Supplementary Material for Organic & Biomolecular Chemistry

Electronic Supplementary Information for the communication:

Remarkable incorporation of the first sulphur containing indole derivative: Another piece in the biosynthetic puzzle of crucifer phytoalexins

M. S. C. Pedras* and D. P. O. Okinyo

Department of Chemistry, University of Saskatchewan, 110 Science Place, Saskatoon, SK,

S7N 5C9, Canada. Fax: 1-306-966-4730; Tel: 1-306-966-4772

E-mail: s.pedras@usask.ca

* Corresponding author.

Experimental Section

General

All solvents were HPLC grade and used as such, except for THF and Et_2O (dried over sodium) and chloroform (glass redistilled). Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Flash column chromatography (FCC): silica gel, grade 60, 230-400 μm. Organic extracts were dried over Na_2SO_4 and the solvents were removed using a rotary evaporator.

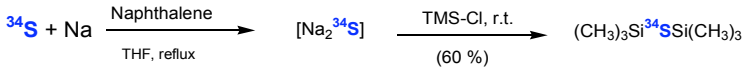
Analytical HPLC was performed with a liquid chromatograph (Agilent 1100 series HPLC system, Agilent Technologies, USA) equipped with a quaternary pump, an automatic injector, a photodiode array detector (DAD), a degasser, and a Hypersil ODS column (5 µm particle size silica, 4.6 i.d. × 200 mm), equipped with an in-line filter. HPLC retention times were obtained under the following conditions: mobile phase, H_2O-CH_3CN (75% : 25%) to CH_3CN (100%) for 35 min, linear gradient and a flow rate of 1.0 ml/min. For LC-ESI-MS, an Agilent 1100 series HPLC system equipped with an autosampler, binary pump, degasser, a photodiode array detector connected directly to a mass detector (Agilent G2440A MSD-Trap-XCT ion trap mass spectrometer) with an ESI source was used. Chromatographic separation was carried out at room temperature using an Eclipse XSB C-18 column (5 μm particle size silica, 150 mm × 4.6 mm I.D.). The mobile phase consisted of a gradient of 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B) (75% A to 75% B in 35 min, to 100% B in 5 min) and a flow rate of 1.0 ml/min. The ion mode was set as positive and negative. The interface and MSD parameters were as follows: nebuliser pressure, 70.0 psi (N_2); dry gas, N_2 (12.0 litres/min); dry gas temperature, 350 °C; spray capillary voltage 3500 V; skimmer voltage, 40.0 V; ion transfer capillary exit, 100 V; scan range, *m*/*z* 100-500. Ultrahigh pure He was used as the collision gas. Mass Spectral (MS) [high resolution (HR), electron impact

(EI)] data were obtained on a mass spectrometer using a solids probe; HPLC-HRMS-ESI was performed on Agilent HPLC 1100 series directly connected to QSTAR XL Systems mass spectrometer (Hybrid Quadrupole-TOF LC/MS/MS) with turbo spray ESI source.

NMR spectra were recorded on 500 MHz spectrometers. For ^{1}H NMR spectra (500 MHz) the chemical shifts (δ) are reported in parts per million (ppm) relative to TMS. The δ values were referenced to $CDCl_3$ ($CHCl_3$ at 7.28 ppm). For ^{13}C NMR (125.8 MHz) the chemical shifts (δ) were referenced to $CDCl_3$ (77.4 ppm). The multiplicities of the ^{13}C signals refer to the number of attached protons: s = C, d = CH, t = CH_2, q = CH_3. Fourier transform infrared (FT-IR) data were recorded on a spectrometer and spectra were measured by the diffuse reflectance method on samples dispersed in KBr.

Synthesis

^{34}S-Hexamethyldisilathiane

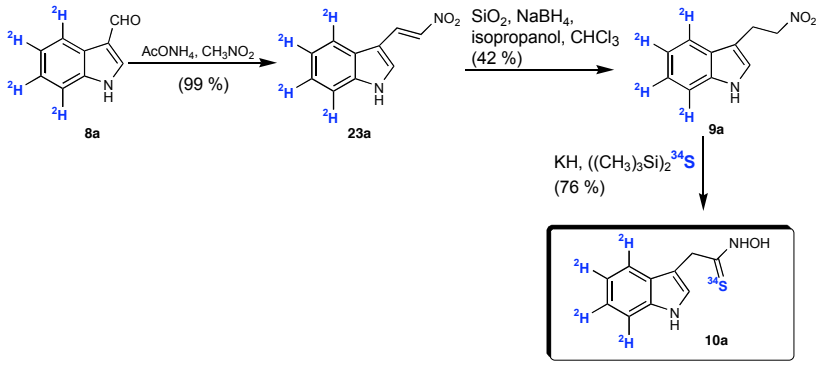


Scheme 1. Synthesis of ^{34}S-hexamethyldisilathiane.

Finely cut pieces of sodium (270 mg, 11.8 mmol) were added to a dry two-necked 50 ml round-bottomed flask charged with sulphur-34 (200 mg, 5.88 mmol) and naphthalene (70.9 mg, 0.553 mmol) in dry THF (6 ml). The reaction mixture was then sonicated for about 2 h during which the sodium and sulphur dissolves. The colour of the reaction mixture changes from yellow to orange to brown to light yellow to green to light green. The reaction mixture was then refluxed for 2 h, was cooled in ice-bath followed by drop wise addition of chlorotrimethylsilane (2.2 ml, 18 mmol) and the reaction mixture turns purple. The reaction mixture was stirred in ice-bath for further 10 min, then at room temperature for 3 h. A short-path distillation apparatus was used to distil off the reaction

mixture at 120–135 ºC to collect fraction 1 containing THF. Vacuum was then applied at 20 kPa to collect fraction 2, then gradually increased from 30 kPa to 68 kPa to collect fraction 3 containing ^{34}S-hexamethyldisilathiane as a colourless vile smelling liquid in 60 % yield based on ^{34}S (633.5 mg). The ^{34}S-hexamethyldisilathiane was analyzed by GC (t_R = 5.15 min), and GC-MS-EI *m/z* (relative abundance) 180 $[M]^+$ (50), 165 (100).

(4',5',6',7'-$^2H_4$)-Indolyl-3-[^{34}S]acetothiohydroxamic acid



Scheme 2. Synthesis of [4',5',6',7'-$^2H_4$]indolyl-3-[^{34}S]acetothiohydroxamic acid (**10a**).

Ammonium acetate (96.4 mg, 1.25 mmol) was added to a suspension of [4',5',6',7'-$^2H_4$]-indole-3-carboxaldehyde (**8a**)[1] (333 mg, 2.23 mmol) in CH_3NO_2 (966 μl, 17.8 mmol).[2] The resulting mixture was refluxed at 128 - 130 $^{\circ}$C for 2 h. The reaction mixture was then diluted with H_2O (20 mL), was extracted with CH_2Cl_2 (35 mL × 3), and the combined organic extract was washed with H_2O (40 mL), then brine (40 mL), was dried over Na_2SO_4 and was concentrated under reduced pressure to yield [4',5',6',7'-$^2H_4$]-3-(2-nitrovinyl)-indole (**23a**) in 99% yield (422 mg). To a mixture of [4',5',6',7'-$^2H_4$]-3-(2-nitrovinyl)-indole (**23a**) (422 mg, 2.20 mmol) and silica gel (4.40 g) in isopropanol (6.4 mL) and chloroform (35 ml) $NaBH_4$ (341 mg, 9.02 mmol) was added in portions of ca. 85 mg over 15 min.[3] The reaction mixture was stirred for further 60 min, during which the intense yellow colour of the reaction mixture disappeared. The excess $NaBH_4$ was then destroyed with 2 M HCl, the reaction mixture was filtered off and the silica gel was rinsed with CH_2Cl_2 (20 ml × 3). The

combined organic extract was washed with H_2O (40 ml), was dried over Na_2SO_4 and was concentrated under reduced pressure. The reaction mixture was subjected to FCC eluting with CH_2Cl_2 to yield [4',5',6',7'-$^2H_4$]3-(2-nitroethyl)indole (**9a**) (178 mg, 42 %) as a brown solid.

[4',5',6',7'-$^2H_4$]3-(2-Nitroethyl)-indole (**9a**) (60.0 mg, 0.309 mmol) in dry THF (4 ml) was added drop wise to potassium hydride (331 mg, 2.47 mmol) in a dry 50 ml round-bottomed flask, cooled in an ice bath. After stirring for 30 min with cooling in an ice bath, the reaction mixture was allowed to warm up to room temperature and stirred for an additional 60 min. The reaction flask was wrapped with aluminum foil and ^{34}S-hexamethyldisilathiane (310 μl, 1.43 mmol) was added drop wise.[4] After 60 min, the reaction mixture was cooled in an ice bath, was diluted with ice-cold H_2O (5 ml), was neutralized with 5 % H_2SO_4 (2 ml), was extracted with CH_2Cl_2 (30 ml × 2), the combined organic extract was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was subjected to FCC (CH_2Cl_2:EtOAc, 100:0, then 60:40) to yield [4',5',6',7'-$^2H_4$]-indolyl-3-[^{34}S]acetothiohydroxamic acid (**10a**) as brownish crystals in 76 % yield (50 mg).

^{1}H NMR (500 MHz $CDCl_3$): δ 8.26 (br s, 1H), 7.20 (s, 1H), 4.27 (s, 2H); HPLC-HRMS-ESI *m/z* measured 213.0797 (213.0795 calcd for $C_{10}H_7{}^2H_4N_2O^{34}S$); HPLC-MS-ESI *m/z* (relative abundance) 213 $[M + H]^+$ (100), 195 (15), 180 (13), 134 (18).

Elicitation and feeding experiments

Plant material: Rutabaga tubers were purchased from local markets.

Feeding and extraction of phytoalexins: Rutabaga (*B. napus* ssp. *rapifera*) root tubers were cut horizontally in 10-15 mm thick discs and cylindrical holes (16 mm in diameter) were made on one side of the discs with a cork-borer.[5] The discs were kept in tightly sealed plastic boxes and incubated at 20 °C in darkness. After 24 h, the discs were UV-irradiated on the surface with holes for 20 min, and were incubated for further 24 h. Precursors (5×10^{-4} M) dissolved in H_2O-CH_3OH-

Tween 80 (95/5/0.05, v/v) were pipetted into each hole (500 μl per hole) and the discs were further incubated at 20 °C in darkness. Following adsorption of precursor solution, the holes were filled with water. The aqueous solution in the holes was withdrawn with a pipette after 72-h incubations and was extracted with EtOAc (30 ml × 2). The combined organic extract was dried over Na_2SO_4 and solvent was removed under reduced pressure. The residue obtained was dissolved in CH_3CN (400 μl) and was analyzed by HPLC-DAD. The residue was then subjected to preliminary clean up by micro-FCC in a Pasteur pipette column packed with silica gel to 35 mm, eluting with 20 ml of CH_2Cl_2 and then 20 ml of CH_2Cl_2-EtOAc (40:60, v/v) to yield two fractions. The phytoalexin containing fractions obtained were analyzed by HPLC-DAD, HPLC-MS-ESI, and HPLC-HRMS-ESI. Control experiments were similarly prepared by incubating rutabaga root tubers with non-labelled precursors or with carrier solution only.

Extraction of glucosinolates: Tissue around the holes was cut, was soaked in MeOH, was ground and was extracted with an aqueous CH_3OH solution. Extraction was carried out overnight on a shaker at 170 rpm, after which the tissue was filtered off and the filtrate was concentrated. The residue obtained was subjected to RP-FCC H_2O-CH_3CN (100:0; 90:10, 80:20, v/v) to yield 3 fractions containing a mixture of indole glucosinolates. The fractions were dissolved in H_2O-CH_3OH (50:50) and were analyzed by HPLC-DAD, HPLC-MS-ESI, and HRMS-ESI.

References

1 M. S. C. Pedras and D. P. O. Okinyo, *J. Label. Compd. Radiopharm.*, 2006, **49**, 33-45.
2 L. Canoira, J. G. Rodriguez, J. B. Subirats, J-A. Escario, I. Jimenez and A. R. Martinez-Fernandez, *J. Med. Chem.,* 1989, **24**, 39-42.
3 A. K. Sinhababu and R. T. Borchardt, *Tetrahedron Lett.,* 1983, **24**, 227-230.
4 J. R. Hwu and S. C. Tsay, *Tetrahedron*, 1990, **46**, 7413-7428.
5 M. S. C. Pedras, S. Montaut and M. Suchy, *J. Org. Chem.,* 2004, **69**, 4471-4476.