Pergamon

Tetrahedron 56 (2000) 295–301

TETRAHEDRON

Syntheses of Novel Hapten–Protein Conjugates for Production of Highly Specific Antibodies to Formononetin, Daidzein and Genistein

Cyril Le Houérou,[a] Catherine Bennetau-Pelissero,[b,c,*] Valérie Lamothe,[c] Françoise Le Menn,[b] Pierre Babin[d] and Bernard Bennetau[a,*]

[a]*Laboratoire de Chimie Organique et Organométallique UMR 5802 CNRS, Université Bordeaux I, 351 cours de la Libération, 33405 Talence, France*

[b]*Laboratoire de Biologie de la Reproduction des Poissons, Université Bordeaux I, Avenue des Facultés, 33405 Talence, France*

[c]*ENITA de Bordeaux, 1 cours du Général de Gaulle, 33175 Gradignan, France*

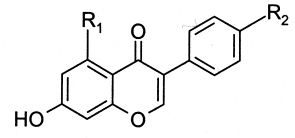
[d]*Laboratoire de Pharmacie Chimique, Université Bordeaux II, Place de la Victoire, 33000 Bordeaux, France*

Received 3 August 1999; accepted 3 November 1999

Abstract—The syntheses of novel hapten–carrier protein conjugates are described. Isoflavones functionalised in C2 were coupled to bovine serum albumin or swine thyroglobulin. The antibodies obtained were used for the development of ELISAs (enzyme-linked immunosorbent assays) to measure the three isoflavones with IC_{50} ranging from 15.6 to 100 ng/mL.

Introduction

Isoflavones such as formononetin **1**, daidzein **2** and genistein **3** are natural oestrogenic compounds present in large amounts (up to 1–4 mg/g)[1] in seeds, fruits and vegetables.[2,3] Phyto-oestrogens have oestrogen-like activity and may interfere with the reproductive cycle in animals.[4–7] However, during the last decade, an interest in the study of these compounds has increased due to their potential effects as health protecting dietary factors in human populations.



1 R_1 = H, R_2 = OMe
2 R_1 = H, R_2 = OH
3 R_1 = OH, R_2 = OH

Epidemiological studies dealt with comparisons between Western and Asian consumers. These showed that vegetarian and Asian consumers were currently exposed to higher phyto-oestrogen concentrations than Western consumers. They also present lower incidence of oestrogen-dependent diseases such as breast, uterus or colon cancer.[8]

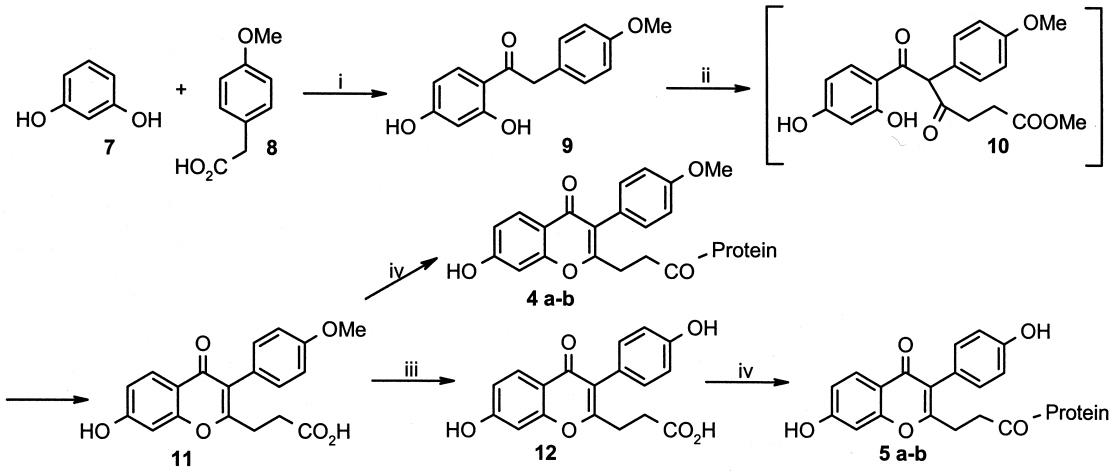
More recently, soy extracts were shown to decrease the incidence of hot flushes[9] and to reduce osteoporosis in menopausal women.[10] Today, soy extracts are commercialised for the prevention of female menopausal troubles. Lately, concern has grown about the potential adverse effects of these compounds in man considering the high amounts of both genistein and daidzein found in infant formula milk substitutes, based on soy.[11,12] Due to the potential effects of phyto-oestrogens as health dietary factors, the demand for analytical methods which are more cost-effective and less time-consuming than GC or HPLC is increasing. This demand could be filled by use of enzyme-linked immunosorbent assays (ELISAs). As a matter of fact, analytical methods based on immunochemistry are widely used in biochemistry, endocrinology or medical chemistry[13] and have been extended to environmental and toxicological areas.[14]

The initial step in the development of an immunoassay is the production of specific antibodies, but small molecules such as isoflavones are non-immunogenic. Hence, it is necessary to design appropriate chemical structures[15] that can be covalently coupled to a carrier protein. Various methods are available for coupling haptens and proteins with carboxylic acid groups in target molecules and amino

Keywords: hapten–protein conjugates; antibodies; isoflavones.

* Corresponding author. Fax: +556-846-646; e-mail: b.bennetau@lcoo.u-bordeaux.fr

0040–4020/00/$ - see front matter
PII: S0040-4020(99)00993-X



Scheme 1. Reagents: (i) $BF_3.Et_2O$ then H_2O, 70%; (ii) LiHMDS, $ClCO(CH_2)_2CO_2Me$, $AcOH/H_2SO_4$, 32%; (iii) BBr_3, CH_2Cl_2, 87%; (iv) coupling with proteins, see ratio in Table 1.

groups in protein.[16] Using these strategies, several authors have described the synthesis of isoflavone–protein conjugates to develop radioimmunoassays (RIAs).[17–20] However a direct conjugation with a functional group in the target molecule and amino groups in protein should be avoided because an important antigenic determinant of the molecule disappears making the resulting antibodies less specific. Moreover, in the RIA technique, radioisotopes are used which are not very convenient for control assays in the diet industry.

Results

Based on these considerations, in order to develop an ELISA test, we designed the synthesis of isoflavone–protein conjugates by introducing an ethylene carboxyl group at C2 carbon for conjugating with BSA or Thyr while all functional groups of isoflavones were preserved (Scheme 1).

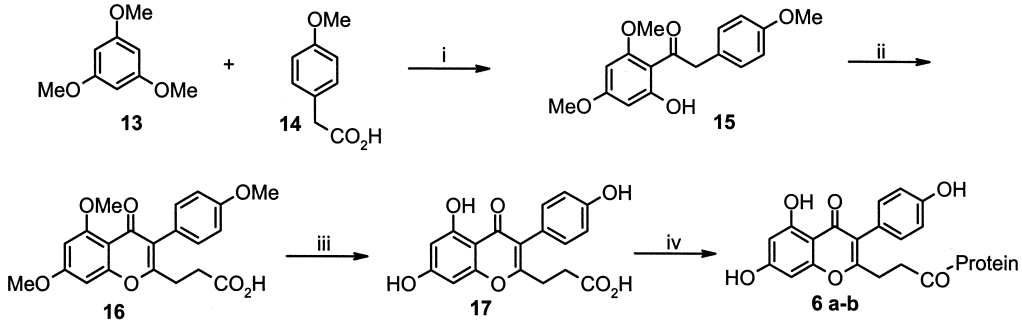
The synthesis of **4** and **5** was achieved by using resorcinol as the starting material. Deoxybenzoin **9** was prepared according to a known procedure[21] in 70% yield with $BF_3.Et_2O$ as catalyst. From the enolate of **9**, generated with lithium hexamethyldisilazane (LiHMDS), addition of methoxycarbonylpropionyl chloride produced the cyclised product **11** in 32% yield. All attempts to isolate the intermediate diketone **10** were unsuccessful. However, treatment of **11** with six equivalents of boron tribromide at low temperature gave the acid **12** in 87% yield.

A similar strategy has been described for the synthesis of hydroxylated flavones[22] with acetophenones and aroyl chlorides.

The synthesis of the hapten **17** of genistein is given in Scheme 2. Initially, it was anticipated that the deoxybenzoin **15** could be made by variation of the known procedure with phloroglucinol but the very poor yields observed prompted us to undertake another process. With the trimethoxyether **13**, the hydroxylated deoxybenzoin **15** was obtained in 33% yield with $BF_3.Et_2O$ as catalyst.

As previously observed, a selective mono-demethylation occurs in the synthesis of chalcones.[23] The acid **17** was finally obtained, after cyclisation and demethylation, following the same procedure as described above. A few examples of isoflavones, with a side chain containing four methylene groups and a carboxylic group, were synthesised by a different method but specific antibodies were not obtained since the acids have not been conjugated with proteins.[24]

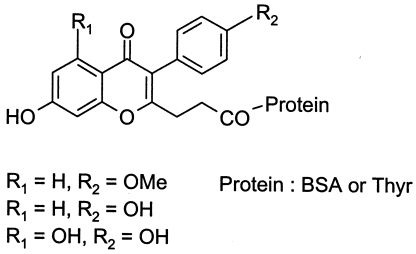
The three haptens (**11**, **12** and **17**) were coupled with BSA (**4**–**6a**) and Thyr (**4**–**6b**). The efficiency of the conjugation was tested spectrophotometrically[25] and is summarised in Table 1.



Scheme 2. Reagents: (i) $BF_3.Et_2O$ then H_2O, 33%; (ii) LiHMDS, $ClCO(CH_2)_2CO_2Me$, $AcOH/H_2SO_4$, 30%; (iii) BBr_3, CH_2Cl_2, 27%; (iv) coupling with proteins, see ratio in Table 1.

Table 1. Conjugation efficiency expressed as the ratio of haptens coupled to (BSA) or (Thyr)

Haptens	Ratio haptens/BSA	Ratio haptens/Thyr
11	48.7 (**4a**)	191.1 (**4b**)
12	44.5 (**5a**)	231 (**5b**)
17	47.2 (**6a**)	200 (**6b**)



4 a-b R_1 = H, R_2 = OMe Protein : BSA or Thyr
5 a-b R_1 = H, R_2 = OH
6 a-b R_1 = OH, R_2 = OH

The BSA conjugates were repeatedly injected individually to New Zealand rabbits affording three polyclonal antibodies. Their titre, specificity and sensitivity were examined by ELISA. The principle of the assay lies in the competition between free isoflavones and coated hapten–Thyr for specific antibodies. All the antibodies exhibited extremely high titres (optimal final dilution 1:60 000; 1:160 000; 1:240 000 for antisera **4a**, **5a** and **6a**, respectively). The optimal assay conditions for each of the antibodies are presented in Table 2.

The sigmoid competition curves obtained from the antibodies produced are shown in Fig. 1.

Once the optimal conditions were determined, the specificities of the antibodies were determined (see Table 3) with the parent compounds (structures given in Fig. 2).

Whereas the titres of our antibodies were extremely high for antisera **4a** and antisera **5a**, the displacement of the standard curve was lower than that of anti ***6a***. This suggests that the antibodies raised against BSA conjugates recognise unspecifically the Thyr conjugates coated onto the plate.

The specificities of our antibodies are good, however, in each case a strong cross-reaction with one of the isoflavones tested was detected. These strong cross-reactions were observed between daidzein and formononetin on the one hand and between genistein and biochanin A on the other hand.

The cross-reactivity values were calculated for each antisera according to the following equation:

$$\frac{IC_{50}\text{ of the isoflavone to which is directed the antibody}}{IC_{50}\text{ of the free phyto-oestrogens}} \times 100.$$

Table 2. Optimal conditions for each of the assay procedures (IC_{50}: concentration of free antigen including 50% displacement of the antibody binding to the coating)

Optimal conditions	Antiserum **4a**	Antiserum **5a**	Antiserum **6a**
Coating conc. (μg/mL)	0.1	0.4	0.1
Std curve limit (ng/mL)	2000–0.97	1000–0.48	250–0.12
IC_{50} (ng/mL)	100	40	15.6
Slope	−0.75 ± 0.19	−0.66 ± 0.11	−0.60 ± 0.08

The data show clearly that the antibodies obtained are valuable for assays of phyto-oestrogens. Because of some high cross-reactions, separation of the compounds prior to assay might be worthwhile. For soy, however, sample treatments are not needed since soy only contains daidzein and genistein and cross-reactions between the two are very low. A simple extraction is therefore sufficient for assay in food and animal tissues. Comparing our sensitivity results with ELISA of two herbicides, Norflurazon® and Bromacil®, described by other authors,[26,27] IC_{50} between 1 and 10 ng/mL, i.e. 1–10 μg/L or 1–10 ppb were reported. In that respect, our ELISAs are of the same order of sensitivity. When we compare our assays to RIAs of phyto-oestrogens we observe that the latter are generally 10–100 times more sensitive than ELISAs are. However, a very high sensitivity is not required. Indeed, previous data[1,28] indicate that phyto-oestrogens are present in high concentrations in plant as well as in animal and human fluids. Moreover, phyto-oestrogens are usually considered to be 1000–10 000 fold less oestrogenic than oestradiol.[28] We thus made the choice of a very easy and convenient method which does not need radioisotopes. This method is suitable to be used by diet manufacturers to control their materials before and after processing, and for detection in animal fluids with correlation to biological activities.

Experimental

Melting points were determined on Kofler apparatus or a Mettler FP 62 capillary melting point apparatus and were uncorrected. Infrared spectra were recorded on a Paragon 1000 Perkin–Elmer infrared spectrophotometer 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 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 were read on a Dynex MRX II microtitration plate reader at 490 nm. The standard curves were semi-log expression of OD sample/OD positive control= *f*(log(concentrations)).

1-(2,4-Dihydroxyphenyl)-2-(4-methoxyphenyl)ethanone 9. $BF_3.Et_2O$ (120 mL, 0.91 mol) was added dropwise to a mixture of resorcinol **7** (5 g, 45.41 mmol) and benzylic acid **8** (7.5 g, 45.13 mmol). The mixture was heated for 12 h at −70°C. After cooling, the mixture was poured onto icy water (800 mL). The product was filtered off and recrystallized from ethanol–water (1:1) to give compound **9** as white crystals (8.2 g, 70%); mp 163°C (Kofler); IR (KBr) 3361, 1623, 1613, 1511, 1435, 1352, 1242, 1175, 1129, 1024; δH (250 MHz, acetone-d_6/$CDCl_3$) 3.29 (s, 2H), 3.69 (s, 2H), 5.88 (d, 1H, *J*=2.4 Hz), 5.96 (dd, 1H,

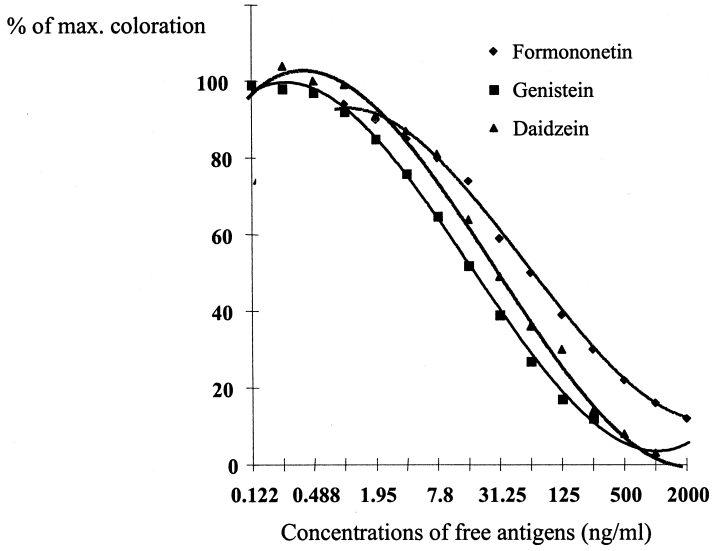


Figure 1. Standard curves obtained with the different antibodies.

J=2.4 Hz, *J*=8.8 Hz), 6.38 (AA′BB′, 2H), 6.74 (AA′BB′, 2H), 7.36 (d, 1H, *J*=8.8 Hz), 8.92 (s, 1H), 12.08 (s, 1H); δC (50 MHz, acetone-d_6/$CDCl_3$ 43.2, 54.4, 102.6, 107.7, 112.2, 113.5, 126.4, 129.9, 132.6, 158.2, 164.2, 165.4, 201.9.

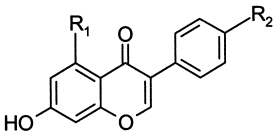
2-(2-Carboxyethyl)-7-hydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one 11. A solution of LiHMDS [prepared by addition of BuLi (2.5 M in hexane, 18.6 mL, 46.47 mmol) to hexamethyldisilazane (9.8 mL, 46.47 mmol) at −70°C] in THF (40 mL) was added dropwise to a solution of the deoxybenzoin **9** (3 g, 11.62 mmol) in THF (40 mL) at 70°C. After return to room temperature, the mixture was cooled to −70°C and a solution of methoxycarbonylpropionyl chloride (1.5 mL, 12.2 mmol) in THF (20 mL) was added dropwise. The reaction mixture was stirred for 4 h, then poured onto water (300 mL) and acidified with HCl (1 M). The solution was extracted with chloroform (3×100 mL). Removal of the organic phase under reduced pressure gave a brown residue that was treated with acetic acid (100 mL) and sulphuric acid (1 mL). The mixture was heated at reflux for 2 h. After removal of the solvent under reduced pressure, the crude product was diluted with chloroform (100 mL). The organic phase was washed with water (3×80 mL) and dried over magnesium sulphate. Removal of the solvent under reduced pressure gave a brown residue which was purified by column chromatography eluting with 20% diethyl ether in dichloromethane and then 20% ethanol in dichloromethane to give a brown powder which was purified again by recrystallisation from ethanol–water (1:1) to give the product **11** as a white powder (1.27 g, 32%): mp 255°C (Mettler); IR (KBr) 3144, 1695, 1626, 1607, 1584, 1510, 1468, 1392, 1294, 1233, 1172; δH (200 MHz, DMSO-d_6) 2.61 (m, 2H), 2.74 (m, 2H), 3.78 (s, 3H), 6.82 (d, 1H, *J*=2.0 Hz), 6.89 (dd, 1H, *J*=8.7 Hz, *J*=2.0 Hz), 6.97 (AA′BB′, 2H), 7.17 (AA′BB′, 2H), 7.86 (d, 1H, *J*=8.7 Hz); 10.75 (s, 1H), 12.3 (s, 1H); δC (62.9 MHz, DMSO-d_6) 27.4, 30.5, 54.9, 101.8, 113.4, 114.8, 115.5, 121.7, 124.8, 127.0, 131.5, 156.9, 158.5, 162.3, 163.5, 173.0, 175.1.

2-(2-Carboxyethyl)-7-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one 12. BBr_3 1 M in dichloromethane (22 mL, 21.5 mmol) was added dropwise with a syringe to a stirred solution of acid **11** (1.2 g, 3.52 mmol) in dichloromethane (60 mL). After complete addition, the mixture was stirred for 24 h at room temperature, then poured onto icy water (400 mL). Filtration of the crude product gave a yellow residue which was recrystallized from ethanol–water (1:1) to give the product **12** as a white powder (1 g, 87%): mp 250–300°C (Mettler); IR (KBr) 3450, 3215, 1712, 1621, 1610, 1562, 1553, 1510, 1401, 1211, 1168; δH (250 MHz, DMSO-d_6) 2.57–2.62 (m, 2H), 2.73–2.78 (m, 2H), 6.79–6.82 (m, 3H), 6.88 (d, 1H, *J*=8.6 Hz), 7.05 (AA′BB′, 2H), 7.86 (d, 1H, *J*=8.6 Hz); 9.52 (s, 1H), 10.75 (s, 1H), 12.3 (s, 1H); δC (62.9 MHz, DMSO-d_6) 27.4, 30.6, 101.8, 114.7, 114.8, 127.0, 131.4, 115.5, 122.0, 123.1, 156.6, 156.9, 162.3, 163.4, 173.1, 175.2; MS *m*/*z* (relative intensity) 326 (56.4 M^+), 281 ($100M^+-CO_2H$), 137 (19.7); $C_{18}H_{14}O_6$ required C 66.26%, H 4.32%, O 29.42%, found C 65.77%, H 4.74%, O 29.29%.

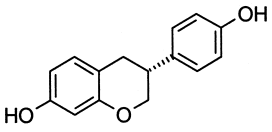
1-(2-Hydroxy-4,6-dimethoxyphenyl)-2-(4-methoxyphenyl) ethanone 15. According to the procedure described above for **9**, trimethoxyether **13** (5 g, 29.73 mmol) was treated with $BF_3.Et_2O$ (80 mL, 0.65 mol) and benzylic acid **14** (4.9 g, 29.5 mmol). The solution was extracted

Table 3. Cross-reactivity between the antibodies and the free compounds (values are % cross-reaction at 50% binding ±SD calculated on three determinations)

Antisera	Formononetin **1**	Daidzein **2**	Equol **19**	Biochanin A **18**	Genistein **3**
4a		65±1	0.36±0.1	7.9±0.03	7.9±0.03
5a	51.5±10		0.19±0.05	1.78±0.5	5.22±1
6a	2.08±1.1	2.05±1.2	0.062±0.02	53±12	



1 R_1 = H, R_2 = OMe
2 R_1 = H, R_2 = OH
3 R_1 = OH, R_2 = OH
18 R_1 = OH, R_2 = OMe



19 Equol

Figure 2. Structures of phyto-oestrogens used for cross-reactivity.

with diethyl ether (2×80 mL). The organic phase was washed with water (2×50 mL) and dried over magnesium sulphate. Removal of the solvent gave a yellow solid residue which was purified by column chromatography eluting with dichloromethane to give a yellow powder product which was recrystallized from ethanol–water (10:1) to give compound **15** as yellow crystals (3 g, 33%): mp 88°C (Kofler); IR (KBr) 3448, 1625, 1590, 1509, 1418, 1279, 1226, 1166, 1145, 1117, 1030; δH (250 MHz, $CDCl_3$) 3.76 (s, 6H), 3.87 (s, 3H), 4.27 (s, 2H), 5.93 (d, 1H, 4*J*=2.1), 6.07 (d, 1H, 4*J*=2.1), 6.89 (AA′BB′, 2H), 7.16 (AA′BB′, 2H), 14.99 (s, 1H); δC (62.9 MHz, $CDCl_3$) 49.4, 55.2, 55.5, 90.8, 93.8, 105.6, 118.8, 127.5, 130.7, 158.4, 162.7, 166.3, 167.9, 203.2.

2-(2-Carboxyethyl)-5,7-dimethoxy-3-(4-methoxyphenyl)-4H-chromen-4-one 16. According to the procedure described above for **11**, compound **15** (3 g, 9.92 mmol) in THF (40 mL) was treated with a solution of LiHMDS [prepared by addition of BuLi (2.5 M in hexane, 12 mL, 29.77 mmol) to hexamethyldisilazane (6.3 mL, 29.77 mmol)] then with a solution of methoxycarbonylpropionyl chloride (2.5 mL, 19.85 mmol) in THF (20 mL). Removal of the solvent under reduced pressure gave a brown residue which was purified by column chromatography eluting with 20% diethyl ether in dichloromethane and then 20% ethanol in dichloromethane to give a brown powder which was purified again by recrystallisation from ethanol–water (1:1) to give the cyclisation product **16** as a white powder (1.1 g, 30%): mp 236°C (Kofler); IR (KBr) 3421, 1712, 1644, 1615, 1572 1514, 1460, 1422, 1246, 1216, 1196, 1176; 1164, 1115; δH (250 MHz, DMSO-d_6) 2.59–2.62 (m, 2H), 2.66–2.69 (m, 2H), 3.87 (s, 6H), 3.87 (s, 3H), 6.47 (d, 1H, *J*=2.1 Hz), 6.64 (d, 1H, *J*=2.1 Hz) 6.96 (AA′BB′, 2H), 7.14 (AA′BB′, 2H), 12.3 (s, 1H); δC (62.9 MHz, DMSO-d_6) 27.0, 30.4, 54.8, 55.7, 55.8, 92.2, 95.8, 107.7, 113.3, 123.0, 125.0, 131.5, 158.4, 158.7, 160.4, 161.0, 163.4, 173.0, 174.1.

2-(2-Carboxyethyl)-5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one 17. According to the procedure described above for **12**, acid **16** (1.7 g, 4.42 mmol) in dichloromethane (60 mL) was treated with BBr_3 1 M in dichloromethane (31 mL, 31 mmol). Filtration of the crude product gave a yellow residue which was recrystallized from ethanol–water (1:1) to give the product **17** as a brown powder (320 mg, 27%): mp 246°C (Kofler); IR (KBr) 3355, 3280, 1710, 1654, 1609, 1562, 1513, 1367, 1215, 1197, 1162; δH (200 MHz, acetone-d_6) 2.71–2.79 (m, 2H), 2.89–2.97 (m, 2H), 6.26 (d, 1H, *J*=2.0 Hz), 6.42 (d, 1H, *J*=2.0 Hz), 6.93 (AA′BB′, 2H), 7.21 (AA′BB′, 2H), 8.46 (s, 1H), 9.57 (s, 1H), 13.0 (s, 2H); δC (50.3 MHz, acetone-d_6) 28.5, 31.1, 94.3, 99.5, 105.1, 116.0, 122.4, 123.7, 132.7, 158.0, 158.6, 163.6, 164.8, 166.1, 173.4, 182.1; MS *m/z* (relative intensity) 342 ($90.6M^+$), 297 ($100M^+-CO_2H$), 153 (25.3); $C_{18}H_{14}O_7$ required C 63.16%, H 4.12%, O 32.72%, found C 62.87%, H 4.31%, O 31.62%.

Preparation of Hapten–Protein conjugates

Haptens were covalently attached to BSA using a variation of two procedures previously reported.[26,27] Peptidic bonds were formed between the carboxylic function of the haptens and the free amino groups of lysine residues present on BSA. A protein solution of BSA (6.1 mg, 0.072 μmol) was prepared in 5 mL borate buffer (0.2M borate–boric buffer, pH 8.7). Tributylamine (17.3 μL, 0.072 mmol), isobutyl chloroformate (4.7 μL, 0.036 mmol) and hapten (0.036 mmol) were mixed in 1 ml DMF for 30 min on an ice bed. The hapten solution in DMF was then added dropwise to the borate buffer solution of protein and continuously mixed for 6 h at room temperature. This solution was then dialysed, first against PBS (0.01 M phosphate buffer with 0.9% NaCl, pH 7.4), and then against distilled water. The conjugates were lyophilised and stored in aliquots at −20°C. The stoichiometric ratio was 1:50.

The coupling of haptens with Thyr (48.2 mg, 0.072 μmol) was realised according to the above experimental procedure. The coupling efficiencies are summarised in Table 1.

Coating antigens and immunisation

Haptens were covalently attached to swine thyroglobulin (Thyr), through their carboxylic function to the lysine residues of Thyr, forming a peptidic bond. The procedure follows the method previously described. In this case, the stoichiometric ratio was 1:500. The coupling efficiency is summarised in Table 1.

Polyclonal antibodies were chosen for their better specificity. Immunisation of the rabbits was done on New Zealand rabbits from CEGAV (France). Rabbits were first earsampled for pre-immunoserum test. For each injection 500 μg of the BSA conjugates were injected. On the first injection antigens were dissolved in 1 mL PBS-complete Freund's adjuvant (v–v). For the subsequent injections anti-

gens were 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 one-week intervals. Two additional injections followed thereafter at three-week intervals. After the fifth injection a test was done on a 5 mL sample of serum to check the specificity and titre of the antibodies. Two last injections were then performed at one-month intervals. Each blood sampling was carried out one week after the previous injection; 50 mL of blood were finally collected. Serum was obtained after clotting for 24 h at 4°C and centrifuged at 3000 g for 10 min at 4°C. Aliquots of sera were stored frozen at −20°C. When in use, the aliquots were diluted v–v with glycerol and stored at −20°C.

ELISA procedure

Microtitration plates were coated with 200 μL/well of a Thyr conjugates solution at the concentrations indicated in Table 2. This operation was performed in coating buffer (0.1 M carbonate bicarbonate buffer, pH 9.6), overnight, at 4°C covered with aluminium sheet. The following day the plates were saturated with PBS–T–PS–DMSO (Phosphate Buffer Saline solution containing Tween, Pork serum and DMSO; 0.01 M phosphate buffer, 0.9% NaCl, 1.6% crude pork serum, pH 7.4, 0.05% Tween 20, 1% DMSO), for 30 min at 37°C and then washed three times in PBS-T-DMSO) (PBS, 0.05% Tween 20%, 1% DMSO). Serial dilutions of the analyte were prepared in PBS–T–PS–DMSO as standard curves and added in a 100 μL/well volume to the coated plates. This operation was followed by the addition of 100 μL/well of antibody solution to PBS–T–PS–DMSO. The final optimal dilutions of the antibodies in the wells are presented in Table 2. After 2 h incubation at 37°C, the plates were washed three times with PBS–T–DMSO. Incubation with 200 μL/well of a second antibody solution (1/1000 in PBS–T–PS–DMSO) was performed. Second antibody is swine immunoglobulin anti-rabbit immunoglobulin coupled to horseradish peroxidase (HRP) (Dako). Incubation lasted 30 min at 37°C. Finally, the plates were washed three times with PBS–T–DMSO, and the revelation of the peroxidase activity was effected by adding 200 μL of *ortho*-phenylenediamine (OPD) 0.5 M, in citrate–phosphate buffer (0.15 M, pH 5) containing 0.025% H_2O_2 30% at room temperature. Revelation lasted for 30 min and the reaction was stopped by adding 50 μL to H_2SO_4 4 M to each well.

Analysis of the antibody titre

The titre of the antibodies in the serum of animals was determined following the ELISA procedure by measuring the binding of a 1/4000 antibody dilution to a 0.25 μg/mL concentration of Thyr conjugates. Coating of Thyr conjugates was performed in coating buffer. The antibody incubation was performed in PBS–T–PS–DMSO.

Cross-reactivity determinations

Stock solutions (1 mg/mL) of the phyto-oestrogens listed in Table 3 were prepared in ethanol. Seria dilutions, from 250 μg/mL to 0.0076 μg/mL (dilution step=4), were prepared in PBS–T–PS–DMSO and each IC_{50} was determined in the assay experiment. The cross-reactivity values were calculated for each antisera according to the following equation: (IC_{50} of the isoflavone to which is directed the antibody/IC_{50} of the phyto-oestrogens listed in Table 3)×100.

Acknowledgements

This work was supported by a grant from the French Ministry of Agriculture, by the French Ministry of 'Recherche et Enseignement Supérieur', by the 'Région Aquitaine' and by the 'ENITA de Bordeaux'.

References

1. Anderson, R. L.; Wolf, W. J. *J. Nutr.* **1995**, *125*, 581S.
2. Farsworth, N. R.; Bingel, A. S.; Cordell, G. A.; Crane, F. A.; Fong, H. H. S. *J. Pharm. Sci.* **1975**, *64*, 535.
3. Farsworth, N. R.; Bingel, A. S.; Cordell, G. A.; Crane, F. A.; Fong, H. H. S. *J. Pharm. Sci.* **1975**, *64*, 717.
4. Hugues Jr., C. L.; Kaldas, R. S.; Weisinger, A. S.; McCants, C. E.; Basham, K. B. *Reprod. Toxicol.* **1991**, *5*, 127.
5. Hugues Jr., C. L.; Chakinala, M. M.; Reece, S. G.; Miller, R. N.; Schomberg Jr., D. W.; Basham, K. B. *Reprod. Toxicol.* **1991**, *5*, 133.
6. Medlock, K. L.; Branham, W. S.; Sheehan, D. M. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 67.
7. Levy, J. R.; Faber, K. A.; Ayash, L.; Hughes Jr., C. L. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 60.
8. Tham, D. M.; Gardner, C. D.; Haskell, W. L. *J. Clin. Endocrinol. Metab.* **1998**, *83*, 2223.
9. Murkies, A. L.; Lombard, C.; Strauss, B. J.; Wilcox, G.; Burger, H. G.; Morton, M. S. *Maturitas* **1995**, *21*, 189.
10. Anderson, J. J.; Garner, S. C. *Baillieres Clin. Endocrinol. Metab.* **1998**, *12*, 543.
11. Setchell, K. D. R.; Zimmer-Nechemias, L.; Cai, J.; Heubi, J. E. *Am. J. Clin. Nutr.* **1998**, *68*, 1453S.
12. Knight, D. C.; Eden, J. A.; Huang, J. L.; Waring, M. A. J. *Paediatr. Child Health* **1998**, *34*, 135.
13. Marco, M.-P.; Hammock, B. D.; Kurth, M. J. *J. Org. Chem.* **1993**, *58*, 7548.
14. Szurdoki, F.; Bekheit, H. K. M.; Marco, M.-P.; Goodrow, M. H.; Hammock, B. D. *New Frontiers in Agrochemical Immunoassay*; Kurtz, D. A.; Skerritt, J. H.; Stanker, L. Eds.; AOAC International: 1995, p 39 and cited references.
15. Sherry, J. P. *Crit. Rev. Anal. Chem.* **1992**, *23*, 217.
16. Erlanger, B. F. *Methods in Enzymology.* **1980**, *70*, 85.
17. Wang, W.; Tanaka, Y.; Han, Z.; Cheng, J. *J. Agric. Food. Chem.* **1994**, *42*, 1584.
18. Hampl, R.; Lapcik, O.; Wahala, K.; Starka, L.; Adlercreutz, H. *Chem. Listy* **1998**, *92*, 44.
19. Lapčík, O.; Hampl, R.; Al-Maharik, N.; Salkla, A.; Wähälä, K.; Adlercreutz, H. *Steroids* **1997**, *62*, 315.
20. Lapčík, O.; Hampl, R.; Hill, M.; Wähälä, K.; Al Maharik, N.; Adlercreutz, H. *J. Steroid Biochem. Mol. Biol.* **1998**, *64*, 261.
21. Wähälä, K.; Hase, T. *J. Chem. Soc., Perkin Trans.* **1991**, *1*, 3005.
22. Cushman, M.; Nagarathnam, D. *Tetrahedron Lett.* **1990**, *31*, 6497.

23. Schiemenz, G. P.; Schmidt, U. *Liebigs Ann. Chem.* **1982**, 1509.
24. Pelter, A.; Ward, R. S.; Whalley, J. L. *Synthesis* **1998**, 1793.
25. Erlanger, B. F.; Borek, F.; Beiser, S. M.; Lieberman, S. *J. Biol. Chem.* **1957**, *228*, 713.
26. Riggles, B.; Dunbar, B. *J. Agric. Food. Chem.* **1990**, *38*, 1922.
27. Szurdoki, F.; Bekheit, H. K. M.; Marco, M.-P.; Goodrow, M. H.; Hammock, B. D. *J. Agric. Food. Chem.* **1992**, *40*, 1459.
28. Setchell, K. D. R. *Estrogens in the Environment II*; McLachlan Ed.; Elsevier: Amsterdam, 1985, p 69.