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Production of Triterpenes from the Callus Tissues of Actinidiaceous Plants

Hiroshi TAKAZAWA[1], Kouichi YOSHIMURA[1], Akira IKUTA[2] * and Kiichiro KAWAGUCHI[3]

[1] *Department of Biological Science and Technology,*
[2] *Research Institutes for Science and Technology, Science University of Tokyo, 2669 Yamazaki, Noda, Chiba 278-8510, Japan,*
[3] *Medicinal Plant Garden, School of Pharmaceutical Science, KitasatoUniversity, Sagamihara, Kanagawa 228-8510, Japan*
**Corresponding author* E-mail address: ikutaaki@rs.noda.tus.ac.jp

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Abstract

Callus tissue from the stems of *Actinidia arguta* (Actinidiaceae) produced the following eight ursane-type triterpenes: α-amyrin; uvaol; ursolaldehyde; ursolic acid; corosolic acid; asiatic acid; $2\alpha, 3\beta, 24$-trihydroxyurs-12-en-28-oic acid (4-epiasiatic acid); and $2\alpha, 3\alpha, 24$-trihydroxyurs-12-en-28-oic acid. The tissue also produced three oleanane-type triterpenes (β-amyrin, oleanolic acid, and maslinic acid), and two phytosterols mixtures (sitosterol and stigmasterol). Seven of the eight ursane-type triterpenes (the exception being asiatic acid), the three oleanane-type triterpenes, and the two phytosterols mixtures were also isolated from *A. chinensis* and *A. polygama* callus tissues. Variations in the production ratios of two of the triterpenes (ursolic acid and oleanolic acid) and the two phytosterols mixtures (sitosterol and stigmasterol) were compared among callus tissues from the three plant species.

Keywords: *Actinidia arguta*, *A. chinensis*, *A. polygama*, Actinidiaceae, callus tissues, chemotaxonomy, triterpenes.

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; KIN, kinetin; MS medium, Murashige and Skoog medium.

Introduction

Actinidia plants are widely distributed in Japan and China. The fruit of *A. polygama* contains volatile components, including nepetalactone and a monoterpene, iridomyr mecin, which causes a well-known specific activity to Felidae (Sakan *et al.*, 1967). Recently, triterpenes were reported in the fruit galls and callus tissues derived from the galls of *A. polygama* (Sashida *et al.*, 1992, 1994). Dihydronepetalactone was produced from the callus tissues as a major constituent of the monoterpene compound, whereas the biologically active monoterpene wasn't produced (Shoyama *et al.*, 1998). During our chemotaxonomic studies comparing the constituents obtained from the callus tissues, we previously reported the presence of triterpenes in the callus tissues of three lardizabalaceous plants (Ikuta, 1995), and three paeoniaceous plant species (Ikuta, 1996). As a part of our continuing chemotaxonomic studies into the chemical constituents of callus tissues, here we report on the callus tissues derived from the stems of three actinidiaceous plant species: *A. arguta*, *A. chinensis*, and *A. polygama*, Several ursane-type triterpenes corresponding to biosynthetic intermediates to ursolic acid were stepwise isolated from the callus tissues of these three plant species. We also succeeded in simutaneous isolation of oleanane-type triterpenes corresponding to the ursane types.

Materials and Methods

Plant Material

The stems of *A. arguta* (Sieb. et Zucc.) Planch. ex Miq., *A. chinensis* Planch. and *A. polygama* (Sieb. et Zucc.) Planch.ex Maxim. (Actinidiaceae) were collected in April 1992 the Medicinal Plant Garden of Kitasato University. Voucher specimens (numbers Acti-91-01~03) were deposited at the herbarium of our institute. Plant materials were identified by Dr. T. Nakamura, Faculty of Pharmaceutical

Sciences, Science University of Tokyo.

Callus Cultures

Callus from the stems of *A. arguta, A. chinensis,* and *A. polygama* were established in April and May, 1992, on glycine-free Murashige and Skoog medium (M&S) (Murashige and Skoog, 1962) containing 2,4-D (1 mg l^{-1} or 3 mg l^{-1}) and kinetin (KIN) (0.1 mg l^{-1}) as plant growth regulators. The callus tissues were subcultured every 5 or 6 weeks onto fresh M&S medium containing 2,4-D (1 mg l^{-1}) and KIN (0.1 mg l^{-1}) at 25 ± 1 ℃ in the dark.

Isolation and Identification of Triterpenes and Phytosterols

The *A. arguta, A. chinensis,* and *A. polygama* callus tissues were subcultured at 5 week intervals over 6 or 7 months on agar M&S medium containing 2,4-D (1 mg l^{-1}) with KIN (0.1 mg l^{-1}) as plant growth regulators. Each callus tissue was harvested at 5 week intervals and preserved in a glass bottle together with adequate MeOH for the investigation of the constituents: *A. arguta* (fresh weight 2 kg, dry weight 36 g), *A. chinensis* (fresh weight 5.4 kg, dry weight 100 g), and *A. polygama* (fresh weight 1.8 kg, dry weight 26 g). The stored total amounts of *A. arguta* callus tissues (fresh weight 2.0 kg, dry weight 36 g) were extracted with cold and hot MeOH. The extracts were concentrated under reduced pressure and the residue was partitioned 4 times repeatedly between H_2O (0.2 l) and $CHCl_3$ (2 l) to obtain the organic soluble fraction. The $CHCl_3$ solutes were evaporated to dryness and the extracts were chromatographed on a column of silica gel by gradient elution using $CHCl_3$ with increasing proportions of MeOH (~100%). The crude triterpene mixtures so obtained were purified repeatedly by column chromatography on a silica gel column eluted with C_6H_6:EtOAc:CH_3CN at 10:1:0.5, C_6H_6:$CHCl_3$ at 5:1, $CHCl_3$:Acetone at 1:1, and $CHCl_3$:MeOH at 1:1, and further were purified repeatedly by column chromatography on silica gel with Hexane:AcOEt:CH_3CN to give the following triterpenoids: **1+9** (2.5 mg), **2** (3.5 mg), **3** (3.5 mg), **4+10** (42 mg), **5+11** (6.5 mg), **6** (4.0 mg), **7** (3.5 mg) and **8** (4.0 mg), and two phytosterol mixtures (sitosterol and stigmasterol). The pure compounds **1, 4, 5,** and **9-11** were obtained by using the preparative HPLC (C18) with an eluent system (80% MeOH (a) and 95% CH_3CN (b)) from the mixtures of each compound **1, 4,** and **5**: (a) tR (min)=(**2**): 8.22, (**4**): 7.71, (**5**): 4.01, (**10**): 7.34, and (**11**): 3.71; (b) t R (min)=(**1**): 10.97 and (**9**): 9.86. Furthermore, the stored callus tissues of *A. chinensis* and *A. polygama* were also extracted and the triterpenes and phytosterols were separated in the same manner as for *A. arguta* callus tissues. These compounds (**1-11**) were identified by their NMR (^1H and ^{13}C) spectra and mass spectra by comparison with published spectral data of each compound, and by HPLC (C 18). HPLC was carried out on a Model 576 (Gasukuro Kogyo, Tokyo); HPLC column type Super-ODS C18, 2 μm, size 4.6 mmϕ x100 mm (Tosho, Tokyo); flow rate 1.0 ml min^{-1}; and eluent MeOH:H_2O =80:20 and 95% CH_3CN. The contents of the triterpene mixtures (ursolic acid and oleanolic acid) and phytosterols (sitosterol and stigmasterol) were determined by a dual-wavelength flying-spot scanner (CS-9300, Shimazu, Tokyo) on a TLC plate. The chromatogram was developed in the solvent system (C_6H_6:EtOAc:CH_3CN at 7:3:0.5) and the TLC plate was heated for 10 min after spring with 10% H_2SO_4. The spots corresponding to the triterpene mixtures and phytosterols were detected at 210 nm (UV).

Isolation of the Mixtures of Ursolic Acid and Oleanolic Acid from the Original Plants.

The stems (610 g of *A. arguta*, 70 g of *A. chinensis*, and 540 g of *A. polygama*) were collected in May 1992, and the samples were repeatedly extracted with cold and hot MeOH (0.5-1 l) separately. The total extract was concentrated at reduced pressure and the $CHCl_3$ solution obtained by partition with H_2O was purified repeatedly by the column chromatography of a silica gel ($CHCl_3$:MeOH at 100~0%, C_6H_6:EtOAc:CH_3CN at 6:4:0.5), and Sephadex LH-20 ($CHCl_3$:MeOH at 1:1) to afford the triterpene mixtures from the individual plants. The triterpenes were identified as mixtures of ursolic acid (**4**) and oleanolic acid (**10**) by the comparison with the authentic samples on HPLC.

Quantitation of Ursolic Acid and Phytosterols

Five standard ursolic acid and phytosterol solutions of different concentrations [mg $(20\ ml)^{-1}$] were prepared and analyzed to establish the calibration curve. The supplied amount of ursolic acid and phytosterols (X) was found to be the proportional to its peak-area ratio (Y). The regression equations were Y=3720.69156 x X-8304.45473 (r=0.999) for ursolic acid, and Y=3643.297529 x X +13853.88496 (r=0.999) for the phytosterols. For the determination of both compounds, the sample of 20 μl was placed onto the TLC. The contents of both mixtures and phytosterols in each sample were calculated from the area under the peak and the regression equation.

Results and Discussion

Constituents of the Callus Tissues

The stored callus tissues of *A. arguta*, *A. chinensis*, and *A. polygama* were successively extracted with cold MeOH and EtOAc. Both extracts were combined and concentrated under reduced pressure, and the residue was partitioned between $CHCl_3$ and H_2O to obtain the organic soluble fraction. The $CHCl_3$ solution from the *A. arguta* callus tissues was investigated first, and in succession the callus tissues of the other two plant species (*A. chinensis* and *A. polygama*) were investigated in the same manner. The extracts from *A. arguta* were chromatographed on a silica gel column using the $CHCl_3$-MeOH and hexane-AcOEt-CH_3CN solvent systems to give eight ursane-type triterpenes **1**-**8**. Nevertheless, compounds **1**, **4**, and **5** showed significant minor peaks in the vicinity of the equivalent peaks in the ^1H and ^{13}C NMR spectrum and on the HPLC (C18). The compounds **1**, **4**, and **5** were mixtures of three oleanane-type triterpenes (**9**-**11**) corresponding to the respective ursane-type triterpenes. It was impossible to separate these mixtures of ursane and the oleanane-type triterpene by the usual purification method using silica gel column chromatography. However, we finally isolated the six triterpenes from the mixtures as pure compounds (**1**, **4**, **5**, and **9**-**11**) by preparative HPLC on C18. Compounds **1**-**11** were determined by comparison with published spectral data: i.e., the ursane-type triterpenes: α-amyrin (**1**), uvaol (**2**) (Piozzi *et al.*, 1986), ursolaldehyde (**3**) (Hota and Bapuji, 1994), ursolic acid (**4**) (Kojima and Ogura, 1986), corosolic acid (**5**) (Kojima and Ogura, 1986), asiatic acid (**6**) (Kojima and Ogura, 1986), 2α, 3β, 24-trihydroxyurs-12-en-28-oic acid (**7**) (4-epi-asiatic acid) (Sun *et al.*, 1992), and 2α, 3α, 24-trihydroxyurs-12-en-28-oic acid (**8**) (Sakakibara and Kaiya, 1983); and oleanane-type triterpenes: β-amyrin (**9**), oleanolic acid (**10**), and maslinic acid (**11**) (Furuya *et al.*, 1987) (**Table 1**). **4** was produced in the highest yield among the triterpenes. A mixture of two phytosterols was also determined to be sitosterol and stigmasterol by comparison with published spectral data (Kojima *et al.*, 1990) and GC-MS. Furthermore, the *A. chinensis* and *A. polygama* callus tissues were compared with the *A. aruguta* callus tissues, and all the triterpenes except for **6**, and the mixtures of sitosterol and stigmasterol (as major sterols) were also isolated from both callus tissues. The three callus tissues produced almost the same constituents except for **6**, and the mixtures of **4** and **10** were produced in relatively large amounts among the triterpenes from all three callus tissues (**Table 1**). Except for **4** and **10** (Yue *et al.*, 1993), this is the first report of these triterpenes being produced from *A. chinensis* and *A. arguta*. Compounds **4**-**8** have been reported from the gall of *A. polygama* and the callus derived from its galls (Sashida *et al.*, 1994). In our study, several ursane-type triterpenes isolated during the early stage of the biosynthesis to ursolic acid were stepwise isolated from the callus tissues of the three plant species.

It is interesting from a biosynthetic point of view that the following biosynthetic sequence for the ursane-type triterpenes (**1**-**8**) can be theoretically presumed based on the co-occurrence of these constituents from the different degrees of oxidation at C-28 or C-2. They are first produced by the oxidation of **1**, and biosynthesized stepwise to **4** after the three step oxidation at C-28, as shown in **Fig. 1**. On the other hand, the hydroxylation of **4** at C-2 produces **5**, while further oxidation of **5** at C-23 produces **6** or the oxidation of **5** at C-24 generates **7**. Compound **8** is probably further biosynthesized from **7** via the 3-ketone by the same

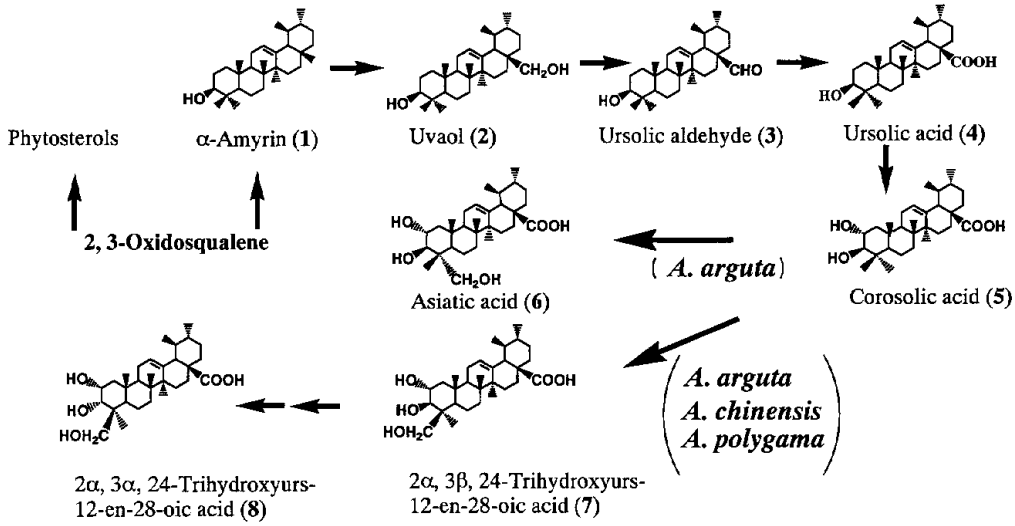

Fig. 1 Biosynthetic pathway of ursane type triterpenes in callus tissues of actinidiaceous plants

Table 1. Distribution of triterpenes in actinidiaceous plant callus tissues and plants.

Plants / Triterpenes	*A. aruguta*		*A. chinensis*		*A. polygama*		
	callus	stems	callus	stems	callus	stems	fruit
Ursane-type triterpenes[a]							
α-Amyrin (**1**)	+	+	+	+	+	+	+
Uvaol (**2**)	+	+	+	+	+	+	+
Ursolaldehyde (**3**)	+	—	+	—	+	—	—
Ursolic acid (**4**)	++	+	++	+	++	+	+
Corosolic acid (**5**)	+	+	+	+	+	+	+
Asiatic acid (**6**)	+	+	—	+	—	+	+
2α, 3β, 24-Trihydroxyurs-12-en-28-oic acid (**7**)	+	+	+	+	+	+	+
2α, 3α, 24-Trihydroxyurs-12-en-28-oic acid (**8**)	+	+	+	+	+	+	+
Oleanane-type triterpenes							
β-Amyrin (9)	+	+	+	+	+	+	+
Oleanolic acid (10)	+	+	+	+	+	+	+
Maslinic acid (11)	+	+	+	+	+	+	+

[a]Triterpenes in plants were detected on tlc, ++ present (large amount), +present, — absent.

sequences as shown for 3-epimaslinic acid biosynthesized from maslinic acid via 3-ketone (Seo *et al.*, 1981). Nevertheless, there are slight differences in the biosynthetic abilities between *A. arguta*, and *A. chinensis* and *A. polygama*, as shown in **Fig. 1** for the production of compounds **6** and **7**, which are biosynthesized from **5** by the independent hydroxylation at C-23 or C-24. The results are consistent with those observed among the callus tissues of three *Paeonia* species (*Paeonia lactiflora*, *P. japonica*, and *P. suffruticosa*) (Ikuta, 1996). This is interesting from both chemotaxonomic and phylogenetic points of view.

Constituents of plants

The occurrences of the triterpenoids in the stems and the fruits of the original plants were further examined and compared with those of the callus tissues for each other on the basis of TLC and HPLC analyses. The results of the comparison between the three actinidiaceous callus tissues and their original plants are shown in **Table 1**. The content of the triterpene mixtures (**4** and **10**) produced from the three callus tissues as the major triterpene compounds were further compared with those of the original plants (**Fig. 2**). The content of the triterpene mixtures in the stems of the *A. arguta* plant was one-twentieth of that produced from the callus tissues after 40 days of cultivation, while the contents of the triterpene mixtures in the stem of the *A. chinensis* and *A. polygama* plants were one-third and one-half, respectively. In these studies,

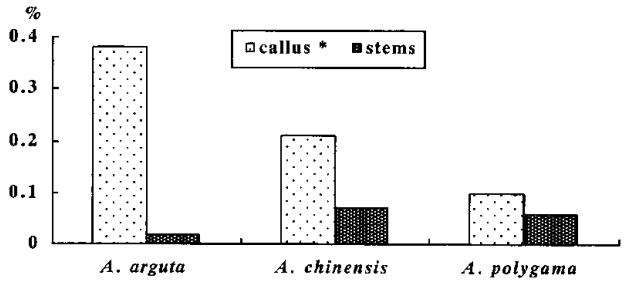

Fig. 2 Comparison of triterpenes** in actinidiaceous callus tissues and stems.
*2,4-D: 1.0 mg l^{-1}, KIN: 0.1 mg l^{-1} at 40 days.
**mixture of ursolic acid and oleanolic acid.

both the three actinidiaceous plant callus tissues and the plants produced many kinds of triterpenes, but with differences in the formation ratios. However, the callus tissues produced especially large amounts of the triterpene mixtures (**4** and **10**) compared to the plants (**Fig. 2**). This further indicated differences between the abilities of callus tissues and the plants to regulate the biosynthesis of the triterpenoids and the phytosterols. Differences in the production of the triterpenes and cholesterol between the callus tissues and the plant of the sunflower have been reported, but no pentacyclic triterpenes were detected in the culture despite it being known that they are produced by the sunflower plant (Nes, 1990).

In the present study, the contents of the triterpene mixtures of **4** and **10** and the phytosterol mixtures (sitosterol and stigmasterol) among the three callus tissues further differed from each other: the phytosterol contents in the *A. polygama* callus tissues was

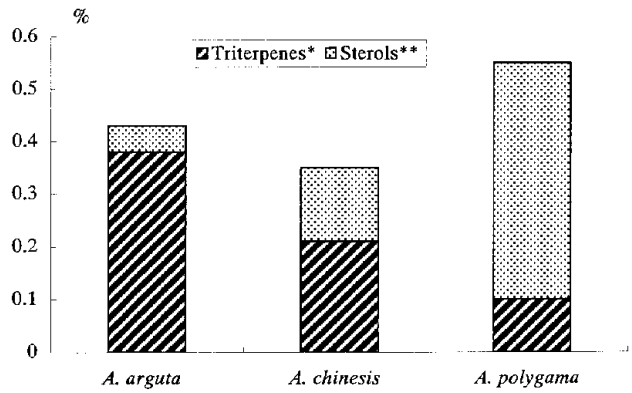

Fig. 3 Comparison of triterpenes and sterols in actinidiaceous callus tissues
*Ursolic acid and oleanolic acid, ** sitsterol and stigmasterol (2,4-D; 1.0 mg l^{-1}, Kin; 0.1 mg l^{-1} at 40 days)

higher than that in the *A. arguta* callus tissues. The production ratio of the triterpene mixtures of **4** and **10** and the phytosterol mixtures (sitosterol and stigmasterol), among the extracts of three callus tissues at 40 days, were compared using TLC. This revealed that the production ratio of the triterpene mixtures (**4** and **10**) and the phytosterols had reversed, to that in *A. polygama* being one-fifth of that in the *A. arguta* callus tissues. On the other hand, in the case of the *A. chinensis* callus tissues, approximately equal amounts of both compounds were produced: i.e., intermediate between the *A. arguta* and *A. polygama* callus tissues (**Fig. 3**). Oleanolic acid and ursolic acid have been shown to exhibit very potent antibacterial activity against both *Escherichia coli* and *Bacillus subtilis* (Liu, 1995), and have recently been known to possess anti-inflammatory, anti-hyperlipidemic, and antitumor-properties (Marwani *et al.*, 1997). The present study shows that the production of oleanolic acid and ursolic acid is approximately 20 times higher in the *A. aruguta* callus tissues are produced ca. 20 times more compared with the stem of then in the original plants suggesting that plant tissues cultures could be useful as a novel source of these pharmacological active compounds.

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