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Biosynthesis of steroidal alkaloids in Solanaceae plants: Involvement of an aldehyde intermediate during C-26 amination

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ABSTRACT

The C-26 amino group of steroidal alkaloids, such as tomatine, is introduced during an early step of their biosynthesis from cholesterol. In the present study, the mechanism of C-26 amination was reinvestigated by administering stable isotope labeled compounds, such as (26,26,26,27,27,27-$^{2}H_6$)cholesterol during biosynthesis of tomatine, solanine and solasonine. The chemical compositions of tomatine and solanine so obtained were analyzed by LC–MS after administering the d_6-cholesterol to a tomato seedling and a potato shoot, respectively. The resulting spectra indicated that two deuterium atoms were eliminated from C-26 of cholesterol during biosynthesis. Furthermore, administration of (6-$^{13}C^{2}H_3$)mevalonate in combination with lovastatin to an eggplant seedling, followed by GC–MS analysis of solasodine after TMS derivatization established that two deuterium atoms were eliminated from C-26 of cholesterol during solasonine biosynthesis. These findings are in contrast to an earlier observation that one hydrogen atom was lost from C-26 during tomatidine biosynthesis, and suggest that C-26 nitrogen atom addition involves an aldehyde intermediate. Thus, it is proposed that the C-26 amination reaction that occurs during steroidal alkaloid biosynthesis proceeds by way of a transamination mechanism.

1. Introduction

Steroidal alkaloids are widely distributed in Solanaceae plants (Harrison, 1990; Helmut, 1998; Petersen et al., 1993). These nitrogen-containing secondary metabolites are categorized into two groups according to the structure of the sidechain: one group has an oxo-aza spiro structure as exemplified by tomatine (**3**) from *Solanum lycopersicum* (tomato) and solasonine from *Solanum melongena* (eggplant), and the other group has a cyclic amine structure, as found in solanine (**5**) and chaconine (**6**) from *Solanum tuberosum* (potato) (see Fig. 1). These steroidal alkaloids are all biosynthesized from cholesterol (**1**) via C-26 oxidation–amination, C-22 oxidation, C-16 oxidation and glycosylation of the C-3 hydroxy group (Friedman, 2002; Ginzberg et al., 2009; Petersen et al., 1993). It is known that the pro-*R* methyl group on C-25 of cholesterol (**1**) (derived from C-2 of mevalonate, and defined as C-26 (Nes, 2011)) is utilized during the biosynthesis of tomatine (**3**) and (**5**) solanine, whereas the pro-*S* methyl group (derived from C-6 of mevalonate, and defined as C-27) is utilized during solasonine (**8**) biosynthesis (Rocchetti and Russo, 1974) (see Fig. 2). In this text, the methyl group that undergoes the amination reaction is described as simply C-26. The biosynthetic pathway shown in Fig. 1 was proposed by linking the naturally occurring compounds that have been characterized from the Liliaceous plant, *Veratrum grandiflorum* (Kaneko et al., 1976). The same pathway can be applied to the steroidal alkaloids of Solanaceae plants based on precursor administration studies that used several radio-isotope labeled compounds, such as 26-aminocholesterol (Tschesche and Brennecke, 1980), 22,26-epiminocholest-5-en-3β-ol and 22,26-epiminocholest-5-ene-3β,16β-diol (Tschesche and Spindler, 1978) respectively. However, the biosynthetic pathway outlined in Fig. 1 requires further refinement using experimental evidence before a detailed and conclusive route can be established for the Solanaceae plants.

Tschesche and coworkers reported that amination at C-26 occurs upon displacement by a "nitrogen" nucleophile (Tschesche et al., 1976). This conclusion was based on their finding that only one hydrogen atom was eliminated from C-26 of cholesterol (**1**) during the biosynthesis of tomatidine (**2**) (aglycone of tomatine (**3**)) when $^{3}H/^{14}C$-cholesterol was applied to the leaves of *S. lycopersicum*. This is the only report describing a C-26 amination mechanism. Furthermore, the H-25 of cholesterol (**1**) was reportedly retained during tomatidine (**2**) biosynthesis in the same species (Tschesche et al., 1976). Arginine was suggested to be a major source for the C-26 nitrogen atom in the biosynthesis of solanidine (**4**) (aglycone of solanine (**5**)) in *V. grandiflorum* (Kaneko et al.,

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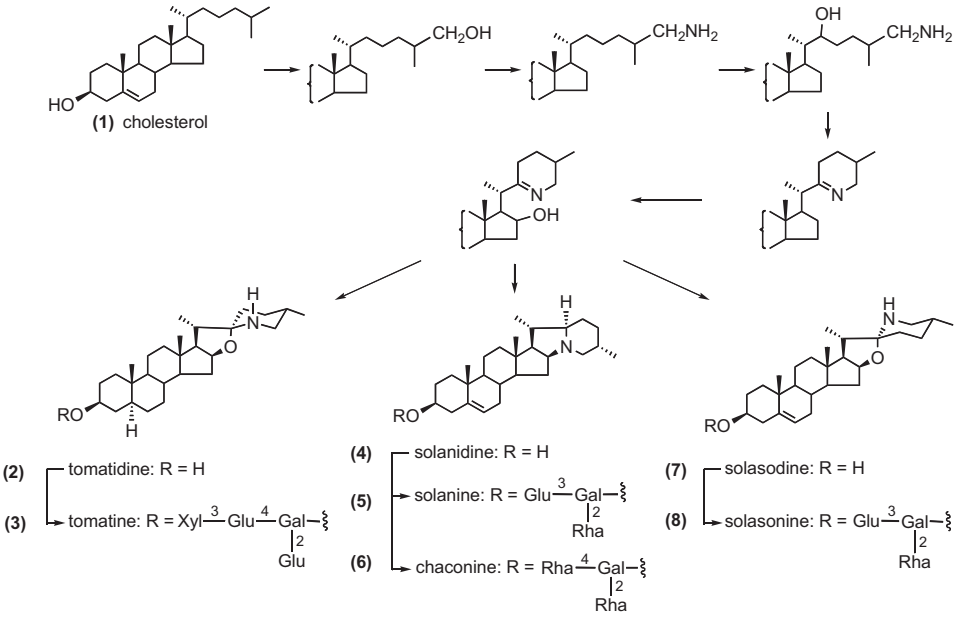

Fig. 1. Biosynthesis of steroidal alkaloids (**2**–**8**) from cholesterol (**1**) in Solanaceae plants.

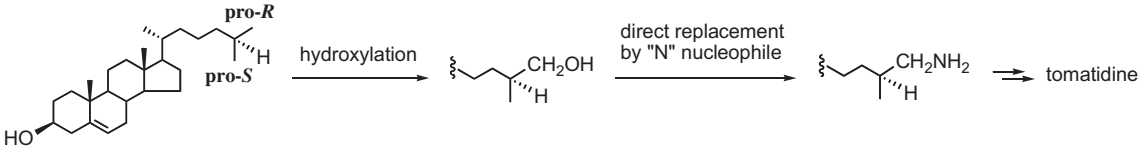

Fig. 2. A mechanism for the C-26 amination step in the biosynthesis of tomatidine (**2**) proposed by Tschesche et al.

1976). The exact sequence of the functionalizations at C-26, C-22 and C-16 remains to be established, although the C-26 functionalization likely occurs first. The mechanism of C-16 functionalization is another intriguing step. It was reported that the 16β-H of cholesterol (**1**) was retained during the biosynthesis of tomatidine (**2**) in *Lycopersicon pimpinellifolium*. By contrast, the 16β-H of cholesterol (**1**) was lost during solanidine (**4**) biosynthesis in *S. tuberosum* (Canonica et al., 1977). A precursor administration study using the appropriate 16α- and 16β-hydroxy compounds is thus required to explain these anomalous results. Furthermore, the timing of aglycone glycosylation has not been fully elucidated. Currently, no biosynthetic gene has been identified as responsible for forming the aglycones of these steroidal alkaloids, although several genes encoding glycosyl transferases, which link the sugar units to solanine (**5**) and tomatine (**3**), have been characterized from *S. lycopersicum*, *S. tuberosum* and *S. aculeatissimum* (Itkin et al., 2011; Kohara et al., 2007; McCue et al., 2006; Osmani et al., 2009).

A research project was thus initiated to revisit steroidal alkaloid biosynthesis, with in study a focus on the mechanism of C-26 amination. It was reasoned that a more popular transamination mechanism involving an aldehyde intermediate was responsible for the C-26 amination, rather than the mechanism proposed by (Tschesche et al., 1976). Our hypothesis could be examined by determining the metabolic fate of the hydrogen atoms at C-26 of cholesterol (**1**). Sterol substrates labeled with deuterium would be more convenient for such tracer experiments than radioisotope-labeled substrates. A literature survey gave no precedent examples of the use of deuterium labeled substrates in steroidal alkaloid biosynthesis. Accordingly, whether Solanaceae plants could uptake and metabolize ^{2}H-labeled cholesterol (**1**) into steroidal alkaloids to such a degree that the fate of the deuterium atom could be followed was first investigated. Promising results were obtained from a preliminary study examining the uptake of commercial (25,26, 26,26,27,27,27-$^{2}H_7$)cholesterol (**1**) when applied to a tomato seedling and a potato shoot. Encouraged by these results, a precursor administration study was carried out to examine the validity of our transamination hypothesis. In this paper, the results of thus study on the mechanism of the C-26 amination in biosynthesis of steroidal alkaloids in the following three Solanaceae plants, tomato, potato and eggplant, are described.

2. Results and discussion

The tomato precursor administration conditions to tomato were optimized using (25,26,26,26,27,27,27-$^{2}H_7$) cholesterol (**1**). Tomato (*S. lycopersicum* cultivar Micro-Tom) seedlings were germinated aseptically on Murashige-Skoog (MS) solid plates, transferred to MS liquid medium (one seedling per 100 mL flask containing 20 mL of medium), and cultivated. On day seven between 0.1 and 2.0 mg of (25,26,26,26,27,27,27-$^{2}H_7$)cholesterol (**1**) was dissolved in acetone (50 μL)–Tween 80 (50 μL) and added to the medium (20 mL). The seedling was grown for two weeks, harvested, lyophilized, ground and extracted with $CHCl_3$–MeOH (1:1). The extract was filtered through an ODS Sep-Pak cartridge using MeOH to removed non-polar substances, and the filtrate was analyzed by LC–MS (ESI, positive ion mode). Endogenous non-labeled tomatine (**3**) was detected by tracing the *m*/*z* 1034 ion $[M+H]^+$, whereas labeled tomatine (**3**) was detected by tracing the *m*/*z* 1039 ion (Fig. 3). It should be noted that labeled tomatine (**3**) had a slightly shorter retention time than the non-labeled tomatine due to the isotope effect of deuterium atoms (Baweja, 1987; Benchekroun et al., 1997). A comparison of the integrated peak areas in the

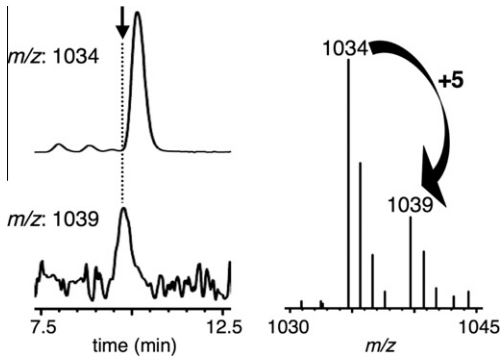

Fig. 3. GC–MS analysis of tomatine (**2**) obtained upon administering (25,26,26,26,27,27,27-$^{2}H_{7}$)cholesterol (**1**) to a tomato seedling. The left two traces are mass-chromatograms for the indicated *m*/*z* ions. The right mass spectrum was obtained at the t_R indicated by the arrow.

two chromatograms established that the ratio of the labeled to non-labeled tomatine (**3**) was approximately 1:50 when 2.0 mg of labeled cholesterol (**1**) was administered. The level of cholesterol (**1**) incorporation into tomatine (**3**), based on the administer substrate, was estimated at 1.0%. A dose of 1–2 mg of the substrate to a seedling growing in 20 mL of the liquid medium furnished satisfactory results. The route of entry of the substrate is most likely from roots. This preliminary study clearly indicated that ^{2}H labeled cholesterol (**1**) could be utilized in the biosynthesis of steroidal alkaloids. This finding allowed us to bypass the tedious work required to validate the use of radioisotope labeled compounds. It is worth noting that labeling the substrate with several deuterium atoms facilitates mass spectroscopic analysis because such a highly labeled metabolite elutes faster than the corresponding non-labeled counterpart in LC and GC chromatograms.

The precursor administration study indicated that five deuterium atoms out of seven were retained in tomatine (**3**) and two were eliminated. Either both deuterium atoms were eliminated from C-26, or they were eliminated separately from C-25 and C-26. The former scenario is more likely because H-25 of cholesterol was reportedly retained during the tomatidine (**2**) biosynthesis in *S. lycopersicum*. The latter possibility was eliminated, based on the results of a precursor administration study using more appropriately labeled cholesterol (**1**). In a similar study, (25,26,26,26,27,27,27-$^{2}H_{7}$)cholesterol (**1**) was administered to a potato (*S. tuberosum*) shoot using the same method described for the tomato seedling. Satisfactory results were obtained from the LC–MS analysis of solanine (**5**) and chaconine (**6**) from the potato shoot, although cholesterol (**1**) incorporation was slightly worse than that of the tomato seedling. The biosynthesized solanine (**5**) and chaconine (**6**) retained five deuterium atoms in agreement with the case of tomatine (**3**) (data not shown).

(26,26,26,27,27,27-$^{2}H_{6}$)Cholesterol (**1**) was selected as the substrate for further tracer experiments, because this compound enabled straightforward monitoring of the C-26 hydrogens atoms. This labeled cholesterol (**1**) was conveniently synthesized, according to the scheme shown in Fig. 4. The tosylate (Westover and Covey, 2006) starting material was prepared from commercially available 3β-hydroxychol-5-en-24-oic acid (Martin et al., 2008). The labeled cholesterol (**1**) was administered to a tomato seedling, and the extract was analyzed by LC–MS in the same manner as described above. As expected, the labeled tomatine (**3**) could be detected by tracing the *m*/*z* 1038 ion (Fig. 5). The mass spectrum clearly indicated that two deuterium atoms were lost during tomatine (**3**) biosynthesis. It is concluded from this result that the two hydrogen atoms are eliminated from C-26 of cholesterol (**1**) during the biosynthesis of tomatine (**3**) in tomato. This finding supports our hypothesis, which is inconsistent with the earlier observation made by Tschesche et al. (1976).

The fates of the C-26 hydrogens during biosynthesis of solanine (**5**) and chaconine (**6**) using a potato shoot was next investigated. As described above, d_6-cholesterol (**1**) was fed to the shoot of a potato, and extracts were analyzed by LC–MS of solanine (**5**) and chaconine (**6**). Labeled and non-labeled (MW 867) solanines (**5**) were detected by monitoring the *m*/*z* 872 and 868 ions $[M+H]^+$, respectively (Fig. 6). The mass spectrum clearly indicated that two deuterium atoms were eliminated during the conversion. Similarly, labeled and non-labeled (MW 851) chaconine (**6**) were detected by monitoring the *m*/*z* 856 and 852 ions $[M+H]^+$, respectively (Fig. 6). This mass spectrum also clearly indicated that two deuterium atoms were eliminated during the conversion. Thus, it was concluded that two hydrogen atoms from C-26 of cholesterol (**1**) were eliminated during biosynthesis of the two steroidal alkaloids having the cyclic amine structure.

To examine the fates of the C-26 hydrogens during solasonine (**8**) biosynthesis in eggplant (*S. melongena*), d_6-cholesterol (**1**) was administer to an eggplant seedling, and the resulting solasonine (**8**) was analyzed by LC–MS in the same manner as described for tomato. Labeled solasonine **(8)** could not be detected by monitoring the *m*/*z* 888 ion $[M+H]^+$, although non-labeled solasonine **(8)** was detected by monitoring the *m*/*z* 884 ion $[M+H]^+$. It appears that eggplants consume exogenous cholesterol (**1**) much less efficiently than tomato and potato. This problem was overcome by administering a labeled form of mevalonate, together with a 3-hydroxy-3-methylglutaryl-(HMG-)CoA reductase inhibitor (Kasahara et al., 2002; Ohyama et al., 2009). This strategy thus used mevalonate as substrate, because the C-6 of mevalonate becomes the pro-*S*-methyl group on C-25 of cholesterol (**1**), eventually leading to the functionalization of solasonine (**8**) at C-26. (6-$^{13}C^{2}H_{3}$)Mevalonate was chosen as substrate, because it was previously used to study plant sterol biosynthesis (Ohyama et al., 2009). Labeled mevalonate (8 mg) in combination with lovastatin (90 mg), an HMG-CoA reductase inhibitor, was administered to an eggplant seedling (20 mL of liquid medium), which was grown for two weeks before extraction. The extract was treated in acidic conditions to cleave the glycosidic bond, yielding solasodine (**7**). The material was trimethylsilylated (the structure of the derivative is a C-22–N double bond form of 22,26-epiiminocholest-5-ene-3β,16-diol di-TMS ether (Laurila et al., 1999), MW 557) and analyzed by GC–MS (EI). The labeled solasodine (**8**) was detected by tracing the *m*/*z* 570 ion, and non-labeled solasodine (**8**) was observed by monitoring the *m*/*z* 557 ion $[M]^+$ (Fig. 7). The increase of 13 mass units was consistent with the loss of two deuterium atoms from C-26 of cholesterol. The increase in mass units would have occurred at C-19 (+3 mass units, note that cycloartenol is an intermediate), C-18 (+4 mass units), C-21 (+4 mass units) and C-26 (+2 mass units).

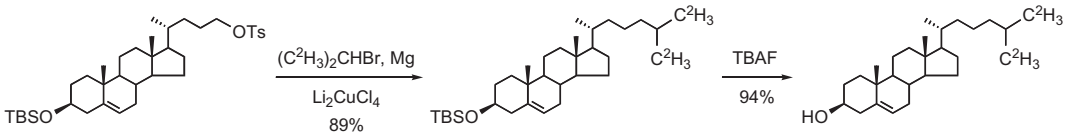

Fig. 4. Synthesis of (26,26,26,27,27,27-$^{2}H_{6}$)cholesterol (**1**).

This conclusion was supported by analyzing the base ion peak at *m*/*z* 125 ($C_8H_{15}N$ corresponds to the C-20 through C-27 moiety + H (Laurila et al., 1999)) for the unlabeled solasodine (**7**) derivative. The corresponding fragment ion for the labeled derivative was observed at *m*/*z* 131, which corresponds to an increase in 6 mass units (Fig. 7). The 6 mass unit increase was ascribed to a 4 mass unit increase at C-21 and a 2 mass unit increase at C-26. It is therefore concluded that two hydrogen atoms were eliminated from the C-26 methyl group of cholesterol (**1**) during the biosynthesis of solasonine (**8**) in eggplant.

In the present study clear evidence is provided that two hydrogen atoms were eliminated from C-26 of cholesterol (**1**) during the biosynthesis of tomatine (**3**) in tomato, solanine (**5**) and chaconine (**6**) in potato, and solasonine (**8**) in eggplant. Our findings clearly ruled out the mechanism proposed by Tschesche et al.

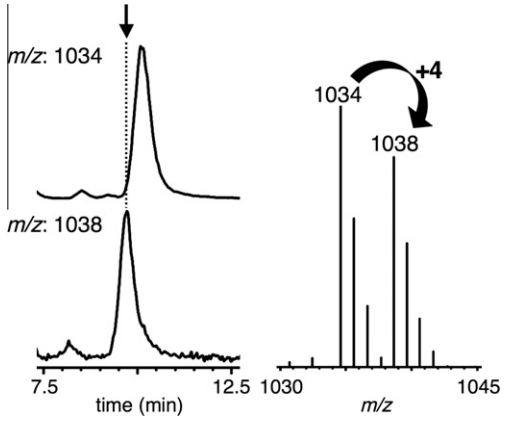

Fig. 5. LC–MS analysis of tomatine (**3**) obtained upon administering (26,26,26,27,27,27-$^2H_7$)cholesterol (**1**) to a tomato seedling. Refer to the Fig. 3 legend.

3. Concluding remarks

It is evident that two hydrogen atoms are eliminated from the pro-*R* methyl group of cholesterol (**1**) during biosynthesis of tomatine (**3**) in tomato, and of solanine (**5**) and chaconine (**6**) in potato, and two hydrogen atoms were eliminated from the pro-*S* methyl group during the biosynthesis of solasonine (**8**) in eggplant. These conclusions are based on this precursor administration study using deuterium labeled substrates. The C-26 amino group in the steroidal alkaloids that exhibit aza-oxo spiro or cyclic amine structures in Solanaceae plants should be introduced in such a mechanism that satisfies the loss of two hydrogen atoms from C-26 of cholesterol (**1**). The most likely mechanism would be a transamination mechanism using pyridoxal as a cofactor (Fig. 8). Accordingly, an intermediate having a 26-aldehyde structure would be involved in this transamination step. Such an aldehyde has never been proposed nor characterized. A precursor administration study using a deuterium labeled aldehyde, such as 3β-hydroxycholest-5-en-26-al, is still necessary. The current study though was unable to identify why the feeding experiments by Tschesche et al. using $^3H/^{14}C$-cholesterol (**1**) on the leaves of *S. lycopersicum* led to their results.

A parallel feeding study using labeled substrate on a red pepper (*Capsicum annuum*) seedling was also carried out to elucidate the fate of the C-26 hydrogens during biosynthesis of tigogenin (aglycone of capsicosides) (Yahara et al., 1994). This study established that only one hydrogen atom was lost from C-26 during the biosynthesis of this steroidal sapogenin (unpublished result). This result is reasonable, because C-26 of the steroidal saponin is at an

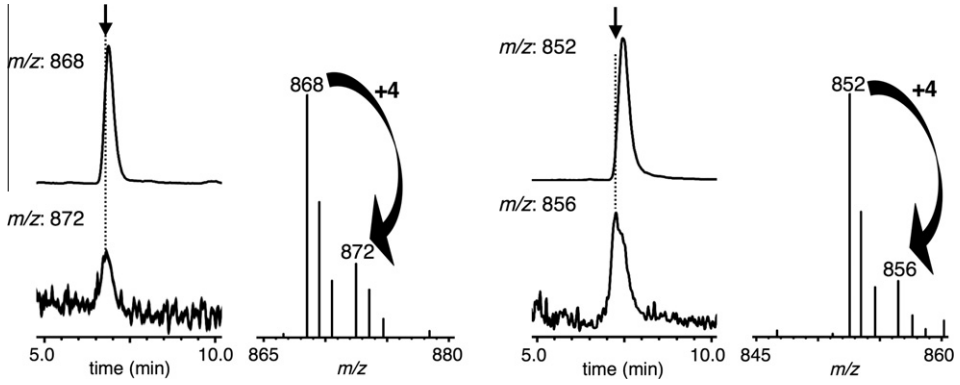

Fig. 6. LC–MS analysis of solanine (**5**) (left) and chaconine (**6**) (right) obtained upon administering (26,26,26,27,27,27-$^2H_7$) cholesterol (**1**) to a potato shoot. Refer to the Fig. 3 legend.

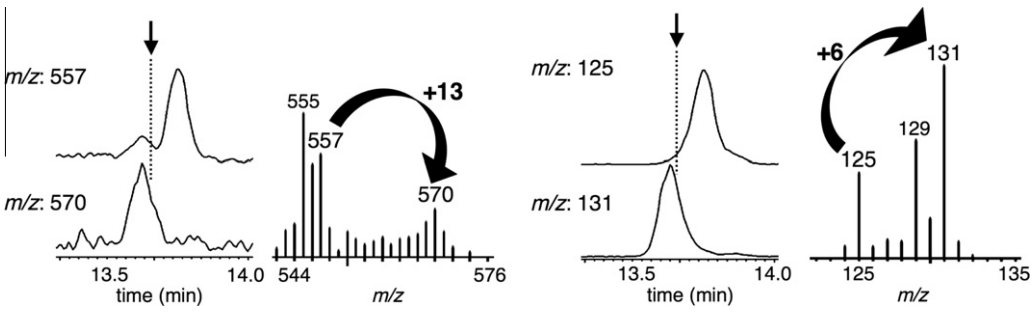

Fig. 7. GC–MS analysis of trimethylsilylated solasodine (**8**) derived from solasonine obtained upon administering (6-$^{13}C^2H_3$)mevalonate to an eggplant seedling. Refer to the Fig. 3 legend.

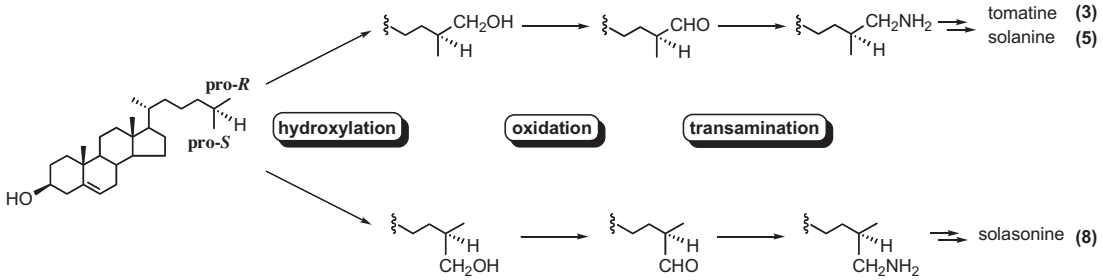

Fig. 8. The proposed mechanism for the C-26 amination reaction in steroidal alkaloid biosynthesis in Solanaceae plants.

alcohol oxidation stage, thus requiring the elimination of one hydrogen atom from C-26 of cholesterol.

The following possibility is speculated despite the current lack of experimental evidence: in a steroidal saponin-producing plant, an enzyme that oxidizes C-26 may only be capable of C-26 hydroxylation and cannot catalyze any further oxidation. In contrast, in steroidal alkaloid-producing plants, an oxidation system composed of either a single enzyme or two distinct enzymes may have evolved to convert the C-26 hydroxy moiety to an aldehyde. P450 enzyme(s) may be responsible for the initial hydroxylation at C-26 of cholesterol (**1**) and the subsequent oxidation to the putative aldehyde intermediate in steroidal alkaloid-producing plants. As described in the introduction, no information regarding enzymology and the genes encoding these enzymes is currently available. The accumulation of such information is essential to elucidating a more detailed biosynthetic mechanism for these steroidal alkaloids. Efforts to identify such P450 genes are currently in progress in our laboratory.

4. Experimental

4.1. General

^{1}H and ^{13}C NMR spectra were recorded on a JEOL AL 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometer in $CDCl_3$ solution. The tetramethylsilane (δ0.00) signal was used as an internal standard for the ^{1}H shifts, and the $CDCl_3$ (δ77.00) signal was used as a reference for ^{13}C shifts. EI-MS (70 eV) spectra were obtained on a JEOL JMS-700 spectrometer. Silica gel 60 N (spherical neutral, 40–100 μm, Kanto Chemical, Japan) was used for column chromatography (CC). LC–MS was performed with a Shimadzu LCMS-2020 apparatus operating in ESI mode attached with a Tosoh TSK gel ODS-80TsQA RP column (15 cm × 2 mm i.d.). GC–MS was conducted using a mass spectrometer (JMS-AM SUN200, JEOL) connected to a gas chromatograph (6890A, Agilent Technologies) with a DB-1 (30 m × 0.25 mm, 0.25 μm film thickness; J&W Scientific) capillary column. Authentic samples of tomatine (**3**), chaconine (**5**), solanine (**6**), and solasodine (**8**) were purchased from Tokyo Kasei, Funakoshi, and Extrasynthase, respectively. The 3β-hydroxychol-5-en-24-oic acid, (25,26,26,26,27,27,27-$^{2}H_7$)cholesterol (**1**), and (1,1,1,3,3,3-$^{2}H_6$)-2-bromopropane were purchased from Tokyo Chemical Industry Co., Ltd., Cambridge Isotope Laboratories, Inc., and Sigma–Aldrich, respectively.

4.2. Plant growth conditions and feeding experiments

Seeds of *S. lycopersicum* cultivar Micro-Tom were sterilized with 0.25% NaOCl for 30 min and germinated on Gelrite (0.8%, Gelrite Gellan Gum, Sigma–Aldrich) plates of 1 × MS medium (Gibco) containing 3% sucrose. A seedling was aseptically transferred to a 100 mL Erlenmeyer flask containing 1 × MS liquid medium (20 mL) supplemented with 3% sucrose and grown on a rotary shaker (60 rpm) at 25 °C in a photoperiodic cycle of 16 h light (50 $mmolm^{-2}\ s^{-1}$)/8 h dark for 7 days. A solution of d_7-cholesterol (**1**) (2.0 mg), dissolved in acetone (50 μL)–Tween 80 (50 μL), was added to the liquid medium and the seedling was grown another two weeks before being harvested. d_6-Cholesterol (**1**) (1.0 mg) was added as a solution in acetone (25 μL)–Tween 80 (25 μL) in the same manner as described above.

Shoots of *S. tuberosum* cultivar Sassy were maintained on the same Gelrite plates as described for *S. lycopersicum*. A shoot of an approximate length of 3–4 cm was aseptically transferred to a 100 mL Erlenmeyer flask and grown as described for *S. lycopersicum*. After 7 days, d_6-cholesterol (**1**) (1.0 mg) was added to the liquid medium and the shoot was grown for two weeks as described above.

Seeds of *S. melongena* cultivar Housui were sterilized and germinated as described for *S. lycopersicum*. A seedling was aseptically transferred to a 100 mL Erlenmeyer flask containing 1 × MS liquid medium (20 mL) supplemented with 3% sucrose, (6-$^{13}C^{2}H_3$)mevalonate (8.0 mg) in H_2O (2.0 mL) and lovastatin (90 μg) in DMSO (10 μL). After two weeks, the seedling was harvested.

4.3. Extraction and analysis of LC–MS and GC–MS

The harvested seedling of *S. lycopersicum* and shoot of *S. tuberosum* were individually lyophilized. A portion of each dried plant tissue (10 mg each) was ground in a motor and a pestle and extracted with $CHCl_3$–MeOH (1:1, 2 mL × 3). After adding Celite® (500 mg) to the extract, the solvent was removed under reduced pressure. The adsorbed material was placed onto an ODS cartridge (Sep-Pak® Vac C18, 500 mg, Waters) and eluted with MeOH (8 mL). The filtrate was concentrated by flushing with N_2, and the residue was suspended in MeOH (400 μL). An aliquot was analyzed by LC–MS using 1:2 mixture of CH_3CN–H_2O containing 5 mM $AcONH_4$ as eluent at a flow rate of 0.2 mL/min.

The harvested eggplant seedling was processed to produce the extract as described above. The extract was treated with 4 M HCl (1.0 mL)–MeOH (1.0 mL) for 1 h at 80 °C. The hydrolyzed reaction mixture was extracted with hexane–EtOAc (1:1, 2 mL × 3), and the solvent was removed under reduced pressure. The residue was trimethylsilylated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide at 80 °C for 30 min, and an aliquot was used for GC–MS analysis. The GC–MS conditions were the same as those described previously (Seki et al., 2008).

4.4. Synthesis of (26,26,26,27,27,27-$^{2}H_6$)cholesterol

The tosylate was prepared from commercially available 3β-hydroxychol-5-en-24-oic acid according to a reported method (bis-TBS (t-butyldimethylsilyl) formation was followed by reduction with $LiAlH_4$ and treatment with tosyl chloride in pyridine) (Martin et al., 2008; Westover and Covey, 2006). Grignard reagent was prepared by adding (1,1,1,3,3,3-$^{2}H_6$)-2-bromopropane

(450 μL, 4.80 mmol, 99.7 atom% ^2H) drop wise to a suspension of Mg (120 mg, 4.94 mmol) in dry THF (4.5 mL) under N_2. The mixture was stirred after completion of the addition for 15 min at room temperature and then cooled to 0 °C. An aliquot (0.64 mL, 64 mmol) of 0.1 M Li_2CuCl_4 solution (prepared from LiCl (8.5 mg) and $CuCl_2$ (13.5 mg) in THF (1 mL)) was added dropwise to the mixture, and the mixture was stirred for a while. A solution of the tosylate (262 mg, 0.42 mmol) in dry THF (4.6 mL) was added to the mixture. The reaction mixture was stirred without cooling for 20 min and diluted with Et_2O and sat. aq. NH_4Cl. The mixture was stirred for 10 min and extracted with Et_2O. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to give a crude product. This product was purified over silica gel eluted with hexane–EtOAc (20:1) to afford the TBS ether (189 mg, 89%) as a white solid. mp. 150–152 °C; ^1H NMR ($CDCl_3$) δ: 5.31 (m, H-5), 3.48 (m, H-3), 1.00 (s, H_3-19), 0.91 (d, J = 6.0 Hz, H_3-21), 0.89 (s, ^{t}Bu), 0.67 (s, H_3-18), 0.05 (s, Me_2Si); ^{13}C NMR ($CDCl_3$) δ: 141.5, 121.2, 72.6, 56.8, 56.2, 50.2, 42.9, 42.3 39.8, 39.4, 37.4, 36.6, 36.2, 35.8, 32.0, 31.9, 28.3 27.5, 26.1 (x3), 25.9, 24.3, 23.9, 21.1, 19.4, 18.7, 18.2, 11.9, −4.6.

To a solution of the TBS ether (178 mg, 0.35 mmol) in THF (1.8 mL) was added 1.0 M TBAF (tetra-n-butylammonium fluoride) in THF (1.8 mL, 1.8 mmol) and the mixture was allowed to stir at room temperature overnight. The mixture was extracted with Et_2O and the organic layer was washed with aq. 2 M HCl, sat. aq. $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated under reduced pressure to give a crude product. The latter was applied to silica gel eluting with hexane–EtOAc (4:1) to afford the labeled cholesterol **2** (130 mg, 94%) as a white solid. Recrystallization from MeOH yielded white crystals (120 mg), mp. 147–149 °C; EI-MS m/z 392 $[M]^+$; The MS spectrum showed that the relative peak intensity for the m/z 392 ion and the 391 ion was at a ratio of 50:1. ^1H NMR ($CDCl_3$) δ: 5.35 (m, H-5), 3.53 (m, H-3), 1.00 (s, H_3-19), 0.91 (d, J = 6.4 Hz, H_3-21), 0.68 (s, H_3-18); ^{13}C NMR ($CDCl_3$) δ: 140.8, 121.7, 71.8, 56.8, 56.2, 50.1, 42.3 (x2), 39.8, 39.4, 37.3, 36.5, 36.2, 35.8, 32.0 (x2), 31.7, 28.2, 27.5, 24.3, 23.8, 21.1, 19.4, 18.7, 11.9.

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