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Rhinovirus Inhibition by Bufadienolides[1a)]

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An evaluation of thirty-four bufadienolides and two related cardenolides against a series of rhinoviruses *in vitro* has been completed. Most of the bufadienolides were found to display some inhibitory activity. Scillarenin and 3-*O*-[*N*-(*tert*-butoxycarbonyl)hydrazido]succinylbufalin were found to be the most active with chemotherapeutic indices of 32 and 16, respectively. In general, the 14β-hydroxy-bufadienolides showed the strongest antiviral activity, and were found more toxic than the corresponding 14β,15β-epoxy-bufadienolides. Introduction of a 16β-hydroxy or 16β-acetoxy substituent into the 14β,15β-epoxybufadienolides enhanced their antiviral activity. Substituents at the 3β-, 5β-, and 19-positions appeared to affect only the level of toxicity.

Keywords——rhinovirus; antiviral; bufadienolide; Ch'an Su

Cancer-causing viruses of the HTLV types lead to a lethal series of human diseases where occurrence is rapidly increasing and becoming a very serious public health problem.[2-4)] A number of other important human viral diseases also lack effective antiviral drugs for curative treatment. Unfortunately, until recently discovery and development of antiviral drugs was very slow. So far the best leads to new types of antiviral drugs have arisen from the relatively small number of animal[5)] and plant[6,7)] constituents screened for antiviral activity. Synthetic modifications of such natural products has provided, *e.g.*, the promising antiviral drugs acyclovir and virazole.[8)]

Based on the observation that a crude extract of the Chinese toad venom preparation Ch'an Su was inhibitory to rhinovirus infection *in vitro*, Sato and Muro[9)] examined the plant bufadienolide scillarenin (SL). Among substances examined in this preliminary study, SL was found the most active against *in vitro* rhinovirus replication. As part of our research directed at discovery of naturally occurring antiviral drugs we have extended this initial study to a representative search of toad venom bufadienolides to assess structure/activity requirements for *in vitro* inhibition of human ribonucleic acid (RNA) viruses of the rhinovirus (picornavirus group)-type.

Materials and Methods

Ch'an Su, Bufadienolides and Cardenolides——Ch'an Su (circle cake), obtained in Hong Kong folk-medical market, was extracted (Soxhlet procedure) with ethyl ether followed by chloroform. The chloroform extract was employed in the present study. The following bufadienolides were isolated from Ch'an Su[10)]: bufalin (BF1), bufotalin (BT1), gamabufotalin (GB1), hellebrigenin (HB), telocinobufagin (TB), resibufogenin (RB1), marinobufagin (MB), resibufagin (RG), cinobufagin (CB1), and desacetylcinobufagin (CB6), as well as the 3-*O*-suberoyl derivatives of bufalin (BF6), cinobufagin (CB3), and resibufogenin (RB3). The 3-*O*-(methyl suberoyl)-bufotalin (BT2) and 3-*O*-(methyl suberoyl)-gamabufotalin (GB2) were prepared by methylating the corresponding suberic acid esters isolated from the Japanese toad (skins), *Bufo formosus* BOULENGER.[11)] We have already described[12)] syntheses of 3-*O*-succinoylcinobufagin (CB2), 3-*O*-acetyl-16-*O*-desacetylcinobufagin (CB7), and 3-*O*-acetylcinobufagin (CB8). For leading references to the remaining bufadienolide synthetic modifications, consult citation 13.

Scillarenin (SL1) was prepared from proscillaridin A.[14] Proscillaridin A (SC2), digitoxigenin (DT1), and k-strophanthin (DT2) were purchased from Fluka Chem. Corp., Hauppauge, N. Y.

Sample Preparation——Each compound was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml, and added to nine volumes of maintenance medium (MSM). These solutions containing 100 μg/ml of test compound were refrigerated prior to use. Further dilutions were carried out with MSM exclusively so that the final concentration of DMSO was less than 2%, and *ca.* 0.01% in most cases. At these low concentrations, DMSO affects neither virus growth nor cell maintenance.

Viruses and Cell Cultures——Human embryonic lung (HEL) cells were cultured with Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum. MSM contained MEM supplemented with 2% heat-inactivated fetal calf serum. M-HeLa cells were cultured with MEM plus 7% calf serum.

Rhinovirus 1A (strain 2060) propagated in HEL cells was used unless otherwise stated. Other rhinoviruses (types 19, 30, 63, and strain 6692) also were propagated with HEL cells. M-HeLa cells were used for propagation of poliovirus 1, rhinovirus 1A, herpes simplex virus (HF), and adenovirus 4. Chick fibroblast cells (CE) were used for Sindbis, Newcastle disease, vesicular stomatitis, and herpes simplex viruses.

Antiviral Test and Cytotoxicity——In the cytopathic effect (CPE) inhibition test, cells were grown in test tubes (3×10^5 cells/ml), and appropriate dilutions of the test compounds were added to the cultures at the time of virus infection with 100 TCD_{50}. Rhinovirus infected cultures were incubated at 34°C on a roller drum operating at 14 rotations per hour. Other virus cultures were maintained at 37°C on a stationary rack. Uninfected control tubes were included to determine toxicity of the test compounds at each dilution. These cultures were examined daily for virus-induced CPE. Antiviral activity was established when infected control cultures containing no test compound showed complete CPE. Minimum inhibitory concentration (MIC) against the virus was determined at the dilution showing 50% inhibition of viral CPE, and minimum toxic concentration (MTC) at the lowest dilution which produced rounding of cells. Antiviral activity was evaluated by determining the chemotherapeutic index (C.I.) *in vitro*, (C.I. = MTC/MIC). The antiviral spectrum of the three especially potent bufadienolides BF9, CB3, and SL1 was further investigated in essentially the same manner, but with additional combinations of cells and virus.

Yield Reduction Test and Plaque Assay——The effect of graded concentrations of BF9 and SL1 on the replication of rhinovirus was examined. HEL cell cultures were infected with rhinovirus 1A at an input multiplicity of 10. After 1 h incubation, unabsorbed virus was removed by washing with Hanks' solution. Next was added MSM containing an appropriate concentration of the bufadienolide. At 10 h after infection, triplicate cultures were frozen and thawed three thimes, clarified by centrifugation, and the supernatants were assayed for virus by counting plaque in HEL monolayers. For the plaque assay of rhinovirus, MSM containing 0.7% agarose (Wehlingberke agarose, Hoechst Research, West Germany) and neutral red at 0.1 mg/ml were used.

Results

Antiviral Activity

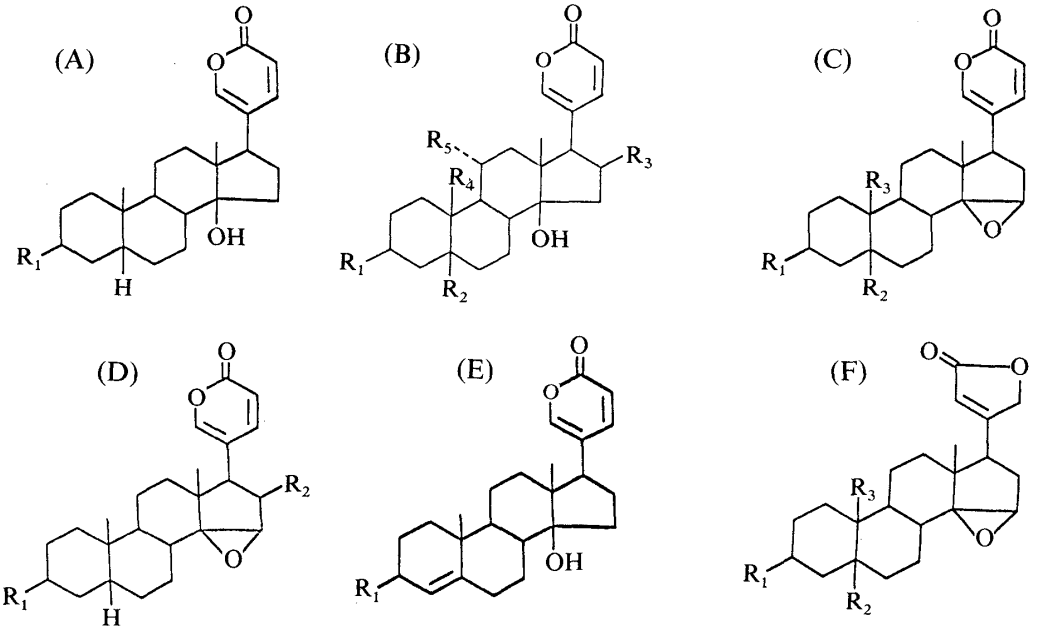
Test materials were considered in seven groups according to their origin and/or structural relationships. Compounds in groups I-V originated from the toad venom preparation Ch'an Su, while those in groups VI and VII were from higher plants.

Group I. Chloroform Extract of Ch'an Su——The extract exhibited pronounced antiviral activity (Table I). The extract at a concentration between 0.06 and 0.5 μg/ml completely inhibited viral CPE and the toxicity of the extract seemed mild compared with certain bufadienolide constituents discussed below.

Group II. BF1 and Derivatives——While the bufadienolides in group II were found to have antiviral activity they were also strongly cytotoxic (Table I). In general, elongation of hydrocarbon chains in esters attached at the 3β-position lowered the toxicity relative to other similarly substituted steroids. The most active compound in this group was BF9 and it inhibited viral CPE completely at doses in the range 31-125 ng/ml with a CI of 16.

Group III. BT1, GB1, HB, TB, and Derivatives——All the compounds in this group were active but, in common with those in group II, showed a high level of toxicity (Table I). Although BT1 (a 16β-acetoxy derivative) was less toxic than BF1 (bufotalin), there was little difference in their CI values. The toxicity of GB1 (a 11α-hydroxy compound) was comparable to that of BF1, but its antiviral activity was only half as great. The toxicities of BT1 and GB1 were reduced when the 3β-hydroxy substituents were converted to 3β-methylsuberoyl (BT2 and GB2) substituents. Interestingly, there were no significant changes in antiviral activity.

TABLE I. *In Vitro* Anti-rhinovirus Activity of Ch'an Su, Bufadienolide Constituents and Related Compounds



Bufadienolide	Substitution	MTC/MIC[a] (ng/ml)	C.I.[b]
(I) Chloroform extract of Ch'an Su (toad poison)		500/16	32
(II) Bufalin and derivatives (A)			
Bufalin (BF1)	$R_1=OH$	20/2.5	8
3-(β-Glucoside) (BF2)	$R_1=\beta$-glucoside	20/20	1
3-*O*-Succinyl (BF3)	$R_1=OCO(CH_2)_2COOH$	63/16	4
3-*O*-Glutaryl sodium salt (BF4)	$R_1=OCO(CH_2)_3COONa$	250/63	4
3-*O*-Adipoyl sodium salt (BF5)	$R_1=OCO(CH_2)_4COONa$	250/63	4
3-*O*-Suberoyl sodium salt (BF6)	$R_1=OCO(CH_2)_6COONa$	250/31	8
3-*O*-Methyl suberoyl (BF7)	$R_1=OCO(CH_2)_6COOCH_3$	250/63	4
3-*O*-(Methyl alanyl)succinyl (BF8)	$R_1=OCO(CH_2)_2CONHCH_2COOCH_3$	500/125	4
3-*O*-[*N*-(*tert*-Butoxycarbonyl) hydrazido]succinyl (BF9)	$R_1=OCO(CH_2)_2CONHNHCOOC(CH_3)_3$	125/8	16
3-*O*-[*N*-(*tert*-Butoxycarbonyl) hydrazido]suberoyl (BF10)	$R_1=OCO(CH_2)_6CONHNHCOOC(CH_3)_3$	125/16	8
(III) Bufotalin, gamabufotalin, hellebrigenin, telocinobufagin and derivatives (B)			
Bufotalin (BT1)	$R_1=OH$, $R_2=H$, $R_3=OCOCH_3$, $R_4=CH_3$, $R_5=H$	100/12	8
3-*O*-(Methyl suberoyl)-bufotalin (BT2)	$R_1=OCO(CH_2)_6COOCH_3$, $R_2=H$, $R_3=OCOCH_3$, $R_4=CH_3$, $R_5=H$	200/25	8
Gamabufotalin (GB1)	$R_1=OH$, $R_2=H$, $R_3=H$, $R_4=CH_3$, $R_5=OH$	31/8	4
3-*O*-(Methyl suberoyl)-gamabufotalin (GB2)	$R_1=OCO(CH_2)_6COOCH_3$, $R_2=H$, $R_3=H$, $R_4=CH_3$, $R_5=OH$	200/50	4
Hellebrigenin (HB)	$R_1=OH$, $R_2=OH$, $R_3=H$, $R_4=CHO$, $R_5=H$	12.5/3.1	4
Telocinobufagin (TB)	$R_1=OH$, $R_2=OH$, $R_3=H$, $R_4=CH_3$, $R_5=H$	50/6.3	8
(IV) Resibufogenin, marinobufagin, and derivatives (C)			
Resibufogenin (BR1)	$R_1=OH$, $R_2=H$, $R_3=CH_3$	1000/500	2

TABLE I. (continued)

Bufadienolide	Substitution	MTC/MIC[a] (ng/ml)	C.I.[b]
3β-Succinyl sodium salt resibufogenin (RB2)	$R_1=OCO(CH_2)_2COONa$, $R_2=H$, $R_3=CH_3$	2000/2000	1
3β-Suberoyl resibufogenin (RB3)	$R_1=OCO(CH_2)_6COOH$, $R_2=H$,	4000/2000	2
3β-(Methyl suberoyl) resibufogenin (RB4)	$R_1=OCO(CH_2)_6COOCH_3$, $R_2=H$, $R_3=CH_3$	2000/500	4
Marinobufagin (MB)	$R_1=OH$, $R_2=OH$, $R_3=CH_3$	2000/250	8
Resibufagin (RG)	$R_1=OH$, $R_2=H$, $R_3=CHO$	1000/250	4
(V) Cinobufagin and derivatives (D)			
3-*O*-Substituted derivatives			
Cinobufagin (CB1)	$R_1=OH$, $R_2=OCOCH_3$	200/25	8
Succinyl (CB2)	$R_1=OCO(CH_2)_2COOH$, $R_2=OCOCH_3$	500/63	8
Suberoyl (CB3)	$R_1=OCO(CH_2)_6COOH$, $R_2=OCOCH_3$	500/63	8
Methyl suberoyl (CB4)	$R_1=OCO(CH_2)_6COOCH_3$, $R_2=OCOCH_3$	125/16	8
Morpholino phosphoryl (CB5)	$R_1=OPO(OH)$-morpholino $R_2=OCOCH_3$	1000/1000	1
Acetyl (CB8)	$R_1=R_2=OCOCH_3$	1000/250	4
Other cinobufagin derivatives			
Desacetyl (CB6)	$R_1=OH$, $R_2=OH$	4000/500	8
3-*O*-Acetyl-16-*O*-desacetyl (CB7)	$R_1=OCOCH_3$, $R_2=OH$	4000/1000	4
3-*O*-Acetyl-16-*O*-desacetyl-16-*O*-glutaryl (CB9)	$R_1=OCOCH_3$ $R_2=OCO(CH_2)_3COOH$	20000/5000	4
3-*O*-Acetyl-16-*O*-desacetyl-16-*O*-adipoyl (CB10)	$R_1=OCOCH_3$, $R_2=OCO(CH_2)_4COOH$	20000/10000	2
(VI) Scillarenin and proscillaridin A (E)			
Scillarenin (SL1)	$R_1=OH$	125/4	32
Proscillaridin A (SL2)	$R_1=$β-rhamnoside	250/63	4
(VII) Digitoxigenin and k-strophanthin (F)			
Digitoxigenin (DT1)	$R_1=OH$, $R_2=H$, $R_3=CH_3$	100/50	2
k-Strophanthin (DT2)	$R_1=$β-glucoside, $R_2=OH$, $R_3=CHO$	6.3/3.1	2

a) Minimum toxic concentration/minimum inhibitory concentration. *b*) Chemotherapeutic index.

While TB, a 5β-hydroxy-steroid, was less toxic than BF1, it displayed equivalent antiviral activity. The 5β-hydroxy-19-oxo-bufadienolide HB was one of the most toxic compounds tested and showed less antiviral activity than BT2 and GB2.

Groups IV and V. RB1, MB, RG, CB1, and Derivatives——The 14β, 15β-epoxy-bufadienolides showed lower toxicity than those with 14β-hydroxy substituents (groups II and III). With the exception of one 5β-hydroxy-derivative (MB), they also showed lower antiviral activity. On the other hand, the 14β, 15β-epoxy-16β-acetoxy-bufadienolides exhibited antiviral activity comparable to the compounds in group III coupled with lower toxicity; an exception was CB5 [a 3β-*O*-(morpholino) phosphoryl derivative]. Similarly, the 14β, 15β-epoxy-16β-hydroxy-bufadienolides CB6 and CB7 were active and even less toxic. Attachment of a glutarate or adipate ester moiety to the 16β-hydroxy group (CB9 and CB10) further reduced toxicity and slightly decreased activity.

Group VI. SL1 and Proscillaridin A (SL2)——Both of these compounds are plant bufadienolides isolated from *Urginea scilla*. Here SL1 was the most active of all the compounds evaluated (Table I) and SL2, the 3β-rhamnoside of SL1, was less active.

Group VII. Digitoxigenin (DT1) and k-Strophanthin (DT2)——The two higher plant cardenolides noted here have a γ-lactone (butenolide-class) at the 17β-position in place of the six-membered δ-lactone ring characteristic of the bufadienolides. Except for the lactone rings both DT1 and BF1 have comparable steroid systems. However, DT1 was less active and less toxic than BF1. These observations suggest that the bufadienolide 5-substituted-2-pyrone ring is important for antiviral activity as well as for toxicity. The cardenolide DT2 (k-strophanthin) showed the highest level of toxicity of the compounds examined.

Antiviral Spectrum of BF9, CB3, and SL1——The three most active bufadienolides in this study—BF9, CB3 and SL1—were found equally active against five types of rhinovirus (Table II), although they varied considerably in their structural features. Importantly, SL1 exhibited a similar level of activity against poliovirus. The other bufadienolides were somewhat less active when host cells were changed from HeLa to HEL. In Table III, properties of SL1 are compared with those of BF1 and TB, two closely related structures lacking the 4,5-double

TABLE II. Antiviral Spectrum of BF9, CB3, and SL1 *in Vitro*[a]

Virus	Cell	Antiviral activity[b]		
		BF9	CB3	SL1
Polio 1	HeLa	+	+	++
Polio 1	HEL	+	+	+++
Rhino 1A	HeLa	+++	++	+++
Rhino 1A	HEL	+++	+++	+++
Rhino 19	HEL	+++	++	+++
Rhino 30	HEL	+++	++	+++
Rhino 63	HEL	+++	+++	+++
Rh. str. 6692	HEL	+++	++	+++
Sindbis	CEC	−	−	−
VSV	CEC	−	−	−
NDV	CEC	−	−	−
Vaccinia	HEL	−	−	−
Vaccinia	CEC	−	−	+
Herpes	HeLa	−	−	−
Herpes	CEC	−	−	−
Adeno 3	HeLa	−	−	−

a) A part of the data has been presented in ref. 1. *b*) Antiviral activity: −, C.I.$\leq$2; +, 2<C.I.$\leq$4; ++, 4<C.I.$\leq$8; +++, C.I.>8.

TABLE III. Effect of Substitution at the Bufadienolide 5β-Position on Antiviral (Rhinovirus) Activity and Toxicity

	Compound		
	BF1	TB	SL1
5β-Position substitution	H	OH	Δ4, 5
Toxicity[a]	625	225	100
Antiviral (rhinovirus) activity	25	25	100

a) The numbers represent toxicity or antiviral activity of the respective compound relative to SL1 (SL1 = 100). The data were selected from Table I.

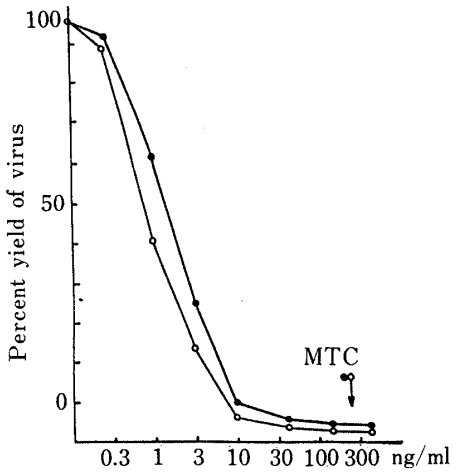


Fig. 1. Effect of BF9 and SL1 on the Yield of Rhinovirus 1A[a)]

a) Part of this data was presented previously in ref. 8. MTC: minimum toxic concentration. ○—○, BF9; ●—●, SL1.

bond. From this limited comparison, it may be inferred that the 4,5-double bond is a structurally specific feature in the favorable C.I. of SL1.

Yield Reduction Test——Concentration dependency of the inhibitory effects of BF9 and SL1 in one-cycle growth of rhinovirus 1A was examined (Fig. 1). Both compounds, at doses of 30 ng/ml or more, reduced the viral production below a thousandth of the control, while 1 ng/ml decreased the production by 35% or 55%, respectively.

Discussion

Although we did not find individual bufadienolides more antiviral and less toxic than the crude chloroform extract of Ch'an Su, most of the compounds examined in this study were found to inhibit viral growth. Also, it was encouraging to discover that some of the bufadienolides showed selective antiviral activity at very low concentrations. The therapeutic index of crude Ch'an Su extract was found to be higher than that of any of its constituents so far examined. Perhaps this is due to unknown synergistic effects or there may be more potent antiviral constituents in Ch'an Su which have yet to be isolated and characterized. Since the potent BF9 is a synthetic intermediate near the original objective bufotoxin this and related toad venom toxins need to be evaluated for antiviral activity.

Some generalizations regarding structure–activity relationships emerge upon analyzing the results summarized in Table I: (1) a δ-lactone of the 2-pyrone-type in the 17β-position confers stronger antiviral activity than with a γ-lactone (butenolide) ring in this position, (2) with other structural features held constant, 14β-hydroxy-bufadienolides appear to be more antiviral, and toxic, than the corresponding 14β, 15β-epoxybufadienolides, (3) with 16β-hydroxy-bufadienolides, the 16β-acetoxy group is variable in its effect on toxicity, (4) carbon chain elongation in ester side-chains derived from the 3β-hydroxyl group generally decreased toxicity without affecting antiviral activity, although certain exceptions were noted, and (5) antiviral activity was increased by introduction of a 4,5-double bond.

The preceding observations suggest that the animal and plant bufadienolides present a useful structural lead for synthesis of structurally simpler, more specific, and less toxic antiviral drugs. In view of the potent antirhinovirus activity shown by the plant flavonoids[7)] chrysosplenol B and C and axillarin the bufadienolide 2-pyrone system appears to be a very important structural feature for synthetic development. In addition, the mechanism of antiviral activity by the bufadienolides needs to be further explored.[9)]

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References and Notes

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