Enzymatic C-4 deacetylation of 10-deacetylbaccatin III

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Second-generation paclitaxel analogues that require replacement of the C-4 acetate by other substituents are in development. An enzyme able to specifically remove the C-4 acetate from paclitaxel could simplify preparation of the analogues. Several strains were isolated from soil samples that contain enzyme activities able to 4-deacetylate 10-DAB (10-deacetylbaccatin III). Selection was made using plates containing 10-DAB as the sole carbon source and screening colonies for deacetylation of 10-DAB. Two strains initially isolated were identified as *Rhodococcus* sp. and deposited with the A.T.C.C. (Manassas, VA, U.S.A.) as strains 202191 and 202192. Whole cells were able to convert 10-DAB into 4,10-DDAB (4-deacetyl-10-deacetylbaccatin III) in 90% yield. The enzyme activity in these strains was not effective with paclitaxel and 10-deacetylpaclitaxel, although 4,10-DDAB was produced from baccatin III. The activity in these strains was associated with an insoluble fraction of cell extracts. Several additional isolates were obtained that were identified as variants of *Stenotrophomonas maltophilia*, and a soluble C-4 deacetylase was purified approx. 218-fold from one of them. The activity of this enzyme was limited to 10-DAB, and the enzyme was not effective with paclitaxel or baccatin III.

Introduction

Paclitaxel, an anticancer compound first isolated by Wani et al. [1], is an approved drug (Taxol®; Bristol-Myers Squibb) for the treatment of ovarian cancer, breast cancer, non-small cell lung cancer and AIDS-related Kaposi's sarcoma [2]. We have previously reported the isolation of microbial strains containing C-13 deacylase [3], C-10 deacetylase [3] and C7-xylosidase [4] enzyme activities, which are able to convert mixtures of taxanes into 10-DAB (10-deacetylbaccatin III), thereby increasing the amount and ease of isolation of this precursor for semisynthesis. We have also reported a strain able to hydroxylate 7-deoxy-10-deacetylbaccatin-III to 6-hydroxy-7-deoxy-10-deacetylbaccatin-III [5].

Chemical modification of the paclitaxel structure at C-4 and other positions has been explored by many groups to determine structure–activity relationships and to try to obtain compounds with efficacy superior to taxol for development as second-generation drugs [5a,5b]. Replacement of the C-4 acetyl group of paclitaxel with other substituents has lead to compounds with improved potency in activity assays [6].

BMS-188797 [7] and BMS-275183 [8] are second-generation paclitaxel analogues that require replacement of the C-4 acetate group with a methyl carbonate group. An enzyme capable of specifically removing the C-4 acetyl group from taxanes could be useful in the synthesis of the C-4-modified paclitaxel analogues to provide starting material to allow incorporation of a methyl carbonate group at this position. Most of the commercially available lipases and esterases hydrolyse esters of primary and secondary alcohols, but have little activity with esters of tertiary alcohols. However, there have been several reports of hydrolysis of esters of tertiary alcohols by lipases and esterases [9–15], and it appears that micro-organisms containing such enzymes may be readily isolated from soil samples using suitable selection and screening techniques [11]. Because the commercially available lipases, proteases and esterases in our collection had no effect on paclitaxel, screening of microbial strains for the desired activity was undertaken. The present study describes the isolation from soil samples of strains that contain enzyme activities able to 4-deacetylate 10-DAB.

Materials and methods

Growth media

Medium A contained 0.5% toasted Nutrisoy, 2% (w/v) glucose, 0.5% yeast extract, 0.5% K_2HPO_4 and 0.5% NaCl, adjusted to pH 7 with HCl. Medium B contained 0.05% K_2HPO_4, 0.05% KH_2PO_4, 0.02% $MgSO_4$, 0.001% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$, 0.1% $(NH_4)_2SO_4$ and 0.5 mg/ml 10-DAB as the sole carbon source adjusted to pH 7. Medium C contained 0.02%

Key words: biotransformation, deacetylation, 10-deacetylbaccatin III, paclitaxel, *Rhodococcus*, *Stenotrophomonas*.

Abbreviations used: 10-DAB, 10-deacetylbaccatin III; 4,10-DDAB, 4-deacetyl-10-deacetylbaccatin III; LB, Luria–Bertani.

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$MgSO_4$, 0.001% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$, 0.1% $(NH_4)_2SO_4$, 0.1% peptone, 1% glycerol and 0.3% K_2HPO_4 adjusted to pH 7 with KH_2PO_4. Modified LB (Luria–Bertani) medium contained 1% tryptone, 0.5% yeast extract and 0.3% K_2HPO_4 adjusted to pH 7 with KH_2PO_4. F7 medium contained 1% malt extract, 1% yeast extract, 0.1% peptone and 2% (w/v) dextrose adjusted to pH 7.

Enrichment and isolation of C-4 deacetylase strains

Soil samples (0.5 g) were suspended in 5 ml of water, and 0.3 ml was added to 10 ml of medium B. After 2–4 days of shaking at 28 °C and 200 rev./min, 0.3 ml was transferred to a fresh flask for a second round of enrichment. After another 2–4 days, 0.1 ml of diluted sample was plated on to medium B containing 1.5% (w/v) Difco™ Agar Noble (agar of increased purity from Becton Dickinson). Colonies were picked and plated on to medium A containing 1.5% (w/v) agar.

Flasks containing 10 ml of medium A were inoculated with a loopful of each isolate and shaken at 28 °C and 200 rev./min. After 1 day, 0.2 ml of methanol containing 2 mg of 10-DAB was added to each flask and the incubation was continued. After a further 2 days, 0.5 ml samples were quenched with 0.5 ml of methanol and samples were assayed by HPLC for hydrolysis of 10-DAB.

Growth of *Stenotrophomonas maltophilia* SC16447 in fermentors

Broth from frozen vials containing *S. maltophilia* SC16447 was used to inoculate F7 medium in flasks. After incubation at 28 °C and 200 rev./min for 1 day, 1 litre of the culture was used to inoculate 100 litres of modified LB medium containing 0.05% SAG antifoam (OSi Specialties) in a fermentor. Fermentation was carried out at 1 vol. · vol.$^{-1}$ · min^{-1} airflow, 250 rev./min agitation and 28 °C. No pH control was used during the fermentation. After 50 h, cell paste (800 g) was collected with a Sharples AS26 centrifuge (Pennwalt) and then stored at −70 °C until used.

HPLC analysis

Samples were analysed with an HP Hypersil ODS (Hewlett–Packard; Agilent) C-18, 5 μm particle size, 200 mm long × 4.6 mm diameter column. The mobile phase was 50% acetonitrile/50% water, the flow rate was 1 ml/min, detection was at 235 nm, the temperature was 40 °C, and injection volume was 10 μl.

Retention times (min) were: paclitaxel, 9.0; 4-deacetylpaclitaxel, 7.3; 10-deacetylpaclitaxel, 6.0; 4-deacetyl-10-deacetylpaclitaxel, 5.0; baccatin III, 4.0; 4-deacetylbaccatin III, 3.2; 10-DAB, 3.0; and 4,10-DDAB (4-deacetyl-10-deacetylbaccatin III), 2.6. Standard compounds were prepared by Bristol-Myers Squibb chemists.

4-Deacetylase assay

Enzyme samples were incubated with 50 mM potassium phosphate buffer containing 0.2 mg/ml 10-DAB and 2% (v/v) methanol at 30 °C (final volume 0.6 ml). Reactions were stopped by adding 0.6 ml of acetonitrile and the mixtures analysed by HPLC.

Purification of 4-deacetylase from *S. maltophilia* SC16447

Frozen cell paste was suspended at a concentration of 20% (w/v) in 50 mM potassium phosphate buffer (pH 7) containing 1 mM dithiothreitol and disrupted by two passages through a microfluidizer. Unbroken cells and debris were removed by centrifugation of the extract at 20434 ***g*** for 20 min. The extract was stored at −20 °C until used for purification. All purification steps were carried out at 4 °C. Frozen extract (83 ml) was thawed and then pumped through 50 ml of Amersham/Pharmacia Q-Sepharose® Fast Flow in a 26-mm-diameter column at 2 ml/min. The column was eluted with a 400 ml 0–0.5 M NaCl gradient with an additional 50 ml of 0.5 M NaCl wash, all in 20 mM potassium phosphate buffer (pH 7) at 2 ml/min. The activity peak fractions containing 24 ml were concentrated to approx. 6 ml using an Amicon YM10 membrane in a stirred cell. The concentrated enzyme was passed at a flow rate of 1 ml/min through 450 ml of Amersham/Pharmacia Sephacryl® S-200 packed in a 2.6 cm × 100 cm column with 50 mM potassium phosphate buffer (pH 7) containing 1 mM dithiothreitol and 0.15 M NaCl. Two 12 ml fractions contained the specific activity peak. A volume of 8 ml of one of the fractions was loaded on to a Bio-Rad UnoQ1 column (1.3 ml) in column buffer (20 mM Tris/HCl, pH 7.4, containing 1 mM dithiothreitol), washed with 5 ml of column buffer and then eluted with a 15 ml 0.15–0.4 M NaCl gradient in the column buffer at a flow rate of 1 ml/min. The activity peak fraction containing 0.5 ml was diluted with 1.8 ml of the column buffer and subjected to a second unoQ chromatography by the same method. The activity peak fraction was concentrated with an Amicon Microcon-10 apparatus to 0.1 ml, then analysed by SDS/PAGE. The major band had an estimated molecular mass of 49000 Da. A gel slice and a PVDF membrane blot containing the band were sent to the W.M. Keck AAA and Protein Sequencing Facility, Yale University (New Haven, CT, U.S.A.), to obtain the sequences of two internal peptides and the N-terminus.

Preparation of 4,10-DDAB

S. maltophilia SC16447 frozen cell paste (20 g) was suspended in 160 ml of 50 mM potassium phosphate buffer (pH 7) with an Ultraturrax homogenizer (Janke and Kunkel) and adjusted to pH 7 with HCl. 10-DAB (200 mg) dissolved in 20 ml of methanol was added to the cell suspension and the mixture was shaken in a 500 ml flask at 28 °C for

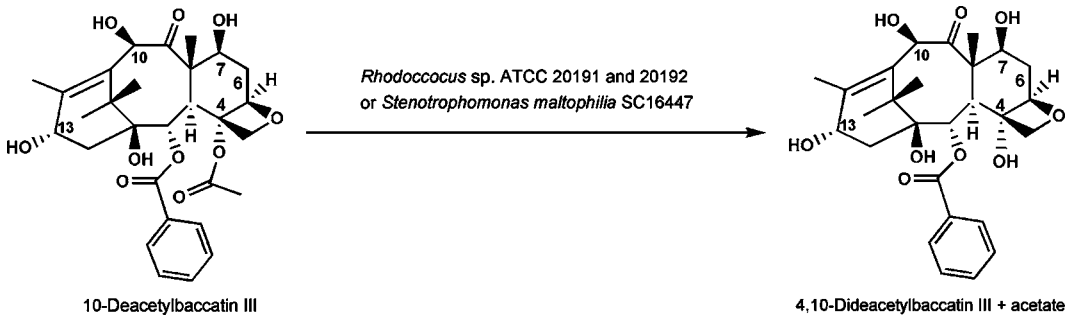

Figure 1 Reaction catalysed by C-4 deacetylase

21 h. The mixture was then adjusted from pH 7 to pH 5.5 with a few drops of acetic acid and the cells were removed by centrifugation. The cell pellet was washed with 20 ml of 10% methanol in water. A portion of the combined supernatants (50 g of 195 g) containing 41 mg of 4,10-DDAB and 4.5 mg of the (presumed) 7-epimer was stirred with 25 ml of ethyl acetate, 15 g of Na_2SO_4 and 2 g of acid-washed celite. The mixture was filtered, and the filter coke was washed with 25 ml of ethyl acetate. The upper phase was separated and the aqueous phase was extracted with an additional 25 ml of ethyl acetate. Concentration of the combined organic extract gave 61 mg of glassy solid that crystallized as needles on suspension in 3 ml of ethyl acetate. Filtration and drying *in vacuo* gave 42.8 mg of colourless solid containing 39.2 mg (95.6% recovery) of 4,10-DDAB and 3.6 mg (8.5%) of the epimer.

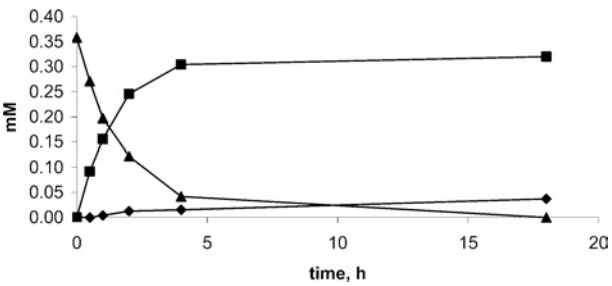

Figure 2 C-4 deacetylation by *Rhodococcus* strain A.T.C.C. 202191

Cells from a single colony were grown for 64 h at 28°C and 200 rev./min on 100 ml of medium A, centrifuged and washed with 50 ml of potassium phosphate buffer (pH 7). A 10% (w/v) cell suspension was prepared in the same buffer and 10-DAB (2 mg; 3.7 μmol) in 0.5 ml of methanol was added to 9.5 ml of the cell suspension in a 50 ml flask. The flask was incubated at 28°C and 200 rev./min for the periods indicated, and 0.5 ml samples were diluted with 0.5 ml of methanol for HPLC assay. ▲, 10-DAB; ■, 4,10-DDAB; ◆, 7-epimer (presumed).

Results and discussion

C-4 deacetylase from *Rhodococcus* sp.

Colonies were picked from plates containing 10-DAB as the sole carbon source. Two isolates from the same soil sample converted 10-DAB into a peak with the same HPLC retention time as a chemically prepared standard of 4,10-DDAB. LC/MS analysis showed $(M + CH_3COO)^- = 561$, consistent with the expected molecular mass of 502 Da. An isomer with a different HPLC retention time was also found at approx. 10% of the concentration of the desired product. The isomer is likely to be the 7-epimer [16]. The deacetylase activity was associated with the cells for both isolates and was not found in a centrifuged broth supernatant. The strains were identified as *Rhodococcus* sp. by using biochemical features and whole-cell fatty-acid analysis and deposited with the A.T.C.C. (Manassas, VA, U.S.A.) as strains 202191 and 202192.

The reaction catalysed by the cells is shown in Figure 1. The time course for conversion of 10-DAB into 4,10-DDAB by strain ATCC 20191 is shown in Figure 2. For strain ATCC 20192, the maximum yield was obtained by the addition of 10% methanol. For strain ATCC 20191, the maximum yield was obtained with 2–5% methanol. The cells also removed the 10-acetate from baccatin III and further converted the product into 4,10-DDAB (Figure 3), but did not hydrolyse the 4-acetate from paclitaxel or 10-deacetylpaclitaxel. The 4-deacetylase enzyme was associated with an insoluble fraction of cell extracts, and efforts to solubilize it with detergents, enzyme treatment or salt washes were not successful. Further screening by the same technique was therefore carried out to find additional strains with 4-deacetylase activity that would be soluble to allow purification and possibly be effective for 4-deacetylation of paclitaxel.

C-4 deacetylase from *S. maltophilia*

A total of 260 isolates were screened, and many strains converted paclitaxel into compounds giving HPLC peaks that appeared to be 10-deacetylpaclitaxel and/or debenzoylpaclitaxel, but the desired 4-deacetylpaclitaxel was not found. However, 17 isolates gave a peak corresponding to 4,10-DDAB. Samples from five of the isolates were analysed by LC/MS and all showed $(M + \text{acetate})^-$ of 561 (molecular mass 502 Da) consistent with 4,10-DDAB.

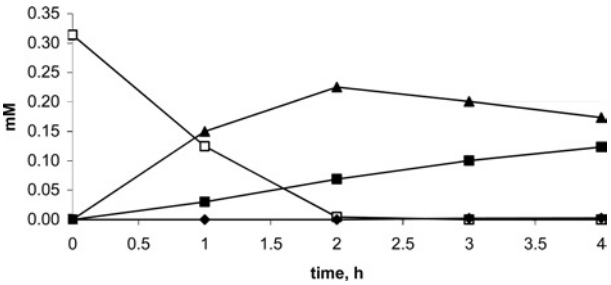

Figure 3 Deacetylation of baccatin III by *Rhodococcus* strain A.T.C.C. 202191

Cells from a 0.2 ml inoculum were grown for 72 h at 28 °C and 200 rev./min on 50 ml of medium A. Baccatin III (2 mg; 3.4 μmol) in 0.5 ml of methanol was added to 9.5 ml of a 2% (w/v) cell suspension in 50 mM potassium phosphate buffer (pH 7) in a 50 ml flask. The flask was incubated at 28 °C and 200 rev./min (the incubation times are indicated in the Figure), and 0.5 ml samples were diluted with 0.5 ml of methanol for HPLC assay. □, baccatin III; ▲, 10-DAB; ■, 4,10-DDAB; ◆, 7-epimer (presumed).

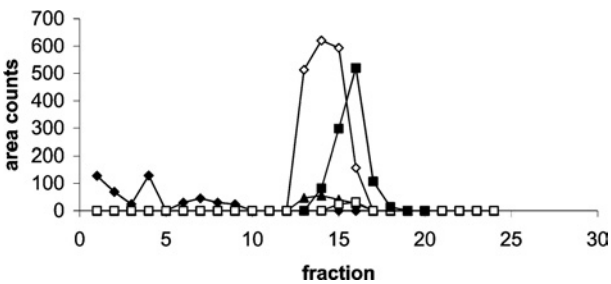

Figure 4 Hydrolysis of paclitaxel and 10-DAB by Q-Sepharose® chromatography fractions of extract of *S. maltophilia* SC 16447

Cells were grown from a 1% inoculum for 3 days at 28 °C and 200 rev./min on 100 ml of medium C, collected by centrifugation and then disrupted by sonication in 25 mM Tris/HCl (pH 7.4) containing 1 mM dithiothreitol. The extract was applied to a 5 ml HiTrap Q (GE Healthcare) column, washed with the same buffer and then eluted with a 25 ml gradient of 0–1 M NaCl in this buffer at 1 ml/min. Fractions (0.2 ml sample) were assayed for 15 h with 0.2 mg of paclitaxel or with 0.2 mg of 10-DAB as described in the Materials and methods section. ◇, 10-Deacetylpaclitaxel; ▲, 10-DAB; ◆, baccatin III; □, 4,10-DDAB from incubation with paclitaxel; ■, 4,10-DDAB from incubation with 10-DAB.

Extracts were prepared from the 17 isolates and were fractionated by chromatography on Q-Sepharose® to try to separate C-4 activity from the C-10 and C-13 hydrolysing activities. Products produced from paclitaxel by the fractions are shown in Figure 4 for extract 111y. Fractions produced baccatin III or mixtures of 10-deacetylpaclitaxel, baccatin III, 10-DAB and 4,10-DDAB. 4-Deacetylpaclitaxel or 4,10-dideacetylpaclitaxel was not observed. Fractions producing 4,10-DDAB from paclitaxel were also assayed with 10-DAB as the substrate. It is apparent that a C-4 activity is eluted at higher salt concentration than the C-13 and C-10 hydrolysing activities. The fractions containing C-4 activity with minimal C-10 activity were again tested for hydrolysis of paclitaxel and 10-deacetylpaclitaxel but 4-deacetylpaclitaxel and 4-deacetyl-10-deacetylpaclitaxel were not observed

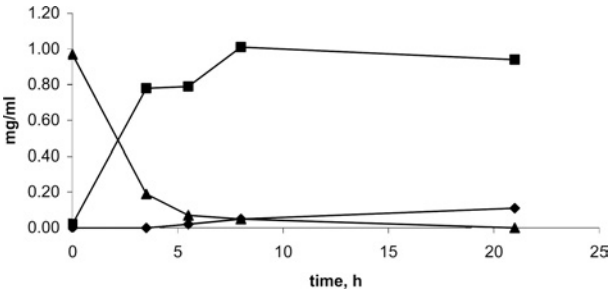

Figure 5 C-4 deacetylation of 10-DAB by *S. maltophilia* strain SC16447

10-DAB (200 mg) was treated with 20 g of cells and the product was isolated as described in the Materials and methods section. ▲, 10-DAB; ■, 4,10-DDAB; ◆, 7-epimer (presumed).

after extended incubation times. Similar activity profiles were also seen after chromatography of the other extracts. Isolate 111y and three other isolates with similar activities were identified using biochemical features as variants of *S. maltophilia*. Isolate 111y was designated as strain SC16447 in the department culture collection (Bristol-Myers Squibb).

We previously reported a C-10 deacetylase that removed the 10-acetyl group from either paclitaxel or baccatin III [3]. Similarly, we found a 7-xylosidase that removed the 7-xylosyl group from 7-xylosylpaclitaxel, 7-xylosyl-10-deacetylpaclitaxel, 7-xylosylbaccatin III and 7-xylosyl-10-deacetylbaccatin III [4]. However, the 4-deacetylases were active only with 10-DAB.

Paclitaxel has been reported to undergo 'hydrophobic collapse' in aqueous/organic solvents with the C-4 acetate sandwiched between the C-13 side chain and the 2-benzoate, whereas these groups are not as close in organic solvents [17]. The purest C-4 deacetylase fractions after chromatography of two of the extracts were freeze-dried and tested for activity against paclitaxel or baccatin III in buffer-saturated toluene. No activity was seen, although the freeze-dried fractions retained activity when tested for hydrolysis of 10-DAB in 50 mM potassium phosphate buffer (pH 7).

Preparation of 4,10-DDAB

Using 10% (w/v) whole cells in 50 mM potassium phosphate buffer (pH 7) containing 10% methanol and 1 mg/ml 10-DAB, 200 mg of 10-DAB was deacetylated (Figure 5). The HPLC yield was 85%, with the remaining material being a product (likely the 7-epimer) that is formed from deacetylated 10-DAB more readily at higher pH. The undesired product was even formed at pH 6 and 5, but not at pH 4. Unfortunately, the enzyme activity decreases rapidly below pH 7. A portion of the reaction product was isolated as described in the Materials and methods section.

Purification of C-4 deacetylase

C-4 deacetylase was purified to near homogeneity from isolate *S. maltophilia* SC16447. Strain SC16447 grew very

Table 1 Purification of the C-4 deacetylase from an extract of *S. maltophilia* SC16447

Step	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (unit/mg)
Extract	83	0.760	273.9	0.003
Q-Sepharose®	24	0.350	22.08	0.016
Sephacryl® S-200	24	0.310	3.6	0.086
Second UnoQ	0.5	0.011	0.015	0.656

poorly on the glycerol/peptone growth medium (medium C) originally used. Four other media were compared with that growth medium, and a modified LB medium (1% tryptone, 0.5% yeast extract and 0.3% K_2HPO_4 adjusted to pH 7 with KH_2PO_4) gave the highest C-4 deacetylase activity and also gave good growth. Growth in F7 medium gave almost exclusively C-10 deacetylase activity.

The enzyme was purified by ion-exchange chromatography on Q-Sepharose® followed by gel filtration on Sephacryl® S-200. Although the enzyme bound tightly to Phenyl-Sepharose after the S-200 step and required 1% Triton X-100 for elution, this did not appear to improve the purity. A portion of the S-200 peak was purified by chromatography twice on UnoQ. The enzyme was purified 218-fold from an extract, as summarized in Table 1. Samples of the predominant 49 000-Da-molecular-mass band seen on SDS/polyacrylamide gels after the purification were sent to a sequencing facility. An N-terminal sequence and two internal sequences were obtained for future cloning and expression of the activity. Initial characterization of the enzyme after partial purification showed that optimum temperature was 40 °C, the optimum pH was approx. 7.5 and the apparent K_m for 10-DAB was 0.14 mM.

Lipases, proteases and esterases have been used extensively for preparation of chiral intermediates in synthetic routes as well as for selective removal of protecting groups. They have been used both in hydrolytic reactions and for acylation reactions carried out in organic solvents. Enzymes are commercially available that may have excellent enantioselectivity and regioselectivity for such reactions, but usually have little activity with esters of tertiary alcohols. The C-4 deacetylase enzyme may find further utility in reactions where esters of tertiary alcohols are involved. Additional studies will be necessary to establish the scope of the enzyme in terms of breadth of substrate specificity and enantioselectivity. The utility of the enzyme for catalysing acylation reactions in organic solvents also remains to be explored.

Acknowledgments

The *Rhodococcus* sp. strains were identified by Dr Jane Tang and Ms Susan Dunham, A.T.C.C. Bacteriology Department (Manassas, VA, U.S.A.), and the *S. maltophilia* strains were identified by Ms Dolores Pirnik, Bristol-Myers Squibb. HPLC standards were provided by Dr Joydeep Kant, Bristol-Myers-Squibb.

References

1 Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P. and McPhail, A. T. (1971) J. Am. Chem. Soc. **93**, 2325–2327
2 Levin, M. (2001) Drugs Today **37**, 57–65
3 Hanson, R. L., Wasylyk, J. M., Nanduri, V. B., Cazzulino, D. L., Patel, R. N. and Szarka, L. J. (1994) J. Biol. Chem. **269**, 22145–22149
4 Hanson, R. L., Howell, J. M., Brzozowski, D. B., Sullivan, S. A., Patel, R. N. and Szarka, L. J. (1997) Biotechnol. Appl. Biochem. **26**, 153–158
5 Hanson, R. L., Kant, J. and Patel, R. N. (2004) Biotechnol. Appl. Biochem. **39**, 209–214
5a Kingston, D. G. I. (2005) Anticancer Agents from Natural Products, pp. 89–122, CRC Press, Boca Raton, FL
5b Fang, W.-S. and Liang, X.-T. (2005) Mini-Reviews in Medicinal Chemistry, Vol. 5, pp. 1–12, Bentham Science Publishers, Pennington, NJ
6 Chen, S., Wei, J., Long, B. H., Fairchild, C. A., Carboni, J., Mamber, S. W., Rose, W. C., Johnston, K., Casazza, A. M., Kadow, J. F. et al. (1995) Biorg. Med. Chem. Lett. **5**, 2741–2746
7 Chen, S. (2002) Front. Biotechnol. Pharm. **3**, 151–171
8 Mastalerz, H., Cook, D., Fairchild, C. R., Hansel, S., Johnson, W., Kadow, J. K., Long, B. H., Rose, W. C., Tarrant, J., Wu, M. et al. (2003) Biorg. Med. Chem. **11**, 4315–4323
9 O'Hagan, D. and Zeidi, N. A. (1992) J. Chem. Soc. Perkin Trans. 1, 947–949
10 Brackenridge, I., McCague, R. M., Roberts, S. M. and Turner, N. J. (1993) J. Chem. Soc. Perkin Trans. **1**, 1093–1094
11 Yeo, S., Nihira, T. and Yamada, Y. (1998) J. Gen. Appl. Microbiol. **44**, 147–152
12 Gudelj, M., Valinger, G., Faber, K. and Schwab, H. (1998) J. Mol. Catalysis B. Enzymatic **5**, 261–266
13 Hotta, Y., Ezaki, S., Atomi, H. and Imanaka, T. (2002) Appl. Environ. Microbiol. **68**, 3925–3931
14 Atomi, H. and Imanaka, T. (2004) Tetrahedron Asymmetry **15**, 2729–2735
15 Schmidt, M., Barbayianni, E., Fotokopoulou, I., Hohne, M., Constantinou-Kokotou, V., Bornscheuer, U. T. and Kokotos, G. (2005) J. Org. Chem. **70**, 3737–3740
16 Fang, W., Fang, Q. and Liang, X. (1997) Synth. Commun. **27**, 2305–2310
17 Vander Velde, D. G., Georg, G. I., Grunewald, G. L., Gunn, C. W. and Mitscher, L. A. (1993) J. Am. Chem. Soc. **115**, 11650–11651

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