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Bioorganic & Medicinal Chemistry Letters 17 (2007) 756–760
Bioorganic & Medicinal Chemistry Letters

Design and synthesis of phenethyl benzo[1,4]oxazine-3-ones as potent inhibitors of PI3Kinaseγ

Thomas B. Lanni, Jr.,[a,*] Keri L. Greene,[a] Christine N. Kolz,[a] Kimberly S. Para,[a] Melean Visnick,[a] James L. Mobley,[b] David T. Dudley,[b] Theodore J. Baginski[b] and Marya B. Liimatta[b]

[a]*Chemistry Department, Pfizer Global Research and Development, 2800 Plymouth Road, Ann Arbor, MI 48105, USA*
[b]*Pharmacology Department, Pfizer Global Research and Development, 2800 Plymouth Road, Ann Arbor, MI 48105, USA*

Received 24 August 2006; revised 25 October 2006; accepted 25 October 2006
Available online 28 October 2006

Abstract—The Type 1 PI3Kinases comprise a family of enzymes, which primarily phosphorylate PIP2 to give the second messenger PIP3, a key player in many intracellular signaling processes [*Science*, **2002**, *296*, 1655; *Trends Pharmacol. Sci.* **2003**, *24*, 366]. Of the four type 1 PI3Ks, the γ-isoform, which is expressed almost exclusively in leukocytes [*Curr. Biol.*, **1997**, *7*, R470], is of particular interest with respect to its role in inflammatory diseases such as rheumatoid arthritis (RA) and chronic obstructive pulmonary disease (COPD) [*Mol. Med. Today*, **2000**, *6*, 347]. Investigation of a series of 4,6-disubstituted-4*H*-benzo[1,4]oxazin-3-ones has led to the identification of single-digit nanomolar inhibitors of PI3Kγ, several of which had good cell based activity and were shown to be active in vivo in an aspectic peritonitis model of inflammatory cell migration.
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The type 1 phosphoinositide 3-kinases (PI3Ks) are heterodimeric enzymes which reside downstream of multiple cell surface receptors.[3] Upon receptor ligation these enzymes are activated through the association of their specific adaptor proteins with phosphorylated intracellular domains of tyrosine kinase and growth factor receptors or β,γ-subunits released from G-protein coupled receptors.[6] Once activated they phosphorylate phosphatidylinositol 4,5 bisphosphate (PIP2) in the 3 position to give the potent second messenger phosphatidylinositol 3,4,5 triphosphate (PIP3).[3,14] This results in the engagement of a multitude of intracellular signal transduction pathways culminating in effects included cell migration, protein synthesis, actin polymerization, and cell proliferation.[10,14]

There are four different isoforms of the type 1 PI3Kinase, and increasing evidence that these different isoforms have specialized functions within specific cells.[11,12] For instance, while the α and β isoforms are ubiquitously expressed, the γ and δ isoforms show much restricted expression patterns. PI3Kγ is primarily restricted to granulocytes, monocytes, and macrophages while the δ isoform is mainly found in T and B cells.[2,9] The migration and accumulation of these cells result in swelling of the joints, which in turn suggests a viable target for inflammation.[6,9]

PI3Kγ knockout mice[8] were injected ip with 10^7 CFU of *Escherichia coli* and demonstrated significantly impaired recruitment of both neutrophils and macrophages to the peritoneum.[1] This model of *E. coli*-induced cell influx was therefore used to test the in vivo efficacy of multiple PI3Kinase inhibitors when administered by oral gavage. Wortmannin[4] (3 mg/kg) potently inhibited cell influx by 79.06% (Fig. 1). LY294002[13] (30 mg/kg) inhibited only 26.86%. Compound **I** (100 mg/kg) inhibited cell influx by 53.84% (Fig. 1). These results strongly indicate that a specific inhibitor of PI3Kγ will have therapeutic value for the treatment of chronic inflammatory diseases such as rheumatoid arthritis (RA) and chronic obstructive pulmonary disease (COPD).[11,12]

To identify chemical matter as substrate for a PI3Kγ inhibitor program, a high-throughput screen (HTS)

Keywords: PI3Kinase; Phosphoinositide 3-kinase; Rheumatoid arthritis; Peritonitis; Inflammatory; Granulocytes; Monocytes; Intracellular; PI3Kγ; Inhibitor.
* Corresponding author. Tel.: +1 734 622 2241; fax: +1 734 622 3107; e-mail: Thomas.Lanni@Pfizer.com

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doi:10.1016/j.bmcl.2006.10.080

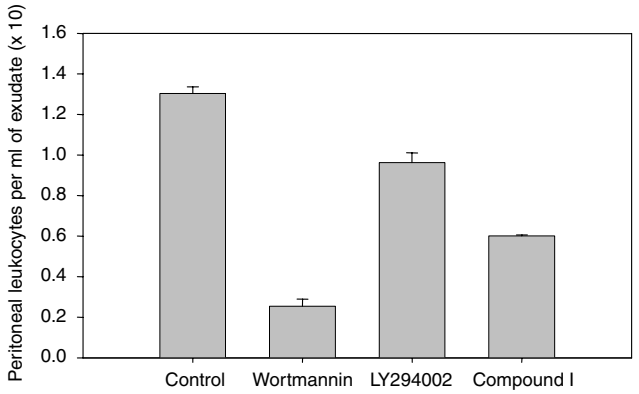


Figure 1. Effects of PI3Kinase inhibitors on a model of *E. coli*-induced peritoneal inflammation. Wortmannin (3 mg/kg), LY294002 (30 mg/kg), and Compound **I** were administered to mice by oral gavage. One hour later, the mice were injected ip with *E. coli* bacteria. Twenty-four hours later, peritoneal lavage was performed, and infiltrating cells counted. Data are averages + SEM for multiple experiments using five mice per group: control ($n = 76$), wortmannin ($n = 9$), LY294002 ($n = 2$) and Compound **I** ($n = 3$).

was conducted on the Pfizer corporate compound collection. A simple rhodanine, Compound **I** (Fig. 2), was identified as a potent inhibitor of PI3Kγ and served as the starting point for our program. We describe herein, our chemistry efforts around increasing the enzymatic and cell based potency in this series along with in vivo results in a cell migration model.

PI3Kγ activity was assessed by incubation of baculoviral co-expressed regulatory and catalytic subunits (p101 and p110) with lipid micelles prepared from phosphatidylethanolamine containing phosphatidylinositol 4,5 bisphosphate (PIP_2) at a molar ratio of 8:1 (approximately 25 μM final PIP_2). Recombinant G-protein βγ-subunits were used to activate the enzyme complex, and the reaction mix contained 20 μM ^{32}P-ATP. Reactions were terminated by addition of cold 75 mM H_3PO_4 and the mixture filtered through glass fiber filter plates and washed with H_3PO_4. ^{32}P-PIP_3 on the filters was then monitored by scintillation counting.[7] Compounds of interest were included in the assay and ability to inhibit formation of ^{32}P-PIP_3 expressed as percentage uninhibited value. IC_{50} values were determined by incubation of the compound at various concentrations (typically a 10 point half-log dose distribution) and curve fitting by standard means (GraphPad Prism). While not routinely evaluated, representative compounds from this class were shown to be competitive with ATP in that relative IC_{50} values increased with increasing ATP concentrations.

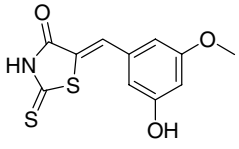
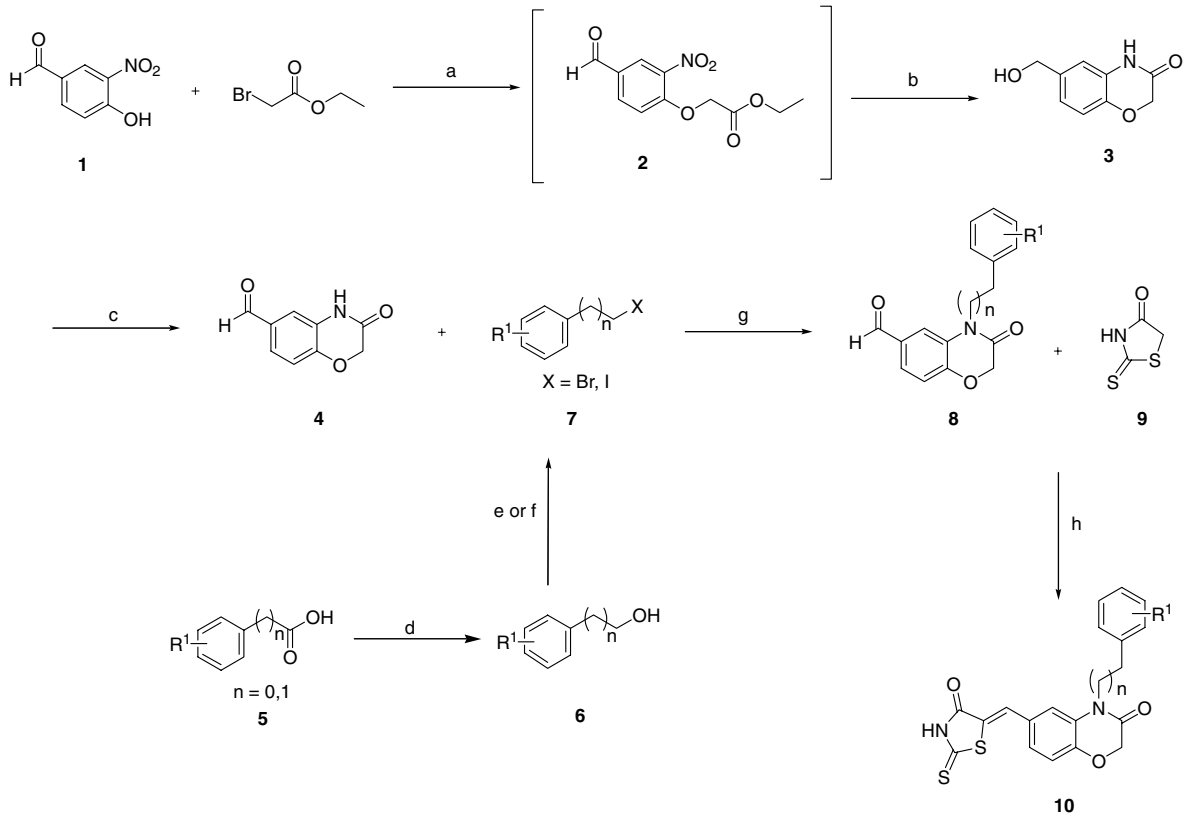


Figure 2.

For determination of compound activity in a cellular setting, the ability to influence stimulated superoxide production in human neutrophils was employed. Activation of this response by the chemotactic peptide *n*-formyl MetLeuPhe has been demonstrated to be dependent on PI3Kγ.[14] Briefly, compounds are incubated with human neutrophils (purified from fresh peripheral blood) in a salt solution containing 120 μM cytochrome *C*. Cells are stimulated by addition of *n*-formyl MetLeuPhe and production of superoxide anion O_2^- is monitored by following reduction of cytochrome *C* at A_{550} nm. Preliminary experiments demonstrated that O_2^- production by this procedure (change in A_{550} nm) was abolished by inclusion of superoxide dismutase. IC_{50} values were determined in a manner similar to the enzyme procedure.

A mouse peritonitis model was employed as a rapid screen to test the efficacy of potential PI3Kinase inhibitors in vivo. Groups of five mice were dosed by oral gavage with the control oral vehicle (0.5% hydroxypropylmethylcellulose/0.25% Tween 80), the positive control PI3Kinase inhibitors wortmannin at 3 mg/kg, or LY294002 at 30 mg/kg, or test compounds at 100 mg/kg. One hour later, the mice were injected ip with *E. coli* bacteria (250 μl at 7.5×10^7 CFU/mL). The bacteria induce a robust, PI3Kinase dependent leukocyte migration into the peritoneum that peaks 24–48 h after injection. Twenty-four hours after *E. coli* administration, the mice were anesthetized and injected ip with 5 mL HBSS/2% FCS, the contents of the peritoneum massaged, and the fluid collected. The cells from this lavage were counted and subjected to differential stain (Data are averages + SEM for multiple experiments using five mice per group). Typical recovery is $1–2 \times 10^6$ cells/mL of lavage fluid, comprised of roughly equivalent numbers of neutrophils and macrophages.

The preparation (Scheme 1) of the benzoxazine–aldehyde template (**4**) begins with commercially available 4-hydroxy-3-nitro-benzaldehyde (**1**) by alkylation with ethyl bromoacetate to yield (4-formyl-2-nitro-phenoxy)-acetic acid ethyl ester (**2**).[15] Subsequent hydrogenation over RaNi gave 6-hydroxymethyl-4*H*-benzo[1,4]oxazin-3-one (**3**)[16] which was re-oxidized with PDC to yield template (**4**).[17] Initial investigation of structure–activity relationships (SAR) involved N-substitution of template (**4**) with a variety of alkyl and benzyl groups followed by conversion to the rhodanine analog. A number of benzyl-substituted analogs were synthesized by either using the commercially available benzyl bromides (**7**, $n = 0$) or reducing benzoic acids (**5**, $n = 0$) to the corresponding alcohols with BH_3–SMe_2. The alcohols were then converted to the benzyl bromide (**7**) using carbon tetrabromide and triphenylphospine, and used in the alkylation of the benzoxazine template (**4**) in respectable yields. Knoevenagel condensation of rhodanine (**9**) with aldehydes provided the target compounds (**10a**–**i**).[5] Binding data showed that *N*-benzyl substitution improved the potency of PI3Kγ binding versus the unalkylated benzoxazine product (PI3Kγ $IC_{50} = 78.0$ nM). The unsubstituted *N*-benzyl analog **10a** was potent at PI3Kγ ($IC_{50} = 23.0$ nM,



Scheme 1. Reagents and conditions: (a) NaH, THF, reflux; (b) RaNi, MeOH, 96%; (c) PDC, CH_2Cl_2, 81%; (d) $Me_2S{:}BH_3$, THF, 76%; (e) CBr_4, PPh_3, CH_2Cl_2; or (f) I_2, PPh_3, Imidazole, 75–90%; (g) K_2CO_3, PTC, CH_3CN, 26–70%; (h) $C_2H_8N_2{\cdot}2(CH_3CO_2H)$, MeOH, 52–80%.

Table 1.

Compound	*n*	R^1	$IC_{50}\gamma$ (nM)	SUOX IC_{50} (nM)	Mouse peritonitis % inhibition
Wortmannin	—	—	19.0	19.00	
LY294002	—	—	1720	1720	
Compound **I**	—	—	77.7 (73.2–82.5, *n* = 4)	1320 (245–7110, *n* = 4)	
10a	0	H	23.0 (13.2–39.9, *n* = 2)	30,000	
10b	0	4-Methyl	29.5 (15.4–56.2, *n* = 2)	30,000	
10c	0	4-*t*-Butyl	2.42 (0.390–15.1, *n* = 2)	19,900	
10d	0	4-Chloro	7.16 (0.939–54.6, *n* = 2)	670	
10e	0	4-Cyano	36.1 (26.8–48.7, *n* = 2)	1570	
10f	0	4-Trifluoromethyl	27.5 (17.6–42.8, *n* = 4)	20,000	
10g	0	3-Methyl	43.7 (10.3–186, *n* = 2)	30,000	
10h	0	3,4-Dimethyl	2.59 (0.179–37.6, *n* = 2)	19,900	
10i	1	H	24.5 (18.9–31.7, *n* = 2)	10,320	
10j	1	3,5-Dimethyl	2.65 (1.43–4.89, *n* = 2)	1620 (90.9–28,800, *n* = 2)	
10k	1	3,5-Dimethoxy	2.79 (1.13–6.93, *n* = 2)	840	
10l	1	4-Chloro	4.76 (0.021–1090, *n* = 2)	363 (211–608, *n* = 3)	25.63
10m	1	4-Methoxy	6.57 (0.337–128, *n* = 2)	372 (233–595, *n* = 3)	43.69
10n	1	4-Methyl	8.83 (0.011–7370, *n* = 2)	1250 (1.64–74,000, *n* = 2)	48.15
10o	1	4-Bromo	2.34 (1.41–3.90, *n* = 4)	565	30.78
10p	1	3-Chloro	4.27 (0.001–35,500, *n* = 2)	424 (25.3–7110, *n* = 2)	41.27
10q	1	3,4-Dichloro	4.36 (0.184–104, *n* = 2)	285 (59.0–1360, *n* = 2)	35.25
10r	1	4-*t*-Butyl	33.2 (0.028–39,700, *n* = 2)	2140	
10s	1	4-Trifluoromethyl	1.92 (0.642–5.75, *n* = 2)	370 (0.379–27,600, *n* = 2)	36.94
10t	1	2,4-Dichloro	4.28 (0.047–388, *n* = 2)	610	
10u	1	3-Methyl	4.96 (1.07–23.0, *n* = 2)	1030	
10v	1	3-Methoxy	2.66 (1.35–5.27, *n* = 3)	375 (0.837–13,500, *n* = 2)	−13.97
10w	1	3,4-Difluoro	4.84 (0.011–2090, *n* = 2)	520	

Table 1), however, it was not active in the superoxide inhibition assay (SUOX IC_{50} = 25,000 nM). Further substitution of the benzyl ring (Table 1) produced potent analogs at PI3Kγ with the most potent analogs having of 4- and 3,4-substitution in the benzyl series. Most of the analogs synthesized in the benzyl series showed no

activity in the superoxide inhibition assay except for **10d** (R = 4-Cl, SUOX IC_{50} = 670 nM). To further explore the SAR, a series of phenethyl analogs were synthesized in a similar manner to the benzoic acids. Commercially available phenyl-acetic acids (**5**) were reduced to the corresponding alcohols (**6**, $n = 1$) using BH_3–SMe_2 and the resultant alcohols were then converted to the primary phenethyl iodides (**7j**–**w**).[9,18] The primary iodides were preferred over the primary bromides in the phase transfer alkylation of the template (**4**), affording modest yields of the N-substituted benzoxazine–aldehyde templates (**8j**–**w**).[19] Knoevenagel condensation of rhodanine (**9**) with aldehydes (**8j**–**w**) provided the target compounds (**10j**–**w**).[5,20] Table 1 summarizes the SAR around the phenethyl analogs. Various substitution of the phenyl ring gave potent analogs for PI3Kγ with limited SAR. The only analog that exhibited a loss of activity was the 4-*t*-butyl analog, **10r** ($IC_{50}\gamma$ = 39.0 nM). *Para* substitution of the phenyl ring gave potent analogs in this series, especially **10o** (R = 4-Br, $IC_{50}\gamma$ = 2.00 nM) and **10s** (R = 4-CF_3, $IC_{50}\gamma$ = 2.00 nM). 2,4- and 3,4-substitution of the phenyl ring also gave potent PI3Kγ inhibition, for instance **10q** (R = 3,4-Cl, $IC_{50}\gamma$ = 4.00 nM) and **10t** (R = 2,4-Cl, $IC_{50}\gamma$ = 5.00 nM). Subsequent testing in cellular assays of superoxide inhibition showed that *para* substitution afforded good potency. Compound **10q** (R = 3,4-Cl, SUOX IC_{50} = 285 nM) was the most potent compound in cell based assays. A few analogs were tested in the mouse peritonitis model with three of them (**10m**, **10n**, and **10p**) showing promising activity, while others exhibited insignificant inhibition. This series will be further evaluated by extending the alkyl linker and with various substitution of the phenyl ring.

In conclusion, a series of benzoxazine–rhodanines have been developed as potent inhibitors of PI3Kγ in enzymatic and cell based assays. The most promising compounds were subsequently profiled in vivo in an aseptic peritonitis model of inflammatory cell migration and showed significant inhibition of neutrophil and monocyte migration to the infected area.

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15. Preparation of intermediate **1**: To a solution of 4-hydroxy-3-nitrobenzaldehyde (10.0 g, 59.8 mmol) in tetrahydrofuran (600 mL) and DMF (240 mL) were added dry sodium hydride (1.58 g, 65.82 mmol) and ethyl bromoacetate (7.30 mL, 65.82 mmol). The reaction mixture was refluxed for 24 h. The reaction was then concentrated and diluted with ethyl acetate (500 mL) and acidified to pH 2 with 1 N HCl. The organic layer was then washed with saturated sodium bicarbonate (2× 200 mL), sodium chloride (2× 200 mL), dried over magnesium sulfate, filtered, and concentrated under reduced pressure to yield a dark red oil. The oil was used in the next step without purification. ^{1}H NMR (d_6-DMSO) δ 1.18 (t, 3H), 4.15 (q, 2H), 5.14 (s, 2H), 7.49 (d, 1H), 8.11 (dd, 1H), 8.41 (s, 1H), 9.93 (s, 1H). MS: M^{-1} = 252 Da.
16. Preparation of intermediate **2**: To a solution of intermediate 1 (6.40 g, 1.67 mmol) in methanol (100 mL) was added Raney nickel (3.0 g). The reaction was then pressurized under an atmosphere of hydrogen to 48 psi for 23 h. The solution was then filtered through a pad of Celite. The Celite cake was washed with methanol (200 mL) and concentrated under reduced pressure to yield a brown solid. ^{1}H NMR (d_6-DMSO) δ 4.36 (s, 2H), 4.50 (s, 2H), 5.12 (s, 1H), 6.62–6.86 (m, 3H). MS: M^{-1} = 178 Da.
17. Preparation of intermediate **3**: To a solution of intermediate 2 (2.0 g, 11.2 mmol) in dichloromethane (80 mL) at room temperature was added pyridinium dichromate (6.31 g, 16.8 mmol). The reaction mixture was stirred for 24 h. The reaction mixture was then filtered through a pad of Celite. The Celite cake was washed with ethyl ether (100 mL) and ethyl acetate (100 mL). The solution was then concentrated under reduced pressure to yield a dark red solid. ^{1}H NMR (d_6- DMSO) δ 4.67 (s, 2H), 7.09 (d, 1H), 7.33 (s, 1H), 7.49 (d, 1H), 9.79 (s, 1H). MS: M^{-1} = 175 Da.
18. Preparation of intermediate **7**: To a room temperature solution of PPh_3 (5.78 g, 22.0 mmol) and I_2 (5.59 g, 22.0 mmol) in dry CH_2Cl_2 (100 mL) after 10 min of stirring was added imidazole (2.50 g, 36.7). Following another 10 min of stirring 2-*m*-tolyl-ethanol (2.00 g, 14.7 mmol) was added and the reaction was monitored by TLC. The reaction was quenched with aqueous sodium metabisulfite and then extracted with Et_2O (3× 30 mL). The organics were dried over $MgSO_4$, filtered, and concentrated down under reduced pressure. The product was chromatographed with EtOAc/hexane (1:3) to yield 2.70 g of product. 1H NMR (d_6-DMSO) δ 2.20 (s, 3H), 3.00–3.15 (m, 2H), 3.40–3.45 (m, 2H), 6.95–7.10 (m, 3H), 7.15–7.20 (m, 1H) ppm.
19. Preparation of intermediate **8**: To a suspension of benzoxazine (0.500 g, 2.82 mmol) and K_2CO_3 (0.971 g,

7.05 mmol) in dry CH_3CN (100 mL) was added 1-(2-iodo-ethyl)-3-methyl-benzene (1.39 g, 5.64 mmol). Benzyltriethylammonium chloride (0.321 g, 1.41 mmol) was then added and the suspension was heated to reflux overnight with good stirring. The heat was turned off and the suspension was allowed to cool to room temperature. EtOAc was added and the reaction was extracted with 1 N HCl (3× 30 mL) and washed with brine (3× 30 mL). The organic layers were dried over $MgSO_4$, filtered, and concentrated down under reduced pressure. The product was chromatographed with EtOAc/hexane (1:3) to yield 0.520 g of product. 1H NMR (d_6-DMSO) δ 2.20 (s, 3H), 2.75–2.85 (m, 2H), 4.15–4.20 (m, 2H), 4.70 (s, 2H), 6.90–7.00 (m, 3H), 7.10–7.18 (m, 2H), 7.55 (d, 1H), 7.62 (s, 1H), 9.85 (s, 1H) ppm. MS: M^{+1} = 296 Da.

20. Preparation of **10g**: To a room temperature solution of 3-oxo-4-(2-*m*-tolyl-ethyl)-3,4-dihydro-2*H*-benzo[1,4]-oxazine-6-carbaldehyde (0.300 g, 1.02 mmol) in MeOH (20 mL) was added 2-thioxo-thiazolidin-4-one (0.135 g, 1.02 mmol) followed by ethylene diamine diacetate (0.183 g, 1.02 mmol). The suspension was allowed to stir overnight at room temperature. The suspension was filtered, then washed with MeOH (30 mL) and Et_2O (30 mL). The yellow product was placed in a drying oven under reduced pressure overnight to yield 0.221 g of product. 1H NMR (d_6-DMSO) δ 2.20 (s, 1H) 2.90 (t, 3H), 4.15 (t, 3H), 4.70 (s, 2H), 7.005 (d, 1H), 7.15 (d, 1H), 7.18–7.20 (m, 4H), 7.40 (s, 1H), 7.70 (s, 1H) ppm. Microanalysis: $C_{21}H_{18}N_2O_3S_2$; calcd: C, 61.44; H, 4.42; N, 6.82; S, 15.62; found: C, 61.27; H, 4.30; N, 6.70; S, 15.75. MS: M^{+1} = 411 Da.