APPLIED MICROBIOLOGY, Oct. 1969, p. 660-667

Vol. 18, No. 4
Printed in U.S.A.

Antiviral and Antitumor Antibiotics

XX. Effects of Rotenone, Deguelin, and Related Compounds on Animal and Plant Viruses

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Received for publication 11 July 1969

Rotenoids and related compounds were investigated for their effects on animal and plant viruses. Of 35 compounds examined, rotenone, rotenone norketone, acetylrotenone, acetylrotenone norketone, deguelin, deguelic acid, dehydrodeguelin, and isotubanol norketone, all used at low concentrations, suppressed the growth of Newcastle disease and herpes simplex viruses as determined by the agar diffusion, plaque inhibition method. Most of the compounds likewise decreased the number of necrotic spots on tobacco mosaic virus-infected leaf discs. Only derrisic acid completely inhibited the local lesion formation at subphytotoxic concentrations. Correlation of antiviral activity with respiratory inhibition of these compounds is discussed.

In searching for antiviral antibiotics, we have noticed that some antibiotics produced by fungi inhibit mitochondrial respiration. On the basis of this observation, we have investigated the effects of some respiratory inhibitors such as ascochlorin, rotenone, antimycin A_3, piericidin A, pentachlorophenol, 2,4-dinitrophenol, and fatty acids on the multiplication of Newcastle disease virus (NDV; 13). Rotenone was found to have a profound effect on virus growth when determined by the agar diffusion, plaque inhibition method. Rotenoids and related compounds were synthesized and tested for their effects on certain animal and plant viruses. Some of the compounds were found to inhibit plaque or local lesion formation in vitro. The antiviral activities of these compounds will be discussed in comparison with their effects on mitochondrial respiration.

MATERIALS AND METHODS

Assay of anti-animal-virus activity. The Miyadera strain of NDV and the HF strain of herpes simplex virus (HSV) were used. Confluent monolayer cultures of primary chick embryo fibroblasts in petri dishes were infected with the viruses, and antiviral activity was tested by the agar diffusion, plaque inhibition method of Herrmann et al. (2) as reported previously (12).

Assay of anti-plant-virus activity. Coleoptiles of 20- to 25-day-grown beans (*Phaseolus vulgaris* L. "Pinto") were inoculated with tobacco mosaic virus (TMV) with the aid of carborundum and cotton swabs. The inoculum was obtained from TMV-infected tobacco leaves (*Nicotiana tobacum* L. Bright Yellow). Leaf discs (12 mm in diameter) were prepared by punching infected coleoptiles. Four discs were immersed in drug solution in each petri dish (3 cm in diameter), and two dishes were used for each drug concentration. They were incubated at 21 C under continuous fluorescent illumination. After 3 days of incubation, viral necrotic spots formed on the leaf discs were counted and expressed in per cent of controls.

Effect on mitochondrial respiration. Mitochondria were prepared from rat liver, and their respiratory potency was measured polarographically by the method of Hagihara (1). Glutamic acid and adenosine diphosphate were employed as substrates. The degree of drug effect on the mitochondrial respiration was expressed as the minimal drug concentration required for complete inhibition of oxygen consumption (see below).

Chemicals used. Rotenoids and the related compounds used in this experiment were obtained from derris roots or chemically synthesized as reported previously (4-11).

RESULTS AND DISCUSSION

Anti-animal-virus activity. As reported elsewhere (13), rotenone has a profound effect on the growth of NDV when examined by the agar diffusion, plaque inhibition method. Anti-NDV

TABLE 1. *Effect of rotenone, deguelin, and their derivatives on NDV growth as determined by the agar diffusion, plaque inhibition method*

No.	Compound	Zone	Concn (μg/ml)							
			20,000	4,000	800	160	32	6.4	1.3	0.3
I	Rotenone	AVZ[a]	52[c]	40	36	28	23	19	14	—
		CTZ[b]	39	33	24	19	—	—	—	—
II	Acetylrotenone	AVZ	L[d]	L	L	44	37	20	18	—
		CTZ	L	L	21	—	—	—	—	—
III	Dehydrorotenone	AVZ	ND[e]	25	17	13	±	—	—	—
		CTZ	ND	18	±	—	—	—	—	—
IV	Deoxyrotenone	AVZ	17	17	15	13	±	—	—	—
		CTZ	—	—	—	—	—	—	—	—
V	Rotenone norketone	AVZ	L	53	45	33	26	17	12	—
		CTZ	L	30	27	20	—	—	—	—
VI	Acetylrotenone norketone	AVZ	27	22	23	20	17	—	—	—
		CTZ	—	—	—	—	—	—	—	—
VII	Deguelin	AVZ	L	L	L	45	31	12	—	—
		CTZ	L	L	25	—	—	—	—	—
VIII	Dehydrodeguelin	AVZ	L	35	26	16	11	—	—	—
		CTZ	L	+	12	—	—	—	—	—

[a] Plaque-free antiviral zone.
[b] Cytotoxicity zone inside the AVZ.
[c] Values are zone diameters expressed in millimeters.
[d] Large.
[e] Not determined.

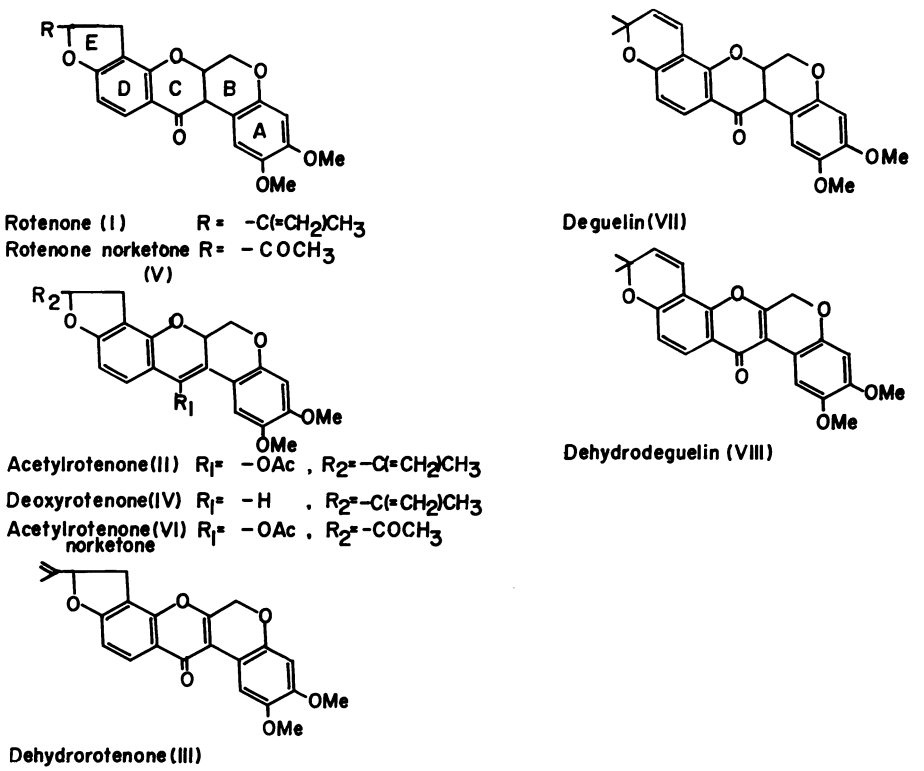


FIG. 1. *Structures of rotenone, deguelin, and their derivatives.*

activity of the related compounds was investigated by the same method (Tables 1–5).

Rotenone and deguelin have been isolated from derris roots and were found to be two of the most effective compounds among all those tested on NDV growth. Dehydrogenation of rotenone and deguelin and conversion to dehydrorotenone and dehydrodeguelin, respectively, decreased the antiviral activity by 5- to 125-fold, but replacement of the isopropenyl group on the dihydrofuran ring with methylketone did not change the activity (Table 1).

Esterification of carboxyl groups, in general, decreased both cytotoxicity and antiviral activity (Tables 2, 4, and 5). A rather evident exception was tephrosic acid (Table 4). Esterification of tephrosic acid strengthened the antiviral activity, but cytotoxicity seemed to be decreased as was the case with other compounds. Acetylrotenone (rotenone enolacetate) was more effective in suppressing NDV growth than was rotenone (Table 1), but, in this case, a strict comparison with the esterification of other compounds was not possi-

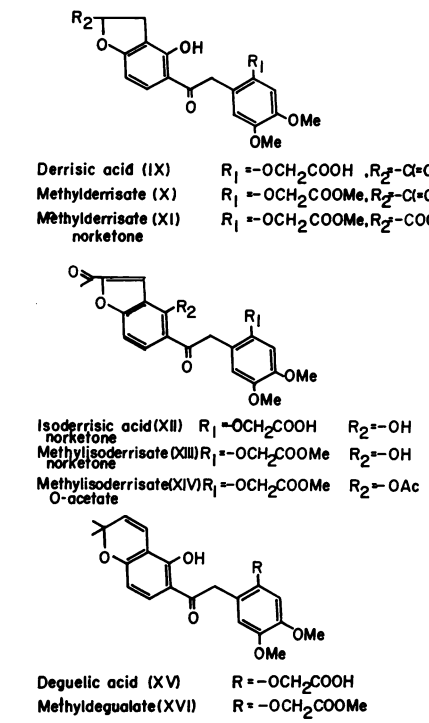


FIG. 2. *Structures of derrisic acid, deguelic acid, and their derivatives.*

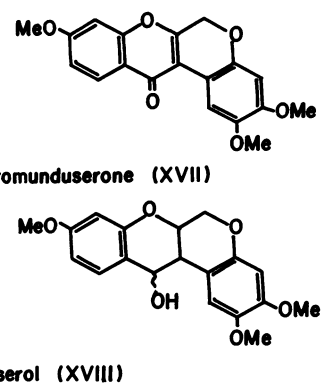


FIG. 3. *Structures of dehydromunduserone and munduserol.*

TABLE 2. *Effect of derrisic acid, deguelic acid, and their derivatives on NDV growth*[a]

No.	Compound	Zone	Concn (μg/ml)							
			20,000	4,000	800	160	32	6.4	1.3	0.3
IX	Derrisic acid	AVZ	55	47	19	15	—	—	—	—
		CTZ	+	15	—	—	—	—	—	—
X	Methylderrisate	AVZ	25	14	—	—	—	—	—	—
		CTZ	—	—	—	—	—	—	—	—
XI	Methylderrisate nor-	AVZ	21	14	13	—	—	—	—	—
	ketone	CTZ	—	—	—	—	—	—	—	—
XII	Isoderrisic acid nor-	AVZ	23	13	12	—	—	—	—	—
	ketone	CTZ	—	—	—	—	—	—	—	—
XIII	Methylisoderrisate	AVZ	ND	16	—	—	—	—	—	—
	norketone	CTZ	ND	—	—	—	—	—	—	—
XIV	Methylisoderrisate	AVZ	28	20	13	—	—	—	—	—
	O-acetate	CTZ	—	—	—	—	—	—	—	—
XV	Deguelic acid	AVZ	L	42	27	13	12	—	—	—
		CTZ	36	27	—	—	—	—	—	—
XVI	Methyldeguelate	AVZ		18	15	12	—	—	—	—
		CTZ		—	—	—	—	—	—	—

[a] Same as in Table 1.

ble, since not only esterification but also a double bond was introduced.

Methylation or acetylation of hydroxyl groups seemed to have slight effect on both cytotoxicity and antiviral activity as was shown with tephrosic acid (Table 4).

The cleavage of the two rings (B and C) adjacent to the dimethylated benzene of rotenone and deguelin to derrisic acid type compounds affected their cytotoxic and antiviral activities as was shown in comparing rotenone and deguelin with derrisic acid and deguelic acid, respectively (Tables 1 and 2). Removal of the A ring from derrisic acid had no effect on the antiviral activity, and tubaic acid had a similar activity (Tables 2 and 5).

Replacement of the methylene group on the dihydrofuran ring of rotenone with an oxo group had only a slight effect on the antiviral activity (Table 1), but a similar conversion in tubaic acid gave a profound effect on the activity (Table 5). Presence of a double bond in the dihydrofuran ring in addition to the keto group markedly decreased the antiviral activity as was shown with isoderrisic acid norketone (Table 2) and isotubaic acid norketone (Table 5). On the contrary, the antiviral activity was increased in the case of isotubanol norketone, in which situation a keto group and a double bond were introduced and the carboxyl group at C-5 was removed from tubaic acid (Table 5).

In conclusion, acetylrotenone (rotenone enol-acetate) showed the most effective activity, of all the compounds tested, on NDV growth and lack of cytotoxicity to cultured cells. Rotenone, rote-

TABLE 3. *Effect of dehydromunduserone and munduserol on NDV growth as determined by the agar diffusion, plaque inhibition method*[a]

No.	Compound	Zone	Concentration (μg/ml)							
			20,000	4,000	800	160	32	6.4	1.3	0.3
XVII	Dehydromundu-serone	AVZ	ND	16	13	±	—	—	—	—
		CTZ	ND	—	—	—	—	—	—	—
XVIII	Munduserol	AVZ	ND	19	15	11	—	—	—	—
		CTZ	ND	—	—	—	—	—	—	

[a] Same as in Table 1.

TABLE 4. *Effect of tephrosic acid, apotoxicalic acid, and their derivatives on NDV growth as determined by the agar diffusion, plaque inhibition method*[a]

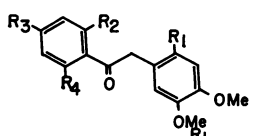
No.	Compound	Zone	Concentration (μg/ml)				
			20,000	4,000	800	160	32
XIX	Tephrosic acid	AVZ	15	—	—	—	—
		CTZ	—	—	—	—	—
XX	Methyltephrosate	AVZ	23	15	13	—	—
		CTZ	—	—	—	—	—
XXI	Ethyltephrosate	AVZ	22	13	—	—	—
		CTZ	—	—	—	—	—
XXII	Tephrosic acid mono-methylether	AVZ	18	—	—	—	—
		CTZ	—	—	—	—	—
XXIII	Methyltephrosate mono-methylether	AVZ	ND	—	—	—	—
		CTZ	ND	—	—	—	—
XXIV	Methyltephrosate dimethyl-ether	AVZ	13	—	—	—	—
		CTZ	—	—	—	—	—
XXV	Tephrosic acid monoace-tate	AVZ	12	—	—	—	—
		CTZ	—	—	—	—	—
XXVI	Tephrosic acid diacetate	AVZ		—	—	—	—
		CTZ		—	—	—	—
XXVII	Apotoxicalic acid	AVZ	18	±	—	—	—
		CTZ	—	—	—	—	—
XXVIII	Methylapotoxicalate	AVZ	17	—	—	—	—
		CTZ	—	—	—	—	—

[a] Same as in Table 1.

TABLE 5. *Effect of tubaic acid and its derivatives on NDV growth as determined by the agar diffusion, plaque inhibition method*[a]

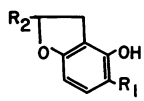
No.	Compound	Zone	Concn (μg/ml)							
			20,000	4,000	800	160	32	6.4	1.3	0.3
XXIX	Tubaic acid	AVZ[a]	ND	30	23	18	±	—	—	—
		CTZ[a]	ND	—	—	—	—	—	—	—
XXX	Methyltubate	AVZ	16	—	—	—	—	—	—	—
		CTZ	—	—	—	—	—	—	—	—
XXXI	Tubaic acid norketone	AVZ	ND	—	—	—	—	—	—	—
		CTZ	ND	—	—	—	—	—	—	—
XXXII	Isotubaic acid norketone	AVZ	ND	—	—	—	—	—	—	—
		CTZ	ND	—	—	—	—	—	—	—
XXXIII	Methylisotubate norketone	AVZ	±	—	—	—	—	—	—	—
		CTZ	—	—	—	—	—	—	—	—
XXXIV	Isotubanol norketone	AVZ	28	28	27	18	15	±	—	—
		CTZ	21	17	12	—	—	—	—	—
XXXV	2-Carboethoxy-4-hydroxy-5-carbomethoxy-cumarone	AVZ	12	—	—	—	—	—	—	—
		CTZ	—	—	—	—	—	—	—	—

[a] Same as in Table 1.

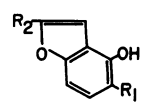


	R1	R2	R3	R4
Tephrosic acid (XIX)	$-OCH_2COOH$	-OH	-OH	-H
Methyltephrosate (XX)	$-OCH_2COOMe$	-OH	-OH	-H
Ethyltephrosate (XXI)	$-OCH_2COOEt$	-OH	-OH	-H
Tephrosic acid (XXII) monomethylether	$-OCH_2COOH$	-OH	-OMe	-H
Methyltephrosate (XXIII) monomethylether	$-OCH_2COOMe$	-OH	-OMe	-H
Methyltephrosate (XXIV) dimethylether	$-OCH_2COOMe$	-OMe	-OMe	-H
Tephrosic acid (XXV) monoacetate	$-OCH_2COOH$	-OH	-OAc	-H
Tephrosic acid (XXVI) diacetate	$-OCH_2COOH$	-OAc	-OAc	-H
Apotoxicalic acid (XXVII)	$-OCH_2COOH$	-OH	-OH	-OH
Methylapotoxicalate (XXVIII)	$-OCH_2COOMe$	-OH	-OH	-OH

FIG. 4. *Structures of tephrosic acid, apotoxicalic acid, and their derivatives.*



	R1	R2
Tubaic acid (XXIX)	-COOH	$-C(=CH_2)CH_3$
Methyltubate (XXX)	-COOMe	$-C(=CH_2)CH_3$
Tubaic acid norketone (XXXI)	-COOH	$-COCH_3$



Isotubaic acid norketone (XXXII)	-COOH	$-COCH_3$
Methylisotubate norketone (XXXIII)	-COOMe	$-COCH_3$
Isotubanol norketone (XXXIV)	-H	$-COCH_3$
2-Carboethoxy-4-hydroxy-5-carbomethoxycumarone (XXXV)	-COOMe	-COOEt

FIG. 5. *Structures of tubaic acid and its derivatives.*

none norketone, and deguelin likewise had a profound and similar effect on NDV growth and on cultured cells but were not as active as acetylrotenone. Acetylrotenone norketone, dehydrodeguelin, deguelic acid, and isotubanol norketone were next most active to the above mentioned compounds. Most of the other compounds were also active on NDV but their effects were not distinct because of the relatively high concentrations of drug needed to produce an effect.

Rotenone, acetylrotenone, rotenone norketone, deguelic acid, deguelin, and isotubanol norketone were found to suppress plaque formation by HSV when tested by the agar diffusion, plaque inhibition method, and their effect on HSV was similar to that found on NDV.

Anti-plant-virus activity. Rotenone is used practically as a pesticide. If it or any of its derivatives are found to have activities on plant viruses, their value as a pesticide may be increased. Therefore, their effect on a plant virus, TMV, was investigated (Table 6).

All the compounds tested at 100 μg/ml had effects to some extent on the formation of necrotic spots after TMV inoculation. However, only derrisic acid completely inhibited local-lesion formation at subphytotoxic concentrations. Munduserol

TABLE 6. *Effect of rotenoids and related compounds on TMV growth as determined by the in vitro local lesion method*

Compounds	Concn (μg/ml)					
	100		10		1	
	LL[a]	T[b]	LL	T	LL	T
Group 1						
Rotenone (I)	32	−	70	−	89	−
Acetylrotenone (II)	19	−	37	−	59	−
Dehydrorotenone (III)	27	−	59	−	77	−
Deoxyrotenone (IV)	59	−	78	−	101	−
Rotenone norketone (V)	20	−	55	−	89	−
Acetylrotenone norketone (VI)	40	−	57	−	72	−
Deguelin (VII)	28	−	69	−	101	−
Dehydrodeguelin (VIII)	26	−	41	−	79	−
Group 2						
Derrisic acid (IX)	0	−	63	−	74	−
Methylderrisate (X)	42	−	58	−	100	−
Methylderrisate norketone (XI)	68	−	90	−	100	−
Isoderrisic acid norketone (XII)	26	−	68	−	84	−
Methylisoderrisate norketone (XIII)	5	−	58	−	147	−
Deguelic acid (XV)	34	−	59	−	84	−
Group 3						
Dehydromunduserone (XVII)	5	++	68	−	211	−
Munduserol (XVIII)	0	+	68	−	100	−
Group 4						
Tephrosic acid (XIX)	58	−	90	−	90	−
Methyltephrosate (XX)	5	+	74	−	126	−
Ethyltephrosate (XXI)	11	+	73	−	142	−
Methyltephrosate dimethylether (XXIV)	58	−	105	−	89	−
Apotoxicalic acid (XXVII)	0	++	68	−	78	−
Methylapotoxicalate (XXVIII)	42	+	90	−	100	−
Group 5						
Tubaic acid (XXIX)	0	+++	61	−	80	−
Isotubaic acid norketone (XXXII)	0	++	100	−	110	−
Methylisotubate norketone (XXXIII)	0	+	47	−	142	−
Isotubanol norketone (XXXIV)	11	−	63	−	95	−
2-Carboethoxy-4-hydroxy-5-carbomethoxy-benzofumarone (XXXV)	47	−	116	−	95	−

[a] Number of local lesions formed on leaf discs expressed in per cent of controls.

[b] Degree of phytotoxicity expressed as: −, no phytotoxicity; +, slight phytotoxicity; ++, moderate phytotoxicity; and +++, severe phytotoxicity.

and methylisotubate norketone also suppressed local lesion formation at 100 μg/ml, but slight phytotoxicities were observed at this concentration.

Esterification had no general effect of decreased activity on anti-TMV activity as it had on anti-NDV activity, and some esterified compounds showed similar or, in some cases, increased activity on TMV. Acetylrotenone, and methyl- and ethyltephrosate were more effective than the corresponding non-enolacetylated and nonesterified compounds as was the case for these compounds in anti-NDV activity.

Complete parallelism was not observed for the respective anti-NDV and anti-TMV activities. In the case of anti-NDV activity, the compounds belonging to group 1 in Table 6 were the most effective, whereas groups 4 and 5 were less active, with some exceptions, than were the compounds listed in the other groups. On the contrary, the compounds listed in groups 3 and 5 were more effective on TMV than were the others. These

differences may be partly explained by the differences found in the host cells. Of special importance is the presence of protective layers such as the cuticule layer on the surface of plant leaves and the resulting difference in permeability to drugs that may be involved. In spite of the lack of complete parallelism, a relatively close relationship was observed in the activities of these compounds on NDV and TMV, both of which are RNA viruses. In addition to the rotenoids and the related compounds described in this paper, 47 anti-NDV compounds, including antibiotics such as trichothecin, brefeldin A, verrucarin A, cycloheximide, puromycin, blasticidin S, quinomycin B, bihoromycin, and several newly isolated antibiotics, were tested. Forty were found active for TMV at concentrations less than 100 μg/ml as determined by the in vitro local lesion method (A. Takatsuki et al., J. Antibiot. **22A**, *in press*; *unpublished observation*). Compared with the previously mentioned antibiotics, the rotenoids and the related compounds investigated were not distinct in their anti-TMV activity. They could not suppress completely the local lesion formation by TMV when used at 100 μg/ml. Some exceptions were found, such as derrisic acid and antibiotics such as trichothecin, bihoromycin, blasticidin S, and some newly isolated antibiotics, which effectively inhibited TMV when used at concentrations lower than 1 μg/ml.

Correlation of the antiviral activities with respiratory inhibitory activity. Rotenone is a well-known inhibitor of electron transfer in mitochondrial respiration. The site of rotenone inhibition has been reported by Lindahl and Öberg (3). A possibility existed that the antiviral activities of the rotenoids and the related compounds on NDV and TMV were due to the inhibition of respiration. To examine this possibility, the effect of these compounds on mitochondrial respiration was investigated, employing the polarographic method of Hagihara (1).

All the test compounds were poorly soluble in water, and examination of their effect on mitochondrial respiration at high drug concentrations (50 to 100 μg/ml) was difficult. Some of the compounds slightly affected the rate of oxygen consumption by mitochondria but did not completely stop the oxygen consumption at the highest drug concentrations tested. The purpose of this experiment was to compare the antiviral activities with respiratoy inhibition activity; then the minimal drug concentration required for complete inhibition of the oxygen consumption may be used as a tentative index.

The compounds listed in group 1 (Table 7) effectively inhibited mitochondrial respiration when used at concentrations lower than 20 μg/ml.

TABLE 7. *Effect of rotenoids and related compounds on mitochondrial respiration*

Compounds	Minimal concentration required for complete inhibition of respiration (μg/ml)
Group 1	
Rotenone (I)	0.1
Acetylrotenone (II)	10
Dehydrorotenone (III)	0.5
Deoxyrotenone (IV)	20
Rotenone norketone (V)	0.2
Deguelin (VII)	0.4
Group 2	
Derrisic acid (IX)	33
Methylderrisate (X)	200
Methylderrisate norketone (XI)	>25
Isoderrisic acid norketone (XII)	>25
Isomethylderrisate norketone (XIII)	>33
Deguelic acid (XV)	>6
Group 3	
Dehydromunduserol (XVII)	>100
Group 4	
Tephrosic acid (XIX)	>100
Methyltephrosate (XX)	>33
Ethyltephrosate (XXI)	>100
Methyltephrosate dimethylether (XXIV)	>100
Apotoxicalic acid (XXVII)	>100
Methylapotoxicalate (XXVIII)	>70
Group 5	
Tubaic acid (XXIX)	>100
Isotubaic acid norketone (XXXII)	>25
Methylisotubate norketone (XXXIII)	>25
Isotubanol norketone (XXXIV)	>100
2-Carboethoxy-4-hydroxy-5-carbomethoxy-benzofumarone (XXXV)	>100

The compounds in groups 2 to 5, except derrisic acid and methylderrisate, were not active on mitochondrial respiration at the highest drug concentrations tested.

The values indicated in Table 7 are too high to explain the antiviral activities of the rotenoids and the related compounds as being due solely to their effect on mitochondrial respiration. The drug concentrations shown in Tables 1 to 5 represent the original drug solutions, and the paper discs used in these experiments absorbed about 0.025 ml of solution per disc. In addition, the

drugs absorbed with the paper discs should first diffuse through the agar overlayers to the cell sheets formed on the bottom of the petri dishes before they could act on NDV growth. Thus, the drug concentrations on the infected cell sheets are much lower (more than 40-fold at least) than the values given in Tables 1 to 5. However, it should be mentioned again that the drug concentrations in Table 7 are the minimal drug concentrations required for the complete inhibition of mitochondrial respiration, and some compounds slightly influenced the respiration but did not suppress it completely. Thus, the possibility that the inhibition of respiratory activity may be the cause of the antiviral activity of the rotenoids and the related compounds cannot be excluded completely at present. Nevertheless, strict parallelism between the degree of antiviral and respiratory inhibitory activities was not observed. For example, acetylrotenone was more effective on NDV than was rotenone (Table 1), but the reverse was shown to be the case for mitochondrial respiratory inhibition (Table 7). Some compounds, such as isotubanol norketone, had no effect on respiratory activity at 100 μg/ml, but they effectively inhibited NDV growth at 30 to 160 μg/ml in the agar diffusion, plaque inhibition method (Table 5). In the case of anti-TMV activity, the compounds in groups 3 and 5 were more effective than those in group 1, but, for more potent inhibitors of respiration, the reverse was true. Ascochlorin, an antiviral antibiotic with potent respiratory inhibitory activity, showed no effect on the local lesions induced by TMV, even when used at 100 μg/ml (A. Takatsuki et al., J. Antibiot., *in press*). Indeed, many factors must be taken into consideration in comparing the antiviral activities and the effects on respiration of the rotenoids and the related compounds. However, the above results seem to indicate that the inhibition of respiration may not be the sole explanation for the antiviral activities of the compounds and that they may have some other activities, perhaps enzymatic in nature, which result in inhibition of virus multiplication, both in cultured animal cells and plant cells.

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