PLANT ANTIVIRAL AGENTS. III[1]. ISOLATION OF ALKALOIDS FROM *CLIVIA MINIATA* REGEL (AMARYLLIDACEAE)

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ABSTRACT.—Lycorine (**1**), one of the main alkaloids of the Amaryllidaceae family, was found to be responsible for the pronounced antiviral activity of the crude extracts from the roots and leaves of *Clivia miniata* Regel. It was shown that the inhibitory activity of lycorine on the cytopathogenic effect caused by a DNA and several RNA viruses on VERO cells was not virucidal. Poliomyelitis virus inhibition occurred at lycorine concentrations as low as 1 μg/ml, whereas concentrations exceeding 25 μg/ml were found to be cytotoxic.
Clivimine (**2**), clivonine (**3**) and cliviamartine (**4**) were also isolated and characterized, but these alkaloids exhibited no antiviral properties.

In an antiviral screening program with crude extracts of antimicrobially active higher plants, it was found that some of the Amaryllidaceae family exhibited a pronounced antiviral effect against several of the test viruses (1, 2). The observation that *Clivia miniata* (Hook) Regel was one of the most active antiviral Amaryllidaceae plants, being active in tissue cultures against poliomyelitis, Coxsackie, Semliki forest, measles and herpes simplex viruses prompted us to investigate this plant for its active constituents (2). We now report the results of this study.

EXPERIMENTAL

PLANT MATERIAL.—The plants were obtained from the National Botanical Garden of Belgium. Voucher specimens, prepared under the direction of Mr. E. Lammens, have been deposited at the herbarium of the National Botanical Garden of Belgium.

CELL CULTURES, TISSUE CULTURE MEDIUM AND TEST VIRUSES.—Cell cultures, culture conditions, tissue culture media and test viruses were used as previously described (2). Viral titers were estimated by the method of plaque formation on VERO monolayers as described in the previous paper (2). The viral titers were respectively 10^8 PFU/ml for poliomyelitis virus, 10^7 PFU/ml for Coxsackie virus, 10^6 PFU/ml for measles virus, 10^6 TCD_{50}/ml for herpes virus and 10^7 TCD_{50}/ml for Semliki forest virus.

EQUIPMENT.—Melting points were determined on a Büchi SMP-20 apparatus and were corrected. Ultraviolet spectra were obtained on a Beckman Acta C III UV-instrument, and infra-red spectra were recorded on a Beckman Acculab TM 4 instrument. ^1H and ^{13}C-nmr spectra were recorded on a Jeol JNM-PFT 100 spectrometer operating at 100 MHz and 25.15 MHz, respectively. Chemical shifts (δ) are given in ppm values from TMS as internal standard. Multiplicities in an off-resonance experiment are given in parentheses.

Mass spectra were determined on a Jeol 01 SG II double-focusing instrument coupled to a JEC-6 spectrum computer and operated at 70 eV. The relative intensities of the peaks are given in parentheses.

Optical rotations were determined with a Perkin-Elmer 241-MC polarimeter. For tlc, Merck precoated silica gel F-254 plates in chloroform-methanol-diethylamine (92:3:5) were used. Spots were located by uv illumination and by spraying with Dragendorff and iodoplatinate reagents.

For analytical-scale hplc, the model Hupe and Busch 1010B solvent delivery system from Hewlett Packard and the model U6K injector with 2 ml loop from Waters Associates were used. Detection at 254 nm was made with a type 1030 B variable uv-detector from Schoeffel Instrument Corp. For preparative semi-hplc a Jobin Yvon Chromatospac Prep apparatus, equipped with a flow-through microcell (2850 psi and an optical path of 10 mm) from Instrumentation Specialities Company (ISCO) and a model 450 variable wavelength detector from Waters Associates were used.

CHEMICAL FRACTIONATION.—Fresh *Clivia miniata* leaves (40 g) were cut into small pieces, macerated in an aqueous ammonia solution (100 ml, 0.5%) for 1 hr and then filtered off.

[1]For the previous paper in this series see reference 2.

The marc was percolated with an ammonia solution and extracted until the marc was exhausted (*ca.* 150 ml, 5%). The filtrate and percolate were combined and extracted with chloroform (4 x 60 ml). The combined chloroform layers were concentrated to *ca.* 50 ml under water pump pressure at about 40°. The residue was extracted with hydrochloric acid (5 x 50 ml, 0.5 N) and the resulting aqueous acidic layers were combined and concentrated to *ca.* 5 ml to give fraction 1. The other layers, *viz.* the ammoniacal aqueous layer and the organic layer, were also concentrated to *ca.* 5 ml to afford, respectively, fraction 2 and fraction 3. The three fractions were tested for their antiviral activity (table 1).

ION EXCHANGE COLUMN CHROMATOGRAPHY.—Fresh cut *Clivia miniata* leaves (50 g) were macerated in aqueous ethanol (150 ml, 80%) for 2 hr and then filtered off. The marc was percolated with the same solvent until the extraction was complete (*ca.* 350 ml). The combined filtrate and percolate was concentrated to a thick residue under water pump pressure at about 40°. An aliquot of this concentrated ethanolic extract (1 ml) was submitted to column chromatography on two glass columns respectively packed with the weakly basic anion exchangers Amberlite IR-45 (20–50 mesh) and Cellex E (20–50 mesh), both in the hydroxide form.

Columns of 5 cm height and 10 mm internal diameter were used; 20 ml fractions were collected. Elution with distilled water (40 ml) afforded fractions 4 and 5. Elution of the resins with hydrochloric acid (40 ml, 1 N) gave fractions 6 and 7. An analogous extract was chromatographed on two glass columns packed, respectively, with the weakly acidic cation exhanger Amberlite IRC-50 (32–45 mesh) and the strongly acidic cation exchanger Dowex 50W (100–200 mesh), both in the hydrogen form. Elution with distilled water (40 ml) afforded fractions 8 and 9; the resins, when eluted with aqueous ammonia (40 ml, 1.5 N), gave fractions 10 and 11. All these fractions were tested for antiviral activity (table 1).

ISOLATION OF LYCORINE (**1**) AND CLIVIMINE (**2**).—A concentrated ethanolic extract prepared from fresh *Clivia miniata* leaves (3 kg) according to the procedure described earlier was acidified with sulfuric acid (100 ml, 0.1 N), filtered, and washed with diethylether (3 x 100 ml) in order to remove the chlorophyll.

Extraction of the aqueous layer with chloroform (3 x 100 ml) transferred the neutral material but also transferred some alkaloids to the organic layer because Amaryllidaceae alkaloids not containing free -OH or -NH-groups usually form chloroform soluble salts (3). Therefore, the combined chloroform fractions were extracted with hydrochloric acid (18 x 50 ml, 2 N). Careful basification of the combined acidic aqueous fractions with aqueous ammonia solution (1.5 N) yielded a precipitate which could not completely be dissolved by addition of an equal volume of chloroform.

The precipitate was removed by centrifugation, washed twice with chloroform (50 ml) and dried *in vacuo* yielding 5.2 g (0.17%, wet weight of the leaves) of crude chloroform-insoluble alkaloids. The solid was dissolved in hot aqueous acetic acid (100 ml, 10%) and reprecipitated by addition of an ammonia solution (10%).

The insoluble basic material was removed by filtration, dissolved in a minimal amount of hot methanol and set aside in a refrigerator overnight; 1.3 g (0.043%) of lycorine was obtained as white prisms. The identity was confirmed by a direct comparison with an authentic sample of lycorine (mmp, $[\alpha]^{20}D$, tlc behavior, uv (EtOH), ir (KBr), pmr and mass spectra). ^{13}C nmr (25.15 MHz, d_6-DMSO) δ 145.8 and 145.3 (s, C-9 and C-10), 141.8 (s, C-3a), 129.9 and 129.7 (s, C-7a and C-11a), 118.6 (d, C-3), 107.2 (d, C-8), 105.2 (d, C-11), 100.7 (t, C-12), 71.8 (d, C-1), 70.3 (d, C-2), 60.9 (d, C-11c), 56.8 (t, C-7), 53.4 (t, C-5), around 40.3 (C-11b obscured by solvent peaks), 28.3 (t, C-4).

The chloroform filtrate was separated from the aqueous ammoniacal filtrate and the latter was extracted with chloroform (2 x 100 ml). The organic layers were combined, acidified with sulfuric acid (50 ml, 0.1 N), washed twice with chloroform (25 ml), and rebasified with an aqueous ammonia solution (1.5 N, pH 9.0).

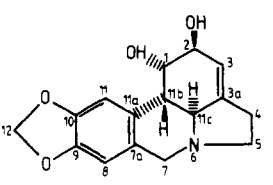
Extraction of the basic aqueous solution with chloroform (4 x 50 ml) and evaporation of the combined chloroform fractions, upon cooling, yielded a precipitate. The precipitate was collected and crystallized from ethanol after standing in a refrigerator overnight; 0.254 g (0.0085%) of clivimine was obtained as white needles. The identity was confirmed by a direct comparison with an authentic sample of clivimine (mmp, $[\alpha]^{20}D$, tlc behavior, uv (EtOH), ir (KBr), pmr and mass spectra).

The chloroform filtrate, named residual alkaloidal fraction, exhibited at least ten spots on tlc in several solvent systems and visualization with Dragendorff- and iodoplatinate reagents.

ISOLATION OF CLIVONINE (**3**) AND CLIVIAMARTINE (**4**).—The residual alkaloid fraction, prepared as above from *Clivia miniata* leaves (3 kg), was evaporated *in vacuo* below 40° to a thick residue, which was dissolved in a mixture of methanol and chloroform (1:1). This solution was chromatographed on a preparative semi-hplc Merck Lobar type B column of 31 cm height and 2.5 cm internal diameter, packed with Lichroprep Si60 (40–63 μ diameter).

The column was developed with a gradient of diethylether-methanol-diethylamine (99:1:0.5) changing to (90:10:0.5) at a flow rate of 1.1 ml/min and a pressure of about 10 kg/cm^2. After elution of a void volume of 150 ml, collection of about 15 ml (300 drops) fractions was begun; a total of 400 fractions was obtained. Monitoring was done with a uv-detector at 254 nm and by tlc analysis of every second fraction.

The second band, corresponding to fractions 47–77, yielded upon concentration *in vacuo* below 40° a precipitate which was filtered off and crystallized from a mixture of dichloromethane-methanol; 40.0 mg (0.0013%) of clivonine was obtained as colorless needles. The compound was identified by comparison of its mp, $[\alpha]^{23}D$, tlc behaviour uv, ir, pmr and mass spectral data with those reported in the literature. It gave ^{13}C-nmr (25.15 MHz, $CDCl_3$),



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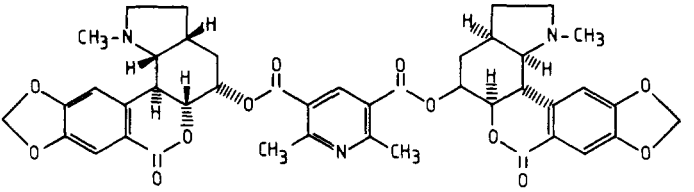

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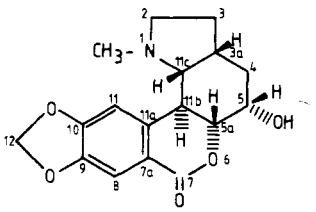

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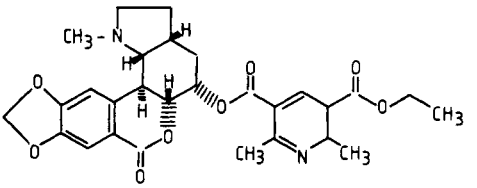

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164.7 (s, C-7), 152.8 (s, C-10), 146.5 (s, C-9), 141.1 (s, C-11a), 119.3 (s, C-7a), 109.5 (d, C-8), 107.7 (d, C-11), 102.5 (t, C-12), 82.7 (d, C-5a), 70.4 (d, C-11c), 68.5 (d, C-5), 53.7 (t, C-2), 46.1 (q, C-1), 34.1 (d, C-3a), 33.8 (s, C-11b), 32.0 (t, C-3), 29.4 (t, C-4).

The first band, corresponding to fractions 30–44 yielded upon evaporation *in vacuo* below 40° an amorphous solid, which was crystallized from acetone to give 6.0 mg (0.0002%) of cliviamartine as colorless needles, mp 170–172° (decomp.); tlc Rf 0.74; uv λmax (MeOH) 271 nm (log ε 3.99), 307 (log ε 3.76); ir νmax (KBr) 1720 cm^{-1} 1220, 1180 (estercarbonyl, aryl conjugated δ-lactone), 1620, 1590, 1510, 1480, 1130, 1040, 926, 860 (aromatic methylenedioxy-group), 1260 (alicyclic-CN); ^{1}H-nmr (100 MHz, $CDCl_3$) δ 8.67 (s, 1H, H-4'), 7.84 (s, 1H, H-11), 7.51 (s, 1H, H-8), 6.05 (s, 2H, H-12), 5.62 (d, 1H, H-5), 4.33 (q, 2H, *J* 7 cps, O-CH_2-CH_3), 4.28 (dd, 1H, H-5a), 3.33–1.65 (m, 9H, H-11b, H-11c, H-2, H-3, H-4 and H-3a), 2.86 and 2.85 (s, 6H, 2' and 6'-CH_3); 2.58 (s, 3H, N-CH_3), 1.32 (t; 3H, *J* 7 cps, O-CH_2-CH_3); ms (70 eV) *m/z* (rel. int.) 522 (M^+; 11), 477 (M^+ -45, 3.5), 316 (9.5), 299 (13.5), 206 (5.1), 126 (8.5), 96 (51.5), 83 (100) and 82 (18.5).

The sixth band, corresponding to fractions 120–140, yielded upon concentration *in vacuo* below 40° a white solid, which, when crystallized from acetone, gave 45.0 mg of clivimine as white needles.

The eighth band, corresponding to fractions 222–260, yielded upon concentration *in vacuo* below 40° a white solid, which could be easily crystallized from methanol to yield 18.0 mg of lycorine.

TOXICITY AND ANTIVIRAL TESTING.—Each of the fractions obtained in the chemical fractionation and ion-exchange chromatography schemes (fractions 1–11) were concentrated *in vacuo* at a temperature below 40° to *ca.* 5 ml for the aqueous layers and to dryness for the organic layers. The aqueous fractions were diluted with tissue culture medium M-2, adjusted with sodium hydroxide (1 N) or hydrochloric acid (1 N) to pH 7.2 and to 10 ml.

The residues of the organic fractions were dissolved in dimethylsulfoxide (1 ml), diluted with tissue culture medium M-2 and adjusted to pH 7.2 and 10 ml.

The diluted extracts were filtered off on a Whatman GF-2 paper disc, sterilized by filtration on a Millipore membrane filter (0.2 μ) and tested for toxicity and antiviral activity on confluent cultures of VERO-cells in microtiter plates according to the method described in a previous paper (2).

The isolated alkaloids were dissolved in hydrochloric acid (0.2 ml, 0.1 N) and diluted with tissue culture medium M-2 in order to obtain concentrations of 1 mg/ml. Serial twofold dilutions in M-2 medium were used for toxicity and antiviral activity.

CYTOPATHOGENIC EFFECT INHIBITION TEST OF LYCORINE ON POLIOMYELITIS VIRUS.—In the CPE inhibition test, VERO-cells were grown in microtiter plates (2 days prior to assay), as previously described (2). For virus assay, serial tenfold dilutions (50 μl) of the poliovirus were used to inoculate pre-emptied confluent monolayers.

The virus was allowed to absorb for 90 min at 37° after which lycorine solutions of appropriate dilutions were added to the cultures. It has been reported that, under these conditions, polio virus biosynthesis starts before the addition of lycorine, so the real antiviral effect of the compound can be tested (4). The dilutions were prepared from a stock solution of lycorine (1 mg/ml) in tissue culture medium M-2. Each lycorine dilution was put into 4 different wells. Virus control (normal polio virus titration), tissue culture control (unaffected VERO-cells in M-2 medium), and an uninfected control (VERO-cells in lycorine dilutions) were included in each test to determine the toxicity of lycorine at each dilution. All these experiments were done in triplicate. All cultures were incubated at 37° and were examined daily for CPE or for toxic effects. Toxic doses of lycorine were considered to be the dilutions which caused destruction or degeneration of the monolayer.

The antiviral activity of the compound was determined when control cultures (infected without lycorine) showed complete CPE, i.e., complete destruction of the monolayer. The virus titers were calculated and expressed as TCD_{50} per ml.

LYCORINE TOXICITY ON VERO CELLS.—VERO cells were grown in complete M-2 medium supplemented with different concentrations of lycorine. Growth curves were determined in serial cell transfers with and without lycorine over a three month period. For lycorine concentrations higher than 10 μg/ml, viability tests (trypan blue exclusion) were done every day.

EXTRACELLULAR VIRUS INACTIVATION.—Lycorine was dissolved at a concentration of 1 mg/ml and dilutions of 50 and 5 μg/ml were made with complete tissue culture medium M-2. For each of the viruses tested, an inoculum of 1 ml was added in duplicate to screw-capped bottles containing 9 ml of lycorine solutions of 50 μg/ml or 5 μg/ml.

After initial shaking of these mixtures, the suspensions were incubated at 37° for 96 hours. Virus control contained no lycorine. For virus infectivity, 1 ml portions of each suspension were taken at regular intervals till the 4th day after incubation. Titer of residual virus was determined after serial tenfold dilutions in M-2 medium. The cell cultures were inspected microscopically every day for a period of five days. CPE was evaluated and virus titers were expressed as TCD_{50}/ml.

RESULTS AND DISCUSSION

A search through the literature showed that all biological properties, as well as toxic effects, of the Amaryllidaceae plants were due to the presence of alkaloids (5). Therefore, it was decided to separate the crude extracts prepared from the roots and the leaves of *Clivia miniata* into alkaloid and non-alkaloid portions in order to determine if the antiviral activity was due to alkaloids or to other constituents of this plant. In a preliminary experiment, the constituents were fractionated into gross chemical classes, and the distribution of the antiviral activity was followed by the tissue culture assay on the various residues obtained.

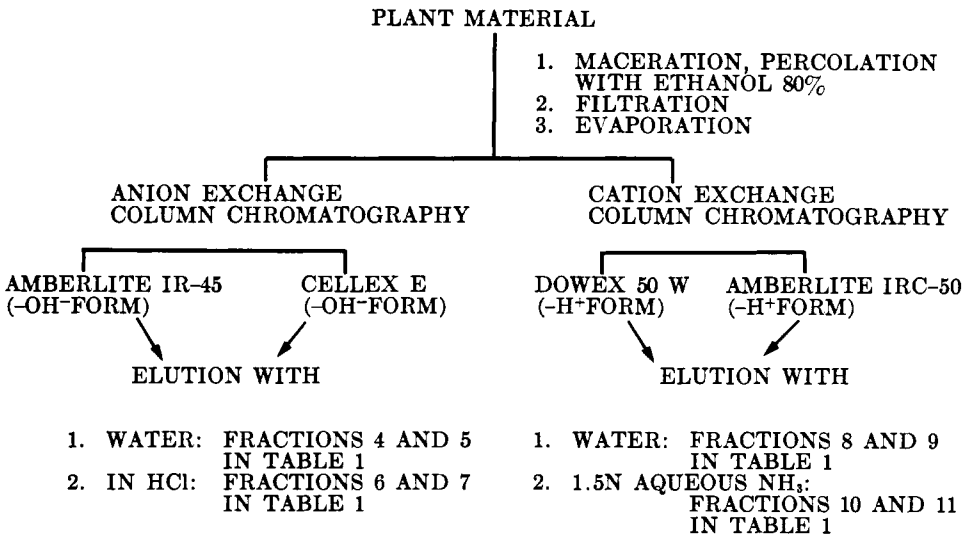
To test for alkaloids, the different fractions were chromatographed on thin layer and sprayed with Dragendorff's reagent. The results of the antiviral screen showed that only the alkaloid containing fraction exhibited an antiviral effect against Semliki forest, herpes simplex and poliomyelitis viruses (table 1).

TABLE 1. Antiviral activity of fractions from *Clivia miniata* Regel in tissue culture expressed as the reduction factor of the viral titer (2).

Fraction Number	Sample	Antiviral activity against	
		Semliki forest Herpes	Polio
1	Aqueous acidic phase containing alkaloids	1000	10,000
2	Aqueous phase containing acids, glycosides, sugars	1	10
3	Chloroform phase containing fats and waxes	1	1
4	Aqueous eluate of the anion exchanger Amberlite IR 45	1000	1000
5	Aqueous eluate of the anion exchanger Cellex E	1000	10,000
6	Acidic eluate of the anion exchanger Amberlite IR 45	1	1
7	Acidic eluate of the anion exchanger Cellex E	1	1
8	Aqueous eluate of the cation exchanger Dowex 50 W	1	10
9	Aqueous eluate of the cation exchanger Amberlite IRC 50	10	1000
10	Ammoniacal eluate of the cation exchanger Dowex 50 W	100	1000
11	Ammoniacal eluate of the cation exchanger Amberlite IRC 50	1000	1000

This was confirmed in a second experiment in which the alkaloids were separated from other compounds by means of ion exchange column chromatography according to the procedure outlined in scheme 1.

Tlc analysis showed that alkaloids were present only in the ammoniacal eluates from the cationic exchange resins and in the aqueous fractions from the anionic ex-



SCHEME 1. Ion exchange chromatography of the crude extract of *Clivia miniata*.

change resins. As shown by the results in table 1, only the alkaloid containing eluates possess significant antiviral properties. These findings strongly indicate that the compounds responsible for the antiviral activity of extracts of *Clivia miniata* must be alkaloids.

Although no less than 150 alkaloids containing six distinct fundamental ring systems have been identified in the Amaryllidaceae family (6), only six alkaloids were isolated from *Clivia miniata* (7–10). Besides lycorine, all six alkaloids are derived from the (2)-benzopyrano [3,4-g] indole nucleus and, consequently, belong to the lycorenine group. A large scale extraction of the alkaloids from the leaves or the roots of *Clivia miniata* was carried out according to the slightly modified procedures described by Briggs *et al.* (9) and Boit *et al.* (10). Lycorine (**1**) and clivimine (**2**), the two major alkaloidal constituents of this plant, were isolated in 0.043% and 0.0085% yield (based on the wet weight of the leaves), respectively. These compounds were identified by direct comparison with authentic samples of **1** and **2**.

After the isolation of these two alkaloids, the chloroform filtrate, named residual alkaloidal fraction, exhibited at least ten spots on tlc in several solvent systems and after visualization with Dragendorff and iodoplatinate reagents. Following several unsuccessful attempts to resolve this mixture by means of conventional absorption chromatography and preparative tlc, the material was subjected to hplc analysis.

Analytical-scale hplc on a 5μ Lichrosorb Si60 prepacked column (Merck) with diethylamine-methanol-diethylether mixtures as mobile phase and monitoring with a variable wavelength uv-detector operating at 254 nm resulted in the resolution of the residual alkaloidal fraction into two major and about eleven minor compounds. Fig. 1 shows a typical elution pattern of the different alkaloids with diethylamine-methanol-diethylether (0.5:3:97) at a flow rate of 1.1 ml/min and at a pressure of 40 kg/cm^2. From the retention times of 7.6 min for clivimine and 9.0 min for lycorine, it can be concluded that one of the major compounds present in the remaining alkaloid fraction was clivimine (**2**) and one of the minor compounds was lycorine (**1**). In an attempt to prepare sufficient material for structural analysis and antiviral testing, preparative semi-hplc was performed on a Lichroprep Si60 prepacked column (Merck Lobar type B) eluted with increasing concentrations of methanol in diethylether and diethylamine.

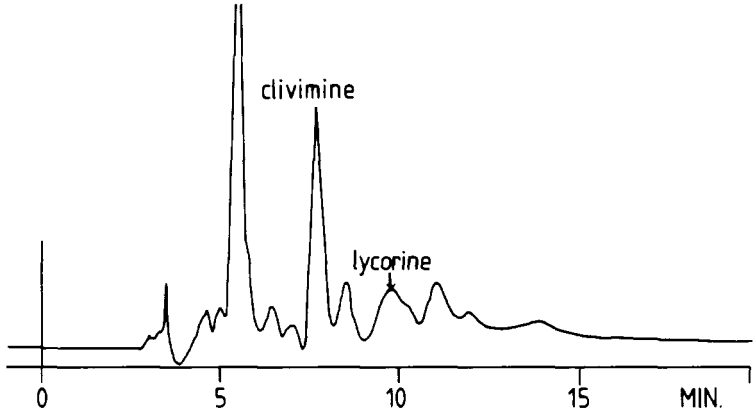


FIGURE 1. Analytical-scale hplc of the residual alkaloidal fraction of *Clivia miniata*.

Fig. 2 shows the preparative separation of the alkaloids, which were eluted at a flow rate of 1.1 ml/min and at a pressure of ±10 kg/cm². Separation of the components was monitored at 254 nm, allowing the collection of ten primary fractions labelled 1–10 according to their order of elution. Concentration of fractions 1, 2, 6 and 8 resulted in the recovery of four white solids, which could be readily crystallized.

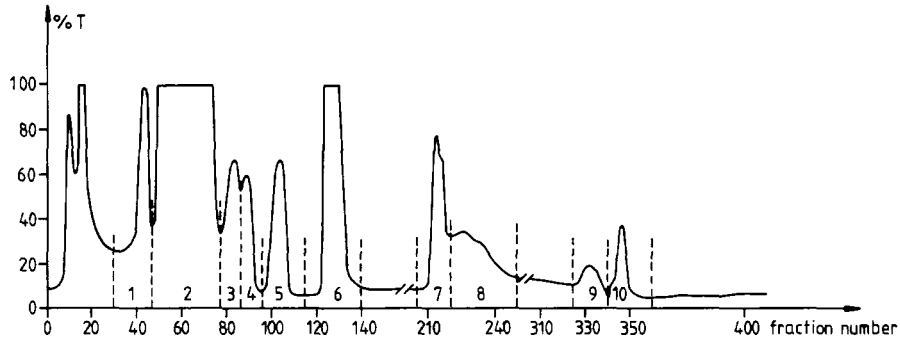


FIGURE 2. Preparative semi-hplc of the residual alkaloidal fraction of *Clivia miniata*

The other fractions did not crystallize and, since they had no antiviral activity, they were not examined further. Fraction 2 was identified as clivonine (**3**) by comparison of mp, uv, ir, ^{1}H- and ^{13}C-nmr, mass spectral and optical rotation values with those reported in the literature (9, 12–17).

Fraction 1 yielded an unknown compound, **4**, which was assigned the molecular formula $C_{28}H_{30}N_2O_8$ on the basis of high resolution mass spectrometry. The uv [λmax 271 (log ε 3.99) and 307 (log ε 3.76)] and ir spectra (1720, aryl-conjugated δ-lactone, 1620, 1590, 1510, 1480, 1130, 1040, 926, 860, aromatic methylenedioxy-group, 1260 alicyclic-CN) as well as the general appearance of the pmr spectrum all were in close agreement with those of clivimine (**2**).

Noteworthy in the proton-nmr spectrum, however, were a two-proton quadruplet at δ 4.33 and a three-proton triplet at δ 1.32 corresponding to an ethyl-ester function. Aside from the one-proton singlet at low field δ 8.67, which was ascribed to the signal from the C(4) hydrogen on the 2.6-lutidine-3.5 di-ester residue, and the three-proton singlets at δ 2.86 and 2.85, attributed to the methyl resonances of the lutidine system, the remaining features of the spectrum were similar to the spectrum of *O*-acetyl-clivonine (15).

Information on the stereochemical relationship of the different rings was obtained from the close resemblance with the spectra of clivonine and clivimine (14).

The mass spectral data provided an additional proof of the structure. The fragmentation pattern is given in fig. 3 and was also very similar to those of **2** and **3**. The most intense peak in the mass-spectrum of **4** occurred at *m/z* 83. The structures ascribed to this ion and also to other prominent ions at *m/z* 82, 96 and 126 are given in fig. 3.

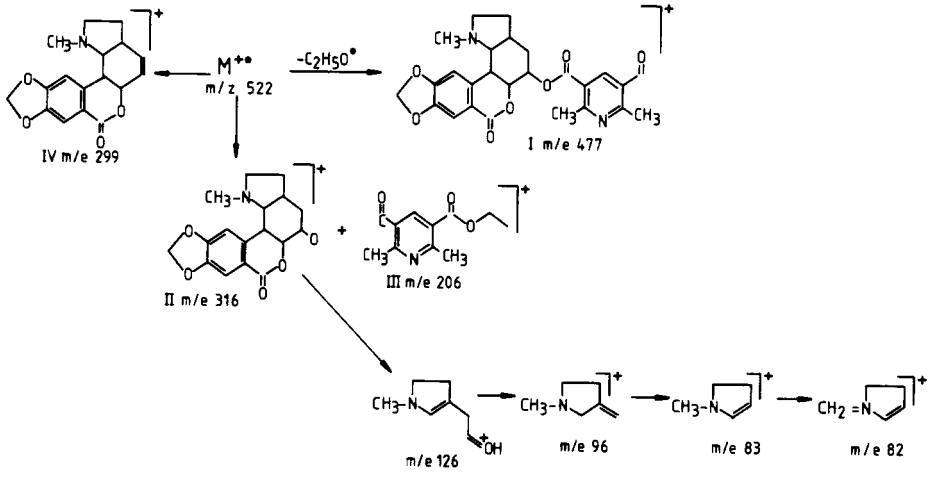


FIGURE 3. Diagnostic mass fragmentations of cliviamartine.

Cleavage of the C–5–OR bond accounts for the peak at *m/z* 477, while the fragment ion detected at *m/z* 299 can be explained by a McLafferty rearrangement process out of the molecular ion M+. The fragment ion at *m/z* 206 is attributed to the 2,6-lutidine-3,5-dicarboxylic ester residue. The peaks at *m/z* 126, 96, 83 and 82 are also present in the mass spectrum of clivonine.

From the above results it would appear that the isolate has structure **4** and should, consequently, be named 3-carboxyclivoninyl-5-carboxyethyl-2,6-lutidine.

During the course of this work, the same product has independently been isolated from the same plant by Döpke, who named it cliviamartine (11).

TABLE 2. Antiviral activity of some isolated *Clivia miniata* alkaloids in VERO–cells expressed as the reduction factor of the viral titer (2).

	Dose in μg/ml	Herpes simplex	Semliki forest	Polio	Measles
Lycorine	100	T	T	T	T
	50	T	T	T	T
	20	1000	10,000	10,000	1000
	10	1000	1000	1000	1000
	5	1000	1000	100	100
	2.5	100	100	100	100
Clivimine	100	1	10	10	1
	50	1	1	1	1
	20	1	1	1	1
	10	1	1	1	1
	5	1	1	1	1
	2.5	1	1	1	1
Clivonine	100	10	10	10	1
	50	1	1	1	1
Cliviamartine	100	10	10	10	1
	50	10	1	1	1

T=Toxic.

Concentration of fractions 6 and 8 resulted in the crystallization of clivimine (2) and lycorine (1), respectively. The antiviral properties of the isolated alkaloids are given in table 2. Evaluation of VERO cells growth patterns in the presence of different lycorine concentrations showed that the growth was already altered at 0.2 μg/ml of lycorine. At 1 μg/ml, cell generation time was doubled; and at 10 μg/ml, cells did not grow. At concentrations of 25 μg/ml or more, cell degeneration quickly occurred; and at concentrations above 50 μg/ml, cells died after 24 hr at 37° in complete medium (T = toxic). In contrast to the pronounced antiviral effect of lycorine on the four viruses tested, the other three alkaloids were devoid of significant antiviral potency. Therefore, only lycorine was further investigated.

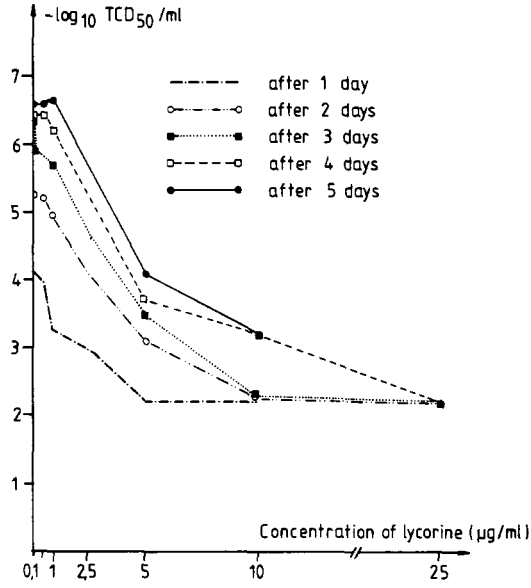


FIGURE 4. Dose-response curve of the lycorine-activity on the CPE of poliovirus on vero cells.

Fig. 5 shows the influence of different lycorine concentrations on the CPE caused by poliovirus on monolayers of VERO-cells expressed as a dose-response curve. The viral titer, expressed as the negative logarithm of the 50 percent tissue culture infective dose (TCD_{50}) per ml, was determined as a function of different lycorine concentrations, which were tested in comparison with polio controls without lycorine. It is clear from these results that in concentrations of 2.5 μg/ml or more the viral CPE of poliovirus on VERO-cells was strongly inhibited.

On the other hand, concentrations lower than 1 μg/ml had no effect on the viral titer, whereas in the presence of 1 μg/ml lycorine the viral CPE was only reduced during the first 4 days after infection. After that the viral titer reached the same maximum as the control without lycorine. These experiments demonstrate that there exists a direct relationship between the viral CPE and the concentration of lycorine used. However non-infected cell-cultures were strongly damaged by lycorine in concentrations of 25 μg/ml or more.

In the presence of 10 μg/ml lycorine, cells did not grow but were still viable.

On the contrary, with concentrations of less than 1 μg/ml, no inhibition of the viral CPE could be observed.

Consequently, lycorine has an effective range (maximum non-toxic dose to minimum effective dose) of 10 to 1 μg/ml on the viruses tested. That is, the therapeutic index based on the viability of the cells has been determined as about 10.

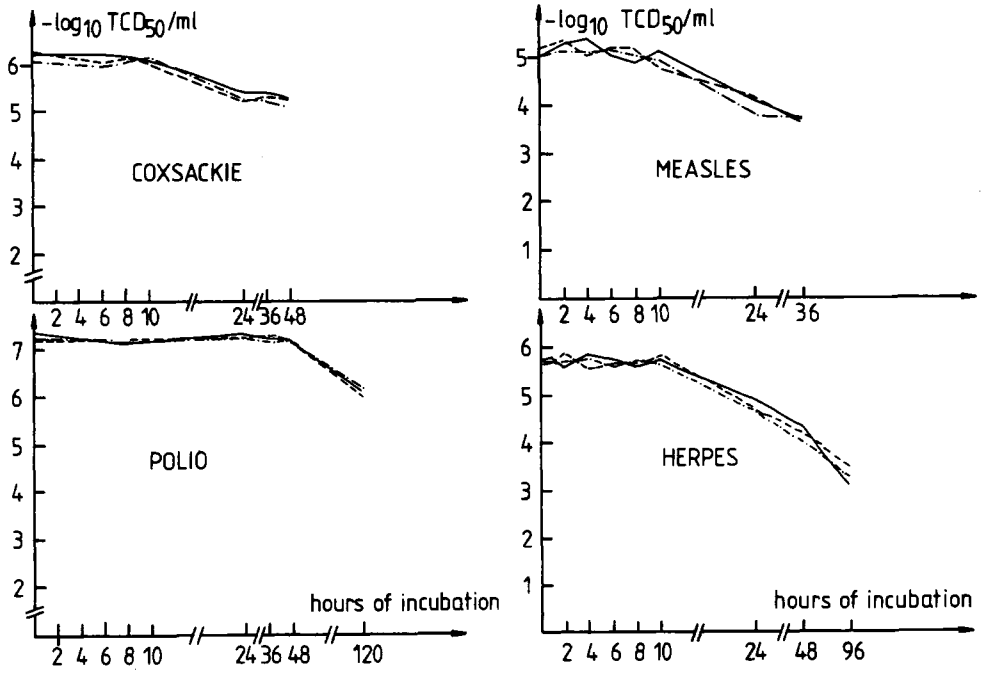


FIGURE 5. Extracellular activity of lycorine against different viruses ———control, — — — lycorine 50 μg/ml, — — — lycorine 5 μg/ml.

Experiments were carried out to determine whether lycorine has any direct action on the extracellular virus. Fig. 5 shows a comparison of the residual infectivity after incubation of several viruses in the presence of different concentrations of lycorine versus a control without the alkaloid. The results clearly indicate that lycorine did not inactivate the viruses directly, not even at concentrations as high as 50 μg/ml, a dose which is sufficiently high to exert cytotoxic activity. The decrease of the viral titers, which was observed especially for measles and herpes viruses, was not due to the presence of lycorine because the viral titers of the controls of these rather unstable viruses were decreased to the same extent. From these experiments, it can be concluded that lycorine showed no extracellular inactivation of the tested viruses.

The inhibition of the viral CPE on monolayers of VERO-cells should, consequently, be explained by another mechanism. Therefore, the influence of lycorine on the biosynthesis of poliomyelitis virus was further investigated. The results of this study will be published later.

In conclusion, our studies showed that the antiviral activity seen in screening studies of *Clivia miniata* Regel was exclusively due to the alkaloid lycorine and that this activity was not due to extracellular inactivation of the viruses.

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