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The flavonoid herbacetin diglucoside as a constituent of the lignan macromolecule from flaxseed hulls

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Abstract

Lignans in flaxseed are known to be part of a macromolecule in which they are connected through the linker-molecule hydroxymethyl-glutaric acid (HMGA). In this study, the lignan macromolecule was extracted from flaxseed hulls and degraded to its monomeric constituents by complete saponification. Besides secoisolariciresinol diglucoside (SDG), the phenolic compounds *p*-coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG) were isolated, which was expected based on indications from the literature. Also the flavonoid herbacetin diglucoside (HDG) was found. The presence of HDG was confirmed by NMR following preparative RP-HPLC purification. Also the presence of the three other constituents (CouAG, FeAG and SDG) was confirmed by NMR.

To prove that HDG is a substructure of the lignan macromolecule, the macromolecule was fragmented by partial saponification. A fragment consisting of HDG and HMGA was indicated. This fragment was isolated by preparative RP-HPLC and its identity was confirmed by NMR. It is concluded that the flavonoid HDG is a substructure of the lignan macromolecule from flaxseed hulls and that it is incorporated in the macromolecule via the same linker-molecule as SDG.

Keywords: Flaxseed hull; Lignan macromolecule; Flavonoid; Secoisolariciresinol diglucoside (SDG); Herbacetin diglucoside (HDG)

1. Introduction

Flaxseed is increasingly used as an ingredient in food products (Oomah, 2001), because of its high α-linolenic acid and dietary fiber content (Bhatty and Cherdkiatgumchai, 1990; Bhatty, 1995), but recently also because of its secondary metabolites. Important secondary metabolites are lignans, which are present in flaxseed in a higher concentration than in other edible sources (Liggins et al., 2000; Mazur and Adlercreutz, 1998; Milder et al., 2005). Lignans are reported to exhibit protective effects against hormone-related types of cancer like breast cancer (Boccardo et al., 2004; Chen et al., 2002; Chen and Thompson, 2003; Thompson et al., 1996a,b) and against non-hormone related colon cancer (Sung et al., 1998). Furthermore, they lower the risk of cardiovascular diseases (Lucas et al., 2004; Vanharanta et al., 1999).

The main lignan in flaxseed is secoisolariciresinol diglucoside (SDG), which is present in defatted flaxseed flour in concentrations up to 3% (w/w) (Bakke and Klosterman, 1956; Johnsson et al., 2000). Other lignans present in flaxseed are matairesinol (MAT) (Liggins et al., 2000), isolariciresinol (isoLARI) (Meagher et al., 1999), pinoresinol (PINO) (Meagher et al., 1999), and lariciresinol (LARI) (Sicilia et al., 2003). Other phenolic compounds reported in flaxseed, which might contribute to the health effects ascribed to flaxseeds, are hydroxycinnamic acids like *p*-coumaric acid (Klosterman et al., 1955), ferulic acid, sinapic acid, caffeic acid (Dabrowski and Sosulski, 1984) and their glucosides, and the flavonoids herbacetin diglucoside (HDG) and kaempferol diglucoside (KDG) (Qiu et al., 1999). The structures of the most relevant compounds are shown in Fig. 1a.

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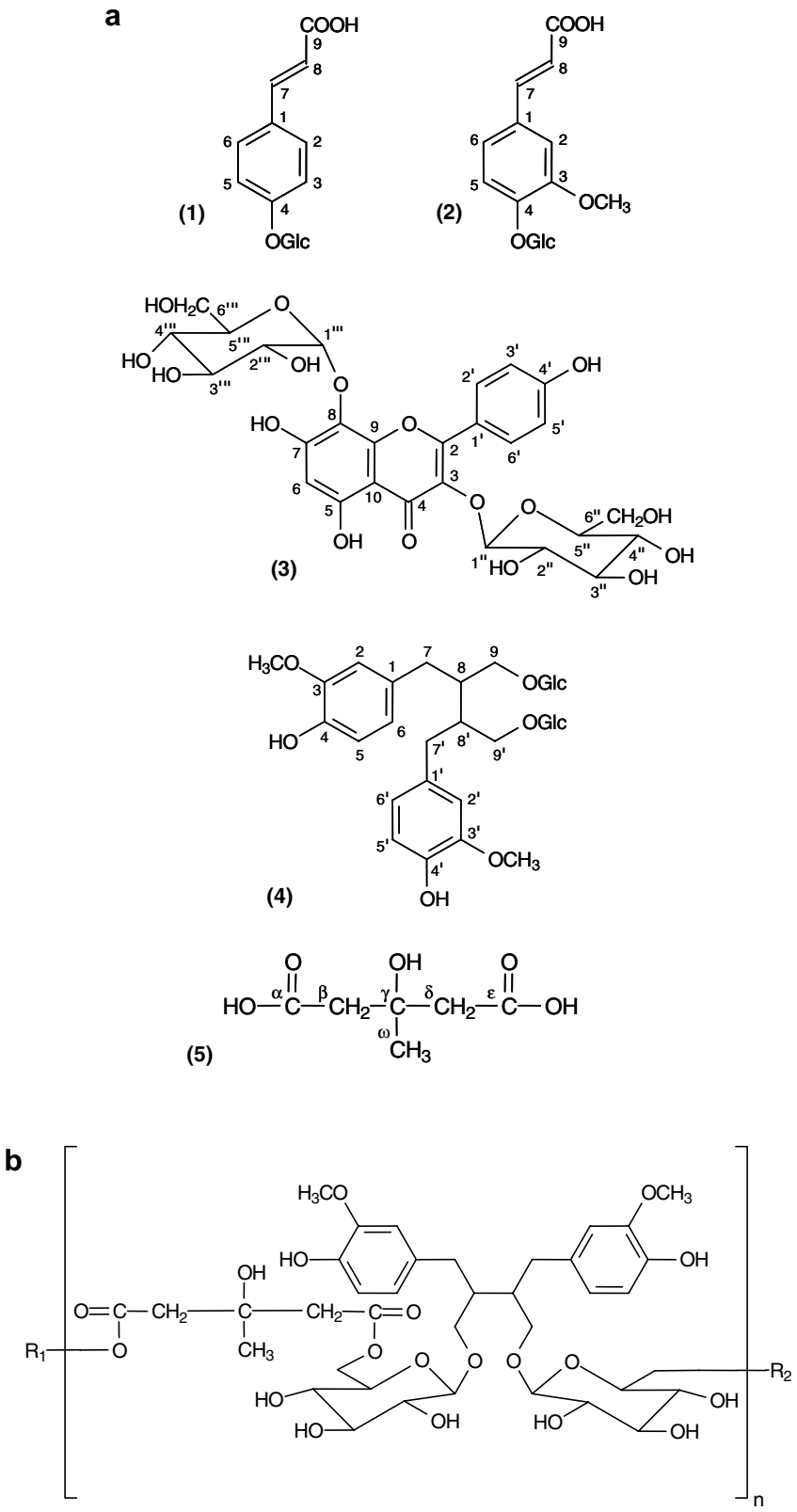

Fig. 1. (a) Structures of the main phenolic constituents of flaxseed: **1** = CouAG, **2** = FeAG, **3** = HDG, **4** = SDG, **5** = HMGA. All glucosyl-moieties are coupled via their C-6. Annotations will be used throughout the text. (b) Structure of the lignan macromolecule from flaxseed: R_1 = H or SDG, R_2 = OH or HMGA.

The lignans in flaxseed are part of an oligomeric structure (Kamal-Eldin et al., 2001), which is referred to as the lignan macromolecule. It is reported (Kamal-Eldin et al., 2001; Klosterman and Smith, 1954) that this lignan macromolecule consists of SDG units that are ester-linked by hydroxy-methyl-glutaric acid (HMGA) (see Fig. 1b). The molecular weight of this lignan macromolecule was estimated to be around 4000 based on the intensity of NMR signals (Kamal-Eldin et al., 2001), but further experimental evidence is lacking. In addition to SDG, *p*-coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG) were reported to be released from the lignan macromolecule after alkali treatment (Johnsson et al., 2002; Kamal-Eldin et al., 2001). However, these components were neither included (Kamal-Eldin et al., 2001) nor annotated (Johnsson et al., 2002) as integral constituents of the lignan macromolecule. For the other compounds identified in flaxseed, there are no indications that they are part of the lignan macromolecule. The hulls, which comprise about 40% of the seed, are enriched in SDG compared to the cotyledons (Madhusudhan et al., 2000; Oomah and Mazza, 1997; Wiesenborn et al., 2003). However, no information is available concerning the presence of a lignan macromolecule in flaxseed hulls. In this study, It is shown that the lignan macromolecule is present in flaxseed hulls and that it contains flavonoidic constituents besides the already known constituents SDG, CouAG en FeAG.

2. Results

2.1. Identification of the constituents of the lignan macromolecule

Flaxseed hulls were used as a source of lignans because of their high lignan content (Oomah and Mazza, 1997). Hulls were extracted with aq. EtOH aiming at the extraction of a lignan macromolecule. From 400 g flaxseed hulls, 29.9 g of extract was obtained, accounting for 7.5% (w/w) of the dry weight of the hulls. To obtain information about the composition of this extract, it was degraded by alkali treatment. Under alkaline conditions the ester-linkages are degraded while the glycosidic bonds are stable. In this way SDG and other glycosides are released, providing information on the native constituents of the lignan macromolecule.

Analysis of the untreated extract (0 mM NaOH) on GPC (Fig. 2) and RP-HPLC (Fig. 3) showed that the extract eluted as one broad peak indicating a heterogenic macromolecular structure. Saponification with increasing concentrations of NaOH resulted in the progressive degradation of this macromolecule as shown by the GPC (Fig. 2) and the RP-HPLC/MS results (Fig. 3). With increasing concentrations of NaOH (1–10 mM), the retention time of the saponified macromolecule on GPC (Fig. 2) was increased as compared to the retention time of the intact macromolecule, pointing at a decrease in fragment size

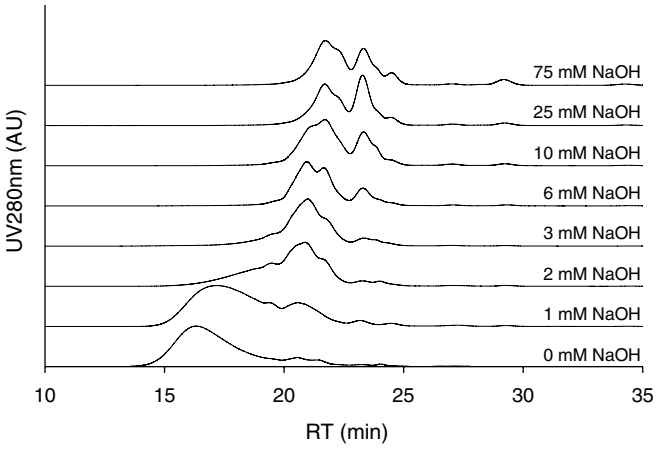

Fig. 2. GPC elution profile of lignan macromolecule saponified with a range of NaOH concentrations.

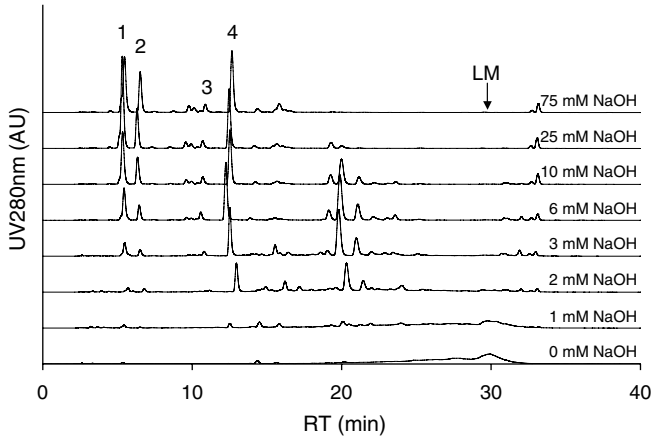

Fig. 3. Analytical RP-HPLC elution profiles of lignan macromolecule saponified with a range of NaOH concentrations: **1** = CouAG; **2** = FeAG; **3** = HDG; **4** = SDG; LM = lignan macromolecule.

upon saponification. Except for a small increase of the peak eluting around 29 min, the chromatogram of the macromolecule saponified with 75 mM NaOH was similar to the chromatogram of the macromolecule saponified with 25 mM NaOH. Therefore, it was assumed that with 75 mM NaOH the macromolecule was degraded completely or could not be degraded further.

Also on RP-HPLC (Fig. 3), the sample saponified with 75 mM NaOH showed a similar fragmentation pattern compared to the pattern of sample saponified with 25 mM NaOH, apart from some variation in the ratio between peak 1 and 2, which was also observed in Fig. 4 (see further). The MS analysis of the compounds separated on RP-HPLC showed that upon saponification with 75 mM NaOH the monomeric constituents were liberated. Peak **1** was annotated as CouAG ($m/z = 325.0$ $[M-H]^-$), peak **2** as FeAG ($m/z = 354.9$ $[M-H]^-$), peak **3** as HDG ($m/z = 625.2$ $[M-H]^-$), and peak **4** as SDG ($m/z = 685.3$ $[M-H]^-$).

The heterogeneity of the lignan macromolecule was confirmed by MALDI-TOF MS analysis of the unsaponified

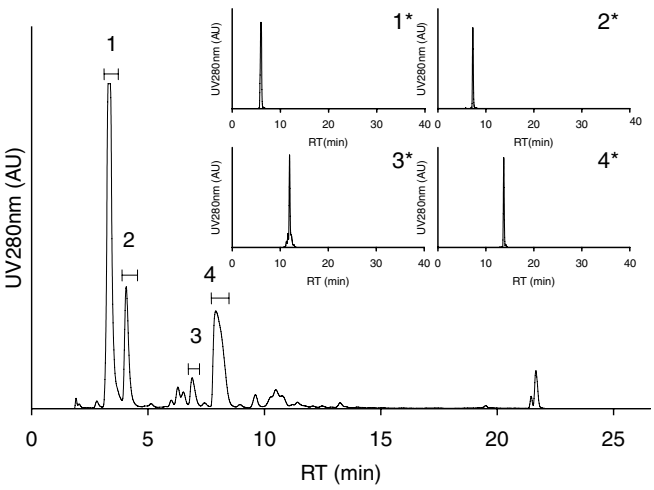

Fig. 4. Preparative RP-HPLC elution profiles of fully saponified lignan macromolecule (75 mM NaOH). The collected fractions are indicated; fraction 1 = CouAG, fraction 2 = FeAG, fraction 3 = HDG, fraction 4 = SDG. In the inserts, the analytical RP-HPLC elution profiles of the four fractions after semi-preparative purification are shown.

extract. A complex spectrum (not shown) was obtained showing clusters of peaks in which molecules ranging from 2SDG + 1HMGA ($m/z = 1521.8$ $[M + Na]^+$) to 5SDG+ 5HMGA ($m/z = 4104.8$ $[M + Na]^+$) could be annotated.

These results showed that the aq. EtOH extract consisted of a heterogenic macromolecule with SDG as one of its constituents, showing that a lignan macromolecule was present in flaxseed hulls. Next, the annotation of the flavonoid HDG is noteworthy, since HDG was isolated from flaxseeds only once before (Qiu et al., 1999).

To be able to confirm the identity of HDG and the other monomeric constituents by NMR, they were purified from the fully saponified lignan macromolecule. Therefore, the analytical RP-HPLC procedure was up-scaled. The preparative RP-HPLC elution profile (Fig. 4) showed high similarity with the one obtained by analytical chromatography (Fig. 3). Fractions of interest were collected as indicated in Fig. 4. The identity of the 4 fractions was confirmed based on their retention time on analytical RP-HPLC and MS analysis. Starting with 2.5 g fully saponified lignan macromolecule, 0.3 g CouAG (fraction 1, $m/z = 325.0$ $[M-H]^-$), 0.1 g FeAG (fraction 2, $m/z = 354.9$ $[M-H]^-$), 0.1 g HDG (fraction 3, $m/z = 625.2$ $[M-H]^-$), and 0.9 g SDG (fraction 4, $m/z = 685.3$ $[M-H]^-$) was collected after preparative RP-HPLC.

The purity of the fractions was determined by analytical RP-HPLC. Based on the area percentage of the main peak, most fractions were not sufficiently pure for NMR analysis (purity ranging from 62% to 91%). Therefore, a second purification step was performed on semi-preparative RP-HPLC. The compounds of interest and the impurities could be separated resulting in more pure fractions. This is shown in the inserts of Fig. 4 in which the analytical RP-HPLC profiles of the purified fractions collected by semi-preparative purification are presented. FeAG (fraction 2*) and SDG (fraction 4*) were 96% and 95% pure, respectively, based on the $UV_{280\ nm}$ responses. In addition, the MS results showed solely m/z-ratios corresponding to FeAG and SDG. CouAG (fraction 1*) was 97% pure based on $UV_{280\ nm}$ responses but MS analysis showed that besides CouAG ($m/z = 324.8$ $[M-H]^-$), a compound with an m/z-ratio of 340.9 ($[M-H]^-$) was present. This compound was annotated as caffeic acid glucoside. The purity of the main peak of HDG (fraction 3*) based on $UV_{280\ nm}$ response was 71%. This is explained by the presence of two shoulders flanking the main peak of HDG. MS analysis of fraction 3* showed the presence of only HDG.

Despite the remaining impurities, the identity of these components could be confirmed by NMR. The chemical shifts observed for FeAG, CouAG, and SDG (see spectral data in Section 4) were as reported in the literature (Johns-

Table 1
NMR data of herbacetin-di-β-glucopyranoside and herbacetin-di-β-glucopyranoside+hydroxy-methyl-glutaric acid+ethanol

Position*	HDG		HDG + HMGA + EtOH	
	^{1}H δ, mult., J (Hz)	^{13}C δ	^{1}H δ, mult., J (Hz)	^{13}C δ
2	–	159.0	–	159.0
3	–	135.3	–	135.4
4	–	nd	–	nd
5	–	158.8	–	158.9
6	6.28, *s*	100.1	6.30, *s*	100.3
7	–	158.8	–	158.9
8	–	126.4	–	126.6
9	–	nd	–	nd
10	–	105.8	–	105.7
1′	–	122.7	–	122.5
2′	8.37, *d*, 8.8	133.1	8.39, *d*, 8.9	133.1
3′	6.92, *d*, 8.8	116.0	6.92, *d*, 8.9	116.2
4′	–	161.6	–	161.8
5′	6.92, *d*, 8.8	116.0	6.92, *d*, 8.9	116.2
6′	8.37, *d*, 8.8	133.1	8.39, *d*, 8.9	133.1
1″	5.32, *d*, 7.6	103.8	5.30, *d*, 7.7	103.9
2″	3.48	75.8	nd	75.5
3″	3.41	77.9	nd	77.7
4″	3.33	71.6	nd	71.0
5″	nd	78.6	nd	78.3
6a″	3.64	62.7	4.27	64.7
6b″	3.77		4.12	
1‴	4.74, *d*, 7.9	107.7	4.75, *d*, 7.8	107.8
2‴	3.58	75.3	nd	75.9
3‴	3.43	77.9	nd	77.9
4‴	3.53	70.8	nd	71.2
5‴	3.42	78.6	nd	78.1
6a‴	3.74	62.3	3.74	62.3
6b‴	3.82		3.83	
α			–	172.0
β*a*			2.56	45.9
β*b*			2.42	
γ			–	70.7
δ*a*			2.47	45.9
δ*b*			2.38	
ε			–	172.7
ωa			1.12, *s*	27.9
CH_2			4.02	61.6
CH_3			1.17, *t*, 7.2	14.6

* In Fig. 1, the annotations are given. nd = not detected.

son et al., 2002; Kamal-Eldin et al., 2001). For HDG, both the ^{1}H and ^{13}C chemical shifts are shown in Table 1 (see Fig. 1 for the annotations). The chemical shifts for the aglycon were similar as reported by Qiu and co-workers (1999). The positions of both β-glucopyranose moieties on the C-3 and C-8 of the aglycone were proven by the presence of a cross-peak in the HMBC spectrum between C-3 and H-1″ and C-8 and H-1‴. It has to be noted that there is a slight difference in chemical shifts compared to the literature since the spectra were obtained in CD_3OD instead of DMSO-d_6 solution.

These results showed that besides SDG, CouAG and FeAG, HDG was released from the lignan macromolecule after alkali treatment. This suggests that also flavonoids are part of the macromolecular lignan structure.

2.2. Identification of HDG as a part of the lignan macromolecule

To obtain further proof for HDG being part of the lignan macromolecule, an oligomeric fragment containing HDG was needed. Therefore, partially fragmented lignan macromolecule was subjected to preparative RP-HPLC. For this, lignan macromolecule partially saponified with 2 mM NaOH was selected, because it resulted in the broadest range of fragments (Fig. 3). In the samples treated with less than 2 mM NaOH, intact macromolecule was still present. In the samples saponified with 6 mM NaOH or more, the monomeric constituents became predominant and the structural diversity was less.

During preparative RP-HPLC of the partially saponified lignan macromolecule, fractions were collected as indicated in Fig. 5. Based on MS analysis, fraction 3 was annotated as monomeric HDG. In fraction 4, an m/z-ratio of 769.1 ($[M-H]^-$) was found which could be annotated as HDG + HMGA. As this fraction was dominated by SDG it was not investigated further. In fraction 5, a fragment with an m/z-ratio of 797.2 ($[M-H]^-$) was annotated as HDG + HMGA + EtOH.

Fraction 5 was not pure and was subjected to semi-preparative purification. The result of the analytical analysis of the fraction, which included the HDG containing fragment, obtained after semi-preparative purification is shown in the insert in Fig. 5. This fraction (fraction 5*) was 92% pure based on the $UV_{280\ nm}$ response. MS analysis showed the presence of several m/z-ratios (data not shown), one of them corresponding to the fragment of HDG + HMGA + EtOH ($m/z = 797.1$ $[M-H]^-$). No further efforts were made to further purify this fraction.

The NMR-data of the fragment of HDG + HMGA + EtOH are shown in Table 1 (see Fig. 1 for the annotations). The presence of HDG in the fragment was proven by obtaining the same chemical shifts for the aglycone as in monomeric HDG. Again, the β-glucopyranose was linked to both the C-3 and the C-8 of the aglycone. HMGA was recognized by the peaks between 2.6 and 2.3 ppm (β and δ) and the presence of cross-peaks in the HMBC spectrum between these peaks and C-α and ε at 172 ppm. These two downfield shifted carbons are typical for an esterified carboxylic acid group.

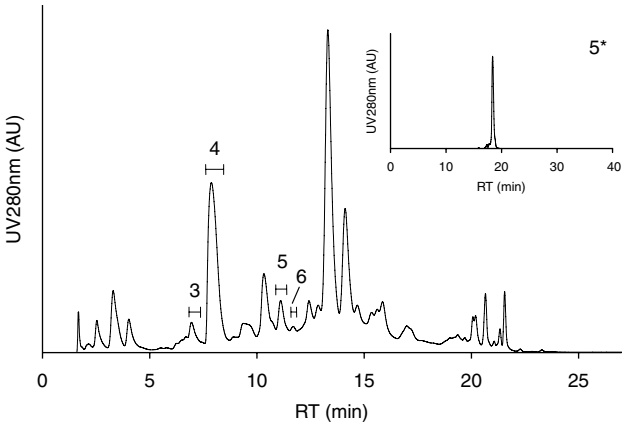

Fig. 5. Preparative RP-HPLC elution profiles of partially saponified lignan macromolecule (2 mM NaOH). The collected fractions are indicated; fraction 3 = HDG, fraction 4 = SDG, fraction 5 = HDG + HMGA + EtOH, fraction 6 = SDG + 2HMGA. In the insert, the analytical RP-HPLC profile of the HDG containing fragment (fraction 5) after semi-preparative purification is shown.

The HMGA molecule is linked to the glucose at C-6 via an ester linkage between C-α and C-6″ proven by the presence of 2 cross-peaks in the HMBC spectrum between C-α and H6a″ and b″, and by the significantly downfield shifted proton and carbon chemical shifts of H6a″ and b″. HMGA is esterified with EtOH via C-ε as shown by the presence of a cross-peak in the HMBC spectrum between C-ε and the CH_2 belonging to ethanol.

To obtain further information about the linkage of HDG via HMGA to the lignan macromolecule, the samples obtained by partial saponification were screened for m/z-ratios corresponding to HDG + 2HMGA (913.2 $[M-H]^-$) and for HDG + HMGA + SDG (1437.3 $[M-H]^-$). No m/z-ratio of 913.2 +/− 0.5 ($[M-H]^-$) was found, but in fraction 6 (Fig. 5) the m/z-ratio of 1437.3 ($[M-H]^-$) was present. Fraction 6 was subjected to MS/MS fragmentation. Fragments with m/z-ratios of 463.1, 625.2, and 1275.3 $[M-H]^-$, annotated as herbacetin monoglucoside, HDG, and HDG + HMGA + SDG minus a glucosyl residue, respectively, were identified. These results are in accordance with the annotation of this m/z-ratio as HDG + HMGA + SDG. Since this fragment was present in low quantities, it was not purified. In the peaks eluting between 12 and 15 min (Fig. 5), no indications of fragments containing HDG were found, and therefore these fragments were not investigated further.

3. Discussion

This study identified the flavonol HDG as one of the constituents of the lignan macromolecule in flax seed hulls. Qiu and co-workers (1999) already reported that the flavonols HDG and KDG were present in flax, but no data were

published pointing at their presence in an oligomeric structure.

Literature reported an HDG content of 0.01% (w/w) in flaxseed (Qiu et al., 1999), which is much lower than the 0.2% (w/w) HDG in flaxseed hulls found in the present study. Flaxseed hulls might be a richer source of HDG compared to flaxseed, but also the extraction procedure used might explain the difference. Extraction under acidic conditions (Qiu et al., 1999) instead of under alkali conditions (this study) might lead to acid hydrolysis of HDG: deglucosylation (Qiu et al., 1999) takes place and a reactive herbacetin carbocation can be formed (Eklund et al., 2004). When water reacts with this carbocation, the less polar herbacetin can be formed. Because of the lower polarity of this herbacetin, it might be lost during the extraction procedure resulting in the lower yield. Alternatively, when organic solvents like MeOH or EtOH react with the carbocations, herbacetin ethers can be formed (Eklund et al., 2004). Qiu and co-workers (1999) indeed identified a herbacetin dimethylether. In the present study, upon full saponification, only HDG and no herbacetin or herbacetin ethers were found. So, both the loss of aglycons and the ether formation might explain the lower HDG content reported in the literature in addition to the possible differences in HDG content between flaxseed and flaxseed hulls.

KDG, the other flavonol identified by Qiu and co-workers (1999), was not found in this study. Due to the low amount of sample material used, this was not expected.

Besides the release of HDG from the lignan macromolecule after alkali treatment, a fragment was isolated in which HDG was linked to HMGA, suggesting that HDG is part of the same macromolecular structure as lignans. This fragment was present as an ethylester, which was assumed to be formed as an intermediate product during saponification in the presence of ethanol (ethanolysis instead of hydrolysis) (Ford et al., 2001). Upon more severe saponification conditions the ethylesters were hydrolyzed again. During ethanolysis, transesterification between a macromolecular constituent and EtOH is assumed to take place. So, the identification of HDG + HMGA + EtOH suggests that at least one of the glucosyl residues of HDG is, via HMGA, ester-linked to another constituent of the macromolecule. Due to the low concentration of HDG it cannot be excluded that fragments of HDG with 2 HMGA moieties are present. Therefore, it is still unclear whether in the lignan macromolecule HDG is present as constituent of the backbone or as a substituent.

The presence of HDG in the macromolecular structure is consistent with the biosynthesis of the lignan macromolecule. It has been proposed that SDG is incorporated in the lignan macromolecule by coupling of the glucosyl moieties with coenzyme A-activated HMGA (Ford et al., 2001). Since the glucosyl moieties and not the lignan itself are involved in the assembly of the macromolecule, it is hypothesized that also the glucosyl moieties of HDG could be a target of HMGA coupling.

Of all lignans described in flaxseed only SDG was found in this study. Because of varying concentrations of constituents due to variations in cultivar (Liggins et al., 2000) and growing conditions (Bhatty and Cherdkiatgumchai, 1990), and because of the use of flaxseed hulls instead of whole seeds, it was not expected that all compounds, especially those present in low concentrations in whole seeds, would be found in hulls. It was remarkable, however, that no MAT was found, MAT being the second most abundant lignan present in flaxseeds (Liggins et al., 2000; Mazur and Adlercreutz, 1998).

In analogy to the transformations described for hydroxymatairesinol (HMR) (Eklund et al., 2004), it is suggested that MAT is unstable under the alkaline conditions used in this study to obtain individual lignans. It is hypothesized that a reactive quinone was formed followed by the opening of the lactone-ring (Eklund et al., 2004). However, the expected reaction products were not identified by MS analysis. The browning of the reaction mixture, which was observed during saponification, might be attributed to quinone methide intermediates (Ekman et al., 1979) supporting the hypothesis of MAT quinone formation. In addition, it has been shown that lignan extraction without alkaline treatment could result in a higher yield of MAT (Milder et al., 2004). This indicates that the isolation of constituents from the lignan macromolecule is strongly dependent on the conditions applied.

In conclusion, the flavonol HDG is identified being present in flaxseed hulls as a subunit of a macromolecular structure, as shown in Fig. 6. HDG is incorporated in the macromolecule by HMGA, the same molecule that inter-

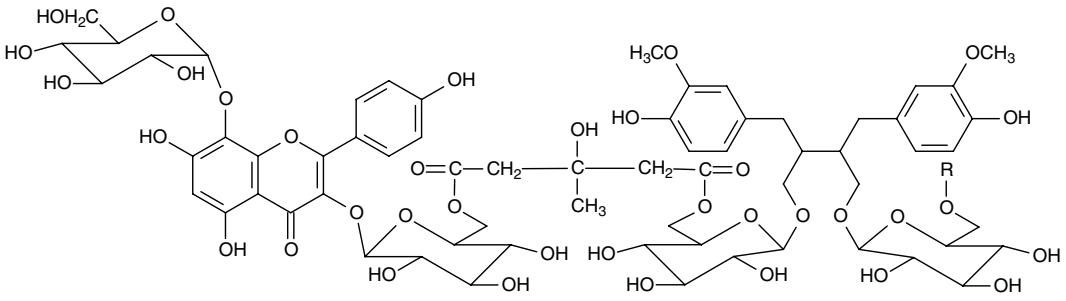

Fig. 6. The chemical structure of HDG + HMGA + SDG; a structural element of the lignan macromolecule from flaxseed hulls.

connects SDG. This shows that HMGA coupling is not limited to lignans, and suggests that more compounds carrying a glucosyl moiety might be incorporated in the lignan macromolecule.

4. Experimental

4.1. Lignan extraction from flaxseed hulls

Flaxseed hulls, kindly provided by Acatris Specialities Holding B.V. (Giessen, The Netherlands), were defatted by soxhlet extraction. In order to defat, 400 g of hulls were extracted with 3 l of *n*-hexane, resulting in 93.5 g oil extracted. The lignan macromolecule was extracted from the defatted hulls by a three-step sequential extraction with 63% (v/v) aq. EtOH. In the first step, 9 ml of aq. EtOH per gram of defatted flaxseed hulls was used, in the second step 3.6 ml/g and in the last step 2.3 ml/g. The first two extractions were performed for 4 h at room temperature while stirring, the last extraction was performed overnight. The extracts and the hulls were separated by filtration on a 595 round paper filter (Schleicher & Schuell). After evaporation of the EtOH, the extract was lyophilized yielding 29.9 g of lignan macromolecule.

4.2. Saponification of the lignan macromolecule

Solutions of 2 mg/ml lignan macromolecule in 63% (v/v) aq. EtOH containing various concentrations NaOH were (partially) saponified to obtain lignan macromolecular fragments of various sizes. NaOH concentrations ranging from 1 to 25 mM NaOH were used for partial saponification. For full saponification 75 mM NaOH was used. Saponification was performed at room temperature while stirring. After 24 h, the reaction was stopped by lowering the pH to 6.5–7.0 with HOAc (20 mM–1.5 M for analytical purposes, glacial HOAc for preparative purification purposes). For analytical purposes, the incubation volume was 1 ml, for preparative purification purposes a volume of 2.5 l was used.

4.3. Sample clean up of saponified lignan macromolecule

Low molecular weight polar material was removed from the (partially) saponified samples by solid phase extraction (SPE; SepPak Vac, 20 cc/5 g, C18 cartridge, Waters). Prior to loading samples onto the SPE cartridge, the EtOH concentration in the reaction mixture was reduced: the 1 ml samples were diluted with 25 ml water, whereas for the 2.5 l incubations the EtOH was evaporated and the remaining solution was concentrated further to max. 5 mg/ml. Insoluble particles formed during concentration, were removed by centrifugation (28,100*g*, 4 °C, 30 min) and the supernatant was applied onto the cartridge. Resolubilisation of the insoluble material in 63% (v/v) aq. EtOH and analysis of this material on analytical RP-HPLC did not show any peaks corresponding to peaks annotated as constituents of the lignan macromolecule (Fig. 3).

After activation of the SepPak cartridge with successively 2 column volumes of MeOH and 2 column volumes of water, a maximum of 150 mg (partially) saponified lignan macromolecule (supernatant) was loaded onto the cartridge. After a wash step with 1 column volume of water, lignans were eluted from the cartridge with 3 column volumes of MeOH of which the first column volume was discarded. For analytical purposes, the MeOH was evaporated under a stream of air to a final volume of 1 ml. For purification purposes, all MeOH was evaporated and the (partially) saponified lignans were lyophilized. Aliquots of the samples were analyzed on analytical RP-HPLC. After full and partial saponification (2 mM NaOH) of 5.0 g lyophilized lignan macromolecule and sample clean up, 2.5 g and 2.8 g of fully and partially saponified lignan macromolecule was obtained, respectively.

4.4. Analytical reversed phase HPLC (RP-HPLC)

Samples were analyzed on an X-Terra C18 MS column (Waters; 3.5 μm particle size, 4.6 × 150 mm) with an X-Terra C18 MS guard column (Waters; 3.5 μm particle size, 4.6 × 10 mm) run on a Thermo Separation Products HPLC system equipped with a membrane degasser, P4000 pump, AS3000 autosampler, and UV3000 detector. Water and acetonitrile (ACN), both acidified with 0.1% (v/v) HOAc, were used as eluents. The flow rate was 0.7 ml/min. The following linear gradient was used: 0–25 min, 10–30% ACN; 25–30 min, 30–50% ACN; 30–40 min, isocratic on 50% ACN; 40–42 min, 50–100% ACN; 42–47 min, isocratic on 100% ACN; 47–50 min, 100–10% ACN; 50–60 min, isocratic on 10% ACN. The eluate was monitored at 280 nm. The injection volume was 20 μl. Samples were injected being solved in the solvent in which they were extracted or eluted (water, MeOH, 63% (v/v) aq. EtOH or ACN:water mixtures).

4.5. Preparative reversed phase HPLC

Lignans and fragments of the lignan macromolecule were purified by preparative RP-HPLC on an X-Terra C18 MS column (Waters; 5 μm particle size, 50 × 100 mm, OBD) with an X-Terra C18 MS guard column (Waters; 5 μm particle size, 19 × 10 mm) ran on a Waters preparative HPLC system equipped with a 2525 pump, 2767 sample manager, Fluid Organizer and 2996 photodiode array detector. Water and ACN, both acidified with 0.01% TFA, were used as eluents. The flow rate was 80 ml/min. The linear gradient was as follows: 0–16.6 min, 10–30% ACN; 16.6–20 min, 30–50% ACN; 20–26.6 min, isocratic on 50% ACN; 26.6–28 min, 50–100% ACN; 28–31.3 min, isocratic on 100% ACN; 31.3–33.3 min, 100–10% ACN; 33.3–40 min, isocratic on 10% ACN.

Up to 600 mg of lignans (2 mg partially or fully saponified lignan macromolecule/ml water) were loaded per run.

Prior to loading, the sample was filtered over a 0.2 μm cellulose acetate filter (Schleicher & Schuell) to remove residual insoluble particles. Sample was loaded using the Waters reagent manager with a maximum flow rate of 2 ml/min.

Based on the response at 280 nm, fractions were collected (leading edge gradient = 5%, peak terminates when valley = 0). Appropriate fractions were pooled, as were the corresponding pools from subsequent runs. ACN was evaporated and the fractions were lyophilized. The purity of the fractions pooled was determined on analytical HPLC.

4.6. Semi-preparative reversed phase HPLC

For purification of 100 mg or less lignan macromolecule constituents, the samples were separated on a semi-preparative X-Terra C18 MS column (Waters; 5 μm particle size, 29 × 150 mm, OBD) with an X-Terra C18 MS guard column (Waters; 5 μm particle size, 19 × 10 mm) ran on a Waters preparative HPLC system as specified above. The gradient was similar to the gradient of analytical RP-HPLC. The flow rate was 12 ml/min. Fractions were collected based on the response at 280 nm as described for preparative RP-HPLC. The purity of the fractions was determined using analytical RP-HPLC and the purified components were lyophilized after evaporation of ACN.

4.7. Liquid chromatography coupled on-line to mass spectrometry (LC-MS)

The molecular mass of the lignans and fragments of the macromolecule were determined on a Thermo Finnigan LCQ Classic coupled on-line to the analytical RP-HPLC. The flow from the analytical RP-HPLC was split: 1/10th was directed to the MS. The MS was equipped with an ESI injector. Spectra were obtained in the negative ion mode over an m/z range of 150–2000 Da. The capillary temperature was 270 °C, the capillary voltage was −7.00 V, the ion spray voltage was set on 4.50 kV and helium was used as sheath gas. MS/MS analysis was performed with a normalized collision energy of 27%.

4.8. Gel permeation chromatography (GPC)

To determine the molecular weight distribution of the lignan macromolecule and the saponified lignans, aliquots of 50 μl (2 mg/ml) were analyzed on a similar HPLC system as described for analytical RP-HPLC, equipped with a Tricorn Superdex Peptide 10/300 GL column (Amersham Bioscience; 10 × 300–310 mm, bed volume = 24 ml, optimum separation range 100–7000 Da). The column was run isocratically at a flow rate of 0.8 ml/min with 40% (v/v) aq. ACN + 0.1% (v/v) TFA as eluent. The eluate was monitored at 280 nm. The included volume (V_{inc} = 16.5 ml) was determined with water. It should be noted that part of the compounds eluted later than the included volume indicating that aspecific interactions occurred. No further calibration was performed since no suitable calibration compounds were available.

4.9. Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

For the qualification of the molecular weight distribution of unsaponified lignan macromolecule, this sample was analyzed on a Ultraflex MALDI-TOF MS (Bruker Daltronics GmbH). As matrix 2,5-dihydroxybenzoic acid dissolved in 50% aq. ACN (15 mg/ml) was used. 2 μl of sample mixture consisting of 10 μl 2 mg/ml unsaponified (0 mM) lignan macromolecule, 10 μl matrix-solution and 1 μl 1 mM NaAc pH 5 was spotted on a gold plate. The MALDI-TOF MS was calibrated with a mixture of malto dextrins (mass range 365–4092). The system was used in the positive reflector mode.

4.10. Nuclear magnetic resonance (NMR)

Prior to NMR analyses, the samples were solved in 99.8% CD_3OD + 0.05% (v/v) TMS (Cambridge Isotope Laboratories, USA). NMR spectra were recorded at a probe temperature of 25 °C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. Chemical shifts were expressed in ppm relative to TMS at 0.00 ppm.

The ^1H and ^{13}C proton decoupled spectra were recorded at 500.13 MHz and 125.77 Hz, respectively. All 2 D COSY spectra were acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker.

For the 2 D HMBC spectrum a standard gradient enhanced 2 D HMQC pulse sequence delivered by Bruker, was changed into a HMBC sequence by setting the delay between the first proton and carbon pulse to 50 ms.

4.11. Spectral data of constituents of the lignan macromolecule

trans-p-Coumaric acid β-glucopyranoside (**1**): ^1H NMR (500.13 MHz, CD_3OD) δ 7.55 (2 H, *d*, J = 8.61 Hz, H-2/6), 7.12 (2 H, *d*, J = 8.56 Hz, H-3/5), 7.63 (1 H, *d*, J = 15.94 Hz, H-7), 6.36 (1 H, *d*, J = 15.94 Hz, H-8), 4.97 (1 H, *d*, J = 6.68 Hz, H-1′), 3.46 (3 H, *m*, H-2′/3′/5′), 3.40 (1 H, *m*, H-4′), 3.90 (1 H, *dd*, J = 12.14, 1.46 Hz, 1 H, H-6a′), 3.70 (1 H, *dd*, J = 12.06, 5.52 Hz, 1 H, H6b′).

^{13}C NMR (125.77 MHz, CD_3OD) δ 130.0 (C-1), 130.9 (C-2 and 6), 117.8 (C-3 and 5), 160.9 (C-4), 146.1 (C-7), 117.4 (C-8), 170.4 (COOD), 101.7 (C-1′), 74.8 (C-2′), 77.8 (C-3′), 71.3 (C-4′), 78.2 (C-5′), 62.6 (C-6′).

trans-Ferulic acid β-glucopyranoside (**2**): ^1H NMR (500 MHz, CD_3OD) δ 7.61 (1 H, *d*, J = 15.93 Hz, H-7), 7.24 (1 H, *d*, J < 1 Hz, H-2), 7.15 (1 H, *dd*, J = 8.46, 1.50 Hz, H-6), 7.18 (1 H, *d*, J = 8.37 Hz, H-5), 6.39 (1 H, *d*, J = 15.92 Hz, H-8), 4.97 (1 H, *d*, J = 7.38 Hz, H-1′),

3.90 (1 H, *s*, CH_3), 3.88 (1 H, *dd*, $J = 12.06$, 1.89 Hz, H-6a′), 3.69 (1 H, *dd*, $J = 12.06$, 5.21 Hz, H6b′).

^{13}C NMR (125.77 MHz, CD_3OD) δ 130.5 (C-1), 112.5 (C-2), 151.0 (C-3), 149.9 (C-4), 117.3 (C-5), 123.6 (C-6) 146.2 (C-7), 117.9 (C-8), 170.5 (COOD), 56.6 (CH_3), 102.3 (C-1′), 74.9 (C-2′), 77.9 (C-3′), 71.4 (C-4′), 78.4 (C-5′), 62.5 (C-6′).

Secoisolariciresinol-di-β-glucopyranoside (**4**): ^{1}H NMR (500.13 MHz, CD_3OD) δ 6.58 (2 H, *s*, H-2/2′), 6.65 (2 H, *d*, $J = 7.9$ Hz, H-5/5′), 6.56 (2 H, *d*, $J = 7.98$ Hz, H-6/6′), 2.69 (2 H, *dd*, $J = 13.72$, 6.58 Hz, H-7a/7a′), 2.61 (2 H, *dd*, $J = 13.62$, 8.07 Hz, H7b/7 b′), 2.12 (2 H, *m*, H-8/8′), 4.08 (2 H, *dd*, $J = 9.83$, 5.50 Hz, H-9a/9a′), 3.47 (2 H, *dd*, $J = 9.75$, 6.56 Hz, H-9 b/1 b′), 3.73 (6 H, *s*, OCH_3), 4.24 (2 H, *d*, $J = 7.79$ Hz, H-1″/1‴), 3.215 (2 H, H-2″/2‴), 3.341 (2 H, H-3″/3‴), 3.302 (2-H, H-4″/4‴), 3.253 (2 H, H-5″/5‴), 3.85 (2 H, *dd*, $J = 11.80$, 1.83 Hz, H-6a″/6a‴), 3.69 (2 H, *dd*, $J = 11.84$, 5.54 Hz, H-6 b″/6 b‴).

^{13}C NMR (125.77 MHz, CD_3OD) δ 134.0 (C-1/1′), 113.6 (C-2/2′), 148.8 (C-3/3′), 145.4 (C-4/4′), 115.7 (C-5/5′), 122.9 (C-6/6′), 35.7 (C-7/7′), 41.3 (C-8/8′), 71.2 (C-9/9′), 104.8 (C-1″/1‴), 75.3 (C-2″/2‴), 78.2 (C-3″/3‴), 71.7 (C-4″/4‴), 77.9 (C-5″/5‴), 62.9 (C-6″/6‴).

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