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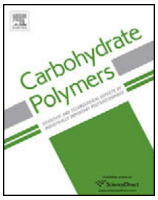

Modification of agarose: 6-Aminoagarose mediated syntheses of fluorogenic pyridine carboxylic acid amides

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ABSTRACT

A facile 6-aminoagarose (AA) mediated synthesis of new fluorogenic amides of agarose with nicotinic (AA-NA) and picolinic acids (AA-PA) employing carbodiimide chemistry have been described. 6-Amino agarose (AA) was synthesized in a facile Mitsunobu-inspired microwave mediated method involving the reaction of agarose with phthalimide in presence of diisopropyl azodicarboxylate and triphenylphosphene (DIAD/TPP) followed by hydrazinolysis. All compounds were characterized by GPC, UV spectrophotometry, fluorescence spectroscopy, FT-IR, ^{1}H and ^{13}C NMR spectra. The fluorescence emissions (λ_{max} 430 and 412 nm) of 1×10^{-3} M solutions of AA-NA and AA-PA in water were significantly higher (ca. 82% and ca. 90%) than those of the molar equivalents (0.2 mg) of NA and PA present in the 1×10^{-3} M solutions of the amides, respectively. These fluorogenic pyridine carboxylic acid amides of agarose may find applications as sensors in biomedical and pharmaceutical industries.

1. Introduction

Fluorescent polysaccharides and their conjugates were prepared with an eye to identifying biomolecules; sensing pH as well as preparing cellulose based organic light emitting diode (Karakawa et al., 2007; Kobayashi, Urayama, & Ichishima, 1990; Qiu, Xu, Zhu, & Qiu, 2005; Schulz et al., 2009; Suizhou et al., 2003). Urreaga and De la Orden (2007), has reported modification of cellulose with amino compounds and their fluorescence properties. Chhatbar, Meena, Prasad, Chejara, and Siddhanta (2011), have reported the facile microwave-assisted method of synthesis of water-soluble fluorescent alginic acid amides employing four diamines as well as their crosslinked products. The synthesis of alginic acid amide conjugates using N,N-dicyclohexylcarbodiimide (DCC) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) as activating agents for the carboxylic acid group is widely studied and reported (Abu-Rabeah, Polyak, Ionescu, Cosnier, & Marks, 2005; Chiu, Lee, Chu, Chang, & Wang, 2008; Polyak, Geresh, & Marks, 2004). Johnston and co-workers have recently described a unique method of amide synthesis, harnessing reversed polarities of both the acid and amine moieties, which is totally different from the traditional approach based on the direct condensation of negatively charged amine and positively charged carbonyl of the carboxyl group. The process of formation of amide bonds is extremely important since this is the cornerstone of protein synthesis. Work is also going on to prepare polymeric fluorescent derivatives, which are essentially used as sensory materials, screening assays for enzymes, detection of peroxides, plastic colorants, electro-luminescence devices etc. (Cumming et al., 2001; Sanchez & Trogler, 2008; Wang, Huang, Xia, Gao, & Yan, 2002; Xia et al., 2004). Furthermore, water-soluble fluorescent polymers are particularly useful tracers for their potential to act as analogs in understanding the behavior of biological macromolecules (Gao, Wang, Yang, & Fu, 2004).

Agarose, the red seaweed polysaccharide is widely used in biomedical and bioengineering applications. The basic disaccharide repeating units of agarose consists of (1,3) linked β-D-galactose (G) and (1,4) linked α-L-3,6-anhydrogalactose (A) (Araki, 1966). Several methods for the synthesis of amino group containing polysaccharide derivatives i.e. 6-amino-6-deoxycellulose, amino cellulose esters and amino starch have been reported (Matsui, Ishikawa, Kamitakahara, Takano, & Nakatsubo, 2005; Pinto Zarth, Koschell, Pfeifer, Dorn, & Heinze, 2011; Dong et al., 2010). Cellulose of different degrees of polymerization was converted to amino group containing esters under homogeneous reaction conditions by ring-opening reaction of various lactams in the presence of p-toluene sulphonic acid chloride (TosCl) (Pinto Zarth et al., 2011). Synthesis

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of an aminopolysaccharide via polymerization of 6-amino-6-deoxy-D-glucose and 2,6-diamino-2,6-dideoxy-D-glucose reported by Kadokawa, Hino, Karasu, Tagaya, and Chiba (2000). The resulted aminopolysaccharide was expected to be useful in the development of glyco-technology (Kadokawa et al., 2000). Carbohydrates functionalized with groups e.g. —SH, —NH_2 and —COOH can be used as synthetic tools for the isolation and affinity-based purification of carbohydrate-binding proteins as well as determination of their structural specificity (Daniel, Eddie, Matthew, & Peter, 2004).

A very useful bioactive molecule is nicotinic acid (pyridine-3-carboxylic acid) or also known as niacin, vitamin B3, and vitamin PP, a well-known cholesterol-lowering agent, was selected for this investigation. Nicotinic acid, which occurs naturally in food when used as a medicine, can improve levels of blood fats (lipids) such as cholesterol and triglycerides (Carlson, 2005; Gille, Bodor, Ahmed, & Offermanns, 2008). Likewise, picolinic acid (pyridine-2-carboxylic acid), an isomer of nicotinic acid was also selected for this study. Picolinic acid is used as an intermediate to produce pharmaceuticals (especially local anesthetics) and its metal salts for the application of nutritional supplements. The acid is believed to form a complex with zinc that may facilitate the passage of zinc through the gastrointestinal wall and into the circulatory system. A study of rats found that dietary picolinic acid increases the turnover and excretion of zinc (Evans & Johnson, 1981). Picolinic acid is an endogenous metabolite of L-tryptophan (TRP) that has been reported to possess a wide range of neuroprotective, immunological, and anti-proliferative affects within the body (Grant, Coggan, & Smythe, 2009).

As part of a continuing program of functional modification of seaweed polysaccharides in our laboratory (Chhatbar, Godiya, & Siddhanta, 2012; Kondaveeti, Prasad & Siddhanta, 2013; Mehta, Kondaveeti, & Siddhanta, 2011; Oza, Meena, & Siddhanta, 2012), we report herein a facile 6-aminoagarose mediated synthesis of new fluorogenic amides of agarose with pyridine carboxylic acids viz. nicotinic and picolinic acids.

2. Materials and methods

Agarose was extracted from the red seaweed *Gracilaria dura* of Indian waters following the method reported in the literature (Meena et al., 2007). The gel strength, sulphate and ash contents of the agarose were 950 g cm^{-2} (0.5% gel), $\leq$0.25% and 0.9%. Other chemicals e.g. phthalimide, diisopropyl azodicarboxylate (DIAD), triphenyl phosphine (TPP), and hydrazine were purchased from M/s. Spectrochem Chemicals Ltd., Mumbai, India. Nicotinic acid (NA) and picolinic acids (PA) were purchased from M/s. SRL Chemicals Ltd., Mumbai, India. 1-Ethyl-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), 2-[N-morpholino]-ethanesulfonic acid (MES) buffer and N-hydroxysuccinimide (NHS) were purchased from M/s Sigma Aldrich, Mumbai, India. The naturally occurring cross linker genipin was purchased from M/s. Challenge Bioproducts Co. Ltd., Taiwan. Milestone Start-S (Italy) programmable microwave reactor (Model Start-S; Terminal T260; Line Voltage 230 V; Magnetron S.N. 131528; Frequency 2450 Hz) was used for the reactions.

The characterizations were done by FT-IR analysis using a Perkin-Elmer FT-IR machine (Spectrum GX, USA) on a KBr disk (2.0 mg sample in 600 mg KBr). ^{13}C NMR spectra were recorded on a Bruker Avance-II 500 (Ultra shield) Spectrometer, Switzerland, at 125 MHz. Samples (Aminoagarose, AA-NA and AA-PA) were dissolved in D_2O (60 mg mL^{-1}) and spectra were recorded at 70 °C, d_6-DMSO was used as the internal standard (ca. δ 39.5 ppm) for AA-NA and AA-PA samples while it was d_3-acetonitrile for AA in the measurement of carbon-13 NMR spectra. A-Ph was dissolved in d_6-DMSO and the spectra were recorded at 70 °C and d_3-acetonitrile was used as internal standard. The ^{13}C NMR spectrum of nicotinic acid (NA) and picolinic acid (PA) were dissolved in d_6-DMSO (60 mg mL^{-1}) and spectra were recorded at ambient temperature. The ^{1}H NMR spectra of Agarose, AA, AA-NA and AA-PA derivatives were recorded (10 mg mL^{-1} of D_2O) at ambient temperature. The ^{1}H NMR spectra of Nicotinic acid and picolinic acid were recorded (10 mg mL^{-1} of d_6-DMSO) at ambient temperature.

The molecular weights (M_n, M_w) and polydispersity index (PDI) were measured by gel permeation Chromatography (GPC) on a Waters Alliance 2695 machine with RI 2414 detector. Columns (Ultra hydragel 120, Ultra hydragel 500) were eluted with 0.1 M $NaNO_3$ at a flow rate of 0.5 mL min^{-1}. Oven and flow cell temperatures were maintained at 45 °C for all measurements. Dextrans of different molecular weights e.g. 4.4×10^3, 4.3×10^4, 1.96×10^5, and 4.01×10^5 Da were used as standards for calibration.

Differential scanning calorimetric (DSC) was measurements were done on a Mettler Toledo DSC822 equipment (Switzerland), using a rate 1 °C min^{-1} in the temperature range −10 °C to 110 °C. Gel strengths of agarose, A-Ph (DS 0.24) and G-AA (DS 0.20) were measured in 0.5% (w/v) gel at 20 °C on a Nikkansui-type gel tester (Kiya Seisakusho Ltd., Japan). The gelling and melting temperatures of gel samples were recorded as reported earlier (Mehta et al., 2011).

2.1. Scanning electron microscopy (SEM)

The changes in morphology of the different samples (AA-NA and Ag-PA) were determined using a scanning electron microscope (Carl-Zeiss), model LEO 1430 VP, Germany, at an accelerating voltage of 20 kV. A small amount of sample was mounted on aluminum stub and placed it in the vacuum chamber. All images were recorded at the same magnification.

2.2. Total nitrogen and degree of substitution (DS)

Total nitrogen was estimated by Kjeldahl method on a KEL PLUS-KES 201 Digestion unit attached to a KEL PLUS-CLASSIC DX Distillation unit (M/s PELICAN equipments, Chennai, India). The degree of substitution (DS) on agarose backbone was calculated on the basis of nitrogen contents of products, hence, it may be noted that isolated agarose did not contain nitrogen.

2.3. Synthesis of 6-amino agarose (AA)

In a typical batch, the dried agarose (306 mg, 1 mmol) was dissolved in N,N-dimethylformamide (DMF) in a microwave reactor (80 °C for 3 min). The agarose solution was cooled to 0 °C followed by the addition of phthalimide (73.56 mg, 0.5 mmol), triphenylphosphine (1049.16 mg, 4 mmol) and diisopropyl azodicarboxylate (787.57 µL, 4 mmol) and reaction was carried out under microwave irradiation at 100 °C for 15 min. The agarose phthalimide (A-Ph) derivative was isolated by precipitation with isopropyl alcohol (IPA) (1:2 v/v), washed with IPA and cold (10 °C) diethyl ether (10 mL × 4; 20 min of each) and dried in a vacuum desiccator at ambient temperature. A-Ph was subjected to hydrazinolysis with hydrazine hydrate (20 mmol) in 5.0 mL ethanol in a microwave reactor at 65 °C for 30 min (Scheme 1). The product (AA) was filtered and washed with hot (50 °C) ethanol (10 mL × 3) and dichloromethane (10 mL × 3) dried in a vacuum desiccators.

2.4. Synthesis of nicotinic acid (pyridine-3-carboxylic acid) and picolinic acid (pyridine-2-carboxylic acid) amide of agarose derivatives

0.036 g (0.5 mmol) of nicotinic acid was added to a 1% (w/v) solution of aminoagarose DS 0.20 (20 mL solution) in 0.1 M MES buffer,