

# Antiviral Activity of 2-( $\alpha$ -Hydroxybenzyl)-Benzimidazole and other 2-Substituted Benzimidazoles against TMV in Tomato Leaf Discs

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Z. Naturforsch. **37 c**, 390–393 (1982); received January 21, 1982

TMV, 2-Hydroxybenzimidazole, Antiviral Chemotherapy

2-( $\alpha$ -hydroxybenzyl)-, 2-benzyl-, 2-phenyl-, 2-methyl- and 2-hydroxymethyl-benzimidazoles were screened for antiviral activity against TMV in tomato leaf discs, for cytokinin activity in the *amaranthus* betacyanin bioassay and the soya bean callus bioassay and for antagonism in the latter bioassay. None of the test compounds showed either cytokinin activity or antagonism. 2-( $\alpha$ -hydroxybenzyl)-benzimidazole at 23.3  $\mu$ M inhibited infectious virus production by 100% and no virus antigen was detectable. None of the other compounds showed similar antiviral activity. The relationship between the substituent at the 2-position and antiviral activity was as previously reported for 2-substituted benzimidazoles in animal virus chemotherapy.

## Introduction

Benomyl (methyl-N-{1-(butylcarbamy)-2-benzimidazole}) and methyl benzimidazole-2-yl-carbamate (MBC, carbendazim), its aqueous decomposition product, have been shown to affect virus replication in plants [1–3] either increasing or decreasing virus production. Benomyl has been shown to exhibit cytokinin activity in the *amaranthus* betacyanin bioassay [4]. Synthetic and natural cytokinins have also been shown to stimulate and inhibit virus production in plants depending on the hormone concentration and virus/host combination used [2, 5–8]. Benzimidazole does not inhibit virus replication in plants [6] but has some cytokinin properties [9].

This investigation was undertaken to determine whether 2-( $\alpha$ -hydroxybenzyl)-benzimidazole (2-HBB), an inhibitor of the replication of some picornaviruses in animal tissue cultures [10] has antiviral activity in plants. Other 2-substituted benzimidazoles previously screened for antiviral activity in animal cells [11] were also tested. The cytokinin activity and possible cytokinin antagonism of these compounds was investigated in an attempt to clarify the relationship between cytokinin activity/antag-

onism and antiviral activity of 2-substituted benzimidazoles in plants.

## Materials and Methods

TMV strain O was purified as described previously [12]. Tomato plants cv. Potentate were inoculated with purified virus (21  $\mu$ g/ml) suspended in 0.05 M sodium phosphate buffer pH 7.2. Plants were grown in John Innes No. 1 compost supplemented with peat 1:1 in pots 15 cm in diameter in the glasshouse.

The test chemicals used were: 2-( $\alpha$ -hydroxybenzyl)-benzimidazole (2-HBB), 2-benzylbenzimidazole, 2-phenylbenzimidazole, 2-methylbenzimidazole and 2-hydroxymethyl benzimidazole (Aldrich Chemical Co., London, U.K.). The chemical structures are shown in Fig. 1.

## Test methods

Leaves of approximately 6–6.5 cm in length were taken from two month old plants. The leaves were rubbed gently with celite abrasive (3 mg/ml) then, using a 1.5 cm diameter cork borer, discs were punched from different leaves and randomised between petri dishes containing Hoaglands nutrient solution [13], and nutrient solution containing the test compounds. The remaining leaves were inoculated with TMV and air dried. Discs were then

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0341-0382/82/0500-0390 \$ 01.30/0



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## 2-SUBSTITUTED BENZIMIDAZOLE TEST COMPOUNDS

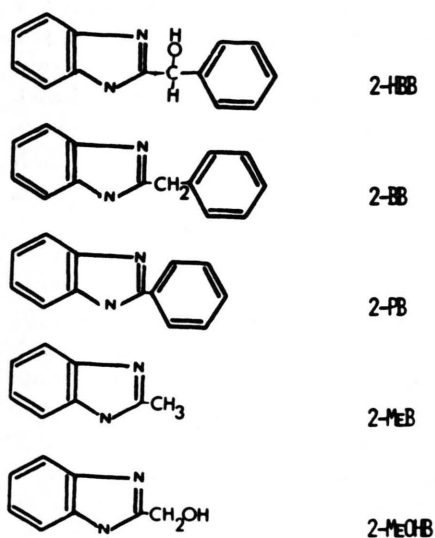


Fig. 1. Structures of the 2-substituted benzimidazole test compounds. 2-HBB, 2-( $\alpha$ -hydroxybenzyl)-benzimidazole; 2-BB, 2-benzyl-benzimidazole; 2-PB, 2-phenylbenzimidazole; 2-MeB, 2-methylbenzimidazole; 2-MeOHB, 2-hydroxy-methylbenzimidazole.

punched from the inoculated leaves and similarly floated on the nutrient solution and nutrient solution containing the test compounds. Four discs were put in each 7 cm diameter petri dish.

The petri dishes were incubated in a growthroom for seven days at 20 °C, 15 h photoperiod and a light intensity of 7 Wm<sup>-2</sup>. At the end of the experiment the discs were ground up in a pestle and mortar, one disc in 0.5 ml of 0.9% w/v NaCl in

10 mM sodium phosphate buffer pH 7.0. The extracts were filtered through nylon and assayed for infectious virus or virus antigen as described below.

Virus infectivity was determined by local lesion assay in *Nicotiana glutinosa* as follows. Four week old *N. glutinosa* plants were selected for uniformity, the top five expanded leaves were selected and the rest removed. All the leaves were then rubbed with celite suspension (3 mg/ml in water). Then 0.1 ml of the extract from the inoculated control discs was used as a standard and inoculated randomly to a different half leaf on each plant. The opposite half leaves were then inoculated randomly with extracts from leaves floated on the test solutions so that each treatment appeared once on every plant but at a different leaf position. Lesion numbers were counted after two days and after transformation [14] the lesion numbers for the test compounds were expressed as a percentage of the control.

The amount of antigen in the disc extracts was determined semi-quantitatively by microimmunodiffusion. The setting up of the slides was as described previously [15]. After three days incubation in a humid chamber at 22 °C, slides were stained in 0.1% coomassie blue in 25% isopropanol 10% acetic acid aq. (v:v:v) for twenty minutes and destained in 14% acetic acid, 7% methanol aq. (v:v), the slides were stabilized in 2% acetic acid, 10% glycerol aq. (v:v) for ten minutes and allowed to dry flat on blotting paper at room temperature. Relative estimates were made by measuring the maximum width of the peak of each precipitin band.

Cytokinin activity was determined by the soya bean callus assay [16]. The test compounds were screened at the same concentrations used for anti-

Table I. Infectivity of tobacco mosaic virus-inoculated tomato leaf discs treated with 2-substituted benzimidazole test compounds.

Chemical Conc. [μM]		Dilution of chemical									
		10 <sup>0</sup>		10 <sup>-1</sup>		10 <sup>-2</sup>		10 <sup>-3</sup>		10 <sup>-4</sup>	
		A	B	A	B	A	B	A	B	A	B
2-HBB	233	0.0000	100 *	0.0000	100 *	2.9808	43 *	5.0659	6 *	5.3371	0
2-BB	27	3.3883	28 *	4.5339	20 *	4.8848	11 *	5.0334	9 *	5.0939	7 *
2-PB	103	5.0541	12 *	5.0061	14 *	5.2369	10 *	4.8543	17 *	5.0283	14 *
2-MeB	129	5.2289	10 *	5.3552	7 *	5.1143	12 *	5.2048	10 *	5.1243	12 *
2-MeOHB	203	5.4084	5 *	5.3353	7 *	5.3931	5 *	5.3585	5 *	5.5881	2

A: Transformed lesion numbers based on mean number of lesions per half leaf in five *N. glutinosa* leaves.

B: Lesion numbers expressed as a percentage reduction compared to the opposite half leaf inoculated with control.

\* Indicates significant difference at P = 0.05.

viral screening (see Table I). Cytokinin antagonism was tested by incorporating 1 ml of each of the test compounds at the highest concentration used in 20 ml of the callus bioassay medium containing  $22.2 \times 10^{-9}$  M benzyladenine. Cytokinin activity was also screened in the amaranthus betacyanin bioassay [17]. Compounds were tested over the range of concentrations used to screen for antiviral activity (Table I).

## Results

The results of the screening for antiviral activity based on local lesion assay are shown in Table 1. Of the compounds screened, 2-HBB showed the greatest inhibitory effect on TMV replication. At 233 and  $23.3 \mu\text{M}$  2-HBB inhibited virus replication by 100%, at  $2.3 \mu\text{M}$  virus was inhibited by 43% and at  $0.23 \mu\text{M}$  by 6%. At  $0.023 \mu\text{M}$  2-HBB did not show any inhibitory activity. The next most inhibitory compound, which was much less active than 2-HBB, was 2-BB. 2-BB was less soluble than 2-HBB; at the maximum concentration used of  $27 \mu\text{M}$ , TMV was inhibited by 28%, at  $2.7 \mu\text{M}$ , inhibition was 20% and of the order of 7–11% at  $0.0027$ – $0.27 \mu\text{M}$ .

The antiviral activity of 2-PB, 2-MeB and 2-MeOHb was not great enough to indicate any potential role in antiviral chemotherapy.

The serological test results parallel the antiviral activity reported above. No antigen was detected in extracts from 2-HBB treatments which resulted in 100% reduction in lesion numbers or in the highest concentration of 2-BB used. Antigen was detected in all other cases.

None of the test compounds showed significant cytokinin activity in either the amaranthus betacyanin bioassay or in the soya bean callus bioassay. None of the test compounds appeared to antagonise 6-benzyl-adenine in the soya bean callus bioassay. The chlorophyll content of inoculated and non-inoculated discs from the treatments did not differ significantly from the control or from each other.

## Discussion

Present data show that 2-HBB inhibits TMV replication in tomato leaf discs. Both infectious virus and virus antigen are reduced. At 233 and  $23.3 \mu\text{M}$  virus antigen was detected. At lower concentrations

inhibition was reduced. No inhibition was detected at  $0.023 \mu\text{M}$  2-HBB.

Of the other 2-substituted benzimidazoles tested, only 2-BB showed a comparable level of inhibition. The solubility of 2-BB in nutrient solution was approximately 10% of that of 2-HBB. At approximately equimolar concentration (approx.  $27 \mu\text{M}$ ) it inhibited TMV by 28% compared to 100% with 2-HBB. Neither 2-PB, 2-MeB nor 2-MeOHb caused inhibition approaching that of 2-HBB.

These data confirm that 2-HBB inhibits a + RNA plant virus similar in putative replication strategy to those animal viruses similarly inhibited [10]. Further, the importance of the substituent at the 2-position,  $\alpha$ -hydroxybenzyl-, is in agreement with similar structure/activity relationships determined in animal cell viral chemotherapy [11].

None of the 2-substituted benzimidazoles screened in this work showed cytokinin activity in either the soya bean callus bioassay or in the amaranthus betacyanin bioassay, nor were they antagonists of 6-benzyl-adenine in the soya bean callus assay. The chlorophyll content of the discs was not affected by the treatments. Thus it would seem that cytokinin activity, as determined in the bioassays described here, is not a prerequisite for antiviral activity in 2-substituted benzimidazoles, and consequently some undesirable side effects of hormone action may be avoidable in 2-substituted benzimidazole antiviral chemotherapy. 2-HBB is reported to act as an inhibitor of RNA-dependent RNA polymerase induced in encephalomyocarditis (EMC) virus in Krebs-II ascites carcinoma cells [18]. In the case of TMV replication in tobacco cells (in which 2-HBB is also inhibitory (Cassells and Long, unpublished)), TMV appears to stimulate the synthesis of a host coded RNA-dependent RNA polymerase [19]. It would be interesting to determine whether 2-HBB inhibits the latter enzyme.

Resistance to 2-HBB has frequently been reported in animal cell systems (*e.g.* see 20). If the drug target in these cases is a virus-coded enzyme or a virus-coded protein which acts as a modifier of a host enzyme, then 2-HBB might be acting as a selection for mutant virus strains. If 2-HBB inhibits a host-coded enzyme, including the catalytic site of a host enzyme modified on virus infection, resistance should be infrequent offering a practical possibility for field use of 2-HBB. It should be stressed that some RNA-dependent replicases found in virus infected cells are

believed to be virus-coded *e.g.* in the case of cucumber mosaic virus infection in cucumber [21].

Caution must be exercised in extrapolating from the present model studies on leaf discs to whole plants when dealing with reversibly inhibitors for

reasons discussed previously [22] but the potential for chemotherapeutants like 2-HBB in plant tissue cultures is considerable [23], as inhibitor concentrations can be maintained, effectively undiluted with growth and development.

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