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[a002]

4-(Hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-β-alanine conjugate: synthesis and photocleavage

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Abstract: The novel functionalised oxygen heterocycle 4-(hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran was synthesised and used in the preparation of a fluorescent β-alanine conjugate. In order to evaluate its photosensitivity, photocleavage reaction in methanol/HEPES buffer (80:20) solution at different wavelengths of irradiation (250, 300 and 350 nm) was carried out and photocleavage kinetic data were obtained.

Keywords: Photocleavable protecting groups; Benzopyrans; Neurotransmitter amino acids.

1. Introduction

Protecting groups are of special importance in organic synthesis and manipulation of polyfunctional molecules, since they avoid the formation of undesired bonds and side reactions.[1]

Photoremovable protecting groups are an interesting alternative to the conventional groups, revealing various advantages, namely the relatively soft conditions required for their cleavage and orthogonality with respect to acid- or base-sensitive groups.[2] They have been reported for convenient and controlled release of functional molecules in organic synthesis,[2] as well as in the caging and release of biologically significant compounds.[2,3] Representative exemples of photoremovable protecting groups include the *o*-nitrobenzyl esters and ethers, benzoins, phenacyl esters, and coumarin (trivial name for benzopyrans) derivatives.[2]

Neuroactive amino acids are a class of biomolecules where caging strategy has been applied. Among them is β-alanine (β-Ala), a physiological transmitter, being the rate-limiting precursor of carnosine, which is a β-alanine-histidine dipeptide present in muscle and brain tissues.[4]

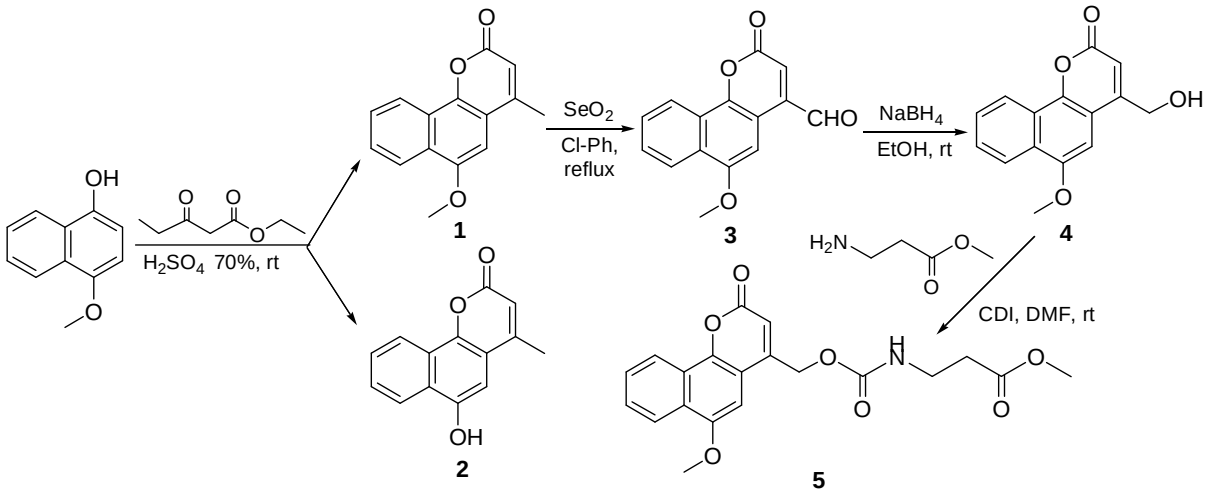
Considering these facts in connection with our current research interests in the development of different (hetero)aromatic fluorophores as photocleavable protecting groups, applied to amino acids, including neurotransmitters[5-7] we now report the synthesis of 4-(hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran and its use in the preparation of a

fluorescent conjugate of β-alanine, with the aim of evaluating the sensitivity to light of the bioconjugate.

2. Results and Discussion

4-Methoxy-1-naphthol was reacted with ethyl acetoacetate through a Pechmann reaction, catalysed by sulphuric acid at room temperature, yielding 6-methoxy-4-methyl-2-oxo-2*H*-benzo[*h*]benzopyran **1** together with the unexpected demethylated derivative, 6-hydroxy-4-methyl-2-oxo-2*H*-benzo[*h*]benzopyran **2**.

By reaction of compound **1** with selenium dioxide, the methyl group was oxidised to the aldehyde **3**, which was then reacted with sodium borohydride, affording the 4-(hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran **4** (Scheme 1, Table 1).



Scheme 1. Synthesis of benzo[*h*]benzopyran **4** and the corresponding β-alanine conjugate **5**.

The functionalised fluorophore **4** was used in the preparation of an inhibitory neurotransmitter amino acid conjugate through an urethane linkage. Thus, β-alanine methyl ester was derivatised at the N-*terminus* with 1,1'-carbonyldiimidazole (CDI) by a carbonyl transfer reaction,[8] in DMF, at room temperature, resulting in the expected bioconjugate **5** in 42% yield (Scheme 1, Table 1).

All compounds synthesised were fully characterised by high resolution mass spectrometry, IR, ^{1}H and ^{13}C NMR, as well as UV/Visible and fluorescence spectroscopy. The IR spectra of conjugate **5** showed bands due to stretching vibrations of the different carbonyl groups present at the fluorophore-amino acid conjugate at 1739 and 1711 cm^{-1}. ^{1}H NMR spectra showed signals of the amino acid residue, such as α-CH_2 (δ 2.62 ppm) and β-CH_2 (δ 3.50 to 3.62 ppm), in addition to the fluorophore methylene group (δ 5.38 ppm). Also the characteristic aromatic protons, H-3 and H-5, of the oxobenzopyran ring were present at 6.56 and 6.65 ppm,

respectively. The confirmation of the presence of the newly formed urethane linkage was also supported by ^{13}C NMR spectra signals of the carbonyl group, which was found at δ 155.27 ppm.

Table 1. Yields, UV/Visible and fluorescence data for compounds **1**-**5** in absolute ethanol.

Compound	Yield (%)	λ_{max} (nm)	log ε	λ_{em} (nm)	Φ_F	Stokes' shift (nm)
1	15	371	3.57	463	0.33	72
2	12	380	3.71	459	0.17	79
3	79	377	3.73	461	0.49	84
4	47	365	3.69	460	0.45	95
5	42	374	3.70	472	0.31	98

The UV/Visible absorption and emission spectra of degassed 10^{-5} M solutions in absolute ethanol of conjugate **5**, in comparison with tag **4** and its precursors, were measured, absorption and emission maxima, molar absorptivities and relative fluorescence quantum yields are also reported (Table 1). Relative fluorescence quantum yields were calculated using 9,10-diphenylanthracene as standard (Φ_F = 0.95 in ethanol).[9] For the Φ_F determination, the fluorescence standard was excited at the wavelengths of maximum absorption found for each one of the compounds to be tested and in all fluorimetric measurements the absorbance of the solution did not exceed 0.1.

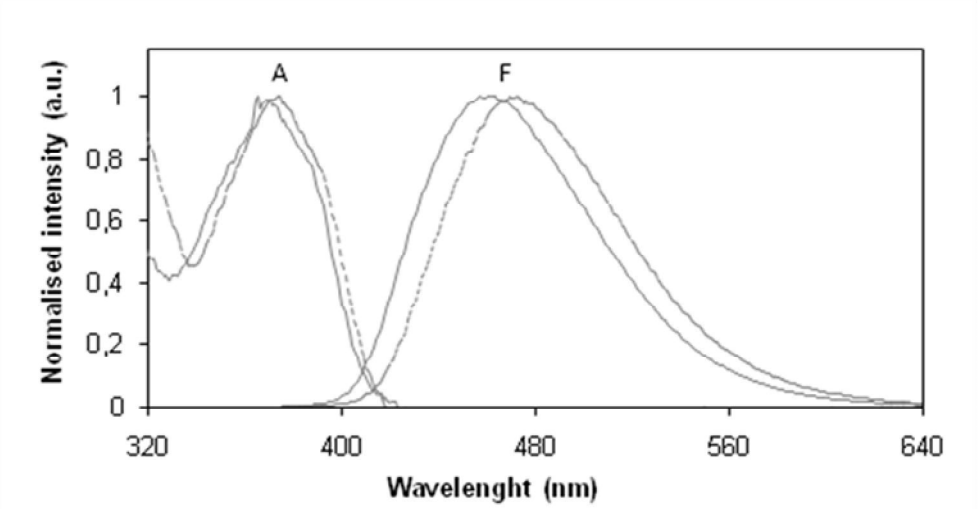


Figure 1. Normalised UV/Visible absorption (A) and fluorescence (F) spectra of precursor **4** and conjugate **5** in ethanol (**4**, λ_{exc} = 365 nm; **5**, λ_{exc} = 374 nm) (**4**, black full line; **5**, black spaced line).

Bioconjugate **5** displayed emission maxima at 472 nm, with large Stokes' shifts (98 nm), which is an important feature in fluorescent labelling for bioapplications. By comparison of precursor **4** and the corresponding conjugate **5**, it was observed a decrease in the fluorescence quantum yield (Φ_F 0.45, **4**; 0.31, **5**), as well as a batochromic shift from **4** to **5** (12 nm). Figure 1 illustrates the absorption and fluorescence normalised spectra of label **4** and the β-alanine conjugate **5** in ethanol.

The sensitivity of benzo[*h*]benzopyran conjugate **5** towards UV-Visible irradiation was evaluated by exposing its solutions in methanol/HEPES buffer (80:20) solution in a Rayonet RPR-100 reactor at 254, 300 and 350 nm. The course of the photocleavage reaction was followed by reverse phase HPLC with UV detection. The plots of peak area (*A*) of the starting material *versus* irradiation time were obtained, at the considered wavelengths. Peak areas were determined by HPLC, which revealed a gradual decrease with time, and were the average of 3 runs. The determined irradiation time represents the time necessary for the consumption of the starting materials until less than 5% of the initial area was detected (Table 2).

Based on HPLC data, the plot of ln *A versus* irradiation time showed a linear correlation for the disappearance of the starting material, which suggested a first order reaction, obtained by the linear least squares methodology for a straight line. The corresponding rate constants were calculated and are presented in Table 2.

Concerning the influence of the wavelength of irradiation on the rate of photocleavage reactions of conjugate **5** in methanol/HEPES buffer (80:20) solution, and accordingly to the lamp power, it was found that irradiation times at 300 and 350 nm were equal, the best results being obtained at 254 nm.

Table 2. Irradiation times (in min) and rate constant ($\times 10^{-2}$ min^{-1}) for the photolysis of conjugates **5** and **6**[10] at different wavelengths in methanol/HEPES buffer (80:20) solution. Flu means fluorophore.

	Compound	254 nm		300 nm		350 nm	
		Irr time	*k*	Irr time	*k*	Irr time	*k*
5	Flu-β-Ala-OMe	75	4.10	140	1.50	140	1.87
6[10]	Z-β-Ala-OFlu	52	6.16	592	0.48	438	0.62

By comparison of β-alanine conjugates **5** and **6**,[10] which differ in the type of linkage between the fluorophore and the neurotransmitter, it was found that at 300 and 350 nm the urethane bond (**5**) cleaved significantly faster than the ester bond (**6**).

3. Conclusions

4-(Hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran **4**, synthesised through a few steps of simple synthetic modifications, was used in the synthesis of a fluorescent β-alanine urethane conjugate **5**. The photophysical characterisation suggested that this novel benzo[*h*]benzopyran is a useful fluorophore for the derivatisation of non-fluorescent amino acids, namely β-alanine, yielding the corresponding bioconjugate with good relative fluorescence quantum yield.

Regarding the photocleavage studies in methanol/ HEPES buffer (80:20) solution, it was found that the fluorescent β-alanine-conjugate cleaves at 300 and 350 nm after a short irradiation time, which is equal at both wavelengths. Owing to these promising results, further studies will be carried out for expanding the applicability of the new fused benzopyran as photocleavable protecting group of others neurotransmitter amino acids.

4. Experimental Section

4.1. Synthesis of 6-methoxy-4-methyl-2-oxo-2*H*-benzo[*h*]benzopyran (1). To a solution of 4-methoxy-1-naphthol (0.612 g, 3.51×10^{-3} mol) in 70% aqueous sulphuric acid (6 mL), ethyl acetoacetate (1.4 mL, 1.1×10^{-2} mol) was added and stirred at room temperature for 3 hours. The reaction mixture was poured into ice water and stirred for 2 hours to give a fine pinkish precipitate. The solid was collected by filtration, washed with cold water and dried in a vacuum oven. After purification by dry flash chromatography, using ethyl acetate/ *n*-hexane, mixtures of increasing polarity as eluent, compound **1** was obtained as a beige solid (0.130 g, 15 %). mp = 131.1 - 133.4 ºC. TLC (ethyl acetate / *n*-hexane, 1:1): R_f = 0.65. ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H = 2.49 (s, 3 H, CH_3), 4.04 (s, 3 H, OCH_3), 6.35 (d, J = 1.2 Hz, 1 H, H-3), 6.73 (s, 1 H, H-5), 7.58-7.70 (m, 2 H, H-8 and H-9), 8.20-8.28 (m, 1 H, H-7), 8.46-8.53 (m, 1 H, H-10). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C = 19.34 (CH_3), 55.70 (OCH_3), 96.55 (C-5), 114.46 (C-3), 114.94 (C-4a), 122.12 (C-7), 122.37 (C-10), 123.90 (C-6b), 127.22 (C-6a), 127.64 (C-9 or C-8), 128.04 (C-8 or C-9), 145.23 (C-4b), 151.93 (C-6), 153.17 (C-4), 161.13 (C-2). IR (KBr 1%, cm^{-1}): ν = 3417, 2926, 1725, 1610, 1596, 1564, 1506, 1469, 1453, 1429, 1386, 1384, 1272, 1248, 1234, 1208, 1176, 1151, 1110, 1083, 1032, 990, 944, 933, 873, 851, 815. UV/Vis (ethanol, nm): λ_{max} (log ε) = 371 (3.57). HRMS (EI): calcd for $C_{15}H_{12}O_3$ [M^+]: 240.0786; found: 240.0791.

In the same preparation, 6-hydroxy-4-methyl-2-oxo-2*H*-benzo[*h*]benzopyran (**2**) was also isolated in 12% yield and the experimental data confirmed its structure.[11]

4.2. Synthesis of 6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-carbaldehyde (3). Compound **1** (0.108 g, 4.50×10^{-4} mol) was reacted with selenium dioxide (0.299 g, 2.70×10^{-3} mol) in chlorobenzene (5 mL), at reflux for 36h. The mixture was filtered hot and the solvent was removed by rotary evaporation. After purification by dry flash chromatography, using ethyl acetate / *n*-hexane, mixtures of increasing polarity as eluent, compound **3** was obtained as an orange solid (0.090 g, 79%). mp = 222.5 - 224.8 ºC. TLC (chloroform / methanol, 9.5:0.5): R_f = 0.69. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 4.08 (s, 3 H, OCH_3), 6.94 (s, 1 H, H-5), 7.65-7.72 (m, 2 H, H-8 and H-9), 7.89 (s, 1 H, H-3), 8.25-8.32 (m, 1 H, H-7), 8.48-8.54 (m, 1 H, H-10), 10.16 (s, 1 H, CHO). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 55.86 (OCH_3), 97.28 (C-3), 110.48 (C-4a), 122.27 (C-10), 122.40 (C-7), 123.51 (C-6b), 125.19 (C-5), 127.62 (C-6a), 127.87 (C-9), 128.92 (C-8), 144.19 (C-4b), 147.09 (C-6), 152.66 (C-4), 160.51 (C-2), 192.13 (CHO). IR (KBr 1%, cm^{-1}): ν = 3430, 3066, 2962, 2926, 2850, 1731, 1712, 1630, 1594, 1561, 1504, 1472, 1450, 1418, 1381, 1272, 1247, 1183, 1138, 1110, 1083, 1028, 985, 928, 893, 867, 855, 775. UV/Vis (ethanol, nm): λ_{max} (log ε) = 377 (3.73). HRMS (EI): calcd for $C_{15}H_{10}O_6$ [M^+]: 254.0579; found: 254.0589.

4.3. 4-(Hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran (4). Compound **3** (0.098 g, 3.85×10^{-4} mol) was reacted with sodium borohydride (0.016 g, 4.28×10^{-4}mol) in chloroform (7 mL) and ethanol (17 mL) for 3 days at room temperature. After purification by dry flash chromatography, using ethyl acetate / *n*-hexane, mixtures of increasing polarity as eluent, compound **4** was obtained as a light yellow solid (0.046 g, 47%). mp = 219.1 – 220.7 ºC. TLC (acetate / *n*-hexane, 7:3): R_f = 0.60. ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H = 4.02 (s, 3 H, OCH_3), 4.86 (dd, J = 4.2 and 1.2 Hz, 2 H, CH_2OH), 5.73 (t, J = 4.2 Hz, 1 H, OH), 6.55 (t, J = 1.2 Hz, 1 H, H-3), 6.93 (s, 1 H, H-5), 7.67-7.75 (m, 2 H, H-8 and H-9), 8.19 (dd, J = 8.0 and 1.5 Hz, 1 H, H-7), 8.32 (dd, J = 7.6 and 1.6 Hz, 1 H, H-10). ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ_C = 56.01 (OCH_3), 59.58 (CH_2OH), 97.12 (C-5), 110.22 (C-3), 112.78 (C-4a), 121.60 (C-10), 121.90 (C-7), 123.03 (C-6b), 126.28 (C-6a), 127.92 (C-9), 128.23 (C-8), 144.17 (C-4b), 151.08 (C-6), 157.37 (C-4), 160.12 (C-2). IR (KBr 1%, cm^{-1}): ν = 3410, 2924, 2853, 1707, 1602, 1567, 1506, 1474, 1451, 1422, 1384, 1355, 1315, 1275, 1250, 1143, 1117, 1097, 1061, 987, 950, 856, 762, 736, 666. UV/Vis (ethanol, nm): λ_{max} (log ε) = 365 (3.69). HRMS (EI): calcd for $C_{15}H_{12}O_4$ [M^+]: 256.0736; found: 256.0745.

4.4. *N*-[(6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-yl)methyloxycarbonyl]-L-β-alanine methyl ester (5). To a solution of CDI (0.059 g, 3.64×10^{-4} mol) in DMF (1 mL), 4-

(hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran (**4**) (0.060 g, 2.43×10^{-4} mol) dissolved in dry DMF (3 mL) was added and the reaction mixture was stirred at room temperature for 3 hours. After the addition of β-alanine methyl ester (0.025 g, 2.44×10^{-4} mol) the mixture was keep reacting in the same conditions for 12 hours. The solvent was evaporated and purification by dry flash chromatography, using chloroform/ methanol, mixtures of increasing polarity as eluent, gave compound **5** as a light yellow solid (0.038 g, 42%). mp = 175.7 - 177.9 ºC. TLC (chloroform/ methanol, 9.5:0.5): R_f = 0.75. ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H = 2.62 (t, J = 5.7 Hz, 2 H, α-CH_2 β-Ala), 3.50-3.62 (m, 2 H, β-CH_2 β-Ala), 3.74 (s, 3 H, OCH_3 β-Ala), 4.04 (s, 3 H, OCH_3), 5.38 (s, 2 H, CH_2), 5.59 (t, J = 5.7 Hz, 1 H, NH β-Ala), 6.56 (s, 1 H, H-3), 6.65 (s, 1 H, H-5), 7.64-7.72 (m, 2 H, H-8 and H-9), 8.24-8.32 (m, 1 H, H-7), 8.48-8.56 (m, 1 H, H-10). ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C = 34.02 (α-CH_2 β-Ala), 36.74 (β-CH_2 β-Ala), 51.9 (OCH_3 β-Ala), 55.87 (OCH_3), 62.01 (CH_2), 95.29 (C-5), 112.10 (C-4a), 112.21 (C-3), 122.22 (C-10), 122.40 (C-7), 123.97 (C-6b), 127.31 (C-6a), 127.88 (C-9), 128.37 (C-8), 145.63 (C-4b), 150.52 (C-4), 152.24 (C-6), 155.27 (C=O urethane), 160.90 (C-2), 172.68 (C=O ester). IR (KBr 1%, cm^{-1}): ν = 3324, 3081, 2925, 2854, 1739, 1711, 1612, 1598, 1562, 1554, 1505, 1474, 1451, 1420, 1382, 1324, 1312, 1252, 1197, 1174, 1145, 1110, 1083, 1052, 1016, 986, 952, 890, 876, 811, 734. UV/Vis (ethanol, nm): λ_{max} (log ε) = 374 (3.70). HRMS (EI): calcd for $C_{20}H_{20}NO_7$ [M^+]: 386.12455; found: 386.12343.

4.5. Photolysis procedure

A 1×10^{-4} M methanol/HEPES (80:20) solution of compound **5** (5 mL) was placed in a quartz tube and irradiated in a Rayonet RPR-100 reactor at the desired wavelength. The lamps used for irradiation were of 254, 300 and 350 ± 10 nm.

Aliquots of 100 μL were taken at regular intervals and analysed by RP-HPLC. The eluent was acetonitrile/water, 3:1, at a flow rate of 0.8 mL/min, previously filtered through a Millipore, type HN 0.45 μm filter and degassed by ultra-sound for 30 min. The chromatograms were traced by detecting UV absorption at the wavelength of maximum absorption for the compound (retention time: 4.7 min.).

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References

1- Isidro-Llobet, A.; Alvarez, M.; Albericio, F. *Chem. Rev.* **2009**, *109*, 2455-504.

2- a) Pelliccioli, A. P.; Wirz, J. *Photochem. Photobiol.* **2002**, *1*, 441-458. b) Bochet, C. G. J. *Chem. Soc., Perkin Trans. 1* **2002**, 125–142.

3- Mayer, G.; Heckel, A. *Angew. Chem. Int. Ed.* **2006**, *45*, 4900-4921.

4- Hill, C. A.; Harris, R. C.; Kim, H. J.; Harris, B. D.; Sale, C.; Boobis, L. H.; Kim, C. K.; Wise, J. A. *Amino Acids* **2007**, *32*, 225-233.

5- Fernandes, M. J. G.; Gonçalves, M. S. T.; Costa, S. P. G. *Tetrahedron* **2007**, *63*, 10133-10139.

6- Fernandes, M. J. G.; Gonçalves, M. S. T.; Costa, S. P. G. *Tetrahedron* **2008**, *64*, 3032-3038.

7- Fernandes, M. J. G.; Gonçalves, M. S. T.; Costa, S. P. G. *Tetrahedron* **2008**, *64*, 11175-11179.

8- D'Addona, D.; Bochet, C. G. *Tetrahedron Lett*. **2001**, *42*, 5227-5229.

9- Morris, J. V.; Mahaney, M. A.; Huber, J. R. *J. Phys. Chem.* **1976**, *80*, 969-974.

10- Soares, A. M.; Costa, S. P. G.; Gonçalves, M. S. T. "Synthesis and photophysical properties of new fluorescent benzobenzopyran bioconjugates of neurotransmitter amino acids", poster communication (P1.179), ESOC 2009, "The Sixteenth European Symposium on Organic Chemistry", Prague, Czech Republic, 12 to 16 July 2009.

11- Gangadasu, B.; Narender, P.; Raju, B. C.; Rao, V. J. *J. Chem. Res.* **2004**, *7*, 480-481.