Design, Synthesis and Biological Evaluation of Novel Dimeric and Tetrameric cRGD-Paclitaxel Conjugates for Integrin-Assisted Drug Delivery

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† These authors contributed equally to the project.

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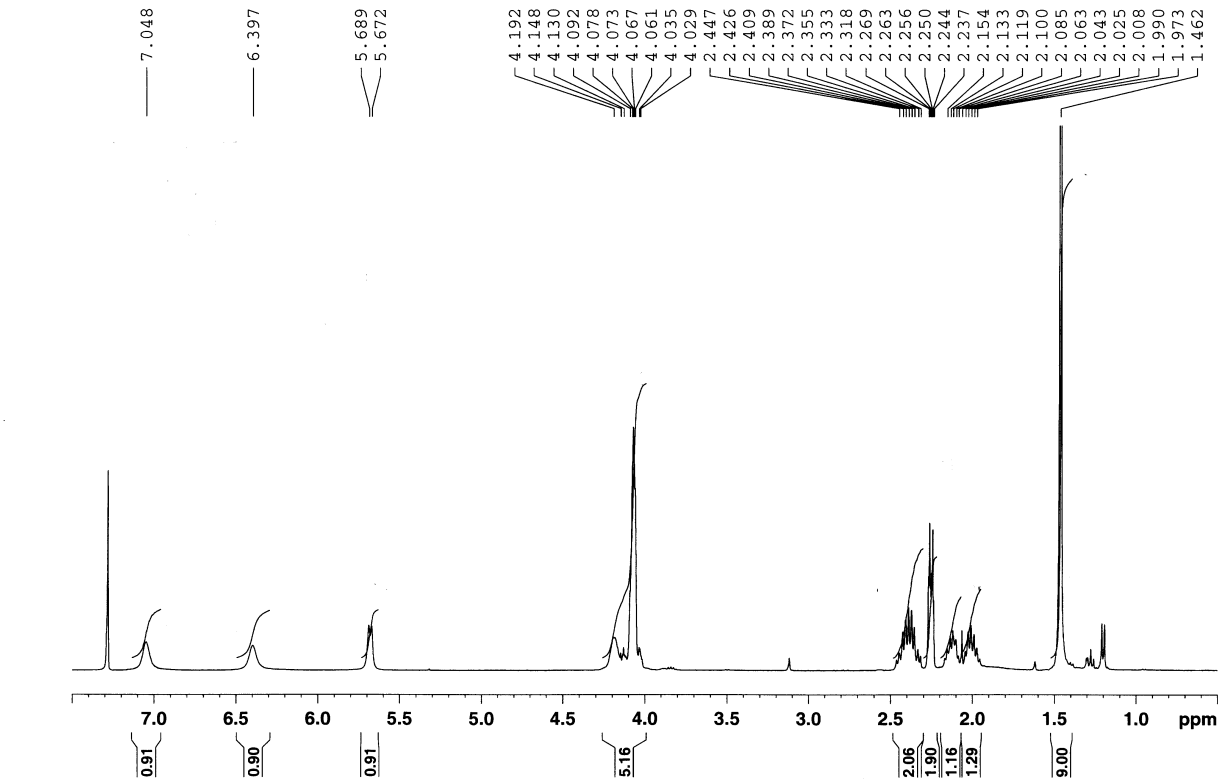
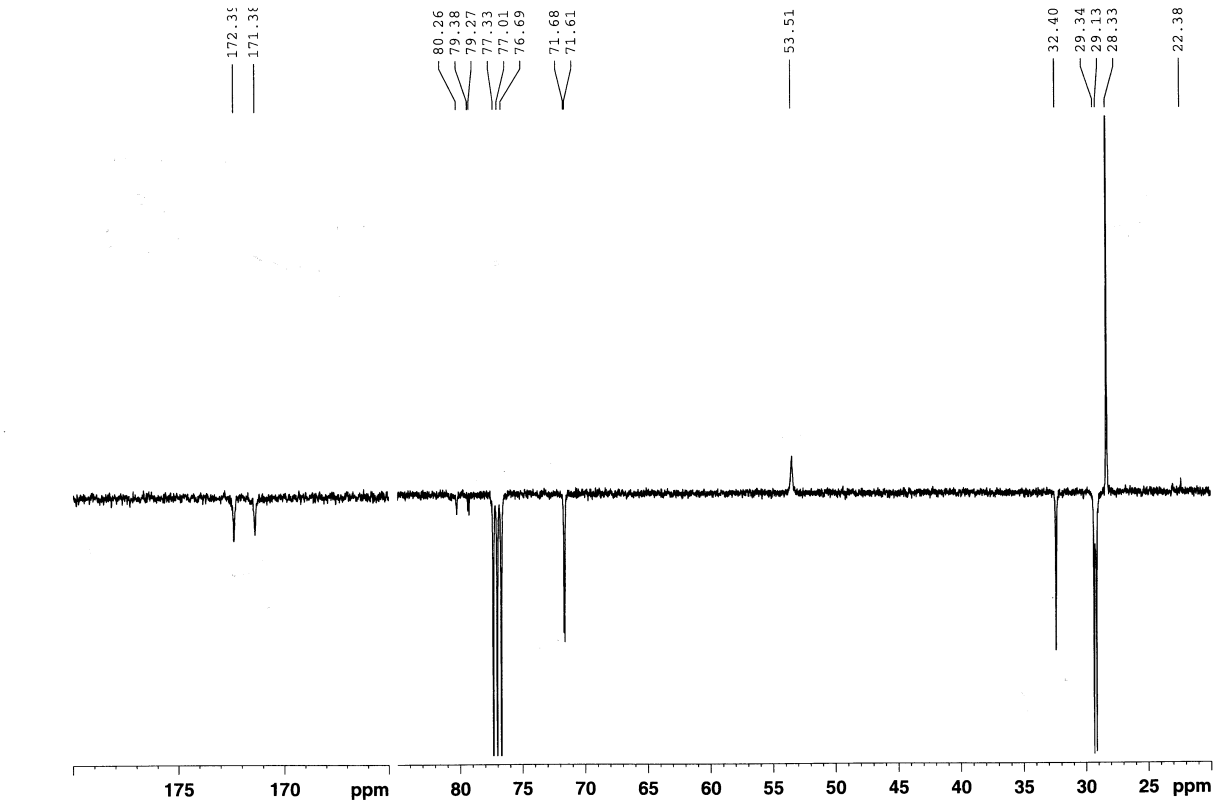
Compound 5. ^{1}H-NMR ($CDCl_3$, 400 MHz)



Compound 5. ^{13}C-NMR ($CDCl_3$, 100.6 MHz)



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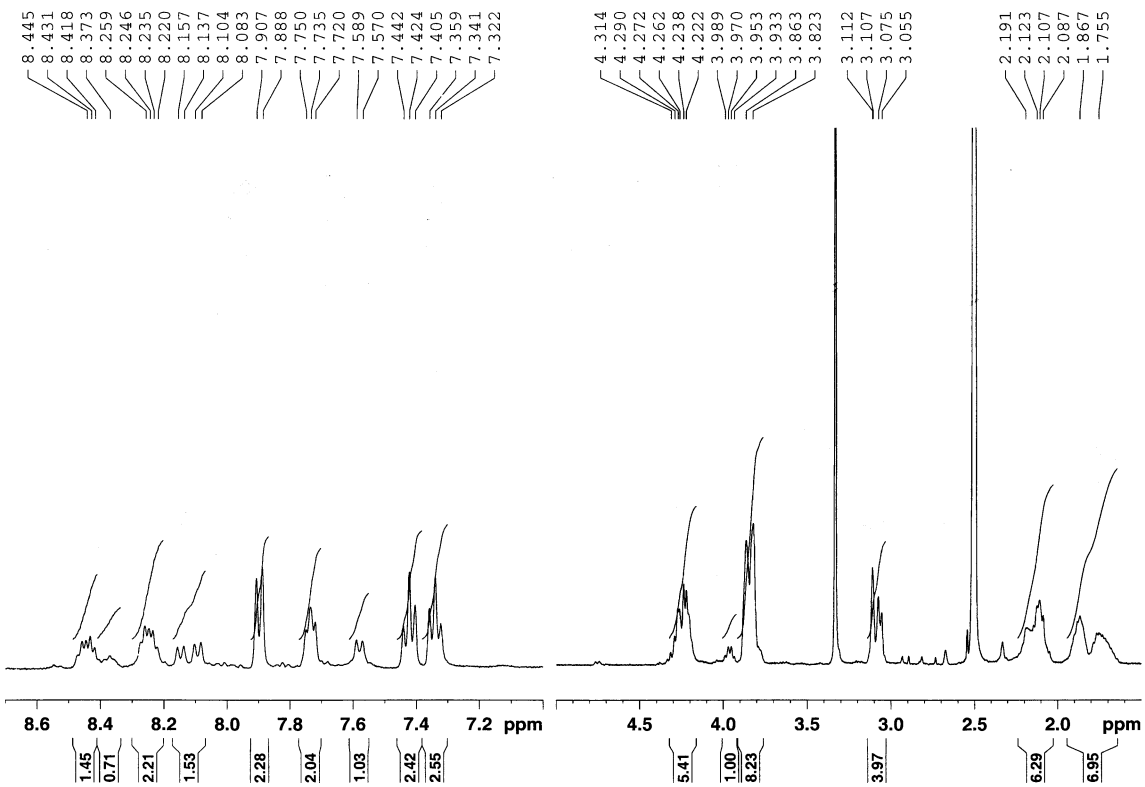

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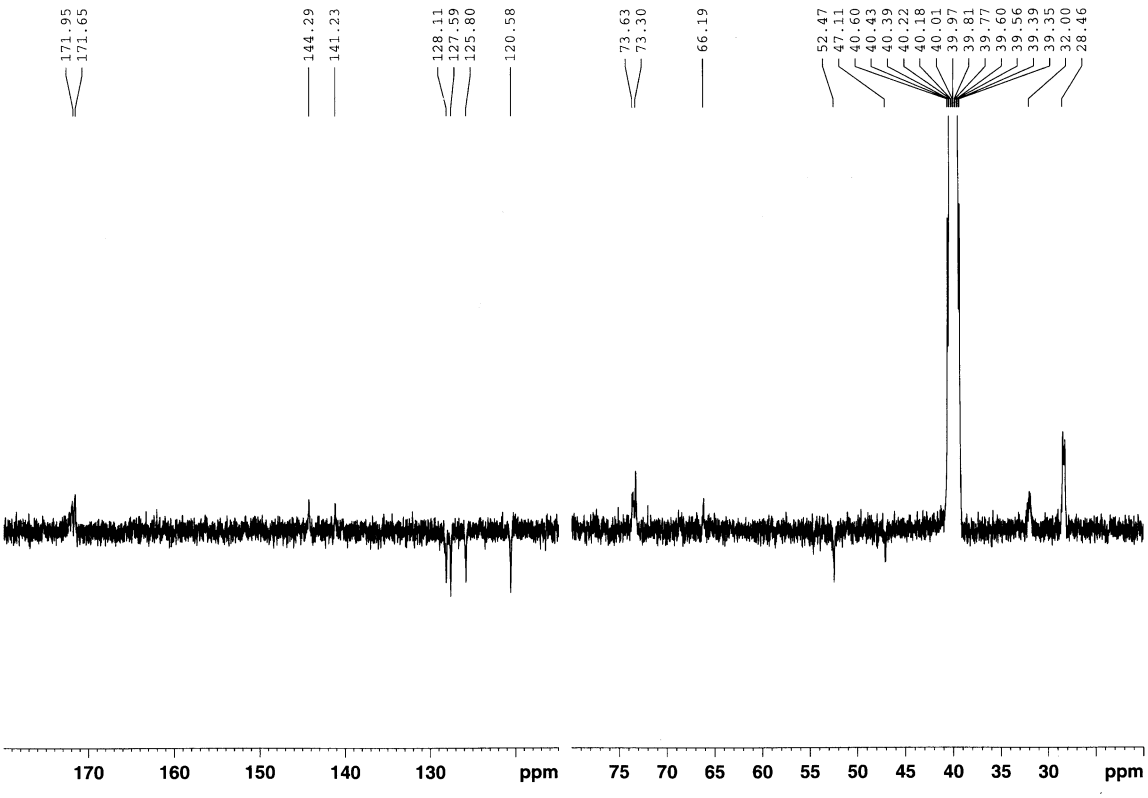

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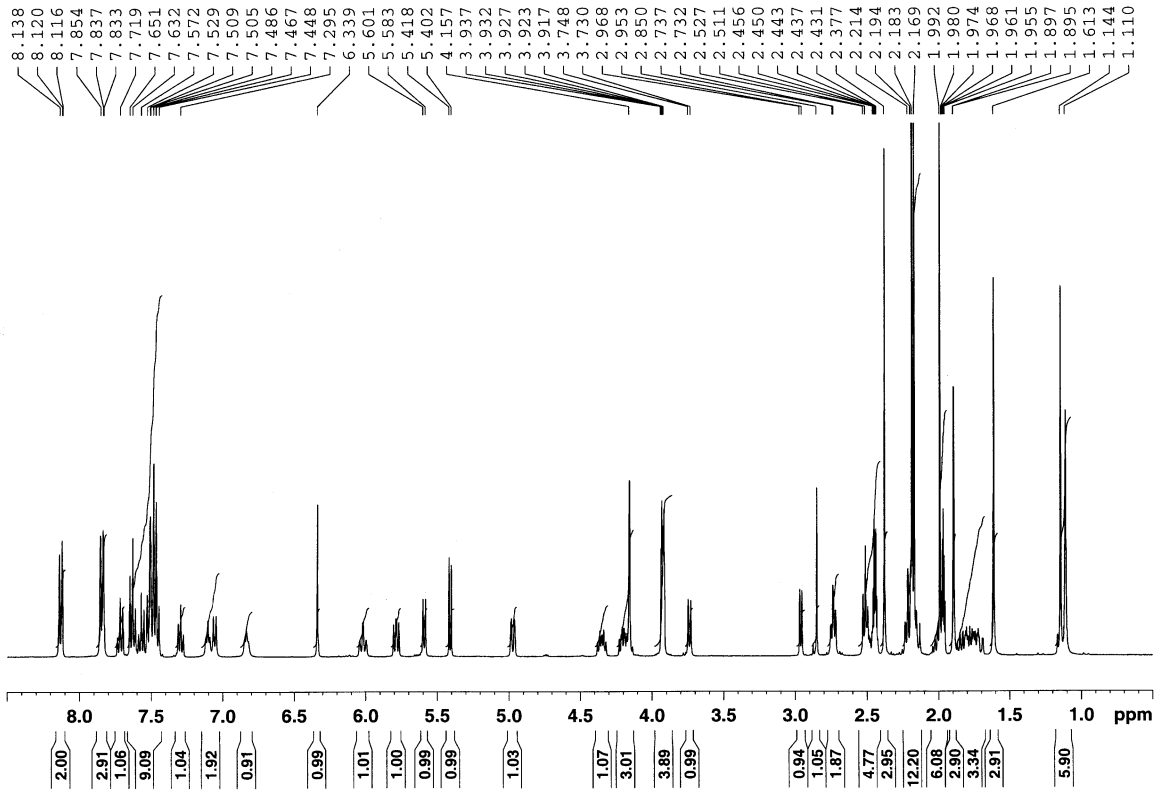

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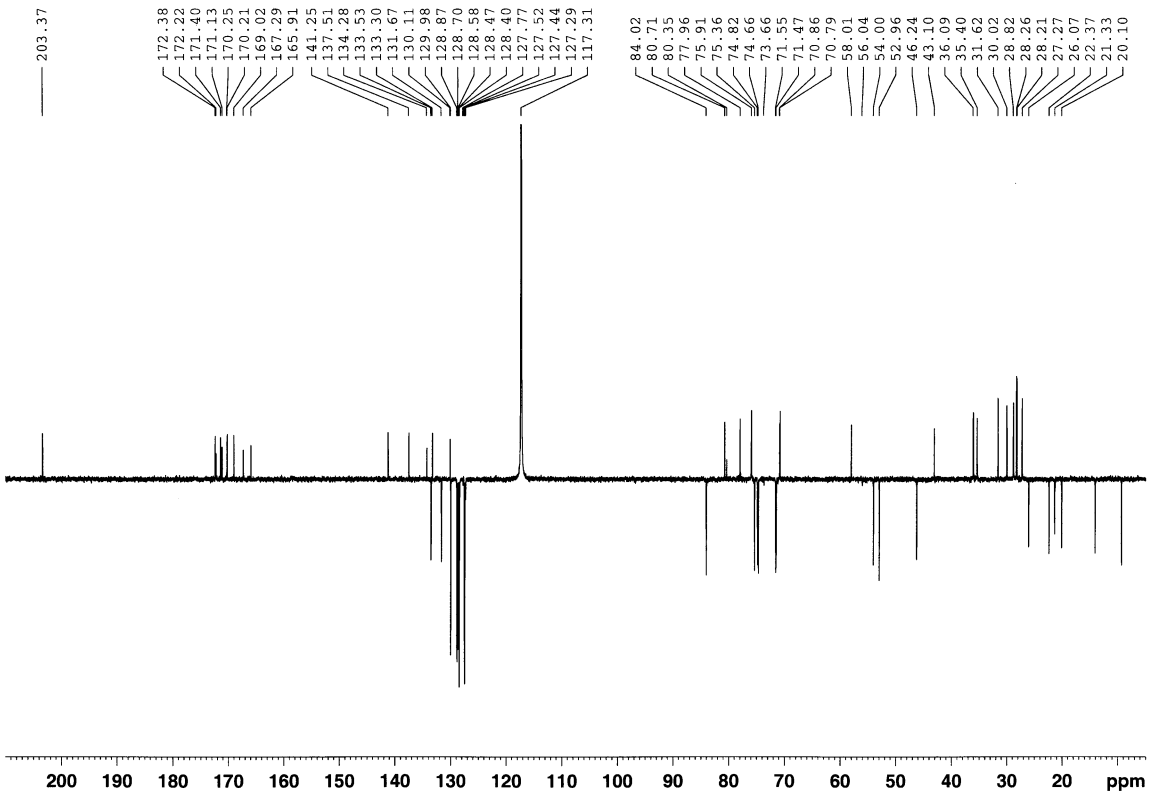

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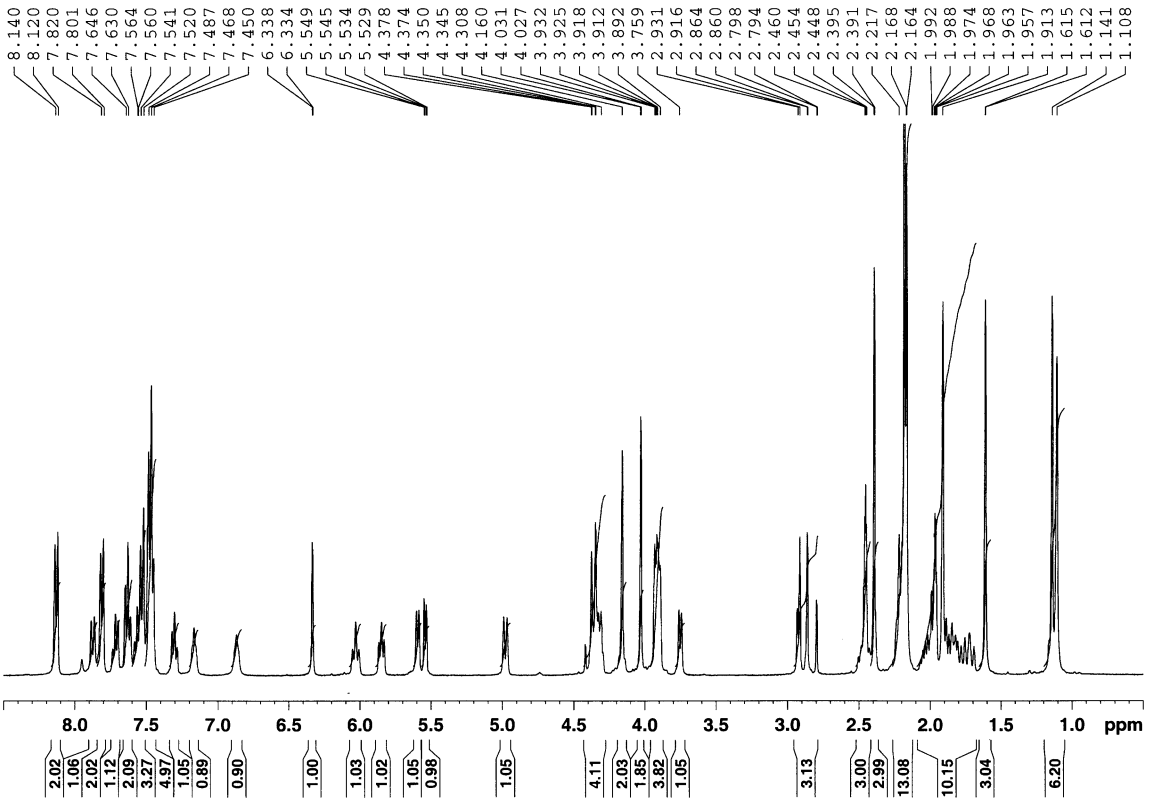

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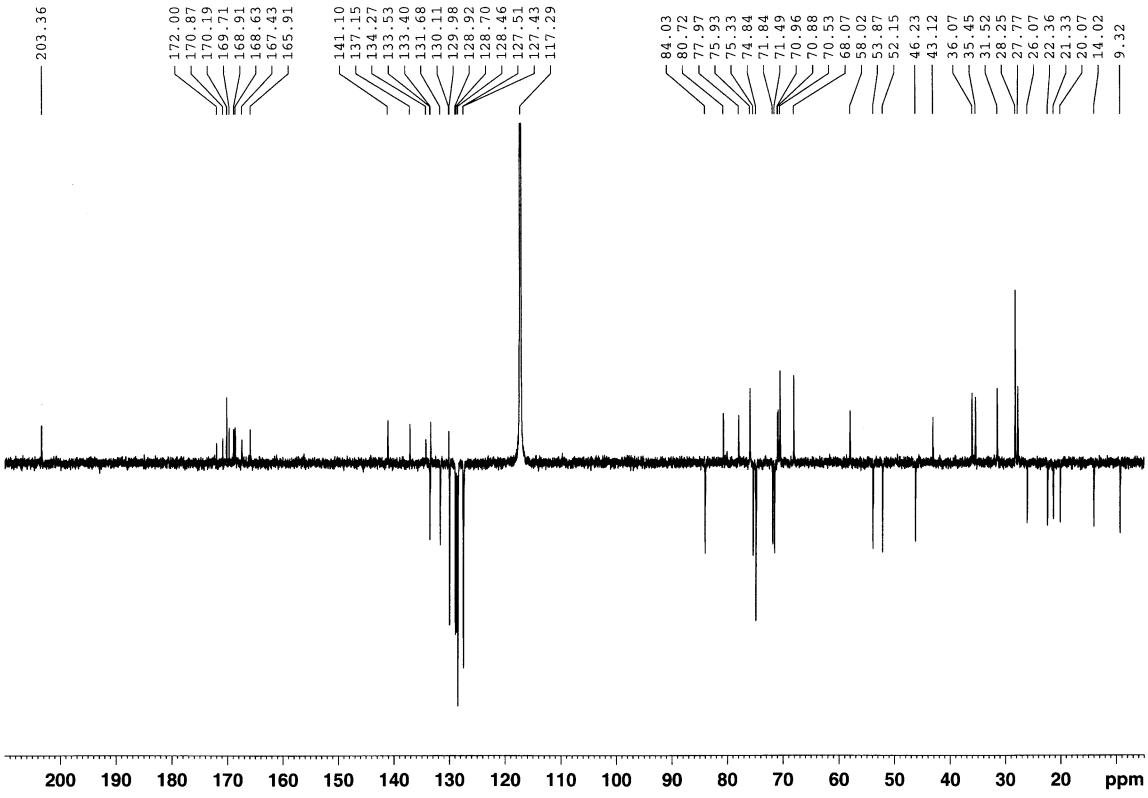

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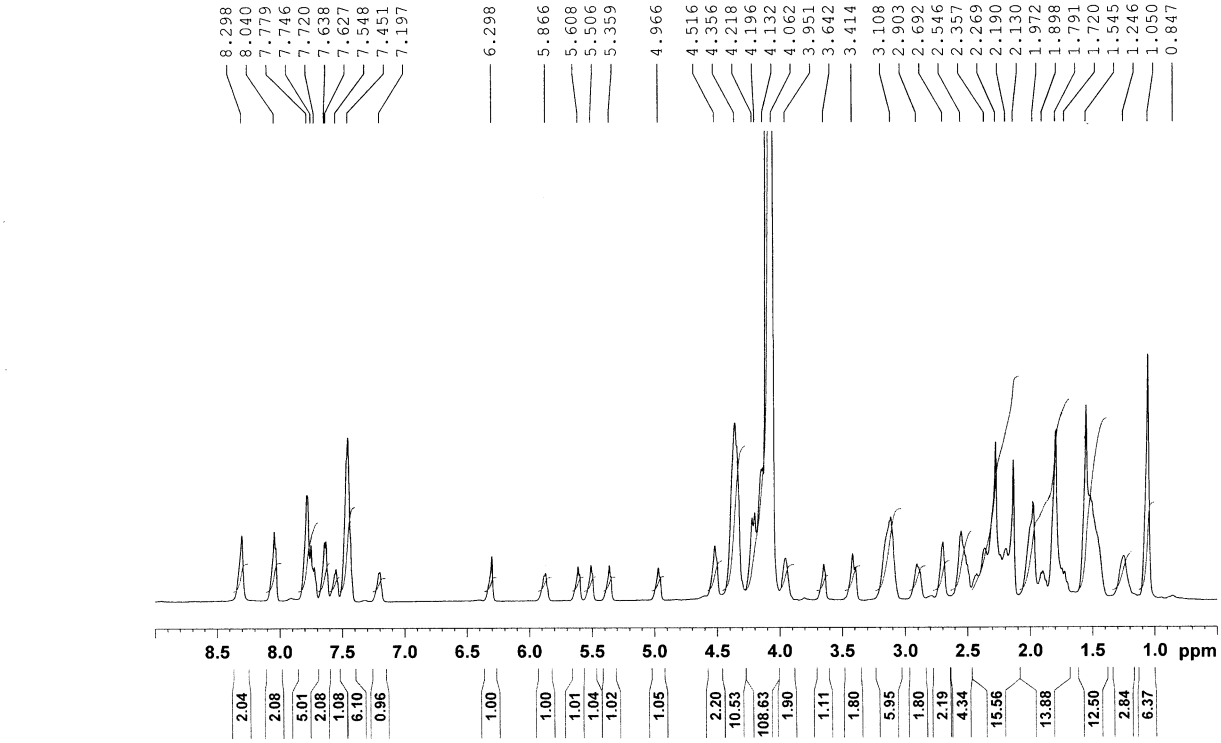

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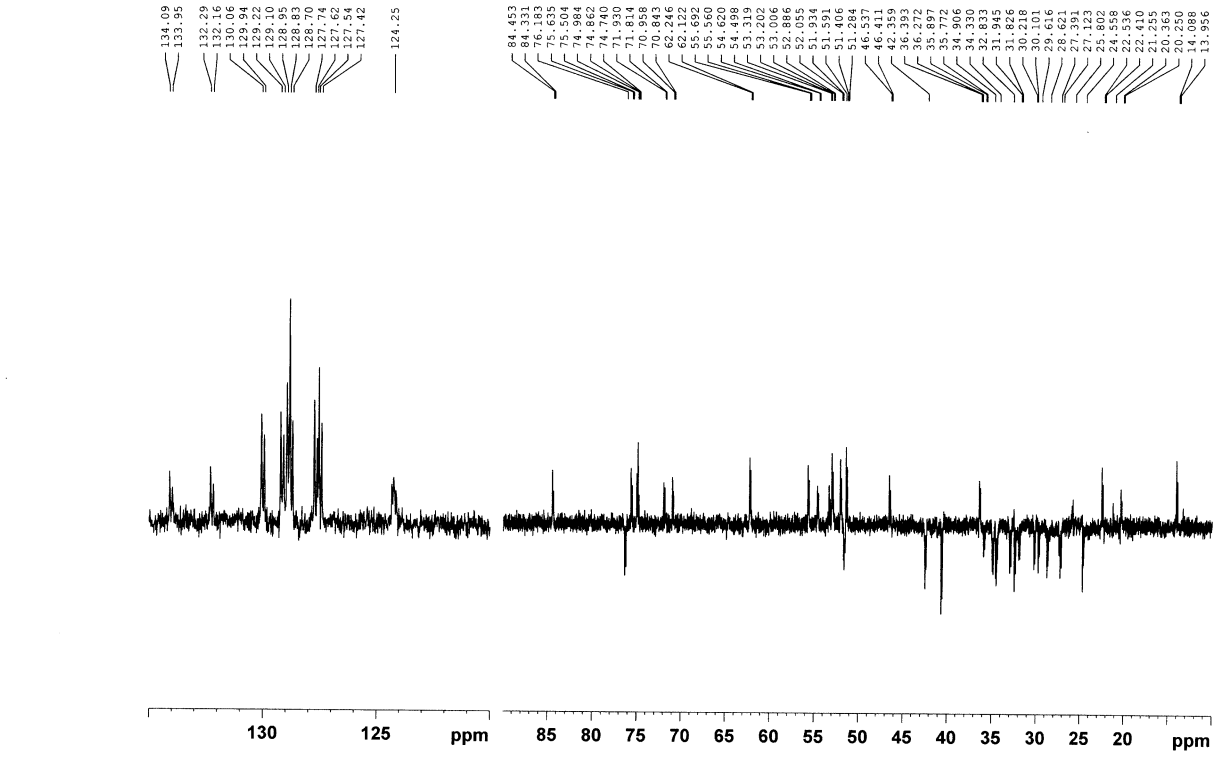

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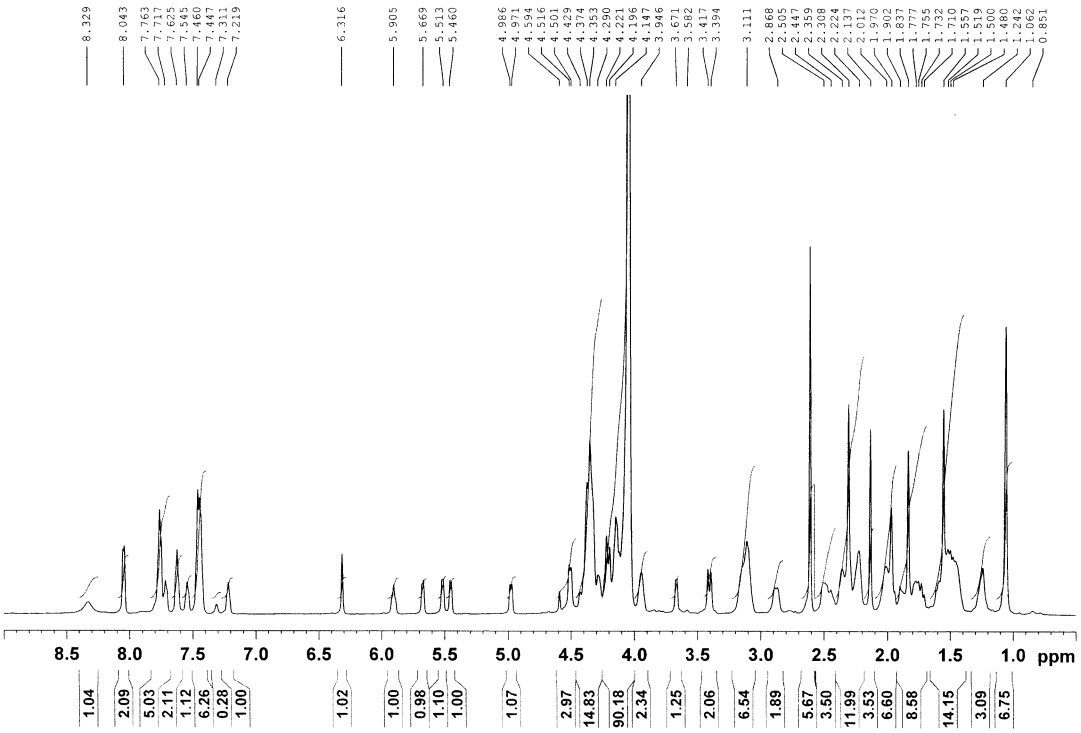

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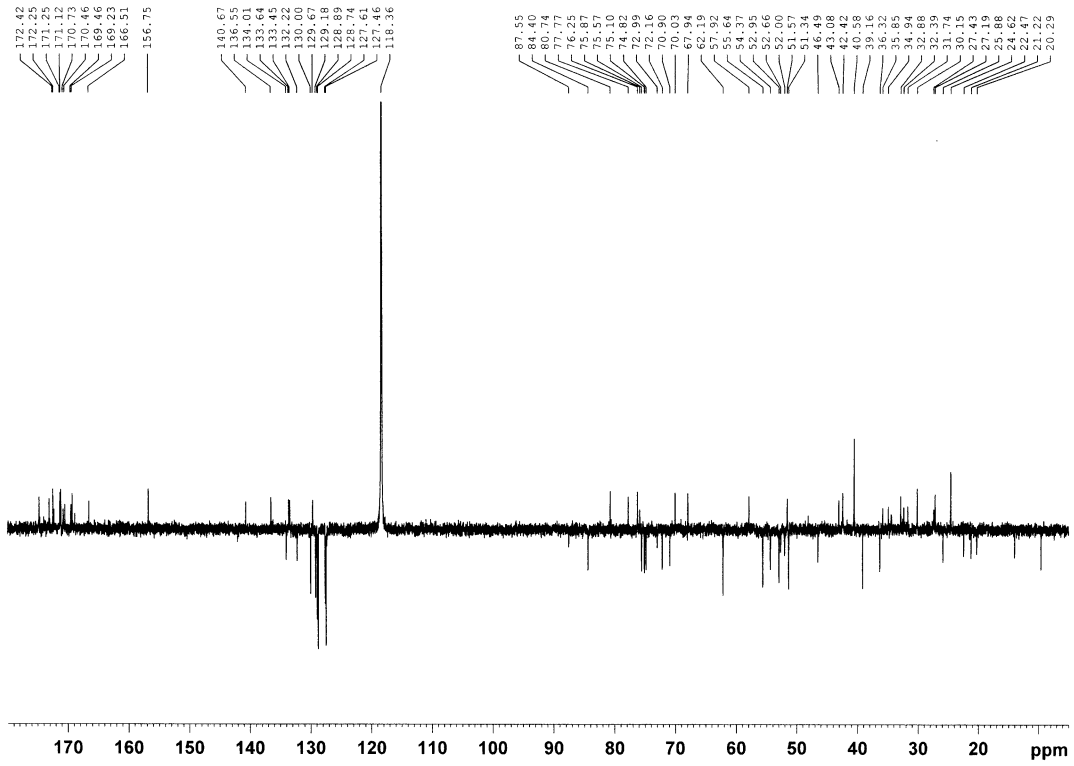

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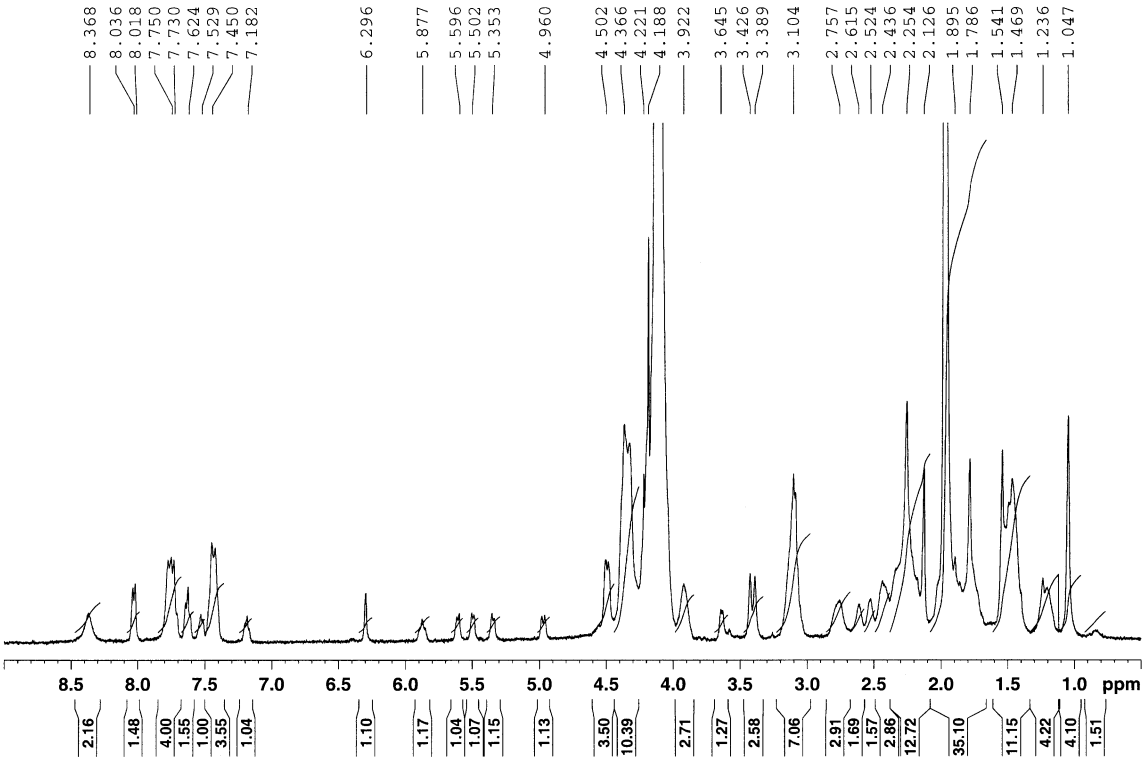
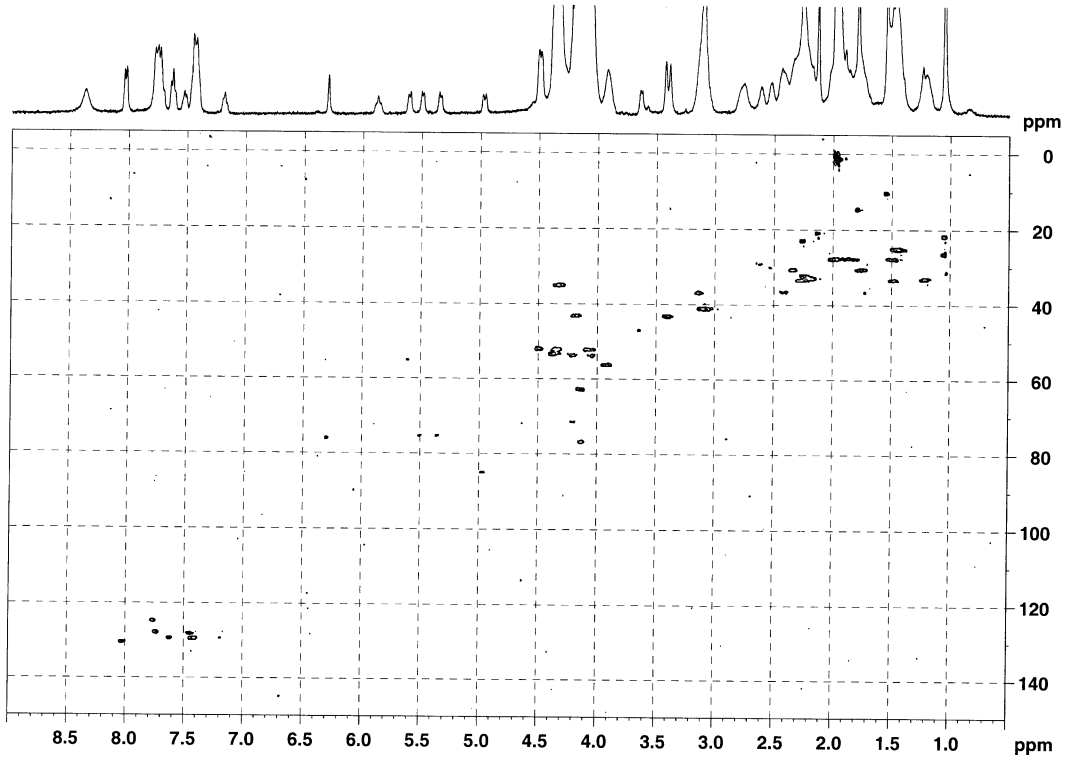


Compound 3c. ^{13}C-NMR HSQC (3:2 CD_3CN/D_2O, 100.6 MHz)



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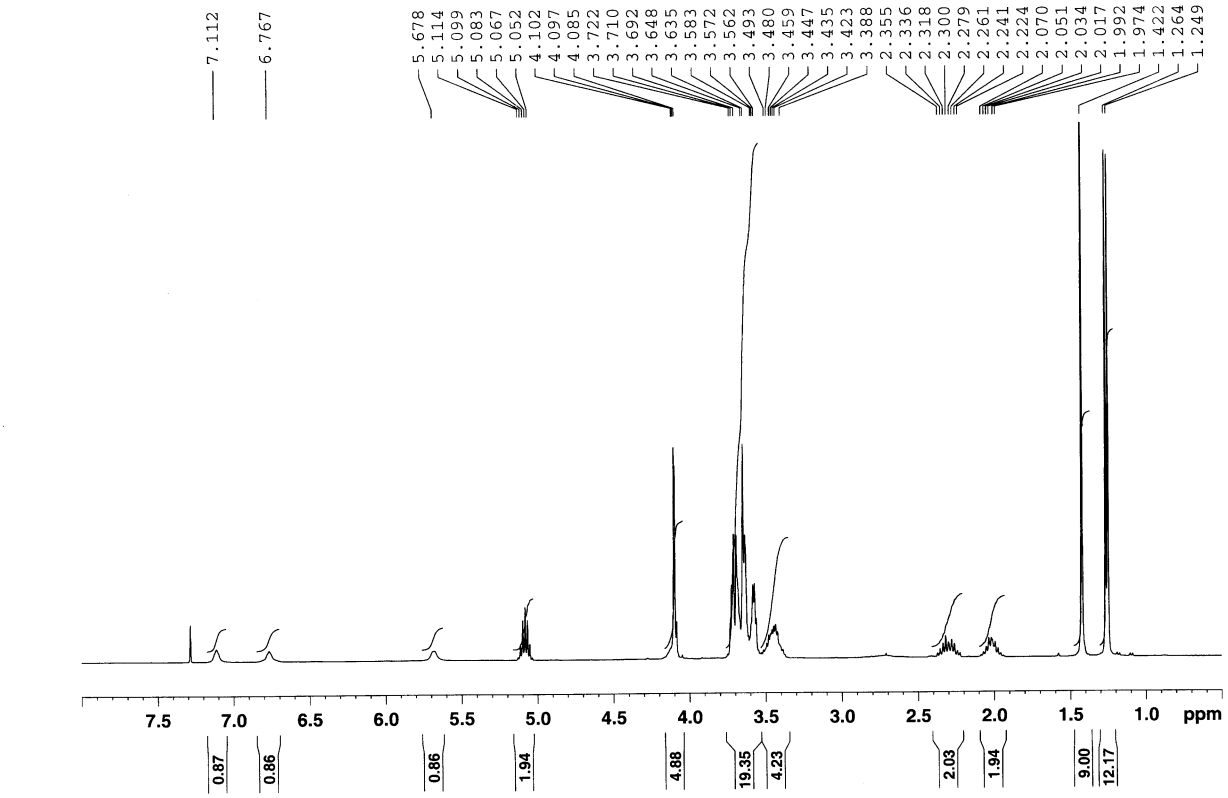

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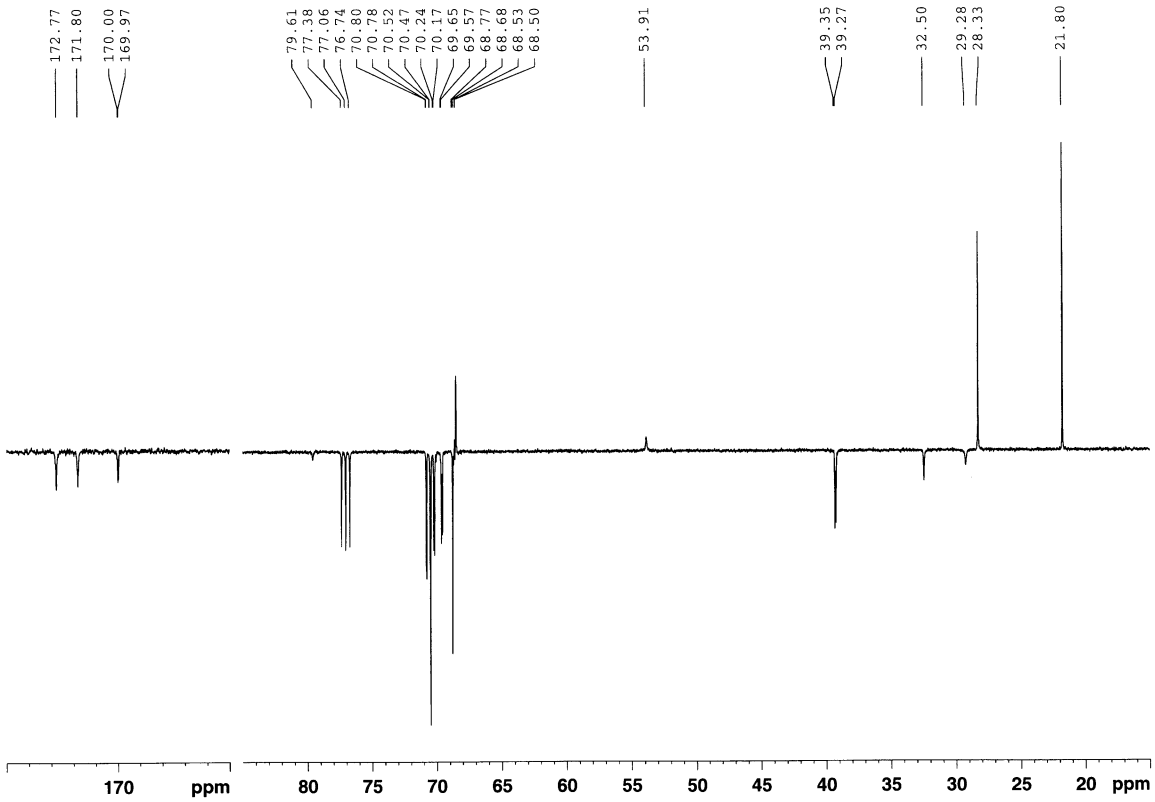

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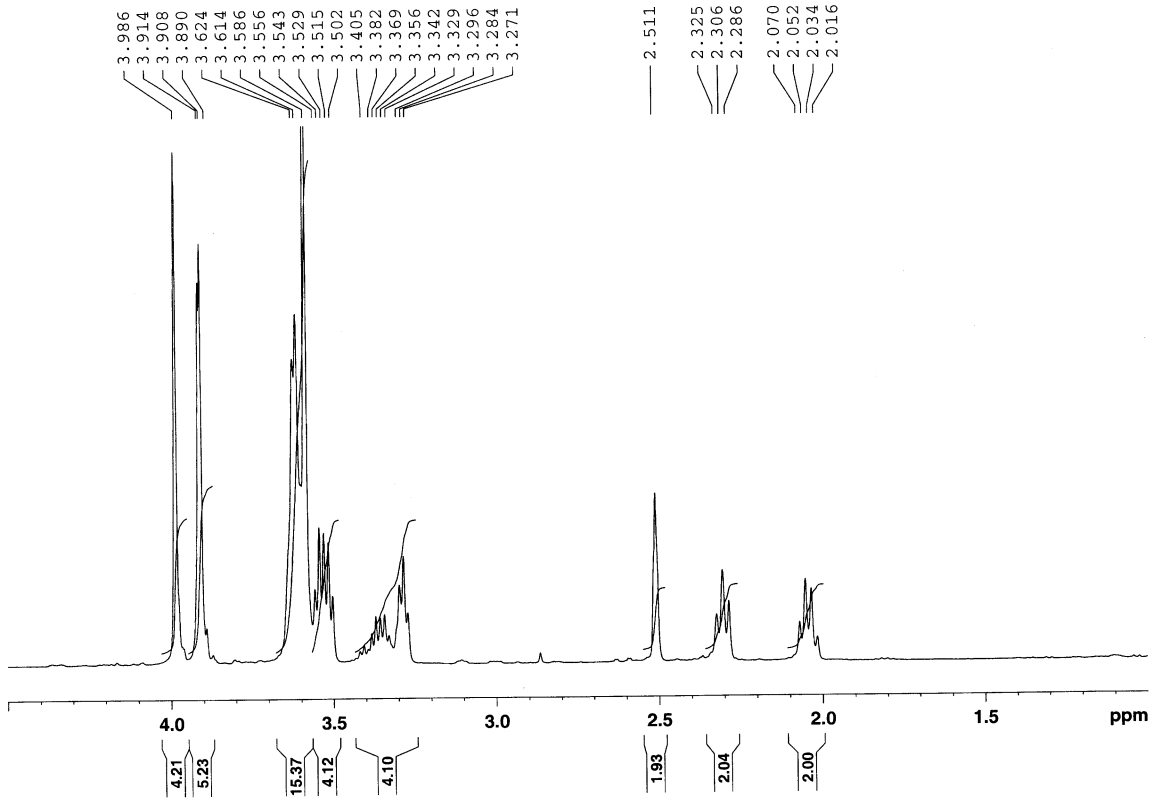
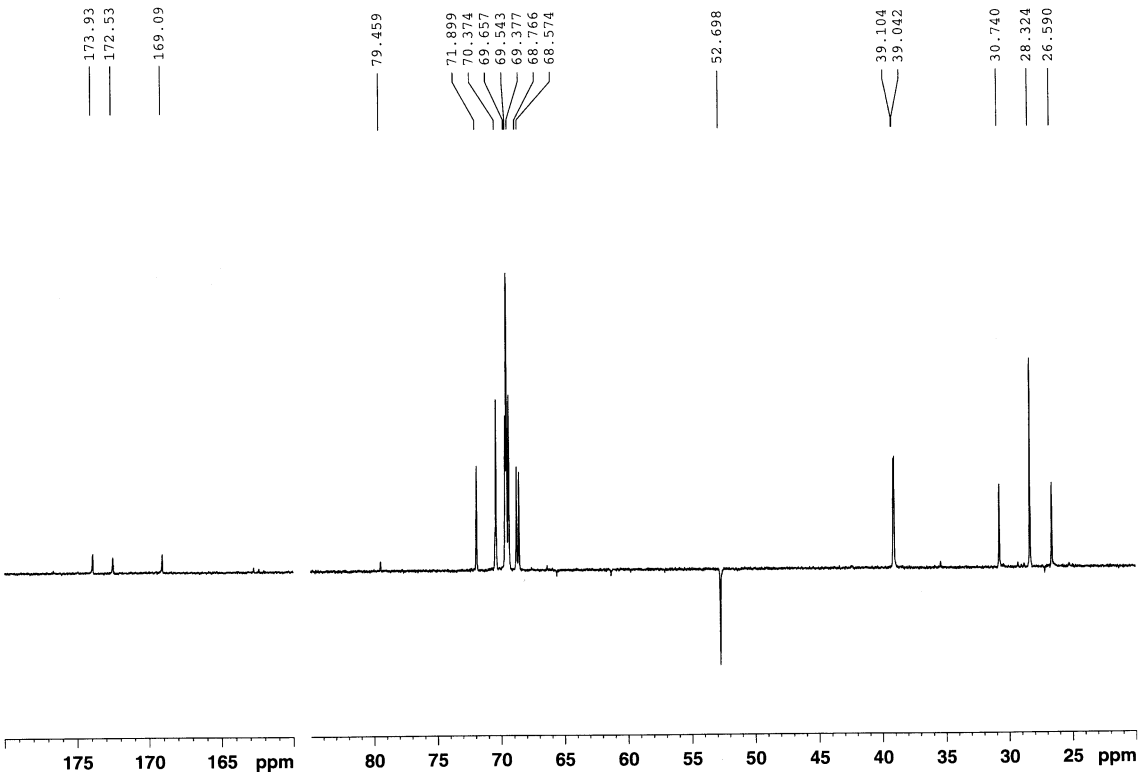


Compound 14. ^{13}C-NMR (D_2O, 100.6 MHz)



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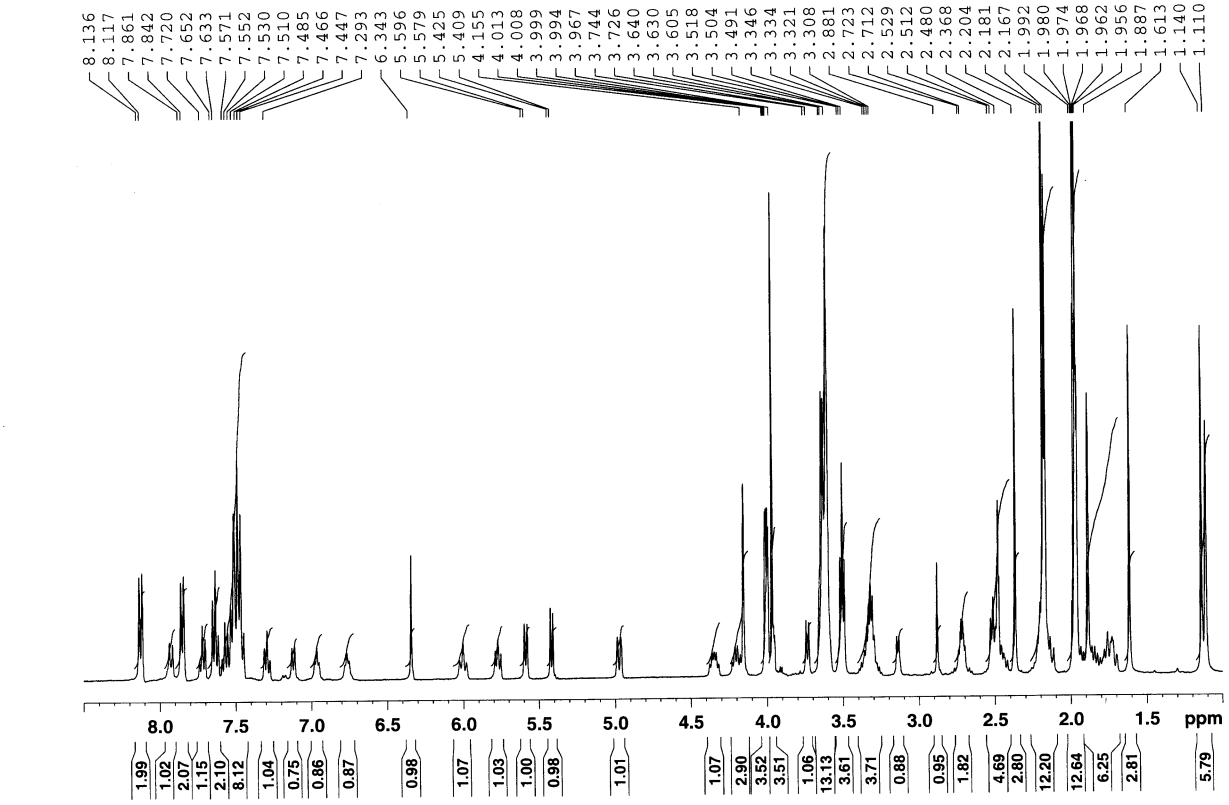
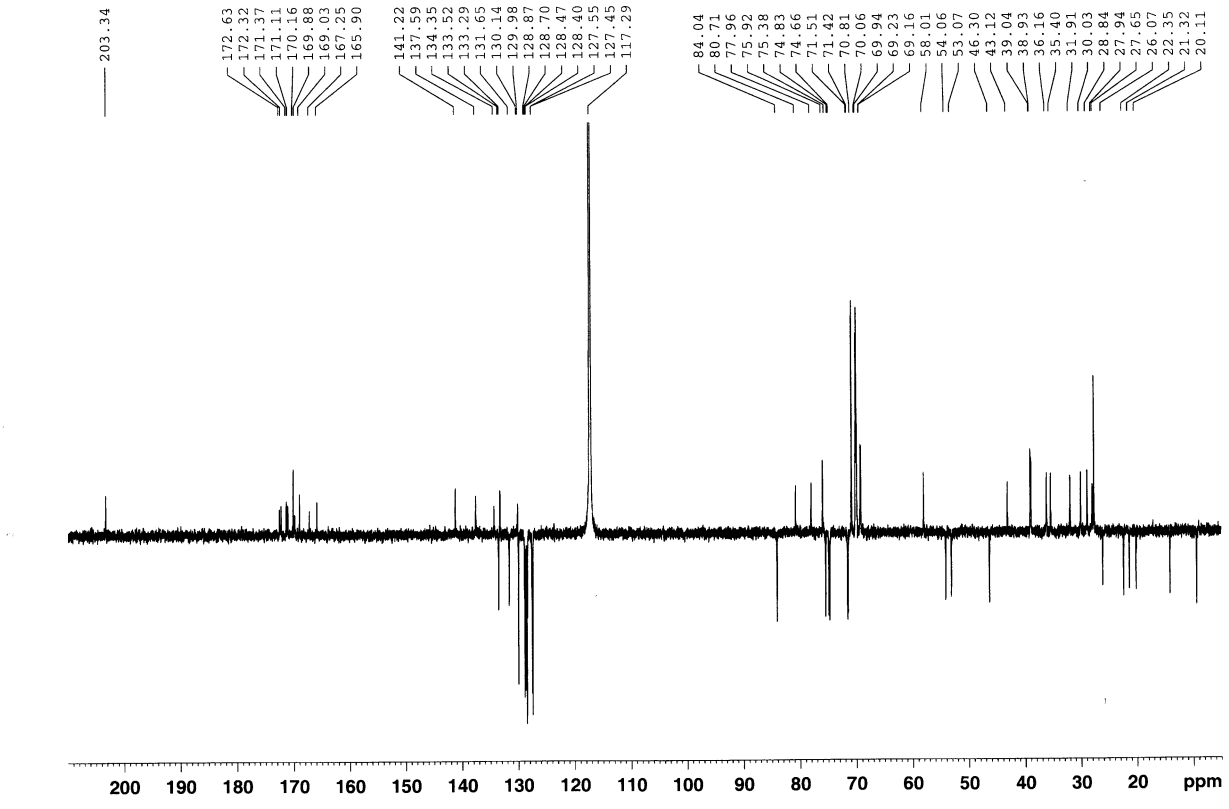
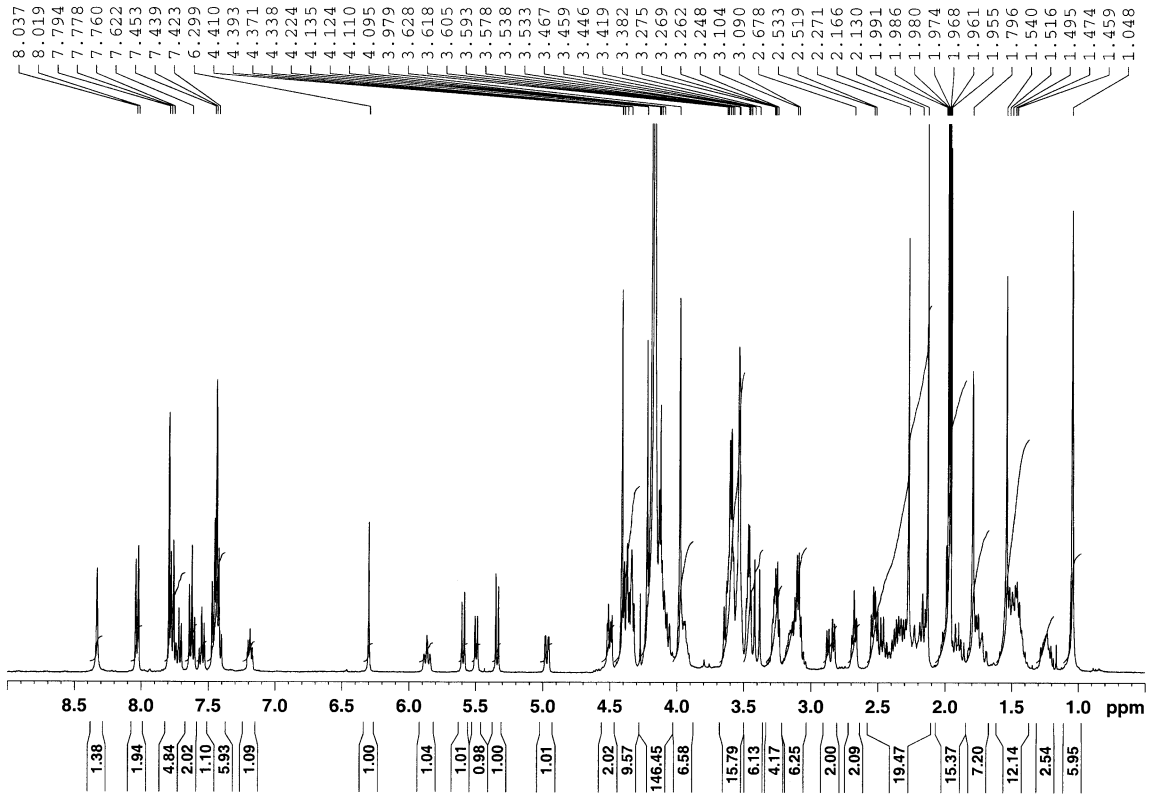


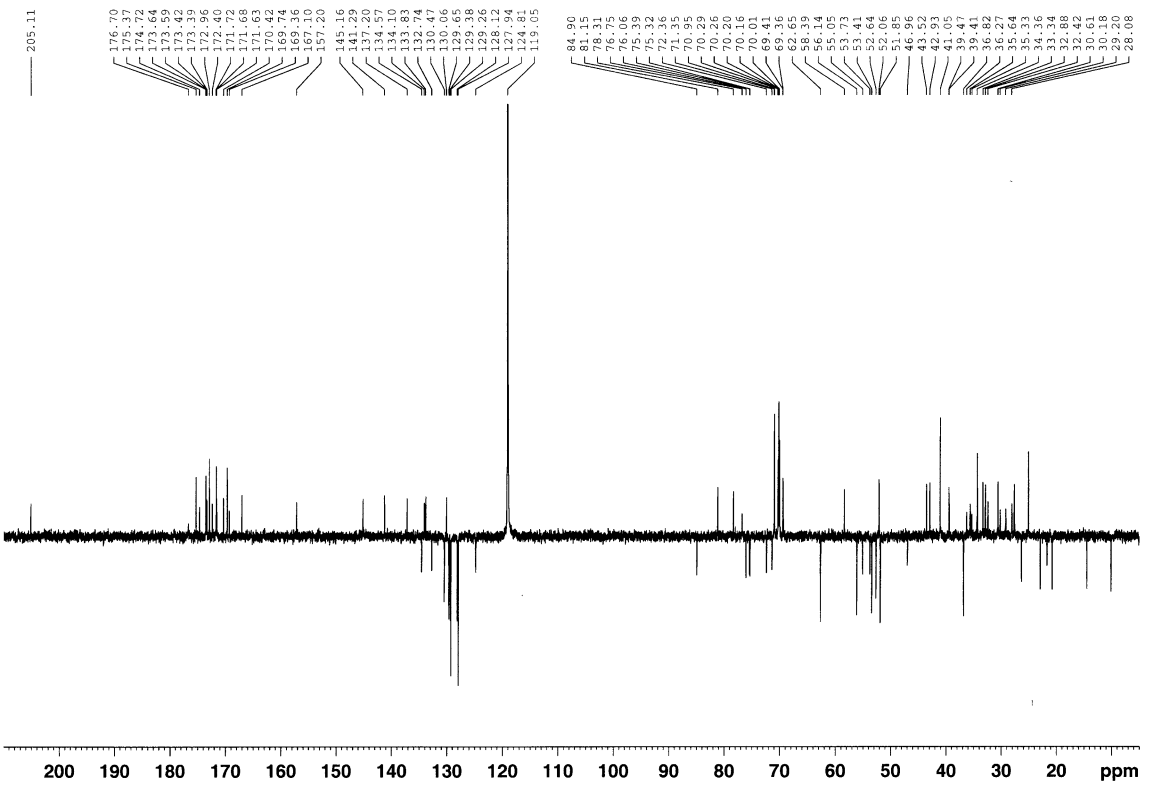
Compound 15. ^{13}C-NMR (CD_3CN, 100.6 MHz)



Compound 4. ^{1}H-NMR (3:2 CD_3CN/D_2O, 400 MHz)



Compound 4. ^{13}C-NMR DEPT (3:2 CD_3CN/D_2O, 100.6 MHz)



HPLC Purification

Purification of single compounds was obtained by means of an MS-assisted preparative HPLC (Waters Co, Milford MA, USA) composed of a 2525 binary gradient pump, a 2996 diode array UV detector, a 2767 fraction collector and autosampler, a CFO column selector, a 515 make-up pump and a single quadrupole ZQ Micromass detector. Under the control of the Waters Fractionlynx software, this apparatus is capable of automatically selecting LC fractions on the basis of the experimentally determined mass values if in agreement with pre-defined molecular ions. The fraction collection phase of the purification is thus automated, leaving to the operator the selection of the appropriate chromatographic method (column, gradient, mobile phase modifiers) and the pooling of best fractions.

Compound 3a

Column: X-Terra RP18, 100 x 19 mm, 5 μm Waters
Flow = 20 mL/min
Phase A (weak solvent) = water MilliQ + 0.1% HCOOH
Phase B (strong solvent) = acetonitrile Chromasolv Sigma-Aldrich +0,1% HCOOH
HPLC run gradient

T(min)	%A	%B
0	80	20
1	80	20
8	60	40
8.2	0	100
10	0	100

MS method parameters : 0 – 10 min MS monitoring scan 100-1500 uma
Collection at m/z = 1144

Compound 3b

Column: Atlantis RP18, 100 x 19 mm, 5 μm Waters
Flow = 20 mL/min
Phase A (weak solvent) = water MilliQ + 0.1% HCOOH
Phase B (strong solvent) = acetonitrile Chromasolv Sigma-Aldrich +0,1% HCOOH
HPLC run gradient

T(min)	%A	%B
0	70	30
1	70	30
8	65	35
8.2	0	100
10	0	100

MS method parameters : 0 – 10 min MS monitoring scan 100-1500 uma
Collection at m/z = 1151

Compound 3c

Column: Symmetry RP18, 300 x 19 mm, 7 μm Waters
Flow = 20 mL/min
Phase A (weak solvent) = water MilliQ + 0.1% HCOOH
Phase B (strong solvent) = acetonitrile Chromasolv Sigma-Aldrich +0,1% HCOOH
HPLC run gradient

T(min)	%A	%B
0	85	15
2	85	15
20	75	25
20.5	0	100
25	0	100

MS method parameters : 0 – 10 min MS monitoring scan 100-1500 uma
Collection at m/z = 936

Compound 4

Column: Symmetry RP18, 300 x 19 mm, 7 μm Waters
Flow = 20 mL/min
Phase A (weak solvent) = water MilliQ + 0.1% HCOOH
Phase B (strong solvent) = acetonitrile Chromasolv Sigma-Aldrich +0,1% HCOOH
HPLC run gradient

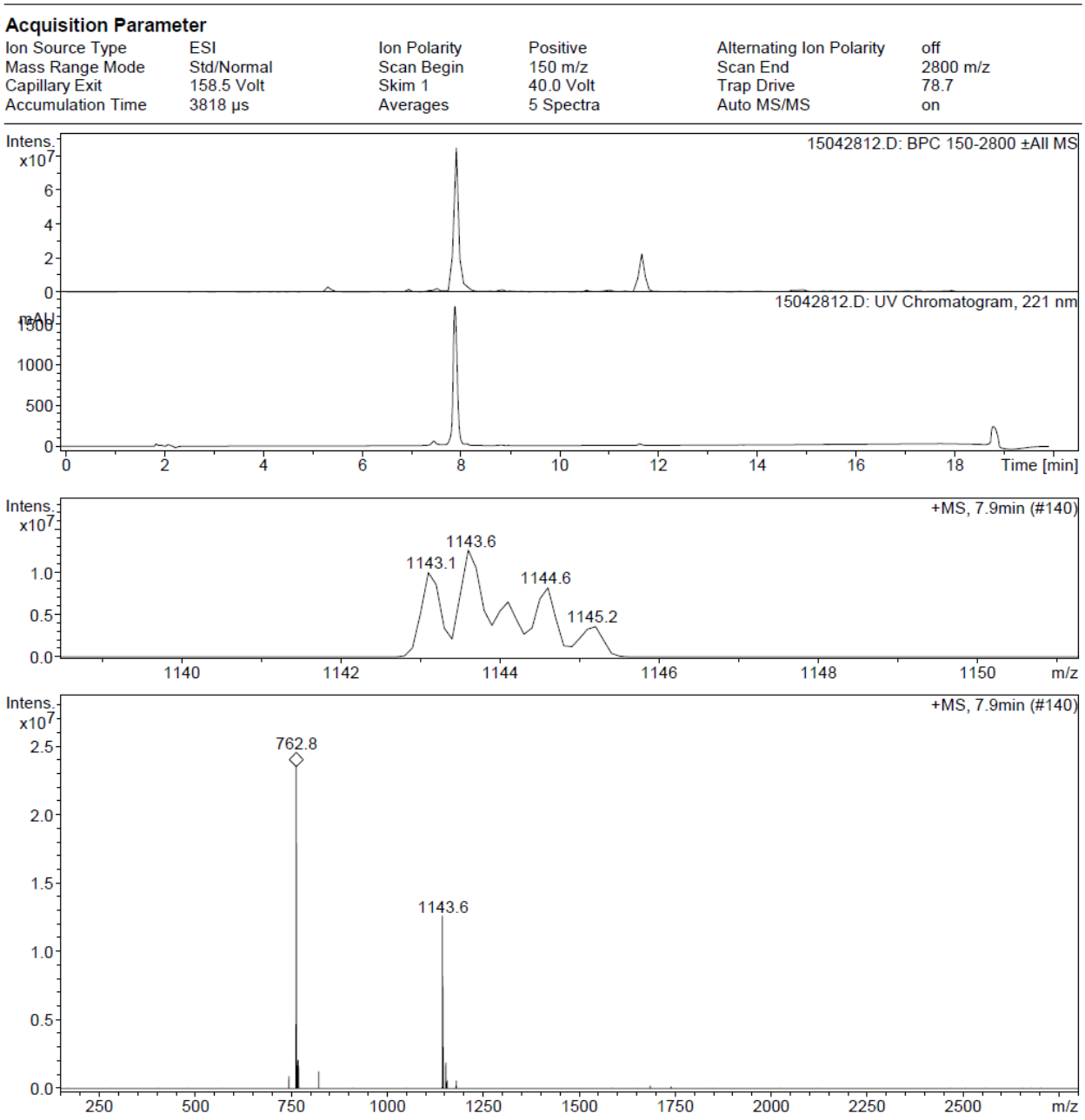
T(min)	%A	%B
0	85	15
2	85	15
20	65	35
20.5	0	100
25	0	100

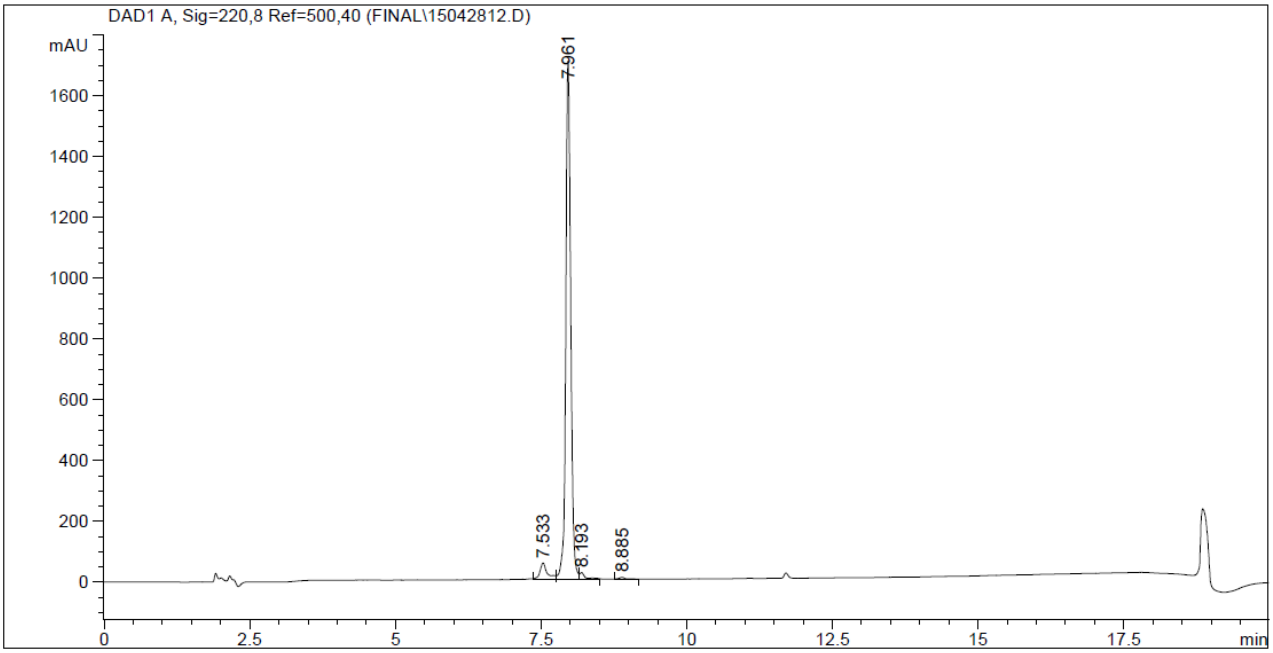
MS method parameters : 0 – 10 min MS monitoring scan 100-1500 uma
Collection at m/z = 889

General method for analytical HPLC

Column: Supelco Discovery, 150 x 4.6 mm, 5 µm
Flow = 1 mL/min
Phase A (weak solvent) = water MilliQ + 0.05% TFA
Phase B (strong solvent) = acetonitrile Chromasolv Sigma-Aldrich + 0.05% TFA
HPLC run gradient: from 20% B to 90% B in 15 min, washing at 100% B for 1 min and equilibration at 20% B in the next 4 min.
λ = 221 nm

Compound 3a



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   2   7.961 VV    0.0910 1.00556e4  1700.93188  94.2287
   3   8.193 VB    0.0846  123.20153   20.95245   1.1545
   4   8.885 BP    0.1001   44.52004    6.32455   0.4172

Totals :                  1.06715e4  1781.16050
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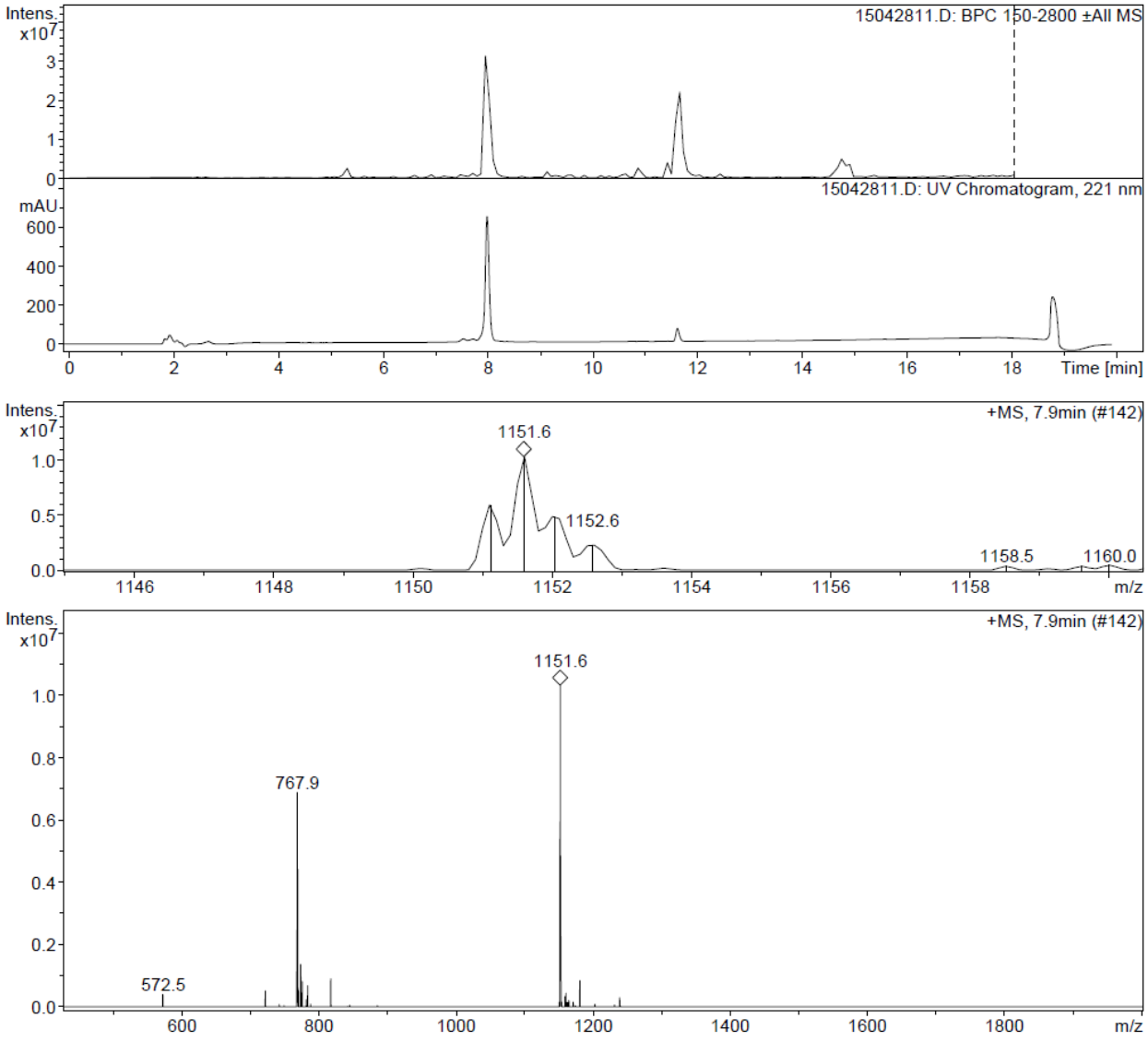
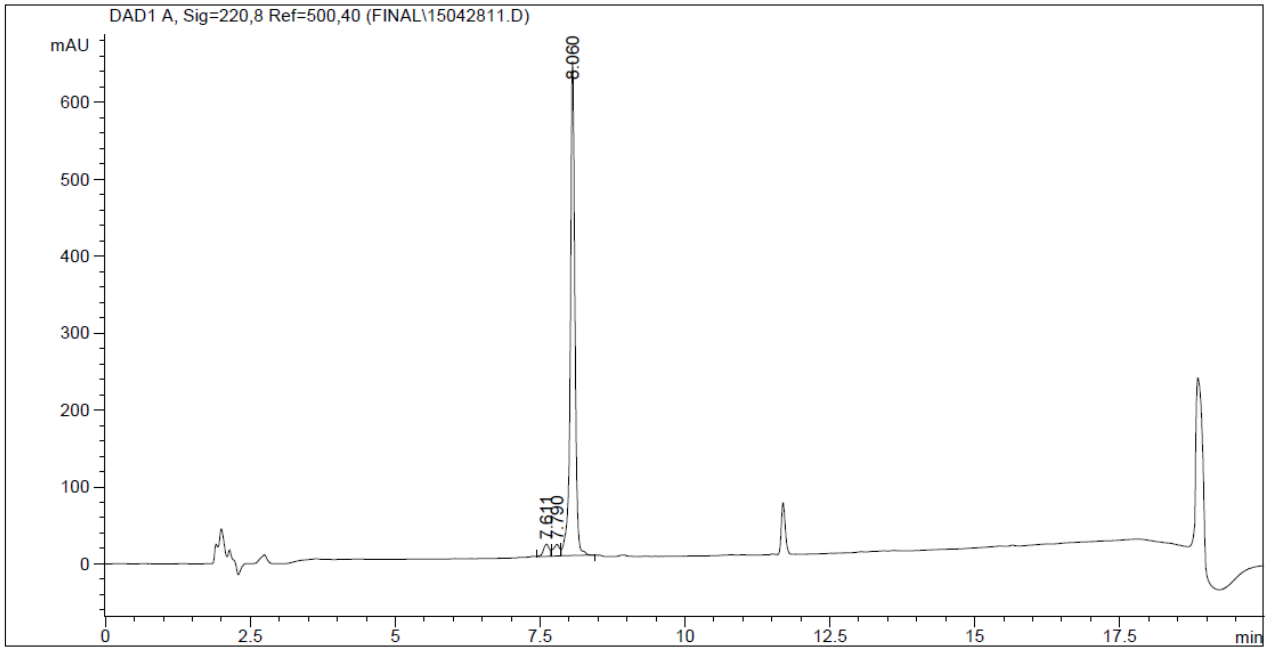
Compound 3b

Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	off
Mass Range Mode	Std/Normal	Scan Begin	150 m/z	Scan End	2800 m/z
Capillary Exit	158.5 Volt	Skim 1	40.0 Volt	Trap Drive	78.7
Accumulation Time	4378 µs	Averages	5 Spectra	Auto MS/MS	on



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Signal 1: DAD1 A, Sig=220,8 Ref=500,40

Peak RetTime Type  Width     Area      Height     Area
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   2   7.790 VV    0.1106  116.20490   14.95651   3.0926
   3   8.060 VB    0.0823 3531.62769  641.04938  93.9894

Totals :                   3757.47419  671.80980

 Results obtained with enhanced integrator!
```

Compound 3c

Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	off
Mass Range Mode	Std/Normal	Scan Begin	150 m/z	Scan End	2800 m/z
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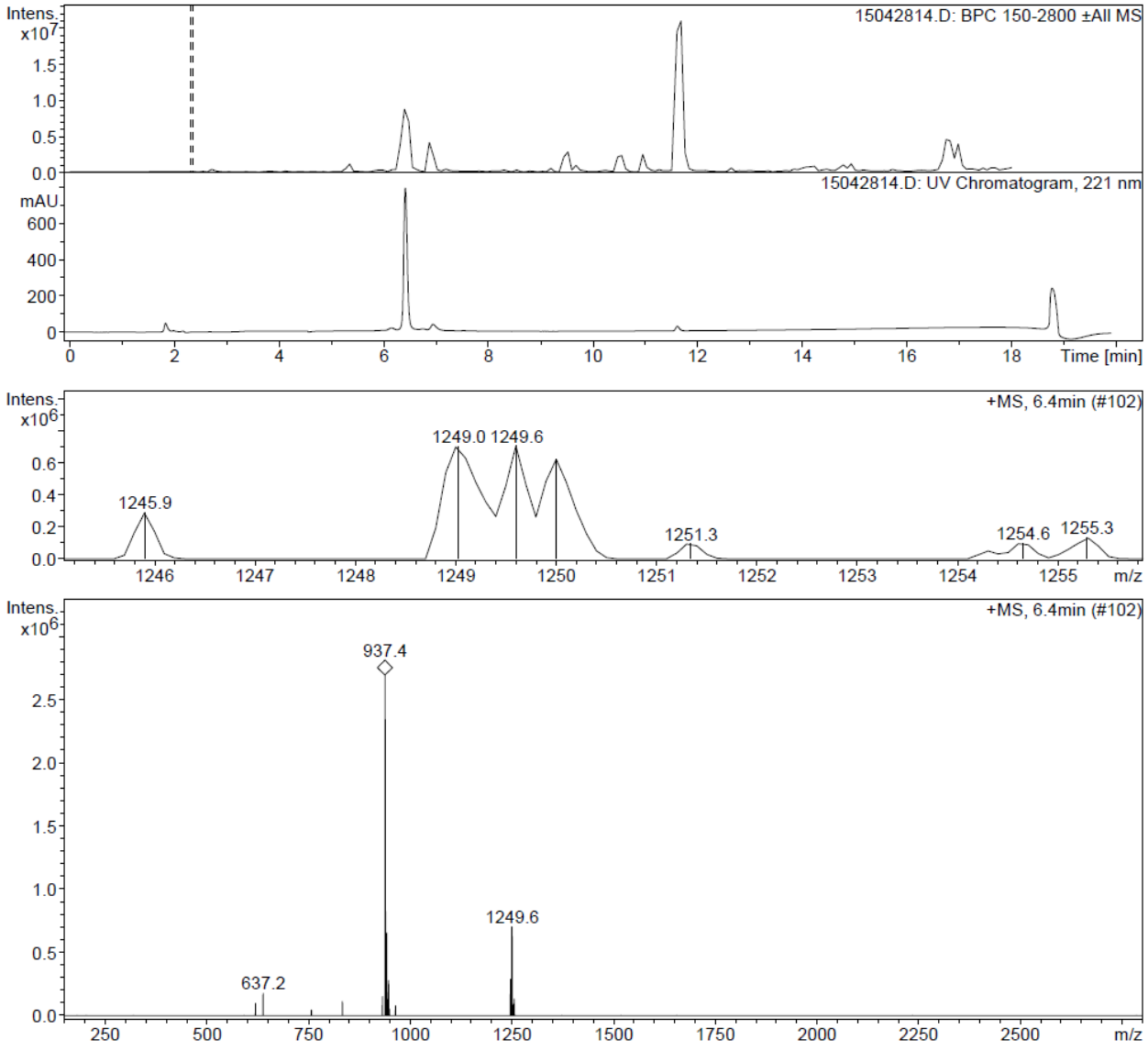

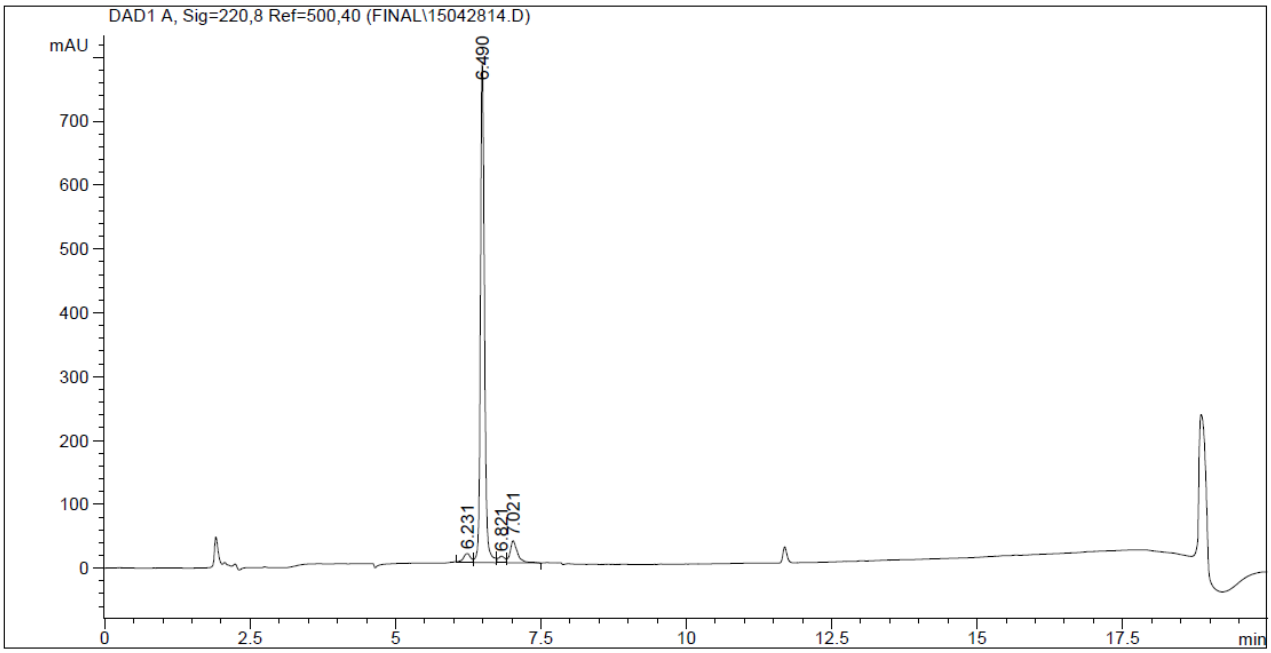


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Use Multiplier & Dilution Factor with ISTDs


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   2   6.490 VV    0.0745 3804.76880  785.07361  88.3713
   3   6.821 VV    0.1105   78.53095   10.11899   1.8240
   4   7.021 VP    0.1262  302.25931   34.49945   7.0204

Totals :                  4305.43712  843.58903
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Compound 4

Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	off
Mass Range Mode	Std/Normal	Scan Begin	150 m/z	Scan End	2800 m/z
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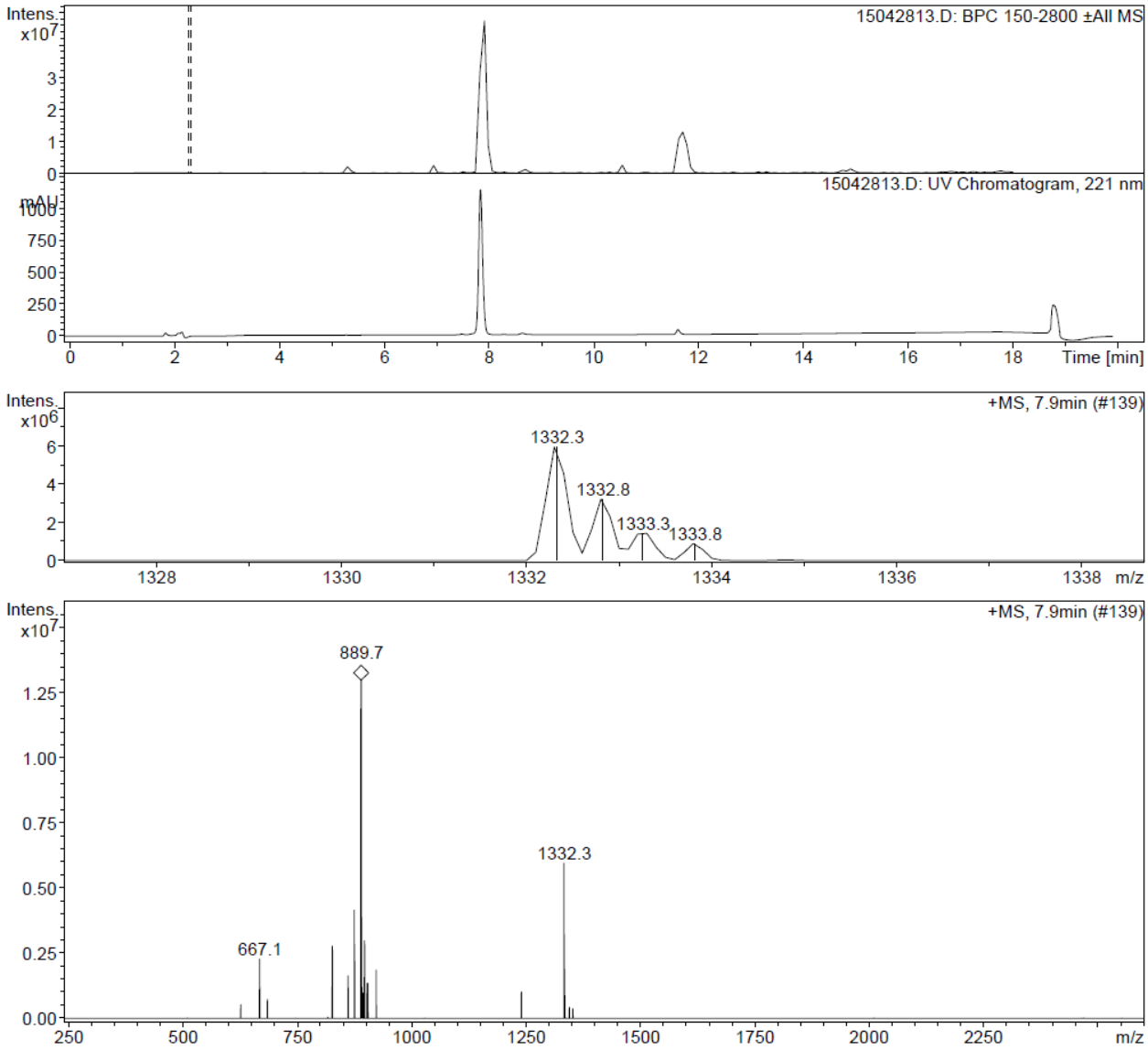
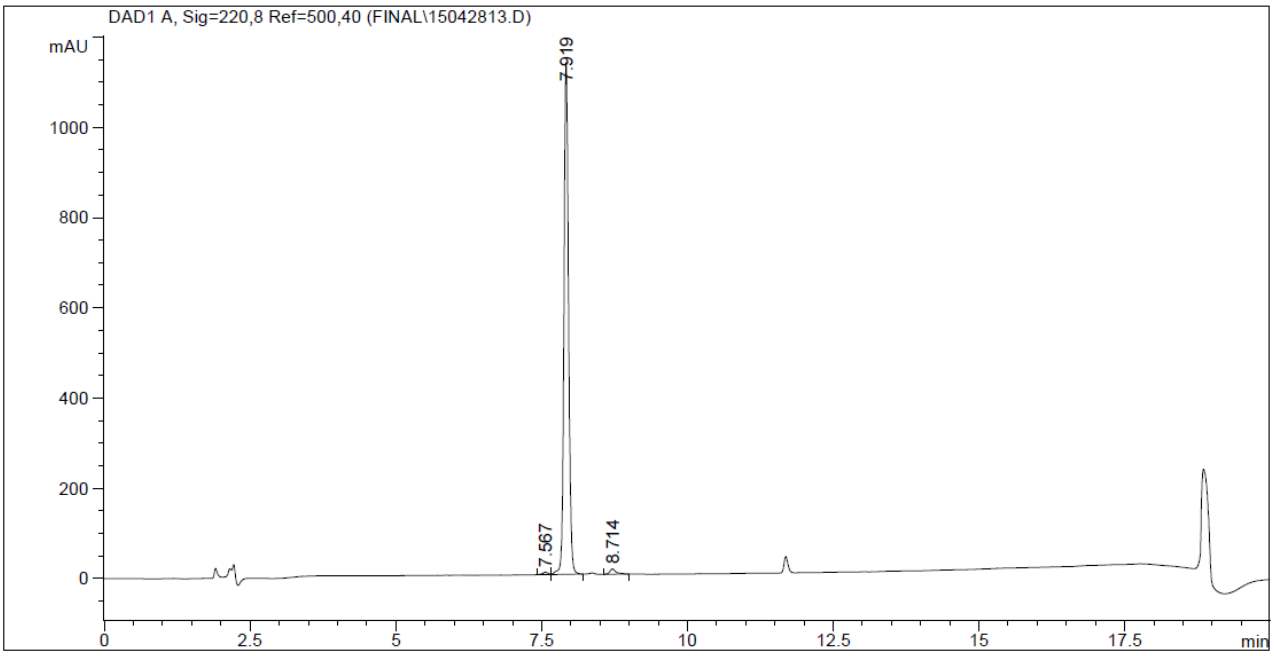
Accumulation Time	3343 µs	Averages	5 Spectra	Auto MS/MS	on



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Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=220,8 Ref=500,40

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
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   1   7.567 BV    0.0942   34.20601    5.30564   0.5412
   2   7.919 VP    0.0859 6198.38477 1132.29382  98.0746
   3   8.714 PB    0.1100   87.48347   11.59283   1.3842

Totals :                  6320.07425 1149.19229

 Results obtained with enhanced integrator!
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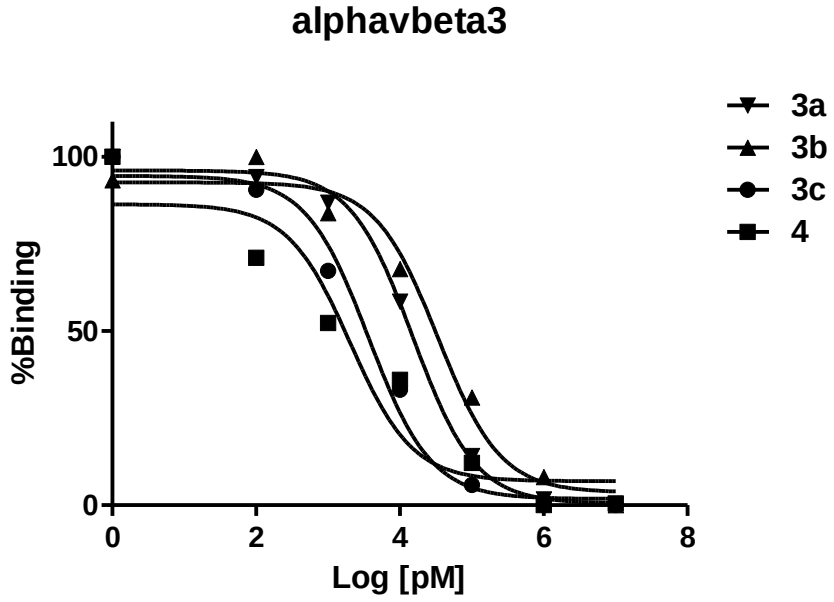
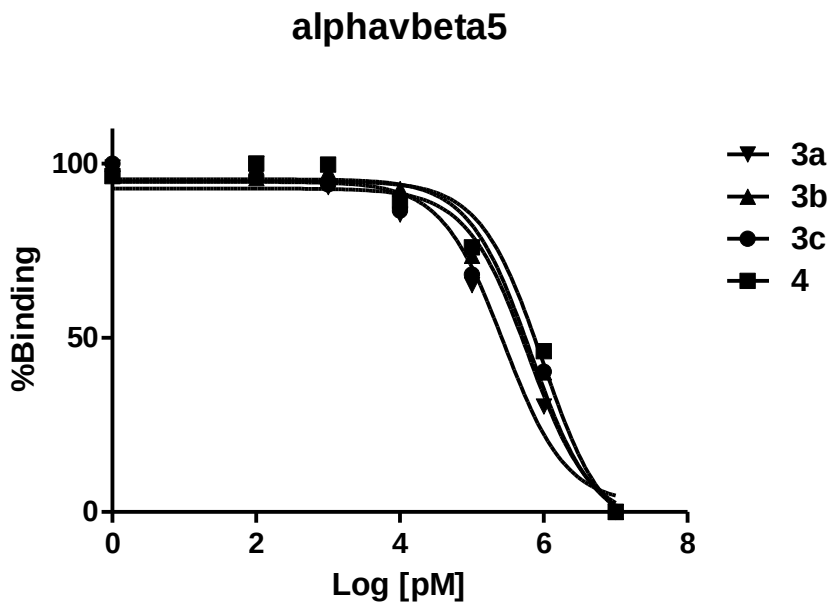
Biology

Solid-phase receptor binding assay. Purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors (Chemicon International, Inc., Temecula, CA, USA) were diluted to 0.5 μg/mL in coating buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MnCl_2$, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. An aliquot of diluted receptors (100 μL/well) was added to 96-well microtiter plates (NUNC MW 96F Medisorp Straight) and incubated overnight at 4 °C. The plates were then incubated with blocking solution (coating buffer plus 1% bovine serum albumin) for an additional 2 h at room temperature to block nonspecific binding, followed by 3 h incubation at room temperature with various concentrations (10^{-5}–10^{-12} M) of test compounds in the presence of biotinylated vitronectin (1 μg/mL). Biotinylation was performed using an EZ-Link Sulfo-NHS-Biotinylation kit (Pierce, Rockford, IL, USA). After washing, the plates were incubated for 1 h at room temperature with biotinylated streptavidin–peroxidase complex (Amersham Biosciences, Uppsala, Sweden) followed by 30 min incubation with 100 μL/well Substrate Reagent Solution (R&D Systems, Minneapolis, MN) before stopping the reaction with the addition of 50 μL/well 2N H_2SO_4. Absorbance at 415 nm was read in a SynergyTM HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.). Each data point represents the average of triplicate wells; data analysis was carried out by nonlinear regression analysis with GraphPad Prism 5.0 software. Each experiment was repeated in triplicate.



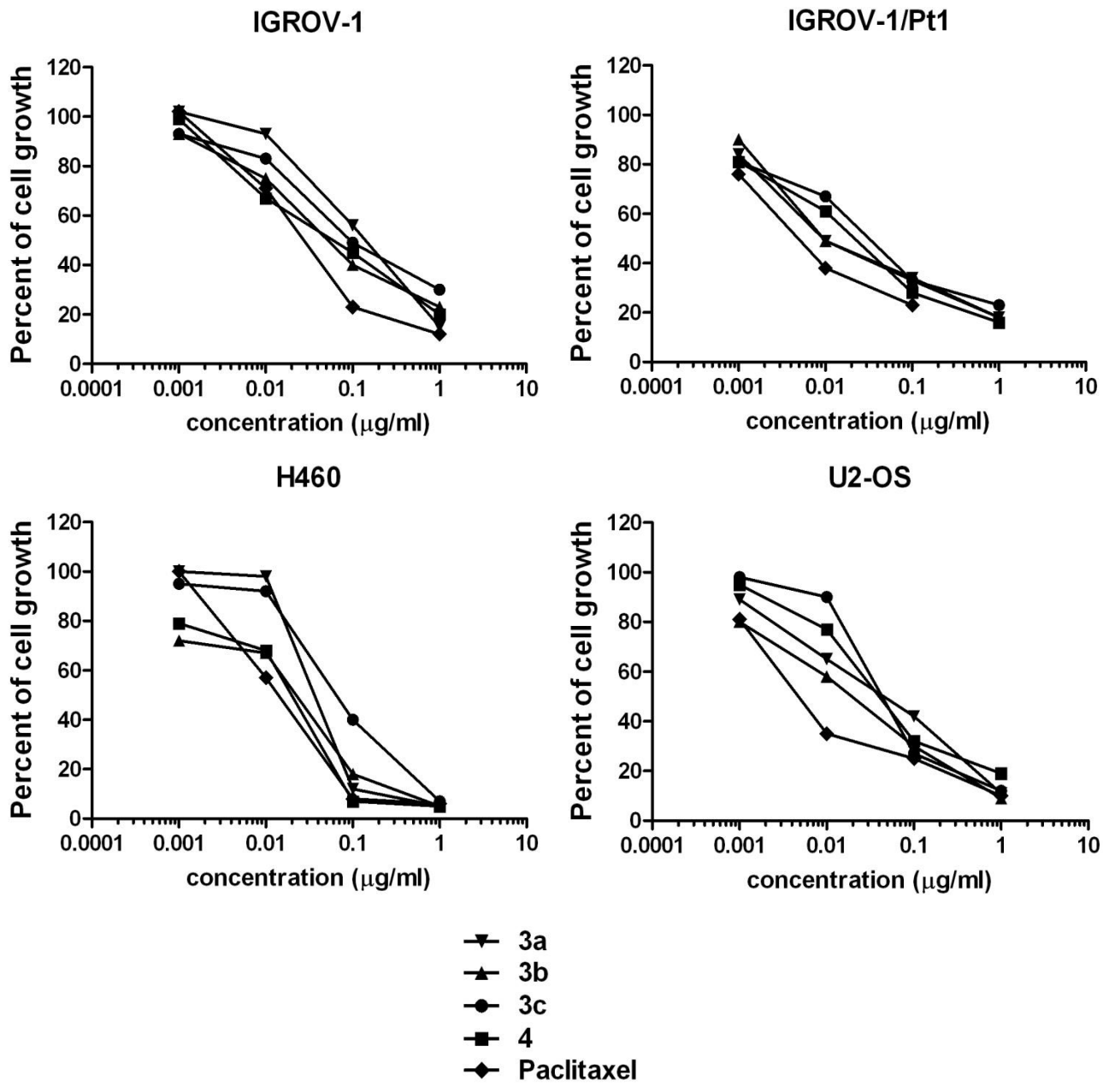


SD was below 10% for each data point.

Drugs. For *in vitro* studies, PTX and compounds under investigation were dissolved in dimethylsulfoxide (DMSO) and then added to culture medium. DMSO concentration in medium never exceeded 0.25%. For *in vivo* studies PTX was dissolved in a mixture of ethanol and cremophor ELP (50+50%) and kept at 4 °C. At treatment the drug was diluted in 90% of cold saline after magnetic stirring and administered i.v. keeping the vial in ice. Compound **3b** was dissolved and administered like PTX at room temperature.

Cell lines and growth conditions. The human ovarian carcinoma IGROV-1 cell line,[1] the cisplatin-resistant IGROV-1/Pt1 subline[2] and the human large cell lung H460 carcinoma cell line (ATCC, HTB-177) were cultured in RPMI-1640 medium; the human osteosarcoma U2-OS cell line (ATCC, HTB-96) was grown in Mc Coy's 5A medium. Medium was supplemented with 10% fetal calf serum.

Cell sensitivity to drugs. The cell sensitivity to antitumor agents was measured by using the growth-inhibition assay based on cell counting. Cells were seeded in duplicates into 6-well plates and exposed to drug 24 h later. After 72 h of drug incubation, cells were harvested for counting with a cell counter. IC_{50} is defined as the drug concentration producing 50% decrease of cell growth. At least three independent experiments were performed.



Cell sensitivity to the compounds.

The cell sensitivity was assessed after 72h of incubation with each compound. Paclitaxel was used as reference compound. A representative experiment for each cell line is shown. SD was below 10% for each data point.

Antitumor activity studies. All experiments of antitumor activity were carried out using female athymic Swiss nude mice, 8-10 weeks-old (Charles River, Calco, Italy). Mice were maintained in laminar flow rooms keeping temperature and humidity constant. Mice had free access to food and water. Experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan according to institutional guidelines. The IGROV-1/Pt1 human tumor xenograft, derived from cultures of the ovarian carcinoma cell line,[2] was used. Exponentially growing cells (10^7 /mouse) were s.c. injected into the right flank of athymic nude mice and the tumor line was achieved by serial s.c. passages of fragments of regrowing tumors into healthy mice. Groups of five mice bearing bilateral s.c. tumors were employed. Tumor fragments were implanted on day 0 and tumor growth was followed by biweekly measurements of tumor diameters with a Vernier caliper. Tumor volume (TV) was calculated according to the formula: TV (mm^3) = $d^2 \times D/2$ where d and D are the shortest and the longest diameter, respectively. Drugs were delivered i.v. and administered every 4 days for 4 times (q4d×4). Treatment started three days after tumor implant, when tumors were just palpable. The efficacy of the drug treatment was assessed as tumor volume inhibition percentage (TVI%) in treated versus control mice, calculated as: TVI% = 100-(mean TV treated/mean TV control × 100), and complete regression rate (CR), i.e. disappearance of tumor lasting at least ten days after the end of treatment; log cell kill, evaluated by the formula: LCK= (T-C)/3.32×DT, where T and C are the times (days) to reach a tumor volume in treated and control mice, respectively, and DT is the doubling time of control tumors. Tumor DT (days) was obtained from semi log best-fit curves of mean tumor volumes in untreated control mice plotted against time. The toxicity of the drug treatment was determined as body weight loss and lethal toxicity. Student t test (two tailed) exact test was used for statistical comparison of tumor volumes in mice.

1. Benard, J., Da Silva, J., De Blois, M. C., Boyer, P., Duvillard, P., Chiric, E., and Riou, G. (1985) Characterization of a human ovarian adenocarcinoma line, IGROV1, in tissue culture and in nude mice. *Cancer Res*. *45*, 4970-4979.
2. Perego, P., Giarola, M., Righetti, S. C., Supino, R., Caserini, C., Delia, D., Pierotti, M. A., Miyashita, T., Reed, J. C., and Zunino, F. (1996) Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. *Cancer Res*. *56*, 556-562.