Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

Design, synthesis and evaluation of 1-(1,5-bis(4-substituted phenyl)-2-methyl-1H-pyrrol-3-yl)-N-methylmethanamines as SERT inhibitors with potential antidepressant action.

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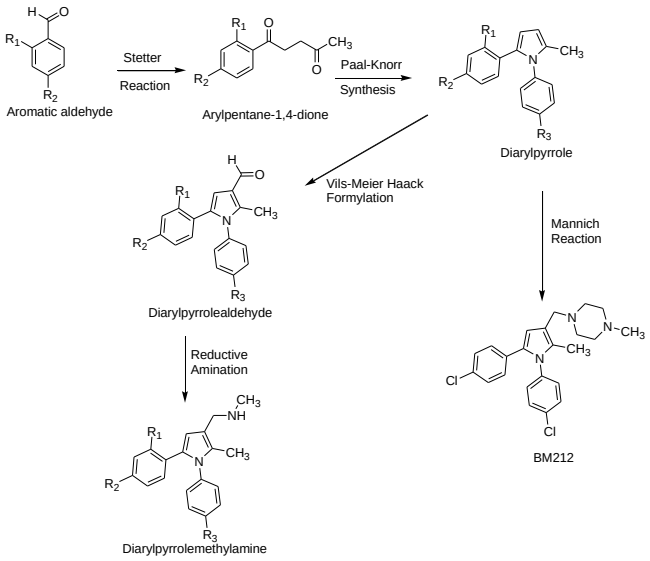
1 Synthesis of BM212 and 1-(1,5-bis(4-substituted phenyl)-2-methyl-1H-pyrrol-3-yl)-N-methylmethanamine [1 - 9]



1.1 Materials and methods

All the reagents used are of reagent grade. The reactions were monitored with pre-coated silica gel TLC plate using appropriate mobile phase. Visualization of the spots was done with the help of UV at 254nm. Melting points were noted using EZMELT-120 (Stanford Research Systems, USA) and are uncorrected. IR spectral data is recorded on Bruker (ALPHA-T) FTIR system using KBr pellet method or ATR ZnSe method as applicable. Opus 7.0 was the version of software used for processing IR data. NMR spectral data were recorded on Bruker-400 MHz instrument in appropriate deuterated solvent using TMS as internal standard. The NMR data was processed using Topshim 3.5 software. Elemental analysis experiments were conducted using Carlo Erba elemental analyzer. ESI-MS mass spectral studies were done on Agilent 6120 Quadrupole LC-MS.

Stetter reaction

The desired 1, 4-diones were synthesized by Stetter reaction. Appropriate aromatic aldehydes (1 m. mole) were treated with methylvinylketone (3 m. moles) with the use of 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazoliumchloride (0.12 m. moles) as a catalyst in the presence of triethylamine (1.5 m. moles) and sodium acetate (0.12 m. moles) using dimethylformamide as a solvent. The reaction mixture was refluxed under nitrogen for a period of 8- 12 hours. The reaction was monitored by TLC and quenched by adding cold water. The mixture was extracted with three successive quantities of ethyl acetate. The combined ethyl acetate fractions were dried over anhydrous sodium sulfate and concentrated. The concentrate was subjected to column chromatography to obtain the desired compounds in a pure form.

Paal-Knorr synthesis

Primary aromatic amine (2 m. mol) was added to arylpentan-1, 4-dione (1m. mole) taken in a round bottom flask. Glacial acetic acid was added to this mixture and refluxed on a sand bath for 6 – 8 hours. Progress of the reaction was observed by TLC. The reaction mixture was neutralized by adding dilute sodium bicarbonate solution and extracted with successive volumes of ethyl acetate. The ethyl acetate layers were combined and concentrated. Pure diaryl pyrrole was obtained by purification using column chromatography.

Mannich reaction

Diaryl pyrrole (1 m. mole) was dissolved in acetonitrile. A solution of secondary aliphatic amine (5 m. mole) in 40 % formaldehyde (5 m. mole) and glacial acetic acid was added drop wise while stirring to the solution of diaryl pyrrole. The reaction mixture was stirred at room temperature for about twelve hours. Completion of the reaction was assessed by TLC. Upon completion of the reaction; a solution of 20 percent sodium hydroxide was added drop wise to the reaction mixture while stirring until a precipitate was observed. The obtained compound was filtered, washed thoroughly with cold water and purified by recrystallization from diethyl ether.

Vilsmeier Haack formylation

Diarylpyrrole (1 m. mole) was dissolved in chloroform. Vilsmeier reagent was prepared by adding dimethyl formamide to phosphorus pentachloride (1 m. mole) taken in a beaker. The reagent was stirred on an ice bath for a few minutes. The solution of diarylpyrrole was added slowly to the Vilsmeier reagent while stirring on a water bath. After completion of addition the reaction mixture was stirred at room temperature for two to three hours. Completion of the reaction (reaction 3.4) was monitored through TLC. After completion, the reaction was quenched by the addition of chilled water. The aqueous layer was extracted with three quantities of ethyl acetate. The ethyl acetate fractions were combined, dried by the addition of anhydrous sodium sulfate and concentrated. The resulting 1, 5- diarylpyrrole-3-carbaldehyde was purified by column chromatography.

Reductive amination

The diarylpyrrole aldehyde (1 m.mol.) was dissolved in 5 ml of ethanol and 2 m. mol. of methylamine was added to this solution. The reaction mixture was stirred at room temperature for three to four hours. One m.mol of sodiumborohydride was added to this reaction mixture and stirring was continued for a period of six to twelve hours. The progress of the reaction was monitored by TLC. Once the reaction was complete, the reaction mixture was poured into cold water and extracted with ethylacetate. The ethylacetate layer was separated, dried over anhydrous sodium sulphate and concentrated. The compounds were washed with diethylether to remove any traces of impurities.

2 NMR DATA

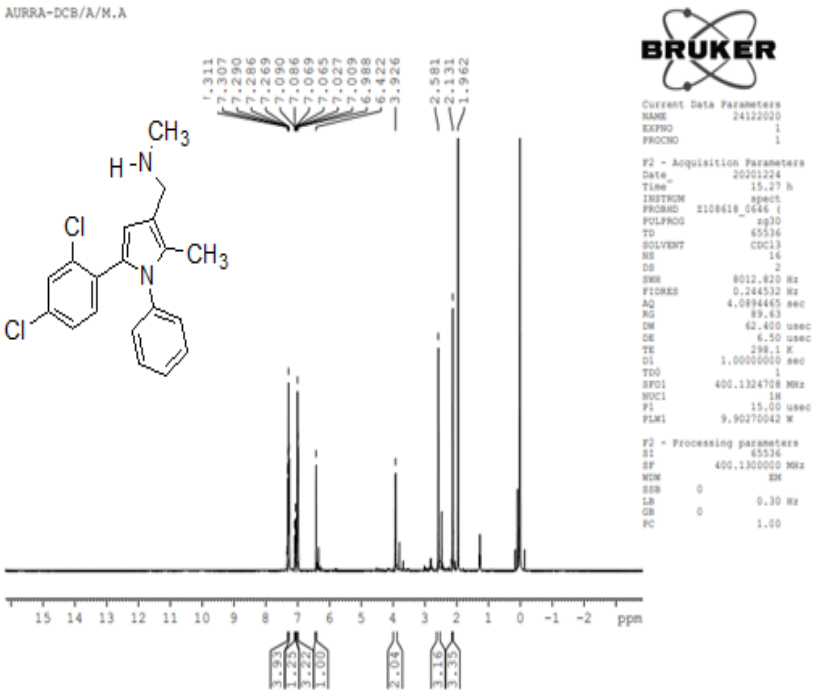


Fig 1: [1]HNMR Spectrum of [5-(2,4-Dichlorophenyl)-2-methyl-1-phenyl-1H-pyrrol-3ylmethyl]methylamine (SA-1).

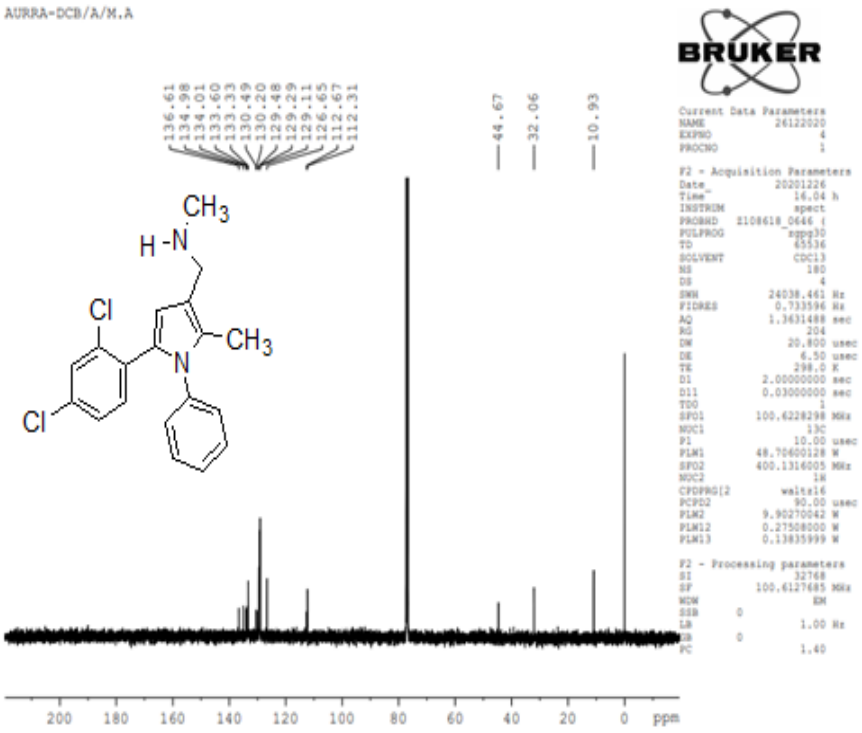


Fig 2: [13]C NMR Spectrum of [5-(2,4-Dichlorophenyl)-2-methyl-1-phenyl-1H-pyrrol-3ylmethyl]methylamine (SA-1).

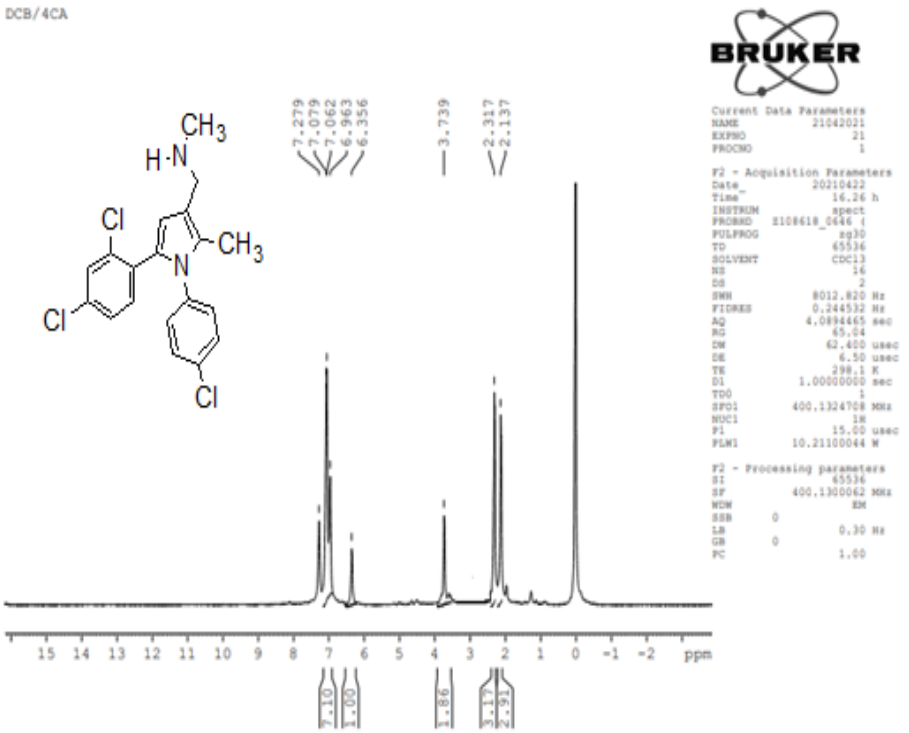


Fig 3: [1]HNMR Spectrum of [1-(4-Chlorophenyl)-5-(2,4-Dichlorophenyl)-2-methyl-1H-pyrrol-3ylmethyl]methylamine (SA-2)

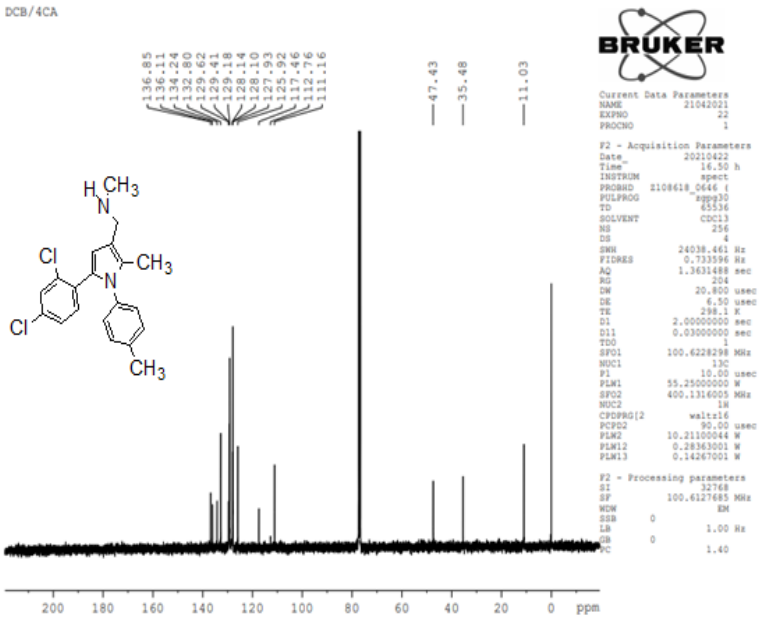


Fig. 4: [13]C NMR Spectrum of [1-(4-Chlorophenyl)-5-(2,4-Dichlorophenyl)-2-methyl-1H-pyrrol-3ylmethyl]methylamine (SA-2).

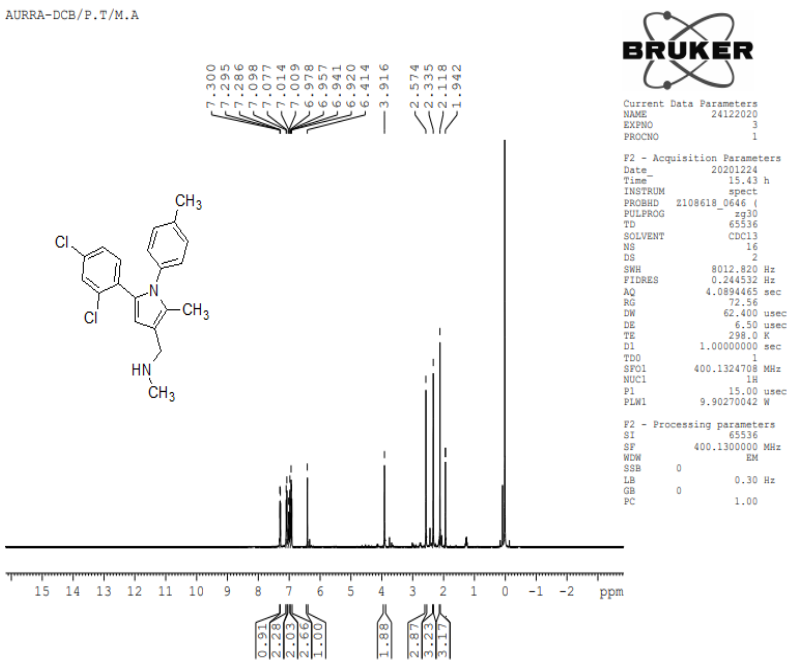


Fig 5: [1]HNMR Spectrum of [5-(2,4-Dichlorophenyl)-2-methyl-1-p-tolyl-1H-pyrrol-3ylmethyl]methylamine (SA-3).

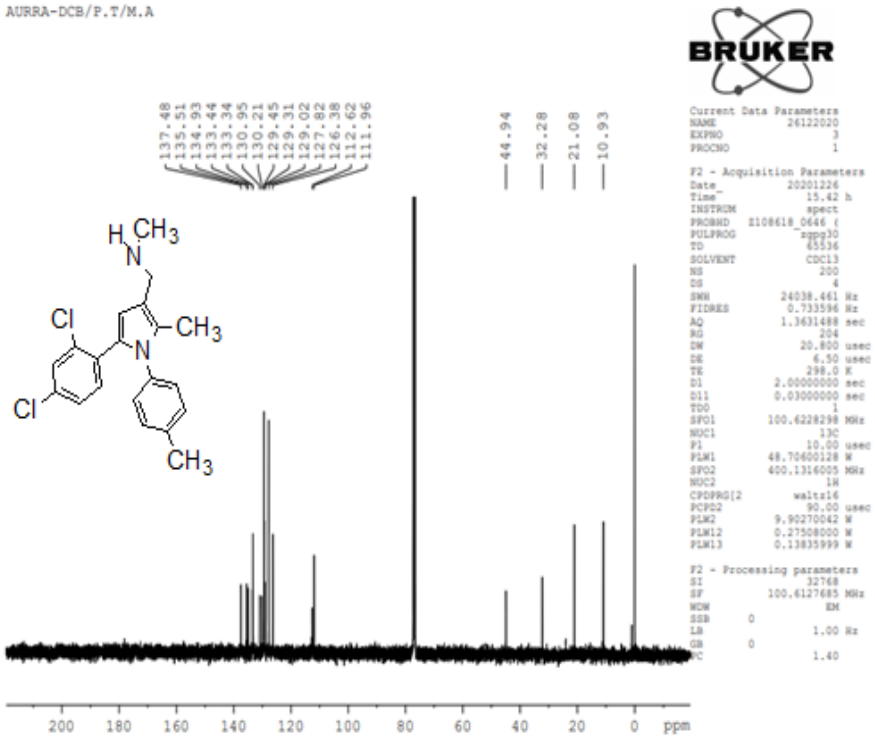


Fig. 6: [13]C NMR Spectrum of [5-(2,4-Dichlorophenyl)-2-methyl-1-p-tolyl-1H-pyrrol-3ylmethyl]methylamine (SA-3).

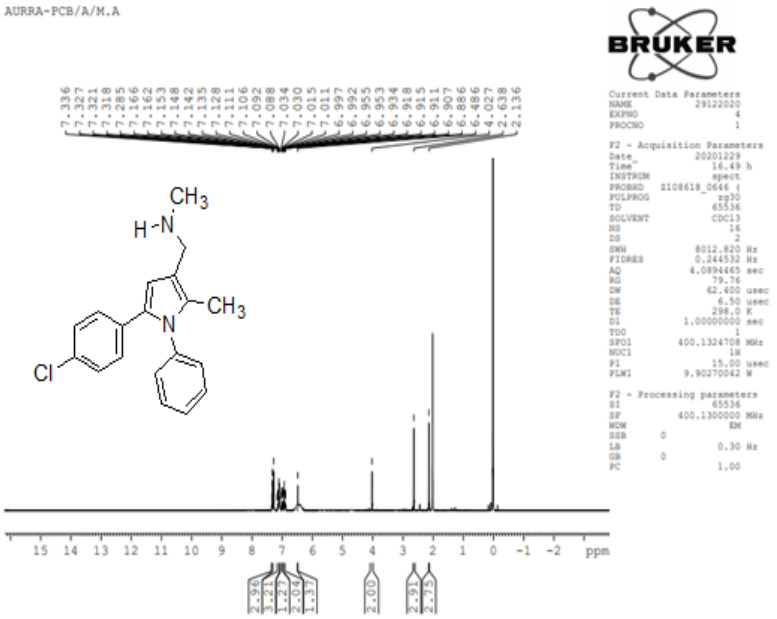


Fig. 7: [1]HNMR Spectrum of [5-(4-Chlorophenyl)-2-methyl-1-phenyl-1H-pyrrol-3ylmethyl]methylamine (SA-4).

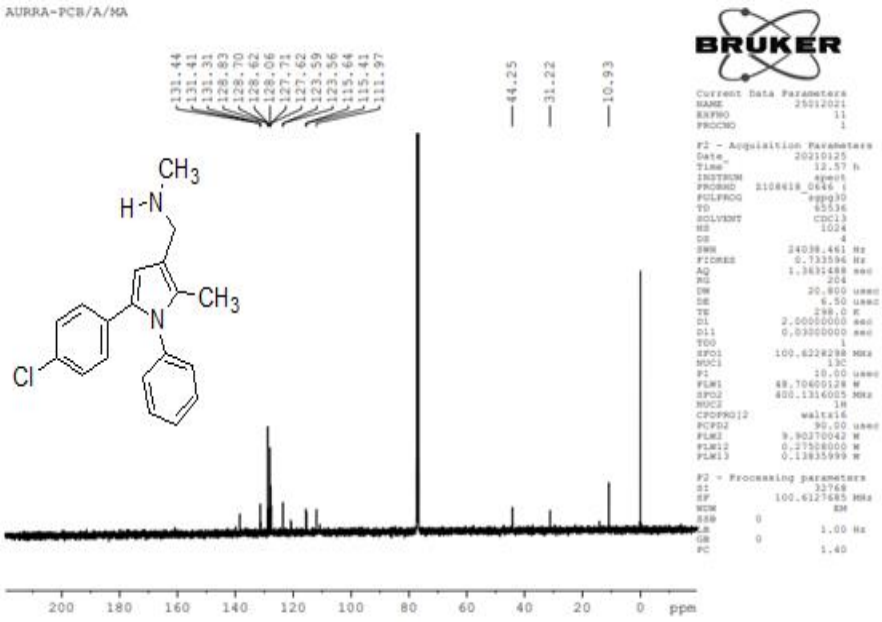


Fig. 8: [13]C NMR Spectrum of [5-(4-Chlorophenyl)-2-methyl-1-phenyl-1H-pyrrol-3ylmethyl]methylamine (SA-4).

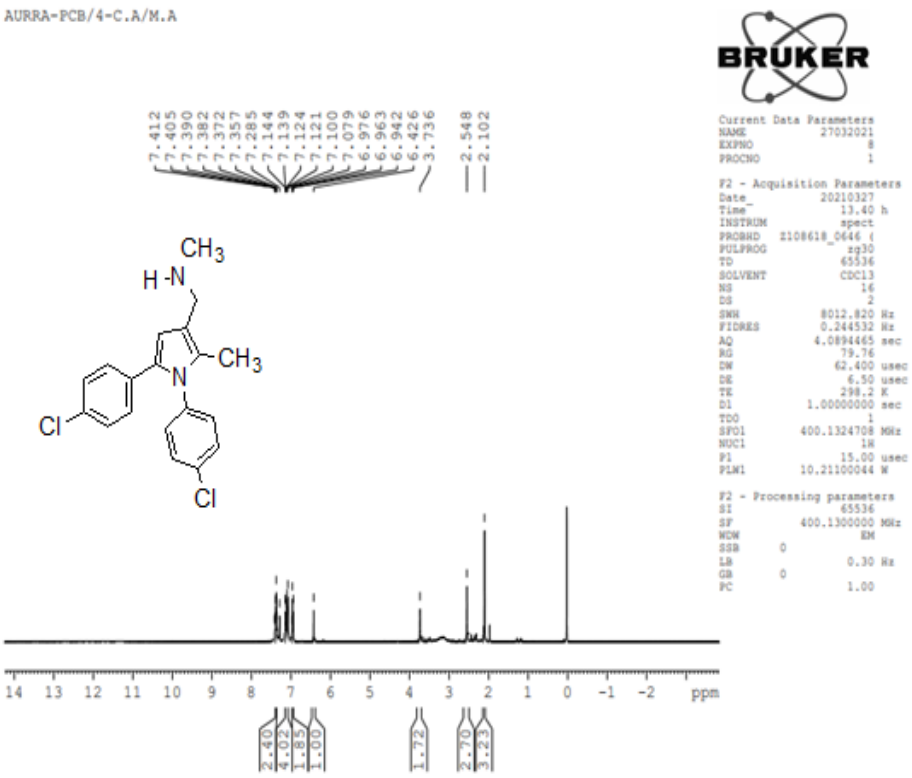


Fig. 9: [1]HNMR Spectrum of [1,5-Bis-(4-chlorophenyl)- 2-methyl-1H-pyrrol-3ylmethyl]methylamine (SA-5).

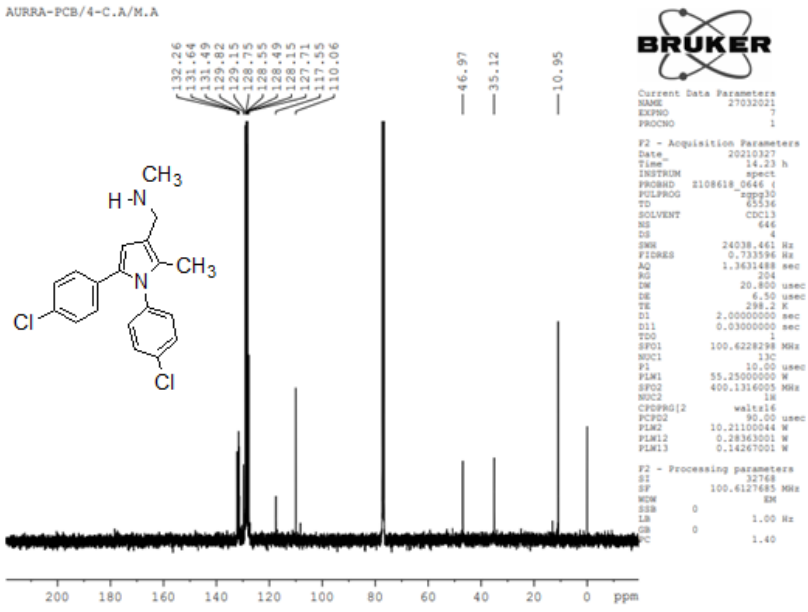


Fig. 10: [13]C NMR Spectrum of [1,5-Bis-(4-chlorophenyl)- 2-methyl-1H-pyrrol-3ylmethyl]methylamine (SA-5).

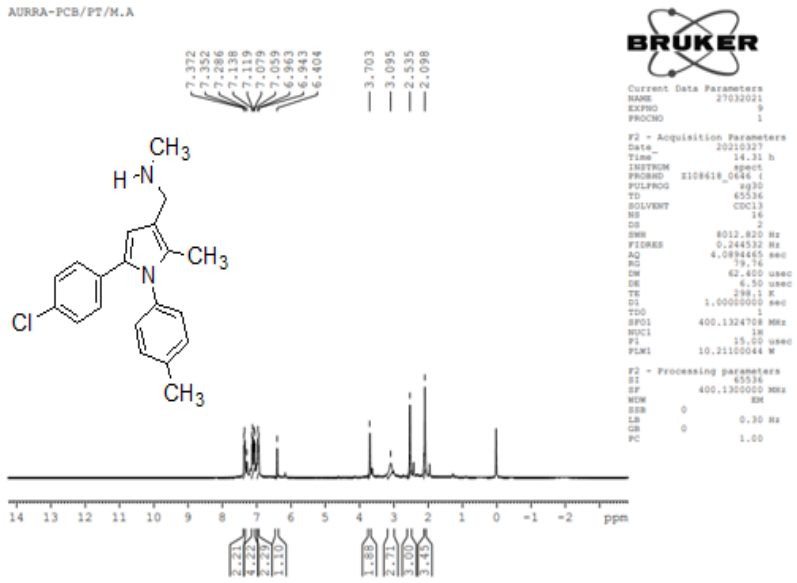


Fig. 11: [1]HNMR Spectrum of [5-(4-Chlorophenyl)-2-methyl-1-p-tolyl-1H-pyrrol-3ylmethyl]methylamine (SA-6).

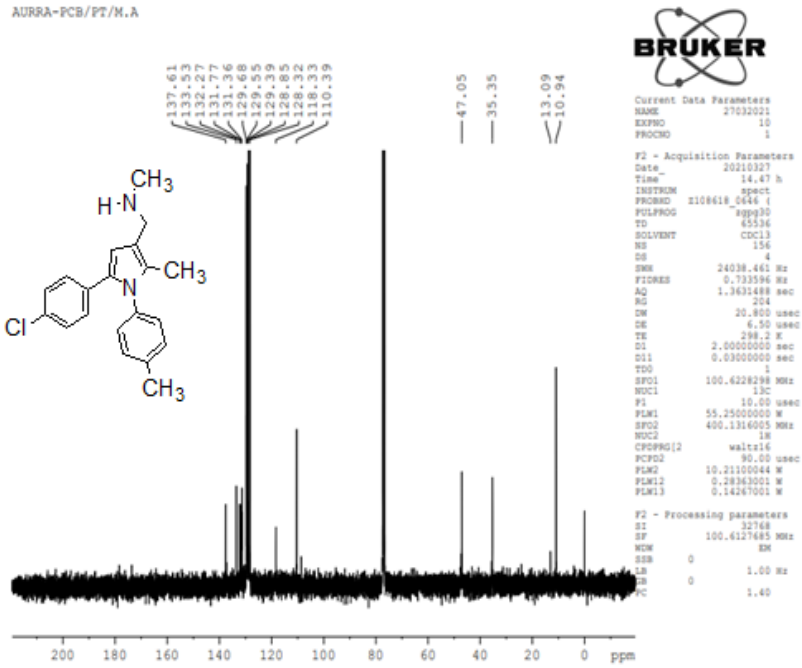


Fig. 12: [13]C NMR Spectrum of [5-(4-Chlorophenyl)-2-methyl-1-p-tolyl-1H-pyrrol-3ylmethyl]methylamine (SA-6).

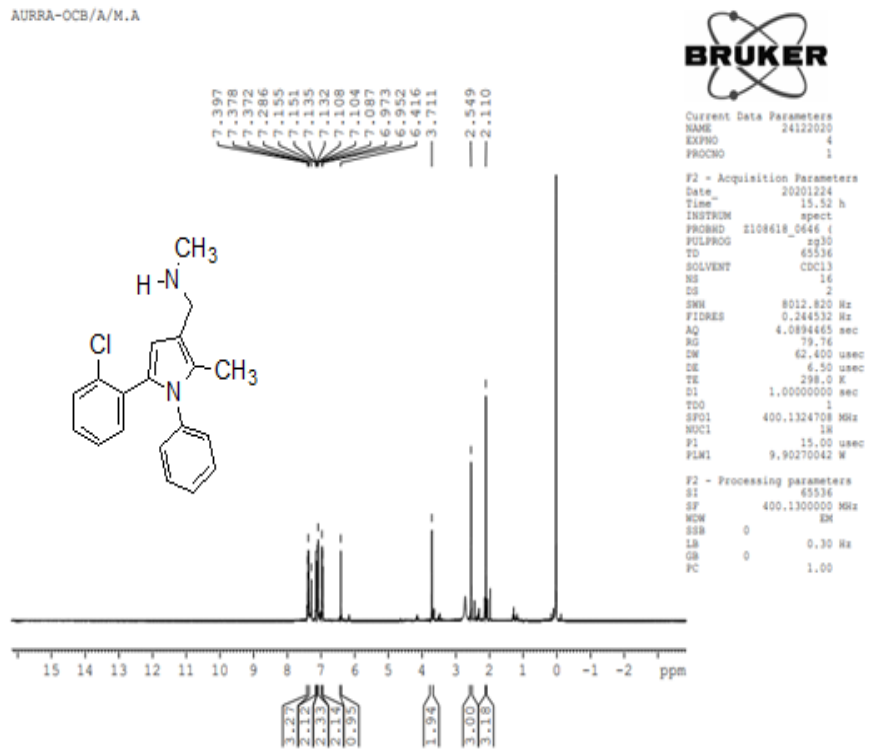


Fig. 13: [1]HNMR Spectrum of [5-(2-Chlorophenyl)-2-methyl-1-phenyl-1H-pyrrol-3ylmethyl]methylamine (SA-7).

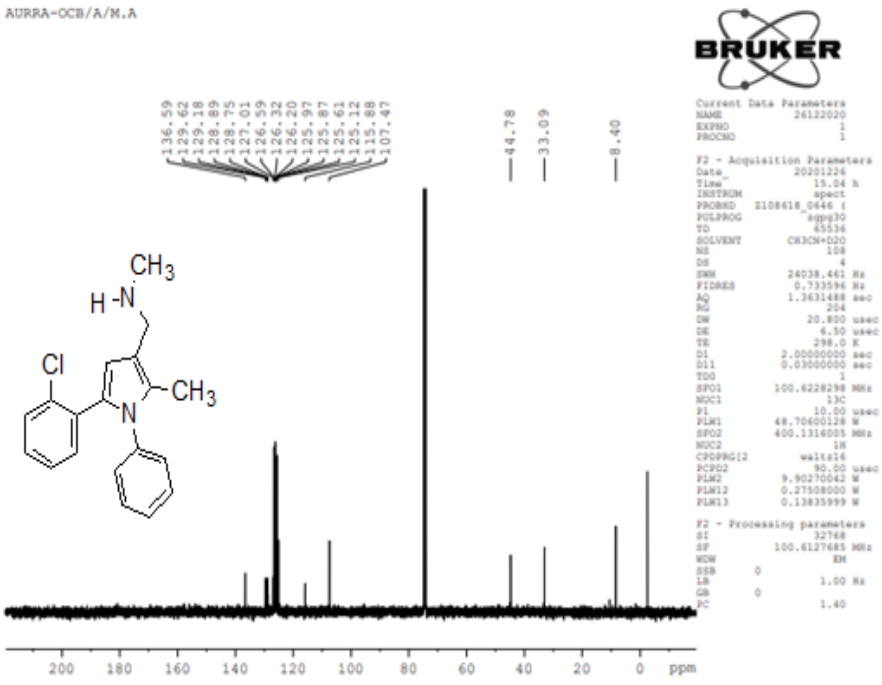


Fig. 14: [13]C NMR Spectrum of [5-(2-Chlorophenyl)-2-methyl-1-phenyl-1H-pyrrol-3ylmethyl]methylamine (SA-7).

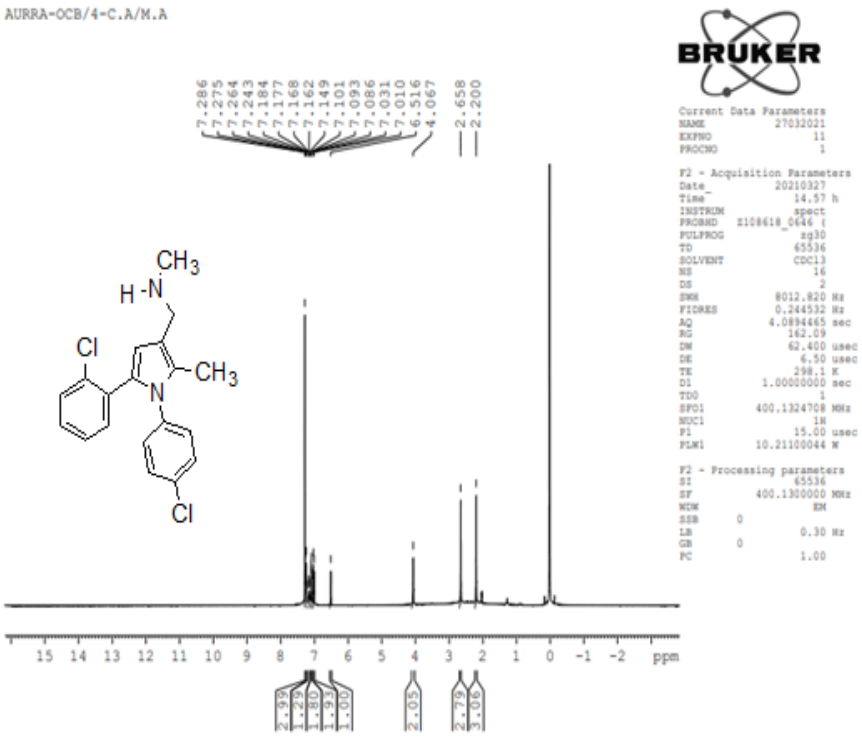


Fig. 15: [1]HNMR Spectrum of [1-(4-Chlorophenyl)-5-(2-chlorophenyl)-2-methyl-1H-pyrrol-3ylmethyl]methylamine (SA-8).

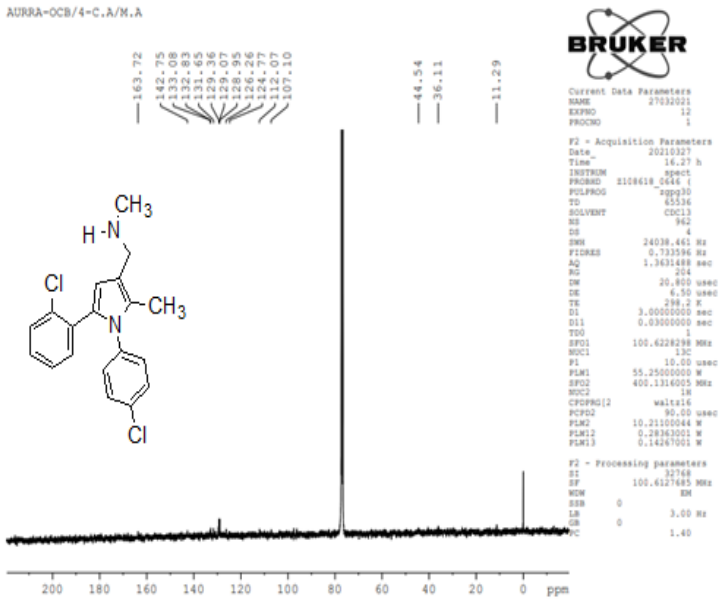


Fig. 16: [13]C NMR Spectrum of [1-(4-Chlorophenyl)-5-(2-chlorophenyl)-2-methyl-1H-pyrrol-3ylmethyl]methylamine (SA- 8)

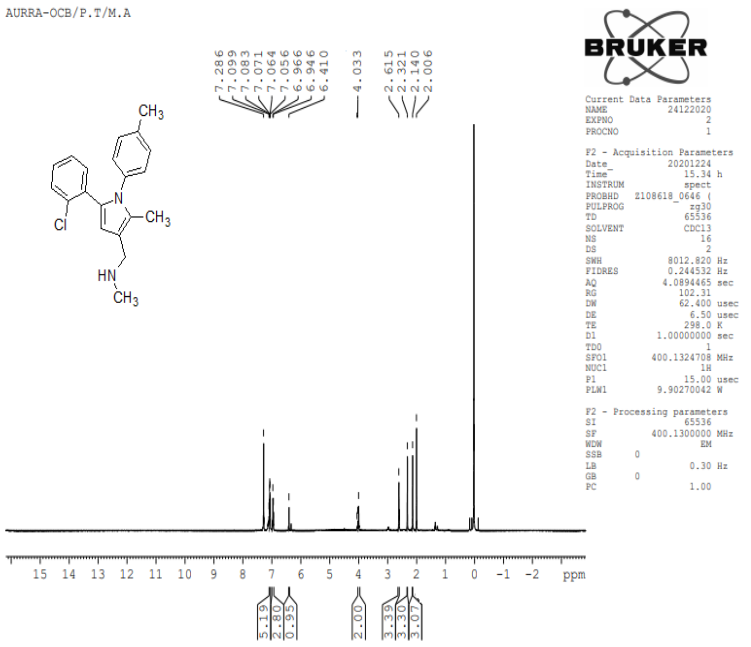


Fig. 17: [1]HNMR Spectrum of [5-(2- Chlorophenyl)-2-methyl-1-p-tolyll-1H-pyrrol-3ylmethyl]methylamine (SA-9).

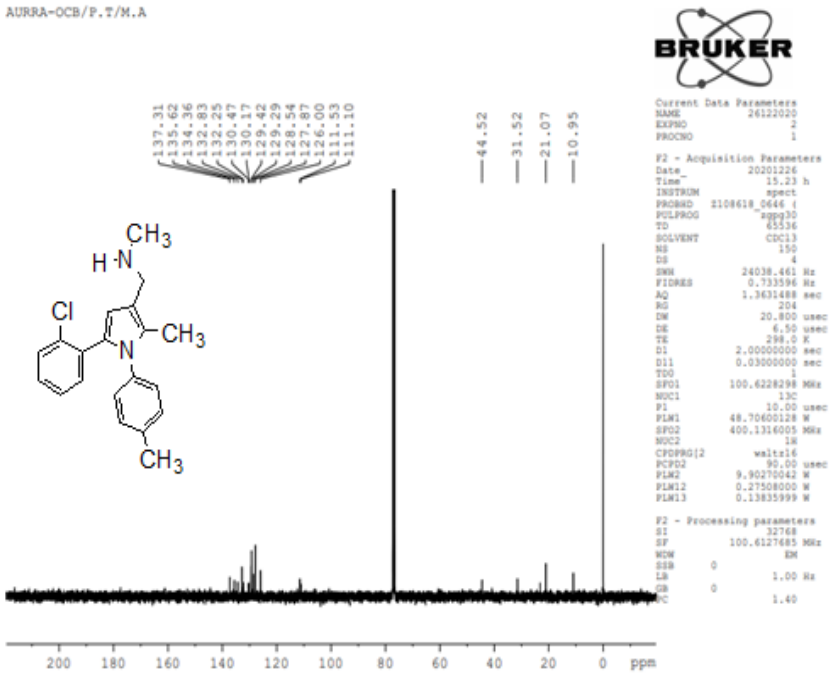


Fig 18: [13]C NMR Spectrum of [5-(2-Chlorophenyl)-2-methyl-1-p-tolyll-1H-pyrrol-3ylmethyl]methylamine (SA-9).

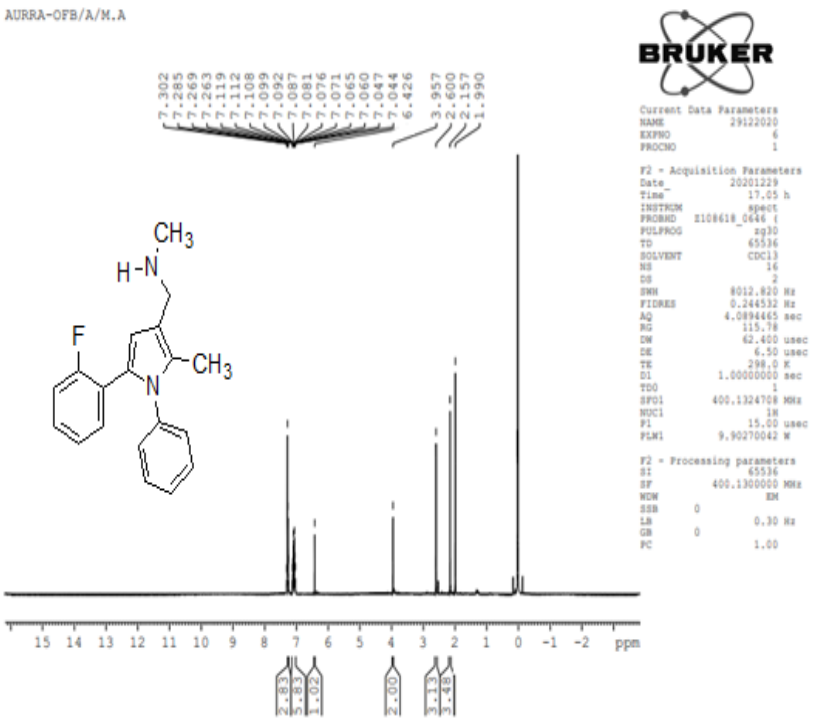


Fig. 19: [1]HNMR Spectrum of [5-(2-Fluorophenyl)-2-methyl-1-phenyl-1H-pyrrol-3ylmethyl]methylamine (SA-10).

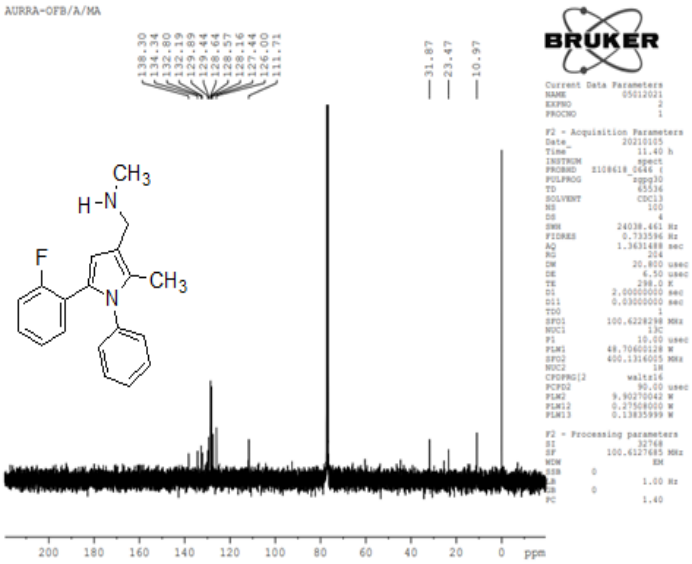


Fig. 20: [13]C NMR Spectrum of [5-(2-Fluorophenyl)-2-methyl-1-phenyl-1H-pyrrol-3ylmethyl]methylamine (SA-10).

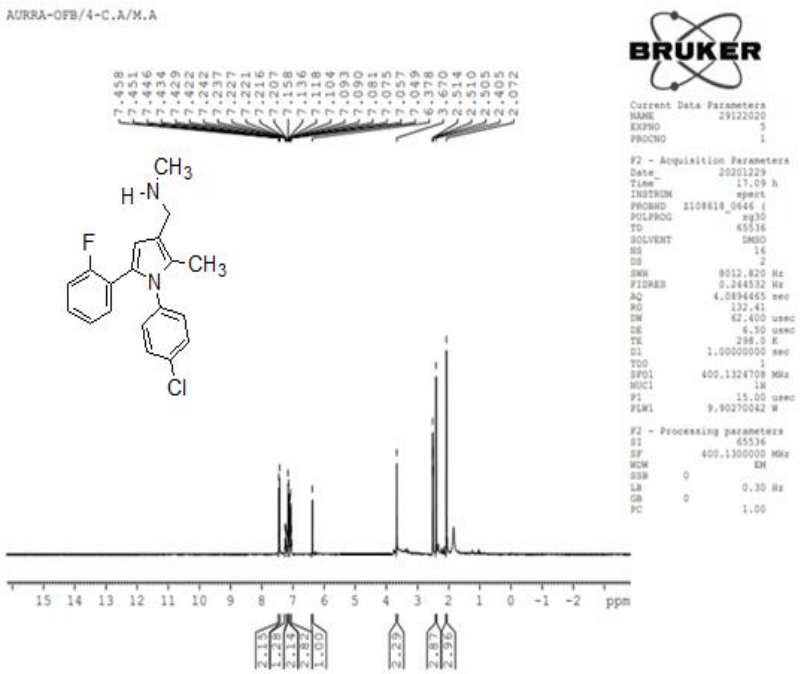


Fig. 21: [1]HNMR Spectrum of [1-(4-Chlorophenyl)-5-(2-fluorophenyl)-2-methyl-1H-pyrrol-3ylmethyl]methylamine (SA-11).

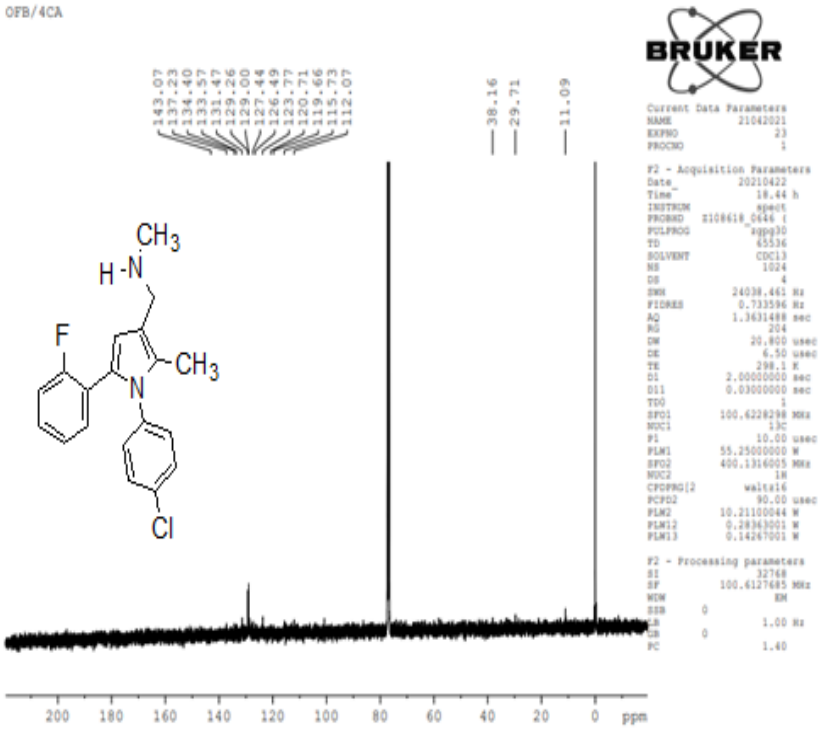


Fig. 22: [13]C NMR Spectrum of [1-(4-Chlorophenyl)-5-(2-fluorophenyl)-2-methyl-1H-pyrrol-3ylmethyl]methylamine (SA-11).

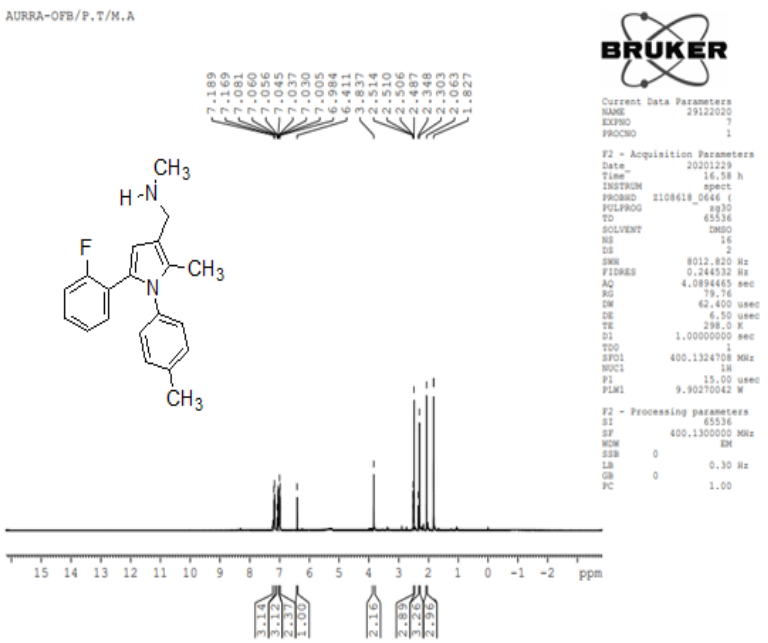


Fig. 23: [1]HNMR Spectrum of 1-[5-(2-Fluorophenyl)-2-methyl-1-p-tolyl-1H-pyrrol-3-ylmethyl]methylamine (SA12).

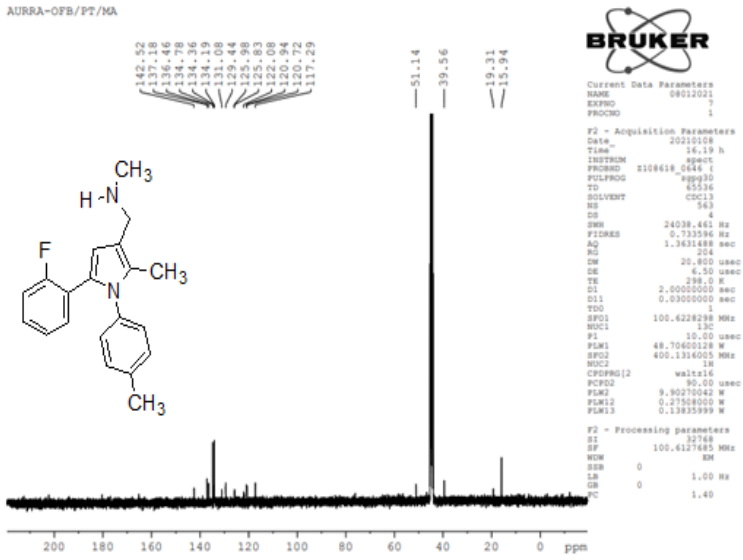


Fig. 24: [13]C NMR Spectrum of 1-[5-(2-Fluorophenyl)-2-methyl-1-p-tolyl-1H-pyrrol-3-ylmethyl]methylamine (SA12).

3 Procedure for *in vitro* screening[10]

Platelet rich plasma (about 10 ml) was obtained from blood drawn from a healthy volunteer. Sertraline HCl was obtained as a gift sample from Harika Drugs Private Limited, Hyderabad, India. Human serotonin ELISA kit was obtained from MyBiosource – MBS160020 (figure 4.3, 48 tests). Alere AM1200 microplate reader was used for reading the results

1. PRP (platelet rich plasma) was separated from the blood collected from a healthy volunteer.

2. The PRP (200 µl) was transferred to eppendorf tubes and washed with twice the original volume (400 µl) of phosphate buffered saline and centrifuged at 2500 r.p.m for 10 minutes. The procedure was repeated twice.

3. The platelet pellet was resuspended back to the original volume (200 µl) using phosphate buffered saline.

4. The platelets were incubated with test/standard compound at a concentration of 100 ng/ml for 10 min followed by incubation with 100 ng/ml 5-HT at room temperature for 10 minutes; to include only the initial linear phase of transport in human platelets.

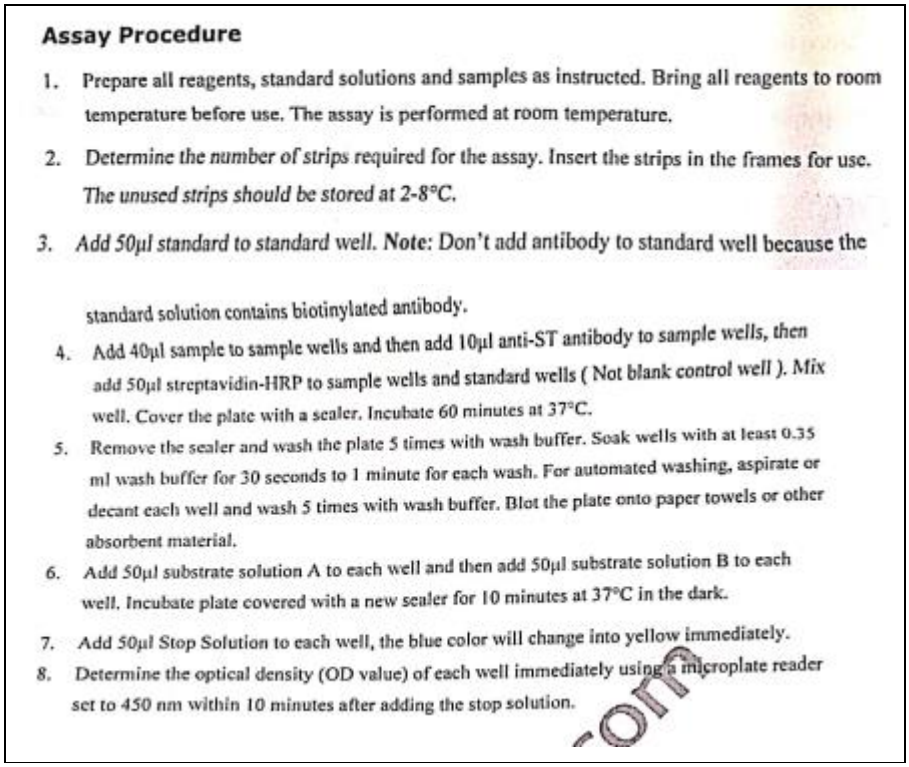
5. The platelet suspension was centrifuged at 2500 r.p.m for ten minutes to separate the platelet pellet. The supernatant was removed with a micropipette and the platelet pellet was washed with phosphate buffered saline

6. Platelets were lysed using ice cold 4M perchloric acid to ensure a final concentration of 1M in the sample and the contents were incubated on ice for 5 min. The samples were centrifuged at 13,000 rpm for 2 min in a cold centrifuge.

7. The supernatant was neutralized by adding ice-cold 2M KOH, and the final pH was adjusted to neutral. The pH was ascertained with the help of a pH paper.

8. The samples were centrifuged at 13,000 rpm for 5 min in a cold centrifuge and the supernatant was retained.

The value of 5-HT in platelets in 200 µl of platelet rich plasma was determined. The platelets were incubated with increasing concentrations of 5-HT (50 ng/ml and 200 ng/ml) to identify uptake by platelets. The platelets were incubated with sertraline (100 ng/ml), a selective serotonin reuptake inhibitor and 5-HT (200 ng/ml) to identify the level of 5-HT uptake inhibition. Platelets were incubated with the test compounds (SA1-SA12) at a concentration of 100 ng/ml and 5-HT (200 ng/ml) to observe the effect on 5-HT uptake. The concentration of 5-HT to be used for incubation was chosen to fall within the range of the constructed standard curve. The assay procedure given in the insert (provided along with the ELISA kit, figure 25) was followed.



Assay Procedure

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2. Determine the *number of strips* required for the assay. Insert the strips in the frames for use. *The unused strips should be stored at 2-8°C.*
3. *Add 50µl standard to standard well.* **Note:** Don't add antibody to standard well because the standard solution contains biotinylated antibody.
4. Add 40µl sample to sample wells and then add 10µl anti-ST antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blot the plate onto paper towels or other absorbent material.
6. Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Add 50µl Stop Solution to each well, the blue color will change into yellow immediately.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Fig. 25: A figure of the assay procedure given in the insert.

4. In vivo assay

Swiss Albino mice were selected for the study. The animals, animal feed and bedding were procured from Mahaveer Enterprises, Hyderabad, India. The animals were housed in hygenic conditions with room temperature regulated with the help of an air conditioner. The standard drug Sertraline was obtained as a gift sample from Harika Drugs Private Limited, Hyderabad, India. The test compounds BM212 and SA-5 were synthesized, purified and characterized by procedures mentioned in chapter III. In the first step acute oral toxicity studies were carried out. After establishing the safe level of dosing in the animal; *in vivo* studies were performed to assess the pharmacological activity of the selected molecules. Graph Pad Prism version 9.0 software was used for statistical analysis and graphical illustraation of results.

The UCMS protocol published by Burstein et al for induction of depression was followed[11]. Three weeks old mice were obtained and were randomly divided into six treatment groups of six animals each after a one week acclimation period. The animals from each cage were marked with food colour at different places for identification. Groups 1, 3, 4, 5 and 6 received treatment wiith standard (sertraline, 10 mg/kg bodyweight), BM212 (low dose, 10mg/kg body weight), BM212 (high dose, 20 mg/kg bodyweight), SA-5 (low dose, 10mg/kg body weight) and SA-5 (high dose, 20 mg/kg bodyweight) respectively. Animals from group two were the control group and were administered with the vehicle. The animals were group housed six animals per cage, each animal belonging to a different treatment group. The stress protocol has been summarized in table 1.

Table 1 : UCMS protocol adopted from Burstein et al.

Stressor	Description
Wet cage	All the mice from a cage were shifted from their home cage to an empty cage. Water was added to the cage upto a depth of 1 cm. Mice were dried gently on a paper towel and returned to the home cage after four hours.
Dampened sawdust	Water was added to the homecage to dampen the bedding. After four hours the mice were transferred to a clean cage with fresh bedding.
Tilted cage	The cages containing the mice were tilted against a wall at an angle of 45º.
Empty cage	Mice are transferred along with their cage mates into an empty cage for four hours.
Social stress	Mice were transferred to a soiled cage inhabited by a different group of mice. After four hours the mice were transferred back to their home cage.
Mice restraint	Mice were restrained individually for four hours in a small plastic restrainer (diameter – 5 cm, height – 7 cm) with holes drilled for air circulation.
Disruption of dark/light cycle	The mice were transferred in their home cage to the stressor room and subjected to 24 hours of illumination from a tube light.

*Food was withheld during adminstration of the stressors except during disruption of the dark/light cycle. Water was not withheld.

*The animals were monitored for every thirty minutes during the first six stressors for any sign of distress and were relieved immediately in case of distress.

The stressors were administered for a period of four weeks and the animals were monitored for change in weight once in three days. The coat state of the animals was evaluated and a well groomed coat was given a score of one as against a score of zero for a dirty coat during the four weeks of stress administration.

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