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APPLICATIONS OF BORONATE DERIVATIVES IN THE STUDY OF CERAMIDES BY GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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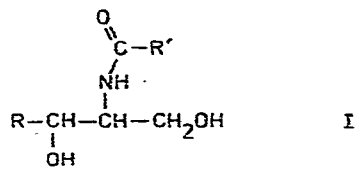
SUMMARY

Methaneboronate derivatives of ceramides possess excellent gas–liquid chromatographic properties and give informative mass spectra. Molecular ions, present at high abundance where an unsaturated acyl substituent is present, are accompanied in the electron impact mass spectra by fragment ions which denote the acyl group and long-chain base. In the gas–liquid chromatographic–mass spectrometric analyses of natural ceramides, as methaneboronate derivatives, the properties of open-tubular columns are exploited to give good separations in moderate analysis times. An application is made to the analysis of ceramides derived from sphingomyelin of human arterial tissue.

INTRODUCTION

Ceramides (N-acyl sphingosines, I) are important components of natural lipid mixtures, both in the free form and as structural constituents of sphingomyelin, cerebrosides, gangliosides, etc. Analyses of ceramides are usually reported in terms of the constituent fatty acids and long-chain bases (sphingosines); less commonly, molecular species are analysed by gas–liquid chromatography (GLC) and combined gas–liquid chromatography–mass spectrometry (GLC–MS)[1–7]. For the purposes of GLC and GLC–MS, ceramides have generally been converted to the bis-O-trimethylsilyl (TMS) ethers; the mass spectra of the derivatives have been extensively studied and major features satisfactorily explained[2,3,5,7]. MS studies have also been reported of ceramides in the free form[8,9], and as acetylated[8] or methylated[9] derivatives.

The 2-amido-1,3-diol structure of ceramides is amenable to the preparation of



I

R,R′=alkyl or alkenyl or hydroxyalkyl

cyclic boronate esters. Since their introduction in 1967 as derivatives for GLC and GLC–MS[10,11], boronates have found wide application in the analysis of diols and amino alcohols. Methaneboronates[12,13] are particularly useful by virtue of the low mass increment accompanying derivative formation, and consequently short GLC retention times. We recently reported[14,15] an evaluation of boronates and related derivatives for the GLC–MS of sphingosines; satisfactory GLC and MS properties were observed for the derivatives, which in certain respects, such as the intensity of molecular ions in electron impact mass spectra, possessed advantages over the corresponding TMS compounds. In a preliminary communication[14], we have also described the preparation and GLC–MS properties of a synthetic ceramide boronate, but these derivatives have not been previously applied in the GLC and GLC–MS analyses of ceramide mixtures. (Complexing of the 1,3-diol function with borate ions has, however, been utilised in the separation of ceramides on columns and thin layers of borate-impregnated silica[16].) In the present paper we report GLC and MS data for synthetic ceramide boronates and describe the application of boronate derivatives to the analysis of natural ceramide mixtures.

GLC and GLC–MS analyses of ceramide mixtures, as TMS derivatives, have hitherto relied on the use of conventional short packed columns of limited separation efficiency (*e.g.*, refs. 1–5). Moreover, the high temperatures required for the elution of high-molecular-weight ceramide derivatives have resulted in high column "bleed", impairing the interpretation of mass spectra of minor components recorded during GLC–MS[17]. Accordingly, in the analyses of ceramide boronate mixtures reported here, we have exploited the ability of open-tubular columns to provide good separation efficiency combined with relatively short analysis times[18]. Furthermore, the use of comparatively wide-bore (*ca.* 0.55 mm) columns has enabled a greater sample loading with a consequent increase in the range of concentrations of sample components over which useful spectra may be obtained during GLC–MS.

EXPERIMENTAL

Materials

N-Palmitoyl sphinganine and N-stearoyl sphinganine were obtained from Miles-Seravac (Slough, Great Britain); other ceramides were prepared from the methaneboronates of sphinganine (Miles-Seravac) or 4-sphingenine (Supelco, Bellefonte, Pa., U.S.A.) and the appropriate acyl chloride (Nu-Chek-Prep, Elysian, Minn., U.S.A.) by a method to be described elsewhere[19]. A mixture of ceramides from bovine brain was obtained from Sigma (Kingston-upon-Thames, Great Britain).

Preparation of methaneboronates

Methaneboronates of sphingosines and ceramides were prepared in pyridine solution by the addition of 1.1 molar proportions of methaneboronic acid (Alfa Inorganics, Ventron-Hicol, Rotterdam, The Netherlands)[13]. For ceramides derived from 2-hydroxyacids, 2.2 molar proportions of reagent were used.

Isolation of ceramides from sphingomyelin of human arterial tissue

Human arterial tissue, obtained within 24 h *post mortem*, was washed with saline solution and broadly classified according to the severity of the atherosclerotic

lesions. Total lipids were obtained by chloroform–methanol (2:1) extraction, and phospholipids were separated from neutral components on a silica gel column. The crude phospholipid mixture was subjected to phospholipase C hydrolysis[20]; ceramides were separated from other products (mainly diglycerides) by preparative thin-layer chromatography (TLC) on Anasil B (Analabs, North Haven, Conn., U.S.A.) using chloroform–ethyl acetate (3:1) as the mobile phase.

Gas–liquid chromatography

Open-tubular glass spirals were drawn from 6-mm light-walled Pyrex glass tubing, in a device constructed according to the design of Desty *et al.*[21], to an internal diameter of *ca.* 0.55 mm. Column blanks (30–80 m) were deactivated by a gas phase silanization method[22] and by passage of a solution of dimethyldichlorosilane in toluene. Column coating with 6–10 μm Silanox (Cabot, Billericia, Mass., U.S.A.) and OV-1 liquid phase was performed essentially according to the method of German and Horning[23].

Columns were installed in a Pye 104 gas chromatograph equipped with a "falling needle" type dry injector device[24]. The column inlet was inserted directly into the flash heater zone and was connected to the flame ionisation detector via a short length of glass-lined stainless-steel tubing (Scientific Glass Engineering, London, Great Britain). Helium was used as carrier gas at a flow-rate of 15 ml/min. Analyses were performed at 300°.

Gas–liquid chromatography–mass spectrometry

GLC–MS, employing an open-tubular column (40 m) of the type described, was carried out using an LKB9000 instrument, fitted with a dry-injection device. Helium was added to the effluent of the column to afford optimum flow-rate (30 ml/min) to the two-stage jet separator. Spectra were recorded at 70 eV, with a separator temperature of 280° and an ion source temperature of 270°. Reference electron impact mass spectra of synthetic ceramide methaneboronates were obtained by analyses on short glass columns (1 m × 3.5 mm I.D.) packed with 1% SE-30 on 100–120 mesh Gas-Chrom Q, installed in the LKB9000 or a DuPont 21-490F instrument. In the latter instance, the ion source temperature was 240°.

RESULTS

Methaneboronate derivatives of ceramides were readily formed by addition of 1.1 molar proportions of methaneboronic acid in pyridine solution. Quantitative conversion to the cyclic boronates, as judged by TLC, occurred within 10 min. With chloroform–ethyl acetate (3:1) as the mobile phase, the derivatives migrated as well defined spots. The derivatives were stable to storage under refrigeration in ethyl acetate solution for a period of several weeks.

Ceramide methaneboronates were found to possess excellent GLC properties. Figs. 1a and b illustrate the analyses, carried out on an open-tubular Silanox-type glass column coated with OV-1 liquid phase, of a mixture of synthetic N-palmitoleoyl and N-palmitoyl sphinganine methaneboronates, and a mixture of synthetic N-palmitoleoyl sphinganine and N-palmitoleoyl 4-sphingenine methaneboronates,

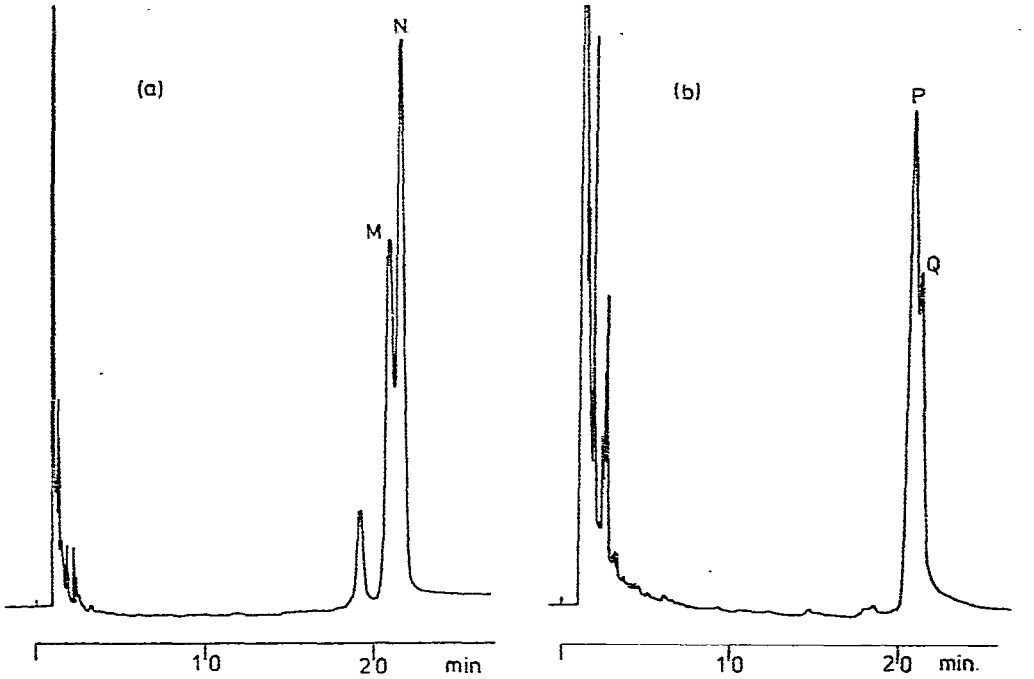


Fig. 1. Open-tubular gas–liquid chromatograms of methaneboronates of synthetic ceramides. Column 40 m × 0.55 mm I.D., coated with OV-1 on Silanox; carrier gas (helium) flow-rate, 15 ml/min; temperature, 300°. (a) Derivatives of N-palmitoleoyl sphinganine (M) and N-palmitoyl sphinganine (N). The minor peak of lower retention time is associated with peak N which was derived from a commercial sample. (b) Derivatives of N-palmitoleoyl sphinganine (P) and N-palmitoleoyl 4-sphingenine (Q). Retention index values are cited in Table I.

TABLE I

GLC RETENTION DATA, USING OV-1 AS THE LIQUID PHASE, FOR SYNTHETIC CERAMIDE METHANEBORONATES

Retention indices were determined on an open-tubular column (40 m × 0.55 mm I.D.) coated with OV-1 on Silanox, at 300°, with a carrier gas (helium) flow-rate of 15 ml/min. Ceramide methaneboronates were co-injected with n-C_{38} and n-C_{42} alkanes.

Ceramide	*Abbreviation**	*Retention index (I)*
N-Myristoyl sphinganine	(LCB 18:0; 14:0)	3702
N-Palmitoyl sphinganine	(LCB 18:0; 16:0)	3896
N-Palmitoleoyl sphinganine	(LCB 18:0; 16:1)	3886
N-Stearoyl sphinganine	(LCB 18:0; 18:0)	4097
N-Oleoyl sphinganine	(LCB 18:0; 18:1)	4073
N-Linoleoyl sphinganine	(LCB 18:0; 18:2)	4074
N-Palmitoyl 4-sphingenine	(LCB 18:1; 16:0)	3912
N-Palmitoleoyl 4-sphingenine	(LCB 18:1; 16:1)	3898

* Abbreviated ceramide names indicate carbon number and degree of unsaturation of the long-chain base (LCB) followed by the carbon number and degree of unsaturation of the N-acyl substituent.

TABLE II

MS DATA FOR SYNTHETIC CERAMIDE METHANEBORONATES

*Ceramide**		*EI mass spectrum (70 eV)***					
LCB	*acyl*	$M^{+}\cdot$	$[M-15]^{+}$	*a*	*b*	*c*	*d*
18:0	14:0	535 (4)	520 (27)	228 (100)	295 (18)	324 (9)	308 (3)
18:0	16:0	563 (1)	548 (13)	256 (100)	323 (9)	352 (7)	308 (1)
18:0	16:1	561 (74)	546 (28)	254 (50)	321 (5)	350 (5)	308 (4)
18:0	18:0	591 (2)	576 (30)	284 (100)	351 (5)	380 (4)	—
18:0	18:1	589 (98)	574 (34)	282 (62)	349 (4)	378 (4)	308 (5)
18:0	18:2	587 (100)	572 (10)	280 (9)	347 (2)	376 (1)	308 (3)
18:1	16:0	561 (3)	546 (8)	256 (100)	323 (37)	—	306 (46)
18:1	16:1	559 (30)	544 (6)	254 (88)	321 (42)	350 (1)	306 (44)

* For nomenclature, see Table I.

** Characteristic fragment ions (Fig. 2) specified as *m/e* (relative intensity); the base peak refers to ions above *m/e* 50, and was *m/e* 55 except as specified above.

respectively. Table I records Kováts' retention indices for a number of synthetic ceramide methaneboronates.

Salient features of the electron impact (EI) mass spectra of synthetic methaneboronates are recorded in Table II. Molecular ions were observed in all spectra, though relative abundances were low for ceramide methaneboronates containing a saturated N-acyl substituent. The high relative intensity of molecular ions in the spectra of ceramide boronates containing an unsaturated N-acyl substituent is noteworthy. N-Acyl groups are characterised by fragment ions a and b (Fig. 2). Mass spectra of ceramide methaneboronates incorporating sphinganine and 4-sphingenine are distinguished by the presence in the former of ions c and in the latter of ions d (Fig. 2). A full discussion of the MS characterisation of ceramide methaneboronates will be presented elsewhere[19].

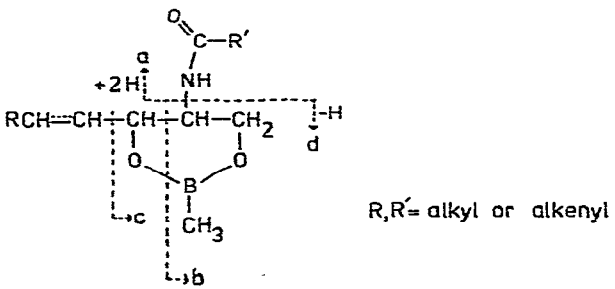


Fig. 2. Principal fragmentation modes of ceramide methaneboronates under electron impact.

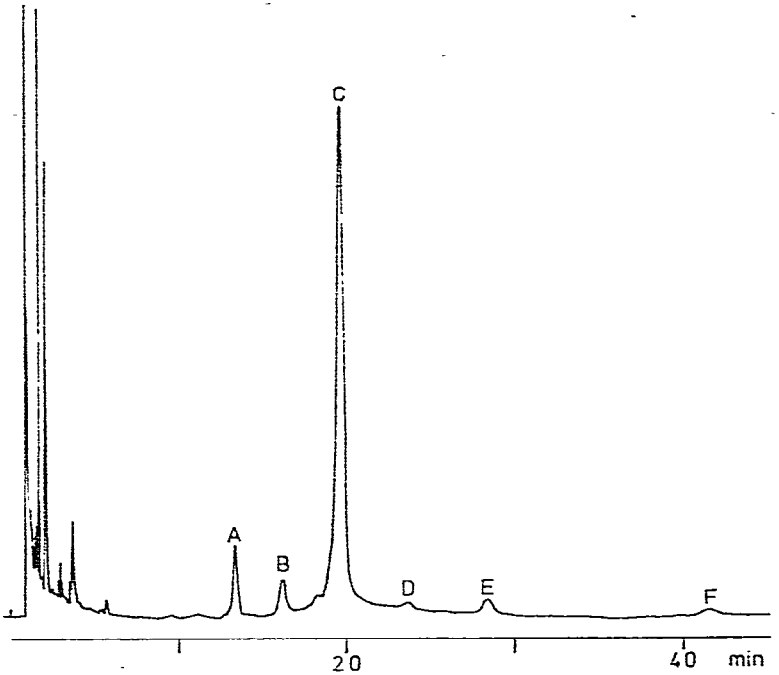


Fig. 3. Open-tubular gas–liquid chromatogram of methaneboronates of ceramides derived from sphingomyelin of human arterial tissue. Conditions, as for Fig. 1. Proposed structural assignments are given in Table III.

Analysis of ceramide mixtures

The application of methaneboronate derivatives to the analysis of natural ceramide mixtures is illustrated by the separation, shown in Fig. 3, of ceramides derived from sphingomyelin of human arterial tissue. The tissue, containing raised fibrous plaques, formed part of a severely atherosclerotic artery. Structural assignments to the components of the ceramide mixture are given in Table III. The low background "bleed" level from the open-tubular column during GLC–MS enabled the recording and interpretation of mass spectra of components of low concentration. The abundances of fragment ions of type d (Fig. 2) in each of the spectra indicated Δ^4-unsaturated long-chain bases; fragmentation of the 2,3-bond is promoted by the presence of the allylic double bond. Spectra recorded during the emergence of peak A (Fig. 3) indicated the presence of two unresolved compounds, assigned the structures N-palmitoyl hexadeca-4-sphingenine methaneboronate and N-myristoyl 4-sphingenine methaneboronate. The mass spectrum of component B, N-palmitoyl heptadeca-4-sphingenine methaneboronate, is shown in Fig. 4. The low concentration in the arterial mixture of ceramides containing very long-chain ($>C_{20}$) acyl substituents is surprising, although an increase in the relative proportions of shorter-chain acyl constituents with increasing severity of atherosclerosis has been reported[25].

Fig. 5 shows the GLC analysis of the methaneboronate derivatives of a mixture of ceramides derived from bovine brain. The separation efficiency is modest by the standards of open-tubular column GLC but analyses of very-high-molecular-weight

TABLE III

CONSTITUENTS OF A MIXTURE OF CERAMIDES DERIVED FROM HUMAN ARTERIAL SPHINGOMYELIN, ANALYSED AS THE METHANEBORONATES

*Constituent**	*Retention index***	*EI mass spectrum****						*Assignment*	
		$M^{+}\cdot$	$[M-15]^{-}$	*a*	*b*	*c*	*d*	*LCB*	*acyl*
A_1	3710	533 (6)	518 (10)	256 (100)	323 (33)	—	278 (34)	16:1	16:0
A_2				228 (64)	295 (22)	324 (6)	306 (29)	18:1	14:0
B	3815	547 (2)	532 (5)	256 (100)	323 (32)	352 (1)	292 (29)	17:1	16:0
C	3913	561 (2)	547 (7)	256 (100)	323 (34)	—	306 (35)	18:1	16:0
D	4012	575 (2)	560 (5)	270 (100)	337 (31)	366 (4)	306 (30)	18:1	17:0
E	4109	589 (3)	574 (3)	284 (100)	351 (29)	380 (2)	306 (44)	18:1	18:0
F§	4307	615 (2)	600 (5)	312 (100)	379 (35)	408 (1)	304 (25)	18:2	20:0

* See Fig. 3.

** Recorded on a glass open-tubular column (40 m × 0.55 mm I.D.), coated with OV-1 on Silanox, at 300°, with a carrier gas (helium) flow-rate of 15 ml/min.

*** For nomenclature of fragmentation, see Fig. 2. Fragment ions given as *m/e* (relative intensity).

§ Tentative assignment; low-intensity mass spectrum.

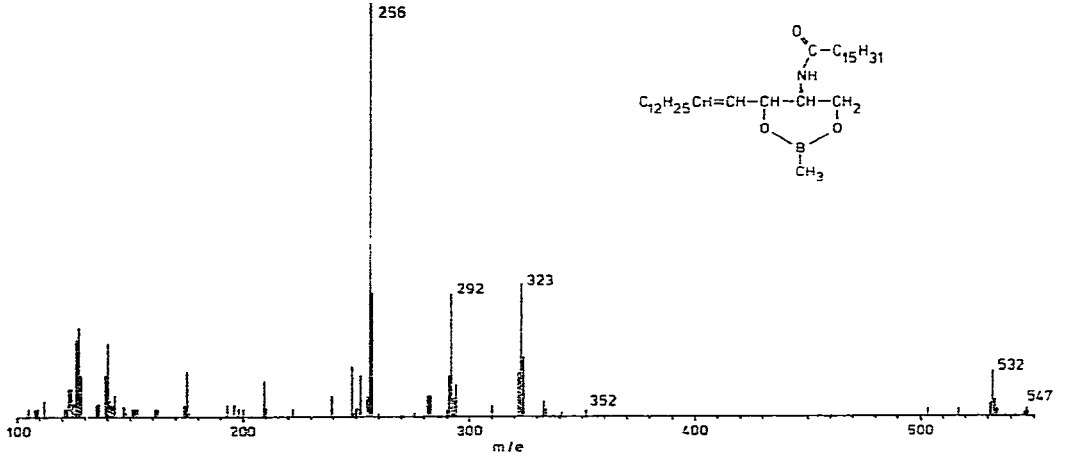


Fig. 4. Electron impact (70 eV) mass spectrum of component B (I = 3815) in the gas chromatogram (Fig. 3) of methaneboronates of ceramides derived from human arterial sphingomyelin. The proposed structural assignment is indicated.

components are achieved in relatively short times at moderate temperatures. The mass spectrum of component X is shown in Fig. 6 together with a tentative assignment of structure as the bis(methaneboronate) of N-2-hydroxystearoyl 4-sphingenine. Prominent ions are attributable to fragmentations analogous to those observed for the methaneboronates of ceramides containing unsubstituted acyl groups.

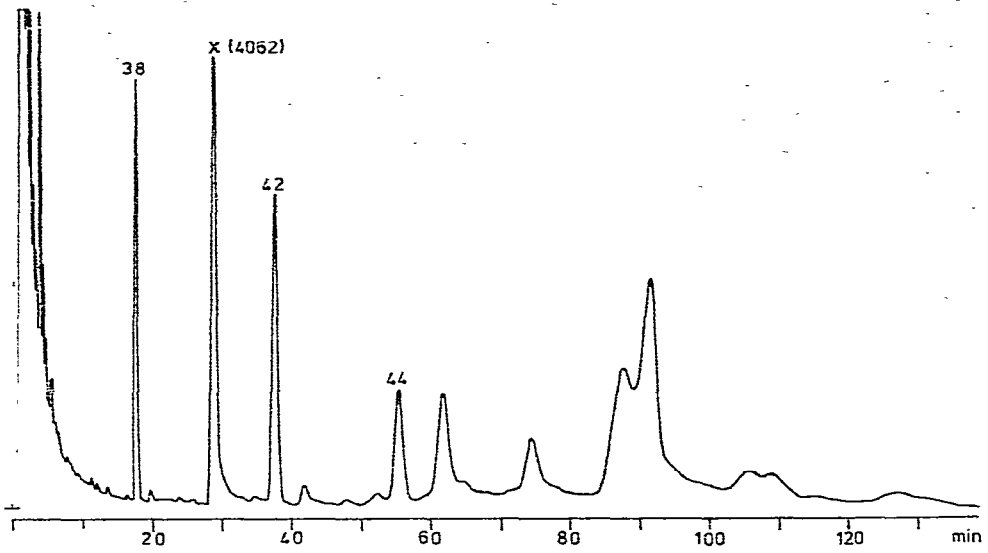


Fig. 5. Open-tubular gas–liquid chromatogram of methaneboronates of ceramides derived by enzymic hydrolysis of sphingolipids of bovine brain. Conditions, as for Fig. 1. Co-injected *n*-alkanes are labelled with the appropriate carbon number. The mass spectrum of component X is shown in Fig. 6.

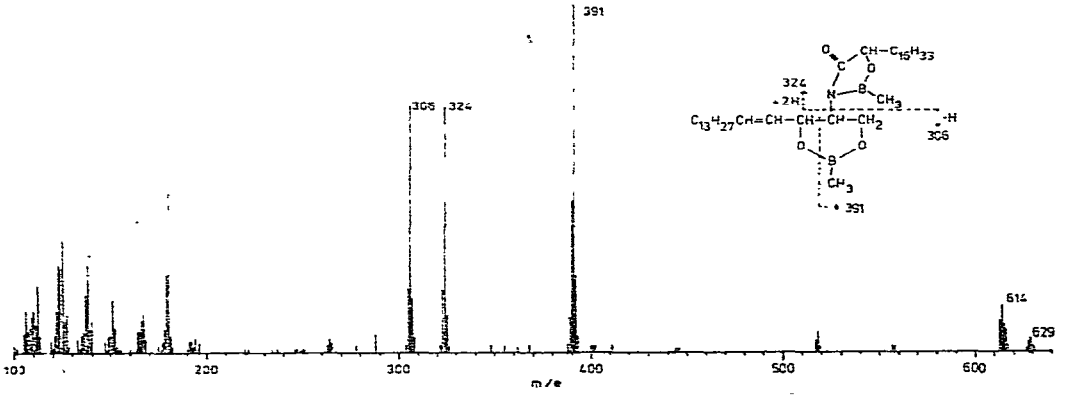


Fig. 6. Electron impact (70 eV) mass spectrum of component X (I = 4062) in the gas chromatogram (Fig. 5) of methaneboronates of ceramides from bovine brain. A tentative structural assignment is indicated.

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