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Paclitaxel Esters of Malic Acid as Prodrugs with Improved Water Solubility

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Abstract—The synthesis of paclitaxel esters of malic acid is described. These compounds were found to have improved water solubility and are stable in solution at neutral pH. The C2′ modified compounds behave as prodrugs, that is, paclitaxel is generated upon exposure to human plasma, whereas the C7 modified derivatives do not. 2′-Malyl paclitaxel sodium salt demonstrated enhanced antitumour activity and less toxicity in a P388 murine leukaemia in vivo model when compared to paclitaxel.

Introduction

Paclitaxel[1] (Taxol®) **1** is a potent anticancer agent used clinically to treat advanced ovarian, breast and non-small cell lung cancer. Although paclitaxel has demonstrated to be a unique antitumour agent it has several disadvantages. One of the major problems is its poor water solubility. Paclitaxel is administered in a vehicle containing ethanol and Cremophor EL®, which is considered to cause some hypersensitivity reactions.[2] In addressing the solubility problems, many research groups have reported syntheses and biological evaluations of water soluble paclitaxel derivatives. These analogues have polar substituents coupled to paclitaxel either at the C2′- or at the C7-hydroxyl group. The solubilising moieties can be (salts of) carboxylic acids,[3–5] phosphates,[6–8] sulphonates,[9] amines,[10,11] sugar derivatives,[12,13] or polyethylene glycol.[14–16] In most cases these moieties are coupled to paclitaxel via an ester or carbonate linkage.

Synthesis

We report the synthesis and biological evaluation of malic acid[17] paclitaxel derivatives. In contrast to many other prodrugs reported the solubilising moiety used is known to be non-toxic, since it is an acid of the Krebs cycle. We are aware that 2′-succinyl paclitaxel and salts thereof have already been published[3,5] and that these compounds have a slightly improved in vivo activity. A major drawback is the instability of these compounds in slightly basic aqueous solution which may very well hamper the clinical application of these compounds. This instability may be caused by assistance of the carboxylate in the hydrolysis of the 2′-ester functionality.[5] Our efforts focus on malic acid derivatives of paclitaxel. We rationalised that the additional hydroxyl functionality may improve stability by forming an intramolecular hydrogen bond between either carbonyl which induces conformational rigidity, thereby retarding hydrolysis. We realised that, for the same reason, this might lead to a slightly diminished water solubility. For preparing our target compounds it is obvious that the anhydride strategy as described for the succinyl derivatives cannot be used for malic acid. 2′-Malyl paclitaxel **3** was prepared in excellent yield by condensing paclitaxel with protected malic acid[18] in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) at −10 °C, followed by deprotection. Upon addition of sodium hydrogen carbonate the sodium salt **4** was obtained. The same reaction, carried out with an excess of protected malic acid in refluxing dichloromethane, afforded 2′,7-dimalyl paclitaxel **6** (Scheme 1).

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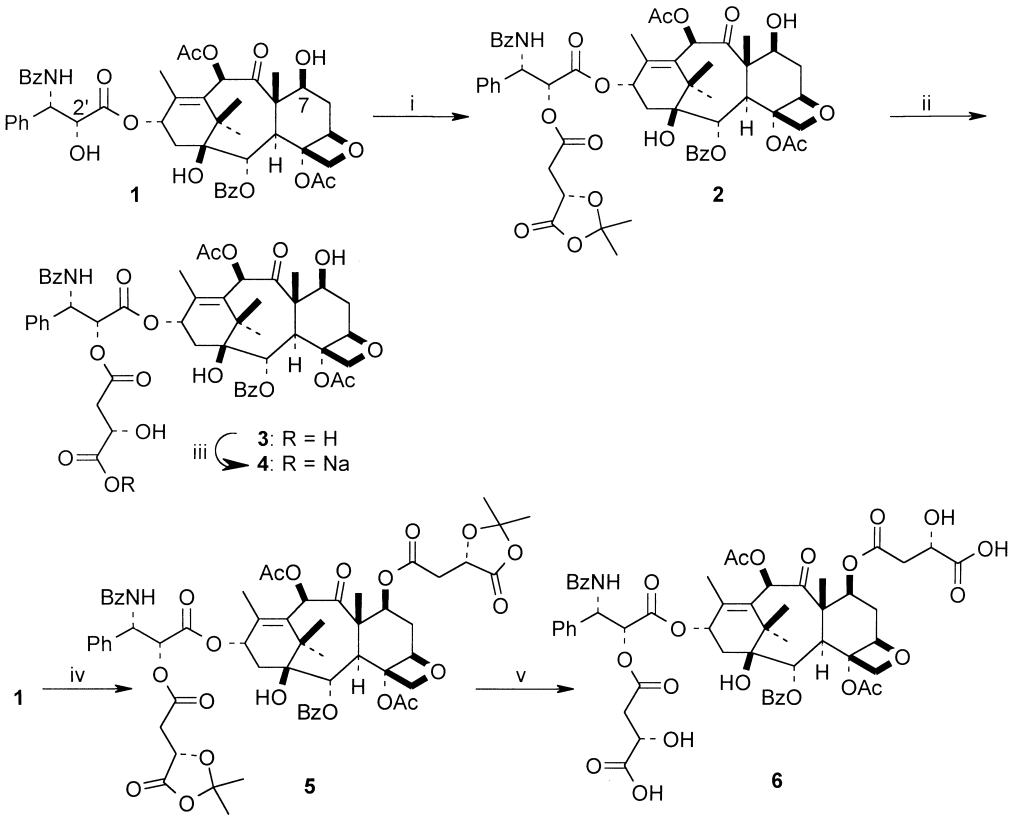
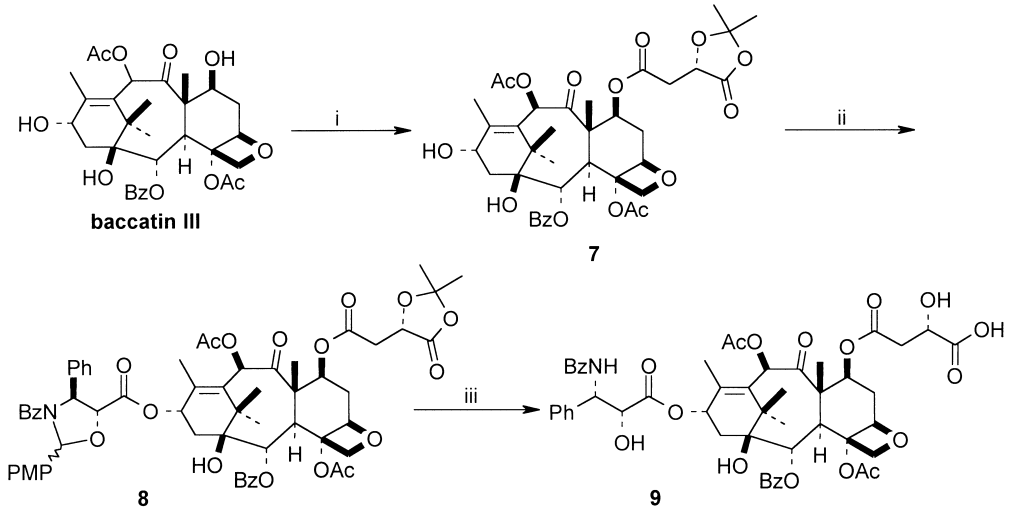
7-Malyl paclitaxel **9** was synthesised starting from baccatin III.[19] This is the first example of a semisynthetic water soluble derivative, since it was not prepared from paclitaxel itself. After reaction of baccatin III with protected malic acid as described above the side chain was introduced using the oxazolidine method.[20] Removal of the protective groups resulted in the desired compound (Scheme 2).

Biological Activity

All derivatives demonstrated improved water solubility (20–60 times) when compared to paclitaxel and proved to be stable when incubated in PBS buffer (pH 7.4) at 37 °C for 48 h, since no liberated paclitaxel was detected (HPLC). In contrast to compounds **6** and **9**, derivatives **3** and **4** generated paclitaxel when incubated in human



Scheme 1. Synthesis of derivatives **3**, **4** and **6**. Reagents: (i) (*S*)-1,2-*O*-isopropylidene-malic acid, DCC, DMAP, CH_2Cl_2, −10 °C, 3 h, 99%; (ii) HOAc:THF:water (1:1:1), r.t., 20 h, 94%; (iii) $NaHCO_3$, acetone, water, 100%; (iv) (*S*)-1,2-*O*-isopropylidene-malic acid, DIC, DMAP,CH_2Cl_2, reflux, 48 h, 72%. (v) HOAc:THF:water (1:1:1), 89%.



Scheme 2. Synthesis of 7-malyl paclitaxel. Reagents: (i) (*S*)-1,2-*O*-isopropylidene-malic acid, DCC, DMAP, CH_2Cl_2, r.t., 24 h, 86% at 81% conversion; (ii) (4*R*,5*S*)-3-benzoyl-2,4-diphenyl-1,3-oxazolidine-5-carboxylic acid, DCC, DMAP, CH_2Cl_2, r.t., 3 h, 74%; (iii) HCl, EtOAc, r.t., 15 min, 89%.

plasma. Therefore 2′-malyl paclitaxel and its sodium salt act as prodrugs and the 7-malyl and 2′,7-dimalyl derivatives do not. Hydrolysis rate of sodium salt **4** in human plasma is particularly encouraging. All compounds were tested for cytotoxicity using defined human tumour cell lines. The results are summarised in Table 1.

Derivatives **6** and **9**, bearing a 7-malyl group, were significantly less active than paclitaxel. Compounds **3** and **4** showed similar activity when compared to paclitaxel.

Prodrug **4** was subjected to in vivo evaluation against the murine leukaemia P388 tumour. The maximum tolerated dose (MTD) was established at approximately 100 mg/kg once i.p. (MTD of paclitaxel: 50 mg/kg once ip). From this MTD the doses for the therapeutic experiments were derived. The results are depicted in Table 2.

Compound **4** caused a dose-dependent increase of survival time. The optimum effect was obtained with 25 mg/kg/inj in a qd 1–4 schedule. This effect was significantly higher than the best result with the reference paclitaxel (T/C 204), which is higher than the range T/C 118–194 reported in literature.[21,22] In the paclitaxel test there were no 30-days survivors, whereas compound **4** showed 2/6 45-days survivors.

In summary, we have synthesised in good yields some novel water soluble paclitaxel derivatives and have demonstrated that 2′-malyl paclitaxel **3** and its sodium salt **4** act as prodrugs. Most interestingly, in addition to the excellent water solubility, 2′-malyl paclitaxel sodium salt **4** was found to be much more active than paclitaxel in the in vivo p388 tumour model on the basis of a higher therapeutic index than paclitaxel and the presence of long term survivors.

Experimental

General methods

1,2-*O*-Isopropylidene-malic acid,[18] baccatin III,[19] and the protected paclitaxel side chain ((4*R*,5*S*)-3-benzoyl-2,4-diphenyl-1,3-oxazolane-5-carboxylic acid[20] were prepared as described in literature. Dichloromethane was distilled from calcium hydride prior to use. All other materials were obtained from commercial sources and used without further purification. Paclitaxel was a generous gift from Pharmachemie B.V. Haarlem.

NMR spectra were recorded on a Bruker AC300 (300 MHz) or a Bruker AM400 (400 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to TMS as internal standard. The purity of the prodrugs was checked with RP-HPLC. All compounds were at least 90% pure, with some small impurities present, which were integrated as less than 2%.

HPLC: Rheodyne injection valve (20 μL loop); Lichrospher 5RP18 column (200×3 mM, Chrompack); UV-detector (Model 759A, Applied Biosystems); eluent: CH_3CN:MeOH:H_2O: 5:1:4 in 10 mM NH_4OAc (pH 5.0). The detection of the (pro)drugs was performed at

Table 1. Water solubility, stability and cytotoxicity of malic acid paclitaxel derivatives

Compd	Water solubility (mg/mL)	$T_{1/2}$[a] (h)		IC_{50}[b] (ng/mL)					
		PH 7.4	Plasma	MCF7	EVSA-T	WIDR	IGROV	M19	A498
Paclitaxel	0.01	—	—	< 3	< 3	< 3	10	< 3	< 3
3	0.2	> 24	20	< 3	< 3	< 3	< 3	3	39
4	0.6	No pacl.[c]	4	< 3	< 3	< 3	233	6	13
6	0.5	No pacl.[c]	No pacl.[c]	69	59	167	49	311	436
9	0.3	No pacl.[c]	No pacl.[c]	390	300	589	241	1344	1485

[a]Half-live values ($T_{1/2}$), time in which 50% of the derivative is degraded to paclitaxel.
[b]IC_{50}, drug concentration required to inhibit cell proliferation to 50% versus untreated cells (37 °C, 72 h).
[c]No paclitaxel detected (HPLC).

Table 2. In vivo test results for prodrug 4 against a murine P388 tumour model

Compd	Mice	Treatment (days)	Dose (mg/kg/inj.)	Toxic deaths (days)	BWC[a] (%) (day 1–8)	Mean survival (days) ± SD[d]	T/C[c] (%)	45 days survivor
Saline	6	1–4			9	9.2 ± 0.4		0/6
4	6	1–4	50	1 (2)	15	11.2 ± 4.7	122	0/6
4	6	1–4	25		−6[b]	32.5 ± 12.3	353	2/6
4	6	1–4	12.5		22	9.7 ± 0.5	105	0/6
4	6	1	100	1 (6)	−10[b]	12.8 ± 4.6	139	0/6
4	6	1	75		12	11.3 ± 1.0	123	0/6
4	6	1	50		16	10.8 ± 1.5	117	0/6
Paclitaxel	6	1–4	12.5		8	18.8 ± 4.8	204	0/6

[a]Body weight loss relative to control.
[b]The relative increase in body weight is due to ascites development.
[c]Treated over control.
[d]Standard deviation.

226 nM, where it is supposed that the extinction coefficients of paclitaxel and paclitaxel prodrugs are equal. The concentrations were determined by measuring the relative area of paclitaxel or the paclitaxel prodrugs.

TLC analysis was performed on Merck precoated silica gel 60 F-254 plates. Spots were visualised with UV light and a 10% H_2SO_4 solution (1 L) containing ammonium molybdate (25 g) and ceric ammonium sulfate (10 g) followed by charring. Column chromatography was carried out on Merck Kieselgel 60.

Mass spectra were obtained with a double focusing VG 7070E spectrometer.

2′-1,2-*O*-Isopropylidene-malyl) paclitaxel (2). A solution of paclitaxel (853 mg, 1.00 mmol) and 1,2-*O*-isopropylidene-malic acid (191 mg, 1.10 mmol) in CH_2Cl_2 (25 mL) was stirred at −10 °C. Next, DCC (227 mg, 1.10 mmol) and DMAP (15 mg, 0.12 mmol) were added. After stirring for 3 h at −10 °C, the mixture was filtered over Hyflo, diluted with EtOAc (75 mL) and washed with a saturated $NaHCO_3$ solution, demineralised water and brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified via column chromatography (EtOAc:hexane, 1:1), yielding **2** (999 mg, 0.99 mmol, 99%). mp 138 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.15 (2H, d, J = 7.2 Hz, H-Ph), 7.80 (2H, d, J = 7.4 Hz, H-Ph), 7.61 (1H, t, J = 7.2 Hz, H-Ph), 7.45 (10H, m, H-Ph), 7.01 (1H, d, $J_{NH\text{-}3'}$ = 9.2 Hz, NH), 6.29 (1H, s, H10), 6.26 (1H, m, H13), 6.00 (1H, dd, $J_{3'\text{-}NH}$ = 9.2 Hz, $J_{3'\text{-}2'}$ = 3.0 Hz, H3′), 5.69 (1H, d, $J_{2\text{-}3}$ = 7.1 Hz, H2), 5.52 (1H, d, $J_{2'\text{-}3'}$ = 3.0 Hz, H2′), 4.97 (1H, d, $J_{5\text{-}6}$ = 8.0, H5), 4.44 (1H, m, H7), 4.32 (1H, d, $J_{20a\text{-}20b}$ = 8.4 Hz, H20a), 4.21 (1H, d, $J_{20b\text{-}20a}$ = 8.4 Hz, H20b), 4.12 (1H, m, CH-malyl), 3.82 (1H, d, $J_{3\text{-}2}$ = 7.1 Hz, H3), 2.96 (2H, m, CH_2-malyl), 2.51 (1H, m, H6), 2.46 (3H, s, $OCOCH_3$), 2.36 (1H, m, H14a), 2.23 (3H, s, $OCOCH_3$), 2.19 (1H, m, H14b), 2.03 (1H, m, H6), 1.93 (1H, s, H18), 1.69 (3H, s, H16), 1.57 (3H, s, acetonide), 1.51 (3H, s, acetonide), 1.25 (3H, s, H17), 1.13 (3H, s, H19); FABMS, 1010 $[M+H]^+$, 1032 $[M+Na]^+$.

2′-Malyl paclitaxel (3). Compound **2** (500 mg, 0.496 mmol) was dissolved in a mixture of HOAc:THF:H_2O (25:25:25 mL). The mixture was stirred at room temperature for 6 h. Next, the organic solvents were removed by evaporation in vacuo. The residue was diluted by water (50 mL) and freeze dried, yielding **2** (451 mg, 0.466 mmol, 94%). Mp 148–151 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.16 (2H, d, J = 7.6 Hz, H-Ph), 7.93 (2H, d, J = 7.6 Hz, H-Ph), 7.61 (1H, t, J = 7.3 Hz, H-Ph), 7.36 (11H, m, H-Ph and NH), 6.30 (1H, s, H10), 6.28 (1H, m, H13), 6.08 (1H, dd, $J_{3'\text{-}NH}$ = 9.2 Hz, $J_{3'\text{-}2'}$ = 2.8 Hz, H3′), 5.68 (1H, d, $J_{2\text{-}3}$ = 7.3 Hz, H2), 5.51 (1H, d, $J_{2'\text{-}3'}$ = 2.8 Hz, H2′), 4.99 (1H, d, $J_{5\text{-}6}$ = 8.0, H5), 4.46 (1H, m, H7), 4.33 (1H, d, $J_{20a\text{-}20b}$ = 8.4 Hz, H20a), 4.27 (1H, m, CH-malyl), 4.22 (1H, d, $J_{20b\text{-}20a}$ = 8.4 Hz, H20b), 3.82 (1H, d, $J_{3\text{-}2}$ = 7.3 Hz, H3), 3.03 (2H, m, CH_2-malyl), 2.55 (1H, m, H6), 2.53 (3H, s, $OCOCH_3$), 2.40 (1H, m, H14a), 2.23 (3H, s, $OCOCH_3$), 2.13 (1H, m, H14b), 1.93 (1H, s, H18), 1.88 (1H, m, H6), 1.69 (3H, s, H16), 1.21 (3H, s, H17), 1.14 (3H, s, H19); FABMS, 992 $[M+Na]^+$.

2′-Malyl paclitaxel sodium salt (4). To a solution of 2′-malyl paclitaxel (**3**) (250.9 mg, 0.259 mmol) in acetone (10 mL) was added a solution of $NaHCO_3$ (21.7 mg, 0.259 mmol) in demineralised water (20 mL). The reaction mixture was stirred at room temperature for 1 h. Sodium salt **4** (256 mg, 0.259 mmol, 100%) was isolated after removal of the acetone in vacuo and freeze drying. mp 195 °C; ^{1}H NMR (300 MHz, $CDCl_3$): in accordance with the structure of compound **3**; FABMS, 992 $[M+H]^+$, 1014 $[M+Na]^+$.

2′,7-Bis-(1,2-*O*-isopropylidene-malyl)-paclitaxel (5). A solution of paclitaxel (50 mg, 0.059 mmol) and 1,2-*O*-isopropylidene-malic acid (51 mg, 0.29 mmol) in CH_2Cl_2 (5 mL) was stirred at 0 °C. Next, diisopropylcarbodiimide (DIC) (100 μL, 0.64 mmol) and DMAP (7.5 mg, 0.061 mmol) were added. After 1 h, the mixture was heated to reflux temperature and stirred for 3 days. The mixture was filtered over Hyflo. The filtrate was diluted with CH_2Cl_2 (30 mL) and washed with a saturated $NaHCO_3$ solution, demineralised water, 0.5 N $KHSO_4$ solution and brine. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified via column chromatography (EtOAc:hexane, 1:1), yielding **5** (49 mg, 0.0421 mmol, 72%). mp 139 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.14 (2H, d, J = 7.6 Hz, H-Ph), 7.80 (2H, d, J = 7.6 Hz, H-Ph), 7.61 (1H, t, J = 7.4 Hz, H-Ph), 7.39 (10H, m, H-Ph), 7.02 (1H, d, $J_{NH\text{-}3'}$ = 9.2 Hz, NH), 6.24 (1H, s, H10), 6.23 (1H, m, H13), 5.99 (1H, dd, $J_{3'\text{-}NH}$ = 9.2 Hz, $J_{3'\text{-}2'}$ = 3.0 Hz, H3′), 5.69 (1H, d, $J_{2\text{-}3}$ = 6.9 Hz, H2), 5.66 (1H, m, H7), 5.55 (1H, d, $J_{2'\text{-}3'}$ = 3.0 Hz, H2′), 4.97 (1H, d, $J_{5\text{-}6}$ = 9.4, H5), 4.84 (1H, m, CH-malyl), 4.64 (1H, m, CH-malyl), 4.33 (1H, d, $J_{20a\text{-}20b}$ = 8.4 Hz, H20a), 4.19 (1H, d, $J_{20b\text{-}20a}$ = 8.4 Hz, H20b), 3.94 (1H, d, $J_{3\text{-}2}$ = 6.9 Hz, H3), 3.10 (2H, m, CH_2-malyl), 2.97 (2H, m, CH_2-malyl), 2.60 (1H, m, H6), 2.45 (3H, s, $OCOCH_3$), 2.35 (1H, m, H14a), 2.25 (1H, m, H14b), 2.21 (3H, s, $OCOCH_3$), 1.97 (1H, s, H18), 1.85 (1H, m, H6), 1.81 (3H, s, H16), 1.58 (6H, s, acetonide), 1.56 (6H, s, acetonide), 1.21 (3H, s, H17), 1.16 (3H, s, H19); FABMS, 1166 $[M+H]^+$.

2′,7-Bis-(malyl) paclitaxel (6). Compound **5** (40 mg, 0.0343 mmol) was dissolved in a mixture of HOAc:THF:H_2O (4:1:2 mL). The mixture was stirred at 45 °C for 6 h. Next, the organic solvents were removed by evaporation in vacuo. The residue was diluted by water (50 mL) and freeze dried, yielding **5** (33 mg, 0.0304 mmol, 89%). mp 166–168 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.11 (2H, d, J = 7.6 Hz, H-Ph), 7.84 (2H, d, J = 7.5 Hz, H-Ph), 7.63 (1H, t, J = 7.5 Hz, H-Ph), 7.37 (11H, m, H-Ph and NH), 6.25 (1H, s, H10), 6.16 (1H, m, H13), 5.99 (1H, dd, $J_{3'\text{-}NH}$ = 8.8 Hz, $J_{3'\text{-}2'}$ = 2.9 Hz, H3′), 5.67 (1H, d, $J_{2\text{-}3}$ = 6.4 Hz, H2), 5.66 (1H, m, H7), 5.63 (1H, d, $J_{2'\text{-}3'}$ = 2.8 Hz, H2′), 4.94 (1H, d, $J_{5\text{-}6}$ = 7.6, H5), 4.48 (2H, m, CH-malyl), 4.32 (1H, d, $J_{20a\text{-}20b}$ = 7.9 Hz, H20a), 4.17 (1H, d, $J_{20b\text{-}20a}$ = 7.9 Hz, H20b), 3.88 (1H, d, $J_{3\text{-}2}$ = 6.4 Hz, H3), 2.97 (4H, m, CH_2-malyl), 2.51 (1H, m, H6), 2.44 (3H, s, $OCOCH_3$), 2.36 (2H, m, H14), 2.07 (3H, s, $OCOCH_3$), 1.93 (1H, s, H18), 1.86 (1H, m, H6), 1.79 (3H, s, H16), 1.20 (3H, s, H17), 1.18 (3H, s, H19); FABMS, 1108 $[M+Na]^+$.

7-(1,2-*O*-Isopropylidene-malyl) baccatin III (7). To a solution of baccatin III (107 mg, 0.183 mmol) and 1,2-*O*-isopropylidene-malic acid (43 mg, 0.274 mmol) in CH_2Cl_2 (5 mL) were added DCC (60 mg, 0.292 mmol) and DMAP (7.5 mg, 0.06 mmol). After stirring for 20 h another portion of 1,2-*O*-isopropylidene-malic acid (43 mg, 0.274 mmol) and DCC (60 mg, 0.292 mmol) were added. After stirring for another 4 h still some starting material was present, but also the formation of a different compound (possibly 7,13-bis-(1,2-*O*-isopropylidene-malyl) baccatin III) was observed on TLC. The mixture was diluted with EtOAc (25 mL) and washed with a saturated $NaHCO_3$ solution. The organic layer was washed with water, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified via column chromatography (EtOAc:hexane, 1:1), yielding baccatin III (20 mg 0.034 mmol) and **7** (88 mg, 0.121 mmol, 66%). Mp 160–163 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.10 (2H, d, J = 7.2 Hz, H-Ph), 7.57 (1H, t, J = 7.2 Hz, H-Ph), 7.47 (2H, d, J = 7.2 Hz, H-Ph), 6.26 (1H, s, H10), 5.67 (2H, m, H2, H7), 4.91 (4H, m, H13 H5 CH-malyl H2′), 4.20 (1H, d, $J_{20a\text{-}20b}$ = 8.4 Hz, H20a), 4.13 (1H, d, $J_{20b\text{-}20a}$ = 8.4 Hz, H20b), 3.95 (1H, d, $J_{3\text{-}2}$ = 7.1 Hz, H3), 2.92 (1H, dd, J_{gem} = 17.2 Hz, $J_{CH\text{-}malyl}$ = 3.0 Hz, CH_2a-malyl), 2.56 (1H, dd, J_{gem} = 17.2 Hz, $J_{CH\text{-}malyl}$ = 9.0 Hz, CH_2b-malyl), 2.51 (1H, m, H6a), 2.46 (3H, s, $OCOCH_3$), 2.36 (1H, m, H14a), 2.23 (3H, s, $OCOCH_3$), 2.19 (1H, m, H14b), 2.03 (1H, m, H6b), 1.93 (1H, s, H18), 1.69 (3H, s, H16), 1.60 (3H, s, acetonide), 1.56 (3H, s, acetonide), 1.21 (3H, s, H17), 1.08 (3H, s, H19); FABMS, 744 $[M+H]^+$, 766 $[M+Na]^+$.

2′,3′-*O*,*N*-((4-methoxy)benzylidene-7-1,2-*O*-isopropylidene-malyl) paclitaxel (8). A solution of **7** (68 mg, 0.094 mmol) and (4*R*,5*S*)-3-benzoyl-2,4-diphenyl-1,3-oxazolidine-5-carboxylic acid (57 mg, 0.141 mmol) in CH_2Cl_2 (5 mL) was stirred at 0 °C. Next, DCC (31 mg, 0.150 mmol) and a few crystals of DMAP were added. After stirring for 8 h at room temperature, the mixture was diluted with EtOAc (25 mL) and washed with a saturated $NaHCO_3$ solution, demineralised water, 0.5 N $KHSO_4$ solution and brine. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified via column chromatography (EtOAc: hexane, 1:1), yielding **8** (77 mg, 0.692 mmol, 74%). Mp 128–130 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.04 (2H, d, J = 7.2 Hz, H-Ph), 7.60 (1H, t, J = 7.2 Hz, H-Ph), 7.46 (3H, m, H-Ph), 7.35 (3H, m, H-Ph), 7.30 (8H, m, H-Ph), 7.91 (1H, d, J = 8.6 Hz, H-Ph-OMe), 6.85 (1H, s, oxazolidine) 6.31 (1H, t, $J_{13\text{-}14}$ = 8.8, H13), 6.26 (1H, s, H10), 5.66 (2H, m, H2, H7), 5.55 (1H, br s, H3′), 4.85 (3H, m, H5 CH-malyl H2′), 4.20 (1H, d, $J_{20a\text{-}20b}$ = 8.4 Hz, H20a), 4.13 (1H, d, $J_{20b\text{-}20a}$ = 8.4 Hz, H20b), 3.95 (1H, d, $J_{3\text{-}2}$ = 7.1 Hz, H3), 3.85 (3H, s, Ph-OMe) 3.09 (1H, dd, J_{gem} = 17.2 Hz, $J_{CH\text{-}malyl}$ = 3.0 Hz, CH_2a-malyl), 2.62 (1H, dd, J_{gem} = 17.2 Hz, $J_{CH\text{-}malyl}$ = 9.0 Hz, CH_2b-malyl), 2.51 (1H, m, H6a), 2.46 (3H, s, $OCOCH_3$), 2.36 (1H, m, H14a), 2.23 (3H, s, $OCOCH_3$), 2.19 (1H, m, H14b), 2.03 (1H, m, H6b), 1.93 (1H, s, H18), 1.69 (3H, s, H16), 1.60 (3H, s, acetonide), 1.57 (3H, s, acetonide), 1.25 (3H, s, H17), 1.13 (3H, s, H19); FABMS, 1151 $[M+Na]^+$.

7-Malyl paclitaxel (9). Compound **8** (74 mg, 0.0.067 mmol) was dissolved in EtOAc (5 mL). Next, an 3.5 N HCl solution in EtOAc (1 mL) was added. The mixture was stirred at room temperature for 15 min. The solvents were removed by evaporation in vacuo. The residue was diluted by water (50 mL) and freeze dried, yielding **9** (64 mg, 0.059 mmol, 89%). Mp 166–168 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.11 (2H, d, J = 7.6 Hz, H-Ph), 7.84 (2H, d, J = 7.5 Hz, H-Ph), 7.63 (1H, t, J = 7.5 Hz, H-Ph), 7.37 (11H, m, H-Ph and NH), 6.25 (1H, s, H10), 6.16 (1H, m, H13), 5.99 (1H, dd, $J_{3'\text{-}NH}$ = 8.8 Hz, $J_{3'\text{-}2'}$ = 2.9 Hz, H3′), 5.67 (1H, d, $J_{2\text{-}3}$ = 6.4 Hz, H2), 5.66 (1H, m, H7), 5.63 (1H, d, $J_{2'\text{-}3'}$ = 2.8 Hz, H2′), 4.94 (1H, d, $J_{5\text{-}6}$ = 7.6, H5), 4.48 (2H, m, CH-malyl), 4.32 (1H, d, $J_{20a\text{-}20b}$ = 7.9 Hz, H20a), 4.17 (1H, d, $J_{20b\text{-}20a}$ = 7.9 Hz, H20b), 3.88 (1H, d, $J_{3\text{-}2}$ = 6.4 Hz, H3), 2.97 (4H, m, CH_2-malyl), 2.51 (1H, m, H6), 2.44 (3H, s, $OCOCH_3$), 2.36 (2H, m, H14), 2.07 (3H, s, $OCOCH_3$), 1.93 (1H, s, H18), 1.86 (1H, m, H6), 1.79 (3H, s, H16), 1.20 (3H, s, H17), 1.18 (3H, s, H19); FABMS, 1108 $[M+Na]^+$.

Biological activity

Water solubility.[23] Paclitaxel and paclitaxel derivatives were suspended in water or PBS-buffer (pH 7.4) until a concentration was reached of 2 mg/mL. The suspensions were sonicated for 15 min and centrifuged (13,000 *g*) for 10 min. The above fluid was analysed, using HPLC.

Stability in human plasma and PBS-buffer. The paclitaxel prodrugs (**3**, **4**, **6** and **9**) were dissolved in water, sonicated and centrifuged. A 100 μL of the above fluid was mixed with 400 μL of plasma (heparin) or PBS-buffer (pH 7.4). The solutions were incubated at 37 °C and on different points in time (T = 0, 0.5, 1, 4, 20, 48 h) 50 μL was extracted with 150 μL of EtOAc. After mixing for 2 min (using a vortex), this mixture was centrifuged (2 min, 13,000 *g*) and 100 μL EtOAc was evaporated (30 min, in vacuo). The (pro)drugs were dissolved in 50 μL eluent and analysed by HPLC.

In vitro cytotoxicity assay. The compounds under study were dissolved to a concentration of 177147 ng/mL in 5% DMSO in full RPMI growth medium. On day 0, 200 μL of trypsinised tumour cells (2×10^3 cells/well) were plated in 96-wells flatbottom microtiter plates (Costar, no. 3799, Badhoevedorp, The Netherlands). The plates were preincubated 24 h at 37 °C, 5% CO_2 to allow the cells to adhere.

On day 2, 100 μL of the highest drug concentration was added to the wells of column 12 and from there diluted 3-fold to column 3 by serial transfer of 100 μL using an eight channel micropipette. The final volume of column 3 was adjusted to 200 μL with PBS. Column 2 was used for the blank. To column 1 PBS was added to diminish interfering evaporation.

On day 7 the incubation was terminated by washing the plates twice with PBS. Subsequently the cells were fixed with 10% trichloroacetic acid in Milli Q water (Millipore, Etten Leur, The Netherlands) and placed at 4 °C for 1 h.

After five washings with tap water, the cells were stained for at least 15 min with 0.4% SRB, dissolved in 1% acetic acid, and subsequently washed with 1% acetic acid to remove the unbound stain. The plates were air dried and the bound protein was dissolved by using 150 μL 10 mmol/L tris base. The absorbance was read at 540 nM using an automatic microplate reader (Titertec, Flow Laboratories Ltd., Irvine, UK). Data were used for construction of concentration–response curves and determination of the IC_{50}-value (for more information on the test methodology, see refs 24–26).

Approximative toxicity. Two mice received 25, 50 100, and 200 mg/kg of compound **4** once intraperitonealy (ip). Mice treated with 200 mg/kg died 3 days later. The substance caused a dose-dependent body weight reduction. The maximum tolerated dose (MTD) is approximative 100 mg/kg once ip. From this dose dosages in the therapeutic experiments were derived.

In vivo P388 leukaemia screen. Paclitaxel and 2′-malyl paclitaxel sodium salt **4** were administered i.p. as aqueous solutions. Female B6D2F1 mice were implanted i.p. with P388 cells (10^6 cells/mouse) on day 0. The mice were then treated ip with paclitaxel or the prodrug for 4 consecutive days (for dose see Table 2). Control groups received a saline solution. Medial survival times of compound-treated (T) mice were compared to the medial survival time of the control (C) mice. The ratio of the two values for each compound treated group of mice was multiplied by 100 and expressed as a percentage (i.e. T/C%). These test were conducted by EPO-GmbH, Berlin, under the supervision of Dr. I. Fichtner.[27]

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