Supporting Information
for

In-vivo toxicity of zebrafish larvae, antioxidant and antimicrobial potential: An investigation of biological applications in sulphonated succinimide

Mathiyazhagan Sivanantham,[a] Gopal Chandru Senadi,[a] Chinnasamy Ragavendran,[c] Mohankumar Ramasamy[a,b]* and Chinnaperumal Kamaraj[b]*

[a] Department of Chemistry, Faculty of Engineering and Technology, SRM Institute of Science and Technology, SRM Nagar, Kattankulathur - 603 203, Chengalpattu District, Tamil Nadu, India.

[b] Interdisciplinary Institute of Indian System of Medicine, SRM Institute of Science and Technology, SRM Nagar, Kattankulathur - 603 203, Chengalpattu District, Tamil Nadu, India.

[c] Department of Conservative Dentistry and Endodontics, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Chennai-600 077, India.

Email: mohankur@srmist.edu.in, kamarajc@srmist.edu.in

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1. General Information

Those compounds **4aa**-**4al**, **4ba**-**4fa**, **4ha**-**4ma**, **6**, and **7** were used from our previous work.[1] All the compounds NMR, HR-MS and Single crystal XRD of compound **4aa** data were already reported in our previous work[1] and their methodology were developed already. Further, we want to explore the biological importance of our sulphonated succinimide compounds. All commercial chemicals and solvents were purchased from Merck, Avra, Carbanio, BLD pharm and SRL.

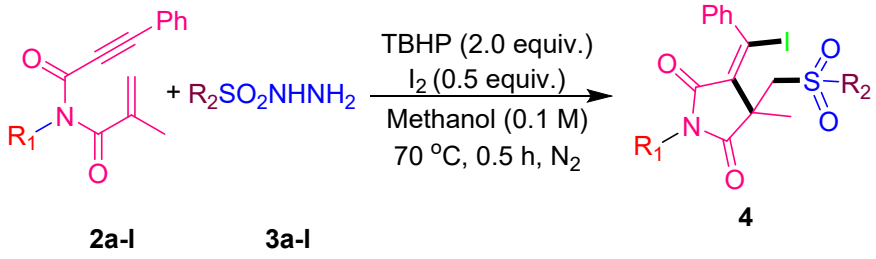
2. Antioxidant enzyme assay in larvae

For biochemical assays, zebrafish larvae (96 hpf) was exposed to compounds **4**, **6** and **7**. The different exposure groups were collected and homogenized with 0.1 M ice-cold PBS (pH 7.4). The homogenate was then centrifuged at 5000 rpm for 15 min. Protein estimation, SOD, and CAT assays were all performed on the supernatant. 30 larvae per group were used in the biochemical experiments, and each experiment was done in triplicate. The Bradford method was used to determine the protein level of the sample. The activity of SOD was measured as previously described.[2] Briefly, the supernatant (50 µL) was added to the reaction mixture containing 50 mM Tris·HCl buffer (pH 8.4), 1 mM EDTA and 2.64 mM pyrogallol. The absorbance was measured at 420 nm in a micro plate reader. The activity was expressed as U/mg protein. CAT assay was done by the method described previously.[3] The supernatant (50 µL) was added to the reaction mixture (3 mL) containing glacial acetic acid, 5% potassium dichromate, and 10 mM phosphate buffer. Further, the reaction mixture was kept in a water bath (70°C) for 20 min, and the absorbance was measured in a micro plate reader at 570 nm.

3. Minimum inhibitory concentration Method

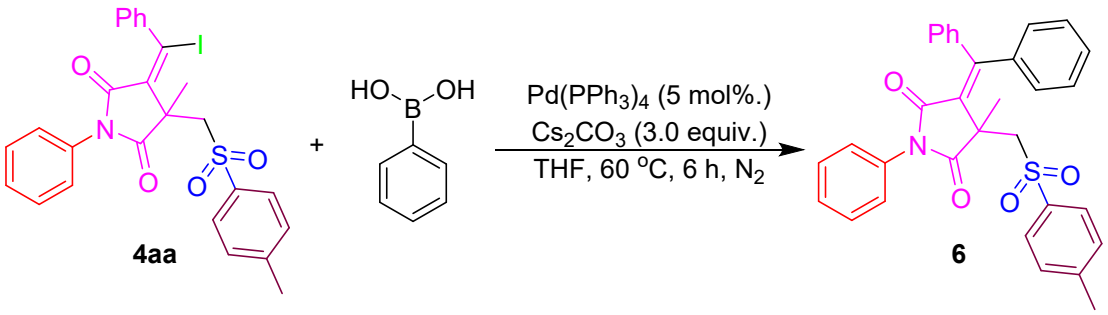
MICs of **4aa**, **4ac**, **4af**, **4ag**, **4ca**, **4ea**, **6**, and **7** against different bacterial strains were determined using an amended broth macro-dilution method.[4] For the estimation of MIC, the stock solutions of **4aa**, **4ac**, **4af**, **4ag**, **4ca**, **4ea**, **6**, and **7** (0.5, 1, 2.5, 5, 7.5, 10, 12.5, 15, 25, 50, 100, and 200 µg/mL) were prepared. The sterile Mueller–Hinton broth was transferred into a sugar test tube (13 × 100 mm) containing 2.0 mL of bacterial inoculum (culture density of 5 × 10^5 CFU/mL) in two sets for each bacterial strain. Subsequently, each test tube was mixed with 2.0 mL individual concentrations of **4aa**, **4ac**, **4af**, **4ag**, **4ca**, **4ea**, **6**, and **7** limiting the final tube volume to 4.0 mL, resulting in a 1:2 dilution followed by incubation for 24 h at 37 °C. The optical density (O.D.) of microbial growth was measured at 600 nm. The lowest dose of **4aa**, **4ac**, **4af**, **4ag**, **4ca**, **4ea**, **6**, and **7** exhibiting no growth after incubation was considered the MIC endpoint.

4. General procedure for the synthesis of 4aa-al and 4ba-ma.[1]



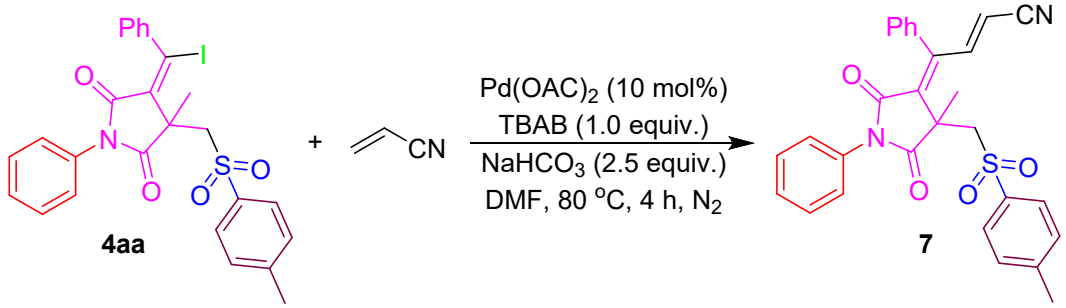
A round-bottom flask equipped with a magnetic stir bar was charged with **2a-l** (0.5 mmol), **3a-l** (1.0 mmol) and I_2 (0.25 mmol), sealed with a septum, and degassed by alternating vacuum evacuation and N_2 back filling. Then TBHP (1.0 mmol) and methanol (0.1M) was charged under N_2 atmosphere. Then the reaction mixture was heated to 70 °C with stirring for 0.5 h (monitored reactions by TLC). After the completion, the precipitated solid was filtered and washed with methanol (2x3 mL) to afford the pure compounds **4aa-al** and **4ba-ma** as an off-white to white solid.

5. Synthesis of 4-(diphenylmethylene)-3-methyl-1-phenyl-3-(tosylmethyl)pyrrolidine-2,5-dione (6).[1]



To a round-bottom flask added **4aa** (0.1 mmol), phenyl boronic acid (0.2 mmol), $Pd(PPh_3)_4$ (5 mol %), Cs_2CO_3 (0.3 mmol) and degassed by alternating vacuum evacuation and N_2 back filling. Then, THF (1 mL) was added under N_2 atmosphere.Then the reaction mixture was heated to 60 ºC with stirring for 6 h (monitored reactions by TLC). The mixture was diluted with H_2O (20 mL) and extracted with ethyl acetate (3×25 mL). The organic layers were dried with Na_2SO_4 and the solvent was then removed under reduced pressure with the aid of a rotary evaporator. The crude material was purified by silica gel column chromatography (Hex:EA=8:2) to afford the corresponding product **6** as white solid.

6. Synthesis of (2*E*,4*Z*)-4-(4-methyl-2,5-dioxo-1-phenyl-4-(tosylmethyl)pyrrolidin-3-ylidene)-4-phenylbut-2-enenitrile (7).[1]



To a round-bottom flask added compound **4aa** (0.1 mmol), $Pd(OAc)_2$ (0.01 mmol), $(Bu)_4NBr$ (0.1 mmol), $NaHCO_3$ (0.25 mmol) and degassed by alternating vacuum evacuation and N_2 back filling. Then, acrylonirile (0.2 mmol), DMF (1.0 mL) were added under N_2 atmosphere and the reaction mixture was heated to 80 ºC with stirring for 4h (monitored reactions by TLC). The mixture was diluted with H_2O (20 mL) and extracted with ethyl acetate (3×25 mL). The organic layers were dried with Na_2SO_4 and the solvent was then removed under reduced pressure with the aid of a rotary evaporator. The crude material was purified by silica gel column chromatography (Hex:EA=7:3) to afford the corresponding product **7** as white solid.

7. IC_{50} values of compounds 4, 6, and 7

Table S1. IC_{50} values in the DPPH, and ABTS radical-scavenging activity assay of succinimide derivatives and SE values

Name of the compounds	[a]DPPH assay [c]IC_{50} ± [d]SE (µM)	[b]ABTS assay IC_{50} ± SE (µM)
Control (Trolox)	20.93 ± 0.56	27.63 ± 0.85
4aa	27.55 ± 0.55	32.38 ± 0.45
4ab	65.49 ± 1.24	126.04 ± 2.11
4ac	49.87 ± 0.76	39.46 ± 0.45
4ad	70.33 ± 1.03	111.20 ± 1.00
4ae	55.30 ± 0.67	56.33 ± 0.65
4af	58.72 ± 1.07	65.14 ± 0.81
4ag	114.66 ± 2.32	122.5 ± 1.53
4ah	105.51 ± 2.17	47.17 ± 0.52

4ai	96.39 ± 1.89	116.67 ± 0.98
4aj	96.91 ± 1.57	107.92 ± 1.20
4ak	114.89 ± 2.71	141.40 ± 1.57
4al	73.99 ± 1.48	94.85 ± 1.02
4ba	104.84 ± 1.74	114.79 ± 1.67
4ca	41.89 ± 0.83	40.68 ± 0.72
4da	138.13 ± 2.21	149.57 ± 1.43
4ea	38.85 ± 0.44	40.95 ± 0.54
4fa	124.93 ± 1.74	136.80 ± 0.99
4ha	138.13 ± 2.38	120.08 ± 2.11
4ia	156.96 ± 2.76	217.41 ± 1.34
4ja	88.22 ± 1.43	158.78 ± 1.94
4ka	102.99 ± 1.97	245.64 ± 2.21
4la	95.44 ± 1.12	196.29 ± 2.54
4ma	154.86 ± 1.73	180.88 ± 2.23
6	24.60 ± 0.33	28.09 ± 0.53
7	21.87 ± 0.87	25.00 ± 0.73

[a]DPPH, and [b]ABTS radical-scavenging activity assay of succinimide derivatives. [c]Inhibitory concentration is estimated to inhibit 50% of specific assay activity. [d] Standard error.

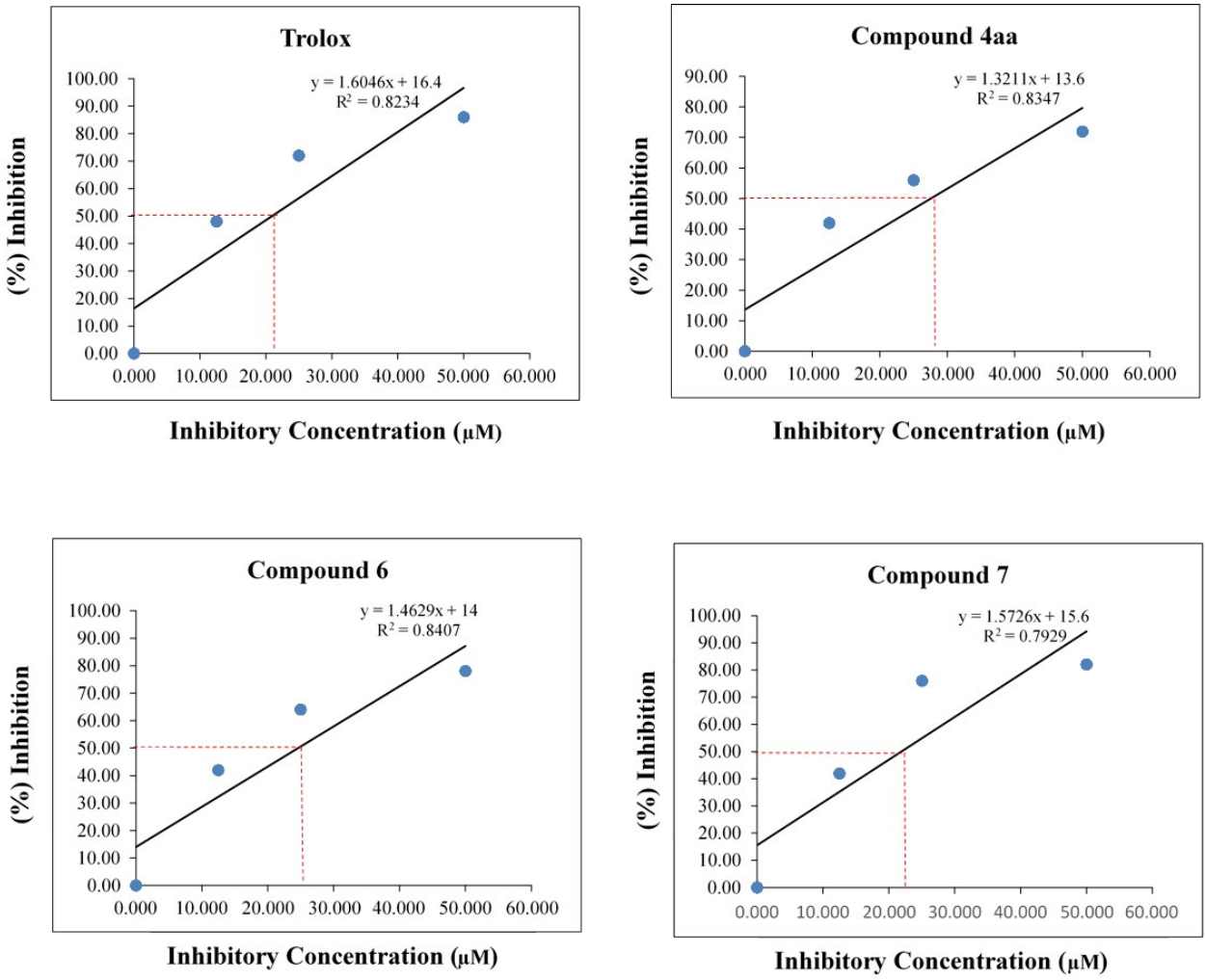


Figure S1. IC_{50} values in the DPPH radical-scavenging activity assay of highly active succinimide derivatives.

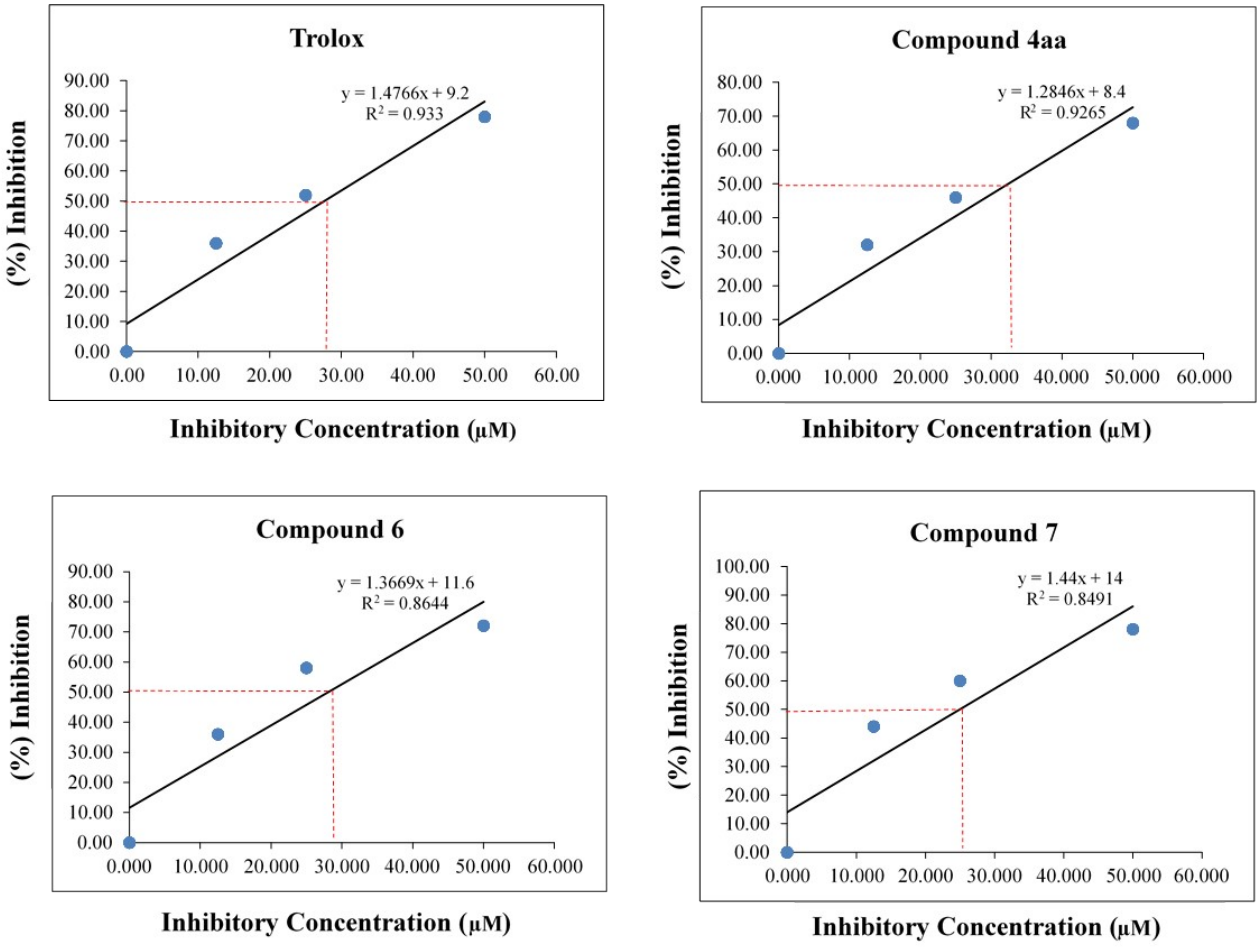


Figure S2. IC_{50} values in the ABTS radical-scavenging activity assay of highly active succinimide derivatives.

8. HPLC analysis

The HPLC analysis were performed on a Shimadzu LC-20AD HPLC system equipped with LC10ADVP binary pump (Shimadzu, Japan) and Rheodyne 7725 injection valve furnished with 20µL loop. The sample was separated in Phenomenex C18 column (RP, 250x4.6 mm, 5µm) using Acetonitrile:Water (0.1% formic acid) as mobile phase in the ratio

of (75:25). Injection volume as 20 µl and flow rate was set at 1.0 mL/min. Detection was done at a wavelength (λ) of 238nm using an SPD-M20A photodiode array detector and column was maintained at ambient temperature. All the data were collected and processed by using Lab Solution Software.

9. References

1. M. Sivanantham, A. Jennifer G, E. Varathan, M. Ramasamy and G. C. Senadi, *Org. Biomol. Chem.*, 2022, **20**, 7942-7948.
2. A. Sannasimuthu, V. Kumaresan, S. Anilkumar, M. Pasupuleti, M. R. Ganesh, K. Mala, B. A. Paray, M. K. Al-Sadoon, M. F. Albeshr and J. Arockiaraj, *Free Radical Biol. Med.,* 2019, **135**, 198-209.
3. A. Sannasimuthu, V. Kumaresan, M. Pasupuleti, B. A. Paray, M. K. Al-Sadoon and J. Arockiaraj, *Algal Res.,* 2018, **35**, 519-529.
4. (a) JF. Hernandez-Sierra, F. Ruiz, D. C. C. Pena, F. Martinez-Gutierrez, A. E. Martinez, A. D. J. P. Guillen, H. Tapia-Perez and G. M. Castanon, *Nanomed. Nanotechnol. Biol. Med.*, 2008, **4**, 237–240. (b) G.-A. Martinez-Castanon, N. Nino-Martinez, F. Martinez-Gutierrez, J. R. Martinez-Mendoza and F. Ruiz, *J. Nanopart. Res.*, 2008, **10**, 1343–1348.

Sample Information

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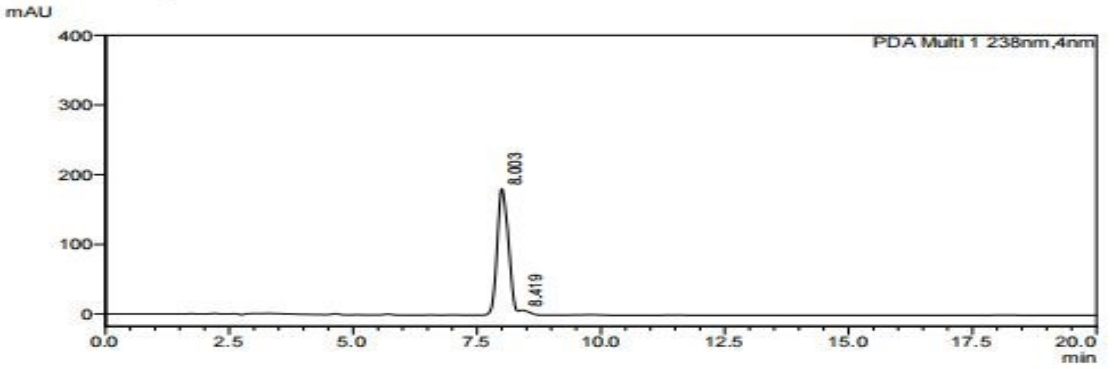

Peak Table

PDA Ch1 238nm

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1	8.003	2703146	177537	99.029
2	8.419	26516	2247	0.971
Total		2729661	179784	100.000

Figure S3. HPLC data of (*E*)-4-(iodo(phenyl)methylene)-3-methyl-1-phenyl-3-(tosyl methyl)pyrrolidine-2,5-dione **(4aa)**

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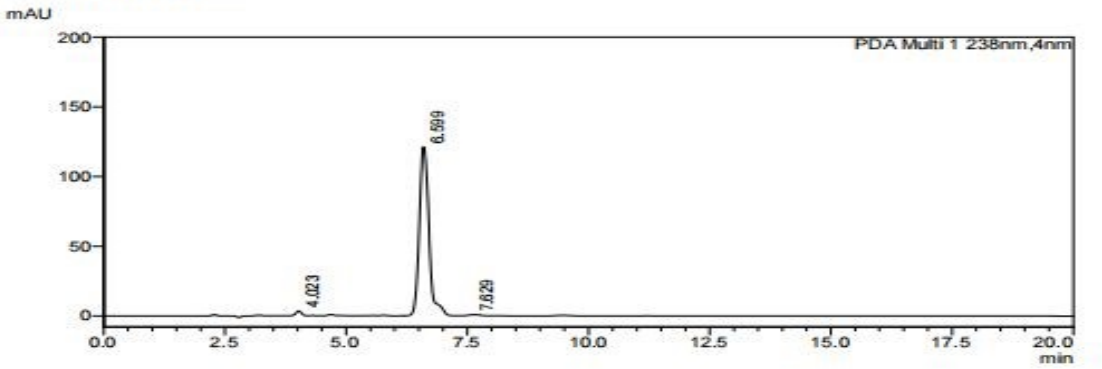

Peak Table

PDA Ch1 238nm

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1	4.023	15243	2510	0.903
2	6.599	1664578	120705	98.625
3	7.629	7965	626	0.472
Total		1687786	123841	100.000

Figure S4. HPLC data of (*E*)-4-(iodo(phenyl)methylene)-3-(((4-methoxyphenyl) sulphonyl)methyl)-3-methyl-1-phenyl pyrrolidine-2,5-dione **(4ac)**

Sample Information

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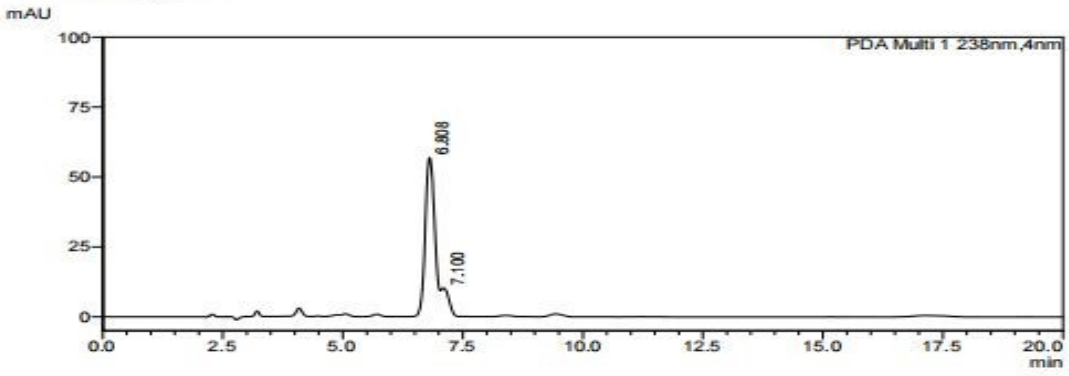

Peak Table

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1	6.808	640071	51605	99.070
2	7.100	6009	1272	0.930
Total		646080	52877	100.000

Figure S5. HPLC data of (*E*)-3-(((4-fluorophenyl)sulphonyl)methyl)-4-(iodo(phenyl) methylene)-3-methyl-1-phenyl pyrrolidine-2,5-dione **(4af)**

Sample Information

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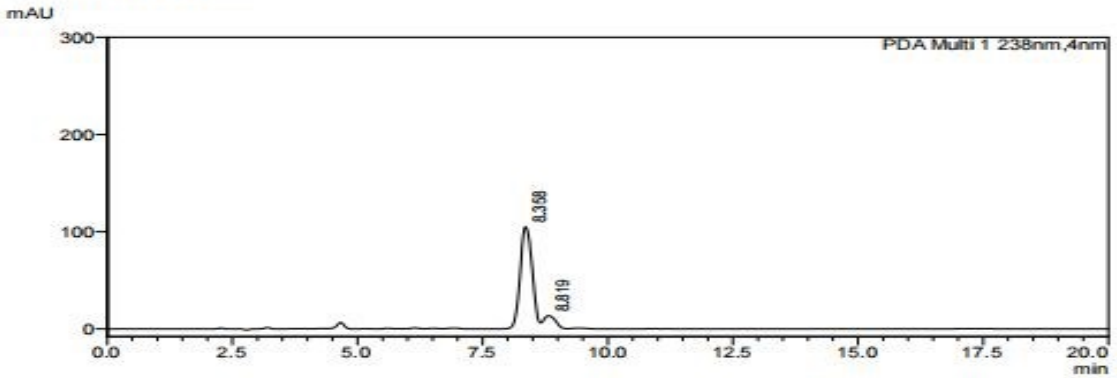

Peak Table

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2	8.819	38910	4487	2.339
Total		1663515	105937	100.000

Figure S6. HPLC data of (*E*)-3-(((4-chlorophenyl)sulphonyl)methyl)-4-(iodo(phenyl) methylene)-3-methyl-1-phenyl pyrrolidine-2,5-dione **(4ag)**

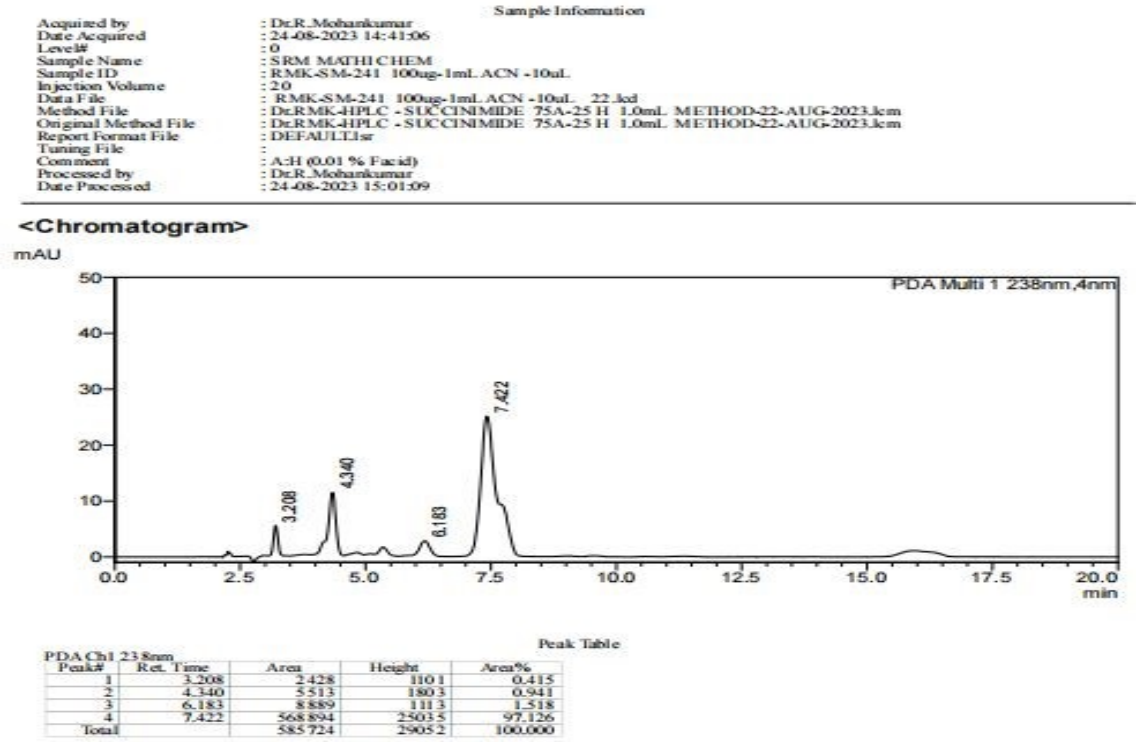

Figure S7. HPLC data of (*E*)-4-(iodo(phenyl)methylene)-1-(2-methoxyphenyl)-3-methyl-3-(tosyl methyl) pyrrolidine-2,5-dione **(4ca)**

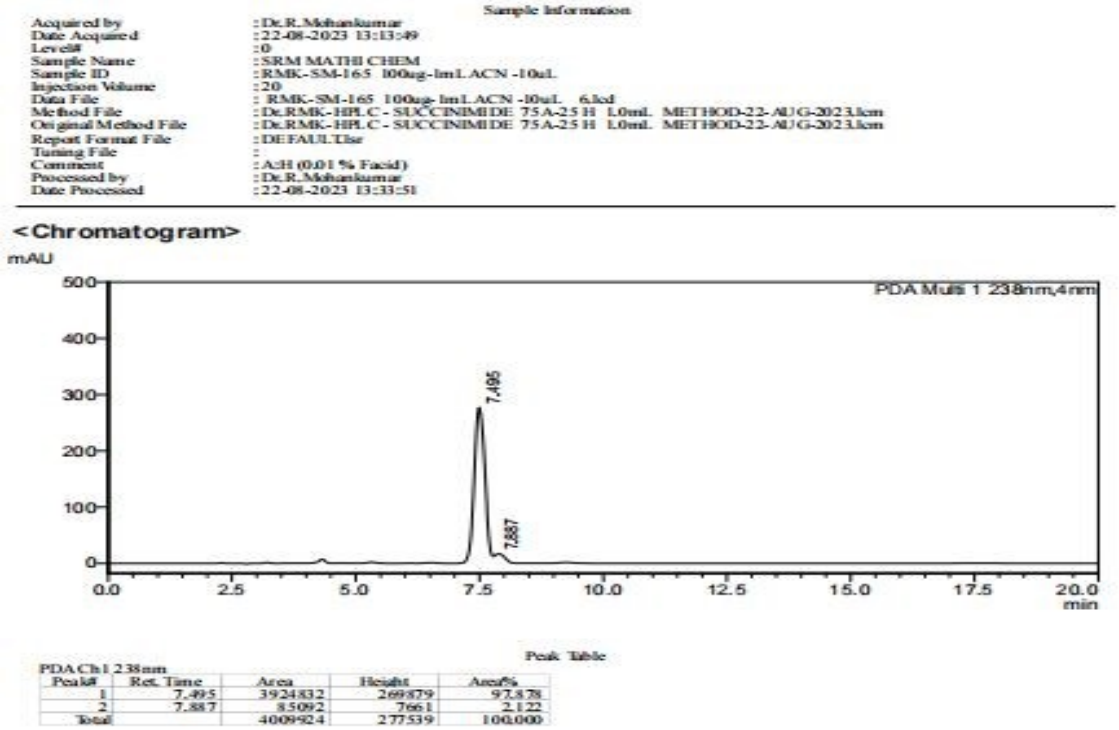

Figure S8. HPLC data of (*E*)-4-(iodo(phenyl)methylene)-1-(4-methoxyphenyl)-3-methyl-3-(tosylmethyl) pyrrolidine-2,5-dione **(4ea)**

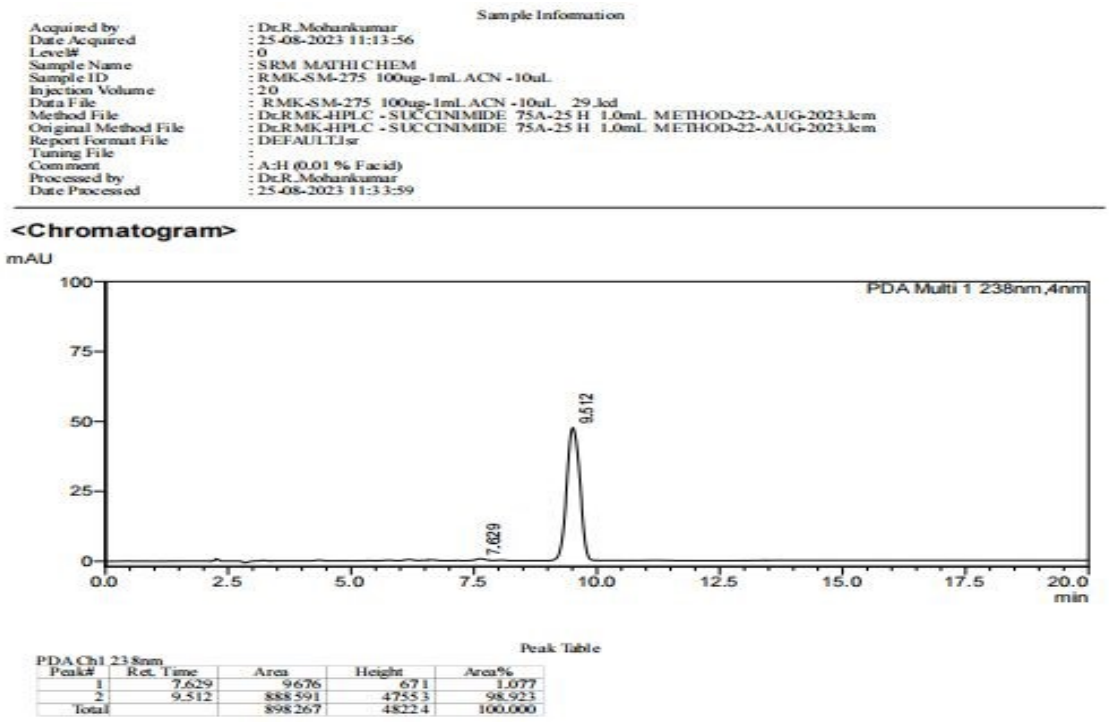

Figure S9. HPLC data of 4-(diphenylmethylene)-3-methyl-1-phenyl-3-(tosylmethyl) pyrrolidine-2,5-dione **(6)**

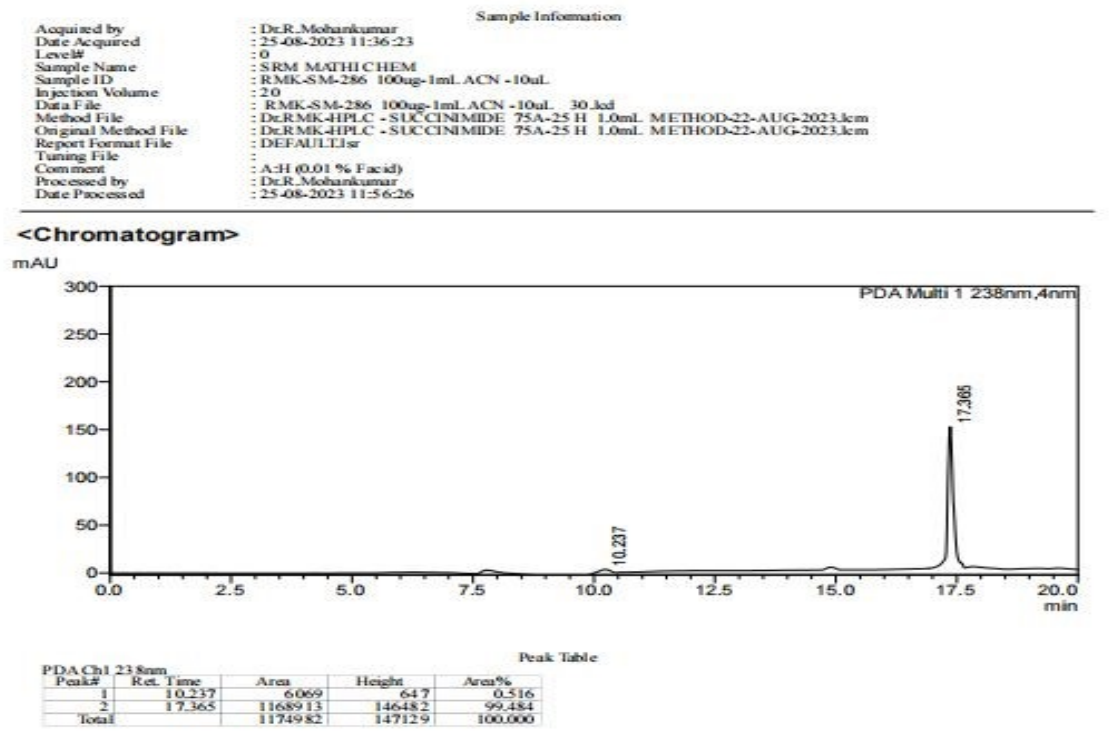

Figure S10. HPLC data of (2*E*,4*Z*)-4-(4-methyl-2,5-dioxo-1-phenyl-4-(tosylmethyl) pyrrolidin-3-ylidene)-4-phenyl but-2-enenitrile **(7)**