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Conjugates of the fungal cytotoxin illudin M with improved tumour specificity

Rainer Schobert [a,*], Bernhard Biersack [a], Sebastian Knauer [a], Matthias Ocker [b]

[a] Organic Chemistry Laboratory, University of Bayreuth, Universitätsstraße 30, 95440 Bayreuth, Germany
[b] Department of Medicine 1, University Hospital Erlangen, Friedrich-Alexander-University Erlangen-Nuernberg, 91054 Erlangen, Germany

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ABSTRACT

A simplified procedure for the isolation of gram quantities of illudin M from culture broths of basidiomycete *Omphalotus olearius* is described. Esters of illudin M with docosahexaenoic acid, chlorambucil, demethylcantharidinic acid (endothall) and 2,2′-bipyridyl-5,5′-dicarboxylic acid were synthesised and tested for cytotoxicity and induction of apoptosis in two clinically relevant tumour cell lines (Panc-1 pancreas carcinoma and HT-29 colon carcinoma) and in non-malignant human foreskin fibroblasts. The demethylcantharidin and the bipyridine conjugates retained the cytotoxicity of the parent illudin M while displaying an improved specificity for the tumour cells over the fibroblasts.

1. Introduction

A frequent drawback of cytotoxic agents that rules them out as candidates for further clinical studies is their lack of specificity for tumour cells over normal body tissue, that is, their small therapeutic index. An often-applied strategy to improve this specificity is the covalent attachment of cancer cell seeking shuttle groups. For instance, the conjugation of clinically established drugs like taxol, camptothecin and doxorubicin to ω-3-fatty acids such as docosahexaenoic acid dramatically improved their efficacy in mice bearing various solid tumours.[1–3]

Illudin M (**1**) is a cytotoxic fungal sesquiterpene of the illudane class comprising strongly electrophilic spirocyclopropane and enone systems. It is amenable to alkylation of bionucleophiles such as DNA, RNA and proteins via a two-stage process (Fig. 1). Together with its congener, illudin S (**2**), it was isolated from the culture medium of *Omphalotus olearius* (formerly known as *Clitocybe illudens* or *Omphalotus illudens*) mushrooms.[4–6] The illudins were found to be highly cytotoxic in various drug resistant cancer cells with increased nucleotide excision repair or ABC drug transporter activity. In some cell lines, one-digit nanomolar IC_{50} concentrations were measured by the established colorimetric cytotoxicity assays, employing 3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) or sulforhodamine B (SRB) as dyes. Yet, the unfavourable toxicity profiles and narrow therapeutic windows of the illudins had prevented clinical application.[7] Limited sets of structurally varied derivatives of **1** and **2** were prepared to improve these pharmacological shortcomings. McMorris et al. reported[8] improved therapeutic indices for some derivatives albeit at the expense of antiproliferative efficacy. Kinder et al. found that an intact indane skeleton is not a prerequisite for antitumoural activity and that some structurally simplified bicyclic analogues of illudins S and M even displayed a better discrimination between different tumour cell lines.[9] Irofulven (**3**), another semisynthetic derivative by the McMorris group,[10] was assessed by the NCI and underwent several phase II clinical trials in hormone refractory prostate cancer, ovarian, pancreatic, renal, colorectal, lung and breast cancer.[11] However, it turned out to be rather ineffective in most of the trials except for some prostate and pancreatic cancer

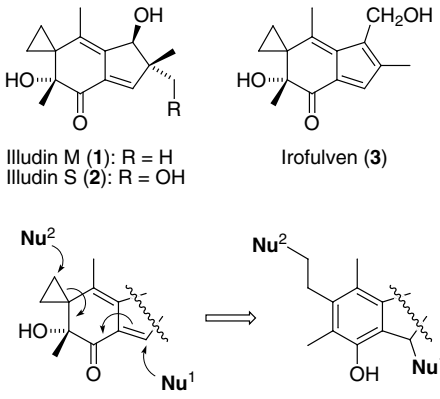

Figure 1. Natural cytotoxins illudin M (**1**) and illudin S (**2**), semisynthetic anticancer drug irofulven (**3**) and their general two-stage reaction with bionucleophiles **Nu**.

* Corresponding author. Fax: +49 0 921 552671.
E-mail address: Rainer.Schobert@uni-bayreuth.de (R. Schobert).

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cases, while severe side effects such as retinotoxicity were frequently observed.[12–19] Specific DNA adducts of **3** were structurally characterised, recently.[20] The rather daunting clinical results were an incentive for us to investigate other derivatives of illudin M, in particular those with an unmodified illudin M core linked to unsaturated fatty acid shuttles, or other potentially synergetic effector components such as alkylating agents or compounds targeting the nucleotide excision repair (NER) machinery. Herein, we report their syntheses and antiproliferative properties in two clinically relevant tumour cell lines, Panc-1 pancreas carcinoma and HT-29 colon carcinoma, as well as in non-malignant human foreskin fibroblasts. We also disclose a simplified procedure for the isolation of gram quantities of illudin M from culture broths of *Omphalotus olearius*.

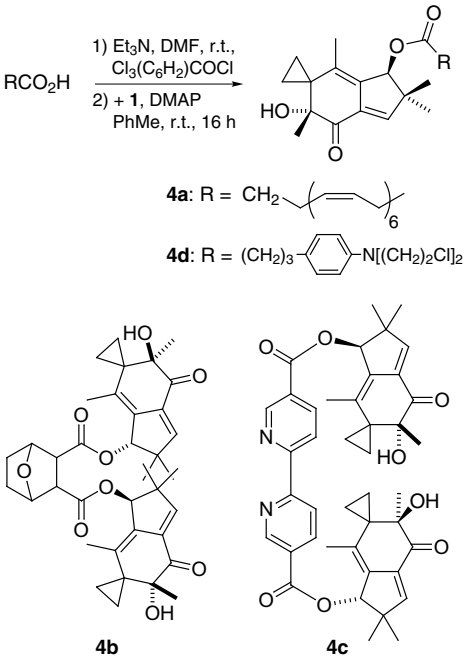
2. Results and discussion

2.1. Isolation of illudin M

Omphalotus olearius (DSM 3398) was grown at 20 °C in a sterile 20 L plastic flask containing HA-medium[21] (72 g yeast extract, 180 g malt extract, 72 g glucose, 18 L water, pH 5.5) under a continuous flow of sterile air. After about 4 weeks, the culture liquid was extracted with ethyl acetate (six to seven extractions, each with 10% of the volume) and the combined and dried extracts were concentrated and purified by column chromatography (silica gel 60; hexane/ethyl acetate 2:1; R_f 0.35). Fractions containing pure illudin M were pooled and concentrated to afford ca. 800 mg of crystalline illudin M (**1**) upon standing. Very little illudin S (**2**) and other minor side products were detected by analytical HPLC[22] in the ethyl acetate extracts, and were easily removed during the chromatographical work-up.

2.2. Ester conjugates of illudin M

Illudin M (**1**) was esterified with various mono- and dicarboxylic acids at its secondary OH-group, according to the protocol of Yamaguchi (Scheme 1).[23] The respective carboxylic acid or sodium salt thereof was first treated with Et_3N and 2,4,6-trichlorobenzoyl chloride to give a mixed anhydride within ca. 20 min. This was treated with **1** and 4-(*N*-dimethylamino)pyridine (DMAP) to afford the corresponding ester **4** in yields ranging from 30% to 75%. Ester **4a** was obtained from docosahexaenoic acid (DHA), ester **4b** from one equivalent of endothall disodium salt and two equivalents of **1**, ester **4c** likewise from 2,2′-bipyridyl-5,5′-dicarboxylic acid, and ester **4d** from chlorambucil. The products were purified by column chromatography on silica gel.



Scheme 1. Synthesis of illudin M esters **4**.

These carboxylic acids were chosen because tumour-specific activities were anticipated of their esters **4**. Polyunsaturated fatty acids such as DHA are known to play an important role in the delay of the progression of cancers caused by reactive oxygen species (ROS), presumably by modulating hormone receptors, Akt kinase and the nuclear factor κ_B (NF-κ_B). $n-3$ fatty acids, in particular, were shown to inhibit the formation of the tumour growth promoter 13-hydroxy-octadecadienoic acid (13-HODE) by lowering the uptake of linoleic acid, which is mediated via G_i protein-coupled signal transduction pathways.[24–26] Endothall (1-oxabicyclo-[2.2.1.]-heptane-2,3-dicarboxylic acid) is the diacid form of the demethyl analogue of cantharidin, the active principle of *Epicanta gorhami* or *Mylabris* ('blister beetles'), used in traditional Chinese medicine for the treatment of liver, lung, intestinal and digestive tract tumours. In clinical trials, cantharidin had shown therapeutic effects on patients with primary hepatoma, but also occasional severe side effects.[27] Endothall was found to be far less toxic while still active against tumours. The mode of action of the cantharidin family members is thought to be related to their inhibition of the serine–threonine protein phosphatase 2A (PP2A)[28] which plays a role in the tumourigenic transformation,[29–31] the control of the cell cycle[32] and in cell proliferation.[33] Other literature findings suggest that the PP2A activity might be essential for the nuclear excisson repair of DNA lesions induced by alkylants.[34] Kelner, McMorris, Jaspers et al. had reported that 90% of illudin's lethal effects in human fibroblasts can be prevented by an active nucleotide excision repair (NER) system.[35] In in vitro experiments, the levels of PP2A in various organs paralleled the cytotoxicity of cantharidin derivatives. Intestinal organs are particularly rich in PP2A. It is also worthy of note that endothall inhibited the growth of primary hepatocellular carcinoma cell lines more than that of normal hepatocytes.[36] Thus conjugation of illudin M to endothall as in **4b** should increase the organ selectivity and tumour specificity. 2,2′-Bipyridyl-5,5′-dicarboxylic acid is a dye-sensitiser and intercalator to DNA. More recently, it has been attached via ester bonds to sugar-type tumour markers with the intention to amplify the bioactivity by forming oligomeric cluster complexes with metal ions available under physiological conditions, such as Cu(II) or Fe(III).[37] Similar ancillary effects were expected from the bipyridine moiety of illudin M conjugate **4c**. Chlorambucil is a clinically used bifunctional cross-linking N-mustard, preferentially alkylating the N-7 atoms of guanine residues in DNA. The conjugate **4d** was therefore hoped to lead to DNA lesions of unusual topology and so to altered responses by proteins involved in the identification and repair of DNA defects.

2.3. Biological evaluation

To assess the therapeutic window of the new illudin M esters **4** relative to the parent compound **1**, cells of Panc-1 pancreatic carcinoma and HT-29 colon adenocarcinoma, as well as non-malignant human foreskin fibroblasts (HFs) were incubated for up to 120 h with **1** and **4** at concentrations ranging from 0.01 to 10 μM, and finally stained with Trypan blue. The number of unstained (vital) cells was counted and expressed as relative cell numbers compared to untreated controls (Figure 2 shows results with 0.1, 1 and 10 μM concentrations of the test compounds.). Although affording seemingly lower activity values, the Trypan blue method

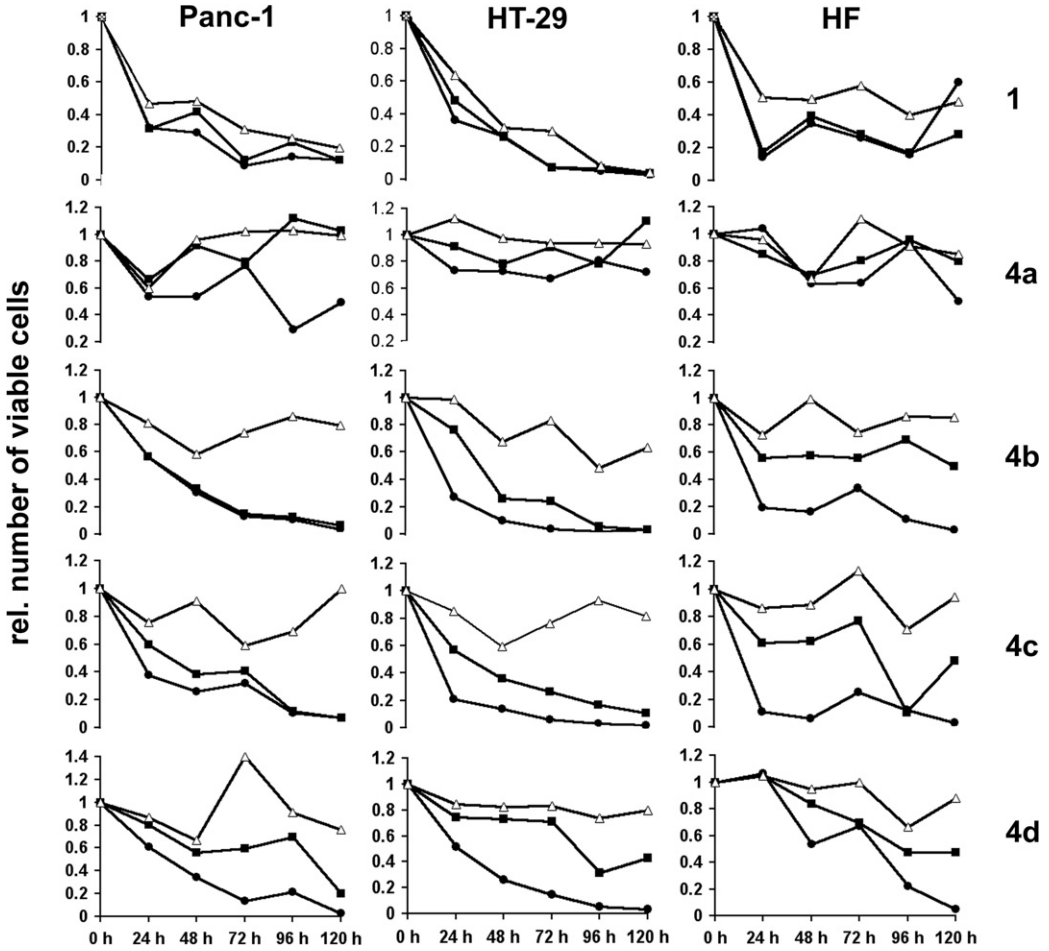


Figure 2. Cytotoxic effects of illudin M (**1**) and its conjugates **4a–d** at various concentrations (●: 10 μM; ■: 1 μM; △: 0.1 μM) in cells of Panc-1 pancreatic carcinoma, HT-29 colon adenocarcinoma and non-malignant human foreskin fibroblasts (HFs) upon incubation for 24–120 h. *Y*-axis shows number of viable cells relative to untreated controls (100%) as ascertained by the Trypan blue exclusion assay. Values represent means of three independent experiments.

is more accurate than MTT or SRB assays since only truly dead cells get stained, and since it allows to directly determine cell proliferation numbers rather than surrogate parameters. The DHA ester **4a** was only very weakly active in all three kinds of cells at any concentration. In contrast, the bis(illudinyl M) demethylcantharidinate **4b** and the bipyridine **4c** exhibited a cytotoxic effect in the tumour cells at concentrations between 1 and 10 μM which was more pronounced and which set on more rapidly than that of **1**. For instance, cell numbers were quickly reduced to 33% (Panc-1) or 26% (HT-29), respectively, after incubation with 1 μM of **4b** for 48 h and to 6% (Panc-1) or 3% (HT-29), respectively, after 120 h. The non-malignant fibroblasts showed a merely moderate response to **4b** at 1 μM, with a drop of the number of viable cells to 57% of untreated controls after 48 h and to 49% after 120 h. Illudin M (**1**) under the same conditions caused cell number reductions to 42% (Panc-1) or 26% (HT-29) or 39% (HF), respectively, after 48 h and to 12% (Panc-1) or 4% (HT-29) or 28% (HF), respectively, after 120 h. Thus, conjugate **4b** had a greater cytotoxic impact on the tumour cells while affecting the non-malignant HF cells less when compared with the parent illudin M. This is also a strong indication of **4b** reaching its targets in an intact form rather than decomposing beforehand into two equivalents of illudin M. The cytotoxicity profile of the bipyridyl conjugate **4c** resembled that of **4b**. At a concentration of 1 μM **4c** reduced the numbers of Panc-1/HT-29/HF cells to 38%/36%/62% after 48 h of exposure and to 7%/11%/48% after 120 h. The chlorambucil conjugate **4d** when applied at medium concentrations, for example, at 0.1 to 1 μM, had a smaller and comparable cytotoxic effect in all three cell lines. With respect to potential clinical applicability, conjugates **4b** and **4c** are clearly the most interesting ones.

The ability of compounds **4** to induce apoptosis in tumour cells was investigated next. Treated cells were stained with propidium iodide, and the labelled nuclei were analysed using a fluorescence-activated cell sorter (FACS). The percentage of apoptotic cells was determined by measuring the fraction of nuclei with a sub-diploid DNA content (Fig. 3). While compounds **4a** and **4d** caused apoptosis rates of less than 10% in Panc-1 and HT-29 cells upon incubation for up to 96 h at concentration of 1 μM, an increase in sub-diploid events was observed in FACS analysis for the same concentration of conjugates **4b** and **4c** reaching up to 74% after 120 h in the case of the demethylcantharidin conjugate **4b** acting on Panc-1 cells. In HT-29 cells, 1 μM of compound **4b** gave rise to 53% apoptosis after 120 h. Compound **4c** was less efficacious, eliciting 62% apoptosis in Panc-1 cells and 21% apoptosis in HT-29 cells upon incubation at 1 μM for 120 h. Non-transformed HFs were more resistant to apoptosis induced by **4b** (33%, 1 μM, 120 h) and **4c** (14%, 1 μM, 120 h). The magnitude of apoptosis induction as well as the spread between induction in the tumour versus the non-malignant cells was the greatest for conjugate **4b**. In comparison, illudin M (**1**) showed a comparable induction of apoptosis in the tumour cells (86% in Panc-1; 48% in HT-29), but an even greater one in the HF (89%). Only at 0.1 μM, illudin M caused a higher apoptosis rate in Panc-1 cells than in HF. Thus, in terms of both cytotoxicity and apoptosis induction, at a concen-

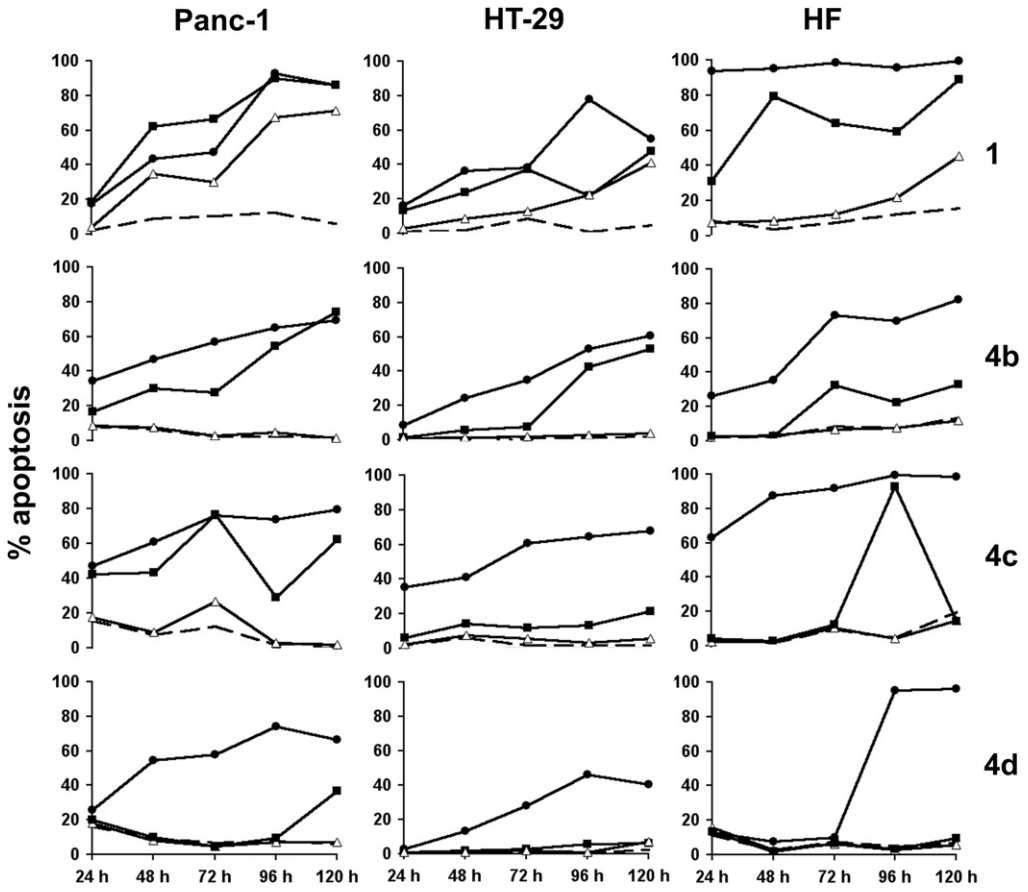


Figure 3. Induction (in%) of apoptosis in Panc-1 cells, HT-29 cells and HF after treatment with different concentrations (●: 10 μM; ■: 1 μM; △: 0.1 μM; – –: control) of illudin M (**1**) and its conjugates **4b–d** by measuring the fraction of nuclei with a sub-diploid DNA content in a FACS. Values represent means of three independent experiments.

Table 1
Vital cells (in%)[a] and apoptotic cells (in%)[b] of Panc-1 pancreatic carcinoma, HT-29 colon adenocarcinoma and non-malignant human foreskin fibroblasts upon exposure for 120 h to 1 μM of compounds **1** and **4a–d**

	Panc-1	HT-29	HF
Vital cells (%)			
1	12.3 ± 9.6	3.8 ± 11.4	28.1 ± 14.6
4a	102.8 ± 1.4	110.6 ± 4.1	80.0 ± 10.2
4b	6.4 ± 7.4	3.0 ± 0.0	49.3 ± 10.3
4c	7.3 ± 5.0	10.8 ± 3.9	47.8 ± 16.5
4d	20.0 ± 3.4	42.7 ± 1.2	47.5 ± 3.6
Apoptosis (%)			
1	86.0 ± 1.5	47.9 ± 0.1	88.9 ± 0.8
4a	2.6 ± 0.9	1.9 ± 0.2	4.4 ± 1.9
4b	74.0 ± 1.5	52.9 ± 1.4	33.1 ± 12.8
4c	62.1 ± 15.6	21.3 ± 1.9	14.3 ± 0.5
4d	36.8 ± 2.9	6.0 ± 0.9	9.5 ± 1.9

[a] Values are derived from concentration–response curves obtained by measuring the percentage of vital cells relative to untreated controls (100%) after 120 h exposure to test compounds (1 μM) in the Trypan blue exclusion assay. Values represent means of three independent experiments ± standard deviation.

[b] Values are derived from the numbers of apoptotic cells as determined by measuring the fraction of nuclei with a sub-diploid DNA content in a FACS relative to untreated controls (100%); they represent means of three independent experiments ± standard deviation.

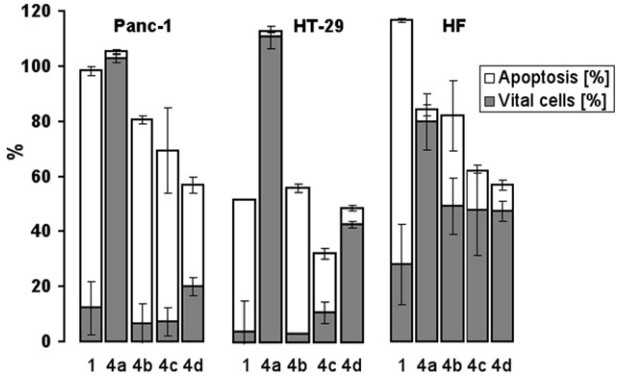


Figure 4. Percentage of vital and apoptotic Panc-1 pancreatic carcinoma cells, HT-29 colon adenocarcinoma cells and non-malignant human foreskin fibroblasts upon exposure for 120 h to 1 μM of compounds **1** and **4a–d**. Conditions as stated in Table 1.

tration of 1 μM, the ester **4b** was as efficacious as illudin M itself, but distinctly more tumour-specific (Table 1 and Fig. 4). Although irofulven (**3**) showed a comparably good selectivity in apoptosis induction in breast cancer cells over normal mammary epithelial cells at 1 μM concentration, its antiproliferative effect as ascertained by the MTT assay was much weaker (ca 70% viable cells upon exposure to 1–10 μM for 24–48 h).[38]

2.4. Conclusions

The finding that the esters of illudin M with endothall (**4b**) and 2,2′-bipyridyl dicarboxylic acid (**4c**) retained the cytotoxicity of the parent illudin M while displaying a significantly improved specificity for tumour cells over normal fibroblasts is encouraging. As endothall is known to target the liver, conjugate **4b** will shortly be tested on various liver cancers in vitro and in vivo. The failure

of the DHA conjugate **4a** did not come as a big surprise. DHA conjugates of other cytotoxic drugs such as taxol also exhibited little if any activity in tumour cell studies but were quite efficacious in corresponding animal models with tumour xenografts. With our simplified procedure for the production of gram quantities of illudin M at hand, larger arrays of ester conjugates can be readily prepared and screened now. This should allow us to pinpoint those properties of the effector groups attached to illudin M which most distinctly improve its therapeutic index and tumour specificity.

3. Experimental

3.1. General

IR-spectra were recorded on a Perkin-Elmer One FT-IR spectrophotometer. Magnetic resonance (NMR) spectra were recorded under conditions as indicated on a Bruker Avance 300 spectrometer. Chemical shifts (δ) are given in parts per million downfield from TMS as internal standard. Mass spectra were recorded using a Varian MAT 311A (EI). Elemental analyses were carried out with a Perkin-Elmer 2400 CHN elemental analyser. For column chromatography, Merck silica gel 60 (230–400 mesh) was used. Starting compounds and components of the culture medium for *Omphalotus olearius* were purchased from the usual sources, and were used without further purification.

3.2. Chemistry

3.2.1. Docosahexaenoyl illudin M (4a)

Docosahexaenoic acid (100 mg, 0.31 mmol) was dissolved in dry DMF (2 mL) and treated with NEt_3 (49 μL, 0.36 mmol) and 2,4,6-trichlorobenzoyl chloride (55 μL, 0.36 mmol). The resulting suspension was stirred at room temperature under an atmosphere of argon for 20 min. A solution of illudin M (76 mg, 0.31 mmol) and DMAP (75 mg, 0.62 mmol) in dry toluene (5 mL) was added and the resulting mixture was stirred for 16 h. After dilution with ethyl acetate and washing with water, the organic phase was dried over Na_2SO_4 and concentrated in vacuum. The residue was purified by column chromatography (silica gel 60; ethyl acetate/*n*-hexane 1:9; R_f 0.31) to leave the product as a colourless oil (109 mg, 0.20 mmol, 65%); ν_{max}(ATR)/cm^{-1} 3491, 3013, 2964, 1733, 1699, 1609, 1445, 1362, 1255, 1148, 1106, 945, 706; δ_H (300 MHz, $CDCl_3$) 0.3–0.4 (1H, m), 0.7–1.0 (5H, m), 1.05 (3H, s), 1.0–1.1 (1H, m), 1.16 (3H, s), 1.33 (3H, s), 1.48 (3H, s), 2.0–2.1 (2H, m), 2.3–2.4 (4H, m), 2.7–2.9 (10H, m), 3.54 (1H, s), 5.2–5.4 (12H, m), 5.64 (1H, m), 6.49 (1H, s); δ_C (75 MHz; $CDCl_3$) 5.9, 8.7, 14.2, 14.5, 20.5, 20.6, 22.8, 24.7, 25.5, 25.6, 26.7, 31.4, 34.1, 48.7, 76.0, 78.7, 126.9, 127.7, 127.8, 127.9, 128.0, 128.2, 128.3, 128.5, 129.4, 132.0, 133.5, 135.1, 135.3, 146.1, 172.7, 200.0; *m/z* (EI, 70 eV) 558 (M^+, 2%), 265 (5%), 231 (24%), 203 (26%), 105 (28%), 79 (65%), 67 (53%), 43 (100%). Anal. Calcd for $C_{37}H_{50}O_4$: C, 79.5; H, 9.0. Found: C, 79.3; H, 8.9%.

3.2.2. Bis(illudinyl M) endothallate (4b)

Analogously to the synthesis of ester **4a**, compound **4b** (75 mg, 30%) was obtained from disodium endothallate (93 mg, 0.40 mmol), NEt_3 (130 μL, 0.94 mmol), 2,4,6-trichlorobenzoyl chloride (144 μL, 0.94 mmol), illudin M (200 mg, 0.81 mmol) and DMAP (199 mg, 1.62 mmol) as a colourless oil; R_f 0.44 (ethyl acetate/*n*-hexane 1:3); ν_{max}(ATR)/cm^{-1} 3489, 2961, 1727, 1689, 1607, 1467, 1447, 1362, 1254, 1238, 1202, 1172, 1106, 1052, 944, 928, 909, 819, 730; δ_H (300 MHz, $CDCl_3$) 0.3–0.5 (2H, m), 0.7–1.8 (34H, m), 3.0–3.1 (2H, m), 3.4–3.6 (4H, m), 4.7–4.9 (2H, m), 5.63 (1H, s), 5.65 (1H, s), 6.49 (1H, s), 6.51 (1H, s); δ_C (75 MHz; $CDCl_3$) 6.1, 8.9, 14.1, 14.5, 14.6, 14.9, 20.6, 20.8, 21.0, 24.6, 24.7, 24.8, 25.6, 26.6, 26.7, 29.3, 29.5, 31.4, 31.5, 48.7, 48.8, 51.0, 51.2, 51.3, 51.8, 75.9, 76.0, 77.8, 78.9, 79.6, 79.7, 79.8, 80.5, 80.6, 133.4, 133.5, 134.9, 135.8, 136.0, 145.9, 146.1, 146.2, 170.8, 171.8, 199.7, 199.9; *m/z* (EI, 70 eV) 646 (M^+, 3%), 629 (3%), 416 (7%), 248 (7%), 231 (100%), 215 (77%), 187 (80%), 43 (69%); accurate mass (EIMS) for $C_{38}H_{46}O_9$: calcd 646.31418, obsd 646.31400.

3.2.3. 2,2′-Bipyridyl-5,5′-dicarboxylic acid bis(illudinyl M) ester (4c).

Analogously to the synthesis of ester **4a**, compound **4c** (184 mg, 51%) was obtained from 2,2′-bipyridyl-5,5′-dicarboxylic acid (125 mg, 0.51 mmol), NEt_3 (163 μL, 1.11 mmol), 2,4,6-trichlorobenzoyl chloride (182 μL, 1.11 mmol), illudin M (273 mg, 1.11 mmol) and DMAP (251 mg, 2.04 mmol) as a yellow oil; R_f 0.24 (ethyl acetate/*n*-hexane 1:4); ν_{max}(ATR)/cm^{-1} 3480, 2967, 2928, 2867, 1717, 1699, 1592, 1362, 1264, 1104, 1023, 944, 762, 730; δ_H (300 MHz; $CDCl_3$) 0.92–0.40 (4H, m), 1.15 (3H, s), 1.24 (3H, s), 1.39 (3H, s), 1.52 (3H, s), 3.60 (1H, s), 5.91 (1H, s), 6.56 (1H, s), 8.39 (1H, d, J = 8.3 Hz), 8.56 (1H, d, J = 8.3 Hz), 9.24 (1H, s); δ_C (75 MHz; $CDCl_3$) 6.0, 8.9, 14.6, 20.8, 24.8, 26.7, 31.5, 49.2, 76.0, 80.0, 121.4, 126.0, 133.6, 134.7, 136.3, 138.2, 146.0, 150.5, 158.4, 164.6, 199.8; *m/z* (EI, 70 eV) 704 (M^+, 4%), 268 (8%), 244 (51%), 227 (57%), 187 (100%), 159 (39%), 128 (35%), 115 (36%), 77 (21); accurate mass (EIMS) for $C_{42}H_{44}N_2O_8$: calcd 704.30977, obsd 704.30920.

3.2.4. (Illudinyl M) 4-[4′-di(chloroethyl)aminophenyl]-butanoate (4d)

Analogously to the synthesis of ester **4a**, compound **4d** (132 mg, 66%) was obtained from chlorambucil (114 mg, 0.38 mmol), NEt_3 (60 μL, 0.44 mmol), 2,4,6-trichlorobenzoyl chloride (67 μL, 0.44 mmol), illudin M (93 mg, 0.38 mmol) and DMAP (93 mg, 0.75 mmol) as a yellow oil; R_f 0.23 (ethyl acetate/*n*-hexane/NEt_3 1:5:0.06); $[\alpha]_D^2$5 −25.2 (*c* 0.5, $CHCl_3$); ν_{max}(ATR)/cm^{-1} 3495, 2962, 2928, 2865, 1726, 1696, 1612, 1518, 1446, 1360, 1252, 1179, 1143, 1105, 945, 909, 821, 802, 730; δ_H (300 MHz; $CDCl_3$) 0.3–0.5 (1H, m), 0.7–1.0 (2H, m), 1.0–1.1 (4H, m), 1.17 (3H, s), 1.35 (3H, s), 1.50 (3H, s), 1.8–2.0 (2H, m), 2.34 (2H, t, J = 7.4 Hz), 2.55 (2H, t, J = 7.6 Hz), 3.5–3.7 (8H, m), 5.66 (1H, s), 6.50 (1H, s), 6.62 (2H, d, J = 6.7 Hz), 7.04 (2H, d, J = 6.7 Hz); δ_C (75 MHz; $CDCl_3$) 6.0, 8.8, 14.5, 20.7, 24.7, 26.7, 26.9, 31.4, 33.5, 34.0, 40.4, 48.8, 53.6, 76.0, 78.7, 112.3, 129.6, 130.6, 133.5, 135.2, 135.3, 144.2, 146.2, 173.2, 200.0; *m/z* (EI, 70 eV) 533 (M^+, 35%), 464 (13%), 302 (100%), 254 (68%), 231 (26%), 215 (22%), 118 (39%); accurate mass (EIMS) for $C_{29}H_{37}{}^{35}Cl_2NO_4$: calcd 533.20996, obsd 533.20990.

3.3. Cytotoxicity assays: cell lines and culture conditions

Panc-1 pancreatic carcinoma cells and HT-29 colon carcinoma cells were cultured on six-well tissue culture plates (Becton Dickinson, Mannheim, Germany) in RPMI-1640 medium (Biochrom, Berlin, Germany), containing 10% foetal calf serum (FCS, Biochrom), penicillin (10^7 U/L) and streptomycin (10 mg/L) at 37 °C and 5% CO_2. Human foreskin fibroblasts (HFs) served as non-malignant controls and were cultured in Dulbecco's modified Eagle's medium (DMEM, Biochrom) with the same supplements. All cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Determination of cell viability. Cells were harvested after the incubation period and stained with Trypan blue (Biochrom, Germany). The number of unstained (intact) cells was counted in a Neubauer chamber and expressed as relative cell numbers compared to untreated controls (= 100%).

3.4. Flow cytometric analysis of apoptosis

For quantification of apoptosis, culture supernatants were collected and the cells were washed two times with PBS, trypsinised

and lysed in a hypotonic solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/mL propidium iodide (Sigma). Analysis of labelled nuclei was performed on a Calibur fluorescence-activated cell sorter (FACS) using CELLQuest software (both from Becton Dickinson). The percentage of apoptotic cells was determined by measuring the fraction of nuclei with a sub-diploid DNA content.

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