食品中化学物質のリスク評価について
—自社製品についてリスクプロファイルを作成しよう

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要　旨

食品は化学的には無数の化合物のかたまりであり、その中には構造や性質がわかっているものから全く不明のものまでが多数存在する。私たちはその全てについて知っているわけではないが、それでも製品を安全だとして販売したり、購入して調理して食べたりしている。加工食品の場合、製造業者は食品をゼロから合成するわけではなく原材料を購入し、加工して販売しているのであるが、消費者にとってはその製品の安全性については製造・販売業者に全責任があると考えられるだろう。実際に商品の原材料や加工工程について最も多くの情報をもっているのは製造業者であろう。従ってその製品にとって何がリスクになりうるのか、どういう注意が必要なのかについては事業者自らが評価しておくことが望ましい。カラメル色素の不純物を例に、リスクプロファイルの作成を薦める。

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<Summary>

Food consists of many chemical components which properties are known or unknown. We don't know them exactly, though we are selling, buying, cooking and consuming them as deemed safe. Food manufacturers produce prepackaged foods from many ingredients bought from other companies or farmers. They do not synthesize food from pure chemical, but it is manufactures' responsibility for safety and quality of the products from consumer's point of view. Actually, it is the manufacture that knows best about the products, for example, how they are processed, what ingredients are used and so on. It is the reason why manufactures are recommended to assess the risk and provide risk management procedure ahead. This paper presents an example, 4-methylimidazole, a contaminants in caramel colors.

1．はじめに

食品には意図的に加えられたものも、そうでないものもあわせて無数の化合物が含まれているが、その全てを知ることは不可能である。現代の日本でも食中毒はそれなりの頻度で発生しているのだが、消費者の間には食べることが時に命の危険を伴うものだという認識はほとんどなく、食品は「安全で安心」なのが当然だという感覚が広がっているようだ。そして近年の分析技術の進歩もあって、時に思いがけないものが食品から検出されたということがニュースになる。問題なのはそれがどの程度の量でどの範囲の食品に入っているか、なのであるが、検出されただけで危険だ、許されない、といった論調が相変わらず多い。食品中に含まれる化学物質のリスク

Risk Assessment of Chemicals in Food - Recommendation of Voluntary Assessment of Your Own Products

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は、残留農薬や食品添加物のように意図的に使用されるものについては極めて低くなるように管理されている。しかし意図せず含まれるものについては必ずしもそうではなく、さらにリスク評価ができるほどのデータが無い場合もある。それでもその食品の製造・販売業者には消費者の疑問に答える、あるいは不安に対応する必要がある。そこで推奨したいのが自社製品に存在しうるハザードについて、リスクプロファイルを準備しておくことである。もともと製品についてよく知っているのは事業者のはずである。食品の安全性を担当している行政機関は、どんなに優れた能力を持っていたとしても、個別製品の製造工程や原材料の入手先などの詳細については企業からの情報提供がない限り、わからないのである。最も良く知っているところが最も良いプロファイルを作ることができるはずである。

2．カラメル色素の 4-MEI を例に

最近米国で話題になり、韓国や日本でも一部が取り上げたカラメル色素中の不純物である 4-メチルイミダゾール（4-MEI）を例にリスクプロファイルを考えてみよう。なおこれは、筆者が公開されているデータのみをもとに試作したものであり、事業者であれば個別の製品の実際の使用量などのデータが使えるはずである。

(1) レベル 1 ― 一目でわかる簡単なまとめ

できるだけ簡単に、一目で全体が把握できるようなものを作る。順番として最初にもってきたのは、情報を知りたい人（例えば消費者相談窓口のオペレーターが消費者からの質問にすぐに答えたい時など）にとってそれが真っ先にあると便利だからで、作成手順としては詳細データをレビューした上で、最後になる。

BfR（ドイツ連邦リスク評価研究所）のリスクプロファイルの様式を一部改変して使うと表 1 のようになる。このフォーマットは全ての化合物に共通で、どこに色が付いているかで判断する。コーラ飲料に使われるカラメル色素中の不純物としての 4-MEI については比較的簡単に、安全上問題となることはないと判断できる。

(2) レベル 2 ―リスクプロファイルシートの作成

よく使われるリスクプロファイルシートの形（表 2）を使ってみた。

表 1　カラメル色素の 4-MEI
Table 1　Risk Profiles of 4-MEI in Caramel Colors（short version）

対象となる集団	コーラを飲む一般人			
健康被害の可能性	通常の摂取で健康被害がでる可能性はない			
健康被害の重症度	なし	僅かな影響 可逆／不可逆	中程度の影響 可逆／不可逆	重大な影響 可逆／不可逆
入手できるデータの信頼性	高い 最も重要なデータがあり 整合性がある	平均的 いくつかの重要なデータが不足しているあるいは矛盾がある		低い 重要なデータが無い、 あるいは矛盾する
消費者によるコントロール	コントロールの必要はない	予防的措置で コントロールできる	摂取を差し控えることで コントロールできる	コントロールできない

表 2　4-MEI の安全性に関するリスクプロファイルシート
Table 2　Risk Profiles of 4-MEI in Caramel Colors（full version）

（○年○月○日）

項　目	内　容
ハザードの名称	4-メチルイミダゾール 4-methylimidazole CAS 番号　822-36-6 化学式　C4H6N2 分子量　82.11 同義語　1H-Imidazole, 4-methyl (9CI) ; imidazole, 4-methyl; 4 (5) -methylglyoxaline; 4 (5), 4 (5) -methylimidazole; 5-methylimidazole （他に構造式など）

由来	・食品　カラメル色素 E150c（クラスⅢ）と E150d（クラスⅣ）の不純物。 ・発酵食品の副生成物　アンモニアと還元糖の反応で生じる。 （注：カラメルⅢ：砂糖またはブドウ糖に代表される食用炭水化物に、アンモニウム化合物を加えて、またはこれに酸もしくはアルカリを加えて熱処理を行う。 カラメルⅣ：砂糖またはブドウ糖に代表される食用炭水化物に、アンモニウム化合物および亜硫酸化合物を加えて、またはこれに酸もしくはアルカリを加えて熱処理を行う。） ・食品以外　医薬品、写真用化合物、色素、ゴムなど多様な製品の成分である。 ・タバコの煙に含まれる。
基準値、その他のリスク管理措置	
EFSA（欧州食品安全機関）	・食品添加物のカラメル色素については EU グループ ADI（一日摂取許容量）300 mg/kg 体重 / 日（カラメル色素そのものの NOAEL（最大無毒性量）は数～数十 g/kg 体重 / 日）。 ・カラメル色素中の 4-MEI の含有量？については指令 2008/128/EC により最大 250 mg/kg 以下。 ・4-MEI の NOAEL は 80 mg/kg 体重 / 日（NTP（米国国家毒性プログラム）の試験結果から。遺伝毒性はないと評価。仮に安全係数 100 を採用すると TDI（耐容一日摂取量）は 0.8 mg/kg 体重 / 日になる）。
JECFA（FAO/WHO 合同食品添加物専門家会議）	・カラメル色素としてのがん原性や変異原性は陰性。
カリフォルニア州	・Prop65（Propositio65 Safe Drinking Water and Toxic Enforcement Act of 1986；プロポジション 65）でヒト発がん物質の疑い有りで NSRL（No Significant Risk Level；無有意リスク量）29 μg/day（2012 年 6 月）と評価し 1 日の摂取量がこれを超えるものは発がん性の警告表示が必要。
米国、豪州	・カラメル色素は GMP に則って製造したものが食品添加物として各種食品に使用することが認められている。
TDI、ADI および ARfD（急性参照用量）の設定根拠	・F344 ラット雌雄　発がん性なし。 ・B6C3F1 マウス オス　投与量　0, 312, 625, 1,250 ppm（摂取量で約 40, 80, 170 mg/kg 体重）肺胞／細気管支腺腫あるいはがん（合計）(9/50, 13/50, 16/50, 22/50) メス　0, 312, 625, 1,250 ppm　肺胞／細気管支腺腫あるいはがん（合計）(3/50, 8/50, 17/50, 14/50)
毒性	・目と皮膚刺激性。
吸収、分布、排出および代謝	・ヒツジに 20 mg/kg 経口投与した場合の半減期は 9.37 hr。吸収は早く (t1/2abs =1.52 hr) 分布容量は大きい (65.8 L)。 ・ラットに 2.5 g/kg 体重経口投与すると主な排泄は糞便。96 時間以内に 99 % 以上排泄される。 ・乳牛に数 g 投与するとミルクにも検出される。
急性毒性	・LD50 ニワトリ腹腔　210 mg/kg ニワトリ経口　590 mg/kg マウス腹腔　165 mg/kg マウス経口　370 mg/kg ウサギ腹腔　120 mg/kg ラット経口　751 mg/kg
反復投与毒性／発がん性／生殖発生毒性等	・NTP　2 年間経口投与 F344/N 雄ラットではがん原性について根拠無し。 F344/N 雌ラットでは単核球白血病（9/50, 7/50, 16/50, 20/50）の増加を根拠にはっきりしない（Equivocal）根拠。 ラットの餌の用量 0, 1,250, 2,500, or 5,000 ppm。 B6C3F1 雄マウスでは肺がん（2/50, 4/50, 4/50, 8/50）肺胞／細気管腺腫およびがんの合計（9/50, 13/50, 16/50, 22/50）で明確な根拠。 B6C3F1 雌マウスでは肺がん（0/50, 8/50, 16/50, 8/50）、肺胞 / 細気管がん（3/50, 0/50, 2/50, 7/50）、肺胞／細気管腺腫およびがんの合計（3/50, 8/50, 17/50, 14/50）で明確な根拠。 マウスの餌の用量 0, 312, 625, or 1,250 ppm。
遺伝毒性	・*Salmonella typhimurium* 遺伝子突然変異試験： TA97, TA98, TA100, および TA1535 で S9 有り・無しどちらも陰性。 ・小核赤血球： ラット骨髄 *in vivo*、腹腔内投与、陰性 マウス骨髄 *in vivo*、腹腔内投与、陰性 マウス末梢血 *in vivo*、雌雄、陰性
推定摂取量	・CSPI（公益科学センター）の調査で日本のコーラ 0.2 mg/kg、体重 50kg の人が 1 日 1 L 飲むとすると 0.2/50 = 0.004 mg/kg 体重 / 日。
MOE（暴露マージン）	・0.2 ppm の 4-MEI を含むコーラ 500 mL を体重 50 kg のヒトが飲むとすると、摂取量は 0.2 mg/kg × 0.5 kg/50 kg = 0.002 mg/kg 体重。NOAEL 80 mg/kg 体重 / 日を POD（Point of Departure；用量反応曲線の出発点）とすると MOE = 40,000。1 L 飲むと 20,000。
添加物が使用される食品	・カラメル色素としての使用。 着色料としては使用量が最も多い。 清涼飲料水、アルコール飲料、漬物、醤油、ソース、みそ、菓子、乳製品、加工食品、薬品、化粧品、ペットフード等。
リスク管理を進める上で不足しているデータ等	特になし。
消費者の関心・認識	・現時点では日本でそれほど関心が高いようには見えない
その他	・4-MEI が含まれるカラメル色素を使って醸造したビールからは検出されない。 ・EU の HPV Chemicals（高生産量化学物質）、ESIS（European chemical Substances Information System；欧州化学物質情報システム）データベース収載。 http://esis.jrc.ec.europa.eu/
注目されるようになった経緯	・カリフォルニア州の Prop65 によりヒト発がん物質の疑い有りで NSRL29 μg/day（2012 年 6 月）と評価し、1 日の摂取量がこれを超えるものは発がん性の警告表示が必要となった。これはカリフォルニア OEHHA（環境保健有害性評価局）が遺伝毒性発がん性についての懸念が明確に否定されなければ遺伝毒性ありとみなすこと、動物実験の投与量のヒトへの換算を体重あたりではなく体表面積あたりでする、などの独特の評価をしているためである。そのためコカコーラ社が製品の組成をみなおした。 ・米国の NPO である CSPI がコーラの濃度を調査し、アメリカは 0.4 ppm, カナダ・イギリスは 0.4 ～ 0.45 ppm, 日本は 0.2 ppm, ブラジルは 0.75 ppm と発表。 ・日本では特定保健用食品のコーラが話題になった時に一部で報道された。

(3) レベル3―参考文献

リスクプロファイルシート作成に用いた文献等を示す。必要に応じ原本の要約や翻訳などを準備しておくと、いざというときに役にたつかもしれない。

カラメル色素の4-MEI関連の参考文献等は、下記を参照。

3．安全のために、リスクについて語る

近年の食品安全確保のための考え方は、事件や事故があったらその原因を調べて対応する、のではなく、ハザードになりそうなものを予め想定して事前に措置を講じるというものである。食品については、農場から食卓まで、全ての段階でそれぞれの関係者が自分の責任をもつ部分についてしっかり責任を果たして初めて安全性が確保される。これは消費者にも明確な責任があるということである。例えばある食品を食べ過ぎて消費者が肥満になったとして、それはメーカーだけに責任があるとは言えないであろう。ただしメーカーにも栄養成分表示や健康的な使用方法の説明などのある程度の責任はもちろんある。経営者のなかには、自社製品のリスクについて語ることそのものに拒否感があるかもしれないが、それでは社会的責任が果たせないのである。社内でも対外的にも、この商品にはこういうところにリスクがあるのでそこに気を付けようと常に言える文化が望ましい。もともと全ての食品にはリスクがあるのである。一部の事業者が「無添加」や「無農薬」のような根拠のない安全性宣伝をすることで一部の消費者の誤解による支持を得ているのは、食品そのもののもつリスクについて消費者に十分な情報が提供されていないことも理由の一つである。現に存在するリスクについて見て見ぬふりをすることは「安全神話」と言われる。「食品は100％安全であるべき」「食品成分だから安全です」といった言説がまさに「安全神話」であり、真の安全性確保とはほど遠い。リスクに向き合ってこそ管理が可能になる。「安全のためにリスクを語る」ことを日常の風景にしたい。

＜参考文献・サイト＞

EFSA

- カラメル色素：消費者暴露量は先に推定されたものより低い
  Caramel colours: consumer exposure lower than previously estimated
  19 December 2012
  http://www.efsa.europa.eu/en/press/news/121219.htm
- カラメル色素（E 150a, c, d）の詳細暴露評価
  Refined exposure assessment for caramel colours (E 150a, c, d)
  EFSA Journal 2012; 10 (12) : 3030 [39 pp.].
  19 December 2012
  http://www.efsa.europa.eu/en/efsajournal/pub/3030.htm
- EFSAはカラメル色素の安全性を評価
  EFSA reviews safety of caramel colours
  8 March 2011
  http://www.efsa.europa.eu/en/press/news/ans110308.htm
- 食品添加物としてのカラメル色素（E 150 a, b, c, d）の再評価に関する科学的意見
  Scientific Opinion on the re-evaluation of caramel colours (E 150 a, b, c, d) as food additives
  EFSA Journal 2011; 9 (3) :2 004 [103 pp.]
  08 March 2011
  http://www.efsa.europa.eu/en/efsajournal/pub/2004.htm
  E 150cについて低いADIを設定したのはその成分の1つである2-アセチル-4-テトラヒドロキシブチルイミダゾール（THI）の免疫系への影響に不確実性があることを考慮したもの。
- ChemIDplus - 4-Methylimidazole - Chemical information with searchable synonyms, structures, and formulas
  TOXNETから検索
  http://toxnet.nlm.nih.gov/

JECFA

- CARAMEL COLOURS
  http://www.inchem.org/documents/jecfa/jecmono/v20je11.htm
  カラメル色素としてのがん原性や変異原性は陰性。

NTP

- Abstract for TR-535 - 4-Methylimidazole (CASRN

822-36-6)
Toxicology and Carcinogenesis Studies of 4-Methylimidazole (CAS No. 822-36-6) in F344/N Rats and B6C3F1 Mice (Feed Studies)
http://ntp.niehs.nih.gov/index.cfm?objectid=9B956B07-F1F6-975E-79BBCDCCD57001C8

KFDA（韓国食品医薬品安全庁）（当時；現在 MFDS（食品医薬品安全処））

・韓国内流通中の‘コーラ’4-MI 検査結果発表
添加物基準課 / 釜山庁有害物質分析課 2012.08.08
http://www.kfda.go.kr/index.kfda?mid=56&pageNo=2&seq=18386&cmd=v
食品医薬品安全庁が 7 月、コカコーラ、ペプシコーラなど国内流通中の 8 社 16 コーラ製品を対象に 4-MI 含量を検査した結果、平均 0.26ppm（最小 0.021 ～最大 0.659ppm）だった。
コカコーラは最小 0.188ppm、最大 0.234ppm、ペプシコーラは最小 0.247ppm で最大 0.459ppm だった

メディア報道等

・コカコーラの発がん物質レベルは世界で多様
CSPI
Tests Show Carcinogen Levels in Coca-Cola Vary Worldwide
June 26, 2012
http://www.cspinet.org/new/201206261.html

・Prop65　2012 年 6 月バージョン
http://oehha.ca.gov/prop65/pdf/2012StatusReportJune.pdf
参考までに Prop65 では：
アクリルアミドは NSR 0.2μg
カドミウム（生殖発生毒性）1 日 4.1μg

・コークとペプシは「発がん性」表示を避けるために色素を変える
Coca-Cola and Pepsi ‘change recipe to avoid putting a cancer warning on their labels’
March 8, 2012
http://cnews.canoe.ca/CNEWS/World/2012/03/08/19478241.html
http://www.dailymail.co.uk/news/article-2112335/Coke-Pepsi-change-recipe-avoid-putting-cancer-warning-labels.html
コカコーラとペプシは、カリフォルニアの法律により「発がん性」と表示しなければならなくなるのを避けるために、製造工程を見直すだろう。

・米国の規制機関は発がん性炭酸飲料という知見に異議を唱える
US regulators dispute finding of cancer-causing soda
March 5
http://www.reuters.com/article/2012/03/06/soda-fda-idUSL2E8E5DSB20120306
米国の NPO、CSPI が、コカコーラやペプシコーラなどに使われているカラメル色素に安全でない量の発がん物質が含まれていると報告したが、FDA は健康リスクはないと言っている。

・キリンメッツコーラは発がん物質入り　「発がんコーラ」はトクホにふさわしくない
08/22 2012
http://www.mynewsjapan.com/reports/1681

・キリンビバレッジ
キリンメッツコーラに含まれるカラメル色素の安全性について。
2012 年 8 月 22 日
http://www.beverage.co.jp/csr/hinshitsu/topics/detail.php?topics=16

・コーラに発がん性？　また同じトリックが使われている
2012 年 8 月 28 日
http://www.foocom.net/column/editor/7568/

・日本カラメル工業会
http://www.morita-fs.co.jp/caramel/pdf/caramel-a4.pdf

BfR

・現在の科学的知見からは食品中の 5-HMF 濃度は安全上の懸念とはならない
17.08.2011
According to the current state of scientific knowledge 5-HMF concentrations occurring in foods do not give rise to safety concerns
http://www.bfr.bund.de/cm/349/according-to-the-current-state-of-scientific-knowledge-5-hmf-concentrations-occuring-in-foods-do-not-give-rise-to-safety.pdf

FDA

・Color Additive Status List
http://www.fda.gov/ForIndustry/ColorAdditives/ColorAdditiveInventories/ucm106626.htm
カラメルは　LIST 4。
Color additives exempt from certification and permanently listed for FOOD use.

FSANZ（豪州・ニュージーランド食品基準機関）

・Australia New Zealand Food Standards Code - Standard 1.3.1 - Food Additives
Colours permitted in accordance with GMP in processed foods specified in Schedule 1
http://www.foodstandards.gov.au/code/Pages/default.aspx
食品添加物として記載。

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Article

Characterization of Natural Aryl Hydrocarbon Receptor Agonists from Cassia Seed and Rosemary

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Abstract: Many recent studies have suggested that activation of the aryl hydrocarbon receptor (AhR) reduces immune responses, thus suppressing allergies and autoimmune diseases. In our continuing study on natural AhR agonists in foods, we examined the influence of 37 health food materials on the AhR using a reporter gene assay, and found that aqueous ethanol extracts of cassia seed and rosemary had particularly high AhR activity. To characterize the AhR-activating substances in these samples, the chemical constituents of the respective extracts were identified. From an active ethyl acetate fraction of the cassia seed extract, eight aromatic compounds were isolated. Among these compounds, aurantio-obtusin, an anthraquinone, elicited marked AhR activation. Chromatographic separation of an active ethyl acetate fraction of the rosemary extract gave nine compounds. Among these compounds, cirsimaritin induced AhR activity at $10–10^2$ μM, and nepitrin and homoplantagenin, which are flavone glucosides, showed marked AhR activation at $10–10^3$ μM.

Keywords: aryl hydrocarbon receptor; health food; cassia seed; rosemary; reporter gene assay

1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that is present in mammalian cells and tissues. The AhR has also been referred to as dioxin receptor because it binds environmental pollutants (e.g., dioxins) and is involved in biotoxicity linked to xenobiotic AhR ligand exposure in animals, including cancer, reproductive impairment, and immunological impairment [1–3]. Although studies have identified numerous xenobiotic ligands for the AhR, such as dioxins, the essential functions of the AhR are largely unknown; therefore, the AhR is still regarded as an orphan receptor.

Functional elucidation of AhR activation by non-toxic ligands such as food constituents has been reported in recent years [4–6]. The AhR has been identified as a target of several signaling pathways that cross-talk with its own regulatory pathway, such as proteasomal degradation, redox-sensitive transcription factors, and mitogen-activated protein kinases (MAPKs) [7,8]. Several studies have also found that the AhR plays an important role in immune system function [9–12]. For example, activation of the AhR is associated with various effects on dendritic cells (DCs) and regulatory T cells and has been shown to mediate the Th1/Th2 cell balance. These cells play a major role in the development of food allergies, an increasing health problem in both humans and animals. Despite existing knowledge regarding the risk factors of and cellular mechanisms underlying food allergies, no approved treatments are yet available. Activation of the AhR by dioxin-like compounds has been shown to suppress allergic sensitization by reducing the absolute number of precursor and effector T cells, preserving $CD4^+$ $CD25^+$ $Foxp^{3+}$ T_{reg} cells, and affecting DCs and their interactions with effector T cells. Additionally, tranilast, an anti-allergy drug, has been shown to cause significant upregulation of *microRNA* (*miR*)-*302* by activation of the AhR [13]. Thus, dietary ligands of the AhR may have anti-inflammatory, anti-allergy, anti-cancer, and immunoregulatory effects. However, while although the role of the AhR in the response to environmental toxins is widely accepted, its broader role in adapting the response to natural ligands is limited. Therefore, it is necessary to characterize various natural AhR ligands.

In the current study, we sought to further characterize AhR agonists present in foods. We examined the AhR activities of 37 health food materials using an *in vitro* reporter gene assay called the chemical-activated luciferase gene expression (CALUX) assay [14–16]. Active sample extracts were subsequently fractionated, and chromatography was performed to characterize the fractions containing AhR activity and associated individual constituents.

2. Results and Discussion

2.1. AhR Activities of Health Food Materials

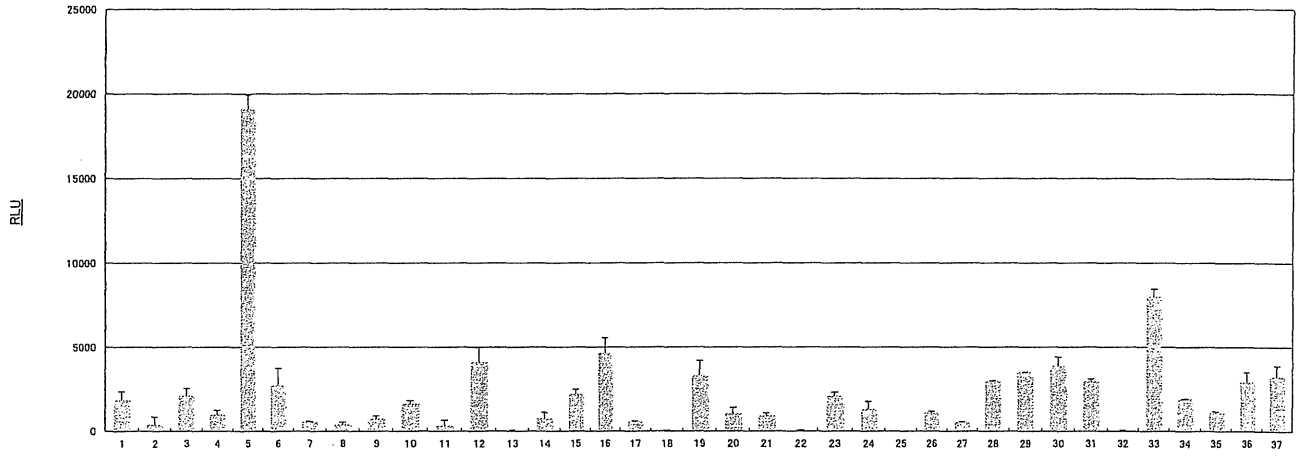
The *in vitro* AhR activation potencies of 37 samples, including the fruits and herbs listed in Table 1, were estimated using the CALUX assay, and the results are shown in Figure 1. Of the samples tested, sample 5 (cassia seed extract) showed the most remarkable induction of luciferase activity, followed

by sample 33 (rosemary extract), with luciferase activity producing more 8,000 relative light units (RLU). Samples 12 (*Eleutherococcus senticosus* rhizome), 16 (fenugreek), 19 (giant crape-myrtle), 29 (parsley), 30 (perilla herb), and 37 (yarrow) also exhibited luciferase activity higher than 3,000 RLU. The data suggest that cassia seed and rosemary may contain significant natural AhR agonists.

Table 1. List of health food materials used for the estimation of AhR activity

No.	Materials
1	Ashitaba (Japanese name) (*Angelica keiskei*)
2	Aloe (*Aloe arborescens*)
3	Amachazuru (Japanese name) (*Gynostemma pentaphyllum*)
4	Bitter melon (*Momordica charantia*)
5	Cassia seed (*Cassia obtusifolia*)
6	Celery (*Apium graveolens*)
7	Coix seed (*Coix lacryma-jobi*)
8	Cornus fruit (Cornus officinalis)
9	Crataegus fruit (*Crataegus cuneata*)
10	Echinacea (*Echinacea purpurea*)
11	Elder (*Sambucus racemosa*)
12	*Eleutherococcus senticosus* rhizome (*Eleutherococcus senticosus*)
13	Eucalyptus leaf (*Eucalyptus globulus*)
14	Eucommia bark (*Eucommia ulmoides*)
15	Fennel (*Foeniculum vulgare*)
16	Fenugreek (*Trigonella foenum-graecum*)
17	Field horsetail (*Equisetum arvense*)
18	Garcinia (*Garcinia verrucosa*)
19	Giant crape-myrtle (*Lagerstroemia speciosa*)
20	Ginger (*Zingiber officinale*)
21	Ginkgo (*Ginkgo biloba*)
22	Gymnema (*Gymnema sylvestre*)
23	Kaki persimmon (*Diospyros kaki*)
24	Lemon balm (*Melissa officinalis*)
25	Lemon grass (*Cymbopogon citratus*)
26	Linden (*Tilia europaea*)
27	Maca (*Lepidium meyenii*)
28	Mugwort (*Artemisia indica*)
29	Parsley (*Petroselinum crispum*)
30	Perilla herb (*Perilla frutescens*)
31	Plantago herb (*Plantago asiatica*)
32	Rabdosia herba (*Rabdosia japonica*)
33	Rosemary (*Rosmarinus officinalis*)
34	Sesame (*Sesamum indicum*)
35	Star anise (*Illicium verum*)
36	Sweet hydrangea leaf (*Hydrangea macrophylla*)
37	Yarrow (*Achillea millefolium*)

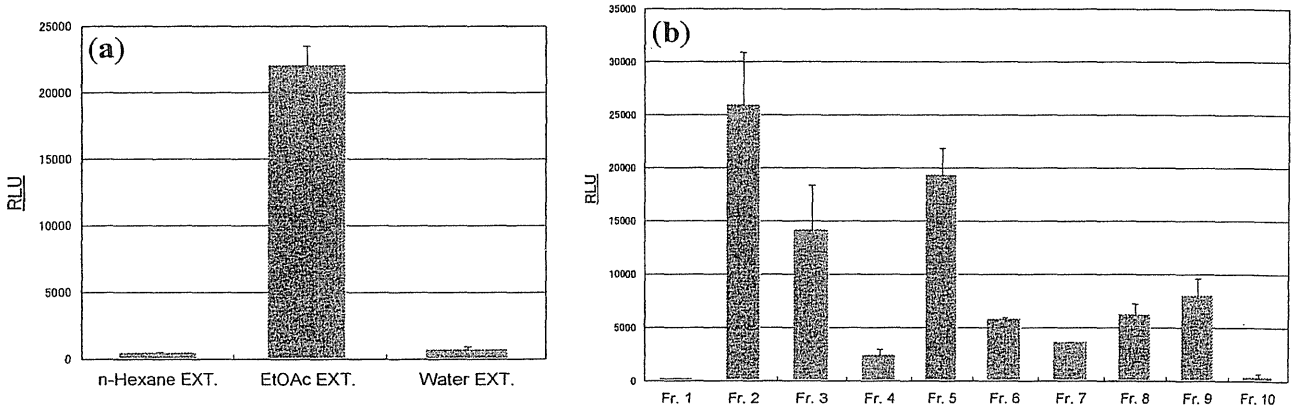
Figure 1. Induction of luciferase activity by health food materials in the CALUX assay. The numbers on the x-axis describe the components listed in Table 1. Sample extracts were used at a final concentration of 100 μg/mL. Results are expressed as means ± SDs.



2.2. Identification and AhR Activity of Constituents

To characterize the active components in sample 5 (cassia seed extract), the extract was first partitioned with organic solvent for separation into *n*-hexane-, ethyl acetate-, and water-soluble fractions. As shown in Figure 2a, AhR activity was present only in the ethyl acetate extract, which was separated by chromatography over Sephadex LH-20 with ethanol to afford 10 fractions (Frs. 1–10).

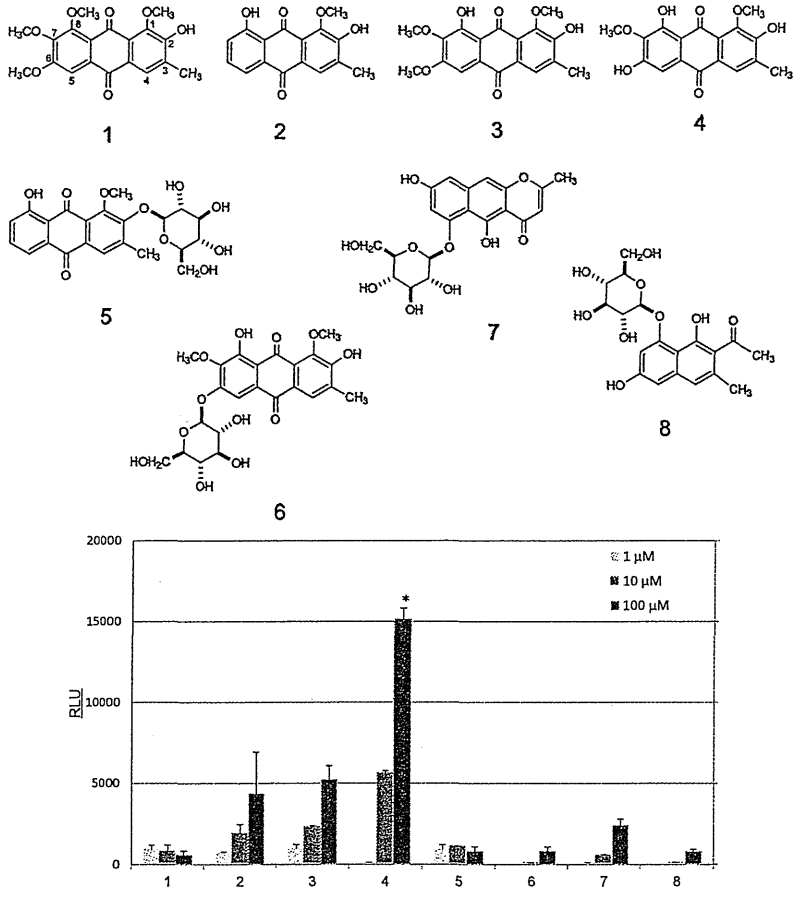
Figure 2. Induction of luciferase activity by cassia seed extracts in the CALUX assay. (**a**) Extracts from cassia seed. (**b**) Fractions from ethyl acetate extracts. Sample extracts were used at a final concentration of 100 μg/mL. Results are expressed as means ± SDs.



Fractions 2, 3, and 5, which exhibited marked AhR activation (Figure 2b), were purified by preparative TLC to afford eight compounds: chryso-obtusin (**1**), obtusifolin (**2**), obtusin (**3**), aurantio-obtusin (**4**), obtusin 2-*O*-glucoside (**5**), aurantio-obtusin 6-*O*-glucoside (**6**), nor-rubrofusarin 6-*O*-glucoside (**7**), and 6-hydroxymusizin 8-*O*-glucoside (**8**). Among these isolates, aurantio-obtusin (**4**) elicited marked AhR activation, followed by obtusifolin (**2**) and obtusin (**3**). In contrast, the glycosides [obtusifolin 2-*O*-glucoside (**5**), aurantio-obtusin 6-*O*-glucoside (**6**), nor-rubrofusarin 6-*O*-glucoside (**7**), and 6-hydroxymusizin 8-*O*-glucoside (**8**)] showed only slight activation of AhR (Figure 3). The

influence of this glycosidic feature on the activity of the related anthraquinones was similar to our previous findings that the AhR activity of isoflavones tended to be weakened by glycosidation [4]. It is notable that the presence of a hydroxyl group at C-8 on the anthraquinone skeleton is necessary for AhR activation.

Figure 3. Induction of luciferase activity in the CALUX assay of compounds isolated from cassia seeds. **1**, chryso-obtusin; **2**. obtusifolin; **3**. obtusin; **4**. aurantio-obtusin; **5**. obtusin 2-*O*-glucoside; **6**. aurantio-obtusin 6-*O*-glucoside; **7**. nor-rubrofusarin 6-*O*-glucoside; **8**. 6-hydroxymusizin 8-*O*-glucoside. * $p < 0.05$ *vs.* IAA.



Additionally, aurantio-obtusin (**4**), which was the most active compound, had a hydroxyl group at C-7 and C-9, which may also contribute to AhR activation. However, to discuss the structure-activity relationships in anthraquinones, additional data from more compounds are required. The results of the present study revealed that AhR activation by the cassia seed extract is associated with anthraquinones and that aurantio-obtusin (**4**) may be an important natural AhR agonist.

For the rosemary extract, AhR activation was also shown by the ethyl acetate-soluble fraction (Figure 4a). To identify the active compounds present, the ethyl acetate extract was subjected to chromatographic purification and chromatographed over a Sephadex LH-20 column with ethanol to afford eight fractions (Frs. 1–8). Fractions 2–8, which exhibited marked AhR activation (Figure 4b), were purified using a MCI-gel CHP-20P and YMC gel ODS-AQ column to give rosmarinic acid (**11**) as a major component and other eight compounds, *i.e.*, vanillic acid (**9**), caffeic acid (**10**), cirsimaritin (**12**),

ladanein (**13**), salvigenin (**14**), nepitrin (**15**), homoplantaginin (**16**), and 6″-*O*-(*E*)-feruloylnepitrin (**17**), as UV-sensitive constituents (Figure 5).

Figure 4. Induction of luciferase activity by rosemary extracts in the CALUX assay. (**a**) Extracts from rosemary. (**b**) Fractions from ethyl acetate extracts. Sample extracts were used at a final concentration of 100 μg/mL. Results are expressed as means ± SDs.

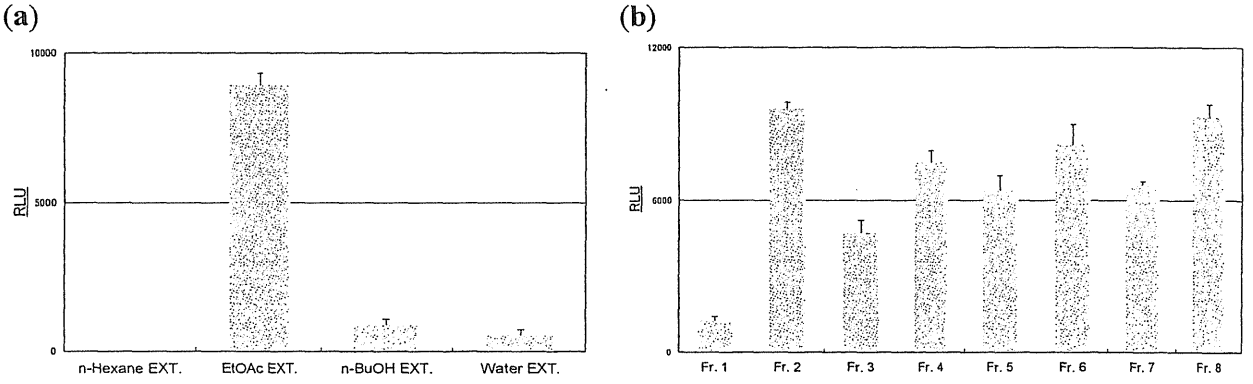
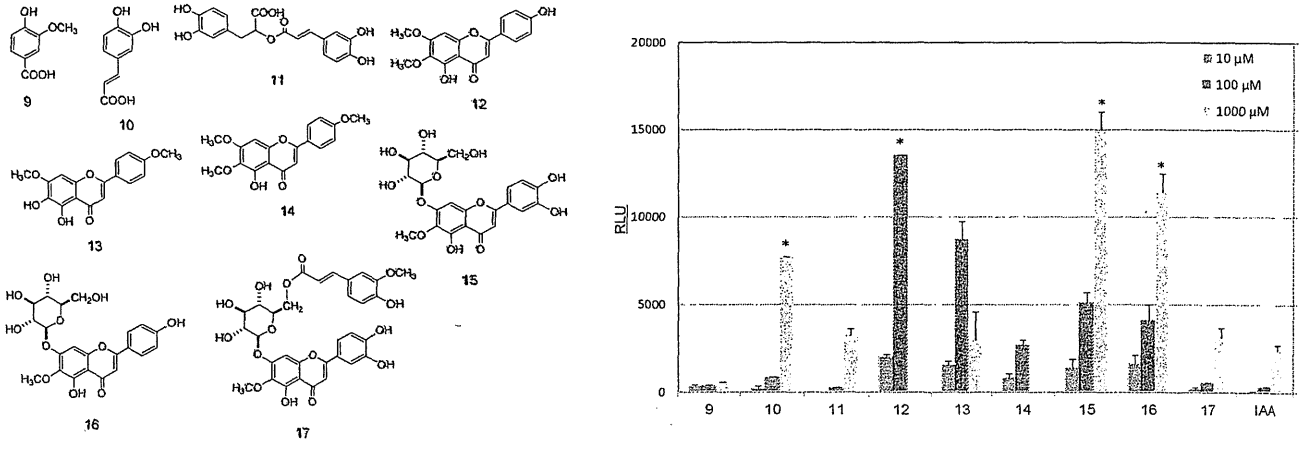


Figure 5. Induction of luciferase activity by compounds isolated from rosemary. The CALUX assay was used to measure luciferase activity. **9**. vanillic acid; **10**. caffeic acid; **11**. rosmarinic acid; **12**. cirsimaritin; **13**. ladanein; **14**. salvigenin, **15**: nepitrin, **16**. homoplantaginin; **17**. 6″-*O*-(*E*)-feruloylnepitrin; IAA. indole 3-acetic acid. * $p < 0.05$ *vs.* IAA.



The ability of compounds **9**–**15**, isolated from rosemary extract, to activate the AhR were examined using reporter gene assays. As shown in Figure 5, cirsimaritin (**12**) and ladanein (**13**) exhibited significant AhR activation at 10–10^2 μM. In contrast, compounds **12**–**14** induced cell death at 10^3 μM (Figure 5). Moreover, nepitrin (**15**) and homoplantagenin (**16**), which are flavone glucosides, showed marked AhR-binding activity at concentrations ranging from 10–10^3 μM lower than those required for binding by indole 3-acetic acid (IAA), a typical natural AhR ligand [8].

As mentioned earlier, AhR activation tends to be weakened by glycosidation of the parent AhR ligand. This tendency has been observed even for flavonoid ligands [4]. In the present study, nepitrin (**15**) and homoplantagenin (**16**), which are flavone glucosides, were found to have noticeable AhR activity.

Some compounds characterized as potential AhR agonist candidates in the current study have been reported to have various biological functions beneficial to human health. For example, lipolytic, antilipogenic, and antiproliferative activities have been identified as biological properties of cirsimaritin (**14**) [17], and nepitrin (**15**) has been reported to have anti-inflammatory and gastroprotective activity [18,19]. Recently, several studies have reported that activation of AhR may be involved in various immune responses as described above; therefore, natural AhR ligands are expected to have beneficial regulatory roles in humans, mediating anti-allergy and anti-cancer effects. Further studies on AhR-activating ingredients derived from natural foods may clarify both the physiological significance of the AhR and the benefits derived from food constituents.

3. Experimental

3.1. General

^{1}H- and ^{13}C-NMR spectra (500 MHz for ^{1}H and 126 MHz for ^{13}C) were recorded on a Bruker AVANCE 500 instrument (Bruker BioSpin, Billerica, MA, USA), and chemical shifts are given in ppm values relative to those of the solvents [chloroform-*d* (δ_H 7.26; δ_C 77.16), methanol-d_4 (δ_H 3.30; δ_C 49.0), dimethylsulfoxide (DMSO)-d_6 (δ_H 2.50; δ_C 39.5), and acetone-d_6 (δ_H 2.04; δ_C 49.0)] on a tetramethylsilane scale. The standard pulse sequences programmed for the instrument (AVANCE 500) were used for each 2D measurement (COSY, HSQC, and HMBC). J_{CH} was set at 10 Hz in HMBC. Electrospray ionization (ESI)-MS, and high-resolution (HR) ESI-MS spectra were obtained using a micrOTOF-Q (Bruker Daltonics, Billerica, MA, USA) mass spectrometer with acetonitrile as the solvent. UV spectra were recorded on a Shimadzu UVmini-1240 system (Shimadzu, Kyoto, Japan).

The reversed-phase (RP) HPLC conditions were as follows: column, L-column ODS (5 μm, 150 × 2.1 mm i.d.) (Chemicals Evaluation and Research Institute, Tokyo, Japan); mobile phase, 5% acetic acid (solvent A) and acetonitrile (solvent B) (0–30 min, 0%–50% B in A; 30–35 min, 50%–85% B in A; 35–40 min, 85%–85% B in A); injection volume, 2 μL; column temperature, 40 °C; flow rate, 0.3 mL/min; and detection, 200–400 nm. TLC was performed on Silica Gel 60 F_{254} plates (Merck, Darmstadt, Germany), and the spots were visualized under a UV lamp (254 nm). Column chromatography was conducted using Sephadex LH-20 (GE Healthcare, Little Chalfont, England), MCI Gel CHP-20P (75–150 μm) (Mitsubishi Chemical Co., Tokyo, Japan), YMC GEL ODS-AQ (AQ12S50) (YMC Co., Ltd., Kyoto, Japan), and Silica Gel 60 (Nacalai Tesque, Kyoto, Japan) columns.

3.2. Samples and Reagents

The reagents used in the present study were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Nacalai Tesque, and 37 health food materials, as shown in Table 1, were obtained from Uchida Wakanyaku Ltd. (Tokyo, Japan), Tochimoto Tenkaido Ltd. (Osaka, Japan), and Nagaoka Perfumery Ltd. (Osaka, Japan). The species were identified by the Herbarium of the College of Pharmaceutical Sciences, Matsuyama University, where the voucher specimens were deposited. All other chemicals were of analytical reagent grade.

3.3. Extraction

The health food samples were prepared as follows: The materials (1 g) were homogenized in aqueous ethanol [ethanol/water (4:1)] (30 mL) for 10 min and filtered. The filtrates were concentrated under reduced pressure and freeze-dried.

3.4. Isolation of Compounds from Cassia Seeds

Cassia seeds (400 g) purchased from Uchida Wakanyaku Ltd. were homogenized in 80% ethanol [ethanol/H_2O (8:3)] (4 L), and a concentrated solution (*ca.* 0.15 L) was extracted successively with *n*-hexane (0.45 L) and ethyl acetate (0.45 L) to obtain the respective *n*-hexane (6.14 g), ethyl acetate (1.54 g), and water (34.47 g) extracts.

The ethyl acetate extract (0.7 g) was chromatographed over a Sephadex LH-20 column with ethanol to give 10 fractions (Frs. 1–10). Frs. 2 and 3 (50 mg) were subjected to preparative TLC [ethyl acetate/methanol (3:1), *n*-hexane/ethyl acetate/acetic acid (10:5:2), and then chloroform/methanol (95:5)] to give chryso-obtusin (**1**) (2 mg), obtusifolin (**2**) (2 mg), obtusin (**3**) (2 mg), aurantio-obtusin (**4**) (2 mg), and obtusin 2-*O*-glucoside (**5**) (2.6 mg). Fr. 5 (100 mg) was similarly purified with preparative TLC [chloroform/methanol/H_2O (14:6:1)] to afford aurantio-obtusin 6-*O*-glucoside (**6**) (2.7 mg), nor-rubrofusarin 6-*O*-glucoside (**7**) (11 mg), and 6-hydroxymusizin 8-*O*-glucoside (**8**) (2.1 mg). Fr. 4 (180 mg) was subjected to column chromatography over silica gel 60 (ϕ 2.0 × 20 cm) with chloroform/methanol (9:1) to give obtusin 2-*O*-glucoside (**5**) (4.1 mg). These known compounds were identified by direct comparison with valid standards or by comparison of their spectral data with those reported in the literature [20,21].

3.5. Isolation of Compounds from Rosemary

Rosemary leaves (526 g) provided by Nagaoka Perfumery Co. Ltd. were homogenized in 80% ethanol (ethanol/H_2O 8:2) (5 L), and a concentrated solution (*ca.* 0.15 L) was extracted successively with *n*-hexane (4 L), ethyl acetate (4 L), and *n*-butanol (4 L) to give the respective *n*-hexane (6.14 g), ethyl acetate (1.54 g), *n*-butanol (14.84 g), and water (34.47 g) extracts. The ethyl acetate extract (1 g) was chromatographed over Sephadex LH-20 with ethanol to give eight fractions (Frs.1–8). Frs. 2–8 (876 mg in total) were combined and further subjected to column chromatography over YMC GEL ODS-AQ and MCI Gel CHP-20P columns with aqueous methanol to yield vanillic acid (**9**) (2 mg), caffeic acid (**10**) (2 mg), rosmarinic acid (**11**) (82.8 mg), cirsimaritin (**12**) (2 mg), ladanein (**13**) (2 mg), salvigenin (**14**) (1.5 mg), nepitrin (**15**) (13.5 mg), homoplantaginin (**16**) (6 mg), and 6″-*O*-(*E*)-feruloylnepitrin (**17**) (2 mg). These compounds were identified by direct comparison with authentic specimens or by comparison of their spectral data with those reported in the literature [22–25].

3.6. Estimation of AhR Ligand Activity

The extracts and compounds were dissolved in DMSO and evaluated for AhR-binding activity using a luciferase assay (CALUX assay). The CALUX assay for AhR ligand activity was conducted as follows. Mouse hepatoma H1L1 cells (*ca.* 1.5×10^5 cells/well) were cultured in 96-well culture plates, and the samples were dissolved in DMSO and then added at final concentrations of $1–10^2$ μg/mL (or

μM in compound)] in three steps in fractions. The final DMSO concentration was 1% in the cell culture medium. The plates were incubated at 37 °C in 5% CO_2 for 24 h for optimal expression of luciferase activity. After incubation, cell viability was confirmed using a microscope. Subsequently, the medium was removed and the cells were lysed. After addition of luciferin as the substrate, luciferase activity was determined using a luminometer (Centro LB960; Berthold, Bad Wildbad, Germany) and recorded as RLUs. The values represent the mean ± SD of at least two or three independent determinations for each experiment. Statistical significance was analysed using the Student's *t* test.

4. Conclusions

In this study, we examined the effects of 37 health food materials on AhR activity using a reporter gene assay and found that cassia seed and rosemary extracts elicited notable AhR activation. To characterize the AhR-activating substances within these extracts, the respective extracts were subjected to fractionation followed by estimation of AhR activity. Eight compounds were isolated and identified from the active fractions of the cassia seed extract. Among them, aurantio-obtusin (**4**), an anthraquinone, was characterized as an effective AhR-activating ligand. In rosemary, nine compounds were isolated from the active extract. Nepitrin (**15**) and homoplantagenin (**16**), which are flavone glucosides, showed marked AhR-binding activity.

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Author Contributions

The listed authors contributed as follows: MT and HT carried out the extraction and isolation. MY and TY participated in the structural elucidation. MN and HH conducted the CALUX assay and analyzed the data. TT, RM, and RT helped interpreting the results. YA organized the study and participated in the structural elucidation. All authors approved the final version.

Conflicts of Interest

The authors have declared that there are no conflicts of interest associated with this study.

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Sample Availability: Samples of the compound **11** are available from the authors.

DETERMINATION OF HEXABROMOCYCLODODECANE IN FISH SAMPLES COLLECTED FROM JAPANESE MARKETS

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Introduction

Hexabromocyclododecane (HBCD) is a brominated flame retardant (BFR) that for many years has been used in plastics and textile coatings around the world. In Japan, domestic use of HBCDs has recently increased, as it has increasingly replaced or supplemented other BFRs. A total of 2,600 t of HBCD was produced in or imported into Japan in 2011.

HBCD's toxicity and the environmental threat it poses are subjects of current discussion. The chemical has been identified in environmental samples from birds, mammals, fish and other aquatic organisms, as well as in soil and sediment. In October 2012, the Persistent Organic Pollutants Review Committee, a subsidiary of the Stockholm Convention on Persistent Organic Pollutants (POPs), adopted a recommendation to include HBCD in the Convention's Annex A for elimination, with specific exemptions for the expanded and extruded polystyrene needed to give countries time to phase-in safer substitutes.

We previously reported that the Japanese populace is exposed to HBCD mostly via fish among the market-basket food group samples investigated[1,2]. Therefore, it is important to clarify the status of seafood pollution by HBCD. In the present study, we analyzed HBCD in fish samples collected from Japanese markets.

Materials and methods

Chemicals

Non-labeled and $^{13}C_{12}$-labeled α-, β- and γ-HBCD analytical standards were purchased from Cambridge Isotope Laboratories. Dichloromethane, n-hexane, acetone, methanol and distilled water (washed with hexane) of dioxin or pesticide analysis grade were purchased from Kanto Chemical. A 44% sulfuric acid-impregnated silica gel was purchased from Wako Pure Chemical Industries.

Samples

The fish samples for the HBCD analysis were purchased from fish markets in the Kyushu district, western Japan during the period 2013-2014. The edible parts were collected, cut into small pieces and blended. The samples were maintained at temperatures below –20°C until analysis.

Sample Preparation

The protocol for the HBCD analysis is illustrated in Fig. 1. Each 5-g fish sample was homogenized with the addition of $^{13}C_{12}$ α-, β- and γ-HBCD, which were then extracted with methanol, a methanol and dichloromethane/hexane(1:9) mixture, and dichloromethane/hexane(1:9), in turn, using a POLYTRON PT 3100 homogenizer (Kinematica). The extracts were shaken with 5% sodium chloride solution, and the organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was dissolved in 10mL of acetone/cyclohexane(3:7), and 2-mL of this solution was subjected to GPC. HBCD was fractionated over 12.5 to 18.5 min after large molecules such as crude fatty acids eluted in 10 to 12 min. The fraction was concentrated and dissolved in 0.3mL of hexane, re-purified with a 1g 44% sulfuric acid-impregnated silica gel packed mini-column, and then reconstituted to 50μL using methanol.

Analytical Methods and Instrumentation

HBCD concentrations were determined using liquid chromatography/mass spectrometry (LC/MS) performed on a Quattro Ultima Pt (Waters) connected to a 2695 LC (Waters). The HPLC column was a 150mm x 2.1mm i.d. 5μm Inertsil ODS-3 (GL-Science). The detection limit of both α- and γ-HBCD was 0.02 ng/g wet weight (ww); that for β-HBCD was 0.01 ng/g ww.

Results and discussion

Table 2 presents the results of the HBCD analysis of the fish samples. HBCD was detected in all fish samples collected in this study. The concentrations of detected total HBCD were 0.12 ng/g –22 ng/g wet weight; mean concentration was 3.1 ng/g when ND was assumed to be zero. The HBCD levels found in this study were higher than those in our previous study of same region (ND –1.4 ng/g)[3]. Fig. 2 shows the concentrations for all the samples. In this study, tuna, yellowtail and one of a mackerel samples showed particularly high concentrations relative to other fish species.

Fig. 3 shows the ratio of HBCD isomers in fish samples. The analysis of isomers detected α-HBCD in all 20 samples, but γ- and β-HBCD in only 11 and 6 samples, respectively. The concentrations of the isomers of all fish samples were in the order: α- > γ- > β-HBCD.

The relationship between total HBCD concentration in the whole wet basis and the fat content (%) is shown in Fig. 4. We observed a correlation between the HBCD concentration and fat content (R^2=0.529).

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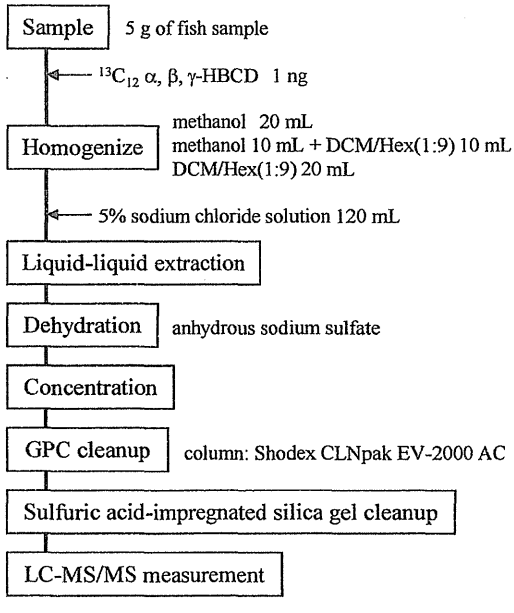

Fig.1 Protocol for HBCD analysis

Table 1 Concentrations of HBCD in fish samples

No.	Fish	Production regions of Japan	Fat content (%)	α-HBCD (ng/g ww)	β-HBCD (ng/g ww)	γ-HBCD (ng/g ww)	Total HBCD (ng/g ww)
1	Sardine	Chugoku-Shikoku	1.3	0.33	ND	0.02	0.35
2	Mackerel -1	Kyushu	3.8	0.60	0.01	0.06	0.67
3	Mackerel -2	Kyushu	4.1	14	0.11	5.2	19
4	Yellowtail -1	Kyushu	3.5	4.2	ND	0.02	4.2
5	Yellowtail -2	Kyushu	11	3.5	0.02	0.15	3.7
6	Yellowtail -3	Chugoku-Shikoku	1.7	2.9	0.01	0.15	3.1
7	Japanese seabass -1	Kyushu	0.54	0.22	ND	0.02	0.24
8	Japanese seabass -2	Kyushu	0.41	0.62	ND	0.04	0.66
9	Sea bream -1	Chugoku-Shikoku	5.0	0.34	ND	ND	0.35
10	Sea bream -2	Kyushu	0.96	0.13	ND	ND	0.13
11	Tuna -1	Kyushu	18	16	0.10	6.2	22
12	Tuna -2	Kyushu	2.8	3.3	0.02	1.1	4.4
13	Horse mackerel -1	Kyushu	0.39	0.12	ND	ND	0.12
14	Horse mackerel -2	Kyushu	0.11	0.20	ND	ND	0.20
15	Horse mackerel -3	Kyushu	0.32	0.23	ND	ND	0.23
16	Horse mackerel -4	Kyushu	1.4	0.70	ND	ND	0.70
17	Cod	Tohoku	0.078	0.18	ND	ND	0.18
18	Largehead hairtail	Kyushu	2.9	0.21	ND	0.02	0.23
19	Young yellowtail	Kyushu	0.14	0.42	ND	ND	0.42
20	Greater amberjack	Kyushu	0.029	0.13	ND	ND	0.13

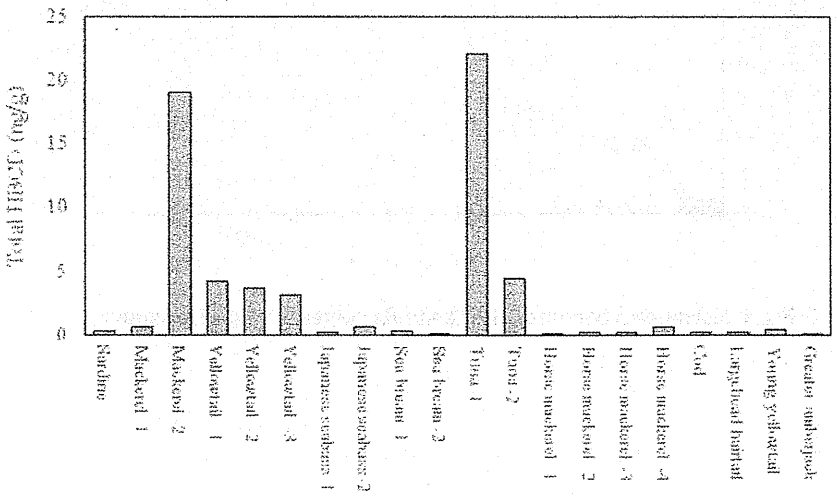

Fig. 2 Concentrations of HBCD in fish samples