J Wood Sci (2005) 51:181–184
DOI 10.1007/s10086-004-0635-1

NOTE

Sanro Tachibana · Hiroko Ishikawa · Kazutaka Itoh

Antifungal activities of compounds isolated from the leaves of *Taxus cuspidata* var. *nana* against plant pathogenic fungi

Received: September 12, 2003 / Accepted: April 7, 2004

Abstract Antifungal activities of seven compounds, taxinine (**1**), paclitaxel (**2**), phenylisoserine methyl ester (**3**), sciadopitysin (**4**), ginkgetin (**5**), isorhamnetin (**6**), and quercetin (**7**), isolated from the leaves of kyaraboku, *Taxus cuspidata* var. *nana*, against five plant pathogenic fungi, *Gibberella fujikuroi*, *Cladosporium cucumeninum*, *Fusarium oxysporum*, *Colletotrichum fragariae*, and *Corynespora cassiicola*, were investigated for utilization of extractives from trees of the genus *Taxus*. Also, the amounts of compounds **2** and **3** on the leaf surface was measured in relation to the antifungal activities of compounds. Taxinine (**1**) showed antifungal activity against *G. fujikuroi*, *C. cucumeninum*, *F. oxysporum*, and *C. cassiicola*. The minimum inhibitory concentration of taxinine for the four fungi was 0.4 μmol. In addition, from the results of antifungal tests, it may be concluded that paclitaxel on the leaves and stem of *T. cuspidata* var. *nana* does not play an important role as an antifungicide in the resistance of trees to plant pathogenic fungal attack.

Key words Antifungal activity · Plant pathogenic fungi · *Taxus cuspidata* var. *nana* · Taxinine · Paclitaxel

Introduction

Yew trees contain many biologically active substances, however, only the utilization of paclitaxel has been examined.[1] In continuation of our recent research on the isolation of compounds from kyaraboku, *Taxus cuspidata* var. *nana*,[2] and Ichii, *T. cuspidata*,[3] on the antifungal activities of the isolated compounds against plant pathogenic fungi,[4,5] and on the production of paclitaxel in tissue cultures of *T. cuspidata* var. *nana*,[6,7] the isolation of compounds from the leaves of *T. cuspidata* var. *nana* was reported in our previous report.[8] In the course of this research, it was noticed that *T. cuspidata* var. *nana* trees are very resistant to plant pathogenic fungi and insects. Daniewski et al.[9] reported that leaf extracts from yew trees (*Taxus baccata*) exhibit very strong resistance to insect pests. Zobel et al.[10] reported that paclitaxel is present on the surface of leaves of three yew trees, *T. baccata*, *T. cuspidata*, and *Taxus media*. Young et al.[11] investigated the antimicrobial properties of paclitaxel and its various analogs, and reported that paclitaxel shows toxicity against fungi belonging to Oomycetes and Basidiomycetes.

In the present report, we describe the antifungal activities of seven compounds, which were isolated from Kyraboku leaves in the previous study,[8] against five plant pathogenic fungi, *Gibberella fujikuroi*, *Cladosporium cucumeninum*, *Fusarium oxysporum*, *Colletotrichum fragariae*, and *Corynespora cassiicola.* We also discuss the role of paclitaxel on the surface of the leaves of *T. cuspidata* var. *nana* in connection with the antifungal activities.

S. Tachibana (✉) · H. Ishikawa · K. Itoh
Department of Applied Bioscience, Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama 790-8566, Japan
Tel. +81-89-946-9864; Fax +81-89-977-4364
e-mail: tatibana@agr.ehime-u.ac.jp

Material and methods

Plant material

Fresh leaves of *Taxus cuspidata* var. *nana* were collected in July 2000, on the outskirts of Matsuyama City, Ehime Prefecture, Japan.

Measurement of amount of paclitaxel and taxinine on the surface of leaves of *Taxus cuspidata* var. *nana*

Twigs having leaves of *T. cuspidata* var. *nana* (1300 g) were cut into pieces 15 cm long, containing first-year and second-year leaves. These materials were dipped into near-boiling water in a 1-liter beaker for 5 s to remove melted wax and any compounds embedded in it.[10] After cooling, the water was extracted twice with chloroform. The chloroform solution was evaporated to dryness under reduced pressure. The residue (259.4 mg) was dissolved in methanol and the solution was diluted to 20 ml with methanol. The amount of paclitaxel in the solution was determined by high perfor-

mance liquid chromatography (HPLC) performed on a reverse-phase column (Supelcosil™ LC-F) in a Shimadzu LC 10A liquid chromatograph equipped with an ultraviolet (UV) detector (wavelength 227 nm) by isocratic elution with acetonitrile/tetrahydrofuran/water (17:28:55 v/v). The flow rate was 1.5 ml/min, and all chromatograms were plotted at the absorbance maximum of paclitaxel, at 227 nm. A 10-μl aliquot of the solution was injected into the column. A calibration curve was obtained using authentic paclitaxel.

In the case of taxinine, twigs having leaves of *T. cuspidata* var. *nana* (230 g) were treated in the same manner as described above. The wax obtained (40.7 mg) was dissolved in methanol and the solution was diluted to 4 ml with methanol. The amount of taxinine in the solution was determined by HPLC as described above. In this case, the wavelength of the UV detector was set at 278 nm, the absorbance maximum of taxinine, and a 5-μl aliquot of the solution was injected into the column. The calibration curve was obtained using authentic taxinine.

The paclitaxel and taxinine in the solution were identified by comparing the retention times with those of authentic paclitaxel and taxinine isolated from *T. cuspidata* var. *nana*.[2] Furthermore, paclitaxel and taxinine isolated from the wax were identified by comparing the mass spectra with those of authentic paclitaxel and taxinine isolated from the leaves of *T. cuspidata* var. *nana*.[2]

For comparison with the amount of paclitaxel on the surface of the twigs, intact leaves were ground with a Waring blender and were extracted with methanol. The methanol solution was evaporated to dryness under reduced pressure. The methanolic extracts were partitioned between dichloromethane and water by the method of Witherup et al.[12] and the dichloromethane-soluble fraction was subjected to HPLC analysis.

Extraction and isolation of paclitaxel and taxinine from the wax on the surface of *Taxus cuspidata* var. *nana*

Twigs having leaves of *T. cuspidata* var. *nana* (8510 g) were cut into pieces 15 cm long which were then dipped into near-boiling water in a 5-l beaker for 5 s as mentioned above. Similar treatment was conducted with a further 7360 g of twigs and leaves of *T. cuspidata* var. *nana* in the same manner as described above. After cooling, the water was combined and extracted twice with chloroform. The chloroform solution was evaporated to dryness under reduced pressure. The residue (3.66 g) was chromatographed on a silica gel column using a chloroform–methanol solvent system as described previously.[2] Fractions containing taxinine and paclitaxel were separated.

The fraction containing taxinine (72.8 mg) was chromatographed on a silica gel column using an *n*-hexane–acetone solvent system. The waxy solid (25.1 mg) obtained from the initial eluate was rechromatographed by preparative thin-layer chromatography (TLC) with *n*-hexane–acetone (3:1 v/v). This yielded taxinine (1.2 mg) after recrystallization from dichloromethane and ethyl acetate, mp 265°–267°C (lit. mp 266°–268°C).[13] FAB-MS (fast-atom bombardment mass spectrum) *m/z*: 607 (M^++H), 606 (M^+), 547, 459, 307, 289, 154 (100%), 131, 107. The mass spectrum of taxinine isolated from the wax was identical with that of authentic taxinine isolated from *T. cuspidata* var. *nana*.[2] The mixed-melting point test of the isolated taxinine with authentic sample showed no depression of melting point.

The fraction containing paclitaxel (35.8 mg) was chromatographed on a silica gel column using a chloroform–methanol solvent gradient and yielded the fraction containing paclitaxel (10.7 mg). The fraction was rechromatographed by preparative TLC with chloroform–methanol (10:1 v/v). This yielded paclitaxel (0.5 mg) after recrystallization from methanol and water, mp 213°–215°C (lit. mp 213°–216°C).[14] FD-MS (field desorption MS) *m/z*: 854 (M^++H), 853 (M^+), 568, 210 (100%), 105, 43. The mass spectrum of paclitaxel isolated from the wax was identical with that of authentic paclitaxel isolated from *T. cuspidata* var. *nana*.[2] The mixed-melting point test of the isolated paclitaxel with authentic sample showed no depression of melting point.

Antifugal activities of the compounds against plant pathogenic fungi

Plant pathogenic fungi

Gibberella fujikuroi, *Cladosporium cucumeninum*, *Fusarium oxysporum*, *Colletotrichum fragariae*, and *Corynespora cassiicola* were obtained from the Ehime Prefectural Agriculture Center. The five fungi were incubated on a slant that had 15 ml of a potato dextrose agar (PDA) medium for 7 days at 25°C in the dark.

Test compounds

The seven compounds, **1** to **7**, isolated in the previous study[8] were used as test compounds. The seven compounds isolated from the fresh leaves of *T. cuspidata* var. *nana* (800 g) as shown in the previous study[8] were: taxinine (**1**) (244.7 mg), mp 266°–268°C (0.0651% on dried leaf); paclitaxel (**2**) (5.4 mg), mp 213°–215°C (0.0014% on dried leaf); phenylisoserine methyl ester (**3**) (4.4 mg), mp 213°–215°C (0.0012% on dried leaf); sciadopitysin (**4**) (94.8 mg), mp 294°–296°C (0.0252% on dried leaf); ginkgetin (**5**) (5.7 mg), mp >300°C (0.0015% on dried leaf); isorhamnetin (**6**) (9.6 mg), mp 303°–305°C (0.0026% on dried leaf); quercetin (**7**) (30.1 mg), mp 312°–314°C (0.0080% on dried leaf). The chemical structures of compounds **1** to **7** are shown in Fig. 1. They were dissolved in a small volume of dimethylsulfoxide (DMSO) or methanol to a fixed concentration (1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, or 0.013 μmol). Each solution (0.1 ml) was added to a filter paper disk (10-mm diameter). In the case of the control, only solvent was added to the disk.

Medium

The PDA medium contained potato (200 g), glucose (20 g), agar (20 g), and distilled water (1000 ml).[15] The pH of the medium was adjusted to 5.6 with 1N HCl or NaOH. A 20-ml portion of the medium was added to a plastic Petri dish and prepared as the test agar medium.

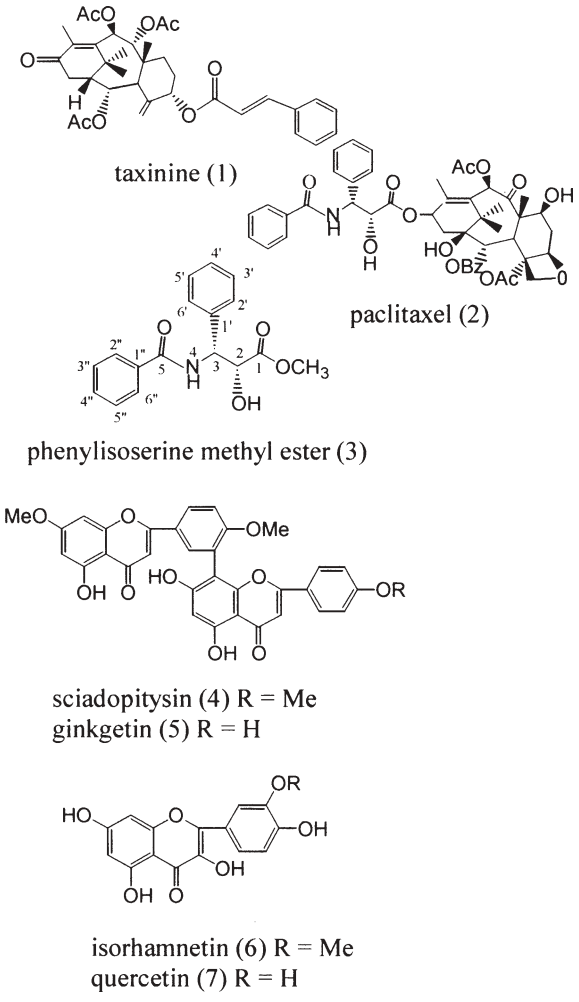

Fig. 1. Chemical structures of compounds **1–7** used for testing

Antifugal test

Ten milliliters of sterilized water was added to a slant containing one of the plant pathogenic fungi, and the fungus was suspended with an inoculating loop. The suspension was filtered with gauze, and a spore suspension (3×10^5/ml) was prepared. A 0.25-ml aliquot of the suspension was added to the agar plate and was spread uniformly. A paper disk prepared as described above was placed on the agar plate and incubated for 3 days at 28°C. After the incubation, the diameter of the inhibition zone for each test compound was measured. In the control, solvent alone was added to the disk and incubated as described above. The antifungal activity of each compound was evaluated as the minimal inhibitory concentration.

Results and discussion

Amount of paclitaxel and taxinine on the leaves of *Taxus cuspidata* var. *nana*

The results showing the content of paclitaxel and taxinine on and in the leaves are shown in Table 1. Paclitaxel was detected in the wax from the surface of twigs having leaves of *Taxus cuspidata* var. *nana* in a yield of 0.000004% on dry material. This amounted to 0.008% of the value inside the leaves. Zobel et al.[10] reported that paclitaxel was present on the surface and inside of leaves of three species (*Taxus baccata*, *Taxus cuspidata*, and *Taxus media*) and two varieties (*Taxus baccata* var. *elegantissima* and *Taxus media* var. *hatfieldii*) of the genus *Taxus*, and paclitaxel concentration on the surface of each genus was less than 0.58%–0.085%. The difference in paclitaxel content obtained in the present study and by Zobel et al.[10] may be ascribed to climate and growing conditions. In addition, Glowniak et al.[16] reported seasonal changes in the concentration of four taxoids including paclitaxel on the surface and inside the leaves of *T. baccata*.

Table 1. Paclitaxel and taxinine content of each part of *Taxus cuspidata* var. *nana*

Sample	Content (% of dry weight)	
	Paclitaxel	Taxinine
Wax[a]	0.000004 (0.013)	0.000020 (0.07)
Leaves	0.053	0.104

Numbers in parentheses show percentage of each compound to weight of wax obtained from twigs with leaves by extraction with hot water
[a] Obtained from the surface of the twigs and leaves

Taxinine was also detected in the wax from the surface of twigs having leaves of *T. cuspidata* var. *nana* in a yield of 0.000020% on dry material. This amounted to 0.019% of the value inside the leaves. There is no report that taxinine is present on the surface of the leaves of *T. cuspidata* var. *nana.* However, Tachibana et al.[2] reported that taxinine was present inside the leaves.

The significance of the presence of paclitaxel and taxinine on the surface of leaves in the genus *Taxus* has not been elucidated. However, Young et al.[11] reported that paclitaxel shows antimicrobial activity against fungi belonging to Oomycetes and Basidiomycetes like *Aphanomyces cochilioides* and *Streum purpureum.* Muranaka et al.[4,5] reported that taxinine showed antifungal activity against some plant pathogenic fungi. Therefore, the paclitaxel and taxinine on the surface of the leaves may protect the trees against microbial attack.

Antifungal activities of the compounds isolated from the leaves of *Taxus cuspidata* var. *nana*

The minimum inhibitory concentrations (MIC) of seven compounds used to test against five kinds of fungi are shown in Table 2. Taxinine showed antifungal activity against *Gibberella fujikuroi*, *Cladosporium cucumeninum*, *Fusarium oxysporum*, and *Corynespora cassiicola*. However, it did not show antifungal activity against *Colletotrichum fragariae*. Taxinine affected all four fungi at the concentration of 0.4μmol. The MIC of the six compounds except taxinine was above 1.6μmol, and the antifungal activities of the six compounds against all five fungi were weak. The relationship between content of each isolated compound and the value of MIC was not recognized. However, taxinine present in relatively higher concentra-

Table 2. Minimum inhibitory concentration of antifungal activity of taxinine against five plant pathogenic fungi

Plant pathogenic fungus	MIC (μmol)
Gibberella fujikuroi	0.4
Cladosporium cucumeninium	0.4
Fusarium oxysporum	0.4
Colletotrichum fragariae	–
Corynespore cassiicola	0.4

MIC, minimum inhibitory concentration

tion than the other six compounds showed stronger antifungal activity than those of the six compounds. However, the activity was weaker than those of a practical antimicrobial agent, itraconazol (0.05–0.0006μmol)[17] and an antifungal agent, methyl 2-benzimidazolecarbamate·HCl (MBC·HCl) (0.01–0.004μmol).[18] Muranaka et al.[4,5] reported that taxinine isolated from *T. cuspidata* var. *nana* showed strong antifungal activity against three plant pathogenic fungi, *Botrytis cinerea*, *Colletotrichum fragariae*, and *Cochliobolus miyabeanus*, at 0.4, 0.05 and 0.2μmol, respectively, and also showed weak antifungal activity against *Altenaria kikuchiana* at 2.0μmol.

However, paclitaxel did not show antifungal activity against the five fungi used here. Young et al.[11] investigated the antimicrobial activity of paclitaxel and its various analogs, and reported that paclitaxel was the most toxic of the test compounds against test fungi belonging to Oomycetes, Basidiomycetes, Deuteromycetes, and Ascomycetes. The median effective concentration (EC_{50}) values for growth inhibition of fungi belonging to Oomycetes, e.g., *Aphanomyces cochilioides*, by taxol ranged from 0.4 to 5.9μmol.[11] They also reported that all test fungi belonging to Basidiomycetes, Deuteromycetes, and Ascomycetes were not sensitive ($EC_{50} > 50\mu$mol), except *Streum purpureum* which was relatively sensitive (9.6μmol).[11] From the results obtained by Young et al.[11] and here, it is also thought that the antifungal activity of paclitaxel against the five fungi used here was weak. In addition, Daniewski et al.[9] reported that leaf extracts from yew trees (*Taxus baccata*)exhibit very strong resistance to insect pests, and two compounds, 10-deacetylbaccatin III and 10-deacetylbaccatin V, are the most responsible for the resistance. Therefore, paclitaxel may play a part but not an important role as a strong antifungicide in the resistance of trees to attack by plant pathogenic fungi.

On the contrary, taxinine showed antifungal activity against all fungi used here except for *Collectotrichum fragariae*. Because taxinine was present on the surface of *T. cuspidata* var. *nana*, taxinine in leaf wax may provide resistance for *T. cuspidata* var. *nana* to plant pathogenic fungi. It is considered that investigation of antifungal compounds in the leaf extracts from *T. cuspidata* var. *nana* is necessary.

From the results obtained here and previously, it is concluded that taxinine has antifungal activity against plant pathogenic fungi. It is also suggested that taxinine present on the surface of leaves contributes to the protection of leaves of *T. cuspidata* var. *nana* against plant pathogenic fungi. However, further investigation will be necessary for clarification of the resistance.

Acknowledgments We thank Koukichi Shinkai, Akinori Nakamura, and the late Tatsuo Tamagawa for the fresh leaves and twigs of *Taxus cuspidata* var. *nana*.

References

1. Tachibana S (1995) Utilization of biologically active substances in trees (in Japanese). Mokuzai Gakkaishi 41:967–977
2. Tachibana S, Matsuo A, Itoh K, Oki T (1994) Extractives in the leaves and bark of *Taxus cuspidata* Sieb. et. Zucc. var. *nana* Rehder. Mokuzai Gakkaishi 40:1008–1013
3. Kurose K, Itoh K, Tachibana S, Irie K, Oki T (1995) Extractives of *Taxus cuspidata* Sieb. et Zucc. leaves and chemotaxonomy of trees of genus *Taxus.* Bull Ehime Univ Forest 33:1–16
4. Muranaka T, Ueno J, Itoh K, Matsumoto I, Tachibana S (1997) Studies on utilization of extractives from the genus *Taxus* tree (2). Antifungal activities of taxinine and its derivatives against *Colletotrichum fragariae*, *Botrytis cinerea*, and *Corynespora cassiicola.* Bull Ehime Univ Forest 35:45–53
5. Muranaka T, Kurose K, Itoh K, Tachibana S (1999) Utilization of extractives from genus *Taxus* tree I. Antifungal activities of flavonoids, taxinine, and its derivatives against *Cochiliobolus miyabeanus* and *Altenaria kikuchiana* (in Japanese). Mokuzai Gakkaishi 45:42–50
6. Tachibana S, Watanabe E, Itoh K, Oki T (1994) Formation of taxol in *Taxus cuspidata* Sieb. et Zucc. var. *nana* Rehder callus cultures. Mokuzai Gakkaishi 40:1254–1258
7. Yoshida M, Muranaka T, Kurose K, Itoh K, Tachibana S (2002) Stimulation of the production of taxol by oligosaccharides in *Taxus cuspidata* var. *nana* callus cultures. Pak J Bio Sci 5:461–465
8. Tachibana S, Watanabe E, Ueno J, Tokubuchi K, Itoh K (2005) Isolation of phenylisoserine methyl ester from the leaves of *Taxus cuspidata* var. *nana.* J Wood Sci 51:176–180
9. Daniewski WM, Gumulka M, Anczewski W, Masnyk M, Bloszyk E, Gupta KK (1998) Why the yew tree (*Taxus baccata*) is not attacked by insects. Phytochemistry 49:1279–1282
10. Zobel AM, Furmanowa M, Glowniak K, Cragg C (1996) Taxol on the surface of leaves and inside the needles of three species and two varieties of *Taxus*. Phytomedicine 3:287–291
11. Young DH, Lichelotti EL, Swindell CS, Krauss NE (1992) Antifungal properties of taxol and various analogues. Experientia 48:882–885
12. Witherup KM, Look SA, Stasko MW, Ghiorzi TJ, Muschik GM (1990) *Taxus* spp. needles contain amounts of taxol comparable to the bark of *Taxus brevifolia.* Analysis and isolation. J Nat Prod 53:1249–1255
13. Wani MC, Taylor HL, Wall ME (1971) Plant antitumor agents. VI. The isolation and structure of taxol, a novel antitumor agent from *Taxus brevifolia.* J Am Chem Soc 93:2325–2327
14. Yoshizaki F, Madarame M, Takahashi C (1986) Principal constituents of the seeds of Japanese yew (*Taxus cuspidata*). Shoyakugaku Zasshi 40:429–431
15. Ando K, Ono Y, Kakishima M, Katsuya K, Sato S, Sato T (1983) Preparation of medium. In: Sato S, Goto M, Doi Y (eds) Experimental methods of plant pathology. Kodannsha Scientific, Tokyo, pp 11–18
16. Glowniak K, Mroczek T, Zobel AM (1999) Seasonal changes in the concentrations of four taxoids in *Taxus baccata* L. during the autumn–spring period. Phytomedicine 6:135–140
17. Ohzeki T, Mori K (2003) Synthetic racemate and enantiomers of cytosporone E, a metabolite of an endophytic fungus, show indistinguishably weak antimicrobial activity. Biosci Biotechnol Biochem 67:2584–2590
18. Tabata T, Kondo T (1977) Protection of shiitake-fungus from mycoparasite by fungicidal agents. III. Difference of antifungal activity of organic compounds containing nitrogen and sulfur in molecule upon the mycelium of shiitake-fungus and its mycoparasites (in Japanese). Mokuzai Gakkaishi 23:209–213