Electronic Supplementary Material (ESI) for RSC Advances

Synthesis of 1-substituted epibatidine analogues and their *in vitro* and *in vivo* evaluation as α4β2 nicotinic acetylcholine receptor ligands.

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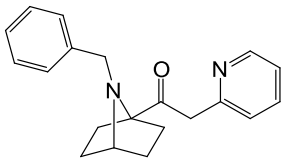
Contents

Synthesis

The synthesis of the compounds **5a-c** was performed as previously reported.[1] The yield of compound **5b** was improved by the use of an alternate purification method. It was isolated from the crude reaction mixture by column chromatography using Al_2O_3 as the stationary phase and CH_2Cl_2/MeOH (95/5) as the eluent in 52% yield.

Synthesis of 1-(7-benzyl-7-azabicyclo[2.2.1]heptan-1-yl)-2-(pyridin-2-yl)-ethanone 11

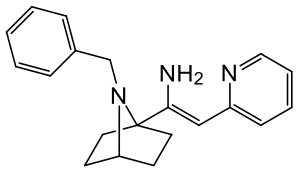
In a dry 100 ml flask 0.88g 2-picoline (9.420 mmol, 2 equiv) is dissolved in 30 ml dry THF. The mixture is placed under inert atmosphere, cooled to -20°C and 4.71 ml of a 2M BuLi solution (9.420 mmol, 2 equiv) is added dropwise. The resulting mixture is stirred at room temperature for 1.5h, after which a solution of 1.0g 7-benzyl-7-azabicyclo[2.2.1]heptane-1-carbonitrile **8b** (4.710 mmol, 1 equiv) in 20 ml dry THF is added. The reaction is continued for 43 hours at room temperature and quenched by adding 25 ml 1.5M HCl. After 1h of hydrolysis, the mixture is neutralised by the addition of solid $NaHCO_3$. Extraction is performed by means of CH_2Cl_2, the combined organic fractions are dried using $MgSO_4$ and the volatiles are evaporated. Column chromatography delivers ketone **11** in 94% yield as a viscous yellow oil as a 3/2 mixture of the keto- and enolforms.



^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 1,38-1,46 (2H, m, 2x $C\underline{H}_aH_bC_q$); 1,59-1,75 (2H, m, 2x $CH_{endo}\underline{H}_{exo}$); 1,91-1,98 (2H, m, 2x $CH_a\underline{H}_bC_q$); 2,13-2,22 (2H, m, 2x $C\underline{H}_{endo}H_{exo}$); 3,30 (1H, t, J=4,4 Hz, $CH_2C\underline{H}CH_2$); 3,52 (2H, s, $NC\underline{H}_2$); 4,19 (1H, $C\underline{H}_2C{=}O$); 5,66 (1H, $HOC_qC\underline{H}$); 6,89-6,97 (1H, d, $NC_qC\underline{H}$ pyr.); 7,09-7,13 (1H, dxd, $NCHC\underline{H}$ pyr.); 7,17-7,24 (1H, m, $NCHC\underline{H}$ pyr.); 7,27-7,34 (4H, m, 2x $C_qC\underline{H}$ fen., $C_qCHCHC\underline{H}$ fen.); 7,42 (2H, t, J=5,5Hz, 2x $C_qCHC\underline{H}$ fen.); 7,50-7,54 (1H, m, $NC_qCHC\underline{H}$ pyr.); 7,56-7,63 (1H, m, $NC_qCHC\underline{H}$ pyr.); 8,19 (1H, d, J=5,50 Hz, $NC\underline{H}$ pyr.); 8,51 (1H, d, J=5,0 Hz, $NC\underline{H}$ pyr.). **^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm):** 28,5 (2x $C_qC\underline{H}_2$ ring); 33,4 (2x $CHC\underline{H}_2$ ring); 47,6 ($C\underline{H}_2C{=}O$); 49,6 ($NC\underline{H}_2$); 50,3 ($NC\underline{H}_2$); 59,2 ($C\underline{H}$ ring); 59,7 ($C\underline{H}$ ring); 72,0 (C_q ring); 96,0 ($C\underline{H}C_qOH$); 118,4 ($NC_qC\underline{H}$ pyr.); 121,2 ($NC_qC\underline{H}$ pyr.); 121,8 ($NCHC\underline{H}$ pyr. enolvorm); 124,6 ($NCHC\underline{H}$ pyr., keto); 126,7 (C_q Ph); 127,1 ($C\underline{H}CHCHC_q$ Ph); 128,2 ($C\underline{H}CHC_q$ Ph); 128,4 ($C\underline{H}CHC_q$ Ph); 128,8 ($CHC\underline{H}C_q$ Ph); 128,9 ($CHC\underline{H}C_q$ Ph); 136,4 ($NC_qCHC\underline{H}$); 137,2 ($NC_qCHC\underline{H}$); 139,9 (C_q Ph) ; 140,7 (C_q Ph); 144,2 ($NC\underline{H}$ pyr.); 149,5 ($NC\underline{H}$ pyr.); 155,4 (C_q pyr. keto) ; 158,5 (pyr. C_q enol); 170,0 (C_qOH); 209,0 ($C_q{=}O$). **IR γ (cm^{-1}):** 1708 (C=O). **MS^{ES} m/z (%):** 307,3 ($M+H^+$; 100). **Chromatography:** 66/33/1 PE/EtOAc/NEt_3: R_f=0,18.

Synthesis of 1-(7-benzyl-7-azabicyclo[2.2.1]heptan-1-yl)-2-(pyridin-2-yl)-ethylene amine 12

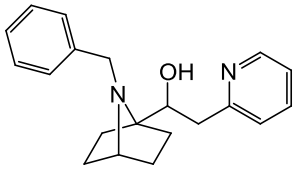
In a dry 100 ml flask 0.88g 2-picoline (9.420 mmol, 2 equiv) is dissolved in 30 ml dry THF. The mixture is placed under inert atmosphere, cooled to -20°C and 4.71 ml of a 2M BuLi solution (9.420 mmol, 2 equiv) is added dropwise. The resulting mixture is stirred at room temperature for 1.5h, after which a solution of 1.0g 7-benzyl-7-azabicyclo[2.2.1]heptane-1-carbonitrile **8b** (4.710 mmol, 1 equiv) in 20 ml dry THF is added. The reaction is continued for 43 hours at room temperature and quenched by adding 30 ml water. Extraction is performed by means of CH_2Cl_2 (4x50ml), the combined organic fractions are dried using $MgSO_4$ and the volatiles are evaporated. Crystallisation in EtOH delivers enamine 12 in 65% Yield. Due to hindered rotation along the neopentylic ethylene substituent, significant broadening of the peaks was observed in the NMR analysis. This was the case for the four CH_2's of the bicyclic core in ^{13}C-NMR and for the benzylic CH_2 in ^{1}H-NMR. Peak identification was performed based on spectra recorded at 50°C in deuterated chloroform, and at 100°C in deuterated DMSO (^{13}C only, see below).



^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.36-1.56 (2H, m, 2x$NCHC\underline{H}_{endo}H_{exo}$); 1.59-1.79 (2H, m, 2x$NC_qC\underline{H}_aH_b$); 1.92 (2H, br. s, 2x$NCHCH_{endo}\underline{H}_{exo}$); 2.02-2.21 (2H, m, 2x$NC_qCH_a\underline{H}_b$); 3.29 (1H, br. s, NCH ring); 3.47 (2H, br. s, NCH_2); 5.14 (1H, s, $NH_2C_qC\underline{H}$); 6.80 (1H, dxd, *J*=7.2Hz, 5.0Hz, $NCHC\underline{H}$ pyr.); 6.94 (1H, d, *J*=7.7Hz, NC_qCH pyr.); 7.22 (1H, t, *J*=7.2Hz, $C_qCHCHC\underline{H}$ Ph); 7.30 (2H, t, *J*=7.2Hz, 2x$C_qCHC\underline{H}$ Ph); 7.36 (2H, d, *J*=7.2Hz, C_qCH Ph); 7.46 (1H, dxd, *J*=7.7Hz, 7.2Hz, $NC_qCHC\underline{H}$ pyr.); 8.40 (1H, d, *J*=5.0Hz, NCH pyr.). **^{13}C-NMR (75 MHz, $CDCl_3$): δ** 26.1-31.5 (2x$NCHC\underline{H}_2$ ring); 36.2-39.8 (2x$NC_qC\underline{H}_2$ ring); 48.9 (NCH_2); 58.9 (NCH ring); 72.4 (NC_q ring); 94.1 ($NH_2C_qC\underline{H}$); 117.1 ($NCHC\underline{H}$ pyr.); 121.6 ($NC_qC\underline{H}$ pyr.); 126.7 ($C_qCHCHC\underline{H}$ Ph); 128.3 (2x$C_qCHC\underline{H}$ Ph); 128.4 (2x$C_qC\underline{H}$ Ph); 135.5 ($NC_qC\underline{H}CH$ pyr.); 140.8 (C_q Ph); 147.7 ($NC\underline{H}$ pyr.); 152.3 (NH_2C_q); 160.1 (C_q pyr.). **^{13}C-NMR (100 MHz, DMSO, 373K): δ** 28.7 (2x$NCHC\underline{H}_2$ ring); 34.1 (2x$NC_qC\underline{H}_2$ ring); 48.8 (NCH_2); 59.1 (NCH ring); 72.4 (NC_q ring); 93.6 ($NH_2C_qC\underline{H}$); 117.2 ($NCHC\underline{H}$ pyr.); 121.7 ($NC_qC\underline{H}$ pyr.); 126.8 ($C_qCHCHC\underline{H}$ Ph); 128.4 (2x$C_qCHC\underline{H}$ Ph); 128.7 (2x$C_qC\underline{H}$ Ph); 135.9 ($NC_qC\underline{H}CH$ pyr.); 141.2 (C_q Ph); 147.7 ($NC\underline{H}$ pyr.); 152.7 (NH_2C_q); 160.4 (C_q pyr.). **IR (cm^{-1}):** ν_{NH2} = 3456, 1622, 1592; ν_{max} = 1538, 1405, 1553, 792, 745, 724, 697. **MS (70 eV): m/z (%)** 306 ($M+H^+$, 100). **HRMS (ESI):** $C_{20}H_{23}N_3$, 306.1970 (calcd, $M+H^+$), 306.1971 (found). **MP:** 112.0°C. **Yield:** 65%.

Synthesis of 1-(7-benzyl-7-azabicyclo[2.2.1]heptan-1-yl)-2-(pyridin-2-yl)-ethanol 13

In a 250 ml flask 1.22 g 1-(7-benzyl-7-azabicyclo[2.2.1]heptan-1-yl)-2-(pyridin-2-yl)-ethanone **11** (3.984 mmol, 1 equiv) is dissolved in 100 ml MeOH. The solution is cooled to 0°C and placed under inert atmosphere, ofter which 165.8 mg $NaBH_4$ (4.383 mmol, 1.1 equiv) is added in small portions. The reaction is stirred for 24h at room temperature. The solvent is removed, leaving approximately one quarter and 70 ml saturated aqueous $NaHCO_3$ is added. The compound is extracted using three portions EtOAc, dried by means of $MgSO_4$, filtered and evaporated. Alcohol **13** is isolated as a viscous yellow oil in 93% yield.

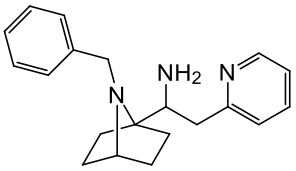


^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.27-1.57 (4H, m, 2x$NC_qC\underline{H}_aH_b$, 2x$NCHC\underline{H}_{endo}H_{exo}$); 1.63-1.83 (2H, m, $NC_qCH_a\underline{H}_b$, $NCHCH_{endo}\underline{H}_{exo}$); 1.90-2.10 (2H, m, $NC_qCH_a\underline{H}_b$, $NCHCH_{endo}\underline{H}_{exo}$); 2.96 (2H, d, *J*=6.1Hz, $HOCHC\underline{H}_2$); 3.18 (1H, t, *J*=4.4Hz, NCH ring); 3.32 (1H, d, *J*=13.2Hz, $NC\underline{H}_aH_b$); 3.72 (1H, d, *J*=13.2Hz, $NCH_a\underline{H}_b$); 3.97 (1H, br. s, OH); 4.39 (1H, t, *J*=6.1Hz, $HOC\underline{H}$); 7.15 (1H, dxd, *J*=7.2Hz, 5.0Hz, $NCHC\underline{H}$ pyr.); 7.18-7.25 (2H, m, NC_qCH pyr., $C_qCHCHC\underline{H}$ Ph); 7.29 (2H, dxd, *J*=7.7Hz, 7.2Hz, $C_qCHC\underline{H}$ Ph);

7.36 (2H, d, *J*=7.2Hz, C_qCH Ph); 7.62 (1H, dxd, *J*=7.7Hz, 7.2Hz, NC_qCHCH pyr.); 8.54 (1H, d, *J*=5.0Hz, NCH pyr.). **^{13}C-NMR (75 MHz, $CDCl_3$): δ** 28.4 (CH_2 ring); 28.5 (CH_2 ring); 29.3 (CH_2 ring); 30.1 (CH_2 ring); 41.1 ($HOCHCH_2$); 49.0 (NCH_2); 59.4 (NCH ring); 71.3 (HOCH); 71.9 (NC_q ring); 121.6 ($NCHCH$ pyr.); 124.1 (NC_qCH pyr.); 126.9 ($C_qCHCHCH$ Ph); 128.4 ($2xC_qCHCH$ Ph); 128.9 ($2xC_qCH$ Ph); 136.6 (NC_qCHCH pyr.); 140.2 (C_q Ph); 149.1 (NCH pyr.); 160.1 (C_q pyr.). **IR (cm^{-1}):** ν_{OH} = 3337; ν_{max} = 2954, 1594, 1436, 1070, 750, 724, 696. **MS (70 eV): m/z (%)** 309 ($M+H^+$, 100). **HRMS (ESI):** $C_{20}H_{24}N_2O$, 309.1967 (calcd, $M+H^+$), 309.1970 (found). **Yield:** 93%.

Synthesis of 1-(7-benzyl-7-azabicyclo[2.2.1]heptan-1-yl)-2-(pyridin-2-yl)-ethane amine 14

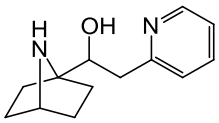
To a solution of 0.15 g 1-(7-benzyl-7-azabicyclo[2.2.1]heptan-1-yl)-2-(pyridin-2-yl)-ethlylene amine **12** (0.491 mmol, 1 equiv) in 20 ml MeOH, 30.9 mg $NaBH_3CN$ (0.491 mmol, 1 equiv) and 29.5 mg acetic acid (0.491 mmol, 1 equiv) are added. The mixture is stirred for 115h at room temperature, after which 40 ml aqueaous saturated $NaHCO_3$ is added. The compound is extracted using five portions of EtOAc (20ml). The combined organic phases are dried, filtered and evaporated, yielding the desired 1-(7-benzyl-7-azabicyclo[2.2.1]heptan-1-yl)-2-(pyridin-2-yl)ethane amine **14** as a slightly yellow oil (95%).



^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.23-1.47 (3H, m, $NC_qCH_aH_b$, $NCHCH_{endo}H_{exo}$, $NC_qCH_aH_b$ or $NCHCH_{endo}H_{exo}$); 1.48-1.62 (1H, m, $NC_qCH_aH_b$ or $NCHCH_{endo}H_{exo}$); 1.64-1.81 (2H, m, $NC_qCH_aH_b$, $NCHCH_{endo}H_{exo}$); 1.90-2.06 (2H, m, $NC_qCH_aH_b$, $NCHCH_{endo}H_{exo}$); 2.69 (1H, dxd, *J*=13.8Hz, 10.5Hz, $NH_2CHCH_aH_b$); 3.06 (1H, d, *J*=13.8Hz, $NH_2CHCH_aH_b$); 3.17 (1H, t, *J*=4.4Hz, NCH ring); 3.32 (1H, d, *J*=11.6Hz, NCH_aH_b); 3.48 (2H, s, NH_2); 3.66 (1H, d, *J*=11.6Hz, NCH_aH_b); 3.66 (1H, d, *J*=10.5Hz, $CHNH_2$); 7.14 (1H, dxd, *J*=7.2Hz, 5.0Hz, $NCHCH$ pyr.); 7.19-7.25 (2H, m, NC_qCH pyr., $C_qCHCHCH$, Ph); 7.30 (2H, dxd, *J*=7.7Hz, 7.2Hz, $2xC_qCHCH$, Ph); 7.37 (2H, d, *J*=7.2Hz, C_qCH, Ph); 7.61 (1H, dxd, *J*=7.7Hz, 7.2Hz, NC_qCHCH pyr.); 8.57 (1H, d, *J*=5.0Hz, NCH pyr.). **^{13}C-NMR (75 MHz, $CDCl_3$): δ** 28.1 (CH_2 ring); 28.5 (CH_2 ring); 28.9 (CH_2 ring); 30.5 (CH_2 ring); 42.5 (NH_2CHCH_2); 48.5 (NCH_2); 52.3 ($CHNH_2$); 58.9 (NCH ring); 71.8 (NC_q ring); 121.3 ($NCHCH$ pyr.); 124.0 (NC_qCH pyr.); 126.6 ($C_qCHCHCH$, Ph); 128.2 ($2xC_qCHCH$, Ph); 128.6 ($2xC_qCH$, Ph); 136.3 (NC_qCHCH pyr.); 140.3 (C_q, Ph); 149.4 (NCH pyr.); 160.2 (C_q pyr.). **IR (cm^{-1}):** ν_{NH} = 3376, 1591; ν_{max} = 2951, 1474, 1435, 1097, 750, 722, 697. **MS (70 eV): m/z (%)** 308 ($M+H^+$, 100). **HRMS (ESI):** $C_{20}H_{25}N_3$, 308.2127 (calcd, $M+H^+$), 308.2130 (found). **Yield:** 95%.

Synthesis of 1-(7-azabicyclo[2.2.1]heptan-1-yl)-2-(pyridin-2-yl)ethanol 5d

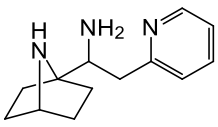
In 100 ml flask 0.59 g 1-(7-benzyl-7-azabicyclo[2.2.1]heptan-1-yl)-2-(pyridin-2-yl)-ethanol **13** (1.923 mmol, 1 equiv) and 0.62 g 1,3-cyclohexadiene (7.691 mmol, 4 equiv) are dissolved in 60 ml methanol. The flask is placed under inert Ar-atmosphere and 0.30 g Pd/C is added (the Pd/C used contained 20% Pd m/m, resulting in 10% catalyst loading). The suspension is heated to reflux for 3h. The catalyst is removed by filtration over celite and the filtrate is evaporated. The resulting oil is crystallized in Et_2O, yielding brownish crystals of 5d in 69% Yield.



^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.30-1.59 (5H, m, $2xNC_qCH_aH_b$, $NC_qCH_aH_b$, $2xNCHCH_{endo}H_{exo}$); 1.59-1.84 (3H, m, $NC_qCH_aH_b$, $2xNCHCH_{endo}H_{exo}$); 2.89 (1H, dxd, *J*=14.3Hz, 9.4Hz, $HOCHCH_aH_b$); 2.97 (1H, dxd, *J*=14.3Hz, 3.3Hz, $HOCHCH_aH_b$); 3.56 (1H, t, *J*=4.4Hz, NCH ring); 4.33 (1H, dxd, *J*=9.4Hz, 3.3Hz, HOCH); 7.15 (1H, dxd, *J*=7.2Hz, 5.0Hz, $NCHCH$ pyr.); 7.20 (1H, d, *J*=7.7Hz, NC_qCH pyr.); 7.62 (1H, dxd, *J*=7.7Hz, 7.2Hz, NC_qCHCH pyr.); 8.49 (1H, d, *J*=5.0Hz, NCH pyr.). **^{13}C-NMR (75 MHz, $CDCl_3$): δ** 29.0 ($NCHC_aH_2$ ring); 31.5 ($NCHC_bH_2$ ring); 32.0 ($NC_qC_aH_2$ ring); 32.4 ($NC_qC_bH_2$ ring); 40.4 ($HOCHCH_2$); 56.5 (NCH ring); 70.7 (NC_q ring); 73.4 (HOCH); 121.7 ($NCHCH$ pyr.); 123.9 (NC_qCH pyr.); 136.8 (NC_qCHCH pyr.); 148.8 (NCH pyr.); 160.4 (C_q pyr.). **IR (cm^{-1}):** $\nu_{NH/OH}$ = 3084; ν_{max} = 2960, 1591, 1472, 1436, 1343, 1212, 1068, 1045, 964, 944, 767, 741. **MS (70 eV): m/z (%)** 219 ($M+H^+$, 100). **HRMS (ESI):** $C_{13}H_{18}N_2O$, 219.1497 (calcd, $M+H^+$), 219.1494 (found). **MP (Et_2O):** 76.5°C. **Yield:** 69%.

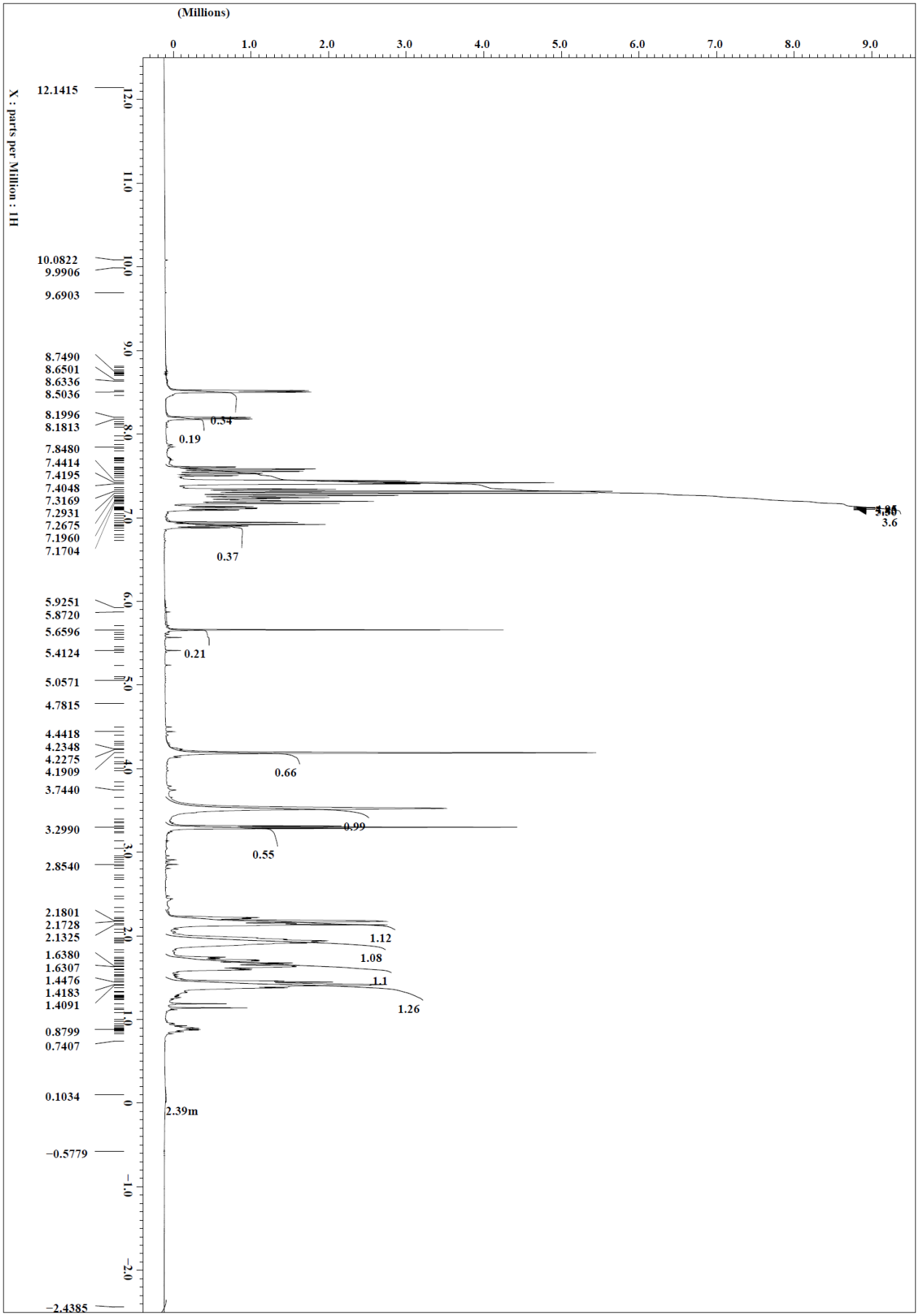
Synthesis of 1-(7-azabicyclo[2.2.1]heptan-1-yl)-2-(pyridin-2-yl)ethane amine 5e

In a 50 ml flask 74.0 mg 1-(7-benzyl-7-azabicyclo[2.2.1]-heptan-1-yl)-2-(pyridin-2-yl)-ethane amine **14** (0.241 mmol, 1 equiv) and 60.7 mg $NH_4^+HCO_2^-$ (0.963 mmol, 4 equiv) are dissolved in 25 ml MeOH. The catalyst, 37.0 mg Pd/C, is added under inert Ar-atmosphere (the Pd/C used contained 20% Pd m/m, resulting in 10% catalyst loading). After heating to reflux for 7h, the catalyst is removed by filtration over celite and the solvent is evaporated. The compound is dissolved in 20 ml distilled water, and washed with two 15 ml portions of Et_2O, EtOAc and CH_2Cl_2. The aquous phase is concentrated by rotary evaporation, yielding compound **5e** in 42% yield as a viscous yellow oil.

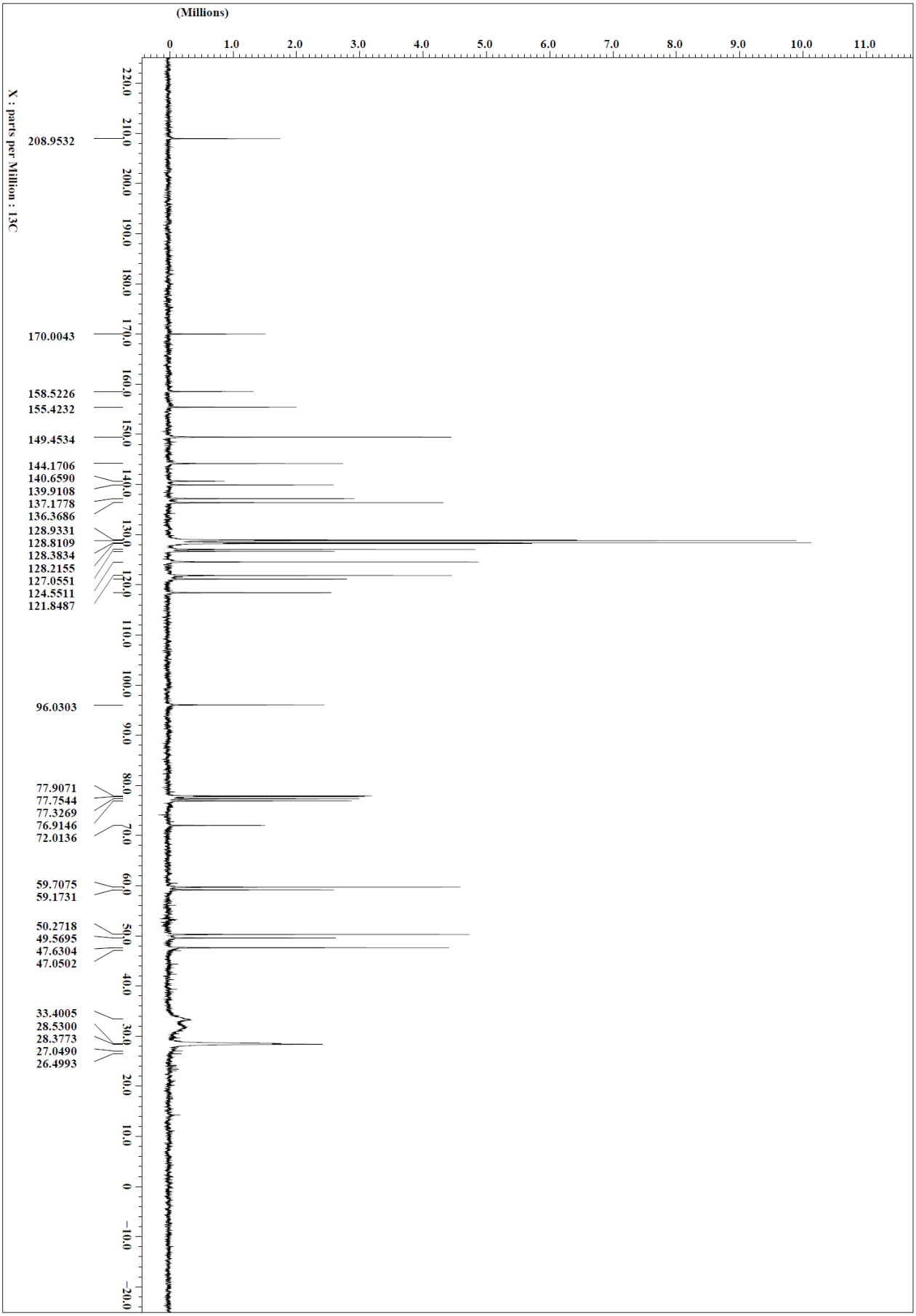


^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.38-1.53 (1H, m, $NCHCH_{endo}H_{exo}$); 1.54-1.72 (3H, m, $2xNC_qCH_aH_b$, $NCHCH_{endo}H_{exo}$); 1.73-1.88 (1H, m, $NC_qCH_aH_b$); 1.94-2.13 (3H, m, $NC_qCH_aH_b$, $2xNCHCH_{endo}H_{exo}$); 2.71 (1H, dxd, *J*=13.8Hz, 9.9Hz, $NH_2CHCH_aH_b$); 3.06 (1H, dxd, *J*=13.8Hz, 2.8Hz, $NH_2CHCH_aH_b$); 3.76 (1H, dxd, *J*=9.9Hz, 2.8Hz, $CHNH_2$); 3.87 (1H, t, *J*=4.4Hz, NCH ring); 4.50 (3H, s, NH, NH_2); 7.15 (1H, dxd, *J*=7.2Hz, 5.0Hz, $NCHCH$ pyr.); 7.22 (1H, d, *J*=7.7Hz, NC_qCH pyr.); 7.62 (1H, dxd, *J*=7.7Hz, 7.2Hz, NC_qCHCH pyr.); 8.51 (1H, d, *J*=5.0Hz, NCH pyr.). **^{13}C-NMR (75 MHz, $CDCl_3$): δ** 27.6 ($NCHC_aH_2$ ring); 29.6 ($NCHC_bH_2$ ring); 30.3 ($NC_qC_aH_2$ ring); 32.4 ($NC_qC_bH_2$ ring); 42.0 (NH_2CHCH_2); 53.9 ($CHNH_2$); 57.0 (NCH ring); 73.3 (NC_q ring); 121.7 ($NCHCH$ pyr.); 124.0 (NC_qCH pyr.); 136.7 (NC_qCHCH pyr.); 149.4 (NCH pyr.); 159.1 (C_q pyr.). **IR (cm^{-1}):** ν_{NH} = 3261, 1588; ν_{max} = 2951, 1476, 1436, 1347, 762. **MS (70 eV): m/z (%)** 218 ($M+H^+$, 100). **Yield:** 42%.

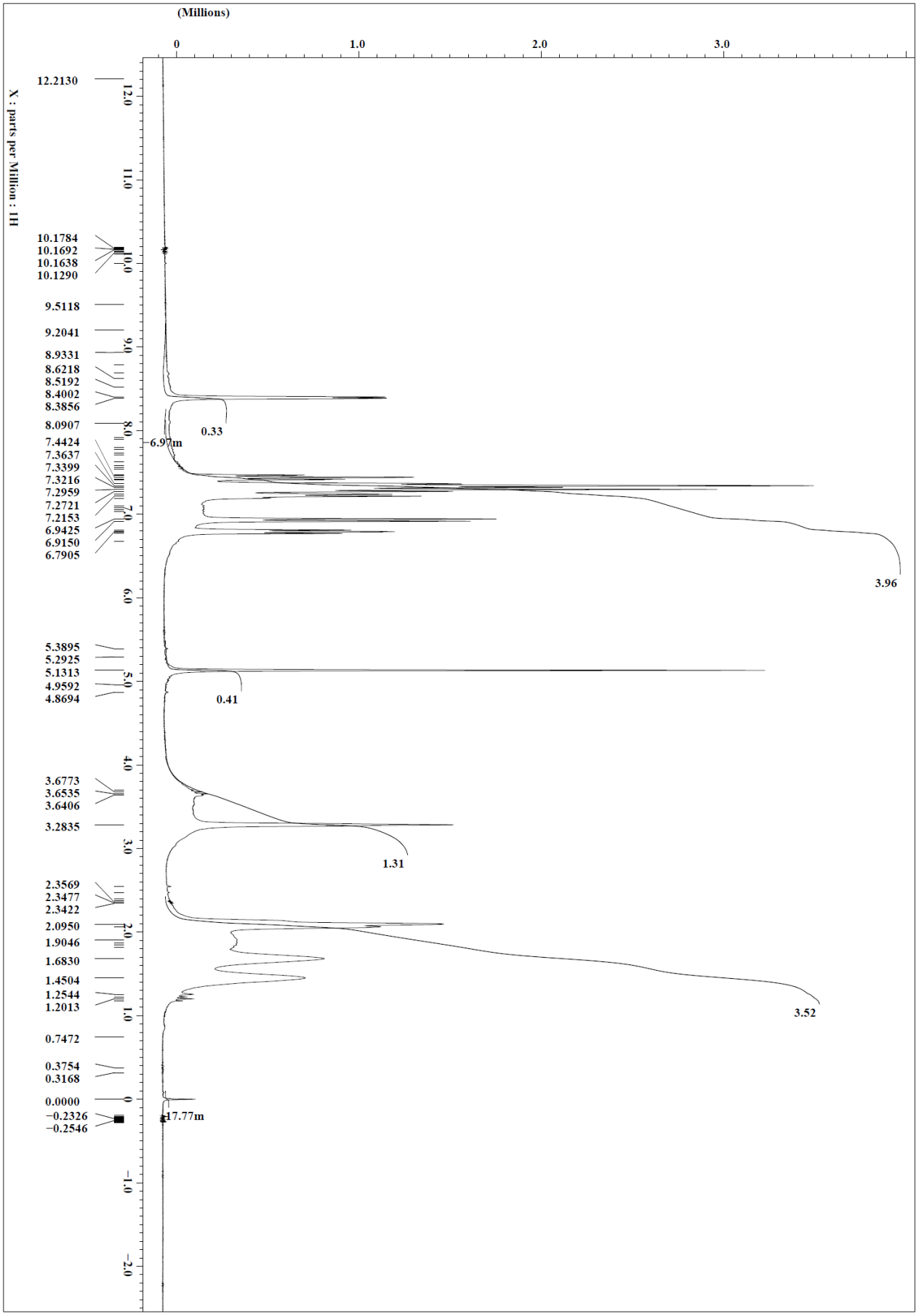
^{1}H-NMR compound 11



^{13}C-NMR compound 11



^{1}H-NMR compound 12



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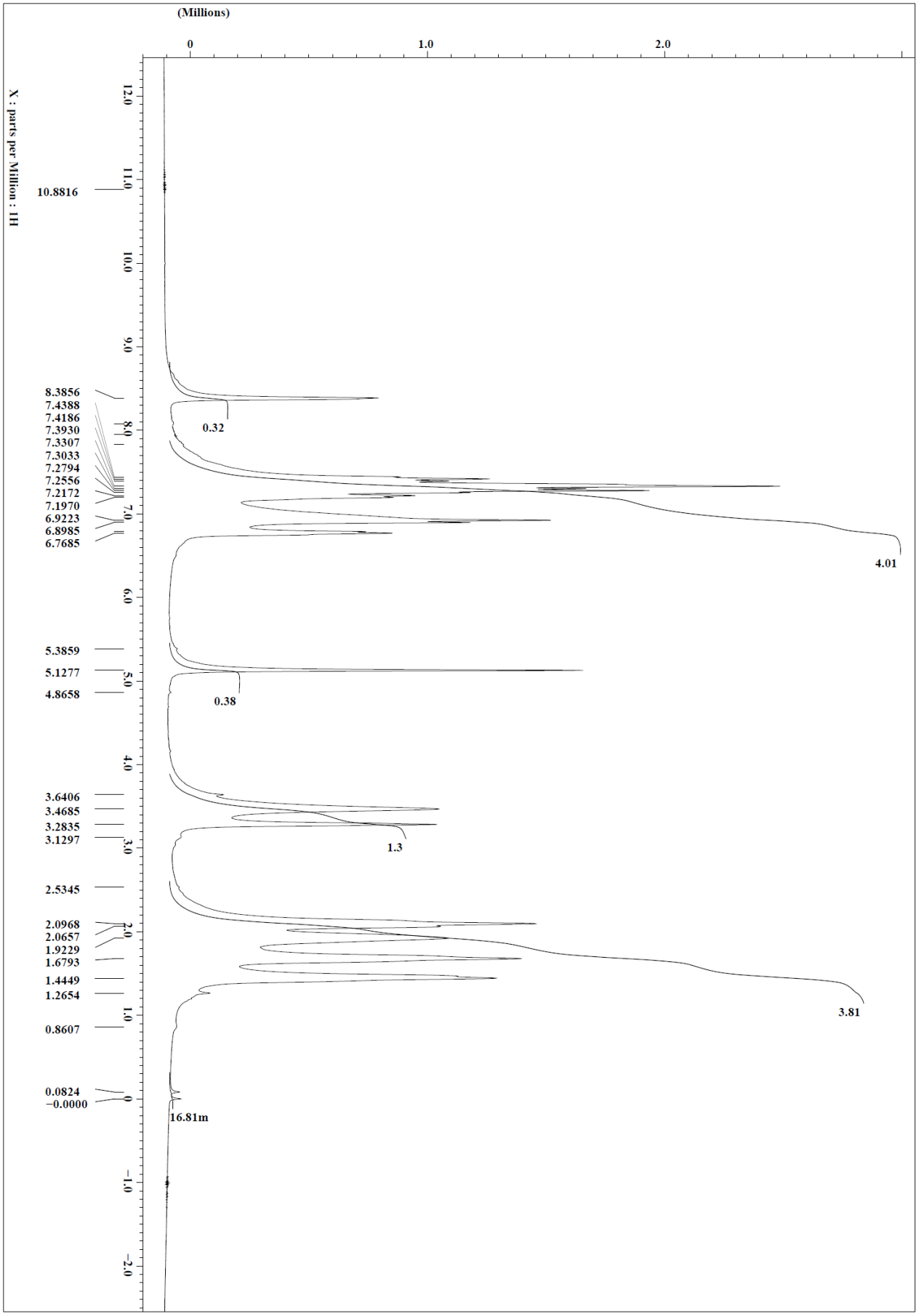

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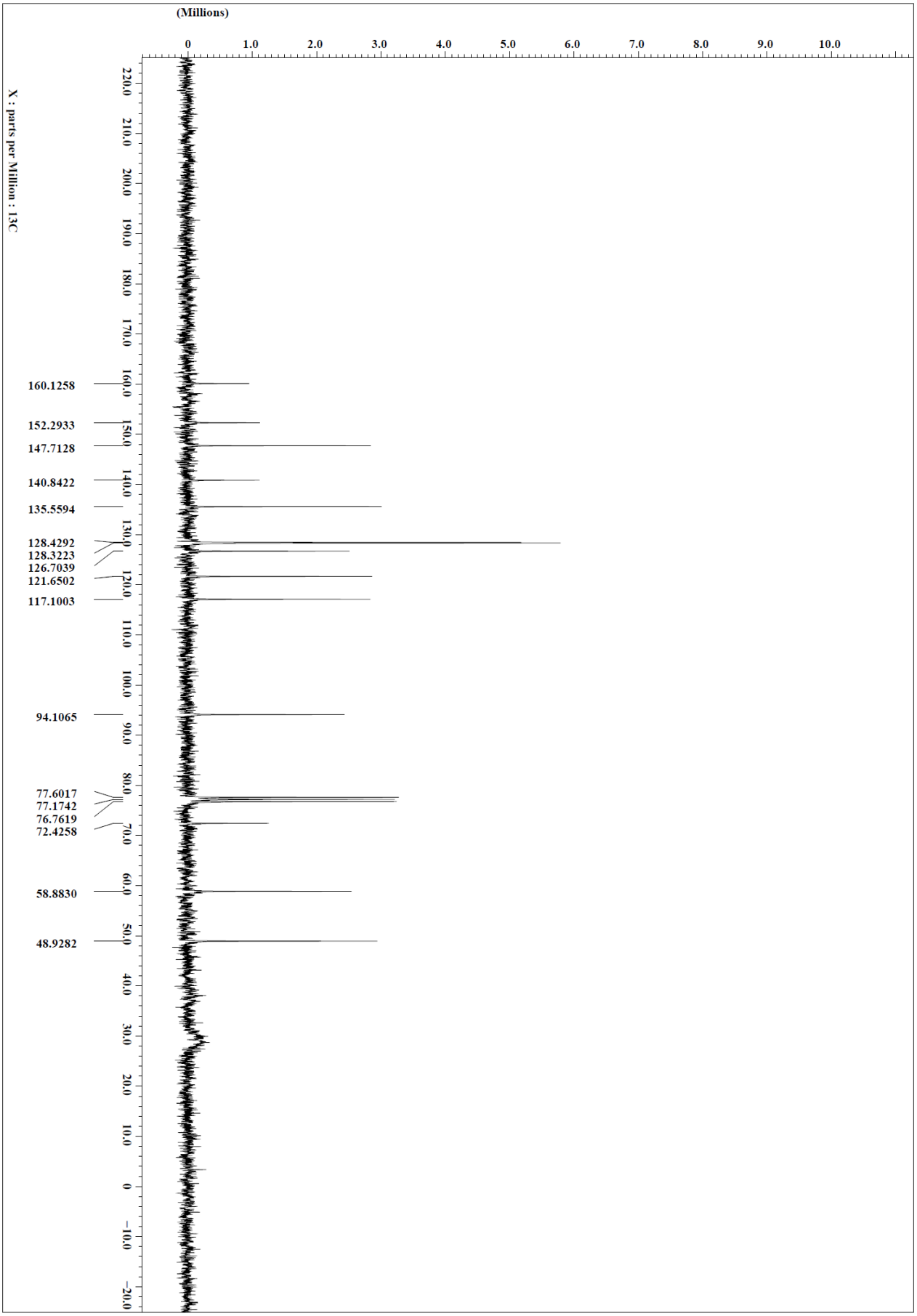

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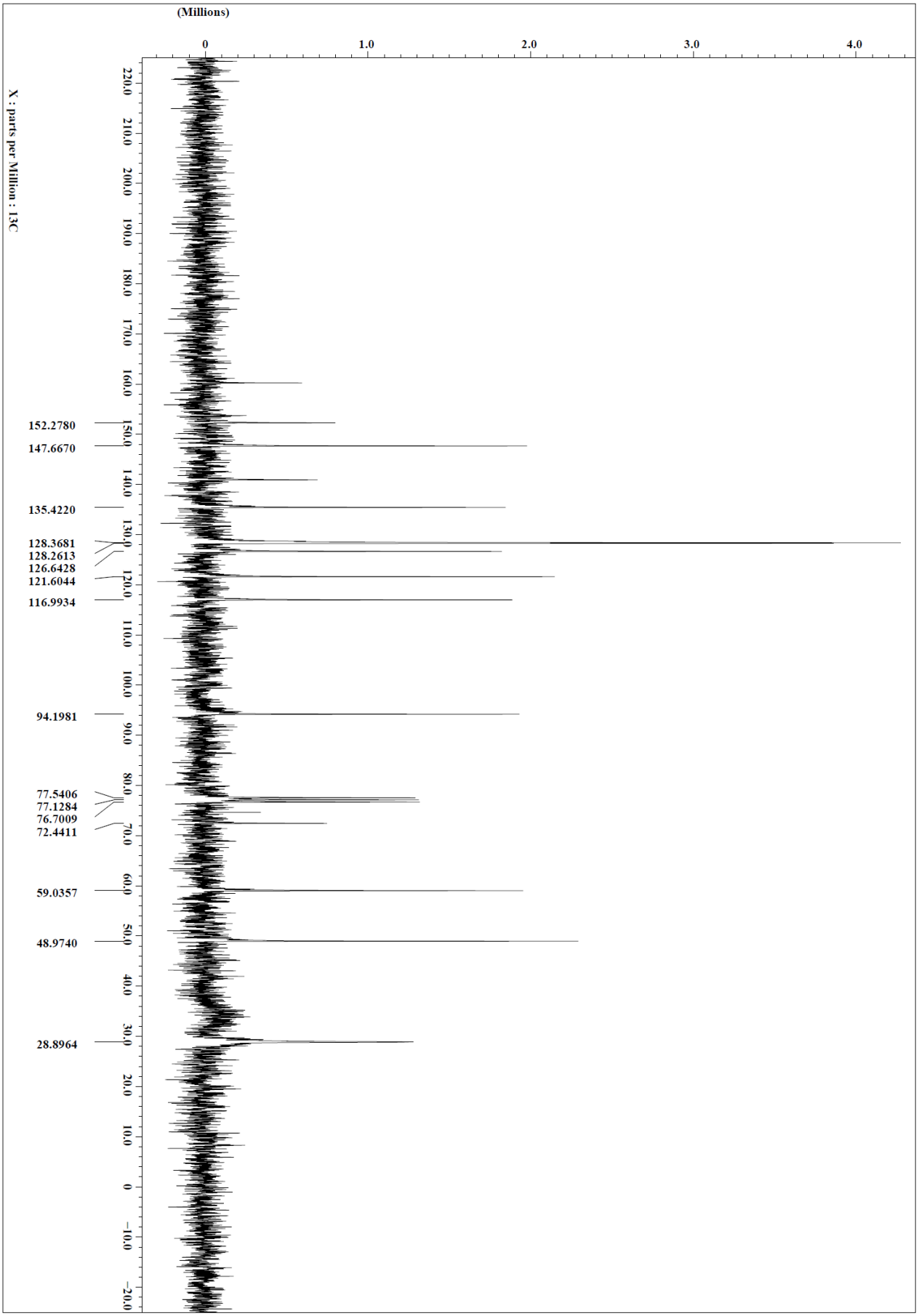

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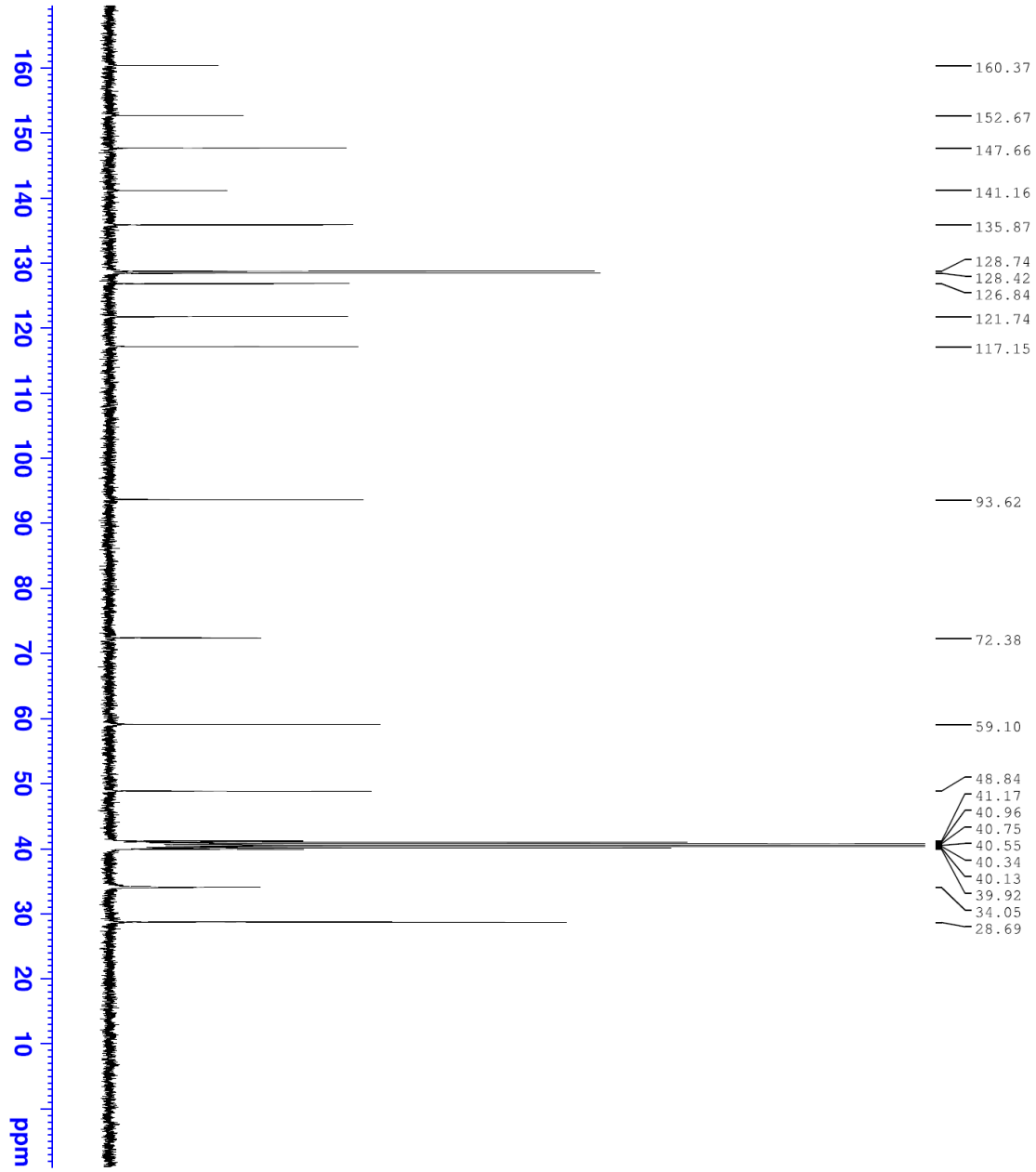


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PROCNO                      1

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PLW13           0.17788000 W

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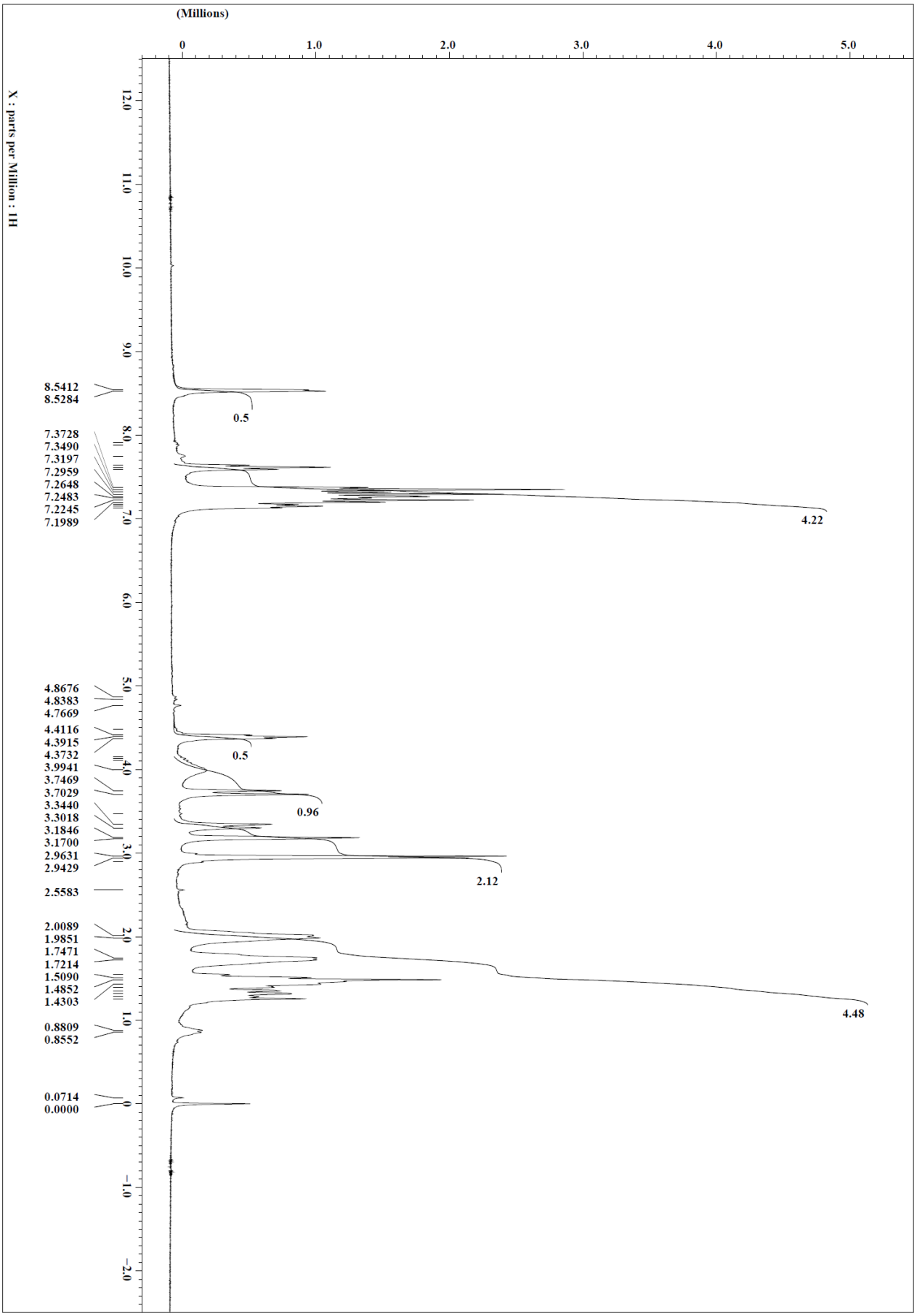

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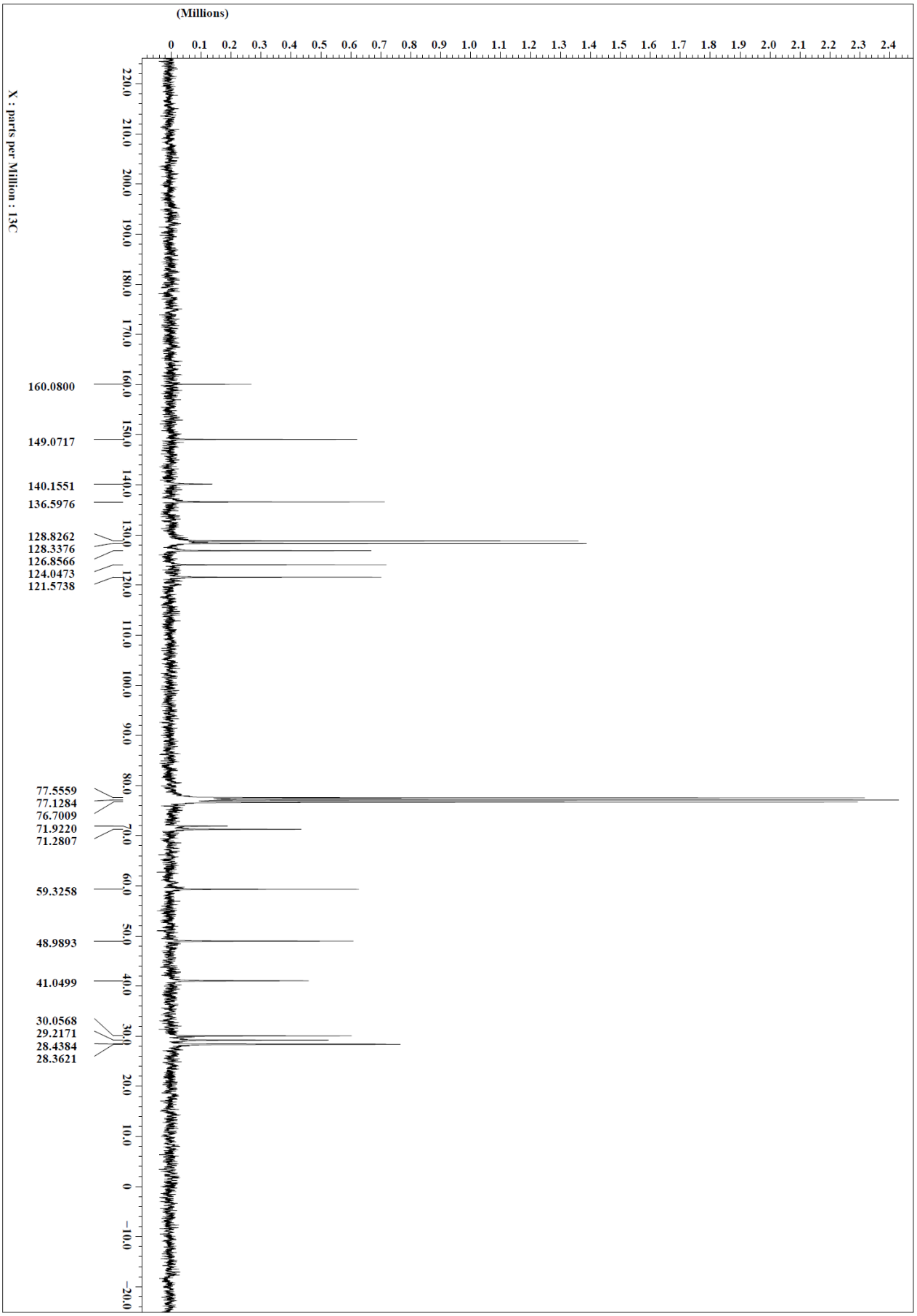

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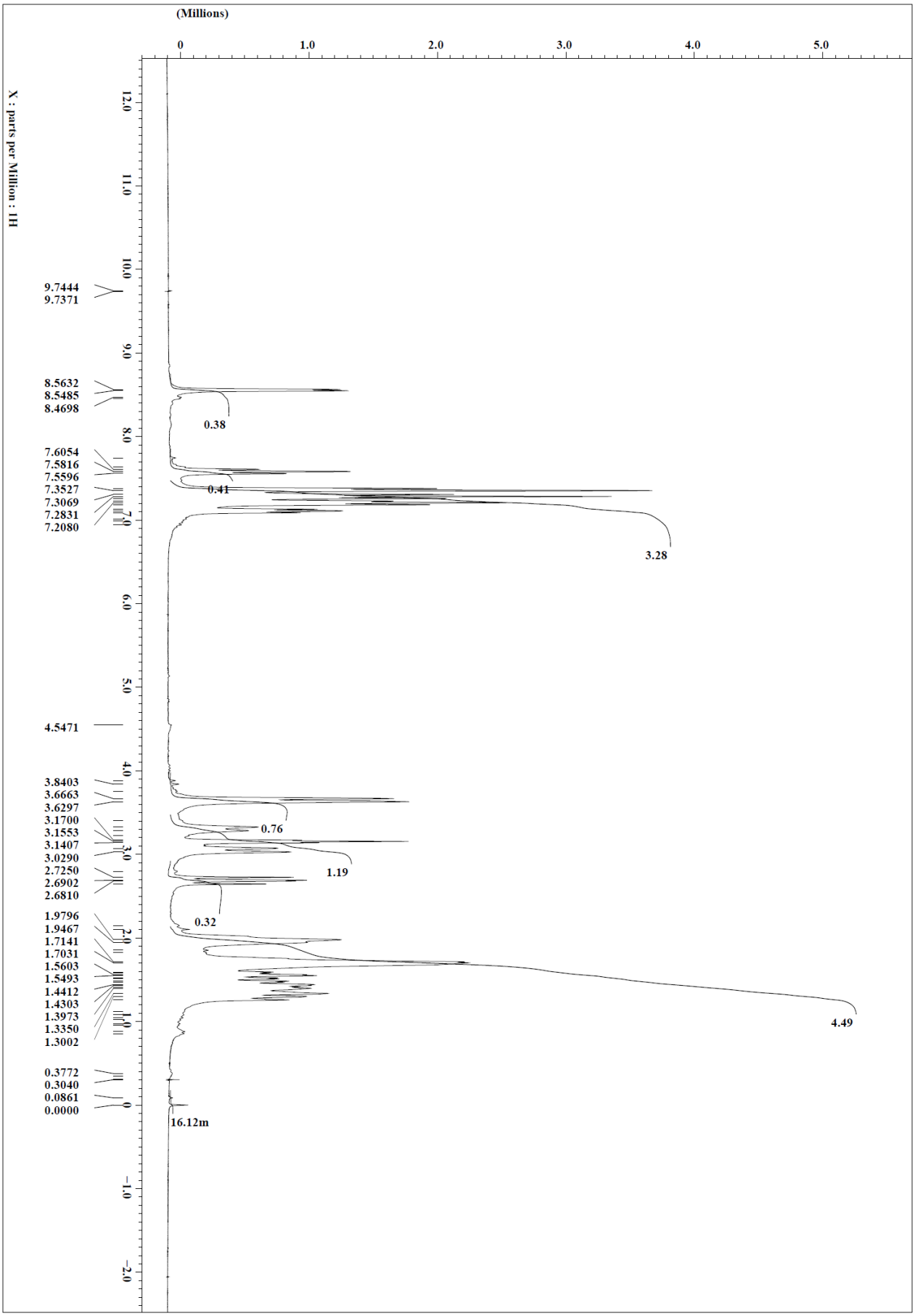

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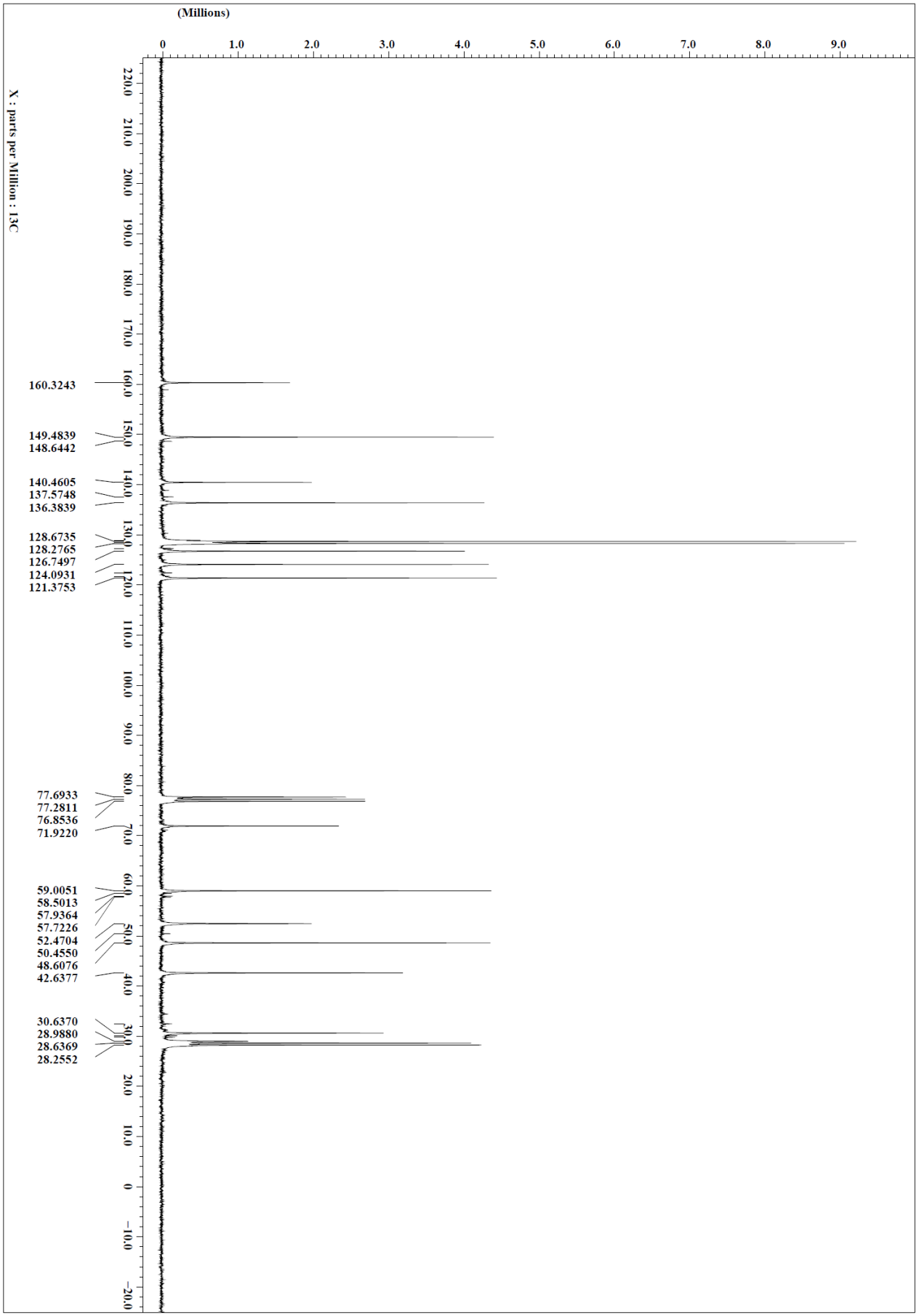

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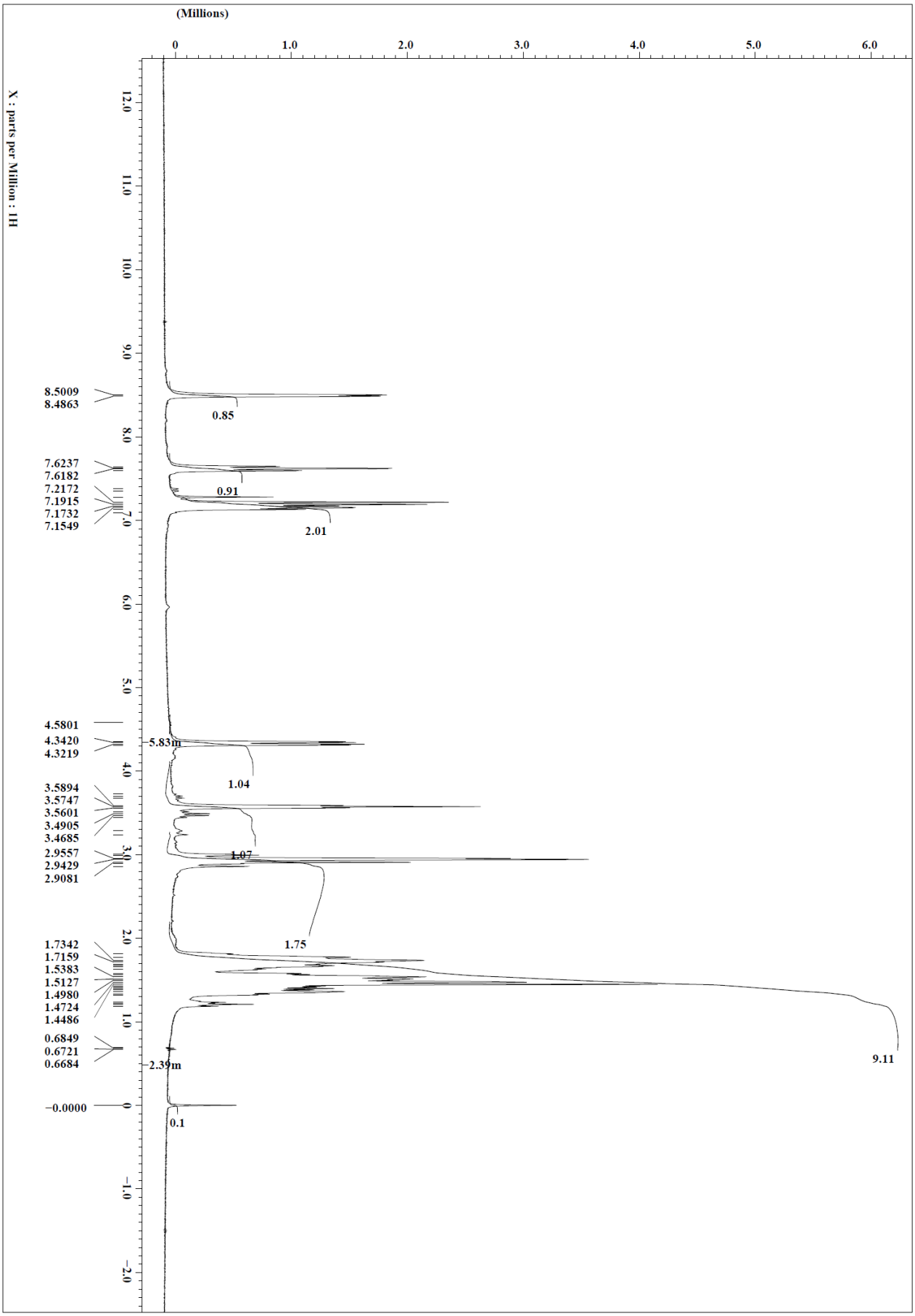

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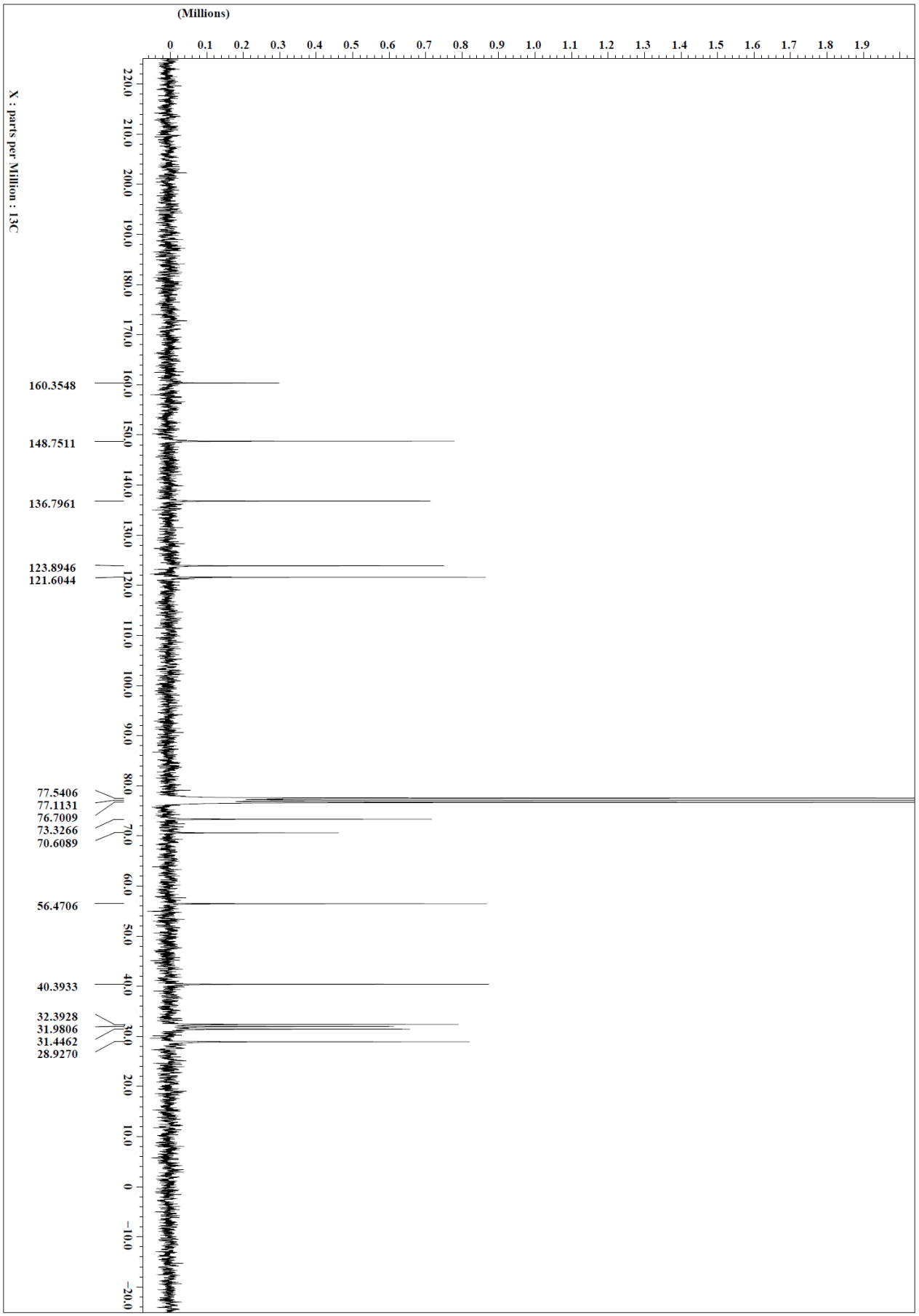

LC-trace compound 5d

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Sample Name: MVO epibat alcohol

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                                              Inj Volume : 2.0 µl
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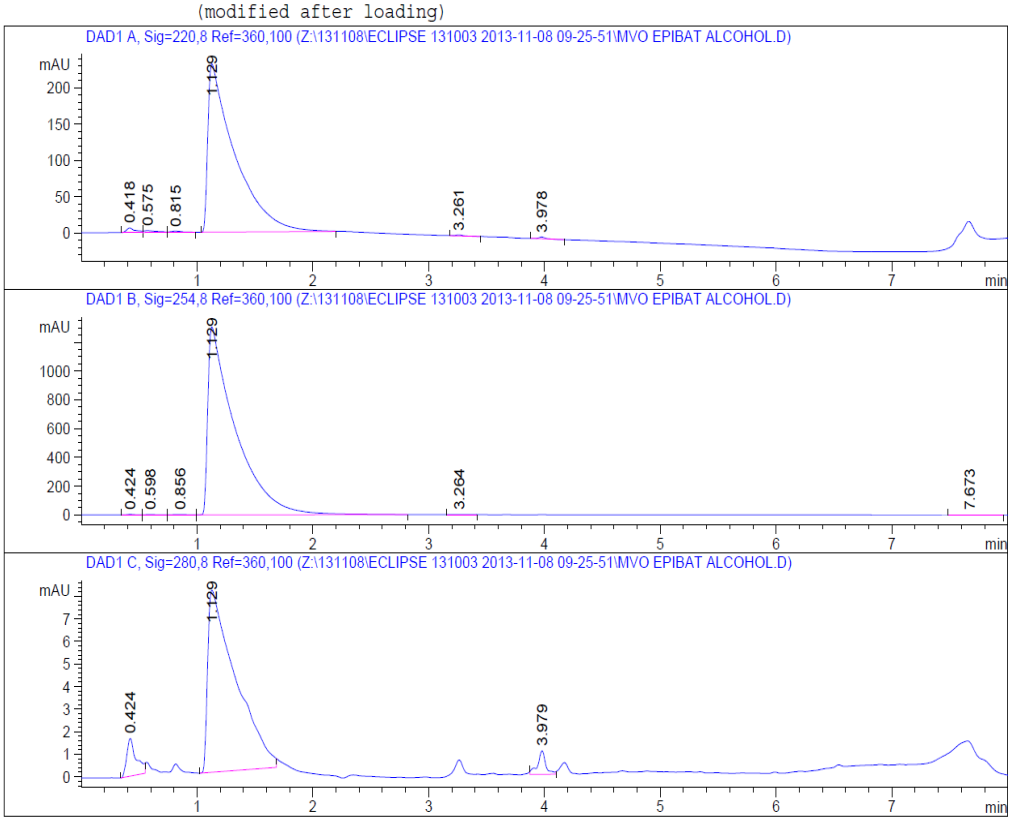


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=====================================================================
                        Area Percent Report
=====================================================================

Sorted By             :      Signal
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Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=220,8 Ref=360,100
```

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
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2	0.575	VV	0.1012	18.59474	2.43231	0.4883
3	0.815	VB	0.0896	9.99410	1.54242	0.2625
4	1.129	BB	0.2096	3729.21973	231.97588	97.9374
5	3.261	BB	0.0799	7.55273	1.37893	0.1984
6	3.978	BB	0.0640	10.33008	2.40853	0.2713

Data File Z:\131108\ECLIPSE 131003 2013-11-08 09-25-51\MVO EPIBAT ALCOHOL.D
Sample Name: MVO epibat alcohol

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
Totals :				3807.75737	245.94446	

Signal 2: DAD1 B, Sig=254,8 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	0.424	BV	0.0669	18.07413	3.83935	0.0842
2	0.598	VV	0.0937	16.53380	2.54734	0.0770
3	0.856	VV	0.0981	20.58427	2.85447	0.0958
4	1.129	VB	0.2129	2.13915e4	1307.02515	99.5995
5	3.264	BB	0.0734	5.93323	1.20628	0.0276
6	7.673	BB	0.1732	24.88946	1.89530	0.1159
Totals :				2.14775e4	1319.36789	

Signal 3: DAD1 C, Sig=280,8 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	0.424	BB	0.0799	9.47329	1.67658	6.3276
2	1.129	BB	0.2191	135.16161	8.08287	90.2803
3	3.979	BB	0.0706	5.07848	1.04644	3.3921
Totals :				149.71337	10.80588	

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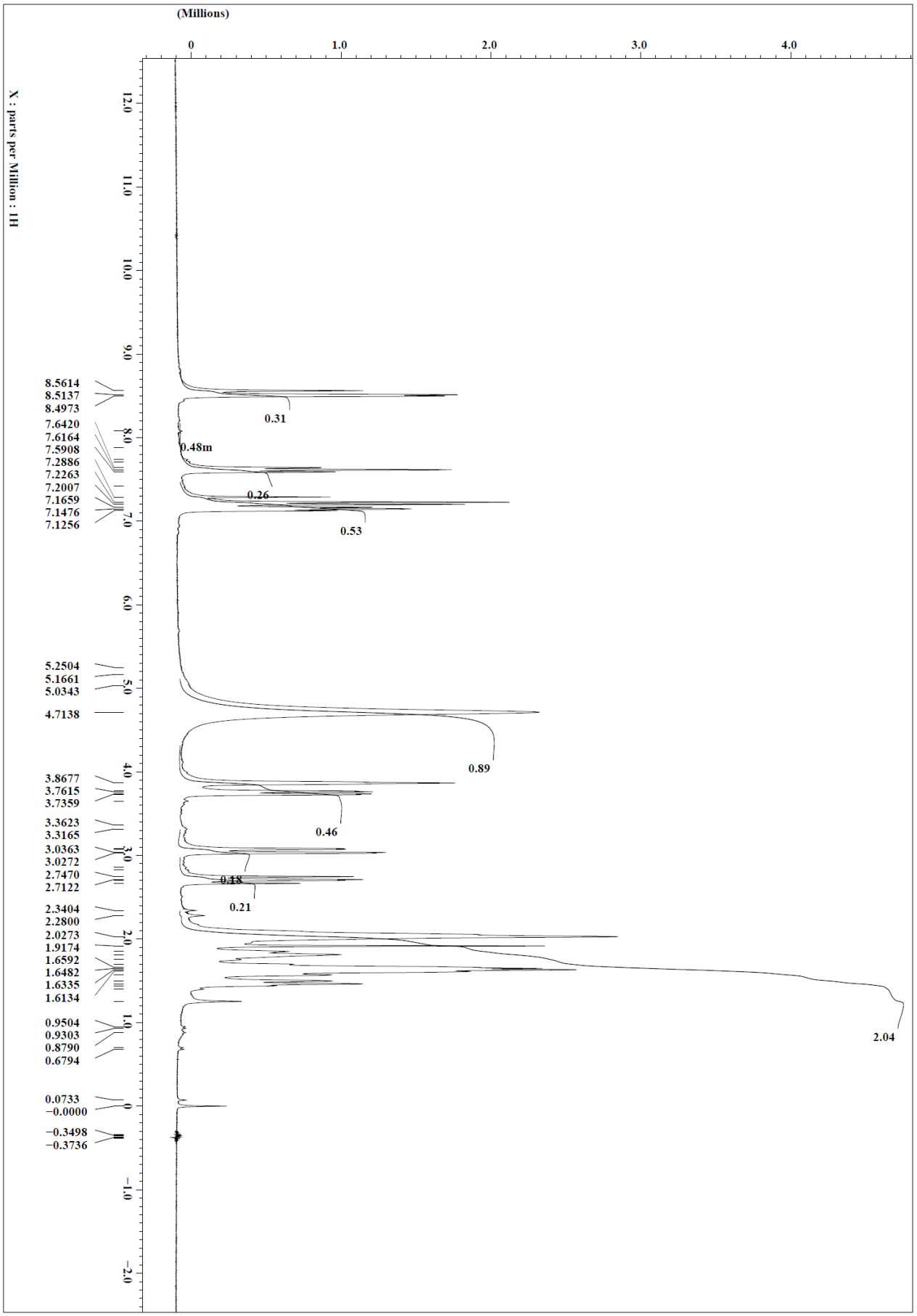
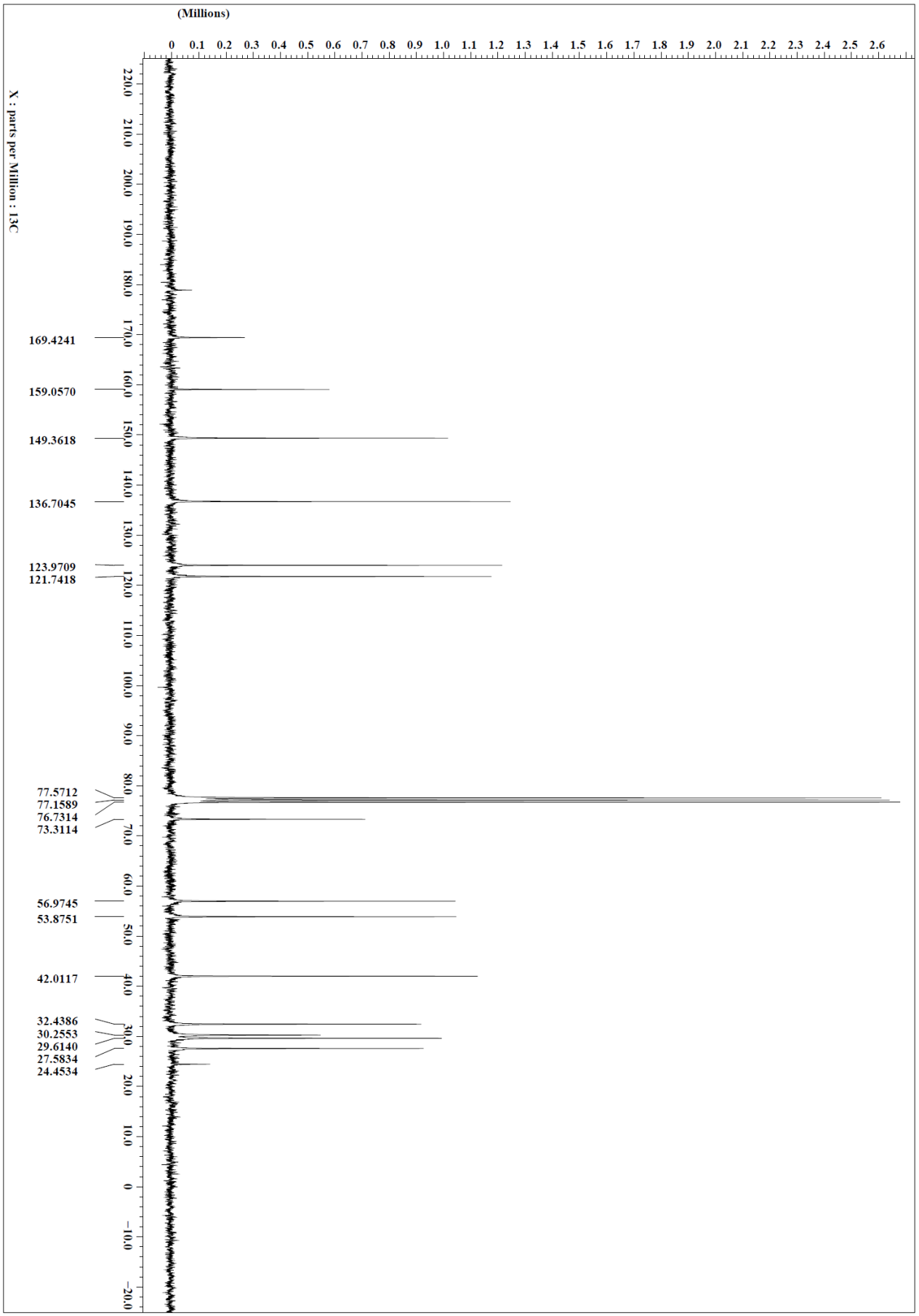
*** End of Report ***

^{1}H-NMR compound 5e



^{13}C-NMR compound 5e



LC-trace compound 5d

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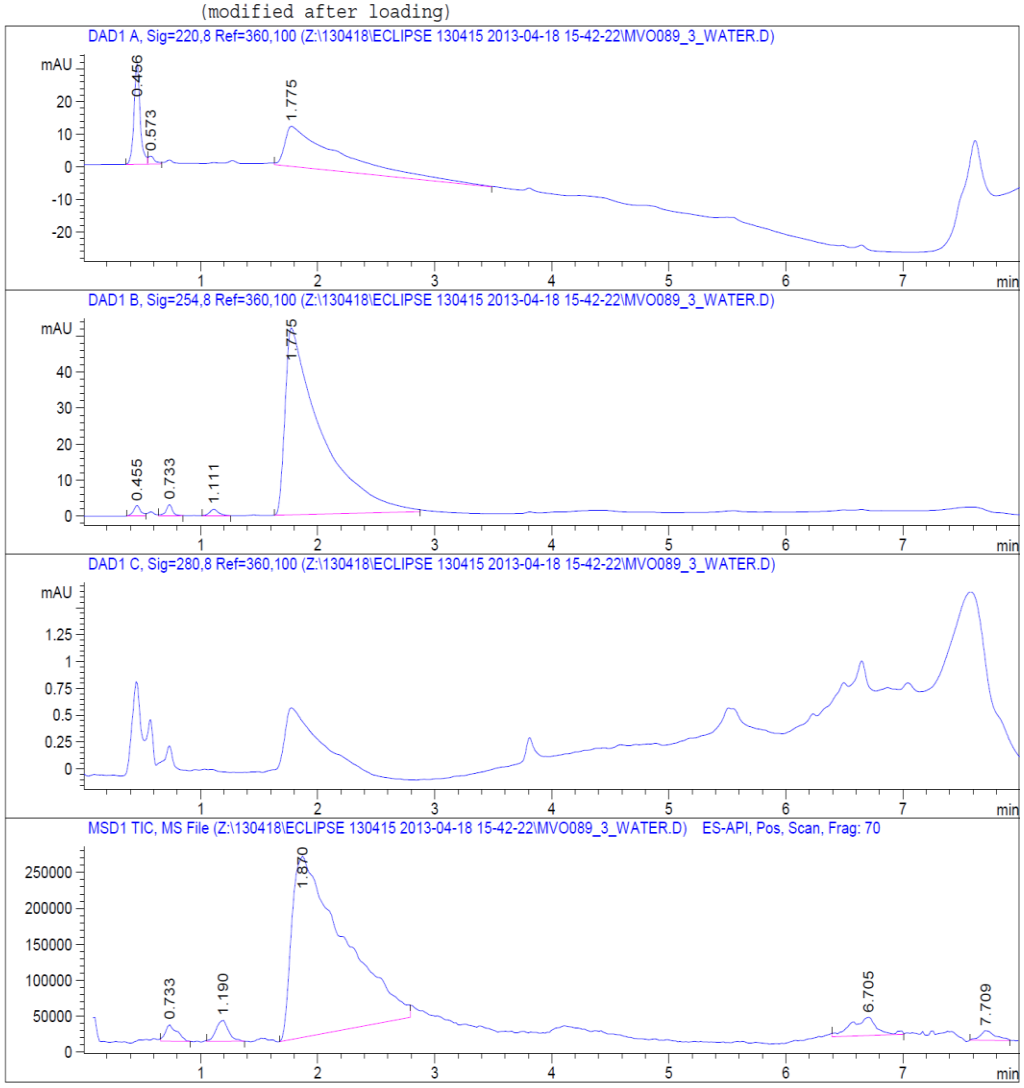


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======================================================================
                        Area Percent Report
======================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs
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Data File Z:\130418\ECLIPSE 130415 2013-04-18 15-42-22\MVO089_3_WATER.D
Sample Name: MVO089_3_water

Signal 1: DAD1 A, Sig=220,8 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	0.456	BV	0.0566	112.23438	30.74329	21.4546
2	0.573	VV	0.0578	9.54221	2.43046	1.8241
3	1.775	BB	0.4117	401.34723	12.26720	76.7213
Totals :				523.12382	45.44095	

Signal 2: DAD1 B, Sig=254,8 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	0.455	BV	0.0642	12.60551	2.92962	1.1005
2	0.733	VB	0.0582	12.41299	3.13163	1.0837
3	1.111	BB	0.0776	9.44084	1.78800	0.8242
4	1.775	BB	0.2792	1110.99866	52.01002	96.9917
Totals :				1145.45801	59.85926	

Signal 3: DAD1 C, Sig=280,8 Ref=360,100

Signal 4: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	0.733	BB	0.0914	1.59577e5	2.30521e4	1.8555
2	1.190	BB	0.1111	2.14776e5	2.96319e4	2.4973
3	1.870	BB	0.3993	7.71077e6	2.52161e5	89.6555
4	6.705	BB	0.2022	3.93103e5	2.53454e4	4.5707
5	7.709	BB	0.1481	1.22217e5	1.37502e4	1.4211
Totals :				8.60044e6	3.43941e5	

*** End of Report ***

Enzyme binding and IC_{50} determination protocols

Enzyme binding tests and IC_{50} determinations were performed at Cerep, France. Procedures are as follows (as reported by cerep):

Adrenergic alpha 1 (non-selective) : antagonist radioligand

Membrane homogenates of cerebral cortex (160 µg protein) are incubated for 60 min at 22°C with 0.25 nM [^{3}H]prazosin in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.1% BSA. Nonspecific binding is determined in the presence of 0.5 µM prazosin. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is prazosin.

Adrenergic alpha 2 (non-selective) : antagonist radioligand

Membrane homogenates of cerebral cortex (160 µg protein) are incubated for 60 min at 22°C with 0.5 nM [^{3}H]RX 821002 in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$ and 1 mM EDTA. Nonspecific binding is determined in the presence of 100 µM(-)epinephrine. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is yohimbine.

Adrenergic beta 1 (human sequence) : agonist radioligand

Cell membrane homogenates (5 µg protein) are incubated for 60 min at 22°C with 0.15 nM [^{3}H]CGP 12177 in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 2 mM EDTA and 0.1% BSA.Nonspecific binding is determined in the presence of 50 µM alprenolol. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is atenolol.

Adrenergic beta 2 (human sequence) : agonist radioligand

Cell membrane homogenates (32 µg protein) are incubated for 120 min at 22°C with 0.3 nM [^{3}H]CGP 12177 in the absence or presence of the test compound in a buffer containing 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4), 85 mM NaCl, 30 mM KCl, 1 mM MgSO4, 5.5 mM glucose, 0.005% bacitracin and 0.1% BSA. Nonspecific binding is determined in the presence of 50 µM alprenolol. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is ICI 118551.

GABA (non-selective) : agonist radioligand

Membrane homogenates of cerebral cortex (120 µg protein) are incubated for 60 min at 22°C with 10 nM [^{3}H]GABA in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4) and 2.5 mM $CaCl_2$. Nonspecific binding is determined in the presence of 100 µM GABA. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is GABA.

Cl^- channel (GABA-gated) : antagonist radioligand

Membrane homogenates of cerebral cortex (120 µg protein) are incubated for 120 min at 22°C with 3 nM [^{35}S]TBPS in the absence or presence of the test compound in a buffer containing 50 mM Na_2HPO_4/KH_2PO_4 (pH 7.4) and 500 mM NaCl. Nonspecific binding is determined in the presence of 20 µM picrotoxinin. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is picrotoxinin.

BZD (central) : agonist radioligand

Membrane homogenates of cerebral cortex (80 µg protein) are incubated for 60 min at 4°C with 0.4 nM [^{3}H]flunitrazepam in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.7). Nonspecific binding is determined in the presence of 3 µM diazepam. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is diazepam.

CB1 (human sequence) : agonist radioligand

Cell membrane homogenates (20 µg protein) are incubated for 120 min at 37°C with 0.5 nM [^{3}H]CP 55940 in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2.5 mM EDTA and 0.3% BSA. Nonspecific binding is determined in the presence of 10 µM WIN 55212-2. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with an ice-cold buffer containing 50 mM Tris-HCl (pH 7.4) and 0.5% BSA using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is CP 55940.

D1 (human sequence) : antagonist radioligand

Cell membrane homogenates (48 µg protein) are incubated for 60 min at 22°C with 0.3 nM [^{3}H]SCH 23390 in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM KCl, 5 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 5 mM EDTA. Nonspecific binding is determined in the presence of 1 µM SCH 23390. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is SCH 23390.

D2S (human sequence) : antagonist radioligand

Cell membrane homogenates (8 µg protein) are incubated for 60 min at 22°C with 0.3 nM [^{3}H]spiperone in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$ and 1 mM EDTA. Nonspecific binding is determined in the presence of 10 µM (+)butaclamol. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is (+)butaclamol.

glycine (strychnine-sensitive) : antagonist radioligand

Membrane homogenates of cerebral cortex (300 µg protein) are incubated for 45 min at 0°C with 0.5 nM [^{3}H]MDL 105,519 in the absence or presence of the test compound in a buffer containing 50 mM Hepes/KOH (pH 7.5). Nonspecific binding is determined in the presence of 1 mM glycine. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (Filtermat A, Wallac) and rinsed several times with ice-cold 50 mM Hepes/KOH using a 48-sample cell harvester (Mach II, Tomtec). The filters are dried then counted for radioactivity in a scintillation counter (Betaplate 1204, Wallac) using a solid scintillator (Meltilex B/HS, Wallac). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is glycine.

M1(human sequence) : antagonist radioligand

Cell membrane homogenates (45 µg protein) are incubated for 60 min at 22°C with 2 nM [^{3}H]pirenzepine in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$ and 1 mM EDTA. Nonspecific binding is determined in the presence of 1 µM atropine. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is pirenzepine.

M2(human sequence) : antagonist radioligand

Cell membrane homogenates (60 µg protein) are incubated for 60 min at 22°C with 2 nM [^{3}H]AF-DX 384 in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$ and 1 mM EDTA. Nonspecific binding is determined in the presence of 1 µM atropine. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is methoctramine.

M3(human sequence) : antagonist radioligand

Cell membrane homogenates (8 µg protein) are incubated for 60 min at 22°C with 0.2 nM [^{3}H]4-DAMP in the absence or presence of the test compound in a buffer containing 10 mM Tris-HCl (pH 7.4) and 2 mM EDTA. Nonspecific binding is determined in the presence of 1 µM atropine. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is 4-DAMP.

nAChR neuronal α-BGTX-insensitive (α4β2) : agonist radioligand

Membrane homogenates of cerebral cortex (800 µg protein) are incubated for 75 min at 4°C with 1.5 nM [^{3}H]cytisine in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 2 mM $CaCl_2$. Nonspecific binding is determined in the presence of 10 µM nicotine. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (Filtermat B, Wallac) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 48-sample cell harvester (Mach II, Tomtec). The filters are dried then counted for radioactivity in a scintillation counter (Betaplate 1204, Wallac) using a solid scintillator (Meltilex B/HS, Wallac). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is nicotine.

nAChR neuronal α-BGTX-sensitive (α7) : antagonist radioligand

Membrane homogenates of cerebral cortex (400 µg protein) are incubated for 150 min at 37°C with 1 nM [^{125}I]α-bungarotoxin in the absence or presence of the test compound in a buffer containing 50 mM K_2HPO_4/KH_2PO_4 (pH 7.4), 10 mM $MgCl_2$ and 0.1% BSA. Nonspecific binding is determined in the presence of 1 µM α-bungarotoxin. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (Filtermat B, Wallac) presoaked with 0.3% PEI and rinsed several times with an ice-cold buffer containing 50 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl and 0.1% BSA using a 48-sample cell harvester (Mach II, Tomtec). The filters are dried then counted for radioactivity in a scintillation counter (Betaplate 1204, Wallac) using a solid scintillator (Meltilex B/HS, Wallac). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is α-bungarotoxin.

nAChR muscle-type (human sequence) : antagonist radioligand

Cell membrane homogenates (60 µg protein) are incubated for 120 min at 22°C with 2.5 nM [^{125}I]α-bungarotoxin in the absence or presence of the test compound in a buffer containing 20 mM Hepes/NaOH (pH 7.3), 118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 0.1% BSA. Nonspecific binding is determined in the presence of 5 µM α-bungarotoxin. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with an ice-cold buffer containing 50 mM Tris-HCl, 150 mM NaCl and 0.1% BSA using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is α-bungarotoxin.

5-HT (non-selective) : agonist radioligand

Membrane homogenates of cerebral cortex (144 µg protein) are incubated for 60 min at 37°C with 2 nM [^{3}H]serotonin in the absence or presence of the test compound in a buffer containing 50 mM Tris-HCl (pH 7.4), 4 mM $CaCl_2$, 10 µM pargyline and 1 g/l ascorbic acid. Nonspecific binding is determined in the presence of 10 µM serotonin. Following incubation, the samples are filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). The filters are dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results are expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound is serotonin.

Off-target receptor binding

Table 1. off-target receptor binding of **5b** and **5c** at 1 μM concentration

	5b	**5c**
Adrenergic alpha 1 (non-selective)[c]	-8	-3
Adrenergic alpha 2 (non-selective)[c]	1	8
Adrenergic beta 1 (h)[b]	3	7
Adrenergic beta 2 (h)[b]	9	7
GABA (non-selective)[b]	20	8
Cl- channel (GABA-gated)[c]	0	1
BZD (central)[b]	-10	-18
CB1 (h)[b]	13	-1
D1 (h)[c]	13	4
D2S (h)[c]	1	2
glycine (strychnine-sensitive)[c]	-12	-30
M1 (h)[c]	3	-3
M2 (h)[c]	8	13
M3 (h)[c]	-7	12
nAChR neuronal α-BGTX-insensitive (α4β2)[b]	49	82
nAChR neuronal α-BGTX-sensitive (α7)[c]	-17	0
nAChR muscle-type (h)[c]	-3	-4
5-HT (non-selective)[b]	6	-4

(h) human sequence; (non-selective) various subtypes included; [a] average of two measurements, values below 30% are considered as not significant; [b] agonist radioligand; [c] antagonist radioligand

Cytotoxicity assays

Cytotoxicity assays were performed at Argenta, UK. HepG2 is a human liver carcinoma cell line (ATCC No. HB-8065). HepG2 is a perpetual cell line which was derived from the liver tissue of a 15 year old Caucasian American male. These cells are epithelial in morphology. H4-II-E-C3 is a rat liver carcinoma cell line (ATCC No. CRL-1600**)**. H4-I-E-C3 is a perpetual cell line which was derived from the liver tissue of *Rattus norvegicus* with a hepatocellular carcinoma. These cells are epithelial in morphology. IMR32 is a human fibroblast/neuroblastoma cell line (ATCC No. CCL 127). IMR32 is a perpetual cell line which was derived from a 13 month old Caucasian male. B65 is a rat neuronal cell line (ECACC 85042305). B65 is a perpetual cell line which was derived from rat nervous tissue.

Cells were seeded into 96 well MTP plates at day -1, allowed to adhere and equilibrate overnight. Medium was removed and compounds added in 200 µl culture medium at the concentrations indicated. Final DMSO concentration in assay was 1%. Duplicate wells per concentration. Cells incubated at 37degc/5% CO_2 for 72 h. Cell viability assessed by metabolic capability (formazan dye formation, MTS – Promega) in mitochondria and cell membrane integrity (LDH release – Promega) Compound solubility in the medium was assessed by direct observation. Positive controls: Terfenadine, Tamoxifen

Determination of metabolic stability

Incubation with rat liver microsomes

These experiments were performed at the CREATe ADME/Tox and DSSc BA groups at Janssen Pharmaceuticals inc. Compounds were supplied as dry powder and dissolved in DMSO (2 mM). Rat liver microsomes were prepared according to method described by Gorrod et al.[2]

Incubations of test compounds in liver microsomes were performed using an automated procedure on the Tecan robotic liquid handler. Final incubation conditions were 1 µM test compound, 1 mg/ml microsomal protein, 2 mM NADPH and 0.1 M phosphate buffer (pH 7.4). The final solvent concentration in the incubation was 0.05% DMSO and 0.15% acetonitrile. Incubations were serially sampled (5 µL) at 0, 2.5, 5, 10, 15, 30, 45 and 60 minutes and the aliquots were terminated by quenching with 200 µL acetonitile/water 1/1. Samples were subsequently centrifuged at 4000 rpm for 10 minutes (4°C). All experiments were performed in triplicate. Of the resulting supernatant, 10 µL was analysed by LC-MS. The reference compound loperamide was included as an experimental control compound (1 µM).

The *in vitro* metabolic half-life ($t_{1/2}$) is calculated using the slope of the log-linear regression from the percentage parent compound remaining versus time relationship (κ),

$t_{1/2}$ = - ln(2)/ κ.

The in vitro intrinsic clearance (Cl_{int}) (ml/min/mg microsomal protein) is calculated using the following formula:

$$Cl_{int} = \frac{0.693}{t_{1/2}} \times \frac{V_{inc}}{W_{mic\ prot,inc}}$$

Where: V_{inc} = incubation volume,
$W_{mic\ prot,inc}$ = weight of microsomal protein in the incubation

The hepatic intrinsic clearance ($Cl_{int\ H}$) (ml/min/kg) is calculated using the following formula:

$$Cl_{int,H} = Cl_{int} \times MPPGL \times \frac{W_{liver}}{W_{body}}$$

Where: *MPPGL* is the microsomal protein per gram liver i.e. $\frac{W_{mic\ prot,liver}}{W_{liver}}$
W_{body} = body weight
W_{liver} = liver weight

The estimated *in vivo* hepatic clearance (Cl_H) (ml/min/kg) may be estimated using the equation for the Well Stirred Model.

$$Cl_{H,blood} = \frac{Q_H \times \left(\frac{Cl_{int,H}}{fu_{mic}} \times \frac{fu_p}{\mathrm{B/P}}\right)}{Q_H + \left(\frac{Cl_{int,H}}{fu_{mic}} \times \frac{fu_p}{\mathrm{B/P}}\right)}$$

Where: Q_H = hepatic blood flow
fu_p = the unbound fraction in plasma.
fu_{mic} = the unbound fraction in microsomes
B/P = blood to plasma ratio

The simplified well-stirred model is used where the values remain uncorrected for binding to both plasma and microsomal proteins (ie fu_p = fu_{mic}) and the blood to plasma ratio is assumed to be 1. As such, the calculation for the in vivo hepatic clearance (ml/min/kg) becomes:

$$Cl_H = \frac{Q_H \times Cl_{int,H}}{Q_H + Cl_{int,H}}$$

Hepatic blood values for the fed state for rat were used: W_{liver} / W_{body} = 40 g/kg, Q_H = 70 ml/min/kg, MPPGL = 45.

Isolation of rat hepatocytes

These experiments were performed at the Laboratory for Pharmacotechnology and Biopharmacy, Gasthuisberg, KU Leuven. Hepatocytes were isolated from male Wistar rats using a collagenase perfusion, as described previously.[3] After isolation, cells were centrifuged (50 *g*)

for 3 min at 4°C and the pellet was resuspended in William's E medium containing 5 % FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin. Hepatocytes were counted using a hemocytometer and cell viability was determined using Trypan blue. Freshly-isolated rat hepatocytes were resuspended in William's E medium containing 2 mM L-glutamine and 4 µg/ml insulin and kept on ice until initiation of the experiment.

Incubation of compounds with rat hepatocytes

These experiments were performed at the Laboratory for Pharmacotechnology and Biopharmacy, Gasthuisberg, KU Leuven. Incubations were performed according to a previously published protocol.[4] Prior to starting uptake experiments, 550 µl of a double-concentrated cell suspension was sampled into 12-well plates and incubated in a thermomixer at 37°C, 100 rpm for 15 min. Subsequently, 550 µl of a pre-warmed 2-fold concentrated compound solution was added to initiate the incubation which lasted for 120 min at 37°C. **5b** and **5c** were incubated at a final concentration of 5 and 50 µM and the final cell density was 1 x 10^6 cell mL^{-1}. Verapamil was used as positive control and was incubated at a final concentration of 5 µM. Samples were taken at 0, 30, 60 and 120 min. Incubations without hepatocytes were used as negative control and samples were taken at 30 and 120 min. At each time point, triplicate 250 µl aliquots of the incubate were transferred to 1.5 ml ice-cold Eppendorf tubes, containing 500 µl of methanol to stop the reaction and precipitate hepatocellular proteins. After centrifugation for 5 min at 20817 g, the supernatant was transferred to a clean Eppendorf tube and kept until analysis at – 20°C.

Sample preparation

Prior to analysis, samples were thawed and 500 µL was transferred into autosampler vials and analysed by LC-fluo (Verapamil) or by LC-MS/MS (**5b** and **5c**) as described below.

Analysis of verapamil

These experiments were performed at the Laboratory for Pharmacotechnology and Biopharmacy, Gasthuisberg, KU Leuven. Verapamil concentrations were determined by reversed phase HPLC with fluorescence detection. The HPLC system consisted of a Water Alliance 2695 separations module and a Novapak C18 column under radial compression (Waters, Milford, MA). Fluorescence (excitation: 230 nm, emission 312 nm) was monitored by a Jasco fluorescence detector. The mobile phase consisted of 50 mM sodium acetate buffer (pH 5.0) and methanol (35:65 v/v). The flow was maintained at a rate of 1 ml/min. Under these conditions, the retention time of verapamil was 5.6 min and the observed peaks were integrated using Empower Pro (Empower 2) software. The calibration curve was linear over the concentration range of 10-0.1 µM.

LC-MS/MS of 5b and 5c

These experiments were performed at the Laboratory for Pharmacotechnology and Biopharmacy, Gasthuisberg, KU Leuven. All experiments were performed on a Thermo Scientific (San Jose, USA) LC–MS/MS system comprising an Accela® autosampler, an Accela® pump and a TSQ Quantum Access® triple quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) source. Data acquisition and peak integration were performed with the Xcalibur® 2.0.7 and LCquan® 2.5.6 software packages, respectively.

An Acquity CSH® C_{18} column (50 mm × 3.0 mm, 1.7 µm) (Waters Corporation, Millford, MA), protected by a VanGuard™ Pre-Column (Waters Corporation, Millford, MA) was used for chromatographic separation. The mobile phase consisted of a 1 mM ammonium bicarbonate buffer (pH 9) (A) and methanol (B). Gradient elution at a constant flow rate of 0.4 mL/min was performed as follows: 95 % A decreased linearly to 5 % A in 0.5 min; constant flow of 5 % A for 3.0 min; linear increase back to 95 % A in 10 sec; re-equilibration for 1.0 min with 95 % A before the next injection. The total run time was 4.0 min and the injection volume was 25 µL (full loop mode). Under these conditions, the retention times of **5b** and **5c** were 2.75 and 2.64 min, respectively.

The mass spectrometer was operated in the positive electrospray mode. The spray voltage was 3000 V and the capillary temperature was set at 200 °C. The vaporiser temperature was 300 °C. Nitrogen was used as the sheath gas (50 arbitrary units) and auxiliary gas (20 arbitrary units). Argon was used as the collision gas at a pressure of 1.5 mTorr. The mass spectrometer was operated in a 3-channel selected reaction monitoring (SRM) mode. Four precursor–product ion pairs were used for detection of **5b**: m/z 205.2 → 78.4 (collision energy: 35 V), m/z 205.2 → 106.2 (23 V), m/z 205.2 → 159.2 (16 V) and m/z 205.2 → 170.2 (17 V). Two ion pairs were used for detection of **5c**: m/z 204.2 → 95.2 (21 V) and m/z 204.2 → 110.3 (19 V). A scan time of 100 ms was used per ion pair. Both Q1 and Q3 were set at 0.7 unit mass resolution. An on-line motorised divert valve was used to introduce the eluent to the mass spectrometer over the period of 1.0 – 4.0 min for data acquisition. The eluent flow outside this interval was diverted to the waste.

Calibration curves were linear over the concentration ranges of 0.1-10 µM. The intraday precision (relative standard deviation; n = 5) of standards with low (0.2 µM), and high (2 µM) concentration amounted to 2.9 % and 1.7 %, respectively. The intraday accuracy (bias) of said standards was -5.1 % and 6 %, respectively.

Plasma Protein Binding

These experiments were performed at the CREATe ADME/Tox and DSSc BA groups at Janssen Pharmaceuticals inc. Plasma protein binding, to determine the drug concentration unbound to rat plasma proteins, was studied using the rapid equilibrium dialysis device. Rat plasma, containing test compound (**5b**, **5c**) at 5 µM, was incubated at 37ºC in the Rapid Equilibrium Dialysis (RED) Device. The RED device consists of a Teflon 48-well base plate which contains disposable inserts. These inserts are bisected by a semi-permeable (MWCO= 8 kD) membrane, creating two chambers. Aliquots (300 µl) of spiked rat plasma are loaded in to one chamber and phosphate buffered saline (PBS pH=7.4, 500µl) is loaded into the other. The plate is then sealed and placed in a shaking incubator at 37 °C for 4.5 h. At the end of the incubation period, samples are removed and analyzed from both the buffer and plasma side by LC/MS/MS (in Multiple Reaction

Monitoring (MRM) mode) to obtain free and bound concentrations. These concentrations are then used to calculate the bound fraction of the drug (f_b) using the equation below:

$$f_b = \frac{2 * [\,plasma\,] - [\,buffer\,]}{2 * [\,plasma\,]}$$

where [plasma] is the concentration measured in the plasma and [buffer] the concentration measured in the buffer. Plasma concentrations are multiplied by a factor 2 as twice the volume of buffer as plasma is used for the concentration determination. Subsequently the unbound fraction (fu) is determined as follows:

$$f_u = 1 - f_b$$

Determination of blood/brain distribution

Brain tissue homogenate binding

These experiments were performed at the CREATe ADME/Tox and DSSc BA groups at Janssen Pharmaceuticals inc. Brain tissue binding, to determine the drug concentration unbound to brain tissue, was studied using the rapid equilibrium dialysis device. 1 in 10 diluted rat brain tissue homogenate was prepared by adding 9 mL PBS (pH=7.4) to 1 g of brain tissue. Brain tissue homogenate, containing test compound (**5b**, **5c**) at 5 µM, was incubated at 37ºC in the Rapid Equilibrium Dialysis (RED) Device. The RED device consists of a Teflon 48-well base plate which contains disposable inserts. These inserts are bisected by a semi-permeable (MWCO= 8 kD) membrane, creating two chambers. Aliquots (300 µl) of spiked 1 in 10 diluted brain tissue homogenate are loaded in to one chamber and phosphate buffered saline (PBS pH=7.4, 500µl) is loaded into the other. The plate is then sealed and placed in a shaking incubator at 37 °C for 5 h. After 5 h, samples are removed and analyzed from both the buffer and brain tissue homogenate side by LC/MS/MS to obtain free and bound concentrations. These concentrations are then used to calculate the percentage compound bound to brain tissue (%BTB) using the equations below.

Apparent unbound fraction ($f_{u,app}$) is as follows:

$$f_{u,app} = \frac{[A]_{buffer}}{[A]_{homogenate}}$$

where $[A]_{homogenate}$ is the concentration measured in the homogenate and $[A]_{buffer}$ the concentration measured in the buffer.

As homogenates are diluted (10 times) the $f_{u,app}$ has to be corrected for the dilution factor to obtain the real unbound fraction in brain tissue ($f_{u,brain}$). This is according to the the following formula:

$$f_{u,brain} = \frac{f_{u\ app}}{D + f_{u\ app} - D \times f_{u\ app}}$$

Where D is the dilution factor. Subsequently, the percentage compound bound to brain tissue (%BTB) is determined as follows:

$$\%\ BTB = (1 - f_{u,brain}) \times 100\ \%$$

Plasma and Brain concentration deteminations

These experiments were performed at the CREATe ADME/Tox and DSSc BA groups at Janssen Pharmaceuticals inc. The plasma and brain pharmacokinetics of **5b** and **5c** were studied in satellite groups of male Sprague Dawley rats (~230g, food and water available *ad libitum*). Compound **5b** was administered at 10 mg/kg using a solution of 0.9% Sodium Chloride, pH 8.85 and dosing route (IP, dosing volume 5 ml/kg) similar to the main pharmacological studies. Compound **5c** was administered at 1 mg/kg using a solution of 0.9% Sodium Chloride, pH 9 and dosing route (IP, dosing volume 5 ml/kg). From each individual animal, (n=3 per time point), blood samples were collected at 30 min, 1 and 2 hr after dose administration. Animals were sacrificed under anaesthesia and blood was collected into 10 ml BD vacutainers™ K3E (Becton Dickinson). Samples were placed immediately on melting ice and plasma was obtained following centrifugation at 4°C for 10 min at 1900 *x g*. All samples were shielded from daylight and stored at ≤ -18 °C prior to analysis. Tissue samples were homogenized in water (1/9 w/v). Plasma and tissue samples were analyzed using a qualified LC-MS/MS method. The lower limit of quantification (LLOQ) was 1 ng/ml for plasma and 10 ng/g for brain samples for compound **5b**, and 0.5 ng/ml for plasma and 1.0 ng/ml for brain samples with compound **5c**. A limited pharmacokinetic analysis was performed using WinNonlin™ Professional (Version 5.1).

Table 2. Blood/Brain distribution of 5b

6a						
	Plasma (ng/ml)					
Time (h)	Individual			Mean		StDev
0	0	0	0	0		
0,5	3140	195	2780	2038	±	1606
1	300	1370	331	667	±	609
2	246	288	625	386	±	208
$AUC_{0\text{-last}}$ (ng.h/ml)				1713		
	Brain (ng/g)					
Time (h)	Individual			Mean		StDev
0	0	0	0	0		
0,5	218	22,1	216	152	±	113
1	30,7	116	31,8	59,5	±	48,9
2	21,3	21,4	50,1	30,9	±	16,6
$AUC_{0\text{-last}}$ (ng.h/g)				136		
	Brain/Plasma ratio					
Time (h)	Individual			Mean		StDev
0,5	0,07	0,11	0,08	0,09	±	0,02
1	0,10	0,08	0,10	0,09	±	0,01
2	0,09	0,07	0,08	0,08	±	0,01

Table 3. Blood/Brain distribution of 5c

6b						
	Plasma (ng/ml)					
Time (h)	Individual			Mean		StDev
0	0	0	0	0		
0,5	28,6	1,81	2,33	10,9	±	15,3
1	8,49	12,6	10,2	10,4	±	2,1
2	1,04	0,878	1,81	1,24	±	0,50
$AUC_{0\text{-last}}$ (ng.h/ml)				13,9		
	Brain (ng/g)					
Time (h)	Individual			Mean		StDev
0	0	0	0	0		
0,5	49,8	3,39	3,17	18,8	±	26,9
1	48,9	48,5	53,9	50,4	±	3,0
2	4,99	4,05	30,2	13,1	±	14,8
$AUC_{0\text{-last}}$ (ng.h/g)				53,8		
	Brain/Plasma ratio					
Time (h)	Individual			Mean		StDev
0,5	1,7	1,9	1,4	1,7	±	0,3
1	5,8	3,8	5,3	5,0	±	1,0
2	4,8	4,6	17	8,7	±	6,9

Rat tail flick tests

These experiments were performed at the Department of Cell Biology, Faculty of Sciences, University of Malaga. The rats were placed in a plastic restrainer tube and the tail placed over the platform of the stimulus unit in the tail-flick apparatus (LE7106, Panlab, Barcelona, Spain). A radiant heat from a halogen lamp was applied directly to the tail and the latency to reflexive withdrawal of the tail was measured. The heat intensity was adjusted such that the tail-flick baseline latencies were 3.0-5.0 s and cut-off was imposed at 15 s to prevent tissue damage. In the acute experiment, tail-flick latencies were recorder 30 min prior drug injection and at 0, 30, 60, 90, 120 and 150 min after drug treatment. The percentage of maximal possible antinociceptive effect (%MPAE) was calculated by comparing the test latency before (basal response) and after a drug administration using the equation: %MPAE = [(test response time–basal response time)/(cut off time–basal response time)]x100.

Table 4. Rat tail flick tests of compound 5b

Saline	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	-11,63	-22,28	-30,91	-28,46	-23,65	-19,04
	0,00	3,85	-9,16	-5,74	-10,10	-8,48	-11,39
	0,00	3,45	-36,01	-39,06	-3,44	-12,60	-39,31
	0,00	5,60	-6,36	5,44	8,45	11,63	-6,28
	0,00	-6,90	-18,71	-12,67	-23,29	-23,20	-15,01
	0,00	-7,85	0,31	6,07	3,74	17,43	4,28
	0,00	10,00	7,60	-19,78	-13,39	7,08	8,46
	0,00	5,32	-19,22	-6,84	0,00	24,24	3,03
	0,00	8,40	-1,60	-2,64	-1,52	18,07	6,55
	0,00	6,74	3,90	1,45	0,38	3,44	5,89
	0,00	-0,23	4,68	6,48	7,71	5,82	11,40
	0,00	-2,36	-24,19	-25,76	-32,57	-34,35	-20,42
	0,00	5,91	-4,65	142,43	-8,79	-9,30	-10,74
	0,00	-16,60	-4,38	-6,17	-9,38	-3,59	3,91
MEAN	0,00	0,26	-9,29	0,88	-7,90	-1,96	-5,62
SEM	0,00	2,18	3,44	11,53	3,43	4,69	3,86

Morphine	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	3,25	100,00	100,00	100,00	-53,57	4,87
	0,00	0,15	100,00	100,00	100,00	-14,19	-1,12
	0,00	-12,60	30,00	18,43	44,90	-11,38	
	0,00	-10,54	100,00	100,00	-5,35	-1,56	-10,12
	0,00	15,20	100,00	100,00	15,05	6,03	0,08
	0,00	10,60	100,00	40,83	4,87	0,35	-10,34
	0,00	-12,69	100,00	50,04	0,97	-9,99	-6,42
	0,00	-19,23	100,00	100,00	26,63	-7,67	-1,40
	0,00	-2,30	100,00	100,00	13,95	144,96	8,53
	0,00	-0,14	100,00	100,00	4,98	18,42	11,16
	0,00	2,26	100,00	100,00	0,32	-4,34	11,65
	0,00	6,17	100,00	100,00	100,00	93,62	83,83
	0,00	8,97	100,00	100,00	100,00	92,26	80,39
MEAN	0,00	-0,84	94,62	85,33	38,95	19,46	14,26
SEM	0,00	2,86	5,39	7,95	12,28	15,43	9,40

5b (0,01 mg/kg)	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	14,30	-35,51	-70,00	-72,05	-34,23	-12,31
	0,00	12,25	6,29	-5,22	-5,14	6,29	4,65
	0,00	-5,30	9,30	11,98	11,31	45,68	9,38
	0,00	-6,47	-2,56	-6,65	0,08	12,18	-2,96
	0,00	2,30	35,27				
	0,00	2,63	16,58				
	0,00	11,36	2,70	2,86	5,00	-2,70	-0,08
	0,00	9,60	5,00	13,60	3,38	4,07	5,92
	0,00	-2,70	5,08	-8,50		-10,41	
	0,00	-5,30	12,84	1,01		-5,84	
	0,00	-2,30	-11,27	-6,73	-6,81	0,24	
	0,00	6,30	-10,97	-8,48	-8,48	-3,08	
	0,00	5,12	-31,35	-47,08	-31,24	-31,46	
	0,00	4,23	-8,45	-3,06	-0,48	1,69	
	0,00	7,30	5,79	100,00	-5,79	-4,13	
	0,00	-11,00	12,03	100,00	23,08	9,17	
MEAN	0,00	2,65	0,67	5,27	-7,26	-0,89	0,77
SEM	0,00	1,88	4,38	12,26	6,97	5,08	3,17

5b (0,1 mg/kg)	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	3,20	5,52	-4,84	0,76	15,72	-14,19
	0,00	7,63	11,76	5,76	11,68	-1,38	15,57
	0,00	4,00	34,30	-4,50	10,93	16,40	9,79
	0,00	-2,30	22,21	-7,54	1,80	0,49	5,74
	0,00	-9,31	-31,51	-23,85	-36,00	-6,35	-26,70
	0,00	-1,23	-14,32	4,89	-7,16	-5,76	18,69
	0,00	5,63	-0,49	-2,84	-3,24		
	0,00	4,97	5,68	9,51	8,33		
	0,00	7,80	4,90	10,64	1,77	-7,26	
	0,00	-5,14	1,41	-4,75	-6,25	-0,35	
	0,00	-4,12	-15,08	9,44	-9,52	-6,99	
	0,00	3,21	1,08	19,81	-0,85	0,15	
	0,00	5,93	33,91	44,21	19,31	-4,64	
	0,00	6,34	18,25	14,64	14,43	25,77	
	0,00	9,21	1,04	19,02	16,79	0,59	
	0,00	3,20	17,59	2,38	-1,58	-0,32	
MEAN	0,00	2,44	6,02	5,75	1,33	1,86	1,48
SEM	0,00	1,33	4,35	3,79	3,33	2,70	7,35

5b (1 mg/kg)	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	-6,30	6,33	4,33	-0,17	17,15	-24,90
	0,00	-14,32	7,40	2,12	3,01	23,84	-22,05
	0,00	10,30	-108,73	-107,08			
	0,00	-12,30	-16,55	-5,84			
	0,00	-0,36	-12,36	-3,51	-20,02	-14,58	-10,70
	0,00	0,98	-0,08	5,82	3,29	-1,07	1,23
	0,00	9,80	4,97	16,64	-6,10	-1,05	
	0,00	8,24	-16,65	-15,12	-10,62	-15,42	
	0,00	7,32	-6,53	13,87		-1,54	
	0,00	5,04	9,49	4,44		-18,47	
	0,00	-2,07	-19,29	-7,55	-9,37	-19,20	
	0,00	-3,50	-3,99	-8,64	0,16	-4,97	
	0,00	5,60	3,05	-4,30	-4,84	-34,41	
	0,00	-3,10	8,59	27,34	18,92	-19,24	
	0,00	-4,20	-10,31	-5,53	-2,57	-7,41	-14,11
MEAN	0,00	0,08	-10,31	-5,53	-2,57	-7,41	-14,11
SEM	0,00	1,98	7,48	7,79	2,98	4,35	4,62

5b (10 mg/kg)	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	3,23	16,93	3,43	2,70	57,32	18,89
	0,00	9,54	6,82	-2,81	5,14	6,82	-4,57
	0,00	-4,23	6,79	6,46	-0,08		
	0,00	-7,14	10,33	-10,15	-8,86		
	0,00	15,30	6,45	13,64			
	0,00	17,00	-13,95	2,62			
	0,00	0,23	-80,50	-29,66	-40,45	-30,61	-40,73
	0,00	5,60	-8,16	7,73	-11,46	21,35	18,40
	0,00	4,30	-44,67	-40,12	-34,25	-38,92	
	0,00	-9,00	-4,21	0,16	16,69	-3,18	
	0,00	-4,12	-1,99	7,96	-1,53		
	0,00	0,98	-9,18	-13,94	-9,52		
	0,00	2,14	50,92				
	0,00	3,47	1,98				
	0,00	8,90	28,65				
	0,00	9,25	-12,85				
	0,00	4,12	-2,91	-4,56	-8,16	2,13	-2,00
MEAN	0,00	3,50	-2,91	-4,56	-8,16	2,13	-2,00
SEM	0,00	1,75	6,87	4,32	5,01	12,17	10,86

Table 5. Rat tail flick tests of compound 5c

Saline	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	-14,05	-10,79	-22,71	-8,96	-19,35	-15,07
	0,00	-52,28	-41,59	-32,21	-50,24	-38,34	-34,62
	0,00	2,08	0,00	12,50	5,67	-0,83	4,33
	0,00	-21,83	-26,06	-23,69	-4,12	-20,49	-28,32
	0,00	-0,97	-9,11	-13,66	-10,47	-9,69	-16,28
	0,00	13,45	-1,09	-1,82	-8,18	-7,00	0,00
	0,00	29,06	-7,44	-0,17	-8,29	-9,74	-6,15
	0,00	2,42	-9,59	-2,42	-8,38	-10,45	-10,10
	0,00	12,84	-1,47	6,97	-8,44	-13,39	-5,60
	0,00	1,69	-0,93	11,36	-5,17	-0,42	-6,02
	0,00	13,04	-6,70	-10,36	-5,71	-9,64	4,82
	0,00	0,00	7,63	-7,45	-10,38	-12,69	-10,91
	0,00	0,00	0,00	-1,69	34,07	1,16	17,53
	0,00	0,00	0,00	0,50	0,25	-4,77	-4,10
MEAN	0,00	-1,04	-7,65	-6,06	-6,31	-11,12	-7,89
SEM	0,00	5,12	3,35	3,55	4,58	2,71	3,57

Morphine	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	30,12	44,61	29,14	-0,97	-10,27	-8,06
	0,00	23,94	43,96	24,63	-9,82	-4,05	3,10
	0,00	-9,33	100,00	100,00	79,95	-6,02	-8,98
	0,00	-18,46	100,00	48,45	-1,50	-17,53	-15,37
	0,00	21,49	89,79	21,41	-1,24	8,71	10,37
	0,00	-10,87	83,23	26,22	2,72	1,84	-7,80
	0,00	-2,77	100,00	57,97	100,00	100,00	100,00
	0,00	-21,25	100,00	88,00	100,00	15,61	100,00
	0,00	-6,74	100,00	100,00	100,00	-27,87	100,00
	0,00	-8,45	100,00	100,00	100,00	-19,58	100,00
	0,00	17,68	86,65	8,23	10,57	-7,11	-12,72
	0,00	-3,73	88,74	6,41	-5,83	-24,47	22,08
	0,00	0,00	100,00	46,21	100,00	100,00	3,02
	0,00	0,00	100,00	100,00	100,00	100,00	-34,44
MEAN	0,00	0,83	88,36	54,05	48,13	14,95	25,09
SEM	0,00	4,31	5,24	9,78	13,71	12,73	13,59

5c (0,01 mg/kg)	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	29,98	1,73	9,97	-1,48	-2,80	13,92
	0,00	38,96	16,96	11,66	1,59	5,83	
	0,00	10,48	2,31	28,51	6,75	3,20	7,28
	0,00	-20,80	29,15	-6,33	-13,67	-19,60	-20,00
	0,00	-10,17	-5,80	-10,58	-12,92	2,44	-6,00
	0,00	-1,78	-5,64	-14,00	-23,30	-4,49	-18,08
	0,00	4,64	-8,65	-8,11	-1,09	-5,65	-4,10
	0,00	3,07	0,65	16,07	7,92	1,45	8,48
	0,00	-12,12	-1,72	-4,75	-2,53	-13,64	-8,08
	0,00	4,80	1,97	9,18	-6,09	-1,20	-1,46
	0,00	-25,88	-33,65	-5,88	8,35	-12,71	17,53
	0,00	-3,97	3,50	4,16	5,77	-10,11	7,56
	0,00	11,18	5,36	0,00	-16,91	-4,91	11,36
	0,00	3,08	-3,08	16,70	-5,78	-5,32	-10,54
	0,00	1,35	49,82	-0,98	-40,76	47,49	-83,60
	0,00	0,00	24,10	6,55	-18,50	-0,28	21,44
MEAN	0,00	2,05	4,81	3,26	-7,04	-1,27	-4,28
SEM	0,00	4,09	4,64	2,91	3,35	3,67	6,53

5c (0,1 mg/kg)	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	31,50	35,28	32,36	7,66	13,65	36,32
	0,00	13,63	10,00	1,15	-9,02	0,10	1,15
	0,00	7,91	11,28	6,75	18,23	4,53	24,20
	0,00	-6,96	15,33	-14,30	-11,29	-12,32	4,70
	0,00	-4,89	-16,11	-2,68	-7,38	-7,38	3,16
	0,00	24,01	2,19	-0,41	3,49	-0,57	12,41
	0,00	5,26	3,68	-18,42	15,00	52,63	25,26
	0,00	18,00	8,00	-4,80	-10,00	-7,00	-15,00
	0,00	-14,29	7,95	-7,68	-9,64	-1,52	-1,61
	0,00	-12,39	-7,61	-2,74	-8,67	-0,62	-9,47
	0,00	-11,74	-17,17	-12,42	-11,45	-1,65	-4,66
	0,00	-31,48	-26,12	-32,33	-29,66	-26,12	-30,51
	0,00	0,91	10,99	-5,12	-4,96	10,50	3,64
	0,00	-31,08	-16,10	-31,69	-25,13	-31,49	-31,49
	0,00	-1,58	54,59	-18,16	-0,65	36,79	10,00
	0,00	20,93	-8,79	6,81	-16,19	-21,03	-3,26
MEAN	0,00	0,48	4,21	-6,48	-6,23	0,53	1,55
SEM	0,00	4,62	5,12	3,91	3,21	5,33	4,60

5c							
(1 mg/kg)	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	12,37	-7,19	7,86	3,68	18,31	4,68
	0,00	-18,93	-27,16	-15,36	-14,67	-25,17	-25,47
	0,00	6,12	-2,34	2,79	5,31	2,25	8,27
	0,00	2,41	-2,87	-8,62	-1,48	0,00	0,00
	0,00	-7,96	-1,76	0,00	0,37	-17,59	-16,67
	0,00	-42,44	-18,67	-34,44	-66,67	-66,67	-66,67
	0,00	32,52	28,78	11,30	18,37	37,40	62,60
	0,00	-1,74	35,22	13,04	4,35	16,52	33,04
	0,00	-17,20	-15,30	-17,90	-2,10	-6,60	-12,10
	0,00	-58,01	-29,34	-16,82	-58,68	-63,12	-41,45
	0,00	-18,47	-23,06	-45,97	-46,53	-51,67	-49,03
	0,00	-17,63	-11,00	-33,75	-37,25	-19,63	-26,00
	0,00	7,43	-24,63	-16,77	-34,08	-26,54	-21,34
	0,00	-1,69	3,91	-9,68	-16,52	-11,55	-11,28
	0,00	15,43	-18,77	-21,85	-6,42	-1,73	-85,19
	0,00	0,00	5,98	-10,93	-37,57	-61,12	-25,61
MEAN	0,00	-6,74	-6,76	-12,32	-18,12	-17,31	-17,01
SEM	0,00	5,54	4,68	4,22	6,36	7,71	8,96

Table 6. Rat tail flick tests of +/- epibatidine

epibatidine							
(4 μg/kg)	basal (-30 min)	t0	t30	t60	t90	t120	t150
	0,00	12,23	100,00	25,11	22,55	33,95	32,80
	0,00	-0,31	100,00	76,04	100,00	11,80	21,66
	0,00	2,36	90,17	52,70	65,22	24,98	3,04
	0,00	5,98	82,89	80,11	98,86	69,78	9,25
	0,00	6,47	100,00	84,71	30,81	45,39	41,53
	0,00	-8,20	92,78	96,99	26,76	27,42	23,91
	0,00	-4,12	100,00	88,21	89,45	60,12	1,50
	0,00	8,32	100,04	65,80	100,04	66,78	20,92
	0,00	13,11	80,59	27,73	80,16	37,86	30,48
	0,00	4,56	70,56	21,78	37,85	33,04	44,44
	0,00	-7,23	100,00	100,00	100,00	100,00	89,57
	0,00	-6,10	100,00	100,00	100,00	100,00	69,91
	0,00	7,20	97,63	99,59	61,64	24,36	6,85
	0,00	0,56	86,75	74,94	59,66	45,00	17,30
	0,00	2,34	100,00	100,00	100,00	81,54	43,79
MEAN	0,00	2,48	93,43	72,91	71,53	50,80	30,46
SEM	0,00	1,74	2,41	7,35	7,77	7,16	6,38

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