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Anticonvulsant screening and brine shrimp lethality test of some para-substituted-N-(2,6-diisopropylphenyl) benzamides

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Abstract
Many antiepileptic agents are available but a search for compounds with more selectivity and lower toxicity continues to be an area of intensive investigation in drug development. Among the benzamides, N-(2,6-dimethylphenyl)-4-aminobenzamide, (Ameltolide®, LY201116) is the most potent antiepileptic agent that is yet to reach the market. In view of this, some N-(2,6-diisopropylphenyl) benzamides were synthesized and screened for anticonvulsant activity in accordance with the Antiepileptic Drug Development Program (ADD) of National Institute of Health (NIH), USA. All the four compounds screened offer partial protection against chemically induced seizure of intra peritoneal metrazol on mice but the 4-chloro compound proved to be the best among the series with a protective index of 50.21 on the brine shrimp lethality test scale.

Keywords: Anticonvulsant, Benzamide, Epilepsy and Seizures.

INTRODUCTION

Epilepsy is a neurological disorder associated with excessive temporary neuronal discharge, characterized by discrete recurrent episodes which results in disturbance of movement, sensation, behaviour, perception and consciousness (Guelen and van der Kleijn, 1978). Epilepsy affects about 0.5-1% of the world's population (Daniels and Jorgensen, 1982).

There are many drugs used in the management of epilepsy. Among these anticonvulsants armamentarium, the benzamides have shown some promising evidence in anticonvulsant properties. Structurally, compounds possessing anticonvulsant activities, e.g. Valproate, are carboxylic acids and their amides (Murray and Kier, 1977). However the basic event responsible for epilepsy should be the rational approach to the development of any anticonvulsant drug.

It is known for instance that the benzamides have a unique behavioral profile and they appear to exert their pharmacological activity selectively at the D_2 dopamine receptors. This D_2 receptor subtype is responsible for their therapeutic use as anti-schizophrenic. Clark *et al.*, (1984) demonstrated that substituted-aminobenzamides show a high level of protection against maximal electrostatic-induced convulsions in animal models.

In a more recent study, it was indicated that active amides act as allosteric modulators of γ-aminobutyric acid ($GABA_A$)

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complex and have affinity to voltage-sensitive calcium channel receptors. Also from SAR studies of α-substituted N-benzylamides of γ-hydroxybutyric acid, Malawaska (2005) defined some structural elements responsible for the anticonvulsant activity as: (i) the presence of the N-benzylamide fragment, (ii) a hydrophobic unit (e.g. a phenyl ring) as a distal binding site and (iii) a group which could act as an H-bond donor. The OH group in γ-hydroxybutyric acid was necessary for MES activity, and the more lipophilic a compound the better anticonvulsant properties it may have (Malawaska, 2003).

Our model compound is Ameltolide, 4-amino-(N-2,6-dimethylphenyl)benzamide (Barnes 1987). It is a potent anticonvulsant in inhibition of MES induced seizures, but it is ineffective against chemically induced seizures. The SAR studies on benzamides shows that 4-aminobenzamide is MES inactive (Clark *et al.,* 1984), but the introduction of an alkyl or aryl substituent unto the amide nitrogen atom confers MES activity. Also a phenyl ring or a cyclic substituent yields greater MES potency than a non cyclic analogue like N-hexyl chain.

In view of this we synthesized the N-(2,6-diisopropyl)phenyl-4-substituted benzamides as analogues of ameltolide. They were aimed at increasing the hydrophobicity / lipophilicity, of ameltolide. A review of the calculated clog P and Log P derived from R_f-values of thin layer chromatography shows that replacing the methyl groups at positions 2 and 6 with isopropyl groups increased hydrophobicity of ameltolide. In this report four *N*-(2,6-diisopropyl)phenyl para-substituted benzamides were prepared, screened and evaluated for their anticonvulsant activity (*see* Table 1).

EXPERIMENTAL

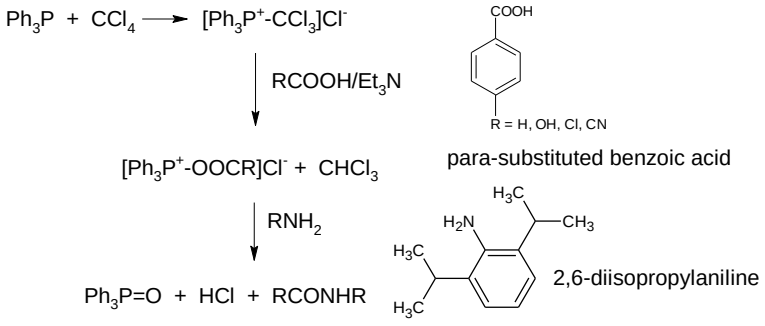
Animals. Mice (25-40 g) were obtained from National Veterinary Research Institute, Vom, Nigeria. The animals were kept in the Animal house of the Department of Pharmacology, University of Jos, Nigeria for 2 days to acclimatize to laboratory condition before the commencement of experiment. They were fed with standard feed and water *ad libitum*.

A typical procedure for the synthesis is as follows: A mixture of 2.623g (0.01M) triphenylphosphine $(C_6H_5)_3P$, 9.6mls (0.1M) carbon tetrachloride (CCl_4) and 30mls of tetrahydrofuran (THF) was refluxed for thirty (30) minutes. The solution was cooled in an ice bath to 5^oC and a mixture of 1.565g (0.01M) 4-chlorobenzoic acid and 1.4mls (0.01M) triethylamine was added and allowed to stand at 5^oC for ten (10) minutes. One equivalent of the amine (0.01M) was added and the mixture was heated and refluxed for forty-five (45) minutes. The precipitated triphenylphosphite was removed by filtration while the solvents were removed under vacuum. The product was purified with preparative TLC on silica, while its CHN analysis agreed within $\pm$ 0.04% range from the expected formula. Its melting point was determined and recorded uncorrected.

Anticonvulsant test. Animals were pretreated with the compounds for 30 minutes and later pentylenetetrazol (85mg/Kg) was administered intraperitoneally. The animals were observed for 30 minutes. Failure to observe a single episode of clonic spasms for at least 5 seconds duration was considered as protection, and the results expressed as number of animals protected/number of animals tested.

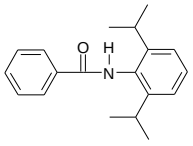
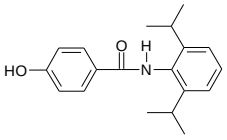
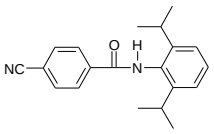
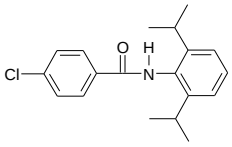
The observed activity of the compounds (see Table 1) may not be phenytoin- or carbamazepine-like.

Synthesis of compounds



Wittig Reaction with Ester-Exchange

TABLE 1: Anticonvulsant Activity of para-substituted-N-(2,6-diisopropylphenyl)benzamides

Para-substituted-N-(2,6-diisopropylphenyl)benzamide	Dose	Onset of Seizure(s)	Episode of Seizure	Time of Death (s)	Number Survived
	30mg/Kg	60.8 ± 5.6	4	160.8 ± 77.3	0/4
	100mg/Kg	67.5 ± 2.7	2	78.8 ± 41.1	0/4
	300mg/Kg	49.8 ± 3.7	3	149.8 ± 36.3	0/4
4-H-DISPA (mp --^{o}C) N-(2,6-diisopropylphenyl)benzamides					
	30mg/Kg	64.8 ± 12.8	3	170.8 ± 87.1	0/4
	100mg/Kg	63.3 ± 8.4	2	138.3 ± 33.5	0/4
	300mg/Kg	54.3 ± 2.6	2	119.0 ± 10.8	0/4
4-OH-DISPA (mp --^{o}C) Para-hydroxyl-N-(2,6-diisopropylphenyl)benzamide					
	30mg/Kg	32.0 ± 1.0	3	136.3 ± 46.1	0/4
	100mg/Kg	37.0 ± 1.0	4	228.3 ± 15.8	0/4
	300mg/Kg	Lethal dose			
4-CN-DISPA (mp 118^{o}C) Para-cyano-N-(2,6-diisopropylphenyl)benzamide					
	30mg/Kg	109 ± 42.7	3	172.3 ± 46.1	0/4
	100mg/Kg	71.5 ± 2.1	6	229.0 ± 64.7	0/4
	300mg/Kg	179.5 ± 12.3	3	207.8 ± 67.2	0/4
4-Cl-DISPA (mp 91^{o}C) Para-chloro-N-(2,6-diisopropylphenyl)benzamide					
(-)C {NEGATIVE CONTROL (ipMTZ only)}	90mg/Kg	64.0 ± 7.0	7	222.5 ± 56.6	0/4
(+)C {POSITIVE CONTROL (Carbamazepine + ipMTZ)}	10mg/Kg	33.75 ± 5.9	4	646.25 ± 50.3	0/4

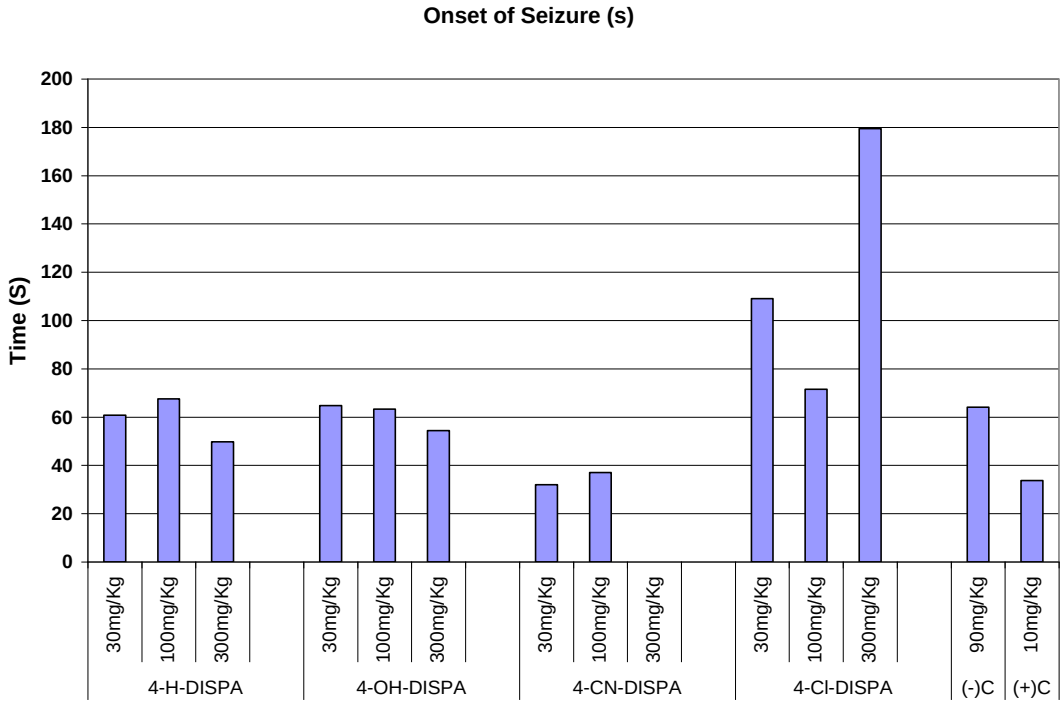


Figure 1: Onset of Seizure(s) of para-substituted-N-(2,6-diisopropylphenyl)benzamides

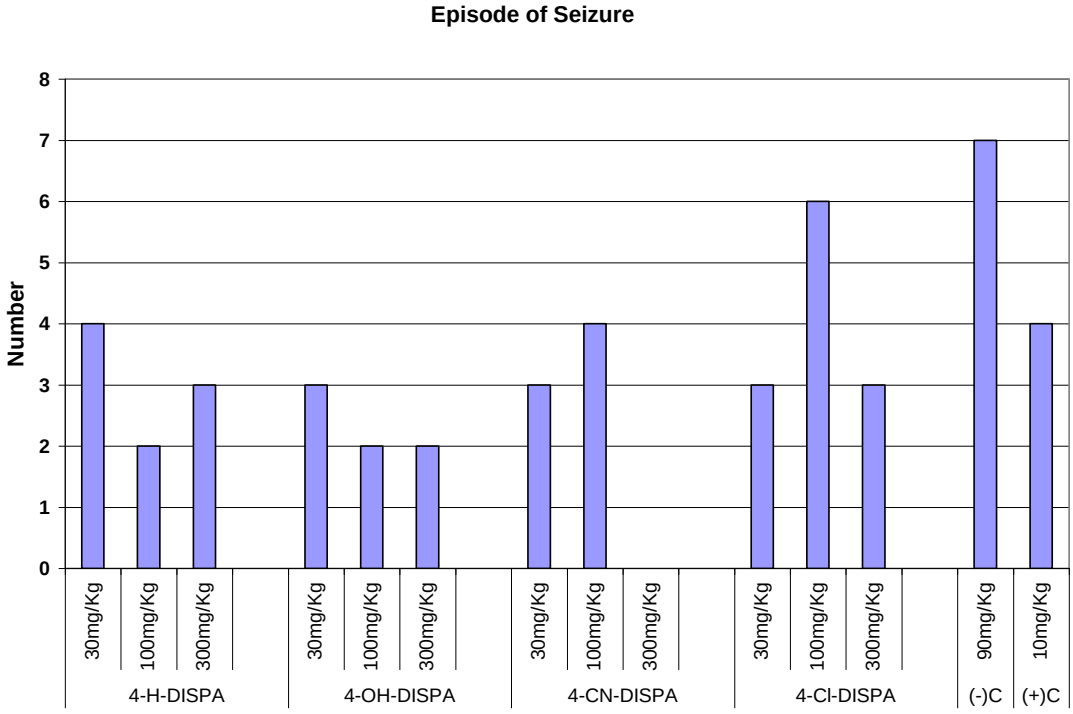


Figure 2: Episode of Seizure of para-substituted-N-(2,6-diisopropylphenyl)benzamides

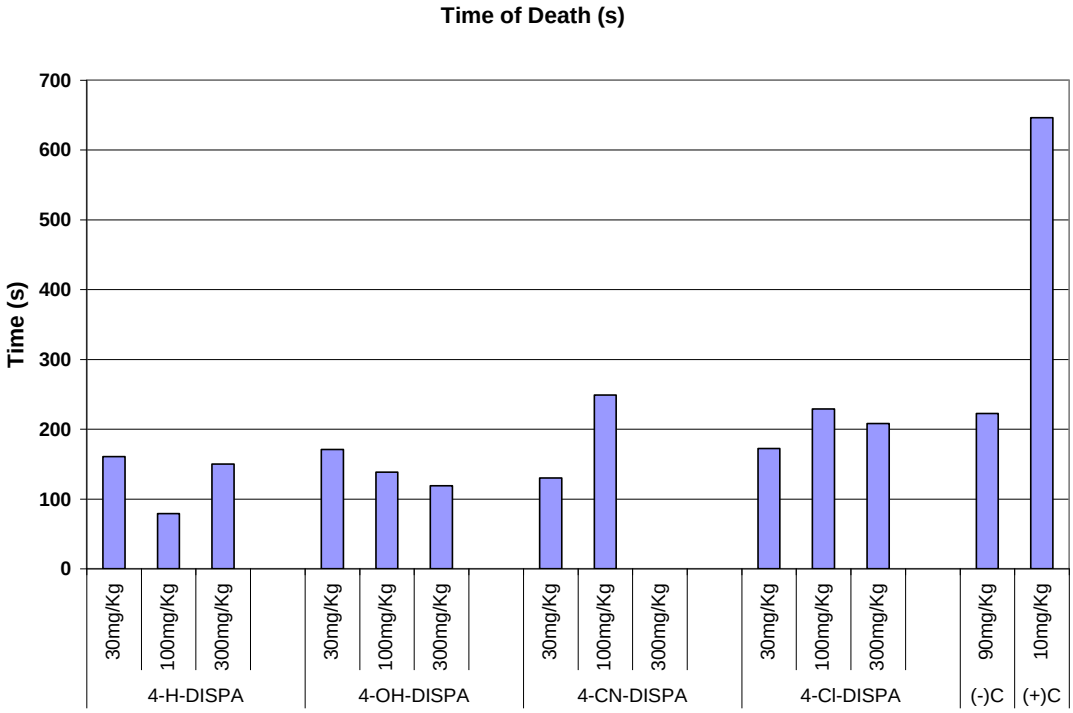


Figure 3: Time of Death for para-substituted-N-(2,6-diisopropylphenyl)benzamides

This is because convulsant activity of maximal electro-shock is blocked, rather than pentylenetetrazole-induced seizure. Swinyard *et al.* (1989) demonstrated that some compounds are termed "preventing seizure spread" rather than "raising seizure threshold". In this experiment, the compounds are seen to increase seizure threshold, probably through GABA pathway.

Pharmacological and toxicological evaluations as well as further Structure Activity Relationship studies on the benzamides are currently being conducted in our laboratory.

Brine Shrimp test. Brine shrimp (*Artemia salina* Leach) eggs were placed in saline water (1% $^{w}/_{v}$ NaCl) in a soap dish. After 48h incubation at room temperature the hatched larvae (nauplii) were attracted to one side of the vessel by a light source and collected with a pipette. Ten nauplii were transferred with some seawater, *ca* 2ml, into each test tube. Specific volumes of sample solution, prepared in sea water, were added to the nauplii such that final concentration of 10, 100 and 1000µg/mL in a total volume of 5mL per test tube will be obtained. Each treatment was carried out in duplicate with saline water as control. After incubating for 24h the number of dead nauplii in each test tube was counted and recorded for the lethality test. Also the number of nauplii with sluggish motion was counted for the sedative estimate. The data was processed using Finney Probit Analysis programme to calculate median Effective Dose (ED_{50}) values with 95% confidence intervals for statistically significant comparisons of potency (McLaughlin *et al.*, 1991).

RESULTS/ DISCUSSION

The 4-chloro-substituted analog of N-(2,6-diisopropyl)phenyl-para-substituted benzamide (DISPA) was found to give better protection for PTZ-induced seizure in mice (*see* Table 1, and Figures 1-3) than all the other four para-substituted analogues of DISPA. The significant increase in

hydrophobicity/lipophilicity introduced by replacing methyl groups with isopropyl groups at positions 2 and 6 on the phenyl ring of ameltolide has not produced any corresponding significant change in its anticonvulsant activity. The 4-CN-DISPA showed a very narrow protective index range. At lower doses of 30 and 100 mg/Kg showed similar anticonvulsant activity with cabamazepine, the positive control but the higher dose is lethal at 300 mg/Kg. 4-OH-DISPA also confirms this observation by showing a dose dependent increased toxicity by its decreasing Onset of seizure time(s) whereas the unsubstituted analog, 4-H-DISPA did not show any significant improvement over the negative control.

The number of Episode of seizure parameter within the DISPA-series gave a better indicator of protective index for anticonvulsant activity compared with that of the negative control. All the compounds tested had lower number of Episode of seizure than the negative control. 4-OH-DISPA had the least number of Episodes of seizure even better than cabamazepine, the positive control.

The time of death of all the four compounds tested at doses less than 100 mg/Kg is not significantly better than that of the negative control. 4-Cl-DISPA gave the time of 229.0$\pm$64.7s at 100mg/Kg which is slightly higher than that of the negative control.

In view of the observed narrow protective index of the DISPA-series, we conducted the brine shrimp toxicity test. Results showed that their ED_{50} protective index is 50.21

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