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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Antiproliferative activity and synthesis of 8-prenylnaringenin derivatives by demethylation of 7-*O*- and 4′-*O*-substituted isoxanthohumols

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Abstract Several analogues of 7-*O*- and 4′-*O*-substituted isoxanthohumol and 8-prenylnaringenin, the strongest known phytoestrogen and potential anticancerogenic agent, were synthesized. Acyl, alkyl, and allyl derivatives of isoxanthohumol underwent the demethylation process using $MgI_2 \times 2Et_2O$ in anhydrous THF with the yields of 61–89%. Some of the compounds approached the international criteria of antiproliferative activity (4 μg/ml) for synthetic agents against the human cancer cell lines.

Keywords Hop flavonoids · Phytoestrogens · Antitumor agents · Antiproliferative activity · MCF-7 · HT-29 · CCRF/CEM

Introduction

Hops (*Humulus lupulus* L.) are used in the brewing industry to add flavor and bitterness to beer. They consist of many prenylated chalcones and flavanones (Stevens and Page, 2004). Among them, xanthohumol (**1**) has received much attention in recent years as an anti-cancer (Colgate *et al*., 2007; Drenzek *et al*., 2011; Okano *et al*., 2011), antioxidant (Delmulle *et al*., 2006; Jacob *et al*., 2011), and anti-HIV (Cos *et al*., 2008) agent. It is readily accessible from carbon dioxide-extracted-hops (spent hop) where its content ranges up to 1% of dry matter. Spent hop is an important by-product of the process of hop extraction in the beer brewing industry, which is usually used as a fertilizer or as an animal feed in the U.S. However, in order to increase the added value of spent hops, hop processing industries have been looking for an alternative utilization of spent hops (Faltermeier *et al*., 2006; Oosterveld *et al*., 2002). Other flavonoids, isoxanthohumol (**2**) and 8-prenylnaringenin (**3**) are also present in hops, but in ten to one hundred times lower concentrations than the content of **1** (Stevens *et al*., 2000). Compound (**3**) is the potential drug in menopausal hormone therapy and the strongest phytoestrogen known in the nature (Borrelli and Ernst, 2010; Böttner, 2008; Chadwick *et al*., 2006; Hyun *et al*., 2008; Overk *et al*. 2008). The compounds (**1**–**3**) have also anti-breast cancer activity (Brunelli *et al*., 2007; Monteiro *et al*., 2007; Wesolowska *et al*., 2010a, b). Prenylflavonoid (**3**) can be synthesized in high yield from xanthohumol (**1**). It requires the cyclization of **1** to isoxanthohumol (**2**) in basic conditions and demethylation of **2**–**3** with $MgI_2 \times 2Et_2O$ (Anioł *et al*., 2008).

Wilhelm and Wessjohann, (2006) studied demethylation of **2**–**3** with $AlBr_3$, BBr_3 or $MeAlCl_2$ in collidine; $ZnBr_2$, CuI, $ZnBr_2$/CuI $Yb_2(SO_4)_3$/KI or CuI, $Sm(OTf)_3$/KI, $CeCl_3$/LiI. Product (**3**) was not detected or obtained with low yield. Hydroxyl groups of **2** were also protected with chlorotriisopropylsilane, demethylated with $AlBr_3$ and deprotected with $(n\text{-}Bu)_4NF$ to obtain 8-prenylnaringenin (**3**) with 73% yield. The best result was obtained for $Sc(OTf)_3$/KI (92%).

Magnesium iodide etherate was previously applied in the regioselective demethylation of 5-acetyl-4,6-dimethoxy-2-isopropenyl-2,3-dihydrobenzofuran (Yamaguchi *et al*., 1987) and substituted 2,6-dimethoxybenzaldehydes (Yamaguchi *et al*., 1999).

Only a few studies can be found in the literature that reported 8-prenylnaringenin and isoxanthohumol derivative synthesis. Methylation of 8-prenylnaringenin (**3**) with

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Me_2SO_4 resulted in the formation of di-*O*-methyl derivatives of **1** and **2** (Jain *et al*., 1978). The synthesis of 7,4′-di-*O*-acetyl-8-prenylnaringenin was carried out using 7,4′-di-*O*-acetylnaringenin as a substrate via its 4-*O*-prenyl ether, which undertook the Claisen–Cope rearrangement (Gester *et al*., 2001). The preparation of chiral 7,4′-dimethyl- or diacetyl- isoxanthohumols and 8-prenylnaringenins was achieved by reducing a carbonyl group to a hydroxyl group with a mixture of formic acid and a base in the presence of chiral catalyst. Separation of the non-transferred enantiomer (2S) or (2R) of the reduced 8-prenylnaringenin diacetyl derivative and splitting the acyl residues in enantiomers by enzyme catalyst solvolysis gave (2S)-8-prenylnaringenin or (2R)-8-prenylnaringenin. The second enantiomers (2R) or (2S) of 8-prenylnaringenin diacetyl derivative was recovered by oxygenation of a hydroxyl group (Metz and Schwab, 2007). Starting from **3**, several carboxylic acid haptenes of this compound were also synthesized. Five linkers [–$(CH_2)_n$COOH, $n = 1, 3, 5, 6$, and 9] were coupled to the C7–OH or C4′–OH group of 8-prenylnaringenin to obtain five derivatives (Schaefer *et al*., 2005).

In this article, we report methods of synthesis of 7-*O*- and 4′-*O*-substituted alkyl, alkenyl and acyl isoxanthohumol derivatives and their demethylation using magnesium iodide etherate. This research is connected with utilization of the spent hop, obtained after extraction with supercritical carbon dioxide. This waste product of the hop industry is rich in xanthohumol, the starting compound in the synthesis of all the compounds described in this article.

Materials and methods

Chemistry

General

All the reactions were carried out under a dry nitrogen atmosphere. The organic solvents were dried and purified according to the standard procedures. The reagents were purchased from Fluka. Isoxanthohumol (**2**) was obtained from xanthohumol (**1**) by dissolving in 1% NaOH and acidification of the reaction mixture as it was described previously (Anioł *et al*., 2008). Analytical thin-layer chromatography was carried out on DC-Alufolien Kieselgel 60 F_{254} silica gel (0.2 mm; Merck) with chloroform: methanol (96:4) as the developing solvent. Visualization was effected 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 accomplished using silica gel (Kiesel 60, 230–400 mesh; Merck) columns. Proton NMR spectra were recorded on a Bruker AMX 300 instrument at 300 MHz with acetone-d_6 as the solvent and TMS as an internal standard. The infrared (IR) spectra in KBr were recorded on a Mattson IR 300 spectrometer.

Synthesis of isoxanthohumol derivatives

*7,4′-Di-O-methylisoxanthohumol (**4**) and 7-O-methylisoxanthohumol (**5**)* A mixture of isoxanthohumol (100 mg, 0.282 mmol), anhydrous K_2CO_3 (232 mg, 1.68 mmol), and methyl iodide (0.5 ml) in 5 ml of anhydrous acetone was stirred for 12 h at room temperature. Acetone was evaporated and the resultant reaction mixture was treated with 10 ml of a saturated NaCl solution and extracted with Et_2O (3 × 10 ml). The organic phase was dried over anhydrous Na_2SO_4, concentrated and was subjected to column chromatography ($CHCl_3$:MeOH, 99:1) to provide 74.9 mg (69.4%) of light yellow solid (mp = 37–39°C, $R_f = 0.60$, $CHCl_3$:MeOH, 98:2) of 7,4′-di-*O*-methylisoxanthohumol (**4**) and 9.1 mg (8.8%) of white solid (mp = 181–184°C, $R_f = 0.21$, $CHCl_3$: MeOH, 98:2) of 7-*O*-methylisoxanthohumol (**5**). ^{1}H NMR and IR spectroscopic data were in agreement with those reported in the literature (Metz and Schwab, 2007; Stevens *et al*., 2000).

*7-O-n-pentylisoxanthohumol (**6**) and 7,4′-di-O-n-pentyl-8-isoxanthohumol (**7**)* The reaction was carried out exactly in the same way as it is described for compounds (**4** and **5**) but 1 ml of *n*-pentyl iodide was used instead of methyl iodide. The product (33.5 mg, 27.6%) 7-*O*-*n*-pentylisoxanthohumol (**6**) was obtained as a pale yellow solid (mp = 140–142°C, $R_f = 0.61$, $CHCl_3$:MeOH, 97:3). The ^{1}H NMR (300 MHz, acetone-d_6) for compound (**6**): δ (ppm): 0.93 (*t*, 3H, $J = 7.1$ Hz, C-7–O$(CH_2)_4CH_3$); 1.33–1.54 (m, 4H, C-7–O$(CH_2)_2CH_2CH_2CH_3$); 1.61 (d, 6H, $J = 1.3$ Hz, CH_3-4″ and CH_3-5″); 1.78–1.87 (m, 2H, C7–O$CH_2CH_2(CH_2)_2CH_3$); 2.63 (dd, 1H, $J = 16.4$ Hz, $J = 3.0$ Hz, CH-3); 2.93 (dd, 1H, $J = 16.4$ Hz, $J = 12.5$ Hz, CH-3); 3.26 (d, 2H, $J = 7.1$ Hz, CH_2-1″); 3.84 (s, 3H, C-5–OCH_3); 4.13 (*t*, 2H, $J = 6.3$ Hz, C-7–O$CH_2(CH_2)_3CH_3$); 5.16 (t_{sept}, 1H, $J = 7.1$ Hz, $J = 1.3$ Hz, CH-2″); 5.36 (dd, 1H, $J = 12.5$ Hz, $J = 3.0$ Hz, CH-2); 6.34 (s, 1H, CH-6); 6.89(d, 2H, $J = 8.6$ Hz, CH-3′ and CH-5′); 7.38 (d, 2H, $J = 8.6$ Hz, CH-2′ i CH-6′); 8.53 (s, 1H, C-4′–OH). IR (KBr) cm^{-1}: 2957, 2931, 2856, 1665, 1599, 1570, 1520, 1458, 1262, 1103, 798. $C_{26}H_{32}O_5$ (424.54): calcd. C 73.56, H 7.60; found C 73.67, H 6.75. The compound 7,4′-di-*O*-*n*-pentyl-8-isoxanthohumol (**7**) was also isolated (18.4 mg, 13.6% yield) as a light yellow solid (mp = 70–75°C, $R_f = 0.87$, $CHCl_3$:MeOH, 97:3). ^{1}H NMR (300 MHz, acetone-d_6) δ (ppm): 0.93 (*t*, 6H, J = 7.1 Hz, C-7- and C-4″–O$(CH_2)_4CH_3$); 1.34-1.54 (m, 8H, C-7- and C-4′-O$(CH_2)_2CH_2CH_2CH_3$); 1.62 (d, 6H, $J = 1.3$ Hz, CH_3-4″ and CH_3-5″); 1.74–1.87 (m, 4H, C-7- and C4′–O$CH_2CH_2(CH_2)_2CH_3$); 2.65 (dd, 1H, $J = 16.3$ Hz,

$J = 3.0$ Hz, CH-3); 2.95 (dd, 1H, $J = 16.3$ Hz, $J = 12.5$ Hz, CH-3); 3.28 (d, 2H, $J = 7.1$ Hz, CH_2-1″); 3.84 (s, 3H, C-5–OCH_3); 4.02 (*t*, 2H, $J = 6.5$ Hz, C-4′–$OCH_2(CH_2)_3CH_3$); 4.13 (*t*, 2H, $J = 6.3$ Hz, C-7–$OCH_2(CH_2)_3CH_3$); 5.17 (t_{sept}, 1H, $J = 7.1$ Hz, $J = 1.3$ Hz, CH-2″); 5.43 (dd, 1H, $J = 12.5$ Hz, $J = 3.0$ Hz, CH-2); 6.34 (s, 1H, CH-6); 6.98 (d, 2H, $J = 8.7$ Hz, CH-3′ and CH-5′); 7.46 (d, 2H, $J = 8.7$ Hz, CH-2′ and CH-6′). IR (KBr) cm^{-1}: 3064, 2952, 2936, 2870, 1675, 1601, 1577, 1517, 1465, 1346, 1253, 1113, 827. $C_{31}H_{42}O_5$ (494.68): calcd. C 75.27, H 8.56; found C 75.51, H 8.44.

*7,4′-Di-O-allylisoxanthohumol (**8**)* The reaction was carried out similarly as it is described for compounds (**4** and **5**) but 1 ml of allyl bromide and 6 ml of anhydrous THF were used instead methyl iodide and acetone. The product was purified by column chromatography ($CHCl_3$:MeOH, 99.3:0.7) to give 100.2 mg of 7, 4′-di-*O*-allylisoxanthohumol (**8**) as a light yellow solid (mp = 79–83°C, $R_f = 0.85$, $CHCl_3$:MeOH, 95:5) with 81.2% yield. ^{1}H NMR (300 MHz, acetone-d_6) δ (ppm): 1.61 (d, 6H, $J = 1.4$ Hz, CH_3-4″ and CH_3-5″); 2.66 (dd, 1H, $J = 16.3$ Hz, J = 3.1 Hz, CH-3); 2.95 (dd, 1H, $J = 16.3$ Hz, $J = 12.5$ Hz, CH-3); 3.28 (d, 2H, $J = 7.2$ Hz, CH_2-1″); 3.84 (s, 3H, C-5–OCH_3); 4.61 and 4.73 (two ddd, 4H, $J = 5.2$ Hz, $J = 1.7$ Hz, $J = 1.5$ Hz, C-7- and C-4′–$OCH_2CH{=}CH_2$); 5.18 (t_{sept}, 1H, $J = 7.2$ Hz, $J = 1.4$ Hz, CH–2″); 5.25 and 5.29 (two dq, 2H, $J = 10.4$ Hz, $J = 1.5$ Hz and $J = 10.4$ Hz, $J = 1.5$ Hz, *trans*-C-7- and *trans*-C-4′–$OCH_2CH{=}CH_2$); 5.42 (dd, 1H, $J = 12.5$ Hz, $J = 3.1$ Hz, CH-2); 5.41 and 5.47 (two dq, 2H, $J = 8.8$ Hz, 1.7 Hz, $J = 8.8$ Hz, 1.7 Hz, *cis*-C-7- and *cis*-C-4′-$OCH_2CH{=}CH_2$); 6.09 and 6.11 (two ddt, 2H, $J = 10.4$ Hz, $J = 8.8$ Hz, 5.2 Hz i $J = 10.4$ Hz, $J = 8.8$ Hz, 5.2 Hz, C-7- i C-4′–$OCH_2CH{=}CH_2$); 6.36 (s, 1H, CH-6); 7.01(d, 2H, $J = 8.7$ Hz, CH-3′ and CH-5′); 7.48 (d, 2H, $J = 8.7$ Hz, CH-2′ and CH-6′). IR (KBr) cm^{-1}: 3080, 2985, 2962, 2915, 2852, 1678, 1604, 1574, 1515, 1272, 1116, 1018, 932, 821. $C_{27}H_{30}O_5$ (434.54): calcd. C 74.63, H 6.96; found C 74.70, H 7.02.

*7,4′-Di-O-acetylisoxanthohumol (**9**)* To a solution of 100 mg (0.282 mmol) of isoxanthohumol and 0.37 ml (2.8 mmol) of Et_3N in 7.4 ml of anhydrous THF was added dropwise acetic anhydride 0.13 ml, 1.4 mmol). After 12 h of stirring at room temperature, the reaction medium was shaken with 36 ml of cooled water. The precipitated crystals were separated, washed twice with 3 ml of water, and dried using vacuum. The crude product was purified by the crystallization from methanol to provide 7, 4′-di-*O*-acetylisoxanthohumol (**9**) as white crystals (92.1 mg, 74.1% yield, mp = 140–141°C, $R_f = 0.68$, $CHCl_3$:MeOH, 99:1). ^{1}H NMR (300 MHz, acetone-d_6) δ (ppm): 1.58 and 1.61 (d, 6H, $J = 1.4$ Hz, CH_3-4″ and CH_3-5″); 2.27 (s, 3H, C-4′–$COOCH_3$); 2.31 (s, 3H, C-7–$COOCH_3$); 2.78 (dd, 1H, $J = 16.3$ Hz, $J = 3.1$ Hz, CH-3); 3.06 (dd, 1H, $J = 16.3$ Hz, $J = 12.9$ Hz, CH-3); 3.19 (d, 2H, $J = 7.02$ Hz, CH_2-1″); 3.80 (s, 3H, C- 5–O–CH_3); 5.09 (t_{sept}, 1H, $J = 7.1$ Hz, $J = 1.4$ Hz, CH-2″); 5.59 (dd, 1H, $J = 12.9$ Hz, $J = 2.9$ Hz, CH-2); 6.49 (s, 1H, CH-6); 7.21 (d, 2H, $J = 8.6$ Hz, CH-3′ and CH-5′); 7.62 (d, 2H, $J = 8.5$ Hz, CH-2′ and CH-6′). IR (KBr) cm^{-1}: 2964, 2927, 1759, 1687, 1593, 1510, 1477, 1369, 1213, 1170, 1093, 837. $C_{25}H_{26}O_7$ (438.48): calcd. C 68.48, H 5.98; found C 68.58, H 6.10.

*7,4′-Di-O-palmitoylisoxanthohumol (**10**)* To a solution of 100 mg (0.282 mmol) of isoxanthohumol and 0.28 ml (2.1 mmol) of Et_3N in 5.7 ml of anhydrous THF was added dropwise palmitoyl chloride (155 mg, 0.594 mmol). After 12 h of stirring at room temperature the reaction medium was shaken with 30 ml of cold water (~0°C), extracted with diethyl ether (3 × 10 ml), dried over anhydrous Na_2SO_4, and concentrated. The resulting residue was purified by column chromatography (hexane:Et_2O:MeOH, 5:5:1) to give 191.2 mg (81.6% yield) of 7,4′-di-*O*-palmitoylisoxanthohumol (**10**) as white crystals (mp = 71–73°C, $R_f = 0.86$, $CHCl_3$:MeOH, 95:5). ^{1}H NMR (300 MHz, acetone-d_6) δ (ppm): 0.87 (*t*, 6H, $J = 6.9$ Hz, C-7- and C-4′–$OOC(CH_2)_{14}CH_3$); 1.28 (s, 44H, C-7- and C-4′–$OOC(CH_2)_3(CH_2)_{11}CH_3$); 1.40 (m, 4H, $J = 6.9$ Hz, C-7- and C-4′–$OOC(CH_2)_2CH_2(CH_2)_{11}CH_3$); 1.59 (d, 6H, $J = 1.2$ Hz, CH_3-4″ and CH_3-5″); 1.73 (kwintet, 4H, $J = 7.3$ Hz, C-7- and C-4′–$OOCCH_2CH_2(CH_2)_{12}CH_3$); 2.60 and 2.64 (two *t*, 4H, $J = 7.3$ Hz, C-7- and C-4′–$OOCCH_2(CH_2)_{13}CH_3$); 2.78 (dd, 1H, $J = 16.3$ Hz, $J = 3.0$ Hz, CH-3); 3.07 (dd, 1H, $J = 16.3$ Hz, $J = 12.9$ Hz, CH-3); 3.19 (d, 2H, $J = 6.7$ Hz, CH_2-1″); 3.80 (s, 3H, C-5–OCH_3); 5.08 (t_{sept}, 1H, $J = 6.7$ Hz, $J = 1.2$ Hz, CH-2″); 5.60 (dd, 1H, $J = 12.9$ Hz, $J = 3.0$ Hz, CH-2); 6.47 (s, 1H, CH-6); 7.20 (d, 2H, $J = 8.5$ Hz, CH-3′ and CH-5′); 7.62 (d, 2H, $J = 8.5$ Hz, CH-2′ and CH-6′). IR (KBr) cm^{-1}: 3184, 2919, 2850, 1759, 1688, 1589, 1510, 1468, 1376, 1265, 1139, 1102, 844, 721. $C_{53}H_{82}O_7$ (831.24): calcd. C 76.58, H 9.94; found C 76.48, H 10.14.

Demethylation of isoxanthohumol derivatives

General procedure Each time 50 mg of compounds (**4**–**10**) were demethylated.

A solution of I_2 (3 eq., 99.5 mg, 0.393 mmol) in anhydrous Et_2O (3.5 ml) and Mg (6 eq., 19.1 mg, 0.786 mmol), taken in the round-bottomed flask and protected from light, was stirred at room temperature until the reaction mixture turned colorless (1.5 h). The resulting mixture of magnesium iodide etherate was separated from unreacted Mg and transferred via syringe under N_2 into the two-neck flask (50 ml), equipped with condenser, containing 50 mg of substrate [**4** (1 eq., 0.131 mmol)-**10**] in anhydrous THF

(9 ml). The reaction mixture was stirred and refluxed for 12 h and afterward the solvent was evaporated under reduced pressure. Then, 1 ml of THF and saturated solution of NH_4Cl (10 ml) were added and the whole mixture was extracted with CH_2Cl_2 (3 × 5 ml). The combined extracts were dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure to give crude product. After purification by column chromatography on silica gel (see Table 1) the products (**11**–**15**) were obtained.

*7,4′-di-O-methyl-8-prenylnaringenin (**11**)* Yield 61.3%, mp = 105–107°C, $R_f = 0.32$ ($CHCl_3$:hexane, 7:3), light-white solid. The ^{1}H NMR and IR spectroscopic data were in agreement with those reported in the literature (Cano *et al.*, 2006; Siddiqui *et al.*, 2003).

*7-O-pentyl-8-prenylnaringenin (**12**)* Yield 84.8%, mp = 132–134°C, $R_f = 0.67$ ($CHCl_3$:MeOH), 97:3, white crystals. ^{1}H NMR (300 MHz, acetone-d_6) δ (ppm): 0.93 (*t*, 3H, $J = 7.3$ Hz, C-7–O$(CH_2)_4CH_3$); 1.41 (m, 2H, C-7–O$(CH_2)_3CH_2CH_3$); 1.49 (m, 2H, C-7–O$(CH_2)_2CH_2CH_2CH_3$); 1.61 (d, 6H, $J = 1.4$ Hz, CH_3-4″ and CH_3-5″); 1.82 (m, 2H, C7–O$CH_2CH_2(CH_2)_2CH_3$); 2.79 (dd, 1H, $J = 17.0$ Hz, $J = 3.0$ Hz, CH-3); 3.16 (dd, 1H, $J = 17.0$ Hz, $J = 12.6$ Hz, CH-3); 3.22 (d, 2H, $J = 7.2$ Hz, CH_2-1″); 4.08 (*t*, 2H, $J = 6.3$ Hz, C-7–O$CH_2(CH_2)_3CH_3$); 5.15 (t_{sept}, 1H, $J = 7.2$ Hz, $J = 1.4$ Hz, CH-2″); 5.46 (dd, 1H, $J = 12.6$ Hz, $J = 3.0$ Hz, CH-2); 6.12 (s, 1H, CH-6); 6.90 (d, 2H, $J = 8.5$ Hz, CH-3′ and CH-5′); 7.41 (d, 2H, $J = 8.5$ Hz, CH-2′ and CH-6′); 8.51 (s, 1H, C-4′–OH); 12.24 (s, 1H, C-5–OH). IR (KBr) cm^{-1}: 3260, 2955, 2926, 2855, 1638, 1616, 1592, 1520, 1467, 1364, 1229, 1094, 832. $C_{25}H_{30}O_5$ (410.51): calcd. C 73.15, H 7.37; found C 73.32, H 7.54.

*7,4′-Di-O-allyl-8-prenylnaringenin (**13**)* Yield 78.9%, mp = 103–105°C, $R_f = 0.84$ ($CHCl_3$:MeOH, 99.3:0.7), pale yellow solid. ^{1}H NMR (300 MHz, acetone-d_6) δ (ppm): 1.60 (d, 6H, $J = 1.3$ Hz, CH_3-4″ and CH_3-5″); 2.82 (dd, 1H, $J = 17.1$ Hz, $J = 3.1$ Hz, CH-3); 3.18 (dd, 1H, $J = 17.1$ Hz, $J = 12.5$ Hz, CH-3); 3.24 (d, 2H, $J = 7.2$ Hz, CH_2-1″); 4.59 and 4.65 (two ddd, 4H, $J = 5.1$ Hz, $J = 1.7$ Hz, $J = 1.5$ Hz, C-7- and C-4′–O$CH_2CH{=}CH_2$); 5.16 (t_{sept}, 1H, $J = 7.2$ Hz, $J = 1.3$ Hz, CH–2″); 5.23–5.31 (m, 2H, *trans*-C-7- and *trans*-C-4′–O$CH_2CH{=}CH_2$); 5.51 (dd, 1H, $J = 12.5$ Hz, $J = 3.1$ Hz, CH-2); 5.39–5.48 (m, 2H, *cis*-C-7- and *cis*-C-4′–O$CH_2CH{=}CH_2$); 6.02–6.16 (m, 2H, C-7- and C-4′–O$CH_2CH{=}CH_2$); 6.12 (s, 1H, CH-6); 7.02 (d, 2H, $J = 8.8$ Hz, CH-3′ and CH-5′); 7.50 (d, 2H, $J = 8.8$ Hz, CH-2′ and CH-6′). IR (KBr) cm^{-1}: 2967, 2911, 2857, 1636, 1587, 1517, 1448, 1378, 1255, 1178, 1118, 1021, 921, 829. $C_{26}H_{28}O_5$ (420.51): calcd. C 74.26, H 6.71; found C 74.09, H 6.88.

*7,4′-Di-O-acetyl-8-prenylnaringenin (**14**)* Yield 88.4%, mp = 139–140°C, $R_f = 0.84$ ($CHCl_3$:MeOH, 98:2), white solid. ^{1}HNMR and IR spectroscopic data were in agreement with those reported in the literature (Gester *et al.*, 2001; Huempel *et al.*, 2005; Metz and Schwab, 2007; Schaefer *et al.*, 2005).

*7,4′-Di-O-palmitoyl-8-prenylnaringenin (**15**)* Yield 74.6%, mp = 67–69°C, $R_f = 0.91$ (hexane:Et_2O:MeOH, 5:5:0.1), white crystals. ^{1}H NMR (300 MHz, acetone-d_6) δ (ppm): 0.87 (*t*, 6H, $J = 6.9$ Hz, C-7- and C-4′–O$OC(CH_2)_{14}$–CH_3); 1.29 (s, 44H, C-7- and C-4′–O$OC(CH_2)_3(CH_2)_{11}$–CH_3); 1.40 (m, 4H, $J = 6.9$ Hz, C-7- and C-4′–O$OC(CH_2)_2CH_2(CH_2)_{11}$–$CH_3$); 1.60 (d, 6H, $J = 1.3$ Hz, CH_3-4″ and CH_3-5″); 1.73 (quintet, 4H, $J = 6.9$ Hz, C-7- and C-4′–O$OCCH_2CH_2(CH_2)_{12}$–CH_3); 2.60 and 2.64 (two *t*, 4H, $J = 7.4$ Hz, C-7- and C-4′–O$OCCH_2(CH_2)_{13}$–CH_3); 2.96 (dd, 1H, $J = 17.2$ Hz, $J = 3.0$ Hz, CH-3); 3.17 (d, 2H, $J = 6.8$ Hz, CH_2-1″); 3.32 (dd, 1H, $J = 17.2$ Hz, $J = 13.1$ Hz, CH-3); 5.07 (t_{sept}, 1H, $J = 6.8$ Hz, $J = 1.3$ Hz, CH-2″); 5.71 (dd, 1H, $J = 13.1$ Hz, $J = 3.0$ Hz, CH-2); 6.30 (s, 1H, CH-6); 7.22 (d, 2H, $J = 8.5$ Hz, CH-3′ and CH-5′); 7.65 (d, 2H, $J = 8.5$ Hz, CH-2′ and CH-6′); 11.87 (s, 1H, C-5–OH). IR (KBr) cm^{-1}: 3437, 2918, 2850, 1751, 1648, 1624, 1592, 1512, 1469, 1379, 1264, 1149, 1077, 840, 722. $C_{52}H_{80}O_7$ (817.21): calcd. C 76.43, H 9.87; found C 76.22, H 10.01.

Antiproliferative activity

The human cell lines of breast cancer (MCF-7), colon adenocarcinoma (HT-29), and leukemia (CCRF/CEM) were obtained from American Type Culture Collection (Rockville, Maryland, USA) and maintained in the Cell Culture Collection at the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. The cells at the density of 10^5/ml were cultivated in 96-well plates (Sarstedt, Germany) in 100 µl of culture medium at 37°C in humid atmosphere containing 5% CO_2. In the case of MCF-7 cell lines, the culture medium

Table 1 Eluents for column chromatography for the purification of compounds (**11–15**)

Compound	**11**	**12**	**13**	**14**	**15**
Eluent	$CHCl_3$:hexane 70:30	$CHCl_3$:MeOH 99.2:0.8	$CHCl_3$ 100	$CHCl_3$:MeOH 99.5:0.5	$CHCl_3$:Et_2O 90:10

consisted of Eagle's medium (IIET, Wroclaw, Poland) with addition of 10% fetal bovine serum (FBS, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 100 μg/ml streptomycin (Jelfa, Jelenia Góra, Poland), 100 U/ml penicillin (Jelfa, Jelenia Góra, Poland), 2 mM L-glutamine (Gibco, Warsaw, Poland), 1.0 mM sodium pyruvate, 1% amino acid, and 0.8 mg/l insulin. The cells of HT-29 line were cultured in the RPMI 1640 and Opti-MEM (1:1) (both from Gibco) medium with addition of *5% FBS,* 100 μg/ml streptomycin, 100 U/ml penicillin, 1 mM sodium pyruvate, and 2 mM L-glutamine. CCRF/CEM culture medium consisted RPMI 1640, 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine.

The compounds were dissolved in acetone (**1–4**, **8**, and **10**) or absolute ethanol (**5–7**, **9**, **11–13**) to the concentration of 10 mg/ml, stored at 4°C, and diluted in the culture medium to obtain concentrations from 0.1 to 100 μg/ml. The controls contained acetone or ethanol at the appropriate concentrations. The solutions of the synthesized compounds in 100 μl of culture medium were added after 24 h of incubation. The sulphorhodamine B (SRB, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) assay for MCF-7 and HT-29 cells and the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assay (Sigma–Aldrich, Germany) for CCRF/CEM cells were executed. Assays were performed after 72 h of continuous exposure of the cultivated cells to varying concentrations of test compounds according to the methods described by Skehan *et al.* (1990) and Marcinkowska *et al.* (1998), using a Multiskan RC photometer (Labsystems, Helsinki, Finland). The readings were recorded at 540 and 570 nm, respectively. Each compound at all the concentrations was investigated in triplicates. Each set of experiments was repeated 3–5 times.

SRB assay

The cells were attached to the bottom of plastic wells by gently layering cold 50% trichloroacetic acid (TCA) on the top of the culture medium in each well. The plates were stored at 4°C for 1 h and washed five times with tap water. The cells fixed with TCA were treated for 30 min with 0.4% solution of sulforhodamine B in 1% acetic acid. Then, the cells were washed four times with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base. Optical density ($\lambda = 540$ nm) was determined in a microplate reader Multiskan RC photometer.

MTT assay

Culture medium was gently removed from each well and cells were incubated for 4 h at 37°C with 20 μl MTT solution (5 mg/ml). Then, 80 μl of the mixture that contained 67.5 g sodium dodecyl sulfate and 225 ml dimethylformamide in 275 ml distilled water were added. After 24 h crystals of formazan were solubilized and the optical densities of the samples were read on a Multiskan RC photometer at 570 nm.

Results and discussion

Chemistry

The main goal of this research was investigation of the demethylation reaction of substituted isoxanthohumols (**4–10**) to provide 8-prenylnaringenins (**11–15**). The investigated reactions are shown in Fig. 1 and the results are summarized in Table 2.

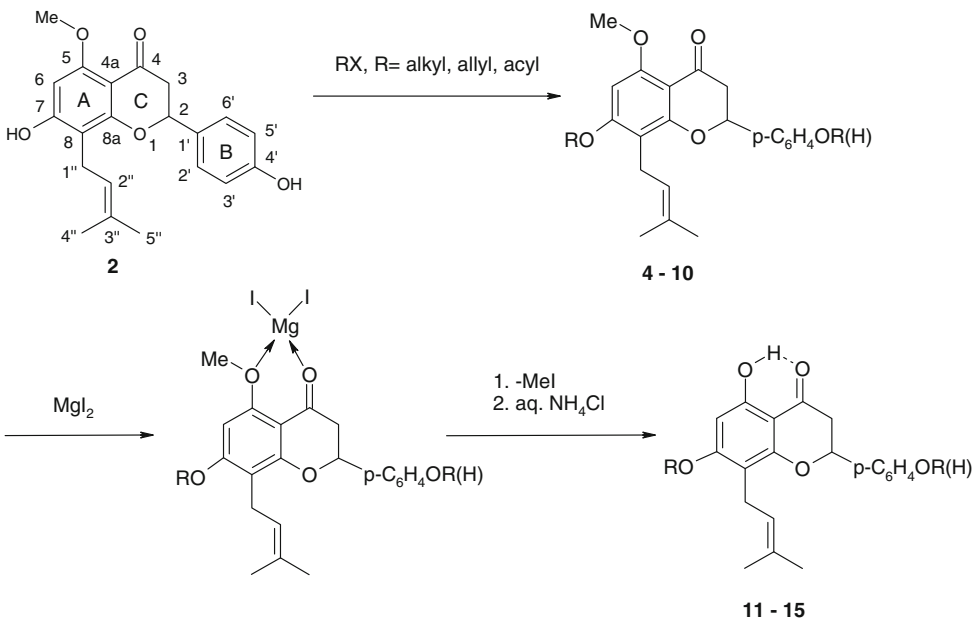

Fig. 1 Synthesis of the isoxanthohumol derivatives (**4–10**) and 8-prenylnaringenin derivatives (**11–15**) from isoxanthohumol (**2**)

Table 2 Synthesis of 7-*O*- and 4′-*O*-substituted isoxanthohumols (**4–10**), their demethylation to 8-prenylnaringenins (**11–15**) and antiproliferative activity in vitro

Entry	Substrate	Product	Yield[a] [%)]	7-*O*-R	4′-*O*-R	Cell line/ID_{50} (μg/ml)±SD		
						MCF-7	HT-29	CCRF/CEM
	–	**1**	–	–	–	4.7 ± 0.6	3.8 ± 0.6	4.1 ± 0.5
	1	**2**	–	–	–	9.4 ± 0.4	32.6 ± 0.3	18.2 ± 1.9
	2	**3**	–	–	–	19.4 ± 1.9	33.2 ± 0.8	24.2 ± 1.4
1a	**2**	**4**	69.4	Me	Me	6.6 ± 0.6	6.0 ± 1.2	5.0 ± 1.7
1b	**2**	**5**	8.8	Me	H	Not tested	Not tested	Not tested
2a	**2**	**6**	27.6	Pentyl	H	8.3 ± 1.2	6.9 ± 0.8	5.4 ± 0.9
2b	**2**	**7**	13.6	Pentyl	Pentyl	7.1 ± 0.6	8.2 ± 1.3	4.3 ± 0.7
3	**2**	**8**	81.2	Allyl	Allyl	5.2 ± 0.1	6.2 ± 1.1	2.7 ± 0.5
4	**2**	**9**	74.1	Ac	Ac	16.9 ± 2.3	32.1 ± 0.7	23.3 ± 1.1
5	**2**	**10**	81.6	Palmitoyl	Palmitoyl	Negative	Negative	Negative
6	**4**	**11**	61.3	Me	Me	36.9 ± 6.2	Negative	Negative
7	**6**	**12**	84.8	Pentyl	H	3.9 ± 0.2	10.0 ± 2.9	4.8 ± 0.4
8	**8**	**13**	78.9	Allyl	Allyl	Negative	Negative	Negative
9	**9**	**14**	88.4	Ac	Ac	28.0 ± 2.6	36.1 ± 3.8	37.0 ± 3.5
10	**10**	**15**	74.6	Palmitoyl	Palmitoyl	Negative	Negative	Negative

Negative Negative in the concentration used

[a] Isolated yield

Xanthohumol, the substrate in the isoxanthohumol synthesis, was isolated from carbon dioxide-extracted-hops (Marynka variety), purified and transformed into isoxanthohumol as described previously *(*Anioł *et al*., 2008*)*.

As model substrates for demethylation, methyl, *n*-pentyl, allyl, acetyl, and palmitoyl derivatives of **2** were selected. They had different chain lengths. It was assumed that the reactivity of homologous series of compounds should be similar, as well as reactivity of monosubstituted isoxanthohumol derivatives in comparison to disubstituted. For this reason, alkylating and acylating agents were used in high quantity to obtain disubstituted derivatives of **2** as a goal of synthesis.

Methyl ethers (**4** and **5**) were synthesized using excess of methyl iodide with 69.4 and 8.8% yield, respectively (Table 2, Entries **1a** and **1b**). During the course of reaction, it was observed that the formation of 7-*O*-methyl compound (**5**), which was methylated to get a dimethyl compound (**4**). There was a characteristic shift of the signal for C-6 proton of substrate (**2**) from 6.21 to 6.36 ppm for compound (**5**) on the NMR spectrum. It was caused by the substitution of C-7–OH group by a methoxy group. The chemical shifts of C-3′-, C-5′- and C-2′-, C-6′-protons were exactly the same in both the compounds ($\delta = 6.89$ and 7.38 ppm, respectively). The formation of products of cleavage of C ring leading to xanthohumol derivatives, as reported for methylation of 8-prenylnaringenin with Me_2SO_4 (Jain *et al.*, 1978). In case of prenylation (Table 2, Entries **2a** and **2b**), the order of alkylation was the same as that of compounds (**4** and **5**). The first product, 7-*O*-pentylisoxanthohumol (**6**) was formed with 27.6% yield ($\delta = 6.34$ (CH-6), 6.89 (CH-3′, CH-5′) and 7.38 ppm (CH-2′, CH-6′), and 7, 4′-*O*-dipentylisoxanthohumol (**7**) with 13.6% yield. The best yield of alkylation was observed during the synthesis of the diallyl compound (**8**, Table 2, Entry 3). Diacyl compounds (**9** and **10**) were obtained with 74.1 and 81.6% yield, respectively (Table 2, Entries **4** and **5**).

Demethylation reactions were carried out according to published procedure *(*Anioł *et al*., 2008*)*. Each time 50 mg of substrate was taken. The rest of the reagents were used proportionally in molar quantities. Demethylation of trimethoxy derivative (**4**) confirmed that the reaction of methyl-aryl ethers with magnesium iodide etherate occurred mainly at *ortho*-position in relation to acyl group. The main product of demethylation (**11**) was obtained with yield of 61.3% (Table 2, Entry **6**) but during the reaction course, the formation of complicated mixture of by-products was observed, which was confirmed by TLC and HPLC. This reaction was not as clean as that of demethylation of isoxanthohumol *(*Anioł *et al*., 2008). The ^{1}H NMR spectrum of **11** showed the lack of signal of C-8–OCH_3 protons at 3.86 ppm, and the presence of signal at 12.25 ppm for the proton of C-8–OH group involved in a strong intramolecular hydrogen bond. A quite similar effect as above was observed for the rest of the synthesized 8-prenylnaringenin derivatives. All the spectra were recorded within 1–2 h after the sample preparation in

acetone-d_6. When the spectrum was accumulated on the next day or later the signals for the hydroxyl protons disappeared because of the hydrogen deuterium exchange. Compound (**11**) was also isolated from *Azadirachta indica* (Siddiqui *et al.*, 2003) and *Esenbeckia berlandieri* ssp. *Acapulcensis* (Cano *et al.*, 2006). Substrate (**4**) used in the above reaction was present in hops in low quantity (Faltermeier *et al.*, 2006; Oosterveld *et al.*, 2002). For testing whether the demethylation depends on chain length of alkyl group, pentyl derivative of isoxanthohumol (**6**) was synthesized.

Demethylation of 7-*O*-pentylisoxanthohumol (**6**) to product (**12**) occurred with high yield of 84.8% (Table 2, Entry 7).

Cleavage of allyl ethers of alcohols and phenols was observed using lewis acids such as the $CeCl_3$–NaI system (Bartoli *et al.*, 2001; Thomas *et al.*, 1999). Compound (**8**) was synthesized to check whether its demethylation was affected by deallylation. There was a possibility that MgI_2, composed with magnesium (typical Lewis acid) and iodine (strong nucleophile) could be similar in action to $CeCl_3$–NaI system. We did not observe the allyl–aryl ether cleavage and the desired product (**13**) were obtained with good 78.9% yield (Table 2, Entry **7**). As in the case of alkyl ethers of isoxanthohumol, for testing whether the yield of demethylation depends on chain length of acyl group, diacetyl and dipalmitoyl derivatives of isoxanthohumol (**9** and **10**) were synthesized. These compounds, as representatives of esters, commonly applied as prodrugs, underwent demethylation with magnesium iodide etherate (Table 2, Entries **9** and **10**). The products, 8-prenylnaringenins (**14** and **15**) were obtained with 88.4 and 74.6% yield, respectively. Thus, introduction of alkyl, allyl or acyl group into isoxanthohumol moiety did not significantly influence the demethylation reaction and all the synthesized compounds were stable during the course of reactions. Nevertheless, during the optimization of the isoxanthohumol demethylation (Anioł *et al.*, 2008) to 8-prenylnaringenin the instability of reagents was observed, which could be associated with the known low stability of flavonoids.

Investigations conducted by a group of Wilhelm and Wessjohann (2006) showed that demethylation of such compounds as isoxanthohumol was very difficult to carry out. Among the 17 demethylating agents only $Sc(OTf)_3$/KI system worked with high yield. Our previous investigations demonstrated that this system could be replaced with $MgI_2 \times 2Et_2O$ to obtain 8-prenylnaringenin with 93% of yield. Now, we showed that this cheap, non toxic, easy to prepare and use agent could be applied in demethylation of acyl, alkyl, and allyl derivatives of isoxanthohumol.

Antiproliferative activity, in vitro

The synthesized compounds were examined for their antiproliferative activity in vitro against the human cell lines of breast cancer (MCF-7), colon adenocarcinoma (HT-29), and leukemia (CCRF/CEM). The results presented in Table 2 are expressed as the concentration in μg/ml leading to 50% inhibition of tumor cells proliferation (ID_{50}-dose) in comparison with the untreated ones. Acetone or ethanol, which was used as solvents, did not show any inhibitory effect on cell proliferation, even in the largest concentrations used. Xanthohumol (**1**), isoxanthohumol (**2**), and 8-prenylnaringenin (**3**), studied previously against selected tumor cell lines (Brunelli *et al.*, 2007, 2009; Monteiro *et al.*, 2007; Zanoli and Zavatti, 2008), were used as reference compounds. The two newly synthesized compounds (**8** and **12**) exhibited higher antiproliferative activity than the most active xanthohumol (**1**) against CCRF/CEM (2.7 μg/ml) and MCF-7 (3.9 μg/ml) cell lines and approaching the cytotoxic activity criterion $ID_{50} \leq 4$ μg/ml for new anticancer synthetic substances. The conducted investigations showed that, 7,4′-di-*O*-methyl-, 7,4′-di-*O*-pentyl-, and 7,4′-di-*O*-allyl- derivatives of isoxanthohumol (**4**, **7**, **8**) were significantly more active than parental isoxanthohumol (**2**) (9.4–32.6 μg/ml) against all investigated cells (2.7–6.6 μg/ml). On the other hand, diacyl derivatives (**9**: 16.9–32.1 μg/ml and **10**: $ID_{50} >$ 100 μg/ml) did not show any significant activity. Among the 8-prenylnaringenin derivatives, the most active compound was 7-*O*-pentyl-8-prenylnaringenin (**12**). This compound possessed the activity against the cells of MCF-7 (3.9 μg/ml), HT-29 (10.0 μg/ml), and CCRF/CEM (4.8 μg/ml) more than three times higher than 8-prenylnaringenin (**3**), 19.4, 33.2, 24.2 μg/ml, respectively. The rest of the derivatives of 8-prenylnaringenin (**11**, **13–15**) possessed low activity or were inactive ($ID_{50} > 100$ μg/ml).

Conclusion

In conclusion, the presented simple methodology of demethylation of isoxanthohumol derivatives via the formation of magnesium iodide etherate, offers an easy transformation route for 8-prenylnaringenin derivatives synthesis using xanthohumol as a starting material, which can be applied to several functional groups. Although the yields obtained (61.3–88.4%) were not as good as in case of demethylation of unsubstituted isoxanthohumol, the method was still easy, cheap and could be carried out in mild conditions. The synthesized compounds showed antiproliferative activity against the human cell lines of breast cancer (MCF-7), colon adenocarcinoma (HT-29), and leukemia (CCRF/CEM). The most active compound possessed activity of 2.7 μg/ml against leukemia cell lines. The developed demethylation protocol could be used in the synthesis of various potentially bioactive 8-prenylnaringenin derivatives and can be of use in the combinatorial

chemistry to prepare libraries of such compounds. It would also help in proper utilization of the spent hops, the waste product of hop industry.

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