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SYNTHESIS OF CONJUGATES OF HYALURONIC AND NICOTINIC ACIDS

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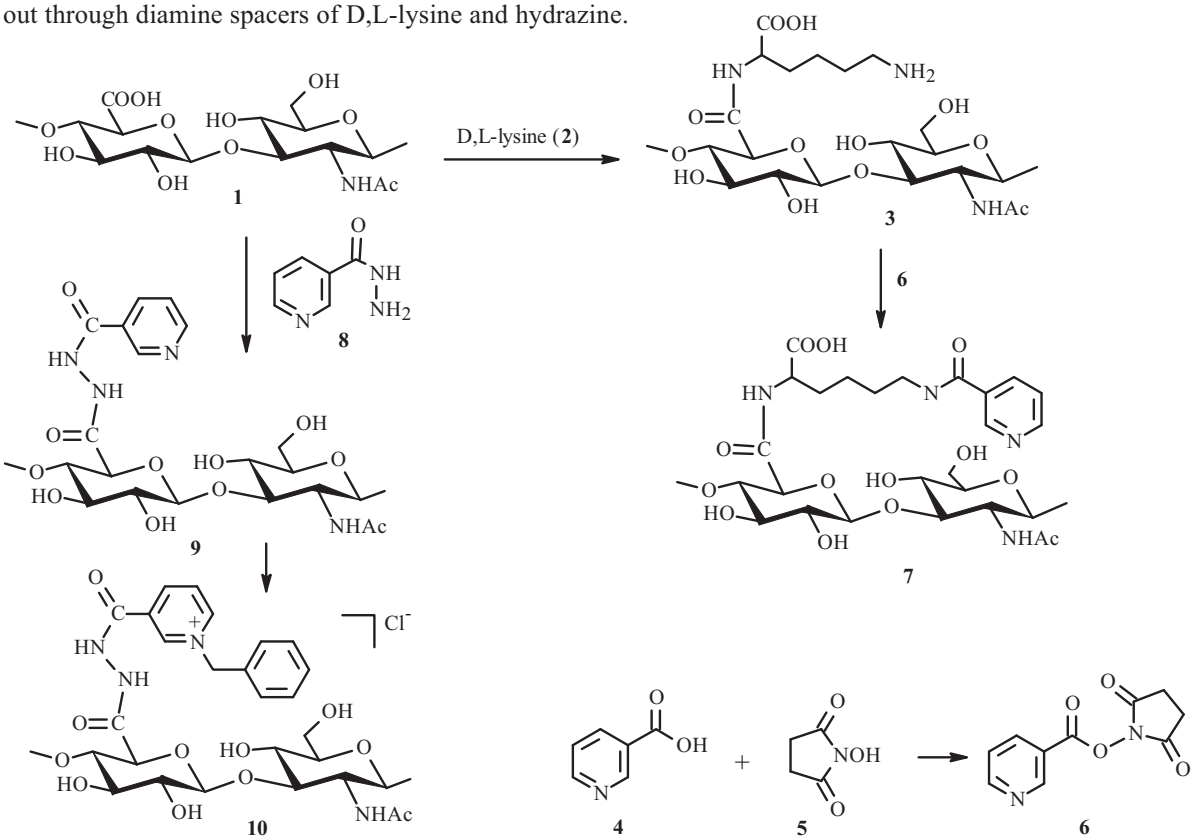
Conjugates with nicotinic acid of hyaluronic acid carboxylic and hydroxyl groups were synthesized and exhibited polyampholyte properties.

Keywords: hyaluronic acid, nicotinic acid, conjugates, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, polyampholytes.

Hyaluronic acid (HA) has recently been more widely applied in various areas of medicine and cosmetics as a macromolecular matrix for drugs and biocompatible material with reparative-regenerative properties. HA is modified chemically by compounds of natural origin in order to change its physicochemical properties and expand its spectrum of biopharmacological activity [1, 2]. Nicotinic acid, which is known for its unique physiological and pharmacological properties [3], is interesting in this respect. Incorporation of it into the HA structure can impart new properties to HA conjugates (including polyampholyte [4]) and lead to the creation of high-molecular-weight models of NAD^+ and NADH [5, 6].

We modified HA (**1**) at both the carboxylic and hydroxyl groups. Conjugation at the carboxylic groups of HA was carried out through diamine spacers of D,L-lysine and hydrazine.



Scheme 1

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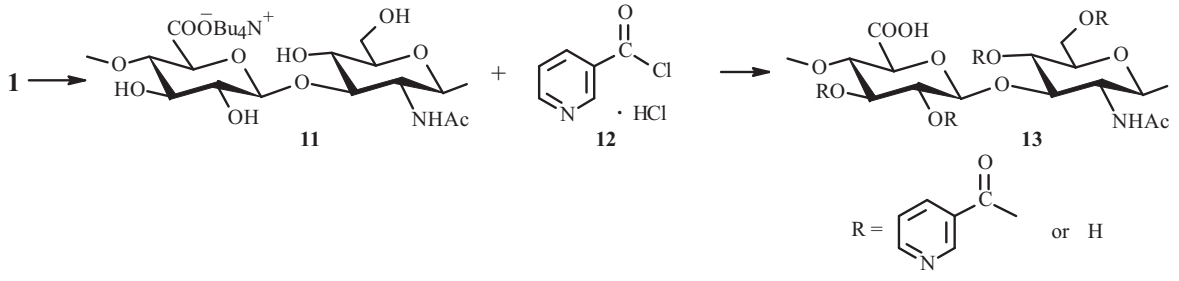
Conjugate **3** (Scheme 1) was obtained via reaction of **1** with D,L-lysine (**2**) in aqueous medium in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and *N*-hydroxybenzotriazole (HBT). The reaction did not occur without HBT. Conjugate **3** was purified of low-molecular-weight reagents by dialysis and represented the amide of HA bonded to lysine through its α-amine. This was consistent with PMR spectral data. Resonances of the terminal lysine methylene were located in the HSQC spectrum (δ_H 2.91 ppm, δ_C 39.2 ppm) in the same region as in the spectrum of α-*N*-acetyllysine and indicated that the ε-NH_2 was free. The ratio of intensities for the proton resonances of this methylene (δ 2.91 ppm) and the methyl protons of the acetamide (δ 1.92 ppm) suggested that the content of lysine-modified units in **3** reached 30% (calculated per 100 disaccharide units of HA).

The carboxylic group of nicotinic acid (**4**) was activated by conversion to *N*-succinimidyl **6** by reaction with *N*-hydroxysuccinimide (**5**) in the presence of dicyclohexylcarbodiimide (DCC) in anhydrous DMF in order to conjugate **3** to **4**. Combination of **3** and **6** in aqueous DMF in the presence of $NaHCO_3$ gave the desired conjugate **7** in which HA and nicotinic acid were linked through a lysine bridge. Resonances of the ε-CH_2NH_2 methylene protons (δ 2.91 ppm) were missing in the PMR spectrum of **7**, indicating that it had been exhaustively acylated. Therefore, **7**, like lysine-modified product **3**, contained 30% modified units, which was confirmed by the ratio of total intensity for resonances of Py protons (δ 7.5–9.0 ppm) and the acetamide singlet (δ 1.92 ppm).

The conjugate of HA (**1**) with nicotinic acid hydrazide (**8**) was synthesized by the action of EDC and HBT (Scheme 1). Conjugates **9a** and **9b** with 20 and 52%, respectively, modified units were obtained depending on the ratio of reagents. The extent of modification was determined from the relative total intensity for resonances of Py protons (δ 7.54–8.90 ppm) and CH_3CON methyl protons (δ 1.92 ppm).

Conjugates **9a** and **9b** were converted to *N*-benzylpyridinium chlorides (**10a** and **b**) by benzylchloride in DMF at 80°C (Scheme 1). PMR spectra of **10a** and **b** contained resonances for benzyl methylene (δ_H 5.85 ppm) and aromatic (δ 7.41 and 7.45 ppm) protons (in a 2:5 ratio). Resonances of Py protons were observed at weaker field (δ 8.1–9.4 ppm) than the same resonances in the spectra of conjugates **9a** and **9b**. The singlet for the acetamide methyl protons was split by the influence of the magnetically anisotropic benzyl group that was close to the neighboring *N*-acetylglucopyranose moiety. A multiplet consisting of seven resonances appeared. The absorption maximum of **10a** and **b** in the UV spectrum was shifted to long wavelength (λ_{max} 276 and a shoulder at 290 nm) relative to conjugates **9a** and **9b** (λ_{max} 263 nm). This was due to an increase of the conjugation chain in the *N*-benzylpyridinium moiety.

The acid-chloride method was the most effective one of those studied by us for synthesizing *O*-esters of HA with nicotinic acid [7]. HA nicotinate (**13**) was obtained by reaction of the tetrabutylammonium salt of HA (**11**) with freshly prepared nicotinic acid chloride (**12**) (Scheme 2).



Scheme 2

The PMR spectrum of **13** in the region of the Py protons (δ 7.50–9.50 ppm) exhibited resonances of different intensity and corresponded apparently to nicotinoyl substituents on all four OH groups of the disaccharide unit. The region of methyl protons contained a singlet for MeCON at δ 1.92 ppm and a strong-field resonance with δ 1.26 ppm that correlated, like the first singlet, with a resonance with δ 25 ppm in the HSQC spectrum and; therefore, corresponded to MeCON methyl protons. The strong-field shift was most probably due to the shielding effect of the magnetically anisotropic Py ring [8], which was located close to the MeCON group, i.e., on C-2 of the glucuronic acid pyranose ring. The degree of substitution in **13** was found to be 0.93 calculated for all four OH groups of the disaccharide unit (or the HA disaccharide unit) (the maximum possible degree of substitution taking into account all four OH groups in the HA disaccharide unit is four) based on the ratio of integrated intensities of the Py protons and both MeCON resonances adjusted to one proton.

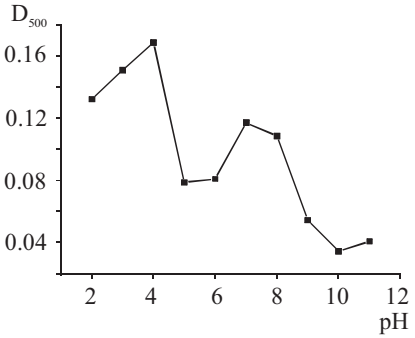


Fig. 1. Absorption of solutions of conjugate **9b** at 500 nm as a function of pH (c = 4 mg/mL).

The ability of Py bases to give salts with acids could produce conjugates **7**, **9a**, **9b**, **10a**, **10b**, and **13** with properties of polyampholytes. Polyampholytes in aqueous solutions are known to be sensitive to a change of ionic strength and pH. They form dispersions of colloidal particles under certain conditions. The absorption of these can be measured spectrophotometrically at 500 nm [4]. It can be seen using **9b** as an example (Fig. 1) that the absorption of the solutions changed as a function of pH (with maxima in the regions of pH 4 and 7). This was indicative of the formation of a complex salt of the Py base of nicotinic acid with a non-conjugated glucuronic acid and confirmed the properties of **9b** as a polyampholyte.

EXPERIMENTAL

PMR and ^{13}C NMR spectra (δ, ppm) were recorded in D_2O solutions with acetone internal standard on a Bruker Avance 400 spectrometer (operating frequency 400.13 MHz for ^1H and 100.58 MHz for ^{13}C). Electronic spectra of aqueous solutions were taken on a Specord M-40 spectrophotometer. Solution pH values were monitored using a pH-340 pH-meter.

We used pharmaceutical grade nicotinic acid (India); thionylchloride (Becton); and tetrabutylammonium iodide, *N*-hydroxybenzotriazole, *N*-hydroxysuccinimide, *N*,*N*-dimethylaminopyridine, D,L-lysine, α-*N*-acetyllysine, dichclohexylcarbodiimide, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, and hydrazine hydrate (Aldrich). Benzylchloride was purified by distillation. HA was prepared as before [8]. Nicotinic acid hydrazide was obtained from the reaction with hydrazine hydrate [9]; the chloride (as the hydrochloride), from the reaction with thionylchloride [10].

Tetrabutylammonium Hydroxide (Bu_4NOH, prepared by the literature method [11] with our modification). A solution of $AgNO_3$ (2 g, 11.77 mmol) in H_2O (10 mL) was treated with NaOH solution (1 N) until Ag_2O stopped precipitating. The precipitate was filtered off, washed with distilled H_2O, suspended in H_2O (20 mL), and treated with Bu_4NI (4.34 g, 11.77 mmol). The mixture was stirred at room temperature until the black Ag_2O solid changed to yellow AgI, which was filtered off. Then, the aqueous solution of Bu_4NOH was used to prepare tetrabutylammonium salt **11**.

Conjugate 3, ratio of reagents **1**:**2**:EDC:HBT 1:5:2:2. A mixture of HA (**1**, 60.0 mg, 0.15 mmol), D,L-lysine (**2**, 137.0 mg, 0.75 mmol), and HBT (40.5 mg, 0.3 mmol) in H_2O (15 mL) was treated with NaOH solution (1 N) until the pH was 6.8, heated to 40°C, stirred vigorously, and treated with dry EDC (57.5 mg, 0.3 mmol). The mixture was heated for 2 h and left for 24 h at room temperature. When the reaction was finished, the mixture was dialyzed against H_2O (24 h) and then treated with EtOH (45–50 mL). The resulting precipitate was separated by centrifugation, washed with EtOH (3 × 5 mL) and Et_2O (2 × 5 mL), and dried at <60°C and reduced pressure to afford an amorphous white powder (62 mg) that was soluble in H_2O and contained 30% lysine-modified units. PMR spectrum (characteristic resonances*): δ 1.2–1.8 (6H, β, γ, δ-CH_2), 1.92 (3H, s, MeCON), 2.91 (2H, m, $C\underline{H}_2NH_2$), 3.3–4.5 (12H in GlcA and GlcNAc units, H-1–H-6).

***N*-Succinimidyl Nicotinic Acid (6)** [12]. Acid **4** (86.1 mg, 0.70 mmol) and *N*-hydroxysuccinimide (80.5 mg, 0.70 mmol) in DMF (1.5 mL) was stirred vigorously, treated with ground dry DCC (158.6 mg, 0.77 mmol), and stirred at 20–25°C for 3–4 h. The course of the reaction was monitored by TLC on Sorbfil plates (Sorbpolimer, Krasnodar), R_f 0.87 (EtOAc:petroleum ether, 2:1). Compound **6** was used without isolation from the reaction mixture in the reaction with lysine-modified HA **3**.

*Here and henceforth characteristic resonances that are important for determining the content of modified units in the conjugates are given.

Conjugate 7. A solution of **6** (154 mg, 0.70 mmol) in DMF (5 mL) was stirred, treated dropwise with a solution of conjugate **3** (60 mg, 0.14 mmol) in aqueous $NaHCO_3$ (5 mL, 0.1 N) at pH 8.5, stirred at 20–25°C for 10–12 h, and dialyzed against H_2O for 24 h. Then the dialysate was treated with a three-fold excess of MeOH. The resulting precipitate was separated by centrifugation, washed with MeOH (3 × 5 mL) and Et_2O (2 × 5 mL), and dried at <60°C and reduced pressure to afford an amorphous white powder (54 mg) that was soluble in H_2O. PMR spectrum: δ 1.2–1.8 (6H, β, γ, δ-CH_2), 1.92 (3H, s, MeCON), 3.15 (2H, m, C$\underline{H_2}$NH), 3.3–4.5 (12H in GlcA and GlcNAc units, H1-H6), 7.5–9.0 (4H, H_{Py}).

Conjugate 9a, ratio of reagents **1**:**8**:EDC:HBT 1:1.2:0.75:1. Compound **1** (60 mg, 0.15 mmol) and nicotinoylhydrazide (**8**, 24.7 mg, 0.18 mmol) were dissolved in H_2O (15 mL), stirred vigorously, and treated with EDC (21.6 mg, 0.11 mmol) (20–25°C). The pH was adjusted to 4.75 using HCl solution (0.1 N). After 2 h HCl (1 N, 2–3 drops) and EtOH (45 mL) were added. The resulting precipitate was worked up as above for conjugate **7** to afford a product (56 mg) containing 20% modified units.

Conjugate 9b, ratio of reagents **1**:**8**:EDC:HBT 1:1.5:1.5:1. A mixture of HA (60 mg, 0.15 mmol) and **8** (30.8 mg, 0.23 mmol) in H_2O (15 mL) was stirred and treated with EDC (43.1 mg, 0.15 mmol). The reaction was carried out and worked up as for **9a** to afford a product (59 mg) containing 52% modified units. UV spectrum (H_2O, λ_{max}, nm): 263. PMR spectrum: δ 1.92 (3H, s, MeCON), 3.3–4.5 (12H in GlcA and GlcNAc units, H1-H6), 7.5–8.9 (4H, H_{Py}).

Conjugates 10a and b. The appropriate conjugate **9a** or **9b** (60 mg, 0.14 mmol) was suspended in anhydrous DMF (4.5 mL), treated with an excess (0.05–0.10 mL) of benzylchloride, and stirred for 20 h at 80°C. When the reaction was finished the DMF and benzylchloride were removed by Et_2O. The product was dissolved in H_2O and dialyzed against H_2O for 24 h in order to purify it further of the reagents. The conjugates were precipitated from the aqueous solutions by adding a three-fold volume of EtOH to afford a water-soluble yellow powder (56–60 mg). UV spectrum (H_2O, λ_{max}, nm): 276 and a shoulder at 290. PMR spectrum: δ 1.85, 1.86, 1.89, 1.92, 1.93, 1.94, 1.95 (3H, m, MeCON), 3.3–4.5 (12H in GlcA and GlcNAc units, H1-H6), 5.85 (2H, m, CH_2Ph), 7.4–7.5 (5H, H_{Ph}), 8.1–9.3 (4H, H_{Py}).

Tetrabutylammonium Salt of HA (11). Compound **1** (300 mg, 0.75 mmol) was dissolved in H_2O (15–20 mL), placed onto a column (300 × 25 mm) with Dowex 50WX4 in the H^+-form, and eluted by H_2O. The fraction with an acidic pH value was collected in a plastic beaker and neutralized by Bu_4NOH. Salt **11** was dried at 80°C. The resulting film was dissolved in MeOH (5 mL) and treated with Et_2O (15 mL). The resulting precipitate was separated by centrifugation, washed with Et_2O (2 × 3 mL), and dried at reduced pressure (60°C) to afford **11** (410 mg), 88% yield.

HA Nicotinate (13). A solution of **11** (87.4 mg, 0.14 mmol) in anhydrous DMF (5 mL) was stirred, treated with freshly prepared nicotinoyl chloride hydrochloride (**12**, 199.4 mg, 1.12 mmol, two-fold excess relative to each HA OH group) in Py (3 mL) and a catalytic amount of *N*,*N*-dimethylaminopyridine, heated at 60°C for 2 h, and left at room temperature overnight. The mixture was worked up as for conjugate **3** to afford a product (80 mg) with 0.93 degree of substitution by nicotinic units. UV spectrum (H_2O, λ_{max}, nm): 267. PMR spectrum: δ 1.26, 1.92 (3H, MeCON), 3.0–4.5 (12H in GlcA and GlcNAc units, H1-H6), 7.5–9.5 (4H, H_{Py}).

Polyampholyte Properties of Conjugate 9b. Several aqueous solutions of the conjugate at concentration 4 mg/mL were prepared. The pH of each solution was adjusted using NaOH (0.1 N) or HCl (0.1 N) to a certain value (from 2 to 11). The absorption at 500 nm was measured in a 1-cm cuvette [4].

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