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ANTIFUNGAL ACTIVITY OF SUBSTITUTED NITROANILINES AND RELATED COMPOUNDS

By N. G. CLARK and A. F. HAMS

The preparation of a group of nitroanilines and some related compounds is described, together with two of the biological methods adopted for the assessment of their antifungal activity. Certain of these compounds show high activity against *Botrytis cinerea*, and one of them, 2,6-dichloro-4-nitroaniline, has been developed as a commercial fungicide (' Allisan ').

Introduction

While a number of Botrytis dusts are commercially available, none is entirely satisfactory and a search for an improved material has been pursued in these laboratories for some years. In the course of routine testing it soon became clear that a group of nitroanilines was highly active in certain tests.

This paper contains a description of the biological methods employed for initial screening and a discussion of the structure/activity relationships which emerged from the testing results. Since this work was completed, one of the compounds has been formulated and marketed under the name ' Allisan '.[1] This is a dust containing 2,6-dichloro-4-nitroaniline, used for the control of *Botrytis* in lettuce grown under glass during the winter. Its practical applications and overall efficacy form the subject of another paper.

Experimental

Preparation of materials

All the chemicals used in this investigation are known in the literature and were prepared by standard methods. The preparation of some of them, however, raised points of interest, and these are now reported briefly.

2,4,6-*Trichloro*-3-*nitroaniline*.—A poor yield of the desired material, highly contaminated with tar, was obtained by the action of chlorine on a solution of *m*-nitroaniline in hydrochloric acid, but nitration of acet-2,4,6-trichloroanilide[2] and then hydrolysis afforded a satisfactory yield.

6-*Bromo*-4-*chloro*-2-*nitroaniline*.—The only previous references to this compound appear to be by Körner,[3] who gives a m.p. of 106·4°, and by Orton[4] who quotes a value of 114–115°. The present work yielded a compound melting at 107–107·5° (Found : C, 28·9 ; H, 1·5. Calc. for $C_6H_4BrClN_2O_2$: C, 28·6 ; H, 1·6%).

4-*Bromo*-6-*chloro*-2-*nitroaniline*.—This compound is best prepared by direct chlorination of 4-bromo-2-nitroaniline.[5] A solution of 4-bromo-2-nitroaniline (25 g.) in benzene (750 ml.) was maintained below 30° while a slow stream of chlorine was bubbled in for about 1 h. The resulting solid was collected, washed with a little benzene, stirred with cold water for 10 min., and again collected. Two crystallisations from ethanol gave 9·1 g. of material m.p. 117–118°. Orton[4] gives m.p. 114°.

4,6-*Dichloro*-N-*methyl*-2-*nitroaniline*.—A more convenient method of preparing this compound is as follows. A solution of 4-chloro-*N*-methyl-2-nitroaniline[6] (10 g.) in glacial acetic acid (100 ml.) was treated with conc. hydrochloric acid (50 ml.) ; an aqueous solution of potassium chlorate (3·1 g. in 50 ml.) was then dropped in during 1 h. After being stirred for a further 2 h. during which time nearly all the solid dissolved, the liquid was filtered, diluted with water, and the resulting solid collected. Thorough washing with water, followed by two crystallisations from ethanol, gave the product (4·2 g.) as orange-red crystals m.p. 78–79°. Blanksma[7] quotes m.p. 80°.

2,3,5,6-*Tetrachloro*-4-*nitroanisole*.—The only previous references to this compound are by Berckmans & Holleman,[2] who give a m.p. of 105–106°, and by Peters *et al.*,[8] who quote a value of 112–113°. The present work yielded a compound melting at 104–105° (Found : C, 29·1 ; H, 1·2. Calc. for $C_7H_3Cl_4NO_3$: C, 28·9 ; H, 1·0%).

4-*Chloro*-NN-*dimethyl*-2-*nitroaniline*.—A more convenient method of preparation appears to be the following. A mixture of 2,5-dichloro-nitrobenzene (90 g.), methanolic dimethylamine solution (120 g., 30% w/w) and ethanol (120 ml.) was heated in an autoclave for 4 h. at 160°. After being cooled, the solid product was removed, washed with a little methanol, and recrystallised from the same solvent. Yield, 75 g., m.p. 54–55°. Clemo & Smith[9] give m.p. 56°.

2,6-*Dichloro*-N-*methyl*-4-*nitroaniline*.—This material is conveniently obtained as follows. A solution of 2-chloro-*N*-methyl-4-nitroaniline[10] (10 g.) in a mixture of glacial acetic acid (100 ml.) and conc. hydrochloric acid (50 ml.) was stirred during 30 min. while an aqueous solution of potassium chlorate (3·1 g. in 50 ml.) was added dropwise. After being stirred for a further 2 h., the reaction mixture was diluted with water, the precipitate collected, washed thoroughly with water, and crystallised twice from ethanol. Yield, 3·4 g., m.p. 83–84°. Qvist & Nermes[11] report a m.p. of 82·5–83°.

2,3,5,6-*Tetrachloro*-4-*nitrophenol*.—A mixture of 2,3,5,6-tetrachloro-1,4-dinitrobenzene (20 g.), aqueous sodium hydroxide solution (140 ml. of 1N) and water (140 ml.) was refluxed for 3 days, cooled and filtered. The deep red filtrate was acidified with hydrochloric acid, the precipitate collected, washed with a little water, and recrystallised from aqueous acetic acid. The yield of colourless needles was 5·4 g., m.p. 147–148° (decomp.). This compound has been obtained previously only as a by-product, m.p. 148–149° (decomp.).[8]

Methods of testing

(a) *Impregnation test*

This test is designed to assess the effect of chemicals upon the mycelial growth of fungi by physical contact and also, where the compounds are volatile, by vapour-phase action.

Agar containing 2% malt extract is prepared, melted and divided into 20-ml. portions each in a separate tube ; these are then sterilised for 20 min. at 20 p.s.i. steam pressure. Calculated quantities of stock acetone solutions of the test chemicals are added to the tubes of hot agar to give an initial concentration of 10 p.p.m. of chemical ; most of the acetone evaporates. The contents of the tubes are poured into sterilised Petri dishes, which are agitated to remove

the last traces of acetone and to mix the chemical thoroughly with the agar. When set, these plates are inoculated with a loopful of a spore suspension of *Botrytis cinerea*. This is done with the plates inverted so that the agar surface is facing downwards at the time of inoculation. The plates are incubated in this position for 6–7 days at 23°, by which time the diameter of the fungus colony in the control plates (without chemical) is approximately 80 mm. Assessment is based on the mean colony diameter, measured in two directions at right-angles, for each of three replicates.

The percentage degree of inhibition of mycelial growth is given by the formula $100(C-T)/C$, where C and T are the diameters of the untreated (control) and treated colony, respectively. Where any concentration of chemical produces a control of greater than 50%, the compound is re-tested at lower concentrations down to 1 p.p.m.

(b) *Spore germination test*

A detailed description of this technique has already been given.[12] The test requires drops of an aqueous spore suspension of the test fungus to be placed on glass slides previously coated with a thin layer of the chemical under investigation. After incubation for 18 h., the effect of the chemical upon spore germination is assessed microscopically. The antifungal activity is expressed as percentage inhibition of germination, and is related to the strength of the acetone solution of the chemical used to coat the glass slide (not, directly, to the density of the deposit).

Since the previous publication, the range of fungi has been altered, and now consists of *Botrytis cinerea*, *Cladosporium fulvum* and *Venturia inaequalis*. Also, the highest concentration of test chemical in acetone has been reduced to 80 p.p.m., giving a dilution sequence of 80, 16, 8, 4, 2.

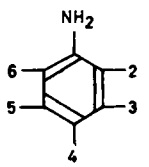
Results

Table I contains a list of the 48 compounds examined for antifungal activity, and includes

Table I

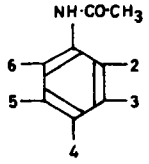
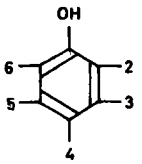
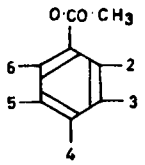
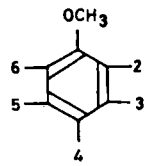
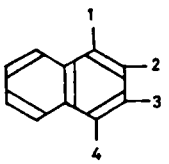
Impregnation test

Nitroanilines



	Substituents						% Control at a concentration of		
	N	2	3	4	5	6	5 p.p.m.	2·5 p.p.m.	1 p.p.m.
1		Cl	CH_3	Cl		NO_2	73	86	10
2		Cl		Cl		NO_2	98	83	44
3		Cl		NO_2		Cl	86	74	42
4		Br		Cl		NO_2	84	72	52
5		Cl		Br		NO_2	80	63	40
6		Br		NO_2		Cl	98	77	47
7		Br		NO_2		Br	78	49	—
8		I		NO_2		I	52	39	—
9	CH_3	Cl		Cl		NO_2	82	58	37
10	CH_3	Cl		NO_2		Cl	81	58	40
11	$2CH_3$	Cl		NO_2		Cl	68	17	—
12		Cl		NO_2	Cl		61	43	—
13		Cl	NO_2	Cl		Cl	82	68	29
14		Cl	Cl	NO_2	Cl	Cl	37*		
15		Cl		NO_2		NO_2	79	42	—
16		NO_2		NO_2		NO_2	55	24	—
17		NO_2		Cl			52	4	—
18		Cl		NO_2			16	—	—
19		NO_2		Br			24	—	—
20		Br		NO_2			47	—	—
21	CH_3	NO_2		Cl			33	—	—
22	CH_3	Cl		NO_2			37	—	—
23	$2CH_3$	NO_2		Cl			21	—	—
24	$2CH_3$	Cl		NO_2			16	—	—
25		Cl		NO_2		CH_3	45	—	—

Table I (*continued*)

	Substituents						% Control at a concentration of		
	N	2	3	4	5	6	5 p.p.m.	2·5 p.p.m.	1 p.p.m.
26		Cl		NO_2		CH_3O	23	—	—
27		NO_2		CH_3			5	—	—
28		NO_2		CH_3O			23*	—	—
29		CH_3		NO_2			38*	—	—
30		CH_3O		NO_2			13*	—	—
Standard	(T.C.N.B.)						80	58	45
				Acetanilides					
31		Cl		Cl		NO_2	33*	—	—
32		Cl		NO_2		Cl	41*	—	—
33		Cl		NO_2		NO_2	24*	—	—
34		NO_2		Cl			14	—	—
35		NO_2		CH_3			50	—	—
36		NO_2		CH_3O			12*	—	—
37		CH_3O		NO_2			0	—	—
				Phenols					
38		Cl		NO_2		Cl	27	—	—
39		Cl	Cl	NO_2	Cl	Cl	56	34	—
40		Cl		NO_2			61	9	—
				Phenyl acetates					
41		Cl		NO_2		Cl	23	—	—
42		Cl	Cl	NO_2	Cl	Cl	51	14	—
43		Cl		NO_2			32	—	—
				Anisoles					
44		Cl		NO_2		Cl	83	38	—
45		Cl	Cl	NO_2	Cl	Cl	76	27	—
				Naphthalenes					
46		1-Amino-4-chloro-2-nitro-					28	—	—
47		1-Acetamido-4-chloro-2-nitro-					0	—	—
48		1-Amino-2,4-dinitro-					25	—	—

* At a concentration of 10 p.p.m.

their chemical structure and biological activity in the impregnation test. The results obtained with 2,3,5,6-tetrachloronitrobenzene (T.C.N.B.), used as a standard, are also included. Because of the generally high activity displayed in this test, as distinct from the poor response in the other test, the compounds are arranged in Table I in a manner best designed to illustrate the relationship between chemical structure and activity against *Botrytis cinerea*. The same numerical arrangement is retained for the other test.

Table II shows the results of the spore germination test. These are presented as the approximate strength of an acetone solution of the chemical which would effect a 50% inhibition of spore germination (i.e., the approx. LD_{50}).

Table II

Spore germination test

(results are approx. LD_{50} in p.p.m.)

Compound no.	*Botrytis cinerea*	*Cladosporium fulvum*	*Venturia inaequalis*	Compound no.	*Botrytis cinerea*	*Cladosporium fulvum*	*Venturia inaequalis*
1	>80	>80	>80	25	>80	>80	>80
2	>80	>80	>80	26	>80	>80	>80
3	>80	>80	>80	27	>80	>80	>80
4	50	>80	>80	28	>80	>80	>80
5	>80	>80	>80	29	>80	>80	>80
6	>80	>80	>80	30	>80	>80	>80
7	>80	>80	>80	31	80	>80	>80
8	>80	>80	>80	32	80	>80	>80
9	>80	>80	>80	33	>80	>80	>80
10	50	>80	>80	34	50	>80	>80
11	>80	60	>80	35	>80	>80	>80
12	>80	50	>80	36	>80	>80	>80
13	50	60	50	37	>80	>80	>80
14	>80	>80	>80	38	70	>80	70
15	50	>80	50	39	50	60	2
16	60	12	50	40	50	60	5
17	>80	>80	>80	41	50	>80	14
18	>80	>80	>80	42	>80	16	14
19	50	>80	70	43	50	50	50
20	70	70	70	44	>80	>80	>80
21	>80	>80	>80	45	>80	70	>80
22	>80	>80	>80	46	>80	>80	>80
23	>80	>80	>80	47	>80	>80	>80
24	>80	>80	>80	48	>80	>80	>80

Table III presents a typical selection of results from greenhouse trials. Five of the compounds giving the best control of *Botrytis cinerea* in the impregnation test were formulated at 4% dusts and used for treating commercially grown lettuce. Results on untreated lettuces are included.

Table III

Greenhouse trials

Compound no.	No. of survivors after 15 weeks (max. 140)	% marketable	Average weight per plant, oz.
2	140	46	1·3
3	130	94	1·8
4	137	97	1·1
5	125	89	1·2
6	133	90	2·1
Untreated	74	40	1·5

Discussion

(a) *Impregnation test*

The present series of compounds was prepared and tested as a result of the high activity exhibited in the impregnation test by compound 1, an intermediate submitted for routine

screening. The methyl group makes this compound relatively inaccessible, but the lower homologue (without the methyl group) is readily available. It was not surprising to find, therefore, that omission of the inert methyl group (compound 2) caused no loss of activity. A comparable level of activity was also exhibited by an isomer, 2,6-dichloro-4-nitroaniline (compound 3), which was ultimately developed as a commercial product.[13] Taking compounds 2 and 3 as models, a group of analogues was prepared in which one or both of the chlorine atoms has been replaced by bromine or iodine (compounds 4–8). High activity was again evident, although there were signs of this diminishing in the high-molecular-weight di-bromo and di-iodo derivatives.

Mono-methylation of compounds 2 and 3 in the amino-group (compounds 9 and 10) had no effect on the activity, but dimethylation caused a diminution in antifungal power (compound 11).

Another isomer of compounds 2 and 3 had good activity (compound 12); this was also demonstrated by compound 13 containing three chlorine atoms. Compound 14, however, containing four chlorine atoms, was almost devoid of activity. The presence of halogen appeared to be essential for high activity, since partial deactivation occurred when one or both of the chlorine atoms in compound 3 were replaced by nitro-groups (compounds 15 and 16).

Few other structural modifications could be effected without greatly impairing the biological efficacy of the compounds. Thus, omission of one of the halogen atoms, leaving one halogen and one nitro-group, reduced activity to a low level (compounds 17–24), while replacement of one of the two chlorine atoms of compound 3 by a methyl or methoxyl group had a similar effect (compounds 25 and 26). Some other mono-substituted nitroanilines were almost devoid of activity (compounds 27–30).

The essential nature of the amino-group, if activity is to be retained, is revealed by the poor biological activity of the remaining compounds in Table I. Acetylation of compounds 2, 3 and 15 almost eliminated the activity (compounds 31–33), and did not improve a poor activity in other cases (compounds 34–37). Replacement of the amino-group by hydroxyl gave a group of phenols (compounds 38–40), but neither they nor their acetates (compounds 41–43) showed the same general activity that the related anilines exhibited. Two anisole derivatives, in which the original amino-group has been replaced by methoxyl, showed moderate activity (compounds 44–45). There appeared to be no compound of promise in the naphthalene series (compounds 46–48).

It is unwise to suggest a possible mode of action of these compounds based on the present limited evidence, but the presence of a free amino-group bearing at least one hydrogen atom seems essential for high activity. If this feature is destroyed (by acetylation or dimethylation) or masked (by two massive iodine atoms or nitro-groups in the *ortho* positions), then biological activity is markedly diminished.

(b) *Spore germination test*

In this test, the chemicals presented an almost uniform picture of inactivity, although compounds 39 and 40 showed high activity against one of the fungi.

(c) *Greenhouse trials*

Results from the impregnation test showed that nine compounds (1–6, 9, 10 and 13) exhibited high activity against *Botrytis cinerea*, and warranted further testing. Because of the relatively difficult syntheses involved (which would have resulted in too expensive a final product), four of these were omitted (compounds 1, 9, 10, 13). The remainder, in the form of 4% dusts, were assessed for their control of *Botrytis cinerea* on winter lettuce grown on a commercial scale. Some typical results from randomised block trials are given in Table III.

Only compounds 3 and 6 produced a satisfactory crop of lettuce, considering both numbers and size. The former, 2,6-dichloro-4-nitroaniline, was preferred to the latter, the closely related 2-bromo-6-chloro-4-nitroaniline, because it was more economical to manufacture. 2,6-Dichloro-4-nitroaniline has now been developed commercially.

Acknowledgments

The authors are grateful to Mr. A. W. Billitt and Dr. H. A. Stevenson for their encouragement and interest in the work, and to Mr. A. Watkinson, Miss G. Oram and Mr. A. J. Haywood for technical assistance.

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Received 18 April, 1961

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EFFECT OF SUPPLEMENTING WITH METHIONINE, CYSTEINE AND DERIVATIVES OF THIAZOLIDINE-4-CARBOXYLIC ACID ON THE NUTRITIVE VALUE OF HERRING-MEAL PROTEIN

By L. R. NJAA

The nutritive value of herring-meal protein was improved by supplementation with methionine, cysteine and derivatives of thiazolidine-4-carboxylic acid.

The effects with graded amounts of methionine were greater than would be expected from the contents of methionine and cystine in herring-meal and from the requirement of the young rat for these amino-acids.

Methionine supplementation also improved the nutritive value of an acetone-dried meal prepared from herring fillets.

The results are explained primarily by the fact that methionine + cystine is the first limiting factor in herring protein. Destruction of amino-acids during meal production seems to be small, although some destruction of the *S*-amino-acids cannot be completely excluded.

Introduction

The utilisation of fish-meal protein by the young rat is improved by supplementing it with methionine. Miller[1] explained this by assuming that protein damage during fish-meal production was due to reactions of the Maillard[2] type.

The present work was undertaken to test whether, and to what extent, methionine was the limiting amino-acid in herring-meal. Supplementation with cysteine, and with some reaction products of cysteine and some aldehydes, was also tested. The latter products, which have been referred to as colourless Maillard compounds, are probably derivatives of thiazolidine-4-carboxylic acid.[3–5]