Limonoids in Miyamashikimi (*Skimmia japonica* Thunb.)

Shin Hasegawa¹, Masaki Miyake², George H. Robertson¹ and Mark Berhow³

¹Western Regional Research Center, Agricultural Research Service, U. S. Department of Agriculture, 800 Buchanan Street, Albany, CA 94710, USA

²Wakayama Nokyo Foods Industry Co., Ltd., 1294 Hikata-Shinhama, Kainan, Wakayama 642-0002

³National Center for Agricultural Utilization Research, Agricultural Research Service, U. S. Department of Agriculture, Peoria, IL 61604, USA

Summary

Miyamashikimi (*Skimmia japonica* Thunb.) seeds contain the following limonoids that are found in seeds of *Citrus* species: deacetylnomilin, nomilin, limonin, obacunone, deacetylnomilinic acid, nomilinic acid, isoobacunoic acid; and those found in seeds of *Fortunella* species: retrocalamin, calamin, cyclocalamin, methyl isoobacunoate diosphenol and isoobacunoate diosphenolic acid; and those found in seeds of *Citrus ichangensis*: ichangensin. They also contain 17- β -D-glucopyranoside of limonin, nomilin, obacunone, deacetylnomilin, calamin, retrocalamin, cyclocalamin and ichangensin. *Skimmia japonica* is the only plant which contains all of these limonoids. These data reveal unusual and unexpectedly high levels of citrus-like limonoids which suggest that *Skimmia japonica* is taxonomically closer to *Citrus* than previously believed.

Key Words: limonoids, *Skimmia japonica*, Rutaceae, papeda, citrus.

Introduction

Limonoids, a group of chemically related triterpenoids present in the Rutaceae and Meliaceae families, are one of the two bitter constituents in citrus juices and processed citrus products. A natural debittering process occurs in maturing citrus fruits in which the bitter limonoid aglycones are glucosidated to form the nonbitter limonoid glucosides (Hasegawa et al., 1991; Fong et al., 1992). Limonoid glucosides, one of the major secondary metabolites in *Citrus* (Fong et al., 1990; Ozaki et al., 1991a), play an important role in fruit quality and human health. Limonoids promote certain anticancer activities in laboratory animals (Lam et al., 1989; Miller et al., 1989), and act as natural pest control agents (Klocke and Kubo, 1982; Mendel et al., 1993).

The taxonomic characterization of citrus species and their relatives within the Rutaceae family is of interest throughout the world. The citrus breeders' search for new rootstocks and new genetic traits can be assisted by limonoid analyses. Limonoids are excellent taxonomic markers because they are specific for the Rutaceae and Meliaceae plants. Certain limonoid biosynthetic pathways are unique to a specific genus and species, and the identity of these compounds can be easily determined.

In this study, taxonomic analyses were conducted on Rutaceae species that could be used in a citrus breeding

program as sources for rootstock or other genetic materials. The taxonomy of these limonoids is important because their composition can be used for classifying Rutaceae plants.

Materials and Methods

Seeds were obtained from mature fruit of plants endemic to the high mountains of Wakayama Prefecture, Japan and seeds of Miyamashikimi (*Skimmia japonica* Thunb.) supplied by Tsukuba Medical Plant Research Station, National Institute of Hygienic Sciences, Tsukuba-city, Ibaraki, Japan.

Seeds were dried at 55 °C for 72 hr and ground by a Retsch-mill (Brinkmann, Westbury, NY). The meal (200-250 g) was placed in a Soxhlet extractor, washed thoroughly with hexane to remove oily materials, extracted with acetone to obtain limonoid aglycones, and then extracted with MeOH to obtain limonoid glucosides and any remaining aglycones.

The hexane extract contained no limonoids, whereas the acetone extract contained about 80% of the total aglycones. The MeOH extract was dried and extracted with H₂O-CH₂Cl₂ (1 : 1). The CH₂Cl₂ fraction, containing aglycones, was pooled with the acetone extract and analysed of limonoid aglycones. The H₂O fraction contained the limonoid glucosides.

Limonoid glucosides were separated by XAD, XAD-2, and DEAE Sephacel column chromatography as described previously (Hasegawa et al., 1989). Aglycones were separated on a silica gel column (Bennett

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and Hasegawa, 1981; Bennett and Hasegawa, 1982; Hashinaga et al., 1990; Ozaki et al., 1991a). Limonoid aglycones were identified with TLC and NMR (Bennett and Hasegawa, 1981; Hasegawa et al., 1986; Hashinaga et al., 1990; Ozaki et al., 1991a), and the glucosides were identified with HPLC and NMR, using procedures previously reported (Bennett et al., 1989; Hasegawa et al., 1989). They were also quantitatively analyzed with HPLC and TLC (Ozaki et al., 1991a). The above procedures, from extraction to identification, were repeated five times to confirm the results. Repeated analyses for limonoids in a given sample showed very little variation.

Result and Discussion

We identified 13 limonoid aglycones and 8 limonoid glucosides in seeds of Miyamashikimi, an unusual combination of limonoids, including those found in *Citrus* and *Fortunella*, and *Citrus* subgenus *Papeda*. We correlated this limonoid data with those of citrus biotypes (Swingle and Reece, 1967; Barrett and Rhodes, 1976; Scora, 1988).

Thirty-six limonoids have been isolated from *Citrus* and its hybrids. Based on biochemical and compositional data accumulated thus far on the limonoids of various citrus species and hybrids, we classified them into the basic limonin biosynthetic pathway group and three species-specific biosynthetic pathway groups (Hasegawa and Herman, 1992) as listed below. See Fig. 1 for chemical structures.

The limonin group (I): this group is found in all citrus species and their hybrids that have been examined so far. Thus, we consider this to be the basic metabolic group. The limonoids synthesized by this pathway, include limonin, nomilin, obacunone, deacetylnomilin, ichangin, limonol, 7 α -obacunol, deoxylimonol, citrusin, nomilinic acid, deacetylnomilinic acid, isoobacunoic acid, epiisoobacunoic acid, isolimononic acid, limonoic acid A-ring lactone, deoxylimonic acid, 17-dehydrolimononic acid A-ring lactone, obacunoic acid, and 19-hydroxydeacetylnomilinic acid.

The calamin group (II): this group is found in *Fortunella* and its hybrids, such as calamondin. The limonoids, synthesized by this pathway, include calamin, retrocalamin, cyclocalamin, methyl isoobacunoate diosphenol, 6-keto-7 β -deacetylnomilol, isocyclocalamin, 6-keto-7 β -nomilin, methyl deacetylnomilinate, calaminic acid, retrocalaminic acid, cyclocalaminic acid, and isoobacunoic acid diosphenol.

The ichangensin group (III): this group exists in *Citrus ichangensis* and its hybrids, such as Yuzu (*Citrus junos* Siebold ex Tanaka), Sudachi (*Citrus sudachi* hort. ex Shirai), Kabosu (*Citrus sphaerocarpa* hort. ex Tanaka), and Hanayu (*Citrus hanaju* hort. ex Shirai). The *C. ichangensis* group also contains relatively high concentrations of deacetylnomilin and deacetylnomilinic acid.

The 7-acetate limonoid group (IV): this group is

Fig. 1. Structures of limonoids.

found in *Poncirus* and its hybrids. This limonoid group includes 1-(10-19)abeo-obacun-9(11)-en-7 α -yl acetate, 1-(10-19)abeo-7 α -acetoxyl-10 β -hydroxyisoobacunoic acid 3, 10-lactone, limonol acetate, and 7 α -obacunyl acetate.

The basic limonin group is present in all citrus species and their hybrids, and in many other non-citrus members of the Rutaceae family plants. In the genus *Citrus*, mandarin, pummelo, and citron are considered to be the most ancient citrus species. These species and their hybrids contain only the limonin group. Non-citrus members of the Rutaceae plants such as *Fortunella* and *Poncirus*, and *Citrus* subgenus *Papeda* have been successfully crossbred with citrus species. We previously found that *Fortunella* and its hybrids with other citrus species contain the calamin group (Bennett and Hasegawa, 1981; Hasegawa et al., 1986). *Citrus ichangensis* (*Citrus* subgenus *Papeda*) has been shown to contain the ichangensin group (Bennett et al., 1988; Herman et al., 1989), and its hybrids with other citrus species also possess ichangensin (Hashinaga et al., 1990; Ozaki et al., 1991b). *Poncirus* and its hybrid with sweet orange has contain 7-acetate limonoid group (Bennett and Hasegawa, 1982). Therefore, the limonoid analytical

data clearly support the existing citrus classification.

The analysis of limonoid aglycones in *Skimmia japonica* seeds showed that they contain limonoids of three of the four biosynthetic groups found in *Citrus* and *Fortunella*, and *Citrus* subgenus *Papeda* (Table 1). They contain: 1) the limonin group including limonin, nomilin, obacunone, deacetylnomilin, deacetylnomilinic acid, nomilinic acid isoobacunoic acid, 2) the ichangensin group including ichangensin, and 3) the calamin group such as calamin, retrocalamin, cyclocalamin, methyl isoobacunoate diosphenol, and isoobacunoate diosphenol.

The presence of the three groups of limonoids in *Skimmia japonica* seeds were also confirmed by the presence of the 17- β -D-glucopyranoside derivatives of ichangensin (the ichangensin group), and obacunone, deacetylnomilin and limonin (V) (the limonin group), and retrocalamin, calamin and cyclocalamin (the calamin group) (Table 2).

Table 1. Limonoids in seeds of Miyamashikimi (*Skimmia Japonica* Thunb.).

Limonoids	Concentration (ppm) ^z
Limonin group	
Limonin	25
Nomilin	535
Deacetylnomilin	670
Obacunone	75
Deacetylnomilinic acid	5
Nomilinic acid	5
Isoobacunoic acid	trace
Calamin group	
Calamin	30
Retrocalamin	60
Cyclocalamin	15
Me-isoobacunoate diosphenol	trace
Isoobacunoate diosphenolic acid	trace
Ichangensin group	
Ichangensin	1540
Unknown	—

^z Values on a dry weight basis.

Table 2. Limonoid glucosides in seeds of Miyamashikimi (*Skimmia Japonica* Thunb.).

17- β -D-glucopyranosides of	Concentrations (ppm) ^z
Ichangensin group	
Ichangensin	300
Calamin group	
Calamin	17
Retrocalamin	30
Cyclocalamin	trace
Limonin group	
Limonin	18
Nomilin	16
Obacunone	32
Deacetylnomilin	15

^z Values on a dry weight basis.

The quantitative estimate of the limonoid composition of *Skimmia japonica* seeds showed that over 50% of total limonoid aglycones was ichangensin and that its glucoside made up over 70% of the total limonoid glucosides. Hence, the major biosynthetic pathway present in *Skimmia japonica* is the ichangensin pathway; nomilin-deacetylnomilin-deacetylnomilinic acid-ichangensin (Bennett et al., 1988; Herman et al., 1989). High concentrations of both nomilin and deacetylnomilin are also present, whereas the calamin group comprises only 3% of the total limonoid aglycones.

Skimmia japonica, a cold-hardy native to the Orient, grows in cold climates. It is the only plant which has been found to contain more than two biosynthetic groups of limonoids. This species is currently classified in the Rutaceae subfamily Toddalioidae and is considered to be fairly remote from *Citrus*. However, our limonoid analysis shows that the species is genetically similar to *Citrus* and *Fortunella*, and the *Citrus* subgenus *Papeda*. *Fortunella* and *Papeda* have been successfully crossbred with citrus species, creating hybrids which have been commercialized. Yuzu, Sudachi, and Kabosu which are the hybrids between *Citrus* and *Citrus* subgenus *Papeda* possess unique acidic flavors and are commercially grown in Asia, particularly in Japan. Calamondin, a hybrid between *Fortunella* and *Citrus* is commercially important in the Philippines. It may be possible to hybridize *Skimmia* species with *Citrus* or *Fortunella* species to create new, unique cold hardy rootstocks.

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ミヤマシキミ (*Skimmia japonica* Thunb.) のリモノイド

長谷川信¹・三宅正起²・George H. Robertson¹・Mark Berhow³

¹米国農務省 (Western Regional Research Center) 94710 Buchanan Street Albany California USA

²和歌山ノーキョー食品工業株式会社 642-0002 海南市日方新浜

³米国農務省 (National Center for Agricultural Utilization Research) 61604 Peoria Illinois USA

摘 要

ミヤマシキミ (*Skimmia japonica* Thunb.) の種子中にはカンキツ類の種子に広く分布するリモノイド (deacetylnomilin, nomilin, limonin, obacunone, deacetylnomilinic acid, nomilinic acid, isoobacunoic acid) が存在した。また, *Citrus ichangensis* および *Fortunella* にそれぞれ特異的に存在するリモノイド (ichangensin および calamin, retrocalamin, cyclocalamin, methyl isoobacunoate diosphenol, isoobacunoate diosphenolic acid) が含有されていた。さらに, ミヤマシキミの種子中には limonin, nomilin, obacu-

none, deacetylnomilin, retrocalamin, calamin, cyclocalamin, ichangensin のそれぞれ配糖体 (17- β -D-glucopyranoside) も検出された。

ミヤマシキミは上記のリモノイドがすべて存在する唯一の植物であり, このことは植物分類上, ミヤマシキミがカンキツ類と近縁種であるというこれまでの説を支持するものである。

²現在: 株式会社島津製作所, 京都市中京区