Facile Synthesis of Tilmicosin and Tylosin Related Haptens for

Use as Protein Conjugates

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Synthesis is described for the haptens 23-demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)-aminotilmicosin (6) from 5-O-mycaminosyltylonolide (OMT) and for 23-demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)-amino-20-dihydrotylosin (10) from demycinosyltylosin (DMT), respectively. The mild reaction conditions used to synthesize the second hapten, DMT derivative 10, were necessary to overcome instabilities and acid lability of DMT. The haptens synthesized here may be further used to produce protein conjugates useful in developing antibodies against the antibiotics tilmicosin and tylosin.

Tilmicosin (1, Fig. 1) is a semi-synthetic macrolide antibiotic approved for veterinary use in the U.S. for cattle, swine, and poultry to combat respiratory disease. It also is used with sheep in various areas of the world. Tilmicosin (1) is derived from the macrolide antibiotic tylosin (2, Fig. 1), which is produced by *Streptomyces fradiae*. The synthesis of 1 from 2 has been described[1,2]. Most antimicrobial agents have limited ability for cellular penetration, but 1 was shown to accumulate in bovine lung tissue[3]. To investigate potential tissue binding sites of these molecules, it would be advantageous to produce monoclonal antibodies specific for 1 and 2.

Previous attempts have been made to produce antibodies to these macrolides. JACKMAN et al. reported preparation of polyclonal antibodies against desmycosin, conjugated through the C20 aldehyde group, for use in determining residues of 1 in feeds and tissues[4]. SILVERLIGHT et al. reported that such polyclonal antibodies had 96% cross-reactivity with 2[5]. WICKER et al. reported the use of polyclonal antibodies to 2 in a fluorescence immunoassay for detecting 2 in premix and feeds[6]. The antibodies produced to 2 also cross-reacted with 1. YAO and MAHONEY produced polyclonal antibodies to 23-deoxy-23-amino-OMT; however, these antibodies demonstrated reactivity

with 12-, 14-, and 16-membered macrolides possessing amino-substituted sugar moieties, regardless of the presence of neutral sugar residues[7].
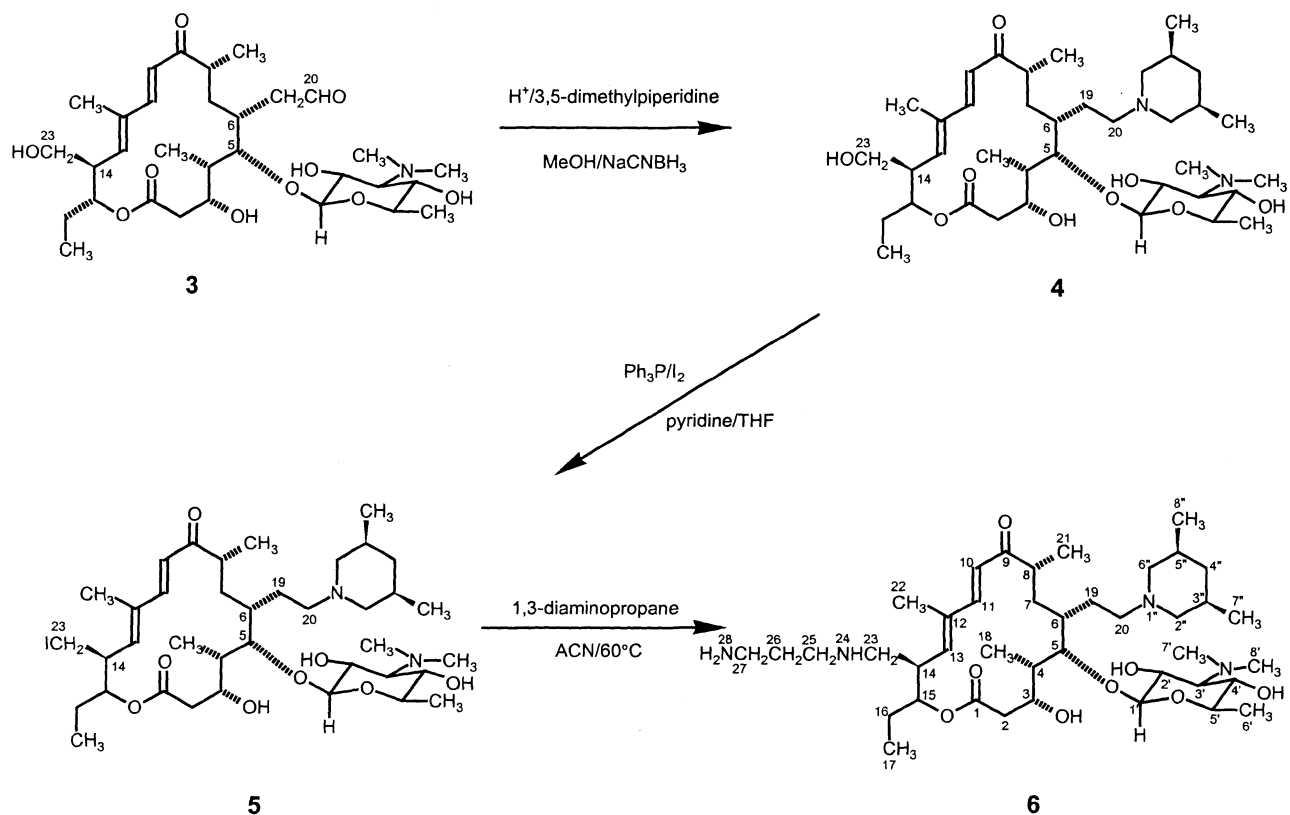
For our experimental purposes, we required monoclonal antibodies that had high specificity. In order to develop antibodies specific for tilmicosin and tylosin, protein conjugates of derivatives 1 and 2 were required that would present immunologically unique portions of the molecules to the immune system. Therefore, the C20 aldehyde group would not be a good choice for the linkage site. Rather, we considered the C23 position to be a good linkage site that would present the best structural differences of 1 and 2 to the immune system. The selection of antibodies with the best specificity may then be accomplished through the monoclonal screening process.

We expected that the synthesis of analogues of 1 and 2 containing 1,3-diaminopropane linker arms would provide suitable haptens. The amino group was selected since it can be utilized with a number of standard synthetic strategies to produce protein conjugates suitable for antibody production, whereas the linker length also provides sufficient spacing to result in favorable antibody production. This paper describes the synthesis of 23-demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)-

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Fig. 1.   Chemical structures of tilmicosin (1), and tylosin (2).

aminotilmicosin (6) from 5-O-mycaminosyltylonolide (OMT) (3) and 23-demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)-amino-20-dihydrotylosin (10) from the acid labile demycinosyltylosin (DMT) (7), which also contains a reactive aldehyde group.

Results

The starting material for the synthesis of 6 was the fermentation product OMT (3) (Scheme 1), which was reductively aminated following the literature procedure of KIRST et al. using 3,5-dimethylpiperidine in MeOH at room temperature[8]. This gave 23-demycinosyl-tilmicosin (4), which was extracted into pH 4 buffer, treated with base, reextracted with CH_2Cl_2, and used without further purification. The product was converted to its 20-iodo analog (5) following the literature procedure of KIRST et al.[9], employing a slow addition of iodine in dry THF at 0°C into triphenylphosphine in dry THF/dry pyridine. 23-Demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)-aminotilmicosin (6) was produced by displacement of the 23-iodo group with 1,3-diaminopropane in ACN at room temperature in an overall yield of 14% according to the method of KIRST et al.[9].

Synthetic manipulations of the macrolide intermediate, DMT, to produce the desired hapten suitable for protein linkage proved to be a difficult endeavor for two reasons. The aldehyde group at C20 of 2 is reactive and can form unwanted by-products during the required chemical reactions or purification steps. In addition, the linkage between the carbohydrate residues (i.e., between the amino sugar and the mycarosyl residue) is acid labile.

The Streptomyces fradiae GS48 fermentation product[9], DMT (7), was reacted with triphenylphosphine and iodine according to a modified method of SAKAMOTO et al.[10], employing pyridine in the presence of 4 Å molecular sieves at 0°C, to obtain 23-deoxy-23-iodo-DMT (8) (Scheme 2). There was some initial confusion during the iodination reaction using triphenylphosphine. The reaction was followed by ion spray mass spectral analysis, and showed a large peak for triphenyphosphine oxide ($Ph_3P=O$) at m/z 279 (Fig. 2). The spectrum also showed several dominant peaks at m/z 557 ($Ph_3P=O)_2{}^+$, m/z 579 ($Ph_3P=O)_2Na^+$, m/z 588 ($Ph_3P=O)_2OMe^+$, m/z 602 ($Ph_3P=O)_2Na_2{}^+$, and m/z 857 ($Ph_3P=O)_3Na^+$ which dwarfed the product, 23-deoxy-23-iodo-DMT, peak at m/z 852. These peaks were evident whenever triphenylphosphine was used in the reaction. This result was verified by ion spray mass spectrometry of the isolated and crystallized $Ph_3P=O$. Product 8 was not

Scheme 1. Synthesis of 23-demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)-aminotilmicosin (6) from OMT (3).

Scheme 2. Synthesis of 23-demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)-amino-20-dihydrotylosin (10) from DMT (7).
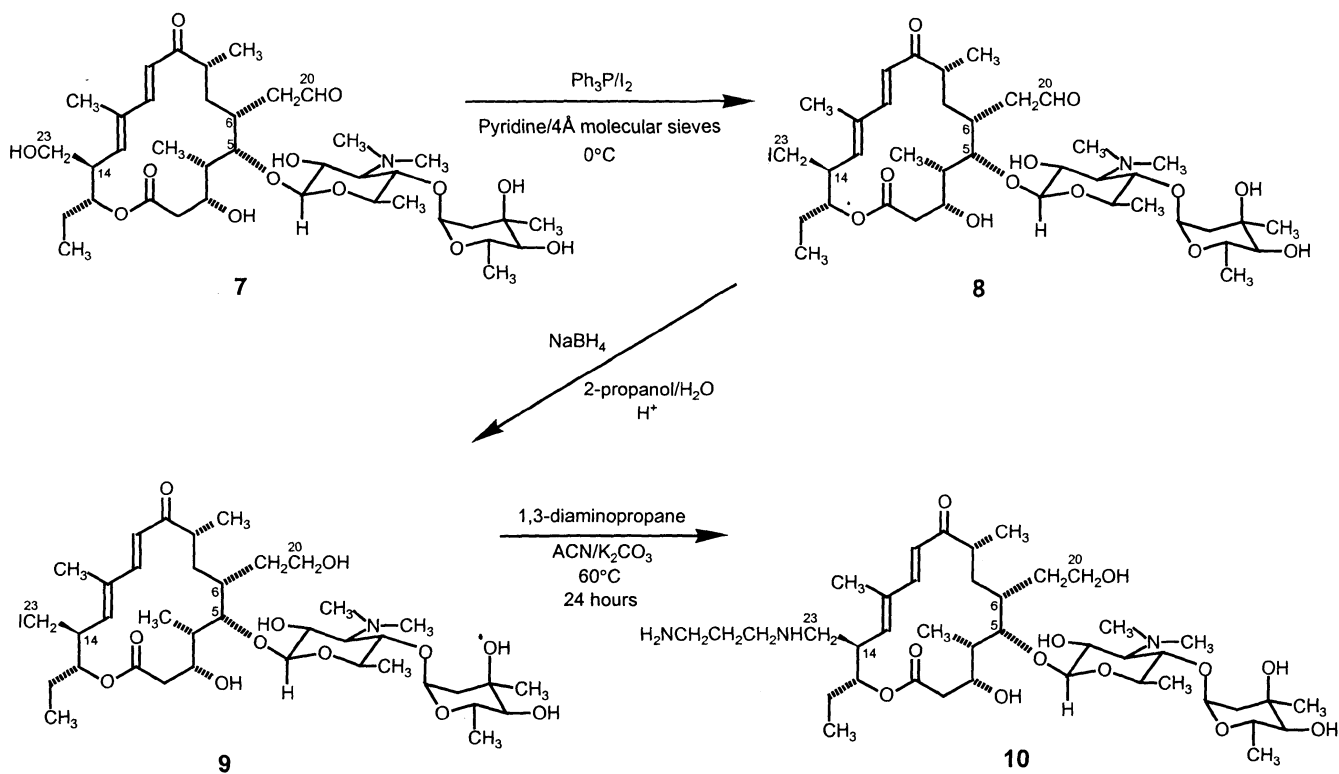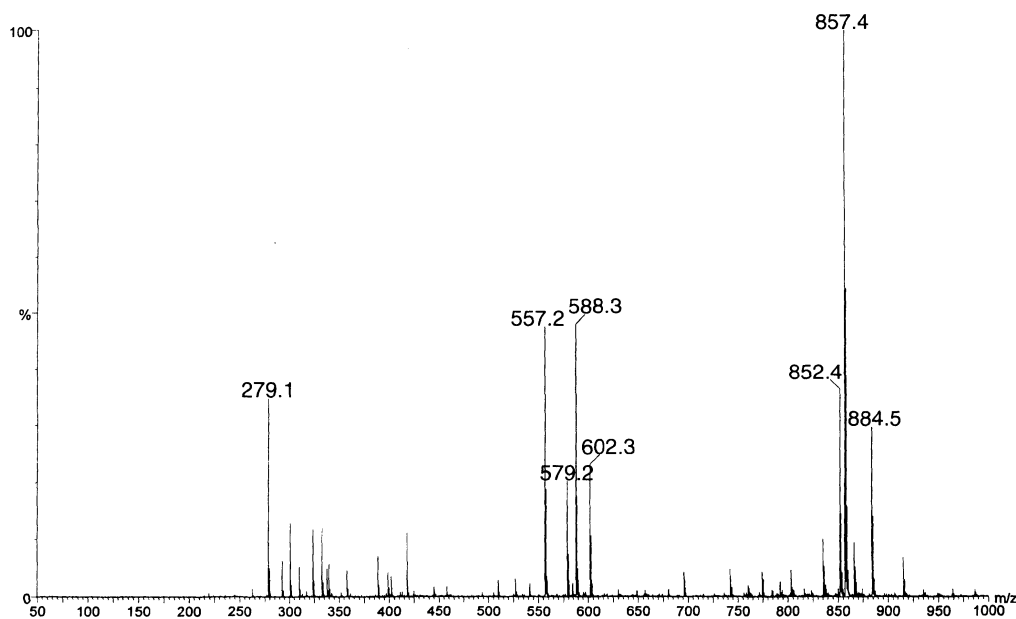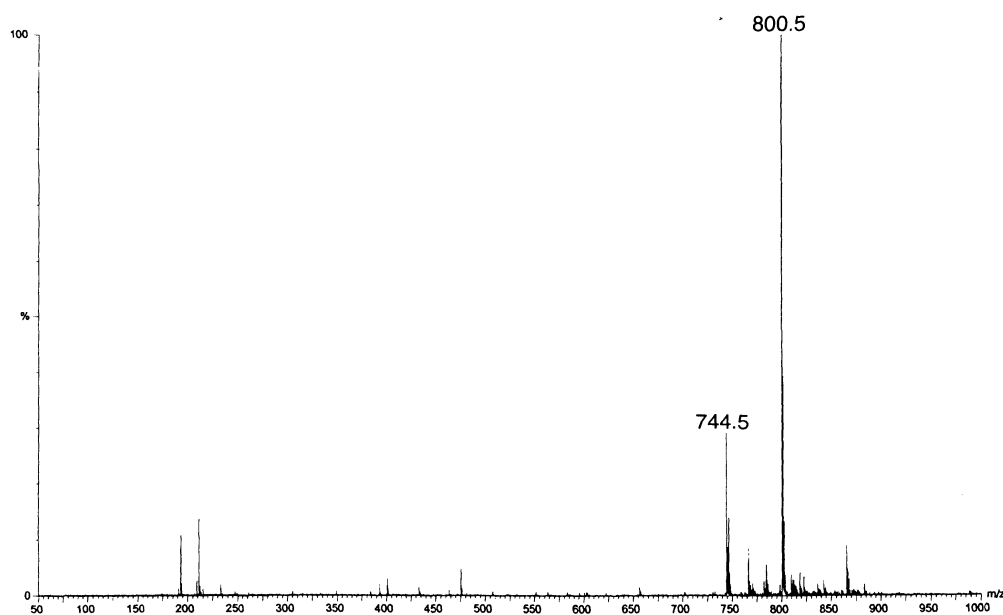
Fig. 2.   ESI-TOF-MS of DMT iodination reaction products.

Fig. 3.   ESI-TOF-MS of crude product 10.

purified due both to the reactivity of the C20 aldehyde group and the acid lability of the linkage between the two carbohydrate groups. Rather, reducing it to the alcohol immediately deactivated the aldehyde group at C20 of 8[11]. Thus, product 8 was reacted with sodium borohydride in isopropanol and water to form the alcohol 9 according to

the method of KIRST et al.[11]. Upon evaporation of CH_2Cl_2 from the product 9, a glass formed, which was directly used in the next reaction. The desired hapten 10 was achieved in overall 11% yield from 7 via an iodide displacement reaction by employing a five-fold excess of 1,3-diaminopropane in ACN in the presence of potassium

carbonate, a modified method of KIRST et al.[9]. The ESI-TOF-MS of the crude product 10 is shown in Fig. 3.

Since the resulting two haptens, 6 and 10 contain an NH_2 linker arm, they can be conjugated to proteins under a number of commonly used conditions. The length of the linker arm is appropriate for favorable recognition and antibody production by the immune system. Experiments to this end are on-going.

Experimental

General Analytical Methods

NMR data were acquired on a Bruker Avance 500 NMR spectrometer, and the following experiments were performed: 1D ^{1}H, 1D ^{13}C, ^{1}H-^{1}H 2D COSY, ^{1}H-^{1}H 2D ROESY, ^{1}H-^{1}H 2D TOCSY, ^{1}H-^{13}C 2D g-HSQC, and ^{1}H-^{13}C 2D g-HMBC. Positive ion electrospray ionization (ESI) mass spectra were acquired using either a Micromass Quattro LC mass spectrometer or a Micromass LCT time-of-flight mass spectrometer. Most of the chemicals were purchased from Aldrich Chemical Company or prepared by the Eli Lilly and Company Natural Products Division and were used without further purification. Semi-preparative high performance liquid chromatography (HPLC) was accomplished using a Supelcosil LC-18-DB Semi-Prep 25 cm×10 mm ID column by Supelco, Inc. (Bellefonte, PA) with the solvent system of ACN/MeOH/^{1}/$_2$% NH$_4$OAc buffer (56^1/$_2$:41:2^1/$_2$, v/v/v) at 4.75 ml/minute using Waters M-6000 pumps and a Waters 996 photodiode Array Detector (Waters Corporation, Milford, MA). Flash chromatography was carried out using silica gel EM Science Kielselgel 60 (230~400 mesh ASTM).

23-Demycinosyl-tilmicosin (4)

OMT (3; 3.0 g, 5.0 mmol) was dissolved in anhydrous methanol (30 ml). 3,5-dimethylpiperidine (1.3 ml, 9.8 mmol) was added followed by sodium cyanoborohydride (0.64 g, 10.1 mmol). This mixture was allowed to stir at room temperature for 24 hours and was monitored by HPLC. The mixture was then diluted with EtOAc and extracted with commercially available pH 4 buffer (3 times). The buffer was brought to pH 12 with a 50% NaOH soln. The aqueous layer was then extracted with CH$_2$Cl$_2$ (3 times). The CH$_2$Cl$_2$ layers were combined, washed with brine, and then dried over anhydrous sodium sulfate. The soln was then evaporated at room temperature under reduced pressure to give 4 as a pale yellow solid (2.7 g; 78% yield). This material was used without further purification.

23-Demycinosyl-23-deoxy-23-iodo-tilmicosin (5)

23-Demycinosyl-tilmicosin (4; 7.8 g, 11.2 mmol) was dissolved in anhydrous THF (40 ml). Triphenylphosphine (4.4 g, 16.8 mmol) was added to this soln followed by pyridine (10 ml), and the mixture was cooled on an ice bath. Iodine (4.2 g, 16.5 mmol) in anhydrous THF (15 ml) was added drop-wise to the ice-cold soln. When the addition was complete the mixture was allowed to stir at room temperature for 18 hours. The reaction was then quenched by addition of 5% sodium thiosulfate soln drop-wise until the iodine color dissipated. The mixture was then diluted with CH$_2$Cl$_2$ and washed with satd NaHCO$_3$. The CH$_2$Cl$_2$ was then washed with brine, dried over anhydrous sodium sulfate and evaporated at room temperature under reduced pressure to a small volume. This material was diluted with fresh CH$_2$Cl$_2$ and the resulting soln was extracted with 1 N HCl. The aqueous soln was made alkaline with 5 N NaOH and reextracted with EtOAc. The EtOAc soln was washed with brine, dried over anhydrous sodium sulfate and evaporated at room temperature under reduced pressure. This gave 5 as a light yellow solid (2.7 g, 30% yield). This material was used without further purification; HPLC showed that the product was approximately 68% pure.

23-Demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)-aminotilmicosin (6)

Crude 23-demycinosyl-23-deoxy-23-iodo-tilmicosin (5; 3.0 g, 1.9 mmol) was dissolved in ACN (50 ml). Commercially available 1,3-diaminopropane (780 µl, 9.4 mmol) was added to this soln and the mixture was stirred at 60°C for 19 hours. The reaction mixture was cooled to room temperature and evaporated under reduced pressure. The crude product was taken up between EtOAc and freshly prepared pH 4 buffer (Note: avoid commercial buffers containing formaldehyde). The phases were separated and the aqueous phase was washed with fresh EtOAc until no more UV active material was removed. The buffer was brought to pH 12 with 5 N NaOH and reextracted with CH$_2$Cl$_2$. The CH$_2$Cl$_2$ was dried over anhydrous sodium sulfate and evaporated at room temperature at reduced pressure giving 6 (0.84 g, 60% yield based on purity of 5). To obtain ultra pure material 6 (120 mg) was purified by semi-prep HPLC, as described in the general analytical methods section. The collected material was diluted with pH 10 NaHCO$_3$ buffer (75 ml), and extracted with fresh CH$_2$Cl$_2$ (3×30 ml). The combined CH$_2$Cl$_2$ layers were dried over sodium sulfate, filtered through Whatman #41 filter paper, and evaporated under vacuum at room temperature to obtain 54 mg 6 at greater than 99% purity. ESI-MS m/z 751.6 (M+1)$^+$; ^{1}H- and ^{13}C-

Table 1. ¹H- and ¹³C-NMR data for product **6**.

Position	¹³C	¹H	Position	¹³C	¹H
1	173.03	—	23	51.25	2.75, 2.69
2	40.17	2.30, 1.97	24	—	—
3	67.09	3.78	25	49.10	2.66
4	42.78	1.59	26	N.A.	N.A.
5	80.00	3.60	27	N.A.	N.A.
6	32.64	1.63	28	—	—
7	34.60	1.74, 1.48	1'	105.58	4.27
8	45.85	2.60	2'	71.25	3.37
9	203.72	—	3'	71.66	2.37
10	119.39	6.50	4'	71.82	3.08
11	148.12	7.21	5'	73.92	3.31
12	135.80	—	6'	18.24	1.21
13	145.14	5.71	7', 8'	42.04	2.51
14	45.94	2.93	1"	—	—
15	75.80	4.76	2"	59.76	2.87, 1.06
16	25.93	1.88, 1.60	3"	30.87	1.83
17	9.97	0.92	4"	43.48	1.70, 0.47
18	9.29	0.99	5"	30.66	1.85
19	25.21	1.71	6"	64.38	2.68, 1.41
20	55.40	2.76, 1.99	7"	19.88	0.97
21	17.91	1.19	8"	19.98	0.80
22	13.19	1.85			

N.A. = Not Assigned

NMR data are presented in Table 1.

23-Deoxy-23-(3-aminoprop-1-yl)-amino-20-dihydro-DMT (10)

DMT (7; 0.45 g, 0.61 mmol) was dissolved in anhydrous pyridine (3 ml). Triphenylphosphine (0.32 g, 1.2 mmol) was added, followed by 4 Å molecular sieves (pyridine just covered the molecular sieves) and this mixture was stirred for 80 minutes at room temperature under dry argon. The mixture was cooled on an ice bath, and a soln of I_2 (0.46 g, 1.8 mmol) in anhydrous pyridine (1 ml) was added dropwise *via* a syringe. The mixture, having a noticeable brown

I_2 color, was stirred for 80 minutes in the ice bath and then at room temperature for 1 hour. The mixture was filtered through a double-layered sandwich of Whatman #2 and #41 filter paper with the aid of CH_2Cl_2 (40 ml). The filtrate was washed with satd $NaHCO_3$ (75 ml), and extracted three successive times with fresh CH_2Cl_2 (40 ml, 2×30 ml), and the CH_2Cl_2 layers were combined. The combined layers were washed with 1 M sodium thiosulfate (80 ml), and the aqueous layer was reextracted with fresh CH_2Cl_2 (2×30 ml). The CH_2Cl_2 layers were combined, dried over anhydrous sodium sulfate, and evaporated under vacuum at room temperature to afford 23-deoxy-23-iodo-DMT (8).

This material was dissolved in 2-propanol (6 ml) and H_2O (4 ml), and sodium borohydride (5.8 mg, 0.15 mmol) was added in small portions. Forty-five minutes after the addition was complete, the pH of the reaction mixture was adjusted to 7.0 by addition of 0.1 N H_2SO_4. The neutralized soln was evaporated to a small volume to remove the isopropanol, and then treated with satd $NaHCO_3$ (30 ml). The mixture was extracted with fresh CH_2Cl_2 (3×20 ml), and the combined CH_2Cl_2 extracts were washed with satd NaCl (35 ml), then dried over anhydrous sodium sulfate, filtered, and evaporated under vacuum at room temperature to give 23-deoxy-23-iodo-20-dihydro-DMT (9). This material was dissolved in ACN (5 ml) in a 10 ml Reacti-vial (Pierce, Rockford, IL). Potassium carbonate (0.17 g, 1.2 mmol) was added to the reaction mixture, followed by 1,3-diaminopropane (1.7 mmol, 0.14 ml). The reaction mixture was stirred at 60°C, and the course of the reaction was followed by TLC on HPTLC-Fertigplatten, Kieselgel 60 F_{254} with a concentration zone (EM Science, Gibbstown, NJ) using EtOAc/EtOH (80:20, v/v). After 24 hours at 60°C, the mixture was cooled to room temperature, and filtered through a sandwich filter of Whatman #2 and #41 to remove the potassium carbonate. CH_2Cl_2 was used to aid filtration. The filtrate was evaporated under vacuum at room temperature. The residue was taken up with fresh CH_2Cl_2 (40 ml) and washed with satd $NaHCO_3$ (75 ml). The $NaHCO_3$ layer was extracted with fresh CH_2Cl_2 (2×30 ml), and the combined CH_2Cl_2 layers were extracted with pH 3.0 buffer (100 ml). The buffer layer was then washed with fresh CH_2Cl_2 (4×40 ml). The pH was raised to 10 with 4 N NaOH, extracted with fresh CH_2Cl_2 (40 ml), twice more with fresh CH_2Cl_2 (30 ml), and the combined CH_2Cl_2 layers were dried over anhydrous sodium sulfate, filtered, and evaporated under vacuum at room temperature to afford 51 mg of 23-deoxy-23-(3-aminoprop-1-yl)-amino-20-dihydro-DMT (10) in an overall 11% yield from DMT (7). ESI-MS m/z 800.5 $(M+1)^+$ (base), 744.5 $(base-H_2NCH_2-CH=CH)^+$ (Fig. 3).

Acknowledgement

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