Carbohydrate Research, 105 (1982) 205–213
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

CHEMICAL FEATURES OF AGGREGATABLE HEPARAN SULPHATES FROM HUMAN-LUNG FIBROBLASTS

LARS-ÅKE FRANSSON AND INGRID SJÖBERG
Department of Physiological Chemistry 2, University of Lund, P.O. Box 750, S-220 07 Lund 7 (Sweden)
(Received October 13th, 1981; accepted for publication, November 23rd, 1981)

ABSTRACT

The ability of fibroblast heparan sulphate to aggregate was examined by affinity chromatography on agarose gels substituted with heparan sulphate variants from beef-lung tissue (designated HS2-A, HS3-A, and HS4-A) having different tendencies for association. Fibroblast heparan sulphate bound only to HS4-A agarose. The cell-derived material was subdivided into low-, medium-, and high-affinity species by affinity chromatography on HS4-A-agarose. High-affinity variants contained a larger proportion of chains of high molecular weight. Degradation of heparan sulphate by selective periodate-oxidation of glucuronate in regions rich in 2-acetamido-2-deoxyglucose followed by scission in alkali produced fragments comprising the highly irregular, iduronate-rich and *N*-sulphate-rich domains. The latter were slightly larger in the high-affinity form. By re-oxidation of the irregular domains, the location of glucuronate residues was identified. The degradation pattern, *i.e.*, the distribution of iduronate-containing repeats, was markedly similar in the high-affinity variant and in the HS4-A chains that were used as affinity ligands. It is suggested that the strength and specificity of heparan sulphate self-association is dependent on complementary and co-operative associations between a number of contact zones.

INTRODUCTION

The glycocalyx of many eukaryotic cells contains a variety of glycoconjugates, *i.e.*, glycolipids, glycoproteins, and proteoglycans. Among the last group, proteo-heparan sulphates are characteristic members. The side chains of these macro-molecules, *i.e.*, the heparan sulphate *per se*, are long and linear polysaccharides based on a backbone of $-(HexA-GlcN)_{\sim 30}$ repeating-units. Certain regions of these chains have a constant composition, *i.e.*, only GlcA–GlcNAc repeating-units, whereas others are highly irregular. The latter regions comprise GlcA–GlcNAc, IdoA–GlcNAc, IdoA–$GlcNSO_3$, and GlcA–$GlcNSO_3$ repeating-units in different proportions and arrangements[1–3]. The distribution of the IdoA and GlcA residues seems to be controlled both by the arrangement of GlcNAc and $GlcNSO_3$ residues and by the

extent of ester sulphation in $(HexA–GlcNSO_3)_n$-block regions (for a more extensive discussion, see ref. 4).

We have shown, using gel-permeation and affinity chromatography as well as light-scattering methods, that certain heparan sulphate chains are able to self-associate[5–7]. This property is correlated with the presence of alternating or mixed IdoA–GlcN and GlcA–GlcN repeating-units[6]. The results of affinity chromatography of various free heparan sulphate chains on agarose gels substituted with the same species showed that the chain–chain associations may be quite specific[7]. In a preceding study[3], three specifically self-associating variants of heparan sulphate from bovine lung (HS2-A, HS3-A, and HS4-A in Table I) were subjected to structural analyses, using two methods of chemical degradation, one degrading the *N*-sulphate-rich segments (HNO_2 treatment) and the other degrading the $–(GlcA–GlcNAc)_n$-regions (periodate oxidation–alkaline elimination). The same methods have previously been used to characterise heparan sulphate chains associated with the cell surface of cultured, human-lung fibroblasts[2]. The object of the present study was to examine the aggregatability of fibroblast heparan sulphate by affinity chromatography on agarose gels substituted with different variants of heparan sulphate. The relationship between binding and chemical parameters, such as chain-length and copolymeric features, was evaluated.

EXPERIMENTAL

Materials. — The methods for purifying and fractionating beef-lung heparan sulphate have been described in detail[1]. In this procedure, a series of fractions (HS1–5) is obtained; these have a progressively increasing content of sulphate and IdoA. Each of these fractions is further separated into more or less association-prone variants by gel-permeation chromatography[3,5,6].

[^{3}H]Heparan sulphate was prepared from human-lung fibroblasts according to methods described previously[2]. The cells, grown in monolayer, were allowed to incorporate 2-amino-2-deoxy-D-[^{3}H]glucose into macromolecular, anionic polysaccharides for 12 h. After depolymerisation of the galactosamino-glycan components by digestion with chondroitinase ABC, the heparan sulphates were separated from split products by gel chromatography on Sephadex G-50 (void-volume fraction).

The procedure for immobilising heparan sulphates on agarose gels was outlined previously[7]. In brief, partially periodate-oxidised chains (~5% destruction of uronate residues) were coupled to adipic acid dihydrazide-substituted Sepharose 4B, and the resulting aldimines were stabilised by reduction.

Methods. — Binding studies (affinity chromatography) were performed at room temperature on columns (6 × 100 mm) containing agarose gels substituted with various self-associating species of heparan sulphate (HS2-A, HS3-A, or HS4-A). The columns were equilibrated with 0.15M NaCl, and samples (~100 μg) were applied in 100 μL of 0.15M NaCl. Elution was with a linear gradient of guanidinium chloride (see legend to Fig. 1).

TABLE I

STRUCTURAL FEATURES[a] OF HEPARAN SULPHATES FROM BOVINE LUNG AND LUNG FIBROBLASTS

Code	*Source*	*–(GlcA–GlcNAc)$_n$–*		*–IdoA–GlcNAc–GlcA–GlcNSO$_3$–*		*(IdoA|GlcA–GlcNSO$_3$)$_n$*		
		Amount (%)	*Size* (n)	*Amount* (%)	*Frequency*	*Amount* (%)	*Size* (n)	*HexA*
HS2-A	Bovine lung	~80	⩾5	10–15	1–2	5–10	1–3	IdoA
HS3-A	Bovine lung	~65	1–10	~25	2–4	~10	5–7	IdoA
HS4-A	Bovine lung	~55	1–7	~25	2–4	~20	6–8	IdoA + GlcA
LF-HS	Fibroblasts	~20	3–4	~10	0–1	~70	1–5	IdoA + GlcA

[a]The data are compiled from earlier studies[2,3,6]. HS2–4 represent fractions obtained by stepwise precipitation with cetylpyridinium chloride at increasing ionic strengths (from 2–4). A connotes association-prone variants obtained by gel-permeation chromatography.

Periodate oxidation of GlcA in $(GlcA–GlcNAc)_n$-block regions of heparan sulphate[1,8] was carried out in 0.02M $NaIO_4$/0.05M sodium formate (pH 3.0) at 4° for 24 h. Scission of oxyheparan sulphates was accomplished by treatment with alkali (pH 12, 20°, 30 min) (see also Scheme 2 of the preceding paper[3]).

Degradation products were separated by gel-permeation chromatography (see Figs. 3–5) on Sephadex G-50 (superfine). Effluents were analysed for uronate by an automated carbazole–borate procedure[9] or for ^3H with a Packard 2650 liquid scintillation counter using Insta-gel (Packard; 0.5 ml of sample mixed with 5 ml of liquid) as scintillator.

RESULTS AND DISCUSSION

Comparisons between heparan sulphates of beef-lung tissue and human-lung fibroblasts. — The structural features of heparan sulphates from beef-lung tissue and human-lung fibroblasts have been investigated previously[2,3,6] and the results are summarised in Table I. Beef-lung heparan sulphates, which are probably derived from several cell-types, may be separated into three major association-prone subfractions, *i.e.*, HS2-A, HS3-A, and HS4-A. The HS2-A species is distinguished by much longer and more frequent $(GlcA–GlcNAc)_n$-segments than the other two species. The latter are characterised by the nature of the *N*-sulphate-containing regions. In HS3-A, there is a larger relative contribution from –IdoA–GlcNAc–GlcA–$GlcNSO_3$-segments than in HS4-A. In HS3-A, the $(HexA–GlcNSO_3)_n$-segments contain chiefly IdoA, whereas the same segments in HS4-A carry both IdoA and GlcA.

Heparan sulphate of human-lung fibroblast has[2] a charge density intermediate between those of HS3 and HS4. Nevertheless, the fibroblast material (LF-HS in

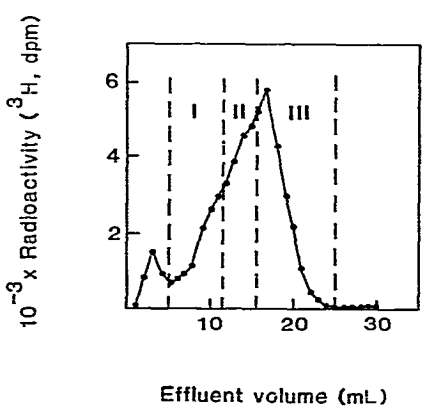

Fig. 1. Affinity chromatography of [^3H]heparan sulphate on HS4-A-agarose. The material was applied in 0.15M NaCl, and elution was performed with a linear gradient of 0.15M NaCl → 1.5M guanidinium chloride. The column size was 6 × 100 mm and the total elution volume was 100 mL. The shape of the gradient was checked by conductivity measurements, and fractions (I–III) were combined, as indicated by vertical, broken lines.

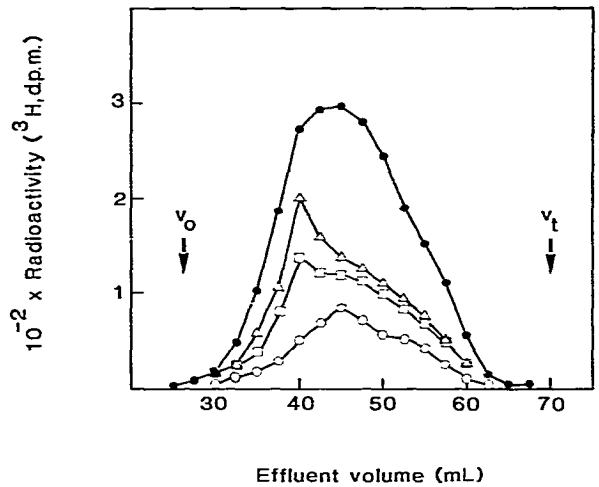

Fig. 2. Gel chromatography on Sepharose CL6B of total, cell-derived [^{3}H]heparan sulphate (—●—), sub-fraction I (—○—), sub-fraction II (—□—), and sub-fraction III (—△—) obtained by affinity chromatography. The total, cell-derived [^{3}H]heparan sulphate was sub-fractionated as described in Fig. 1, and each fraction was chromatographed separately. The column was 8 × 1500 mm, the eluent was 0.75M guanidinium chloride (pH 7.0), and the elution rate was 6 mL/h: V_o, void volume; V_t, total volume. The eluting solvent used would inhibit self-association between individual chains[5].

Table I) contains a very high proportion (~70%) of $(HexA–GlcNSO_3)_n$ regions. These regions comprise both $IdoA–GlcNSO_3$ and $GlcA–GlcNSO_3$ repeats with an exceedingly low degree of ester-sulphation[2]. Hence, the fibroblast heparan sulphate is chemically closest to HS4-A from the lung tissue.

Affinity chromatography and chemical structure. — Heparan sulphate (^{3}H-labelled) from lung fibroblast was subjected to affinity chromatography on various heparan sulphate–agaroses. It was bound only to HS4-A-agarose (Fig. 1), and not to HS2-A or HS3-A-agarose (not shown). The material was subdivided into three fractions (I–III) of increasing affinity for HS4-A-agarose. As the strength of association could be dependent on size (co-operative interaction between a number of contact zones), the three sub-fractions of [^{3}H]heparan sulphate were analysed by gel-permeation chromatography on Sepharose CL6B (Fig. 2). It is seen that the high-affinity variants contained a larger proportion of chains of high molecular weight than did low-affinity ones.

Affinity between heparan sulphates could also depend on the detailed chemical structure and the sequential arrangement of the putative contact zones. To investigate this possibility, the [^{3}H]heparan sulphate sub-fractions I–III were subjected to periodate oxidation–alkaline elimination, which selectively degrades the $(GlcA–GlcNAc)_n$-segments, whereby the *N*-sulphate-rich segments are released in oligosaccharide form [general structure $GlcN–(HexA–GlcN)_n–R$, where R is the remnant of oxidised and cleaved GlcA residues]. The fragments were fractionated by gel-permeation chromatography on Sephadex G-50 (Fig. 3), and the results showed

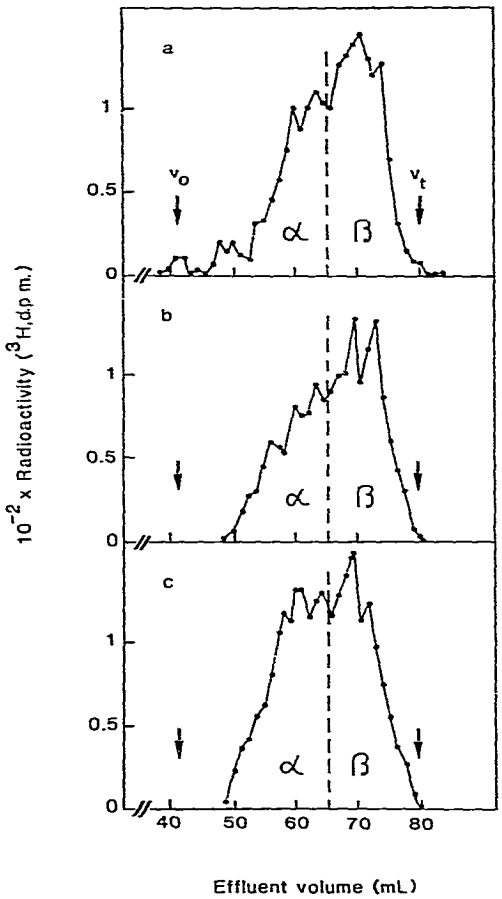

Fig. 3. Gel chromatography of periodate oxidation–alkaline elimination products from [^{3}H]heparan sulphate sub-fractions (a) I, (b) II, and (c) III on Sephadex G-50. The materials were oxidised with periodate (pH 3.0, 4°, 24 h), dialysed, treated with alkali (pH 12, 20°, 30 min), and applied to a column (8 × 1500 mm) of Sephadex G-50, which was eluted with 0.2M pyridine acetate (pH 5.0) at 6 mL/h. Fractions (α and β) were combined, as indicated by the vertical, broken line.

that oligomers with n 3–5 were slightly more prevalent in high-affinity variants (α ~50% in Fig. 3c) than in the two low-affinity ones (α ~40% in Figs. 3a and 3b).

The oligosaccharide fractions labelled α in Fig. 3 and derived from low-, medium-, and high-affinity variants of [^{3}H]heparan sulphate were re-oxidised with periodate, to identify the location of GlcA–GlcNSO$_3$ repeats in these fragments[3]. The results showed (Fig. 4) that such repeats were present in each fraction, both as consecutive elements (n = 0 corresponds to such structures) and intercalated between single IdoA–GlcNSO$_3$ repeats (n = 1). The latter were also present as consecutive elements (n = 3–5).

The degradation patterns obtained by periodate oxidation–alkaline elimination

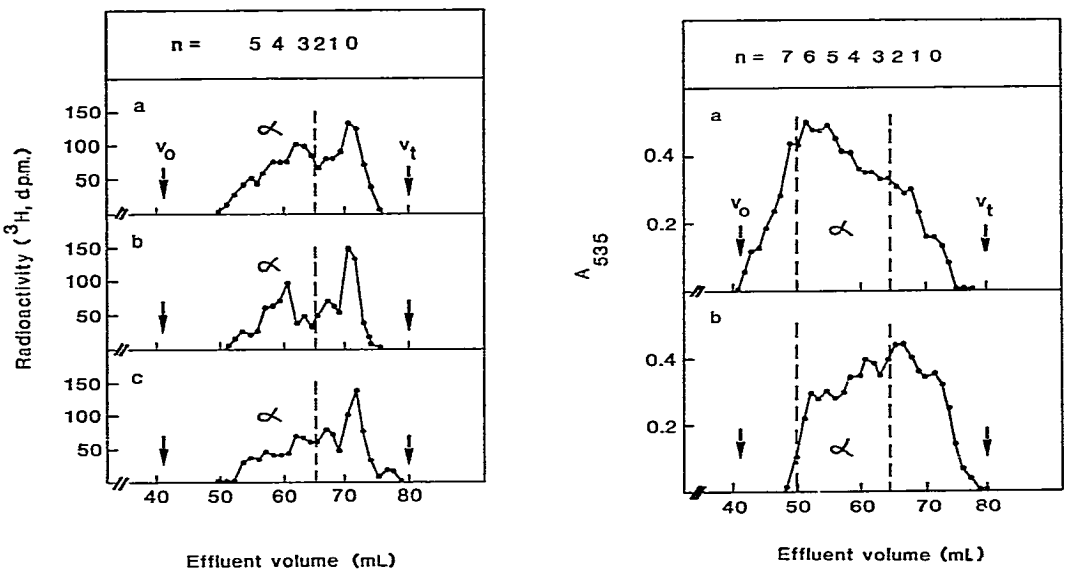

Fig. 4. Gel chromatography of periodate oxidation–alkaline elimination products from the oligosaccharide fraction α of (a) sub-fractions I, (b) II, and (c) III from [^{3}H]heparan sulphate. The sub-fractions I–III of cell-derived [^{3}H]heparan sulphate obtained by affinity chromatography (Fig. 1) were separately oxidised and degraded (Fig. 3), to yield oligosaccharide fractions α and β. The former fractions, comprising oligosaccharides of the general structure GlcN–(HexA–GlcN)$_{3-5}$–R (see also Fig. 5), were re-oxidised with periodate, cleaved with alkali, and re-chromatographed on the same column (see Fig. 3). The broken line indicates the upper limit of the elution volume of α.

Fig. 5. Gel chromatography on Sephadex G-50 of periodate oxidation–alkaline elimination products from heparan sulphate HS4-A after (a) the initial degradation and (b) the re-oxidation of oligosaccharide fraction α. Fraction HS4-A from beef lung was oxidised with periodate (pH 3.0, 4°, 24 h), dialysed, treated with alkali (pH 12, 20°, 30 min), and applied to the same column as in Fig. 3. The fragments have the general structure GlcN–(HexA–GlcN)$_n$–R and ranged from $n = 0$ to $n \sim 9$. The position of the various fragments indicated in the top panel is deduced from the ratio of reducing power/uronate content, as discussed elsewhere[1]. In the A_{535}-profile, the proportion of fragments $n = 0$ is heavily underestimated, as GlcN–R carries no uronic acid; the colour yield is due to an unspecific reaction. The oligosaccharide fraction α in (a) was collected, re-oxidised, cleaved, and re-chromatographed on the same column (b).

(in two stages) of low-, medium-, and high-affinity variants of [^{3}H]heparan sulphate should be compared with the structural features of HS4-A, *i.e.*, the chains on the affinity matrix to which it was bound. The results of the initial degradation of HS4-A are shown in Fig. 5a. The oligosaccharides of type GlcN–(HexA–GlcN)$_n$–R, with $n = 5$–6, were the most prominent, unlike the situation with the fibroblast heparan sulphate (Fig. 3) where fragments with $n = 0$–3 were preponderant. However, re-oxidation and cleavage of these oligomers (α in Figs. 3c and 5a) produced markedly similar degradation patterns for HS4-A (Fig. 5b) and high-affinity [^{3}H]heparan sulphate (Fig. 4c). Thus, segments like (IdoA–GlcNSO$_3$)$_n$ showed the same relative distribution, with decreasing proportions in going from $n = 1$ to $n = 5$. The fragments derived from low- and medium-affinity variants (Fig. 4a and 4b) displayed

much more irregular profiles. It should be noted that the proportions of $n = 0$ cannot be compared between the labelled and unlabelled heparan sulphate, because, in the former (Fig. 4), all fragments carry [^{3}H]GlcN, whereas $n = 0$ is heavily underestimated in the latter (Fig. 5) due to the absence of HexA in this fragment.

CONCLUSION

As pointed out in the preceding report[3], heparan sulphates may be distinguished by at least three structural criteria, namely, the size distribution of the $(GlcA–GlcNAc)_n$ regions, the occurrence of such alternating or mixed segments as $–(GlcNSO_3–IdoA/GlcA)_m–(GlcNAc–GlcA)_n–$, and the variation of IdoA and GlcA in $(HexA–GlcNSO_3)_n$-block regions. Heparan sulphate from human-lung fibroblast is dominated by the segments $(HexA–GlcNSO_3)_{1-5}$, which account for ~70% of all the repeating disaccharides. Within these segments, a large variety of arrangements of the $IdoA–GlcNSO_3$ and $GlcA–GlcNSO_3$ repeats probably exist (see Figs. 3 and 4). Alternating sequences like $–GlcNSO_3–IdoA/GlcA–GlcNAc–GlcA–GlcNSO_3–$ are rare (~10%) and the $(GlcA–GlcNAc)_n$-segments that separate the $(HexA–GlcNSO_3)_n$-block regions have a narrow distribution of size, *i.e.*, $n = 3–4$. Assuming an average chain-length of 30–40 disaccharides [the result of gel chromatography (Fig. 2) is compatible with this assumption], there is only scope for two $(GlcA–GlcNAc)_{3-4}$-segments/chain. Consequently, there should be three *N*-sulphate-rich domains/chain, each with $n = 8–10$. As the fragments isolated after periodate oxidation had $n = 1–5$, the presence of a few $–HexA–GlcNAc–GlcA–GlcNSO_3$-sequences within these larger domains may be postulated, because the GlcA residue in these sequences might be periodate-sensitive[1,8]. The proportion of such tetrasaccharide sequences (~10%) is in keeping with an incidence of 3–4/chain, *i.e.*, ~1/domain.

The calculations presented above represent an average picture of the copolymeric structure of fibroblast heparan sulphate, and the extent of variation may be quite considerable. Nevertheless, this material represents a much narrower range of variants than those seen in the heparan sulphate fraction from beef-lung tissue. Among these variants, HS4-A has a significant overlap with the fibroblast material in terms of chemical structure. This is illustrated by the size-distribution of the $(GlcA–GlcNAc)_n$-segments ($n = 1–7$ and 3–4, respectively) and by the presence of both IdoA and GlcA in $(HexA–GlcNSO_3)_n$-domains.

The affinity chromatography experiments showed that chain–chain interaction could take place between fibroblast heparan sulphate and the structurally related, beef-lung preparation HS4-A. Sub-fractionation of fibroblast heparan sulphate by affinity chromatography on HS4-A-agarose revealed further structural complementarity. Variants with a high affinity for HS4-A–agarose contained larger proportions of longer $(HexA–GlcNSO_3)_n$-domains, *i.e.*, greater agreement with HS4-A. Furthermore, the size-distribution of the $(IdoA–GlcNSO_3)_n$-segments, present within these larger domains, showed an even more striking similarity in the two cases (*cf.* Figs. 4c and 5b). These findings strongly suggest that the strength and specificity of the

interaction is dependent on complementary and co-operative associations between a number of contact zones. Maximal fit is achieved if the arrangement of IdoA and GlcA within the contact zones and the spacing of the contact zones, *i.e.*, the size of the intervening $(GlcA–GlcNAc)_n$-segments, is similar in the interacting chains.

ACKNOWLEDGMENTS

The authors thank Mrs. Birgitta Havsmark for expert technical assistance, and Mrs. Ruth Lovén for typing the manuscript. The work was supported by grants from the Swedish Medical Research Council (567), Greta och Johan Kocks Stiftelser, Alfred Österlunds Stiftelse, Gustav V:s 80-årsfond, Riksföreningen mot Reumatism, and the Medical Faculty, University of Lund.

REFERENCES

1 L.-Å. Fransson, I. Sjöberg, and B. Havsmark, *Eur. J. Biochem.*, 106 (1980) 59–69.
2 I. Sjöberg and L.-Å. Fransson, *Biochem. J.*, 191 (1980) 103–110.
3 L.-Å. Fransson and B. Havsmark, *Carbohydr. Res.*, 105 (1982) 215–225.
4 I. Jacobsson and U. Lindahl, *J. Biol. Chem.*, 255 (1980) 5094–5100.
5 L.-Å. Fransson, I. A. Nieduszynski, and J. K. Sheehan, *Biochim. Biophys. Acta*, 630 (1980) 287–300.
6 L.-Å. Fransson, B. Havsmark, I. A. Nieduszynski, and T. N. Huckerby, *Biochim. Biophys. Acta*, 633 (1980) 95–104.
7 L.-Å. Fransson, B. Havsmark, and J. K. Sheehan, *J. Biol. Chem.*, (1981) 13039–13043.
8 L.-Å. Fransson, A. Malmström, I. Sjöberg, and T. N. Huckerby, *Carbohydr. Res.*, 80 (1980) 131–145.
9 D. Heinegård, *Chem. Scr.*, 4 (1973) 199–201.