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ABSTRACT:

Context: The European white-berry mistletoe [*Viscum album* L. (Loranthaceae)] is among the oldest known medicinal plants. At present the most important application of mistletoe extracts is treatment of cancer. However, natural products specific to mistletoe have rarely been encountered in current literature.

Objective: To discover novel natural products specific to European mistletoe.

Materials and methods: European mistletoe was extracted with methanol, purified to partition against diethyl ether and further purified with XAD-7 column chromatography. Pure compounds were separated by Sephadex column chromatography and preparative HPLC. The structures of the novel compounds were established using a combination of several 2D NMR spectroscopic techniques and mass spectrometry.

Results: A new type of natural product derived from the methyl ester of gammahydroxybutyric acid (GHB) coupled to hydroxybenzoic acids, namely 3-(3′-carbomethoxypropyl) gallic acid and 3-(3′-carbomethoxypropyl)-7→3″-protocatechoyl galloate were characterized from European white-berry mistletoe. Condensation of the 3-hydroxyl of gallic acid with the 4-hydroxyl of GHB significantly reduced the radical scavenging properties of the former compound.

Discussion and conclusion: The compounds characterized define a novel group of natural products that may be of particular interest because it appears that the two new compounds are not closely related to any known natural product.

Introduction

For thousands of years, secondary metabolites from plants have continued to play an important role in medicine (Ji et al. 2009). Recently, Newman (2008) estimated that approximately 60% of the drugs available are either directly or indirectly derived from natural products. In line with this, there is currently an increased interest in the study of traditional medicinal plants as sources of new drug candidates (Muthu et al. 2006). European white-berry mistletoe [*Viscum album* L. (Loranthaceae)], order Santalales (APG3, 2009) is an evergreen dioecious small shrub growing half parasitically on a tree host in temperate Europe and western Asia. European mistletoe has been utilized as an important medicinal plant for millennia. Conditions treated with European mistletoe include hypertension, diabetes, arthrosis, epilepsy and cancer (Büssing, 2000 and references therein). In particular, the anticancer activity of mistletoe extracts has been extensively studied. The anticancer activity of mistletoe extracts has been ascribed to the presence of lectins (Franz 1986), viscotoxins (Romagnoli et al. 2000) and alkaloids (Khwaja et al. 1980 and 1986; Park et al. 1998, 1999; Chen et al. 2005 and references therein; Dong et al. 2009; Ge et al. 2009; Zhou et al. 2010). Although European mistletoe has remained an important medicinal plant for millennia, limited information is available about the content of secondary metabolites. The chemical composition of European mistletoe extracts is rather complex (Bar-Sela 2011 and references therein) and only a very limited number of natural products have been identified therefrom. Recently we identified two novel aminoalkaloids from European mistletoe, which were the first alkaloids ever to be identified from this species (Amer et al. 2012). The major objective of the current study was to isolate novel compounds from European mistletoe which may have potential as future drug candidates. In this paper we report on the isolation and characterisation of a novel group of natural products derived from gammahydroxybutyric acid (GHB) coupled to phenolic acids isolated from *Viscum album*.

Materials and methods

Extraction of plant material

Plant material and host tree *V. album* [source: Lille, France, host tree: a hybrid of Populus nigra L. with P. deltoids Bartram ex Marshall = P. x canadensis Moench (Salicaceae)] was identified by Professor Frederic Dupont, Department of Botany, Faculty of Pharmacy, University of Lille 2. The European mistletoe used in this work grows in the botanical garden of Department of Botany, Faculty of Pharmacy, University of Lille 2. A voucher specimen has been deposited (accession number LIP-BOTA-12070201). Fresh plant material of *Viscum album* (1.5 kg) was chopped using a blender and extracted three times with methanol (6 L) at room temperature for 24 h. The extracts were stored in a refrigerator at 4°C. The crude extract was filtered through a glass wool filter. Water in the plant was approximately 60%, since 10 g of the fresh plant material lost 6 g after drying in oven at 110°C for 30 h. The methanol extract was concentrated under reduced pressure on a rotavapor and purified by partition against diethyl ether to remove non-polar aliphatic compounds. A dark brown solution was left after removal of the latter.

Instruments and equipment

Concentration under reduced pressure was performed with a Büchi Rotavapor, R-205. Adsorption column chromatography was performed with a 105 x 5 cm Amberlite XAD-7 column (Amberlite XAD-7HP, Particle size: 20-60 mesh, wet, Sigma Aldrich). Gel filtration chromatography was performed on a Sephadex LH-20 column (100 x 5 cm). Analytical HPLC was performed with an Agilent 1100 HPLC equipped with a HP 1050 mutidiode array detector, a 20 µl loop and a 250 x 4.6 mm, 5 µm Hypersil GOLD column. Preparative HPLC was performed with a Gilson 321 HPLC with UV detection (Dionex UltiMate 3000 Variable Wavelength Detector). The system was equipped with a 250 x 22 mm, C18 Altech column.

High resolution mass spectra were recorded using a JEOL AccuTOF JMS T100LC instrument fitted with a DART inlet from Ion Sense. The NMR experiments were performed on a Bruker 600 MHz instrument equipped with a cryogenic probe.

Amberlite XAD-7 column chromatography

The concentrated purified aqueous extract was applied to an Amberlite XAD-7 column. The column was eluted with distilled water (5 L) to remove polar aliphatic constituents. The purified extract was eluted with methanol (5 L). Elution with methanol (5 L) furnished a purified fraction that was concentrated under reduced pressure using a rotavapor.

Sephadex LH-20 column chromatography

The concentrated XAD-purified extract was applied to a Sephadex LH-20 column. The column was eluted with a solvent gradient. Fractions 1-18 were eluted using 3.6 L of methanol-distilled water-TFA (60:40:0.2; v/v) as mobile phase while fractions 19-43 were obtained using 2 L of methanol-distilled water-TFA (70:30:0.2; v/v) as mobile phase. Fractions 44-48 were eluted using 1 L of methanol-distilled water-TFA (75:25:0.2; v/v) as mobile phase. The last fractions (49-58) were eluted using 1 L of methanol-distilled water-TFA (80:20:0.2; v/v) as mobile phase. The eluted fractions were concentrated under reduced pressure using a rotavapor and checked for purity by analytical HPLC. Pure compound **1** (400 mg) was obtained from fraction 29. Fraction 35 contained 25 mg pure compound **2**. Compound **3** was isolated from fraction 47 on preparative HPLC as described below.

Analytical HPLC

Two solvents; A, distilled water and 0.5 % TFA (trifluoroacetic acid) and B, acetonitrile and 0.5 % TFA (trifluoroacetic acid) were used for elution. The elution profile consisted of 90% A

and 10% B, followed by gradient elution for 10 min (14% B), isocratic elution for 10–14 min, and the subsequent gradient conditions: 18 min (16% B), 22 min (18% B), 26 min (23% B), 31 min (28% B), and 32 min (40% B), isocratic elution for 32–40 min, gradient elution for 40–43 min (10% B) and final linear elution for 43–46 min (10% B). The flow rate was 1.0 ml/min, and aliquots of 15 µl were injected with a Micro Auto-sampler (Agilent 1100 Series).

Preparative HPLC

Two solvents; A, distilled water and 1.0% TFA (trifluoroacetic acid) and B, methanol and 1.0% TFA (trifluoroacetic acid) were used for elution. The elution profile consisted of 90% A and 10% B for 0-4 min, followed by gradient elution for 4-40 min (10–90% B), isocratic elution for 44-57 min and final linear elution for 57-58 min (90–10% B). The flow rate was 15.0 ml/min, and aliquots of 1000 µl were injected with a microliter syringe. Following this procedure compound **3** (2 mg) was eluted after 29.8 min.

Spectroscopy

i) UV spectroscopy

UV-Vis absorption spectra were recorded on-line during HPLC analysis over the wavelength range 240-600 nm in steps of 2 nm.

ii) Mass spectrometry

The isolated compounds were dissolved in methanol. A glass needle was soaked in the analyte solution, and thereafter placed in the source at atmospheric pressure. The analysis was performed at a helium flow rate 0.76 L/min, gas temperature 300°C and a needle voltage of approximately 3000 V. Mass spectral data were recorded at orifice 1 voltage 19 V, orifice 2 voltage 7 V, ring lens voltage 8 V and detector voltage 2300 V. The spectrum was recorded over the mass range 50-1000 m/z.

iii) NMR spectroscopy

NMR samples were prepared by dissolving the isolated compounds in deuterated dimethylsulphoxide (99.9 atom % D, Sigma-Aldrich). The 1D ^{1}H, the 1D ^{13}C CAPT and the 2D ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HSQC, ^{1}H-^{1}H COSY and ^{1}H-^{1}H ROESY NMR experiments were obtained at 600.13 MHz and 150.90 MHz for ^{1}H and ^{13}C, respectively, at 298K.

DPPH scavenging activity

These experiments were performed in accordance with the procedure described by Glavind (1963) and Lyckander and Malterud (1996), with some modifications outlined below.

Methanol solutions of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (190 µl, 45 µg/ ml) were mixed with the test substances 3-(3′-carbomethoxypropyl)gallic acid and gallic acid (10 µl) in 96 well UV-transparent microtiter plates (Greiner; cyclic olefin copolymer (COC) flat bottom). The decrease in absorption at 517 nm was measured over a period of 30 min using an EON Biotek absorbance microplate reader. All experiments were performed at 25°C.

Results

Fresh plant material of *Viscum album* L. was extracted with methanol. After removal of methanol under reduced pressure, the concentrated extract was purified by partition against diethyl ether. The aqueous phase was purified with XAD-7 Amberlite adsorption chromatography. Pure compounds, **1** (400 mg) and **2** (25 mg), were isolated by Sephadex LH-20 chromatography. Compound **3** (2 mg) was isolated from a Sephadex fraction on preparative HPLC.

The absorption maxima of the UV-Vis spectra of **1**-**3** at ~ 250-260 nm were compatible with aromatic compounds based on substituted hydroxybenzoic acid core structures.

Compound **1** was identified as gallic acid (3,4,5-trihydroxybenzoic acid) by 1D and 2D NMR spectroscopy (Table 1 and figure 2). A quasi-molecular ion $[MH]^+$ at *m/*z 171.029 in the high resolution mass spectrum of **1** corresponding to $C_7H_5O_5$ (calc. 171.029) confirmed this identity. From 1.5 kg fresh plant material 400 mg of the pure substance was isolated. The water content of the fresh plant material was determined to be 60 % of the total weight. Thus, gallic acid comprises approximately 0.07 % of the dry weight of European mistletoe. The substance has previously been identified in *Viscum album* by Popova (1991) along with chlorogenic, caffeic and ferulic acids.

The aromatic region of the 1D ^{1}H NMR spectrum of **2** showed a 2H AB system at δ 7.14 (d 2.0 Hz, H-6) and δ 7.06 (d 2.0 Hz, H-2) and 1H singlets at δ 9.51 (5-OH) and δ 8.76 (4-OH), which is in accordance with an asymmetrically monosubstituted derivative of gallic acid. The aliphatic region of the 1D ^{1}H NMR spectrum showed a singlet at δ 3.55 (OCH3), in addition to three connected -CH_2- groups at δ 2.37 (*t* 7.4 Hz, H-2′), δ 1.69 (*p* 7.4 Hz, H-3′) and δ 3.44 (*t* 7.4 Hz, H-4′), all of which agree with identification of the compound as the methyl ester of gammahydroxybutyric acid. The identification of gallic acid and the methyl ester of gammahydroxybutyric acid as sub-units of **2** was confirmed by the observations of the twelve ^{13}C signals in the 1D ^{13}C CAPT spectrum (Table 1). The individual ^{1}H signals of the methyl ester of gammahydroxybutyric acid subunit were assigned by the 2D ^{1}H-^{1}H COSY experiment (Table 1). A crosspeak at δ 3.44/136.5 (H-4′/C-3) observed in the 2D ^{1}H-^{13}C HMBC spectrum confirmed the identity of **2** to be the novel natural product 3-(3′-carbomethoxypropyl) gallic acid (figure 2). A quasimolecular ion $[MH^+]$ corresponding to $C_{12}H_{15}O_7$ confirmed this identity. The NMR spectra of **3** shared many similarities to that of **2** showing the presence of a unit of gallic acid substituted with a 3′-carbomethoxypropyl unit at the 3-hydroxyl (Table 1). In addition, the carboxyl group of the galloyl subunit was esterified with a hydroxyl of 3,4-dihydroxybenzoic acid, which showed a 1H singlet at δ 9.86 (4″-OH)

and a 3H ABX system at δ 7.48 (*dd* 2.1, 8.3 Hz, H-6′), δ 7.44 (*d* 2.1 Hz, H-2″) and δ 6.82 (*d* 8.3 Hz, H-5″) in the aromatic region of the 1D ^{1}H NMR spectrum of **3**, which is in accord with a mono *O*-substituted unit of protocatechuic acid (Table 1). The identification was supported by the observation of 19 individual ^{13}C resonances in the 1D ^{13}C CAPT spectrum of **3**. All ^{13}C signals were assigned by the 2D ^{1}H-^{13}C HSQC and HMBC spectra. The individual ^{1}H signals of the methyl ester of gammahydroxybutyric acid subunit were assigned by the 2D ^{1}H-^{1}H COSY experiment (Table 1). The strong crosspeak at δ 9.86/6.82 (4″-OH/H-5″) observed in the 2D ^{1}H ROESY spectrum of **3** showed that the only remaining phenolic OH group of the substituted protocatechuic acid subunit was positioned at the 4″-position. Based thereupon, the position of esterification between the carbonyl carbon of the galloyl subunit and protocatechuic acid subunit was determined to be at the 3″-position (figure 2). Moreover, a crosspeak at δ 3.44/136.1 (H-4′/C-3) observed in the 2D ^{1}H-^{13}C HMBC spectrum confirmed the linkage between the 3′-carbomethoxypropyl unit and the galloyl moiety to be at the 3-hydroxyl. Thus, the structure of **3** was established as the novel natural product 3-(3′-carbomethoxypropyl)-7→3″-protocatechoyl galloate. A molecular ion *m/z* 406.092 corresponding to $C_{19}H_{18}O_{10}$, (calc. 406.089) and ions due to simple losses from this of CO_2 at *m/z* 362.100 (calc. 362.099) and $CH_2CH_2CO_2Me$ at m/z 319.057 (calc. 319.045) were in accord with this identity. Fragment ions due to rearrangement scission of the ester bond corresponding in mass to the loss of *ortho*quinone-4-carboxylic acid at *m/z* 254.078 (calc. 254.078), and of methanol from the latter at *m/z* 222.051 (calc. 222.052), together with observation of a fragment corresponding to orthoquinone-4-carboxylate at m/z 151.055 (calc. 151.003) in the mass spectrum of **3** lent further support to this identification (Figure 3).

The radical scavenging properties of compounds **1** and **2** were determined with the DPPH (Diphenylpicrylhydrazyl) assay (Glavind 1963; Lyckander & Malterud, 1996). Gallic acid has previously been reported to be a potent radical scavenger (Masaki et al. 1994) and the

calculated IC_{50} for this compound was 27.7 μM. Substitution of the 3-hydroxyl of gallic acid with methyl ester of GHB, which is the case for compound **2**, reduced the radical scavenging properties twenty-fold compared with that of gallic acid (IC_{50} for compound **2** = 222 μM; Table 2).

Discussion

Since the 1920s extracts of European mistletoe (*Viscum album*) have been used in the treatment of cancer (Lev et al. 2011 and references therein). The anticancer activity of these extracts has previously been ascribed to three main groups of compounds, namely (1) lectins, a group of proteins with relatively high molecular weight which become denatured and loose their activity upon heating, apparently due to disruption of their tertiary structure (Franz 1986), (2) viscotoxins, a group of more heat-stable proteins with considerably lower molecular weights (Romagnoli et al. 2000), and (3) alkaloids, which we identified very recently (Amer et al. 2012).

In this paper we report on the identification in European mistletoe of two novel compounds consisting of phenolic acids merged with the methyl ester of gammahydroxybutyric acid. In addition, a substantial amount of gallic acid, which is one of the main building blocks of these compounds, was isolated from the same source. Gallic acid, and some derivatives thereof have previously been reported to exhibit promising anticancer activity against different cancer cell lines including prostate cancer, mammary gland and cervix adenocarcinomas and lymphoblastic leukemia cancer cell lines (Gomes et al. 2003; Indap et al. 2006; Agarwal et al. 2006; Saxena et al. 2008; Kaur et al. 2009). Gammahydroxybutyric acid has previously been reported to exhibit anti-angiogenic activities and antitumor effect in animal trials (Basaki et al. 2002). Future analysis to reveal the therapeutic potential of the compounds reported in this paper is already planned.

Conclusion

The discovery of several novel natural products from European mistletoe justifies further investigations into this traditional medicinal plant. European mistletoe has been used for treatment of cancer with success although the chemical composition of preparations from this plant have been poorly characterised. This is particularly valid with respect to medium-sized organic molecules belonging to the main molecular regime relevant in the search for new drug candidates (Chin et al. 2006). The methods used in the current paper open possibilities for isolation of new compounds from this source that has previously been regarded as having an intractable complex composition.

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Figure legends

Figure 1. European white-berry mistletoe (*Viscum album*) photographed in Hamburg 11.11.2005. Photo: Professor Frédéric Dupont.

Figure 2. Structures of compounds **1**-**3** isolated from European mistletoe (*Viscum album*)

Figure 3. Mechanisms for formation of important fragments observed in the mass spectrum of compound **3**

Table 1. ^{1}H and ^{13}C NMR chemical shift values (ppm) and coupling constants (Hz) of compounds **1-3** isolated from European mistletoe (*Viscum album*).

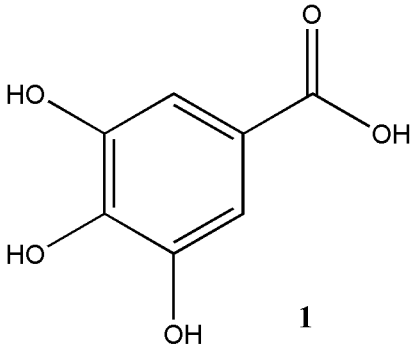
Compound	**3** δ ^{1}H	**3** δ ^{13}C	**2** δ ^{1}H	**2** δ ^{13}C	**1** δ ^{1}H	**1** δ ^{13}C
Galloyl						
1		120.65		120.7		120.50
2	7.07 *d* 2.0	116.80	7.06 *d* 2.0	116.6	6.90 *s*	108.79
3		136.09		136.5		145.49
3-OH					9.16 *s*	
4		144.56		144.5		138.06
4-OH	8.76 *s*		8.76 *s*		8.80 *s*	
5		145.30		145.4		145.49
5-OH	9.55 *s*		9.51 *s*		9.16 *s*	
6	7.14 *d* 2.0	112.06	7.14 *d* 2.0	112.1	6.90 *s*	108.79
7		167.40		167.3		167.54
3-GHB-OMe						
1’		173.32		173.3		
2’	2.38 *t* 7.4	30.75	2.37 *t* 7.4	30.8		
3’	1.70 *p* 7.4	22.96	1.69 *p* 7.4	23.1		
4’	3.44 *t* 7.4	50.79	3.44 *t* 7.4	51.2		
OMe	3.55 *s*	51.31	3.55 *s*	51.3		
7→3’’-PCA						
1’’		121.70				
2’’	7.44 *d* 2.1	124.71				
3’’		136.44				
4’’		155.71				
4’’-OH	9.86 *s*					
5’’	6.82 *d* 8.3	115.76				
6’’	7.48 *dd* 2.1, 8.3	125.82				
7’’		167.30				

Table 2. DPPH scavenging activity of gallic acid (**1**). Values presented are from two series of experiments.

[**1**] μM	Series 1 % DPPH scavenged	Series 2 % DPPH scavenged	Mean value DPPH scavenged	Estimated deviation from the mean	Gallic acid : DPPH Molar ratio (both series)
2.1	3.3	12.0	7.7	6.2	0.004
4.1	5.8	8.2	7.0	1.7	0.007
6.2	13.6	20.1	16.9	4.7	0.011
8.2	19.8	19.2	19.5	0.5	0.015
10.3	19.2	21.3	20.2	1.4	0.019
20.6	42.5	46.0	44.2	2.5	0.037
41.1	79.1	86.1	82.6	5.0	0.074
61.7	94.5	93.9	94.2	0.4	0.112

Table 3. DPPH scavenging activity of 3-(3'-carbomethoxypropyl) gallic acid(**2**). Values presented are from two series of experiments.

[**2**] μM	Series 1 % DPPH scavenged	Series 2 % DPPH scavenged	Mean value DPPH scavenged	Estimated deviation from the mean	Gallic acid : DPPH Molar ratio (both series)
63.5	21.1	20.5	20.8	0.4	0.278
105.8	30.7	30.6	30.7	0.1	0.464
169.3	42.8	42.6	42.7	0.1	0.742
211.6	48.8	47.1	47.9	1.2	0.927



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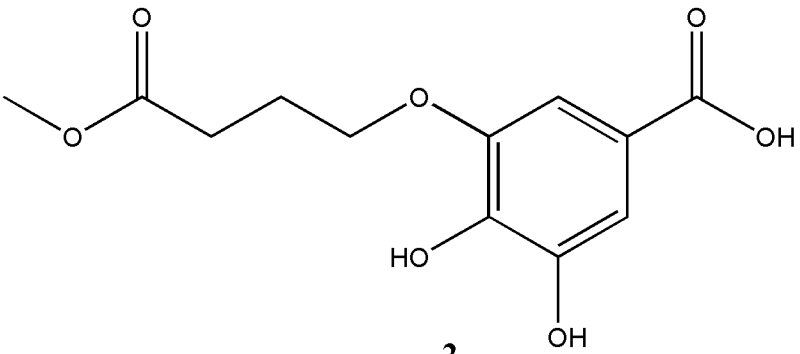

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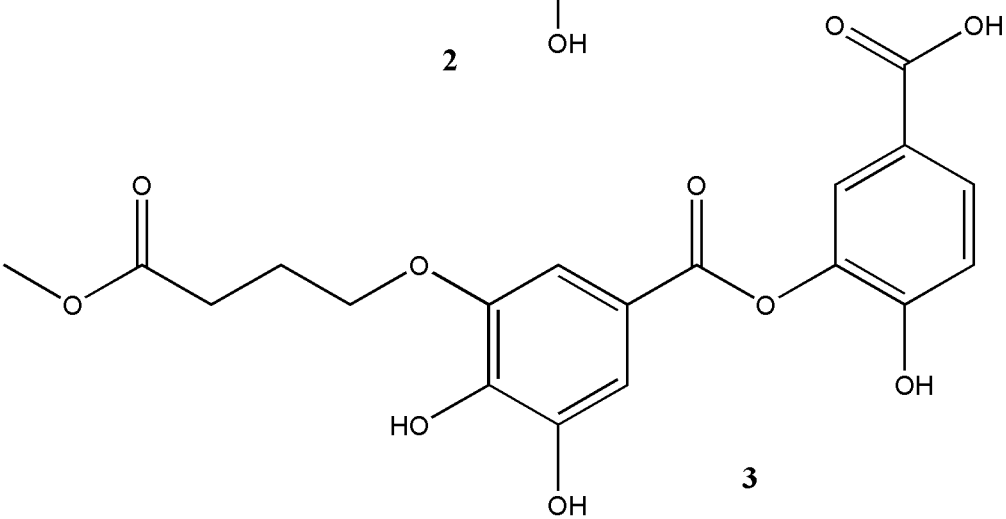
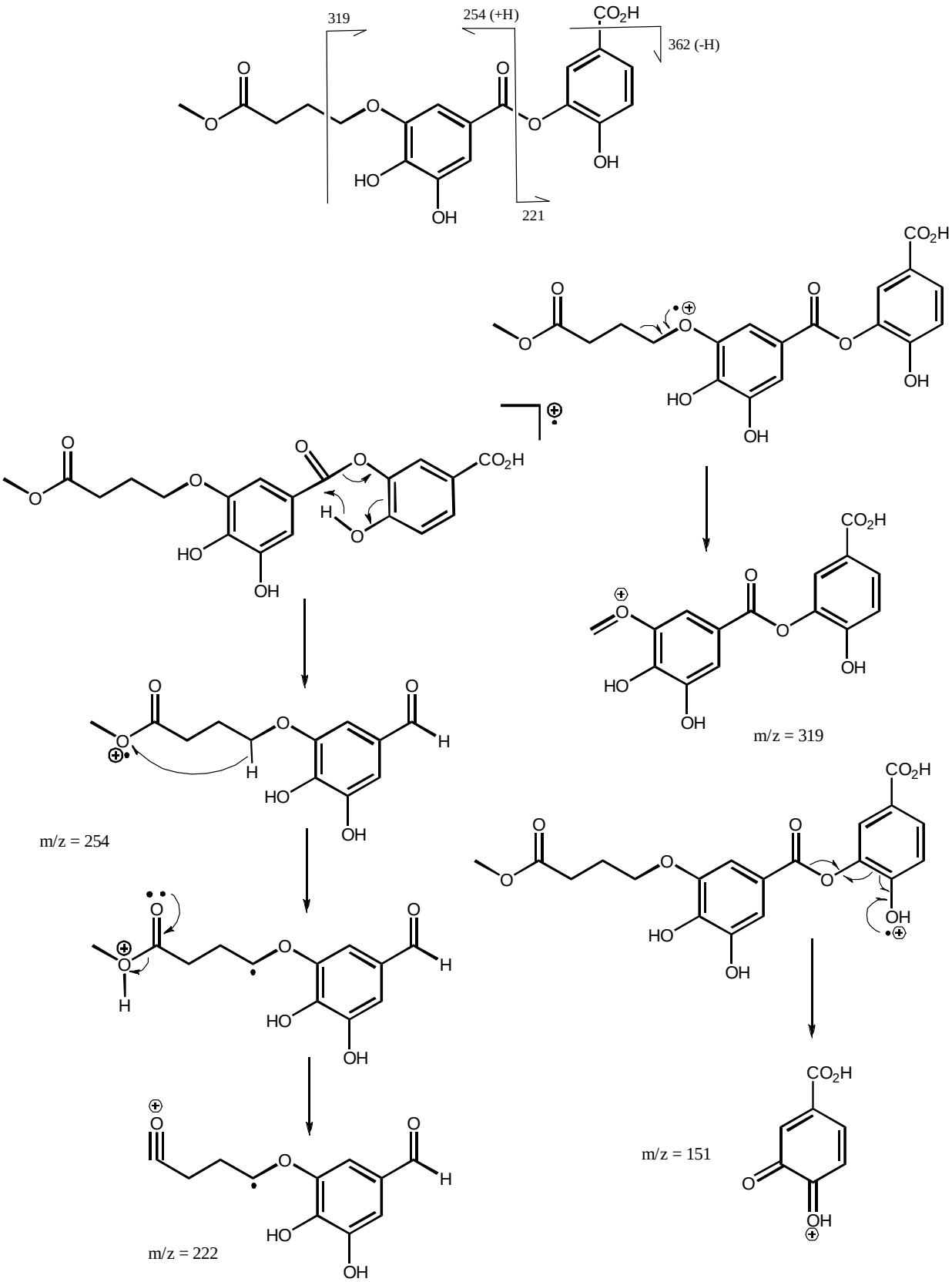


3


319
254 (+H)
362 (-H)
221
m/z = 319
m/z = 254
m/z = 222
m/z = 151