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Structure Determination of the Major Component of the Starfish Ganglioside Molecular Species LG-2 by Tandem Mass Spectrometry[1]

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Abstract: The biologically active ganglioside molecular species, LG-2, which has been obtained from the starfish *Astropecten latespinosus*, was completely methylated followed by separation using reversed phase HPLC to give a methylated major component, LG-2M-5, in the pure state. The ceramide moiety of LG-2M-5 was indicated by the positive ion fast atom bombardment tandem mass spectrometry (FABMS/MS) of LG-2M-5. This fact was verified from the measurements of the positive ion FABMS/MS of the permethylates prepared from four known starfish cerebrosides.

INTRODUCTION

During the course of our search for the biologically active gangliosides from starfish, the ganglioside molecular species, GP-2, which has been obtained from the starfish *Asterina pectinifera*, has been found to support the survival of cultured neuronal cells.[2] GAA-7, obtained from the starfish *Asterias amurensis versicolor* showed neuritogenic and growth-inhibitory activities toward the mouse neuroblastoma cell line (Neuro 2a),[3] and another ganglioside molecular species (LG-2) obtained from the starfish *Astropecten latespinosus* revealed mild antitumor activity against murine lymphoma L1210 cells *in vitro*.[4] On the other hand, based on the considerable interest and importance in determining the molecular species composition of glycosphingolipids, a series of studies on the isolation and structure elucidation of pure glycosphigolipids possessing a homogeneous ceramide moiety from the starfish have been performed in our laboratory.[5-10] Continuing the preceding study,[4] we conducted the isolation and structure elucidation of pure ganglioside from the above mentioned biologically active ganglioside molecular species LG-2. In the present paper, we report the structure determination of the major component of the starfish ganglioside molecular species, LG-2, by fast atom bombardment tandem mass spectrometry (FABMS/MS).

RESULTS AND DISCUSSION

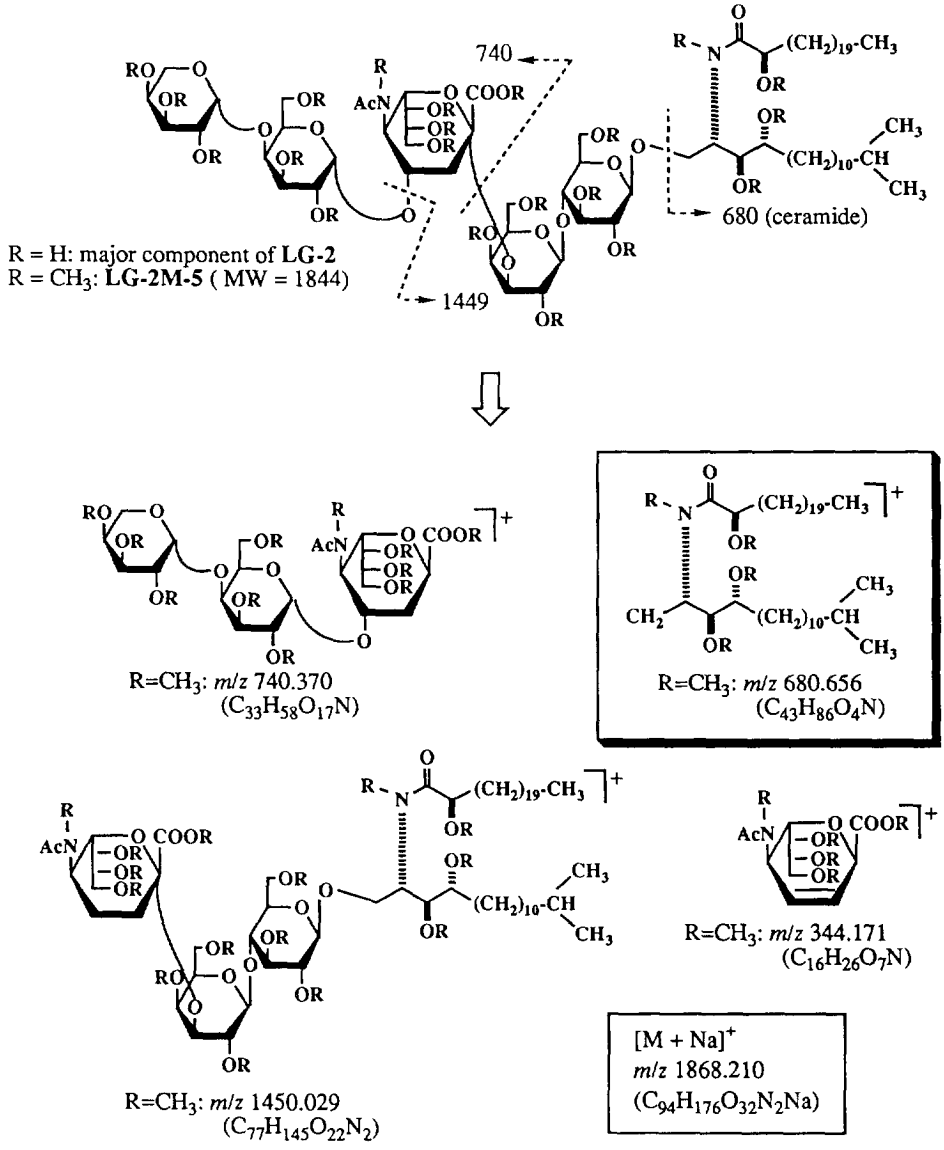
In the preceding paper, [4] we reported the structure of two new ganglioside molecular species, LG-1 and LG-2, obtained from the whole bodies of the starfish *Astropecten latespinosus*. Namely, the structure of the carbohydrate part, the absolute structure of the core part of the ceramide, and the heterogeneous 2-hydroxy fatty acid and long-chain base (phytosphingosine) components of both LG-1 and LG-2 have already been characterized. Following the preceding studies, we attempted the isolation of the pure ganglioside possessing the homogeneous ceramide moiety from the biologically active ganglioside molecular species, LG-2, by means

of high performance liquid chromatography (HPLC). However, the ideal conditions for separation of the native ganglioside molecular species were not obtained. Therefore, we examined the separation of the ganglioside molecular species (LG-2) into individual components by using its permethylated derivative, LG-2M, prepared via the Hakomori's method[11] used in the preceding studies.[4]

LG-2M was separated by reversed-phase HPLC into seven peaks, and could be recovered to give the major component, LG-2M-5, revealing a single peak upon HPLC. The structure of LG-2M-5, the permethylated derivative of the major component of LG-2, was determined as follows by the aid of the mass spectrometry because of the lack of substance. In the positive ion FAB mass spectrum (MS) of LG-2M-5 [Fig. 1(a)], the molecular ion species due to $[M + Na]^+$ and $[M + H]^+$ and fragment ion peaks were observed at *m/z* 1867, 1845, 1449, 740, 680 and 344, respectively and the structures of the ions except *m/z* 1845 were verified from the high resolution positive ion FABMS as shown in Scheme 1. In these peaks, the peak at *m/z* 680 must be derived from the ceramide moiety, however, its fatty acid and long-chain base component could not be characterized. In addition, since no other ion peaks giving useful information about the ceramide composition were obtained in the positive ion FABMS, positive ion FAB tandem mass spectrometry (MS/MS) for LG-2M-5 was conducted. Namely, to clarify the structure of the ceramide moiety of LG-2M-5, the ion at *m/z* 680 was selected as a precursor ion and the collision-activated dissociation (CAD) spectrum was measured.

The positive ion CAD spectrum [Fig. 1(b)] showed characteristic fragment ion peaks at *m/z* 296, 325, 382, 384, 408 and 438, together with the groups of peaks spaced 14 mass units apart (corresponding to a difference in CH_2) due to the loss of the methylene groups in the alkyl groups of the fatty acid and long-chain base moieties in the range over 450 mass units. In the characteristic fragment ion peaks, the peak at *m/z* 438 (ion *a*) is presumed to be formed by the A cleavage of the parent ion *m/z* 680 and that at *m/z* 325 (ion *e*) by the fission of the α–ketoalcohol (E cleavage) of ion *a*. Ion *b* at *m/z* 408 must arise from the cleavage between C-2 and C-3 of the long-chain base part (B cleavage). On the basis of these three fragment ions, *m/z* 325 (*e*), 408 (*b*) and 438 (*a*), indicating the 2-hydroxy fatty acid moiety of the ceramide part of LG-2M-5, the fatty acid moiety is regarded as a 2-hydroxydocosanoic acid derivative. On the other hand, the long-chain base (phytosphingosine) moiety was characterized by three other fragment ion peaks at *m/z* 296, 382 and 384. The peak at *m/z* 296 (*c* - H ion) presumably arose from fission of the C-N bond (C cleavage) followed by elimination of one hydrogen. The ions at *m/z* 384 and 382 can be attributed to the long-chain base residue and its dehydro analog, respectively, due to D cleavage (ion *d* and *d* - 2H). Thus, the long-chain base moiety of the ceramide part of LG-2M-5 is suggested to be a 2-amino-15-methyl-1,3,4-hexadecanetriol derivative.

Meanwhile, to verify the above mentioned fragmentation of the ceramide ion, the measurements of positive ion FABMS/MS of the known cerebroside permethylates were conducted. Four known pure starfish glucocerebrosides, astrocerebroside C,[9] acanthacerebroside B,[5,7] asteriacerebroside D[10] and acanthacerebroside C,[5] possessing the same ceramide framework as that of LG-2 but consisting of different fatty acid and long-chain base components from those of LG-2, were methylated using Hakomori's method[11] to produce their permethylated derivatives **1**, **2**, **3** and **4** (Fig. 2). Compounds **1**, **2**, **3** and **4**, respectively, revealed the important fragment ion peak due to ceramide residue at *m/z* 708, 666, 650 and 664, like the ion at *m/z* 680 of LG-2M-5, together with the molecular ion peaks in their positive ion FABMS. When each ceramide ion (*m/z* 708, 666, 650 and 664) was selected as a precursor ion, and the positive ion CAD spectrum of the each ceramide ion was measured, the characteristic fragment ion peaks (*a*, *b*, *c* - H, *d*, *d* - 2H



Scheme 1 Positive ion FAB mass spectral fragmentation of **LG-2M-5**

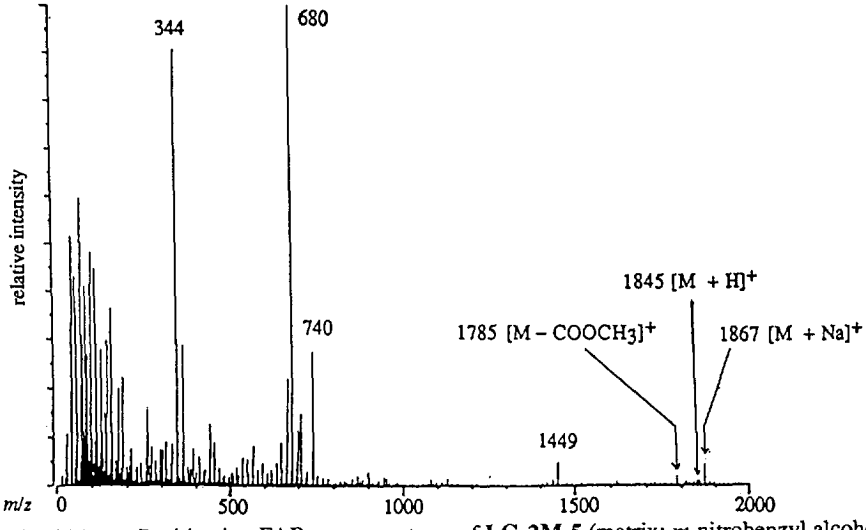


Fig. 1(a) Positive ion FAB mass spectrum of **LG-2M-5** (matrix: *m*-nitrobenzyl alcohol)

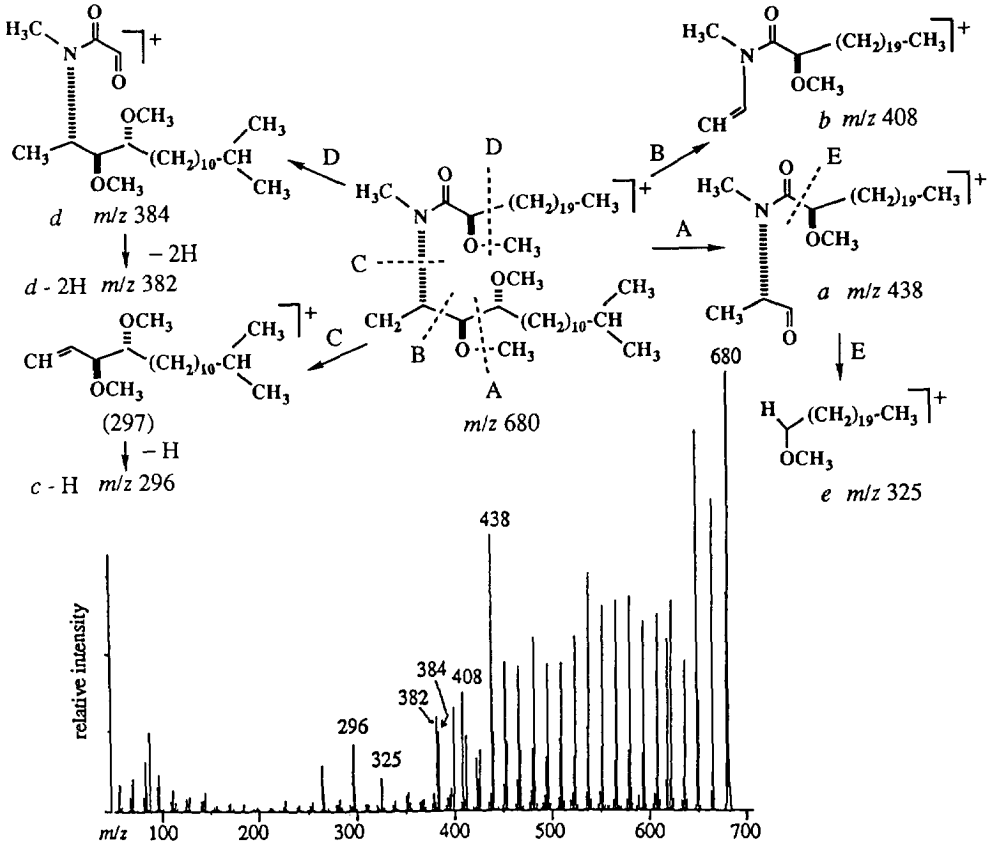


Fig. 1(b) Positive ion CAD spectrum of **LG-2M-5**
The ion at *m/z* 680 was selected as the precursor ion.

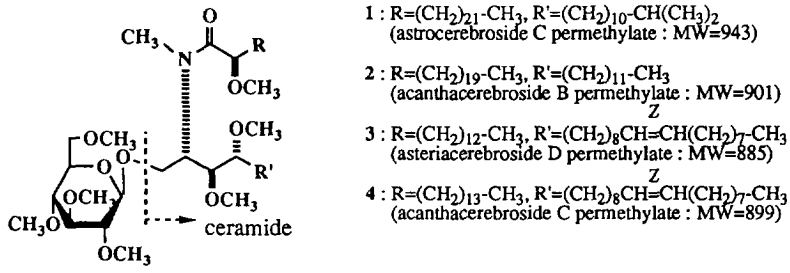
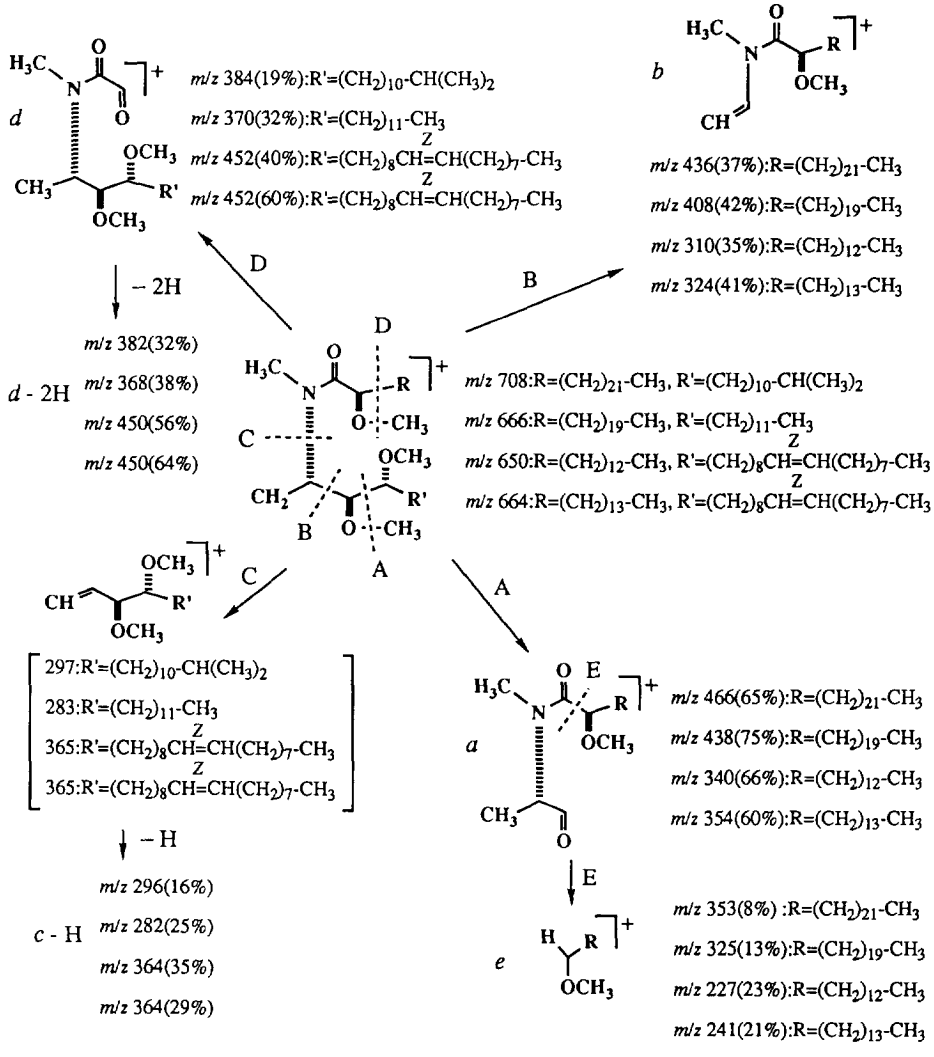


Fig. 2 Structures of cerebroside permethylates



Scheme 2 Positive ion CAD spectral fragmentation of Cerebroside Permethylates. The ions at *m/z* 708, 666, 650 and 664 were selected as precursor ions. ( ): relative intensities

and *e*), which have been observed in the CAD spectrum of the ceramide ion from LG-2M-5, were observed apparently from all four various ceramide ions as shown in Scheme 2. Consequently, the proposed fragmentation of the ceramide ion due to A, B, C, D and E cleavage is reasonable.

Accordingly, the structure of the ceramide moiety of LG-2M-5 is considered to be the 2-(2-hydroxydocosanoyl amino)-15-methyl-1,3,4-hexadecanetriol derivative, and therefore, the structure of the LG-2M-5, the permethylated derivative of the major component of LG-2, and the corresponding native major component of LG-2 are determined as revealed in Scheme 1.

As for the application of the tandem mass spectrometry to the structure elucidation of glycosphingolipids, several useful studies have been reported.[12-18] According to the application of tandem mass spectrometry for the analysis of permethylated glycosphingolipids, our study at this time, to the author's knowledge, follows the studies by Costello[15] and Her[17]. However, with regard to the analysis of the fragmentation of the permethylated ceramide ion (for example *m/z* 680) consisting of the phytosphigosine base and 2-hydroxy fatty acid residue in the positive ion FABMS/MS, we believe this study has been made for the first time and must be useful for the structure elucidation of glycosphingolipids. Structure determination of the major component of the biologically active starfish ganglioside molecular species by tandem mass spectrometry is worthy to note.

EXPERIMENTAL

Positive ion FABMS. All mass spectra (FABMS and FABMS/MS) were acquired using a JEOL SX/SX102A tandem mass spectrometer of BEBE geometry, which was controlled by a JEOL DA-7000 data system. Positive ion FABMS were obtained using only the first spectrometer (MS1), and the spectra were measured with the following conditions: xenon atom beam, 5 kV; ion source accelerating potential, 10 kV; matrix, m-nitrobenzyl alcohol.

Positive ion FABMS/MS . The fragment ions generated by positive ion FABMS were selected as precursor ions, and collided with argon molecules in the third field-free region. The argon pressure was sufficient to attenuate the primary ion beam by 50 %. The fragment ions were dispersed by the second spectrometer (MS2) and the spectra were recorded as the CAD spectra.

Separation of LG-2M. LG-2M, permethylate of LG-2, prepared using Hakomori's method in the preceding studies[4] showed seven peaks in the reversed-phase HPLC [column, cosmosil 5C18-AR (4.6 x 250 mm); solvent 90% EtOH; flow rate 0.8 ml/min; detection, RI detector] : t_R[min](ratio of peak areas) = 16.2 (3), 25.7 (8), 29.5 (5), 32.0 (6), 34.1 (35), 41.3 (9), 48.7 (6). 1 mg of LG-2M was separated by HPLC into the above mentioned seven fractions using the conditions described above and the major fraction (fraction 5, LG-2M-5, 0.3 mg) was obtained.

LG-2M-5. Positive ion FABMS[see Fig.1(a)]: *m/z* (%) 1867 (4) $[M + Na]^+$, 1845 (1) $[M + H]^+$, 1785 (2) $[M - COOCH_3]^+$, 1449 (5), 740 (27), 680 (100) (ceramide ion), 344 (91). HR Positive ion FABMS (see Scheme 1): *m/z* 1868.210 $[M + Na]^+$ (calcd for $C_{94}H_{176}O_{32}N_2Na$, 1868.213), 1450.029 (calcd for $C_{77}H_{145}O_{22}N_2$, 1450.031), 740.370 (calcd for $C_{33}H_{58}O_{17}N$, 740.373), 680.656 (calcd for $C_{43}H_{86}O_4N$, 680.658), 344.171 (calcd for $C_{16}H_{26}O_7N$, 344.172). Positive ion FABMS/MS [CAD of *m/z*

680, see Fig. 1(b)]: *m/z* (%) 438 (73) (ion *a*), 408 (30) (ion *b*), 384 (19) (ion *d*), 382 (24) (ion *d* - 2H), 325 (9) (ion *e*), 296 (16) (ion *c* - H).

Methylation of cerebrosides. Four known cerebrosides, astrocerebroside C,[9] acanthacerebroside B,[5,7] asteriacerebroside D[10] and acanthacerebroside C,[5] were methylated using Hakomori's method[11] as described in the preceding paper[4] to give their permethylates, astrocerebroside C permethylate (**1**), acanthacerebroside B permethylate (**2**), asteriacerebroside D permethylate (**3**) and acanthacerebroside C permethylate (**4**), respectively, as shown in scheme 2.

Astrocerebroside C permethylate (**1**). Positive ion FABMS: *m/z* (%) 966 (5) $[M + Na]^+$, 944 (17) $[M + H]^+$, 708 (100) (ceramide ion). Positive ion FABMS/MS (CAD of *m/z* 708, see Scheme 2): *m/z* (%) 466 (65) (ion *a*), 436 (37) (ion *b*), 384 (19) (ion *d*), 382 (32) (ion *d* - 2H), 353 (8) (ion *e*), 296 (16) (ion *c* - H).

Acanthacerebroside B permethylate (**2**). Positive ion FABMS: *m/z* (%) 924 (15) $[M + Na]^+$, 902 (16) $[M + H]^+$, 666 (100) (ceramide ion). Positive ion FABMS/MS (CAD of *m/z* 666, see Scheme 2): *m/z* (%) 438 (75) (ion *a*), 408 (42) (ion *b*), 370 (32) (ion *d*), 368 (38) (ion *d* - 2H), 325 (13) (ion *e*), 282 (25) (ion *c* - H).

Asteriacerebroside D permethylate (**3**). Positive ion FABMS: *m/z* (%) 908 (7) $[M + Na]^+$, 886 (27) $[M + H]^+$, 650 (100) (ceramide ion). Positive ion FABMS/MS (CAD of *m/z* 650, see Scheme 2): *m/z* (%) 340 (66) (ion *a*), 310 (35) (ion *b*), 452 (40) (ion *d*), 450 (56) (ion *d* - 2H), 227 (23) (ion *e*), 364 (35) (ion *c* - H).

Acanthacerebroside C permethylate (**4**). Positive ion FABMS: *m/z* (%) 922 (14) $[M + Na]^+$, 900 (24) $[M + H]^+$, 664 (100) (ceramide ion). Positive ion FABMS/MS (CAD of *m/z* 664, see Scheme 2): *m/z* (%) 354 (60) (ion *a*), 324 (41) (ion *b*), 452 (60) (ion *d*), 450 (64) (ion *d* - 2H), 241 (21) (ion *e*), 364 (29) (ion *c* - H).

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