Synthesis, DNA-binding, and Antiviral Activity of Certain Pyrazolo[3,4-*d*]pyrimidine Derivatives

E. R. El-Bendary*[a] and F. A. Badria[b]

Departments of [a] Medicinal Chemistry and [b] Pharmacognosy, Faculty of Pharmacy, University of Mansoura, Mansoura, 35516 Egypt

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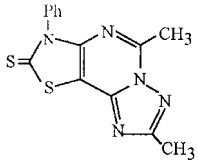
Summary

Some new pyrazolo[3,4-*d*]pyrimidine derivatives have been prepared and tested for their antiviral and DNA-binding activities. Compounds **4**, **5**, and **6** showed high binding affinity to DNA at concentrations of 19, 27, and 28 μg/ml, respectively. On the other hand, compounds **6** and **10** reduced the number of viral plaques of *Herpes simplex* type-1 (HSV-1) by 66 and 41%, respectively. The detailed synthesis and spectroscopic and biological data are reported.

Introduction

Pyrimidines and condensed pyrimidines have received much attention over the years because of their interesting pharmacological properties [1–6]. In our laboratory considerable effort has recently been invested in the preparation and evaluation of heterocyclic compounds containing the pyrimidine nucleus, leading to numerous compounds with promising antiviral and antitumor activities [7–10]. Moreover, some substituted pyrazolopyrimidines have been documented as adenosine antagonists [1–3]. These facts, in addition to our previously obtained results about the remarkable activity of thiazolotriazolopyrimidines[11] (structure A), encouraged the synthesis of new derivatives of the pyrazolo-[3,4-*d*]pyrimidine nucleus as purine isosteres and the evaluation of their DNA-binding and antiviral activities.



Structure A

Results and Discussion

Chemistry

The preparation of 5-amino-4,5-dihydro-1-phenyl-6-phenylamino-pyrazolo[3,4-*d*]pyrimidin-4-one **3** necessary for this study is shown in Scheme 1. 5-Amino-1-phenyl-1*H*-pyrazole-4-carboxylic acid ethyl ester **1** was prepared through the reaction of ethyl (ethoxymethylene) cyanoacetate with phenylhydrazine[3,12]. Treatment of **1** with phenyl isothiocyanate afforded ethyl 5-(phenylthiourido)-1-phenyl-pyrazole-4-carboxylate **2** [13], which was then cyclized by refluxing in neat hydrazine hydrate to produce the target compound **3** in 47% yield. The synthetic pathways employed in the preparation of compounds **4**–**8** are reported in Scheme 1. Reaction of pyrazolo[3,4-*d*]pyrimidin-4-one **3** with either triethylorthoformate or triethylorthoacetate, in the presence of *p*-toluenesulfonic acid, yielded 1,8-diphenyl-pyrazolo[3,4-*d*][1,2,4]triazolo[2,3-*a*]pyrimidin-4-one **4** and 7-methyl-1,8-diphenylpyrazolo[3,4-*d*][1,2,4]triazolo[2,3-*a*]-pyrimidin-4-one **5**, whereas 7-thioxo derivative **6** was obtained by the reaction of **3** with carbon disulphide and potassium hydroxide. 1-Phenyl-6-phenylamino-5-(2-thienyl-carboxamido)pyrazolo[3,4-*d*]pyrimidin-4-one **7** was prepared through the reaction of 2-thenoylchloride, in presence of triethylamine, with the starting material **3**. Furthermore, reaction of **3** with phenyl isothiocyanate yielded 1-phenyl-6-phenylamino-5-(*N*-phenylthiourido)pyrazolo[3,4-*d*]pyrimidin-4-one **8**.

Condensation of **3** with certain aromatic aldehydes, gave the desired 5-(4-halobenzylideneamino)pyrazolo[3,4-*d*]-pyrimidin-4-one derivatives **9**–**11** (Scheme 2). Compound **3** was also utilized by allowing it to react with certain α-bromoacetophenones in ethanol in the presence of *p*-toluenesulfonic acid as a catalyst for prolonged time to afford the cyclized product 1,9-diphenyl-7-(4-halophenyl)-8,9-dihydropyrazolo[3,4-*d*]pyrimidino[1,2-*b*][1,2,4]triazin-4-ones **12**–**14** (Scheme 2).

Biology

The antitumor activity of the newly synthesized compounds was determined using DNA binding assay [14] and methyl green DNA displacement assay [15].

DNA Binding Assay

In this method, a fixed amount of the ligand is spotted on the RP-18 TLC plates followed by addition of known amount of DNA on the same spot. The plate was then developed and the position of unbound DNA was determined by spraying the plates with anisaldehyde reagent. The free DNA was detected as a blue spot (R_f, MeOH–H_2O, 8:2) on RP-18 TLC. It was demonstrated that, when DNA was mixed with compounds known to interact with it, e.g. ethidium bromide, the complex was retained at the origin. Compounds with high binding affinity to DNA remained on the base line or migrated for a very short distance, while compounds with poor binding affinity did not cause DNA to be retained at the origin.

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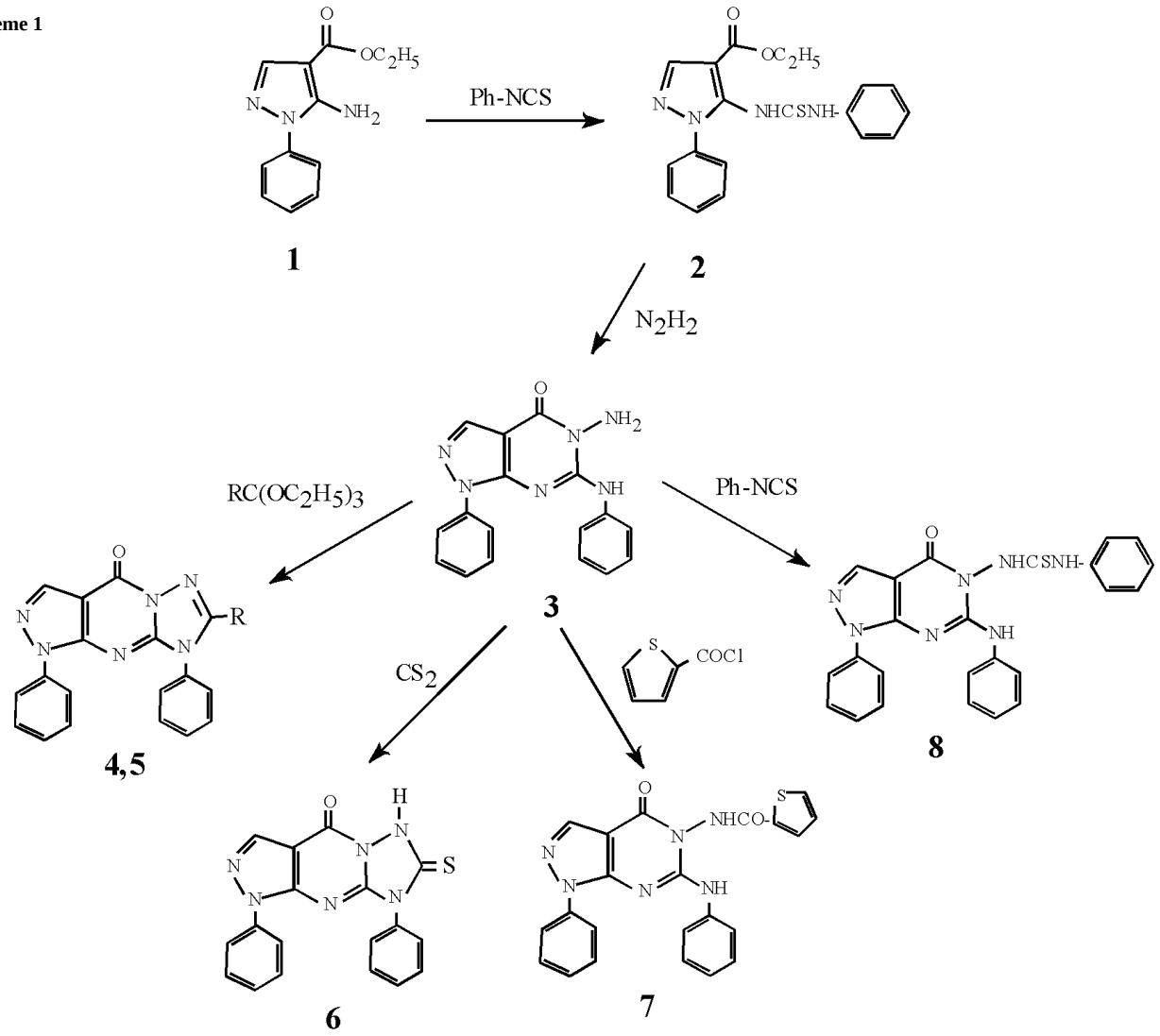
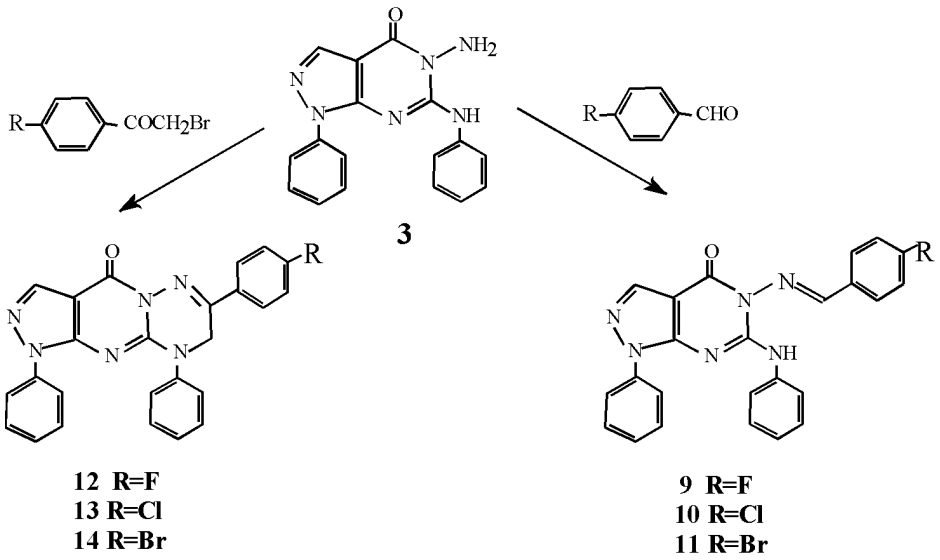
Scheme 1



Scheme 2



Methyl Green-DNA Displacement Assay

Methyl green reversibly binds polymerized DNA forming a stable complex at neutral pH. The absorption maximum for the DNA/methyl green complex is 642.5–645 nm. This assay was used to measure the displacement of methyl green from DNA by compounds having ability to bind with DNA. The degree of displacement was determined spectrophotometrically by measuring the change in initial absorbance of the DNA/methyl green solution in the presence of reference compound. The activity of compounds which showed high affinity for DNA have been determined and expressed as CC_{50} (Concentration required for 50% decrease in the initial absorbance of the DNA/methyl green solution). These results indicated that compounds **4**,**5**, and **6** showed high binding affinity for DNA and their structures are characterized by a pyrazolotriazolopyrmidine moiety. On the other hand, replacement of the triazole nucleus by 1,2,4-triazine led to compounds **12**, **13**, and **14** that exhibited moderate affinity for DNA (Table 2).

In vitro Anti-Herpes simplex-1 Virus (HSV-1)

The synthesized compounds were tested *in vitro* for their antiviral activity using *Herpes simplex* type-1 (HSV-1) as a viral model. The antiviral testing was performed using Vero cells (cells isolated from the kidneys of green monkeys), HSV-1, and Aphidicolin as a positive control [16]. The viral culture and test solutions were incubated for 66 h. At the end of the incubation period, the percentage reduction in the number of virus plaques were recorded and compared to the positive control. Compounds **6** and **10** showed the highest activity among the tested compounds and reduced the number of HSV-1 plaques by 66 and 41% respectively while compounds **4**, **5**, **11**, **9**, **12**, **14**, and **13** showed 33, 31, 30, 22, 21, 20, and 15% reduction, respectively. Compounds **7** and **8** proved to lack any antiviral activity (Table 3).

Cytotoxicity Assay

Cultured mammalian cells have been utilized to determine the response of tumor cells isolated from cancer patients to various chemotherapeutic agents[17, 18] as well as the antiviral potency of the synthesized compounds using *Herpes Simplex* as a viral model[16]. CC_{50} (the compound concentration that cause 50% reduction in cell density) was recorded and compared to the positive control. Regarding the cytotoxicity of the tested compounds, the results obtained revealed that these compounds have weak cytotoxicity which demonstrated a selective DNA binding and consequently a safe antiviral activity.

Conclusion

The basic structure pyrazolo[3,4-*d*]pyrimidine **9**–**11** possesses promising antiviral activity with lower affinity for DNA, while the pyrazolotriazolopyrimidine compounds **4**–**6** showed higher potency towards HSV-1 and high binding affinity for DNA. On the other hand, formation of pyrazolopyrimidinotriazines moieties reduced the antiviral potency and afforded compounds **12**–**14** with moderate affinity to DNA.

Experimental Part

Chemistry

The melting points (°C, uncorrected) were determined in open glass capillaries on a Mel-temp II melting point apparatus. ^{1}H-NMR spectra were recorded on a varian EM 360 (90 MHz) instrument using TMS as an internal standard (Chemical shift in δ ppm). Microanalytical data (C,H,N) were in agreement with the proposed structures within ±0.4% of the theoretical values were performed at the Microanalytical Center, Cairo University, Egypt.

*5-Amino-4,5-dihydro-1-phenyl-6-phenylaminopyrazolo[3,4-d]pyrimidin-4-one (**3**)*

A mixture of ethyl 5-(phenylthiourido)-1-phenylpyrazole-4-carboxylate **2** (3.66 g, 0.01 mol) and hydrazine hydrate (10 g, 0.2 mol) was heated under reflux for 10 h. The solid that separated upon cooling was filtered, washed with water and recrystallized from acetic acid to yield 1.5 g (47%) of **3**, mp: 173–175°C. Analysis for ($C_{17}H_{14}N_6O$): C,H,N. ^{1}H-NMR (DMSO-d_6): 6.05(s, 2H, NH_2, exchangeable), 7.15–7.45 (m, 10 H, ArH), 7.65 (s, 1H, pyrazole), 8.8 (br s, 1H, NH, exchangeable).

*1,8-Diphenylpyrazolo[3,4-d][1,2,4]triazolo[2,3-a]pyrimidin-4-one (4) and 7-methyl-1,8-diphenylpyrazolo[3,4-d][1,2,4]triazolo[2,3-a] pyrimidin-4-one (**5**)*

A mixture of **3** (3.8 g, 0.012 mol), *p*-toluenesulfonic acid (3.8 g, 0.02 mol) and triethylorthoformate or triethylorthoacetate (45 ml) was heated under reflux for 20 h. The solution was concentrated in *vacuo* and the obtained residue was triturated with ice-cold water, filtered, dried and recrystallized from aqueous ethanol (Table 1). ^{1}H-NMR ($CDCl_3$) **4:** δ 7.08–7.40 (m, 6H, ArH), 7.71–7.90 (m, 4H, ArH), 8.05 (s, 1H, pyrazole), 8.5 (s, 1 H, triazole). **5**: δ 2.40 (s, 3H, CH_3), 7.10–7.41 (m, 6H, ArH), 7.90–8.0 (m, 4H, ArH), 8.07 (s, 1H, pyrazole).

*1,8-Diphenylpyrazolo[3,4-d][1,2,4]triazolo[2,3-a]pyrimidin-4-one-7-thione (**6**)*

Carbon disulphide (3 ml, 0.05 mol) was added dropwise to a mixture of **3** (1.6 g, 0.005 mol) and potassium hydroxide (0.28 g, 0.005 mol) in ethanol (15 ml). After complete addition, the reaction mixture was stirred at room temperature for 1 h. followed by heating under reflux for 8 h. The solvent was evaporated in *vacuo*. Water was added to the residue and the alkaline solution was filtered. The clear filtrate was acidified with dilute hydrochloric acid and the separated solid was collected and recrystallized from chloroform-ethanol to give 0.7 g (46%) of **6**; mp: 255–257 °C. Analysis ($C_{18}H_{12}N_6OS$); C,H,N. ^{1}H-NMR (DMSO-d_6),: δ 7.20–7.50 (m, 10 H, ArH), 7.7 (s, 1H, pyrazole), 14.08 (br s, 1H, NH, exchangeable).

*1-Phenyl-6-phenylamino-5-(2-thienylcarboxamido)pyrazolo-[3,4-d] pyrimidin-4-one (**7**)*

2-Thenoylchloride (0.73 g, 0.005 mol) was added dropwise to a stirred solution of **3** (1.6 g, 0.005 mol) and triethylamine (0.51 g, 0.005 mol) in dichloromethane (10 ml). The reaction mixture was heated at 70 °C for 3h. After cooling, the separated solid was collected by filteration, washed with ice-water, dried and recrystallized from chloroform to produce 0.86g (40%) of **7**; mp: 128–130 °C. Analysis ($C_{22}H_{16}N_6O_2S$); C,H,N. ^{1}H-NMR (DMSO-D_6): δ 6.55 (s, 1H, NH-CO exchangeable), 6.85 – 7.70 (m, 13 H, ArH), 7.8 (s, 1H, NH exchangeable), 8.06(s, 1H, pyrazole).

*1-Phenyl-6-phenylamino-5-(N-phenylthiourido)pyrazolo[3,4-d]pyrimidin-4-one (**8**)*

Phenylisothiocynate (1.35 g, 0.01 mol) was added dropwise to a solution of **3** (1.6 g, 0.005 mol) in ethanol (20 ml). The mixture was heated under reflux for 6 h. After cooling, the mixture was poured into water. The precipitated solid was collected by filtration and recrystallized from methanol to yield 1.2 g (53%) of the title compound **8**; mp: 105–107 °C. Analysis ($C_{24}H_{19}N_7OS$); C,H,N. ^{1}H-NMR (DMSO-d_6): δ 6.90 (s, 1H, NH exchange-

able) 7.2–7.55 (m, 15H, ArH), 7.80 (s, 1H, pyrazole), 7.90 (s, 1H, NH exchangeable), 9.4 (s, 1H, NH exchangeable).

*1-Phenyl-6-phenylamino-5-(4-halobenzylideneamino)pyrazolo-[3,4-d]pyrimidin-4-one (**9–11**)*

A suspension of **3** (1.6 g, 0.005 mol) in acetic acid (12 ml) was heated at reflux with the appropriate aldehyde (0.005 mol) for 4 h. The solid separated upon cooling was collected by filtration, dried and recrystallized [**9** (HOAc) and **10**,**11** (HOAc-H_2O] (Table 1). ^{1}H-NMR (DMSO-d_6) **9:** δ 7.21–7.60 (m, 14 H, ArH), 7.80 (s, 1H, pyrazole), 10.09 (s, 1H, CH), 11.18 (s, 1H, NH exchangeable). ^{1}H-NMR (DMSO-d_6) **10:** δ 7.28–7.56 (m, 14H, ArH), 7.85 (s, 1H, pyrazole), 9.9 (s, 1H, CH), 10.09 (s, 1H, NH exchangeable). ^{1}H-NMR (DMSO-d_6) **11**: δ 7.28–7.59 (m, 14H, ArH), 8.0 (s, 1H, pyrazole), 10.0 (s, 1H, CH), 11.18 (s, 1H, NH exchangeable).

*1,9-Diphenyl-7-(4-halophenyl)-8,9-dihydropyrazolo[3,4-d]pyrimidino-[1,2-b] [1,2,4]triazin-4-one (**12–14**)*

A mixture of **3** (3.8 g, 0.012 mol), 4-halo-α-bromoacetophenone (0.012 mol) and *p*-toluenesulfonic acid (3.8 g, 0.2 mol) in ethanol (20 ml) was heated at reflux for 24 h. After cooling, the mixture was poured into ice-water and the obtained product was filtered, dried and recrystallized from a chloroform-pet. ether mixture (Table 1). ^{1}H-NMR ($CDCl_3$) **12:** δ 5.3 (s, 2H, CH_2), 6.98–7.75 (m, 14 H, ArH), 7.95 (s, 1H, pyrazole). **13:** δ 5.28 (s, 2 H, CH_2), 7–7.73 (m, 14H, ArH), 8.06 (s, 1H, pyrazole). **14:** δ 5.3 (s, 2H, CH_2), 7.0–7.74 (m, 14H, ArH), 8.05(s, 1H, pyrazole).

Biological Investigation

The following instruments and materials were used in the biological evaluation; hemocytometer (Bright line, AO instrument Co. Buffalo, NY, USA), inverted microscope (Nikon Inc., Instrument Division, Garden City, NY, USA), Dulbecco's modified Eagles medium (Gibco, Grand Island, NY, USA), supplement with 10% calf serum (Gibco), antibiotic and trypsin (Sigma, St. Louis, MO, USA), Giemsa stain (Fisher Scientific Co., Fair Lawn, NY, USA). TLC plates (RP-18F_{254}; 0.25 mm, Merck), DNA (calf thymus type I, Sigma, 100 μg/ml), DNA/methyl green (Sigma, St. Louis, MO, USA).

DNA Binding Assay

Analysis of the DNA binding affinity of the tested compounds was performed using RP-TLC. TLC plates (RP-18 F_{254}; 0.25 mm; Merck) were predeveloped with MeOH-H_2O (8:2). Test compounds were then applied (5 mg/ml in MeOH) at the origin, followed by the addition of DNA (1 mg/ml in H_2O-MeOH mixture) at the same positions at the origin. The plates were then developed with the same solvent system and the position of the DNA was determined by the spraying with anisaldehyde reagent. The reagent yields a blue color spot with DNA, and the intensity of the color was proportional to the quantity of DNA added to the plate. Ethidium bromide was used as a positive control.

Table 1. Melting points, yield percentages, and molecular formulae of the new compounds.

Compound	R	Mp (°C)	Yield %	Molecular formulae
4	H	173–175	56	$C_{18}H_{12}N_6O$
5	CH_3	115–117	65	$C_{19}H_{14}N_6O$
9	F	210–212	71	$C_{24}H_{17}FN_6O$
10	Cl	145–147	78	$C_{24}H_{17}ClN_6O$
11	Br	198–200	86	$C_{24}H_{17}BrN_6O$
12	F	83–85	39	$C_{25}H_{17}FN_6O$
13	Cl	87–89	42	$C_{25}H_{17}ClN_6O$
14	Br	96–98	48	$C_{25}H_{17}BrN_6O$

Colorimetric Assay for Compounds that Bind to DNA (Methyl Green DNA Displacement Assay)

DNA methyl green (20 mg, Sigma. St. Louis, MO, USA) was suspended in 100 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 7.5 mM $MgSO_4$ and stirred at 37 °C for 24 h. Unless otherwise indicated, samples to be tested were dissolved in EtOH in Eppendorff tubes. Solvent was removed in *vacuo*, and 200 μl of the DNA/methyl green solution was added to each tube. The absorption maximum for the DNA/methyl green complex is 642.5–645 nm. Samples were incubated in the dark at ambient temperature. After 24 h, the final absorbance of samples was determined. Readings were corrected for initial absorbance and normalized as a % of the untreated DNA/methyl green absorbance value. CC_{50} were determined for each compound as shown in Table 2.

Antiviral Screening

Microtiter trays with confluent cultures of vero cells were inverted and the medium was shaken out. A serial dilution of sterile samples dissolved in the medium was then added (100 μl in each well).Tray wells were then inoculated with 30 plaque forming units of *Herpes simplex*-type 1 (HSV-1) virus

Table 2. Activity of compounds on DNA-methyl green assay.

Compound	CC_{50} (μg/ml)
Ethidium bromide	79
4*	19
5*	27
6*	28
9	40
10	61
11	52
12	41
13	30
14	38

Values represented the concentration required for 50% decrease in the initial absorbance of the DNA-methyl green solution.
* Compounds which possess high DNA affinity.

Table 3. Antiviral and cytotoxic activity of tested compounds.

Compound	% reduction in number of plaques	[b]CC_{50} (μg/ml)
Aphidicolin[a]	100	220
4	33	220
5	31	100
6	66	220
7	None	220
8	None	220
9	22	150
10	41	220
11	30	100
12	21	220
13	15	100
14	20	100

[a] Positive control. The minimum antiviral concentration of Aphidicolin is 5 μg/ml and of the tested compounds is 100 μg/ml.
[b] CC_{50} values (Concentration of compound resulting in 50% reduction in cell density) were estimated visually from triplicate cultures.

in 100 µl medium containing 10% (*v/v*) calf serum. In each tray, the last row of wells was reserved for controls consisting of vero cells treated with virus but not test compounds. The trays were incubated under normal culture conditions (37 °C, 5% CO_2 atmosphere) for 66 h. The trays were inverted onto a pad of paper towels and the remaining cells were rinsed carefully with the medium and fixed with 3.7% (*v/v*) formaldehyde in saline solution for 20 min, then rinsed with water, stained with 0.5% (*w/v*) crystal violet solution in 20% aqueous ethanol for 30 min and rinsed with water again. The trays were examined visually, antiviral activity was estimated as the % reduction in the number of virus plaques (Table 3).

References

[1] L. P. Davies, D. J. Brown, S.C. Chow, G. A. R. Johnston, *Neuro Sci. Lett.* **1983**, *41*, 189.

[2] L. P. Davies, S.C. Chow, J. H. Skeritt, D. J. Brown, G. A. R. Johnston, *Life Sci.* **1984**, *34*, 2177.

[3] N. P. Peet, N. L. Lentz, S. Sunder, M. W. Dudley, A. M. L. Ogden, *J. Med. Chem.* **1992**, *35*, 3263.

[4] M. Z. Rizk, A. Z. Abdel-Hamid, L.H. Feddah, *Egypt. J. Pharm. Sci.* **1993**, *34*, 57.

[5] J. B. Jiang, D. P. Hesson, B. A. Dusak, D. L. Dexter, G. J. Kang, E. Hamel, *J. Med. Chem.* **1990**, *33*, 1721.

[6] E.S. Badway, S. M. Rida, A. A. Hazza, H. T. Fahmy, Y. M. Gohar, *Eur. J. Med. Chem.* **1993**, *28*, 91, 97.

[7] M. A. El-Sherbeny, M. B. El-Shmawy, H. I. El-Subbagh, A. A. El-Emam, F. A. Badria, *Eur. J. Med. Chem.* **1995**, *30*, 445.

[8] H. I. El-Subbagh, M. A. El-Sherbeny, M.N.Nasr, F. E. Goda, F. A. Badria, *Boll. Chim. Farm.* **1995**, *134*, 80.

[9] M. A. El-Sherbeny, F. A. Badria, S. A. Kheira, *Med. Chem. Res.* **1996**, *6* , 28.

[10] I. A. Shehata, H. I. El-Subbagh, A. M. Abdelal, M. A. El-Sherbeny, A. A. Al-Obaid, *Med. Chem Res.* **1996**, *7*, 148–163.

[11] E.R. El-Bendary, M. A. El-Sherbeny, F. A. Badria, *Boll. Chim. Farm.* **1998**, *137*, 115.

[12] P. Schmidt, J. Druey, *Helv. Chim. Acta* **1956**, *39*, 986.

[13] H. M. Vrupakshaiah, M. P. Talawar, U. V. Laddi, Y. S. Somannovar, V. V. Badiger, S. C. Bennur, *Indian J. Heterocycl. Chem.* **1996**, *6*, 63.

[14] J. M. Pezzuto, S. K. Antosiak, W. M. Messmer, M. B. Slaytor, G. R. Honig, *Chem. Biol. Interact.* **1983**, *43*, 323.

[15] J. M. Pezzuto, C. T. Che, D. D. Mepherson, J.I. Zhu, G. Topeu, C. A. J. Erdelmeier,, G. A. Cordell, *J. Nat. Prod.* **1991**, *54*, 1522.

[16] M. Abou-Karam, W. T. Shier, *J. Nat. Prod.* **1990**, *53*, 340–345.

[17] G. M. William, V. C. Dunkel, V. A. Ray, (eds) *Ann. NY. Acad. Sci.* **1983**, *407*, 1–484.

[18] L. D. Hafford, F. A. Badria, M. Abou-Karam, W. T. Sheir; R. D. Rogers, *J. Nat. Prod.* **1991**, *54*, 1543–1552.

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