Reactivity of Some Nucleophiles and Amino-acid Derivatives towards 4-Dimethylamino-1-methoxycarbonylpyridinium Chloride †

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The kinetics of the reaction of 4-dimethylamino-1-methoxycarbonylpyridinium chloride ($MeOCOPy^+NMe_2$) with water and nucleophiles have been examined as a model reaction of 1-acyl-4-dimethylaminopyridinium ions with amino-acid residues of proteins.

Second-order rate constants determined for a series of 11 L-amino-acids allow the calculation of the relative reactivity of the different functional groups as well as the prediction of the selectivity of the reagent at various pH values (thiol, phenol, and imidazole functions are much more reactive than primary amines).

The reagent $MeOCOPy^+NMe_2$ is particularly insensitive to hydrolysis in the pH range 5—7, a region in which hydrolysis is often competitive with nucleophilic reactions. A Brönsted correlation is observed with primary amines; the slope is $\beta = 1$, a value which is usually interpreted as a rate-determining breakdown of the tetrahedral intermediate. For thiol and phenol functional groups, the attack is the rate-determining step.

A comparison with $AcImH^+$ reactivity shows a similar behaviour for both substrates although $MeOCOPy^+NMe_2$ is much less reactive.

WATER-SOLUBLE reagents are widely used in biochemistry for the modification of proteins [1] because organic co-solvents can cause the denaturation of the protein structure. They may also be used in peptide semi-synthesis, for instance in the acylation of water-soluble fragments.

In spite of their high reactivity, *N*-acetylpyridinium ions display a high degree of selectivity with respect to factors other than basicity for reactions with nucleophiles [2] but their instability has limited their use for *in situ* reactions.[3]

Acylated derivative of 4-dimethylaminopyridine (DMAP) are much more stable than their unsubstituted analogues and are generally isolable compounds. The stabilisation is due to the electron-donation effect of the dimethylamino-substituent leading to a delocalisation of the positive charge.[4]

The water-soluble 1-acyl, -alkoxycarbonyl, -phosphoryl, -sulphonyl, and -cyano -4-dimethylaminopyridinium salts may be used for the rapid modification of proteins.[5] Knowledge of the relative reactivities of the various nucleophilic functionalities may be very useful for the selective modification of these functional groups.

A quantitative investigation of 4-dimethylamino-1-methoxycarbonylpyridinium chloride ($MeOCOPy^+NMe_2$) behaviour was undertaken since this compound is the first analogue of the series of alkoxycarbonyl derivatives which are more stable than the acetyl derivatives. Thus, this compound was isolated and the direct nucleophilic reaction was studied.

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The only previous evaluation of the reactivity of this compound was made by Moodie and his co-workers who estimated the first-order rate constant for the hydrolysis of $MeOCOPy^+NMe_2$ produced *in situ* from the reaction of 4-dimethylaminopyridine with methyl chloroformate.[6]

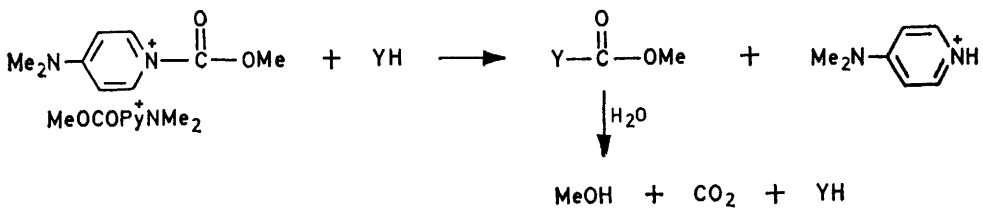
As models of protein residues, we studied the reactivity of amino-acids bearing various nucleophilic functional groups (primary amine, thiol, phenol, imidazole). From these results we can predict which functional groups will be preferentially methoxycarbonylated by $MeOCOPy^+NMe_2$ at a given pH.

In order to obtain more information about the reaction mechanism, we have also studied the reactivity of some common nucleophiles. These results can be compared with similar studies on some acyl heterocyclic amides [7] which have been examined as potential acylating agents (*N*-acetyl-imidazolium [8] and -pyridinium [2] ions, *N*-acetylsuccinimide,[9] *N*-acetyl-1,2,4-benzotriazole [10,11]).

This study enables us to give structure–reactivity relationships in a reaction where both the nucleophile and the leaving group are amines.

RESULTS

In the presence of a nucleophile, $MeOCOPy^+NMe_2$ gives the corresponding methoxycarbonyl derivative, the hydrolysis of which is slow. For some nucleophiles (phenol, imidazole, and phenylalanine) methoxycarbonyl derivatives have been isolated on a preparative scale (see Experimental section).



SCHEME 1

The observed rate equation is given in equation (1).

$$-d[MeOCOPy^+NMe_2]/dt = k_{obs}.[MeOCOPy^+NMe_2] \quad (1)$$

Second-order rate constants k_n for reactions of nucleophiles with $MeOCOPy^+NMe_2$ at 25 °C and ionic strength 0.4

	Nucleophiles	pK'_a [a]	pH	[Y] + [YH]/M [b]	Number of runs [c]	k_n/dm³ mol⁻¹ s⁻¹
Amines						
1	Trifluoroethylamine	5.72	5.27—6.29	0.027—0.16	7	$(8.3 \pm 0.4) \times 10^{-4}$
2	Histidine	6.20	5.68—6.66	0.05—0.08	6	$(7.3 \pm 0.4) \times 10^{-2}$
3	*N*-α-Acetylhistidine	7.15	6.66—7.75	0.04—0.08	6	1.10 ± 0.05
4	Imidazole	7.18	6.70—7.70	0.04—0.08	6	1.17 ± 0.05
5	Glycine ethyl ester	7.85	7.17—7.87	0.05—0.4	12	0.20 ± 0.01
6	Glycylglycine	8.24	7.76—8.76	0.005—0.12	18	0.32 ± 0.01
7	Methionine	9.18	8.71—9.67	0.005—0.045	8	1.04 ± 0.04
8	Phenylalanine	9.20	8.71—9.69	0.015—0.065	9	1.00 ± 0.04
9	*N*-ε-Acetyl-lysine	9.66	9.17—9.65	0.02—0.06	7	2.4 ± 0.2
10	Glycine	9.68	9.30—10.25	0.01—0.34	16	9.0 ± 0.4
11	n-Propylamine	10.89	10.52—10.82	0.02—0.08	7	$(1.2 \pm 0.1) \times 10^2$
12	Lysine	10.89	10.85—11.29	0.02—0.08	8	$(1.4 \pm 0.2) \times 10^2$
Miscellaneous						
13	Mercaptoethanol	9.53	9.23—9.78	0.002—0.008	7	$(9.5 \pm 0.5) \times 10^2$
14	N-Acetylcysteine	9.55	9.30—9.79	0.001—0.006	10	$(1.40 \pm 0.15) \times 10^3$
15	Phenol	9.83	9.35—10.24	0.002—0.008	8	$(3.20 \pm 0.15) \times 10^2$
16	*N*-Acetyltyrosine	9.86	9.49—10.37	0.0007—0.005	13	$(4.0 \pm 0.1) \times 10^2$
17	H_2O		2.18—5.27		4	$(3.4 \pm 0.1) \times 10^{-7}$
18	HO^-		8.71—12.12		7	$(6.5 \pm 0.5) \times 10^2$

[a] pK of the conjugate acid of the nucleophile (ionic strength 0.4) measured as described in the Experimental section. [b] Total concentration of nucleophile. [c] In stopped-flow experiments, each run represents 3—5 identical kinetic curves.

The pseudo-first-order rate constants were determined by spectrophotometric observation of the disappearance of $MeOCOPy^+NMe_2$, at 305 nm, in the presence of an excess of nucleophile. In all experiments, the temperature was maintained at 25 ± 0.1 °C and the ionic strength at 0.4 by the addition of KCl.

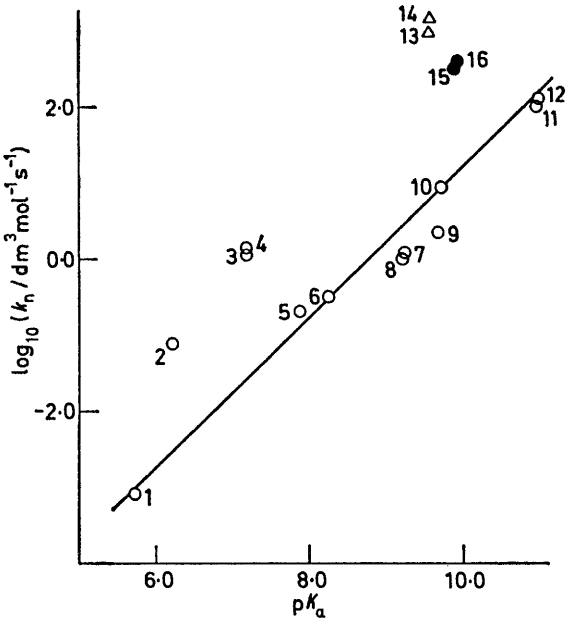


FIGURE 1 Brönsted plot for reaction of various nucleophiles with $MeOCOPy^+NMe_2$ at 25 °C and ionic strength 0.4. The numbering is the same as in the Table

At high pH, there is a contribution of hydrolysis ($k_{hyd.}$) to the overall rate constant ($k_{obs.}$).

The hydrolysis constant was determined in the absence of nucleophiles, in 0.001, 0.005, and 0.01M-hydrochloric acid solutions (k_{H_2O}) and in 0.005, 0.01, and 0.02M-sodium hydroxide solutions (k_{HO^-}). In between these extreme ranges of pH, $k_{hyd.}$ was determined by plotting $k_{obs.}$ *versus* the total concentration of nucleophile [Y + YH] at constant pH and extrapolating to [Y + YH] = 0. The nucleophilic constants (k_n) were determined by plotting ($k_{obs.} - k_{hyd.}$) *versus* the concentration of free nucleophile [Y]; the results are well fitted by equation (2).

$$k_{obs.} - k_{hyd.} = k_n[Y] \quad (2)$$

The second-order rate constants are given in the Table.

A Brönsted type plot of log k_n *versus* pK_a of the conjugate acid of the nucleophile is given in Figure 1. The data for primary amines which usually show a ' normal ' behaviour (trifluoroethylamine, glycine ethyl ester, glycylglycine, glycine, and propylamine) fall on a line with a slope $\beta = 1$. Lysine also falls on this line but *N*-ε-acetyl-lysine (α-NH_2 is the reactive amine), phenylalanine, and methionine exhibit a negative deviation from this line. This lower reactivity can be attributed to steric hindrance for the α-NH_2 function of amino-acids in which the CH_2 residue is substituted by an alkyl or functional group.

Imidazole and the histidine and *N*-α-acetylhistidine imidazoles exhibit an enhanced reactivity (35 times) compared to primary amines of the same basicity.

N-α-Acetyltyrosine has exactly the same reactivity as phenol (both having the same pK_a) but is 300 times more reactive than phenylalanine which can be used to give an order of magnitude estimate of the tyrosine α-NH_2 reactivity.

Similarly, *N*-α-acetylcysteine, which is slightly more reactive than mercaptoethanol, is much more reactive than primary amines.

DISCUSSION

Selectivity of the Reaction with Nucleophiles.—In order to compare the relative reactivities of the various nucleophilic groups of amino-acids and their *N*-acetyl derivatives in the pH range 5—9, we have calculated the apparent rate constant:

$$k_n' = \frac{k_n[Y]}{[Y + YH]} \text{ and the ratio: } r = \frac{k_n'}{k'_{n(Gly)}}$$

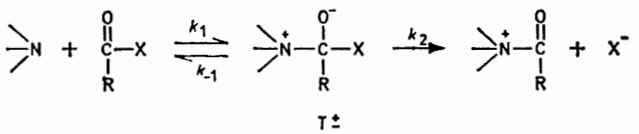
using the reactivity of the α-NH_2 group of glycine as a reference.

We observe the following. (a) Acetylcysteine is highly reactive in the whole pH range ($r = 200$). (b) *N*-Acetyltyrosine and *N*-acetylhistidine have comparable reactivities at pH 5 and 7 ($r \sim 30$) but the former becomes much more reactive at pH 9 ($r = 30$ for acetyltyrosine and $r = 0.7$ for acetylhistidine). (c) In the pH range 5—9, the amino-groups of amino-acids are about as reactive as glycine ($0.3 < r < 1.2$).

The plateau corresponding to spontaneous hydrolysis is observed until pH 5.5 where the rate of hydrolysis of $MeOCOPy^+NMe_2$ ($k_{hyd.} = k_{H_2O} + k_{HO}\text{-}[HO^-]$) begins to increase slowly. However, it is still not competitive with the nucleophilic reactions below pH 7. Above pH 7 a line of slope equal to 1 corresponding to alkaline hydrolysis is observed. This substrate then is particularly interesting in the pH range 5.5—7 where the spontaneous hydrolysis of the commonly used acylating agents is more competitive with nucleophilic reactions.

If $MeOCOPy^+NMe_2$ is to be used as a reagent for selective modification of proteins, these results may be very useful, but it must be kept in mind that (i) the reactivities of the residues in a protein depend on their unique environments and may be quite different from the reactivities of model compounds [1] and (ii) factors such as the sensitivity to hydrolysis of the modified residues must be taken into account.

Mechanism of the Nucleophilic Reaction.—A number of studies involving structure–reactivity relationships in acyl-transfer reactions provide strong evidence for a two-step mechanism with a tetrahedral intermediate (Scheme 2).[12] This two-step mechanism has been shown to occur even in the unfavourable case where the intermediate possesses two good leaving groups.[6,13] Therefore, it should be even more likely with dimethylaminopyridine which is not a very good leaving group (pK_a = 9.79).



$T^{\pm}$

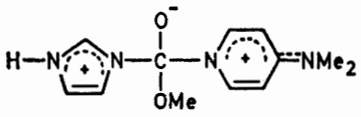
SCHEME 2

Dimethylamino-1-methoxycarbonylpyridinium ion, like other methoxycarbonylated derivatives of heterocyclic amines (imidazole and pyridine [14]) is much less reactive than its acetylated analogue. For instance, the ratio of the spontaneous hydrolysis rate constants of 1-acetyl- and 1-methoxycarbonyl-4-methylpyridinium ion is 190; [15] for the same derivatives of 4-dimethylaminopyridinium ion this ratio is 160 (comparison of the data of the Table with the rate constant for $AcPy^+NMe_2$).[4] This is also true, although to a smaller extent, for the reaction of propylamine with acetyl and methoxycarbonyl derivatives of 4-methylpyridine. The situation is completely different for esters and carbonates. For instance, the ratios of the rate constants for the reaction of nucleophiles with *p*-nitrophenyl acetate and *p*-nitrophenylmethyl carbonate vary from 1.3 to 2.4.[15]

The nature of the rate-determining step of aminolysis is dependent on the relative abilities of N and X^- to cleave from $T^{\pm}$ ($k_2 : k_{-1}$). In the case of aminolysis of $MeOCOPy^+NMe_2$ we have to compare the leaving-group abilities of primary amines and a substituted pyridine, a comparison which, to our knowledge, has been done in only a few cases.[2,15]

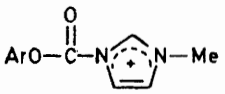
A slope $\beta = 1$ of the Brönsted plot for this reaction strongly suggests that the breakdown of the intermediate is the rate-determining step (k_2). This value of β is in good agreement with the Brönsted slopes found for the aminolysis of esters,[16] carbonates,[17] chloroformate,[6] and heterocyclic amides.[2,8] We do not observe a curvature in the Brönsted plot for the most basic amines. This would be expected for the following reasons: (a) curvatures are generally observed for substrates more reactive than $MeOCOPy^+NMe_2$ and (b) propylamine is only 1.5 pK units more basic than DMAP and, therefore, there is no reason for a change in the slow step.

Imidazole is more reactive than primary amines towards $MeOCOPy^+NMe_2$. This enhanced reactivity is observed for substrates of intermediate reactivity such as *p*-nitrophenyl acetate which is about as reactive as our substrate towards amines.[18] In those cases, the rate-determining step is believed to be the breakdown of the intermediate. This means that, in the intermediate T_2, imidazole is a better leaving group than DMAP.

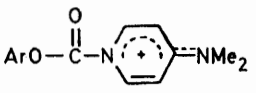


T_2

It has been recently shown by Gresser and Jencks [19] that in the reaction of phenol with compounds (1) and (2), the ratio k_N (corresponding to expulsion of amine) to k_O (expulsion of *p*-nitrophenoxide ion) is equal to 9 for *N*-methylimidazole and 4 for DMAP, showing that, in this case, DMAP is a worse leaving group than *N*-methylimidazole.



(1) Ar = $p\text{-}NO_2C_6H_4$



(2) Ar = $p\text{-}NO_2C_6H_4$

(2′) Ar = Ph

In both the cases with oxygen nucleophiles (phenol and *N*-α-acetyltyrosine) and sulphur nucleophiles (mercaptoethanol and *N*-α-acetylcysteine) the rate-determining step is the formation of the intermediate. These nucleophiles are very reactive towards $MeOCOPy^+NMe_2$. The reactivity of mercaptoethanol towards this substrate is of the same order of magnitude as it is towards 2,4-dinitrophenylacetate (DNPA).[20] Structure–reactivity correlations for the reaction of thiols with DNPA [20] show

that mercaptoethanol falls on a line with a low value of β which is interpreted as a rate-determining attack of the nucleophile. Gresser and Jencks [19] have found that the ratio k_N (expulsion of DMAP) to k_O (expulsion of phenoxide ion) is equal to 20 in the reaction of MeOH with (2'). Thus, there is no doubt that DMAP is a better leaving group than phenol and that the slow step is the phenoxide attack.

Figure 2 shows a plot of log k_n for $MeOCOPy^+NMe_2$ *versus* log k_n for $AcImH^+$ with a variety of nucleophiles. This plot gives a line with a slope equal to unity indicating that the mechanisms are probably very close and that

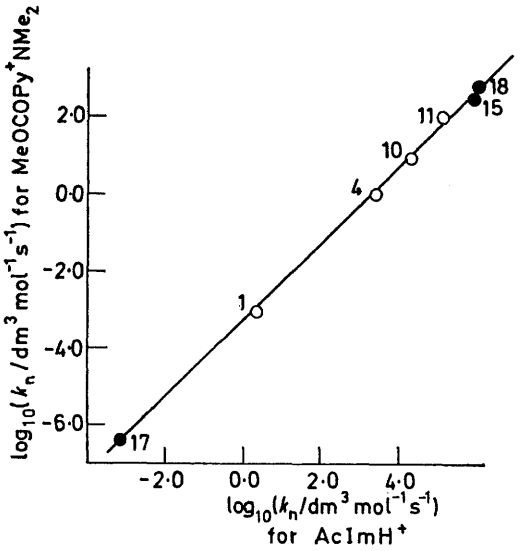


FIGURE 2 Plot of $\log_{10}k_n$ ($dm^3\ mol^{-1}\ s^{-1}$) for reaction of nucleophiles with $MeOCOPy^+NMe_2$ at 25 °C and ionic strength 0.4 (this study) *versus* the same quantity for reaction of the same nucleophiles with 1-acetylimidazolium ($AcImH^+$).[8b,c] The numbering of the nucleophiles is the same as in the Table (with $AcImH^+$, 4 is *N*-methylimidazole and 11 ethylamine)

the selectivity of these derivatives is the same. However, the slower spontaneous hydrolysis of $MeOCOPy^+NMe_2$ between pH 5.5 and 7 and the greater stability of methoxycarbonyl derivatives compared to the acetyl analogues makes it a more interesting acylating agent than acetylimidazole.

EXPERIMENTAL

Materials.—The methyl chloroformate, L-amino-acids (Flüka puriss), *N*-acetyl derivatives of the L-amino-acids (Sigma), and acetonitrile (Merck for spectroscopy) were used as supplied. 4-Dimethylaminopyridine (Cilag Chemie) was recrystallised from ethyl acetate. Trifluoroethylamine hydrochloride and imidazole were purified by recrystallisation. Phenol and n-propylamine were purified by distillation.

4-Dimethylamino-1-methoxycarbonylpyridinium Chloride.—Methyl chloroformate (0.12 cm^3, 1.5 mmol) in dry ethyl acetate (5 cm^3) were added at 0 °C to a stirred solution of DMAP (122 mg, 1 mmol) in ethyl acetate (5 cm^3). The precipitate of *4-dimethylamino-1-methoxycarbonylpyridinium chloride* (217 mg) was recrystallised from acetonitrile–ethyl acetate, m.p. 89 °C; $\nu_{max.}$ (Nujol) 1 775 (C=O) and 1 650 cm^{-1} (C=N); n.m.r. (D_2O) δ 3.39 [s, 6 H, $N(CH_3)_2$], 4.16 (s, 3 H, OCH_3), 7.0 (m, 2 H, H_β), and 8.6 (m, 2 H, H_α); u.v. $\lambda_{max.}$ (CH_3CN) 300 nm (ε 20 600); $\lambda_{max.}$ (H_2O) 302 nm (ε 19 600) (Found: C, 47.5; H, 6.7; N, 12.85. $C_9H_{13}ClN_2O_2$ requires C, 49.82; H, 6.04; N, 12.91%).

Product Studies.—Methyl phenyl carbonate. To a solution of phenol (0.01 mol, 0.94 g) and Na_2CO_3 (1 g) in H_2O (10 cm^3), $MeOCOPy^+NMe_2$ (0.015 mol, 3.24 g) was added. The mixture was stirred for 10 min and acidified to pH 3. After evaporation, the residue was extracted with ethyl acetate (1.30 g, 88% yield) and distilled, b.p. (20 mmHg), 117—118 °C [lit.,[21] b.p. (38 mmHg), 120—121 °C)]; $\nu_{max.}$ (film) 1 755 cm^{-1} (C=O) (lit.,[21] 1 754).

N-Methoxycarbonyl-L-phenylalanine Dicyclohexylammonium Salt.—To a solution of phenylalanine (0.01 mol, 1.65 g) in 1N-NaOH (10 cm^3), $MeOCOPy^+NMe_2$ (0.015 mol) was added. After the same treatment as above, the residue (2 g) was dissolved in dry ether (50 cm^3) and dicyclohexylamine (0.01 mol, 1.81 g) was added. After 2 h, the *N-methoxycarbonyl-L-phenylalanine dicyclohexylammonium salt* (4.5 g, 90% yield) was filtered, m.p. 169—170 °C (Found: C, 68.3; H, 8.85; N, 6.85. $C_{23}H_{36}N_2O_4$ requires C, 68.30; H, 8.96; N, 6.92%).

1-Methoxycarbonylimidazole.—To a solution of imidazole (0.01 mol, 0.68g) in H_2O (5 cm^3), $MeOCOPy^+NMe_2$ (0.015 mol) was added. The mixture was stirred for 10 min and evaporated to dryness. The residue was extracted with ethyl acetate and recrystallised from ether–hexane (0.52 g, 40% yield), m.p. 41—42 °C (lit.,[14] m.p. 41—42 °C).

Kinetic Methods.—Solutions were buffered by a mixture of the free base of the nucleophile Y and its conjugated acid YH ([Y] : [YH] = 1 : 3, 1, 3) without any external buffer.

Most experiments were monitored by a stopped-flow technique. The two limbs of the D 110 Durrum stopped-flow apparatus were loaded respectively with the buffer solution at twice the required concentration and an aqueous solution of $McOCOPy^+NMe_2$ containing 4% (v/v) of acetonitrile. For both solutions the ionic strength was adjusted to 0.4 by addition of potassium chloride. For slower reactions, 30 μl of an acetonitrile solution of $MeOCOPy^+NMe_2$ were added at zero time to the buffer solution (at ionic strength 0.4) in a thermostatted cell of a Cary 15 spectrophotometer containing 3 ml of solution. In the case of glycine ethyl ester, experiments were carried out on both sets of apparatus and results were in good agreement. The disappearance of $MeOCOPy^+NMe_2$ was monitored at 305 nm (315 nm for phenolate); good first-order rates were observed over *ca.* 3 half lives. The pH of each run was measured and the known concentration of Y was checked by the relation $pK'_a - pH = -\log[Y] : [YH^+]$, pK'_a being the apparent constant at ionic strength 0.4. At high pH and low concentration of nucleophile, [Y] was corrected for the concentration of hydroxy ions using $pK'_w = 14.00$—0.18 (0.18 is the value of the logarithm of the activity coefficient of potassium chloride at $\mu = 0.4$ extrapolated from Harned and Owen's values [22]).

Determinations of pK_a.—A Radiometer PHM 64 pH-meter, together with an REC 61 Servograph, autoburette ABU 12, and glass micro-electrode Radiometer type G 2222 B, was used. pK_a was calculated from pH measurements at several concentrations around the half neutralisation point. The ionic strength was maintained at 0.4 with KCl. Activity coefficient corrections on the measured values of the pK'_a give the thermodynamic pK'_a which are in good agreement with literature values.[23]

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