Polymorphism

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Structure Elucidation and Characterization of Different Thyroxine Polymorphs

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Abstract: *Thyroid hormones regulate almost every process in the body, including body temperature, growth, and heart rate. They influence carbohydrate metabolism, protein synthesis and breakdown, and cardiovascular, renal, and brain function. Two new polymorphs of synthetic* L*-thyroxine (T4) are reported and the effect of polymorphism on the solubility of this important hormone is shown. Conformational changes were also discovered to have a remarkable effect on the strength of halogen bonding and the reactivity of the C–I bonds, which could have a significant effect on the hormone activity.*

Thyroxine (T4), the main thyroid hormone that regulates human metabolism, is synthesized from thyroglobulin by thyroid peroxidase (TPO) in the presence of hydrogen peroxide and iodide (Figure 1).[1] Thyroid hormones act on every cell in the body and their action alters the basal metabolic rate, protein synthesis, regulation of bone growth and neuronal maturation, and sensitivity to other hormones.[2] They are important for proper development and the differentiation of all cells and they are known to regulate protein, fat, and carbohydrate metabolism. Humans with low T4 levels, a condition known as hypothyroidism, suffer mental slowness, weight gain, depression, and fatigue.[3] The structure of T4 is unusual in that a diiodophenolic group is connected to a diiodotyrosyl moiety through an oxygen atom. The ether linkage appears to control the unique conformation and biological activity of T4.[4] The phenolic ring is perpendicular to the tyrosyl ring, with one of the iodine atoms of the phenolic ring occupying a position close to the tyrosyl ring (Figure 1).

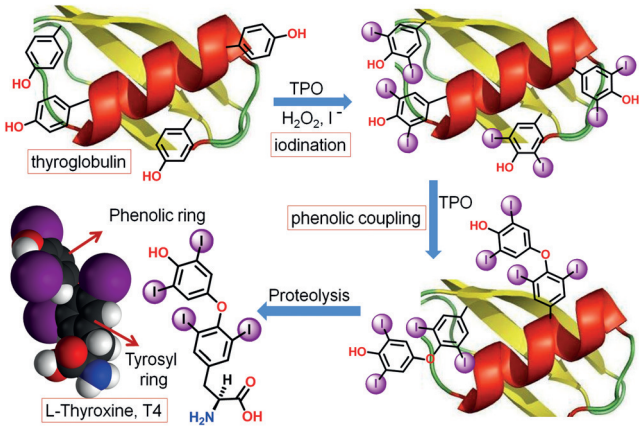

Figure 1. A) Biosynthesis of L-thyroxine (T4) from thyroglobulin by thyroid peroxidase (TPO) in the presence of H_2O_2 and iodide. The space-filling model indicates the relative orientation of the two iodinated phenyl rings in T4.

The synthetic form of T4 is a lifesaver for the large number of people with thyroid disorders. More than 200 million people around the world suffer from thyroid disorders and most of them take T4, which is a drug with a narrow therapeutic index.[3] Since thyroid hormone metabolism is tightly regulated, minor variations in the inert ingredients, manufacturing process, and solubility can affect the absorption and bioavailability of the drug. There have been reports of adverse effects with generic T4, and the American Thyroid Association (ATA), The Endocrine Society (TES), and the American Association of Clinical Endocrinologists (AACE), which represent more than 4600 clinical endocrinologists, issued a joint statement suggesting that different brands of T4 are not bioequivalent, thus leading to differences in the bioavailability of the drug.[5] The difference in bioavailability has been ascribed to variation in the drug formulation. However, it is not known whether a change in the crystalline conformation of T4 can alter its physical and pharmacological properties. Herein, we report on new polymorphs of T4, which differ in terms of molecular conformation in the crystal form and solubility. We also show that the halogen-bonding[6] ability of the iodine atoms in T4 depends strongly on the molecular conformation. Understanding the effect of conformational change on the reactivity of the C–I bond is crucial since T4 is a pro-hormone and the selective removal of iodine atoms from T4 by three selenoenzymes, the iodothyronine deiodinases Dio1, Dio2, and Dio3 (Figure S2 in the Supporting Information), is a key step in maintaining its biological activity through receptor binding and thyroid hormone homeostasis.[7]

Earlier structural studies of different salts and co-crystals of T4[8] indicated that this compound can exist in both *cisoid* and *transoid* conformations, which are determined by the relative orientation of the diiodophenolic ring with respect to the amino acid moiety. The structure of T4 in a salt-free zwitterionic form is still unknown. When we crystallized T4 from a mixture of methanol and ammonia, rhomboid-shaped triclinic crystals (Form I) in the P1 space group were obtained (Figure 2).[9] Interestingly, when the compound was crystallized from a mixture of acetonitrile and ammonia, relatively larger rhomboid-shaped monoclinic crystals (Form II) in the $P2_1$ space group were obtained. The X-ray structures of these

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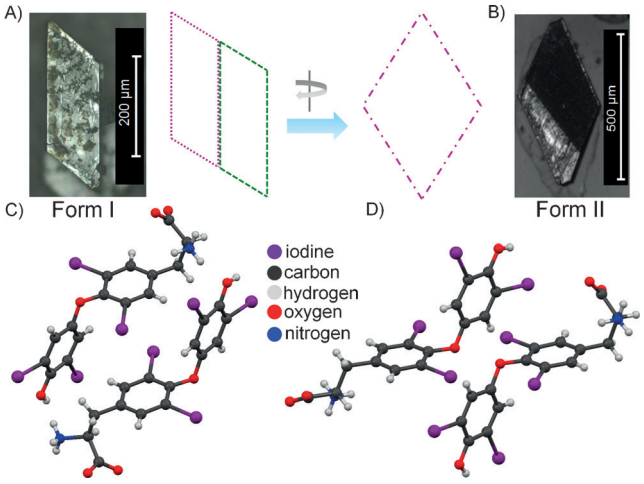


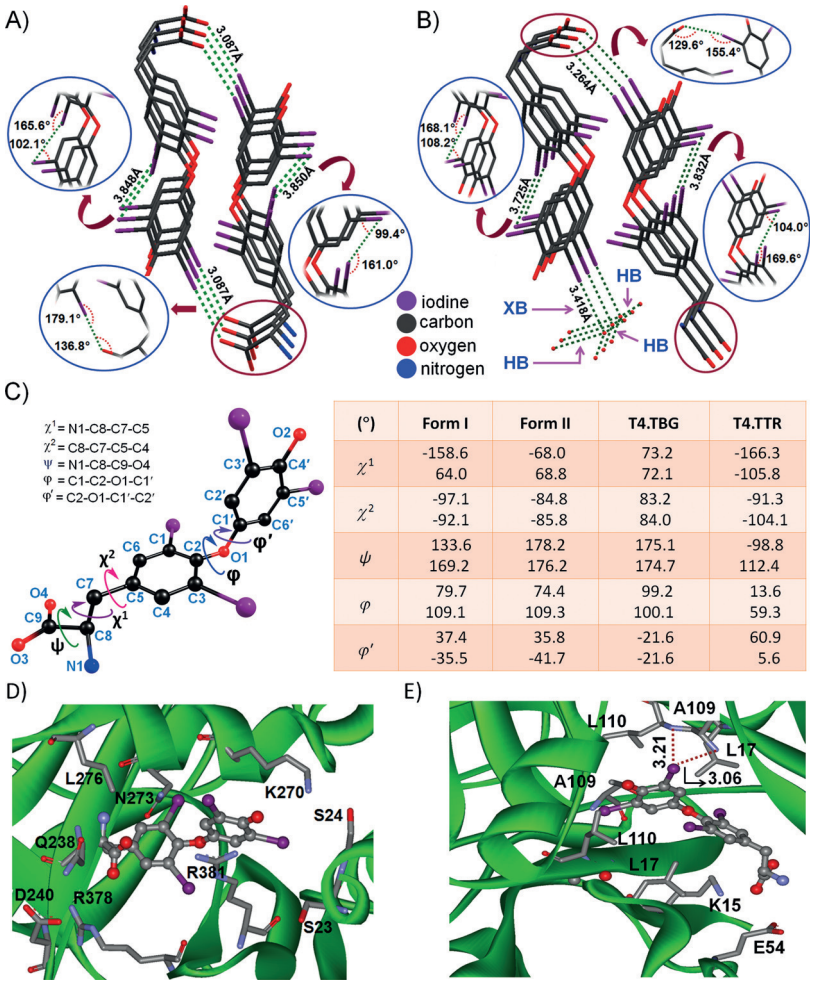
Figure 2. A, B) Rhomboid-shaped crystals obtained for T4 from methanol and acetonitrile, respectively. C,D) Single-crystal X-ray structures of two polymorphs (Forms I and II), indicating two different conformations in the asymmetric unit. Water molecules are omitted for clarity. ORTEP diagrams with full atom labeling are given in the Supporting Information (Figure S1).

two forms indicate that T4 exists in a *transoid* conformation. The unit cell parameters (Table S1 in the Supporting Information) for these two crystals are different from those reported earlier for the salts and co-crystals.[8] The present study indicates that the *transoid* conformer of T4 itself can exist in different polymorphic forms.

Interestingly, the two molecules present in the asymmetric unit of each polymorph are not identical (Figure 2C and D). This is probably due to different intermolecular interactions in the crystals and the orientation of the amino and carboxylic groups with respect to the diphenylether moiety. The crystal packing diagrams for Form I and II show an extensive halogen bonding (XB) network involving iodine and oxygen atoms (Figure 3A and B). Weak interactions between the phenolic ring iodine of one T4 molecule and the tyrosyl ring iodine of neighboring T4 are observed in Forms I and II, although the average I···I distance in Form II (3.779 Å) is shorter than that in Form I (3.849 Å). In these cases, the directional XB is formed through interaction of one of the iodine atoms with the positive electrostatic potential (σ-hole)[10] of another iodine atom along the extension of the C–I bond. In both forms, one of the phenolic-ring iodine atoms interacts with the carboxylic acid moiety of an adjacent unit to form a relatively stronger XB (I···O: 3.087 Å in Form I and 3.264 in Form II). Interestingly, in Form II, one of the phenolic-ring iodine atoms is halogen bonded to a water molecule, with an I···O_w distance of 3.418 Å and a C–I···O_w angle of 178.5°. The water is in fact involved in hydrogen bonding (HB) with other water molecules (Figure 3B). It should be noted that XB with water molecules is extremely rare, and a literature search indicated few nontrivial C–X···O_W contacts.[11] It has been shown that XB controls the selectivity of FRET substrate probes for matrix metalloproteinase (MMP-9), with a stabilizing contribution from the I···O_W-H bridge.[12] The interaction of iodine atoms in T4 with water molecules at the active site of deiodinases may modulate C–I bond cleavage. Unfortunately, there is no structural information available for the binding of T4 or its metabolites at the active site of Dio1, Dio2, or Dio3, since these enzymes are membrane-bound proteins and their expression and purification are extremely difficult. However, the crystal structure of the catalytic domain of Dio3 has recently been solved and the binding site of T4 was modelled by superimposing the binding site of T3 in the T3 receptor on Dio3.[13]

A comparison of the conformational parameters (ψ, χ^1, χ^2, ϕ, ϕ') between the two different forms leads to some interesting observations (Figure 3C). While the ϕ and ϕ' values are very similar in the two structures, there are significant differences in the χ^1, χ^2 and ψ values, which is probably responsible for the difference in the interaction between the phenolic-ring iodine and the carboxylate moiety (Figure 3A and B). To understand the effect of the conformation of T4 upon binding to proteins, we analyzed the structure of T4 bound to the two major transport proteins thyroxine binding globulin (TBG)[14] and transthyretin (TTR;[15] Figure 3D and E). Structure analysis indicates that T4 binds to TBG in a *cisoid* geometry and the conformations of the two T4 molecules at the two different sites are identical (Figure 3C and Figure S10 in the Supporting Information). By contrast, T4 binds to TTR in a *transoid* geometry and the conformation of the T4 bound at one site (A site; Figure 3E) is different to that of T4 bound at the other site (B site; Figure S11 in the Supporting Information). Furthermore, the molecular conformations of T4 bound to these sites are different to those of Form I, Form II, and TBG-bound T4 (T4.TBG), thus indicating that binding to amino acid residues can alter the conformation. As a result, one of the phenolic-ring iodine atoms of T4 at the A site forms two strong XB contacts with backbone nitrogen of L110 and A109, with I···N distances of 3.21 Å and 3.06 Å, respectively, whereas no such XB interactions can be observed at the B site (Figure S11 in the Supporting Information). These observations suggest that conformation changes can alter the binding and release of thyroid hormones at the binding sites of transport proteins such as TBG and TTR.

High-resolution powder X-ray diffraction (PXRD), Raman spectroscopy, solid-state nuclear magnetic resonance (SS-NMR) spectroscopy, and differential scanning calorimetry (DSC) are generally used to characterize different polymorphs of drugs and bioactive organic compounds.[16] The PXRD peaks were sharp and highly intense for Form I and bulk T4, whereas weak and broad peaks were observed for Form II (Figure 4A). The peaks at 2θ values of 31.85°, 32.50° and 39.10° observed for Form I were not detected for Form II. The Raman spectra of Forms I and II showed significant difference in the stretching frequencies (Figure 4B). In particular, the frequencies of C9=O, C8–H and N1–H groups for Form I are shifted 48, 7 and 13 cm^{-1}, respectively, with respect to the corresponding peaks for Form II, thus indicating that the arrangements of the carboxylate and amino groups are different in the two polymorphs. The ^{13}C SS-NMR spectra for Form I and Form II (Figure 4C) show significant differences in the chemical shifts of the



(°)	Form I	Form II	T4.TBG	T4.TTR
χ^1	-158.6 64.0	-68.0 68.8	73.2 72.1	-166.3 -105.8
χ^2	-97.1 -92.1	-84.8 -85.8	83.2 84.0	-91.3 -104.1
ψ	133.6 169.2	178.2 176.2	175.1 174.7	-98.8 112.4
φ	79.7 109.1	74.4 109.3	99.2 100.1	13.6 59.3
φ'	37.4 -35.5	35.8 -41.7	-21.6 -21.6	60.9 5.6

Figure 3. A, B) Packing in the crystal structures of Form I and Form II, respectively, showing extensive halogen bonding (XB). The circles indicate the different orientations of carboxyl and amine groups around the chiral center. Hydrogen atoms are omitted for clarity. C) Conformational parameters of T4 in various forms. The two values for each parameter for Form I and Form II correspond to two molecules in the asymmetric unit of each polymorph, and for T4.TBG and T4.TTR, they correspond to two independent protein-bound T4 molecules in each protein. D, E) Crystal structures of T4 in complex with TBG (PDB: 2CEO) and TTR (PDB:1ICT), respectively, indicating the binding of T4 in different conformations.

carbon atom of the acid group (157.8–159.5 ppm for Form I and 173.4–176.2 ppm for For II), the chiral carbon center (C8) (42.3–43.6 ppm for Form I and 56.7–57.9 ppm for Form II), and C7 (22.5 ppm for Form I and 32.3–38.9 ppm for Form II). Interestingly, the chemical shifts for the carbon atom of the C–I bonds in Form I (74.5–80.9 ppm) are remarkably different to those of Form II (92.3–97.1 ppm), thus suggesting that the reactivity of the C–I bonds may be different for the two polymorphs in the solid state. This leads to an assumption that the reactivity of the C–I bonds at various conformations of T4 should be different when these conformations are stabilized in solution, for example, by protein binding. DSC analysis indicated that T4 decomposes at temperatures above 200 °C and the decomposition temperature for Form I is almost identical to that for Form II, although the DSC profiles for these two polymorphs were different to that of bulk T4 (Figure S8 in the Supporting Information).

To understand the solution behavior of the two different forms of T4, we studied the optical activity of these polymorphs and compared them to that of the bulk T4. Interestingly, the specific rotation of Form I in methanol is remarkably different to that of Form II in acetonitrile (Figure 5 A), thus indicating that these solvents stabilize different conformations in solution. The identical specific rotations for Form I and bulk in methanol indicate that the bulk T4 is converted exclusively to Form I in methanol. By contrast, different specific rotations were observed for Form II and bulk T4 in acetonitrile (Figure 5 A), which indicates that one or more other polymorphs with higher specific rotations may be formed in addition to Form II. Since only Form II could be obtained from the crystallization of T4 in acetonitrile, the other polymorphs may be metastable in nature. The formation of such metastable polymorphs has been reported.[17] Attempts to isolate the third form of T4 were unsuccessful. However, we were able to obtain the unit cell parameters for Form III at low temperatures (Table S1 in the Supporting Information). Interestingly, at pH 4, the solubility of Form I ($1.22 \pm 0.02\ \mu g\,mL^{-1}$) was found to be significantly higher than that of Form II ($0.86 \pm 0.02\ \mu g\,mL^{-1}$) and bulk T4 ($0.77 \pm 0.01\ \mu g\,mL^{-1}$), thus indicating that the solubility of Form I is about 42 % and 45 % greater than that of than Form II and bulk T4, respectively. By contrast, at pH 9, the solubility of Form II ($21.42 \pm 0.78\ \mu g\,mL^{-1}$) was found to be 38 % and 42 % higher than that of Form I ($16.23 \pm 0.66\ \mu g\,mL^{-1}$) and bulk T4 ($14.97 \pm 0.23\ \mu g\,mL^{-1}$), respectively (Figure 5 B and Table S2 in the Supporting Information). Since T4 has a narrow therapeutic index, such changes in the pH-dependent solubility could lead to significant changes in properties such as oral bioavailability and absorption, which could alter the therapeutic outcome of this clinically useful drug.

Another remarkable and hitherto unexplored feature of the T4 structure is the ability of this molecule to form conformation-dependent XB with various donor atoms (halogen-bond acceptors). It is known that the Dio1 enzyme mediates the deiodination of both phenolic and tyrosyl rings, whereas the other two isoforms are selective for either phenolic rings (Dio2) or tyrosyl rings (Dio3; Figure S2 in the Supporting Information). The origin of such selectivity is still not known. When the XB energy was calculated for different T4 conformers by density functional theory (DFT)[18] with methylselenolate ($MeSe^-$) as the halogen-bond acceptor (Figure S12 in the Supporting Information), different sets of energy values were obtained for the phenolic- and tyrosyl-ring iodine atoms (Figure 5 C). While the tyrosyl-ring iodine

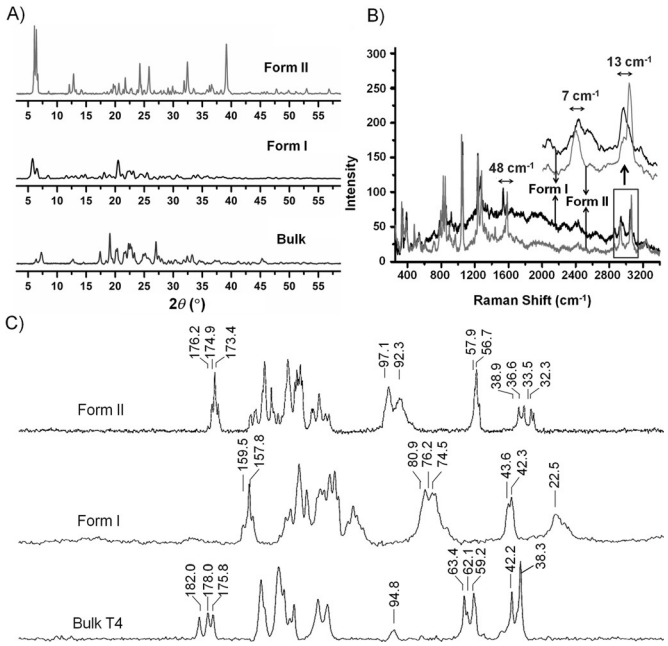


Figure 4. A) Powder X-ray diffraction pattern. B) FT-Raman spectra of Form I and Form II. C) Solid-state ^{13}C NMR spectra of T4 in bulk, Form I, and Form II.

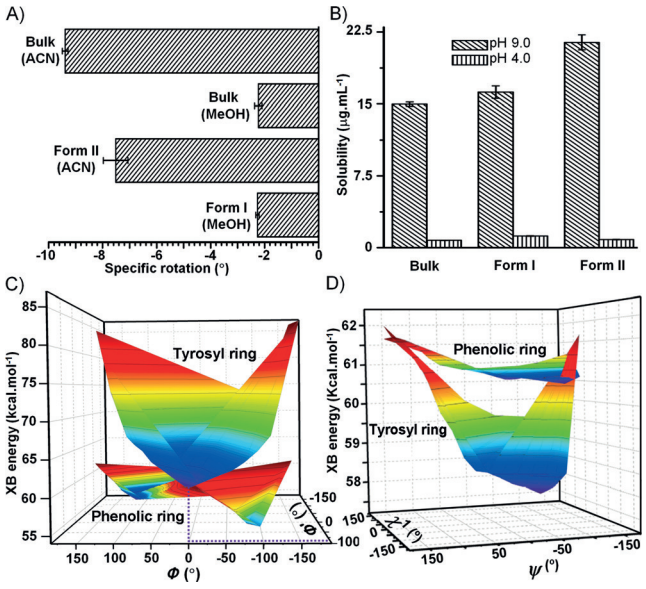


Figure 5. A) Optical activity (specific rotation) of T4 in bulk, Form I, and Form II in acetonitrile and methanol. B) Solubility of different forms of T4 at different pH values. C) Halogen bonding (XB) energy between selenium ($MeSe^-$) and iodine determined for the tyrosyl and phenolic rings for different conformations of T4. The dotted lines indicate that the XB energies for the iodine atoms in the two rings are almost identical at $\varphi = 0°$ and $\varphi' = 94°$, thus indicating that T4 can undergo both 5- and 5′-deiodination in this conformation. D) The variation in the XB energy when χ^1 and ψ were changed at $\varphi = 0°$.

atom forms stronger XB than that of phenolic-ring iodine atom at several conformations,[19] the change in torsional angles φ and φ' (see Figure 3C), can alter the strength of I···Se interactions such that the XB energy for the phenolic-ring iodine becomes identical to that of the tyrosyl-ring iodine atoms. When XB energy was plotted against these torsional angles, the strength of I···Se interaction was found to be almost identical for both phenolic- and tyrosyl-ring iodine atoms at a conformation, with φ, φ' values of 0° and 94°, respectively (Figure 5C). When the orientation of the amino acid moiety (determined by χ^1 and ψ) was changed at the above φ value, that is, 0°, the XB energy for the phenolic-ring iodine was higher than that of the tyrosyl-ring iodine atoms for several T4 conformations (Figure 5D). These results suggest that the selenoenzymes Dio1, Dio2, and Dio3 may control the regioselectivity for tyrosyl- vs phenolic-ring deiodination by altering the T4 conformation at the active sites.

In summary, we showed that T4 can exist in different polymorphic forms, with different solubility, optical activity and spectroscopic characteristics. In this study, we used two polymorphs of T4 to understand how the relative orientation of tyrosyl and phenolic rings alters the ability of the iodine atoms to form halogen bonding (XB) with donor atoms. The mechanism of dehalogenation by deiodinases remains elusive despite a large number of enzyme mimetic, mutational, and enzymatic studies. The results reported herein suggest that not only the strength of XB, but also the reactivity of the C–I bonds in T4 can be altered by changing the molecular conformation, which may provide valuable insight into the role of amino acids residues at the active sites of iodothyronine deiodinases in controlling the regioselectivity of deiodination.

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[1] a) "Biosynthesis and Secretion of Thyroid Hormones": F. Gentile, R. DiLauro, G. Salvatore in *Endocrinology*, 3rd ed. (Ed.: L. J. DeGroot), Philadelphia, WB Saunders Company, **1995**, pp. 517–542; b) "Hormone synthesis": A. Taurog in *The Thyroid* (Eds.: L. E. Braverman, R. Utiger), Philadelphia, Lippincott Williams & Wilkins, **2000**; pp. 61–85.

[2] a) P. M. Yen, *Physiol. Rev.* **2001**, *81*, 1097–1142; b) J. H. Oppenheimer, H. L. Schwartz, C. N. Mariash, W. B. Kinlaw, N. C. W. Wong, H. C. Freake, *Endocr. Rev.* **1987**, *8*, 288–308.

[3] a) J. R. Garber, R. H. Cobin, H. Gharib, J. V. Hennessey, I. Klein, J. I. Mechanick, R. Pessah-Pollack, P. A. Singer, K. A. Woeber, *Thyroid* **2012**, *22*, 1200–1235.

[4] a) H. A. Selenkow, S. P. Asper, Jr., *Physiol. Rev.* **1955**, *35*, 426–474; b) E. C. Jorgensen, *Pharmacol. Ther. Part B* **1976**, *2*, 661–682.

[5] a) J. M. Carswell, J. H. Gordon, E. Popovsky, A. Hale, R. S. Brown, *J. Clin. Endocrinol. Metab.* **2013**, *98*, 610–617; b) Thyroxine. In *Top pharmaceuticals*, E. Wilson, *Chem. Eng. News* **2005**, *83*, 5; c) American Thyroid Association, The Endocrine

Society, American Association of Clinical Endocrinologists, *Thyroid* **2004**, *14*, 486.
[6] a) P. Metrangolo, H. Neukirch, T. Pilati, G. Resnati, *Acc. Chem. Res.* **2005**, *38*, 386 – 395; b) P. Metrangolo, F. Meyer, T. Pilati, G. Resnati, G. Terraneo, *Angew. Chem. Int. Ed.* **2008**, *47*, 6114 – 6127; *Angew. Chem.* **2008**, *120*, 6206 – 6220; c) D. Manna, G. Mugesh, *J. Am. Chem. Soc.* **2012**, *134*, 4269 – 4279; d) P. Metrangolo, G. Resnati, *Nat. Chem.* **2012**, *4*, 437 – 438.
[7] a) G. G. J. M. Kuiper, M. H. A. Kester, R. P. Peeters, T. J. Visser, *Thyroid* **2005**, *15*, 787 – 798; b) J. Köhrle, *Mol. Cell. Endocrinol.* **1999**, *151*, 103 – 119; c) A. C. Bianco, D. Salvatore, B. Gereben, M. J. Berry, P. R. Larsen, *Endocr. Rev.* **2002**, *23*, 38 – 89; d) J. Köhrle, *Methods Enzymol.* **2002**, *347*, 125 – 167.
[8] a) N. Joel, F. G. Canepa, *Acta Crystallogr.* **1951**, *4*, 283; b) V. Cody, *Acta Crystallogr. Sect. B* **1981**, *37*, 1685 – 1689; c) V. Cody, M. Greiner, E, DeJarnette, *J. Appl. Crystallogr.* **1972**, *5*, 140 – 141; d) A. Katrusiak, A. Katrusiak, *J. Pharm. Sci.* **2004**, *93*, 3066 – 3075.
[9] CCDC 1403849 (T4, Form I), 1403850 (T4, Form II) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre.
[10] An Overview of σ-Hole Bonding, an Important and Widely-Occurring Noncovalent Interaction"": P. Politzer, J. S. Murray in *Practical Aspects of Computational Chemistry Methods, Concepts, and Applications* (Eds.: J. Leszczynski, M. K. Shukla), Springer, Heidelberg, **2009**, chap. 6.
[11] P. Auffinger, F. A. Hays, E. Westhof, P. S. Ho, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 16789 – 16794.
[12] I. Tranchant, L. Vera, B. Czarny, M. Amoura, E. Cassar, F. Beau, E. A. Stura, V. Dive, *Chem. Biol.* **2014**, *21*, 408 – 413.
[13] U. Schweizer, C. Schlicker, D. Braun, J. Köhrle, C. Steegborn, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 10526 – 10531.
[14] A. Zhou, Z. Wei, R. J. Read, R. W. Carrell, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 13321 – 13326.
[15] A. Wojtczak, P. Neumanna, V. Cody, *Acta Crystallogr. Sect. D* **2001**, *52*, 957 – 967.
[16] a) L. Yu, G. A. Stephenson, C. A. Mitchell, C. A. Bunnell, S. V. Snorek, J. J. Bowyer, T. B. Borchardt, J. G. Stowell, S. R. Byrn, *J. Am. Chem. Soc.* **2000**, *122*, 585 – 591; b) J. Bernstein, *Polymorphism in Molecular Crystals*, Clarendon Press, Oxford UK, **2002**; c) T. Ono, J. H. Horst, P. J. Jansens, *Cryst. Growth Des.* **2004**, *4*, 465 – 469; d) J. Gath, L. Bousset, B. Habenstein, R. Melki, A. Böckmann, B. H. Meier, *PLoS One* **2014**, *9*, e90659.
[17] a) D. Braga, F. Grepionia, L. Mainia, *Chem. Commun.* **2010**, *46*, 6232 – 6242; b) H. Miura, T. Ushio, K. Nagai, D. Fujimoto, Z. Lepp, H. Takahashi, R. Tamura, *Cryst. Growth Des.* **2003**, *3*, 959 – 965.
[18] Gaussian 09, Revision A.1, M. J. Frisch et al. Gaussian, Inc., Wallingford CT, **2009**. Full references are given in the Supporting Information.
[19] In agreement with this, most of the synthetic mimetics of deiodinases remove an iodine atom from the tyrosyl ring; a) D. Manna, G. Mugesh, *Angew. Chem. Int. Ed.* **2010**, *49*, 9246 – 9249; *Angew. Chem.* **2010**, *122*, 9432 – 9435; b) D. Manna, G. Mugesh, *J. Am. Chem. Soc.* **2011**, *133*, 9980 – 9983; c) S. Mondal, G. Mugesh, *Chem. Eur. J.* **2014**, *20*, 11120 – 11128; d) D. Manna, S. Mondal, G. Mugesh, *Chem. Eur. J.* **2015**, *21*, 2409 – 2416.

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