Supplementary Information for:

Cyclic peptide-based potent human SIRT6 inhibitors

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Contents:

Experimental

General. The following materials were obtained from commercial sources for the compound preparation, and were used as received without further treatment. Sigma-Aldrich China: N-methylmorpholine (NMM), trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF), Rink Amide resin, hydrazine, Lawesson's reagent, triethylamine, tetrahydrofuran (THF); TCI Shanghai: suberic acid, succinic acid, myristic acid, tartaric acid, Fmoc-OSu, 1,4-dioxane; Alfa Aesar China: 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBt), phenol, thioanisole, ethanedithiol, 1-dodecyl isothiocyanate, 1-tetradecyl isothiocyanate, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC-HCl), N,N-diisopropylethylamine, $NaHCO_3$, Na_2CO_3, anhydrous Na_2SO_4, LiOH; Honeywell China: acetonitrile, dichloromethane (DCM), MeOH, ethyl acetate; Sinopharm Chemical Reagent Co., Ltd.: piperidine, acetic anhydride, diethyl ether; Shanghai Jianglai Co., Ltd. (http://jianglai.company.lookchem.cn): Boc-Lys-OMe • HCl; Shanghai Plus Bio-Sci & Tech Co., Ltd.: MBHA resin; GL Biochem (Shanghai) Ltd.: 2-chlorotrityl chloride resin; The N^{α}-Fmoc-protected amino acids were purchased from TCI Shanghai, GL Biochem (Shanghai) Ltd., or Shanghai Plus Bio-Sci & Tech Co., Ltd.

Routine unit-resolution mass spectrometry was performed on a Thermo LXQ LC-ion trap mass spectrometer at Jiangsu University. High-resolution mass spectrometry (HRMS) was performed on an AB 5600+ Q TOF high-resolution mass spectrometer at the Pharmacy School of Fudan University.

The following materials were obtained from commercial sources for the sirtuin inhibition assay and the pronase digestion assay, and were used as received without further treatment. Sigma-Aldrich China: the active human recombinant His_6-SIRT1, Hepes, Trizma, β-NAD^+, a 1.0

M solution of $MgCl_2$ (molecular-biology grade), the pronase from *Streptomyces griseus*; Cayman Chemical: the active human recombinant GST-SIRT1, the active human recombinant His_6-SIRT2, the active human recombinant His_6-SIRT3, the active human recombinant GST-SIRT5, the active human recombinant His_6-SIRT6; TCI Shanghai: DL-Dithiothreitol (DTT); Alfa Aesar China: NaCl, KCl.

The peptide substrates prepared and used in the sirtuin inhibition assay were: the SIRT1/2/3 substrate H_2N-HK-[N^{ε}-acetyl-lysine]-LM-COOH corresponding to amino acids 380-384 of the human p53 protein acetylated at K^{382}; the SIRT5 substrate CH_3CONH-AR-[N^{ε}-succinyl-lysine]-ST-$CONH_2$ corresponding to amino acids 7-11 of the human histone H3 protein succinylated at K^9; the SIRT6 substrate H_2N-EALPK-[N^{ε}-myristoyl-lysine]-TGGPQ-$CONH_2$ corresponding to amino acids 15-25 of the human tumor necrosis factor α (TNFα) myristoylated at K^{20}.

Synthesis of 4-7 (Scheme 1). These compounds were prepared by the Fmoc chemistry-based manual solid phase peptide synthesis (SPPS) on the Rink amide resin. For each amino acid coupling reaction, 4 equivalents of a N^{α}-Fmoc-protected amino acid, 3.8 equivalents of the coupling reagent HBTU and the additive HOBt were used in the presence of 0.4 M NMM/DMF, and the coupling reaction was allowed to proceed at room temperature for 1h. A 20% (v/v) piperidine/DMF solution was used for Fmoc removal. After the completion of the on-resin amino acid assembling and the N-terminal α-amino group acetylation with acetic anhydride in the presence of 0.4 M NMM/DMF, the side chain Mtt protecting group from two Lys(Mtt) residues were selectively removed with a 1% (v/v) TFA/DMF solution before the two exposed free amino groups were acylated with Fmoc-glycine under peptide coupling reaction condition. After the Fmoc removal from the two incorporated Fmoc-glycine residues with a 20% (v/v) piperidine/DMF solution, the two newly exposed free amino groups were then acylated at

room temperature for 1h with a diacid (suberic acid or succinic acid) under peptide coupling reaction condition. The corresponding resin-bound cyclized peptides thus obtained were then treated with a 2% (v/v) solution of hydrazine (NH_2NH_2) in DMF, the now exposed free amino group at the central position was then reacted with 1-dodecyl isothiocyanate or 1-tetradecyl isothiocyanate, completing the synthesis of the resin-bound cyclic pentapeptides. The final treatment with reagent K (83.6% (v/v) TFA, 5.9% (v/v) phenol, 4.2% (v/v) ddH_2O, 4.2% (v/v) thioanisole, 2.1% (v/v) ethanedithiol) at room temperature for 4h cleaved the crude **4**-**7** from the resin and removed the side chain Pbf and ^{t}Bu protecting groups as well. Following the concentration of the cleavage filtrate and the precipitation of the crude **4**-**7** in cold diethyl ether, the crude **4**-**7** were purified by reversed-phase high pressure liquid chromatography (RP-HPLC) on a semi-preparative C18 column (1 x 25 cm, 5 μm). The column was eluted with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 4.5mL/min and was monitored at 214 nm. The pooled HPLC fractions were concentrated *in vacuo* to remove acetonitrile and the remaining aqueous solution was lyophilized to give all the compounds as puffy white solids. The purified **4**-**7** were >95% pure based on RP-HPLC analysis on an analytical C18 column (0.46 x 25 cm, 5 μm). The exact masses of the purified **4**-**7** were confirmed by high-resolution mass spectrometry (HRMS) analysis (see **Table 1**).

Synthesis of 8 and **9 (Scheme 2).** These two compounds were prepared in the same manner as that of compounds **4**-**7** (see above), with the exception of the lack of incorporation of two glycine residues in compounds **8** and **9**. The crude **8** and **9** were also purified by semi-preparative RP-HPLC as above described. The purified **8** and **9** were also >95% pure based on RP-HPLC analysis on an analytical C18 column (0.46 x 25 cm, 5 μm). The exact masses of the purified **8** and **9** were also confirmed by HRMS analysis (see **Table 1**).

Synthesis of 1 (Scheme S1). (a) To a stirred, ice-cooled mixture of Boc-Lys-OMe • HCl (296.8 mg, 1.0 mmol), myristic acid (251.2 mg, 1.1 mmol) in DCM (15 mL) was added triethylamine (139.4 μL, 1.0 mmol), followed by the addition in small portions of EDC-HCl (210.9 mg, 1.1 mmol). After the addition was complete, the reaction mixture was stirred at room temperature overnight before the addition of ddH_2O (15 mL). The organic layer was isolated and the aqueous layer was extracted once with DCM (15 mL). The combined organics were washed successively with 10% (w/w) tartaric acid (2 x 15 mL), 1.0 M aqueous $NaHCO_3$ solution (2 x 15 mL), ddH_2O (2 x 15 mL), and brine until the washing became neutral; and were then dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. To the resulting residue was then added at 0 ^oC LiOH (211.0 mg, 1.0 mmol), ddH_2O (4.2 mL), and MeOH (12 mL), and the reaction mixture was stirred at 4 ^oC for 2 days. The reaction mixture was neutralized at 0 ^oC with TFA and concentrated under reduced pressure, which was followed by the lyophilization of the aqueous residue. To the lyophilized residue was subsequently added TFA (5 mL) at 0 ^oC and the reaction mixture was stirred at room temperature for 3h before being concentrated under reduced pressure. The resulting residue was then dissolved in ddH_2O (5 mL) and the solution was neutralized with a 10% (w/v) aqueous Na_2CO_3 solution before the addition of another batch of the 10% (w/v) aqueous Na_2CO_3 solution (5 mL). To the resulting solution was subsequently added dropwise at 0 ^oC a solution of Fmoc-OSu (675 mg, 2.0 mmol) in 1,4-dioxane (5 mL). After the addition was complete, the reaction mixture was stirred at room temperature for 5h before the addition of ddH_2O (50 mL). Following the removal of the excess Fmoc-Osu with diethyl ether extraction (2 x 100 mL) and the acidification at 0 °C of the aqueous layer with a 6.0 M aqueous HCl solution to pH ~1, the resulting mixture was extracted with ethyl acetate (3 x 100 mL). The combined organics were then dried with anhydrous Na_2SO_4 and concentrated under reduced

pressure. The obtained residue was subsequently dissolved in THF (20 mL), and to the solution was added Lawesson's reagent (404.7 mg, 1.0 mmol) at room temperature and the reaction mixture was then stirred at room temperature overnight. Of note, this step of reaction was performed according to the literature procedure by Lin and co-workers.[1] Following the concentration of the reaction mixture under reduced pressure, the resulting oily residue was further dried under high vacuum and subjected to both unit-resolution and high-resolution mass spectrometric analysis. It was found that compound **1a** (i.e. N^{α}-Fmoc-N^{ε}-thiomyristoyl-lysine) depicted in **Scheme S1** was the predominant species in this material: HRMS (ESI) calcd for $C_{35}H_{51}N_2O_4S$ ($[M+H]^+$) 595.3564; found: 595.3568. Therefore, it was directly used for the following SPPS. **(b)** To 2-chlorotrityl chloride resin (40.8 mg, 0.04 mmol) was added Fmoc-Thr(tBu)-OH (10 mg, 0.025 mmol), N,N-diisopropylethylamine (8.65 μL, 0.05 mmol), and DCM (1.0 mL), and the whole mixture was then stirred for 10 min at 4 ^{o}C and 45 min at room temperature. To the reaction mixture was added MeOH (180 μL) and N,N-diisopropylethylamine (20 μL), and the resulting mixture was stirred at room temperature for 15 min before being filtered. The obtained resin was then washed with DMF (3 x) and DCM (3 x), affording the 2-chlorotrityl chloride resin loaded with Fmoc-Thr(tBu) which was subsequently used for the sequential incorporation of Ser(tBu), N^{ε}-thiomyristoyl-lysine, Arg(pbf), and Ala under standard Fmoc chemistry-based SPPS. After the Fmoc removal from the incorporated Fmoc-Ala, the whole peptidyl resin was treated with a 90% (v/v) TFA solution (TFA/DCM/H_2O=18/1/1, v/v) (1.45 mL) at room temperature for 4h before being filtered. Following the concentration of the filtrate with a stream of N_2 gas in a fuming hood, the crude **1** was precipitated out from cold diethyl ether. The crude **1** was also purified by semi-preparative RP-HPLC as above described. The purified **1** was also >95% pure based on RP-HPLC analysis on an analytical C18 column

(0.46 x 25 cm, 5 μm). The exact mass of the purified **1** was also confirmed by HRMS analysis (see **Table 1**).

Synthesis of 10 (Scheme S2). This synthesis followed the standard Fmoc-chemistry based manual SPPS described above. The orthogonal deprotection of the Mtt protecting group on lysine side chain and the ensuing reaction of the exposed free amino group with 1-tetradecyl isothiocyanate were performed in the same manner as that described above for the synthesis of compounds **4**-**7**. Following the cleavage of the crude **10** from the resin with a 90% (v/v) TFA solution (TFA/DCM/H_2O=18/1/1, v/v) at room temperature for 4h and its precipitation from cold diethyl ether, the crude **10** was also purified with semi-preparative RP-HPLC as described above. The purified **10** was also >95% pure based on RP-HPLC analysis on an analytical C18 column (0.46 x 25 cm, 5 μm). The exact mass of the purified **10** was also confirmed by a HRMS analysis (see **Table 1**).

***In vitro* sirtuin inhibition assay.** The HPLC-based sirtuin inhibition assay that our laboratory has been using over past several years was employed in the current study and was performed as described previously.[2] An assay solution (50 μL) contained the following components: 50 mM Hepes (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, β-NAD^+ (0.5 mM for the SIRT1 and SIRT2 assays, 3.5 mM for the SIRT3 assay, 0.8 mM for the SIRT5 assay, or 0.2 mM for the SIRT6 assay), the peptide substrate (0.3 mM of the above-mentioned SIRT1/2/3 substrate for the SIRT1 assay, 0.39 mM of the above-mentioned SIRT1/2/3 substrate for the SIRT2 assay, 0.105 mM of the above-mentioned SIRT1/2/3 substrate for the SIRT3 assay, 0.88 mM of the above-mentioned SIRT5 substrate, or 0.02 mM of the above-mentioned SIRT6 substrate), one test compound (**1**, **4**, **5**, **6**, **7**, **8**, **9**, or **10**) with varied concentrations including 0, and a sirtuin (His_6-SIRT1 or GST-SIRT1, 320 nM; His_6-SIRT2, 309

nM; His_6-SIRT3, 320 nM; GST-SIRT5, 370 nM; or His_6-SIRT6, 313 nM). It should be noted that the same [S]/Km ratios for both substrates (~3.2 for the peptide substrates and ~5.6 for β-NAD^+) were employed for the inhibition assays with all the five human sirtuins (SIRT1/2/3/5/6) employed in the current study. An enzymatic reaction was initiated by the addition of a sirtuin at 37 °C and was allowed to be incubated at 37 °C for 10 min (for the SIRT1 assay), 12 min (for the SIRT2 assay), 10 min (for the SIRT3 assay), 5 min (for the SIRT5 assay), or 12 min (for the SIRT6 assay) until quenched with the following stop solution: 100 mM HCl and 0.16 M acetic acid. The quenched assay solutions were directly injected into a reversed-phase C18 column (0.46 x 25 cm, 5 μm) which was eluted with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile containing 0.05% (v/v) TFA at 1 mL/min with UV monitoring at 214 nm. Turnover of the limiting substrate was maintained at <10%. Stock solutions of the test compounds were all prepared in ddH_2O. IC_{50} values were estimated from the Dixon plots ($1/v_0$ *vs.*[inhibitor])[3] as an indication of the inhibitory potency.

Pronase digestion assay. This assay was also performed as described previously by our laboratory.[2] Fifty (50) μL of a solution of a test compound (**9**, **10**, or the linear pentapeptide control H_2N-HK-(N^{ε}-acetyl-lysine)-LM-COOH) in ddH_2O (160 μM) was mixed thoroughly with 50 μL of a pronase solution in 100 mM Tris•HCl (pH 7.3) (8 ng/μL), and the resulting solution was incubated at 37 °C until quenched with a solution of acetic acid in ddH_2O (1.0 M) at 0, 1.5, 3, and 6 min (for the control) or at 0, 10, 20, 40, and 80 min (for **9** and **10**). At each time point, 20 μL of a pronase digestion mixture was taken and treated with 40 μL of the 1.0 M acetic acid solution, and the whole mixture was vigorously vortexed, centrifuged, and the supernatant was directly injected into a reversed-phase C18 analytical HPLC column (0.46 x 25 cm, 5 μm). The C18 column was eluted with a gradient of ddH_2O containing 0.05% (v/v) TFA and acetonitrile

containing 0.05% (v/v) TFA at 1 mL/min with UV monitoring at 214 nm. The HPLC peak areas obtained for a given test compound at different time points were used to estimate the percentage remaining for this test compound with the digestion time. The graph of the percentage remaining *versus* time was used to compare the proteolytic stability of different test compounds, as shown in **Figure 4**.

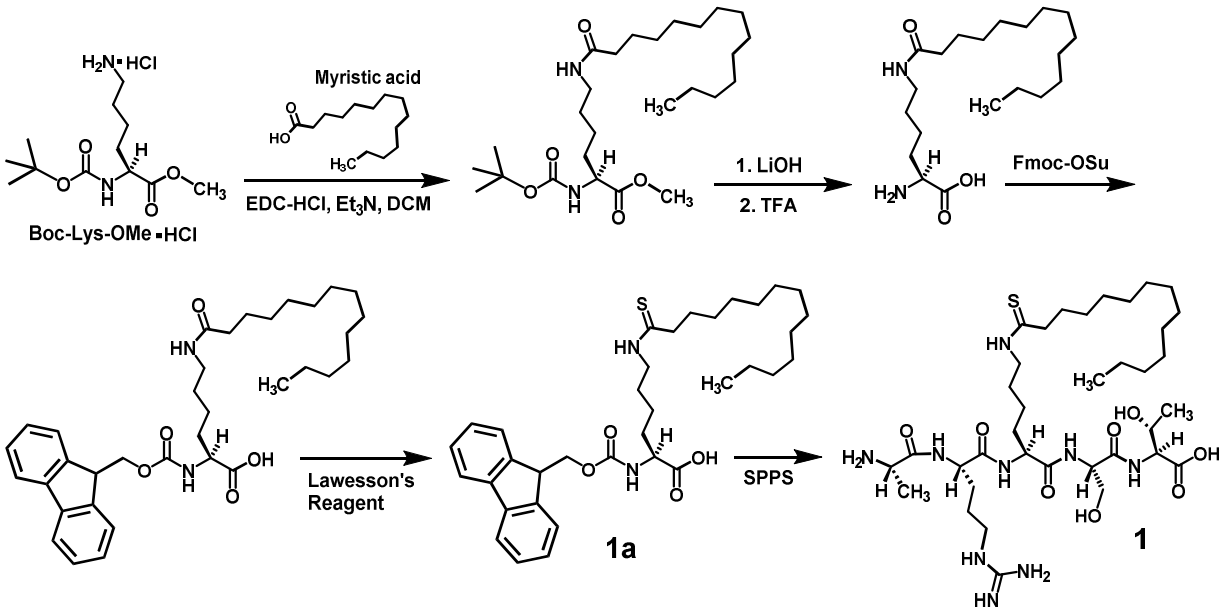
Western blotting analysis. BxPC-3 cells (from Procell, Wuhan, China) were cultured and subsequently treated for 18h with compound **9** (stock solution prepared in ddH_2O) at different concentrations (0, 0.5, 2, 10 μM) before cell lysis and SDS-PAGE. Proteins on a SDS-PAGE gel were subsequently transferred onto a polyvinyl difluoride (PVDF) membrane. After having been blocked, the transblotted membrane was incubated overnight at 4 °C with the following primary antibodies: anti-acetylated H3K9 (from Sigma-Aldrich, United States), anti-histone H3 (from Cell Signaling Technology, United States), anti-GADPH (from ZSGB-BIO, Beijing, China). After the treatment with the above primary antibodies, the transblotted membrane was treated with the goat anti-rabbit IgG-horseradish peroxidase conjugate (from ZSGB-BIO, Beijing, China) for 1h at room temperature, and the immunoblots (for K9-acetylated histone H3 protein, total histone H3 protein, and GADPH) were visualized by chemiluminescence. Band intensities were determined with ImageJ2x 2.1.4.7.

References:

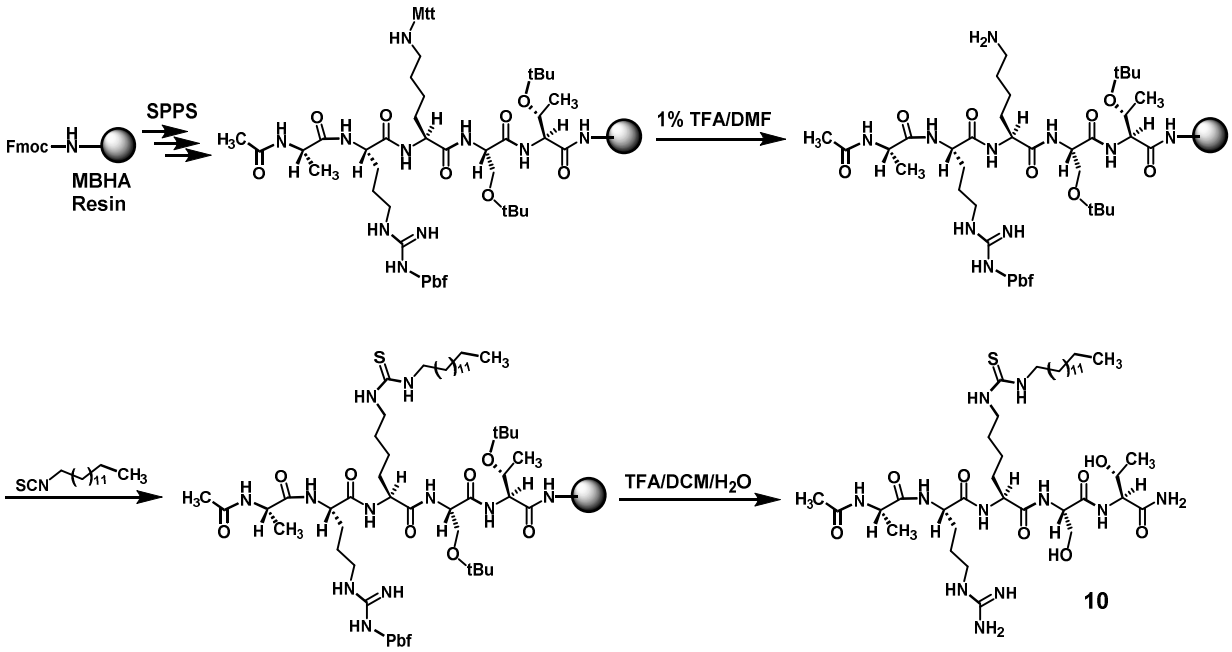
1. B. He, J. Hu, X. Zhang and H. Lin, *Org. Biomol. Chem*., 2014, **12**, 7498.

2. B. M. Hirsch, C. A. Gallo, Z. Du, Z. Wang and W. Zheng, *MedChemComm*, 2010, **1**, 233.

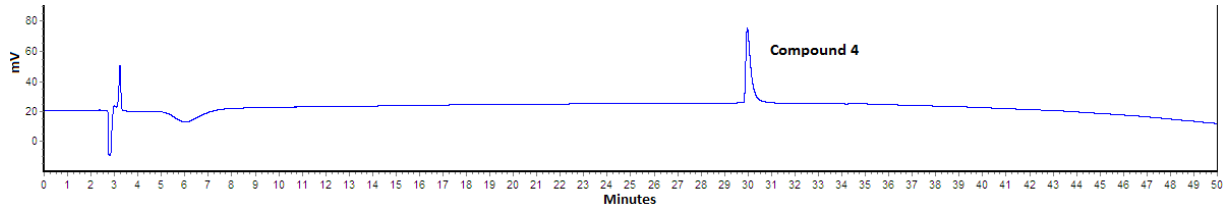
3. M. Dixon, *Biochem. J*., 1953, **55**, 170.



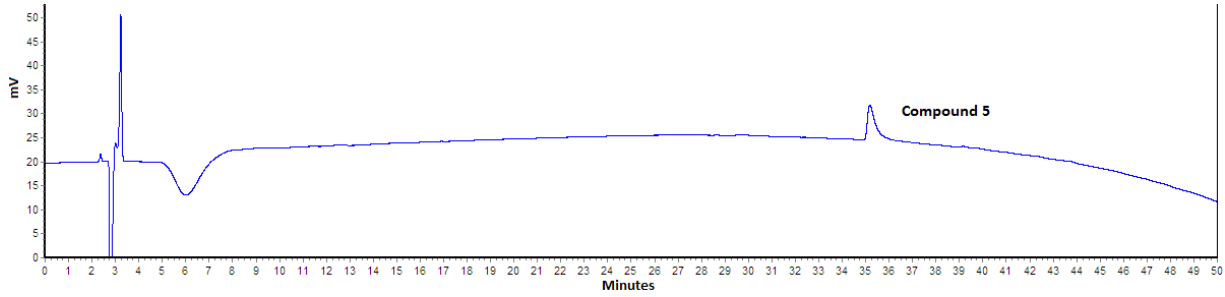
Scheme S1. The synthesis of compound **1**. SPPS, solid phase peptide synthesis.



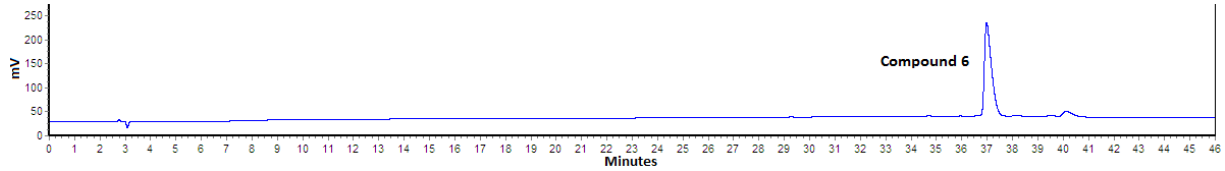
Scheme S2. The synthesis of compound **10**.



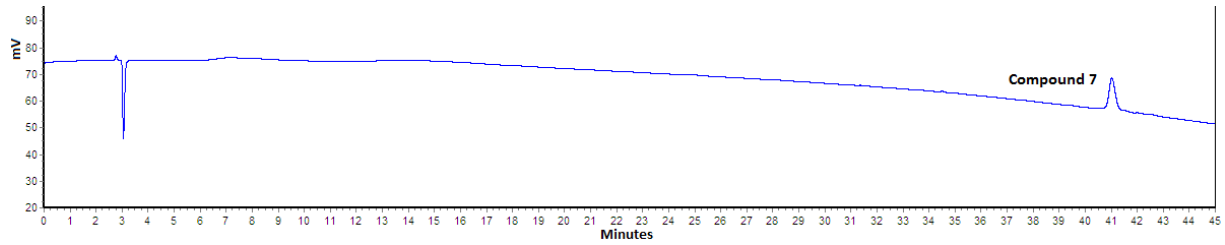
The RP-HPLC analysis of the purified compound **4**.



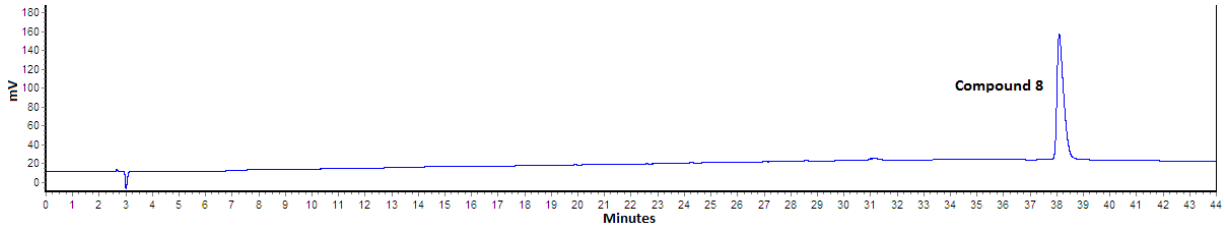
The RP-HPLC analysis of the purified compound **5**.



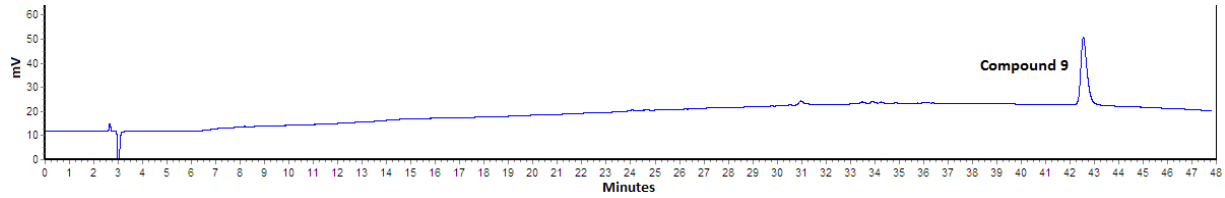
The RP-HPLC analysis of the purified compound **6**.



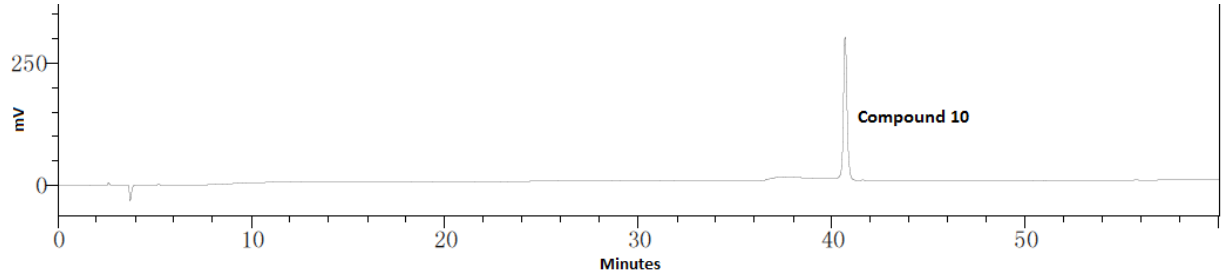
The RP-HPLC analysis of the purified compound **7**.



The RP-HPLC analysis of the purified compound **8**.



The RP-HPLC analysis of the purified compound **9**.



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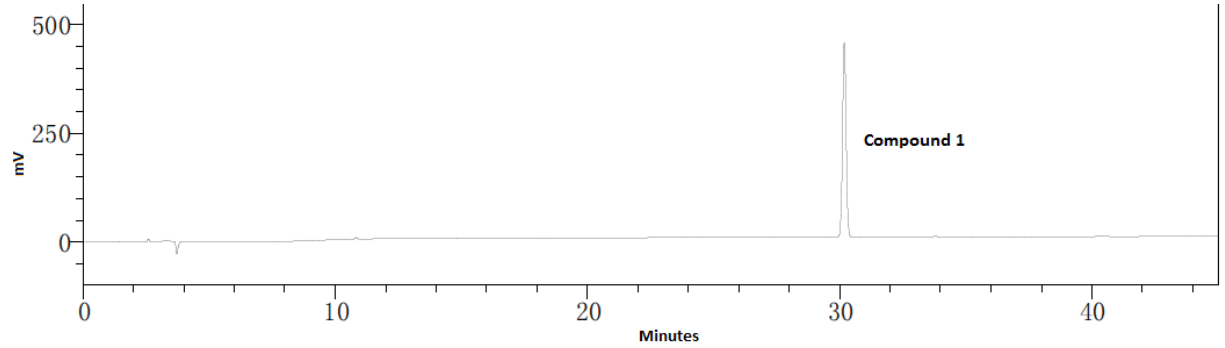
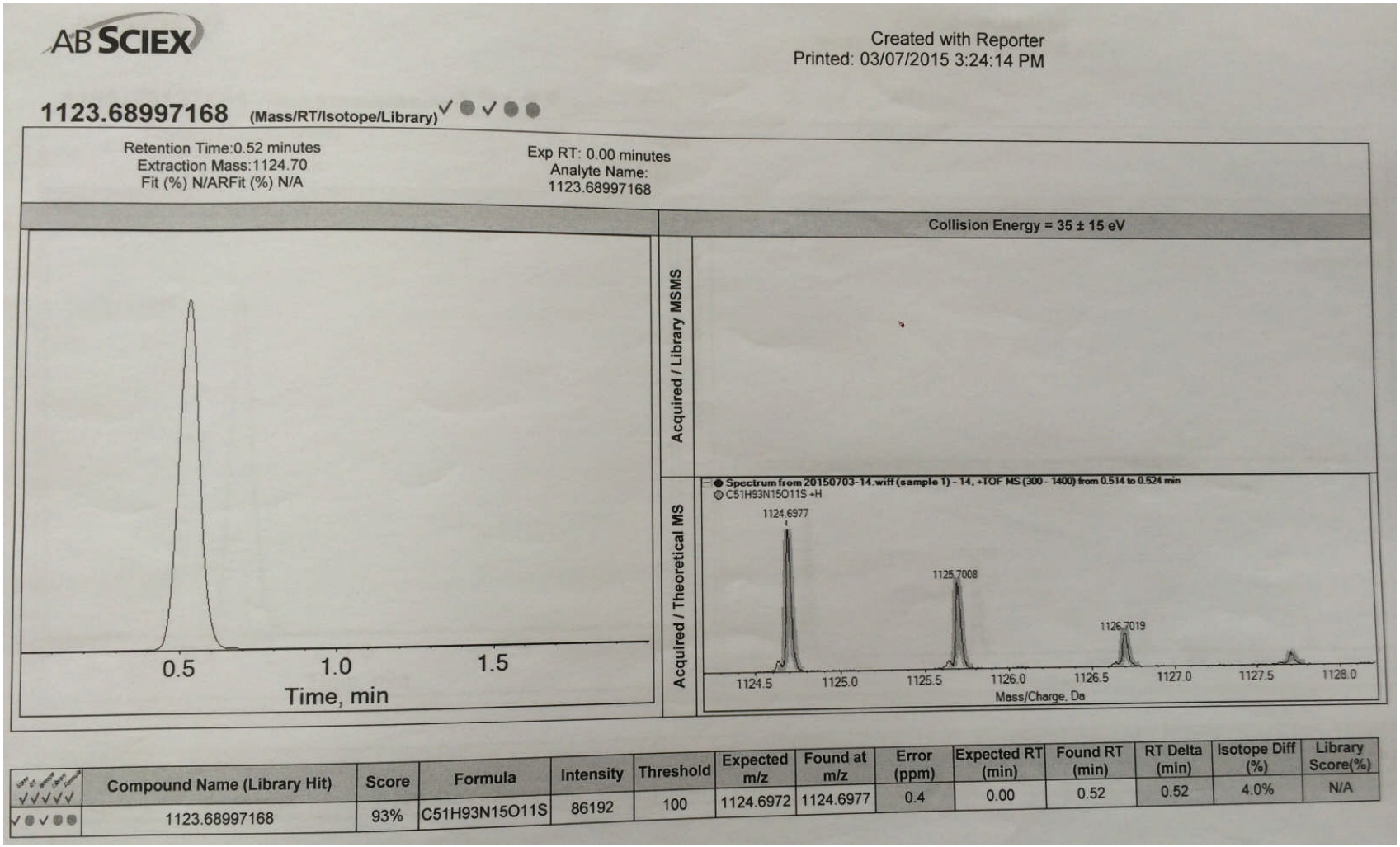
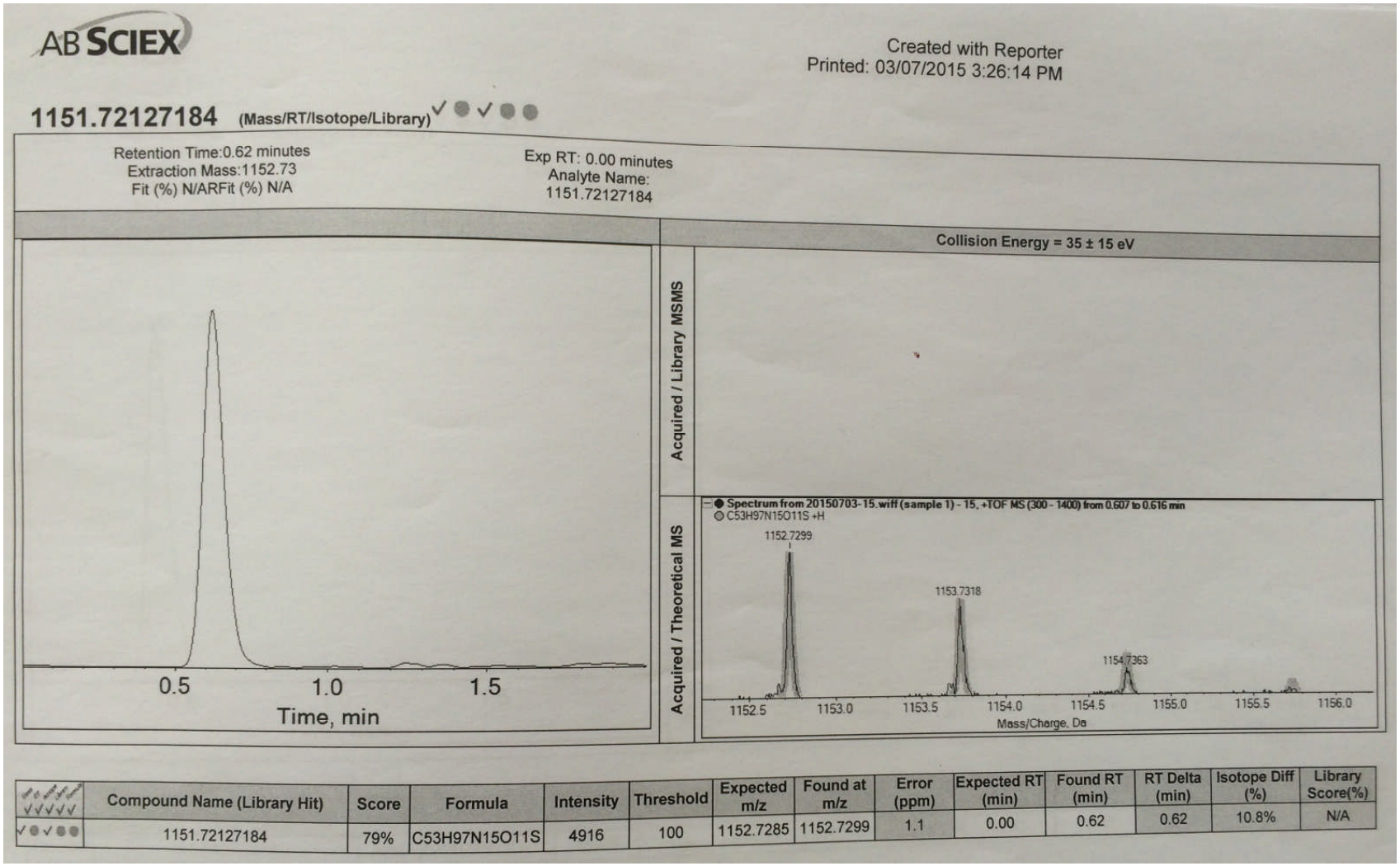


The RP-HPLC analysis of the purified compound **1**.



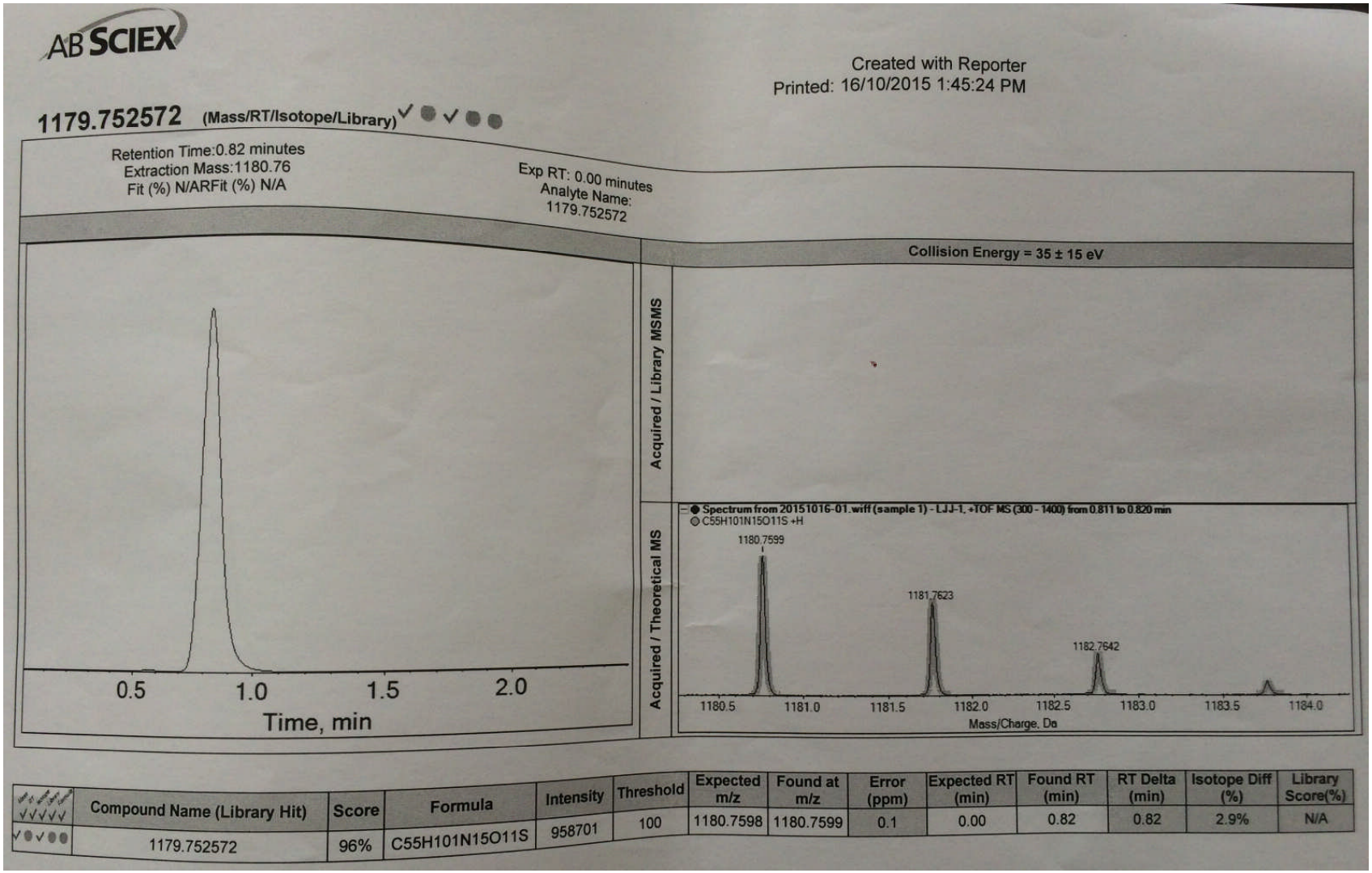
Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score(%)
1123.68997168	93%	C51H93N15O11S	86192	100	1124.6972	1124.6977	0.4	0.00	0.52	0.52	4.0%	N/A

The HRMS spectrum for the purified compound **4**.



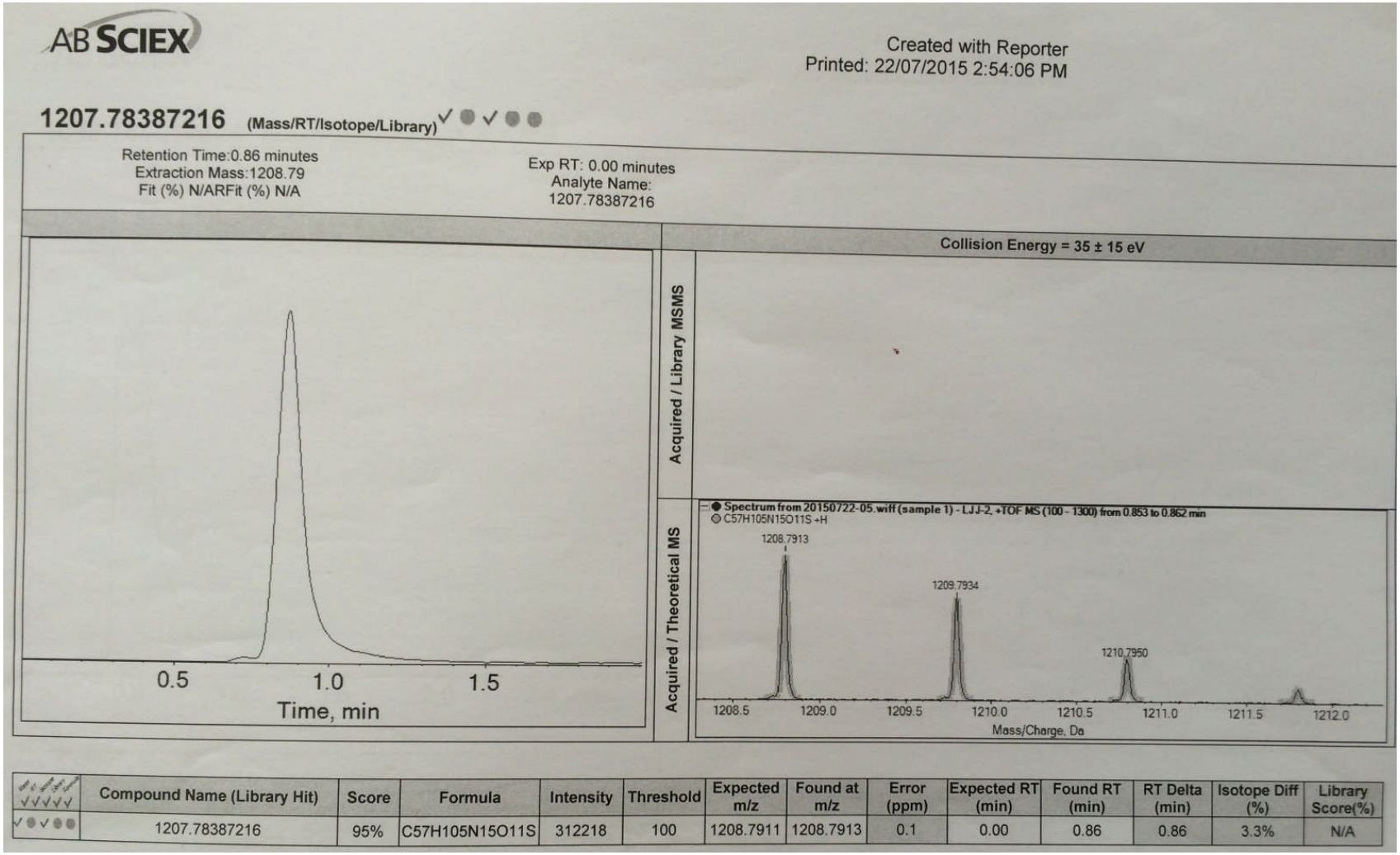
Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score(%)
1151.72127184	79%	C53H97N15O11S	4916	100	1152.7285	1152.7299	1.1	0.00	0.62	0.62	10.8%	N/A

The HRMS spectrum for the purified compound **5**.



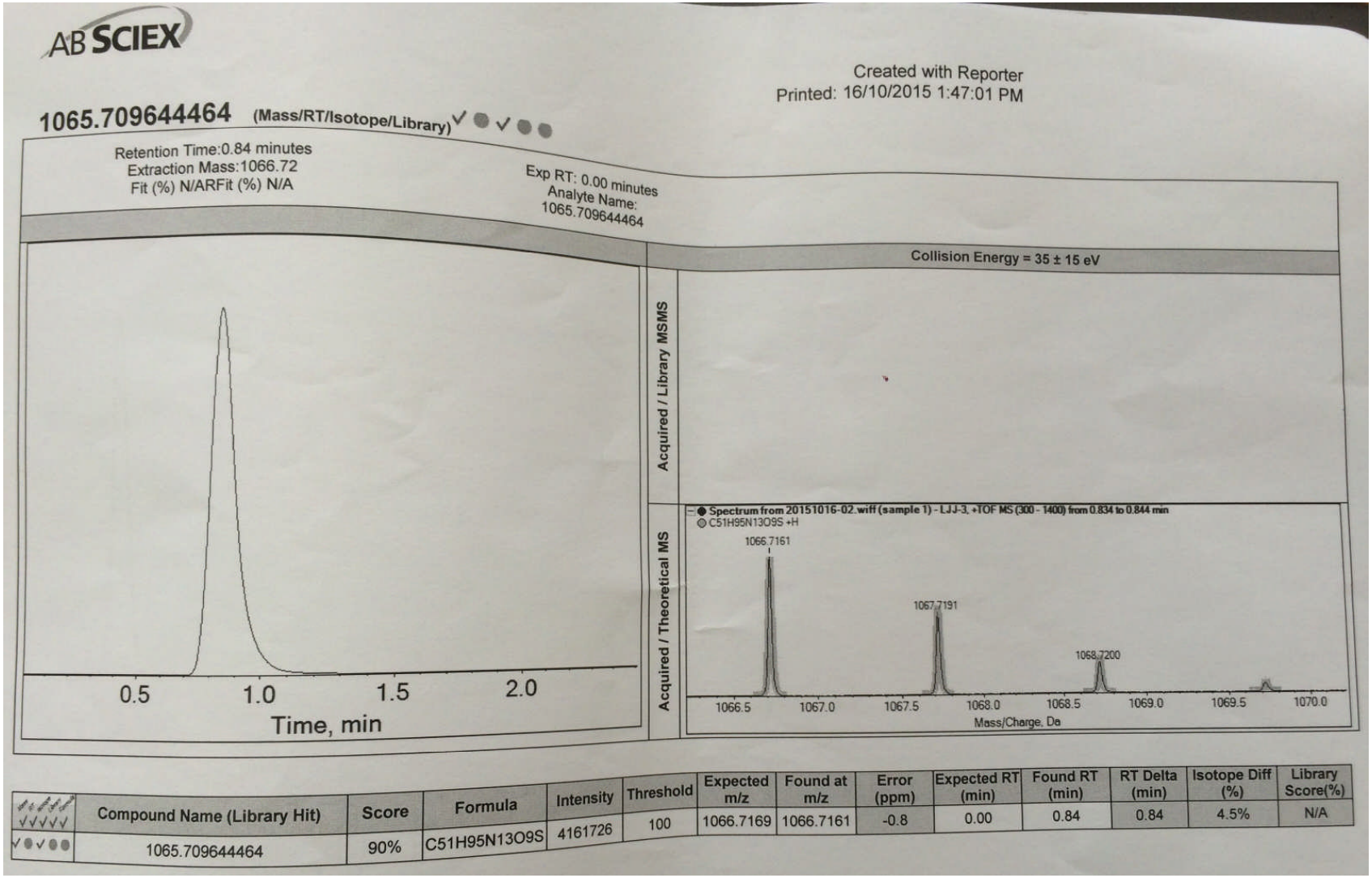
Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score(%)
1179.752572	96%	C55H101N15O11S	958701	100	1180.7598	1180.7599	0.1	0.00	0.82	0.82	2.9%	N/A

The HRMS spectrum for the purified compound **6**.



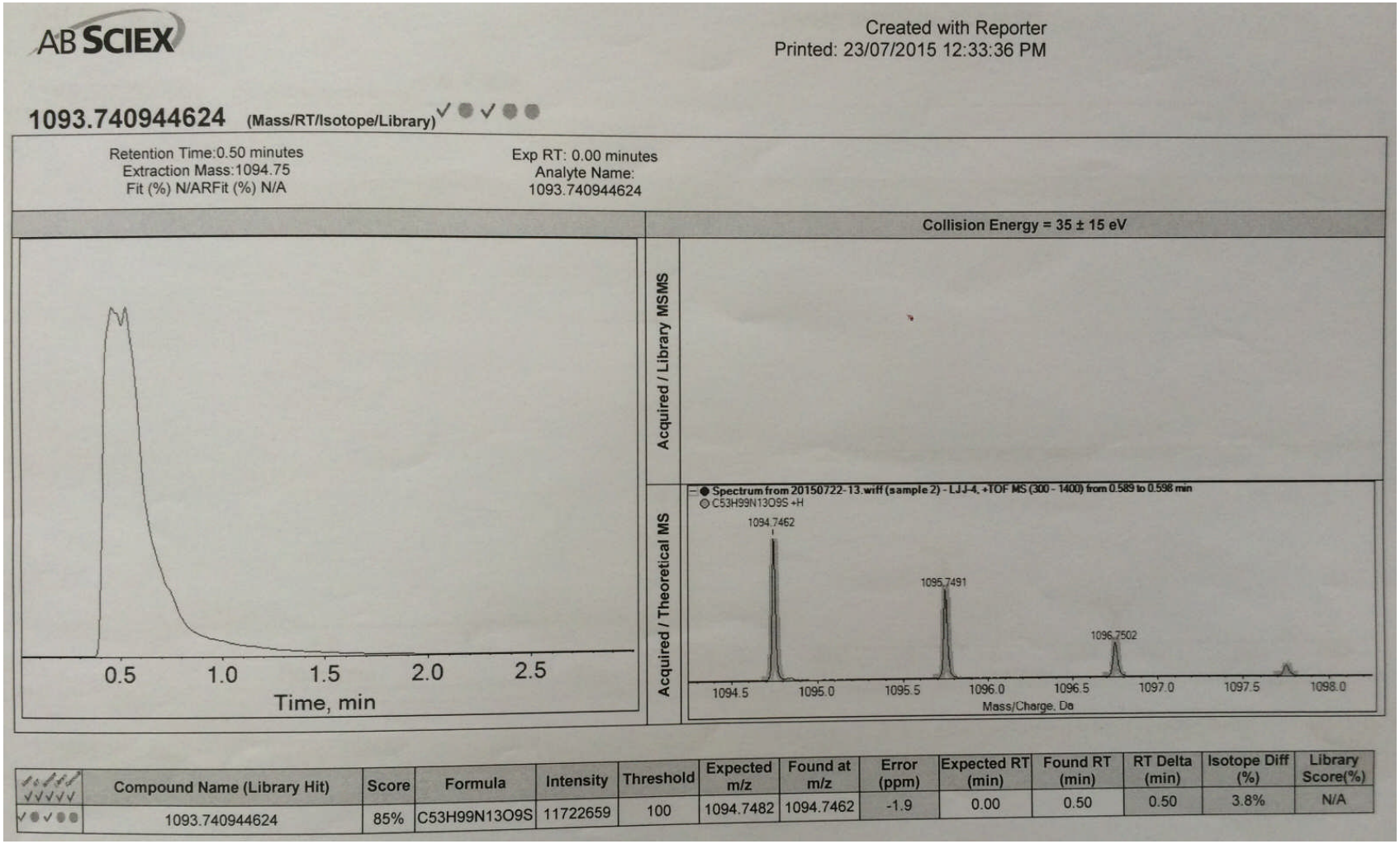
Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score(%)
1207.78387216	95%	C57H105N15O11S	312218	100	1208.7911	1208.7913	0.1	0.00	0.86	0.86	3.3%	N/A

The HRMS spectrum for the purified compound **7**.



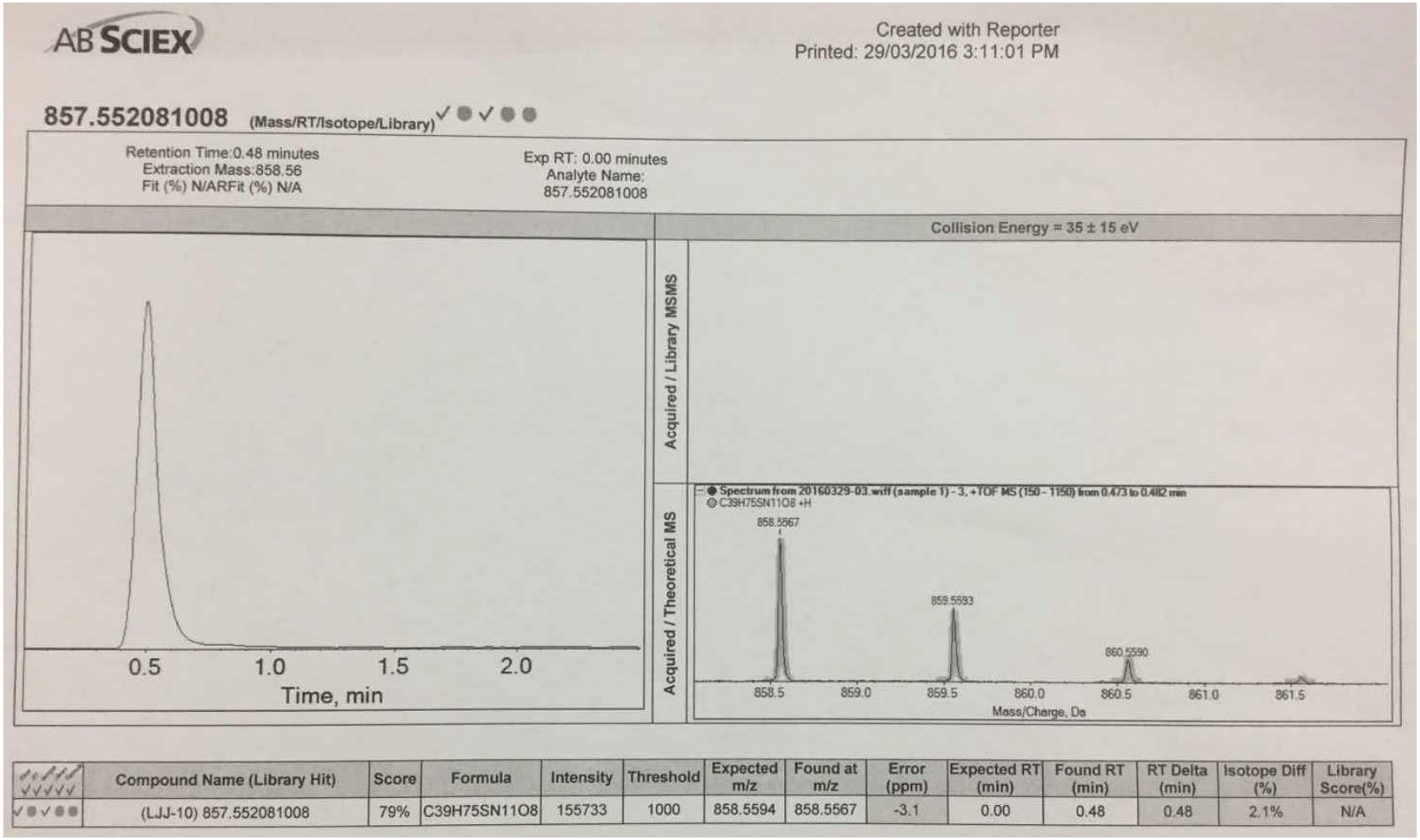
Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score(%)
1065.709644464	90%	C51H95N13O9S	4161726	100	1066.7169	1066.7161	-0.8	0.00	0.84	0.84	4.5%	N/A

The HRMS spectrum for the purified compound **8**.



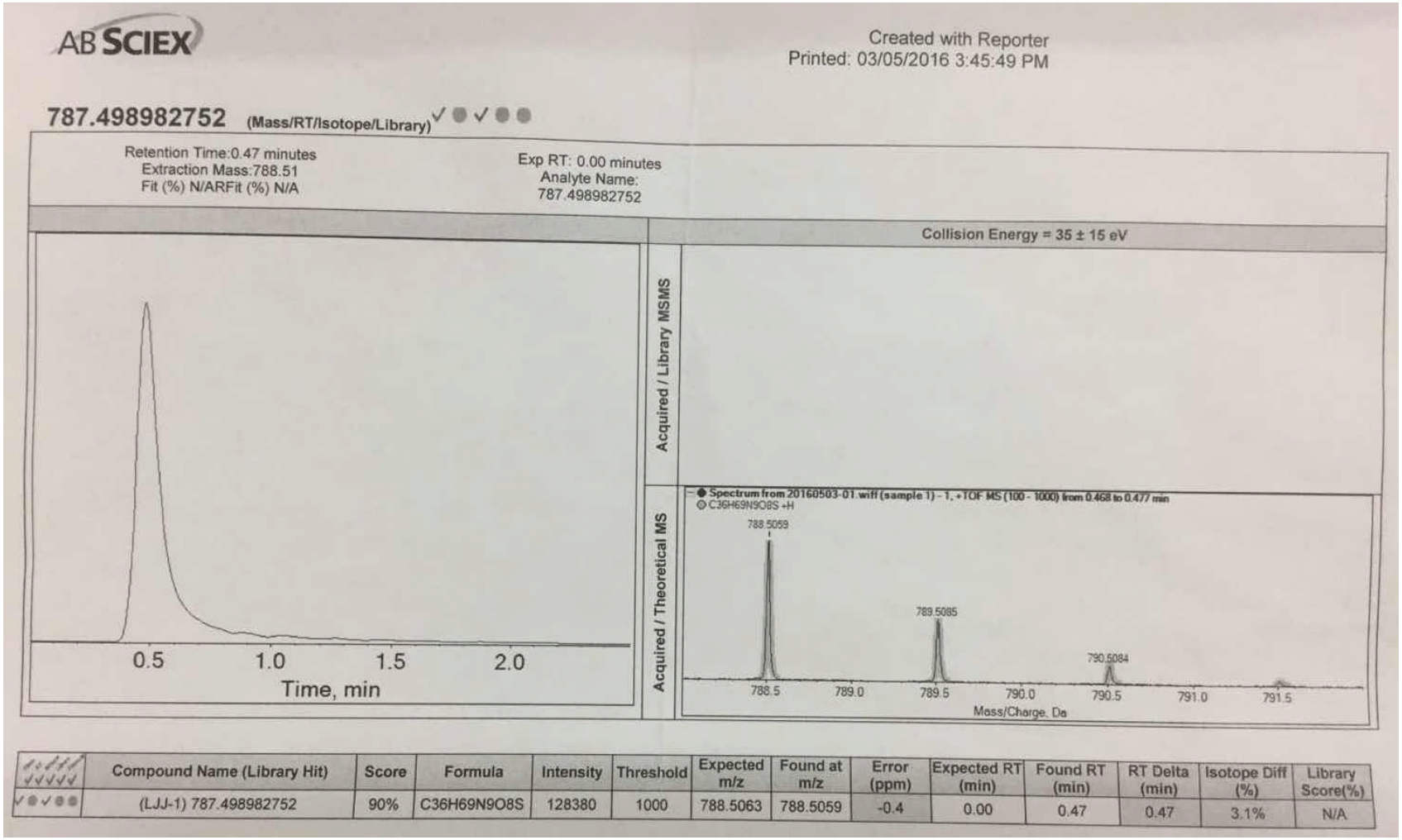
Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score(%)
1093.740944624	85%	C53H99N13O9S	11722659	100	1094.7482	1094.7462	-1.9	0.00	0.50	0.50	3.8%	N/A

The HRMS spectrum for the purified compound **9**.



Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score(%)
(LJJ-10) 857.552081008	79%	C39H75SN11O8	155733	1000	858.5594	858.5567	-3.1	0.00	0.48	0.48	2.1%	N/A

The HRMS spectrum for the purified compound **10**.



Compound Name (Library Hit)	Score	Formula	Intensity	Threshold	Expected m/z	Found at m/z	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Isotope Diff (%)	Library Score(%)
(LJJ-1) 787.498982752	90%	C36H69N9O8S	128380	1000	788.5063	788.5059	-0.4	0.00	0.47	0.47	3.1%	N/A

The HRMS spectrum for the purified compound **1**.