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Entropy effect in the evaluation of the proton affinity of N-3-benzoyl-2-deoxycytidines by the kinetic method

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Abstract

The kinetic method has been applied, in its extended form, to the evaluation of the entropy effect in the determination of the proton affinity (PA_N) of *N*-3-para substituted benzoyl 2′-deoxycytidines. The latter, mixed with nucleosides of known PA, afforded the appropriate proton-bound heterodimers when subjected to electrospray ionization. Their collisional induced dissociations were studied in a Q*q*TOF hybrid instrument as a function of both the gas pressure and the collision energy of the dimers entering *q*. The value of ΔS for each reaction was evaluated and correlated with the effect of the substituent at the para position of the benzoyl group. For each reaction, a Hammett plot of ΔPA_N vs. σ values of the substituents on the benzoyl group shows that the carbonyl group participates, in the transition state, to the stabilization/destabilization of the N-3-protonated species. In the cases examined, one can rule out that the formation of intramolecular hydrogen bonds occurs under thermodynamic control. The activation energy for the back reaction can, therefore, be neglected. [See chart on next page.] (Int J Mass Spectrom 210/211 (2001) 165–172) © 2001 Elsevier Science B.V.

Keywords: Proton affinity of benzoylcytidines; Kinetic method; σ-Hammett proton affinity correlation

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1. Introduction

In an earlier application of the kinetic method [1], eqn. 1 was used to determine the PA_N of a given nucleoside or nucleobase relative to the PA_a of a reference amine [2]. This equation was derived from the simplified form of the rate constant equations for the energy-dependent competing dissociations of the proton bound $[dN + a + H]^+$ ions, equations 2a and 2b.

$$\ln(k_N/k_a) = \ln \nu_N/\ln \nu_a + A(s - 1)(\Delta E_0/E) \qquad (1)$$

Equation 1 holds for the dissociation of proton-bound dimers, which competitively dissociate into each of the singly protonated species illustrated in Equations 2a and 2b. The equation can be written in the form of equation 3 which gives the PA_N unknown species:

$$[N - H - a]^+ \xrightarrow{k_N} [NH]^+ + a \qquad (2a)$$

$$[N - H - a]^+ \xrightarrow{k_N} [aH]^+ + N \qquad (2b)$$

$$PA_N = \frac{[\ln(k_N/k_{a_1})]PA_2 - [\ln(k_N/k_{a_2})]PA_1}{\ln(k_N/k_{a_2}) - \ln(k_N k_{a_1})} \qquad (3)$$

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Dedicated to Professor Nico Nibbering on the occasion of his retirement.

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Table 1
Relative percentage of the product ions (12 scans average) of the proton-bound dimers formed between BCYT and TPA, TBA, TPEA, DIPEA whose PA values[a] are 236.90, 238.39, 238.87, 237.57 kcal/mol, respectively

	$[\text{BCYT-H}]^+$	$[\text{TPA-H}]^+$	$[\text{TBA-H}]^+$	$[\text{TPEA-H}]^+$	$[\text{DIPEA-H}]^+$
$[\text{BCYT-H-TPA}]^+$	71.3	28.7			
$[\text{BCYT-H-TBA}]^+$	16.4		83.6		
$[\text{BCYT-H-TPEA}]^+$	2.9			97.1	
$[\text{BCYT-H-DIPEA}]^+$	50				50

[a] See 10.

Equation 3 is simply derived from Equation 1 if the reverse activation energy, if any, for each single reaction path (Equations 2a and 2b) is neglected and if the two competing processes are characterized by similar entropy changes. These assumptions require that the interacting species possess similar structures and hence similar internal energies and equivalent effective numbers of oscillators. This approach was used to determine the PA of a series of nucleosides [3, 4].

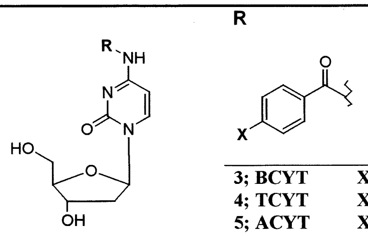
The many applications of the Cooks method [5, 6] reported in the last two decades allows one to conclude that for loosely bound species, as exemplified by Equations 2a and 2b, which react through product-like transition states, the assumptions made in deriving Equation 1 hold. A more comprehensive application of the method considers the evaluation of the thermodynamic parameters effective temperature (T_{eff}) [7] and apparent affinity (ΔG^{app}) [8], to be used in the Armentrout approach [9] for the determination of the thermodynamic parameters of interest.

2. Experimental

2.1. Mass spectrometry

Fast atom bombardment (FAB) experiments were performed on a B-E sector instrument (VG-ZAB-2F) operating at the accelerating potential of 8 keV using a standard FAB gun. A neutral Xe beam of 8 keV energy and a neutral flux derived from an ion current of ~10 μA were employed. The proton-bound dimers were produced by bombardment of 1–2 μL of saturated solution of glycerol containing the given deoxycytidine and the reference amine. The spectra were recorded at a resolving power of 1000 by scanning the magnetic field. The mass-analyzed ion kinetic energy (MIKE) spectra were acquired by scanning the electrostatic sector potential upwards. The relative abundance of the product ion peaks (Table 1) corresponds to the average of the best 12 spectra. The standard deviation in the detection of each peak area was in the range of 0.09–0.2 mm^2.

The electrospray ionization (ESI) experiments were carried out in a hybrid Q*q*TOF mass spectrometer (PE SCIEX-QSTAR) equipped with an ion spray ionization source. Proton-bound dimers were generated by direct infusion (5 μL/min) of a solution containing the appropriate modified 2′-deoxycitidine (**3–8,** as seen in) and the given reference compound (10 pmol/μL, dissolved in solution 0.1% acetic acid, methanol/water 50/50) at the optimum ion spray voltage of 4500 V. The nitrogen gas flow was set at 30 psi (pounds per square inch) and the potentials of the orifice, the focusing ring and the skimmer were kept at 30, 50, and 25 V relative to ground, respectively. MS/MS experiments were performed in the collision cell *q* on the isotopically pure (^{12}C) peak of the



3; BCYT	**X = H**
4; TCYT	**X = CH3**
5; ACYT	**X = OCH3**
6; CBCYT	**X = Cl**
7; NCYT	**X = NO2**
8; PACYT	**X = Ph Ac**

Chart

Table 2
Experimental data for the determination of the proton affinity by ESI-MS/MS of nucleosides 3–6 and 8

	PA	$\ln(k_N/K_{ref})$				Plot $\ln(k_N/k_{ref})$ versus $PA\text{-}PA_{avg}$	
Reference bases for BCYT	(kcal/mol)	CAD 1	CAD 2	CAD 3	CAD 4	slope	intercept
2′,3′-ddC	237.60[4]	0.602	0.608	0.709	0.719	−2.1134	2.1408
2′-dA	237.00[4]	2.231	2.363	2.374	2.314	−2.3669	2.24
A	236.40[4]	3.476	3.476	3.604	3.573	−2.4014	2.3347
3′-dA	236.80[4]	2.442	2.512	2.653	2.686	−2.3797	2.3231
Reference bases for TCYT							
2′,3′-ddC	237.60[4]	1.889	1.926	1.836	1.835	−2.8911	2.8837
BCYT	237.62	1.829	1.815	1.815	1.900	−2.9634	2.9253
2′-dA	237.00[4]	3.604	3.705	3.808	3.823	−2.9119	2.8824
3′-dA	236.80[4]	4.212	4.254	4.069	4.254	−3.0204	2.9534
Reference bases for ACYT							
2′,3′-ddC	237.60[4]	2.568	2.689	2.769	2.791	−8.0256	1.6467
BCYT	237.62	2.327	2.171	2.231	2.050	−8.0411	1.6347
TCYT	237.84	0.514	0.507	0.575	0.532	−8.1489	1.69
PACYT	237.79	1.176	1.172	1.184	1.153	−8.0068	1.6317
Reference bases for CBCYT							
G	236.80[2]	0.328	0.374	0.239	0.391	−2.1674	−0.0116
2′-dA	237.00[4]	0.160	−0.160	−0.120	−0.020	−2.3797	−0.0759
2′,3′-ddC	237.60[4]	−1.616	−1.671	−1.578	−1.434	−2.2976	−0.0679
A	236.40[4]	1.046	1.154	1.190	1.128	−2.1495	0.0163
Reference bases for PACYT							
2′,3′-ddC	237.60[4]	1.534	1.298	1.348	1.475	−3.4272	1.5083
BCYT	237.62	0.219	0.219	0.199	0.221	−3.5127	1.5337
2′-dA	237.00[4]	2.634	2.658	2.836	2.889	−3.5569	1.5636
3′-dA	236.80[4]	2.863	2.944	2.891	2.934	−3.5893	1.5989
Reference bases for NCYT							
C	234.8[4]	−1.036					
2′,5′-ddC	234.6[4]	−0.848					

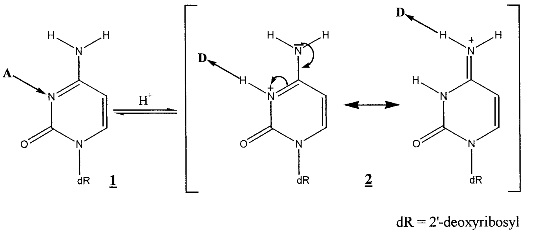
selected precursor ions by keeping the first quadrupole analyzer at 19 V relative to ground and operating at unit resolution, and scanning the time-of-flight (TOF) analyzer.

The collisional induced dissociations (CID) measurements, at variable collision gas pressure, were performed at the instrumental parameters CAD1, CAD2, CAD3, and CAD4, which correspond to 9.48×10^{-4}, 2.84×10^{-3}, 5.53×10^{-3}, 6.16×10^{-3} torr and 6.26×10^{14}, 1.88×10^{15}, 3.50×10^{15}, 4.07×10^{15} molecules/cm^2 gas thickness, respectively. All the acquisitions were averaged over 20 scans at a TOF resolving power of 8000 with a standard deviation of 0.01 cps (Table 2).

The CID measurements, at constant gas pressure (CAD1), were performed at the *q* voltages of 10,12, 14, 16, 18, and 20 eV, corresponding to the collision energy calculated from $E_{cm} = E_{lab}(m/[m + M])$

2.2. Chemicals

Guanosine (G), 2′-deoxyadenosine (2′-dA), Adenosine (A), 2′,3′-dideoxycytidine (2′,3′-ddC), 3′-deoxyadenosine (3′-dA), cytidine (C) and 2′,5′-dideoxycytidine (2′-5′-ddC) and reference amines were commercially available. The parasubstituted benzoyl cytidines **3–8** were synthesized in our labo-



Scheme 1

ratory by standard procedures [11] from commercially available 2′-deoxycytidine

3. Results and Discussions

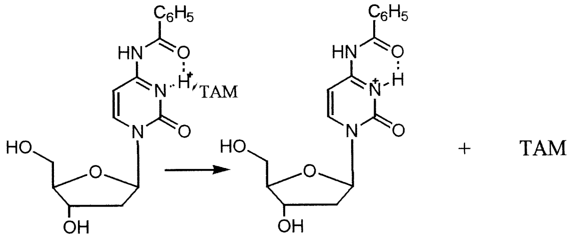
The PA of natural and modified nucleosides can be determined directly by applying the kinetic method [2, 5, 6]. Nucleic acid molecules are highly basic compounds requiring a set of reference bases larger than that provided by tertiary amines alone [2].

Preliminary results [4] show that nucleosides themselves can be used as references for the evaluation of the unknown PA of other nucleic acid molecules. The thermodynamic properties of a pair of interacting nucleosides slightly differing in the sugar ring structure should be very similar, thus justifying most of the assumptions made in deriving Equation 3. The main concern is, however, related to the possible formation of multiple hydrogen bonds within the interacting species, which may affect the entropy changes.

The horizontal interactions of neutral nucleic acids, within doubly heliced DNA, are described by the classic Watson–Crick or Hoogsteen pairs, which have been experimentally proved even for simple nucleosides both in solution [12] and in the solid state [13]. The gas-phase provides a unique environment for dealing with interacting species sharing a common proton. Under these conditions, the donor (D)/acceptor (A) functions present on the neutral (1, Scheme 1) are considerably modified in its conjugated acid 2.

In the case of pairs of nucleosides sharing a proton, one can postulate that they interact primarily through a single hydrogen bond.

Other structural motifs, which may affect the accuracy of the PA determination by the proposed method, are related to the formation of intramolecular hydrogen bonds within the protonated species, after the partitioning of the proton [14]. In this case, in fact, the energy for the back reaction, i.e. that released by the protonated species when it “cyclizes”, is not considered in Equation 3.



TAM= Tertiary amines

Scheme 2

A set of N-3 parasubstituted-benzoyl-2′-deoxycytydines (**3**–**8**, as seen in Chart) was chosen to evaluate, by the kinetic method, the entropy effect and the effect of intramolecular hydrogen bond formation on the measured PAs.

The PA of BCYT (3) was first evaluated from the unimolecular dissociations of four proton bound dimers formed with tertiary amines (TAM) by FAB (Table 1) [15]. The linear correlation ($R^2=0.956$) of the PA vs. ln k_{BCYT}/k_{TAM} plot provided the PA value of 237.5 kcal/mol which is 1.3 kcal/mol higher than that of the corresponding unsubstituted 2′-deoxycytidine [2, 4]. The PAs of 1,4 butanediamine[16] and lysine [14] are 17.9 and 12.7 kcal/mol, respectively, higher than that of a primary amine. This enhancement was ascribed to the formation of intramolecular hydrogen bonds.

The PA enhancement observed for benzoylcytidine, although small, has to be attributed to the participation of the vicinal carbonyl group in the stabilization of the expected [17] *N*-3 protonated site of the nucleobase (Scheme 2). If the benzoyl group were not involved in the suggested interaction through space, a lower PA than that of the corresponding 2′-deoxycytidine (1) would be expected as a consequence of the electron-withdrawing effect of the nucleobase.

The ΔPA between **1** and **3** might, however, be

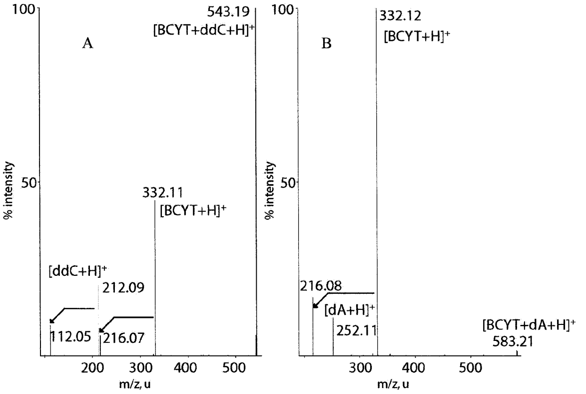


Fig. 1. CID spectra of proton-bound dimmers obtained from electrosprayed solution of BCYT and reference nucleosides, at 25 eV skimmer and 2.84 × 10^{-3} Torr of nitrogen gas in the collision cell (*q*) of a high resolution QqTOF instrument. (A) Product ion scan of $[BCYT + ddC + H]^+$ at *m/z* 543.19. The ions at *m/z* 332.11 and 212.09 correspond to $[BCYT + H]^+$ and $[ddC + H]^+$ protonated species. The species at *m/z* 216.07 and 112.05 correspond to the protonated nucleobases obtained from BCYT and ddC, respectively.

underestimated if the achievement of the "cyclic" protonated structure (Scheme 2) did not occur in the transition state. A comprehensive evaluation of the PA of **3**–**8** was, therefore, performed using as reference compounds, nucleosides whose PA values were independently determined by the same method. Data were processed by applying the kinetic method in its extended forms [6, 9].

Proton affinity of N-3-benzoyl-2′-deoxy-cytidine(BCYT); the case of BCYT will be discussed in detail. Proton-bound heterodimers of this nucleoside with 2′, 3′-dideoxycytine (ddC), 2′-deoxyadenosine (2′-dA), adenosine (A) and 3′-deoxyadenosine (3′-dA) were formed by electrospray in a Q*q*TOF hybrid instrument (Table 2). Different experimental conditions were set for the reaction chamber (*q*) either by varying the collision gas pressure or by modulating the collision energy of the clusters. A typical CID spectrum of the species $[BCYT + ddC + H]^+$ displays the two protonated nucleosides at *m/z* 332 and 212, respectively [Fig. 1(A)]. Consecutive dissociation of the initially formed product ions depends on the structure of the clusters, under the same experimental conditions, or on its internal energy when the reactions of the same reacting species are considered. In fact, the species $[BCYT + dA + H]^+$ displays the product ion $[dA + H]^+$ which did not undergo further fragmentation [Fig. 1(B)].

The determination of the kinetic constant for the two competing channels leading to the protonated species (Equation 3) included the intensities of the ions formed in consecutive fragmentations where applicable. In the case of the spectrum reported in Fig. 1A, the relative intensities of protonated nucleoside (I_N) and reference (I_{ref}) were obtained from the sum of the intensities of the two correlated pairs, i.e. $I_N = I_{332} + I_{216}$ and $I_{ref} = I_{212} + I_{112}$, respectively.

The measurements were carried out at four different gas pressures (CAD1 to CAD4, see Section 2) and the $\ln(k_N/k_{ref})$ vs. PA-PA_{avg} plot [Fig. 2(A), Table 2] provided the quantity $-1/RT_{eff}$. PA_{avg} represents the average of the measured PA at the four different experimental conditions. The plot shown in Fig. 2(B) provided the thermodynamic quantities of BCYT. The PA of 237.62 ± 0.02 kcal/mol obtained from this plot is equal to that determined [15] when tertiary amines were used as references in the standard implementation of the kinetic method [2]. It is noteworthy that a value of 237.4 kcal/mol was evaluated from the spontaneous dissociations, in a B-E instrument, of only two proton bound species, i.e. those formed by BCYT with tripropylamine (TPA) and tributylamine (TBA) (Fig. 3)

ESI experiments at different internal energy of the reacting ions and the extensive data treatment [9] provide [Fig. 2(B)] ΔS value of 1.34 ± 0.03 cal/mol °K. This ensures that entropy changes do not significantly affect the determination of the PA of the examined nucleosides even when other nucleosides, which might undergo multiple hydrogen bonding formation, are selected as reference compounds.

Finally, the breakdown of each of the proton-bound dimers examined was followed in the same Q*q*TOF instrument as a function of the kinetic energy of the impinging species by keeping constant the gas pressure in the collision cell. The plot of ln K_N/K_{ref} vs. the kinetic energy of the ions entering the cell (the collision energy), i.e. [Fig. 4(A)] and the plot of ln K_N/K_{ref}vs. PA-PA_{avg} [Fig. 4(B)] provided the PA and

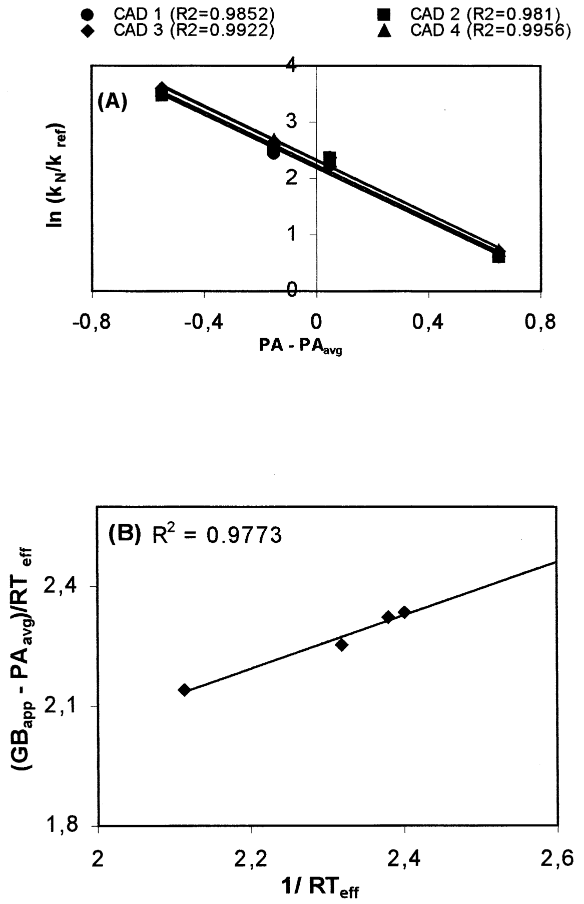


Fig. 2. (A) Evaluation of $-1/RT_{eff}$ (from the slope) and $(GB_{app}$-$PA_{avg})/1/RT_{eff}$ (from the intercept) parameters at four different gas pressures (CAD1 to CAD4, see Section 2) in the collision cell *q* of a QqTOF instrument. The $\ln(k_N/k_{ref})$ values were obtained, for each experimental consition, from the proton-bound dimmers generated by ESI from BCYT and the reference nucleosides ddC, dA, A, 3′-dA. The CID spectra were recorded at 25 V skimmer voltage and 8000 TOF resolution. (B) Evaluation of the thermodynamic quantities of BCYT from the parameters obtained from the linear regression of (A). PA = 237.62 ± 0.02 kcal/mol, ΔS = 1.34 ± 0.03 cal/mol°K.

ΔS values of 237.65 ± 0.15 kcal/mol and 1.02 ± 0.03 cal/mol °K, respectively. The latter differs by 0.32 kcal/mol °K from that determined by varying the pressure of the collision gas cell which was 1.34 ± 0.03 cal/mol °K. This value is probably more accurate since it is obtained in experimental conditions that allow one to examine the breakdown of the parent species over a broader range of internal energies.

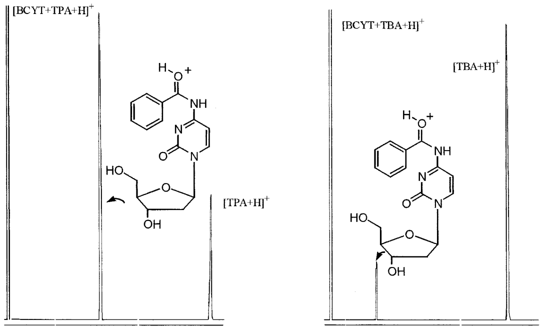


Fig. 3. MIKE spectra of $[BCYT + TPA + H]^+$. The proton-bound dimer was produced by 8 KeV Xe ion bombardment of the mixture of reagents suspended in glycerol.

The procedure outlined in Fig. 2 was used for the determination of the thermodynamic parameters of benzoylated cytidine **3**–**6** and **8** (Table 1). Only two reference compounds, cytidine (C) and 2′,5′-dideoxycytidine (2,5′-ddC), were available for NCYT (**7**) and the PA of 233.7 kcal/mol was obtained by the approach described above (Table 2) [4]. The PAs of the benzoylated deoxycytidines **3**–**8** seem to be correlated with the electronic effect of the substituent present in the para position of the aromatic ring.

Values of ΔPA of ~ −1 and −4 kcal/mol were in fact observed between the nucleosides bearing an unsubstituted phenyl ring and the corresponding *p*-chloro and *p*-nitro derivatives, respectively. Electron donating groups cause a continuous enhancement of the PA, even if the effect, in this direction, is less pronounced (Scheme 2). The results clearly support the interpretation outlined in , i.e. that the PAs of the benzoylated cytidines examined are affected by the benzoyl group through the formation of intramolecular hydrogen bonds.

If the formation of the “cyclic” protonated structure took place after the partitioning of the proton the energy for the back reaction could not be neglected. In this case, however, the involvement of the benzoyl group in the stabilization/destabilization of the transition state should not be affected by the electronic effect of the substituent. The ΔPA_s of *p*-benzoyl substituted $[PA_{N(R)}]$ and unsubstituted BCYT $[PA_{N(H)}]$ were therefore matched with the σ_p values of the substituent [18] by applying the Hammett free-energy relationship (Eq. 5). Compound **8** was not

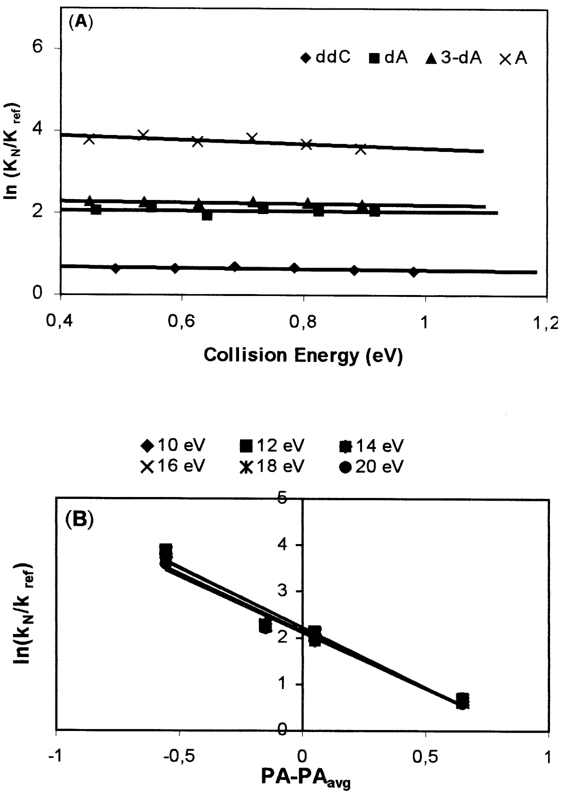


Fig. 4. Kinetics of dissociations of [BCYT + N + H]$^+$ proton-bound dimers as a function of their translational energy in a QqTOF instrument. (A) ln k_N/k_{ref} vs. collision energy (eV) plot for ions of translational energy in the range of 10–20 eV. (B) ln k_N/k_{ref} vs. PA-PA$_{avg}$ of the same [BCYT + N + H]$^+$ proton-bound dimmers.

included, because the σ_p value for a phenylacetyl group is not available. This group, however, behaves, as expected on the basis of elementary chemistry, as an electron donating species.

$$PA_{N(R)} - PA_{N(H)} = \rho\sigma_p \tag{4}$$

A $\rho = -4.18$ was obtained from the linear correlation of ΔPas vs. σ_p of compounds (**3**–**7**) [Fig. 5(A)]. The high and negative value of ρ shows that the benzoyl group participates in the transition state stabilization of the protonated species and that the effect is directly proportional to the electron releasing ability of the para substituent group. It can be assumed therefore that the initially formed proton bound dimers of the type (BCYT + N + H)$^+$ release the neutral nucleoside reference unimolecularly, through a reactive configuration where the benzoyl group approaches the proton in the transition state.

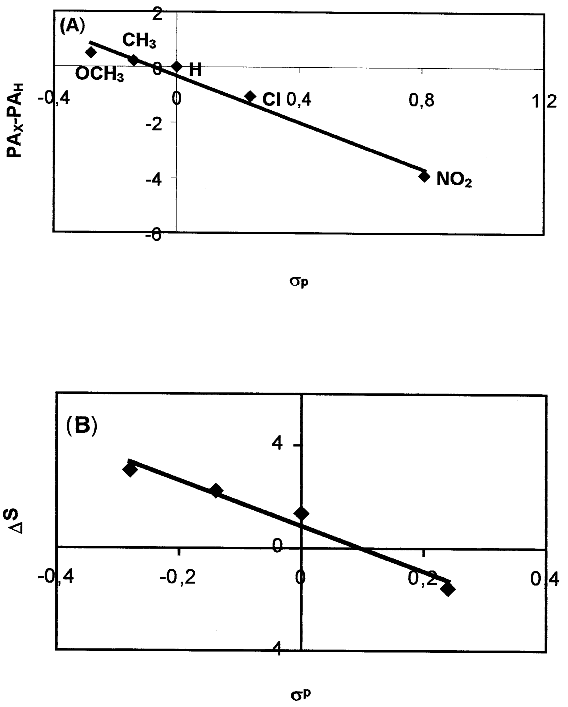


Fig. 5. (A) Value of ρ = -4.18 ± 0.08 is obtained from the linear correlation of ΔPa_s vs. σ_p (R^2 = 0.9738). (B) Entropy changes (ΔS) are linearly (R^2=0.9639) correlated with the σ_p of 2′-deoxycytidine **3**–**6**.

A further interesting observation can be drawn from the linear correlation [Fig. 5(B)]. ΔS and σ_p values were obtained as previously described (Table 3). If the entropy changes are interpreted in terms of the effective number of oscillators available at the threshold [19], the larger and more positive ΔS, the higher is the number of rotors that begin to freeze.

Table 3
PA and ΔS value of compounds 3–8

N-3-Benzoyl-2-deoxycytidines	PA (kcal/mol)	ΔS (cal/mol °K)
3	237.62 ± 0.02	1.34 ± 0.03
4	237.84 ± 0.01	2.21 ± 0.16
5	238.11 ± 0.01	3.02 ± 0.04
6	236.56 ± 0.04	−1.57 ± 0.03
7	233.70 ± 0.10	
8	237.79 ± 0.02	0.06 ± 0.02

This has a favorable effect on the ΔH of the reaction since it provides more stabilization to the transition state leading to the partitioning of the proton, thus enhancing the PA of the given nucleoside.

4. Conclusion

In conclusion, the determination of the proton affinity of nucleic acid molecules by the kinetic method provides accurate values, which cannot be determined at the present time by any other means. The recent approaches, which require prolonged experiments and extensive data treatment, provide more refined thermodynamic data,which at least for the determination of PAs, did not differ significantly from those obtained by faster and simpler methods.

The use of electrospray in high resolution Q*q*TOF instruments enables one to set a number of different experimental conditions which allow accurate estimations of the thermodynamic quantities of nucleosides to be made.

Nucleosides can be used as references for the determination of PAs of other nucleosides. The formation of species stabilized by intramolecular hydrogen bonds after the partitioning of the proton in the dissociation of the proton-bound precursors, does not necessarily occur under thermodynamic control. The energy for the back reaction can therefore be neglected even in the particular cases reported here, where the formation of intramolecular hydrogen bonded species has been clearly demonstrated.

Acknowledgement

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