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# Preparation of fluorescent labeled gentamicin as biological tracer and its characterization by liquid chromatography and high resolution mass spectrometry☆

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ABSTRACT

This work deals with the preparation of single-labeled bioconjugates of the antibiotic Gentamicin (GT) with the sulforhodamine-derived fluorescence dye Texas Red®-X (TR), its purification by high-performance liquid chromatography (HPLC) and its characterization by high-resolution mass spectrometry. Aminoglycosides such as GT are efficient antibiotics, but also problematic due to severe side effects such as nephro- and ototoxicity. Fluorescent labeled GT is used to visualize cellular uptake and distribution of the antibiotic to finally understand the mechanisms of serious adverse drug reactions. Pharmaceutically administered GT is a mixture of mainly four different components, which exhibit three (GT(C1)) or four (GT(C1a), GT(C2), GT(C2a)) primary amino functional groups which can be coupled with the labeling reagent TR. Thus, multiple labeling could be envisaged which was assumed to be detrimental for uptake studies by fluorescence imaging. The proposed synthesis aimed at preparation of single labeled product and together with the employed purification strategy indeed yielded single labeled GT as product. Analytical control of the reaction product was carried out by means of mass spectrometry (UHPLC-ESI-QTOF-MS/MS) to rule out over-labeling of GT, which would alter the physicochemical characteristics of GT and its cellular uptake significantly. Moreover, LC–MS/MS analysis gave valuable insights into structural diversity of single labeled products. Further, high-resolution MS and MS/MS spectra of underivatized GT are provided as well. The analytical information on preparation strategy and structure diversity is valuable for studies with a clinical focus on research of aminoglycoside toxicity. Furthermore, it is deemed to be useful for the development of LC–MS/MS assays for the determination of aminoglycosides or the fast screening of synthetic biology samples from biotechnological drug discovery.

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## 1. Introduction

Gentamicin (GT) is a prominent member of the essential group of aminoglycosides which are broad spectrum antibiotics and highly efficient, especially in the treatment of life-threatening gram negative bacterial infections and tuberculosis. Aminoglycosides attack the small ribosomal subunit and bind to the 16s-RNA causing disruption of the translation process in protein biosynthesis [1]. Although the mechanism of action in bacteria was thoroughly clarified by means of crystal structures [2], the cellular mechanisms causing side effects are largely unknown. Especially ototoxicity and nephrotoxicity are limiting factors to their wider therapeutic use [3,4]. Gentamicin labeled with fluorescence dyes [5] was applied in several studies to understand drug trafficking in kidney proximal tubule cells [6,7] and inner ear sensory cells [8–10].

Gentamicin is isolated by fermentation from *Micromonospora purpurea* and therefore, possesses an inherent structural diversity of several components. In the present study, the pharmaceutically employed substance was utilized. It constitutes a mixture of gentamicin components $C_1$, $C_{1a}$, $C_2$ and $C_{2a}$ (GT(C1), GT(C1a), GT(C2) and GT(C2a)) (Fig. 1a). In general, the highly hydrophilic molecules contain the aminocyclitol 2-desoxystreptamine (B-ring) as common central element, which is also referred to as aglycone. It is connected pseudo-glycosidically to the amino sugar garosamine (amino sugar I) (C-ring) at position 6 and the amino sugar purpurosamin (amino sugar II, purpurosamin components

*Abbreviations:* GT, gentamicin; TR, Texas Red®-X; GTTR, Texas Red®-X labeled gentamicin; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography-mass spectrometry; SPE, solid phase extraction; ACN, acetonitrile; CE, collision energy; DP, declustering potential; IDA, information-dependent acquisition; XIC, extracted ion chromatogram; BPC, base peak chromatogram.

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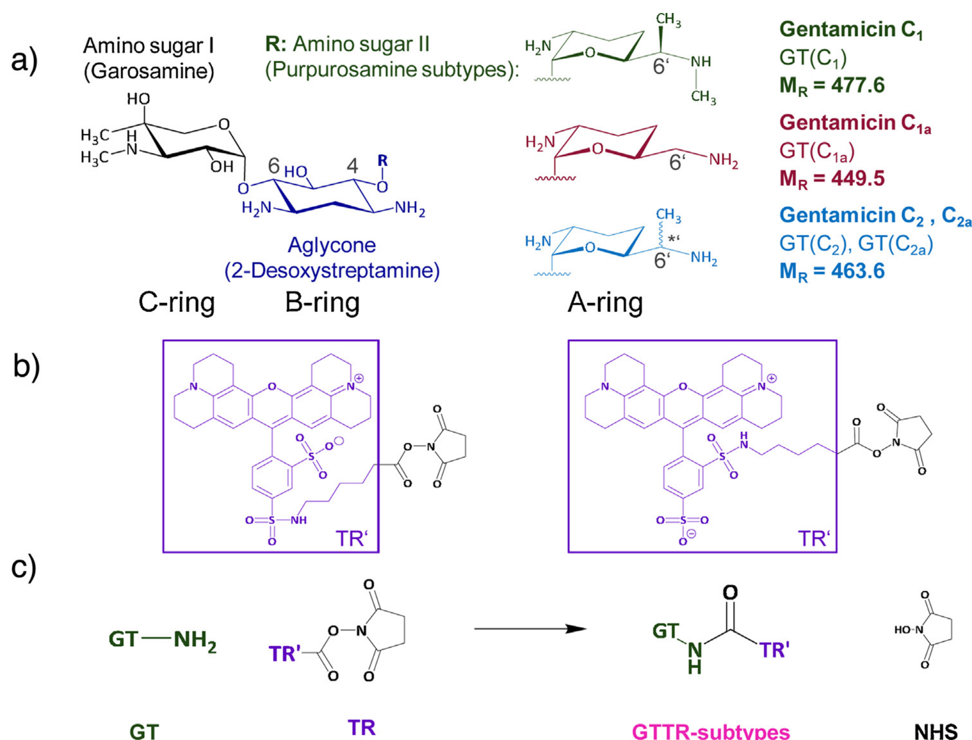


**Fig. 1.** (a) Structure of Gentamincin (GT) C1, C1a, C2 and C2a, (b) both isomeric forms of the fluorescence-label Texas Red®-X succinimidyl ester (TR) and (c) coupling reaction of GT with TR.

A–C) (A-ring) at position 4 which is the characteristic element of the gentamicin components under investigation. Having the opposite configuration exclusively with respect to the 6′ carbon of amino sugar II gentamicin $C_2$ (6′R) and $C_{2a}$ (6′S) are epimers of each other. The first objective in this study was to prepare gentamicin derivatized with a single fluorescently labeled dye as molecular probe for drug trafficking studies. The selected amine reactive labeling reagent Texas Red is a sulforhodamine derived fluorescence dye that is used to produce probes for confocal microscopy. The fluorogenic label can be attached to the substrates via an amide-linkage formed by coupling a primary amino-group of GT with the *N*-hydroxysuccinimide (NHS) activated carboxylic acid group of Texas Red®-X (TR) (Fig. 1c). Unfortunately, TR is a mixture of two isomeric forms (Fig. 1b), which adds to the structural complexity of the product mixture. Along with the distinct forms of GT present in the product and possessing either three (GT(C1)) or four (GT(C1a), GT(C2) and GT(C2a)) primary amino functions, the derivatization with TR may in principle yield a theoretical number of 30 possible single-labeled products (Supplemental information, Table S-1). To preserve the pharmacokinetic properties of GT a single labeling of GT was intended by a high molar excess of GT in the reaction mixture [7–12]. The mono-labeled conjugates used as biological tracers are supposed to undergo a relatively similar cellular uptake and distribution as GT itself [6]. Hence, it was of importance and goal of this study i) to develop an optimized protocol for synthesis of single labeled derivatives, ii) their preparative purification into one product fraction (for sake of yield) yet being free from excess of underivatized GT, free TR, or TR-derived products without GT, and iii) develop an adequate quality control method by hyphenation of UHPLC with high resolution mass spectrometry using an ESI-QTOF-MS/MS instrument. Furthermore, this method should allow for structural characterization of the bioconjugate and underivatized GT as well, for which high-resolution mass spectra were not discussed in detail yet in the literature. Accurate mass MS and MS/MS data of precursor and fragment ions are of utmost importance for identification of those compounds in untargeted profiling methods.

Analytical studies on gentamicin aminoglycoside were mainly related to the detection of this antibiotic agent (without derivatization) in food and environmental samples or biomatrices [13–15]. A few studies dealt with structural characterization of GT and other aminoglycoside antibiotics in the context of impurity profiling using ion-trap MS instruments [16–19]. High resolution MS characterizations of GT have not been broadly discussed in the literature and there are just a few articles dealing with general drug residue screening by HR-MS technology with major focus on detection but not structural characterization of GT [20,21]. Considering this lack of information on general fragmentation patterns, the presented results which support fragmentation patterns by accurate mass fragments might be of a more general interest in aminoglycoside antibiotics research.

## 2. Materials and methods

### 2.1. Materials

Texas Red®-X succinimidyl ester (TR) was obtained from Life Technologies (Eugene, OR, USA). Gentamicin sulfate (GT), formic acid, glacial acetic acid and ammonia were purchased from Sigma–Aldrich (Munich, Germany). *N*,*N*-Dimethylformamide (DMF), potassium hydrogen carbonate and potassium carbonate were supplied by Acros Organics (Geel, Belgium). Acetonitrile (ACN) was from Roth (Karlsruhe, Germany) and methanol from VWR (Fontenay-sous-Bois, France). All solvents were HPLC grade or ultra-mass spectrometry grade for LC–MS, respectively. Highly purified water was prepared with an Elga PureLab Ultra purification system (Celle, Germany).

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