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Tyramine Pathways in Citrus Plant Defense: Glycoconjugates of Tyramine and Its *N*-Methylated Derivatives

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ABSTRACT: Glucosylated forms of tyramine and some of its *N*-methylated derivatives are here reported for the first time to occur in *Citrus* genus plants. The compounds tyramine-*O*-*β*-D-glucoside, *N*-methyltyramine-*O*-*β*-D-glucoside, and *N*,*N*-dimethyltyramine-*O*-*β*-D-glucoside were detected in juice and leaves of sweet orange, bitter orange, bergamot, citron, lemon, mandarin, and pomelo. The compounds were identified by mass spectrometric analysis, enzymatic synthesis, and comparison with extracts of *Stapelia hirsuta* L., a plant belonging to the *Apocynaceae* family in which *N*,*N*-dimethyltyramine-*O*-*β*-D-glucoside was identified by others. Interestingly, in *Stapelia hirsuta* we discovered also tyramine-*O*-*β*-D-glucoside, *N*-methyltyramine-*O*-*β*-D-glucoside, and the tyramine metabolite, *N*,*N*,*N*-trimethyltyramine-*O*-*β*-glucoside. However, the latter tyramine metabolite, never described before, was not detected in any of the *Citrus* plants included in this study. The presence of *N*-methylated tyramine derivatives and their glucosylated forms in *Citrus* plants, together with octopamine and synephrine, also deriving from tyramine, supports the hypothesis of specific biosynthetic pathways of adrenergic compounds aimed to defend against biotic stress.

KEYWORDS: *Citrus plant, tyramine-glucoside, N-methyltyramine-glucoside, hordenine-glucoside, candicine-glucoside, Stapelia hirsuta, biotic stress*

INTRODUCTION

The enzymatic decarboxylation of the aromatic amino acids phenylalanine, tyrosine, and tryptophan in plants represents a physiological process which operates as an interface between the primary and secondary metabolism aimed at production of defensive substances in response to attacks by insects, herbivores, and pathogens.[1] In citrus plants, two pathways for the biosynthesis of secondary metabolites, which likely play defensive roles, have been recently highlighted.[2,3] The first pathway starts with tryptophan decarboxylation by the enzyme trypthophan decarboxylase (TDC), producing the biogenic amine tryptamine, which then, by successive hydroxylation, is transformed into serotonin (5-hydroxytryptamine). We reported the occurrence in *Citrus* genus plants of tryptamine, serotonin, and all their *N*-methyl derivatives, that is, *N*-methyltryptamine, *N*,*N*-dimethyltryptamine, *N*,*N*,*N*-trimethyltryptamine, 5-hydroxy-*N*-methyltryptamine, 5-hydroxy-*N*,*N*-dimethyltryptamine (bufotenine), and 5-hydroxy-*N*,*N*,*N*-trimethyltryptamine (bufotenidine).[2] Among these compounds, *N*,*N*,*N*-trimethyltryptamine and 5-hydroxy-*N*,*N*,*N*-trimethyltryptamine are nicotine-like cholinergic agents by exerting their action on acetylcholine receptors. 5-Hydroxy-*N*,*N*,*N*-trimethyltryptamine is a cholinergic agent about 10-fold more powerful than *N*,*N*,*N*-trimethyltryptamine.[4] These pharmacological properties led us to hypothesize that both compounds could represent the goal of a biosynthetic pathway targeted to the defense of citrus plants against aggressors. The second defensive pathway starts with tyrosine decarboxylation, catalyzed by the enzyme tyrosine decarboxylase (TYDC), producing tyramine, from which octopamine (4-(2-amino-1-hydroxy-ethyl)phenol) is derived by subsequent hydroxylation by the enzyme tyramine-*β*-hydroxylase (T*β*H).[3] Both tyramine and octopamine, which exert adrenergic effects in mammals, are able to function in plants as neurotransmitters and neuromodulators by acting on specific receptors of plant attackers such as insects and herbivores. In this way, these compounds modify behavior and metabolism of plant attackers through the activation of G protein-coupled receptor-mediated signal transduction pathways.[5−8] Four octopamine receptors (Oamb, Oct*β*1R, Oct*β*2R, Oct*β*3R) and three tyramine receptor (TyrR, TyrRII, TyrRIII) modulate metabolic functions and behavior of invertebrates, such as flying ability, learning capacity, memory, and sleep/wake cycle, and also influence fertility.[7,9−11] In some plant genera, including *Citrus* genus,[12−16] successive *N*-methylation of tyramine by the same or different methyltransferase(s) produces *N*-methyltyramine and *N*,*N*-dimethyltyramine (hordenine). In citrus plants, synephrine (*N*-methyloctopamine), a powerful adrenergic amine, originates from the hydroxylation of *N*-methyltyramine, whereas in animals it is formed through the *N*-methylation of octopamine.[14]

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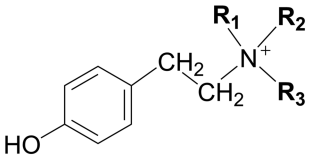
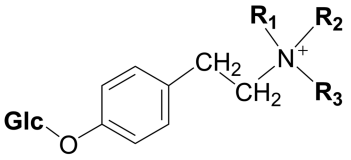
Structural representation	R_1	R_2	R_3	No.	Compound	Transition monitored
tyramine derivatives	H	H	H	**1**	tyramine	**138.1→121**
	CH_3	H	H	**2**	*N*-methyltyramine	**152.1→121**
	CH_3	CH_3	H	**3**	*N,N*-dimethyltyramine (*hordenine*)	**166.1→121**
	CH_3	CH_3	CH_3	**4**	*N,N,N*-trimethyltyramine (*candicine*)	**180.1→121**
glucosylated tyramine derivatives	H	H	H	**5**	tyramine-*O*-β-D-glucoside	**300.1→121**
	CH_3	H	H	**6**	*N*-methyltyramine-*O*-β-D-glucoside	**314.2→121**
	CH_3	CH_3	H	**7**	*N,N*-dimethyltyramine-*O*-β-D-glucoside (*hordenine-O*-β-D-*glucoside*)	**328.2→121**
	CH_3	CH_3	CH_3	**8**	*N,N,N*-trimethyltyramine-*O*-β-D-glucoside (*candicine-O*-β-D-*glucoside*)	**342.2→121**

Figure 1. Structural representations and MS^2 transitions utilized for analyses of tyramine derivatives by HPLC-ESI-MS/MS.

When we analyzed the distribution of methylated tyramine derivatives in leaves and fruits of several citrus species, we revealed for the first time the occurrence of *N,N,N*-trimethyltyramine, a quaternary ammonium compound, known as candicine or maltoxin.[17] This substance, first detected in barley rootlets (*Hordeum distichon* L.),[18] acts in animals as a powerful depolarizing neuromuscular blocker and shows muscarine-like and sympathomimetic effects.[19,20] In this context, candicine may represent the final step of a biosynthetic process which, starting from tyramine, is aimed to improve plant defense.[17,21] Recently, we identified a newly metabolic pathway in *Citrus* plants leading to the formation of serotonin 5-*O*-*β*-glucoside and all its *N*-methylated derivatives, that is, *N*-methylserotonin 5-*O*-*β*-glucoside, *N,N*-dimethylserotonin (bufotenine) 5-*O*-*β*-glucoside, and *N,N,N*-trimethylserotonin (bufotenidine) 5-*O*-*β*-glucoside.[22] It is well-known that many plants store their defense metabolites as inactive glycosides that can be hydrolyzed by specific glycosidases to release, when necessary, their toxic defensive aglycons. In this respect, our results showed that in citrus plants such a glycosylation mechanism can occur for serotonin and its methylated derivatives. These recent findings of the occurrence of glucosylated derivatives of serotonin and all its *N*-methylated derivatives in several *Citrus* genus plants[22] prompted us to investigate the possibility that a similar modification might also occur for tyramine and its *N*-methylated derivatives, that is, *N*-methyltyramine, *N,N*-dimethyltyramine (hordenine), and *N,N,N*-trimethyltyramine (candicine), whose presence was previously detected in leaves and fruits of various citrus plants.[17]

MATERIALS AND METHODS

Reagents. Tyramine, *N,N*-dimethyltyramine, *β*-glucosidase from almonds, and 0.1% solution of formic acid in water were purchased from Sigma-Aldrich (Milan, Italy). *N,N,N*-Trimethyltyramine was synthesized as reported.[17] A Discovery-C_8 analytical column, 250 × 3.0 mm inner diameter, 5 *μ*m particle size, with guard column of the same material, was obtained from Supelco (Milan, Italy). All other chemicals were of analytical reagent grade. Purified water was obtained from a Milli-Q system from Millipore (Milan, Italy).

Citrus Leaf Extracts. Citrus leaves of sweet orange (*Citrus sinensis*), bitter orange (*Citrus aurantium*), bergamot (*Citrus bergamia Risso & Poit*), citron (*Citrus medica*), lemon (*Citrus limon*), mandarin (*Citrus reticulata*), and pomelo (*Citrus maxima*) were harvested in October and December 2014 from the Research Centre for Citrus Crops and Mediterranean (CRA-ACM), arboretum of Reggio Calabria (Italy). For each species, 25 g of leaves, finely chopped, was homogenized in a blender with 100 mL of 0.1% formic acid in Milli-Q-grade water and then kept under stirring for 30 min. Finally homogenates were centrifuged at 18000*g* for 30 min, and supernatants were stored in 20 mL vials at −20 °C.

Citrus Juice Preparation. The endocarp (the edible part of the fruit constituted of carpels containing the juice cells) of sweet orange, bitter orange, bergamot, lemon, and mandarin deprived of seeds was homogenized in a mixer and then centrifuged at 18000*g* for 30 min. Supernatants were stored at −20 °C until used for the subsequent determinations.

***Stapelia hirsuta* Extracts.** *Stapelia hirsuta* L. was purchased in a local plant market. The plant aerial part was used to obtain the extracts. Slices of the stem (25 g) were homogenized in a blender with 100 mL of 0.1% formic acid in Milli-Q-grade water and then kept under stirring for 30 min. The homogenate was centrifuged at 18000*g* for 30 min and the supernatant stored in 20 mL vials at −20 °C.

HPLC-ESI-MS/MS Analyses. HPLC-ESI-MS/MS analyses were performed with an HPLC Agilent 1100 series coupled online with an Agilent LC-MSD SL quadrupole ion trap. MS acquisition was performed by using electrospray ionization (ESI) in positive-ion mode. Nitrogen, as both a drying and a nebulizing gas, was used at a flow rate of 7 L/min and a pressure of 30 psi. The nebulizer temperature was set at 350 °C. The ion charge control (ICC) was applied with a target set at 30000 and maximum accumulation time at 20 ms. Measurements were performed from the peak area of the extracted ion chromatogram (EIC). Multiple reaction monitoring (MRM) mode was used for detection of analytes. The MS^2 transitions utilized were 138.1 → 121 for tyramine, 152.1 → 121 for *N*-methyltyramine, 166.1 → 121 for *N,N*-dimethyltyramine, 180.1 → 121 for *N,N,N*-trimethyltyramine, 300.1 → 121 for tyramine-*O*-*β*-glucoside, 314.2 → 121 for *N*-methyltyramine-*O*-*β*-glucoside, 328.2 → 121 for *N,N*-dimethyltyramine-*O*-*β*-glucoside, and 342.2 → 121 for *N,N,N*-trimethyltyramine-*O*-*β*-glucoside. HPLC-ESI-MS^2 analyses were performed with a Discovery-C8 analytical column (Supelco), 250 mm × 3.0 mm i.d., 5 *μ*m particle size, eluted isocratically with 0.1% formic

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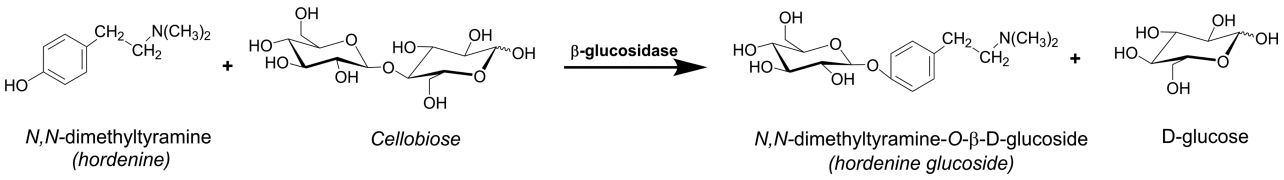

Figure 2. Transglucosylation reaction of cellobiose with hordenine catalyzed by almond β-glucosidase.

acid in water, at a constant flow rate of 0.1 mL/min. The injection volumes were 10−20 μL for each sample or standard solutions.

Enzymatic Synthesis of β-D-Glucosylated Forms of *N*-Methylated Tyramine Derivatives. As β-D-glucosides of tyramine and its *N*-methylated forms were not commercially available, we prepared the reference standard substances through a transglucosylation reaction with cellobiose catalyzed by the β-glucosidase from almonds. Moreover, only *N*,*N*-dimethyltyramine (hordenine) was commercially available, whereas *N*-methyltyramine and *N*,*N*,*N*-trimethyltyramine were not. Therefore, the transglycosylation reaction was conducted only for hordenine by using the substance to be glucosylated in pure form (Figure 2). The reaction mixture for the synthesis of hordenine-*O*-β-D-glucoside contained 100 mg of cellobiose, 10 mg of hordenine, and 10 U of β-glucosidase from almonds in sodium acetate buffer 50 mM at pH 5.0. The reaction was conducted at 37 °C, and the formation of the product was followed over time by analyzing 5 μL aliquots of the reaction mixture at 1 h intervals by HPLC-ESI-MS^2 by monitoring the MS^2 transition 328.2 → 121. In order to obtain the glucosylated derivatives of *N*-methyltyramine and *N*,*N*,*N*-trimethyltyramine, we first subjected tyramine to controlled methylation with iodomethane. The reaction was conducted as previously described,[17] and the formation of the products was monitored over time by HPLC-ESI-MS^2. When the various *N*-methylated tyramines reached convenient levels, the methylation reaction was stopped by removing under vacuum the solvent and excess of iodomethane. Successively, 10−20 mg of the dried material was subjected to the transglucosylation reaction, as reported above for hordenine. The formation of products was monitored by analyzing in MRM mode by HPLC-ESI-MS^2 5 μL aliquots of the reaction mixture every hour through the MS^2 transitions 300.1 → 121 for tyramine-*O*-β-glucoside, 314.2 → 121 for *N*-methyltyramine-*O*-β-glucoside, 328.2 → 121 for *N*,*N*-dimethyltyramine-*O*-β-glucoside, and 342.2 → 121 for *N*,*N*,*N*-trimethyltyramine-*O*-β-glucoside.

Statistical Analysis. Data are expressed as mean ± std of $n = 4$ determinations, each in duplicate. Differences were assessed by *t* test, and a *p* value less than 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Identification and Characterization of the Glycosylated Forms of Tyramine and Its *N*-Methylated Derivatives. In the light of our previous findings,[17,22] we investigated whether tyramine and its *N*-methylated derivatives (Figure 1) may be subjected to glycosylation in citrus plants as it happens for serotonin and its *N*-methylated derivatives.

To this aim, we first subjected leaf extracts of citrus plants to HPLC-ESI tandem mass analysis. The ESI-MS^2 chromatogram of a bergamot leaf extract was obtained in multiple reaction monitoring (MRM) mode by isolating the ion masses at *m*/*z* 138.1, 152.1, 166.1, 180.1, which coincide with those of the protonated tyramine, *N*-methyltyramine, *N*,*N*-dimethyltyramine, and *N*,*N*,*N*-trimethyltyramine, and the ion masses at *m*/*z* 300.1, 314.2, 328.2, and 342.2, which coincide with those of their protonated glycosylated derivatives, respectively (Figure 3). In the chromatographic conditions employed, tyramine, *N*-methyltyramine, *N*,*N*-dimethyltyramine, and *N*,*N*,*N*-trimethyltyramine eluted well resolved. Their identities were assigned from the MS^2 fragmentation patterns (Figure 3, track A, panels A1−A4) and coelution with authentic standards.[17] The retention times were 20.5 min for tyramine, 24.0 min for *N*-methyltyramine, 28.7 min for *N*,*N*-dimethyltyramine, and 32.5 min for *N*,*N*,*N*-trimethyltyramine. Interestingly, three more peaks were observed in the chromatogram (Figure 3, track B). The first (peak B1), at t_R 17.2 min, corresponds to *m*/*z* 300.1, the second (peak B2), at t_R 19.5 min, corresponds to *m*/*z* 314.2, and the third (peak B3), at t_R 22.5 min, corresponds to *m*/*z* 328.2. All three peaks showed a common ion fragment at *m*/*z* 121 in their MS^2 fragmentation patterns (Figure 3, track B, panels B1−B3), which led us to suppose that the compounds of peaks B1, B2, and B3 were structurally related to tyramine, *N*-methyltyramine, and *N*,*N*-dimethyltyramine, compounds known to form an ion fragment at *m*/*z* 121 in their MS^2 fragmentation patterns (Figure 3, panels A1−A4 from track A). As matter of fact, the MS^2 fragment at 121 is generated by neutral loss of ammonia, methylamine, and *N*,*N*-dimethyltyramine, from tyramine, *N*-methyltyramine, and *N*,*N*-dimethyltyramine, respectively.[23] It is worth noting that neither in bergamot leaf extracts nor in leaf extracts of other citrus species was the peak at *m*/*z* 342.2 with a MS^2 fragment at *m*/*z* 121, corresponding to glycosylated *N*,*N*,*N*-trimethyltyramine, detected.

The common structural motif of the compounds of peaks B1, B2, and B3 was further supported by the identity of their MS^3 fragmentation patterns obtained by isolating the MS^2 fragment at *m*/*z* 121 from each of them (Figure 3, panel C). On the other hand, also the MS^3 fragmentation patterns obtained by isolating the MS^2 fragment at *m*/*z* 121 of tyramine, *N*-methyltyramine, and *N*,*N*-dimethyltyramine were identical to those of the three unknown peaks (Figure 3, panel C).[17] Furthermore, all three unknown substances showed in their MS^2 fragmentation patterns ion fragments having mass/charge ratios coinciding with those of protonated masses of tyramine, *N*-methyltyramine, and *N*,*N*-dimethyltyramine, that is, *m*/*z* 138.1 for peak B1, 152.1 for peak B2, and 166.1 for peak B3 (Figure 3, panels B1−B3 from track B), which likely arise from the neutral loss of a hexose residue (162 amu) from the parent ions.[23,24] Therefore, taken together, the MS^2 fragmentation patterns of the peaks B1, B2, and B3 led us to reasonably infer that the unknown compounds were *O*-glycosylated forms of tyramine, *N*-methyltyramine, and *N*,*N*-dimethyltyramine, respectively.

Mass Spectrometric Characterization of the Glycosylated Forms of Tyramine and Its *N*-Methylated Derivatives in *Stapelia hirsuta*. On the basis of the above considerations, we sought to investigate the chemical structures of the putative glycosylated derivatives of tyramine and its *N*-methylated forms, which likely occurred in citrus plants. As such compounds were not commercially available, we took advantage of a study conducted on *Stapelia hirsuta* L., a cactus-like plant belonging to the *Apocynaceae* family, in which hordenine, candicine, and hordenine-*O*-β-D-glucoside were detected.[25] Actually, with the exception of the study of Shabana

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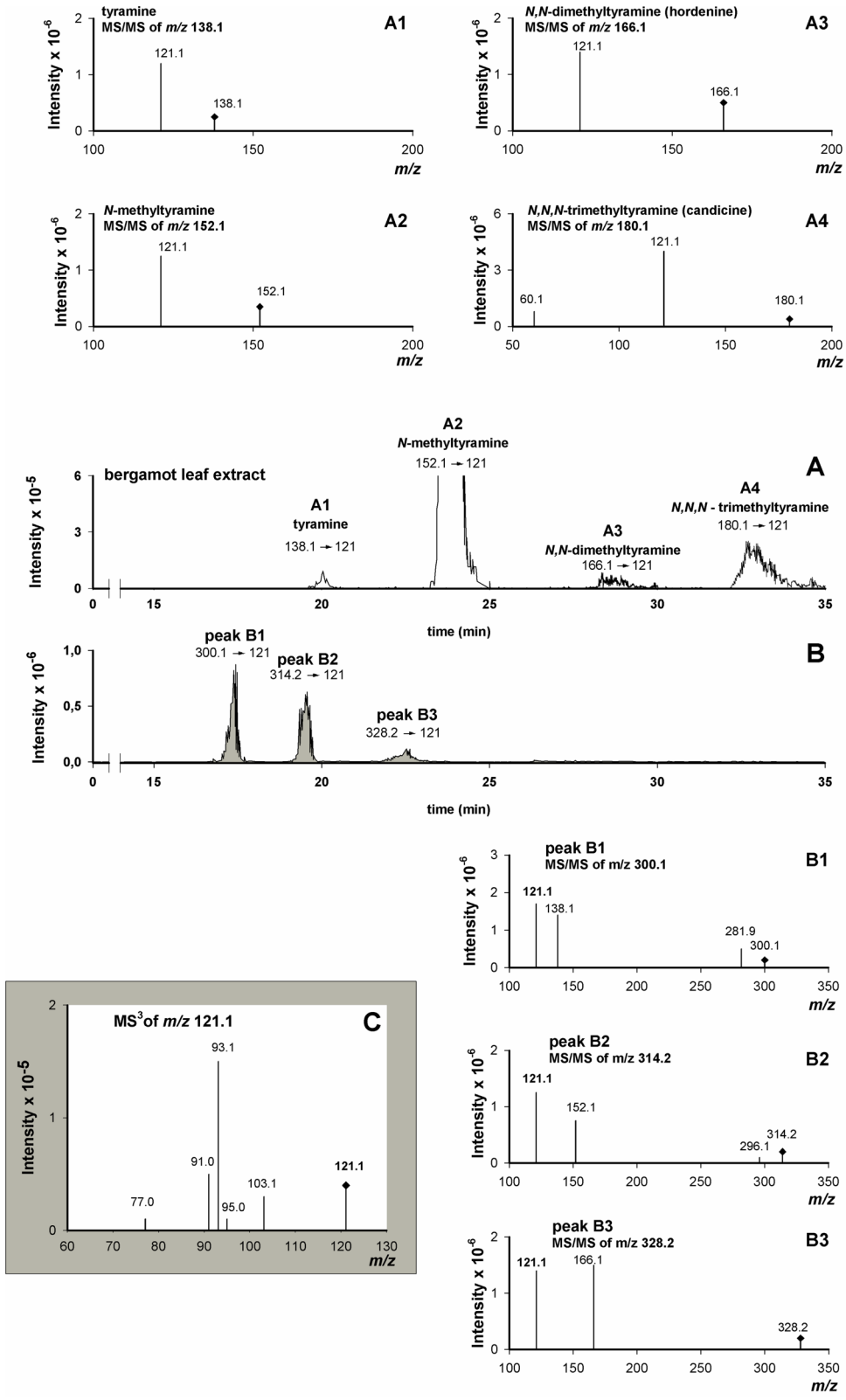

Figure 3. Representative HPLC-ESI-MS^2 chromatogram of a bergamot leaf extract. Track A: The chromatogram was obtained in multiple reaction monitoring (MRM) mode by isolating the ion masses at m/z 138.1, 152.1, 166.1, and 180.1, which coincide with those of the protonated tyramine, *N*-methyltyramine, *N*,*N*-dimethyltyramine (hordenine), and *N*,*N*,*N*-trimethyltyramine (candicine), respectively, and (track B) by isolating the ion masses at m/z 300.1, 314.2, 328.2, and 342.2, which coincide with those of their protonated glycosylated derivatives, respectively. Panels A1−A4: MS^2 fragmentation patterns of the peaks in track A. Panels B1−B3: MS^2 fragmentation patterns of the peaks in track B. Panel C: MS^3 fragmentation pattern of the MS^2 fragment ion (m/z 121.1) common to peaks in tracks A and B. The chromatographic conditions are reported under Materials and Methods.

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et al.[25] and a more recent NMR metabolomic study concerning the probable occurrence of *O*-glucosyltyramine in cell cultures of *Cannabis sativa* L.,[26] as far as we know, the presence of *O*-glycosylated derivatives of tyramine or its *N*-methylated derivatives in other sources has not been described. Therefore, with the aim to obtain at least one reference standard to compare with the unknown substances in citrus plants, extracts from the aerial parts of *Stapelia hirsuta* were subjected to mass spectrometric characterization. Also in this case, the chromatogram (Figure 4) was obtained in multiple reaction monitoring (MRM) mode by isolating the ion masses at *m/z* 138.1, 152.1, 166.1, and 180.1, which coincide with those of the protonated tyramine, *N*-methyltyramine, *N,N*-dimethyltyramine, and *N,N,N*-trimethyltyramine, and the ion masses at *m/z* 300.1, 314.2, 328.2, and 342.2, which coincide with those of their protonated glycosylated derivatives, respectively. Results showed the occurrence of hordenine and candicine in the extract of *Stapelia hirsuta* (Figure 4, track A), thus confirming what was previously reported.[25] However, two intense peaks corresponding to tyramine and *N*-methyltyramine were detected. The identity of these compounds, not previously mentioned,[25] was confirmed by comparison with authentic standards. As for glycosyltyramine derivatives, four well resolved peaks were observed in the chromatogram (Figure 4, track B). The first (peak B1), at t_R 17.2 min, corresponds to ion mass of *m/z* 300.1, the second (peak B2), at t_R 19.5 min, corresponds to ion mass of *m/z* 314.2, the third (peak B3), at t_R 22.5 min, corresponds to ion mass of *m/z* 328.2, and the fourth (peak B4), at t_R 26 min, corresponds to ion mass of *m/z* 342.2. The peak B3 at t_R 22.5 min, isolated at *m/z* 318.2, shows a MS^2 fragmentation pattern (Figure 3, panel B3) which confirms the previously reported presence of hordenine-*O*-*β*-D-glucoside in the *Stapelia* extract.[25] In fact, the MS^2 fragment at *m/z* 166 (corresponding to protonated hordenine) is generated by the neutral loss of the glucosyl residue from the protonated compound, while the MS^2 fragment at *m/z* 121 arises from the dimethylamine neutral loss (45 amu) from the fragment at *m/z* 166. Furthermore, the MS^3 analysis conducted by isolating the MS^2 fragment at *m/z* 121 showed the same fragmentation pattern as that obtained by isolating the fragments at *m/z* 121 which arise from MS^2 fragmentation of tyramine and its *N*-methyl derivatives (data not shown). As for peaks B1 and B2 (Figure 4, track B), similar considerations to those reported above reveal their possibly identities as tyramine-*O*-*β*-D-glucoside (peak B1) and *N*-methyltyramine-*O*-*β*-D-glucoside (peak B2), so far not reported in this plant. Notably, peaks B1 and B2 showed the same retention times and MS^2 fragmentation pattern as peaks B1 and B2 found in the extracts of citrus plants (Figure 3, panels B1−B3 from track B). It is also interesting to note the peak B4 at *m/z* 342.2, whose MS^2 fragmentation pattern is consistent with that of *N,N,N*-trimethyltyramine-*O*-*β*-D-glucoside (Figure 4, panel B4). In fact, the MS^2 fragment at *m/z* 180 (corresponding to *m/z* of *N,N,N*-trimethyltyramine) is generated by the neutral loss of the glucosyl residue from the parent ion, while the MS^2 fragment at *m/z* 121 arises from the trimethylamine neutral loss (59 amu) from the fragment at *m/z* 180. This was further substantiated by the MS^3 fragmentation pattern obtained by isolating the MS^2 fragment at *m/z* 180, where, besides the MS^3 fragment at *m/z* 121, also present is a fragment at *m/z* 60 corresponding to the trimethylammonium ion.

Mass Spectrometric Characterization of the Glucosylated Forms of Tyramine and Its *N*-Methylated Derivatives Produced by Enzymatic Synthesis.

The identity of peak B3 in the chromatogram of the bergamot leaf extract (Figure 3) as hordenine-*O*-*β*-D-glucoside was ascertained by its retention time and MS^2 fragmentation pattern, which were the same as those of peak B3 in the chromatogram of the *Stapelia* extract (Figure 4, track B), where the occurrence of hordenine-*O*-*β*-D-glucoside was known.[25] Moreover, peaks B1 and B2 in the *Stapelia* extract chromatogram (Figure 4, track B) show the same retention times and

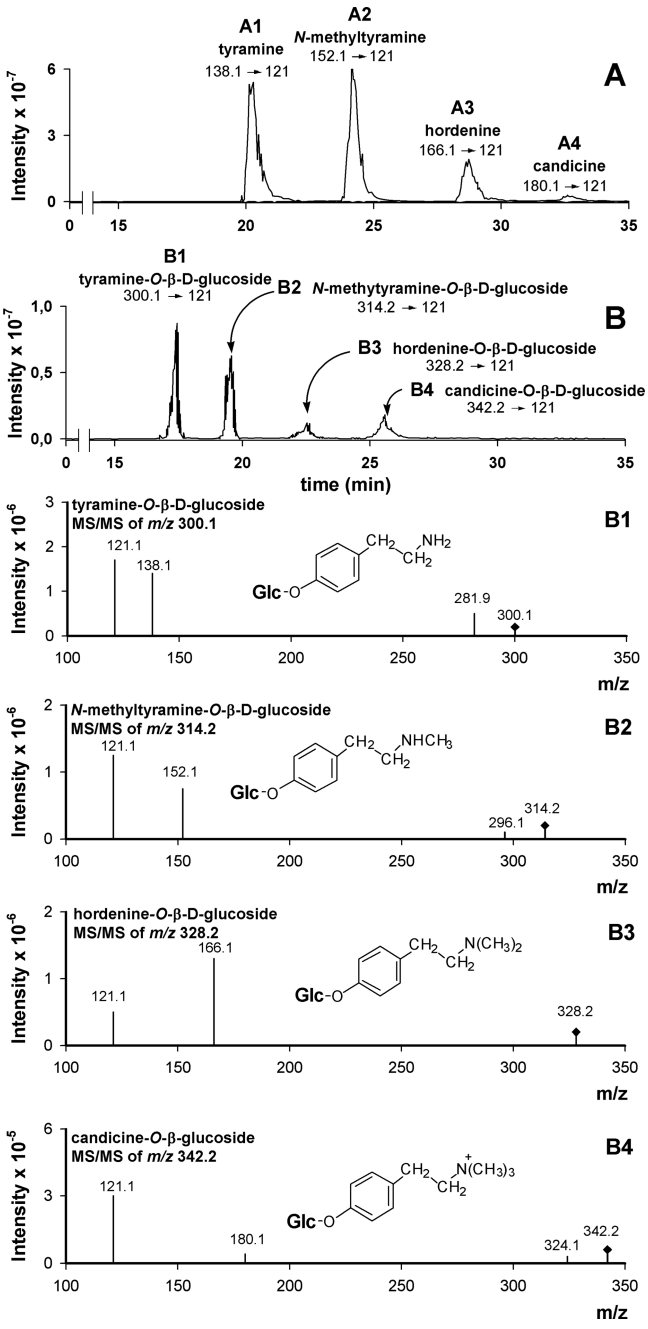

Figure 4. Representative HPLC-ESI-MS^2 chromatogram of a *Stapelia hirsuta* extract. Track A: The occurrence of tyramine and all its *N*-methylated derivatives (peaks A1−A4) was confirmed by comparison with authentic standards. Track B: MS^2 fragmentation patterns of the peaks B1 (t_R 17.2 min, *m/z* 300.1), B2 (t_R 19.5 min, *m/z* 314.2), B3 (at t_R 22.5 min, *m/z* 328.2), and B4 (at t_R 26 min, *m/z* 342.2). The chromatographic conditions are reported under Materials and Methods.

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MS^2 fragmentation patterns as peaks B1 and B2 of the bergamot leaf extract (Figure 3, track B) which, on the basis of their MS^2 fragmentation patterns, led us to suppose that they were tyramine-*β*-D-glucoside and *N*-methyltyramine-*β*-D-glucoside. Furthermore, peak B4 (Figure 4, panel B) showed a MS^2 fragmentation pattern which was consistent with its identity as *N,N,N*-trimethyltyramine-*O*-*β*-D-glucoside. Indeed, we did not observe a peak analogous to peak B4 in any citrus plant extract. In order to confirm the identities of these three substances in *Stapelia*, we performed their enzymatic synthesis by a transglycosylation reaction with cellobiose catalyzed by *β*-glucosidase from almonds. The HPLC-ESI-MS^2 time course of the transglycosylation reaction showed that all the expected glucosides of tyramine and its *N*-methylated derivatives were formed and showed the same chromatographic retention times and MS^2 fragmentation patterns as the corresponding peaks in the chromatograms of the extracts from citrus plants and *Stapelia* (Figure 5).

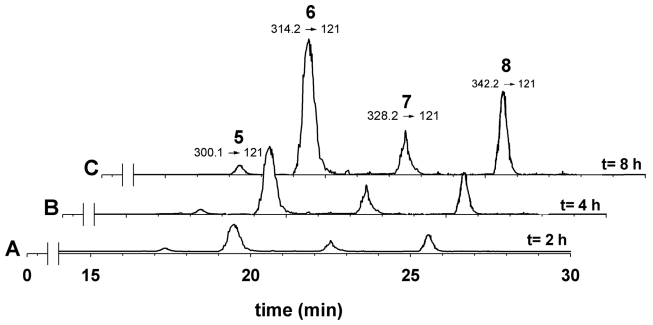

Figure 5. Time course of transglucosylation reaction catalyzed by almond *β*-glucosidase. Transglucosylation reaction mixture, besides cellobiose, tyramine, and enzyme, contained *N*-methyltyramine, *N,N*-dimethyltyramine, and *N,N,N*-trimethyltyramine previously produced by controlled methylation of tyramine with iodomethane (see Materials and Methods). The MS^2 extracted ion chromatograms in MRM mode, obtained by isolating the ion masses at *m/z* 300.1, 314.2, 328.2, and 342.2, show the production over time of glucosylated tyramine and its *N*-methylated derivatives, respectively. The *m/z* of the parent and extracted ions are the same as on the corresponding peaks in Figure 4, track B. (A) 2 h, (B) 4 h, and (C) 8 h of incubation. The four peaks showed the same chromatographic retention times and MS^2 fragmentation patterns as the corresponding peaks in the chromatograms of the extracts from citrus plants and *Stapelia hirsuta*.

Distribution of Tyramine Glucosylated Derivatives in Citrus Plants. The levels of the glucosylated tyramine and its *N*-methylated derivatives were determined in leaves and juices of sweet and bitter orange, bergamot, mandarin, lemon, citron, and pomelo (Figure 6, panels A–C). For each citrus species, the levels of the glucosylated tyramine and its *N*-methylated derivatives are normalized to 100% for the plant species that showed the highest level of the specific metabolite (Figure 6, panels A–C). It appears evident that leaves are the tissue part of plants where these substances accumulate mostly. In juices, the basal levels of these substances were very low or even zero, as in the case of lemon juice (Figure 6, right sides of panels A–C). The glucosylated tyramine derivatives reached the highest levels in sweet and bitter orange leaves, whereas such levels are mainly low in all other species (Figure 6, panels A–C). A noticeable diversity between citrus plants and *Stapelia* consists in the fact that, although *N,N,N*-trimethyltyramine (candicine) has been detected in leaves of citrus plants,[17] its glucosylated

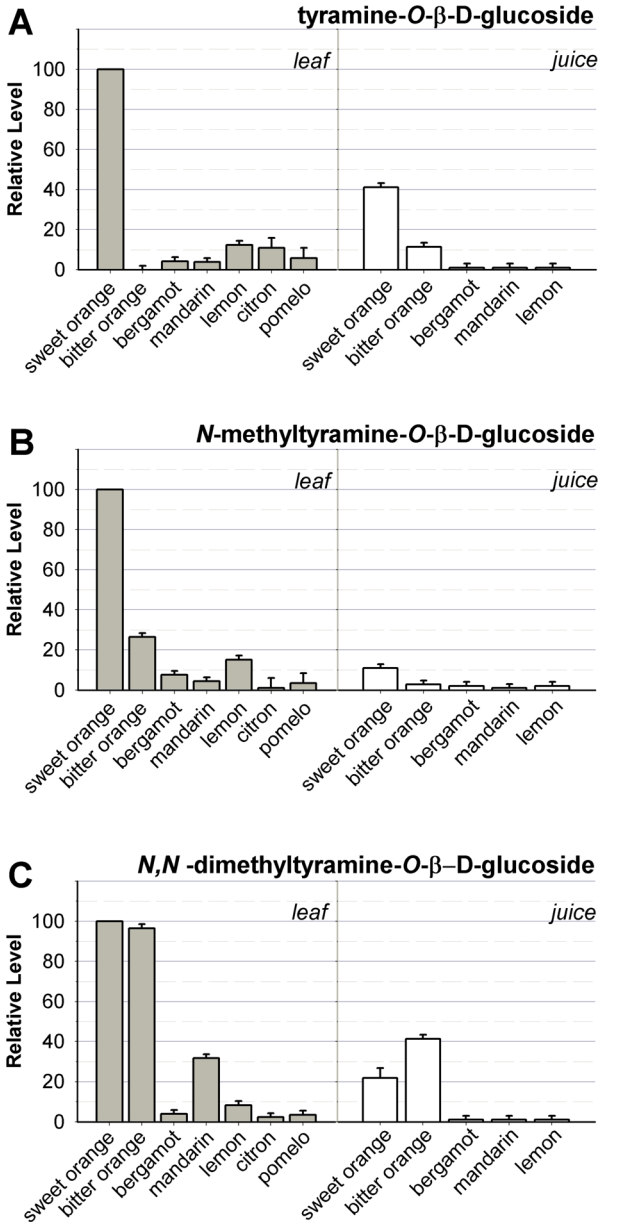

Figure 6. Relative levels of glucosylated tyramine and its *N*-methylated derivatives in various *Citrus* species. Graphs are normalized to 100% representing the level of the specific compound in the top productive species. Panel A: tyramine-*O*-*β*-D-glucoside. Panel B: *N*-methyltyramine-*O*-*β*-D-glucoside. Panel C: *N,N*-dimethyltyramine-*O*-*β*-D-glucoside. The left side of each panel shows relative levels of the specific compound in leaves, the right side, in juice. The level of each compound is the average of four determinations. Bars represent the standard deviations.

derivative was not observed in any of them, whereas, as seen above, in the *Stapelia* extracts it occurs together with all other glucosylated tyramine derivatives. This observation suggests the absence in citrus plant genus of the biosynthetic machinery for the two possible pathways that may produce this substance, that is, *N*-methylation of hordenine-1-*O*-*β*-D-glucoside or glucosylation of candicine (Figure 7).

Interrelationship of the Tyramine Derivatives in *Citrus* Genus. In this paper we show for the first time the presence of glucosylated forms of tyramine and its *N*-methylated derivatives in *Citrus* genus plants. Noticeably, these results highlight the

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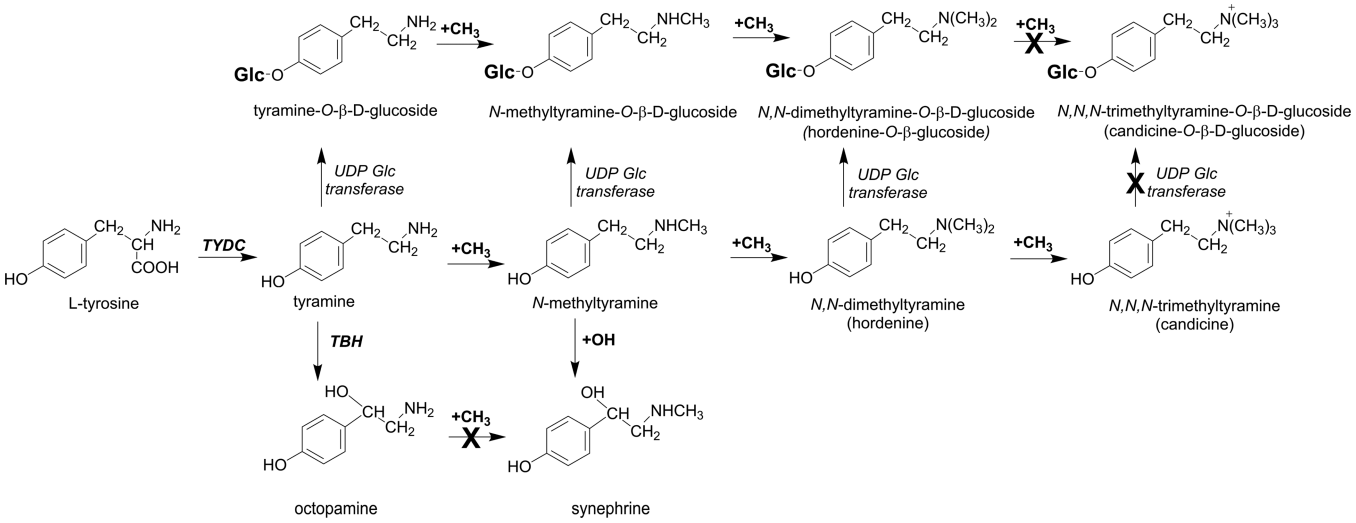

Figure 7. Scheme of the putative metabolic pathways for the biosynthesis of the tyramine derivatives in citrus plants. First step of the pathways is the formation of tyramine from tyrosine, catalyzed by the enzyme tyrosine decarboxylase (TYDC). Successively, *N*-methylation of tyramine is probably accomplished by the action of the same or different methyltransferase(s) acting in consecutive manner. Then, each methylated intermediate could be transformed in the corresponding glucoside by reactions likely catalyzed by UDP glucosyltransferase(s), which transfer the sugar residue from UDP-glucose, acting as a donor molecule. Candicine is reported to occur in citrus plants, but the corresponding glucoside was not detected. This is likely due to the absence of the biosynthetic machinery for the two possible pathways that may produce this substance, that is, *N*-methylation of hordenine-1-*O*-*β*-D-glucoside or glucosylation of candicine. Octopamine originates by hydroxylation of tyramine catalyzed by tyramine *β*-hydroxylase (TBH). It is worth noting that, in citrus plants, the adrenergic amine synephrine (*N*-methyloctopamine) is not produced by methylation of octopamine, as in animals, but by hydroxylation of *N*-methyltyramine.

parallelism between the metabolic pathways of tyramine and serotonin in this plant genus. Indeed, in our previous studies we showed the occurrence of serotonin (5-hydroxytryptamine) and all its *N*-methylated derivatives[2,3] and, successively, also that of their glucosylated forms.[22] Likewise, tyramine and its *N*-methylated derivatives (*N*-methyltyramine, *N*,*N*-dimethyltyramine, and *N*,*N*,*N*-trimethyltyramine) were found to occur in citrus plants[17] and, as here reported, also the glucosylated derivatives of tyramine, *N*-methyltyramine, and *N*,*N*-dimethyltyramine but not the glucosylated derivative of *N*,*N*,*N*-trimethyltyramine. As for the biosynthesis of those compounds, at present, there are no specific studies on the subject. However, we hypothesize that a putative metabolic pathway may comprise two steps of methylation and glycosylation of tyramine, involving a sequence of reactions starting with the formation of tyramine from tyrosine, catalyzed by the enzyme tyrosine decarboxylase (TYDC) (Figure 7). Afterward, tyramine might be *N*-methylated by the action of the same or different methyltransferase(s) in consecutive steps (Figure 7). The pathway could end by the transfer of the sugar residue to tyramine and its *N*-methylated derivatives. Alternatively, tyramine-*O*-*β*-D-glucoside could be *N*-methylated to produce in a consecutive manner the forms at higher methylation degree (Figure 7).

The distribution of *N*-methylated derivatives of tyramine[17] and its glycosylated forms resembles that of other *Citrus* metabolites involved in defense and resistance mechanisms,[27−30] such as limonoids and flavanones, which act as powerful natural pesticides[31] and antifungal agents.[27,30]

From a physiological point of view, the biosynthesis of the glucosylated metabolites could be likely linked to the role played in the plant defense by their aglycons, which affect processes essential for plant aggressors, such as fertility, locomotion, spawning, and hatching rate.[8] Some aglycons even act as antimicrobial metabolites which, as in the case of serotonin, accumulate in plants in response to the infection by fungal pathogen.[32] It is well-known that glycosylation, by making many compounds less reactive and more water-soluble, is a way to prevent cell damage and safely store toxic compounds employed by plants for defensive aims. We found a greater presence of the glucosylated tyramine derivatives in leaves than in fruits (Figure 6, panels A−C), which supports the proposed role of glycosides in plant defense.[33−36] In fact, leaves are the part of plants more susceptible to aggression by herbivores and pathogens and, for this reason, they require effective means of defense. As known, when leafy tissues are damaged by attackers, the glycosides, compartmentalized mostly in the vacuoles, undergo the action of specific glycosidases, which can occur either in the plant or also in the aggressors' tissues.[35,36] Consequently, the aglycons are released and can play their deterrence and toxicity roles.[35,36] In this respect, the coexistence in citrus plants of tyramine and serotonin derivatives suggests the common finality of all these secondary metabolites to the defensive response against biotic stress in this important plant genus.

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Notes

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