

## Aliphatic Amine-based Ionic Liquids for Effective Production of Paclitaxel and Related Taxanes in Plant Cell Culture

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We report here hydrophobic ionic liquids (ILs) which enhance the productivity of paclitaxel and the related taxanes of 10-deacetyl baccatin III, baccatin III and cepharomannine with *in situ* extraction from an aqueous medium in suspension cell culture of *Taxus cuspidata* in the IL-medium two phase culture system. Two aliphatic amine-based ILs of N-methyl-n-propyl-piperidinium bis(trifluoromethane-sulfonyl)imide (PP13-TFSI) and N-methyl-n-butylpyrrolidinium bis(trifluoromethanesulfonyl)imide (P14-TFSI) were used. It was found that the two ILs had no cytotoxicity and enhanced productivity of paclitaxel and the related taxanes in the cell culture. In particular, more hydrophobic P14-TFSI, which enhanced the productivity of paclitaxel and the total taxanes by a factor of more than 2 compared to PP13-TFSI, could be an effective extractant.

### 1. Introduction

Paclitaxel (PX) is an expensive anticancer drug because the conventional semi-synthetic method requires many steps of reactions of precursors such as baccatin III or 10-deacetyl baccatin III extracted from yew tree needles. A culture using callus induced from the needles is one of the promising methods for the cost-effective production of paclitaxel. However, there is a problem of feedback-inhibition of the paclitaxel produced in the culture. In order to reduce the paclitaxel's inhibition, two phase culture system using water-immiscible organic solvents, have been proposed for the *in situ* extraction of hydrophobic paclitaxel from the culture medium [1-2]. Thus, we investigated the use of hydrophobic ionic liquids (ILs) for the *in situ* extraction of paclitaxel from the aqueous medium [3] and reported that 1-hexyl-3-methylimidazolium hexafluorophosphate (HMIN-PF<sub>6</sub>) extracted paclitaxel from the aqueous medium [4]. ILs have gained considerable attention as safer solvents in contrast to conventional organic solvents because of their unique properties such as negligible volatility, high thermal stability, and selective solubility. ILs have been applied for extraction and separation of bioactive compounds [5-9]. It is reported that HMIN-PF<sub>6</sub> extracted hydrophobic 3-indole-butyric acid from pea plants [10]. Ferulic acid and caffeine acid found in various plants could be readily extracted with HMIN-PF<sub>6</sub> [11]. Recently, higher extraction efficiencies were obtained for DNA molecules using magnetic ILs such as the benzyltrioctylammonium bromotrichloroferrate (III) ( $[(C_8)_3BnN^+][FeCl_3Br^-]$ ) and 1,12-di(3-hexadecylbenzimidazolium) dodecane bis[(trifluoromethyl)-sulfonyl]imide bromotrichloroferrate (III) ( $[(C_{16}BnIM)_2C_{12}^{2+}][NTf_2^-, FeCl_3Br^-]$ ) [12]. Though there is a report on enhancement of extraction efficiency of paclitaxel from

biomass taken from the culture broth using a co-solvent made of 1-butyl-3-methylimidazolium tetrafluorophosphate (BMIN-BF<sub>4</sub>) and methanol under acidic conditions [13], there is no report on the *in situ* extraction of paclitaxel and the related taxanes of 10-deacetyl baccatin III (10-DAB), baccatin III (BIII) and cepharomannine (CM) in the plant cell culture with the ILs except our study [4]. Figure 1 shows the main metabolic pathway of PX and the related taxanes from geranylgeranyl diphosphate [14].

In the present research the effect of two hydrophobic aliphatic amine-based ILs of N-methyl-n-propylpiperidinium bis(trifluoromethanesulfonyl) imide (PP13-TFSI) and N-methyl-n-butylpyrrolidinium bis(trifluoromethanesulfonyl) imide (P14-TFSI) rather than HMIN-PF<sub>6</sub> on the enhancement of the productivity of PX and the related taxanes of 10-DAB, BIII and CM by the *in situ* extraction in plant cell culture were investigated considering their cytotoxicity.

## 2. Experimental

### 2.1 Reagents

Two aliphatic amine-based ILs of N-methyl-n-propylpiperidinium bis(trifluoromethanesulfonyl)imide (PP13-TFSI) and N-methyl-n-butylpyrrolidinium bis(trifluoromethanesulfonyl)imide (P14-TFSI) purchased from Kanto Chemical Co. (Tokyo, Japan) were used. Their physical properties and chemical formulas are shown in Table 1. 1-Hexyl-3-methylimidazolium hexafluorophosphate (HMIN-PF<sub>6</sub>, TCI, Tokyo, Japan), which was found to enhance the productivity of the taxanes in the previous report [14], was used to compare the efficiency of the taxane production to the two ILs.

### 2.2 Plant cell culture

Callus induced from the needles of *Taxus cuspidata* and a modified Gamborg's B5 medium [1] were used for the culture. Suspension culture inoculated by the precultured cells was carried out in a 100 cm<sup>3</sup> Erlenmeyer flask containing 20 cm<sup>3</sup> of the B5 medium and 1 cm<sup>3</sup> of the ILs (5 vol%) on a rotary shaker (NR-150, Taitec, Saitama, Japan) at 110 rpm in the dark at 26 °C. During the culture the amounts of fresh cells, paclitaxel and the related taxanes in the culture flask were measured.

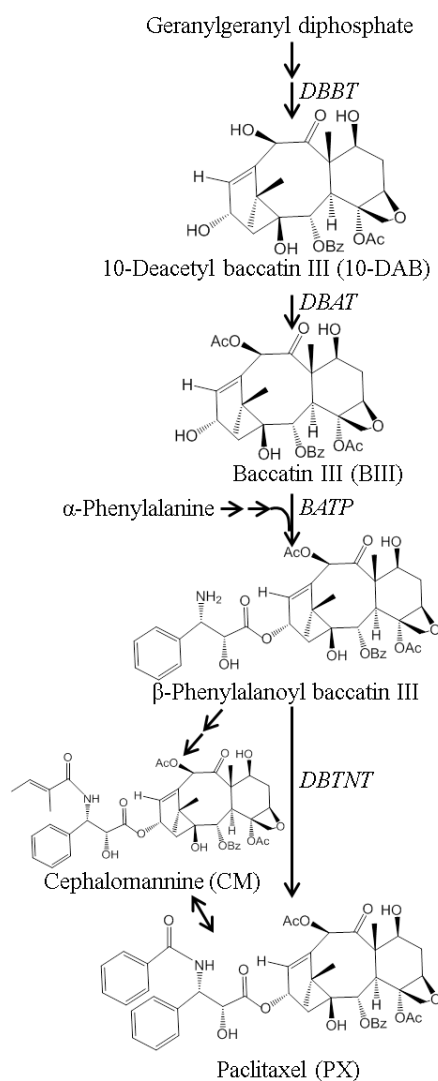


Figure 1 Main metabolic pathway of paclitaxel and the related taxanes from geranylgeranyl diphosphate [14].

*DBBT*: taxane 2a-O-benzoyl transferase, *DBAT*: 10-deacetyl baccatin III-10-O-acetyltransferase, *BAPT*: baccatin III: 3- amino-3- phenylpropanoylltransferase, *DBTNBT*: 3'-N-debenzoyl-2'-deoxytaxol-N-benzoyltransferase c.

### 2.3 Evaluation of cytotoxicity and effectiveness for taxane production of ILs

To examine the cytotoxicity and the effectiveness for the taxanes production of ILs, the suspension cell culture with *in situ* extraction with 5 vol% IL was carried out. The suspension cell culture in the absence of IL was conducted as the control culture.

For examination of the cytotoxicity, relative cell growth rate,  $R_{FCW}$ , were defined as follows,

$$R_{FCW} [-] = FCW_{IL}/FCW_C \quad (1)$$

where  $FCW_{IL}$  and  $FCW_C$  are the fresh cell weight in the cultures including IL and that in the control after 7 d culture periods, respectively.

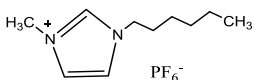
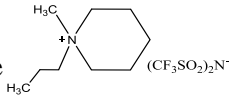
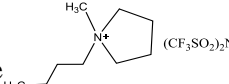
If an IL has cytotoxicity against the cells, the value of  $R_{FCW}$  value will be less than 1.

For evaluation of the effectiveness of the IL on the productivity of the taxanes, the specific production rate of the taxane,  $E_{taxane}$  was defined as follows,

$$E_{taxane} [\mu\text{g}/(\text{g-cell} \cdot \text{d})] = \Delta P / (x \cdot \Delta t) \quad (2)$$

where  $\Delta P$  is the amount of produced taxane (10-DAB, BIII, PX, CM and total amount of these taxanes (total)) during a culture period of  $\Delta t$ ,  $\Delta t$  is 7 d and  $x$  is fresh cells weight.

Table 1 Chemical formula and properties of ILs used in this reseach

IL	Chemical formula	Abbreviation	Solubility in aqueous medium [mM]	Partition coefficient of paclitaxel in IL-medium two phase system [-]
1-Hexyl-3-methylimidazolium hexafluorophosphate		HMIN-PF <sub>6</sub>	29.5	160
N-Methyl-n-propylpiperidinium bis(trifluoromethanesulfonyl)imide		PP13-TFSI	19.9	37.1
N-Methyl-n-butylpyrrolidinium bis(trifluoromethanesulfonyl)imide		P14-TFSI	16.1	45.4

### 2.4 Analysis

Cells were harvested from cultures, washed with water, blotted on filter paper to remove excess liquid, and then weighed to determine FCW. The amounts of the paclitaxel and the related taxanes in the medium phase, the IL phase and the cells in all samples were analyzed by using a reversed-phase HPLC system according to the analytical procedures as described previously [2].

## 3. Results and Discussion

### 3.1 Cytotoxicity of ILs

The weight of the fresh cells in the culture including each IL after a 7 d culture period is shown in Figure 2. Regardless of the kinds of ILs, the fresh cells weight in the culture with the IL was as similar as that in the contorol culture and the values of  $R_{FCW}$  under all the culture conditions were

almost 1, indicating that all the ILs used in the present research have no cytotoxicity.

### 3.2 Effects of ILs on the productivities of paclitaxel and the related taxanes

Figure 3 shows the effect of ILs on the productivity of PX,  $P_{PX}$ , which is defined by Equation (2). The values of  $P_{PX}$  in the cultures including the ILs increased compared to that in the control culture. This increase might be caused by partition of the produced PX into the ILs. The  $P_{PX}$  value of P14-TFSI was 2 times higher than that of PP13-TFSI. P14-TFSI was more effective to extract and produce PX than PP13-TFSI and HMIN-PF<sub>6</sub> (Figure 3), resulted from its stronger hydrophobicity than PP13-TFSI due to the lowered solubility in the aqueous medium than that of PP13-TFSI (Table 1).

The ILs might affect the activities of enzymes related to the biosynthesis of PX. Thus, the productivities of the related taxanes of 10-DAB, BIII and CM (Figure 1) in the culture including the ILs were examined. As shown in Figure 3, the productivities of the taxanes in the culture with the ILs were greater than those of the control culture. Greatest productivities of the total taxanes, which mean the total sum of the taxanes, in the culture with P14-TFSI were observed. The value of  $E_{total}$  in the culture with P14-TFSI was 2 times greater than that with PP13-TFSI. These results suggest that P14-TFSI could be an excellent extractant of PX and a stimulator for the enzymes

related to the metabolic pathway of paclitaxel synthesis in the plant cell culture. Though the reason for the stimulation of the metabolism was still unclear, this should be further studied. An effective method for the back-extraction of the target taxanes from the ILs should be explored. A back-extraction and recovery of the taxanes from the ILs by adjustment of pH was under consideration because adjustment of the medium pH contributed to the efficient back-extraction of hydrophobic ferulic acid and caffeine acid from HMIN-PF<sub>6</sub> [11]

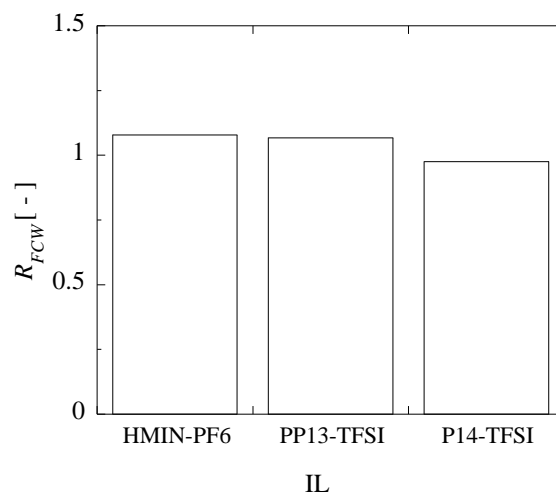


Figure 2 Value of  $R_{FCW}$  after a 7 d culture period in the culture including 5 vol% IL.

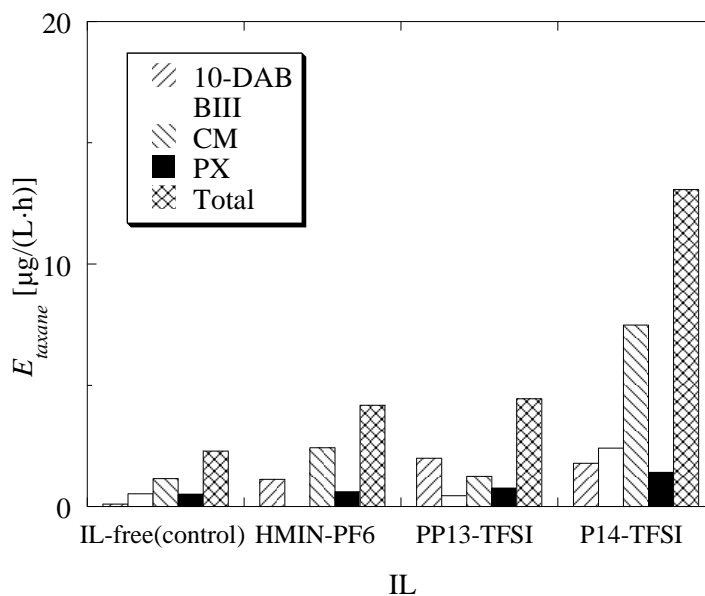


Figure 3 Productivities of 10-DAB, BIII, CM and PX after a 7 d culture period in the culture including 5 vol% IL. "Total" means the productivities of total sum of the taxanes.

#### 4. Conclusion

For effective *in situ* extraction of the anticancer drug, paclitaxel, from the aqueous medium in the suspension cell culture of *T. cuspidata*, two aliphatic amine-based ILs of N-methyl-n-propyl-piperidinium bis(trifluoromethanesulfonyl)imide (PP13-TFSI) and N-methyl-n-butylpyrrolidinium bis(trifluoromethanesulfonyl)imide (P14-TFSI) as extractants were used in the cell culture. The two ILs were found to have no cytotoxicity and increased the productivities of paclitaxel and the related taxanes of 10-DAB, BIII and CM. Greatest productivities of paclitaxel and the total taxanes in the culture with P14-TFSI was observed, suggesting that P14-TFSI could be an excellent extractant and stimulator for the enzymes related to the metabolic pathway of paclitaxel synthesis.

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