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ANTIVIRAL (RNA) ACTIVITY OF SELECTED AMARYLLIDACEAE ISOQUINOLINE CONSTITUENTS AND SYNTHESIS OF RELATED SUBSTANCES[1]

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ABSTRACT.—A series of 23 Amaryllidaceae isoquinoline alkaloids and related synthetic analogues were isolated or synthesized and subsequently evaluated in cell culture against the RNA-containing flaviviruses (Japanese encephalitis, yellow fever, and dengue viruses), bunyaviruses (Punta Toro, sandfly fever, and Rift Valley fever viruses), alphavirus (Venezuelan equine encephalomyelitis virus), lentivirus (human immunodeficiency virus-type 1) and the DNA-containing vaccinia virus. Narciclasine [**1**], lycoricidine [**2**], pancratistatin [**4**], 7-deoxypancratistatin [**5**], and acetates **6–8**, isonarciclasine [**13a**], *cis*-dihydronarciclasine [**14a**], *trans*-dihydronarciclasine [**15a**], their 7-deoxy analogues **13b–15b**, lycorines **16** and **17**, and pretazettine [**18**] exhibited consistent in vitro activity against all three flaviviruses and against the bunyaviruses, Punta Toro and Rift Valley fever virus. Activity against sandfly fever virus was only observed with 7-deoxy analogues. In most cases, however, selectivity of the active compounds was low, with toxicity in uninfected cells (TC_{50}) occurring at concentrations within 10-fold that of the viral inhibitory concentrations (IC_{50}). No activity was observed against human immunodeficiency virus-type 1, Venezuelan equine encephalomyelitis virus, or vaccinia viruses. Pancratistatin [**4**] and its 7-deoxy analogue **5** were evaluated in two murine Japanese encephalitis mouse models (differing in viral dose challenge, among other factors). In two experiments (low LD_{50} viral challenge, variant I), prophylactic administration of **4** at 4 and 6 mg/kg/day (2% EtOH/saline, sc, once daily for 7 days, day −1 to +5) increased survival of Japanese-encephalitis-virus-infected mice to 100% and 90%, respectively. In the same model, prophylactic administration of **5** at 40 mg/kg/day in hydroxypropylcellulose (sc, once daily for 7 days, day −1 to +5) increased survival of Japanese-encephalitis-virus-infected mice to 80%. In a second variant (high LD_{50} viral challenge), administration of **4** at 6 mg/kg/day (ip, twice daily for 9 days, day −1 to +7) resulted in a 50% survival rate. In all cases, there was no survival in the diluent-treated control mice. Thus, **4** and **5** demonstrated activity in mice infected with Japanese encephalitis virus but only at near toxic concentrations. To our knowledge, however, this represents a rare demonstration of chemotherapeutic efficacy (by a substance other than an interferon inducer) in a Japanese-encephalitis-virus-infected mouse model.

Numerous chemotherapeutic agents have their origins in terrestrial plant and marine organism sources (1–3). For example, plants of the Amaryllidaceae have yielded over 100 different alkaloids with diverse biological properties (2). The medicinal prop-

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erties of extracts from the daffodil *Narcissus poeticus* L. were known to the Greek physician Hippocrates of Cos as early as 300 B.C. and to the Romans of the first century A.D. Over the past two decades, a number of potentially important constituents of this family have been identified as isocarbostyrils, such as narciclasine [**1**] [whose extraction and antimitotic properties have been described by Ceriotti (4)], lycoricidine (7-deoxynarciclasine) [**2**], pancratistatin (5–7) [**4**], and the alkaloids lycorine [**16**], pseudolycorine [**17**], and pretazettine [**18**]. Pancratistatin [**4**] and 7-deoxynarciclasine [**2**] have been isolated from the bulbs of Hawaiian *Pancratium littorale* Jacq. and *Zephyranthes grandiflora* and their structures elucidated (7). Considerable efforts (8,9) have recently culminated in the total synthesis of racemic pancratistatin by Danishefsky and Lee (8). *trans*-Dihydronarciclasine [**15a**], the principal antiviral and cytostatic constituent of the Chinese medicinal plant *Zephyranthes candida*, has also been recently isolated (10). The numbered ring structure for compounds including **1**, **2**, and **4** is depicted in Figure 1.

Biological properties, syntheses, and mechanisms of action (if known) of the narciclasine- and lycorine-type Amaryllidaceae alkaloids have been summarized (2, 3, 11–15). For example, lycorine [**16**] was found to be responsible for the antiviral activity of leaf and root extracts of the Amaryllidaceae plant *Clivia miniata* Regel against Herpes simplex, Semliki forest, polio, Coxsackie, and measles viruses in Vero cells (16). Such biosynthetic products exert their biological activities mainly by inhibiting protein synthesis at the step of peptide bond formation. A recent study of the effect of lycorine on viral protein formation in poliovirus-infected HeLa cells serves as a useful illustration (17). Pseudolycorine [**17**] and pretazettine [**18**] similarly inhibit protein synthesis and have manifested activity against murine Rauscher leukemia virus and neurotropic RNA viruses (18). While some reports (19) describe the antiviral properties of crude extracts containing the isocarbostyril-type compounds against neurotropic RNA viral infections in mice with Japanese encephalitis virus and lymphocytic choriomeningitis (LCM) virus (20–22), to our knowledge no systematic study has been undertaken to evaluate the antiviral (RNA) activities of the pure, naturally occurring compounds and synthetic derivatives. Structure-activity studies have been limited by the synthetic strategies required.

A group of RNA viruses belonging to the families Flaviviridae, Bunyaviridae, and Togaviridae cause hemorrhagic, encephalitic, or febrile disease throughout large areas of the world (23). Vaccines exist to control some flavivirus diseases (24–26); however, with the exception of interferon and its inducer poly (ICLC), a nuclease-resistant complex of polyriboinosinic-polyribocytidylic acid, poly-1-lysine, and carboxymethylcellulose, (27), no specific antiviral chemotherapeutic agents have demonstrated efficacy against flaviviral infections (28). Ribavirin, 1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide, already approved for therapy against respiratory syncytial virus in children, has shown efficacy against some bunyavirus infections (29,30). Our preliminary evaluation of certain *Amaryllis* constituents gave antiviral screening data that indicated

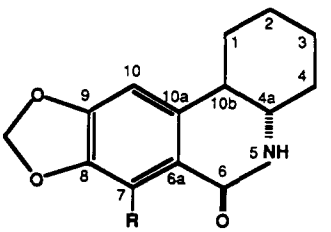
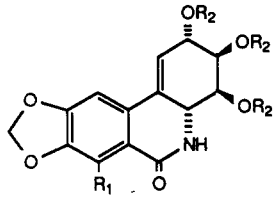
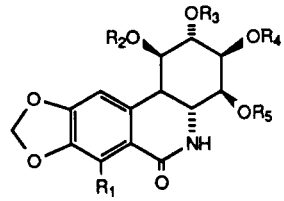


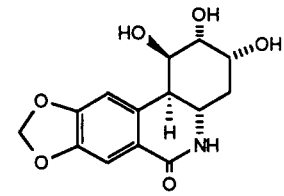
FIGURE 1. Numbered narciclasine/pancratistatin ring structure.



1 R_1=OH, R_2=H
2 R_1=R_2=H
3 R_1=H, R_2=Ac



4 R_1=OH, R_2-R_5=H
5 R_1-R_5=H
6 R_1=R_2=R_4=R_5=H, R_3=Ac
7 R_1=H, R_2-R_5=Ac
8 R_1=R_2=H, R_3=R_4=R_5=Ac



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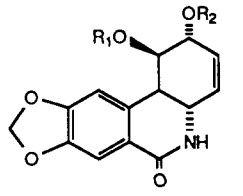
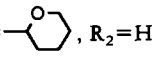
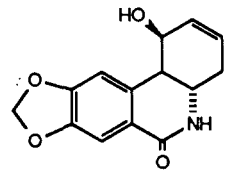
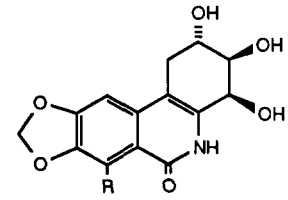


10 R_1=  , R_2=H

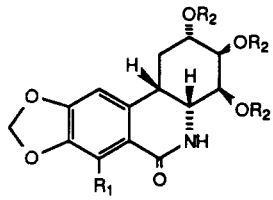
11 R_1=R_2=H



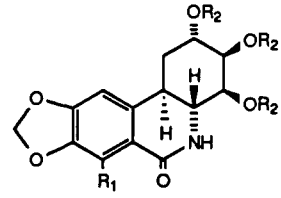
12



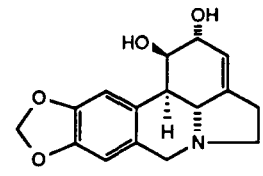
13a R=OH
13b R=H



14a R_1=OH, R_2=H
14b R_1=R_2=H
14c R_1=OAc, R_2=Ac
14d R_1=H, R_2=Ac



15a R_1=OH, R_2=H
15b R_1=R_2=H
15c R_1=OAc, R_2=Ac
15d R_1=H, R_2=Ac



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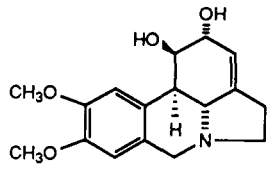
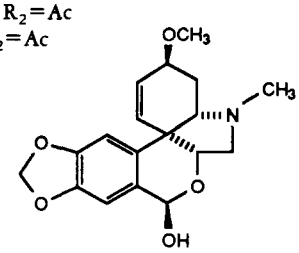


17



18

the presence of in vitro activities against Flaviviridae and Bunyaviridae. In order to explore this lead, compounds **1–18** have been obtained and their antiviral (RNA) activities evaluated both in vitro and in appropriate available animal models.

RESULTS AND DISCUSSION

CHEMISTRY.—Compounds **1–18** used in this study were either synthesized, isolated from natural sources, or furnished for testing as acknowledged (see Experimental).

Narciclasine [**1**] and lycorine [**16**] were obtained by extraction of the bulbs of *Nar-*

cissus incomparabilis (31). Syntheses have been reported for (+)-lycoricidine (32,33), for racemic lycorine (34–38), and pancratistatin (8) as well as for the isonarciclasines **13a** and **13b** (39–41). An elegant, highly-convergent synthesis of racemic lycorine has been reported (42) based in part upon synthetic strategy described by Stork and Morgans (43). For this study, 7-deoxynarciclasine [**2**] (lycoricidine) and pancratistatin [**4**] were isolated from *P. littorale* (7). No specific synthesis of narciclasine has appeared but techniques used for isolating pancratistatin (8) were very effective. Lycoricidine triacetate [**3**], lycoricidine monoacetate [**6**], lycoricidine triacetate [**8**], and their hydroxy analogues **10–12** were synthesized by methods initially described by Ohta and Kimoto (44) and subsequently modified by Ugarkar *et al.* (45). 7-Deoxypancratistatin [**5**] (44,46) and triol **9** were obtained by synthesis from intermediates **10** and **12**. Acetylation of **5** with Ac_2O and 4-dimethylaminopyridine (DMAP) gave tetraacetate **7** (44).

Isonarciclasine [**13a**] *cis*-dihydronarciclasine [**14a**], and *trans*-dihydronarciclasine [**15a**] were obtained from narciclasine by catalytic hydrogenation on Adam's catalyst, as described by Mondon and Krohn (47) with modifications described herein. The double bond-isomerized, sparingly soluble product, isonarciclasine [**13a**], was isolated when pyridine was used in place of dimethylformamide as solvent. The mixture of *cis*- and *trans*-dihydronarciclasines **14a** and **15a**, present in the pyridine filtrate, was acetylated with Ac_2O to afford a mixture of the tetraacetates **14c** and **15c**. Purification by cc on Si gel and removal of the acetate protecting groups (31) by heating with 2 N aqueous $Ba(OH)_2$ solution gave the pure *cis*- and *trans*-dihydronarciclasines. The *trans*-dihydronarciclasine was also isolated from *Z. candida* (10). When 7-deoxynarciclasine [**2**] was treated by a similar hydrogenation/acetylation procedure, 7-deoxyisonarciclasine [**13b**] and the 7-deoxy-*cis*- and *trans*-dihydronarciclasines **14b** and **15b** were obtained from triacetates **14d** and **15d**. The enantiospecific total synthesis of *trans*-dihydrolycoricidine [**15b**] was recently reported (48). The spectroscopic properties of the synthetic analogues have been established by 300 MHz and 2D (COSY) nmr spectroscopy (see Experimental).

ANTIVIRAL ACTIVITY.—*In vitro studies.*—The isolated alkaloids narciclasine [**1**], 7-deoxynarciclasine [**2**], pancratistatin [**4**], lycorines **16** and **17**, and pretazettine [**18**] and synthesized analogues **3**, **5–15** were evaluated to determine their in vitro inhibitory properties against the RNA-containing flaviviruses [Japanese encephalitis (JE), yellow fever (YF), and dengue type 4 viruses]; bunyaviruses [Punta Toro (PT), sandfly fever-Sicilian (SF), and Rift Valley fever (RVF) viruses]; the alphavirus (family Togaviridae), Venezuelan equine encephalomyelitis (VEE) virus, the lentivirus, human immunodeficiency virus type 1 (HIV-1), and the DNA-containing vaccinia virus (VV). The antiviral assays used determine the 50% inhibition (IC_{50}) of virus-induced cytopathic effect by an MTT assay (53,54) except for dengue and RVF viruses, where activity was determined by a plaque reduction assay (50,51,55). The concentration of test compound that was cytotoxic to 50% of uninfected cells (TC_{50}) was also determined, as was the ratio of these two values, expressed as a therapeutic index (TI).

In general, antiviral activity was consistently observed against the flaviviruses tested (JE, YF, dengue-4) and to a slightly lesser degree against the bunyaviruses (PT, SF, RVF). The IC_{50}, TC_{50}, and TI data are summarized in Tables 1 and 2. All compounds tested had anti-flavivirus activity except lycoricidine triacetate [**3**] (which could be considered a prodrug of **2**), triol **9**, alcohols **10–12**, and 7-deoxyisonarciclasine [**13b**] (vs. JE virus). Generally, however, the selectivity of the agents was low. Toxicity in uninfected cells (TC_{50}) generally occurred at concentrations within 10-fold (or less) that of the viral inhibitory concentration (IC_{50}). Exceptions included the mono- and triacetates **6** and **8**, which could be considered precursors of 7-deoxypancratistatin [**5**]. No activity was observed against VEE, HIV-1, or VV (data not shown).

TABLE 1. Antiviral Activity In Vitro Against Flaviviruses: Japanese Encephalitis, Yellow Fever and Dengue-Type 4.

Compound	Virus								
	Japanese Encephalitis[a]			Yellow Fever[a]			Dengue-4[b]		
	TC_{50}[c]	IC_{50}[c]	TI_{50}	TC_{50}	IC_{50}	TI_{50}	TC_{50}	IC_{50}	TI_{50}
1	0.031	0.008	4.08	0.037	0.006	6.1	0.06	0.015	4.0
2	0.27	0.056	4.9	0.29	0.053	5.6	0.25	0.059	4.2
3	>1.0	inactive		>1.0	inactive		>25	inactive	
4	0.092	0.022	4.2	0.079	0.016	4.9	0.5	0.063	8.0
5	2.8	0.48	5.9	2.6	0.4	6.6	2.5	0.67	3.7
6	10.8	3.3	3.3	21.0	4.8	4.3	50.0	1.5	33.3
7	2020	724	2.8	>1000	262	3.8	not tested		
8	16.4	4.5	3.6	8.3	2.2	3.8	100	<5.0	>20.0
9	2000	inactive		1800	inactive		not tested		
10	>100	inactive		>100	inactive		not tested		
11	630	inactive		500	inactive		not tested		
12	>100	inactive		>100	inactive		not tested		
13a	1.5	0.72	2.1	0.90	0.22	4.1	5.0	0.27	18.5
13b	>100	inactive		23.8	5.7	4.2	50.0	8.5	5.9
14a	4.9	0.96	5.1	5.2	1.3	3.9	>5.0	2.5	>2.0
14b	62.5	12.7	4.9	64.0	9.6	6.6	25.0	4.4	5.7
14c	29.0	8.1	3.5	97.0	[d]	[d]	not tested		
15a	0.025	0.004	5.6	0.027	<0.003	>8.5	0.063	0.015	4.2
15b	0.22	0.039	5.6	0.28	0.037	7.5	2.5	0.5	5.0
16	2.7	0.33	8.2	2.04	0.28	7.3	2.5	0.24	10.4
17	1.4	0.28	5.0	1.3	0.35	3.7	1.0	0.39	2.6
18	2.3	0.60	3.8	2.8	0.50	5.6	not tested		

[a] TC_{50} and IC_{50} obtained by MTT assay.
[b] IC_{50} measured by plaque reduction; TC_{50} measured by cytopathic effect.
[c] In ug/ml.
[d] Viral cytopathic effect reduced 25–49% only.

The pattern of in-vitro activity exhibited against all flaviviruses was not observed against the three bunyaviruses. Most of the compounds **1–18** inhibited PT and RVF viruses but generally with low selectivity. Pancratistatin [**4**] failed to reduce the viral cytopathic effect by 50% against PT virus. Acetate precursors **3** and **7**, triol **9**, and alcohols **10–12** were inactive against RVF and PT viruses. Less activity was observed against SF virus. Only 7-deoxynarciclasine [**2**], 7-deoxypancratistatin [**5**], 7-deoxy-*cis*-dihydronarciclasine [**14b**], and pretazettine [**18**] showed marginal activity (TI_{50}<4.5). It is of interest to note that of the four compounds exhibiting activity against SF virus, all lack the C-7 hydroxy group.

Further structure/activity correlations are also evident. Whereas narciclasine [**1**] and its 7-deoxy analogue **2** were the most toxic to host cells of any compounds in this series (in addition to pancratistatin [**4**] and the *trans*-dihydro analogues **15a** and **15b**), shifting of the double bond from the C-1–C-10b position of the C-ring to the C-10b–C-4a position (isonarciclasines **13a** and **13b**) increased the TC_{50} values by 24–200-fold. This change is reflected in the fact that isonarciclasine [**13a**] exhibited the highest selectivity (TI_{50}) against dengue virus of any of the unacetylated compounds. Similarly, the presence of a cis-fused C ring (**14a–14c**) in place of a trans-fused C ring (**15a–15c**) resulted in decreases in toxicity (increased TC_{50}) by factors of 200–280. The presence or absence of a 7-hydroxy substituent was also manifested in the TC_{50} values.

TABLE 2. Antiviral Activity In Vitro Against Bunyaviruses: Punta Toro, Rift Valley Fever and Sandfly Fever-Sicilian.

Compound	Virus								
	Punta Toro[a]			Rift Valley Fever[b]			Sandfly Fever-Sicilian[a]		
	TC_{50}[c]	IC_{50}[c]	TI_{50}	TC_{50}	IC_{50}	TI_{50}	TC_{50}	IC_{50}	TI_{50}
1	0.029	0.0074	3.9	0.022	inactive		0.028	inactive	
2	0.27	0.042	6.3	0.83	0.15	5.5	0.26	0.058	4.5
3	>1.0	inactive		<2.5	inactive		>1.0	inactive	
4	0.10	[d]	[d]	0.5	0.16	3.1	0.13	inactive	
5	2.9	0.66	4.3	21.5	5.1	4.3	4.5	1.7	2.7
6	9.6	4.7	2.1	24.0	5.5	4.4	5.2	inactive	
7	>320	inactive		>250	inactive		>320	inactive	
8	10.6	2.5	4.2	<250	inactive		21.5	inactive	
9	1200	inactive		>250	inactive		>320	inactive	
10	>100	inactive		<250	inactive		>100	inactive	
11	500	inactive		<250	inactive		>320	inactive	
12	>320	inactive		250	inactive		>320	inactive	
13a	1.4	0.28	5.1	25.0	3.3	7.6	0.72	inactive	
13b	26.2	7.2	3.7	50.0	10.0	5.0	17.6	inactive	
14a	8.0	2.2	3.6	5.0	1.4	3.6	8.0	[d]	[d]
14b	68.0	14.0	4.8	not tested			73.0	25	3.0
14c	77.0	12.0	6.4	not tested			21	[d]	[d]
15a	0.026	0.008	3.3	not tested			0.027	inactive	
15b	0.34	0.057	5.9	0.5	0.25	2.0	0.25	inactive	
16	2.3	0.50	4.6	5.0	0.93	5.4	1.4	inactive	
17	2.3	0.60	3.9	3.8	0.63	6.0	2.5	inactive	
18	2.3	0.61	3.7	10.0	2.9	3.5	2.5	0.82	3.0

[a]TC_{50} and IC_{50} obtained by MTT assay.
[b]IC_{50} measured by plaque reduction; TC_{50} measured by cytopathic effect.
[c]In μg/ml.
[d]Viral cytopathic effect reduced 25–49% only.

When comparing pancratistatin [**4**] with its 7-deoxy analogue **5** (and similarly narciclasine [**1**] with lycoricidine [**2**]), host cell toxicity (TC_{50}) was reduced by 8–32-fold when the 7-hydroxy substituent was replaced by hydrogen. Successive esterification of the hydroxy groups and C-1–C-4 (compounds **5–8**) also served to increase the TC_{50} values with increasing esterification. Finally, the effects of C-ring hydroxylation on antiviral efficacy were ascertained by comparing triol **9**, diol **11**, and mono-hydroxy **12** with 7-deoxypancratistatin [**5**]. Only the latter compound **5** showed any antiviral activity. Thus, if one disregards the configurational change of the C-3 hydroxy group in **5** and **9**, then the presence of a hydroxy group at C-4 becomes necessary for antiviral activity.

In-vivo studies.—Pancratistatin [**4**] and its 7-deoxy analogue **5** were evaluated in one or both of the variants of the murine JE virus models described (see Experimental). In variant I (low viral dose challenge model, $9LD_{50}$), prophylactic administration of 7-deoxypancratistatin [**5**] in hydroxypropylcellulose (HPC) at 40 mg/kg/day (sc, once daily for 7 days, day −1 to +5) significantly ($P = 3.6 \times 10^{-4}$) increased survival to 80% in virus-infected mice compared to the diluent-treated control animals (0% survival). At 20 mg/kg/day, an increase in survival rate to 60% and a prolonged average day of death (ADD) were observed while a dose of 10 mg/kg/day was ineffective in reducing mortality or increasing the ADD. Toxicity control animals receiving 40 mg/kg/day lost weight during the treatment period; no toxicity was observed at lower doses (see Table 3). Thus, prophylaxis with 7-deoxypancratistatin partially protected against JE viral

TABLE 3. Evaluation of Pancratistatin [4] and 7-Deoxypancratistatin [5] in the Murine Japanese Encephalitis (Low Virus Dose Challenge) Model.

Treatment	Dose (mg/kg/day)	Uninfected		Infected				
		(# Dead/Total)	ADD ± SD	(# Dead/Total)	P^b	ADD ± SD[a]	P^c	GMTD[d]
Pancratistatin [4]	6	3/5	2.7 ± 0.6	7/10	0.105	4.0 ± 2.4	ND[e]	6.5
	4	0/5		0/10	<0.001	—	NA[f]	28.0
	2	0/5		8/10	0.24	16.3 ± 1.9	<0.001	18.0
Untreated		0/5		10/10	NA	14.0 ± 1.8	NA	13.9
2% EtOH/saline		0/5		10/10	NA	12.8 ± 1.3	NA	12.7
Pancratistatin [4]	6	0/5		1/10	<0.001	15.0 ± 0.0	0.36	26.3
	4	0/5		7/10	0.105	15.4 ± 2.0	0.02	18.4
	2	1/5	28.0 ± 0.0	9/10	0.50	11.6 ± 1.4	ND	12.5
Untreated		0/5		10/10	NA	12.2 ± 2.5	NA	11.9
2% EtOH/saline		0/5		10/10	NA	12.4 ± 2.6	NA	12.1
7-Deoxypancratistatin [5]	40	0/5		2/10	<0.001	9.5 ± 4.9	0.06	22.2
	20	0/5		4/10	0.005	15.0 ± 2.6	0.03	21.7
	10	0/5		9/10	0.50	13.6 ± 1.9	0.21	14.4
Untreated		0/5		10/10	NA	12.2 ± 2.5	NA	11.9
Hydroxypropylcellulose		0/5		10/10	NA	12.6 ± 1.2	NA	12.6

[a]Average day of death ± standard deviation.
[b]*P* value by Fisher's Exact Test comparing mortality in drug-treated to diluent-treated mice.
[c]*P* value by Student's *t*-test comparing days of death in drug-treated to diluent-treated mice.
[d]Geometric mean time to death.
[e]ND = not done.
[f]NA = not applicable.

infections in mice. However, efficacy was primarily observed at dose levels approaching toxicity. Pancratistatin [**4**] exhibited similar results. Prophylactic administration of a 2% EtOH saline solution of pancratistatin with variant I (Table 3) at 4 mg/kg/day (2% EtOH saline, sc, once daily for 7 days, day −1 to +5) resulted in 100% survival of JE-virus-infected mice as compared to the diluent-treated controls (0% survival). At 6 mg/kg/day, pancratistatin was toxic to 60% of the uninfected mice while a dose of 2 mg/kg/day did not significantly reduce JE virus-induced mortality. However, it did prolong the ADD. In a second experiment using the same model, **4** was administered at 6 mg/kg/day and significantly increased survival (to 90%) in JE-virus-challenged mice compared to the diluent treated controls (0% survival). No mortality occurred in the toxicity control animals. At 4 mg/kg/day, survival was reduced to 30%, while the 2 mg/kg/day dose level had no significant effect on the JE viral challenge.

In variant II (high viral challenge model, 100 LD_{50}), prophylactic administration of pancratistatin (Table 4) at 2–8 mg/kg/day, (ip, twice daily for 9 days, day −1 to +7) resulted in only 50% survival at the 6 mg/kg/day dose as compared to 0% survival in the diluent-treated mice. While this represents a significant increase in the number of survivors, there was no significant increase in the mean time to death of the non-surviving animals, although the value corresponding to the 8 mg/kg dose approached significance.

TABLE 4. Evaluation of Pancratistatin [**4**] in the Murine Japanese Encephalitis (High Virus Dose Challenge) Model.

Treatment	Dose (mg/kg/day)	Wt. change (Day +7)	Uninfected (No. dead/total)	Infected (No. dead/total)	ADD ± SD (days)
Pancratistatin	8	1.5	2/5	7/10	12.9 ± 0.5
	6	1.3	1/5	5/10[a]	11.8 ± 0.1
	5	1.4	1/5	7/10	10.4 ± 0.5
	4	1.8	0/5	9/9	12.3
	3	2.1	0/5	9/10	12.6
	2	2.8	0/5	9/10	12.6
Untreated		2.7	0/10	10/10	11.5
2% EtOH/saline		1.8	0/10	10/10	12.6

[a]Significant at $p = 0.05$; 2-tailed compared to 2% EtOH/saline treatment. Survival by Fisher's Exact Test; average day of death (ADD) by Student's *t*-test.

Insolubility of pancratistatin in aqueous media and limited bioavailability may be partially responsible for poor reproducibility of pharmacokinetics which may have produced the variable in vivo test results. Differences between the two murine models include age of mice, administration routes of drug, and virus, as well as schedule and the LD_{50} of viral challenge. We did not determine which of these differences accounted for the experimental variations. However, these results are significant in that they represent one of the few example of chemotherapy of JE viral infections in animal systems. [One earlier study (20) describes the effects of narcissidine, 4-methoxypseudolycorine (22), derived from *Narcissus tazetta*, on JE virus-infected mice, which had prolonged survival times, but there were no significant differences in mortality of treated versus control animals.] Further syntheses and evaluations of additional analogues are underway in an effort to broaden the therapeutic margin between efficacy and toxicity.

CONCLUSIONS.—We found that a series of naturally occurring or synthetic Amaryllidaceae alkaloids **1–18** related to narciclasine and lycorine possess antiviral

efficacy with accompanying low selectivity in vitro against three flaviviruses, JE, YF, and dengue viruses; against the bunyaviruses, PT and RVF viruses; and, to a lesser extent, against SF virus. We found prophylactic efficacy of pancratistatin and 7-deoxypancratistatin in JE-virus-infected mice; however, chemotherapeutic doses bordered on toxic doses. Therefore, further structure/activity-guided synthetic studies will be pursued in an effort to produce a more effective compound with less toxicity.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All solvents were distilled before use, dried when necessary, and all chemicals were reagent grade. Evaporations were conducted at bath temperatures $\leq 30°$ with a Büchi rotary evaporator under water aspirator or mechanical oil pump vacuum. Melting points were determined with a Thomas Hoover capillary apparatus and are uncorrected. Microanalyses were performed by Atlantic Microlab, Inc., Atlanta, GA. Results agreed within $\pm 0.4\%$ of theory. The ^{1}H-nmr and ^{13}C-nmr spectra were recorded on a Varian XL200 spectrometer with ADVANCE data system operating at 200 MHz and 50.3 MHz, respectively. Additional ^{1}H-nmr spectra were recorded on a Nicolet NT300WB spectrometer with 1280 data system operating at 300 MHz. Standard Varian COSY determined proton-proton connectivity. Mass spectral data were obtained by employing an MAT 312 mass spectrometer. Chemical shifts are expressed in ppm and referenced to TMS (^{1}H nmr) or DMSO at 39.7 ppm (^{13}C nmr). Ir spectra were recorded by a Perkin-Elmer Model 1310 spectrophotometer as Nujol mulls or KBr pellets. Tlc was performed on Woelm F Si gel sheets (254/366) with detection of products under a short wavelength uv lamp and/or spraying with 40% methanolic H_2SO_4 and charring.

Pseudolycorine [**17**] and pretazettine [**18**] were obtained from both the National Cancer Institute and the University of Hawaii. Narciclasine [**1**], lycoricidine [**2**], pancratistatin [**4**], and lycorine [**16**] were obtained by isolation (7,31). Lycoricidine analogues **3**, **6**, **8**, **10**, **11**, and **12** were prepared by Schubert's modification (45) of the earlier procedure of Ohta and Kimoto (44). Isonarciclasine [**13a**], *cis*- and *trans*-dihydronarciclasines **14a** and **15a**, and their respective tetraacetates **14c** and **15c** were synthesized from narciclasine according to procedures described by Mondon and Krohn (39–41, 47).

4aH-r,1H-trans,2H-cis,3H-trans,4H-trans,10bH-trans-1,2,3,4-Tetrahydroxy-8,9-methylenedioxy-1,2,3,4,4a,10b-hexahydro-6(5H)-phenanthridone (7-deoxypancratistatin) [**5**].—OsO_4 (100 mg, 0.39 mmol) was added to a solution of *N*-methylmorpholine-*N*-oxide (2.2 g, 19 mmol) dissolved in *t*-BuOH (25 ml), Me_2CO (25 ml), and H_2O (20 ml), and the mixture was stirred for 10 min. A warm solution of racemic intermediate (45) **10** (4 g, 11 mmol) in *t*-BuOH/Me_2CO (200 ml each) was added over 5 min. The mixture was stirred for 24 h at 25°, at which point tlc [$CHCl_3$-MeOH (6:1)] indicated ca. 60% reaction. Additional OsO_4 (50 mg, 0.195 mmol) and *N*-methyl-morpholine-*N*-oxide (500 mg) were added. After 24 h (95% completion), the solvents were removed in vacuo at 35°. Trituration of the residue with EtOH (25 ml), removal of solvent in vacuo, a second treatment with EtOH (25 ml), and filtration gave the 1-(2′-tetrahydropyranyloxy)-2,3,4-trihydroxy analogue as a pale-yellow solid, 3.6 g (82%), mp 222–224°. Without further purification, the tetrahydropyranyl group was removed by heating at reflux a mixture of the pyranyl ether (3.4 g, 8.6 mmol) and *p*-toluenesulfonic acid monohydrate (200 mg) in EtOH (150 ml) for 3 h. Cooling to 0°, filtration, concentration of the filtrate in vacuo, and a second cooling and filtration gave two crops of tetraol **5**. Recrystallizations from HOAc followed by H_2O gave 1.51 g (57%) of alcohol **5** as an analytically pure, off-white amorphous powder, mp 308° (dec) [lit. (44) mp 305°]. Nmr and ir spectral data agreed with literature values (44).

4aH-r,1H-trans,2H-cis,3H-trans,4H-trans,10bH-trans-1,2,3,4-Tetraacetoxy-8,9-methylenedioxy-1,2,3,4,4a,10b-hexahydro-6(5H)-phenanthridone (7-deoxypancratistatin-1,2,3,4-tetraacetate) [**7**].—A solution of the 1-(2′-tetrahydropyranyloxy)-2,3,4-trihydroxy analogue prepared above (3.4 g, 9.5 mmol) and *p*-toluenesulfonic acid monohydrate (500 mg) in EtOH (300 ml) was heated at reflux for 12 h. The volume was reduced in vacuo to 40 ml, and the mixture was cooled. The resulting product was collected by filtration, dissolved in Ac_2O (200 ml) containing DMAP (400 mg), and heated at reflux for 10 h. Ac_2O was removed by distillation under reduced pressure followed by co-evaporation with EtOH (2×50 ml). The solid residue was recrystallized from Me_2CO (200 ml) yielding 2.1 g (51%) of acetate **7**, mp 306° [lit. (44) mp 300°]. Nmr and ir spectral data agreed with literature values (44).

4aH-r,1H-trans,2H-cis,3H-cis,10bH-trans-1,2,3-Trihydroxy-8,9-methylenedioxy-1,2,3,4,4a,10b-hexahydro-6(5H)-phenanthridone [**9**].—OsO_4 (12.5 mg, 0.048 mmol) was added to a solution of *N*-methylmorpholine-*N*-oxide (0.78 g, 6.6 mmol) in *t*-BuOH (2.4 ml), Me_2CO (2.4 ml), and H_2O (1 ml). A solution of olefin (44) **12** (1 g, 3.86 mmol) in 50% *t*-BuOH/Me_2CO was added over 5 min. The reaction mixture was stirred at 25° for 6 days until tlc [$CHCl_3$-MeOH (6:1)] showed complete disappearance of starting material **12**. The solution was treated with charcoal, stirred for 3 h at 25°, and filtered through Celite (pre-

pared in aqueous Me_2CO), and the solvent was removed in vacuo. Two recrystallizations from H_2O gave 620 mg (49%) of triol **9**, mp 288–290°; ^1H nmr (DMSO-d_6) δ 7.69 (s, 1H, exchanges with D_2O, NH), 7.32 (s, 1H, H-7, uncoupled in COSY), 6.91 (s, 1H, H-10, coupled to H-10b), 6.06 (AB, J = 0.96 Hz, 2H, CH_2O), 5.12, 4.87, 4.67 (d, J = 4.7, 3.3, 6.4 Hz, respectively, 1H each, exchange with D_2O, 1-OH, 2-OH, 3-OH, respectively), 4.37 (d, J = 2.5 Hz, 1H, H-1, coupled to H-2, H-10b, 1-OH), 3.86 (dd, 1H, H-3, coupled to H-2, H-4), 3.81 (dd, overlapping partially with H-3, 1H, H-2, coupled to H-1, H-3), 3.55 (dt, J = 12.4, 3.8 Hz, 1H, H-4a, coupled to H-4, H-10b), 2.85 (dd, J = 12.6, 1.5 Hz, 1H, H-10b, coupled to H-10, H-4a, H-1), 1.8 (dd, $J_{ea} = J_{ae}$ = 3.8 Hz, 1H, H_{eq}-4), 1.7 (dd, J_{aa} = 11.7 Hz, 1H, H_{ax}-4); ^{13}C nmr (tentative assignments) δ 164.04 (C=O), 150.15, 145.63, 135.60, and 124.23 (aryl), 106.75 and 105.21 (C-7, C-10), 101.34 (CH_2O), 72.13, 68.03, and 65.30 (C-1, C-2, C-3), 47.85 (C-4b), 40.15 (C-10b), 33.89 (C-4). *Anal.* found C 57.25, H 5.19, N 4.74; $C_{14}H_{15}NO_6$ requires C 57.34, H 5.16, N 4.78.

7-Deoxyisonarciclasine **[13b]** *and 7-deoxy-*cis- *and* trans-*dihydronarciclasine triacetates* **14d** *and* **15d**.—A solution of 7-deoxynarciclasine **2** (1.02 g, 3.4 mmol) in a MeOH-EtOH (1:1) (400 ml) was degassed with N_2 followed by addition of platinum oxide (57 mg). The mixture was hydrogenated at ambient temperature and pressure for 24 h, producing a precipitate which was collected with catalyst by filtration through Celite. The residue was heated in pyridine and the solution filtered through Celite. Concentration of the filtrate in vacuo gave a dark brown solid (150 mg), which crystallized from pyridine/hexane to give 7-deoxyisonarciclasine (39–41) **[13b]**: mp >300° (100 mg, 9.8%); ir (KBr) 3400, 3284, 3237, 1653, 1629, 1590, 1487, 1472, 1460, 1064, 1042 cm^{-1}; ^1H nmr (DMSO-d_6) δ 1.95, 1.99, 2.12 (br s, 1H each, OH), 2.38 (dd, J = 16.2, 6.5 Hz, 1H, H-1), 2.94 (dd, J = 16.2, 5.3, 1H, H-1), 3.61 (dd, J = 7.6, 3.3 Hz, 1H, H-3), 4.00 (dd, J = 12.9, 6.3 Hz, 1H, H-2), 4.50 (d, J = 2.2 Hz, 1H, H-4), 6.16 (br s, 2H, OCH_2O), 7.1 (s, 1H, H-7), 7.54 (s, 1H, H-10), 8.58 (br s, 1, NH); ^{13}C nmr (DMSO-d_6) δ 30.33 (C-1), 66.02 (CH), 66.21 (CH), 71.74 (CH), 101.19 (C-7), 101.84 (CH_2), 104.41 (C-10), 106.11 (C-10b), 120.71 (C-4a), 134.60 (C-6a, C-10a), 146.7 (C-8), 151.68 (C-9), 160.70 (C-6); eims *m/z* $[M]^+$ 291 (100%), 275 (20), 255 (32), 244 (20), 231 (28), 203 (55).

The original filtrate obtained after removing precipitate and catalyst was concentrated to dryness in vacuo and treated with Ac_2O (7 ml)/pyridine (10 ml) at 60° for 6 h. Pyridine was removed by azeotropic distillation with MeOH and cyclohexane, and the residue was concentrated to dryness. Flash chromatography on a Si gel column and elution with CH_2Cl_2-MeOH (99.4:0.6) followed by crystallization from Me_2CO/hexane gave 150 mg (10%) of 7-deoxy-*trans*-dihydronarciclasine triacetate (40) **[15d]**: mp 148–149°; ir (KBr) 3420, 1757, 1661, 1502, 1474, 1462, 1245, 1224, 1190, 1061, 1038 cm^{-1}; ^1H nmr ($CDCl_3$) δ 1.92 (m, 1H, H-1), 2.08 (s, 3H, Ac), 2.13 (s, 3H, Me), 2.14 (s, 3H, Me), 2.48 (dt, d = 14.4, 3.3 Hz, 1H, H-1), 3.17 (td, J = 12.6, 4.2 Hz, 1H, H-10_b), 3.82 (dd, J = 12.6, 10.7 Hz, 1H, H-4a), 5.18–5.45 (m, 3H, C*H*OCOCH$_3$), 6.03 (s, 2H, OCH_2O), 6.72 (s, 1H, H-10), 6.94 (br s, 1H, NH), 7.52 (s, 1H, H-7); eims *m/z* $[M]^+$ 419 (40%), 360 (30), 297 (20), 255 (98), 239 (100). Continued elution of the column yielded a mixture of cis and trans triacetates **14d** and **15d** (374 mg, 26%) followed by pure 7-deoxy-*cis*-dihydronarciclasine triacetate (40) **[14d]**, 440 mg (31%). Crystallization from Me_2O/hexane gave an amorphous powder: mp 144–146°; ir (NaCl) 3300, 1750, 1671, 1481, 1467, 1371, 1246, 1231, 1053, 1038, 756 cm^{-1}; ^1H nmr ($CDCl_3$) δ 1.80–2.30 (m, 1H, H-1), 2.01 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.16 (s, 3H, Ac), 3.20 (dt, J = 14.7, 3.8 Hz, 1H, H-10_b), 3.92 (t, J = 3.8 Hz, 1H, H-4a), 5.20–5.56 (m, 3H, C*H*OCOCH$_3$), 6.02 (s, 2H, OCH_2O), 6.24 (br s, 1H, NH), 6.67 (s, 1H, H-10), 7.84 (s, 1H, H-7); eims *m/z* $[M]^+$ 419 (15%), 299 (20), 257 (42), 239 (100).

*7-Deoxy-*trans-*dihydronarciclasine* **[15b]**.—A mixture of 7-deoxy-*trans*-dihydronarciclasine triacetate **[15d]** (60 mg, 0.14 mmol) in MeOH (15 ml) and aqueous 1 M $Ba(OH)_2$ solution (5 ml) was heated on a steam bath for 15 min, cooled to 25°, saturated with solid CO_2, stirred at 25° overnight, and filtered. The filtrate was evaporated to dryness, the residue was redissolved in MeOH, and the solution was filtered. The solvent was removed in vacuo and the residue crystallized from MeOH to give 35 mg (83.4%) of 7-deoxy-*trans*-dihydronarciclasine **[15b]**: mp 320–322° [lit. (40) mp >300°]; ir (KBr) 3555, 3491, 3454, 3427, 1671, 1464, 1268, 1074, 1047, 1036 cm^{-1}; ^1H nmr (DMSO-d_6) δ 1.63 (td, J = 13.0, 2.4 Hz, 1H, H-1), 2.13 (dt, J = 13.0, 3.0 Hz, 1H, H-1), 2.872 (td, J = 12.0, 3.6 Hz, 1H, H-10b), 3.30 (m, 1H, H-4a, signal flanked with H_2O in DMSO), 3.705 (br s, 2H, 2 × OH), 3.869 (br s, 1H, OH), 4.825 (br d, J = 3.3 Hz, 1H, -C*H*OH), 4.940 (br d, J = 5.8 Hz, 1H, -C*H*OH), 4.976 (br d, J = 3.4 Hz, 1H, -C*H*OH), 6.065 (br s, 2H, OCH_2O), 6.924 (s, 1H, H-10), 6.930 (br s, 1H, NH), 7.290 (s, 1H, H-7); eims *m/z* $[M]^+$ 293 (72%), 202 (100), 189 (60).

*7-Deoxy-*cis*-dihydronarciclasine* **[14b]**.—A solution of 7-deoxy-*cis*-dihydronarciclasine triacetate **[14d]** (440 mg, 1.05 mmol) in MeOH (50 ml) containing K_2CO_3 (200 mg) was stirred at 25° for 2 h and filtered through a Sephadex LH-20 column. The column was eluted with CH_2Cl_2-MeOH (3:2), and the product was crystallized from MeOH/Me_2CO to give 7-deoxy-*cis*-dihydronarciclasine **[14b]** (250 mg, 81%) as an amorphous powder: mp >300° [lit. (40) mp >300°]; ir (KBr) 3300, 1653, 1610, 1468, 1404,

1387, 1357 cm^{-1}; ^1H nmr (DMSO-d_6) δ 1.300 (q, J = 12.5 Hz, 1H, H-1), 1.685 (dt, J = 12.5, 4.0 Hz, 1H, H-1), 3.030 (dt, J = 12.5, 4.0 Hz, 1H, H-10b), 3.300 (m, 1H, H-4a, signal flanked with H_2O in DMSO), 3.595 (t, J = 3.6 Hz, 1H, -C*H*OH), 3.638 (dd, J = 11.0, 4.5 Hz, 1H, -C*H*OH), 3.929 (t, J = 3.0 Hz, 1H, -C*H*OH), 4.400–5.100 (br s, 3H, 3 × OH), 6.040 (m, 2H, OCH_2O), 6.949 (s, 1H, H-10), 7.231 (s, 1H, H-7), 7.751 (br s, 1H, NH); eims *m/z* $[M]^+$ 293 (50%), 202 (45), 189 (18).

IN-VITRO ANTIVIRAL AND CYTOTOXICITY ASSAYS.—The in vitro antiviral and cytotoxic effects of a test compound were measured (49) either: (a) by observing inhibition of viral cytopathic effect (50–52) by using an MTT assay [JE, YF, SF, PT, VEE, VV, and HIV-1 viruses (53,54)] or (b) by a general plaque reduction assay [dengue virus (55), RVF virus (56)]. Compounds were evaluated for antiviral efficacy against the following viruses (viral strain): JE virus (Nakayama), YF virus (Ashibi), SF virus (Sicilian), PT virus (Adames), VEE virus (Trinidad donkey), VV, (Lederle vaccine). All assays were carried out in Vero cells except for the use of MT-2 and CEM cells in the HIV-1 assay (53,54). Compounds to be assayed were dissolved in DMSO and diluted with H_2O to a final volume of 2% except for **1**, **2**, **5–8**, **14c**, and **15c**, which were solubilized in aqueous EtOH.

A general plaque reduction assay (55,56) was used to test for antiviral activity of candidate compounds against dengue virus. Each drug to be tested was dissolved in appropriate diluent (DMSO or EtOH), brought to twice the highest concentration to be tested in cell culture maintainance medium (Hank's basal salt solution-Hepes containing 2% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 10 mg/ml streptomycin), and sterilized by filtration. Five twofold dilutions of 2 × drug were prepared in cell culture medium. For each drug, 12 wells of a 24-well plastic tissue culture plate containing confluent monolayer cultures of LLCMK2 cells were used. Six wells were infected by removing growth medium and adding 100 μl of dengue 4 virus (Caribbean strain) containing 50–100 plaque-forming units (pfu). The remaining six wells received medium without virus. After adsorption for 1 h, 0.5 ml each 2 × concentration of drug was added to duplicate (infected and control) wells. Medium without drug was added as a control. Cultures were then overlaid with 2.5% agarose in nutrient medium and incubated for 6 days, at which time they were stained by adding 2 ml of 5% neutral red. Wells were decanted after 4 h, and plaques were counted. The IC_{50} was determined as the concentration of drug-reducing plaques by 50% over the untreated control, while the minimum toxic concentration (MTC) was estimated visually by inspection of uninfected drug-treated wells.

Basic measurements and definitions used throughout these studies include: (a) Cellular toxic concentraton 50% (TC_{50}), defined as the drug concentration (μg/ml) that reduces cell numbers and their metabolic activity by 50% as compared to the viability of uninfected control cells in duplicate test wells in the MTT assay; (b) Viral inhibitory concentration 50% (IC_{50}), defined as the drug concentration (μg/ml) at which 50% reduction of viral cytopathic effect (CPE) is observed in triplicate test wells; (c) therapeutic (or antiviral) index (TI), a value proportional to the overall in vitro activity, calculated as a ratio of (TC_{50}/IC_{50}). It is a single drug concentration measurement of the relative anticellular and antiviral effectiveness of a compound during the same test and time period. All in vitro MTT assay results given represent an average of 2–6 individual test results.

IN VIVO STUDIES.—*Murine Japanese encephalitis virus model, variant I. Low LD_{50} viral challenge.*—Groups of 15 C57Bl/6 mice (VAF+, Charles River Laboratories), weighing 14 to 16 g, were treated sc with phosphate-buffered saline (PBS) or drug once daily on a 7-day schedule with the first dose administered on the day (day −1) preceding viral challenge. Ten of the 15 animals in each group were infected ip with 9 LD_{50} (adequate to produce 90–100° mortality in the diluent controls) of JE virus (Beijing strain) 2 h after the second dose of compound was administered (day 0). Controls included untreated, uninfected mice; untreated, virus-infected mice; diluent-treated, virus-infected mice; and diluent-treated, uninfected mice. Pancratistatin was solubilized in EtOH and diluted in sterile saline to a final EtOH concentration of 2%; 7-deoxypancratistatin was suspended in NCI-hydroxypropylcellulose, HPC. Compounds were prepared at concentrations appropriate for dosing at 0.1 ml of compound per 10 g of body wt. Animals were monitored for 28 days post-virus infection. Body weights were recorded on days −1 through 7, 14, and 21. Weight change was determined as a measure of drug toxicity. The average day of death (ADD) and geometric mean time to death (GMTD) were calculated. An in vivo virus rating (VR) for each drug concentration was calculated by dividing the GMTD in treated animals by that in diluent-treated controls. The statistical significance of differences in the mortality rates for the drug-treated, virus-infected animals compared to the diluent-treated, virus-infected animals was compared by Fishers' Exact test. Differences in the ADD for drug-treated, virus-infected animals compared to the diluent-treated, virus-infected animals were compared by Student's *t*-test.

Variant II. High LD_{50} viral challenge.—Groups of 15 C57Bl/6 mice (VAF+, Charles River Laboratories) weighing 12–14 g were treated ip with PBS or drug twice daily on a 9-day schedule, with the first dose administered on the day (day −1) preceding viral challenge. Ten of the 15 animals in each group were in-

fected sc with 100 LD_{50} of JE virus (Beijing strain, adequate to produce 100% mortality in the diluent controls) 6 h after the second dose of compound was administered (day 0). Controls included untreated, uninfected mice; untreated, virus-infected mice; diluent-treated virus-infected (and uninfected) mice. Animals were monitored for 28 days post-virus infection. Body weights were recorded on days −1 through +7. Weight change was used as a measure of drug toxicity.

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