Synthesis of Haptens and Conjugates for ELISAs of Phytoestrogens. Development of the Immunological Tests

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Seven carboxylic acid haptens of isoflavonoids were synthesized, with the spacer arm on the oxygen atom at the C7 position for one series, with formononetin, daidzein, equol, biochanin A, and genistein, and at the C8 position for a second series, with only formononetin and daidzein. The different haptens were coupled to bovine serum albumin (BSA) and to swine thyroglobulin (Thyr). Polyclonal antibodies were generated against the BSA conjugates. Enzyme-linked immunosorbent assays (ELISAs) were developed based on competition between free phytoestrogens and the Thyr−hapten conjugates for specific antibodies. IC_{50} values of the standard curves ranged between 0.8 and 20 ng/mL that is, 0.3 and 9.2 pmol/well. The antibodies obtained should be useful for assays in vegetable matter as well as in biological fluids after a separation step. These ELISAs should be valuable also in the food industry to control phytoestrogen concentrations prior to and after processing.

Keywords: *Phytoestrogens; isoflavonoids; haptens; polyclonal antibodies; ELISA*

INTRODUCTION

Phytoestrogens are natural estrogens present in large amounts (up to 1−4 mg/g; Anderson and Wolf, 1995) in numerous vegetables, some of which enter animal and human food (Farnsworth et al., 1975a,b). They were demonstrated to be responsible for adverse effects on animal reproduction and development in mammalian species, such as sheep (Bennetts et al., 1946), and rats (Medlock et al., 1995; Levy et al., 1995). Indeed, in sheep grazing subterranean or red clover, fertility was found to decrease (Bennetts et al., 1946). A thorough examination of the endocrine reproductive cycle showed endocrine disruptions such as progesterone level impairments, lower rates of embryo implantation (Obst and Seamark, 1970), or reduced surge of the gonadotrophin hormone (Findlay et al., 1973; Hughes et al., 1991a,b). The animals generally exhibited persistent estrous and increased uterine weight (Braden et al., 1967).

In man, because of lower exposure, only beneficial effects have been documented so far. Most of the epidemiological studies compared Western and Asian consumers. They showed that vegetarian and Asian consumers, exposed to higher phytoestrogen concentrations, presented lower incidence of estrogen-dependent diseases such as breast or uterine cancer (Tham, 1998). More recently soy extracts were shown to decrease the incidence of menopausal women's hot flushes (Murkies et al., 1995). Animal studies also led to the conclusion that phytoestrogens can protect menopausal women against osteoporosis (Anderson and Garner, 1998). In vitro, binding to steroid binding protein (hSHBG and human αFeto-protein) was shown (Martin et al., 1978; Garreau et al., 1991). More recently, Kuiper et al. (1998) showed that genistein **4** (Figure 1) bound to the β form of the mammalian estradiol receptor. Lately, concern has grown about potential adverse effects of these compounds in man, considering the very high levels of phytoestrogens found in infant formulas of milk substitutes based on soy (Setchell et al., 1998; Knight et al., 1998). Indeed, the effect previously demonstrated in rats after in utero (Hilakivi-Clarke et al., 1998; Golden et al., 1998) or neonatal exposure (Medlock et al., 1995; Santti et al., 1998) were examined cautiously. This issue is still under investigation.

New tools for phytoestrogen assays were developed recently to measure animal and human exposure, which is likely to vary from one diet culture to another. Most of them were immunological tests. Radioimmunoassays (RIAs) were developed on formononetin **1** (Wang et al., 1994; Hampl et al., 1998), daidzein **2** (Lapèík et al., 1997), and **4** (Lapèík et al., 1998) with haptens to which a spacer arm was attached on the oxygen atom at the C7 or C4′ position. To develop an ELISA, we tried to reproduce the previously reported syntheses. However, instead of the required acids, complex mixtures were recovered. Moreover, in the previous syntheses of haptens **8** and **11**, the physical and spectroscopic data are not given. As a result of this lack of information and difficulty in reproducing the reactions, we decided to undertake a new process to synthesize the required haptens with the spacer arm on the oxygen atom at the C7 position and to synthesize new haptens with the spacer arm at the C8 position.

In this paper we report the synthesis of haptens **8**−**11** of isoflavones **1**, **2**, biochanin A **3**, **4**, and equol **5**. Because RIAs use radioisotopes, which are not convenient for control assays in the food industry, we also

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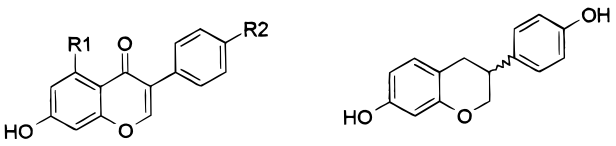

R1 = H R2 = OMe **1**
R1 = H R2 = OH **2**
R1 = OH R2 = OMe **3**
R1 = OH R2 = OH **4**

equol **5**

Figure 1. Structures of isoflavonoids.

present the synthesis of hapten−protein conjugates, the production of the corresponding antibodies, and the ELISA tests. The cross-reactivity of the antibodies will be also discussed.

EXPERIMENTAL PROCEDURES

Chemicals. Organic starting materials for the hapten synthesis were obtained from Sigma-Aldrich Chimie s.a.r.l. (St. Quentin Fallavier, France), Acros Organics France (Noisy, France), and Lancaster Synthesis Ltd. (Bishheim, France). Thin-layer chromatography (TLC) was performed on 0.25 mm precoated silica gel 60 F254 plastic sheets from SDS (Peypin, France). Column chromatography was carried out on silica gel (SDS 200, 60 Å CC, 35−70 μm, Peypin, France). Diethyl ether and dichloromethane were dried over 4 Å molecular sieves (SDS, Peypin France) and then distilled from calcium hydride. Ethanol was dried by treatment with magnesium followed by heating under reflux and distillation. Dimethylformamide and pyridine were distilled from barium oxide. Acetone was dried over 4 Å molecular sieves and then distilled under nitrogen. All reagents for biological work were purchased from Sigma-Aldrich Chimie s.a.r.l. except for the second antibody, that is, swine immunoglobulin anti-rabbit immunoglobulin coupled to horseradish peroxidase purchased from Dako (Trappes, France).

Instruments. Melting points were determined on a Kofler apparatus or Mettler FP 62 capillary melting point apparatus and are uncorrected. Infrared spectra were recorded on a Paragon 1000 Perkin-Elmer with polystyrene as standard; wavenumber (cm^{-1}) values are given. Proton magnetic resonance spectra (^1H NMR) were recorded on a Bruker AC 200 at 200 MHz or on a Bruker AC 250 spectrometer at 250 MHz. Carbon magnetic resonance spectra (^{13}C NMR) were recorded on a Bruker AC 250 spectrometer at 62.9 MHz or on a Bruker AC 200 at 50.3 MHz. Chemical shifts (δ) are given in parts per million (ppm) using tetramethylsilane as the internal standard at 0.00 ppm. Electron impact mass spectra were determined on a VG Auto Spec-Q apparatus at 70 eV; data are reported as *m*/*z* (relative intensity). The ELISA technique was carried out on microtitration plates (NUNC maxisorp) with 96 wells. The optical densities (OD) were read on a Dynex MRX II microtitration plate reader at 490 nm. Positive controls were wells receiving coating and specific antibody without any free phytoestrogen. The standard curves were expressed according to the formula $OD_{sample}/OD_{positivecontrol} = f[\log(\text{concentrations})]$.

Syntheses of Haptens. The syntheses of haptens **8**−**12** are described in Figure 2, and the syntheses of haptens **20** and **21** are shown in Figure 3. The structures of the compounds were verified by spectral methods.

*7-Ethoxycarbonylmethoxy-3-(4-methoxyphenyl)-4H-chromen-4-one (**6**).* A solution of **1** (3 g, 11.2 mmol), ethyl bromoacetate (3.8 g, 22.7 mmol), and powdered potassium carbonate (3 g, 22 mmol) in acetone (50 mL) was refluxed for 48 h (the reaction was monitored by TLC). After cooling, removal of the solvent under reduced pressure gave a crude product, which was filtered off and purified by washing with water (50 mL), NaOH (1 M, 30 mL), and water again (30 mL) to give the product **6** as a white powder (3.2 g, 81%): mp 145 °C; IR (KBr) 1747, 1637, 1605, 1252; δ_H (250 MHz, acetone-d_6) 1.27 (t, 3H, J = 7.1 Hz), 3.83 (s, 3H), 4.25 (q, 2H, J = 7.1 Hz), 4.94 (s, 2H), 6.98 (AA′BB′, 2H), 7.06 (d, 1H, J = 2.4 Hz), 7.12 (dd, 1H, J = 8.8 Hz, J = 2.4 Hz), 7.57 (AA′BB′, 2H), 8.13 (d, 1H, J = 8.8 Hz), 8.22 (s, 1H); δ_C (62.9 MHz, acetone-d_6) 14.7, 55.8, 62.2, 66.3, 102.5, 114.6, 115.7, 117.3, 125.4, 125.6, 128.5, 131.3, 153.9, 158.8, 160.8, 163.5, 168.9, 206.1.

*7-Ethoxycarbonylmethoxy-5-hydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one (**7**).* A solution of ***3*** (3 g, 10.5 mmol), ethyl bromoacetate (3 g, 17.9 mmol), and powdered potassium carbonate (2.9 g, 21 mmol) in acetone (50 mL) was refluxed for 48 h (the reaction was monitored by TLC). After cooling, removal of acetone under reduced pressure gave a crude product, which was filtered off and purified by washing with water (50 mL), NaOH (1 M, 30 mL), and water again (30 mL) to give the product **7** as a white powder (3.7 g, 95%): mp 158 °C; IR (KBr) 3469, 1745, 1656, 1610, 1249, 1182; δ_H (250 MHz, DMSO-d_6) 1.23 (t, 3H, J = 7.1 Hz), 3.79 (s, 3H), 4.20 (q, 2H, J = 7.1 Hz), 4.96 (s, 2H), 6.45 (d, 1H, J = 2.3 Hz), 6.71 (d, 1H, J = 2.3 Hz), 7.02 (AA′BB′, 2H), 7.52 (AA′BB′, 2H), 8.47 (s, 1H), 12.94 (s, 1H); δ_C (62.9 MHz, DMSO-d_6) 13.9, 55.1, 60.8, 65.0, 93.1, 98.4, 105.8, 113.7, 122.2, 122.7, 130.1, 154.8, 157.3, 159.2, 161.7, 163.5, 168.2, 206.7.

*7-Carboxymethoxy-3-(4-methoxyphenyl)-4H-chromen-4-one (**8**).* A solution of sodium carbonate (5% aqueous, 8 mL, excess) was added to a solution of ester **6** (1 g, 2.82 mmol) in acetone (50 mL). The mixture was refluxed for 48 h (the reaction was monitored by TLC). After cooling, removal of the solvent under reduced pressure, and acidification with HCl (1 M) gave a crude product, which was filtered off and purified by washing with water (50 mL) to give the product **8** as a white powder (800 mg, 87%): mp 214 °C; IR (KBr) 3448, 1736, 1624, 1598, 1249; δ_H (250 MHz, DMSO-d_6) 3.79 (s, 3H), 4.89 (s, 2H), 7.00 (AA′BB′, 2H), 7.09 (d, 1H, J = 2.3 Hz), 7.14 (dd, 1H, J = 8.8 Hz, J = 2.3 Hz), 7.52 (AA′BB′, 2H), 8.04 (s, 1H, J = 8.8 Hz), 8.42 (s, 1H); δ_C (50.3 MHz, DMSO-d_6) 55.2, 65.2, 101.6, 113.7, 114.9, 118.0, 123.4, 124.1, 127.1, 130.1, 153.6, 157.3, 159.1, 162.2, 169.6, 174.7.

*7-Carboxymethoxy-5-hydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one (**9**).* To a solution of ester **7** (850 mg, 2.29 mmol) in acetone (25 mL) was added sodium carbonate (5% aqueous, 10 mL, excess). The mixture was refluxed for 48 h (the reaction was monitored by TLC). After cooling, removal of acetone under reduced pressure and acidification with HCl (1 M) gave a crude product, which was filtered off and purified by washing with water (50 mL) to give the product **9** as a white powder (710 mg, 91%): mp > 250 °C; IR (KBr) 3426, 1766, 1736, 1648, 1613, 1249, 1179; δ_H (250 MHz, DMSO-d_6) 3.71 (s, 3H), 4.74 (s, 2H), 6.33 (d, 1H, J = 2.2 Hz), 6.57 (d, 1H, J = 2.3 Hz), 6.93 (AA′BB′, 2H), 7.43 (AA′BB′, 2H), 8.37 (s, 1H), 12.85 (s, 1H), 13.2 (s,1H); δ_C (62.9 MHz, DMSO-d_6) 55.1, 65.1, 93.1, 98.4, 105.6, 113.7, 122.1, 122.7, 130.1, 154.7, 157.3, 159.1, 161.6, 163.7, 169.4, 180.2.

*7-Carboxymethoxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**10**).* BBr_3 (1 M in dichloromethane, 54 mL, 53.68 mmol) was added dropwise with a syringe to a stirred solution of ester **6** (3.17 g, 8.95 mmol) in dichloromethane (30 mL). After complete addition, the mixture was stirred for 24 h at room temperature and then was poured onto icy water (500 mL). Filtration of the crude product gave a yellow residue, which was purified by chromatography on a column of silica gel eluted with 50% diethyl ether in dichloromethane and then with 20% ethanol in dichloromethane to give the product **10** as a yellow powder (2 g, 71%): mp > 250 °C; IR (KBr) 3265, 1741, 1716, 1616, 1599, 1252; δ_H (250 MHz, DMSO-d_6) 4.90 (s, 2H), 6.83 (AA′BB′, 2H), 7.11 (dd, 1H, J = 8.8 Hz, J = 2.4 Hz), 7.15 (d, 1H, J = 2.4 Hz), 7.41 (AA′BB′, 2H), 8.04 (d, 1H, J = 8.8 Hz), 8.38 (s, 1H), 9.57 (s, 1H), 12.4 (s, 1H); δ_C (50.3 MHz, DMSO-d_6) 65.0, 101.4, 114.7, 114.9, 126.9, 130.0, 153.2, 117.9, 122.3, 123.6, 157.1, 157.2, 162.0, 169.5, 174.6; MS, *m*/*z* (relative intensity) 312 (100 M^+), 254 (11.9 $M^+ - CH_2CO_2$), 195 (46.5), 137 (15.8), 118 (40.4). $C_{17}H_{12}O_6$ required: C, 65.39%; H, 3.87%; O, 30.74%. Found: C, 65.85%; H, 3.89%; O, 28.79%.

*7-Carboxymethoxy-5-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**11**).* BBr_3 (1 M in dichloromethane, 38 mL, 37.8 mmol) was added dropwise with a syringe to a stirred solution of ester **7** (2 g, 5.4 mmol) in dichloromethane (40 mL). After complete addition, the mixture was stirred for 24 h at room

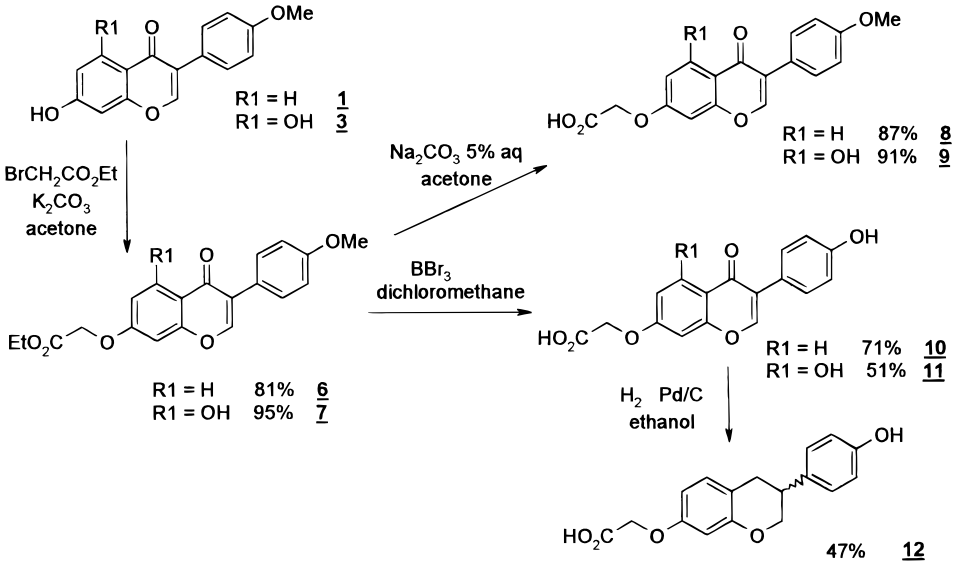

Figure 2. Syntheses of haptens **8**−**12**.

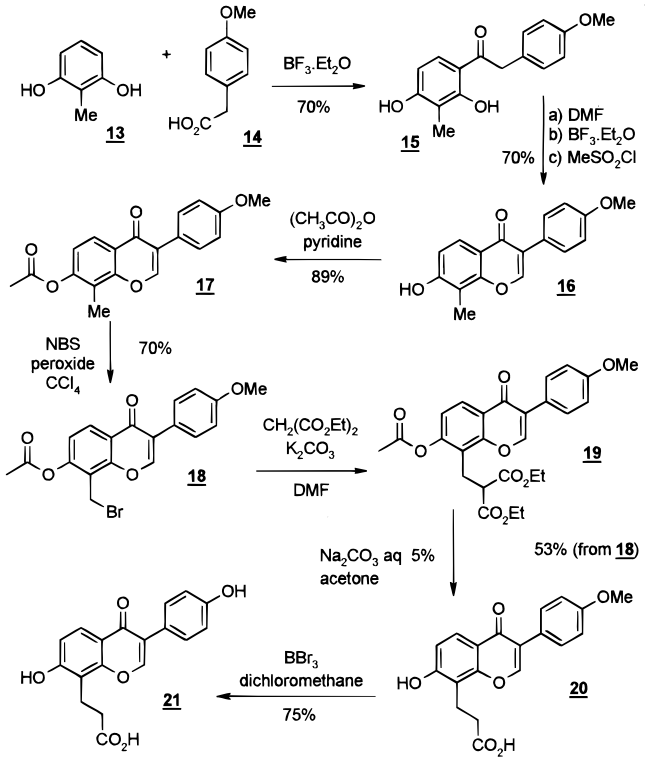

Figure 3. Syntheses of haptens **20** and **21**.

temperature and then was poured onto icy water (400 mL). Filtration of the crude product gave a yellow residue, which was purified by chromatography on a column of silica gel eluted with 50% diethyl ether in dichloromethane and then with 20% ethanol in dichloromethane to give the product **11** as a yellow powder (2 g, 71%): mp > 250 °C; IR (KBr) 3105, 1721, 1660, 1611, 1250, 1177; δ_H (250 MHz, DMSO-d_6) 4.70 (s, 2H), 6.37 (d, 1H, J = 1.8 Hz), 6.58 (d, 1H, J = 1.8 Hz), 6.82 (AA′BB′, 2H), 7.38 (AA′BB′, 2H), 8.38 (s, 1H), 9.43 (s, 1H), 12.92 (s, 1H), 13.1 (s, 1H); δ_C (62.9 MHz, DMSO-d_6) 66.6, 92.9, 98.5, 105.2, 115.0, 120.9, 122.4, 130.0, 157.3, 157.5, 161.5, 164.4, 169.7, 180.3; MS, *m*/*z* (relative intensity) 328 (100 M^+), 270 (7.3 $M^+ - CH_2CO_2$), 211 (21.2), 153 (8.6), 118 (21.7). $C_{17}H_{12}O_7$ required: C, 62.20%; H, 3.68%; O, 34.12%. Found: C, 62.57%; H, 3.78%; O, 33.47%.

*7-Carboxymethoxy-3,4-dihydro-3-(4-hydroxyphenyl)-2H-chromene (**12**).* A mixture of acid **10** (765 mg, 2.45 mmol) and palladium (10% on charcoal, 2 g, 5 mol %) was stirred overnight under hydrogen atmosphere. Because of highly inflammable properties, hydrogen was manipulated according to the classic safety instructions. The mixture was filtered and the filtrate evaporated to give a white solid residue, which was purified by chromatography on a column of silica gel, eluted with 40% methanol in dichloromethane, to give the product **12** as a white powder (350 mg, 47%): mp 217 °C; IR (KBr) 3333, 1742, 1613, 1240, 1164; δ_H (250 MHz, acetone-d_6) 2.78−2.94 (m, 2H), 3.03−3.11 (m, 1H), 3.91−4.00 (m, 1H), 4.16−4.24 (m, 1H), 4.66 (s, 2H), 6.37 (d, 1H, J = 2.6 Hz), 6.48 (dd, 1H, J = 8.4 Hz, J = 2.6 Hz), 6.82 (AA′BB′, 2H), 7.00 (d, 1H, J = 8.4 Hz), 7.16 (AA′BB′, 2H), 9.32 (s, 1H), 13.0 (s, 1H); δ_C (62.9 MHz, DMSO-d_6) 31.1, 36.9, 64.5, 70.3, 101.7, 107.1, 114.8, 115.2, 128.3, 130.1, 131.4, 154.5, 156.1, 157.0, 170.2.

*1-(2,4-Dihydroxy-3-methylphenyl)-2-(4-methoxyphenyl)ethanone (**15**).* $BF_3 \cdot Et_2O$ (200 mL, 1.61 mol) was added dropwise to a mixture of 2-methylresorcinol ***13*** (10 g, 80.55 mmol) and benzylic acid **14** (13.4 g, 80.63 mmol). The mixture was heated for 12 h at 70 °C. After cooling, the mixture was poured onto icy water. The product was filtered off and recrystallized from chloroform to give the deoxybenzoin **15** as beige crystals (15.3 g, 70%): mp 176−178 °C; IR (KBr) 3284, 1622, 1609, 1584, 1514, 1496, 1359, 1327, 1312, 1247, 1219, 1104, 1031; δ_H (250 MHz, acetone-d_6) 2.05 (s, 3H), 3.75 (s, 3H), 4.19 (s, 2H), 6.50 (d, 1H, J = 8.8 Hz), 6.87 (AA′BB′, 2H), 7.25 (AA′BB′, 2H), 7.46 (d, 1H, J = 8.8 Hz), 9.42 (s, 1H), 13.11 (s, 1H); δ_C (62.9 MHz, acetone-d_6) 7.7, 44.2, 55.5, 107.9, 114.7, 130.9, 131.2, 112.0, 113.1, 128.2, 159.5, 163.1, 164.5, 203.9.

*7-Hydroxy-3-(4-methoxyphenyl)-8-methyl-4H-chromen-4-one (**16**).* $BF_3 \cdot Et_2O$ (45 mL, 224.76 mmol) was added dropwise to a stirred solution of deoxybenzoin **15** (15.3 g, 56.2 mmol) in dimethylformamide (DMF) (150 mL) at room temperature. The mixture was heated to 50 °C, and mesyl chloride (15 mL, 168.57 mmol) was added with a syringe. The solution was warmed to 70 °C and stirred for 3 h. After cooling, the mixture was poured onto icy water (700 mL). Filtration of the crude product gave a yellow residue, which was recrystallized from chloroform to give the isoflavone **16** as a white powder (11 g, 70%): mp 292 °C; IR (KBr) 3166, 1620, 1593, 1575, 1510, 1417, 1291, 1268, 1245, 1176; δ_H (250 MHz, DMSO-d_6) 2.23 (s, 3H), 3.78 (s, 3H), 6.99 (AA′BB′, 2H), 7.01 (d, 1H, J = 8.7 Hz), 7.52 (AA′BB′, 2H), 7.83 (d, 1H, J = 8.7 Hz), 8.40 (s, 1H), 9.67 (s, 1H); δ_C (62.9 MHz, DMSO-d_6) 7.9, 55.1, 110.8, 113.5, 113.7, 116.6, 122.7, 123.7, 124.3, 130.0, 153.1, 155.4, 158.8, 159.9, 174.9.

*7-Acetoxy-3-(4-methoxyphenyl)-8-methyl-4H-chromen-4-one (**17**).* Acetic anhydride (25 g, 244.9 mmol) was added dropwise to a stirred solution of isoflavone **16** (5 g, 17.71 mmol) in pyridine (80 mL). The mixture was heated to 120 °C for 24 h.

After cooling and removal of the solvent under reduced pressure, a brown solid residue was obtained and then purified by column chromatography, eluting with 10% ethyl acetate in dichloromethane to give the product **17** as a white powder (5.1 g, 89%): mp 168 °C; IR (KBr) 1755, 1636, 1607, 1584, 1511, 1430, 1369, 1293, 1248, 1219, 1208, 1187, 1179; δ_H (250 MHz, DMSO-d_6) 2.27 (s, 3H), 2.38 (s, 3H), 3.79 (s, 3H), 7.01 (AA′BB′, 2H), 7.29 (d, 1H, J = 8.7 Hz), 7.53 (AA′BB′, 2H), 8.02 (d, 1H, J = 8.7 Hz), 8.56 (s, 1H); δ_C (62.9 MHz, DMSO-d_6) 8.8, 20.5, 55.1, 113.6, 120.1, 123.6, 130.0, 154.0, 119.7, 121.6, 123.3, 123.7, 152.4, 154.6, 159.0, 168.5, 175.0.

*8-Bromomethyl-7-acetoxy-3-(4-methoxyphenyl)-4H-chromen-4-one (**18**).* A well-stirred mixture of acetate **17** (9 g, 27.75 mmol), *N*-bromosuccinimide (5 g, 28.05 mmol), and benzoyl peroxide (0.5 g) in dry carbon tetrachloride (100 mL) was refluxed for 4 h. After cooling, the mixture was filtered, and the filtrate was evaporated to give a yellow solid residue, which was purified by column chromatography, eluting with 5% diethyl ether in dichloromethane to give the product **18** as a white powder (10.6 g, 70%): mp 182 °C; IR (KBr) 1763, 1642, 1610, 1513, 1432, 1174, 1142, 1026; δ_H (250 MHz, DMSO-d_6) 2.03 (s, 3H), 3.77 (s, 3H), 5.25 (s, 2H), 6.96 (AA′BB′, 2H), 7.09 (d, 1H, J = 8.9 Hz), 7.50 (AA′BB′, 2H), 8.00 (d, 1H, J = 8.9 Hz), 8.37 (s, 1H); δ_C (62.9 MHz, DMSO-d_6) 20.6, 29.7, 55.1, 108.8, 113.5, 114.4, 116.5, 123.1, 124.0, 127.4, 130.0, 152.9, 156.0, 158.9, 161.3, 170.4, 174.6.

*7-Acetoxy-8-[2,2-bis(ethoxycarbonyl)ethyl]-3-(4-methoxyphenyl)-4H-chromen-4-one (**19**).* A solution of bromide **18** (6 g, 14.88 mmol), diethyl malonate (8 g, 50 mmol), and powdered potassium carbonate (6.6 g, 47.7 mmol) in DMF (60 mL) was stirred at room temperature for 4 days (the reaction was monitored by TLC). The mixture was diluted with water and dichloromethane (100 mL and 100 mL) and acidified (pH 1−2) with HCl (1 M). The organic layer was washed with water (2 × 50 mL) and dried over magnesium sulfate. Removal of the solvent gave a yellow solid residue, which was used to obtain hapten **20**. A small fraction was purified by column chromatography, eluting with 5% diethyl ether in dichloromethane to give **19** as a yellow solid (10 g): mp 122−124 °C; IR (NaCl) 1731, 1649, 1609, 1584, 1514, 1432, 1370, 1251, 1176, 1037; δ_H (200 MHz, acetone-d_6) 1.40 (t, 6H, J = 7.2 Hz), 2.62 (s, 3H), 3.68 (d, 2H, J = 7.6 Hz), 4.07 (s, 3H), 4.08 (t, 1H, J = 7.6 Hz), 4.37 (m, 4H), 7.23 (AA′BB′, 2H), 7.53 (d, 1H, J = 8.8 Hz), 7.83 (AA′BB′, 2H), 8.38 (d, 1H, J = 8.8 Hz), 8.59 (s, 1H); δ_C (62.9 MHz, acetone-d_6) 14.3, 21.0, 23.7, 51.6, 55.6, 62.1, 114.5, 121.3, 125.2, 131.0, 153.9, 120.8, 123.1, 125.0, 125.2, 156.8, 160.7, 169.2, 169.3, 175.9.

*8-(2-Carboxyethyl)-7-hydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one (**20**).* To a solution of **19** (10 g) in acetone (70 mL) was added sodium carbonate (5% aqueous, 100 mL, excess). The mixture was refluxed for 24 h (the reaction was monitored by TLC). After cooling, removal of acetone under reduced pressure and acidification with HCl (1 M) gave a crude product, which was filtered off and purified by washing with dichloromethane (50 mL) to give the hapten **20** as a white powder (2,7 g, 53% from **18**): mp > 250 °C; IR (KBr) 3327, 1702, 1621, 1611, 1578, 1512, 1439, 1268, 1254, 1239, 1178, 1044; δ_H (250 MHz, DMSO-d_6) 2.45 (m, 2H), 3.02 (m, 2H), 3.78 (s, 3H), 6.98 (AA′BB′, 2H), 7.02 (d, 1H, J = 8.8 Hz), 7.53 (AA′BB′, 2H), 7.86 (d, 1H, J = 8.8 Hz), 8.40 (s, 1H), 10.79 (s, 1H), 12.2 (s, 1H); δ_C (62.9 MHz, DMSO-d_6) 18.3, 32.8, 55.1, 113.6, 113.8, 114.1; 116.7, 122.7, 124.3, 124.5, 130.0, 153.1, 155.3, 158.9, 160.0, 173.9, 174.9.

*8-(2-Carboxyethyl)-7-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one (**21**).* BBr_3 (1 M in dichloromethane, 22 mL, 21.5 mmol) was added dropwise with a syringe to a stirred solution of hapten **20** (1.22 g, 3.58 mmol) in dichloromethane (30 mL). After complete addition, the mixture was stirred for 24 h at room temperature and then was poured onto icy water (300 mL). Filtration of the crude product gave a yellow residue, which was recrystallized from ethanol/water (1:1, v/v) to give the product **21** as a yellow powder (875 mg, 75%): mp > 300 °C; IR (KBr) 3243, 1702, 1623, 1514, 1437, 1293, 1265, 1213; δ_H (250 MHz, DMSO-d_6) 2.51 (m, 2H), 3.05 (m, 2H), 6.85 (AA′BB′, 2H), 7.04 (d, 1H, J = 8.8 Hz), 7.42 (AA′BB′, 2H), 7.90 (d, 1H, J = 8.8 Hz), 8.39 (s, 1H), 9.57 (s, 1H), 10.78 (s, 1H), 12.2 (s, 1H); δ_C (62.9 MHz, DMSO-d_6) 18.3, 32.8, 113.8, 114.1, 114.9, 116.7, 122.6, 123.0, 124.5, 130.0, 152.8, 155.3, 157.1, 159.9, 173.8, 175.0; MS, *m/z* (relative intensity) 308 (100 $M^+ - H_2O$), 266 (11.6 $M^+ - H_2O - CH_2CO$), 191 (11.7), 149 (17.8), 118 (34.7). $C_{18}H_{14}O_6$ required: C, 66.26%; H, 4.32%; O, 29.42%. Found: C, 65.64%; H, 4.59%; O, 28.59%.

Table 1. Titer Evaluated from OD at 490 nm with 2.5 μg/mL Coating and 1/4000 Antibody Dilution[a]

antiserum	hapten	titer
P 01234	**8**	+++
P 49636	**9**	+
P 14422	**10**	+++
P 37159	**11**	−
P 01114	**12**	++
P 49639	**20**	+++
P 49550	**21**	+++

[a] Low titer, −, OD < 1; moderate titer, +, 1 ≤ OD < 1.5; high titer, ++, 1.5 ≤ OD < 2; extremely high titer, +++, 2 ≤ OD < 2.5.

Preparation of Conjugates. Haptens **8**−**12**, **20**, and **21** were coupled either to BSA (for injection to rabbits) or to Thyr (for coating). The conjugation procedure was adapted from those of Riggles et al. (1990) and Szurdoki et al. (1992). Briefly, 0.072 mmol of tributylamine (17.3 μL), 0.036 mmol of isobutyl chloroformate (4.7 μL), and 0.036 mmol of hapten were dissolved in 1 mL of DMF at 4 °C and then stirred on an ice bath for 30 min. The resulting activated hapten solution in DMF was added dropwise to the protein solution (BSA, 0.72 μmol, 6.1 mg; Thyr, 0.072 μmol, 48.24 mg) in 5 mL of borate buffer (0.2 M borate−boric buffer, pH 8.7). The mixture was stirred for 6 h at room temperature and then dialyzed, against phosphate-buffered saline (PBS; 0.01 M, pH 7.4, 0.9% NaCl) and then against distilled water. The hapten−protein conjugates were lyophilized and stored at −20 °C.

Immunization of the Rabbits. Immunization was achieved on New Zealand rabbits from CEGAV (France). Rabbits were first ear-sampled for pre-immunserum test. Hapten−BSA conjugates (500 μg) were injected each time. For the first injection the conjugate was dissolved in 1 mL of PBS−complete Freund's adjuvant (v/v). For subsequent injections the antigen was dissolved in PBS−incomplete Freund's adjuvant (v/v). Injections were performed at multiple points according to the following injection schedule. The first three injections were performed at 1-week intervals. Two additional injections followed thereafter at a 3-week interval. One week after the fifth injection, a test was performed on a 5 mL blood sample to check the specificities and titers of antibodies. Two final injections were then performed at a 1-month interval. Fifty milliliter blood samples were collected 1 week after the last injection. Serum was obtained after blood clotting at 4 °C, for 24 h, and centrifuged at 3000*g* for 10 min at 4 °C. Sera were stored at −20 °C in small aliquots. The efficiency of the immunization was tested in ELISA (as described below) by a direct binding of the antibody onto the coated conjugate. Titers of all antisera were compared using the same Thyr−conjugate coating antigens (2.5 μg/mL) and comparing the OD for a 1/4000 antibody dilution (see Table 2).

Assay Procedure. Coating of the wells was performed with the Thyr−hapten conjugates (200 μL/well) in solution in carbonate buffer (0.05 M, pH 9.6) at 4 °C, overnight. The same hapten was used for coating as for immunization; that is, the assays were hapten homologous. Concentrations of conjugates are listed in Table 3. The wells were then saturated with PBS−T−PS−DMSO (PBS containing 0.1% porcine serum, 0.05% Tween 20, and 1% DMSO) at 37 °C for 30 min. Plates were rinsed three times with PBS−T−DMSO (PBS, 0.05% Tween 20, 1% DMSO). Serial dilutions of the analyte in PBS−T−PS−DMSO were prepared as standard curves, and 100 μL/well was added to the plate (see Table 3). Specific antibodies (see concentrations in Table 3 in PBS−T−PS−DMSO) were then added (100 μL/well). The incubation lasted for 2 h at 37 °C. The plates were washed three times with PBS−T−DMSO. Then 200 μL/well of the second antibody was added in PBS−

Table 2. Optimal Conditions for Each Assay Procedure

antiserum	P 01234	P 14422	P 01114	P 49636	P 37159	P 49639	P 49550
coating (μg/mL)	0.1	0.08	0.5	0.15	2.5	0.1	0.5
specific antibody dilution	1/30000	1/50000	1/12000	1/50000	1/2000	1/60000	1/110000
competitor	**1**	**2**	**5**	**3**	**4**	**1**	**2**
standard curve limit (ng/mL)	250−0.122	62.5−0.03	250−0.122	250−0.122	250−0.122	500−0.24	1000−0.48
IC_{50}[a]	5	0.8	10	20	5	7.8	15.6
slope	0.54 ± 0.09	0.51 ± 0.08	0.55 ± 0.08	0.35 ± 0.11	0.49 ± 0.17	0.73 ± 0.09	0.59 ± 0.06
second antibody dilution	1/2000	1/1000	1/1000	1/1000	1/2000	1/1000	1/1000

[a] IC_{50}, concentration of the analyte required for 50% inhibition of the antibody binding to the coating antigen.

Table 3. Specificity of the ELISAs toward Selected Phytoestrogens[a]

antiserum	**1**	**2**	**5**	**3**	**4**
P 01234 antiserum − compound **1**		1.75 ± 0.25	0.013 ± 0.005	51 ± 10	0.012 ± 0.001
P 14422 antiserum − compound **2**	12 ± 8		0.26 ± 0.15	6.1 ± 3.0	45 ± 10
P 01114 antiserum − compound **5**	0.013 ± 0.005	0.12 ± 0.03		0.025 ± 0.005	0.015 ± 0.004
P 49636 antiserum − compound **3**	2.0 ± 0.5	0.75 ± 0.12	0.04 ± 0.002		0.8 ± 0.2
P 37159 antiserum − compound **4**	0.60 ± 0.15	40 ± 15	0.054 ± 0.006	20 ± 5	
P 49639 antiserum − compound **1**		23.6 ± 0.32	0.026 ± 0.002	26.6 ± 2.8	3.9 ± 0.7
P 49550 antiserum − compound **2**	50 ± 8		0.12 ± 0.05	15.9 ± 6.5	41.3 ± 6.0

[a] Results are expressed as percent cross-reactivity values: (IC_{50} of parent compound/IC_{50} of compound) × 100 (±SD calculated on three tests).

T−PS−DMSO (see Table 3 for concentrations). The incubation was performed at 37 °C for 30 min. To measure peroxidase activity, 200 μL/well of substrate solution containing 0.005 M *o*-phenylenediamine (10 mg in 20 mL) and 0.00025% H_2O_2 30% (5 μL in 20 mL) in citrate−phosphate buffer (0.15 M, pH 5.0) was added. The reaction took place at room temperature for 30 min and was stopped with 50 μL/well of 4 M H_2SO_4. OD values were read at 490 nm. The standard curves counted 12 points in duplicates with a 2-fold increase between concentrations.

Cross-Reactivity Tests. The cross-reactivities of the antibodies were tested in the competitive way, according to the optimal conditions summarized in Table 3. In that case besides a standard curve obtained with the analyte, the antibody was exposed to serial dilutions of other phytoestrogens in PBS−T−PS−DMSO. The concentrations of the competitors varied from 50 to 0.003 μg/mL with a 4-fold decrease between concentrations. Results are given in Table 3.

RESULTS AND DISCUSSION

Synthetic Work. Five carboxylic acid haptens of isoflavonoids were synthesized with the spacer arm at the O7 position (**8**−**12**) and two haptens (**20** and **21**) with the spacer arm at the C8 position.

Hapten Series with Spacer Arm on the Oxygen Atom at C7. As mentioned earlier, the attempted reactions, following previous procedures, to synthesize the required acids did not provide the expected haptens and, instead, complex mixtures were recovered. Moreover, a previous report on hapten syntheses by regioselective alkylation of genistein at either the 4′-O or 7-O position did not present detailed experimental procedures (Lapèík et al., 1998, and references cited therein). Surprisingly, the cited reference (Wähälä et al., 1995), in the experimental procedure for chemicals, deals only with the synthesis and labeling of isoflavones. Therefore, we turned our attention to devising a new process to synthesize the required acids.

Starting from **1** and **3** (Figure 2), obtained as previously reported (Pelissero et al., 1991), the spacer arm was bonded to the oxygen atom at the C7 position using ethyl bromoacetate with potassium carbonate in acetone. Saponification of esters **6** and **7** afforded the corresponding acids **8** and **9**. With **3**, the C7 hydroxyl group was alkylated selectively according to ^{2}D NMR data. This could be explained by the presence of a hydrogen bond between the hydroxyl group and the B ring carbonyl. Haptens **10** and **11** were obtained in good yields by selective demethylation with boron tribromide (Bhatt and Kulkarni, 1983). Hydrogenation of **10** afforded the hapten **12**, suitable for the production of antibodies against equo **5**.

C8 Hapten Syntheses. To improve the specificity of antibodies, we tried to avoid a direct conjugation using a hydroxyl group of isoflavoneS, which is an important antigenic determinant of this class of compounds. By a variation of the Wähälä method (Wähälä and Hase, 1991), we obtained the methylated deoxybenzoin **15** (Figure 3). After protection of hydroxyl groups with acetic anhydride, the brominated compound **18** was obtained by bromination with *N*-bromosuccinimide and a catalytic amount of benzoyl peroxide in carbon tetrachloride (Zammattio et al., 1991). A malonic ester synthesis [see, for example, Szurdoki et al. (1992) and cited references] afforded the hapten **20**. Treatment of the latter with boron tribromide, in the same conditions as above, gave the hapten **21**.

Conjugation Tests. The conjugations obtained, according to the method described above, were tested for their efficiency following the spectrophotometric method described by Erlanger et al. (1957).

Immunization Tests. According to Table 1, the titers of the antibodies were extremely high except that of P 37159. In that case we injected four different rabbits (not listed here) with the same results.

ELISA Tests. The optimal conditions of the ELISAs are shown in Table 2. The standard sigmoid curves obtained for each of the antibodies raised are presented in Figures 4 and 5. In the series functionalized on the oxygen atom at the C7 position, the sensitivities of the assays are generally good with standard curve limits between 250 and 0.244 ng/mL and IC_{50} around 10 ng/mL. In that respect P 14422 is somehow different because its standard curve limits are 62.5 and 0.0304 ng/mL and its IC_{50} is at 0.8 ng/mL. This could be related to the conjugation ratio obtained with BSA, which is the best of the series. The slopes obtained in this series were all rather similar (Figure 4). However, in some cases, the variability calculated on nine different assays is high, possibly due to an adsorption phenomenon on glassware or microtitration plates. As a matter of fact, we used silicone-coated glassware, silicone-coated tips, and buffers with 1% DMSO to reduce the adsorption phenomenon.

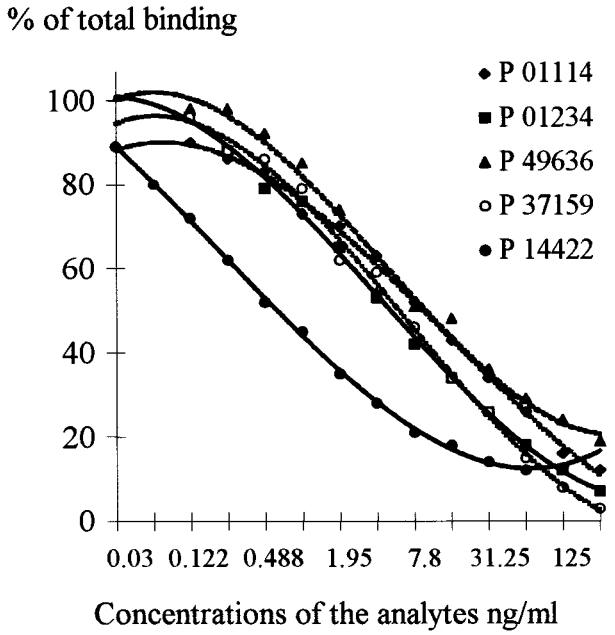

Figure 4. Standard curves obtained with the different antibodies in the O7 series. Points are the mean of duplicates. Analyte−antibody combinations are listed in Table 2.

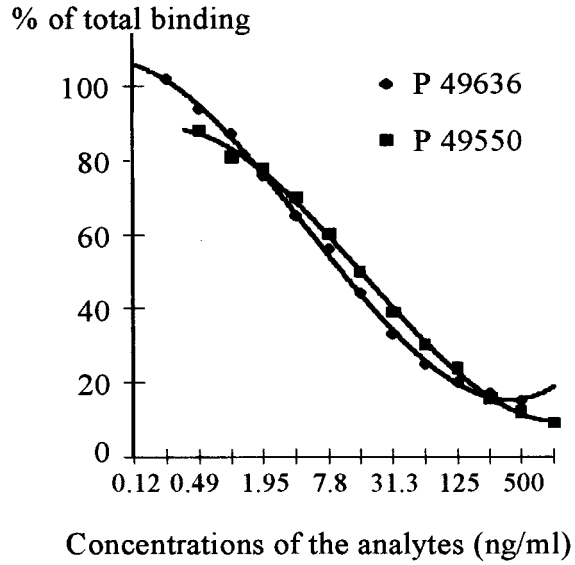

Figure 5. Standard curves obtained with the different antibodies in the C8 series. Points are the mean of duplicates. Analyte−antibody combinations are listed in Table 2.

In the C8 series, the sensitivities are lower. For P 49550 the standard curve limits are 1000 and 0.488 ng/mL and the IC_{50} is at 15.6 ng/mL, and for P 49639 the standard curve limits are 500 and 0.244 ng/mL with an IC_{50} at 7.8 ng/mL. This could be due to a nonspecific recognition of the Thyr−hapten conjugates. Our sensitivity results are of the same order as those obtained with ELISA of two herbicides (norflurazon and bromacil), described by Riggles et al. (1990) and Szurdoki et al. (1992). Indeed, they obtained IC_{50} values between 1 and 10 ng/mL, that is, 1−10 μg/L or 1−10 ppb, as we did. When we compared our ELISAs to RIAs of phytoestrogens, we observed that the latter are generally 10−100 times more sensitive than the former. Lapèík et al. (1997) obtained for **2** an IC_{50} of 0.170 ng/mL and for **4** an IC_{50} of 0.311 or 0.150 ng/mL depending on the antibody used (Lapèík et al., 1998). In addition, Wang et al. (1994) reported for **1** an IC_{50} of 0.4 ng/mL. However, previous data indicated that phytoestrogens were present in high concentrations in food (Anderson and Wolf, 1995) as well as in animal and human fluids (Setchell, 1985). Moreover, phytoestrogens were considered to be 1000−10000-fold less estrogenic than estradiol (Setchell, 1985). Thus, a very high sensitivity assay is not required for quantification in food or measurements of physiological relevance.

Cross-Reactivity of the Antibodies. The results obtained on cross-reactivities are presented in Table 3. It can be seen that cross-reactions of all phytoestrogens with P 14422 are low. Inversely, the other antisera exhibit very low cross-reactions with **5**. This can be interpreted in terms of the steric and electronic structural features of **5**, which are very different from those of isoflavones. On the other hand, high levels of cross-reactions are recorded between other compounds from the two series. In the group of haptens functionalized on the oxygen atom at the C7 position, the highest cross-reactions are registered between **2** and **4**; P 37159 recognizes **2** at 45%, and P 14422 recognizes **4** at 40%. This means that the additional hydroxyl group in **11** on the C5 position is not recognized by the antibodies. The same phenomenon is observed with P 49636, which recognizes **1**, at 51%. Wang et al. (1994) obtained the same results with their antibody anti-formononetin 7-*O*-carboxymethyl ether.

In the group of haptens functionalized at the C8 position, the specificity is worse. High levels of cross-reactions are observed between P 49550, **1**, and **4**, whereas P 49639 strongly recognizes **2** and **3**. In that case the cross-reaction between **2** and **1** could be expected with respect to the similarity between OMe and OH groups at C4′ (Sherry, 1992). However, this cross-reactivity was higher in the C8 series than in the O7 series. As a matter of fact, in the first series, the short spacer arm at position 7 projected the opposite moiety (position 4′) of the hapten molecule for strong recognition in the immunizing conjugate. Thus, the spacer arm directed the specificity of the O7-derived antibody series toward the immunodominant part (position 4′) of the hapten (Szurdoki et al., 1995) In the C8 series, the position of the linker at the hapten appears to allow more conformational freedom of the hapten in the immunizing conjugates; thus, the selectivity of the resulting antisera is lower. Lapèík et al. (1994, 1998) worked with the molecules substituted in 4′ and on the oxygen atom at C7. As we showed in the C8 series, they obtained high cross-reactions between **1** and **2** as well as between **3** and **4**.

In conclusion, the antibodies obtained are valuable for assays of phytoestrogens (Picherit et al., unpublished results). However, because of some high cross-reactions, separation of the compounds, prior to assay, should be worthwhile. In addition, because of high phytoestrogen levels in foods and because of low biological activities of these compounds, the sensitivity of our assays appears to be sufficient. Moreover, ELISAs are easier to use than RIAs because they do not need radioisotopes. All of these facts make our ELISAs very easy and suitable for measurements of phytoestrogen levels of physiological relevance in animal fluids and for diet manufacturers to control their materials before and after processing.

ABBREVIATIONS USED

BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; IC_{50}, inhibiting concentration 50%; IR, infrared spectroscopy; NBS, *N*-bromosuccinimide; NMR, nuclear magnetic resonance; OD, optical density; PBS, phosphate-buffered saline; ppb, parts per billion; ppm, parts per million; PS, pork serum; RIA, radioimmunoassay; T, Tween 20; TLC, thin-layer chromatography; Thyr, thyroglobulin.

LITERATURE CITED

Anderson, J. J.; Garner, S. C. Phytoestrogens and bone. *Baillieres Clin. Endocrinol. Metab.* **1998**, *12*, 543−557.

Anderson, R. L.; Wolf, W. J. Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *J. Nutr.* **1995**, *125*, 581S−588S.

Bennetts, H. W.; Underwood, E. J.; Shier, F. L. A specific breeding problem of sheep on subterranean clover pastures in western Australia. *Aust. Vet. J.* **1946**, *22*, 2−12.

Bhatt, V.; Kulkarni, S. U. Cleavage of ethers. *Synthesis* **1983**, 149−182.

Braden, A. W. H.; Hart, N. K.; Lamberton, J. A. The œstrogenic activity and metabolism of certain isoflavones in sheep. *Aust. J. Agric. Res.* **1967**, *18*, 335−348.

Erlanger, B. F.; Borek, F.; Beiser, S. M.; Lieberman, S. Steroid-protein conjugates. I. Preparation and characterisation of conjugates of bovine serum albumin with testosterone and with cortisone. *J. Biol. Chem.* **1957**, *228*, 713−727.

Farnsworth, N. R.; Bingel, A. S.; Cordell, G. A.; Crane, F. A.; Fong, H. H. S. Potential value of plants as sources of new antifertility agents I. *J. Pharm. Sci.* **1975a**, *64*, 535−598.

Farnsworth, N. R.; Bingel, A. S.; Cordell, G. A.; Crane, F. A.; Fong, H. H. S. Potential value of plants as sources of new antifertility agents II. *J. Pharm. Sci.* **1975b**, *64*, 717−754.

Findlay, J. K.; Buckmaster, M.; Chamley, W. A.; Cumming, I. A.; Hearnshaw, H.; Goding, J. R. Release of luteinising hormone by œstradiol 17β and a gonadotrophin-releasing hormone in ewes affected with clover disease. *Neuroendocrinology* **1973**, *11*, 57−66.

Garreau, B.; Valette, G.; Adlercreutz, H.; Wähälä, K.; Makelä, T.; Benassayag, C.; Nunez, E. Phytoestrogens: new ligand for rat and human α fetoprotein. *Biochim. Biophys. Acta* **1991**, *1094*, 339−345.

Golden, R. J.; Noller, K. L.; Titus-Ernstoff, L.; Kaufman, R. H.; Mittendorf, R.; Stillman, R.; Reese, E. A. Environmental endocrine modulators and human health: an assessment of the biological evidence. *Crit. Rev. Toxicol.* **1998**, *28*, 109−227.

Hampl, R.; Lapèík, O.; Wähälä, K.; Starka, L.; Adlercreutz, H. Radioimmunoassay analysis of phytoestrogens of isoflavonoid series. *Chem. Listy* **1998**, *92*, 44−50.

Hilakivi-Clarke, L.; Cho, E.; Clarke, R. Maternal genistein exposure mimics the effects of estrogen on mammary gland development in female mouse offspring. *Oncol. Rep.* **1998**, *5*, 609−616.

Hughes, C. L., Jr.; Kaldas, R. S.; Weisinger, A. S.; McCants, C. E.; Basham, K. B. Acute and subacute effects of naturally occurring estrogens on luteinizing hormone secretion in the ovariectomized rat: Part 1. *Reprod. Toxicol.* **1991a**, *5*, 127−132.

Hughes, C. L., Jr.; Chakinala, M. M.; Reece, S. G.; Miller, R. N.; Schomberg, D. W., Jr.; Basham, K. B. Acute and subacute effects of naturally occurring estrogens on luteinizing hormone secretion in the ovariectomized rat: Part 2. *Reprod. Toxicol.* **1991b**, *5*, 133−137.

Knight, D. C.; Eden, J. A.; Huang, J. L.; Waring, M. A. J. Isoflavone content of infant foods and formulas. *Pediatr. Child Health.* **1998**, *34*, 135−138.

Kuiper, G. G.; Lemmen, J. G.; Carlsson, B.; Corton, J. C.; Safe, S. H.; van der Saag, P. T.; van der Burg, B.; Gustafsson, J. A. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **1998**, *139*, 4252−4563.

Lapèík, O.; Hampl, R.; Al-Maharik, N.; Salkla, A.; Wähälä, K.; Adlercreutz, H. A novel radioimmunoassay for daidzein. *Steroids* **1997**, *62*, 315−320.

Lapèík, O.; Hampl, R.; Hill, M.; Wähälä, K.; Al Maharik, N.; Adlercreutz, H. Radioimmunoassay of free genistein in human serum. *J. Steroid. Biochem. Mol. Biol.* **1998**, *64*, 261−268.

Levy, J. R.; Faber, K. A.; Ayash, L.; Hughes, C. L., Jr. The effect of prenatal exposure to the phytoestrogen genistein on sexual differentiation in rats. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 60−66.

Martin, P. M.; Horwitz, K. B.; Ryan, D. S.; McGuire, W. L. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. *Endocrinology* **1978**, *103*, 1860−1867.

Medlock, K. L.; Branham, W. S.; Sheehan, D. M. Effects of coumestrol and equol on the developing tract of the rat. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 67−71.

Murkies, A. L.; Lombard, C.; Strauss, B. J.; Wilcox, G.; Burger, H. G.; Morton, M. S. Dietary flour supplementation decreases post-menopausal hot flushes: effect of soy and wheat. *Maturitas* **1995**, *21*, 189−195.

Obst, J. M.; Seamark, R. F. Plasma progesterone concentrations during the reproductive cycle of ewes grazing yarloop clover. *J. Reprod. Fert.* **1970**, *21*, 545−547.

Pelissero, C.; Bennetau, B.; Babin, P.; Le Menn, F.; Dunoguès, J. The estrogenicity of certain isoflavones in the Siberian sturgeon *Acipenser baeri*. *J. Steroid. Biochem. Mol. Biol.* **1991**, *38*, 293−299.

Riggles, B.; Dunbar, B. Development of enzyme immunoassay for the detection of the herbicide Norflurazon. *J. Agric. Food Chem.* **1990**, *38*, 1922−1925.

Santti, R.; Makelä, S.; Strauss, L.; Korkman, J.; Kostian, M. L. Phytoestrogens: potential endocrine disruptors in males. *Toxicol. Ind. Health* **1998**, *14*, 223−237.

Setchell, K. D. R. Naturally occurring non-steroidal estrogens of dietary origin. In *Estrogens in the Environment II*; McLachlan, Ed.; Elsevier Publishing: Amsterdam, The Netherlands, 1985; pp 69−85.

Setchell, K. D. R.; Zimmer-Nechemias, L.; Cai, J.; Heubi, J. E. Isoflavone content of infant formulas and the metabolic fate of theses phytoestrogens in early life. *Am. J. Clin. Nutr.* **1998**, *68*, 1453S−1461S.

Sherry, J. P. Environmental chemistry: the immunoassay option. *Crit. Rev. Anal. Chem.* **1992**, *23*, 217−300.

Szurdoki, F.; Bekheit, H. K. M.; Marco M. P.; Goodrow, M. H.; Hammock, B. D. Synthesis of haptens and conjugates for an enzyme immunoassay for analysis of the herbicide Bromacil. *J. Agric. Food Chem.* **1992**, *40*, 1459−1465.

Szurdoki, F.; Bekheit, H. K. M.; Marco, M.-P.; Goodrow, M. H.; Hammock, B. D. In *New Frontiers in Agrochemical Immunoassay*; Kurtz, D. A., Skerritt, J. H., Stanker, L., Eds.; AOAC International: Arlington, VA, 1995; pp 39−63.

Tham, D. M.; Gardner, C. D.; Haskell, W. L. Clinical review 97: Potential health benefits of dietary phytoestrogens: a review of the clinical epidemiological and mechanistic evidence. *J. Clin. Endocrinol. Metab.* **1998**, *83*, 2223−2235.

Wähälä, K.; Hase, T. Expedient synthesis of polyhydroxyisoflavones. *J. Chem. Soc., Perkin Trans. 1* **1991**, 3005−3008.

Wähälä, K.; Hase, T.; Adlercreutz, H. Synthesis and labeling of isoflavone phytoestrogens, including daidzein and genistein. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 27−32.

Wang, W.; Tanaka, Y.; Han, Z.; Cheng, J. Radioimmunoassay for quantitative analysis of formononetin in blood plasma and rumen fluid of mothers fed red clover. *J. Agric. Food Chem.* **1994**, *42*, 1584−1587.

Zammattio, F.; Brion, J. D.; Belachmi, L.; Le Baut, G. A convenient synthesis of (2-styrylchromo-8-yl)acetic acids. *J. Heterocycl. Chem.* **1991**, *28*, 2013−2019.

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