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ORIGINAL PAPER

A Spectroscopic Study of the Interaction of the Fluorescent β-Carboline-3-carboxylic Acid *N*-methylamide with DNA Constituents: Nucleobases, Nucleosides and Nucleotides

Iñigo X. García-Zubiri · Hugh D. Burrows · Joao S. Seixas de Melo · María Monteserín · Antonio Arroyo · María J. Tapia

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Abstract Interaction between β-carboline-3-carboxylic acid *N*-methylamide, βCMAM, and nucleobases, nucleosides and nucleotides is studied in the ground state with UV-visible, ^{1}H NMR and ^{31}P NMR spectroscopies and in the first excited state, with steady-state and time-resolved fluorescence spectroscopy. Job plots show a predominant 1:1 interaction in both electronic states. Association constants are estimated from changes in the absorption spectra, and show that the strongest interaction is produced with the nucleosides: 2′-deoxyadenosine (dAdo) and thymidine (Thd), and with the mononucleotides: 2′-deoxycytidine 5′- monophosphate (5′-dCMP) and uridine 5′- monophosphate (5′-UMP). These results are corroborated by the upfield shifts of two ^{1}H NMR resonances of the βCMAM indole group. The ^{31}P NMR resonance of nucleotides is shifted downfield, suggesting the presence of electrostatic or hydrogen bond interaction with βCMAM. In the first electronic singlet excited state, static and dynamic quenching of βCMAM emission is achieved upon addition of nucleobases, nucleosides and nucleotides. This has been analysed using Stern–Volmer kinetics.

Keywords Fluorescence · Absorption · β-carboline · Nucleobases · Nucleosides · Nucleotides

I. X. García-Zubiri · M. Monteserín · A. Arroyo · M. J. Tapia (✉)
Departamento de Química, Universidad de Burgos,
Plaza Misael Bañuelos s/n,
Burgos 09001, Spain
e-mail: mjtapia@ubu.es

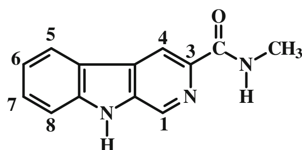
H. D. Burrows (✉) · J. S. Seixas de Melo
Departamento de Química, Universidade de Coimbra,
3004-535 Coimbra, Portugal
e-mail: burrows@ci.uc.pt

Introduction

β-carbolines are a group of synthetic and naturally occurring alkaloids, which can be found in plants [1–5] and foodstuffs, [6, 7] where they are formed as protein pyrolysis products through cooking at high temperature (frying or grilling, particularly on an open flame) [1, 8] They are also found in alcoholic beverages, [1] tobacco smoke, [1, 6] and animal fluids and tissues, [9] sometimes with an endogenous origin. [1] β-Carbolines have interesting pharmacological, [10] and medical [11] properties and have been used as antiviral, [10] antimicrobial, [10] hallucinogenic, [12] antiallergic, [3] potential insulin secretagogues, [13] antimalarial [14] or anti-tumour agents, [14–17] and as photosensitizers with potential application in photodynamic therapy [18] or as antioxidants, [9, 19] although the mutagenic and co-mutagenic properties of this class of compounds has been also indicated. [20–28]

Since β-carbolines are widespread and are of pharmacological importance, particularly in cancer treatment, much attention and numerous interdisciplinary studies have been focused on their biological effects. Many aspects of the biological activity of β-carbolines are related to their interaction with DNA, which is reported to occur by intercalation. [29, 30] The planar β-carboline ring is suggested to be sandwiched between adjacent base pairs of double stranded DNA (dsDNA), stabilized by extensive van der Waals and hydrogen-bonding interaction along the groove of the DNA helix. [29, 30] The interaction shows high selectivity towards the G-C pairs. [31] However, we have recently shown that β-carboline-3-carboxylic acid *N*-methylamide (βCMAM, Fig. 1) also interacts with single stranded DNA, ssDNA, and with the polynucleotides:

Fig. 1 Structure of βCMAM



polyadenylic (Poly A), polycytidylic (Poly C), polyguanylic (Poly G), polythymidylic (Poly T) and polyuridylic (Poly U) acids. [32] The interaction is stronger with dsDNA than with ssDNA, and shows selectivity towards Poly G, suggesting that intercalation may involve some specific interactions, such as with guanine in the G-C base pair. [32] However, the fact that βCMAM also interacts with ssDNA and oligonucleotides show that intercalation is not the only mechanism involved in βCMAM-polynucleotide interactions.

The goal of this work is to obtain further insight into the nature of other alternative mechanisms to intercalation involved in βCMAM-polynucleotide interactions through the study of βCMAM complexation with nucleobase, nucleoside and nucleotide constituents using electronic (UV-visible and fluorescence), ^{1}H NMR and ^{31}P NMR spectroscopies. Fluorescence is particularly valuable for studying these interactions since the poor aqueous solubility of some of these compounds (particularly nucleobases) demands the use of very sensitive techniques that allow quantitative measurements at low concentrations. [33] In addition, β-carbolines are good fluorophores, [34–37] as can be seen by their use as fluorescence standards, [38, 39] and detailed information has been presented on the photophysical properties and acid-base behaviour in the ground and first excited state of βCMAM in aqueous solution. [40] Moreover βCMAM is a pharmacologically active β-carboline derivative known to act as an inverse agonist of the benzodiazepine/GABA/channel chloride receptor complex [41] and to have anxiogenic effects on tonic immobility in poultry. [42]

Experimental

Reagents

β-Carboline-3-carboxylic acid *N*-methylamide (βCMAM), norharmane, the nucleobases adenine (Ade), cytosine (Cyt), guanine (Gua), thymine (Thy), uracil (Ura), the mononucleosides 2′-deoxyadenosine (dAdo), 2′-deoxycytidine (dCyd), 2′-deoxyguanosine (dGuo), and the mononucleotides 2′-deoxyadenosine 5′- monophosphate (5′-dAMP), 2′-deoxycytidine 5′- monophosphate (5′-dCMP), 2′-deoxyguanosine 5′- monophosphate (5′-dGMP), thymidine 5′- monophosphate (5′-TMP) and uridine 5′- monophosphate (5′-UMP) were purchased from Sigma; the mononucleosides thymidine (Thd) and uridine (Urd) were purchased from Aldrich. Stated purity was ≥98%; all compounds were used without further treatment. Structures are shown in Fig. 2.

The solvents used were spectrophotometric grade methanol from Fluka, deuterium oxide from Merck and Millipore MilliQ water. Values of pH of mononucleotide solutions were adjusted to 6 (natural pH of βCMAM solution) by addition of an aqueous sodium hydroxide solutions, to avoid βCMAM acid-base equilibrium to compete with βCMAM-mononucleotide complex formation. For spectroscopic studies, to avoid re-absorption phenomena, measurements were made on 10^{-4} M solutions in 1% (*v*/*v*) methanol–water, obtained by preparing 10 mM stock solutions in methanol and diluting with Millipore water. Various volumes of nucleobase, nucleoside and nucleotide solutions were added directly to cuvettes containing βCMAM solutions (10^{-4} M). Solutions for the photophysical studies were degassed by bubbling with argon for around 15 min.

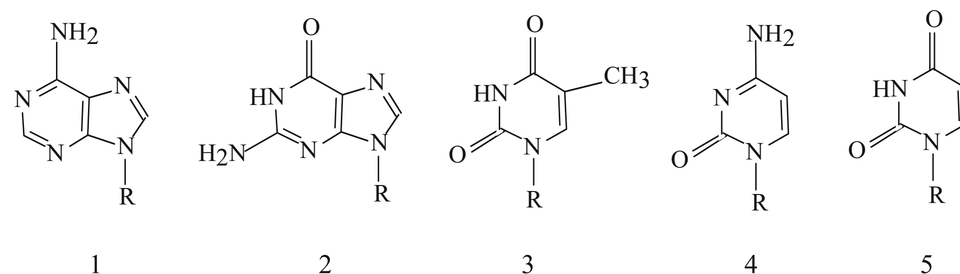
For NMR experiments, βCMAM solutions (10^{-3} M) were prepared in deuterated methanol (CD_3OD)-deuterium oxide (D_2O) mixtures (20% *v*/*v*). For ^{1}H NMR experiments, a few drops of tetramethylsilane were added as reference.

Apparatus and methods

Absorption spectra were recorded in 1 cm quartz cuvettes on a Shimadzu UV-2501 PC spectrophotometer. The steady-state fluorescence spectra were measured with excitation at 335 nm using a Shimazdu RF-5301 PC spectrofluorimeter (3.0 nm excitation bandwidth and 1.5 nm emission bandwidth, except for studies with guanine, where an emission bandwidth of 3 nm was necessary). All spectral measurements were made at 298 K. The quantum yields (ϕ) for the neutral and the cationic forms of βCMAM in aqueous solution were determined using norharmane in benzene (ϕ=0.30) [37] and in 0.05 M H_2SO_4 (ϕ=0.60), respectively, as standards, [39] respectively. Fluorescence decays were measured using a home-built Time-Correlated Single Photon Counting apparatus consisting of an IBH NanoLED (λ_{exc}=339 nm) as the excitation source, Jobin-Ivon monochromator, Philips XP2020Q photomultiplier, and Canberra instruments Time-to-amplitude converter and Multichannel Analyser. Alternate measurements (1,000 counts per cycle), controlled by Decay® software (Biodinâmica-Portugal), of the pulse profile at 339 nm and the sample emission were performed until $1–2\times10^{4}$ counts at the maximum were reached. [43] The fluorescence decays were analysed using the modulating functions method of Striker with automatic correction for the photomultiplier “wavelength shift”. [44] All experiments were carried out at room temperature (293 K).

^{1}H NMR and ^{31}P NMR spectra were recorded with a Varian Inova 400 spectrometer operating at 399.92 and 161.9 MHz, respectively.

Fig. 2 Structure of nucleobases, nucleosides and nucleotides



R	1	2	3	4	5
H	Ade	Gua	Thy	Cyt	Ura
HOH$_2$C, O, OH	dAdo	dGuo	Thd	dCyd	
HOH$_2$C, O, OH OH					Urd
O, HO—P—O—CH$_2$, O, OH, OH	5′-dAMP	5`-dGMP	5′-TMP	5′-dCMP	
O, HO—P—O—CH$_2$, O, OH, OH OH					5′-UMP

Results and discussion

Interaction of βCMAM with nucleobases, nucleosides and nucleotides was studied both in the ground electronic state, using UV-Visible absorption spectroscopy, ^{1}H and ^{31}P NMR spectroscopies, and in the first excited electronic state using steady-state and time-resolved fluorescence spectroscopy.

Ground state studies

UV-Visible absorption spectroscopy

Absorption spectra were measured on solutions at pH 6 containing varying amounts of βCMAM and the appropriate nucleobases, nucleosides or nucleotides but with the same total overall concentration (10^{-4} M). This gave molar fractions of the components varying between 0 and 1. In the case of Gua, due to its poor solubility, the concentration of βCMAM was 6×10^{-6} M, while the guanine concentration varied from 6×10^{-6} to 3.6×10^{-5} M. The corresponding Job plots of the absorbance at the maximum (335 nm) versus βCMAM molar fraction indicate predominant formation of a 1:1 complex (βCMAM: nucleobases, nucleosides or nucleotides) in all cases.

Association constants were obtained from absorption spectra of samples with a constant βCMAM concentration (10^{-4} or 6×10^{-6} M with guanine) and various concentrations of nucleobases, nucleosides or nucleotides. Evidence for complexation between βCMAM and the nucleobases, nucleosides or nucleotide was obtained by considering the decreases in absorbance at the two βCMAM maxima (335

and 347 nm) and increasing absorbance at wavelengths above 355 nm (the isosbestic point). These changes are qualitatively similar to those observed upon addition of oligonucleotides or DNA to βCMAM solutions. In all cases these can be attributed to complexation rather than an acid-base equilibrium since the pH remained constant (ca. 6). [32]

From the spectroscopic changes, the association constants in the ground state, K_G^{BH}, were calculated using the Benesi–Hildebrand equation in the following form for complexes with 1:1 stoichiometry: [33, 45]

$$\Delta OD = \frac{(\varepsilon_{\beta CMAM\text{-}N} - \varepsilon_{\beta CMAM})\,[\beta CMAM]_0 K_G^{BH}[N]}{1 + K_G^{BH}[N]} \qquad (1)$$

where $\varepsilon_{\beta CMAM\text{-}N}$ and $\varepsilon_{\beta CMAM}$ are the molar extinction coefficients of complexed and free βCMAM, respectively at the analytical wavelength, $[\beta CMAM]_0$ is its initial concentration, [N] is the ligand concentration (nucleobases, nucleosides or nucleotides) and ΔOD is the change of absorbance relative to free βCMAM at the absorption maximum, 335 nm, chosen as analytical wavelength since this is where the biggest change is observed upon complexation (Fig. 3).

The association constants shown in Table 1 were obtained from non-linear fittings of Eq. 1 for systems in which sufficiently large spectroscopic changes were observed. The poor solubility of nucleobases and the small spectral changes limit the complexation study in other cases.

The association constants are of the same order of magnitude, but possibly slightly higher, compared with those found for complexation of harmane with these compounds. [33] They are about one order of magnitude lower than those for complexation of βCMAM with polynucleotides, such as the sodium salts of polyadenylic (Poly A) or polyguanylic (Poly G) acids, or double stranded DNA at natural pH. [32] When the ligand has several positions for binding, as with the polynucleotides, the interaction with βCMAM is stronger probably due to cooperative effects.

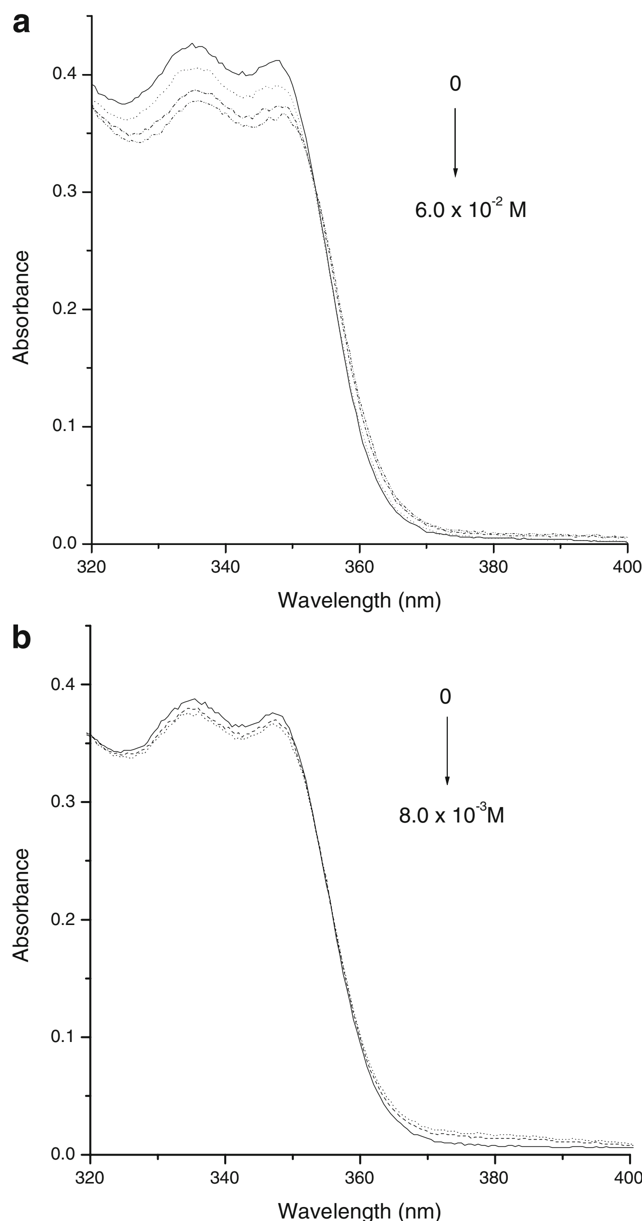


Fig. 3 Absorption spectra of βCMAM with different concentrations of: **a** thymidine, Thd, (0, 2.7×10^{-3}, 9.6×10^{-3}, 3.9×10^{-2}, 6.0×10^{-2} M) and **b** 2′-deoxycytidine 5′- monophosphate, 5′-dCMP, (0, 3.0×10^{-3}, 8.0×10^{-3} M)

¹H NMR spectroscopy

Further evidences for the interaction between βCMAM and nucleosides or nucleotides, were obtained from ^{1}H NMR and ^{31}P NMR experiments. No signal of ^{13}C for βCMAM was detected in our experimental conditions. The poor solubility of nucleobases did not allow us to obtain information on βCMAM binding with them using this technique.

^{1}H NMR spectra of samples of the β-carboline (ca 1 mM) with different concentrations of nucleosides or nucleotides with βCMAM:ligand ratios varying from 1:1 to excess of ligand (limited by the solubility of the compounds) were obtained in deuterated methanol (CD_4)-deuterium oxide (D_2O) mixtures (20% *v*/*v*).

The βCMAM ^{1}H NMR peaks at 8.54 and 8.66 ppm in the aromatic region were found to be the most sensitive to additions of nucleosides and nucleotides. These peaks are attributed to protons 5 and 8 of the indol group (Fig. 1), respectively. [46] In Trypargine, a β-carboline derivative which lacks the charge withdrawing methylamide group, these two proton peaks appear at 7.51 and 7.62 ppm, respectively. [47] In this spectral region there are no nucleoside or nucleotide signals, which facilitates the study

Table 1 Benesi–Hildebrand association constants for the ground state K_G^{BH}, calculated according to Eq. 1

Compound	dAdo	Thd	dCyd	Urd	5′-dCMP	5′-UMP
K_G^{BH}(mol^{-1} L)	48±17	47±8	11±5	5±3	59±40	25±22

of the effect of complexation in this region. For most of the systems, an upfield chemical shift was seen in the peaks at 8.54 and 8.66 ppm, as seen in Fig. 4 for βCMAM-Thd. In a few cases, there was no effect or a slight downfield shift (≤0.05 ppm) in these peaks.

The chemical shift increments, $\Delta\delta$ ($\Delta\delta = \delta_{\beta CMAM} - \delta_{\beta CMAM-ligand}$) at several βCMAM:ligand ratios are shown in Fig. 5 for the peaks at 8.66 and 8.54 ppm.

From both UV-Visible and ^{1}H NMR, the strength of the complex formed in the ground state was studied. Larger $\Delta\delta$ values (≥0.05 for the βCMAM:ligand ratio 1:1) indicate formation of a stronger complex (K_G^{BH} ≥25 mol^{-1} L). From this, the strongest interactions seem to be between βCMAM and the mononucleosides: dAdo, Thd and the mononucleotides: 5′-dCMP and 5′-UMP, which is in excellent agreement with the association constants obtained from UV-Visible spectroscopy (Table 1) However, absorption spectroscopy seems to be slightly more sensitive than ^{1}H NMR to detect complex formation for dCyd and Urd since association constants were determined from the changes in absorption spectra (K_G^{BH}=11 and 5 mol^{-1} L, respectively) although these nucleosides show $\Delta\delta\approx 0$. For all the other systems with $\Delta\delta\approx$ 0, it was not possible to determine association constants due to the small changes in the absorption spectra, showing, with two different spectroscopic techniques, that the interaction is very weak.

From data shown in Fig. 5 it can be seen that the maximum $\Delta\delta$ values are larger for nucleotides than for nucleosides (the upper limits of *Y* axe scale are: 0.40 and 0.20, respectively) and the highest K_G^{BH} constants are also higher for nucleotides (59 mol^{-1} L for 5′-dCM) than for nucleosides (48 mol^{-1} L for dAdo), suggesting that the maximum interaction is stronger for nucleotides than for nucleosides. In addition, the peak at around 8.54 ppm seems to be slightly more sensitive to complex formation than that at 8.66 ppm.

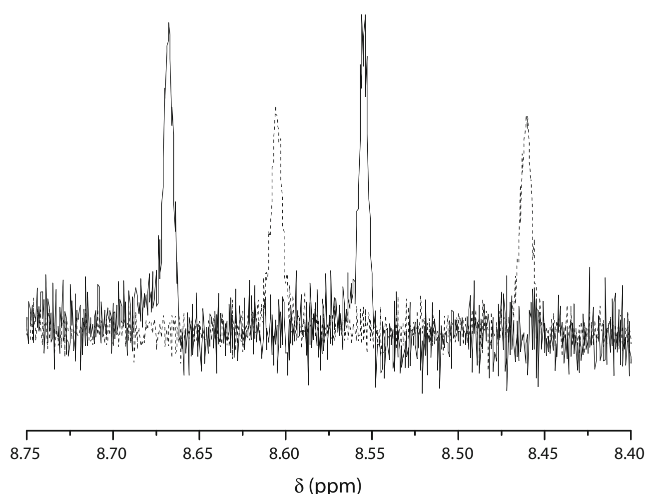


Fig. 4 ^{1}H NMR of βCMAM (2.5×10^{-3} M), *solid line*, and βCMAM (2.5×10^{-3} M) with Thd (10^{-1} M), *dotted line*, in deuterated methanol (CD_3OD)-deuterium oxide (D_2O) mixtures (20% *v/v*)

In the literature it has been indicated that indole protons of indole-3-acetate are shifted up to 0.45 ppm upfield on formation of complexes with [Pt(bpm)(L-Arg)] in D_2O, and this has been attributed to the stacking of the indole ring with the bpm moiety coordinated to platinum. [48] We believe that similar effects could be involved in the upfield shift of the βCMAM signals, due to stacking of βCMAM with the base of nucleosides or nucleotides, as suggested for the interaction of harmane with nucleobases, nucleosides and nucleotides. [33] Such upfield shifts of Ade, Thy, Gua and Cyt resonances, accompanied by signal broadening, have been considered to be indicative of intercalative binding of β-cabolines to DNA by π-stacking. [31]

Roughly speaking, we could say that, in the ground state, βCMAM interaction is favoured with nucleosides or nucleotides containing pyrimidine bases with respect to those ones containing purine ones. In the latter case, the association constant was only determined for dAdo. These results could be explained by π-stacking between βCMAM and the bases that would be favoured in the cases of pyrimidine bases (smaller than purine ones).

However, as different $\Delta\delta$ values are obtained for the two peaks considered and these values do not follow a common pattern with the type of base involved in the binding (for nucleosides and nucleotides interaction is stronger for dAdo, Thd, 5′-dCMP and 5′-UMP) we believe that other interactions may also be involved in the complex formation. Possible candidates for these interactions include hydrogen bonding and charge transfer interactions between the purine or pyrimidine bases and βCMAM.

^{31}P NMR spectroscopy

Upon addition of βCMAM, the ^{31}P NMR signals of the nucleotides are shifted downfield, and in many cases are slightly broadened, as shown in Fig. 6. The maximum downfield shift is generally observed for a 1:1 βCMAM: nucleotide ratio, confirming results from Job plots of absorption spectra of the stoichiometry of complexes. With excess nucleotide, the ^{31}P NMR signal sharpens and shifts

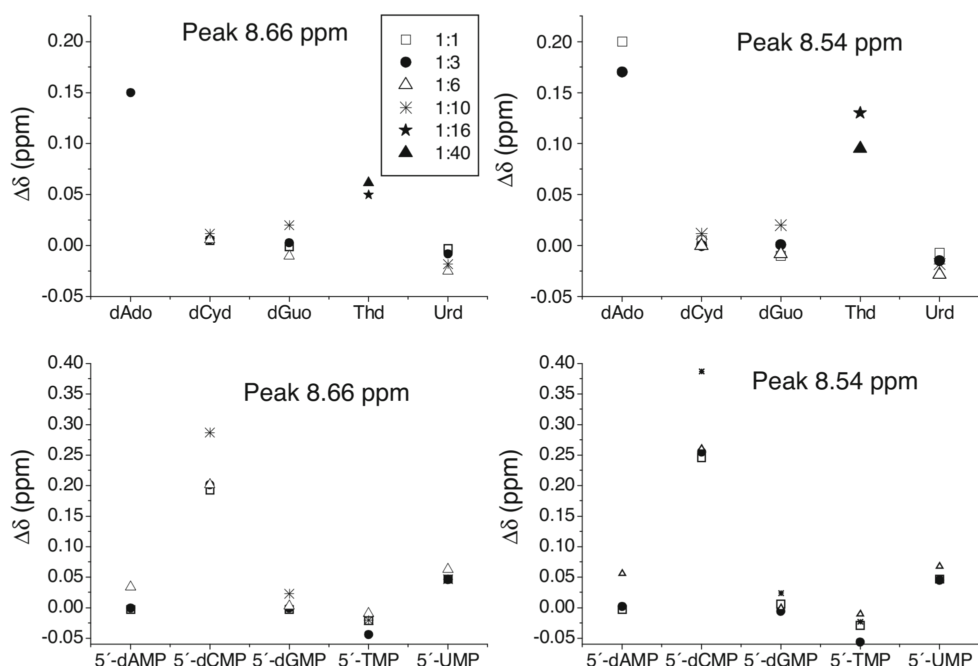


Fig. 5 $\Delta\delta$ ($\Delta\delta = \delta_{\beta\mathrm{CMAM}} - \delta_{\beta\mathrm{CMAM-ligand}}$) for the peaks at 8.66 and 8.54 ppm at several βCMAM:ligand ratios

back to the value for the pure nucleotide. The fact that only a single peak is observed suggests fast exchange, and that we are seeing an average of complexed and free nucleotides.

No direct correlation is seen between the changes in chemical shift of ^{31}P NMR and ^{1}H NMR resonances, which indicates we are seeing different facets of the complexation of βCMAM with nucleotides.

The ^{31}P NMR signal of free nucleotides is also shifted downfield upon increasing pH which indicates that any interaction involving weakening of the PO–H bond of the partially protonated phosphate groups at pH 6 (pK_a=0.7–1.0 and 6.1–6.4 for the phosphate groups of ribonucleotides) [49] either by electrostatic interaction or hydrogen bonding with βCMAM, would explain the downfield shift of phosphorous peak. Similar downfield shifts of the ^{31}P NMR resonance of nucleotides have been described for complexes with azoniacyclophanes containing phenyl-, biphenyl- or bipyridyl-units and attributed either to electrostatic interaction or to polyammonium group interactions with phosphate group. [50]

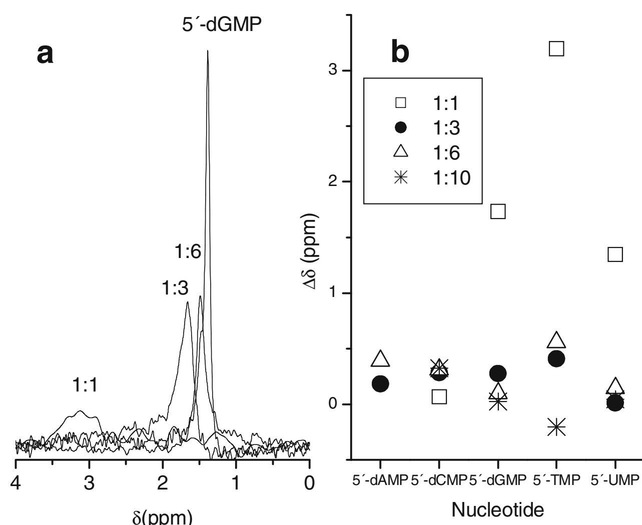


Fig. 6 **a** ^{31}P NMR spectra of 5′-dGMP (10^{-2} M) and βCMAM (10^{-3} M) with 5′-dGMP (10^{-3}, 3×10^{-3}, 6×10^{-3} and 10^{-2} M) in deuterated methanol (CD_4)-deuterium oxide (D_2O) mixtures (20% *v/v*; curve for βCMAM:5′-dGMP, ratio1:1, has been multiplied by 2), **b** $\Delta\delta$ ($\Delta\delta=\delta_{\mathrm{Nucleotide\text{-}ligand}}-\delta_{\mathrm{Nucleotide}}$) at several βCMAM:ligand ratios

The presence of interactions between βCMAM and both the phosphate groups of nucleotides and the bases could explain the slightly higher association constants observed with nucleotides compared to nucleosides (Table 1) in addition to the larger $\Delta\delta$ values seen in ^{1}H NMR experiments (Fig. 5)

First excited electronic state

Fluorescence spectroscopy

Upon adding nucleobases, nucleosides or nucleotides to a solution of βCMAM in methanol-water (1% *v/v*), quenching of the emission is observed. This has been analysed by a Job plot of fluorescence intensity versus βCMAM molar fraction, and indicates that 1:1 interaction is also predominant in the first excited state.

To gain insight into the nature of the quenching process, steady state and time-resolved fluorescence experiments were carried out in the presence of various concentrations of substrate solutions keeping constant βCMAM molar concentration (10^{-4} M). The non-stationary experiments

were carried out exciting at 339 nm and recording the emission at the maximum (387 nm) Decays of βCMAM as either the isolated compound or in the presence of nucleobases, nucleosides and nucleotides were fitted to a single exponential. This is in agreement with the fact that, under our experimental conditions, only the emission of the neutral form of βCMAM is observed.

From the steady state experiments, good Stern–Volmer plots were obtained according to Eq. 2, while for the time resolved measurements data were treated using Eq. 3:

$$\frac{F_0}{F} = 1 + K_{\mathrm{SV}}^{\mathrm{s}}[N] \quad (2)$$

$$\frac{\tau_0}{\tau} = 1 + K_{\mathrm{SV}}^{\mathrm{n-s}}[N] \quad (3)$$

Here F_0 and F are the emission intensities in absence and presence of quencher (nucleobase, nucleoside or nucleotide), $[N]$ is the molar concentration of quencher, τ_0 and τ are the βCMAM lifetimes in absence and presence of quencher, respectively. $K_{\mathrm{SV}}^{\mathrm{s}}$ and $K_{\mathrm{SV}}^{\mathrm{n-s}}$ are the Stern–Volmer constants obtained from the steady state and time resolved experiments are summarised in Table 2.

Good linear fittings were obtained plotting the ratio F_0/F versus $[N]$ for all the substrates (from zero to millimolar concentrations, except for guanine, where lower concentrations were used due to poor solubility; up to 5.6×10^{-5} M). With cytosine both absorbance at 335 nm and emission intensity slightly increase, probably due to experimental errors such as scattering effects. When cytosine is added to βCMAM solutions Rayleigh scattering at 335 nm grows from 5 to 21 in arbitrary units (cytosine concentration from 0 to 1×10^{-4} M, respectively) while for systems such as βCMAM-thymidine, scattering is kept around 5 in arbitrary units for a higher thymidine concentration range (0 to 1.5×10^{-2} M). Good Stern–Volmer plots were also observed plotting τ_0/τ versus $[N]$. However, in general, the slopes of the dynamic quenching plots are smaller than those from steady-state fluorescence measurements, suggesting that both static and dynamic quenching of βCMAM emission by nucleobases, nucleosides and nucleotides may be involved. The exceptions seem to be with guanine and cytosine. However, the lack of effect of guanine concentration on the lifetime is due probably to the narrow range of concentrations used.

Second order rate constants, k, have been calculated by plotting $1/\tau$ against substrate molar concentration, $[N]$. Good linear fittings were obtained with most of the systems. The second order rate constant (the slopes of the linear fittings) are shown in Table 2. Except for dAdo and dCyd, the second rate constant values obtained are between around 2×10^9 and 4×10^9 L mol^{-1} s^{-1}, close, although slightly lower, than the rate of diffusion controlled processes given by Murov for uncharged species of the same size at 25 °C in water, 7.4×10^9 L mol^{-1} s^{-1}. [51] This seems to indicate that, in most cases, dynamic quenching has an important contribution. This is corroborated by the fact that, dAdo and dCyd, which have lower second rate constant values, show also lower $K_{\mathrm{SV}}^{\mathrm{s}}$ values. However, with the steady state results for Gua, dGuo or 5′-dGMP in Table 2 we can conclude that the quenching is predominantly static in these systems since the steady state rate constants $K_{\mathrm{SV}}^{\mathrm{s}}$, which depend on both static and dynamic quenching, are much higher than for the other systems while $K_{\mathrm{SV}}^{\mathrm{n-s}}$ are similar. In addition, in these systems no strong ground state complexes seem to be formed and taking into account that guanine is the most easily

Table 2 Steady-state, $K_{\mathrm{SV}}^{\mathrm{s}}$, dynamic, $K_{\mathrm{SV}}^{\mathrm{n-s}}$, Stern–Volmer constants and second order rate constant, k

Compound	$K_{\mathrm{SV}}^{\mathrm{s}}$ mol^{-1} L	$K_{\mathrm{SV}}^{\mathrm{n-s}}$ mol^{-1} L	k L mol^{-1} s^{-1}
Ade	24±1	5.4±0.1	$2.4\times10^9\pm7\times10^8$
Cyt	Not determined	13.7±1.5	$2.8\times10^9\pm3\times10^8$
Gua	552±30	Not determined	Not determined
Thy	21±1	16.1±1.6	$4.3\times10^9\pm2\times10^8$
Ura	18±1	7.6±0.2	$2.6\times10^9\pm5\times10^8$
dAdo	15±1	0.6±0.1	$1.3\times10^8\pm1\times10^7$
dCyd	5.0±0.1	2.9±0.1	$6.2\times10^8\pm2\times10^7$
dGuo	95±1	14.4±1.1	$3.0\times10^9\pm2\times10^8$
Thd	47±1	14.9±0.4	$3.1\times10^9\pm8\times10^7$
Urd	34±1	10.5±0.1	$2.2\times10^9\pm3\times10^7$
5′-dAMP	27±1	13.6±0.5	$2.1\times10^9\pm6\times10^8$
5′-dCMP	13.1±0.1	7.8±0.3	$2.1\times10^9\pm6\times10^7$
5′-dGMP	65±1	9.6±1.3	$2.0\times10^9\pm3\times10^8$
5′-TMP	29±1	8.2±0.8	$1.7\times10^9\pm2\times10^8$
5′-UMP	23.5±0.2	8.0±0.6	$1.7\times10^9\pm1\times10^8$

oxidizable of the nucleobases, [52, 53] a possible explanation for the strongest static quenching would be a charge transfer to the first excited electronic state of βCMAM. Compounds that contain guanine base also seem to be more efficient quencher of harmane fluorescence than the phosphate groups. [33]

With harmane, static and dynamic quenching were generally only reported with nucleotides and in these cases, the dynamic quenching was associated with electrostatic interactions of harmane with the phosphate dianions. [33] However, we think that in our systems, other groups are also involved in the quenching, since dynamic quenching is not only observed for βCMAM- nucleotides systems, but also with nucleosides and nucleobases.

To analyze the possible effect of charge transfer on the quenching processes the steady-state, K_{SV}^{s} and dynamic, K_{SV}^{n-s}, Stern–Volmer constants of βCMAM with nucleobases, nucleosides and nucleotides are plotted versus the experimental reduction potential of the nucleobases, E(Red) [52] (Fig. 7).

The same reduction potential was used for nucleobases, nucleosides and nucleotides with the same base component, which seems to be a reasonable assumption because, the differences between E(Red) of these compounds for several nucleobase derivatives are normally below 0.05 V, as has been shown by Steenken *et al.* [54] in the case of uracil, uridine, uridine-5′-phosphate, thymine, thymidine, thymidine-5′-phosphate, cytosine, cytidine, and cytidine-5′-phosphate at pH between 8 and 9.

Dynamic Stern–Volmer constants, K_{SV}^{n-s} do not follow any clear tendency with E(Red); however, the steady-state Stern–Volmer constants are dependent on these potentials. K_{SV}^{s} constants decrease approximately exponentially with the increase of their E(Red) in the case of nucleobases (Fig. 7a). Two sets of K_{SV}^{s} constants can be distinguished whose values decrease linearly with the increase of nucleobases E(Red) for nucleosides and nucleotides (Fig. 7b and c, respectively). For nucleosides, the two sets are: dGuo > dAdo > dCyd and dGuo > Thd > Urd; and for nucleotides: 5′-dGMP > 5′-dAMP > 5′-dCMP and 5′-dGMP > 5′-TMP > 5′-UMP.

Seidel *et al.* concluded that the quenching of fluorescent dyes follows the sequence: Gua > Ade > Cyt > Thy ≥ Ura if the nucleobases are oxidized. [52] This is mainly what we observe in our systems for nucleobases (when data are available). For nucleosides and nucleotides the behaviour is

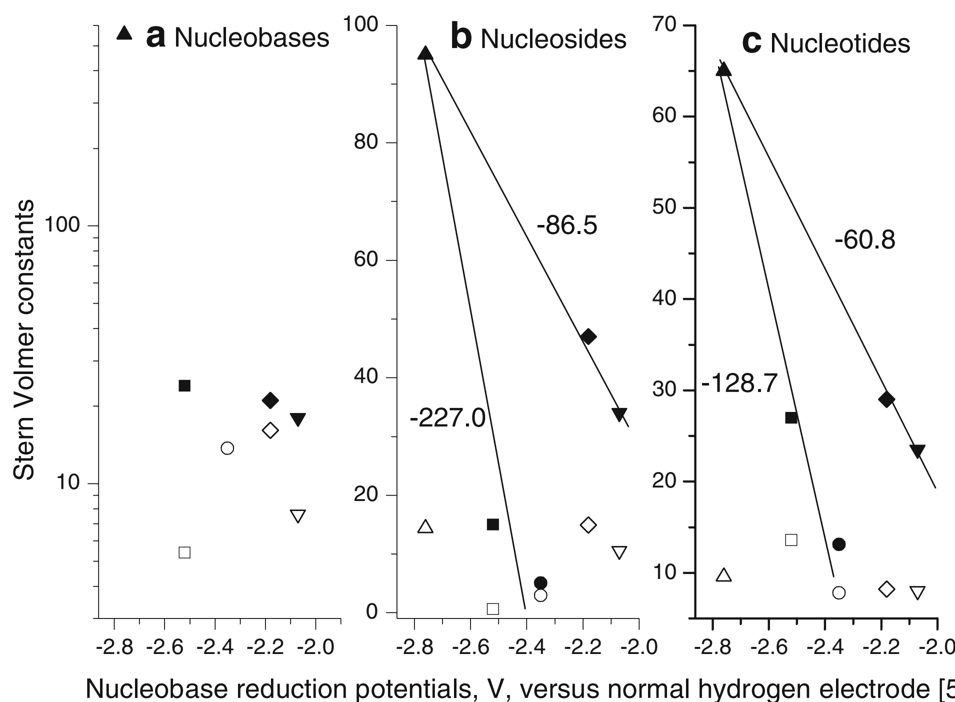


Fig. 7 Steady-state, K_{SV}^{s} (*full symbols*) and dynamic, K_{SV}^{n-s} (*empty symbols*), Stern–Volmer constants of βCMAM versus the nucleobases reduction potentials [52]. **a** Nucleobases: (*squares*) Ade, (*circles*) Cyt, (*upright triangles*) Gua, (*diamonds*) Thy, (*inverted triangles*) Urd; **b** Nucleosides: (*squares*) dAdo, (*circles*) dCyd, (*upright triangles*) dGuo, (*diamonds*) Thd, (*inverted triangles*) Urd; **c** Nucleotides: (*squares*) 5′-dAMP, (*circles*) 5′-dCMP, (*upright triangles*) 5′-dGMP, (*diamonds*) 5′-TMP, (*inverted triangles*) 5′-UMP. For A-Nucleobases Stern–Volmer constants are plotted in logarithmic scale due to the high value of K_{SV}^{s} of guanine. For nucleosides and nucleotides the slopes of the linear fittings are also shown

close to that predicted by Seidel *et al.* but with the compounds grouped in two sets, according to their nucleobase component: Gua > Ade > Cyt and Gua > Thy > Ura. According to this, we can suggest that nucleobases, nucleosides and nucleotides may be oxidized by βCMAM in the first excited electronic state.

We notice, that except for Gua, the other two elements of the series have in common a similar six atom ring structures (Ade-Cyt and Thy-Ura) (Fig. 2). Moreover, the higher the structural differences between the structure of the non-common series elements of the two series (Ade-Cyt > Thy-Ura, Fig. 2) the higher the dependence of the K^{s}_{SV} Stern–Volmer constants on the E(Red), as it can be concluded from the slopes of the linear fittings of these constants versus E(Red), shown in Fig. 7b and c. This means that βCMAM static quenching constants are showing selectivity for the nucleoside and nucleotide six atom ring structures through the charge transfer from nucleobase constituents to βCMAM first excited state.

From the comparison of the slopes of the linear fitting of K^{s}_{SV} Stern–Volmer constants of nucleosides and nucleotides with E(Red) it seems that the dependence of the K^{s}_{SV} constants on the charge transfer to βCMAM first excited state is stronger for nucleosides than for nucleotides, indicating that possibly, for nucleotides, other factors apart from the charge transfer process, are involved. Seidel et al. indicate that a coupled proton transfer and a hydrophobic effect should also be considered [52].

From our ^{31}P NMR results (Fig. 6) and the static quenching constants of nucleosides and nucleotides shown in Table 2, we believe that the electrostatic interaction and/or hydrogen bonding between βCMAM and PO-H are also important factors in the βCMAM steady-state quenching by some nucleotides. In fact, those nucleotides with higher ^{31}P NMR signal chemical shifts (5′-TMP > 5′-GMP > 5′-UMP, Fig. 6) show a higher decrease of K^{s}_{SV} in comparison with the same constant of the respective nucleosides. The higher the ^{31}P NMR signal shift, the higher the decrease as it is probed by the ratio between the K^{s}_{SV} constant of nucleotide and the K^{s}_{SV} constant of its counterpart nucleoside that are below one: 5′-TMP/Thd (0.61) > 5′-dGMP/dGuo (0.68) > 5′-UMP/Urd (0.69). However, for those nucleotides with hardly any shift in ^{31}P NMR signal (5′-dAMP and 5′-dCMP, Fig. 6), the same K^{s}_{SV} constant ratios are higher than one [5′-dAMP/dAdo (1.8) >5′-dCMP/dCyd (2.6)] showing that in these cases the quenching is more effective for the nucleotide derivatives. It could be concluded that the establishment of additional electrostatic interaction and/or hydrogen bonding between βCMAM and PO-H decrease the efficiency of the photoinduced electron charge transfer from nucleotides to the first excited state of βCMAM. These interactions can also be expected to occur in polynucleotides (sodium salts of the: polyadenylic (Poly A), polycytidylic (Poly C), polyguanylic (Poly G), polythymidylic (Poly T) and polyuridylic (Poly U) acids) and single strand deoxyribonucleic acid (ssDNA) at pH 6 [32] as can be induced from the very reasonable agreement between the steady-state Stern–Volmer constants of nucleotides, polynucleotides and ssDNA (Table 3). K^{s}_{SV} constants for double strand deoxyribonucleic acid (dsDNA) at pH 6 are also presented.

From the data shown in Table 3, it is worthy of note that, except for dsDNA in which intercalative binding of βCMAM to DNA by π-stacking is possible and poly G with a high contribution of electron transfer to βCMAM, probably favoured by cooperative effects, the other systems for which data are available have comparable K^{s}_{SV} constants. Nucleotide and polynucleotide steady state Stern–Volmer constants are very close and the value for ss-DNA is around the average value of those ones of the nucleotides (5′-dAMP, 5′-dCMP, 5′-dGMP and 5′-TMP). This suggests that the interaction between βCMAM and ssDNA is quite similar to the interaction between βCMAM and the DNA constituent nucleotides showing the same specific interactions (electrostatic interaction and/or hydrogen bonding). These interactions seem to be relevant when no high electron transfer or π-stacking are involved (Poly G and dsDNA, respectively).

Conclusions

Experimental evidence for the interaction between β-carboline-3-carboxylic acid *N*-methylamide (βCMAM) and nucleobases, nucleosides and nucleotides is obtained

Table 3 Steady-state, K^{s}_{SV}, Stern–Volmer constants of the quenching of βCMAM emission by nucleotides, polynucleotides (Poly A, Poly C, Poly G, Poly T and Poly U), ssDNA and dsDNA at pH 6 [32]

Nucleotides	K^{s}_{SV} mol^{-1} L	Polynucleotides	K^{s}_{SV} mol^{-1} L	DNA pH=6	K^{s}_{SV} mol^{-1} L
5′-dAMP	27±1	Poly A	31.2±0.5	ssDNA	41.5±1
5′-dCMP	13.1±0.1	Poly C	10.5±0.5	dsDNA	1,394.6±23
5′-dGMP	65±1	Poly G	631.4±21		
5′-TMP	29±1	Poly T	–		
5′-UMP	23.5±0.2	Poly U	14.2±0.1		

by the hypochromism in the absorption spectra of βCMAM with the appearance of an isosbestic point at 355 nm (indicative of an association equilibrium), by an upfield shift of ^{1}H NMR resonance of protons 5 and 8 of the indole group of βCMAM, a downfield shift of the ^{31}P NMR signal of the phosphorus atom of nucleotides and the quenching of βCMAM fluorescence. Job plots of both absorption and emission data, coupled with ^{31}P NMR shift increments (for several nucleotides) show that a 1:1 (βCMAM:ligand) interaction is predominant for all the systems studied in both ground and first excited states.

From UV-visible and ^{1}H NMR spectral data, the strongest interactions are observed with the nucleosides dAdo and Thd (48±17 and 47±8 mol^{-1}L) and with the nucleotides 5′-dCMP and 5′-UMP (59±40 and 25±22 mol^{-1}L). Association constants have been calculated using Benesi-Hildebrand equation for a 1:1 complex and seem to be slightly higher for compounds containing pyrimidine bases (with the exception of dAdo) probably due to π stacking contributions as can be induced from the shifts of ^{1}H NMR signals, although hydrogen bond with carbonyl group could be also involved. Electrostatic interaction and/or hydrogen bond between βCMAM and nucleotide would explain shifts of ^{31}P NMR signal of phosphorus in nucleotides upon complexation with βCMAM. This probes that other type of interactions are involved in the complexation of βCMAM with DNA constituents, apart from intercalation.

Static and dynamic quenching of βCMAM fluorescence is observed upon complexation with nucleobases, nucleosides and nucleotides and Stern–Volmer constants are calculated from both the steady-state and time-resolved experiments. The compounds with the guanine ring are the most efficient quenchers and their steady-state Stern–Volmer constant are higher than for the compounds with other nucleobases indicating a major contribution of a static component in the quenching in spite of the poor interaction of these compounds with βCMAM in the ground state. This is suggested to be due to a charge transfer from Gua, dGuo or 5′-dGMP to βCMAM in the first excited electronic state since it is well-known that guanine is the most easily oxidizable of the nucleobases. Although, a charge transfer process may be more important for Gua, dGuo and 5′-dGMP, it also has an effect on the steady state quenching of βCMAM emission by the other nucleobases, nucleosides and nucleotides. For nucleosides and nucleotides, a linear decrease of the steady-state Stern–Volmer constants is observed for the series: dGuo > dAdo > dCyd and dGuo > Thd > Urd (nucleosides) and 5′-dGMP > 5′-dAMP > 5′-dCMP and 5′-dGMP > 5′-TMP > 5′-UMP (nucleotides). From this, it was concluded that nucleobases, nucleosides and nucleotides are oxidized by βCMAM in the first excited electronic state, showing certain selectivity for the structural similarity between the nucleobase six atom ring.

For nucleotides, electrostatic interactions and/or hydrogen bonding between βCMAM and PO-H are also important for explaining the quenching of βCMAM emission. When this kind of interactions are probed by the ^{31}P NMR signal shift, a decrease of the steady-state Stern–Volmer constants of 5′-TMP, 5′-dGMP and 5′-UMP nucleotides in comparison with the correspondent Thd, Guo, Urd nucleosides are observed. The opposite behaviour is observed for 5′-dAMP and 5′-dCMP in comparison to dAdo and dCyd, when no strong interaction between βCMAM and PO-H group of the nucleotide can be induced from the ^{31}P NMR signal shift.

βCMAM steady-state Stern–Volmer constants with polynucleotides (Poly A, Poly C, Poly T and Poly U) and ssDNA are very close to those calculated with the nucleotides indicating that similar electrostatic and hydrogen bonding interactions take place. For Poly G and dsDNA additional cooperative charge transfer and π-stacking contributions increase dramatically the βCMAM steady-state Stern–Volmer constant in one and two orders of magnitude, respectively.

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