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Synthesis of a doxycycline-[$^{13}CD_3$] standard

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A stable isotope labelled mass spectrometry internal standard of the antibiotic doxycycline was prepared to assist in pharmacokinetic analyses. Our approach was to first *N*-demethylate doxycycline using a non-classical Polonovski reaction and then re-methylate using methyl-[$^{13}CD_3$] iodide, which gave doxycycline-[$^{13}CD_3$] with an isotopic purity of 99%.

Keywords: MS internal standard; doxycycline; antibiotic; demethylation

Introduction

The tetracycline family, which was discovered as a result of the screening of soil samples for antibiotics, has been effectively used for decades. This drug class is recognized as having interesting pleiotropic properties,[1] and currently, there are over 200 clinical trials underway.[2] Based on this renewed interest, the study of potential uses of tetracyclines is expanding. As a member of the tetracycline antibiotic class, doxycycline is used for the treatment of a variety of infections, including sinusitis, pelvic inflammatory disease, acne and rosacea. Prophylactically, it is used against malaria, by preventing the development of parasites in the blood that cause malaria.[3,4] Doxycycline is also used prophylactically and therapeutically for the prevention and treatment of anthrax and plague, and for the treatment of the tick borne diseases such as Lyme[5] and Rocky Mountain spotted fever.[6]

The therapeutic potential and applications of tetracyclines are being redefined and expanded, and as such, stable isotope labelled doxycycline is a valuable research tool for studying its biodistribution and mechanism of action.

Results and discussion

A mass spectroscopy (MS) analytical standard of doxycycline with M + 4 isotopic incorporation was prepared as a chemical probe to assist in pharmacokinetic analyses. Our approach for the efficient synthesis of stable isotope labelled doxycycline-[$^{13}CD_3$] **4** was to first *N*-demethylate doxycycline and then re-methylate using iodomethane-[$^{13}CD_3$].

This work extends the utility of the non-classical Polonovski reaction from alkaloids to tetracyclines.[7,8] Formation of the demethylated tetracyclines is an important intermediate for the synthesis of novel tetracyclines as well as labelled standards. Utilizing a modified Polonovski reaction in a two-step process as shown in Scheme 1, the free base of doxycycline **1** was readily converted to the *N*-oxide **2** using 3-chloroperoxybenzoic acid (*m*-CPBA). The *N*-oxide **2** was 95% pure by LC/MS (liquid chromatography mass spectrometry). After conversion of the *N*-oxide **2** to the hydrochloride, treatment with iron ($Fe(0)/FeCl_3$) formed *N*-desmethyl product **3** in 79% yield by LC/MS. A side reaction gave 21% doxycycline **1** as a byproduct by LC/MS, which was readily removed during the workup and purification procedure. Purification with ethylenediaminetetraacetic acid to remove the iron salts, followed by reverse phase flash chromatography and crystallization gave 99% pure *N*-desmethyldoxycycline **3** with 0.6% doxycycline **1** in 9% overall yield.

We determined that the best approach for methylation of *N*-desmethyldoxycycline **3**, a secondary amine, with labelled methyl iodide-[$^{13}CD_3$] was to use polymer-supported triphenylphosphine and diisopropyl azodicarboxylate in tetrahydrofuran as shown in Scheme 2. These reaction conditions provided the corresponding tertiary amine product **4**. With this method, only trace amounts of the quaternization product were observed even in the presence of a large excess of methyl iodide (three equivalents).[9] Purification using reverse phase chromatography followed by crystallization from ethanol/water and then treatment with HCl provided highly pure doxycycline-[$^{13}CD_3$] hyclate with an isotopic purity of 99% (33% yield).

Conclusion

This approach for the synthesis of stable isotope labelled doxycycline **4** by first *N*-demethylation and then re-methylation with stable isotope labelled iodomethane provides an efficient synthesis of doxycycline-[$^{13}CD_3$] **4**, which is not readily accessible by other routes.

Experimental

General methods

All reactions were carried out under inert argon atmosphere using commercial reagents of the highest grade and without further purification. Doxycycline hyclate was purchased from Sigma-Aldrich (St. Louis, MO). Methyl iodide-[$^{13}CD_3$] was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Intermediates were analysed by nuclear magnetic resonance (NMR) (Varian Mercury VX 300 MHz or Varian 400 MHz). All chemical shifts are reported in parts per million (δ) downfield from

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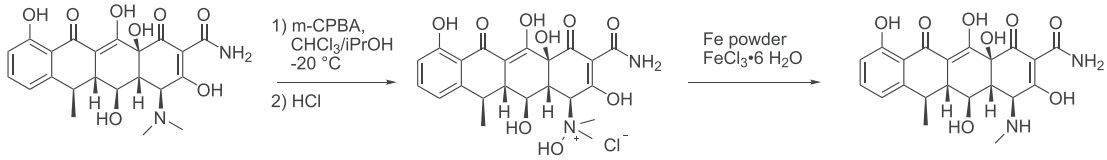
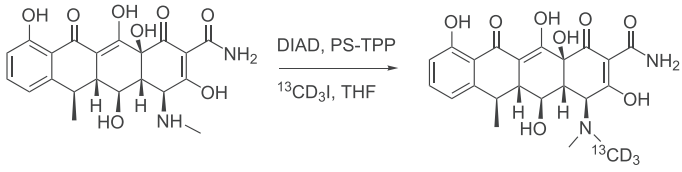


1 2 3

Scheme 1. N-demethylation of doxycycline



3 4

Scheme 2. Synthesis of stable isotope labelled doxycycline

tetramethylsilane. High resolution mass spectra (HRMS) were run using a Thermo LTQ-Orbitrap XL hybrid ion-trap mass spectrometer. LC/MS data were obtained using a Thermo LCQ Fleet and Finnigan Surveyor System. Reverse phase chromatography was performed using Silicycle C_{18} silica gel (40–63 μ) on a Biotage Isolera IV preparative flash chromatography system.

N-desmethyldoxycycline [(4S,4aR,5S,5aR,6R,12aS)-3,5,10,12,12a-pentahydroxy-6-methyl-4-(methylamino)-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide] (**3**)

Doxycycline hyclate (4.08 g, 7.95 mmol) was free based in methanol with sodium hydroxide (1 equiv) and evaporated *in vacuo*. Doxycycline (*free base*) **1** plus NaCl was suspended in $CHCl_3$/isopropanol (3:1) (240 mL) and then warmed to 50 °C to maximize solubility. The resulting mixture was cooled to −78 °C and then treated with *m*-CPBA (97%, 1.72 g, 1.25 equiv) in one portion. The resulting suspension was stirred at −20 °C to −10 °C over 45 min to form the *N*-oxide **2**. The formation of the *N*-oxide **2** was monitored by MS. The *N*-oxide mixture was acidified with 1.23 M ethereal HCl (6.46 mL, 7.95 mmol). The mixture at −20 °C was treated with iron powder (66.6 mg, 325 mesh, 97%, 0.15 equiv catalyst) and a solution of $FeCl_3$•6 H_2O (64.5 mg, 0.03 equiv catalyst) in isopropanol (1.5 mL). The stirred mixture was allowed to warm to RT (room temperature) over 2 h. The LC/MS showed conversion to *N*-desmethyl product **3** in 79% yield with 21% doxycycline **1** byproduct contaminant. The resulting solution was cooled in an ice bath and then poured into 10 mM ethylenediaminetetraacetic acid tetrasodium salt (pH 10) (400 mL), stirring for 15 min at RT lowered the pH to 3. The phases were separated, and the aqueous phase was washed with $CHCl_3$ (2 × 50 mL). The aqueous solution was adjusted to pH 7 with 1 M NaOH (14 mL) and then filtered through a plug of reverse phase C_{18} silica gel (25 g) rinsing with water. Lyophilization gave a crude solid (4 g), which was dissolved in water and injected onto a column of 50 g of Silicycle C_{18} eluting with a gradient of 100% water to 100% CH_3CN giving 560 mg of crude product. This material was crystallized from ethanol/water (1/1) leaving 302 mg (8.8% yield) of 99.4% pure *N*-desmethyldoxycycline **3** with 0.6% doxycycline **1**. ^{1}H NMR: (300 MHz, CD_3OD) δ 7.46 (t, 1H, *J* = 8.2 Hz), 6.93 (d, 1H, *J* = 8.2 Hz), 6.82 (d, 1H, *J* = 8.2 Hz), 3.76–3.65 (m, 2H), 2.82–2.66 (m, 1H), 2.77 (s, 3H), 2.50–2.40 (m, 2H), 1.51 (d, 3H, *J* = 6.9 Hz). FTIR: (Solid Phase ATR) 3205, 2529, 2160, 1977, 1567, 1455, 1394, 1323, 1244, 1220, 1114, 1044, 1001, 933, 886, 858, 809, 709 cm^{-1}. UV: (MeOH) λ_{max} 360.0 nm (ε□14,335), 274.9 (14,663). Optical rotation: (MeOH) c = 0.051, [α] = −174°. Elemental analysis: calculated for $C_{21}H_{22}N_2O_8$•2.7 H_2O: C, 52.65; H, 5.76; N, 5.85; found: C, 52.76; H, 5.76; N, 5.78. MS: (ESI) m/z (relative intensity) 431 (M + H, 100), 445 (0.62). HPLC: Varian Pursuit C_{18} (3 μ), 50 × 2 mm; 0.1% formic acid in water/0.1% formic acid in CH_3CN 100/0 to 60/40 over 5 min then hold; 350 nm; R_t = 5.60 min; purity >99%.

*Doxycycline-[$^{13}CD_3$] hyclate [(4S,4aR,5S,5aR,6R,12aS)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide] (**4**)*

A suspension of triphenylphosphine, polymer bound (200–400 mesh, ~3.0 mmol/g, 174.3 mg, 0.523 mmol, 1.5 equiv) in THF (20 mL) was stirred for 1 h at RT. Next, diisopropyl azodicarboxylate (DIAD) (103.1 ⊠L, d 1.027, 105.8 mg, 0.523 mmol, 1.5 equiv), *N*-desmethyldoxycycline (150 mg, 0.349 mmol) and iodomethane-$^{13}CD_3$ (^{13}C, 99%; D_3, 99%, stabilized with copper wire) (0.065 mL, d 2.34, 152.3 mg, 1.044 mmol, 3 equiv) were added. The reaction mixture was stirred at RT for 4 days and then diluted with ethyl ether (20 mL) and extracted with water (3 × 20 mL). The combined aqueous phases were washed with ethyl ether (20 mL), evaporated *in vacuo* to remove residual volatile organic solvent and then lyophilized to give crude product (133 mg). This material was filtered through a plug of Silicycle C_{18} (700 mg) eluting with CH_3CN/water (4/6). Evaporation gave 91 mg of a solid, which was acidified with 1 N HCl (0.2 mL) and crystallized from EtOH/water (1/1) (4 × 0.33 mL) at 0 °C. Further evaporation *in vacuo* gave the hyclate product (67 mg). This material was triturated with hexane (2 × 1 mL) and ether (2 × 1 mL) and evaporated *in vacuo* giving 60 mg of pure product **4** (33% yield) with an isotopic purity of 99%. ^{1}H NMR: (400 MHz, CD_3OD) δ 7.49 (t, 1H, *J* = 8.2 Hz), 6.95 (d, 1H, *J* = 8.2 Hz), 6.84 (d, 1H, *J* = 8.2 Hz), 4.42 (d, 1H, *J* = 2.8 Hz), 3.63–3.54 (m, 2H), 3.00–2.89 (m, 3H), 2.82–2.70 (m, 2H), 2.61–2.54 (m, 1H), 1.54 (d, 3H, *J* = 6.8 Hz), 1.17 (t, 1.5H, *J* = 7.0 Hz, hyclate ethanol methyl peak). ^{13}C NMR: (75 MHz, DMSO-d_6) δ 192.54, 173.89, 171.71, 161.10, 147.85, 136.75, 115.93, 115.66, 115.49, 107.23, 95.15, 73.11, 67.99, 64.50, 63.04, 55.97, 53.81, 45.25, 44.00–40.00 (m, 4C), 18.52, 15.85. HRMS: (ESI, Full-scan) m/z (relative intensity) calculated, 449.18273; found, 449.18266 (M + H, 100). FTIR: (Solid Phase ATR) 3205, 2529, 2160, 1977, 1574, 1455, 1243, 1002, 885, 710 cm^{-1}. UV: (MeOH) λ_{max} 355.0 nm (ε14,746), 270.1 (14,888). Optical Rotation: (MeOH) c = 0.099, [α] = −100°. HPLC: Varian Pursuit C_{18} (3 μ), 50 × 2 mm; 0.1% formic acid in water/0.1% formic acid in CH_3CN 100/0 to 60/40 over 5 min then hold; 360 nm; R_t = 5.80 min; UV purity >99%.

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Conflict of interest

The authors did not report any conflict of interest.

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