Carbohydrate Research, 197 (1990) 15–32
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

OLIGOSACCHARIDE MICROANALYSIS BY C.D. SPECTROSCOPY. REFERENCE CURVES FOR D-MANNOSE DERIVATIVES*

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(Received February 12th, 1989; accepted for publication, May 8th, 1989)

ABSTRACT

This study completes the spectroscopic basis for a novel oligosaccharide microanalytical method, wherein a derivatization sequence provides monosaccharide subunits bearing two types of exciton-coupling chromophore groups ("bichromophoric") for circular dichroic spectroscopy, namely 4-bromobenzoate (λ_{max} 245 nm) and 4-methoxycinnamate (λ_{max} 311 nm). The 24 possible derivatives of methyl α-D-mannopyranoside bearing two chromophore and two acetate groups have been prepared and their c.d. spectra recorded. These spectra have been used to calculate the c.d. curves of the 14 possible tetrachromophoric derivatives according to the pairwise additivity rule. The accuracy of these calculations has been demonstrated for six representative derivatives. The c.d. curves obtained here, together with curves of the corresponding D-glucose and D-galactose derivatives, comprise a set of 42 unique and distinctive spectra which can be utilized to identify sugars and their linkage patterns, and thus represent the basis for an alternative to methylation analysis which does not rely upon synthetic standards.

INTRODUCTION

Recent years have seen a rapid growth in the understanding of the important and varied roles of oligosaccharides from glycoproteins and glycolipids[1]. Because these oligosaccharides are encountered more and more frequently by researchers in many areas of biology and biochemistry, an oligosaccharide microanalytical method accessible to non-specialists would be highly desirable.

Determination of linkage structure in oligosaccharides has long relied upon methylation analyis by g.l.c.–m.s. of partially methylated alditol acetates[2]. Linkage analysis generally follows a sugar component analysis, and an additional analysis for determination of monosaccharide absolute configuration may be performed.

Considerable advances in capillary g.l.c. have reduced the quantities required

*This work was supported by a grant (GM-34 509) from the Institute of General Medicine, National Institutes of Health.
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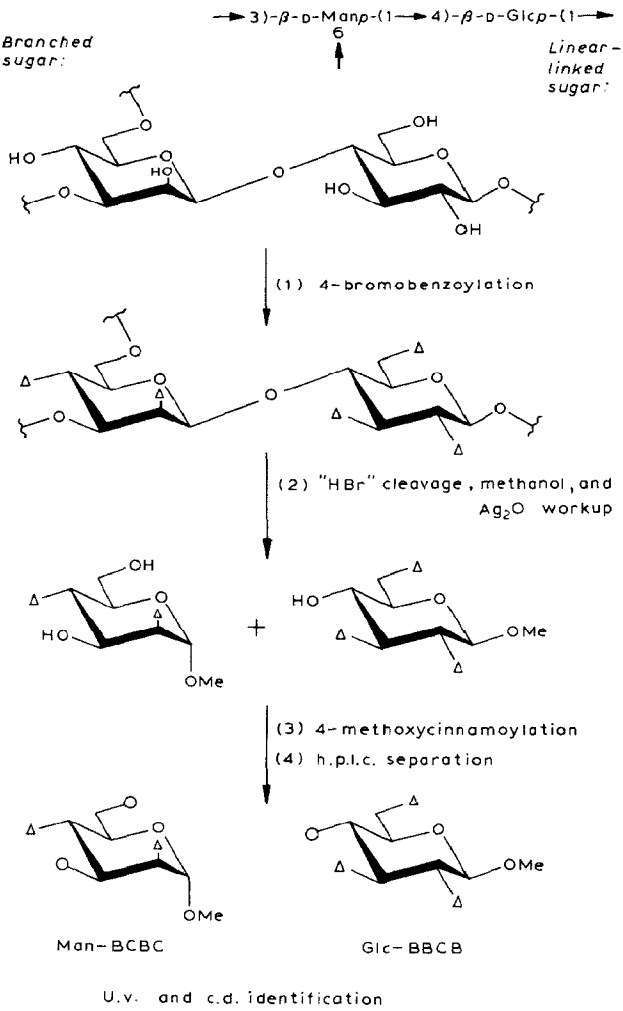
0008-6215/90/$ 03.50

for methylation analysis to the subnanomolar range[3]. Great improvements have been made in the areas of m.s. and chemical derivatization techniques as well, and yet the basic strategy has remained unchanged. Although m.s. can distinguish between positional isomers of partially methylated alditol acetates and indicate their purity, enantiomeric and diastereomeric derivatives are not differentiated. Hence, much of the analysis relies on chromatographic separation and comparison of g.l.c. retention times with a large bank of synthetic standards. This requirement



Scheme 1. "Bichromophoric" oligosaccharide derivatization procedure for a hypothetical disaccharide subunit. After perbenzoylation (step 1) of the oligosaccharide, "HBr" cleavage followed by treatment with methanol and Ag_2O affords methyl glycosides that contain free hydroxyl groups at the linkage positions. Subsequent cinnamoylation (step 3) affords bichromophoric derivatives, separable by h.p.l.c. (Step 4), for u.v. and c.d. analysis. Derivatization of the four hydroxyl groups is indicated in the order 2,3,4,6 by: (B) 4-bromobenzoate (△), and (C) 4-methoxycinnamate (○).

for dozens of standards renders methylation analysis inaccessible to the non-specialist.

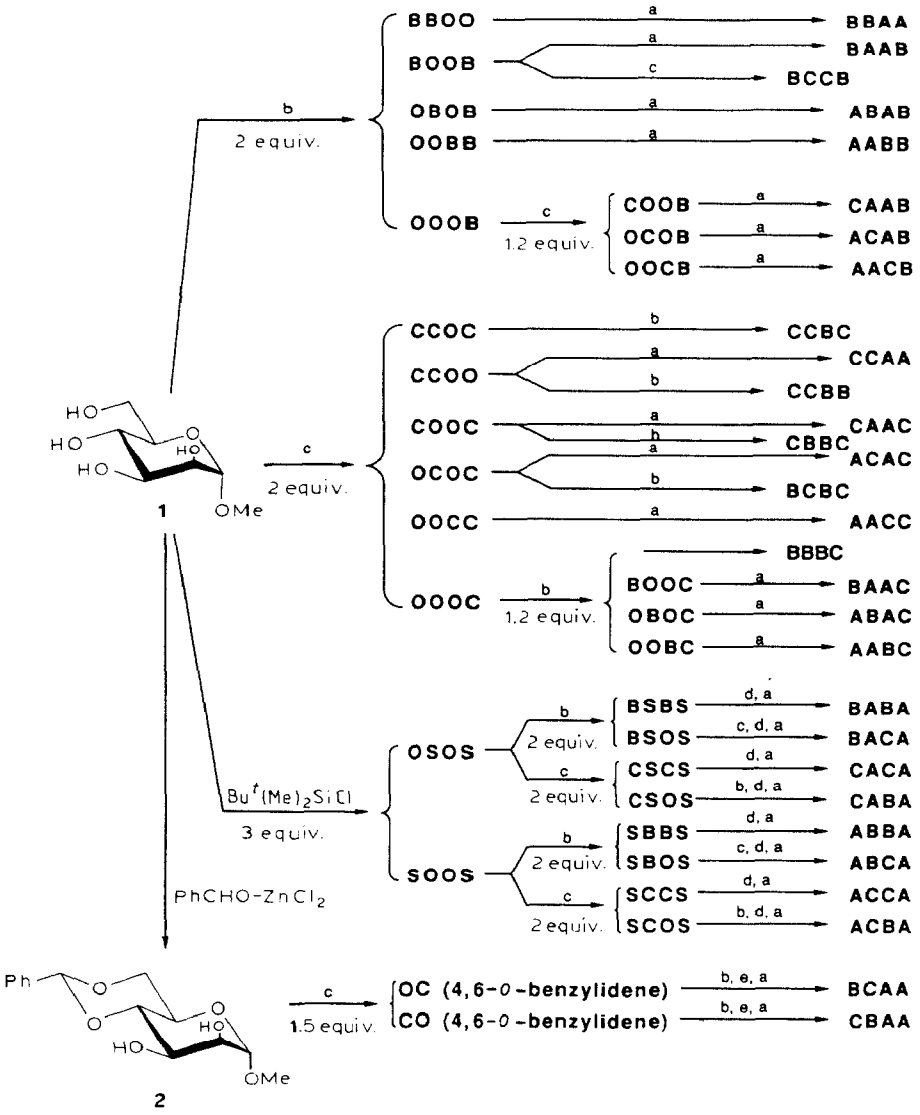
A novel alternative to methylation analysis for carbohydrate-linkage point determination is based upon circular dichroism, in particular exciton coupling[4], a spectroscopic method accessible to both biochemists and natural product chemists alike. In this approach (Scheme 1), permethylation is replaced with perbromobenzoylation, thus tagging the free hydroxyl groups with the strongly absorbing exciton chromophore, 4-bromobenzoate (λ_{max} 245 nm) group. Upon cleavage of an oligosaccharide into its monosaccharide components, methoxycinnamoylation of the mixture tags the liberated hydroxyl groups that were involved in glycosidic linkages with a second, red-shifted exciton-chromophore, 4-methoxycinnamate (λ_{max} 311 nm) group. Separation by h.p.l.c. affords the monosaccharide components which are "bichromophoric", *i.e.*, derivatized with two types of exciton-chromophore groups. The ratio of the two chromophore groups is readily determined by u.v. spectrometry, and the strong exciton coupling between the chromophore groups makes these derivatives ideally suited for analysis by circular dichroism at nanomole levels.

We recently prepared all 14 possible bichromophoric derivatives of both methyl α-D-glucopyranoside[5] and methyl α-D-galactopyranoside[6], and it was found that the c.d. curves were unique and characteristic for each derivative, thus indicating the sugar-linkage pattern and absolute configuration. Furthermore, the c.d. spectra of all 28 derivatives examined could be accurately calculated by summation of the six "basis" spectra representing the six pairwise, interchromophoric interactions found for each derivative, thus demonstrating the general validity of pairwise additivity in these systems.

In the present study, we utilize this principle of pairwise additivity to empirically calculate c.d. curves for all possible bichromophoric derivatives of methyl α-D-mannopyranoside. The accuracy of these calculations was confirmed for six representative tetrachromophoric derivatives which were prepared synthetically to provide experimental c.d. curves for comparison. The c.d. curves reported herein, together with curves of the corresponding D-glucose[5] and D-galactose[6] derivatives, comprise a set of 42 unique and distinctive spectra which serve as a reference library to identify unknown derivatives, and thus represent the spectroscopic basis for an oligosaccharide microanalytical method which does not rely upon synthetic standards.

RESULTS

Analogous to previous studies[5,6], all 24 permutational isomers of methyl α-D-mannopyranoside containing two exciton-chromophore and two acetate groups were prepared (Scheme 2). The normalized c.d. spectra of these 24 compounds represent a "basis set" for pairwise interactions in bichromophoric D-mannopyranoside derivatives possessing three or four chromophore groups. This set



Scheme 2. Preparation of dichromophoric diacetates (basis set) of methyl α-D-mannopyranoside (1), as well as several tri- and tetra-chromophoric compounds by way of a sequential acylation either with or without protection–deprotection steps. Derivatization of the four hydroxyl groups is indicated in the order 2,3,4,6 by: (A) acetate, (B) 4-bromobenzoate, (C) 4-methoxycinnamate, (O) free hydroxyl group, and (S) *tert*-butyldimethylsilyl group. Steps: (a) Excess acetic anhydride–pyridine–4-dimethylaminopyridine; (b) excess 4-bromobenzoyl chloride or 4-bromobenzoic triflic anhydride (unless indicated otherwise)–pyridine–4-dimethylaminopyridine; (c) excess 4-methoxycinnamoyl chloride or 4-methoxycinnamic triflic anhydride (unless indicated otherwise)–pyridine–4-dimethylaminopyridine; (d) HF in aqueous acetonitrile; and (e) 4-toluenesulfonic acid in methanol.

TABLE I

C.D. DATA FOR HOMOINTERACTIONS. THE SIX DIACETATE DIBENZOATE ESTERS AND THE SIX DIACETATE DICINNAMATE ESTERS OF THE BASIS SET

Compound[a]	λ^b ($\Delta\varepsilon$)	λ ($\Delta\varepsilon$)	$\Delta\varepsilon = 0^c$	*Amplitude*[d]
BBAA	233 (+8)	252 (−35)	239	−43
BABA	233 (+2)	251 (−21)	238	−23
BAAB	224 (+1)	250 (−8)	236	−9
ABBA	235 (+21)	252 (−51)	243	−72
ABAB	233 (−1)	249 (+12)	238	+13
AABB	234 8−3)	250 (+18)	239	+21
CCAA	287 (+15)	322 (−31)	304	−46
CACA	288 (+4)	321 (−6)	307	−10
CAAC	296 (−3)		238	−3[e]
ACCA	288 (+39)	323 (−69)	306	−108
ACAC	286 (−5)	321 (+11)	299	+16
AACC	281 (−8)	322 (+14)	302	+22

[a]Derivatization of the four positions is represented in the order 2,3,4,6 by: (A) acetate, (B) 4-bromobenzoate, and (C) 4-methoxycinnamate. [b]Extrema in nm. [c]Point where $\Delta\varepsilon$ changes sign, in nm. [d]Amplitude difference at extrema. [e]No split Cotton effect observed.

includes spectra of six diacetate dibenzoate and six diacetate dicinnamate compounds, which represent the degenerate or "homo" pairwise interactions (Table I). Also included in this basis set are the spectra of 12 diacetatemonobenzoatemonocinnamate compounds, representing nondegenerate or "hetero" pairwise interactions (Table II). The acetate groups, which make a negligible contribution to the c.d. curves, were used in the present study to insure that ring conformations of the

TABLE II

C.D. DATA FOR HETEROINTERACTIONS. THE TWELVE DIACETATE MONOBENZOATE MONOCINNAMATE ESTERS OF THE BASIS SET

Compound[a]	λ^b ($\Delta\varepsilon$)	λ ($\Delta\varepsilon$)	λ ($\Delta\varepsilon$)	$\Delta\varepsilon = 0^c$
BCAA	238 (−5)	253 (−1)	286 (−8)	
BACA	241 (−4)	256 (−1)	309 (−4)	226
BAAC	245 (−5)		306 (+1)	268
ABCA	246 (+12)		307 (−12)	259
ABAC	244 (−5)		304 (+3)	265
AABC	248 (−2)		296 (+5)	256
CBAA	245 (+9)		311 (−11)	259
CABA	247 (+4)		311 (−10)	261
CAAB	251 (−1)		311 (−3)	238
ACBA	248 (+13)		308 (−14)	260
ACAB		251 (−3)	308 (+2)	234, 264
AACB	233 (+2)	250 (−2)	306 (+4)	242, 256

[a]Derivatization of the four positions is represented in the order 2,3,4,6 by: (A) acetate, (B) 4-bromobenzoate, and (C) 4-metoxycinnamate. [b]Extrema in nm. [c]Point where $\Delta\varepsilon$ changes sign, in nm.

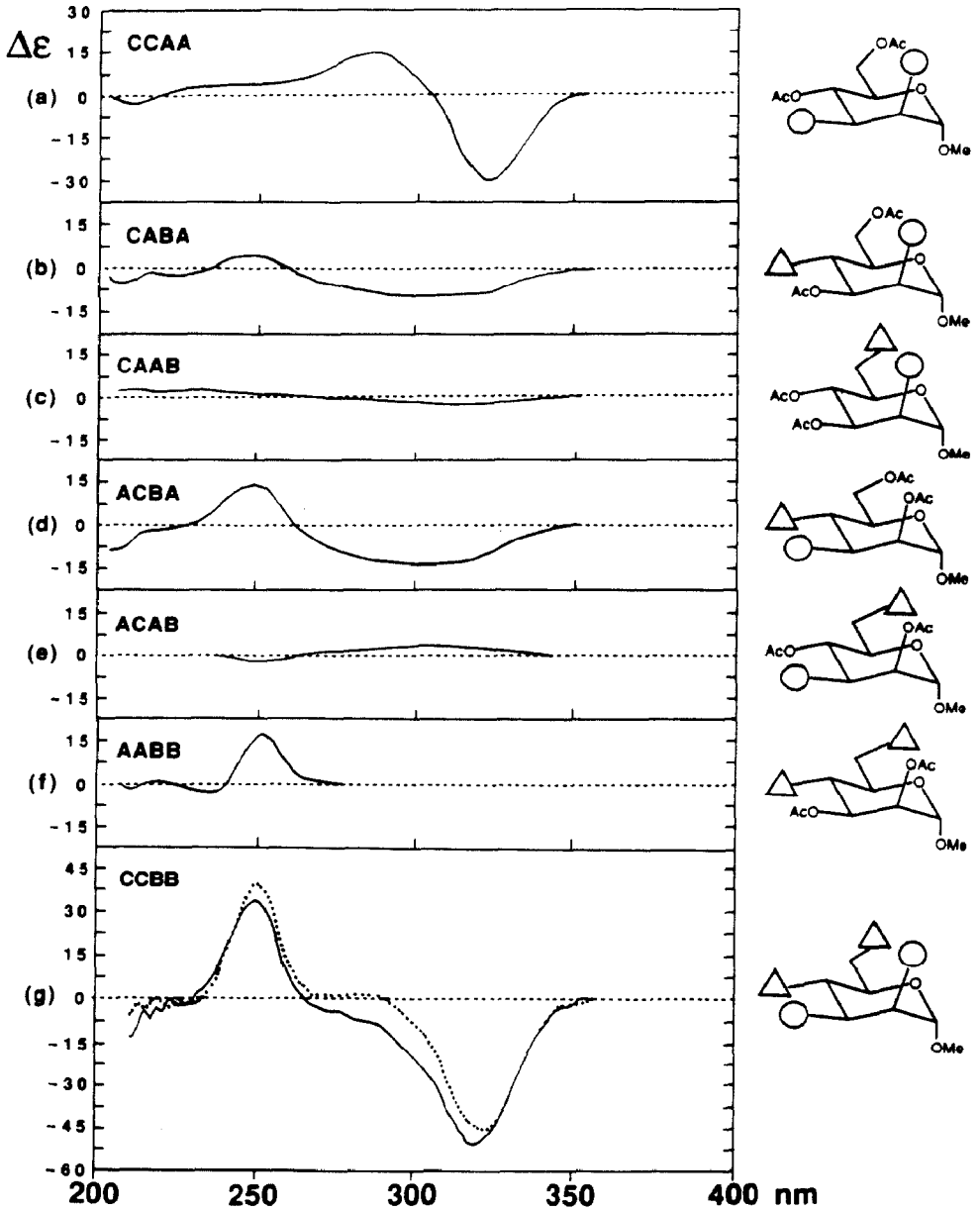


Fig. 1. Pairwise additive calculation of the c.d. curve of methyl 4,6-di-*O*-(4-bromobenzoyl)-2,3-di-*O*-(4-methoxycinnamoyl)-α-D-mannopyranoside (BBCC): (a–f) The observed c.d. spectra of the six di-chromophoric "basis-set" derivatives representing the six pairwise interactions contributing to the spectrum of CCBB; (g) summation of the six contributing interactions [Σ(a)–(f)] gives the empirically calculated c.d. spectrum of CCBB. (—) The experimentally observed spectrum (·····) is superimposed to illustrate pairwise additivity. (△) 4-Bromobenzoate, and (○) 4-methoxycinnamate group.

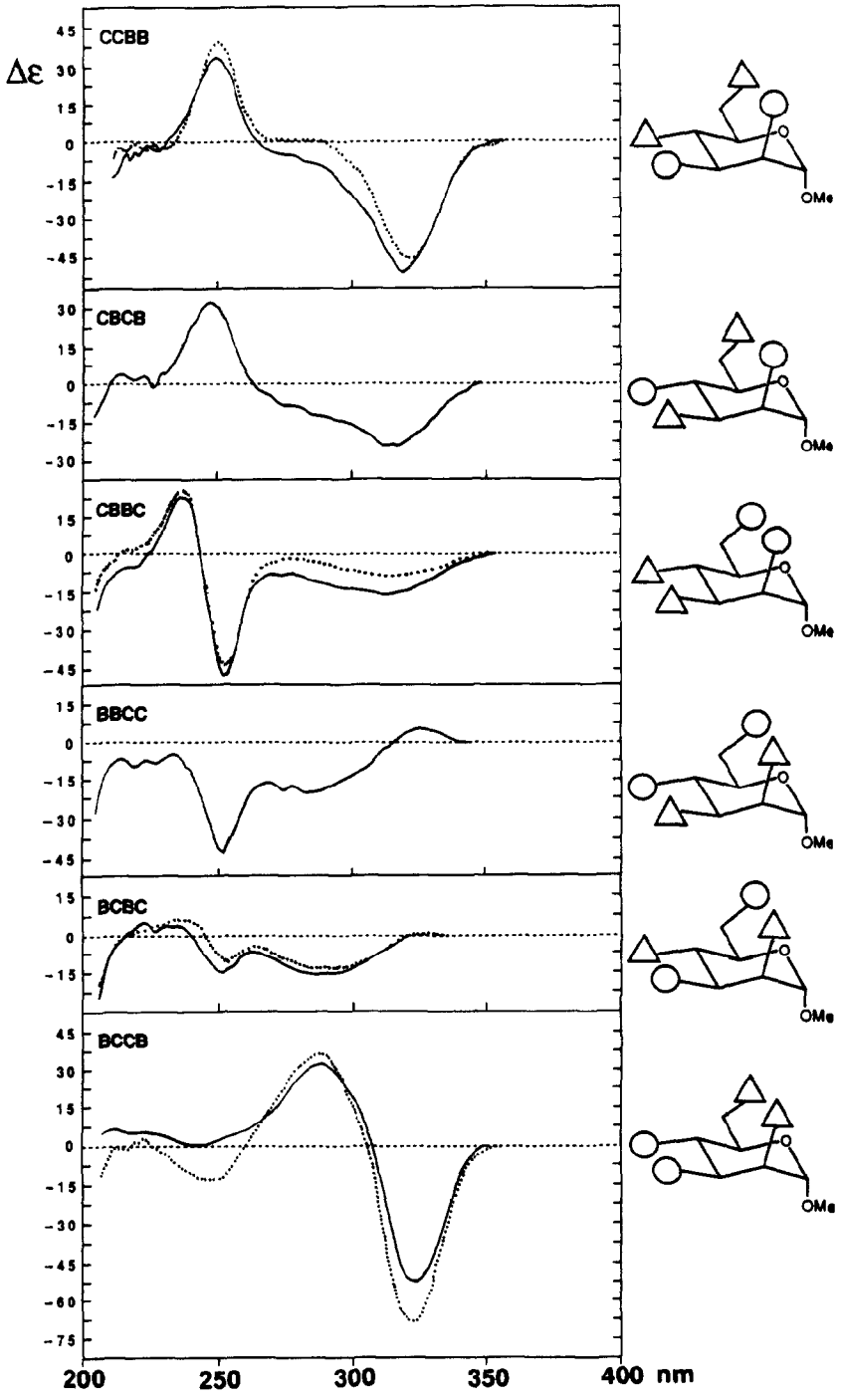


Fig. 2. Calculated (——) c.d. spectra for the six dibenzoate dicinnamate derivatives of methyl α-D-mannopyranoside. Included for comparison are the observed (·····) spectra of CCBB, CBBC, BCBC, and BCCB. (△) 4-Bromobenzoate, and (○) 4-methoxycinnamate group.

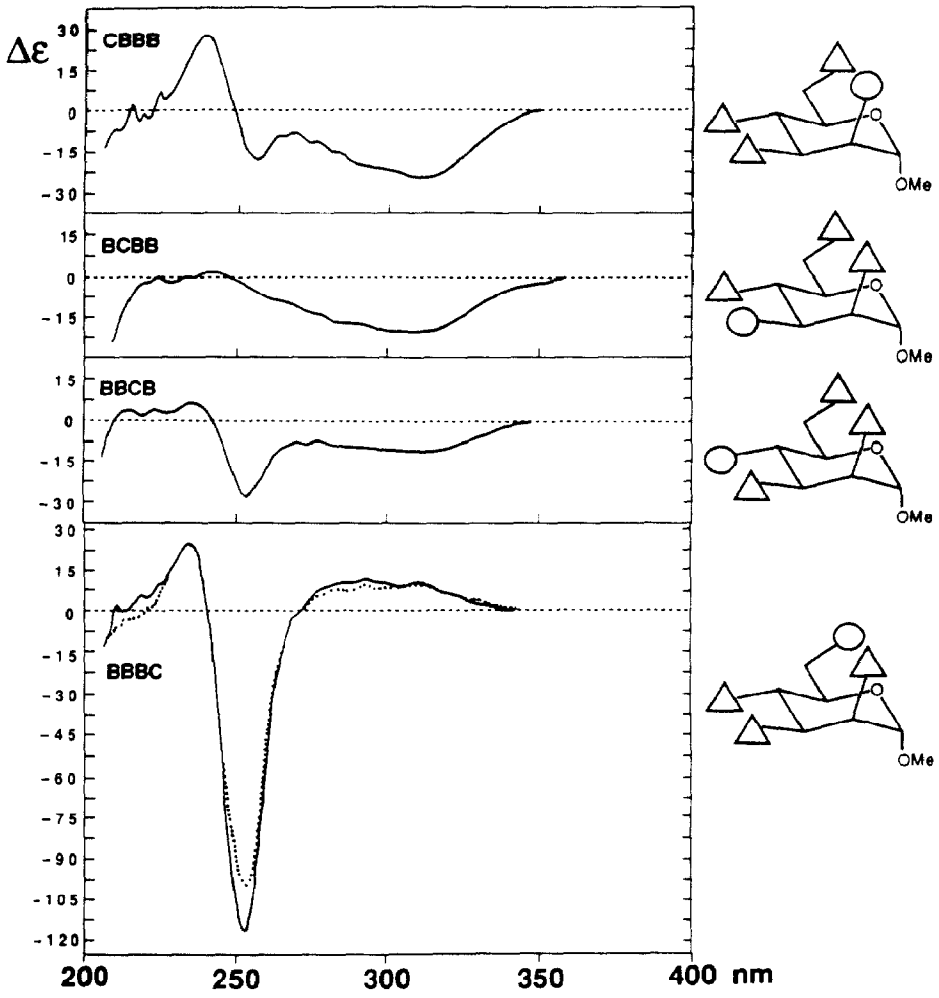


Fig. 3. Calculated (——) c.d. spectra for the four tribenzoate monocinnamate derivatives of methyl α-D-mannopyranoside, including the observed (·····) spectrum of BBBC for comparison. (△) 4-Bromobenzoate, and (○) 4-methoxycinnamate group.

basis set compounds match conformations of the tetrachromophoric tetraesters for which c.d. curves will be generated.

Empirical calculations of the c.d. spectra for tetrachromophoric derivatives, obtainable from a bichromophoric derivatization of oligosaccharides (Scheme 1), were performed by summation of the six component basis set spectra representing the six contributing pairwise interactions. An example is shown in Fig. 1 for calculation of the c.d. curve of the 2,3 dicinnamate 4,6-dibenzoate derivative, "CCBB". Owing to the large number of derivatives of the parent D-mannoside, simple four-letter abbreviations for the substitution patterns are used throughout

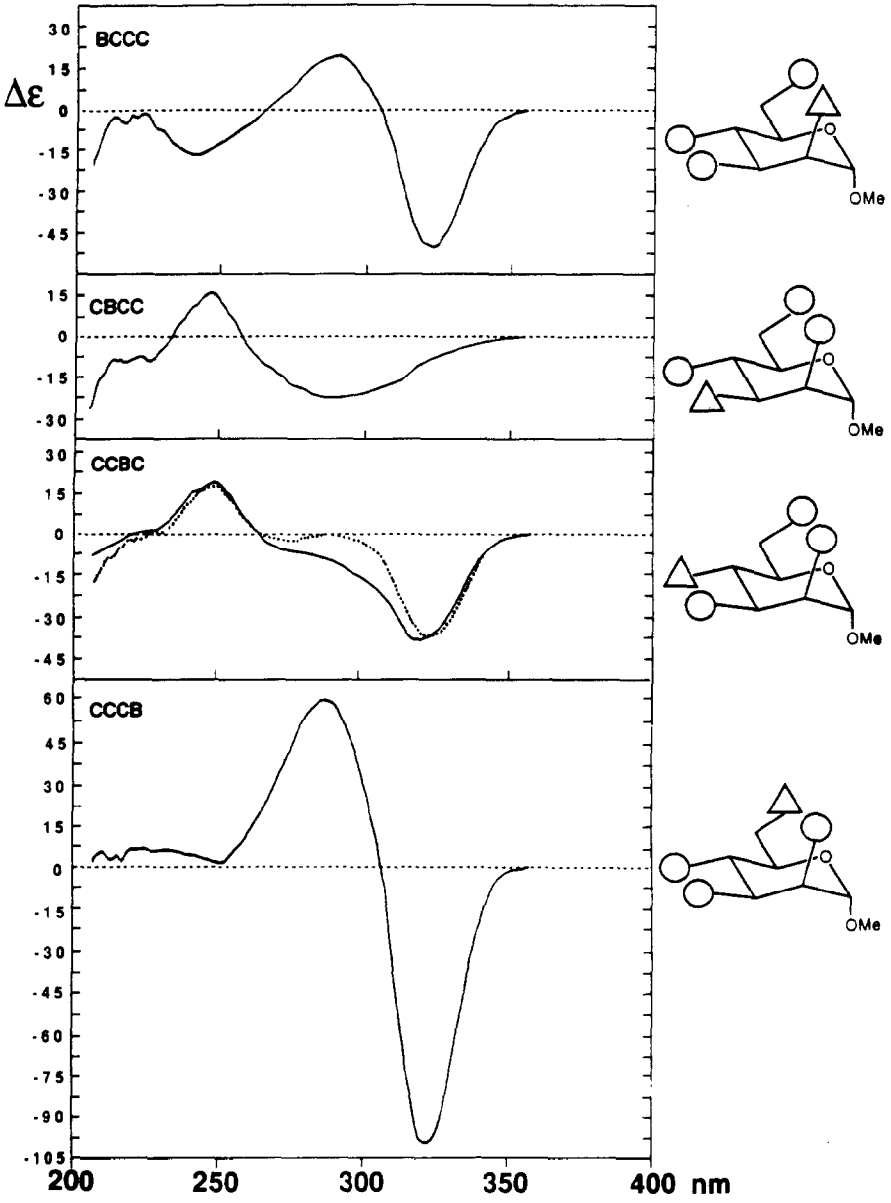


Fig. 4. Calculated (——) c.d. spectra for the four monobenzoate tricinnamate derivatives of methyl α-D-mannopyranoside, including the observed (·····) spectrum of CCBC for comparison. (△) 4-Bromobenzoate, and (○) 4-methoxycinnamate group.

TABLE III

C.D. DATA FOR TETRACHROMOPHORIC DERIVATIVES

Compound[a]	λ^b ($\Delta\varepsilon$)	λ ($\Delta\varepsilon$)	λ ($\Delta\varepsilon$)	λ ($\Delta\varepsilon$)	$\Delta\varepsilon = 0^c$
CBBB (calc.)	240 (+27)	257 (−19)	268 (−9)	311 (−25)	249
BCBB (calc.)	238 (+2)	310 (−20)			252
BBCB (calc.)	235 (+6)	253 (−28)	314 (−11)		242
BBBC (calc.)	234 (+25)	252 (−118)	293 (+11)		240, 270
BBBC (obs.)	234 (+27)	252 (−98)	302 (+10)		241, 269
BCCC (calc.)	241 (−17)	291 (+21)	324 (−51)		266, 306
CBCC (calc.)	247 (+15)	290 (−23)			258
CCBC (calc.)	248 (+19)	319 (−39)			264
CCBC (obs.)	248 (+18)	323 (−38)			266
CCCB (calc.)	288 (+63)	323 (−101)			307
CCBB (calc.)	250 (+34)	319 (−51)			265
CCBB (obs.)	251 (+41)	322 (−46)			280
CBCB (calc.)	247 (+33)	318 (−25)			264
CBBC (calc.)	237 (+22)	252 (−48)	269 (−8)	313 (−17)	244
CBBC (obs.)	237 (+26)	254 (−47)	276 (−2)	314 (−9)	244
BBCC (calc.)	234 (−6)	252 (−44)	285 (−21)	325 (+5)	315
BCBC (calc.)	236 (+4)	252 (−14)	264 (−6)	287 (−14)	241
BCBC (obs.)	238 (+8)	253 (−11)	265 (−5)	294 (−10)	245
BCCB (calc.)	249 (−15)	289 (+36)	324 (−70)		262, 306
BCCB (obs.)	240 (−2)	289 (+31)	324 (−56)		307

[a]Derivatization of the four positions is represented in the order 2,3,4,6 by: (A) acetate, (B) 4-bromobenzoate, and (C) 4-methoxycinnamate. [b]Extrema in nm. [c]Point where $\Delta\varepsilon$ changes sign, in nm.

the tables, figures, schemes, and text. Derivatization of the four hydroxyl groups is indicated in the order 2,3,4, and 6 by A = acetate, B = 4-bromobenzoate, C = 4-methoxycinnamate, O = free hydroxyl, and S = *tert*-butyldimethylsilyl group. Thus, the 2,3-dicinamate 4,6-dibenzoate derivative is conveniently denoted CCBB. The six basis set spectra representing the pairwise interactions in CCBB are shown in Figs. 1a–f. These include spectra of CCAA (Fig. 1a) and AABB (Fig. 1f) representing the contributing "homo" interactions, as well as spectra of CABA, CAAB, ACBA, and ACAB (Fig. 1b–e), which represent the "hetero" benzoate cinnamate exciton-coupled interactions. Summation of these six spectra provides an empirically calculated c.d. curve that accurately simulates the observed curve for CCBB (Fig. 1g).

Calculated spectra for all six possible dibenzoate dicinnamate compounds are shown in Fig. 2. In addition to the synthetic CCBB described above, three other compounds in this class, CBBC, BCBC, and BCCB, were prepared to demonstrate the accuracy of the calculations. The observed c.d. spectra for these compounds are shown superimposed with the calculated spectra. Similarly, the spectrum of the synthetic tribenzoate monocinnamate, BBBC, is shown in Fig. 3, together with the calculated spectra for the four derivatives in this class. Likewise, the observed spectrum of the monobenzoate tricinnamate, BCCC, is shown in Fig. 4 with the calculated spectra for the four derivatives in this third class (the c.d. data for these

TABLE IV

^{1}H-N.M.R. DATA[a]

Compound[b]	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*OAc*	*OMe-α*
Basis-set compounds								
BBAA	4.90 (1.1)	5.52–5.69 (m, H-2–H-4)			4.11 (m)	4.36 (4.7, 12.2)	2.14	3.48
						4.20 (2.1, 12.2)	1.96	
BABA	4.87 (1.6)	5.47 (1.6, 3.1)	5.60–5.65 (m, H-3–H-4)		4.16 (m)	4.29 (5.1, 12.4)	2.07	3.47
						4.20 (3.1, 12.4)	1.85	
BAAB	4.83 (1.5)	5.45 (1.5, 3.2)	5.44 (2.8, 9.8)	5.54 (9.8)	4.13 (2.1, 4.3, 9.8)	4.64 (2.3, 12.3)	2.06	3.44
						4.35 (4.6, 12.3)	1.95	
ABBA	4.83 (1.6)	5.42 (br s)	5.70 (m, H-3–H-4)		4.22 (m)	4.34 (5.7, 12.4)	2.05	3.48
						4.22 (m, 1 H)	1.87	
ABAB	4.77 (1.1)	5.37 (1.1, 3.1)	5.52 (3.1, 10.1)	5.57 (10.1)	4.14 (2.3, 5.0, 10.1)	4.53 (2.3, 12.1)	2.07	3.43
						4.43 (5.0, 12.1)	1.95	
AABB	4.75 (1.4)	5.27 (1.4, 2.8)	5.55 (2.8, 9.9)	5.63 (9.9)	4.22 (3.6, 5.1, 9.9)	4.53 (3.6, 12.2)	2.14	3.44
						4.39 (5.1, 12.2)	1.88	
CCAA	4.82 (1.5)	5.42 (1.5, 3.1)	5.52 (3.1, 9.8)	5.45 (9.8)	4.05 (2.3, 4.9, 9.8)	4.33 (4.9, 12.2)	2.12	3.44
						4.16 (2.3, 12.2)	2.01	
CACA	4.74 (1.5)	5.28 (1.5, 2.8)	5.46 (2.8, 9.0)	5.42 (9.0)	4.06 (m)	4.27 (m, 2 H)	2.13	3.38
							2.00	
CAAC	4.82 (1.3)	5.38–5.46 (m, H-2–H-4)			4.08 (m)	4.35–4.40 (m, 2 H)	2.08	3.45
							2.00	
ACCA	4.79 (1.5)	5.34 (1.5, 2.8)	5.34–5.59 (m, H-3–H-4)		4.10 (m)	4.32 (5.4, 12.7)	2.18	3.45
						4.20 (2.3, 12.7)	2.11	
ACAC	4.77 (1.5)	5.29 (1.5, 2.4)	5.47 (2.4, 10.0)	5.42 (10.0)	4.09 (m)	4.34 (d, 4.1, 2 H)	2.14	3.44
							2.03	
AACC	4.74 (1.4)	5.26 (1.4, 2.3)	5.41–5.52 (m, H-3–H-4)		4.12 (m)	4.35 (d, 4.7, 2 H)	2.15	3.43
							1.96	
BCAA	4.87 (1.4)	5.51 (1.4, 3.3)	5.56 (2.7, 10.1)	5.50 (10.1)	4.07 (2.3, 4.9, 10.1)	4.33 (4.9, 12.2)	2.14	3.46
						4.18 (2.3, 12.2)	2.02	
BACA	4.88 (1.7)	5.50 (1.7, 2.9)	5.54–5.56 (m, H-3–H-4)		4.11 (m)	4.31 (5.1, 12.2)	2.13	3.48
						4.23 (2.7, 12.2)	1.93	

Table IV (continued)

BAAC	4.87 (1.4)	5.51–5.44 (m, H-2–H-4)			4.10 (m)	4.47 (2.4, 12.2) 4.33 (4.5, 12.2)	2.08 1.97	3.47
ABCA	4.79 (1.7)	5.40 (1.7, 2.8)	5.52–5.65 (m, H-3–H-4)		4.14 (m)	4.29 (5.8, 12.3) 4.17 (2.8, 12.3)	2.12 2.10	3.45
ABAC	4.80 (1.6)	5.39 (1.6, 2.4)	5.54 (m, H-3–H-4)		4.14 (1.4, 3.4, 9.3)	4.40 (3.4, 12.2) 4.36 (1.4, 12.2)	2.13 1.99	3.46
AABC	4.78 (1.6)	5.29 (1.6, 3.0)	5.56–5.58 (m, H-3–H-4)		4.19 (m)	4.36–4.38 (m, 2 H)	2.19 1.91	3.48
CBAA	4.84 (1.1)	5.50 (1.1, 2.0)	5.47–5.58 (m, H-3–H-4)		4.08 (m)	4.37 (5.4, 12.3) 4.18 (2.1, 12.3)	2.14 1.95	3.45
CABA	4.84 (1.6)	5.42 (1.6, 2.6)	5.58–5.61 (m, H-3–H-4)		4.15 (m)	4.33 (5.7, 12.2) 4.20 (2.9, 12.2)	2.06 1.90	3.47
CAAB	4.81 (1.6)	5.38 (1.6, 3.3)	5.43 (3.3, 9.9)	5.54 (9.9)	4.12 (2.4, 4.6, 9.9)	4.57 (2.4, 12.2) 4.40 (4.6, 12.2)	2.06 2.01	3.44
ACBA	4.81 (1.7)	5.35 (1.7, 3.0)	5.66 (3.0, 0.1)	5.61 (10.1)	4.16 (2.8, 5.8, 10.1)	4.32 (5.8, 12.3) 4.19 (2.8, 12.3)	2.20 2.06	3.47
ACAB	4.77 (1.7)	5.31 (1.7, 2.6)	5.49–5.53 (m, H-3–H-4)		4.13 (2.6, 5.1, 10.0)	4.52 (2.6, 12.1) 4.41 (5.1, 12.1)	2.14 2.02	3.44
AACB	4.75 (1.8)	5.28 (1.8, 3.0)	5.48 (3.0, 10.1)	5.56 (10.1)	4.17 (2.9, 5.1, 10.1)	4.55 (2.9, 12.1) 4.41 (5.1, 12.1)	2.15 1.98	3.44
Tri- and tetra-chromophoric derivatives								
BCBC	4.96 (1.8)	5.58 (1.8, 2.7)	5.77 (2.7, 10.1)	5.84 (10.1)	4.27 (3.5, 4.5, 10.1)	4.51 (3.5, 12.0) 4.38 (4.5, 12.0)		3.52
BCCB	4.93 (1.7)	5.57 (1.7, 3.1)	5.70 (3.1, 10.2)	6.81 (10.2)	4.27 (2.6, 4.5, 10.2)	4.72 (2.6, 12.1) 4.43 (4.5, 12.1)		3.50
CCBB	4.87 (1.5)	5.48 (1.5, 3.5)	5.74 (3.5, 10.3)	5.84 (10.3)	4.29 (m)	4.62 (3.0, 11.8) 4.44 (4.2, 11.8)		3.47
CBBC	4.88 (1.7)	5.54 (1.7, 3.1)	5.76 (3.1, 9.9)	5.84 (dd, 9.3, 9.9)	4.28 (4.8, 4.8, 9.3)	4.43 (m, 2 H)		3.50
BBBC	4.98 (1.7)	5.65 (1.7, 3.1)	5.82 (3.1, 10.2)	5.92 (10.2)	4.33 (3.5, 4.5, 10.2)	4.53 (3.5, 12.0) 4.42 (3.5, 12.0)		3.54
CCBC	4.89 (1.7)	5.48 (1.7, 2.7)	5.74 (2.7, 10.1)	5.77 (10.1)	4.27 (m)	4.43 (d, 4.4, 2 H)		3.51

[a] δ Values. In parentheses, J values in Hertz. If not indicated, the following multiplicities were observed: H-1, d; H-2, dd; H-3, dd;, H-4, t; H-5, ddd; and H-6, ABX. [b] Derivatization of the four positions is represented in the order 2,3,4,6 by: (A) acetate, (B) 4-bromobenzoate, and (C) 4-methoxycinnamate.

calculated and observed spectra is presented in a more precise form in Table III). The agreement between calculated and observed spectra confirmed the thoroughly demonstrated principle of pairwise additivity, thus serving to add further confidence to the accuracy of the remaining spectral calculations.

DISCUSSION

Inspection of these 14 c.d. spectra of bichromophoric D-mannose derivatives (Figs. 2–4), together with the corresponding D-glucose[5] and D-galactose[6] derivatives, revealed that each of the total of 42 compounds has a unique curve related to the configuration, chromophore ratio, and substitution pattern of the sugar. In cases where the curve shape appears similar, *e.g.*, compare BCBC (Fig. 2) and BBCB (Fig. 3), significant differences appear in the magnitudes of the $\Delta\varepsilon$ values for a given λ_{max} value (see Table III). This reference library of distinctive, "fingerprint" c.d. spectra can be used to identify unknown hexopyranose derivatives obtained from a bichromophoric derivatization of an oligosaccharide. Thus, the identity and linkage positions of the sugar subunits can be established without recourse to synthetic standards.

The 24 basis-set spectra reported herein, together with the corresponding D-glucose[5] and D-galactose[6] sets, can be similarly used to calculate a complete library of c.d. curves for the many possible derivatives of deoxypyranose sugars, such as fucose and rhamnose[7]. All of these derivatives contain three instead of four chromophores, and thus reflect different chromophore ratios in their u.v. spectra. The c.d. curves of 2-acetamido-2-deoxy sugar derivatives are similar to those of the corresponding 2-deoxy sugars, and those of *N*-benzoylamino or *N*-cinnamoylamino sugars resulting from amino sugars resemble the curves of the oxygenated counterparts[7]. Thus, the reference curves are also applicable to aminodeoxy sugars, and the basis-set spectra are representitative of the dideoxypyranoses. Future work can expand the reference library as other pyranose sugar classes are studied by c.d. spectroscopy, namely the uronic and sialic acids commonly found in glycoconjugates. As the pairwise additivity principle has been amply demonstrated, successful application to most, if not all, pyranose sugars found in Nature is anticipated.

Utilization of this c.d. method for oligosaccharide-linkage, point determination requires an efficient, convenient derivatization procedure amenable to microscale work. Recently, a glycoside-cleavage reaction was developed that meets these criteria[8]. Performed on perbromobenzoylated oligosaccharides, a hydrogen bromide-catalyzed cleavage reaction cleanly liberates each monosaccharide component without benzoyl migration or loss of acyl groups, thus maintaining the integrity of the linkage points (Scheme 1, step 2). Typically, the acylated oligosaccharide is dissolved in a haloacetic acid (*e.g.*, trifluoroacetic acid) containing hydrobromic acid, and allowed to react with warming to generate a mixture of glycosyl bromides. The bromosugars provided by this modified version of the

"acetobrominolysis" reaction[9] are then converted into the corresponding methyl glycosides with silver oxide and methanol. The hydroxyl groups liberated from glycosidic linkages are then tagged with cinnamate groups (Step 3) to give bichromophoric derivatives. Terminal sugar residues, however, provide perbromobenzoylated derivatives without the cinnamate chromophore. After h.p.l.c. separation and purification (Step 4), the monosaccharide constituents are readily identified by u.v., c.d., and m.s. analysis. Comparison with the reference library curves and tabulated data unambiguously establishes the configuration, linkage, and branch points of carbohydrate subunits of oligosaccharides.

EXPERIMENTAL

General methods. — All intermediates and final products were characterized by ^{1}H-n.m.r. spectroscopy (Bruker WM 250 operated at 250 MHz) in $CDCl_3$ solution (see Table IV). Purification of all tetraacylates prior to u.v. and c.d. measurements was performed by h.p.l.c. employing a normal phase analytical column (Phenomenex IB-Sil 5 SIL, 250 × 4.6 mm) and 7:3 hexane–ethyl acetate or 97:3 hexane–2-propanol as the eluents. The u.v. absorbance measurements were performed with a Perkin–Elmer Lambda 4C spectrophotometer to calculate sample concentrations. Acetonitrile solutions were prepared at concentrations of 5–15 μM, which were determined on the basis of the average 4-methoxycinnamate u.v. ε values, experimentally determined at 311 nm (where bromobenzoate groups are transparent): mono, ε_{mM} 24.00; di, 45.00; and tri, 68.00. In the case of the diacetate dibenzoate esters, the average value of ε_{mM} 38.20 at 245 nm was used. The c.d. measurements were recorded with a JASCO 500A spectropolarimeter driven by a JASCO DP500N data processor utilizing four scans between 200 and 400 nm. An IBM-PC computer operated with JASCO software was used to normalize all c.d. spectra to a concentration of 10μM, as well as to perform spectral summations of basis set components for generating empirical c.d. spectra of tri- and tetrachromophoric compounds.

All solvents and reagents were prepared or purified as follows. Dichloromethane and pyridine were distilled from CaH_2, and 4-dimethylaminopyridine was recrystallized from hexane–benzene. The reagents, 4-bromobenzoyl chloride and 4-methoxycinnamoyl chloride, were used for acylation reactions without recrystallization. 4-Methoxycinnamoyl chloride was prepared from the acid and thionyl chloride (1.2 equiv.) in refluxing benzene (2 h). Benzene and excess reagent were removed *in vacuo,* and distillation in a sublimation apparatus (140°/13 Pa) afforded the pure acid chloride.

All compounds were prepared by various sequences of partial bromobenzoylation, 4-methoxycinnamoylation, acetylation, and in certain cases partial trimethylsilyl protection of the parent methyl α-D-mannopyranoside (**1**). The sequences of reaction followed the typical reaction order for esterification of the four hydroxyl groups, namely 6 ≫ 3 > 2 > 4. For example, cinnamoylation of **1**

(226 mg) with 2.3 equivs. of cinnamoyl chloride gave a mixture of the acylated derivatives CCCC, COCC, CCOC, CCOO, COOC, OCOC, OOCC, and OOOC, which were isolated as follows. After removal of pyridine *in vacuo,* column chromatography of the reaction mixture (silica, methanol–dichloromethane solvent system, gradient elution from 1:99 to 1:24) provided five fractions (I–V). Half of fraction I (264 mg total) was loaded onto four, 20 × 20 cm, 100-μ preparative t.l.c. plates, which were developed with 3:97 methanol–dichloromethane to give the tetracinnamate CCCC (uppermost band, 12.5 mg), and the two tricinnamates CCOC (second band, 13 mg) and COCC (third band, 102 mg).

*^{1}H-N.m.r. characterization of the methyl glycosides investigated and m.s. data of some of the compounds prepared. — Methyl 2,3,4,6-tetra-*O*-(4-methoxycinnamoyl)-α-*D*-mannopyranoside (CCCC).* Partial ^{1}H-n.m.r.: δ 5.67 (dd, 1 H, *J* 8.8, 10.0 Hz, H-4), 5.63 (dd, 1 H, *J* 3.5, 10.0 Hz, H-3), 5.47 (dd, 1 H, *J* 1.5, 3.5 Hz, H-2), 4.87 (d, 1 H, *J* 1.5 Hz, H-1), 4.43 (bd, 2 H, *J* 3.9 Hz, H_2-6), 4.20 (dt, 1 H, *J* 3.9, 8.8 Hz, H-5), 3.83 (s, 3 H, 4-MeO), 3.80 (s, 6 H, 3 4-MeO), 3.77 (s, 3 H, 4-MeO), and 3.48 (s, 3 H, OMe).

*Methyl 2,3,6-tri-*O*-(4-methoxycinnamoyl)-α-*D*-mannopyranoside (CCOC).* Partial ^{1}H-n.m.r.: δ 5.4 (m, 24 H, H-2,3), 4.84 (d, 1 H, *J* 1.3 Hz, H-1), 4.80 (dd, 1 H, *J* 3.8, 12.1 Hz, H-6a), 4.44 (dd, 1 H, *J* 1.9, 8, 12.1 Hz, H-6b), 4.05 (ddd, 1 H, H-4), 3.95 (ddd, 1 H, H-5), 3.80 (s, 3 H, 4-MeO), 3.78 (s, 3 H, 4-MeO), 3.77 (s, 3 H, 4-MeO), 3.43 (s, 3 H, MeO), and 3.27 (d, 1 H, *J* 4.2 Hz, OH-4).

*Methyl 2,4,6-tri-*O*-(4-methoxycinnamoyl)-α-*D*-mannopyranoside (COOC).* Partial ^{1}H-n.m.r.: δ 5.34 (dd, 1 H, *J* 9.9, 10.0 Hz, H-4), 5.24 (dd, 1 H, *J* 1.5, 3.5 Hz, H-2), 4.87 (d, 1 H, *J* 1.5 Hz, H-1), 4.44–4.35 (m, 2 H, H-6ab), 4.22 (m, 1 H, H-3), 4.10 (m, 1 H, H-5), 3.81 (s, 6 H, 4-MeO), 3.80 (s, 3 H, 4-MeO), and 3.44 (s, 3 H, OMe).

*Methyl 3,6-di-*O*-(4-methoxycinnamoyl)-α-*D*-mannopyranoside (OCOC).* Fraction III was pure OCOC (292 mg, 47%), the major product of the partial cinnamoylation; partial ^{1}H-n.m.r.: δ 5.18 (dd, 1 H, *J* 3.2, 9.6 Hz, H-3), 4.73 (d, 1 H, *J* 1.4 Hz, H-1), 4.63 (dd, 1 H, *J* 4.5, 12.1 Hz, H-6a), 4.38 (dd, 1 H, *J* 1.9, 12.1 Hz, H-6b), 4.07 (dd, 1 H, *J* 1.4, 3.2 Hz, H-2), 3.96 (dd, 1 H, *J* 9.6, 9.8 Hz, H-4), 4.84 (ddd, 1 H, *J* 1.9, 4.5, 9.8 Hz, H-5), 3.77 (s, 3 H, 4-MeO), 3.76 (s, 3 H, 4-MeO), and 3.37 (s, 3 H, OMe).

*Methyl 2,6-di-*O*-(4-methoxycinnamoyl)-α-*D*-mannopyranoside (COOC).* Fraction IV was purified by silica column chromatography with an ethyl acetate–hexane gradient from 9:11 to 11:9. The first component to be eluted was the major component of this fraction, COOC (48.4 mg); partial ^{1}H-n.m.r.: δ 5.23 (dd, 1 H, *J* 1.6, 3.4 Hz, H-2), 4.79 (d, 1 H, *J* 1.6 Hz, H-1), 4.77 (dd, 1 H, *J* 3.1, 12.2 Hz, H-6a), 4.34 (dd, 1 H, *J* 1.9, 12.2 Hz, H-6b), 4.08 (m, 1 H, H-3), 3.80 (s, 3 H, 4-MeO), 3.75 (m, 1 H, H-5), 3.73 (s, 3 H, 4-MeO), and 4.38 (s, 3 H, OMe).

*Methyl 6-*O*-(4-methoxycinnamoyl)-α-*D*-mannopyranoside (OOOC).* Fraction V was identified as the monocinnamate OOOC; partial ^{1}H-n.m.r.: δ 4.78 (bs, 1 H, H-1), 4.76 (dd, 1 H, *J* 3.8, 12.3 Hz, H-6a), 4.32 (dd, 1 H, *J* 1.8, 12.3 Hz, H-6b),

3.97 (bs, 1 H, H-2), 3.86 (m, 1 H, H-5), 3.83 (s, 3 H, 4-MeO), 3.75 (bd, 1 H, *J* 9.2 Hz, H-3), 3.65 (dd, 1 H, *J* 9.2 Hz, H-4), and 3.38 (s, 3 H, OMe).

Similarly, partial benzoylation of **1** (2 equiv. of 4-bromobenzoyl chloride in pyridine with 4-dimethylaminopyridine as a catalyst at room temperature and overnight) afforded the 3,6-dibenzoate derivative OBOB (O = OH group) as the major product, along with the 2,6-dibenzoate BOOB (Scheme 2). Minor products which were isolated included BBOO, OOBB, and OOOB. Separation of these partially acylated intermediates was performed as described above, similarly to the separations for the analogous series of methyl α-D-glucopyranoside[5] and methyl α-D-galactopyranoside[6] derivatives previously described. Subsequent acetylation of the dibenzoates with excess acetic anhydride provided the homobasis-set derivatives BBAA, BAAB, ABAB, and AABB.

*Methyl 4,6-di-*O*-acetyl-2,3-di-*O*-(4-bromobenzoyl)-α-*D*-mannopyranoside (BBAA).* Partial ^{1}H-n.m.r., see Table IV; m.s.: *m/z* 642 (M^+) and 611 ($M^+ - OMe$).

Anal. Calc. for $C_{24}H_{21}Br_2O_9$: $M^+ - OMe$, 610.9553. Found: 610.9587.

*Methyl 3,4-di-*O*-acetyl-2,6-di-*O*-(4-bromobenzoyl)-α-*D*-mannopyranoside (BAAB).* Partial ^{1}H-n.m.r., see Table IV; m.s.: *m/z* 642 (M^+) and 611 ($M^+ - OMe$).

Anal. Calc. for $C_{25}H_{24}Br_2O_{10}$: M^+, 641.9736. Found: 641.9741.

*Methyl 2,6-di-*O*-acetyl-3,4-di-*O*-(4-bromobenzoyl)-α-*D*-mannopyranoside (ABBA).* Partial ^{1}H-n.m.r., see Table IV; m.s.: *m/z* 642 (M^+) and 611 ($M^+ - OMe$).

Anal. Calc. for $C_{24}H_{21}Br_2O_9$: $M^+ - OMe$, 610.9553. Found: 610.9618.

4-Methoxycinnamoylation of BOOB with excess cinnamoyl chloride gave the bichromophoric compound BCCB. Partial cinnamoylation of OOOB (1.2 equiv. of 4-methoxycinnamoyl chloride) and subsequent acetylation gave the heterobasis-set derivatives CAAB, ACAB, and AACB.

Acetylations with acetic anhydride–pyridine–4-dimethylaminopyridine for the basis-set compounds were performed as the final steps, as the acetate groups are prone to migrate under the basic reaction conditions of benzoylation or cinnamoylation. Therefore, a trimethylsilyl ether protecting group was used for the preparation of the 2,4- and 3,4-bichromophoric derivatives as shown in Scheme 2. Namely, the 2,4- and 3,4-bichromophoric derivatives, BABA, BACA, CACA, ABBA, ABCA, ACCA, and ACBA, were prepared by appropriate sequences of partial benzoylation or cinnamoylation of the 3,6- and 2,6-bis(*tert*-butyldimethylsilyl) derivatives of **1**, respectively.

*Methyl 2,6- and 3,6-di-*O*-(tert-butyldimethylsilyl)-α-*D*-mannopyranoside (SOOS and OSOS).* To a solution of **1** (549 mg, 2.83 mmol) in dry *N,N*-dimethylformamide (3 mL) were added imidazole (1.15 g, 16.9 mmol) and *tert*-butylchlorodimethylsilane (1.28 g, 8.48 mmol), and the mixture was stirred for 2 h at room temperature[10]. The mixture was dissolved in 3:1 hexane–ethyl acetate (50 mL) and washed with water (2 × 20 mL) and then NaCl solution (10 mL). The

organic layer was dried ($MgSO_4$) and concentrated to give a crude oil (1.5 g) which was purified by column chromatography on silica gel (50 g). The stepwise gradient elution with 5:1 to 2:1 hexane–ethyl acetate gave a mixture of trisilylated compounds (620 mg), 3,6-disilyl compound (OSOS, 600 mg), and a crude 2,6-disilyl compound (SOOS, 100 mg). Crude SOOS was purified by silica gel (10 g) column chromatography (3:1 hexane–ethyl acetate) to give pure SOOS (65 mg); ^{1}H-n.m.r. (OSOS): δ 4.73 (d, 1 H, J 1.5 Hz, H-1), 3.87 (bd, 2 H, J 5.3 Hz, H-6), 3.84 (dd, 1 H, J 3.6, 8.6 Hz, H-3), 3.76 (m, 1 H, H-2), 3.71 (dd, 1 H, J 8.6, 9.5 Hz, H-4), 3.56 (dt, 1 H, J 9.5, 5.3 Hz, H-5), 3.37 (s, 3 H, OMe), 0.92, 0.91 (Bu^t), 0.16 (3 H), 0.14 (3 H), and 0.10 (6 H, Me); m.s.: m/z 423 (M^++1), 391.

Anal. Calc. for $C_{19}H_{43}O_6Si_2$: M^++1, 423.2598. Found: 423.2645.

^{1}H-N.m.r. (SOOS): δ 4.57 (d, 1 H, J 1.4 Hz, H-1), 3.89 (dd, 1 H, J 3.0, 1.4 Hz, H-2), 3.89 (dd, 1 H, J 5.0, 10.5 Hz, H-6a), 3.84 (1 H, J 4.7, 10.5 Hz, H-6b), 3.70 (m, 2 H, H-3,4), 3.51 (ddd, 1 H, J 9.5, 5.0, 4.7 Hz, H-5), 3.34 (s, 3 H, OMe), 0.90 (18 H, 2 Bu^t), 0.11 (3 H), and 0.09 (9 H, 4 Me).

*Methyl 2,4-di-*O*-(4-bromobenzoyl)-3,6-di-*O*-(*tert*-butyldimethylsilyl)-α-*D*-mannopyranoside (BSBS) and methyl 2-*O*-(4-bromobenzoyl)-3,6-di-*O*-(*tert*-butyldimethylsilyl)-α-*D*-mannopyranoside (BSOS).* To a solution of OSOS (21.2 mg, 0.05 mmol) in dry pyridine (0.5 mL) was added 4-dimethylaminopyridine (6 mg, 0.05 mmol) and 4-bromobenzoyl chloride (22 mg, 0.1 mmol), and the mixture was stirred under Ar for 30 h at room temperature. After adding a drop of water, the mixture was stirred for additional 5 h and concentrated to dryness. The residue was diluted with 3:1 hexane–ethyl acetate and passed through a pipet column of neutral alumina (activity II–III, 1 g) which was then washed with 3:1 hexane–ethyl acetate. The filtrate and washings were concentrated and separated by silica gel (5 g) column chromatography (benzene, then 49:1 benzene–ethyl acetate) to give BSBS (2.5 mg) and BSOS (20 mg); partial ^{1}H-n.m.r. (BSBS): δ 5.54 (dd, 1 H, J 9.5, 9.7 Hz, H-4), 5.32 (dd, 1 H, J 1.8, 3.4 Hz, H-2), 4.82 (d, 1 H, J 1.8 Hz, H-1), 4.31 (dd, 1 H, J 3.5, 9.5 Hz, H-3), 3.90 (ddd, 1 H, J 9.5, 5.0, 4.7 Hz, H-5), 3.75 (m, 2 H, H-6), 3.45 (s, 3 H, OMe), 0.86, 0.61 (2 Bu^t), 0.02 (3 H), −0.02 (6 H), and −0.17 (3 H, 4 Me); partial ^{1}H-n.m.r. (BSOS): δ 5.23 (dd, 1 H, J 1.8, 3.4 Hz, H-2), 4.76 (d, 1 H, J 1.8 Hz, H-1), 4.06 (dd, 1 H, J 3.4, 9.1 Hz, H-3), 3.93 (ddd, 1 H, J 9.1, 9.4, 2.2 Hz, H-4), 3.91 (m, 2 H, H-6), 3.63 (ddd, 1 H, J 9.4, 4.0, 4.0 Hz, H-5), 3.39 (s, 3 H, OMe), 0.93 (9 H), 0.79 (9 H, 2 Bu^t), 0.12, 0.11, 0.10, and 0.09 (Me).

*Methyl 2-*O*-(4-bromobenzoyl)-3,6-di-*O*-(*tert*-butyldimethylsilyl)-4-*O*-(4-methoxycinnamoyl)-α-*D*-mannopyranoside (BSCS).* To a solution of BSOS (20 mg, 0.033 mmol) in dry pyridine (0.5 mL) was added 4-dimethylaminopyridine (5 mg), silver triflate (20 mg, 0.078 mmol), and 4-methoxycinnamoyl chloride (20 mg, 0.1 mmol), and the mixture was stirred under Ar for 12 h at room temperature[6,12]. After a workup similar to that just described using an alumina column, the crude product was purified by silica gel (2 g) column chromatography (benzene) to give BSCS (20 mg); partial ^{1}H-n.m.r.: δ 5.34 (dd, 1 H, J 9.0, 9.4 Hz, H-4), 5.30 (dd, 1 H, J 1.7, 3.5 Hz, H-2), 4.81 (d, 1 H, J 1.7 Hz, H-1), 4.25 (dd, 1 H, J 3.5, 9.4 Hz,

H-3), 3.84 (s, 3 H, 4-MeO), 3.76 (m, 3 H, H-5,6a,6b), 3.43 (s, 3 H, OMe), 0.89, 0.68 (2 Bu^t), 0.05, 0.04, 0.03, and 0.01 (Me).

Methyl 2-O-(4-bromobenzoyl)-4-O-(4-methoxycinnamoyl)-α-D-mannopyranoside (BOCO). To a solution of BSCS (20 mg) in acetonitrile (0.8 mL) was added 47% HF (0.2 mL). The mixture was stirred for 12 h at room temperature[11], and then it was poured dropwise into saturated $NaHCO_3$ solution (5 mL) and extracted with 2:1 hexane–ethyl acetate (3 × 10 mL). The extract was purified by silica gel (2 g) column chromatography (2:1 to 1:1 hexane–ethyl acetate) to give BOCO (15 mg); partial ^{1}H-n.m.r.: δ 5.38 (dd, 1 H, *J* 1.6, 3.5 Hz, H-2), 5.31 (dd, 1 H, *J* 9.9 Hz, H-4), 4.91 (d, 1 H, *J* 1.6 Hz, H-1), 4.34 (dd, 1 H, *J* 3.5, 9.9 Hz, H-3), 3.85 (s, 3 H, MeO-4), 3.78 (m, 3 H, H-5,6a,6b), and 3.44 (s, 3 H, OMe).

The 2,3-heterobichromophoric derivatives, BCAA and CBAA, were prepared by monocinnamoylation of the 4,6-*O*-benzylidene derivative followed by benzoylation and subsequent deprotection with 4-toluenesulfonic acid (1%) in methanol for 6 h at 0°.

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