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IMIDAZOL-1-YLALKANOATE ESTERS AND THEIR CORRESPONDING ACIDS. A NOVEL SERIES OF EXTRINSIC ^{1}H NMR PROBES FOR INTRACELLULAR pH

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Abstract: A novel series of extrinsic probes for intracellular pH (pH_i) determination by ^{1}H NMR is described. Imidazol-1-ylacetate, malonate, 3-glutarate and 2-succinate esters were synthesized by reaction of imidazole either with α-bromoesters or with α,β-unsaturated esters. The corresponding acids were prepared by hydrolysis.

Progress in our understanding of the physiological role of intracellular pH (pH_i)[1] has been favored by the progressive development of a number of physico-chemical techniques. In particular, methodologies like equilibrium distribution of radioactive weak acids and bases[2], pH microelectrodes[1a], fluorescent probes[3] and NMR spectroscopy[4] have been applied.

In the last decade, the NMR approach has experienced a spectacular development primarily because of its ability to perform repetitive, non invasive measurements of pH_i in a variety of biological systems; including intracellular organelles, isolated or cultivated cells, perfused organs or even intact animals and human beings. Among the various NMR methodologies, ^{31}P NMR spectroscopy is probably the most widely used[5], although ^{19}F and ^{13}C NMR techniques[5,6] have also been reported. In contrast, the use of ^{1}H NMR to determine pH_i has received considerable less attention[7], in spite of its inherent advantages over ^{31}P, ^{19}F or ^{13}C NMR, in terms of sensitivity; or its additional versatility in the detection of a larger number of metabolites *in situ*.

In 1982 Rabenstein and Isab[8] proposed the use of imidazole **1** as an extrinsic probe for pH_i determination by ^{1}H NMR, remaining until now as the only example of a ^{1}H NMR probe for pH_i described in the literature. The method was based on the general theory of equilibrium distribution of weak acids and bases between the intracellular and extracellular environments[2] and on the large titration range (0.93 ppm) of the chemical shift of the imidazole H-2 proton within the physiological pH. Shortly after the addition of imidazole to an erythrocyte suspension, two signals from the H-2 proton were observed, arising respectively, from imidazole molecules distributed between the intracellular and extracellular compartments. Notably the method

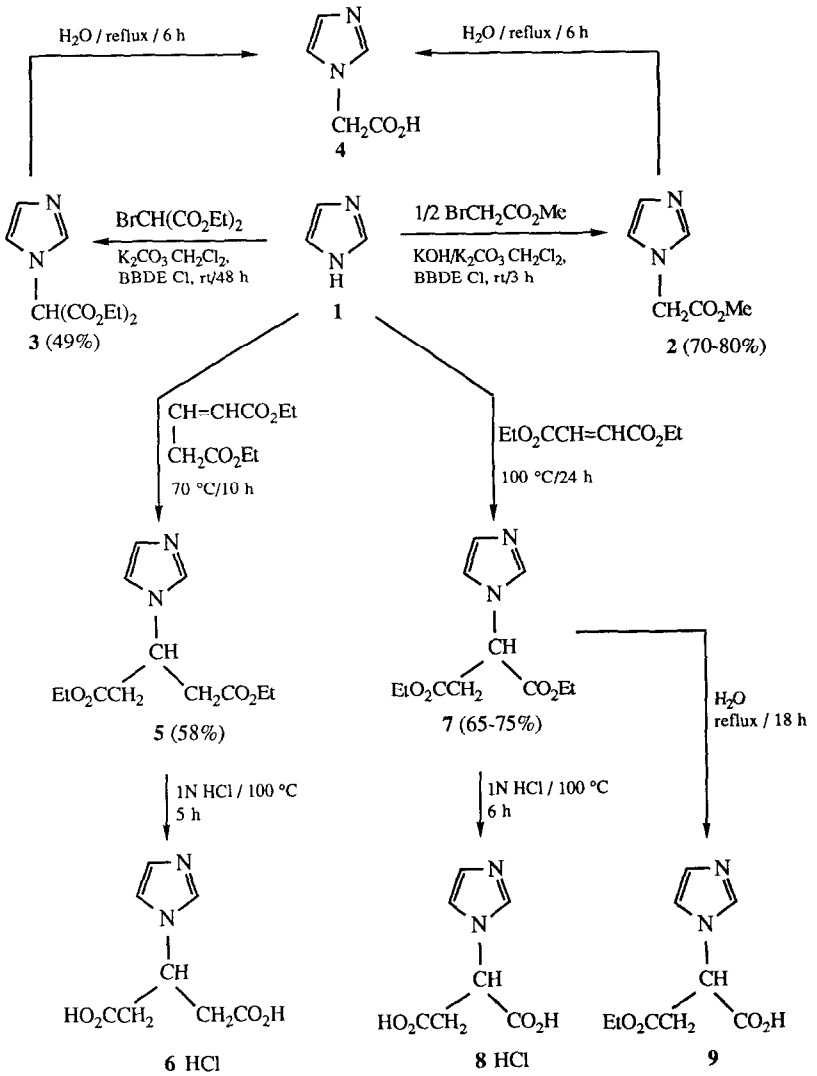
remained limited because the two H-2 signals initially observed coalesced with time into a single resonance, revealing a collapse in the transmembrane pH gradient. This effect was most probably caused by the significant permeability of the erythrocyte membrane to both, ionized and non ionized forms of imidazole; a fact which eventually resulted in an equilibration of pH between the inside and outside compartments.

In this report, we communicate several modifications of the basic structure of the imidazole molecule designed to decrease its permeability through biological membranes and improve its performance as an extrinsic pH_i probe. We have introduced either acetate, malonate, succinate or glutarate esters at the position 1 of the imidazole and further increased the negative charge of the corresponding anions at physiological pH, by preparing the corresponding acids.

Syntheses (Scheme I). Methyl imidazol-1-ylacetate **2** (70-80 %) was prepared by alkylation of imidazole **1** with methyl bromoacetate under solid-liquid phase transfer catalysis (PTC) conditions in methylene chloride and 1,5-bis-(N,N-diethylammonium)diethylether, dichloride (BBDE Cl)[9] as catalyst. The same conditions were used to synthesize diethyl imidazol-1-ylmalonate **3** (49 %) using diethyl bromomalonate as alkylating agent. This alkylation was also performed with triethylamine in acetonitrile[10] but the yield did not change significantly (53 %). The procedure described here is a convenient alternative to those reported previously for the preparation of imidazol-1-ylacetates and malonates[11]. Diethyl 3-(imidazol-1-yl)glutarate **5** could not be prepared by the PTC alkylation method because diethyl 3-bromoglutarate[12] underwent an elimination process in the reaction medium to give diethyl glutaconate. For this reason, ester **5** was obtained by addition of imidazole to the α,β-unsaturated ester (58 %). This reaction was also used to prepare diethyl 2-(imidazol-1-yl)succinate **7** by using diethyl fumarate as unsaturated ester (75 %). Imidazole also added to diethyl maleate to give a slightly lower yield (65 %) of compound **7** Esters were hydrolysed to the corresponding acids **4**, **6**, **8** and **9**. Compounds were characterized by elemental analysis, ^1H (200.13 MHz), ^{13}C (50.33 MHz) NMR and IR spectroscopy[13].

pK_a determinations. The pK_a of the H-2 proton in compounds **1-9** was determined by ^1H NMR spectroscopy (*Table I*). Observed changes in the chemical shift of the H-2 proton are derived from protonation/deprotonation at N-3 nitrogen atom. pH titrations (22 ^0C) were performed using 10 mM solutions of the appropriate compound in D_2O, varying the pH with additions of NaOD or DCl (TSP was used as internal reference). For every compound, the dependence of the H-2 chemical shift (δ) with respect to pH was obtained and computer fitted to the Henderson-Hasselbalch equation, $pH = pK_a - \log [(\delta - \delta_1) / (\delta_2 - \delta)]$; where δ_1 and δ_2 refer to the inferior (anionic) and superior (protonated) chemical shift limits of the H-2 titration curve. A three parameter, non-linear regression algorithm based on least squares minimization, allowed to determine optimal values for δ_1, δ_2 and pK_a.

As indicated in *Table I*, the pK_a for the H-2 proton in the ester molecules was always smaller than in the corresponding acids. Moreover, the pK_a of the diacid **8** was significantly higher than the pK_a of the monoacid **9** and the latter, higher than the pK_a of the corresponding diester **7**. It is important to note here that compounds **2-9** provide the biochemist with a series of novel molecules which have; (i) a very convenient range of pK_a's near the physiological value, and (ii) superior ^1H NMR titration ranges than imidazole.



Scheme 1

Table 1. pK_a values, titration limits (δ_1, δ_2), and pH-titration ranges ($\Delta\ \delta$) of the H-2 proton in the compounds described in Scheme I.

Compound	H-2 pKa	δ_1	δ_2	$\Delta\ \delta$
1	7.14	7.775	8.701	0.926
2	7.10	7.645	8.700	1.055
3	5.02	7.860	9.069	1.209
4	7 23	7.641	8.713	1.072
5	6.45	7.799	8.972	1.173
6	7.37	7.705	8.772	1.067
7	6 02	7.818	8.944	1.126
8	7.32	7.696	8.799	1.103
9	6.86	7.745	8.860	1.115

Preparation of erythrocytes, incubation conditions and experimental design. Erythrocytes were prepared from the blood of adult male Sprague-Dawley rats fed ad libitum. Whole blood taken from the inferior caval vein, was centrifuged at 5000 g and washed three times with isotonic saline solution (155 mM NaCl). After the last wash, the erythrocyte pellet was resuspended to approximately 65% hematocrite in 155 mM NaCl (20% D_2O) prior to use. The erythrocyte suspension was placed in 5 mm NMR tubes and compounds **1**, **2**, **5** and **7** were added to a final concentration of 4 mM in a total incubation volume of 0.5 mL. Routinely one ^1H NMR[14] spectrum from the erythrocyte suspension was taken before the addition of the probe, and one immediately after. Then the erythrocyte suspension containing the probe was incubated for 60 minutes at 37 °C in a separate circulating water bath, and a final ^1H NMR spectrum taken immediately after the incubation period.

Determination of intracellular and extracellular pH by ^1H NMR. pH_i and extracellular pH (pH_e) were determined by ^1H NMR measuring the chemical shift of the H-2 proton (δ). This δ value was substituted in the Henderson-Hasselbalch equation described above, using pK_a, δ_1 and δ_2 previously obtained (*Table I*) pH_e was confirmed after ^1H NMR spectroscopy, by measuring the pH of the suspension using a combined glass electrode connected to a conventional pH meter.

Figure 1 shows an illustrative comparison of the use of compounds **1** (left panels) and **7** (right panels) as pH_i probes. A, C and B, D depict the 7.0 – 8.2 ppm region of ^1H NMR spectra obtained respectively, before and after the incubation period.

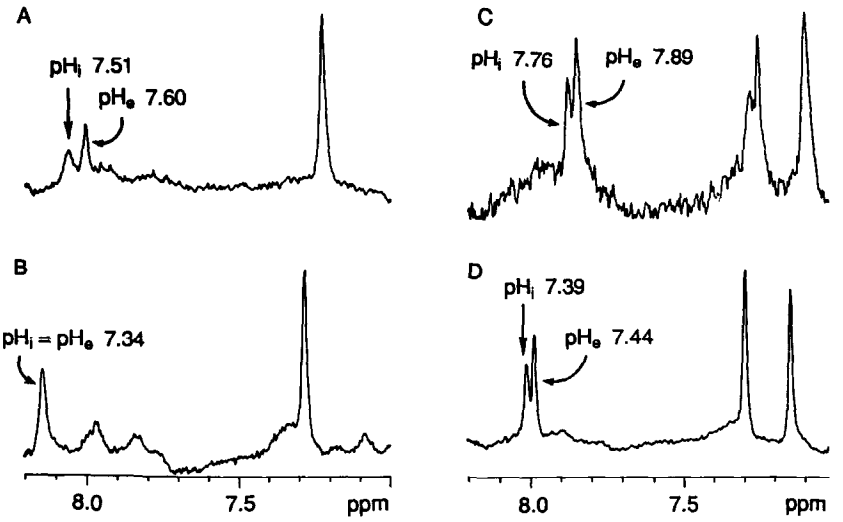


Figure 1. Use of compounds **1** (left panels) and **7** (right panels) as extrinsic pH probes. Spectra shown in the top were obtained immediately after the addition of **1** (A) or **7** (C) to the erythrocyte suspension. Spectra B and D were obtained respectively, from the erythrocyte suspensions used in spectra A and C, after 1 hour incubation at 37 °C.

Notably, only signals from **9** were observed after the addition of **7** to the erythrocyte suspension (C), indicating a rapid and total regioselective hydrolysis of **7** to its half-ester form. Two H-2 signals derived from the pH_i and pH_e compartments were observed for compounds **1** (A) and **9** (C), before the incubation period. However after 60 minutes incubation at 37 °C, only one signal was observed for compound **1** (B), revealing the complete equilibration of pH_i and pH_e. In contrast, both internal and external H-2 resonances were clearly distinguisable in compound **9** (D), a fact consistent with the conservation of the transmembrane pH gradient. In both cases, the smaller values for pH_i and pH_e obtained after incubation are due to lactic acid production from endogenous stores of glucose or glycogen. Similar results to those obtained with **7** and **9** were reproduced with **2** and **4**. In general, esters were hydrolysed in the incubation medium or intracellularly yielding the corresponding acids, with the exception of **5** which suffered no detectable hydrolysis nor penetrated the intracellular space. No further metabolic transformations of these compounds were observed during the time frame covered by these experiments. In summary compounds **2**, **4**, **7** and **9** were shown to be especially useful as novel extrinsic ^1H NMR probes for the measurement of pH_i in erythrocytes.

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13. **2**: Isolated through a silica gel column (9:1 CH_2Cl_2:Ethanol), mp 54-55 °C (from hexane) Lit.[15] oil, IR (KBr) cm^{-1} 1720 (CO), ^1H NMR ($CDCl_3$) δ 3.79 (3H, s, CH_3), 4.75 (2H, s, CH_2), 6.97 (1H, bs, H-5), 7.11 (1H, bs, H-4), 7.65 (1H, bs, H-2), ^{13}C NMR ($CDCl_3$) δ 47.3 (CH_2), 52.2 (CH_3), 119.6 (C5), 128.9 (C4), 137.5 (C2), 167.7 (CO). Anal. Calcd. for $C_6H_8N_2O_2$, C 51.42, H 5.75, N 19.99; found, C 51.59, H 5.66, N 20.19. **3**: Isolated through a silica gel column (9:1 CH_2Cl_2:Ethanol), $bp_{0.1}$ (Kugelrohr) 165-200 °C, IR (film) cm^{-1} 1730 (CO), ^1H NMR ($CDCl_3$) δ 1.31 (6H, t, J = 7.1 Hz, CH_3), 4.25-4.36 (4H, m, CH_2), 5.53 (1H, s, CH), 7.11 (1H, bs, H-5), 7.16 (1H, bs, H-4), 7.72 (1H, bs, H-2), ^{13}C NMR ($CDCl_3$) δ 13.4 (CH_3), 61.7 (CH), 62.2 (CH_2), 119.1 (C5), 128.8 (C4), 137.2 (C2), 164.2 (CO). **3** picrate: mp 118-119 °C (from ethanol). Anal. Calcd. for $C_{16}H_{17}N_5O_{11}$, C 42.21, H 3.76, N 15.38; found, C 42.30, H 3.74, N 15.37. **5**: Isolated through a silica gel column (97:3 CH_2Cl_2:Ethanol), $bp_{0.1}$ (Kugelrohr) 150-200 °C, IR (film) cm^{-1} 1715 (CO), ^1H NMR ($CDCl_3$) δ 1.19 (6H, t, J = 7.1 Hz, CH_3), 2.75-2.96 (4H, m, CH_2), 4.09 (4H, q, J = 7.1 Hz, CH_2), 5.01-5.12 (1H, m, CH), 6.96 (1H, bs, H-5), 7.04 (1H, bs, H-4), 7.56 (1H, bs, H-2), ^{13}C NMR ($CDCl_3$) δ 13.6 (CH_3), 39.8 (CH_2), 50.5 (CH), 60.7 (CH_2), 116.3 (C5), 129.2 (C4), 136.3 (C2), 169.1 (CO). **5** picrate: mp 111 °C (from ethanol). Anal. Calcd. for $C_{18}H_{21}N_5O_{11}$, C 44.73, H 4.38, N 14.49; found, C 44.57, H, 4.03, N 14.42. **7**: Isolated through a silica gel column (98:2 CH_2Cl_2:Ethanol), IR (film) cm^{-1} 1720 (CO), ^1H NMR ($CDCl_3$) δ 1.22 (3H, t, J = 7.2 Hz, CH_3), 1.25 (3H, t, J = 7.1 Hz, CH_3), 3.10 (2H, AB, J = 16.3, 7.7 Hz, CH_2), 4.14 (q, 2H, J = 7.1, OCH_2), 4.22 (2H, q, J = 7.2 Hz, OCH_2), 5.22 (1H, X (apparent t) J = 7.7 Hz, CH), 7.07 (1H, bs, H-5), 7.23 (1H, bs, H-4), 7.57 (1H, bs, H-2),^{13}C NMR ($CDCl_3$) δ 13.6 (CH_3), 13.7 (CH_3), 37.3 (CH_2), 55.6 (CH), 61.1 (CH_2), 62.1 (CH_2), 117.7 (C5), 129.4 (C4), 136.8 (C2), 168.2 (CO), 168.9 (CO). **7** picrate: mp 95 °C (from ethanol). Anal. Calcd. for $C_{17}H_{19}N_5O_{11}$, C 43.50, H 4.08, N 14.92; found, C 43.47, H 3.94, N 14.89. **4**: mp 257-258 °C (decomp) Lit.[16] 269 °C, ^1H NMR (D_2O) δ 4.76 (2H, s, CH_2), 7.36 (2H, bs, H-4, H-5), 8.61 (1H, bs, H-2), ^{13}C NMR (DMSO-d_6/H_2O) δ 52.8 (CH_2), 120.7 (C5), 124.1 (C4), 136.6 (C2), 171.4 (COOH). **6**HCl (hygroscopic) ^1H NMR (D_2O) δ 3.03 (4H, d, J = 7.1 Hz, CH_2), 5.20 (1H, q, J = 7.1, CH), 7.40 (1H, bs, H-4), 7.60 (1H, bs, H-5), 8.86 (1H, bs, H-2), ^{13}C NMR (DMSO-d_6/H_2O) δ 38.8 (CH_2), 54.2 (CH), 120.7 (C5), 121.0 (C4), 136.4 (C2), 172.0 (COOH). **8**HCl: mp 249-250 °C (decomp), ^1H NMR (D_2O) δ 3.28-3.33 (2H, m, CH_2), 5.55 (1H, dd, J = 7.2, 5.2 Hz, CH), 7.42 (1H, bs, H-4), 7.55 (1H, bs, H-5), 8.84 (1H, bs, H-2). **9**: mp 136-38 °C (from ethanol), ^1H NMR (D_2O) δ 1.12 (3H, t, J = 7.2 Hz, CH_3), 3.18-3.26 (2H, m, CH_2), 4.07 (2H, q, J = 7.2 Hz, OCH_2), 5.32 (1H, dd, J = 8.7, 5.3 Hz, CH), 7.41 (1H, bs, H-4), 7.51 (1H, bs, H-5), 8.79 (1H, bs, H-2), ^{13}C NMR (DMSO-d_6/H_2O) δ 13.4 (CH_3), 36.9 (CH_2), 56.2 (CH), 59.9 (OCH_2), 119.2 (C5), 126.7 (C4), 137.4 (C2), 169.1 (CO ester), 169.4 (COOH).
14. ^1H NMR spectra (22 °C) of the erythrocyte suspensions were acquired at 360 MHz using 90° pulses, 16K data table, 3.6 KHz spectral width (1.7 s acquisition time) and 3 s total recycle time. The water signal was attenuated using a 1 s presaturating pulse applied with the decoupler.
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