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LC-MS based quantification of 2′-ribosylated nucleosides Ar(p) and Gr(p) in tRNA†

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RNA nucleosides are often naturally modified into complex non-canonical structures with key biological functions. Here we report LC-MS quantification of the Ar(p) and Gr(p) 2′-ribosylated nucleosides in tRNA using deuterium labelled standards, and the first detection of Gr(p) in complex fungi.

RNA possesses a number of chemically modified bases that are involved in key biological processes, such as codon-anticodon binding and stabilization of the RNA structure.[1–5] To date, over 100 modified RNA nucleosides have been identified from natural sources.[6] The functions and biosyntheses of these interesting structures have been elucidated to some extent; however, few studies have attempted to quantify natural levels of modification or to investigate the biological relevance or regulation of these levels. In order to address these issues, we have begun to quantify the modified bases using an LC-MS based quantification method using isotope labelled standards.[7]

The Ar(p) **1** and Gr(p) **2** nucleosides are unusual 2′-phosphoribosylated modifications of the canonical bases adenosine (A) and guanosine (G) (Fig. 1).[8–10] Both nucleosides have been detected at position 64 (in the T arm) of initiator $tRNA^{Met}$ in yeasts and plants, and appear to distinguish this tRNA from elongator $tRNA^{Met}$, which is unmodified in this position.[11,12] The nucleosides are also postulated to generally occur in fungi based on tRNA sequences,[11] but have not yet been detected in complex fungi. Both nucleosides possess a characteristic 2′-phosphoribose modification; however, under typical digest conditions needed to detect these compounds, the phosphate group is hydrolysed to give the simpler Ar **3** or Gr **4** nucleoside. Here we report syntheses of deuterium labelled analogues in order to characterize the distribution of these specially modified bases in various tissues and in particular in higher fungi. Our study shows that in the higher fungi investigated by us it is Gr(p) and not Ar(p) that is present in the tRNAs in this position, suggesting that Gr(p) is more commonly utilised.

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† Electronic supplementary information (ESI) available: Fig. S1, experimental data and NMR spectra. See DOI: 10.1039/c1cc11011j

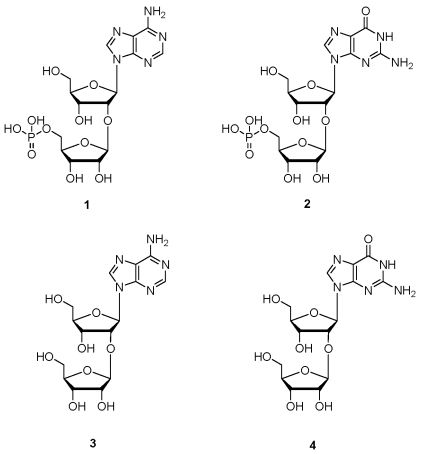
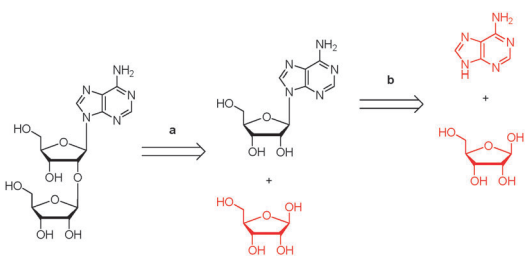


Fig. 1 2′-phosphoribosylated nucleosides Ar(p) **1** and Gr(p) **2** and the corresponding dephosphorylated forms Ar **3** and Gr **4** resulting from enzymatic RNA digestion.

Syntheses of both nucleosides have been reported involving Vorbrüggen glycosylation reactions between protected A or G nucleosides and an appropriately reactive ribose.[13–15] Based on these available synthetic routes, we decided that deuterated ribose would be the most practical precursor for a practical late stage incorporation[16] of the label. Scheme 1 shows the most practical retrosyntheses of **3**, revealing that step (a) is the best option for incorporation of the (cost) limiting labelled reagent. Late stage incorporation of an isotope labelled base (adenine or guanine) was expected to be more synthetically challenging, and was therefore not attempted. As an isotope labelled ribose, we chose 5,5-d_2-ribose **5** (Fig. 2), which can be



Scheme 1 Retrosynthesis of Ar showing possible stages for incorporation of an isotope label. Potentially labelled starting materials are coloured in red.

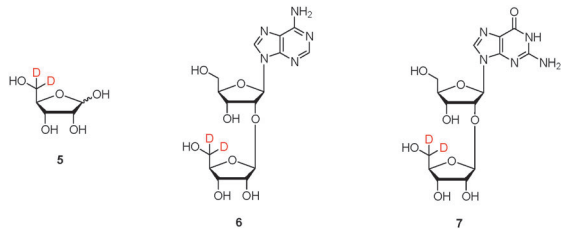
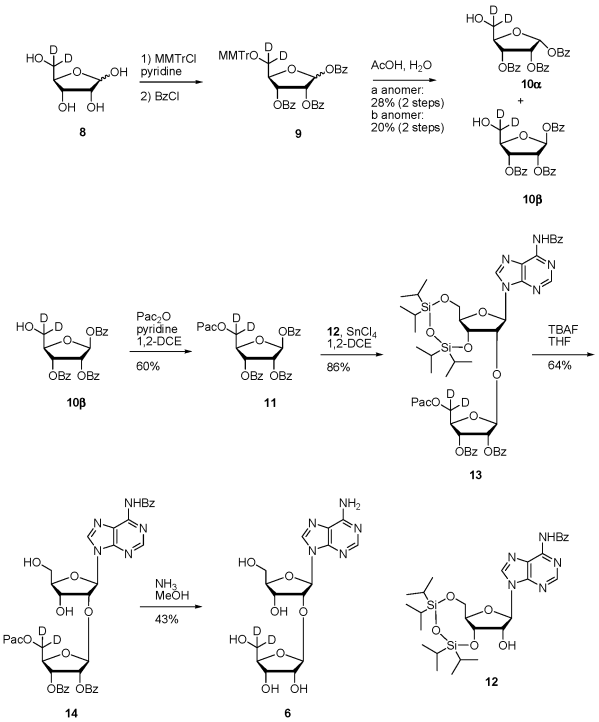


Fig. 2 Isotope-labelled ribose **5** and target deuterated nucleosides **6** and **7**. Labelled atoms are coloured in red.

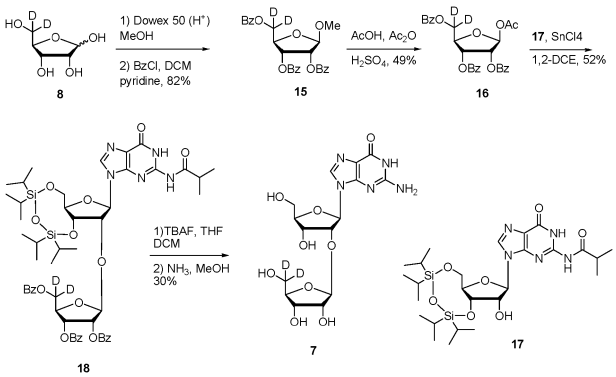


Scheme 2 Synthesis of d_2-Ar **6**.

synthesized in 6 steps[17] and is also commercially available, for the preparation of our target deuterated nucleosides **6** and **7** (Fig. 2).

In order to allow optional incorporation of the phosphate group present in natural Ar(p), we initially undertook the synthesis of d_2-Ar starting with a 5-Pac (phenoxyacetyl) protected ribose[15] (Scheme 2). Here, d_2-ribose **8** was protected with a MMTr (monomethoxytrityl) group at the 5-hydroxyl group, then Bz (benzoyl) groups at the 1, 2 and 3 hydroxyl groups to give protected ribose **9**. Subsequently, the MMTr group was removed under acid conditions, at which point the α and β anomers of the product **10** were separated. The 5-hydroxy group of **10β** was then protected with a phenoxyacetyl group to give fully protected d_2-ribose **11**. The key step, Vorbrüggen glycosylation of **11** with the 3′,5′-protected A derivative **12**[18] was carried out in moderate yield (60%), and finally all protecting groups were removed with TBAF followed by NH_3/MeOH to give d_2-Ar **6** in 43% yield after RP-HPLC purification.

Initially, the same strategy was attempted for d_2-Gr **7** using protected ribose **11** as the Vorbrüggen coupling partner, based



Scheme 3 Synthesis of d_2-Gr **7**.

on the strategy reported for the non-deuterated nucleoside.[13] However, in our hands this reaction gave consistently poor yields. Instead, the simpler 1-Ac-2,3,5-Bz-protected d_2-ribose **16** was used (Scheme 3).[14] Several strategies were investigated for the effective preparation of this precursor. The most effective was determined to be methylation of the 1-hydroxyl group of d_2-ribose using Dowex 50 cation exchange resin (H^+ form) in MeOH,[19] followed by benzoylation then acetylation in $AcOH/Ac_2O/H_2SO_4$[20] to give protected ribose **16**. This precursor was then reacted with protected guanosine **17**[21] and $SnCl_4$ to give **18** in 52% yield. Protecting groups were finally removed, again using TBAF then NH_3/MeOH to give d_2-Gr **7** in 30% yield after RP-HPLC purification.

Following successful preparation of deuterium labelled Ar and Gr, LC-MS quantifications of Ar(p) and Gr(p) were carried out using these standards and our reported method.[7] Briefly, the isotope labelled nucleosides were added to unknown nucleoside samples from enzymatic digestion of tRNA, LC-MS was measured, then the integrals of the LC-MS peaks for the labelled and natural nucleosides were compared to quantify the natural nucleosides. Initially, calibration curves comparing the LC-MS integrals for the labelled and natural nucleosides[13] were determined (Fig. S1, ESI†). These give excellent linear fits, confirming the applicability of the deuterated standards to quantification of nucleoside samples. Subsequently, LC-MS quantifications of Ar(p) and Gr(p) in tRNA isolated from various tissues were carried out (Table 1).

To our surprise, we observed that the distribution of the nucleotides Ar(p) and Gr(p) strongly varies between the investigated species. Ar(p) was only detected in *Saccharomyces cerevisiae*, while neither of the two nucleosides were found in

Table 1 Levels of Ar(p) and Gr(p) in various cell and tissues. n.d., not detected

Species/cell type	Ar(p) (per 100 tRNA molecules)	Gr(p) (per 100 tRNA molecules)
E. coli	n.d.	n.d.
S. cerevisiae	1.25 ± 0.03	n.d.
C. albicans	n.d.	1.55 ± 0.07
C. nebularis	n.d.	2.4 ± 0.1
F. fomentarius	n.d.	1.25 ± 0.06
S. scrofa	n.d.	n.d.
HeLa	n.d.	n.d.

bacteria (*Escherichia coli*) or in mammalian cells (*Sus scrofa* and HeLa). The nucleoside Gr(p) in contrast is widely distributed. Most importantly we for the first time investigated the modified nucleosides in complex fungi such as *Clitocybe nebularis* and *Fomes fomentarius*. Here we detected only Gr(p), indicating that this modification is more commonly used to distinguish between initiator and elongator tRNA. In both higher fungi the nucleoside Ar(p) was clearly not present since not even traces could be detected. This result is in agreement with a study in plants and other yeast species showing that Gr(p) is of widespread use.[8]

The detected levels of Ar(p) and Gr(p) are relatively low (1–3 modifications per 100 tRNA molecules) compared to the levels of other modified nucleosides.[7] Analysis of a previous quantification of yeast tRNA[22] reveals that initiator tRNA makes up approximately 2.5% of all tRNA present in cells. Because we detected the modification at a level between 1 and 3%, our results show that even in complex fungi, the modification is likely found only in the initiator tRNA and that it is absent in all other tRNA molecules present in the cell. The quantitative data therefore support the idea that the modifications Ar(p) and particularly Gr(p) are key components of initiator tRNA needed to correctly start the translational process.

In summary, we report the synthesis of isotope labelled Ar and Gr and performed the first quantitative analysis directly in higher fungi. The results support the idea that Gr(p) is more widely distributed and that both modifications are only present in initiator tRNA.

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