Deacylation of 2-*N*-Isobutyryl- and 2-*N*-Isobutyryl-6-*O*-methyl-2'-deoxyguanosine in the Condensed and Gas Phase. A Kinetic Investigation

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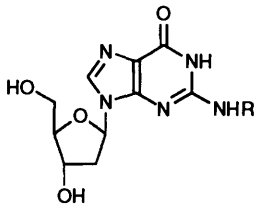
The kinetics of deacyclation of 2-*N*-isobutyryl- **2** and 2-*N*-isobutyryl-6-*O*-methyl-2'-deoxyguanosine **4** show that the former can be deblocked by ammonolysis, whereas the latter requires strong alkaline conditions. This different behaviour is correlated with the availability of the enolizable lactam function of the guanine ring. Similar effects are in operation in the unimolecular gas-phase dissociations of the same species.

The isobutyryl group has been extensively used in the protection of the 2-*N*-amino function of guanosine and deoxyguanosine, in oligonucleotide synthesis,[1,2] to obtain modified deoxyguanosine nucleosides[3,4] and in the post-synthetic modification of guanine containing oligonucleotides.[5] A number of experiments have proved, however, that the isobutyryl group is fairly resistant to mild deprotection. It has, in fact, been observed that overnight heating, in ammonia–methanol, of protected DNA oligomers, containing 2-*N*-isobutyryl deoxyguanosines, does not ensure a complete removal of the protecting group;[6] the deprotection of the same function in 6-*O* modified guanine residues is very slow in conc. ammonia,[5] whereas, in similar conditions, the complete deacylation of 2-*N*-isobutyryl-6-*O*-*p*-nitrophenylethyl-2'-deoxyguanosine requires prolonged treatment.[7] The reported[8] half-time ($t_{1/2}$) for the hydrolysis of 2-*N*-isobutyryldeoxyguanosine **2** in 0.2 mol dm^{-3} NaOH–methanol (1:1, v/v) is 271 min, thus showing that in strongly alkaline conditions, more than one day is needed for its complete deprotection. Finally we have found (*vide infra*) that ammonolysis does not allow a complete conversion of 2-*N*-isobutyryl-6-*O*-methyl-2'-deoxyguanosine **4** into the expected[3] deacylated derivative **3**. It seems, therefore, that the removal of the isobutyryl group is even more difficult when the guanine ring is protected at the 6-*O* moiety. The role of the enolizable function of the lactam moiety of the guanine ring, in the kinetics of deprotection of 2-*N*-isobutyryl derivatives of deoxyguanosine **2** and **4** was therefore evaluated in different environments. The kinetic data obtained in solution were then matched with those gathered, in the gas phase, from the unimolecular dissociations of the conjugated bases of the same compounds.



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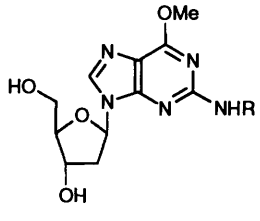
2 R = Pr^{i}CO



3 R = H
4 R = Pr^{i}CO

Results and Discussion

Solution-phase Experiments.—Two derivatives of deoxyguanosine **1**, *i.e.* 2-*N*-isobutyryldeoxyguanosine **2** and 2-*N*-isobutyryl-6-*O*-methyldeoxyguanosine **4**, were used as model compounds to investigate the kinetics of deprotection of the exocyclic amino function. The nucleoside **2** is usually present in synthetic protocols leading to oligonucleotides, whereas **4** is a typical modified unit, which can be incorporated into oligomers designed for studies related to the biological functions of modified DNAs. The deacylation of **2** and **4** was checked in two different environments, monitoring by HPLC the composition of the reaction mixture as a function of time. The kinetic constants (k) of the reactions were determined by conventional methods[9] from the slope of the straight lines obtained by plotting $\ln[A_0]/[A_x]$ *vs.* time (t), where $[A_0]$ and $[A_x]$ are the concentrations of the reactant at $t = 0$ and $t = x$ min, respectively. The structure of the deacylated products **1** and **3** thus obtained was determined unambiguously by NMR and fast atom bombardment (FAB) mass spectrometry.

The deprotection of **2** in 8 mol dm^{-3} ammonia–methanol at 50 °C (Table 1) was nearly four times faster than that of the same substrate in 0.2 mol dm^{-3} sodium hydroxide–methanol, which was characterised[8] by a $t_{1/2}$ of 271 min and an estimated k of 2.56×10^{-3}. The R_f value of the final compound was identical with that of the commercial 2'-deoxyguanosine **1**, while its FAB and ^{1}H NMR spectra confirmed the structure. The stability of acyl protected deoxyguanosines in strongly alkaline media has been attributed to the formation of the conjugated base of the amide function.[1] The complete deprotection of nucleoside **2** can be, therefore, achieved, at the monomer level, in nearly 7 h. On the contrary, in the same environment *ca.* 10% conversion of **4** into **3** was observed after 24 h, thus making this deprotection procedure quite unsuccessful even at the monomer level. It is worth mentioning that while **3** and **4** can easily be distinguished in the HPLC analysis, they give overlapping spots on SiO_2 TLC plates, using a wide variety of eluents.

When the deprotection of **2** and **4** was carried out in 1 mol dm^{-3} potassium hydroxide–methanol at 22 °C (Table 1), the deacylation of the 6-*O*-methyl derivative **4** was much faster than that of the nucleoside **2**. The kinetic parameters of the latter reaction agreed with the data already obtained in a similar environment.[8] In strongly alkaline media, therefore, the 6-*O*-protected isomer is more labile than the unprotected nucleoside towards deacylation of the 2-*N*-amide function. In this condition, in fact, it is possible to convert the fully base-protected nucleoside **4** into 6-*O*-methyl-2'-deoxyguanosine **3** in quantitative yields after 7 h (see Experimental).

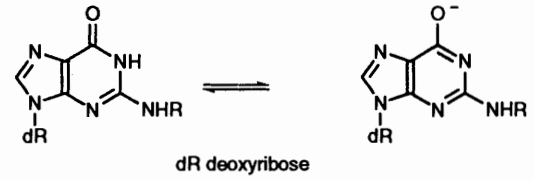
The kinetic data reported above suggest possible deacylation mechanisms in operation in the different environments used, and allows the evaluation of the role of the enolizable function in the 6 position of the guanine ring. The pK_a of deoxyguanosine **1**, in water is 9.26[10] and is associated with removal of the 2-*N* proton which leads to a stable conjugated base (Scheme 1). The ease of 6-*O* functionalization of 2-*N*-protected deoxyguanosines in mildly basic conditions[3,4–11,12]

Table 1 Kinetic data for the deprotection of **2** and **4** in the condensed phase

Reaction	Conversion (%)	$t_{1/2}$/min[c]	$k/10^{-3}$ min^{-1}
dG[Ib] **2** $\xrightarrow{a}$ dG **1**	96	69.5	9.68 ± 0.03
dG[Ib] **2** $\xrightarrow{b}$ dG **1**	40	156.7	4.24 ± 0.04
6-O-Me-dG[Ib] **4** $\xrightarrow{b}$ 6-O-Me-dG **3**	80	66.0	10.50 ± 0.04

[a] 8 mol dm^{-3} NH_3–MeOH, 50 °C. [b] 1 mol dm^{-3} KOH–MeOH, 22 °C. [c] Evaluated from $t_{1/2} = \ln 2/k$.

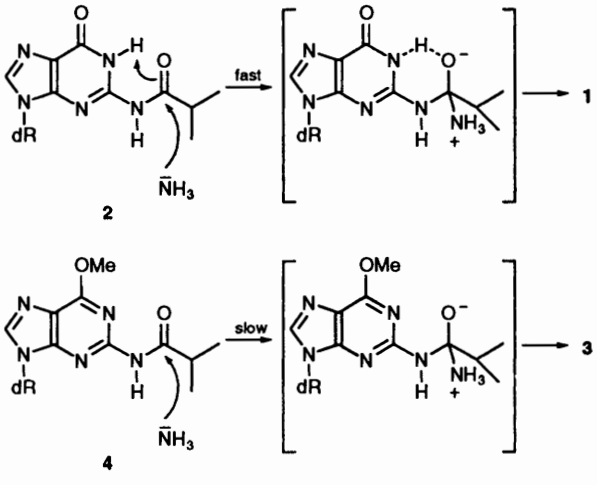
ensures that the preferred deprotonation site in basic media of the isomer **2** is still associated with the enolizable lactam moiety (Scheme 1). The unknown p*K*a of **2** should fall in the



1 R = H
2 R = Pr^{i}CO

Scheme 1

same range as that of **1**. The p*K*a of ammonium ions in water changes[13] from 9.25 to 9.28 going from 28 to 50 °C. Assuming that the replacement of water with methanol affects to a similar extent the pK_as of **2** and ammonia, it can be considered that the prototropic equilibrium reported in Scheme 1 is still in operation during the ammonolysis of the amide group. It can be suggested, therefore, that the 1-*N* proton of the guanine ring assists the deprotection of the substrate through intramolecular general-acid catalysis, which has been widely documented in similar systems,[14] by lowering the critical energy for the formation of the tetrahedral intermediate (Scheme 2). A

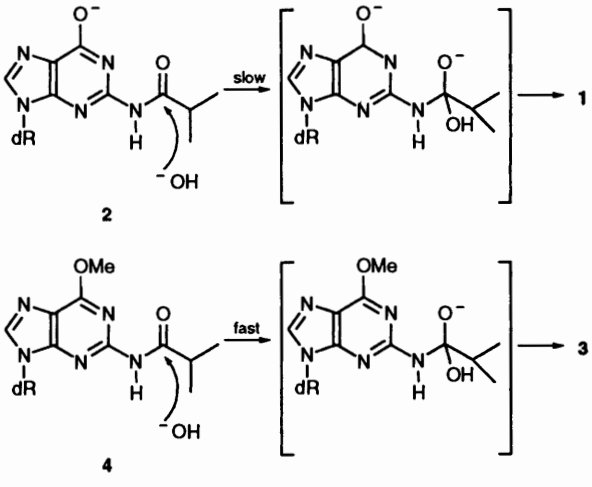


Scheme 2

complete conversion of **2** into **1** was achieved in less than 7 h. Ammonia is not a good nucleophile for the transformation of **4** into **3**, a 10% conversion was observed, in fact, after 24 h. This result supports indirectly the mechanism of ammonolysis previously suggested. The lack of enolizable protons in **4** means that there is no activation of the carboxy moiety *via* intramolecular protonation in the transition state, thus rising the critical energy required to obtain the tetrahedral intermediate (Scheme 2).

The kinetics of deprotection of **2** are therefore in agreement with many experimental findings,[1,2] which have shown that the isobutyryl group can be used for the protection of deoxyguanosine in oligonucleotide synthesis, although its removal in conditions which do not cause degradation of the oligomer (ammonia–methanol) can pose some problems at the macromolecular level.[6] However, the kinetics of deprotection of **4** indicate that the incorporation of 6-*O*-protected 2-*N*-isobutyryl-2′-deoxyguanosine units into modified oligonucleotide strands is not advisable[5] because the required final deprotection of the amino function is difficult to achieve in mild conditions.

The different behaviour of **2** and **4** in a strongly alkaline medium (1 mol dm^{-3} KOH–MeOH) is associated with the availability of the 1-*N* proton in the guanine ring. The kinetic constants for the hydrolysis of the amide function differ by more than one order of magnitude (see above) and in this condition the faster reaction is that leading to 6-*O*-methyl-2′-deoxyguanosine **3** from compound **4** (Scheme 3). The pH of the medium should now shift the prototropic equilibrium reported in Scheme 1 to the right, therefore, it can be assumed that the substrate undergoing hydrolysis is now the conjugated base of 2-*N*-isobutyryldeoxyguanosine **2** (Scheme 3). The formation of



Scheme 3

the tetrahedral intermediate develops, now, a charged oxide moiety close to the ionised oximino-function thus causing an enhancement of the critical energy. However, the reaction proceeds smoothly when the cyclic amide function is blocked by methylation thus allowing a complete conversion of **4** into **3** in less than 7 h. These conditions are satisfactory for the preparation of the modified nucleoside **3**, unfortunately, they cannot be used for the synthesis of modified oligonucleotides containing 6-*O* methylated guanine moieties.

Gas-phase Experiments.—The kinetics of deacylation of modified deoxyguanosines **2** and **4** clearly show that the enolizable proton at the 1 position of the guanine ring plays a major role and that, in strongly alkaline media, the nucleosides **1** and **2** should be present as their conjugated bases (Scheme 1). The latter can be produced by FAB[15] and released intact into the gas phase.[16–18] These gaseous species are sampled in a non-interacting environment ($p = 10^{-7}$ Torr, *ca.* 1.33×10^{-5} N m^2) and shown not to undergo bimolecular reactions. However, they are produced with an average internal energy of nearly 150 kJ mol^{-1},[19,20] and therefore they can dissociate unimolecularly

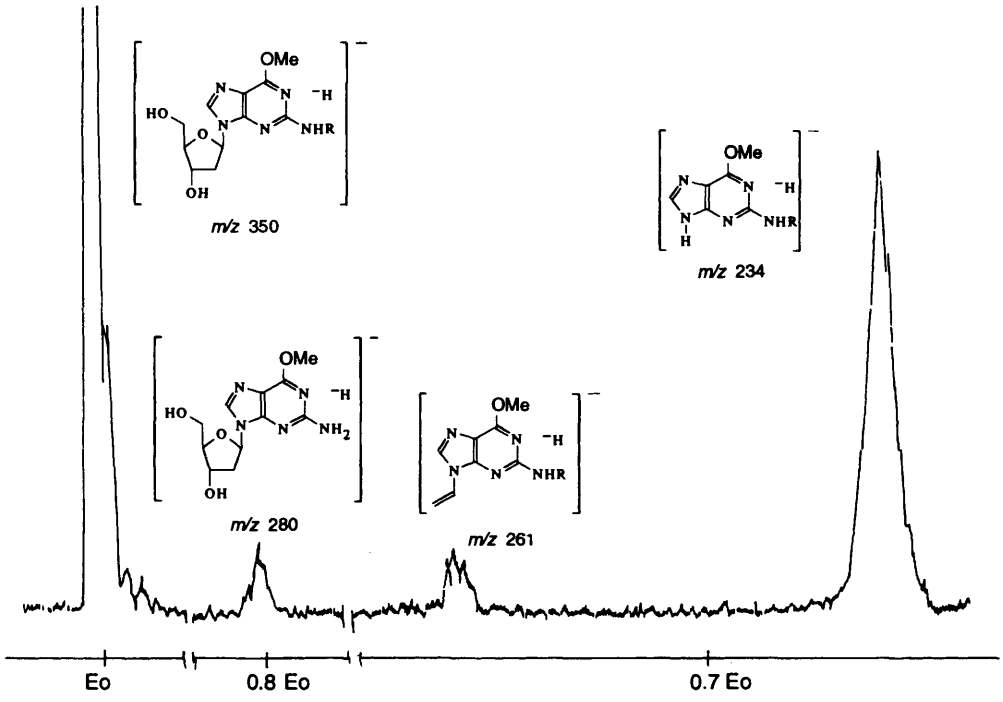


Fig. 1 FAB-MIKE spectrum of the $[M - H]^-$ of **4**

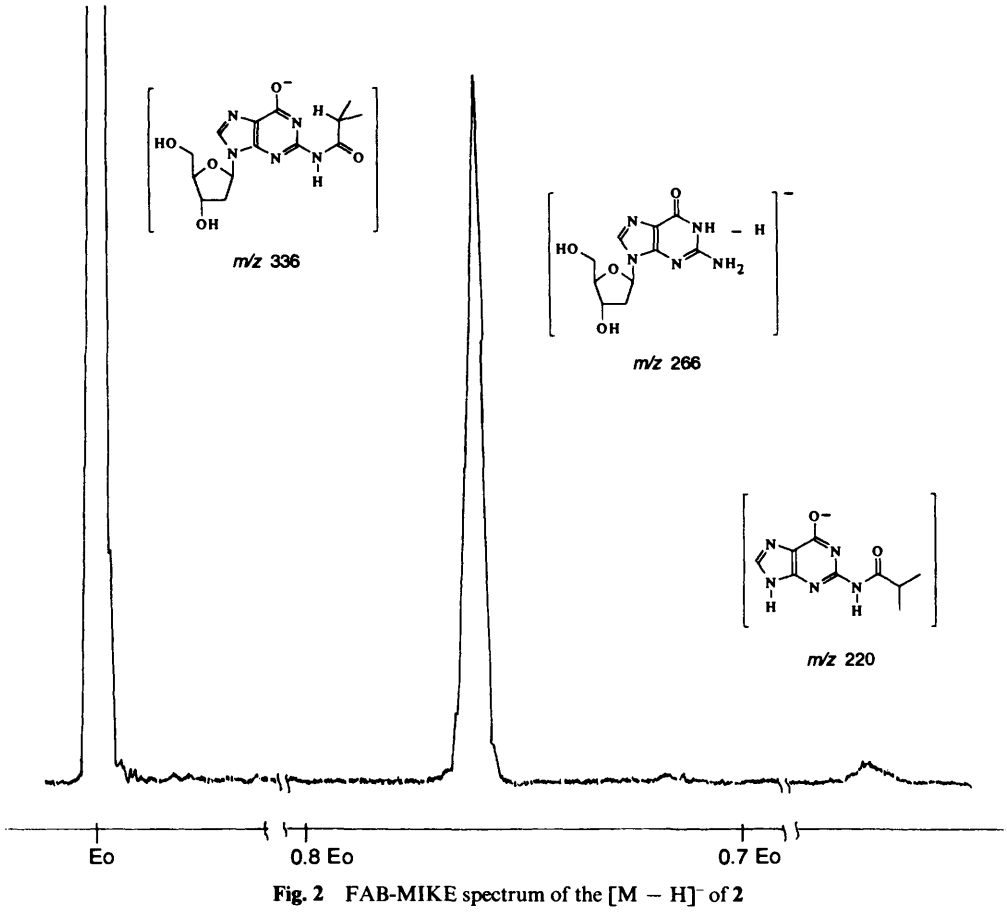


Fig. 2 FAB-MIKE spectrum of the $[M - H]^-$ of **2**

in the time scale of the mass spectrometric experiment (*ca.* 10^{-5} s).[21] The kinetics of the competing unimolecular fragmentations undergone by a given reactant can be monitored, in a B–E sector instrument, *i.e.* a double-focusing mass spectrometer with a magnetic analyser preceding the electrostatic sector.[22,23] In a typical experiment the analyte is dissolved in glycerol and exposed to 8 KeV Xenon atom bombardment. The deprotonated molecular ions $(M - H)^-$ thus formed are then transferred by the magnet into the reaction chamber preceding the electrostatic analyser and allowed to dissociate there. The reaction products are selectively recorded according to their kinetic energy (MIKE spectra, Figs. 1 and 2).[21,22]

In this experimental condition, the $(M - H)^-$ of species **1**, at *m/z* 266 affords mainly the conjugated base of guanine **5**, at *m/z*

150, with 81.0% relative yield *via* a gas-phase 'depurination'[24] of the nucleoside, occurring remote from the charge site[18] (Scheme 4). The other competing reaction paths give rise to its 9-*N*-vinyl derivative, at *m/z* 176, and to water loss elimination with 15.6 and 3.4% yield, respectively. Similar behaviour was exhibited by the 6-*O*-methylated isomer **4**, at *m/z* 350 (Fig. 1, Scheme 4) which afforded **6**, at *m/z* 234 with 82.0% yield, its 9-*N*-vinyl derivative, at *m/z* 260 (8.5%), and the 2-*N*-deacylated species, at *m/z* 280 (9.5%), by formal loss of neutral dimethylketene. However, the $(M - H)^-$ species **2** (Fig. 2, Scheme 4), at *m/z* 336, preferentially undergoes deacylation at the exocyclic amide group affording **1**, at *m/z* 266 (98%) and the conjugated base of 2-*N*-isobutyryl-6-*O*-methylguanine at *m/z* 220 (2%).

The conjugated bases of **1**, **2** and **4** are formed by prototropic equilibria which involve the removal of a proton from different sites, and the corresponding reaction profiles should be characterised by different enthalpy changes (ΔH). However, their formation and desorption into the gas phase occurs in a dynamic region of the ion source where collisions with other components of the excited system randomise in part their internal energy content.[25] In other words, the available internal energy of the reacting ions does not simply depend on the ΔH of the acid–base reaction. A consequence of this mechanism of ion formation is that the internal energy distribution of the $[M - H]^-$ species is similar and centred, as previously mentioned, around a maximum of *ca.* 150 kJ mol^{-1}. The reaction enthalpies ($\Delta_r H$) for the transformations of **1** into **5** and **4** into **6** (Scheme 4) are given by the eqns. (1) and (2), respectively, where $\Delta_f H°$s are the heats of formation of the various species and $\Delta_f H$(N) is that of the released neutral, which is common to all processes.

$$\Delta_r H^1 = \Delta_f H°(\mathbf{1}) - \Delta_f H°(\mathbf{5}) - \Delta_f H°(\mathrm{N}) \quad (1)$$

$$\Delta_r H^2 = \Delta_f H°(\mathbf{4}) - \Delta_f H°(\mathbf{6}) - \Delta_f H°(\mathrm{N}) \quad (2)$$

Eqns. (1) and (2) hold if the critical energy for the back reaction, if any, is negligible or similar in the two different processes. With this assumption the difference in the reaction enthalpy changes ($\Delta\Delta_r H$) is given by the eqn. (3).

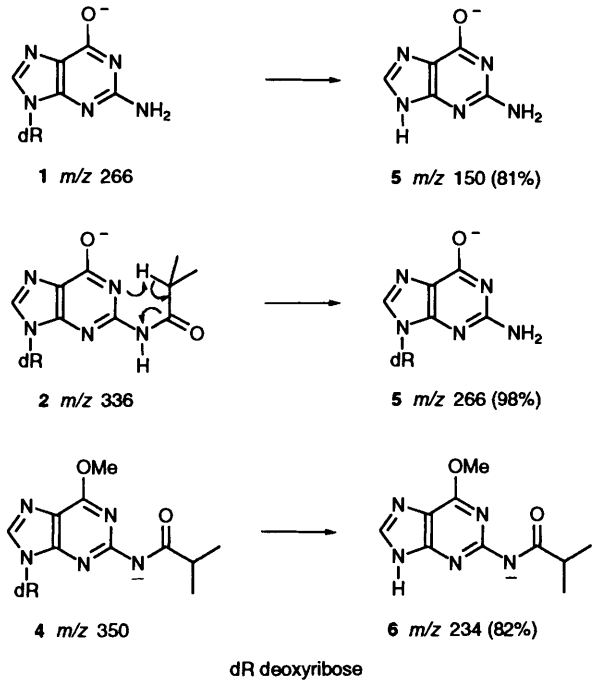
$$\Delta\Delta_r H = [\Delta_f H°(\mathbf{1}) - \Delta_f H°(\mathbf{5})] - [\Delta_f H°(\mathbf{4}) - \Delta_f H°(\mathbf{6})] \quad (3)$$

Assuming that the electronic effects, if any, of the sugar moiety in the stabilisation of the negatively charged nucleobase are negligible, or similar for the two ionised nucleosides, the $\Delta_f H°$ of **1** and **4** differ by an additive term[26] due to the heat of formation of the common sugar moiety [$\Delta_f H°$(S)] and leads to eqns. (4) and (5).

$$\Delta_f H°(\mathbf{1}) = \Delta_f H°(\mathbf{5}) + \Delta_f H°(\mathrm{S}) \quad (4)$$

$$\Delta_f H°(\mathbf{4}) = \Delta_f H°(\mathbf{6}) + \Delta_f H°(\mathrm{S}) \quad (5)$$

If the $\Delta_f H°$ of **1** and **4** [eqns. (4) and (5)] are inserted into eqn. (3), the $\Delta\Delta_r H$ becomes nearly zero and the depurination of **1** and **4**, leading to ionised guanines **5** and **6**, proceeds through reaction paths of similar critical energies. The ionised 2-*N*-isobutyryldeoxyguanosine **2** undergoes preferential deacylation, probably assisted by the ionised oximino function of the guanine residue (Scheme 4). The critical energy of the concomitant depurination process, occurring with 2% relative yield only, should be similar to that of the same processes taken by **1** and **4**, if the average internal energy of the reactants **1**, **2** and **4** is similar and the same thermochemical considerations of eqns. (1)–(3) are applied. The preferred elimination of a formal dimethylketene unit from **2** with respect to depurination processes can be ascribed, therefore, to the availability of the enolizable function on the guanine base which drives the formation of the reaction product *via* a six-membered transition state. On the other hand, if the critical energies for the depurination of the ionised nucleosides reported in Scheme 4 are similar, it can be concluded that the lack of deacylation of **4**, in this experimental condition, is due to structural effects related to the presence of the blocked oximino-function on the nucleobase.



Scheme 4

Experimental

General Methods and Materials.—2-*N*-Isobutyryl-2′-deoxyguanosine **2** was prepared as reported previously.[27] Solvents were purified and dried by conventional methods. Standard methanolic solutions of ammonia and trimethylamine were prepared from saturated solutions of the appropriate dry amines in dry methanol, by back titration with 1 mol dm^{-3} hydrochloric acid and Methyl Orange as indicator. Silica gel-precoated plates were used for TLC, and Kieselgel 60 H without gypsum was used for short-column chromatography. HPLC Violet PM 700 apparatus, equipped with an UV detector SP-430 and on-line data acquisition and processing, was used for the kinetic experiments in solution. NMR spectra were measured at 300 MHz with tetramethylsilane as internal standard and [$^2H_6$]dimethyl sulfoxide as solvent. *J* values are given in Hz. FAB Mass spectra were obtained on a B–E type sector instrument, from a 2 μl glycerol solution of sample, a neutral xenon beam of 8 keV and a neutral current of 10 μA. MIKE spectra were obtained by upwards scanning of the electrostatic analyser at a constant accelerating potential of *ca.* 8 kV, fixing the magnetic field to the appropriate value for the transmission of the selected reactant ions into the second field-free-region of the instrument. The kinetic data, either in solution or in the gas phase have been obtained from five independent measurements.

Ammonolysis of 2-N-*isobutyryl-2′-deoxyguanosine* **2**. A solution of **2** (10 mg, 0.03 mmol) in 8 mol dm^{-3} ammonia–methanol (15 cm^3) was allowed to react in a screw-capped septum vial, at 50 ± 1 °C. The reaction was followed up to 96% conversion and the samples, taken by syringe at regular

intervals of time, were immediately frozen in liquid nitrogen. The samples collected were analysed by HPLC [Chrompack Chromospher C-18 column, methanol–water (6:4 v/v), as eluent, 1 cm^3 min^{-1}]. The retention times (t_r) of **2** and of the deacylated compound **1** were 462 and 386 s, respectively. Plotting $\ln[A]/[A_0]$ *vs.* time, the straight line $f(x) = -9.68 \times 10^{-3}x - 4.77 \times 10^{-1}$ was obtained from which the k and $t_{1/2}$ values of $9.97 \pm 0.03 \times 10^{-3}$ min^{-1} and of 69.5 min, respectively, were obtained.

Alkaline hydrolysis of 2-N-*isobutyryl-2'-deoxyguanosine* **2**. A solution of **2** (50 mg, 0.15 mmol) in 1 mol dm^{-3} KOH–methanol (5 cm^3) was allowed to react in a stoppered flask at 22 ± 1 °C. The reaction was followed up to a 40% conversion and the samples, taken at regular intervals of time, were analysed as previously described. Plotting $\ln[A]/[A_0]$ *vs.* time, the straight line $f(x) = -4.24 \times 10^{-3}x - 2.30 \times 10^{-1}$ was obtained from which the k and $t_{1/2}$ values of $4.24 \pm 0.04 \times 10^{-3}$ min^{-1} and 156.7 min, respectively, were obtained.

Alkaline hydrolysis of 2-N-*isobutyryl*-6-O-*methyl-2'-deoxyguanosine* **4**. A solution of **4** (52.5 mg, 0.15 mmol) in 1 mol dm^{-3} KOH–methanol (5 cm^3) was allowed to react in the same conditions as for **2**. The reaction was followed up to 80% conversion and the samples, taken at regular intervals of time, were analysed by HPLC [Chrompack Lichrosorb 10-NH_2 column, acetonitrile–water (7:3 v/v), as eluent, 1 cm^3 min^{-1}]. The t_r of **4** and of the deacylated product **3** were 318 and 329 s, respectively. Plotting $\ln[A]/[A_0]$ *vs.* time, the straight line $f(x) = -1.05 \times 10^{-2}x - 1.06 \times 10^{-1}$ was obtained from which the k and $t_{1/2}$ values of $10.50 \pm 0.04 \times 10^{-3}$ min^{-1} and 66.0 min, respectively, were obtained.

2-N-*Isobutyryl*-6-O-*methyl-2'-deoxyguanosine*. The synthetic scheme reported by Jones *et al.*,[3] was followed with some modifications. Peracylation[28] of 2'-deoxyguanosine afforded 2-*N*-3',5'-*O*-triisobutyryl-2'deoxyguanosine, with 80% isolated yield. The latter was sulfonylated according to the reported method[3] to give 2-*N*-3',5'-*O*-triisobutyryl-6-*O*-(2,4,6-triisopropylbenzensulfonyl)-2'-deoxyguanosine with 84% isolated yield. A solution of the latter sulfonylated compound (1.90 g, 2.56 mmol) and 2 mol dm^{-3} trimethylamine–methanol solution (4.3 cm^3, 8.6 mmol) in dry dichloromethane (3 cm^3) was kept at 0 °C under magnetic stirring. After 15 min, 1,8-diazabicyclo-[5.4.0]undecene (DBU, 0.6 cm^3, 3.84 mmol) was added and the reaction was stirred at 0 °C for 2 h. TLC monitoring of the reaction mixture [chloroform–methanol–conc. aq. ammonia, (80:18:2 v/v/v)] showed the complete conversion of the reactant and the presence of a mixture of compounds.[3] 1 mol dm^{-3} Sodium hydroxide (6 cm^3) was then added after 45 min under magnetic stirring at room temp. A single TLC spot was detected after alkaline treatment. The reaction mixture was buffered by addition of an equivalent volume of 1 mol dm^{-3} aqueous ammonium chloride. The crude mixture was concentrated and partitioned between water and ethyl acetate (10 cm^3). The organic layers were washed three times with brine (3 × 10 cm^3) and the combined water layers were re-extracted with ethyl acetate (3 × 10 cm^3). The combined organic layers, dried and evaporated to dryness afforded a solid residue which was purified by short-column chromatography [chloroform–methanol–aq. conc ammonia (93.5:4.5:2, v/v/v)] to give 2-*N*-isobutyryl-6-*O*-methyl-2'-deoxyguanosine **4** (94%); δ_H 8.45 (1 H, s, NH), 8.33 (1 H, s, 8-H), 6.40 (1 H, dd, *J* 5.7 and 6.2, 1'-H), 5.29 (1 H, d, *J* 3.5, 3'-OH), 4.90 (1 H, t, *J* 5.6, 5'-OH), 4.42 (1 H, m, 3'-H), 3.85 (1 H, m, 4'-H), 2.93 [1 H, q, *J* 8.0, 2-C*H*$(CH_3)_2$], 2.66 (1 H, m, 2'-H), 2.24 (1 H, m, 2'-H) and 1.11 [6 H, *J* 8.0, 2-CH$(CH_3)_2$]; FABMS (−) *m/z* 350 [100%, $(M - H)^-$], 280 (33%), 234 (90%) and 164 (35%).

6-O-*Methyl-2'-deoxyguanosine* **3**. A solution of compound **4** (500 mg, 1.42 mmol) in 1 mol dm^{-3} KOH–methanol (50 cm^3) was stirred at room temp. for 8 h. The reaction was monitored by HPLC as described previously, because starting and final products gave overlapping spots by TLC using all possible combination of eluent mixtures. A 0.4 mol dm^{-3} solution of ammonium chloride–methanol (250 cm^3) was then added and the resulting mixture was centrifuged. The crude residue was washed several times with methanol and acetone until all soluble materials were removed. 395 mg (99%) of the insoluble 6-*O*-methyl-2'-deoxyguanosine **4** was obtained. The NMR spectrum was consistent with that published.[4] FABMS (+) *m/z* 282 (32%), 268 (57%), 166 (64%) and 152 (100%).

Conclusions

The kinetic of deacylation of the modified deoxyguanosines **2** and **4** depends on the experimental conditions. Mild procedures, such as ammonolysis, can be applied for the complete deblocking of isomer **2**, whereas the transformation of **4** into 6-*O*-methyl-2'-deoxyguanosine **3** requires the use of a strongly alkaline environment. The kinetic data obtained in the condensed phase suggest that a major role is played by the enolizable lactam function of the guanine ring. The participation of the acidic proton at the 1 position of the ring in the ammonlysis of **2** can be considered, in fact, to be the driving force of the process (Scheme 2).

The kinetics of the gas-phase reactions discussed above suggest that the reactivity of the conjugated bases of the same isomers, **2** and **4**, in an isolated environment, is still related to the availability of the acidic hydrogen of the guanine ring. Finally, the data presented above are in agreement with the observation that the preparation of oligonucleotides containing modified guanines at the 6-position is better carried out by post-synthetic modifications[5] of suitable reactive guanine monomers initially incorporated into the oligomers.

Acknowledgements

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