Improved Method for the Synthesis of *trans*-Feruloyl-*β*-sitostanol

Anthony M. Condo, Jr.,[†] David C. Baker,*[,†] Robert A. Moreau,[‡] and Kevin B. Hicks[‡]

Department of Chemistry, University of Tennessee, Knoxville, Tennessee 37996-1600, and Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038

Phytosterols and phytostanols are known to lower low-density lipoprotein−cholesterol (LDL-C) levels in humans by up to 15%, and at least two products, Benecol and Take Control, are now on the market as naturally derived fatty acid esters of phytostanols (stanol esters) and phytosterols (sterol esters), respectively. A synthetic process was developed to synthesize gram quantities of *trans*-feruloyl-*β*-sitostanol from ferulic acid and *β*-sitostanol, with high purity and yields of ∼60%. The process involves (a) condensation of *trans*-4-*O*-acetylferulic acid with the appropriate phytostanol or phytostanol mixture in the presence of *N*,*N*-dicyclohexylcarbodiimide and 4-(dimethylamino)-pyridine, (b) separation of the *trans*-4-*O*-acetylferuloyl products by preparative liquid chromatography, (c) selective deacetylation of the feruloyl acetate, and (d) chromatographic purification of the feruloylated phytostanols. The process was successfully applied to synthesize stanol *trans*-feruloyl esters from "Vegetable Stanols", a mixture of ∼70:30 *β*-sitostanol and *β*-campestanol, in comparable purity and yield.

Keywords: *β-Sitostanol; trans-feruloyl-β-sitostanol; phytostanols; stanol esters*

INTRODUCTION

Two of the most popular cholesterol-lowering "functional" foods currently on the market are Benecol and Take Control, with the former containing synthetic fatty acid esters of phytostanols (stanol esters) derived from tall oil and the latter containing synthetic fatty acid esters of phytosterols (sterol esters) derived from soybean oil (*1*). Phytosterols and phytostanols are known to lower low-density lipoprotein−cholesterol (LDL-C) levels in humans by up to 15%, and the FDA has recently permitted a rare health claim for their use in low-fat diets (*2*). Phytosterols and phytostanols are thought to lower LDL-C by inhibiting the absorption of cholesterol from the intestine (*3*). Free phytosterols and phytostanols are poorly soluble in most foods, and this has led to problems with formulations and inconsistencies in efficacy. To solve this problem, they are usually esterified with fatty acids to make them soluble in margarines and other high-fat food products (*4*). In the intestine, fatty acid esters are hydrolyzed by digestive enzymes, producing the physiologically active free sterol or stanol (*4*). We at the USDA laboratories have recently discovered and patented a natural phytosterol-containing oil from corn fiber (corn fiber oil) that contains high levels of natural phytosterol and phytostanol fatty acid esters and a unique class of stanol esters, the stanol ferulate esters (also called ferulate phytostanol esters) (*5*, *6*). Although many clinical studies have been performed on fatty acid esters of stanols and sterols, no studies have examined the efficacy of stanol ferulate esters. Because these compounds contain a ferulic acid moiety, which is a powerful antioxidant, it is proposed that in addition to reducing LDL-C levels, they might also reduce the oxidation of LDL-C, further lowering the risk of coronary heart disease. To test this hypothesis, quantities of pure stanol ferulate esters are required. A method that uses 4-*O*-acetylferuloyl chloride as the acylating agent has been published for the synthesis of these types of esters (*7*, *8*). The present study was undertaken to develop an improved method for the synthesis of *β*-sitostanol ferulate (**5a**). Because "soy stanols" are a more readily available commercial product, containing ∼70% *β*-sitostanol (**3a**) and 30% *β*-campestanol (**3b**), the ferulate ester synthesis was also evaluated with this mixed substrate (**3a** + **3b**).

The published procedures (*7*, *8*) for the chemical synthesis of *trans*-feruloyl-*β*-sitostanol is limited by two steps: (1) the required preparation of the highly reactive *trans*-4-*O*-acetylferuloyl chloride, which is difficult to purify and handle, and (2) the deprotection step that uses sodium borohydride to remove the acetyl protective group on the *trans*-feruloylated product. Our goal in the present work was to develop a more workable synthesis of the compounds.

MATERIALS AND METHODS

General Procedures. All reactions were conducted under a nitrogen atmosphere unless otherwise indicated. Anhydrous solvents were dried prior to distillation as follows: dichloromethane, chloroform, and pyridine were refluxed over calcium hydride; THF was refluxed over sodium benzophenone ketyl; and methanol was refluxed with magnesium metal shavings. *trans*-Ferulic acid and acetic anhydride were purchased from Aldrich Chemical Co. and used without further purification. *β*-Sitostanol (**3a**) was purchased from Research Plus, Inc., and used without further purification. To maximize the yield of the coupled products **4a** and **4b**, the starting material was dried for 18 h to remove residual ethanol (78 °C, <1 Torr). "Vegetable Stanols" were obtained from AC Humko, Inc. (Memphis, TN) and used without further purification. Thin-layer chromatography (TLC) was carried out on E. Merck Silica Gel 60 aluminum-backed plates. Visualization was

* Corresponding author [telephone (865) 974-1066; fax (865) 974-1536; e-mail dcbaker@utk.edu].

[†] University of Tennessee.

[‡] U.S. Department of Agriculture.

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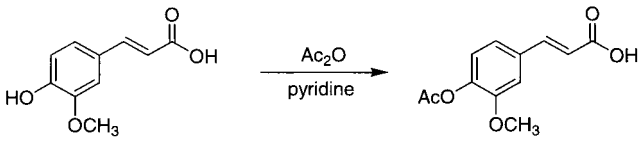
Scheme 1. Preparation of *trans*-4-*O*-Acetylferulic Acid



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accomplished by a combination of UV exposure (254 nm) and treatment with a phosphomolybdic acid dip. Solvents were evaporated at aspirator vacuum at 25 °C unless otherwise indicated. Preparative liquid chromatography (PLC) was carried out on a Waters Prep LC/System 500A with a flow rate of 250 mL/min of the indicated solvent system and with refractive index detection. Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were measured at the sodium D line at 20 °C using a Perkin-Elmer 241 polarimeter (1-dm cell). ^{1}H and ^{13}C NMR spectra were recorded at 300.087 and 75.464 MHz, respectively, on a Varian Mercury series spectrometer as solutions in $CDCl_3$ with an internal standard of TMS. ^{1}H chemical shifts are reported as δ (parts per million) downfield from the TMS standard. ^{13}C chemical shifts are reported as δ (parts per million) relative to $CDCl_3$ (77.00 ppm) as standard. Infrared spectra were obtained on a Bomem MB series spectrometer as KBr pellets. Elemental analyses were furnished by Atlantic Microlab, Inc., of Norcross, GA, and are within ±0.4% of the theoretical values. Electron-impact mass spectra (EIMS) were obtained on a VG-ZAB-EQ mass spectrometer (Manchester, U.K.) using an ionization potential of 70 eV.

HPLC Conditions. Ester products [β-sitostanol ferulate (**5a**) and β-campestanol ferulate (**5b**), with retention times of 35.4 and 33.9 min, respectively] were separated and quantified via a reversed-phase HPLC system consisting of a Hewlett-Packard (Avondale, PA) model 1100 pump and autosampler, with the effluent entering first a Hewlett-Packard model 1100 UV detector at 320 nm and then an Alltech-Varex model MKIII evaporative light-scattering detector (Deerfield, IL). The column (100 × 3 mm) was a Chromsep glass cartridge packed with LiChrosorb7RP18 (7 μm) by Varian-Chrompack (Bridgewater, NJ). The 60-min solvent gradient program (0.5 mL/min) was for a ternary solvent system of solvents A/B/C: at 0 min, 10:10:80; at 10 min, 10:10:80; at 40 min, 10:0:90; at 50 min, 10:0:90; at 52 min, 10:10:90; and at 60 min, = 10:10:90, A/B/C (v/v/v), where A = 2-PrOH, B = water, and C = MeOH. Normal-phase HPLC was used to separate various stanol esters from the free-OH stanols. Normal-phase HPLC was carried out using the same HPLC system and flow rate as above with a LiChrosorb Diol column (100 × 3 mm) and a 60-min solvent program consisting of solvents A/B: at 0 min, 100:0; at 8 min, 100:0; at 10 min, 75:25; at 40 min, 75:25; at 41 min, 100:0; and at 60 min, 100:0, where A = 1000:1 hexane/HOAc and B = 100:1 hexane/2-PrOH.

***trans*-4-*O*-Acetylferulic Acid (2).** *trans*-Ferulic acid (**1**, 25.0 g, 128.7 mmol) was dissolved in distilled pyridine (100 mL) in a 250-mL round-bottom flask (Scheme 1). Acetic anhydride (65 mL) was added, and the mixture was stirred overnight in the dark. The solution was diluted with toluene (50 mL) and evaporated to yield a yellow syrup. Subsequent addition and evaporation of toluene (3 × 50 mL) yielded an off-white solid that was dried for 18 h (78 °C, <1 Torr) prior to use. ^{1}H and ^{13}C NMR data were consistent with published literature values (*9*).

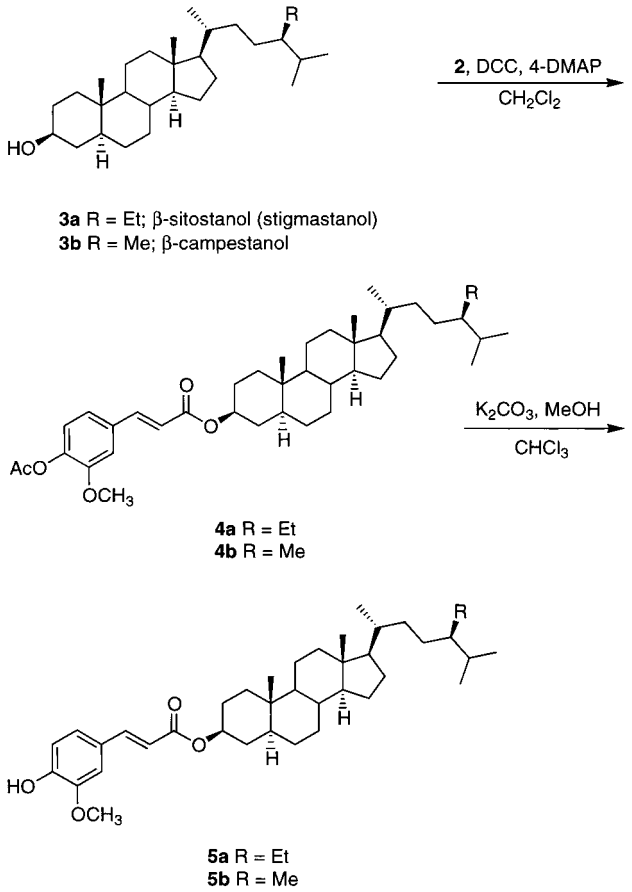
3-*O*-(*trans*-4-*O*-Acetylferuloyl)-β-sitostanol (4a). *trans*-4-*O*-Acetylferulic acid (**2**, 23.2 g; 98.2 mmol) and β-sitostanol (**3a**, 49.0 g; 108 mmol; 1.1 equiv) were dissolved in dry CH_2Cl_2 (750 mL). DCC (1.0 M in CH_2Cl_2, 108.0 mL; 108 mmol; 1.1 equiv) and DMAP (1.20 g; 9.82 mmol; 0.10 equiv) were added, and the mixture was stirred for 18 h. The DCU that formed was filtered off, and the solution was extracted with H_2O (2 × 150 mL), 10% HOAc (2 × 150 mL), and again with H_2O (2 × 150 mL). The organic extracts were combined and dried over anhydrous $MgSO_4$ and evaporated to yield a white solid. The solid was dissolved in a minimal amount of THF and chilled at 0 °C overnight to precipitate any residual DCU. The solution was filtered, the solvent was evaporated, and the residual solid was chromatographed using PLC with 2:4:1 chloroform/hexane/EtOAc as the eluent to yield **4a** as a white solid (29.0 g, 43%). Two additional products were recovered and identified as ethyl 4-*O*-acetylferulate (5.8 g, 22%) and unreacted β-sitostanol (**3a**, 11.9 g, 29%). ^{1}H and ^{13}C NMR as well as EIMS were used to characterize the minor products. Characterization data for the major product **4a** are as follows: TLC R_f (5:1 petroleum ether/EtOAc) 0.24; mp 162 °C [lit. (*7*) 156 °C]; $[\alpha]_D^{20}$ +20.6° (*c* 0.7, $CHCl_3$); IR (cm^{-1}) 2938, 2867, 1760, 1711, 1639, 1600, 1512, 1155, 1073; ^{1}H NMR δ 0.6-2.0 (β-sitostanol moiety, ~50H), 2.33 (s, 3H), 3.86 (s, 3H), 4.83 (m, 1H), 6.37 (d, 1H, J = 16.2 Hz), 7.10 (m, 3H), 7.62 (d, 1H, J = 15.9 Hz); ^{13}C NMR δ 12.06, 12.15, 12.34, 18.80, 19.09, 19.91, 20.76, 21.27, 23.08, 24.28, 26.04, 27.63, 28.33, 28.67, 29.12, 32.04, 33.92, 34.15, 35.51, 36.21, 36.80, 39.98, 42.60, 44.68, 45.79, 54.19, 55.86, 56.12, 56.39, 73.96, 110.95, 118.88, 121.08, 123.08, 133.36, 141.06, 143.41, 151.11, 166.15, 168.64; EIMS (70 eV), *m*/*z* 634.5 [M$^+$], 592.4 [M$^+$ − CH_3CO], 398.4 [M$^+$ − $C_{12}H_{12}O_5$]. Anal. Calcd for $C_{41}H_{62}O_5$: C, 77.56; H, 9.84. Found: C, 77.58; H, 9.88. ^{1}H and ^{13}C NMR data are consistent with published literature values (*7*).

In a similar experiment, using a sample (633 mg, 1.52 mmol) of **3a** that had been dried for 18 h (78 °C, <1 Torr), 489 mg (61%) of coupled product **4a** was obtained, and no ethyl 4-*O*-acetylferulate was detected.

3-*O*-(*trans*-4-Feruloyl)-β-sitostanol (5a). Compound **4a** (28.5 g, 44.9 mmol) was dissolved in a 2:1 $CHCl_3$-MeOH solvent mixture (450 mL). K_2CO_3 (1.24 g, 8.98 mmol, 0.20 equiv) was added, and the mixture was refluxed for 6 h. TLC analysis revealed the consumption of the starting material and the appearance of a new spot with a decreased R_f value. The reaction was quenched by the addition of satd aq NH_4Cl (100 mL). The layers were separated, and the organic layer was washed with H_2O (2 × 100 mL) and dried over $MgSO_4$. PLC using 2:4:1 $CHCl_3$/hexane/EtOAc as the eluent yielded a white solid (23.5 g, 88%) that was recrystallized ($CHCl_3$-MeOH) to give the final product **5a** as fine white crystals (19.0 g, 71%): TLC: R_f (5:1 petroleum ether/EtOAc) 0.14; HPLC (normal phase): t_R 26.2 min; mp 156−157 °C (lit. (*7*) 156 °C); $[\alpha]_D^{20}$ +22.3° (*c* 1.33, $CHCl_3$); IR (cm^{-1}): 3528, 3408, 2938, 2867, 1707, 1634, 1594, 1514, 1173, 1074; ^{1}H NMR: δ 0.6−2.0 (β-sitostanol moiety, ~50H), 3.91 (s, 3H), 4.83 (m, 1H), 5.96 (s, 1H), 6.2 (d, J = 15.9 Hz, 1H), 6.91 (d, J = 7.8 Hz, 1H), 7.05 (m, 2H), 7.60 (d, J = 16.2 Hz, 1H); ^{13}C NMR: δ 12.06, 12.14, 12.33, 18.79, 19.09, 19.91, 21.26, 23.08, 24.27, 26.04, 27.67, 28.33, 28.67, 29.12, 32.03, 33.91, 34.19, 35.50, 36.20, 36.81, 39.97, 42.58, 44.66, 45.78, 54.19, 55.86, 56.12, 56.38, 73.68, 109.07, 114.57, 115.99, 122.88, 126.93, 144.25, 146.54, 147.63, 166.63; EIMS (70 eV) *m*/*z*: 592.4 [M$^+$], 398.4 [M$^+$ − $C_{10}H_{10}O_4$]. Anal. Calcd for $C_{39}H_{60}O_4$: C, 79.01; H, 10.20. Found: C, 78.73; H, 10.21.

3-*O*-(*trans*-4-*O*-Acetylferuloyl)-β-sitostanol (4a) and 3-*O*-(*trans*-4-*O*-acetylferuloyl)-β-campestanol (4b). By the same procedure used for the preparation of **4a**, *trans*-4-*O*-acetylferulic acid (**2**, 8.86 g, 37.5 mmol) and Vegetable Stanols (69.0% **3a** and 30.4% **3b**, 17.2 g, 41.3 mmol, 1.1 equiv) were dissolved in dry CH_2Cl_2 (75 mL) and reacted with DCC (8.52 g; 41.3 mmol; 1.1 equiv) and DMAP (504 mg; 4.13 mmol; 0.10 equiv). Workup as for **4a** gave a residual solid that upon PLC (5:1 petroleum ether/EtOAc) gave **4a** and **4b** as a white solid (9.25 g, 39%) that was an inseparable mixture by chromatographic techniques. The characterization data for the mixture are as follows: TLC R_f (5:1 petroleum ether/EtOAc) 0.24; mp 160−161 °C; $[\alpha]_D^{20}$ +22.2° (*c* 0.18, $CHCl_3$); IR (cm^{-1}) 2946, 2862, 1764, 1710, 1639, 1596, 1514, 1155, 1078; ^{1}H NMR δ 0.6−2.0 (β-sitostanol/β-campestanol moieties, ~50H), 2.32 (s, 3H), 3.85 (s, 3H), 4.83 (m, 1H), 6.36 (d, J = 16.2 Hz, 1H), 7.10 (m, 3H), 7.61 (d, J = 15.9 Hz, 1H); ^{13}C NMR δ 12.05, 12.14, 12.33, 15.44*, 15.51*, 17.65*, 18.32*, 18.72*, 18.80*, 18.91*, 19.10,

Scheme 2. Preparation of 3-*O*-(*trans*-Feruloyl)-*β*-sitostanol and -*β*-campestanol



19.89, 20.28*, 20.59*, 20.70, 21.27, 23.10, 24.27, 26.09, 27.63, 28.32, 28.67, 29.16, 30.32*, 30.61*, 31.47*, 32.03, 32.44*, 33.71*, 33.93, 34.16, 35.51, 35.93*, 36.20, 36.81, 38.83*, 39.07*, 39.99, 42.60, 44.68, 45.81, 54.21, 55.84, 56.14, 56.19*, 56.40, 79.92, 110.99, 118.89, 121.04, 123.05, 133.35, 141.10, 143.37, 151.13, 166.10, 168.55; EIMS (70 eV), *m/z* (**4a**) 634.4 [M^+], 592.4 [$M^+ - CH_3CO$], 398.4 [$M^+ - C_{12}H_{12}O_5$], (**4b**) 620.4 [M^+], 578.4 [$M^+ - CH_3CO$], 384.4 [$M^+ - C_{12}H_{12}O_5$]. Anal. Calcd for 70% $C_{41}H_{62}O_5$ (**4a**) and 30% $C_{40}H_{60}O_5$ (**4b**): C, 77.51; H, 9.81. Found: C, 77.51; H, 9.94. Note: Resonances in the ^{13}C spectrum attributable to the minor component **4b** are denoted by an asterisk (*).

3-*O*-(*trans*-4-Feruloyl)-*β*-sitostanol (5a) and 3-*O*-(*trans*-4-Feruloyl)-*β*-campestanol (5b). Compounds **4a** and **4b** (9.20 g, 14.6 mmol) were deacylated [1:1 $CHCl_3$/MeOH (100 mL), K_2CO_3 (202 mg, 1.46 mmol)] according to the procedure used for **4a** to give the final products **5a** and **5b** (Scheme 2) as fine white crystals (7.30 g, 84%) after PLC (5:1 petroleum ether/EtOAc) and recrystallization ($CHCl_3$/MeOH). The characterization data for the mixture of isomers are as follows: TLC R_f (5:1 petroleum ether/ethyl acetate) 0.14; HPLC (reversed-phase) t_R 33.9 min (**5b**) and t_R 35.4 min (**5a**); mp 157 °C; $[\alpha]_D^{20}$ +19.8° (*c* 0.23, $CHCl_3$); IR (cm^{-1}) 3531, 3408, 2936, 2867, 1705, 1633, 1594, 1513, 1172, 1074; ^1H NMR δ 0.6−2.0 (*β*-sitostanol/*β*-campestanol moieties, ∼50H), 3.88 (s, 3H), 4.82 (m, 1H), 5.86 (s, 1H), 6.27 (d, J = 15.9 Hz, 1H), 6.91 (d, J = 7.8 Hz, 1H), 7.05 (m, 2H), 7.59 (d, J = 15.9 Hz, 1H); ^{13}C NMR δ 12.08, 12.18, 12.37, 15.47*, 15.54*, 17.67*, 18.34*, 18.74*, 18.82, 18.93*, 19.11, 19.92, 20.31*, 20.62*, 21.29, 23.12, 24.30, 26.11, 27.70, 28.35, 28.71, 29.18, 30.34*, 30.64*, 31.51*, 32.07, 32.48*, 33.74*, 33.96, 34.24, 35.55, 35.96*, 36.23, 36.85, 38.87*, 39.10*, 40.02, 42.63, 44.72, 45.85, 54.25, 55.92, 56.10*, 56.17, 56.22*, 56.43, 73.70, 109.10, 114.57, 116.10, 122.91, 127.01, 144.23, 146.56, 147.63, 166.63; EIMS (70 eV), *m/z* (**5a**) 592.4 [M^+], 398.4 [$M^+ - C_{10}H_{10}O_4$], (**5b**) 578.4 [M^+], 384.4 [$M^+ - C_{10}H_{10}O_4$]. Anal. Calcd for 70% $C_{39}H_{60}O_4$ (**4a**) and 30% $C_{38}H_{58}O_4$ (**4b**): C, 78.96; H, 10.17. Found: C, 78.98; H, 10.24. Note: Resonances in the ^{13}C spectrum attributable to the minor component **5b** are denoted by an (*).

RESULTS AND DISCUSSION

The process described herein proceeds directly from *trans*-4-*O*-acetylferulic acid (**2**), which is easily prepared by acetylation of ferulic acid (**1**) in acetic anhydride/pyridine, to give a product identical with that reported by Ebenezer (*9*). Thus, activation of **2** with *N*,*N*-dicyclohexylcarbodiimide (*10*) and condensation with *β*-sitostanol (**3a**) in the presence of 4-(dimethylamino)-pyridine proceeded smoothly to give the coupled product, *trans*-4-*O*-acetylferuloyl-*β*-sitostanol (**4a**). The coupled product was purified by crystallization and filtration of the byproduct, *N*,*N*-dicyclohexylurea, and the crude product was subjected to preparative liquid chromatography (normal phase, silica gel), which served to separate the unreacted starting material and another byproduct, ethyl *trans*-4-*O*-acetylferulate. The origin of the latter was traced to ethanol, which was present in the *β*-sitostanol starting material (33 mol % ethanol; Research Plus, Inc.). Removal of the ethanol by thorough drying of the *β*-sitostanol raised the yield from 43 to 61% of pure **4a** and eliminated the ethyl ester byproduct.

Removal of the acetate protecting group required a selective hydrolytic procedure that favored acetate hydrolysis over hydrolysis of the *trans*-ferulate ester linkage. The published procedure (*7*, *8*) used sodium borohydride, a hydride reducing agent, which is not a common reagent to accomplish ester reductions, owing to the resistance of nonactivated esters to the action of borohydride. We found that a simple transesterification process that makes use of potassium carbonate in refluxing chloroform/methanol was ideal for the task (*11*). Thus, refluxing the acetate-protected **4a** with potassium carbonate in a 2:1 mixture of chloroform/anhydrous methanol proceeded to give the product **5a** as an analytically pure material in 71% yield after recrystallization. The product was characterized by NMR spectroscopy, by mass spectrometry, and by elemental analysis. Normal-phase (silica gel) HPLC and TLC (silica gel) of the product showed a single substance.

The acylation process was adapted to the processing of a mixture of stanols, termed Vegetable Stanols (AC Humko, Inc.), that was made up of a 7:3 mixture of *β*-sitostanol (**3a**) and *β*-campestanol (**3b**) (reversed-phase HPLC analysis showed 69.0% **3a** and 30.4% **3b**). Soy oil phytosterols are typically comprised of about 51% *β*-sitosterol, 16% stigmasterol, and 22% *β*-campesterol (*12*), and upon hydrogenation to reduce sterols to stanols, the composition of vegetable (soy) stanols is about 67% *β*-sitostanol and 22% *β*-campestanol. Processing the mixture as for pure *β*-sitostanol was carried out to give a ∼7:3 mixture of the protected coupled products **4a** and **4b**, as well as the free-hydroxy *trans*-feruloyl esters **5a** and **5b,** in comparable yield. At no stage in the synthesis were the products separable by either crystallization or normal-phase chromatography (silica gel); an analytical-scale separation was achieved by reversed-phase HPLC using a ternary solvent gradient. The products, either **4a** and **4b** or **5a** and **5b**, could be discerned most readily from one another by inspection of their ^{13}C NMR spectra, which gave separate signals for a number of the resonances.

The process described herein offers a straightforward process to *trans*-feruloylated *β*-sitostanol, as well as to

a mixture of β-sitostanol and β-campestanol esters. The process should be amenable to modest-sized synthesis of related products. Compounds **5a** and **5b** are undergoing evaluation as cholesterol-lowering agents. Details of those studies will be reported elsewhere.

ABBREVIATIONS AND NOMENCLATURE USED

Nomenclature: β-Sitostanol (also known as stigmastanol) is (3β,5α)-stigmastan-3-ol [CAS Registry No. 83-45-4]; β-campestanol is (3β,5α,24R)-ergostan-3-ol [CAS Registry No. 474-60-2]; *trans*-ferulic acid is 4-hydroxy-3-methoxy-*trans*-cinnamic acid [CAS Registry No. 537-98-4]. (CAS Registry No. were provided by the author.)

Abbreviations: DCC, *N*,*N*-dicyclohexylcarbodiimide; DCU, *N*,*N*-dicyclohexylurea; DMAP, 4-(dimethylamino)-pyridine; LDL-C, low-density lipoprotein−cholesterol.

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