Supporting Information for:

Solid Phase Synthesis of Acridine-Peptide Conjugates and Their Analysis by Tandem Mass Spectrometry

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Experimental Section

Materials. All reagents were obtained from commercial sources and were used without further purification unless noted otherwise. Glassware for all reactions was oven dried at 125 °C overnight and cooled in a dessicator prior to use. Reactions were carried out under an atmosphere of dry nitrogen. Liquid reagents were introduced by oven-dried microsyringes. Tetrahydrofuran was distilled from sodium metal and benzophenone and dichloromethane from CaH_2. To monitor the progress of reactions, thin layer chromatography (TLC) was performed with Merck silica gel 60 F254 precoated plates, eluting with the solvents indicated. Yields were calculated for material which appeared as a single spot by TLC and homogeneous by ^{1}H NMR. Short and long wave visualization was performed with a Mineralight multiband ultraviolet lamp at 254 and 365 nanometers, respectively. Flash column chromatography was carried out using Mallinckrodt Baker silica gel 150 (60-200 mesh) or Acros aluminum oxide (basic, 50-200 micron). EM Science silica gel 60 PF254 containing gypsum was used for purification on a Harrison Research Model 7924T Chromatotron. ^{1}H and ^{13}C Nuclear Magnetic Resonance spectra of pure compounds were acquired on a Varian VXR-300 spectrometer at 300 and 75 MHz, respectively. Chemical shifts for proton and carbon NMR are reported in parts per million in reference to the solvent peak. The abbreviations s, d, dd, t, td, q, m, and brs stand for singlet, doublet, doublet of doublets, triplet, triplet of doublets, quartet, mulitplet, and broad singlet, in that order. High resolution, FAB, and EI mass spectra were recorded on a Finnigan MAT 95 mass spectrometer, and in each case a $[M]^+$ or $[M+H]^+$ peak was found.

Preparation of 9(10*H*)-acridone-4-carboxylic acid (1). Prepared according to Rewcastle and Denny *Synthesis*, **1985**, 217-220.

Preparation of *iso*-propyl-9(10*H*)-acridone-4-carboxylate. To a solution of acid **1** (0.905 g, 3.75 mmol) in freshly distilled tetrahydrofuran (THF, 20 mL), was added carbonyldiimidazole (CDI, 1.25 g, 7.5 mmol) and isopropyl alcohol (37 mmol, 10 equiv). The resulting solution was stirred at room temperature for 6 h, and TLC (toluene/acetic acid; 91:9) indicated complete conversion. The solvent was then removed under reduced pressure. The residue was dissolved in dichloromethane, extracted with 1 N NH_4OH (aq), and washed twice with water. The organic layer was dried over anhydrous Na_2SO_4, and solvent was removed under reduced pressure to yield 1.05 g (95%) of ester **3** as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.74 (dd, 1H), δ 8.44 (dd, 1H), δ 8.22 (dd, 1H), δ 7.85 (dd, 1H), δ 7.70 (td, 1H), δ 7.32 (td, 1H), δ 7.27 (td, 1H), δ 5.35 (m, 1H), δ 1.46 (d, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.4, 168.5, 142.3, 140.7, 136.8, 134.3, 133.7, 130.3, 128.1, 127.1, 122.6, 121.9, 120.2, 118.1, 114.9, 70.0, 22.2; HRMS (EI) calcd mass for $C_{17}H_{15}NO_3$: 281.11, found $[M]^+$ 281.11.

Preparation of *iso*-propyl-9-(1,2,4-triazol-1-yl)acridine-4-carboxylate (2). To a solution of 1,2,4-triazole (0.320g, 4.62 mmol, 10 equiv) in dry acetonitrile (8 mL) at 0 °C (ice-bath), was added dropwise freshly distilled phosphoryl chloride (130 μL, 1.39 mmol, 3 equiv) and distilled triethylamine (TEA, 0.775 mL, 5.55 mmol, 12 equiv) and stirred for 30 min. A solution of the previous ester (0.130 g, 0.462 mmol) in dichloromethane (2 mL) was added to the phosphoryl tristriazolide reagent mixture and refluxed for 96 h. TLC (EtOAc/hexanes/methanol; 90:9:1) indicated complete conversion, and TEA (12 equiv) and water (200 μL) were then added to quench the reaction. After 10 min, the mixture was cooled and concentrated under reduced pressure. The residue was redissolved in dichloromethane (50 mL), extracted with saturated aqueous $NaHCO_3$ (2 x 25 mL), and washed with brine (50 mL). The aqueous layers were

back extracted with 50 mL of dichloromethane. The orange colored organic layers were combined and dried over anhydrous Na_2SO_4, and then evaporated under reduced pressure. The residual gum containing intermediate **2** was ready for the subsequent substitution reaction at the 9-position. This product was not further characterized.

Preparation of *iso*-propyl-9-anilinoacridine-4-carboxylate (3). To a solution of intermediate ester **2** (0.5 mmol) in dry acetonitrile (3mL), was added distilled aniline (0.45 mL, 5 mmol, 10 equiv) and TEA (1 mL, 7.5 mmol, 15 equiv). The resulting mixture was refluxed for 24 h. When the reaction was determined to be complete by TLC (EtOAc/hexanes/methanol; 90:9:1), the solution was allowed to cool, and the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography on a column of alumina oxide, eluting with a gradient of hexanes/acetone/TEA (99.5:0:0.5 to 90:8:2) to give 0.155 g (94% over 2 steps) of **5** as an orange solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (bs, 1H), δ 7.23 (m, 4H), δ 7.05 (d, 2H), δ 6.93 (t, 2H), δ 6.75 (d, 2H), δ 6.63 (d, 1H), δ 6.57 (t, 1H), δ 5.42 (m, 1H), δ 1.32 (d, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.9, 153.5, 151.2, 141.2, 140.4, 134.2, 133.5, 131.6, 129.9, 129.5, 123.6, 122.1, 120.7, 119.8, 118.5, 117.7, 116.5, 113.1, 69.2, 22.1; HRMS (FAB) calcd mass for $C_{23}H_{20}N_2O_2$: 356.15, found $[M+H]^+$ 357.16.

Preparation of 9-anilinoacridine-4-carboxylic acid (4). To a solution of ester **3** (0.100 g, 0.28 mmol) in THF (3 mL), was added lithium hydroxide monohydrate (0.025 g, 0.60 mmol, 2 equiv) in water (0.6 M). The resulting solution was stirred at room temperature overnight. The reaction was then neutralized with 2 N HCl, the solvent was evaporated under reduced pressure, and the brick red residual oil was dissolved in chloroform. The salt precipitates were filtered away, and the organic solution was concentrated. The solvent was removed under reduced pressure to give 0.088 g (99%) of acid **4** as an orange solid. ^{1}H NMR (300 MHz, methanol-d_4) δ 8.39 (d, 1H), δ 8.09 (d, 1H), δ 7.86 (d, 1H), δ 7.62 (t, 1H), δ 7.51 (t, 1H), δ 7.40 (t, 2H), δ 7.18 (t, 1H), δ7.06 (m, 4H); ^{13}C NMR (75 MHz, methanol-d_4) δ 168.0, 153.4, 151.1, 141.2, 140.5, 134.2, 133.5, 131.6, 130.0, 129.5, 123.6, 122.2, 120.7, 119.9, 118.5, 117.8, 116.6, 113.1; HRMS (FAB) calcd mass for $C_{20}H_{14}N_2O_2$: 314.11, found $[M+H]^+$ 315.11.

Preparation of *N*-hydroxysuccinimidyl-9-anilinoacridine-4-carboxylate (5). To a solution of acid **4** (0.050g, 0.16 mmol) and *N*-hydroxysuccinimide (NHS, 0.037 g, 0.32 mmol, 1.25 equiv) in THF (2 mL), was added slowly 1,3-dicyclohexylcarbodiimide (DCC, 0.066 g, 0.32 mmol, 1.25 equiv). The mixture was allowed to stir at room temperature overnight. The dicyclohexylurea (DCU) precipitate that formed during the course of the reaction was filtered away, washed (2x) with THF, and the filtrate was collected and concentrated under reduced pressure. The residue containing intermediate **5** was ready for the subsequent amidation reaction at the 4-position. This product was not further characterized.

Preparation of (glycine-alanine-alanine)-9-anilinoacridine-4-carboxamide. Several fixed peptide sequences, including **7** (ACR-GRS, see Scheme 2 and Figure 2), were synthesized and analyzed by electrospray mass spectrometry prior to generation of the library **8**. The following procedure was used for the solid phase synthesis of the acridine-peptide conjugate ACR-GAA, a representative member of the library. Synthesis of the library was carried out in the same manner, however, a split and pool strategy was employed to generate diversity. Protected amino acids used in these syntheses were Fmoc-Arg-(Pbf)-OH, Fmoc-Lys-(Boc)-OH, Fmoc-Ser-(Trt)-OH.

Rink Amide AM resin (NovaBiochem, ~0.69 mmol/g loading, 60 mg, 0.04 mmol) was added to a 15 mL fritted-glass filter flask reactor equipped with a screw cap and teflon stopcock. The resin was pre-swelled with DMF and then shaken for 30 min on a Burrell Model 75 Wrist Action™ shaker with 50% piperidine / DMF (8 mL) to deprotect the Fmoc group. The resin was next washed with DMF (3 x 8 mL), MeOH (3 x 8 mL), and again with DMF (3 x 8 mL). Activated amino acids were prepared by dissolving the Fmoc amino acid (Fmoc-Ala-OH, 32 mg, 0.1 mmol, 2.5 equiv) and HOBt (15 mg, 0.1 mmol, 2.5 equiv) in ~1 mL DMF. PyBOP (52 mg, 0.1 mmol, 2.5 equiv) and DIPEA (20 µL, 0.1 mmol, 2.5 equiv) were then added, and the resulting solution was stirred at 4 °C for 30 min. The resin was suspended in DMF (5 mL) and the activated amino acid solution was added to the reactor. The coupling lasted ~ 3 h and was monitored by ninhydrin (Kaiser test). Double-coupling of the first residue to the resin was

performed to ensure efficiency. When a negative Kaiser test was obtained, the resin was rinsed with DMF (3 x 8 mL), MeOH (3 x 8 mL), CH_2Cl_2 (3 x 8 mL) and again with DMF (3 x 8 mL), preparing the resin for the next round of coupling.

The above procedure was repeated with Fmoc-Ala-OH then Fmoc-Gly-OH (30 mg, 0.1 mmol, 2.5 equiv), and the resulting tripeptide ready to be capped with the activated acridine intercalator. Compound **5** (3 equiv) was delivered as a THF solution to the resin. The mixture was shaken overnight in a reactor, and the resin was filtered, washing away unreacted acridine **5**. Acid **4** could be recovered from this solution later. The resin was washed with THF (3 x 8 mL), and then prepared for cleavage from the resin. Rinses with DMF (3 x 8 mL), acetic acid (1 x 8 mL), DCM (3 x 8 mL), and finally MeOH (3 x 8 mL) were performed, and the reactor was placed under high vacuum overnight. The dry resin (similar to **6**) was then suspended in a "cleavage cocktail" solution of trifluoroacetic acid/triisopropylsilane/water (TFA/TIS/H_20; 95:2.5:2.5, 8 mL) and shaken for 18 h to effect cleavage from the resin (and amino acid side chain deprotection if necessary). The resulting solution containing ACR-GAA-NH_2 was collected and concentrated under reduced pressure.

At this point, purification of the single acridine-peptide conjugate or entire combinatorial library was usually performed by a procedure detailed in Guelev, V.M. et al. *Chem. Biol*. **2000**, *7*, 1-8. Ether precipitation of the residue, followed by extraction with water, and subsequent neutralization of the the acidic aqueous layer with triethylamine gave the product(s). Following lyophilization, the residue was dissolved in water, further purified on a Waters Sep-Pak cartridge, eluting with a 50:50 mixture of MeOH/H_20 (0.1% TFA), and again concentrated. This purification gave a sample that could be analyzed by electrospray mass spectrometry. To obtain an analytically pure acridine-peptide conjugate, ACR-GAA was purified by flash chromatography on a column of silica gel eluting with chloroform/methanol/TEA (94.5:5:0.5) to give 7 mg (33% over 2 steps) of an orange solid. ^{1}H NMR (300 MHz, methanol-d_4) δ 8.40 (d, 1H), δ 8.16 (d, 1H), δ 8.07 (d, 1H), δ 8.01 (t, 1H), δ 7.55 (m, 4H), δ 7.48 (m, 4H), δ 4.4 (q, 1H), δ 4.37 (q, 1H), δ 4.27 (bs, 1H), δ 1.45 (d, 3H), δ 1.42 (d, 3H); ES-MS calcd mass for $C_{28}H_{28}N_6O_4$: 512.2, found [MH^+] 513.3; ES-MS/MS of 513.3: 496.1, 425.1, 354.1, 326.1, and 297.3; Molar extinction coefficient (ε) at λ_{max}= 441 nm was determined to be 4000 $M^{-1}cm^{-1}$ for ACR-GAA and used to approximate concentrations of all other acridine-peptide conjugates.

Mass Spectrometric Analysis of Acridine-Peptide Conjugates. Electrospray mass spectrometry (ES-MS) is a soft ionization technique that predominantly produces molecular ions. The use of electrospray tandem mass spectrometry (ES-MS/MS) enables collision-induced dissociation of a selected molecular ion, resulting in a fragmentation pattern that contains structural information regarding the selected ion. All ES-MS and ES-MS/MS experiments were performed on a Finnigan LC-Q Deca electrospray ion-trap mass spectrometer (San Jose, CA) in the positive ion mode. The acridine-peptide conjugate (or intact library) was dissolved in a 50:50 mixture of MeOH/H_2O with 1% formic acid and directly infused into the instrument at a flow rate of 8 μL/min. Typical ES-MS and ES-MS/MS conditions utilized a capillary voltage of 5 V at 275 °C. Ultrapure nitrogen was used as a sheath gas at a flow rate of 1 L/min. ES-MS/MS was carried out using ultrapure helium gas (40 psi) with a trapping time of 85 ms, and allowed for sequencing of peptides attached to the acridine. Xcaliber software was used to run the instrument and analyze the data.