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Development of a Radioimmunoassay for the Quantitative Determination of 8-Prenylnaringenin in Biological Matrices

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Seven carboxylic acid haptens of 8-prenylnaringenin (8-PN) were synthesized, coupled to cationized bovine serum albumin, and employed to raise specific antisera in rabbits. Two linkers of different lengths (C_3H_6COOH and $C_6H_{12}COOH$) were coupled to the C7-OH group and separated into their respective enantiomers yielding the first four haptens. Racemic derivatives with C4′-OH coupled linkers $C_5H_{10}COOH$ and $C_9H_{18}COOH$ were synthesized carrying a methylated C7-OH. Another racemic C4′-OH hapten (CH_2COOH) was prepared starting from naringenin. The haptens elicited variable antibody titers dependent on linker lengths, with short linkers giving the best results. Three antisera were characterized in detail: *anti*-C7-carboxy-propyloxy-2*S*-(−)-8-PN (*anti*-H-11), *anti*-C7-carboxy-propyloxy-2*R*-(+)-8-PN (*anti*-H-10), and *anti*-C4′-carboxy-methoxy-*rac*-8-PN (*anti*-H-25). *anti*-H-10 and *anti*-H-11 showed about 9% enantiomeric cross-reactivity, and *anti*-H-11 did not discriminate between isoxanthohumol (IX) and 8-PN (84% cross-reactivity). For *anti*-H-10, cross-reactivities in the range of 2−5% were found for xanthohumol, IX, and 6-prenylnaringenin. Respective numbers for *anti*-H-25 were 0.02, 0.1, and 0.2%. Tritiated 8-PN was synthesized yielding a ^{3}H-tracer of high specific radioactivity (2.22 GBq/mg). A radioimmunoassay using *anti*-H-25 and ^{3}H-8-PN was established and used for the quantitative determination of 8-PN in various beer brands and in the urine of six men after the consumption of three different brands of beer. Furthermore, the dose-dependent excretion of 8-PN was tested after the consumption of a higher volume of a single beer brand with and without spiking with 8-PN and a small oral dose of authentic 8-PN, respectively. Conflicting results led to a pilot test on the in vivo conversion (demethylation) of IX into 8-PN in two men. Conversion rates of 1.9 and 4.4% were estimated. Thus, the total 8-PN dose in beer brands spiced with natural hop or hop products seems to be the sum of the 8-PN amount in a consumed volume and the amount arising from the conversion of IX.

KEYWORDS: Phytoestrogens; 8-prenylnaringenin; RIA; beer; isoxanthohumol

INTRODUCTION

Phytoestrogens are polyphenolic, secondary metabolites found in leafy plants and fungi that exhibit estrogenic activity both in vivo and in vitro. Initially discovered by their negative impact on the fertility of domestic sheep feeding on *Trifolium subterraneum* (red clover) (*1*), phytoestrogens are subdivided by their chemical structures into the classes of isoflavones, flavanones, coumestans, and resorcylic acid lactones. Epidemiological evidence suggests that phytoestrogens ingested with foodstuffs may have beneficial effects on the prevention of breast cancer, perimenopausal symptoms, and atherosclerosis, although potential negative effects have been suggested as well (*2*). However, the estrogenic potency of most of these compounds is very low as compared to endogenous 17β-estradiol. The prenylated flavanone 8-prenylnaringenin (8-PN) was recently isolated from the wood of the tropical tree *Anaxagorea luzonensis* (*3*) and also identified as the estrogenic component in hops (*Humulus lupulus*) and beer (*4*, *5*). As 8-PN was identified as the most potent phytoestrogen, it has become the subject of various research activities in chemistry (*6*), receptor binding and transactivation (*7*), plant biochemistry (*8*, *9*), in vitro pharmacology (*3*, *10*−*13*), and analytical methods (*14*−*17*).

Because broad strata of the population are widely exposed to 8-PN by consuming beer, which is known to contain different amounts of this compound depending on the brand (from 0 to 100 μg/L) (*4*, *14*), it is of considerable interest for public health

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to have a sensitive, specific, cheap, and rapid analytical method at hand to detect different exposures in consumers and to analyze 8-PN concentrations in different beer brands and production batches. Immunological assays normally meet these criteria although specificity needs to be demonstrated for each matrix. Earlier attempts to raise polyclonal antisera for the detection of the isoflavonoids formononetin, daidzein, biochanin-A, and genistein were only partly successful, and separation steps were necessary prior to enzyme-linked immunosorbent assay (ELISA) quantification of one specific compound (*18*). Two main criteria had to be met in the case of 8-PN: First, the antiserum needed to discriminate between 8-PN and structurally similar isoxanthohumol (IX) because the latter is present in beer at concentrations exceeding those of 8-PN about 100-fold. Second, after absorption and metabolism of 8-PN in man, the antiserum needed to discriminate between parent compound and metabolites, which are not yet known but most probably contain, besides phase I metabolites, conjugates with glucuronic and/or sulfuric acid.

Two sites of the molecule (C7-OH and C4′-OH) were targeted for derivative syntheses in order to obtain suitable haptens (for structures see Figure 1). Linker lengths were varied (C_3 and C_6 at C7-OH and C_6 and C_9 at C4′-OH) to achieve maximum immunogenicity. Linkers at C4′-OH could not be directly introduced into 8-PN without covalent modification (i.e., methylation) of the C7-OH group since various attempts to reversibly protect the C7-OH group within the 8-PN molecule failed. Then, C7-OH haptens were synthesized and purified as both 2*S*-(−)- and 2*R*-(+)-enantiomers. Another approach was followed by starting the hapten synthesis from naringenin resulting in a racemic C_1-linked C4′-OH 8-PN with a free C7-OH group. Because the peroxidase (POD) conjugates generated from the haptens did not show sufficient chemical stability, a tritiated tracer was subsequently synthesized to extend the use of antisera to a radioimmunoassay (RIA). Possible applications for the most specific antiserum will be reported in the context of beer analyses and human exposure of 8-PN by beer consumption.

EXPERIMENTAL PROCEDURES

Chemicals. Nonradioactive syntheses were performed using commercially available chemicals from Sigma-Aldrich (Germany), Roth (Karlsruhe, Germany), and Merck (Darmstadt, Germany). Naringenin was purchased from Senn-Chemicals (Dielsdorf, Switzerland). Detailed syntheses, ^{1}H NMR, and mass spectra data of new compounds are available electronically as Supporting Information. Cationized bovine serum albumin (cBSA) and immunization adjuvant were prepared by Biogenes (Berlin); ovalbumin (OVA) and POD (EIA grade) were obtained from Roche; dimethyl formamide (DMF) was obtained from Fluka; and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich.

Instruments. Extinction (ELISA) was measured at 450 nm in a OpsysMR (Dynex Technologies) microplate reader, and radioactivity (RIA) was counted for 4 min in a scintillation counter (Packard 1900 TR TriCarbo). Extraction of samples with methyl *tert*-butyl ether (MTBE) was performed with a head-to-head rotor (Heidolph) and low speed centrifugation (Heraeus-Christ).

Hapten Syntheses. The syntheses of haptens **9**−**12**, **15**, and **17** are shown in **Figure 2**. The starting compound in these reactions was racemic 8-PN **4** that was obtained by a synthesis described before (*6*). The synthesis of hapten **25**, starting from racemic naringenin, is shown in **Figure 3**.

Preparation of Conjugates. Hapten−protein conjugates were synthesized using the carbodiimide/NHS (EDAC/NHS) method adapted from Bauminger et al. (*19*). The haptens **9**, **11**, **15**, **17**, and **25** were coupled to cBSA for immunization of rabbits, to OVA for serum titer evaluation, and to POD for enzymatic quantification (ELISA). Carboxylic acid haptens were activated in dry DMF using NHS and EDAC and subsequently coupled to the respective protein dissolved in phosphate-buffered saline (PBS, pH 7.5).

OVA conjugates were purified via Sephadex G-25 gel filtration whereas dialysis against PBS (pH 7.5) was used for purification of cBSA conjugates. The coupling efficiency was determined by spectrophotometric analysis. Stock solutions of cBSA and OVA conjugates were stored at −20 °C. POD conjugates were purified by Sephadex G-25 gel filtration and stored at +4 °C.

Synthesis of Tritiated Tracer. The synthesis of the ^{3}H-labeled tracer of 8-PN is shown in **Figure 4**. First, the 3′,5′-dibromo derivative **36** of 8-PN was prepared starting from racemic naringenin. Both bromine atoms were exchanged to ^{3}H in a final, palladium-catalyzed step leading to the tritiated tracer **37**.

Immunization of Rabbits. Two rabbits were immunized for each hapten−cBSA conjugate. All animals were ear-sampled for preimmune serum testing prior to immunization. One milligram of hapten−cBSA conjugate in 0.5 mL of PBS (pH 7.5), emulsified in 1.0 mL of Freund's adjuvant (Biogenes, Berlin), was applied to each animal by intramuscular injection. Subsequent injections were performed at different body areas according to the following schedule: 1 and 2, injection in 1 week intervals; 3, injection after a 2 week interval; 4 and 5, injection in 4 week intervals; 6, injection after a 6 week interval; and 7, injection after a 12 week interval. Blood samples were drawn at various intervals to monitor serum titer development. Animals were sacrificed 1 week after the last injection, and 50 mL of blood was collected from each rabbit. Serum was obtained after blood clotting at +4 °C for 24 h and centrifugation at 3000*g* for 10 min. IgG fractions were isolated from serum aliquots of 10 mL by protein A sepharose affinity chromatography. For storage, IgG fractions were dialyzed against PBS (pH 7.5), lyophilized, and stored at −20 °C.

Titer Development. Interim and final bleedings of animals were checked for immune response. Serial dilutions of antisera were incubated with the respective OVA conjugates and coated onto 96 well microtiter plates. Bound antibodies were detected with an anti-rabbit-IgG−POD conjugate. The POD−IgG loading ratio was estimated by spectrometry at 450 nm.

RIA Procedures. Assay volumes were 1.0 mL consisting of 0.1 mL of standards (75−10000 pg/mL) plus 0.7 mL of buffer or 0.8 mL of extracts of unknowns, 0.1 mL of tracer (10000 dpm/vial), and 0.1 mL of antiserum solution (final vial dilution 1:100000). The mixture was incubated at +4 °C overnight. Adhesive separation of unbound antigen was achieved by admixture of 0.2 mL of 1.25% dextran-coated charcoal suspension at 0 °C for 15 min, subsequent low speed centrifugation (2300*g*), and decantation of the supernatant. The radioactivity was counted in a liquid scintillation counter after admixture of 4 mL of Atomlight Plus to the supernatant for 4 min.

Evaluation. TECAN easyWIN fitting Version 6.1 was used to fit the standard curve and to read the concentration of unknowns. Cross-reactivity was calculated as a percentage of that concentration of test substance able to displace the tracer by 50% in relation to the respective 8-PN concentration.

Sample Preparation. Aliquots of urine samples of each individual were diluted with physiological saline to give 0.5 mL and then extracted with 2.5 mL of MTBE for 50 min (Heidolph-Rotor). After the aqueous phase was frozen at −20 °C, the organic layer was separated into a new vial and evaporated to dryness under nitrogen flow. Extract residues were redissolved in 0.8 mL of assay buffer, and the solution was directly used for RIA analysis.

Cleavage of conjugates was carried out using β-glucuronidase/arylsulfatase as described earlier (*23*). After cleavage, the samples were extracted as described before and analyzed by RIA.

Cross-Reactivity. Cross-reactivity was tested for 2*S*-(−)-6-prenylnaringenin (6-PN), 2*R*-(+)-6-PN, xanthohumol (XH), and IX, all compounds that are also found in hops. In addition, selected endogenous sex steroids (estradiol, estrone, estriol, testosterone, and progesterone) were tested for cross-reactivity. Ten-fold dilutions of 10 or 100 μg/mL solutions of compounds were tested.

After enzymatic cleavage of conjugates, ether extracts were separated by high-performance liquid chromatography (HPLC) and collected

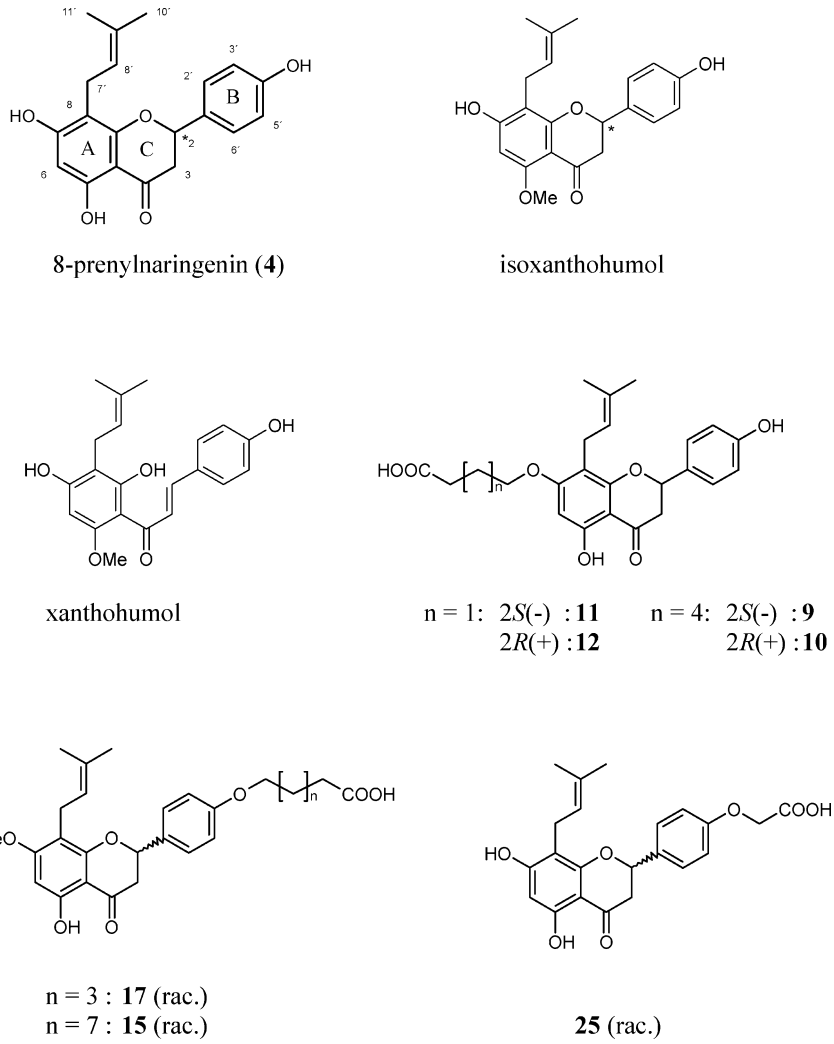

Figure 1. Chemical structures of 8-PN, XH, and IX and synthesized haptens **9**–**12**, **15**, **17**, and **25**.

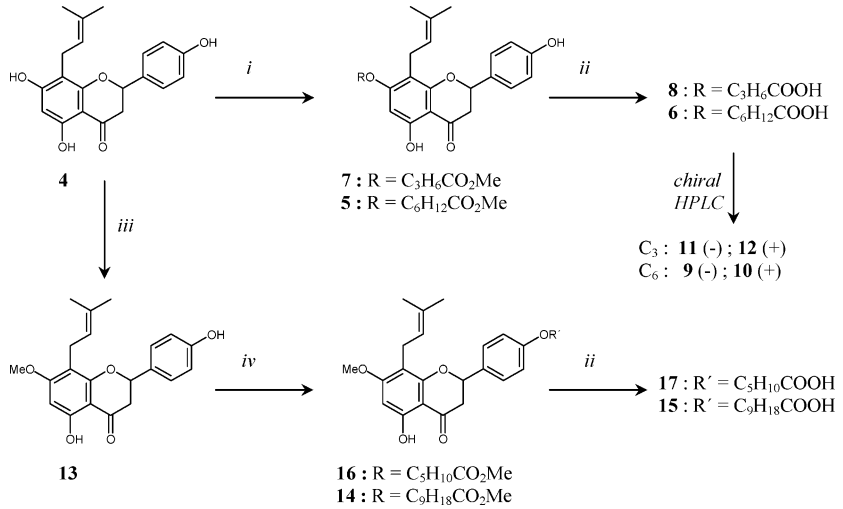

Figure 2. Scheme of synthesis of haptens **9**–**12**, **15**, and **17**. (i) Acetone, K_2CO_3, RBr, reflux. (ii) LiI, 2,4,6-collidine, reflux. (iii) MeI, K_2CO_3, acetone, reflux. (iv) $P(Ph)_3$, DIAD, THF, ROH.

fractions were subsequently analyzed by RIA in order to verify specificity of 8-PN analysis in urine collected within the beer drinking and IX conversion tests. The HPLC system consisted of a Kromasil C_{18} 5 μm (4.6 mm × 250 mm) column operated at room temperature and a steady flow of 1 mL/min. Elution was achieved by 20% acetonitrile in 0.2% acetic acid for 1 min followed by a linear gradient to 80% acetonitrile in 0.2% acetic acid within 19 min. Thereafter, the column was reconstituted by a linear gradient (4 min) and isocratic elution with 20% acetonitrile in acetic acid (1 min). The retention time of 8-PN was 18.2 min. Residues of 0.5 min fractions were quantified by RIA.

Beer Consumption. Six healthy men (39 ± 12 years, 83 ± 6 kg, and 185 ± 7 cm) participated in this experimental setting and were informed about the aims of the test and signed a written protocol. They were also informed about the outcome of a clinical single dose study, in which 50, 250, and 750 mg of 8-PN was given to each of six

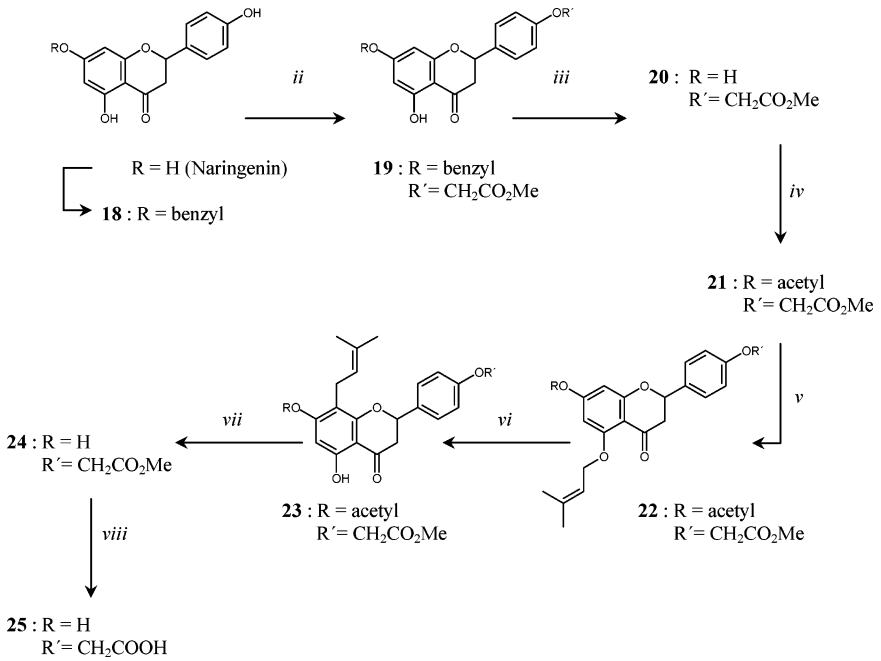

Figure 3. Scheme of the synthesis of hapten **25**. (i) $PhCH_2Br$, acetonitrile, K_2CO_3, reflux. (ii) $P(Ph)_3$, DIAD, THF, $HOCH_2CO_2Me$. (iii) EtOH, THF, Pd/C, H_2. (iv) Pyridine, $(CH_3CO)_2O$. (v) $P(Ph)_3$, DEAD, $(CH_3)_2CH{=}CHCH_2OH$, THF. (vi) Decaline, reflux. (vii) K_2CO_3, MeOH, H_2O. (viii) LiI, 2,4,6-collidine, reflux.

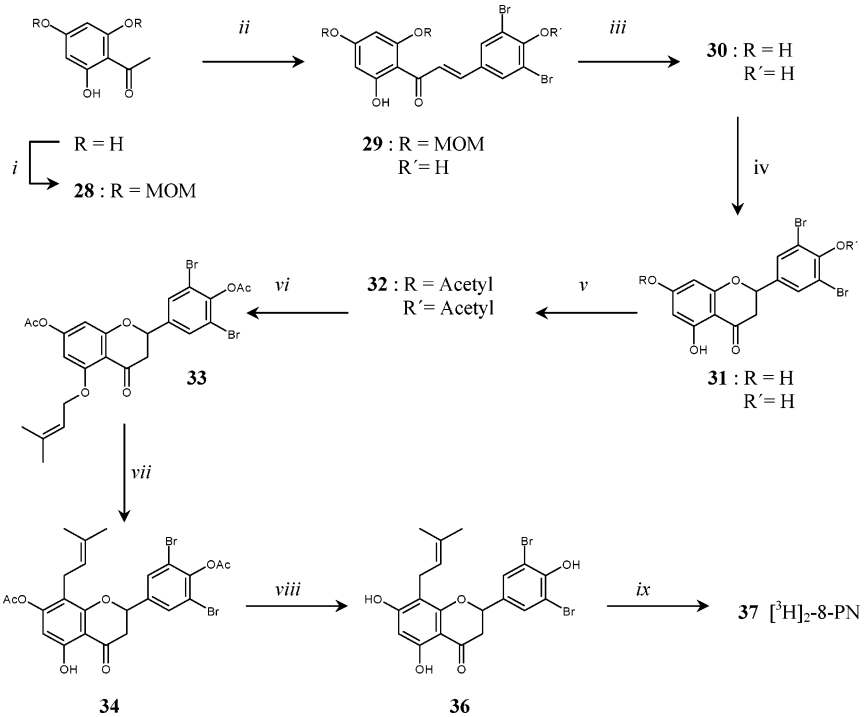

Figure 4. Scheme of the synthesis of ^3H-tracer **37**. (i) MOMCl, K_2CO_3, acetone, reflux. (ii) *p*-OH-3,5-dibromobenzaldehyde, EtOH, THF, KOH. (iii) HCl, MeOH, reflux. (iv) NaOAc, MeOH, reflux. (v) $(CH_3CO)_2O$, pyridine. (vi) $P(Ph)_3$, DIAD, $(CH_3)_2CH{=}CHCH_2OH$, THF. (vii) $CHCl_3$, $Eu[FOD]_3$, reflux. (viii) K_2CO_3, MeOH, H_2O. (ix) Acetic acid ethyl.

menopausal women without any drug-related side effects. This information was provided since each participant had to take 0.25 mg of authentic substance on test days (TDs) 5 and 6. The spiking dose corresponded to 0.03% of the highest tolerated dose of the clinical study. The authentic compound was taken from a GMP production batch.

The test was in six sessions, and urine was to be collected prior to (blank sample) and for 24 h after each consumption. After thorough mixing, the total volume was recorded by participants and an aliquot was kept at −16 °C in a plastic screw vial until analysis. Three days before each test, beer consumption but not other alcohol was prohibited. No other restrictions were made for daily life activities or diet habits. Three beer brands were used, and bottles of identical production batches were distributed to the participants. Beer A was spiced with CO_2-extracted bitter acids and thus did not contain 8-PN or IX. Analysis indeed revealed a concentration of <1.6 μg 8-PN/L. Beer B had a medium concentration of 8-PN (about 15 μg/L), and beer C had a higher concentration (33 μg/L).

In sessions 1−3, participants consumed 0.5 L of beers A−C within 50 min; in session 4, they consumed 1 L of beer C within 90 min; and in session 5, they consumed 1 L of beer C, which was spiked with 0.25 mg of 8-PN as aqueous/ethanolic (1:1) solution. In session 6, participants ingested only 0.25 mg of 8-PN as a 5% (v/v) alcoholic

solution without beer. All participants finished all test consumptions in the order 1−6 except participant MH, who followed the order 1, 2, 3, 6, 4, and 5.

RIA Analysis of Beer Brands. Sixteen beer brands (**Table 6**) were analyzed. Beer aliquots were degassed at +20 °C and 50 mbar, of which 0.1 mL was mixed with 0.4 mL of physiological saline and then extracted with ether as described above. RIA analysis was carried out as duplicates.

Test for IX Conversion. Two of the six subjects participating in the beer drinking test (MH and OS) ingested 10 mg of IX mixed into a small volume of a high percentage alcoholic beverage and then collected urine at 12 h intervals for 3−4 days. Urine volumes were recorded as before, and small samples were kept deep frozen until analysis. Both participants did not drink any beer at least 3 days before ingestion of IX and during the urine collection period. IX normally is present in beer at a concentration of 2 mg/L. Thus, both volunteers were administrated an IX dose equivalent to that present in 5 L of beer. IX was purchased from Phytochem (Ichenhausen, purity >95%).

RESULTS AND DISCUSSION

Synthetic Chemistry. Four carboxylic acid haptens of 8-PN were synthesized with a linker at the C7-O position (**9**−**12**) and three haptens bearing a spacer arm at the C4′-O position (**15**, **17**, and **25**). A tritiated tracer (**37**) was furthermore synthesized by halogen to tritium substitution of a dibromo derivative of 8-PN (**36**).

Series of Haptens with Linker on C7-O. Starting with racemic 8-PN, which was obtained employing the route of synthesis described by Gester et al. (*6*), the respective methyl esters **5** and **7** were obtained by classic William's ether synthesis. Subsequent saponification to the free racemic acids **6** and **8** was carried out with good yield using lithium iodine in collidine to avoid either basic or acidic aqueous conditions to which 8-PN responds with elimination/ring fission, described previously as Wesley-Moser rearrangement of flavonoids (*20*). Optically pure haptens **9**−**12** were isolated by chiral HPLC (for details, see the Supporting Information).

Haptens with Linker on C4′-O. The syntheses of a first set of C4′-O haptens started from reacemic 8-PN as well. The highly nucleophilic C7-OH group had to be protected by methylation in order to obtain the respective methyl esters **14** and **16** via Mitsunobu ether synthesis. Common protection group chemistry for phenols failed due to the above-mentioned pH sensibility of the molecule. Free acids **15** and **17** had therefore a C7-methoxy group instead of a phenol. Signficantly reduced immunogenicity of these haptens led to the choice of a second synthetic approach starting with naringenin. The nucleophilic C7-OH was initially benzyl protected (**18**) and the linker was coupled at the free C4′-OH via Mitsunobu (**19**). The prenyl group was then introduced as described before (*6*), following deprotection of C7-OH. Methyl ester cleavage under the same conditions as mentioned before gave hapten **25**.

Tritiated Tracer. Pd-catalyzed halogen to tritium substition is a suitable method for radiolabeling of small organic compounds. Treatment of 8-PN with either *N*-bromo- or *N*-iodosuccinimide (NBS/NIS) in THF resulted in the respective bromo/iodo derivative at C6. Still, the subsequently introduced tritium lable at this position instantly exchanged protons with protic solvents. Excess equivalents of either NBS or NIS led to halogenation of the aliphatic prenyl side chain instead of the aromatic B ring. We therefore pursued the approach to introduce bromine residues to the B ring by de novo synthesis. Methoxy methyl protected 2,4,6-tridydroxyacetophenone (**28**) and 3,5-dibromo-4-hydroxybenzyaldehyde were used for an aldol condensation and subsequent catalytic ring closure to give the 3′,5′-dibromo-naringenin derivative **31**. From there on, the prenyl side chain was introduced as described above. Double bromine to tritium exchange of **36** gave the ^{3}H tracer **37** with good yield and very high stability of the tritium lable. The specific activity was 2.22 GBq/mg.

Table 1. Cross-Reactivity of Three Antisera against 8-PN Enantiomers and Structurally Similar Hop Constituents

	cross-reactivity (%)		
analyte	*anti*-H-11	*anti*-H-10	*anti*-H-25
2*S*-(−)-8-PN	100	9.1	100
2*R*-(+)-8-PN	8.6	100	98
XH	0.6	2.2	0.02
IX	84	2.2	0.10
2*S*-(−)-6-PN	0.2	4.6	0.15

Conjugation Tests. The conjugates obtained were tested for hapten loading by spectrophotometric methods. According to chemical instability of conjugates, ELISA development was abandoned in favor of the RIA (see the Supporting Information).

Immunization of Rabbits. Immunogenicity and titer developments were clearly different for the antigenes investigated. Gernerally, long linkers such as *anti*-H-15 (C_9 linker) or *anti*-H-17 (C_5 linker) caused no or very little immune response. Both of these antigenes were methylated at C7-OH, which most likely further reduced immunogenicity. *anti*-H-9 and *anti*-H-10 (both carrying C_6 linkers at C7-O) showed a high immune response, which was sufficient for detailed evaluation of the antisera. A very high immunogenicity was obtained with short linkers such as *anti*-H-11/12 (C_3 linker at position C7-O) and *anti*-H-25 (C_1 linker at position C′4-O). Long linkers presumably hid the antigen within the protein matrix or did not provide a strong antigen presentation and therefore caused the loss of immunogenicity.

Cross-Reactivity. Three antisera representing different linking strategies were tested in detail for cross-reactivities (**Table 1**). The stereoselectivity of *anti*-H-10 and *anti*-H-11 was pronounced, but the cross-reactivity with the opposite enantiomer was still about 9%. Thus, the stereoselectivity of both antisera was not sufficient for quantitative determination of a single enantiomer. In addition, *anti*-H-11 showed an extensive cross-reaction with IX while *anti*-H-10 did not sufficiently discriminate 6-PN. The most specific antiserum was *anti*-H-25, showing a low cross-reactivity with XH, IX, and 6-PN. This antiserum was also tested in the RIA set up for cross-reaction with endogenous sex steroids. As expected, no cross-reactivity (<0.001%) was observed for estradiol, estrone, estriol, testosterone, and progesterone. Hapten 11, linked at position C7-O via a short C_3 residue, led to an antigen, which was unable to present the free C5-OH as a determinant while the open ring structure of XH was well-discriminated by the respective antiserum. On the other hand, hapten **10** linked at the same position with a long (C_6) residue and gave an antigen that was able to present the respective C5-OH group more efficiently. However, the improvement in specificity against IX was reached on the expense of lower specificities against 6-PN and XH. Thus, *anti*-H-10 presumably did not only show a somewhat lower immunogenicity as compared to *anti*-H-11 but also might contain a higher variety of antibodies due to the higher flexibility of presented hapten. The higher immunogenicity and specificity of the short-linked *anti*-H-11 and *anti*-H-25 found in the present experiments is in agreement with respective data found for isoflavonoid haptens (*18*, *21*, *22*). Stereoselectivity of antisera was found to be insufficient for both antisera tested. Because racemization during coupling and immunization can be ne-

glected, it is true that stereoisomers were important but not exclusively determinant for the (flexible) antigens presented.

On the basis of specificity data, *anti*-H-25 was selected and developed as RIA for the quantitative determination of racemic 8-PN in biological matrices. It must be emphasized at this point, however, that also *anti*-H-11 represents a valuable tool for the quantitative determination of 8-PN when racemic 8-PN is used for standardization and the presence of XH and IX in samples can be excluded. This is the case when the pharmacokinetics and metabolic fate of 8-PN as an authentic compound need to be followed.

A high cross-reactivity to metabolites had to be taken into account for assay development, according to the linking position of the antigen leading to *anti*-H-25 (C4′) and the anticipated occurrence of C4′-glucuronides or -sulfates of 8-PN in animal species and humans. This problem was solved by determination of ether extracts instead of direct quantification in biological fluids.

RIA Tests. *Sample Preparation.* Aliquots of urine samples (untreated or deconjugated blank or pooled samples) were diluted with physiological saline to give 0.5 mL and then extracted with 2.5 mL of MTBE for 50 min (Heidolph-Rotor). After the aqueous phase was frozen, the organic layer was transferred into a new vial and evaporated to dryness under nitrogen. Extract residues were redissolved in 0.8 mL of assay buffer, and the solution was directly used for RIA analysis.

Duplicate analysis was carried out for all tests. Cleavage of conjugates in serum and urine samples was carried out using β-glucuronidase/arylsulfatase as described earlier (*23*).

Recovery by Ether Extraction. Extraction of 8-PN either by means of diethyl ether or MTBE was complete, as shown by the fact that extracted and not extracted standard curves were superimposable (**Figure 4**). The linearity of extraction was shown at two concentrations (0.3 and 3.0 ng/mL) of spiked blank urine samples. No matrix interference was seen up to an extracted volume of 300 μL.

Robustness and Stability of Agents. Stock solutions (in assay buffer) of standards, tracer, and antiserum were stable for more than 2 months. No degradation of the tracer in ethanolic solution was found over a period of 6 months when kept at −20 °C. In principle, extractions of urine samples can be carried out with either diethyl ether or MTBE. However, the assay is sensitive (elevated blanks) against peroxides known to form in diethyl ether; therefore, extraction was performed with MTBE.

Precision (Inter- and Intraassay Variation). Blank urine samples were spiked with 8-PN to give nominal concentrations of 0.3 and 1 ng/mL. Samples were analyzed 7-fold in one assay and at least in duplicate in eight different assays.

The intraassay coefficient of variation (CV) was 8.8 and 7.0% for 0.3 or 1.0 ng/mL urine, respectively. The interassay CV was 26.8% for 0.3 ng/mL urine and 17.6% for 1.0 ng/mL sample. Thus, for both concentrations, the intraassay precision was within the accepted range for analytical methods (CV < 20%) while the interassay precision was met only for the higher concentration.

Accuracy. Blank urine samples were spiked with 1 and 3 ng 8-PN/mL and analyzed 5-fold. Found concentrations were 1.36 ± 0.14 and 3.29 ± 0.24 ng/mL, indicating an overestimation of roughly 30% in these experiments.

Sensitivity. The RIA system described has a sensitivity limit of 0.3 ng 8-PN/mL urine using 0.1 mL of sample. In case of free analyte in urine, 0.3 mL were extracted and the sensitivity was increased to 0.1 ng/mL. Thus, the lower limit of quantification was set to 0.1 ng/mL.

Table 2. Time Intervals between Testing Days and Concentrations of Free 8-PN in Blank Urine Samples of Six Individuals[a]

	MH[b]		AS		OS		DS		FW		JF	
TD	A	B	A	B	A	B	A	B	A	B	A	B
1		<min		<min		<min		104		<min		<min
2	1	<min	0	128	0	<min	1	<min	0	<min	0	<min
3	1	<min	0	647	8	139	2	102	0	<min	0	<min
4	1	755	0	930	0	218	0	485	11	<min	7	<min
5	4	138	1	933	0	1014	1	170	0	171	9	<min
6	1	<min	1	190	0	322	2	1551	0	636	0	1988

[a] A = time interval between TDs in days; B = blank urine concentration in pg/mL; and min = detection limit of 100 pg/mL (300 μL extracted). [b] MH changed the follow-up of TDs (1–3, 6, 4, 5).

Table 3. Time Intervals between TDs and Concentrations of Free Plus Conjugated 8-PN in Blank Urine Samples of Six Individuals[a]

	MH[b]		AS		OS		DS		FW		JF	
TD	A	B	A	B	A	B	A	B	A	B	A	B
1		0.78		0.39		0.72		1.25		0.31		0.58
2	1	0.55	0	0.65	0	0.37	1	0.98	0	<min	0	0.72
3	1	2.1	0	0.93	8	1.02	2	1.31	0	0.30	0	1.81
4	1	5.06	0	3.34	0	1.93	0	3.85	11	<min	7	1.98
5	4	1.47	1	6.34	0	4.68	1	3.44	0	0.96	9	1.43
6	1	3.25	1	3.97	0	5.59	2	9.10	0	1.53	0	20.8

[a] A = time interval between TDs in days; B = blank urine concentration in ng/mL; min = detection limit of 0.3 ng/mL. [b] MH changed the follow-up of TDs (1–3, 6, 4, 5).

In summary, with the *anti*-H-25 antiserum and tritiated 8-PN, we developed a RIA suitable for the quantitative determination of free and conjugated (after enzymatic cleavage of conjugates) 8-PN in urine. Almost identical quality criteria for the assay were found for the determination in human serum. Data will be described elsewhere (*24*).

Renal Excretion in Beer Consumption Tests. Urine samples collected during the beer drinking tests were analyzed both prior and after enzymatic cleavage of conjugates. Results were calculated as excretion per 24 h.

Because the experimental design did not demand a specific time interval between testing days, this question was handled differently by individuals (**Table 2**). Concentrations of free 8-PN before the first TD were below the detection limit of 0.1 ng/mL. However, concentrations in urine samples collected directly before beer consumption increased remarkably and reached values of 1−2 ng/mL in individual samples. This can be explained by increasing doses of 8-PN at testing days 1−6 and an overlap of incomplete excretion and new 8-PN dose. Thus, it turned out that excretion of 8-PN is slower than expected and an interval of several days between TDs would have been necessary to avoid accumulation of 8-PN during the study. Following enzymatic cleavage of conjugates, 0.3−1.25 ng/mL were found in blank urines (**Table 3**). Although it cannot be excluded that beer consumption 4 days before TD 1 might have led to elevated blank values, there were two participants who hardly ever consume beer. Thus, it is likely that there are other sources of 8-PN (or IX, see below) in regular diet. Preconsumption concentrations in urine markedly increased with TDs and short intervals between TDs. The lowest values were found for participant FW at TD 2 and TD 4 (after an interval of 11 days) and the highest (20.8 ng/mL) for JF at TD 6 (no interval to TD 5). On the basis of these data, it is clear that there was a varying overlap of 8-PN excretion from the foregoing TDs.

Table 4. 24 h Renal Excretion of Free Plus Conjugated 8-PN at TD 1–6 (in μg)

TD	MH[a]	AS	OS	DS	FW	JF	mean	SD
1	2.56	2.23	1.55	1.39	0.57	1.47	1.63	0.70
2	9.14	2.53	2.21	1.25		2.88	3.60	3.20
3	12.9	3.55	3.62	2.78	0.59	2.26	4.28	4.36
4	49.9	18.0	3.88	14.4		6.31	18.5	18.5
5	68.2	40.3	11.4	63.8	24.0	35.4	40.5	22.2
6	66.1	23.6	15.5	43.3	20.6	38.9	33.0	18.8

[a] MH changed the follow-up of TDs (1–3, 6, 4, 5).

Table 5. Renal Excretion of RIA Reactive Material Following a Single Oral Intake of 10 mg of IX in Two Individuals; Data Are Given as μg 8-PN Per 12 or 24 h[a]

	MH		OS	
time interval (h)	μg/0.5 days	μg/day	μg/0.5 days	μg/day
–12 (prevalue)	0.703	1.41	0.615	1.23
0–12	4.25 NT		3.68 DT	
12–24	9.66 DT	13.9	1.42 NT	5.10
24–36	9.07 NT		2.37 DT	
36–48	3.17 DT	12.4	3.12 NT	5.49
48–60	2.97 NT		2.53 DT	
60–72	1.12 DT	4.09	0.96 NT	3.49
72–84	2.02 NT		1.26 DT	
84–96	1.17 DT	3.19		
96–108	1.87 NT			
SUM	35.3	33.6	15.3	14.0
$t_{1/2}$ (h)	22.3	31 (4 dp)	nc	30 (2 dp)

[a] nc = not calculable; DT = daytime; NT = nighttime; and dp = data points.

To validate specificity of determined 8-PN in enzyme treated urine samples, extracts of two urine samples collected directly before beer consumption were separated by HPLC and eluates were collected in fractions. Residues were then analyzed by RIA. As shown in **Figure 6**, only negligible interference was observed and the assay can therefore be regarded as highly specific.

The mean (± SD) value of excretion of free 8-PN was 0.08 ± 0.06 μg for TD 1 (no 8-PN dose) and then increased to 0.14 ± 0.04, 0.54 ± 0.38, and 0.85 ± 0.53 μg for TD 2−4 with nominal 8-PN doses of 7.5, 16.5, and 33 μg, respectively. Excreted amounts correlate with increasing 8-PN doses for TD 2−4. Following consumption of the spiked beer (TD 5) and authentic 8-PN (TD 6), about 3.5 μg was excreted, which is less than half of the amount expected by linear extrapolation of dose increases (ca. 13 μg).

8-PN conjugates are the primary renal excrements. Mean values of 8-PN conjugates plus free 8-PN excretion were 10−20-fold higher than for free compound alone (**Table 4**). Again, there was a detectable excretion of 8-PN at TD 1. On TD 1, an average 1.6 ± 0.7 μg was found although no 8-PN or IX had been administered. This mean value corresponds well to preconsumption excretion of 1.4 or 1.2 μg of 8-PN in the IX conversion test (**Table 5**). It therefore appears most likely that, beside beer, there is one or more hitherto unknown dietary source of 8-PN or IX, which itself can be converted into 8-PN. Following TDs 2−4, an increase in excreted amounts was observed with increasing doses. However, interindividual variations were high leading to a CV of means of above 100% (**Table 4**). This points to a nonnormal distribution of values or to outliers. Indeed, if values of participant MH were not included into mean value calculations, CVs ranged from 50 to 60%. On TDs 2−4, the mean 8-PN excretion accounted for 41, 26, and 50% of nominal 8-PN dose, respectively. Excretion of nominal

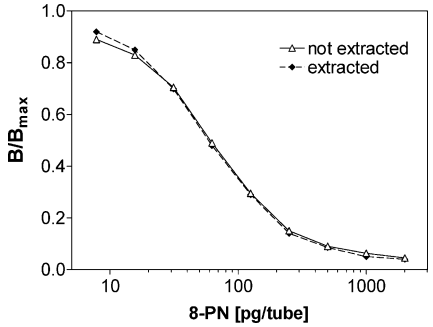

Figure 5. RIA using *anti*-H-25 (total IgG fraction) and ^{3}H-8-PN as tracers; shown are nonextracted and ether-extracted standard curves.

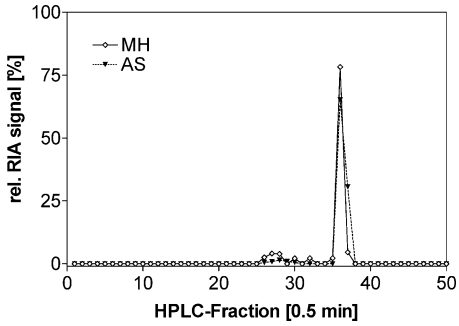

Figure 6. HPLC separation of blank urine extracts following cleavage of conjugates and subsequent quantification of residues per eluent fraction by RIA. Amounts per fraction were converted into % of amounts found in total chromatography. 8-PN elutes in fractions 36–37. MH (AS) means blank sample before TD 4 (*5*).

doses of TDs 5 and 6 (283 and 250 μg) was much lower with values of 16 and 13%, respectively.

Adding up all 8-PN excreted per participant for TD 1−TD 6, the mean amount was 99 ± 62 μg (range: 38−209 μg). This corresponds to 17% of total 8-PN dose (590 μg).

Renal Excretion in IX Conversion Test. Two participants of the beer consumption test (MH and OS) volunteered for an oral intake of 10 mg of IX. Twelve hour urine fractions were collected for 4.5 (MH) and 3.5 (OS) days. 8-PN was quantified in urine after cleavage of conjugates (**Table 5**).

Again, there were positive pretest 8-PN signals in the RIA, suggesting a daily excretion of 1.2−1.4 μg of 8-PN not related to beer consumption. In one subject, 35 μg of 8-PN was found in 4.5 days of urine and roughly the half (15 μg) in the 3.5 days urine of the other subject. Generally, after the first day, a higher proportion of the daily excreted amount was found in urine collected overnight. On the basis of 12 h data, the excretion half-life for MH was determined to 22.3 h, and a somewhat slower elimination was estimated using daily excreted amounts of both subjects.

Because it is known that the RIA has a low but measurable cross-reactivity to IX (0.15%), the specificity of analysis was verified as described above. As shown in **Figure 7**, there were minor elevations in fractions 21 and 28 (OS) or 26 and 30 (MH), which probably reflect large amounts of IX in the urine. However, about 90% of the RIA detection was due to 8-PN eluting in fraction 36. Thus, although specificity of RIA measurements for 8-PN in urine after intake of IX was not 100%, data can be used for further considerations and clearly demonstrate an in vivo conversion of IX into 8-PN.

Conclusions on In Vivo Tests. The availability of a highly specific and sensitive RIA for 8-PN detection was the basis for the present two pilot studies in which uptake of different 8-PN

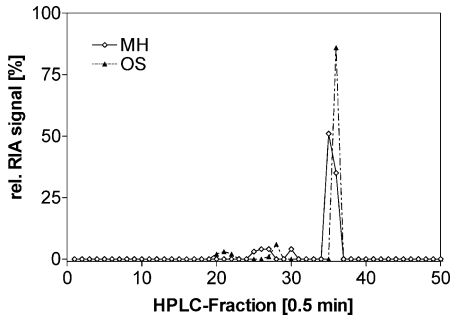

Figure 7. HPLC separation of ether extracts of two urine samples after cleavage of conjugates and subsequent RIA analysis of residues of HPLC fractions; sample MH (OS) means 12–24 h (0–12 h) collection period after oral intake of 10 mg of IX; 8-PN elutes in fraction 36; data are shown as a percentage per fraction of total HPLC run.

doses in beer and the conversion of IX into 8-PN were tested. Excretion data can be interpreted within the context of renal excretion of free and conjugated 8-PN elaborated within a clinical phase Ia study (*24*). The study revealed that only 0.23% of a 50 mg oral dose was renally excreted as free 8-PN and about 8% as conjugates. In contrast to these figures, markedly higher mean values of excreted free and conjugated 8-PN were estimated after beer consumption. On TDs 2−6, free 8-PN excretion accounted for 1.8, 3.2, 2.5, 1.3, and 1.4% of nominal 8-PN dose. A respective calculation on the basis of excreted conjugates (**Table 4**) reveals similar high values, i.e., 41, 26, 50, 16, and 13% of the nominal dose. Thus, both data sets clearly show much higher than expected excretion on the basis of phase Ia study data. Possible explanations include different pharmacokinetics of 8-PN after a high (50 mg) acute oral dose and low (μg range) doses in beer. However, it is rather improbable that routes of elimination have changed with different doses, because complete absorption of the high dose was found in the clinical study. The degree of metabolism could have changed with different doses, but in this scenario, the low dose (beer) would have been metabolized to a higher extent than the high dose. Therefore, differences in pharmacokinetics at different 8-PN doses do not offer a plausible explanation for the present results.

There is, however, another possible source for 8-PN in beer. Beer spiced with hop products contains about 2 mg IX/L. Because concentrations of 8-PN (ca. 20 μg/L) and IX in beer differ by a factor of 100, a low in vivo conversion of IX into 8-PN (by oxidative *O*-demethylation) could significantly increase the total 8-PN dose. Very recently, it was shown that human liver microsomes indeed convert IX into 8-PN in vitro (*25*). The pilot test in two individuals ingesting 10 mg of IX showed that this conversion indeed has occurred. Estimated conversion rates were 1.9 (OS) and 4.4% (MH) of the IX dose and generated 8-PN was excreted with a half-life of about 1 day, which—at a daily consumption of beer—would lead to an 1.6-fold accumulation of 8-PN. Obviously, the amount of 8-PN in beer is not the only and main source of estrogenicity but also the conversion of IX needs to be considered.

Whether consumption of high amounts of beer over a long period of time causes estrogenic effects and might feminize men has been the subject of numerous discussions. Clearly, with the presented limited data, this question cannot be answered but rather helps us to focus the ongoing debate by developing a "worst case scenario" for the exposure to 8-PN. With an assumed individual IX conversion rate of 5%, a daily consumption of 3 L of beer (50 μg 8-PN/L, 2 mg IX/L) and calculating with an accumulation factor of 2, the resulting steady state body burden of 8-PN can be approximated to 1 mg/day. More studies on steady state 8-PN burden and variations in IX conversion need to be done in order to get more information and a proper basis for estimating various consumer scenarios. The developed RIA has been proven to constitue an excellent tool for respective future investigations.

Table 6. Concentrations of 8-PN in Various Beer Brands and Their Spicing Methods[a]

code (origin)	type	8-PN (μg/L)	spicing
A (D)	nonalcoholic	< 1.6	I
B (D)	pilsner	15.1	I + II
C (D)	pilsner	33.0	I + II + III
D (D)	pilsner	7.3	I + III
E (D)	pilsner	16.0	III
F (D)	pilsner	16.6	II + III
G (D)	pilsner	8.1	I + III
H (D)	cryst. wheat	12.0	III
I (D)	cryst. wheat	10.3	III
K (D)	diet	7.1	I + III
L (CZ)	pilsner	16.3	nr
M (D)	stout	24.3	III
N (D)	stout	98.4	II + III
O (IR)	stout	46.2	I + III
P (IR)	stout	138.5	nr
Q (FIN)	porter	41.9	I + III

[a] Explanation: Origin D, CZ, IRL, FIN = Germany, Czech Republic, Ireland, Finland; nr = no response from brewery; spicing methods: I = CO_2 extract, II = ethanolic extract, and III = hop or hop pellets.

As a result of the high sensitivity of the RIA, we found elevated blank 8-PN values and a daily "normal" excretion of about 1.5 μg of 8-PN. We conclude that beer is not the only source of 8-PN in the diet. The present data show that there are at least two possible sources for 8-PN in the diet or in beverages, i.e., 8-PN itself and IX. This conclusion is in conflict with the literature, which negate other sources for 8-PN except hop. However, even low concentrations in vegetarian foodstuffs escaping the used detection methods or in meat of animals fed with hop tresters from CO_2 extracts might have been sufficient to raise pretreatment blank values. The RIA and the cleavage of conjugates represent a very powerful system for detection of low 8-PN concentrations in urine. It is estimated that a daily dose of less than 1 μg of 8-PN can be detected by our system.

8-PN Concentrations in Beer Brands. RIA results (**Table 6**) show that 8-PN concentrations vary widely between different brands. Most beers tested contained less than 40 μg/L, but in some stouts, 8-PN concentrations were close to or even above 100 μg/L. Beer brands A−C were used in the drinking test described above.

Principal techniques of beer spicing employ CO_2 extracts of hop lacking both 8-PN and IX, ethanolic hop extracts containing both compounds or pellets of dried hop. Most beers listed in **Table 6** were spiced by combined methods and often by a combination of CO_2 extract and dried hop. Respective 8-PN concentrations (beers D, G, K, O, and Q) were either below 10 or above 40 μg/L, reflecting different amounts of used hop products. In agreement with the soley applied CO_2 spicing, no 8-PN was found in beer A. However, no clear correlation was seen between the method of beer spicing and the found 8-PN concentrations because too many other variables were not known.

ABBREVIATIONS USED

8-PN, 8-prenylnaringenin; 6-PN, 6-prenylnaringenin; IX, isoxanthohumol; XH, xanthohumol; cBSA, cationized bovine

serum albumin; POD, peroxidase; OVA, ovalbumin; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; MTBE, methyl *tert*-butyl ether.

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Supporting Information Available: Details on syntheses and spectrometric data of reported new compounds; coupling efficiency of haptens to cBSA, OVA, and POD; and titer development of antigenes of 8-PN in rabbits. This material is available free of charge via the Internet at http://pubs.acs.org.

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