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W. Schänzer  
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A. Gotzmann  
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Kairong Cui, Moutian Wu\*, Xin Liu, Yinong Zhang and Shan Wang

## **Study on the Metabolites of Bromantane in Urine Using GC/MS and GC/Tandem MS/MS**

China Doping Control Center, Beijing, China

### **ABSTRACT**

This paper presents a study on the urinary metabolites of bromantane in man using GC/MS and GC/tandem MS/MS. Through comparison of the chromatograms between blank urine and bromantane positive urine, the estimated molecular weights of the possible metabolites, and the isotopic ions with element bromine, the proposed metabolites, including monohydroxybromantane, dihydroxybromantane, dehydromonohydroxybromantane and dehydrodihydroxybromantane were detected and characterized. The fragmentation pathways were studied by GC/MS/MS both in parent mode and in daughter mode.

### **1. INTRODUCTION**

Though bromantane is banned by IOC we could find few publications about it. Some of them were presentations given by Dr. Ayotte and Dr. Semenov in the workshop in Cologne respectively, in which they showed that the major metabolites of bromantane were hydroxylated bromantane<sup>(1,2)</sup>. From the mass spectra<sup>(3)</sup> we can see there may be dehydrohydroxybromantane in bromantane positive urine.

Our study was focused on trying to estimate in which ring, in the aromatic ring or/and in the adamantane-ring, the hydroxylation of bromantane could occur during the metabolism of bromantane. Before the study we used GC/NPD and GC/MS to check the purity of the bromantane tablet used for excretion study. The gas chromatogram and TIC of GC/MS showed that the purity of the bromantane tablet was good enough for our excretion study. We used GC/

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\*: corresponding author

MSD to obtain the normal EI mass spectra of the possible metabolites of bromantane and further on used GC/tandem MS/MS to study the fragments of the proposed metabolites because the tandem MS/MS can eliminate the interference of biomatrixes to a great extent and supply the explication for the structure of the fragments. According to the comparison of ion chromatograms between blank urine and the bromantane positive urine 13 possible metabolites of bromantane were found using the estimated molecular weights with the characteristic isotopic bromine element.

## 2. EXPERIMENTAL

### 2.1 *Subject and Sample collection*

A 53 year old volunteer, healthy Chinese male, received orally a single tablet (50 mg Bromantane Hydrochloride kind gift from the Moscow Doping Laboratory). 0~106 hr urine samples were collected and stored at -20°C till analysis.

### 2.2 *GC/MS and GC/MS/MS analysis*

A gas chromatograph model 6890 (Hewlett-Packard) fitted with a model 7673A auto-sampler was connected to a mass selective detector 5973 (Hewlett-Packard). For GC/MS determination the separation was carried out with HP-1 column (17m, 0.2 mm I.D., 0.33  $\mu$ m filmthickness). The injector, operated in split mode (1:5 split ratio) and the interface were both maintained at 280°C. The oven temperature program was: initial 180°C, rate 1: 3.3°C/min to 231°C, rate 2: 30°C/min to 310°C and maintained for 2min at 310°C. Helium was used as a carrier gas at flow rate 0.8ml/min (at 180°C). During the running the pressure was kept constant automatically. The mass spectra were obtained in full scan mode from m/z 50 to m/z 550 in 1 second.

A gas chromatograph model 5890 series II plus (Hewlett-Packard) was connected to the TSQ-7000 (Finnigan) for GC/MS/MS determination. The same gas chromatographic column and temperature program was used as described above for GC/MSD. The transfer line was set at 300°C. The injector, operated in split mode (1:15 split ratio) and the interface were maintained both at 280°C. For GC/MS/MS the first MS worked in EI mode, (electron energy 70 eV), manifold was set at 70°C, ion source at 180 °C, conversion dynode at 13 kV, scan rate of the

daughter mass spectra at 500 a.m.u./s, dwell time in RIM mode at 100 ms/per ion. Emission current was 400  $\mu$ A, electron multiplier voltage between 1100~1300. PFTBA was used as the calibrate for tuning. Collision gas was argon gas at 1.8~2 mTorr. Collision energy depended on substances in the range of -15 ~ -25 V. The correct factor (MSMSC) was 0.7.

### 2.3 *Extraction and Derivatization*

5 ml of urine sample were added to an Amberlite XAD-2 column. After the column was washed with 5 ml distilled water, 50  $\mu$ l methyltestosterone in methanol (1 ng/ $\mu$ l) as an internal standard was added to the column. The absorbed fraction was eluted with 2 ml methanol. The methanolic eluate was evaporated to dryness. The residue was dissolved in 1 ml of 0.2 M phosphate buffer pH 6.8 and hydrolyzed with 100  $\mu$ l  $\beta$ -glucuronidase from E.coli (12500 unit/3 ml phosphate buffer). After hydrolysis at 55°C for 3 h about 100 mg of solid carbonate buffer were added to alkalize the hydrolyzed solution. The freed bromantane metabolites were extracted with 5 ml ether. After centrifugation the ethereal layer was transferred and evaporated to dryness. The residues were derivatized with 50  $\mu$ l of MSTFA/TMSI/dithioerythritol 1000:3:1 (v/v/w) and heated at 70°C for 30 min. 1  $\mu$ l of the derivatized solution was injected into the GC/MS or GC/MS/MS.

## 3. RESULTS AND DISCUSSION

The first kind of bromantane metabolism is monohydroxylation. The molecular isotopic ions 393 and 395 with bromine were used to find the monohydroxybromantanes, which were expected as metabolites of bromantane in human urine. We have found five possible monohydroxybromantanes (1-I~1-V) with the retention times of 10.49, 11.01, 11.66, 12.44 and 12.60 minutes respectively. (See Fig. 1.) The five possible metabolites of bromantane have the same molecular ion m/z 393 (395). It was confirmed by GC-MS/MS in parent mode that these ions (393/395) did not come from any other mother ions. The mass spectra of 1-I~1-V were showed in Fig. 2. In the EI mass spectra obtained by GC-MS the ions m/z 73, 91, 133, 223 and the molecular ions 393 (395) were common ions. It evidenced that the five possible monohydroxybromantane have a similar structure. Furthermore, from the daughter mass spectra of ions 393 and 395 showed in Fig.

3 respectively, the fragments with or without isotope of bromine could be clearly identified by the mass difference of  $m/z$  2 and almost equal abundance ratio in the correspondent ions. With MS/MS in daughter mode these ions  $m/z$  393 and  $m/z$  395 produced common daughter ions  $m/z$  223,  $m/z$  171 (173) and  $m/z$  184 (186). Based on these data the proposed fragmentation pathway of monohydroxybromantane was showed in Fig. 4. Such fragmentation pattern clearly inferred that all of the monohydroxyl group substituted in the adamantane-ring.

The second kind of bromantane metabolism we proposed is dehydromonohydroxylation. The molecular bromine containing isotopic ions 391 and 395 were used to find the dehydromonohydroxybromantane (2-I). The chromatogram and the EI mass spectrum of 2-I with the retention time 9.85 min obtained by GC/MSD were showed in Fig 5. The isotope ions of  $m/z$  391/393,  $m/z$  376/378,  $m/z$  302/304,  $m/z$  234/236 and  $m/z$  184/186 both in EI mass spectra and in daughter mass spectra (See Fig.6) confirmed that the element of bromine was included in the chemical structures of these ions. The common ions of 223 and 131 in daughter mass spectra of ions  $m/z$  391 and  $m/z$  393 implied that there was no element of bromine in the structures of ions  $m/z$  223 and 131. So the possible fragmentation pathway of 2-I was suggested in Fig.7. The dehydro-monohydroxylation could be related to the adamantane-ring. However, it is not clear where the ion  $m/z$  223 instead of 221 came from.

The third kind of possible bromantane metabolism is dihydroxylation. The molecular ion of these metabolites (3-I~3-VI) is 481 and ion 483 should be the corresponding isotopic one. Fig. 8 showed the ion  $m/z$  481/483 chromatograms and Fig 9 the EI mass spectra of 3-I~3-VI obtained by GC/MSD. The daughter mass spectra of ions  $m/z$  481/483 of 3-I~3-VI (except 3-III) in Fig. 10 showed that the common couple ions with isotope of element bromine were 481/483, 259/261 and 272/274. The common ions 223 and 133 indicated the same fragments as the fragments of monohydroxybromantane. It was also confirmed by the same daughter mass spectra of ions  $m/z$  223 of 1-I and 3-V in Fig. 11. This was the evidence that only one hydroxyl group substitution took place in the admantane-ring of 3-I, 3-II, 3-IV, 3-V and 3-VI. The daughter mass spectra of ions  $m/z$  481/483 of 3-III showed the couples of ions, 378/380, 336/338 and the common ions 312, 222, 131 (See also Fig. 10). The possible mechanism of fragmentation of dihydroxy-

bromantane was suggested in Fig.12. The dihydroxylation could be introduced in two different ways: either both of the two hydroxyl groups substituted in the adamantane-ring or one hydroxyl group in the adamantane-ring and the other in the aromatic ring. The exact substituted positions in these rings are not clear.

The last kind of possible bromantane metabolism is dehydrodihydroxylation. We have found a possible metabolite (4-I) with the molecular ion of 479 and its isotopic ion 481. The gas chromatogram and its EI mass spectrum were showed in Fig. 13. The couples of ions 479/481, 464/466, 243/245 and so forth were seen clearly in Fig. 14. In MS/MS daughter mode we can see more couples of ions and the common ion 221. The structure and the fragmentation pathway of 4-I were suggested in Fig.15. The  $m/z$  243/245 and 221 are very characteristic. They clearly inferred that one hydroxylation occurred in the aromatic-ring and the other hydroxylation and the double band in the adamantane-ring.

The retention times of 13 possible metabolites of bromantane, which could be divided into 4 groups, were summarized in Tab.1

Tab.1 The Retention Time and Relative Retention Time in GC/MSD and GC/MS/MS

Possible Met.	Rt (min, MSD)	RRt	Rt (min, MS/MS)	RRt	Hydroxylation
1-I	11.01	0.72	10.89	0.71	OH in the adamantane ring
1-II	11.66	0.76	11.68	0.77	OH in the adamantane ring
1-III	12.44	0.81	12.41	0.81	OH in the adamantane ring
1-IV	12.60	0.82	12.89	0.84	OH in the adamantane ring
1-V	10.49	0.70	10.41	0.68	OH in the adamantane ring
2-I	9.82	0.65	9.57	0.63	OH in the adamantane ring
3-I	15.35	0.99	15.11	0.98	one OH in each ring
3-II	15.68	1.01	15.49	1.01	one OH in each ring
3-III	16.52	1.07	16.67	1.09	Both OH in the adamantane ring
3-IV	11.29	0.73	10.99	0.73	one OH in each ring
3-V	13.97	0.90	13.76	0.90	one OH in each ring
3-VI	14.78	0.96	14.56	0.95	one OH in each ring
4-I	13.11	0.85	12.95	0.84	one OH in each ring
I.S.	15.42	-	15.27	-	-

As examples the urinary excretion variations of some main metabolites (1-II, 1-III and 1-IV) were showed in Fig. 16. The concentrations of 1-II, 1-III and 1-IV in urine were relative high and they were detectable until 72 hr. after application of bromantane. The concentrations of others are lower.

### References

1. C.Ayotte, We can't escape it, 15<sup>th</sup> Cologne Workshop on Dope Analysis, 1997
2. V.F.Sizoi, S.L.Bolotiv and V.A.Semenov, Study of Bromantan Metabolites Structure, Recent Advances in Doping Analysis (5), Sport & Buch Strauss, 1998, pp 287-300
3. Private Communication with Prague Doping Control Laboratory, 1997

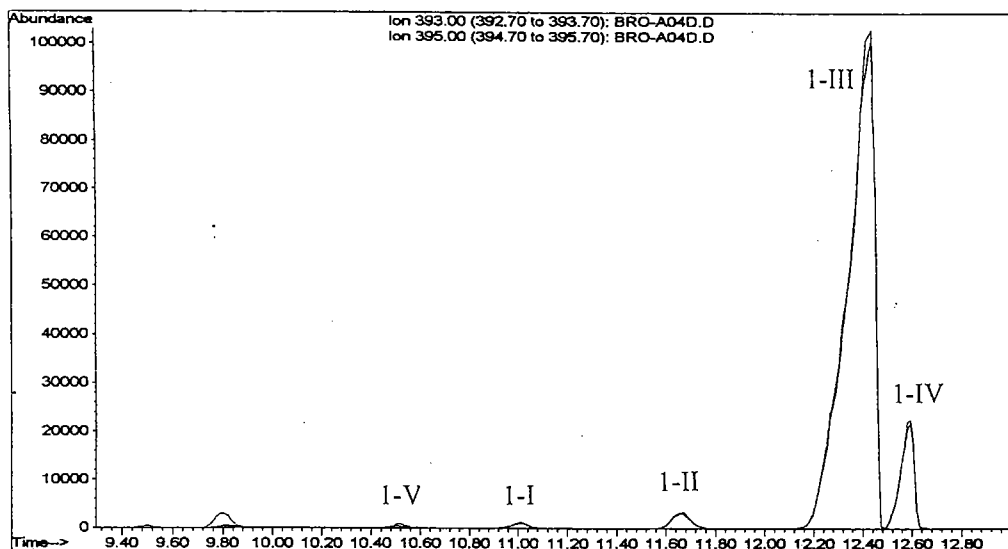
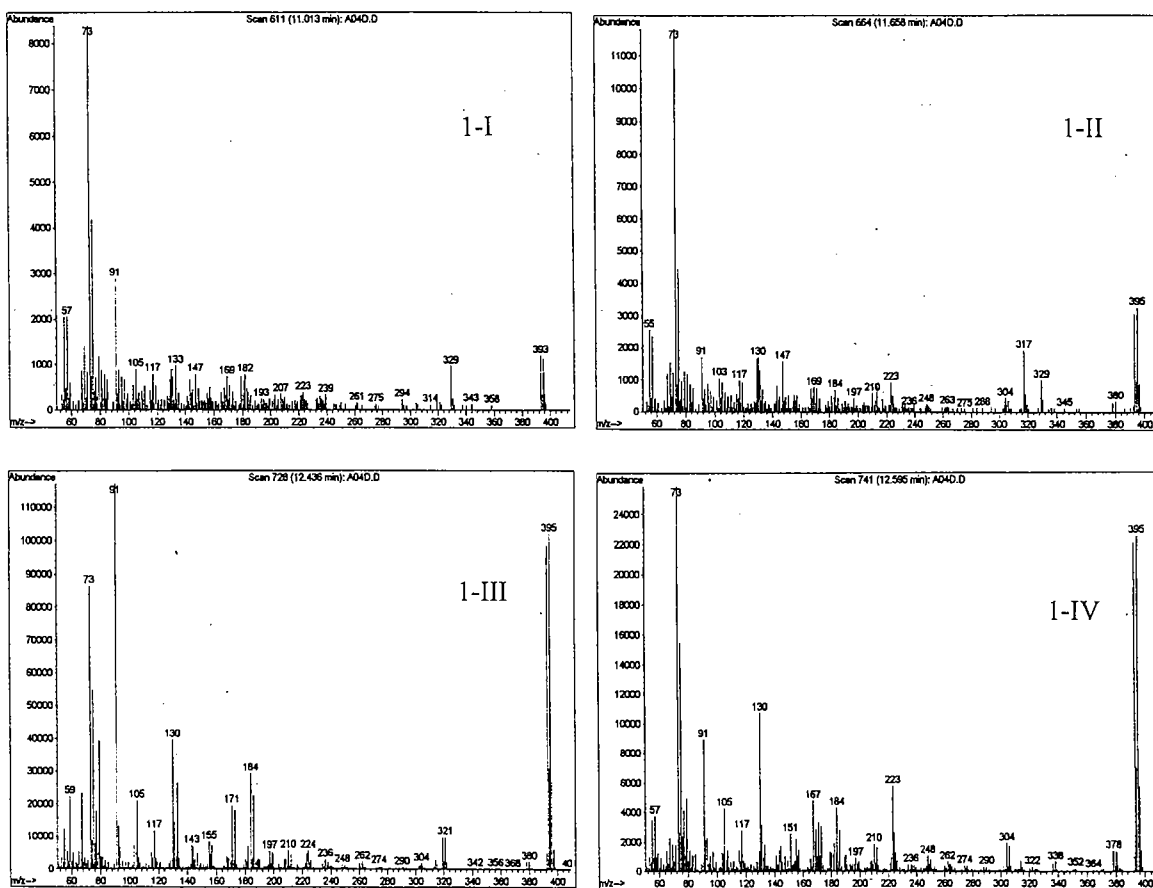


Fig.1 The Chromatograms of Monohydroxybromantane 1-I~1-V



(Fig.2 continuing)



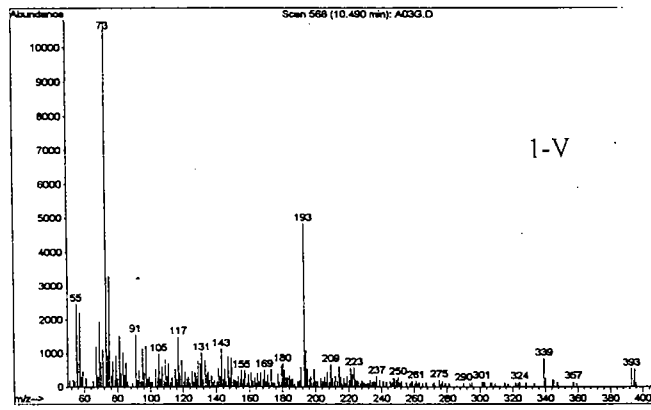
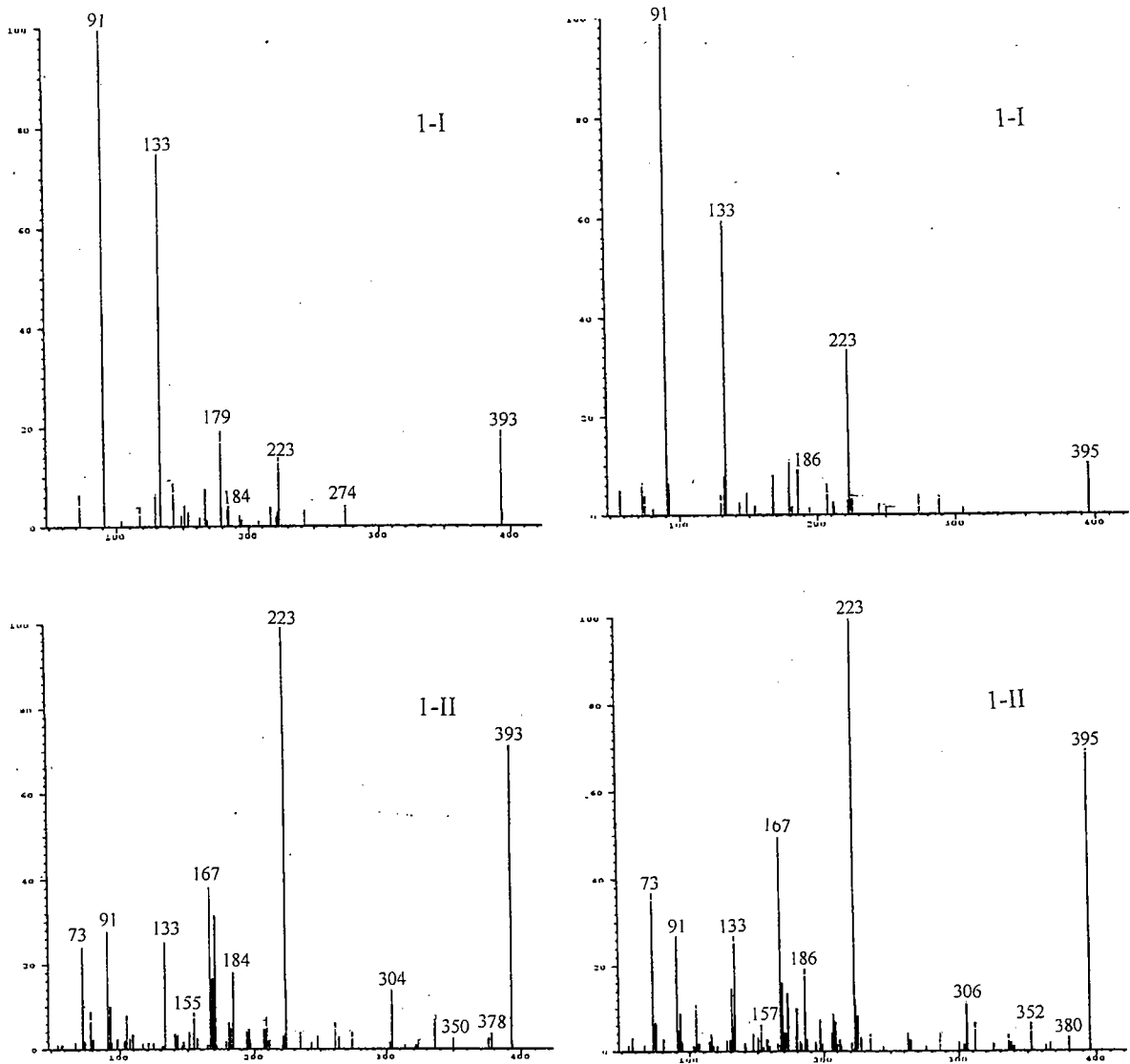


Fig.2 The Mass Spectra of Monohydroxybromantane 1-I~1-V



(Fig.3 continuing)

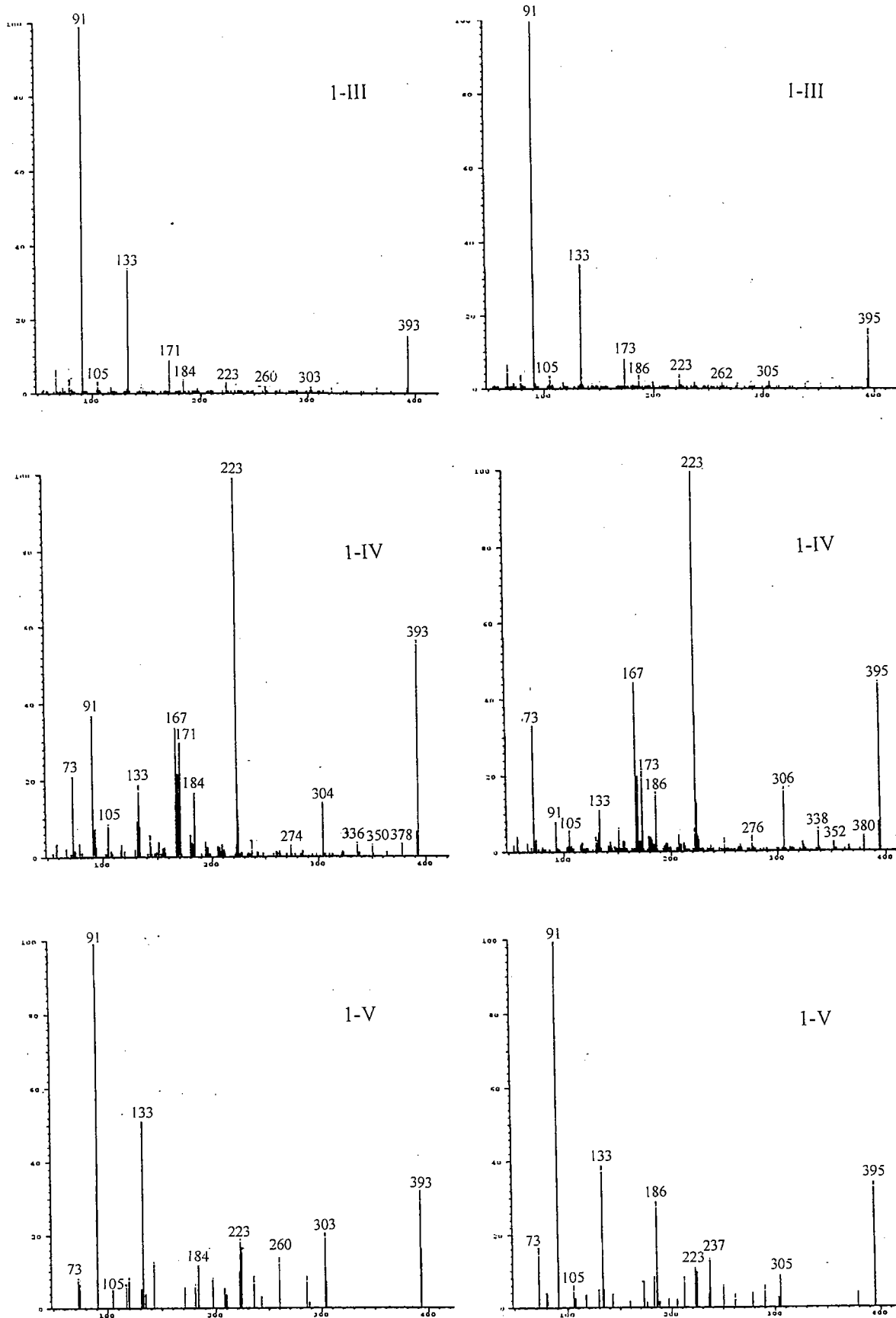


Fig. 3 The Daughter Mass Spectra of Monohydroxybromantanes 1-I~1-V  
(Left: m/z 393, Right: m/z 395)

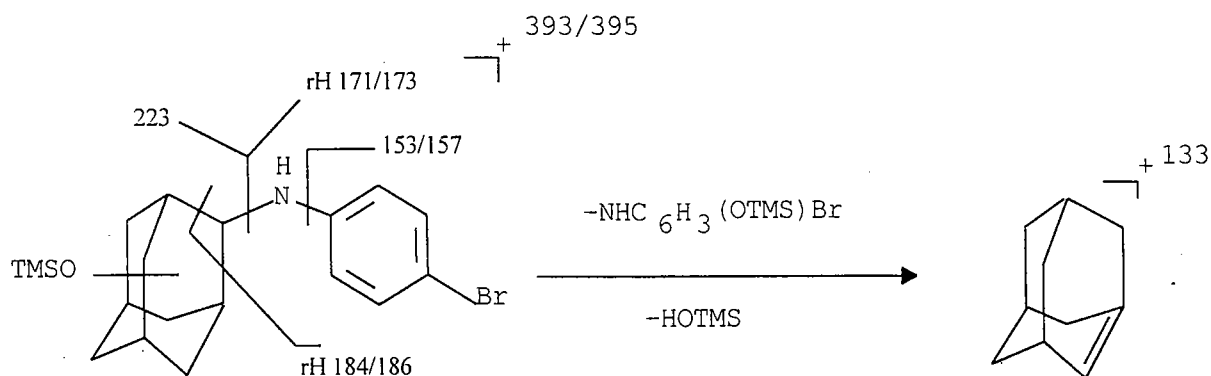


Fig.4 The possible Fragmentation Pathway of Monohydroxybromantanes

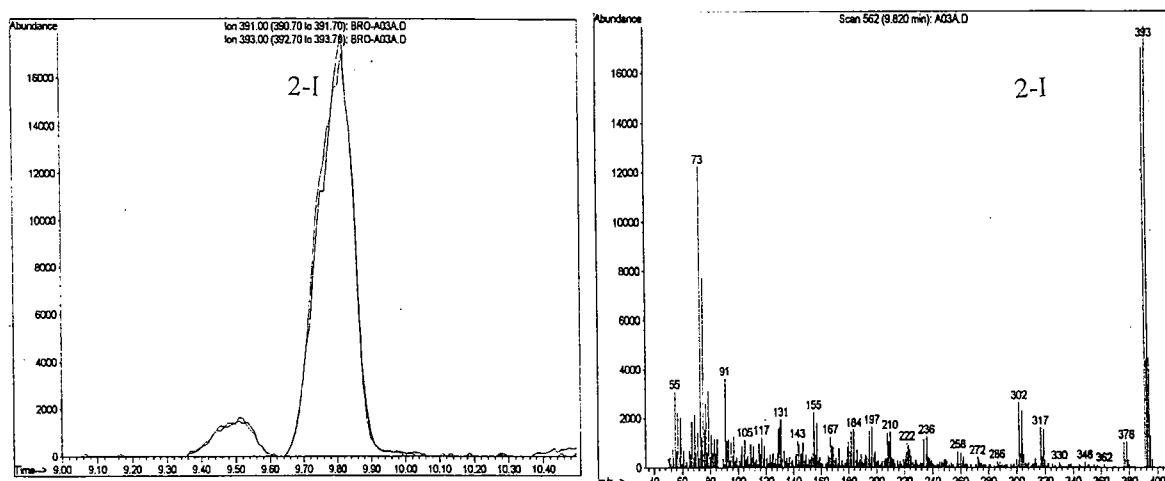


Fig.5 The Chromatogram and EI Mass Spectrum of 2-I

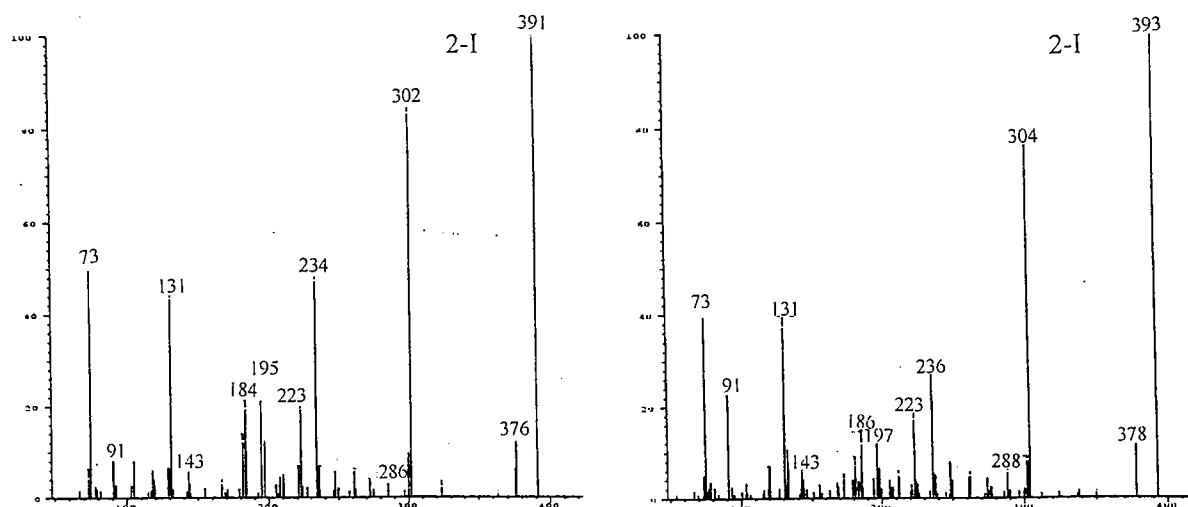


Fig.6 The Daughter Mass Spectra of 2-I (Left: m/z 391, Right: m/z 393)

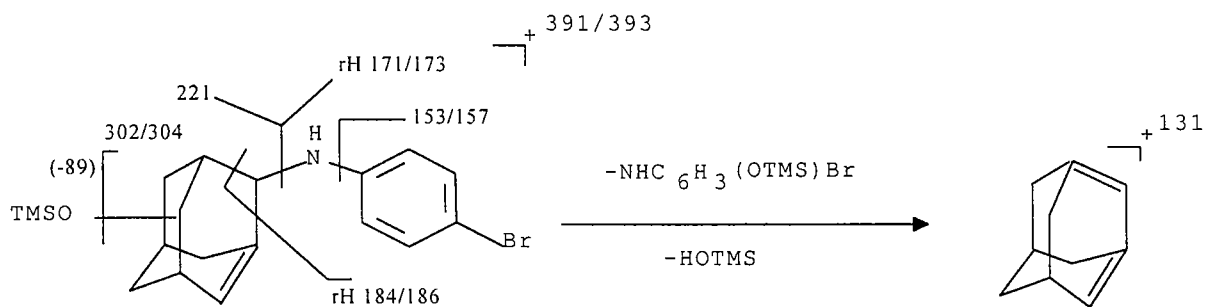


Fig.7 The Possible Fragmentation Pathway of 2-I

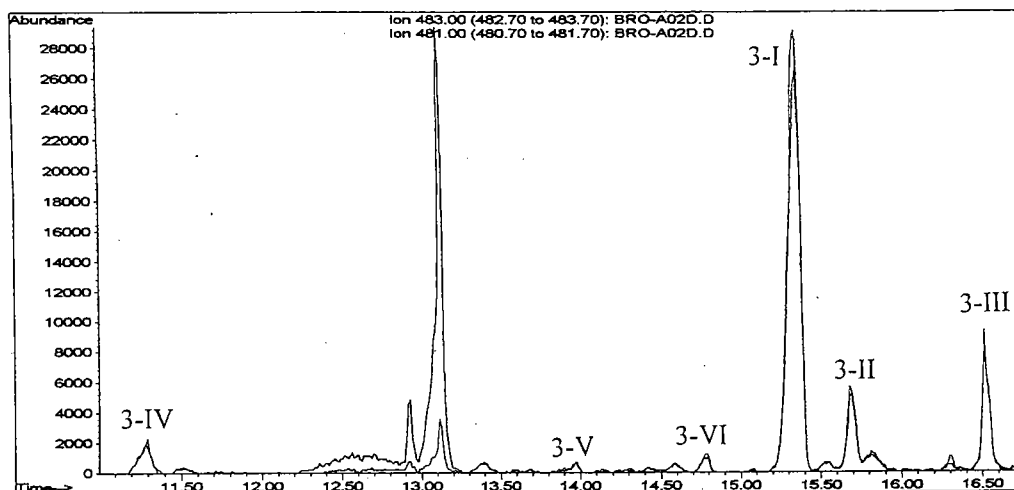
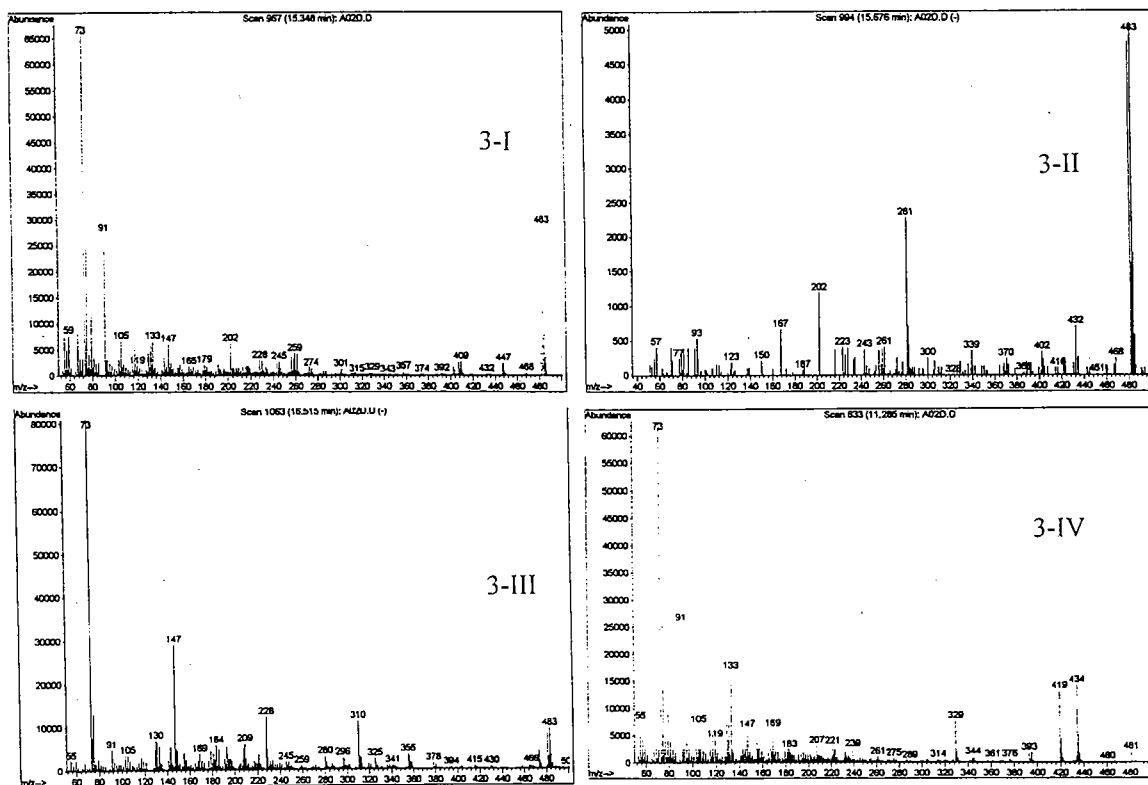


Fig.8 The Chromatograms of Dihydroxybromantanes 3-I~3-VI



(Fig.9 continueing)

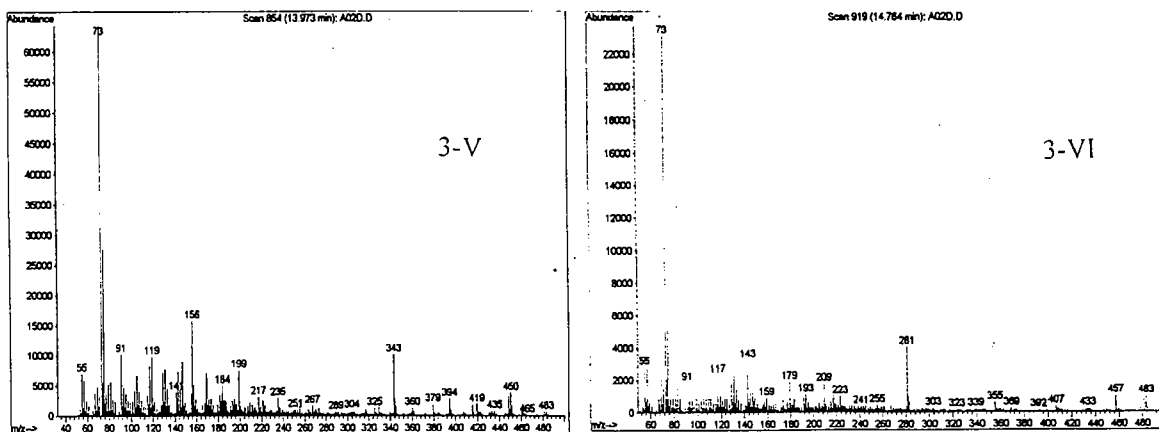
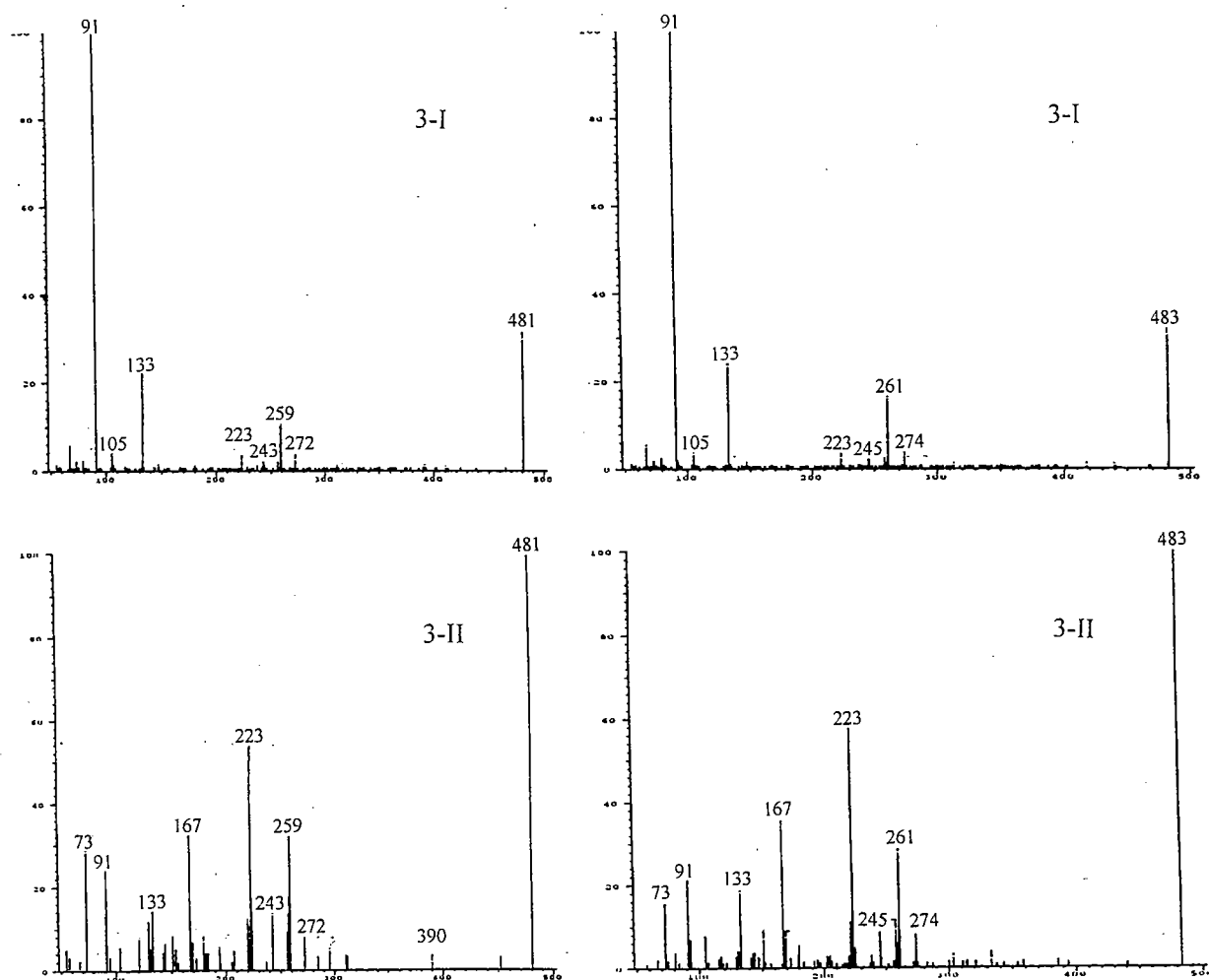
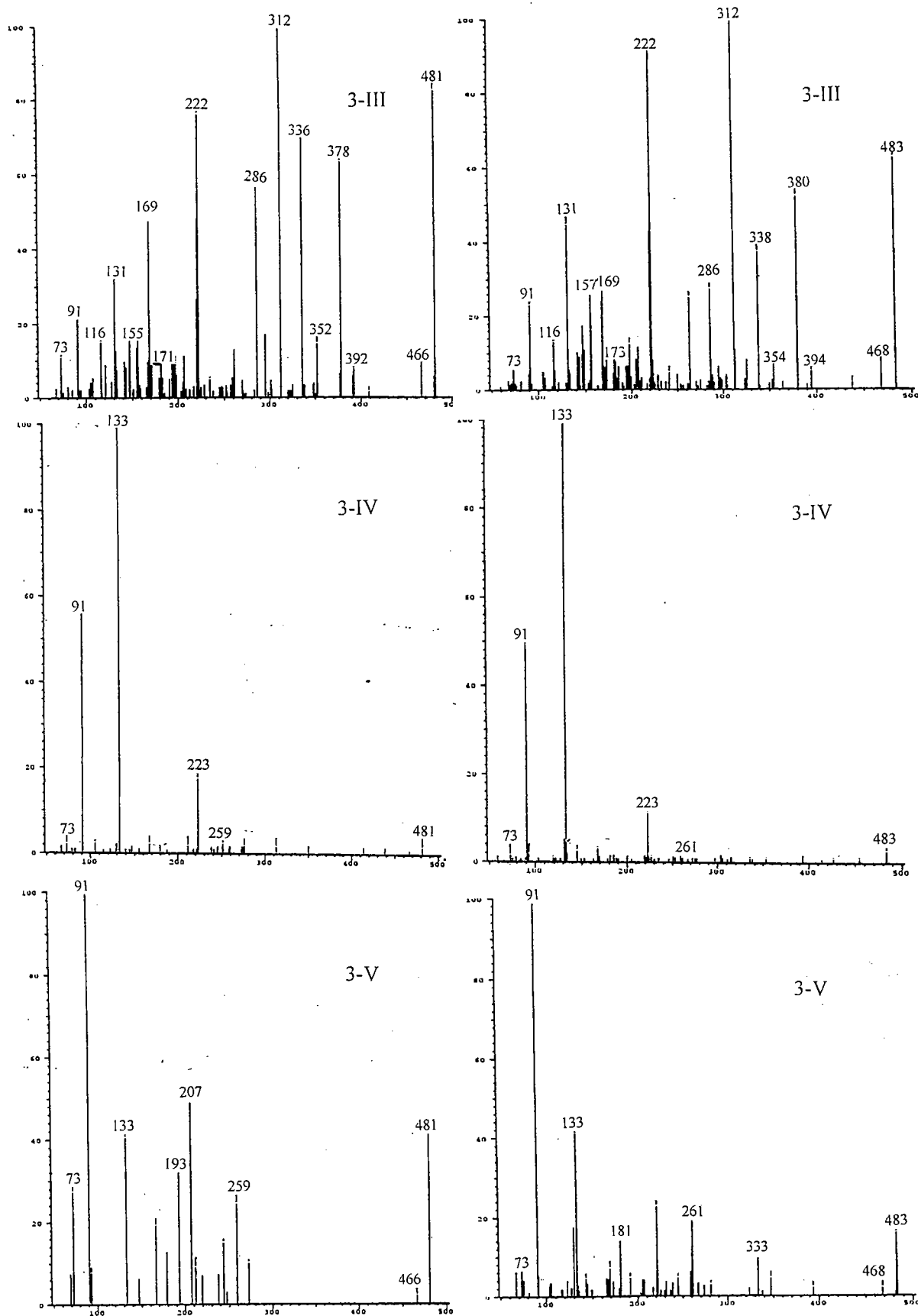


Fig.9 The EI Mass Spectra of Dihydroxybromantane 3-I~3-VI



(Fig.10 continueing)



(Fig. 10 continuing)

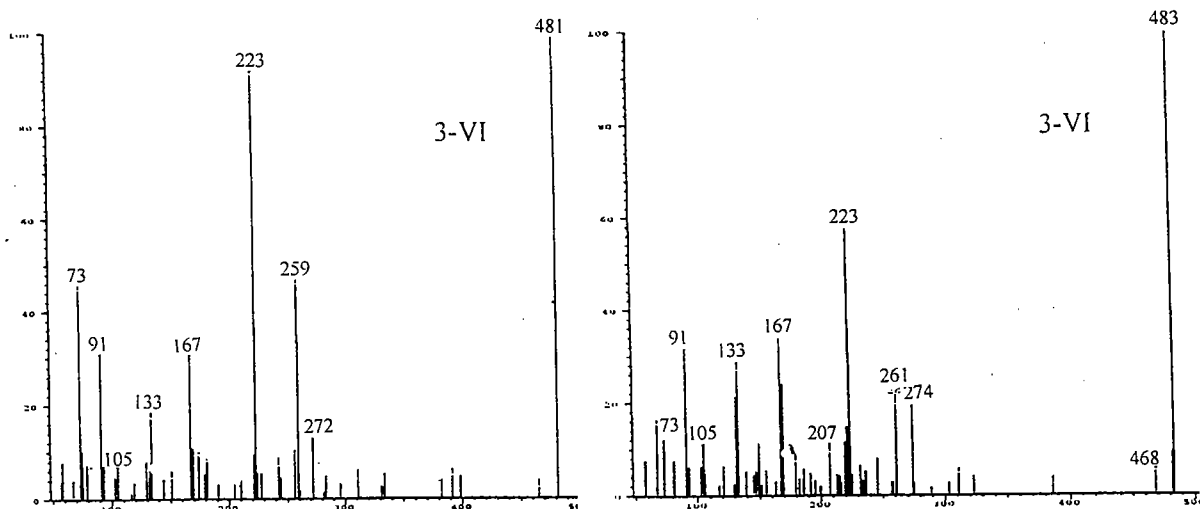


Fig.10 The Daughter Mass Spectra of Dihydroxybromantanes 3-I~3-VI  
(Left: m/z 481, Right: m/z 483)

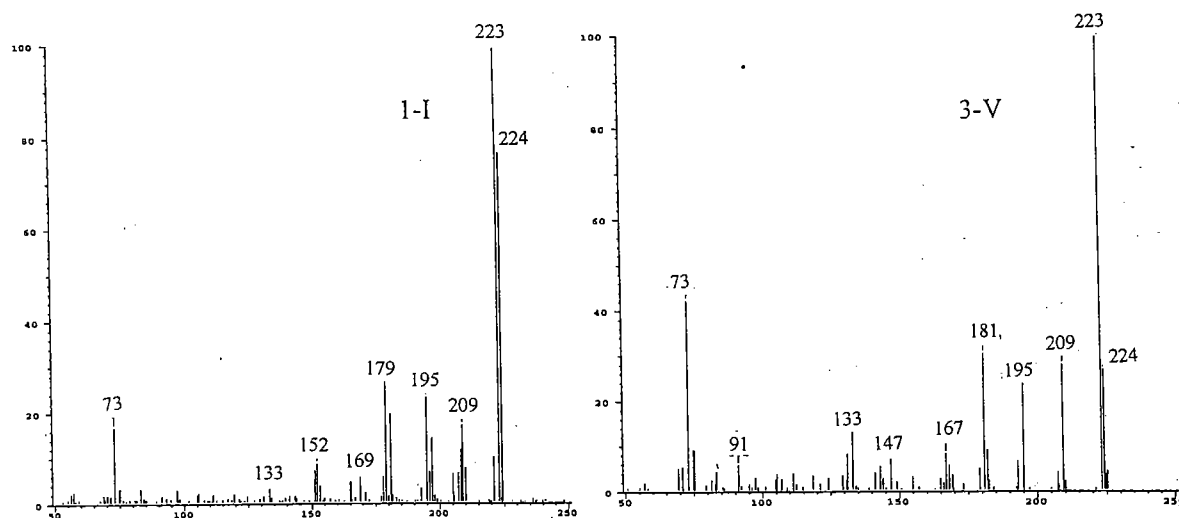
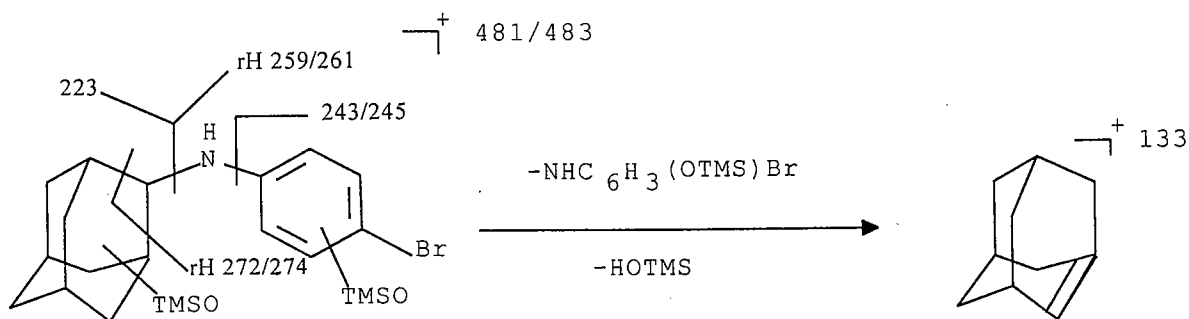
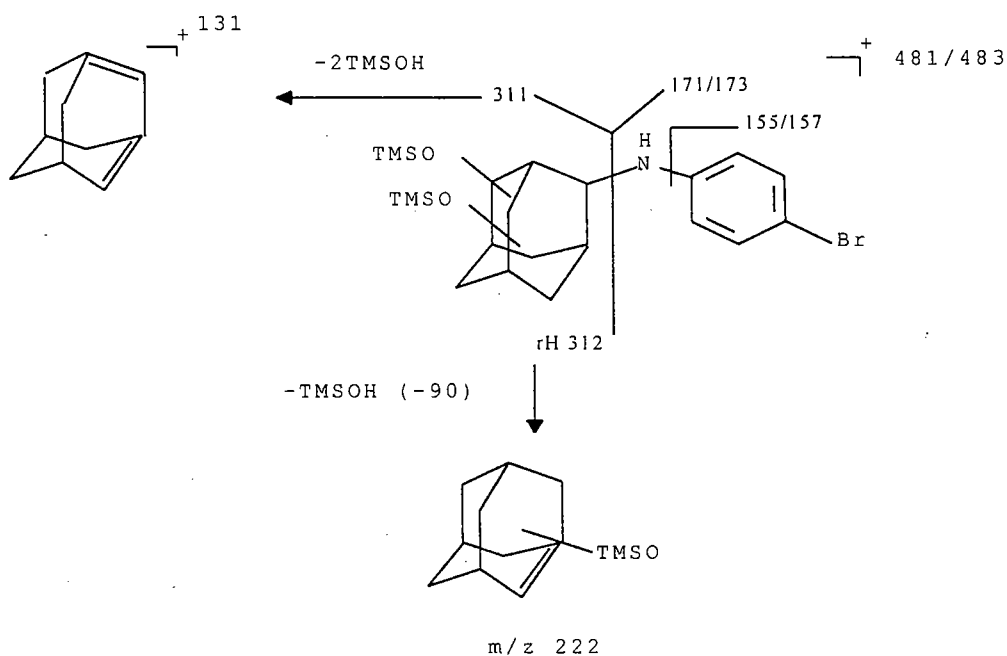


Fig.11 The Daughter Mass Spectra of Ions m/z 223 from 1-I and 3-V (Unsubtracted)



The Fragmentation Pathway of 3-I, 3-II, 3-IV, 3-V and 3-VI

(Fig.12 continueing)



The Fragmentation Pathway of 3-III

Fig.12 The Possible Fragmentation Pathway of Dihydroxybromantanes 3-I~3-VI

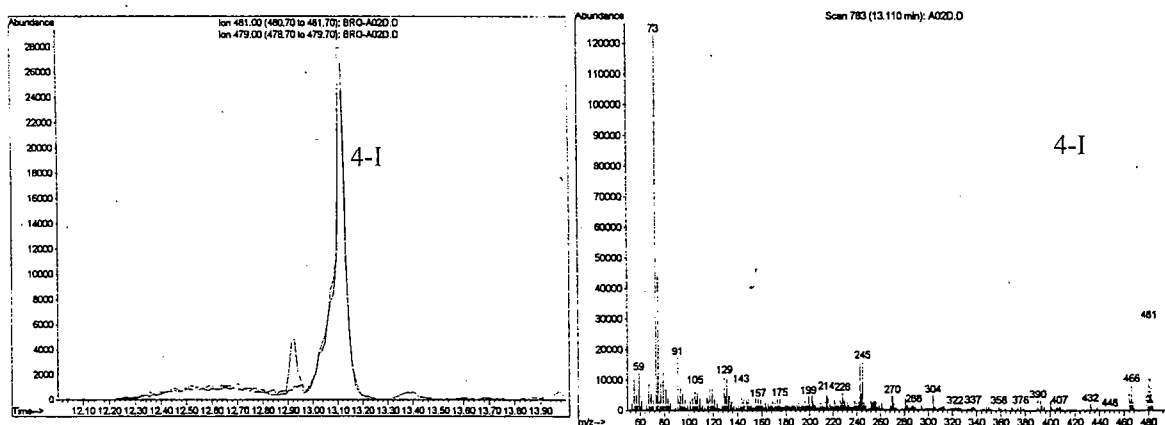


Fig.13 The Gas Chromatogram and EI Mass Spectrum of 4-I

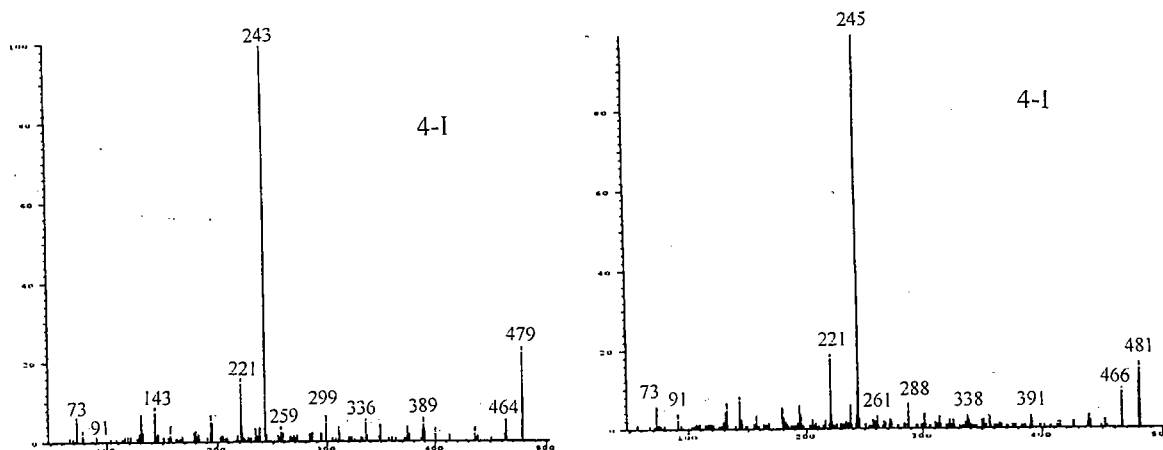


Fig.14 The Daughter Mass Spectra of 4-I (Left: m/z 479, Right: m/z 481)



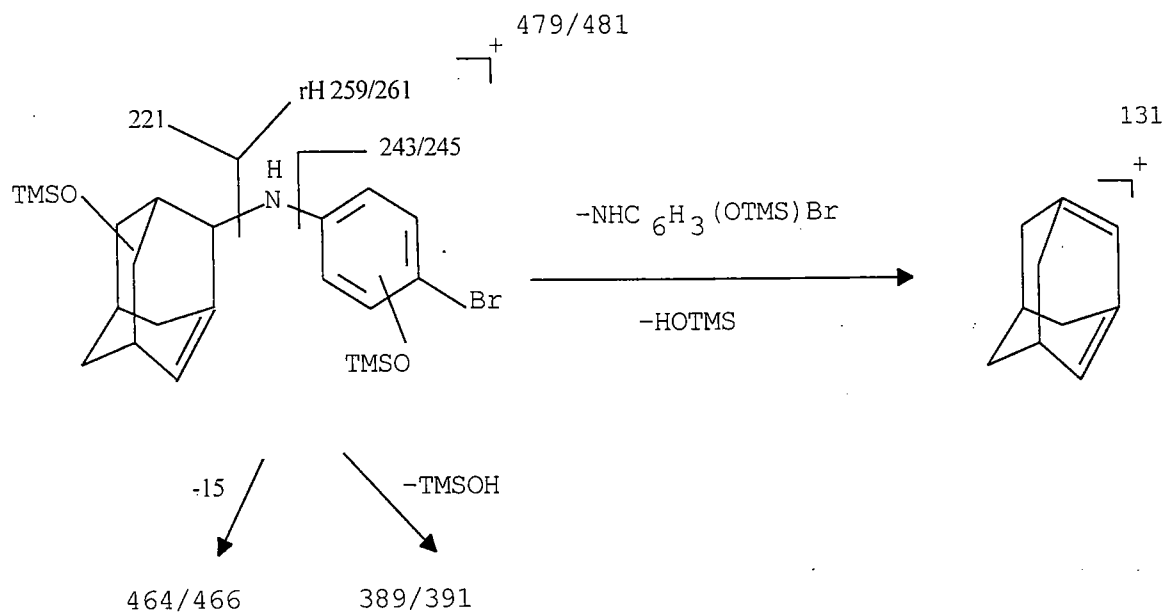


Fig.15 The Possible Fragmentation Pathway of 4-I

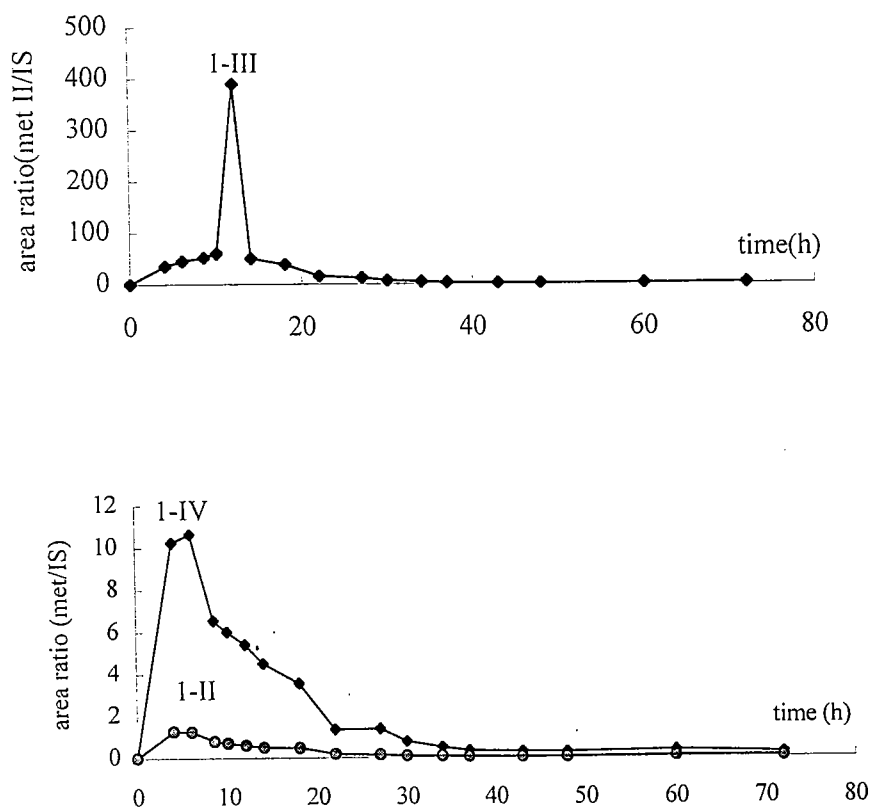


Fig.16 The Excretion Variations of Bromantane Metabolites 1-II, 1-III and 1-IV