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Discovery of potent and selective rhodanine type IKKβ inhibitors by hit-to-lead strategy

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ABSTRACT

Regulation of NF-κB activation through the inhibition of IKKβ has been identified as a promising target for the treatment of inflammatory and autoimmune disease such as rheumatoid arthritis. In order to develop novel IKKβ inhibitors, we performed high throughput screening toward around 8000 library compounds, and identified a hit compound containing rhodanine moiety. We modified the structure of hit compound to obtain potent and selective IKKβ inhibitors. Throughout hit-to-lead studies, we have discovered optimized compounds which possess blocking effect toward NF-κB activation and TNFα production in cell as well as inhibition activity against IKKβ. Among them, compound **3q** showed the potent inhibitory activity against IKKβ, and excellent selectivity over other kinases such as p38α, p38β, JNK1, JNK2, and JNK3 as well as IKKα.

NF-κB is a multifunctional transcription factor and plays central role in the regulation of a variety of genes associated with inflammation, anti-apoptosis, and proliferation.[1] NF-κB exists as an inactive form bound with inhibitory κB (IκB) protein in the cytoplasm. Upon stimulation by proinflammatory cytokine, IκB is phosphorylated, polyubiquitinated, and degraded through the S26 proteasome pathway, thus NF-κB sets free from IκB and translocates to the nucleus where it binds to κB site and activates target genes.[2] Phosphorylation of IκB is catalyzed by IκB kinase (IKK) complex protein which is composed of two catalytic subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit, IKKγ (NEMO).[3] IKKα and IKKβ are both serine–threonine kinase, but IKKα is considered to regulate the duration of NF-κB response with prolonged expression of proinflammatory cytokines observed in IKKα-deficient cells.[4] In this reason, development of selective inhibitors of IKKβ over IKKα is advantageous to anti-inflammatory and autoimmune disease such as rheumatoid arthritis.[5]

As a program of searching compounds for showing an inhibitory activity on IKKβ in our in-house library, high-throughput screening (HTS) is employed for the generation of novel hit structure. In this process, we have identified several hit compounds. Among them, a hit compound (**1**) was selected from druggable and transformative points of view. Selected compound **1** has rhodanine ring as a core structure with diethylaminoalkoxyphenyl moiety in western part and phenylmethylene moiety in eastern part. In the present study, we replaced western and eastern parts of parent compound (**1**) with various substituents to obtain the maximum inhibitory activity against IKKβ. Compound **3q** was found to have more than 100-folds increased inhibitory activity compared to hit compound, and to show excellent selectivity over other kinases such as IKKα, p38α, p38β, JNK1, JNK2, and JNK3. Also NF-κB activation and TNFα production were efficiently blocked by compound **3q** in cell-based assay. All these findings suggest that optimized compound **3q** has high possibility as a potential candidate for treatment of disease associated with NF-κB activation such as rheumatoid arthritis, chronic obstructive pulmonary disease (COPD), and cancer.

Hit compound **1** has two distinct substituents on the rhodanine nucleus, diethylaminopropanoxyphenyl and 4-hydroxyphenylmethylene group (Fig. 1). In our preliminary fragment analysis study of hit compound **1**, we found that deletion of the aminoalkyl group at the western part resulted in a complete loss of activity. It suggests that the aminoalkyl group is essential for keeping inhibitory activity against IKKβ. On the basis of fragment study of **1**, we first tried to investigate the substituent effect of eastern part fixing the western part with diethylaminopropanoxyphenyl group. We

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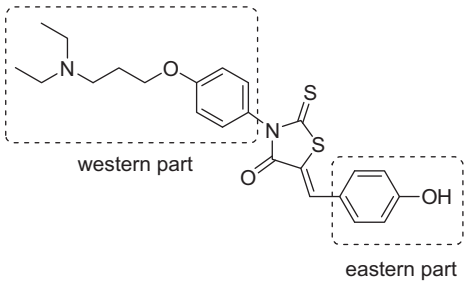


Figure 1. Hit structure and its screening data.

replaced phenylmethylene moiety with other aryl groups involving heteroaromatics for the eastern part, and examined their enzyme inhibitory activities against IKKβ and suppression abilities of NF-κB activation and TNFα production in cell-based assay. The synthesis of this series of compounds was described in Scheme 1. Alkylation of 4-nitrophenol with dibromopropane gave corresponding monoalkyl product, which was reacted with diethylamine in the presence of K_2CO_3 to afford 4-(diethylaminopropanoxy)-1-nitrobenzene (**5**). Reduction of nitro compound **5** by catalytic hydrogenation over Pd/C in methanol provided aniline **6**. Aniline **6** was converted to rhodanine **7** by reaction with bis(carboxymethyl)trithiocarbonate in ethanol and water (4:3),[6] which was condensed with various aryl aldehydes in the presence of sodium acetate in acetic acid to afford compounds **2a–r**.[7]

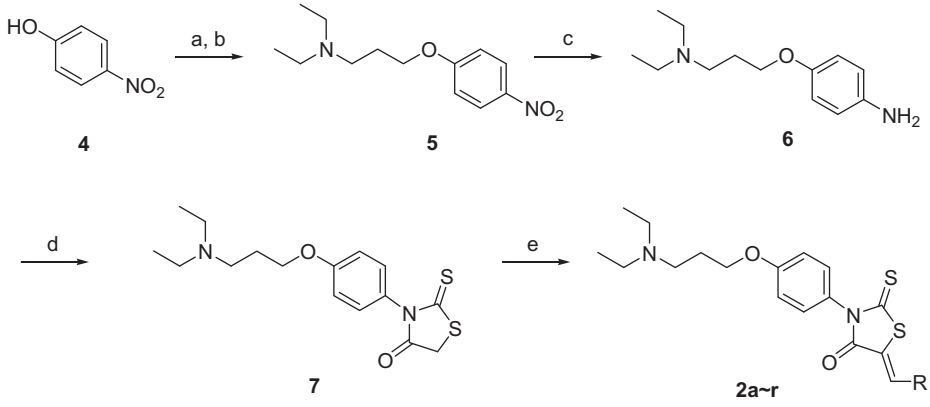
Based on the aromatic nature of size and physicochemical properties, a series of 18 compounds were synthesized and their biological activities were evaluated. Inhibitory enzymatic activity of IKKβ was measured by TR-FRET (time-resolved fluorescence resonance energy transfer) method on IMAP® platform,[8] and NF-κB report gene assay was performed to evaluate inhibitory ability of synthesized compounds toward NF-κB activation.[9] Additionally, inhibition study of TNFα production was carried out with the spleen cell of collagen induced arthritis (CIA) mouse model.[10]

Various aromatic analogues such as simple monocyclic aromatics, heteroaromatics, and bulky bicyclic and phenoxyphenyl aromatics were introduced to the eastern part. Table 1 shows representative enzymatic and subsequent cell-based assay results from the analogues of eastern part. Simple aromatic and heteroaromatic compounds possessing either electron donating or electron withdrawing group did not show any inhibition activity compared to *p*-hydroxyphenyl ring of hit compound. Only **2b** compound which has 3,4-dichloro substituent was improved in activity compared to hit compound, but did not display subsequent activity in cell-based assay. In bulky aromatic analogues, phenoxyphenyl (**2m–2p**) and 5-phenylfuranyl (**2q**, **2r**) were found to be useful replacement for the eastern part. Notably, carboxamido substituted phenoxyphenyl and phenylfuranyl analogues except *o*-carboxamidophenoxyphenyl (**2n**) provided a considerable improvement in enzymatic activities compared to hit compound, and also blocked NF-κB activation and TNFα production in cell-based assay. However, they were still inferior to reference compound[11] in enzymatic and cell-based activities.

In order to obtain the optimized compound, structure activity relationship of the western part was investigated. With the fixed carboxamidophenoxyphenyl and carboxamidophenylfuranyl in eastern part, we next focused our efforts on the structural modification and activity evaluation for a variety of aminoalkoxyphenyl derivatives in western part. We have diversified diethylamino group to other amino group such as pyrrolidine, morpholine, and piperazine, and we also gave variety to carbon chain length ranging from 2 to 5. We made this series of compounds as the same manners with Scheme 1, and the method for preparation of **3a–s** was depicted in Scheme 2.

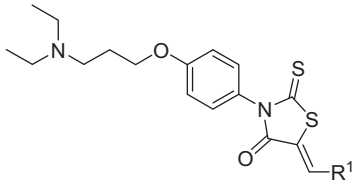
The inhibitory activities of various aminoalkoxyphenyl derivativs in western part of rhodanine ring are summarized in Table 2. The results showed that the IKKβ inhibitory activities were mainly influenced by amino groups and carbon length between nitrogen and oxygen in western part, and the position of carboxamido substituent in eastern part. As the length of carbon chain goes to increase, proportional positive effect was observed in IKKβ inhibitory activity (**3s** > **3r** > **3q**). Especially **3s** was as potent as reference compound. However, **3s** and **3r** which has 4 and 5 carbons as a linker, respectively, did not show inhibitory activity against NF-κB activation and TNFα production in cell-based assay. These results seemed to be caused by poor cell permeability of compounds **3s** and **3r**. In the case of 2 and 3 carbon length linker, compound with 3 carbons linker (**2n**, **2o** and **2p**) was moderately superior to the corresponding compounds with 2 carbons linker (**3a**, **3b** and **3c**) in IKKβ inhibitory activities. These results indicate that optimal carbon length is three carbons apart from oxygen to nitrogen in western part.

As we can see in Table 2, structural modification of amino group has considerably affected the potency of inhibitors. We have replaced diethylamino group with cyclic secondary amino groups such as pyrrolidine, piperidine, morpholine, and 4-methylpiperazine. In analogues of pyrrolidine and piperidine with *p*-$CONH_2$ substituent in eastern part (**3f**, **3i**), their inhibitory activities were equipotent to parent diethylamino compound (**2p**). The most noticeable improvement effect resulted from the introduction of 4-methylpiperazine moiety, which led to around 100-folds in-



Scheme 1. Reagents and conditions: (a) Dibromopropane, K_2CO_3, CH_3CN, 80 °C, 78%; (b) diethylamine, K_2CO_3, CH_3CN, 80 °C, 53%; (c) Pd/C (10%), H_2, MeOH, 98%; (d) bis(carboxymethyl)trithiocarbonate, EtOH/H_2O (4:3), reflux, 52%; (e) aldehydes, NaOAc, AcOH, reflux, 57–88%.

Table 1
IKKβ inhibitory activities of rhodanine derivatives **2a**–**2r**



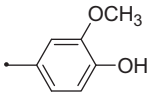
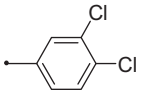
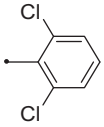
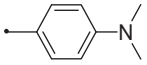
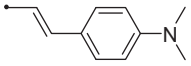
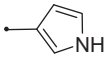
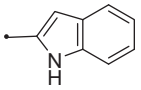
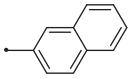
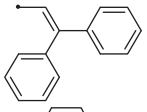
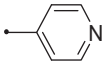
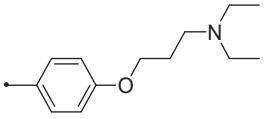
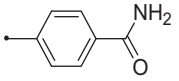
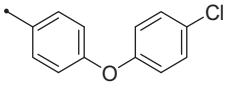
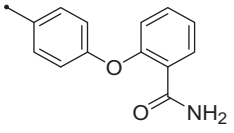
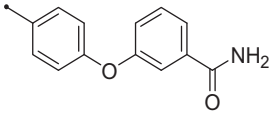
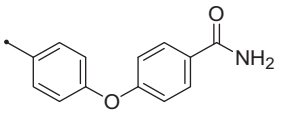
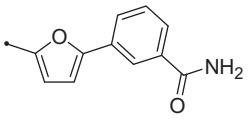
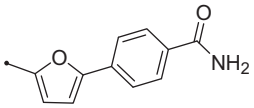
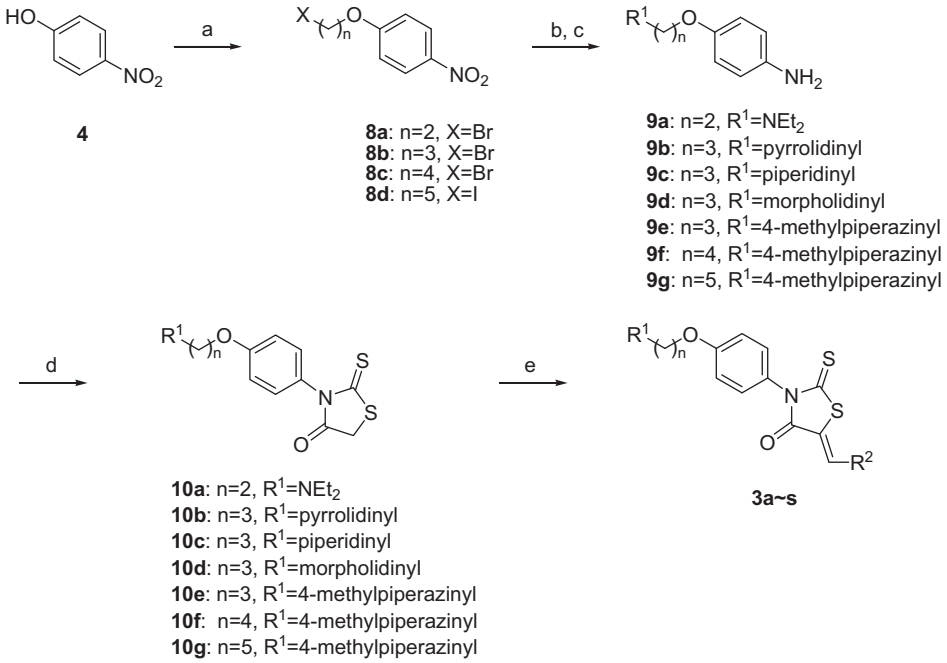
Compd.	R^1	Enzyme assay		Cell-based assay	
		% Inhibition of IKKβ (10 μM)	IKKβ IC_{50} (μM)	% Inhibition of NF-κB (10 μM)	TNFα IC_{50} (μM)
2a		–	–	–	–
2b		61.8	9.3	–	–
2c		3.4	–	–	–
2d		–	–	–	–
2e		28.1	–	–	–
2f		–	–	–	–
2g		–	–	–	–
2h		23.4	–	–	–
2i		52.6	14.1	–	–
2j		3.2	–	–	–
2k		–	–	–	–
2l		15.5	–	–	–
2m		36.5	15.6	71.4	13
2n		4.8	–	–	–
2o		84.9	7.3	39.3	10
2p		95.7	2.6	46.6	8

Table 1 (*continued*)

Compd.	R^1	Enzyme assay		Cell-based assay	
		% Inhibition of IKKβ (10 μM)	IKKβ IC_{50} (μM)	% Inhibition of NF-κB (10 μM)	TNFα IC_{50} (μM)
2q		87.1	5.7	59.1	–
2r		77.2	3.3	57.4	–
Reference compound		88.7	0.17	73.9	1.8



Scheme 2. Reagents and conditions: (a) Dihaloalkane, K_2CO_3, CH_3CN, 80 °C, 64–82%; (b) amines, K_2CO_3, CH_3CN, 80 °C, 48–76%; (c) Pd/C (10%), H_2, MeOH, 90–99%; (d) bis(carboxymethyl)trithiocarbonate, EtOH/H_2O (4:3), reflux, 33–64%; (e) aldehydes, NaOAc, AcOH, reflux, 62–86%.

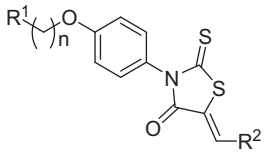
crease in enzymatic inhibitory activity compared to hit compound (**1**). Interestingly, replacement of diethylamino group with morpholine ring resulted in the complete loss of inhibitory activity.

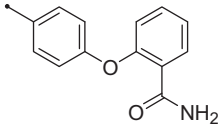
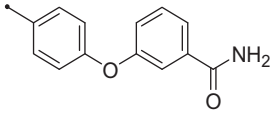
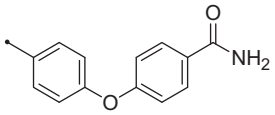
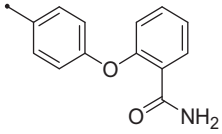
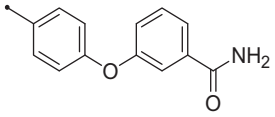
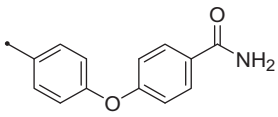
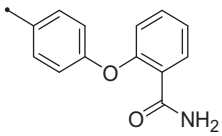
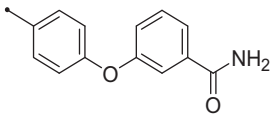
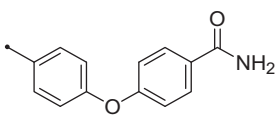
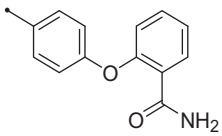
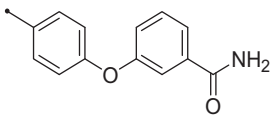
Considering the biological effects on the position of carboxamido substituent in eastern part, there was a declining tendency of inhibitory activities as an order of *para* > *meta* > *ortho* whatever aminoalkoxy substituents were attached in western part. One exception was observed in morpholine substituent (**3i**–**3n**), in this case every compounds having morpholine moiety were inactive against IKKβ.

There are lots of kinases in human biological system. To reduce side effects possibly occurring on inhibiting other kinases, selectivity for target kinase is needed. In this reason, we performed kinase panel test against IKKα and other kinases such as p38α, p38β, JNK1, JNK2 and JNK3 with selected compounds (**3f**, **3p** and **3q**) which possessed good inhibitory activities in enzymatic and cell-based assay, and the results are summarized in Table 3. Selected compounds showed excellent selectivity over p38α, p38β, JNK1, JNK2, and JNK3 as well as IKKα. It is noteworthy that IKKα/IKKβ IC_{50} ratio of **3f**, **3p** and **3q** is over 20 compared with reference compound.

In summary, from high throughput screening technology, we have identified a hit compound **1** which has rhodanine nucleus as a core structure. Starting from this hit compound, structural modification was carried out in eastern and western part of rhodanine nucleus in order to obtain potent and selective IKKβ inhibitors. Compound **3q**[12] which possesses *p*-carboxamidophenoxyphenyl moiety in eastern part and 4-methylpiperazinylpropoxyphenyl group in western part was found as a potent inhibitor against IKKβ, and showed balanced inhibitory activity against NF-κB activation and TNFα production in cell-based assay. Moreover, compound **3q** has excellent selectivity over other kinases such as IKKα, p38α, p38β, JNK1, JNK2, and JNK3. Taken together, optimized compound **3q** has high possibility as a potential candidate for treatment of disease associated with NF-κB activation such as rheumatoid arthritis, chronic obstructive pulmonary disease (COPD), and cancer.

Table 2
IKKβ inhibitory activities of rhodanine derivatives **3a–3s**



Compd	*n.*	R^1	R^2	Enzyme assay		Cell-based assay	
				% Inhibition of IKKβ (10 μM)	IKKβ IC_{50} (μM)	% Inhibition of NF-κB (10 μM)	TNFα IC_{50} (μM)
3a	2	NEt_2		1.6	–	–	–
3b	2	NEt_2		17.1	–	57.0	7
3c	2	NEt_2		62.6	8.7	58.5	8
3d	3			21.3	–	–	–
3e	3			57.3	6.3	57.0	7
3f	3			85.3	1.5	58.5	5
3g	3			18.6	–	–	–
3h	3			83.0	6.3	42.1	30
3i	3			81.2	4.2	48.2	30
3j	3			–	–	–	–
3k	3			48.8	–	–	–

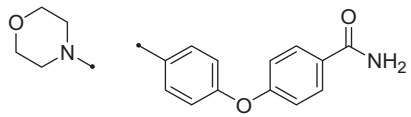
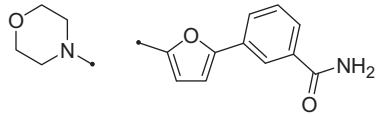
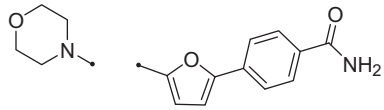
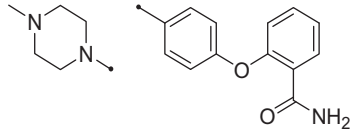
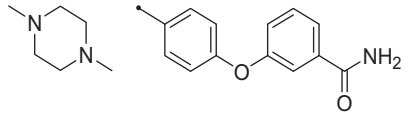
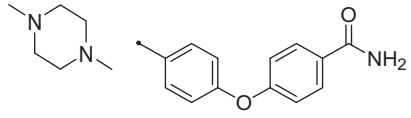
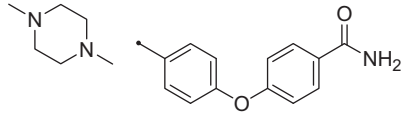
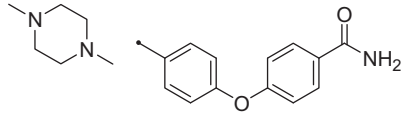
3l	3		–	–	–	–
3m	3		2.1	–	–	–
3n	3		–	–	–	–
3o	3		52.7	8.5	42.4	12
3p	3		86.8	1.5	64.7	5
3q	3		90.1	0.35	65.4	2
3r	4		90.4	0.23	–	4
3s	5		89.3	0.16	–	–
Reference compound			88.7	0.17	73.9	1.8

Table 3
Selectivity data of **3f**, **3p** and **3q** over other kinases (IC_{50} values in μM)

Compd.	IKKβ	IKKα	p38α	p38β	JNK1	JNK2	JNK3
3f	1.5	>30	>30	>30	>30	>30	>30
3p	1.5	>30	>30	>30	>30	>30	>30
3q	0.38	>30	>30	>30	>30	>30	>30
Reference compound	0.17	0.64	–	–	–	–	–

Acknowledgments

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References and notes

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9. For NF-κB report gene assay, HeLa cells were transfected with 1 μg of NF-κB-Luc reporter plasmid DNA using calcium phosphate precipitation methods. Cells were pre-treated with 10 μM of inhibitors for 1 h and further incubated with TPA (50 ng/mL) for 8 h. Luciferase activity was determined and normalized to the protein content of each extract. Results are presented as relative luciferase unit (RLU) per mg compared to non-treated cell ($n = 3$).
10. Eight weeks after the primary type II collagen immunization, the mouse spleens were collected for cell preparation and washed twice with PBS. The spleens were minced and the red blood cells were lysed with 0.83% ammonium chloride. The cells were filtered through a cell strainer and centrifuged at 1300 rpm at 4 °C for 5 min. The cell pellets were re-suspended in RPMI 1640 medium and plated in 24-well plates (Corning, NY, USA) at a concentration of 1×106 cells/well. Isolated splenocytes were cultured with inhibitors for 72 h. The amounts of TNFα in the culture supernatants were measured by ELISA. Antibodies directed against mouse TNFα and against biotinylated anti-mouse TNFα were used as the capture and detection antibodies, respectively. Alkaline phosphatase (Sigma) was used for the chromogenic reaction. The amounts of TNFα present in the test samples were determined from standard curves constructed with serial dilutions of recombinant murine TNFα (R&D Systems). The absorbance was determined with an ELISA microplate reader at 405 nm.

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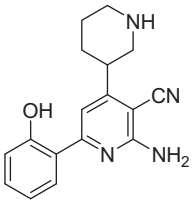

12. 2HCl salt form of **3q**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.17–2.18 (m, 2H), 2.81 (bs, 3H), 3.53–3.60 (m, 10H), 4.13 (t, J = 5.7 Hz, 2H), 7.09 (d, J = 9.0 Hz, 2H), 7.16 (d, J = 8.8 Hz, 2H), 7.21 (d, J = 8.8 Hz, 2H), 7.33 (d, J = 9.0 Hz, 2H), 7.36 (bs, 1H), 7.74 (d, J = 8.8 Hz, 2H), 7.95 (d, J = 8.8 Hz, 2H), 7.98 (bs, 1H), 11.55 (bs, 2H); ^{13}H NMR (100 MHz, DMSO-d_6) δ 23.9, 42.5, 48.8, 50.2, 53.6, 65.6, 115.4, 118.6, 118.9, 119.3, 119.7, 122.3, 128.2, 128.9, 130.3, 130.7, 132.4, 133.4, 158.2, 158.8, 159.2, 167.5, 194.5: HRMS Calcd for $C_{31}H_{32}N_4O_4S_2$ $(M+H)^+$ 589.1937. Found 589.1986.