Supporting Information

Identification of 3-Hydroxy-2-(3-Hydroxyphenyl)-4*H*-1-Benzopyran-4-ones as Isoform-Selective PKC-ζ Inhibitors and Potential Therapeutics for Psychostimulant Abuse

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In vitro Kinase Assay

After incubating purified PKC-ζ (Millipore) with 5 μM (final concentration) of heterocyclic small molecule libraries and additional PKC reaction components (50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 100 μM Na_3VO_4, 100 μM $Na_4P_2O_7$, 1 mM NaF, 100 μM PMSF, 2 μg phosphatidylserine, and 40 μM PKC substrate), 50 μM-ATP (final concentration) was added to initiate the reactions (50 μL total reaction volume). After 30-min incubation at 30 °C, 50 μl of Kinase-Glo Plus Reagent (Promega) was added to each well. The plate was mixed and incubated at room temperature for 10 min. The luminescence signal was measured on a Perkin Elmer Victor Multilabel Plate Reader. Chelerythrine, a potent PKC-ζ inhibitor, was used in the assay as a positive control.

Cellular Viability Assay

Cos-7 cells were seeded in 48-well culture plates (2×10^4 per well), 24 h later cells were treated with drugs (15 μM) or vehicle control (DMSO) for 24 and 48 h in DMEM supplemented with 10% serum. At the end of the incubation period, the survival of cells was determined by the MTT assay, as per manufacturer's recommendation (Cayman Biochemicals, Ann Arbor, MI). Briefly, 10% volume of MTT was added to each well and samples were incubated for 3 h, culture medium was aspirated and replaced with 100 μL of formazan dissolving solution. Colorimetric intensity was quantified using an ELISA reader at 570 nm. Values of were obtained after subtraction of matched blanks and the ODs of vehicle controls were taken as 100% and values for drug treatment are expressed as % of control.

PKC Immunoprecipitation Enzymatic Assay

One million Cos-7 cells were plated on 10 cm tissue culture dishes and allowed to attach for 6 h. Cells were then infected with adenovirus harboring the human PKC-ζ gene at an moi of 10 in complete DMEM. After 3 h, media was replaced with fresh complete DMEM and cells were allowed to grow an additional 21 h. PKC-ζ expressing Cos-7 cells were subsequently harvested by scraping and incubated for 30 min at 4 °C in lysis buffer A [20 mM HEPES (pH 7.4), 10 mM EDTA, 125 mM NaCl, 1 mM DTT, 1 mM sodium orthovanadate, 0.5 mg/ml benzamidine,1% NP40] in the presence of protease inhibitors [0.1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 2.5 μg/ml pepstatin, 5 μg/ml leupeptin]. Samples were centrifuged at 12,000 × *g* for 15

min, and 10 mg of supernatant was used for immunoprecipitation using 1 μg of PKC-ζ polyclonal antibody (C-20 epitope; Santa Cruz) and 20 μL of protein A-Sepharose beads (Roche). PKC-containing immunoprecipitates were washed three times in buffer A, followed by incubation at 32 °C for 15 min in a total volume of 40 μL with buffer B [0.5 mM EGTA, 12.5 mM $MgCl_2$, 20 mM HEPES (pH 7.5)] containing test compounds (15 μM), 30 μg of PKC-ζ specific substrate (MBP4-14; Calbiochem), and 10 μCi [γ-^{32}P] ATP (1.5 μCi/tube). Samples were immobilized onto P81 phosphocellulose paper and washed extensively in 0.75% phosphoric acid buffer and retained radioactivity was quantitated by liquid scintillation.

Docking Simulation

The structures for the compounds were energy minimized and conformational analysis was carried out using the Catalyst ConFirm algorithm implemented in the Accelrys Discovery Studio 2.1 suite from Accelrys Inc. (San Diego, CA, www.accelrys.com). Docking studies were also performed using the LigandFit programs implemented in the Accelrys Discovery Studio 2.1. In the current docking studies using LigandFit, the binding site was generated from the co-crystallized ligand within the targeted protein from the protein data bank (code : 1ZRZ).[1] The co-crystallized structure of PKC-ι and BIM1 was used as a reference structure for docking studies because of the similarity with PKC-ζ.[1] LigandFit parameters were set to the default values. Interaction energies were estimated using the Drieding force field with a non-bonded interaction cutoff distance of 10.0 Å and a distance dependent dielectric constant. A maximum of 10 poses with interaction energies $\leq$ 20.0 kcal/mol and RMS similarity threshold of 1.5 Å for each compound were saved. Each of optimal poses was evaluated and prioritized according to the DockScore function based on the Piecewise Linear Potential function (PLP).

QM/MM Calculation

The algorithm used the 1ZRZ.pdb[1] file from the PDB repository. Only two water molecules (those closest to the active site) from the X-ray crystal structure were included. All other water molecules were removed. Hydrogen atoms were added and side chain flips of GLN, HIS, and ASN were corrected with molprobity.[2] 3D structures of the inhibitors were built using SMILES as input to the BALLOON program[3] that creates 3-D conformers of each ligand. The geometry of each conformer was optimized using the semi-empirical AM1 method, as implemented in

CHARMM.[4] The optimized geometry was then superimposed on the BIM1 original ligand located in the 1ZRZ.pdb active site. Two orientations were examined for each conformer (**Fig. S1)**. In CHARMM, the position of the protein hydrogen atoms were energy minimized (while all other atom positions were constrained). Next, the atomic positions of the inhibitor conformer were geometry optimized based on AM1 energies (while all other protein atoms were constrained). Throughout the calculation, the protein was described using the CHARMM force field, the inhibitor with an AM1 Hamiltonian, and the water atoms with the TIP3 force field.

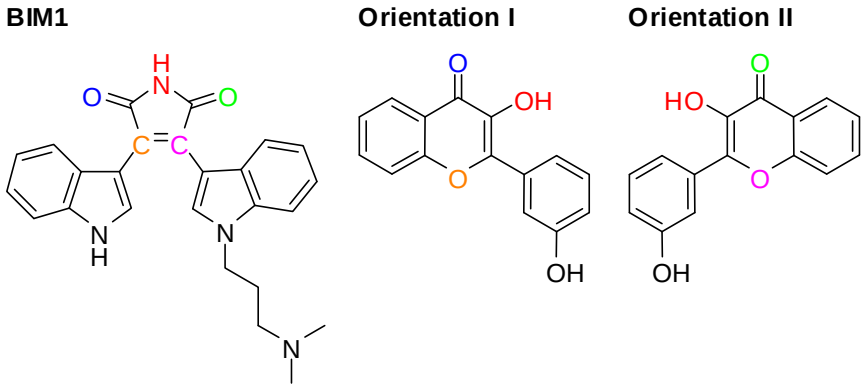
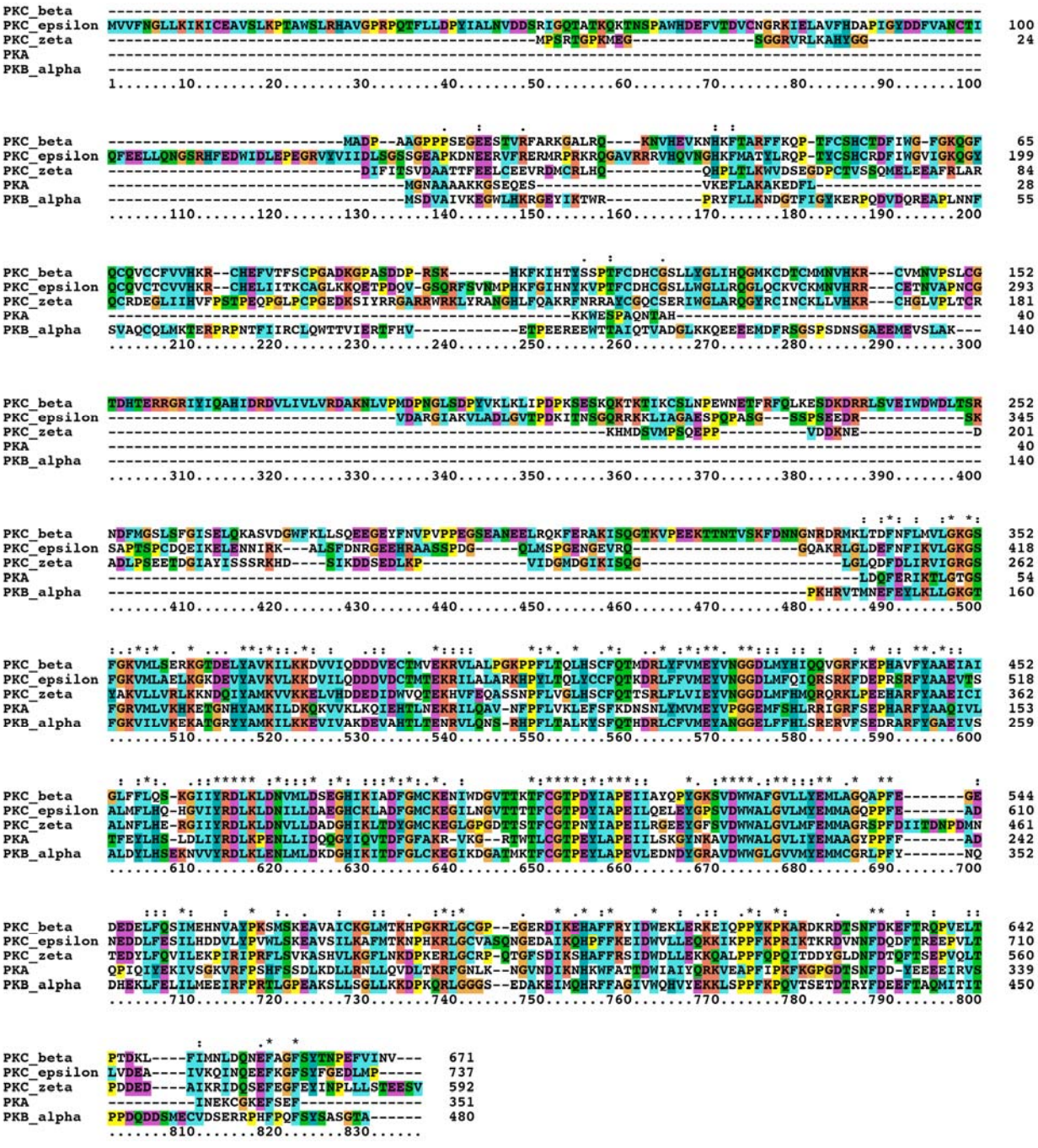


Fig. S1 The two poses were tested for each of **1**–**3** in the QM/MM analysis. Atoms were overlapped based on color coding, for example, the 4-keto group (C=O) was placed on top of the BIM1 oxygen with the same color. For simplicity, shown here is only compound **2.**

References

1. A. Messerschmidt, S. Macieira, M. B. Velarde, C. Benda, A. Jestel, T. N. Brandstetter and M. Blaesse, *J. Mol. Biol.*, 2005, **352**, 918.
2. I. W. Davis, L. W. Murray, J. S. Richardson and D. C. Richardson, *Nucleic Acids Res.,* 2007, **35**, W375.
3. M. J. Vainio and M. S. Johnson, *J. Chem. Info. Mod.*, 2007, **47**, 2462.
4. B. R. Brooks, R. E. Bruccoleri, B. D. Olafson, D. J. States, S. Swaminathan and M. Karplus, *J. Comp. Chem.,* 1983, **4**, 187; A. D. MacKerell, Jr., B. Brooks, C. L. Brooks, III, L. Nilsson, B. Roux, Y. Won and M. Karplus, in *The Encyclopedia of Computational Chemistry*, eds. P. v. R. Schleyer et al., John Wiley & Sons, Chichester, 1998, vol. 1, pp. 271–277.

ClustalW2 Multiple Sequence Alignment



1. Database Accession Number

   PKC-β2: GenBank X07109; PKC-ε: GenBank X65293; PKC-ζ: GenBank BC014270; PKA: GenBank X07767; PKB-α: SWISS-PROT P31749

2. The following symbols denotes the degree of conservation observed in each column:
   - "*" means that the residues or nucleotides in that column are identical in all sequences in the alignment.
   - ":" means that conserved substitutions have been observed, according to the COLOR table below.
   - "." means that semi-conserved substitutions are observed.
3. The red color is used for the small and hydrophobic amino acids, AVFPMILW; the blue color is used for acidic amino acids, DE; the magenta color is used for basic amino acid, RK; and the green color is used for the rest amino acids, STYHCNGQ.

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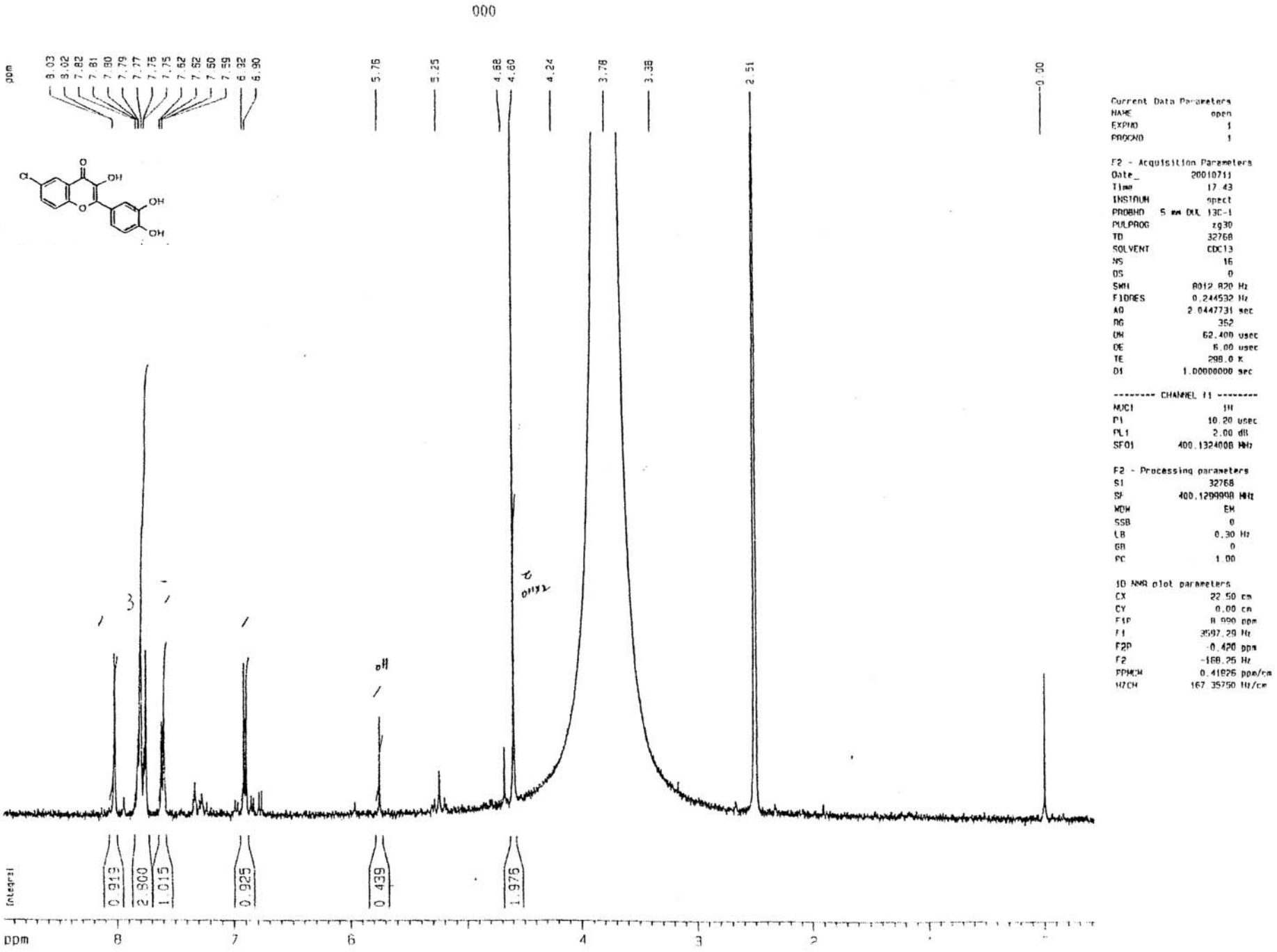
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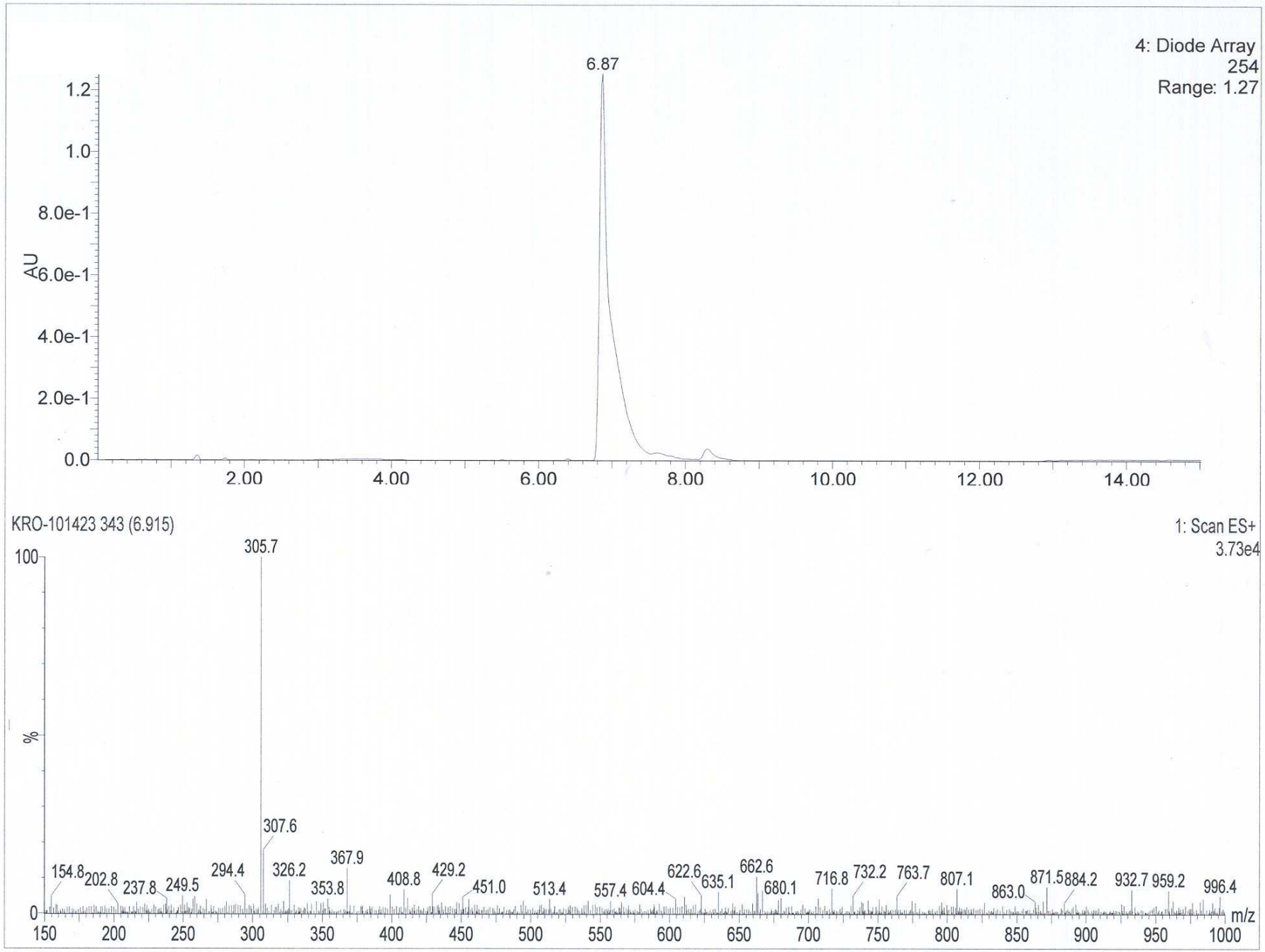

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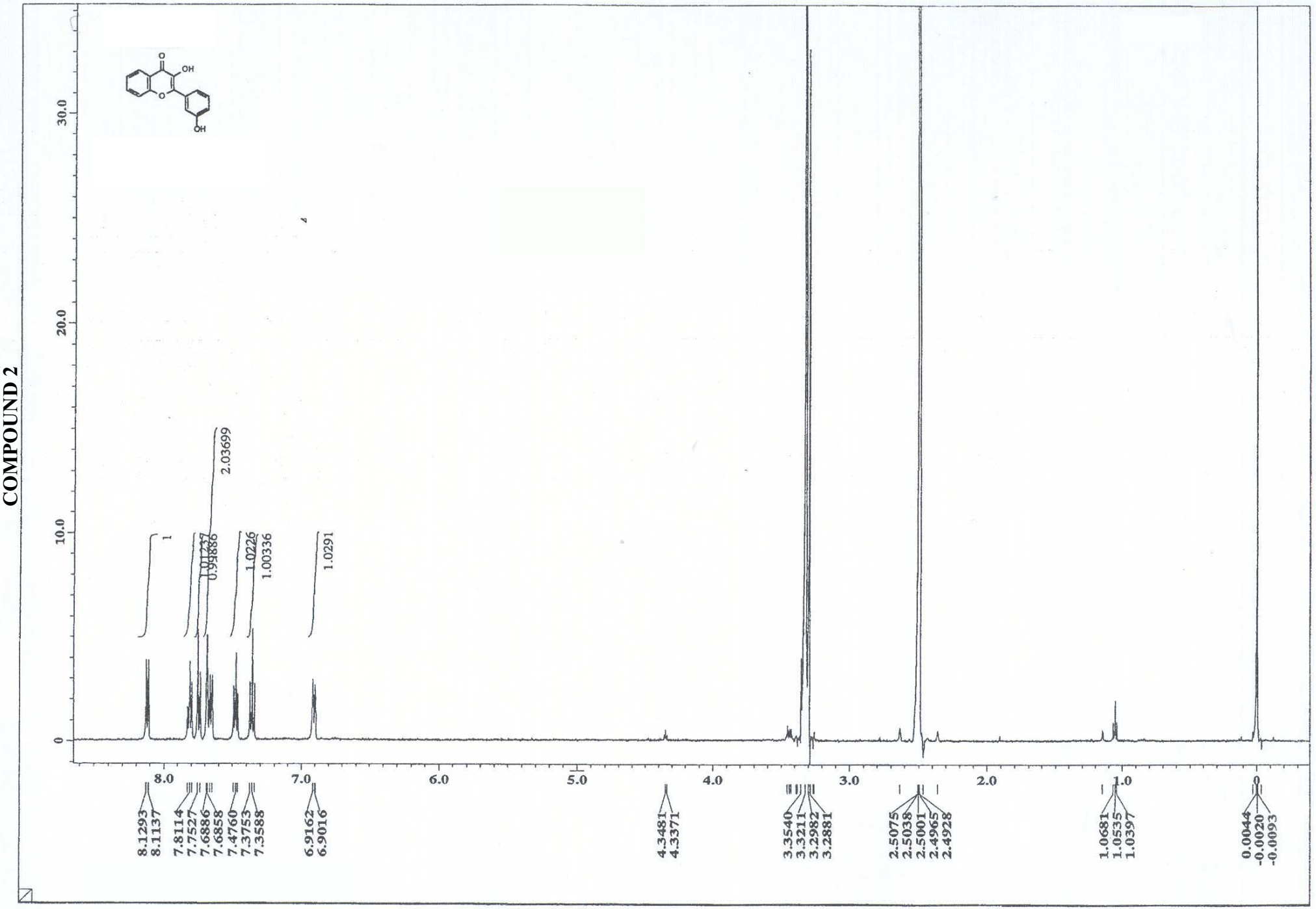
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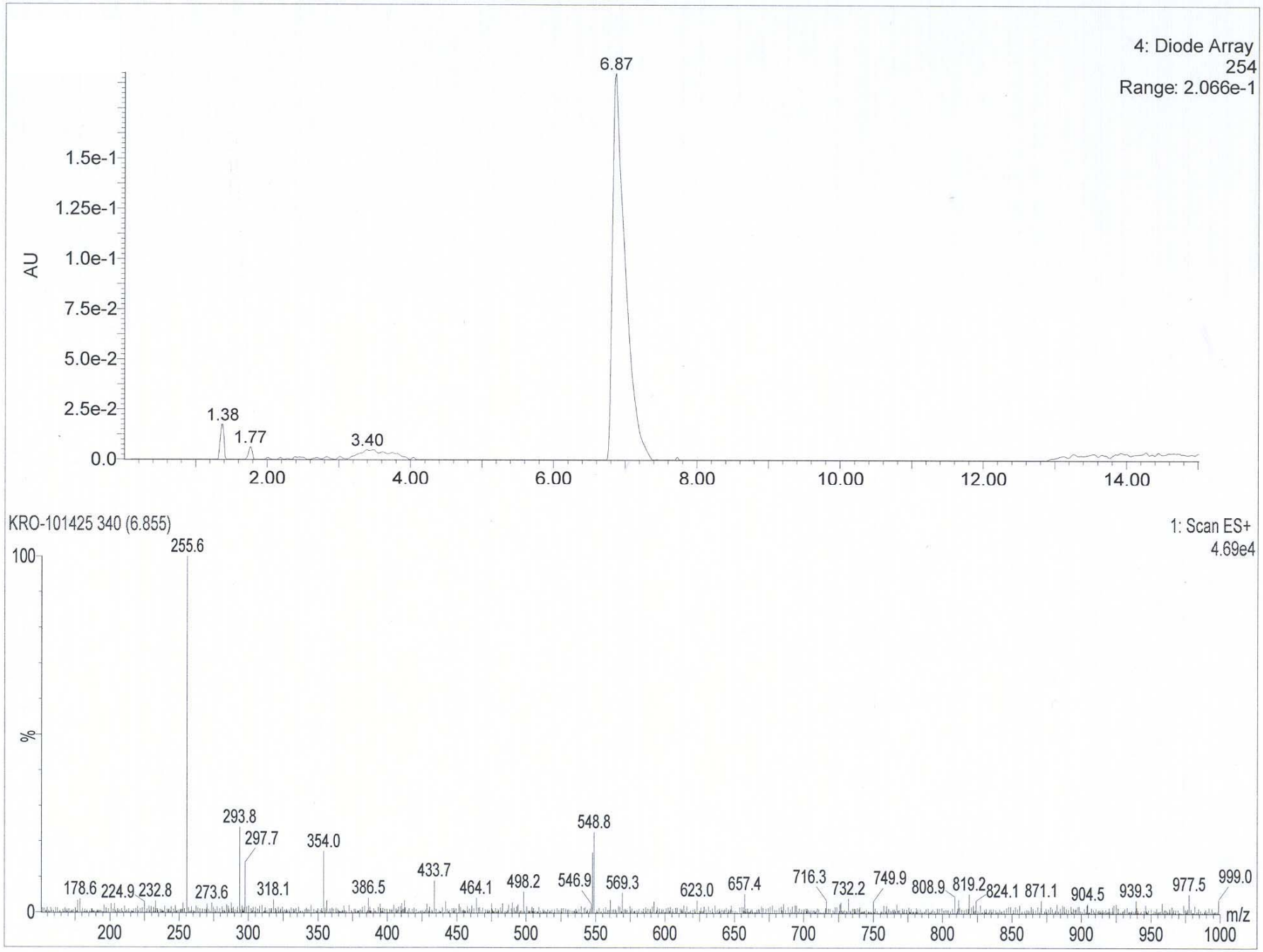
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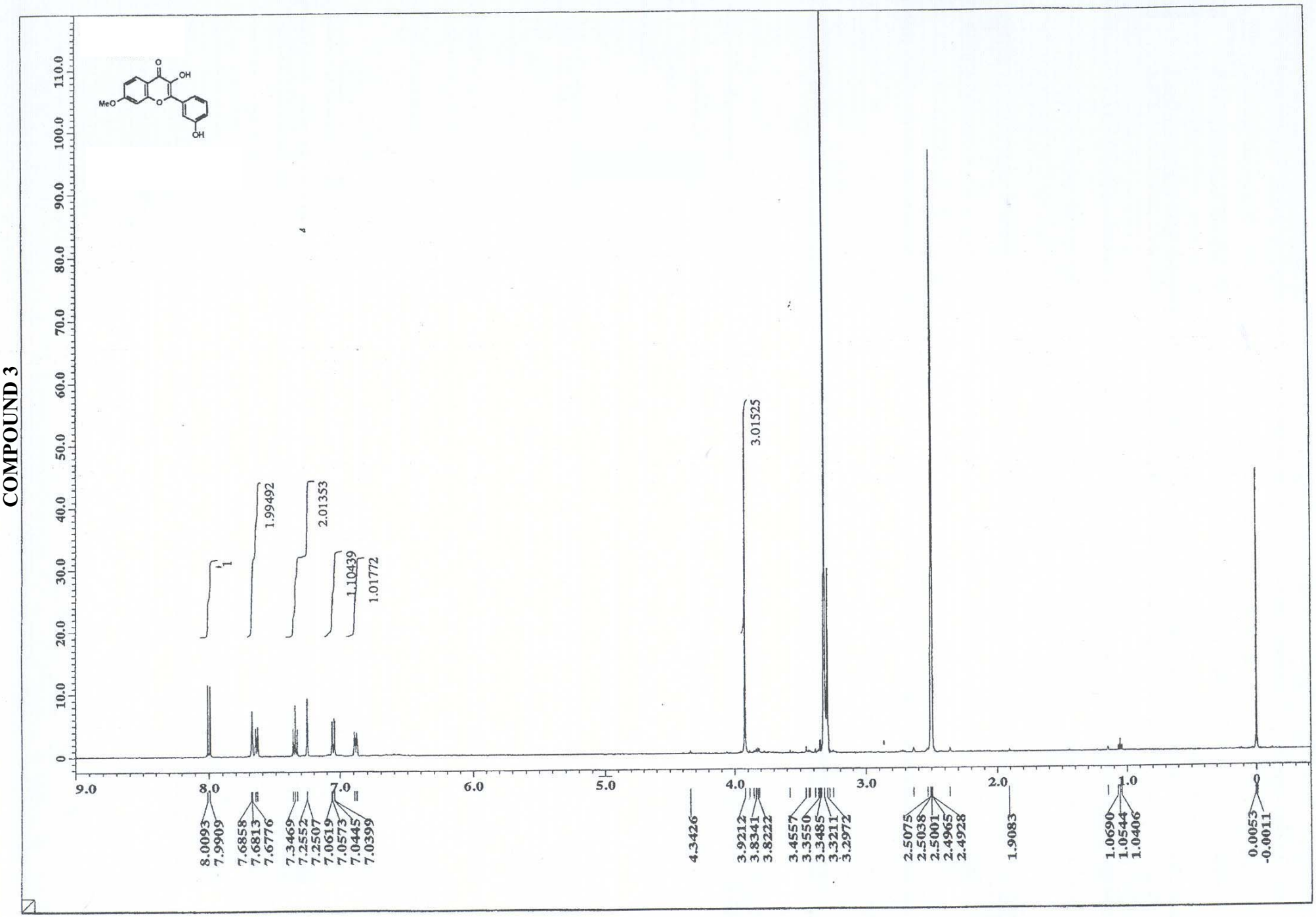
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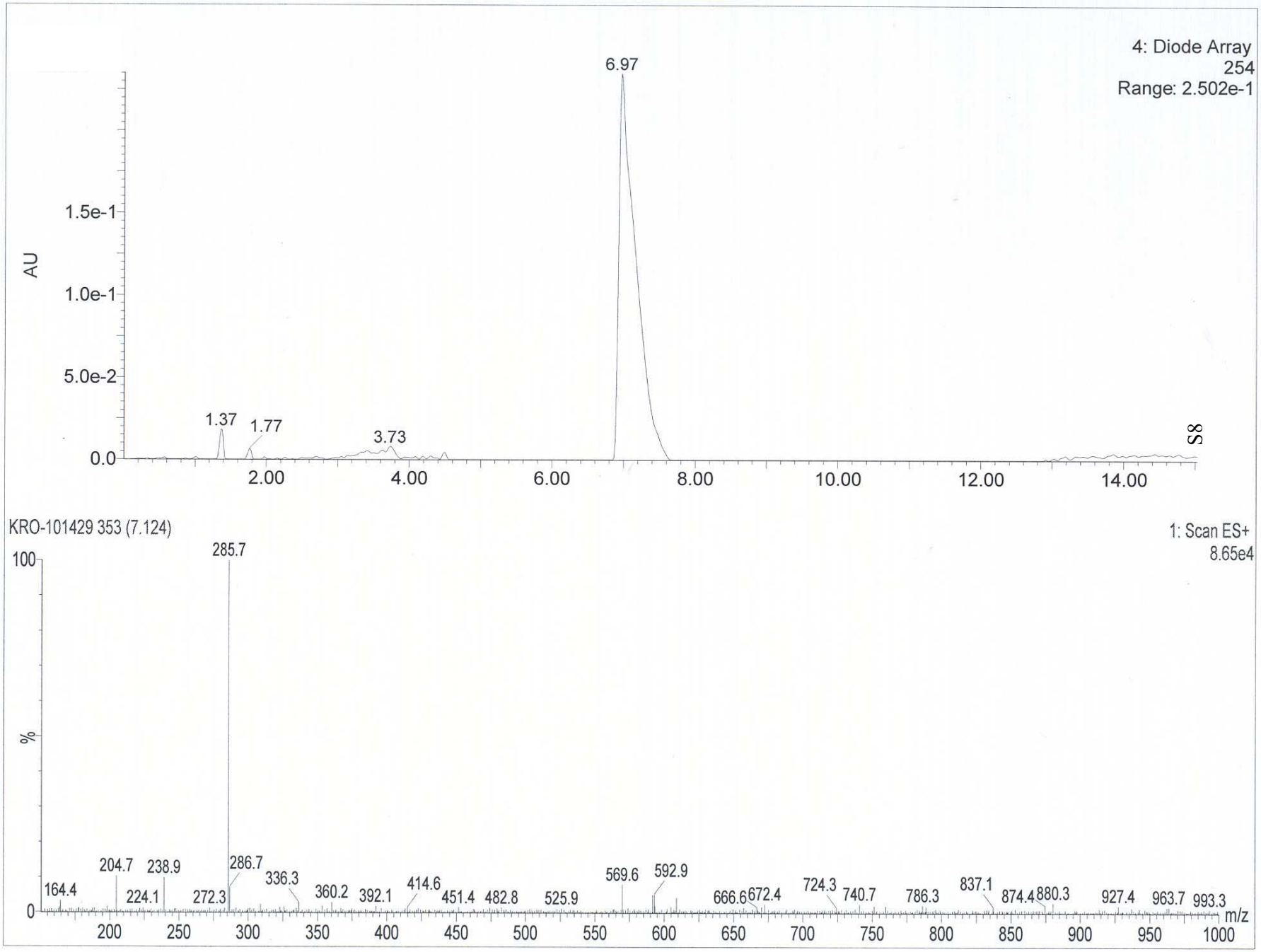
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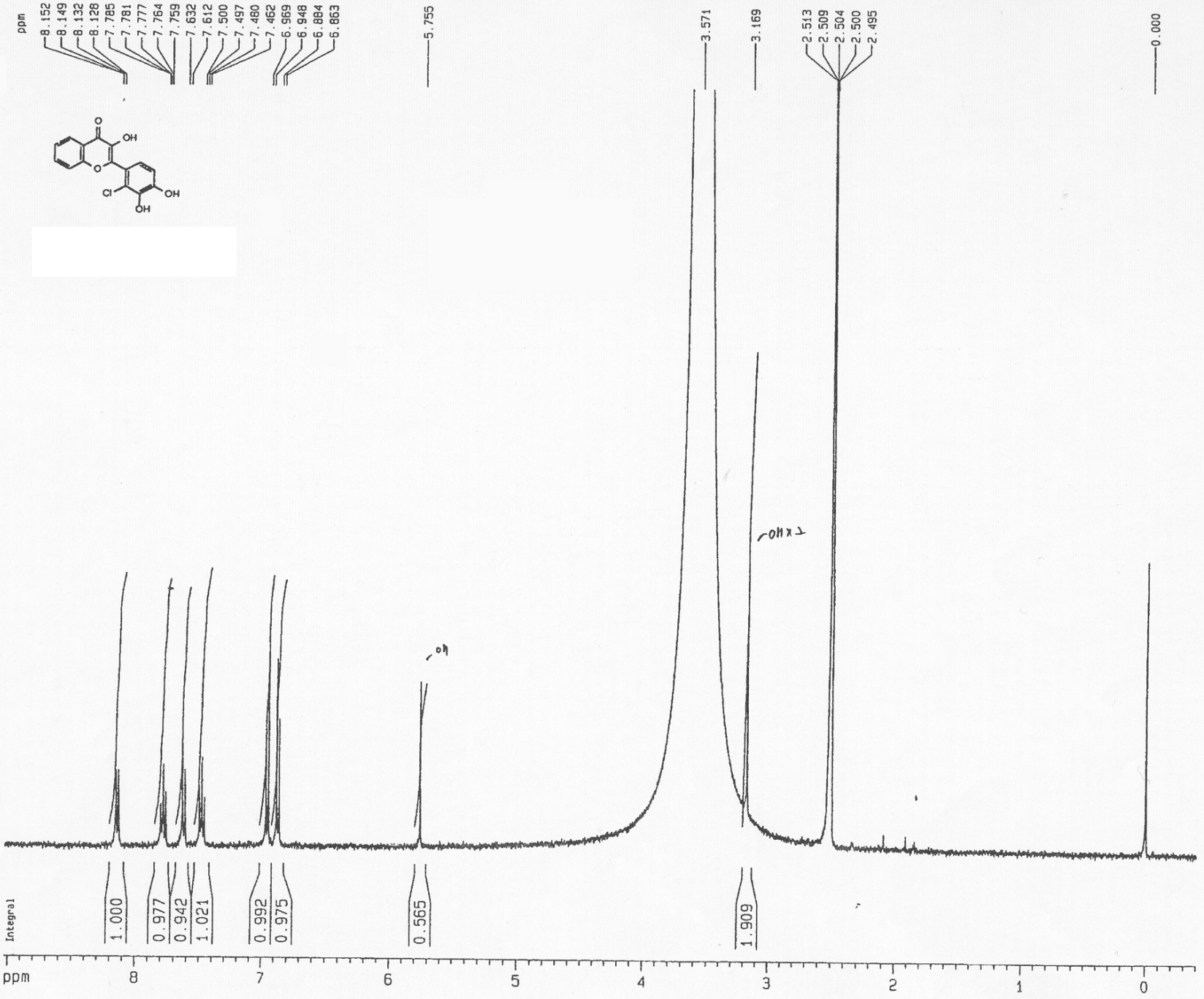

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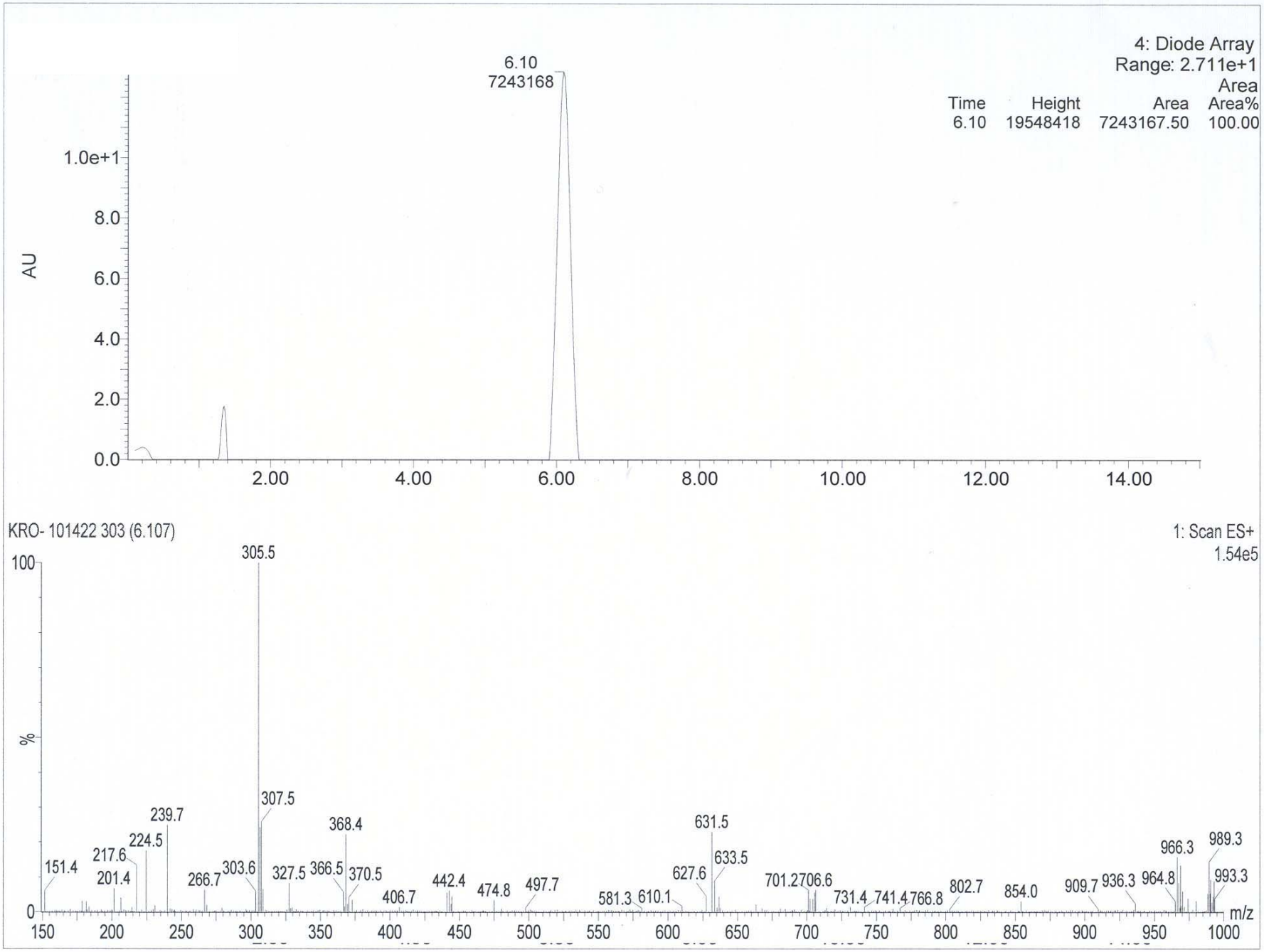
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