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Communication

Synthesis and Structure Elucidation of Glutamyl-Queuosine

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ABSTRACT: Queuosine is one of the most complex hypermodified RNA nucleosides found in the Wobble position of tRNAs. In addition to Queuosine itself, several further modified derivatives are known, where the cyclopentene ring structure is additionally modified by a galactosyl-, a mannosyl-, or a glutamyl-residue. While sugar-modified Queuosine derivatives are found in the tRNAs of vertebrates, glutamylated Queuosine (gluQ) is only known in bacteria. The exact structure of gluQ, particularly with respect to how and where the glutamyl side chain is connected to the Queuosine cyclopentene side chain, is unknown. Here we report the first synthesis of gluQ and, using UHPLC-MS-*co*injection and NMR studies, we show that the isolated natural gluQ is the *α*-allyl-connected gluQ compound.

In addition to the four canonical nucleosides Adenosine (A), Cytidine (C), Guanosine (G), and Uridine (U) RNA molecules, particularly tRNAs (tRNA), contain a large variety of modified noncanonical nucleosides[1] which influence their functions and properties.[2−4] Among these noncanonical nucleosides, Queuosine (Q, **1**, Figure 1) is one of the most

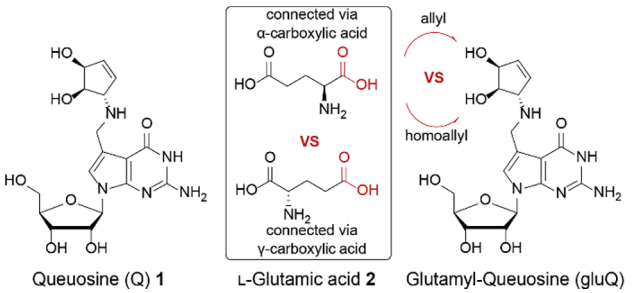

Figure 1. Depiction of hypermodified nucleoside Q **1** and potential structure of glutamylated Q-derivatives.

complex and highly modified.[5−7] It is found in cytosolic and mitochondrial tRNA of bacteria and eukaryotes and is located at position 34 in the anticodon loop, which is also called the "Wobble position".[8] The Q-nucleoside is replacing G in GUN-anticodons and is therefore found in $tRNA^{Tyr}$, $tRNA^{Asp}$, $tRNA^{His}$, and $tRNA^{Asn}$.[9,10]

Queuosine **1** (Figure 1) is chemically derived from Guanosine (G). The main feature of the noncanonical base is the replacement of the N^7 nitrogen atom by a C^7 carbon, to which an unusual 1(*S*)-amino-2(*R*),3(*S*)-dihydroxycyclopent-4-ene unit is attached via a methylene linker.[7,11] The noncanonical nucleoside Q allows the tRNAs to decode synonymous codons by wobble base pairing.[12,13] In particular, it enables the decoding of both C and U nucleosides at the third position in the mRNA (mRNA) codon triplet during translation. In addition to altering decoding properties, Queuosine has also been reported to influence translational speed[14] and decoding fidelity.[15] In the absence of sufficient levels of the amino acid tyrosine, dietary supply of Queuine, which is the nucleobase of Queuosine, has been shown to be essential for survival in mice.[16,17]

In vertebrates further glycosylated derivatives of Queuosine are known, namely Galactosyl-Queuosine (galQ) and Mannosyl-Queuosine (manQ), whose structures were already elucidated.[5,18,19] In bacteria, a glutamylated version of Q (gluQ) was detected.[20,21] However, the function and exact structure of gluQ have not been fully elucidated. It is known that gluQ is an ester formed upon reaction of one of the free cyclopentene hydroxyl groups with L-glutamic acid **2**.[20] It is, however, not known which carboxylic acid (*α* versus *γ*) is connected to which OH-group (allyl versus homoallyl, Figure 1).

To elucidate the structure, we performed a classical total synthesis approach of different gluQ derivatives and compared the compounds with natural gluQ isolated from *E. coli* RNA. The data allowed us to conclude that, in natural gluQ, the *α*-COOH of L-glutamic acid is connected to the allyl-OH group.

For the synthesis of the different gluQ derivatives, we first prepared 7-formyl-7-deazaguanosine nucleoside precursor **3** (Scheme 1). Reaction of formic acid methylester **4** with chloroacetonitrile and NaOMe afforded formyl chloroacetonitrile, which was coupled to 1,3-diamino-5-hydroxypyrimidine **5** in a Hantzsch pyrrole synthesis to generate $preQ_0$ **6**.[22] In a two-step procedure, **6** was treated with Boc_2O to afford the double N^2-Boc- and O^6-*tert*-butyl protected derivative **7**.[23] A subsequent nucleosidation reaction was performed with an *α*-1-chloro-2,3,5-tri-*O*-benzylribose derivative **8**, which was

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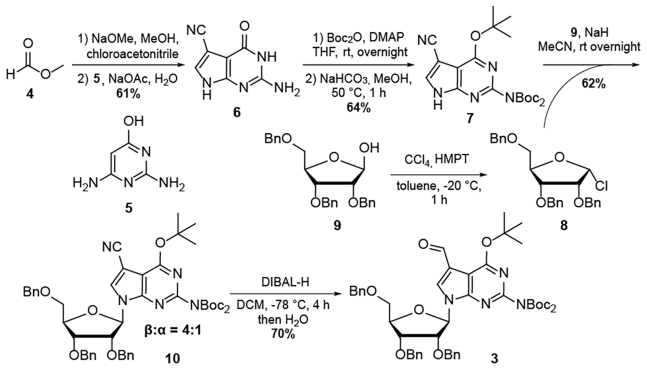
Scheme 1. Synthesis of the Key Nucleoside Precursor 3 via Nucleosidation of α-1-Chloro-2,3,5-tri-*O*-benzylribofuranose 8 with Protected preQ$_0$ 7



preformed from 2,3,5-tri-*O*-benzylribose **9** in an Appel chlorination reaction. After deprotonation of the heterocycle with NaH this reaction furnished the protected-preQ$_0$ nucleoside **10** with good stereoselectivity (α/β = 1:4). Finally, the nitrile was reduced to the imine with DIBAL-H, which yielded the aldehyde **3** after hydrolysis.[24]

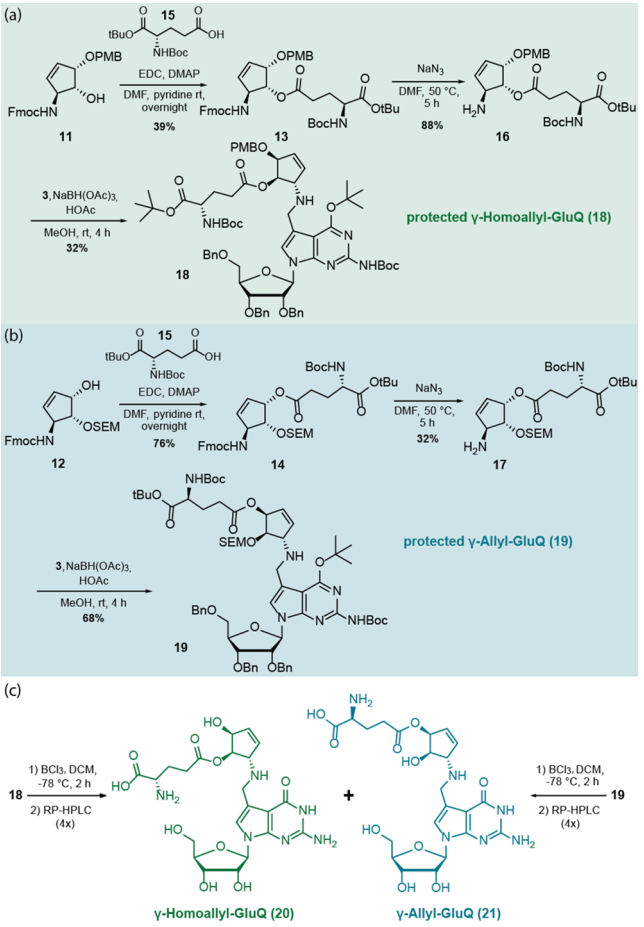
This key 7-formyl-7-deazaguanosine nucleoside **3** was subsequently subjected to a reductive amination with different glutamylated 1(*S*)-amino-2(*R*),3(*S*)-dihydroxycyclopent-4-enes (Schemes 2 and 3).

For the synthesis of the allyl- and homoallyl-gluQ derivatives, in which the glutamyl unit is connected via the γ-COOH group, we used the syntheses outlined in Scheme 2. Starting points were the 4-O-PMB and 5-O-SEM protected cyclopentene derivatives **11** and **12**. The compounds **11** and **12** were prepared as recently reported by us.[18] To obtain the correct regioisomers **13** and **14** (Scheme 2a,b), we used the *N*-Boc and α-COOtBu protected L-glutamic acid **15** derivative for the EDC induced coupling. The subsequent Fmoc deprotection with NaN_3[25] to avoid basic conditions yielded the corresponding amino compounds **16** and **17**, which were used in a reductive amination affording **18** and **19**.

Subsequent deprotection with BCl_3 furnished the γ-COOH coupled homoallyl and allyl gluQ derivatives **20** and **21** (Scheme 2c). However, under these harsh deprotection conditions, the majority of the compound hydrolyzed to Queuosine. Additionally, while the protected compounds (**18** and **19**) could be isolated in pure form, the HPL chromatogram of the deprotected compounds provided only a single, but identical peak for both **20** and **21**. NMR analysis of the individual peaks showed, however, signals from two mixed molecular species. With the assistance of 2D-NMR, we could assign both possible regioisomers of the γ-COOH coupled gluQ in both samples (Figure 2). The preparation of pure regioisomers seemed difficult at this stage.

Before starting extensive attempts to separate the γ-connected homoallyl- and allyl-compounds **20** and **21**, we decided to compare the mixture with the natural product to see if one of the compounds corresponds to the natural material. To this end, we harvested *E. coli* cells and isolated small RNAs using standard procedures. After breaking down the RNA to the nucleoside level, using enzymatic digestion at slightly acidic conditions, we performed UHPLC-MS-coinjection experiments with the mixture of **20** and **21**. First, upon injection of only the *E. coli* isolated and digested RNA into the UHPLC-MS device, we could detect a signal at 15.37 min

Scheme 2. Synthesis of the Protected gluQ Derivatives, in Which the γ-COOH Group Is Connected to (a) the Homoallyl-OH Group and (b) the Allyl-OH Group; (c) Full Deprotection of the Allyl- and Homoallyl Connected Versions of gluQ



corresponding to natural gluQ (Figure 3a, b). When we next coinjected the mixture of the allyl/homoallyl γ-gluQ (**20** and **21**) and the digested RNA, we detected two clearly distinguishable signals at 15.32 and 15.61 min. These data show that neither of the two coeluting γ-gluQ compounds (Supp. Figure 1) is identical with the natural product (Figure 3c).

The synthesis of the allyl- and homoallyl-α-carboxylic ester derivatives was performed, as shown in Scheme 3a and b. For the α-connected homoallyl compound (Scheme 3a), we again started with the 4-*O*-PMB-protected cyclopentene unit **11**. Esterification of a *N*-Boc and γ-COOtBu protected L-glutamate derivative **22** generated amino acid coupled cyclopentene **23**. Subsequent removal of the Fmoc group under nonbasic conditions with NaN_3[25] gave the free amine **24**, which was used to perform the reductive amination reaction with **3** to obtain the protected α-homoallyl-gluQ derivative **25**. For the synthesis of the α-COOH esterified allyl-gluQ derivative (Scheme 3b), we again used the 5-*O*-SEM-protected cyclopentene **12**. The subsequent esterification to **26**, Fmoc-deprotection to **27**, and reductive amination with **3** provided the protected α-allyl-gluQ derivative **28**. We subsequently carefully deprotected both compounds using BCl_3 at −90 °C, followed by mild neutralization of the reagent with MeOH.

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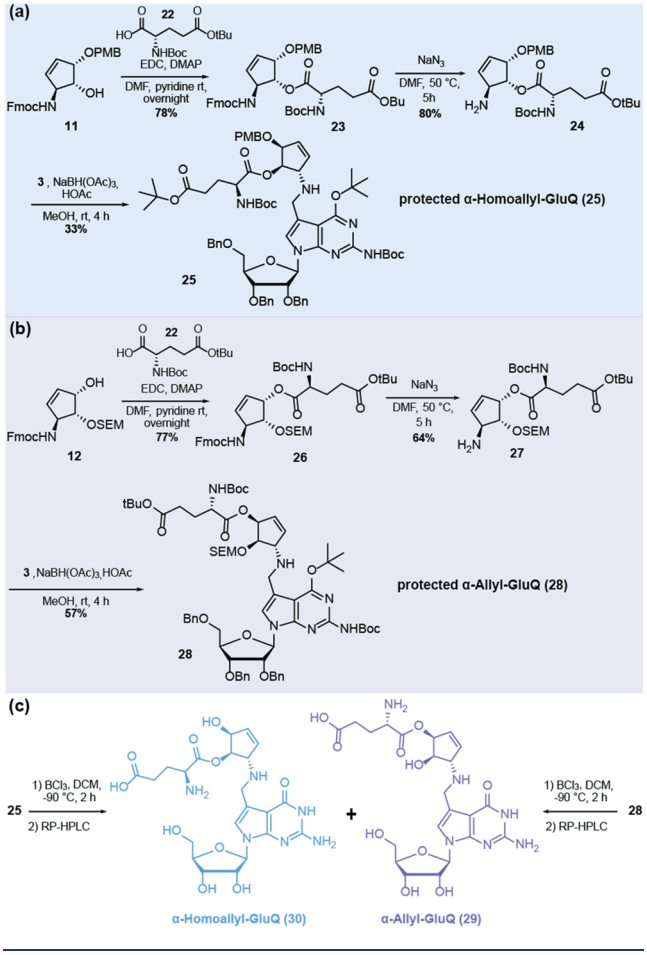
Scheme 3. Synthesis of the Protected gluQ Derivatives in Which the α-COOH Group Is Connected to (a) the Homoallyl-OH Group and (b) the Allyl-OH Group; (c) Full Deprotection of the Allyl- and Homoallyl Connected Versions of gluQ



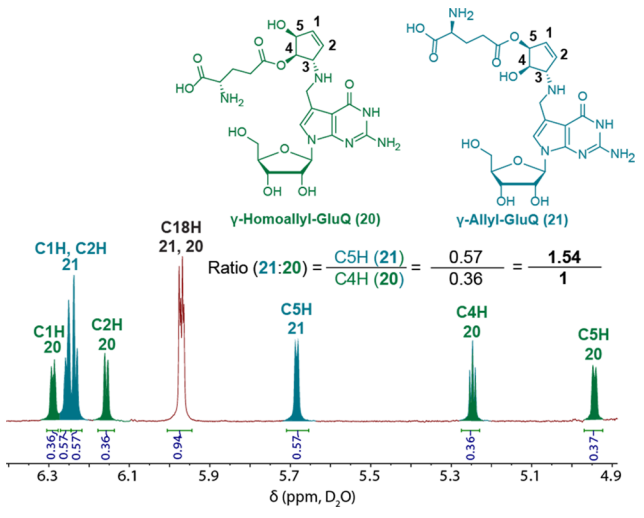


Figure 2. Zoom into the NMR-spectrum (D_2O) of deprotected **18**, yielding an allyl- and homoallyl-mixture of γ-gluQ derivatives connected via the γ-carboxylic acid. Isomeric ratio is determined by the integration of isolated signals. Full NMR spectra can be found in the Supporting Information.

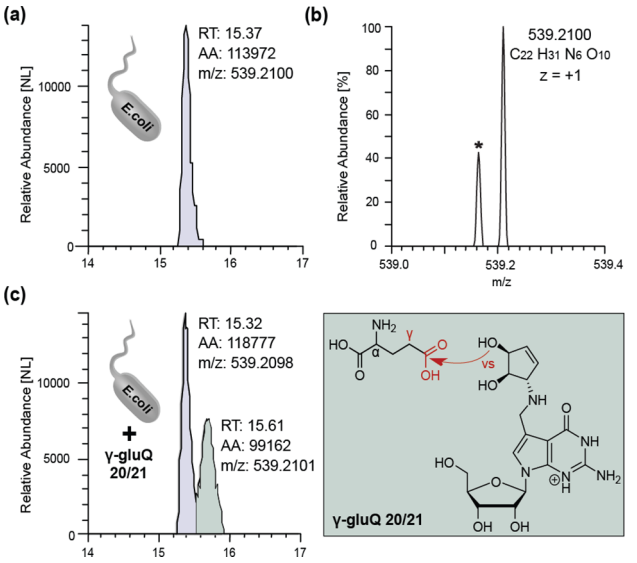


Figure 3. Results of the UHPLC-MS-*co*injection experiments of γ-gluQ with natural gluQ obtained from *E. coli* RNA. (a) Digested RNA from *E. coli* only. (b) Mass spectra of the natural gluQ compound. *Co-eluting background signal. (c) Digested RNA from *E. coli* + γ-gluQ mixture **20** and **21**.

This ensured the integrity of the labile ester bond during deprotection, avoiding most of the degradation to Queuosine (Scheme 3c).

Again, both compounds proved to be indistinguishable from each other in the chromatographic analysis. Subsequent NMR analysis showed again the presence of a mixture of both α-allyl and α-homoallyl species. Coinjection with the natural material, however, showed a perfect overlap of the peaks, proving that one of the two α-regioisomers had to be the correct compound (Supp. Figure 2). We therefore tried to separate the two synthetic isomers first using CE-MS, this however without success (Supp. Figure 3). With the working hypothesis that a potential interconversion of the two isomers might be a very fast process in aqueous solutions, therefore making separation in the liquid phase extremely difficult, we attempted analysis in the gas phase by TIMS-TOF MS. Indeed, using this technique, we could detect two signals corresponding to the two gluQ isomers (Supp. Figure 4). Upon further development of the HPLC-MS method, however, we could finally achieve separation of the α-allyl-gluQ from the α-homoallyl-gluQ also in liquid phase.

With the adjusted HPLC conditions, we were then able to obtain the synthetic α-allyl- and α-homoallyl compounds **29** and **30** in pure form and assigned the structures using $^{13}C/^{1}H$ and $^{15}N/^{1}H$ HMBC spectra. Characteristic for the two compounds were the ^{3}J-couplings between the bridging N atom and the tertiary homoallyl H atom and the ^{3}J-coupling of this H atom to the C atom of the carboxyl group of the amino acid residue.

To our surprise, under the optimized deprotection conditions the deprotection of both the allyl compound **28** and the homoallyl compound **25** gave a mixture of allyl:homoallyl compounds in a ratio of about 25:3 as determined by HPLC (Supp. Figure 5). As expected, the amino acid side chain can obviously fluctuate between the two hydroxyl groups with a preference for the allyl-isomer under the given deprotection conditions. We noted, however, that both are rather unstable in water because they degrade quickly

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to Queuosine, potentially because of the neighboring hydroxyl group assisting the hydrolysis. The instability of gluQ has previously been reported.[26] We found, however, that the compounds can be stabilized in an aqueous solution at a pH range between pH = 3.5 and 4.0. Under these conditions, only little degradation to Q and very little isomerization between allyl and homoallyl remained observable.

In order to study which of the compounds corresponds to the natural material, we next repeated the coinjection experiment using the improved HPLC method. While we detected two separate signals when we coinjected the α-homoallyl-gluQ **30**, only one amplified signal was detected with the α-allyl-gluQ compound **29** upon coinjection (Figure 4b). To exclude the possibility of gluQ isomerizing during purification from *E. coli* or digestion of the RNA we subjected α-homoallyl-gluQ to the purification and digestion procedures without observing any isomerization (Supp. Figure 6), proving that α-allyl-gluQ is indeed the correct structure of the natural material. In addition, the natural material was observed to isomerize to the α-homoallyl-gluQ after isolation, confirming an identical behavior to our synthetic gluQ standard (Supp. Figure 7).

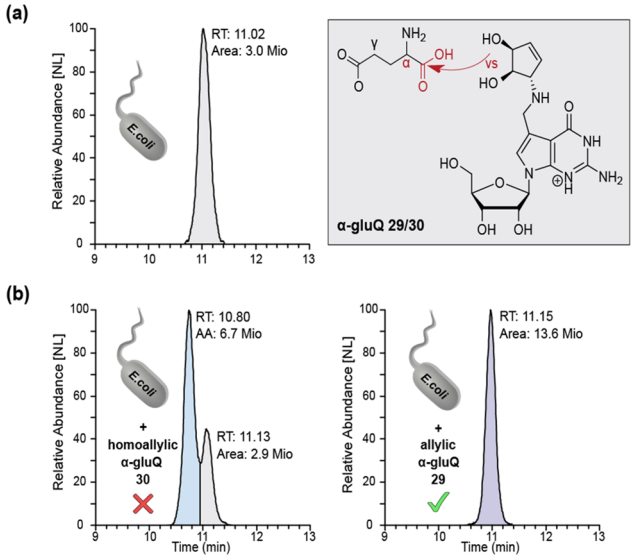

Figure 4. Results of the UHPLC-MS-coinjection experiments of α-allyl-gluQ and α-homoallyl-gluQ with natural gluQ obtained from *E. coli* RNA. (a) Digested RNA from *E. coli* only. (b) Left: digested RNA from *E. coli* with α-homoallyl-gluQ, resulting in two distinct signals. Right: digested RNA from *E. coli* + α-allyl-gluQ resulting in one amplified signal.

In summary, we present here the first total synthesis of natural gluQ. Our experiments show that the natural gluQ is the α-connected allyl species with the chemical structure **29**. Interesting is the observation that the compound is very much prone to hydrolysis, even at neutral pH values. This is potentially catalyzed by the neighboring OH-group. We show furthermore that the gluQ compound can interconvert between the allyl and the homoallyl form under specific conditions. Given the fact that hydrolysis is the dominant process in a physiological environment and isomerization—based on our final results—is very slow, we can be confident that the allyl position is the favored substrate for the truncated aminoacyl tRNA synthetase responsible for the formation of gluQ. Why does nature use an unstable modified nucleoside in a position of the anticodon loop that is critical for decoding genetic information? We believe that our improved protocols and structural elucidation can provide the basis for studies of the conformational dynamics of this unusual nucleoside during translation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c10075.

Synthetic procedures, NMR-spectra, CE-MS and TIMS-TOF data, Isolation of RNA from *E. coli*, further sample preparation and LC-MS parameters are attached in the Supporting Information. (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Q, Queuosine; manQ, Mannosyl-Queuosine; galQ, Galactosyl-Queuosine; gluQ, Glutamyl-Queuosine; CE-MS, Capillary Electrophoresis–Mass Spectrometry; TIMS-TOF, Trapped Ion Mobility Spectrometry Time of Flight

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