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Alkaloids of *Cynanchum vincetoxicum*: Efficacy against MDA-MB-231 Mammary Carcinoma Cells

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Alkaloids **1-4** from *Cynanchum vincetoxicum* (asclepiadaceae) (Scheme 1) do not have affinity to the oestrogen receptor but they inhibit the growth of the hormone-independent mammary carcinoma cells MDA-MB-231 (Fig. 1) and bind to nucleosides and nucleotides (Table 1). Intercalation was not observed.

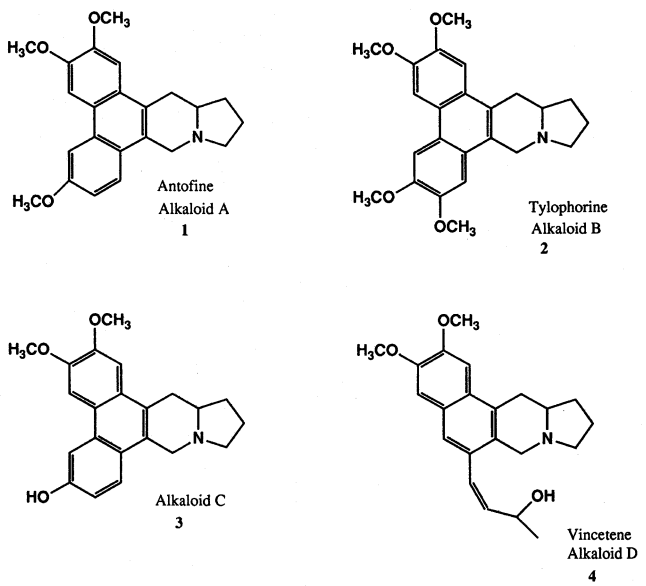
Alkaloide aus *Cynanchum vincetoxicum*: Wirkung auf MDA-MB 231 Mammakarzinom-Zellen

Die Alkaloide **1-4** aus *Cynanchum vincetoxicum* (Asclepiadaceae) (Scheme 1) zeigen keine Affinität zum Oestrogen-Rezeptor, hemmen aber das Wachstum der hormonunabhängigen Mammakarzinom-Zellen MDA-MB-231 und binden an Nukleoside und Nukleotide (Table 1). Interkalation wurde nicht beobachtet.

In former times *Cynanchum vincetoxicum* had been used against mammary carcinoma[1]. First citations of this therapy are already found in the 6th century[2]. Also in medieval times preparations of *Cynanchum vincetoxicum* (ointments, cataplasms, lotions) had been used for the treatment of external breast cancer[3,4].

We wanted to know whether the alkaloids **1**, **2**, **3**, and **4** (Scheme 1) of this plant might contribute to the claimed cytostatic activity of this plant. Therefore, we tested whether these alkaloids[5] show affinity to the oestrogen receptor of hormone dependent mammary carcinoma cells[6,7,8] in comparison with [^{3}H]-oestradiol: according to our experiments none of these alkaloids nor the natural mixture of alkaloids from *C. vincetoxicum* show affinity. On the other hand these alkaloids inhibit growth of the hormone independent breast cancer cells MDA-MB 231[9] (Fig. 1): Whilst Alkaloid A shows only weak activity at 1 µM concentration, Alkaloid B and Alkaloid C are most effective: within the biological scattering (0.8% and 1.1%, respectively) no cell growth was observed, whilst Alkaloid D shows $T/C_{corr.} = 6.7\%$ (biological scattering: 2.5%).

In order to test whether this growth inhibition is caused by interaction (intercalation, e.g.) with DNA we used UV-difference spectroscopy[10,11,12]: all the alkaloids interact with



Scheme 1

+) Dedicated to Prof. Dr. *J. Slavík*, Brno, on the occasion of his 70th birthday.

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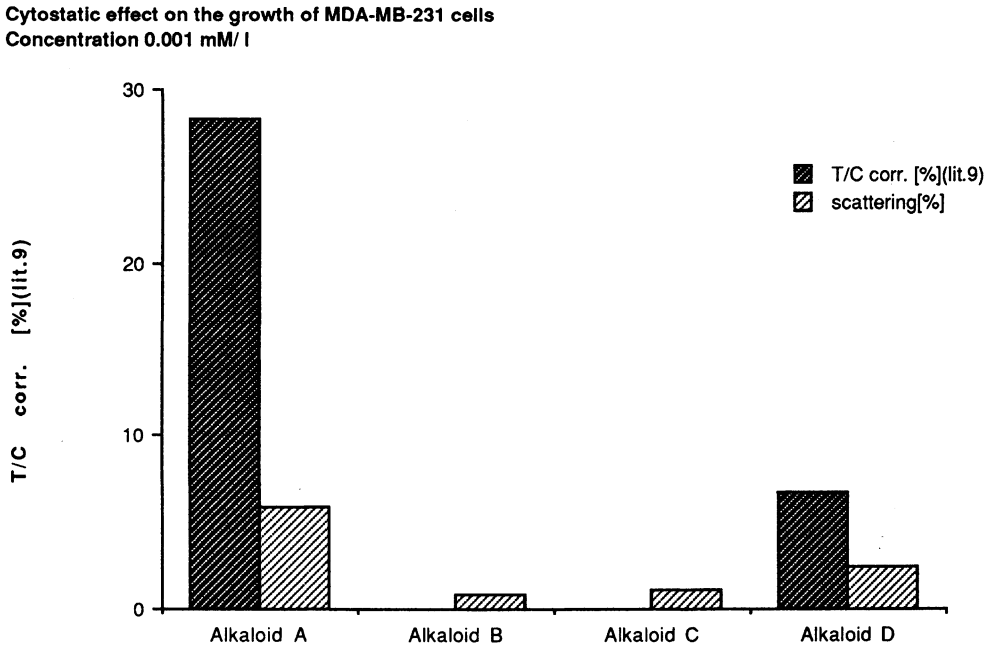
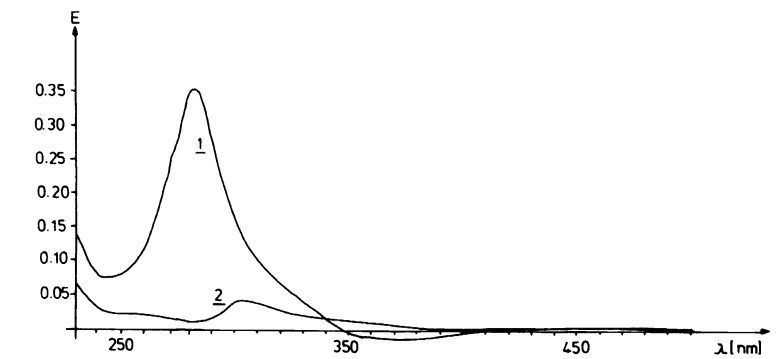
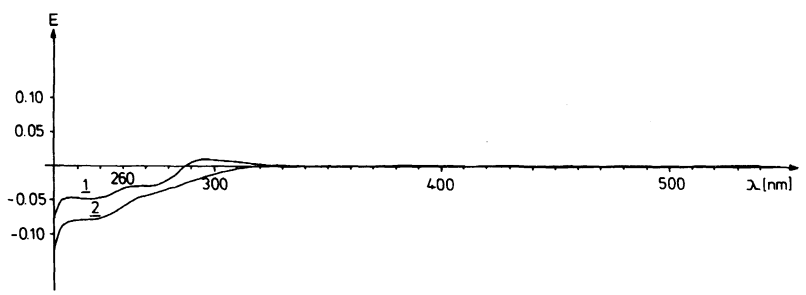


Figure 1



Difference spectra for 1: Ethidium bromide with native DNA;
2: Ethidium bromide with sonicated DNA;
R=[Ethidium bromide]/[DNA]=1.33.



Difference spectra for 1:Alkaloid D with sonicated DNA;
2:AlkaloidD with native DNA;
R=[Alkaloid D]/[DNA]=1.33.

Figure 2

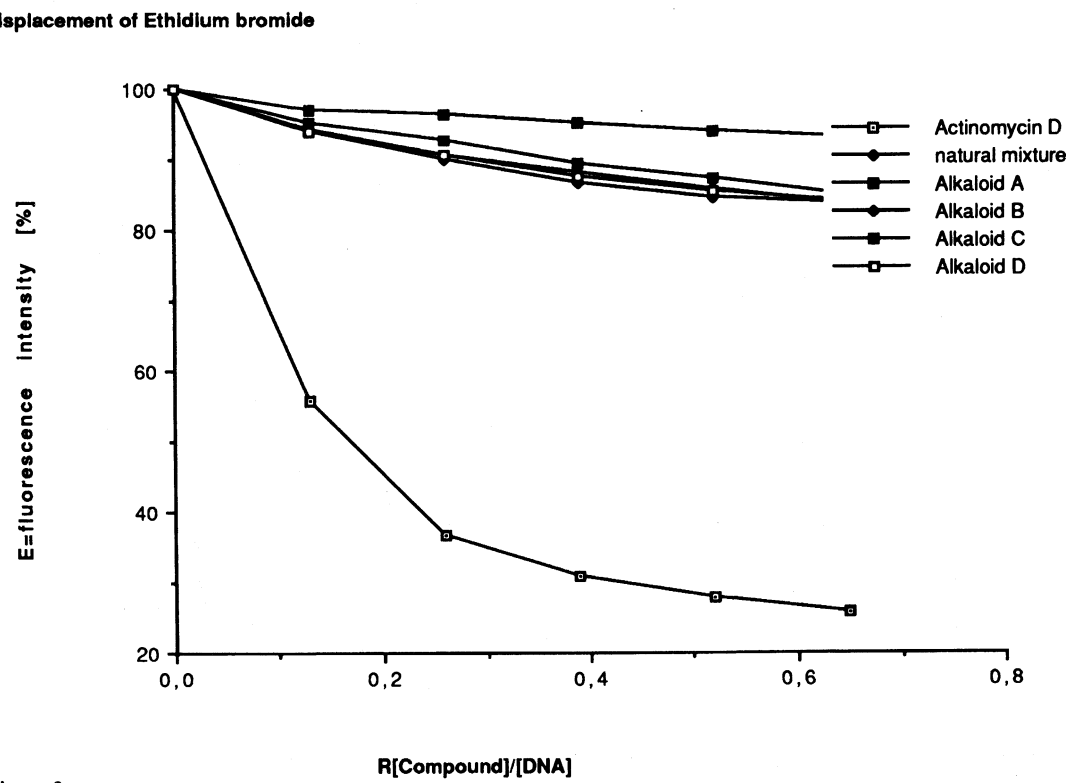


Figure 3

native as well as with sonicated DNA (Fig. 2). Therefore, intercalation cannot contribute much to this efficacy. This is corroborated by the negative results of ethidium bromide displacement[13] (Fig. 3).

The degree of interaction of the alkaloids with the respective DNA-nucleosides (Fig. 4) and DNA-nucleotides (Fig. 5), however, is very different and points towards an unspecific effect (Table 1).

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Experimental Part

UV-spectroscopy: Uvikon 810 (Kontron), tandem quartz cuvettes.- *Fluorescence spectroscopy*: Hitachi F 3000, fluorescence cuvettes.- *Chromatography*: TLC: SiO_2, Merck 5554, TLC Al-foils, silica 60 F_{254}. Detection: *Dragendorff* reagent (*Munier/Machboef*).- Column chromatography: SiO_2, Merck 7734 silica 60 (70-230 mesh).- Drying of org. phases: Na_2SO_4.- All temp. in °C.

Extraction of Alkaloids[5]

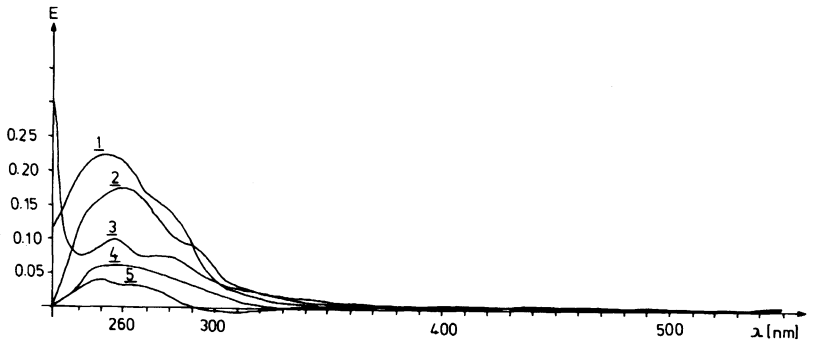
4.6 kg of coarsely milled*), dried overground parts of *C. vincetoxicum* from the surroundings of Regensburg were macerated 4 times with a total of 40 l of MeOH for 24 h at room temp.- After squeezing the pertinent liquid was combined with the extract, evaporated *in vacuo*, acidified by dil. HCl to pH 2-3, and filtered after 12 h at 4°. In order to remove non-

*) We thank Iso-Werk, Regensburg, for kind assistance.

Table 1: UV-difference spectroscopy of alkaloids A, B, C, D and natural mixture. R^1 = [Alk.]/[Nuc.] = 0.5; R^2 = [Alk.]/[Nuc.] = 1.0. Values at l = 260 nm.

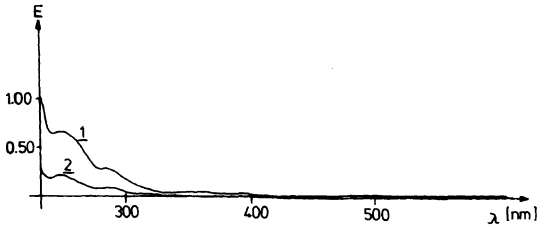
Nucleosid	Alkaloid A	Alkaloid B	Alkaloid C	Alkaloid D	natural mixture
2`-Deoxy-adenosine	R^1=.007 R^2=.113	R^1=.064 R^2=.184	R^1=.059 R^2=.063	R^1=.146 R^2=.116	R^1=.062 R^2=.157
2`-Deoxy-cytidine	R^1=.015 R^2=.080	R^1=.089 R^2=.089	R^1=.089 R^2=.090	R^1=.095 R^2=.010	R^1=.042 R^2=.230
2`-Deoxy-guanosine	R^1=.064 R^2=.018	R^1=.153 R^2=.000	R^1=.031 R^2=.171	R^1=.031 R^2=.175	R^1=.304 R^2=.241
2`-Deoxy-thymidine	R^1=.100 R^2=.050	R^1=.063 R^2=.131	R^1=.075 R^2=.059	R^1=.060 R^2=.028	R^1=.014 R^2=.172
2`-Deoxy-uridine	R^1=.014 R^2=.025	R^1=.074 R^2=.061	R^1=.002 R^2=.005	R^1=.110 R^2=.135	R^1=.180 R^2=.044

Nucleotid	Alkaloid A	Alkaloid B	Alkaloid C	Alkaloid D
2`-Deoxy-adenosine-5`-phosphate	R^1=.032 R^2=.037	R^1=.075 R^2=.100	R^1=.110 R^2=.190	R^1=.008 R^2=.007
2`-Deoxy-cytidine-5`-phosphate	R^1=.018 R^2=.084	R^1=.254 R^2=.244	R^1=.017 R^2=.146	R^1=.030 R^2=.165
2`-Deoxy-guanosine-5`-phosphate	R^1=.065 R^2=.020	R^1=.020 R^2=.025	R^1=.016 R^2=.050	R^1=.145 R^2=.010
2`-Deoxy-thymidine-5`-phosphate	R^1=.020 R^2=.040	R^1=.044 R^2=.050	R^1=.010 R^2=.180	R^1=.010 R^2=.120
2`-Deoxy-uridine-5`-phosphate	R^1=.005 R^2=.050	R^1=.080 R^2=.180	R^1=.115 R^2=.200	R^1=.005 R^2=.075



Difference spectra for 1: natural alkaloid mixture; R=[nat.mixture]/[2`-Deoxyguanosine]=0.5;
2:Alkaloid B;R=[Alkaloid B]/[2`-Deoxyguanosine]=0.5;
3:Alkaloid D;R=[Alkaloid D]/[2`-Deoxyguanosine]=0.5;
4:Alkaloid A;R=[Alkaloid A]/[2`-Deoxyguanosine]=0.5;
5:Alkaloid C;R=[Alkaloid C]/[2`-Deoxyguanosine]=0.5;

Figure 4



Difference spectra for 1: Alkaloid A;[R=Alkaloid A]/[2`-Deoxyguanosine-5`-phosphate]=1;
2: Alkaloid A;[R=Alkaloid A]/[2`-Deoxyguanosine-5`-phosphate]=0.5;

Figure 5

basic materials the aqueous phase was extracted 5-6 times with half its volume of Et_2O, basified with NH_3 to pH 9, and the bases were extracted 6 times by about 1/3 of its volume with Et_2O. After drying ether was removed *in vacuo*: 0.06% of the dry plant material were obtained as partially crystalline mixture of crude alkaloids.- For CC the crude alkaloids were dissolved in $CHCl_3$ and adsorbed at 4 times its weight of SiO_2 *in vacuo*.- Alkaloids **1-4** were separated using SiO_2 and MeOH/acetone/benzene 5/30/65. The separation process was controlled by TLC.

Specific Rotations

Alkaloid A:
c = 0.10 g/100 ml MeOH; T = 24°.

Wavelength λ [nm]	α [°]	[α] [°·ml /g·dm]	Δ[α] [°·ml /g·dm]
589	-0.037	-37.9	-4.0
578	-0.039	-40.0	-4.1
546	-0.047	-48.2	-4.5
436	-0.095	-97.4	-7.0

Alkaloid B:
c = 0.10 g/100 ml MeOH; T = 24°.

Wavelength λ [nm]	α [°]	[α] [°·ml /g·dm]	Δ[α] [°·ml /g·dm]
589	-0.099	-94.8	-6.5
578	-0.104	-99.0	-6.7
546	-0.120	-114.3	-7.5
436	-0.224	-213.3	-12.3

Alkaloid C:
c = 0.10 g/100 ml MeOH; T = 24°.

Wavelength λ [nm]	α [°]	[α] [°·ml /g·dm]	Δ[α] [°·ml /g·dm]
589	-0.102	-103.0	-7.2
578	-0.108	-109.1	-7.5
546	-0.125	-126.3	-8.4
436	-0.236	-238.4	-14.0

Alkaloid D:
c = 0.11 g/100 ml MeOH; T = 24°.

Wavelength λ [nm]	α [°]	[α] [°·ml /g·dm]	Δ[α] [°·ml /g·dm]
589	-0.021	-19.4	-2.8
578	-0.022	-20.4	-2.8
546	-0.025	-23.1	-3.0

Affinity to the oestrogen receptor

The uteri of freshly slaughtered calves were stored in ice cold physiological NaCl solution until preparation of the cytosol. All preparations were done at 4°: the longitudinally cut horns of the uteri were freed from fat, parametrium, and perimetrium. Remainings of blood and mucus were removed by washing with physiological NaCl solution. After adding of tris-buffer (10 mM tris, 1.5 mM EDTA, 3 mM NaN_3, pH 7.5) the horns were cut by a scissors. 10 g of tissue were suspended in 10 ml of tris-buffer and homogenized (Ultraturrax, 3 times 10 s, then glass homogenizer). Then the homogenate was centrifuged (10 500 x g, 60 min, +4°). The clear supernatant was taken off by a pipette without the fatty material.- Protein content: 10-20 mg/ml cytosol.

Binding affinity to the oestrogen receptor was determined indirectly because no radioactive inhibitor was available: According to the recommendations of EORTC[8] we used the charcoal adsorption technique. The interaction of [^{3}H]-oestradiol, reduced by an inhibitor (run B), is compared with the control (no inhibitor, run A), and the unspecifically bound amount of [^{3}H]-oestradiol is determined in run C:

solution (µl)	run A	run B	run C
[^{3}H]-oestradiol 0.5 pM	100	100	100
inhibitor		100	
17β-oestradiol 2.0 nM			100
tris-buffer	300	200	200
cytosol	100	100	100

Runs A, B, and C were incubated at +4° for 16 h under gentle shaking. The non-bound amounts of [^{3}H]-oestradiol and of inhibitor were removed by incubation with 0.5 ml of charcoal suspension (0.8% activated charcoal Norit, 0.008% dextrane in tris-buffer pH 7.5) for 1.5 h at 4°. Adsorbing material was removed by centrifugation (700 x g, 10 min). The determination was done in minivials: 100 µl of the supernatant were mixed with 2 ml of scintillation liquid. The rate of bound [^{3}H]-oestradiol was determined as the average of three measurements. The percental rate of [^{3}H]-oestradiol is plotted against the logarithm of the molar concentration of the inhibitor. We have chosen six concentrations of inhibitor in order to cover the range of 10-90% of bound [^{3}H]-oestradiol. From the diagram, that concentration of inhibitor is determined which inhibits the binding of [^{3}H]-oestradiol to the receptor for 50%. The RBA-value is calculated as follows: RBA = [oestradiol] · 100/[inhibitor]; $RBA_{oestradiol} = 100\%$.

Efficacy against MDA-MB 231 cells [9,14]

These cells have been obtained from ATCC and were cultivated as monolayers in dishes (Costar) in a water steam saturated atmosphere containing 5% of CO_2. We used *McCoy* medium, enriched with 10% of newborn calf serum, gentamycine (40 µg/ml) and $NaHCO_3$ (2.2 g/l, pH 7.35). Just before confluence the cells were harvested with 0.05% trypsin - 0.02% EDTA in 0.15 M NaCl. Already a few h after distribution the cells continued to grow as monolayers. Cell growth was determined by the mikrotitre assay: 100 µl of cell suspension/well were switched to 96-well mikrotitration plates. By counting under the microscope the cell density was determined (10-15 cells/visual field). After 2-3 days of incubation at 37° in the atmosphere mentioned above the medium was changed at alkaloid containing medium: to this end stock solutions of pertinent concentrations were prepared, which after 1:1000 dilution by medium led to the desired final concentrations. 16 control wells/dish contained 0.1% of the solvent used for the preparation of the stock solutions. The initial cell density was determined by addition of vinblastine (10^{-7} M) in 18 wells. After three days of incubation the medium was removed. Then 100 µl of 1% glutaraldehyde in PBS were added for fixation. After 15 min the aldehyde solution was decanted and the affixed cells were coloured with 100 µl of 0.02% crystal violet solution. After 25 min the dying solution was decanted, the excess of crystal violet was washed off with water by suspending the cells for 15 min. After decanting 100 µl of 70% EtOH were added. The mikrotitre dishes were shaken for 1-2 h, until all the dye stuff was dissolved. The optical density of the coloured solutions was determined in a mikrotitre dish autoreader EL 309 (Biotek) at λ = 578 nm. Data were processed on an Olivetti M 24 PC.

Interaction of the alkaloids with DNA (UV-difference spectroscopy)

Solutions: tris buffer pH 7.4: 12.1 g tris and 5.85 g NaCl in 700 ml water, N-HCl was added for adjusting to pH 7.4, then filled up to 1 l by water.- DNA-solution: 3.7 mg of calf thymus DNA type I (Sigma) were dissolved in 100 ml of tris buffer pH 7.4 by gentle stirring for 12 h at 4°.- Stock solutions of test compounds: 10^{-2} M in DMSO.- Concentration of DNA was determined by measurements at 260 and 280 nm, respectively (content of phosphorus).- For $E_{280}/E_{260} < 1$, *Lambert-Beer's* law is valid:

$c_{DNA} = E_{260}/\varepsilon_{DNA} \cdot d = 0.498/6600 = 7.5 \cdot 10^{-5}$ M

$\varepsilon_{DNA} = 6600\ M^{-1}$ (P) cm^{-1} at λ = 260 nm; d = 1 cm; E_{260} = extinction of DNA solution at 260 nm.

We used tandem cuvettes. For determination of the base line, 1 ml of DNA solution was pipetted into the sample cuvette, 1 ml of tris-buffer was given into the reference cuvette. For getting the difference spectrum 1 µl of alkaloid solution was added to the DNA and to the blank, respectively. The volume differences in both cuvettes were balanced by 1 µl of DMSO. After gentle mixing extinction differences were determined between 550 and 230 nm. The quotient [alkaloid]/[DNA] is plotted against the extinction. A linear increase points towards a dependence on the concentration.

Interaction with sonicated DNA

In order to destroy the DNA structure, the DNA solutions (*vide supra*) were sonicated for 10 min at room temp. The UV-difference spectra of native and sonicated DNA were compared.

Interactions with phosphorylated and non-phosphorylated DNA-increments

Instead of DNA solutions stock solutions of the nucleosides 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine, 2'-deoxythymidine, and 2'-deoxyuridine or of the corresponding nucleotides were used. The base line was determined analogously (*vide supra*) using 5 µl of nucleoside- or nucleotide stock solution, respectively. Consequently 5 µl of DMSO were needed for volume balance. The difference spectra were determined using 2.5 and 5 µl of test solutions added to the nucleosides or nucleotides, respectively.- Determination parameters: scan: 550-230 nm; speed: 100 nm/min; zero suppression: -0.4; recorder scale: 0.8; chart speed: 10 cm/min.

Ethidium bromide displacement

The methodology of *LePecq*[13] was used.- Excitation: 546 nm, emission: 590 nm.- Tris-buffer pH 7.4 and DNA-solution: *vide supra*.- Concentration of DNA-solution (determination at 260 nm): $7.56 \cdot 10^{-5}$ M (P).- Ethidium bromide stock solution: 10^{-2} M in DMSO.- Control: actinomycin D, 10^{-2} M in DMSO.- Test substances: alkaloids **1**-**4**.

1 ml of ethidium bromide solution - [DNA]/[Ethidium bromide] = 1.5 - was pipetted into the fluorescence cuvette and the maximal fluorescence (100%) was determined. After addition of varying amounts of alkaloid solution (1-5 µl) or actinomycin D, respectively, the solutions were mixed gently and the fluorescence was determined (mean value of three determinations). For graphic evaluation the molar ratio [substance]/[DNA] or the added quantity of test substance [µl], respectively, is plotted against the fluorescence intensity [%].- Parameters: excitement wavelength: 546 nm; emission wavelength: 590 nm; excitement bandpass: 5 nm; emission bandpass: 5 nm.

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