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Synthesis and Evaluation of Anthranilic Acid-Based Transthyretin Amyloid Fibril Inhibitors

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Abstract. Eight small molecules were synthesized to evaluate the structure activity relationships (SAR) of N-substituted anthranilic acids. The molecules were synthesized by benzylation or arylation of methyl anthranilate. A light scattering-based amyloid fibril formation assay was used to evaluate potential inhibitors of transthyretin (TTR) amyloid fibril formation *in vitro*. The *m*-carboxyphenylated and *o*-trifluoromethylphenylated anthranilic acids are potent inhibitors that will be subjected to further SAR and structural analysis.

Introduction One common feature of neurodegenerative diseases classified as amyloid disorders is the deposition of insoluble, high molecular weight cross-β-sheet fibrils derived from the self-assembly of one of twenty human proteins.[1-8] Wild type transthyretin (TTR) amyloid fibrils appear to cause senile systemic amyloidosis (SSA), whereas deposition of one of sixty different single site TTR variants is associated with a variety of disorders collectively referred to as familial amyloid polyneuropathy (FAP).[7,9] These diseases do not involve the brain, but instead involve peripheral organs and nerves. These diseases have been classified as protein misfolding diseases, whereby tetrameric transthyretin dissociates to an alternatively folded monomer that self-associates into amyloid fibrils.[1-3,10-16] Tetrameric TTR binds to two molecules of thyroxine with negative cooperativity and high affinity (K_{A1} 10^8, K_{A2} 10^6) in the cerebral spinal fluid. However in the blood plasma, transthyretin has a largely unoccupied T_4 binding site because thyroid binding globulin is the primary T_4 carrier (K_A 6 x 10^9). Our strategy for inhibiting TTR amyloid fibril formation is to identify ligands that will bind to transthyretin in plasma using this binding site. Ligand binding stabilizes TTR and increases the activation barrier associated with the tetramer to monomeric amyloidogenic intermediate transition that allows amyloid fibril formation.[16-18] Previous results from our laboratory demonstrate that thyroxine and flufenamic acid (Flu, **1**) are capable of stabilizing the tetrameric form of transthyretin, preventing amyloid fibril formation at a pH below 5.5 where TTR normally dissociates into an alternatively folded monomer that self-associates into amyloid fibrils.[16-18]

Chemistry Methyl anthranilate was covalently modified by arylation of the aniline nitrogen by a variety of trifluoromethylated aryl iodides and carboxymethylated aryl bromides employing a Pd-catalyzed aryl

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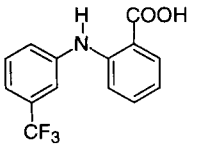
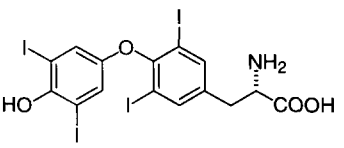


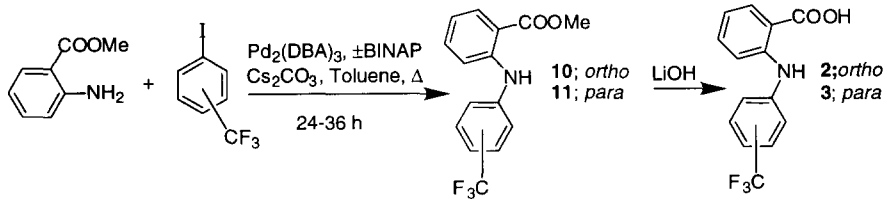
1, Flufenamic Acid



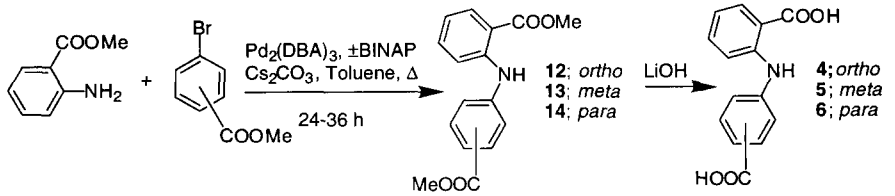
Thyroxine (T_4)

coupling reaction developed in the Buckwald laboratory, **Schemes 1A & 1B**.[19,20] The resulting diesters were hydrolyzed to the diacids utilizing LiOH in THF:methanol:water; 3:1:1.

Scheme1A

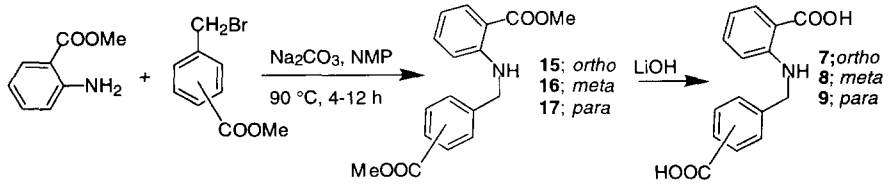


Scheme 1B



Methyl anthranilate was also covalently modified by N-alkylation with carboxymethylated benzyl bromides to afford three benzyl anthranilate diesters. The diesters were hydrolyzed as described above to afford the diacids **7-9**. The characterization data for all compounds is provided.[21]

Scheme 2



Biological Assay The stagnant light scattering assay employed previously to quantify the extent of TTR amyloid fibril formation will be used herein to evaluate TTR amyloid fibril inhibitors.[12,16] This assay was used to discover Flu, **1**, an excellent TTR amyloid fibril inhibitor that will be used as a positive control in the

screening studies herein.[17] Flufenamic acid binds to wild type TTR with negative cooperativity (K_{D1} = 30±14 nM, K_{D2} = 255±97 nM), completely inhibiting amyloid fibril formation at pH 4.4 at a concentration of 10.8 μM, 3x the physiological concentration of TTR (3.6 μM).[17] The light scattering assay (400 nm) subjects TTR (3.6 μM) to acidic partial denaturation at pH 4.4 (maximal amyloid fibril formation is observed at this pH, acetate buffer, 37°C) in either the absence (assigned to be 100% fibril conversion) or in the presence of a potential amyloid fibril inhibitor. Hence, 0% TTR fibril formation indicates complete inhibition of amyloid fibril formation by the compound being evaluated.

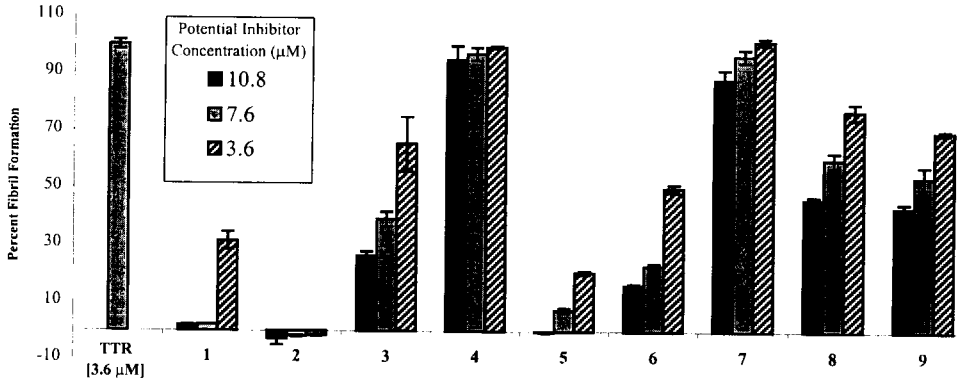


Fig. 1 The extent of TTR amyloid fibril formation measured by light scattering at 400 nm as a function of inhibitor concentration. The left most bar represents the extent of TTR fibril formation (37°C, 72 h, pH 4.4) in the absence of inhibitor.

Results and Discussion A collaborative effort between our group and the Sacchettini laboratory at Texas A&M has led to a 2.0 Å resolution structure of flufenamic acid bound to transthyretin as depicted by the cover figure.[17] This structure provides insight into the design of analogs of Flu screened herein. The extent of transthyretin amyloid fibril formation in the presence of the Flu analogs was evaluated by light scattering as described briefly above and in detail in the references that follow.[12,16] **Figure 1** demonstrates that *o*-trifluoromethylphenyl substituted anthranilic acid **2** and *m*-carboxyphenyl substituted anthranilic acid **5** are excellent inhibitors. The remainder of the analogs are not nearly as effective. It appears that the presence of a methylene spacer in **7**, **8** and **9** is detrimental to inhibition of TTR fibril formation.

It is likely that **2** binds to TTR using similar molecular interactions as those which allow Flu to bind to TTR. The TTR•Flu_2 structure is shown of the cover of this journal. Comparison of the *o*- and *p*-CF_3 isomers to the *m*-trifluoromethylphenyl ring of Flu reveals that the *o*-CF_3 substituent in **2** leads to a more active inhibitor. Additional structural and binding information is required to fully explain this exciting result.

Compound **5** is likely to bind to TTR differently than does Flu. Flufenamic acid positions its CF_3 substituted phenyl ring in an inner hydrophobic binding pocket that is just large enough to accommodate this functionality. The carboxylated ring of Flu is positioned in the much larger outer binding pocket of TTR

which is large enough to bind both carboxylated phenyl rings of **5**. We speculate that this inhibitor will bind using the outer binding site exclusively, as this binding mode will permit the two carboxylates of **5** to interact with the two Lys-15 ε-ammonium groups flanking the outer binding site of TTR.[18] The Kelly and Sacchettini laboratories are working together to obtain detailed structural data to further explain the efficacy of **2** and **5**.

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21. All reagents and solvents were purchased from Aldrich, Lancaster, Acros or Advanced ChemTech. Dry methyl anthranilate was obtained by distillation over $CaCl_2$, and was stored over 4 Å molecular sieves. All reactions were performed under argon except for the hydrolysis reactions. Reaction progress was monitored by Thin Layer Chromatography (TLC) on silica gel $60F_{254}$ coated aluminum TLC plates from EM Science. Unless stated otherwise, product purification was performed by flash chromatography using silica gel (230-400 mesh) purchased from EM Science. ^1H NMR spectra were obtained on Bruker spectrometers at 400 or 600 MHz and ^{13}C NMR spectra were acquired at 75, 100 or 150 MHz. Chemical shifts are reported in ppm relative to $CDCl_3$ (7.27 ppm for ^1H and 77.00 ppm for ^{13}C) or CD_3OD (3.30 ppm for ^1H NMR). Mass spectrometry data were obtained at the Scripps Research Institute. The fast atom bombardment (FAB) data were obtained using NBA/NaI matrix. EA refers to ethyl acetate, Hex to hexanes, and TFA to trifluoroacetic acid.

General Procedure for Aryl Coupling to Prepare Compounds 10-14: An oven dried flask containing a sir bar was attached to a water condenser capped with a rubber septum. A needle attached to a vacuum line was used to evacuate the apparatus for 30 min. The set up was then flame dried and cooled to room temperature under vacuum. The vacuum was filled using an argon filled balloon. The appropriate aryl bromide (1.2 eq), Cs_2CO_3 (1.4 eq), $Pd_2(DBA)_3$ (4.5 mol%), BINAP (racemic, 3 mol%), and methyl anthranilate (1 eq.) were added in that order, respectively. Finally, toluene was added through the reflux condenser and the reaction mixture (0.5 M methyl anthranilate) was heated to 100 °C for 24-36 h. The reaction mixture was cooled to room temperature and filtered through celite. The filtrate was concentrated under vacuum and the crude product was purified by flash chromatography using a gradient of ethyl acetate and hexanes to obtain a 40-95% yield. Characterization data is summarized below for the compounds prepared:

Compound 10: $^1H(CDCl_3)$(400 MHz): δ 9.60 (bs, 1H), 7.91 (dd, *J* = 7.96, 1.60 Hz, 1H), 7.58 (d, *J* = 7.56 Hz, 1H), 7.47 (d, *J* = 8.10 Hz, 1H), 7.38 (t, *J* = 7.56 Hz, 1H), 7.24 (m, 1H), 7.07 (m, 2H), 6.72 (m, 1H), 3.83 (s, 3H); $^{13}C(CDCl_3)$(300 MHz): δ 168.00, 146.73, 139.42, 133.93, 132.40, 131.65, 129.51, 127.03, 126.97, 125.90, 123.36, 122.95, 118.31, 114.74, 113.36; HRMS (FAB) calcd for $C_{15}H_{12}F_3NO_2$ $(MH)^+$ 296.0898, found 296.0901, R_f= 0.54 (EA/Hex, 1:5).

Compound 11: $^1H(CDCl_3)$(400 MHz): δ 9.65 (bs, 1H), 8.0 (ddd, *J* = 7.70, 1.50, 0.50 Hz, 1H), 7.56 (d, *J* = 8.40 Hz, 2H), 7.40 (m, 2H), 7.30 (d, *J* = 8.60 Hz, 2H), 6.86 (m, 1H), 3.92 (s, 3H); $^{13}C(CDCl_3)$(300 MHz): δ 168.71, 145.83, 144.31, 134.05, 131.74, 126.62, 126.56, 126.51, 119.75, 118.77, 114.98, 113.51, 51.90; HRMS (FAB) calcd for $C_{15}H_{12}F_3NO_2$ $(MH)^+$ 296.0898, found 296.0901, R_f= 0.52 (EA/Hex, 1:5).

Compound 12: $^1H(CDCl_3)$(400 MHz): δ 7.69 (dd, *J* = 7.30, 2.10 Hz, 2H), 7.56 (dd, *J* = 7.83, 1.35 Hz, 2H), 7.24 (m, 2H), 3.84 (s, 3H); $^{13}C(CDCl_3)$(300 MHz): δ 166.51, 134.23, 132.49, 131.97, 131.22, 127.06, 121.54, 126.51, 52.40; HRMS (FAB) calcd for $C_{15}H_{12}F_3NO_2$ Na $(MNa)^+$ 308.0899, found 308.0906, R_f= 0.26 (Hex/EA, 9:1).

Compound 13: $^1H(CDCl_3)$(400 MHz): δ 9.48 (bs, 1H), 7.89 (d, *J* = 8.08 Hz, 1H), 7.84 (s, 1H), 7.65 (dd, *J* = 5.40, 1.64 Hz, 1H), 7.31 (m, 3H), 7.17 (d, *J* = 7.56 Hz, 1H), 6.69 (m, 1H), 3.83 (s, 3H), 3.82 (s, 3H); $^{13}C(CDCl_3)$(300 MHz): δ 168.67, 166.67, 146.97, 140.98, 134.08, 133.93, 131.21, 129.26, 126.08, 124.13, 122.54, 117.65, 113.89, 112.27, 52.05, 51.72; LRMS (ESI) *m/e* 286 $(MH)^+$, 308 $(MNa)^+$, R_f= 0.31 (EA/Hex, 1:5).

Compound 14: $^1H(CDCl_3)$ (400 MHz): δ 9.70 (bs, 1H), 7.99 (m, 3H), 7.46 (d, *J* = 8.60 Hz, 1H), 7.40 (m, 1H), 7.23 (d, *J* = 8.60 Hz, 2H), 6.86 (m, 1H), 3.91 (s, 3H), δ 3.89 (s, 3H), $^{13}C(CDCl_3)$(300 MHz): δ 168.80, 166.90, 145.49, 145.28, 133.93, 131.62, 131.10, 123.25, 118.94, 118.53, 115.52, 113.8, 51.88, 51.75; HRMS (FAB) calcd for $C_{16}H_{15}NO_4$ $(MH)^+$ 286.1076, found 286.1089, R_f= 0.5 (EA/Hex, 1:5).

General Procedure for Alkylation to Prepare Compounds 15-17: To a flame dried flask with a stir bar, methyl anthranilate (1 eq.) and sodium carbonate (1.1 eq.) were added. The appropriate substituted benzyl halide (1.1 eq.) and NMP (3 mL) were added. The mixture was then heated at 90°C for 4-12 h. The reaction mixture was transferred to a separatory funnel using 1:1 mixture of water and ethyl acetate. The product was extracted into the organic phase using ethyl acetate (3 x 20 mL). The combined organic phases were washed with water (2 x 20 mL) to remove NMP. The organic phase was then dried with Na_2SO_4 (anhydrous), filtered and concentrated under vacuum to obtain the crude product which was purified by flash chromatography (ethyl acetate/hexanes gradient) to obtain a 70-75% yield. The characterization data for this series is summarized below:

Compound 15: $^1H(CDCl_3)$(400 MHz): δ 7.98 (dd, *J* = 6.48, 1.08 Hz, 1H), 7.91 (dd, *J* = 6.48, 1.60 Hz, 1H), 7.46 (m, 2H), 7.30 (ddd, *J* = 6.48, 1.32, 0.84 Hz, 1H), 7.25 (m, 1H), 6.55 (m, 2H), 4.85 (s, 2H), 3.92 (s, 3H), 3.87 (s, 3H); $^{13}C(CDCl_3)$(300 MHz): δ 169.07, 167.75, 150.92, 141.01, 134.56, 132.48, 131.63, 131.09, 128.07, 126.90, 114.81, 111.64, 52.11, 51.52, 45.41; LRMS (ESI) for $C_{17}H_{17}NO_4$, *m/e* 300 $(MH)^+$, 322 $(MNa)^+$, R_f= 0.45 (EA/Hex, 1:5).

Compound 16: ^{1}H($CDCl_3$)(400 MHz): δ 8.23 (bs, 1H), 8.03 (s, 1H), 7.93 (m, 2H), 7.55 (d, *J* = 7.30 Hz, 2H), 7.40 (dd, *J* = 7.56, 7.56 Hz, 1H), 7.27 (ddd, *J* = 7.80, 7.68, 1.36 Hz, 1H), 6.59 (2H, m), 4.49 (d, *J* = 5.40 Hz, 2H), 3.90 (s, 3H), 3.86 (s, 3H); ^{13}C($CDCl_3$)(300 MHz): δ 168.38, 166.19, 150.18, 139.05, 134.04, 131.12, 130.93, 129.98, 128.21, 127.85, 127.69, 114.57, 111.03, 109.78, 51.43, 50.88, 45.98; HRMS (FAB) calcd for $C_{17}H_{17}NO_4$ $(MH)^+$ 300.1236, found 300.1246, R_f = 0.54 (EA/Hex, 1:5).

Compound 17: ^{1}H($CDCl_3$)(400 MHz): δ 8.26 (bs, 1H), 8.00 (d, *J* = 8.08 Hz, 2H), 7.93 (dd, *J* = 8.10, 1.60 Hz, 1H), 7.41 (d, *J* = 8.10 Hz, 2H), 7.26 (m, 1H), 6.6 (t, *J* = 7.00 Hz, 1H), 6.52 (d, *J* = 8.36 Hz, 1H), 4.51 (d, *J* = 5.70 Hz, 2H), 3.90 (s, 3H), 3.87 (s, 3H); ^{13}C($CDCl_3$)(300 MHz): δ 168.25, 166.00, 149.89, 143.67, 133.82, 130.90, 129.19, 128.24, 126.03, 114.41, 110.82, 109.58, 51.21, 50.75, 45.83; LRMS (ESI) *m/e* 300 $(MH)^+$, 298 $(M\text{-}H)^-$, R_f = 0.41 (EA/Hex, 1:5).

General Procedure for Hydrolysis to Obtain 2-9 from 10-17 Respectively: The appropriate monomethyl ester or dimethyl ester was placed in a round bottom flask and dissolved in THF : Methanol : Water (3:1:1, 0.074 M ester). LiOH (4 eq. for each methyl ester) was added and the resultant solution was stirred at room temperature, monitoring the reaction by TLC. After allowing the reaction to proceed overnight, the mixture was acidified to pH 2 (1N HCl) and the product was extracted into the organic phase using ethyl acetate (3 x 25 mL). The organic layer was dried using Na_2SO_4 (anhydrous), filtered, and the solvent removed under vacuum. The crude product was purified either by reverse phase HPLC (C18 column, using a 20-80% gradient of solvent B over 20 minutes, 1mL/min flow rate (solvent A = 94.5% distilled water, 5 % CH_3CN, 0.5% TFA; Solvent B = 94.5% CH_3CN, 5% water, 0.5 % TFA) or by recrystallization using ethyl acetate, methanol and hexanes) to obtain a 52-85% yield of the desired product. The characterization of these compounds is summarized below:

Compound 2: ^{1}H(CD_3OD)(400 MHz): δ 8.00 (dd, *J* = 7.57, 1.88 Hz, 1H), 7.68 (d, *J* = 8.10 Hz, 1H), 7.57 (m, 2H), 7.35 (dd, *J* = 7.56, 1.60 Hz, 1H), 7.21 (t, *J* = 7.40 Hz, 1H), 7.14 (d, *J* = 8.80 Hz, 1H), 6.82 (dd, *J* = 7.10, 1.08 Hz, 1H); HRMS (FAB) calcd for $C_{14}H_{10}F_3NO_2$ $(MH)^+$ 282.0742, found 282.0750, R_f = 0.32 (EA/Hex, 1:5).

Compound 3: ^{1}H(CD_3OD)(400 MHz): δ 8.02 (d, *J* = 8.00 Hz, 1H), 7.57 (d, *J* = 8.64 Hz, 2H), 7.40 (m, 2H), 7.34 (d, *J* = 8.40 Hz, 2H), 6.88 (m, 1H); HRMS (FAB) calcd for $C_{14}H_{10}F_3NO_2$ $(MH)^+$ 282.0742, found 282.0749, R_f = 0.25 (EA/Hex, 1:5).

Compound 4: ^{1}H(CD_3OD)(600 MHz): δ 7.78 (dd, *J* = 7.46, 2.10 Hz, 2H), 7.67 (dd, *J* = 8.11, 0.90 Hz, 2H), 7.41 (td, *J* = 6.04, 1.32 Hz, 2H), 7.37 (td, *J* = 5.70, 1.70 Hz, 2H), HRMS (FAB) calcd for $C_{14}H_{10}F_3NO_2$ $(MH)^+$ 282.0742, found 282.0749, R_f = 0.63 (Hex/EA/TFA 3/2/0.1%).

Compound 5: ^{1}H(CD_3OD)(400 MHz): δ 8.00 (dd, *J* = 8.10, 1.60 Hz, 1H), 7.87 (s, 1H), 7.69 (m, 1H), 7.44 (m, 2H), 7.38 (m, 1H), 7.28 (d, *J* = 8.60 Hz, 1H), 6.79 (t, *J* = 7.80 Hz, 1H), HRMS (FAB) calcd for $C_{14}H_{10}NO_4Na$ $(MNa)^+$ 280.0586, found 280.0581, R_f = 0.33 (100% EA).

Compound 6: ^{1}H(CD_3OD)(400 MHz): δ 8.02 (dd, *J* = 6.48, 1.36 Hz, 1H), 7.96 (dd, *J* = 8.92, 2.16 Hz, 2H), 7.45 (m, 2H), 7.26 (dd, *J* = 8.92, 2.16 Hz, 2H), 6.88 (ddd, *J* = 7.00, 1.36, 1.08 Hz, 1H), HRMS (FAB) calcd for $C_{14}H_{11}NO_4$ $(MH)^+$ 258.0766, found 258.0769. R_f = 0.62 (100% EA).

Compound 7: ^{1}H(CD_3OD)(400 MHz): δ 8.00 (dd, *J* = 6.73, 1.09 Hz, 1H), 7.91 (dd, *J* = 6.34, 1.65 Hz, 1H), 7.46 (m, 2H), 7.35 (m, 1H), 7.30 (m, 1H), 6.72 (dd, *J* = 7.45, 0.88 Hz, 1H), 6.67 (m, 1H), 4.8 (s, 2H), LRMS (ESI) *m/e* 272 $(MH)^+$, 270 $(M\text{-}H)^-$, 294 $(MNa)^+$, R_f = 0.6 (100% EA).

Compound 8: ^{1}H(CD_3OD)(400 MHz): δ 7.99 (s, 1H), 7.87 (m, 2H), 7.50 (d, *J* = 7.80 Hz, 1H), 7.37 (t, *J* = 7.56 Hz, 1H), 7.22 (m, 1H), 6.62 (d, *J* = 8.10 Hz, 1H), 6.54 (t, *J* = 7.10 Hz, 1H), 4.49 (s, 2H), HRMS (FAB) calcd for $C_{15}H_{13}NO_4$ $(MH)^+$ 272.0923, found 272.0916, R_f = 0.56 (100% EA).

Compound 9: ^{1}H(CD_3OD)(400 MHz): δ 7.97 (d, *J* = 8.40 Hz, 2H), 7.90 (d, *J* = 7.84 Hz, 1H), 7.45 (d, *J* = 7.80 Hz, 2H), 7.25 (m, 1H), 6.58 (m, 2H), 4.55 (s, 2H), LRMS (ESI) *m/e* 272 $(MH)^+$, 270 $(M\text{-}H)^-$, R_f = 0.56 (100% EA).