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Synthesis of galactosyl-queuosine and distribution of hypermodified Q-nucleosides in mouse tissues

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Abstract: Queuosine (Q) is a hypermodified RNA nucleoside that is found in tRNA^{His}, tRNA^{Asn}, tRNA^{Tyr}, and tRNA^{Asp}. It is located at the wobble position of the tRNA anticodon loop, where it can interact both with U or C bases located at the respective position of the corresponding mRNA codons. In higher eukaryotes, including humans, the Q base is for yet unknown reasons further modified in tRNA^{Tyr} and tRNA^{Asp} by the addition of a galactose or mannose sugar, respectively. The reason for this additional modification, and how the sugar modification is orchestrated with Q-formation and insertion, is unknown. Here, we report a total synthesis of the hypermodified nucleoside galactosyl-queuosine (galQ). The availability of the compound enabled us to study the absolute levels of the Q-family nucleosides in six different organs of new-born and adult mice, and also in human cytosolic tRNA. Our synthesis now paves the way to a more detailed analysis of the biological function of the Q-nucleoside family.

In all three domains of life, RNA contains next to the canonical bases (A, C, G, and U) a large variety of modified nucleosides.^[1] Most of these are chemically simple derivatives of the canonical nucleosides. They often carry methylations at various positions of the heterocycle or the sugar, but others are heavily modified involving multi-step biosynthesis pathways. Queuosine **1** (Q) is one of the most complex of these so-called hypermodified nucleosides. (Fig. 1). It is found in a large number of different species and also present in the cytosolic and mitochondrial tRNA^{Tyr}, tRNA^{Asp}, tRNA^{His} and tRNA^{Asn} of humans.^[2-8] Interestingly, in the human cytosolic tRNA^{Tyr} and tRNA^{Asp}, Q is further modified with galactose (galQ) or mannose (manQ), respectively.^[9, 10] In these tRNAs, the sugar is proposed to be attached to the homoallylic hydroxyl-group of the cyclopentene ring system that is linked to the 7-deazaheterocycle.^[11] While the chemical synthesis of Q has been achieved,^[12-14] no reports exist about the preparation of its sugar-modified derivatives galQ **2** and manQ **3**, which has hampered investigations of their biological role. Accordingly, the exact function of galQ and manQ as part of the human cytosolic tRNA^{Tyr} and tRNA^{Asp}

is unknown. In addition, we do not know to which extent the corresponding tRNAs are modified with different Q-family nucleosides, and how the G/Q-exchange process and the sugar derivatisation is orchestrated. Furthermore, quantitative data about Q-modification levels in different organs is also lacking.

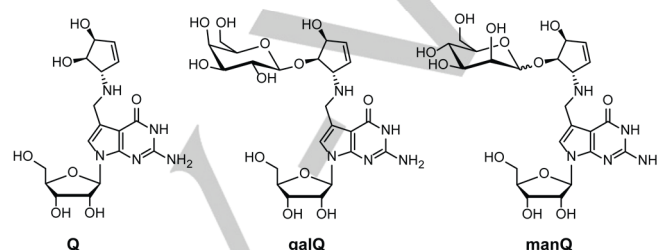


Figure 1. Depiction of the hypermodified base queuosine (**1**, Q) and of the galactosylated and mannosylated Q-derivatives galQ (**2**) and manQ (**3**) present in human cytosolic tRNA^{Asp} and tRNA^{Tyr}, respectively.

To address these questions, we performed the first total synthesis of galactosyl-queuosine **2**. This allowed us to confirm its proposed structure, and to report the absolute levels of all Q-family members in different tissues of new-born and adult mice. Finally, we were able to measure to which extent human cytosolic tRNAs are modified with the three Q-family nucleosides.

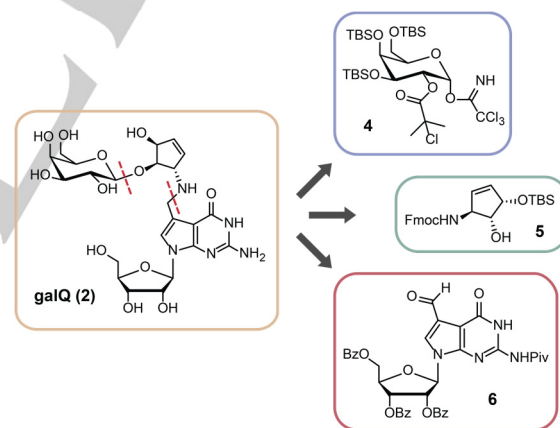


Figure 2. Retrosynthetic analysis for galQ **2**, showing the three key-precursors **4**, **5**, and **6**.

Galactosyl-Q **2** was constructed from three appropriately protected parts (Fig. 2): The 7-formyl-7-deazaguanosine **6** was prepared, as reported by us, with Bz-protected hydroxyl-groups at the ribose, and a pivaloylate protection group at the 2-amino residue.^[14] The galactose sugar was introduced as a TBS- and 2-chloroisobutryl-protected trichloroacetimidate **4**, and the cyclopentene unit **5** was used with Fmoc-protected allyl amine and a TBS-protected allylic alcohol. We choose the 2-chlorobutryl protecting group for the sugar-donor **4** because of its bulkiness in order to avoid unwanted orthoester

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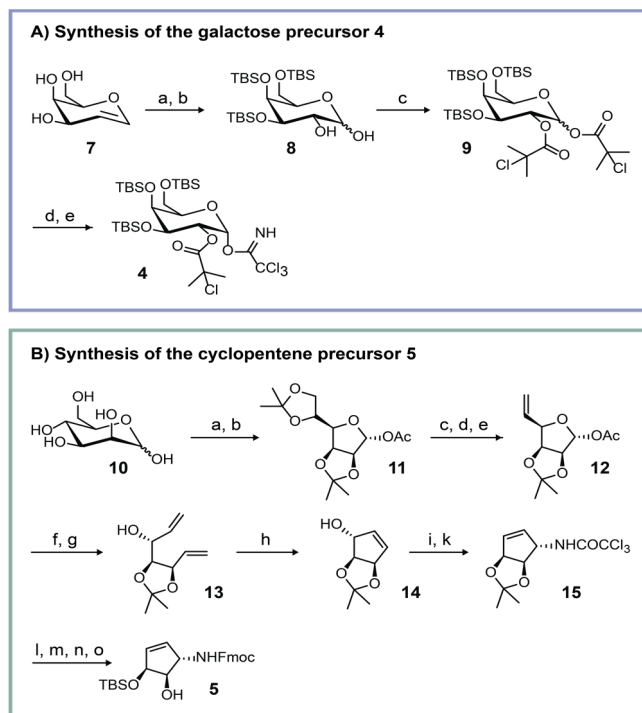
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formation as the main product of the glycosylation reaction as shown by Szpilman *et al.*^[15]

The galactosyl-donor **4** itself was prepared from D-galactal **7**, which was first TBS-protected (**Scheme 1A**).^[16] *cis*-dihydroxylation of the double bond from the sterically less demanding side furnished compound **8**.^[17] This step was followed by protection of the two newly-introduced hydroxyl-groups with 2-chlorobutyric acid to give the galactose-donor-precursor **9**. Deprotection of the anomeric hydroxyl-group with hydrazine provided the galactose precursor with a free anomeric hydroxyl-group which was subsequently converted into the trichloroacetimidate donor **4** using a standard procedure.

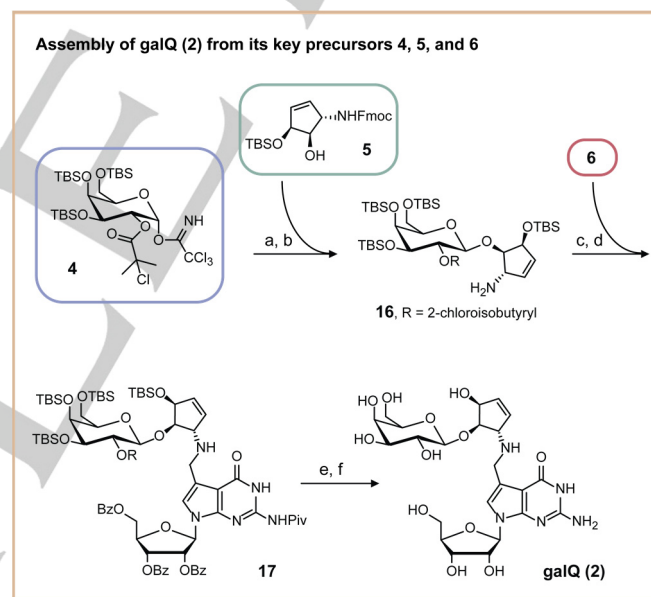


Scheme 1. Synthesis of the key precursors **4** and **5**. **A)** Synthesis of the galactose precursor **4**: a) TBSCl, imidazole, DMF, 55 °C, 2 d; b) $K_2OsO_4 \cdot 2 H_2O$, NMO, THF, *t*-BuOH, H_2O , rt, 4 h; c) 2-chloroisobutyric acid, DIC, DMAP, 0 °C, 30 min \rightarrow rt, 2 h; d) $N_2H_4 \cdot AcOH$, DMF, -40 °C to rt, 3 h; e) Cl_3CCN , CS_2CO_3 , DCM, rt, 4 h; **B)** Synthesis of the cyclopentene precursor **5**: a) 2,2-dimethoxypropane, acetone, *p*-TsOH, rt, 1 h; b) Ac_2O , pyridine, 0 °C \rightarrow rt, 18 h; c) aq. $AcOH$ (66%), 55 °C, 4 h; d) triethyl-orthoformate, 100 °C, 30 min; e) Ac_2O , 130 °C, 5 h; f) *t*-BuOK, MeOH, 20 min; g) NaH, DMSO, Ph_3PMeBr , THF, rt \rightarrow 68 °C, 2 h; h) Grubbs-(I)-catalyst, DCM, rt, 26 h; i) Cl_3CCN , DBU, DCM, rt, 20 min; j) α -xylene, 150 °C, 5 h; l) NaOH, MeOH, rt, overnight; m) Fmoc-OSu, $NaHCO_3$, H_2O , 1,4-dioxane; n) $AcOH$, H_2O , EtOAc, 50 °C, 24 h; o) TBSOTf, DMF, -55 °C, 15 min.

Scheme 1B shows the synthesis of the protected 5(*S*)-amino-3(*S*),4(*R*)-dihydroxycyclopent-1-ene **5**. Starting point was mannose **10**^[18] which was converted as reported into the double-acetonide-protected mannofuranoside **11** with an acetyl-protected anomeric center in two steps. Selective cleavage of the acetonide protecting group at the primary hydroxyl-group, followed by an orthoester-based elimination, allowed introduction of a terminal double bond (**12**). Anomeric deprotection, followed by a *Wittig*-reaction, provided the precursor **13** for the ring-closing metathesis reaction. The free hydroxyl-group in **14** was then the starting point for an *Overman*-rearrangement, providing the amine protected as a trichloroacetamide **15**.^[19] Cleavage of this protecting group with NaOH

was followed by Fmoc-protection of the free amine using a standard procedure. We finally opened the acetal and protected the allylic hydroxyl-group selectively with TBS-OTf in DMF at -55 °C. In this reaction, the temperature is particularly important. If the reaction was performed at higher temperatures and with prolonged reaction times, we noted selective protection of the homoallylic position.

Assembly of the galQ nucleoside **2** from the precursors **4–6** is shown in **Scheme 2**. We first galactosylated the cyclopentene derivative. This sterically demanding step was successfully achieved by activation of the trichloroacetimidate with 2-chloro-6-methylpyridinium triflate in dichloromethane at room temperature.^[15, 20] We achieved selective formation of the β -configured galactoside due to the neighboring-group effect. Subsequent cleavage of the Fmoc-protection group gave product **16**, which was followed by a two-step reductive amination. First, the imine was formed in benzene, subsequently followed by reduction of the imine with $NaBH_4$ in methanol to afford protected galQ **17**. A two-step deprotection protocol, in which we first removed the TBS-groups with $HF \cdot NEt_3$, was followed by cleavage of ester-type protecting groups under *Zemplén*-conditions. For the cleaving of the pivaloyl-amide protecting group, we needed to use 0.5 M NaOMe in methanol. This strategy provided the target compound **2** with a total yield of 0.5 % in 20 linear steps from the mannose starting molecule for the cyclopentene unit. The synthesis provided a sufficient amount of material for all further investigations.



Scheme 2. Depiction of the galQ **2** assembly from the three key-precursors **4**, **5**, and **6**. a) 2-chloro-6-methylpyridinium triflate, DCM, rt, 2 h; b) DBU, MeCN, rt, 1.25 h; c) benzene, rt, 5 h; d) $NaBH_4$, MeOH, 0 °C, 1 h; e) $HF \cdot NEt_3$, DCM, rt, 4 d; f) NaOMe, MeOH, rt, 2 d.

We next investigated if our synthetic β -homoallylic galQ **2** is identical with the natural product, because analytical data available for galQ was very limited.^[11] For this experiment, we isolated total RNA from mouse liver and performed an enzymatic digestion of the isolated RNA to the nucleoside level. This nucleoside mixture was analyzed by HPLC-MS. Indeed, under our HPLC-conditions, we detected two signals with the appropriate *m/z* value for galQ and manQ in the

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extracted ion chromatogram with a retention time of around 32 and 35 min. In the same m/z -range, no other peaks were present. We next co-injected our synthetic β -homoallylic galQ **2**, which led to a marked increase of the second signal with a retention time of about 35 min (Fig. 3). This result unambiguously showed that our synthetic compound galQ **2** and the co-eluting natural compound with the same retention time are identical. Therefore, this natural compound is indeed a β -galactosylated Q-derivative. Taken together, our experiment confirms the proposed chemical structure of galQ, in which the bond between the homoallylic hydroxyl-group of queuosine and galactose is in β -configuration.

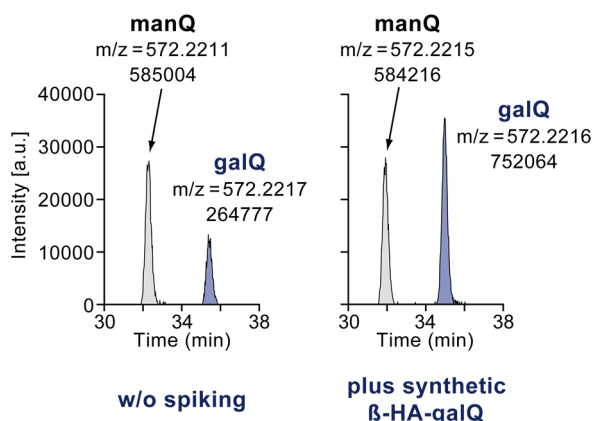


Figure 3. Results of a co-injection study confirming the identity of our synthetic β -homoallylic-galQ **2** with the natural product. Depicted are the extracted-ion chromatograms ($m/z = 572.2148$ – 572.2248) of two HPLC-MS analyses, either with (right) or without (left) prior spiking of the synthetic galQ **2**. The exact mass $[M+H]^+$ of galQ (and manQ) is 572.2198 u, showing a perfect match of less than 4 ppm deviation with the two MS-peaks observed by us.

Having identified the HPLC-retention time of galQ and therefore also of manQ, we finally were able to determine the absolute levels of galQ, manQ, and Q in different tissues of new-born (post-natal day 1; pd1) and adult mice (post-natal month 3; pm3). For an initial broad study, we measured the respective nucleoside levels in cortex, cerebellum, liver, kidney, heart, and spleen, using the same RNA-isolation and digestion protocol as for the co-injection experiment (Fig. 4A). From our data it is clearly evident that the levels of all three modifications (galQ, manQ, and Q) generally increase with age. This effect is by far most pronounced with Q, while galQ and manQ only show a modest increase, if at all. Furthermore, and for all three modifications investigated, we see differences between the six organs at the same age. Heart, followed by brain tissues, contain the largest levels of Q and its sugar-modified derivatives, followed by kidney, liver and spleen. At gross, the changes of modification levels observed by us positively correlate with the respective organ-specific protein-synthesis demands, as we have shown before.^[7] Nevertheless, there are some prominent outliers. These outliers (e.g. heart tissue), seem to rather correlate with the organ-specific density of mitochondria. It was shown before that the Q-base in mitochondrial tRNA^{Tyr} and tRNA^{Asp} is not sugar-modified.^[8] We therefore speculate that the organ-specific differences in the levels of galQ, manQ, and Q are due to an overlay of two independent effects: The organ-specific protein-synthesis ratio, and the organ-specific mitochondrial density.

It is well-established that for biosynthesis of Q (and its sugar-modified derivatives), eukaryotes have to take up the queuine-base from their

diet,^[21, 22] mammals thereby profiting from their gut microbiome.^[23] We therefore speculate that the low levels of Q-family nucleosides in new-born mice observed here may be caused by a lack of queuine-supply in new-born mice, which only later establish their microbiome. Furthermore, high rates of cell division and tissue development in young mice may cause additional queuine supply problems.

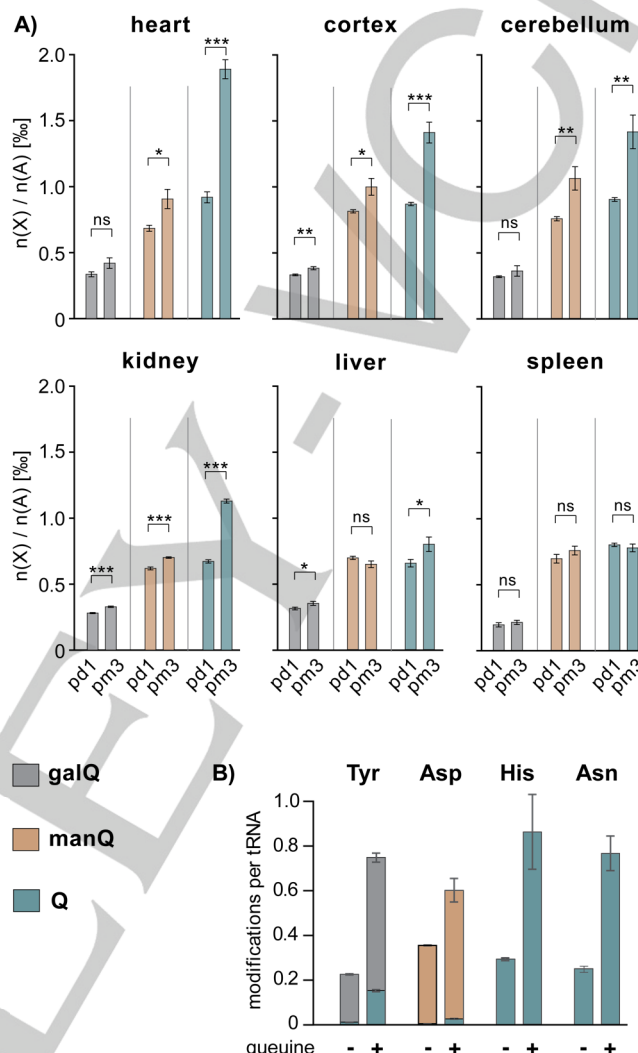


Figure 4. A) Absolute levels of galQ **2**, manQ **3**, and Q **1**, in six different organs of new-born (post-natal day 1, pd 1) and adult (post-natal month 3, pm 3) mice. Values are given as number of xQ-modifications $n(X)$ per 1000 adenosine nucleosides $n(A)$. Error bars represent the standard deviation of three biological replicates. For statistical analysis, Student's unpaired two-tailed t-test was used. n.s.: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. **B)** Number of galQ-, manQ-, and Q-modifications per cytosolic tRNAs^{Asn}, tRNAs^{His}, tRNAs^{Asp}, and tRNAs^{Tyr} from human HEK 293T cells, respectively. queuine+: cells grown in queuine-enriched medium, queuine-: cells grown in standard medium. Values are given as average modifications per tRNA. Error bars represent the standard deviation of three biological replicates.

To further study the influence of queuine-availability on Q-family modification levels, cell culture experiments were performed: *Human embryonic kidney 293T* (HEK 293T) cells were grown either in culture medium supplemented with 20 nM of the queuine enriched medium) or in medium without additional queuine (standard medium). Queuine is the substrate of the TGT-enzyme, which performs the exchange of a guanine base by the queuine heterocycle during tRNA-

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maturation.^[24-26] From both cell populations, cytosolic *tRNA*^{Tyr}, *tRNA*^{Asp}, *tRNA*^{His}, and *tRNA*^{Asn} were isolated and digested to the nucleoside level. For each of these four individual tRNA-species, the number of galQ-, manQ-, and Q-modifications per tRNA was then determined by a mass spectrometry-based isotope-dilution method using the reference compound synthesized here (see supporting information). Indeed, our data show that the extent of Q-modification in the wobble position of cytosolic *tRNA*^{Tyr}, *tRNA*^{Asp}, *tRNA*^{His}, and *tRNA*^{Asn} is strongly dependent on queuine availability (Fig. 4B).^[27] In case of *tRNA*^{Tyr} (galQ), *tRNA*^{His} and *tRNA*^{Asn} (Q), the difference in modification extent between cells grown in enriched versus standard medium is threefold, while for *tRNA*^{Asp} (manQ) it is 1.7 fold. These results are well in line with our hypothesis and might therefore explain the lower modification levels in new-born mice. Of note, in all of our experiments even a sufficient queuine-supply did not lead to fully modified tRNAs. This might again be an indication of the modification machinery lagging behind the *de novo*-synthesis of tRNA in highly proliferating cells. Furthermore, we detected a Q-only-modified *tRNA*^{Tyr}-form in our experiments lacking the galactose sugar, while *tRNA*^{Asp} was always found to be either modified with manQ or completely unmodified. It seems that, in our experimental setup, mannosylation of *tRNA*^{Asp} may be more tightly connected to G/Q-exchange than the galactosylation of Q-only-bearing *tRNA*^{Tyr}. Testing this exciting hypothesis is an interesting starting point for future studies.

In summary, we here report the first total synthesis of the human natural product galactosyl-queuosine **2**. Our synthetic material allowed us to confirm the proposed galQ structure by direct comparison with natural material, and we show that this hypermodified nucleoside is present in all tissues of new-born and adult mice. We furthermore report the absolute levels of all three Q-family-members in six different mouse organs and in human cytosolic tRNAs. Taken together, our results confirm the crucial importance of tRNA galQ- and manQ-modification.

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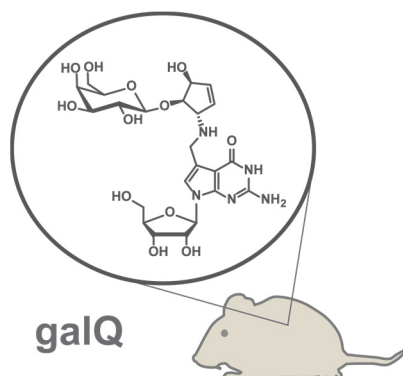
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The first total synthesis of the hypermodified RNA nucleoside galactosyl-queuosine (galQ) was achieved. Moreover, the distribution of this nucleoside, and, in addition, of mannosyl-queuosine and queuosine, was determined in tissues of new-born and adult mice, and in human cytosolic tRNAs.



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