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Microscale structure determination of oligosaccharides by the exciton chirality method

Mayland Chang,[a] Harold V. Meyers,[a] Koji Nakanishi,[a,b] Makoto Ojika,[a] Jeong Hill Park,[b] Myung Hwan Park,[a,b] Reiji Takeda,[a,b] Jésus T.Vázquez[a] and William T. Wiesler[a]

[a]Department of Chemistry, Columbia University, New York, New York 10027, USA
[b]Suntory Institute for Bioorganic Research, Shimamoto, Mishima, Osaka, Japan

Abstract - Application of the circular dichroic exciton chirality method, a powerful chiroptical tool for the determination of absolute configuration at stereocenters in organic molecules, to oligosaccharide structure elucidation is described. The general strategy is to exploit the highly sensitive CD spectra of sugars derivatized with exciton chromophores to differentiate the hydroxyl groups that are free from those that are involved in glycosidic linkages, without the need for authentic standards. One derivatization scheme involves the phenylbenzyl chromophore, which withstands methanolysis conditions, and can be oxidized to the more sensitive phenylbenzoates (PhBn/PhBz scheme). Alternatively, the free hydroxyl groups can be tagged with *p*-bromobenzoate, followed by cleavage of the peracylated oligosaccharide with 48% HBr, trifluoroacetic acid anhydride and oxalyl bromide (HBr scheme, monochromophoric). More highly characteristic spectra can be obtained if the newly liberated hydroxyl groups—indicating the site of glycosidic linkage—are derivatized with a second chromophore, *p*-methoxycinnamate (HBr scheme, bichromophoric). In all these approaches, the resulting sugar derivatives are separated by HPLC, followed by CD analysis and comparison to published reference spectra to unambigously determine the identity of the sugars, linkage patterns and absolute configurations.

INTRODUCTION

The availability of simple microscale methods for the analyses of peptides and nucleic acids is a major factor contributing to the dramatic progress in molecular biology and related sciences. On the other hand, the structure elucidation of oligosaccharides is a more complex task because of the far greater number of isomeric structure possibilities. For instance, in a tetramer WXYZ there are only 24 possible isomers for a peptide or nucleotide, however this figure increases to 34,560 in the case of a saccharide. In addition, the fact that oligosaccharide moieties in glycoconjugates are heterogeneous and difficult to purify due to their hydrophilic properties exacerbates the problem.

The classical method for structural studies of oligosaccharides is methylation analysis in which the saccharide is permethylated, methanolyzed, reduced with sodium borodeuteride to the corresponding alditols and acetylated prior to GC/MS analysis. Comparison of retention times and fragmentation patterns with authentic specimens determines the identity of the sugars and glycosidic linkage positions. Development of modern analytical instrumentation, including HPLC and MS, has rendered this approach an extremely powerful and general method (ref. 1). However, a library of authentic standards is needed—a requirement not easy to fulfill in view of the variety of saccharides present in different sources, including saponins and antibiotics. In addition, methylation analysis does not afford information regarding the absolute configuration of the sugars.

Over the past several years we have focused our attention on the development of a complementary method for the determination of glycosidic linkages of sugar units in oligosaccharides based on the circular dichroic exciton chirality method (ref. 2), which in turn finds precedent in the coupled oscillator theory (ref. 3). The current status of this method is summarized below.

EXCITON CHIRALITY METHOD

When two or more chromophores which are chirally disposed are positioned nearby in space—intra- or intermolecularly—the various electric transition moments will interact and give rise to coupled circular dichroic curves ("split CD curves"). For the commonly used *p*-substituted benzoate chromophore (Fig. 1) there exist two major transitions, the longitudinal La and transversal L_b bands. The strongest and most important interaction between two benzoates results from coupling of the L_a bands, while that involving the L_b transitions is much weaker because of its substantially lower UV ε value and, consequently, plays a minor role. However, these transitions become increasingly relevant in defining the shape of an entire CD curve in the interaction between two different chromophores (*vide infra*).

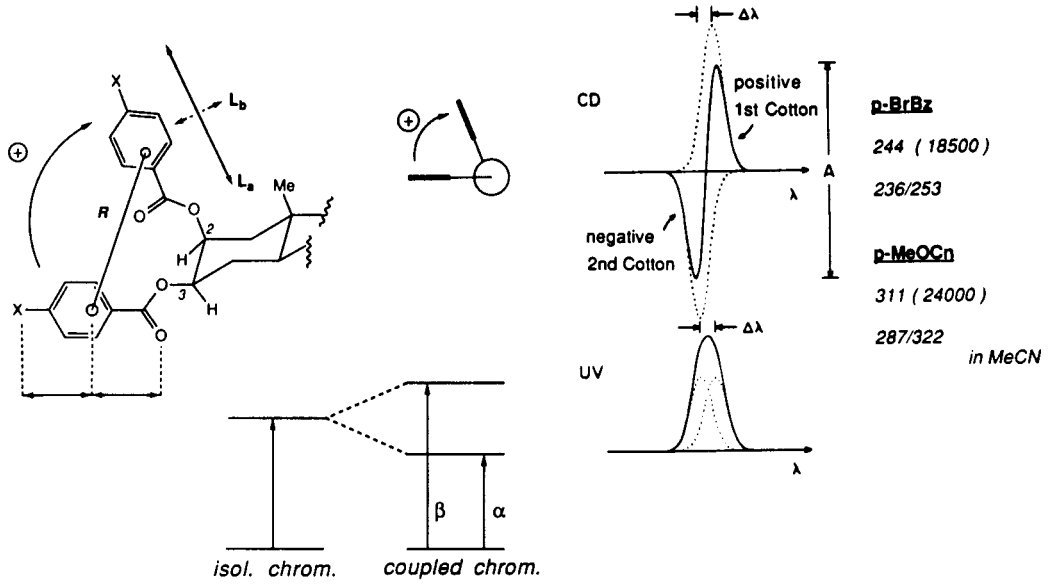


Fig. 1. Coupling of benzoate L_a transitions results in a bisignate or "split" CD curve.

For a molecule containing two benzoate groups—which could be as far apart as 13 Å, such as a 3,15-dibenzoate on a steroid skeleton (ref. 1)—the two L_a bands couple to give two excited states, leading to red-shifted α and blue-shifted β transitions of opposite signs. In electronic spectroscopy, these absorptions have only one sign—usually depicted as positive bands—and hence the red- and blue-shifted transitions give simply a summation curve twice as intense as the integrated intensity of a single chromophore. In contrast, in circular dichroic spectroscopy, if the absolute chirality or sense of twist of the two transition moments is *clockwise*—as depicted in Fig. 1—then, according to the nonempirical coupled oscillator theory, this interaction leads to a split CD curve in which the Cotton effect at the longer wavelength (first CE) is without exception *positive* , while the second CE is negative. The distance between the maximum (peal) and minimum (trough) of the split CD curve is defined as the *amplitude* and is expressed in Δε units. Since the directions of the L_a transitions and the C-O bonds (C_2-O and C_3-O in Fig. 1) are approximately parallel to each other, the sign of the split CD curve reflects the absolute stereochemistry of the diol group. This approach constitutes the dibenzoate chirality method (ref. 4). The *p*-bromobenzoate chromophore (λ_{max} 244 nm, ε 18,500 in MeCN) gives rise to split CD spectra with extrema at 236 nm and 253 nm and amplitudes in the order of Δε = 62 in α-dibenzoate pyranoses (see Fig. 2); the signs of the bisignate curves reflect the chirality. Similarly, the *p*-methoxycinnamate group (λ_{max} 311 nm, ε 24,000 in MeCN) affords split CD curves at 287 nm and 322 nm, Δε ca. 98. These very large amplitudes necessitate sample levels of only a few micrograms, thus rendering this chiroptical method a very sensitive physical tool for the structure elucidation of oligosaccharides.

Different features of the exciton chirality method in the analysis of oligosaccharide structure are discussed below (ref. 1):

1. The sign of the split CD curve is determined by the relative chirality of the chromophore transition moments.
2. Any chromophore with a large ε and known direction of the electric transition moment can be used.
3. A linear relation exists between the UV ε and CD amplitude; namely, *the stronger the absorption, the larger the amplitude* and the less sample required.
4. In a vicinal dibenzoate, the amplitude attains a maximum value when the dihedral angle between the two chromophores is ca. 70°; no split CD is observed when the angle is 0° or 180° (i.e., when there is no chirality).
5. The extent of the coupling, or amplitude of the bisignate CD curves, is *inversely proportional to the square of the interchromophoric distance R.*
6. Two *p*-bromobenzoates are coupled even when the chromophores are 13 Å apart with a dihedral angle of 60°.
7. Coupling is still observed between two chromophores whose absorption maxima are separated by 100 nm .
8. The amplitude of the split CD curve for a compound containing two or more identical chromophores can be approximated by the summation of each interacting basis pair (Fig. 2, "additivity" relation) (ref. 5). For example, in the 2,3,4-trisacylate shown in Fig. 3, the total amplitude is equal to the summation of the amplitudes of the 2,3-, 2,4- and 3,4-triacylates. As indicated in Fig. 4, there is an excellent agreement between the observed and calculated amplitudes for the various sugars—including amino, deoxy and N-acetylated sugars (ref. 6)—that are commonly found in nature.

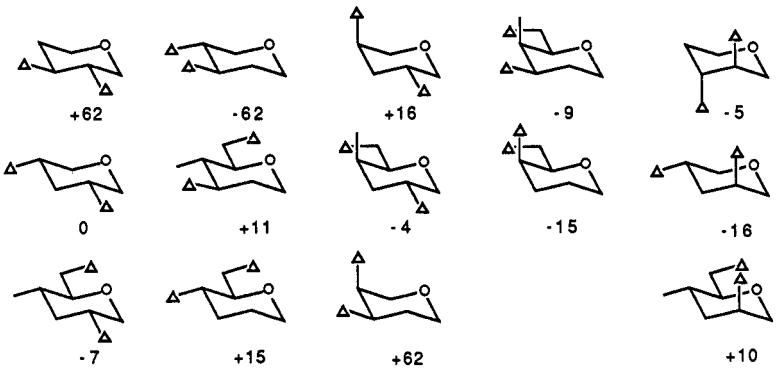
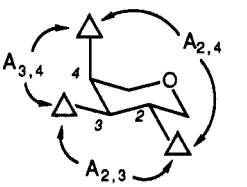


Fig. 2. Standard amplitudes of all possible bis-*p*-bromobenzoate interactions in pyranose sugars.



Total A ≅ $A_{2,3} + A_{2,4} + A_{3,4}$

calcd A = (+62) + (+16) + (+62) = 140

Fig. 3. Additivity in amplitudes of pyranose benzoates, see L-ara, L-rha and L-fuc (Fig. 4) for observed values.

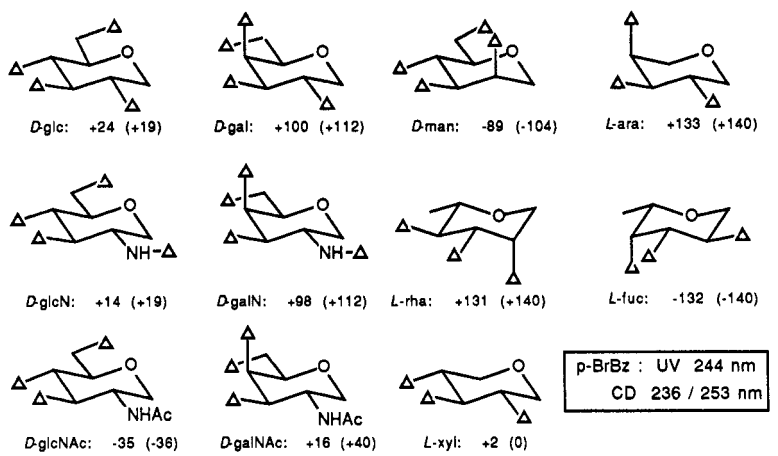


Fig. 4. Observed and calculated (in parentheses) A values of tri- and tetra-*p*-bromobenzoates of the most commonly occurring pyranoside sugars (ref. 6).

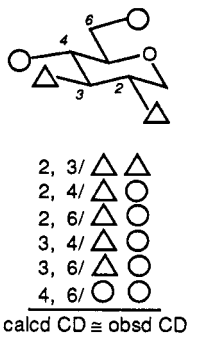


Fig.5. Additivity in CD spectra of pyranoside containing two different chromophores.

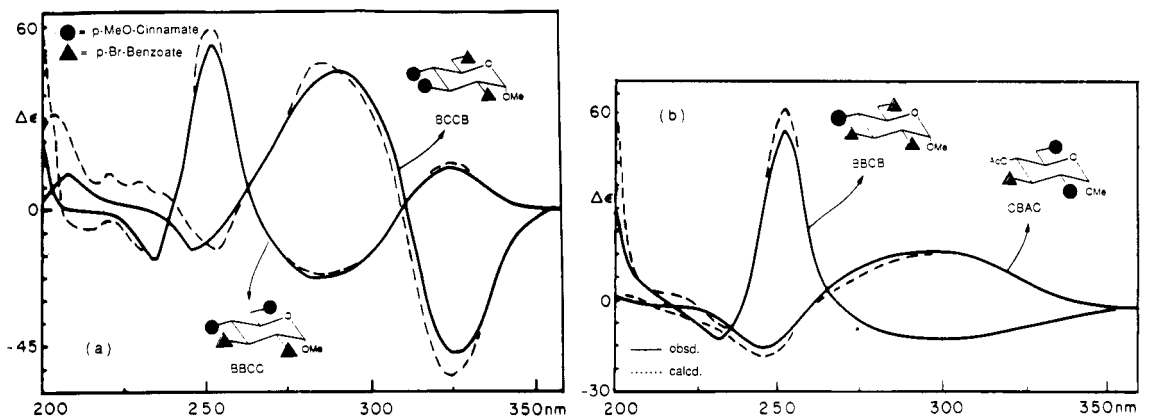


Fig. 6. Comparison of observed to calculated spectra in bichromophoric glucoside derivatives.

9. The additivity relation applies—not only for the monochromatic amplitude—but for the entire CD curve as well, when two different chromophores are present. As an example, the calculated CD spectrum for glucose BBCC represents the summation of the CD curves of two "homo" (2, 3-B/B and 4, 6-C/C) and four "hetero" B/C (2, 4-; 2, 6-; 3,4- and 3,6-) interactions (Fig. 5) (ref. 7, 8). Sugar derivatives are given a four letter code representing substitutions at positions 2, 3, 4 and 6 by A = acetate, B = *p*-bromobenzoate and C = *p*-methoxycinnamate. As shown in Fig. 6, an excellent agreement is found between the sum of the six basis set curves (calculated) and the observed curve. Similarly, the same principle applies to the other three glucose examples in Fig. 6.

For glucose pyranoside, there exist twelve possible homo interactions, six bisbenzoates (B/B) and six biscinnamates (C/C), as well as 12 permutations of hetero interactions (B/C) (Fig. 7). Thus, there is a total of *24 basis set curves* for each glucose (ref. 7), galactose (ref. 8) and mannose pyranoses (ref. 9). The additivity relation indicates that the CD spectrum of a tetra- or a triacylate can be simulated by the summation curve of the component basis set spectra. This has been demonstrated by preparing all 14 possible hetero tetra- and several triacylates of glucose (ref. 7) and galactose (ref. 8), together with a few of the tetra- and triacylates in the mannose series (ref. 9). Without exception, we found that the additivity relation holds in every sample examined. Furthermore, each curve is diagnostic and can be regarded as a characteristic fingerprint spectrum (Fig. 8, unpublished) consisting of extrema around 245/305 nm, accompanied by weak but characteristic shoulders around 285 and 310 nm, the latter are due to coupling involving the L_b transitions bands. *The 72 basis set data are stored in a computer and the desired summation curves for the tetra- and triacylates are generated by simple manipulation of the stored data. On the other hand, a library of the 114 tetra- and triacylated [3 sugars x (14 tetra- + 24 tri-)] CD spectra for the commonly occurring hexoses should serve as a useful reference that will identify the sugars, the substitution patterns of the benzoates and cinnamates—which in turn establishes the positions of the free and glycosylated hydroxyl groups in an oligosaccharide—as well as the absolute configurations.* These observed and calculated curves will also apply to the deoxy-, N-acetyl- and aminopyranoses, since the NAc groups do not interact with the benzoates or cinnamates, and the CD spectra of NBz and NCin groups can be approximated from those of the OBz and OCin groups.

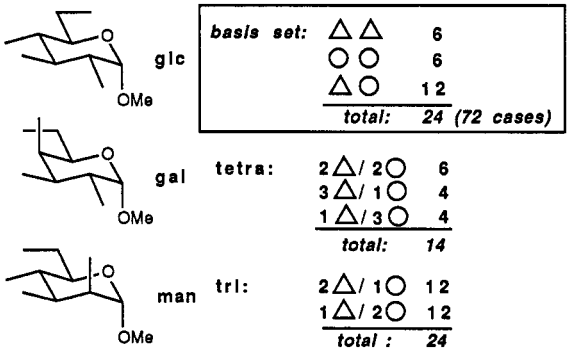


Fig 7. Homo and hetero interactions (basis set) of glucose, galactose and mannose from which the tetra- and triacylated CD curves are obtained.

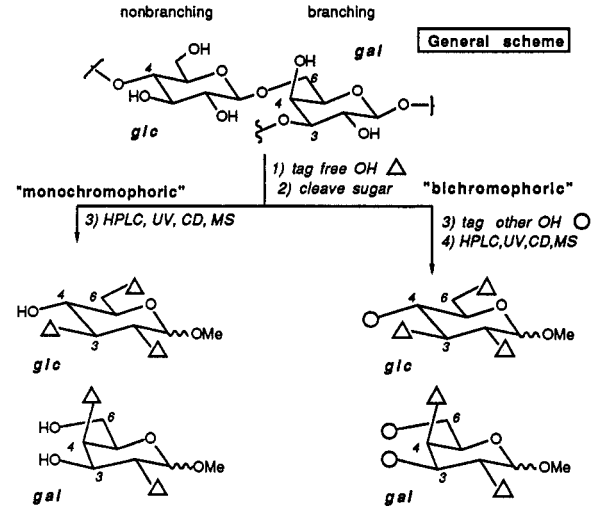


Fig. 9. General scheme for application of chiroptical method.

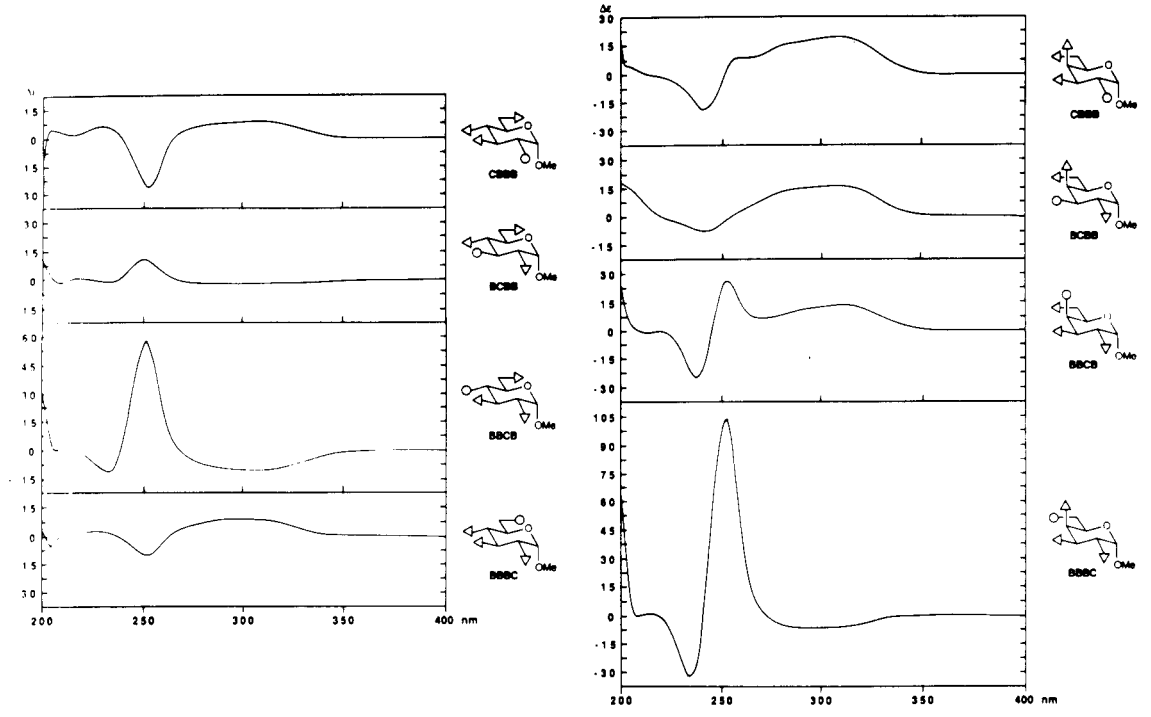


Fig. 8. Circular dichroic spectra of tribenzoate/monocinnamate sugar derivatives.

GENERAL SCHEME FOR APPLICATION OF CHIROPTICAL METHOD

The general strategy in oligosaccharide structure elucidation is to differentiate the hydroxyl groups that are free from those that are involved in glycosidic linkages (Fig. 9). This can be accomplished by tagging the free OH's with one chromophore, followed by methanolysis of the oligosaccharide into its monosaccharide components and subsequent separation by HPLC and analysis by CD (monochromophoric scheme). The resulting CD curves are then compared with values derived by adding the amplitudes of the possible basis set units in that sugar. However, until our recent finding (*vide infra*), protecting groups were required in the initial step because glycosidic bonds in peracylated oligosaccharides are resistant to hydrolysis/methanolysis and, even if such hydrolysis could be effected, these moieties would be lost or undergo acyl migration under these cleavage conditions.

THE PHENYLBENZYL/PHENYLBENZOATE SCHEME

Search for a chromophore that would be stable to acid hydrolysis, as well as show valid additivity relationships, led to the *p*-phenylbenzyl ether group (λ_{max} 253 nm, ε 20,300 in MeCN) (ref. 10). All 18 bis-*p*-phenylbenzylates of glucose, galactose and mannose were prepared and the amplitudes of the split CD curves were measured (ref. 10). Our studies revealed that *the additivity relation holds for all the possible tris- and tetra-phenylbenzylates*, despite the presence of a methylene group. Thus, the preferred conformation of the -O-CH_2- group in the phenylbenzyl groups is similar to that of the -O-CO- moiety of the bromobenzoates. Therefore, the PhBn scheme to determine the sites of glysosidic linkages (Fig. 10) was developed. This approach can be a practical method for oligosaccharide studies, however the amplitudes of the split CD spectra of phenylbenzylates are weak compared to those of the corresponding benzoates because of the longer interchromophoric distances R in the former, as a result of the intervening methylene moiety. A 5-9 fold increase in the amplitudes and retention of all additivity relations (ref. 10, data mostly unpublished) was achieved by acetylation of the hydroxyl groups at the glycosidic linkage positions, followed by direct oxidation with $RuCl_3/NaIO_4$ of Ph-C_6H_4-CH_2-O- to Ph-C_6H_4-CO-O- in 60% yield (PhBz scheme, Fig. 10). All 18 possible PhBz basis sets of Glc, Gal and Man were prepared to cover all the pyranose series; the amplitudes of the split CD curves centered at 264 nm (unpublished). This approach has yet to be applied to the structure elucidation of unknown oligosaccharide samples due to the development of a simpler scheme (*vide infra*) that eliminates the very difficult perbenzylation step, which must be carried out in strict anhydrous conditions and whose yield is much lower than that for peracylation. Nevertheless, the extensive investigations carried out with the phenylbenzyl series led to two useful findings which are described below.

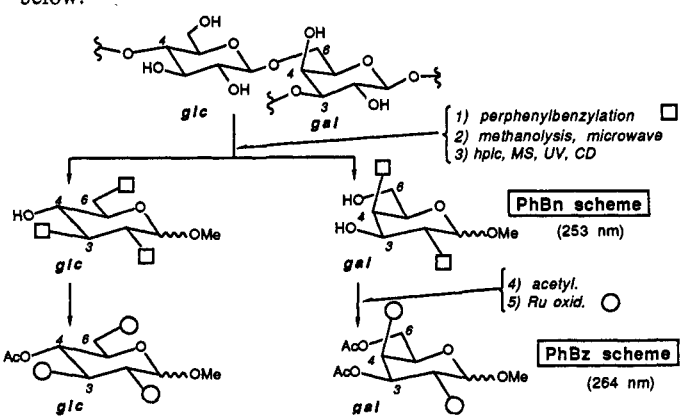


Fig. 10.
PhBn/PhBz schemes for oligosaccharide structure determination.

Methanolysis in microwave oven leads to anomeric inversion

Methanolysis of oligosaccharides typically involves overnight reflux in 1N HCl/MeOH. Cleavage of the glycosidic linkages in some of the perphenylbenzylates, however, requires more vigorous conditions, presumably due to hydrophobic shielding of the bulky phenylbenzyl groups. Fortunately, we found that phenylbenzylates, as well as permethylated saccharides, are cleaved within 15 minutes when this reaction is carried out in an ordinary microwave oven (ref. 11). Typically, the saccharide is dissolved in 5% HCl-MeOH/dioxane (3:1) and placed in a custom-made, airtight teflon vessel with a screwcap; the course of the reaction can be followed every few minutes as desired (i.e., carrying out partial hydrolysis). Although the use of a microwave oven in organic synthesis finds precedent in the literature (ref. 12), this method has not been used extensively; the possibility of explosion precludes its widespread use. However, in the microgram scale used in the present studies this need not be considered. One very important feature of the methanolysis reaction in the microwave oven is that *the major product is the anomer having the inverted configuration.* As depicted in Fig. 11, both α, α- and β, β-diglucosides afford the β- and α-isomers, respectively, as the major anomers. All examples examined so far follow this general rule. The paucity of methodology for the determinination of anomeric configurations—except for ^1H NMR which is not applicable to microgram quantities of oligosaccharides—could make this approach very attractive. In this method, methanolysis of underivatized oligosaccharides in the microwave oven, followed by per-*p*-bromobenzoylation (ref. 6) and HPLC of the product mixture leads to a *nanogram scale identification of the sugar moieties and anomeric configurations* (ref. 11). The HPLC analysis can be simplified by addition of a third step involving anomerization of the β-methyl-per-*p*-bromobenzoylated glycosides to the corresponding α-sugars with anhydrous $FeCl_3$ in CH_2Cl_2 (ref. 13).

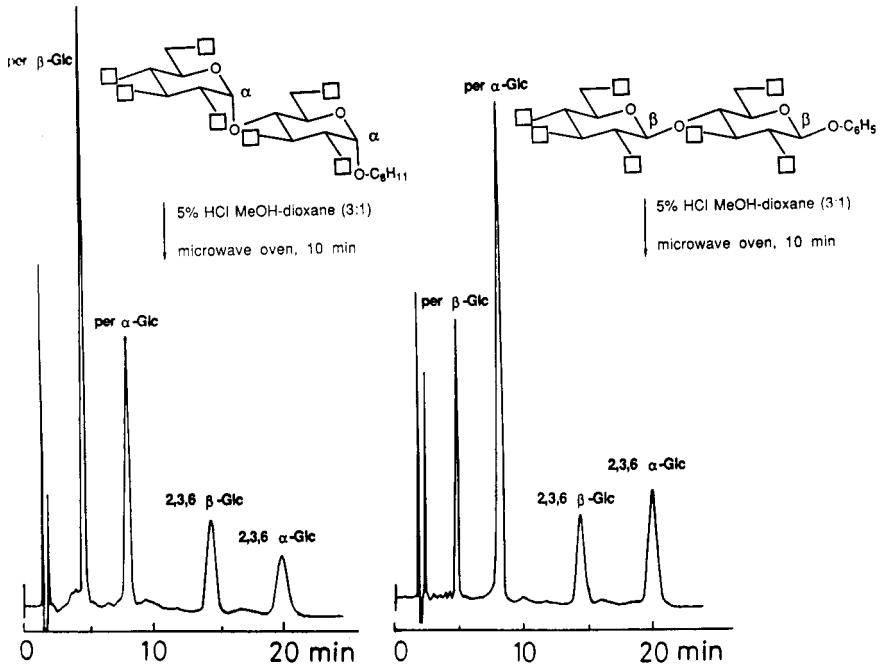


Fig. 11. Anomeric inversion of glucoside perphenylbenzylate during methanolysis in the microwave oven.

Determination of configurations of hydroxyl groups by phenylbenzylation

The absolute configuration of naturally occurring compounds containing two or more hydroxyl moieties can be easily ascertained from the phenylbenzylated derivatives due to the fact that benzylates and benzoates can be treated similarly in exciton chirality problems. This strategy is particularly useful in the case of hydroxyl groups that are resistant to acylation (e.g., tertiary or hindered hydroxyl moieties) (ref. 14).

A COMBINED MONOCHROMOPHORIC AND BICHROMOPHORIC/HBr SCHEME

The entire oligosaccharide structure determination scheme can be greatly simplified if conditions were found in which the glycosidic linkages could be cleaved in the presence of benzoate groups. This can be accomplished—in all cases examined to date—by heating the *p*-bromobenzoylated oligosaccharide in a capped vial for 30 min (step 2, Fig. 12) with 48% HBr, trifluoroacetic anhydride and oxalyl bromide (ref. 15). Apparently, oxalyl bromide reacts with water in 48% HBr to generate HBr, CO_2 and CO, while the trifluoroacetyl group serves as a labile protecting group for the newly liberated hydroxyl groups that prevents migration of existing benzoates. The CO present may also play a simiral role by generating an unstable formate ester under these conditions. Because the side products generated in this reaction are volatile, evaporation *in vacuo* leaves only the acylated 1-α-bromo sugars, which are then converted with Ag_2O/MeOH to the β-methyl glycosides in high yields.

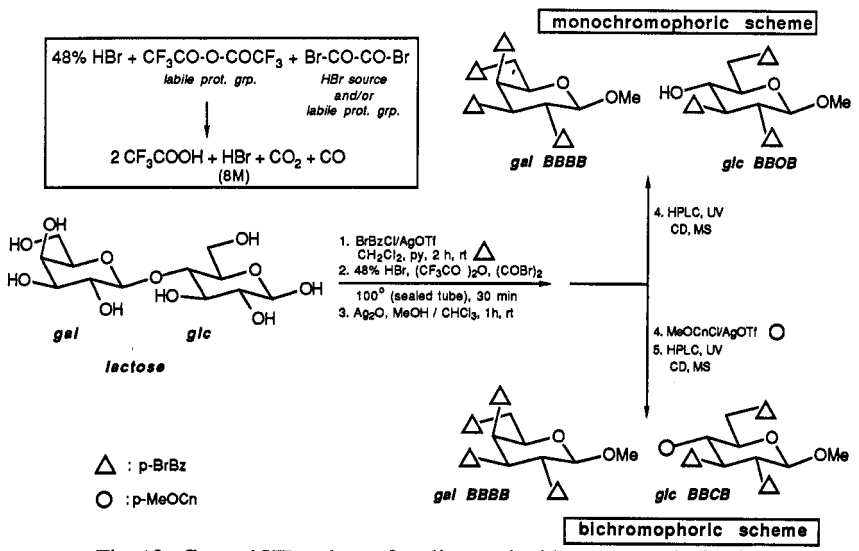


Fig. 12. General HBr scheme for oligosaccharide structure elucidation.

Monochromophoric scheme

After HPLC separation of the mixture of methyl glycosides, each peak is analyzed by MS and UV to determine the identity of the sugar (hexose, N-Ac sugar, deoxy sugar, etc.) and concentration of the sample (from the known UV ε values), respectively. In turn, the CD spectra are normalized and the amplitude of the benzoate band determines the spatial disposition of the benzoate groups (i.e., linkage positions), identity of the sugars and absolute configurations.

Bichromophoric scheme

More characteristic spectra can be obtained if after methyl glycoside formation (step 3, Fig. 12), the mixture is cinnamoylated and then separated by HPLC. UV measurements of each HPLC peak determine the ratio of benzoate (λ_{max} 244 nm) to cinnamate groups (λ_{max} 310 nm) and the concentration of the sample. Subsequent comparison of the resulting CD curves with the 114 reference curves—together with the M^+ peaks—identifies the sugar (Glc, Gal, Man, deoxy, NAc, NH_2-sugar, etc.), the location of the benzoate (free OH's in the original saccharide) and cinnamate groups (glycosidic linkages), as well as absolute configuration.

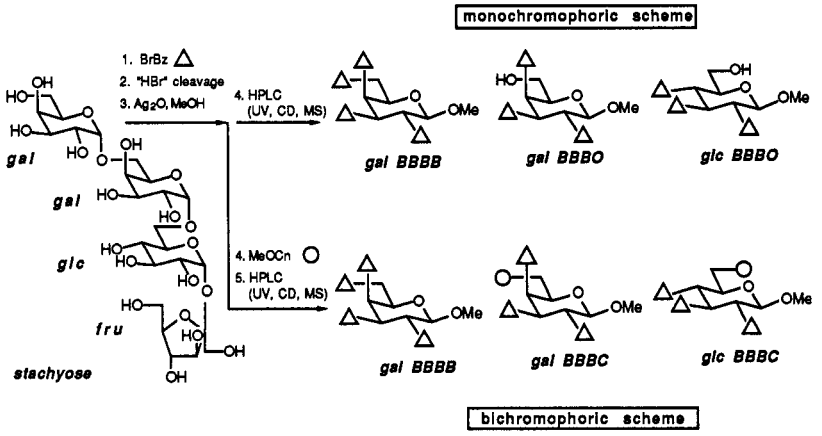


Fig. 13. Application of HBr scheme to stachyose.

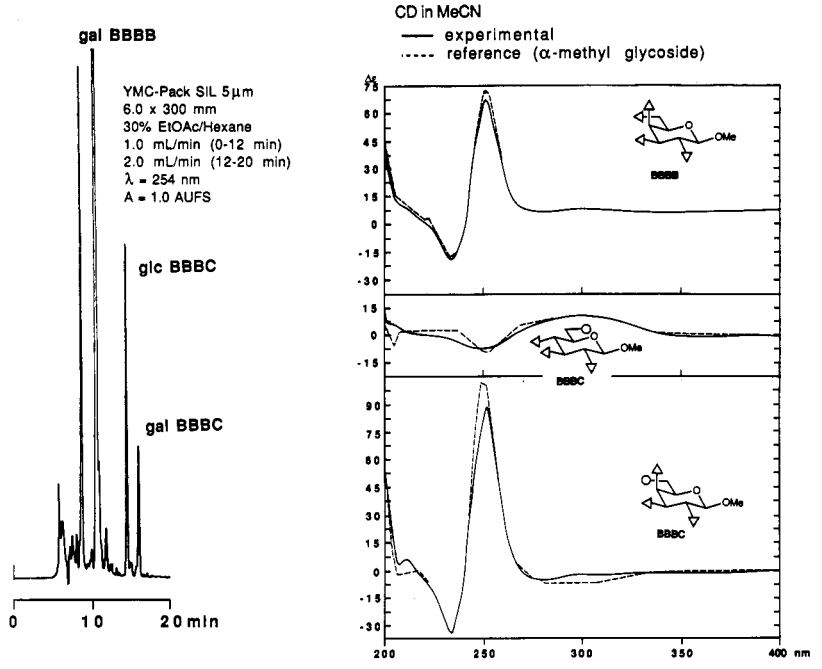


Fig. 14. HPLC separation and CD spectra of HBr scheme applied to stachyose.

STACHYOSE

The schemes indicated in Fig. 12 have been applied to a few oligosaccharides. An example carried out with ~200 μg of stachyose is illustrated in Fig. 13 and 14. Comparison of the CD curves of the isolated HPLC peaks (Fig. 14) to published reference spectra (Fig. 8) unambigously determines that the sugars are galactose BBBB, glucose BBBC and galactose BBBC. At present, we ignore treatment of furanoses (i.e., fructose, ribose, etc.) because the conformations of the five-membered rings are not rigid; that is the chirality of ribose 2,3-bisbenzoate is reversed in the α- and β-methyl ribosides. Therefore, the current method would be applicable to furanoses on an empirical basis only, however the various derivatives have not yet been prepared.

More complex oligosaccharides—rather than simple known samples—are currently under study. However, the basic concepts and procedures have been demonstrated, and all reference amplitudes and curves necessary for both the mono- and bichromophoric schemes are now available. These studies would allow for the complete structure determination of oligosaccharides, save for sequencing of sugar residues, which has to be clarified by modern FAB-MS techniques (ref. 16).

Acknowledgements

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