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Distribution of Methoxyl Groups in Apple Pectic Substances

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ABSTRACT

The distribution of methoxyl groups in apple pectic substances was investigated by means of fractionation on ion-exchange and gel-filtration columns and by means of degradation of pectin fractions by pectin lyase and pectate lyase. Pectin fragments thus obtained were fractionated by gel-permeation chromatography and high-pressure liquid chromatography. It was concluded that a heterogeneous intermolecular distribution of the methoxyl groups exists with peaks at degrees of esterification of about 50%, 70% and 95%. The intramolecular distribution of the methoxyl groups cannot be distinguished from a random distribution. Since plant pectin esterases cause a blockwise de-esterification, it is unlikely that the biosynthesis of apple pectic substances passes through a stage of 100% esterification after which partial de-esterification by pectin esterase occurs.

INTRODUCTION

In the preceding papers (de Vries *et al.*, 1981, 1982, 1983) a model of apple pectin molecules was presented. According to this model the molecules consist of homogalacturonan regions ('smooth' regions) and heterogalacturonan regions ('hairy' regions). The degree of esterification (DE) of the heterogalacturonan regions is almost 100%. The long homogalacturonan regions (representing over 90% of the galacturonate residues) have an average degree of esterification of about 70%.

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This paper deals with the distribution of the methoxyl groups in the homogalacturonan regions. Extensive fractionation by different methods was performed to study the intermolecular distribution. The intramolecular distribution was studied by extensive degradation of pectin fractions with purified pectolytic enzymes. Because the side chains are located in a few short regions (de Vries *et al.*, 1981), it can be concluded that the effect of the side chains on the enzyme activities can be neglected in these studies. Pectin fractions extracted from apple were compared with pectins prepared by either alkaline or enzymic saponification of fully esterified pectin. Degraded pectin fractions were fractionated by high-pressure liquid or by gel-permeation chromatography.

MATERIALS AND METHODS

Materials

Pectic substances were extracted from apple alcohol insoluble solids (AIS) as described by de Vries *et al.* (1981). Commercial apple pectin was obtained from Obipectin Ltd, Bischofszell, Switzerland (Brown Ribbon pectin). Esterification of this pectin was performed in cold acidified absolute methanol (2°C) for two weeks as described by Heri *et al.* (1961); in the text this esterified pectin is referred to as 'pectin (DE = 95%)'. Saponification (de-esterification) to a final DE of 71% of this fully esterified apple pectin was performed in cold aqueous solutions (0°C) for 48 h by addition of a calculated amount of 0·1 N KOH. In the text the pectin thus obtained is referred to as 'trans-esterified' pectin. Complete de-esterification of Brown Ribbon pectin was performed by repeated addition of 0·1 N KOH.

Analytical methods

The anhydrouronic acid (AUA, $MW = 176$) content of pectin fractions was determined by an automated carbazole sulphuric acid assay (van Deventer-Schriemer & Pilnik, 1976). The neutral sugars were analysed by gas chromatography as their alditol-acetates (Albersheim *et al.*, 1967; Darvill *et al.*, 1975). The methoxyl content was determined by gas-chromatographic analysis of the methanol released on alkaline

de-esterification (1 h at room temperature, 0·1 N KOH). Methanol was converted to methyl nitrite and determined according to the method of Versteeg (1979).

Gel filtration

A sample of (degraded) pectin (10–50 mg in 2 ml of buffer) was applied to a Sephacryl S-300 column (80 × 2·5 cm) and eluted with 0·05 M sodium phosphate buffer, pH = 5·6 (unless otherwise stated). The flow rate (0·3 ml min^{-1}) was controlled by an LKB peristaltic pump and the whole procedure was conducted at room temperature. The void volume (Blue Dextran) of the column was 120 ml; the included molecules (glucose) appeared at an elution volume of 300 ml.

Enzymic degradation

Highly purified pectin lyase (type 2, van Houdenhoven, 1975) and pectate lyase (Rombouts *et al.*, 1978) were used. Enzyme reaction conditions were as follows:

Pectin lyase (EC 4.2.2.10; poly(methoxygalacturonide) lyase): 0·2 mg ml^{-1} substrate and 0·01 (limited degradation) or 2 (extensive degradation) units ml^{-1} in 10 mM sodium citrate or phosphate buffer pH = 5·2 at 30°C for 4–10 h.

Endo-pectate lyase (EC 4.2.2.2; poly(1,4-α-D-galacturonide) lyase: 0·1 mg ml^{-1} substrate and 5–15 units ml^{-1} enzyme in 10 mM sodium carbonate buffer pH = 6·9 at 30°C for 4 h.

The extent of degradation (% bonds broken) was determined spectrophotometrically at 234 nm, assuming $\epsilon_{235} = 4800$ M^{-1} cm^{-1} (MacMillan & Vaughn, 1964) for the de-esterified unsaturated product and $\epsilon_{235} = 5500$ M^{-1} cm^{-1} (Edstrom & Phaff, 1964) for the esterified unsaturated product. In the case of extensive degradation, it was supposed that the degradation was complete when additional portions of enzyme did not result in an increase in the absorbance at 235 nm. The absorbance values of substrate blanks did not increase under the conditions mentioned above.

Ion-exchange

10–100 mg of pectin sample were dissolved in 5 mM sodium phosphate buffer, pH = 5·1 (unless otherwise stated) and applied to a 10 × 0·4 cm

column of DEAE-cellulose (Whatman DE 52). The pectins were eluted from the column with a linear gradient of 5–300 mM sodium phosphate buffer of pH = 5·1 (200 ml) (unless otherwise stated). Experiments were performed at room temperature.

High-pressure liquid chromatography (HPLC)

A Spectra Physics liquid chromatograph (SP 8000) equipped with a Schoeffel 770 variable wavelength detector was used. A 250 × 4·6 mm internal diameter LiChrosorb 10 NH_2 (Merck) with a Vydac 501 SC quard column (100 × 2·1 mm internal diameter, 37–44 μ, Chrompack) was eluted with sodium acetate buffers of varying pH and concentration (Voragen *et al.*, 1982). The exact conditions depended on the age and the condition of the column.

Calculations

Given a pectin with a known DE and assuming a random distribution of the methoxyl groups along the chain molecules the frequencies of certain sequences of galacturonate residues or esterified galacturonate residues (e.g. a sequence of exactly four esterified galacturonate residues) can be calculated using the statistics developed by Leegwater (1972) for the distribution of the functional groups in modified starches. The fraction of esterified residues present in sequences of exactly n esterified residues is:

$$(1 - DE)\, DE^n$$

where for DE an infinite chain length is assumed. With these statistics we can also calculate the theoretical amounts of pectin fragments with certain sequences released from pectin polymers by enzymic degradation taking into account the mode of action of the enzyme. This may be illustrated with the example of pectin lyase degraded pectin. HPLC analysis of the digest shows peaks for oligomers with 0, 1, 2 or more non-esterified residues (Fig. 5(a)). Peak c represents all oligomers which have two non-esterified residues.

Assuming that pectin lyase can only split bonds in regions where there are sequences of at least four esterified galacturonate residues pectin fragments containing the following four sequences can occur in peak c:

A-A

A-E-A

A-E-E-A

A-E-E-E-A

in which E = esterified galacturonate residue and A = non-esterified galacturonate residue. The quantities of each of these four sequences can be calculated using the formula mentioned above. However, not all pectin fragments containing these sequences occur in peak c; other fragments containing the same sequence but having more than two non-esterified residues occur in peak d (Table 2). For instance from the fragments containing the sequence A-E-A fragments containing the following sequences occur in peak d:

A-A-E-A-A

A-E-A-E-A-E-A

A-E-A-E-A-E-A-E-A-E-A

A-E-E-A-E-A-E-A-E-A-E-E-A

A-E-A-E-A-E-A-E-A-E-E-A, etc.

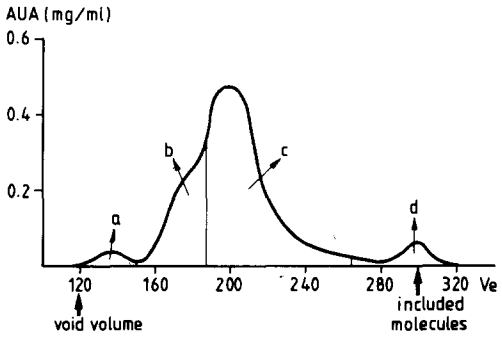
With the following formula the frequencies of the oligomers in peak c can be calculated:

$$b^{-1}\{(b^2a-2(ab+a^2b+a^3b)\,b^2a\}+a^{-1}\{(ab-2(b^2a+a^2b+a^3b)\,ab\}$$
$$+a^{-1}\{(a^2b-2(b^2a+ab+a^3b)\,a^2b\}+a^{-1}\{(a^3b-2(b^2a+ab+a^2b)\,a^3b\}$$

where a = DE and $b = 1 - \mathrm{DE}$ and in which the terms $-2(ab + \ldots)$, etc., correct for those fragments which have more than two non-esterified residues. The factors $a^{-1} + b^{-1}$ make the results apply to the total amount of galacturonate residues and not to the amount of esterified or non-esterified residues only.

RESULTS AND DISCUSSION

Figure 1 shows the typical gel-filtration pattern of a pectate lyase degraded pectin fraction. Three peaks can be observed, the first peak containing the hairy regions (de Vries *et al.*, 1982) and the two others being galacturonan chain fragments. This typical pattern can be ex-



	a	b	c	d
Degree of esterification (%)	95	81	75	50
% of AUA	7	31	54	8
neutral sugar content (moles/mole of galacturonic acid residues)	1.33	.03	.02	.01
moles rhamnose/mole arabinose	.08	.06	.10	.05
moles xylose/mole arabinose	.09	.09	.10	.07
moles galactose/mole arabinose	.87	.71	3.0	6.1
moles glucose/mole arabinose	.10	.06	.10	.09

Fig. 1. Gel filtration of a pectate lyase degraded pectin. Ve = elution volume. The substrate was a DEAE-cellulose purified pectin from the cold buffer extract of AIS from ripe apples. Degradation with pectate lyase as described in the text. Extent of degradation was 4%. The eluent was water.

plained in three ways. The first possibility is that the pectolytic enzymes have a certain degree of multiple attack (French & Robyt, 1967). The fact that the gel-filtration pattern of pectate lyase degraded 'trans-esterified' pectin (see under 'Materials') shows a single peak (Fig. 2) rules out this possibility. It can also be concluded from Fig. 2 that the native distribution of the methoxyl and carboxyl groups differs from the distribution in trans-esterified pectin.

The other two possible explanations are that the pattern shown in Fig. 1 is the result of an intermolecular or an intramolecular distribution of the methoxyl groups.

Intermolecular distribution

Data about the intermolecular distribution of the methoxyl groups can only be obtained by extensive fractionation as represented in Fig. 3 and

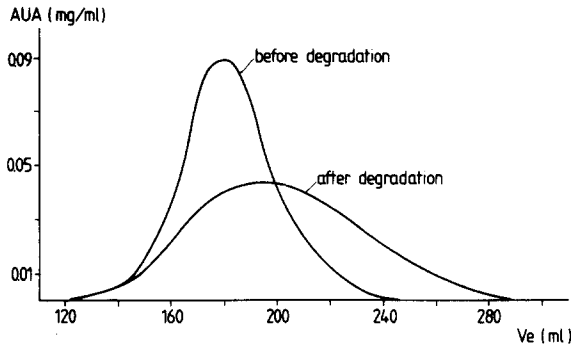


Fig. 2. Gel filtration of trans-esterified pectin before and after pectate lyase degradation. Ve = elution volume. The substrate was a commercial apple pectin which was esterified and subsequently alkali saponified to DE = 71% as described in the text. Degradation with pectate lyase as described in the text. Extent of degradation was 3%. The eluent was 0·05 M sodium phosphate buffer, pH = 5·6.

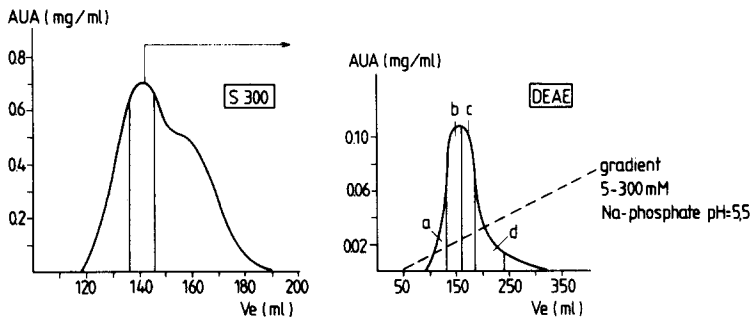


Fig. 3. Repeated fractionation of pectin fractions on gel-filtration and ion-exchange columns. Ve = elution volume. The substrate was a DEAE purified pectin fraction from the hot buffer extract of AIS from ripe apples. Sephacryl S-300: the eluent was 0·05 M sodium phosphate buffer, pH = 5·6.

Table 1. It was observed that an intermolecular distribution is present. Almost all the pectin fractions studied (de Vries *et al.*, 1981) have a DE of 70–80%. From extensive fractionation, however, it can be concluded that all the pectin fractions contain a small number of molecules with a DE of about 50% as well as a small number with a DE of about 95%. The construction of a distribution curve for the intermolecular distribution of the methoxyl groups, however, would demand a series of time-consuming fractionation experiments.

TABLE 1
DE of Pectin Fractions after Repeated Fractionation, as Shown in Fig. 3

Fraction	*AUA (%)*	*DE (%)*
a	19	86
b	44	78
c	29	71
d	7	65

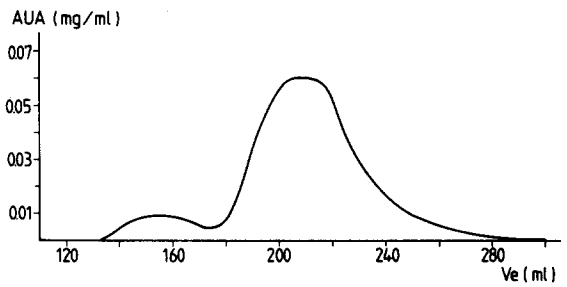


Fig. 4. Gel filtration of pectate lyase degraded pectin fraction c (see Table 1 and Fig. 3). Ve = elution volume. Degradation with pectate lyase as described in the text. The extent of degradation was 3%. The eluent was 0·05 M sodium phosphate buffer, pH = 5·6.

As shown in a previous paper (de Vries *et al.*, 1983), the fraction with DE = 95 has a neutral sugar side chain constitution different from that of the other fractions. It can be concluded from the extensive fractionation experiments that the typical pattern of Fig. 1 (three peaks) is caused by an intermolecular distribution present. Indeed, an extensively purified pectin fraction does not show this behaviour (Fig. 4).

Intramolecular distribution

The next question to be answered is: what is the intramolecular distribution? Extensive degradation by pectate lyase and pectin lyase and

subsequent fractionation of the resulting oligomeric partially esterified galacturonides on HPLC resulted in information about the intramolecular distribution. In the fractionation on HPLC an ion-exchange mechanism is involved (Voragen *et al.*, 1982). The chromatograms of partially esterified tri-galacturonic acid (Tjan *et al.*, 1974) show four peaks, namely of trimers with zero, one, two and three free carboxyl groups. It may therefore be concluded that partially esterified oligogalacturonides are separated according to the number of free carboxyl groups and not according to chain length. Some chromatograms are given in Fig. 5. The peaks indicated as a, b and c represent oligogalacturonides with zero, one and two free carboxyl groups. It can be seen in Fig. 6 that these peaks contain several oligogalacturonides. In this figure a comparison can be made between pectin with a native distribution of methoxyl groups and pectin with a modified distribution (trans-esterified pectin). Only small differences between the two cases are observed, suggesting that the native distribution and the modified distribution are similar. This hypothesis has been confirmed by an analysis of the results of enzymic degradation as represented in Table 2. In this table experimental results as found by HPLC are compared with theoretical results. The theoretical results are based on assumptions concerning the mode of action of the enzyme and on assumptions

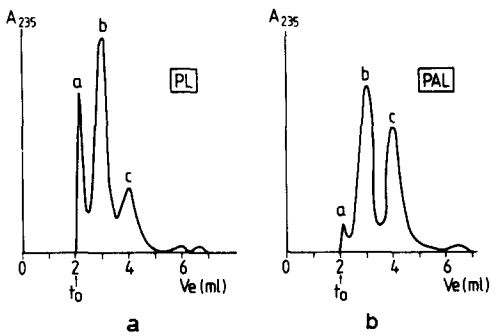


Fig. 5. High-pressure liquid chromatograms of pectin fractions degraded with (a) pectin lyase and (b) pectate lyase to degradation limits. A_{235} = absorbance at 235 nm; Ve = elution volume. HPLC conditions as described in the text. The substrate was a pectin fraction from the oxalate extract of AIS from ripe apples (de Vries *et al.*, 1981). Enzymic degradation as described in the text. Extent of degradation: (a) 18% and (b) 7%.

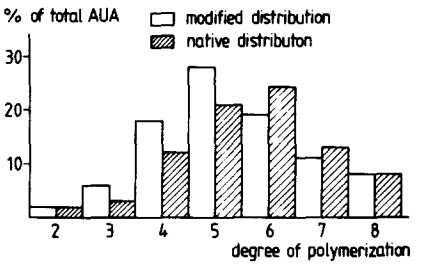


Fig. 6. Distribution of the degree of polymerization of pectin oligomers in peak b (as shown in Fig. 5(a)) in two different cases. The peaks b of several runs were collected and completely saponified (in alkali); the degree of polymerization of oligogalacturonides present was determined by HPLC as described by Voragen *et al.* (1982). Modified distribution: the substrate was trans-esterified pectin (see text). Native distribution: substrate as in Fig. 5.

concerning the intramolecular distribution of methoxyl groups. With the help of the statistics as developed by Leegwater (1972) for the distribution of functional groups in modified starches, frequencies of occurrence of certain sequences (e.g. fully esterified tetramers) can be calculated. Infinite chain length and a random distribution of methoxyl groups are assumed. The problem is that in order to draw conclusions about the distribution of methoxyl groups, assumptions must be made about the mode of action of the enzymes and vice versa. In Table 2 two sets of assumptions have been worked out. The calculations have been illustrated under 'Materials and Methods'. From Table 2 some conclusions can be drawn. The differences between extracted pectins and trans-esterified pectins are relatively small. Although the trans-esterified pectin was not fractionated extensively, intermolecular differences are not likely to exist because the de-esterification reaction proceeds randomly (although not completely – see below). It can be concluded that the intramolecular distribution of the methoxyl groups in both cases (extracted pectins and trans-esterified pectin) may very well be similar. However, a note of caution should be sounded. The degree of polymerization of extracted pectins and of trans-esterified pectins differ substantially (as was concluded from their gel-filtration patterns) and this certainly results in differences in the relative amounts of oligomers produced (boundary effects). Another problem is the instability of esterified oligomers: even at pH 5·5 a pectin lyase degraded

TABLE 2
Percentage of Total AUA Present in the Peaks of the High-Pressure Liquid Chromatograms of Pectin Lyase Degraded Substrate

Peak	*'4'*	*'3'*	*Extracted pectin*[a]	*AIS*[b]	*Trans-esterified pectin*[c]
a	6	6	13	19	7
b	22	31	48	42	35
c	17	38	30	24	39
d	55	25	9	15	8

[a] Pectin fraction from the oxalate extract of AIS from ripe apples (de Vries *et al.*, 1981). Figure 5(a) gives the corresponding high-pressure liquid chromatogram.
[b] Alcohol insoluble solids from ripe apples (de Vries *et al.*, 1981).
[c] See text under 'Materials'.
a, b and c, Pectin fragments with zero, one or two non-esterified galacturonate residues, respectively (Fig. 5).
d, Pectin fragments with three or more non-esterified galacturonate residues (theoretical values obtained using $d = 100 - (a + b + c)$.
'3' and '4', Theoretical values based on the assumption that pectin lyase can only split bonds in regions where there are sequences of at least three or four esterified galacturonate residues. See 'Materials and Methods'.
A random distribution of methoxyl groups is assumed and infinite chain length.
DE assumed = 70%.

pectin undergoes some de-esterification. A third problem is the occurrence of product inhibition (Voragen, 1972) which may be different in both cases. In the extraction procedure applied only 40–50% of the total AUA present in the AIS is extracted. Comparison of the columns 'extracted pectin' and 'AIS' in Table 2 indicates that the differences between extracted pectins and non-extractable pectins are small. In addition, the average DEs of the pectin extracted and of the residual pectin do not differ. Comparison of theoretical data (columns '3' and '4' in Table 2) with experimental data ('extracted pectin') suggests that pectin lyase can split wherever a sequence of at least three esterified

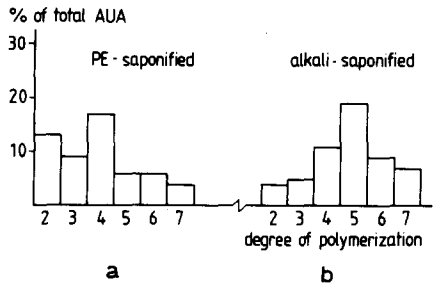


Fig. 7. Distribution of the degree of polymerization of pectate lyase degraded pectins with different distributions of the methoxyl groups. PE = pectin esterase. Extensive degradation with pectate lyase as described in the text. The pectate lyase digests were completely de-esterified (in alkali) and the degree of polymerization of oligogalacturonides was determined by HPLC as described by Voragen *et al.* (1982). (a) Pectin (DE = 95%) was de-esterified by citrus PE (Versteeg, 1979) to DE = 60%. (b) Pectin (DE = 95%) was de-esterified by alkali to DE = 60%. The resulting pectins were the substrates for the pectate lyase. Extent of degradation: (a) 12% and (b) 8%.

galacturonate residues occurs in a chain. It has been observed by Voragen (1972) that this pectin lyase cannot degrade fully esterified trimers. This shows that the mode of action of enzymes in the case of oligomers can differ substantially from that in the case of polymers.

In Fig. 7 it is shown that the action of pectin esterase (PE) can be detected by pectate lyase degradation and subsequent de-esterification of the substrate. In the case of PE action the amounts of dimers and trimers present are relatively high due to the preferential attack on the de-esterified regions by pectate lyase. In this way commercial citrus pectin was shown to be affected by PE. Apple pectins, however, appear not to be influenced by PE action. The pectins from the oxalate extracts did not differ from pectin fractions from the other extracts in this respect. Neither is the small fraction of molecules with a DE of about 50% affected by PE. This indicates that the role of plant PE is not influencing the distribution of methoxyl groups in the pectin of the plant. The role of PE *in vivo* is certainly not clearly understood (Versteeg, 1979). Figure 5 also provides information about the mode of action of pectate lyase. The chromatograms of pectate lyase degraded pectins show the presence of oligomers with only one non-esterified galacturonate residue. This implies that pectate lyase can split not only bonds between free (non-esterified) galacturonate residues, but also

other bonds, perhaps between free and esterified galacturonate residues. It can be concluded from Fig. 5 and Table 2 that the distribution of methoxyl groups may very well be a random one. It must be emphasized, however, that it is very hard to distinguish between a random distribution and a distribution characterized by short-range regularity. The distribution of methoxyl groups in the case of trans-esterified pectins is not a completely random one. The expected amounts of blocks of de-esterified residues could not be detected in the chromatograms, which indicates that the distribution of the methoxyl groups is more regular. This is not unexpected: the rate of de-esterification decreases with decreasing DE (Deuel & Stutz, 1958). Investigations of the mode of action of enzymes involved in the biosynthesis of highly esterified pectins may produce more information about this subject than can be obtained by enzymic degradation.

According to the literature (Joslyn, 1962; Darvill *et al.*, 1980) calcium bridges between pectin molecules play a role in the structure of plant cell walls. Calcium binding of pectins, however, may only occur when blocks of non-esterified galacturonate residues are present (Rees, 1969; Kohn, 1975). Blocks of more than three non-esterified galacturonate residues appeared to be practically absent in apple fruit cell walls, as was shown by the analysis of pectin lyase digests of apple AIS in this study.

Literature on the distribution of methoxyl groups in pectic substances is scarce. Boothby (1980) claimed a continuous distribution for plum fruit pectic substances; in electrophoretic separation, however, not only the DE but also the molecular weight and the neutral sugar content are important parameters. Fielding (1975) found some evidence in favour of a random distribution of methoxyl groups. Kohn (1975) showed that the calcium binding of extracted pectins could not be distinguished from trans-esterified pectins. Attempts are being made to obtain more information about the distribution of methoxyl groups in the oligogalacturonides by proton NMR studies according to the method of Tjan *et al.* (1974).

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