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Synthetic and mechanistic insight into nosylation of glycine residues†

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The Fukuyama–Mitsunobu alkylation procedure is widely used to introduce alkyl substituents to amino groups in general and *N*-alkylation of peptides in particular. Here we have investigated the procedure in detail for *N*-alkylation of peptides with N-terminal glycine residues, based on the observation that standard conditions lead to substantial bis-nosylation of the glycine amino group. A systematic evaluation of this observation was carried out and it was demonstrated that for peptides with alanine, β-alanine or γ-aminobutyric acid (GABA) as N-terminal residues mono-nosylation was observed under the same conditions. Moreover, bis-nosylation was independent of the type of resin, neighboring amino acid and nature of the peptide. Calculations suggest that the reason for the bis-nosylation is the fact that the deprotonated mono-nosyl species is particularly stable in the case of the terminal Gly residue because the N^- residue can become closer to the SO_2 unit. Finally, the mono-nosylated N-terminal glycine could be obtained by careful optimization of the procedure, adding only one equivalent of 2-nitrobenzenesulfonyl chloride.

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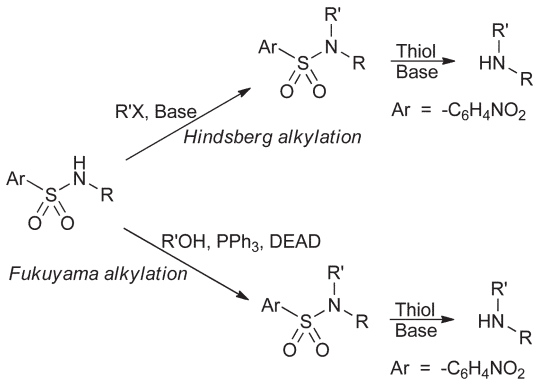
Introduction

In 1890 Hindsberg[1] first reported a N–C coupling method for smooth alkylation of amines *via* the corresponding arylsulfonamides, thereby avoiding bis-alkylation or even quaternation of the nitrogen atom (Scheme 1). A highly efficient variant of the Hindsberg method is the Fukuyama–Mitsunobu method, which was developed as an alternative utilizing an alcohol as the alkylating agent.[2–4] Here the 2-nitrobenzenesulfonamide is reacted with alcohol under Mitsunobu conditions, conveniently providing the *N*-alkylated 2-nitrobenzenesulfonamide. This procedure has wide applications[5] since the arylsulfonyl group, for ease the 2-nitrobenzenesulfonyl group, is efficiently removed after alkylation in the presence of thiols under basic conditions affording solely the mono-alkylated product (Scheme 1).



Scheme 1

This Fukuyama–Mitsunobu protocol was extended to solid-phase applications and has been extensively utilized for *N*-alkylations on solid phase. Specifically, facile *N*-alkylation of peptides using the Fukuyama protocol was demonstrated by Miller and Scanlan[6] and has subsequently been extensively used in the synthesis of peptides and peptide derivatives.[7] Particularly *N*-methylation in peptides is highly efficient.[8,9]

We have utilized the Fukuyama–Mitsunobu method extensively, both for generating polyamines in the synthesis of spider polyamine toxins on a solid phase[10–15] and, recently, for *N*-alkylation of small peptides on a solid phase.[16,17] During the latter studies, we observed that having a glycine residue in the N-terminal of a small peptide furnished strongly reduced yields of the desired *N*-alkyl peptides and realized that this was due to consistent bis-nosylation of the amino group of glycine. Here we report a thorough investigation into the nature of this bis-nosylation. Finally, we also provide a means to avoid bis-nosylation, which should be a notable precaution

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for performing *N*-alkylation of glycine residues, particularly considering the widespread use of the Fukuyama–Mitsunobu protocol.

Results and discussion

As a continuation of investigations aimed at extended applications of Fukuyama–Mitsunobu solid-phase *N*-alkylations of peptides, we introduced 2-nitrobenzenesulfonamide (nosyl) groups at the N-terminals of dipeptides terminating with amino acids such as glycine, alanine and valine using a standard protocol including four equivalents of nosyl chloride, ensuring almost quantitative sulfonation of amino groups in analogy to previously reported procedures[2] including our own work.[17] In order to check whether the arylsulfonation reactions had run to completion the product composition from test cleavages with TFA was examined by LC-MS. Clean mono-nosylation was observed in all but one case. Glycine was the notable exception, since in addition to obtaining the desired mono-nosylated product, *o*-Ns-Gly-Gly-OH (**1**, Scheme 2), LC-MS also revealed the presence of the corresponding bis-nosylated product, $(o\text{-Ns})_2$-Gly-Gly-OH (**2**, Scheme 2). Compounds **1** and **2** were formed in a ratio of approximately 1 : 1 (Scheme 1, Fig. 1 and Table 1, entry 1), which was verified by subsequent work-up of the cleavage mixtures affording **1** and **2** in 39% and 42% yields, respectively (Scheme 2).

Notably, when alanine or valine residues were present in the N-terminal and they were treated with nosyl chloride under identical conditions no bis-nosylation was observed and exclusively the corresponding mono-nosylated products were obtained (Fig. 1 and Table 2, entries 2 and 3, respectively). An obvious rationalization of this phenomenon would be that bis-nosylation was strictly related to the steric bulk at the α-carbon of the N-terminal residue. We therefore investigated nosylation with β-alanine and γ-aminobutyric acid (GABA) in the N-terminal of a dipeptide under identical conditions. However, in both cases no bis-nosylation was observed and solely the mono-nosylated products could be detected by LC-MS analysis of the TFA cleaved reaction mixtures (Fig. 1 and Table 1, entries 4 and 5). This clearly suggests that steric bulk of the α-carbon could not be a determining factor for the difference between glycine and alanine or valine in the degree of bis-nosylation.

In all experiments so far, we employed a dipeptide with glycine in the C-terminal and speculated that the neighboring amino acid could play a decisive role. We therefore replaced

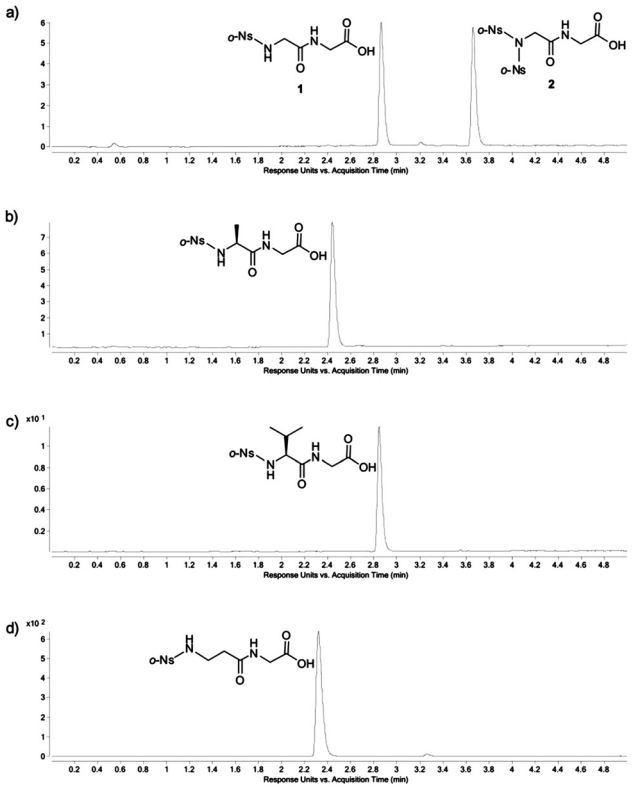
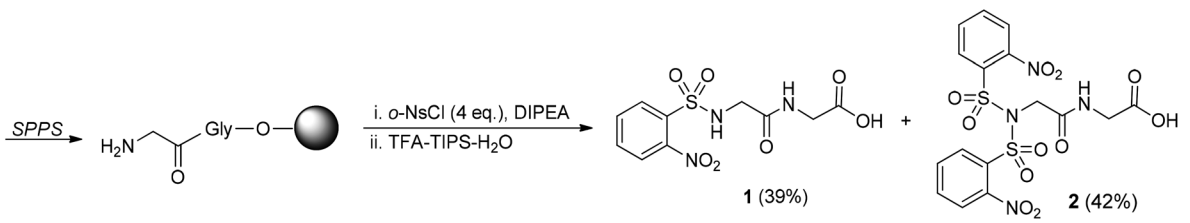


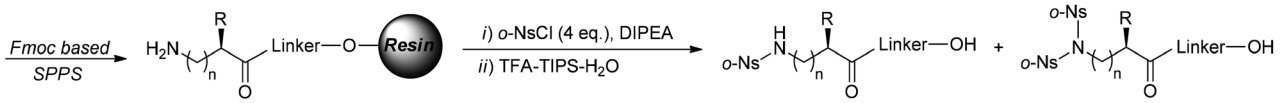
Fig. 1 Nosylation of resin-bound dipeptides with 4 equivalents of 2-nitrobenzenesulfonyl chloride followed by cleavage (TFA–TIPS–H_2O): (a) Gly–Gly (Table 1, entry 1); (b) Ala–Gly (Table 1, entry 2); (c) Val–Gly (Table 1, entry 3); (d) β-Ala–Gly (Table 1, entry 4).

the C-terminal glycine with valine, but observed that the mono- to bis-nosylation ratio was independent of the neighboring amino acid (Table 1, entry 6), suggesting that the steric influence of the neighboring amino acid had no impact on the nosylation ratio. We then speculated whether bis-nosylation of glycine resides was dependent on the resin and/or linker employed, and therefore performed the solid-phase nosylations on a Wang resin instead of on a 2-chlorotrityl resin, and obtained similar results (Table 1, entries 7 and 8). Moreover, to ensure that the observed bis-nosylation was not restricted to dipeptides, we prepared two model 15-mer peptides including all proteogenic amino acids bearing side chain functionalities. When a glycine was the N-terminal residue, the mono- to bis-nosylation ratio was approximately 1 : 1 whereas with an N-terminal alanine only mono-nosylation was observed



Scheme 2

Table 1 *N*-Terminal mono *vs.* bis *o*-nitrobenzenesulfonation of resin bound primary amines



Entry	R	*n*	Linker	Resin	Mono to bis nosylation ratio[a]
1	H	0	Gly	2-Chlorotrityl	50 : 50
2	Me	0	Gly	2-Chlorotrityl	100 : 0
3	Pr^{i}	0	Gly	2-Chlorotrityl	100 : 0
4	H	1	Gly	2-Chlorotrityl	100 : 0
5	H	2	Gly	2-Chlorotrityl	100 : 0
6	H	0	Val	2-Chlorotrityl	50 : 50
7	H	0	Gly	Wang	50 : 50
8	H	0	Val	Wang	50 : 50
9	H	0	YWTSRQPNKHGDCV	2-Chlorotrityl	50 : 50
10	Me	0	YWTSRQPNKHGDCV	2-Chlorotrityl	100 : 0
11	H	0	–OCH_2CH_2C═O–	2-Chlorotrityl	100 : 0
12	H	0	Sar[b]	2-Chlorotrityl	50 : 50

o-Ns = 2-Nitrobenzenesulfonyl (2-$O_2NC_6H_4SO_2$–).[a] Approximated by HPLC analysis of the cleavage mixture. [b] Sar (sarcosine) = *N*-methylglycine.

Table 2 PCM-G3MP2 calculated energies for deprotonation[a]

Reaction	Δ*E*	Δ*G*
	1186.7	1190.5
	1190.5	1194.3
	1208.1	1210.1
	1203.7	1210.2
	1202.5	1200.4

[a] 0 K values in kJ mol^{-1}.

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(Table 1, entries 9 and 10), which confirms that this seems to be a general phenomenon for peptides regardless of their length.

Finally, we turned our attention to the role of the amide bond between the glycine and the neighboring amino acid. To address this, we first examined the isosteric replacement of the amide functionality with an ester function, thereby removing the NH moiety and reducing the electronegativity of the carbonyl group. Performing the nosylation on a Gly–Gly depsipeptide resulted in pure mono-nosylation and the bis-nosylated product could not be observed by LC-MS (Table 1, entry 11). This unequivocally demonstrates that the carbonyl group, the NH moiety or both play a decisive role in the observed bis-nosylation. To examine this further, and specifically address the influence of the amide NH moiety, the *N*-methylated amino acid, sarcosine (*N*-methylglycine), was introduced next to an N-terminal glycine and nosylation was performed. This resulted in the same mono- to bis-nosylation ratio of approximately 1 : 1 (Table 1, entry 12), as observed in our initial Gly–Gly dipeptide, thus highlighting the decisive role of the carbonyl group of the amide bond.

To examine the relevance of these observations to synthesis in solution (DMF), we performed nosylations using the same conditions applying a large excess of nosyl chloride on *tert*-butylamides of glycine (H-Gly-NHBu-*t*), alanine (H-Ala-NHBu-*t*) and valine (H-Val-NHBu-*t*). We observed the same phenomenon as on solid phase: For glycine bis-nosylation occurred to a very large degree whereas the amides of alanine and valine gave pure mono-nosyl products.

Thus, we observed that bis-nosylation occurs for peptides with a glycine in the N-terminal. Generally, if a glycine residue is nosylated, it will result in a significantly detrimental yield of the corresponding alkylated peptide. On the other hand, for all other investigated residues there seems to be no risk of obtaining bis-nosylated products when utilizing an excess of nosyl chloride, as generally employed in solid-phase synthesis. We therefore investigated how the bis-nosylation of glycine residues could be avoided using nosylation of the 15 amino acid peptide GWYTSRQPNKHEDCV on trityl resin as an example. We found that reducing the number of equivalents of nosyl chloride from 4 to 1.1 only led to a very low degree of

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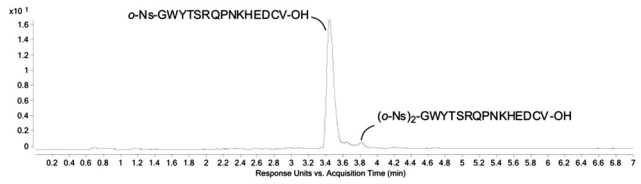
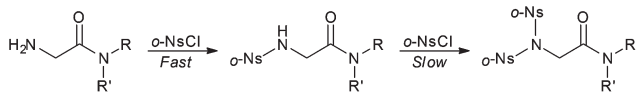


Fig. 2 Nosylation of a resin-bound 15 amino acid peptide GWYTSRQPNKHEDCV with 1.1 equivalents of 2-nitrobenzenesulfonyl chloride leading to an almost pure mono-nosylated product.



Scheme 3

bis-nosylation, affording the crude *o*-Ns-GWYTSRQPNKHEDCV in high purity (Fig. 2) and a good yield of the mono-nosylated product (76%) after HPLC purification.

It is important to avoid adding an excess of nosyl chloride during nosylation of only glycine terminated peptides. Because of the low amount of bis-nosylated product formed with addition of 1.1 equivalent (Fig. 2) it is assumed that reaction with the first nosyl chloride molecule is faster than introduction of the second nosyl group (Scheme 3).

Calculations

In order to understand these observations in more detail, we employed a computational approach. The 0 K energy was calculated at the G3(MP2) level which is known to be accurate within chemical accuracy (2.0 kcal mol^{-1}).[18] This method is based on an MP2(FULL)/6-31G(d) geometry calculation and an HF/6-31G(d) frequency calculation. We verified that all of the calculated geometries do correspond to a genuine minimum (zero imaginary frequencies, Fig. 3). To address solvent effects, we invoked the polarizable continuum model (PCM) by Tomasi.[19] All calculations were carried out with the GAUSSIAN03 suite of programs.[20]

We investigated the central effects in mono- and bis-nosylation processes computationally to address why a bis-nosylated product is obtained only in the case of glycine (Table 1) whereas mono-substitution is obtained for all other amino acids. We focused on the energy requirements for deprotonation of the mono-nosylated species as the reaction is carried out under conditions that are sufficiently basic to deprotonate the sulfamide and because our observations indicate that the bis-nosylation is related to thermodynamic properties of the process rather than to kinetics. The results are shown in Table 2 and they are consistent in the sense that the lowest energy of deprotonation is indeed found for the glycine terminal, and thus it is only in this case that the mono-nosyl species can become deprotonated to become sufficiently nucleophilic so as to be attacked by a second equivalent of NsCl. We observed that bis-nosylation took place when this N–H was methylated (Table 1, entry 12). This finding is not in line with the calculated energy for deprotonation in Table 2. We attribute this discrepancy to the fact that PCM does not take into account specific solvent interactions and thus the N–H hydrogen bond to the negatively charged nitrogen will be essential for anion stability and *N*-methylation will increase the energy for deprotonation too much compared to a situation where the solvent can hydrogen bond to the negatively charged nitrogen atom.

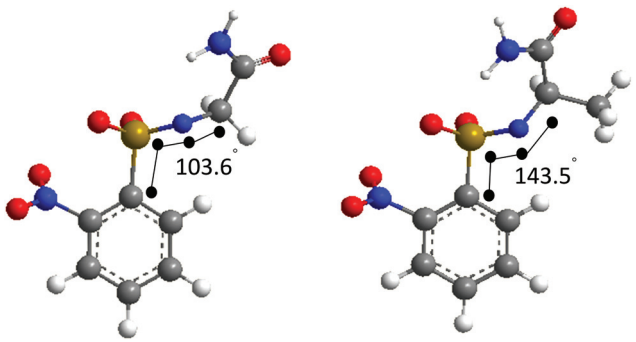


Fig. 3 Calculated geometries (MP2(FULL)/6-31G(d)) for the sulfamide of Gly and an example of a substituted analogue. The characteristic increase in the marked dihedral angle upon substitution is noted.

Conclusions

In conclusion, we identified that bis-nosylation is an important issue in treatment of peptides having an N-terminal glycine with an excess of nosyl chloride. From studies based on systematic variations of the peptide structure it was clear that this phenomenon was not caused by steric factors. Notably, N-terminal glycine exclusively caused bis-nosylation whereas other amino acid motifs, even with a less hindered amide functionality, underwent clean mono-nosylation. Introducing an amide-to-ester substitution next to an N-terminal glycine cleanly resulted in mono-nosylation whereas an N–Me group did not, suggesting the carbonyl group of the amide moiety to be decisive. Calculations indicated that the energy requirement for deprotonation of the mono-nosylated species is the key. This energy is determined by the anion stability which is closely related to the structure as the more substituted anions are more congested and the N^- to S overlap is thus less easy to form. For use in Fukuyama–Mitsunobu reactions, the formation of bis-nosylated by-products seriously reduces yield by truncation of the peptide. However, the bis-nosylation problem could be overcome by using one equivalent of nosyl chloride since introduction of the first nosyl group is sufficiently faster than introduction of the second group.

Experimental section

Chemistry

All reagents were obtained from commercial suppliers and used without further purification. Proton (^{1}H) and carbon

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(^{13}C) NMR spectra were recorded on Bruker Avance (400 MHz). Chemical shifts (δ) are reported in parts per million (ppm) with reference to tetramethylsilane (TMS) as an internal standard. NMR experiments were carried out in $CDCl_3$. The following abbreviations are used for the proton spectra multiplicities: s, singlet; br s, broad singlet; d, doublet; dd, double doublet, triplet; q, quartet; sep, septet m, multiplet. Coupling constants (J) are reported in Hertz (Hz). TLC analysis was performed on silica gel F_{254} (Merck) and detection was carried out by examination under UV light and staining with potassium permanganate. Flash column chromatography was performed on silica gel with a solvent of HPLC grade. Elemental analyses were performed by Mr J. Theiner, Department of Physical Chemistry, University of Vienna, Austria. Preparative HPLC was performed on an Agilent 1100 system using a C18 reverse-phase column (Zorbax 300 SB-C18, 21.2 × 250 mm) with a linear gradient of the binary solvent system of H_2O–ACN–TFA (A: 95/5/0.1 and B: 5/95/0.1) and a flow rate of 20 mL min^{-1}. Analytical HPLC was performed on an Agilent 1100 system with a C18 reverse-phase column (Zorbax 300 SB-C18 column, 4.6 × 150 mm) with a flow rate of 1 mL min^{-1} and a linear gradient of the binary solvent system of H_2O–ACN–TFA (A: 95/5/0.1 and B: 5/95/0.1). Mass spectra were obtained with an Agilent 6410 Triple Quadrupole Mass Spectrometer instrument using electron spray coupled to an Agilent 1200 HPLC system (ESI-LC/MS) with a C18 reverse-phase column (Zorbax Eclipse XBD-C18, 4.6 × 50 mm), an auto-sampler and diode-array detector using a linear gradient of the binary solvent system of H_2O–ACN–formic acid (A: 95/5/0.1 and B: 5/95/0.086) with a flow rate of 1 mL min^{-1}. During ESI-LC/MS analysis evaporative light scattering (ELS) traces were obtained with a Sedere Sedex 85 Light Scattering Detector. The identity of all tested compounds was confirmed by ESI-LC/MS, which also provided purity data (all >95%; UV and ELSD). High-resolution mass spectra (HRMS) were obtained using a Micromass Q-Tof 2 instrument and were all within ±5 ppm of theoretical values.

Solid-phase synthesis nosylated peptides; general procedure. Peptides were manually synthesized by Fmoc-based solid-phase peptide synthesis (SPPS) using a MiniBlock (Mettler-Toledo, Columbus, OH, USA). The 2-chlorotrityl chloride polystyrene resin (1–2% DVB cross linking, 100–200 mesh) was used as a solid support, and after the resin was swelled in dry DCM for 15–30 min, the first amino acid was loaded to the resin using diisopropylethylamine (DIPEA) (resin/amino acid/DIPEA in 1 : 4 : 8) in DCM for 1.5 h, followed by capping with methanol (DCM–MeOH–DIPEA 17 : 2 : 1). Fmoc deprotection was performed with 20% piperidine in DMF (1 × 5 and 1 × 15 min; wash step in between) and coupling of the consecutive amino acid was carried out with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and DIPEA (resin/amino acid/HBTU/DIPEA 1 : 4 : 4 : 4) in dry DMF (2 mL) for 30 min. After attachment of the last amino acid Fmoc deprotection was performed, followed by washing and drying of the resin with DCM. The resin was swelled in DIPEA (6 equiv.) in THF (2.5 mL) for *ca.* 30 min. 2-Nitrobenzenesulfonyl (Ns) chloride (4 equiv.) in DCM (1 mL) was added slowly while agitating the solution for 1 h followed by draining and washing with THF, MeOH, and DCM. An inhomogeneous mixture of TFA–triisopropylsilane (TIPS)–H_2O (18 : 1 : 1, 4 mL) was added and the mixture was shaken for 2 h. The peptide-containing solution was filtered into a flask and combined with two successive washes with DCM. After gentle evaporation in a stream of nitrogen the crude peptide was analyzed by LC-MS and eventually purified by HPLC.

Nosylation of H-Gly-Gly-2ClTrt-resin (Scheme 2 and Table 1, entry 1)

An approximately 1 : 1 mixture of mono- and bis-nosylated products was obtained by following the procedure above. Separation by HPLC gave *o*-Ns-Gly-Gly-OH 1 and (*o*-Ns)$_2$-Gly-Gly-OH 2.

***o*-Ns-Gly-Gly-OH 1 (Scheme 2)**

The mono-nosylated dipeptide **1** (0.031 g, 39%) was obtained as colorless crystals. Mp: 168–170 °C. Anal. calcd for $C_{10}H_{11}N_3O_7S$: C, 37.86; H, 3.49; N, 13.24. Found: C, 37.67; H, 3.23; N, 13.12. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.83 (s, 2H), 3.86 (s, 2H), 7.78–7.84 (m, 2H), 7.88–7.92 (m, 1H), 8.07–8.11 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 41.74, 46.60, 126.27, 131.76, 133.83, 134.56, 135.19, 149.45, 170.92, 172.70.

(*o*-Ns)$_2$-Gly-Gly-OH 2 (Scheme 2)

The bis-nosylated dipeptide **2** (0.053 g, 42%) was obtained as colorless crystals. Mp: 218–222 °C (dec.). Anal. calcd for $C_{16}H_{14}O_{11}S_2$: C, 38.25; H, 2.81; N, 11.15. Found: C, 38.39; H, 2.61; N, 11.01. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.60 (d, J = 5.5 Hz, 2H), 4.54 (s, 2H), 7.89–8.00 (m, 4H), 8.35 (d, J = 8.0 Hz, 2H), 8.43–8.47 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 40.94, 50.72, 124.67, 130.95, 131.80, 132.75, 136.36, 147.42, 165.62, 170.75.

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