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NEW DERIVATIVES FOR THE ANALYSIS OF SPHINGOSINE LONG-CHAIN BASES BY GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Cyclic derivatives formed by reaction of sphingosines with methane-, *n*-butane- and benzeneboronic acid have been examined by gas–liquid chromatography (GLC) and by combined GLC–mass spectrometry (MS). Analogous compounds have also been studied, in which the amino group has been protected, for example as an acetone Schiff base or N,N-dimethylaminomethylene derivative. Phytosphingosine (4D-hydroxysphinganine) yielded bis-alkaneboronates. Most of the derivatives were satisfactory for GLC. Molecular ions were apparent (1–100% relative abundance) in all electron impact mass spectra, and (as $[M + 1]^+$ ions) were the base peaks in isobutane chemical ionisation mass spectra.

INTRODUCTION

Sphingosines* derived from naturally occurring sphingolipids may consist of highly complex mixtures containing as many as thirty individual components[3]. Though many studies of sphingosine mixtures have involved examination of oxidation products, combined gas–liquid chromatography–mass spectrometry (GLC–MS) has been extensively applied in the analysis of derivatives of the intact bases. The derivatives most frequently used for GLC and GLC–MS have been bis-O-trimethylsilyl (TMS) ethers[4–6] or N-acetyl bis-O-TMS ethers[5,6]. Both of these have the disadvantage, for the purposes of GLC–MS, of the absence or very low abundance of molecular ions in the electron impact (EI) mass spectra. The 2-amino-1,3-diol system typical of the sphingosines is eminently suited to the formation of cyclic boronate esters. The value of butaneboronates and benzeneboronates for the characterisation of 1,2- and 1,3-diols by GLC–MS was first demonstrated by Brooks and Watson in 1967[7,8], and has since been widely exploited. In particular, the derivatives are useful for their selectivity, their good chromatographic properties, and their mass spectra, which contain well-defined molecular ions —frequently in high abundance.

* In this paper "sphingosine" is used as a generic term to describe sphingolipid long-chain bases. Individual sphingosines are named in accordance with the proposals of the IUPAC-IUB Commission on Biochemical Nomenclature[1,2]. Thus, sphinganine: D-*erythro*-1,3-dihydroxy-2-amino-octadecane; 4-sphingenine: D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-octadecene; 4D-hydroxysphinganine: D-*ribo*-1,3,4-trihydroxy-2-aminooctadecane.

Accordingly, we have examined the utility for analysis by GLC and GLC–MS, of boronates of sphingosines, and of related derivatives obtained by protection of the amino group. In the present paper, we report GLC and MS data from an initial study of these derivatives, evaluated with a view to their use in the analysis of sphingosines from human arterial tissue.

EXPERIMENTAL

Materials

Sources of sphingosines and reagents were as follows: sphinganine (dihydrosphingosine), Miles-Seravac (Maidenhead, Great Britain); 4-sphingenine (sphingosine), Supelco (Bellefonte, Pa., U.S.A.); 4D-hydroxysphinganine (phytosphingosine), Sigma London (Kingston-upon-Thames, Great Britain); methaneboronic acid, Alfa Inorganics (Ventron-Hicol, Rotterdam, The Netherlands); butaneboronic acid, Callery (Callery, Pa., U.S.A.); benzeneboronic acid, Aldrich (Gillingham, Dorset, Great Britain); N,N-dimethylformamide dimethylacetal, Pierce and Warriner (Chester, Great Britain).

Preparation of derivatives

Boronates. Methane-, butane- and benzeneboronates of sphingosines were prepared in pyridine solution by addition of 1 molar equivalent of the appropriate boronic acid[7–9]. Reactions were complete within 10 min at room temperature. Pyridine was removed under nitrogen, and the derivatives were dissolved in ethyl acetate for GLC analysis and subsequent storage.

N,N-dimethylaminomethylene derivatives. Conversion of boronates (*ca.* 100 μg) to N,N-dimethylaminomethylene (DMAM) derivatives[10] was achieved by addition of a solution (20 μl; 2 mmole/ml) of N,N-dimethylformamide dimethylacetal in pyridine. After several minutes at room temperature, reagent and solvent were removed under nitrogen and the derivative was dissolved in ethyl acetate.

N-Acetylation. Two methods were employed in the preparation of N-acetyl sphingosine boronates. Selective N-acetylation of sphinganine (5 mg) was performed by dissolution in methanol (500 μl) and addition of acetic anhydride (150 μl)[11]. The mixture was left overnight at room temperature and the reagents were removed by evaporation. Subsequent conversion to boronates was carried out as described above. Alternatively, boronate formation preceded an acetylation step in which the boronate (100 μg) was dissolved in a 20:1 mixture of pyridine and acetic anhydride (100 μl) and kept overnight at room temperature. Reagents were removed under a stream of nitrogen and the derivative was dissolved in ethyl acetate for GLC and GLC–MS analyses.

Acetone Schiff base derivatives. Sphingosine boronates were converted to the corresponding acetone Schiff bases[12,13] by dissolution in acetone and heating at 60° for 10 min. Derivatisation was complete as judged by thin-layer chromatography (TLC) and GLC.

Gas–liquid chromatography and combined gas–liquid chromatography–mass spectrometry

GLC analyses employing conventional packed columns were performed on

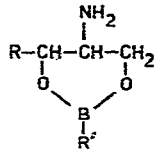
Pye 104 or Perkin-Elmer F33 instruments, equipped with glass columns (2 m × 3 mm) packed with 1% OV-1 or 1% OV-17 on Gas-Chrom Q (100–120 mesh). Nitrogen (50 ml/min) was the carrier gas. High-resolution GLC was carried out with the Pye 104 instrument fitted with an open-tubular glass column (30 m × 0.5 ± 0.1 mm I.D.) coated with OV-1 on Silanox, prepared according to the method of German and Horning[14], as previously described[15]. Packed column GLC employed conventional liquid sample injection whereas a "falling-needle" type dry injection assembly[16] was used for open-tubular columns.

GLC–MS was carried out with a Varian 2700 gas chromatograph interfaced via an all-glass single-stage jet separator to a DuPont 21-490F mass spectrometer. Separations were performed using packed columns similar to those employed in GLC analyses. The accelerating voltage was 1.4 kV, the electron energy 70 eV, and the ion source temperature 250°. For the recording of chemical ionisation mass spectra using isobutane as reagent gas, appropriate conditions were established by reference to the spectrum of *m*-xylene[17].

RESULTS AND DISCUSSION

Cyclic boronate derivatives of sphingosine long-chain bases were rapidly formed under mild conditions. Derivative formation was confirmed by GLC and GLC–MS analyses (discussed below). Mild trimethylsilylation of the sphingosine boronates failed to affect the GLC or MS properties, indicating the absence of a free hydroxyl group. Moreover, derivatisation of free amino groups by microreaction of the boronates confirmed the initial structure as a dioxaborinane (I) rather than the possible alternative 1,3,2-oxazaborolidines. The sphingosine boronates studied were stable during storage in ethyl acetate solution over periods of several weeks. Destruction of the derivatives resulted within 1 min of the addition of several molar proportions of propane-1,3-diol.

4D-Hydroxysphinganine also yielded a derivative under similar reaction conditions; GLC–MS analysis indicated a bis-boronate of probable structure II.



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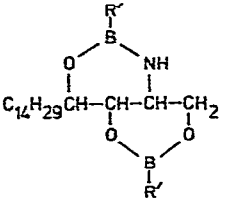


II

Fig. 1 shows derivatisation procedures, including boronate formation, that have been performed for sphinganine. The stability of the boronates is such that derivatisation of the amino group can be achieved without affecting the dioxaborinane ring. The stability of boronates of certain 1,2- and 1,3-diols to the conditions of trimethylsilylation, oximation and acetylation is well known (see refs. 7–9 and papers there cited). N-Acetylation of sphinganine methaneboronate was performed under standard conditions. N-Acylation of sphingosine boronates with long-chain acyl

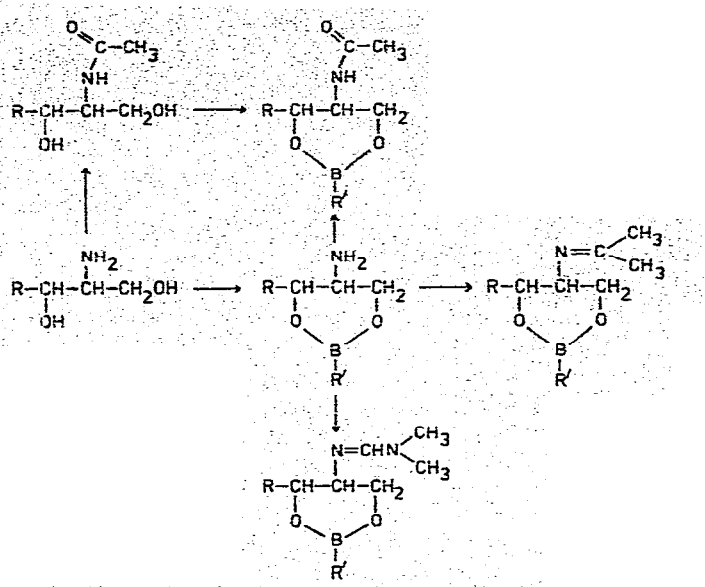


Fig. 1. Derivatisation of sphingosine long-chain bases.

chlorides constitutes a useful method for the preparation of ceramides on the microscale[18]. N-Acetyl sphinganine boronates were also prepared by selective N-acetylation[11] followed by boronate formation. Imine preparation[12,13] has been demonstrated to be valuable for the protection of primary amine groups for chromatographic analyses. Sphingosine boronates were readily converted to acetone Schiff base or N,N-dimethylaminomethylene derivatives under mild conditions.

Gas chromatography of sphingosine boronates

Sphingosine boronates prepared by the addition of 1 molar proportion of boronic acid were satisfactory derivatives for GLC employing conventional packed columns, though some peak tailing was observed. Addition of excess boronic acid, however, resulted in a pronounced drop in peak height and marked peak tailing; the effect is attributable to the formation of acyclic boronate derivatives of the free amino group, analogous to observations with steroids containing isolated hydroxyl groups[9]. Peak height was restored and peak shape improved by the conversion of the $-NH_2$ to acetyl, DMAM or acetone Schiff base derivatives.

Sphingosine boronate derivatives were further evaluated using an open-tubular column of OV-1 liquid phase coated over Silanox. The use of a column of moderate length (30 m) enabled separations of high resolution (*ca.* 30,000 theoretical plates) to be performed in reasonably short analysis times, a factor of particular importance in the GLC of ceramide boronates[17] conducted in parallel with the present work. Fig. 2 shows open-tubular GLC analyses of benzeneboronate derivatives of sphinganine. The improvement in chromatographic behaviour afforded by protection of the primary amino group is evident. Such protection is effectively achieved during boronate formation with 4D-hydroxysphinganine. GLC analysis of the bis-methaneboronate of 4D-hydroxysphinganine is shown in Fig. 3.

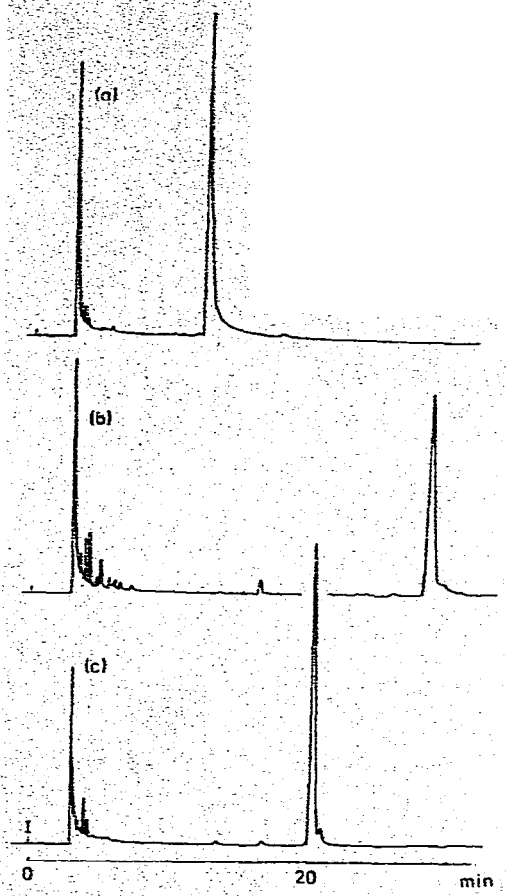


Fig. 2. Gas chromatography of benzeneboronates of sphinganine. Column, open-tubular glass, 30 m × 0.5 ± 0.1 mm I.D., coated with OV-1 on Silanox; temperature, 250°. (a) Amino benzeneboronate; (b) N-acetyl benzeneboronate; (c) benzeneboronate acetone Schiff base.

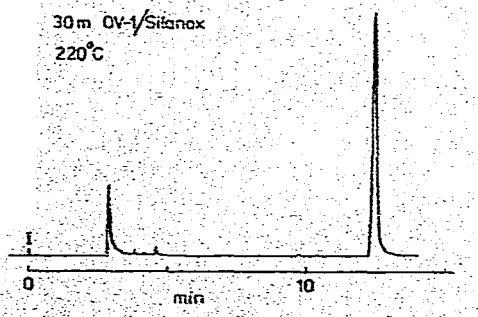


Fig. 3. Gas chromatography of 4D-hydroxysphinganine bis-methaneboronate. Column, as in Fig. 2; temperature, 220°.

Table I records retention index data (recorded on packed columns) for twelve derivatives of sphinganine, together with data for selected derivatives of 4-sphingenine and 4D-hydroxysphinganine. The methaneboronates are notable for their short retention times arising from the small mass increments associated with derivative formation[9,19]. Preparation of N-acetyl or DMAM derivatives results in substantial increases in retention index (*ca.* 250 units on OV-1 liquid phase). In contrast, conversion of $-NH_2$ to the acetone Schiff base leads to a modest increase (less than 100 units on OV-1 and OV-17 phases): this, together with the improved peak shape, makes these derivatives very suitable for GLC.

TABLE I

GAS CHROMATOGRAPHIC DATA FOR SPHINGOSINE BORONATE DERIVATIVES

Compound	*Derivative*	*Δm* *	*Temperature (°C)*	*I*	
				OV-1	*OV-17*
Sphinganine	Methaneboronate	24	200	2315	2480
	Butaneboronate	66	230	2590	2740
	Benzeneboronate	86	230	2940	3220
	Methaneboronate DMAM**	79	250	2570	2765
	Butaneboronate DMAM	121	230	2840	3020
	Benzeneboronate DMAM	141	270	3260	3535
	N-Acetyl methaneboronate	66	230	2600	2835
	N-Acetyl butaneboronate	108	270	2850	3080
	N-Acetyl benzeneboronate	128	270	3210	3595
	Methaneboronate acetone Schiff base	64	230	2420	2565
	Butaneboronate acetone Schiff base	106	230	2675	2815
	Benzeneboronate acetone Schiff base	126	270	3040	3310
4-Sphingenine	Methaneboronate	24	200	2305	2485
	Benzeneboronate	86	230	2940	3270
	Methaneboronate acetone Schiff base	64	230	2420	2580
	Benzeneboronate acetone Schiff base	126	270	3030	3360
4D-Hydroxysphinganine	Bis-methaneboronate	48	230	2425	2570

* Mass increment associated with derivative formation.
** DMAM = N,N-dimethylaminomethylene.

Corresponding boronates of sphinganine and 4-sphingenine are not separated on OV-1 liquid phase; co-injection on to an OV-1 open-tubular column results in a peak broadening indicative of only slight differences in retention time. This contrasts with the N-acetyl bis-O-TMS derivatives of sphinganine and 4-sphingenine which are readily separated on OV-1[5,6], the latter being eluted first[20]. Formation of the boronate ring evidently reduces the effect of the allylic double bond. Sphinganine and 4-sphingenine benzeneboronates are, however, easily separated on OV-17 phase; in this instance the saturated analogue has the shorter retention time.

Mass spectrometry of sphingosine boronates

Outline data for the EI and isobutane chemical ionisation (CI) mass spectra of a number of derivatives of sphinganine, 4-sphingenine and 4D-hydroxysphinganine

are recorded in Table II. EI spectra of the boronates of sphinganine (*e.g.*, methaneboronate, Fig. 4a) were notable for the abundance of molecular ions, which were the base peaks in each case. This contrasts with the EI spectra of the bis-O-TMS and N-acetyl bis-O-TMS derivatives, where molecular ions are absent or of very low intensity. Little fragmentation of the amino boronates occurred under EI, but prominent ions were invariably observed attributable to scission of the boron-containing ring with concomitant hydrogen migration to the neutral fragment as depicted in III.

TABLE II

ELECTRON IMPACT AND CHEMICAL IONISATION MASS SPECTROMETRIC DATA FOR SPHINGOSINE BORONATE DERIVATIVES

Compound	*Derivative (mol. wt. in parentheses)*	*Mass spectrum*			
		EI			*CI*
		Relative intensity (%)		*Base peak (m/e)*	*Relative intensity (%)** $[M-R]^+$*
		$M^{+\cdot}$	$[M-R]^{+*}$		
Sphinganine	Methaneboronate (325)	100	2	325	1
	Butaneboronate (367)	100	10	367	1
	Benzeneboronate (387)	100	2	387	3
	Methaneboronate DMAM (380)	4	9	71	9
	Butaneboronate DMAM (422)	8	58	73	10
	Benzeneboronate DMAM (442)	15	87	98	15
	N-Acetyl methaneboronate (367)	2	8	85	4
	N-Acetyl butaneboronate (409)	1	12	85	14
	N-Acetyl benzeneboronate (429)	1	8	60	53
	Methaneboronate acetone Schiff base (365)	7	24	58	6
	Butaneboronate acetone Schiff base (407)	1	9	58	7
	Benzeneboronate acetone Schiff base (427)	2	19	58	7
4-Sphingenine	Methaneboronate (323)	10	1	84	2
	Benzeneboronate acetone Schiff base (425)	2	2	58	1
4D-Hydroxy-sphinganine	Bis-methaneboronate (365)	8	1	84	1
	Bis-butaneboronate (449)	13	1	126	2

* R = Methyl, butyl or phenyl, of respective boronates.
** The base peak was $[M+1]^+$ in every case.

M/e 84 constituted the base peak of the EI spectrum of 4-sphingenine methaneboronate (Fig. 4b); this ion is analogous to that of *m/e* 85 arising from triol methaneboronates[21]. The presence of a double bond allylic to the boronate ring promotes fragmentation of the molecule. The EI mass spectrum (Fig. 4c) of 4D-hydroxysphinganine bis-methaneboronate included a molecular ion of moderate abundance, together with a fragment ion, *m/e* 168, attributable to fragmentation adjacent to the oxazaborolidine ring (*cf.* IV).

Molecular ions were present in the EI spectra of all sphinganine boron-

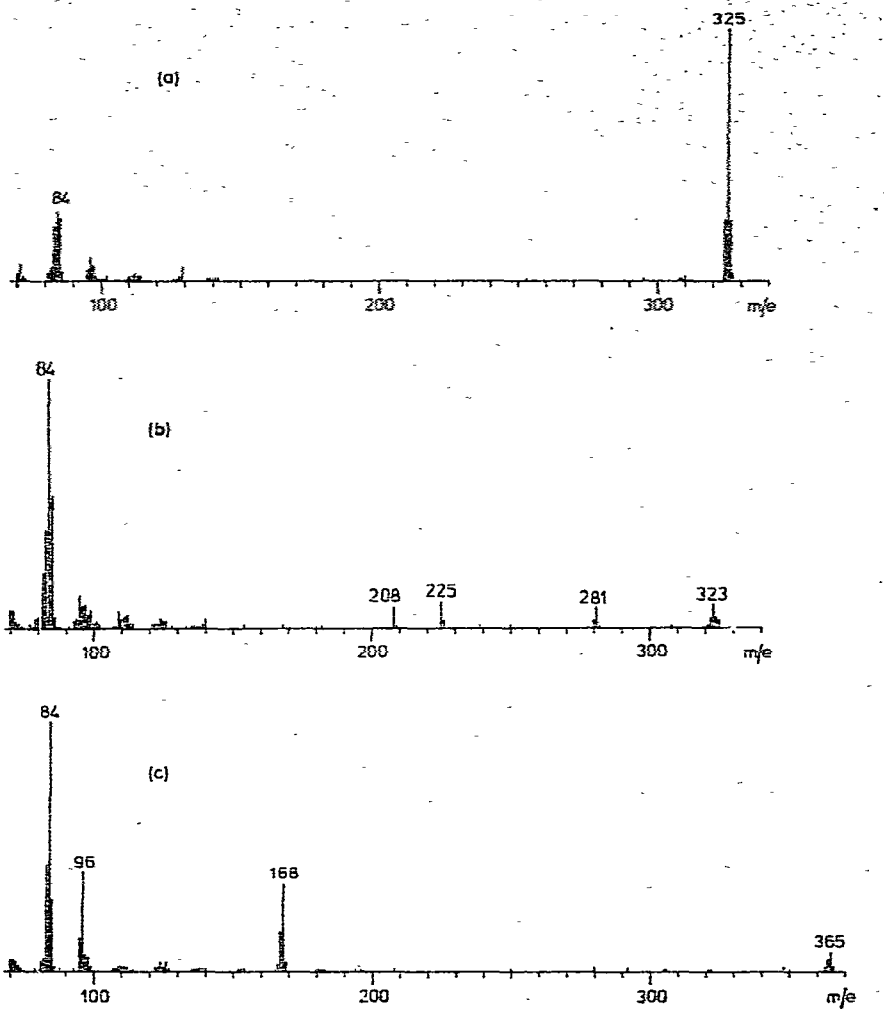
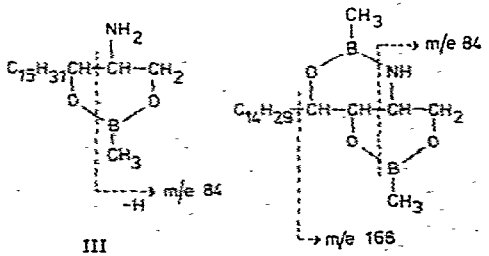


Fig. 4. Electron impact mass spectra of sphingosine methaneboronates (70 eV). (a) sphinganine methaneboronate; (b) 4-sphingenine methaneboronate; (c) 4D-hydroxysphinganine bis-methaneboronate.



ate derivatives in which the NH_2 group was protected, though relative abundances were substantially decreased in comparison with the amino boronates. Parallel analyses by GLC–CI-MS, however, led to unequivocal molecular weight assignment: quasi-molecular ions, $[M+1]^+$, appeared as base peaks in the isobutane CI spectra of all the derivatives examined. The EI spectrum of sphinganine benzeneboronate DMAM included an intense ion (*m/e* 201; 43% rel. int.) attributable to the fragmentation across the boronate ring observed in the spectra of the amino boronates. The corresponding ions in the spectra of methane- and butaneboronate DMAM analogues appeared at only low intensity. EI spectra of N-acetyl and of acetone Schiff base derivatives of each sphinganine boronate included ions attributable to similar fragmentation but without associated hydrogen migration. Further aspects of the mass spectrometry of sphingosine boronates will be discussed elsewhere.

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