

Cognitive enhancing and neuroprotective effect of tricyclic cage 3,7-diazabicyclo[3.3.1]nonane derivative

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1. Chemistry

NMR spectra were recorded on the Agilent 400-MR spectrometer (400.0 MHz for ^1H ; 100.6 MHz for ^{13}C) at room temperature; chemical shifts (δ) were measured with reference to the solvents: CDCl_3 for ^1H ($\delta = 7.26$ ppm), ^{13}C ($\delta = 77.16$ ppm), $\text{DMSO}-d_6$ for ^1H ($\delta = 2.50$ ppm), ^{13}C ($\delta = 39.50$ ppm) and CD_3OD for ^1H ($\delta = 3.31$ ppm), ^{13}C ($\delta = 49.00$ ppm). Chemical shifts (δ) are given in ppm; J values are given in Hz. When necessary, assignments of signals in NMR spectra were made using 2D techniques. The measurements were done in a positive ion mode (interface capillary voltage 4500 V). Analytical thin layer chromatography was carried out with Merck Silica Gel 60 plates (supported on aluminum); the detection was done by UV lamp (254 and 365 nm) and chemical staining (solution of ninhydrin in EtOH). Column chromatography was performed on Merck Silica Gel 60.

N-(5-Cyano-2,3-dihydro-1*H*-inden-2-yl)benzo[*d*][1,3]dioxole-5-carboxamide (**3**)

To a solution of 2-amino-2,3-dihydro-1*H*-indene-5-carbonitrile **2** (930 mg, 5.88 mmol) in absolute acetonitrile, K_2CO_3 (1.61 g, 11.7 mmol) and benzo[*d*][1,3]dioxole-5-carboxylic acid chloride (1.13 g, 6.12 mmol) were added. The resulting mixture was stirred under heating at 50 °C for 9 hours until complete consumption of the starting amine (TLC control $R_f = 0.07$ in $\text{CHCl}_3/\text{EtOH}$ (50:1) system). After that the reaction mixture was cooled to room temperature, and filtered off. The solid residue was washed with acetonitrile until no more product was washed out (TLC control). The filtrates were combined and the solvent was distilled off. The resulting product was purified by column chromatography (eluents CHCl_3 , $\text{CHCl}_3/\text{EtOH}$ (100:1)). This yielded pure *N*-(5-cyano-2,3-dihydro-1*H*-inden-2-yl)benzo[*d*][1,3]dioxole-5-carboxamide **3** (1.60 g, 5.22 mmol, 89%) as a white crystalline product. mp: 187–188 °C. ^1H NMR (CDCl_3), δ : 2.96–3.02 (m, 2H), 3.40–3.48 (m, 2H), 4.93 (m, 1H), 6.02 (s, 2H), 6.25 (br. d, 1H, J 7.0 Hz), 6.80 (d, 1H, J 7.8 Hz), 7.24–7.26 (m, 2H), 7.34 (d, 1H, J 7.8 Hz), 7.49 (d, 1H, J 7.8 Hz), 7.52 (s, 1H). ^{13}C NMR (CDCl_3), δ : 39.65, 40.34, 50.93, 101.73, 107.59, 107.94, 110.59, 119.20, 121.59, 125.63, 128.34, 131.06, 142.30, 146.83, 147.97, 150.45, 166.68. Melting point and spectral data corresponded to those published previously.^{S1}

N-(5-(Aminomethyl)-2,3-dihydro-1*H*-inden-2-yl)benzo[d][1,3]dioxole-5-carboxamide (**4**)

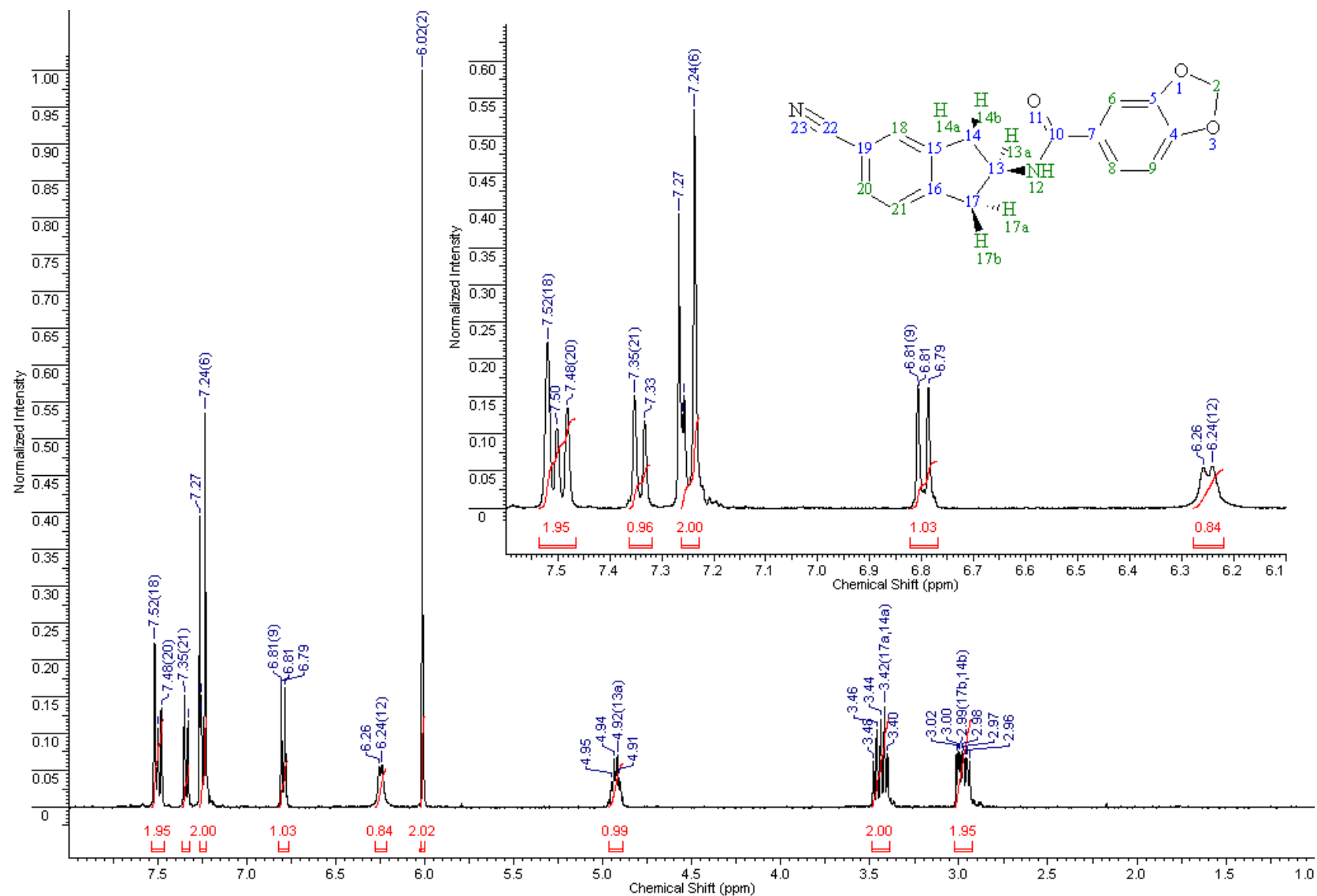
A mixture of *N*-(5-cyano-2,3-dihydro-1*H*-inden-2-yl)benzo[d][1,3]dioxole-5-carboxamide **3** (800 mg, 2.61 mmol), 10% Pd/C (172 mg, 6 mol. %) and HCl_{conc} (ω = 35%, 0.58 ml) in absolute ethanol (40 ml) was placed into a glass autoclave and hydrogen was passed through (p = 5 atm) while stirring at room temperature for 9 hours, then under heating at 50 °C for 4 hours until complete consumption of the starting compound **3** (TLC control R_f =0.48 in CHCl₃/EtOH (20:1) system). The reaction mixture was filtered through celite and washed with ethanol until no more product was washed out (TLC control). The filtrates were combined and the solvent was distilled off. The crude product was suspended into distilled water, and a 20% aqueous solution of KOH was added dropwise up to pH 11. The resulting mixture was extracted with CH₂Cl₂, the combined organic phases were dried over anhydrous Na₂SO₄, then filtered off and the solvent was distilled off. The resulting product was purified by flash chromatography (eluents CHCl₃/EtOH (20:1), EtOH, EtOH/NH₃ (20:1)). This yielded pure *N*-(5-(aminomethyl)-2,3-dihydro-1*H*-inden-2-yl)benzo[d][1,3]dioxole-5-carboxamide **4** (676 mg, 2.18 mmol, 83%) as a white crystalline product. mp: 239–241 °C (in the form of hydrochloride). ¹H NMR (CD₃OD, in the form of hydrochloride), δ : 3.02 (dd, 2H, ²J 15.9 Hz, ³J 7.3 Hz), 3.35 (dd, 2H, ²J 16.3 Hz, ³J 8.4 Hz), 4.08 (s, 2H), 4.79 (m, 1H), 6.03 (s, 2H), 6.87 (d, 1H, J 8.1 Hz), 7.25 (d, 1H, J 8.2 Hz), 7.31–7.33 (m, 3H), 7.42 (dd, 1H, ³J 8.1 Hz, ⁴J 1.7 Hz). ¹³C NMR (DMSO-*d*₆, in the form of hydrochloride), δ : 38.44, 38.68, 42.07, 50.82, 101.72, 107.47, 107.80, 122.41, 124.55, 125.14, 126.41, 127.37, 128.33, 132.15, 141.76, 147.24, 149.62, 165.26. Melting point and spectral data corresponded to those published previously^{S1}.

N-(5-((1,11-Dimethyl-4,8,12-trioxo-3,6,9-triazatricyclo[7.3.1.1^{3,11}]tetradecan-6-yl)methyl)-2,3-dihydro-1*H*-inden-2-yl)benzo[d][1,3]dioxole-5-carboxamide (**5**)

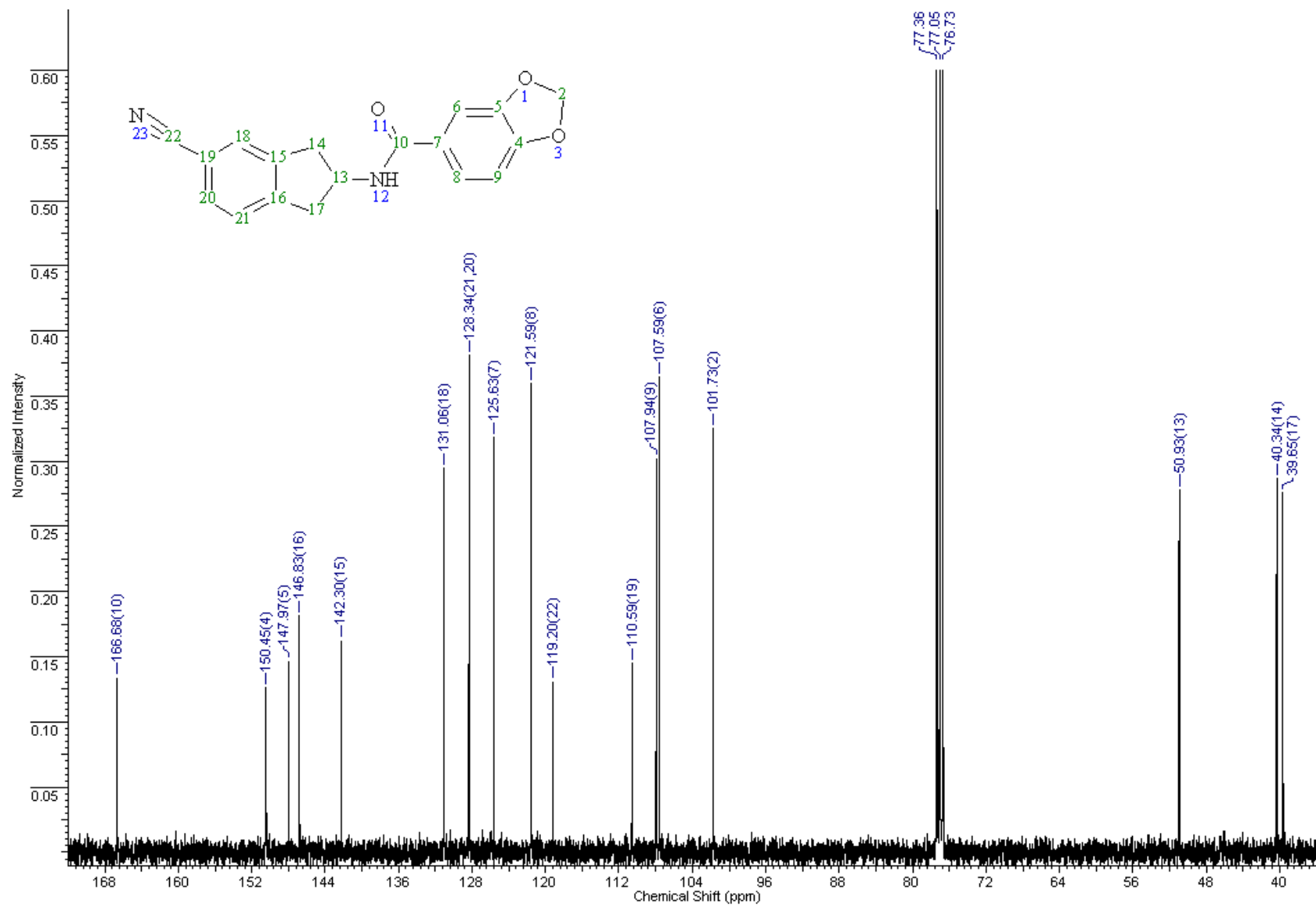
To a solution of *N*-(5-(aminomethyl)-2,3-dihydro-1*H*-inden-2-yl)benzo[d][1,3]dioxole-5-carboxamide **4** (985 mg, 3.17 mmol) in absolute acetonitrile (450 ml), K₂CO₃ (3.51 g, 25.4 mmol) and 3,7-bis(chloroacetyl)-1,5-dimethyl-3,7-diazabicyclo[3.3.1]nonan-9-one (1.17 g, 3.65 mmol) were added. The reaction mixture was stirred under heating at 70 °C for 26 hours until complete consumption of the starting amine (TLC control R_f = 0.05 in CHCl₃/EtOH (10:1) system). After that the reaction mixture was cooled to room temperature and filtered off. The solid residue was washed with acetonitrile until no more product was washed out (TLC control). The filtrates were combined and the solvent was distilled off. The resulting product was purified by double column chromatography (eluents first CHCl₃/EtOH (100:1), CHCl₃/EtOH (50:1), CHCl₃/EtOH (20:1); second AcOEt/EtOH (50:1), AcOEt/EtOH (20:1)). This yielded pure *N*-(5-((1,11-dimethyl-4,8,12-trioxo-3,6,9-triazatricyclo[7.3.1.1^{3,11}]tetradecan-6-yl)methyl)-2,3-dihydro-1*H*-inden-2-yl)benzo[d][1,3]dioxole-5-carboxamide **5** (1.50 g, 2.68 mmol, 84%) as a white crystalline product. mp: 173–174 °C. ¹H NMR (CDCl₃), δ : 1.01 (s, 3H), 1.09 (s, 3H), 2.74 (d, 2H, ²J 13.4 Hz), 2.90–2.95 (m, 2H), 3.02 (d, 2H, ²J 13.4 Hz), 3.21–3.27 (m, 2H), 3.39–3.46 (m, 2H), 3.59 (s, 2H), 3.78 (m, 2H), 4.93 (m, 4H), 6.02 (s, 2H), 6.17 (d, 1H, ³J 7.2 Hz), 6.81 (d, 1H, ³J 8.3 Hz), 7.15–7.29 (m, 5H). ¹³C NMR (CDCl₃), δ : 15.41, 15.80, 39.36, 39.53, 44.94, 45.39, 50.85, 53.91, 55.04, 59.91, 60.00, 62.01, 101.29, 107.19, 107.54, 121.16, 124.69, 126.05, 127.91, 128.14, 134.05, 141.01, 141.42, 147.52, 149.93, 166.24, 167.79, 210.52. Melting point and spectral data corresponded to those published previously^{S1}.

2. NMR spectra

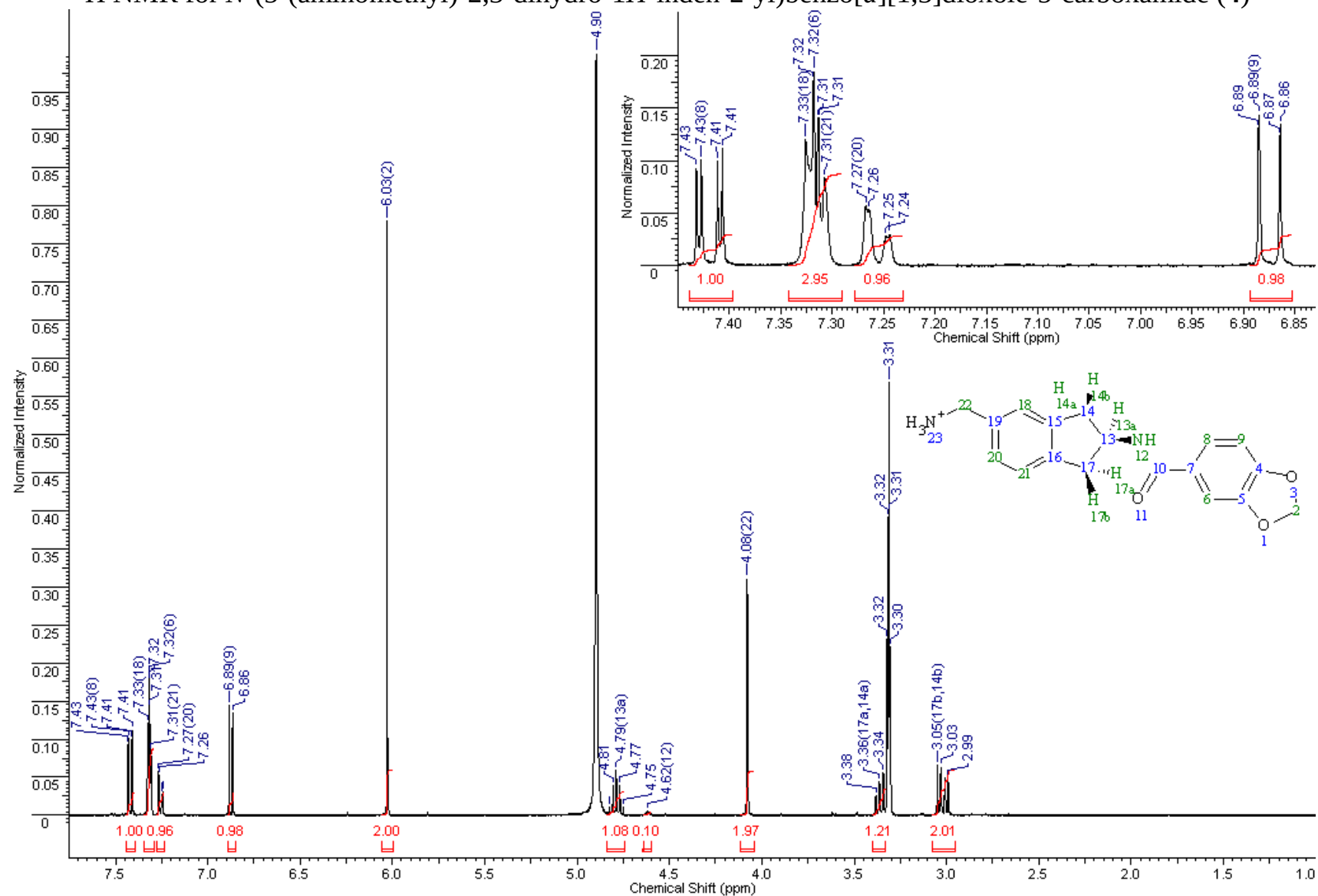
^1H NMR for *N*-(5-cyano-2,3-dihydro-1*H*-inden-2-yl)benzo[*d*][1,3]dioxole-5-carboxamide (**3**)



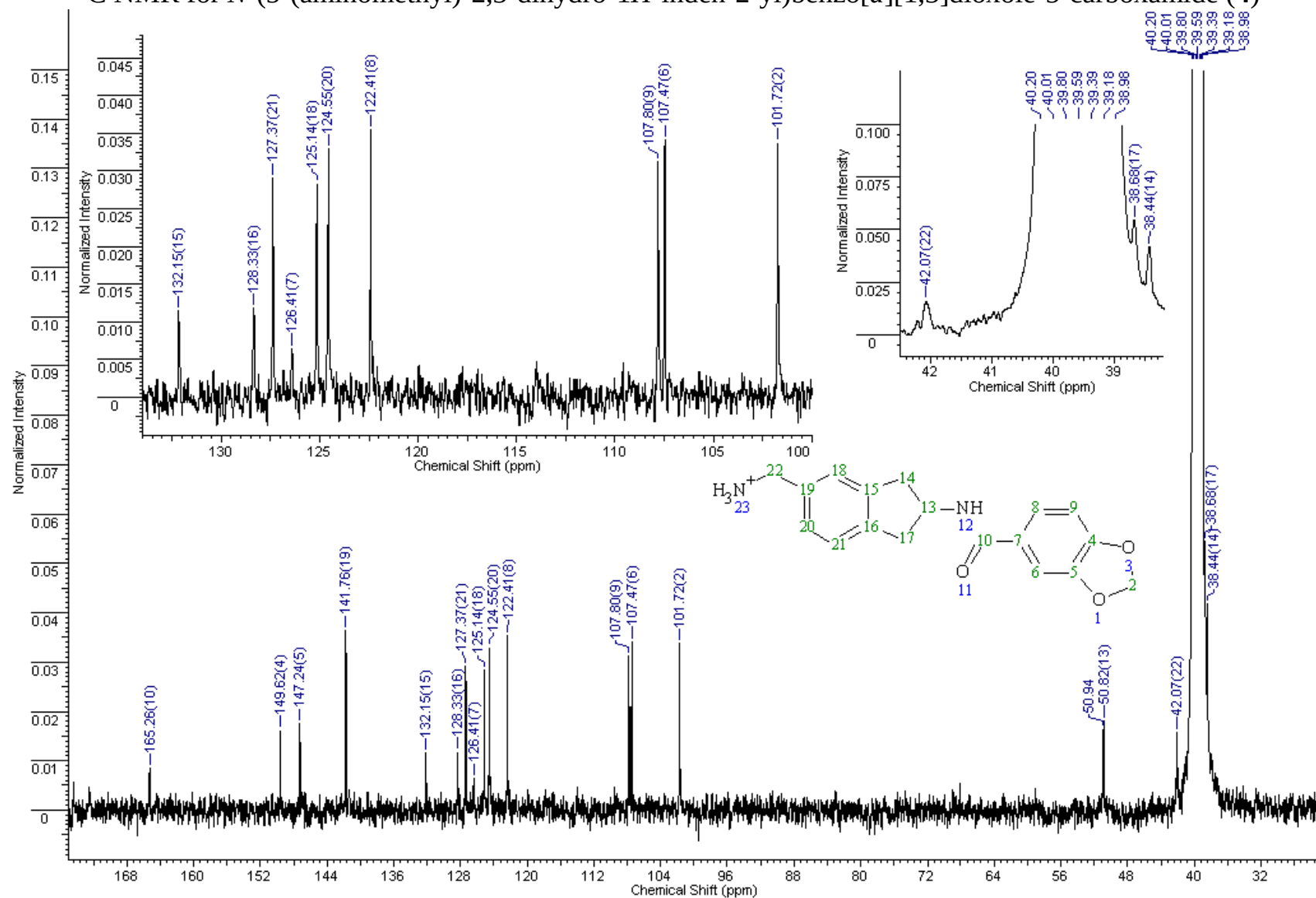
¹³C NMR for *N*-(5-cyano-2,3-dihydro-1*H*-inden-2-yl)benzo[*d*][1,3]dioxole-5-carboxamide (3)



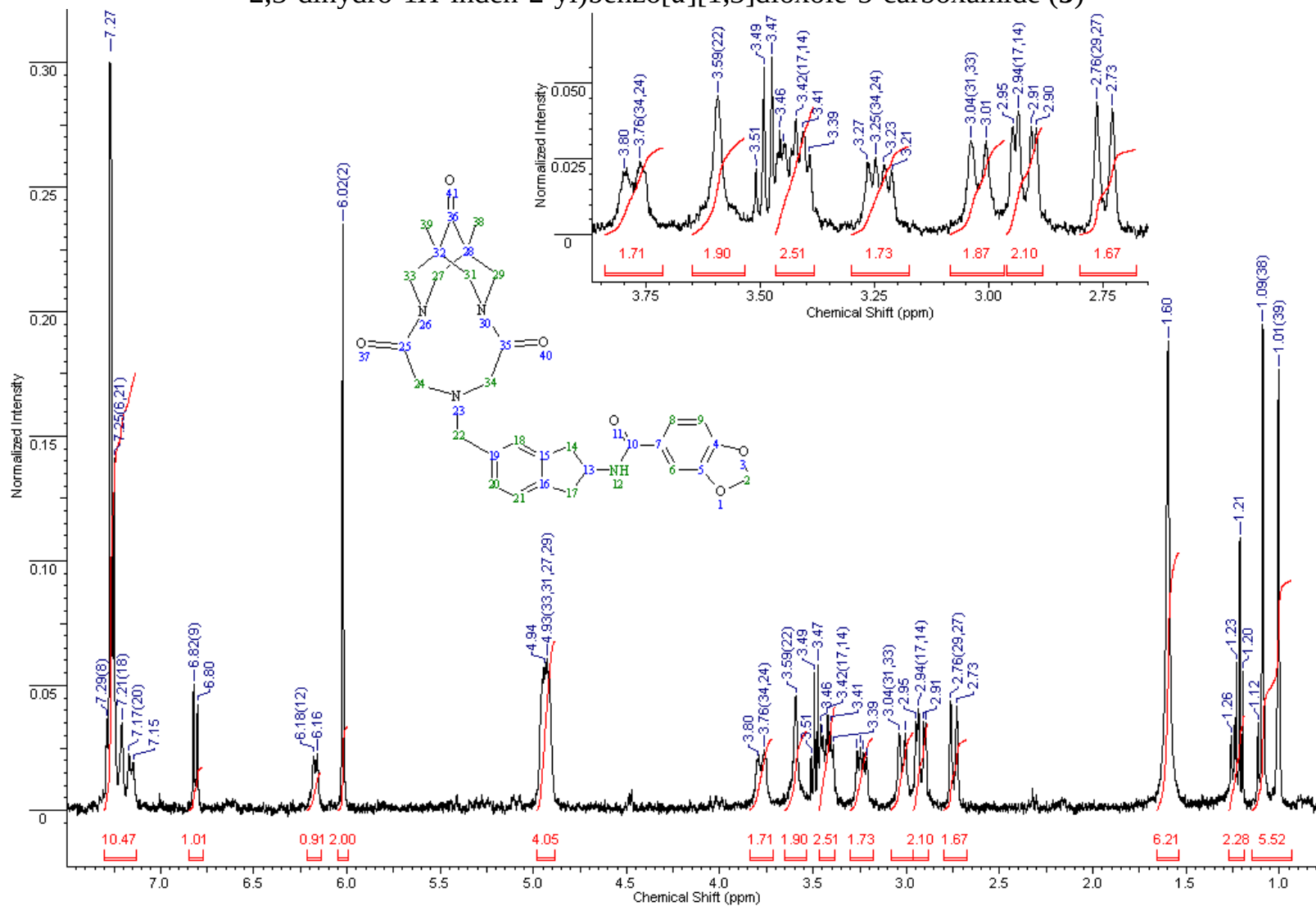
^1H NMR for *N*-(5-(aminomethyl)-2,3-dihydro-1*H*-inden-2-yl)benzo[*d*][1,3]dioxole-5-carboxamide (**4**)



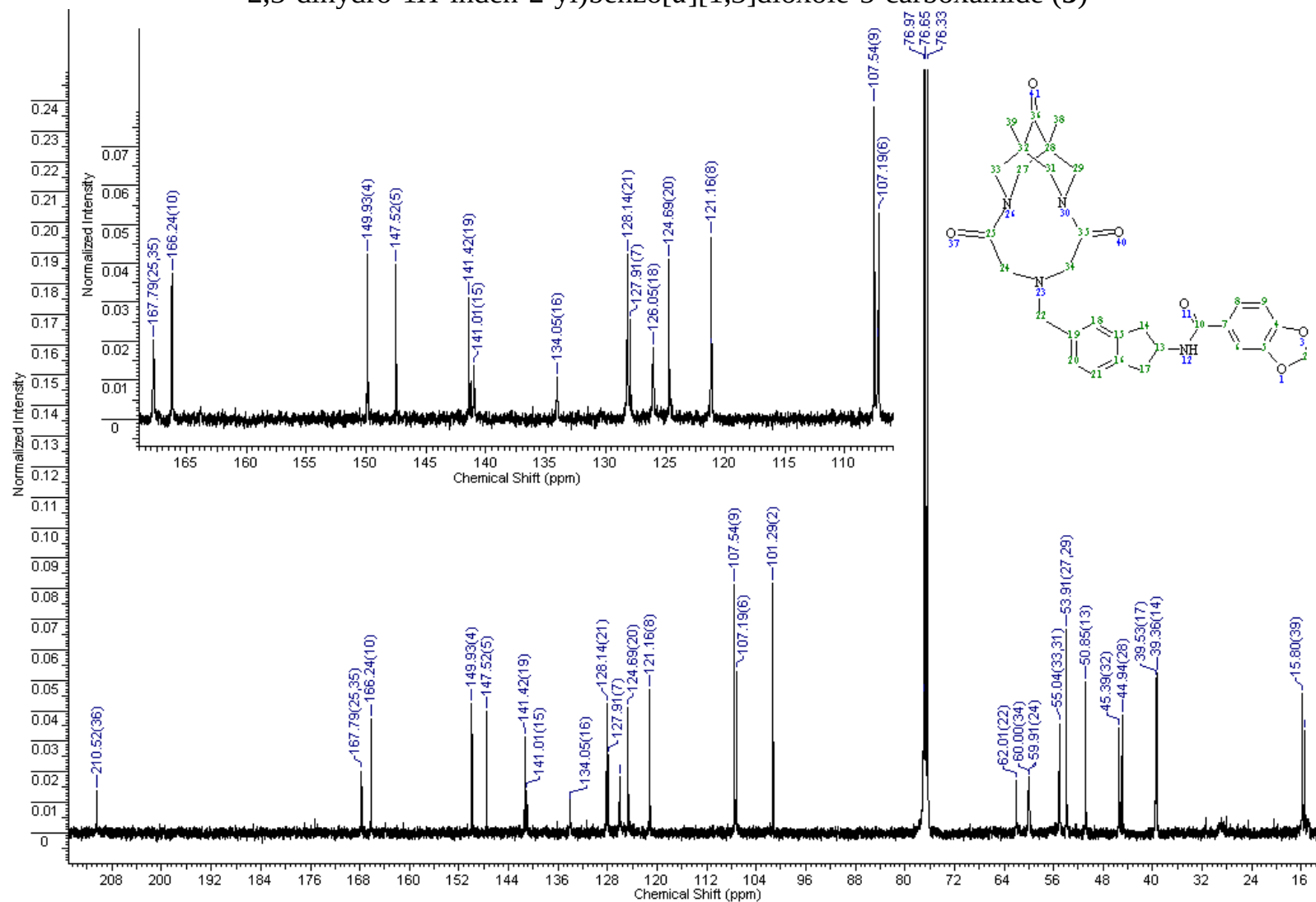
^{13}C NMR for *N*-(5-(aminomethyl)-2,3-dihydro-1*H*-inden-2-yl)benzo[*d*][1,3]dioxole-5-carboxamide (**4**)



^1H NMR for *N*-(5-((1,11-dimethyl-4,8,12-trioxo-3,6,9-triazatricyclo[7.3.1.1^{3,11}]tetradecan-6-yl)methyl)-2,3-dihydro-1*H*-inden-2-yl)benzo[*d*][1,3]dioxole-5-carboxamide (**5**)



^{13}C NMR for *N*-(5-((1,11-dimethyl-4,8,12-trioxo-3,6,9-triazatricyclo[7.3.1.1^{3,11}]tetradecan-6-yl)methyl)-2,3-dihydro-1*H*-inden-2-yl)benzo[*d*][1,3]dioxole-5-carboxamide (**5**)



3. X-ray diffraction study of target compound 5

Single crystals of target compound **5** were grown from ethyl acetate by its slow evaporation. The X-ray diffraction data were collected by using STOE diffractometer, Pilatus100K detector, focusing mirror collimation Cu K α (1.54086Å) radiation, rotation method mode. STOE X-Area software was used for cells refinement and data reduction. Data collection and image processing was performed with X-Area 1.67 (STOE & Cie GmbH, Darmstadt, Germany, 2013). Intensity data were scaled with LANA (part of X-Area) in order to minimize differences of intensities of symmetry-equivalent reflections (multi-scan method). The structures were solved and refined with SHELX^{S2} program. The non-hydrogen atoms were refined by using the anisotropic full matrix least-square procedure. All hydrogen atoms were placed in the calculated positions and allowed to ride on their parent atoms [C-H 0.93-0.98; Uiso 1.2 Ueq(parent atom)]. Refinement was made against 1752 reflections. 349 parameters were refined using 253 restraints. The final R 0.064 against 641 F²>2 σ (F²). Molecular geometry calculations were performed with the SHELX program, and the molecular graphics were prepared by using DIAMOND^{S3} software. Selected crystallographic data can be seen in Table 1.

Table S1 Crystal data and structure refinement for target compound **5**.

Parameter	Value
Empirical formula	C ₃₁ H ₃₄ N ₄ O ₆
Formula weight	558.62
Temperature	295(2) K
Wavelength	1.54186 Å
Crystal system	Orthorhombic
Space group	<i>Pca</i> 2 ₁
Unit cell dimensions	$a = 15.9960(10)$ Å $b = 21.592(2)$ Å $c = 9.5342(7)$ Å $\alpha = 90^\circ$ $\beta = 90^\circ$ $\gamma = 90^\circ$
Volume	3293.0(4) Å ³
Z	4
Density (calculated)	1.127 Mg m ⁻³
Absorption coefficient	0.647 mm ⁻¹
F(000)	1184
Crystal size	0.12 × 0.08 × 0.06 mm ³
θ range for data collection	5.778° to 44.973°
Index ranges	$-14 \leq h \leq 14$, $-19 \leq k \leq 19$, $-4 \leq l \leq 8$
Reflections collected	13796
Independent reflections	1752 [R(int) = 0.1775]

Completeness to $\theta = 44.973^\circ$	98.9 %
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	1752 / 253 / 348
Goodness-of-fit on F^2	0.791
Final R indices [$I > 2\sigma(I)$]	$R_1 = 0.0644$, $wR_2 = 0.1532$
R indices (all data)	$R_1 = 0.1571$, $wR_2 = 0.1787$
Absolute structure parameter	$-1.4(10)$
Largest diff. peak and hole	0.150 and $-0.201 \text{ e} \cdot \text{\AA}^{-3}$

Individual atoms of the molecule are disordered (O5/O6/C31) as a result of the superposition of different conformations of the five-membered ring. This is reflected in the Figure 1 by the large thermal vibration values of the oxygen and carbon atoms. Furthermore, the cell contains a disordered solvent molecule. The squeeze procedure was used to refine the positional and thermal parameters of atoms in the molecule. Due to the disorder and small crystal size, a rapid decrease in reflection intensity is observed with increasing reflection angle. Therefore, some statistical parameters fall outside of the standard range. CCDC-2516029 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

4. Pharmacological/biological assays

In vitro toxicity

MSCs (multipotent mesenchymal stromal cells) from rat adipose tissue were cultured in DMEM/F12 growth medium (PanEco, Russia) with 10% fetal bovine serum (PAA laboratories), 0.584 mg/ml L-glutamine (PanEco), 5000 U/ml streptomycin (PanEco) and 5000 U/ml penicillin (PanEco) in Petri dishes under standard culture conditions (37 °C, 5% CO₂). Subculture was performed when 70% confluence was reached. MSCs were removed from the surface of Petri dishes using a Versen solution (PanEco) with the addition of 0.25% trypsin (PanEco) and seeded into 24-well plates (SPL Lifesciences, Korea) at a density of 50 thousand cells per well. After 24 hours, solutions of the test compound were added to the cells. To dissolve the test compound, a minimal volume of DMSO (PanEco) was used, therefore, cells incubated in a growth medium with the addition of DMSO were also studied. Cells cultured in growth medium without the addition of the test compound or DMSO were used as a control. After 24 hours of cell incubation, the cell viability was determined using the MTT test. The medium was removed, MTT (PanEco) was added at a concentration of 0.5 mg/ml and incubated for 2 hours at 37 °C, visually monitoring the color development. Formazan crystals were dissolved with DMSO by shaking on a shaker for 20 min. The absorbance of formazan was estimated by measuring the optical density of the solution in each well and subtracting the background value. Measurements were performed using a BioRad Reader xMark tablet reader (Bio-Rad Laboratories, USA).

In vitro neuroprotective effect

The experiments were performed on HT-22 mouse hippocampal cells (Utrecht, the Netherlands). The cells were cultured in DMEM growth medium (HyClone, USA), with 5% fetal bovine serum (Gibco, USA), in a CO₂ incubator at 37 °C in an atmosphere containing 5% CO₂ until a monolayer formed. The test compound was dissolved at a concentration of 1 mM in 2 ml of pure DMSO (AppliChem, Panreac, Germany); 50 μ l of the resulting solution were diluted in 50 ml of sterile deionized water. A series of dilutions for application to cell culture was prepared in a 0.1%

DMSO solution. The test compound was added 24 hours before and immediately after H₂O₂ at final concentrations of 10⁻⁸–10⁻¹¹ M. 0.1% DMSO solution was also added to the Control and H₂O₂ groups. To model oxidative stress, the cells were incubated in the presence of H₂O₂ (PFC "Renewal", Russia), at a final concentration of 1.5 mM in an atmosphere of 5% CO₂ for 30 min at 37 °C in DMEM medium containing 5% fetal bovine serum and 2 mM L-glutamine (ICN Pharmaceuticals, USA). Then, the culture medium containing H₂O₂ was replaced with normal medium and after 4 hours the cell viability was determined using the MTT test. At the end of the experiment, the culture medium was replaced with 0.5 mg/ml MTT solution (AppliChem, Panreac, Germany) and incubated for 30 minutes at 37 °C. The MTT solution was then removed from the wells and DMSO was added to dissolve the formazan. After 15 minutes, the light absorption was measured using a Multiscan spectrophotometer (Thermo) at a wavelength of 600 nm. To assess intergroup differences when comparing two groups, the Kruskal-Wallis test followed by Dunn's post hoc test (ANOVA) was used. Data were considered significant at $p \leq 0.05$.

In vivo behavioral tests

The study used 40 male C57BL/6 mice, weighing 18–20 grams, aged 7–8 weeks. The animals were obtained from the Andreevka branch of the Federal State Budgetary Scientific Institution Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency of Russia. After arrival, the animals were kept in quarantine for 7 days, in accordance with the SOP "Admission, quarantine and clinical examination of animals". During the experiment, the animals were housed in polycarbonate cages with 5 animals per cage under controlled conditions: at a temperature of 23±3°C, relative humidity of 55±15%, with 12-hour light-dark cycles; the animals had free access to food and water. All animal procedures were performed in strict compliance with the State Standard GOST 33044-2014 "Principles of Good Laboratory Practice of the Eurasian Economic Union in the Field of Medicinal Products Circulation," adopted on November 3, 2016, No. 81, and the State Standard GOST 33215-2014 "Guidelines for the Care and Maintenance of Laboratory Animals. Rules for equipping facilities and organizing procedures," adopted on July 1, 2016. The experiment was carried out in accordance with ethical principles and was approved by the Bioethics Commission of Sechenov University (protocol No. 184 of 10.21.2024).

The animals were divided into four groups of ten mice. The test compound was administered orally once a day for 7 days at doses 0.01 mg, 0.1 mg, and 1 mg per mouse (equivalent to 0.5, 5, and 50 mg/kg, respectively), as well as placebo for control group. Following the 7-day course of administration, behavioral tests were conducted. During the behavioral tests, administration of the test compound was continued until the end of the experiment.

The Open field test was conducted in two stages. In the first stage, two identical objects were placed in the open field arena. A mouse was placed in the center of the arena for three minutes. In the second stage (24 hours later), one of the objects was replaced, and the mouse was placed in the arena for three minutes. The recognition index was calculated as the ratio of the time the mouse spent near the old object to the time the mouse spent near the new object. During the test, animals were administered the test compound orally 1 hour before the start of each stage.

The Elevated plus maze test used a 4-arm maze and was conducted in two stages. In the first stage, two identical objects were placed opposite each other in the two arms of the maze. A mouse was placed in the maze for 3 minutes. In the second stage (after 24 hours), one of the objects was replaced, and the mouse was placed in the maze for 3 minutes. The recognition index was calculated as the ratio of the time the mouse spent near the old object to the time the mouse spent near the new object. During the test, animals were administered the test compound orally 1 hour before the start of the first stage, and the test compound 1 hour before the start of the second stage.

The Morris water maze consisted of a circular pool (150 cm in diameter) filled with opaque water, into which a small platform (10 cm in diameter) was submerged, invisible to the animal. The test protocol consisted of five training sessions, each with five trials. The sessions were conducted at the same time of day, 24 hours apart. A mouse was placed at the edge of the pool. The maximum duration of the first session was 90 seconds. If the mouse was unable to find the platform on its

own, the experimenter assisted it. After finding the platform, the mouse remained on it for 30 seconds. The subsequent four sessions were 60 seconds long. If the platform was not found, the animal was removed from the pool and left outside the maze for 30 seconds. The platform's location remained constant throughout the test and was located in the opposite sector from where the animal was launched, at the edge of the thigmotaxis zone. The time it took the animal to find the platform and climb onto it was recorded. During the test, animals were administered the test compound orally 1 hour before the start of each training session.

For all quantitative data, the group mean and standard deviation were calculated using the Microsoft Excel data analysis package (version 14.0.4760.1000, 32-bit). Statistical processing of data was carried out using SPSS Statistics 17.0 software (version 17.0.0.236), and graphs (if necessary) were plotted in Statsoft Statistica 12 for Windows 10 (version 12.5.192.0).

5. Molecular modeling

The interactions between positive modulator **5** and the AMPA receptor were modeled using molecular docking and molecular dynamics simulations. The previously published^{S4} general computational protocol was significantly modified in order to simulate the dynamic environment of the full tetrameric receptor. The structure of the dimeric ligand-binding domain of the rat GluA2 AMPA receptor was obtained from the Protein Data Bank (PDB: 4FAT)^{S5} and allowed to relax in the simulation. The ligand structures were converted to 3D and preoptimized in the MMFF94 force field using Avogadro 1.2.0 software^{S6}. Both enantiomers were constructed and analyzed separately. The initial models of the protein–ligand complexes were obtained by molecular docking into the positive allosteric modulator binding site with AutoDock Vina 1.1.2 software^{S7}. The poses with the best scoring function value and ligand position were selected and the complex model was built using the UCSF Chimera 1.18 software^{S8}.

The system models were prepared for molecular dynamics using the Ligand Reader & Modeler and Solution Builder modules of the CHARMM-GUI web service^{S9,S10} and the simulations were performed in the CHARMM36/CGenFF 4.6 force field^{S11,S12} employing the GROMACS 2024.3 software^{S13}. Since the dynamics of the isolated ligand-binding domain dimer (in particular, in terms of the three distances between the key secondary structure elements at the subunit interface) is substantially different from the full-size tetrameric receptor where they interact with the transmembrane and amino terminal domains and with other subunits, the distributions of the distances were analyzed using 30 ns section of the 100 ns trajectory for the model GluA2 tetramer (UniProt: P19491-2, PDB: 6DLZ) in the POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) membrane. From comparison of the distributions, the required bias potentials were determined as combinations of steep harmonic walls with a soft central harmonic term. For the analysis and visualization of the results, the cpptraj software^{S14} in the AmberTools 23 package^{S15} and UCSF Chimera were used. The binding free energies were estimated over the stable portion of the trajectories (last 20 ns, 101 frames at 200 ps interval) using the MM/GBSA approach implemented in the gmx_MMPBSA 1.6.1 software^{S16}. The internal dielectric constant $\epsilon = 4$ and the Interaction Entropy model for the conformation entropy contribution were used.

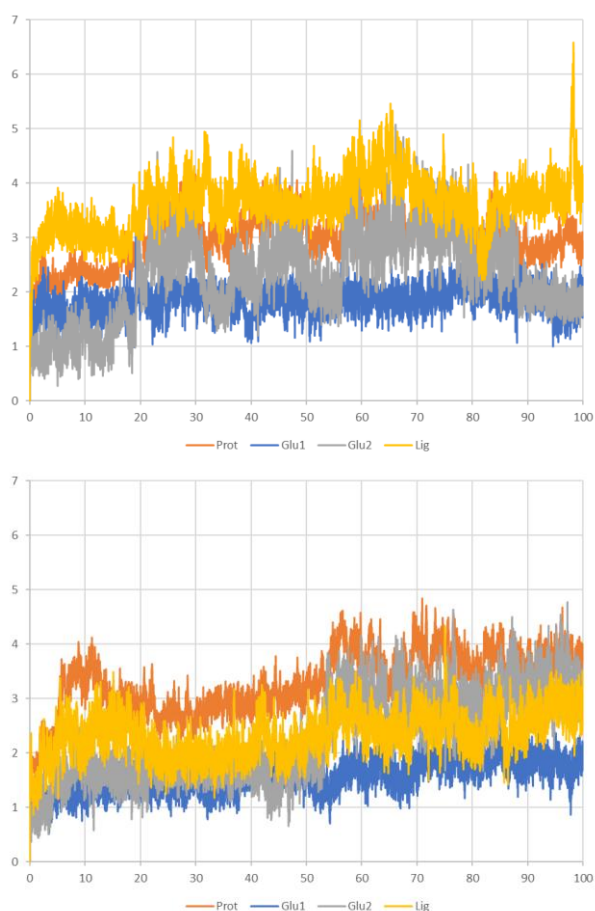


Figure S1 Root-mean-square deviations (RMSD, Å) of non-hydrogen atoms of the protein, glutamate Glu1 and Glu2, and the ligand for two enantiomers of compound **5** (top, *R*-**5**; bottom, *S*-**5**) during MD simulations of the complex of the modulator with the dimeric GluA2 ligand-binding domain of the AMPA receptor.

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