DNA-Catalyzed Hydrolysis of Esters and Aromatic Amides

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Oligonucleotides and oligonucleotide conjugates

DNA oligonucleotides and oligonucleotide conjugates were obtained from Integrated DNA Technologies (Coralville, IA) or prepared by solid-phase synthesis on an ABI 394 instrument using reagents from Glen Research. All oligonucleotides and conjugates were purified by 7 M urea denaturing PAGE with running buffer 1× TBE (89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3), extracted from the polyacrylamide with TEN buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 300 mM NaCl), and precipitated with ethanol as described previously.[1] See final sections of the supporting information for all solid-phase and small-molecule synthesis procedures.

In vitro selection procedure

The selection procedure, cloning, and initial analysis of individual clones were performed essentially as described previously,[1a,2] but with a different ligation step[3] and with a new capture step based on DMT-MM–promoted amide bond formation. An overview of the key selection and capture steps of each round is shown in Figure 1. A depiction of the capture step with nucleotide details and validation data is shown in Figure S1. The random deoxyribozyme pool was 5′-CGAAGCGCTAGAACAT-N_x-AGTACATGAGACTTAGCT-GATCCTGATGG-3′ (x = 20, 30, or 40). For a reselection experiment the pool was partially (25%) randomized, prepared using phosphoramidite mixtures as described previously.[4] PCR primers were 5′-CGAAGCGCTAGAACAT-3′ (forward primer) and 5′-$(AAC)_4$XCCATCAGGATCAGCTAAGTCTCATGTACT-3′, where X is the HEG spacer to stop Taq polymerase (reverse primer). In each round, the ligation step to attach the deoxyribozyme pool at its 3′-end with the 5′-end of the carbonyl-based substrate conjugate was performed using a DNA splint and T4 DNA ligase. The splint sequence was 5′-AAGTACATGAGACTTTTCC-ATCAGGATCAGCTAAGTCTCATGTACT-3′, where the underlined T is included to account for the untemplated A nucleotide that is added at the 3′-end of each PCR product by Taq polymerase. This T nucleotide was omitted from the splint used for ligation of the initially random N_x pool, which was prepared by solid-phase synthesis without the untemplated A. Nucleotide sequences of the DNA anchor oligonucleotide, the deoxyribozyme binding arms, the 5′-amino modified capture oligonucleotide, and the capture splint are shown in Fig. S1.

Procedure for ligation step in round 1. A 25 μL sample containing 1.2 nmol of DNA pool, 900 pmol of DNA splint, and 600 pmol of carbonyl-based substrate was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. To this solution was added 3 μL of 10× T4 DNA ligase buffer (Fermentas) and 2 μL of 5 U/μL T4 DNA ligase (Fermentas). The sample was incubated at 37 °C for 12 h and purified by 8% PAGE.

Procedure for ligation step in subsequent rounds. A 17 μL sample containing the PCR-amplified DNA pool (~5–10 pmol), 30 pmol of DNA splint, and 50 pmol of carbonyl-based substrate was annealed in 5 mM Tris, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. To this solution was added 2 μL of 10× T4 DNA ligase buffer (Fermentas) and 1 μL of 1 U/μL T4 DNA ligase (Fermentas). The sample was incubated at 37 °C for 12 h and purified by 8% PAGE.

Procedure for selection step in round 1. Each selection experiment was initiated with 200 pmol of the ligated pool. A 20 μL sample containing 200 pmol of ligated pool was annealed in (conditions *A*) 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA or (conditions *B*) 5 mM CHES, pH 9.0, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The selection reaction was initiated by bringing the sample to 40 μL total volume containing (conditions *A*) 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl or (conditions *B*) 50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl. The Mn^{2+} was added from a 10× stock solution containing 200 mM $MnCl_2$. The Zn^{2+} was added from a 10× stock solution containing 10 mM $ZnCl_2$, 20 mM HNO_3, and 200 mM HEPES at pH 7.5; this stock solution was freshly prepared from a 100× stock of 100 mM $ZnCl_2$ in 200 mM HNO_3. The metal ion stocks were added last to the final sample. The sample was incubated at 37 °C for 14 h and precipitated with ethanol.

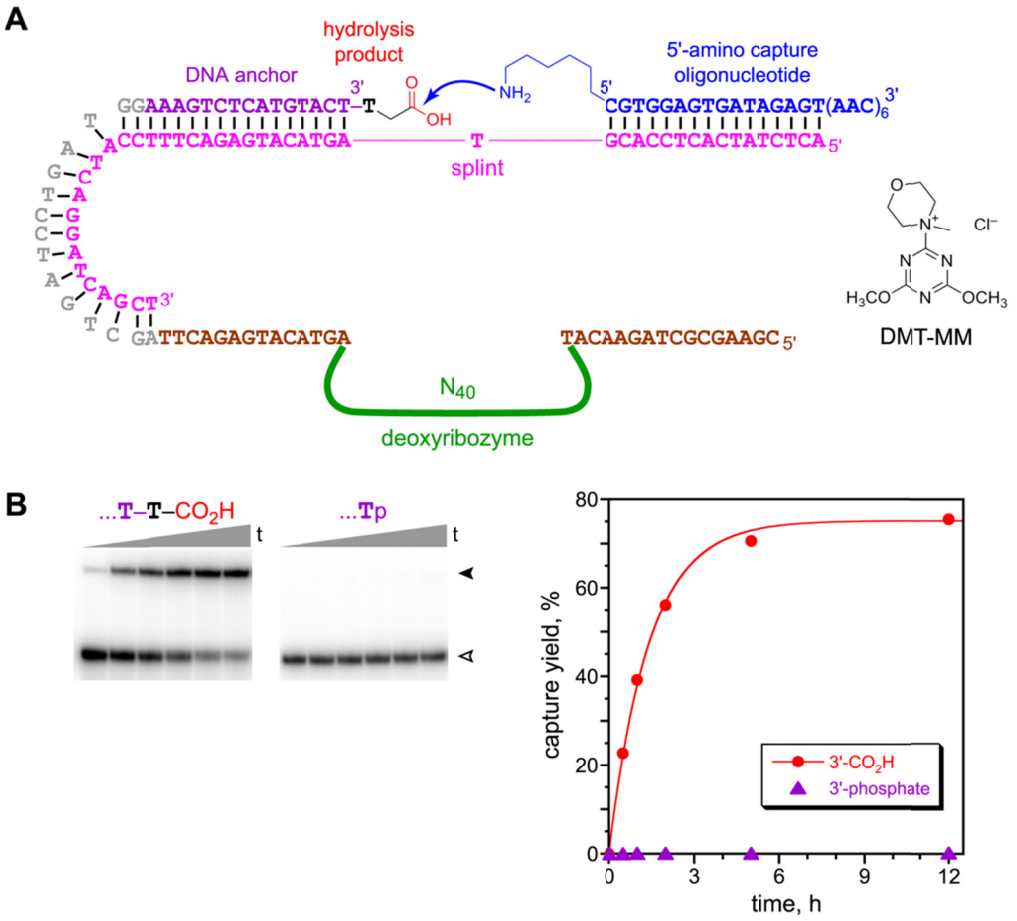


Figure S1. Capture step during in vitro selection. (A) Capture of the carboxylic acid hydrolysis product by a 5′-amino oligonucleotide with DMT-MM as activator. (B) Experimental validation of the capture reaction, along with a negative control experiment to show that the 3′-phosphate product of phosphodiester hydrolysis at the 5′-side of the 3′-terminal thymidine is not detectably captured.

Procedure for selection step in subsequent rounds. A 10 µL sample containing the ligated pool was annealed in (conditions *A*) 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA or (conditions *B*) 5 mM CHES, pH 9.0, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The selection reaction was initiated by bringing the sample to 20 µL total volume containing (conditions *A*) 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl or (conditions *B*) 50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 14 h and precipitated with ethanol.

Procedure for capture step in round 1. A 90 µL sample containing the selection product, 300 pmol of DNA splint, and 400 pmol of 5′-amino capture oligonucleotide was annealed in 100 mM MOPS, pH 7.0, and 1 M NaCl by heating at 95 °C for 3 min and cooling on ice for 5 min. The capture reaction was initiated by bringing the sample to 100 µL total volume containing 100 mM MOPS, pH 7.0, 1 M NaCl, and 100 mM DMT-MM. The sample was incubated at 37 °C for 12 h.

Procedure for capture step in subsequent rounds. A 22.5 µL sample containing the selection product, 30 pmol of DNA splint, and 50 pmol of 5′-amino capture oligonucleotide was annealed in 100 mM MOPS, pH 7.0, and 1 M NaCl by heating at 95 °C for 3 min and cooling on ice for 5 min. The capture reaction was initiated by bringing the sample to 25 µL total volume containing 100 mM MOPS, pH 7.0, 1 M NaCl, and 100 mM DMT-MM. The sample was incubated at 37 °C for 12 h. A parallel capture

standard reaction was performed in each round using the entirely random pool. The yield of each such reaction is plotted as "control" in Figure S3; the position of this capture product was used as a marker for excising the appropriate gel bands.

Procedure for PCR in subsequent rounds. In each selection round, two PCR reactions were performed, 10-cycle PCR followed by 30-cycle PCR. First, a 100 μL sample was prepared containing the PAGE-purified selection product, 200 pmol of forward primer, 50 pmol of reverse primer, 20 nmol of each dNTP, and 10 μL of 10× Taq polymerase buffer (1× = 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton X-100). This sample was cycled 10 times according to the following PCR program: 94 °C for 2 min, 10× (94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min. Taq polymerase was removed by phenol/chloroform extraction. Second, a 50 μL sample was prepared containing 1 μL of the 10-cycle PCR product, 100 pmol of forward primer, 25 pmol of reverse primer, 10 nmol of each dNTP, 20 μCi of α-^{32}P-dCTP (800 Ci/mmol), and 5 μL of 10× Taq polymerase buffer. This sample was cycled 30 times according to the following PCR program: 94 °C for 2 min, 30× (94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min. Samples were separated by 8% PAGE.

Procedure for cloning and initial screening. From the desired selection round, PCR was performed using the same procedure as described above, using primers 5′-CGAAGCGCTAGAACAT-3′ (forward primer) and 5′-TAATTAATTAATTACCCATCAGGATCAGCT-3′ (reverse primer), where the extensions with TAA stop codons in each frame were included to suppress false negatives in blue-white screening.[5] The PCR product was cloned using a TOPO TA cloning kit (Invitrogen). Initial screening of individual deoxyribozyme clones was performed using DNA strands prepared by PCR from miniprep DNA derived from individual *E. coli* colonies. The miniprep DNA samples were first assayed by digestion with EcoRI to ascertain the presence of the expected insert. The concentration of each PAGE-purified deoxyribozyme strand was estimated from the UV shadowing intensity relative to suitable standards. Each screening assay used ~0.2 pmol of 5′-^{32}P-radiolabeled ester or anilide substrate and ~20 pmol of deoxyribozyme and the single-turnover assay procedure described in a subsequent section of this document.

Structures of ester and amide substrates

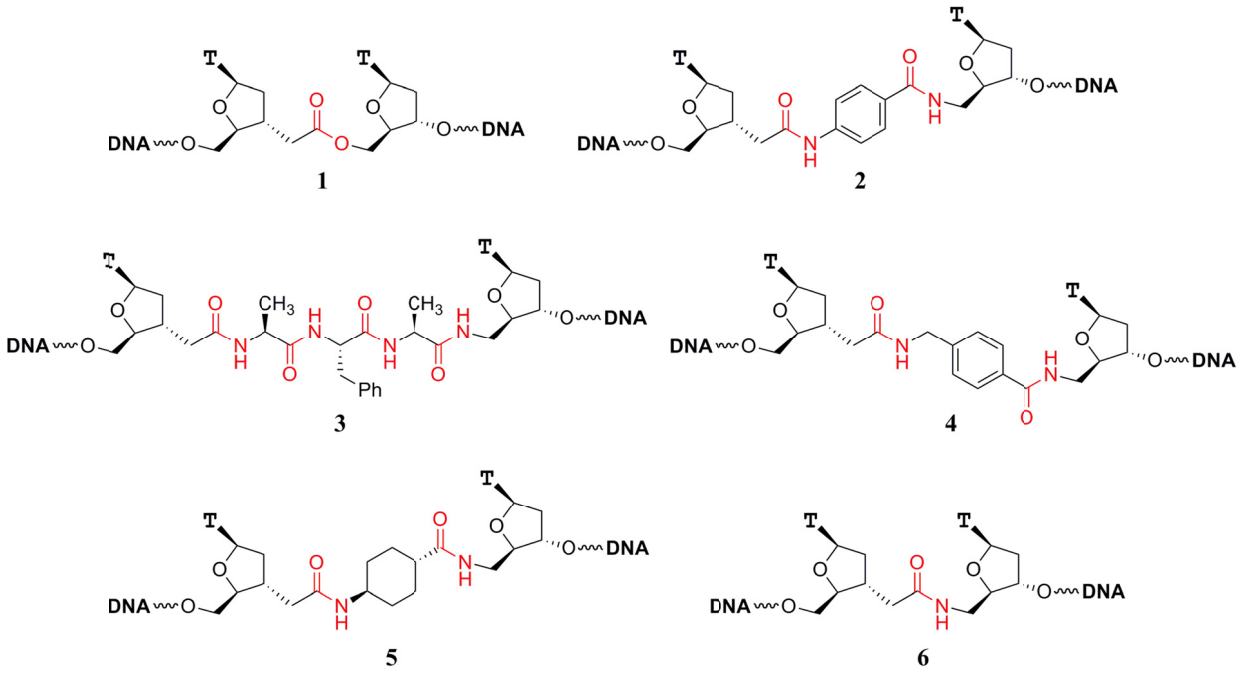


Figure S2. Full chemical structures of the ester and amide substrates shown in Figure 2, showing connections to DNA. The first thymidine (T) nucleoside on each side is drawn. The DNA sequence for the 5′-anchor segment is 5′-AAAGTCTCATGAACTT-3′, where the 3′-terminal T is illustrated. The DNA sequence for the 3′-anchor segment is 5′-TATGTTCTAGCGCTTCG-3′, where the 5′-terminal T is illustrated.

Selection progressions

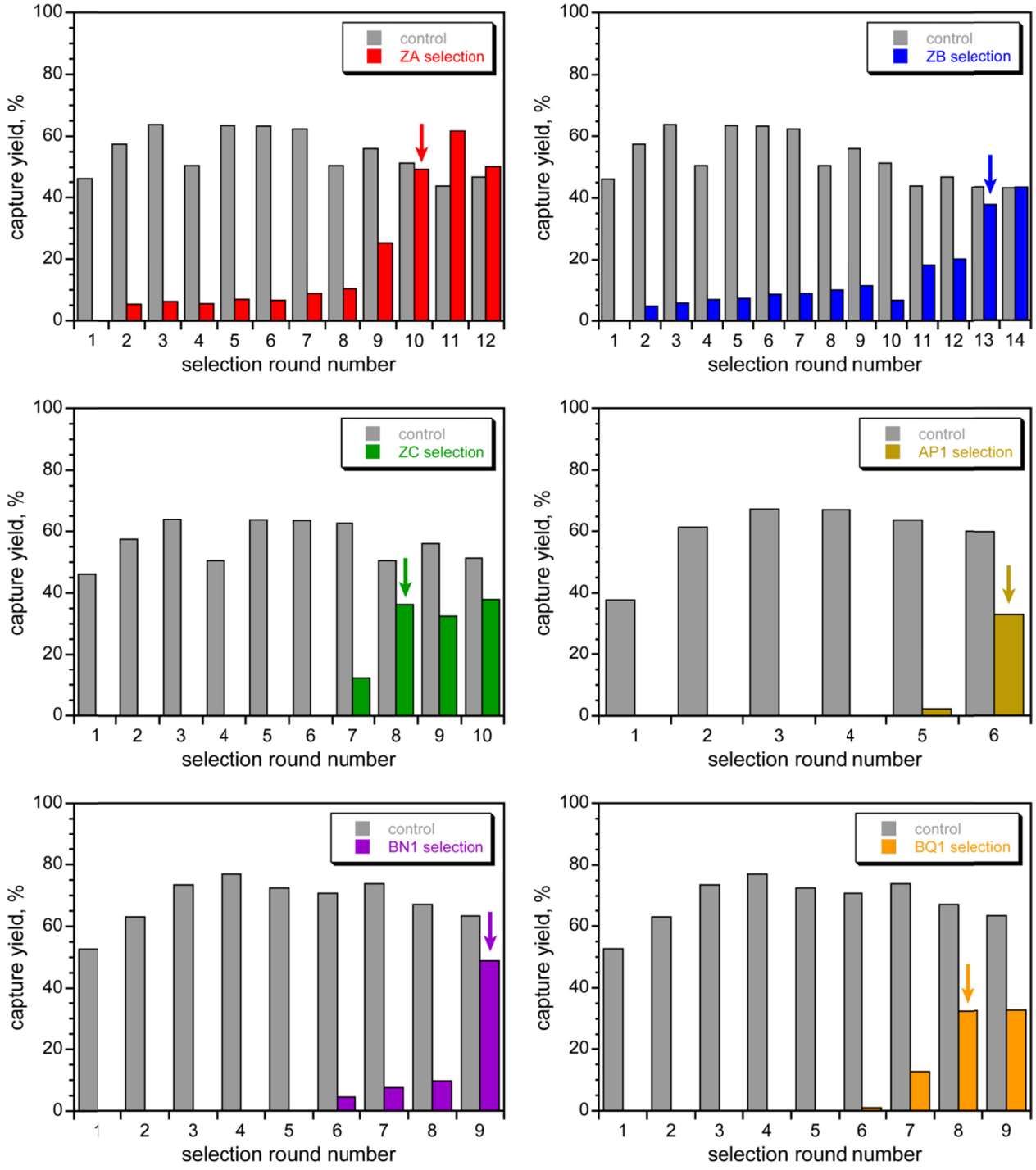


Figure S3. Progressions of the in vitro selection experiments. In each round, "control" refers to the yield for the DMT-MM–promoted capture reaction using the control 3′-CO_2H substrate as illustrated in Figure S1A, and "selection" refers to the yield for the DMT-MM–promoted capture reaction using the deoxyribozyme pool for that round. Arrows mark the cloned rounds. Round 1 pool yields were not measured because the pool was not radiolabeled. The ZA and ZB selections were for ester hydrolysis under conditions *A* and *B*, respectively, using an N_{40} random region. The ZC selection was for anilide hydrolysis under conditions *A* using an N_{40} random region. The AP1 selection was the reselection of 8ZC9 under conditions *A*. The BN1 and BQ1 selections were for anilide hydrolysis under conditions *A* using N_{20} and N_{30} random regions, respectively.

Sequences of individual deoxyribozymes

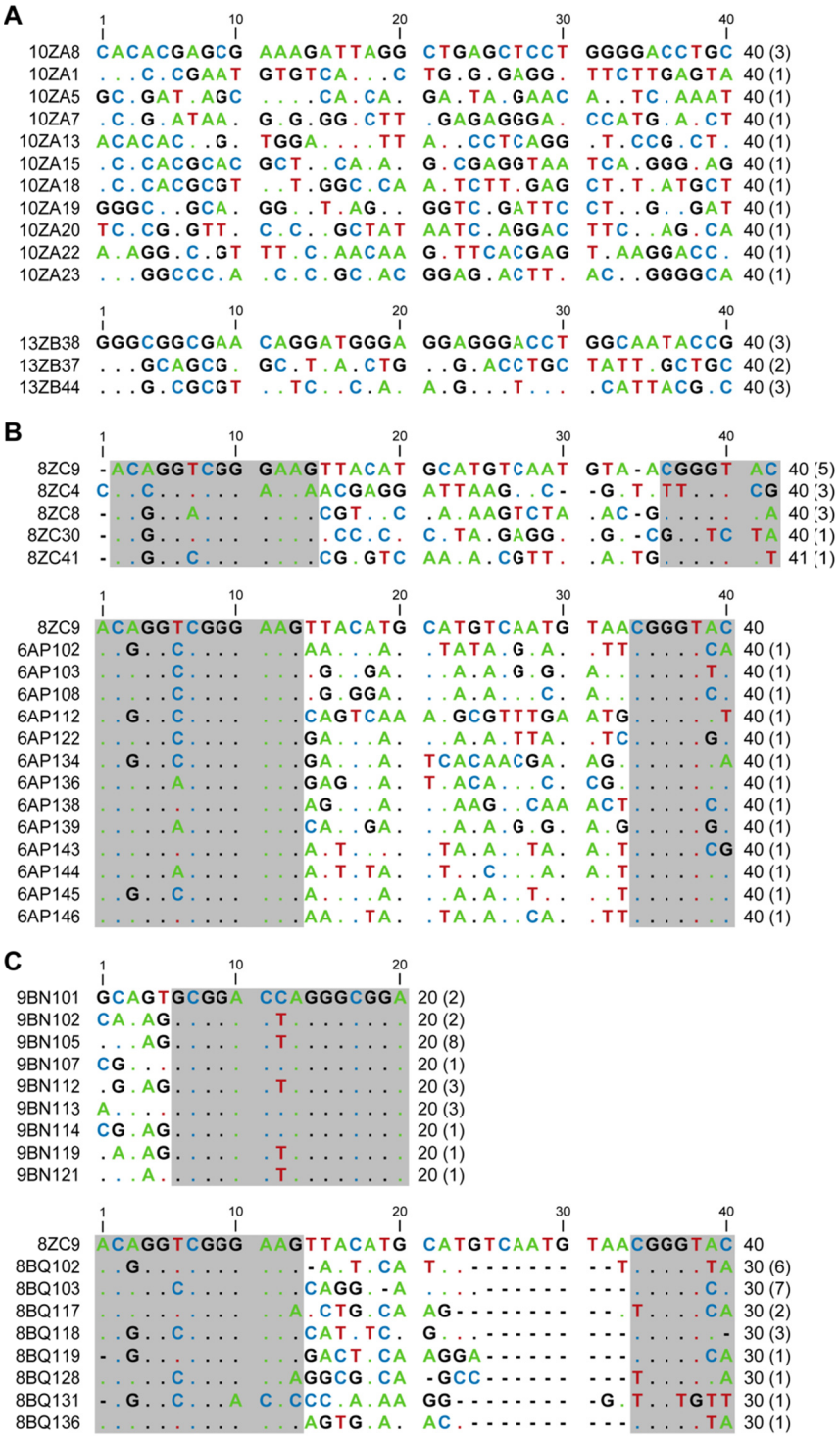


Figure S4. Sequences of the initially random regions of the new deoxyribozymes. All deoxyribozymes were used as 5′-CGAAGCGCTAGAACAT-N_x-AGTACATGAGACTTT-3′, where N_x represents the specific x nucleotides (x = 20, 30, 40) of the initially random region as determined through the selection process. All alignments show only the initially random region. Grey boxes denote conserved sequence elements. A dot denotes conservation, i.e., the same nucleotide as in the uppermost sequence; a dash denotes a gap. In both sets of sequences, next to the sequence length in nucleotides on the far right is shown (in parentheses) the number of times that sequence was found during cloning. (A) Ester-hydrolyzing N_{40} deoxyribozymes. (B) Anilide-hydrolyzing N_{40} deoxyribozymes. (C) Anilide-hydrolyzing N_{20} and N_{30} deoxyribozymes.

Single-turnover deoxyribozyme assay procedure

The ester or anilide substrate was 5′-^{32}P-radiolabeled using γ-^{32}P-ATP and polynucleotide kinase (Fermentas). A 10 μL sample containing 0.2 pmol of 5′-^{32}P-radiolabeled ester or anilide substrate and 20 pmol of deoxyribozyme was annealed in (conditions *A*) 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA or (conditions *B*) 5 mM CHES, pH 9.0, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The DNA-catalyzed hydrolysis reaction was initiated by bringing the sample to 20 μL total volume containing (conditions *A*) 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl or (conditions *B*) 50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C. At appropriate time points, 2 μL aliquots were quenched with 5 μL stop solution (80% formamide, 1× TBE [89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3], 50 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol). Samples were separated by 20% PAGE and quantified with a PhosphorImager. Values of k_{obs} were obtained by fitting the yield versus time data directly to first-order kinetics; i.e., yield = $Y \bullet (1 - e^{-kt})$, where $k = k_{obs}$ and Y is the final yield. Each k_{obs} value is reported with error calculated as the standard deviation from the indicated number of independent determinations. When k_{obs} was sufficiently low such that an exponential fit was not meaningful, the initial points were fit to a straight line, and k_{obs} was taken as the slope of the line.

Mass spectrometry to establish product identities

The cleavage products of a representative deoxyribozyme from each indicated selection experiment were analyzed by MALDI mass spectrometry. The cleavage product was prepared from a 50 µL sample containing 100 pmol of ester or anilide substrate and 200 pmol of the indicated deoxyribozyme. The sample was annealed in (conditions *A*) 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA or (conditions *B*) 5 mM CHES, pH 9.0, 15 mM NaCl, and 0.1 M EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The DNA-catalyzed hydrolysis reaction was initiated by bringing the sample to 100 µL total volume containing (conditions *A*) 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl or (conditions *B*) 70 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 24 h, precipitated with ethanol, desalted on Sephadex G-25 gel filtration resin and Millipore C_{18} ZipTip, and analyzed by MALDI mass spectrometry (matrix 3-hydroxypicolinic acid) on a Bruker UltrafleXtreme MALDI TOF/TOF mass spectrometer.

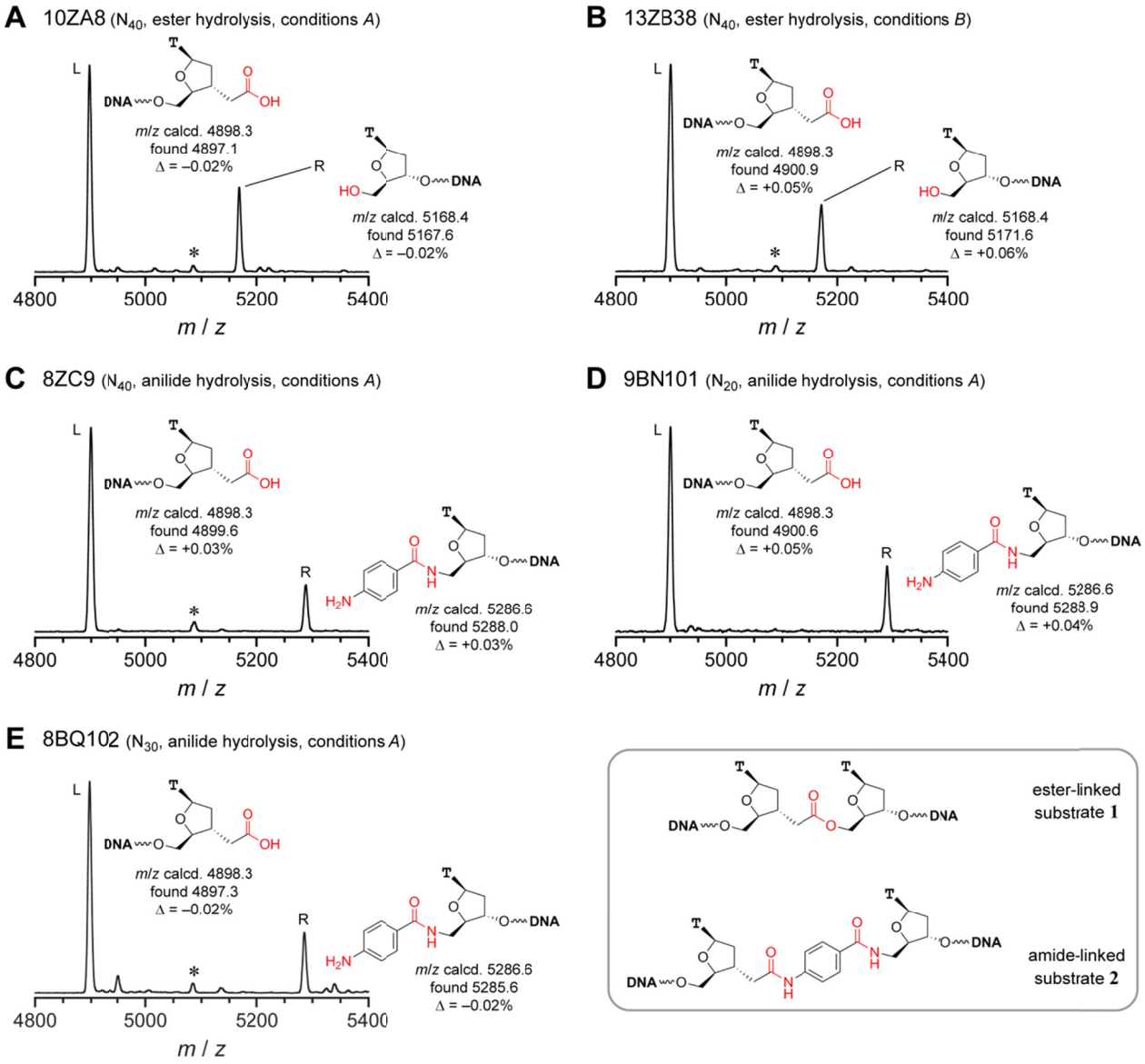


Figure S5. MALDI mass spectrometry to establish identities of cleavage products from representative deoxyribozymes. L denotes the left-hand cleavage product; R denotes the right-hand cleavage product. An asterisk denotes the peak for uncleaved substrate with $z = 2$. Ester-linked substrate **1**: (A) 10ZA8, (B) 13ZB38. Anilide-linked substrate **2**: (C) 8ZC9, (D) 9BN101, (E) 8BQ102. All *m*/*z* values are $[M+H]^+$.

Kinetic plots for additional ester-hydrolyzing 10ZA deoxyribozymes

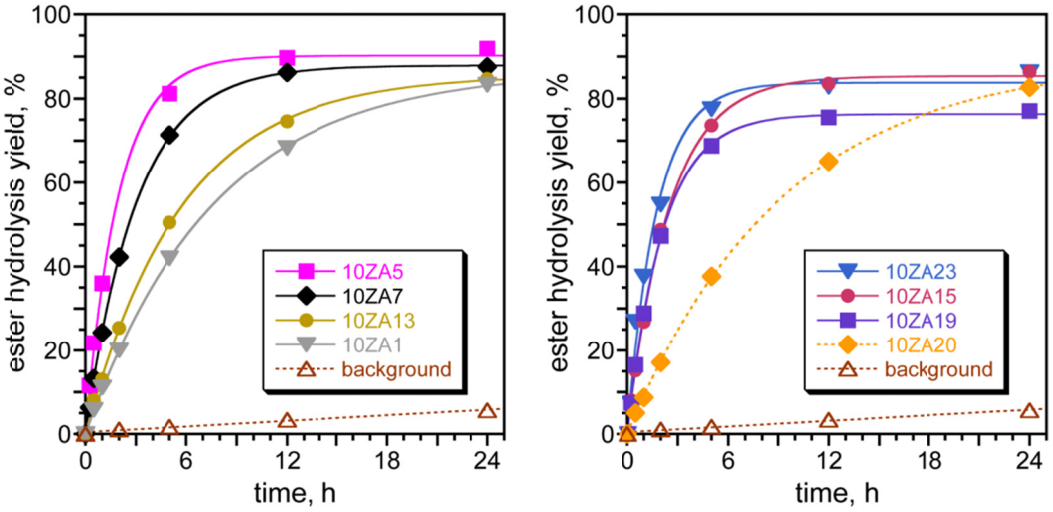


Figure S6. Kinetic plots for the eight additional ester-hydrolyzing 10ZA deoxyribozymes, in addition to the three 10ZA deoxyribozymes of Figure 3. Incubation conditions *A*: 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C. k_{obs} values for the first plot, top to bottom: 0.51, 0.33, 0.18, 0.13 h^{-1}. k_{obs} values for the second plot, top to bottom: 0.57, 0.40, 0.48, 0.11 h^{-1}. k_{bkgd} = 0.0024 h^{-1}.

Dependence of ester-hydrolyzing 10ZA and 13ZB deoxyribozymes on pH

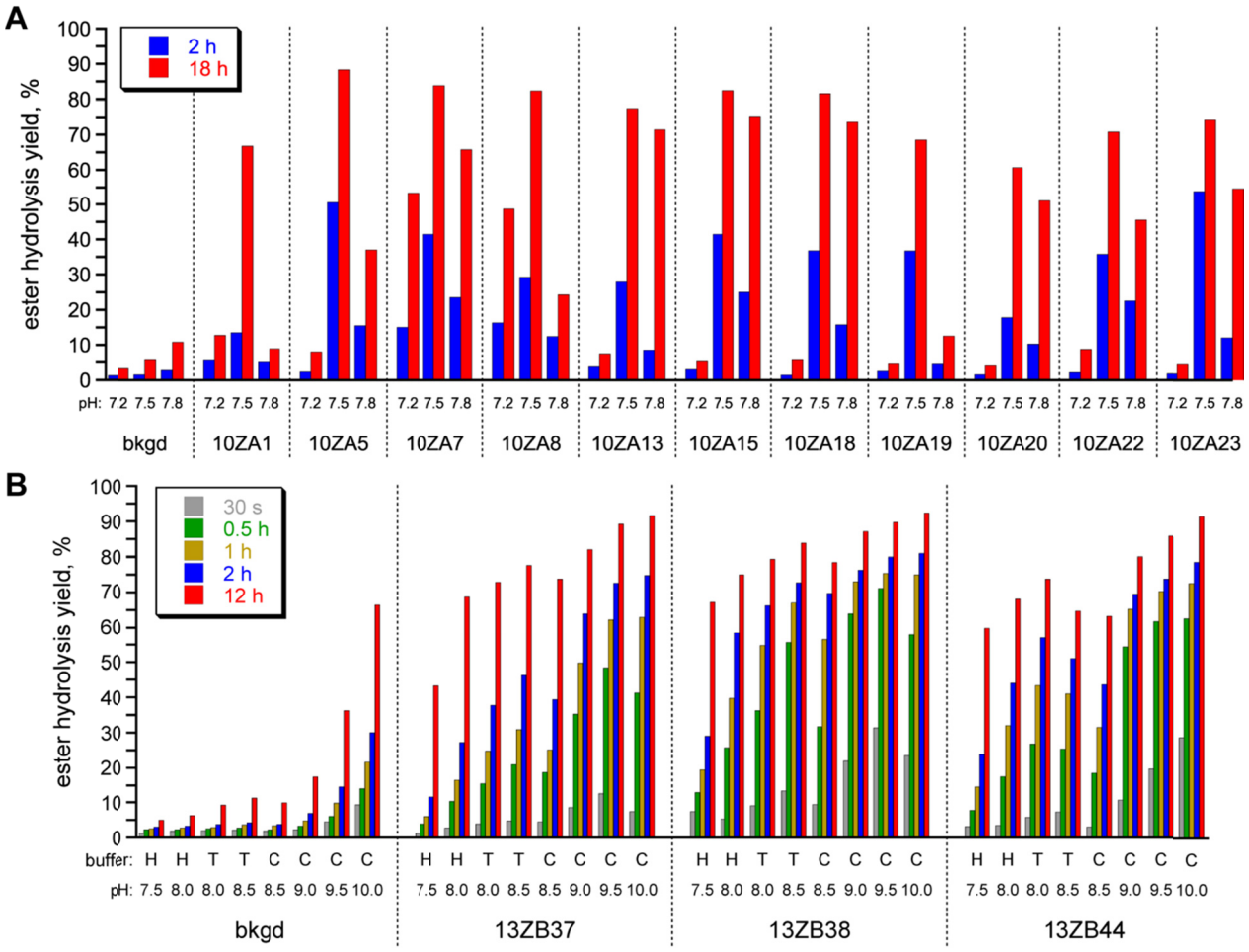


Figure S7. Dependence of ester-hydrolyzing deoxyribozymes on pH. bkgd = background ester hydrolysis in the absence of any deoxyribozyme. (A) The eleven 10ZA deoxyribozymes from conditions *A* (70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C). (B) The three 13ZB deoxyribozymes from conditions *B* (50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C). Buffer compounds are abbreviated H = HEPES, T = TAPS, C = CHES.

Dependence of ester-hydrolyzing 10ZA and 13ZB deoxyribozymes on metal ions

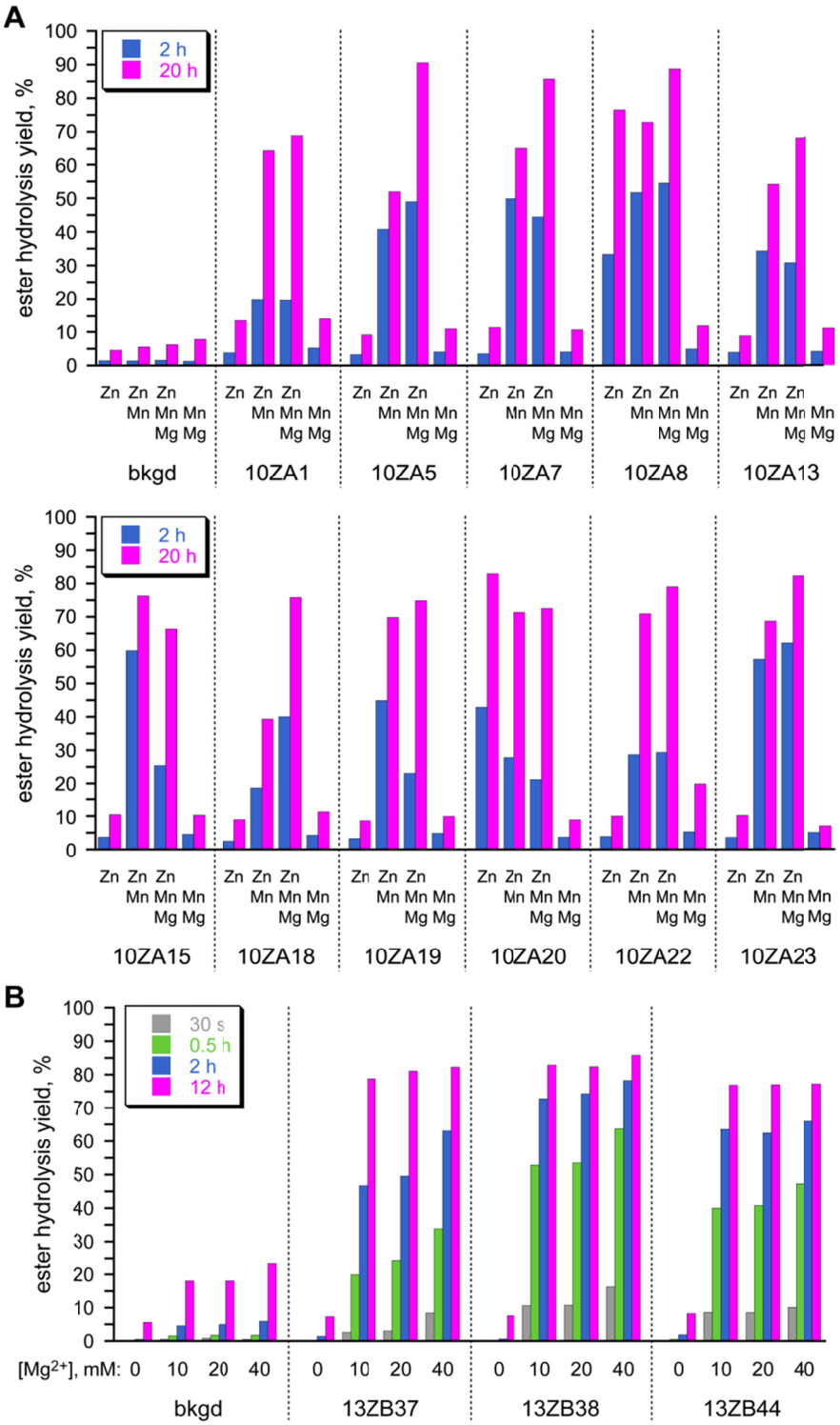


Figure S8. Dependence of ester-hydrolyzing deoxyribozymes on metal ions. bkgd = background ester hydrolysis in the absence of any deoxyribozyme. (A) The eleven 10ZA deoxyribozymes from conditions *A* (70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C). Of the 11 deoxyribozymes, all required Zn^{2+}. Seven (10ZA1,7,13,15,19,22,23) additionally required Mn^{2+}, and two (10ZA5,18) additionally required both Mn^{2+} and Mg^{2+} for optimal activity. (B) The three 13ZB deoxyribozymes from conditions *B* (50 mM CHES, pH 9.0, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C). The apparent K_d for Mg^{2+} is <10 mM for all three 13ZB deoxyribozymes.

Dependence of anilide-hydrolyzing 8ZC deoxyribozymes on pH

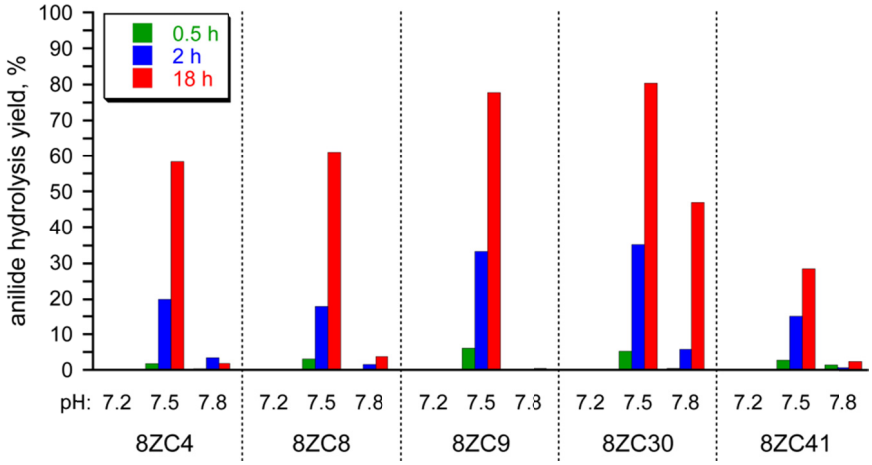


Figure S9. Dependence of the five 8ZC anilide-hydrolyzing deoxyribozymes on pH. Incubation conditions derived from conditions *A*: 70 mM HEPES, pH 7.2, 7.5, or 7.8 as indicated, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C.

Dependence of anilide-hydrolyzing 8ZC deoxyribozymes on metal ions

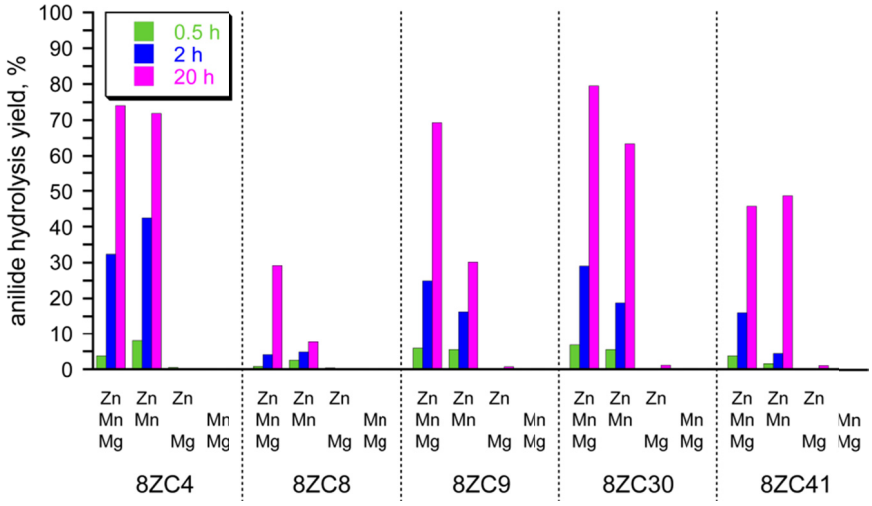


Figure S10. Dependence of the five anilide-hydrolyzing 8ZC deoxyribozymes on metal ions. Incubation conditions derived from conditions *A*: 70 mM HEPES, pH 7.5, combinations of 1 mM $ZnCl_2$, 20 mM $MnCl_2$, and 40 mM $MgCl_2$ as indicated, and 150 mM NaCl at 37 °C. Of the five deoxyribozymes, all required both Zn^{2+} and Mn^{2+}; two (8ZC8 and 8ZC9) additionally required Mg^{2+} for optimal activity.

Activities of anilide-hydrolyzing deoxyribozymes identified from N_{20} and N_{30} random pools

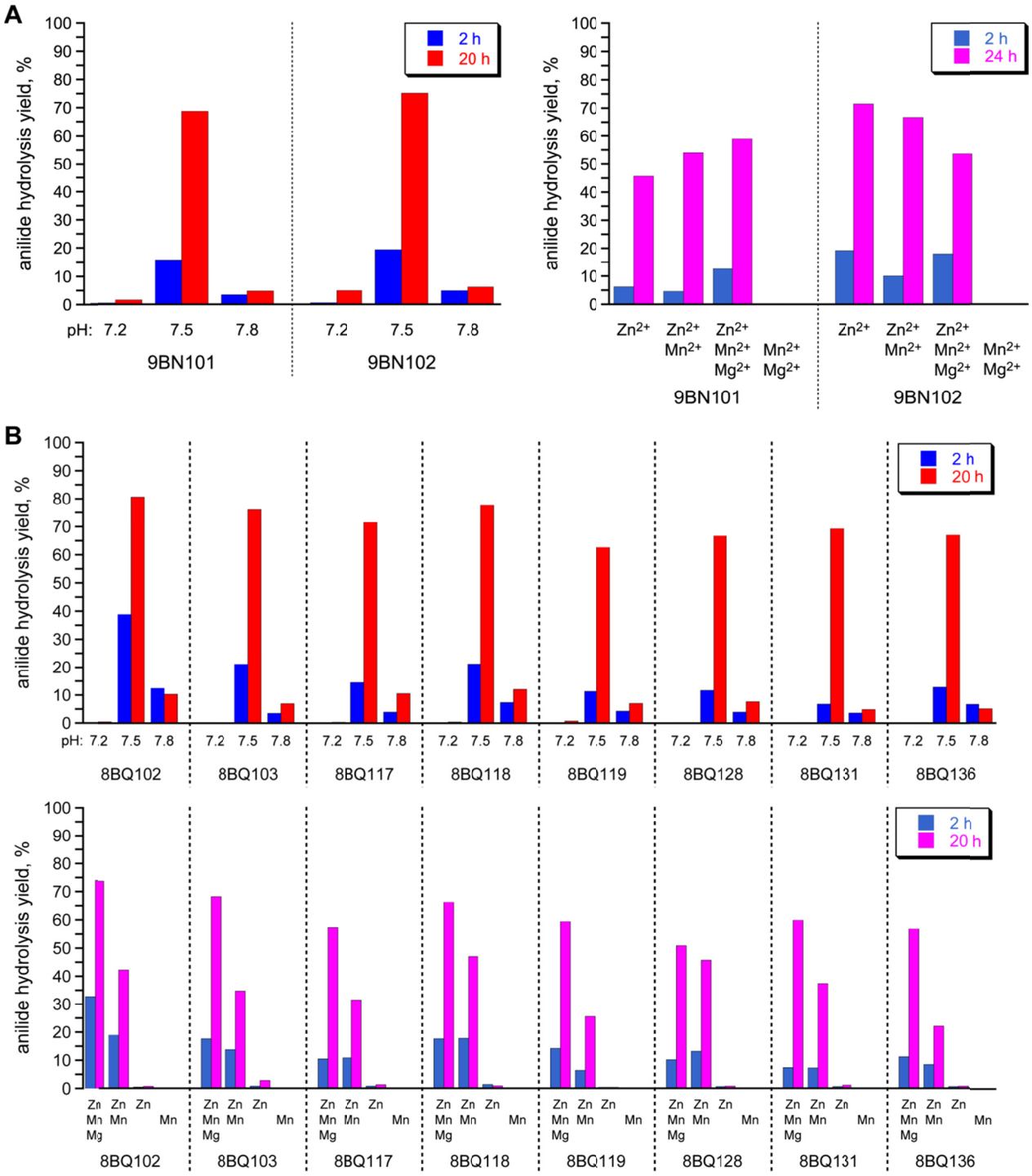


Figure S11. Activities of 9BN1 and 8BQ1 anilide-hydrolyzing deoxyribozymes identified from N_{20} and N_{30} random pools: dependence on pH and metal ions. Incubation conditions *A*: 70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C. (A) The two 9BN1 deoxyribozymes from the N_{20} pool. (B) The eight 8BQ1 deoxyribozymes from the N_{30} pool.

Linear free energy relationship (LFER) data for anilide-hydrolyzing 8ZC deoxyribozymes

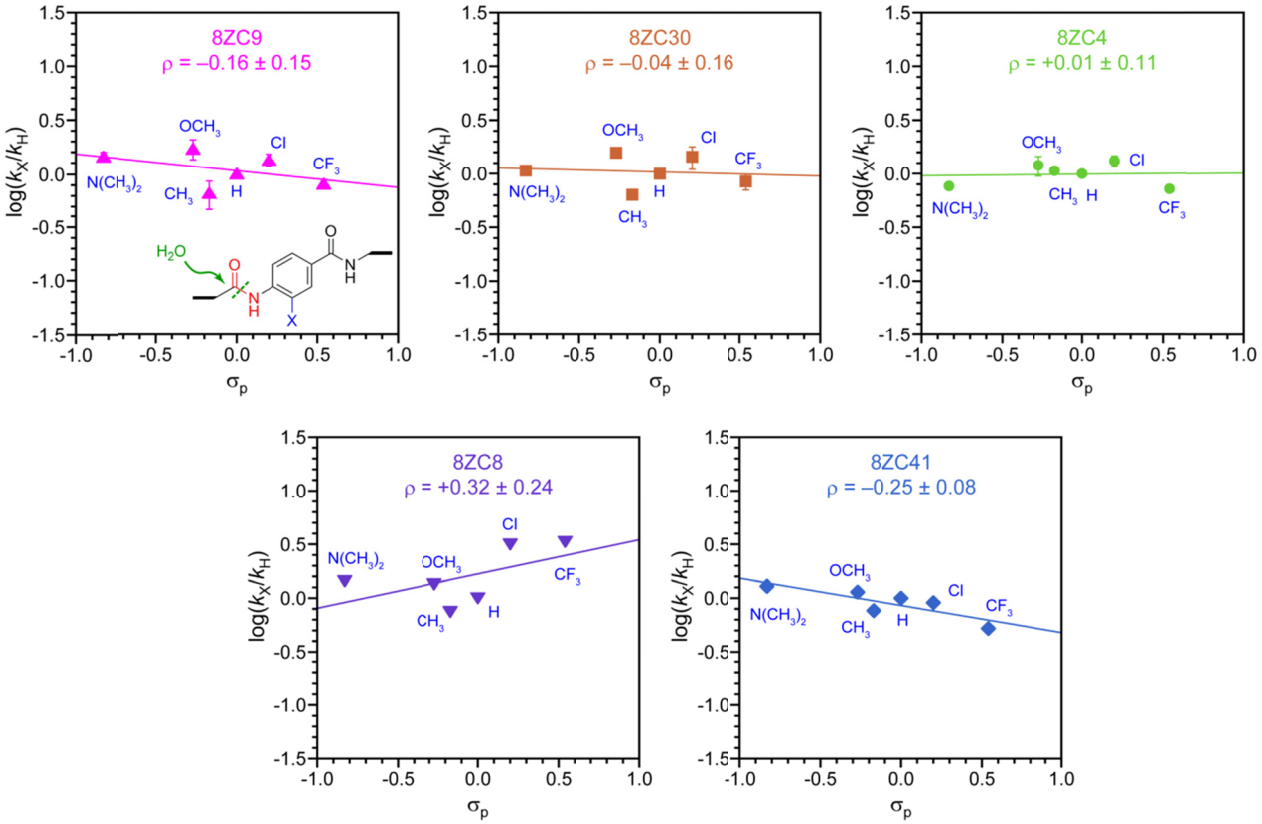


Figure S12. Linear free energy relationship (LFER) data for anilide-hydrolyzing deoxyribozymes. All k_{obs} values were determined under conditions *A* (70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C), similar to the data in Figure 4. For each of 8ZC9, 8ZC30, and 8ZC4, k_{obs} for all six substrates was determined multiple times ($n = 3$); each k_{obs} value was calculated as mean ± standard deviation. For each of 8ZC8 and 8ZC41, k_{obs} was determined once. For X = Cl or CF_3, a small amount of nonspecific hydrolysis (~2%) was observed at 30 s; the hydrolysis yield at 30 s was substrated from the yield at all later timepoints. For all X other than CF_3, yields at 24 h were similar to those for X = H as shown in Figure 4. For X = CF_3, yields at 24 h were 13–21%, but k_{obs} could still be determined reliably. Error bars correspond to the quadratic sum of the fractional uncertainties in k_X and k_H.[6] Values of σ_p were used as tabulated.[7] Unweighted linear fits to the data points provided the indicated ρ values and standard deviations.

Mechanistic implications of the LFER data for DNA-catalyzed anilide hydrolysis

Two addition-elimination mechanisms for aromatic amide (anilide) hydrolysis are shown in Figure S13. Panel A depicts a three-step mechanism in which addition of water is followed by a discrete nitrogen protonation step and finally elimination of the aniline leaving group. If either the addition or elimination step is rate-determining, then we expect $\rho >> 0$ for substituents placed on the aniline ring. For rate-determining addition, an electron-withdrawing group will increase the carbonyl electrophilicity; for rate-determining elimination, an electron-withdrawing group will increase the leaving group ability. In contrast, if the nitrogen protonation step is rate-determining, then we expect $\rho << 0$ due to the increase in positive charge (i.e., decrease in electron density) on the aniline upon protonation. Rate-determining proton transfer to nitrogen has been invoked for hydroxide-catalyzed anilide hydrolysis on the basis of a negative ρ value.[8] Panel B depicts a related two-step mechanism in which the protonation and elimination reactions of the three-step mechanism occur at the same time. For the addition step we still expect $\rho >> 0$, but for the elimination step with concomitant nitrogen protonation, we now expect a cancellation of substituent effects because protonation (which would lead to $\rho << 0$) and elimination (which would lead to $\rho >> 0$) respond in opposite directions. Of the various mechanistic possibilities, the experimental observation of $\rho \approx 0$ is consistent only with the two-step mechanism of panel B with a rate-determining protonation/elimination step. Determining the identity of the general acid awaits further structural and mechanistic analysis of these deoxyribozymes. Nucleobases themselves can be general acids in ribozymes,[9] and metal-bound water molecules are other reasonable candidates. We also note that other mechanistic possibilities, e.g., a rate-determining conformational change, are also consistent with the LFER data.

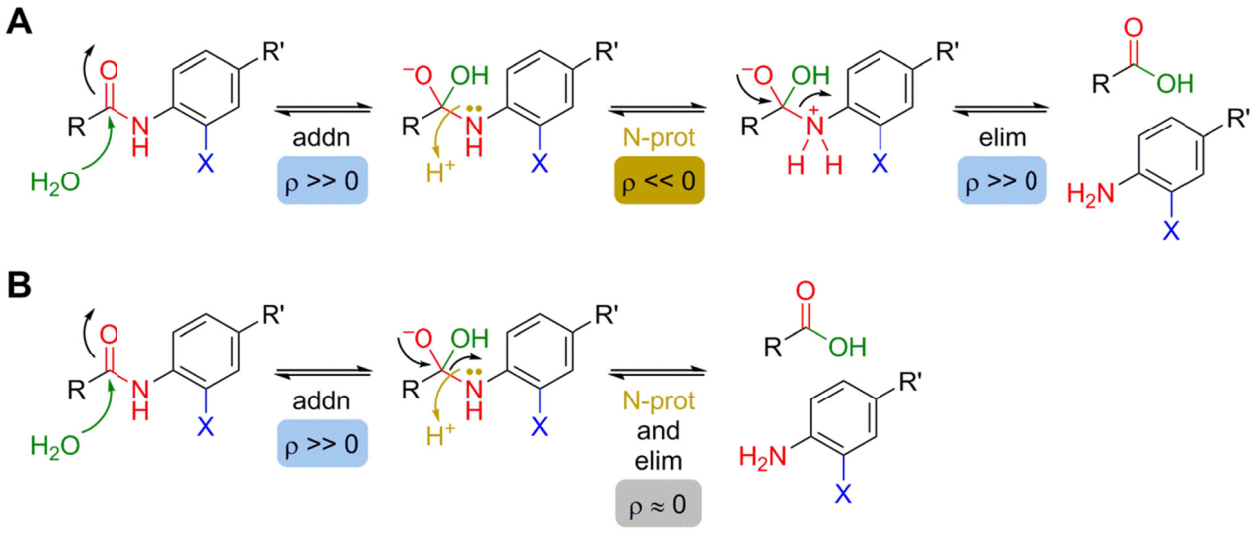


Figure S13. Two addition-elimination mechanisms for aromatic amide (anilide) hydrolysis and expected substituent effects. For each step is shown the expected LFER ρ value if that step is rate-determining. (A) Three-step mechanism involving addition of water, protonation of nitrogen, and elimination of aniline. (B) Two-step mechanism in which the protonation and elimination reactions occur at the same time. In this panel, note that "$\rho \approx 0$" represents one possible specific outcome due to cancellation of substituent effects upon protonation ($\rho << 0$) versus elimination ($\rho >> 0$). If the cancellation were not complete, then the ρ value would not be ~0. However, the experimental observation of $\rho \approx 0$ is consistent only with this step as rate-determining.

Activities of anilide-hydrolyzing 8ZC deoxyribozymes with a phenyl ester substrate

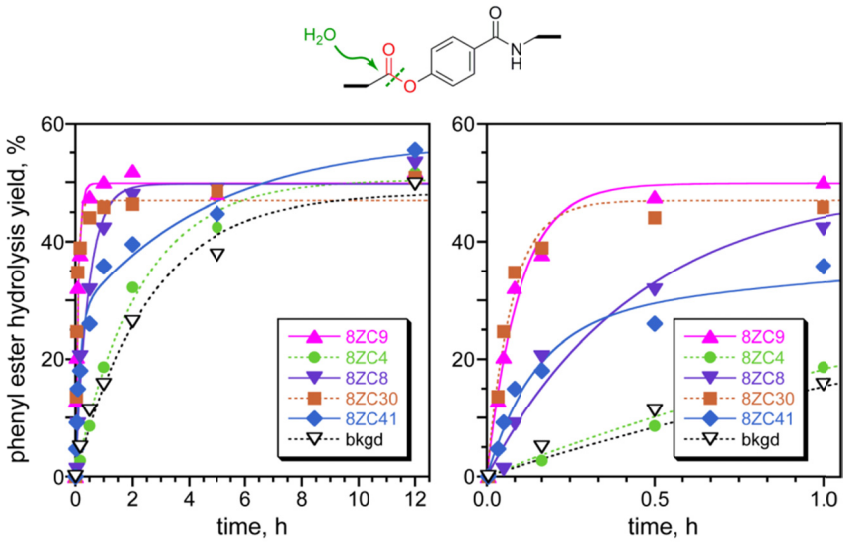
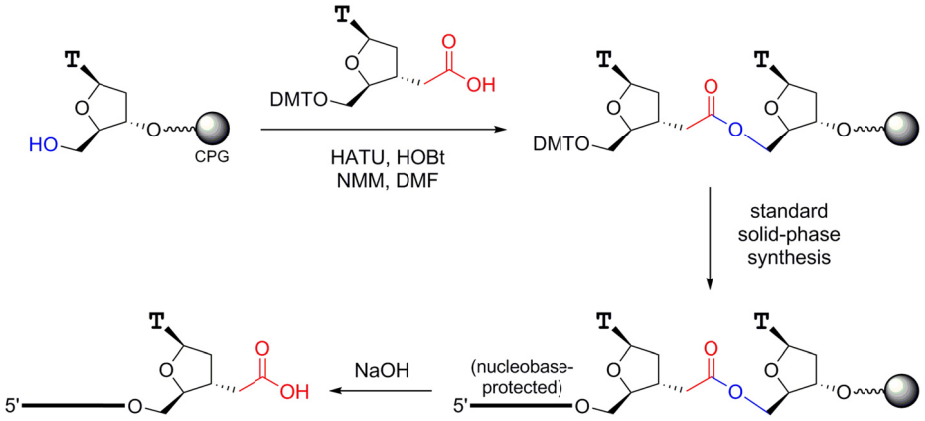


Figure S14. Activities of the five anilide-hydrolyzing 8ZC deoxyribozymes with a phenyl ester substrate. The illustrated phenyl ester substrate (in which the anilide nitrogen of **2** is replaced by oxygen) was hydrolyzed by each deoxyribozyme under conditions *A* (70 mM HEPES, pH 7.5, 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C). The phenyl ester substrate hydrolyzed substantially (~40%) when extracted from polyacrylamide gel with standard TEN buffer. Therefore, extraction was performed with 10 mM NaOAc, pH 4.6, 1 mM EDTA, and 300 mM NaCl followed by ethanol precipitation, which decreased the hydrolysis to ~4%. All data values were corrected for this nonspecific hydrolysis that occurred during substrate preparation. The illustrated data are representative of two independent experiments. The plot on the right shows the same data as on the plot on the left, simply expanded on the time axis. All data were fit to single-exponential kinetics except for 8ZC41, which reproducibly required double-exponential kinetics. The phenyl ester substrate has a rather high background hydrolysis rate constant under the incubation conditions (k_{bkgd} = 0.4 h^{-1}). The k_{obs} for 8ZC4 was indistinguishable from k_{bkgd}. The k_{obs} for 8ZC8 was 2 h^{-1}, which is 5-fold higher than k_{bkgd}. The k_{obs} for 8ZC9 and 8ZC30 were 10 h^{-1} and 13 h^{-1}, which are 25-fold and 33-fold higher than k_{bkgd}. 8ZC41 had higher k_{obs} of 7 h^{-1} (48% of total fit amplitude) and lower k_{obs} of 0.2 h^{-1} (52% of total fit amplitude).

Solid-phase synthesis

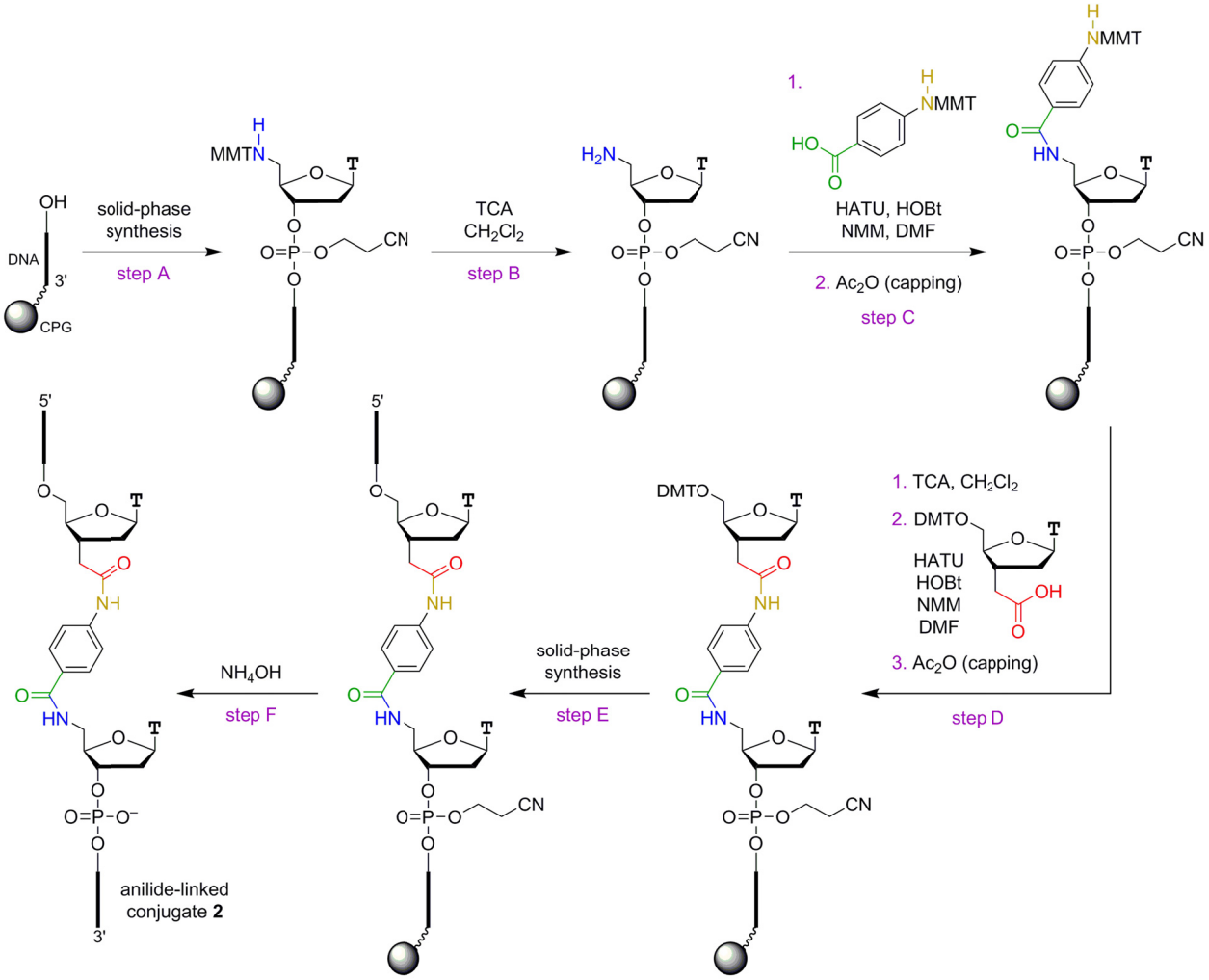
Synthesis of ester-linked conjugate 1



Synthesis of 3′-CO_2H oligonucleotide. 5′-DMT-thymidine-derivatized CPG solid support (40 µmol/g; 200 nmol) was deprotected with TCA/CH_2Cl_2 (3 × 1 mL) and washed with CH_2Cl_2 (3 × 5 mL). The support was rinsed with 2% (v/v) NMM/CH_2Cl_2 and CH_2Cl_2 (3 × 5 mL) to improve the efficiency of the next coupling step and dried under a stream of nitrogen. 5′-DMT-3′-CO_2H thymidine analogue (64 mg, 100 µmol), HATU (38 mg, 100 µmol), and HOBt (14 mg, 100 µmol) were dissolved in 500 µL of dry DMF. NMM (26 µL, 230 µmol) was added to this solution, which was introduced into the DNA synthesis column via two syringes. The coupling step was performed for 18 h with mixing every 5 min for the first 2 h and no mixing for the remaining 16 h. The column was washed with DMF (3 × 5 mL) and CH_2Cl_2 (3 × 5 mL) and dried under a stream of nitrogen. The remainder of the oligonucleotide was synthesized on the ABI 394 instrument using standard procedures. Cleavage of the oligonucleotide from the CPG and nucleobase deprotection were performed using 500 µL of 400 mM NaOH in 4:1 MeOH:H_2O for 24 h at room temperature. The sample was diluted to 2 mL with water and desalted using a PD10 column (GE Healthcare). The fractions containing desalted oligonucleotide were concentrated and purified by 20% PAGE.

Solution-phase coupling of 3′-CO_2H oligonucleotide and 5′-OH oligonucleotide. An 80 µL sample containing 1.0 nmol of 3′-CO_2H oligonuclotide, 1.5 nmol of 5′-OH oligonucleotide, and 1.25 nmol of DNA splint was annealed in 125 mM MES, pH 6.0, and 125 mM NaCl by heating at 95 °C for 3 min and cooling on ice for 5 min. The sequence of the DNA splint was 5′-CGAAGCGCTAGAACATAAAGTACATG-AGACTTTAACAACAACAACAAC-3′, where the underlined region was included to shift the splint away from the desired product on PAGE. The reaction was initiated by bringing the sample to 100 µL volume containing 100 mM MES, pH 6.0, 100 mM NaCl, 40 mM EDC, 30 mM HOBt, and 10% (v/v) DMF, which was required to dissolve the HOBt. The sample was incubated at room temperature for 24 h, precipitated with ethanol, and purified by 20% PAGE.

Synthesis of anilide-linked conjugate 2



Synthesis and deprotection of 5′-amino-5′-deoxythymidine oligonucleotide (steps A and B). Oliogonucleotide synthesis was performed on the ABI 394 instrument using standard procedures, providing a CPG-supported oligonucleotide terminating in a *N*-MMT-5′-amino-5′-deoxythymidine residue. The MMT protecting group was removed with 3% TCA/ CH_2Cl_2 (3 × 1 mL), and the support was rinsed with CH_2Cl_2 (3 × 5 mL). The support was rinsed with 2% (v/v) NMM/CH_2Cl_2 and CH_2Cl_2 (3 × 5 mL) to improve the efficiency of the next coupling step and dried under a stream of nitrogen.

Coupling of N*-MMT-4-aminobenzoic acid (step C1).* *N*-MMT-4-aminobenzoic acid (56 mg, 100 µmol), HATU (38 mg, 100 µmol), and HOBt (14 mg, 100 µmol) were dissolved in 500 µL of dry DMF. NMM (26 µL, 230 µmol) was added to this solution, which was introduced into the DNA synthesis column via two syringes. The coupling step was performed for 2 h with mixing every 5 min. The support was washed with DMF (3 × 5 mL) and CH_2Cl_2 (3 × 5 mL) and dried under a stream of nitrogen.

Capping of unreacted 5′-amino groups (step C2). Capping of unreacted amino groups was performed using 500 µL of Glen Research Cap Mix A (10% Ac_2O/10% pyridine in THF) and 500 µL of Glen Research Cap Mix B (10% 1-methylimidazole in THF). Cap Mix A and Cap Mix B were combined and introduced into the DNA synthesis column via two syringes. The capping step was performed for 30 min with mixing every 5 min. The support was washed with CH_2Cl_2 (3 x 5 mL) and dried under a stream of nitrogen.

Deprotection of N-*MMT group (step D1)*. Deprotection with 3% TCA/CH_2Cl_2 was performed as described above for deprotection of 5′-amino groups (step B).

Coupling of 5′-DMT-3′-CO_2H thymidine nucleoside analogue to aniline moiety (step D2). 5′-DMT-3′-CO_2H thymidine nucleoside analogue was coupled to the terminal aniline moiety using the coupling step described above (step C1), using the 5′-DMT-3′-CO_2H thymidine nucleoside analogue in place of *N*-MMT-4-aminobenzoic acid and extending the coupling reaction from 2 h to 18 h.

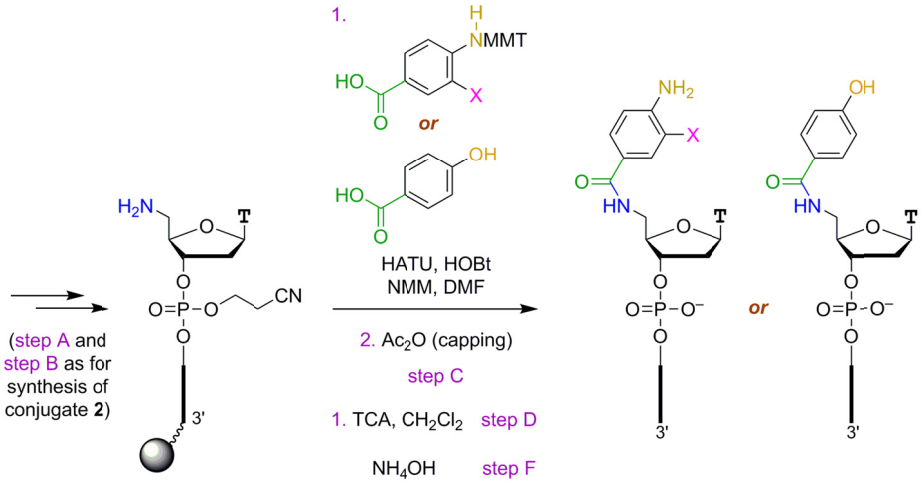
Capping of unreacted amino groups (step D3). Capping with acetic anhydride was performed as described above for capping of unreacted 5′-amino groups (step C2).

Completion of synthesis (steps E and F). The remainder of the conjugate was synthesized on the ABI 394 instrument using standard procedures. The conjugate was cleaved from the solid support using 500 µL of 30% NH_4OH by incubating at 55 °C for 16 h. The NH_4OH was evaporated, and the final conjugate was purified by 20% PAGE.

Synthesis of amide-linked conjugates 3, 4, 5, and 6

Conjugate **3** was synthesized as described for anilide-linked conjugate **2** with modifications. The Ala-Phe-Ala tripeptide was incorporated via three cycles of steps B and C, using *N*-MMT-Ala, *N*-MMT-Phe, and *N*-MMT-Ala in place of *N*-MMT-4-aminobenzoic acid in the first, second, and third step C1, respectively. The coupling time in step D2 was 2 h instead of 16 h. Conjugates **4** and **5** were synthesized as described for anilide-linked conjugate **2**, except using the appropriate *N*-MMT-protected amino carboxylic acid compound in step C1. The coupling time in step D2 was 2 h instead of 16 h. Conjugate **6** was synthesized as described for anilide-linked conjugate **2**, except omitting steps B and C. The coupling time in step D2 was 2 h instead of 16 h.

Synthesis of ring-substituted analogues of anilide-linked conjugate 2 and the phenyl ester analogue

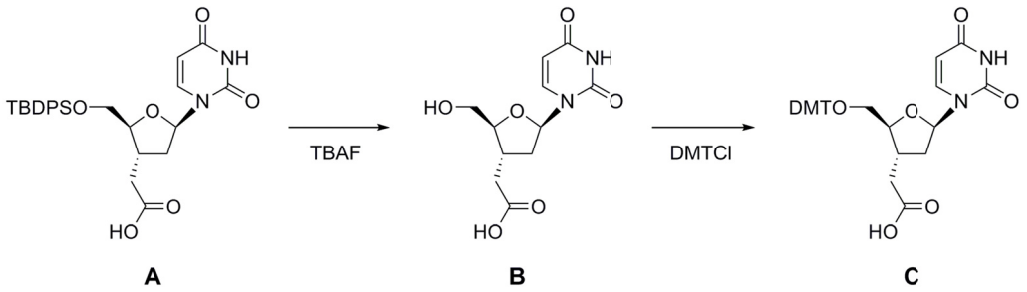


Ring-substituted anilide analogues of **2** as well as the phenyl ester analogue were synthesized by a convergent approach, using the 3′-CO_2H oligonucleotide from the synthesis of ester-linked conjugate **1**. This approach was adopted to conserve the synthetic 3′-CO_2H thymidine nucleoside analogue. The procedure for synthesis of anilide-linked conjugate **2** was followed through step C1, using the appropriately ring-substituted *N*-MMT-4-aminobenzoic acid or 4-hydroxybenzoic acid in place of *N*-MMT-4-aminobenzoic acid in step C1. For the anilide analogues only, step D1 was performed. For all conjugates, step F was then performed. The 5′-aniline/phenol-terminated oligonucleotide was purified by HPLC, using a Phenomenex Gemini C_{18} semi-prep column (250 × 10 mm) using a gradient of 15–30% solvent A in solvent B over 45 min (A: 50% MeCN/50% 20 mM TEAA, pH 7.0; B: 20 mM TEAA, pH 7.0). Fractions containing the 5′-aniline/phenol-terminated oligonucleotide were pooled and evaporated. Residual TEAA was removed by dissolving the sample in 100 μL of water and evaporating (3×).

The 3′-CO_2H oligonucleotide and 5′aniline/phenol-terminated oligonucleotide were joined by solution-phase coupling using the procedure for ester-linked conjugate **1**, except using MOPS, pH 7.0 at 37 °C (aniline) or MOPS, pH 7.0 at 25 °C (phenol).

Small-molecule synthesis

Reagents were commercial grade and used without purification unless otherwise indicated. Dry solvents were obtained from Aldrich Sure/Seal or Acros Acroseal bottles or by drying over freshly activated 4Å molecular sieves overnight. All reactions were performed under argon unless otherwise noted. Thin-layer chromatography (TLC) was performed on silica gel plates pre-coated with fluorescent indicator with visualization by UV light (254 nm). Flash column chromatography was performed with silica gel (230-400 mesh). For all compounds that contain the 4,4′-dimethoxytrityl (DMT) group or 4-monomethoxytrityl (MMT) group, silica gel columns were packed with the initial solvent additionally containing 2% (v/v) triethylamine. ^{1}H and ^{13}C spectra were recorded on a Varian Unity 500 or Varian Unity 500 VXR instrument. The chemical shifts in parts per million (δ) are reported downfield from TMS (0 ppm) and referenced to the residual proton signal of the deuterated solvent, as follows: $CDCl_3$ (7.26 ppm), CD_3OD (3.31 ppm) for ^{1}H NMR spectra; $CDCl_3$ (77.2 ppm), CD_3OD (49.2 ppm) for ^{13}C NMR spectra. Multiplicities of ^{1}H NMR spin couplings are reported as s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), or m (multiplet and overlapping spin systems). Values for apparent coupling constants (*J*, Hz) are reported. Mass spectrometry data were obtained at the UIUC School of Chemical Sciences mass spectrometry laboratory using a Waters Quattro II instrument (LR-ESI).



5′-DMT-3′-CO_2H thymidine nucleoside analogue (C)

The 5′-TBDPS-3′-CO_2H thymidine nucleoside analogue **A** was prepared in six steps from thymidine as reported.[10] Compound **A** was deprotected with TBAF to form the 3′-CO_2H thymidine nucleoside analogue **B** according to the procedure reported for the TBDMS analogue,[11] followed by 5′-OH protection using DMTCl to form the 5′-DMT-3′-CO_2H thymidine nucleoside analogue **C** as reported.[11] All compounds on this route were previously reported;[10-11] ^{1}H NMR spectra of each compound were consistent with the reported spectra.

General procedure for MMT protection of amino acids, including aniline derivatives. The specific procedure described here was used for MMT protection of 4-aminobenzoic acid; for the other synthesis procedures, all reagent amounts were scaled linearly. Portions of TMSCl (1.4 mL, 11.0 mmol, 3.7 equiv) and Et_3N (1.6 mL, 11.4 mmol, 3.8 equiv) were added to a suspension of 4-aminobenzoic acid (413 mg, 3.0 mmol, 1.0 equiv) in CH_2Cl_2 (25 mL) at room temperature. The mixture was heated at reflux (bath temperature 70 °C) for 2 h. After cooling to room temperature, MMTCl (920 mg, 3.0 mmol, 1.0 equiv) was added, and the solution was heated at reflux for 2 h (aliphatic amines) or 16 h (aromatic amines). After cooling to room temperature, the solution was cooled further in an ice bath, and Et_3N (4 mL) and MeOH (4 mL) were added to quench any unreacted MMTCl. After 30 min, the solution was diluted with CH_2Cl_2 (150 mL) and washed with 5% (w/v) aqueous citric acid (30 mL) and saturated NaCl (30 mL). The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The oily residue was purified by silica gel column chromatography, eluting with 0–4% CH_3OH in CH_2Cl_2 containing 2% (v/v) Et_3N.

***N*-MMT-4-aminobenzoic acid triethylammonium salt**

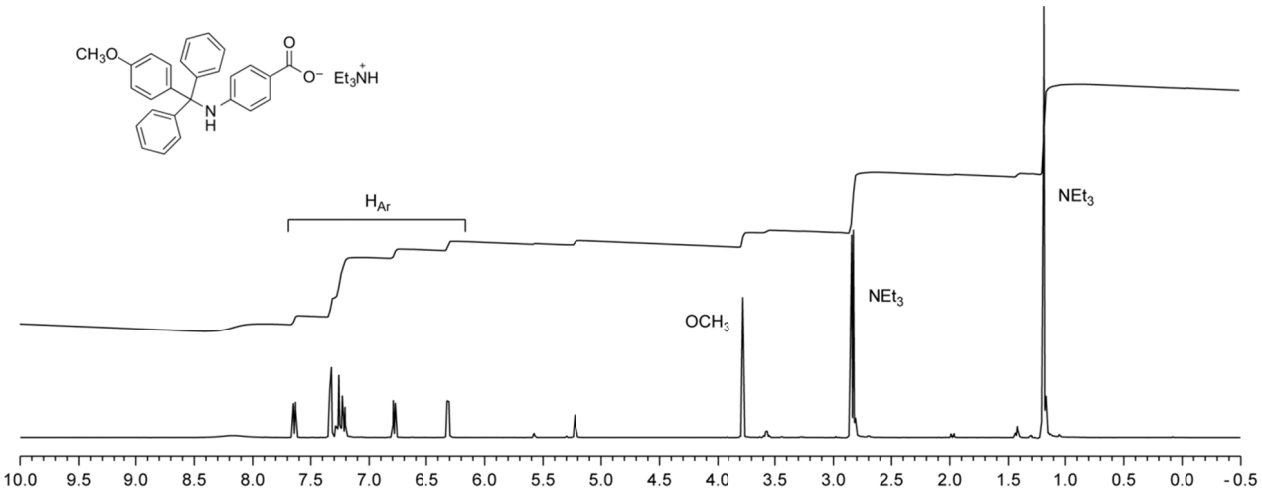
Following the general MMT protection procedure, from 413 mg (3.0 mmol) of 4-aminobenzoic acid (Aldrich), 1.19 g (77%) of product was isolated as a white solid.

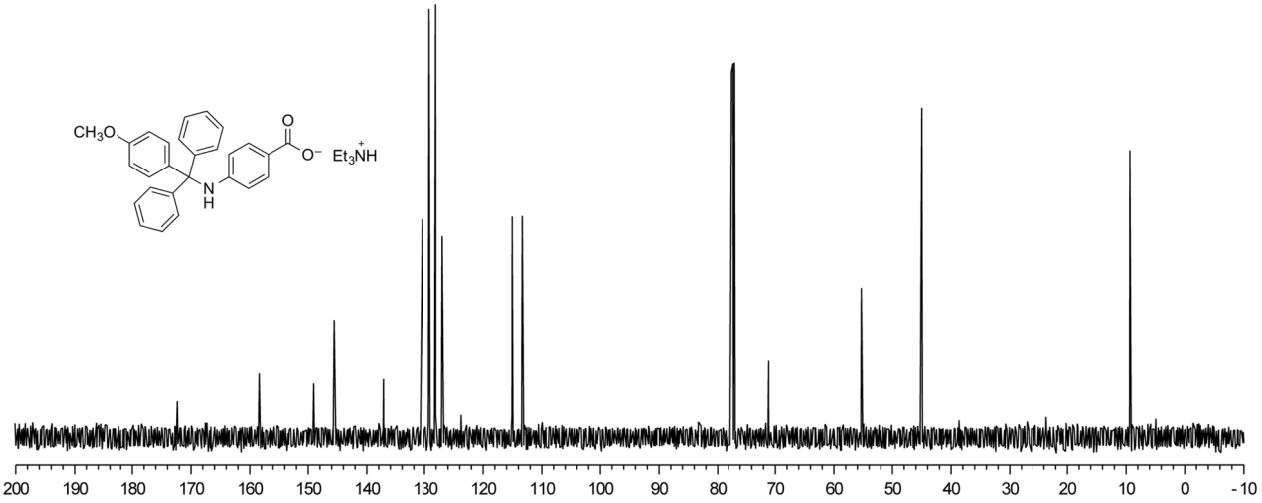
TLC: R_f = 0.19 [4% CH_3OH in CH_2Cl_2 with 2% (v/v) Et_3N].

^{1}H NMR: (500 MHz, $CDCl_3$) δ 7.64 (d, *J* = 8.6 Hz, 2H), 7.33 (d, *J* = 7.5 Hz, 4H), 7.27-7.19 (m, 8H), 6.79 (d, *J* = 8.8 Hz, 2H), 6.31 (d, *J* = 8.6 Hz, 2H), 3.78 (s, 3H), 2.84 (q, *J* = 7.2 Hz, 6H), 1.18 (t, *J* = 7.2 Hz, 9H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 172.3, 158.4, 149.1, 145.6, 137.2, 130.5, 130.4, 129.2, 128.1, 127.0, 123.8, 115.1, 113.3, 71.1, 55.3, 45.0, 9.4 ppm.

MS: *m*/*z* calcd. for $C_{27}H_{22}NO_3$ $[M-H]^-$: 408.2; found: 408.1.





***N*-MMT-Ala-OH•Et_3N**

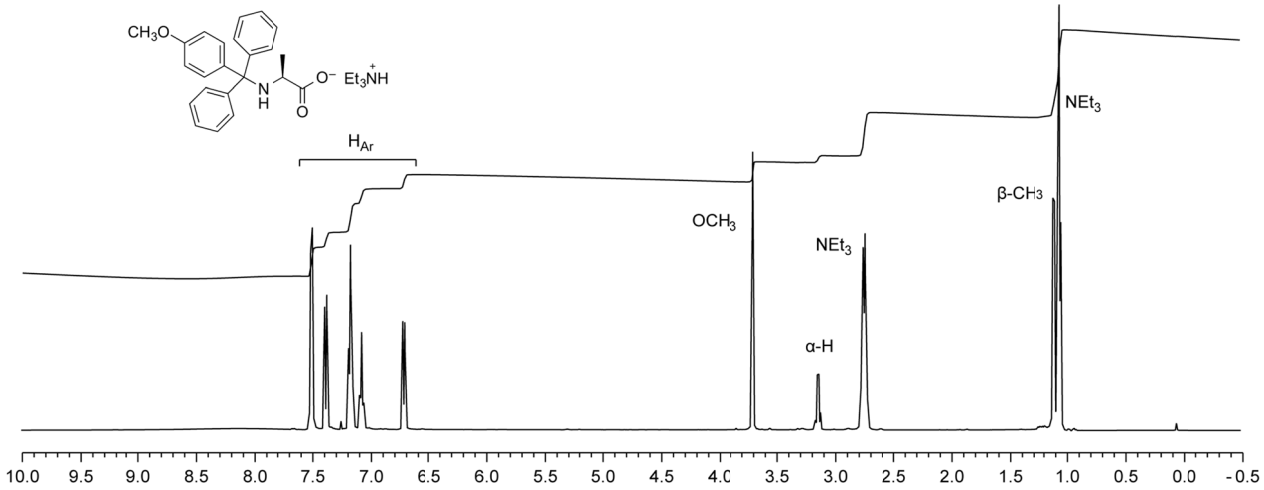
From 265 mg (3.0 mmol) of L-alanine (Acros), 1.10 g (79%) of product was isolated as a white solid.

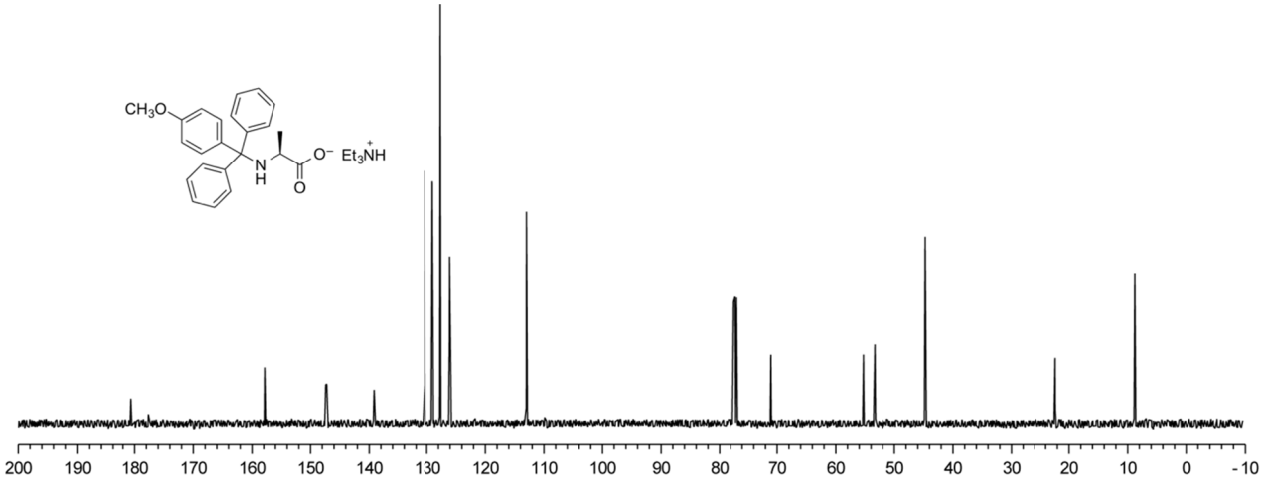
TLC: R_f = 0.35 [4% CH_3OH in CH_2Cl_2 with 2% (v/v) Et_3N].

^{1}H NMR: (500 MHz, $CDCl_3$) δ 7.51 (d, *J* = 8.1 Hz, 4H), 7.39 (d, *J* = 8.1 Hz, 2H), 7.18 (t, *J* = 7.1 Hz, 4H), 7.08 (t, *J* = 7.0 Hz, 2H), 6.72 (t, *J* = 8.7 Hz, 2H), 3.71 (s, 3H), 3.15 (q, *J* = 6.9 Hz, 1H), 2.76 (q, *J* = 7.2 Hz, 6H), 1.12 (d, *J* = 6.9 Hz, 3H), 1.07 (t, *J* = 7.3 Hz, 9H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 180.6, 157.8, 147.4, 139.2, 130.5, 129.1, 127.7, 126.1, 113.0, 71.1, 55.3, 53.3, 44.7, 22.4, 8.8 ppm ppm.

MS: *m*/*z* calcd. for $C_{23}H_{23}NO_3$ $[M+Et_3NH]^+$: 463.3; found: 463.3.





***N*-MMT-Phe-OH•Et_3N**

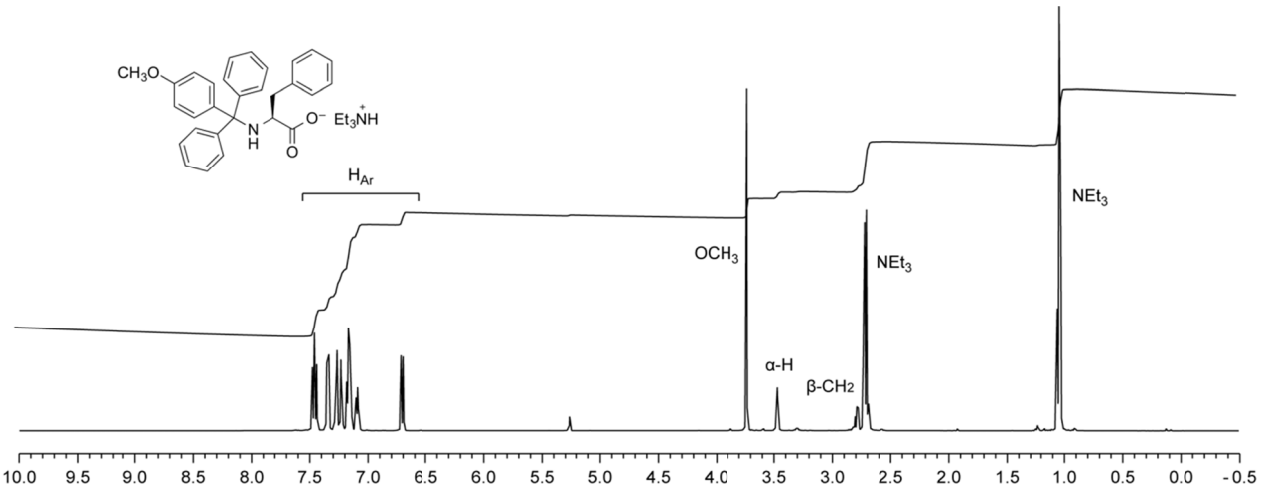
From 498 mg (3.0 mmol) of L-phenylalanine (Aldrich), 1.37 g (85%) of product was isolated as a white solid.

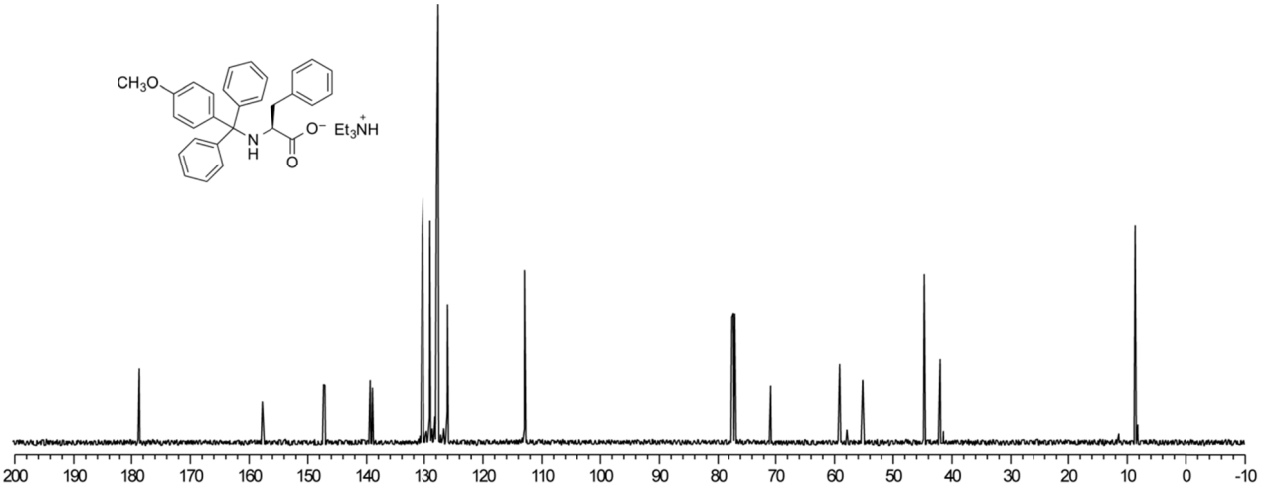
TLC: R_f = 0.51 [4% CH_3OH in CH_2Cl_2 with 2% (v/v) Et_3N].

^{1}H NMR: (500 MHz, $CDCl_3$) δ 7.48-7.44 (m, 4H), 7.35 (d, *J* = 8.9 Hz, 2H), 7.27 (d, *J* = 7.9 Hz, 2H), 7.23 (t, *J* = 7.4 Hz, 2H), 7.18-7.14 (m, 5H), 7.10-7.07 (m, 2H), 6.71 (d, *J* = 8.9 Hz, 2H), 3.73 (s, 3H), 3.47 (t, *J* = 6.0 Hz, 1H), 2.80 (dd, *J* = 13.2, 6.0 Hz, 1H), 2.72 (q, *J* = 7.3 Hz, 6H), 1.04 (t, *J* = 7.3 Hz, 9H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 178.7, 157.8, 147.3, 147.1, 139.4, 139.0, 130.5, 130.3, 129.1, 129.0, 127.9, 127.7, 126.1, 126.0, 112.9, 70.9, 59.1, 55.2, 44.7, 42.0, 8.7 ppm.

MS: *m*/*z* calcd. for $C_{29}H_{26}NO_3$ $[M–H]^–$: 436.1; found: 436.2.





***N*-MMT-4-(aminomethyl)benzoate triethylammonium salt**

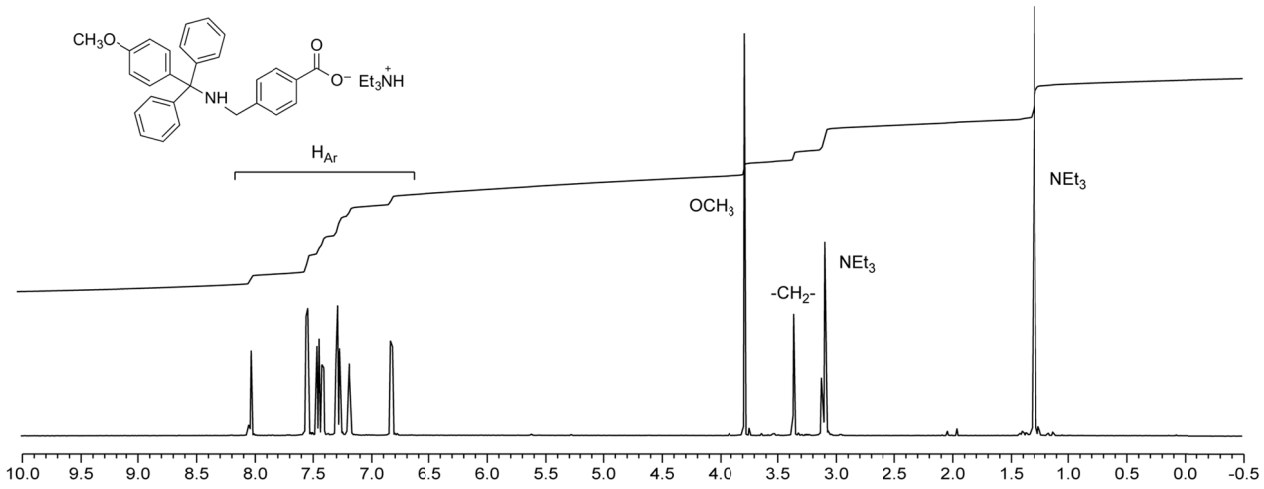
From 150 mg (1.0 mmol) of 4-(aminomethyl)benzoic acid (Aldrich), 371 mg (71%) of product was isolated as a white solid.

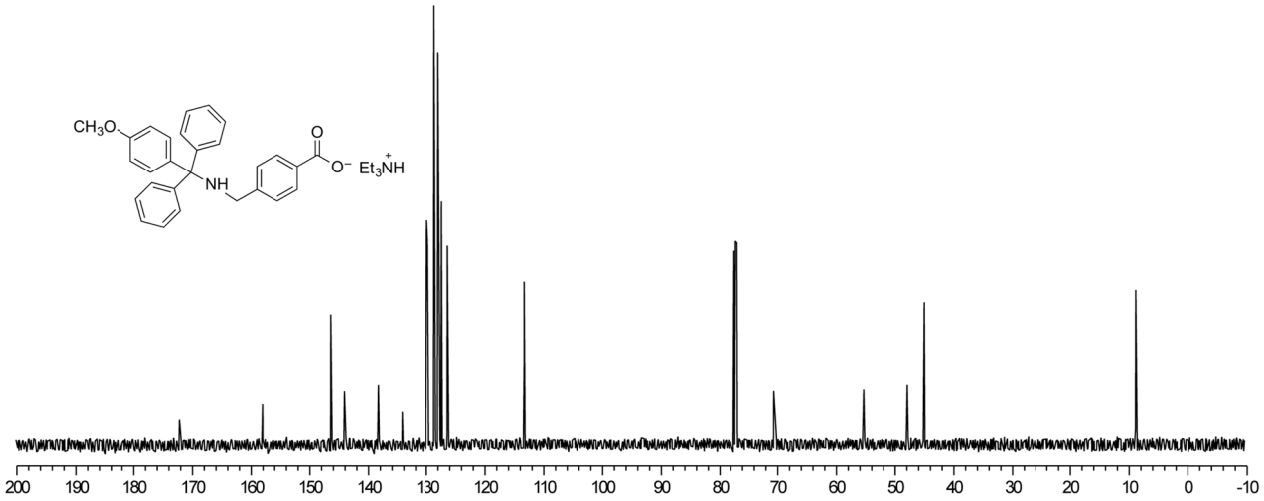
TLC: R_f = 0.24 [4% CH_3OH in CH_2Cl_2 with 2% (v/v) Et_3N].

^{1}H NMR: (500 MHz, $CDCl_3$) δ 8.04 (d, *J* = 8.1 Hz, 2H), 7.55 (d, *J* = 7.2 Hz, 4H), 7.46 (d, *J* = 8.9 Hz, 2H), 7.42 (d, *J* = 8.2 Hz, 2H), 7.29 (t, *J* = 7.4 Hz, 4H), 7.19 (t, *J* = 7.3 Hz, 2H), 6.83 (d, *J* = 8.9 Hz, 2H), 3.79 (s, 3H), 3.36 (s, 2H), 3.10 (q, *J* = 7.3 Hz, 6H), 1.30 (t, *J* = 7.3 Hz, 9H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 172.2, 158.1, 146.4, 144.1, 138.3, 134.3, 130.0, 129.9, 128.7, 128.0, 127.4, 126.4, 113.4, 70.7, 55.4, 48.0, 45.0, 8.9 ppm.

MS: *m*/*z* calcd. for $C_{28}H_{24}NO_3$ $[M-H]^-$: 422.2; found: 422.1.





***N*-MMT-*trans*-4-aminocyclohexanecarboxylate triethylammonium salt**

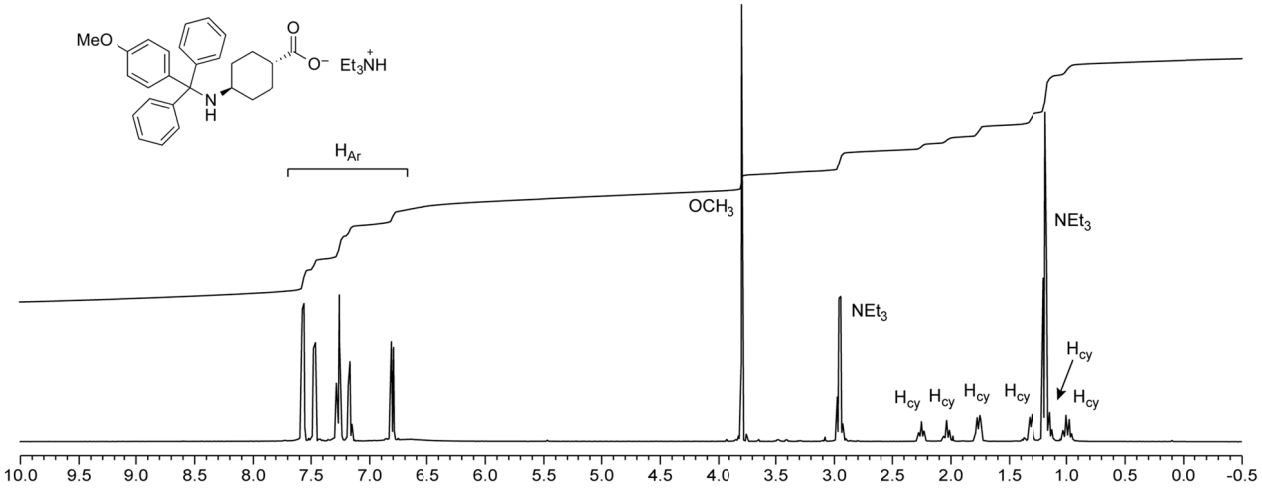
From 145 mg (1.0 mmol) of *trans*-4-aminocyclohexanecarboxylic acid (TCI America), 337 mg (65%) of product was isolated as a white solid.

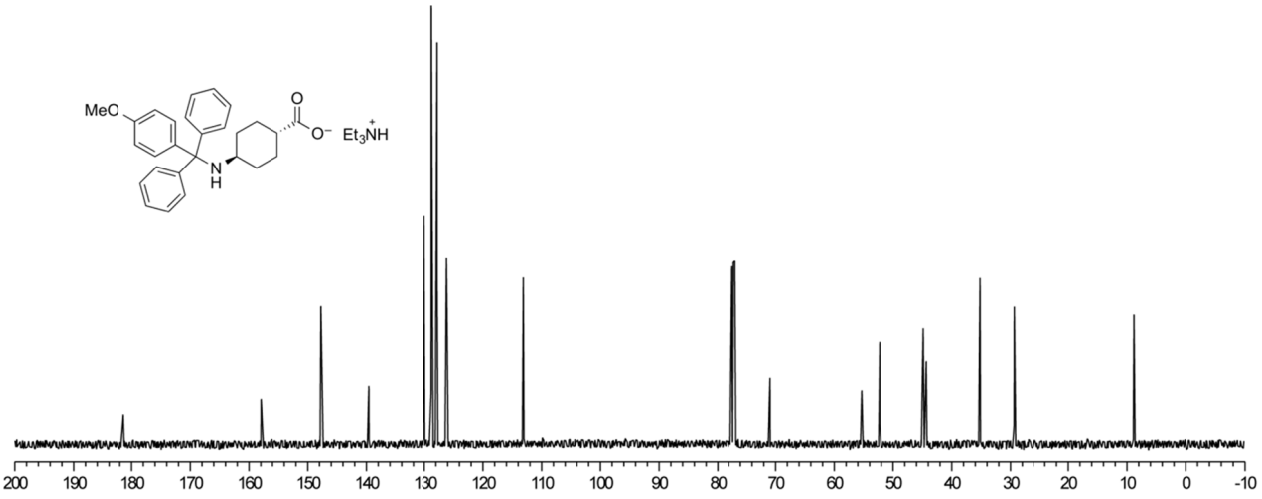
TLC: R_f = 0.27 [4% CH_3OH in CH_2Cl_2 with 2% (v/v) Et_3N].

^{1}H NMR: (500 MHz, $CDCl_3$) δ 7.54 (d, *J* = 7.4 Hz, 4H), 7.44 (d, *J* = 8.8 Hz, 2H), 7.23 (t, *J* = 7.7 Hz, 4H), 7.14 (t, *J* = 7.3 Hz, 2H), 6.78 (d, 8.8 Hz, 2H), 3.77 (s, 3H), 2.91 (q, *J* = 7.3 Hz, 6H), 2.25-2.19 (m, 1H), 2.06-2.00 (m, 1H), 1.76-1.72 (m, 2H), 1.30-1.27 (m, 2H), 1.16 (t, *J* = 7.3 Hz, 9H), 1.17-1.10 (m, 2H), 1.00-0.92 (m, 2H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 181.4, 158.0, 147.8, 139.6, 130.1, 128.8, 127.8, 126.2, 113.1, 71.0, 55.3, 52.3, 44.9, 44.3, 35.2, 29.4, 8.8 ppm.

MS: *m*/*z* calcd. for $C_{27}H_{28}NO_3$ $[M–H]^-$: 414.2; found: 414.1.





***N*-MMT-4-amino-3-(trifluoromethyl)benzoic acid triethylammonium salt**

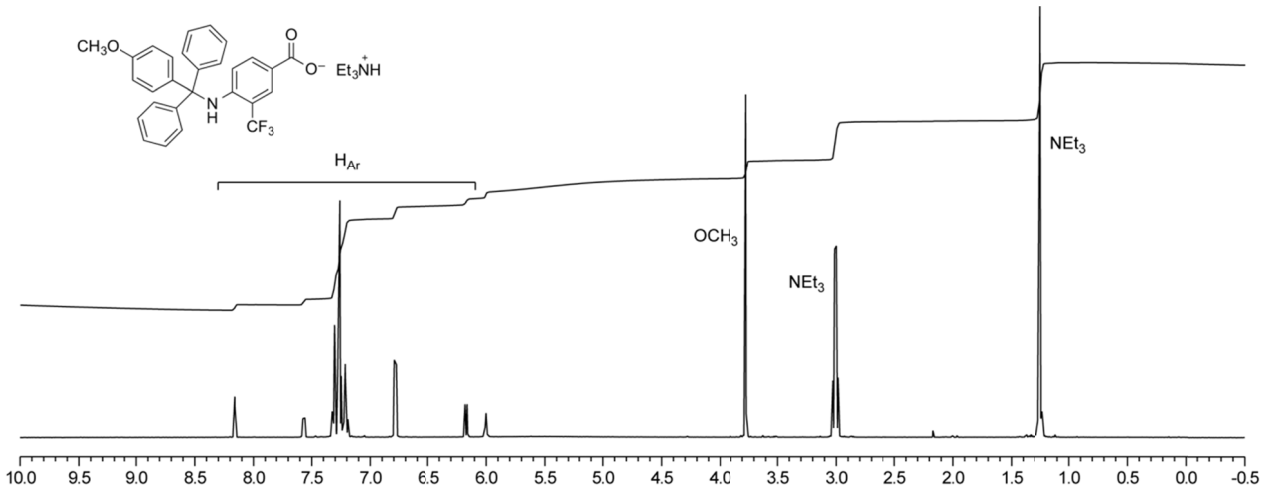
From 203 mg (1.0 mmol) of 4-amino-3-(trifluoromethyl)benzoic acid (Matrix Scientific), 272 mg (47%) of product was isolated as a white solid.

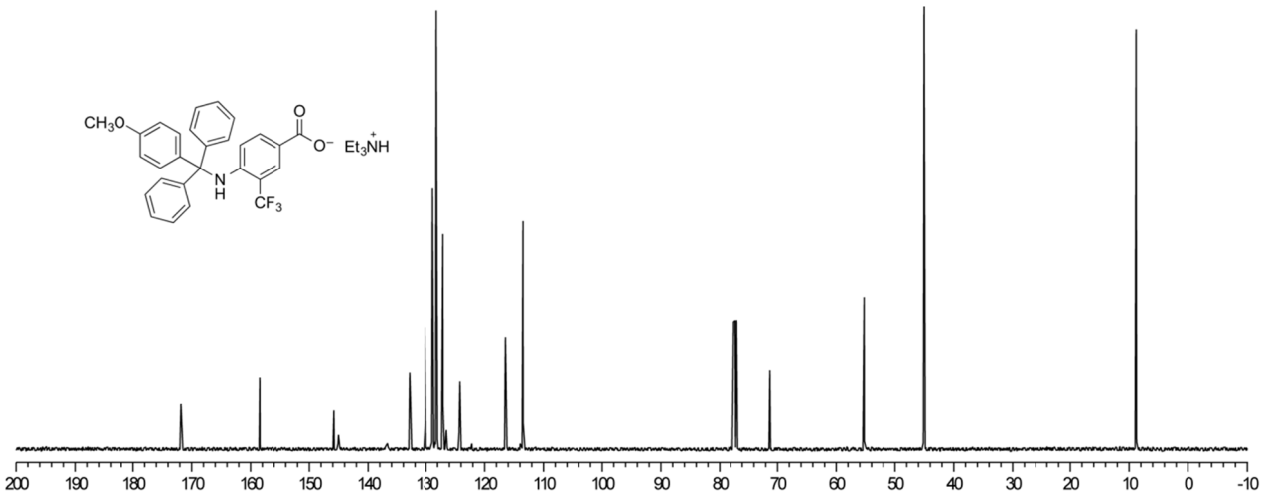
TLC: R_f = 0.28 [4% CH_3OH in CH_2Cl_2 with 2% (v/v) Et_3N].

^{1}H NMR: (500 MHz, $CDCl_3$) δ 8.16 (d, *J* = 2.0 Hz, 1H), 7.57 (dd, *J* = 8.9, 2.0 Hz, 1H), 7.32-7.19 (m, 12H), 6.79 (d, *J* = 8.9 Hz, 2H), 6.17 (d, *J* = 8.8 Hz, 1H), 3.77 (s, 3H), 3.01 (q, *J* = 7.3 Hz, 6H), 1.25 (t, *J* = 7.3 Hz, 9H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 171.8, 158.4, 145.8, 145.0, 136.7, 132.9, 130.2, 128.9, 128.2, 127.1, 126.5, 124.3, 124.2, 116.5, 113.5, 71.3, 55.3, 45.0, 8.8 ppm (one expected peak not observed in aromatic region).

MS: *m*/*z* calcd. for $C_{28}H_{23}F_3NO_3$ $[M+H]^+$: 478.2; found: 478.2.





***N*-MMT-4-amino-3-chlorobenzoic acid triethylammonium salt**

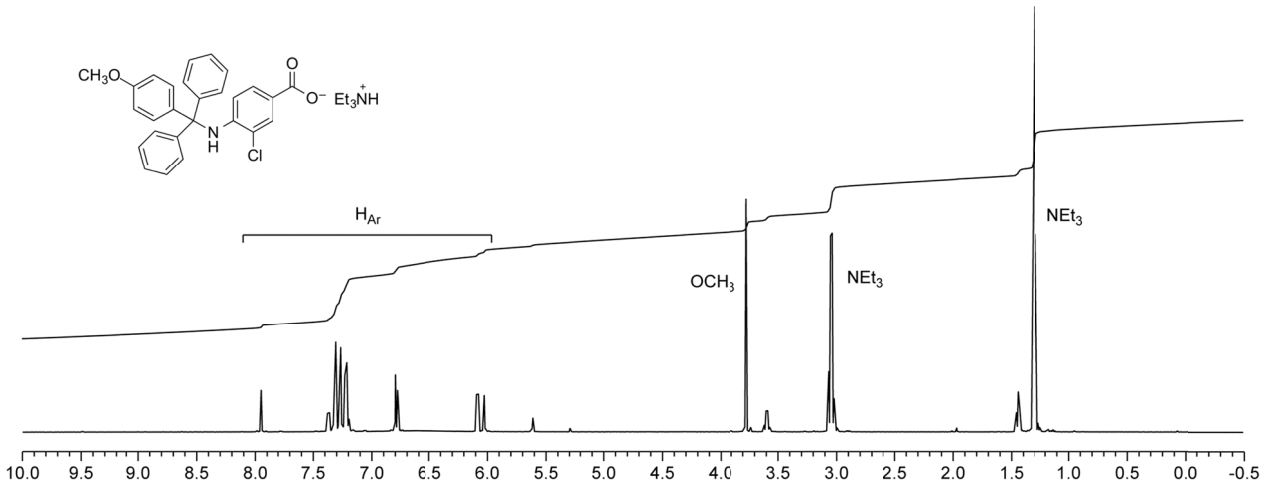
From 170 mg (1.0 mmol) of 4-amino-chlorobenzoic acid (Aldrich), 245 mg (55%) of product was isolated as a white solid.

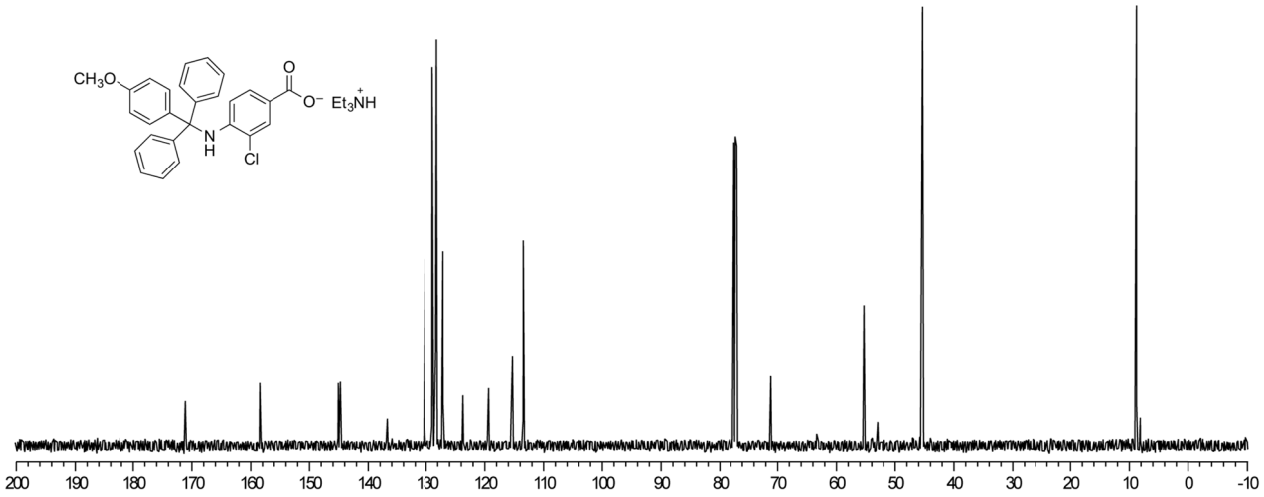
TLC: R_f = 0.22 [4% CH_3OH in CH_2Cl_2 with 2% (v/v) Et_3N].

^{1}H NMR: (500 MHz, $CDCl_3$) δ 7.94 (d, *J* = 2.0 Hz, 1H), 7.36 (dd, *J* = 8.6, 2.0 Hz, 1H), 7.31-7.19 (m, 12H), 6.78 (d, *J* = 8.9 Hz, 2H), 6.08 (d, *J* = 8.7 Hz, 1H), 6.05 (bs, 1H), 3.76 (s, 3H), 3.06 (q, *J* = 7.3 Hz, 6H), 1.30 (t, *J* = 7.3 Hz, 9H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 171.1, 158.5, 145.1, 144.8, 136.8, 130.4, 130.3, 129.0, 128.4, 128.2, 127.1, 123.7, 119.4, 115.3, 113.5, 71.2, 55.3, 45.3, 8.8 ppm.

MS: *m*/*z* calcd. for $C_{27}H_{22}NaClNO_3$ $[M+Na]^+$: 466.1; found: 466.1.





***N*-MMT-4-amino-3-methylbenzoic acid triethylammonium salt**

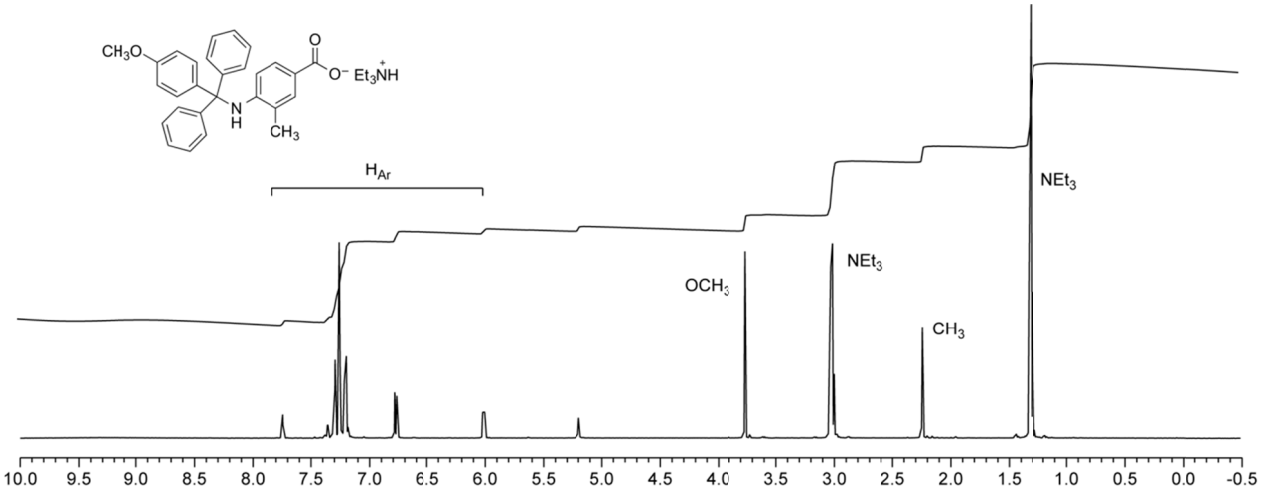
From 300 mg (2.0 mmol) of 4-amino-3-methylbenzoic acid (Aldrich), 766 mg (73%) of product was isolated as a white solid.

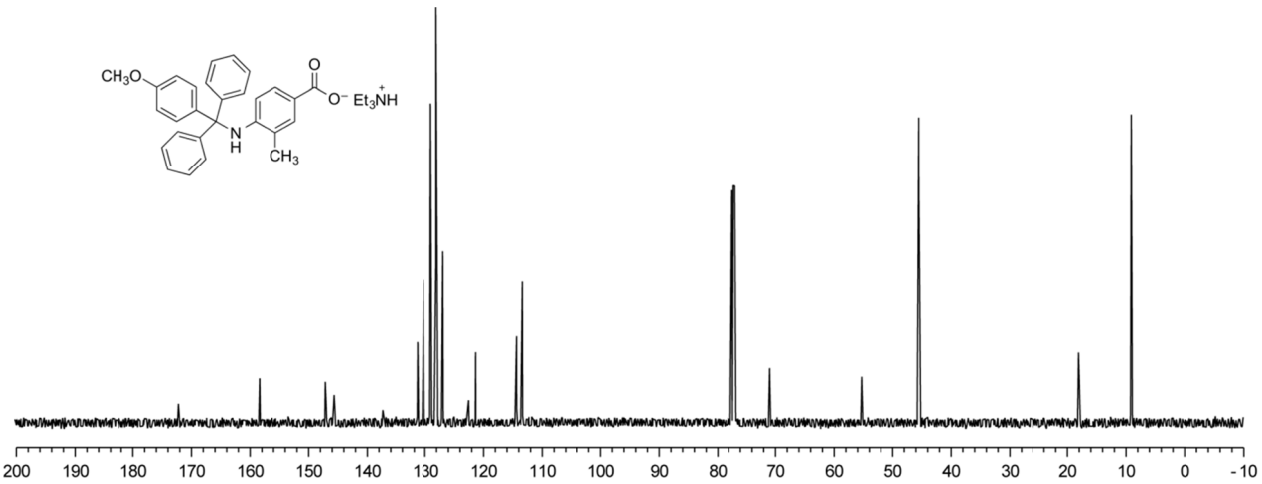
TLC: R_f = 0.25 [4% CH_3OH in CH_2Cl_2 with 2% (v/v) Et_3N].

^{1}H NMR: (500 MHz, $CDCl_3$) δ 7.74 (d, *J* = 1.1 Hz, 1H), 7.37 (dd, *J* = 8.6, 2.1 Hz, 1H), 7.31-7.18 (m, 12H), 6.78 (d, *J* = 8.9 Hz, 2H), 6.01 (d, *J* = 8.6 Hz, 1H), 3.76 (s, 3H), 3.02 (q, *J* = 7.3 Hz, 6H), 2.24 (s, 3H), 1.32 (t, *J* = 7.3 Hz, 9H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 172.2, 158.4, 147.2, 145.7, 137.4, 131.4, 130.3, 129.0, 128.1, 126.9, 122.6, 121.4, 114.4, 113.4, 71.0, 55.3, 45.5, 18.1, 9.1 ppm (one expected peak not observed in aromatic region).

MS: *m*/*z* calcd. for $C_{28}H_{25}NaNO_3$ $[M+Na]^+$: 446.2; found: 446.3.





***N*-MMT-4-amino-3-methoxybenzoic acid triethylammonium salt**

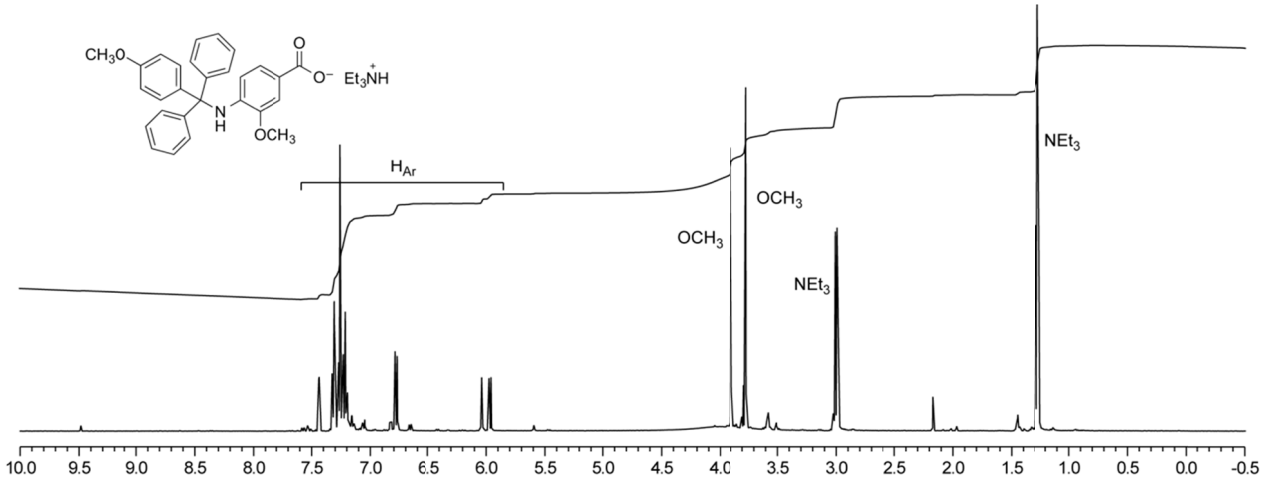
From 330 mg (2.0 mmol) of 4-amino-3-methoxybenzoic acid (Acros), 530 mg (49%) of product was isolated as a white solid.

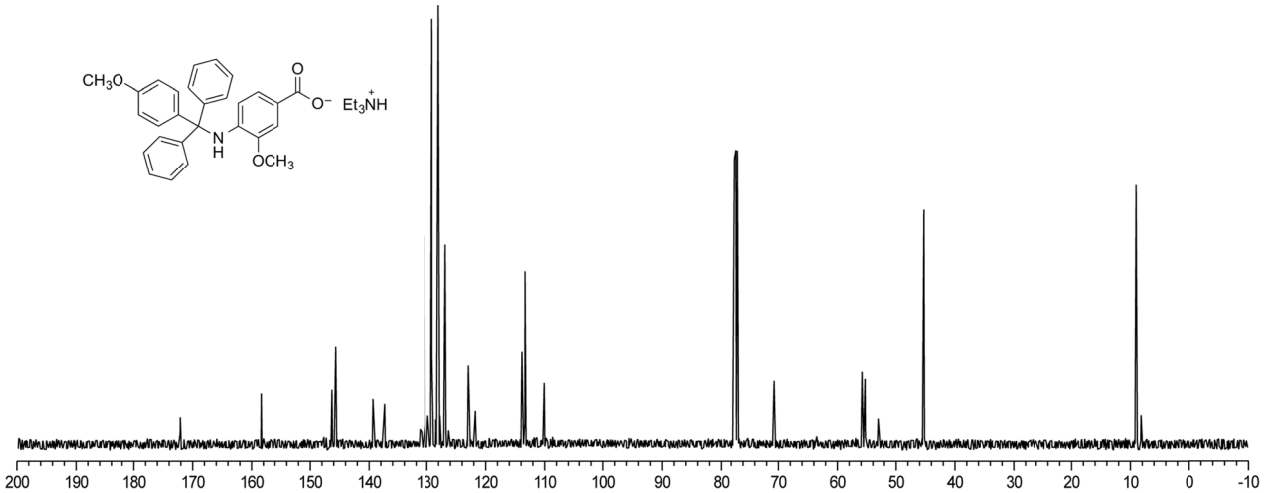
TLC: R_f = 0.25 [4% CH_3OH in CH_2Cl_2 with 2% (v/v) Et_3N].

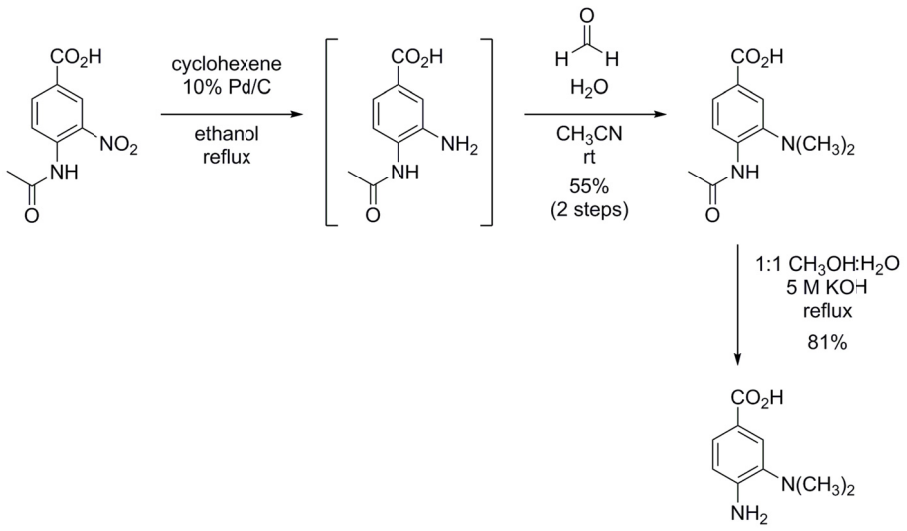
^{1}H NMR: (500 MHz, $CDCl_3$) δ 7.44 (d, *J* = 1.8 Hz, 1H), 7.32-7.18 (m, 13H), 6.78 (d, *J* = 8.9 Hz, 2H), 5.97 (d, *J* = 8.4 Hz, 1H), 3.91 (s, 3H), 3.77 (s, 3H), 3.00 (q, *J* = 7.3 Hz, 6H), 1.27 (t, *J* = 7.3 Hz, 9H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 172.1, 158.4, 146.3, 145.7, 139.4, 137.4, 130.5, 129.2, 128.1, 126.9, 122.9, 121.8, 113.9, 113.3, 110.1, 70.7, 55.8, 55.3, 45.3, 9.0 ppm.

MS: *m*/*z* calcd. for $C_{28}H_{25}NaNO_4$ $[M+Na]^+$: 462.2; found: 462.2.







4-Acetamido-3-aminobenzoic acid

To a solution of 4-acetamido-3-nitrobenzoic acid (498 mg, 2.2 mmol, Acros) in ethanol (30 mL) was added cyclohexene (1.4 mL, 13.8 mmol) and 10% Pd/C (157 mg). The mixture was heated at reflux for 4 h (bath temperature 120 °C). The hot reaction mixture was filtered through a bed of Celite in a 60 mL coarse fritted funnel, and the Celite cake was washed with boiling EtOH (3 × 30 mL). The flow-through was concentrated under reduced pressure to give a mixture of starting material and product that was used for the next step without further purification.

4-Acetamido-3-(dimethylamino)benzoic acid

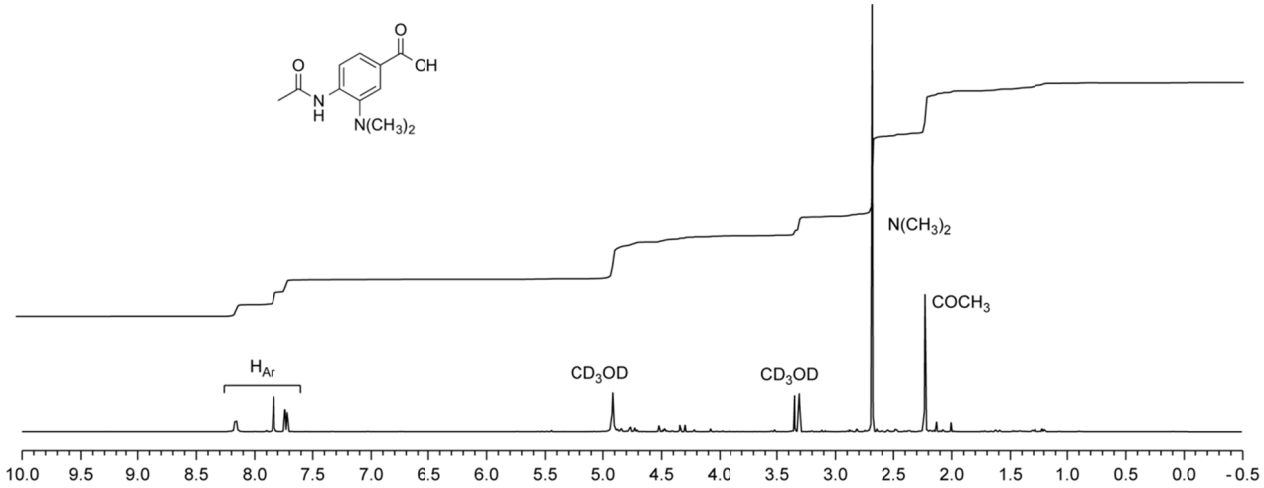
The 4-acetamido-3-aminobenzoic acid from the previous reaction step was dissolved in CH_3CN (X mL). A solution of 37% aqueous formaldehyde (formalin, 3.0 mL) was added, and the clear colorless solution was stirred at room temperature for 1 h. Glacial acetic acid (0.5 mL, 8.7 mmol) added, and the sample was stirred for 24 h. The sample was diluted with water (50 mL) and extracted with ethyl acetate (5 × 30 mL). The organic layer was washed with saturated NaCl, dried over Mg_2SO_4, and concentrated under reduced pressure. The product was purified by silica gel column chromatography, eluting with 1–4% CH_3OH in CH_2Cl_2. Appropriate product fractions were combined and evaporated to provide 266 mg (55% over two steps) of the product as a white solid.

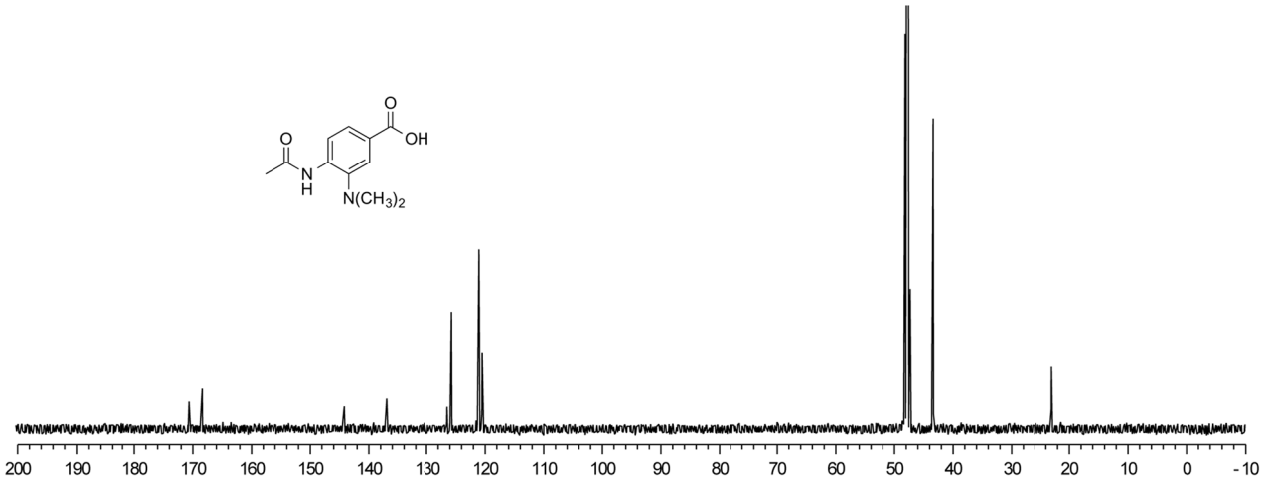
TLC: R_f = 0.37 (4% CH_3OH in CH_2Cl_2).

^{1}H NMR: (500 MHz, CD_3OD) δ 7.56 (d, *J* = 1.9 Hz, 1H), 7.56 (dd, J = 1.9, 8.3 Hz, 1H), 6.70 (d, *J* = 8.3 Hz, 1H), 2.65 (s, 6H) ppm, 2.23 (s, 3H) ppm.

^{13}C NMR: (125 MHz, CD_3OD) δ 170.0, 169.8, 145.6, 138.3, 127.9, 127.1, 122.4, 121.9, 44.8, 24.4 ppm.

MS: *m*/*z* calcd. for $C_{11}H_{15}N_2O_3$ $[M+H]^+$: 223.1; found: 223.1.

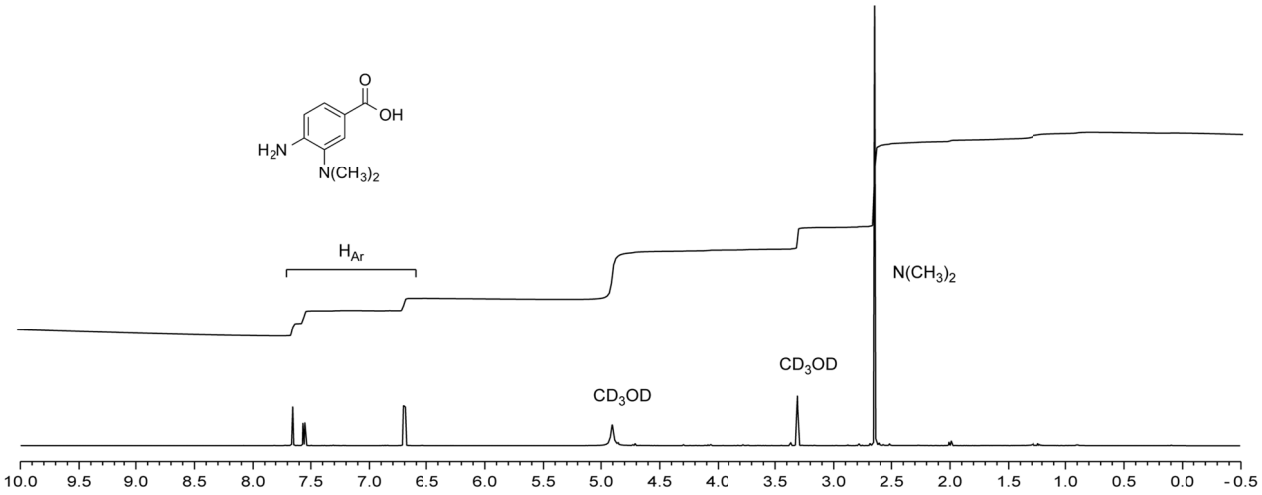


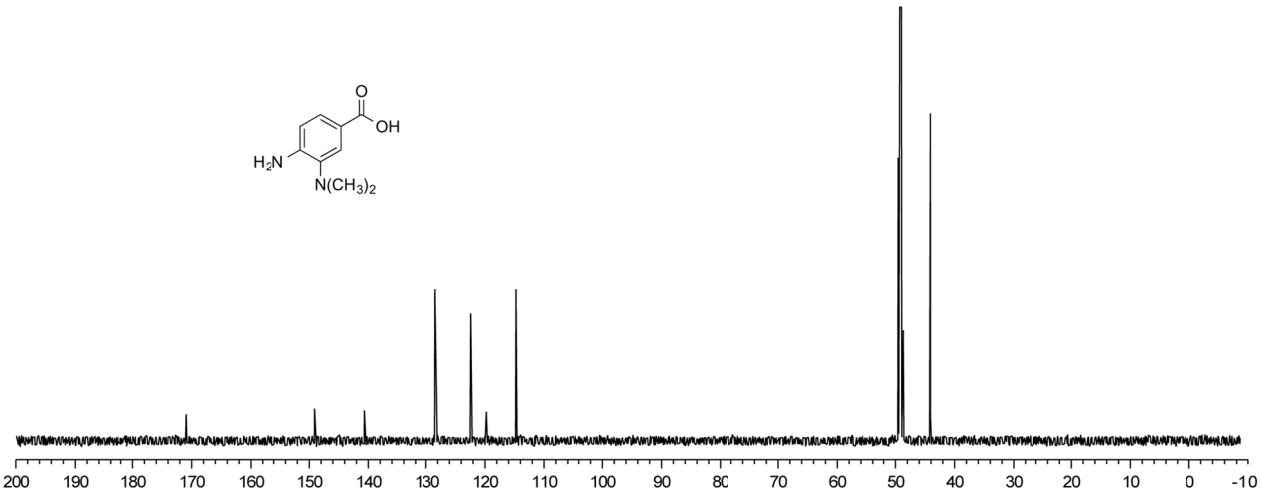


4-Amino-3-(dimethylamino)benzoic acid

4-acetamido-3-(dimethylamino)benzoic acid (200 mg, 0.9 mmol) was heated at reflux (bath temperature 120 °C) in a solution of 1:1 CH_3OH:H_2O containing 5 M KOH (10 mL) for 12 h. The clear colorless solution was diluted with water (50 mL) and neutralized with conc. HCl. The neutralized solution was extracted with ethyl acetate (3 × 30 mL), washed with saturated NaCl (30 mL), and dried over $MgSO_4$. The solution was evaporated to provide 132 mg (81%) of the product as a brown solid.

TLC: R_f = 0.29 (4% CH_3OH in CH_2Cl_2).
^{1}H NMR: (500 MHz, $CDCl_3$) δ 7.66 (d, J = 1.9 Hz, 1H), 7.56 (dd, J = 8.3, 1.9 Hz, 1H), 6.70 (d, J = 8.3 Hz, 1H), 2.65 (s, 6H) ppm.
^{13}C NMR: (125 MHz, $CDCl_3$) δ 170.9, 149.1, 140.7, 128.5, 122.4, 119.8, 114.7, 44.1 ppm.
MS: m/z calcd. for $C_9H_{13}N_2O_2$ $[M+H]^+$: 181.1; found: 181.1.





***N*-MMT-4-amino-3-(dimethylamino)benzoic acid triethylammonium salt**

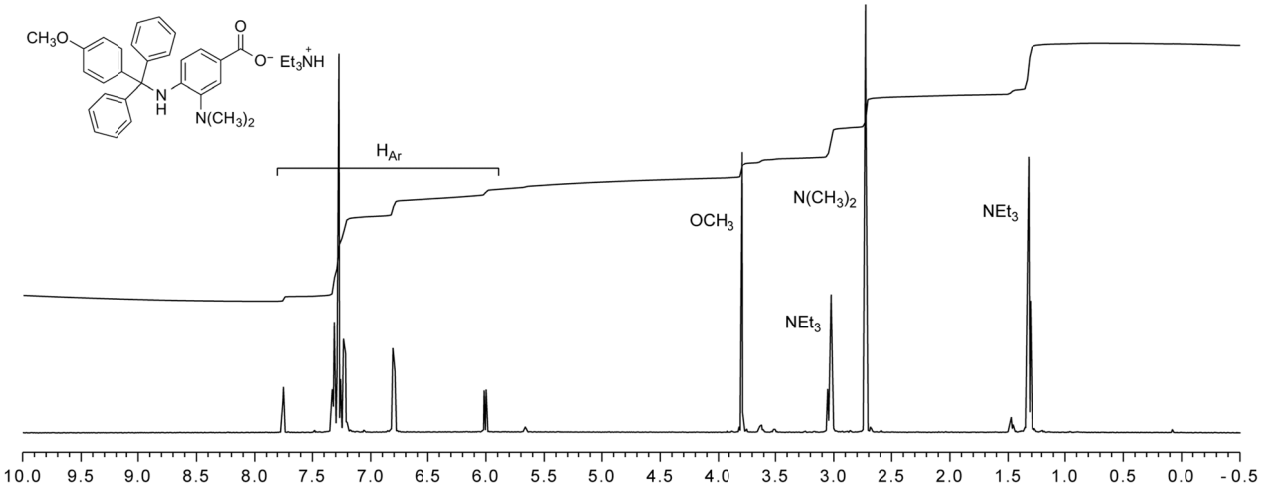
Following the general MMT protection procedure, from 132 mg (0.73 mmol) of 4-amino-3-(dimethylamino)benzoic acid, 168 mg (41%) of product was isolated as an off-white solid.

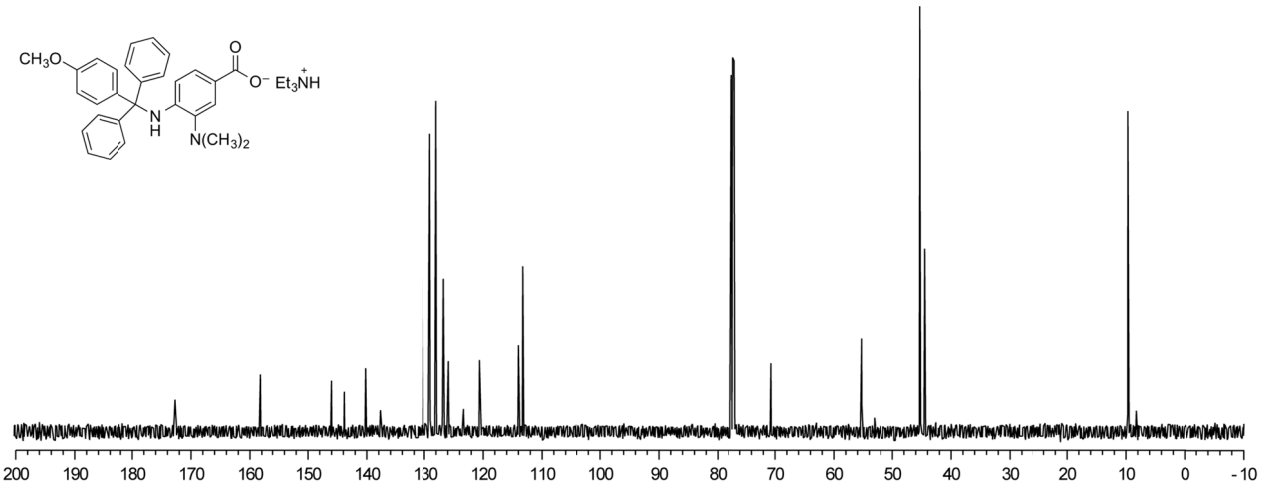
TLC: R_f = 0.25 [4% CH_3OH in CH_2Cl_2 with 2% (v/v) Et_3N].

^{1}H NMR: (500 MHz, $CDCl_3$) δ 7.74 (d, *J* = 2.0 Hz, 1H), 7.31-7.19 (m, 13H), 6.79 (d, *J* = 8.9 Hz, 2H), 5.99 (d, *J* = 8.6 Hz, 1H), 3.77 (s, 3H), 3.02 (q, *J* = 7.3 Hz, 6H), 2.71 (s, 6H), 1.31 (t, *J* = 7.3 Hz, 9H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 172.7, 158.2, 146.0, 143.8, 140.2, 137.7, 130.4, 129.1, 128.0, 126.8, 125.9, 123.3, 120.6, 114.0, 113.3, 70.0, 55.3, 45.3, 44.5, 9.7 ppm.

MS: *m*/*z* calcd. for $C_{29}H_{28}NaN_2O_3$ $[M+Na]^+$: 475.2; found: 475.4.





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