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PAPER

7-Deazapurine biosynthesis: NMR study of toyocamycin biosynthesis in *Streptomyces rimosus* using 2-^{13}C-7-^{15}N-adenine†

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Although 7-deazapurines are well known and feature in the hypermodified RNA base queuosine, and in a range of nucleoside antibiotics such as toyocamycin, a mechanistic understanding of their biosynthesis is a longstanding problem. In particular, the obligatory loss of the N-7 nitrogen atom is puzzling, and in order to address this mechanistic conundrum a novel doubly labeled purine, [2-^{13}C, 7-^{15}N]-adenine, has been prepared and used as a biosynthetic precursor to toyocamycin in *Streptomyces rimosus*. NMR spectroscopy and mass spectrometry clearly showed incorporation of ^{13}C but loss of ^{15}N in the toyocamycin produced.

Introduction

Before it becomes fully functional in protein synthesis, transfer RNA (tRNA) undergoes a number of cellular post-transcriptional processing and maturation events,[1] including modification of the nucleo-bases. Over 120 modified nucleosides have now been identified and characterised,[2] together with a number of genes that encode the tRNA modifying enzymes.[3] Although the nucleoside modifications include simple methylation reactions of the base or ribose ring, it is the more extensive structural changes, often termed hypermodifications, involving multiple enzymatic steps that are more interesting. The most widely studied hypermodified base is queuine that forms the queuosine nucleoside **1**, a 7-deazaguanosine derivative (Fig. 1), which is found in the wobble position of bacterial and eukaryotic tRNAs coding for Asn, Asp, His and Tyr. Although a complete picture of the physiological role of queuosine (and queuine) is yet to emerge, it is thought to play a fundamental role in enhancing translational fidelity, and has been implicated in a number of processes, including regulation of mitotic signalling pathways in cancer.[4] Queuosine is biosynthesised from GTP and the details of the pathway, in which $PreQ_0$ (7-cyano-7-deazaguanine) is a key intermediate (see Scheme 1), have begun to be adumbrated. The initial step has been long known, and involves the ring opening of the 5-membered purine ring with loss of C-8 (as formate),[5] in a step common with other pathways such as folate biosynthesis. This is followed by a number of steps, for which some individual *que*-genes and

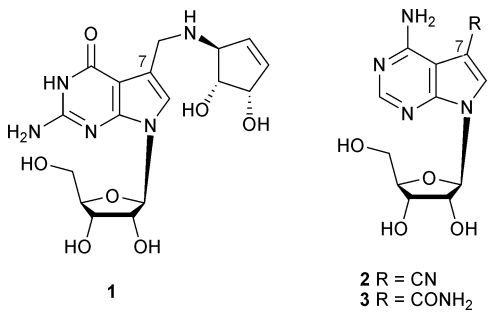

Fig. 1 Structures of 7-deazapurines: (a) the hypermodified tRNA nucleoside queuosine **1**; (b) the nucleoside antibiotics toyocamycin **2** and sangivamycin **3**.

Que-enzymes have been identified, including the first ever nitrile reductase enzyme.[6–16]

7-Deazapurines (pyrrolo[2,3-*d*]pyrimidines) also occur in a number of *Streptomyces* secondary metabolites such as the nucleoside antibiotics toyocamycin **2** and sangivamycin **3** (Fig. 1) isolated from *S. rimosus*. Almost four decades ago it was established by classical radiolabeling techniques that C-2, but not C-8, of purines is incorporated into the pyrrolopyrimidine, whilst the two 5-membered pyrrole ring carbons *and* the nitrile carbon of toyocamycin derive from ribose.[17–19] The clusters of *toy*-genes involved in toyocamycin biosynthesis have been identified, and shown to be homologous with certain *que*-genes involved in queuosine biosynthesis.[14,15] As a result of these recent studies, a scheme for the biosynthesis of the hypermodified tRNA component queuosine and antibiotics such as toyocamycin has emerged as outlined in Scheme 1.

However, despite the aforementioned elegant studies, mechanistic puzzles remain, particularly the fascinating and substantial rearrangement required to convert pterin type structures into the 7-deazapurine $PreQ_0$. One critical question is the fate of N-7. In the early work it was apparently assumed that since C-8

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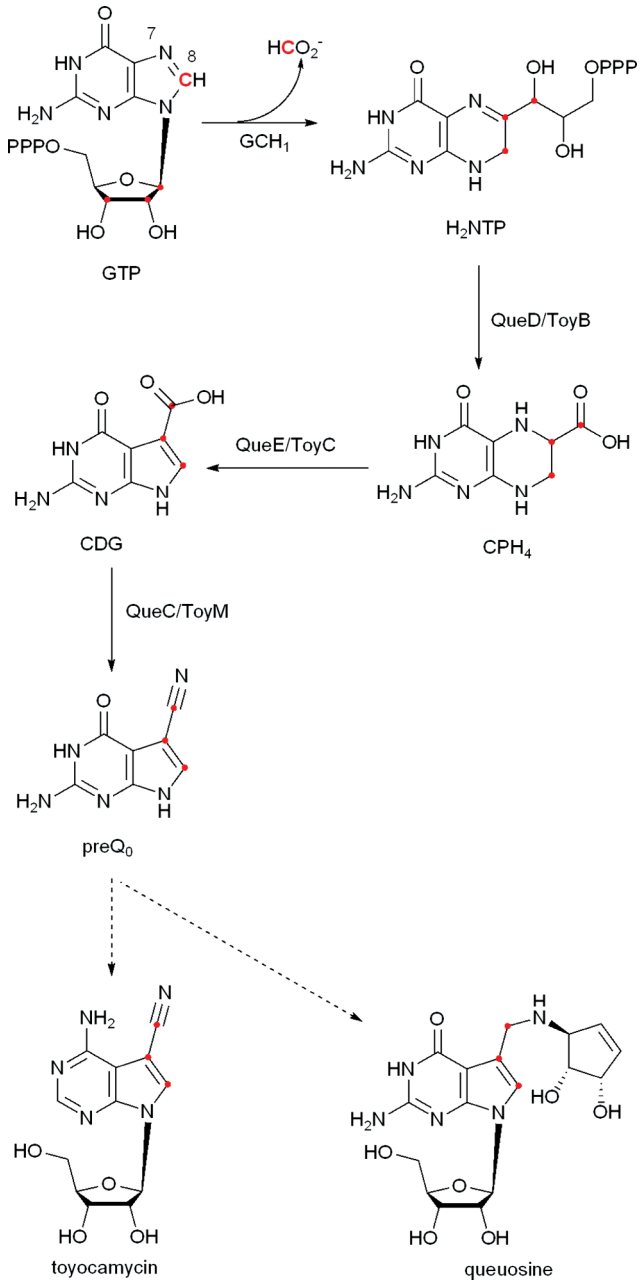


Scheme 1 Biosynthetic routes from GTP to the hypermodified tRNA component queuosine and the nucleoside antibiotic toyocamycin.[14,15] *Abbreviations*: GCH1, GTP cyclohydrolase type I; H_2NTP, 7,8-dihydroneopterin triphosphate; CPH_4, 6-carboxy-5,6,7,8-tetrahydropterin; CDG, 7-carboxy-7-deazaguanine; Que, Toy, refer to the homologous enzymes involved in queuosine and toyocamycin biosynthesis. The indicated carbon atoms represent the labeling pattern established by early radioisotopic experiments.[5,17–19]

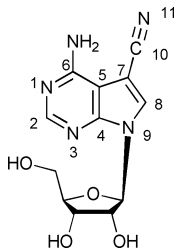
was lost, N-7 must also be lost in the biosynthetic pathway.[17–19] However, there was no evidence to sustain this hypothesis and as recently as 2008,[14] it was acknowledged that the source of the cyano nitrogen atom in $PreQ_0$ (and toyocamycin) was not known – was it derived from N-7 of the starting purine or was N-7 lost, and the cyano nitrogen incorporated from elsewhere? In view of this uncertainty, we decided to study the problem using a purine precursor isotopically labeled at N-7, and follow its fate by NMR spectroscopy in the biosynthesis of toyocamycin in *S. rimosus*. Whilst our investigation was in progress, a further paper from the Bandarian group showed that if [$^{15}N_5$]-labeled GTP was used as a precursor, one ^{15}N atom is lost *en route* to $preQ_0$, and also that the cyano nitrogen was derived from external (^{15}N-labeled) ammonia.[16] The results described herein, obtained by an entirely different approach that starts with an N-7 labeled purine and demonstrates the subsequent elimination of the label, complement these recent data.

Results and discussion

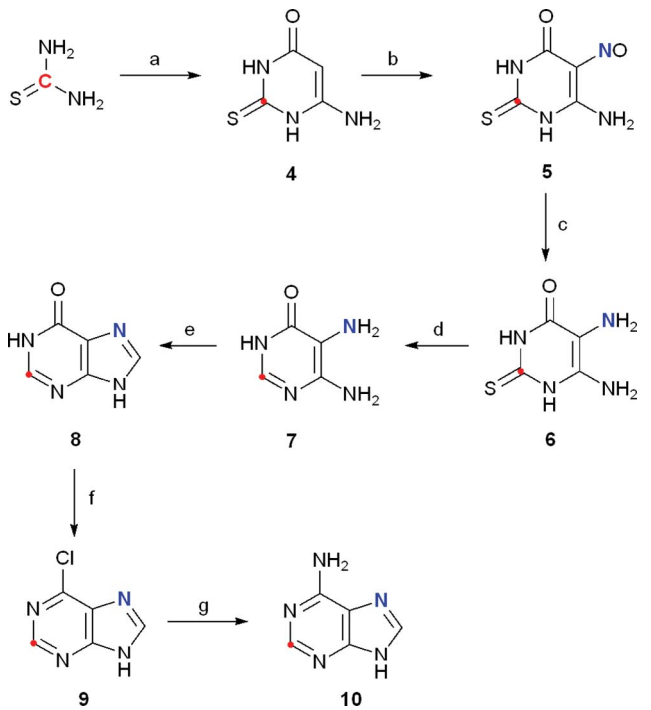
Our approach required the synthesis of a purine precursor specifically labeled with ^{15}N, an NMR active isotope, at N-7. As an internal control, the precursor also required a label at a position that was known to be retained in 7-deazapurine biosynthesis, and therefore we chose to incorporate a ^{13}C label at C-2. Even though earlier work had shown that both radioactive guanine and adenine were incorporated into toyocamycin by *S. rimosus*,[17] for ease of synthesis, we elected to use labeled adenine. Although a number of labeled adenine derivatives are known,[20–22] the specific combination of 2-^{13}C and 7-^{15}N required for this study has not previously been reported. Having trialled all the reactions on unlabeled starting materials to optimise conditions and to provide NMR spectra of unlabeled materials for comparison, we embarked on the synthesis of [2-^{13}C, 7-^{15}N]-adenine. Thus reaction of ethyl cyanoacetate with [^{13}C]-thiourea gave the [2-^{13}C]-6-amino-2-thioxopyrimidone **4**, nitrosation of which using sodium ^{15}N-nitrite introduced the second isotopic label. Reduction of the nitroso group was followed by desulfurisation over RANEY® nickel to deliver the double labeled diaminopyrimidone **7**. Conversion into hypoxanthine **8** was readily achieved using the literature protocol with formic acid in DMF in the presence of diethoxymethyl acetate,[21] and this was followed by reaction with phosphorus oxychloride to give 6-chloropurine **9**. Finally reaction with ammonia in ethanol, under microwave conditions that reduced the reaction time, gave the desired doubly labeled adenine **10** (Scheme 2). The labeled compounds displayed the expected NMR spectroscopic properties, with ^{13}C spectra run under standard conditions exhibiting just one strong signal for the labeled carbon with the natural abundance ^{13}C carbon atoms essentially absent. In the ^1H NMR spectra, H-2 protons attached directly to the ^{13}C-labeled carbon showed the expected large J_1 coupling (*ca.* 200 Hz), and H-8 protons the smaller J_2 couplings to the ^{15}N-label (*ca.* 8–12 Hz). ^{15}N NMR spectroscopy was also carried out on compounds **6**, **7**, **9** and **10**, with the observed chemical shifts being closely matching literature values.[21]

With the desired isotopically labeled precursor to hand, attention turned to the production of toyocamycin by fermentation of *S. rimosus*. Reproducible growth conditions were readily found (see Methods) and following harvesting of the cells, toyocamycin was isolated and purified by chromatography as a colourless solid in a yield of 30–40 mg per L of fermentation broth. Both ^1H and ^{13}C NMR spectra could be fully assigned and were in accord with those reported in the literature.[23] Next, the fermentation was repeated in minimal media containing ^{15}N ammonium chloride as the sole source of nitrogen, and, as expected,[24] gave toyocamycin ^{15}N-labeled at the position of all nitrogen atoms. The ^{15}N NMR spectrum clearly showed five signals, one for each of the labeled

Table 1 Mass spectrometric analysis of toyocamycin obtained from feeding [2-^{13}C, 7-^{15}N]-adenine to *Streptomyces rimosus*



M	$[M+H]^+$ found	$[M+H]^+$ theoretical	$[M+Na]^+$ found	$[M+Na]^+$ theoretical
[2-^{12}C, 11-^{14}N]-toyocamycin	292.1040	292.1043	314.0870	314.0863
[2-^{13}C, 11-^{14}N]-toyocamycin	293.1110	293.1077	315.0899	315.0897
[2-^{12}C, 11-^{15}N]-toyocamycin	293.1110	293.1013	315.0899	315.0833
[2-^{13}C, 11-^{15}N]-toyocamycin	294.9388	294.1047	—	316.0867



Scheme 2 Synthesis of [2-^{13}C, 7-^{15}N]-adenine **10**. *Reagents and conditions*. (a) EtO_2CCH_2CN, NaOEt, EtOH, reflux (95%); (b) $NaNO_2$, 1 M HCl, 0 °C (90%); (c) $Na_2S_2O_4$, aq $NaHCO_3$ solution (94%); (d) RANEY® nickel, aq ammonia, reflux (96%); (e) $MeCO_2CH(OEt)_2$, HCO_2H, DMF, 130 °C (94%); (f) $POCl_3$, $PhNMe_2$, reflux (67%); (g) NH_3, EtOH, 150 °C, microwave (300 W) (82%).

nitrogens. Importantly, it was also possible to assign the five signals based on the comparison with fully labeled adenosine,[25] and the fact that there was only one signal at δ –116.8 (referenced to nitromethane) in the nitrile region of the spectrum.[26] In addition, the ^{13}C NMR spectrum of fully ^{15}N-labeled toyocamycin showed the expected coupling between the C and N of the nitrile group at *ca.* δ 115.

When the critical feeding experiment was done in minimal media containing [2-^{13}C, 7-^{15}N]-adenine **10**, toyocamycin was again produced, and was carefully analysed for the presence or absence of both ^{13}C and ^{15}N labels. The inclusion of the ^{13}C atom was clearly seen in the ^{13}C NMR spectrum, with the peak for C-2 (purine numbering) at δ –153.6 increasing in intensity by *ca.* three times compared to the spectrum of unlabeled compound, whilst the nitrile carbon at *ca.* δ 115 remained a singlet, precluding the possibility of ^{15}N being present in the nitrile group. In addition, there was no evidence for a nitrile ^{15}N signal (at δ –116.8) in direct-observe mode. The presence or absence of ^{15}N in the toyocamycin produced by fermentation in the presence of [2-^{13}C, 7-^{15}N]-adenine **10** was also studied by mass spectrometry (for spectrum, see the ESI†). By high resolution MS analysis of the fermentation product, peaks for both labelled and unlabeled toyocamycin were observed with the peaks due to the unlabeled compound being *ca.* four times the intensity of those due to labeled compound. Thus peaks were observed at *m/z* 292.1040 and 293.1110 ($[M+H]^+$) with the corresponding ($[M+Na]^+$) peaks at 314.0870 and 315.0899. As shown in Table 1, these data correspond closely to the theoretical values confirming the incorporation of ^{13}C, but not of ^{15}N. As also shown in Table 1, incorporation of an additional ^{15}N atom in the metabolite would have resulted in a peak at 294.1047 for the protonated adduct, or at 316.0867 for the adduct with sodium. Alternatively the presence of ^{15}N but with no ^{13}C would have resulted in a peak at 293.1013 for the protonated adduct, or at 315.0833 for the adduct with sodium. None of these peaks were present in the spectrum.

Hence, our spectroscopic experiments demonstrate that N-7 is indeed lost in the conversion of adenine into toyocamycin, and from this definitive evidence from the two independent approaches described herein and elsewhere,[16] we assume that this represents a general biosynthetic pathway for all 7-deazapurines.

Experimental section

Commercially available reagents were used throughout without further purification unless otherwise stated. Anhydrous DMF, DMSO, methanol were purchased from Aldrich. All other solvents were obtained as spectroscopic grade and used as received. Light petroleum refers to the fraction of petroleum boiling in the range 40–60 °C. All reactions were carried out under a nitrogen or an argon atmosphere. Microwave reactions were carried out in a CEM Discover™ S-class (300 W) microwave reactor with IR temperature sensor.

Thin layer chromatography was carried out using aluminium backed plates coated with silica gel. The plates were visualised under UV light at 254 nm. Flash chromatography was carried out using silica gel with eluent specified.

Melting points were measured on a hot stage apparatus and are uncorrected. IR spectra were recorded in the range 4000–600 cm^{-1} as solids in attenuated total reflectance (ATR) mode. Specific rotations are quoted in $10^{-1}degcm^2g^{-1}$. Mass spectra were carried out on a TOF spectrometer, using electrospray (ES) technique. ^1H NMR and ^{13}C NMR spectra were recorded using at 300, 400 or 500 MHz (^1H frequencies; corresponding ^{13}C frequencies are 75, 100 and 125 MHz respectively). Chemical shifts are quoted in parts per million (ppm) and are referenced against residual protonated solvent. ^{15}N-NMR spectra were recorded at 40.5 MHz on a 400 MHz (^1H) instrument and were referenced to $CH_3{}^{15}NO_2$ and are recorded using inverse gated ^1H-decoupling technique.

[2-^{13}C]-6-Amino-2-thioxo-1,2-dihydro-4(3*H*)-pyrimidinone 4

Ethyl cyanoacetate (1.063 g, 9.4 mmol) was added to a solution of sodium ethoxide in ethanol (21% wt; 5 mL). After about 1 min, a colourless suspension was formed. [^{13}C]-Thiourea (0.708 g, 9.3 mmol) was added, and the mixture was then heated under reflux for 2 h. The ethanol was evaporated under reduced pressure, and the residue was dissolved in water (15 mL). Acetic acid was added to precipitate a solid that was collected by filtration, washed with water (10 mL), ethanol (10 mL) and acetone (10 mL) and dried to yield a pearl white solid (1.268 g, 8.9 mmol; 95%); mp >300 °C (lit.,[27] unlabeled, mp >300 °C); (Found: $M+Na^+$, 167.0074. $C_3{}^{13}CH_5N_3OS+Na^+$ requires 167.0085); ν_{max} (solid)/cm^{-1} 3422, 3326, 2921, 1618, 1588, 1544, 1179, 789; δ_H (400 MHz; DMSO-d_6) 6.36 (4H, br. s, NH and SH), 4.69 (1H, s, H-5); δ_C (100 MHz; DMSO-d_6) 174.6 (strong signal, C-2), 161.6 (C-4), 154.3 (C-6), 78.2 (C-5); *m/z* (ESI) 145 ($M+H^+$), 167 ($M+Na^+$). NMR data are consistent with the literature.[22]

[2-^{13}C, 5-^{15}N]-6-Amino-5-nitroso-2-thioxo-1,2-dihydro-4(3*H*)-pyrimidinone 5

[2-^{13}C]-6-Amino-2-thioxo-1,2-dihydro-4(3*H*)-pyrimidinone **4** (2.444 g, 17.0 mmol) was suspended in hydrochloric acid (1 M; 65 mL) and cooled in ice. A solution of ^{15}N-sodium nitrite (1.239 g, 18.0 mmol) in water (10 mL) was added. The mixture was stirred overnight in ice, and the resulting red suspension was filtered and washed with water and ethanol to give the title compound as a red solid (2.628 g, 15.3 mmol; 90%); mp 275–289 °C (lit.,[21] [5-^{15}N]-labeled, mp 270–285 °C decomp.); (Found: $M+Na^+$, 196.9959. $C_3{}^{13}CH_4N_3{}^{15}NO_2S+Na^+$ requires 196.9957); ν_{max} (solid)/cm^{-1} 1689, 1645, 1485, 1276, 1165, 745; δ_H (400 MHz; DMSO-d_6) 12.56 (2H, s, NH), 11.25 (1H, s, SH), 7.72 (2H, br. s, NH_2); δ_C (100 MHz; DMSO-d_6) 176.4 (strong signal, C-2); *m/z* (ESI) 197 ($M+Na^+$), 175 ($M+H^+$).

All NMR data are consistent with those reported for the [2-^{13}C]-labeled compound,[22] and the [5-^{15}N]-labeled compound.[21]

For reference, the unlabeled compound has the following NMR data; δ_H (400 MHz; DMSO-d_6) 12.56 (2H, s, NH), 11.23 (1H, s, SH), 7.69 (2H, br. s, NH_2); δ_C (100 MHz; DMSO-d_6) 176.4 (C-2), 159.4 (C-4), 142.6 (C-6), 140.4 (C-5).

[2-^{13}C, 5-^{15}N]-5,6-Diamino-2-thioxo-1,2-dihydro-4(1*H*)-pyrimidinone 6

A suspension of [2-^{13}C, 5-^{15}N]-6-amino-5-nitroso-2-thioxo-1,2-dihydro-4(3*H*)-pyrimidinone **5** (2.912 g, 16.9 mmol) in saturated sodium hydrogen carbonate solution (70 mL) was cooled in ice. Sodium dithionite (7.660 g, 44 mmol) was added in four portions to the stirred suspension, and stirring was continued for 6 h. Acetic acid was added slowly to the yellow suspension, and the resulting precipitate was collected by filtration. The solid was washed with water and ethanol and dried to give the title compound (2.505 g, 15.9 mmol, 94%); mp >300 °C (lit.,[21] [5-^{15}N]-labeled, mp >300 °C decomp.); (Found: $M+H^+$, 161.0331. $C_3{}^{13}CH_6N_3{}^{15}NOS+H^+$ requires 161.0344); ν_{max} (solid)/cm^{-1} 3403, 3309, 2968, 2899, 1631, 1568, 1458, 1269, 1175; δ_H (400 MHz; DMSO-d_6) 5.67 (6H, br. s, NH and NH_2); δ_C (100 MHz; DMSO-d_6) 167.5 (strong signal, C-2); δ_N (40.5 MHz; DMSO-d_6) - 356.8 (5-NH_2); *m/z* (ESI) 183 ($M+Na^+$), 161 ($M+H^+$).

All NMR data are consistent with those reported for the [2-^{13}C]-labeled compound,[22] and the [5-^{15}N]-labeled compound.[21]

For reference, the unlabeled compound has the following NMR data; δ_H (400 MHz; DMSO-d_6) 5.68 (br. s); δ_C (100 MHz; DMSO-d_6) 167.6 (C-2), 157.9 (C-4), 140.5 (C-6), 102.5 (C-5).

[2-^{13}C, 5-^{15}N]-5,6-Diamino-4(3*H*)-pyrimidinone 7

[2-^{13}C, 5-^{15}N]-5,6-Diamino-2-thioxo-1,2-dihydro-4(1*H*)-pyrimidinone **6** (2.205 g, 14.0 mmol) was dissolved in aqueous ammonia (5%; 60 mL). RANEY® nickel (7.5 mL of a 50% slurry in water) was added to this solution, and the reaction mixture was heated under reflux for 2 h. The black mixture was filtered hot through a bed of Celite, and the filter cake was washed with boiling water. The filtrate was concentrated under reduced pressure to give the title compound as a yellow solid (1.688 g, 13.4 mmol; 96%); mp 226–227 °C (lit.,[21] [5-^{15}N]-labeled, mp 226–227 °C); (Found: $M+H^+$, 129.0607. $C_3{}^{13}CH_6N_3{}^{15}NO+H^+$ requires 129.0623); ν_{max} (solid)/cm^{-1} 3405, 3317, 2359, 1635, 1580, 1458, 1272, 1177; δ_H (400 MHz; DMSO-d_6) 7.43 (1H, d, *J* 200 Hz, H-2), 5.59 (5H, br. s, NH and NH_2); δ_C (100 MHz; DMSO-d_6) 138.2 (strong signal, C-2); δ_N (40.5 MHz; DMSO-d_6) -346.3 (5-NH_2); *m/z* (ESI) 151 ($M+Na^+$), 129 ($M+H^+$).

All NMR data are consistent with those reported for the [2-^{13}C]-labeled compound,[22] and the [5-^{15}N]-labeled compound.[21]

For reference, the unlabeled compound has the following NMR data; δ_H (400 MHz; DMSO-d_6) 7.42 (1H, s, H-2), 5.59 (5H, br. s, NH and NH_2); δ_C (100 MHz; DMSO-d_6) 156.4 (C-4), 147.1 (C-6), 138.1 (C-2), 110.4 (C-5).

[2-^{13}C, 7-^{15}N]-Hypoxanthine 8

A suspension of [2-^{13}C, 5-^{15}N]-5,6-diamino-4(3*H*)-pyrimidinone **7** (500 mg, 4.0 mmol) in formic acid (3 mL) was heated under reflux for 1 h. The solution was then concentrated *in vacuo* to give a yellow solid. Diethoxymethyl acetate (1.30 mL, 8 mmol), formic acid (0.2 mL, 5 mmol), and DMF (13 mL) were added, and the resulting suspension was heated at 130 °C for 4 h. The mixture was then concentrated to a brown solid, which was resuspended in boiling acetonitrile (8 mL) for 10 min, chilled (0 °C), filtered, and then dried to give the title compound (509 mg, 3.7 mmol, 94%); mp >300 °C (lit.,[21] [7-^{15}N]-labeled, mp >300 °C); (Found:

M+H$^+$, 139.0463. $C_4{}^{13}CH_4N_3{}^{15}NO$+H$^+$ requires 139.0467); ν_{max} (solid)/cm^{-1} 3419, 1667, 1580, 1421, 1214, 1137, 965, 891; δ_H (400 MHz; DMSO-d_6) 8.08 (1H, d, *J* 8, H-8), 7.96 (1H, d, *J* 200, H-2); δ_C (100 MHz; DMSO-d_6) 144.5 (strong signal, C-2); *m*/*z* (ESI) 161 (M+Na$^+$), 139 (M+H$^+$).

All NMR data are consistent with those reported for the [2-^{13}C]-labeled compound,[22] and the [7-^{15}N]-labeled compound.[21]

For reference, the unlabeled compound has the following NMR data; δ_H (400 MHz; DMSO-d_6) 8.12 (1H, s, H-2), 7.98 (1H, s, H-8); δ_C (100 MHz; DMSO-d_6) 155.4 (C-5), 153.4 (C-8), 144.6 (C-2), 140.3 (C-4), 119.2 (C-6).

[2-^{13}C, 7-^{15}N]-6-Chloropurine 9

[2-^{13}C, 7-^{15}N]-Hypoxanthine **8** (300 mg, 2.2 mmol) was dissolved in a mixture of $POCl_3$ (9 mL) and N,N-dimethylaniline (0.75 mL, 5.9 mmol). The mixture was stirred and heated under reflux until a homogenous black solution was obtained. The solution was allowed to cool and concentrated under vacuum to a black gum. Excess of $POCl_3$ was removed with an oil pump. The residue was purified using a chromatography (silica gel, MeOH–CH_2Cl_2 10 : 90 v/v) to give the title compound (229 mg, 1.5 mmol, 67%); mp >300 °C (lit.,[21] [7-^{15}N]-labeled, mp >300 °C); (Found: M+H$^+$, 157.0125. $C_4{}^{13}CH_3N_3{}^{15}N^{35}Cl$+H$^+$ requires 157.0133); ν_{max} (solid)/cm^{-1} 3100, 3050, 1766, 1563, 1383, 1315, 1224, 845; δ_H (400 MHz; DMSO-d_6) 8.73 (1H, d, *J* 208, H-2), 8.69 (1H, d, *J* 12, H-8); δ_C (100 MHz; DMSO-d_6) 151.4 (strong signal, C-2); δ_N (40.5 MHz; DMSO-d_6) -152.1 (*N*-7); *m*/*z* (ESI) 179 (M+Na$^+$), 157 (M+H$^+$).

All NMR data are consistent with those reported for the [2-^{13}C]-labeled compound,[22] and the [7-^{15}N]-labeled compound.[21]

For reference, the unlabeled compound has the following NMR data; δ_H (400 MHz; DMSO-d_6) 8.73 (1H, s, H-2), 8.68 (1H, s, H-8); δ_C (100 MHz; DMSO-d_6) 154.3 (C-6), 151.4 (C-2), 147.7 (C-4), 146.4 (C-8), 129.4 (C-5).

[2-^{13}C, 7-^{15}N]-Adenine 10

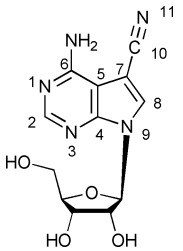
[2-^{13}C, 7-^{15}N]-6-Chloropurine **9** (50 mg, 0.3 mmol) was dissolved in ethanolic ammonia (2 M; 3 mL) and heated at 150 °C in a sealed tube in a microwave reactor (300 W) for 2 h. After standing overnight a precipitate is formed. After filtration, and evaporation of the filtrate, the residue was purified by chromatography (silica gel, MeOH–CH_2Cl_2, 10 : 90 v/v) to give the title compound as a colourless solid (36 mg, 0.26 mmol, 82%); mp> 300 °C (lit.,[28] unlabeled, mp >360 °C); (Found: M+H$^+$, 138.0632. $C_4{}^{13}CH_5N_4{}^{15}N$+H$^+$ requires 138.0433); ν_{max} (solid)/cm^{-1} 3346, 3107, 1672, 1600, 1418, 1307, 1252, 939; δ_H (400 MHz; DMSO-d_6) 8.10 (1H, d, *J* 200 Hz, H-2), 8.08 (1H, d, *J* 12, H-8), 7.10 (2 H, s, NH); δ_C (100 MHz; DMSO-d_6) 152.4 (strong signal, C-2); δ_N (40.5 MHz; DMSO-d_6) -138.6 (*N*-7); *m*/*z* (ESI) 160 (M+Na$^+$), 138 (M+H$^+$).

All NMR data are consistent with those reported for the [2-^{13}C]-labeled compound,[22] and the [7-^{15}N]-labeled compound.[21]

For reference, the unlabeled compound has the following NMR data; δ_H (400 MHz; DMSO-d_6) 8.11 (1H, s, 2-H), 8.09 (1H, s, 8-H), 7.08 (2 H, br. s, NH); δ_C (125 MHz; DMSO-d_6) 155.5 (C-6), 152.9 (C-2), 151.1 (C-4), 140.0 (C-8), 117.7 (C-5).

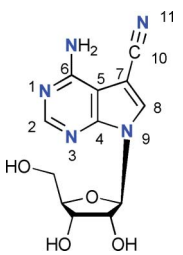
Toyocamycin

Growth conditions. *Streptomyces rimosus* (ATCC No 14500) was purchased, grown on ATCC medium 73 (YGC medium) agar plates for 14 days, harvested and maintained as a 30% glycerol stock. Tryptone soy broth (Oxoid) (100 mL) was innoculated with 100 μL of stock and grown at 28 °C for 48 h. This seed flask (1 mL) was used to innoculate YGC medium (300 mL) that was grown for 72 h before toyocamycin was isolated. For the incorporation of the labels, after 55 h the cells were harvested (10 min, 1000 g) and resuspended in M9 minimal media (300 mL) containing the label of interest and grown for a further 17 h before isolation.



Isolation of toyocamycin. The media was centrifuged (10 min, 3000 g) and the supernatant adjusted to pH 2 with hydrochloric acid, centrifuged (30 min, 35000 g) and the supernatant adjusted to pH 7 with sodium hydroxide before lyophilisation to a third of its original volume. Toyocamycin was then isolated by extraction with 1-butanol (3 × 300 mL). The organic layer was evaporated to dryness and the residue was dissolved in water (100 mL) at 70 °C. The insoluble material was removed by filtration and the filtrate was lyophilised to dryness. The resulting solid was purified by flash chromatography (silica gel, CH_2Cl_2–MeOH, 80 : 20 v/v) to give toyocamycin (average yield 30–40 mg per L of fermentation broth); mp 242–245 °C (lit.,[29] mp 243 °C); $[\alpha]_D^{23}$ −41.0 (*c* 1.0, 0.1 M HCl) (lit.,[29] $[\alpha]_D^{25}$ −55.6 (*c* 1.0, 0.1 M HCl)); (Found: M+H$^+$, 292.1043. $C_{12}H_{13}N_5O_4$+H$^+$ requires 292.0968); δ_H (400 MHz; DMSO-d_6) 8.45 (1H, s, H-8), 8.23 (1H, s, H-2), 6.92 (2H, br. s, NH_2), 6.06 (1H, d, *J* 5.5 Hz, H-1′), 5.49 (1H, d, *J* 6.1 Hz, 2′-OH), 5.21 (2H, br. s, 3′-OH and 5′-OH), 4.37 (1H, m, H-2′), 4.10 (1H, m, H-3′), 3.94 (1H, dd, *J* 3.7 Hz, 7.3, H-4′), 3.67 (1H, m, H-5′), 3.56 (1H, m, H-5′); δ_C (100 MHz; DMSO-d_6) 157.0 (C-6), 153.5 (C-2), 150.1 (C-4), 132.4 (C-8), 115.3 (CN), 101.2 (C-5), 87.8 (C-1′), 85.5 (C-4′), 83.0 (C-7), 74.2 (C-2′), 70.2 (C-3′), 61.2 (C-5′); *m*/*z* (ESI) 314 (M+Na$^+$), 292 (M+H$^+$). NMR data are consistent with the literature.[23]

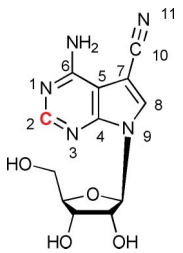
[^{15}N]$_5$- Toyocamycin



The same procedure reported for toyocamycin was used in order to obtain the *title compound*. After 55 h, the cells were harvested (10 min, 1000 g) and resuspended in M9 minimal media

(300 mL) containing ^{15}N-ammonium chloride at 1 g L^{-1} and grown for a further 17 h before isolation; (Found: M+H$^+$, 297.0909. $C_{12}H_{13}{}^{15}N_5O_4$+H$^+$ requires 297.0893); δ_H (400 MHz; DMSO-d_6) 8.45 (1H, s, H-8), 8.22 (1H, dd, *J* 7.4 Hz, 22.8, H-2), 6.90 (2H, d, *J* 88.1 Hz, NH_2), 6.06 (1H, d, *J* 5.7 Hz, H-1′), 5.48 (1H, d, *J* 6.1 Hz, 2′-OH), 5.22 (2H, br. s, 3′-OH and 5′-OH), 4.37 (1H, t, *J* 5.3 Hz, H-2′), 4.11 (1H, t, *J* 3.9 Hz, H-3′), 3.94 (1H, dd, *J* 3.7 Hz, 7.3, H-4′), 3.67 (1H, m, H-5′), 3.56 (1H, m, H-5′); δ_C (100 MHz; DMSO-d_6) 157.0 (1C, d, *J* 19.8 Hz, C-6), 153.5 (1C, s, C-2), 150.2 (1C, s, C-4), 132.4 (1C, d, *J* 14.5 Hz, C-8), 115.4 (1C, d, *J* 17.5 Hz, CN), 101.2 (1C, s, C-5), 87.8 (1C, d, *J* 10.6, C-1′), 85.5 (C-4′), 83.0 (C-7), 74.3 (C-2′), 70.2 (C-3′), 61.2 (C-5′); δ_N (40.5 MHz; DMSO-d_6) –116.8 (CN), –143.5 (*N*-1), –154.5 (*N*-3), –216.0 (*N*-9), –296.5 (NH_2); *m*/*z* (ESI) 297 (M+H$^+$), 319 (M+Na$^+$).

[2-^{13}C]- Toyocamycin



The same procedure reported for toyocamycin was used in order to obtain the *title compound*. After 55 h, the cells were harvested (10 min, 1000 g) and resuspended in M9 minimal media (300 mL) containing [2-^{13}C, 7-^{15}N]-adenine and grown for a further 17 h before isolation; (Found: M+H$^+$, 293.1110. $C_{11}{}^{13}CH_{13}N_5O_4$+H$^+$ requires 293.1077); δ_C (100 MHz; DMSO-d_6) 157.0 (C-6), 153.6 (strong signal, C-2), 150.1 (C-4), 132.4 (C-8), 115.3 (CN), 101.2 (C-5), 87.7 (C-1′), 85.5 (C-4′), 83.0 (C-7), 74.2 (C-2′), 70.2 (C-3′), 61.2 (C-5′); *m*/*z* (ESI) 315 (M+Na$^+$), 293 (M+H$^+$).

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