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ARTICLE

C22 Isomerization in α-Tomatine-to-Esculeoside A Conversion during Tomato Ripening Is Driven by C27 Hydroxylation of Triterpenoidal Skeleton

Takao Yamanaka,[†,‡] Jean-Paul Vincken,[†] Han Zuilhof,[§] Aagje Legger,[†] Norihisa Takada,[‡] and Harry Gruppen*,[†]

[†]Laboratory of Food Chemistry, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands, [‡]Nippon Del Monte Corporation, 3748 Shimizu-cho, Numata, Gunma 378-0016, Japan and [§]Laboratory of Organic Chemistry, Wageningen University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands

Compositional analysis by liquid chromatography/mass spectrometry of triterpenoid glycosides in different tomato cultivars, ripening stages, and parts of fruits showed that α-tomatine was generally most abundant in the flesh of the mature green stage, whereas esculeoside A was predominant in that of the red ripe stage. The sum of these glycoalkaloids was more or less constant, suggesting that α-tomatine is converted to esculeoside A during ripening. Besides various substitutions, the C22αN → C22βN isomerization is an important step in this transformation. By quantum chemical calculations it was shown that hydroxylation at C27 of the triterpenoidal skeleton is the driving force behind the isomerization. For the protonated form of the glycoalkaloid (predominant at the pH of tomato tissue), the C22βN configuration becomes more favorable than that of C22αN, through the extra energy provided by the hydrogen bond between the protonated nitrogen and the lone pair of the oxygen of the C27-OH.

KEYWORDS: Glycoalkaloid; tomato; chromatography; mass spectrometry; quantum chemical calculation

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is a well-known source of health-promoting substances such as vitamin C, vitamin E, flavonoids, and carotenoids (*1*). Many of these compounds have been investigated with respect to their accumulation in different tomato varieties, stages of maturity, and tissues. The carotenoid content has been shown to range from 5 to 140 mg/kg of fresh weight in various cultivars, with lycopene being the most abundant carotenoid (*2*, *3*). Lycopene has been shown to accumulate progressively during ripening (*3*–*5*). Furthermore, accumulation of metabolites seems to be tissue-dependent. In the domesticated tomato, *L. esculentum*, naringenin chalcone is exclusively and abundantly present in the peel of certain cultivars, whereas the flesh contains only minor amounts of quercetin-3-rutinoside (*6*).

Green tomatoes, in particular, also contain other abundant secondary metabolites, such as the steroidal glycoalkaloid α-tomatine. The structure of its aglycone, tomatidine, is shown in **Figure 1**. Regular varieties accumulate up to 800 mg/kg of fresh weight in green tomatoes, whereas mini-tomatoes can accumulate even 20-fold that (*7*). Also, it has been found that the α-tomatine content is strongly cultivar-dependent (*2*, *8*–*10*). α-Tomatine has been known as a natural compound, involved in disease resistance of tomato plants (*11*, *12*). Its health-beneficial effects have also been reported, for example, lowering plasma LDL, inhibitory effect on growth of cancer cells, and enhancement of the immune system (*11*, *12*).

In recent studies, several new glycoalkaloids were found, mainly in ripe and overripe tomato fruits (*3*, *13*–*18*); most of their structural diversity resides in the E- and F-rings. Furthermore, an abundant furostanol glycoside, tomatoside A, and its less abundant dehydro analogue were identified using mass and NMR spectroscopy (*19*). Some of these also possess potential health-beneficial properties (*15*, *20*), whereas others show antiphytoparasitic activity (*21*).

It is well-known that the α-tomatine content decreases during tomato fruit ripening (*9*, *22*–*24*), and it has been suggested that α-tomatine is the precursor of esculeoside A (*25*, *26*). At present, no quantitative compositional data for the major triterpenoid glycosides in unripe and ripe tomato fruits are available to support this speculation. Furthermore, the mechanism behind the isomerization of the F-ring, essential in the α-tomatine to esculeoside A transformation, has never been addressed. In the present study, the major triterpenoid glycosides are quantified in different tomato cultivars, ripening stages, and fruit parts. A possible mechanism for the conversion of α-tomatine into esculeoside A is proposed and substantiated by quantum chemical calculations.

MATERIALS AND METHODS

Materials. Tomato fruits of the cultivars 'Shanon' and 'TP-0136' were kindly provided by Nunhems Netherlands B.V.

*Corresponding author (telephone +31-317-482888; fax +31-317-484893; e-mail harry.gruppen@wur.nl).

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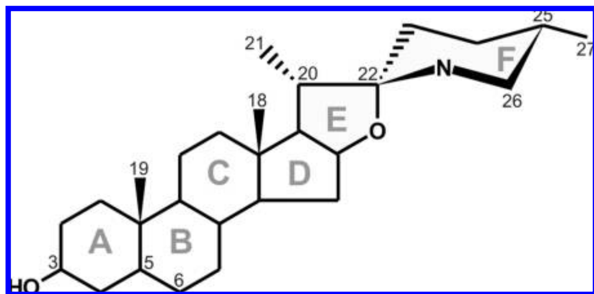

Figure 1. Structure of tomatidine. Annotation of the rings (A−F) and relevant carbon numbers are indicated. The so-called lycotetraose glycosyl chain, consisting of Glc→[Xyl→]Glc→Gal-, is attached to *O*-3. Compounds with a C5−C6 double bond (Δ5-unsaturation) are referred to as dehydro compounds.

(Nunhem, The Netherlands). Tomato fruits of the cultivars ‘Claree’ and ‘Elanto’ were kindly provided by Oudshoorn (Nootdorp, The Netherlands) and van Luijk (Nootdorp, The Netherlands), respectively. α-Tomatine was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Extraction of Triterpenoid Glycosides from Tomato Fruits. Tomato fruits were cut in halves and separated into a flesh and a jelly parenchyma (including the seeds) part. Each fraction was homogenized using a blender (Braun GmbH, Kronberg, Germany), prior to freeze-drying. Afterward, freeze-dried samples were ground into a fine powder with a particle size of ∼0.7 mm using a type A70 grinder (Framo, Eisenbach, Germany), cooled with liquid nitrogen. This powder was stored at −20 °C until use.

Freeze-dried tomato powder (5 g) was extracted (2 h, continuous stirring at 200 rpm, room temperature) with 400 mL of 70% (v/v) aqueous ethanol, containing 0.5% (v/v) acetic acid (*19*). After filtration through no. 589/2 filter paper (Schleicher & Schuell, Dassel, Germany), the residue was re-extracted with 100 mL of the same extractant, under similar conditions. After filtration over filter paper, the residue on the filter paper was rinsed with 10 mL of the same extractant. The combined filtrates were concentrated under reduced pressure with a rotary evaporator (below 40 °C), until all ethanol was removed. *n*-Hexane was added to the residual aqueous phase to a ratio of 1:1 to remove pigments from the extract. Subsequently, the aqueous phase was adjusted to pH 8.0 with ammonia to neutralize the cationic nitrogen atom of the glycoalkaloids. Low molecular weight polar compounds were removed from the extract using solid phase extraction with Sep-Pak tC18 vac 35 cm^3/10 g, cartridges (Waters, Etten-Leur, The Netherlands). The cartridge was preconditioned with 35 mL of methanol, followed by 35 mL of water. After sample loading, the cartridge was washed with 35 mL of water and eluted with 50 mL of methanol. The eluate was evaporated until dryness under reduced pressure. The extract obtained was suspended in 2 mL of 0.1% (v/v) aqueous acetic acid. Samples were centrifuged at 14000*g* for 5 min prior to RP-HPLC analysis.

Determination of Triterpenoid Glycosides by Reversed-Phase High-Performance Liquid Chromatography Mass Spectrometry (RP-HPLC-MS). RP-HPLC, in combination with evaporative light-scattering detection (ELSD), was used for analysis of tomato triterpenoid glycosides. Separation was performed on a 150 mm × 4.6 mm i.d., 3.5 μm, XTerra RP18 column (Waters, Milford, MA) with a 10 mm × 4.6 mm i.d., 3.5 μm, XTerra RP18 guard column run on a Spectra System HPLC (Thermo Separation Products, Fremont, CA). The solvents used were water/acetic acid (100:0.1, v/v) (A) and acetonitrile/acetic acid (100:0.1, v/v) (B). The following elution program was used: 0 → 5 min, 0% B (isocratic); 5 → 30 min, 0 → 40% B (linear gradient); 30 → 45 min, 40 → 100% B (linear); 45 → 50 min, 100% B (isocratic); 50 → 52 min, 100 → 0% B (linear); 52 → 60 min, 0% B (isocratic). Samples (20 μL) were injected. The flow was split into three directions: 250 μL/min to the Alltech ELSD 2000 detector (Deerfield, IL), 50 μL/min to the LCQ Deca XP max MS (Thermo Finnigan, San Jose, CA), and 700 μL/min to the waste. The nebulizer temperature of the ELSD was set at 115 °C with a gas flow of 3.2 L/min. The molecular mass of the triterpenoid glycosides was identified by mass spectrometry. MS analysis was performed using electrospray ionization and detection in the positive ion mode, with a spray voltage of 5.5 kV, a capillary voltage of 15 V, and a capillary temperature of 200 °C, according to the method of Cataldi et al. (*13*). Total ion current was used to record the abundances of the protonated triterpenoid glycosides and their fragments. A full-scan mass spectrum over a range of *m*/*z* 150−1500 was recorded. The control of the instrument and data processing were achieved with Xcalibur software (Thermo Finnigan, San Jose, CA). The triterpenoid glycosides were quantified using commercial α-tomatine as a reference compound. A calibration curve was made with α-tomatine (0.25−2.00 mg/mL; R^2 with ELSD was 0.997), from which the amounts of the various triterpenoid glycosides were calculated, assuming that their response factors were similar.

Quantum Chemical Calculations. The molecular geometries of several α-tomatine analogues and their hydrogen-bonded complexes have been fully optimized, using density functional theory (DFT) with the B3LYP functional, as implemented in GAUSSIAN 03 (*27*), and the 6−311G(d,p) basis set. Solvent effects were mimicked by placing the solute in a dielectric medium mimicking water, using the IEF-PCM model (*28*, *29*). Both geometry optimization and energy calculations were performed in this medium.

RESULTS AND DISCUSSION

Separation of Tomato Triterpenoid Glycosides. Prior to quantitative analysis of tomato triterpenoid glycosides, the extraction conditions were optimized with respect to completeness of recovery of native compounds (*19*). Subsequently, the extract was analyzed by RP-HPLC-ELSD-MS. Elution profiles of the various tomato extracts are shown in **Figures 2** and **3** for material derived from ‘TP-0136’. The other extracts showed similar peaks, but their intensities differed considerably from those of ‘TP-0136’. The various triterpenoid glycosides were annotated on the basis of their $[M + H]^+$ molecular ions and their fragments in the MS mode (**Table 1**). Dehydrotomatine coeluted with α-tomatine at a retention time of 23.4 min with *m*/*z* 1032.5 and 1034.5, respectively. No further attempts were done to separate these compounds, because they were clearly identified by their MS spectra. A compound with *m*/*z* 1050.5 eluted at a retention time 20.5 min. On the basis of its mass, this compound might represent lycoperoside H (*17*). However, it cannot be excluded that this peak corresponds to hydroxytomatine (one hydroxyl substituent in the F-ring). Hydroxytomatine has not been described in the literature, but it is likely to be an intermediate product in the biosynthesis of esculeoside A. The same can be stated for compound *m*/*z* 1108.7 at a retention time 21.6 min. On the basis of MS, this peak might correspond to esculeoside A without glucosylation at *O*-26, although this compound has not been described as such in the literature. Furthermore, a compound with *m*/*z* 1228.6 eluted at a retention time 18.0 min. This compound was tentatively annotated esculeoside B, based on MS.

A compound with *m*/*z* 1065.6 was predominantly found in the jelly parenchyma of tomatoes. This compound has been characterized as the furostanol glycoside tomatoside A (*19*) and does not belong to the class of glycoalkaloids. To investigate where this compound accumulates predominantly, freeze-dried seeds from ‘TP-0136’ were separated manually from freeze-dried jelly parenchyma (including the seeds).

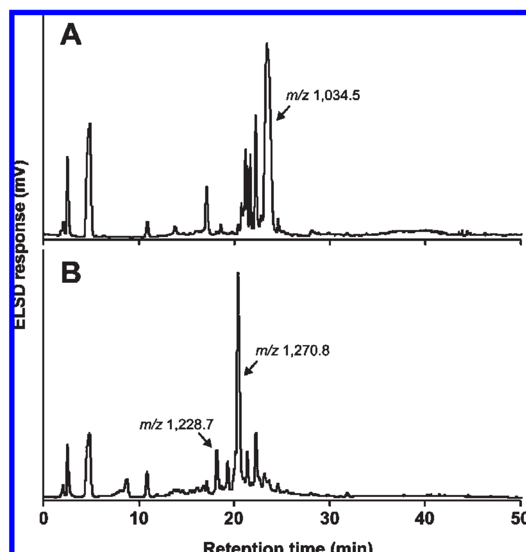

Figure 2. RP-HPLC elution profiles of tomato triterpenoid glycosides extracted from flesh part of (**A**) green mature and (**B**) red ripe tomatoes ('TP-0136').

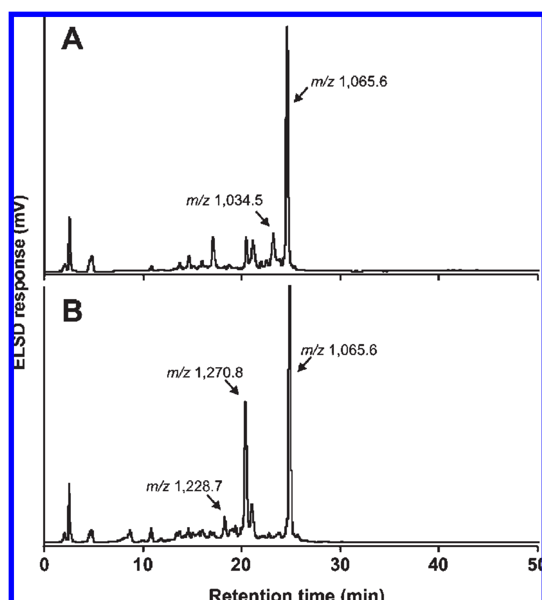

Figure 3. RP-HPLC elution profiles of tomato triterpenoid glycosides extracted from jelly parenchyma (including the seeds) of (**A**) green mature and (**B**) red ripe tomato ('TP-0136').

Table 1. Molecular Masses and Fragmentation Patterns of Known Tomato Triterpenoid Glycosides

annotation	m/z [M + H]$^+$	fragments (MS mode)	refs
α-tomatine	1034.5	902.5, 740.5, 578.5, 416.4	*11*
dehydrotomatine	1032.5	900.5, 738.5, 576.5, 414.4	*7*
esculeoside A	1270.8	1138.7, 1108.7, 814.7, 652.4	*15*
esculeoside B	1228.6	934.9, 772.6, 610.6	*15,17*
lycoperoside F	1270.8	1138.7, 1108.7, 814.7, 652.4	*17*
lycoperoside G	1270.8	1138.7, 1108.7, 814.7, 652.4	*17*
lycoperoside H	1050.5	918.5, 756.5, 594.5	*17*
dehydrotomatoside	1063.6	901.5, 739.3, 577.4, 415.2	*19*
tomatoside A	1065.6	903.7, 741.7, 579.4, 417,3	*19*

Both fractions (seeds and jelly) were ground and extracted, and the extracts were analyzed by LC-MS. In accordance with Moco and co-workers (*3*), it was confirmed that tomatoside A was predominant in seeds.

Triterpenoid Glycoside Composition in Tomato Fruits. The compounds eluting in the various peaks of the RP-HPLC profiles were quantified, using α-tomatine as a standard, with the assumption that all triterpenoid glycosides have response factors similar to that of α-tomatine. The results are summarized in **Tables 2** and **3**, for four different cultivars, for both flesh and the jelly parenchyma, with two maturity stages each.

Triterpenoid Glycosides in Tomato Flesh. The total amount of triterpenoid glycosides in the flesh remained more or less constant during maturation, except for the cultivar 'Claree'. However, for all four cultivars, the triterpenoid glycoside composition was markedly different at the two stages of maturation. The most striking difference was the decrease in α-tomatine (the predominant triterpenoid glycoside in the green mature stage), and the increase in esculeoside A (the major triterpenoid glycoside in the red ripe stage). The opposite trends in α-tomatine and esculeoside A contents, together with the rather constant total amount of triterpenoid glycosides, point at the possibility that α-tomatine might be converted to esculeoside A during the ripening, as has been hypothesized by Nohara et al. (*25*) and Iijima et al. (*26*). Furthermore, the esculeoside B content increased concomitantly with that of esculeoside A. This is not unexpected, as it has been suggested by Fujiwara et al. (*15*) that esculeoside A might be a precursor of esculeoside B. In accordance with the literature (*8*−*10*, *30*), we have observed that cherry-type tomatoes ('Claree') are richer in α-tomatine than the noncherry types. Also, 'TP-0136' (medium-size cultivar) showed a relatively higher α-tomatine content.

Triterpenoid Glycosides in Jelly Parenchyma of Tomato. The jelly parenchyma (including the seeds) always showed higher levels of triterpenoid glycosides than the flesh. The same trends were visible with respect to α-tomatine, esculeoside A, and esculeoside B. Besides, the jelly parenchyma contained the predominant tomatoside A. Typically, the total amount of triterpenoid glycosides seemed to fluctuate more for the jelly parenchyma than for the tomato flesh. During ripening, the triterpenoid glycoside content remained rather constant for 'TP-0136' and 'Elanto', whereas it increased for 'Claree' and decreased for 'Shanon'. To a large extent, this is due to changes in the tomatoside A content (and esculeoside A for 'Claree').

From α-Tomatine to Esculeoside A: C22αN → C22βN Isomerization. Our results on the quantification of glycoalkaloids in the green mature and red ripe stage for four tomato cultivars suggest that the total amount of glycoalkaloids remains more or less constant during ripening. This further supports the hypothesis that α-tomatine, the major glycoalkaloid in the green mature stage, might be converted during ripening to esculeoside A, the major glycoalkaloid in the red ripe stage, as previously hypothesized by Nohara and coworkers (*25*). For such a transformation to occur, isomerization of the F-ring is required, but the driving force behind this reaction is unclear. Energy calculations on intermediates of the α-tomatine to esculeoside A pathway might provide clues on this.

Figure 4 summarizes possible reactions on the F-ring of α-tomatine. It has been reported that the pK_a value of solasodine, the C-22 isomer of dehydrotomatidine, is 7.7 (*31*). Assuming a similar pK_a value for α-tomatine, and given the pH of tomato fruit of approximately 4.5, α-tomatine will reside mainly in the protonated form in tomato fruit. Under these conditions, ring opening can occur, and the resonance-stabilized carbocation can react back to α-tomatine or,

Table 2. Triterpenoid Glycoside Composition of the Flesh Part of Tomato Fruits from Four Tomato Cultivars at Different Ripening Stages[a]

cultivar	ripening stage[b]	α-tomatine (1034.5)	esculeoside B (1228.6)	esculeoside A (1270.8)	1050.5	1108.7	tomatoside A (1065.6)	total
'Shanon'	gm	800.7 (±9.3)	386.0 (±8.7)	469.1 (±4.7)	nd[c]	629.3 (±9.4)	nd	2285.1 (±32.1)
	rr	nd	469.2 (±10.9)	1783.8 (±8.4)	211.9 (±2.5)	nd	nd	2464.9 (±21.8)
'TP-0136'	gm	1314.2 (±9.8)	nd	192.1 (±1.3)	210.6 (±10.7)	282.6 (±11.9)	205.3 (±2.7)	2204.8 (±36.4)
	rr	nd	406.7 (±13.8)	1709.2 (±10.8)	nd	nd	221.3 (±11.2)	2337.6 (±35.8)
'Claree'	gm	1613.0 (±9.8)	nd	136.4 (±0.5)	154.0 (±13.5)	139.8 (±4.7)	nd	2043.2 (±28.5)
	rr	nd	181.8 (±2.6)	794.3 (±13.7)	132.0 (±5.8)	nd	137.5 (±4.7)	1245.6 (±26.8)
'Elanto'	gm	868.0 (±14.3)	nd	nd	131.9 (±2.9)	nd	nd	999.9 (±17.2)
	rr	nd	162.5 (±3.3)	794.7 (±5.3)	nd	122.0 (±0.6)	nd	1079.2 (±9.2)

[a] n=3, mg/kg of DW. Standard deviation is indicated in parentheses. [b] gm, green mature; rr, red ripe. [c] nd, not detected.

Table 3. Triterpenoid Glycoside Composition of the Jelly Parenchyma (Including the Seeds) of Tomato Fruits from Four Tomato Cultivars at Different Ripening Stages[a]

cultivar	ripening stage[b]	α-tomatine (1034.5)	esculeoside B (1228.6)	esculeoside A (1270.8)	1050.5	1108.7	tomatoside A (1065.6)	total
'Shanon'	gm	690.5 (±10.8)	231.2 (±6.1)	1531.9 (±10.8)	nd[c]	322.3 (±6.8)	1118.7 (±14.2)	3894.6 (±48.7)
	rr	nd	365.1 (±19.2)	1995.3 (±9.5)	nd	nd	248.3 (±8.3)	2608.7 (±37.0)
'TP-0136'	gm	1184.4 (±20.0)	nd	578.5 (±14.3)	728.1 (±14.4)	287.3 (±17.9)	3304.3 (±9.3)	6082.6 (±75.9)
	rr	nd	452.8 (±6.2)	1972.3 (±6.6)	669.5 (±16.1)	nd	3327.3 (±12.8)	6421.9 (±41.7)
'Claree'	gm	2693.4 (±4.5)	nd	188.9 (±2.3)	669.3 (±15.3)	147.0 (±4.9)	1198.5 (±4.2)	4897.1 (±31.2)
	rr	630.7 (±18.2)	278.4 (±14.8)	3501.1 (±12.9)	nd	191.5 (±17.0)	2255.7 (±16.1)	6857.4 (±79.0)
'Elanto'	gm	1345.1 (±2.5)	nd	228.9 (±2.5)	274.8 (±7.2)	130.6 (±1.6)	1717.1 (±2.2)	3696.5 (±16.0)
	rr	178.1 (±10.2)	216.9 (±8.5)	1501.9 (±12.6)	nd	nd	1766.4 (±8.5)	3663.3 (±39.8)

[a] n = 3, mg/kg of DW. Standard deviation is indicated in parentheses. [b] gm, green mature; rr, red ripe. [c] nd, not detected.

further, to its isomer, filotomatine. Given the low abundance of filotomatine in tomato fruits (*13*), the reaction to α-tomatine seems to be preferred. Our energy calculations showed that α-tomatine was 3.7 kcal/mol more stable than filotomatine. This indicated that the axial position of the C27 methyl group is energetically unfavorable.

If α-tomatine is the precursor of esculeoside A, then the biosynthesis of the latter is a multistep conversion, involving 23- and 27-hydroxylation. It is still unclear which of these hydroxylation steps occurs first. In **Figure 4**, we assumed that 27-hydroxylation precedes that of C23. To our knowledge, singly hydroxylated precursors of esculeoside A have not been described in the literature. Possibly, one of these precursors is represented by the compound with m/z 1050.5 eluting at a retention time of 20.5 min, but given the small quantity of this compound this was not further established. Alternatively, the compound with m/z 1050.5 might correspond to lycoperoside H. Thus, it is difficult to deduce the driving force for isomerization from the C22 configuration of intermediate products of esculeoside A.

After isomerization, the 27-OH might be in close proximity of the nitrogen atom. Our working hypothesis was that hydrogen bonding between the lone pair of the nitrogen and the 27-OH (N···H−O-27) might compensate for the unfavorable axial position of C27. This was verified by energy calculations, which showed that 27-OH-filotomatine was only 0.2 kcal/mol less stable than 27-OH-tomatine. When the experimental error of the energy calculations is taken into account, the energy of the two molecules can be regarded as similar. Thus, the formation of the hydrogen bond in 27-OH-filotomatine (**Figure 5A**) compensated the 3.5 kcal/mol penalty of filotomatine to reside in the C22αN configuration with the axially oriented methyl group. However, it is insufficient to explain why esculeoside A has been found only in the C22βN configuration. The energy gain corresponds well to a hydrogen bond of intermediate strength, which might be expected from its geometric parameters such as binding distance and angle (*32*). Due to the pH of tomato fruit and the expected pK_a value of tomatine-like compounds, it is expected that the major proportion of the glycoalkaloids is present in the protonated state. Therefore, the energy of protonated 27-OH-filotomatine (**Figure 5B**) was also calculated and compared with that of protonated 27-OH-tomatine. It appeared that the former was in fact 2.7 kcal/mol more stable than the latter. This indicates that C27 hydroxylation is the driving force behind the C22αN → C22βN isomerization, as the β-isomer was found to be energetically more favorable than the α-isomer after hydroxylation, at least in the protonated state, a condition likely to be predominant in tomato fruit.

The major change in glycoalkaloid composition during ripening of tomato was the increase in esculeoside A at the expense of α-tomatine, whereas the total amount of glycoalkaloids remained more or less constant. This observation provides additional evidence for the idea that α-tomatine is a precursor of esculeoside A. The conversion of α-tomatine to esculeoside A requires a C22αN → C22βN isomerization. By quantum chemical calculations, it is shown that hydroxylation at C27 of the triterpenoidal skeleton enables the formation of an intramolecular hydrogen bond, which increases the likelihood of F-ring rearrangement to the β-isomer. When protonation of the F-ring nitrogen (the predominant form at tomato pH) is accounted for, the β-isomer becomes the energetically preferred configuration with the hydrogen bond between the protonated nitrogen and the lone pair of the oxygen of the C27-OH.

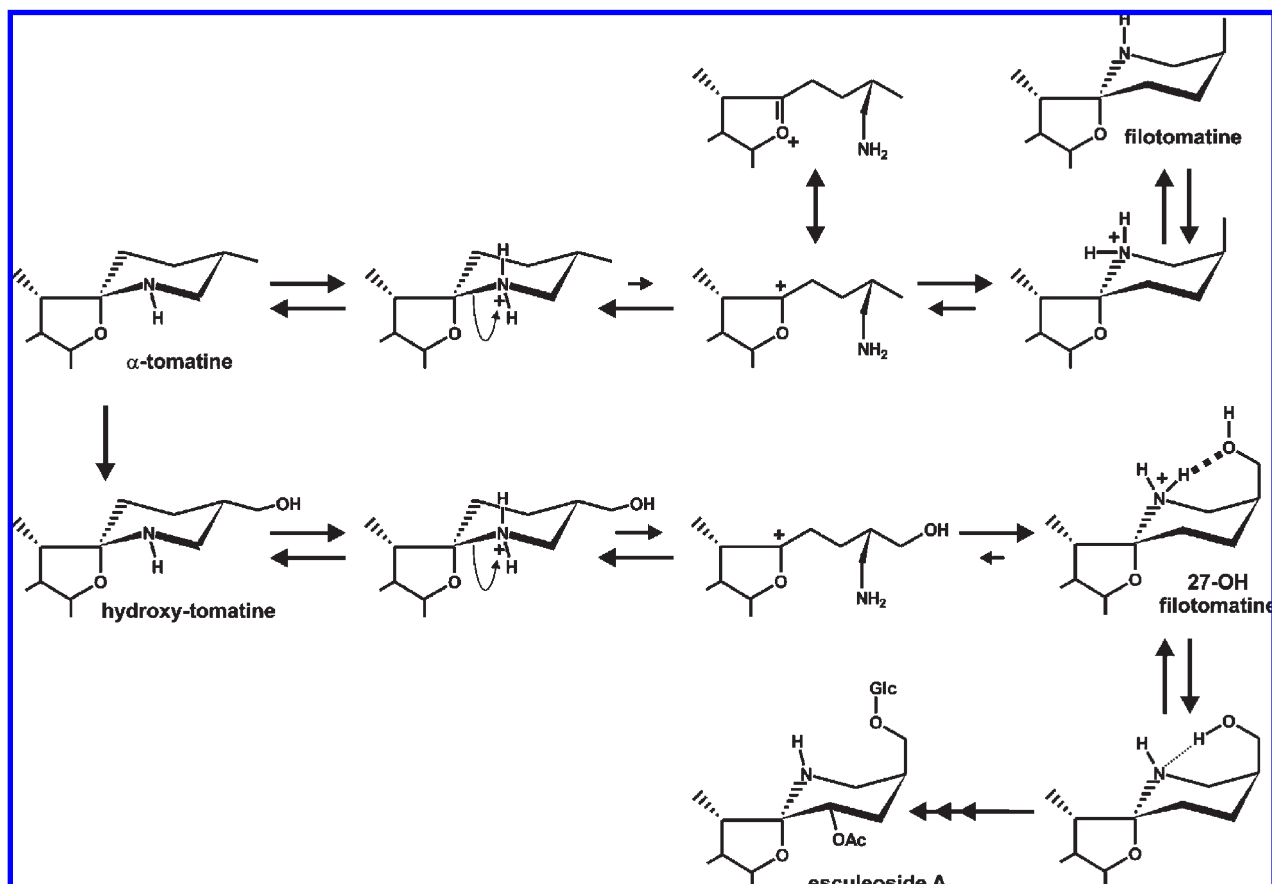

Figure 4. Scheme for the hypothetical conversion of α-tomatine to esculeoside A during tomato ripening.

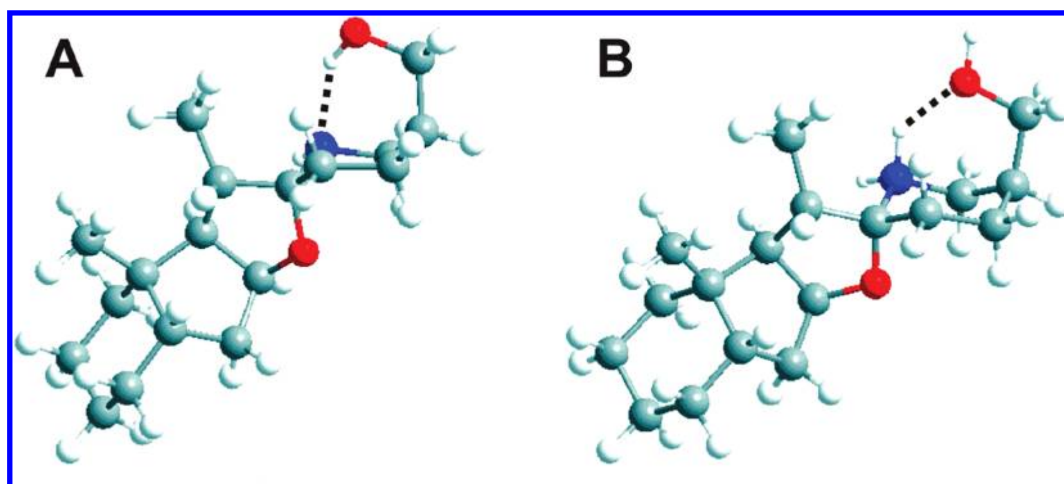

Figure 5. Model of truncated 27-OH-filotomatine, with C- and D-rings of the steroidal skeleton included to properly mimic the steric interactions and rigidity of the furan ring. All structures are optimized with density functional theory (B3LYP/6−311G(d,p)), in combination with a self-consistent reaction field approximation of water. Gray, red, blue, and white atoms indicate carbon, oxygen, nitrogen, and hydrogen, respectively.

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