Comparative Activities and Toxicities IV

Dinitro- and Dichloronitro- Phenylamino Acids and Esters

By WILLIAM O. FOYE and DAVID B. MORTENSON

N-2,4-Dinitrophenyl- and N-4,5-dichloro-2-nitrophenyl-α-amino acids and esters have been synthesized in an attempt to remove the toxic effects of the nitro and dichloronitro benzene nucleus to the host without removing antimicrobial activity. The esters were more effective than the free amino acids in reducing acute toxicity to mice. Some antibacterial and antifungal activity was retained in these derivatives, particularly the N-4,5-dichloro-2-nitrophenyl α-amino acids.

BELLENGHI and Wittgens (1) have reviewed the antimycotic and antibacterial effects of nitrovinyl derivatives in the benzene, thiophene, and furan series and shown that the presence of the nitro group in these compounds generally enhanced these activities. Other nitro-containing furans (2), quinolines (3), and phenols (4–6) have been tested for antibacterial action, and the introduction of the nitro group has generally enhanced the effectiveness of the compounds against bacteria. Serious toxic effects may be observed from the use of nitro-containing compounds, however, such as Tien, *et al.* (7), found in a series of N-arylglycines that showed good antitubercular activity *in vitro*.

Previously, in this laboratory, successful use was made of D-glucamine as a detoxifying agent for nitro-containing and halogenated benzene derivatives (8), but with the exception of the 2-nitro-4,5-dichlorophenyl residue, antibacterial activity was also removed by introduction of the glucamine moiety. Introduction of the glycine and glycine ethyl ester residues into the nitro benzenes did not cause such a striking detoxication effect, but neither was the antibacterial spectrum diminished so drastically. Since then, a series of N-2,4-dinitrophenyl and N-2-nitro-4,5-dichlorophenyl derivatives of several representative α-amino acids and their ethyl esters have been prepared, and we now can report the effects these amino acids and esters have on the toxicities and antibacterial spectra of the dinitro and dichloronitro benzene residues. The physical properties of the compounds prepared are listed in Table I.

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The relative detoxifying effects of the various amino acids and esters may be seen in Table II, where the acute i.p. toxicities in mice are recorded. With the exception of the serine derivative, the N-2,4-dinitrophenyl amino acids were found to be lethal to mice within the dose range of 400–800 mg./Kg. A greater range of toxicities, and generally greater toxicity, was found with the N-4,5-dichloro-2-nitrophenyl amino acids, the glycine and serine derivatives conferring the largest detoxifying effects. The amino acid esters showed a more dramatic detoxifying effect, on the 2-nitro-4,5-dichlorophenyl residue, the lethal doses to mice exceeding 800 mg./Kg. The leucine ester was not obtained in crystalline form for comparison.

Antibacterial screening of the compounds prepared (with the exception of the glycine ester derivative) was carried out by using a small quantity of the dry, powdered material placed on an agar plate. These tests (Table III) showed that in approximately two-thirds of the cases (10 organisms were used) growth inhibition resulted, although the success of this method is dependent upon the ability of the compounds to diffuse through agar. Lack of solubility of the compounds in suitable solvents prevented the use of more customary procedures.

Antimicrobial testing against a larger number of bacteria and several fungal plant pathogens was carried out at the Lilly Research Laboratories with some of the compounds, using the agar dilution technique (see Table IV). N-(2,4-Dinitrophenyl)-methionine, N-(2,4-dinitrophenyl)-leucine, and N-(2,4-dinitrophenyl)-phenylalanine showed no growth inhibition of the organisms tested below a concentration of 200 mcg./ml., but the results obtained with the other compounds tested are shown in Table IV. The 2,4-dinitrophenyl amino acids, the most consistent inhibitors on the agar plate, were found to be without antibacterial activity at the dilutions employed. The 4,5-dichloro-2-nitrophenyl

TABLE I.—N-2,4-DINITROPHENYL- AND N-4,5-DICHLORO-2-NITROPHENYL-DL-AMINO ACIDS AND ESTERS

Compound	M.p., °C.	Purification Solvent	Yield, %	Formula	Analyses, %—Calcd.	Found
N-(2,4-Dinitrophenyl)-glycine	203.5–205.5[a]	Aq. methanol	44	$C_8H_7N_3O_6$	...	...
N-(2,4-Dinitrophenyl)-DL-phenylalanine	217.5–219[b]	Ether-pet. ether	19	$C_{15}H_{13}N_3O_6$	...	...
N-(2,4-Dinitrophenyl)-DL-leucine	134.5–135.5[c]	Ether-pet. ether	32	$C_{12}H_{15}N_3O_6$	...	...
N-(2,4-Dinitrophenyl)-DL-methionine	122–123.5[d]	Ether-pet. ether	17	$C_{11}H_{13}N_3O_6S$	...	...
N-(2,4-Dinitrophenyl)-DL-serine	204–206[e]	Ether-pet. ether	33	$C_9H_9N_3O_7$	...	...
N-(4,5-Dichloro-2-nitrophenyl)-glycine	198–199	Aq. ethanol	31	$C_8H_6Cl_2N_2O_4$	C, 36.30 H, 2.30	36.50 2.30
N-(4,5-Dichloro-2-nitrophenyl)-DL-phenylalanine	193–194	Aq. ethanol	8	$C_{15}H_{12}Cl_2N_2O_4$	C, 50.70 H, 3.40	51.15 3.34
N-(4,5-Dichloro-2-nitrophenyl)-DL-leucine	172.5–173	Aq. ethanol	21	$C_{12}H_{14}Cl_2N_2O_4$	C, 44.88 H, 4.39	45.07 4.48
N-(4,5-Dichloro-2-nitrophenyl)-DL-methionine	135–136.5	Aq. ethanol	26	$C_{11}H_{12}Cl_2N_2O_4S$	C, 38.94 H, 3.56	39.08 3.77
N-(4,5-Dichloro-2-nitrophenyl)-DL-serine	203.5–204	Aq. methanol	39	$C_9H_8Cl_2N_2O_5$	C, 36.63 H, 2.73	36.67 2.96
Ethyl N-(4,5-dichloro-2-nitrophenyl)-glycinate	122–123	Aq. ethanol	24	$C_{10}H_{10}Cl_2N_2O_4$	C, 40.80 H, 3.40	41.07 3.58
Ethyl N-(4,5-dichloro-2-nitrophenyl)-DL-phenylalaninate	104–106	Aq. ethanol	58	$C_{17}H_{16}Cl_2N_2O_4$	C, 51.90 H, 4.10	52.88 4.22
Ethyl N-(4,5-dichloro-2-nitrophenyl)-DL-methioninate	67– 69	Abs. ethanol	22	$C_{13}H_{16}Cl_2N_2O_4S$	C, 42.50 H, 4.40	42.01 4.58
Ethyl N-(4,5-dichloro-2-nitrophenyl)-DL-serinate	127–128	Aq. ethanol	19	$C_{11}H_{12}Cl_2N_2O_5$	C, 40.90 H, 3.70	40.80 3.70

[a] Lit. (12) m.p. 203–204°. [b] Lit. (12) m.p. 204–206°. [c] Lit. (13) m.p. 133°. [d] Lit. (12) m.p. 117–118°. [e] Lit. (12) m.p. 200–202°.

TABLE II.—ACUTE INTRAPERITONEAL TOXICITIES[a] IN MICE

No.	Compound	Dose, mg./Kg.	No. Died/No. Used	Toxic Signs[b]
1	N-(2,4-Dinitrophenyl)-glycine	400	1/5	Died in 6 hr.
		800	5/5	Died in 3 hr.
2	N-(4,5-Dichloro-2-nitrophenyl)-glycine	400	2/5	Died in 6 hr.
		800	5/5	Died, 1–3 hr.
3	N-(4,5-Dichloro-2-nitrophenyl)-glycine ethyl ester	400	0/5	
		800	1/5	Died overnight
4	N-(2,4-Dinitrophenyl)-DL-phenylalanine	400	3/5	Died in 8 hr.
		800	5/5	Died, 10–30 min.
5	N-(4,5-Dichloro-2-nitrophenyl)-DL-phenylalanine	200	2/5	Died in 48 hr.
		400	5/5	Died in 1 hr.
6	N-(4,5-Dichloro-2-nitrophenyl)-DL-phenylalanine ethyl ester	400	1/5	Died in 2 days
		800	1/5	Died in 2 days
7	N-(2,4-Dinitrophenyl)-DL-leucine	400	1/5	Died in 2 days
		800	5/5	Died in 6 hr.
8	N-(4,5-Dichloro-2-nitrophenyl)-DL-leucine	200	1/5	Died in 3 days
		400	5/5	Died in 8 hr.
9	N-(2,4-Dinitrophenyl)-DL-methionine	400	1/5	Died in 6 hr.
		800	5/5	Died in 3 hr.
10	N-(4,5-Dichloro-2-nitrophenyl)-DL-methionine	100	3/5	Died overnight
		200	4/5	Died in 6 hr.
11	N-(4,5-Dichloro-2-nitrophenyl)-DL-methionine ethyl ester	400	0/5	
		800	0/5	Some ataxia
12	N-(2,4-Dinitrophenyl)-DL-serine	400	0/5	
		800	1/5	Died overnight
13	N-(4,5-Dichloro-2-nitrophenyl)-DL-serine	400	1/5	Died overnight
		800	5/5	Died, 3–4 hr.
14	N-(4,5-Dichloro-2-nitrophenyl)-DL-serine ethyl ester	400	0/5	
		800	0/5	None

[a] Determined at the Lilly Research Laboratories by C. L. Rose. [b] Observations were made for 1 week after single injections of compound.

amino acids in general showed some antibacterial activity, although none approached the breadth of spectrum previously found for N-(4,5-dichloro-2-nitrophenyl)-D-glucamine (8). Only two of the esters were tested, the glycine and phenylalanine derivatives. The former showed no activity while the latter showed some activity, particularly against the fungal plant pathogens. N-(2,4-Dinitrophenyl)-glycine was also tested in mice against two types of virus, *Streptococcus pyogenes*, *Proteus vulgaris*, and typhoid organisms, but was found ineffective.

TABLE III.—ANTIBACTERIAL SCREENING[a]

Test Organism	Compound Number 1	2	4	5	6	7	8	9	10	11	12	13	14
Staphylococcus aureus	−	−	−	−	+	−	−	−	−	+	−	−	+
Sarcina lutea	−	−	−	−	−	−	−	−	−	−	−	−	+
Bacillus subtilis	−	+	+	+	+	−	+	−	+	+	−	−	+
Escherichia coli	−	+	−	+	+	−	−	−	−	+	−	−	+
Aerobacter aerogenes	−	+	+	+	+	−	+	−	+	+	−	−	+
Serratia marcescens	−	+	+	+	+	−	+	−	+	+	−	−	+
Alcaligenes faecalis	−	−	−	−	+	−	−	−	−	−	−	−	+
Klebsiella pneumoniae	−	−	−	−	+	−	−	−	−	−	−	−	+
Proteus vulgaris	−	−	−	−	−	−	−	−	−	−	−	−	+
Pseudomonas aeruginosa	−	−	−	+	+	−	+	−	+	+	−	+	+

[a] A negative sign denotes inhibition of growth, which was usually over a radius of 2–3 mm. on the agar plate. A small quantity of the dry, powdered compound was placed on the plate.

TABLE IV.—ANTIBIOTIC SPECTRA[a]

Test Organism	Inhibitory Concentration, mcg./ml.[b] Compd. No. 5	6	8	10	13
Staphylococcus aureus	200	...	50	100	..
Staphylococcus albus	...	...	50	100	..
Bacillus subtilis	200	...	50	100	..
Sarcina lutea	200	...	50	100	..
Mycobacterium tuberculosis	200	200	50	50	50
Mycobacterium avium	...	...	100	100	..
Escherichia coli	...	...	...	...	..
Proteus vulgaris	...	...	...	100	..
Pseudomonas aeruginosa	...	...	...	[c]	..
Aerobacter aerogenes	...	...	...	...	..
Klebsiella pneumoniae	...	...	...	...	..
Salmonella enteritidis	...	...	...	...	..
Shigella paradysenteriae	...	...	...	...	..
Saccharomyces pastorianus	...	...	...	...	..
Candida albicans	...	...	...	...	..
Trichophyton rubrum	...	200	...	...	..
Trichophyton interdigitale	...	200	100	...	..
Brucella bronchiseptica	...	...	...	5	..
Vibrio metschnikovii	...	...	100	...	..
Alternaria solani	200	...	...	...	..
Aspergillus niger	...	200	...	...	..
Ceratostomella ulmi	...	200	...	...	..
Colletotrichum lagenarium	...	...	...	...	..
Colletotrichum phomoides	...	200	...	...	..
Colletotrichum pisi	...	200	...	...	..
Endoconidiophora fagacearum	...	200	...	...	..
Sclerotinia fructicola	...	200	...	...	..

[a] Carried out at the Lilly Research Laboratories by M. Clarkson and D. Fleming. [b] The agar dilution technique was used, the bacteria being observed for 48 hours and the fungal plant pathogens for 72 hours. [c] Test organism did not grow sufficiently well to establish an end point; where no inhibitory concentration is reported, the organism was not inhibited by 200 mcg./ml. of compound.

It may be concluded from these comparisons that the introduction of the α-amino acid residue into the *m*-dinitrobenzene molecule shows a small but definite detoxifying effect (*m*-dinitrobenzene killed four of five mice used at 400 mg./Kg., and 2,4-dinitroaniline killed all of five mice at 400 mg./Kg.). The amino acid esters, however, showed a greater ability to detoxify, when attached to the 4,5-dichloro-2-nitrophenyl nucleus. Again, as in our previous study (8), loss of toxicity to mice generally paralleled loss of toxicity to microorganisms, although use of the amino acid ester derivatives holds some promise that a selective detoxifying effect without corresponding loss of antimicrobial ability may be achieved.

EXPERIMENTAL

The melting points were taken on a Fisher-Johns block and are uncorrected. Analyses were done either by Weiler and Strauss, Oxford, England, or by Carol K. Fitz, Needham, Mass.

N-2,4-Dinitrophenylamino Acids.—These compounds were prepared either by the method of Abderhalden and Blumberg (9) or Sanger (10). A representative synthesis follows. DL-Phenylalanine (2.0 Gm., 0.012 mole) and sodium bicarbonate (4.0 Gm., 0.048 mole) were dissolved in 50 ml. of water, and 2.5 ml. (0.220 mole) of 2,4-dinitrofluorobenzene in 100 ml. of ethanol was added. The mixture was shaken at room temperature for 2 hours, and the ethanol was then removed by evaporation. The resulting mass was dissolved in water, shaken three times with ether, and the aqueous solution was acidified. The yellow precipitate was collected, washed with ice water, and dissolved in acetone. The acetone solution was shaken with anhydrous sodium sulfate, filtered, and evaporated to a small volume. An equal volume of benzene was added, followed by petroleum ether until the product precipitated. The compound was dried in air, dissolved in ether, and reprecipitated with petroleum ether. This precipitation procedure was repeated several times until well-formed crystals were obtained. The yield was 0.78 Gm. (19%) of yellow crystalline compound, m.p. 217.5–219° [lit. (10) m.p. 204–206°].

N-4,5-Dichloro-2-nitrophenylamino Acids.—A typical procedure follows. Glycine (0.75 Gm., 0.01 mole) and sodium bicarbonate (1.68 Gm., 0.20 mole) were dissolved in 50 ml. of warm water and 50 ml. of warm ethanol was added. 1,2-Dinitro-4,5-dichlorobenzene (2.37 Gm., 0.01 mole) [prepared by the procedure of Hartley and Cohen (11)] was dissolved in 100 ml. of warm aqueous ethanol (50:50). The two solutions were mixed and boiled until no odor of ethanol could be detected. The solution was cooled and filtered, and the residue was dissolved in warm water. The aqueous solution was acidified,

and the yellow product was filtered and recrystallized from aqueous ethanol. The yield was 0.83 Gm. (31%); m.p. 198–199°.

N-4,5-Dichloro-2-nitrophenylamino Acid Ethyl Esters.—The following procedure is representative. N-4,5-Dichloro-2-nitrophenyl-DL-serine (3.0 Gm., 0.008 mole) was dissolved in 100 ml. of absolute ethanol. The solution was heated to 70°, and dry hydrogen chloride was introduced, with stirring, for a period of 2 hours. The resulting solution was evaporated to a small volume and cooled. Water was added until the ester precipitated, and the precipitate was collected. This precipitation from ethanol was repeated, and the product was dried over calcium chloride. The yield was 0.60 Gm. (19%); m.p. 127–128°.

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Phytochemical Investigation of *Amphipterygium adstringens*

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A general phytochemical investigation was conducted on *Amphipterygium adstringens*, Schlecht. Numerous qualitative studies were conducted and several conventional techniques were employed in the detection, isolation, purification, and chemical characterization of constituents. Qualitative chemical tests showed the presence of phytosterols, glycosides, and tannins, and the absence of alkaloids and phloroglucides. An organic acid and a steroidal saponin were isolated from the diethyl ether and *n*-butanol extracts, respectively. The *p*-nitrobenzyl and *p*-phenylphenacyl derivatives of the carboxylic acid were prepared. The neutralization equivalent and infrared spectrum of the isolated acid were established. Chromatographic and infrared studies of the saponin have been reported.

THIS PHYTOCHEMICAL study was initiated primarily because preliminary screening tests indicated that *Amphipterygium adstringens*, Schlecht (Fam. *Julianiaceae*) possessed a certain degree of anticancer activity (1). This observation, together with a survey of the available literature which revealed no apparent report on the exact chemistry of this botanical species, stimulated interest and prompted this investigation. Furthermore, since Martinez (2) briefly makes note of *Amphipterygium adstringens* in the second part of his book which deals only with medicinal plants lacking scientific investigation, it was felt that the project was worthy of exploration.

A survey of botanical literature revealed disagreements in the scientific nomenclature of *Amphipterygium adstringens*, Schlecht. Some of the early reports refer to the species as *Hypopterygium adstringens*. In the more recent literature the generic name is given as *Juliania*, family *Julianiaceae*. *Amphipterygium adstringens*, commonly known as "Cuachalalate," is a small tree indigenous to Mexico, growing from Michoacán to Morelos and Puebla to Oaxaca. The assumed medicinal effects of the bark are briefly described by Martinez (2). Concoctions of the bark are used extensively for alleviating numerous conditions, including malaria, intermittent fever, ulcers, and cancer of the gastrointestinal tract.

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