CHAPTER III
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EXPERIMENTAL

Purification of reagents

Purification of 1-chloro-2,4-dinitrobenzene (Dinitrochlorobenzene – DNCB).

1-Chloro-2,4-dinitrobenzene (LOBA) was recrystallized from ethanol. The melting point of the recrystallized sample is 324K.

Purification of pyrimidine – 2,4,6 (1H, 3H, 5H)-trione (barbituric acid)

Barbituric acid (LOBA) was recrystallized from ethanol-water mixture (50:50 v/v; mp. 518K).

Purification of triethylamine

Triethylamine (Riedel) was distilled and the fraction boiling at 362K was collected and used.

Purification of tri-n-butylamine

Tri-n-butylamine (Kemphasol) was distilled and used (bp. 489K).

Purification of ethanol

Clean dry magnesium turnings (BDH, LR, 2.5g), iodine (Thomas Baker, AR, 0.25g) and commercial absolute alcohol (Hayman, AR, 30 ml) were placed in a round-bottomed flask fitted with a reflux condenser and a calcium chloride guard tube. The mixture was refluxed in a water bath till the iodine colour disappeared and 450ml of absolute alcohol was added and refluxed for 1h. The fraction boiling at 351K was collected and used.

Purification of ether

Ether (Reachem, LR) was dried over sodium bits and then distilled by circulating ice cold water (bp. 308K).

Purification of DMSO

DMSO (Merck, LR) was dried over molecular sieves (type 4A), distilled and used (bp. 462K).
Purification of water

Deionized water was distilled first from small quantities of potassium permanganate and the distillate obtained was again distilled and used (bp. 373K).

Purification of acetone

Acetone (Qualigen) of analar grade was used as such.

Purification of ethyl acetate

Ethyl acetate (BDH, LR) was dried over molecular sieves (type 4A) and the fraction boiling at 350K was collected and used.

Purification of 2-methylaniline

2-Methylaniline (BDH, LR) was distilled and the fraction boiling at 473K was collected and used.

Purification of 2-methoxyaniline

2-Methoxyaniline (BDH, LR) was distilled and the fraction boiling at 498K was collected and used.

Purification of tetrahydro-1,4-oxazine (morpholine)

Morpholine (Qualigen) of analar grade was used as such.

Purification of chloroform

Chloroform of spectroscopic grade was dried over molecular sieves (type 4A) and distilled. The fraction distilled over at its boiling point (334K) was collected and used.

Preparation of 1,3,5-trinitrobenzene (TNB)

TNB is prepared from p-nitrotoluene. It involves three stages.

i. Conversion of p-nitrotoluene to 2,4,6-trinitrotoluene$^{171}$

ii. Oxidation of 2,4,6-trinitrotoluene to 2,4,6-trinitrobenzoic acid$^{172}$

iii. Decarboxylation of 2,4,6-trinitrobenzoic acid to TNB$^{173}$

(i) Preparation of 2,4,6-trinitrotoluene

p-Nitrotoluene (Riedel, 25g) was added to a mixture of concentrated sulphuric acid (110ml, d 1.5) and fuming nitric acid (40ml, 85%) maintained at 323K. The
temperature was gradually raised to 383K during 5h. The product was isolated by pouring into an excess of water, filtered and dried (yield, 34g. mp. 353K).

(ii) **Oxidation of 2,4,6-trinitrotoluene to 2,4,6-trinitrobenzoic acid**

To 180g (100 ml) of concentrated sulphuric acid in a three necked flask, placed in an empty water bath, were added 2,4,6-trinitrotoluene (18g), while the mixture was stirred mechanically. Sodium dichromate (BDH, LR, 27g) was then added in small quantities with constant stirring until the temperature reached 313K. The empty water bath was then filled with cold water and the addition of sodium dichromate continued at such a rate that the temperature remained between 318 and 328K. The addition took one hour after which the thick mass was stirred for two hours and poured into a beaker containing crushed ice (~200g). The insoluble trinitrobenzoic acid was filtered off and carefully washed with cold water until it was free from chromium salts.

The product was then mixed with distilled water (100ml) at 328K and 15% sodium hydroxide solution was dropped in with continuous stirring until a faint red colour was just produced. When it persisted for a minute, the colour was discharged by the addition of a few drops of acetic acid and the insoluble trinitrotoluene was filtered off and washed with little water. The filtrate was used directly for the next stage.

(iii) **Decarboxylation of 2,4,6 – trinitrobenzoic acid**

The filtrate obtained above was mixed with glacial acetic acid (15 ml) and heated gently with continuous stirring when trinitrobenzene separated in crystalline form and floated on the surface of the liquid as a frothy layer. The process was carried on till no more solid separated out. The solution was filtered and the solid was recrystallized first from acetic acid and from alcohol twice (yield 26g, mp. 396K).

**Preparation of 2,4,6-trinitro-1,3-benzenediol (styphnic acid)**

In a beaker mounted in an ice bath was placed 15.4g (0.14 mol) of resorcinol (Merck) 400ml of water and 75g of crushed ice. Then 16ml (0.3 mol) of
concentrated sulphuric acid was added with stirring followed by an ice cold solution of sodium nitrite (20.5g, 0.3mol) in 150 ml of water in 1-2 ml proportions over a period of 45 minutes while maintaining the reaction temperature between 273 and 277K. The mixture was allowed to stand in the ice bath for three hours to complete the reaction. The product heavily contaminated with sodium sulphate was collected on Whatman number 41 paper and allowed to dry overnight.

The next step was done in a hood owing to the evolution of nitric oxide. A 500 ml beaker was charged with 70 ml of 71% concentrated nitric acid and the pulverized crude product of the first step was added in small amounts with stirring for 60-70 minutes, keeping the temperature between 273 and 277K. The ice bath was removed to allow the mixture to attain the room temperature. After 30 minutes the mixture was heated slowly to 348K for 45 minutes to complete the reaction. The mixture was cooled to room temperature and the product was filtered off with Whatman number 41 paper and washed with water. The product 2,4,6-trinitroresorcinol was dried [yield 21g (70%); mp. 448K). Purity was improved by recrystallization from ethyl acetate (mp. 452K).

**Preparation of ethyl 2-(phenylmethyl)-3-oxobutanoate**

Absolute alcohol (19ml) was introduced into a 250ml round-bottomed flask fitted with a reflux condenser and 1.5g of sodium was added in bits. Freshly distilled ethyl acetoacetate (E.Merck, AG, 16.25g) was added after all the sodium had dissolved. Benzylchloride (Ranbaxy, LR, 8g) was added later and the mixture was kept at room temperature for an hour after which it was refluxed for another hour. The condensation product was distilled under reduced pressure (14 mm). The title ester distills over at 437K.

**Preparation of ethyl 2-(4-nitrophenylmethyl)-3-oxobutanoate**

Several attempts were made to purify the title ester by fractional distillation under reduced pressure but they were unsuccessful on account of p-nitrobenzylchloride persistently adhering to it. The ester contaminated with a little of p-nitrobenzylchloride was used as such for the preparation of the bicyclic complex.
Preparation of the carbon-bonded sigma complex from 1-chloro-2,4-dinitrobenzene, barbituric acid and triethylamine

DNFCB (0.01mol) in absolute ethanol was mixed with 0.01mol of barbituric acid in absolute ethanol. Triethylamine (0.02mol) was then added and the mixture was shaken well for 5 to 6 hrs. The solution was filtered and kept as such for 48 hrs. On standing, coloured crystals come out from the solution. The crystals were powdered well and washed with absolute ethanol and copious amount of dry ether and recrystallized from absolute alcohol [yield of pure crystals 60%, mp. 535-537K (decomposes at its melting point)].

Preparation of the carbon-bonded sigma complex from 1-chloro-2,4-dinitrobenzene, barbituric acid and tri-n-butylamine

The procedure specified under the preparation of complex from 1-chloro-2,4-dinitrobenzene, barbituric acid and triethylamine was used to prepare complex from 1-chloro-2,4-dinitrobenzene, barbituric acid and tri-n-butylamine also [yield of pure crystals 70%, mp. 521-523K (decomposes at its melting point)].

Isolation of the tetranitro diphenyl esters from 1-chloro-2,4-dinitrobenzene, ethyl 3-oxobutanoate/methyl 3-oxobutanoate/tert-butyl 3-oxobutanoate and triethylamine

DNFCB (0.01 mol) in absolute ethanol was mixed with alkyl 3-oxobutanoate (0.01 mol) in absolute ethanol. Triethylamine (0.05 mol) was then added and the mixture was shaken well for 4 hours. On standing pale yellow crystals come out from the solution after a week time. The crystals were filtered and washed well with distilled water to dissolve triethylammonium chloride formed during the reaction and dried. The dried crystals were powdered and washed with copious amount of ether to remove the unreacted reactants and then with little absolute alcohol. The crystals obtained after washing were recrystallized either from ethylacetate or from chloroform [ethyl 2,2-bis (2,4-dinitrophenyl) ethanoate (yield 70%, mp. 420K), methyl 2,2-bis(2,4-dinitrophenyl) ethanoate (yield 75%, mp. 428K), tert-butyl 2,2-bis(2,4-dinitrophenyl) ethanoate (yield 70%, mp. 427K)].
Preparation of the bicyclic complex from TNB, ethyl 2-(phenylmethyl)-3-oxobutanoate and triethylamine

The bicyclic complex was prepared by dissolving 1,3,5-trinitrobenzene (0.01mol) and ethyl 2-(phenylmethyl)-3-oxobutanoate in ethanol to give a saturated solution. A three fold excess of triethylamine was added, and the intensely coloured solution was kept at 300K for 24h. The solution was concentrated under vacuum giving an oily mass. The oil was washed with copious amounts of dry ether and redissolved in ether-ethanol solution which, after standing for 12h at 280K, deposited crystals of the complex. The complex was recrystallized twice from absolute ethanol (yield 60%). Single crystals of bicyclic complex were obtained from ethanol at room temperature by slow evaporation (mp. 404K).

Preparation of the bicyclic complex from TNB, ethyl 2-(4-nitrophenylmethyl)-3-oxobutanoate and triethylamine

Symmetrical 1,3,5-trinitrobenzene (0.01 mol) and ethyl 2-(4-nitrophenylmethyl)-3-oxobutanoate (~0.01 mol) were mixed together and ground well in a mortar to get a mull. To this mull a three fold excess of triethylamine was added and again ground well. The resulting dark coloured mass was extracted with hot absolute alcohol and kept as such for 24h. The solution was concentrated in vacuum to give an oily mass. This oily mass was repeatedly washed with copious amount of dry ether and redissolved in absolute alcohol. To the alcoholic solution, 50 fold excess of dry ether was added and refrigerated between 273K and 283K for 6h to get the crystals of the bicyclic complex. The crystals obtained were recrystallized from absolute alcohol (yield 60%). Single crystals were obtained from ethanol at room temperature by slow evaporation. (mp. 433K)

Preparation of donor-acceptor adduct from styphnic acid and 2-methylaniline

Styphnic acid (2.45g, 0.01 mol) was dissolved in minimum quantity of absolute ethanol. 2-Methylaniline (1.07g, 0.01 mol) dissolved in minimum amount of ethanol was added to styphnic acid solution. The solution was kept over night and
then poured into ice cold water with stirring. The adduct formed was filtered and washed with water and dried. The dried adduct was washed several times with ether and then recrystallized from water. Ethanol / ethylacetate could also be used as recrystallizing solvents (yield 70%, mp. 473K).

**Preparation of donor-acceptor adduct of styphnic acid and 2-methoxyaniline**

Styphnic acid (2.45g, 0.01 mol) and 2-methoxyaniline (1.23g, 0.01 mol) were dissolved in minimum amount of DMSO. These two solutions were mixed and stirred for 24 hrs. Dark viscous solution obtained after stirring was poured into ice cold water with stirring. The adduct formed was filtered and washed with water and dried. The dried adduct was washed several times with ether and then recrystallized from water. Ethanol and ethylacetate could also be used as recrystallizing solvents (yield 70%, mp. 455K). Single crystals were prepared by slow evaporation of the solvent at room temperature.

**Preparation of donor-acceptor adduct from styphnic acid and morpholine**

Styphnic acid (0.01 mol) and morpholine (0.01 mol) were used for the preparation. Both the procedures described under the preparation of donor-acceptor adduct from styphnic acid and 2-methylaniline / 2-methoxyaniline, yielded good amount of the product (75%). Pure crystals were obtained through recrystallization using either ethylacetate or ethanol (mp. 481K).

**Spectral Studies**

The visible data were obtained on a Perkin-Elmer Lambda 15 / Lambda 25 UV/VIS spectrometer. The IR spectra were recorded using Perkin-Elmer RXI infrared spectrophotometer as KBr pellets. The PMR spectra were obtained from Bruker DRX – 300 spectrometer with (DMSO)d$_6$ / CDCl$_3$ as solvents and TMS as an internal reference. The $^{13}$C NMR was recorded using NMR spectrometer Bruker DRX-300 (300 MHz, FT NMR) with (DMSO)d$_6$ / CDCl$_3$ as solvents. $^1$H–$^1$H COSY spectra were obtained from NMR spectrometer Bruker DRX-300 (300 MHz FT
NMR) using CDCl$_3$ solvent. The FAB mass spectra were recorded on a JEOL SX
102/DA-6000 mass spectrometer using Xenon (6kV, 10mA) as the FAB gas. The
accelerating voltage was 10kV and the spectra were recorded at room temperature.
m-Nitrobenzyl alcohol was used as matrix.

Thin Layer Chromatographic studies

This study was undertaken to check the purity of the isolated molecules. The
adsorbent used was silicagel. The slurry of silicagel in chloroform was coated
uniformly on a TLC plate. The complex solution was prepared using pure absolute
ethanol and spotted on the plate. The developing solvent used was the mixture of
tert.butanol and ethylacetate in the ratio 3:1.

Crystal structure determination

Suitable crystals of the compounds were selected for single crystal X-ray
diffraction experiments and mounted on Bruker AXS diffractometer/ Siemens AED
single crystal diffractometer with graphite monochromated MoK$_\alpha$ /CuK$_\alpha$ radiation.
The structure was solved by direct methods and refined by full-matrix least square
method. The non-hydrogen atoms were refined anisotropically. All the hydrogens
were placed in their idealized positions and refined as riding on their carrier atoms.
The programs used for the crystal structure determination are – Data collection :
APEX2 (Bruker, 2004),\textsuperscript{176} cell refinement : SAINT-Plus (Bruker, 2004),\textsuperscript{176} data
reduction : SAINT-Plus and XPREP (Bruker, 2004)\textsuperscript{176}; structure solving : SIR92
(Altornare et al., 1993)\textsuperscript{177}; structure refinement : SHELXL97 (Sheldrick, 2008)\textsuperscript{178};
molecular graphics : ORTEP (Farrugia, 1997)\textsuperscript{179} and Mercury (Macrae et al.,
2006).\textsuperscript{180}

Thermal Analysis

TGA/DTA data were collected employing Perkin Elmer (Pyris Diamond
model) from 303K to 873K at a heating rate of 5K/min under N$_2$(g) purge using
alumina powder as reference. Instrument (NETZSCH STA 409 C/CD) was used for
the TG/DTA studies, from 303K to 1673K at the heating rate of 10K/min and
20K/min under N$_2$(g) purge with alumina powder as reference. The impact sensitivity
was determined by the fall hammer method using a 2.0kg drop weight. The activation energies of exothermic decomposition reactions were determined by Kissinger\textsuperscript{181} and Ozawa\textsuperscript{182} methods.

**Biological activity studies**

Biological activity studies were carried out in the Department of Pharmacology, Periyar College of Pharmaceutical Sciences, Tiruchirappalli-620 021, Tamil Nadu, India. Animal facility of this institution is approved by the Committee for the Purpose of Control and Supervision of Experimental Animals (registration number CPCSEA/265). The experimental procedures adopted for the evaluation of the biological activities have been approved by the Institutional Animal Ethics Committee and conducted according to the guidelines of Indian National Science Academy for the use and care of experimental animals. The animals were maintained at a well ventilated, temperature controlled (303 ± 1K) animal room for 7 days prior to the experimental period and provided with food and water. The animals were acclimatized to laboratory conditions before the test. Each animal was used only once.

**Anticonvulsant activity studies**

Maximal Electro Shock (MES) method\textsuperscript{183, 184} was followed to study the anticonvulsant activity. Albino rats of either sex weighing 150-200g were divided into groups of six animals each. The positive control used was phenytoin (25mg/kg). The control group was fed with normal saline (5ml/kg). All the other groups received the synthesized molecules (25-100 mg/kg). All drugs were administered orally. The drugs were given one hour before the induction of MES (150mA/0.2sec). The current was applied to the animal using the corneal electrodes of electroconvulsometer (model 100-3, INCO). The different stages of convulsions such as tonic flexion, tonic extensor, clonus convulsion, stupor and recovery/death were noted. The time spent by the animal in each of these phases was noted. Reduction in time in extensor phase of convulsion is an indication of anticonvulsant activity. The mean value for each group was calculated and compared with control. The results were expressed as mean ± standard error. The test of significance was analysed by student’s t-test\textsuperscript{185}. 
Evaluation of hypnotic action

Albino mice of either sex weighing 25-30g were divided into groups of six animals each. The positive control used was pentobarbital 20mg/kg. The control group was given normal saline (1ml/kg). The other groups received synthesized molecules (100mg/kg). From the time of administration, time of loss of righting reflex, time of onset of action and time of recovery, the hypnotic action of the synthesized molecules has been established.

Evaluation of Wound healing activity\textsuperscript{186,187}

Male albino rats of 150-200g body weight were selected. The animals were starved for 12 hours prior to wounding. Under light ether anaesthesia wounding was performed. The animals were divided into groups of six animals each. A circular wound of about 2.5 cm diameter was made on depilated thoracic of rats under light ether anaesthesia in aseptic condition and observed throughout the study. The animals were housed individually. The test samples were formulated as an ointment in simple ointment base. The formulated ointment (0.5g) was applied on the wound only one time daily for 12 days starting from the day of wounding. The observations of percentage wound closure were made on 4\textsuperscript{th}, 8\textsuperscript{th} and 12\textsuperscript{th} post wounding days. Data are expressed as mean ± SEM and subjected to student’s t-test by comparing with the control. The standard drug used for comparison was 0.2% nitrofurazone ointment.

Antimicrobial susceptibility test by Disc Diffusion Technique\textsuperscript{188-192}

Disc impregnated with known concentration of antibiotic are placed on agar plate that has been inoculated uniformly over the entire plate with culture of the bacterium to be tested. The plate is incubated for 18-24 hrs at 310K for bacteria (for fungi the plate is incubated for 24-48 hrs at 298K). During this period, the antimicrobial agent diffuses through the agar and may prevent the growth of the organism. Effectiveness of susceptibility is proportional to the diameter of zone of inhibition.

Preparation of inoculum

The test microorganism were obtained from National Chemical Laboratory (NCL), Pune and maintained by periodical subculturing on Nutrient agar and
Sabouraud Dextrose medium for bacteria and fungi respectively. These microbial strains are inoculated in peptone water and Sabouraud Dextrose broth and incubated at 310K and 298K for 6 to 18 hrs for bacteria and fungi respectively.

**Standardisation of Inoculum**

Reproducibility of the disc-diffusion test largely depends on the size of the inoculum used. The zone of inhibition decreases with increasing size of the inoculums, because the antimicrobial agent has to react with a greater number of bacteria. Hence the inoculum size should be standardized. Standardization of inoculums is done by comparing with the turbidity of the inoculums. The standard roughly contains $1\times10^8$ organisms/ml or 2 organisms seen on the smear under oil immersion objective.

**Preparation of standard**

Barium chloride (0.5ml, 0.048M) is mixed with sulphuric acid (99.5 ml, 0.36N). The resultant suspension of barium sulphate precipitate is used as the standard ($1\times10^8$ cells/ml).

**Matching with the standard**

Barium sulphate standard is placed beside the broth culture in an adequate light against a white background with a contrasting line. Turbidity of the broth is compared with the standard. If the turbidity is less than the standard, incubation is continued for a longer period. If it is more turbid, sterile broth is added until it matches the turbidity.

**Experimental procedure**

A Muller Hinton Agar plate is recommended for susceptibility test by disc diffusion technique.

1. The prepared plates were dried before use and labeled at the bottom with name of culture, sample and standard.

2. A sterile cotton swab on a wooden applicator stick was dipped into a bacterial suspension. Excess fluid was removed by rotating the swab.

3. Uniform distribution of the inoculums were obtained by rubbing gently over the plate in several directions.
4. The sterile disc was placed on in the inoculated plate (15mm from the edge of the plate and 24mm in between the centre of the discs). Same procedure is followed for placing the standard disc.

5. Level of the sample was adjusted in the sterile disc using micropipette.

6. The plates were incubated at 310K in an incubator with up right position. The diameter of the zone of inhibition of growth was measured.

The bacterial pathogens such as *Staphylococcus aureus* (NCIM 2079), *Escherichia coli* (NCIM 2065), *Bacillus subtilis* (NCIM 2063) and *Pseudomonas aeruginosa* (NCIM 2036) and fungal pathogens such as *Candida albicans* (NCIM 3102) and *Aspergillus niger* (NCIM 3105) are used for the present study. Ciprofloxacin (5μg/disc) and amphotericin B (100 units/disc) are used as standards for bacterial and fungal studies respectively.

**Toxicity studies**

Albino mice were fasted overnight, weighed and divided into groups, each group consisting of ten animals. Test samples were administered by oral route. After administration of the compounds, the animals were observed for death due to acute toxicity.

**Gross behavioural studies**

The procedure involved an initial phase of undisturbed observation and a later manipulative phase during which animals were subjected to the least provoking stimuli. In the initial phase, the animal was observed for body position, locomotion, rearing, respiration, heart rate, salivation, urination, skin colour, tremors, staggering and gait. In the later phase, the effect on grip strength, limb tone, abdominal tone, passivity, writhing reflex, pinnal reflex, corneal reflex, pupil size, pain response and lacrimation were also observed.

**Scoring**

The effects of the test substance on the animal were scored with the use of nine degrees, with a scale ranging from 0 to 8. Scoring was performed at the time of peak. The basal score for normal signs or effects was 4, scores below 4 were given for subnormal responses, those above 4, for supranormal. The basal score for
abnormal signs is 0 and the maximal score was 8. The profile was divided into two parts – behavioral and neurological.

**Behavioral profile**

**Awareness**

The alertness or stupor was recorded. The mouse was grasped with thumb and index finger in order to hold the dorsal skin. In this situation, the mouse is held in a walking position. The scores were as follows.

Score 0: An unaffected mouse moves its head and limbs and tries to escape.

Score 2: If the mouse, still grasped in the same manner, held in vertical position, it struggles.

Score 4: When the unaffected mouse is placed in the supine position on the back of the observer’s hand held in fist so that the thumb can support the mouse’s head, it tries to escape.

Score 6: The unaffected mouse tries to escape when held vertically by one fore paw.

Score 8: The unaffected mouse tries to escape when held vertically by one fore paw or by one hind paw.

**Mood**

The unaffected mouse grooms itself frequently. Its grooming was scored 4. Vocalization (basal score 0) may point to a noxious stimulus. Restlessness (basal score 0) was absent in unaffected mouse. Irritability (basal score 0) is an extension of restlessness. Fearfulness (basal score 0) was also recognized.

**Motor activity**

This includes spontaneous activity (basal score 4) of the mice when placed in a bell jar. If the animal sleeps score is 0. If there is a little activity the score is 1. Lesser activities are scored as 2 and 3. Excessive activity is scored as 6, constant walking (score 6), walking with some running (score 7), agitated spurts (score 8). A similar test was performed with the same scoring, where the animals were removed and placed on a table. This is called reactivity (basal score 4). The touch response (basal score 4) when the animal response to the touch with a pencil or forceps at
various parts. The pain response (basal score 4) was graded when a small artery clamp was attached to the base of the tail.

**Neurological profile**

**Central excitation**

The startle response (basal score 0) of the animal to a loud noise was recorded. The degree of elevation of tail was recorded as strauber response (basal score 0). The degree of tremor (basal score 0) and the convulsions (basal score 0) were also recorded.

**Motor incoordination**

The body position (basal score 4) and the limb position (basal score 4) were noted. A staggering gait (basal score 0) may indicate ataxia, abnormal gait (basal score 0) may indicate muscular relaxation or may be related to ataxia.

The somersault test (basal score 0) was also performed. The mouse was picked up by tail and tossed in the air so that a somersault of 2 and 3 turns made before landing upon a pad of sponge rubber. This procedure was repeated until 5 trials had been made, the scoring was as follows. Standing on four feet in all trials: \( \frac{3}{2} \), score 0

Lying on one side: If \( \frac{3}{2} \) or \( \frac{1}{2} \), score is 1
If \( \frac{3}{2} \) or \( \frac{1}{2} \), score is 2
If \( \frac{3}{2} \), the score is 3.

Lying on the back: If \( \frac{3}{2} \) or \( \frac{1}{2} \), the score is 4
If \( \frac{3}{2} \) or \( \frac{1}{2} \), the score is 5
If \( \frac{3}{2} \), the score is 6.

Slowly regaining from a supine or side position, the score is 7. Remaining on the back, the score is 8.
Muscle tone

The limb tone (basal score 4) was estimated by grasping a forepaw of the mouse and noting the resistance to extension of the paw. The grip strength (basal score 4) was measured by allowing the animal to grasp a pencil in the horizontal position and noting the ease with which the animal drops to the table. The body tone (basal score 4) and the abdominal tone (basal score 4) were estimated by noting the muscle tension in comparison with control animals.

Reflexes

The pinnal (basal score 4) reflex was tested by touching the center of the pinna with a hair or other fine instrument. To elicit the corneal reflex (basal score 4), the cornea was touched with a stiff hair which causes the animal to withdraw.