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Metabolite profiling of alkaloids and strictosidine synthase activity in camptothecin producing plants

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

Camptothecin derivatives are clinically used anti-neoplastic alkaloids that biogenetically belong to monoterpenoid indole alkaloids. Camptothecin-related alkaloids from the methanol extracts of *Ophiorrhiza pumila*, *Camptotheca acuminata* and *Nothapodytes foetida* plants were profiled and identified using a reverse-phase high performance liquid chromatography coupled with on-line photodiode array detection and electrospray-ionization ion-trap mass spectrometry. A natural 10-glycosyloxy camptothecin, chaboside, was accumulated in tissues of *O. pumila* but not in *C. acuminata* and *N. foetida*. Anthraquinones regarded as phytoalexins were present in the extracts of hairy roots and calli but not in the differentiated plants of *O. pumila*. These findings demonstrated a remarkable difference in the constituents between the differentiated plants and the hairy roots or calli tissues. The activity of strictosidine synthase, a key enzyme of camptothecin biosynthesis, was detected in the protein extracts of stems and roots of *O. pumila*, being correlated with the pattern of strictosidine synthase mRNA expression.

Keywords: *Ophiorrhiza pumila*; *Camptotheca acuminata*; *Nothapodytes foetida*; Rubiaceae; Nyssaceae; Icacinaceae; Anti-tumor alkaloid; Camptothecin; Anthraquinone; Strictosidine synthase (STR)

1. Introduction

Camptothecin (**1**), a quinoline alkaloid, was firstly isolated from *Camptotheca acuminata* (Nyssaceae) (Wall et al., 1966). This alkaloid is produced also in some other plant species, such as *Ervatamia heyneana* (Apocynaceae) (Gunasekera et al., 1979), *Nothapodytes foetida* (Govindachari and Viswanathan, 1972) and *Merrilliodendron megacarpum* (Icacinaceae) (Arisawa et al., 1981), and in some species of the genus *Ophiorrhiza* (Rubiaceae) (Tafur et al., 1976; Aimi et al., 1989). Camptothecin (**1**) exhibits an anti-tumor action due to its inhibitory activity to DNA topoisomerase I (Hsiang et al., 1985). At present, semi-synthetic water-soluble camptothecin analogues, topotecan (**2**) and irinotecan (**3**), are used as clinical anti-tumor agents throughout the world (Fig. 1). In spite of the rapidly growing pharmaceutical market of these camptothecin analogues, they are synthesized from natural camptothecin (**1**), which is obtained by extraction from the intact plants of *C. acuminata* and *N. foetida*. The production of secondary metabolites by genetically engineered plant cell cultures has become a keen issue because of a shortage of natural resources and subsequent environmental concerns (Springob and Saito, 2002). Recently, a hairy root culture of *O. pumila* has been established by infection with *Agrobacterium rhizogenes* (Saito et al., 2001). The hairy root produces high levels of camptothecin (**1**) and excretes it into the culture medium in large quantities (Saito et al., 2001; Sudo et al., 2002). For better production of this pharmaceutically important alkaloid, it is necessary to compare the accumulation pattern of alkaloids and other secondary metabolites in *O. pumila* with already known camptothecin producing plants, such as *C. acuminata* and *N. foetida*, and to elucidate tissue specificity of accumulation.

Abbreviations: MS, mass spectrometry; TIA, monoterpenoid indole alkaloid; STR, strictosidine synthase; DAD, photodiode array detection; ESI, electrospray ionization; MS/MS, tandem mass spectrometry.

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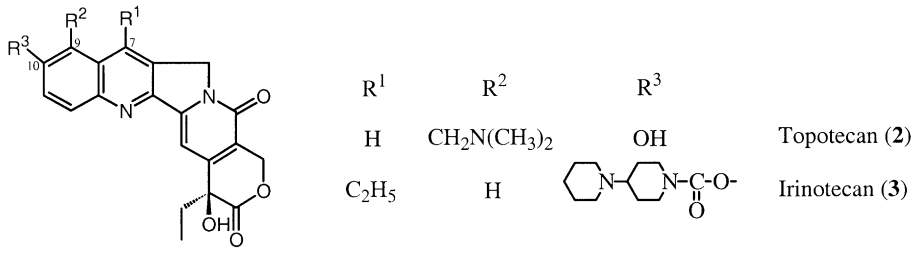
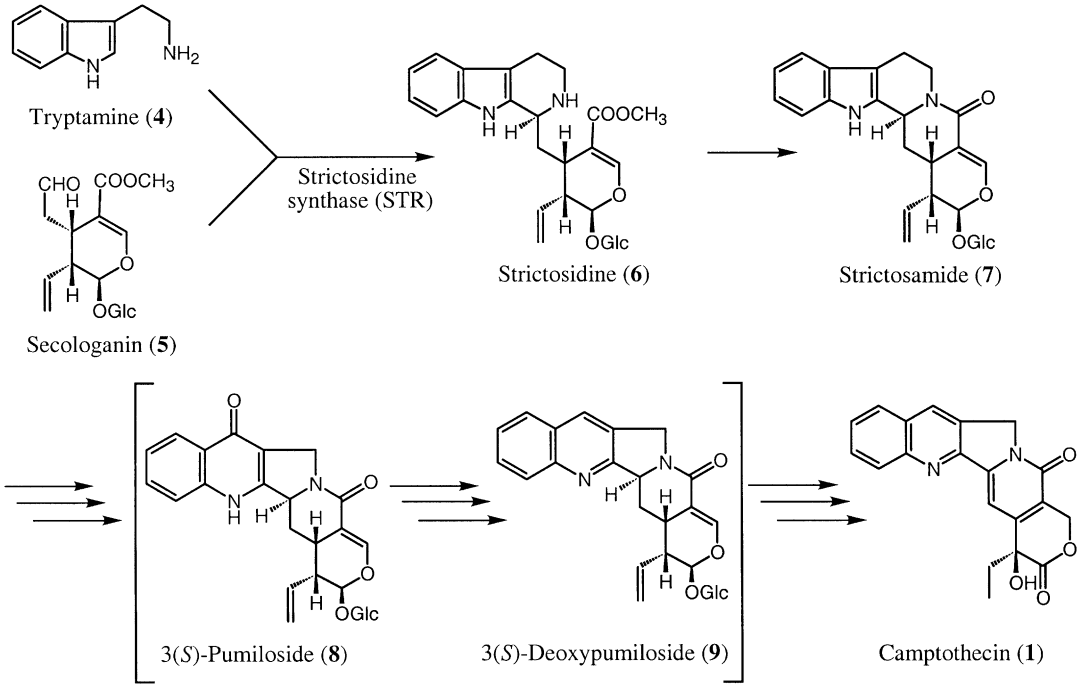


Fig. 1. Clinically used camptothecin derivatives, topotecan (**2**) and irinotecan (**3**).

For the phytochemical analysis of alkaloids, traditionally, they are purified by chromatography and then analyzed by physicochemical methods such as mass spectrometry (MS), NMR, etc. Recently, a simple, sensitive and selective analysis alternative, high performance liquid chromatography coupled to mass selective detection (LC/MS) has been improved (Niessen, 1999). This LC/MS method can be applicable for fast and non-targeted metabolite profiling of particular cells (metabolomics analysis) (Huhman and Sumner, 2002).

Although camptothecin (**1**) is structurally grouped in quinoline alkaloids, it is biogenetically a modified monoterpenoid indole alkaloid (TIA). The common intermediate, from which a variety of TIAs are formed, is strictosidine (**6**). Strictosidine (**6**) is formed by the condensation of tryptamine (**4**) with the iridoid glucoside, secologanin (**5**) (Scheme 1) (Stöckigt and Zenk, 1977). This condensation is catalyzed by strictosidine synthase (STR) (EC 4.3.3.2) (Scott and Lee, 1975; Stöckigt and Ruppert, 1999). Subsequently, intramolecular cyclization of strictosidine (**6**) yields strictosamide (**7**), a penultimate precursor of camptothecin formation in *C. acuminata* (Hutchinson et al., 1979). As an alternative pathway from strictosidine (**6**), a variety of TIAs are derived from deglucosylated strictosidine, and the resulting reactive dialdehyde produces different types of TIAs such as cathenamine in *Catharanthus roseus* (Hemscheidt and Zenk, 1980). The cDNA encoding STR was first isolated from *Rauvolfia serpentina* (Kutchan et al., 1988) and subsequently from *C. roseus* (McKnight et al., 1990), and the catalytically functional proteins were heterologously expressed (Kutchan, 1989; McKnight et al., 1991; Roessner et al., 1992; Kutchan et al., 1994). Recently, cDNA encoding STR was isolated and characterized from *O. pumila* (Yamazaki et al., submitted).

In the present study, metabolite accumulation in camptothecin-producing plants, *O. pumila*, *C. acuminata* and *N. foetida*, was profiled using a reverse-phase HPLC-photodiode array detection and electrospray ionization mass spectrometry (HPLC/DAD/ESI/MS). The metabolite analysis was also carried out with different cell



Scheme 1. The predicted biosynthetic pathway of camptothecin (**1**) in *Ophiorrhiza pumila*. The enzyme is: STR, strictosidine synthase. Plausible biogenetic intermediates are put in parentheses.

types of *O. pumila*. Besides, camptothecin-accumulation sites in the differentiated *O. pumila* plants and the distribution of STR enzymatic activity were investigated.

2. Results

2.1. Metabolite profiling of secondary products in *O. pumila*, *C. acuminata* and *N. foetida*

The reverse-phase HPLC/DAD/ESI/positive-ion MS system was used to profile the alkaloids in the MeOH extracts of *O. pumila*, *C. acuminata* and *N. foetida* plants and the different types of *O. pumila* tissues. The standard alkaloids and anthraquinones could be separated using reverse-phase HPLC/DAD (Fig. 2) by the method with a little modification of previous report (Graham, 1991).

The MeOH extracts of plant materials investigated in the present study contained mainly alkaloids and anthraquinones. These compounds were identified with the standard compounds according to their R_t values, UV spectra and mass spectra as summarized in Table 1. Fig. 3 shows HPLC chromatograms of the MeOH extracts of six plant materials examined in this study.

Camptothecin (**1**) was identified in the extracts of *O. pumila*, *C. acuminata* and *N. foetida* plants and *O. pumila* hairy roots but not in *O. pumila* calli. Whole tissues of *O. pumila* produce not only camptothecin (**1**) but also related alkaloids, such as strictosamide (**7**), 3(*S*)-pumiloside (**8**), 3(*S*)-, 3(*R*)-deoxypumiloside (**9**, **10**), chaboside (**11**), 9-methoxycamptothecin (**12**) and strictosidinic acid (**14**), that are not produced in *C. acuminata* (Figs. 3 and 4, Table 1). Among them, chaboside (**11**) is the first isolated natural camptothecin glucoside (Aimi et al., 1990). 3(*S*)-Pumiloside (**8**) and 3(*S*)-deoxypumiloside (**9**) are thought to be biogenetic intermediates in the formation of camptothecin from strictosamide (**7**) (Scheme 1) (Aimi et al., 1989; Kitajima et al., 1997a). Strictosidinic acid (**14**) is probably formed by a branched pathway from the route to camptothecin (**1**). Regarding pot-growing or sterile condition for *O. pumila* plants, these growth conditions may affect only on quantity of each alkaloid, but the metabolite patterns are quite similar (Fig. 3A and B, Table 1). No anthraquinones were detected in differentiated plants of *O. pumila*. Hairy roots of *O. pumila* produced camptothecin (**1**) and 3(*S*)-pumiloside (**8**) (Fig. 3C, Table 1). Interestingly they also contained at least six anthraquinones, including four unidentified anthraquinones deduced from their UV spectra (Figs. 3C and 5, Table 1). The calli of *O. pumila* produced no alkaloids but only anthraquinones (Figs. 3D and 5, Table 1). It is

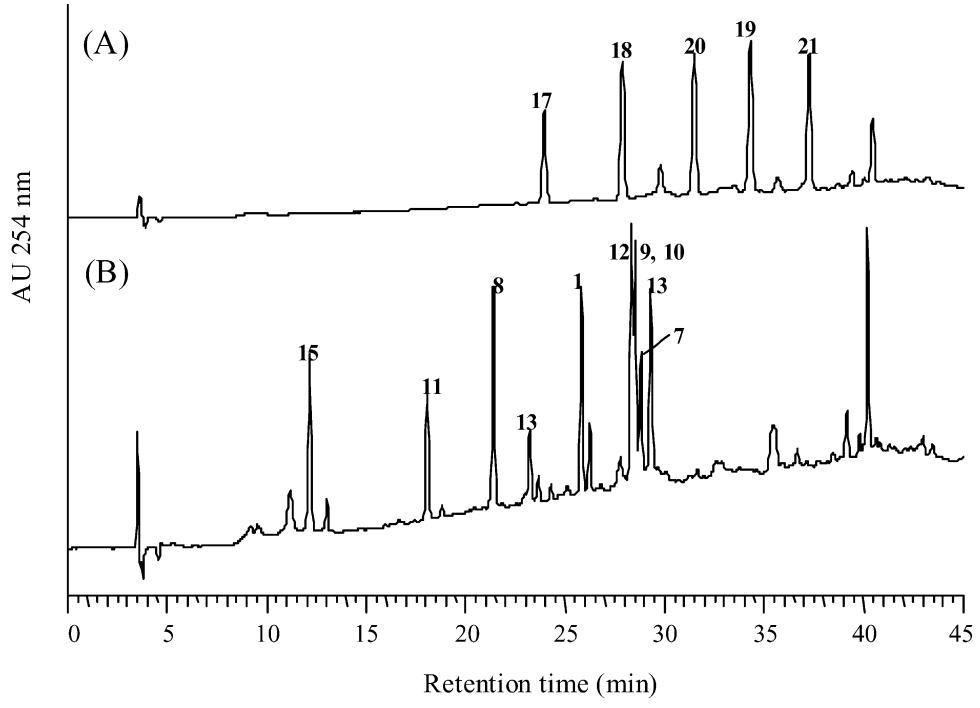


Fig. 2. HPLC/DAD chromatograms of standards compounds detected at 254 nm. (A) Chromatograms of standard anthraquinones. (B) Chromatograms of standard alkaloids. **1**, camptothecin; **7**, strictosamide; **8**, 3(*S*)-pumiloside; **9**, 3(*S*)-deoxypumiloside; **10**, 3(*R*)-deoxypumiloside; **11**, chaboside; **12**, 9-methoxycamptothecin; **13**, 10-hydroxycamptothecin; **14**, lyalosidic acid; **16**, mappicine; **17**, lucidin 3-*O*-β-purimeveroside; **18**, 3-hydroxy-2-hydroxymethylanthraquinone; **19**, 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone; **20**, 2-hydroxymethyl-3-methoxyanthraquinone; **21**, 1,3-sihydroxy-2-methoxymethylanthraquinone. The peak numbers corresponding to the compound numbers in Table 1. Mobile phase: 0–35 min linear gradient from solvent A [H_2O–HOAc–MeOH (79.8:0.2:20)] to solvent B [H_2O–HOAc–MeOH (9.975:0.025:90)], 35–40 min isocratic at 100% of solvent B.

Table 1
Alkaloids and anthraquinones detected in *O. pumila*, *C. acuminata* and *N. foetida*

Compound No.[a]	R_t[b] (min)	λ_{max} (nm)	ESI/MS(/MS) (*m*/*z*)	*O. pumila*				*C. acuminata*	*N. foetida*
				Plant	Sterile plant	Hairy root	Callus	Plant	Plant
1 Camptothecin	25.84	251, 288, 364	349.18 $[M+H]^+$; 320.08 $[M-C_2H_5+H]^+$ [c]; 305.21 $[M-CO_2+H]^+$ [c]	0.030[d]	0.051[d]	0.024[d]		0.146[d]	0.048[d]
6 Strictosidine	14.92	228, 239, 271, 280	531.18 $[M+H]^+$; 514.21 $[M-OH+H]^+$ [c]; 369.16 $[M-Glc+H]^+$ [c]						
7 Strictosamide	28.80	242, 280, 290	499.08 $[M+H]^+$; 337.20 $[M-Glc+H]^+$ [c]	+	+				
8 3(*S*)-Pumiloside	21.39	243, 315, 327	513.05 $[M+H]^+$; 351.22 $[M-Glc+H]^+$ [c]	+	+	+			
9 3(*S*)-Deoxypumiloside **10** 3(*R*)-Deoxypumiloside	28.53[e]	238, 307, 319	497.09 $[M+H]^+$; 335.08 $[M-Glc+H]^+$	+	+				
11 Chaboside	18.08	240, 267, 293, 325, 370	557.14 $[M+H]^+$; 395.18 $[M-Glc+H]^+$ [f]	0.069[d]	0.030[d]				
12 9-Methoxycamptothecin	28.35	261, 304, 319, 358	379.14 $[M+H]^+$; 335.24 $[M-CO_2+H]^+$	0.007[d]	0.014[d]				0.069[d]
13 10-Hydroxycamptothecin	23.23	240, 265, 313, 327, 377	365.19 $[M+H]^+$						
14 Strictosidinic acid	15.04	223, 271, 280, 288	517.12 $[M+H]^+$; 500.18 $[M-OH+H]^+$	+	+				
15 Lyalosidic acid	12.19	246, 304, 374	513.06 $[M+H]^+$; 351.12 $[M-Glc+H]^+$ [f]						
16 Mappicine	29.31	245, 287, 367	307.28 $[M+H]^+$						
17 Lucidin 3-*O*-β-purimeveroside	23.55	245, 265, 405	565.34 $[M+H]^+$; 547.16 $[M-H_2O+H]^+$			+			
18 3-Hydroxy-2-hydroxymethyl-anthraquinone	27.47	245, 276, 336	255.11 $[M+H]^+$; 237.01 $[M-H_2O+H]^+$ [f]			+			
19 1-Hydroxy-2-hydroxymethyl-3-methoxyanthraquinone	33.92	245, 273, 405	285.29 $[M+H]^+$				+		
20 2-Hydroxymethyl-3-methoxy-anthraquinone	31.09	243, 273, 334	269.19 $[M+H]^+$						
21 1,3-Dihydroxy-2-methoxy-methylanthraquinone	36.85	245, 281, 409	285.17 $[M+H]^+$; 253.49 $[M-CH_3OH+H]^+$ [c]						
22 3-*O*-Caffeoylquinic acid	11.69	219, 235, 297, 325	355.07 $[M+H]^+$ [c]; 163.05 $[M-C_7H_{12}O_6+H]^+$ [c]	+	+	+		+	
U-1[g]	27.05	239, 305, 320		+	+	+			
Q-1[h]	25.12	245, 276, 336				+			
Q-2[h]	29.16	243, 273, 405				+			
Q-3[h]	32.72	245, 280, 409				+			
Q-4[h]	35.84	245, 276, 334				+			
Q-5[h]	27.17	255, 281, 334, 405					+		
Q-6[h]	31.43	243, 271, 339					+		
Q-7[h]	33.09	245, 276, 331, 384					+		
Q-8[h]	33.47	251, 281, 384, 400					+		

[a] Structure in figures shows only a plausible enantiomer. However, the presence of the opposite enantiomer cannot be ruled out.
[b] R_t, retention time (min).
[c] *m*/*z* Values of the major fragment obtained both mass and tandem mass data.
[d] Numbers are alkaloid content (%) (dry weight).
[e] 3(*S*)-Deoxypumiloside (**9**) and 3(*R*)-deoxypumiloside (**10**) cannot be separated in this condition.
[f] *m*/*z* Values of the major fragment obtained tandem mass data.
[g] Unknown compound.
[h] Unknown compound having anthraquinone skeleton deduced from UV spectra.

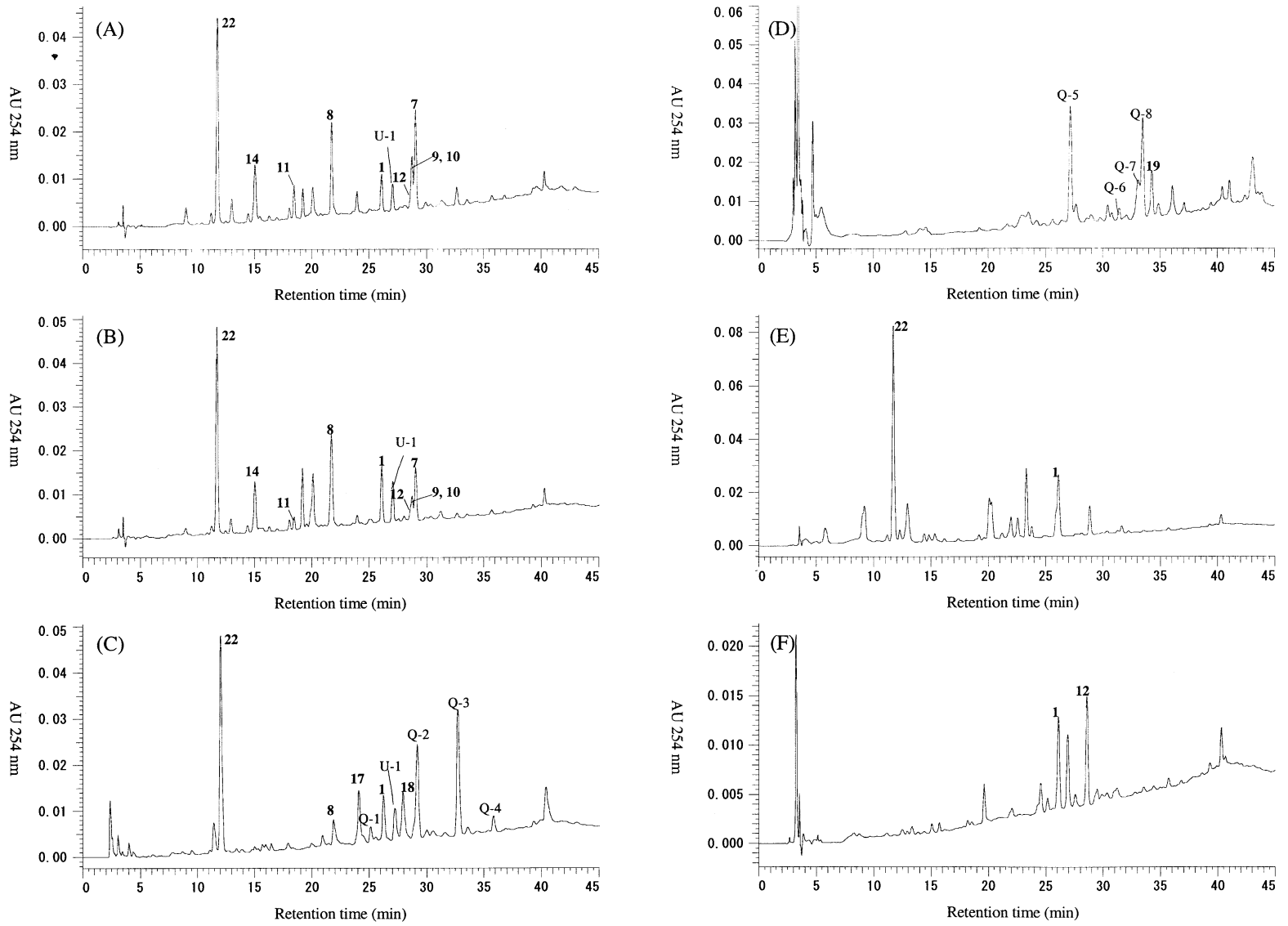


Fig. 3. HPLC/DAD chromatograms of the plant extracts detected at 254 nm. (A) *O. pumila* pot-growing plant extracts, (B) *O. pumila* sterile plant extracts, (C) *O. pumila* hairy root extracts, (D) *O. pumila* callus extracts, (E) *Camptotheca acuminata* plant extracts, (F) *Nothapodytes foetida* plant extracts. The peak numbers correspond to the compounds listed in Table 1.

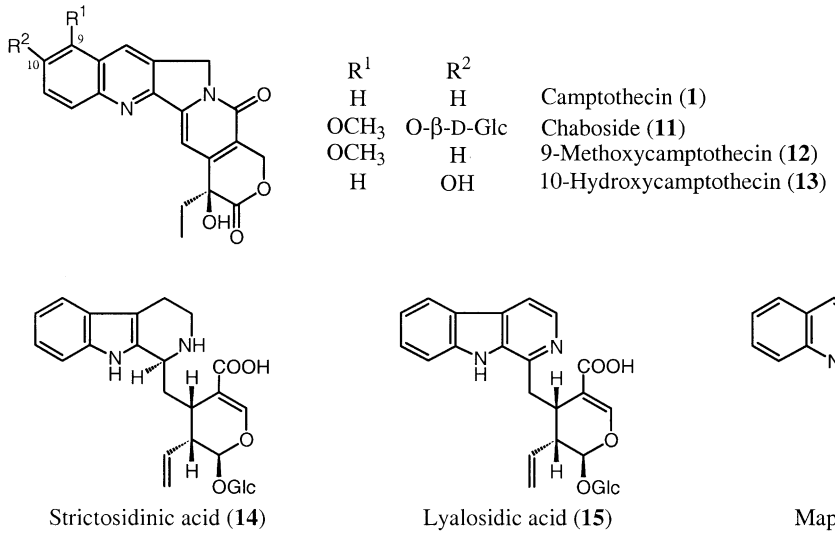


Fig. 4. Chemical structures of camptothecin-related alkaloids.

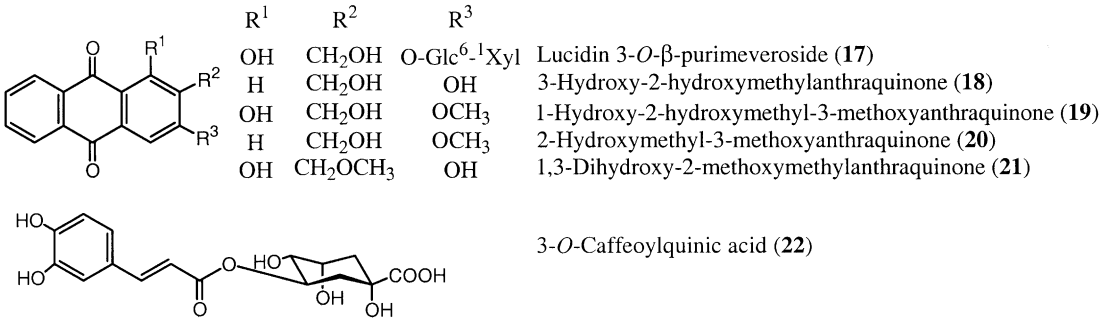


Fig. 5. Chemical structures of anthraquinones and 3-*O*-caffeoylquinic acid (**22**).

notable that anthraquinones produced in calli are different from those in hairy roots, suggesting that distinct anthraquinone biosynthetic enzymes are induced in these tissues. The alkaloid pattern of extracts of leaves of *C. acuminata* and *N. foetida* are relatively simple (Fig. 3E and F, Table 1). In *C. acuminata*, camptothecin (**1**) was the major alkaloid. 10-Hydroxycamptothecin (**13**) has been reported to be present in *C. acuminata* (Wani and Wall, 1968). However, we did not detect this compound in our sample. In *N. foetida*, camptothecin (**1**) and 9-methoxycamptothecin (**12**) were present in almost equal amounts.

2.2. Distribution of camptothecin (*1*) in different tissues of *O. pumila* plant

Camptothecin (**1**) was extracted from different parts of 6-month-old *O. pumila* plants (Fig. 6) and 3-week-old hairy roots, and quantified by HPLC/DAD. The highest levels of camptothecin accumulation were found in flower buds followed by young leaves, stems and roots. Old leaves contained lower levels of camptothecin (**1**). When compared to per leaf basis, the top leaves contain 2-fold camptothecin (**1**) /leaf to the old leaves. Also in *C. acuminata*, the similar distribution was observed (López-Meyer et al., 1994). In hairy roots of *O. pumila*, camptothecin accumulated at the similar level with that in roots.

2.3. Distribution of STR activity in different tissues of *O. pumila*

The STR activity was measured in the protein extracts of different tissues of plants and hairy roots of *O. pumila* (Table 2). The STR enzymatic activities were detected in the extracts of stems, roots and hairy roots, however, no activity was detected in the leaf and callus extracts. The distribution of STR activity was well correlated with the pattern of mRNA accumulation by northern blot analysis (Yamazaki et al., submitted). The patterns of enzyme activity of STR and mRNA expression were roughly correlated with camptothecin accumulation in tissues of *O. pumila*, except for young leaves, suggesting that roots and stems are presumably the main tissues for camptothecin biosynthesis. In the protein extracts from *C. acuminata* and *N. foetida*, no STR activity was detected in the roots, stems and leaves.

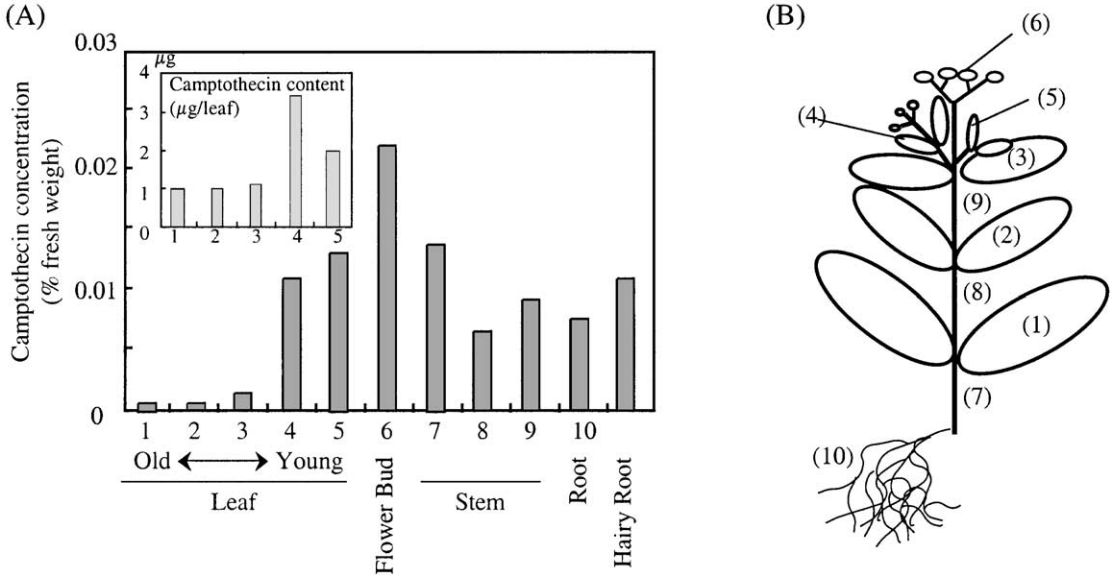


Fig. 6. Distribution of camptothecin content in 6-month-old *O. pumila* plants and 3-week-old hairy roots. (A) Camptothecin accumulation in different parts of the plants and hairy roots. (B) Different parts of the plants examined. Numbers correspond to those in (A).

Table 2
STR enzyme activities in protein extracts and expression of the *STR* mRNA in *O. pumila* tissues

	Leaf	Stem	Root	Hairy root	Callus
STR enzymatic activity (pkat/mg±S.D.[a])	<5	43±6	49±1	324±16	<5
STR mRNA expression[b]	–	++	++	+++	n.d.[c]

[a] S.D., standard deviation ($n=3$).
[b] Yamazaki et al., submitted.
[c] n.d., Not determined.

2.4. Enzymatic characterization of STR in O. pumila

The crude enzyme preparation extracted from hairy roots and plants was found to form stereospecifically strictosidine (**6**) from tryptamine (**4**) and secologanin (**5**) as the substrates, and no formation of vincoside, 3β(*R*)-epimer of strictosidine (**6**), was detected. In addition, no strictosamide (**7**) was produced under the experimental condition. It should be noted that strictosamide (**7**) is formed non-enzymatically from strictosidine (**6**) under basic condition, the conversion was completed at pH 11 within 10 min at room temperature. The production of strictosidine (**6**) was not affected by the presence of glucono δ-lactone, an inhibitor of the activity of glucosidase, suggesting no further glucosidase-catalyzed reaction takes place for strictosidine (**6**) by the protein extracts.

The apparent K_m values for hairy root protein extracts were 0.50 and 0.90 mM for tryptamine (**4**) and secologanin (**5**), respectively, and V_{max} value was 430 pkat/mg of protein. The K_m values for the plant protein extracts were 0.40 and 0.60 mM, respectively, and V_{max} value was 46.4 pkat/mg of protein. The substrate inhibition was observed for neither tryptamine (**4**) nor secologanin (**5**).

3. Discussion

Although total synthesis of camptothecin (**1**) has been already achieved, the clinically formulated anti-tumor medicines, topotecan (**2**) and irinotecan (**3**), are currently semi-synthesized using natural camptothecin (**1**) as a starting material extracted from *C. acuminata* and *N. foetida*. This semi-synthetic process requires multiple steps of chemical conversion to these clinically used compounds. In this context, chaboside (**11**), 9-methoxy and 10-glucoside of camptothecin (**1**), might be a promising candidate for efficient precursor of topotecan (**2**), irinotecan (**3**) and other possible new anti-tumor compounds with C-10 hydroxyl moiety. In plants of *O. pumila*, chaboside (**9**) was found in approximately 65% of total camptothecin-related compounds (Table 1). Unfortunately, hairy roots of *O. pumila* did not produce chaboside (**11**) (Fig. 3C, Table 1). 3-*O*-Caffeoylquinic acid (**22**) was mainly detected in the extracts of differentiated plants of *O. pumila* and *C. acuminata*. Anthraquinones, that are inducibly produced phytoalexins as a defense response, were detected in hairy roots and calli with the different compound patterns (Fig. 3C, D, Table 1). These findings indicate that the hairy roots and calli were under stress conditions to result in production of anthraquinone as phytoalexin. In Rubiaceae plants, anthraquinones are regarded as phytoalexins and reported to be produced under stress conditions such as tissue culture conditions (Wijnsma et al., 1985, 1986).

The highest accumulation of camptothecin (**1**) in young leaves, flower buds and roots (Fig. 6), which are the important parts of reproduction and growing of a plant, is presumably benefitable to plant for the prevention from attacks by insects and fungi. The observed discrepancy of plants parts, in particular young leaves, between STR enzyme activity and camptothecin accumulation may be caused by that camptothecin biosynthesis may occur in the limited parts where expression of mRNA and enzyme activity takes place and then camptothecin (**1**) is transported to other parts in spite of its very low solubility. The precise mechanism of transport and storage of camptothecin (**1**) remains unclear. One possible mechanism for transport of such insoluble compound is conversion into a soluble compound such as camptothecin glucoside, e.g., chaboside (**11**), and transport to other parts.

In cell-free extracts from hairy root of *O. pumila*, STR only catalyzes the stereospecific synthesis of strictosidine (**6**). The conversion of strictosidine (**6**) to strictosamide (**7**) remains unclear whether it is an enzyme-catalyzed reaction or simple non-enzymatic removal of methanol. At a subcellular level, strictosidine (**6**) is likely formed in the vacuole deduced from the structure of *O. pumila* STR enzyme (Yamazaki et al., submitted). Although the transport of strictosidine (**6**) itself has not been studied yet, strictosidine (**6**) may need to be translocated to other organelles for further conversion into camptothecin (**1**). Interestingly, no strictosidine (**6**) could be detected in the extracts of any tissue of *O. pumila* (Fig. 3), suggesting the rapid conversion of strictosidine (**6**) in vivo.

4. Conclusions

The reverse-phase HPLC/DAD/ESI/MS method was applied to allow the separation and identification of alkaloids as well as anthraquinones from the MeOH extracts of *O. pumila*, *C. acuminata* and *N. foetida* plants and different tissues. Since *O. pumila* produces a variety of camptothecin-related alkaloids, it may be a promising resource of camptothecin (**1**) and related alkaloids that can be new lead compounds for anticancer drug development.

STR activity detected in *O. pumila* tissues only catalyzed synthesis of strictosidine (**6**). The tissue-specific distribution of STR activity was well correlated with *STR* mRNA expression in *O. pumila*.

5. Experimental

5.1. Plant materials

Sterilized plants (Kitajima et al., 1997b), hairy roots (Saito et al., 2001) and calli (Kitajima et al., 1998) of *O. pumila* were cultured as described previously. The wild plants of *O. pumila* were collected in Amami-oshima Island, Kagoshima, Japan in March 2002, and *N. foetida* was collected in Ishigaki-Island, Okinawa, Japan in July 2002, and grown in a plant growth chamber with a photoperiod of 18 h light (4500 lx)/6 h dark at 25 °C. Voucher specimens are deposited at the Herbarium of the Medicinal Botanical Gardens of Chiba University. Young leaves of *C. acuminata* were collected at the Medicinal Botanical Gardens of Chiba University, Japan in May 2002.

5.2. Chemicals

Standard compounds, camptothecin (**1**), strictosidine (**6**), strictosamide (**7**), 3(*S*)-pumiloside (**8**), 3(*S*)-deoxypumiloside (**9**), 3(*R*)-deoxypumiloside (**10**), chaboside (**11**), 9-methoxycamptothecin (**12**), strictosidinic acid (**14**), lyalosidic acid (**15**), lucidin 3-*O*-β-purimeveroside (**17**), 3-hydroxy-2-hydroxymethylanthraquinone (**18**), 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone (**19**), 2-hydroxymethyl-3-methoxyanthraquinone (**20**), 1,3-dihydroxy-2-methoxymethylanthraquinone (**21**) and 3-*O*-caffeoylquinic acid (**22**) were extracted and purified from *O. pumila* and *O. kuroiwai* (Govindachari and Viswanathan, 1972; Kelley et al., 1976; Hutchinson et al., 1979; Itokawa et al., 1983; Chang and Lee, 1984; Aimi et al., 1989, 1990; Kitajima et al., 1997a,b, 1998). Standard strictosidine (**6**) was synthesized from tryptamine (**4**) and secologanin (**5**) (Battersby et al., 1968; Blackstock et al., 1972). Standard 10-hydroxycamptothecin (**13**) was chemically synthesized (Aimi et al., unpublished). Standard mappicine (**16**) was semi-synthesized from camptothecin (**1**) (Fortunak et al., 1994; Boger and Hong, 1998). Tryptamine (**4**) and secologanin (**5**) were purchased from Nacalai tesque (Kyoto) and Sigma-Aldrich (St. Louis, MO), respectively.

5.3. Analytical methods

The plant materials were homogenized in a mortar and pestle. Per gram of plant material, 10 ml MeOH was added. After extracting in a ultrasonic bath for 15 min, the resulting homogenate was centrifuged at 10,000×*g* for 10 min. The supernatants were filtered through a 45 μm filter (Corning, Plaza Corning, NY) and analyzed by HPLC/DAD and HPLC/DAD/ESI/MS.

All mass spectra were acquired using ThermoQuest model LCQ^{DECA} (San Jose, CA) ion-trap mass spectrometer equipped with an ESI source. The instrument was coupled with HPLC binary pump (G1312B), DAD (G1315B) and auto sampler (G1329A) (Agilent, Palo Alto, CA). The separation was carried out on a Mightysil-RP18 column (5 μm, 250×4.6mm; Kanto Chem. Tokyo) at a flow rate of 0.8 ml/min. Elution gradient

program was as follows: 0–35 min linear gradient from solvent A [H_2O–HOAc–MeOH (79.8:0.2:20)] to solvent B [H_2O–HOAc–MeOH (9.975:0.025:90)], 35–40 min isocratic at 100% of solvent B. The positive-ion ESI was performed with an ion source voltage of 5.0 kV and a capillary offset voltage of 10 V. Nitrogen was used both as drying and auxiliary gas, and capillary temperature was 350 °C. Mass spectra were recorded over the range 210–600 m/z at 2 scans/s. Alkaloids and anthraquinones were identified by their MS spectra, their UV spectra and by their retention times compared with the data obtained with the standards.

Quantitative analysis was performed by HPLC/DAD system (binary pump, L-7100; DAD, L-7455; auto sampler, L-7200; Hitachi, Tokyo) using the same column as HPLC/DAD/ESI/MS analysis. Quantification of alkaloids was achieved by integration of UV 365 nm chromatograms by calibration with the standard compounds.

5.4. Protein extraction from plant

The plant materials were immediately frozen in liquid nitrogen. Frozen tissues were grinded to fine powder in a mortar and pestle. Per gram of frozen powdered plant material, 5% polyvinylpolypyrrolidone and 2 ml buffer A (0.1 M sodium phosphate buffer pH 7.2, 2 mM EDTA, 4 mM 1,4-dithiothreitol) were added. After thawing and homogenizing, the resulting homogenate was centrifuged at 10,000×g for 5 min. The supernatant was desalted and buffer-changed to buffer B (50 mM sodium phosphate buffer pH 6.8, 1 mM phenylmethanesulfonyl fluoride, 5 mM β-mercaptoethanol, 150 mM sodium chloride, 0.1% Triton X-100) on Sephadex G-25 (PD-10 column, Amersham Pharmacia Biotech, Uppsala). The buffer-changed protein was concentrated to 1 ml using Centricon YM-30 (Millipore, Bedford, MA). The protein concentration was determined using BioRad's adaptation of the Bradford dye assay (Bradford, 1976) with bovine serum albumin as standard.

5.5. Enzyme assay of STR in protein extracts

The STR activity in the protein extracts was measured as the formation of strictosidine (**6**) from tryptamine (**4**) and secologanin (**5**) as described previously (Pennings et al., 1989). The standard 100 μl reaction mixture contained 1 mM tryptamine (**4**) (hydrochloric salt), 5 mM secologanin (**5**), buffer B and 350–500 μg proteins. The assay mixtures were incubated at 37 °C for 20 min. The reaction was terminated by cooling on ice prior to HPLC analysis. The quantity of strictosidine (**6**) formed was determined by HPLC. The $K_{m(app)}$ values were determined from Michaelis-Menten plots using six different substrate concentrations.

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