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CEREBROSIDES IN MATURE-GREEN AND RED-RIPE BELL PEPPER AND TOMATO FRUITS

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Key Word Index—*Capsicum annum*; *Lycopersicon esculentum*; Solanaceae; bell pepper; tomato; cerebrosides; fruits; ripening; HPLC analysis.

Abstract—Cerebrosides (CBs) isolated from pericarp lipids of green and red bell pepper and tomato fruits were separated by a new HPLC method into ≥ six components. HPLC profiles were similar for the two fruits and changed little with ripening. CB 1 was the major peak, CB 2 was found only in bell pepper, and CBs 4, 5 and 6 were more abundant in pepper than in tomato. Total CBs and HPLC CB fractions were cleaved for analysis of fatty acid (FA), sphingoid (SP) and sugar moieties. FAs of total CBs were >95% saturated 2-hydroxyl. In CBs 1 and 2, 2-hydroxyl 16:0 was ≥98% of the total FAs, whereas 2-hydroxyl 22:0, 23:0 and 24:0 predominated in CBs 4–6. The SPs of CBs 1 and 2 were tentatively identified as the 8-*cis* and 8-*trans* isomers of 4,8-sphingadienine and 8-sphingenine, respectively, whereas the SPs of CBs 4–6 were identified as isomers of 4-hydroxy-8-sphingenine. Glucose was the only sugar in bell pepper and tomato CBs. The major CB in both fruits was deduced to be 1-*O*-β-glucosyl-*N*-(2′hydroxypalmitoyl)-4-*trans*-8-*cis*-sphingadienine.

INTRODUCTION

Cerebrosides (CBs) are a group of glycosphingolipids composed of a hexose, a long-chain aminoalcohol (sphingoid; SP) and an amide-linked long-chain fatty acid (FA). Galactocerebrosides were first isolated from brain tissue in the late 1800s [1]. These mammalian CBs include a mix of saturated and monoenoic *n*-alkyl- and 2-hydroxy-FAs, with sphingosine (4-*trans*-sphingenine, a 1,3-dihydroxy-2-aminoalcohol) as the major SP [1]. Over 30 years ago, glucocerebrosides (glucoCBs) were identified as a minor (<1%) lipid class in plant tissues [2]. Perhaps because they constitute such a small portion of the total tissue lipids, plant glucoCBs received little attention prior to the recent finding (following development of the two-phase membrane partitioning method) that they can represent a major fraction of the total lipids in both the plasma membrane and tonoplast [3–6]. Several reports have also implicated glucoCBs as playing a role in chilling injury [5] and in acclimation to cold and water-deficit stress [3, 7] in different plant species.

The most thorough chemical and physical characterization has been carried out with CBs isolated from seeds and/or leaves of a few monocot and dicot crop species, including rice, oats, winter rye, soybean, mung bean and spinach [6]. The CBs from all plant sources examined thus far have glucose as the sole hexose in a β-1 linkage with the 1-hydroxyl of the SP. It also appears that plant CBs typically include mostly 2-hydroxy-FAs, saturated or monoenoic, ranging from C_{14} to C_{26}. The major SPs are typically the dihydroxyl bases sphinganine, 8-sphingenine and 4,8-sphingadienine, and the trihydroxyl bases 4-hydroxysphinganine (phytosphingosine) and 4-hydroxy-8-sphingenine. The most detailed information on plant CB structure is available for leaves and grains of cereal crops. One generalization from these studies is that a higher degree of SP hydroxylation occurs in leaf compared with seed CBs [6]. The proposed involvement of CBs in chilling injury and in the capacity to acclimate to cold is based on the demonstration that these glycosphingolipids exhibit a phase-transition temperature (T_m) well above ambient (35–56°) and also elevate the T_m of membrane phospholipids [5, 8].

In vegetables and fruits, CBs have been isolated from tubers of white yam [9], sweet potato [10], potato [11] and also from apples [12]. CBs from potatoes and apples were shown to contain only glucose and mostly or exclusively 2-hydroxy-FAs; the SPs were not identified [11, 12]. In the present study, CBs were isolated from outer pericarp tissue of two solanaceous fruits (bell pepper and tomato) at two stages of ripening (mature-green and red-ripe) and characterized after separation by a newly devised reverse-phase HPLC method employing a C_6 (hexyl) column.

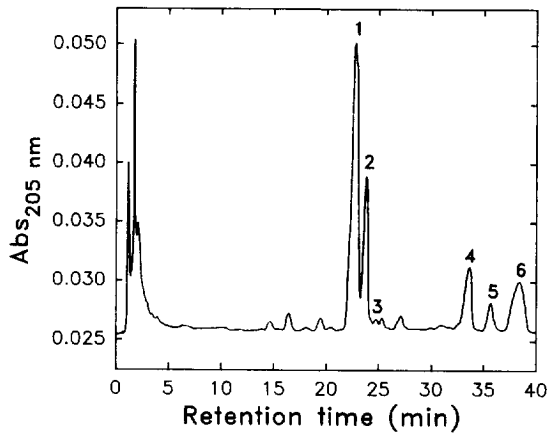

Fig. 1. HPLC profile of cerebrosides (CBs) isolated from pericarp tissue of mature-green bell pepper fruit. The six CB peaks detected by UV monitoring at 205 nm over a 40 min run are indicated by the numerals 1–6. Several minor components were not quantified or characterized.

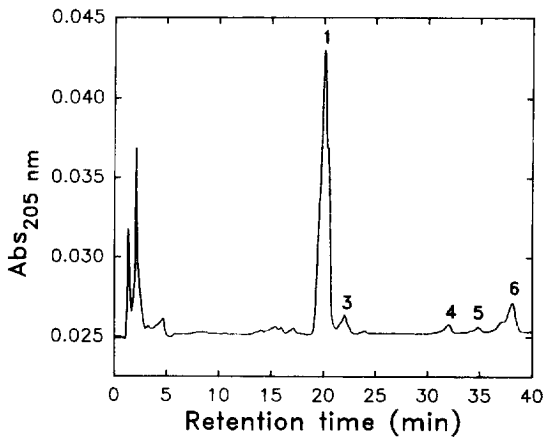

Fig. 2. HPLC profile of cerebrosides (CBs) isolated from pericarp tissue of mature-green tomato fruit. The five CB peaks detected by UV monitoring at 205 nm over a 40 min run are indicated by the numerals 1, 3 and 4–6, corresponding to the same peaks noted in the profile of bell pepper fruit CBs (Fig. 1).

RESULTS

The HPLC profiles of CBs isolated from pericarp tissue of mature-green bell pepper fruit are shown in Fig. 1. The major component (CB 1) eluted after *ca* 22 min and was followed *ca* 1 min later by a second prominent peak (CB 2). The remaining three sizeable peaks (CBs 4–6) eluted between 31 and 39 min. The minor components preceding CB 1 are steryl glycoside contaminants. The proportions of the major CBs changed only slightly with ripening (Table 1). A small decrease in the amounts of CBs 1 and 2 was offset by small increases in CBs 3–5. Several minor CBs that eluted between 25 and 31 min (Fig. 1) composed *ca* 2–4% of the total, but these were not individually quantified or characterized. HPLC profiles of CBs from green tomato fruit are shown in Fig. 2. With the exception of CB 2, which was absent, the major CBs in tomato were the same as those found in bell pepper. CB 1 was predominant in tomato, and CB 6 was second in order of abundance. Changes in the proportions of individual CBs with ripening were more pronounced in tomato than in bell pepper (Table 1). A decrease in CB 1 was balanced by increases in CBs 3, 5 and 6. The HPLC profile in Fig. 2 shows a splitting of the CB 1 peak, indicating the presence of two components. This split was more clearly evident in the CB profiles of both red-ripe tomato and bell pepper (data not shown). The other major CB peaks also generally appear to have one or more 'shoulders'.

Quantification of total CBs per g fr. wt of pericarp tissue was accomplished by determining the amount of glucose and the amount of 2-hydroxy-FAs (using 2-hydroxyl 18:0 methyl ester as internal standard) recovered after HCl methanolysis of CB samples. The CB concentration was *ca* four-fold higher in bell pepper compared with tomato pericarp. With ripening from green to red, CBs declined from 45 to 36 nmol g^{-1} fr. wt in bell pepper and from 10.1 to 9.8 nmol g^{-1} fr. wt in tomato (mean values of three determinations).

The 2-hydroxy-FA compositions of total CBs from green and red fruits and of HPLC-purified CB fractions are shown in Table 2. Six FAs were detected in amounts exceeding *ca* 0.8 wt % of the total. These included 2-hydroxyl 16:0 (16:0h) and both odd and even carbon-chain FAs ranging from 2-hydroxyl 22:0

Table 1. Cerebroside (CB) composition of pericarp tissue from mature-green (MG) and red-ripe (RR) bell pepper and tomato fruits determined by C_6 HPLC. Values indicate the area percentage of total CB peaks measured at 205 nm and represent the mean of three determinations ±s.d.

CB peak	HPLC R_t time (min)	Bell pepper fruit		Tomato fruit	
		MG	RR	MG	RR
1	21.8	48±4	45±3	74±5	76±4
2	23.0	18±3	17±2	—	—
3	24.3	2±1	4±1	4±1	7±2
4	32.2	13±2	15±3	4±1	4±1
5	35.3	4±1	5±1	4±1	5±1
6	38.4	15±2	14±2	14±3	18±3

Table 2. Fatty acid composition of total cerebrosides (CBs) and HPLC CB fractions from pericarp tissue of mature-green (MG) and red-ripe (RR) bell pepper and tomato fruits. Fatty acids were all 2-hydroxyl and were analysed as their methyl esters by FID-GC. Values are expressed as wt % of total methyl esters and represent the mean of three determinations ±s.d. HPLC CB fractions were pooled from both MG and RR fruits

Fatty acid	Bell pepper fruit						Tomato fruit		
	MG-CBs	RR-CBs	CB 1	CB 2	CBs 4 + 5	CB 6	MG-CBs	RR-CBs	CB 1
16:0h	63±4	60±4	97±1	96±1	4±1	—	75±4	70±3	96±1
22:0h	13±1	15±2	1±<1	<1±<1	70±4	<1±<1	5±1	5±1	<1±<1
23:0h	4±1	5±1	<1±<1	1±<1	14±2	1±<1	3±1	4±1	1±<1
24:0h	17±3	17±2	2±<1	3±1	11±2	98±1	15±2	19±2	3±1
25:0h	2±<1	2±<1	—	—	1±<1	—	1±<1	2±<1	—
26:0h	1±<1	1±<1	—	—	<1±<1	—	<1±<1	<1±<1	—

to 26:0 (i.e. 22:0h, 23:0h, 24:0h, 25:0h and 26:0h). For both bell pepper and tomato, 16:0h was the predominant FA in total CBs, regardless of the stage of ripening. Both 22:0h and 24:0h were also major FAs in bell pepper CBs, whereas 24:0h was the only other major FA in tomato CBs. The FAs of CBs 1 and 2 from bell pepper, as well as CB 1 from tomato, consisted almost exclusively of 16:0h ($\geq$96%). FAs of the combined HPLC fraction of CBs 4 and 5 from bell pepper were mainly 22:0h and 23:Oh in a ratio of *ca* 2:1, whereas FAs of CB 6 from bell pepper were composed almost entirely of 24:0h.

Peracetates of three sphingoid standards (sphinganine, 4-*trans*-sphingenine and 4-hydroxy-sphinganine) and of the SPs derived from tomato CB 1, bell pepper CB 1, bell pepper CB 2 and bell pepper CBs 4 + 5 + 6 (pooled) were purified by HPLC and analysed by capillary FID–GC. The order of HPLC retention times of the various SP acetates was tomato CB 1 = bell pepper CB 1 (7.8 min) < bell pepper CBs 4 + 5 + 6 (8.2 min) < bell pepper CB 2 (8.6 min) < 4-*trans*-sphingenine (9.0 min) < 4-hydroxysphinganine (9.8 min) < sphinganine (10.7 min). Thus, the SP acetates from CB 1, CB 2, and CBs 4 + 5 + 6 differed in their HPLC retention times; all eluted earlier than any of the SP acetate standards. As with HPLC, the GC retention times of SP acetates from the fruit CB fractions did not match those of any of the three standards. Among the standards, introduction of the 4-*trans* double bond into sphinganine decreased the GC retention time slightly, whereas addition of the 4-hydroxyl increased the retention time substantially. The D,L-sphinganine standard, which was a racemic mixture of *erythro*- and *threo*-isomers, gave two GC peaks in a ratio of *ca* 1:2, whereas the all D-*erythro*-4-*trans*-sphingenine and 4-hydroxysphinganine standards gave only one GC peak. SP acetates from each of the fruit CB fractions gave two, close-running GC peaks with a peak area ratio of *ca* 3:2. The order of GC retention times of the various SP acetates was tomato CB 1 = bell pepper CB 1 (15.9 + 16.3 min) < bell pepper CB 2 (16.2 + 16.8 min) < 4-*trans*-sphingenine (17.4 min) < sphinganine (17.6 + 17.9 min) < bell pepper CBs 4 + 5 + 6 (20.0 + 20.4 min) < 4-hydroxysphinganine (22.4 min).

The three SP acetate standards, as well as SP acetates from tomato CB 1, bell pepper CB 2 and bell pepper CBs 4 + 5 + 6, were also analysed by ammonia chemical ionization GC–mass spectrometry. For each of the SP acetate samples, the three major ions were $[M + NH_4]^+$, $[M + H]^+$, and $[M - CO_2CH_3]^+$. As expected, these three ions were all 2 mu lower in the spectrum of 4-*trans*-sphingenine triacetate (*m/z* = 443, 426 and 366), compared with that of sphinganine triacetate (*m/z* 445, 428 and 368), reflecting loss of two protons with introduction of the double bond. In the spectrum of 4-hydroxysphinganine tetraacetate, the three major ions (*m/z* 503, 486 and 426) were all 58 mu higher than those in the spectrum of sphinganine triacetate (replacement of a proton by an acetate moiety). The two isomers of the SP acetate from tomato CB 1 had almost identical spectra and the three major ions (*m/z* 441, 424 and 364) were all 4 mu lower than those of sphinganine triacetate, indicating a subtraction of four protons and, hence, the presence of two double bonds. The two SP acetate isomers from bell pepper CB 2 also had very similar spectra and the major ions (*m/z* 443, 426 and 366) matched those of 4-*trans*-sphingenine. Finally, the isomers of the SP acetate from bell pepper CBs 4 + 5 + 6 again had closely similar spectra and each of the three major ions (*m/z* 501, 484 and 424) were 2 mu lower than those in the spectrum of 4-hydroxysphinganine, indicating a difference of one double bond. The conclusions from the CI GC–mass spectral data are that the SPs from tomato CB 1 are isomers of sphingadienine, the SPs from CB 2 of bell pepper are isomers of sphingenine, and the SPs from CBs 4 + 5 + 6 of bell pepper are isomers of 4-hydroxysphingenine.

Infrared spectra over the range 4000–650 cm^{-1} were generated for HPLC-purified CB fractions including tomato CB 1, bell pepper CB 1, bell pepper CB 2 and bell pepper CB 6. The IR spectra for tomato and bell pepper CB 1 were virtually identical and showed two distinct peaks at 966 and 720 cm^{-1}, indicative of *trans* and *cis* double bonds, respectively. The IR spectrum of bell pepper CB 2 closely resembled those of bell pepper and tomato CB 1; peaks indicative of *trans* and *cis* double bonds were evident. Finally, although much less pronounced than in the spectra of CBs 1 and 2, peaks at

ca 964 and 720 cm^{-1} were evident in the IR spectrum of CB 6, indicating the presence of *trans*- and *cis*-isomers.

DISCUSSION

Two previous studies of plant CBs have included reverse-phase HPLC analysis of CB molecular species [13, 14]. One involved separation of a complex mixture of glucoCBs from leaf tissue and leaf plasma membrane of rye on a 25 cm C_{18} column [13]. Individual CBs were eluted over a span of *ca* 9–62 min with a 3:2 mixture of cyanomethane and methanol. The second included HPLC separation of glucoCBs isolated from seeds of two *Phaseolus* species [14]. This method also used a 25 cm C_{18} column, but elution was effected with methanol–H_2O (25:1) over *ca* 20–130 min with the column held at 40°. In both studies, CBs separated by HPLC were detected by UV absorbance (at 210 and 220 nm, respectively). In contrast, the HPLC method developed in the present study used a 15 cm C_6 column and gradient elution of CBs with cyanomethane and H_2O. This technique has the advantages of greater sensitivity (UV monitoring at 205 nm, with a high signal-to-noise ratio and a detection limit of *ca* 1 nmol) and a shorter run time. Concerning the latter point, minor CB molecular species with 25:0h and 26:0h FAs had not yet eluted at 40 min, but continuing the gradient for another 10 min allowed elution of all CBs by 50 min total run-time. The new method can also be used for simultaneous analysis of CBs and steryl glycosides; glucosides of stigmasterol and sitosterol elute ≥4 min prior to CB 1. Finally, the C_6 column may serve to segregate CB species which include diversus trihydroxy SPs, whereas the C_{18} column clearly does not [13, 14]. CBs from bell pepper and tomato which include sphingadienine (d18:2) and sphingenine (d18:1) were separated from those which include 4-hydroxysphingenine (t18:1) by ≥8 min. However, this may have been at least partly a function of the much longer chain-length FAs associated with the latter.

The GC and GC–mass spectrometry data indicate that CBs from pericarp tissue of mature-green and red-ripe bell pepper and tomato fruits include isomers of sphingadienine, sphingenine and 4-hydroxysphingenine as the predominant SPs. The FA 16:0h was almost exclusively associated with d18:2 and d18:1 SPs, whereas 22:0h, 23:0h and 24:0h were primarily associated with t18:1 SPs. Similar specificity of the amide-linked FAs has been reported for CBs from seeds and leaves of several dicotyledonous species, including seeds [15] and leaves [16] of soybean, seeds of two *Phaseolus* spp. [14] and leaves of spinach [17]. Unpublished data from this laboratory indicate that this also holds for CBs from tissues of apple and muskmelon fruits.

Although no positional analysis was performed, it is concluded from previous reports on plant CBs [6, 13–17] that the d18:2 SPs of bell pepper and tomato fruit CBs are isomers of 4,8-sphingadienine and that the t18:1 SPs are isomers of 4-hydroxy-8-sphingenine. The IR spectra of bell pepper and tomato CB 1 indicate that the major d18:2 SP is 4-*trans*-8-*cis*-sphingadienine and, hence, the major CB molecular species is 1 - *O* - *β* - glucosyl - *N* - (2′ - hydroxypalmitoyl) - 4 - *trans* - 8 - *cis*-sphingadienine. This is in contrast with reports on CBs from seeds of soybean [15] and leaves of spinach [17], which found the major (>80%) d18:2 SP to be the 4-*trans*-8-*trans*-isomer. The IR spectrum of bell pepper CB 6 showed that both *cis*- and *trans*-isomers of 4-hydroxy-8-sphingenine are present. This is in accord with the study of spinach leaf CBs [17], which showed roughly equal amounts of *cis*- and *trans*-t18:1 SPs. The IR spectrum of bell pepper CB 2, along with the HPLC, GC and GC–mass spectral data, supports the conclusion that the SPs of bell pepper CB 2 are *cis*- and *trans*-isomers of 8-sphingenine.

Several facts support the conclusion that the two GC peaks obtained with each of the SP acetate fractions from CB 1, CB 2 and CBs 4 + 5 + 6 represent the 8-*cis*- and 8-*trans*-isomers. Although acid methanolysis can cause artefacts such as epimerization of SPs from *erythro*- to *threo*-isomers [18], the conditions used in this work reportedly yield a minimum of 'by-products' [1]; 4-*trans*-sphingenine triacetate derived from bovine galactoCB by this procedure gave only one GC peak. Furthermore, splitting of the CB 1 peak was noted in HPLC profiles and since glucose and 16:0h are virtually the only hexose and FA moieties in CB 1, different isomers of a d18:2 SP must be responsible for this. The IR spectra showed that SPs from CB 1, CB 2 and CB 6 included both *cis* and *trans* double bonds. For the monoenoic SPs of CBs 2 and 6, this can only mean that both 8-*cis*- and 8-*trans*-isomers were present; hence, it is probable that CB 1 included 8-*cis*- and 8-*trans*-isomers of d18:2, which accounts for the split peak on HPLC.

In part, this study was conducted to determine whether changes in CB content and/or composition that occur with ripening of bell pepper and tomato fruits could be involved in the increase in membrane permeability and decrease in chilling-sensitivity [19–22] during this developmental stage. On the whole, a stronger case can be made for significance of CB changes with ripening of bell pepper compared with tomato fruit. First, the amount of CBs per g fr. wt was over four-fold greater in bell pepper pericarp tissue and second, during ripening, CB content declined by 20% in bell pepper, but only 3% in tomato. A relationship between sensitivity to chilling and CBs with a high proportion of 8-*trans*-SPs has been proposed based on the fact that, compared with the 8-*cis*-isomers, 8-*trans*-SPs yield CB molecular species which undergo a cooling exotherm at a much higher temperature [14, 23]. In mature-green bell pepper and tomato fruits there appears to be a majority of CBs with 8-*cis*-SPs and this changes little with ripening. Finally, the small increase in the proportion of CBs with trihydroxy-SPs during ripening of tomato should increase membrane

stability and decrease permeability rather than the reverse [23].

EXPERIMENTAL

Plant material. Tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) and bell pepper (*Capsicum annuum* L. v. Gator Bell) plants were grown in cultivar trial field plots at Beltsville, using black plastic mulch and trickle irrigation. Fruits were hand-harvested at the mature-green and red-ripe stages of development (determined visually) and were processed within a few hr after harvest. Segments of outer pericarp tissue were freed of seeds and locular material, frozen in liquid N_2 and stored in plastic bags at −80° until used. Samples of tissue (10 g fr. wt), each from a single fruit, were lyophilized overnight prior to lipid extraction.

Lipid extraction and isolation of cerebrosides. Dried tissue samples were pulverized and extracted with 25 ml $CHCl_3$–MeOH (2:1) using 2 × 30 sec bursts of a Polytron homogenizer. The homogenate was filtered through a sintered glass funnel, the tissue-cake rinsed with 5 ml $CHCl_3$ and the filtrate given a Folch wash. The washed $CHCl_3$phase was evapd under a stream of N_2. Total pericarp lipids were redissolved in 2 ml $CHCl_3$ and stored under N_2 at −80° until fractionated. Total lipids were sepd into neutral, glyco- and phospholipid frs by silicic acid CC. After elution of neutral lipids with $CHCl_3$–Me_2CO (20:1) two glycolipid frs (GL1 and GL2) were sequentially eluted with $CHCl_3$–Me_2CO (1:1) and Me_2CO–MeOH (20:1). CBs were found only in GL2, which was dissolved in MeOH–$CHCl_3$ (9:1) containing 0.6 M KOH and shaken for 4 hr to achieve alkaline methanolysis of contaminant glycerolipids. Non-saponifiable lipids derived from GL2 were sepd by TLC on silica gel 60 (EM Science, Darmstadt) using $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (10:4:2:2:1). CBs ran as a 1-cm-wide band from R_f 0.59 to 0.64 and were eluted from the adsorbent with $CHCl_3$–MeOH (2:1) followed by a Folch wash. This TLC-purified fr. included 96–99% CB with 1–4% steryl glycoside as contaminant.

HPLC separation and analysis of cerebrosides. The HPLC column used for CB analyses was C_6 (hexyl) reverse-phase on 5 μm Spherisorb, 150 × 4.6 mm i.d., from Phase Separations, Inc. (Norwalk, CT). Sepn of CBs was effected using a linear gradient of MeCN–H_2O starting at a 11:9, increasing to 17:3 over 35 min, then returning to 11:9 from 35 to 40 min. Flow was maintained at 0.9 ml min^{-1} throughout. Detection was at 205 nm. CB samples were dissolved in MeOH and passed through a 0.45 μm PTFE membrane filter prior to injection.

Analysis of fatty acid, sphingoid and hexose moieties. CB samples were cleaved by refluxing for 18 hr at 75° in 2 N HCl in MeOH. After addition of H_2O, 2-hydroxyl fatty acid Me esters (2-hydroxy-FAME) and SPs were extracted with hexane and EtOAc, respectively. The remaining MeOH–H_2O–HCl phase was evapd in a stream of N_2 and the residue refluxed in 2 N aq. HCl to yield a free hexose fr. Hexose was converted into the alditol acetate and analysed by FID-GC as previously described [24]. Analysis of 2-hydroxy-FAME was performed by FID-GC on a 0.25 mm i.d. × 15 m SP 2330 fused silica capillary column (Supelco). The column oven was programmed to increase from 160° to 220° at 3° min^{-1}; injector and detector temps were 225° and 250°, respectively, and the He head pressure was 69 kPa. Standards of 2-hydroxy-FAME were obtained from Matreya, Inc. (Pleasant Gap). SPs were initially purified by TLC using the same system as that for CBs. SP bands were located by ninhydrin-staining. After peracetylation by refluxing in Ac_2O–pyridine (1:1) SPs were purified and isolated by C_6 reverse-phase HPLC as described above, except that the gradient ran from 62.5 to 80% MeCN over 25 min, returning to 62.5% from 25 to 30 min. HPLC-purified SP acetates were then analysed and identified by FID-GC and GC-MS. FID-GC was performed isothermally at 240° on a 0.25 mm i.d. × 30 m SPB-1 fused silica capillary column (Supelco). Injector and detector temps were 280° and 320°, respectively; the He head pressure was 171 kPa. NH_3 CI GC–MS was carried out at the Insect Neurobiology and Hormone Laboratory, Beltsville, using a 0.32 mm i.d. × 30 m DB-1 fused silica capillary column (J & W Scientific). Operation was isothermal at 240°, with a source temp. of 150° and a He head pressure of 171 kPa.

IR spectra of intact, HPLC-purified CBs were recorded by dissolving samples in $CHCl_3$ and applying them to the surface of 2.5-mm-diam. KBr discs followed by evapn of the solvent in a stream of N_2.

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