Characterization of Light-harvesting Pigments of *Chloroflexus aurantiacus*. Two New Chlorophylls: Oleyl (Octadec-9-enyl) and Cetyl (Hexadecanyl) Bacteriochlorophyllides-*c*

Frédéric Fages, Nils Griebenow,[a] Kai Griebenow, Alfred R. Holzwarth,* and Kurt Schaffner
Max-Planck-Institut für Strahlenchemie, D-4330 Mülheim a. d. Ruhr, Federal Republic of Germany

The bacteriochlorophyll-*c* pigment contained in the chlorosomes of the phototrophic and thermophilic bacterium *Chloroflexus aurantiacus* is shown to comprise, in the least, 10 constituents, *viz.* the 2a-epimeric pairs [*i.e.*, the (2a*R*,7*S*,8*S*)- and (2a*S*,7*S*,8*S*)-diastereoisomers in 2:1 ratio] of five bacteriochlorophyllide-*c* esters. All compounds were fully characterized by ^{1}H and ^{13}C NMR spectroscopy, and by mass spectra. They include the bacteriochlorophyllides-*c* of geranylgeranio, phytol, and stearol(octadecan-1-ol), which have been reported previously but have not yet been completely characterized, and two novel bacteriochlorophylls-*c*, *viz.* the bacteriochlorophyllides-*c* of the non-isoprenoid alcohols 'cetol' (hexadecan-1-ol) and 'oleol' (octadec-9-en-1-ol).

Bacteriochlorophyll(BChl †)-*c* is the major chlorophyll in the phototrophic bacteria families Chlorobiaceae and Chloroflexaceae. It is contained in the main light-harvesting antennae, the so-called chlorosomes, of these bacteria.[1] These chlorosomes are extra-membraneous vesicles, assembling up to 16 000 BChl-*c* molecules.[2] In connection with our investigation of the mechanism and kinetics of the exciton transfer in the chlorosomes of the thermophilic *Chloroflexus aurantiacus*, we have recently found[3,4] that the organizational principle of this antenna appears to be direct chromophore–chromophore aggregation which does not require a protein matrix.‡ Chlorosome preparations which were essentially free of proteins still fulfilled all criteria establishing the specific structural and functional integrity of the BChl-*c* organization in intact chlorosomes, *viz.* the characteristics of the absorption[6a] and of the stationary[6a,b] and time-resolved fluorescence,[6c,d] the unaltered electron-micrographic dimensions,[7] and the unchanged linear dichroism.[8] Thus, these chlorosomes were the first example of a photosynthetic antenna found not to contain chromophore–protein complexes as the constituent elements. This organization differs from all other cases where proteins play the decisive structural role either by way of covalent binding to the chromophores as in phycobiliproteins,[9] or by non-covalent complexing as with chlorophylls.[10,11]

These findings have prompted us to analyse the BChl-*c* pigments which are contained in the chlorosomes of *C. aurantiacus*, and which are capable of assembling in aggregates functioning as efficient light-harvesting antennae.

In contrast to BChl-*c* from green sulphur bacteria (Chlorobiaceae), which have been extensively investigated,[12] the chemical characterization of the isolated BChl-*c* molecules from *C. aurantiacus* has received only little attention, and it lacks full structural assignments. Gloe and Risch isolated a new BChl representative, octadecanyl ('stearyl') bacteriophyllide-*c* (**4**), from four strains of *C. aurantiacus*.[13] Furthermore, they identified the three bacteriopheophytins-*c* (**5**), (**7**), and (**8**) from strain OK-70-fl of *C. aurantiacus*,[14] with compound (**7**) not separated from a binary mixture with compound (**8**). The same methyl ester derivative (**9**) was obtained from all three bacteriopheophytins, with the (*R*) configuration at C(2a) on the basis of Horeau analysis. More recently, Brune *et al.*[15] showed the chlorosomes of strain J-10-fl to contain, *inter alia*, four major BChl-*c* components, including compound (**4**) which proved to be a mixture of 2a-epimers, *i.e.* the (2a*R*,7*S*,8*S*)- and (2a*S*,7*S*,8*S*)-diastereoisomers.

We now report the resolution, into their components, of the four chromatographic BChl-*c* fractions observed with the strain OK-70-fl of *C. aurantiacus*. Three of these fractions, compounds (**1**), (**3**),§ and (**4**), were resolved into the 2a-epimeric components, and one, compound (**2**), was found to be composed of the 2a-epimers of two new bacteriochlorophylls-*c*, *i.e.* the hexadecanyl ('cetyl') and octadec-9-enyl ('oleyl') bacteriochlorophyllides-*c*.

Results and Discussion

Isolation of the Bacteriochlorophylls-c (**1**)–(**4**) *and Configurational Analysis.*—Methanol extraction of the cells of *C. aurantiacus* (strain OK-70-fl) afforded, after removal of most of the carotenoids by precipitation with water, a mixture composed primarily of BChl-*c* and BChl-*a*. Four major BChl-*c* fractions (Figure 1) could be isolated from this extract by two consecutive reverse-phase HPLC separations. The relative areas of each of these bands, determined by analytical HPLC, are compiled in Table 1. These values were reproducible for a large number of extractions over a period of *ca.* 2 years.

The visible absorption spectra of the fractions (3) and (4) in Figure 1 were identical and characteristic of uncontaminated BChl-*c*-type pigments (maxima at 435 and 668 nm in methanol), whereas the spectra of fractions (1) and (2) revealed that the BChl-*c* pigments of these samples were still contaminated with BChl-*a* and carotenoids which could be removed by normal-phase HPLC (see Figures 2 and 3).

The ensemble of analytical data shows that the pigments (**1**)–(**4**) naturally occur, in each case, as mixtures of BChl-*c* 2a-epimers. Pheophytinization and transesterification of the

[a] Predoctoral student of the Philipps University, Marburg.

† Abbreviations used in this paper: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; Chl, chlorophyll; mBPh, methyl bacteriopheophorbide; GEF, gel-electrophoretic filtration; DEPT, distorsionless enhancement by polarisation transfer; COSY, correlation spectroscopy; mPh, methyl bacteriopheophorbide.

‡ For early suggestions that chromophore–chromophore aggregation may be another organizational principle of photosynthetic antennae, see ref. 5.

§ The phytyl group in compound (**3**) appears to be configurationally homogeneous by NMR spectroscopy.

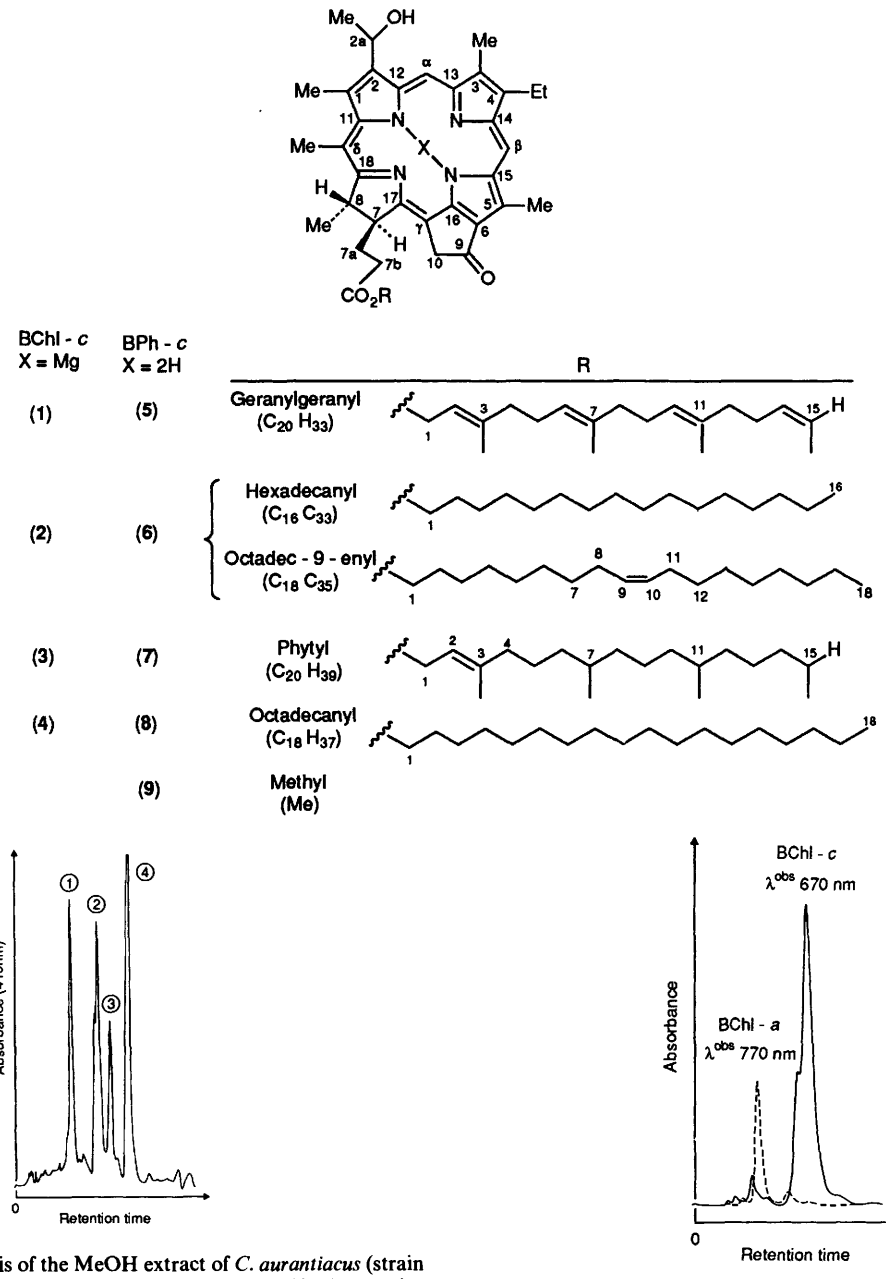

Figure 1. HPLC analysis of the MeOH extract of *C. aurantiacus* (strain OK-70-fl) after first preparative, reverse-phase HPLC. The four major fractions (1)–(4) are mainly composed of the BChl-*c* 2a-epimers of (1) (1) (geranylgeranyl bacteriochlorophyllide-*c*), (2) (2) (mixture of cetyl and oleyl esters), (3) (3) (phytyl ester), and (4) (4) (stearyl ester).

Figure 2. Analytical normal-phase HPLC of fraction (1), containing BChl-*a* and BChl-*c* (1), after isolation by a second preparative reverse-phase HPLC separation from the mixture shown in Figure 1 (0.8 ml/min; 50 bar; for solvent phase see Experimental section).

mixture of epimeric pairs (1)–(4) (comprising 10 BChl-*c* components) to a binary mixture of the known[14,15] mBPh-*c* epimers (9) established that all five BChl-*c* compounds have the same chlorin moiety in common.

Following the crystallographic and synthetic work of Smith *et al.*,[12b,16] the (2a*R*) configuration was attributed to those epimers in the Mg-free mixtures which are chromatographically (reverse-phase HPLC) more mobile, and whose α-*meso* ^{1}H NMR resonances in $CDCl_3$ are more shielded. In the case of compounds (5)–(9) these are the major components. Since epimerization should not have occurred during pheophytinization and transesterification, the major BChl-*c* components should also be the (2a*R*) epimers (Figure 3). In HPLC these epimers are now less mobile (normal-phase columns), and in the ^{1}H NMR spectrum the α-*meso* shifts in CD_4OD are now located at lower field. This was confirmed by the chromatographic and NMR features of artificial mixtures of BChl-*c* epimers before and after pheophytinization.

Integration of the HPLC peaks (assuming identical absorption coefficients for both epimers) and ^{1}H NMR signals of the α-*meso* protons (*cf.* Figure 3) consistently gave a (2a*R*:2a*S*) ratio of 2:1 for all four BChl-*c* fractions. This ratio proved to be constant for three independent batches of cell extractions.* Moreover, preparative, normal-phase HPLC

* *Note added in proof*: For a very recent report on similar (2a*R*:2a*S*) ratios of ensemble BChl-*c* from *C. aurantiacus* (strain J-10-fl), see F. W. Bobe, N. Pfennig, K. L. Swanson, and K. M. Smith, *Biochemistry*, 1990, **29**, 4340.

Table 1. Retention times and relative areas of the major BChl-*c* fractions (1)–(4) in Figure 1.[a]

	Fraction			
	(1)	(2)	(3)	(4)
Total membrane preparation[b,c]				
Retention time (min)	12.6 (0.1)	15.4 (0.1)	17.0 (0.1)	18.8 (0.3)
Relative ratio (%)[d]	27.1 (2.9)	25.0 (2.0)	23.3 (3.2)	100
GEF Chlorosome preparation[b,c]				
Retention time (min)	12.9 (0.4)	16.1 (0.4)	17.7 (0.4)	19.8 (0.5)
Relative ratio (%)[d]	32.8 (2.4)	24.5 (2.5)	20.3 (2.3)	100

[a] Data obtained by analytical HPLC, Nucleosil 5-C-18 column/MeOH. [b] Refs. 3 and 6*a*. [c] Standard deviations are given in parentheses and were obtained from several independent preparations. The differences in retention time between total membrane and GEF chlorosome preparations, which in part exceed the error limit, reflect the additional uncertainty in the reproducibility of the HPLC conditions for measurements performed throughout a period of 2 years. [d] Relative ratios normalized to the area of fraction (4) = 100%.

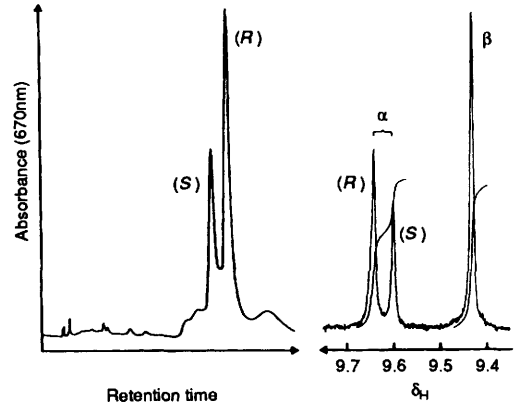

Figure 3. Preparative normal-phase HPLC (left; 2 ml/min; 26 bar; for solvent phase see Experimental section) and ^{1}H NMR analysis (right) of the BChl-*c* (**1**) portion isolated by preparative normal-phase HPLC from fraction (1) (and corresponding to the BChl-*c* peak shown in Figure 2): mixture of (2a*R*) and (2a*S*) epimers of compound (**1**).

allowed us to resolve fractions (1), (3), and (4) into their pure epimeric constituents. Fraction (2) gave two mixtures, each consisting of either the (*R*) or the (*S*) epimers of the 'cetyl' and 'oleyl' bacteriochlorophyllides-*c*.

The ^{1}H NMR values (Tables 2 and 3) agree satisfactorily with the respective literature data for the 2a-epimeric mixture of BChl-*c* (**4**) [the occurrence of both epimers of (**3**) has been demonstrated by NMR spectroscopy (Brune *et al.*[15])], and for the pheophytin derivatives of the pure 2a-epimers of compounds (**1**), (**3**), and (**4**), *i.e.* BPh-*c* (2a*R*)-(**5**), (2a*R*)-**7**), and (2a*R*)-(**8**) (Risch *et al.*[14]).

Structure Elucidation of the Bacteriochlorophylls-c (**1**), (**3**), *and* (**4**).—The mass spectra of each mixture of the corresponding BPh-*c* epimers (**5**), (**7**), and (**3**) exhibited the appropriate parent molecular ions (at *m/z* 838, 844, and 818, respectively) and fragmentation patterns.[14] Tables 2 and 3 list the ^{1}H NMR resonances of all six BChl-*c* components. Among the features characteristic of the bacteriochlorophyllide-*c* moiety are the ^{1}H AX_3 spectra for the 2-(1-hydroxyethyl) group, and the multiplets for the methine and secondary methyl protons at C(7) and C(8) of the reduced ring D. As has been reported for related chlorins bearing a 2-(1-hydroxyethyl) group,[18] most of the inherently non-isochronous resonances of the protons attached to the ring system of the epimers of compounds (**1**), (**3**), and (**4**) appear distinctly doubled. The shift differences of the more remote protons in the geranylgeranyl and phytyl side-chains [at C(2) and C(1,2,3a), respectively], however, which exceed any error limit, can hardly be ascribed solely to unimolecular diastereotopy. Rather, a contribution by diastereoisomeric BChl-*c* aggregates is quite likely.[19] Accordingly, the α-*meso* signals of the epimers of compound (**3**) remained split when measured in [$^2H_5$]pyridine–[^{2}H]chloroform (4:1), whereas the phytyl resonances coalesced to a broad singlet at δ 1.49. Pyridine is expected to act as a particularly strong ligand to magnesium and thus to prevent aggregation. The inherent α-*meso* shift differences may even be augmented by asymmetric ligation of pyridine to magnesium brought about by the asymmetric substitution of C(7) and C(8).

Table 4 shows the chemical shifts of the full ^{13}C NMR spectrum of the mBPh-*c* epimers, obtained from a mixture of (2a*R*)- and (2a*S*)-(**9**). The data of the high-field region fully agree with those reported by Risch *et al.*,[14] and all shifts are also satisfactorily compatible with the corresponding literature values for derivatives of mBPh-*c* and -*d*.[12a,20] Heteronuclear shift correlation (^{1}H–^{13}C COSY) confirmed the previous assignments of carbons bound to hydrogen. Our assignments of the quaternary carbons were adopted from the literature data.[12a,20]

The ^{13}C NMR spectra of the BChl-*c* derivatives (**1**), (**3**), and (**4**) were obtained from the pure (2a*R*) epimers in all cases, and also from the mixtures more abundant in the (2a*R*) epimer in the case of compounds (**3**) and (**4**). The resonances of the hydrogen-bound carbons (Table 4) were assigned on the basis of DEPT measurements of all three samples, and of a ^{1}H–^{13}C COSY experiment with compound (**4**) (Figure 4).

Apart from a Chl-*a*[21] and a BChl-*a* study,[22] the ^{13}C NMR spectra of the Mg-containing chlorophylls and bacteriochlorophylls have been investigated much less widely than those of the free-base derivatives. The assignment of the quaternary carbons of compounds (**1**), (**3**), and (**4**) by direct comparison with literature data was not possible. We assigned the lowest-field resonances at δ 175.05/175.07 and δ 199.78 (Table 4) to the ester carbonyl carbon C(7c) and to the ketone carbon C(9), respectively. The signals of all other quaternary carbons were assigned on the basis of a comparison with the most recently revised[20,21] ^{13}C chemical-shift differences between Chl-*a* and mPh-*a*. When these differences were combined with the values of mBPh-*c* (**9**) (Table 4), an acceptable correlation with the experimental shifts for compounds BChl-*c* (**1**), (**3**), and (**4**) was obtained.

Finally, Table 5 lists all ^{13}C resonances of the alcohol side-chains of compounds (**1**), (**3**), and (**4**). They are in good agreement with the limited data published,[14,22] and the geranylgeranyl shifts are also consistent with the values known for the free alcohol.[23] The signals of the olefinic quaternary ^{13}C signals of the phytyl and geranylgeranyl groups were distinguished from those of the chlorin moiety by comparison with literature data,[14,22,23] and the assignments were further confirmed by the trends exhibited by the three compounds,

Table 2. ^{1}H NMR data of the 2a-epimeric chlorin moieties of the bacteriochlorophylls-*c* (**1**)–(**4**).[a,b]

	(1)		(2)		(3)		(4)	
	(2a*R*)	(2a*S*)	(2a*R*)[c]	(2a*S*)[c]	(2a*R*)	(2a*S*)	(2a*R*)	(2a*S*)
α-H	9.68 (s)	9.63 (s)	9.68 (s)	9.63 (s)	9.68 (s)	9.63 (s)	9.68 (s)	9.63 (s)
β-H	9.45 (s)		9.45 (s)		9.45 (s)		9.45 (s)	
2a-H	6.30 (q, 6.7)	6.33 (q, 6.8)		6.33 (q, 6.7)	6.31 (q, 6.7)	6.32 (q, 6.6)		6.34 (q, 6.5)
10-H_2	5.17, 5.08 (AB, 20.0)	5.18, 5.09 (AB, 20.0)	5.21, 5.06 (AB, 20.0)	5.19, 5.09 (AB, 20.0)	5.17, 5.12 (AB, 20.0)	5.21, 5.10 (AB, 20.0)	5.22, 5.12 (AB, 19.6)	5.21, 5.10 (AB, 19.6)
8-H	4.58 (m)	4.60 (m)		4.57 (m)	4.60 (m)	4.62 (m)		4.63 (m)
7-H	4.08 (m)	4.10 (m)	4.10 (m)	4.11 (m)	4.10 (m)	4.09 (m)		4.15 (m)
δ-Me	3.80 (s)		3.80 (s)		3.80 (s)		3.83 (s)	
4a-H_2	3.74 (q, 7.7)		3.74 (q, 7.8)		3.74 (q, 7.7)		3.76 (q, 8.2)	
5a-Me	3.57 (s)		3.57 (s)		3.57 (s)		3.59 (s)	
1-Me	3.30 (s)	3.32 (s)	3.32 (s)	3.33 (s)	3.32 (s)	3.33 (s)	3.33 (s)	3.34 (s)
3-Me	3.25 (s)		3.25 (s)		3.25 (s)		3.30 (s)	
7b-H_2	2.40–2.50 (m)		2.30–2.50 (m)		2.40–2.50 (m)		2.40–2.55 (m)	
7a-H_2	2.10–2.20 (m)		2.00–2.30 (m)		2.10–2.25 (m)		2.15–2.30 (m)	
2-CH*Me*	2.07 (d, 6.6)	2.09 (d, 6.6)	2.07 (d, 6.6)	2.09 (d, 6.7)	2.07 (d, 6.6)	2.09 (d, 6.6)	2.07 (d, 6.6)	2.09 (d, 6.6)
4-CH_2*Me*	1.70 (t, 7.5)		1.70 (t, 7.5)		1.70 (t, 7.6)		1.70 (t, 7.7)	
8-Me	1.51 (d, 6.6)	1.50 (d, 6.5)	1.51 (d, 6.9)	1.50 (d, 7.1)	1.51 (d, 7.0)	1.50 (d, 7.0)	1.52 (d, 7.0)	1.50 (d, 7.0)

[a] Designations of spin multiplicities (s, d, t, q, m, AB) and coupling constants (in Hz) in parentheses. Concentration 15 mM in CD_3OD. See ref. 15 for comparison of part of these data with literature values. [b] Chemical shifts (δ). Interposed values refer to chemical shifts identical for both the (2a*R*) and (2a*S*) epimers. [c] The shifts for (2a*R*)- and (2a*S*)-(**2**) were determined with mixtures of the corresponding 'cetyl' and 'oleyl' epimers.

Table 3. ^{1}H NMR data of the alcohol side-chains of the 2a-epimeric bacteriochlorophyllides-*c* (**1**)–(**4**).[a]

	Geranylgeranyl (**1**)			'Cetyl' and 'oleyl' (**2**)			Phytyl (**3**)			'Stearyl' (**4**)	
	(2a*R*)	(2a*S*)		(2a*R*)	(2a*S*)		(2a*R*)	(2a*S*)		(2a*R*)	(2a*S*)
2-H	5.01 (m)	5.11 (m)	9-, 10-H[b]	5.21 (m)	5.22 (m)	2-H	5.06 (m)	5.14 (m)	1-H_2	3.73 (t, 6.5)	3.74 (t, 6.6)
1-H_2	4.30 (m)	4.39 (m)	1-H_2[b,c]	3.74 (m)		1-H_2	4.36 (m)	4.44 (m)	-H_2 2–17	1.11–1.28 (m)	
4-, 5-, 8-, 9-, 12-, 13-H_2	1.79–2.02 (m)	1.78–2.00 (m)	8- and 11-H_2[b]	1.88 (m)		4-H_2	1.84 (m)	1.91 (m)	18-H_3	0.87 (t, 6.9)	
3-, 7-, 11-, 15-, 15-Me	1.40–1.62 (m)		7- and 12-H_2[b]	1.53 (m)		3-Me	1.54 (s)	1.60 (s)			
			2–6[b,c], 7–15[c] and 13–17[b] H_2	1.10–1.40 (m)		7-, 11-, 15-, 15-Me	0.67–0.81[d]	0.68–0.82[d]			
			16[c], 18[b] H_3	0.82 (t, 6.8)							

[a] See footnotes *a* and *b* of Table 2; see also ref. 17 for further literature data. [b] 'Oleyl'. [c] 'Cetyl'. [d] Values for signals of the methyl doublets. Chemical shifts were not determined.

considering the fact that compound (**4**) contains no such carbons.

Structure Elucidation of the Two New Bacteriochlorophylls-c (**2**).—Hydrolysis of compound (**2**) with potassium hydroxide in methanol afforded a 3:7 mixture of 'cetol' (hexadecan-1-ol) and 'oleol' (octadec-9-en-1-ol), readily identified with authentic samples by gas chromatography and mass spectrometry. The NMR (Tables 2–5), mass, and IR spectra are fully consistent with this assignment. The presence of a *cis*-1,2-disubstituted double bond in the alcohol side-chains of one of the BChl-*c* components, (**2**), was additionally demonstrated by NMR signals for two olefinic (δ 5.22) and four allylic protons (δ 1.88) and two olefinic carbons (δ_C 130.78 and 130.71). The absence of a *trans*-out-of-plane deformation band in the IR spectrum between 960–970 cm^{-1} and of *trans*-allylic resonances in the ^{13}C

Table 4. ^{13}C NMR chemical shifts of the 2a-epimeric chlorin moieties of the bacteriochlorophylls-*c* (**1**)–(**4**) and of bacteriopheophorbide-*c* methyl ester (**9**).[a]

Carbon	BChl-*c* (**1**)–(**4**)[b] (2a*R*)	BChl-*c* (**1**)–(**4**)[b] (2a*S*)[d]	mBPh-*c* (**9**)[c] (2a*R* and *S*)
3-*Me*	11.13		10.91
5-Me	12.74		11.70
1-Me	17.65	17.73	16.37
4-CH_2*Me*	18.08		17.09
4-*C*H_2Me	20.42		19.21
δ-Me	21.96		20.15
8-Me	21.42	21.38	20.68
2-CH*Me*	26.68		25.49
7b	31.25	31.18	29.81
7a	31.93	31.87	30.85
8	49.84		48.27
10	49.92		48.49
7	51.76	51.79	51.42
2a	66.34	66.40	64.97
α	101.51	101.32	97.53
β	106.65		102.53
δ	106.38	106.42	105.53
γ	106.06		105.84
5	134.78		128.14
6	131.95		130.32
1	136.55	136.50	131.46
3	133.70		133.60
12		147.15	136.60
15	147.21	148.99	138.68
11		155.37	140.66
2	155.40	147.11	142.73
4	147.04	144.73	144.25
16		155.65	147.46
14		147.60	151.42
13		152.38	153.32
17		162.35	159.08
18		169.41	172.79
CO_2R	169.46	175.06	173.69
9	175.05	199.78	197.10
MeO			51.45

[a] See footnote *b* of Table 2. Chemical shifts relative to internal tetramethylsilane [for compounds (**1**)–(**4**)] and to the centre of $CDCl_3$ at δ 77.0 [for compound (**9**)]. See ref. 14 for comparison of part of the data for compound (**9**) with literature values. [b] Concentration 15 mM in CD_3OD. See also footnote *c* in Table 2. [c] Concentration 15 mM in $CDCl_3$–CD_3OD (4:1). [d] Determined only for compounds (**3**) and (**4**) in (2a*R*,*S*) mixtures.

NMR spectrum at δ_C ca. 33–34 is consistent with the assigned *cis* configuration [in the spectrum of compound (**2**) the *cis*-allylic ^{13}C resonances overlap with those of the other aliphatic carbons at δ 29.3–31.7].[24] Integration of the allylic and olefinic NMR proton signals in compound (**2**) again gave results consistent with a 3:7 ratio of cetyl and oleyl side-chains. The double-bond position was ascertained by its oxidative cleavage with osmium tetraoxide and potassium periodate in the alcohol fraction of the hydrolysate, which furnished nonanal. Together with the identification of the chlorin as a 2a-epimeric mixture of bacteriochlorophyllides-*c* (*vide supra*), the structure of the components of compound (**2**) was thus unequivocally established.

Conclusions.—The five bacteriochlorophyllides-*c* in the chlorosomes of *C. aurantiacus* (strain OK-70-fl) differ only with regard to the alcohol side-chain, but not also with regard to the substitution of the chlorin nucleus as is the case with other phototrophic bacteria such as the Chlorobiaceae.[12] Noteworthy are the predominance (*ca.* 70%) of non-isoprenoid linear alcohol side-chains including the two novel derivatives of compound (**2**), and the 2:1 ratio of 2a-epimers, both in particular with regard to a possible relevance to the aggregate structure within the chlorosomes.

Experimental

A Merck L-6200 solvent-delivery system and a Shimadzu SPD 6AV absorbance detector served for analytical HPLC. The normal-phase column (125 × 4.6 mm) was packed with Lichrospher-10μ-diol-100 [cyclohexane–propan-2-ol (33:1) as the eluant], and the reverse-phase column (125 × 4.6 mm) with Nucleosil 5-C-18 (MeOH). Preparative HPLC was carried out with a Gilson instrument consisting of a Spectrochrom M absorbance detector, a Model 303 solvent-delivery system, and a Model 302 injector. Preparative normal-phase columns (250 × 8 mm; cyclohexane + 2–7% propan-2-ol) had the same packing as the corresponding analytical columns. The preparative reverse-phase columns (250 × 20 mm; MeOH) were packed either with Nucleosil 5-C-18 or with Lichroprep RP-18. In preparative flash chromatography 26 mm columns packed with Merck silica gel 60 (0.042–0.060 mm) were used. Gas chromatographic analyses were performed with a Packard 427 instrument equipped with a 20 m column packed with Carbowax 20M (0.8 atm H_2; column temperature programmed from 70–250 °C at 6 °C/min; injection port and detector temperatures 180 and 285 °C, respectively). High-field ^1H and broadband decoupled ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer, operating at 400.136 MHz for ^1H and 100.614 MHz for ^{13}C. DEPT and COSY two-dimensional ^1H–^{13}C correlation spectra were run on a Bruker AC-270 spectrometer (at 270.134 MHz for ^1H and 67.925 MHz for ^{13}C). All spectra were measured at 25 °C in 1 ml solutions in a 5-mm-wide tube. Concentrations and solvents were 0.035M in $(CD_3)_2CO$–CD_3OD (4:1) for the COSY spectra, and 0.015M in CD_3OD for all other spectra. The chemical shifts were calibrated with tetramethylsilane; see also footnotes *a* and *b* of Table 2. After NMR experiments requiring long acquisition times, the purity of the samples was analysed by visible absorption spectroscopy and HPLC. The visible absorption spectra were measured with Cary 17 and Hitachi 100-80 spectrometers. Mass spectra were acquired with a Varian Mat-CH5 spectrometer in the electron-impact mode (direct-insertion probe, 70 eV; source temperature from 200–300 °C). Coupled gas chromatography–mass spectrometry was performed with a Hewlett-Packard MSD 5870 instrument using the column and conditions described for gas chromatography, and IR spectra were measured with a Perkin-Elmer 580 spectrophotometer (data not given). Centrifugations were carried out with a Beckmann JA-10 rotor.

Chemicals and Organism.—All solvents were distilled prior to use. [$^2H_6$]Acetone (99.8%), [^2H]chloroform (99.5%), [$^2H_4$]methanol (99.5%), oleol (octadec-9-en-1-ol), and [$^2H_5$]pyridine (99%) were obtained from Merck, and cetol (hexadecan-1-ol) from Sigma. *Chloroflexus aurantiacus*, strain OK-70-fl, was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig (no. 636).

Cell Growth and Pigment Extraction.—Strain OK-70-fl of *C. aurantiacus* was grown in batch cultures as described previously.[6a] The cells were harvested by centrifugation (20 min; 8 000 rpm; 5 °C) and stored at −80 °C. The total BChl-*c* was extracted with MeOH, and most of the carotenoids were precipitated by addition of water (10% v/v) according to the method of Brune *et al.*[15] Further purification of the supernatant extract (containing BChl-*a*, -*c*, and residual carotenoids) first involved two consecutive reverse-phase HPLC steps:

Table 5. ^{13}C NMR chemical shifts of the alcohol side-chains of the 2a-epimeric bacteriochlorophyllides-*c* (**1**)–(**4**).[a]

Geranylgeranyl [(2a*R*)-(**1**)]		'Cetyl' and 'oleyl' [(2a*R*)-(**2**)]			Phytyl (**3**) (2a*R*)	Phytyl (**3**) (2a*S*)		'Stearyl' (**4**) (2a*R*)	'Stearyl' (**4**) (Sa*S*)
C(1)	62.16	C(9, 10)[b]	130.78	C(1)	62.17	62.13	C(1)	65.55	65.62
C(2)	119.58		130.71	C(2)	119.50	119.55	C(2)	29.36	29.45
C(3)	143.20	C(1)	65.55	C(3)		143.70	C(3)	26.78	26.85
C(4)	40.34	C(3)	26.77	C(4)	40.59	40.63	C(4–15)	29.3–30.7	
C(5)	26.99	C(16,[c] 18[b])	14.42	C(5)	25.88	25.92	C(16)	33.05	
C(6)	124.85	C(15,[c] 17[b])	23.68	C(6)	33.44	33.38	C(17)	23.71	
C(7)	136.22	C(14,[c] 16[b])	32.99	C(7)	33.62	33.60	C(18)	14.43	
C(8)	40.62		29.34–31.71	C(8)		38.20			
C(9)	27.38			C(9)		25.31			
C(10)	125.29			C(10, 12)		38.30			
C(11)	135.69					38.28			
C(12)	40.70			C(11)		33.76			
C(13)	27.71			C(13)		25.77			
C(14)	125.39			C(14)		40.43			
C(15)	131.91			C(15)		29.08			
C(16)	25.85			C(3-*Me*)	16.28		16.30		
C(3-*Me*)	16.36			C(7-, 11-*Me*)		20.08			
						20.10			
C(7-, 11-*Me*)	15.99			C(15—Me_2)		22.99			
	16.02					23.09			
C(15-Me_2)	17.71								

[a] See footnotes *a* and *b* of Table 4. See refs. 14, 22–24 for comparison with part of these data. [b] 'Oleyl'. [c] 'Cetyl'.

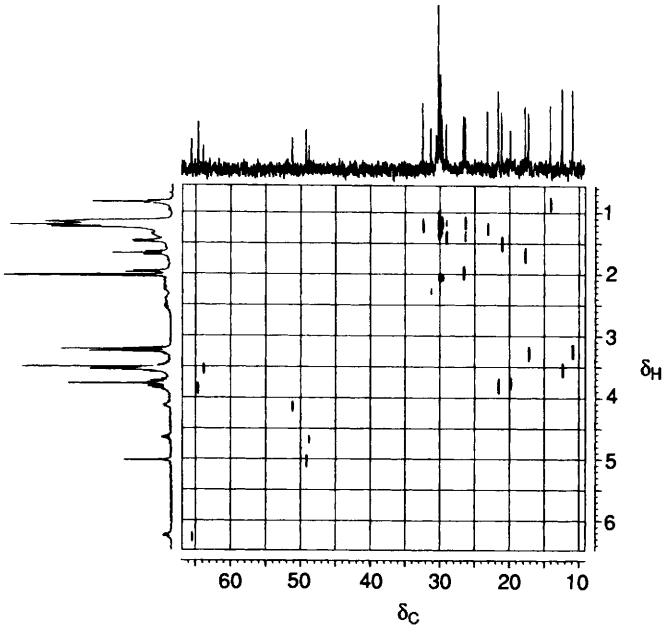

Figure 4. ^1H–^{13}C COSY of the (2a*R*)-BChl-*c* epimer (**4**) [30 mM in $(CD_3)_2CO$–CD_3OD (4:1); 25 °C] between δ 0.5–6.3 for the proton resonances, and between δ_C 10–65 for the carbon resonances.

Lichroprep RP-18 with MeOH–water (9:1) removed much of the residual carotenoids, and on Nucleosil 5-C-18 with MeOH the BChl-*c* fractions (1)–(4) (Figure 1) were then separated. While the fractions (3) and (4) proved to represent homogenous samples of BChl-*c* (**3**) and (**4**), respectively, fractions (1) and (2) were still contaminated by BChl-*a* and carotenoids (see Figure 2) which could now be removed by normal-phase HPLC on Lichrospher-diol (Figure 3). This normal-phase HPLC was used further to isolate the pure 2a-epimers of compounds (**1**)–(**4**). Each BChl-*c* fraction was then taken to dryness, the residues were dissolved in a minimal volume of CH_2Cl_2, precipitated by addition of light petroleum (b.p. range 40–60 °C) at 0 °C, and the precipitates were collected by centrifugation. All operations were performed in dim light. From wet cells (80 g), the four BChl-*c* fractions were obtained in the following amounts: (**1**) (19.5 mg), (**2**) (14.5 mg), (**3**) (12 mg), and (**4**) (59 mg). For the NMR data see Tables 2 and 3 (^1H), 4 and 5 (^{13}C), and Figure 4.

Bacteriopheophytins-c (**5**)–(**9**).—To a solution of each fraction (5 mg) of BChl-*c* (**2a**)-epimers (**1**)–(**4**) in CH_2Cl_2 (50 ml) was added an equal volume of 0.1 M-HCl. After being shaken, the CH_2Cl_2 layer was decanted, washed with 10% aq. Na_2CO_3, dried over anhydrous Na_2SO_4, and evaporated to dryness. Flash chromatography on silica gel [CH_2Cl_2–MeOH (49:1)] afforded the corresponding pure BPh-*c* samples (**5**)–(**8**) in *ca.* 80% yield each. The mass and ^1H NMR data of compounds (**5**), (**7**) and (**8**) are in agreement with the published spectra;[14] compound (**6**) showed *m/z* 816 [M^+ ($C_{52}H_{72}N_4O_4$), 100%], 798 (45), 790 [M^+ ($C_{50}H_{70}N_4O_2$), 45], and 772 (21); $\delta_{chlorin}$ (3 mg/0.6 ml $CDCl_3$) 9.93 (2a*S*, s) + 9.91 (2a*R*, s) (α-H), 9.45 (s) (β-H), 6.51 (q, *J* 6.5 Hz) + 2.15 (*S*, d, *J* 6.5 Hz) + 2.13 (*R*, d, *J* 6.5 Hz) [CH(2a)Me], 5.19 (m, 10-H_2), 4.57 (m, 8-H), 4.17 (m, 7-H), 3.87 (s, δ-Me), 3.69 (q, *J* 7.6 Hz) + 1.68 (t, *J* 7.6 Hz) (4-Et, 3.65 + 3.50 + 3.27 (3 s) (5-, 1-, + 3-Me, resp.), 2.47 + 2.15 (2 m) [7-$(CH_2)_2$], and 1.47 (*S*, d, *J* 7.5 Hz) + 1.48 (*R*, d, *J* 7.2 Hz) (8a-Me); $\delta_{cetyl',oleyl'}$ 5.28 (m, CH=$CH_{oleyl'}$), 3.93 (m, 2 × 1-H_2), 1.93 (m, 8- and 11-$H_{2\text{-}oleyl'}$), 1.58 (m, 7- and 12-$H_{2\text{-}oleyl'}$), 1.0–1.4 [m, $(CH_2)_{14}$ + 2 × $(CH_2)_5$], and 0.82 (t, 6.8 Hz, 2 × Me).

Bacteriopheophorbide-*c* methyl ester (**9**) was obtained by standard transesterification procedures.[14] Preparative reverse-phase HPLC with MeOH–water (9:1) resolved the epimers in yields of 85 mg (2a*R*)-mBPh and 45 mg (2a*S*)-mBPh from wet cells (80 g). For the ^{13}C NMR data see Table 4, and for the mass spectrum, ref. 14.

Ester Hydrolysis of the Mixture of 'Cetyl' (Hexadecanyl) and 'Oleyl' (Octadec-9-enyl) Bacteriochlorophyllides-c (**2**).—Compound (**2**) (10 mg) was dissolved in 1% KOH in MeOH (100 ml) and kept overnight under N_2 at room temperature. After addition of water (50 ml) the reaction mixture was extracted with diethyl ether (3 × 50 ml). The combined organic portions were washed with water, dried over Na_2SO_4, and evaporated

under reduced pressure. Chromatography of the crude alcohol mixture on silica gel with light petroleum–diethyl ether (1:1) gave a waxy, colourless, 3:7 mixture (2 mg) of cetol (hexadecan-1-ol) and oleol [(Z)-octadec-9-en-1-ol], identified by comparison with authentic samples by IR and GC–MS analysis.

Oxidative Double-bond Cleavage in the Hydrolysate.—The above alcohol mixture (1 mg) was treated at room temperature with a catalytic amount of OsO_4 in the presence of $NaIO_4$ in 1,4-dioxane–water (1:1 v/v).[25] Work-up gave a crude product which, according to coupled GC–MS, contained nonanal (*m/z* 142, M^+, $C_9H_{18}O$) as a new product. The aldehyde was identified with the product of a cleavage of authentic oleol under the same conditions.

Acknowledgements

We thank S. Emunds and D. Pagirnus (growth of cells), M. Trinoga and H. Selbach (HPLC), U. Schappert, D. Kreft, J. Bitter, and W. Riemer (NMR) for technical assistance. Part of this work will be included in the Ph.D thesis of K.G. (Heinrich-Heine-Universität, Düsseldorf). The Alexander-von-Humboldt Foundation supported F.F. with a postdoctoral fellowship.

References

1 J. M. Olson, *Biochim. Biophys. Acta*, 1980, **594**, 33.
2 K. Schmidt, *Arch. Microbiol.*, 1980, **124**, 21.
3 A. R. Holzwarth, K. Griebenow, and K. Schaffner, *Z. Naturforsch., Teil C*, 1990, **45**, 35.
4 K. Griebenow, A. R. Holzwarth, and K. Schaffner, *Z. Naturforsch., Teil C*, 1990, **45**, in the press.
5 D. W. Kupke and C. S. French, 'Encyclopedia of Plant Physiology,' ed. W. Ruhland, Springer Verlag, Berlin, 1960, vol. V, p. 298; D. L. Worcester, T. J. Michalski, and J. J. Katz, *Proc. Natl. Acad. Sci. USA*, 1986, **83**, 3791.
6 (*a*) K. Griebenow and A. R. Holzwarth, *Biochim. Biophys. Acta*, 1989, **973**, 235; (*b*) K. Griebenow and A. R. Holzwarth in 'Molecular Biology of Membrane-bound Complexes in Phototrophic Bacteria,' ed. G. Drews, in the press; (*c*) K. Griebenow, M. G. Müller, and A. R. Holzwarth, *ibid.*; (*d*) A. R. Holzwarth, M. G. Müller, and K. Griebenow, *J. Photochem. Photobiol. B*, 1990, **5**, 457.
7 K. Griebenow, Diploma Thesis, Philipps-Universität, Marburg/MPI Strahlenchemie, Mülheim a.d. Ruhr, 1988.
8 V. van Mourik, K. Griebenow, B. van Haeringen, A. R. Holzwarth, and R. van Grondelle, 'Current Research in Photosynthesis,' ed. M. Baltscheffsky, Kluwer, Dordrecht, 1990, vol. 2, p. 141.
9 T. Schirmer, W. Bode, R. Huber, W. Sidler, and H. Zuber, *J. Mol. Biol.*, 1985, **184**, 257.
10 B. W. Matthews and R. E. Fenna, *Acc. Chem. Res.*, 1980, **13**, 309.
11 H. Zuber, *Photochem. Photobiol.*, 1985, **42**, 821; *Trends Biochem. Sci.*, 1986, **11**, 414.
12 (*a*) K. M. Smith, M. J. Bushell, J. Rimmer, and J. F. Unsworth, *J. Am. Chem. Soc.*, 1980, **102**, 2437; (*b*) K. M. Smith, L. A. Kehres, and H. D. Tabba, *ibid.*, p. 7149; (*c*) M. B. Caple, H.-C. Chow, and C. E. Strouse, *J. Biol. Chem.*, 1978, **253**, 6730.
13 A. Gloe and N. Risch, *Arch. Microbiol.*, 1978, **118**, 153.
14 N. Risch, H. Brockmann, and A. Gloe, *Liebigs Ann. Chem.*, 1979, 408.
15 D. C. Brune, T. Nazawa, and R. E. Blankenship, *Biochemistry*, 1987, **26**, 8644.
16 K. M. Smith, G. M. F. Bisset, and M. J. Bushell, *J. Org. Chem.*, 1980, **45**, 2218; K. M. Smith, D. A. Goff, J. Fajez, and K. M. Barkigia, *J. Am. Chem. Soc.*, 1982, **104**, 3747.
17 J. J. Katz, L. L. Shipman, T. M. Cotton, and T. R. Janson, 'The Porphyrins,' ed. D. Dolphin, Academic, New York, 1978, vol. V, p. 401.
18 H. Scheer and J. J. Katz, 'Porphyrins and Metalloporphyrins,' ed. K. M. Smith, Elsevier, Amsterdam, 1975, p. 399.
19 J. K. M. Sanders, J. C. Waterton, and I. S. Denniss, *J. Chem. Soc., Perkin Trans. 1*, 1978, 1150.
20 K. M. Smith and D. A. Goff, *J. Chem. Soc., Perkin Trans. 1*, 1985, 1099.
21 S. G. Boxer, G. L. Closs, and J. J. Katz, *J. Am. Chem. Soc.*, 1974, **96**, 7058.
22 R. G. Brereton and J. K. M. Sanders, *J. Chem. Soc., Perkin Trans. 1*, 1983, 435.
23 Y. Tanaka, H. Sato, A. Kageyu, and T. Tomita, *J. Chromatogr.*, 1985, **347**, 275.
24 E. Bascetta and F. D. Gunstone, *Chem. Phys. Lipids*, 1985, **36**, 253.
25 L. F. Fieser and M. Fieser, 'Reagents for Organic Synthesis,' Wiley, New York, 1967, vol. 1, p. 809.

Paper 0/01556C
Received 6*th April* 1990
Accepted 14*th June* 1990