Supporting Information

for

Acidic Hydrolysis of *N*-ethoxybenzylimidazoles (NEBIs): Potential Applications as pH-Sensitive Linkers for Drug Delivery

Seong Deok Kong,[†] Alice Luong,[†] Gerald Manorek,[‡] Stephen B. Howell, [‡] and Jerry Yang[†,*]

†Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla CA 92093-0358, and ‡Department of Medicine and the Rebecca and John Moores Cancer Center, University of California, San Diego, 3855 Health Sciences Drive, MC 0819, La Jolla, 92093

General Comments

All reagents were purchased from Sigma-Aldrich, Inc., TCI, or Alfa Aesar and used without further purification. Doxorubicin (AKA Adriamycin) was from Bedford Laboratories. NMR spectra were recorded on a Varian 400 (FT, 400 MHz, ^{1}H; 100 MHz, ^{13}C) spectrometer. HRMS (high-resolution mass spectra) were obtained in the Department of Chemistry & Biochemistry, University of California, San Diego. Kinetic analysis by reverse-phase high performance liquid chromatography (RP-HPLC) was performed with a Agilent 1100 Series HPLC using an analytical reverse-phase column (SPHERI-5 Phenyl 5 micron, 250 x 4.6 mm).

General Procedure for the synthesis of *N*-ethoxybenzylimidazoles (1-8)

A solution of benzaldehyde (6.82 mmol), triethyl orthoformate (3.3 g, 22.5 mmol), and conc. HCl (10 µL, 90 µmol) was refluxed in 3.7 mL of ethanol for 24 hrs. Diethyl ether was added and the organic layer was extracted with 2 M NaOH. The organic layer was dried over anhydrous Na_2SO_4. After removal of diethyl ether under reduced pressure, the crude mixture was distilled under vacuum (55-65 °C/ 0.5 mmHg) to give pure benzaldehyde diethylacetal (82-91 % yield). All acetals were characterized by ^{1}H-NMR and used without further purification.

A mixture of imidazole (1.0 g, 14.7 mmol), benzaldehyde diethylacetal (58.8 mmol), and *p*-toluenesulfonic acid (84 mg, 0.441 mmol) was heated neat at 110 °C for 1-3 days, accompanied by concurrent distillation of ethanol. After cooling, sodium carbonate (0.47 g, 4.41 mmol) was added and the crude mixture was distilled under

vacuum (150-170 °C/ 0.5 mmHg) to give *N*-ethoxybenzylimidazole derivatives (**1**-**8**) (15-75 % yield).

Characterization of **1**: ^{1}H NMR ($CDCl_3$, 400 MHz) 1.258 (t, 3 H), 3.539 (m, 2 H), 6.178 (s, 1 H), 6.955 (s, 1 H), 7.078 (s, 1 H), 7.290-7.327 (m, 5 H), 7.673 (s, 1 H); ^{13}C NMR ($CDCl_3$, 100 MHz) 14.839, 64.469, 86.854, 116.970, 125.654, 128.357, 128.766, 129.647, 136.249, 137.616; HRMS (*m/z*) calcd for $C_{12}H_{14}N_2O$ (M^+), 202.1101; found, 202.1102.

Characterization of **2**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.254 (t, 3 H), 3.523 (m, 2 H), 6.136 (s, 1 H), 6.920 (s, 1 H), 7.091 (s, 1 H), 7.177 (d, 2 H), 7.474 (d, 2 H), 7.673 (s, 1 H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.954, 64.686, 86.308, 116.948, 123.043, 127.563, 130.088, 131.631, 136.398, 136.885; HRMS (*m/z*) calcd for $C_{12}H_{13}BrN_2O$ (M^+), 280.0206; found, 280.0207.

Characterization of **3**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ1.297 (t, 3 H), 3.573 (m, 2 H), 6.268 (s, 1 H), 6.927 (s, 1 H), 7.132 (s, 1 H), 7.506 (d, 2 H), 7.724 (s, 1 H), 8.217 (d, 2 H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.979, 65.019, 85.813, 116.881, 123.804,127.031, 130.682, 136.535, 144.476, 148.155; HRMS (*m/z*) calcd for $C_{12}H_{13}N3O_3$ (M^+), 247.0951; found, 247.0954.

Characterization of **4**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.902 (t, 3 H), 1.260 (t, 3 H), 1.322 (m, 2 H), 1.562 (m, 2 H), 2.588 (t, 2 H), 3.547 (m, 2 H), 6.162 (s, 1 H), 6.974 (s, 1 H), 7.085 (s, 1 H), 7.157 (d, 2 H), 7.211 (d, 2 H), 7.677 (s, 1 H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.998, 14.882, 22.346, 33.539, 35.324, 64.428, 86.955, 116.981, 125.596, 128.392, 129.707, 135.003, 136.274, 143.633; HRMS (*m/z*) calcd for $C_{16}H_{22}N_2O$ (M^+), 258.1727; found, 258.1728.

Characterization of **5**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.258 (t, 3 H), 3.583 (m, 2 H), 6.417 (s, 1 H), 6.959 (s, 1 H), 7.049 (s, 1 H), 7.218 (t, 1 H), 7.370 (t, 1 H), 7.536 (d, 1 H), 7.645 (d, 1 H), 7.701 (s, 1 H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.874, 64.754, 85.960, 116.979, 122.396, 127.356, 127.605, 129.400, 130.410, 132.916, 136.490, 136.632; HRMS (*m/z*) calcd for $C_{12}H_{13}BrN_2O$ (M^+), 280.0206; found, 280.0203.

Characterization of **6**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.253 (t, 3 H), 3.549 (m, 2 H), 3.747 (s, 1 H), 6.472 (s, 1 H), 6.841 (d, 1 H), 6.966-7.340 (m, 4 H), 7.638-7.667 (m, 2 H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.257, 55.762, 64.738, 82.219, 110.872, 117.093, 120.806, 126.206, 126.532, 129.261, 130.293, 136.770, 156.288; HRMS (*m/z*) calcd for $C_{13}H_{16}N_2O_2$ (M^+), 232.1206; found, 232.1209.

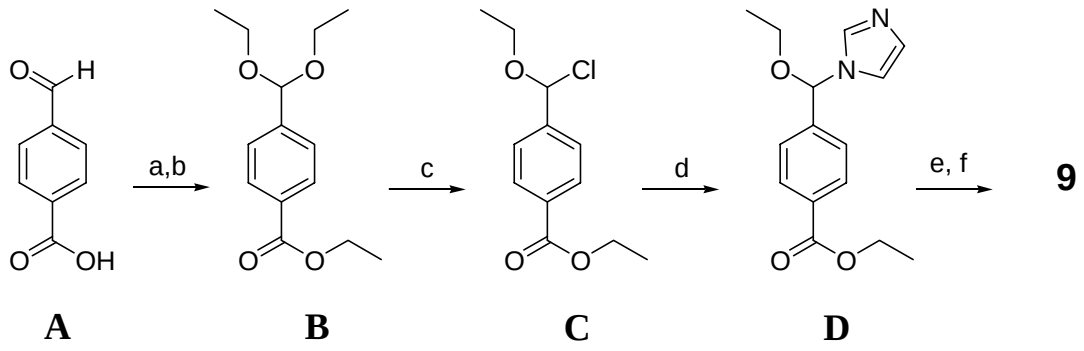
Characterization of **7**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.300 (t, 3 H), 3.664 (m, 2 H), 6.491 (s, 1 H), 7.059 (s, 1 H), 7.127 (s, 1 H), 7.450-7.546 (m, 2 H), 7.615-7.706 (m, 2

H), 7.763 (s,, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.926, 65.643, 85.159, 111.088, 116.643, 117.062, 126.209, 129.568, 130.139, 133.254, 136.386, 141.222; ^{13}C NMR (Acetone-d_6, 100 MHz) δ 14.495, 65.035, 84.882, 111.161, 116.563, 117.125, 126.592, 129.744, 129.846, 133.435, 133.606, 137.071, 141.789; HRMS (*m/z*) calcd for $C_{13}H_{13}N_3O$ (M^+), 227.1053; found, 227.1055.

Characterization of **8**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.213 (t, 3 H), 3.583 (m, 2 H), 6.907 (s, 1 H), 7.030 (s, 1 H), 7.134 (s, 1 H), 7.289 (d, 1 H), 7.517-7.627 (m, 2 H), 7.725 (s,, 1 H), 7.933 (d, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.683, 65.873, 83.735, 117.508, 125.062, 127.500, 130.140, 130.241, 132.873, 133.443, 136.940, 148.102; HRMS (*m/z*) calcd for $C_{12}H_{13}N_3O_3$ (M^+), 247.0951; found, 247.0950.

Synthesis of *N*-ethoxybenzylimidazole-doxorubicin conjugate (9)

We synthesized compound **9** using a synthetic route outlined in Scheme S1.



Scheme S1. Synthetic scheme for *N*-ethoxybenzylimidazole-doxorubicin conjugate **9**. Reagents: a) $SOCl_2$, ethanol; b) triethyl orthoformate, HCl, ethanol, 78 % yield for two steps; c) $SOCl_2$, HOAc, neat; d) NaH, imidazole, THF, 33% yield for two steps; e) NaOH, THF/H_2O, 90% yield; f) doxorubicin, EDC, THF, 54% yield.

4-carboxylbenzaldehyde (**A**) (13.5 mmols) and thionyl chloride (27 mmols) was refluxed in 50 mL anhydrous dichloromethane (DCM) and 20mL distilled ethanol for 12 hours. An additional 3 mL of thionyl chloride (41 mmols) was added and the solution was allowed to reflux for another 24 hours. The solution was cooled and the solvents were removed under reduced pressure. The product mixture was dissolved in DCM and washed with 10% sodium bicarbonate. The organic layer was dried over anhydrous Na_2SO_4. Crude NMR indicated a mixture of **B** and ethyl 4-formylbenzoate and was taken on without further purification.

The crude mixture of **B** and ethyl 4-formylbenzoate was combined with triethyl orthoformate (44.5 mmol) and conc. HCl (19.5 µL, 178 µmol) in 2.59 mL of absolute ethanol and allowed to reflux for 24 hours. After removal of ethanol and excess orthoformic acid under reduced pressure, the crude mixture was taken up in diethyl ether and the solution was washed with 2M NaOH. The organic layer was dried over anhydrous Na_2SO_4, and the diethyl ether was removed under reduced pressure. Compound **B** was distilled from the crude mixture under vacuum (141 ºC, 0.7 Torr) to give pure compound (78 % isolated yield from **A**). Characterization of **B**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.170 (t, 6H), 1.318 (t, 3H), 3.481 (m, 4 H), 4.295 (q, 2H), 5.476 (s,

1H), 7.478 (d, 2H), 7.969 (d, 2H) ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.465, 15.316, 61.157, 100.956, 126.834, 129.587, 130.526, 144.045, 166.411 ESI-MS: 224.96 (M^+ - ethyl), 206.99 (M^+ - ethoxy).

The synthesis of **C** was performed in using a modified procedure as previously reported (*1*). A solution of **B** (1.13 mmol), freshly distilled acetyl chloride (1.8 mmol) and thionyl chloride (0.227 mmol) was refluxed for one hour under N_2. The excess thionyl chloride and acetic acid were removed under reduced pressure and the crude mixture was characterized by ^{1}H NMR. ^{1}H-NMR (in $CDCl_3$) indicated a new peak at δ = 6.203 ppm, presumably corresponding to the benzylic H in **C**. The crude yield of **C** by ^{1}H-NMR was ~54%, with the remainder of the material identified as starting material **B** (~8%) as well as a large amount (~38%) of ethyl 4-formylbenzoate. The crude material was immediately taken on to the next step without further purification. **Note**: we observed hydrolysis of **C** (presumably with moisture in the air) over the course of several hours when left open to air. Compound **C** should, therefore, be prepared fresh and used immediately.

In a dry flask, NaH (0.612 mmol) and imidazole (0.612 mmol) was allowed to stir for one hour in 0.5 mL of anhydrous THF. A solution of a crude mixture of **C** (0.612 mmols) was added to the imidazole solution. The solution was stirred for 12 hours at 23 ºC. After removal of the solvent under reduced pressure, **D** was isolated by silica chromatography using as eluent a 95:5 mixture of DCM:methanol. The isolated yield of **D** was 33%. Characterization of E: ^{1}H NMR (MeOH, 400 MHz) δ 1.270 (t, 3H), 1.368 (t, 3H), 3.526 (m, 1H), 3.674 (m, 1H), 4.358 (q, 2H), 6.527 (s, 1H), 7.089 (d, 2H), 7.509 (d, 2H), 7.946 (s, 1H), 8.034 (d, 2H). ^{13}C NMR (MeOH, 100 MHz) δ 15.621, 15.158,

62.349, 65.760, 87.583, 118.570, 127.252, 129.949, 130.777, 132.216, 138.103, 144.535, 167.464 ESI-MS: 274.54 ($M+H^+$)

A solution of the **D** (0.151 mmol) and LiOH (0.151 mmol) in 0.8 mL of a 5:3 tetrahydrofuran (THF):water solution was allowed to stir for 12 hrs at 23 ºC. The THF and water were removed under reduced pressure and the crude solid was washed with chloroform. ^{1}H-NMR of the crude in CD_3OD indicated only the desired carboxylate was present (presumably as the lithium salt). The yield was estimated as 90% by weight.

The crude mixture from ester hydrolysis of **D** (.134mmol) was dissolved in 1.5 mL dimethylformamide (DMF). Doxorubicin (16.7 µmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 66.8mmol) were added to the DMF solution and stirred for 12 hours at 23 ºC. Product **9** was isolated by silica chromatography using as eluent a 92:8 mixture of DCM:methanol. The isolated yield of **9** was 54%**.** Characterization of **9**: ^{1}H NMR (MeOH, 400 MHz) δ 1.242 (t, 3H), 1.283 (m, 6H), 1.833 (d, 1H), 2.136 (m, 2H), 2.356 (d, 1H), 2.915 (d,1H), 3.017 (d, 1H), 3.491 (m, 1H), 3.635 (m, 1H), 3.741 (s, 1H), 3.955 (s, 3H), 4.326 (t, 2H), 4.753 (d, 2 H), 5.069 (s, 1H), 5.435 (s, 1H), 5.492 (s, 1H), 6.471 (s, 1H), 6.990(s, 1H), 7.081 (s, 1H), 7.433 (m, 3 H), 7.740 (t, 1H0, 7.809 (d, 3H), 7.885 (s, 1H). HRMS (*m/z*) calcd for $C_{40}H_{42}O_{13}N_3$ ($M+H^+$) 772.2712; found, 772.2721.

Synthesis of *N*-(4-formylbenzoyl)doxorubicin (10)

Doxorubicin (6.9µmol) was stirred in 660µL of acetonitrile and 340µL of water. 4-carboxybenzaldehyde (13.8 µmol), and EDC (17.6 µmol) were added to the solution and

the solution was stirred for 18 hours at 23 ºC. Product **10** was isolated by silica chromatography using as eluent a 90:10 mixture of DCM:methanol. The isolated yield of **10** was 51%**.**

Characterization of **10**: ^{1}H NMR ($CDCl_3$, 300 MHz) 1.341 (s, 3H) 1.873 (d, 1H), 2.004(m, 2H), 2.172 (d, 1H), 2.227 (d,1H), 2.353 (d, 1H), 3.103 (d, 1H), 3.301 (d, 1H), 3.760 (s, 1H), 4.266 (m, 1H), 4.788 (s, 3H), 5.337 (d, 1H), 5.556 (d, 1H), 6.519 (d, 1H), 7.416 (d, 1H), 7.800 (t, 1H), 7.919 (m, 5H), 8.051 (d, 1H), 10.064 (s, 1H). ESI-MS: 698.01 ($M+Na^+$)

General procedure for hydrolysis of *N*-ethoxybenzylimidazole derivatives (1-8)

1-8 (0.05 mmol) were placed in 0.5 mL of 0.5 M 2-[N-morpholino]ethanesulfonic acid (MES) buffer (pH=5.5) or 0.5 M N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffer (pH=7.4) containing 20-40% DMSO-d_6 (v/v) and incubated at 37 °C in a constant temperature bath. The rates of hydrolyses were obtained by ^{1}H-NMR measurements. The relative ^{1}H NMR integrations of the benzylic protons of **1-8** and the aldehyde proton of the benzaldehyde product resulting from hydrolysis were compared over the time in order to estimate the rate of hydrolysis for the *N*-ethoxybenzylimidazoles.

Hydrolysis of *N*-ethoxybenzylimidazole conjugated with Doxorubicin (9)

The *N*-ethoxybenzylimidazole conjugated with Doxorubicin (**9**) (0.37 µmol) was dissolved in 0.5 mL of 18.6 mM MES buffer (pH=5.5) or 18.6 mM HEPES buffer (pH=7.4) containing 30% DMSO (v/v) and incubated at 37 °C in a constant temperature bath. The hydrolysis of **9** at pH=5.5 and pH=7.4 was monitored by RP-HPLC by

injection of small aliquots (20 μL) of the solutions at regular time intervals and analyzing the chromatograms at λ= 470 nm. The products were eluted with an isocratic solvent mixture of 70% MeOH and 30% H_2O with a flow rate of 1 mL/min. The retention time of **9** and **10** were 6.25 minutes and 8.11 minutes, respectively. We determined the rates of hydrolysis of the *N*-ethoxybenzylimidazole moiety in **9** by comparison of the relative integrated HPLC peak areas of **9** and **10** at each time point.

Cytotoxicity studies of (10) on human ovarian carcinoma 2008 cells

Human ovarian carcinoma 2008 cells were plated in 6-well plates at a density of 200 cells in 3 mL of media (RPMI-1640 + 10% Fetal Bovine Serum) per well and incubated overnight to allow the cells to adhere to the bottom of the wells. After overnight incubation the media was removed from each well and fresh growth media containing different concentrations of **10** was added to the wells. The cells were incubated for 1 hour in the presence of **10**. Following the removal of the solutions containing **10**, the cells were incubated with fresh media for 10 days to allow surviving cells to form colonies. After removal of media, the plates were washed with 2 mL of room temperature PBS buffer (pH 7.4, 0.138 M NaCl, 0.003 mM KCl, 14.2 mM potassium phosphate), and then fixed and stained for 15 minutes with 1 mL of 0.1% crystal violet solution that contains 10% methanol. Clusters containing >50 cells were scored as colonies using an AlphaInontech Imager. We performed this colony formation assay in triplicate on 3 independent occasions.

(1) Capon, B., Nimmo, K. (1975) General acid-catalyzed hydrolysis of benzaldehyde aryl methyl acetals *J. Chem. Soc., Perkin Trans. 2*, *10*, 1113-1118.

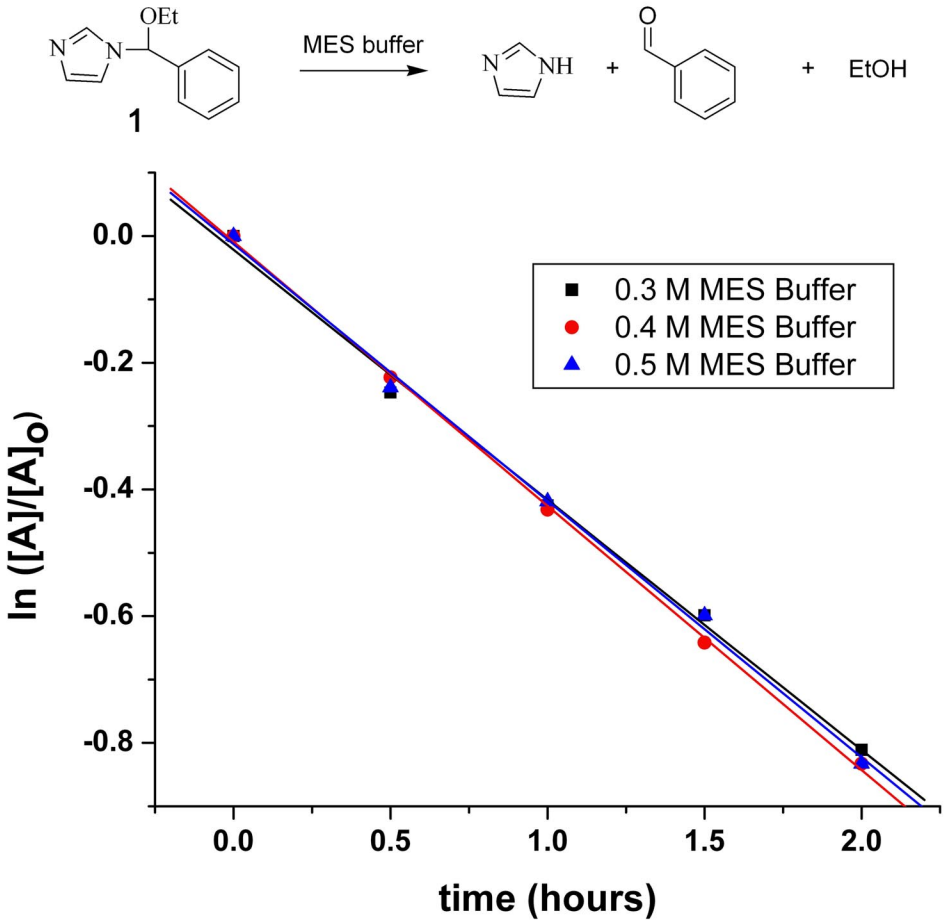


Figure S1. The dependence of the concentration of MES buffer on the rates of hydrolysis of *N*-ethoxybenzylimidazole **1**. NaCl was added to solutions containing less than 0.5 M MES buffer to maintain a constant ionic strength for all measurements. Solutions of **1** (50 mM) in buffered D_2O solutions containing 20% d_6-DMSO (v/v) were incubated in a 37°C constant temperature bath. Rates of hydrolyses were obtained by ^{1}H-NMR (400 MHz) measurements.