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Oligosaccharins — a new class of signalling molecules in plants

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Abstract. The review deals with oligosaccharins — biologically active oligosaccharides of the following structural types: the (1 → 6, 1 → 3)-β-D-glucan fragments of the fungal cell wall; the chitin and chitosan fragments; polygalacturonic acid fragments; the xyloglucan fragments of the plant cell wall; lipooligosaccharides synthesised by bacteria — symbionts of leguminous plants. The isolation, determination of the structure, and total syntheses of these complex compounds as well as the results of the study of their biological role as inductors of the immunity of plants to attack by pathogenic fungi and bacteria and as regulators of the morphogenesis in the development processes in plants and of the interaction of the latter with symbiont bacteria are described. The bibliography includes 197 references.

I. Introduction

Biologically active oligosaccharides, fulfilling signalling functions in plants, were discovered about 15 years ago.[1] Several years later, it was suggested that these oligosaccharide bio-regulators be called 'oligosaccharins', the suffix of which was chosen by analogy with the names of the most important phytohormones — auxins, gibberellins, and cytokinins.[2] Oligosaccharins are formed as a result of the partial cleavage of carbohydrate-containing biopolymers of the cell walls of plants or phytopathogenic fungi, act at extremely low concentrations, and participate in biological processes of the greatest importance for plants such as protection against attack by pathogenic fungi or bacteria, regulation of growth and morphogenesis, and interaction with bacterial symbionts.[3] The number of studies devoted to oligosaccharins has been growing at an increasing rate. The interest in these substances is associated not only with the possibility of investigating with the aid of oligosaccharins the detailed mechanisms of intercellular interaction and the regulation of plant metabolism but also with the very attractive prospect of the practical employment of this new class of regulators in the biotechnology of plants in agriculture.[4]

The low availability of oligosaccharins required considerable efforts on the part of carbohydrate chemists to improve the methodological procedures used in the isolation and the determination of the structure of oligosaccharins and the development of new effective approaches to the synthesis of these complex oligosaccharides. Without exaggerating, one may say that the high current level of the analytical and synthetic research in the field of complex oligomeric carbohydrates is in many respects determined by the brilliant studies on the chemistry of oligosaccharins.

There exist in the literature several excellent reviews devoted principally to the biological properties of oligosaccharins,[5–10] but there are no reviews on the chemical synthesis of these substances. There exists only one review in Russian,[11] the authors of which are the founders of this field — Albersheim and Darvill at the Complex Carbohydrate Research Center (Athens, Georgia, USA); it is devoted to the problems and advances in the early stages of the studies on oligosaccharins. The aim of the present review is to give an account of the present state of research into oligosaccharins not so much from the standpoint of the plant physiology as from the standpoint of carbohydrate chemistry in order to draw the attention of Russian investigators to this extremely interesting and practically important problem.

II. Oligosaccharins in the reactions of plants with phytopathogenic fungi and bacteria

1. The oligosaccharide fragment of the fungal cell wall β-glucan

One of the most thoroughly investigated mechanisms of the self-protection of plants against fungal infection is that of the formation of phytoalexins,[12–14] which serve as a kind of plant antibiotic. As a rule, they are hydrophobic, have low molecular masses, and are toxic to a wide range of microorganisms. Several hundreds of phytoalexins have already been isolated from plants. From the chemical standpoint, they belong to different classes of secondary metabolites — flavonoids, terpenoids, polyunsaturated compounds, etc.[15] It is essential to emphasise that there are virtually no phytoalexins in a healthy plant and their appearance in the infected plant is a response to a fully defined signal. Such signals are called elicitors of phytoalexins and they can be of abiotic or biotic origin. Abiotic factors frequently damage the plant cell wall, while biological elicitors are either enzymes which hydrolyse the cell wall biopolymers or fragments of these biopolymers; sometimes the nature of these substances is that of lipids. The accumulation of phytoalexins in plants is a result of the *de novo* formation of enzymes ensuring their synthesis and of RNA coding for these enzymes. Thus the phytoalexin elicitors regulate the metabolism at the level of the

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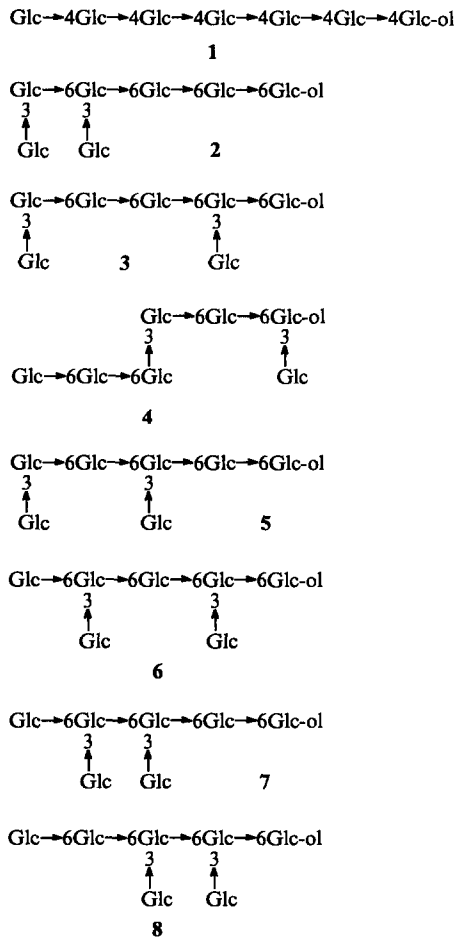
transcription of DNA. For many phytoalexins, there exist sensitive specific detection and quantitative determination methods, so that the elicitor—phytoalexin system can serve as an extremely reliable criterion for the estimation of the elicitor activity of a wide variety of chemical compounds.

The first oligosaccharin was discovered in the study of the responses of plants to attack by phytopathogenic fungi. When soya bean is infected by the pathogenic fungus *Phytophthora megasperma f. sp. glycinea*, the plant begins to produce phytoalexins (glyceollin and glycinol), which are derivatives of pterocarpan, the cell walls of the fungus, consisting mainly of carbohydrates, exhibiting a powerful elicitor activity.[16] The main component of these cell walls is a β-glucan, a sparingly soluble high-molecular-mass polysaccharide, having a branched structure with 1→6 and 1→3 bonds between β-D-glucopyranose residues.[17,18] In order to isolate a specific chemical substance, produced as a result of the elicitor activity, the fungal cell walls were subjected to partial acid hydrolysis, which led to the dissolution of ~50% of the initial material.[19] A mixture of oligosaccharides with degrees of polymerisation of 5–9, in which an appreciable elicitor activity persisted, was obtained with the aid of low-resolution gel chromatography of the hydrolysate in P-2 biogel. The testing technique involved the spectrophotometric determination of carbohydrates by reaction with anthrone, while that of pterocarpan derivatives—based on their intrinsic absorption at 285 nm[20]—enabled the authors to determine the content and activity of the elicitors in all the chromatographic fractions. Next the mixture was separated in terms of degree of polymerisation with the aid of high performance liquid chromatography (HPLC) on P-2 biogel and the heptasaccharide fraction was reduced with sodium tetrahydroborate to polyols (this did not affect the biological activity but facilitated significantly the subsequent separation) and was again separated with the aid of HPLC on a Whatman PAC column. Unfortunately such treatment led to the irreversible loss of about 50% of the oligosaccharides. The active fraction was then separated by reversed-phase chromatography on a Supelco Spherisorb ODS column, eluting with water, which again led to the loss of half the material but made it possible to concentrate the activity in a single peak. The final separation of the components of this peak on an Altex Ultrasphere ODS column (elution with a 2% solution of acetonitrile in water) made it possible to obtain seven components, six of which represented individual reduced heptasaccharides, while the seventh was shown by subsequent studies to be a mixture of such compounds. Only compound **6** (Scheme 1) exhibited elicitor activity.

Thus, in the course of the isolation of the elicitor **6**, the authors[19] were able to solve a problem of truly unique complexity: the only active oligosaccharide was isolated from a mixture of hundreds of closely related compounds. Although the authors of the above investigation had an unlimited supply of the initial cell wall material, the immensely laborious isolation procedure provided the final heptasaccharides in amounts of the order of 50 μg only. In order to establish the structures of these substances, it was necessary to develop a special strategy and methods for structural analysis (see, for example, Darvill and coworkers[21,22]), which made it possible to limit the requirements for the test substances to very small amounts.

The degree of polymerisation of both unsubstituted and methylated oligosaccharides was determined by mass spectrometry (MS) with bombardment by fast atoms. The monosaccharide composition (and at the same time the absolute configuration) was analysed with the aid of the GLC of the trimethylsilyl derivatives of (+)-2-butylglycosides of the hydrolysate[23] (only D-glucose and sorbitol were found in all cases). The pyranose forms of the monosaccharide residues and the β-configurations of all the glycosidic centres were demonstrated with the aid of ^{1}H NMR spectra of the methylated oligosaccharides. Despite the extremely small amounts of the

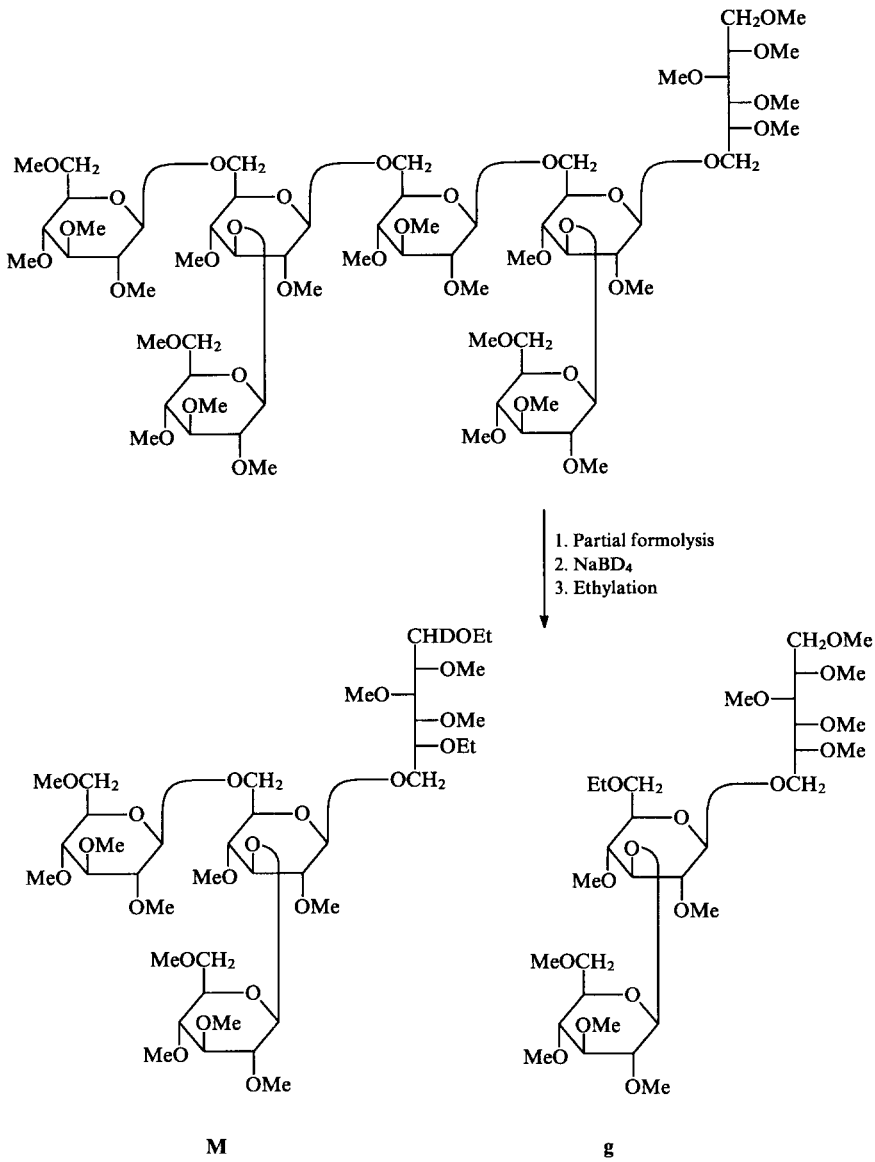
Scheme 1



test compounds, it was possible to obtain clear signals due to the anomeric protons and to make sure that the spectral range 5.0–5.5 ppm, in which the signals of the H(1) protons of the α-glucopyranose residues are observed, is unoccupied; furthermore, the H(1) signals observed in the spectrum had spin–spin coupling constants (SSCC) greater than 7.2 Hz, which is characteristic of *trans*-diaxial protons. A four-proton signal at 4.37 ppm, assigned to the anomeric protons of the glucose residues attached to O(6), and a two-proton signal at 4.73 ppm, characteristic of the anomeric protons of the residues bound to O(3) in a neighbouring unit, were observed in the spectra of the methylated compounds **2**–**8**. These signals were assigned by analogy with the methylated gentiobioside and laminaribioside respectively.[24]

The most complex problem, namely the elucidation of the disposition of the intermonomer bonds and the sequence of monosaccharide residues in the oligosaccharides, was solved by means of a mass-spectrometric study of the methylation products. The methylation stage itself was carried out by Hakamori's method modified for microamounts of the test substance,[21,22] while the methylated oligosaccharides were isolated from the reaction mixture with the aid of Sep-Pak C-18 cartridges.[25] The analysis of the derivatives obtained included partial formolysis, the reduction of the formolysis products with $NaBD_4$, ethylation of the reduced fragments at the liberated hydroxy-groups, and the determination of their structure with the aid of GLC-MS. As a result of the application of the method of derivative formation based on the incorporation of a deuterium atom, it was possible to distinguish all the fragments the reducing properties of which are due to elimination from the initial molecule on formolysis; the hydroxyls liberated in this process were marked by ethyl groups (Scheme 2).[26]

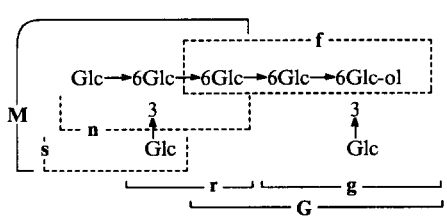
Scheme 2



M g

Naturally partial formolyis results in the formation, apart from the fragments **M** and **g** indicated in Scheme 2, also of many other substances. Overall, in the case of the oligosaccharide **6**, for example, trisaccharide (**f**, **g**, **n**, **r**, and **s**) and tetrasaccharide (**G** and **M**) cleavage products, shown in Scheme 3, were identified.

Scheme 3

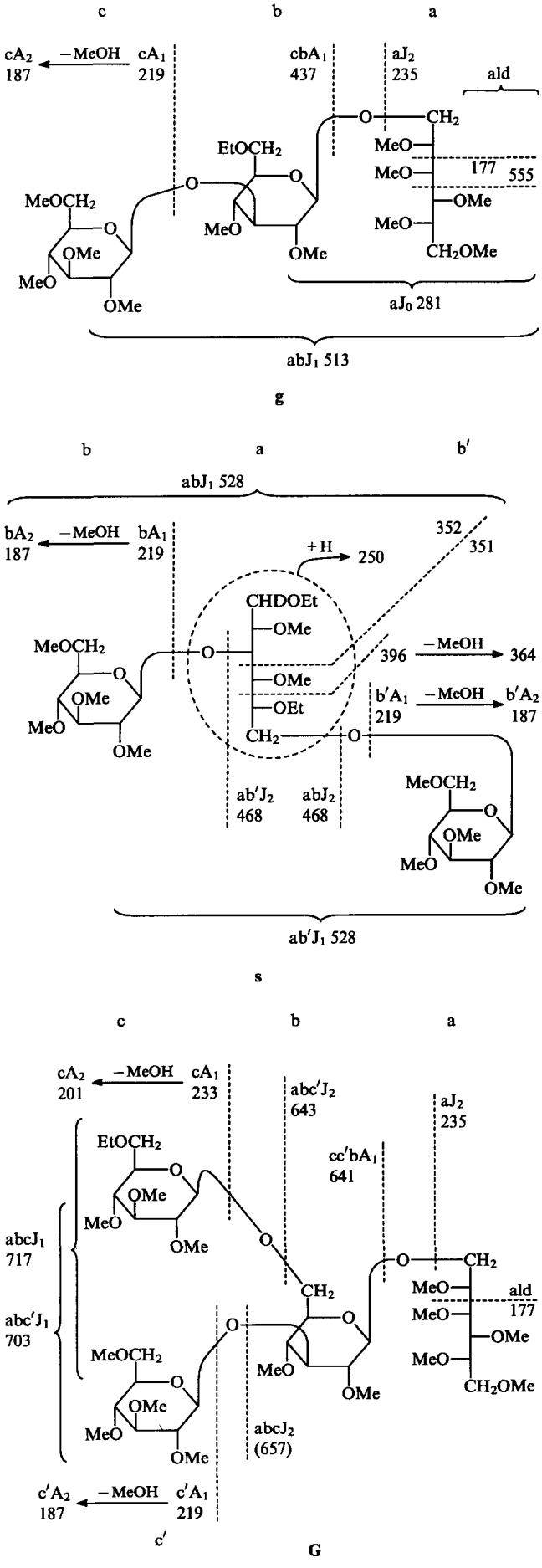


Analogous fragments are formed also from other methylated heptasaccharides. They can all be divided into three structural types: linear oligosaccharides, fragments branched at the polyol group, and substances with a branch at an inner glucose residue. The structures of the entire set of fragments, characteristic of each oligosaccharide, can be established with the aid of GLC-MS. Scheme 4 (see over) presents the most probable paths leading to the mass-spectrometric fragmentation and the diagnostic ions for representatives of each of the three structural types mentioned above (**g**, **s**, and **G**). The diagnostic ions in the mass spectrum belong to the A, J, and ald series. The nomenclature used for the fragments is the same as in the communication of Chizhov and Kochetkov[27] and in Aspinall's book.[28]

By applying the methods listed above in succession to the compounds isolated, Sharp et al.[26] were able to establish for them the structures shown in Scheme 1 (compounds **3** and **4** were not separated and their structures were determined by analysing the mixture).

The first of the heptasaccharides isolated is a fragment of the cellulose molecule and therefore differs by its origin from all the other heptasaccharides which are fragments of the β-glucan molecules in the fungal cell wall. Despite the evident similarity between the structures of these heptasaccharides, it was found that only one of them exhibits an elicitor activity and that this activity is observed clearly when 1 pmol of compound **6** per millilitre of the plant tissue is used (the highest of the known elicitor activities) and does not change in the presence of a twentyfold excess of the isomeric oligosaccharides. This surprising result demonstrated for the first time that the signalling molecules, in response to the appearance of which the protecting systems of the host plant are mobilised following attack by pathogenic fungi, may consist of purely carbohydrate fragments of the biopolymers in the fungal cell wall, while the

Scheme 4



system receiving the signal is capable of distinguishing clearly even between oligosaccharides which are structurally very similar.

The method of preparation of the heptasaccharide **6** from a natural source entailed, in principle, the possibility that the carrier of biological activity is some other substance, the chromatographic properties of which are only fortuitously identical with those of the heptasaccharide. In order to rule out this hypothesis, an unambiguous synthesis[29] of the heptasaccharide fragment of the fungal β-glucan was undertaken. Its successful completion coincided with the final determination of the structure of the natural product **6**.[26] Communications concerning the isolation,[19] determination of the structure,[26] and the synthesis[29] of this oligosaccharin and the comparative study of the biological activities of the natural and synthetic oligosaccharins[30] were published simultaneously in 1984.

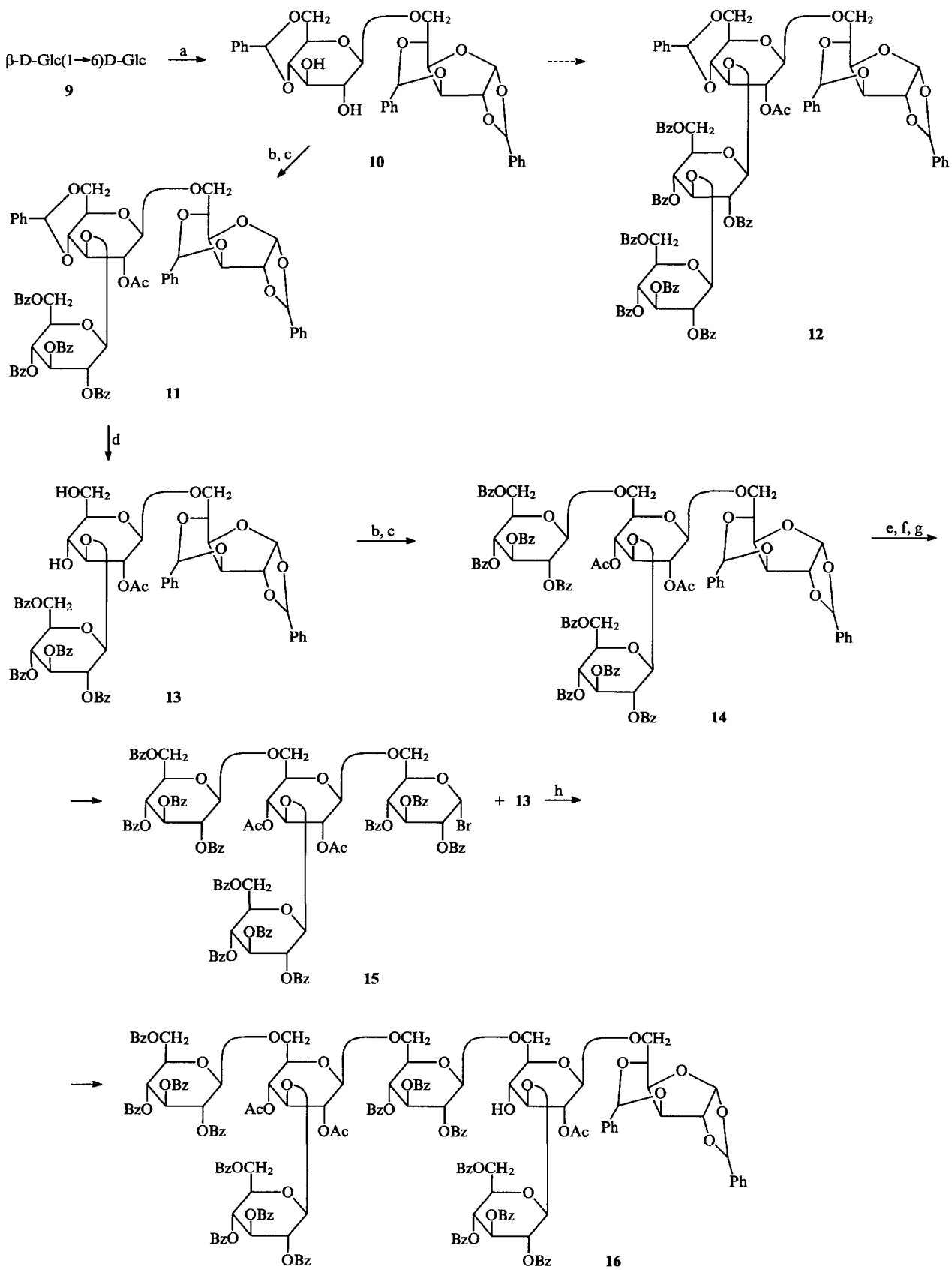
An elegant and extremely rational scheme (Scheme 5) has been developed for the synthesis of compound **6**. Its starting material was gentiobiose **9**. The condensation of this disaccharide with benzaldehyde led to the tri-*O*-benzylidene derivative **10**, containing two free hydroxy-groups. Partial acetylation of this substance afforded a mixture of the 2-*O*- and 3-*O*-acetyl derivatives, but glycosylation by treatment with 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide in the presence of silver triflate occurred regiospecifically in the 3-position. Subsequent acetylation afforded the protected trisaccharide **11**. The tetrasaccharide analogue **12** was synthesised similarly, but the glycosylating agent was hepta-*O*-benzoyl-α-laminaribiosyl bromide.

Hydrolysis of compound **11** under fairly unusual conditions—on treatment with a solution of dichloroacetic acid in aqueous acetic acid—led to the selective removal of one of the three benzylidene groups occupying the 4- and 6-positions. This resulted in the formation of a derivative of the trisaccharide **13**—the key compound in the entire synthesis. Indeed, on the one hand, compound **13** served as a precursor of the reducing end of the future heptasaccharide, and, on the other, its glucosylation, occurring selectively at the primary alcoholic group, and subsequent acetylation afford the tetrasaccharide precursor **14** for the nonreducing end of the target heptasaccharide.

The removal of the benzylidene groups from compound **14** was probably accompanied by the partial hydrolytic cleavage of the 1→6 bonds, because the halogenose **15** was obtained in only 24% yield. However, other methods of synthesis of this compound were ineffective. Condensation of the glycosyl halide **15** with the partially protected trisaccharide **13** under glycosylation conditions, which had already been frequently used previously in this synthesis, led to a protected derivative of the required heptasaccharide **16**. The unblocking of the latter afforded the heptasaccharide **17**, which was reduced to a polyol indistinguishable in terms of its chemical properties and biological activity from the substance isolated as a result of the hydrolysis of the cell wall of the fungus *Phytophthora megasperma*.[30] The use of compound **12** instead of compound **11** in the same sequence of synthetic operations led to the octasaccharide **18**; the biological activity of the corresponding polyol was one-third of that of the heptasaccharide elicitor.

The synthesis of the biologically active oligosaccharide removed all doubt concerning the chemical nature of the new class of signalling compounds. Next, it was of great interest to elucidate the mechanism of its signalling activity and the structural elements of the oligosaccharin necessary for the manifestation of biological properties. The first data concerning the variation of their structure and biological activity, obtained on comparing compound **6** with its analogues **2**–**5**, **7**, and **8**, were extremely approximate, since the authors did not have the oligosaccharides in amounts sufficient for a detailed study. After the successful synthesis of compound **6**, it became possible to hope that analogues of this compound could be synthesised.

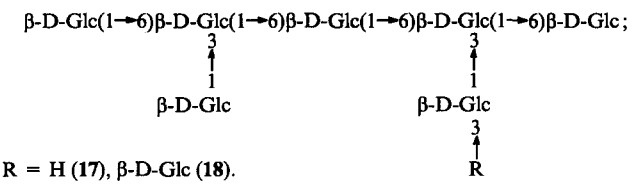
Scheme 5



(a) PhCHO, $ZnCl_2$; (b) tetra-*O*-benzoyl-α-D-glucopyranosyl bromide, CF_3SO_2OAg, 4 Å sieve, CH_2Cl_2;
(c) Ac_2O, Py; (d) $CHCl_2COOH - AcOH - H_2O$; (e) 90% AcOH; (f) BzCl, Py; (g) HBr, $AcOH - CH_2Cl_2$;
(h) CF_3SO_2OAg, 4 Å sieve, CH_2Cl_2

Scheme 5 continued over.

Scheme 5 (continued)



R = H (**17**), β-D-Glc (**18**).

Admittedly, it was necessary to develop for this purpose new synthetic procedures, because the elegant specific method of synthesis of the oligosaccharin **6** was rather unsuitable for the synthesis of its structural analogues.

Çhree years later, a communication concerning new methods of synthesis of the oligosaccharide **17** appeared[31] in which the authors used extremely effectively the latest advances in glycoside synthesis.[32] In the first stages of the construction of this complex molecule, as in the first synthesis, use was made of benzoylated glycosyl halides, which ensured the maximum yields and the stereospecificity of the glycosylation as a result of the simultaneous involvement of the benzoyl group at C(2) [33] and of silver triflate as the promoting agent.[34] In the last stages of the block synthesis of the heptasaccharide, the glycosylating agents were thioglycosides in the presence of methyl triflate.[35,36] As regards protecting groups, mention should be made of the extremely successful employment in this synthesis of the regiospecific opening of the 4,6-*O*-benzylidene acetals by reductive cleavage on treatment with the borane—trimethylamine complex and aluminium chloride[37] and also of the chloroacetyl groups attached to the primary hydroxyls, which are selectively removed in the presence of other ester groups on treatment with hydrazine dithiocarbonate[38] or thiourea.[39] The sequence of synthetic operations is clear from Scheme 6.

The starting material for the synthesis of the trisaccharide glycoside donor **22** was the 4,6-*O*-benzylidene derivative of methylthio-β-glycopyranoside **19**. Its glycosylation with 2,3,4,6-tetra-*O*-benzoyl-α-D-glycopyranosyl bromide and subsequent benzoylation led to the protected disaccharide **20**. The subsequent reductive cleavage of the acetal group and the repeated glycosylation by the same reagent afforded the required derivative of the branched trisaccharide **22**. In the synthesis of the tetrasaccharide glycosyl acceptor **26**, compound **20** was used as the glycosylating agent. Its condensation with benzyl-2,3,4-tri-*O*-benzoyl-β-D-glucopyranosyl bromide **23**, the subsequent reductive cleavage of the acetal group, and the glycosylation of the liberated primary hydroxyl with 2,3,4-tri-*O*-benzoyl-6-*O*-chloroacetyl-α-D-glucopyranosyl bromide **24** afforded the tetrasaccharide derivative **25**. The selective removal of the chloroacetyl group led to the acceptor **26**, the glycosylation of which with the thioglycoside donor **22** and subsequent removal of the protecting groups made it possible to obtain the synthetic heptasaccharide **17**, which was identical with the substance synthesised previously in terms of all the parameters.[29]

In order to produce the heptasaccharide in gramme amounts, the method of synthesis was modified (Scheme 7). Compound **21** was glycosylated with the chloroacetylated bromide **24** and the resulting chloroacetylated thioglycoside **27** was used both for the synthesis of the tetrasaccharide glycosyl acceptor **28** and as the glycosyl donor for the glycosylation of compound **28**.

Compared with the first synthesis of the heptasaccharide **17**,[29] the new approach provided incomparably greater possibilities for the preparation of analogues and derivatives of the compound. It is therefore not surprising that the communication of Fugedi et al.[31] was soon followed by new papers in which this approach was developed. The first of these[40] was devoted to the synthesis of the heptasaccharide **30**—an analogue of the oligosaccharin **17** differing from the latter by the position of the branching points. All the intermediates **20** and **22**–**24**, the glycosylation methods, and the combination of protecting groups described above were used in this synthesis (Scheme 8).

However, the use of the chloroacetylated thioglycosides **27** for the successive introduction of branched trisaccharide units with the required structure into the molecules being synthesised proved to be the most fruitful. In the next study,[41] this approach was employed to obtain two very interesting compounds. One of these is the glycoside of the biologically active heptasaccharide, where the aglycone is the methyl ω-hydroxynonanoate residue (Scheme 9). Evidently, this aglycone can be used as the spacer in the synthesis of affine sorbents designed for the isolation of cell components which exhibit a specific affinity for structure **6** or **17**, i.e. potential receptors which receive the chemical signal.

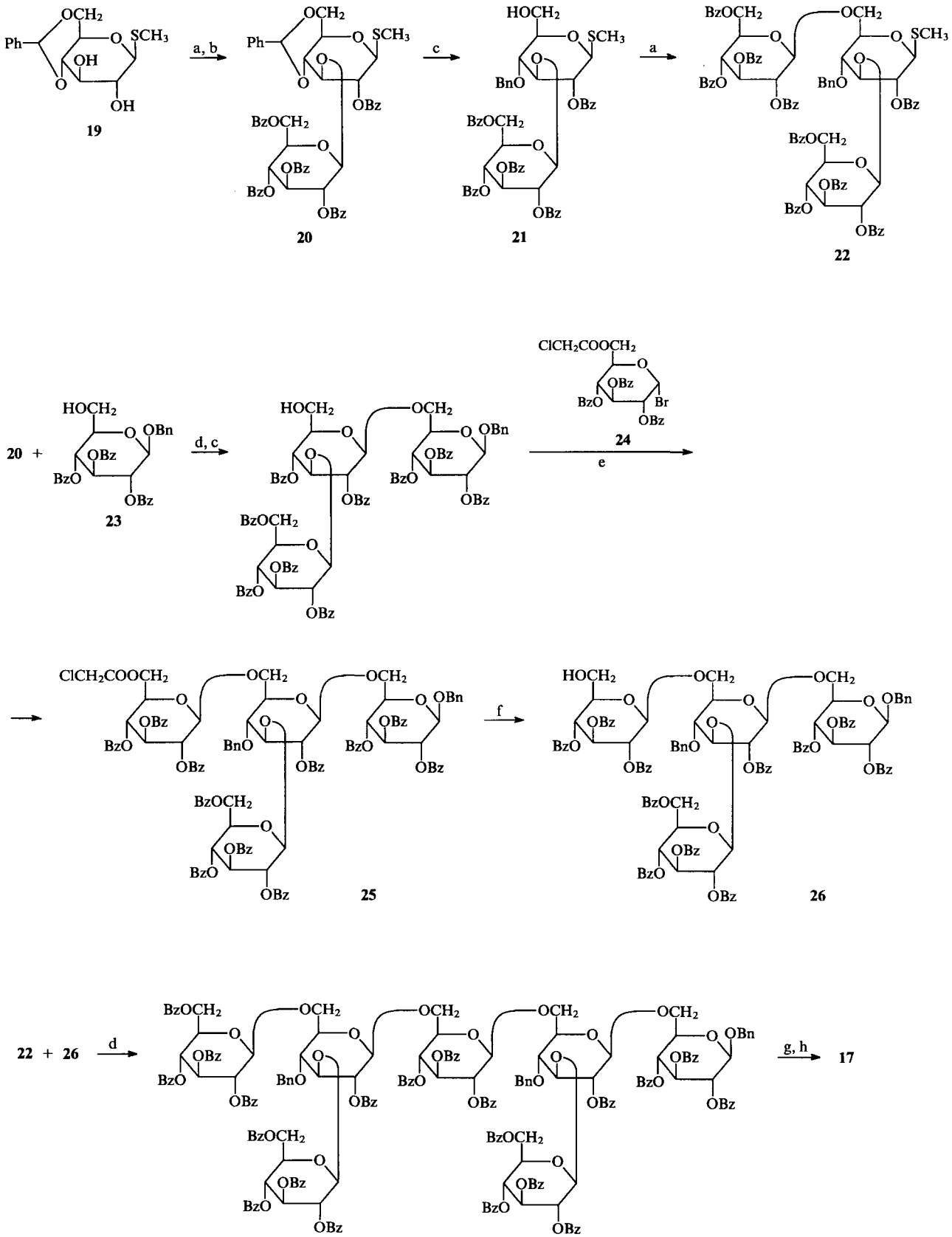
The second compound synthesised by Birberg et al.[41] is the decasaccharide **31** in which the trisaccharide structural element of compound **17** is repeated yet again. The problem involving the construction of such a complex molecule was solved by the threefold glycosylation of the corresponding precursors of the derivative of the branched trisaccharide **27** (Scheme 10).

A similar approach, in which use was made of the trisaccharide block **32** where the chloroacetyl group is attached to the primary hydroxyl of the glucose residue joined in the 6-position of a neighbouring unit and the other protecting groups are combined with the other hydroxyls, with another method (the trichloroacetimidate method) employed for the construction of the glycosidic linkage,[42] enabled Japanese investigators to obtain allyl α-glycosides of the hexasaccharide **33** and the nonasaccharide **34**, the carbohydrate chains of which are constructed in accordance with the same plan as in compound **6** (Scheme 11).[43] The same workers obtained analogues of the hexasaccharide **33**, containing the residues of β-D-xylose, *N*-acetyl-β-D-glucosamine, or β-D-glucosamine as one of the terminal nonreducing units.[44]

The first observations of the influence of structural modifications on the elicitor activity of the fungal β-glucan fragments date back to the period of the isolation of the heptasaccharide fractions[17] when it was established that the reduction of the heptasaccharides to the corresponding polyols does not affect the activity. This implied that the reducing end of the active heptasaccharide can apparently be subjected to appreciable changes without weakening the elicitor properties. This feature of the heptasaccharide **17** was used by two groups of investigators[44,45] to synthesise its conjugates with tyramine **35** and 2-(4-aminophenyl)ethylamine **37** via the reductive amination reaction. Iodination of these conjugates with radioactive iodine made it possible to obtain the labelled iodo-derivatives **36** and **38**, which are very essential for the investigation of the mechanism of the action of the oligosaccharin in the plant cell and of its fate (Scheme 12).

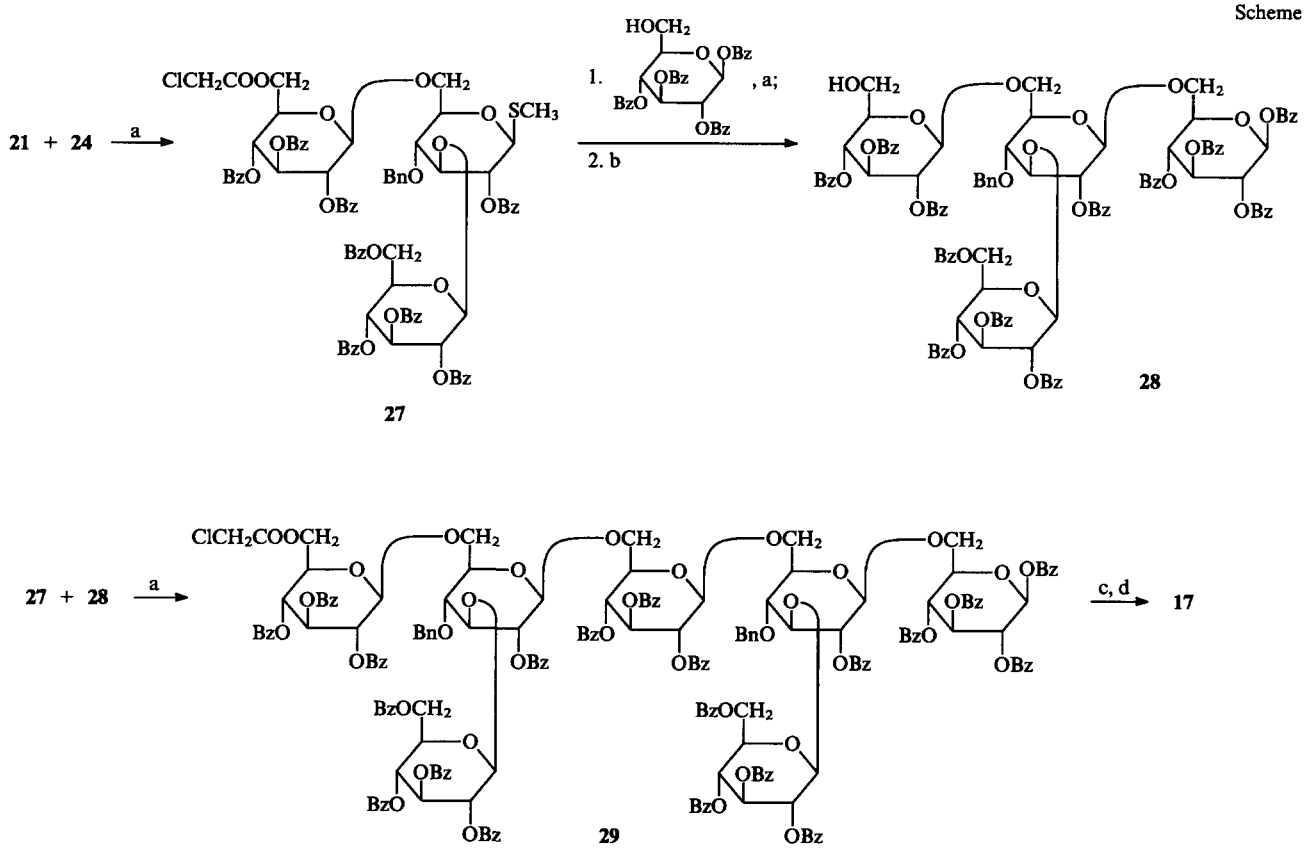
A comparative study of the elicitor activities of the majority of the synthetic analogues of the natural oligosaccharin mentioned above has been described.[44] As a result of quantitative measurements, it was established that the hexasaccharide allyl glycoside **33** is the smallest active molecule; virtually the same activity is exhibited by the heptasaccharide **17** and the higher oligosaccharides **31** and **34**. The branched trisaccharide fragment of the non-reducing end in these molecules is extremely important for the manifestation of the elicitor activity. Its modification, for example substitution of one of the terminal glucose residues by glucosamine or *N*-acetylglucosamine residues, lowers the activity by a factor of 10–1000. The activity also falls by a factor of 1000 if the glucose side residues are arranged next to one another, as in compound **30**, and are not separated by one unit, as in the natural elicitor. On the other hand, a wide variety of modifications of the reducing end of the heptasaccharide are permissible. Thus the activity is retained in the allyl α-glycosides of the hexasaccharide **33**; the conjugation of the heptasaccharide **17** with tyramine and subsequent iodination also have virtually no effect on the activity.

Scheme 6



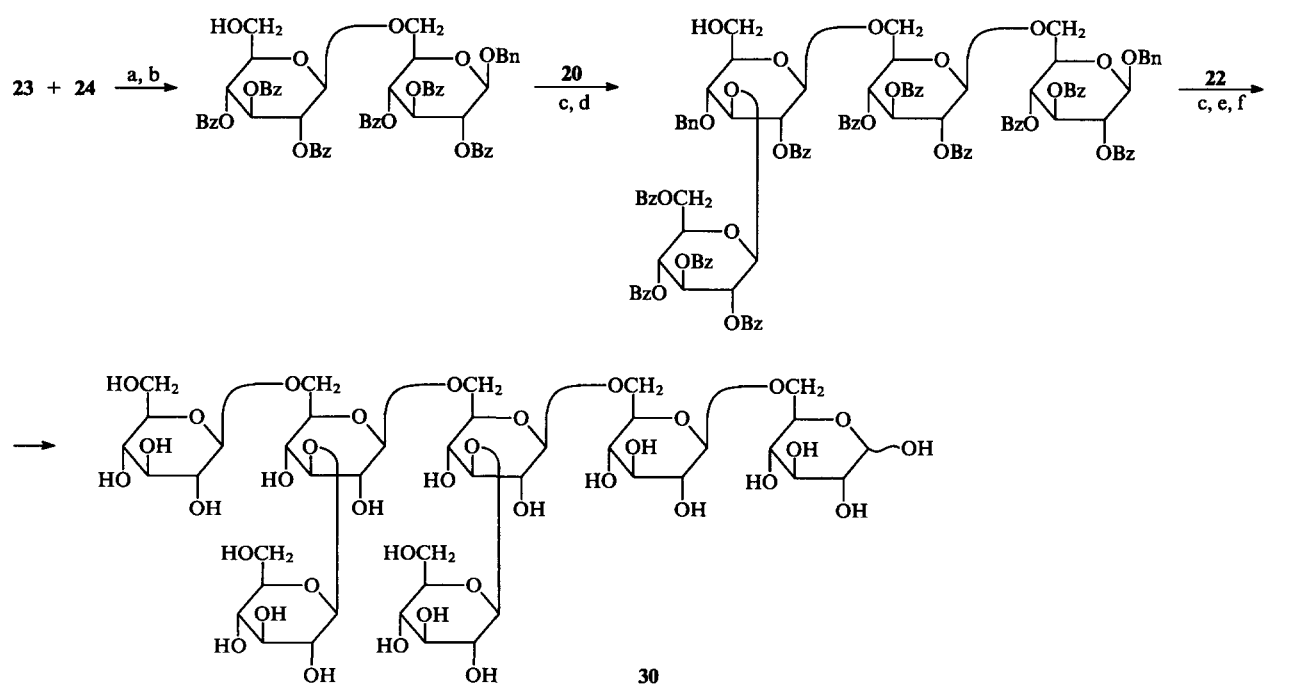
(a) tetra-*O*-benzoyl-α-D-glucopyranosyl bromide, CF_3SO_2OAg; (b) BzCl, Py; (c) $BH_3 \cdot Me_3N$, $AlCl_3$; (d) CF_3SO_2OMe, 4 Å sieve, CH_2Cl_2; (e) CF_3SO_2OAg; (f) $H_2NNH_2 \cdot H_2COS_2$; (g) MeONa, MeOH; (h) H_2/Pd – C.

Scheme 7



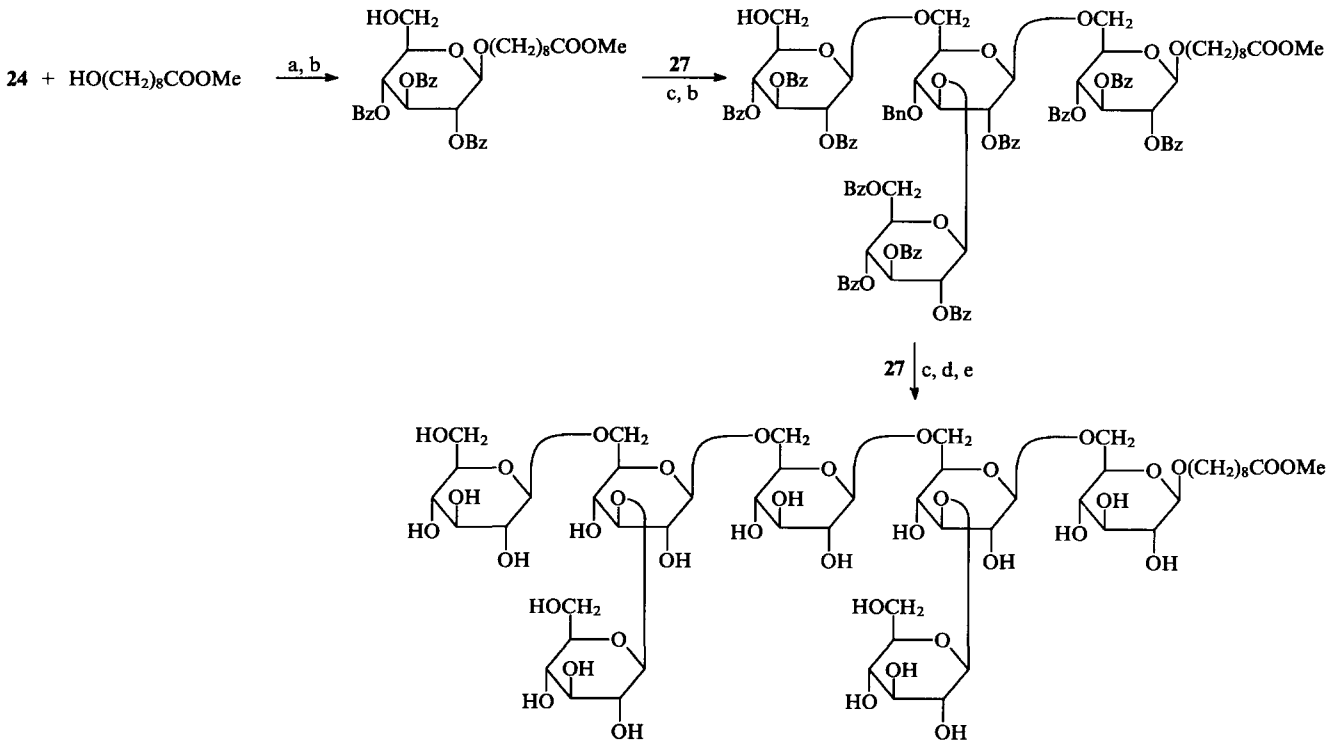
(a) CF_3SO_2OMe, 4 Å sieve, CH_2Cl_2; (b) $H_2NNH_2 \cdot H_2COS_2$; (c) MeONa, MeOH; (d) H_2/Pd–C.

Scheme 8



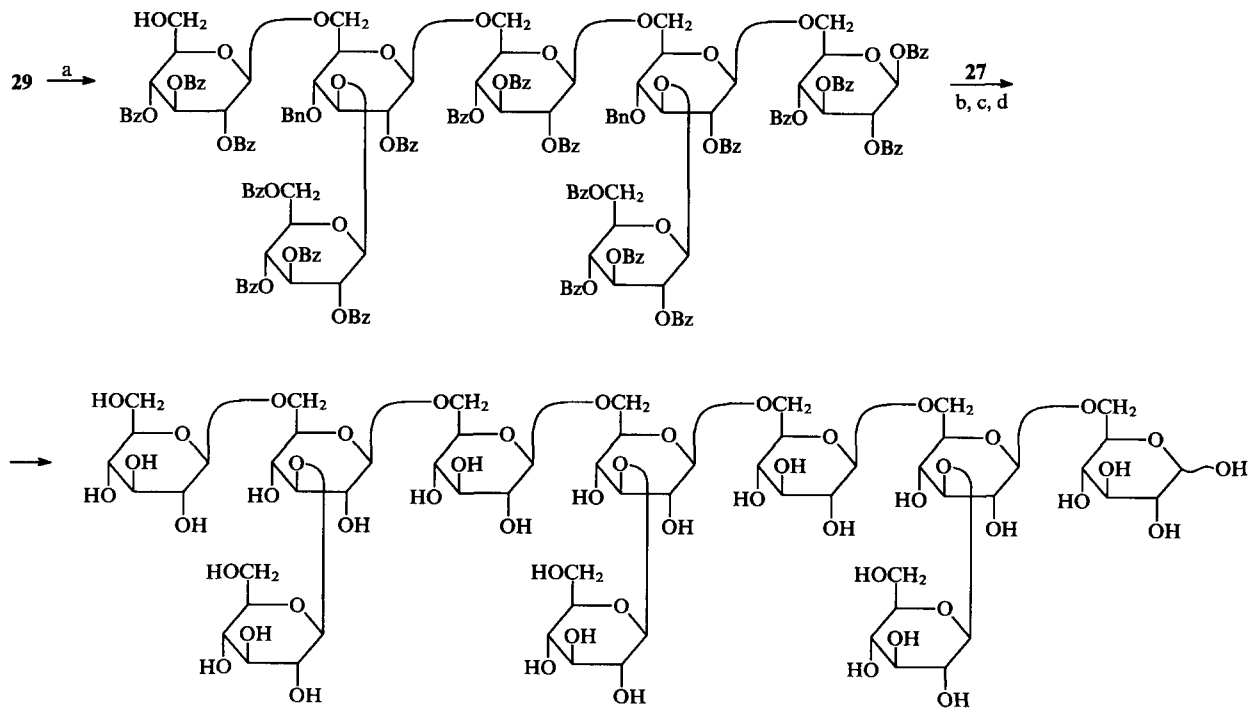
(a) CF_3SO_2OAg, 4 Å sieve, CH_2Cl_2; (b) $H_2NNH_2 \cdot H_2COS_2$; (c) CF_3SO_2OMe, 4 Å sieve, CH_2Cl_2; (d) $BH_3 \cdot Me_3N$, $AlCl_3$; (e) MeONa, MeOH; (f) H_2/Pd–C.

Scheme 9



(a) CF_3SO_2OAg ; (b) $H_2NNH_2 \cdot H_2COS_2$; (c) CF_3SO_2OMe ; (d) MeONa, MeOH ; (e) $H_2/Pd-C$.

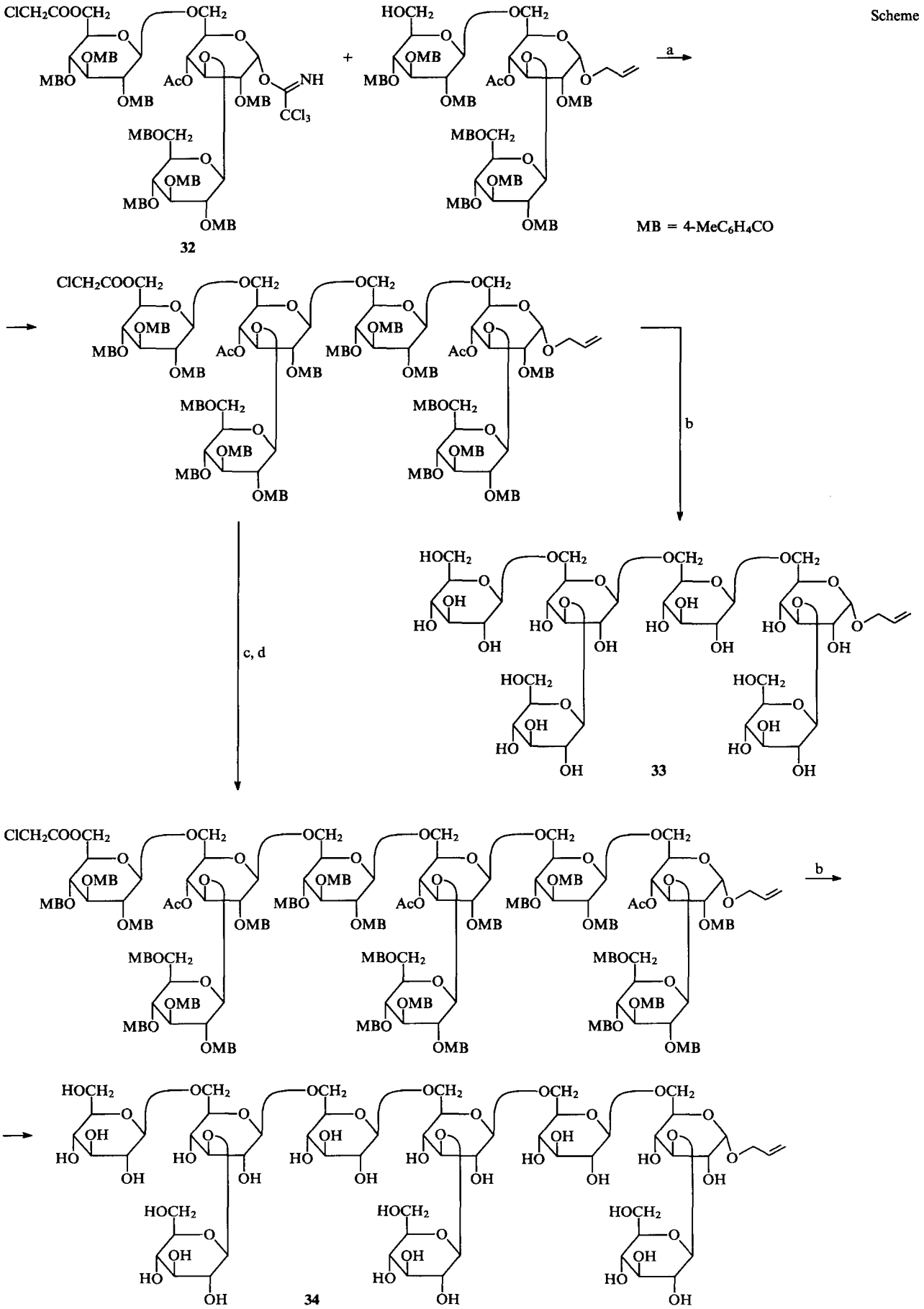
Scheme 10



31

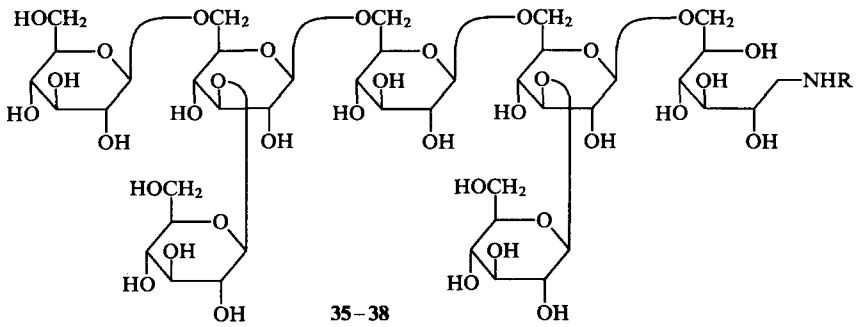
(a) $H_2NNH_2 \cdot H_2COS_2$; (b) CF_3SO_2OMe, 4 Å sieve, CH_2Cl_2 ; (c) MeONa, MeOH + THF; (d) $H_2/Pd-C$.

Scheme 11

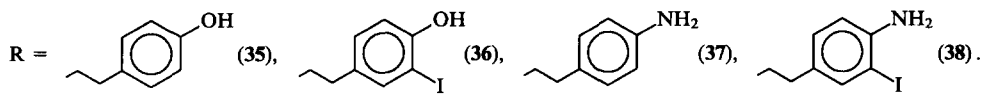


(a) $BF_3 \cdot Et_2O$, AW-300 sieve, $(CH_2Cl)_2$; (b) MeONa, MeOH; (c) H_2NCSNH_2, EtOH; (d) **32**, a.

Scheme 12



35–38



The first attempts to investigate the mechanism of the action of the oligosaccharide fragments of the fungal β-glucan on plant cells were made using a complex heterogeneous mixture of certain fragments,[46–49] but more reliable data were obtained with the aid of synthetic oligosaccharides.[45,50] In the latter studies, ^{125}I-labelled conjugates of the oligosaccharin with tyramine or 2-(4-aminophenyl)ethylamine were used. It was shown[51] that centres with a specific binding of oligosaccharins exist in the membranes of plant cells and that their binding process is characterised by saturation, reversibility, and an affinity ($K_\alpha = 7.5 \times 10^{-10}$ mol dm^{-3}) comparable to the concentration of compound **6** necessary for the manifestation of elicitor activity. All the binding centres are of the same type and are inactivated on proteolysis or on heat treatment, which is evidence in support of their protein (or glycoprotein) nature. The competitive inhibition of the binding process by numerous analogues demonstrates a direct relation between the affinity for the active centre and the elicitor activity. All these data confirm the hypothesis that the membranes of plant cells contain specific receptors for oligosaccharins. The high level of the specificity and of the discriminating capacity of the recognition system can be explained by the fact that the cells must recognise low concentrations of fragments of the foreign polysaccharide against the background of extremely large amounts of the 1 → 4-, 1 → 3-, and 1 → 3, 1 → 4-β-D-glucans present in the healthy plant under normal conditions.

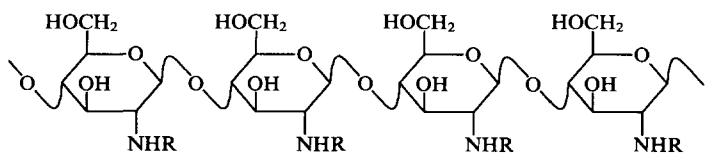
The initiation of the synthesis of phytoalexins is not the only biological effect of the oligosaccharide **17**. Other plant protecting mechanisms are also mobilised simultaneously (for a more detailed description of a multiplicity of the activities of oligosaccharins, see below in the subsequent sections of the review), in particular the synthesis of β-glucanase — the enzyme present also in healthy plant cells which is capable of cleaving the β-glucan of the fungal cell wall with formation of signalling oligomers — is induced. Whereas the protecting function of enzymes of this type had been treated previously as a direct effect of the phytopathogen on the cell wall, limiting its growth, after the discovery of oligosaccharins the role of β-glucanase appears to be more probably that of generating signalling molecules, which stimulate a multiplicity of mechanisms protecting the host plant.

In conclusion of this section, one may note that, as a result of extremely intensive chemical researches, the heptasaccharide **17** and its analogues have become fairly readily available. An excellent practical manual of the methods of synthesis and of the characteristics of these compounds, including the synthesis of conjugates with tyramine and the introduction of the ^{125}I isotope into the latter, has been published.[52] Therefore, new discoveries in the field of the mechanism of the action of substances of the type considered on plant cells are to be expected in the immediate future.

2. Oligosaccharide fragments of chitin and chitosan

Together with β-glucan, the cell walls of phytopathogenic fungi contain chitin — an insoluble high-molecular-mass homopolymer the linear molecules of which are made up of 1 → 4-linked residues of *N*-acetyl-β-D-glucosamine — and chitosan — the product of the deacetylation of chitin (Scheme 13).[53,54] In higher plants, these polymers are absent but fragments capable of cleaving the carbohydrate chains of chitin and chitosan (chitinases and chitosanases) occur very widely.[55,56] Since it is evident that these enzymes do not participate in the primary metabolism of the plants themselves, it has been suggested that their purpose is to protect the plants against pathogenic fungi. Indeed, by disturbing the finely balanced processes in the biosynthesis and lysis of the components of the growing cell walls of the fungus, the chitino-lytic enzymes of the plants can limit its growth.[57] On the other hand, the oligosaccharide fragments formed in the enzymic hydrolysis of chitin and chitosan serve as signalling molecules, in response to the appearance of which the mechanisms for the synthesis of phytoalexins and other protecting responses of the plant are switched on. This phenomenon should play a no less important role in the interaction of plants with pathogenic fungi.

Scheme 13



R = Ac, H.

In contrast to the branched β-glucan, the linear chitin and chitosan, which contain a single type of linkage, afford on fragmentation a comparatively less complex set of oligosaccharides, which can differ from one another only by the degree of polymerisation, the degree of acetylation, and (for incomplete acetylation) by the distribution of the acetyl groups. The oligosaccharide products of the fragmentation of the chitin and chitosan molecules can be obtained from these polysaccharides by chemical methods[58-61] or by enzymic hydrolysis.[62] However, the technical difficulties in the isolation of the individual oligomers are fairly considerable, so that the majority of studies on the biological activities have been carried out using either the whole cell walls of the phytopathogenic fungi or the polymeric chitin and chitosan as elicitors; oligosaccharide fractions containing 2–3 oligomer-homologues are frequently used.

The formation of phytoalexins on treatment with chitosan and its fragments has been observed both in the tissues of the whole plant[63] and in plant cell suspension culture.[64,65] Other protecting systems of the plants are switched on simultaneously—such as the formation of the inhibitors of proteinases[66,67] or the synthesis of the protecting (1 → 3)-β-D-glucan of callose;[65,68] the oligosaccharide fragments of chitin also stimulate the lignification of cell walls.[69-71] The induction of the formation of chitinases[72,73] and chitosanases,[74] which, together with the inhibitors of proteinases and 1,3-β-glucanases, belong to the group of proteins associated with the pathogenesis, is particularly clearly expressed.[75,76] It has been shown that the protecting action of the chitinases does indeed consist both in the direct inhibition of the growth of the fungus[77,78] and in the formation of the elicitors of numerous systems protecting the host plant by cleaving the biopolymers in the cell wall of the phytopathogen.[79] It is of interest to note that the transgenic tobacco plants, carrying the gene of the leguminous plant chitinase, exhibit an increased stability to attack by soil fungi.[80]

As a rule plant chitinases constitute a complex made up of several exo- and endo-enzymes. In the study of the induction of chitinases by individual chitin oligosaccharides, it was established[81] that the hexasaccharide is the best stimulator of the exo-chitinase activity, whereas the endo-chitinase activity is stimulated most effectively by the heptasaccharide; the octa- and nonasaccharides also exhibit an appreciable elicitor activity. The lower oligosaccharides (including the tetramer) and the fraction of higher oligosaccharides with a degree of polymerisation of 13–20 proved to be almost inactive. The response of the plant to the introduction of elicitors was fairly rapid: the maximum activity of the induced chitinases was observed after 12–24 h, the enzymes being detected not only at the site where the oligosaccharides had been introduced (the cotyledons of pumpkin seedlings) but also the remote parts of the plant (leaves). It has been shown that the biosynthesis of chitinases is associated with the activation of the corresponding genes and the formation of mRNA,[82,83] but it has not so far been established whether this phenomenon is due to the permeation of the chitin oligomers into the cell nucleus; the mechanism of the propagation of the signals through the plant tissues is likewise unknown.

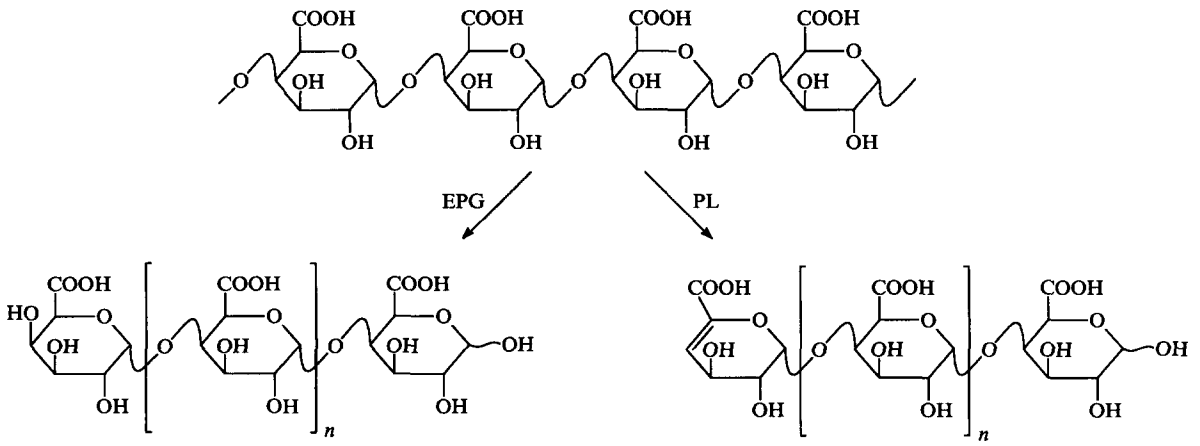
In the manifestation of other types of biological activity, a relation has also been observed beween the effect and the degree of polymerisation or structure of the oligosaccharide, although this problem has not been studied in detail. Thus the lignification of damaged wheat leaves is induced by chitooligosaccharides with a degree of polymerisation in excess of 4 but not by the corresponding fragments of chitosan.[70] However, oligomers of chitosan (degree of polymerisation in excess of 7) and not those of chitin serve as the elicitors of the formation of the phytoalexin pisatin in pea pods.[79,84] However, the deacetylated and partially acetylated oligosaccharides containing more than four monosaccharide residues stimulate equally effectively the formation of the inhibitors of proteinases in tomato and potato leaves.[66,67]

3. Oligogalacturonides

Pectin substances are important structural components of the primary plant cell wall.[5,85] The simplest representative of pectins is homogalacturonan or polygalacturonic acid—a linear polymer consisting of 1 → 4-linked α-D-galacturonic acid residues in the molecules of which some of the carboxy-groups may be present as the methyl ethers. Many microbial phytopathogens (both fungi and bacteria) produce enzymes capable of cleaving homogalacturonan either via a hydrolytic reaction [endopolygalacturonases (EPG)] or via an elimination reaction [pectate lyases (PL)]. The oligosaccharides formed possess the activity of phytoalexin elicitors,[86] the maximum of which corresponds to oligomers with a degree of polymerisation of 12 (Scheme 14).[87-90] Plants contain specific EPG inhibitors,[91] which regulate the activity of the enzymes produced by the phytopathogens, preventing an unduly far reaching cleavage of the polygalacturonic acid and thereby promoting the accumulation of higher oligosaccharides possessing the elicitor activity.[92]

Together with the formation of phytoalexins, other plant protecting mechanisms are switched on under the influence of oligogalacturonides, e.g. endo-(1 → 3)-β-gluconases, chitanases, and lysozyme are induced. They cleave the cell walls of phytopathogenic fungi and bacteria,[93,94] liberating the elicitors of the protecting reactions described in the previous sections. The lignification of protected tissues is also enhanced in the presence of oligogalacturonides.[95,96] It is important to note that the activities of the oligosaccharide elicitors, originating from the fungal or bacterial cell wall, on the one hand, and from the host plant, on the other, exhibit a synergism, i.e. the plant protecting reactions are switched on at lower concentrations of the oligosaccharides of different structure when these act together.[93,97]

Scheme 14



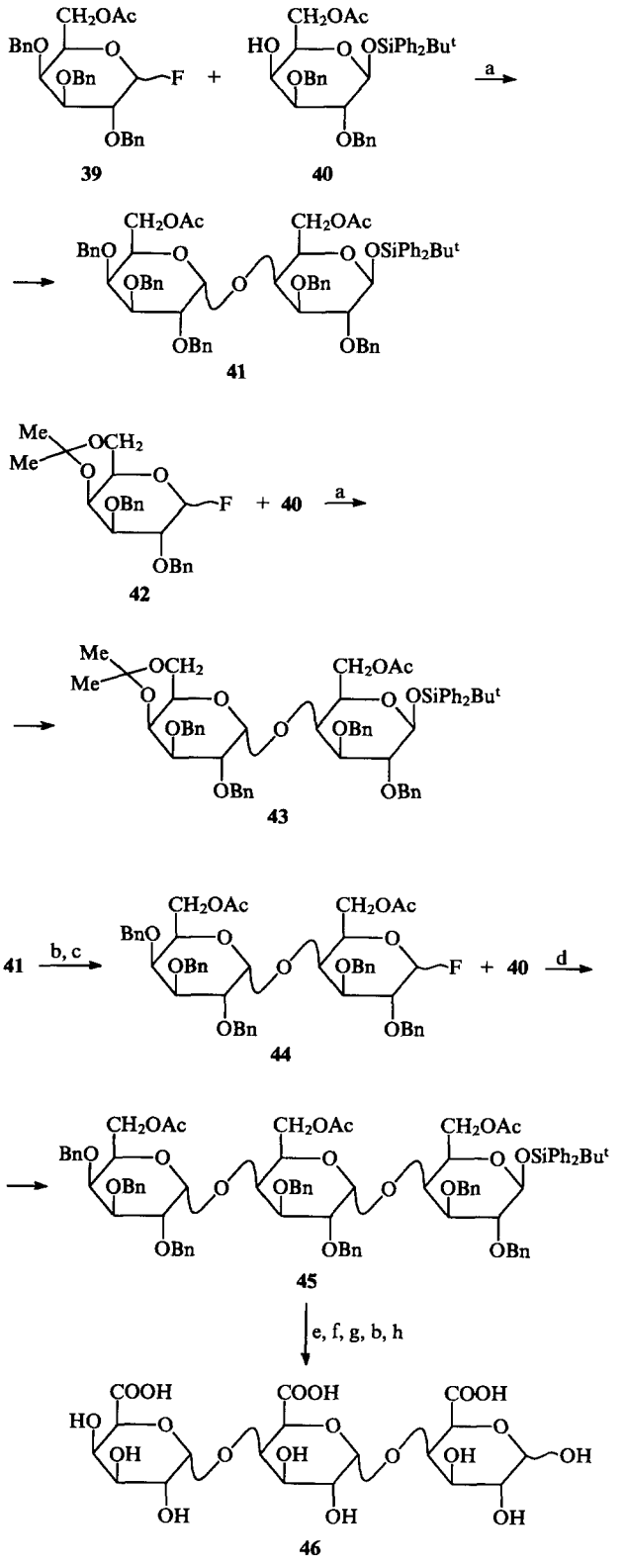
Several other types of biological activity of oligogalacturonides have been described. Compounds with a degree of polymerisation of 2–20 induce the formation of proteins inhibiting insect proteinases; it is believed that the plant is protected in this way against damage inflicted by plant eating insects.[98] In the presence of oligogalacturonides, cell suspension cultures produce endocellular isoperoxidases[96] and hydrogen peroxide.[99] Low concentrations of oligogalacturonides with a degree of polymerisation of 12–15 induce sharp changes in the permeability to K^+ and Ca^{2+} of the cell plasma membrane and its depolarisation.[100] Oligosaccharides with a degree of polymerisation of 14–15 induce the phosphorylation of the 34 kDa protein isolated from the plasma membrane.[101] It still remains to be elucidated to what extent these effects on the cell surface are associated with the elicitor activity of the oligogalacturonides described above.

The structural requirements imposed on the biologically active oligogalacturonides differ significantly from those for the heptasaccharide fragment of the fungal β-glucan described above. Thus the presence of the 'normal' α-D-galacturonic acid residue or even of the Δ^4-unsaturated uronic acid at the nonreducing end of the oligogalacturonide (Scheme 14) hardly affects the activity. On the other hand, reduction with sodium tetrahydroborate to alditol diminishes the activity.[102] The latter may be associated with the general decrease in the charge of the molecule as a result of the lactonisation of the reducing end.[8] The activity also falls on esterification[89] or reduction of the carboxy-groups.[90] A critical degree of polymerisation is apparently necessary for the acquisition by the oligogalacturonide of a stable conformation ensuring a fully defined spatial disposition of the charged groups, because acid oligosaccharides of a different chemical nature (fragments of chondroitin sulphate, hyaluronate) or acid polypeptides (polyglutamate) lack of elicitor properties.[102]

Oligogalacturonides, which are essential for the study of their activity on plant cells, can be obtained in preparative amounts from commercial pectin or polygalacturonic acid preparations in two ways: by partial acid hydrolysis or by enzymic cleavage.[52,103] An advantage of the first method is simplicity and low cost, while that of the second is high yields of the oligosaccharides and the absence of severe influences, which may induce undesirable structural changes in the native molecules. The most difficult task is the separation of the mixture of oligosaccharides with a degree of polymerisation of 10–15 into individual components, which is carried out at the limit of the resolving capacity of the modern chromatographic technique. It has been shown that even in the case of a successful isolation of the homopolymer fractions, the principal target components can contain up to 15% of impurities, which, whilst having the same degree of polymerisation, differ in the structure at the reducing end and also in certain unknown structural elements.[9,10] All this renders the chemical synthesis of oligogalacturonides extremely attractive.

The synthesis of oligomer-homologues of α-D-galacturonic acid appears at first sight to be a completely hopeless task for two reasons: in general, owing to the lack of convenient synthetic methods for the construction of α-glycosidic linkages and, in particular, because the methods for glycosylation by galacturonic acid derivatives have been little investigated. Nevertheless the problem was solved successfully when Nakahara and Ogawa proposed an extremely novel strategy for the synthesis.[104,105] They used data showing that benzylated glycopyranosyl fluorides react with alcohols in the presence of $SnCl_2$ and $AgClO_4$, affording predominantly 1,2-*cis*-glycosides.[106] It was established for galactose derivatives that the protected fluoride **39** interacts under these conditions with the partially protected derivative **40** to form a mixture of α- and β-disaccharides from which the α-isomer **41** was isolated in a satisfactory yield (Scheme 15). The fluoride **42** reacted even more selectively, giving rise almost exclusively to the α-linked

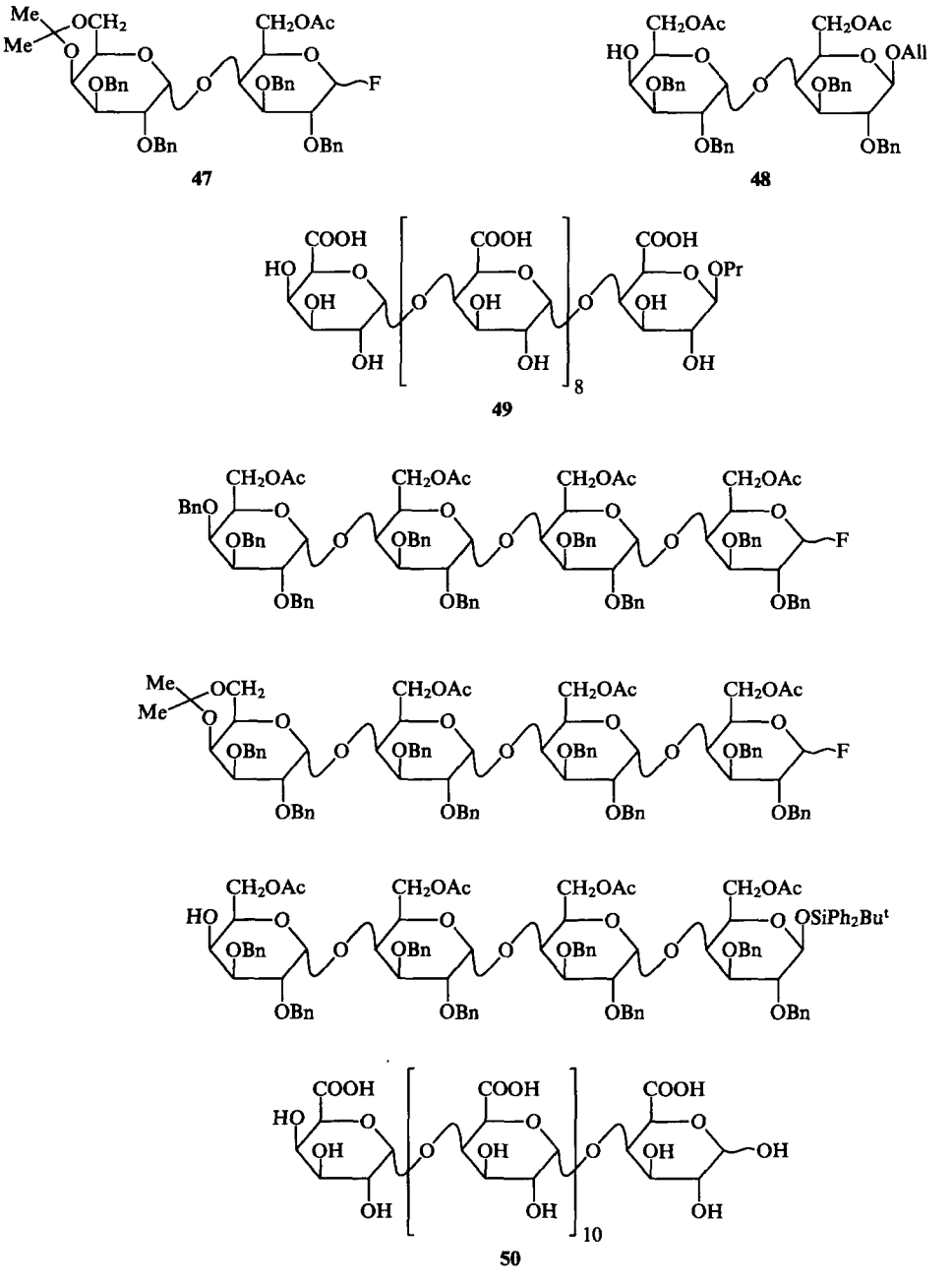
Scheme 15



(a) $Sn(OTf)_2$ or Bu^n_3SnOTf; (b) Bu^n_4NF–AcOH, THF; (c) Et_2NSF_3, THF; (d) $SnCl_2$, $AgClO_4$, Et_2O; (e) MeONa, MeOH; (f) CH_3SOCH_3, $(COCl)_2$; (g) $NaClO_2$; (h) H_2/Pd–C.

protected disaccharide **43**. It proved easy to convert compound **41** into the disaccharide fluoride **44**, the repeated condensation of which with compound **40** led to the α-linked trisaccharide **45**. On deacetylation of this substance with subsequent two-stage oxidation of the three primary alcohol groups, initially to the

Scheme 16



aldehyde groups by treatment with dimethyl sulfoxide and oxalyl chloride,[107,108] and then to carboxy-groups by treatment with sodium chlorite in the presence of hydrogen peroxide[109] yielded the trigalacturonide **46**. Using the same procedures, the authors synthesised subsequently a model disaccharide, which, however, was not obtained in a free form but only in the form of the n-propyl glycoside **49** (Scheme 16). The molecule of this compound was constructed using the disaccharide units **44**, **47**, and **48**.[110,111] Compounds **48** and **44** served as the precursors of the reducing and nonreducing ends of the future decasaccharide. The central part of the molecule was constructed by the threefold employment of compound **47** as the glycoside donor: first in the reaction with compound **48** and then, after each glycosylation, the new glycoside acceptor was prepared by removing the isopropylidene group from the terminal non-reducing galactose residue and by partial acetylation at the primary alcohol group. The final stage of the synthesis was the simultaneous oxidation of all ten primary alcohol groups.

The total synthesis of the dodecasaccharide elicitor **50** may be regarded as the completion of this series of studies. In this case, the molecule was assembled from tetrasaccharide units (Scheme 16).[112,113] However, the amount of the oligosaccharin synthesised in this way was small and data concerning the detailed study and the biological activity of the synthetic specimen have still not been published.

Derivatives containing a radioactive label are necessary for the investigation of the mechanism of the activity of oligogalacturonides. The reductive amination method using tyramine and subsequent introduction of radioactive iodine, used for the heptaglucoside **17** (see above), are unsuitable for oligogalacturonides; instead it has been suggested that hydrazones be obtained by reaction with tyrosine hydrazide and then iodinated.[52,114] It was shown in at least one of the biological tests that the conversion of the dodecagalacturonide into the hydrazone does not affect the biological activity.[114]

* * *

Summarising the above section, we may note that, in the interaction of the plant with phytopathogens, there is a possibility of the enzymic cleavage of the biopolymers in the cell walls of both partners. Some of the oligosaccharides thus liberated possess a specific biological activity and act as the elicitors of the protecting reactions of the host plant, oligosaccharides of different origin manifesting a distinct synergism in their activity. Although only one of the types of the plant protecting reactions is analysed in many experimental studies, it appears that in all cases there is a complex response to the appearance of oligosaccharins, whereupon, together with the synthesis of phytoalexins, enzyme systems cleaving the phytopathogen cell wall are activated and the tissue lignification process is intensified, which creates a barrier to further penetration by the phytopathogen, etc. By virtue of their multiple (pleiotropic) action on plants, oligosaccharins thus resemble the 'classical' phytohormones — auxins, gibberellins, cytokinins, abscisic acid, and ethylene. However, it must be emphasised that oligosaccharins are active at significantly lower concentrations. Another analogy with phytohormones involves the lack of species specificity: a similar activity of oligosaccharins has been demonstrated in many representatives of the plant kingdom, the relationship between which is extremely remote. Although the mechanism of the activity of oligosaccharins has still not been established, it is likely that in most cases a highly specific binding of the oligosaccharin to the membrane receptor protein takes place, which in fact constitutes the 'signal of fear' ['give away signal' in Ref. 8 (Ed. of Translation)] for the switching on of the plant protecting mechanisms.[8-10]

III. Oligosaccharins as regulators of plant growth and morphogenesis

1. Oligogalacturonides

The ability of plant cells to be differentiated and converted into specialised plant organs is controlled by many factors, among which hormones, light, and temperature are of primary importance.[115,116] The influence of various chemical compounds on organogenesis can be followed on explants of tobacco peduncles which, during cultivation on an artificial medium, produce, in the course of 24 days, flowers, vegetative shoots, or roots depending on the auxin and cytokinin concentrations and on the pH.[117] A sensational report stating that the oligosaccharide fragments of plant cell walls (tentatively the fragments of the pectin polysaccharides) alter the specificity of the action of phytohormones in relation to organogenesis appeared in 1985.[118] However, several years elapsed before it was possible to characterise more thoroughly the chemical structure of the active oligosaccharides and to achieve reproducible results in this fairly capricious biological test.[119,120]

It was believed for some time that fairly complex fragments of pectin substances containing, apart from D-galacturonic acid, the residues of neutral monosaccharides serve as regulators of organogenesis. However, it was shown subsequently that the active principle nevertheless comprises the simple linear oligogalacturonides, which have already been considered as elicitors of phytoalexins. It is now known [119-121] that oligogalacturonides with a degree of polymerisation of 10–14 induce the formation of flowers and inhibit the formation of roots under conditions where without the addition of oligosaccharins tobacco explants form only roots or altogether fail to produce new organs. Oligogalacturonides act at concentrations of the order of 10^{-7} M, the highest activity being shown by compounds with a degree of polymerisation of 12–14. The existence of two types of activity in these oligosaccharins is probably associated with the fact that the same groups of genes are activated both in organogenesis in a healthy plant and in the biosynthesis of proteins associated with pathogenesis.[122]

Several other examples of the influence of oligogalacturonides on plant development processes are known. Thus a mixture of these oligosaccharides retards the auxin-induced growth of segments of pea plant stems, acting at a fairly high concentration (10^{-4} M).[123] This type of activity has been investigated in greater detail for xyloglucan fragments (see below). Oligogalacturonides induce the formation of ethylene in tomatoes (tomato fruits)[124,125] and in a pear cell culture suspension.[126] Hence they can participate in the complex process involving the hormonal regulation of the ripening of fruits.[127]

2. Oligosaccharide fragments of xyloglucan

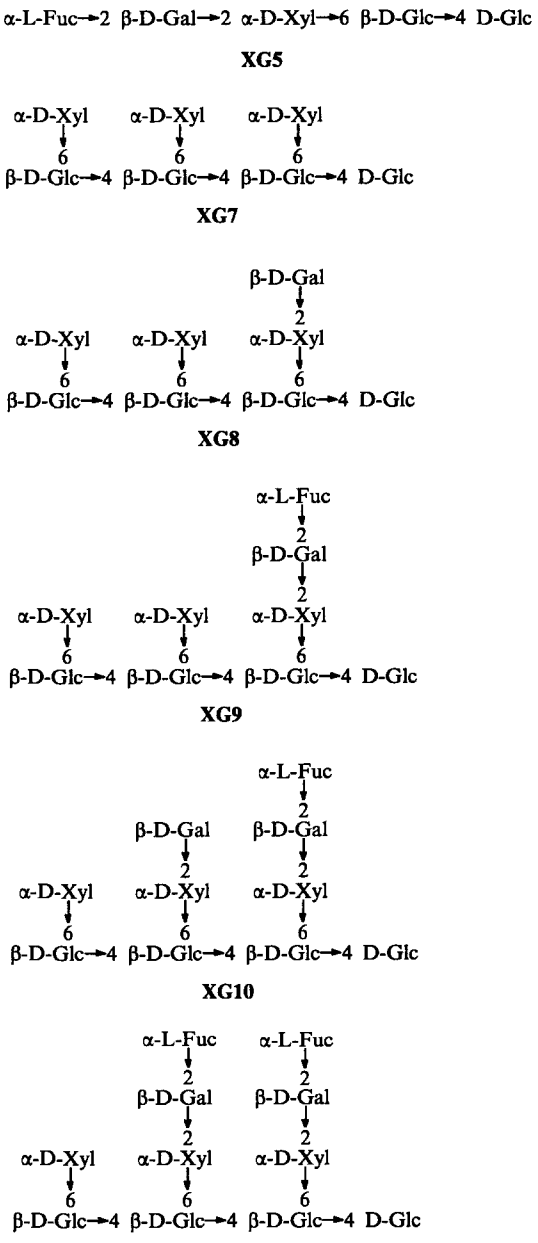
Xyloglucan is a universal component of the hemicelluloses in higher plants.[5,128-130] Its content in the primary cell walls of dicotyledonous plants is usually 20%–25%, while in the *Gramineae* its content may be significantly lower (2%–5%). Xyloglucan is deposited in large amounts in the seeds of certain plants, where it serves as a reserve of nutrient material used during growth. Apart from glucose and xylose, xyloglucans have been found to contain galactose, fucose, and arabinose. Whilst having a common regular design, xyloglucans of different origin can differ in the proportions of monosaccharides. In particular, storage xyloglucans usually virtually lack fucose residues. We shall consider below the structures of typical xyloglucans in dicotyledonous plants, which have been isolated from the cell walls or from the medium cell in suspension cultures.[131]

The basis of the xyloglucan molecule is a linear chain made up of 1 → 4-linked β-D-glucopyranose residues, which have the same structure as cellulose. About 75% of the glucose residues in this chain have been substituted in the 6-position by α-D-xylopyranose residues (a small proportion of these substituted glucose residues carry in addition α-L-arabinofuranose, β-D-xylopyranose, or 3-*O*-α-L-arabinofuranosyl-β-D-xylopyranose residues in the 2-position). From one-third to half of the xylose residues are substituted in their turn in the 2-position by β-D-galactopyranose residues. Finally, many lactose residues have in the 2-position a substituent in the form of a α-L-fucopyranose residue. In those cases where it was possible to isolate the polysaccharide without using alkaline extraction, it was demonstrated that certain galactose residues may carry one or two acetyl groups in the 6-, 3-, or 4-positions.[132,133]

In the structural analysis of xyloglucan, enzymic cleavage has played a most important role. The effect of a mixture of fungal enzymes, known under the commercial name 'Driselase' (Sigma), which results in the formation of 6-*O*-α-D-xylopyranosyl-D-glucose (isoprimeverose), is a diagnostic feature for the polysaccharide.[134] Other carbohydrate components of the plant cell wall do not contain such a structural fragment. Since the above disaccharide can be readily distinguished in terms of its chromatographic behaviour from other disaccharide products of enzyme treatment, the detection of isoprimeverose in the hydrolysate can serve as convenient and reliable evidence for the presence of xyloglucan in the raw material or in the mixture of polysaccharides analysed.

Fungal cellulases cleave xyloglucan at the glycosidic linkages connecting the unsubstituted β-D-glucopyranose in the main chain. The main products of such cleavage are a heptasaccharide and a nonasaccharide [designated in the literature by XG7 and XG9 (Scheme 17)], which demonstrates the considerable regularity of the distribution of substituents in the main chain, where each fourth glucose residue is free. Together with the XG7 and XG9 oligosaccharides, it is possible to detect in the hydrolysate the XG5, XG8, XG10, XG11, and higher oligosaccharides containing the L-arabinose residues, although their amounts are smaller.[21,131,134-138]

Scheme 17



Paper chromatography,[139,140] chromatography in a thin silica gel layer,[141] gel chromatography on P-2 biogel,[131,142] and high performance liquid chromatography (HPLC)[143] have been proposed for the separation of the products of the enzymic hydrolysis of xyloglucan. This includes the separation of the products in the form of pyridylamino-derivatives obtained by reductive ammination using 2-aminopyridine.[144] The determination of the structures of these complex oligosaccharides is due to several groups of investigators. We may note that in this field too the leading place is occupied by the team headed by Prof. Albersheim, who developed the most effective physicochemical methods for the characterisation of the structures of xyloglucan fragments—^{1}H NMR spectroscopy and mass spectrometry with bombardment by fast atoms.[137,138] In order to obtain well resolved NMR spectra, it has been suggested that the oligosaccharides be reduced with sodium tetrahydroborate to the corresponding alditols (this procedure also helps to improve the separation and purification of oligosaccharides with the aid of HPLC). The observed rules governing the positions and forms of the signals due to the anomeric protons[137,145] make it possible to establish the structures of the majority of oligosaccharides from one-dimensional ^{1}H NMR spectra.

Reduced oligosaccharides are also used for mass spectrometry with bombardment by fast atoms. The spectra of the negative ions of compounds which have not been converted into their derivatives then indicate the presence of intense pseudomolecular ions $[M - H]^-$, while the fragment ions formed as a result of the dissociation of the intermonomer bonds originate from the reduced end of the molecule. On the other hand, for acetylated oligosaccharide alditols most information is yielded by the spectrum of the positive ions, which contains the intense pseudomolecular ion $[M + NH_4]^+$, while the fragment ions originate from the nonreducing end of the molecule. The combination of two such mass spectra makes it possible to determine the sequence of the monosaccharide residues in the test compounds.[136,145,146] The effectiveness of these methods has been demonstrated particularly strikingly in the latest studies by the group of investigators mentioned above, which were devoted to the establishment of the structures of the higher oligosaccharide fragments of xyloglucan containing between 17 and 20 monosaccharide residues.[145,146]

The primary wall of a plant cell constitutes an extremely involved complex of biopolymers of several types: celluloses, xyloglucan, polysaccharides of the pectin group, and structural protein.[147,148] In all probability the xyloglucan molecules in the cell wall are bound covalently to the pectins and the latter are bound to the structural protein, whereas the cellulose microfibrils form stable noncovalent complexes with xyloglucan molecules via hydrogen bonds. It has been suggested that cell growth is ensured by the partial cleavage of the xyloglucan molecules, which enables the cellulose microfibrils to be displaced relative to one another. Indeed, it is well known that auxins stimulating cell growth activate cell cellulases and, as stated above, these enzymes are capable of cleaving xyloglucan.[149-151]

In this connection, the discovery of a specific biological activity of the XG9 nonasaccharide proved to be extremely interesting: at a very low concentration of the order of 10^{-8} M, this compound inhibited the auxin-induced growth of segments of pea plant stems.[152] Surprisingly, this effect diminished not only at low but also at high XG9 concentrations; the latter observation implied, incidentally, that the inhibition of growth is not a consequence of the cytotoxic activity of the preparation. The closely related XG7 oligosaccharide completely lacked the antiauxin activity. The hypothesis arose that the biologically active XG9 fragment of the xyloglucan participates in the regulation of cell growth, acting in accordance with the negative feedback principle: an increase in the amount of auxin should induce the appearance of the inhibitor which prevents unrestricted growth.[152]

The test for the antiauxin activity is not unduly reliable and is not very convenient in practice, but the existence of the antiauxin activity of XG9 described above has been confirmed in two other laboratories—both in similar experiments on the inhibition of the lengthening of stem segments[153,154] and in experiments on the regeneration of the cell walls of protoplasts.[155] It has also been possible to observe the formation of the nonasaccharide fragment of xyloglucan *in vivo*.[134] The set of oligosaccharides obtained by the enzymic hydrolysis of xyloglucan made it possible to carry out interesting observations on the relation between structure and biological activity.[142,156]

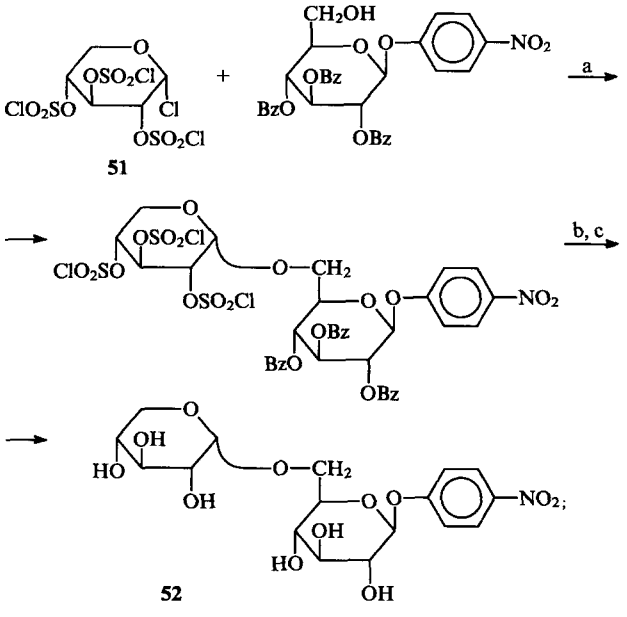
It was shown that the presence of the α-L-fucose residue is a necessary condition for the manifestation of the antiauxin activity, because all oligosaccharides lacking fucose proved to be inactive. However, the XG10 oligosaccharide, containing the fucose residue but having also an additional galactose residue in the neighbouring chain likewise failed to exhibit an antiauxin activity; on the other hand, XG11 with two fucose residues in the molecule had an activity higher than that of XG9.[157]

Other structural elements were less important: the activity of the reduced XG9 did not differ from that of the initial oligosaccharide; the activity did not fall even after the enzymic elimination of the α-D-xylo-pyranose residue most remote from the reducing end of XG9.[157] A biological activity was also shown by the fucose-containing XG5 and 2-*O*-α-L-fucopyranosyl-lactose—a trisaccharide which is not a xyloglucan fragment but has been isolated from human milk; admittedly, these oligosaccharides were active at higher concentrations. In the case of XG5, an optimum in the concentration dependence of the antiauxin activity, so characteristic of XG9, was also not observed.[142,146]

At significantly higher concentrations (1—200 μM), the XG9 oligosaccharide activates cellulase;[158] a similar effect is shown to a smaller extent by XG5 and XG7. This second activity of XG9 helps one to understand why the oligosaccharide loses its anti-auxin activity at relatively high concentrations.[159] At extremely low concentrations (of the order of 1 nM), XG9 and XG10 inhibit the lengthening of segments of the pea plant stems induced by acidification,[160] from which it follows that the anti-auxin activity of XG9 is not associated with the suppression of the entry of hydrogen ions into the cell wall under the influence of auxin[161] but involves some hitherto unknown common stages in the influence of auxin and the pH on cell growth.

The biological activity of the xyloglucan fragments aroused an increased interest by chemists engaged in synthetic chemistry in the problem of the synthesis of these oligosaccharides. As early as 1986, a communication was published concerning the synthesis of *p*-nitrophenyl β-isoprimaveroside **52** (Scheme 18).[162]

Scheme 18



(a) Ag_2CO_3, $AgClO_4$, $CHCl_3$; (b) NaI, MeOH—H_2O; (c) MeONa, MeOH.

The method used by the authors to form the α-xyloside bond is noteworthy: the glycoside donor employed is a tris(chloro-sulfate) of α-xylopyranosyl chloride—a compound containing at the C(2) atom a group incapable of participating in the glycosylation process. It is obtained in one stage from xylose on treatment with sulfuryl chloride. Compound **52** was subsequently used to synthesise an artificial antigen (by reduction to *p*-aminophenyl glycoside, diazotisation, and azo-coupling to bovine serum albumin), with the aid of which antibodies specific to the β-isoprimeverose residue were obtained.[163] Analogous artificial antigens were also prepared using the XG7, XG8, and XG9 oligosaccharides isolated from the enzymic hydrolysates of the natural xyloglucan.[164] The corresponding antibodies can find applications in the study of the location of xyloglucan in plant cell walls, as had been done earlier with the antibiodies to native xyloglucan,[165] and for the quantitative determination of the polysaccharide by the immunosorbent method (ELISA).[166] It is of interest to note that the antibodies specific to XG7 and XG8 (as well as lectins, which bind fucose residues)[167] inhibit the auxin-induced lengthening of segments of the epicotyls of the beans of *Vigna angularis* but do not affect the growth of the segments of the oat coleoptiles[168] despite the fact that in both plants the antibodies prevent the decrease in the molecular mass of xyloglucan under the influence of auxin. These data confirm that the cleavage of xyloglucan in dicotyledonous plants is an important stage in cell growth, but in monocotyledonous plants the cleavage of hemicelluloses of another type can play a more significant role.

A communication concerning the synthesis of the tri- and penta-saccharide fragments of xyloglucan, containing L-fucose residues, appeared in 1990.[169] The benzylated 1,2-orthoacetates **53** and **55** were used to liberate the hydroxy-groups at C(2) in D-xylose and D-galactose derivatives. The trisaccharide **59** was synthesised by the stepwise addition of monosaccharide residues, starting with the reducing end of the molecule (Scheme 19).

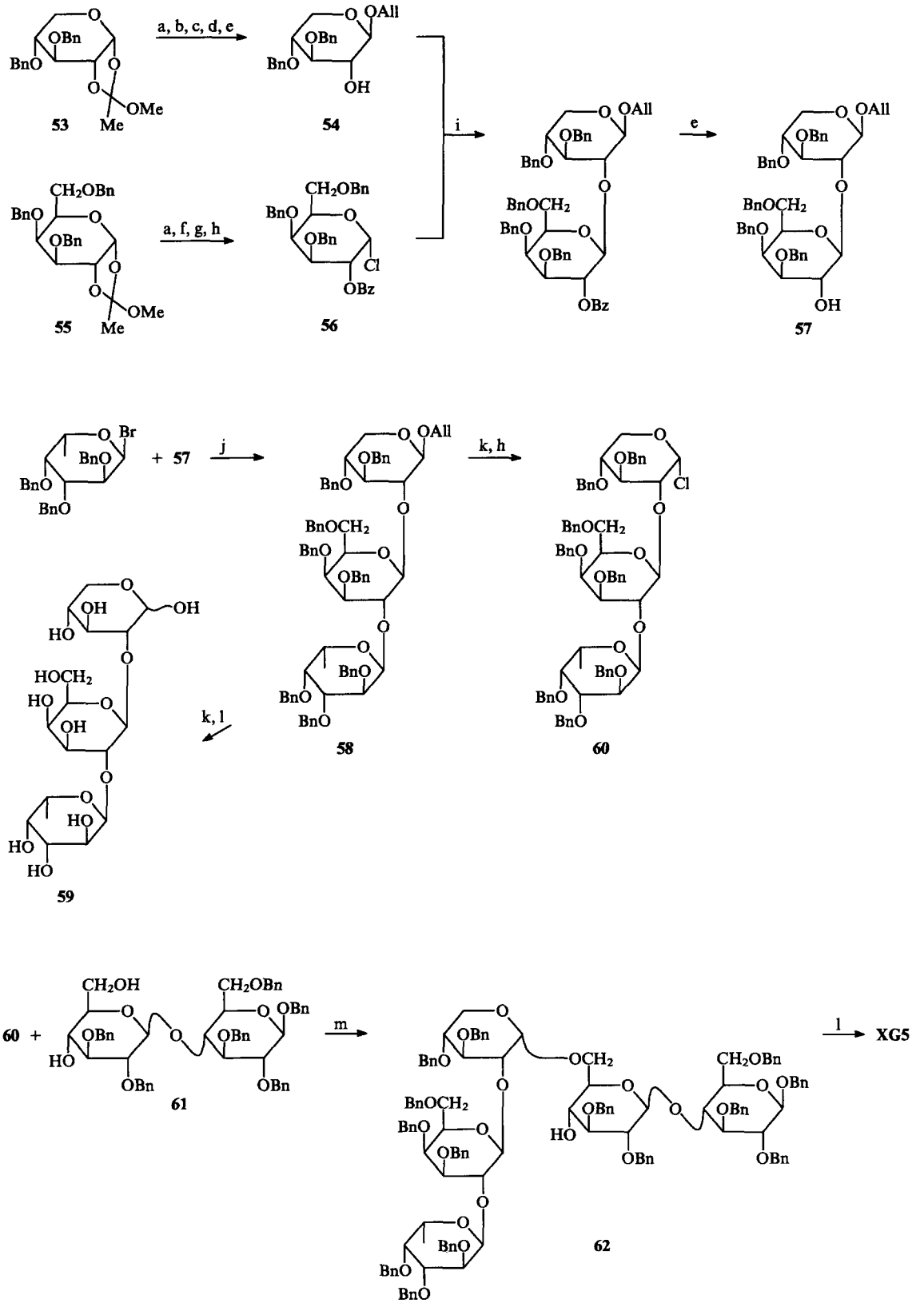
The interaction of the protected allyl xyloside **54** with the galactose derivative **56** and subsequent removal of the benzoyl group afforded the partly protected disaccharide **57**, the fucosylation of which under the conditions of the preferential formation of the α-glycosidic linkage led to the protected disaccharide **58**.[170] As a result of the removal of the protecting groups, this compound gave rise to the free trisaccharide **59** and in addition it was converted into the protected glycosyl chloride **60**, the reaction of which with the partly benzylated benzyl β-cellobioside **61** led to the protected pentasaccharide **62**. The deblocking of the compound afforded XG5.

Biological studies on the synthetic pentasaccharide led to the discovery of new types of activity of this compound.[171] It was found that the oligosaccharin at a concentration of the order of 10^{-8} M inhibits the growth of the fusicoccin (a hormone, the activity of which is close to that of auxins)-stimulated growth of pumpkin cotyledons but does not affect their growth if 6-benzylaminopurine (a hormone belonging to the group of cytokinins) is used as the stimulator. When a tissue culture of wheat germ was acted upon simultaneously by the auxin and the pentasaccharide, the formation of callus tissue was accelerated together with its faster growth, whereas in the absence of the auxin the pentasaccharide induced the vigorous formation of roots instead of the callus. The biological activity of the oligosaccharide fragments of xyloglucan was thereby confirmed on the synthetic specimen and the involvement of these compounds not only in growth processes but also in the processes associated with the morphogenesis of plants was observed.

Whilst developing approaches to the synthesis of XG9, the group of investigators[172] who described this synthesis also reported the synthesis of the second part of the XG9 molecule—6,6′-bis(*O*-α-D-xylopyranosyl)cellobiose. However, at the same time the total syntheses of XG7 and XG9 were achieved by another team of investigators (Scheme 20).[173]

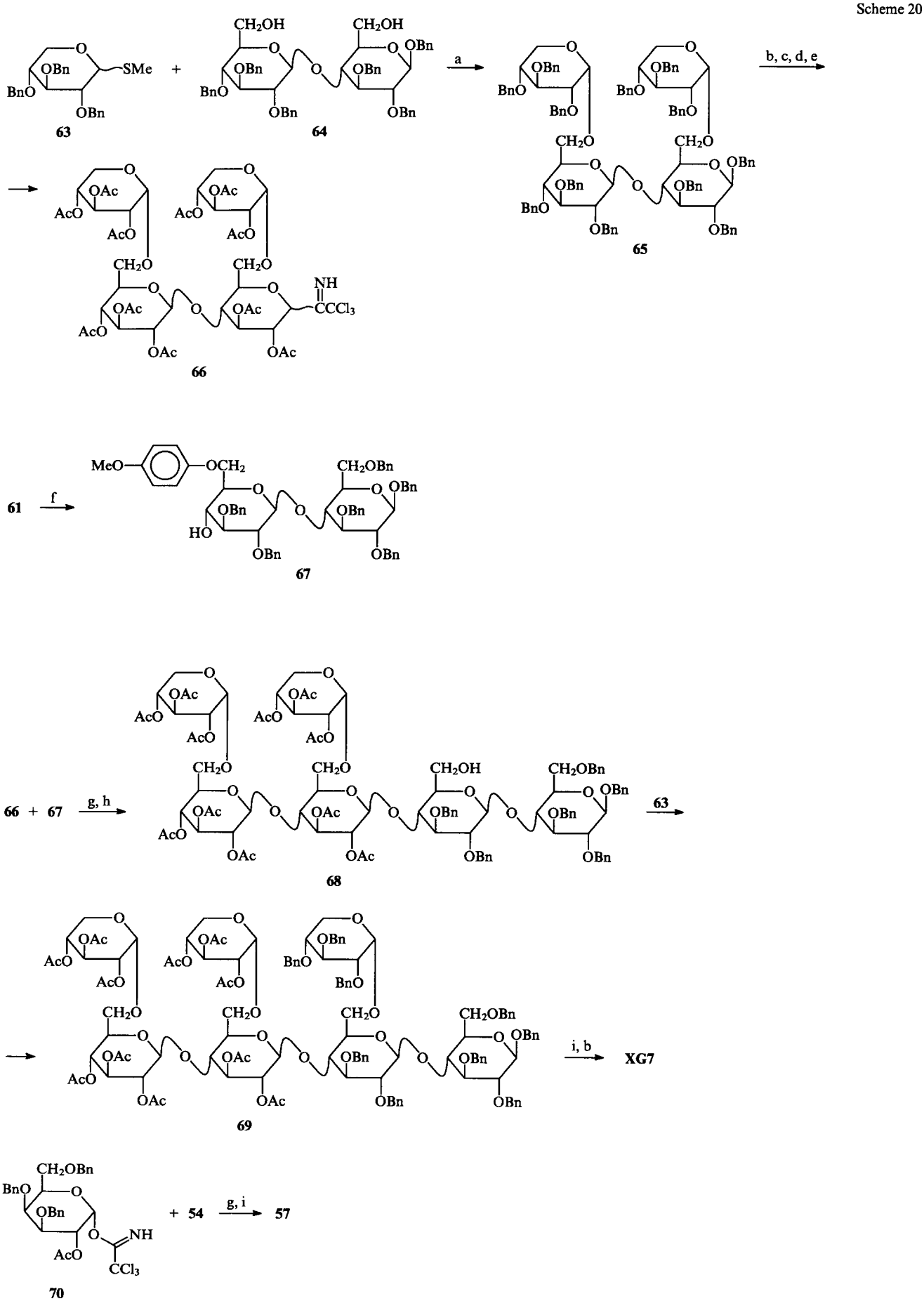
The key compound for the synthesis of both XG7 and XG9 was the hexasaccharide precursor **68**. In order to synthesise it, the derivative **64** of benzyl β-cellobioside with two free primary alcohol groups was glycosylated with the protected methyl thioxyloside **63** under the conditions of the preferential formation of α-glycosidic bonds,[174] which led to the tetra-saccharide derivative **65**. This compound was converted in several stages into the acetylated trichloroacetimidate **66**, which was used for the specific glycosylation of the protected benzyl β-cellobioside **67** obtained from compound **61** (Scheme 19) and *p*-methoxyphenol by Mitsunobu's reaction.[175] The removal of the *p*-methoxyphenyl group from this glycosylation product by

Scheme 19



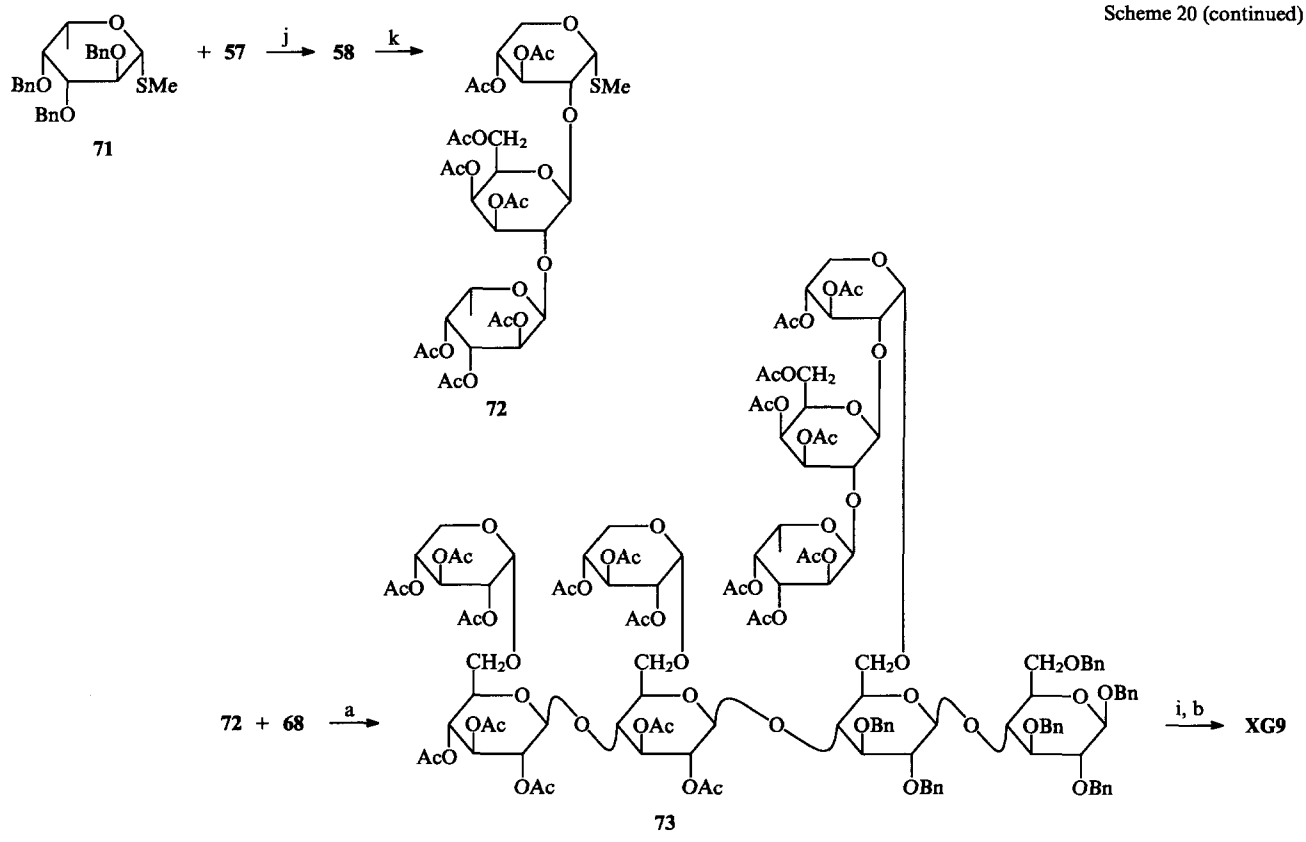
(a) 90% AcOH; (b) Ac_2O, Py; (c) HBr, CH_2Cl_2; (d) $CH_2=CHCH_2OH$, $Hg(CN)_2$, CH_3CN; (e) MeONa, MeOH; (f) BzCl, Py; (g) Et_3N, MeOH, 4 °C; (h) DMF–$(COCl)_2$; (i) $Hg(CN)_2$, CH_3CN; (j) Et_4NBr, 4 Å sieve, DMF–CH_2Cl_2; (k) Bu^tOK, DMSO, and then $Hg(OAc)_2$; (*l*) H_2/Pd–C; (m) CF_3SO_2OAg, 3 Å sieve, *sym*-collidine, −60 °C.

Scheme 20



Scheme 20 continued over.

Scheme 20 (continued)



(a) $CuBr_2$, Bu_4NBr, $HgBr_2$, 4 Å sieve; (b) H_2/Pd–C; (c) Ac_2O, Py; (d) $H_2NNH_2 \cdot AcOH$, DMF; (e) CCl_3CN, DBU, CH_2Cl_2; (f) 4-$MeOC_6H_4OH$, $(EtOCON)_2$, PPh_3, $(CH_2Cl)_2$; (g) $BF_3 \cdot Et_2O$; (h) $CeNH_4(NO_3)_4$, CH_3CN-H_2O (4 : 1); (i) MeONa, MeOH; (j) CF_3SO_2OMe, 4 Å sieve; (k) 7 stages, overall yield 31%.

treatment with cerium ammonium nitrate[176] led to the derivative **68**. Unfortunately, its xylosylation with methyl thioxyloside **63** under the conditions described above afforded a mixture of the α- and β-anomers in proportions of 4 : 3. Nevertheless, the mixture was separated, after which the deblocking of the derivative **69** led to the target heptasaccharide XG7.

The trisaccharide glycosyl donor, necessary for the conversion of compound **68** into the XG9 derivative, was synthesised in the following way. Allyl xyloside **54** and the galactose derivative **70** with the selectively protected O(2) atom were obtained, using the 1,2-orthoesters of the corresponding sugars, by procedures similar to that used by Torgov et al.[169] The disaccharide derivative **57** was glycosylated with the benzylated methyl thio-β-L-fucopyranoside **71**. The α-glycosidic bonds were formed preferentially in this stage (the ratio of the α- and β-anomers was 81 : 7). The trisaccharide derivative **58**, obtained by a fairly long although sufficiently effective method, was converted into the protected methyl thioglycoside **72**. Glycosylation of the hexasaccharide derivate **68** by this thioglycoside again led to a mixture of the α- and β-anomers in proportions of 3 : 1, but the required α-anomer **73** was isolated in a pure state and was converted into XG9 after the removal of the protecting groups.

The biological properties of the synthetic XG7 and XG9 were investigated with the aid of the classical test—inhibition of the auxin-induced growth of segments of the pea plant stems. The results agreed fully with data obtained in the study of fragments of the natural xyloglucan: XG7 was inactive, while the activity of XG9 did not differ from that of the natural specimen.[157]

* * *

In conclusion of this section, one should note that cell growth and the differentiation of plant tissues are accompanied by the partial cleavage of the carbohydrate polymers in the cell walls. Some of the oligosaccharide fragments thus formed play the role of signalling molecules: they control the hormonal influence on growth or include different groups of genes in morphogenesis. The antiauxin activity of the XG9 nonasaccharide fragment of the xyloglucan is the most thoroughly investigated example of such biological activity. The mechanism of this phenomenon is so far unknown, but fairly rigid structural requirements, the fulfillment of which is necessary for the manifestation of biological activity by the substances, have been established. The oligosaccharin XG9 acts at extremely low concentrations. As in the case of auxin,[177] in all probability the specific binding of the oligosaccharide to the membrane cell proteins serves as the signal. Presumably the influence of the xyloglucan fragments on cell growth is fairly universal and depends little on the plant species; only the cells of plants deficient in xyloglucan, the growth of which is evidently ensured by the partial cleavage of hemicelluloses of another type, constitute an exception. The discovery of morphogenetic activity in xyloglucan fragments is of great interest,[171] but these studies are still in the very initial stage.

IV. Interaction of plants with bacterial symbionts

A classical example of symbiosis is the interaction of leguminous plants with bacteria of the *Rhizobium* genus, which leads to the formation of new plant organs ('nodules') and the transformation of bacteria into bacteroids, which affords overall a biological system capable of fixing atmospheric nitrogen.[178,179] This interaction is specific: individual bacterial biovars can settle only on quite definite species of the leguminosae. The formation of nodules (nodulation) requires an extensive and concerted rearrangement of the activity of the gene apparatus of both partners, which is achieved as a result of the exchange of chemical signals. The cells of the root cortex are known to synthesise flavones or isoflavones, in response to the presence of which the bacteria begin to produce the 'nodulation factor'.[179,180] The host plant possesses a full genetic programme for the organogenesis of nodules, which is switched on in response to the appearance of this factor. The chemical structure of the first representative of the nodulation factors was established in 1990. The substance proved to be an extremely unusual lipooligosaccharide.[181,182]

The preparative isolation of this compound required much preliminary work. A reliable test was found, which made it possible to determine the activity of the nodulation factor from the deformation of the root hairs of the host plant.[183] In order to facilitate the processing of the culture liquid, a mutant strain *Rhizobium meliloti*, incapable of producing exocellular polysaccharides, was chosen for cultivation. The production of the nodulation factor by the cells of this strain was increased approximately by a factor of 100 by genetic engineering methods. The extraction of the culture liquid with butanol and subsequent purification of the extract by preparative chromatography on the C(18) reversed phase, on Sephadex LH-20, on DEAE-trisacryl and again with the aid of reversed phase HPLC led to the formation of a substance (about 4 mg from 10 litres of the culture) exerting a specific activity on the alfalfa root hairs. The preparation isolated was designated as NodRm-1.[181,182]

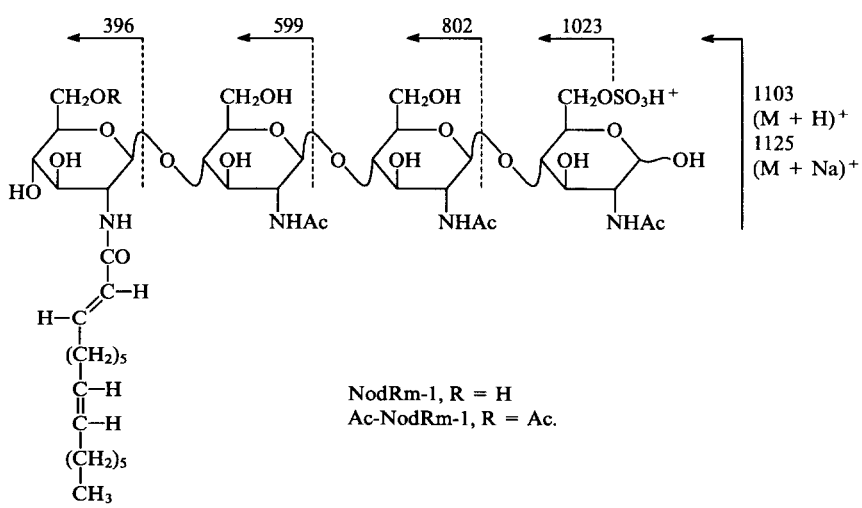
The structure of this substance is illustrated in Scheme 21. It was established using several chemical cleavages as well as mass-spectrometric and NMR spectroscopic data. The presence of the sulfate group was confirmed by the incorporation of the ^{35}S-labelled sulfate in the biosynthesis of the oligosaccharide. Glucosamine, the D-configuration of which was demonstrated with the aid of gas–liquid chromatography (GLC) of (−)-2-butyl glycosides, was identified in the products of the total acid hydrolysis. The nature of the intermonomer bonds was established by the methylation method and the configurations of the glycosidic centres and of the double bonds in the aliphatic acid residue were determined with the aid of the NMR spectra. Mass spectrometry with bombardment by fast atoms made it possible to determine the positions of the double bonds in the aliphatic acid and to find the locations of the substituents in the native oligosaccharides (the mass numbers of the most informative ions for NodRm-1 are indicated in Scheme 21). An admixture of a minor component, the tetrasaccharide Ac-NodRm-1 containing in addition the *O*-acetyl group in the 6-position in the terminal nonreducing glucosamine residue, was detected in the preparation by the same method.

Thus it was found that the signalling function was assumed by a compound having an extremely unusual structure for bacterial metabolites. Its carbohydrate chain is a tetrasaccharide whose structure is identical with that of the corresponding fragment of the molecule of chitin (which is absent from bacteria) and carries the lipophilic substituent in the form of a doubly unsaturated higher aliphatic acid residue at the non-reducing end and a highly polar substituent consisting of the sulfate group at the reducing end. The substance induces the deformation of the root hairs in alfalfa (the host plant for the chosen strain) but not in the closely related vetch, acts at very low concentrations (10^{-8}–10^{-11} Ũ), and is capable of inducing the organogenesis of nodules in the absence of bacteria.[184] The reduction of the double bonds in the aliphatic acid residue or the elimination of the sulfate group fully deprive NodRm-1 of its specific activity.[185] Admittedly, the tetrasaccharide from which the sulfate group has been removed is capable of inducing the deformation of the root hairs and nodulation at relatively high concentrations but only in leguminous plants other than the normal host plant (alfalfa).[184,185]

A more thorough analysis of the signalling oligosaccharide produced by *Rhizobium meliloti* demonstrated the presence of a further three minor components. One of them differed from the compounds described above solely by the structure of the higher aliphatic acid ($C_{16:3}\Delta^{2,4,9}$), containing an additional double bond in the 4-position. Two others differed from one another by the higher aliphatic acids ($C_{16:2}\Delta^{2,9}$ or $C_{16:3}\Delta^{2,4,9}$) and from the three preceding compounds by the fact that the carbohydrate basis of their molecules comprised a pentasaccharide and not a tetrasaccharide fragment of the chitin molecule.[186]

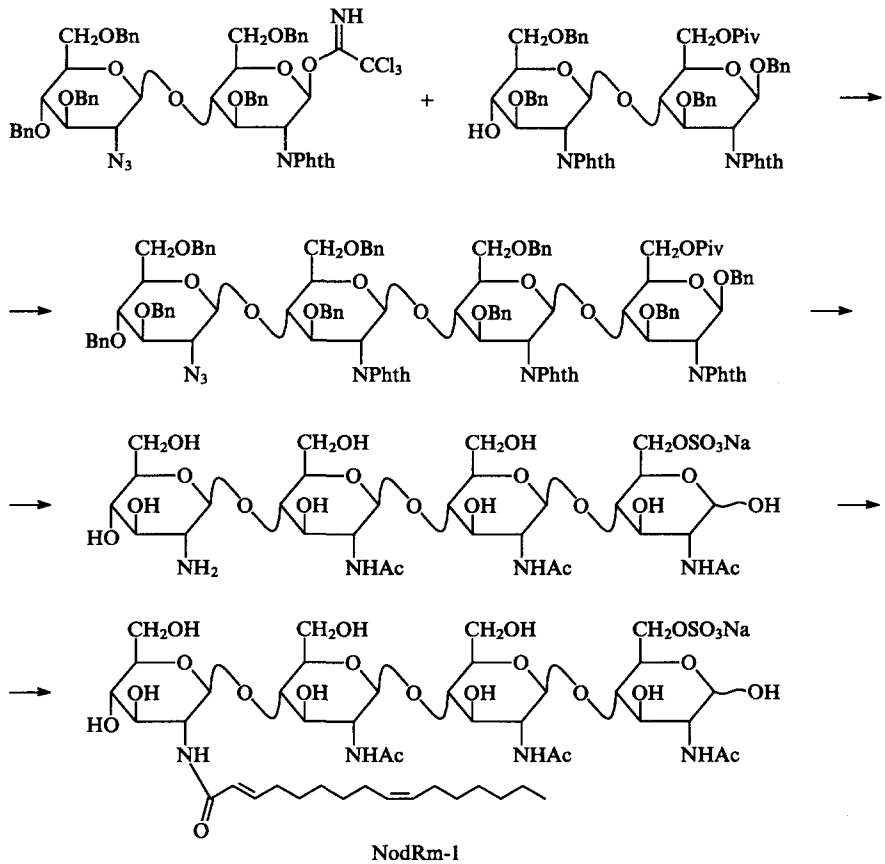
Lipooligosaccharides with a similar molecular structure have been found also in other bacterial species. Thus *R. leguminosarum var. viciae*, a symbiont of the pea plant, produces derivatives of chitotetraose and chitopentaose, lacking sulfate groups, in which the amino-group of the terminal nonreducing glucosamine residue has been acetylated by the $C_{18:4}\Delta^{2,4,6,11}$ or $C_{18:1}\Delta^{11}$ aliphatic acid, while the 6-position in this residue and all other amino-groups are acetylated.[187] The oligosaccharins of *Bradyrhizobium japonicum*, a symbiont of the soya bean plant and certain other leguminous plants, are derivatives of chitopentaose in which there are 2-*O*-methylfucose

Scheme 21



NodRm-1, R = H
Ac-NodRm-1, R = Ac.

Scheme 22



NodRm-1

derivatives in the 6-position at the reducing end instead of sulfate groups; the substances differ in the nature of the higher aliphatic acid residues ($C_{18:1}\Delta^9$, $C_{16:1}$ or $^{*}_{16:0}$) and by the presence or absence of the acetyl group in the 6-position at the nonreducing end.[188-190]

Despite the limited amount of the available information, an impression has thus been created that the symbiotic bacteria are capable of synthesising a chitotetraose or a chitopentaose as a common basis of the future signalling molecules, while the necessary specificity of the structure of oligosaccharin is achieved by the addition of definite substituents to the nonreducing and reducing terminal residues of these oligosaccharides.[191] Since the isolation of the individual signalling compounds produced by bacteria remains a complex task, there is so far no information about the mechanism of the reaction of these substances. The synthesis of these oligosaccharides is also fairly complex. Despite the relative availability of chitotetraose or chitopentaose, which can be obtained by the fragmentation of chitin or chitosan (see above), it has not so far been possible to put forward a satisfactory synthetic scheme which would permit the introduction of specific substituents into the complete chitooligosaccharide molecule. Two total syntheses of the nodulation factors have been published[192,193] (in the form of reports delivered at the XVI International Symposium on Carbohydrates without experimental details). In one of them (Scheme 22) the NodRm-1 molecule is assembled from two disaccharide derivatives substituted in different ways. The successive removal of the protecting groups and reduction of the azide group of the terminal nonreducing residue of the tetrasaccharide molecule permit the introduction of the sulfate group and the higher aliphatic acid residue in the required positions.[192]

In another synthesis (Scheme 23),[193] the molecule is assembled from derivatives of monosaccharides, the necessary **A**–**D** units being obtained from 2-azido-2-deoxy-3,4-di-*O*-benzyllaevoglucosan **75**. This substance was synthesised in only three stages from the D-glucal **74** using oxidative 1,6-iodocyclisation[194] and was converted into the monobenzyl ether **76** by a standard method.[195] Subsequent synthesis of the tetrasaccharide derivative **77** made it possible to place in the required positions not only the sulfate group and the higher aliphatic acid residue but also the acetyl group, so that the final result of the synthesis was the compound Ac-NodRm-1.

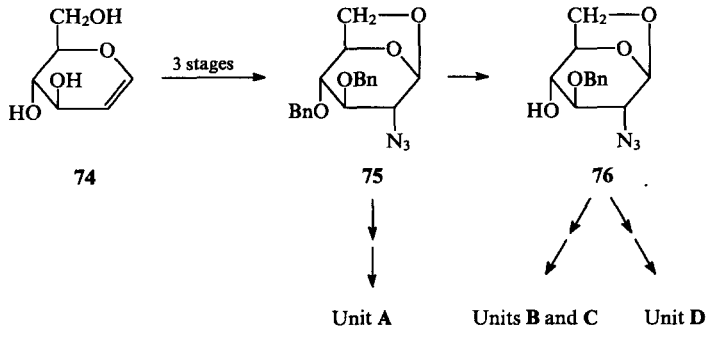
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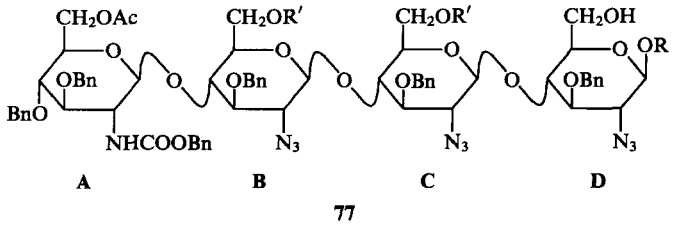
The oligosaccharins described in this section differ significantly from the substances examined in the previous sections of the review. They are not fragments of carbohydrate-containing biopolymers in the cell walls but represent the product of specific biosynthesis: the set of genes controlling this process in the bacterial cell is well known.[181,185,196] The nodulation factors are also characterised by a higher specificity, since their biological activity is manifested only in relation to a very limited number of plant species. It is extremely probable that the part of the plant receptor complex receiving the nodulation signals comprises lectins located on the surface of the cytoplasmic membrane of the cells in the root hairs and capable of the specific binding of oligosaccharins. The transfer of the pea lectin gene to the seedlings of white clover led to the transfer of susceptibility to the infection and nodulation by the *Rhizobium* strain specific to the pea plant.[197]

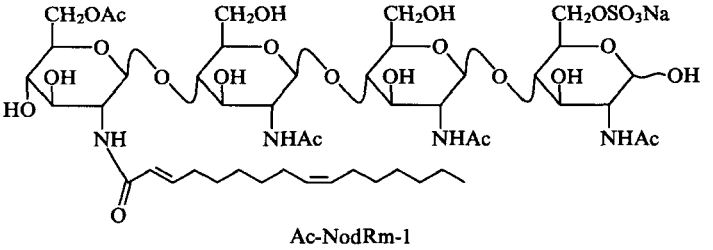
V. Conclusion

In the present review, we have examined five types of oligosaccharins which are used by plants to regulate most important physiological processes, such as growth and morphogenesis, and the relation with phytopathogens and symbiotic bacteria. Impressive results have already been

Scheme 23







achieved in this field of study, although many processes associated with the role of oligosaccharins *in vivo* and with the mechanism of their action still remain to be elucidated. The interest in oligosaccharins can be explained not only by the new possibilities of investigating with their aid the physiology of the plant cell but also with the extremely promising prospects for the creation on their basis of ecologically harmless growth regulators and plant protecting agents. However, oligosaccharins continue to remain difficult to obtain. Further progress in this field will depend in many respects on the development of new economical procedures for the preparation of oligosaccharins from natural sources and methods for the synthesis of both the oligosaccharins themselves and their analogues necessary for the elucidation of the dependence of the biological activity on the chemical structure of these compounds.

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