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Studies on factors influencing stability and recovery of paclitaxel from suspension media and cultures of *Taxus cuspidata* cv *Densiformis* by high-performance liquid chromatography

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Abstract

An HPLC method was developed for quick scanning of taxanes from large numbers of plant cell suspension samples. The method was optimized for analysis of a range of taxanes of differing polarity. Identification of a standard mixture of paclitaxel and 12 related taxanes was achieved in less than 15 min using a gradient mode and a Microsorb-MV C_8 column. The method was used to investigate the influence of several factors on stability and recovery of paclitaxel from suspension media and cultures of *Taxus cuspidata* cv *Densiformis*. Incubation time had the most significant influence on stability of paclitaxel, contributing 88% to the total variation. Shaking contributed 6% to the total variation. Light contributed only 0.25% to the total variation. Analysis of test samples of suspension cultures of *T. cuspidata* cv *Densiformis* over a 4 week period show paclitaxel, 10-deacetylbaccatin III, and baccatin III levels ranging from 0 to 149 μg/L, from 0 to 1.9 mg/L, and from 0 to 583 μg/L, respectively.

Keywords: *Taxus cuspidata*; Paclitaxel; Taxanes

1. Introduction

Paclitaxel is an important antitumor compound that has the ability to bind and stabilize microtubules and block cell replication in the late G2-M phase of the cell cycle [1]. This compound originally was harvested from the bark of the Pacific Yew, *Taxus brevifolia* Nutt.; however, the bark from approximately three to six trees is required to supply enough paclitaxel for the treatment of one patient [3]. Continued harvesting of the bark from these trees eventually would lead to a complete loss of this supply. Paclitaxel has since been isolated from many other species of *Taxus* and from different parts of these plants [2]. For example, paclitaxel and its precursors can be harvested from leaves of yews in concentrations comparable to the bark [4]. Leaves are renewable resources but limited to seasonal growth.

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Other biological sources for supplying paclitaxel being examined include fungi [5], hickory nuts [6], and tissue cultures of various Yews [2]. The goal of using tissue culture as a paclitaxel production method requires fast growing cultures with high and stable productivity. However, production of paclitaxel in these cultures shows considerable variation, and this variability remains the single most problematic issue in using cell cultures [7]. This variation is due to a number of factors including differences in cell lines [7], in genetic stability of the same cell line [8] and in sensitivity of productivity to culture conditions [9].

However, another source of variation, which may limit the total amount of paclitaxel in these cultures, is the stability of this compound in culture media. Paclitaxel was found to be susceptible to hydrolysis and epimerization in animal culture media, with 7-epitaxol being the major derivative, causing the concentration of paclitaxel to decrease with time [10]. Since paclitaxel accumulates over a period of several weeks in cell suspension cultures following stimulation with methyl jasmonate [7], it is important to determine the stability of this compound under these conditions in plant culture media. The purpose of the present study was to examine the influence of incubation time, shaking, and light on stability and recovery of paclitaxel in media used for plant cell suspension cultures. An HPLC method was developed for this purpose which allowed separation and identification of paclitaxel and 12 other taxanes in less than 15 min, using a Microsorb-MV C_8 column with a gradient mode. This method was also applied in the analysis of test samples of suspension cultures of *Taxus cuspidata* Siebold & Zucc. cv *Densiformis* (Taxaceae).

2. Experimental

2.1. Chemicals

Paclitaxel and 10-deacetylbaccatin III standards were obtained from Sigma (St. Louis, MO, USA). A mixture of 13 standard taxanes was supplied by Hauser Chemical Research (Boulder, CO, USA). This mixture consisted of: baccatin III, cephalomannine, 10-deacetylbaccatin III, 10-deacetyltaxol, 7-epi-10-deacetyltaxol, 7-epitaxol, paclitaxel, taxinine M, taxol C, 7-xylosyl-10-deacetyltaxol, 7-xylosyl-10-deacetyltaxol B, 7-xylosyl-10-deacetyltaxol C, and 7-xylosyltaxol.

Acetonitrile, methanol, and methylene chloride all were of analytical grade. Gamborg's B5 medium and α-naphthaleneacetic acid (NAA) were purchased from Carolina Biological Supply (Burlington, NC, USA). Casein hydrolysate and picloram were purchased from Sigma. Water of nanopure quality was used throughout the study.

2.2. Instrumentation

The HPLC system consisted of a manual Rheodyne injector, Waters 600 gradient pump, Waters 486 tunable absorbance detector, and Waters 600 controller. Waters Millennium Chromatography software was used for data acquisition, processing, and reporting. A Varian (Varian Associates, Walnut Creek, CA, USA) Microsorb-MV C_8 column (50×4.6 mm, 5 μm) was used.

The mobile phase composition for the Microsorb-MV C_8 column started with acetonitrile–water (25:75) and progressed to acetonitrile–water (50:50) in 10 min with a flow rate of 2.50 mL/min. The detector wavelength was set at 227 nm, the column was operated at ambient temperature, and the injection volume was 20 μL. Linearity of the detector was determined with triplicate analyses of 10-deacetylbaccatin III, baccatin III, and paclitaxel standard solutions in the range of 0.3125 to 10 μg/mL.

2.3. Recovery and stability tests

Evaluation of our extraction procedure was assessed by spiking suspension media with paclitaxel dissolved in methanol to levels equivalent to 0, 0.2, 1, 5, and 10 mg/L. Flasks were placed on a rotary shaker at 125 rpm with an 8-h photoperiod (3.2 μmol m^{-2} s^{-1}). Samples were tested after 1 h, 1 week and 2 weeks to compare recovery rates. The samples (1 mL of broth) were extracted twice with 1 mL of chloroform, evaporated to dryness, and reconstituted in 1 mL of methanol. Another set of samples was tested directly by mixing 0.5 mL of suspension medium with 0.5 mL of methanol.

A second experiment tested the influence of light (3.2 μmol m^{-2} s^{-1} for 8 h) versus dark incubation in combination with two levels of shaking (0 and 125 rpm) on stability and recovery of paclitaxel. Flasks of suspension media were spiked with paclitaxel dissolved in methanol to provide a level equivalent to 10 mg/L of medium. Samples were taken after 5 min, 30 min, 60 min, and 1 week. All samples were tested directly by mixing 0.5 mL of suspension media with 0.5 mL of methanol.

2.4. Tissue culture

Callus of *T. cuspidata* cv *Densiformis* was grown on Gamborg's B5 medium supplemented with sucrose (20 g/L), casein hydrolysate (2 g/L), agar (8 g/L), picloram (0.5 mg/L), and α-naphthalene acetic acid (1.8 mg/L). Eight milliliters of medium were dispensed into each culture tube (16×125 mm) and autoclaved at 121.5°C for 20 min.

Media for initiation of suspension cultures consisted of Gamborg's B5 media supplemented as above except without agar. Fifty milliliters were then dispensed into 250 mL polycarbonate Erlenmeyer flasks and autoclaved at 121.5°C for 20 min.

For initiation of suspension cultures, approximately 300 mg of 1 year old callus were transferred to each flask and placed on a rotary shaker at 125 rpm without light. Flasks were incubated for 2 weeks at 25°C and subcultured by inoculating 25 mL of the 2 week old culture into 25 mL of fresh media.

To test the influence of shaking on taxane production, one set of cultures was maintained with shaking (125 rpm) and one set without shaking. Both sets were incubated without light. One milliliter of liquid media, including the cells, from each flask was sampled over a period of 28 days. The sample was then extracted four times with 1 mL of methylene chloride. The methylene chloride extract was allowed to evaporate to dryness at room temperature and then reconstituted in 100 μL of methanol for HPLC analysis.

2.5. Statistical procedures

All experiments were performed in triplicate. Data were analyzed using two- and three-way analyses of variance. A square root transformation was used for data with unequal variances. The Newman–Keuls' test ($P = 0.05$) was used to determine significant differences between pairs of means. The contribution of a source of variation was calculated by dividing the sum of squares (SS) by total SS and multiplying the resulting product by 100.

3. Results and discussion

A gradient method was developed using a Microsorb-MV C_8 column that allowed separation of a mixture of 13 taxanes (Fig. 1) in less than 15 min [11]. By comparison, Theodoridis et al. [12] achieved separation of this mixture of 13 taxanes within 30 min using a phenyl column. The speed of the Microsorb-MV method will allow analysis of large numbers of samples for a range of taxanes of differing solubility. The identification and elution order of the 13 taxanes' peaks was determined by LC–MS using electrospray ionization. The elution order of the 13 taxanes' mixture was as follows: 10-deacetylbaccatin III, baccatin III, 7-xylosyl-10-deacetyltaxol B, 7-xylosyl-10-deacetyltaxol, taxinine M, 7-xylosyl-10-deacetyltaxol C, 10-deacetyltaxol, 7-xylosyltaxol, cephalomannine, 7-epi-10-deacetyltaxol, paclitaxel, taxol C, and 7-epitaxol. Elution order was related to molecular size, number of acetylated hydroxyl groups, and substitution of the xylosyl group at the 7-position [13].

Recovery of paclitaxel from suspension media was studied to compare the efficacy of extraction to a direct sampling method (Table 1). The direct sampling procedure, mixing 0.5 mL of suspension medium with 0.5 mL of methanol followed by direct analysis of the sample by HPLC, was a modification of a procedure used by Mirjalili and Linden [14]. A range of concentrations from 0.2 to 10 mg/L was tested, consistent with ranges normally found in cell suspension cultures of different strains [14,15]. Recovery by extraction, based on the 1 hour samples, varied from 60 to 100%. While direct sampling theoretically provides 100% recovery of paclitaxel, it was not as sensitive as the extraction procedure, obviously a result of the inherent dilution with direct sampling. The results also demonstrated significant ($P \geq 0.001$) loss of paclitaxel with length of incuba-

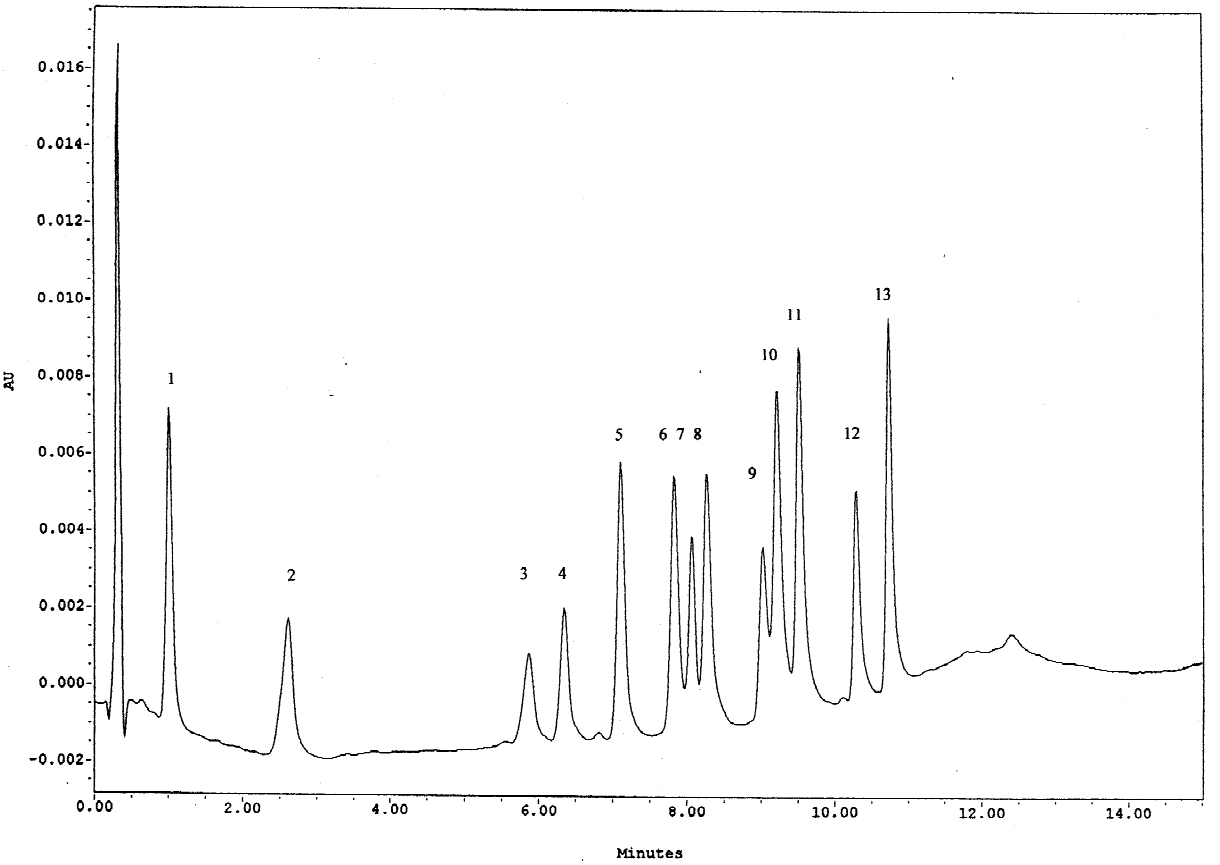

Fig. 1. HPLC chromatogram of standards using a Microsorb-MV C_8 column. Chromatographic conditions as in Experimental. t_R values for compounds 1–13: 1.0, 2.6, 5.9, 6.4, 7.1, 7.8, 8.1, 8.3, 9.0, 9.2, 9.5, 10.3, and 10.7 min, respectively. Peaks: 1=10-deacetylbaccatin III, 2=baccatin III, 3=7-xylosyl-10-deacetyltaxol B, 4=7-xylosyl-10-deacetyltaxol, 5=taxinine M, 6=7-xylosyl-10-deacetyltaxol C, 7=10-deacetyltaxol, 8=7-xylosyltaxol, 9=cephalomannine, 10=7-epi-10-deacetyltaxol, 11=paclitaxel, 12=taxol C, 13=7-epitaxol.

tion in suspension medium. There was 18 to 89% loss of paclitaxel in the first week and 96 to 100% disappearing in the second week. The amount of loss of paclitaxel was concentration dependent ($P \geq 0.001$), at least during the first week, with the percentage loss of paclitaxel decreasing as concen-

Table 1
Stability and recovery of paclitaxel from suspension media using extraction and direct methods[a]. Four concentrations (A=10 mg/L; B=5 mg/L; C=1 mg/L; D=0.2 mg/L) were tested following 1 h, 1 week and 2 weeks of incubation. Values are the average of three replicates±SE

Time	Extraction method				Direct sampling method			
	A	B	C	D	A	B	C	D
1 h	7.5±0.2	3.3±0.2	0.6±0.1	0.2±0.0	9.0±0.3	4.3±0.2	0.6±0.0	0±0
1 week	0.8±0.1	0.9±0.1	0.2±0.0	0.2±0.0	0.8±0.0	0.7±0.0	0±0	0±0
2 weeks	0.3±0.0	0.1±0.1	0±0	0±0	0±0	0±0	0±0	0±0

[a] Extracted samples (1 mL of broth) were shaken twice with 1 mL of chloroform, the chloroform portion evaporated to dryness and reconstituted in 1 mL of methanol. Direct sampling was tested by mixing 0.5 mL of suspension medium with 0.5 mL of methanol.

Table 2
Influence of light and shaking on recovery of paclitaxel from suspension media. Media were spiked to achieve a concentration of 10 mg/L paclitaxel. Four combinations of light and shaking (A = light, no shake; B = dark, no shake; C = light, shake; D = dark, shake) were tested following 0 min, 30 min, 1 h and 1 week of incubation. Values[a] are the average of three replicates ± SE

Time	Treatment			
	A	B	C	D
0 min	10.3±0.2	9.4±0.2	10.2±0.5	9.1±0.3
30 min	7.2±0.1	7.2±0.2	6.8±0.2	6.8±0.2
1 h	7.1±0.2	6.9±0.0	4.5±0.0	5.2±0.1
1 week	4.1±0.1	3.3±0.6	0.7±0.1	0.7±0.0

[a] All samples were tested directly by mixing 0.5 mL of suspension media with 0.5 mL of methanol.

tration decreased. However, by the second week of incubation, paclitaxel was almost completely undetectable in all samples. Contrary to previous studies with animal cell culture media [10], there was no increase in 7-epitaxol or other taxane standards in plant cell culture media that corresponded to loss of paclitaxel. It may be that paclitaxel becomes bound to specific medium ingredients or to cell surfaces, which may prevent extraction, but this hypothesis needs further testing.

The influence of incubation time, shaking, and light on stability of paclitaxel from suspension media was also studied (Table 2). Incubation time had the greatest influence on recovery and stability of paclitaxel ($P \geq 0.001$), contributing 88% to the total variation. During the first 30 min of incubation, there was a 23–33% loss of paclitaxel and a 60–93% loss following 1 week of incubation. Shaking had the next greatest influence ($P \geq 0.001$), contributing 6% to the total variation observed. With shaking, there was a 92–93% loss of paclitaxel compared to cultures not shaken (60–65%) following 1 week of incubation. There also were significant ($P \geq 0.05$) differences detected due to the effect of light, although this factor contributed only 0.25% to the total variation. The influence of light was greatest during the initial incubation when paclitaxel concentration was greatest. These results suggest that incubating suspension cultures in the dark would provide only a partial answer for maximizing recovery of paclitaxel from suspension cultures. Shaking is necessary for good cell growth, presumably due to increased oxygen, but at the same time it stimulates rapid loss of paclitaxel. These results may provide an additional explanation for increased paclitaxel content in cultures exposed to low levels of oxygen [14]. That is, there is not increased production but decreased loss of paclitaxel in these cultures.

Results for the analysis of suspension cultures using the Microsorb-MV C_8 column under gradient conditions are presented in Table 3. A typical chromatogram of these cultures is presented in Fig. 2. There was a high variability in replicate flasks. This is consistent with other studies [8]. Although the amount of paclitaxel varied from day to day ($P \leq 0.05$), the difference between cultures shaken and cultures not shaken was not significant ($P =$

Table 3
Influence of shaking on paclitaxel, 10-deacetylbaccatin III, and baccatin III production[a] in suspension cultures of *Taxus cuspidata* cv *Densiformis*. Values (μg/L) are the average of three replicates ± SE for cultures shaken (A) and not shaken (B)

Day	Paclitaxel		10-Deacetylbaccatin III		Baccatin III	
	A	B	A	B	A	B
0	65±20	64±45	1447±373	783±783	157±50	0±0
1	60±40	63±32	707±116	857±497	163±15	40±40
2	16±2	17±10	1040±455	1163±290	517±372	57±57
3	55±7	34±17	877±283	0±0	143±19	0±0
6	62±50	23±2	1150±361	360±360	257±70	143±119
13	0±0	17±10	507±337	303±121	27±7	287±252
15	0±0	0±0	1933±1242	297±72	377±240	583±513
21	65±44	81±81	0±0	0±0	0±0	0±0
28	149±65	106±54	0±0	0±0	0±0	0±0

[a] Samples of 1 mL of *Taxus cuspidata* suspension cultures were extracted four times with 1 mL of methylene chloride. The dried residue of the methylene chloride extract was reconstituted in 100 μL of methanol for HPLC analysis.

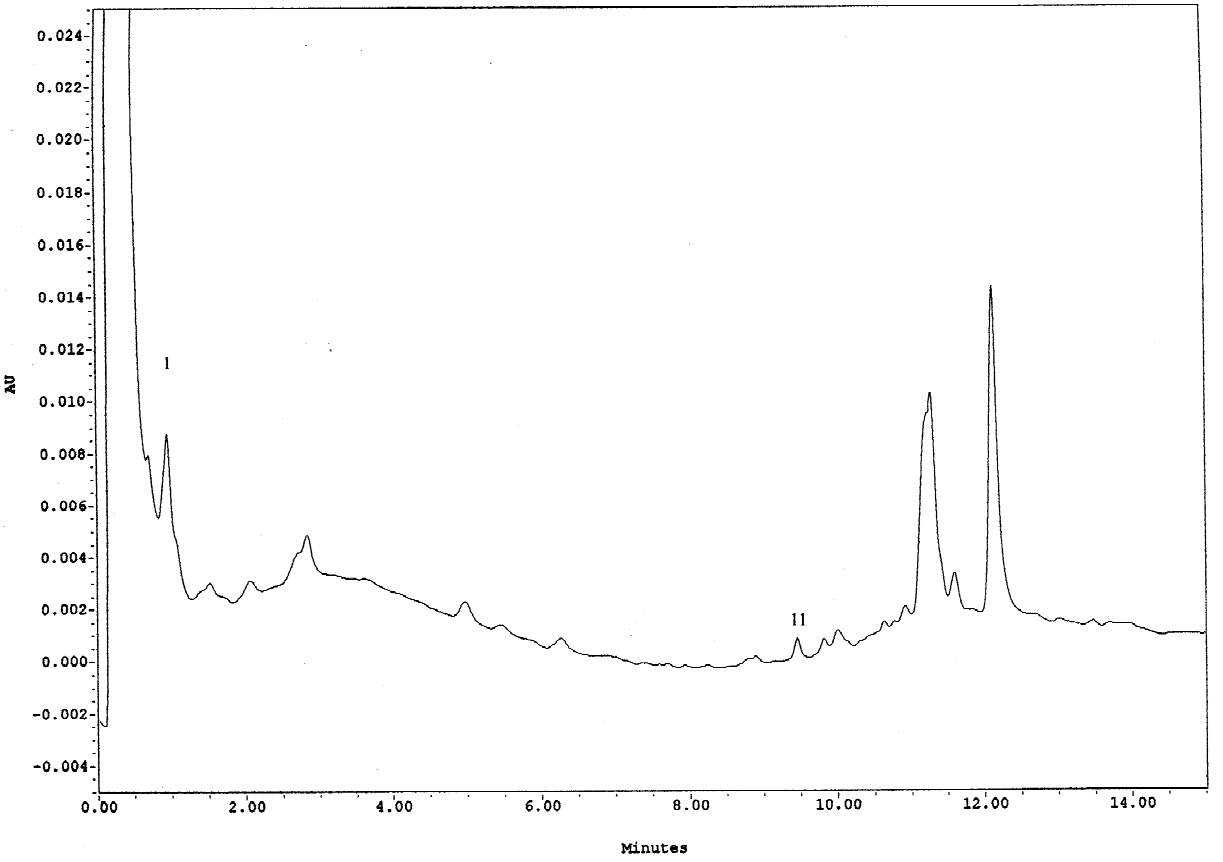

Fig. 2. HPLC chromatogram of crude extract of *Taxus cuspidata* cv *Densiformis* cell suspension culture, showing 10-deacetylbaccatin III (1) and paclitaxel (11). Chromatographic conditions as in Fig. 1.

0.67). Samples maintained on the shaker produced a maximum of 149 μg/L of paclitaxel, while samples not shaken produced a maximum of 106 μg/L of paclitaxel after 4 weeks of culture. The lack of influence of shaking on paclitaxel content of these cultures may be due to the low concentration of this compound (<0.2 mg/L). Paclitaxel was less sensitive to shaking at lower concentrations (Table 1).

Shaking did increase ($P \leq 0.05$) production of 10-deacetylbaccatin III (0–1.9 mg/L or 0–0.00019%). It may be that the lack of increase in paclitaxel is tied to the increase in this compound, since 10 deacetylbaccatin III can be a degradation product of paclitaxel [16]. There also was greater production of baccatin III (0–583 μg/L) than paclitaxel in these cultures (Table 3). These results suggest that suspension cultures may be more useful for production of 10-deacetylbaccatin III and baccatin III. Paclitaxel can be prepared from 10-deacetylbaccatin III using a semisynthetic procedure [17]. Witherup et al. [4] reported quantities of 10-deacetylbaccatin III from 0.002 to 0.02% from leaves and stems of various *Taxus* species. Although the percentage yield of these suspension cultures was low compared to leaves, they may prove to be a better source of compounds such as 10-deacetylbaccatin III and baccatin III, producing them in less time and without the restrictions of seasonal growth.

References

[1] J. Liebmann, J.A. Cook, C. Lipschultz, D. Teagne, J. Fischer, J.B. Mitchell, Cancer Chemother. Pharmacol. 33 (1994) 331.

[2] M. Jaziri, A. Zhiri, Y. Guo, J. Dupont, K. Shimomura, H. Hamada, M. Vanhaelen, J. Homes, Plant Cell Tiss. Org. Cult. 46 (1996) 59.
[3] D. Gibson, R. Ketchum, N. Vance, A. Christen, Plant Cell Rep. 12 (1993) 479.
[4] K.M. Witherup, S.A. Look, M.W. Stasko, J. Nat. Prod. 53 (1990) 1249.
[5] A. Stierle, G. Strobel, D. Stierle, Science 260 (1993) 214.
[6] A. Hoffman, W. Khan, J. Worapong, G. Strobel, D. Griffin, B. Arbogast, D. Barafsky, R.B. Boone, L. Ning, P. Sheng, L. Daley, Spectroscopy 13 (1998) 22.
[7] R.E.B. Ketchum, D.M. Gibson, R.B. Croteau, M.L. Shuler, Biotechnol. Bioeng. 62 (1999) 97.
[8] R.E.B. Ketchum, D.M. Gibson, Plant Cell Tiss. Org. Cult. 46 (1996) 9.
[9] T.J. Hirasuna, L.J. Pestchanker, V. Srinivasan, M.L. Shuler, Plant Cell Tiss. Org. Cult. 44 (1996) 95.
[10] I. Ringel, S.B. Horwitz, J. Pharmacol. Exp. Ther. 242 (1987) 692.
[11] T. Nguyen, G. Gonyea, R.A. Smith, J. Eshraghi, in: Proceedings of the 47th ASMS Conference, 1999.
[12] G. Theodoridis, G. Laskaris, C.F. de Jong, A.J.P. Hofte, R. Verpoorte, J. Chromatogr. A 802 (1998) 297.
[13] L.K. Shao, D.C. Locke, Anal. Chem. 69 (1997) 2008.
[14] N. Mirjalili, J.C. Linden, Biotechnol. Bioeng. 48 (1995) 123.
[15] N. Mirjalili, J.C. Linden, Biotechnol. Prog. 12 (1996) 110.
[16] S.L. Richheimer, D.M. Tinnermeier, D.W. Timmons, Anal. Chem. 64 (1992) 2323.
[17] I. Ojima, I. Habus, M. Zhao, J. Org. Chem. 56 (1991) 1681.