Synthesis and Biological Characterization of Protease-Activated Prodrugs of Doxazolidine

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Supporting Information

Synthesis of aFK-PABC-Doxaz (1)

General. Unless otherwise stated, all general information described in the main text is applicable here, including NMR, mass spectrometry, and HPLC. There are two additional HPLC methods described here. Method S1 uses a gradient of acetonitrile and 0.1% trifluoroacetic acid (TFA) and has ramp times of (acetonitrile percentages): 20% initially until 1.5 min, 30% at 5 min, 70% at 15 min, held isocratic until 20 min, the returning to 20% by 22 min. Method S2 was a gradient of acetonitrile and 20 mM sodium dihydrogen phosphate, pH 4.0 with the following profile (acetonitrile percentages): 20% initially, 35% at 2 min, 60% at 7 min, 75% at 12 min, 20% at 14 min. The machines, columns, and flow rates are the same as described in the main text.

Doxorubicin free base. See main text.

Doxazolidine. See main text.

Fmoc-PABA (3). See main text.

Loading of Fmoc-PABA to polystyrene resin. See main text

Alloc-D-Ala (4). Using a procedure adapted from work of Sakakura and Hayakawa,[1] a mixture of D-alanine (1.78 g, 20.0 mmol) and allyl chloroformate (2.5 mL, 23.6 mmol) in a 1.0 M NaOH solution (20 mL, 20 mmol) was stirred for 3 h. During this period, a 2.0 M NaOH solution (10 mL, 20 mmol) was added in four portions to the reaction mixture. Concentrated HCl (37%) was then added until the pH of the mixture became approximately 1. The resulting solution was extracted with diethyl ether (150 mL x 5). The combined organic layers were washed with brine (100 mL), dried over $MgSO_4$, and concentrated to give N-(allyloxycarbonyl)-D-alanine (alloc-D-Ala, **4** in main text, Scheme 3) (3.45 g, 99% yield) as a colorless oil. Optical rotation and ^{1}H NMR data matched the literature data.[1]

Solid phase peptide synthesis. Identical to main text, except that the SPPS method ended with the addition of **4** to the growing peptide chain, yielding alloc-D-Ala-L-Phe-L-Lys(alloc)-PABA (bis-alloc-aFK-PABA) coupled to polystyrene resin.

Cleavage of bis-alloc-aFK-PABA (5) from the resin and activation to bis-alloc-aFK-PABA-pNP carbonate (6). Method for cleavage is identical to main text. **5** (52 mg, 0.082 mmol) was added to a dry round bottom flask and 2 mL of dry THF and 2 mL dry DCM were added. To the stirring solution, p-nitrophenyl chloroformate (1.5 equiv) was added followed immediately by pyridine (1.5 equiv). The reaction was monitored by HPLC (Method S1); the chromatogram showed the product eluting at 16.9-17.0 min with strong absorption at 280 nm. Ethyl acetate (25 mL) was added and the organic layer was washed once with deionized H_2O, followed by three to four washes with saturated sodium carbonate until the aqueous layer no longer turned yellow upon extraction. The organic layer was then dried over solid $NaSO_4$ and filtered. The solvent was evaporated by rotary evaporation to yield a yellow solid, which was purified by radial chromatography, eluting with chloroform:methanol (30:1), to afford **6** (53 mg, 84%). ^{1}H NMR spectrum of the peptide derivative in $CDCl_3$/CD_3OD matched the literature spectrum.[2]

Bis-alloc-aFK-PABC-Doxaz (7). To a heat-dried round bottom flask that had been cooled under argon, **6** (151 mg 0.19 mmol) and doxazolidine (1 equiv) were added in minimal dry DMSO. The mixture was stirred under argon and the reaction was monitored by HPLC (Method S1) for the appearance of **7**, which eluted as a 480 nm-absorbing shoulder on the peak for **6** (16.9 and 17.0 min, respectively). After 5 days, more doxoazolidine (0.5 equiv) was added. After a total of 7 days, most of **6** had been consumed. The crude product was precipitated by addition of 3 mL of the reaction solution to 12 mL of ice-cold PBS, followed by centrifugation at 0 °C for 10 min at 6,000 rpm (Beckman J2-21 ultracentrifuge, JA-20 rotor, 4350 x g). The supernatant, which was strongly yellowish-orange, was removed. The precipitate was resuspended in another 12 mL of cold PBS, to which was added 3 mL of the reaction mixture, followed by centrifugation. This process was repeated until the entire volume of reaction mixture was used (here, 4 times). Washing of the pellet in cold PBS continued until the supernatant was nearly colorless (approximately three further washes). The pellet was then dissolved in DMSO and subjected to high-vacuum rotary evaporation (10^{-2} Torr) until dry. The crude product was purified by silica gel radial chromatography, eluting with 30:1 $CHCl_3$:MeOH. The total yield of **7** was 79 mg (33%) and of alloc-D-Ala-L-Phe-L-(alloc)Lys-PABC-Dox (15.1 min elution with Method S1) was 26 mg (11%). Both were pure by HPLC and NMR spectroscopy. **7** was characterized by the following spectroscopic data with

NMR assignments made from 1D ^{1}H NMR, homonuclear COSY, NOSY, HSQC, and HMBC spectra: ^{1}H NMR at 59 ºC (500 MHz, $CDCl_3$, 3% CD_3OD) δ 1.23 (d, 3H, J = 6 Hz, A-Me), 1.34 (d, 3H, J = 6 Hz, 5'-Me), 1.34 (m, 2H, K-γ), 1.44 (m, 1H, K-δ), 1.54 (bm, 1H, K-δ), 1.70 (m, 1H, K-β), 1.78 (dt, 1H, J = 15, 6 Hz, 2'), 1.95 (m, 1H, K-β), 2.02 (s, 14-OH), 2.13 (dt, 1H, J = 14, 4 Hz, 8), 2.24 (bm, 1H, 2'), 2.44 (dt, 1H, J = 14, 1 Hz, 8), 3.03 (d, 1H, J = 19 Hz, 10), 3.06 (m, buried, K-ε–NH), 3.04 (bdd, 1H, J = 13, 7 Hz, F-β), 3.12 (bm, 2H, K-ε), 3.21 (bdd, 1H, J = 13, 6 Hz, F-β), 3.25 (dd, 1H, J = 19, 2 Hz, 10), 4.01 (dd, 1H, J = 6, 2 Hz, 4'), 4.04 (m, 1H, Ala-α), 4.05 (s, 3H, 4-OMe), 4.10 (m, 1H, 5'), 4.15 (m, 1H, 3'), 4.30 (bdd, 1H, J = 14, 6 Hz, Ala-alloc), 4.41 (dd, 1H, J = 14, 6 Hz, Ala-alloc), 4.48 (m, 1H, K-α), 4.54 (m, 2H, K-alloc), 4.61 (q, 1H, J = 6 Hz, F-α), 4.68 (9-OH), 4.72 (d, 1H, J = 6 Hz, 14), 4.92 (d, 1H, J = 4 Hz, OCH_2N), 5.00 (bd, 1H, J = 4 Hz, OCH_2N), 5.10 (bd, 1H, J = 12 Hz, Ala-alloc), 5.10 (s, 2H, Bn), 5.15 (d, 1H, J = 18 Hz, Ala-alloc), 5.17 (dd, 1H, J = 12, 2 Hz, K-alloc), 5.27 (dd, 1H, J=18, 2 Hz, K-alloc), 5.29 (bs, 1H, 7), 5.41 (t, 1H, J = 6 Hz, 1'), 5.48 (bd, J = 6 Hz, Ala-NH), 5.71(ddt, 1H, J = 18, 12, 6 Hz, Ala-alloc), 5.90 (ddt, 1H J = 18, 12, 6, Hz, K-alloc), 7.09 (bd, J = 8 Hz, K-α–NH), 7.21 (m, 3H, m-F, p-F), 7.23 (d, 2H, J = 8 Hz, o-F), 7.29 (d, 2H, J = 8 Hz, PABC 2"), 7.39 (d, 1H, J = 8 Hz, 3), 7.60 (d, 2H, J = 8 Hz, PABC 3"), 7.75 (t, 1H, J = 8 Hz, 2), 8.02 (d, 1H, J = 8 Hz, 1), 8.45 (s, PABC-NH), 13.17 (s, phenolic OH), 13.83 (s, phenolic OH) ; ^{13}C NMR chemical shifts from HSQC δ 15.98 (5'-Me), 17.38 (Ala-Me), 22.86 (K γ), 29.57 (2'), 30.50 (K β), 34.09 (10) 37.02 (F β), 35.84 (8), 40.45 (K ε), 50.28 (3'), 51.07 (Ala α), 53.94 (K α), 55.02 (F α), 56.83 (4-OMe), 65.30 (14), 65.60 (5'), 65.61 (K-alloc), 66.12 (Ala-alloc), 67.02 (Bn), 69.12 (7), 77.80 (4'), 79.14 (O-CH_2-N), 99.87 (1'), 117.70 (K-alloc), 117.95 (K-alloc), 118.97 (3), 119.96 (1), 120.14 (PABC 3"), 127.18 (m- or p-F), 128.76 (o-F), 128.95 (PABC 2"), 129.05 (m- or p-F), 135.69 ppm (2); some unprotonated carbons from HMBC δ 132.4 (Ala-alloc-CO), 133.5 (6a), 133.6 (10a), 153.3 (Bn-CO), 171.1 (F-CO), 173.7 (Ala-CO), 213.6 ppm (13); MS-ESI$^+$, observed 1241.4484 (± 4.3 ppm); calculated 1241.4537; ESI-MS, observed m/z = 1241.4484; calculated m/z for (M + Na^+) = 1241.4537.

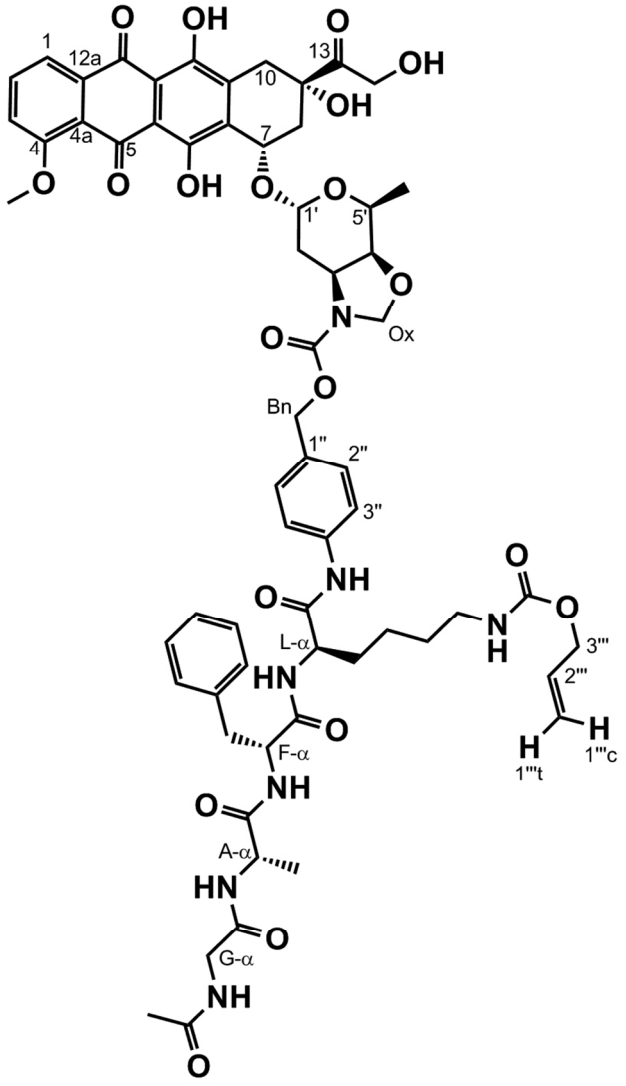
The 15.1 min product was characterized as alloc-D-Ala-L-Phe-L-(alloc)Lys-PABC-Dox from the following spectroscopic data with NMR assignments made from 1-D ^{1}H NMR and homonuclear COSY spectra and reference to the assignments for spectrum of **7**. The spectrum matched the literature spectrum[2] but is reported here at higher resolution and with the benefit of homonuclear COSY for verification of assignments: ^{1}H NMR at 55 ºC ($CDCl_3$, 10% CD_3OD) δ 1.21 (d, 3H, J = 7 Hz, Ala-Me), 1.25 (d, 3H, J = 7 Hz, 5'-Me), 1.34 (m, 2H, K-γ), 1.47 (m, 1H, K-δ), 1.69 (m, 1H, K-β), 1.54 (bm, 1H, K-δ), 1.69 (m, 1H, 2'), 1.80 (m, 1H, 2'), 1.88 (m, 1H, K-β), 2.13 (d, 1H, J = 14 Hz, 8), 2.34 (d, 1H, J = 14 Hz, 8), 2.99 (bm, 1H, F-β), 3.03 (d, 1H, J = 19, 10), 3.12 (bm, 2H, K-ε), 3.20 (bm, 1H, F-β), 3.25 (d, 1H, J = 19 Hz, 10), 3.59 (bs, 1H, 4'), 3.83 (t, 1H, J = 8 Hz, 3'), 4.03 (m, 1H, Ala-α), 4.03 (s, 3H, 4-OMe), 4.10 (m, 1H, 5'), 4.10 (m, 1H, 5'), 4.30 (m, 1H, Ala-alloc), 4.42 (dd, 1H, J = 14, 6 Hz, Ala-alloc), 4.44 (m, 1H, K-α), 4.52 (m, 2H, K-alloc), 4.61 (bs, 1H, F-α), 4.72 (s, 2H, 14), 4.96 (s, 2H, Bn), 5.08 (d, 1H, J = 10 Hz, Ala-alloc), 5.14 (d, 1H, J = 12 Hz, Ala-alloc), 5.16 (d, 1H, J = 10 Hz, K-alloc), 5.25 (d, 1H, J = 17 Hz, K-alloc), 5.46 (bs, 1H, 1'), 5.28 (bs, 1H, 7), 5.72 (ddt, 1H, J = 18, 12, 6 Hz, Ala-alloc), 5.88 (ddt, 1H, J=18, 12, 6 Hz, K-alloc), 7.17 (m, 3H, m-F, p-F), 7.20 (d, 2H, J = 8 Hz, o-F), 7.20 (d, 2H, J = 8 Hz, PABC 2"), 7.36 (d, 1H, J = 8 Hz, 3), 7.51 (d, 2H, J = 8 Hz, PABC 3"), 7.74 (t, 1H, J = 8 Hz, 2), 8.01 (d, 1H, J = 8, 1), 13.2 (s, phenolic OH), and 13.9 ppm (s, phenolic OH).

aFK-PABC-Doxaz, phosphate salt (1b). In a centrifuge tube, **7** (7.2 mg) was dissolved in 3 mL of a 2:1 mixture of DCM/THF. The solvent was degassed with argon followed by addition of morpholine (10 equiv) and tetrakis(triphenylphosphine)palladium (0) (0.2 – 0.5 equiv). The reaction was allowed to proceed for 20 min at room temperature, then centrifuged at 3030 x g for 15 min. The supernatant was removed and stored at -20 ºC. The remaining solids were washed three times with ethyl acetate (10 mL each). The solid was dissolved in 1 mL of 50 mM acetic acid and allowed to stand for 30 min at room temperature. An equal volume of 50 mM sodium acetate was added and volatile material was removed in a speedvac at $4x10^{-2}$ Torr overnight. The reaction supernatant (see above) was extracted twice into 1:1 v/v glacial acetic acid/50 mM sodium acetate (5 mL each), and volatile material was evaporated in a

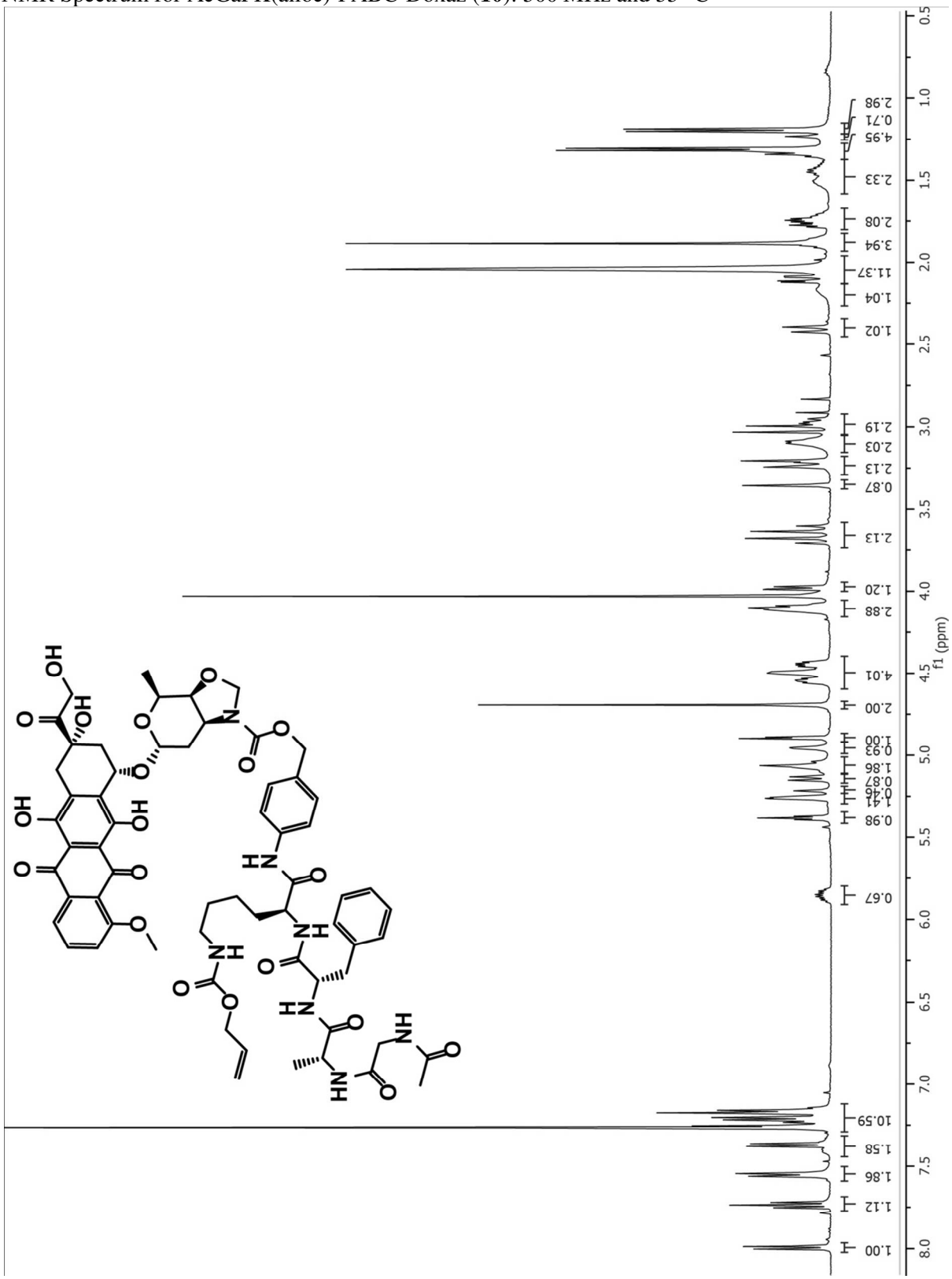
speedvac at $4x10^{-2}$ Torr overnight. Solids from both the pellet fraction and the supernatant fraction were redissolved in DMSO containing 1% v/v acetic acid. All solutions were subjected to preparative HPLC purification (Method S2), collecting the 9.8 min peak. Solvent from combined chromatography collections was evaporated using a speedvac a $4x10^{-2}$ Torr. The solid product was redissolved in DMSO, yielding **1b**. The yield was determined to be in 72% from the optical density at 480 nm of a standard solution in 50% DMSO/ 50% H_2O, assuming a molar absorbtivity of 11,500 M^{-1} cm^{-1} and was characterized from the following spectroscopic data: ^{1}H NMR at 55 ºC (DMSO-d_6) δ 1.08 (d, 3H, J = 7 Hz, Ala-Me), 1.25 (d, 3H, J = 7 Hz, 5'-Me), 1.38-1.50 (m, 2H, K-γ, overlapping patterns), 1.61 (m, 2H, K-δ), 1.7-1.9 (m, 4H, 2'and K-β, overlapping patterns), 2.20 (dd, 1H, J = 14, 6 Hz, 8), 2.24 (dd, 1H, J = 14, 4 Hz, 8), 2.79 (m, 3H, F-β and K-ε overlapping patterns), 2.99 (d, 1H, J = 18, 10), 3.01 (d, 1H, J = 18 Hz, 10), 3.19 (m, 1H, K-β), 3.16 (m, 1H, F-β), 3.83 (m, 1H, Ala-α), 3.97 (dd, 1H, J = 4, 6 Hz, 4'), 4.00 (s, 3H, 4-OMe), 4.07 (q, 1H, J = 7 Hz, 3'), 4.32 (dq, 1H, J = 4, 7 Hz, 5'), 4.40 (m, 1H, K-α), 4.55 (d, 1H, J = 20 Hz, 14), 4.58 (d, 1H, J = 20 Hz, 14), 4.71 (m, 1H, F-α), 4.86 (d, 1H, J = 4 Hz, O-CH_2-N), 4.89 (d, 1H, J = 4 Hz, O-CH_2-N), 5.04 (s, 2H, Bn), 5.05 (m, 1H, 7), 5.28 (t, 1H, J = 4 Hz, 1'), 5.31 (s, 1H, 9-OH), 7.16 (m, 1H, p-F), 7.22 (t, 2H, J = 7 Hz, m-F), 7.26 (d, 2H, J = 7 Hz, o-F), 7.30 (d, 2H, J = 8 Hz, PABC 2"), 7.62 (d, 2H, J = 8 Hz, PABC 3"), 7.65 (m, 1H, 2), 7.94 (m, 2H, 1 and 3 overlapping), 7.7-8.2 (bm, NH_3^+), 8.37 (d, 1H, J = 7 Hz, K-NH), 8.64 (d, 1H, J = 8 Hz, F-NH), 9.21 (bm, NH_3^+), 10.04 (s, 1H, anilide-NH), phenolic OH protons not shown; ESI-MS, observed m/z = 1051.4261; calculated m/z for (M + Na^+) = 1051.4295.

aFK-Dox was also prepared as described by de Groot and co-workers.[2] It was characterized by the following high resolution NMR data that compared favorably with lower resolution data reported by de Groot and co-workers:[2] ^{1}H NMR at ambient temperature (DMSO-d_6) δ 1.00 (d, 3H, J = 7 Hz, Ala-Me), 1.12 (d, 3H, J = 7 Hz, 5'-Me), 1.35 (m, 2H, K-γ), 1.47 (m, 1H, K-β), 1.47 (m, 1H, K-β), 1.60 (m, 2H, K-δ), 1.66-1.82 (m, 2H, 2' overlapping patterns), 1.83 (m, 1H, K-β), 2.12 (dd, 1H, J = 14, 5 Hz, 8), 2.19 (d, 1H, J = 14 Hz, 8), 2.73 (m, 1H, F-β), 2.78 (m, 2H, K-ε), 2.97 (d, 1H, J = 18, 10), 3.01 (d, 1H, J = 18 Hz, 10), 3.16 (m, 1H, F-β), 3.45 (d, 1H, J = 6 Hz, 4'), 3.72 (m, 1H, 3'), 3.78 (m, 1H, Ala-α), 3.98 (s, 3H, 4-OMe), 4.16 (q, 1H, J = 6 Hz, 5'), 4.40 (m, 1H, K-α), 4.58 (d, 2H, J = 6 Hz, 14), 4.71 (m, 1H, F-α), 4.75 (d, 1H, J = 6 Hz, 4'-OH), 4.89 (t, 1H, J = 6 Hz, 14-OH), 4.90 (s, 2H, Bn), 4.95 (m, 1H, 7), 5.22 (m, 1H, 1'), 5.12 (s, 1H, 9-OH), 6.89 (d, 1H, J = 8 Hz, 3'-NH), 7.17 (m, 1H, p-F), 7.24 (t, 2H, J = 8 Hz, m-F), 7.26 (d, 2H, J = 8 Hz, PABC 2"), 7.28 (d, 2H, J = 8 Hz, o-F), 7.62 (d, 2H, J = 8 Hz, PABC 3"), 7.65 (m, 1H, 2), 7.93 (m, 2H, 1 and 3 overlapping), 7.99 (bm, NH_3^+), 8.61 (d, 1H, J = 8 Hz, K-NH), 8.76 (d, 1H, J = 9 Hz, F-NH), 9.21 (bm, NH_3^+), 10.28 (s, 1H, anilide-NH), phenolic OH protons not shown.

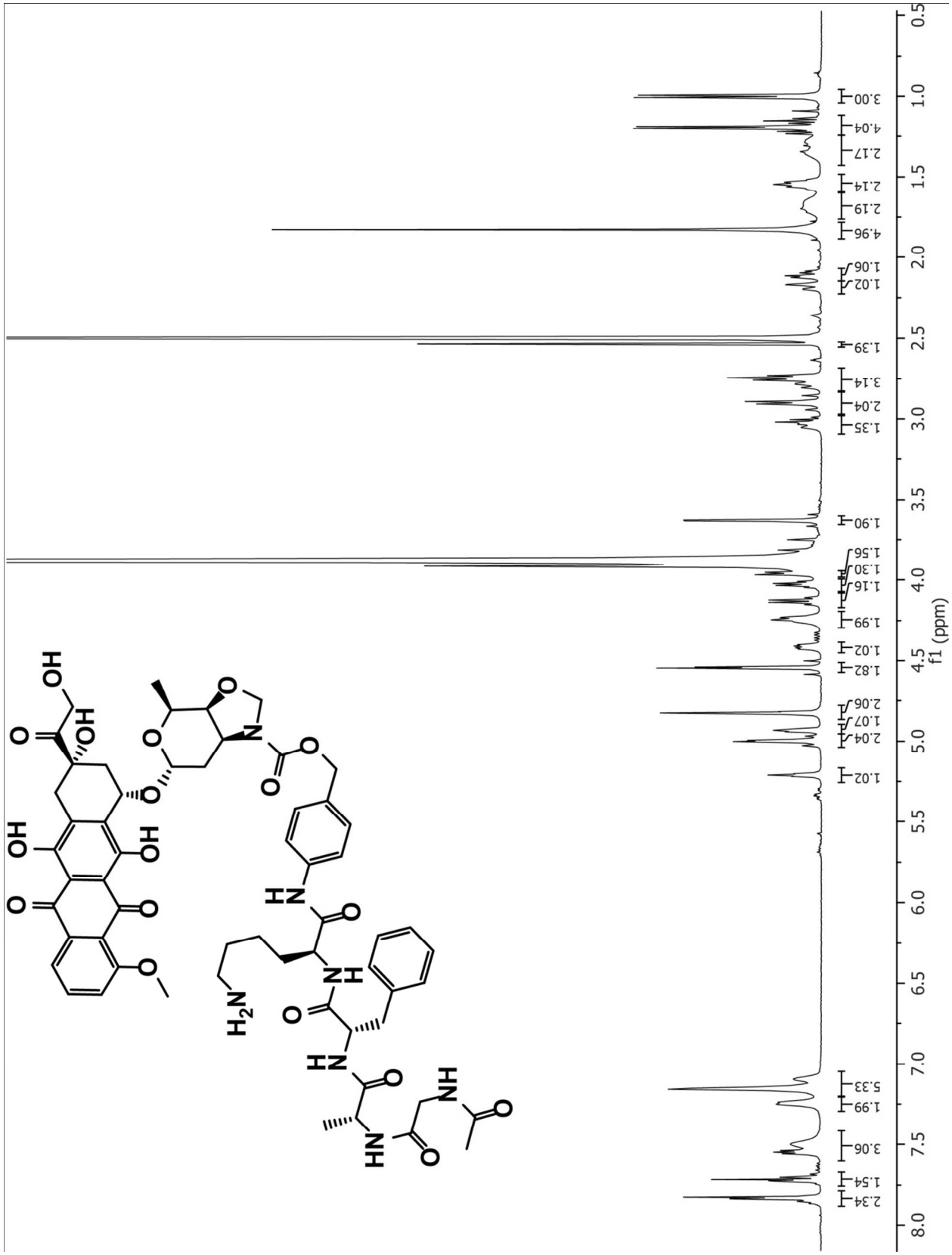
Numbering for protease-activated prodrugs of doxaz. The numbering of Ac-GaFK(alloc)-PABC-Doxaz (**10**) is shown. The numbering for all other prodrugs described in the text is similar.



NMR Spectrum for AcGaFK(alloc)-PABC-Doxaz (**10**): 500 MHz and 55 °C



NMR Spectrum for GaFK-Doxaz (**1b**): 500 MHz at 55 °C



References for Supporting Information

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