Biosynthetic Pathway to the Cancer Chemopreventive Secoisolariciresinol Diglucoside−Hydroxymethyl Glutaryl Ester-Linked Lignan Oligomers in Flax (*Linum usitatissimum)* Seed†

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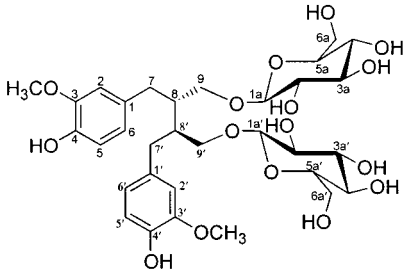
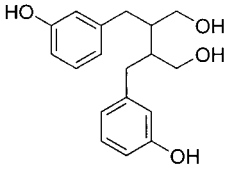
Application of stable and radioisotope precursor/tracer experiments resulted in the identification of various phenylpropanoid, monolignol, and lignan metabolites involved in the biosynthesis of the cancer chemopreventive secoisolariciresinol diglucoside (SDG; **1**)-containing ester-linked "polymer(s)" in flax (*Linum usitatissimum*) seed. Individual analysis of size-segregated flax seed capsules at five early stages of their development provided a metabolic profile of intermediates leading to "biopolymer" biosynthesis. The use of ^{1}H and ^{13}C NMR and HRMS analyses resulted in the identification of 6a-HMG (hydroxymethyl glutaryl) SDG (**17**) and 6a,6a′-di-HMG SDG (**18**) as the two major components of the ester-linked "biopolymer(s)". Based on metabolic tracer analyses and relative radioisotopic incorporations throughout each of these five stages of seed development, a biochemical pathway is proposed from phenylalanine to SDG (**1**), with subsequent mono- and di-substitutions of SDG (**1**) with HMG CoA. These metabolites then serve as precursors for formation of the SDG−HMG ester-linked oligomers. Results from this study will facilitate future isolation and characterization of the proteins and enzymes involved in biosynthesis of the SDG−HMG ester-linked oligomers in flax seed.

Secoisolariciresinol diglucoside (SDG; **1**) is a natural cancer chemopreventive agent effective against the onset of breast, prostate, and colon cancers.[1] Upon ingestion, it is catabolized into the mammalian lignans enterodiol (**2**) and enterolactone (**3**) through the effects of anaerobic fecal microflora.[2,3] Although their modes of cancer chemopreventive action are not yet known definitively, they are considered to be associated with modulating activities related to sex hormone metabolism as well as inhibiting cell proliferation and angiogenesis.[1,4−9] SDG (**1**) additionally displays antioxidant activity, exhibiting an ability to reduce hypercholesterolemic atherosclerosis,[10,11] and helps prevent development of streptozotocin-induced diabetes.[12,13] Both SDG (**1**) and flax seed differentially reduce plasma insulin-like growth factor I levels in rats, with this being correlated with a decreased breast cancer risk, with flax seed alone acting more efficiently than SDG (**1**).[14,15]

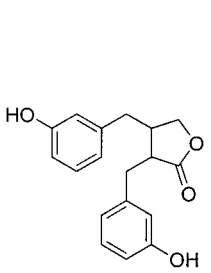
SDG (**1**) is the major alkali releasable lignan present in flax (*Linum usitatissimum* L., Linaceae) seed, and it is estimated to be in amounts 60−700 times greater than that described thus far for any other edible plant.[16,17] Depending upon the cultivar, it can be isolated in overall yields of up to >3% of the seed following base hydrolysis of its aqueous soluble "polymeric"-ester complex. Since its initial isolation and identification by Klosterman and Clagett 47 years ago,[18] various modifications of this method have been reported.[17,19−23] Other lignans reported present in flax seed include a diastereomer of SDG (**4**)[24] with unknown configuration (discussed later), pinoresinol (**5**), isolariciresinol (**6**),[25] and matairesinol (**7**),[25,26] as well as *p*-coumaric (**8a**), sinapic (**9**), and ferulic (**10a**) acids,[27] and a ferulate ester (**11**).[28]



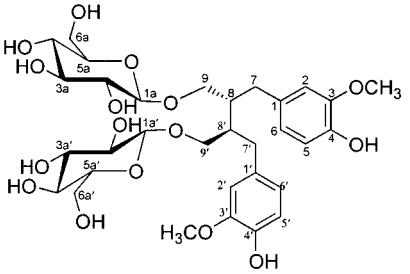
1: Secoisolariciresinol diglucoside (SDG)



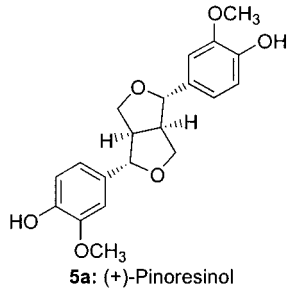
2: Enterodiol



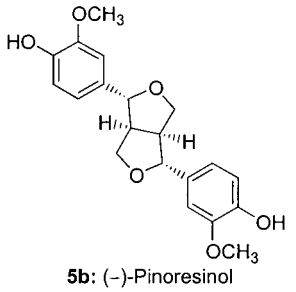
3: Enterolactone



4: SDG diastereomer



5a: (+)-Pinoresinol



5b: (−)-Pinoresinol

There is considerable interest in both defining and biotechnologically employing the SDG (**1**) biosynthetic pathway for its health benefits. Current knowledge of C-8−

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† Abbreviations used are: SDG, secoisolariciresinol diglucoside; UDPG, uridine diphosphate glucose; Bis-Tris, 2-[bis(2-hydroxyethyl) amino]-2−2(hydroxymethyl)-1,3-propanediol; HMG, hydroxy methylglutaryl.

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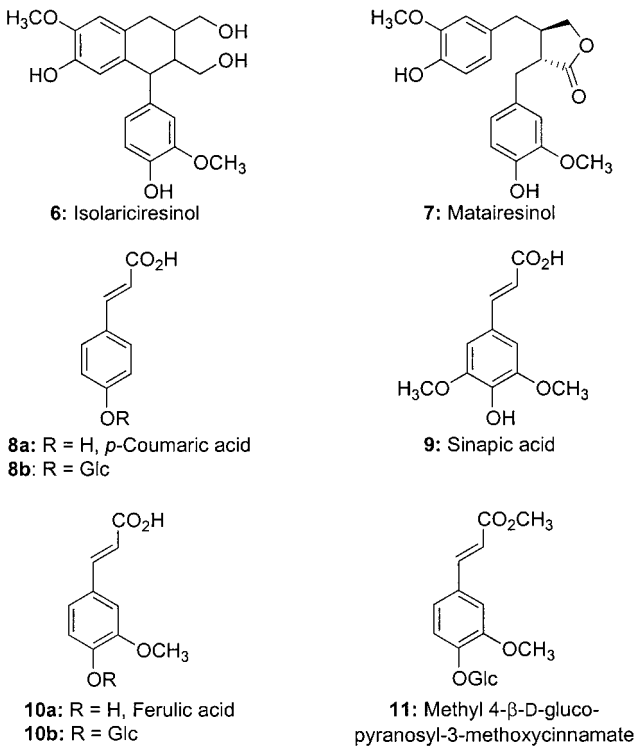


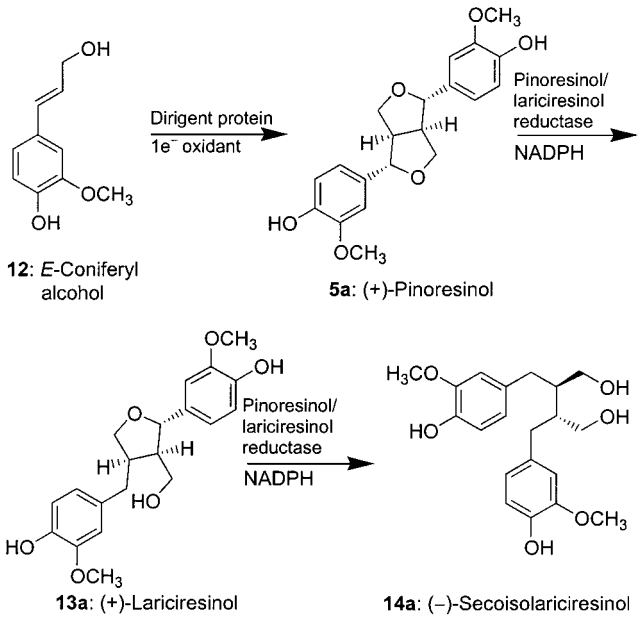


Figure 1. Lignan biosynthetic pathway to (−)-secoisolariciresinol (**14a**) in *Forsythia intermedia*.

C-8′ linked lignan biosynthesis in planta has been established with *Forsythia intermedia*, *Podophyllum peltatum*, *Thuja plicata*, and *Linum flavum*.[29−36] Entry into each pathway involves the dirigent mediated coupling of *E*-coniferyl alcohol (**12**) to afford (+)-pinoresinol (**5a**),[30,35] which is subsequently converted enantiospecifically into (+)-lariciresinol (**13a**) and (−)-secoisolariciresinol (**14a**) via the action of a bifunctional NADPH-dependent pinoresinol/lariciresinol reductase[31,32,35] (Figure 1). Assuming pinoresinol (**5**) is a precursor of SDG (**1**) in flax seed,[25] a pathway similar to that occurring in *F. intermedia* can be envisaged for formation of SDG (**1**) with an additional role by a UDPG:glucosyltransferase, glycosylating the C-9 and C-9′ hydroxyl positions, respectively. At the beginning of this investigation, however, neither the structure(s) nor the biochemical pathway to the SDG ester-linked "biopolymer(s)" in flax seed was known. It had been established that SDG (**1**) and the glucosides **8b** and **10b** of *p*-coumaric and ferulic acids could be released from the aqueous ethanol-soluble "polymer(s)" following alkaline hydrolysis at room temperature, indicating ester-linkage attachments.[18,28,37]

To deduce the metabolic pathway in flax seed responsible for the formation of the SDG ester-linked "polymer(s)", in vivo precursor administration experiments were employed using various stable [^{13}C]- and radio [^{14}C and ^{3}H]-labeled isotopes to flax capsules segregated into five distinct developmental stages. This was followed by radiochemical, high-resolution HPLC-MS and ^{1}H/^{13}C NMR spectroscopic analyses of purified flax seed phenylpropanoid pathway intermediates and lignan metabolites. This resulted in an identification of both the nature of the "polymeric" constituents and the metabolic pathway involved. It was considered particularly important to identify the former, given that these represent the substances ingested and subsequently catabolized from flax seed to give rise to the mammalian lignans **2** and **3**.

Results and Discussion

Flax seed development overall is not synchronized simultaneously, as seeds (or their developing capsules) are present at different developmental stages during growth of each plant's corymbrose inflorescence. To study SDG (**1**) formation as a function of seed development and its subsequent conversion into the "polymer(s)", a methodology was needed to obtain seeds at different development stages. An arbitrary means to conveniently achieve this was to separate seed capsules using different sizing screens. In this way, seeds were segregated into five early stages of capsule development [see Supporting Information and figure therein]. Mature flax seeds (designated as stage 6) were also examined, these representing seeds with completely developed embryos. Note that differences between flax seed stages 5 and 6 are in color and not size; the fully mature seeds of the cultivar used have brown seed coats, whereas those at stage 5 are of a translucent vanilla color with visible cotyledons.

Following capsule segregation and seed extraction as described (see Experimental Section), the SDG (**1**) ester-linked "polymer(s)" from each stage were individually removed by EtOH/H_2O (40:60, v/v) extraction, with all manipulations carried out in duplicate. After successive organic extractions of each aqueous EtOH extract with hexanes, EtOAc, and $CHCl_3$, the remaining aqueous solubles were either treated with 0.25 M NaOH to liberate SDG (**1**) or used directly to quantify free SDG (**1**) amounts. As shown in Figure 2, alkali releasable SDG (**1**) levels are very low at developmental stages 1 and 2, but rapidly increase throughout the various stages of growth, reaching their highest values at maturation (~0.8% w/w for this cultivar). In contrast, untreated samples produced almost undetectable levels of SDG (**1**), with stage 5 exhibiting the most (0.03%) and mature seeds (stage 6) the least (not detectable). These data thus demonstrate that SDG (**1**) [or SDG (**1**) equivalents] is/are increasingly accumulating during different stages of seed development and that free SDG (**1**), if formed, is apparently converted efficiently into the ester-linked "polymer". [For the remainder of this

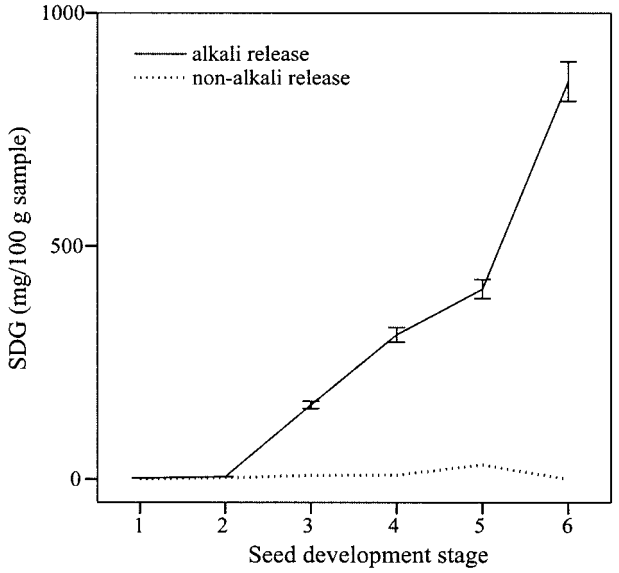
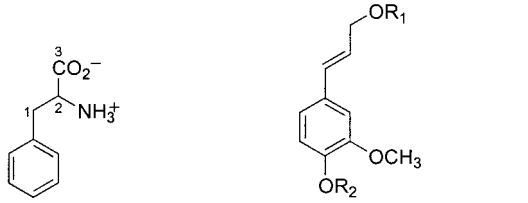


Figure 2. Plot of SDG (**1**) levels, both free and releasable upon alkali hydrolysis, as a function of flax seed development stage. Relative SDG (**1**) levels for seed developmental stage 6 are based on analysis from fully mature flax seeds.

investigation only stages 1−5 were used, since these were involved in the early stages of SDG (**1**) "polymer(s)" formation and seed development.]



15: L-Phenylalanine

16a: R_1 = H, R_2 = Glc, *E*-Coniferin

16b: R_1 = Glc, R_2 = H, *E*-Isoconiferin

Individual administration of L-[U-^{14}C]-phenylalanine (**15**) to seed capsules at each of the five developmental stages resulted in its facile conversion over 24 h into various radiolabeled components. One of these was coincident with the HPLC retention volume of SDG (**1**), both before (extract A2; Figure 3A) and after (extract A3) alkaline hydrolysis (data not shown) of the aqueous EtOH extracts. These data suggested that small amounts of SDG (**1**) are being formed in the developing seeds prior to their incorporation into the SDG-containing "polymer"; radiochemical incorporation levels in free SDG (**1**) reached a maximum value at stage 3 (∼3.9%) under the conditions employed and then steadily declined. [As discussed later, subsequent alkaline hydrolysis of the aqueous EtOH extracts from stage 3 onward resulted in significant increases in the radiochemical levels of SDG (**1**).]

To confirm unambiguously that this radiochemical constituent was indeed SDG (**1**), L-[3-^{13}C]-phenylalanine (**15**) was administered to the developing seeds in stage 4 capsules, with these being used due to their large supply and simplicity of seed extraction. Following 24 h uptake of L-[3-^{13}C]-phenylalanine (**15**) and its subsequent metabolism in the seed capsules (see Experimental Section), the alkali releasable SDG (**1**) was isolated and subjected to ^{13}C NMR spectroscopic analysis. The ^{13}C NMR spectral patterns were identical to that of authentic SDG (**1**), except for a single enriched peak (∼2 × natural abundance) at δ 34.5, corresponding to the C-7 and C-7′ resonances of SDG (**1**) (data not shown).

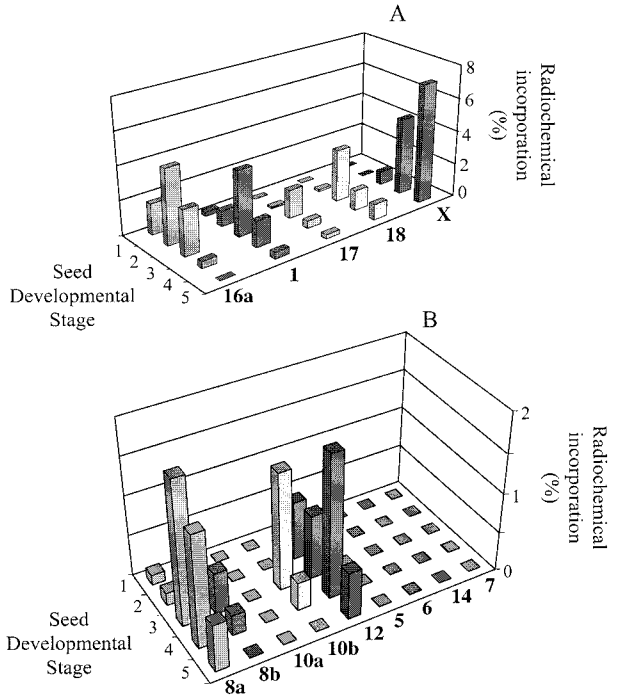


Figure 3. Metabolic profile observed during SDG (**1**) ester-linked "polymer" formation through five early stages of flax seed development (stages 1−5). Percent incorporations are based on conversions of L-[U-^{14}C]-phenylalanine (**15**). (A) Components present in aqueous EtOH-soluble (extract A2) samples at each stage prior to alkali hydrolysis; (B) components present in organic- (extract C) and base-hydrolyzed aqueous EtOH-soluble (extract A3) samples excluding SDG (**1**) and *E*-coniferin (**16a**).

The enantiomeric purity of the secoisolariciresinol (**14**) component of the aforementioned radio-[^{14}C]-labeled SDG (**1**) formed de novo in the developing seeds was next determined. Following its release from stage 5 capsules, the SDG (**1**) was HPLC purified and treated with β-glucuronidase to afford [^{14}C]-secoisolariciresinol (**14**). In this regard, both the (−)- and (+)-enantiomers of natural abundance secoisolariciresinol (**14a/b**) can be readily separated by chiral HPLC analysis (see Figure 4A).[31] Subsequent chiral HPLC analysis of the [^{14}C]-secoisolariciresinol (**14**) from stage 5 developing seeds, following its hydrolytic release, revealed that only the corresponding radiolabeled (+)-enantiomer (**14b**) was present (Figure 4B). Taken together, these data confirmed unambiguously that the fraction containing the isolated SDG (**1**) was derived from the (+)-enantiomer of secoisolariciresinol (**14b**).

Attention was then directed toward identifying the other unknown radiochemical compounds being formed at each stage of flax seed development following uptake and metabolism of L-[U-^{14}C]-phenylalanine (**15**) (see Figure 3A,B). Under the conditions employed, neither pinoresinol (**5**), isolariciresinol (**6**), matairesinol (**7**), nor secoisolariciresinol (**14**) was detected at any of the five stages of flax seed development examined, i.e., whether by HPLC electrospray ionization mass spectrometric (LC-ESI-MS), UV, or radiochemical analyses of either the alkali- or nonalkali-treated aqueous EtOH or the organic-soluble samples (see Figure 3B). This can perhaps best be explained if the pool sizes of these compounds are very low due to their rapid conversion into SDG (**1**) and its congeners.

For identification of the organic-soluble radiolabeled constituents (extract C, Figure 3B), it was found that the two metabolites detected corresponded to *E*-coniferyl alcohol (**12**) (*m*/*z* 179 [M − H]$^-$) and *p*-coumaric acid (**8a**) (*m*/*z* 163 [M − H]$^-$), respectively, this being further confirmed

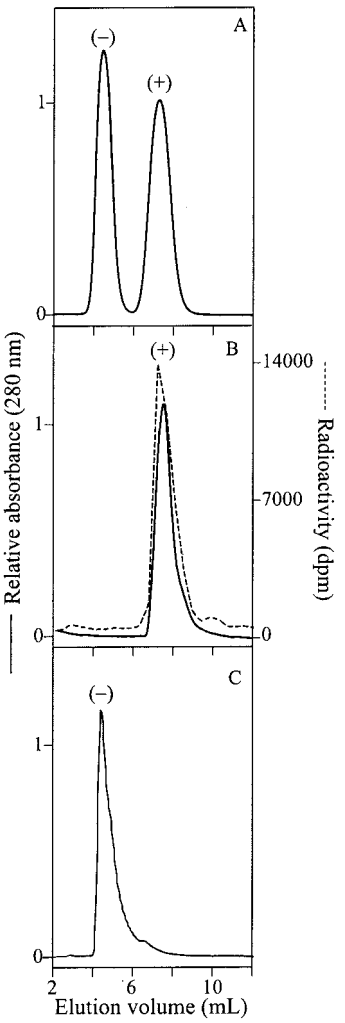


Figure 4. HPLC chiral analyses of various secoisolariciresinol (**14**) samples. (A) Synthetic (±)-secoisolariciresinols (**14a/b**), (B) [^{14}C]-(+)-secoisolariciresinol (**14b**) released following its purification after β-glucuronidase hydrolysis of [^{14}C]-SDG (**1**), and (C) (−)-secoisolariciresinol (**14a**) after β-glucuronidase hydrolysis of the SDG diastereomer (**4**).

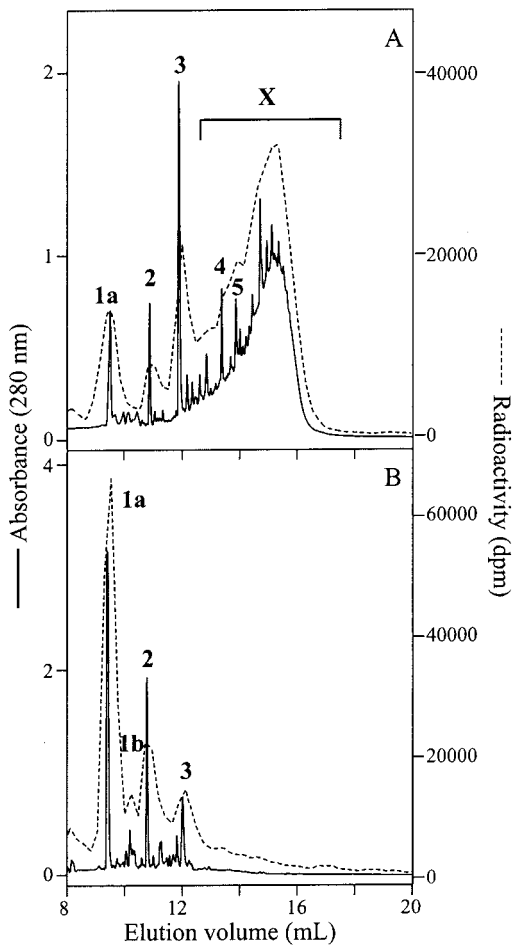


Figure 5. C_{18} reversed-phase HPLC analyses of stage 5 developing flax seed aqueous EtOH solubles following uptake and metabolism of L-[U-^{14}C]-phenylalanine (**15**). (A) Extract A2, including the SDG ester-linked "polymer"; (B) extract A3, aqueous EtOH solubles *after* base hydrolysis [1a = SDG (**1**); 1b = SDG diastereomer (**4**); 2 = 6a-HMG SDG (**17**); 3 = 6a,6a′-di-HMG−SDG (**18**); 4 = dimer-1 (**19**); 5 = dimer-2 (**20**); **X** ≅ ester-linked SDG "polymer(s)" including (**22**)].

by coelution with authentic standards. Interestingly, *p*-coumaric acid (**8a**) had its highest radiochemical incorporation (∼1.8%) at stage 3 and then gradually declined to ∼1.3% by stage 5 (see Figure 3B). On the other hand, *E*-coniferyl alcohol (**12**) was detected at all stages, with a maximum value (∼1.8%) at stage 4. By contrast, no ferulic acid (**10a**) was detected at any stage of the developing seeds.

The aqueous EtOH solubles at each developmental stage (extract A2, following organic solvent extraction) were also subjected to HPLC analysis. Besides SDG (**1**) and its congeners **4**, **17**, **18**, and "**X**" [as discussed later (Figure 3A)], one other abundant radiochemical component was identified as *E*-coniferin (**16a**) by its UV, HPLC retention time, and mass spectral fragmentation pattern (*m*/*z* 341 [M − H]$^-$). Interestingly, *E*-coniferin (**16a**) was present in the aqueous EtOH solubles before and after alkali hydrolysis (stages 1−4); on the other hand, no isoconiferin (**16b**) was detected.

Besides SDG (**1**), HPLC radiochemical analysis of the alkali-hydrolyzed aqueous solubles (extract A3) revealed two other major radiochemical constituents, whose LC-ESI-MS analyses gave molecular ions of *m*/*z* 385 [M + CH_3-COOH − H]$^-$ and 415 [M + CH_3COOH − H]$^-$, respectively. These were found to be the phenylpropanoid glucosides, **8b** and **10b**, previously reported in flax seed[28] (see Experimental Section). The highest radiochemical incorporation of L-[U-^{14}C]-phenylalanine into *p*-coumaric acid glucoside (**8b**) was ∼0.5% at stage 3, whereas for ferulic acid glucoside (**10b**), it was ∼1.6% at stage 3 (Figure 3B). Essentially no radiochemical incorporation into either glucoside was detected at stage 5, although both could be detected by UV and LC-ESI-MS analyses after alkali hydrolysis, indicating that their formation and conversion into the alkali labile components occurred at an earlier stage of seed development.

Attention was finally directed toward identifying the components involved in forming the SDG (**1**) ester-linked "polymer(s)". For illustrative purposes, Figures 5A and 5B show the HPLC profiles of the aqueous EtOH solubles obtained for stage 5 seed development, prior to and following base hydrolysis, respectively. In addition to SDG (**1**) being detected prior to base hydrolysis (Figure 5A, peak 1a), two other radiochemical and UV detectable peaks (2 and 3) were also observed. The majority of the radioactivity was, however, associated with a complex series of components eluting between 12 and 17 mL (designated "**X**"). Subsequent alkali hydrolysis (0.25 M NaOH, 2 h, room temperature) resulted in hydrolytic cleavage of this complex mixture with a concomitant increase in both radiochemical and UV peak intensities of SDG (**1**; peak 1a), and

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for SDG Diastereomer (**4**) and SDG-HMG Analogues **17** and **18**

		4[a]	**17**[b]		**18**[c]	
position		^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1			131.7		134.2	
2		6.64 d (1.8)	112.8	6.61 d (1.9)	113.7	6.60 d (1.0)
3			147.0		148.9	
4			144.2		145.5	
5		6.65 d (8.1)	114.9	6.65 d (8.0)	115.8	6.61 d (8.0)
6		6.59 dd (8.1, 1.8)	121.0	6.57 dd (8.0, 1.9)	123.0	6.48 dd (8.0, 1.0)
7	a	2.72 dd (13.5, 7.0)	32.7	2.59−2.60 m	35.7	2.59 dd (13.8, 5.5)
	b	2.65 dd (13.5, 7.6)				2.38 dd (13.8, 7.2)
8		2.11 t (5.3, 5.0)	39.8	2.13 m	41.8	2.05 m
9	a	3.92 dd (10.0, 5.3)	68.9	3.99 dd (9.9, 6.1)	70.8	3.83 br t (8.3, 7.6)
	b	3.58 dd (10.0, 5.8)		3.48 dd (9.9, 2.7)		3.29 dd (9.0, 5.5)
OCH_3-3		3.75 s	55.3	3.73 s	56.4	3.67 s
1a		4.21 d (7.6)	102.8	4.24 d (7.8)	104.7	4.11 d (7.9)
2a		3.21 t (8.8, 7.6)	73.4	3.16−3.31 m	75.3	2.98 t (8.6, 8.3)
3a		3.36 t (8.8, 8.8)	76.5	3.16−3.31 m	78.3	3.14 t (9.0, 8.6)
4a		3.26 overlap	70.3	3.16−3.31 m	71.8	3.05 t (9.3, 9.3)
5a		3.26 t (7.7, 6.5)	73.6	3.45 overlap	78.1	3.34 t (9.3, 9.3)
6a	a	3.87 br d (10.6)	63.8	4.41 dd (11.8, 2.1)	62.9	3.97dd (11.4, 7.5)
	b	3.67 dd (11.7, 5.3)		4.22 dd (11.8, 5.7)		4.29 br d (10.4)
1b			170.3			
2b	a		45.8	2.68 d (14.3)		2.56 d (14.2)
	b			2.65 d (14.3)		2.47 d (14.2)
3b			68.9			
4b	a		45.9	2.62 d (15.2)		2.43 d (14.4)
	b			2.57 d (15.2)		2.39 d (14.4)
5b			173.3			
6b			27.6	1.35 s		1.23 s
1′						
2′				6.60 d (1.7)		
3′						
4′						
5′				6.64 d (8.0)		
6′				6.56 dd (8.0, 1.7)		
7′	a			2.59−2.60 m		
	b					
8′				2.13 m		
9′	a			4.07 dd (10.2, 6.1)		
	b			3.44 dd (10.2, 1.7)		
OCH_3-3′				3.74 s		
1a′				4.23 d (7.8)		
2a′				3.16−3.31 m		
3a′				3.34 overlap		
4a′				3.16−3.31 m		
5a′				3.16−3.31 m		
6a′	a			3.85 dd (11.8, 2.1)		
	b			3.69 dd (11.8, 5.5)		

[a] Measured in CD_3OD, with all assignments confirmed by ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra; δ in ppm, J in Hz. [b] Measured in CD_3OD. [c] Measured in DMSO, with all assignments confirmed by ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra.

to a much lesser extent in a new peak designated 1b, and in peak 2 (Figure 5B).

The radioactive component, peak 1b, was HPLC purified and subjected to MALDI-TOF and LC-ESI-MS analyses, respectively. It had a UV absorbance and mass spectrum identical with that of SDG (**1**); that is, MALDI-TOF-MS analysis gave a *m*/*z* 709.298 $[M + Na]^+$ (calcd for $C_{32}H_{46}O_{16}$-Na, 709.268) and an LC-ESI-MS of *m*/*z* 745.8 $[M + CH_3$-$COOH - H]^-$. These results suggested that this compound was the SDG (**1**) diastereomer of unknown configuration, previously reported.[24] Following large-scale extraction and its isolation in milligram quantities (see Experimental Section), peak 1b was subjected to ^{1}H and ^{13}C NMR spectroscopic and chiral HPLC analyses.

The ^{13}C NMR spectrum of peak 1b (Table 1) was essentially identical to SDG (**1**),[38] displaying only 16 distinguishable carbon resonances (because of the plane of symmetry in the molecule), including six aromatic and 10 aliphatic signals, with all protonated carbons assigned on the basis of HMQC analyses. Its ^{1}H NMR spectrum was also essentially identical to that of SDG (**1**), except for methylene group resonances at the C-9 and C-9′ positions, which appeared as two double doublets at δ 3.92 and 3.58 (see Table 1), in contrast to δ 4.09 and 3.47 in SDG (**1**).[38] Its HMBC spectrum, after analysis of long-range correlations between H-9(a,b)/C-1a and H-1a/C-9, indicated that the glucose moieties were attached at the C-9 and C-9′ positions of secoisolariciresinol (**14**). Finally, β-D-glucuronidase treatment gave secoisolariciresinol (**14**), whose HPLC chiral analysis established it to be the (−)-enantiomer (**14a**) (Figure 4C). Thus, component 1b was compound **4**, the diastereomer of secoisolariciresinol diglucoside (**1**), presumably originating from (−)-secoisolariciresinol (**14a**). Interestingly, ratios of both diastereomers **4** and **1** are approximately 1:99, suggesting that two distinct biochemical pathways to both (+)- and (−)-enantiomers of secoisolariciresinol (diglucoside) are operative.

Peaks 2 and 3 (Figure 5A,B) were identified as follows: First, both radiolabeled components were individually isolated from stage 4 developing seeds and converted into SDG (**1**) after alkali hydrolysis, indicating that they were SDG (**1**) ester derivatives (data not shown).

Subsequent larger scale extraction, following metabolism of unlabeled L-Phe (**15**), gave peak 3 as a colorless

powder with a UV spectrum similar to that of SDG (**1**). Its high-resolution MALDI-TOF-MS had an *m/z* 997.375 [M + Na]$^+$ corresponding to $C_{44}H_{62}O_{24}Na$. The ^{13}C NMR spectrum (Table 1) was very similar to SDG (**1**) and revealed 22 resonances, including six aromatic, two carboxylic, and 14 aliphatic signals, i.e., indicating a plane of symmetry in the molecule and thus a total of 44 carbons. Relative to SDG **1**, six additional resonances (i.e., 12 carbons) were observed at δ 173.3 and 170.3 (two carboxylic acid and two ester functionalities), 45.9 and 45.8 (four methylene groups), 68.9 (two quaternary carbons), and 27.6 (2 × CH_3 groups): these assignments were verified further by analysis of the corresponding DEPT and HMQC spectra. Analysis of the ^1H NMR spectrum (Table 1) also revealed a SDG-like spectrum except for additional signals corresponding to two methyl groups at δ 3.67 and two sets of AB systems ($^1H-^1H$ COSY spectrum) for four methylene groups at δ 2.56, 2.47 (each 2H, J = 14.2 Hz) and δ 2.43, 2.39 (each 2H, J = 14.4 Hz), respectively. In the HMBC spectrum, long-range correlations were observed between H-2b/C-1b, 3b, 4b, 6b, H-4b/C-2b, 3b, 5b, 6b, H-6b/C-2b, 3b, 4b. Together, these data suggested that the additional structural features were from addition of two hydroxymethyl glutaryl groups (HMG) (**21**) to the SDG (**1**) core. Additionally, long-range correlations between H-1a/C-9 and H-6a/C-1b revealed that the glucose moieties were attached to the C-9 and C-9′ positions of secoisolariciresinol (**14b**), whereas the proposed HMG (**21**) groups were linked to the C-6a and C-6a′ positions. The signals of the two protons on C-6a (and C-6a′) were also shifted downfield at δ 3.97 (1H, dd, J = 11.4, 7.5 Hz) and 4.29 (1H, br d, J = 10.4 Hz), respectively, this again being in accordance with the two HMG (**21**) groups being ester-linked to the C-6a (and C-6a′) positions.

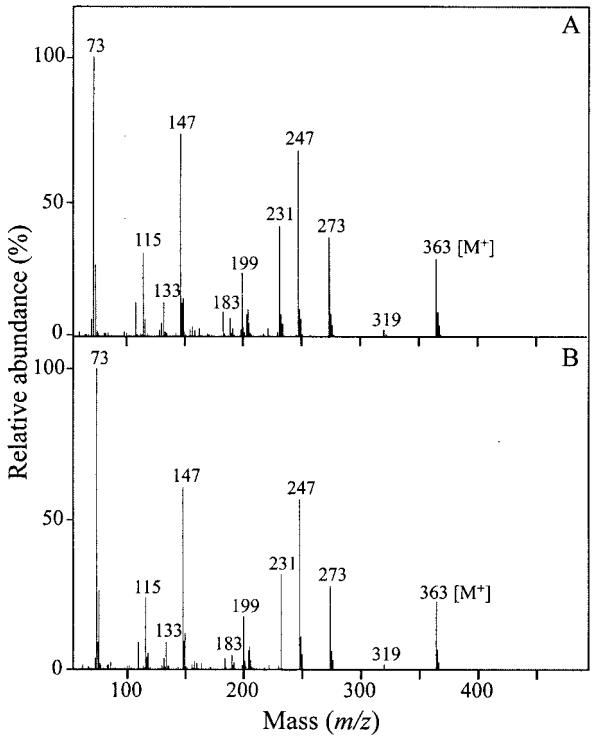
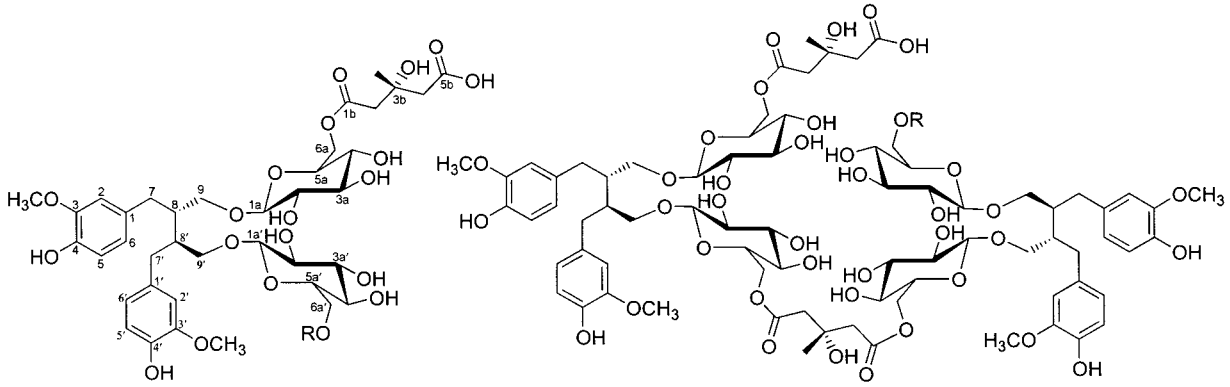


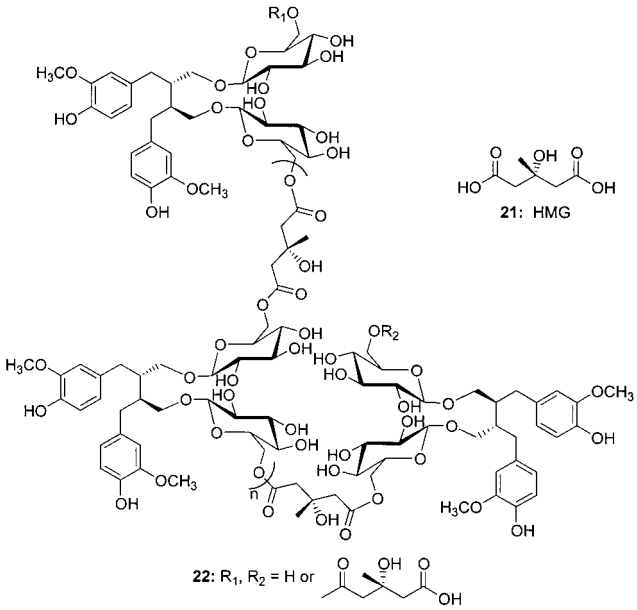
Figure 6. GC/MS analyses of trimethylsilyl-derivatized HMG (**21**). (A) An authentic standard, and (B) that isolated after base hydrolysis of **18**.

Peak 2 (Figure 5) was isolated as above as a colorless powder and also had a UV absorption similar to SDG (**1**). A molecular formula of $C_{38}H_{54}O_{20}$ was deduced from its high-resolution MALDI-TOF-MS *m/z* 853.330 [M + Na]$^+$ (calcd for $C_{38}H_{54}O_{20}Na$, 853.311). Comparing its molecular formula with that of **18**, it had only one HMG group. Its ^1H NMR spectrum had two sets of signals for the CH_2O groups on C-6a and C-6a′: δ 4.41 (1H, dd, J = 11.8, 2.1 Hz) and 4.22 (1H, dd, J = 11.8, 5.7 Hz), and at δ 3.85 (1H, dd, J = 11.8, 2.1 Hz) and 3.69 (1H, dd, J = 11.8, 5.5 Hz), respectively, indicating that only one HMG group was substituted at the C-6a position. Thus, this compound was 6a-HMG−SDG (**17**). It was also noted that the radiochemical incorporation of L-[U-^{14}C]-phenylalanine into 6′-HMG SDG (**17**) reached ~1.7% at stage 3 (Figure 3A) and again steadily declined upon maturation of the flax seed.



Final verification of the HMG structure was achieved by alkaline hydrolysis to give SDG (**1**) and the corresponding acid (**21**). Derivatization (trimethylsilylation) of the latter and its subsequent GC/MS analysis gave a major peak, having a retention time and mass spectral fragmentation pattern (M$^+$ *m/z* 363) identical to that of authentic HMG (**21**) (see Figure 6A,B). Thus, peak 3 was 6a,6a′-di-HMG−SDG (**18**). It was also noted that the incorporation of L-[U-^{14}C]-phenylalanine (**15**) into 6a,6a′-di-HMG−SDG (**18**) was ~3.1% at stage 3 (Figure 4A), but then began to steadily decline as seed maturation and "polymer" formation proceeded.

In addition to compounds **17** and **18**, two other substances were individually isolated from the putative "polymer" fraction "**X**" (see Figure 5A, peaks 4 and 5) from stage 4 seeds. Both had UV spectra similar to that of SDG (**1**), and saponification at room temperature of each again afforded SDG (**1**) (data not shown). MALDI-TOF-MS analyses (see Supporting Information) revealed that both were SDG HMG dimers, with peak 4 having two HMG (**19**)-moieties (*m*/*z* 1666.57 $[M + Na]^+$) and peak 5 containing three HMG (**20**) groups (*m*/*z* 1810.89 $[M + Na]^+$). Since only HMG (**21**) and no other acids could be detected by GC/MS after base hydrolysis, it is proposed that peaks 4 and 5 correspond to the ester-linked derivatives **19** and **20**, respectively, i.e., through ester linkages between **18** and SDG (**1**), and **18** and **17**, respectively. In this way, the SDG-containing "polymers" appear to consist of mixtures of various dimers, trimers, tetramers, and so forth, thereby constituting an ester-linked SDG−HMG-based family of compounds. No other SDG (**1**) esters have yet been detected with HMG (**21**) groups at positions other than the C-6 position of glucose. Hence, it is proposed that both **17** and **18** are the two main building blocks, and a tentative example for the SDG−HMG ester-linked "polymer(s)" **22** is shown. Interestingly, the overall percent radiochemical incorporation into the "polymer(s)" was highest at stage 5 (Figure 3A) with ~7.2% (percentage based on the total amount of L-[U-^{14}C]-phenylalanine administered).



Note, however, that in addition to compounds **17** and **18**, alternative substitutions/linkage patterns may also exist, e.g., HMG linked to positions on glucose other than C-6. Additionally, the glucosides of *p*-coumaric acid (**8b**) and ferulic acid (**10b**) may also be involved in formation of SDG ester-linked "polymer(s)", since like SDG (**1**) they are also liberated during saponification of the aqueous EtOH solubles. Conversely, the previous report of methyl 4-*β*-D-glucopyranosyl-3-methoxycinnamate (**11**),[28] the methyl ester of **10b**, is presumed to be an artifact, resulting from the methanol extraction isolation procedure utilized.

The only report of *β*-hydroxy-*β*-methylglutaric acid (**21**) from flax seed was by Klosterman and Smith in 1954, following isolation after treatment with sodium methoxide.[39] With there being no subsequent reports on HMG in flax seed, it is evident that formation of the SDG−HMG building blocks **17** and **18** appear to have gone unnoticed for almost 50 years. Indeed, the joint bioavailability of SDG (**1**) and HMG (**21**) in flax seed may explain why in some studies seeds are more efficacious than SDG (**1**) alone. HMG elicits significant hypoglycemic responses and is used as an HMG CoA lyase competitive inhibitor to treat *Diabetes mellitus* and hypocholesterolemic atherosclerosis.[40−44] Furthermore, the properties of flax seed are presumably also enhanced by the presence of alkali releasable *p*-coumaric acid (**8b**) and ferulic acid (**10b**) glucosides, both of which exhibit antioxidant and some breast and colon cancer chemopreventive properties.[45,46]

Finally, in attempting to explain the observation made on metabolic buildup (of radiochemical intermediates) during flax seed development, it needs to be recognized that the tissue is also very heterogeneous. That is, a flax seed contains five distinct cell layers in the seed coat alone, of which the inner walls of the mucilaginous epidermal cells are considered to be suberized, and the secondary walls of the fiber cells lignified.[47] Thus, caution must be exercised in interpreting the results of metabolism of various phenylpropanoid intermediates since they can be incorporated into the suberized or lignified layers, as well as the aqueous EtOH-soluble SDG−HMG ester-linked "polymer(s)".

Nevertheless, it is tempting to speculate that similar pathways to lignan biosynthesis in *F. intermedia* are occurring in *L. usitatissimum*, although the data suggest that the primary pathway involves a dirigent mediated coupling of *E*-coniferyl alcohol (**12**) to afford (−)-pinoresinol (**5b**). Indeed, protein preparations from *L. usitatissimum* seeds can convert **12** stereoselectively into **5b** (data not shown). This can then be subsequently converted enantiospecifically to first, (−)-lariciresinol (**13b**), and second, (+)-secoisolariciresinol (**14b**), followed by glucosylation by an UDPG:glucosyltransferase to yield SDG (**1**) (Figure 7). Indeed, an enantiospecific reductase using (−)-pinoresinol (**5b**) as substrate has been cloned and the recombinant protein expressed in functional form (data not shown). Interestingly, on the basis of the presence of the SDG diastereomer (**4**), there also appears to be another pathway to SDG, with the minor route being that to (−)-secoisolariciresinol (**14a**). In support of this contention, the dirigent protein involved in (+)-pinoresinol (**5a**) formation has also been obtained (data not shown). On the other hand, we also examined whether isoconiferin (**16b**) might serve as a precursor to SDG, but no evidence for this was obtained.

On the basis of the radiochemical data, the resulting SDG (**1**) and **4** so formed undergo mono- and diesterifications of HMG CoA to yield **17** and **18**, respectively, these then subsequently being metabolized into formation of the SDG−HMG ester-linked "polymer(s)" at later stages of seed maturity. This is reflected in Figure 3A, where overall incorporation levels into SDG (**1**) and its HMG derivatives **17** and **18** all decrease after stage 3, while the percent radiochemical incorporation into the "polymer" **X** increases up to stage 5. While the physiological functions of these SDG-containing derivatives are as yet unknown in flax seed, they may function as natural antioxidants for protection of the large volume of oils (30% α-linoleic acid)[47] present in the seed.

Precedence for natural product HMG (**21**) derivatives has been observed previously in the cytotoxic triterpenes isolated from the Basidiomycetes *Hebeloma crustuliniforme*.[49] Additionally, analyses of the flax seed SDG−HMG ester-linked "polymer(s)" seem to have some parallels with the ester-linked hydrolyzable tannins, namely, the gallotannins and the ellagitannins. In these cases, however, they are most often linked to *β*-D-glucose through *meta*-

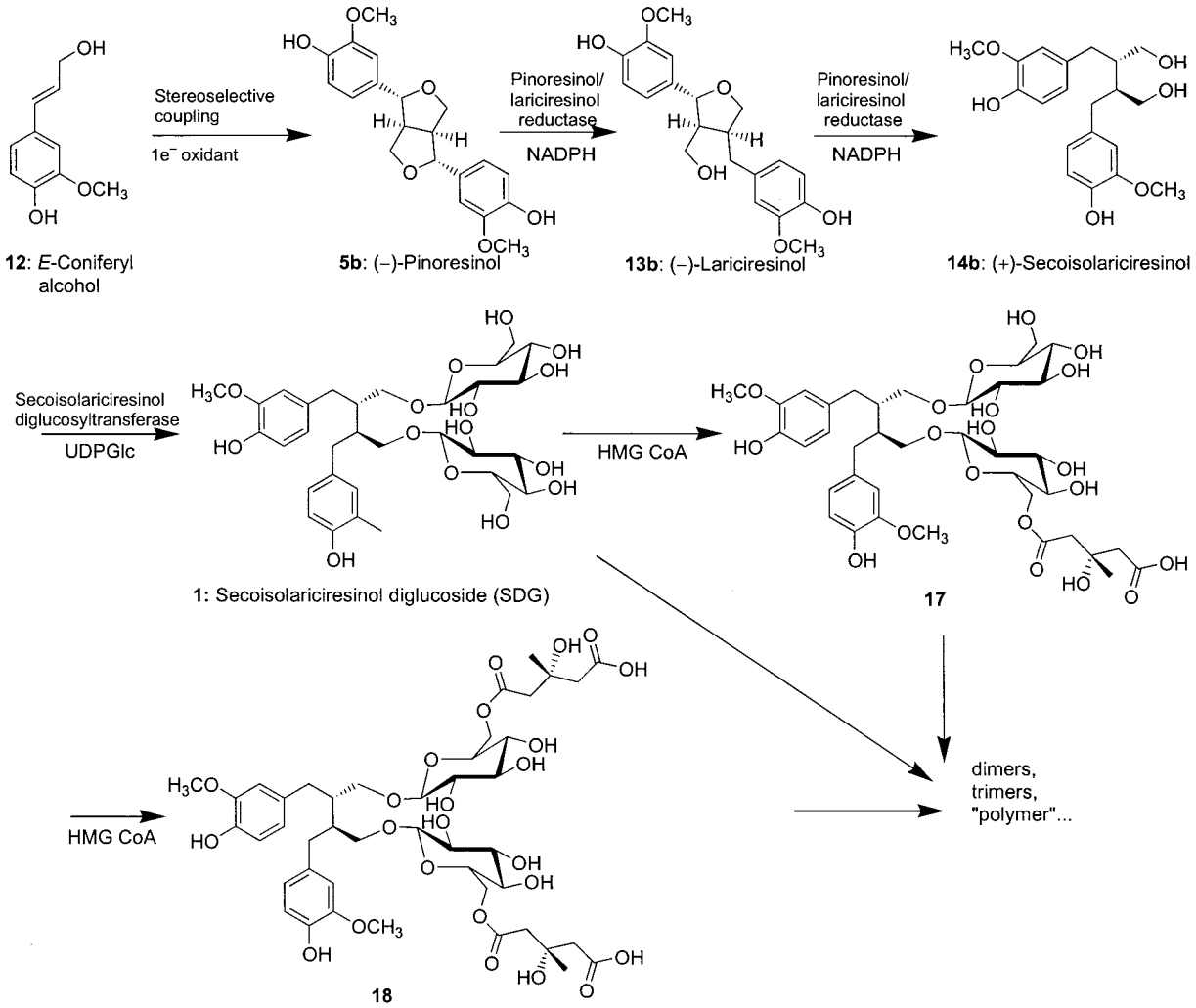


Figure 7. Proposed major biosynthetic pathway to the SDG−HMG ester-linked "biopolymer(s)" in developing flax *L. usitatissimum* seed; note that ~1% of SDG is the diastereomer (**4**).

depside bonds. Their biosynthesis, however, has been documented mainly in leaf tissue.[50−54]

In any event, results from this study will now facilitate a biochemical approach to isolate and characterize further the proteins/enzymes involved in biosynthesis of the SDG−HMG ester-linked "polymer(s)" in flax seed.

Experimental Section

General Experimental Procedures. All solvents and chemicals were either reagent or HPLC grade. β-Glucuronidase (*Helix pomatia*, 600 000 U/g) and 3-hydroxy-3-methylglutaric acid were purchased from Sigma. Radiolabeled L-[U-^{14}C]-phenylalanine (**15**) (16.7 GBq/mM) was obtained from DuPont NEN, and L-[3-^{13}C]-phenylalanine (**15**) (>99 atom % ^{13}C) was from Cambridge Isotope Laboratories. Costar EIA/RIA 96 well polystyrene flat bottom plates were purchased from Corning. A hand testing screen for seed sorting was obtained from Seedburo Equipment Co., Chicago, IL, with seed exclusion sizes 12, 4.76 mm; 14, 5.56 mm; 16, 6.35 mm; and 18, 7.14 mm. Tri-Sil and Silyl-8 silylation reagents were obtained from Pierce.

^{1}H and ^{13}C NMR spectra were obtained on Varian Mercury 300B and Brüker AMX 500 spectrometers, and chemical shifts are given in δ ppm relative to TMS. HPLC electrospray ionization mass spectrometric analyses (LC-ESI-MS) were recorded on a Waters 2690 Alliance/Finnigan MAT LCQ, whereas MALDI-TOF spectra were obtained on a Voyager 2025-DE RP system. The ESI-LC-MS operating parameters were as follows: HPLC system 2 (see below); electrospray capillary temperature at 200 °C, capillary voltage at 4.0 V, sheath gas flow pressure (N_2) set at 60 psi, auxiliary gas flow pressure (He) at 15 psi, spray voltage at 3.5 kV, spray current at 18.41 μA, with the tube lens offset set at 25 V. All spectra were obtained in the negative-ion mode, over an *m/z* range of 150−2000, at one scan every 2 s, and collected in the form of continuum data.

GC/MS analyses employed a Hewlett-Packard 5973 mass selective detector, a 6890 series GC system, and a 7683 series injector equipped with an HP-5 column (5% phenyl methyl siloxane, 30 m × 2.5 μm inner diameter). GC/MS method: GC temperature program initiated at 120 °C, holding for 5 min, rising to 280 °C at a rate of 10 °C/min, and held at 280 °C after 30 min.

HPLC analyses employed a Waters 2690 Alliance system. Reversed-phase HPLC analyses were carried out using either an analytical Nova-Pak C_{18} column (150 × 3.9 mm inner diameter, Waters) [HPLC system 1: flow rate of 1 mL/min, solvent A = H_2O/3% acetic acid and solvent B = CH_3CN; linear gradient A/B from 95:5 to 75:25 between 0 and 40 min, and then to 5:95 in 5 min; HPLC system 2: flow rate of 0.3 mL/min, linear gradient A/B from 95:5 to 75:25 between 0 and 40 min, then to 60:40 in 50 min, and finally to 5:95 in 10 min] or a preparative Nova-Pak HR C_{18} column (300 × 7.8 mm inner diameter, Waters) [HPLC system 3: flow rate of 8 mL/min; linear gradient of solvents A and B as above; linear gradient A/B from 95:5 to 60:40 between 0 and 50 min, then to 5:95 in 50 min], with detection at 280 nm in each case.

Chiral HPLC separations employed a Chiracel OD column (240 × 4.6 mm inner diameter; Chiral Technologies, Inc.)

eluted with hexanes/ethanol (70:30, v/v) at a flow rate of 0.5 mL/min (HPLC system 4).

Radiochemical analyses were conducted in ScintiVerse II (Fischer Scientific) and measured using a Packard Tricarb 2000 CA liquid scintillation counter.

E-[9-^{3}H]-Coniferyl alcohol (1.29 GBq/mol) (**12**)[36] and *E*-[2-^{14}C]-coniferin (11.1kBq/mol) (**16a**) were prepared as previously described.

Plant Material. Flax (*L. usitatissimum*, L.) plants (var. Niche) were maintained at the Washington State University greenhouse facilities.

Correlation of Alkaline Hydrolytic Releasable SDG (1) Levels with Flax Seed Developmental Stage. Developing flax seed capsules (each containing 10 seeds) were collected from 1-month-old greenhouse-grown plants and segregated into five developmental stages using four hand-testing screens. Stage 5 seed development contained the largest and oldest capsules, which were excluded from size 18 screens, whereas stage 1 represented newly developed seeds whose capsules freely passed through size 12 screens. Stages 2, 3, and 4 represented capsules excluded from screen sizes 12, 14, and 16, respectively. Capsules from each stage were individually freeze-dried for 24 h, after which their seeds (~100 mg) were extracted with tweezers and pulverized in a planetary micro mill (Pulverisette Fritsch) using 45 mL bowls (30 min, high speed). Mature seeds (100 mg, designated as stage 6) were also pulverized in a planetary micro mill, followed by a hexanes wash (2 mL, 1 h) with subsequent removal of the hexanes via filtration. The fine powders from each stage were then divided in half, and all were individually extracted with EtOH/H_2O (40:60, v/v, 2 mL) for 4 h at room temperature with orbital shaking (250 rpm). Each residue was then removed by filtration to afford a filtrate containing the soluble SDG ester-linked polymeric extract. The two samples for each stage were placed at room temperature in an orbital shaker (250 rpm), with one sample for each developmental stage treated with NaOH (10 M, 0.5 mL) for alkaline hydrolysis. After 2 h, all samples were extracted successively with hexanes, EtOAc, and $CHCl_3$ (1 mL each), and the remaining aqueous solubles were frozen (−80 °C) and freeze-dried for 24 h. The remaining residue for each sample was resuspended in aqueous MeOH (30%, v/v) and subjected to HPLC analysis (HPLC system 1). To quantify secoisolariciresinol diglucoside (**1**) levels, extrapolation of the SDG (**1**) UV peak area of each sample was carried out using the standard curve slope $y = 2.4073e{-}09x$, $R = 0.99966$.

Administration of L-[U-^{14}C]-Phenylalanine (15) to Flax Capsules at Different Developmental Stages. Secondary inflorescence stems of 1-month-old flax plants were harvested and directly placed in deionized water. Capsules with a remaining peduncle (~1 cm) were pruned from stems and immediately size sorted as described above. Four fresh capsules of each of the five developmental stages were individually placed in wells of a Costar EIA plate containing L-[U-^{14}C]-phenylalanine (**15**) (148 kBq, 1 mM in 100 μL). After 24 h uptake and metabolism of the precursor at room temperature under a Tensor fluorescent lamp (placed at an 18 cm distance from the capsules), capsules from each developmental stage were frozen (−80 °C) and freeze-dried for 48 h. Seeds (40) from each stage were then dissected with tweezers from dried capsules and homogenized in EtOH/H_2O (40:60, v/v, 500 μL) with a 1.5 mL plastic pellet pestle (Kontés) followed by shaking (2 h, room temperature). Each extract was centrifuged (14000*g*, 2 min) and its EtOH/H_2O solubles were removed (extract A1). To each remaining pellet was added EtOAc (500 μL), in which an organic extraction was carried out for 2 h with shaking. After centrifugation (14000*g*, 2 min), the EtOAc organic solubles were removed, affording extract B. Extract A1 from each stage was then back-extracted twice with 1 mL each of hexanes, EtOAc, and $CHCl_3$, and the organic solubles for each sample were combined with the corresponding extract B and evaporated to dryness in vacuo to afford extract C. An aliquot (100 μL) of the remaining aqueous solubles (extract A2) for each stage was then subjected to alkaline hydrolysis (10 M NaOH, 3 μL) for 2 h at room temperature, affording extract A3. The resulting dried extract C from each developmental stage, as well as the remaining extract A2 and base-hydrolyzed extract A3 samples, were reconstituted in MeOH/H_2O (30:70 v/v, 100 μL) and subjected to reversed-phase HPLC analysis (HPLC system 2). Fractions (300 μL) were collected and supplemented with ScintiVerse II for radiochemical analysis.

Chiral HPLC Analysis of [^{14}C]-SDG (1). [^{14}C]-SDG (**1**) recovered after alkaline hydrolysis (extract A3 above) of developmental stage 5 flax seed (purification based on HPLC system 2) was freeze-dried, resuspended in Bis-Tris buffer (20 mM, pH 5, 100 μL, Sigma), and subjected to β-glucuronidase (1000 U) hydrolysis for 12 h (37 °C). The reaction was stopped with addition of EtOAc (500 μL) and centrifuged (14000*g*, 2 min), and the EtOAc layer was removed and evaporated to dryness. The [^{14}C]-secoisolariciresinol (**14**) was resuspended in EtOH (50 μL) and analyzed by chiral HPLC (HPLC system 3). Fractions (500 μL) were collected and supplemented with ScintiVerse II for radiochemical analysis.

Uptake and Metabolism of Natural Abundance Phenylalanine (15) in Developing Flax Seed. Four size-sorted capsules for each stage of development were placed in wells of Costar EIA plates as before, but now containing natural abundance L-phenylalanine (**15**) (1 mM, 100 μL to each). Extraction procedures to give extracts A2, A3, and C were performed as described above, with each extract then subjected to LC-ESI-MS analyses to identify the eluted phenylpropanoid and lignan metabolites.

Administration of L-[3-^{13}C]-Phenylalanine (15) to Developmental Stage 4 Flax Capsules. L-[3-^{13}C]-Phenylalanine (**15**) (1 mM, 99 atom % ^{13}C) was administered to 288 flax capsules (100 μL/capsule) at stage 4 development using three Costar EIA 96 well plates. Following uptake and metabolism for 24 h, the capsules were removed and freeze-dried. The seeds (~5 g) were then excised using tweezers and ground to a powder in a planetary micro mill (15 min), with the resulting powder extracted with EtOH/H_2O (40:60, v/v, 50 mL) for 2 h at room temperature. After filtration, the aqueous EtOH solubles were successively back-extracted with $CHCl_3$, EtOAc, and hexanes (50 mL, twice). The organic solubles were discarded, and the remaining aqueous layer was subjected to alkaline hydrolysis (240 μL, 10 M NaOH) for 2 h with shaking and then freeze-dried. The residue was redissolved in MeOH/H_2O (30:70 v/v, 100 μL) and subjected to HPLC analysis (HPLC system 2), with the fraction corresponding to [7,7′-^{13}C]-SDG **1** being hand collected, freeze-dried, reconstituted in CD_3OD (1 mL, Sigma), and subjected to ^{13}C NMR spectroscopic analysis.

Isolation and Structural Elucidation of SDG (1), SDG Diastereomer (4), *p*-Coumaric Acid Glucoside (8b), and Ferulic Acid Glucoside (10b). Mature flax seed meal (1.2 kg dry wt, previously defatted by cold pressing) was ground to a powder (Waring Blender), washed with hexanes (2 L) to remove residual oils, and extracted with EtOH/H_2O (40:60, 2 L) at room temperature for 24 h. The EtOH/H_2O solubles were concentrated (200 mL) in vacuo, with the resulting solubles then being subjected to alkaline hydrolysis (10 M NaOH, 20 mL) for 2 h with stirring. After acidification (10 M HCl) to pH 5, the extract was freeze-dried and the resulting residue (124 g) was extracted with MeOH (200 mL) for 20 min with stirring. The MeOH extract was decanted, concentrated (20 mL) in vacuo, and subjected to preparative HPLC analyses (HPLC system 4). Hand collection afforded the following phenolics: SDG (**1**) (7.9 g), *p*-coumaric acid glucoside (**8b**) (11.2 g), ferulic acid glucoside (**10b**) (1.8 g), and the SDG diastereomer (**4**) (63.0 mg).

SDG Diastereomer (4): colorless powder; UV λ_{max} 280 nm; ^{1}H and ^{13}C NMR, spectroscopic data, see Table 1; LC-ESI-MS *m*/*z* 745.8 $[M + CH_3COOH - H]^-$, HR MALDI-TOF-MS *m*/*z* 709.298 $[M + Na]^+$ (calcd for $C_{32}H_{46}O_{16}Na$, 709.268).

SDG (1) and SDG Analogue Isolation and Identification from Stage 4 Developing Flax Seed. Freeze-dried flax seeds (27.5 g dry wt) at stage 4 development were ground to a powder in a planetary micro mill (15 min) and extracted with EtOH/H_2O (40:60 v/v, 150 mL × 2) at room temperature for 24 h. After filtration, the aqueous EtOH solubles were extracted with hexanes (150 mL × 2) to remove the oils, and the remaining aqueous layer was freeze-dried. The latter was

then dissolved in MeOH/H_2O (30:70 v/v, 1 mL) and subjected to multiple preparative HPLC (HPLC system 4). In correspondence with the eluted radioactive components previously analyzed above (extract A2), the coincident compounds of interest eluting at the same elution volumes were individually hand-collected and freeze-dried to afford SDG (**1**) (2.3 mg); **17** (1.2 mg); **18** (4.2 mg); **19** (0.4 mg); and **20** (0.2 mg).

SDG (1): colorless powder; UV λ_{max} 280 nm; ^{1}H NMR (300 MHz, in CD_3OD) as described in Chimichi et al.;[38] LC-ESI-MS *m/z* 745.3 $[M + CH_3COOH - H]^-$.

6a-HMG SDG (17): colorless powder; UV λ_{max} 280 nm (log ϵ 3.59); ^{1}H NMR spectroscopic data, see Table 1; low-resolution (LR) MALDI-TOF-MS *m/z* 853.3 $[M + Na]^+$, HR-MALDI-TOF-MS *m/z* 853.330 $[M + Na]^+$ (calcd for $C_{38}H_{54}O_{20}Na$, 853.311); LC-ESI-MS, *m/z* 829.4 $[M - H]^-$.

6a,6a′-di-HMG−SDG (18): colorless powder; UV λ_{max} 280 nm (log ϵ 3.73); ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; LR-MALDI-TOF-MS *m/z* 997.1 $[M + Na]^+$, 973.4 $[M - H]^-$, LC-ESI-MS, *m/z* 973.8 $[M - H]^-$, HR-MALDI-TOF-MS *m/z* 997.375 $[M + Na]^+$ (calcd for $C_{44}H_{62}O_{24}Na$, 997.353).

SDG−HMG Dimer-1 (19): colorless powder; UV λ_{max} 280 nm; LR-MALDI-TOF-MS *m/z* 1666.57 $[M + Na]^+$.

SDG−HMG Dimer-2 (20): colorless powder; UV λ_{max} 280 nm; LR-MALDI-TOF-MS *m/z* 1810.89 $[M + Na]^+$.

GC/MS Identification of Acyclic Components Following Alkaline Hydrolysis of 18. To compound **18** (0.5 mg, 0.5 μM) dissolved in H_2O (100 μL) was added NaOH (10 M, 3 μL). After 2 h shaking at 25 °C, the reaction mixture was acidified to pH 5 (5 M HCl) and freeze-dried. The resulting residue was dissolved in pyridine (100 μL), to which was added bis(trimethylsilyl)trifluoroacetamide (100 μL). After 4 h at room temperature, an aliquot (1 μL) was subjected to GC/MS analysis.

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Supporting Information Available: Representative depiction of a L-[U-^{14}C]-phenylalanine (**15**) administration experiment to developing flax seed and MALDI-TOF analyses of dimers **19** and **20** isolated from flax seed at developmental stage 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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