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SYNTHESIS AND ANTIVIRAL PROPERTIES OF ETHYL(3-ETHYLADAMANT-1-YL)CARBAMATE

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The new adamantine derivative ethyl(3-ethyladamant-1-yl)carbamate was found to have high antiviral activity against herpes simplex virus and vaccinia viruses, as well as adenovirus (EC_{50} = 0.62, 5.15, and 48.5 μg/ml respectively). Activity against the herpesvirus was demonstrated in experiments in various cell cultures using different virus strains, including a variant resistant to acycloguanosine (ACG). Simultaneous application of ethyl(3-ethyladamant-1-yl)carbamate and ACG increased the inhibition of herpesvirus reproduction in cell cultures and decreased the mortality of laboratory animals with experimental herpesvirus neuroinfection as compared with the use of each substance alone. 3-Ethyladamant-1-yl)carbamate was a low-toxicity compound, did not inhibit DNA synthesis in uninfected cell cultures, and its acute intragastric LD_{50} for white mice was 1831.8 mg/kg.

Keywords: adamantine, ethylcarbamate, virus infections, antiviral activity.

Herpesvirus infections are the second most common after influenza and other acute respiratory viral infections. Herpes simplex virus currently infects 60 – 90% of the population [1]. Genital herpes is one of the most widespread infectious pathologies in humans, with all the signs of an epidemic [2]. Resistant variants of herpes simplex virus, despite decades of use of acycloguanosine (ACG – acyclovir, Virolex, Zovirax) and its analogs, account for a few per cent of isolates, and are somewhat more common in patients with immunodeficiencies (4 – 7%) [3, 4]. It should therefore be noted that chronic recurrent herpes is the most frequently seen form of endogenous infection, and that such patients have repeated episodes of using ACG. According to our admittedly limited data, herpes simplex virus, usually type 2, isolated from all the patients with urogenital pathology studied have some degree of resistance to ACG [5].

The importance of finding and developing new substances effectively inhibiting herpesviruses results not only from cases of resistance to ACG as the most widely drug, but also from the existence of types of herpesvirus infection in which ACG lacks adequate efficacy. The use of combinations of antiviral drugs with different mechanisms of action leads to additive or synergistic action; realization of this potential in medical practice requires widening of the range of available drugs.

We describe here the synthesis of a new adamantine derivative, ethyl(3-ethyladamant-1-yl)carbamate, and present the results of studies of its antiviral properties.

The main method used for preparing *N*-substituted esters of carbamic acid consists of attaching alcohols to isocyanates [6, 7]; the reaction can use isocyanates formed in situ from carboxylic acid acylazides or amides in the Curtius or Hofmann reactions. *N*-substituted esters of carbamic acid were also synthesized by the reaction of alcohols with isocyanic acid or chloroformates with amines [8 – 10].

Carbamic acid esters whose structures contain an amide fragment are known to be able to undergo adamantylation in nitric or sulfuric acid [11, 12]. Nitroxy and hydroxy derivatives of adamantine in sulfuric acid easily generates the adamantyl cation, which can attack the unshared electron pair in nitrogen-containing nucleophiles.

We have previously shown that interaction of hydroxy or nitroxy derivatives of adamantine with potassium cyanate

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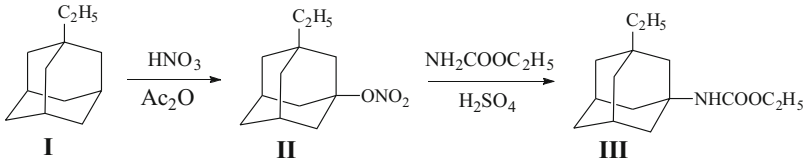
Scheme



and alcohols in concentrated sulfuric acid leads to the production of *N*-adamantylcarbamates 10]. In sulfuric acid, in situ formed isocyanic acid quickly produces the carbamic acid ester, which then undergoes alkylation by the 1-adamantylation. The results showed that the reaction was partially inhibited by addition of alkyl groups into the adamantane carcass, and in this situation the method does not yield adamantylated carbamates at sufficient yield. The starting 3-ethyladamant-1-yl nitrate (II) was synthesized from 1-ethyladamantane (I) as described in [13]. Ethyl(3-ethyladamantan-1-yl)carbamate (III) was obtained by the reaction of compound II with ethylcarbamate in 94% sulfuric acid at high yield (84%).

The ^{1}H NMR spectrum of compound III contained an NH proton signal as a broad singlet with $\delta = 4.51$ ppm. Proton signals as triplets and quartets with $\delta = 0.76$ ppm and 1.13 ppm and a spin-spin coupling constant (SSCC) of 7.8 Hz corresponded to signals from the ethyl group attached to the adamantine carcass. Ethyl group proton signals from the ethyl carbamate fragment were present as a triplet with $\delta = 1.9$ ppm for the methyl group and a quartet with $\delta = 4.0$ ppm for the methylene group, with SSCC = 7.1 Hz. The ^{13}C NMR spectrum contained a signal with $\delta = 154.76$ ppm, corresponding to the carbon atom of the carbonyl group.

EXPERIMENTAL CHEMICAL SECTION

^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM ECX-400 (Japan) spectrometer at a frequency of 400 MHz, with TMS as the internal standard and $CDCl_3$ as solvent. IR spectra were recorded on a Shimadzu IRAffinity-1 spectrophotometer (Japan) in a thin layer on a KBr plate. Mass spectra were taken on a Thermo Finnigan DSQ (USA) chromatomass spectrometer with a mass-selective detector in the electron ionization regime (70 eV), a B-5MS 30 m × 0.32 mm quartz column at a column temperature of 80 – 340°C (heating rate 20°C/min), an evaporator temperature of 300°C, and helium as the carrier gas. UV spectra were recorded on a Shimadzu UV mini 1240 (Japan) UV spectrometer. Elemental analysis was run on a EuroVector EA-3000 CHNS-O elemental analyzer (Italy) with L-cysteine as the standard. Elemental analysis data were consisted with calculated values.

3-Ethyladamant-1-yl nitrate (II) was prepared from 1-ethyladamantane (I) as described in [13] with a yield of 90% and $n_D^{20} = 1.4957$.

Ethyl(3-ethyladamant-1-yl)carbamate (III). Nitrate II (2.6 g, 0.01 mol) was dissolved in 20 ml of 94% sulfuric acid with cooling, 8 g (0.09 mol) of ethylcarbamate was added, and the mix was left overnight. The reaction was poured onto ice and extracted with toluene; the toluene extract was washed with saturated sodium bicarbonate solution and water, and dried over anhydrous Na_2SO_4. The toluene was then removed by evaporation in a vacuum rotary evaporator and the residue was distilled in vacuo. The yield was 2.4 g (84%), the melting temperature was 164°C/15 mm; $n_D^{20} = 1.4998$. The IR spectrum, ν_{max}, cm^{-1}, was: 3385 (NH), 3010, 1710 (C=O), 1520 (C-N). The mass spectrum, *m*/*z* (I_{rel}, %), was: 251 $[M]^+$ (59), 222 (14), 194 (100), 176 (10), 166 (58), 148 (40), 138 (20), 93 (19), 79 (20). The atomic formula was

TABLE 1. Inhibition of the Multiplication of Herpes and Vaccinia Viruses in CEF Cell Cultures in the Presence of Ethyl(3-ethyladamant-1-yl)carbamate (II)

Virus	Zone diameter, screening test, mm		Plaque reduction assay			
	toxicity	suppression of plaque formation	Concentration, μg/ml	virus titer, log pfu/ml	difference from control, log pfu/ml	EC_{50} (I_{95}), μg/ml
Herpes	9	22	50 – 6	< 3.9	> 1.6	0.62 (0.89 – 0.42)
			3	4.9	0.6	
			0	5.5	–	
Vaccinia	18	18	50 – 12	< 2.5	> 1.6	5.15 (6.63 – 3.99)
			6	3.2	0.9	
			0	4.1	–	

pfu – plaque-forming units; I_{95} – confidence interval

$C_{15}H_{25}NO_2$. The ^1H NMR spectrum ($CDCl_3$), δ, ppm, was: 0.76 (t, J 7.8 Hz, 3H, CH_2CH_3), 1.13 (q, J 7.8 Hz, 2H, CH_2CH_3), 1.9 (t, J 7.1 Hz, 3H, CH_2CH_3), 1.36 – 1.37 (m, 4H, $2CH_{2Ad}$), 1.53 – 1.56 (m, 4H, $2CH_{2Ad}$), 1.79 – 1.86 (m, 4H, $2CH_{2Ad}$), 2.08 – 2.09 (m, 2H, $2CH_{Ad}$), 4.00 (q, J 7.1 Hz, 2H, CH_2CH_3), 4.51 (broad s, 1H, NH). The ^{13}C NMR spectrum, δ_C, ppm, was: 7.10, 14.69, 29.71, 34.55, 35.90, 36.02, 40.79, 41.60, 46.20, 51.54, 59.99, 154.76. The UV spectrum was (C_2H_5OH), λ_{max}, nm (lg ε), was: 257 (5.0).

EXPERIMENTAL BIOLOGICAL SECTION

Cell culture studies. Studies were performed using monolayer cultures of primary chick embryo fibroblasts (CEF) and human embryo kidney (HEK), transformed green monkey kidney cells (Vero), herpes simplex virus type 1 (strain 1C) and its variant resistant to the known antiherpetic agent ACG obtained by serial passage of the starting virus in the presence of the drug, vaccinia virus, and type 3 adenovirus. Herpes and vaccinia viruses were used in a screening test in CEF cells and plaque reduction assay in Vero cells under agar; adenovirus was used for assessment of the effects of study drug on the development of the cytoplasmic effect (CPE) in HEK cells. Medium 199 was used as maintenance and nutritive medium, based on a concentrate supplemented with 1% bacteriological agar and 0.005% neutral red (all reagents from Sigma). Multiplicities of infection were 0.00002 cfu/cell in the screening test and the plaque reduction assay and 0.001 $TCID_{50}$/cell (tissue cytopathic infectious dose).

The criteria for antiviral activity were the presence and size of the plaque suppression zone (screening test) and the decrease in the CPE and infectious titer of viruses in the presence of different test substance concentrations. The concentration giving 50% suppression of virus replication in the presence of study compound (EC_{50}) was calculated using a program based on probit analysis using the Finney algorithm [14].

Toxic properties were determined in terms of the ability to induce cytodestructive changes to cells at 48 – 96 h of incubation and impairment of the ability to take up neutral red. The maximum tolerable concentration (MTC) was taken as the highest concentration of substance not producing these changes. In addition, 50% suppression of DNA synthesis in uninfected Vero cell cultures was determined in terms of ^3H-thymidine uptake [15].

Study substances were prepared by dilution in maintenance medium from stock solution (5 mg/ml) in double-distilled water; 10% ethanol was added for compound III. ACG was used as the sodium salt of acyclovir (Virolex powder for intravenous infusions, substance manufactured by The Wellcome Foundation Ltd.).

Laboratory animal studies. Mongrel white mice weighing 6 – 7 g were infected with herpesvirus and its ACG-resistant variant intracerebrally at a dose corresponding to 1000 – 10,000 LD_{50}/0.1 ml or i.p. at a dose of 100 LD_{50}/0.1 ml. Both virus variants, the initial and resistant, were preadapted by passaging by intracerebral infection of white mice.

Drug III was given to animals intragastrically as a suspension in isotonic sodium chloride solution containing 10% Tween-80, starting 3 h after infection twice daily to completion of the experiment (or for seven days for i.p. infection). Animals were observed until death in controls (5 – 6 days from infection) or for 14 days after i.p. infection.

The acute toxicity of compound III was tested on mice of both genders weighing 18 – 20 g; compound III was given intragastrically as a suspension in 10% Tween-80 in five increasing doses starting at 1.6 g/kg with a step of 0.1 g/kg (2 – 4 animals per dose, total 16 animals). Animals were observed for 10 days after inoculation. Results were processed as described for calculation of EC_{50}.

TABLE 2. Inhibition of the Reproduction of Herpes Virus and its ACG-Resistant Variant by Ethyl(3-ethyladamant-1-yl)carbamate (III), ACG, and the Combination of Compound III and ACG in Vero Cells

Group	Concentration, μg/ml	Herpesvirus		ACG-resistant herpesvirus	
		virus titer, lg $TCID_{50}$/ml	difference from control, lg $TCID_{50}$/ml	virus titer, lg $TCID_{50}$/ml	difference from control, lg $TCID_{50}$/ml
III	30	4.0	2.1	3.8	2.4
	15	4.9	1.2	4.5	1.7
	7.5	5.7	0.4	5.6	0.6
ACG	0.4	4.8	1.3	6.2	0
	0.2	5.6	0.5	6.2	0
ACG/III	0.4/15	0	6.1	4.0	2.2
	0.2/7.5	3.0	3.1	5.1	1.1
Control virus	0	6.1	–	6.2	–

RESULTS AND DISCUSSION

Cell culture toxicity studies showed that the MTC of compound III was 50 μg/ml. A 50% level of suppression of DNA synthesis was obtained in uninfected Vero cell cultures (suppression of ^{3}H-thymidine uptake) at 92 μg/ml, which is evidence that III has low toxicity (the value for ACG is 2 μg/ml [14], while the MTC is greater than 1600 μg/ml).

The antiviral activity of III was determined at the MTC and lower concentrations (Table 1). These results show that compound III has a marked ability to suppress herpes and vaccinia virus multiplication in cell cultures.

A decrease in the infective titer of herpesvirus by 1.6 lg cfu/ml or more in the concentration range 50 – 6 μg/ml was demonstrated. Calculated EC_{50} values for III were 0.62 μg/ml for herpesvirus and 5.15 μg/ml for vaccinia virus.

In addition, studies in HEK showed that III suppressed the development of the CPE of adenovirus by 75% at the MTC and ½MTC (EC_{50} = 48.5 (79.5 – 29.6) μg/ml).

Comparison of the antiviral actions of III and ACG against herpesviruses was performed using Vero cells (Table 2). ACG had no inhibitory activity against the resistant variant of the virus. At the same time, compound III at concentrations of 30 and 15 μg/ml suppressed the multiplication of this variant by 2.4 and 1.7 lg $TCID_{50}$ respectively.

Use of low concentrations of III and ACG led to a marked synergistic action in relation to herpesvirus. Thus, the combination of 0.4 μg/ml acycloguanosine and 15 μg/ml III produced 100% arrest ofvirus reproduction. At the same time, individual use of substances at these concentrations decreased the virus titer by no more than 1.3 log $TCID_{50}$/ml. Lower concentrations of both substances, inactive when used alone, i.e., 0.2 μg/ml ACG and 7.5 μg/ml compound III, as

TABLE 3. Efficacies of Ethyl(3-ethyladamant-1-yl)carbamate (III), ACG, and their Combination in Experimental Herpes Meningoencephalitis in White Mice

Compound	Dose, mg/kg	Indicator of efficacy					
		herpesvirus			ACG-resistant herpesvirus		
		number of animals (died/total)	Mortality, ± S_x, %	ED_{50} (I_{95}), mg/kg	number of animals (died/total)	Mortality, ± S_x, %	ED_{50} (I_{95}), mg/kg
Intracerebral infection							
III	200	8/13	61.5 ± 13.4	167.0 (287.5 – 97.0)	6/23	26.1 ± 9.2	79.6 (109.2 – 58.0)
	100	5/13	38.5 ± 13.5		4/16	25.0 ± 10.8	
	50	14/15	93.3 ± 6.4		9/16	56.2 ± 12.4	
	25	15/15	100.0		16/16	100.0	
	0	9/9	100.0		6/6	100.0	
ACG	50	3/20	15.0 ± 8.0	<< 50	17/17	100.0	> 50
	0	8/8	100.0		20/21	95.2 ± 4.7	
I.p. infection							
III	100	2/35	3.9 ± 3.3	< 0.1	2/21	9.5 ± 6.4	< 0.1
	10	7/29	7.9 ± 5.0		5/25	20.0 ± 8.0	
	1	5/24	20.8 ± 8.3À		9/34	26.5 ± 7.6	
	0.1	7/21	33.3 ± 10.3		9/25	36.0 ± 9.6	
	0	33/36	91.7 ± 4.6		39/42	92.8 ± 4.0	
ACG	10	0/12	0	0.13 (0.4 – 0.04)	-	-	-
	1	1/12	8.3 ± 2.6		-	-	
	0.1	4/7	57.1 ± 18.7		-	-	
	0	11/12	91.7 ± 8.0		-	-	
ACG/III	1/0.1	0/3	0	~ 0.013/-	-	-	-
	0.1/0.1	0/4	0		-	-	
	0.01/0.1	3/4	3		-	-	

"-" – not determined

well as 0.1 μg/ml ACG and 3.75 μg/ml compound III, suppressed virus multiplication by 3.1 and 1.7 lg $TCID_{50}$/ml respectively.

The level of suppression of the ACG-resistant herpesvirus variant in the presence of these combinations of ACG and compound III was no greater than the antiviral effect of III used alone.

The antiviral properties of compound III demonstrated in cell cultures were confirmed in experimental herpesvirus infection using different routes of infection. The results (Table 3) provide evidence that compound III can reduce the mortality of mice with infections due to viruses including the ACG-resistant variant by amounts ranging from over 50% to 85%.

The mean effective dose of III was 79.6 mg/kg in infection with the ACG-resistant variant and 167 mg/kg on infection with the initiation herpes variant. Solvent (10% tween-80 in isotonic saline, virus control group, dose 0 in table 3) had no effect on the course of infection.

Compound III had greater efficacy in animals infected via the i.p. route. As shown by the results presented in Table 3, the decrease in mortality from the control level due to III ranged from over 50% to 85%. The mean effective dose of the study compound in this case was less than 0.1 mg/kg, i.e., >70 times smaller than in intracerebral infection of animals.

The data obtained here provide evidence of the significant efficacy of compound III in experimental herpesvirus infection and confirm its activity against the ACG-resistant herpesvirus variant as seen in cell culture experiments.

The decrease in mortality in infected animals given ACG in combination with III reached 19.4 – 94.4% compared with the control level. In these conditions, the decrease in mortality on treatment of animals with 1 mg/kg acyclovir combined with 0.1 mg/kg III was increased in comparison with the group of animals given acyclovir 1 mg/kg alone, from 77.7% to 94.4%, while the increase in the group given 0.1 mg/kg acyclovir was from 37.3% to 94.4%.

Thus, these studies demonstrated a high level of antiviral activity for compound III against herpes simplex and vaccinia viruses, and also against adenovirus. Activity against herpesvirus was demonstrated in experiments on various cell cultures and different virus strains, including an ACG-resistant variant. Combined administration of III and ACG increased the inhibition of herpesvirus reproduction in cell cultures and decreased mortality in laboratory animals with experimental herpesvirus neuroinfection as compared with use of each substance alone.

compound III was a low-toxicity compound. Its LD_{50} for single intragastric doses was 1831.8 (1754.1-1912.9) mg/kg. Thus, the LD_{50}/ED_{50} ratio (k) was 11.0 – 23.0 in infections induced by intracerebral administration of herpesvirus and more than 18,318 in infection following i.p. administration of virus.

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