A ^{1}H Nuclear Magnetic Resonance Spectroscopic Study of Some *N*-Methyl and *N*-Acyl Derivatives of Guanosine. The Structure of *N*,*O*(2′),*O*(3′),*O*(5′)-Tetra-acetylguanosine

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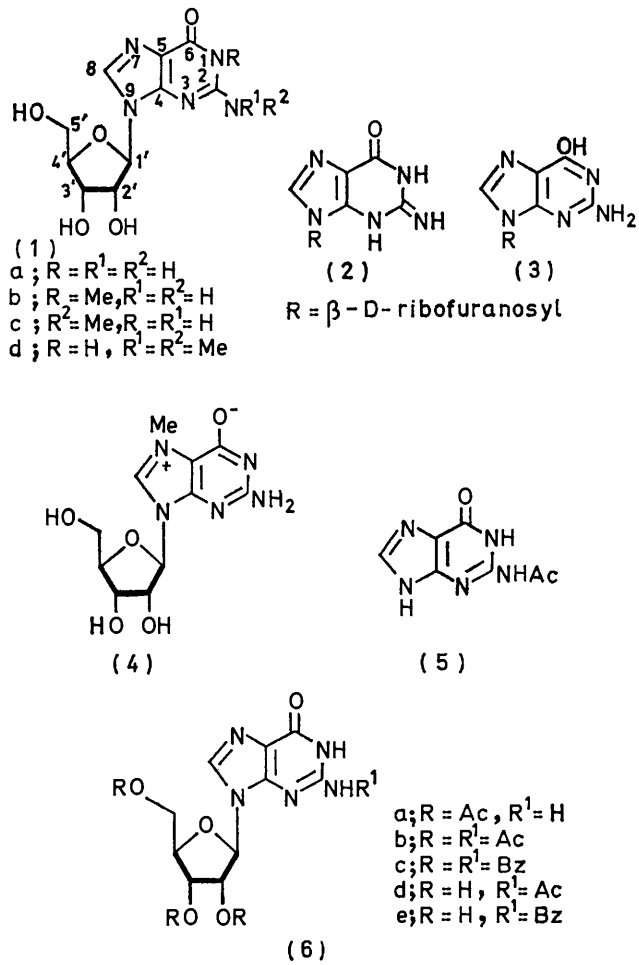
Features of the ^{1}H n.m.r. spectra of guanosine and its *N*(1)-methyl, *N*(2)-methyl, *N*(2),*N*(2)-dimethyl, and *N*(7)-methyl derivatives in anhydrous [$^2H_6$]dimethyl sulphoxide solution are discussed. Possible conclusions, based on the spectral data, relating to the tautomeric and conformational equilibria of these compounds are considered. An unsuccessful attempt to determine the site of *N*-acylation of guanosine by n.m.r. spectroscopy is described; however, the structure of *N*(2),*O*(2′),*O*(3′),*O*(5′)-tetra-acetylguanosine has been established by a chemical method. Rates of deacylation of *N*(2)-acetyl- and *N*(2)-benzoyl-guanosines have been determined.

THE ^{1}H n.m.r. spectrum of guanosine (1a) in anhydrous dimethyl sulphoxide solution (Table 1, spectrum 1) reveals several signals which, since they disappear on the addition of deuterium oxide, may be assigned to exchangeable OH and NH protons. Of particular interest is the broad singlet at δ 6·48 which has been assigned [1-3] to the 2-NH_2 protons. A possible alternative tautomeric structure (2) for guanosine has been excluded [1-3] on the basis of this assignment, as the 1-H and 2-NH signals of structure (2) would be expected to have different chemical shifts. A third possible tautomeric structure (3) † has been excluded [3] on the basis of i.r. spectral data.

Such is the main evidence for (1a) being the preferred tautomeric structure of guanosine; similar arguments have been used [2] for the predominance of the corresponding tautomer of 2′-deoxyguanosine. As these conclusions are of such importance in considerations relating to the secondary structures [4] of nucleic acids, it seemed worthwhile to check them by examining the n.m.r. spectra of some *N*-methyl derivatives of guanosine.‡ All the methylated nucleosides studied have in fact been identified as minor constituents of tRNA digests.[5] It was hoped that n.m.r. spectroscopy could also be used to locate the site at which the guanine portions of guanosine undergoes acylation.

The n.m.r. spectrum of *N*(1)-methylguanosine [6] (1b) (Table 1, spectrum 2) displays a singlet (3H) at δ 3·40, which may be assigned to the *N*-methyl protons, and no low-field signal corresponding to the putative H-1 resonance at δ 10·74 in the n.m.r. spectrum of guanosine (spectrum 1). The only other noticeable difference between the n.m.r. spectra of guanosine and *N*(1)-methylguanosine is that the 2-NH_2 protons of the latter are more deshielded. These data alone do not provide sufficient evidence to support the assignment of structure



(1a) rather than (3) to the principal tautomer of guanosine; indeed, more convincing evidence in favour of structure (1a) is provided by i.r. spectral data [3] and by

† Very recently, G. C. Y. Lee and S. I. Chan (*J. Amer. Chem. Soc.*, 1972, **94**, 3218) have studied the dependence of the widths of the H-8 signals of guanosine and some of its derivatives upon various factors, and have concluded that, in neutral aqueous solution at room temperature, guanosine is an equilibrium mixture of the lactam (1a) and lactim (3) (*ca.* 16%) tautomers.

‡ All the conclusions, based on the present work, which relate to the preferred tautomeric forms of guanosine and its *N*-methyl derivatives, are derived from n.m.r. spectral data obtained for solutions in [$^2H_6$]dimethyl sulphoxide. Although it cannot be assumed that the tautomeric equilibria would be the same in aqueous solution, it is necessary to study solutions in a non-aqueous medium if the signals of the exchangeable protons are to be observed.

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[3] H. T. Miles, F. B. Howard, and J. Frazier, *Science*, 1963, **142**, 1458.
[4] J. D. Watson and F. H. C. Crick, *Nature*, 1953, **171**, 737, 964.
[5] R. H. Hall, 'The Modified Nucleosides in Nucleic Acids,' Columbia University Press, New York and London, 1971.
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the qualitative similarity [7] between the u.v. absorption spectra of guanosine and *N*(1)-methylguanosine at pH 7.

However, the n.m.r. spectrum of *N*(2)-methylguanosine [8] (1c) (Table 1, spectrum 3) provides good additional evidence for the exclusion of (2) as the predominant tautomer of guanosine. The *N*-methyl protons of (1c) resonate as a doublet (δ 2·95, *J* 4·5 Hz), which collapses to a singlet on addition of deuterium oxide. This establishes the presence of an NH·CH_3 group. It follows that the multiplet at δ 6·48 (spectrum 3), which collapses to a singlet on irradiation at δ 2·95 and disappears on addition of deuterium oxide, may be assigned to the 2-NH proton. These considerations support the conclusion [1-3] that the broad singlet at δ 6·48 in the n.m.r. spectrum of guanosine (spectrum 1) may be assigned to the 2-NH_2 protons. Additional evidence that guanosine and *N*(2)-methylguanosine exist predominantely in similar tautomeric forms [as represented by (1a) and (1c), respectively] is provided by the observation that the chemical shifts of the signals assigned to their putative 1-H proton resonances differ by only 0·14 p.p.m.

The n.m.r. spectrum of *N*(2),*N*(2)-dimethylguanosine [8] (1d) (Table 1, spectrum 4) calls for no special comment. As expected, a low-field signal (δ 10·82), corresponding to the signals assigned to the 1-H of guanosine (1a) and *N*(2)-methylguanosine (1c), is seen, but no signal assignable to a 2-NH group is observed. However, the spectrum of 7-methylguanosine [9] (4) (Table 1, spectrum 5) differs appreciably from all the other spectra so far considered: no sugar OH signals are apparent, H-8 resonates more than 1 p.p.m. downfield from the corresponding proton in guanosine and the other *N*-methyl derivatives (Table 1, spectra 1—4), and, as has been reported previously,[10] H-8 readily undergoes exchange. Also, the methyl protons in structure (4) are much more deshielded than the corresponding protons in compounds (1b—d).

The spectra of *N*(2)-methyl- and *N*(2),*N*(2)-dimethylguanosines (1c and 1d) (Table 1, spectra 3 and 4) both display three distinct OH signals: a doublet (respectively, δ 5·47 and 5·45, *J* 6 Hz) tentatively assigned to the 2′-OH, a doublet (respectively, δ 5·24 and 5·22, *J* 4 Hz) tentatively assigned to the 3′-OH, and a triplet (respectively, δ 5·03 and 5·01, *J* *ca.* 5 Hz) assigned to the 5′-OH. However, although the spectra of guanosine and *N*(1)-methylguanosine (1a and b) (spectra 1 and 2, respectively) display doublets (*J* 6 Hz) in the region of δ 5·45, the other two hydroxy-protons of both compounds resonate as multiplets (δ 5·19 and 5·15, respectively). Thus the 5′-OH protons in the latter two compounds (1a and b) are more deshielded than the corresponding protons in (1c) and (1d). A possible explanation of this effect is that one pair of nucleosides prefers to take up the *syn*-conformation [11] (with the base and sugar residues on the same side of the glycosidic bond) and the other prefers the *anti*-conformation [as illustrated in formula (1)]. It seems likely on steric grounds that (1a) and (1b) would prefer the *syn*- and (1c) and (1d) the *anti*-conformation.

TABLE 1

100 MHz ^{1}H N.m.r. spectra of solutions in anhydrous [$^2H_6$]dimethyl sulphoxide at 35° [a]

Spectrum No.	Compound	1-H [b]	2-NH or -NH_2 [b,c]	8-H	N·CH_3 [c]	CO·CH_3 [c]	1-H′ [$J_{1',2'}$] [d]	2′-, 3′-, and 5′-OH [b,c]
1	Guanosine (1a)	10·74s	6·48br,s (2)	7·96s			5·74d [5·5]	5·40d (1), 5·12m(2)
2	*N*(1)-Methylguanosine (1b)		7·06br,s (2)	8·00s	3·40s (3)		5·77d [5·5]	5·43d (1), 5·14m (2)
3	*N*(2)-Methylguanosine (1c)	10·88br,s	6·48m (1)	8·02s	2·95d (3) [e]		5·85d [5·5]	5·48d (1), 5·25 d(1), 5·03t (1)
4	*N*(2),*N*(2)-Dimethylguanosine (1d)	10·82br,s		8·03s	3·18s (6)		5·83d [5·5]	5·45d (1), 5·22d (1), 5·01t (1)
5	*N*(7)-Methylguanosine (4)		6·20br,s (2)	9·19br,s [b]	4·09s		5·91d [5]	
6	*O*(2′),*O*(3′),*O*(5′)-Triacetylguanosine (6a)	10·81br,s	6·56br,s (2)	7·96s		2·18s (3), 2·11s (6)	6·05d [6]	
7	*N*(2),*O*(2′),*O*(3′),*O*(5′)-Tetraacetylguanosine (6b)	11·73br,s [f]	12·21br,s [f] (1)	8·31s		2·31s (3), 2·22s (3), 2·15s (3), 2·14s (3)	6·18d [6]	
8	*N*(2),*O*(2′),*O*(3′),*O*(5′)-Tetrabenzoylguanosine (6c)	11·79s [f]	12·40br,s [f] (1)	8·42s				
9	*N*(2)-Acetylguanosine (6d)			8·36s		2·30s (3)	5·91d [6]	
10	*N*(2)-Benzoylguanosine (6e)			8·36s			6·00d [6]	
11	*N*(2)-Acetylguanine (5)			8·12br,s		2·25s (3)		

[a] Chemical shifts in p.p.m. (δ); t-butyl alcohol as internal standard. [b] Removed by addition of D_2O. [c] Figures in parentheses represent numbers of protons, as estimated by integration. [d] Figures in square brackets represent coupling constants in Hz. [e] This doublet (*J* 4·5 Hz) collapses to a singlet on addition of D_2O. [f] The assignments made for the 1-H and 2-NH resonances are not firm; it is possible that they should be reversed.

[7] Ref. 5, pp. 132, 135.
[8] A. Yamazaki, I. Kumashiro, and T. Takenishi, *J. Org. Chem.*, 1967, **32**, 3032.
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[11] For leading references relating to conformational preferences of nucleosides, see R. E. Schirmer, J. P. Davis, J. H. Noggle, and P. A. Hart, *J. Amer. Chem. Soc.*, 1972, **94**, 2561.

It was hoped that the data obtained (Table 1, spectra 1—5) might help to identify the site of *N*-acylation of guanosine. This is of practical importance, as *N*-acyl derivatives of guanosine and 2′-deoxyguanosine are used as intermediates in oligonucleotide synthesis.[12] Although the product obtained by the acetylation of guanine has been identified as *N*(2)-acetylguanine [13] (5), the evidence [12,14] that guanosine (1a) and its derivatives undergo acylation on N-2 is by no means conclusive. When guanosine reacts with acetic anhydride in pyridine solution at room temperature, *O*(2′),*O*(3′),*O*(5′)-triacetylguanosine [15] (6a) is obtained; however, when it is heated for 2 h under reflux with *ca.* 8 mol. equiv. of acetic anhydride in pyridine, a mixture of (6a) (*ca.* 20%) and a tetra-acetyl derivative (*ca.* 80%) is obtained. The latter, which can be isolated pure by silica gel chromatography, has not yet been induced to crystallize.

The n.m.r. spectrum of this tetra-acetyl compound (Table 1, spectrum 7) reveals two broad one-proton singlets at δ 11·73 and 12·21. As both of these signals disappear on the addition of deuterium oxide, they may be assigned to N–H protons; however, they occur at too low a field to permit a correlation with the N–H signals of guanosine or its *N*-methyl derivatives.* An alternative approach is to compare the n.m.r. spectrum of the tetra-acetyl compound with that of authentic N(2)-acetylguanine [13] (5) (spectrum 11). However the N–H signals of compound (5) could not be observed, even in very dry [$^2H_6$]dimethyl sulphoxide solution. The structure of the tetra-acetyl compound was ultimately established as (6b) by a chemical method; when it was heated with 98% formic acid, under reflux, cleavage of the glycosidic linkage occurred and *N*(2)-acetylguanine (5), identical with authentic material,[13] was obtained in 54% isolated yield.

Although the structure of *N*(2),*O*(2′),*O*(3′),*O*(5′)-tetra-acetylguanosine (6b) cannot be ascertained from its n.m.r. spectrum, this spectrum (Table 1, spectrum 7) is of use in the elucidation of the structure of *N*,*O*(2′),*O*(3′),*O*(5′)-tetrabenzoylguanosine.[14] The spectrum (spectrum 8) of the latter reveals signals at δ 11·79 and 12·40, which may be assigned to exchangeable N–H protons. As the chemical shifts of these signals closely correspond to those of the two N–H protons in the spectrum of (6b), we conclude that the tetrabenzoyl derivative has structure (6c).

N(2)-Benzoylguanosine (6e) has been obtained [14] by treatment of the tetrabenzoyl derivative (6c) with sodium methoxide in methanol–dioxan. Indeed the comparative stability of (6e) to methoxide ion has been offered [14] as evidence for the presence of an ionizable benzamido-group in the molecule and thus for the tetrabenzoyl derivative having the assigned structure (6c). *N*(2)-Acetylguanosine (6d) may similarly be prepared in satisfactory yield from the tetra-acetyl derivative (6b); however, it has not yet been obtained crystalline. The signals of the exchangeable NH and OH protons cannot be observed in the n.m.r. spectra of either *N*(2)-acetyl- or *N*(2)-benzoyl-guanosine (6d and e) (Table 1, spectra 9 and 10) even in thoroughly dried [$^2H_6$]dimethyl sulphoxide solution.

Both compounds (6d) and (6e) are potentially useful intermediates in ribonucleoside chemistry. In the synthesis of oligonucleotides, it is desirable [12] to protect the guanine residues by *N*-acylation in order to avoid difficulties in characterization of the products and to prevent side-reactions during the phosphorylation steps. It is then necessary to remove the *N*-acyl groups at the end of the synthesis. For this reason, procedures for the deacylation of (6d) and (6e) have been examined. The half-times for the deacetylation and debenzoylation, respectively, of the latter compounds in ammonia, methylamine, and dimethylamine solutions are listed in Table 2. As previously reported [12b] deacetylation occurs much more readily than debenzoylation, and methylamine is a more effective deacylating agent than either ammonia or dimethylamine. These results suggest that, in synthetic work, it should be advantageous to protect guanine residues by *N*-acetylation and to remove the protecting groups by treatment with methylamine in a suitable solvent.

TABLE 2

Half-times for the deacylation of *N*(2)-acetyl- and *N*(2)-benzoyl-guanosines in methanolic ammonia, and in ethanolic methylamine and dimethylamine solutions at 20°

	Half-times of deacylation (min)		
Compound	NH_3–MeOH [a]	$MeNH_2$–EtOH [b]	Me_2NH–EtOH [b]
N(2)-Acetyl-guanosine (6d)	42	5	40
N(2)-Benzoyl-guanosine (6e)	1500	200	1200

[a] Half-saturated at 0°. [b] 33% w/w.

EXPERIMENTAL

N.m.r. spectra (100 MHz) of solutions in anhydrous [$^2H_6$]dimethyl sulphoxide (dried by stirring with calcium hydride for 16 h at 20° and then distilling under reduced pressure) and in mixtures of this solvent with deuterium oxide were measured with a Varian HA 100 spectrometer. t-Butyl alcohol (dried by distillation over sodium metal) was used as an internal standard. U.v. spectra were measured with a Cary recording spectrophotometer, model 14M-50. The determinations of deacylation rates were carried out with a Zeiss model PMQII u.v. spectrophotometer.

Ascending paper chromatograms were run on Whatman

* The 2-NH proton of *N*(2),*O*(2′),*O*(3′),*O*(5′)-tetra-acetylguanosine (6b) would be expected to resonate appreciably downfield from the 2-NH_2 protons of guanosine. However, it is difficult to predict whether acetylation on the 2-NH_2 group of guanosine would also have a significant deshielding effect on H-1.

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[13] R. Shapiro, B. I. Cohen, S.-J. Shiney, and H. Maurer, *Biochemistry*, 1969, **8**, 238.

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[15] H. Bredereck, *Chem. Ber.*, 1947, **80**, 401.

no. 1 paper in the following solvent systems: (A) butan-1-ol–acetic acid–water (5 : 2 : 3); (B) propan-2-ol–formic acid–water (65 : 1 : 34). Thin-layer chromatograms were run on glass plates coated with Merck Kieselgel GF_{254} in system (C): chloroform–methanol (4 : 1). Mallinckrodt analytical grade silicic acid (100 mesh) was used for adsorption chromatography.

N(2),O(2′),O(3′),O(5′)-*Tetra-acetylguanosine* (6b).—Guanosine (9·0 g, 32 mmol), acetic anhydride (25·1 ml, 266 mmol), and pyridine (140 ml) were heated together, under reflux, for 2 h. The products were cooled, treated with methanol (40 ml), set aside at 20° for 1 h, and then concentrated under reduced pressure. The oil so obtained was partitioned between chloroform and aqueous sodium hydrogen carbonate. The dried (Na_2SO_4) chloroform layer was evaporated under reduced pressure, the residue dissolved in ethanol, and the solution re-evaporated. The latter procedure was repeated several times to give a brown glass. T.l.c. [system (C)] revealed a minor component (*ca.* 20%) with an R_F value corresponding to that of *O*(2′),*O*(3′),*O*(5′)-triacetylguanosine and a major component (*ca.* 80%) with a higher R_F value.

A solution of this material in dichloromethane was applied to a column (27 cm × 10 cm^2) of silicic acid. The column was first washed with dichloromethane and then eluted with dichloromethane–methanol (98 : 2) to give N(2),O(2′),O(3′),O(5′)-*tetra-acetylguanosine* (8·4 g, 59%). This compound was obtained after rechromatography as a glass (Found: C, 47·6; H, 5·0; N, 15·3. $C_{18}H_{21}N_5O_9$ requires C, 47·9; H, 4·7; N, 15·5%], $\lambda_{max.}$ (95% EtOH) 258 and 282 (ε 14,600 and 12,500), $\lambda_{min.}$ 225 and 271 nm (ε 3600 and 11,500); R_F (A) 0·70, (B) 0·74, (C) 0·83.

N(2)-*Acetylguanosine* (6d).—Methanolic sodium methoxide (2·5M; 65·5 ml, 164 mmol) was added to a stirred solution of *N*(2),*O*(2′),*O*(3′),*O*(5′)-tetra-acetylguanosine (12·3 g, 27·3 mmol) in anhydrous dioxan (450 ml) and anhydrous methanol (450 ml) at 20°. After 10 min, the products were neutralized with an excess of ZeoCarb 225 (pyridinium form) cation-exchange resin. The resin was filtered off and the filtrate concentrated under reduced pressure to give a solid. After the addition and subsequent removal of ethanol by evaporation, *N*(2)-acetylguanosine (5·7 g, 64%) was filtered off. Attempts to recrystallize this material were unsuccessful and it did not have a sharp m.p.; $\lambda_{max.}$ (95% EtOH) 255, 260, and 282, $\lambda_{min.}$ 228, 257, and 272 nm.

Conversion of N(2),O(2′),O(3′),O(5′)-*Tetra-acetylguanosine* (6b) *into* N(2)-*Acetylguanine* (5).—The tetra-acetylguanosine (0·30 g) was heated with 98% formic acid (7 ml) under reflux for 2 h. The products were then cooled and the formic acid was evaporated off under reduced pressure. Paper chromatography [systems (A) and (B)] and t.l.c. [system (C)] revealed a major [corresponding to *N*(2)-acetylguanine] and a minor [R_F (A) 0·70, (B) 0·80, (C) 0·92] u.v.-absorbing component. The products were purified by preparative paper chromatography on Whatman no. 3 MM paper (prewashed with M-acetic acid) in system (A). *N*(2)-Acetylguanine, eluted from the chromatogram with hot water, crystallized from water; yield 0·069 g (54%), m.p. 336° (decomp.) $\lambda_{max.}$ (H_2O) 260 (ε 16,100), $\lambda_{min.}$ 230 nm (ε 4600), R_F (A) 0·27, (B) 0·46, (C) 0·35, and was spectroscopically (u.v. and i.r.) and chromatographically [systems (A), (B), and (C)] identical with authentic *N*(2)-acetylguanine.[13] The minor u.v.-absorbing component has not been identified.

N(2),O(2′),O(3′),O(5′)-*Tetrabenzoylguanosine* (6c).—Anhydrous guanosine (19 g, 67 mmol), benzoyl chloride (57 ml, 491 mmol), and pyridine (250 ml) were stirred together at 20° for 4 h. Water (40 ml) was then added and, after a further 1 h, the products were concentrated under reduced pressure. The residue was partitioned between chloroform and aqueous sodium hydrogen carbonate. The dried (Na_2SO_4) chloroform layer was evaporated, the residue dissolved in ethanol, and the solution re-evaporated. After this procedure had been repeated several times, the resultant residue was crystallized from butan-2-one to give *N*(2),*O*(2′),-*O*(3′),*O*(5′)-tetrabenzoylguanosine (40·0 g, 85%) (Found: C, C, 64·8; H, 4·2; N, 10·0. Calc. for $C_{38}H_{29}N_5O_9$: C, 65·2; H, 4·2; N, 10·0%), m.p. 168—169° (lit.,[14] 162·5—165°), $\lambda_{max.}$ (95% EtOH) 233, 267, and 285 (ε 54,300, 17,100, and 15,200), $\lambda_{infl.}$ 256 and 296 (ε 18,300 and 14,300), $\lambda_{min.}$ 217, 263, and 274 nm (ε 29,500, 16,900, and 13,700).

Rate Studies on the Deacylation of N(2)-*Acetyl- and* N(2)-*Benzoyl-guanosines*.—Rates of deacylation were determined spectrophotometrically in (*a*) methanolic ammonia (half-saturated at 0°), (*b*) methylamine–ethanol (33% w/w), and (*c*) dimethylamine–ethanol (33% w/w) solutions. A solution of the substrate in methanol or ethanol (a few μl of the concentration required to give an optical density of 0·5—0·6 at 305 nm after dilution) was added to the ammonia or amine solution, contained in a 1 cm quartz cuvette at 20°. The stoppered cuvette was thoroughly shaken for 15 s and the change in optical density at 305 nm was measured spectrophotometrically. A blank cell containing the ammonia or amine solution was used. First-order kinetics were observed in all six experiments. The duration of each experiment was *ca.* 10 half-times of deacylation, after which the reaction was assumed to be complete. The results are illustrated in Table 2.

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