Diagnostic Ions for the Analysis of Phenylalanine Adducts of Acrylamide and Styrene by ESI-QTOF Mass Spectrometry

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ABSTRACT: To facilitate the detection of acrylamide or styrene adduct of amino acids by mass spectrometry based techniques, phenylalanine was used as a representative amino acid and pyrolysis was employed in conjunction with isotope labeling technique as a microscale sample preparation tool to generate the reaction products. The residues remaining after the pyrolysis of phenylalanine/styrene, phenylalanine/acrylamide, and phenylalanine/glucose mixtures at 250 °C were analyzed by electrospray quadrupole time-of-flight (ESI-QqTOF) mass spectrometry to identify the adducts. The phenylalanine/acrylamide adduct was independently synthesized for confirmation. Characteristic product ions in the tandem mass spectra were found at m/z 191 for the acrylamide adduct and at m/z 262 and 190 for its double-addition product. On the other hand, an ion at m/z 224 was shown to be diagnostic of the styrene adduct. The ability of the m/z 224 ion to predict the presence of styrene adduct in a heated phenylalanine/glucose model system was tested and verified. Detailed isotope labeling analysis of the phenylalanine/glucose model further indicated the formation of a novel adduct that was consistent with the reaction of the Amadori product with styrene. Such diagnostic ions that are needed to develop MS/MS-based screening methodologies may accelerate in the future the detection of Michael-type adducts in food.

KEYWORDS: *Maillard reaction, styrene, acrylamide, electrospray, quadrupole time-of-flight, mass spectrometry, isotope labeling*

INTRODUCTION

Thermally generated toxicants during the processing of food such as acrylamide have become a major source of concern regarding the safety of prepared foods.[1] Acrylamide and its counterparts are mainly formed in a two-step process involving decarboxylation of the Schiff base adducts of the amino acids with the aid of a carbonyl moiety, normally a sugar, through the formation of a 5-oxazolidinone intermediate followed by a deamination step generating the potentially toxic amino acid derivatives.[2−4] In addition to asparagine, which generates acrylamide, other amino acids also can give rise to similar vinylic compounds analogous to acrylamide such as styrene from phenylalanine and piperidine from lysine.[5,6] Although the origin of such compounds is fairly well understood, their fate in food generally remains unknown.[1] Some studies suggest that acrylamide can undergo Michael-type addition with amino acids such as cysteine, N-acetylcysteine, and phenylalanine[7,8] or with amino compounds derived from amino acids and that such a process may result in decreased acrylamide content in foods.[9] For instance, the levels of acrylamide in fried snacks were found to be reduced when the uncooked products were soaked in the presence of amino acids such as lysine, glycine, and cysteine.[10] This can be explained by the fact that acrylamide can form adducts, such as 3-(alkylamino)propionamides, with amino compounds.[11] In fact, acrylamide was also found to form adducts with pyridoxamine, niacin, and naringenin as well.[12−14] Styrene, generated from phenylalanine in the Maillard reaction,[5,15,16] should also behave similarly. Although this process of conjugating toxic compounds with amino acids may be considered a "mitigation" step, in the absence of detailed knowledge regarding their toxicity and ability to release the toxic compounds during storage or in vivo, such a characterization could be misleading. Although presently such Michael-type adducts have been identified only in model systems, there is a good possibility that they could also be formed in food. To accelerate the development of methodologies for their detection in food, phenylalanine was used as a model amino acid to investigate in detail the MS/MS fragmentation patterns of the corresponding adducts with acrylamide and styrene to facilitate their detection by mass spectrometry based techniques and to demonstrate their further chemical reactivity in Maillard model systems.

MATERIALS AND METHODS

Chemicals. Acrylamide, D-glucose, formic acid, L-phenylalanine, L-phenyl-$^{13}C_6$-alanine, and styrene (>98%) were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). [^{13}C-1]Phenylalanine, [^{15}N]phenylalanine, [$^{13}C_6$-ring]phenylalanine, [^{13}C-1]glucose, [^{13}C-2]glucose, [^{13}C-3]glucose, [^{13}C-4]glucose, [^{13}C-5]glucose, [^{13}C-6]-glucose, and [$^{13}U_6$]glucose were >98% enriched and purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Methanol was of HPLC grade purchased from EMD Chemicals (Gibbstown, NJ, USA). Nanopure water was provided by a Millipore Synergy ultrapure water purification system (Billerica, MA, USA). Infrared spectra were recorded on a Bruker Alpha-P spectrometer (Bruker Optic GmbH, Ettlingen, Germany) equipped with a deuterated triglycine sulfate (DTGS) detector, a temperature-controlled single-bounce diamond

attenuated total reflectance (ATR) crystal, and a pressure application device for solid samples. Processing of the FTIR data was performed using Bruker OPUS software (Bruker Optic GmbH). ^{1}H NMR spectra were acquired in D_2O on a 300 MHz Varian Mercury instrument. Melting points were determined on an OptiMelt automated melting point system (Sunnyvale, CA, USA).

Sample Preparation. Pyrolysis residues were generated using a CDS Analytical Pyroprobe 2000 system (Oxford, PA, USA). About 1 mg of sample mixture (see Table 1) was packed inside a quartz tube

Table 1. Model Systems[a] Used To Generate Phenylalanine (Phe) Adducts Using ESI-QqTOF-MS

model system	measured m/z	theoretical m/z	error (ppm)	molecular formula
Phe-acrylamide[b]	237.1231	237.1234	1.1	$C_{12}H_{16}N_2O_3$ + H$^+$
Phe + acrylamide	237.1226	237.1234	3.2	$C_{12}H_{16}N_2O_3$ + H$^+$
	308.1605	308.1605	0.1	$C_{15}H_{21}N_3O_4$ + H$^+$
Phe + styrene	270.1493	270.1489	1.7	$C_{17}H_{19}NO_2$ + H$^+$
Phe + glucose	394.1648	394.1649	0.2	$C_{23}H_{23}NO_5$ + H$^+$

[a]All of the reaction mixtures used for the pyrolytic generation of the target compounds were equimolar except styrene, which was added in excess. [b]2-[(3′-Aminopropanamide]-3-phenylpropanoic acid.

(0.3 mm thickness), plugged with quartz wool and inserted inside the coil probe, and pyrolyzed for 20 s at a temperature of 250 °C. The quartz tube was then placed inside a microcentrifuge tube, and 1 mL of nanopure water was added to dissolve the nonvolatile residues. The samples were vortexed and then sonicated in a Branson model 2510 ultrasonic bath (Danbury, CT, USA) for 5 min followed by a 10-fold dilution in 50% methanol and 0.1% formic acid prior to their analysis by LC-MS.

High-Resolution Mass Spectrometry. Sample were separated on a Shimadzu Nexera ultrahigh-performance liquid chromatography (uHPLC) system coupled to a Triple TOF 5600 quadrupole time-of-flight instrument (AB Sciex, Concord, ON, Canada). Liquid chromatography was performed on an Agilent XDB-C18 column (50 × 4.6 mm) (Agilent Technologies, Santa Clara, CA, USA) filled with 1.8 μm particles using the following mobile phases: A, 100% water, 0.1% formic acid; B, 100% acetonitrile 0.1% formic acid, using an elution gradient starting at 5% B for 2 min with a linear increase to 80% B at 6 min held for another 2 min at a flow rate of 0.4 mL/min. The volume of injection was 15 μL. Data were acquired in positive electrospray mode using information-dependent acquisition (IDA) with a survey TOF-MS (m/z 115−625, 300 ms accumulation), followed by 3 MS/MS per cycle (m/z 80−615, 250 ms accumulation each) with a total cycle time of 1.1 s and a resolution of approximately 30000. Precursor ions were chosen on the basis of an inclusion list with dynamic background subtraction. A DuoSpray ion source was operated with an ion spray voltage of 5000 V in positive mode with a source temperature of 450 °C. The curtain gas was maintained at 30 psi, and GS1 and GS2 gases were both held at 50 psi. The declustering (DP) and exit (EP) potentials were set at 80 and 10 V, respectively. A collision energy (CE) of 30 ± 10 V was used for MS/MS experiments. High-resolution TOFMS and MS/MS were calibrated automatically every five runs with an automated calibrant delivery system (CDS) using a calibration standard mix with ions ranging from m/z 121 to 609 in positive mode. Data were acquired using Analyst TF 1.5.1, and PeakView 1.2 was used to study the MS/MS spectra.

Synthesis of 2-[(3′-Aminopropanamide]-3-phenylpropanoic Acid. Phenylalanine (0.32g) and acrylamide (0.2g) were dissolved in 10 mL of 0.1 N HCl and heated in an open vial at 75 °C for 7 h under a fume hood. After cooling, the concentrated solution crystallized, and the crystals were suspended in water and filtered. The solid was washed with water followed by methanol and dried. IR (solid) 3371 cm^{-1} (NH$_2$ asymmetric stretch), 3191 cm^{-1} (aromatic C—H stretch), 1621 cm^{-1} (amide C=O stretch), 1587 cm^{-1} (COO asymmetric

stretch), 1436 cm^{-1} (COO symmetric stretch); ^{1}H NMR (300 MHz, D$_2$O) δ 7.10 − 7.3 (m, 5H, aromatic), 3.83 (t, 1H, α-CH), 3.09−3.15 (m, 4H, aliphatic), 2.52−2.57 (m, 2H, aliphatic); mp 226.1−226.7 °C (dec); MS (ESI-QqTOF) m/z 237.1231 [M + H]$^+$ (calcd for $C_{12}H_{16}N_2O_3$ + H$^+$, 237.1234).

RESULTS AND DISCUSSION

The availability of fast analytical techniques for the identification of acrylamide or styrene adducts of amino acids may contribute to our understanding of the fate of these potential toxicants in food. The formation of these adducts may cause an underestimation of their measured amounts in food due to the possibility of their release during storage or postconsumption in the digestive tract. Phenylalanine was chosen as a representative amino acid to study its interactions with acrylamide and styrene, two of the better known thermally generated toxicants derived from amino acids. Pyrolysis was used as a microscale sample preparation tool to generate reaction products to minimize the use of expensive labeled precursors when isotope labeling studies were conducted. The residues remaining after the pyrolysis of phenylalanine/styrene, phenylalanine/acrylamide, and phenylalanine/glucose mixtures at 250 °C were analyzed by electrospray quadrupole-time-of-flight (ESI-QqTOF) mass spectrometry to identify adduct formation in these models. We have demonstrated in the past[17] the use of such pyrolysis probes as a sample preparation tool that can greatly reduce the quantities of labeled precursors needed to perform such analyses. Furthermore, to confirm the principle that amino acid adducts of acrylamide could be formed during pyrolytic sample preparation described above, 2-[(3′-amino-propanamide]-3-phenylpropanoic acid (**1**) (phenylalanine−acrylamide adduct) was chemically synthesized and analyzed by ESI-QqTOF-MS, which indicated the presence of a peak at m/z 237.1231 ($C_{12}H_{16}N_2O_3$ + H$^+$) consistent with the expected adduct formation (see Tables 1 and 2). When a

Table 2. Major MS/MS Fragments[a] of the Peak[b] at m/z 237.1234 ($C_{12}H_{16}N_2O_3$) Generated from Precursors Listed in Table 1

m/z (Da)	relative intensity (%)	error	MS/MS fragments[a]	label incorporation from Phe
105.0703	10	4.1	C_8H_8	none
130.0650	15	1.0	C_9H_7N	1 × N
132.0806	100	1.3	C_9H_9N	1 × N
178.0859	40	2.0	$C_{10}H_{11}NO_2$	1 × N and 1 × C-1
191.1176	62	1.5	$C_{11}H_{14}N_2O$	1 × N
237.1231	56	1.1	$C_{12}H_{16}N_2O_3$	1 × N and 1 × C-1

[a]Collision energy = 25 ± 10 V from QqTOF. [b]Retention time = 3.3 min.

sample prepared by the pyrolysis of a mixture of phenylalanine and acrylamide was similarly analyzed by ESI-QqTOF-MS, a similar peak at m/z 237.1226 was also observed. The two peaks, one from the synthetic sample and the other from the pyrolytic reaction mixture, showed identical molecular formulas, MS/MS fragments (Table 1; Figures 1 and 2), and retention times on the chromatographic column when analyzed by LC-MS (see Table 2). Furthermore, the phenylalanine/acrylamide pyrolytic reaction sample also exhibited a peak at m/z 308.1605 (see Tables 1 and 3) consistent with the double-addition product (**2**) of acrylamide with phenylalanine. Zamora et al.[6] and Arribas-Lorenz et al.[12] also reported similar double-addition adducts of acrylamide with glycine and pyridoxamine,
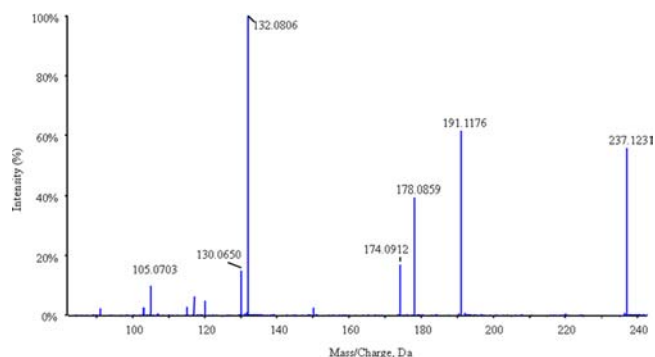
Figure 1. MS/MS spectrum of synthetic 2-[(3′-aminopropanamide]-3-phenylpropanoic acid (**1**).

Table 3. Major MS/MS Fragments[a] of the Peak[b] at *m/z* 308.1605 ($C_{15}H_{21}N_3O_4$) Generated from Precursors Listed in Table 1

m/z (Da)	relative intensity (%)	error	MS/MS fragments[a]	label incorporation from Phe
105.0708	4	11	C_8H_8	none
132.0813	75	4	C_9H_9N	$1 \times N$
178.0863	5	0.3	$C_{10}H_{11}NO_2$	$1 \times N$ and $1 \times C$-1
190.0865	100	1.3	$C_{11}H_{12}NO_2$	$1 \times N$ and $1 \times C$-1
191.1178	38	0.5	$C_{11}H_{14}N_2O$	$1 \times N$
249.1237	40	1.3	$C_{13}H_{16}N_2O_3$	$1 \times N$ and $1 \times C$-1
262.1556	1.6	2.3	$C_{14}H_{19}N_3O_2$	$1 \times N$
308.1605	14	0.1	$C_{15}H_{21}N_3O_4$	$1 \times N$ and $1 \times C$-1

[a]Collision energy = 30 ± 10 V from QqTOF. [b]Retention time = 3.5 min.

Table 4. Major MS/MS Fragments[a] of the Peak[b] *m/z* 270.1489 ($C_{17}H_{19}NO_2$) and Its Relative Peak Intensity

m/z (Da)	relative intensity (%)	error	MS/MS fragments[a]	label incorporation from Phe
105.0703	100	4.0	C_8H_8	none
120.0804	35	3.2	C_8H_9N	$1 \times N$
224.1438	15	1.9	$C_{16}H_{17}N$	$1 \times N$
270.1493	12	1.7	$C_{17}H_{19}NO_2$	$1 \times N$

[a]Collision energy = 30 ± 10 V from QqTOF. [b]Retention time = 4.7 min.

respectively. The ability to generate the adducts at *m/z* 237.1234 and 308.1605 from phenylalanine and acrylamide through pyrolytic sample preparation technique allowed us to introduce labeled precursors such as [^{13}C-1]phenylalanine and [^{15}N]phenylalanine and confirm the presence of nitrogen and C-1 atoms from phenylalanine in these adducts and in the MS/MS fragments. On the basis of the above findings, the phenylalanine and styrene model was similarly analyzed using only the pyrolytic sample preparation technique, and the targeted peak at *m/z* 270.1493 ($C_{17}H_{19}NO_2 + H^+$) was identified in the spectrum (Table 4) and proposed to be the predicted adduct **3**.

MS/MS Fragmentation Patterns of Phenylalanine Adducts of Acrylamide and Styrene. The proposed MS/MS fragmentation pathways for the phenylalanine adducts of acrylamide (**1** and **2**) are shown in Figures 2 and 3, and that of the styrene adduct is shown in Figure 4. The accurate masses

are listed in Tables 2−4 in addition to isotope incorporation data for the acrylamide adducts that was performed due to the multiple sources of nitrogen atom, which in this case cannot be distinguished by the elemental analysis alone compared to the
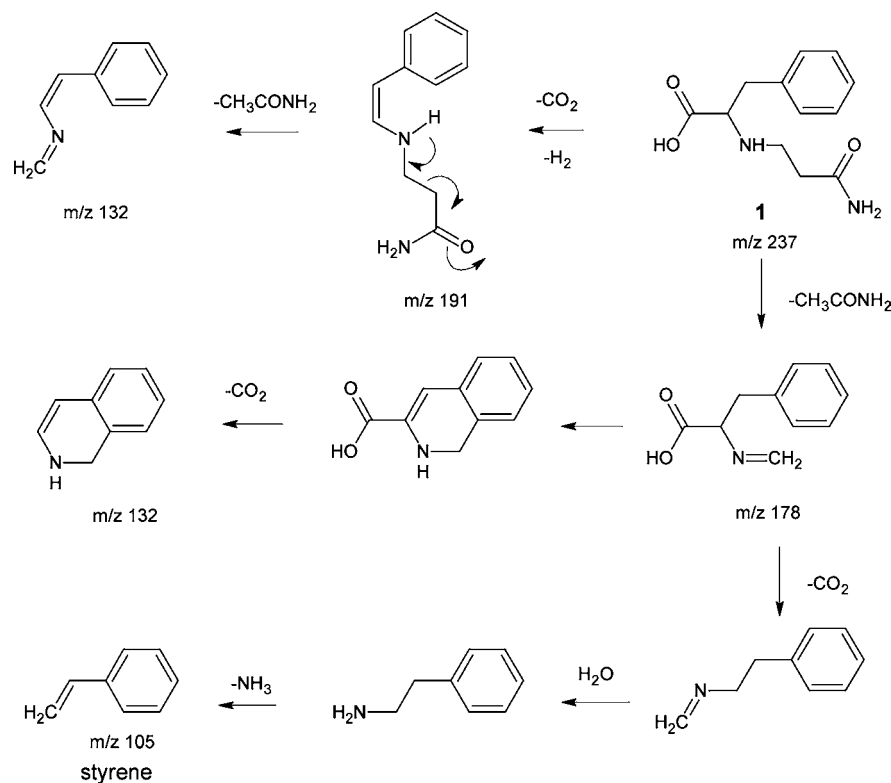

Figure 2. Proposed MS/MS fragmentation pathway of phenylalanine/acrylamide adduct at *m/z* 237 generated from pyrolytic reaction mixtures or from compound **1** (reported *m/z* values are for the protonated ions).
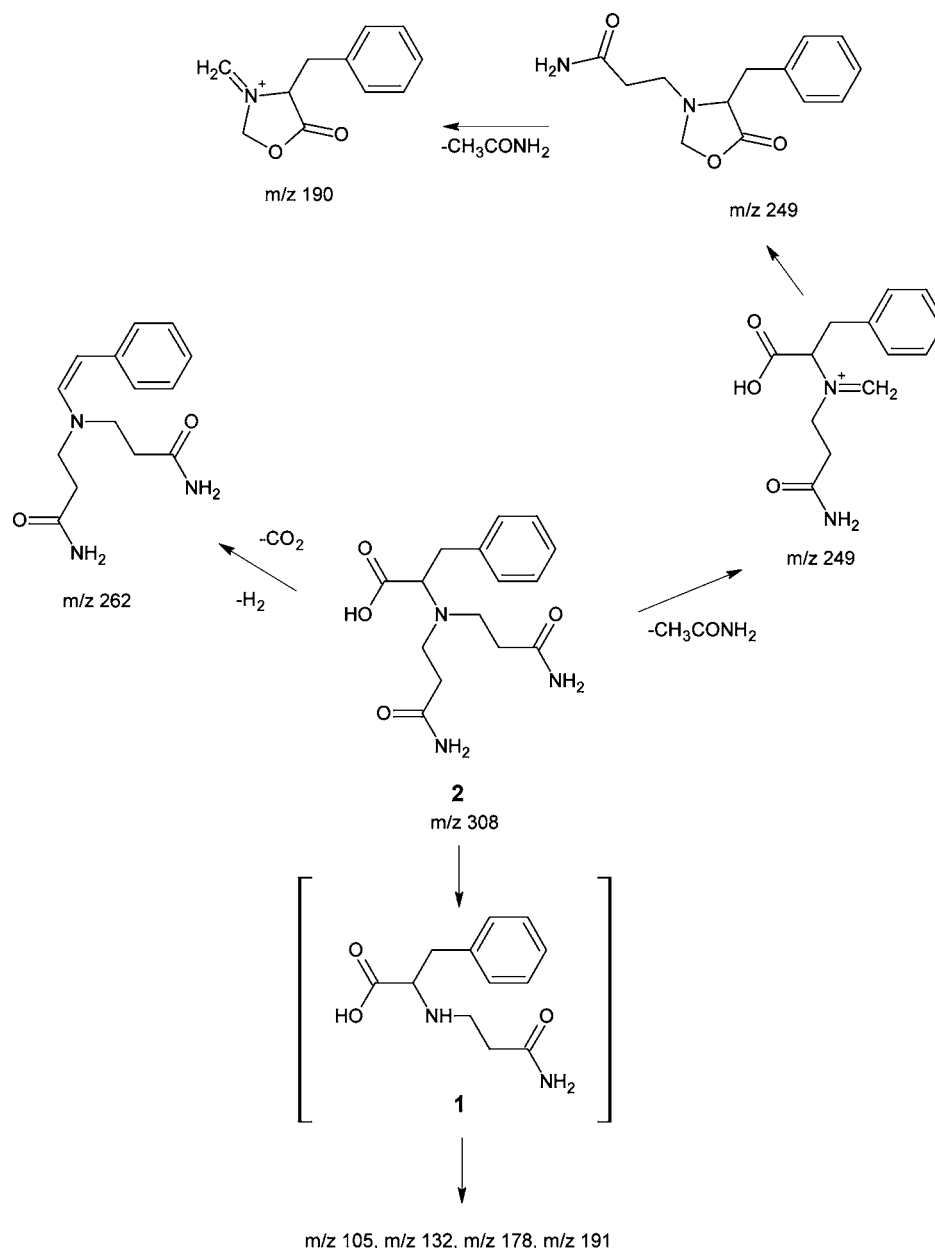
Figure 3. Proposed MS/MS fragmentation of the double-addition phenylanine/acrylamide adduct at m/z 308 generated from pyrolytic reaction mixtures (reported m/z values are for the protonated ions).

styrene adduct. As indicated above, the phenylalanine/acrylamide mixture also generated a double-addition product (**2**) at m/z 308. Comparison of the MS/MS data of the three phenylalanine adducts shows that they follow a similar initial reaction; starting with the parent molecule, they all undergo oxidative decarboxylation to produce ions at m/z 191 and 262 shown in Figures 2 and 3, respectively, for the acrylamide adducts and the ion at m/z 224 shown in Figure 4 in the case of styrene adduct. These three ions could be considered as diagnostic ions for the presence of phenylalanine adducts of styrene and acrylamide. The initially formed m/z 224 in the case of the styrene adduct undergoes elimination reaction to regenerate styrene at m/z 105 ($C_8H_8 + H^+$) and 2-phenylethenamine at m/z 120. On the other hand, MS/MS fragmentation of the acrylamide adducts (**1** and **2**) shows a more complex pattern. Apparently, the double-acrylamide adduct **2** shown in Figure 3 can fragment into **1** (not detected)

on the basis of the observation of common ions at m/z 191, 178, 132, and 105 in the MS/MS spectra of both compounds (see Tables 2 and 3). One of the characteristic reactions observed in the case of acrylamide adducts is the ability of the acrylamide moiety to undergo elimination of the acetamide molecule and generate reactive imine derivatives such as the intrinsically cationic fragment at m/z 249 (Figure 3) and a fragment at m/z 178 (Figure 2). Both ions have the ability to form 5-oxazolidinone intermediates[18,19] and undergo either decarboxylation,[18] as in the case of m/z 178 (Figure 2), or elimination of acetamide, as in the case of m/z 249, to generate a characteristic ion at m/z 190 that can be considered as a diagnostic ion for the double-addition product **2**. After decarboxylation, the ion at m/z 178 can eventually release the phenethylamine followed by deamination to form styrene. If the collision-induced transformation of **1** to styrene (see Figure 2) could be proven to occur in food during processing, a
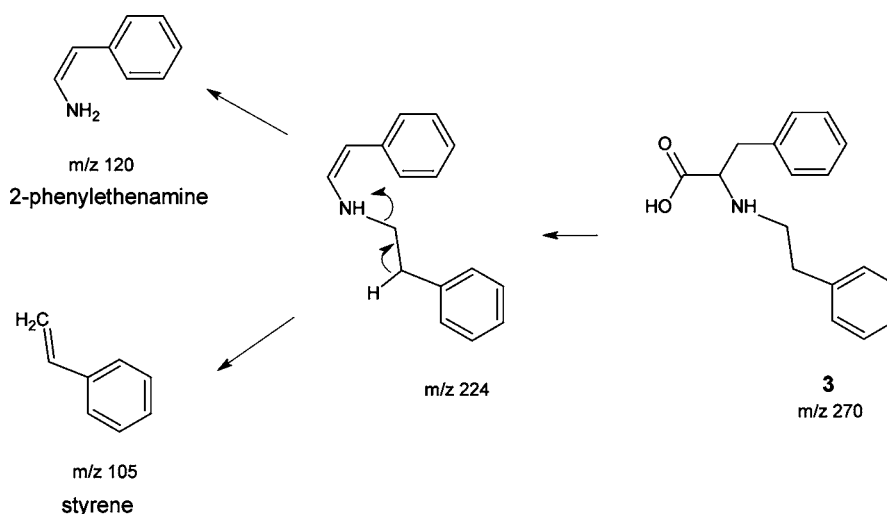
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Figure 4. Proposed MS/MS fragmentation of the phenylalanine/styrene adduct at m/z 270 generated from pyrolytic reaction mixtures (reported m/z values are for the protonated ions).

Table 5. Model Systems Used To Perform Isotope Labeling Experiments for the Identification of the Peak at m/z 394 by ESI-QqTOF-MS

model system	measured m/z (MH$^+$)	theoretical m/z (MH$^+$)	error (ppm)	MH$^+$	no. of labels
glucose + Phe	394.1648	394.1649	0.3	$C_{23}H_{23}NO_5$	NA
[$^{13}U_6$]glucose + Phe	400.1843	400.185	1.8	$^{13}C_6C_{17}H_{23}NO_5$	6 C atoms
glucose + [^{13}C-1]Phe	395.1685	395.1683	0.6	$^{13}C_1C_{22}H_{23}NO_5$	1 C atom
glucose + L-phenyl-$^{13}C_6$-alanine	406.2043	406.2052	2.1	$^{13}C_{12}C_{11}H_{23}NO_5$	12 C atoms
glucose + [^{15}N]Phe	395.1608	395.1619	2.9	$C_{23}H_{23}^{15}NO_5$	1 N atom

highly likely proposition, this finding could have a significant impact on the reported values of acrylamide and styrene in various foods and at the same time demonstrate that one toxicant, in this case, acrylamide, can be converted into another toxicant, styrene, through its reaction with phenylalanine.

Further Reactivity of the Phenylalanine/Styrene Adduct in the Maillard Reaction Model System. Styrene has been detected in phenylalanine Maillard model systems by various groups[5,15] and during pyrolytic reactions;[16] it is, therefore, reasonable to assume that styrene generated in situ in these model systems may undergo a similar addition reaction with the phenylalanine present and form adduct **3** and/or further react with other Maillard reaction products to generate novel adducts. To test the hypothesis that the ion at m/z 224 is diagnostic of the formation of **3** in food or model systems, the ESI-QqTOF mass spectrum of the phenylalanine/glucose pyrolytic reaction mixture was searched for the presence of m/z 270 and 224. Both ions were found in the phenylalanine/glucose reaction mixture, and the MS/MS analysis carried out on m/z 224 generated, as expected, ions at m/z 105 and 120, the characteristic daughter ions of **3** shown in Figure 4, indicating the potential usefulness of the above identified ions.

To search for new adducts of **3**, a series of isotope labeling experiments were performed using [$^{13}U_6$]glucose, [^{13}C-1]-phenylalanine, [^{15}N]phenylalanine, and L-phenyl-$^{13}C_6$-alanine (see Table 5) as precursors in glucose/phenylalanine reaction samples. The data analysis was targeted on the identification of an ion that incorporated two phenyl groups from phenylalanine and carbon atoms from glucose as indicated by the incorporation of an appropriate number of labeled atoms from the specific precursors listed in Table 5, ensuring the presence of phenylalanine, styrene, and sugar moieties. A peak

at m/z 394.1648 was observed with the high-resolution QqTOF instrument. The accurate mass indicated a molecular formula of $C_{23}H_{23}NO_5$ as the protonated molecule MH$^+$. As shown in Table 5, isotope labeling experiments indicated the incorporation of 6 carbon atoms from glucose, 12 carbon atoms from two phenyl rings, and 1 C-1' atom and 1 nitrogen atom from phenylalanine. The balance of the carbon atoms therefore should have come from the incorporation of two sets of C-2' and C-3' atoms from phenylalanine. The proposed structure of this adduct (**4**) shown in Figure 5 is consistent with the isotope label incorporation pattern and is based on detailed MS/MS fragmentation studies (Table 6). As shown in Figure 5, the carbon backbone of such a structure (**4**) could result either from the reaction of styrene with the Amadori product or from the reaction of glucose with **3** to generate structure **4′**. The latter can undergo carbonyl group migration from the C-2 to C-3 atom of glucose through enolization, followed by dehydrations of two hydroxyl groups at C-2 and C-4 positions of glucose, and finally oxidation of the styrene moiety may generate the proposed structure **4**. A peak corresponding to this structure at m/z 328.1393 ($C_{15}H_{21}NO_7 + H^+$) was detected in the phenylalanine/glucose reaction mixture using ESI-QqTOF-MS analysis. Both reactions leading to the formation of **4** in phenylalanine/glucose reaction mixtures shown in Figure 5 can be considered to be feasible pathways.

The MS/MS studies on the adduct formation of phenylalanine with acrylamide and styrene have revealed the diagnostic ions needed to develop MS/MS-based screening methods for their detection in food. Furthermore, these studies have indicated the potential of these adducts to undergo further reactions with other Maillard reaction products or to release free styrene and acrylamide under specific conditions as
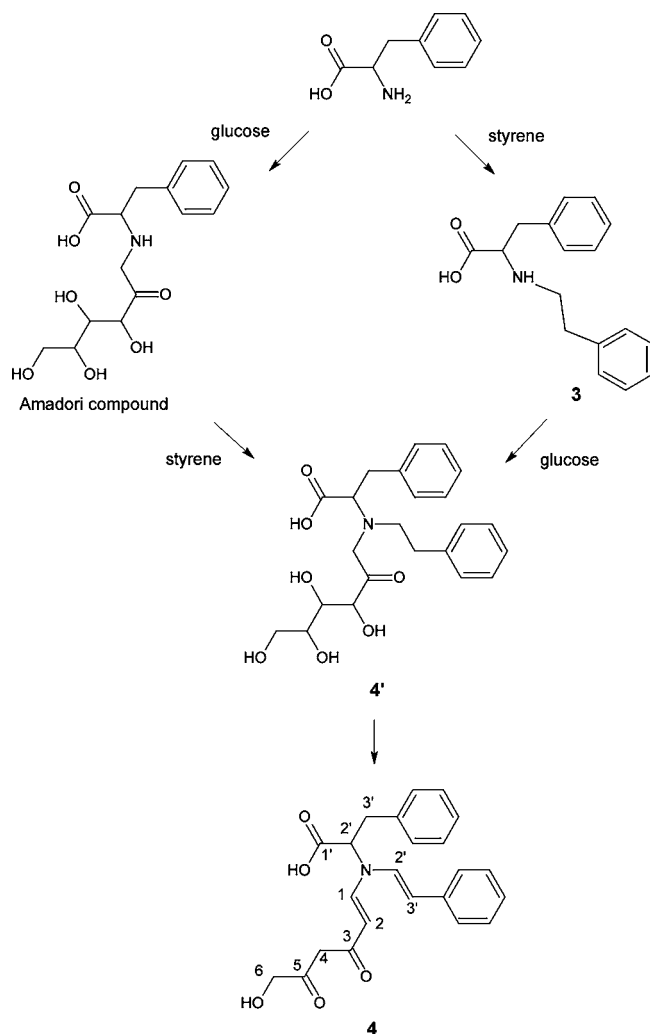
Figure 5. Proposed origin and structure of the unknown compound **4** (at m/z 394) based on isotope labeling data and MS/MS fragmentation studies.

Table 6. MS/MS^a Fragments^b of the Peak^c at m/z 394.1649 ($C_{23}H_{23}NO_5$)

m/z	error (ppm)	MS/MS fragments^a	label incorporation^d
304.1323	3.0	$C_{20}H_{17}NO_2$	C-1 to C-3; 1 × N, 1 × C′-1
316.133	0.7	$C_{21}H_{17}NO_2$	C-1, C-2, C-3, C- 4; 1 × N, 1 × C′-1
330.1489	0.1	$C_{22}H_{19}NO_2$	C-1, C-2, C-3, C-5 C-6; 1 × N, 1 × C′-1
334.1431	2.0	$C_{21}H_{19}NO_3$	C-1, C-2, C-3, C4; 1 × N, 1 × C′-1
346.143	2.2	$C_{22}H_{19}NO_3$	C-1 to C-5; 1 × N, 1 × C′-1
348.1591	0.9	$C_{22}H_{21}NO_3$	C-1 to C-6; 1 × N
358.1433	1.3	$C_{23}H_{19}NO_3$	C-1 to C-6; 1 × N, 1 × C′-1
376.1548	1.2	$C_{23}H_{21}NO_4$	C-1 to C-6; 1 × N, 1 × C′-1
394.1648	0.2	$C_{23}H_{23}NO_5$	C-1 to C-6; 1 × N, 1 × C′-1

^{a}Collision energy = 30 ± 10 V from QqTOF; C = sugar carbons, C′ = phenylalanine carbons. ^{b}MS/MS fragments and label incorporation patterns are consistent with the proposed structure **4**. ^{c}Retention time = 5.8 min. ^{d}All listed fragments incorporated two phenyl rings (12 carbons).

supported by the literature observations[11] in the case of the glycine/acrylamide adduct. This class of thermally generated compounds with unknown toxicity may be represented in various heated foods by as many as 30 different derivatives considering the range of amino acids present in processed foods.

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Notes
The authors declare no competing financial interest.

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