Supplementary Information

for

Discovery of LRE1 as a specific and allosteric inhibitor of soluble adenylyl cyclase

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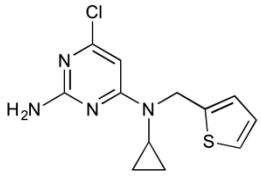
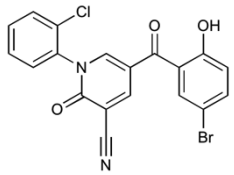
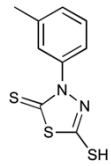
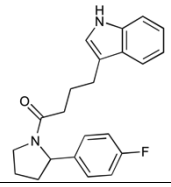
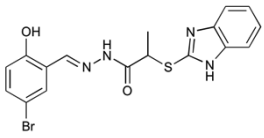
Supplementary Results

Supplementary Table 1. Small molecule screening data.

Category	Parameter	Description
Assay	Type of assay	Purified Enzyme assay
	Target	Soluble adenylyl cyclase
	Primary measurement	Mass spectrometry
	Key reagents	Human sAC enzyme, ATP, $NaHCO_3$
	Assay protocol	Reactions (40 µL) contained 50 mM Tris pH 7.5, 1 mM ATP, 5 mM $MgCl_2$ 5 mM $CaCl_2$, 40 mM $NaHCO_3$, 2 mM DTT and sAC incubated at 37ºC for 3 hours and stopped with 40 µL of 1%(v/v) formic acid. Samples were analyzed in a Rapid-fire automated Solid phase extraction system coupled to TOF mass spectrometer. Order of addition: 1) reaction buffer (10 µL). 2) compounds (80 nL) 3) sAC enzyme solution (5 µL) 4) 20 µL reaction buffer. 5) Reactions were started with the further addition of 5 µL ATP in reaction buffer then stopped after 3 hours. 6) stop solution (40 µL of 1% formic acid).
	Additional comments	Sample Analysis, Column washing and elution: 35 µl of sample was aspirated from each well of the 384-well polypropylene microplate and then injected onto a graphitized carbon SPE column extraction cartridge, washing with 5 mM ammonium acetate, pH 10 and eluting it in 25% acetonitrile, 25% acetone in 5 mM ammonium acetate, pH 10, onto the electrospray-MS, where the mass spectra of each sample were collected. The RapidFire sipper was washed between sample injections using organic (25% acetonitrile, 25% acetone in 5 mM ammonium acetate, pH 10) and then aqueous (5 mM ammonium acetate, pH 10) solvents.
Library	Library size	33,135 pure compounds
	Library composition	Low molecular weight compounds were selected for drug-likeness scores and diversity from a larger collection of 1 million compounds.
	Source	Enamine
	Additional comments	Pipeline pilot software (Biovia inc.) was used to execute a published algorithm for scoring compounds based on drug-likeness, called the quantitative exponent of drug-likeness (Q.E.D.) score [84]. From the 7.87 million commercially available compounds, 3.05 million compounds had a weighted Q.E.D. score greater than 0.7. The "choose diverse" component of the pipeline pilot software utilizes ECFP6 fingerprint descriptors to cluster the compounds into structurally related groups and chooses a single representative of each cluster, based on Tanimoto distances, called "cluster centers". The "choose diverse" component identified the 100,000 most structurally diverse compounds from among these 3.05 million structures. From these 100,000 "cluster centers," 33,135 could be purchased from Enamine in 1 mg quantities at a price which was within our budgetary limits. These compounds were formatted in 10 copies of 5 mM DMSO stocks, frozen at -20C in 384-well deep well plates.
Screen	Format	384-well plate V bottom
	Concentration(s) tested	10 µM; 0.2%DMSO

	Plate controls	DMSO (negative control), denatured enzyme control (positive control)
	Reagent/ compound dispensing system	Multidrop Combi with RapidStack (Thermo Scientific) for reagents; Janus Automated Workstation with nanohead (Perkin-Elmer) for compounds
	Detection instrument and software	RapidFire Mass Spectroscopy (Agilent); Agilent MassHunter Workstation Software
	Assay validation/QC	Hit compounds were re-picked from the original 5mM source plates and retested at 7 serially diluted concentrations; in triplicate and a concentration-response curve fitted to a 4 parameter hyperbolic using CDD software. HPLC-MS performed on all hit compounds to detect at least 90% purity from the primary sample.
	Correction factors	none
	Normalization	Normalized within each assay plate: Percent inhibition (%NPI): $[(\chi - \mu_-) / (\mu_+ - \mu_-)] \times 100\%$; where χ is the sample; μ_- mean of negative control and μ_+ mean of the positive control
	Additional comments	
Post-HTS analysis	Hit criteria	Normalized Percentage Inhibition≥ 35%
	Hit rate	0.139%
	Additional assay(s)	ELISA based detection of enzyme mediated cAMP formation
	Confirmation of hit purity and structure	LC-MS; powders reordered and independently synthesized and re-tested.
	Additional comments	All hit compounds reported were at least 95%b pure by LC-MS.

Supplementary Table 2. Small molecule screen validated hits.

Molecule Name (Q.E.D. score)	Structure	Molecular Weight (g/mol)	Primary Screen % NPI	RF-MSS Assay IC50 (μM)	EIA Assay IC50 (μM)
RU-0204277 **LRE1** (0.872)		280.8	57.0	3.3	6.3
RU-0207148 (0.752)		429.7	67.6	5.7	4.4
RU-0207328 (0.534)		240.7	58.1	> 20	> 20
RU-0206544 (0.707)		350.4	50.0	> 20	> 20
KH7		419.3	-	-	8.0 [Ref:27]

Supplementary Table 3. Crystallography data.

	sAC/LRE1	**sAC/ApCpp/LRE1**
Data collection		
Space group	$P6_3$	$P6_3$
Cell dimensions		
a, *b*, *c* (Å)	99.3, 99.3, 99.4	100.0, 100.0, 98.7
Resolution (Å)	44.42–1.79 (1.90–1.79)[1]	44.61–1.86 (1.98–1.86)
R_{meas}[2]	11.5 (119.2)	12.2 (128.1)
$I / \sigma I$	16.3 (2.1)	10.2 (1.3)
Completeness (%)	99.9 (99.5)	99.9 (99.7)
Redundancy	11.4 (11.2)	4.6 (4.4)
Refinement		
Resolution (Å)	44.42–1.79	44.61–1.86
Unique reflections	49,787	44,694
R_{work}[3] / R_{free}[4]	16.2 / 20.6	17.8 / 22.3
No. atoms	4165	4005
protein	3730	3650
ligand[5]	18	50
solvent[6]	417	305
B factors	30.7	35.5
protein	30.5	34.9
ligand	20.7	40.3
solvent	41.6	41.0
r.m.s. deviations		
Bond lengths (Å)	0.019	0.019
Bond angles (°)	2.08	2.03

[1]Values in parentheses are for highest-resolution shell.

[2]$R_{meas} = \frac{\sum_h \sqrt{\frac{n}{n-1}} \sum |\langle I \rangle - I|}{\sum_h \sum I}$; I is the intensity of an individual measurement, $\langle I \rangle$ the corresponding mean value, and h and n are the indices and redundancies of the reflections.

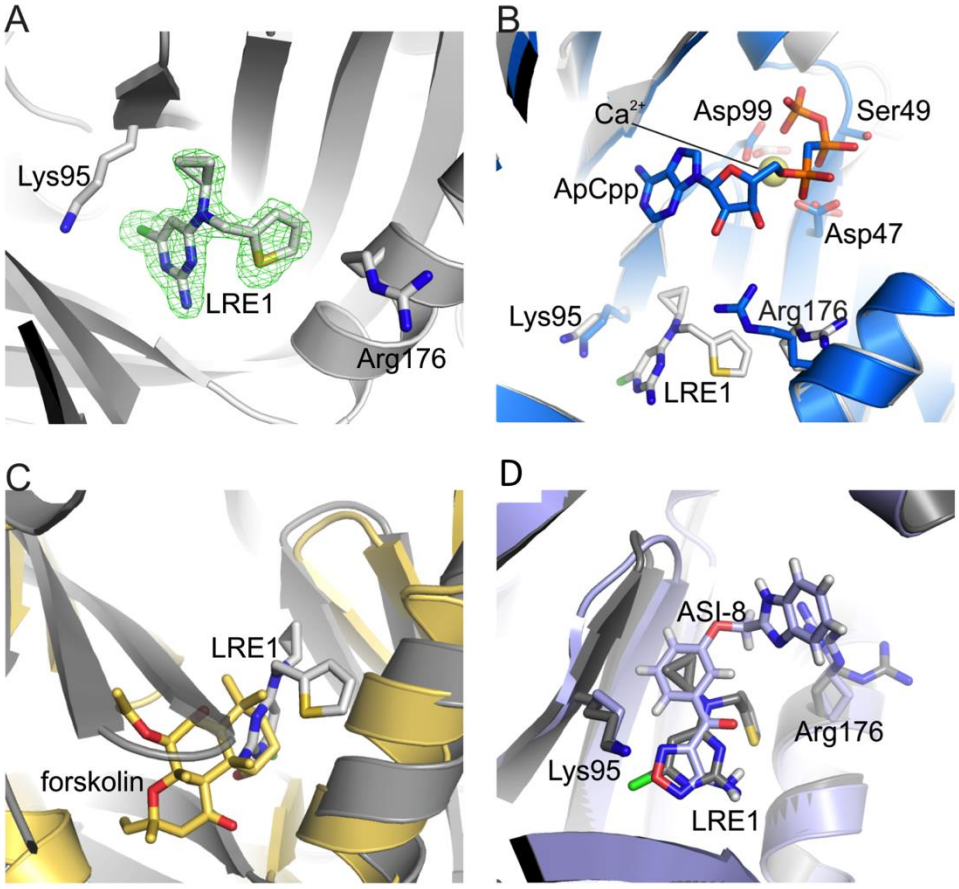
[3]R-factor = $R = \frac{\sum_{hkl} ||F_{obs}| - k|F_{calc}||}{\sum_{hkl} |F_{obs}|}$; $|F_{obs}|$ is the observed and $|F_{calc}|$ the calculated structure factor amplitude.

[4]R_{free} was calculated from 5 % of measured reflections omitted from refinement.

[5]Ligand = inhibitor or inhibitor + substrate analog + Ca^{2+}

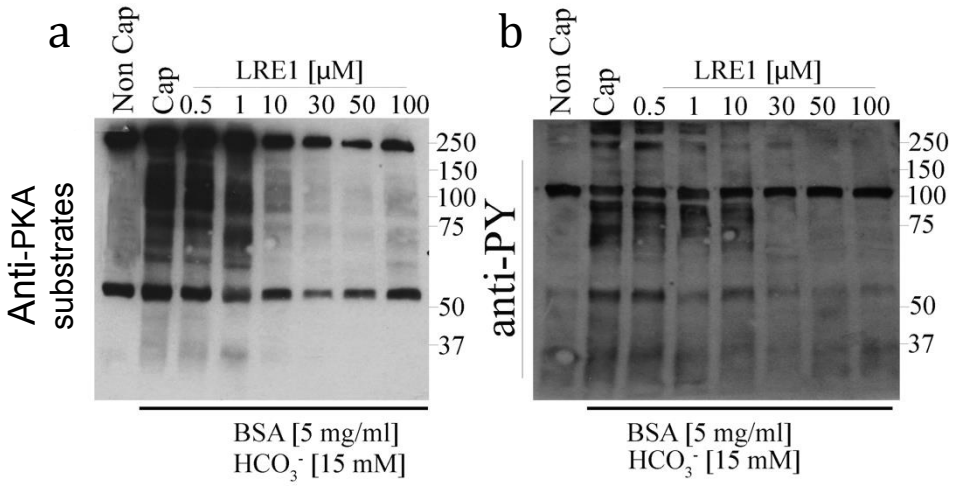
[6]Solvent comprises water and other solvent molecules

Supplementary Figure 1



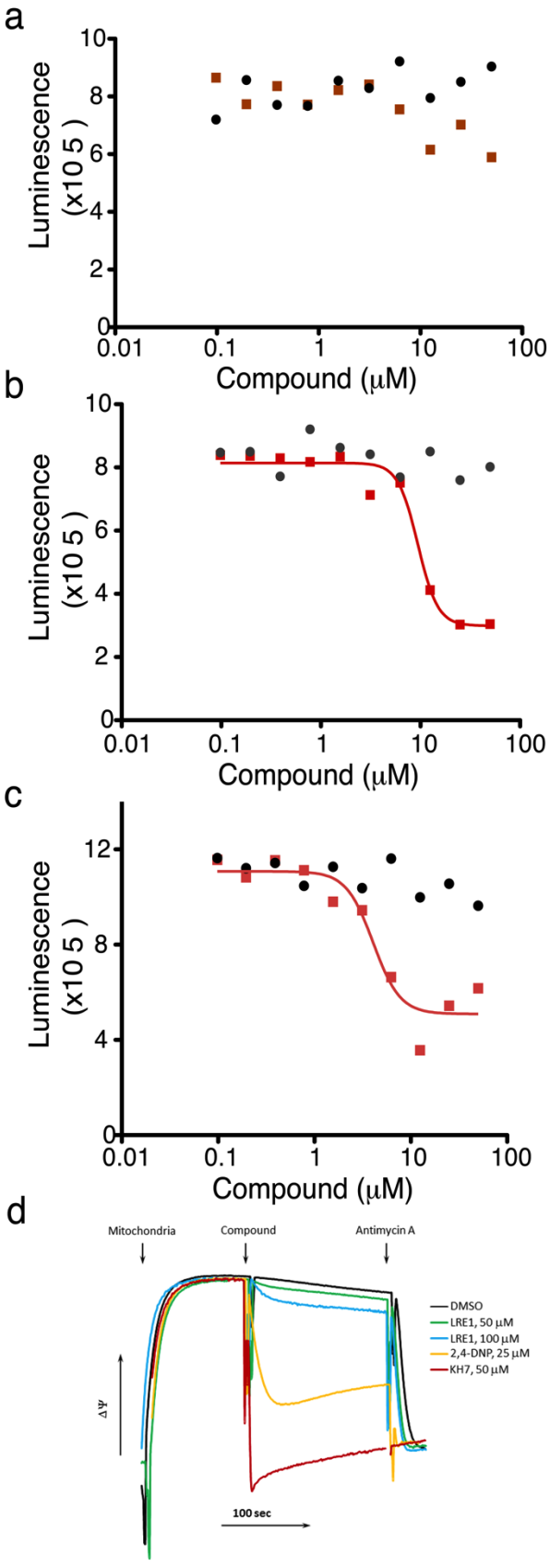
Supplementary Figure 1: Structural analysis of sAC inhibition by LRE1 and comparison to other AC modulators. (a) Close-up of the BBS of the sAC/LRE1 complex. Ligand and key interacting residues are shown as sticks colored according to atom type. LRE1 is overlaid with F_o-F_c omit electron density (blue) contoured at 5σ. **(b)** Overlay of the sAC/LRE1 complex (grey) with a sAC/ApCpp structure (blue). Ligands and interacting residues are shown as sticks (colored according to atom type), and Ca^{2+} as a yellow sphere. **(c)** Overlay of sAC/LRE1 (grey) with a tmAC/forskolin complex (yellow; RMSD 9.7 $Å^2$ for 238 C_α atoms). The ligands are shown as sticks and colored according to atom type. **(d)** Overlay of sAC/LRE1 (grey) with sAC/ASI-8 (blue; RMSD 0.3 $Å^2$ for 366 C_α atoms). Ligands and two key BBS residues are shown as sticks and colored according to atom type.

Supplementary Figure 2.



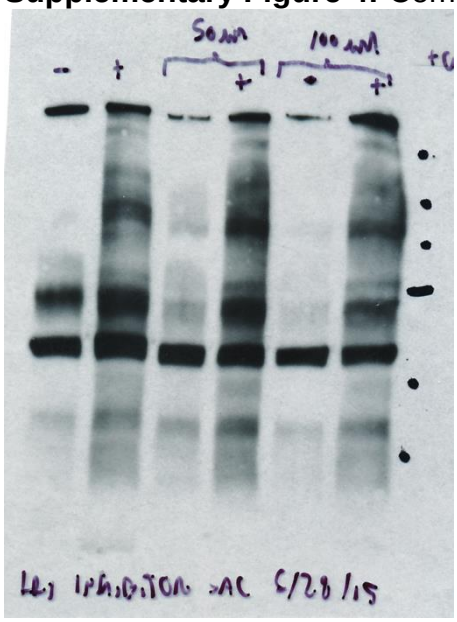
Supplementary Figure 2: LRE1 inhibits sAC dependent sperm capacitation. Mouse cauda sperm obtained in media devoid of Ca^{2+}, HCO_3^-, or BSA were activated by incubation in capacitation media containing 15 mM HCO_3^-, 5 mg/ml BSA, and 0.3 mM EGTA for 60 minutes in the presence of the shown amounts of LRE1. Non Cap = non capacitated negative control. Cap = DMSO treated capacitated positive control. **(a)** Western blot using anti-PKA substrates antibodies. The complete gel is shown in Supplementary Figure 6. **(b)** Western blot using anti-phospho tyrosine antibodies of the same blot as in (a). The complete gel is shown in Supplementary Figure 7. Shown are representative Western blots of experiments repeated three times using independent sperm preparations from different mice. Complete gels used for these images are included below.

Supplementary Figure 3.

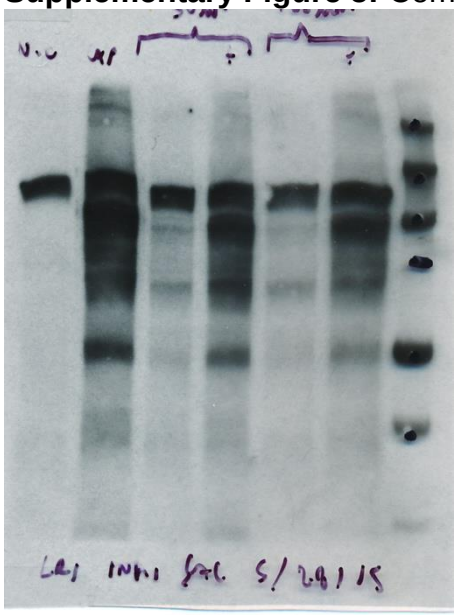


Supplementary Figure 3: **LRE1 is less toxic than KH7.** WT **(a,b)** or sAC KO **(c)** MEFs (1500 cells/well; 384 wells/plate) were grown for 24 **(a)** or 48 hours **(b,c)** in the presence of the indicated concentrations of LRE1 (black circles) or KH7 (red squares). Cell viability, as determined by CellTiter-Glo, is shown as luminescence; values are averages of triplicate determinations ± S.E.M. of a representative experiment repeated at least two times. **(d)** Changes in membrane potential ($\Delta\Psi m$) in mouse brain mitochondria (at 0.2 mg/ml) incubated in 125 mM KCl, 20 mM HEPES pH 7.4, 4 mM KH_2PO_4, 0.5 mM EGTA, 0.2 mg/ml fatty acid free bovine serum albumin, 0.4 uM Safranin O, 5 mM pyruvate, and 2.5 mM malate in the presence of DMSO solvent alone (black line), 50 µM LRE1 (green line), 100 µM LRE1 (cyan line), 50 µM KH7 (red line), or 25 µM of the classical uncoupler 2,4 dinitrophenol (2,4-DNP). Addition of Antimycin A to inhibit the mitochondrial electron transport chain confirms the mitochondrial integrity. Data represent single measurements of a representative experiment repeated at least two times.

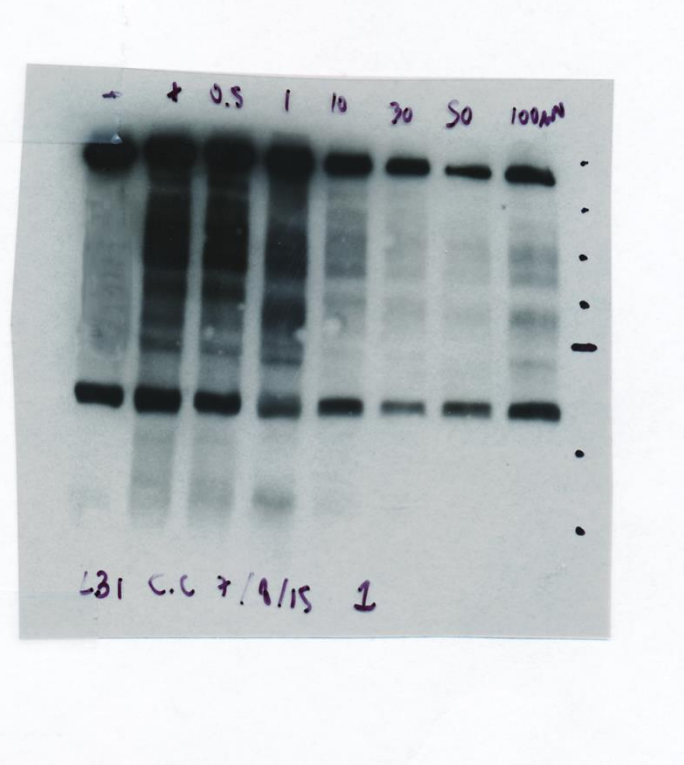
Supplementary Figure 4: Complete Gel used for construction of Figure 5a.



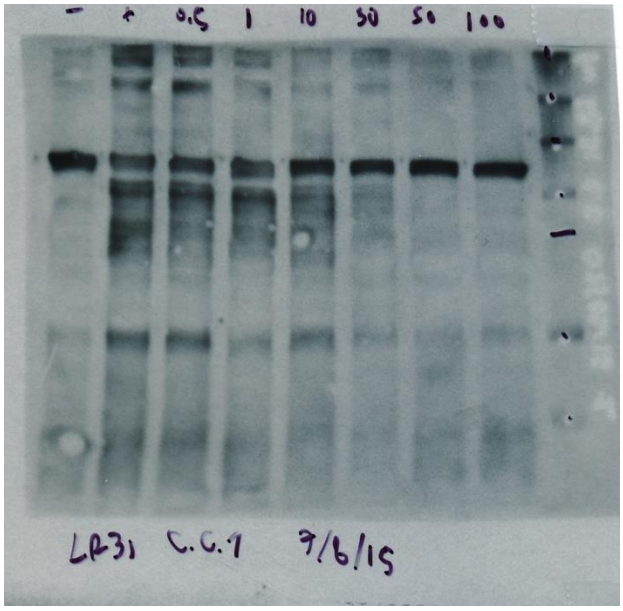
Supplementary Figure 5: Complete Gel used for construction of Figure 5b.



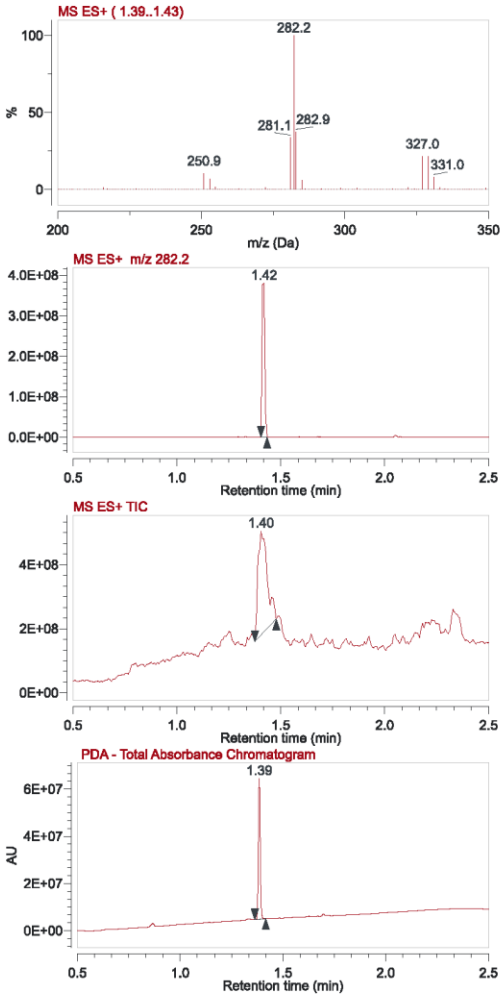
Supplementary Figure 6: Complete Gel used for construction of Supplementary Figure 2a.



Supplementary Figure 7: Complete Gel used for construction of Supplementary Figure 2b.



Supplementary Figure 8: LC/MS Validation of LRE1 structure



Supplementary Figure 9: NMR Validation of LRE1 structure