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Article

Biotinylated xanthohumol: synthesis and *in vitro* biological evaluation for anticancer therapy

Monika Stompor[1*], Rafał Podgórski[1], Marta Świtalska[2], Joanna Wietrzyk[2]

[1]Faculty of Medicine, Centre for Innovative Research in Medical and Natural Sciences, University of Rzeszów, Warzywna 1a, 35-310 Rzeszów, Poland

[2]Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Department of Experimental Oncology, Weigla 12, 53–114 Wrocław, Poland

[*]Corresponding author. Faculty of Medicine, Centre for Innovative Research in Medical and Natural Sciences, University of Rzeszów, Warzywna 1a, 35-310 Rzeszów, Poland Tel.: +48 17 851 68 55. E-mail address: monika.stompor@gmail.com

Abstract: Two biotinylated derivatives of the main hop chalcone xanthohumol (**1**) were prepared by a one-step synthesis via esterification using biotin and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC HCl) and 4-dimethylaminopyridine (DMAP) as coupling reagents. The products were characterized spectroscopically and their antiproliferative activity toward MCF-7, MCF-10A, HepG2, MDA-MB-231, 4T1 and Balb/3T3 cell lines was investigated using the SRB assay. For all three tested compounds the best activity was noted in the case of human (MCF-7) and mice (4T1) breast cancer cell lines (IC_{50} values < 9 μM). Both biotinylated derivatives showed higher anticancer activity than xanthohumol (**1**) towards all types of tested breast cancer cells. Double biotinylated xanthohumol (**3**) proved to be the most active in inhibiting cell growth, with IC_{50} values equal to 5.35 ± 1.5 μM for 4T1 and 8.03 ± 0.53 μM for MCF-7 cell lines. Compound **3** was also more active than **1** and **2** against liver cancer cells HepG2 (IC_{50} = 17.37 ± 5.1 μM), while the IC_{50} values for **1** and **2** were equal to 21.5 ± 2.7 and 22.1 ± 3.9 μM, respectively. 4-*O*-biotinylxanthohumol (**2**) was the second most active growth inhibitor, particularly with respect to MCF-7 (IC_{50} = 6.19 ± 1.7 μM) and 4T1 (IC_{50} = 6.64 ± 0.4 μM) cell lines. The antioxidant activity was evaluated using the 1.1-diphenyl-2-picrylhydrazyl radical (DPPH) method. All tested compounds (**1-3**) have antioxidant activity between 2.73 and 3.38 mM. It was reported for the first time that new prenylated chalcones containing the biotin moiety effectively inhibited proliferation of cancer cells *in vitro*.

Keywords: xanthohumol; biotinylated chalcones; anticancer activity; antioxidants

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1. Introduction

Compounds of natural origin, including plant polyphenols and vitamins are a part of our daily diet. They are found in leaves, flowers, fruits, seeds, roots and bark of plants. Flavonoids have received a great deal of attention recently, due to their valuable medicinal properties. The vitamins which are present in plants have an essential role in many processes involved in rapid cell division in a large number of highly aggressive tumors. As a result, such tumors, in many instances upregulate the processes involved in the uptake of several of these vitamins, namely folate, vitamin B_{12}, and biotin (vitamin H). These vitamins, particularly biotin, have an immense potential to be used as imaging agents and targeting moieties for the delivery of cytotoxic agents to a wide variety of tumors. There is also an enormous potential to use biotin in combination with folate or vitamin B_{12} as dual targeting for tumor cells. Biotin is a form of vitamin B, present in many foods and available as a suplement, which especially during pregnancy is important for normal fetal development.

Xanthohumol (**1**), being one of the chalcone constituents of *Humulus lupulus L.*, *Sophora flavescens L.*, *Medicago sativa L.* and beer has been reported to have a broad spectrum of biological activity, including antiinflammatory, antioxidant, antibacterial and antidiabetic one [1-5]. Due to its strong antioxidant properties xanthohumol is also a promising chemopreventive agent [6]. Numerous *in vitro* studies have shown that this flavonoid inhibited cell proliferation and induced programmed cell death in many cancer cell lines, including breast, ovarian, lung, hepatic, oral, colon, cervical, prostate and leukemia ones. Recent studies revealed that xanthohumol has also the ability to sensitize MCF-7/ADR cells to doxorubicin, which increases cytotoxic effect of this chemotherapy drug [7]. The most often described mechanism of anticancer activity of xanthohumol involves its ability to suppress cell proliferation and induce programmed cell death in cancerous cells [8-9]. Xanthohumol and its structural analogues are also inhibitors of many enzymes, such as cyclooxygenase and lipoxygenase, which can lead to formation of reactive oxygen species. The multiple functionalities make these polyphenols distinguished chelators, through their hydroxyl groups, to the metallic part of the enzymes and thus, act as competitive inhibitors of many enzymes. XN-enriched diet could ameliorate diabetic-associated metabolic disturbances by regulating glucose and lipid pathways [2]. Both XN and its derivative 8PN treatments prevented body weight gain; decreased triglyceride, cholesterol and improved insulin sensitivity [10-11]. Recent study demonstrated that daily intake of xanthohumol might significantly improve HDL function, leading to protection against atherosclerosis [12].

However, some drawbacks, such as low solubility in water, significantly limit xanthohumol (**1**) in potential medical applications. Therefore, many researchers have dedicated to modifying flavonoid structures, so as to obtain novel derivatives with high efficiacy, low toxicity and minimal side effects. In the literature there are only a few studies that report synthesis of XN derivatives and their biological activity [4]. There is need to perform more detailed research that would lead to the development of new, innovative molecularly targeted therapeutic approaches for cancer treatments. In the literature there

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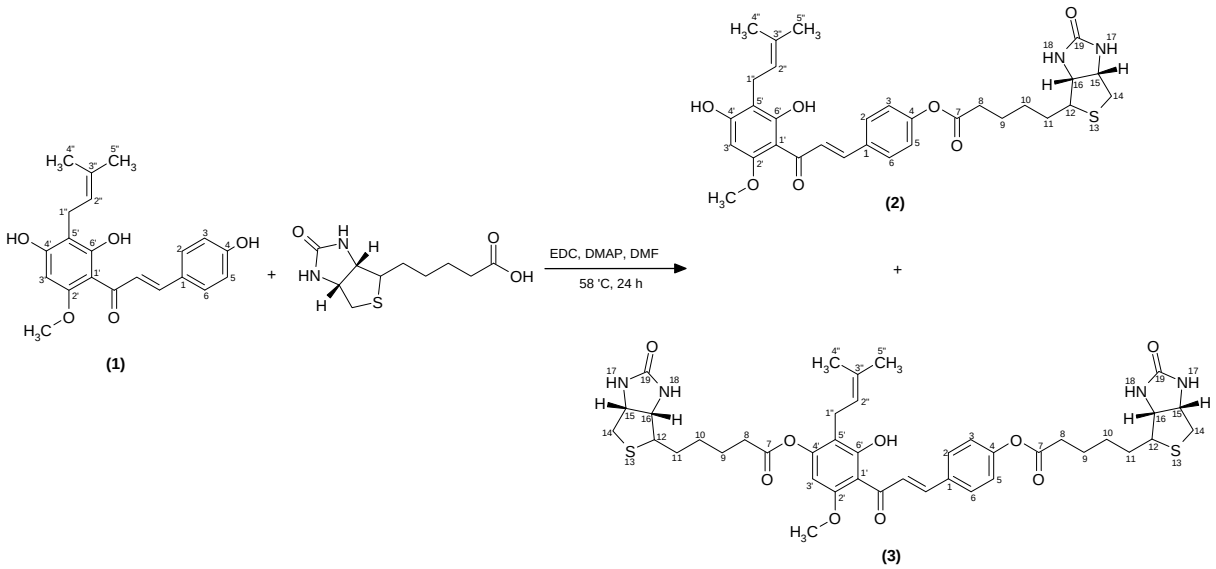
are no examples of synthesis of biologically active derivatives of prenylflavonoids conjugated with biotin. To date, there are also no reports on chemical synthesis of biotinylated xanthohumol (**1**). Also, there is no information about the research comparing antiproliferative activity of **1** and its biotinylated derivatives against cancer cell lines of various origin. Keeping in mind the valuable properties of hop flavonoids, the aim of this study was to obtain and determine the antiproliferative activity of the new biotinylated derivatives of **1**.

2. Results and Discussion

Chemical covalent modification of flavonoids is a way to improve the hydrophobic nature of these compounds. We decided to synthesize biotin derivatives of XN (**1**) so as to investigate their biological activity and to compare it with the activity of the parent compound. Synthesis of biotinylated derivatives of **1** began from isolating XN from spent hops by extraction with acetone. The crude XN was purified by column chromatography, followed by recrystallization from dichloromethane. Treatment of XN (**1**) with biotin in DMF at room temperature gave new esters **2** and **3**, which were purified by column chromatography to afford the pure products in 13 and 29 % yield, respectively. The synthesis of the biotinylated molecules are shown in **Scheme 1**. The structure of newly synthesized compounds were determined on the basis of ^{1}H NMR, ^{13}C NMR, and elemental analyses. To verify that biotin was indeed coupled to the hydroxyl at C-4 position in **1** and not to any other hydroxyl that might have been produced by trans-esterification, we characterized the product by the COSY, HMQC, and HMBC NMR spectra. In the ^{1}H NMR spectrum of **2**, the presence of two hydroxyl groups was indicated by the two one-proton singlets at 14.42 and 8.31 ppm, ascribed to the OH groups in ring A at C-6’ and C-4’ positions, respectively. The signals of the two =NH groups at 6.37 ppm and 6.45 ppm were also observed.



Scheme 1. Biotinylation of xanthohumol (**1**).

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Clinical use of anticancer drugs is limited by their dose-dependent side effects. Food intervention with the use of plants containing bioactive compounds with high antioxidant activity may be a safe and effective way to prevent life-style diseases, including cancers. Due to synergistic action, it could be possible to decrease drug dose, while providing the same therapeutic effect. Additionally, there is the possibility to reduce harmful side effects of chemotherapeutics on normal cells without loss of effectiveness of treatment, because antioxidants can stabilize DNA and contribute to strengthening the antioxidant barrier, which is highly beneficial to chemotherapy.

It is known that the biological activity of flavonoids is determined by their chemical structures and their physicochemical properties such as size of molecules, lipophilicity and water solubility. In the literature there are only few examples of the synthesis of xanthohumol's derivatives [4, 13]. Our previous reports suggest that prenylation increases bioavailability and bioaccumulation of the hop flavonoid 8-prenylnaringenin and therefore may be a promising tool to apply the biological functions of these compounds for medical use [14]. Flavonoids derived from hop can also increase the anti-cancer activity of other estrogenic metabolites. However, no major differences between incubation with xanthohumol (**1**) alone or co-incubation with zearalenone (non-steroidal mycoestrogene) in MCF-7 cells were observed. Only for 8-prenylnaringenin, which is the most active natural phytoestrogen derived from hop, when added to zearalenone in amounts of 14.7 and 29.4 μM, there was a trend towards an increase in the zearalenone-induced breast cancer cell proliferation observed (up to 72%) [15]. The last studies suggested that xanthohumol's antitumor activity may arise from its ability to inhibit multiple target proteins identified in cancer cells and it could be potentially used in anticancer drug target therapy. Based on their diverse biological activity, hop flavonoids may be also potential adjuvant therapeutic agents for co-treatment in cancer therapy [16-17]. Also, other prenylated chalcones (eg. *O*-prenyl derivative of 2′-hydroxy-3,4,4′,5,6′-pentamethoxychalcone) in combination with common anticancer drug, such as paclitaxel, enhanced the effect of cell growth inhibition, as determined by cell viability and proliferation assays [18].

Because biotin is found in some types of cancer cells in higher concentrations than in normal ones, the addition of the biotin group to an anticancer agent may result in better delivery and selectivity toward cancer cells [19-23]. Many different cancer types have previously been found to show increased uptake of the vitamins folate, vitamin B12, and biotin; however, it is not known whether these tumor lines show increased uptake of one or more of the vitamins [24].

Table 1. Antiproliferative activity of biotinylated xanthohumols.

Compounds	**Cell line** IC_{50} [μM]					
	MCF-7	**MDA-MB-231**	**HepG2**	**MCF-10A**	**4T1**	**Balb/3T3**
1	8.8±1.16	18.9±4.6	21.5±2.7	21.1±4.3	8.7±1.3	12.6±8.8
2	6.19±1.7	18.6±1.2	22.1±3.9	19.5±3.1	6.64±0.4	12.3±9.0
3	8.03±0.53	16.9±1.6	17.37±5.1	16.8±2.5	5.35±1.5	16.6±4.0

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biotin	14%*	0%*	0%*	6.2%*	0%*	0%*
cisplatin	26.7±1.7	16.3±2.3	5.7±1.0	10.7±4.0	1.0±0.17	14.0±3.7

IC_{50} – compound concentration leading to 50% inhibition of cell proliferation. Data are presented as mean ± SD of 3-5 independent experiments.

*inhibition of cell proliferation [%] of compound at concentration of 250 μM

Xanhtohumol (**1**), the main prenylated hop chalcone, may inhibit cell growth and induce apoptosis in many types of human cancers, including breast cancer [6].

To better understand the influence of flavonoid compounds on the aggressive kind of triple-negative breast cancer (TNBC), we used 4T1 and MDA-MB-231 cells that do not express the estrogen receptor (ER), progesterone receptor (PgR) or the gene for human epidermal growth factor receptor 2 (HER-2). The non-TNBC MCF-7 cell line and non-tumorigenic human mammary MCF-10A (TNBC) epithelial cell line were used as a control. The cytotoxicity of biotin-flavonoid compounds in comparison with their non biotinylated forms was also evaluated against human liver cancer (HepG2). The mice cell line (Balb/3T3) was used as a non-cancerous control.

In this study we found that both biotinylated xanthohumols (**2** and **3**) demonstrated higher antiproliferaive activity than **1** toward all tested breast cancer cell lines. Compound **3** was slighty more active against HepG2 liver cancer cells (IC_{50} =17.4 μM) than **1** and **2**, for which the IC_{50} doses were comparable (~ 22 μM). Xanthohumol esterified by two molecules of biotin was also active toward normal mice fibroblasts, with the IC_{50} = 16.6 μM. The human breast cancer MCF-7 cell line expresses both types of estrogen receptors with a predominance of ERα. The possible mechanism of action by which the compounds inhibit proliferation of tumor cells is related to their interaction with the estrogen receptor present in MCF-7 breast cancer cells. In this research we studied the effect of xanthohumol and its biotinylated analogs on the ERα-negative MDA-MB 231 cell line, which is more aggressive cancer type than the ERα-positive MCF 7 cell line. Our results showed that all compounds were more active to MCF-7 cells. According the last studies, **1** inhibit cellular proliferation in MDA-MB-231 cells through an intrinsic mitochondrial-dependent pathway [25].

The effect of **1** on MCF-7 breast cancer cells was described earlier by Yoshimaru *et al.* [26], with IC_{50} value = 8.7 μM, which is in accordance with our observations. The authors reported that the oncoprotein brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3) and tumor suppressor prohibitin 2 (PHB2) complex plays a pivotal role in E2 signaling modulation in this ERα-positive breast cancer. XN treatment effectively prevented the BIG3-PHB2 interaction, thereby releasing PHB2 to directly bind to both nuclear- and cytoplasmic ERα. Guerreiro *et al.* [27] suggested that XN may modulate alkaline phosphatase isoenzymes in MCF-7 breast cancer cells, and that alkaline phosphatase loss was associated with increased cell proliferation. According to our results, the differences in the activity of tested compounds may be also due to the presence of C=O, –NH– and –S– groups in biotin molecule, which have a certain degree of hydrophilicity. And the N, H, and S atoms

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belonging to the above-mentioned groups can form hydrogen bonds with the components of the cell membrane, leading to the enhancement of the effect of the biotinylated compounds.

Low water-solubility of flavonoid aglycones coupled with their short residence time in the intestine and their poor absorption do not allow to experience therapeutic effects from the consumption of flavonoids. Hence, development of semisynthetic methods of obtaining their more soluble derivatives is needed. Some publications indicate also a higher bioavailability of flavonoid glucosides compared to the free aglycones, which may be the way to improve the anticancer potential. However, obtained by microbial synthesis glycosides of **1** had lower cytotoxic activity than xanthohumol against the cells of breast (MCF-7) and prostate (PC-3) cancers. Whereas, replacement of the chalcone with a flavanone skeleton resulted in a significant decrease in activity against all tested cancer cell lines: breast (MCF-7), prostate (PC-3) and colon cancer (HT-29) [28].

The last molecular studies using BALB/c-4T1 breast cancer mouse model showed that **1** significantly slowed down tumor growth and inhibited expression of antitumor proliferation protein Ki-67, as well as breast cancer-specific marker – cancer antigen 15-3 (CA15-3). The analysis revealed that XN enhanced the secretion of perforin, granzyme B, increased the ratio of CD8+/CD25+ and deregulated selected cytokines (Th1 and Th2) [29]. In hepatocellular carcinomas HepG2 and Huh7, the inhibitory effect of **1** on cell proliferation was attributed to the induction of apoptosis by decreasing cell migration, interleukin-8 expression and NF-κB/p53-apoptosis signaling pathway activation [30-31]. In our study in the triple-negative breast cancer cell lines the IC_{50} values varied from 18.9 to 16.9 μM and from 8.7 to 5.35 μM, for MDA-MB-231 and 4T1, respectively. These suggest that the antiproliferative activity of tested flavonoids against various cancer cell lines may be determined by their chemical structures and by their physicochemical properties, such as size of molecules, lipophilicity and water solubility. However, molecular weights of the biotinylated compounds (**2** and **3**) are much higher than of the unsubstituted substrate (**1**). Thus, with the increased molecular weight it became more difficult for the biotinylated chalcones to get through the cell membrane, and therefore the amount of biotinylated derivatives entering the cell may be too low. Nevertheless, the best antiproliferative activity was noted for compound **3**, containing two biotins and having the highest molecular weight (about 806.98 g/mol). The second most active compound was **2** with one biotin and M=580.69 g/mol. So, the increase in molecular weight of modified xanthohumols by about 226-452 g/mol did not diminish the anti-proliferative activity, and what is more, this activity was higher than for the parental xantohumol (**1**).

It may be hypothesized that biotin-containing compounds enter biotin-possitive cancer cells more efficiently than in the case of normal fibroblasts (Balb/3T3) and non-cancer breast cells (MCF-10A), allowing the flavonoid moiety to reduce oxygen, which leads to increased ROS generation and, ultimately, cell death. It can also be hypothesized that the vitamin moiety with greater frequency binds to biotin receptors on cancer cells compared to noncancer ones, resulting in preferable conjugate uptake and, as a result, leading to higher inhibition of proliferation. In this aspect, non-TNBT MCF-7 cells which additionally are

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characterized by a predominance of biotinic than folate receptors, were more sensitive to tested compounds than the more aggressive TNBC metastatic human breast tumors, that over-express biotin receptors but are deprived of ER and HER-2 receptors (eg. MDA-MB-231).

Antioxidant potential shown in **Table 2** was defined as a concentration of the antioxidant in mmol/L (mM) that causes 50% loss of the DPPH activity (EC_{50}). DPPH assay revealed that double biotinylation of **1** do not changed its antioxidant potential (EC_{50} for **1** and **3** were comparable and were between 2.73 and 2.76 mM). Lower activity was observed for compound **2** (EC_{50} = 3.38 mM).

Table 2. Antioxidant activity of compounds **1**-**3**.

Compounds	EC_{50} [mM] ± SD
1	2.7284 ± 0.035818
2	3.3788 ± 0.026491
3	2.7624 ± 0.016695
ascorbic acid	0.056402 ± 0.00008

3. Materials and methods

3.1. General procedures

XN (**1**) (3′-[3,3-dimethyl allyl]-2′,4′,4-trihydroxy-6′-methoxychalcone) was isolated from spent hops. As a raw material for isolation of **1**, by-products obtained after extraction of hops with supercritical CO_2 in industrial installations located in the New Chemical Syntheses Institute, in Puławy, Poland were used. Gradient grade purity methanol was purchased from Merck (Darmstadt, Germany). All other reagents, if not mentioned otherwise, were purchased from Sigma Aldrich (St. Louis, MO, USA) and were of analytical grade. Analytical thin-layer chromatography was carried out on silica gel 60 F_{254} plates (Merck) with chloroform:methanol (96:4 v/v) as the developing solvent. Visualization of the compounds was accomplished with a solution of 10 g $Ce(SO_4)_2$ and 20 g phosphomolybdic acid in 1 L of 10% H_2SO_4, followed by heating. Preparative column chromatography was performed using silica gel (Kieselgel 60, 230–400 mesh; Merck). NMR spectra (^1H, ^{13}C, HSQC, DEPT 135) were recorded with a Bruker 500 MHz Ultra Shield TM Plus instrument with DMSO-d_6 as solvent and TMS as an internal standard. High-resolution mass spectra (HR-ESI-MS) were measured with a Bruker apex ultra FT-ICR instrument (Bruker, Billerica, MA, USA).

The purity of synthesized products was monitored by HPLC using an Ultimate 3000 DIONEX chromatograph equipped with a LGP-3400 RS dual-pump fluid control module, a TCC-3100 thermostatted column compartment, a WPS-3000 autosampler, and Diode Array

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Detector DAD-3000 RS (Sunnyvale, CA, USA). The system was controlled and data acquisition was carried out using the Chromeleon 6.80 software (Dionex Corporation). HPLC analysis was carried out using a reverse phase C18 column (ACE 5 C18, 5 µm, 100 Å, 4.6 × 250 mm) connected to a guard column (ACE 5 C18) (Advanced Chromatography Technologies LTD, Scotland). The mobile phase consisted of two components: A–1% HCOOH in H_2O and B– 1% HCOOH in MeCN. The flow rate was set at 1 mL/min and the gradient elution was applied: 0–1 min, 50% B, 1–16 min 50–100% B, 16-21 min 100% B, 21–23 min 100–50% B, 23–28 min 50% B. The total time of analysis was 28 min. The samples temperature was fixed at 12 °C and the column temperature was fixed at 28 °C, the injection volume was 10 µL. The content of **1-3** was detected at 368 nm.

3.2. Chemistry

EDC (230 mg, 1.2 mmol) and DMAP (74 mg, 0.6 mmol) were added to a mixture of xanthohumol (**1**) (100 mg, 0.28 mmol) and biotin (276 mg, 1.13 mmol) in DMF (10 ml). The reaction mixture was stirred at 58 °C for 24 h. The crude products were directly purified by flash chromatography and characterised using spectroscopic methods.

3.2.1. 4-*O*-biotinylxanthohumol (2)

(22 mg, 13%) as a yellow solid; **^{1}H NMR** (500 MHz, DMSO_d_6) δ (ppm): 14.46 (1H, s, 6'-OH), 10.60 (1H, s, 4'-OH), 7.88 (1H, d, *J* = 15.6 Hz, H-α), 7.77 (2H, d, *J* = 8.4 Hz, H-2, H-6), 7.69 (1H, d, *J* = 15.6 Hz, H-β), 7.21 (2H, d, *J* = 8.4 Hz, H-3, H-5), 6.46 (1H, s NH-18), 6.39 (1H, s, NH-17), 6.07 (1H, s, H-3'), 5.14 (1H, t, *J* = 6.0 Hz, H-2"), 4.33-4.31 (1H, m, H-15), 4.17-4.15 (1H, m, H-16), 3.86 (3H, s, -OCH_3), 3.16-3.13 (3H, m, H-1" and H-12), 2.84 (1H, dd, *J* = 12.4 Hz, *J* = 5.0 Hz, H-14), 2.63-2.58 (4H, m, H-8 and H-11), 1.77-1.65 (3H, m, H-9 and H-11), 1.70 (3H, s, H-5"), 1.61 (3H, s, H-4"), 1.57-1.50 (1H, m, H-11'), 1.48-1.37 (2H, m, H-10). **HRMS (ESI)** *m/z*: calcd. for $[C_{31}H_{36}N_2O_7S + H]^+$ = 581.2316; found $[M+H]^+$ = 581.2321. **UV** (MeOH) λ_{max}: 349.4 nm.

3.2.2. 4,4'-di-*O*-biotinylxanthohumol (3)

(67 mg, 29%) as a yellow solid; **^{1}H NMR** (500 MHz, DMSO_d_6) δ (ppm): 13.11 (1H, s, 6'-OH), 7.82 (2H, d, *J* = 8.7 Hz, H-2, H-6), 7.76 (1H, d, *J* = 15.7 Hz, H-α), 7.72 (1H, d, *J* = 15.7 Hz, H-β), 7.22 (2H, d, *J* = 8.7 Hz, H-3, H-5), 6.46 (2H, s, 2 x NH-18), 6.45 (1H, s, H-3'), 6.37 (2H, s, 2 x NH-17), 5.03 (1H, t, *J* = 6.0 Hz, H-2"), 4.33-4.31 (2H, m, 2 x H-15), 4.17-4.15 (2H, m, 2 x H-16), 3.87 (3H, s, -OCH_3), 3.17-3.12 (4H, m, 2 x H-12 and H-1"), 2.84 (2H, dd, *J*= 12.5 Hz, *J*= 4.8 Hz, 2 x H-14), 2.62 (4H, t, *J* = 7.5 Hz, 2 x H-8), 2.59 (2H, 2 x d, *J* = 12.5 Hz, H-14'), 1.77 – 1.64 (6H, m, 2 x H-9 and 2 x H-11), 1.69 (3H, s, H-5"), 1.62 (3H, s, H-4"), 1.57-1.49 (2H, m, 2 x H-11'), 1.48-1.37 (4H, m, 2 x H-10). **^{13}C NMR** (125 MHz, DMSO-d_6) δ (ppm): 193.6, 171.6, 171.0, 162.7, 161.7, 159.0, 154.3, 152.2, 142.1, 132.3, 131.1, 129.9, 127.4, 122.5, 121.7, 114.4, 110.1, 97.9, 61.0, 59.2, 56.5, 55.3, 40.4, 33.3, 33.2, 28.02, 27.98, 27.89, 25.4, 24.31, 24.29, 22.0, 17.7. **HRMS (ESI)** *m/z*: calcd. for $[C_{41}H_{50}N_4O_9S_2 + H]^+$= 807.3091; found $[M+H]^+$ = 807.3084. **UV** (MeOH) λ_{max}: 335.1 nm.

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3.3. Cell culture and sulforhodamine (SRB) assay

The biological studies were performed *in vitro* using biotin receptor-positive human cancer cell lines: MCF-7 (breast cancer), MDA-MB-231 (breast cancer), HepG2 (liver cancer), in comparison to murine mammary biotin receptor-positive gland cancer (4T1), normal mice fibroblasts (BALB/3T3) and human normal breast cells (MCF-10A). The cell lines were obtained from American Type Culture Collection (Rockville, Maryland, U.S.A.) or from European Collection of Authenticated Cell Cultures (MCF-7 cell line; ECACC, Salisbury, UK). The cell lines are maintained in the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

MDA-MB-231 and 4T1 cells were cultured in RPMI1640 medium (IIET, Wroclaw, Poland), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1.0 mM sodium pyruvate (4T1), 4.5 g/L glucose (4T1) (all from Sigma-Aldrich Germany).MCF-7 cells were cultured in Eagle medium (IIET, Wroclaw, Poland), supplemented with 10% fetal bovine serum, 1% mem non-essential amino acid solution, 0.8 mg/L of insulin and 2 mM L-glutamine (all from Sigma-Aldrich, Germany), MCF-10A cells were cultured in the F-12 nutrient mixture (Gibco, Scotland, UK), supplemented with 5% horse serum (Gibco, Scotland, UK), 10 µg/mL of chlorea toxin (*Vibrio cholerae)*, 10 µg/mL of hydrocortisone and 20 ng/mL of epidermal growth factor human (all from Sigma-Aldrich, Germany). HepG2 and BALB/3T3cells were cultured in Dulbecco medium (Gibco, Scotland), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, (all from Sigma-Aldrich Germany). All culture media were supplemented with penicillin (100 units/mL; Polfa Tarchomin S.A., Poland), and streptomycin (100 µg/mL; Sigma-Aldrich, Germany). All cell lines were grown at 37°C in a 5% CO_2 humidified atmosphere.

Prior to usage, the compounds were dissolved in DMSO to achieve the concentration of 50 mM, and subsequently diluted in culture medium to reach the required concentrations (ranging from 0.4 µM to 250 µM).

24 Hours before addition of tested compounds, the cells were placed in 96-well plates (Sarstedt, Germany) at density of 1×10^4 cells per well (4T1 cells were placed at the density of 0.7×10^3 cells per well) in 100 µL of culture medium. An assay was performed after 72 hours of exposure to varying concentrations of the tested agents. Cytotoxic SRB assay was applied against MCF-7, MCF-10A, 4T1, BALB/3T3, HepG2 and MDA-MB-231 cell lines. The test was carried out according to the method described before [32].

The results were calculated as an IC_{50} (inhibitory concentration 50) – the concentration of tested agent that inhibits proliferation of the cell population by 50%. IC_{50} values were calculated for each experiment separately and mean values ± SD are presented in **Table 1**. Each compound in each concentration was tested in triplicate in a single experiment, which was repeated 3–5 times.

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3.4. Antioxidant activity

The antioxidant activity was determined by the method of DPPH according to the modified procedure described earlier. The absorbance was measured using a microplate reader (TECAN Infinite 200). All experiments were performed in triplicate. The radical solution was prepared by dissolving 3.94 mg DPPH in 50 mL of MeOH. For the photometric assay 100 µL DPPH solution and 100 µL antioxidant solution. Depending on the solubility, concentrations of the tested solutions were prepared in range from 0.1 mg/1 mL to 4 mg/1 mL by dissolving in MeOH. The study used the 96-well microtiter plates. After 30 min, the absorbance was measured at 517 nm. The antioxidant ability of tested compound was assessed according to the following formula:

$$\% \text{ DPPH inhibition} = 100 \times (A_0 - A_c)/A_0$$

where A_0 is the absorbance of DPPH solution, A_c is the average absorbance value of the tested solution containing antioxidant at a known concentration.

4. Conclusion

Here, we present a new approach to identify biotinylated analogues of prenylated chalcone xanthohumol (**1**), the main flavonoid of hop (*Humulus lupulus*). The antitumor activity of xanthohumol may arise from its ability to inhibit multiple target proteins, therefore biotinylated chalcones **2** and **3** can be also proposed as tools to identify proteins that **1** may directly bind to in living cancer cells – as a molecular probe in a drug delivery system. The identified derivatives can be prepared in a short, one-step synthesis and can be a useful tool to probe tumor biology in live cells by affinity capture, flow cytometry, confocal microscopy and other imagining methods used in medicine, including the spectroscopic and luminescence methods. To our knowledge, **2** and **3** constitute the only reported biotinylated chalcone probe to have such combined characteristics.

In summary, we have synthesized two novel low-molecular-weight compounds using a simple ester coupling of xanthohumol (**1**) to biotin. Their antiproliferative activity towards selected cancer cell lines was investigated in comparison with **1**. Our results clearly showed that incorporation of biotin moiety into the main hop prenyl chalcone significantly improves its selective cytotoxic activity. At this stage our results concerning the *in vitro* activity of two newly synthesized compounds **2** and **3** are promising and qualify them for further *in vivo* study as anticancer agents. It is also important to continue the investigation on the mechanism of their action. This study may be also helpful in utilization of by-products obtained after extraction of hop cones with supercritical carbon dioxide in the brewing industry (spent hops). This waste material is rich in **1**, which we used as a substrate for the synthesis of **2** and its derivatives.

These studies are in progress. The aim of further work is also to elucidate the mechanism of the cytotoxicity of biotin-modified flavonoids.

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Author contributions:

Monika Stompor conceived the research, designed the experiment, performed synthetic part of experimental work, analyzed the data and wrote the manuscript. Rafał Podgórski measured antioxidant activity. Marta Świtalska performered biological assays. Joanna Wietrzyk was supervising biological assays.

Conflict of interest

The authors declare that they have no competing interests.

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