7 (C7), 32.5 (C6), 39.0 (C1), 47.1 (C4), 199.7 (C=S). MS (ESI) m/z (rel%): 312.12 (72), 270.07 (100), 175.98. $[M + H]^+$ (6), 151.04 (17), 102.19 (15). Anal. calcd for $C_7H_{10}ClNS$: C, 47.86; H, 5.74; N, 7.97; S, 18.25. Found: C, 47.73; H, 5.62, N, 8.05; S. 18.34.

3.1.5. (1S,5S,6R,7R)-7-Chloro-5-methyl-2-azabicyclo[4.1.0]-heptan-3-imine hydrochloride salt **6**

A solution of (−)-*anti-cis*-**5** (0.03 g, 0.15 mmol) in saturated methanolic ammonia (1.00 mL) was stirred at room temperature under an argon atmosphere for 46 h. Concentration of the reaction mixture under reduced pressure gave a residue which was diluted with acetone (1 mL) with formation of a precipitate which was removed by filtration. The solid was converted into its hydrochloride salt by solving in CH_3OH and acidification to pH 3.5 with anhydrous HCl–CH_3OH (0.50 mL, prepared from 0.30 mL of AcCl in 8.00 mL of CH_3OH). After concentration under reduced pressure the residue was disgregated in acetone and filtered to afford the corresponding hydrochloride salt which was disgregated with acetone to afford 0.02 g (45%) of 6 as a white solid. $[\alpha]_D^{25} = +62.12$ (c 0.03, MeOH), (lit. $[\alpha]_D^{25}$ +68.10 (c 1.00, MeOH)). FW = 195.089. Mp = 234–235 °C. IR (KBr) 1690 cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.18 (d, 3H, CH_3, J = 6.7 Hz), 1.43–1.50 (m, 1H, H_6); 1.78–1.86 (m, 1H, CH), 2.44 (d, 2H, CH_2, J = 10.9 Hz), 3.05 (dd, 1H, H_7 J_1 = 8.5 Hz, J_2 = 5.5 Hz), 3.65 (dd, 1H, H_1, J_1 = 7.3 Hz, J_2 = 5.5 Hz), 8.50 (s(a), 1H), 9.20 (s(a), 1H), 9.70 (s(a), 1H). ^{13}C NMR (DMSO-d_6) δ 21.2 (CH_3), 22.7 (C5), 25.2 (C7), 29.3 (C6), 32.7 (C1), 51.7 (C4), 169.17 (C=NH). MS (ESI) m/z (rel%): 225.21 (13), 161.09 (25), 159.09 $[M + H]^+$ (100). Anal. calcd for $C_7H_{12}Cl_2N_2$: C, 43.10; H, 6.20; N, 14.36. Found: C, 43.21; H, 6.36, N, 14.43.

3.2. Biological assays

DMEM, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, U.S.A.). Lipopolysaccharides from *Escherichia coli* 026:B6 (LPS, Sigma, L8274), interferon-gamma from rat (INF-γ), sulforhodamine-B (SRB), Griess reagent, and 2,3,5-triphenyltetrazolium chloride (TTC) were purchased form Sigma (St. Louis, MO U.S.A.). Alzet mini-osmotic pumps (Alzet, model 2004). Isoflurane (Isobar®Vet, Schering-Plough, Middlesex, UK). Recombinant murine iNOS was purchased from Cayman Chemical (Cat. No. 60864). Stock solutions of ONO-1714 were prepared in Dimethyl sulfoxide (DMSO, Appli-Chem). L-[^{14}C]arginine (Amersham), Heparin (Chiesi, 962357.9). Griess' reagent for nitrite (Fluka, 03553).

3.2.1. Animals

Hsd:ICR (CD-1) mouse (n = 24) derived from animals from Charles River Laboratories, Wilmington, Massachusetts, and C57BL/6JOlaHsd male mice (n = 6) of 12 weeks, were purchased from Harlan Laboratories. Animals were kept in a temperature-controlled room with a light/dark cycle 12:12, having access to food and water *ad libitum*. All procedures comply with the rules of the European Union Directive (86/609/EEC) and the protocol approved by the Animal Welfare Committee of the Cajal Institute.

3.2.2. Cell culture

Resident peritoneal macrophages were recovered from CD-1 mice by instilling and withdrawing 9 mL sterile phosphate-buffered saline (PBS) (pH 7.4) solution, containing 0.2% heparin, from the peritoneal cavity. Collected cells were washed, resuspended in culture medium, and seeded (1×10^6 cells/well) in tissue culture plate (Falcon, 24 well), and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with inactivated 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C, for 48 h.

3.2.3. Nitrite assay. NO production and quantification

The inhibitory activity of the test compounds on LPS plus INF-γ induced NO production was evaluated. After 48 h in culture, cell supernatants were replaced with fresh medium containing LPS (100 ng/mL) plus IFN-γ (100 ng/mL) in the presence or absence of test material: medium only (negative control) or IFN-γ and LPS (positive control). After additional 48 h incubation, nitrite released into the supernatants of mouse macrophages was determined by the standard Griess reaction by adding 50 μL of test solution to 96-well flat-bottomed plates containing 50 μL of Griess reagent [1% sulfanilamide/0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride/2.5% H_3PO_4]. The samples were assayed in quadruplicate. After 15 min at room temperature, the absorbance of each well was measured in a FLUOstar OPTIMA-BMG LABTECH microplate reader at 540 nm and the nitrite concentration was determined from a standard curve of sodium nitrite. The percentage inhibition was expressed as: (nitrite level of test samples/nitrite level of vehicle-treated control) × 100. All experiments were repeated at least two times.

3.2.4. Determination of cell viability

In parallel with the Griess assay, macrophages from each well were subjected to a cell viability assay using the SRB test. This method measures the cellular protein content of adherent or suspension cultures, according to a previous report [40]. The SRB is a pink aminoxanteno dye with two sulfonic groups. Sulfonic groups come together, electrostatically with the basic amino acid residues of the protein in mildly acidic conditions. This technique of staining allowed us to study the inhibition of cell growth. The SRB bind to cellular proteins was measured spectrophotometrically at 540 nm, which provides information on the relative cell growth and viability of the cells. Cells were fixed for 1 h at 4 °C by adding 200 μL per well of trichloroacetic acid (TCA) to 12.5% (v/v) in double distilled water. Then, the fixative solution was removed vigorously and the samples thoroughly washed with tap water. Plates were then dried with cold air. Once fixed, the cells were stained with 0.4% SRB (w/v) in 1% acetic acid (putting 100 μL per well) for 30 min stirring at room temperature. After this time, the SRB was removed and the cultures were washed with 1% acetic acid to remove residual dye. After optimum drying, dye bound to proteins was solubilized with 10 mM Trizma base (putting 200 μL per well) for 30 min with stirring at room temperature. Then procedure to read the optical densities was performed by using a plate reader at 540 nm wavelength.

3.2.5. Enzyme assay with recombinant murine iNOS

The concentration of the inhibitor producing 50% inhibition (IC_{50}) was obtained by measuring per cent inhibition at least seven concentrations of inhibitor. The assay media contained 20 μM [^{14}C]-L-arginine. The IC_{50} value is the means of three individual experiments. The iNOS enzyme activity was measured by [^{14}C]-citrulline assay, by monitoring the conversion of [^{14}C]-L-arginine to [^{14}C]-L-citrulline. The details of the procedure have already been described [41]. The radioactivity was then measured in a liquid scintillation

counter (LS 6500, M-P Scintillation Counter, Beckman) and expressed as counts per minute (cpm) per mg of protein per minute (data not shown).

3.2.6. Alzet osmotic pumps. Permanent focal ischemia model

Chronic administration of the compound was performed subcutaneously by using Alzet mini-osmotic pumps (model 2004, 200 μL, 0.25 μL/h) placed subcutaneously. The daily pumping rate was 24 μg/kg of ONO-1714 dissolved in dimethyl sulfoxide (DMSO). For the ischemic control group, pumps were filled with the vehicle. The treatment was dispensed for 21 days before and 48 h after pMCAO. Mice (3 per group) were anesthetized with 3% isoflurane (in 70% N_2O, 30% O_2) for induction and with 2% isoflurane for maintenance. Rectal temperature was maintained at 36.5 °C with use of a heating pad. The frontal branch of the MCA was exposed and occluded permanently by suture ligation as previously reported with modifications [16]. Briefly, an incision perpendicular to the line connecting the lateral canthus of the left eye and the external auditory canal was made to expose and retract the temporalis muscle. A burr hole was drilled, and frontal and parietal branches of the MCA were exposed by cutting and retracting the dura. The frontal branch of the MCA was elevated and ligated with a suture nylon monofilament 8/0. Following ligation, a sharp decrease of blood flow was evinced by a laser Doppler flowmetry (Järfalla, Sweden). We selected exclusively for this study animals that showed post-ligature a drop of blood flow of at least 65% and exclude animals which have undergone surgery for longer than 15 min. Following surgery, subjects were returned to their cages, kept at room temperature and allowed free access to food and water. Physiological parameters: rectal temperature, mean arterial pressure and blood glucose levels, were not significantly different between studied groups. Rectal temperature was controlled at ±37.0 °C throughout experiment by using a temperature-regulated heating pad. Measurements of hemodynamics parameters, taken 15 min after the onset of pMCAO, were: mean blood pressure 84.4 ± 3.2 and blood glucose level 132.4 ± 2.3 mg/dl. The experiments compared an untreated ischemic control group ($n = 3$) with an ischemic group treated with ONO-1714 ($n = 3$).

3.2.7. Determination of infarct size by TTC

Two days after pMCAO, animals were killed by neck dislocation to assess infarct outcome. Brain was removed and cut into seven 1 mm-thick coronal brain slices (Brain Matrix, WPI, UK) and stained with TTC (1% TTC in 0.1 M phosphate buffer) [42]. Infarct volumes were calculated sampling each side of the coronal sections with a digital camera (Pentax Optio S7), and the images were analyzed using ImageJ 1.33u software (National Institutes of Health, Bethesda, MD). The digitized image was displayed on a video monitor. With the observer masked to the experimental conditions, the contralateral hemisphere perimeter was overlapped onto the ipsilateral hemisphere to exclude edema, and infarct borders were delineated with an operator controlled cursor. The area of infarct, which was unstained, was determined by counting the pixels contained within the outlined regions of interest and expressed in square millimeters. Infarct volumes (in mm^3) were integrated from the infarct areas over the extent of the infarct calculated as an orthogonal projection. All animals in the control group displayed infarcts after the occlusion procedure, which included exclusively the cerebral cortex.

3.2.8. Statistical analysis

Results are expressed as the means ± S.E.M. *t*-test was used to determine the statistical significance of differences between the means, and p value of <0.05 was considered significant.

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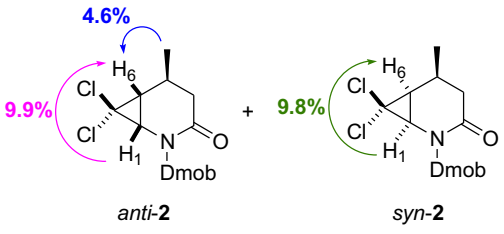
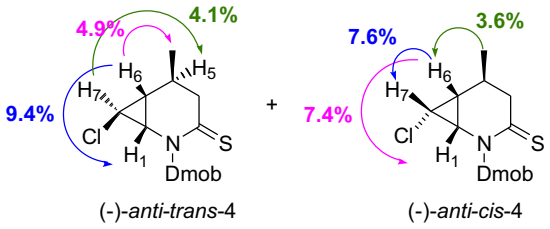
[20] Stererochemical assignment for **2**.



anti-**2** *syn*-**2**

[21] Stererochemical assignment for **4**.



(-)-*anti-trans*-4 (-)-*anti-cis*-4

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