

Neoglycopolymers produced by aqueous ring-opening metathesis polymerization: decreasing saccharide density increases activity

Michael C. Schuster, Kathleen H. Mortell, Adrian D. Hegeman, Laura L. Kiessling *

Department of Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA

Received 5 January 1996; revised 28 April 1996; accepted 30 April 1996

Abstract

Non-natural carbohydrate-bearing polymers are emerging as important materials for the investigation of multivalent carbohydrate–protein interactions. In the present study, neoglycopolymers were generated via aqueous ring-opening metathesis polymerizations. A procedure employing a ruthenium catalyst, preformed by treating a small quantity of the monomer with RuCl_3 , reduced metal contamination in the products. To examine the effect of sugar residue density on polymer function, materials bearing one or two sugar ligands per repeat unit were synthesized. These polymers were tested for their ability to inhibit the erythrocyte agglutinating activity of the carbohydrate-binding protein, concanavalin A. The polymers with lower sugar density were found to be more potent inhibitors than polymers with higher density, a result that can be rationalized by analysis of the protein and polymer structures.

Keywords: Aqueous ring-opening metathesis polymerization; 7-oxanorbornene; Multivalency; Neoglycopolymer; Concanavalin A; Glucose; Mannose

1. Introduction

Many important cell–cell recognition events, including viral and bacterial adhesion to host cells, germ cell recognition in fertilization and leukocyte adhesion in the immune response, are mediated by protein–carbohydrate interactions [1]. Because lectins often bind weakly to monovalent saccharide ligands, nature frequently assembles multiple interactions to facilitate cell–cell recognition. Several factors can influence the affinity and specificity of such multivalent binding events, including: (1) the structure of

the individual saccharide residues; (2) the structural features of the template upon which the saccharide residues are displayed; (3) the relative spatial orientations of the saccharide recognition elements. The contributions of these factors are difficult to assess in natural systems, because of the complexity of both the glycoconjugates and of the cell surface on which they reside. As a result, non-natural carbohydrate-bearing polymers are emerging as promising materials for the study and control of cooperative saccharide binding events [2–4]. To gain information about the spatial distribution of sugar binding sites on a cell surface, the sugar ligand density of such materials has been correlated with their inhibitory potency [2–6]. Al-

* Corresponding author.

though several groups have varied the approximate saccharide density of polyvalent ligands within acrylamide-based polymers, [6,7] few examples of polymerizations designed to control the presentation of saccharide recognition elements exist.

One polymerization method that offers more control over the size and structure of the resulting materials than acrylamide polymerizations is the ring-opening metathesis polymerization (ROMP). We [8] and others [9,10] have demonstrated that ROMP provides access to multivalent saccharide-substituted polymers. Moreover, we have shown that aqueous ROMP of 7-oxanorbornene derivatives generates neoglycopolymers that dramatically inhibit the cell agglutinating properties of concanavalin A [8,11]. To examine the effect of decreasing the sugar residue density displayed by these materials, we compared the inhibitory activities of polymers possessing two recognition elements per monomer to those bearing only one in a concanavalin A-mediated hemagglutination assay. These polymers were synthesized using preformed ruthenium catalyst solutions in order to minimize any deleterious biological side effects due to metal contamination. The polymer with the lower sugar ligand density exhibited the greatest inhibitory potency, a result that can be rationalized by consideration of the concanavalin A and polymer structures.

2. Experimental

2.1. Materials

Reaction solvents were freshly distilled from sodium-benzophenone ketyl (tetrahydrofuran) or calcium hydride (dichloromethane, triethylamine). Chromatography solvents were ACS grade; dichloromethane, acetone and hexanes were distilled. Analytical thin layer chromatography was performed on 0.25 mm Merck pre-coated silica gel plates (60F-254), and flash chromatography on E.M. Science silica gel-60

(230–400 mesh). ^1H - and ^{13}C -NMR spectra were recorded on a Bruker WP-300 or a Bruker AM-500 Fourier Transform spectrometer. All reactions were run under an inert atmosphere of either nitrogen or argon. Ethereal solutions of diazomethane were prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide using fire-polished glassware according to standard procedures [12]. Concanavalin A (Calbiochem) was dissolved in 10 mM HEPES buffer containing 100 mM CaCl_2 (pH 8.5) and dialyzed against phosphate buffered saline (pH 7.4). Dextran standards for gel filtration chromatography were obtained from Polysciences and SF-1000 gel filtration medium from Pharmacia. Other chemicals were obtained from Aldrich and used as supplied.

2.2. Monomer synthesis

Derivatives of 7-oxanorbornene bearing two glucose (1) or mannose (2) residues were synthesized as previously described (Fig. 1) [8,11]. Monosubstituted oxanorbornene (3) was generated as a 1:1 mixture of diastereomers using a sequential coupling procedure. Esterification of 3,6-oxy-1,2,3,6-tetrahydrophthalic anhydride with 1 equivalent of alcohol 9 in the presence of catalytic 4-dimethylaminopyridine produced the monoacid ester adduct as a 1:1 mixture of diastereomers. Treatment of this acid with freshly-generated diazomethane afforded the mixed ester. Removal of the triethylsilyl protecting groups with HF-pyridine complex produced unsymmetrical oxanorbornene 3 (Fig. 2). Mannose-containing oxanorbornene 4, as a 1:1 mixture of diastereomers, was generated by an analogous series of reactions from mannoside alcohol 10.

2.3. Polymerizations

The polymers were generated under two distinct sets of reaction conditions. In one procedure, deoxygenated water was added to a flask containing monomer and ruthenium trichloride,

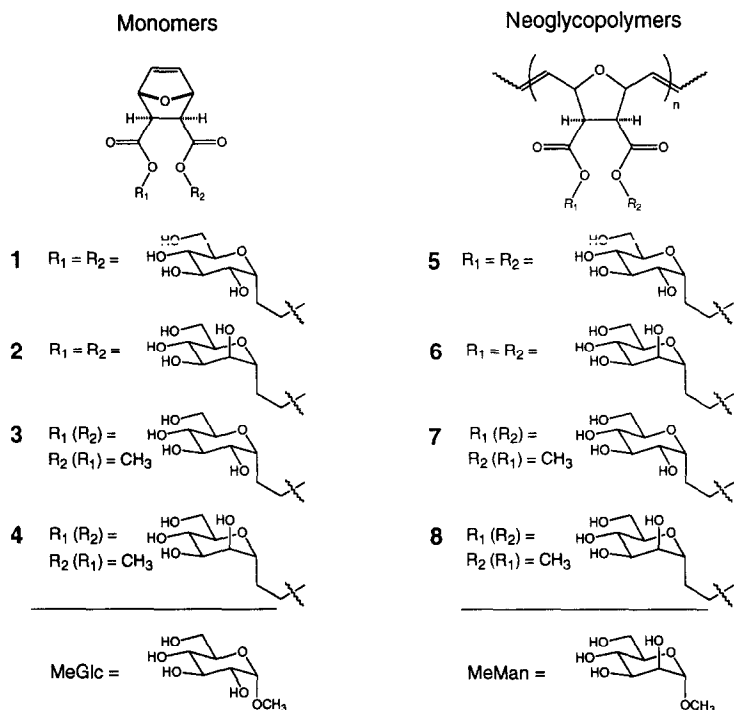


Fig. 1. Oxanorbornene-derived monomers and corresponding polymers produced by aqueous ruthenium-catalyzed ROMP. Oxanorbornenes **3** and **4** were generated as a 1:1 mixture of diastereomers.

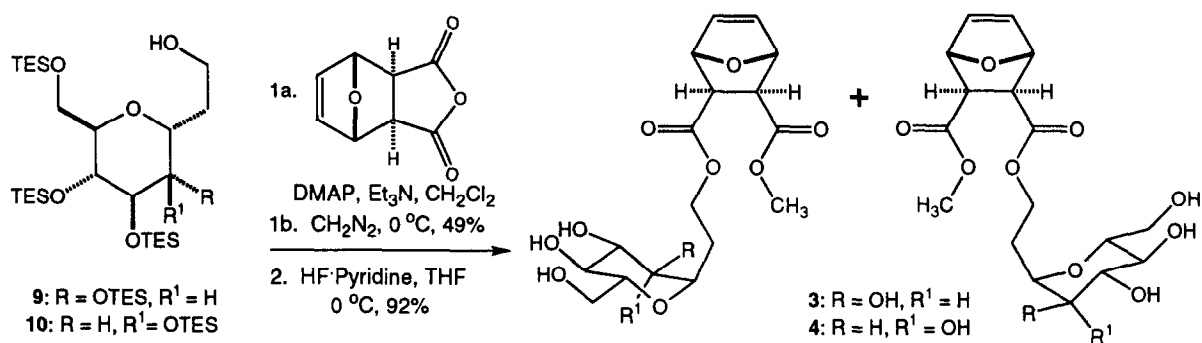


Fig. 2. Synthetic scheme for generation of monomers **3** and **4** from alcohols **9** and **10**.

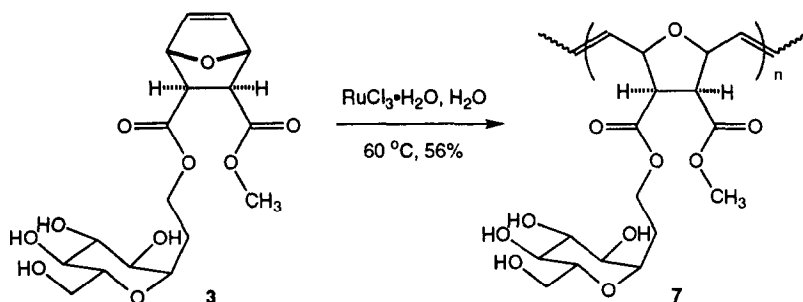


Fig. 3. Scheme depicting the aqueous ring-opening metathesis polymerization of monomer **3** to afford **7**. Similarly, polymer **8** was produced from monomer **4** in 52% yield.

and the resulting solution was stirred at 60°C under N₂ for 12 h. In the alternative protocol, which is related to a method previously employed by Novak and Grubbs [13], the polymerization was initiated with a solution containing preformed catalyst (Fig. 3). To generate the active catalyst, a small portion of the monomer to be polymerized was heated with RuCl₃ · 3H₂O in water for 12 h. The resulting catalyst was diluted with deoxygenated water, and this green solution was transferred to a flask containing the monomer. Upon heating at 60°C for 2 h, the polymerization was complete.

The product polymers were isolated and purified by precipitation and gel filtration chromatography. First, materials generated by ROMP were precipitated and washed with methanol. Following this treatment, polymers were dissolved in water, the resulting solutions concentrated, and the polymers again precipitated by the addition of methanol. The neoglycopolymers were isolated as off white solids. For further purification and to ensure that the bioactivities of polymers of similar molecular masses were compared, the materials were subjected to gel filtration chromatography. The polymers were eluted from columns packed with SF-1000 gel filtration medium with phosphate-buffered saline at pH 7.4. Fractions corresponding to the same molecular mass range were collected for the biological characterization of each polymer sample.

2.4. Hemagglutination inhibition assays

Hemagglutination assays using the mannose/glucose specific lectin concanavalin A were performed according to standard protocols [14]. A solution of concanavalin A in phosphate-buffered saline (pH 7.4) containing 0.5% bovine serum albumin was incubated with decreasing concentrations of inhibitor in a 96 well tray. A 2% solution (v/v) of rabbit red blood cells was added, and after 1 h at 25°C, the wells were examined for agglutination. The minimum saccharide concentration at which each compound inhibited agglutination was determined (Fig. 5).

3. Results and discussion

The development of an efficient method for the synthesis of bioactive, multivalent carbohydrate derivatives is a critical step toward elucidating the roles of saccharides in biological recognition processes. In our early efforts to generate saccharide-substituted polymers, oxanorbornene derivatives were polymerized in aqueous solution using ruthenium trichloride as an initiator. Attempts to optimize the reaction yields and decrease reaction times by increasing the ratio of ruthenium trichloride to monomer were ineffective. Samples prepared in this manner were discolored, indicating they were con-

Table 1
Polymerizations initiated by RuCl₃ · 3H₂O or by a preformed catalyst solution

Monomer	[M] ^a	[RuCl ₃ · 3H ₂ O] (M)	Cat. ^b	Yield (%)	M _{rel} ^c	%trans ^d
1	1.1	0.035		72	10 ⁶	34
1	0.1		x	75	10 ⁶	34
2	0.90	0.019		56	10 ⁶	44
2	0.055		x	85	10 ⁶	45
3	1.0	0.049		43	10 ⁶	39
3	0.1		x	56	10 ⁶	31
4	0.53	0.06		52	10 ⁶	44

^a Monomer concentration (M).

^b To preform the catalyst, monomer (5 mg, 13 μmol) was heated at 60°C with RuCl₃ · 3H₂O (0.5 mg, 1.3 μmol) in water (20 μl). The resulting solution was transferred to a flask containing monomer (25 mg, 64 μmol).

^c Molecular mass estimated by gel filtration chromatography with respect to dextran standards.

^d Percent of backbone olefins in *trans* configuration determined by ¹H-NMR.

taminated with large amounts of ruthenium; as a result they behaved irreproducibly in the hemagglutination inhibition assays [15]. To limit the amount of residual ruthenium in the neoglycopolymers, a second polymerization method, wherein a preformed catalyst was employed in the polymerization, was explored. Both polymerization procedures generate high molecular mass polymers, which possess similar stereochemical heterogeneity (Table 1). Polymerizations using recycled catalyst solutions, however, initiate faster and proceed with higher yields. Most significantly for the preparation of bioactive materials, the use of the preformed catalyst solutions decreases the amount of residual ruthenium in the resulting polymer.

The effectiveness of the neoglycopolymers as multivalent ligands will depend on their molecular masses. To determine this parameter, these materials were subjected to gel filtration chromatography, an operation that also removes excess ruthenium and other contaminants. The molecular mass of each polymer was determined by comparing the migratory aptitude of the polymers relative to dextran standards. A relative molecular mass (M_{rel}) of approximately 10^6 was obtained for all of the polymers, regardless of polymerization method (Table 1).

An important feature of the polymers is their microstructure, since the template on which the sugar ligands are presented undoubtedly influences the ability of these materials to inhibit cell agglutination. All of the polymers share certain features. The conditions used herein for polymerization produce stereochemically inhomogeneous backbone structures, as has been described for other polymers generated in aqueous solution by ruthenium-catalyzed ROMP [16,17]. Thus, the polymer backbones contain a mixture of *cis*- and *trans*-alkene isomers, with the *cis* geometry predominating in some cases (Table 1). We generated polymers derived from two types of monomers, possessing either one or two saccharide units. As described below, each type of monomer gives rise to polymers with distinct local structural variations (Fig. 4).

Some information about the local structure of polymers **5** and **6**, which are generated from bis sugar-substituted monomers **1** and **2**, can be obtained from the ^{13}C -NMR spectra of the polymers. Overall, these polymers have similar structures as judged from their NMR spectra, which correlate except in the region corresponding to the glucose or mannose residues. Two sets of broad overlapping peaks centered at δ 81.2 and 77.8 ppm can be assigned to allylic

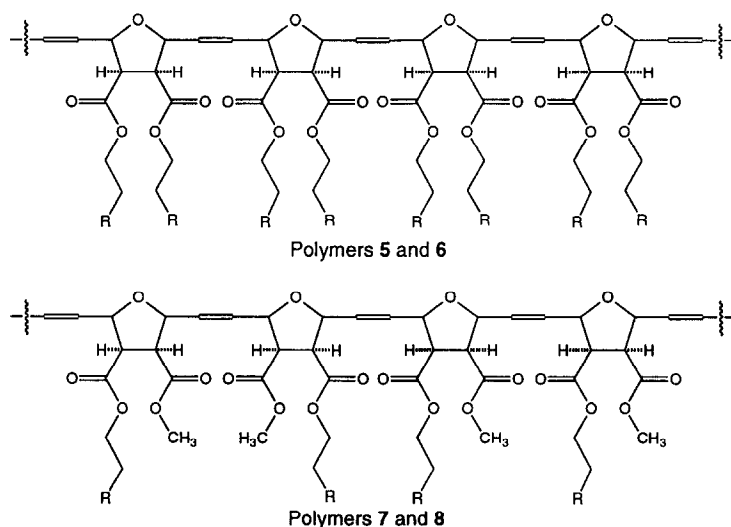


Fig. 4. Schematic view of potential variations in the neoglycopolymers. The *R*-group designates an α -C-glycoside residue of glucose or mannose.

carbons adjacent to a trans olefin (downfield resonance) or a cis olefin (upfield resonance) [18]. Although the overlapping peaks make it difficult to make definitive assignments, these signals likely arise from allylic carbons of differing tacticity. The NMR data suggest that the polymers comprise a mixture of local structures that differ in olefin geometry and tacticity.

Neoglycopolymers **7** and **8** are more stereochemically diverse than are polymers **5** and **6** (Fig. 4); therefore, they present a wider array of saccharide arrangements for protein binding. First, monomers **3** and **4**, which bear a single saccharide residue, were generated and polymerized as mixtures of diastereomers. In addition, oxanorbornene systems with a single substituent can give rise to head-tail isomerism under these polymerization conditions. The ^{13}C -NMR spectra of compounds **7** and **8** show multiple unresolved peaks centered at δ 81.4 and 78.1 ppm, implying, as argued above, that the polymers are atactic. Given these stereochemical issues and that either diastereomer

could occupy each position in the diad, it is clear that the template on which the sugar ligands are arrayed displays saccharide residues in geometries not accessible to the polymers carrying two sugars per repeat unit. Consequently, polymers **7** and **8** present a more diverse set of sugar distances and orientations than do polymers **5** and **6**.

3.1. Hemagglutination inhibition assays

To assess the effect of different polymer structures on the interaction of a carbohydrate-binding protein with its ligand, polymers **5–8** were tested as inhibitors of hemagglutination mediated by a glucose/mannose specific protein, concanavalin A. The tetrameric form of concanavalin A causes agglutination of red blood cells by binding to cell surface glycoproteins. Agglutination facilitated by the protein can be inhibited by monomeric or polymeric derivatives of glucose and mannose [19]. The concentration of saccharide residues required

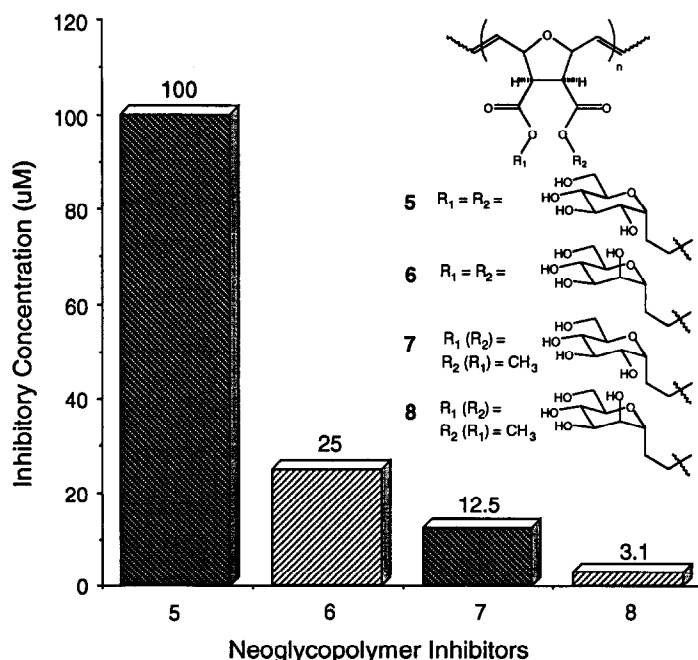


Fig. 5. Concentration of saccharide residues at which polymers **5–8** inhibited concanavalin A-mediated agglutination. Inhibitory concentration for MeGlc: 25 mM; for MeMan: 6 mM.

for inhibition of four agglutinating doses of concanavalin A was determined for each substrate within a 2-fold dilution.

The polymeric structures are approximately 1000-fold more effective inhibitors on a per-saccharide basis than are monomeric α -methyl glucopyranoside (MeGlc) or α -methyl mannoside (MeMan). Concanavalin A shows a four-fold preference for binding MeMan over MeGlc; similarly, bis-mannoside polymer **6** and mono-mannoside polymer **8** inhibited at lower concentrations than did their corresponding glucosides (Fig. 5).

A comparison of the polymers bearing one sugar residue per repeat unit with polymers bearing two per repeat revealed that inhibitory potency increased with a decrease in sugar density (Fig. 5). For both the glucose-bearing polymers **5** and **7**, and the mannose-bearing polymers **6** and **8**, the polymer containing one sugar per repeat unit inhibited at an eight-fold lower saccharide concentration than did the polymers bearing two sugars per repeat unit. A two-fold difference in inhibitory dose can be rationalized by assuming that one of the two sugar ligands on the repeat units of neoglycopolymers **5** and **6** is not accessible to the protein. The additional improvement in inhibitory potency of polymers **7** and **8** must arise from other structural features of these materials.

Neoglycopolymers **7** and **8** are expected to differ from polymers **5** and **6** in the variety of microstructures of the saccharide recognition elements presented, in the local steric environments, and in their solvation properties. Each of these differences may contribute to the enhanced activity of **7** and **8** relative to the more densely functionalized materials. At pH 7.4, concanavalin A exists as a homotetramer with four sugar binding sites, separated by 65 Å [20]. In polymers **5** and **6**, molecular modeling studies indicate that the two saccharides connected to a single repeat unit are too close to bridge two binding sites on the same protein, and certainly too close to interact simultaneously with different proteins [15]. In contrast, poly-

mers **7** and **8** could present sugars on adjacent repeat units at a distance that could span two binding sites on a single protein. The corresponding saccharide residues in **5** and **6** are more sterically crowded, a characteristic that may preclude concanavalin A binding at many sites. In addition, polymers **7** and **8** contain a variety of saccharide–saccharide distances and orientations; with these multivalent ligands, the probability is higher that optimal ligand–ligand distances are presented to the protein.

These results contribute to our investigations of the functional properties of a new class of polyvalent carbohydrate ligands, materials prepared by an aqueous ring-opening metathesis polymerization (ROMP). Previously, we demonstrated that such saccharide-substituted polymers act as polyvalent ligands for carbohydrate-binding proteins [8,11]. The present study shows that a change in ligand density, achieved by modifying the monomer structure, can improve the binding affinity of a neoglycopolymer for a carbohydrate-binding protein. Advances in ruthenium catalyzed ROMP, including the development of well-defined catalysts, are making more polymer structures accessible by this method [21,22]. Access to a variety of polymer structures will aid in not only optimizing ligands but also elucidating the recognition parameters for biologically relevant multivalent interactions.

Acknowledgements

This research was supported in part by the National Institutes of Health (GM-49975). L.L.K. thanks the NSF National Young Investigator Program, the Beckman Young Investigator Program, the American Cancer Society (Junior Faculty Award) and the Milwaukee Foundation (Shaw Scientist Program) for support. M.C.S. is a student in the UW-Madison M.D./Ph.D. Integrated Degree Program. K.H.M. acknowledges the Upjohn Company (ACS Division of Organic Chemistry fellow-

ship) and the N.I.H (Chemistry–Biology Interface, T32 GM08505) for predoctoral fellowships. ADH thanks the N.I.H. for a predoctoral fellowship (Molecular Biosciences, T32 GM07215). We thank Dr. C. Fry (UW-Madison, Chemistry Instrument Center) for helpful discussions and J. Van Stelle (UW-Madison, Animal Care Facility) for assistance obtaining rabbit erythrocytes.

References

- [1] A. Varki, *Glycobiology* 3 (1993) 97.
- [2] Y. Lee and R. Lee, *Acc. Chem. Res.* 28 (1995) 321.
- [3] L.L. Kiessling and N.L. Pohl, *Chem. Biol.* 3 (1996) 71.
- [4] F.D. Tropper, A. Romanowska and R. Roy, *Meth. Enzymol.* 242 (1994) 257.
- [5] W. Spevak, J.O. Nagy, D.H. Charych, M.E. Schaefer, J.H. Gilbert and M.D. Bednarski, *J. Am. Chem. Soc.* 115 (1993) 1146.
- [6] M. Mammen, G. Dahmann and G.M. Whitesides, *J. Med. Chem.* 38 (1995) 4179.
- [7] M.N. Matrosovoch, L.S. Mochalova, V.P. Marinina, N.E. Byramova and N.V. Bovin, *FEBS Lett.* 272 (1990) 209.
- [8] K.H. Mortell, M. Gingras and L.L. Kiessling, *J. Am. Chem. Soc.* 116 (1994) 12053.
- [9] C. Fraser and R.H. Grubbs, *Macromolecules* 28 (1995) 7248.
- [10] K. Nomura and R.R. Schrock, *Macromolecules* 29 (1996) 540.
- [11] K.H. Mortell, R.V. Weatherman and L.L. Kiessling, *J. Am. Chem. Soc.* 110 (1996) 2297.
- [12] T.H. Black, *Aldrichim. Acta* 16 (1983) 3.
- [13] B.M. Novak and R.H. Grubbs, *J. Am. Chem. Soc.* 110 (1988) 7542.
- [14] T. Osawa and I. Matsumoto, *Meth. Enzymol.* 28 (1972) 323.
- [15] R.V. Weatherman, Unpublished results.
- [16] A.D. Benedicto, B.M. Novak and R.H. Grubbs, *Macromolecules* 25 (1992) 5893.
- [17] W.J. Feast and D.B. Harrison, *J. Molec. Catal.* 65 (1991) 63.
- [18] K.J. Ivin, *Olefin Metathesis* (Academic Press, New York, 1983).
- [19] H. Bittiger, *Concanavalin A as a Tool* (Wiley, London, 1976) p. 639.
- [20] Z. Derewenda, J. Yariv, J.R. Helliwell, A.J. Kalb, E.J. Dodson, M.Z. Papiz, T. Wan and J. Campbell, *EMBO J.* 8 (1989) 2189.
- [21] H.H. Fox, M.O. Wolf, R. O'Dell, R.R. Schrock and M.S. Wrighton, *J. Am. Chem. Soc.* 116 (1994) 2827.
- [22] M.A. Hillmyer, W.R. Laredo and R.H. Grubbs, *Macromolecules* 28 (1995) 6311.