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Research paper

Novel routes to either racemic or enantiopure α-amino-(4-hydroxy-pyrrolidin-3-yl)acetic acid derivatives and biological evaluation of a new promising pharmacological scaffold

Samy Cecioni [a], Kaïss Aouadi [a, 1, 2], Julie Guiard [a, 2], Sandrine Parrot [b, 2], Nathalie Strazielle [c], Sandrine Blondel [c], Jean-François Ghersi-Egea [c], Christian Chapelle [e], Luc Denoroy [b, d, *], Jean-Pierre Praly [a]

[a] CNRS UMR 5246, Université Lyon1, Institut de Chimie et Biochimie Moléculaires et Supramoléculaires (ICBMS), F-69622 Villeurbanne, France
[b] INSERM U1028, CNRS UMR5292, Université Lyon 1, Lyon Neuroscience Research Center, NeuroDialyTics Unit, Lyon F-69000, France
[c] INSERM U1028, CNRS UMR5292, Université Lyon 1, Lyon Neuroscience Research Center, Blood Brain Interfaces Exploratory Platform BIP, Lyon F-69000, France
[d] INSERM U1028, CNRS UMR5292, Université Lyon 1, Lyon Neuroscience Research Center, BioRaN, Lyon F-69000, France
[e] Université de Lyon, Pulsalys, F-69000 Lyon, France

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ABSTRACT

Cycloaddition between (+) or (−)-menthone-derived nitrones and *N*-benzyl-3-pyrroline afforded enantiopure spiro-fused heterocycles. The reaction occurred enantio- and diastereo-selectively on the less hindered side of the nitrone, the 3-pyrroline *N*-benzyl group being oriented outwards, thus controlling the configurations of three simultaneously created chiral centers. From either (+) or (−)-menthone, both enantiomeric cycloadducts were synthesized in excellent yield. Removing the chiral auxiliary and the *N*-benzyl group delivered a series of enantiopure 4-hydroxy-3-glycinyl-pyrrolidine derivatives in 3–5 steps and 36 to 81 overall yields. Using two other achiral nitrones, shorter routes to racemic analogues were developed. Two of the synthesized compounds markedly lowered extracellular glutamate level and modestly interacted with cannabinoid type-1 receptors. As these two neuroactive compounds were devoid of *in vitro* toxicity and did not cross the blood brain interface, they might represent potential pharmacological agents to target peripheral organs.

1. Introduction

Naturally occurring and synthetic alkaloids are long known bioactive products which have found numerous medicinal applications. Consequently, their chemistry is well developed, and they are accessible through a number of methods. Among them, cycloaddition reactions between unsaturated moieties acting as dipolarophiles and various dipoles represent direct and popular methods, which can be applied with tight selectivity as exemplified in our recent study using chiral nitrones [1]. In addition to their synthetic interest, 5-membered heterocycles display interesting properties and bioactivities. For example, isoxazolidinyl nucleosides can be found among heterosubstituted nucleoside analogs [2], while a new class of small molecule transcriptional inhibitors includes a series of substituted isoxazolines [3–5]. Pyrrolidine-containing iminosugars are receiving continuous attention as they have high therapeutic potential in the treatment of diseases such as diabetes, cancer, and lysosomal storage disorders [6,7]. Pyrrolidine and proline derivatives have proved useful for the synthesis of inhibitors binding to neuronal nitric oxide synthase [8],

Abbreviations: Glu, glutamate; CB, cannabinoids; CSF, cerebrospinal fluid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione or (IUPAC name) 7-nitro-2,3-dioxo-1,4-dihydroquinoxaline-6-carbonitrile; HPLC, high performance liquid chromatography.

* Corresponding author. CNRS UMR5292, INSERM U1028, Université Lyon 1, Lyon Neuroscience Research Center, BioRaN team, 8 Avenue Rockefeller, F-69373 Lyon Cedex 08, France.
E-mail address: luc.denoroy@univ-lyon1.fr (L. Denoroy).

[1] Present address: Université de Monastir, Laboratoire de Synthèse Hétérocyclique, Produits Naturels et Réactivités, Faculté des Sciences de Monastir, Avenue de l'Environnement, 5000 Monastir, Tunisia.

[2] Author contributions: K.A., J.G., and S.P. contributed equally to the work.

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the development of new drug candidates for hepatitis C treatment [9–11] and the design of insulin-like growth factor-I and its derivatives as potential neuroprotective agents [12,13].

Our studies on the [3 + 2] cycloaddition of menthone-based nitrones [1,14] as glycine equivalents reacting with different substituted alkenes first aimed at the synthesis of stereoisomers of 4-hydroxy-isoleucine [15–17], a naturally-occurring molecule found in fenugreek (*Trigonella foenum-graecum)*, a plant traditionally used as an antidiabetic remedy, due to the insulinotropic properties of (2*S*,3*R*,4*S*)-4-hydroxy-isoleucine. The menthone-derived spiro-bicyclic nitrones react preferentially through their less hindered face, opposite to the nitrone isopropyl group (Scheme 1). With a *Z* symmetrical alkene, the reaction occurs through one preferred transition state (TS) with minimized steric interactions to afford a stereochemically defined cycloadduct [1,15,17]. This design is synthetically attractive because of: a) stereocontrolled creation of up to three stereogenic centers in one step; b) atom economy due to recovery of the chiral auxiliary; c) access to both enantiomers depending on the choice of the nitrone; d) mildness of the reaction conditions. The cycloaddition of such chiral nitrones with either *E*-, or *Z*-1,4-dichloro-but-2-ene afforded enantiopure dichlorinated isoxazolidines suitable for further elaboration. Generating amine groups at two different sites allowed for subsequent cyclizations, to afford two pyrrolidine scaffolds, of either the 3-substituted-4-hydroxy-proline, or 3-glycinyl-4-hydroxy-pyrrolidine type [1]. The first ones appeared to be precursors toward the synthesis of kainic acid and kainoids [1,18]. Kainic acid is a naturally occurring amino acid used *in vivo* for its neuroexcitatory and epileptogenic properties with induction of seizures [19]. The 3-glycinyl-4-hydroxy-pyrrolidine scaffold corresponds to surprisingly unexplored amino acid mimics related to iminocyclitols, and structural analogs of gamma-aminobutyric acid (GABA), an important inhibitory neurotransmitter. Therefore, on a structural basis, these novel molecules are close to classes of synthetically and biologically important molecules (Fig. 1).

Compared to our approach with *Z*-1,4-dichloro-but-2-ene and chiral nitrones [1], accessible 3-pyrrolines were obviously advantageous dipolarophiles for more direct syntheses towards the 3-glycinyl-4-hydroxy-pyrrolidine scaffold, as disclosed in our recent patent [20]. We now describes synthetic routes to 3-glycinyl-4-hydroxy-pyrrolidine derivatives, based on cycloaddition between *N*-protected 3-pyrrolines and either menthone-based nitrones or achiral nitrones, to deliver in few steps enantiopure or racemic products (Scheme 1), along with their biological evaluation.

Because of strong analogies of the designed structures with existing bioactive molecules mimicking amino acids neurotransmitters (Fig. 1), effects on the same targets were expected for these pyrrolidine derivatives. More precisely, these new molecules were hypothesized to act on brain glutamate (Glu) system or on brain GABA system. In order to test this hypothesis, an *in vitro* screening was performed to determine on which receptors some of the new molecules obtained may act. Unexpectedly, none of the compounds

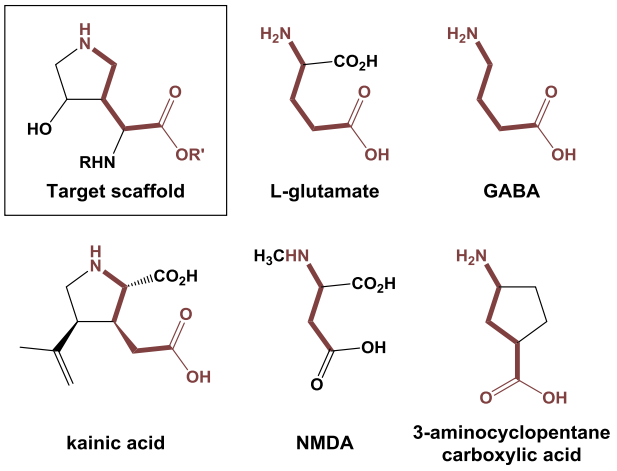


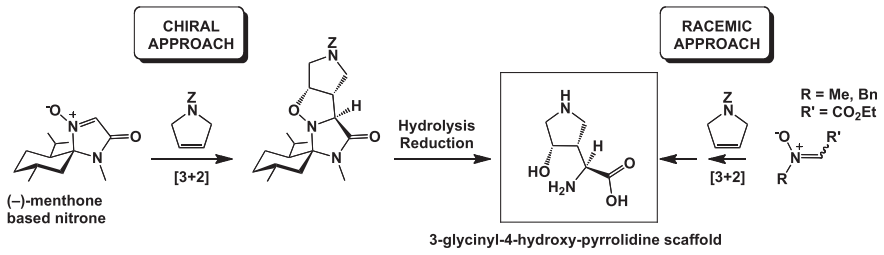
Fig. 1. Target molecules and structural analogs mimicking neurotransmitters as excitatory glutamate and inhibitory GABA.

bound to the various glutamatergic or GABA-ergic sites, but two of them exhibited weak interaction with cannabinoid receptors, a result which nevertheless allowed hypothesizing that they may indirectly alter brain Glu level. A brain microdialysis experiment, performed to test this second hypothesis, revealed that these two compounds induced a marked fall in extracellular Glu concentration. Finally, bidirectional permeability experiments across an *in vitro* blood-brain interface were carried out to investigate whether these two compounds could act at both peripheral and central levels after a systemic administration.

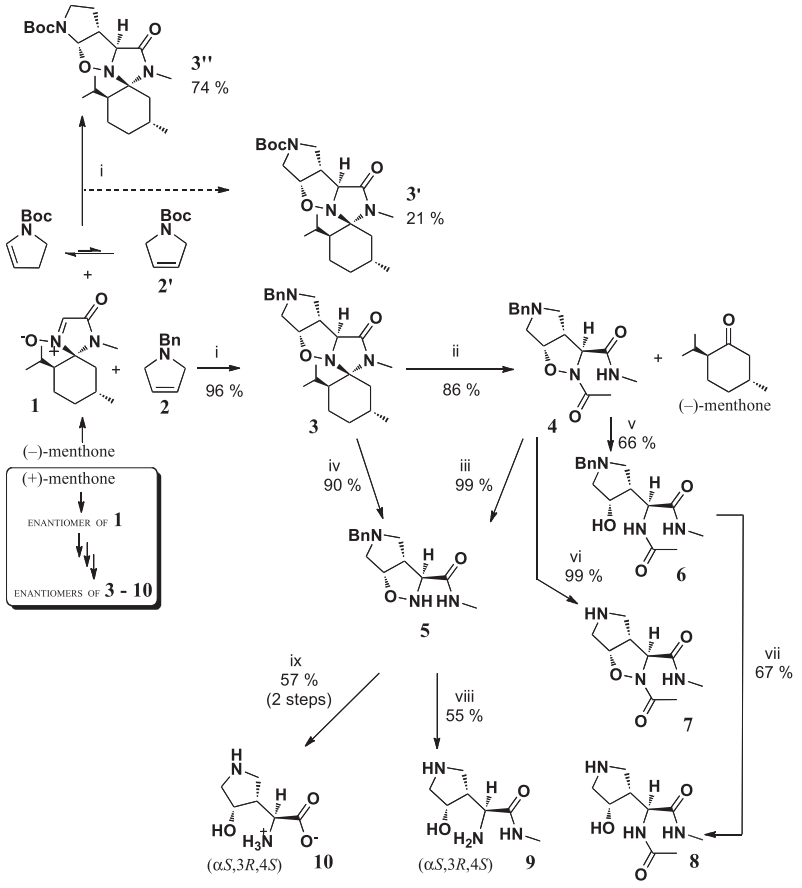
2. Results and discussion

2.1. Chemical synthesis

Cycloaddition between the (−)-menthone-derived nitrone **1** and commercial *N*-benzyl-3-pyrroline (**2**) was completed after overnight reflux in toluene or within 2 h under microwave activation, to afford cycloadduct **3** in 96% yield (Scheme 2). This route was straightforward compared to our recent synthesis of the debenzylated analog of **3** [1]. In a similar cycloaddition (see Supplementary Data, S4 and S5), the less reactive *N*-Boc-3-pyrroline (**2′**) reacted within 10 h (microwave activation), to afford **3′** the *N*-Boc analog of **3** (21% yield), and predominantly an isomeric cycloadduct **3"** (74% yield), arising from a regio-, and stereo-selective cycloaddition of nitrone **1** to *N*-Boc-2-pyrroline (Fig. 2, Scheme 2). Therefore, the synthetic work was further developed with *N*-benzyl protected products derived from readily available cycloadduct **3**. Based on the experience accumulated in previous syntheses, structure elucidation of the cycloadducts **3** and **3′** was straightforward. However, diffraction analysis of crystals



Scheme 1. Routes to 3-glycinyl-4-hydroxy-pyrrolidines: racemic and chiral design from 3-pyrrolines [1].



Scheme 2. Synthesis of enantiopure α-amino-(4-hydroxy-pyrrolidin-3-yl)acetic acid derivatives by cycloaddition of (−)-menthone-derived nitrone **1** and *N*-benzyl-3-pyrroline **2**. Reagents and conditions: (i) toluene, μW, 140 °C, 2 h and 10.5 h with, respectively, **2** and **2'**; (ii) 1/1:1 Ac_2O/AcOH, ε H_2SO_4, 60 °C; 2/5M aq NaOH added dropwise; (iii) $SOCl_2$, MeOH, reflux, 30 min; (iv) 1/1:1 Ac_2O/AcOH, ε H_2SO_4, 46 °C, 6h30; 2/5M aq NaOH added over 2h30, 0 °C; (v) $Mo(CO)_6$, 15:1 CH_3CN/H_2O; (vi) H_2, $Pd(OH)_2$/C, EtOH, CH_2Cl_2, 14 h; (vii) H_2, $Pd(OH)_2$/C, EtOH, 2 h; (viii) H_2, $Pd(OH)_2$/C, EtOH/CH_2Cl_2 and AcOH, ~12 h; (ix) 1/1atm H_2, $Pd(OH)_2$/C, EtOH/CH_2Cl_2 and AcOH, 24 h; 2/LiOH, THF/H_2O, H^+ Dowex resin.

obtained for *N*-benzyl **3** and *N*-Boc **3″** were carried out so as to confirm fully their structures (Fig. 2). That of **3″** is best explained by a cycloaddition taking place between nitrone **1** and the *N*-conjugated 2-pyrroline arising from an unexpected rearrangement of 3-pyrroline **2'** (Scheme 2). Hence, crystal analysis provided easily unambiguous structural proofs for the obtained cycloadducts, in particular for isomeric *N*-Boc **3″** whose structure would have been difficult to establish by NMR analysis only. There was no regioselectivity issue in the cycloadditions of **1** to symmetrical 3-pyrrolines **2** and **2'**. Interestingly, the 74% yield recorded for **3″** denoted a high regioselectivity for the cycloaddition to the 2-pyrroline isomer, probably due to both steric and electronic factors.

Removal of the chiral auxiliary in **3** was attempted by acidic (3N HCl, AcOH, 80 °C) and basic ($LiOH.H_2O$, THF/H_2O) treatments, as developed previously for cycloadducts derived from acyclic alkenes [1,14–17]. However under such acidic treatment at temperature below 60 °C and under other mild acidic conditions, no reaction occurred, while with 3N HCl partial conversion of **3** occurred (31 and 80% at 60, and 85 °C respectively), to afford the desired product **5**, in ~40% yield. Probably the presence of the basic nitrogen atom

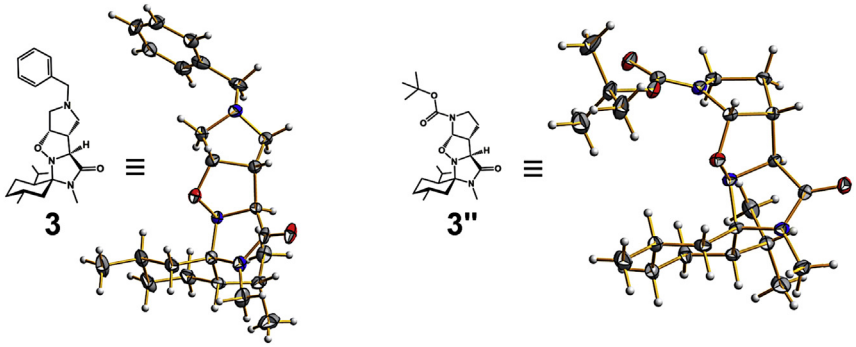


Fig. 2. View of the atomic structures of **3** and **3″** determined by single-crystal X-ray diffraction analysis (displacement ellipsoids are plotted at the 50% probability level).

present in **3** affected the cleavage, which was finally achieved by acetolysis, in a mixture of Ac_2O/AcOH and with a catalytic amount of H_2SO_4. After neutralization with 5M NaOH added dropwise, the *N*-acetyl-methylamide-pyrrolidine **4** was obtained in 86% yield. Gratifyingly, extended reaction times and slower basic quenching (over 2.5 h) afforded directly the methylamide **5** in a 90% yield from **3**. Based on thin-layer chromatography (TLC) monitoring of these reactions, it was assumed that this protocol preserved an acidic medium for a prolonged time, so that protonation of the isoxazolidine nitrogen atom prevented its acetylation in the first period of the treatment before hydrolysis of Ac_2O had occurred. Otherwise, selective *N*-deacetylation of **4** to **5** was quantitatively achieved upon treatment in anhydrous MeOH acidified with $SOCl_2$ with brief heating. The chiral auxiliary (−)-menthone was recovered in yield as high as 80% during the purification process and has been checked to be reusable for the generation of chiral nitrone.

The next steps consisted in reductive treatments for achieving *N*-debenzylation and/or reductive cleavage of the N—O bond. Cleavage of the N—O bond occurred chemoselectively upon treatment with molybdenum hexacarbonyl to afford **6** in 66% yield [21]. *N*-Debenzylation was achieved by standard catalytic hydrogenolysis at atmospheric pressure in the presence of $Pd(OH)_2$/C in EtOH with another cosolvent (CH_2Cl_2, H_2O, AcOH). Interestingly, depending on their structure, the molecules studied displayed various reactivities. For example, the *N*-acetyl methylamide **4** was debenzylated in excellent yield to afford **7** after a 14 h treatment, without reductive opening of the N—O bond, probably more resistant due to *N*-acetylation. In contrast, the *N*-benzyl hydroxypyrrolidine **6** was converted within 2 h to **8** (67% yield). For achieving both *N*-debenzylation and reductive opening of compound **5,** long reaction times were needed. Thus, **5** was converted to the rather polar methylamide **9** within 12 h, while enantiopure 3-glycinyl-4-hydroxy-pyrrolidine **10** required a 24 h reductive treatment. Compound **10** was isolated after a final basic hydrolysis and purification over acidic resin. As stereochemistry can be of crucial importance as regard to bioactivities, the syntheses were also carried out similarly with the (+)-menthone derived nitrone ***ent*-1**, so as to deliver the corresponding enantiomers ***ent*-3** to ***ent*-8** and ***ent*-10**. All the products were studied by NMR spectroscopy (see Supplementary data). No epimerization was observed. Amino acids **10**, ***ent*-10**, and **(±)-10** (*vide infra*) gave identical well resolved ^{1}H NMR spectra in D_2O. When the D_2O solution was made basic by adding LiOH, the ^{1}H NMR spectrum of ***ent*-10** changed significantly (see Supplementary data, Fig. S1), as the zwitterionic form was presumably displaced to the corresponding lithium salt.

We also considered a racemic approach to the target molecules, using easily accessible nitrones, like methyl nitrone **11** [22—24], benzyl nitrone **12** [25,26], and cyano nitrone **13** [27], obtained as *E*/*Z* mixtures representing achiral glycine equivalents (Scheme 3). Cycloaddition between nitrone **11** and *N*-benzyl-3-pyrroline **2** proceeded within 1 h under microwave activation, to deliver cycloadduct **(±)-14** (58% yield) which was subjected to hydrogenolysis to afford **(±)-15** (90% yield) as ester hydrolysis occurred in aqueous THF. Under the similar conditions nitrone **12** led to cycloadduct **(±)-16** (68% yield). Catalytic hydrogenation achieved both bis-debenzylation and reductive opening of the N—O isoxazolidine bond, affording, due to concomitant ester hydrolysis, racemic amino acid **(±)-10** in quantitative yield.

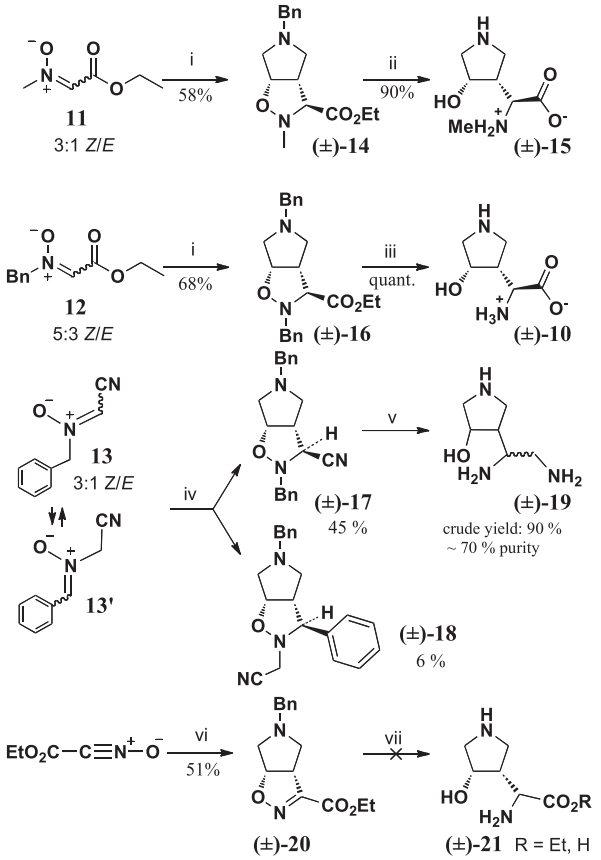
Cycloaddition of nitrone **13** and **2** proceeded also readily within 2 h to afford two cycloadducts **(±)-17** and **(±)-18** (45% and 6% isolated yield). While structure **(±)-17** was expected, that of **(±)-18**, established by 2D NMR spectroscopy, indicated the involvement of an isomeric nitrone (**13′**) in the cycloaddition with **2**. No evidence of the contamination of **13** by **13′** was observed. Catalytic hydrogenation achieved both bis-debenzylation, reductive opening of the N—O isoxazolidine bond, and reduction of the cyano group in **(±)-17** to afford diamino alcohol **(±)-19** as the main product (3:1 ratio with a monobenzylated impurity, among minor byproducts). Deprotection of **(±)-17** was slow even under a 4 bar hydrogen pressure, and the minor product was shown by MS to display a benzyl group. All of the four cycloadducts so obtained showed weak NOE effects between protons H-3 and H-6, thus indicating a *trans* disposition. As dipoles of the nitrile oxide type were expected to afford related isoxazoline-based cycloadducts, other assays (carried out with ethyl chlorooximidoacetate [28]/NEt_3 for generating the corresponding nitrile oxide) afforded the desired product **(±)-20** which was found stable: when exposed to catalytic hydrogenation under various conditions, it reacted slowly and partially, affording a mixture of products, with evidence of the reduction of the benzyl ring to a cyclohexyl ring in one product, as shown by mass spectroscopy [29]. These experiments showed that access to racemic substituted pyrrolidines with achiral dipoles suffered from drawbacks, notably as the nitrones exist as *E* and *Z* forms, and sometimes as other isomers, thus leading to challenging purifications. Such drawbacks did not occur with menthone-based nitrones which are structurally well defined, stable and reactive enough in the presence of a suitable dipolarophile as *N*-benzyl-3-pyrroline. They afforded in high yield stereochemically defined products, with recovery of the chiral auxiliary in the next steps.

Compounds ***ent*-8, 9, 10** and ***ent*-10** were considered for *in vitro* assays on structural bases and availability. Further tests were carried out with compounds selected when they showed results deserving closer investigation.

2.2. Biological activities

In order to investigate the type of receptors which could be targeted by the compounds ***ent*-8, 9, 10** and ***ent*-10,** including Glu or GABA receptors, a search of specific binding sites was then done using Standard Operative Procedures (See Supplementary data, Table S3). Sixty binding sites were examined in duplicate and data revealed that none of these four compounds significantly bound to any site of ionotropic glutamate receptors (AMPA, kainate, NMDA, PCP, glycine-strychnine-insensitive), metabotropic mGluR5 receptors, nor to $GABA_{A/B}$ receptors and benzodiazepine sites of $GABA_A$ receptors. Surprisingly, the compounds **9** and ***ent*-10** altered the specific binding (29% of inhibition) of a control agonist of cannabinoid type 1 (CB-1) receptors, whereas ***ent*-8** altered the binding of an agonist of cholecystokinin type 1 (CCK1) receptors (Table 1). In contrast to its enantiomer ***ent*-10**, compound **10** did not bind to any site of all the receptors tested. Although the percentage of agonist binding inhibition was modest, additional tests were performed to determine the agonist/antagonist property of **9** and ***ent*-10** versus cannabinoid receptor subtypes. Experiments showed that the two compounds were not antagonists for CB-1 and CB-2 receptors (data not shown), nor agonists for CB-2 receptors (Supplementary data Fig. S2 bottom for ***ent*-10**). However, ***ent*-10** led to a moderate inhibition of the hepatic cell response vs CB-1 control agonist (CP55940), suggesting that it could be a partial agonist of CB-1 receptors (Supplementary data Fig. S2 top). In contrast, this approach failed to specify the type of agonistic property of compound **9** towards CB-1 receptors (data not shown).

The lack of any binding of our novel compounds on Glu or GABA receptors was unexpected because their structures exhibited strong analogies with existing molecules mimicking neurotransmitter amino acids. Nevertheless, the two compounds **9** and ***ent*-10** which act on CB-1 receptors may indirectly alter brain Glu system owing to the well-known interactions between Glu system and CB systems via CB-1 receptors [30]. If this hypothesis is valid, these two compounds are expected to exhibit a



Scheme 3. Synthesis of racemic α-amino-(4-hydroxy-pyrrolidin-3-yl)acetic acid derivatives. Reagents and conditions: (i) **2**, μW, 80 °C, 1 h; (ii) H_2, $Pd(OH)_2$/C, 1:1 THF/H_2O, 24 h; (iii) H_2, $Pd(OH)_2$/C, 1:1 THF/H_2O, 2 h (iv) **2**, μW, 80 °C, 2 h; (v) H_2, $Pd(OH)_2$/C, 10:10:1 THF/H_2O/AcOH, 40 h; (vi) **2**, $EtO_2CCCl{=}NOH$, Et_3N, toluene, reflux, overnight; (vii) H_2, pressure from 1 to 10 atm, $Pd(OH)_2$/C, various solvents.

sought pharmacological property, i.e. indirectly modulating brain glutamatergic transmission while being devoid of the side-effects encountered with antagonists of ionotropic Glu receptors [31,32].

In order to determine whether **9** and ***ent*-10** can indirectly affect brain Glu level, an *in vivo* approach, in which all connections between brain areas and neuronal networks are functionally preserved, is needed. For that purpose, the brain microdialysis technique was used, since it allows both the local administration of a drug and the monitoring of drug-induced changes in neurotransmitters concentration in the extracellular medium within the central nervous system of live animals [33–35]. Thus, Glu level was monitored in the striatum, a well-defined brain structure containing glutamatergic nerve terminals, when locally infusing the compound **9** or ***ent*-10** by reverse dialysis of a 1 mM solution. By using the dialysis yield of amino acids previously determined with our microdialysis probe, i.e. around 7–9 % [36], one may assume that the concentration of the perfused **9** and ***ent*-10** compounds in the brain extracellular space is lower than 100 μM. Under such conditions, compounds **9** and ***ent*-10** induced marked 50-60%-decreases in Glu levels (Fig. 3). In order to test whether Glu could retro-modulate this effect by acting on ionotropic Glu receptors, we co-administered 0.2 mM CNQX (an antagonist of kainate-subtype of ionotropic receptors) 10 min after the beginning of the application of each compound (Fig. 3). The data obtained revealed partial reversal of the effects due to ***ent*-10**, suggesting that kainate receptors may have a modulatory role in the mechanism of the ***ent*-10**-induced fall in extracellular Glu. In contrast, no significant reversal of compound **9**-induced change in Glu levels was observed with CNQX (Fig. 3). Such a difference was unlikely to be due a difference in sensitivity of kainate receptors between animals receiving either **9** or ***ent*-10**, since local administration of kainate induced similar effect on Glu levels for those animals.

Despite the absence of any binding to Glu or GABA receptors, the two novel pyrrolidine compounds induced a clear-cut lowering of brain extracellular Glu when infused intracerebrally. These two compounds were not available in a sufficient quantity to determine whether their biological activity extended to the central nervous system after a systemic administration. To address this issue, we examined the ability of the two compounds **9** and ***ent*-10** to cross the blood-brain interface using a differentiated cellular model of the choroid plexus epithelium that reproduces *in vivo* properties of the blood-cerebrospinal fluid (CSF) barrier [37]. These include tight junctions that restrict paracellular permeability, and efflux mechanisms mediated by multispecific transport proteins. Exposure of the basolateral side of choroidal cells to the compounds at a concentration of 150 μM for 60 min did not induce any alteration of the integrity of the monolayer, as assessed by the measurement of [^{14}C]-sucrose permeability across the monolayers (Fig. 4A). The permeability coefficients of the two compounds were low, being close or similar to sucrose coefficient values (Fig. 4B). For comparison, the influx permeability coefficients measured using this model for the highly to mildly lipid soluble compounds caffeine and urea are respectively 12×10^{-3} and 1×10^{-3} cm. min^{-1}. The influx Pe value for the antiretroviral drug AZT, whose penetration through the choroidal epithelium is restricted by an active efflux mechanism is around 0.6×10^{-3} cm. min^{-1} [38].

In order to investigate whether the blood to CSF permeability of our compounds was similarly limited by an efflux process, we compared influx and efflux rates for **9** and ***ent*-10**. For ***ent*-10**, the Pe values measured in the two directions were similar. The efflux permeability for **9** was lower than the influx permeability, and also lower than that of sucrose (not shown). This indicates that the poor permeation of the molecules across the blood-CSF barrier results from their hydrophilic properties rather than from a specific transporter-mediated efflux mechanism. Given the limited transfer (Pe ≈ $0.3–0.4 \times 10^{-3}$ cm min^{-1}) from the blood peripheral compartment into the cerebral compartment for these compounds, and their absence of toxicity toward the barrier cells, major central side-effects are not to be expected.

The permeability study did not reveal alteration of the cellular blood-CSF barrier integrity due to compounds **9** and ***ent*-10**. An additional Standard Operative Procedure was carried out in order to verify the absence of toxicity in another cell line. This was done on hepatic HepG2 cells and data obtained did not reveal any cytotoxicity of the two compounds tested, namely **9** and ***ent*-10** (data not shown).

Table 1
Significant values of the percent inhibition of control specific binding determined at 10 μM (n = 2) for compounds ***ent*-10**, **9** and ***ent*-8**.

% Of inhibition of specific binding	*ent-10*	*9*	*ent-8*
CB1 receptors	+29%	+29%	n.s.
CCK1 receptors	n.s.	n.s.	−46%

n.s. not significant.

3. Conclusion

The cycloaddition between (+) or (−)-menthone-derived nitrones as chiral glycine equivalents and *N*-benzyl-3-pyrroline

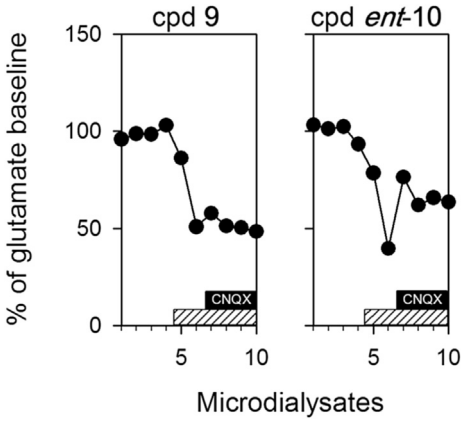


Fig. 3. Effects of compounds **9** and ***ent*-10** on glutamate level (black circles) in the striatum of anesthetized rats. The new synthetic compounds (hatched bars) were administered at 1 mM by reverse dialysis. CNQX, a kainate receptor antagonist, was administered at 0.2 mM (black bars) by reverse dialysis. Data are expressed as percentage of the mean of the four baseline values preceding the beginning of the application of the synthetic compound.

opened a direct access to enantiopure fused heterocycles. In agreement with previous studies, the reaction occurred on the less hindered face of the nitrone, with the 3-pyrroline *N*-benzyl group oriented outwards, thus minimizing steric interactions in the TS. This achieves a tight control of the configurations of the three simultaneously created chiral centers. *N*-Benzyl-3-pyrroline was more reactive than its *N*-Boc analogue, which isomerized to 2-pyrroline and led preferentially to the corresponding cycloadduct. By choosing either (+) or (−)-menthone, both enantiomeric cycloadducts were synthesized in almost quantitative yield. Mild hydrolysis, applied for removing the chiral auxiliary, combined with debenzylation by hydrogenolysis, and sometimes saponification, delivered efficiently a series of enantiopure 4-hydroxy-3-glycinyl-pyrrolidine derivatives (3–5 steps, 36 to 81 overall yields). In the search for shorter routes to racemic analogues, achiral *N*-methyl and *N*-benzyl nitrones derived from ethyl glyoxalate, and a cyano nitrone were reacted also with *N*-benzyl-3-pyrroline. Although the first nitrones allowed for the synthesis of the desired racemic 4-hydroxy-3-glycinyl-pyrrolidine in 2 steps and a 52–68 % overall yield, cycloadditions with the achiral nitrones employed appeared to be less selective. By contrast, the well-defined menthone-based nitrones allowed for a perfect control of the stereochemistry of the cycloadducts and efficient syntheses of enantiopure 4-hydroxy-3-glycinyl-pyrrolidine derivatives.

The biological data obtained with derivatives based on this simple yet unexplored scaffold show some promising pharmacological properties. The two compounds **9** and ***ent*-10** lowered brain Glu levels *in vivo* while not exhibiting any brain or hepatic toxicity. Such a property must be emphasized since it may lead to a novel treatment of some psychiatric disorders which are characterized by central glutamatergic hyperactivity, such as depression or addictive states [35,39–41], while Glu lowering pharmacological agents are presently scarce and actively sought. Although these two compounds did not cross the blood-brain interface, one can assume that the modification of active chemical moieties may lead to ability to cross this barrier while keeping the brain Glu lowering property. On the other hand, **9** and ***ent*-10** could act on Glu signaling systems which are present at the peripheral level, such as adipose tissues, pancreatic beta cells and heart [42–44]. In conclusion, these new accessible scaffolds have a high pharmacological potential that deserves further investigations.

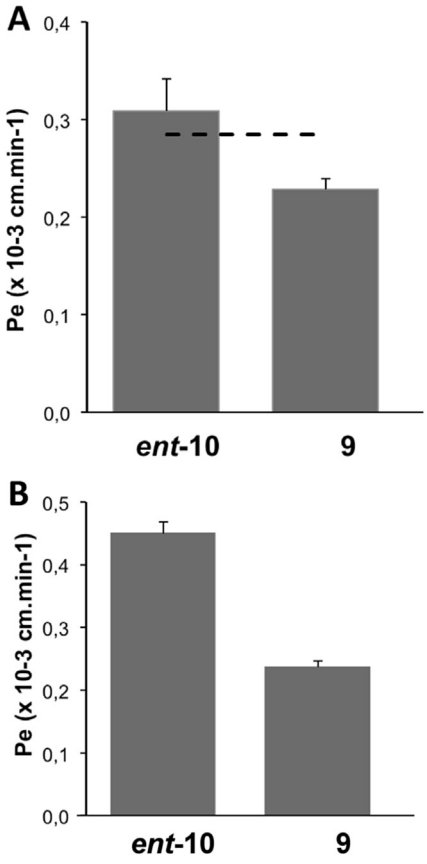


Fig. 4. Blood-to-CSF influx of compounds ***ent*-10** and **9** across the choroidal epithelial cell monolayer. A. The integrity of the physical barrier during exposure of the cells to the compounds at a concentration of 150 μM, assessed by the measurement of the paracellular permeability to sucrose, is preserved. The dotted line represents the mean Pe value for sucrose determined in the absence of any compound. B. Blood-to-CSF permeability coefficients are presented for compounds. They are close or similar to the sucrose permeability coefficients. Data are expressed as mean ± SD in both graphs.

4. Experimental section

4.1. Chemical synthesis

4.1.1. General

All reagents for synthesis commercially available (highest purity available for reagent grade compounds) were used without further purification. Solvents were distilled over CaH_2 (CH_2Cl_2), Mg/I_2 (MeOH), Na/benzophenone (THF) or purchased dry (DMF, DMSO, Toluene). All reactions were performed under an Argon atmosphere. Reactions under microwave activation were performed on a Biotage Initiator system. NMR solvents were purchased from Euriso-Top (Saint Aubin, France). TLC was carried out on aluminum sheets coated with silica gel 60 F_{254} (Merck). TLC plates were inspected by UV light (λ = 254 nm). Three staining reagents were used in order to develop TLC plates [10% H_2SO_4 in EtOH/H_2O (95:5 v/v), Ninhydrin in *n*BuOH and AcOH or *p*-anisaldehyde in EtOH and H_2SO_4] followed by heating. Silica gel column chromatography was performed with silica gel Si 60 (40–63 μm). Optical rotation was measured using a Perkin Elmer polarimeter. NMR spectra (see Supplementary data) were recorded at 293 K, unless otherwise stated, using a 300 MHz or a 400 MHz spectrometer. Shifts are referenced relative to deuterated solvent residual peaks. The NMR signals were identified following the numbering shown (see Table S2 in Supplementary Data). The following abbreviations were used to explain the observed multiplicities: s, singlet; d, doublet; t,

triplet; q, quadruplet; m, multiplet (br denotes a broad signal). Coupling constants (*J*) were expressed in Hertz. Sometimes, geminal (^2J) and vicinal (^3J) coupling constants were identified. When assigned, the vicinal coupled protons were indicated as subscripts. Complete signal assignments from 1D and 2D NMR were based on COSY, HSQC and HMBC correlations. ESI mass spectra were recorded in the positive mode using a Thermo Finnigan LCQ spectrometer. High resolution (HR-ESI-QToF) mass spectra were recorded using a Bruker MicroToF-Q II XL spectrometer.

4.1.2. (1S,2S,5R,3'aR,3'bS,7'aS)-2′-Benzyl-2-isopropyl-5,5'-dimethyl-1H-decahydro-spiro[cyclohexane-1,6′-7'-oxa-2′,5′,6'a-triaza-cyclopenta[a]pentalen-4'-one] (**3**)

In a Biotage Initiator 2–5 mL vial, nitrone **1** (392 mg, 1.64 mmol) and *N*-benzyl-3-pyrroline **2** (314 mg, 1.97 mmol, 1.2 eq) were introduced. The vial was flushed with argon and 2.5 mL of anhydrous toluene was added ($c = 0.66$ mM). The vial was sealed with a septum cap and was sonicated for 20 s. The resulting mixture was irradiated by microwaves (temperature: 140 °C). TLC monitoring (EtOAc) showed full conversion after 2 h. After the crude mixture was concentrated, the crude product was purified by flash silica gel column chromatography (EtOAc) to afford cycloadduct **3** (625 mg, 1.57 mmol, 96%) with no traces of other isomer. Monocrystals of compounds **3** were obtained from a saturated Et_2O solution cooled in a freezer. $R_f = 0.48$ (EtOAc). $[\alpha]_D + 40.4$ (*c* 1.1, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37–7.20 (m, 5H, CH-ar), 4.59 (td, 1H, $J = 7.0$ Hz, $J = 3.0$ Hz, H-4), 3.71–3.49 (m, 3H, NC*H*$_2$Ph, H-6), 3.42 (dd, 1H, $J = 10.3$ Hz, $J = 6.6$ Hz, H-3), 2.78 (dd, 1H, $J = 10.3$ Hz, $J = 3.0$ Hz, H-5), 2.75–2.69 (m, 4H, NC*H*$_3$, H-2), 2.65 (dd, 1H, $J = 9.4$ Hz, $J = 3.7$ Hz, H-2′), 2.58 (dd, 1H, $J = 9.9$ Hz, $J = 6.6$ Hz, H-5′), 2.14–2.08 (m, 1H, H-9), 2.00 (dtt, 1H, $J = 12.9$ Hz, $J = 6.5$ Hz, $J = 3.3$ Hz, H-10), 1.90–1.78 (m, 2H, H-11, H-12), 1.68–1.59 (m, 1H, H-12′), 1.48 (dt, 1H, $J = 13.5$ Hz, $J = 6.7$ Hz, H-15), 1.38 (dd, 1H, $J = 12.1$ Hz, $J = 3.2$ Hz, H-13), 1.18 (t, 1H, $J = 12.3$ Hz, H-9′), 0.95–0.85 (m, 10H, H-11', H-14, H-16). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.8 (C=O), 138.9 (C-ar), 128.6 (CH-ar), 128.3 (CH-ar), 127.1 (CH-ar), 88.0 (C-8), 79.6 (C-4), 71.9 (C-6), 59.6 (NCH_2Ph), 59.4 (C-5), 59.3 (C-2), 49.1 (C-3), 48.2 (C-13), 41.0 (C-9), 35.0 (C-11), 29.0 (C-10), 25.9 (NCH_3), 24.5 (C-15), 24.2 (*C*H$_3$), 22.6 (C-12), 22.4 (*C*H$_3$), 18.7 (*C*H$_3$). HR-ESI-QToF MS (positive mode): *m*/*z* calcd for $C_{24}H_{36}N_3O_2$ $[M+H]^+$: 398.2802, found: 398.2806.

4.1.3. (3S,3aR,6aS)-2-Acetyl-5-benzyl-3-(N-methyl-carboxamide)-hexahydro-pyrrolo[3,4-d]isoxazole (**4**)

In the acetolysis approach for the cleavage of the chiral auxiliary, compound **3** (226 mg, 0.57 mmol, 1 eq.) was placed in a 50 mL round-bottom flask. The flask was flushed with argon and 3 mL of AcOH were added. Under stirring, 3 mL of Ac_2O and 6 drops (~0.3 mL) of H_2SO_4 (95%) were added to the suspension. The mixture was then heated to 60 °C for 6h30. The flask was cooled down to 0 °C and a 5M solution of NaOH was added dropwise until pH 8. The resulting crude mixture was then transferred slowly into 300 mL of saturated $NaHCO_3$. The aqueous medium was extracted with CH_2Cl_2 (5 × 50 mL) and the combined organic layers were dried with Na_2SO_4. After concentration, the crude product was purified by silica gel flash chromatography (product added on dry silica then direct elution with EtOAc) to afford compound **4** (148 mg, 86%). $R_f = 0.28$ (EtOAc). $[\alpha]_D - 14.2$ (*c* 1, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34–7.20 (m, 5H, CH-ar), 6.44 (bs, 1H, N*H*CH$_3$), 4.97 (bs, 1H, H-6), 4.71 (dd, 1H, $J = 7.1$ Hz, $J = 4.8$ Hz, H-4), 3.62 (t, 1H, $J = 7.1$ Hz, H-3), 3.57 (d, 1H, $J = 12.7$ Hz, NC*H*$_2$Ph), 3.33 (d, 1H, $J = 12.7$ Hz, NC*H*$_2$Ph), 3.10–2.99 (m, 2H, H-2, H-5), 2.79 (d, 3H, $J = 4.9$ Hz, NC*H*$_3$), 2.34 (dd, 1H, $J = 10.0$ Hz, $J = 7.1$ Hz, H-2′), 2.19 (dd, 1H, $J = 11.6$ Hz, $J = 4.8$ Hz, H-5′), 2.09 (s, 3H, NC(O)CH$_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.7 (MeN*C* = O), 169.8 (*C*(O)NHCH$_3$), 138.1 (C-ar), 128.9 (CH-ar), 128.5 (CH-ar), 127.5 (CH-ar), 85.0 (C-4), 66.2 (C-6), 60.7 (C-5), 60.4 (C-2), 59.9 (NCH_2Ph), 48.4 (C-3), 26.4 (NCH_3), 21.2 (C(O)*C*H$_3$). HR-ESI-QToF MS (positive mode): *m*/*z* calcd for $C_{16}H_{22}N_3O_3$ $[M+H]^+$: 304.1656, found: 304.1667.

4.1.4. (3S,3aR,6aS)-5-Benzyl-3-(N-methyl-carboxamide)-hexahydro-pyrrolo[3,4-d]isoxazole (**5**)

Synthesis from ***4*** *by N-deacetylation.* In a dry and argon-flushed 25 mL round-bottom flask, 3 mL of freshly distilled methanol were added. The flask was cooled down to 0 °C and 151 μL (5.2 eq.) of $SOCl_2$ were added dropwise. Stirring was maintained for 10 min and compound **4** (122 mg, 0.40 mmol, 1 eq.) in CH_2Cl_2 (4 × 1 mL) was added dropwise over 10 min. After 15 additional min at 0 °C, the mixture was allowed to warm to rt and was heated (refluxing methanol) for 1 h. The crude mixture was cooled to rt and of a 5% Na_2CO_3 solution (4 mL) was added. The aqueous layer was extracted with 60 mL of EtOAc and the combined organic layers were washed twice with Na_2CO_3 (5%, 40 mL). The obtained aqueous phase was extracted with CH_2Cl_2 (2 × 20 mL). Rotary evaporation, vacuum – drying, followed by silica gel flash chromatography (elution gradient from EtOAc to EtOAc/IPA, 1/1) afforded pure **5** (104 mg, 0.4 mmol, 99%). *Shorter synthesis from* **3**: In a shorter acetolysis approach for the cleavage of the chiral auxiliary, compound **3** (542 mg, 1.36 mmol) was placed in a 50 mL round-bottom flask. The flask was flushed with argon and 7.2 mL AcOH were added. Under stirring, 7.2 mL Ac_2O and 0.4 mL H_2SO_4 (95–97 %; dropwise addition) were added to the suspension. The mixture was heated to 46 °C for 4 h and another 0.4 mL portion of H_2SO_4 (95–97 %) was added. The resulting mixture was maintained for 2h30 at 46 °C and then overnight at rt. The flask was cooled down to 0 °C and a 5M solution of NaOH was added dropwise over a period of 2h30 until pH 8. The resulting crude mixture was transferred slowly into 700 mL saturated aqueous $NaHCO_3$. The aqueous medium was extracted with CH_2Cl_2 (5 × 100 mL) and the combined organic phase was dried with Na_2SO_4. After concentration, the crude product (containing trace amount of the *N*-acetyl analog) was purified by silica gel flash chromatography as before to afford **5** (320 mg, 90%). $R_f = 0.74$ (CH_2Cl_2/IPA, 1/1). $[\alpha]_D + 10.9$ (*c* 1.3, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37–7.23 (m, 5H, CH-ar), 7.13 (bs, 1H, N*H*CH$_3$), 6.22 (d, 1H, $J = 3.9$ Hz, ON*H*R), 4.62 (dd, 1H, $J = 6.6$ Hz, $J = 4.8$ Hz, H-4), 3.66 (bs, 1H, H-6), 3.62 (t, 1H, $J = 6.7$ Hz, H-3), 3.48 (d, 2H, $J = 6.1$ Hz, NC*H*$_2$Ph), 3.07 (d, 1H, $J = 10.9$ Hz, H-5), 3.00 (d, 1H, $J = 9.9$ Hz, H-2), 2.79 (d, 3H, $J = 5.0$ Hz, NC*H*$_3$), 2.37 (dd, 1H, $J = 9.9$ Hz, $J = 6.7$ Hz, H-2′), 2.21 (dd, 1H, $J = 10.9$ Hz, $J = 4.9$ Hz, H-5′). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2 (C=O), 138.4 (C-ar), 128.6 (CH-ar), 128.5 (CH-ar), 127.4 (CH-ar), 83.7 (C-4), 71.0 (C-6), 61.8 (C-5), 60.8 (C-2), 59.9 (NCH_2Ph), 50.2 (C-3), 26.0 (NCH_3). HR-ESI-QToF MS (positive mode): *m*/*z* calcd for $C_{14}H_{20}N_3O_2$ $[M+H]^+$: 262.1550, found: 262.1553.

4.1.5. (αS,3R,4S)-α-Acetamido-(1-benzyl-4-hydroxy-pyrrolidin-3-yl)-N-methylacetamide (**6**)

In a 50 mL round-bottom flask containing 600 mg of compound **4** (1.98 mmol), $Mo(CO)_6$ (417 mg, 1.58 mmol, 0.8 eq.) was added and the flask was flushed with argon. Then, 20 mL of freshly distilled MeCN were added. Upon stirring, further 1.3 mL of water (MilliQ quality) was added and the resulting mixture was heated for 3 h with gentle reflux. Meanwhile, the solution turned brown to black. The crude mixture was concentrated after TLC monitoring (CH_2Cl_2/IPA, 8/2) showed completion of the reaction. The crude slurry was diluted in CH_2Cl_2 and filtered through a plug of Celite (CH_2Cl_2/MeOH, 9/1) and concentrated again. The crude product was purified on basic Al_2O_3 (elution gradient CH_2Cl_2 then CH_2Cl_2/MeOH, 8/2) to yield pure **6** (399 mg, 66%). $R_f = 0.18$ (Ace/IPA, 1/1). $[\alpha]_D + 33.1$

(*c* 0.6, DMSO). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35–7.21 (m, 5H, CH-ar), 7.12 (d, 1H, J = 8.0 Hz, N*H*Ac), 6.92 (d, 1H, J = 4.5 Hz, N*H*CH_3), 4.55 (dd, 1H, J = 8.0 Hz, J = 5.4 Hz, H-6), 4.47 (td, 1H, J = 5.4 Hz, J = 2.8 Hz, H-4), 3.68 (d, 1H, J = 12.9 Hz, NC*H*$_2$Ph), 3.57 (d, 1H, J = 12.9 Hz, NC*H*$_2$Ph), 2.93 (dd, 1H, J = 10.3 Hz, J = 5.6 Hz, H-5), 2.77 (d, 3H, J = 4.8 Hz, NC*H*$_3$), 2.66–2.50 (m, 4H, H-2, H-2′, H-3, H-5′), 2.02 (s, 3H, NC(O)C*H*$_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.4 (N*C*(O)CH_3), 171.3 (*C*(O)NHCH_3), 138.5 (C-ar), 128.9 (CH-ar), 128.5 (CH-ar), 127.3 (CH-ar), 71.5 (C-4), 62.0 (C-5), 60.5 (N*C*H$_2$Ph), 55.1 (C-2), 53.4 (C-6), 45.4 (C-3), 26.4 (C(O)CH_3), 23.3 (N*C*H$_3$). HR-ESI-QToF MS (positive mode): *m*/*z* calcd for $C_{16}H_{24}N_3O_3$ $[M+H]^+$: 306.1812, found: 306.1823.

4.1.6. (3S,3aR,6aS)-2-Acetyl-3-(N-methyl-carboxamide)-hexahydro-pyrrolo[3,4-d]isoxazole (**7**)

A 100 mL round-bottom flask containing compound **4** (157 mg, 0.52 mmol) was subjected to three vacuum/argon cycles. Under argon, 6 mL of EtOH and 6 mL of CH_2Cl_2 were added and the resulting mixture was subjected to another five vacuum/argon cycles. Then, 110 mg of $Pd(OH)_2$/C 10% (0.1 mmol, 0.2 eq.) were added. The flask was finally subjected to five vacuum/argon cycles and subsequent five vacuum/hydrogen cycles. Stirring under 1 atm H_2 was maintained overnight. The medium was filtered over a plug of Celite (CH_2Cl_2 and EtOH), concentrated, and the crude product was purified on a C-18 prepacked silica column with a combiflash system (elution gradient from water to water/methanol, 1/1) to yield **7** (110 mg, 99%). R_f = 0.24 (*n*BuOH/H_2O/AcOH, 3/1/1). $[\alpha]_D$ + 32.3 (*c* 0.6, DMSO). ^{1}H NMR (400 MHz, D_2O) δ 5.23 (br s, 1H, H-4), 4.79* (br s, 1H, H-6), 3.75–3.45 (m, 5H, H-2, H-2′, H-3, H-5, H-5′), 2.79 (br s, 3H, NC*H*$_3$), 2.21 (br s, 3H, NC(O)C*H*$_3$). (*signal partially hidden by solvent residual peak: attribution from 2D experiments). ^{13}C NMR (100 MHz, D_2O) δ 170.9 (N*C*(O)CH_3), 170.0 (*C*(O)NHCH_3), 83.5 (C-4), 63.9 (C-6), 49.8 (C-5), 49.6 (C-3), 49.4 (C-2), 26.4 (N*C*H$_3$), 20.2 (NC(O)*C*H$_3$). HR-ESI-QToF MS (positive mode): *m*/*z* calcd for $C_9H_{15}N_3NaO_3$ $[M+Na]^+$: 236.1006, found: 236.1005.

4.1.7. (αS,3R,4S)-α-Acetamido-(4-hydroxy-pyrrolidin-3-yl)-N-methylacetamide (**8**)

A 25 mL round-bottom flask containing compound **6** (19 mg, 0.06 mmol) was subjected to three vacuum/argon cycles. Under argon, 5 mL of EtOH were added and the resulting mixture was subjected to another five vacuum/argon cycles. Then, 7 mg of $Pd(OH)_2$/C 20% (0.012 mmol, 0.2 eq.) were added. The flask was finally subjected to five vacuum/argon cycles and subsequent five vacuum/hydrogen cycles. After stirring for 2 h under 1 atm H_2, the medium was filtered over a plug of Celite (CH_2Cl_2 and EtOH), and concentrated. The crude product was purified on a C-18 prepacked silica column with a combiflash system (elution water) to yield **8** (9 mg, 67%). R_f = 0.31 (*n*BuOH/H_2O/AcOH, 3/1/1). $[\alpha]_D$ + 34.0 (*c* 0.2, DMSO). ^{1}H NMR (400 MHz, D_2O) δ 4.44–4.41 (m, 1H, H-4), 4.39–4.23 (m, 1H, H-6), 3.43–3.16 (m, 2H, H-2, H-5), 3.11–2.89 (m, 2H, H-2′, H-5′), 2.77 (d, 3H, J = 4.1 Hz, NC*H*$_3$), 2.54–2.46 (m, 1H, H-3), 2.07 (bs, 3H, NC(O)C*H*$_3$). ^{13}C NMR (100 MHz, D_2O) δ 174.6 (N*C*(O)CH_3), 174.0 (*C*(O)NHCH_3), 70.8 (C-4), 54.0 (C-5), 52.8 (C-6), 46.2 (C-3), 45.9 (C-2), 26.1 (N*C*H$_3$), 22.0 (NC(O)*C*H$_3$). HRMS ESI-QToF, positive mode: *m*/*z* calcd for $C_9H_{15}N_3NaO_3$ $[M+Na]^+$: 236.1006, found: 236.1005.

4.1.8. (αS,3R,4S)-α-Amino-(4-hydroxy-pyrrolidin-3-yl)-N-methylacetamide (**9**)

A 50 mL round-bottom flask containing compound **5** (100 mg, 0.38 mmol) was subjected to three vacuum/argon cycles. Under argon, 10 mL EtOH and 6 mL CH_2Cl_2 were added and the resulting mixture was subjected to another five vacuum/argon cycles. Then, 100 μL AcOH and 40 mg of $Pd(OH)_2$/C 20% (0.076 mmol, 0.2 eq) were added. The flask was finally subjected to five vacuum/argon cycles and subsequent five vacuum/hydrogen cycles. Stirring was maintained overnight under 1 atm H_2. TLC monitoring showed a complete conversion. The catalyst was removed by filtration over a plug of Celite (EtOH/MeOH). After concentration, addition of MeOH (2 mL) and toluene (5 mL) precipitated the crude **9** as a foam (36.7 mg, 55% yield) containing 10% AcOH. White foam; R_f = 0.19 (*n*-BuOH, CH_3CO_2H, H_2O, 3:1:1); $[\alpha]_D^{22}$ +3.0 (*c* 0.5, H_2O). ^{1}H NMR (D_2O, 400 MHz) δ 4.61 (m, 1H, $J_{3,4}$ = 3.7 Hz, H-4), 3.50 (d, 1H, $J_{3,6}$ = 9.9 Hz, H-6), 3.40 (m, 2H, H-5, H-5′), 3.23 (dd, 1H, $J_{2',3}$ = 8.5 Hz, ^2J = 11.6 Hz, H-2′), 3.20 (t, 1H, $^2J = J_{2,3}$ = 11.8 Hz, H-2), 2.74 (s, 3H, NCH_3), 2.40 (m, 1H, H-3). ^{13}C NMR (D_2O, 100 MHz) δ 175.9 (CO), 69.31 (CH), 53.70 (CH_2), 53.07 (CH), 47.29 (CH), 45.37 (CH_2), 26.07 (CH). MS: ESI positive current: 174.0 $[M+H]^+$, 347.0 $[2M + H]^+$; HRMS calcd for $C_7H_{15}N_3O_2$ Na_1, m/z: 196.1056; found: 196.1051 (also visible: 174.1228 $[M+H]^+$; 212.0788 $[2M + H]^+$).

4.1.9. (αS,3R,4S)-α-Amino-(4-hydroxy-pyrrolidin-3-yl)acetic acid (**10**)

A 100 mL round-bottom flask containing compound **5** (112 mg, 0.43 mmol) was subjected to three vacuum/argon cycles. Under argon, 10 mL of EtOH and 6 mL of CH_2Cl_2 were added and the resulting mixture was subjected to another five vacuum/argon cycles. Then, 45 mg of $Pd(OH)_2$/C 20% (0.086 mmol, 0.2 eq.) were added. The flask was finally subjected to five vacuum/argon cycles and subsequent five vacuum/hydrogen cycles. Stirring was maintained under 1 atm H_2 for 3 h. As TLC monitoring showed an incomplete conversion, 100 μL of AcOH were added (*ca.* 4 eq., additional 5 vacuum/hydrogen cycles) and stirring was maintained for 24 h. The catalyst was removed by filtration over a plug of Celite (EtOH/MeOH). After concentration, the crude product (156 mg, R_f = 0.17 in *n*BuOH/H_2O/AcOH, 3/1/1) was subjected to basic hydrolysis using 180 mg of hydrated LiOH (4.3 mmol, 10 eq.) in 4 mL of THF and 4 mL of ultrapure water in a 50 mL round-bottom flask. Stirring was maintained at rt for 3 h and the solvents were evaporated off. The crude carboxylate salt was purified on a C-18 prepacked silica column with a combiflash system (elution water) and a subsequent purification on a Ion Exchange Resin (Dowex 50W X8). After incorporation of the product into a resin column, the column was extensively washed with water. Addition of 2.5% and then 5% of NH_4OH into the eluent allowed the elution of the carboxylate ammonium. Evaporation and high-vacuum drying provided the zwitterionic form of compound **10** (47 mg, 0.29 mmol, 57% over two steps). R_f = 0.18 (*n*BuOH/H_2O/AcOH, 3/1/1). $[\alpha]_D$ + 4.2 (*c* 0.5, DMSO/H_2O, 4/1). ^{1}H NMR (400 MHz, D_2O) δ 4.58 (t, 1H, J = 3.0 Hz, H-4), 3.54 (d, 1H, J = 9.5 Hz, H-6), 3.44 (dd, 1H, J = 8.6 Hz, J = 11.8 Hz, H-2), 3.39–3.36 (m, 2H, H-5, H-5′), 3.29 (d, 1H, J = 11.8 Hz, H-2′), 2.46 (ddd, 1H, J = 3.8 Hz, J = 9.1 Hz, J = 12.4 Hz, H-3). ^{13}C NMR (100 MHz, D_2O) δ 178.6 (C=O), 69.8 (C-4), 54.0 (C-6), 53.7 (C-5), 46.4 (C-3), 46.1 (C-2). HR-ESI-QToF MS (positive mode): *m*/*z* calcd for $C_6H_{13}N_2O_3$ $[M+H]^+$: 161.0921, found: 161.0924.

4.1.10. (±)-cis 2H-5-Benzyl-2-methyl-3,3a,4,5,6,6a-hexahydro-pyrrolo[3,4-d]isoxazole-3-carboxylic acid, ethyl ester ((±)-**14**)

A solution of nitrone **11** (300 mg, 2.29 mmol) and *N*-benzyl-3-pyrroline **2** (0.8 mL, 4.20 mmol) in toluene (3 mL) was stirred for 1 h at 80 °C under micro-wave irradiation. TLC (1:3 EtOAc/petroleum ether) showed complete conversion of the nitrone into a major product, and unidentified polar byproducts. The solvent was evaporated and the residue was subjected to silica gel column chromatography (mobile phase: 1:4 EtOAc/toluene) to afford cycloadduct **(±)-14** (390 mg, 58%). Colourless oil. R_f = 0.4 (1:4 EtOAc/toluene). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35–7.20 (m, 5H, H–Ar), 4.60 (dd, 1H, J = 4.9 Hz, J = 7.4 Hz, H-4), 4.20 (q, 2H, J = 7.1 Hz, H-8), 3.70 (d, 1H, J = 13.2 Hz, NC*H*$_2$Ph), 3.58 (d, 1H,

J = 13.2 Hz, NCH_2Ph), 3.25 (q, 1H, J = 7.1 Hz, H-3), 3.17 (br s, 1H, H-6), 3.00 (d, 1H, J = 11 Hz, H-5′), 2.94 (d, 1H, J = 9.8 Hz, H-2′), 2.78 (s, 3H, NCH_3), 2.28 (br t, 1H, J = 7.6 Hz, H-2), 2.19 (br d, 1H, J = 6.6 Hz, H-5), 1.27 (t, 3H, J = 7.1 Hz, H-9). 1D NOE experiments with selective irradiations (H-3, H-4, or H-6), showed signals enhancements as follows: H-3 irradiation: enhancements of H-2: 4%, H-2': 1.8%, H-4: 4%; irradiation of H-4: enhancements of H-3: 3.5%, H-5: 3.7%, H-5': 1.9%; irradiation of H-6: enhancements of H-2: 1.3%, H-2′: 2.7%. ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.03 (CO), 138.58 (C-ar), 128.73, 128.41, 127.17 (5CH-ar), 81.03 (C-4), 75.48 (C-6), 61.26 (C-8), 59.08 (C-11), 58.77 (C-5), 56.79 (C-2), 52.54 (C-3), 43.85 (NCH_3), 14.24 (C-9). HRMS-ESI, positive mode: m/z calcd for $C_{16}H_{23}N_2O_3$ $[M+H]^+$: 291.1703; found: 291.1702.

4.1.11. (±)-3,4-cis α-N-Methylamino-(4-hydroxy-pyrrolidin-3-yl) acetic acid ((±)-15)

Cycloadduct **(±)-14** (150 mg, 517 μmol) was dissolved in a 1:1 THF/water mixture (15 mL). The medium was stirred overnight under H_2 atmosphere at rt in the presence of $Pd(OH)_2$/C 20% (20 mg) as catalyst. Under these conditions, fast *N*-debenzylation, slower N–O bond reductive cleavage and hydrolysis of the ethyl ester occurred cleanly, within ~15 h. Solids were removed by filtration over Celite and the solvent was evaporated under reduced pressure. Purification was achieved by reversed phase column chromatography (elution: H_2O, then MeOH/H_2O 1/10) and the material (85 mg, 95% yield) was recrystallized from MeOH to afford **(±)-15** (80 mg, 89%) as white crystals, mp 192 °C (MeOH). ^{1}H NMR (400 MHz, D_2O) δ 4.54 (t, 1H, ^3J = 3.2 Hz, H-4), 3.36 (dd, 1H, ^3J = 3.3 Hz, ^2J = 12.8 Hz, H-5), 3.31 (dd, 1H, ^3J = 0.9 Hz, ^2J = 12.7 Hz, H-5′), 3.26 (dd, 1H, ^3J = 8.5 Hz, ^2J = 11.6 Hz, H-2), 3.18 (t, 1H, ^2J = ^3J = 11.7 Hz, H-2′), 3.17 (d, 1H, J = 10 Hz, H-6), 2.34 (s, 3H, NCH_3), 2.32 (m, 1H, H-3). ^{13}C NMR (101 MHz, D_2O) δ 178.99 (CO), 69.41 (C-4), 62.78 (C-6), 53.87 (C-5), 46.04 (C-3), 45.79 (C-2), 33.47 (NCH_3). HRMS-ESI, positive mode: m/z calcd for $C_7H_{15}N_2O_3$ $[M+H]^+$: 175.1078; found: 175.1077.

4.1.12. (±)-cis 2H-2,5-Dibenzyl-3,3a,4,5,6,6a-hexahydro-pyrrolo [3,4-d]isoxazole-3-carboxylic acid, ethyl ester ((±)-16)

Nitrone **12** (149 mg, 0.72 mmol) and *N*-benzyl-3-pyrroline **2** (170 μL, 0.89 mmol) were dissolved in toluene (2 mL) containing a few drops of CH_2Cl_2 for solubilization. Cycloaddition was completed after stirring for 1 h at 80 °C under micro-wave irradiation. The solvent was evaporated under reduced pressure and purification of the residue by silica gel column chromatography (mobile phase: 1/9 EtOAc/toluene) afforded cycloadduct **(±)-16** (180 mg, 68% yield). Colourless oil; R_f = 0.6 (EtOAc/toluene 1/4). ^{1}H NMR (500 MHz, 50 °C, $CDCl_3$) δ 7.25–7.45 (m, 10H, H–Ar), 4.62 (dd, 1H, J = 4.9 Hz, J = 7.6 Hz, H-4), 4.22 (d, 1H, ^2J = 13.7 Hz, ONCH_2Ph), 4.12 (dq, 2H, $^3J_{8,9}$ = 7.1 Hz, J = 1.6 Hz, H-8), 4.05 (d, 1H, ^2J = 13.7 Hz, ONCH_2Ph), 3.68 (d, 1H, ^2J = 13.2 Hz, NCH_2Ph), 3.61 (d, 1H, ^2J = 13.2 Hz, NCH_2Ph), 3.39 (d, 1H, ^3J = 7.0 Hz, H-6), 3.28 (q, 1H, ^3J = 7.0 Hz, H-3), 3.00 (d, 1H, ^3J = 11 Hz, H-5′), 2.95 (d, 1H, ^3J = 9.6 Hz, H-2′), 2.32 (br s, 1H, H-2), 2.21 (br s, 1H, H-5), 1.24 (t, 3H, ^3J = 7.1 Hz, H-9). ^{13}C NMR (126 MHz, 50 °C, $CDCl_3$) δ 170.28 (C=O), 138.64, 136.93, 129.50, 128.76, 128.44, 128.30, 127.47, 127.23 (12C-ar), 81.05 (C-4), 73.09 (C-6), 61.24 (C-8), 60.92 (C-10), 59.06 (C-11), 58.88 (C-5), 56.94 (C-2), 52.23 (C-3), 14.23 (C-9). 2D NOE correlations were confirmed by 1D NOE experiments with selective irradiations (H-3, H-4, or H-6), showing signals enhancements as follows: H-3 irradiation: enhancements of H-2: 3.7%, H-2': 1.7%, H-4: 5.2%; irradiation of H-4: enhancements of H-3: 3.9%, H-5: 2.9%, H-5': 2.0%, H-6: 1.0%; irradiation of H-6: enhancements of H-2: 0.9%, H-2′: 3.9%, H-4: 1.4%. HRMS-ESI, positive mode: m/z calcd for $C_{22}H_{27}N_2O_3$ $[M+H]^+$: 367.2016; found: 367.2009.

4.1.13. (±)-3,4-cis α-Amino-(4-hydroxy-pyrrolidin-3-yl)-acetic acid ((±)-10)

Cycloadduct **(±)-16** (30 mg, 82 μmol) was dissolved in a 1:1 THF/water mixture (3 mL). After addition of $Pd(OH)_2$/C 20% (10 mg) as catalyst, the medium was stirred overnight under H_2 atmosphere at rt. Solids were removed by filtration over Celite and the solvent was evaporated under reduced pressure to afford a residue (20 mg). HRMS ESI, positive mode: m/z calcd for $C_6H_{13}N_2O_3$ $[M+H]^+$: 161.0921; found: 161.0927. NMR data was found in agreement with **10** and **ent-10** (see Supplementary data, NMR data).

4.1.14. (±)-cis 2H-2,5-Dibenzyl-3-cyano-3,3a,4,5,6,6a-hexahydro-pyrrolo[3,4-d]isoxazole ((±)-17) and (±)-cis 2H-5-Benzyl-2-cyanomethyl-3-phenyl-3,3a,4,5,6,6a-hexahydro-pyrrolo[3,4-d] isoxazole ((±)-18)

Cyanonitrone **13** (550 mg, 3.44 mmol) and *N*-benzyl-3-pyrroline **2** (1.3 mL, 6.83 mmol) were dissolved in toluene (4 mL) and the medium was stirred for 2 h at 80 °C under micro-waves irradiation. The solvent was removed under reduced pressure before purification by silica gel column chromatography (eluent: EtOAc/toluene 1/10). This allowed the separation of a minor more mobile regioisomer (60 mg, 6%) from the main racemic cycloadduct expected **(±)-17** (500 mg, 45%). Compound **(±)-17**: Yellowish oil. R_f = 0.4 (1:4 EtOAc/toluene). ^{1}H NMR (500 MHz, 80 °C, toluene-d_8) δ 7.4–7.0 (m, 10H, H–Ar), 4.28 (ddd, 1H, J = 3.4 Hz, J = 6.0 Hz, J = 7.5 Hz, H-4), 4.15 (d, 1H, J = 13.4 Hz, H-8), 3.80 (d, 1H, J = 13.4 Hz, H-8), 3.29 (d, 1H, J = 13.1 Hz, H-9), 3.23 (d, 1H, J = 13.1 Hz, H-9), 3.07 (d, 1H, J = 3.6 Hz, H-6), 2.79 (ddd, 1H, J = 4.0 Hz, J = 7.8 Hz, J = 11.6 Hz, H-3), 2.48 (dd, 1H, J = 3.1 Hz, J = 10.1 Hz, H-5′), 2.22 (br s, 1H, H-5), 2.17 (br m, 1H, H-2′), 2.09 (br m, 1H, H-2). 1D NOE experiments with selective irradiations (H-3, H-4, or H-6), showed signals enhancements as follows: H-3 irradiation: enhancements of H-2: 2.5%, H-2': 1.9%, H-4: 2%, H-6: 1.1%; irradiation of H-4: enhancements of H-3: 2.1%, H-5: 1.9%, H-5': 1.4%; irradiation of H-6: enhancements of H-2: 2%, H-2': 1.8%. ^{13}C NMR (126 MHz, 80 °C, toluene-d_8): δ 139.8 (Ca–ar), 137.2 (Ca'-ar), 130.2, 129.5, 129.4, 129.3, 128.6, 128.1 (10C-ar), 116.9 (C-7), 81.9 (C-4), 60.0 (C-9), 59.9 (C-6), 59.8, 59.7 (C-5, C-8), 57.5 (C-2), 53.6 (C-3). HRMS-ESI, positive mode: m/z calcd for $C_{20}H_{22}N_3O$ $[M+H]^+$: 320.1757; found: 320.1767.

Compound **(±)-18:** yellow clear oil. R_f = 0.6 (1/4 EtOAc/toluene). ^{1}H NMR (500 MHz, toluene-d_8) δ 7.5–7.0 (m, 10H, H–Ar), 4.40 (dd, 1H, J = 4.7 Hz, J = 7.7 Hz, H-4), 3.71 (d, 1H, J = 8.1 Hz, H-6), 3.39 (s, 2H, H-9), 3.20 (d, 1H, J = 17.2 Hz, H-7), 3.01 (d, 1H, J = 10.8 Hz, H-5′), 2.77 (d, 1H, J = 17.2 Hz, H-7), 2.71 (d, 1H, J = 9.7 Hz, H-2′), 2.64 (app. q, 1H, J = 7.3 Hz, H-3), 1.73 (m, 2H, H-2, H-5). ^{13}C NMR (126 MHz, toluene-d_8) δ 140.02 (Ca–ar), 138.41 (Ca'-ar), 129.84, 129.35, 129.35, 129.31, 128.93, 128.02 (10C-ar), 115.00 (C-8), 82.19 (C-4), 76.02 (C-6), 59.54 (C-9), 59.28 (C-5), 58.04 (C-3), 56.60 (C-2), 42.16 (C-7). 2D NMR (HMBC) showed correlations between H-6 and aromatic carbons, as well as correlations between methylene H-7 and C-8 in the nitrile group. 1D NOE experiments with selective irradiations (H-3, H-4, or H-6), showed signals enhancements as follows: H-3 irradiation: enhancements of H-2: 4.6%, H-4: 4.2%, H-6: 1.1%, H–Ar: 3.5%; irradiation of H-4: enhancements of H-3: 3.8%, H-5: 4.1%, H-5': 1.2%; irradiation of H-6: enhancements of H-2': 2.9%, H-5': 0.3%, H–Ar: 5.4%. HRMS-ESI, positive mode: m/z calcd for $C_{20}H_{22}N_3O$ $[M+H]^+$: 320.1757; found: 320.1750.

4.1.15. (±)-3,4-cis 3-(1,2-Diaminoethyl)-4-hydroxy-pyrrolidine ((±)-19)

The cycloadduct **(±)-17** (40 mg, 125 μmol) was dissolved in a 10:10:1 THF/water/AcOH mixture (3 mL). After adding $Pd(OH)_2$/C 20% (10 mg) as catalyst, the medium was stirred at rt for 40 h under H_2 (4 bars). TLC monitoring (3:1:1 *n*BuOH/water/AcOH) showed the

complete transformation of **(±)-17** into four more polar products. The medium was filtered through a pad of Celite, and the solvent was evaporated under reduced pressure. The crude residue (23 mg, 90% crude yield) showed by mass spectrometry a predominant ion (*m/z* 146[M+H]$^+$) corresponding to the expected amino alcohol **(±)-19** and another one, for a minor benzylated product (*m/z* 221 [M+H]$^+$). ^{13}C NMR confirmed the existence of these 2 products in 1:3 to 1:4 ratio. **(±)-19:** ^{1}H NMR (400 MHz, D_2O) δ 4.59 (br s, 1H, H-4), 3.47 (dd, 1H, ^2J = 11.6 Hz, ^3J = 8.6 Hz, H-2), 3.34 (m, 2H, H-5), 3.21 (dt, 1H, ^3J = 3.2 Hz, ^3J = 9.8 Hz, ^3J = 9.8 Hz, H-6), 3.15 (t, 1H, ^2J = 11.6 Hz, ^3J = 11.6 Hz, H-2), 3.04 (dd, 1H, ^3J = 3.2 Hz, ^2J = 13.3 Hz, H-7), 2.87 (dd, 1H, ^3J = 10.0 Hz, ^2J = 13.3 Hz, H-7), 2.30 (m, 1H, H-3). ^{13}C NMR (101 MHz, D_2O) δ 69.41 (C-4), 53.66 (C-5), 48.04 (C-6), 47.01 (C-3), 45.15 (C-2), 43.38 (C-7). HRMS-ESI, positive mode: *m/z* calculated for $C_6H_{16}N_3O$ [M+H]$^+$: 146.1288; found: 146.1281. The other product showed the following signals: ^{13}C NMR (101 MHz, D_2O) δ 132.9 (1C-ar), 129.5 (2CH-ar), 129.48 (1CH-ar), 129.1 (2CH-ar), 68.81 (CH, C-4), 60.62 (CH_2, C-7), 53.84 (CH_2, C-5), 51.15 (CH, C-6), 45.29 (CH_2, C-2), 43.29 (CH_2-benzyl), 42.24 (CH, C-3). MS *m/z* = 221 [M+H]$^+$ corresponded to $C_{13}H_{20}N_2O$, indicating loss of one amino residue (probably the α-amino group) and the presence of a benzyl group (seen by NMR) probably attached to the pyrrolidine nitrogen.

4.1.16. (±)-cis 3aH-5-N-Benzyl-4,5,6,6a-tetrahydro-pyrrolo[3,4-d]-isoxazole-3-carboxylic acid, ethyl ester ((±)-20)

N-Benzyl-3-pyrroline **2** (504 μL, 2.64 mmol) was added to a solution of ethyl chlorooximidoacetate (1 g, 6.62 mmol, 2.5 eq) in toluene (20 mL). The flask was heated to 110 °C (oil bath) while a solution of Et_3N (276.8 μL, 19.8 mmol, 7.5 eq) in toluene (15 mL) was added over 16 hh using a syringe pump. After concentration under reduced pressure, the crude product was purified by silica gel column chromatography (4:1 petroleum ether/EtOAc) to afford compound **(±)-20** (370 mg, 1.35 mmol, 51%). Yellow oil R_f = 0.52 (7:3 petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26 (m, 5H, phenyl), 5.20 (dd, 1H, $J_{5,4}$ = 4.5 Hz, $J_{3,4}$ = 9.6 Hz, H-4), 4.31 (2 complex q, 2H, *J* = 7.2 Hz, *diastereotopic* OCH_2), 3.91 (dt, 1H, $J_{2',3}$ = 1.0 Hz, $J_{2,3}$ = 7.1 Hz, $J_{3,4}$ = 9.5 Hz, H-3), 3.68 (d, 1H, ^2J = 13.3 Hz, NCH_2Ph), 3.55 (d, 1H, ^2J = 13.3 Hz, NCH_2Ph), 3.24 (d, 1H, ^2J = 11.2 Hz, H-5′), 3.19 (d, 1H, ^2J = 9.9 Hz, H-2′), 2.44 (dd, 1H, $J_{2,3}$ = 7.2 Hz, ^2J = 9.8 Hz, H-2), 2.37 (dd, 1H, $J_{5,4}$ = 4.6 Hz, ^2J = 11.2 Hz, H-5), 1.31 (t, 3H, *J* = 7.2 Hz, Me). ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.7, 152.2 (C=N, C=O), 137.8 (C-ar), 128.5 (2CH-ar), 128.2 (2CH-ar), 127.1 (1CH-ar), 87.4 (C-4), 61.9 (OCH_2), 61.1 (C-5), 58.3 (CH_2 benzyl), 56.9 (C-2), 51.0 (C-3), 14.1 (CH_3). MS *m/z* = 274.13 for $C_{15}H_{18}N_2O_3$; MS-ESI positive mode: 275.1 [M+H]$^+$, 297.1 [M+Na]$^+$.

4.1.17. Attempted synthesis of (±)-3,4-cis α-Amino-(1-benzyl-4-hydroxy-pyrrolidin-3-yl)acetic acid, ethyl ester ((±)-21)

A 25 mL round-bottom flask containing compound **(±)-20** (60 mg, 0.21 mmol) was subjected to three vacuum/argon cycles. Under argon, 6 mL MeOH was added and the resulting mixture was subjected to another five vacuum/argon cycles. $Pd(OH)_2$/C 20% (20 mg, 0.038 mmol) was added. The flask was subjected to five vacuum/argon cycles and five vacuum/hydrogen cycles. Stirring was maintained overnight under H_2 (10 bar) and the medium was filtered over a plug of Celite (H_2O/MeOH). Compound **(±)-21** was not detected by MS, but instead an ion *m/z* 285 [M+H]$^+$ indicated a product formed by reduction of the isoxazoline and displaying a cyclohexyl group (reduction of the benzyl group), while the crude reaction mixture led to complex NMR spectra. When the reduction was attempted without AcOH and under a 4 bar H_2 pressure, the starting material was still present after 4 days, and TLC showed a multicomponent mixture (6 spots).

4.1.18. Synthesis of enantiomers from the (+)-menthone-based nitrone

Appling the protocols reported above for the cycloaddition with the (+)-menthone-based nitrone, and for further reactions delivered in similar yields the corresponding enantiomers, and with identical NMR data with those obtained initially from the (−)-menthone-based nitrone, except for their optical rotations (see Supplementary data Table S1).

4.1.19. X-ray diffraction analysis of compounds 3 and 3″

Suitable crystals of **3** and **3″** were selected and mounted on a Gemini kappa-geometry diffractometer (Agilent Technologies UK Ltd) equipped with an Atlas CCD detector and using Mo radiation (λ = 0.71073 Å). Intensities were collected at 110 K for **3** and 100 K for **3″** by means of the CrysalisPro software (Agilent Technologies, Version 1.171.34.49, release 20-01-2011 CrysAlis171.NET, compiled Jan 20 2011,15:58:25). Reflection indexing, unit-cell parameters refinement, Lorentz-polarization correction, peak integration and background determination were carried out with the CrysalisPro software. An analytical absorption correction was applied using the modeled faces of the crystal [45]. The resulting set of *hkl* was used for structure solution and refinement. The structures were solved by direct methods with SIR97 [46] and the least-square refinement on F^2 was achieved with the CRYSTALS software [47]. All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were all located in a difference map, but those attached to carbon atoms were repositioned geometrically. The H atoms were initially refined with soft restraints on the bond lengths and angles to regularize their geometry (C···H in the range 0.93–0.98 Å) and U_{iso}(H) (in the range 1.2–1.5 times U_{eq} of the parent atom), after which the positions were refined with riding constraints. Crystal data for compound **3**: $C_{24}H_{35}N_3O_2$, Mr = 397.56 g mol^{-1}, Monoclinic, $P2_1$,*a* = 9.718 (1) Å, *b* = 8.2101 (8) Å, *c* = 13.979 (2) Å, $\alpha = \gamma = 90°$ and β = 101.79(1)°, D_{calc} = 1.209 g cm^{-3}, Z = 2, independent reflections = 5111 (R_{int} = 0.038), *R*values [I > 2σ(I), 4575 reflections]: R_1 = 0.056, wR_2 = 0.129; *S* = 0.94. Crystal data for compound **3″**: $C_{22}H_{37}N_3O_4$, Mr = 407.55 g mol^{-1}, Monoclinic, *C*2,*a* = 15.752 (2) Å, *b* = 11.156 (1) Å, *c* = 13.719 (2) Å, $\alpha = \gamma = 90°$ and β = 108.15(1)°, D_{calc} = 1.182 g cm^{-3}, Z = 2, independent reflections = 4822 (R_{int} = 0.043), *R*values [I > 2σ(I), 4001 reflections]: R_1 = 0.054, wR_2 = 0.110; *S* = 0.92. Crystallographic data (excluding structure factors) for the structures **3** and **3″** in this report have been deposited with the Cambridge Crystallographic Data Centre as supplementary publications nos. CCDC 981868 and CCDC 981867. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

4.2. Biological activities

4.2.1. Determination of target receptors

Experiments based on inhibition of specific binding have been conducted *in vitro* on standardized assays for sixty potential pharmacological targets including G-protein coupled receptors, ion channels and transporters (please see Supplementary data, Table S3) using specific radioligands. This study has been carried out by Eurofins Cerep-Panlabs (Celle L'Evescault, France). The percentage of inhibition of specific binding was determined for the 4 compounds at a concentration of 10 μM (n = 2). Results are expressed as percent of control specific binding [(measured specific binding/control specific binding) × 100] and as percent of inhibition of the control specific binding [100-(measured specific binding/control specific binding) × 100], obtained in presence of four synthesis compounds (***ent*-8**, **9**, ***ent*-10** and **10**). Data analysis was performed on the sixty receptor targets using a « box and

whisker » representation being performed for each compound. The properties agonist/antagonist were thereafter determined only for the compounds exhibiting at least 25% of inhibition of specific binding, i.e. for **9** and ***ent*-10** vs both CB1- and CB2-receptors using specific agonists or antagonist ligands and measuring the release of cAMP by human recombinant CHO cells [48]. Data analysis was performed using one-way ANOVA using the software developed by Institut Pierre Louis INSERM UMR S1193 (http://marne.u707.jussieu.fr/biostatgv/?module=tests).

Cytotoxicity was evaluated by Cerep Inc. (Redmond, WA, USA) using a hepatic HepG2 cell line exposed 72 h to each compound at 37 °C and five criteria were followed: cell number, intracellular free calcium, nuclear size, membrane permeability, mitochondrial membrane potential [49].

4.2.2. *In vivo* extracellular glutamate concentration using microdialysis and analysis by capillary electrophoresis

Concentric microdialysis probes were constructed from regenerated cellulose dialysis tubing (Spectra/Por hollow fiber, MWCO 6000 Da, 225 mm o.d., 3 mm active dialysis length; Spectrum Medical Industries, Los Angeles, CA, USA) and fused-silica capillary tubing (40 μm i.d × 105 μm o.d.; Polymicro Technology, Phoenix, AZ, USA), the body of the probe being made of a 3-cm 26G stainless steel tube, as previously designed [50]. Before implantation, the probes were perfused at a rate of 1 μL/min with artificial cerebrospinal fluid (aCSF) (145.0 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.45 mM NaH_2PO_4, 1.55 mM Na_2HPO_4, pH 7.4) using a pump from Harvard (Model 22, South Natick, MA, USA).

In vivo microdialysis experiments were carried out on male Wistar rats (weighting ≈ 300 g; Charles River Laboratories, L'Arbresle, France). The animals were kept and used in experiments according to the European Communities Council Directives. Our protocol was approved by the local ethical committee of Université Lyon 1 (CEEA-55). Rats were anaesthetized with 1.4 g/kg i.p. urethane (Sigma Chemicals, St. Louis, MO, USA) and probes were implanted in the dorsolateral striatum according to the atlas of Paxinos and Watson at the following coordinates (relative to bregma): anterior 0 mm, lateral +3.5 mm, ventral +6.5 mm below the brain surface. The rate of infusion was 1 μL/min. The collection of basal samples was initiated 2 h after microdialysis probe implantation. At the end of the experiment, the rats were killed with a lethal dose of anesthetic.

Samples were collected every 5-min in 0.2 mL PCR tubes (Axygene, Union City, CA, USA) and immediately stored at −30 °C until analysis. New compounds were administered *in situ* at 1 mM through reverse microdialysis by switching the inlet of the probe from normal aCSF to aCSF containing the tested chemicals [50,51].

Glu levels were determined by capillary electrophoresis with laser-induced fluorescence detection [52]. On the day of analysis, 5 μL of sample and 5 μL of standard solutions were derivatized at room temperature by adding 2 μL of a mixture (1:2:1 v/v/v) of (i) internal standard (10^{-4} M cysteic acid in 0.117 M perchloric acid), (ii) a borate/NaCN solution (100:20 v/v mixture of 500 mM borate buffer, pH 8.7, and 87 mM NaCN in water), and (iii) a 2.925 mM solution of naphthalene-2,3-dicarboxaldehyde in acetonitrile/water (50:50 v/v). The samples were diluted in 5 μL of water and then analyzed for amino acid content using an automatic capillary electrophoresis P/ACE™ MDQ system (Beckman, USA) equipped with a ZETALIF laser-induced fluorescence detector (Picometrics, France). Excitation was performed using a laser diode (Oxxius, France) at a wavelength of 410 nm, the emission wavelength being 490 nm. Separations were carried out on a 63 cm × 50 μm i.d. fused-silica capillary (Composite Metal Services, Worcester, England) with an effective length of 52 cm. Each day, before the analyses, the capillary was sequentially flushed with 0.25 M NaOH (15 min), ultra-pure water (15 min), and running buffer (75 mM sodium borate, pH 9.20 ± 0.02) (5 min). The separation conditions were an applied voltage of 25 kV, hydrodynamic sample injection (10 s at 0.2 psi), and a temperature, between 41 and 43 °C. The capillary was sequentially flushed for 30 s each with running buffer between analyses. Electropherograms were acquired at 15 Hz using P/ACE™ MDQ software. The Glu levels in striatal microdialysates were far above the limits of detection and were expressed as percent of the four baseline values preceding compound administration.

All the reagents of the microdialysis experiments (except our synthesis compounds) were purchased from Sigma Chemicals (St Louis, MO, USA). Standard solutions of 1 or 0.1 mM of our synthesized compounds were aliquoted in aCSF and stored at −30 °C. Before administration, they were diluted in aCSF.

4.2.3. Blood-CSF barrier permeability

Primary cell cultures of choroid plexus epithelial cells were prepared from neonate rat choroid plexuses as previously described [38,53] The cells were seeded on microporous Transwell polycarbonate filter inserts (6.5 mm diameter, 0.33 cm^2 surface, 0.4 μm pore size) and grown in culture medium consisting of Ham's F-12 and DMEM (1:1) supplemented with 10% FCS, 2 mM glutamine, 50 μg/mL gentamycine, and several growth factors as previously reported [53]. The medium was renewed every other day until the day of the permeability studies.

Permeability studies were performed on a rotating platform (200 rpm) at 37 °C, in Ringer-Hepes buffer (RH) as previously described [37]. The volumes used in the two compartments of the inserts (the lower one mimicking blood and the upper one mimicking brain CSF) were chosen as to be in equilibrium and to avoid any hydrostatic pressure. Inserts were rinsed with RH buffer before initiating permeability studies. For each synthesized compound, transfer was carried out in the same conditions on three laminin-coated inserts (without cells) and three inserts covered by a confluent monolayer of epithelial cells. Blood-to-CSF flux and CSF-to-blood flux were measured for **9** and **ent-10** as previously described [37,38]. Samples were assayed by HPLC.

[^{14}C]-sucrose permeability was measured as an index of the barrier integrity. At the end of the transfer of the compound of interest, [^{14}C]-sucrose was added in the upper compartment of inserts. The radioactivity of aliquots sampled at regular intervals from each well as well as an aliquot of the upper chamber was determined by liquid scintillation counting using a β-counter (Canberra Packard TRI-CARB 1600 TR).

Data were expressed as permeability coefficient Pe (cm min^{-1}) calculated from the clearance curves obtained from the transfer experiments performed over 1 h, as previously described in details [37,38].

4.2.4. HPLC analysis of two new synthetized compounds

The primary amine of compound ***ent*-10** was derivatized at room temperature by adding one volume of OPA (o-phthaldialdehyde; 5 mg/mL in 100/5/895 (v/v/v) methanol/2β-mercaptoethanol/200 mM borate buffer, pH 9.5). Reaction time was set to 2 min. Derivatized samples were stable for 30–40 min. The analytical HPLC conditions were as follows: RP-C18 column (150 mm, 5 μm), mobile phases with elution gradient in min (A:B): 0–5 (83:17); 6–11 (35:65); 12–17 (83:17), with A being a 90:10 (v:v) mixture of 50 mM potassium phosphate buffer pH 6 and methanol and B being 100% methanol; spectrophotometric detection at 340 nm; flow rate at 1 mL/min; injection volume of 20 μL. The retention time for ***ent*-10** was of 6.1 min.

The secondary amine of compound **9** was derivatized at room temperature with a 0.1 volume of FMOC

(fluorenylmethyloxycarbonyl chloride; 5 mg/mL in acetonitrile) and 20 μL of 200 mM borate buffer, pH 9.5. Reaction time was set to 30 min and derivatized samples were stable for 2 h. The analytical conditions were as follows: RP-C18 column (15 mm × 5 μm), mobile phase with elution gradient in min (A:B): 0 (65:35); 18 (40:60); 19–29 (20:80); 30–35 (35:35), with A being a 90:10 (v:v) mixture of 50 mM sodium acetate buffer pH 4,2 and acetonitrile and B being 100% acetonitrile; spectrophotometric detection at 230 nm; flow rate at 1 mL/min; injection volume of 20 μL. Retention time for derivative **9** was of 14.8 min.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.05.017.

Authors' contribution

JPP and SP designed experiments; SC, KA and JG performed chemical synthesis and analyzed data; SP performed brain microdialysis study; SP and LD analyzed neurochemical data; JFGE, NS and SB performed blood brain interface study and analyzed data; SC, KA, SP, LD, JFGE and JPP wrote the manuscript, JPP and CC supervised the research.

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