Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

Supporting Information

Synthesis and σ receptor affinity of spiro[[2]benzopyran-1,1'-cyclohexanes] with exocyclic amino moiety in 3`-position

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Contents

1. Synthetic procedure

Synthesis of cyclohexane-1,3-dione monoethylene ketal (7)

Monoketal **7** was synthesized according to ref.[1,2]

Cyclohexane-1,3-dione (1.0 g, 8.92 mmol) was dissolved in toluene (200 mL) at130 °C. Subsequently, ethylene glycol (498 µL, 8.92 mmol) and *p*-toluenesulfonic acid (0.17 g, 10 mol %) were added and the mixture was heated to reflux for 1 h in a Dean-Stark apparatus. The reaction mixture was cooled to rt, Et_2O (200 mL) was added and the mixture was washed with saturated $NaHCO_3$ solution (100 mL). The organic layer was dried (K_2CO_3), concentrated in vacuo and the residue was rapidly purified by fc (Ø 8 cm, 20 cm, cyclohexane : ethyl acetate 3 : 1, 65 mL). R_f (cyclohexane : ethyl acetate 2 : 1 = 0.26). Pale yellow oil, yield 0.46 g (33 %). $C_8H_{12}O_3$ (156.2). MS (EI): m/z (%) = 156 [M, 4], 113 [M – $CH_3CH_2CH_2$*, 40], 99 [M – $CH_3C{=}OCH_2$*, 100], 55 [$O^+{\equiv}CCH{=}CH_2$, 45]. IR: $\tilde{\nu}$ (cm^{-1}) = 2956, 2883 (s, v, C-H, alkyl), 1714 (s, v, C=O), 1084 (s, v, C-O). ^{1}H NMR (d_6-DMSO): δ (ppm) = 1.71 – 1.77 (m, 2H, 5-CH_2), 1.85 – 1.88 (m, 2H, 4-CH_2), 2.30 (t, J = 7.0 Hz, 2H, 6-CH_2), 2.58 (s, 2H, 2-CH_2), 3.87 – 3.95 (m, 4H, OCH_2CH_2O).

2. Receptor binding studies

2.1. Materials

Guinea pig brains, rat brains and rat livers were commercially available (Harlan-Winkelmann, Borchen, Germany). Pig brains were a donation of the local slaughterhouse (Coesfeld, Germany). The recombinant L(tk-) cells stably expressing the GluN2B receptor were obtained from Prof. Dr. Dieter Steinhilber (Frankfurt, Germany). Homogenizers: Elvehjem Potter (B. Braun Biotech International, Melsungen, Germany) and Soniprep® 150 (MSE, London, UK). Centrifuges: Cooling centrifuge model Eppendorf 5427R (Eppendorf, Hamburg, Germany) and High-speed cooling centrifuge model Sorvall® RC-5C plus (Thermo Fisher Scientific, Langenselbold, Germany). Multiplates: standard 96 well multiplates (Diagonal, Muenster, Germany). Shaker: self-made device with adjustable temperature and tumbling speed (scientific workshop of the institute). Harvester: MicroBeta® FilterMate 96 Harvester. Filter: Printed Filtermat Typ A and B. Scintillator: Meltilex® (Typ A or B) solid state scintillator. Scintillation analyzer: MicroBeta® Trilux (all Perkin Elmer LAS, Rodgau-Jügesheim, Germany).

2.2. Preparation of membrane homogenates from guinea pig brain

5 guinea pig brains were homogenized with the potter (500-800 rpm, 10 up and down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 23,500 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 7.4) and centrifuged again at 23,500 x g (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5-6 volumes of buffer and frozen (-80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

2.3. Preparation of membrane homogenates from rat liver

Two rat livers were cut into small pieces and homogenized with the potter (500-800 rpm, 10 up and down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 31,000 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 8.0) and incubated at rt for 30 min. After the incubation, the suspension was centrifuged again at 31,000 x g for

20 min at 4 °C. The final pellet was resuspended in 5-6 volumes of buffer and stored at -80 °C in 1.5 mL portions containing about 2 mg protein/mL.

2.4. Protein determination

The protein concentration was determined by the method of Bradford[3] modified by Stoscheck.[4] The Bradford solution was prepared by dissolving 5 mg of Coomassie Brilliant Blue G 250 in 2.5 mL of EtOH (95 %, v/v). 10 mL deionized H_2O and 5 mL phosphoric acid (85 %, m/v) were added to this solution, the mixture was stirred and filled to a total volume of 50 mL with deionized water. The calibration was carried out using bovine serum albumin as a standard in 9 concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 4.0 mg /mL). In a 96 well standard multiplate, 10 µL of the calibration solution or 10 µL of the membrane receptor preparation were mixed with 190 µL of the Bradford solution, respectively. After 5 min, the UV absorption of the protein-dye complex at λ = 595 nm was measured with a plate reader (Tecan Genios®, Tecan, Crailsheim, Germany).

2.5. General procedures for the binding assays

The test compound solutions were prepared by dissolving approximately 10 µmol (usually 2-4 mg) of test compound in DMSO so that a 10 mM stock solution was obtained. To obtain the required test solutions for the assay, the DMSO stock solution was diluted with the respective assay buffer. The filtermats were presoaked in 0.5 % aqueous polyethylenimine solution for 2 h at rt before use. All binding experiments were carried out in duplicates in the 96 well multiplates. The concentrations given are the final concentration in the assay. Generally, the assays were performed by addition of 50 µL of the respective assay buffer, 50 µL of test compound solution in various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L), 50 µL of the corresponding radioligand solution and 50 µL of the respective receptor preparation into each well of the multiplate (total volume 200 µL). The receptor preparation was always added last. During the incubation, the multiplates were shaken at a speed of 500-600 rpm at the specified temperature. Unless otherwise noted, the assays were terminated after 120 min by rapid filtration using the harvester. During the filtration, each well was washed five times with 300 µL of water. Subsequently, the filtermats were dried at 95 °C. The solid scintillator was melted on the dried filtermats at a temperature of 95 °C for 5 min. After solidifying of the scintillator at rt, the trapped radioactivity in the

filtermats was measured with the scintillation analyzer. Each position on the filtermat corresponding to one well of the multiplate was measured for 5 min with the [^{3}H]-counting protocol. The overall counting efficiency was 20 %. The IC_{50} values were calculated with the program GraphPad Prism® 3.0 (GraphPad Software, San Diego, CA, USA) by non-linear regression analysis. Subsequently, the IC_{50} values were transformed into K_i values using the equation of Cheng and Prusoff.[5] The K_i values are given as mean value ± SEM from three independent experiments.

2.6. σ_1 receptor assay

The assay was performed with the radioligand [^{3}H]-(+)-pentazocine (22.0 Ci/mmol; Perkin Elmer). The thawed membrane preparation of guinea pig brain (about 100 µg of the protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H]-(+)-pentazocine, and TRIS buffer (50 mM, pH 7.4) at 37 °C. The non-specific binding was determined with 10 µM unlabeled (+)-pentazocine. The K_d value of (+)-pentazocine is 2.9 nM.[6]

2.7. σ_2 receptor assay

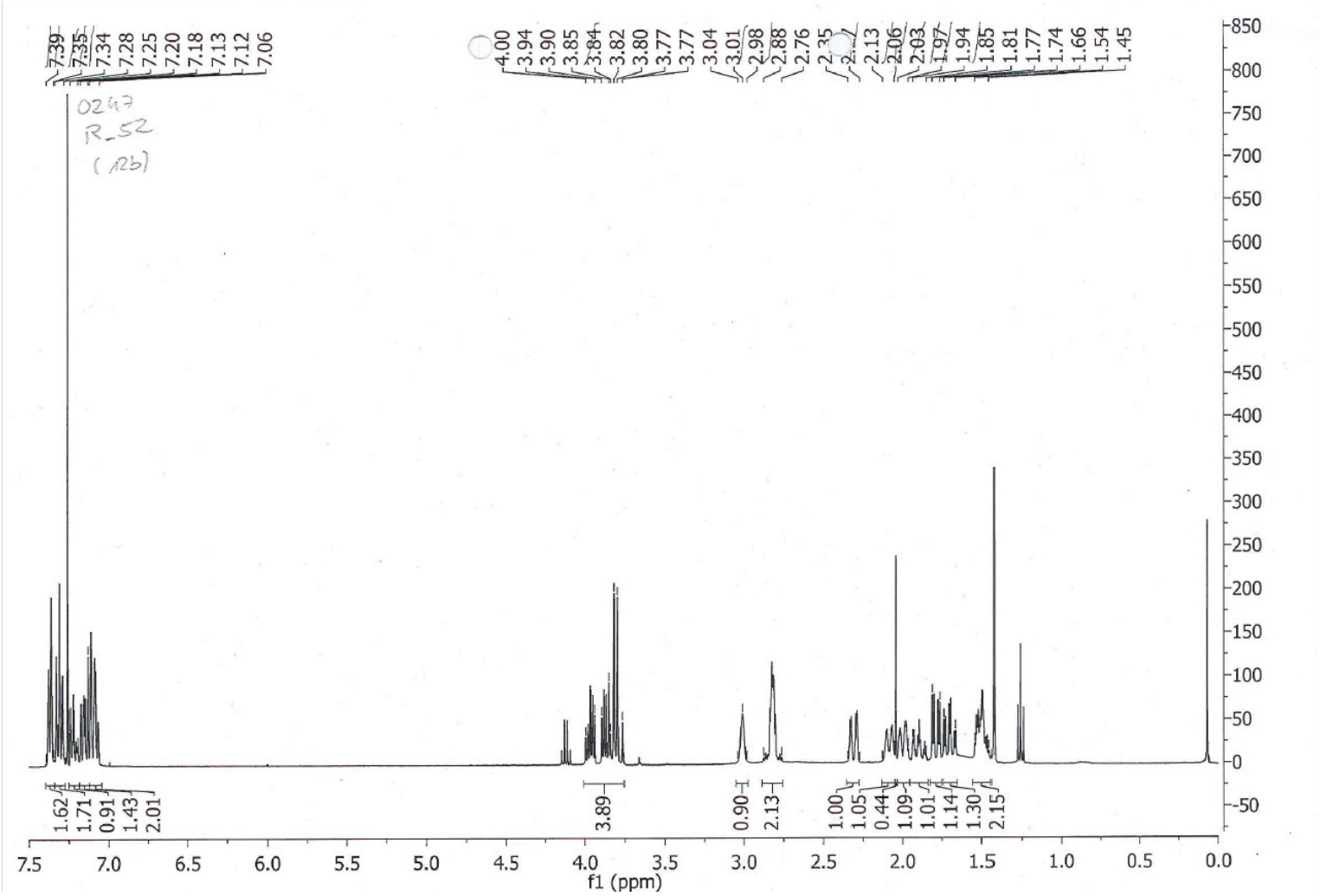
The assays were performed with the radioligand [^{3}H]di-*o*-tolylguanidine (specific activity 50 Ci/mmol; ARC, St. Louis, MO, USA). The thawed rat liver membrane preparation (about 100 µg protein) was incubated with various concentrations of the test compound, 3 nM [^{3}H]di-*o*-tolylguanidine and buffer containing (+)-pentazocine (500 nM (+)-pentazocine in TRIS buffer (50 mM TRIS, pH 8.0)) at rt. The non-specific binding was determined with 10 µM non-labeled di-*o*-tolylguanidine. The K_d value of di-*o*-tolylguanidine is 17.9 nM.[7]

3. References

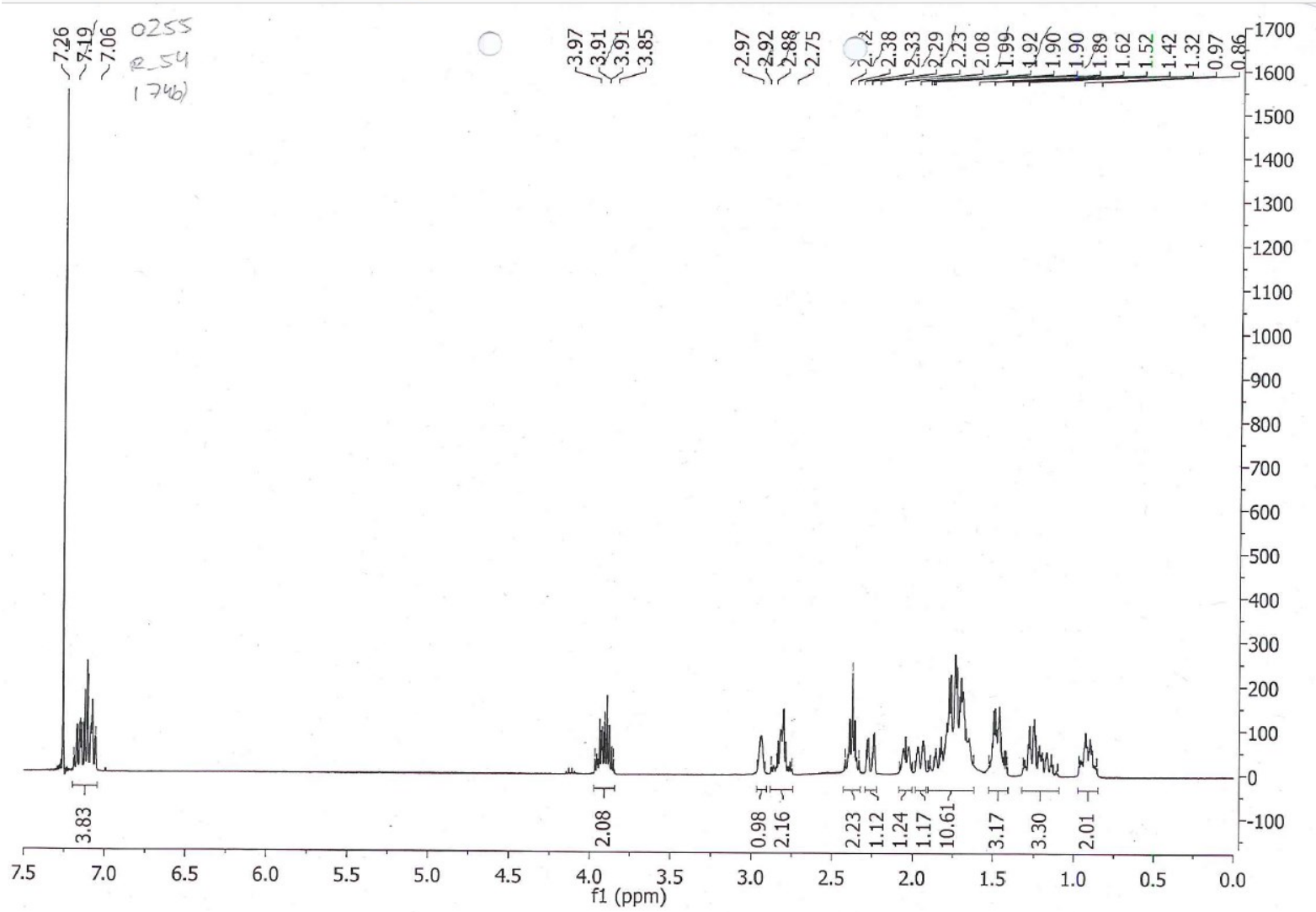
1. Hayashi, T.; Takagi, H.; Masuda, H.; Ogoshi, H. Synthesis and structure of new *cis*-1,3-dihydroxycyclohexane derivative having four convergent hydroxy groups. *J. Chem. Soc. Chem. Commun*. **1993**, 346-365.
2. Takagi, H.; Hayashi, T.; Mizutani, H.; Ogoshi, H. Synthesis and structure of tetraols with convergent and divergent arrays of hydroxy groups. *J.Chem. Soc., Perkin Trans*. **1999**, 1885-1892.
3 Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal*. *Biochem*. **1976,** *72*, 248–254.
4 Stoscheck, C. Quantification of protein, *Methods Enzymol*. **1990,** *182*, 50–68.
5 Cheng, Y.-C.; Prusoff, W. H. Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction*, Biochem. Pharmacol*. **1973,** *22*, 3099–3108.
6 DeHaven-Hudkins, D. L.; Fleissner, L. C.; Ford-Rice, F. Y. Characterization of the binding of [3H](+)-pentazocine to σ recognition sites in guinea pig brain, *Eur. J. Pharmacol. Mol. Pharmacol*. **1992,** *227*, 371–378.
7 Mach, R. H.; Smith, C. R.; Childers, S. R. Ibogaine possesses a selective affinity for σ2 receptors, *Life Sci*. **1995,** *57*, PL57–PL62.

4. NMR spectra

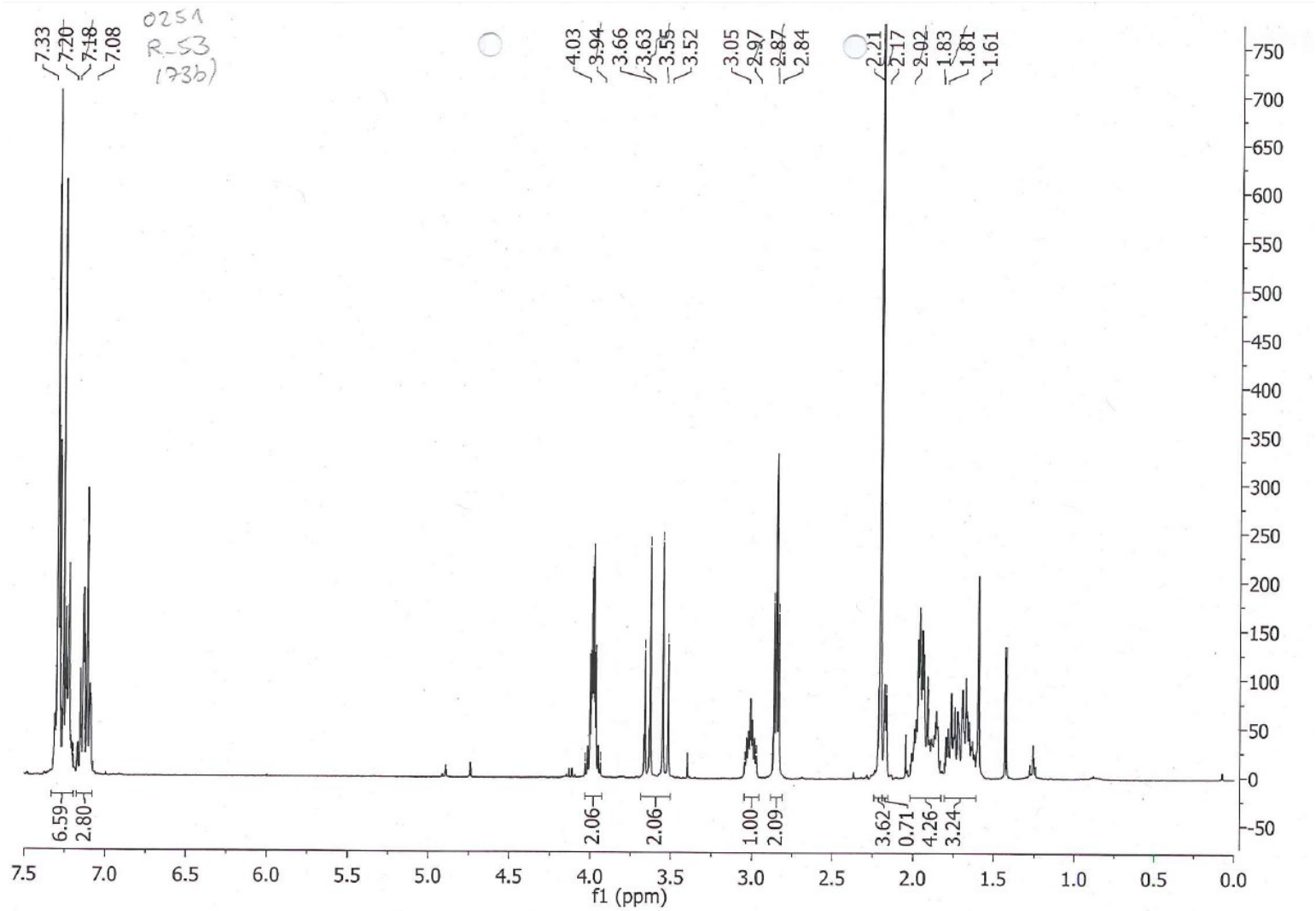
^{1}H NMR spectrum of *cis*-N-benzyl-3,4-dihydrospiro[[2]benzopyran-1,1'-cyclohexan]-3'-amine (*cis*-**4a**)



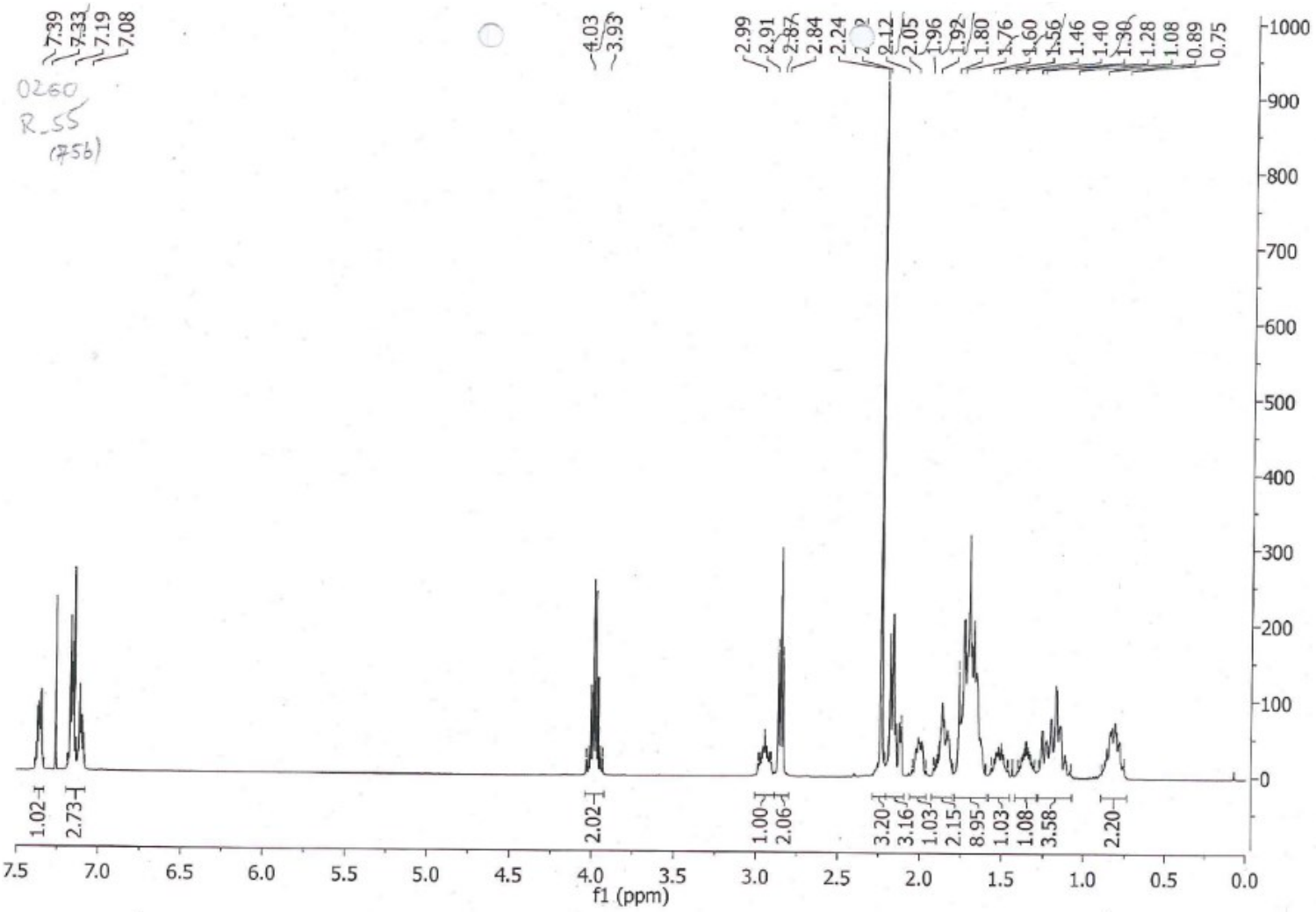
^{1}H NMR spectrum of *cis*-N-(cyclohexylmethyl)-3,4-dihydrospiro[[2]benzopyran-1,1'-cyclohexan]-3'-amine (*cis*-**4b**)



^{1}H NMR spectrum of *cis*-N-benzyl-N-methyl-3,4-dihydrospiro[[2]benzopyran-1,1'-cyclohexan]-3'-amine (*cis*-**5a**)



^{1}H NMR spectrum of *cis*-N-(cyclohexylmethyl)-N-methyl-3,4-dihydrospiro[[2]benzo-pyran-1,1'-cyclohexan]-3'-amine (*cis*-**5b**)



5. HPLC chromtograms

HPLC chromatogram of *cis*-N-benzyl-3,4-dihydrospiro[[2]benzopyran-1,1'-cyclohexan]-3'-amine (*cis*-**4a**)

(12b) MeOH

HPLC

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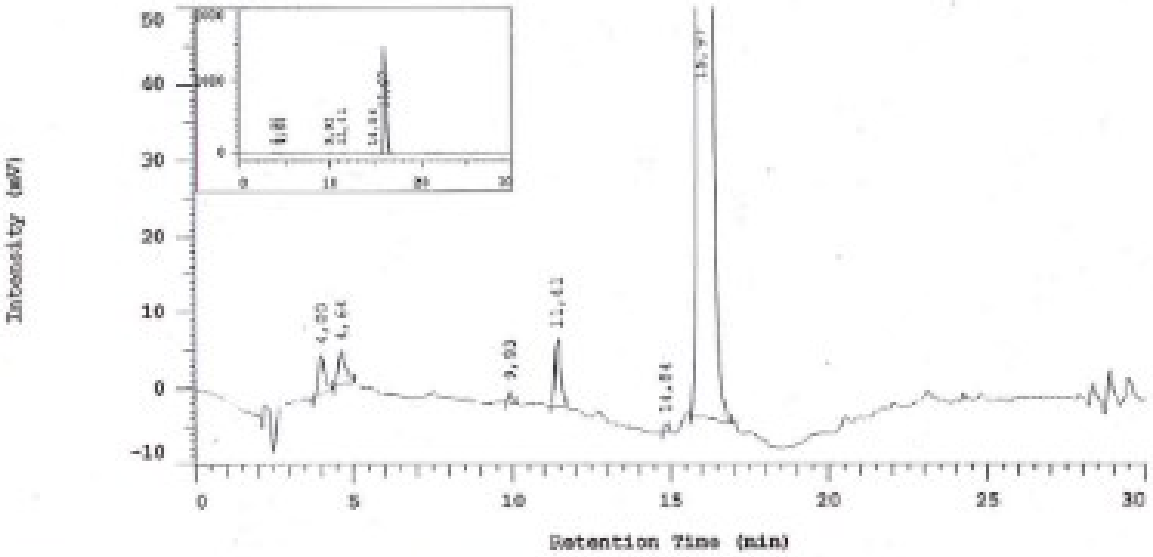
Reported: 05.05.09 08:59
Processed: 05.05.09 08:59

Data Path: D:\WIN32APP\HSM\Chromni\DATA\0195\
Application: Chromni
Sample Name: R_52.1
Injection from this vial: 1 of 1

Series:0195
Vial Number: 11
Vial Type: UNK
Volume: 5,0 ul

Chrom Type: HPLC Channel : 1



Acquisition Method: Chromni MeOH
Blank Subtr Sample Name: MeOH
Column Type: 010
Developed by: Christian
Solvent A: Wasser + 0,05%TFA
Solvent C: MeOH +0,05% TFA

No.	RT	Area	Conc 1	BC
1	4,00	72390	0,253	BB
2	4,64	73607	0,257	MC
3	9,93	14812	0,052	MC
4	11,41	98552	0,344	BB
5	14,84	5869	0,021	MC
6	15,97	28350508	99,073	MC
		28615738	100,000	

Peak rejection level: 0

HPLC chromatogram of of *cis*-N-(cyclohexylmethyl)-3,4-dihydrospiro[[2]benzopyran-1,1'-cyclohexan]-3'-amine (*cis*-**4b**)

(795)

MeOH

HPLC

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Reported: 25.06.09 08:48
Processed: 25.06.09 08:48

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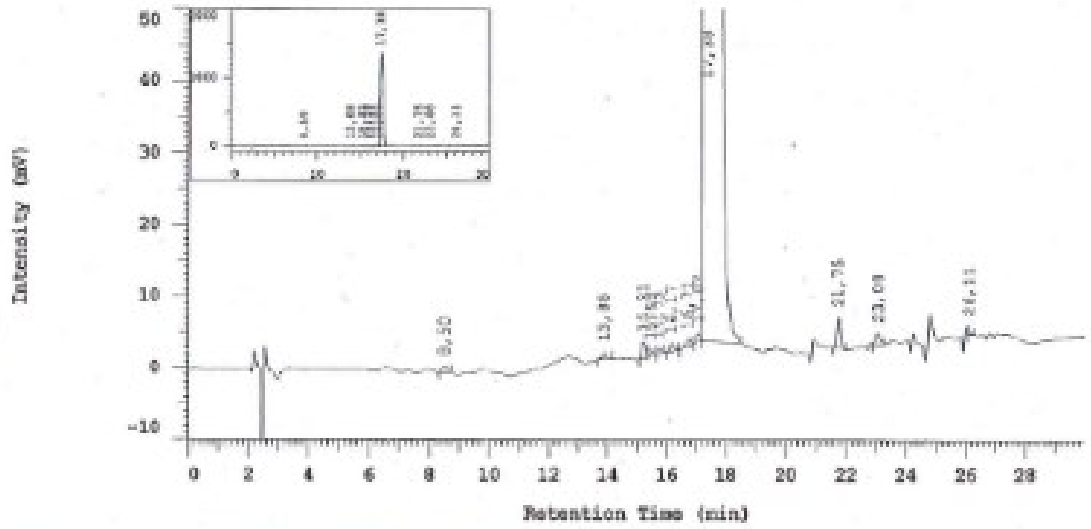
Chrom Type: HPLC Channel : 1



Acquisition Method: Chromni MeOH
Blank Subtr Sample Name: MeOH
Column Type: 010
Developed by: Christian
Solvent A: Wasser + 0,05%TFA
Solvent C: MeOH +0,05% TFA

No.	RT	Area	Conc 1	BC
1	8,50	9512	0,032	MC
2	13,88	9996	0,034	MC
3	15,21	19668	0,066	MC
4	15,43	7115	0,024	MC
5	15,73	4096	0,014	MC
6	16,17	10665	0,036	MC
7	16,71	10697	0,036	MC
8	17,07	8061	0,027	BB
9	17,38	29525849	99,526	MC
10	21,75	33779	0,114	MC
11	23,08	18750	0,063	MC
12	26,11	8337	0,028	MC
		29666525	100,000	

Peak rejection level: 0

HPLC chromatogram of *cis*-N-benzyl-N-methyl-3,4-dihydrospiro[[2]benzopyran-1,1'-

HPLC

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Reported: 25.06.09 08:45
Processed: 25.06.09 08:45

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Series:0336
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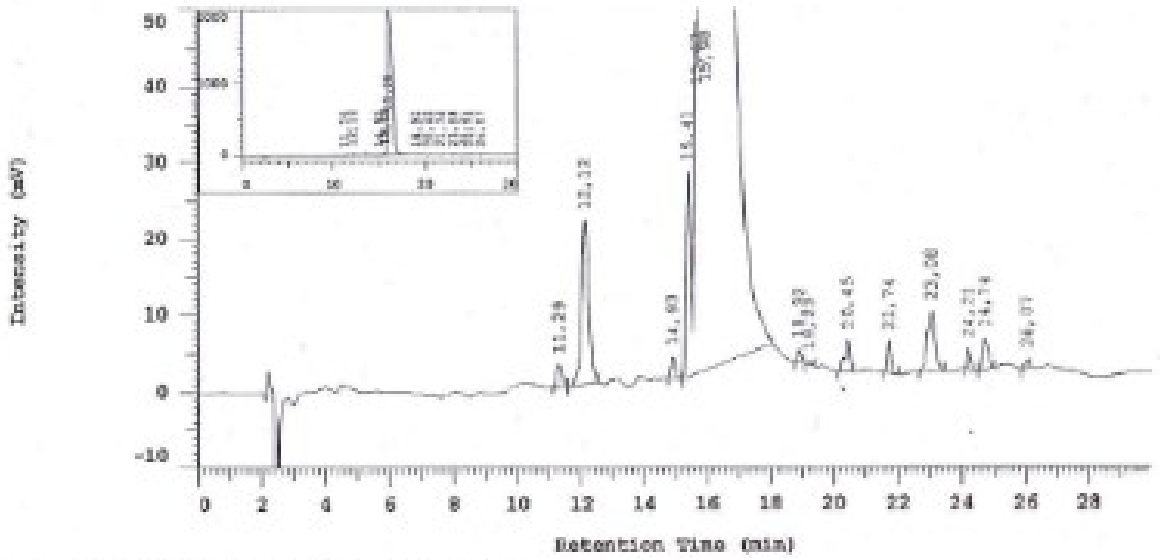
Vial Type: UNK
Volume: 5,0 ul
Chrom Type: HPLC Channel : 1



Acquisition Method: Chromni MeOH
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Developed by: Christian
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Solvent C: MeOH +0,05% TFA

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1	11,29	30640	0,037	BB
2	12,12	311564	0,378	MC
3	14,93	25870	0,031	BB
4	15,41	241507	0,293	MC
5	15,68	305377	0,370	MC
6	15,98	81267936	98,541	MC
7	18,92	11926	0,014	BB
8	19,23	4120	0,005	MC
9	20,45	45642	0,055	BB
10	21,74	32471	0,039	MC
11	23,08	117444	0,142	MC
12	24,21	18400	0,022	BB
13	24,74	48681	0,059	BB
14	26,07	9705	0,012	BB
		82471283	100,000	

Peak rejection level: 0

cyclohexan]-3'-amine (*cis*-**5a**)

HPLC chromatogram of *cis*-N-(cyclohexylmethyl)-N-methyl-3,4-dihydro-spiro[[2]benzopyran-1,1'-cyclohexan]-3'-amine (*cis*-**5b**)

(75b)

HPLC

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Processed: 02.07.09 11:16

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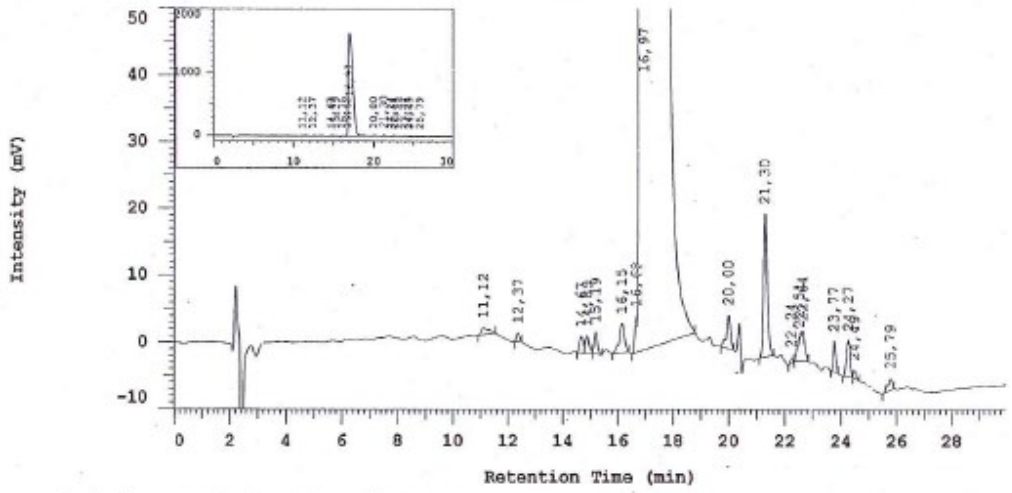
Chrom Type: HPLC Channel : 1



Acquisition Method: Chromni MeOH
Blank Subtr Sample Name: MeOH
Column Type: 010
Developed by: Christian
Solvent A: Wasser + 0,05%TFA
Solvent C: MeOH +0,05% TFA

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2	12,37	9778	0,017	MC
3	14,67	21474	0,037	BV
4	14,89	24990	0,043	VB
5	15,19	28432	0,049	BB
6	16,15	57336	0,099	MC
7	16,68	32153	0,055	MC
8	16,97	57416883	98,972	MC
9	20,00	47078	0,081	MC
10	21,30	179180	0,309	BB
11	22,24	3820	0,007	MC
12	22,51	27309	0,047	MC
13	22,64	41592	0,072	MC
14	23,77	28098	0,048	MC
15	24,27	49288	0,085	MC
16	24,49	7520	0,013	MC
17	25,79	18527	0,032	BB
		58013404	100,000	

Peak rejection level: 0