Supplementary Information

A Robotic System for Automated Chemical Synthesis of Therapeutic Agents

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The PDF file includes:

SUPPLEMENTARY METHODS

Chemicals and Reagents

All target compounds were synthesized on the 400–450-μm-sized 2-chlorotrityl chloride (CTC) resin (1.19 mmol/g, Rapp Polymere GmbH, Tuebingen, Germany). The BMB library was synthesized using commercially available building blocks purchased from Sigma-Aldrich, Acros Organics, Maybridge Chemicals, or TCI America. All solvents and catalysts were purchased from Sigma-Aldrich and used as supplied unless otherwise stated. The amine-functionalized building block, diethyl (4-aminobenzyl)phosphonate, was protected with a tert-butyloxycarbonyl (BOC) group prior to its use for synthesis of the BMB library. All other building blocks were used as purchased without further modification. The BOC protection reaction was performed by conventional synthetic procedures. Briefly, diethyl (4-aminobenzyl)phosphonate (5.0 g, 20.5 mmol) was dissolved in a mixture of tetrahydrofuran (THF)/water (65 mL THF/16 mL water, 80/20 v/v). Di-tert-butyl dicarbonate (4.90 g, 22.5 mmol) and sodium bicarbonate (2.58 g, 30.75 mmol) were added to the reaction mixture, followed by stirring at room temperature for 20 h. The organic solvent was evaporated *in vacuo*, and the aqueous layer was extracted with ethyl acetate (3 × 50 mL). The combined organic phase was washed with 10% citric acid and saturated sodium carbonate brine (3 × 50 mL), dried over anhydrous sodium sulfate, and filtered. The solvent was evaporated *in vacuo*, and the crude product was recrystallized from hot hexane to afford the desired product as white crystals (5.6 g, 79% yield).

Preparation for Automated Synthesis

The BMB library was synthesized using the Heck reaction followed by the Horner–Emmons Wittig reaction. All of the processes including microwave reactions, heating or cooling, shaking, bead collecting and splitting, washing beads, and handling exhaust or waste were conducted using the automated synthesis system. Before running the automated synthesis, 80 reaction tubes were placed in the RR. CTC polystyrene resins (200 mg) were dispersed in 120 mL of dimethyl sulfoxide (DMSO) and placed in the SPBD. The chemical in the tubes were as follows: tubes 1–20: empty tubes; tubes 21–40: Block 1 in dichloromethane (DCM) containing 80 μL diisopropylethylamine (DIPEA); tubes 41–60: bromo-2-methoxybenzaldehyde (Block 2), palladium(II) acetate ($Pd(OAc)_2$), tri(o-tolyl)phosphine ($P(O\text{-}Tol)_3$), and tetrabutylammonium bromide (TBAB) in dimethylformamide (DMF) containing 17 μL triethylamine (Et_3N); and tubes

61–80: Block 3 and potassium t-butoxide in toluene. The solvent volume in each tube was 1 mL. Then, we created the related command sequence, which was then loaded to the automated GUI to drive individual robotics for the automated synthesis.

Roles of Individual Robotic Systems

1. 360° Robot Arm (RA): To replace the role of a chemist, the ability to move reaction tubes from one location of the robotic system to another accurately, repeatedly and in a complicated order is the most important feature and simultaneously the most difficult to achieve. The RA transfers tubes from one location to another using the given coordinates. To maximize this function, the RA is placed at the center of the integrated system and achieves the maximum range of reachable distance. The other robotic systems are positioned such as to minimize the movement of the reaction tubes during chemical reactions surrounding the central RA. The Reagent Rack (RR), designed to hold 121 reaction tubes, is positioned next to the LH to minimize the distance and time of tube movement. The tubes, with or without starting materials and reagents before starting and after completing reactions by the RA, are stored in the RR. Holes for the tubes are equidistant from each other, and only 3-point calibration is employed to generate all possible locations for reaction tubes by subsequently calculating in the 2D Cartesian plane.

2. Capper–Decapper (CAP): The CAP is positioned between the LH and SPBD to open and close tube caps before and after transferring tubes to both robots. To ensure repeated and accurate capping and uncapping, a screw thread and caps that are compatible with the original CEM reaction tube were chosen (**Fig. 1B**). The septum from the commercial tube is reused specifically to be penetrable by Gilson's needle; it also serves to form a tight seal after screwing using the CAP.

3. Split-Pool Bead Dispenser (SPBD): The most important feature of solid-based split-pool synthesis is to aspirate and dispense beads not only accurately, but also relative to the number of tubes. For accomplishing this goal, an SPBD was designed, which is capable of dispensing water or organic solvents (e.g., DMSO) while equally distributing beads into a designated number of tubes. The diameter of the needle, which is used to efficiently and rapidly aspirate or dispense beads along with solvents, is 5 mm. The collection and distribution of beads are achieved by pressurizing and subjecting the solvent reservoir to vacuum for aspiration, the reservoir is

evacuated approximately 2.5 psi, causing the liquid content to flow into the reservoir, while a pressure of approximately 5 psi is applied to the reservoir to push the liquid out. To ensure equal distribution of the beads across the desired number of tubes, a stirring fan is placed inside the reservoir. On aspiration or dispensation, the fan is switched on, and the liquid inside the reservoir is stirred, causing beads to disperse throughout the volume of the liquid.

4. Liquid Handler (LH): The LH is required to handle (aspirate or dispense) reagents and solvents relative to the number of tubes, while preventing beads from traveling from the reaction tube. The probe is designed to 1) aspirate or dispense liquid, 2) vacuum/gas/hold vacuum, and 3) wash or flush tube walls. A special needle with a narrow tip (2.5 mm diameter) is used to filter out beads while aspirating liquids from or dispensing washing solvents into the tube. Moreover, the LH can be also used for transferring solutions of starting material from one tube to another with resin inside. The LH, connected to two valves, is capable of choosing from eight solvents to accurately dispense into each tube. After each reaction step, beads are washed by the repeated aspiration and dispensation of solvents for each tube. Two shaking racks as well as one customized heating or cooling rack is also present in the LH (**Fig. 1C, Fig. S1**) for traditional temperature-sensitive chemical reactions, which is realized by connecting a chiller to control the temperature from −20 °C to 150 °C while shaking.

5. Microwave Reactor (MWR): The MWR is added to expand the chemistry options available to the chemist. The MWR is customized to place reaction tubes on the microwave tube holders using the RA. It can microwave a single tube at a time for any period of time; other features include controllable pressurization, magnet stirring, and rapid cooling. The standard 10 mL CEM microwave tubes, which are made of borosilicate glass so that they can sustain high temperatures and pressure, are used for most chemical reactions. **Fig. 1D** shows every possible movement that can be made by the RA, as well as detailed functions of each robotic system. By integrating all robots into a single computer controller, open communication can be made between each instrument, thereby permitting one computer to handle all jobs and preventing other robots from entering a deadlock or crashing into each other while running simultaneously.

Hardware Integration

The automated robotic chemistry system was integrated with six individual instruments, which communicate with a central PC through RS-232 serial ports. The integration layout was specifically designed for sequential or dynamic command sequences with a 360° Robotic Arm (*RA*; Zymark Corp., Hopkinton, MA) as the center. The RA revolved within the surrounding robotic systems accomplishing the delivery of the reaction tubes. Solvent aspiration and dispensation were conducted using an automated LH system (*LH*; GX-281 Liquid Handler, Gilson, Inc., Middleton, WI), which was configured with a valve actuator (Valvemate II, Gilson, Inc., Middleton, WI) to immediately provide access up to eight solvents. Independent or subsequent microwave reactions were carried out using a CEM Explorer Microwave (*MW*; CEM, Matthews, NC) with enhanced heating capability. The reaction tubes (10 mL Pressure Vial & Septa Acc Kit) were purchased from CEM, and the set screws and caps of the tubes were customized by LAE Technologies (Barrie, ON, Canada). The dispensing and distribution of beads into the reaction tubes were conducted using the SPBD (TechElan LLC, Mountainside, NJ). Before transferring tubes to the SPBD, an air-pressure-based CAP (HyperTask, Hopkinton, MA) was used to open or close specially designed caps (LAE Technologies Inc.). The reaction temperature was controlled by a refrigerated circulating water bath (*CHILL*; JeioTech, Woburn, MA), which was connected to a customized heating or cooling rack (LAE Technologies Inc.) on the LH. After the integration of all of the robotics surrounding the RA, the position of each robotic system was coordinated by the RA based on the Cartesian coordinate plane.

Software Integration

As shown in **Fig. 2A**, custom software is designed to be as simple as possible to enable any chemist to work from simple parallel syntheses to complicated combinatorial syntheses. When the command sequence is loaded into the software, the software reads the file, populates the group of tubes required for the reaction, and asks the user to assign positions for reaction tubes. After all groups are filled with the information on tube positions, the user presses the “run” button, which in turn starts serial reactions. Log files are created as robots go through each step for the user to determine the step at which the user is currently or to determine the time at which the specific reaction is completed.

A series of command sequences are used to drive the robotic system to achieve automated syntheses. Each user can write a command sequence in a text file and then load it into the

automated chemistry software to run serial syntheses. To start, the command sequence begins with a command called "STEP," which is read by the software to allow both the software and the robots to determine the number of reaction steps existing in the entire command sequence file. The software determines the number of groups of tubes in each step and keeps counting the total number of tubes and their positions. In addition, to further reduce the workload of the user, a GUI for the Command Sequence Creator software is developed for the creation and easy access of a command sequence for the synthesis (**Fig. 2B**). Any combination of syntheses can be achieved using the Command Sequence Creator, which takes care of small rules associated with the command sequence file, such as the "STEP" command, required by the first line of every command sequence file.

Microsoft C# was used as the programming language to develop the automation software, and Microsoft Visual Studio 2005 was used to create the main GUI and codes. The main software integrated all sub-robotics by either referencing dynamic link library (DLL) files built in the individual robotic or by manually sending signals to the customized SPBD and CAP through RS-232 serial ports. Each robotic contained its own C# class files, which are incorporated into the custom method files, and compliant DLL files were used to operate each robotics in order. For instance, DLL files for the MW containing specific methods to set desired temperature, pressure, boiling temperature, ramp time, hold time, stirring, and cooling were called by the C# class file to run each tube in the MW reactor. For robotics that require manual signals to be sent, a blueprint was created to communicate through the RS-232 serial port, and operation signals were sent to the robotics using C# codes.

Automated Synthesis

Empty tubes 1–20 were used to prepare CTC resins with 4-aminostyrene. The RA transferred tubes 1–20 from RR to SPBD, and 10 mg of beads (0.01 mmol, as calculated from the loading capacity of the resin) was dispensed in each tube. The RA then put the tubes on rack 2 of LH and put tubes 21–40 on rack 1 of LH. The LH aspirated DMSO in tubes 1–20 and then transferred starting materials from tubes 21–40 to tubes 1–20 one by one. For each reaction, 10 molar equiv of 4-aminostryene (14 μL, 0.12 mmol) and 40 molar equiv of DIPEA (80 μL, 0.48 mmol) in DCM (1 mL) were added to the tube with resins, which were shaken at room temperature overnight. Then, the beads were subsequently washed using methanol, DMF, and DCM (three washes per

solvent, 5 mL per wash). After washing, the RA transferred tubes 21–40 from rack 1 of LH to the RR.

For the Heck reaction, the RA put tubes 41–60 on rack 1 of LH. Then, LH transferred starting materials from tubes 41–60 to tubes 1–20. For each reaction, a suspension of 10 molar equiv of the desired Block 2 (25.6 mg, 0.12 mmol), 1 molar equiv of $Pd(OAc)_2$ (2.7 mg, 0.01 mmol), 1 molar equiv of $P(O\text{-}Tol)_3$ (3.6 mg, 0.01 mmol), 2 molar equiv of TBAB (7.7 mg, 0.02 mmol), and 10 molar equiv of Et_3N (17 μL, 0.12 mmol) in DMF (1 mL) were added to the 4-aminostyrene-loaded CTC beads. The reaction was conducted at 100°C for 24 h, followed by washing with water, DMF, and DCM (three washes per solvent, 5 mL per wash). After washing, the RA transferred tubes 41–60 from rack 1 of LH to the RR.

Block 3 was added under the optimized Wittig reaction. The RA placed tubes 61–80 on rack 1 of LH, and the starting materials were transferred from tubes 61–80 to tubes 1–20. For each reaction, 10 molar equiv of the desired Block 3 (0.12 mmol) and 10 molar equiv of potassium tert-butoxide solution (1 M in THF, 119 μL, 0.12 mmol) in anhydrous toluene (1 mL) were added to the loaded CTC beads in the custom MW tube containing a micro stir bar. MW reactions were performed at 110°C with a power of 300 W for 1 h. Following reaction completion, the RA transferred tubes 1–20 to rack 2, and the beads were washed with water, DMF, and DCM (three washes per solvent, 5 mL per wash). After washing, the RA transferred tubes 61–80 from rack 1 of LH to the RR.

After the Wittig reaction, all molecules were subsequently cleaved from the CTC beads at the linker by suspending the beads in a mixture of 20% trifluoroacetic acid (TFA) and 80% DCM (1 mL/tube). The beads in 20/80 TFA/DCM were subjected to shaking at room temperature for 3 h, and then the cleaved compound was filtered from the beads, dried using a rotary evaporator, and dried under vacuum overnight. The BOC-protecting group on diethyl (4-aminobenzyl)phosphonate was simultaneously cleaved during room-temperature incubation using 20/80 TFA/DCM.

All cleaved compounds were analyzed by an UltraFlex III MALDI TOF/TOF system (Bruker Optics, Billerica, MA) equipped with a SmartBeam laser operating at 100 Hz and set to an extraction voltage of 25 kV in the positive mode. A mixture of 0.5 μL of a 100 mM 2,5-dihydroxybenzoic acid (DHB) solution in DMSO and the same amount of each chemical synthesized were produced on the metal-based 384 circle stainless steel μFocus MALDI plate

(Hudson Surface Technology, Newark, NJ) to prepare the sample for MALDI MS. HPLC analyses were conducted using a Waters ACQUITY UPLC system on a BEH C18 column (2.1×50 mm, 1.7 μm) with an Xevo G2 QTof detector connected to a PC with MassLynx 4.1 software. The mobile phase was solvent A = 0.1% formic acid in water, solvent B = 0.1% formic acid in acetonitrile with a linear gradient from 10% to 90% (from A to B for 5 min). The flow rate was 0.6 mL/min, and all compounds were identified by molecular weight. Purity analysis was completed using the Evaporative Light Scattering Detector spectrum, where the area under each peak was calculated to obtain the total area for all compounds in the crude mixture. The area under the product peak was used to determine the percentage of total peak area accounted for by the desired product, and thus the purity of the sample and yield in milligrams. Prior to biological evaluations, Compounds 1, 4, and 9 from the large batch automated syntheses were purified through preparative TLC (EMD Millipore, Billerica, MA), with hexane/ethyl acetate (1/1, v/v) as eluent. ^{1}H-NMR spectra were recorded using 400 MHz Bruker and 600 MHz Varian high-resolution NMR spectrometer using 5-mm NMR tubes (Wilma 528-PP) in $CDCl_3$, CD_2Cl_2 or DMSO-d_6 (Cambridge Isotope Laboratories, Andover, MA) at 25˚C. All ^{1}H-NMR spectra and chemical shifts are reported as ppm (S7-S9).

Selection of Compounds Using UMAP

The 20 BMB analogs, along with the 230 other nerve-targeting compounds from our previous study, were processed simultaneously for comparison. The fingerprinting method we chose was Morgan bit fingerprinting, and the metric chosen for the Uniform Manifold Approximation and Projection (UMAP) was hamming. Morgan fingerprinting applies the Morgan algorithm to molecules, which means the minute differences in structure among the 20 compounds were noted, and the hamming metric measures distances between bit strings via the number of changes that must be applied to each bit string, which allows for more direct comparisons of structural differences. For the UMAP, the number of nearest neighbors was set to 20, and the minimum distance was set to 0.4. Upon computing the UMAP, an algorithm counted the number of high-performing neighbors in the BMB library within a 0.3 radius of each BMB analog.

Ex Vivo Nerve-Specific Fluorescence Library Screening

Sciatic nerves were harvested from a female Yorkshire pig (E.M. Parsons and Sons, Hadley, MA), fixed in 2% paraformaldehyde (PFA), and flash frozen in optimal cutting temperature (OCT) compound with liquid nitrogen. Nerves were cryosectioned in cross-section at 10 μm, followed by fixing with 2% PFA (15 min) and washing with phosphate-buffered saline (PBS) 3 times (5 min each). A formulation was previously developed for intravenous (IV) administration and was used in the current study to incubate the fluorophores with the nerve tissue.[1, 2] All fluorophores were mixed from the 100 mM stock solution into the IV formulation (D5W containing 5% Cremophor EL) at 100 μM and incubated with the washed nerve tissue sections at room temperature for 20 min. The nerve section was then washed an additional 2 times with PBS (5 min per each), after which coverslips were mounted using Fluoromount-G (Southern Biotech, Birmingham, AL).

NIR fluorescence microscopy for *ex vivo* nerve tissue slides was performed on a Nikon Eclipse TE2000 microscope system as previously described.34, 36 The microscope was equipped with a 100 W mercury light source (Nikon, Melville, NY) passed through a 360/50 nm band pass (BP) excitation filter, a 400 nm long pass (LP) beam splitter, and a 550/50 nm BP emission filter. Images were acquired on an Orca-AG (Hamamatsu, Bridgewater, NJ). Image acquisition and analysis was performed using IPLab software (Scanalytics, Fairfax, VA).

In Vivo Intraoperative Nervous Tissue Imaging

6 weeks old CD-1 mice (male; 20-25 g) were purchased from Charles River Laboratories (Wilmington, MA), and animal studies were performed under the supervision of BIDMC IACUC in accordance with approved institutional protocol #155-2008. Animals were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally (Webster Veterinary, Fort Devens, MA). Preparative TLC-purified compounds representing positive *ex vivo* nerve-specific fluorescence were used for nerve-specific fluorescence imaging followed by systemic administration into CD-1 mice. The 0.5 mg/kg of each compound in D5W was IV injected 4 h prior to imaging, and the imaging interval was chosen from previous nerve imaging studies.[1, 2] The real-time intraoperative dual-NIR fluorescence imaging system was described in detail previously.[1, 3] Herein, two 375 ± 10 nm LEDs (Epitex, Inc., Kyoto, Japan) was used as an excitation light, which created a fluence rate of 0.15 mW/cm^2 on the imaging field. Color images were collected under the white light source (400–650 nm) at 5,500 lux. During fluorescence imaging, the white light was turned off to eliminate high background signals. For each experiment, camera

exposure time and image normalization were held constant. The trigeminal ganglia, optic nerve, and brain were specifically imaged compared to adipose and muscle tissues. After *in vivo* imaging, major organs were resected, imaged, and the signal from each organ/tissue was quantified by measuring radiance (photons/s/cm^2/sr). Statistical analysis was carried out using the unpaired Student's t-test or one-way analysis of variance (ANOVA). Results were presented as mean ± SD and curve fitting was performed using Prism version 4.0a software (GraphPad, San Diego, CA).

References

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2. S. L. Gibbs, Y. Xie, H. L. Goodwill, K. A. Nasr, Y. Ashitate, V. J. Madigan, T. M. Siclovan, M. Zavodszky, C. A. Tan Hehir and J. V. Frangioni, *PLoS One*, 2013, **8**, e73493.
3. H. Hyun, M. H. Park, E. A. Owens, H. Wada, M. Henary, H. J. Handgraaf, A. L. Vahrmeijer, J. V. Frangioni and H. S. Choi, *Nat Med*, 2015, **21**, 192-197.

Table S1. Technical and design comparisons of existing commercial and laboratory custom-made synthetic robots for small molecule building blocks.

Automated system	Strategy	Reaction type	Operating temp.	Capacity	Component module #	Liquid handler	Purity
Science, 2019, 365, eaax1566	Flow chemistry	Coupling, alkylation, reduction, etc	RT to 120 °C	~3-20g total mass of the reactants	9	--	--
Nature, 2020, 583, 237-241.	Reaction optimization	Photochemical reaction	20.6 to 23.5 oC	~200mg total mass of the reactants/vial	9	Up to 14 different washing solvents	--
Mater. Adv. 2024	OBOC	Peptide synthesis, Heck/Wittig reaction	-20 to 150 °C	10-50mg resins/vial	6	Up to six different washing solvents	>50%

Table S2. Expected monoisotopic masses and mass error (ppm) between the calculated and observed MALDI-TOF values of the target compounds.

Compd	MALDI	Calculated	ppm	Compd	MALDI	Calculated	ppm
1	352.204	352.158	130.623186	11	352.232	352.158	210.132952
2	345.173	345.153	57.945317	12	345.227	345.153	214.397673
3	369.250	369.209	111.048214	13	369.280	369.209	192.303005
4	361.176	361.123	146.764399	14	361.200	361.123	213.223749
5	407.136	407.071	159.677304	15	405.152	405.073	195.026575
6	453.164	453.059	231.757895	16	454.066	453.059	2222.668571
7	357.201	357.173	78.393384	17	357.251	357.173	218.381569
8	341.224	341.178	134.826982	18	341.256	341.178	228.619665
9	342.264	342.173	265.947342	19	342.248	342.173	219.187370
10	341.270	341.178	269.653964	20	341.244	341.178	193.447409

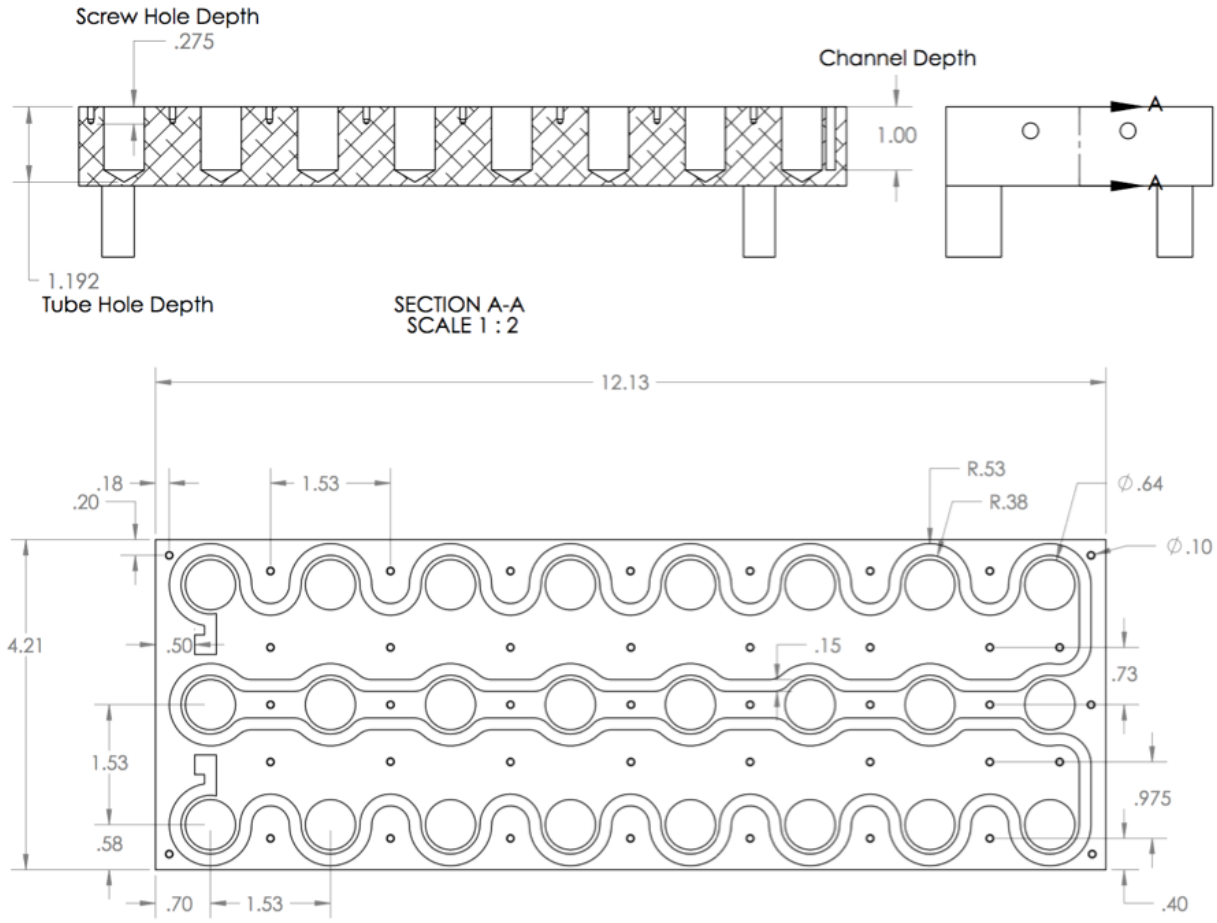

Figure S1. Schematic drawing of the heating/cooling rack. The rack is customized to control temperature between −20 °C and 150 °C by connecting a chiller with adding coolant (50/50 vol% water/ethylene glycol) while shaking.

1. STEP:2
2. GROUP:1
3. SPBD:D:RR:5
4. RA:RR:RACK2
5. LH:A:RACK2
6. GROUP:2
7. RA:RR:RACK1
8. LH:T:RACK1:RACK2:1:2
9. RA:RACK1:RR
10. GROUP:1
11. SHAKE:RACK2:600:300
12. LH:A:RACK2
13. LH:W:RACK2:5:3:2:3
14. LH:W:RACK2:5:1:2:3
15. LH:W:RACK2:5:2:2:3
16. GROUP:2
17. RA:RR:RACK1
18. LH:T:RACK1:RACK2:2:2
19. RA:RACK3:RR
20. GROUP:1
21. CHILL:100
22. SHAKE:RACK1:1440:300
23. CHILL:25
24. LH:A:RACK2
25. LH:W:RACK2:5:5:2:3
26. LH:W:RACK2:5:2:2:3
27. LH:W:RACK2:5:1:2:3
28. GROUP:2
29. RA:RR:RACK1
30. LH:T:RACK1:RACK2:2:2
31. RA:RACK1:RR
32. GROUP:1
33. RA:RACK2:MW
34. MW:10:60:200:110:111:250:TRUE:FALSE // turn on stir
35. RA:MW:RACK2
36. LH:A:RACK2
37. LH:W:RACK2:5:5:2:3
38. LH:W:RACK2:5:2:2:3
39. LH:W:RACK2:5:1:2:3

Figure S2. Command sequences for the automated synthesis of BMB library. The series of command sequences were input into the command sequence creator, which was then loaded to the automated graphical user interface to drive the machine to do automated synthesis.

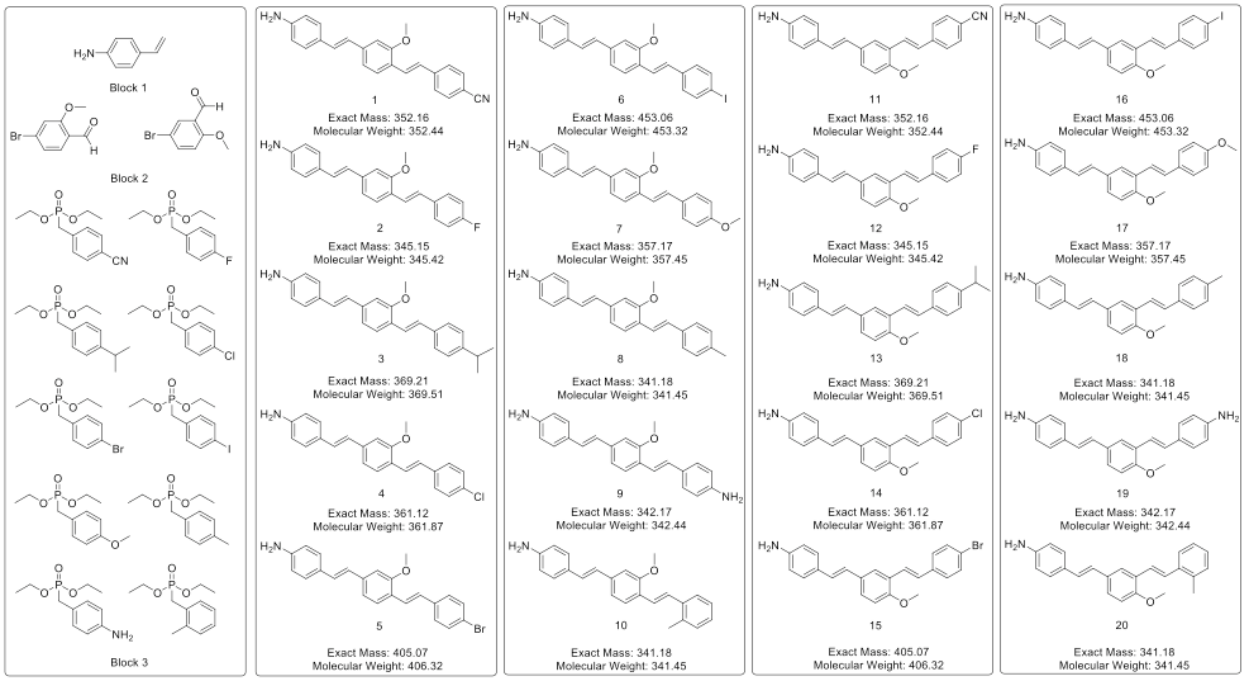

Figure S3. Chemical structures of the BMB library obtained from the automated synthesis. Note: diethyl (4-aminobenzyl)phosphonate in Block 3 was protected with *tert*-butyloxycarbonyl (BOC) prior to the use for BMB library synthesis.

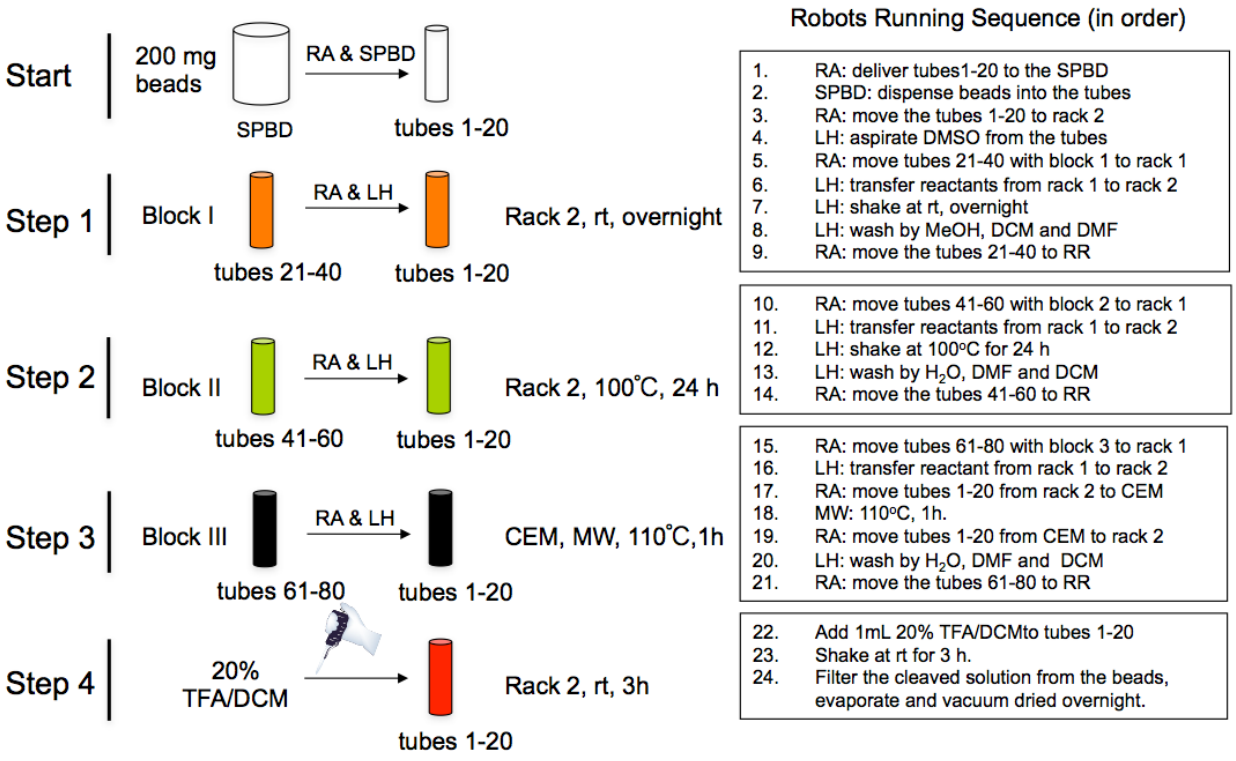

Figure S4. Step-by-step sequence for automated BMB library synthesis. Beads dispense and the following reactions of loading Block 1, Block 2, and Block 3 were automatically accomplished. Cleavage of the target compounds from the beads was achieved manually.

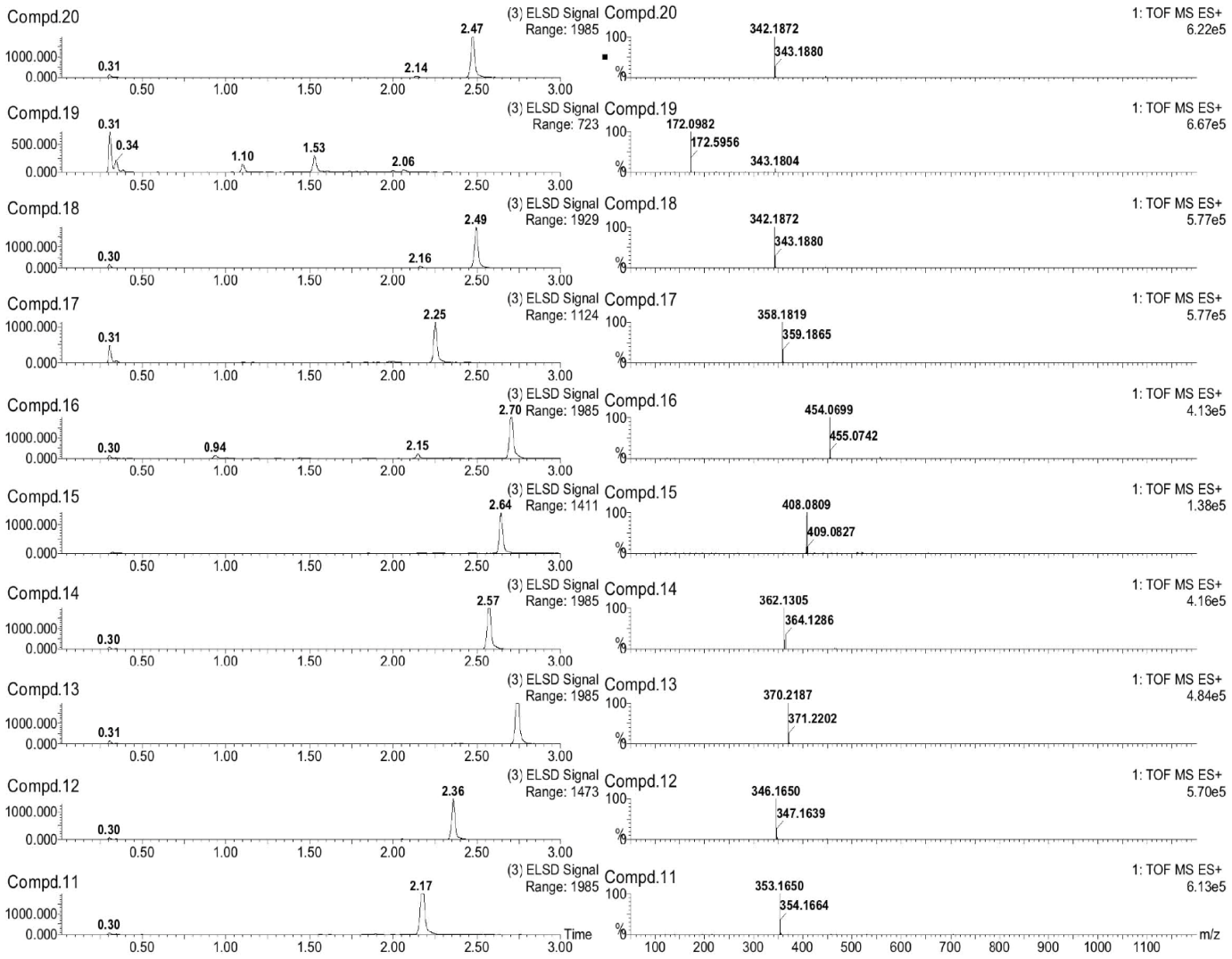

Figure S5. UPLC and ESI MS analyses manually synthesized BMB library. Column: Waters ACQUITY UPLC BEH C18 (2.1 x 50 mm 1.7 µm), gradient: 10% to 90% from solvent A to B for 5 minutes (solvent A = 0.1% formic acid in water, solvent B = 0.1% formic acid in acetonitrile), flow rate: 0.6 ml/min.

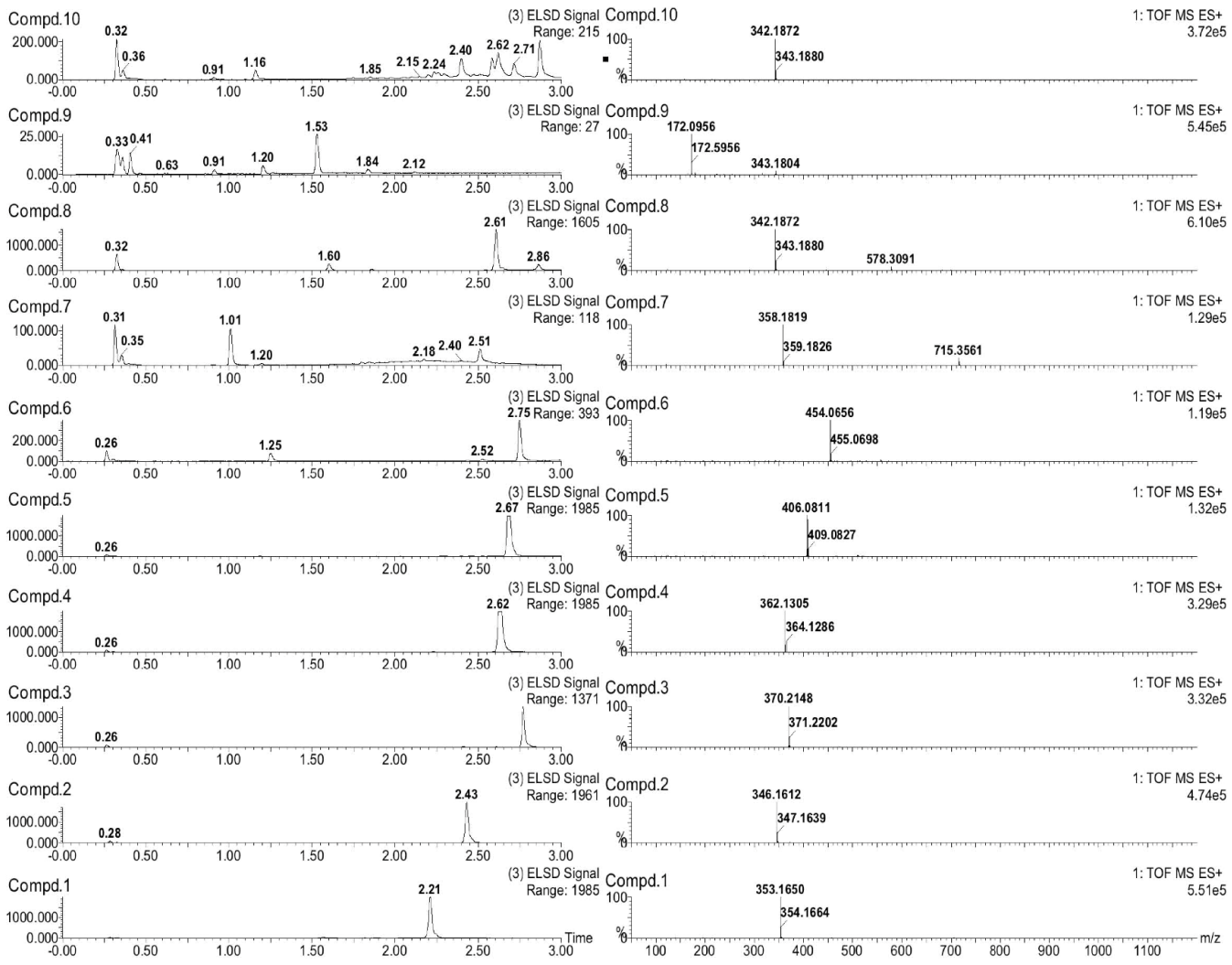

Figure S5 (continued). UPLC and ESI MS analyses manually synthesized BMB library. Column: Waters ACQUITY UPLC BEH C18 (2.1 x 50 mm 1.7 µm), gradient: 10% to 90% from solvent A to B for 5 minutes (solvent A = 0.1% formic acid in water, solvent B = 0.1% formic acid in acetonitrile), flow rate: 0.6 ml/min.

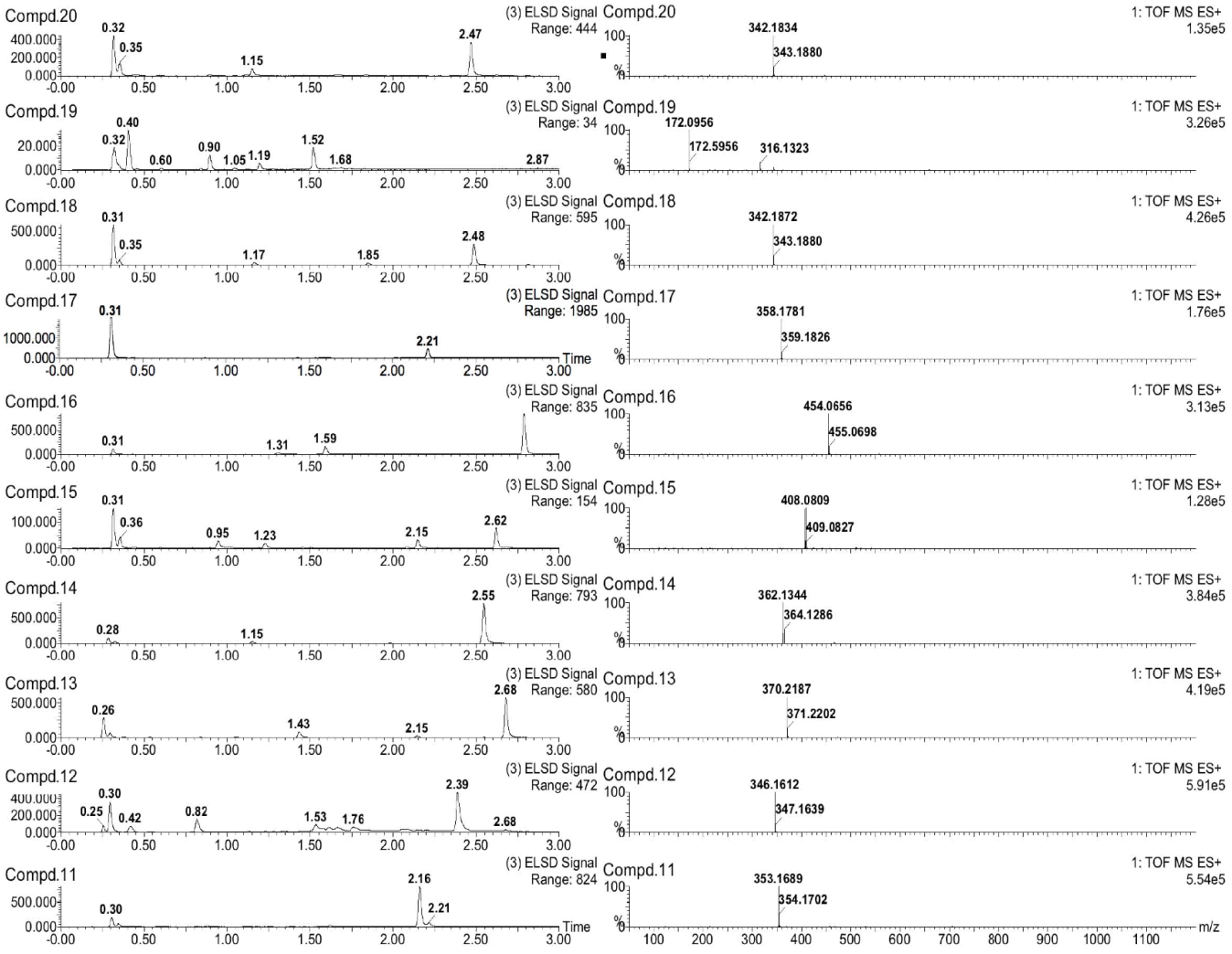

Figure S6. UPLC and ESI MS analyses of automatically synthesized BMB library. Column: Waters ACQUITY UPLC BEH C18 (2.1 x 50 mm 1.7 µm), gradient: 10% to 90% from solvent A to B for 5 minutes (solvent A = 0.1% formic acid in water, solvent B = 0.1% formic acid in acetonitrile), flow rate: 0.6 ml/min.

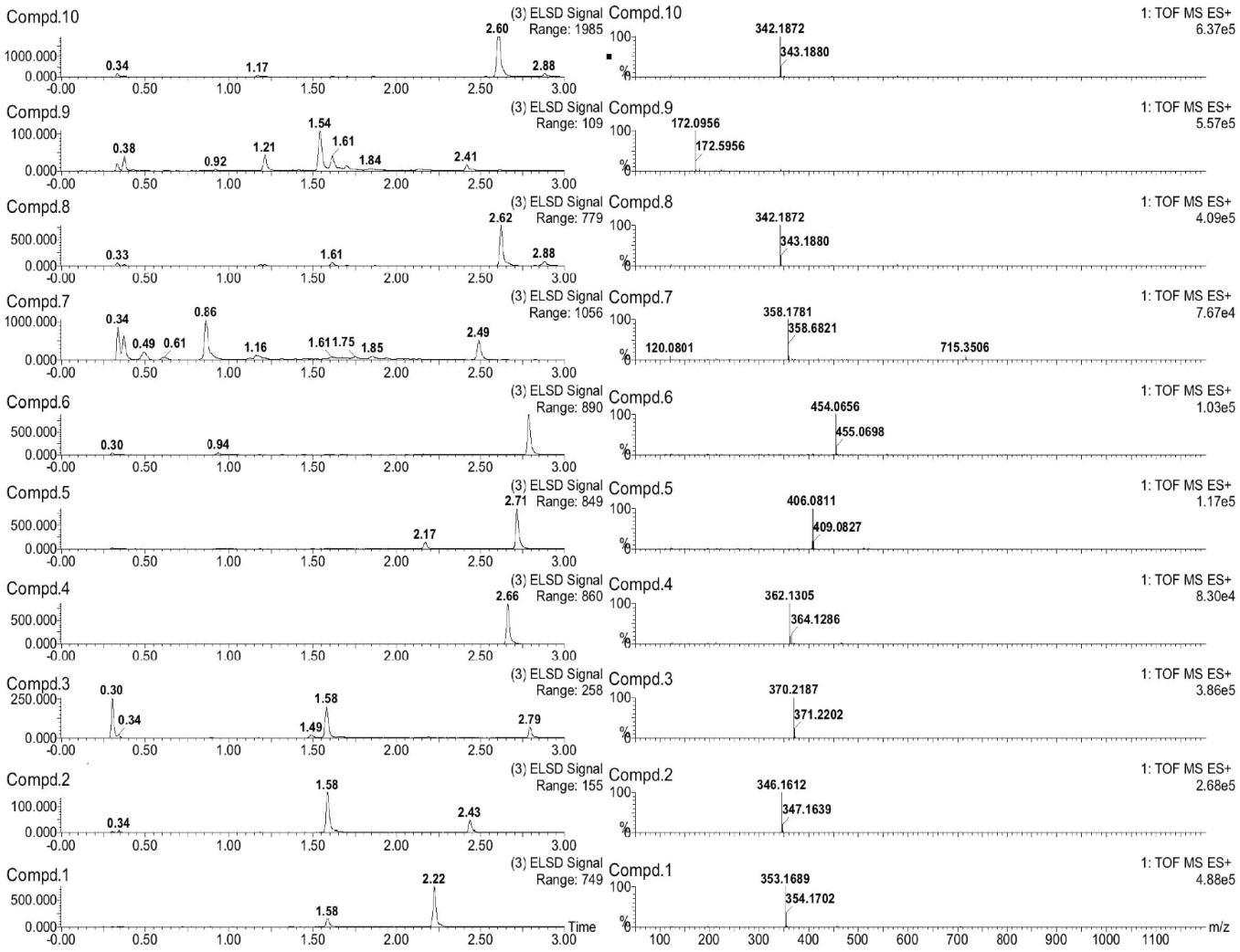

Figure S6 (continued). UPLC and ESI MS analyses of automatically synthesized BMB library. Column: Waters ACQUITY UPLC BEH C18 (2.1 x 50 mm 1.7 µm), gradient: 10% to 90% from solvent A to B for 5 minutes (solvent A = 0.1% formic acid in water, solvent B = 0.1% formic acid in acetonitrile), flow rate: 0.6 ml/min.

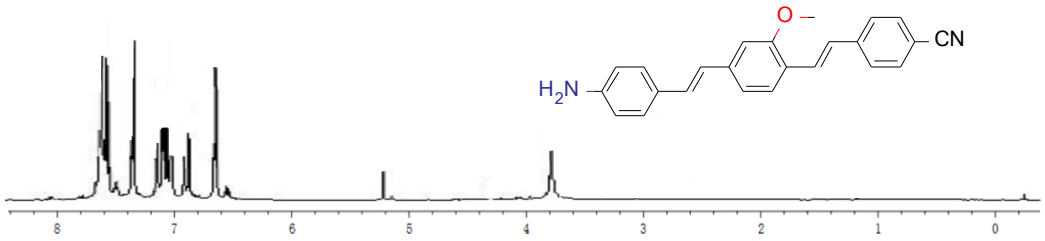

Figure S7. ^{1}H-NMR of Compound 1, 4-((*E*)-4-((*E*)-4-aminostyryl)-2-methoxystyryl) benzonitrile. ^{1}H-NMR (CD_2Cl_2, 400 MHz): δ 7.67-7.58 (m, 6H), 7.40 (d, 2H, *J* = 8 Hz), 7.19-7.08 (m, 4H), 6.93 (d, 1H, *J* = 16 Hz), 6.72 (d, 2H, *J* = 8 Hz), 3.98 (s, 3H).

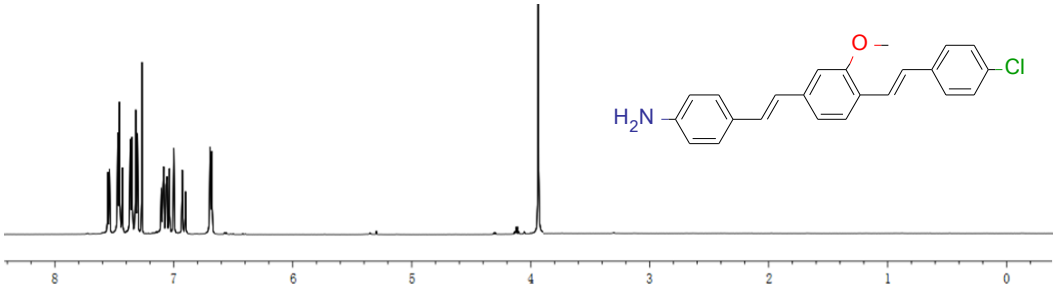

Figure S8. ^{1}H-NMR of Compound 4, 4-(4-(4-chlorostyryl)-3-methoxystyryl)aniline. ^{1}H-NMR ($CDCl_3$, 600 MHz): δ 7.54 (d, 1H, *J* = 8.0 Hz), 7.44 (dd, 3H, *J* = 16.5 Hz, J = 6.8 Hz), 7.35 (d, 2H, *J* = 8.3 Hz,), 7.30 (d, 2H, *J* = 8.4 Hz), 7.11-6.91 (m, 5H), 6.68 (d, 2H, *J* = 8.3 Hz), 3.94 (s, 3H).

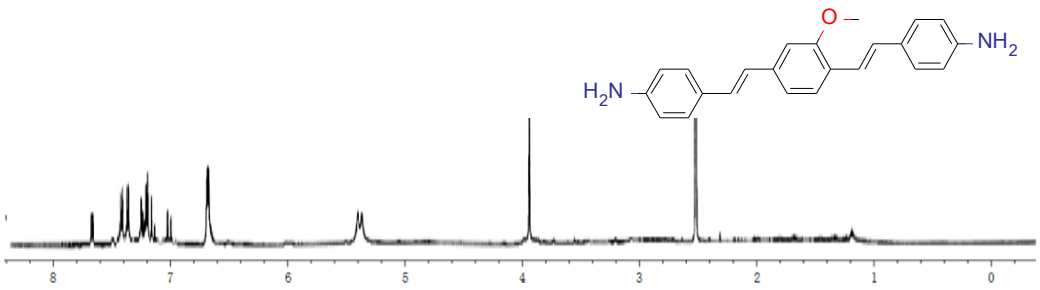

Figure S9. ^{1}H-NMR of Compound 9, 4,4'-(1E,1'E)-2,2'-(2-methoxy-1,4-phenylene)bis(ethene-2,1-diyl)dianiline. ^{1}H-NMR (DMSO-d_6, 600 MHz): δ 7.51 (d, 1H, *J* = 7.8 Hz), 7.26 (d, 2H, *J* = 8.4 Hz), 7.21 (d, 2H, *J* = 8.4 Hz), 7.09-6.98 (m, 5H), 6.87 (d, 1H, *J* = 16.8 Hz), 6.54 (dd, 4H, *J* = 8.4 Hz, *J* = 4.8 Hz), 5.29 (m, 4H), 3.86 (s, 3H).

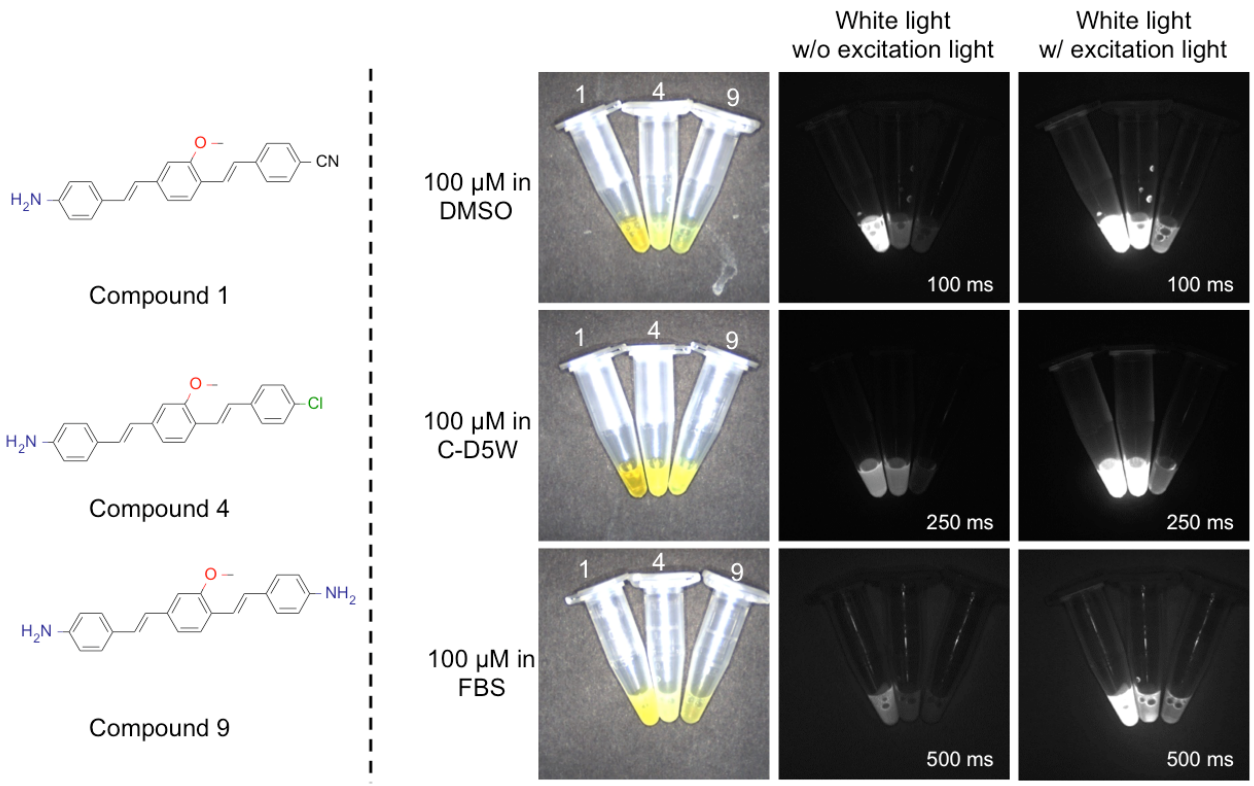

Figure S10. Comparison in *in vitro* brightness of Compounds 1, 4, and 9 in various solvents. Stock solutions were made in DMSO, C-D5W, and FBS at a concentration of 100 µM.

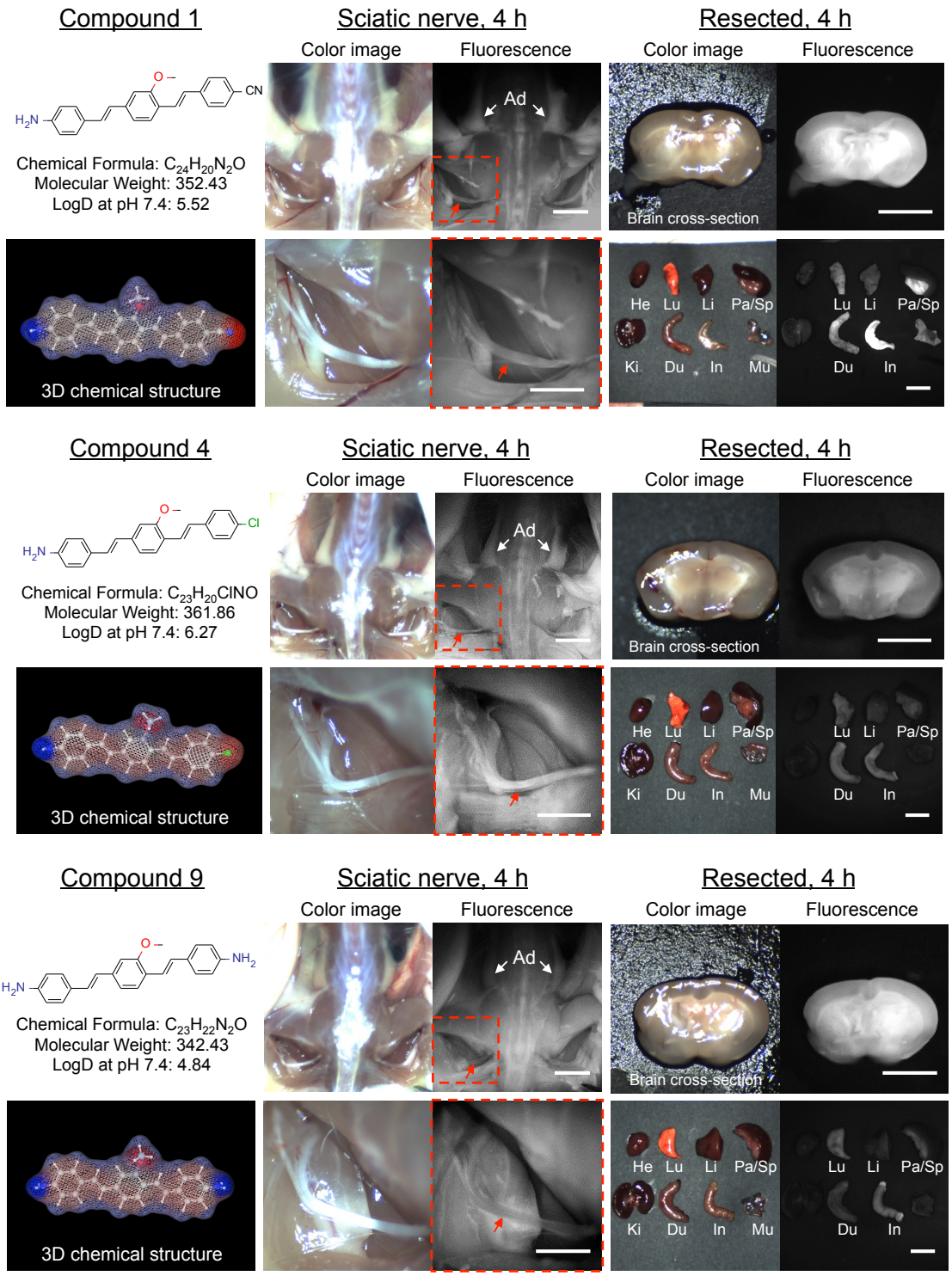

Figure S11. Comparison in *in vivo* brightness of Compounds 1, 4 and 9 in nervous tissue. 0.5 mg/kg of each compound in D5W was injected intravenously into CD-1 mice 4 h prior to imaging. All images were acquired from sciatic nerve and cross-sectioned brain tissue by intraoperative fluorescence imaging system. Abbreviations used are: Ad, adipose; Du, duodenum; He, Heart; In, intestine; Ki, kidney; Li, liver; Lu, lungs; Mu, muscle; Pa, pancreas; Sp, spleen. Scale bar = 0.4 cm.