Synthesis of ^{13}C-labelled Patulin [4-Hydroxy-4H-furo[3,2-c]pyran-2(6H)-one] to be used as Internal Standard in a Stable Isotope Dilution Assay

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Key words: Atmospheric pressure chemical ionization (APCI); electrospray ionization (ESI); patulin; stable isotope dilution assay

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Abstract

A synthetic route was established for the preparation of [13C2]-4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one (patulin) to be used in a stable isotope dilution assay. Mass spectral analyses were performed using electron impact ionization (EI), negative electrospray ionization (ESI), collision-induced dissociation (CID), and atmospheric pressure ionization. Fragmentation routes in the EI mode and in CID were concluded and compared with each other.

Introduction

The mycotoxin patulin [4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one] is produced by different species of Penicillium, Aspergillus, and Byssochlamys (Lovett and Thompson, 1978; Northolt et al., 1978; Roland and Beuchat, 1984). The molds are known to affect fruits (Frank et al., 1977), berries (Lindroth et al., 1978), bread (Reiss, 1973), and meat products (Alperden et al., 1973), and patulin concentrations up to 45 mg/kg have been reported therein (Fritz et al., 1979). Due to its acute toxic (Burghardt, 1992), teratogenic (Sugiyanto et al., 1993), and possible cancerogenic (Dickens and Jones, 1961) potency, the World Health Organization recommends to limit its content in foods to 50 µg/kg (van Egmont, 1989).

High-performance liquid chromatography with UV detection is commonly used in patulin analysis, and detection limits of 1 µg/kg, for example, in apple products, have been reported (Official methods for patulin analysis, 1984). The recoveries lie between 70 and 90% (Bartolomé et al., 1994; Brause et al., 1996).

Especially, physiological studies, for example, on patulin metabolism in the human body (Barhoumi, 1996), require much more sensitive and selective methods. So far, only an enzyme-linked immunosorbent assay (ELISA) has been reported to detect

patulin down to 1 ng/kg on the basis of sample volumes of 0.1 mL (Schecklies, 1993). However, no systematic data on precision and reproducibility of the method were given (Schecklies, 1993).

Similar requirements in the analysis of odorants (Sen et al., 1991), veterinary drugs (Suhre et al., 1981), and migrants (Castle et al., 1988) have proven the superiority of stable isotope dilution assays (SIDA) in which isotopomers of the respective analytes are used to exactly correct losses of analytes during cleanup.

The purpose of the following study was, therefore, to synthesize 13C-labeled patulin for use in a SIDA.

Material and Methods

The following chemicals were obtained commercially from the sources given in parentheses: acetyl chloride, powdered molecular sieves (3 Å), methanesulfonyl chloride, ethyl bromo[13C2]acetate, propylene oxide, and pyridinium chlorochromate (Aldrich, Steinheim, Germany); l-(+)-arabinose, acetone (AR grade), diphenylamine, aniline, triphenylphosphine, sodium hydrogencarbonate, sodium sulfate, and P2O5 (Merck, Darmstadt, Germany); trifluoroacetic acid and 2,2-dimethoxypropane (Sigma, Munich, Germany); Celite (Serva, Heidelberg, Germany).

Diethyl ether was dried over sodium, and dichloromethane and pyridine were purified by distilling over P2O5 or potassium hydroxide, respectively.

NMR Spectroscopy. One-dimensional NMR experiments were performed by means of an AM 360 (Bruker, Karlsruhe, Germany) operating at 360.13 MHz (for 1H) and 90.55 MHz (for 13C). Unless otherwise stated, solutions in deuteriochloroform were monitored. Shifts are expressed in parts per million downfield from tetramethylsilane. Signals were singlets unless specified otherwise, and J values are given in hertz. Heteronuclear 1H−13C multiple-quantum coherence (HMQC) experiments were performed on an AC 200 (Bruker, Karlsruhe, Germany) operating at 200.13 MHz as recently described (Hofmann et al., 1995).

Mass Spectrometry. High-resolution mass spectra were recorded by means of a MAT 95 S (Finnigan MAT, Bremen, Germany). Ionization energy in the electron impact mode was 70 eV.

Syntheses. The synthesis of 13C2-labeled patulin was performed according to a seven-step procedure starting from l-arabinose as outlined in Figure 1 and following a route described by Bennett et al. (1991) for the unlabeled mycotoxin with several modifications.

Methyl-α-l-arabinose (2). l-(+)-Arabinose (1; 50 g, 333 mmol) was dissolved in methanol (400 mL) and, after addition of acetyl chloride (7 mL), refluxed for 7 h. The mixture was stored overnight in a refrigerator (8 °C), and the precipitate was filtered with suction and washed with ice-cold methanol. To increase the yield, the filtrate and washings were refluxed for a further 3 h, concentrated to ∼120 mL, and the precipitate formed was isolated. The remaining filtrate and the washings were concentrated to 50 mL and cooled to 8 °C, then diethyl ether (50 mL) was added, and the solution was cooled to −20 °C. The precipitate was separated and washed. This procedure was repeated five times to obtain 2 as crystals in an overall 64% yield.

Methyl 3,4-O-Isopropylidene-α-l-arabinopyranoside (3). 2 (15 g, 91 mmol) was suspended in acetone (400 mL), then dimethoxypropane (17 mL) and sulfuric acid (0.5 mL, 18 mol/L) were added, and the mixture was stirred under argon for 24 h at room temperature. After addition of ammonia (1.5 mL, 25%), the suspension was filtered over dry Celite (10 g) and washed with acetone (100 mL). The solvent was evaporated, dichloromethane (300 mL) was added, and the solution was dried over Na2SO4. Evaporation of the solvent afforded methyl 3,4-O-isopropylidene-α-l-arabinopyranoside (3; 18.7 g, 100%).

Methyl 3,4-O-Isopropylidene-α-l-erythro-pentopyranosid-2-ulose (4). 3 (30.9 g, 152 mmol) and molecular sieves (3 Å, 85 g) were suspended in dichloromethane (800 mL) under an argon atmosphere. Aliquots of dry pyridinium chlorochromate (PCC; 85 g) were added successively with stirring and cooling and, finally, the mixture was stirred for 3 h at room temperature. After dilution with diethyl ether (500 mL), the solution was filtered over silica (5 g), which was then flushed with a mixture of ethyl acetate and hexane (300 mL, 1 + 1, v/v). The solvent was evaporated and the residue dissolved in hexane (50 mL). The crude product was then purified by flash chromatography (FC) over silica (20 g). After the column was flushed with ethyl acetate/hexane (150 mL, 1 + 4, v/v), the target compound was isolated by elution with ethyl acetate/hexane (200 mL, 1 + 1, v/v) and pooled from three runs (7.04 g, 23%).

The presence of 4 was checked by thin-layer chromatography on silica using ethyl acetate/hexane (1 + 1, v/v) as the solvent mixture. 4 was visualized by spraying with

a solution of diphenylamine/aniline (1% in ethanol/phosphoric acid, 85 + 15, v/v) followed by heating (15 min at 100 °C).
The following 1H NMR data were obtained: *δ 1.40 (CH3), 1.47 (CH3), 3.49 (OCH3), 4.08 (d, J = 13.1, H-6), 4.24 (dd, J = 13.3 and 2.2, H-6'), 4.54 (ddd, J = 5.7, 2.2, and 0.9, H-7), 4.68 (d, J = 5.8, H-7a), 4.70 (H-4).* 13C NMR data of 4 were identical with those reported previously by Bennet et al. (1991).

Methyl 2-Deoxy-3,4-O-isopropylidene-2-C-[(E)- and (Z)-ethoxy[13C]carbonyl[13C]methylene]- α-l-erythro-pentopyranosid-2-ulose (5). A mixture of 4 (888 mg, 4.39 mmol), triphenylphosphine (2.7 g, 10.3 mmol), and ethyl bromo[13C2] acetate (1 g, 5.92 mmol) was dissolved in dry dichloromethane (22 mL) and cooled to 0 °C under an atmosphere of pure nitrogen. After addition of propylene oxide (4.1 mL), the solution was stirred for 4 days at room temperature. Solvent evaporation afforded an oily residue, which was extracted with petroleum ether (boiling range = 40−60 °C, 4 × 25 mL). The extracts were concentrated to 2 mL and purified by flash chromatography on silica (20 g). Elution was performed with ethyl acetate/hexane (200 mL, 1 + 30, v/v), followed by ethyl acetate/hexane (100 mL, 1 + 9, v/v) and finally ethyl acetate (40 mL). 5 was detected in the FD fractions by TLC on silica using ethyl acetate/hexane (2 + 8, v/v) as the mobile phase. The elution volumes from 160 to 340 mL were pooled and evaporated to dryness to yield a mixture of the two geometric isomers methyl 2-deoxy-3,4-O-isopropylidene-2-C-[(E)- and (Z)-ethoxy[13C]carbonyl[13C]methylene]- α-l-erythro-pentopyranosid-2-ulose (5; 951 mg, 79%). The NMR data were identical with those reported by Bennet et al. (1991).

(4S, 7S, 7aR)-7-Hydroxy-4-methoxy-7, 7a-dihydro[$^{13}C_2$]furo[3,2-c]pyran-2-(4H, 6H)-one ***6***
5 (951 mg, 3.47 mmol) was refluxed for 3 h in a mixture of methanol (49 mL) and hydrochloric acid (1 mL, 1.2 mol/L) under an atmosphere of pure nitrogen. The solvent was evaporated and the residue taken up in dichloromethane (20 mL). After extraction with saturated aqueous sodium hydrogencarbonate (20 mL), the organic phase was dried over Na2SO4 and evaporated to dryness. The crude residue was recrystallized from ethanol to obtain 6 as crystals (244 mg, 37%). The NMR data were identical with those reported by Bennet et al. (1991).

(S)-4-Methoxy-4H-[13C2]furo[3,2-c]pyran-2(6H)-one (7). 6 (244 mg, 1.30 mmol) was dissolved in dry pyridine (4.6 mL) and cooled to 0 °C under dry nitrogen. Freshly distilled methanesulfonyl chloride (0.17 mL) was then dropwise added and the mixture stirred for 2 h at room temperature. After addition of chloroform (4 × 10 mL), the mixture was filtered over a paper filter and the combined filtrates were washed with hydrochloric acid (2 mol/L, 5 mL). After the organic phase was washed with brine (7 mL), the solution was dried over Na2SO4 and concentrated to 10 mL for use in the next synthetic step.

4-Hydroxy-4H-[13C2]furo[3,2-c]pyran-2(6H)-one (8). One-tenth of the solution containing 7 was evaporated to dryness and, after addition of a mixture of trifluoroacetic acid/water (1.65 mL; 10 + 1, v/v), heated for 1 h at 50−70 °C. The solution was then concentrated to 0.5 mL and, after addition of ethyl acetate (5 mL), washed with a saturated aqueous solution of sodium hydrogencarbonate (2.5 mL). The organic phases were dried over Na2SO4 and concentrated to 200 µL. The crude product was purified by high-performance liquid chromatography (pump 110 A; Beckman, Munich, Germany) on silica using ethyl acetate/hexane (1 + 1, v/v) as the solvent. The effluent (1 mL/min) was monitored at 275 nm using a UV detector Uvikon 735 LC (Kontron Instruments, Neufahrn, Germany). The target compound was pooled from 30 runs in the elution range of 8.5−10.5 mL and, after evaporation of the solvent, characterized by 1H NMR (total yield of the two last synthetic steps = 50%). The following signals were obtained: 1H NMR (CD3COCD3) δ 2.90 (br s, OH), 4.37 (dd, J = 17.3 and 4.0, H-6), 4.66 (dd, J = 17.7 and 2.9, H-6‘), 6.05 (m, H-7/H-4), 6.07 (dd, J = 185 and 9.3, H-3). 13C NMR (CD3COCD3) δ 110.6 (d, J = 69, C-3), 169.4 (d, J = 68, CO).
Data obtained by high-resolution mass spectrometry of 8 (relative intensities in parentheses): m/z 111.9957 (100%), 54.9517 (61%), 55.9549 (57%), 128.0393 (49%), 83.9646 (46%), 68.9202 (43%), 53.9440 (32%), 42.9997 (31%), 156.0284 (M+, 28%), 138.0393 (23%).

Methyl 2-Deoxy-3,4-O-isopropylidene-2-C-[(E)- and (Z)-ethoxycarbonyl methylene]-α-l-erythro-pentopyranosid-2-ulose. The mixture of the two geometric isomers 9 and 10 was prepared as reported for 13C-labeled 5 by using unlabeled ethyl bromacetate. 9

and 10 were separated by high-performance liquid chromatography on silica using an isocratic HPLC system (pump 110 A; Beckman, Munich, Germany) and ethyl acetate/hexane (2 + 8, v/v) as the mobile phase with a flow of 2 mL/min. The effluent was monitored at 254 nm with a Uvikon 735 LC (Kontron Instruments, Neufahrn, Germany). 9 and 10 were present in a ratio of 1.4 to 1 and were singly pooled from 20 runs in the elution ranges of 7.0−7.4 and 7.9−8.8 mL, respectively.

The two compounds gave the following NMR signals: 1H NMR, E-isomer 9, δ 1.30 (t, J = 7.1, CO2CH2CH3), 1.40 (CH3), 1.53 (CH3), 3.48 (OCH3), 3.64 (dd, J = 13.1 and 1.5, H-5), 3.67 (d, J = 13.1, H-5‘), 4.21 (quart., J = 7.2, CO2CH2CH3), 4.31 (dt, J = 6.0 and 1.8, H-4), 5.23 (d, J = 1.8, H-1), 6.03 (d, J = 7.5, H-3), 6.35 (d, J = 1.8, CH); Z-isomer 10, δ 1.29 (t, J = 7.1, CO2CH2CH3), 1.39 (CH3), 1.51 (CH3), 3.47 (OCH3), 3.98 (d, J = 13.3, H-5), 4.10 (dd, J = 13.0 and 2.6, H-5‘), 4.18 (quart., J = 7.1, CO2CH2CH3), 4.23 (dd, J = 4.4 and 1.8, H-4), 4.76 (dd, J = 5.5 and 2.0, H-3), 6.16 (d, J = 1.8, CH), 6.25 (H-1). 13C NMR 9 δ 14.0 (CO2CH2CH3), 25.1 (CH3), 26.2 (CH3), 55.3 (OCH3), 60.5 (C-5), 62.8 (CO2CH2CH3), 68.4 (C-3), 75.0 (C-4), 97.6 (C-1), 110.3 (CMe2), 123.9 (CH), 147.6 (C-2), 165.3 (CO); 10 δ 15.1 (CO2CH2CH3), 26.2 (CH3), 27.7 (CH3), 55.4 (OCH3), 57.9 (C-5), 60.3 (CO2CH2CH3), 71.5 (C-3), 73.8 (C-4), 95.2 (C-1), 109.6 (CMe2), 118.6 (CH), 150.4 (C-2), 165.0 (CO).

Liquid Chromatography (LC)/Multiple-Stage Mass Spectrometry (MS)^n

LC(MS)n spectra were recorded with an LCQ (Finnigan MAT) ion trap mass spectrometer operating in the negative electrospray ionization mode with a spray needle voltage of −4.5 kV and a spray current of 0.2 μA. The temperature of the capillary was 220 °C and the capillary voltage −6 V. The sheath and auxiliary gas nitrogen nebulized the sample solutions with flows of 2.3 or 3.0 mL/min. The ion trap was run with a helium pressure of 10-3 Torr.

Samples were diluted with methanol/water (98 + 2, v/v) or ethyl acetate to reach concentrations of 50 μg/mL and were introduced by means of a syringe pump operating at a flow of 18 μL/min. For HPLC/MS analysis, 3 μL of a solution was injected onto a spectra series high-performance liquid chromatograph (Thermo Separation Products, San Jose, CA) equipped with a Nucleosil RP18 column (250 × 2.0 mm i.d., 5 μm) and using an isocratic flow of 0.4 mL acetonitrile/water (1 + 9, v/v). LC/(MS)n experiments were performed by collision-induced dissociation (CID), which

was achieved by applying a supplementary radio frequency field on the endcap electrodes of the ion trap. The collision energy was set to 9.5%.
In the negative atmospheric pressure chemical ionization mode (APCI), the temperature of the vaporizer tube was 450 °C, the corona discharge needle was supplied with a voltage of −3 kV, and the discharge current was 5 μA. The capillary temperature was 150 °C and the capillary voltage −4 V. The nitrogen flows were 3.0 or 2.3 mL/min, respectively, for sheath and auxiliary gas.

Results and Discussion

Syntheses. Due to their weak acidity, the five hydrogen atoms in the structure of patulin can be assumed to be subject to protium/deuterium exchange, if a deuterium-labeled standard would be prepared. We, therefore, decided to synthesize a 13C-labeled standard.

In the seven-step procedure shown in Figure 1, [13C2]-patulin (8) was synthesized starting from unlabeled l-(+)-arabinose (1).

Due to the cis-configuration of the hydroxy groups at C-3 and C-4, this sugar can be selectively oxidized at C-2 into the ketone 4 after protection of the remaining hydroxyl functions by o-methyl glycosylation and isopropylidene formation. The original procedure reported by Bennett et al. (1991) was improved by using 2,2-dimethoxypropane in the preparation of the isopropylidene derivative.

A one-pot Wittig reaction of ketone 4 with 13C-labeled ethyl bromoacetate afforded the mixture 5 of two geometric isomers. As Bennett et al. (1991) showed, only the E-compound can react to lactone 6. In a preparation using unlabeled ethyl bromoacetate, the reaction mixture was separated by high-performance liquid chromatography and the unlabeled isomers 9/10 (Figure 2) were characterized by 1H NMR.

Antielimination of water from 6 and hydrolysis of the methoxy group then gave the labeled patulin in an overall yield of 2.3%.

Patulin and its labeled analogue (8) differed in their 1H NMR spectra only in the signal of the hydrogen at C-3, which showed a splitting by coupling with the 13C-labeled carbon-3 (coupling constant of 185 Hz) in [13C2]-patulin.

The 13C NMR spectrum of the unlabeled mycotoxin (Figure 3B) gave seven signals besides the two signals at 29.9 and 206.3 ppm coming from the solvent acetone-d6. As expected, due to the low natural occurrence of carbon-13 (~1.1%) in the 13C NMR spectrum of the labeled patulin, only the signals of the two incorporated carbon-13 atoms at 169.4 (C-2 in Figure 1) and 110.6 ppm (C-3 in Figure 1) appeared showing coupling of 68 Hz (Figure 3A).

Mass Spectral Analysis. In Figure 4, the mass spectrum in the electron impact mode obtained for the labeled patulin (A) is contrasted to the data measured for the unlabeled mycotoxin (Figure 4B), the latter being in good agreement with a spectrum published recently by Seijas et al. (1989). In agreement with the incorporation of the

two labeled carbon atoms, the molecular mass was shifted by 2 mass units to give the M+ ion 156 in the labeled isotopomer (Figure 4A). Further ions showing an incorporation of two labeled carbons were m/z 112, 128, 136, 84, and 71. Chernyshev et al. (1988) have proposed the fragmentation pattern of patulin occurring during mass spectral analysis using electron impact ionization (MS/EI). Application of the suggestions of these authors on the fragmentation of [13C]2-patulin (Figure 5) corroborated the elimination of acetaldehyde (C2H4O) from the M+ ions to give the labeled base ion m/z 112. Further labeled ions are generated by elimination of water (m/z 138) or carbon monoxide (m/z 128). Chernyshev et al. (1988) have assumed that fragment V should be formed from the mother ion IV by elimination of carbon dioxide. If this is true, labeled CO2 should be lost. Our data, however, showed that fragment V contained both labeled carbons, suggesting its formation by elimination of unlabeled CO from fragment II.

Negative Electrospray Ionization. Analysis of the labeled patulin by mass spectrometry in the negative electrospray ionization mode (ESI) showed the quasi-molecular ion (m/z 155; M − H) and its dimer (m/z 311; 2M − H) as well as the ion (m/z 201; M + 46 − H) (Figure 6A). The structure of the latter is unknown as it appears when patulin is dissolved either in methanol or in ethyl acetate. The same fragmentation pattern was obtained for the unlabeled patulin (Figure 6B).
To study the fragmentation of patulin during ESI measurements in more detail, several MSn experiments were performed. CID of the quasi-molecular ion (m/z 155; [M − H]-) of the labeled patulin produced major ions at m/z 110 and 111 and minor ions at m/z 127 and 137 (Figure 7A), showing some agreement with the results obtained by electron impact ionization (cf. Figure 4A). A similar result was obtained for the unlabeled patulin (cf. Figures 7B and 4B); however, no fragment m/z 108 corresponding to m/z 110 obtained for the labeled isotopomer was observed. To clarify the reason for the differences observed for the ions m/z 110 and 111 in the labeled and m/z 109 in the unlabeled patulin, the respective fragments were analyzed after CID. As shown in Table 1, LC(MS)2 of m/z 110 as well as 111 resulted in m/z 82. This indicated that the fragmentation during ESI may follow different ways, for example, a loss of labeled CO2 first, followed by unlabeled CO or, alternatively, a loss of unlabeled acetaldehyde first (as in electron impact measurements), followed

by labeled CO. Due to the same molecular weight of CO2 and acetaldehyde, this, of course, cannot be differentiated from the unlabeled patulin.

In summary, these results suggest that negative ions formed by CID in negative ESI often have their counterparts in metastable positive ions in EI ionization. A similar correlation between CID in positive ESI mode and in fast atom bombardement ionization has been used in structural investigations on the kedarcidin chromophore by Lee et al. (1996). However, our studies on labeled patulin indicate that CID may follow also a completely different way of fragmentation compared to the electron impact mode.

Negative Atmosphere Pressure Ionization (APCI). The spectra recorded in the negative APCI mode contained the ion (m/z 156; [M]-) as the base fragment of the labeled patulin (Figure 8A), which was identical with the corresponding spectrum of the unlabeled compound (Figure 8B).

Conclusions.

A method has been developed for the synthesis of 13C-labeled patulin for use in SIDA which will enable its analysis especially in samples requiring more complex cleanup procedures.

Further studies aimed at quantifying patulin in different foods by using the labeled isotopomer for either high-resolution mass chromatography of the silylated compound or ESI and APCI mass chromatography of the underivatized compounds are under way.

Table 1. Product ions of LC/ $(MS)^n$ of $^{13}C_2$-patulin after collision induced dissociation

process	LC/MS	LC/$(MS)^2$	LC/$(MS)^3$
fragments	m/z	m/z	m/z
	155	**110** (-$^{13}CO_2$)	**82** (-CO)
		111 (-C_2H_4O)	**82** (-^{13}CO)
		127 (- CO)	n.d.
	201	**155**	n.d.
	311	n.d.	n.d.

n.d. not determined

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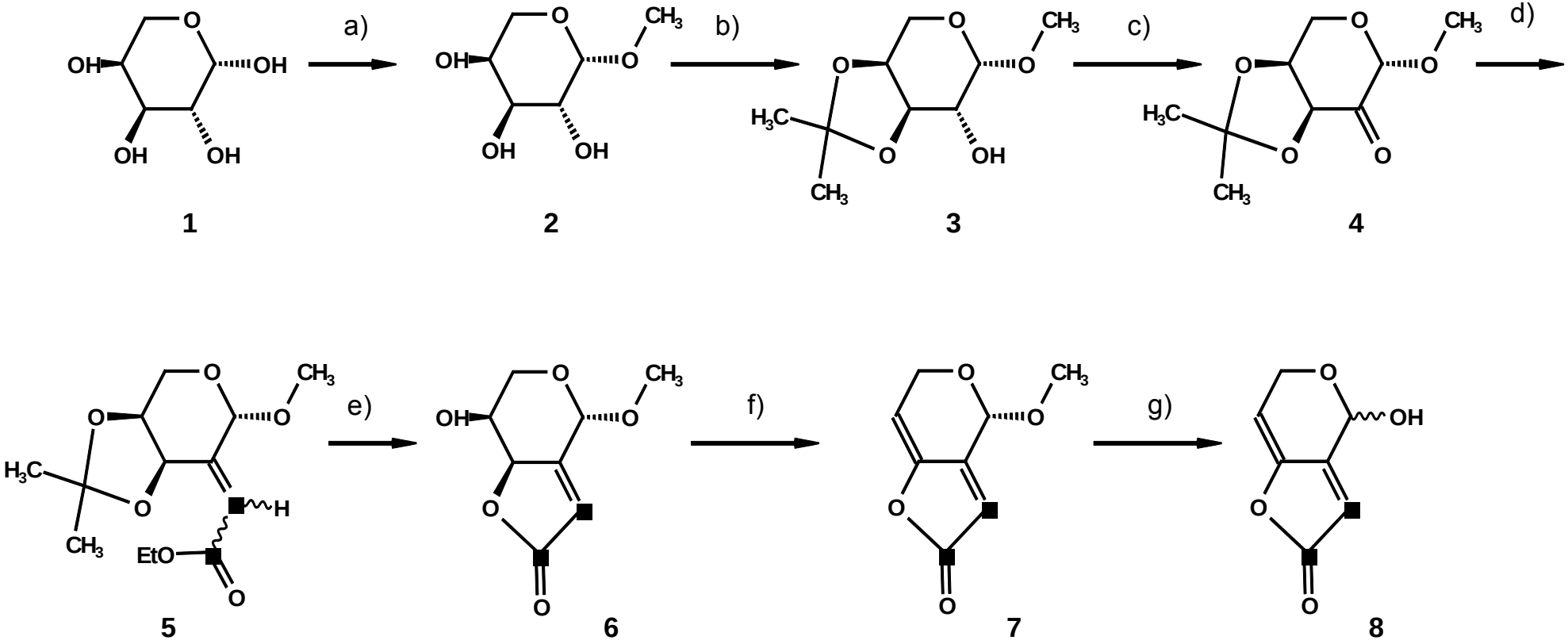
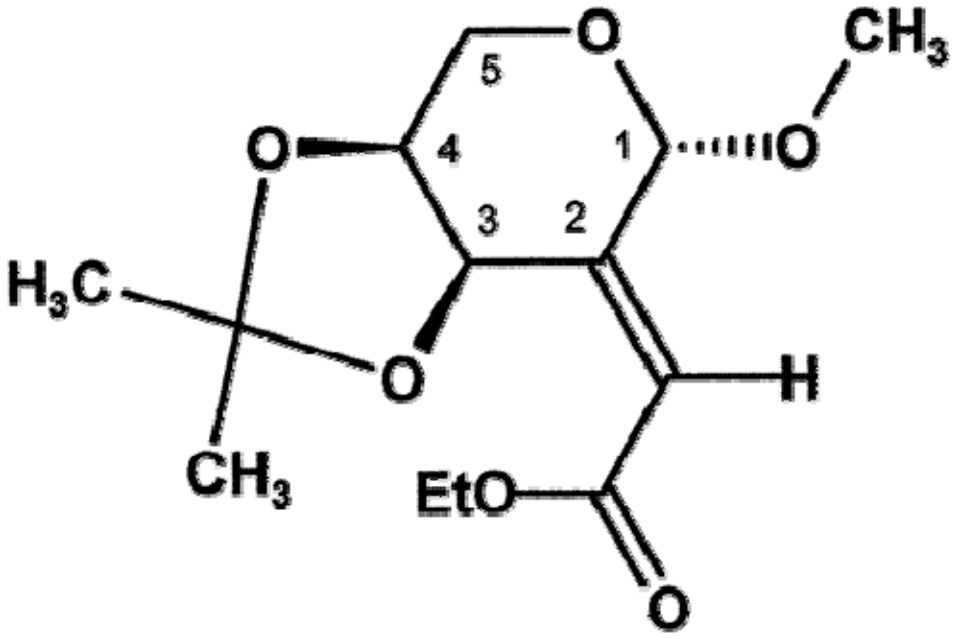


fig. 1. Route of synthesis to [$^{13}C_2$]-patulin,(■) ^{13}C-label : a) HCl/MeOH, b) 2,2-dimethoxypropane, Acetone/ H_2SO_4, c) PCC/ molecular sieves, d) propyleneoxide , ethyl [$^{13}C_2$]-bromoacetate, triphenylphosphine e) HCl/MeOH, f) methanesulfonylchloride g) TFA/ H_2O



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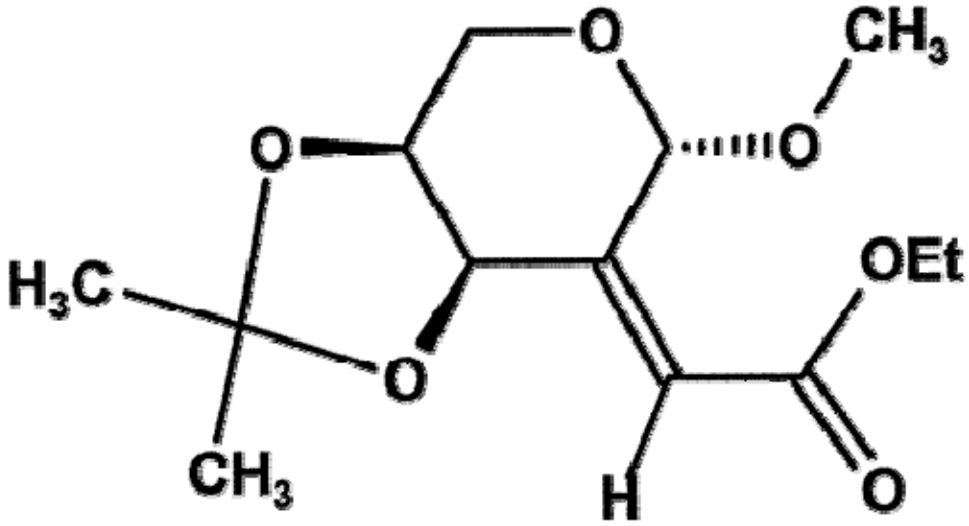

10

Fig 2. Structure of geometric isomers 9/10 formed by reaction of ketone 4 with unlabeled ethyl bromoacetate.

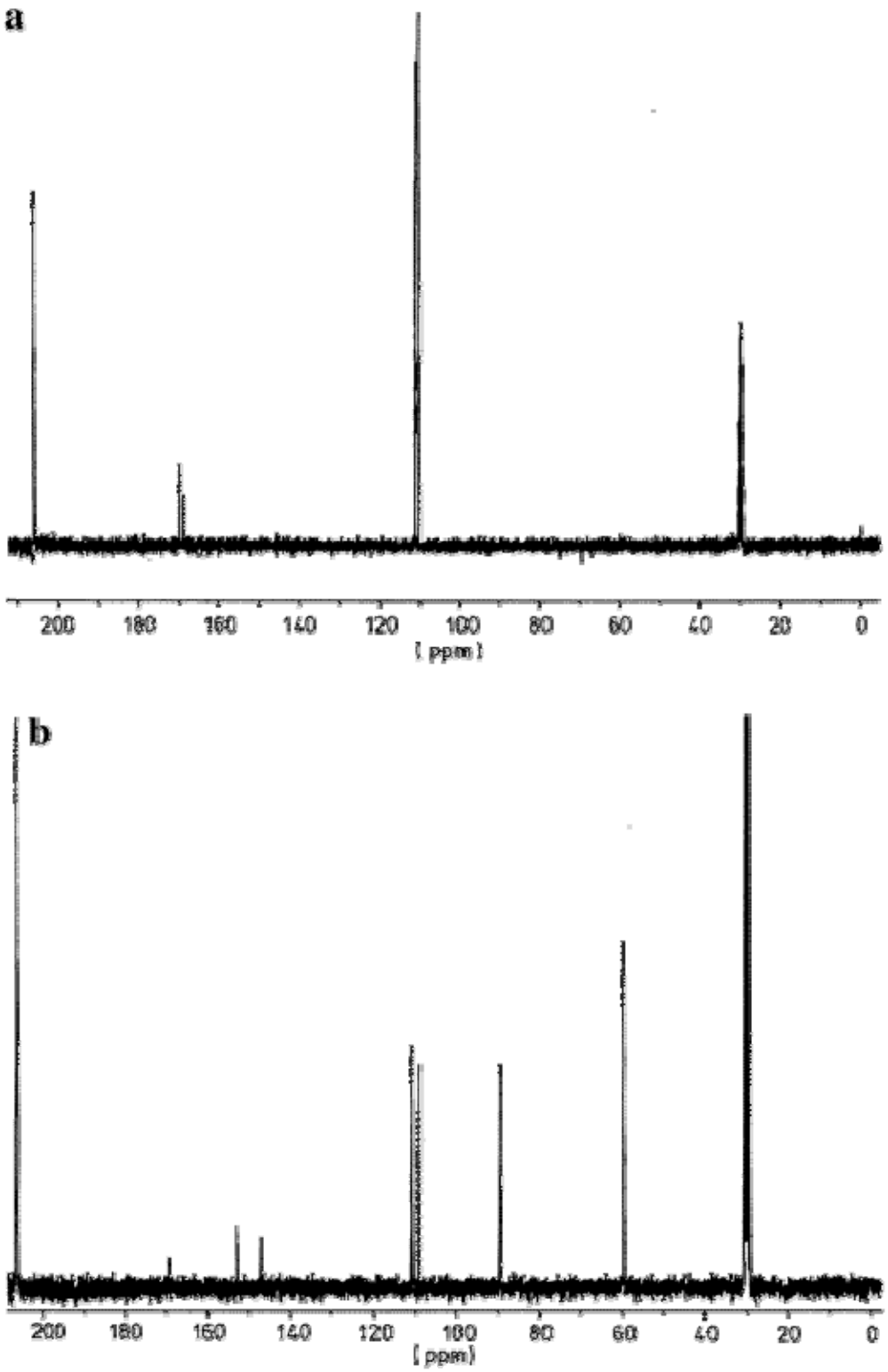

Figure 3 ^{13}C NMR spectrum of labeled (A) and unlabeled (B) patulin. Signals at 29.9 and 206.3 ppm are caused by the solvent acetone-d_6.

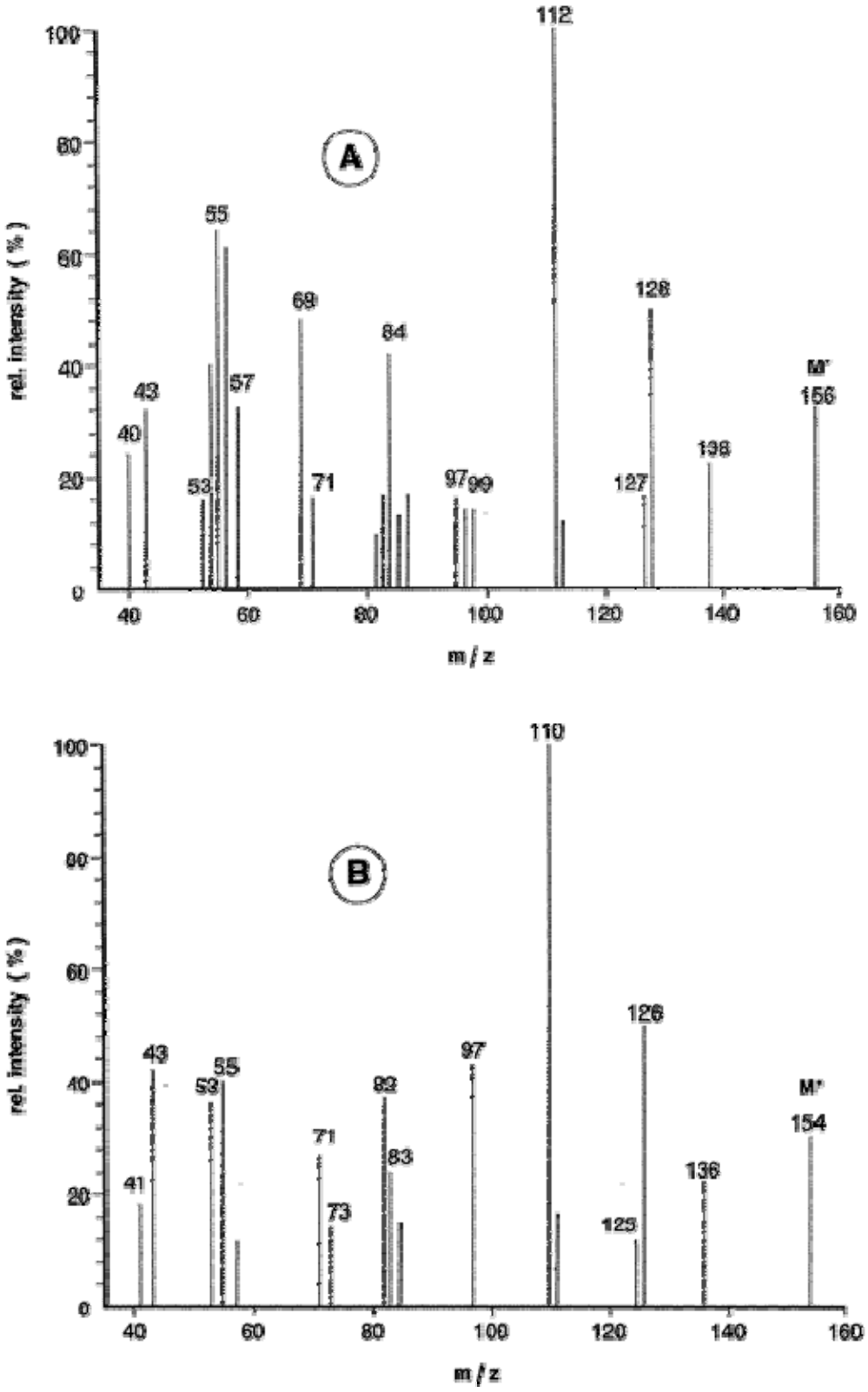

Figure 4 Mass spectrum of labeled (A) and unlabeled patulin (B) obtained by mass spectrometry in the electron impact mode using the direct insertion technique.

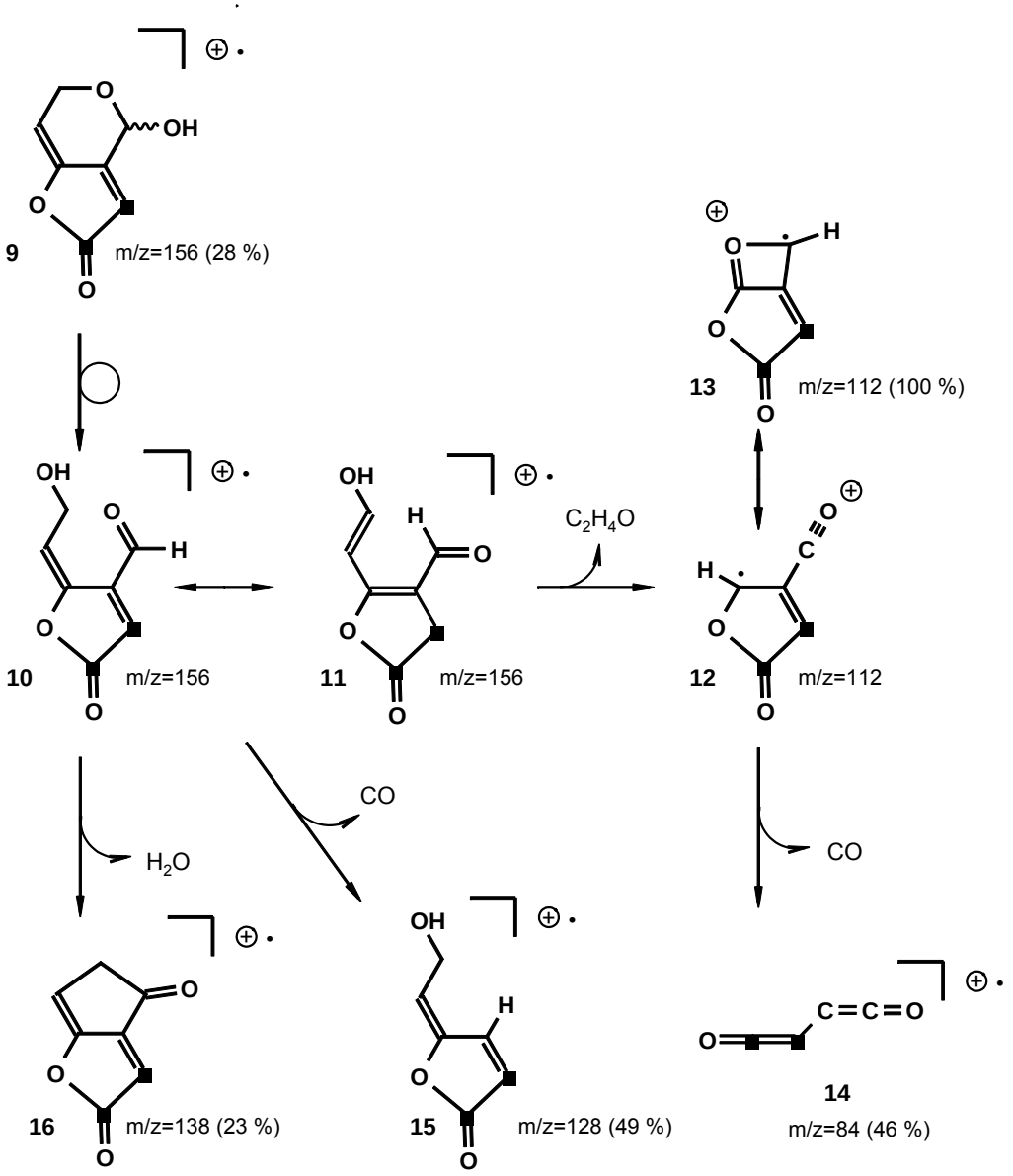

fig. 5. Suggested fragmentation route of [$^{13}C_2$]-patulin in mass spectroscopy, electron impact mode (relative intensities in %),(■) ^{13}C-label

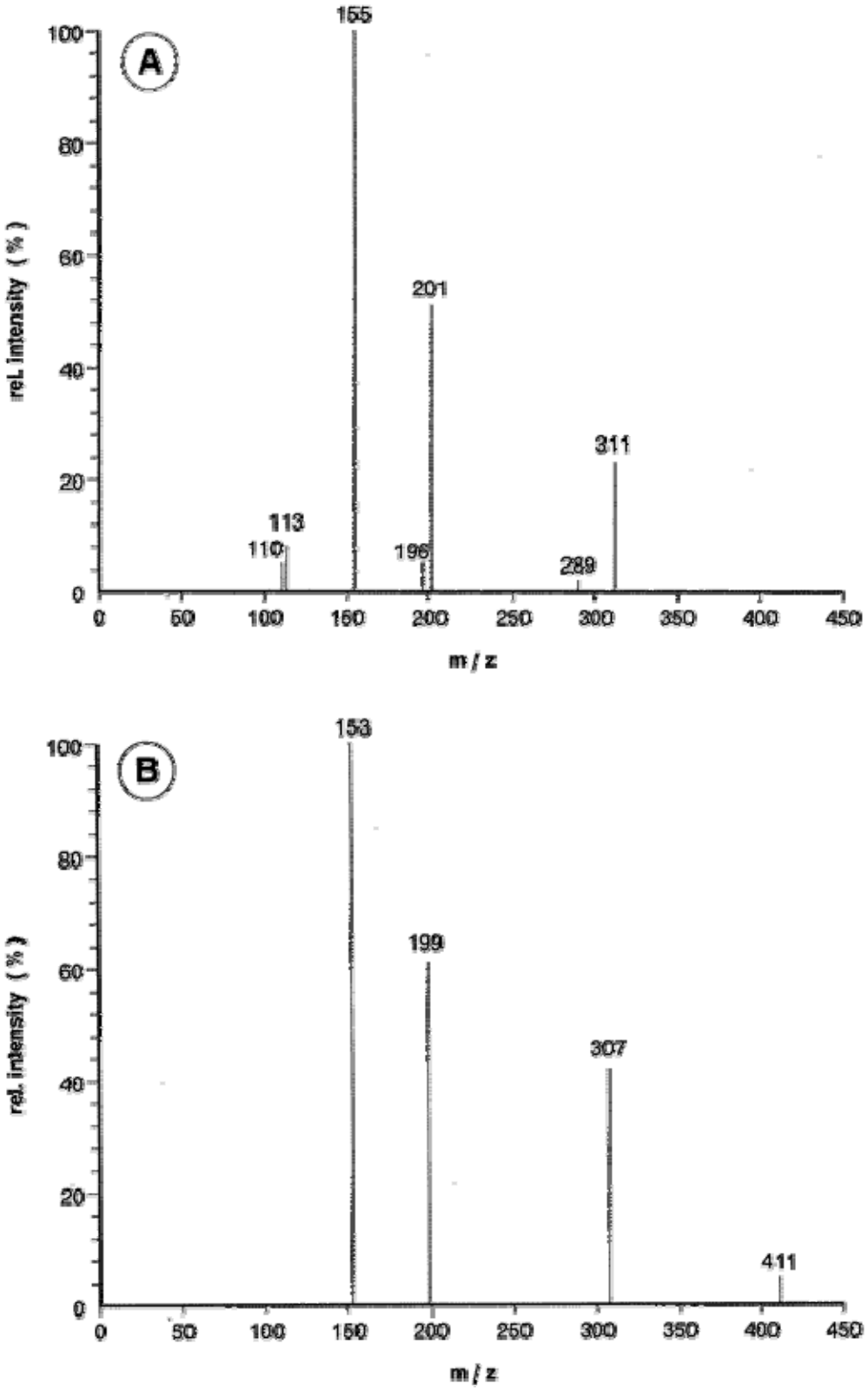

Figure 6 Mass spectra obtained by negative ESI of the labeled (A) and the unlabeled patulin (B).

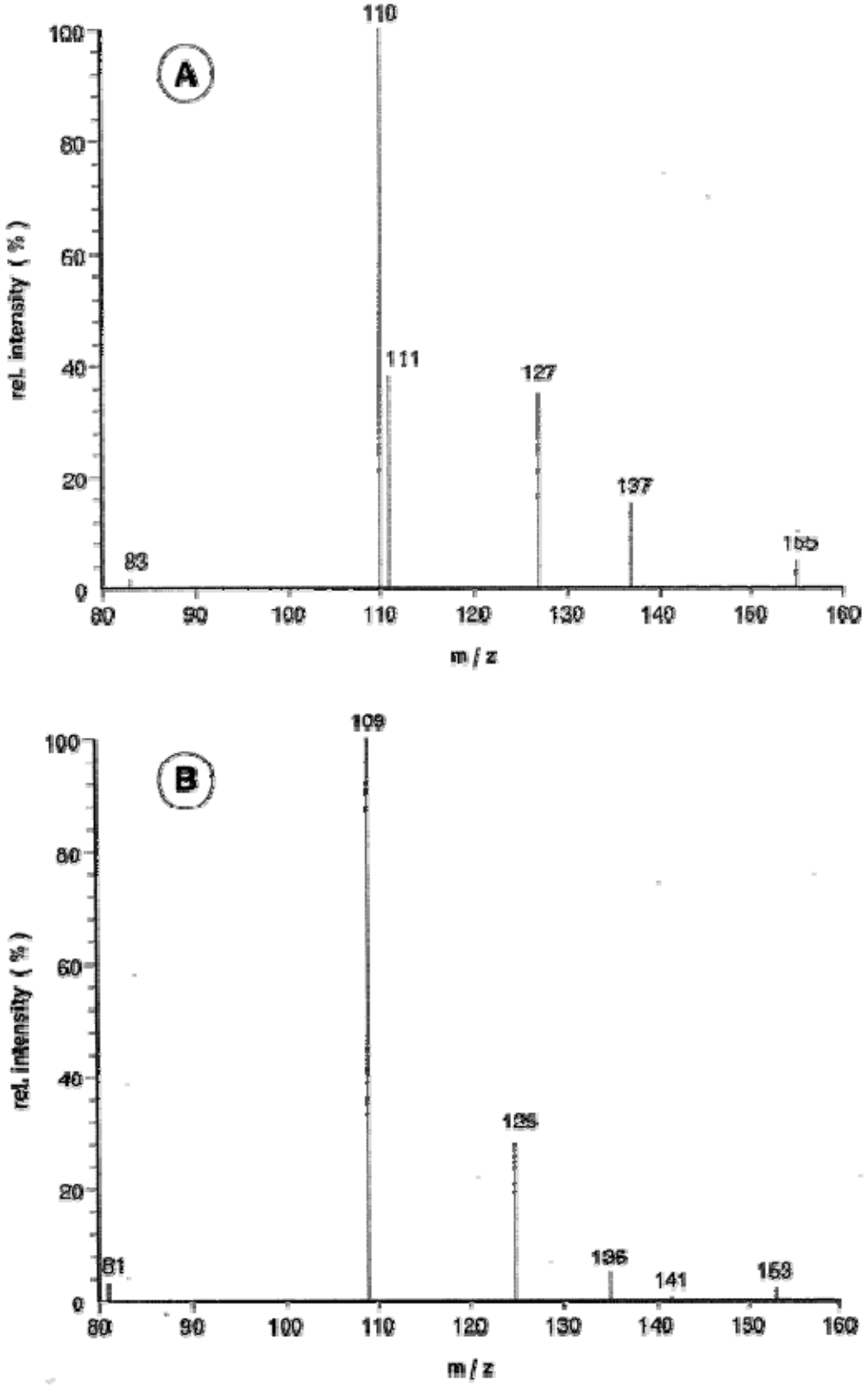

Figure 7 Mass spectra obtained by CID of the molecular ions *m*/*z* 156 (labeled patulin; A) and *m*/*z* 154 (unlabeled patulin; B).