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for

Tetrabutylammonium Fluoride-Assisted Rapid Alkylation Reaction in Microtiter Plates for Discovery of Enzyme Inhibitors in Situ

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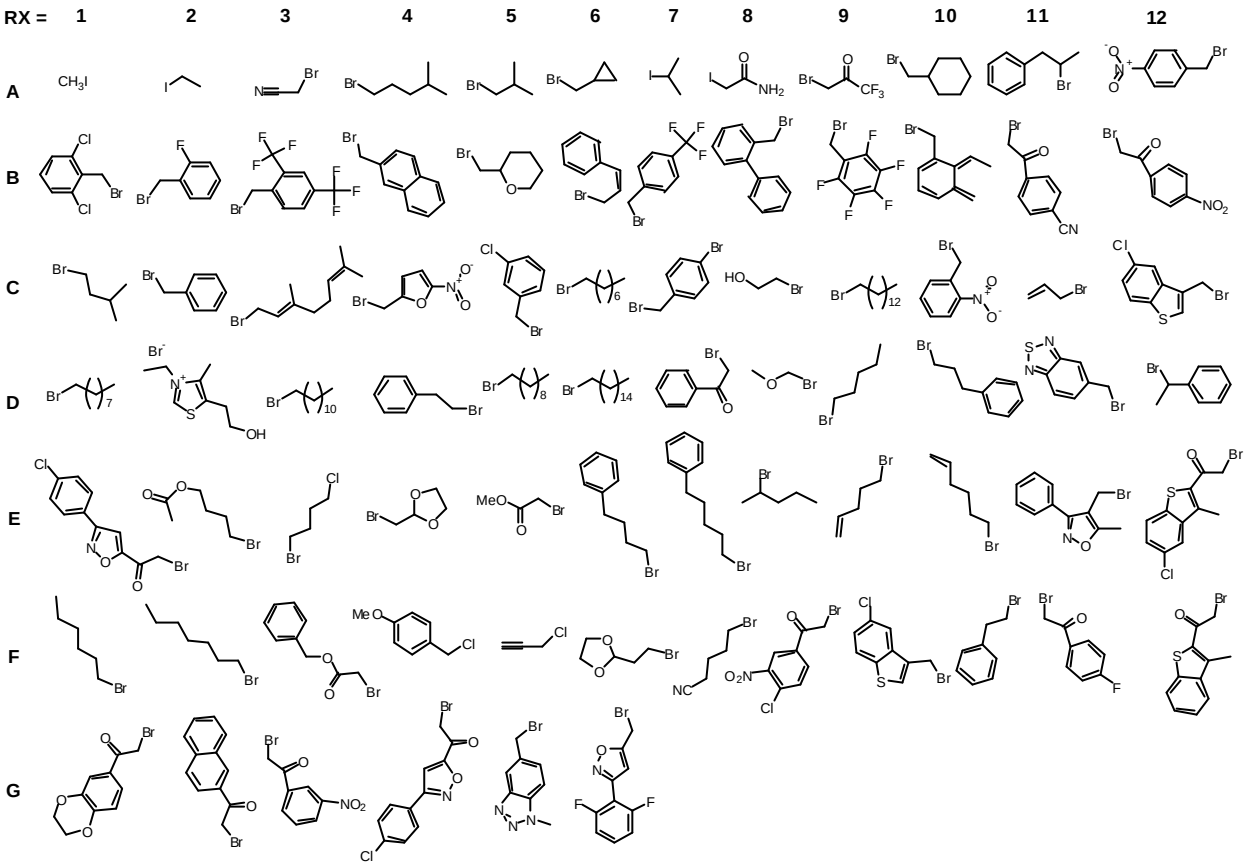
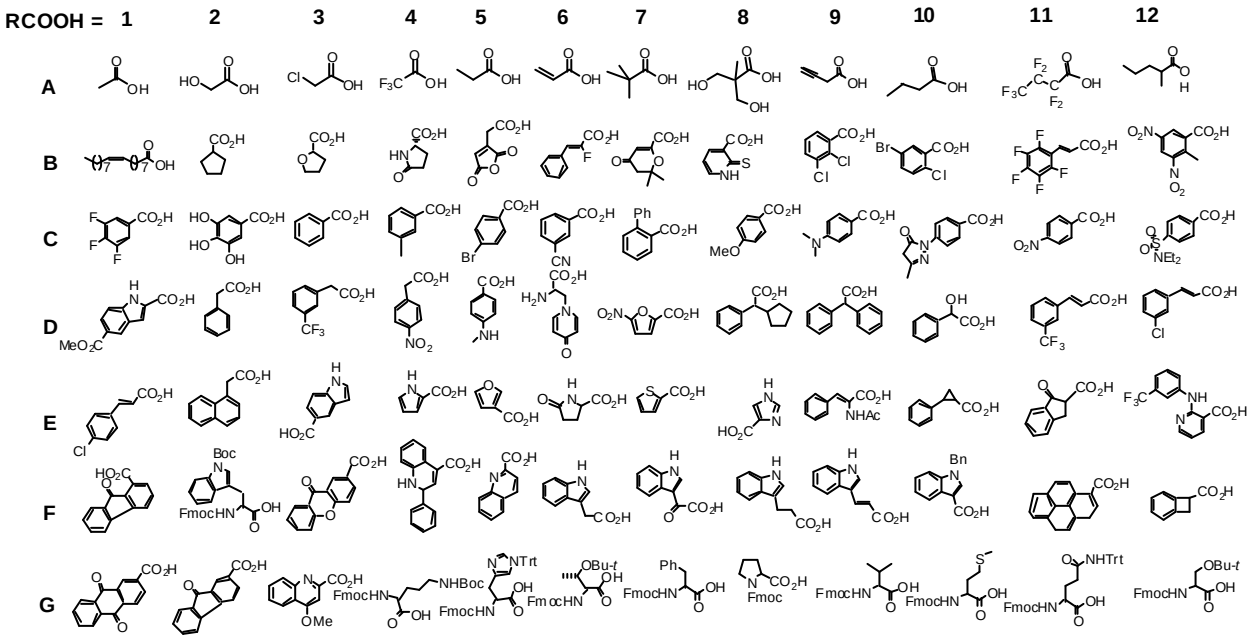
Materials and reagents. Analytical TLC was performed on pre-coated plates (EMD, silica gel $60F_{254}$). Silica gel used for flash column chromatography was EMD silica gel 60 (40-63 μm). Reagents of the highest purity were purchased from Aldrich, Sigma, Acros and Novabiochem. $TBAF{\cdot}3H_2O$ was purchased from Acros. 1M TBAF in THF was purchased from Aldrich.

78 organic halides.



84 organic acids



Experimental Procedures

General procedure for the TBAF assisted ester bond formation (entry 24, Table 2). 4-Dimethylamino-benzoic acid (100 mg, 0.61 mmol) and benzyl bromide (124 mg, 0.73 mmol, 1.2 equiv) were placed in a 10 mL flask with a stirring bar, followed by the addition of TBAF (0.73 mL, 0.73 mmol, 1.2 equiv, 1 M in THF) at room temperature. After being stirred for 4 h at RT, the reaction was directly loaded into the column and the product was eluted with solution of 4:1 hexane: ethyl acetate to yield 129 mg (83%) of product as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.95 (d, *J* = 8.8 Hz, 2H), 7.44-7.31 (m, 5H), 6.62 (d, *J* = 8.8 Hz, 2H), 5.31 (s, 3H), 3.01 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 166.74, 153.31, 136.75, 131.36, 128.43, 127.89, 127.85, 116.76, 110.62, 65.81, 39.97.; ESI-MS calculated for $C_{16}H_{17}NO_2$ 255.13, found 255.1. **(Entry 21):** ^{1}H NMR (500 MHz, $CDCl_3$) δ = 10.77 (brs, 1H), 7.84 (d, *J* = 8.1 Hz, 1H), 7.43-7.31 (m, 6H), 6.95 (d, *J* = 8.4 Hz, 1H), 6.82-6.81 (m, 1H), 5.33 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 169.81, 161.63, 135.68, 135.19, 129.87, 128.56, 128.40, 128.14, 119.04, 117.47, 112.26, 66.81, 28.08.; ESI-MS calculated for $C_{14}H_{12}O_3$ 228.08, found 228.0.

Library preparation for in situ screening: Each well of a 96-well microtiter plate was added 12.0 μL of each alkyl halide (from a 100 mM stock solution in DMF, 1.2 equiv) and 10.0 μL of 100 mM stock solution of core **1**. The reaction was initiated by adding 12.0 μL of 100 mM TBAF in DMF (1.2 equiv) to each well. The reaction mixtures were kept at 25 °C for 4 h and were monitored for completion based on the disappearance of core **1** by TLC with $CHCl_3$/MeOH (10:3, R_f = 0.42) and LC-MS (C8 column). The reaction mixture in each well was then diluted to 200 μM ready for the assay as previously described.[1]

Synthesis of compound 3: Core **1b**[2] (20.0 mg, 0.07 mmol) and 2-(Bromomethyl)-5-nitrofuran (17.3 mg, 0.084 mmol, 1.2 equiv) were placed in a 5 mL flask with a stirring bar. Then, TBAF (0.84 mL, 0.084 mmol, 1.2 equiv, 0.1 M in DMF) was added at 0 ^{o}C. After being stirred for 30 min at 0 ^{o}C, the resulting reaction mixture was poured into water (5 mL) and extracted with ethyl acetate (10 mL x 3). The combined organic extracts were washed with brine and dried over Na_2SO_4. Evaporation of solvents and purification of the residual oil by column chromatography on silica gel (hexane/ethyl acetate = 3:1 as eluant) gave pure ester **3** (25.9 mg, 91%) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 8.27 (s, 1H), 8.24 (d, *J* = 8.5 Hz, 2H), 7.36-7.26 (m, 6H), 6.86 (d, *J* = 8.5 Hz, 1H), 6.7 (s, 1H), 5.34 (s, 2H), 4.98 (s, 2H).; ^{13}C NMR (125 MHz, $CDCl_3$) δ = 182.00, 164.06, 158.13, 154.17, 151.90, 140.06, 133.79, 129.19, 129.17, 128.43, 127.40, 126.92,

124.85, 117.39, 113.81, 111.98, 110.97, 58.07, 44.31.; ESI-MS calculated for $C_{21}H_{14}N_2O_7$ 406.08, found 406.0.

Synthesis of compound 4-Methylamino-benzoic Acid Benzotriazol-1-yl Ester 7.
To a solution of 4-methylamino-benzoic acid (53.5 mg, 0.3539 mmol, 1.0 equiv), *O*-benzotriazol-1yl- *N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU, 147.7 mg, 0.3894 mmol, 1.1 equiv) in DMF (2.0 mL) was added *N*,*N*-diisopropylethylamine (DIEA, 68 μL , 0.3894 mmol, 1.1 equiv). After the solution was stirred at 25 °C for 1.0 h, the reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel to provide the desired Benzotriazole ester **7** (88.3 mg, 0.3291 mmol) in 93% yield as a white solid: TLC R_f = 0.28 ($CHCl_3$ as eluant); ^{1}H NMR ($CDCl_3$, 400 MHz) *d* = 8.07–8.04 (m, 3 ArH, 3 H), 7.53–7.46 (m, 2 ArH, 2 H), 7.41 (t, *J* = 7.6 Hz, ArH, 1 H), 6.63 (d, *J* = 8.7 Hz, 2 ArH, 2 H), 2.92 (d, *J* = 1.5 Hz, CH_3N, 3 H,); ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 162.70, 154.70, 143.52, 133.00, 129.05, 128.43, 124.62, 120.29, 111.52, 111.05, 108.61, 29.91; IR (KBr) 3346 (m, NH), 3010 (m), 2907 (w), 1764 (s, C=O), 1602 (s), 1545 (s), 1497 (s), 1446 (s), 1362 (s), 1238 (s), 1068 (s), 957 (s), 791 (s) cm^{-1}; HRMS [*M* + 1] calcd for $C_{14}H_{13}N_4O_2$: 269.1039, found 269.1032.

4-Dimethylamino-benzoic Acid Benzotriazol-1-yl Ester 8. As described for **7.** Benzotriazole ester **8** was obtained in 93% yield as a light yellow solid: TLC R_f = 0.50 ($CHCl_3$ as eluant); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.07–8.12 (m, 3 × ArH, 3 H), 7.47–7.55 (m, 2 × ArH, 2 H), 7.42 (t, *J* = 6.7 Hz, ArH, 1 H), 6.74 (d, *J* = 8.9 Hz, 2 × ArH, 2 H), 3.13 (s, 2 CH_3, 6 H), ; ^{13}C NMR ($CDCl_3$, 100 MHz) 163.51, 154.38, 143.57, 132.73, 129.11, 128.36, 124.54, 120.35, 110.6, 110.02, 108.64, 40.01; IR (KBr) 2914 (w), 1768 (s, C=O), 1609(s), 1537 (s), 1441 (s), 1386 (s), 1263 (s), 1183 (s), 957 (s), 770 (s) cm^{-1}; HRMS [M + 1] calcd for $C_{15}H_{15}N_4O_2$: 283.1195, found 283.1199.

Screening for cathepsin B inhibition: All enzymatic assays were carried by measuring the increased fluorescence resulted from the hydrolysis of Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride (Sigma) by bovine spleen cathepsin B (Sigma) at RT, with excitation wavelength 380 nm and emission wavelength 460 nm. The assay buffer consisted of 50 mM MES, 200 mM NaCl, 5 mM EDTA, 0.15% Brij 35 and 1mM DTT, pH 6.0 at room temperature. The inhibitory activity for crude reaction mixture from each microtiter plate was screened by using 30 nM cathepsin B, 5 μM substrate and inhibitor concentration as indicated.

Characterization of slow inhibitor against cathepsin B: The protocol of kinetic measurements of a time-dependent inhibitor was followed.[3] The progress curves of the bovine spleen cathepsin B reactions in the presence of varying concentrations of the time-dependent inhibitor were measured using the fluorogenic substrate. The reaction was initiated by adding 0.03 μM enzyme to a mixture containing 5 μM substrate and 0 to 10 μM inhibitor. Over the entire 7 min time window, the uninhibited enzyme showed a linear progress curve, whereas the inhibited enzyme with different concentration of inhibitor illustrated a time-dependent reduction of reaction velocity (Figure 1). The reaction rate was lower at higher inhibitor concentration. This suggests the inhibitor is either slow-binding or slow-inactivating inhibitor.

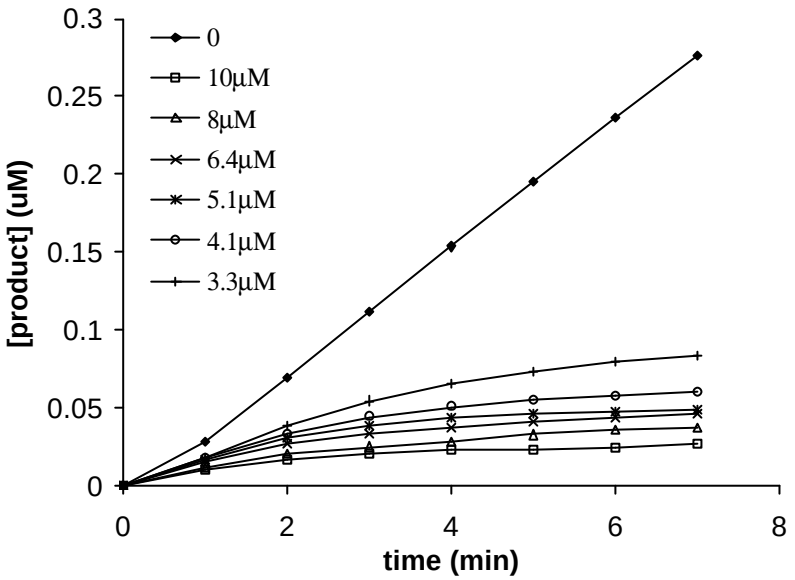


Figure S1. The progress curves in the presence of 3.3–10 μM inhibitor for reactions initiated by adding enzyme (final concentration = 0.04 μM) into mixture of substrate (5 μM) and inhibitor. Over the entire 5 min time window, the uninhibited enzyme displayed a linear progress curve, whereas the inhibited enzyme with different concentration of inhibitor showed a time-dependent reduction of reaction velocity

Measurements of K_i and k_{inact}: The enzyme was preincubated with different concentrations (0-12.5 μM) of inhibitor and then a small aliquots of the solution was taken periodically for initial velocity assay (final enzyme concentration = 0.03 μM) using the 5 μM substrate (Figure 2). For initial velocity measurement, the increase of the product fluorescence was monitored at excitation 380 nm and emission 460 nm for the first 240 s of the reaction. The value of $\ln(v_i/v_0)$ was fitted with the incubation time to obtain the k_{obs} of inactivation at different inhibitor concentration.

The half-life ($t_{1/2}$) can be calculated from the k_{obs} of inactivation (ln2/ k_{obs} = $t_{1/2}$). The half-life for inactivation at each inhibitor concentration is plotted against 1/[inhibitor] (Figure 3).[4] k_{inact} and K_i can be determined from the linear line in the plot. The value of the intercept at the y-axis is ln2/k_{inact} and the x axis intercept is $-1/K_i$.

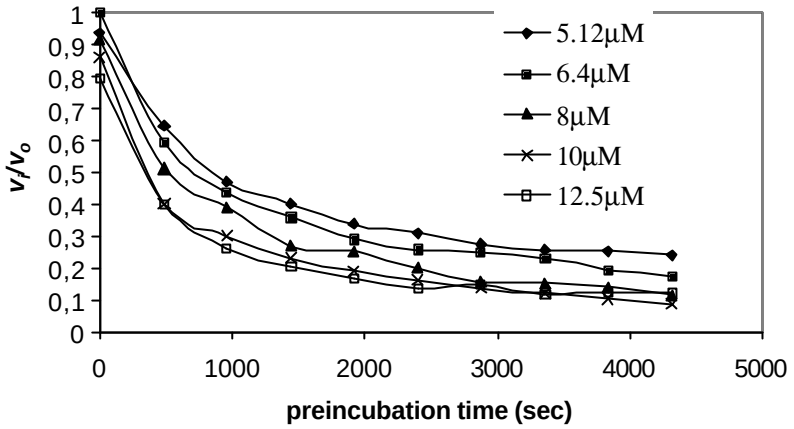


Figure S2. Preincubation time dependence of the fractional velocity of the protease- catalyzed reaction in the presence of 5.12–12.5 µM time-dependent inhibitor **8**.

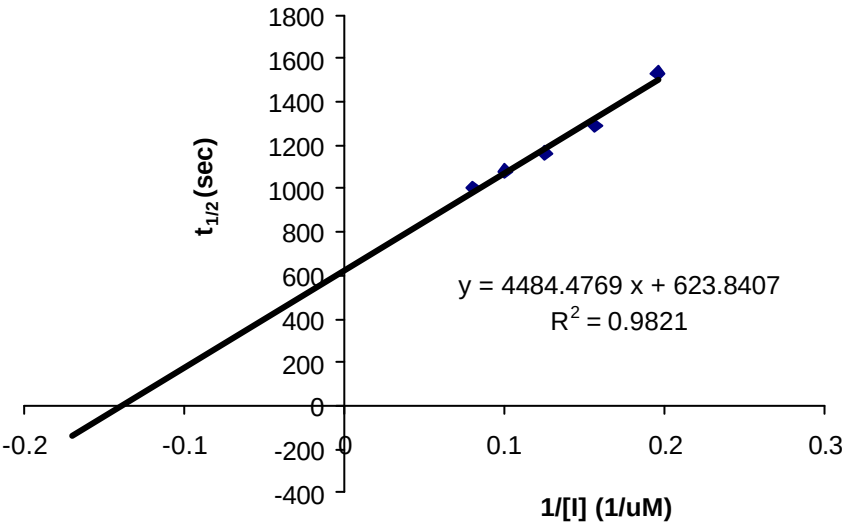
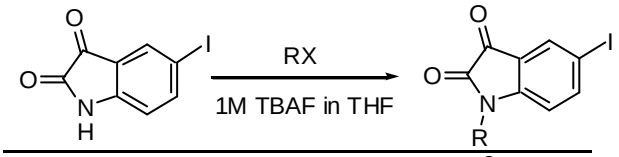
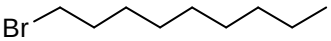


Figure S3. Kitz and Wilson replot of the half-life (t1/2) of enzyme inactivation as a function of the reciprocal of the slow inactivator concentration. The k_{inact} is 1.1 x 10^{-3} s^{-1} and K_i is 7.18 µM for the time-dependent inactivator **8** based on the kinetic data.

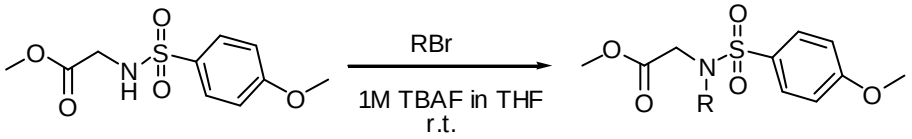
Table S1. Reaction of 5-iodo-isatin and organic halides.

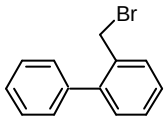
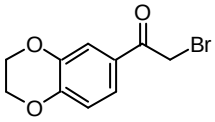
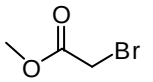
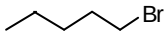
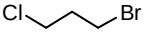
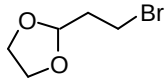
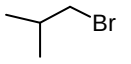


RX	Yield[a] (%)
BnBr	89
CH_3I	95
	77
	60[b]

a. Isolated yield.
b. Reaction at 60 °C.

Table S2. TBAF assisted N-alkylation of sulfonamide..



RX	Reaction Time (min)	Yield[a] (%)
BnBr	10	90
	10	97
	10	92
	10	94
	45	86
	45	88
	45	78
	60	84
	10	86

a. Isolated yield.

General procedure for the TBAF assisted sulfonamide N-alkylation. (Compound **12**): Compound **9** (20 mg, 0.04 mmol) and (4-bromobutyl)benzene (10.2 mg, 0.05 mmol, 1.2 equiv) were placed in a 2 mL flask with a stirring bar followed by the addition of TBAF (50 μL, 0.05 mmol, 1.2 equiv, 1M in THF) at room temperature. After being stirred for 2.5 h at room temperature, the reaction was directly loaded into the column and the product was eluted with solution of 2:1 hexane/ethyl acetate to yield compound **12** (20.5 mg 86%) as a pale yellow liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.68 (d, J = 8.8 Hz, 2H),

7.31-7.10 (m, 10 H), 6.94 (d, J = 8.8 Hz, 2H), 5.11 (brs, 1H), 4.77 (d, J = 8.5 Hz, 1H), 3.86 (s, 3H), 3.82-2.75 (m, 4H), 3.62 (d, J = 10 Hz, 1H), 3.20-2.89 (m, 6H), 2.57 (t, J = 6.6 Hz, 2H), 2.43 (t, J = 6.6 Hz, 1H), 2.09-2.04 (m, 1H), 1.87-1.81 (m, 3H), 1.44-1.25 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.35, 156.57, 142.25, 138.06, 130.59, 129.88, 129.77, 128.95, 128.80, 126.99, 126.30, 114.78, 75.79, 73.65, 72.90, 67.33, 55.50, 52.96, 50.84, 35.83, 35.67, 33.17, 28.67, 28.32.

General procedure for the TBAF assisted Benzotriazole N-alkylation (compound **13**)**:** Benzotriazole (100 mg, 0.84 mmol) and β-bromo-4-(diethylamino)acetophenone (272 mg, 1.00 mmol, 1.2 equiv) were placed in a 5 mL flask with a stirring bar followed by the addition of TBAF (1.00 mL, 0.84 mmol, 1.2 equiv, 1M in THF) at room temperature. After being stirred for 2 h at room temperature, the reaction was directly loaded into the column and the product was eluted with solution of 6:1 hexane: ethyl acetate to yield 217.4 mg (84%) of product as a pale yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (d, *J* = 8.5 Hz, 1H), 7.96 (d, *J* = 9.2 Hz, 2H), 7.50-7.30 (m, 3H), 6.68 (d, *J* = 9.2 Hz, 2H), 5.98 (s, 2H), 3.42 (q, *J* = 7.0 Hz, 4H), 1.19 (t, *J* = 7.0 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 187.92, 152.46, 146.42, 134.38, 131.37, 128.00, 124.21, 121.41, 120.25, 110.91, 110.46, 53.76, 45.06, 12.87; ESI-MS calculated for $C_{18}H_{20}N_4O$ 308.16, found 308.12. (Compound **14**): Pale yellow solid. 89% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.96 (d, *J* = 8.5 Hz, 1H), 7.92 (d, *J* = 8.5 Hz, 1H), 7.77 (d, *J* = 9.0 Hz, 2H), 7.52 (dd, *J* = 7.5 Hz, *J* = 7.5 Hz, 1H), 7.36 (dd, *J* = 7.5 Hz, *J* = 7.5 Hz, 1H), 6.6 (d, *J* = 9.0 Hz, 2H), 5.80 (s, 2H), 3.41 (q, *J* = 7.0 Hz, 4H), 1.19 (t, *J* = 7.0 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 188.65, 151.90, 143.40, 130.40, 127.95, 127.83, 124.55, 120.91, 119.68, 110.43, 110.35, 79.67, 44.53, 12.37; ESI-MS calculated for $C_{18}H_{20}N_4O_2$ 324.16, found 324.11.

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[2] S. E. Webbwe, J. Tikhe, S. T. Worland, S. A. Fuhrman, T. F. Hendrickson, D. A. Matthews, R. A. Love, A. K. Patick, J. W. Meador, R. A. Ferre, E. L. Brown, D. M. Delisle, C. E. Ford, S. L. Binford, *J. Med. Chem.* **1996**, *39*, 5072-5082.

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