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Negative circular dichroism (CD) band of lactones of sialic acid polymers observed at 235 nm

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NeuAc oligomers and polymers (DP2-15), isolated from a commercial colominic acid, were lactonized in 10 mM HCl or glacial acetic acid. All lactonized oligo/polymers showed a negative circular dichroism (CD) band at 235 nm. The determination of lactonized oligo/polymer was performed by capillary electrophoresis. Molar ellipticity per lactone ring was -1.5×10^4 deg·cm^2/dmol, and the molar ellipticity showed additivity with respect to the lactone ring. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Ganglioside lactones have been isolated from animal tissues and cells (Riboni *et al.*, 1986; Terabayashi *et al.*, 1990; Nores *et al.*, 1987) and three types of lactone linkage, NeuAc1–9NeuAc, NeuAc1–2Gal and NeuAc1–4Gal, have been found (Terabayashi *et al.*, 1990; Yu *et al.*, 1985; Acquotti *et al.*, 1987). We found that each type of lactone showed a characteristic negative CD band at 235 nm with a molar ellipticity of -1.4×10^4 deg·cm^2/dmol per lactone ring (Terabayashi *et al.*, 1992). Lactone formation of GD1b, a member of the ganglioside family, was followed accurately by observing the ellipticity change at 235 nm (Tsuda *et al.*, 1994). The lactone formed from the carboxyl group of sialic acid was also found by McGuire and Binkley (1964) in colominic acid, a capsular polysaccharide of *E. coli* which was composed of NeuAc α-(2–8) linked homopolymers with different degrees of polymerization (DP). More recently, lactonization of NeuAc homopolymers including colominic acid was examined *in vitro* (Lifely *et al.*, 1981, 1984). Crescenzi *et al.* (1991) investigated the lactonization of colominic acid by observing optical rotation at 302 nm, and reported CD spectra of the colominic acid lactone. However, no investigation has been carried out on NeuAc polymers with different degrees of polymerization. In particular, the molar ellipticity for a lactone ring in each oligo/polymer has not been measured. Measurement of the ellipticity at 235 nm is a better method than IR or GLC (McGuire & Binkley, 1964; Lifely *et al.*, 1981, 1984) for following lactones formed by sialic acid, because the measurement enables lactone formation to be monitored continuously. In addition, the degree of lactonization can be determined using as little as 1 mg sample and the method is nondestructive (Tsuda *et al.*, 1994). However, the additivity of the molar ellipticity was confirmed in only dilactones formed in disialo-gangliosides GD3 and GD1b (Terabayashi *et al.*, 1992). NeuAc oligo/polymers are suitable samples to characterize the CD band at 235 nm because they contain no hydrophobic moiety such as ceramide to form micelles in water, in contrast to the ganglioside.

In this study, we determined the ellipticity of lactones from each NeuAc oligo/polymer (DP2-10) with the aid of capillary electrophoresis and revealed that the molar ellipticity per residue is -1.5×10^4 deg·cm^2/dmol and additive with respect to the number of lactone rings as in ganglioside lactones. These results indicate that the CD band is useful for analyzing lactones formed in sugar chains containing sialic acid(s).

MATERIALS AND METHODS

Samples

N-acetylneuraminic acid (NeuAc) and colominic acid (from *E. coli*) were purchased from Sigma (St Louis, USA). NeuAc oligomers (DP2-6, from hydrolysate of colominic acid) were purchased from Nacalai tesque (Kyoto, Japan). The purity of each sample was checked by thin layer chromatography (TLC) as described below.

Oligomers and polymers of NeuAc were separated from colominic acid by DEAE-Sephadex A-25 column chromatography (Nomoto *et al.*, 1982).

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Colominic acid (900 mg) was dissolved in 50 ml of 0.01 M Tris-HCl (pH 7.6) and applied on a DEAE-Sephadex A-25 (2.6 × 80 cm, Cl^-) column equilibrated with the same buffer. The column was washed with the starting buffer, and NeuAc oligomers and polymers were eluted with 8000 ml of linear gradient of NaCl (0–0.6 M) in 0.01 M Tris-HCl (pH 7.6).

Lactonization

Oligomers and polymers of NeuAc (1 mg) were lactonized in 0.5 ml of glacial acetic acid or 1–10 mM HCl. Lactonization was monitored from the change of ellipticity at 235 nm. After the lactonization, each sample was lyophilized.

Capillary electrophoresis (CE)

CE was carried out by the method of Ogawa *et al.* (1995) using a Quanta 4000 system from Waters (Milfold, MA, USA) equipped with a UV filter detector adjusted to 214 nm, an automatic injector and a capillary cartridge, fitted with 50 μm i.d × 60–120 cm fused-silica capillary tubes. The running buffer was 25 mM sodium tetraborate–75 mM boric acid (pH 6.9) containing 0.3% methyl cellulose (MC # 8000, Nacalai tesque) and 100 mM SDS. All experiments were carried out at room temperature, and injection of the sample was performed in the hydrostatic mode (10 cm in height) for 30–120 s according to the concentrations of the samples.

Mild alkali treatment

Lactone ring was hydrolyzed in 25 mM sodium tetraborate–75 mM boric acid buffer (pH 8.8) at room temperature for 2 h.

Thin-layer chromatography (TLC)

A small portion (about 1 μg as NeuAc) of the sample solution was applied on a high-performance thin-layer plate (HPTLC, silica gel 60, Merck, Darmstadt). Chromatography was performed in 1-propanol/25% aqueous ammonia/water (6:1:2.5, v/v) (Kitazume *et al.*, 1992) for the analysis of the polymers, DP of which was not larger than 10. The polymers of higher DP were chromatographed with 1-propanol/ethanol/25% aqueous ammonia/0.1%$CaCl_2$ (30:30:10:25, v/v). A neutral solvent system used for the oligomer lactone was ethylacetate/1-propanol/ethanol/water (1:4:1:1, v/v).

CD spectrometry

CD spectra in the range of 200–300 nm were obtained on J-20 and J-720 Spectropolarimeters (JASCO, Tokyo, Japan) using 1 and 2 mm cells.

FT-IR

FT-IR spectra were obtained in the microscopic mode on an FT-530 spectrometer (Horiba, Kyoto, Japan).

Determination of NeuAc

NeuAc was determined by the resorcinol–HCl method of Svennerhorm (1957) as modified by Miettinen and Takki-Luukkainen (1959).

RESULTS AND DISCUSSION

As shown in Fig. 1, a commercial colominic acid fractionated into 15 separated peaks and one large composite peak using DEAE-Sephadex A-25 column chromatography. TLC (Fig. 2) shows that each of these

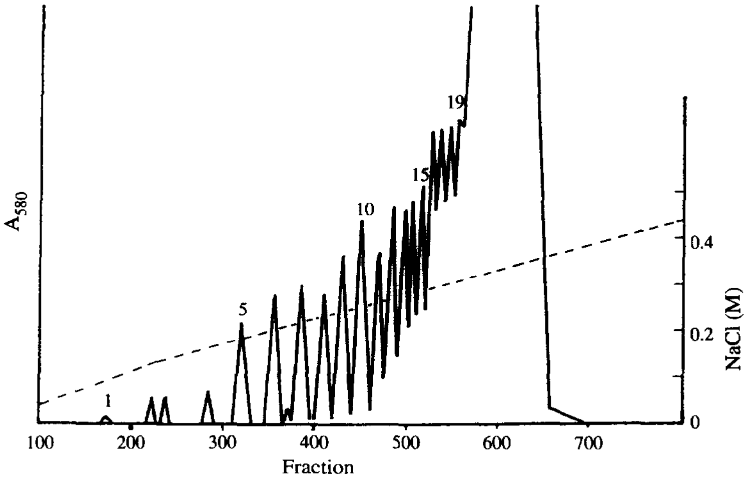

Fig. 1. Chromatographic separation of NeuAc oligo/polymers from colominic acid on a DEAE-Sephadex A-25 column. Peak numbers correspond to DPs of NeuAc oligo/polymers. The content of NeuAc was determined by the resorcinol method.

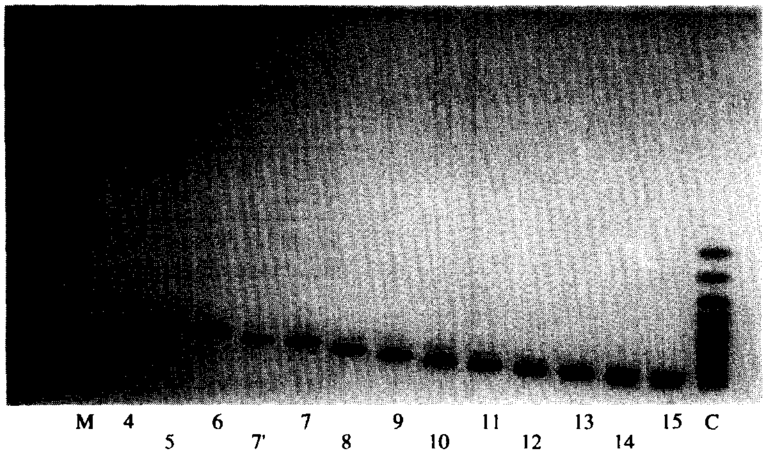

Fig. 2. Thin-layer chromatogram of NeuAc polymers eluted from a DEAE-Sephadex A-25 column. A small portion (about 1 μg as NeuAc) of each peak was applied onto an HPTLC plate and developed with a solvent system, 1-propanol/25% aqueous ammonia/water (6:1:2.5, v/v). M, authentic NeuAc monomer; C, colominic acid. Spots were detected using the resorcinol reagent.

separate peaks consists of a single component and the order of the eluted peaks corresponds to the DP of NeuAc polymers. NeuAc polymers with a DP of more than 19 (after peak 19) could not be separated even if an elution gradient with a more gentle slope was used. Figure 3 shows the CE patterns of NeuAc oligomer (DP4) and its lactone. The lactone migrated faster than the NeuAc monomer. After a mild alkaline treatment of the lactone, however, the mobility became the same as that of the parent oligomer DP4. Since the lactonized oligomer was not hydrolyzed by neuraminidase (from *Cl. perfringens*), the time course of the lactone digestion is not identical with that of the parent oligomer DP4 which is eventually hydrolyzed to a monomer by the enzyme. On a TLC plate (Fig. 2), NeuAc oligo/polymers were separated into identifiable spots. However, lactones of the oligo/polymers were detected as faint bands with pronounced tailing under the chromatographic conditions using an alkaline solvent system. Due to decomposition of the lactone linkage during development, exact determination of degree of lactonization could not be carried out by the TLC-densitometry. In CE analysis, the lactone of each oligo/polymer was detected as a sharp baseline-separated peak (Fig. 3). From this point of view, CE is an excellent method for analysis of lactones of NeuAc oligo/polymers. FT-IR spectra of the lactonized oligomer DP4 (Fig. 4) showed the characteristic absorption for the ester linkage at 1750 cm^{-1}, which is not observed in the parent oligo/polymers. Figure 5 shows the time course of CD spectral change of NeuAc oligomer DP4 during lactonization in 10 mM HCl. The oligomer showed a negative CD band centered at 225 nm. After the addition of HCl, another negative CD band, which was not observed in the parent oligomer, appeared at 235 nm and enlarged with the progress of lactonization as observed in the lactonizations of GD1b (Tsuda *et al.*, 1994) and colominic acid (Crescenzi *et al.*,

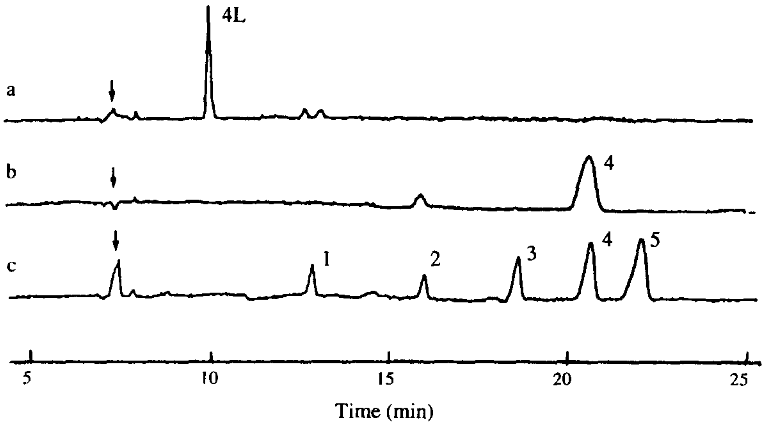

Fig. 3. CE pherograms of NeuAc oligomer DP4 before and after mild alkali treatment. a, lactonized oligomer DP4; b, lactonized oligomer DP4 after mild alkali treatment; c, mixture of standard samples of NeuAc monomer and oligomers DP2-5. Arrows indicate water. Operating conditions as described in the text.

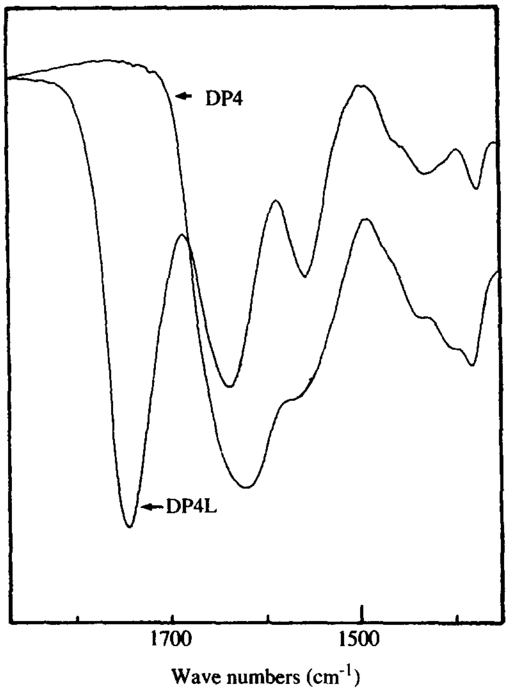

Fig. 4. FT-IR spectra of lactonized oligomer DP4 (DP4L) and its parent oligomer (DP4).

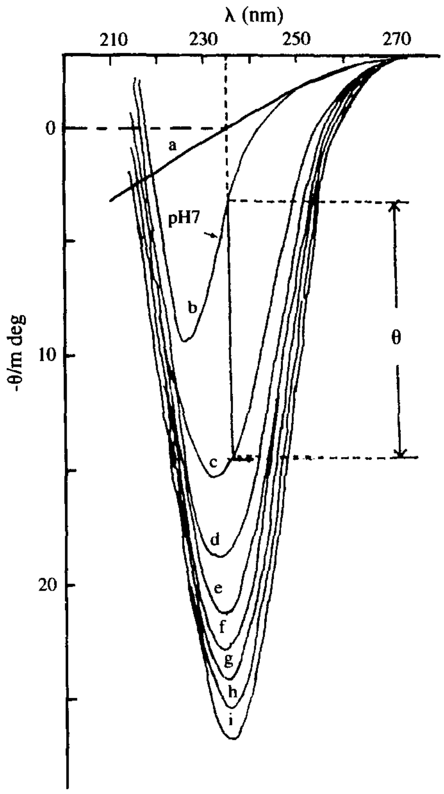

Fig. 5. CD spectral change at 235 nm of NeuAc oligomer DP4 (1 mg/ml) during lactonization. CD spectra were obtained on a J-20 Spectropolarimeter using 2 mm cell at 20°C. a, water; b, NeuAc oligomer DP4 at pH 7,0; c–i, sequential measurements of NeuAc oligomer DP4 at an interval of 8 min after dissolved in 10 mM HCl.

Table 1. Molar ellipticity of NeuAc oligo/polymers at 235 nm

DP	Lactone				
	2	3	4	5	10
-[Θ]	1.47	2.88	4.65	5.84	13.68
-[Θ$_R$]	1.47	1.44	1.55	1.46	1.52

Θ, molar ellipticity of band at 235 nm (10^4 deg · cm^2/dmol). Θ$_R$, molar ellipticity per residue (10^4 deg · cm^2/dmol).

1991). The molar ellipticities are summarized in Table 1. The degrees of lactonization estimated by CE were 88–95%. From these figures, the molar ellipticities per residue, [Θ$_R$], for oligomers and polymers were estimated as $-1.45 \sim -1.55 \times 10^4$ deg·cm^2/dmol. The molar ellipticity showed additivity with respect to the lactone ring. In ganglioside, three types of inner ester linkages (NeuAc1–9NeuAc, NeuAc1–2Gal, and NeuAc1–4Gal) which form six-membered lactone rings have been found (Terabayashi *et al.*, 1990; Yu *et al.*, 1985; Acquotti *et al.*, 1987). We found that ganglioside lactones show a characteristic negative CD band at 235 nm with a molar ellipticity of $-1.4 \sim -1.5 \times 10^4$ deg·cm^2/dmol per lactone ring (Terabayashi *et al.*, 1992). Ganglioside contains a hydrophobic moiety, ceramide, which consists of a long-chain base and a long-chain fatty acid. Since this glycolipid forms micelles in water, the question could be raised as to whether the CD band was an artifact. We revealed that no substantial change in light scattering intensity occurred during lactone formation, while the ellipticity at 235 nm increased (Tsuda *et al.*, 1994). From these results, we concluded that the CD band described above is not an artifact. NeuAc oligo/polymers are suitable samples to characterize the CD band, because the oligo/polymers form only one type of lactone linkage, NeuAc1–9NeuAc (Lifely *et al.*, 1981, 1984), and do not form micelles in water in contrast to ganglioside. The results in this study support our view that the CD band is attributed to the presence of the six-membered lactone ring formed between the carboxyl of a sialic acid residue and the hydroxyl of the neighboring sugar. Measurement of the ellipticity at 235 nm is more useful for analyzing lactones formed in sugar chains containing sialic acid(s) than IR and GLC, because the measurement is nondestructive and enables lactone formation to be monitored continuously. The degree of lactonization can be measured using as little as 1 mg sample.

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