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CRYSTAL STRUCTURE OF PHLORIZIN AND THE IODOTHYRONINE DEIODINASE INHIBITORY ACTIVITY OF PHLORETIN ANALOGUES

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Abstract—Phloretin, a 7,8-dihydrochalcone of plant origin, and the high molecular weight (<15,000) polyphloretinphosphate (PPP) polymers are potent inhibitors of iodothyronine monodeiodinase activity from rat liver microsomal preparations, whereas phlorizin, the 2′-*O*-glucoside of phloretin, is inactive The polymers, differing in degree of phosphorylation-dependent polymerization, exhibited a concentration-dependent, and ultimately complete, inhibition of deiodinase activity with an IC_{50} between 0 2 and 0 5 μg PPP/ml Phloretin inhibition, on the other hand, was cofactor (DTE) competitive, with a K_i = 0 75 μM 2′,4′,6′,3,4-Pentahydroxychalcone, which has a substitution pattern in the A-ring identical to that of phloretin, was the only active inhibitor (IC_{50} = 8 μM) among several derivatives tested The phloretin biodegradation products, phloretic acid and phloroglucinol, and its biosynthetic precursors, monomeric cinnamic acid and cinnamic acid derivatives, were inactive in concentrations up to 100 μM The X-ray crystal structure analysis of phlorizin dihydrate showed that the molecule is planar and fully extended, similar to the conformation observed in chalcone structures that are characterized by an α,β-unsaturated bond between phenol rings Comparison of the planar phlorizin crystal structure with a skewed or antiskewed thyroid hormone conformation revealed that the β-D-glucose moiety does not share any of the thyroid hormone's conformational space, and that the best structural homology is found with the antiskewed conformation of 3′,5′,3-triiodothyronine, the natural deiodinase substrate that also inhibits further deiodination

Iodothyronine monodeiodinases are membrane-bound enzymes responsible for the extrathyroidal metabolism of thyroxine (T_4: 3,5,3′,5′-tetraiodothyronine) In rat liver microsomal preparations, a single deiodinase enzyme catalyzes both the phenolic and tyrosyl monodeiodinations of T_4 resulting in the formation of hormonally active T_3 (3′,3,5-triiodothyronine) or inactive rT_3 (3′,5′,3-triiodothyronine) (Fig. 1). Structure–activity studies further show that this enzyme is inhibited by several classes of phenolic or anionic compounds including iodothyronines, halogenated dyes, contrast media, beta-adrenergic antagonists, and extracts and secondary metabolites of certain plants [1–6]. Additional investigation of plant extracts has revealed that the active components are polycyclic phenolic derivatives of cinnamic acid, the precursor of chalcones in flavonoid biosynthesis (Fig. 2), and that, among flavonoids, the aurones are the most potent inhibitors of rat liver deiodinase [7]

Chalcones are open chain flavones in which the two aromatic rings are joined by a three-carbon α,β-unsaturated carbonyl system. Dihydrochalcones result from the reduction of the double bond. Biosynthetic isomerization, oxidation and hydroxylation reactions of the chalcones give rise to flavones or aurones [8].

The dihydrochalcone phloretin, its 2′-β-D-glucoside phlorizin [2,4,6-trihydroxy-β-(4-hydroxyphenyl-propiophenone], and synthetic high molecular weight polyphloretinphosphate (PPP) polymers (Fig 3) are known to influence membrane fluidity, potential, and transport processes in a variety of systems [9–13]. PPP, but not the monomer phloretin or its glucoside, acts as a prostaglandin antagonist [14–16]. In addition, the PPP polymers inhibit thyroid-stimulating-hormone (TSH) binding and response in human thyroid membranes *in vitro*,‖ and TSH- and LATS (long-acting thyroid stimulator)-stimulated secretions of thyroid hormones *in vivo* [17].

Two mechanisms have been postulated for phloretin effects on membrane processes and enzyme activities: (1) interaction of the polyphenol with membrane (phospho) lipids, which is characterized by a high half-maximum effective concentration (>10 μM), and (2) by specific inhibitory interaction with membrane proteins (IC_{50} 0 1 to 10 μM)

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‖ S M Amir, K Kasagi, M Coretti, M Blank and S H Ingbar, *Endocrinology* **117**, 860 (1985)

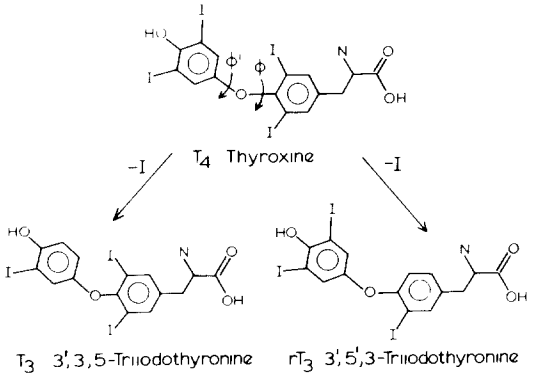

Fig 1 The thyroid hormone thyroxine and its two monodeiodination products, metabolically active T_3, and calorigenically inactive rT_3. The ether bond torsion angles ϕ (C5-C4-O4-C1') and ϕ' (C4-O4-C1'-C6') are also shown

A major source of phlorizin is in commercially grown varieties of apple trees and other *Rosaceae* species, where it has been found in their leaves, bark and root, but not in their fruit [18]. It has also been shown to possess antifungal and bactericidal properties [19, 20].

Therefore, to explore further these structure–activity relationships, the potencies of a series of phloretin derivatives and precursors, including PPP polymers, as inhibitors of rat liver deiodinase activity, were investigated. In addition, the X-ray crystal structure analysis of phlorizin was carried out, and its molecular structure was compared with those of the thyroid hormones [21, 22]. This is also the first report of the structure of a dihydrochalcone.

MATERIALS AND METHODS

Chemicals Phloretin, phlorizin, and 2',4',6',3,4-pentahydroxychalcone were purchased from Roth (Karlsruhe, F. R. G.), phloroglucin and phloretic acid [3-(4-hydroxyphenyl)-propionic acid] were obtained from Fluka AG (Buchs, CH). 2',4-Dihydroxy-

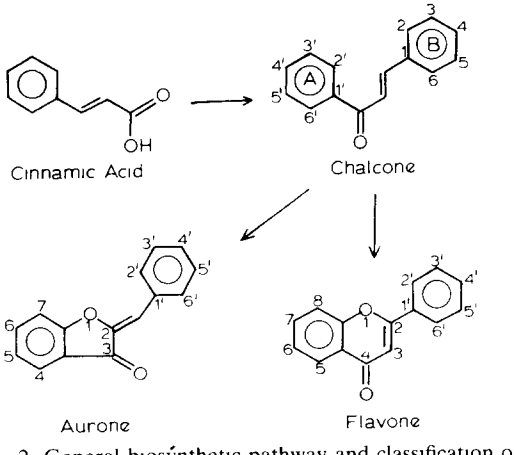

Fig 2 General biosynthetic pathway and classification of flavones. Individual flavones are characterized by their degree of hydroxylation and heterocyclic ring saturation

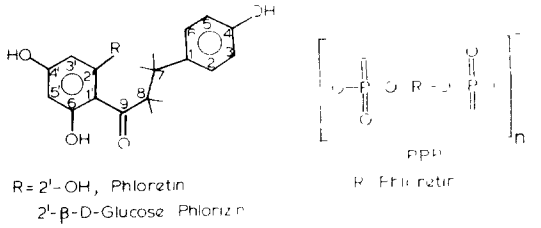

Fig 3 Numbering scheme for phloretin, its 2'-*O*-glucoside phlorizin, and the polymer PPP

chalcone, 2',4',4-trihydroxychalcone and 2',4'-dihydroxy-3,4-methylenedioxychalcone were donated by the late Dr. E. C. Jorgensen, University of California, San Francisco. Polyphloretinphosphate (PPP) preparations were a gift from H. Fex, Leo Aktiebolaget (Sweden). All PPP preparations were in the form of their sodium salts with molecular weights of 15,000 or less, depending on the extent of their phosphate esterification and the number of phenolic hydroxyl groups [10].

[^{125}I]T_3 (sp. act. 750–1250 μCi/μg) was obtained from Amersham-Buchler (Braunschweig, F. R. G.). All other reagents were of the highest purity commercially available.

Iodothyronine deiodinase assay Microsomal fractions were prepared from liver homogenates of adult male Lewis rats [23] and were characterized by marker enzymes and protein determination. The T_4-5'-iodothyronine monodeiodinase assay was performed as previously described [6, 24]. The inhibitory action of the phloretin derivatives in this assay was tested by incubating 50 μl of freshly dissolved aqueous solutions in a total volume of 0.4 ml of the assay mixture containing 10 μM T_4, 10 mM dithioerythreitol (DTE) as cofactor, 100 mM Tris-HCl (pH 7.4), and 100 μg rat liver microsomal protein at 37° for 20 min. All incubations were carried out in duplicate and each phloretin derivative was tested in at least three different concentrations. The enzymic T_4-deiodination products T_3 and 3,3'-T_2 were determined by specific RIAs [25]. From each tube of incubation mixture iodothyronines were estimated in duplicate by these RIA methods. Interference of phloretin derivatives with the RIAs of T_3 and 3,3'-T_2 was routinely checked by omission of T_4 from the incubation mixture. No effect on the maximum binding in either RIA was observed. Zero-time values of incubation representing the cross-reactivity of T_4 in the RIAs as well as contamination of the substrate T_3 by other iodothyronines, were constantly found to be on the order of 15–25% of the specific products formed after 20 min of incubation with enzyme.

Kinetic analysis of enzyme activity was performed using Lineweaver-Burk plots and the least-squares method for determination of linear regression [26]. Half-maximum inhibition (IC_{50}) values for deiodinase activity were calculated from semilogarithmic concentration–response curves using inhibitor concentrations between 0.1 and 100 μM.

Crystallography Crystals of phlorizin (Sigma, St. Louis, MO, U.S.A.) dihydrate grown at room tem-

Table 1 Phlorizin positional and isotropic thermal parameters

Atom	X/a(σ)	Y/b(σ)	Z/c(σ)	$B_{eq}(\sigma)Å^{2*}$
C(1)	0 3374(2)	0 3900(1)	0 8043(7)	3 0(1)
C(2)	0 3648(2)	0 4319(1)	0 9802(8)	3 7(1)
C(3)	0 4190(2)	0 4204(1)	1 1539(9)	3 9(1)
C(4)	0 4481(1)	0 3658(1)	1 1593(8)	3 3(1)
C(5)	0 4236(2)	0 3241(1)	0 9897(8)	3 3(1)
C(6)	0 3679(2)	0 3360(1)	0 8116(8)	3 4(1)
C(7)	0 2771(2)	0 4066(1)	0 6207(7)	3 2(1)
C(8)	0 2452(2)	0 3588(1)	0 4496(7)	3 0(1)
C(9)	0 1871(2)	0 3819(1)	0 2722(7)	3 2(1)
O(9)	0 1755(2)	0 4341(1)	0 2833(7)	5 1(1)
C(1′)	0 1456(1)	0 3464(1)	0 0848(7)	2 9(1)
C(2′)	0 1506(1)	0 2861(1)	0 0476(6)	2 7(1)
C(3′)	0 1105(2)	0 2570(1)	−0 1383(7)	3 0(1)
C(4′)	0 0630(1)	0 2871(1)	−0 3007(6)	2 9(1)
C(5′)	0 0560(2)	0 3462(1)	−0 2747(8)	3 3(1)
C(6′)	0 0952(2)	0 3748(1)	−0 0834(8)	3 3(1)
O(2′)	0 1981(1)	0 2578(1)	0 2112(5)	3 0(1)
C(1*)	0 2150(1)	0 2007(1)	0 1461(6)	2 6(1)
C(2*)	0 2827(1)	0 1864(1)	0 2981(7)	2 9(1)
C(3*)	0 3007(1)	0 1231(1)	0 2531(6)	2 7(1)
C(4*)	0 2379(1)	0 0851(1)	0 3124(6)	2 5(1)
C(5*)	0 1734(1)	0.1062(1)	0 1599(6)	2 5(1)
O(6*)	0 1606(1)	0 1647(1)	0 2380(5)	2 6(1)
O(2*)	0 3390(1)	0 2194(1)	0 1942(9)	4 9(1)
O(3*)	0 3569(1)	0 1091(1)	0 4304(6)	4 0(1)
O(4*)	0 2535(1)	0.0276(1)	0 2320(5)	3 2(1)
C(5*1)	0 1082(2)	0 0713(1)	0 2232(8)	3 5(1)
O(5*2)	0 0976(1)	0 0660(1)	0 5090(6)	3 9(1)
O(4′)	0 0245(1)	0 2562(1)	−0 4787(6)	3 6(1)
O(6′)	0 0849(1)	0 4320(1)	−0 0660(7)	4 6(1)
O(4)	0 5022(1)	0 3559(1)	1 3377(6)	4 1(1)
O(1W)	0 4332(2)	0 1800(2)	0 7554(9)	5 8(1)
O(2W)	0 4412(2)	0.0411(2)	0 1624(15)	9 3(2)

* $B_{eq} = 4/3\Sigma_i\Sigma_j\beta_{ij}(a_i \cdot a_j)$

perature from an aqueous solution, have an orthorhombic crystal system, space group $P2_12_12_1$, with lattice parameters $a = 19.124(1)$, $b = 23.715(4)$, and $c = 4.918(1)$Å and $z = 4$ Precise lattice parameters were calculated by least-squares analysis of the twenty values from 25 reflections, with a range of 60.16–69.56° The intensities of 2664 independent reflections [$2611 > 2\sigma(I)$] were collected with CuKα radiation on a Nonius CAD-4 automated diffractometer with a Ni-filter Corrections were made for Lorentz and polarization effects, but not for absorption or extinction effects The structure was solved by the use of the direct methods programs MULTAN [27] and NQEST [28] and was refined by full-matrix anisotropic least-squares techniques. The function $\Sigma w\|F_o| - |F_c\|^2$ was minimized, where w is based on diffractometer counting statistics. The final residual, $R = \Sigma\|F_o| - |F_c\|/|F_o|$, was 0 062 for 2603 data Atomic scattering factors are from *International Tables for X-ray Crystallography* [29]. Hydrogen atoms were located in difference Fourier syntheses and refined isotropically. The fractional coordinates and isotropic thermal parameters are listed in Table 1.

RESULTS

Inhibition of rat liver microsomal iodothyronine deiodinase activity by each phloretin derivative was examined at different inhibitor concentrations As illustrated (Fig. 4), a concentration-dependent, and ultimately complete, inhibition of deiodinase activity was achieved with three samples of PPP polymers that differed in degree of phosphorylation-dependent polymerization Half-maximum inhibition of deiodinase activity was produced with 0 2 to 0 5 μg/ml PPP, as measured by T_3 and 3,3′-T_2 production. The mean values of three to five experiments were not statistically different among the three molecular weight fractions of PPP.

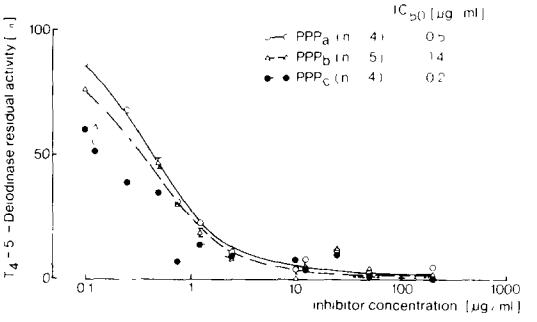

Fig 4 Concentration–response curves for various molecular weight polymers of polyphloretinphosphate

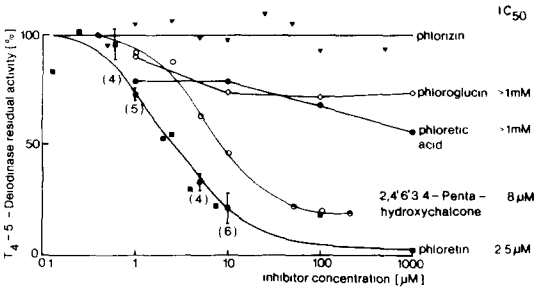

Fig 5 Concentration–response curve showing the inhibition of deiodinase activity by a number of phloretin derivatives

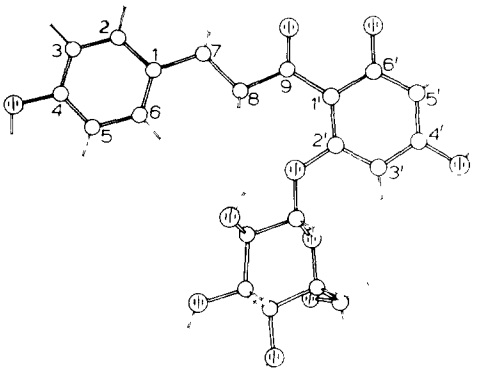

Fig 7 Molecular structure of phlorizin

As shown (Fig. 5), the monomeric dihydrochalcone phloretin inhibited deiodinase activity to half-maximum at 2.5 μM, or 0.7 μg/ml on a weight basis. However, phlorizin, the naturally occurring 2'-*O*-glucoside, had no inhibitory effect on this enzyme. The biodegradation products, phloroglucin and phloretic acid, showed a weak concentration-dependent inhibition. In contrast to the activity of phloretin, no inhibitory effect, up to 100 μM concentration, was found for 2',4-dihydroxychalcone, 2',4',4-trihydroxychalcone or 2',4'-dihydroxy-3,4-methylenedioxychalcone. However, 2',4',6',3,4-pentahydroxychalcone, which has a substitution pattern in the A-ring identical to that of the dihydrochalcone phloretin, was a potent inhibitor (IC_{50} = 8 μM).

A Lineweaver–Burk plot (Fig 6) of cofactor (DTE) concentration versus phloretin concentration shows lines intersecting close to the ordinate. These results suggest a cofactor competitive mechanism of inhibition, similar to that found for the aurones [7] Variation of T_4 concentrations at different phloretin concentrations yielded a non-competitive mechanism of inhibition with respect to the substrate T_4 (data not shown). According to the ping–pong mechanism of the rat liver iodothyronine deiodinase action, such kinetic characteristics are expected and support the cofactor competitive mechanism of action for phloretin.

The molecular conformation of phlorizin (Fig 7) shows the structure to be planar and fully extended, similar to that observed in chalcone structures [30, 31] There are two intramolecular hydrogen bonds between O(9) O(6') (2 44Å) and O(2') . O(2*) (2 84Å) The 2'-*O*-glucoside is coplanar with the phenolic ring, and the torsion angles about the bridging oxygen are about 165° for both C—O bonds The glucoside ring has a half-chair conformation and a gauche–gauche 5-hydroxymethyl conformation There is also an extended network of intermolecular hydrogen bonds among the two water molecules and the phlorizin molecules in the crystal

Calculation of classical potential energies for non-bonded interactions with rotation about the single bonds in phlorizin shows that the overall low energy conformation is similar to that observed in the crystal structure These calculations indicate that there are steep potential energy wells for rotations about the glucoside bonds and broad shallow wells (ΔE = 1 kcal) for rotations about the methylene carbon bonds between C(1), C(7) and C(8) The chalcone structure [30, 31] also have conformations within these energy ranges

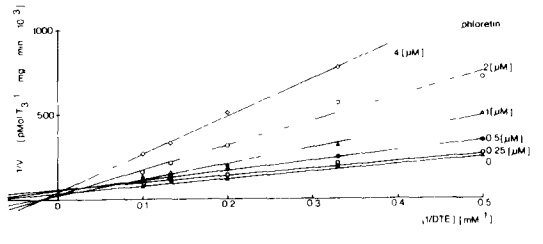

Fig 6 Lineweaver–Burk plot of inhibition of T_4-5'-deiodinase reaction by various phloretin concentrations at different cofactor (DTE) concentrations

DISCUSSION

The aurone plant flavones are potent inhibitors of rat liver microsomal iodothyronine deiodinase activity, with potencies comparable to that of the natural substrate inhibitor, rT_3 [7] This report shows that, like the aurones, the dihydrochalcone phloretin, its polyphloretinphosphate polymers, and 2',4',6',3,4-pentahydroxychalcone are also potent inhibitors of deiodinase activity. Lineweaver–Burk analysis of phloretin inhibition indicates a cofactor (DTE) competitive mechanism, as found for the 4',4,6-trihydroxyaurone. Inhibition by PPP polymers is the first example of a high molecular weight (<15,000) deiodinase inhibitor

While the mechanism of PPP polymer inhibition cannot be explained readily because of the undefined structure of the phosphorylated polymers, structure–activity relationships among flavonoid monomers indicate that inhibitory potency is a function of their molecular structure As reported previously for monocyclic phenols [7], this study also shows that single ring phenolic precursors or biodegradation products of phloretin are inactive, as are the chalcones with an unsaturated bridge linking the two phenolic rings, with the exception of 2',4',6',3,4-pentahydroxychalcone This suggests that the inhibitory potency is not dependent on the α,β-unsaturated

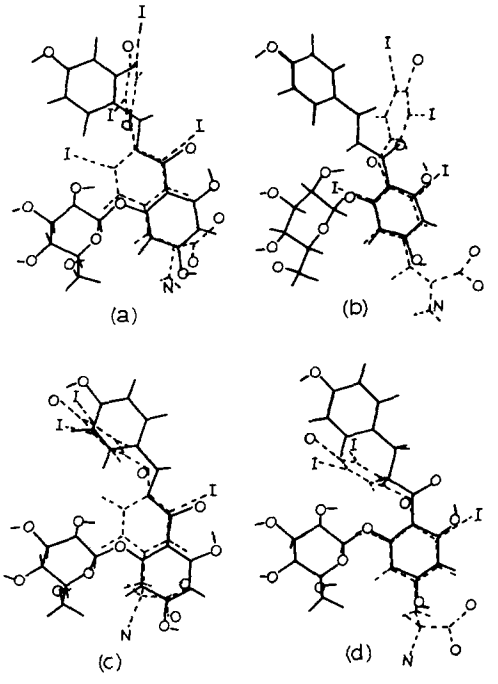

Fig 8 Superimposition of phlorizin (solid line) on T_4 (dashed) in a skewed conformation with the molecules aligned with 09 of phlorizin on that of hormone tyrosyl iodine (a), and (b) with the phenyl ketone on that of the hormone tyrosyl ring Superimposition of phlorizin (solid line) on rT_3 (dashed) in an antiskewed conformation with 09 aligned on the hormone tyrosyl iodine (c), and (d) with the phenyl ketone ring on that of the hormone tyrosyl ring

ketone. The inactivity of phlorizin also implies that the steric bulk of the 2'-sugar is responsible for its ineffectiveness. This is supported by other data indicating that the flavone 3-*O*-glucosides are inactive, while their aglycones have inhibitory potency [7]. Chalcones carrying only 2',4'-dihydroxy substitution in the A ring are inactive as iodothyronine deiodinase inhibitors. Therefore, the presence of a free 6'-OH is required for inhibitory potency since this group may form a strong intramolecular hydrogen bond with the 9-keto function (Fig 7), comparable to the 4-OH of aurones or the 5-OH of flavonoids [32] Thus, the presence of a bulky 2'-glucoside (e g. phlorizin) or the absence of a 6'-OH (e g. inactive chalcones) may prevent an essential ligand–enzyme interaction in this region of the molecule. Potent aurones and flavones have a comparable oxygen function in this region of the molecule, whereas cinnamic acid derivatives lack such an oxygen and are inactive [6, 33–35].

To understand the inhibitory mechanism of these phloretin derivatives as deiodinase substrates, the molecular conformation of phlorizin was compared with those of the thyroid hormones. Analysis of the stereochemical characteristics of the thyroid hormones shows that their conformations fall into two classes, dependent upon the iodine substitution pattern in the diphenyl ether rings [36–38]. The predominant conformation is skewed in which the two iodophenyl rings are mutually perpendicular and bisect about the 120° ether bridge. In this orientation, the torsion angles about the ether bridge are $\phi \sim \pm 90°$, $\phi' \sim 0°$ (Fig. 1) The presence of the 3,5-iodine atoms on the tyrosyl ring provides the steric bulk necessary to lock this conformation Removal of at least one of these tyrosyl iodine atoms permits an alternative antiskewed conformation in which the tyrosyl ring bisects the plane of the phenolic ring (e.g. $\phi \sim 0°$, $\phi' \sim \pm 90°$).

To investigate the structural homologies between phlorizin and the thyroid hormones, their conformational topologies were compared using computer graphic techniques. For example, when the ketone function of phlorizin is matched with the tyrosyl iodine of T_4 in a skewed conformation, a portion of the phenolic glucoside moiety occupies most of the hormone alanine structure (Fig. 8a) However, if the overlap is made between the phlorizin phenolic ring and the tyrosyl ring, there is even less structural homology (Fig 8b). On the other hand, if phlorizin is compared, in an analogous manner, with the antiskewed rT_3 structure, then the degree of structural homology increases (Fig. 8c and d). Since the thyroid hormones are either skewed or antiskewed, there can only be partial structural homology with a planar phlorizin structure. Although the dihydrochalcones have conformational flexibility, potential energy calculations of nonbonded interactions indicate that a planar structure is preferred. However, rotation about the methylene carbon bonds would permit a closer spatial comparison with the hormone structures

We have shown previously that the T_4 binding site of thyroxine-binding prealbumin (TBPA) can serve as a model for the deiodinase active site [33–35] Crystallographic data for the T_4–TBPA complex [39] were used to model phlorizin in the hormone binding site These computer graphic studies indicate that phlorizin does not fit into the hormone site and, as shown (Fig. 9), the glucoside causes steric interference with the TBPA residues near the channel entrance. On the other hand, the aglycone phloretin can be modelled into the hormone binding site *

The enzyme kinetic data show a cofactor competitive inhibition of phloretin in the ITH-D reaction with a K_i similar to that observed for the most potent natural aurone, 4,4',6-trihydroxyaurone, and suggest a different mode of action than the substrate competitive ITH-D analogous [6, 7, 35] The cofactor competitive mechanism of phloretin inhibition of rat liver ITH-D found in kinetic experiments does not exclude binding of this compound to the substrate binding site of ITH-D However, this can only be demonstrated after purification of the detergent soluble membrane-integrated enzyme [40–42] A cofactor competitive mechanism of inhibition of ITH-D was also observed for thiouracil analogues and various SH-directed reagents [43–46]

The very low K_i value for phloretin and the potent ITH-D inhibition by the anionic polyphloretin-phosphate, which is thirty times more active in the ITH-D reaction than in the inhibition of binding of

* Preliminary binding studies indicate that phloretin displaced ^{125}I-labelled T_4 from human TBPA with an IC_{50} of 2 5 μM, comparable to the IC_{50} of ITH-D inhibition

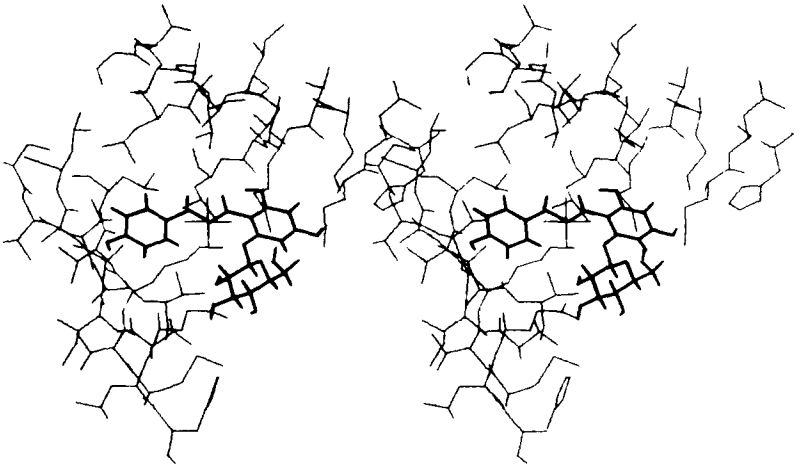

Fig 9 Stereo representation of phlorizin (dark line) modelled on T_4 in TBPA Note that the glucoside does not fit into the binding channel of TBPA This may account for the inactivity of phlorizin, whereas phloretin is a potent deiodinase inhibitor

TSH to human thyroid membranes,* suggest a highly specific interaction of these anions with the ITH-D enzyme and not with membrane lipids, since the latter process should produce higher IC_{50} values compared to those found here for the phloretin and its derivatives [7].

Thus, the results of these studies show that phloretin and its high molecular weight polymers are potent inhibitors of deiodinase activity. Crystallographic analysis of phlorizin shows that the molecule is coplanar, and conformational comparisons indicate that it has structural homologies with the thyroid hormones However, only the agylcone, phloretin, can fit in the hormone binding site of TBPA, suggesting that these flavonoids can have more than one mode of action at the deiodinase active site

Phloretin derivatives, as well as the aurones, may prove to act as antithyroidal drugs based on a cofactor competitive mechanism of inhibition of extrathyroidal local tissue bioactivation (T_3 production) and inactivation (rT_3 formation) of the thyromimetic potency contained in the (pro)hormone L-thyroxine.

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