Title: Avoiding critical nucleoside impurities in Beta-Pseudouridine: A Technical Insight

Introduction:

Modified nucleoside triphosphates, particularly N1-Methyl pseudouridine triphosphate, play an essential role in the formulation of mRNA vaccines. A crucial concern for regents used in IVT is quality of the starting materials. EnginZyme has developed key raw materials for this industry that are produced fully enzymatically enabling lower environmental impact, better cost of goods and equivalent or even superior quality.

In pseudouridine, one key aspect of quality is control over the presence of a **critical impurity**, alpha-pseudouridine, a by-product of the synthesis of beta-pseudouridine can be eliminated with an efficient, enzymatic synthesis. In this document, we highlight the purity of our beta-pseudouridine product, focussing on the absence of the alpha-isomer. The beta-pseudouridine is enzymatically produced while still meeting all regulatory and quality requirements for the beta-pseudouridine pre-RSM material.

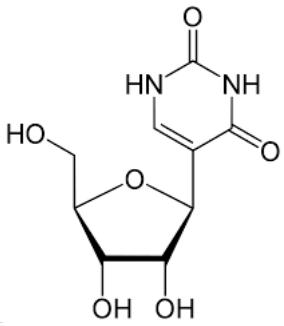

Figure 1 beta-pseudouridine

Technical Background:

Pseudouridine, and isomerised forms of uridine, exist in two isomeric configurations, alpha (α) and beta (β). The distinction between these isomers lies in the orientation of the glycosidic bond. While beta-pseudouridine is commonly found and utilized in RNA modifications, alpha-pseudouridine is a by-product of the chemical synthesis and is regarded as a critical impurity. We have developed a fully enzymatic synthesis of beta-pseudouridine starting from a readily available nucleoside starting material. The enzymatic route and subsequent downstream isolation are extremely efficient affording high-purity beta-pseudouridine without any trace of the critical impurity.

Analysing for alpha-pseudouridine

We developed an analytical method to allow separation alpha and beta-pseudouridine to demonstrate the isomeric purity. As shown in the picture below, Enginzyme-produced material is free of alpha-isomer (with limit of detection of around 1 ppm) corresponding to commercial 100% HPLC purity. As a reference material for alpha-pseudouridine we used commercially available standard.

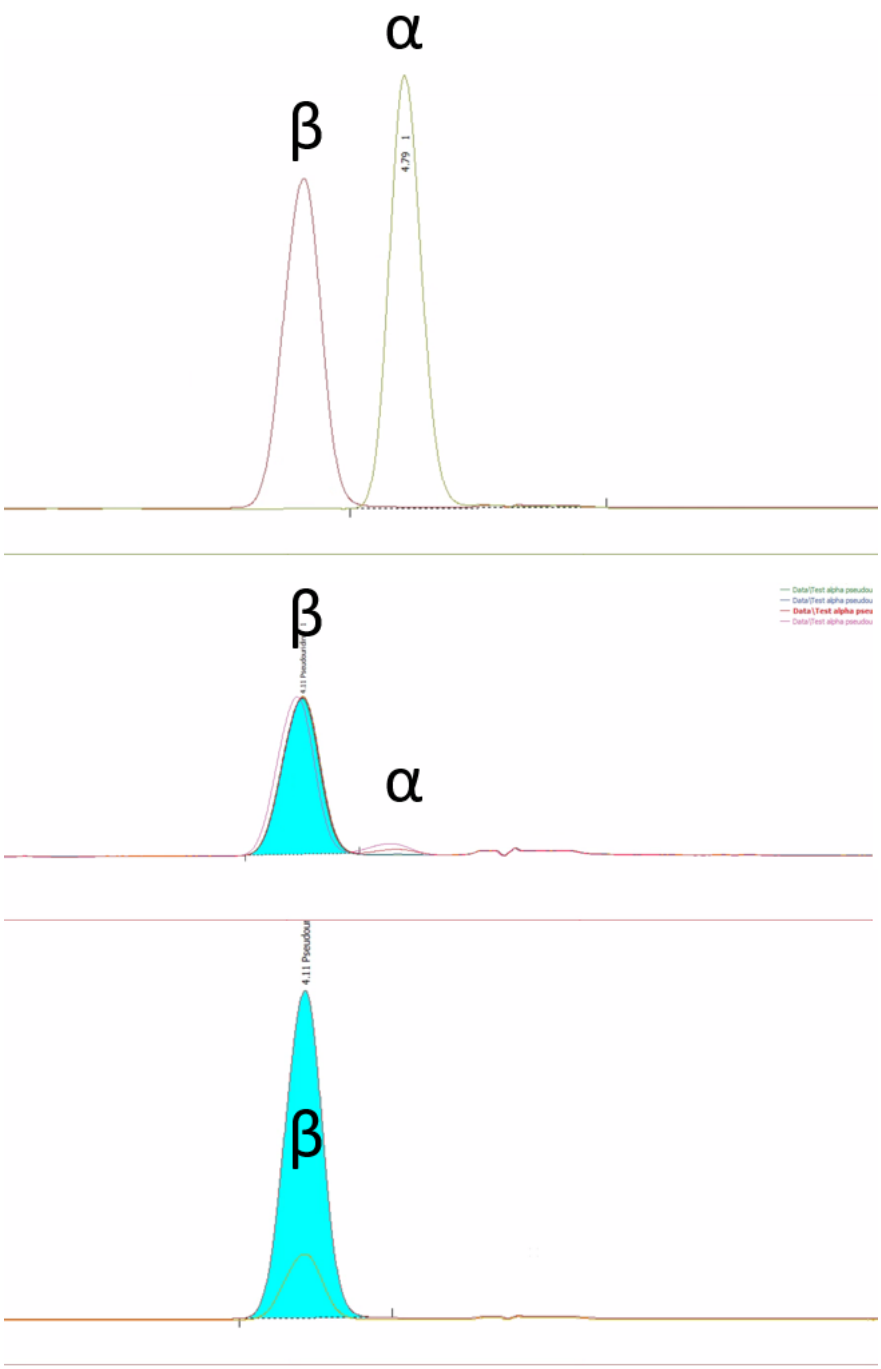

Figure 2 HPLC analysis of commercial standards of alpha and beta pseudouridine, the middle frame shows effect of spiking beta-pseudouridine with alpha. The bottom window is a representative trace for EnginZyme beta-pseudouridine without the presence of criticlal impurity

Mechanistic Insight:

From a mechanistic perspective, unlike common synthetic chemistry routes, the enzymatic synthetic route employed for our beta-pseudouridine product precludes the formation of the alpha isomer. Without delving into exhaustive details (which can be found in Pfeiffer, M., Ribar, A. & Nidetzky, B. *Nat Commun* **14**, 2261 (2023).

https://doi.org/10.1038/s41467-023-37942-7), the way the enzyme necessarily proceeds with a Mannich-type mechanism through the ribose-bound alpha configured intermediate excludes the formation of alpha-pseudouridine mechanistically impossible.

Conclusion:

Our beta-pseudouridine stands out for its unparalleled purity, absence of a known critical impurity and being a fully enzymatic synthesis. Our production takes pace in a fully cGMP compliant facility in Europe. The beta-pseudouridine is available GMP-grade, animal free and in commercial volumes. Samples and CoA of our material are available on request.

For further information, queries, or collaboration opportunities, please contact our technical team.