New Approaches for Enhancing the Selectivity and Activity of Thyroid Hormone Analogs

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Abstract

Thyroid hormones control a wide range of physiological processes in all known vertebrates. These actions are mediated by two different receptor subtypes that have heterogeneous distribution in tissues. While the endogenous hormone does not distinguish between the two subtypes, synthetic ligands have been made that will preferentially activate one subtype over the other *in vitro*.

While there is a wide array of compounds that are selective for the β-subtype, there are no known compounds that are selective for the α-subtype both *in vitro* and *in vivo*. A number of structural factors make this a difficult target, but this study produced a range of compounds with selectivities in the five- to twenty-fold range, many of which have potencies at the α-receptor in cell-based transactivation assays in the hundred-nanomolar range.

The efforts to produce a potent α-selective analog provided structure-activity relationship data that suggested some of the features used to improve the potency of those compounds could be applied to β-selective compounds as well. These changes proved to be synthetically tractable and improved the potency of an already potent and selective compound by roughly ten-fold at both receptors in a cell-based transactivation assay. These results were recapitulated *in vivo* in

the thyroid hormone receptor-mediated expression of an endogenous gene in the brain. The improved β-selective compounds are potential therapeutics for stimulating TR-mediated remyelination in diseases such as X-linked adrenoleukodystrophy and multiple sclerosis.

Chapter 1 – Introduction

1.1 Discovery of the Thyroid Hormone System

Thyroid hormones are the terminal signaling molecules of one of the fundamental endocrine systems found in all vertebrates, exerting influence on every tissue. They are prime drivers of development and also play a critical role in maintaining homeostasis in mature organisms.

The earliest scientific observations of the thyroid hormone system were in ancient Greece and China, where physicians discovered that marine sponges and seaweeds could be used to treat goiter, a swelling of the thyroid gland then most commonly caused by iodine deficiency. [1,2] Courtois' discovery in 1811 that iodine is an elemental component of seaweed provided the chemical basis of this effect. A number of doctors began to treat goiters directly with iodine, such as the Swiss physician Coindet in 1813. [3] However this treatment remained controversial in the medical world well into the 20th century as safe dose ranges were not well understood. Thyrotoxicosis from overdose or hypothyroidism via the Wolff-Chaikoff effect, a paradoxical state of excess iodide inhibiting thyroid hormone synthesis, were frequently induced. The case for iodine was bolstered by Bauman's discovery in 1896 that organically bound iodine was a major constituent of the thyroid gland. [4] The question of whether iodine

supplementation was the solution to goiter was largely resolved through simultaneous clinical trials carried out by Bayard in Switzerland and Marine & Kimball in the United States during the course of the First World War. [1] These led to large-scale public health programs to iodize salt during the 1920s, which largely eliminated iodine deficiency in the developed world.

Iodine supplementation could suppress goiters induced by iodine deficiency, but there were other conditions that it could not solve. Hypothyroidism, a suboptimal level of circulating thyroid hormone, required other solutions as it represents a number of different underlying conditions that all result in impaired production of thyroid hormone by the gland. The first treatment to directly address hypothyroidism was pioneered by George R. Murray in 1891, who orally administered desiccated thyroid gland from sheep, with results that were published nearly three decades later after the patient had demonstrated a sustained recovery. [5] Desiccated thyroid extract became the standard of care for nearly a century, but it presented a number of shortcomings in terms of dosing. Unlike iodine, which is a pure substance, desiccated thyroid extract was a heterogenous mixture of active ingredients including iodine and thyroid hormones. As reliable methods for measuring the constituents did not exist until the 1970s, dosing was based primarily on dry weight. This led to

patients receiving variable mixtures of each component, leading to fluctuations in circulating thyroid hormone levels.

While it had been known since 1896 that some iodine-containing substance was excreted from the thyroid gland[4] and that substance had been isolated in pure form in 1915, [6] determining its exact structure was a significant challenge. Kendall made an attempt, but produced an inaccurate model in 1918. [7] It was not until 1927 that the structure of tetraiodothyronine, also known as thyroxine or T4, was fully elucidated by Harington. [8] While lacking direct experimental evidence at the time, Harington also predicted that the biosynthesis of T4 would be accomplished by the sequential conversion of iodide to iodine, the iodination of tyrosine residues, and the coupling of two diiodotyrosine residues together. The process of elucidating the biological was chronicled by Selenkow in 1955 and confirmed Harington's hunch. [9]

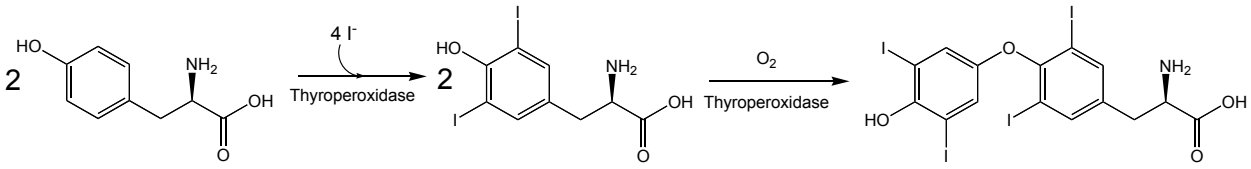

Figure 1.1

Biological synthesis of thyroxine from tyrosine and iodide by thyroperoxidase.

All subsequent development of thyromimetics flowed from understanding the molecular structure of endogenous thyroid hormones.

1.2 Development of Tetraiodothyronine-Based Analogs

Variations on the structure of thyroxine were synthesized and tested to understand the molecular determinants of thyroid hormone structure and with an eye towards producing clinical therapeutics. These early efforts to develop new analogs were constrained by two factors: the theory that T4 was the active form of the hormone and the limited information that could be obtained from the assays available at the time for assessing relative activity based on gross physiological measurements. Multiple pharmacokinetic and pharmacodynamic steps lay between administration of a drug and measurable outcomes, which muddled the structure-activity relationship (SAR) data that researchers used to refine their design strategies.

The first method for assaying thyroid hormone analogs was directly tied to the condition that had led to the discovery of thyroid hormones. Goiter occurs when chronically low serum levels of thyroid hormone lead to decreased levels in the brain, signaling the hypothalamic-pituitary-thyroid (HPT) axis to increase thyroid hormone synthesis by the thyroid. This signaling is ineffective if TH synthesis is blocked, whether from a lack of iodide in the tissue or direct inhibition of the synthetic machinery. Chronic stimulation of the thyroid results in tissue growth and a significant increase in total thyroid mass. During the

1940s a number of compounds were discovered that could induce goiter, [10] allowing researchers to directly study the ability of thyroid hormone and thyroid hormone analogs to suppress goiter formation in model organisms rather than relying on spontaneous cases in human subjects. Thioureas, primarily derivatives of 2-thiouracil, were the most effective goiterogenic drugs. [11] It was determined by Alexander in 1959 that thiouracil-induced goiter results from the inhibition of thyroperoxidase (TPO), the enzyme responsible both for converting dietary iodide into iodine, the addition of iodide to tyrosine residues within the 660 kDa polypeptide precursor of thyroid hormones thyroglobulin (TG), and oxidatively coupling two diiodotyrosine residues together to form thyroxine. [12] Goiter-prevention assays compared thyroid weight between untreated, thiouracil-treated animals administered thyroid hormone and thiouracil-treated animals administered a thyroid hormone analog to give an indication of the relative potency of the analog in comparison to thyroid hormone. While this was a simple and powerful assay technique, it was limited by the lack of understanding of the underlying mechanisms. Thyroid hormone analogs had to be metabolically stable, cross the blood-brain barrier, and activate thyroid hormone receptors in the brain to suppress the HPT axis and reduce goiter formation. So, for instance, an analog with improved target engagement but reduced blood-brain barrier

penetration or metabolic stability would not appear to have any enhanced activity by this assay technique.

The second primary methodology for assaying thyroid hormone analogs was the amphibian metamorphosis assay. Gudernatsch reported in 1912 that feeding thyroid tissue to tadpoles induced precocious metamorphosis. [13] These results were subsequently found to be due to the actions of thyroid hormone and were formally adapted as a quantitative and reproducible method for assaying thyroid hormone analogs by Gaddum in 1927. [14] The assay had the benefit of operational simplicity - tadpoles were immersed in water spiked with a drug - and cheapness, as tadpoles are both less labor-intensive and easier to house than rodents. It was eventually determined that the assay could be further simplified, by studying the rate at which isolated tadpole tail tips shortened in response to thyroid hormones and thyroid hormone analogs as this recapitulated the results from intact tadpoles. [15] But this methodology suffered from many of the same limitations as the goiter-prevention assay, as the relative potencies were compound metrics dependent on uptake, efficacy, and receptor selectivity. Additionally, the results of goiter-prevention assays and tadpole metamorphosis assays rarely agreed due to the increased uptake of exogenous compounds in amphibians and differences in metabolic stability between mammals and amphibians.

Various methods for measuring changes in metabolic rate were frequently used as methods for assaying thyroid hormone analogs. Increased metabolism, heart rate, and oxygen consumption had been observed features of hyperthyroid conditions such as Graves' disease and these effects could be evoked by chronic administration of thyroxine in mammals. [16] Oxygen consumption could be measured directly by placing an animal within a sealed chamber with soda lime to absorb the released carbon dioxide and water, then measuring the amount of oxygen necessary to maintain a constant pressure within the vessel. [17] A modification of this setup developed by Benedict and Homans passed the expelled gases through sulfuric acid to dehydrate them, followed by soda lime, which allowed for the measurement of carbon dioxide production by weighing the soda lime after the experiment was complete. [18] A simpler, but more cruel, method placed treated animals in bell jars and observed the length of time until the animal asphyxiated as a measurement of increased oxygen consumption. [19] Heart rate was measured by standard electrocardiographs. [20] As with other physiological measurements, these produced compound metrics of potency, further complicated by other physiological drivers of metabolism such as endogenous thyroid hormones - unless the animals had first been thyroidectomized - and the nervous system.

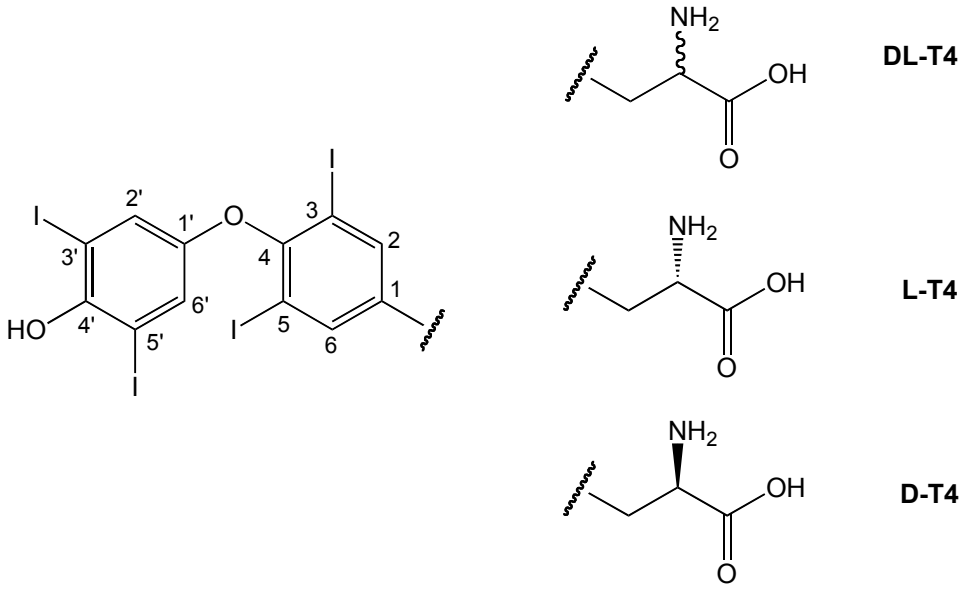

Figure 1.2

The enantiomers of tertraiodothyronine (T4).

The initial methodologies for extracting T4 from the thyroid were extremely harsh - the chemical and biological tools available for liberating thyroxine from its proteinaceous precursor thyroglobulin would almost invariably degrade the T4 by liberating iodine or racemizing the amino acid portion of the molecule. As a result, most early preparations were racemic mixtures of levo- and dextrothyroxine. Harington reported a resolution of synthetic T4 into its L- and D- forms in 1928, which created the first analog. [21] Levothyroxine is synthesized endogenously while the D-form is only formed by synthesis or *in vivo* racemization. Dextrothyroxine would go on to be the first seriously investigated thyromimetic for extrathyroidal clinical applications.

Many of the earliest thyroid hormone analogs were reported by Schuegraf in 1929, who explored alternative halogen substitutuions - tetrabromo, tetrachloro, or mixed dibromo/diiodo or dichloro/diiodo substitutions in the 3,5 and 3',5' positions of DL-thyronine. [22] The pure D-enantiomers of these analogs were later synthesized by Dibbo in 1961. [23] Schuegraf's compounds were initially evaluated by Abderhalden and Wertheimer in 1928 with an amphibian metamorphosis assay - all six analogs were active albeit with reduced potency compared to T4. [24] They found that analogs with iodine in the 3,5-position and bromine in the 3',5'-position were more active than the analogs with iodine in the 3',5'-position and bromine in the 3,5-position, while the opposite held true for chlorine substituted analogs. In all cases potency of bromine substituted analogs were greater than chlorine analogs. Gaddum's report in 1930 briefly suggested that the tetrabromo analog was only weakly active, requiring a 50 mg/kg dose for noticeable effects, [17] but Abderhalden's results were partially corroborated by Leblond and Grad in 1948 with assays of heart rate, heart weight, and oxygen consumption in thyroidectomized rats. [25] Leblond and Grad found one case where their results disagreed with Abderhalden's work, noting that the thyronine with iodine in the 3',5'-position and bromine in the 3,5-position was more active than analog with iodine in the 3,5-position and bromine in the 3',5'-position. This type of

discrepency was relatively common, as different model organisms would often produce significantly different relative activities for thyroid hormone analogs. Other permutations of halogenated D-thyronine analogs were synthesized by Dibbo in 1961 including 3',5'-dibromo-3,5-dichloro, 3,3',5-tribromo-5'-iodo, 3,3',5-trichloro-5'-iodo, and the inverse 3',5,5'-triiodo-3-chloro. [23] The latter two were tested in 1961 by Cuthbertson in serum and liver cholesterol and heart ventricle weight assays in rats. [26] 3,3',5-trichloro-5'-iodo-D-thyronine had significant potency in all three assays, but increased heart ventricle weight occurred at lower doses than those necessary for liver cholesterol reductions, which meant that its cardiac impact was too great to have any clinical utility. 3',5,5'-triiodo-3-chlorothyronine was tested in both the L and D forms with weak but roughly equal potency for reducing serum and liver cholesterol, but the L-thyronine required a significantly higher dosage to evoke increases in ventricular weight, which made it a more promising candidate for therapeutic use. A series of fluorinated thyroxine derivatives were synthesized by Niemann in 1941, [27,28] and assayed for their ability to prevent goiter formation in rats by Cortell in 1949, who found that the 3',5'-difluoro-3,5-diiodothyronine derivative had between 1/30- and 1/60- the activity of T4 and 3'-fluoro-3,5,5'-triiodothyronine had at least 1/3- the activity of T4. [29] These results showed that any other halogen could replace the iodines of T4 and produce an active

compound, but these substitutions invariably reduced the activity of the analogs as the van der Waals radii of the halogens decreased.

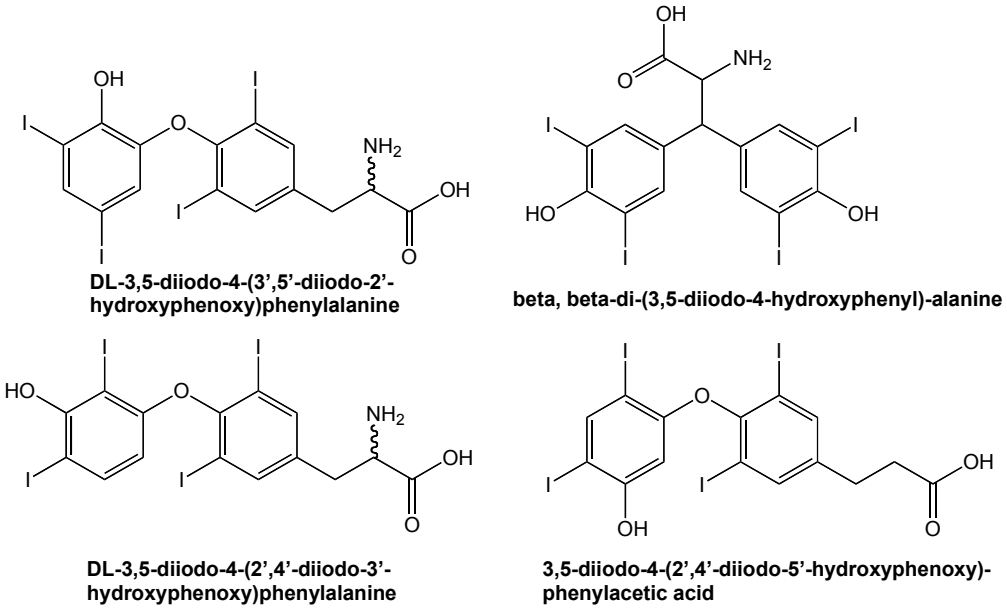

Figure 1.3

Positional isomers of tetraiodothyronine.

Positional isomers of T4 were explored that helped to develop an understanding of the structural constraints required to produce active thyronines. Niemann reported the synthesis and testing of two different isomers (Fig 1.3) in 1941 modifying the outer ring: DL-3,5-diiodo-4-(3',5'-diiodo-2'-hydroxyphenoxy)phenylalanine was between 1/25 and 1/50 as active as DL-T4[30] and DL-3,5-diiodo-4-(2',4'-diiodo-3'-hydroxyphenoxy)phenylalanine was inactive at all tested doses. [31] These results were confirmed by Boyer in 1942. [32] This SAR data created a belief that the efficacy of thyromimetics was dependent on being able to establish an equilibrium with the quinoid form of the molecule,

which would only be possible if the outer ring hydroxyl group was in the 4' or 2' position. Bruice expanded on this work in 1954 and produced a deaminated 'meta' analog (Fig 1.3) with a propionic acid moiety, which was installed to increase the base potency of the analog. 3,5-diiodo-4-(2',4'-diiodo-5'-hydroxyphenoxy)-phenylacetic acid was demonstrated to have 2% of the activity of DL-thyroxine in a tadpole metamorphosis assay. [33] The β-alanine isomer of T4 (Fig 1.3) was synthesized by Cookson in 1952, [34] but no reports of its activity have been published. Harington reported a more extreme modification in 1929 - β,β-di-(3,5-diiodo-4-hydroxyphenyl)-alanine - rearranged the positions of the diaryl ether oxygen and the amino acid. [35] This isomer was inactive at all tested doses. These studies reinforced the theory that it was critical to have a hydroxyl group in the 4'-position, halogens in the 3',5'-positions, and a diaryl ether structure.

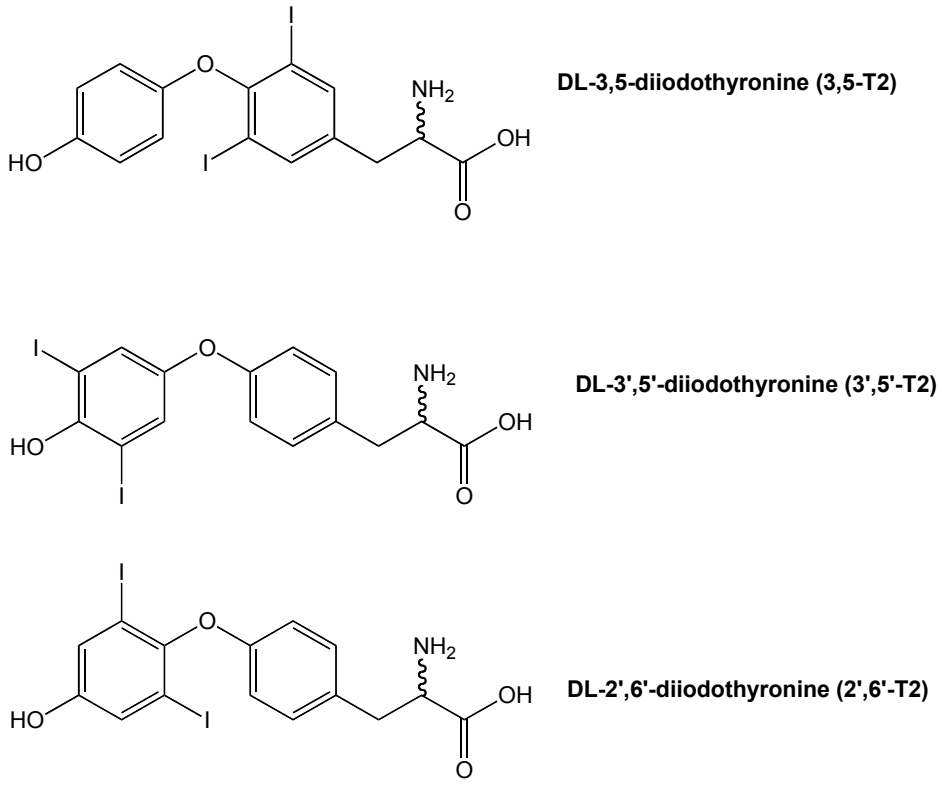

Figure 1.4

Positional isomers of diiodothyronine (T2).

The diiodothyronines (T2) formed a second class of thyronine derivatives studied during the early phase of analog development that helped to refine the structural requirements for halogens. Harington produced 3,5-diiodothyronine (3,5-T2) (Fig 1.4) as part of his synthesis of thyroxine in 1927. [8] 3,5-T2 was assayed by Gaddum in 1930 and found to have a potency roughly fifteen times less than T4 by weight. [17] Block Jr. and Powell produced another metabolite when they synthesized 3',5'-diiodothyronine (3',5'-T2) (Fig 1.4), [36] but preliminary work by William Salter suggested that it was physiologically inactive at doses four times greater than DL-T4. A third isomer, 2',6'-

diiodothyronine (2',6'-T2) (Fig 1.4) was produced by Niemann and McCasland[37] and was found to be physiologically inactive at doses one hundred times greater than DL-T4. [38] The sum of these experiments with alternative halogens, isomers of T4, and isomers of T2 was the information that analogs based on a thyronine core required halogens at the 3,5-position and a hydroxyl group at the 4' position, while full potency required halogens at the 3',5'-position as well. As of 1950 there was no clear mechanistic explanation for these structural requirements.

Other hydrophobic group substitutions for the inner and outer ring iodines of T4 were explored beginning in the 1950s. Replacement of the inner ring T4 iodines by nitro groups, which was a synthetic intermediate in the synthesis of thyroxine, was intentionally synthesized by Clayton in 1951[39] and evaluated by Wiswell in 1955, [40] who found no activity in an oxygen uptake assay. The tetramethyl analog of DL-T4 was synthesized by Bielig in 1957[41] and evaluated by Pittman in 1961, who found that it had no appreciable metabolic activity. [42] Tetramethyl L-T4 was synthesized by Block in 1972[43] and evaluated by Pittman in 1973, who found that it was between 1/10- and 1/100- as potent as T4 in oxygen consumption and heart rate assays in thyroidectomized rats. [44] Intermediate dimethyl-diiodo analogs provided a bridge between thyroxine and tetramethylthyronine. Syntheses of 3',5'-dimethyl-3,5-diiodo DL-thyronine were

reported simultaneously by Barnes[45] and Bruice[46] in 1953. The compound was evaluated by Pittman in 1961 and found to have 88% of the activity of DL-thyroxine. [42] L-3',5'-diiodo-3,5-dimethylthyronine was synthesized by Paul Block Jr. and evaluated for its binding affinity to human plasma prealbumin by Andrea in 1980, who found that it had <0.1% of the affinity of T4. [47] However, prealbumin binding is not strictly comparable to other binding assays used to evaluate thyromimetics. Analogs known to have greater physiological activity than L-T4 demonstrated lower binding affinity for prealbumin, suggesting that this binding affinity does not correlate with physiological activity. These results demonstrated that methyl-substituted thyronines have significantly reduced activity in comparison to iodothyronines, but inner ring substitutions had a greater impact than outer ring substitutions.

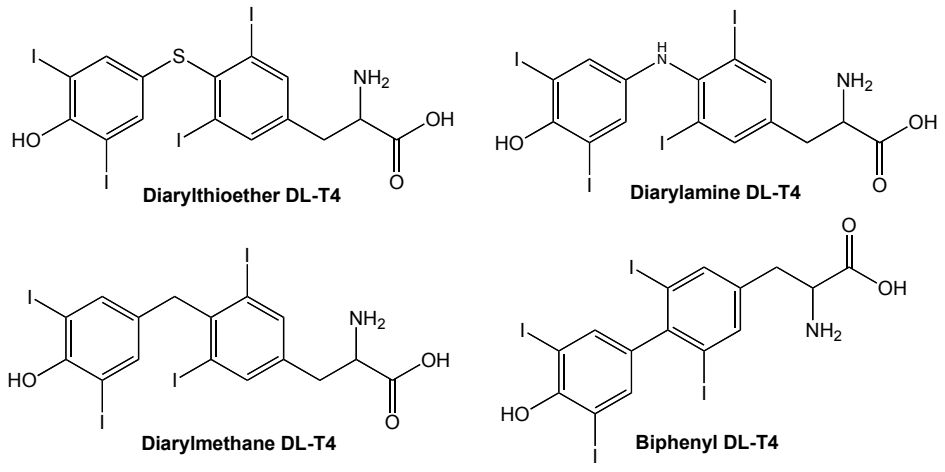

Figure 1.5

Substitutions of the bridging group of tetraiodothyronine (T4).

The first analog modifying the diaryl ether motif was produced in 1930, substituting a diaryl thioether for the diaryl ether of thyronine, the amino acid analog of thyroxine that does not contain any iodines. [48] Nearly two decades passed before the more physiologically relevant thioether substitution was made in thyroxine by Harington, [49] which demonstrated 12% of the activity of T4 in stimulating basal metabolic rate in humans[50]. During the 1950s it was generally accepted that only diaryl ethers and thioethers were tolerated in thyroid hormone analogs, but subsequent work disproved that theory. Methylene-bridged analogs were reported by Tripp[51] and evaluated by Psychoyos in 1973, who found that the methylene-bridge analog of DL-thyroxine was roughly ten-fold less potent than the ether-bridged parent compound in inducing glycerophosphate dehydrogenase activity in liver, heart, and kidney. [52] The diarylamine analog of T4 was produced, along with a number of diaryl amide analogs, by Mukherjee in 1971, [53] but *in vitro* or *in vivo* testing of these compounds has not been published. The necessity of a bridging group was demonstrated by the biphenyl analog of T4, synthesized by Barnes in 1953[45] and evaluated by Barker in 1965, who found that it had less than 0.3% of the activity of T4 to promote increased heart rate in thyroidectomized rats. [54] This cemented one more structural constraint necessary for producing active thyronines,

demonstrating that a bridging group between the two aryl rings was necessary for activity.

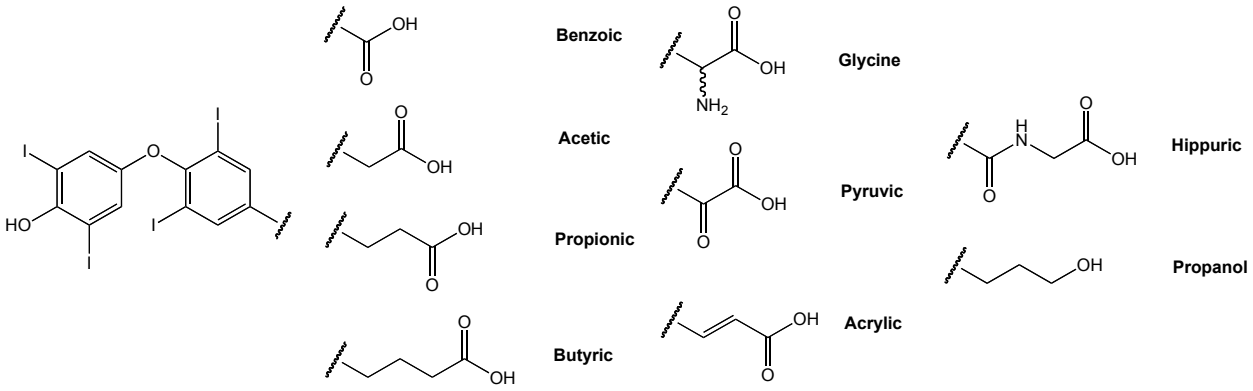

Figure 1.6

Substitutions of the alanyl group of tetraiodothyronine (T4).

The alanine amino acid functionality of T4 was another feature open for modification. Tetraiodothyrobenzoic acid (Fig 1.6) was synthesized by Harington in 1927[8]. It was tested by Frieden and Winzler in 1948 and found to have low but significant effects on amphibian metamorphosis and in a rat-based thiouracil-induced goiter prevention assay. [55] Tetraiodothyroacetic acid (tetrac), which would later be found to be an endogenous metabolite, [56] was synthesized by Harington and Pitt-Rivers in 1952[57] and assayed by Pitt-Rivers in 1953, who found that it had roughly 1/2 the activity of L-T4 in a rat goiter prevention assay. [58] The deaminated analog of thyroxine tetraiodothyropropionic acid (tetraprop) was first reported by Clayton in 1951[39] and was initially found to be inactive in a rat anoxia assay, [19] but was later found to be one-hundred thirty

times more active than DL-T4 in a tadpole metamorphosis assay. [59] Tetraiodothyrobutyric acid was first assayed by Money in 1958 in a tadpole metamorphosis assay and found to be twelve times as potent as L-T4. [60] These results suggested that activity peaked with tetraiodothyropropionic acid, with shorter or longer alkanoic acids demonstrating reduced activity compared to thyroxine. The glycine analog of thyroxine was synthesized by Frieden in 1948[61] and assayed by Bruice in 1953 who found that it had 16% of the activity of DL-T4 in an amphibian metamorphosis assay and 0.2% of the activity of DL-T4 in rodents. [62] The pyruvic acid analog, which was also found to be a metabolite of T4, [63] was synthesized by Canzanelli in 1935 and was found to be roughly 1/4 as active as T4 in stimulating metabolism in dogs. [64] The acrylic acid analog of thyroxine was synthesized by Wawzonk[65] in 1950 and physiologically assayed by Barker in both normal and thyroidectomized rats, which found that it had roughly 1/100 the activity of DL-T4. [66] Niemann and McCasland reported the synthesis of the hippuric acid analog of T4 in 1944[67], which was assayed by Cortell in 1949 and found to possess weak agonist activity in a goiter prevention assay[29]. Two thyroalkonol analogs of T4 were synthesized and assayed for activity in rats by Wechter in 1965, who found that at 5 mg/kg the propanol analog was inactive while the butanol analog produced a small but significant decrease in serum cholesterol. [68] These experiments demonstrated that the amine

functionality of thyroxine was not only dispensable but removal actually increased potency while most other substitutions usually decreased or abolished activity, providing another structural constraint.

1.3 Development of Triiodothyronine-Based Analogs

The development of more potent thyroid hormone analogs followed from the discovery of an unknown endogenous iodothyronine by Gross and Pitt-Rivers in 1952. [69] This work was followed by the isolation from the thyroid gland and synthesis of 3,3',5-triiodothyrnone (T3) in 1953, [70] which confirmed that it was the unknown constituent. Further work by Gross and Pitt-Rivers demonstrated that T3 was more potent than T4 in a number of physiological models of thyroid hormone action. [71] At this point the scientific consensus was that T4 was the primary effector of the thyroid hormone system and it took several decades of work to convince the endocrinology community that T4 was primarily a prohormone for T3.

Many strands of evidence were necessary to effect this change in doctrine. Pitt-Rivers demonstrated in 1955 that radiolabeled T3 could be formed after the administration of radiolabeled T4 in human patients without functional thyroid glands, which demonstrated that the conversion was primarily extrathyroidal. [72]

There was a major setback when Lassiter and Stanbury reported in 1958 that they could not replicate the experimental results of Pitt-Rivers, which resulted in many members of the field concluding that T4 was still the active hormone. [73] The tide started to turn when more convincing evidence for the importance of T3 was provided by a report from Bray and Hildreth in 1967 using two inhibitors, methimazole (MMI) and propylthiouracil (PTU). [74] MMI blocks production of thyroid hormone by the gland, while PTU blocks both production in the gland and the extrathyroidal conversion of T4 into T3 by deiodinases. The experiment showed that T4 or T3 produced increased metabolism when coadministered with MMI, but only T3 was active while T4 was inactive when each was coadministered with PTU. The mechanism behind these results was determined by Oppenheimer[75] in 1972 and confirmed by Bernal[76] in 1974 when it was experimentally demonstrated that PTU inhibits the conversion of T4 to T3 in rats. Similar experiments were carried out in humans by Geffner that confirmed the results of Oppenheimer and Bernal while also demonstrating that PTU amplified the production of TSH. [77] The conversion of unlabeled T4 to T3 in humans was demonstrated directly by Braverman[78] in 1970 and of radiolabeled T4 to T3 in rats by Schwartz[79] in 1971. These data were eventually sufficient to reorient the focus of thyromimetic research towards T3.

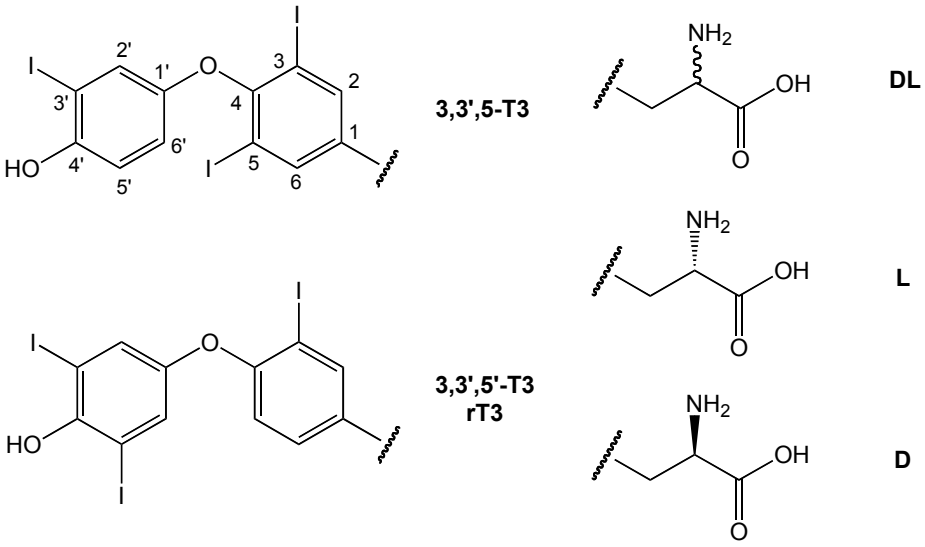

Figure 1.7

Enantiomers of 3,3',5-triiodothyronine (T3) and 3,3',5'-triidothyronine (rT3).

As with T4, positional isomers of T3 were explored. 3,3',5'-triiodothyronine, reverse T3 (rT3) was found to be an endogenous component of blood by Michel in 1956[80], but was demonstrated to be biologically inactive by Pittman in 1962. [81] Jorgensen reported 'ortho' and 'meta' analogs that repositioned the iodine and alanyl groups on the inner ring, which were found to have little to no activity in a rat goiter prevention assay. [82] These results confirmed much of what had been found with T4 analogs - the position of halogens and the amino acid functionality were very sensitive and only certain perturbations could be made while preserving activity.

As with T4 analogs, halogen substitutions were some of the first to be explored. 3,3',5-tribromo-DL-thyronine was first synthesized by Yagi in 1953, [83]

which was demonstrated by Mussett and Pitt-Rivers in 1954 to be more potent than 3,3',5,5'-tetrabromo-DL-thyronine[84]. 3,3',5-trichloro-DL-thyronine was first synthesized and evaluated by Mussett, who found that it was also more potent than the tetrachloro analog, but the trichloro analog had only 1.2% of the activity of T4. [85] Cuthbertson reported in 1961 that 3,3',5-triichloro-D-thyronine produced decreases in serum cholesterol, but also pathological cardiac stimulation and repression of endogenous hormone production when tested in rats. [26] DL-thyronine analogs with 3,5-diiodo substitutions and either 3'-bromo or chloro substitutions had surprising activities in comparison to their T4 analogs. Michel found that the 3'-bromo derivative was more potent than the 3',5'-dibromo analog while the 3'-chloro derivative was less potent than the 3',5'-dichloro analog. [86] The 3,5-diiodo-3'-fluorothyronine analog was synthesized by Niemann in 1941[28] and evaluated by Mussett in 1957, who found that it was more potent than 3,5-diiodo-3',5'-diflurothyronine. [84] The 3,5-dibromo-3'-iodothyronine analog was synthesized by Mussett in 1957 and found to have an activity intermediate between that of 3,3',5-tribromothyronine and 3,5-diiodo-3'-bromothyronine. [85] The 3,3'-diiodo-5-bromo-DL-thyronine analog was synthesized[87] and assayed in rats[88] by Gemmill in 1956, who found that it had roughly half the activity of L-T3 or equal activity to DL-T3 in preventing goiter formation and increasing metabolic activity. D-thyronine analogs with 3'-bromo-

3,5-dichloro and 3'-chloro-3,5-dibromo substitutions were synthesized by Dibbo in 1961, but no assay results have been published. [23]

A range of alkyl substitutions were explored. 3'-Methyl-3,5-diiodo-L-thyronine was first evaluated by Pittman in 1961, who found that it was roughly twice as potent as L-T4 and half as potent as L-T3 at stimulating increased basal metabolic rate in rats. [42] A series of 3'-alkyl substituents ranging from straight chain methyl and ethyl groups to branched chain isopropyl and isobutyl groups to cyclic phenyl and cyclohexyl groups were all synthesized and tested in a rat cholesterol lowering assay by Blank in 1963, who found that the isopropyl substitution was roughly equipotent with L-T3, while the ethyl and methyl substitutions had decreasing activity as the substituents grew smaller, the larger phenyl and cyclohexyl groups also had reduced potency - roughly 30% of L-T3. [89] The study also found that dialkyl substitutions at the 3'- and 5'-positions completely abolished activity. Both of these results were confirmed by Greenberg in 1963, as well as in a number of other physiological assays. [90] The 3'-isopropyl analog (DIIP) was evaluated in humans and found to be roughly twice as potent as L-T4 on a mass basis. [91] The 3'-*tert*-butyl analog of L-T3 was synthesized and assayed by Buess in 1965, who found that it was equipotent with L-T4 in a tadpole metamorphosis assay[92]. The compound was also tested by Jorgensen, who reported in 1965 that it was equipotent was L-T4 in a rat

antigoiter assay. [93] Naphthyl and hydroxynaphthyl ethers of DL-diiodothyronine were synthesized by Jorgensen in 1961 and tested in 1962, with the result that the 4'-hydroxy-1-napthyl analog was found to be as potent as L-T4 in a rat antigoiter assay and 20% as potent as L-T3 in a rat oxygen consumption assay. [94,95] Addition of a bromine at the 3' position reduced its antigoiter activity to 20% of L-T4 and nearly abolished its activity in stimulating oxygen consumption. Alkyl substitutions at the 3,5-positions of DL-3'-iodothyronine were synthesized and tested in a rat goiter prevention assay by Jorgensen in 1962, who found that either a single or double replacement of the inner ring iodines with methyl groups reduced activity to 6-20% that of L-T4 for a single methyl substitution and 1-3% for a double methyl substitution in a rat anti-goiter assay. [96] These results suggested that there was significantly more flexibility for alkyl substitutions at the 3'-position without a loss of potency than at the 3,5-positions.

As with T4 analogs, various substitutions of the biaryl ether moiety have been explored. The thioether analog was synthesized and assayed by Mussett in 1957, who found that it was roughly 1.5 times more active than T4 and 1500 times more active than the T4 thioether analog in a goiter prevention assay. [85] The methylene-bridged analog of DL-T3 was reported by Tripp in 1973[51] and evaluated by Psychoyos in 1973, who found that it was roughly twenty-fold less

potent than L-T3 but nearly equipotent with L-T4 in inducing glycerophosphate dehydrogenase activity in rat liver, heart, and kidney. [52]

As with T4, deaminated analogs were also built on a T3 core. Michel found a general trend of potency increasing by chain length - the propionic acid analog was roughly ten times more potent than the acetic acid analog, which was in turn roughly ten times more potent than the benzoic acid analog. [86] It was also observed that these analogs were almost two orders of magnitude more potent in an amphibian metamorphosis assay than a goiter prevention assay. Tadpole metamorphosis assays with the same set of deaminated analogs plus the butyric acid analog by Money in 1958 confirmed that the potency peaked with the propionic acid analog - the butyric acid analog was only half as potent as the acetic acid analog. [60] The acrylic acid analog of T3 was synthesized and assayed by Roche in 1956, who found that it had 22% of the activity DL-T4 and 3.25 times more activity than the acrylic acid analog of T4 in a rat goiter prevention assay. [97] Thyroalkonols were explored, which reduced the carboxylic acid of various thyroacetic acids to alcohols. Both the one-carbon and two-carbon analogs produced significant reductions in serum sterols and weight gain in comparison to controls in fat fed rats at doses of 0.3 mg/kg. [68] The pyruvic acid analog of T3 was initially found by Roche in 1954 as a oxidatively deaminated metabolite of T3 administration to rats, [63] but was later demonstrated by Roche

in 1955 to be equipotent with DL-thyroxine in an amphibian metamorphosis assay. [98] The results confirmed what had been found with T4 analogs - the amino acid functionality of T3 could be done away with, but analogs without a simple alkanoic acid functionality generally reduced activity.

The importance of the 4'-hydroxy group of T3 was tested by Jorgensen in 1961, who replaced it with a 4'-amino group and found that it had roughly 1.5% the activity of T3 in a rat goiter prevention assay. [99]

The SAR obtained from T3 analogs roughly matched that found for T4 analogs - substitutions of the iodines, biaryl ether, and alanyl moieties were generally active, but, with rare exceptions, less active than the natural active hormone T3.

1.3 Development of Clinical Analogs

Clinical interest in L-T4 and L-T3 as agents for reducing cholesterol and increasing metabolism goes back to the late-19th century and continued on through the mid-20th century. [100] However, it had long been observed that there was no therapeutic window for these hormones - doses sufficient to produce the desired effects were invariably accompanied by suppression of the HPT axis, followed by a rebound in serum cholesterol levels, then by harmful increases in

cardiac output leading to tachycardia and heart failure if the dosage was increased to account for the suppressed endogenous production of thyroid hormones. [101] While biochemical methods for accurately measuring cholesterol levels in serum and tissue were well-established by the 1940s, the challenge of developing new compounds became more complicated as researchers set out to isolate the cholesterol lowering effects of thyroid hormone action from other elements such as cardiotoxicity, suppression of the HPT axis, or increases in basal metabolic rate.

If L-T4 and L-T3 did not offer any way to produce the desired effects without negative side effects, the wide array of analogs being synthesized during the mid-20th century might offer novel patterns of activity. The first hints came from Lerman and Pitt-Rivers, who administered triac to hypothyroid patients and reported in 1955 and 1956 that this analog decreased serum cholesterol without significantly raising basal metabolic rate (BMR). [102,103] However, the authors noted that the results might have been due to the timing of their measurements as there was a significant interval between administration of triac and measurements of BMR, which may have not been appropriately matched to the quick onset of its effects. This work was followed up by Rall in 1956, who weakened the hypothesis by finding that triac had similar nonselective effects to T3 and T4, albeit with reduced potency. [104] Related work reported by Best in

1957 and 1959 found that the benzoic acid analog of thyroxine could produce a significant reduction in liver cholesterol without impacting thyroid weight in fat fed rats treated with thiouracil. [105,106] This led to studies with rats reported by Best and Duncan in 1958 and 1960 demonstrating that both the benzoic acid[107] and D-alanine analogs of 3,3',5-triiodothyronine[108] had more pronounced effects on serum cholesterol than on goiter prevention compared to L-T3, which were reconfirmed by the two researchers in 1962. [109] These results were independently confirmed by Greenberg in 1961, who found that in rats D-T3 had 25% the calorigenic effect of L-T3, but only 8% the anti-goiterogenic effect. [110] Tapley reported in 1959 that L-T4 and D-T4 had significantly different distributions, with D-T4 partitioning selectively to the liver. [111] Duncan and Best extended the work and found that D-T3 also partitioned strongly to the liver, which provided a mechanism for the D isomers' selective effects. [112] However, the same study found that the benzoic acid analog of T4, which had selective effects, partitioned in the same way as the propionic acid analog, which did not have selective effects, ruling that out as a mechanism for the benzoic acid analog's selective effects. They also performed a small scale clinical trial in euthyroid patients, which found that D-thyronines and thyrobenzoic acid could reduce serum cholesterol while having less impact on cardiac output than L-thyronines, suggesting that they might have broader clinical potential for

reducing cardiovascular disease. [113] A similar study with L- and D-thyronines plus tetraiodothyroformic acid and 3,5-diiodo-3',5'-dimethylthyroformic acid was carried out with a small cohort of hypothyroid patients, which further explored their selective effects and found them efficacious and well-tolerated. [109] Searcy reported in 1961 that short-term D-T4 treatment of patients with heart disease significantly reduced the levels of β-lipoproteins but did not significantly affected α-lipoproteins. [114] Barnes reported a clinical trial with D-T4 on euthyroid patients who did not have heart disease or elevated cholesterol in 1962, which found that cholesterol levels dropped in the treated patients compared to those receiving placebo without any increase in basal metabolic rate. [115] Larger clinical studies began concurrently - some trials found that while D-thyronines and the benzoic acid analogs could reduce serum cholesterol in both hypothyroid and euthyroid patients, they did not provide a sufficiently broad therapeutic window to be approved for general use and were especially risky for patients with preexisting cardiac conditions, [116-118] one trial found that D-T4 was neither beneficial nor harmful, [119] while other trials continued to suggest that D-thyronines were effective and acceptably safe. [120,121] D-T4 was eventually approved as a therapeutic drug in 1967 under the trade name Choloxin, [122] with positive reports from clinicians praising the efficacy and safety

of the drug as a treatment for cardiovascular disease appearing soon after it entered the market. [123]

Cracks began to emerge during the Coronary Drug Project. This large scale, longitudinal clinical trial investigated the most effective medications for treating atherosclerosis available when it began in 1965. By 1970, D-T4 had been discontinued for a subgroup of patients who experienced frequent ectopic ventricular beats at baseline leading to increased mortality versus the placebo group. [124] By 1972 D-T4 treatment had been discontinued entirely as the broader patient population being treated with the drug displayed significantly increased mortality over the placebo group. [125] Potential mechanisms for these results began to emerge in the 1980s. Studies in 1984 reported that D-T4 could induce clinical hyperthyroidism at therapeutically relevant doses[126] and the ratio between the dose that reduced cholesterol and the dose that suppressed TSH were the same for D-T4 as for L-T4. [127] More recently it was determined that the D-T4 and D-T3 are unable to competitively inhibit the transport of L-T3 by MCT8, the main transporter of thyronines into the brain, which could have an effect on the ability of D-thyronines to suppress TSH. [128] Another study reported that many preparations of D-T4 were contaminated with L-T4, with further evidence that the majority of the clinical effects could be ascribed to L-T4 rather than D-T4. [129] Additionally, it was demonstrated that D-T3 binds to

the thyroid hormone receptor with equal affinity as L-T3, meaning that any selective physiological effects were not occurring at the level of receptor activation. [130] Further study suggested that D-T4 is preferentially deiodinated to the rT3 form, meaning that the bulk of an administered dose is metabolically deactivated, providing a plausible mechanism for the higher doses of D-T4 necessary to elicit cholesterol lowering compared to L-T4. [131] However, despite this evidence of poor efficacy and significant dangers, D-T4 was still being investigated in clinical trials as late as 1998[132] and was not removed from FDA approval until 2011. [133]

1.4 Early Modern Thyromimetic Development

More modern thyromimetics began to be synthesized once a body of structure-activity relationship data had been built up around analogs that modified a single aspect of the T4 or T3 structure. This slowly developed into the understanding that thyroid hormone analogs can be thought of as modular structures where elements can be mixed and matched to produce new analogs.

T4 analogs with multiple modifications followed from monosubstituted analogs. Replacement of the outer ring iodines in T4 with methyl groups had been made and found to reduce potency only modestly, [45,46] so this was

combined with the replacement of the amino acid functionality with a propionic acid, which had been demonstrated to increase potency. 3',5'-dimethyl-3,5-diiodothyropropionic acid (Me2Pr2) was synthesized by Kharasch in 1956, [134] then assayed by Pittman in 1958[135] and 1961, [42] who determined that it had 30% greater activity than tetraiiodothyropropionic (Pr4) acid in elevating oxygen consumption in thyroidectomized rats. As Pr4 was slightly more potent than L-T4, this rated Me2Pr2 as significantly more potent than L-T4. Instead of a propionic acid group, Wechter reported a thyroalkanol with the 3',5'-dimethyl-3,5-diiodothyronine core and an ethanol polar group that evoked significant reductions in serum sterols at both 5 and 0.3 mg/kg doses in rats. [68] Buess reported a 3,5-diiodothyropropionic acid analog with di-*tert*-butyl groups in the 3',5' positions in 1965, but it was not assayed. [92] Matsuura reported another variant in 1960, with di-*tert*-butyl groups in the 3',5' positions and the inner ring iodines replaced with bromines. [136] However 3',5'-di-tert-butyl-3,5-dibromothyropropionic acid was found to be inactive in both tadpole metamorphosis and rat goiter prevention assays, suggesting a limit on the steric bulk that could be accommodated on a disubstituted outer ring. By inference, this suggests that 3',5'-di-tert-butyl-3,5-diiodothyropropionic acid also would have been inactive, despite the potency boost afforded by iodines in comparison to bromines. Matsuura returned with a new set of analogs in 1964 that utilized

the same thyropropionic acid core with various inner and out ring halogen substitutions including 3,3',5,5'-tetrabromo, 3',5'-dibromo-3,5-diiodo, 3',5'-diiodo-3,5-dibromo, and 3'5'-diiodo-3,5-dichloro, but none were assayed. [137] Tripp synthesized[51] and Psychoyos assayed[52] a methylene-bridged analog of tetraiodothyroacetic acid in 1973, which was found to be more than ten fold less potent than its ether bridged parent compound in assays of GPD activity in rat liver and heart. Ultimately these analogs definitively demonstrated the limits of T4 analogs, which could potentially surpass their parent but could not approach the activity of T3 analogs.

T3 received the same kind of synthetic attention. Buess reported a 3,5-diiodothyropropionic acid analogs with a *tert*-butyl group in the 3' position in 1965. [92] Given that 3'-tert-butyl-3,5-diiodothyronine had activity comparable to T4, it can be assumed that the propionic acid analog would also be as or more active. Blank reported a series of T3 analogs in 1963 that took several new tacks - N,N-diethylaminoethyl esters of 3,5-diiodothyroalkanoic acids with either 4'-hydroxy or 4'-methoxy substitutions were assayed for their ability to reduce serum cholesterol. [138] Of these compounds, the 3'-iodo benzoate and propionate esters were only weakly active, with 3-6% of the activity of L-T3, while the 3'-iodo and 3'-isopropyl acetate analogs demonstrated comparable activity to L-T3. Despite their enhanced activity over their methyl ether counterparts, the

phenolic compounds were not deemed as safe due to greater calorigenic activity and significant increases in heart weight than the free acids when administered to rats. [90]

Synthesizing iodine-free analogs with activity comparable to T3 became a major goal as the role of deiodinases in the metabolism of thyroid hormones became clear. [139] Blank had demonstrated in 1963 that the 3'-iodo group of L-T3 could be replaced by an isopropyl group while retaining full potency. [89] It was a short leap from that data to the work Taylor Jr reported in 1967 to replace the inner ring iodines with bromines. [140] 3'-isopropyl-3,5-dibromo-L-thyronine (DIBIT) was shown to be two to seven times as active as L-T4 in rat heart rate elevation and anti-goiter assays and a tadpole metamorphosis assay. In the metamorphosis assay it was demonstrated to be less active than the 3,5-diiodo analog, but only by two fold. These results confirmed that iodines were not required for significant activity when an analog had a 3' alkyl substitution. Jorgensen and Wright released two reports in 1970 that expanded upon the idea of iodine-free analogs. The first reversed the alkyl and halogen substitutions, placing di-isopropyl or di-*sec*-butyl groups in the 3,5 positions and bromine, iodine, or methyl groups in the 3' position of DL-thyronine. [141] While this seemed like a logical substitution given that 3'-isopropyl groups had been sufficient in other contexts, the compounds were universally inactive in rat heart

rate elevation and tadpole metamorphosis assays. This showed that the constraints on the 3,5 position were tighter than at the 3' position. The second paper focused on DL-thyronine analogs with 3,3',5-alkyl substitutions. [142] Instead of the bromines of DIBIT, the inner ring was substituted with methyl groups to form 3'-isopropyl-3,5-dimethyl-DL-thyronine (DIMIT). While a 3'-iodo-3,5-dimethyl-DL-thyronine analog demonstrated low but measurable activity at 4-5% that of L-T4 in a rat anti-goiter assay, the 3'-isopropyl-3,5-dimethyl-DL-thyronine analog displayed no measurable activity. This suggested that the 3-isopropyl substitution was not always a sufficient mimic of a 3'-iodo substitution - replacement of halogens on the inner ring with methyl groups reduced the activity too much. However, Jorgensen remade the analog on an L-thyronine core and reported in 1974 that it had significantly more activity than the DL-thyronine analog. [143] Contrary to results with the DL analog, in a rat anti-goiter assay L-DIMIT was found to have 18% of the activity of L-T4 while 3'-iodo-3,5-dimethyl-L-thyronine had 5% of the activity of L-T4. The contribution of the 3'-isopropyl group was confirmed by the activity of 3,3',5-trimethyl-L-thyronine, which was only 3% that of L-T4 in the same assay. This demonstrated that while replacement of halogens significantly reduced the activity of thyromimetics, they could still retain a sufficient amount of activity to be useful compounds. This was shown unequivocally by Tamagna in 1979 when

DIMIT was administered to humans and shown to fully suppress thyrotropin (TSH) release after stimulation by thyrotropin-releasing hormone (TRH), but it required a molar dose roughly thirty-seven times greater than that of L-T3 to produce the same physiological effects. [144]

Several analogs expanded on the DIMIT core by further modifying the 3,5 positions. Jorgensen reported the synthesis and testing of 3'-isopropyl-3,5-diethylthio-DL-thyronine and 3'-isopropyl-3,5-diphenylthio-DL-thyronine in 1969, which found that the compounds were completely inactive as agonists or antagonists in rat anti-goiter assays[145]. More conservative modifications were reported by Chopra in 1984 with 3'-isopropyl-3,5-diethylthyronine demonstrating no activity in a rat TSH suppression assay. [146] These two studies further demonstrated the tight constraints on the inner ring 3,5 positions - increases in steric bulk beyond the van der Waals radii of iodines completely abolished activity.

While O-methyl ethers had commonly been used as protecting groups in the synthesis of thyronines, it was unknown whether they would be active *in vivo*. The methyl ether of DL-thyroxine was first synthesized by Myers in 1932, [147] who noted that it acted in a similar fashion to thyroxine in a tadpole metamorphosis assay but had little effect in humans at doses comparable to physiological concentrations. [148] Loeser tested the compound in guinea pigs and

reported in 1938 that it was more potent than thyroxine at increasing oxygen consumption when administered orally. [149] Conversely, Clayton reported in 1950 that it was only half as active at T4 in raising oxygen consumption in mice[150] and Lerman reported in 1952 that it was 5-6% as active as L-T4 at reducing human myxedema. [50] From this somewhat inauspicious start, the methyl ether moiety would be utilized in other thyroid hormone analogs to evoke novel physiological responses. Greenberg reported a methyl ether variant of 3,3',5-triiodothyropropionic acid (triprop) in 1962 that was assayed in rats and found to be nearly equipotent with its parent compound in terms of its antigoiterogenic effects, but had significantly less activity in terms of increasing oxygen consumption or reducing serum cholesterol. [151] This opened the possibilities for new analogs with differential effects. Greenberg also reported activities of the methyl ether analogs of triac and 3'-isopropyl-3,5-diiodothyroacetic acid, which had reduced calorigenic activity in comparison to their phenolic parent compounds, but largely maintained their antigoiterogenic activity. [90] These two sets of compounds were viewed as potential treatments for hyperthyroidism - the separation of anti-goiterogeic and calorigenic activity seemed to offer the possibility of suppressing endogenous thyroid hormone production while avoiding additional peripheral thyrotoxicity. Jorgensen reported a methyl ether of 3'-*tert*-butyl-3,5-diiodo-L-thyronine in 1965, which had <13% the

antigoiterogenic activity of either its parent phenolic compound or L-thyroxine. [93] Later methyl ether compounds took a different tack, emphasizing cholesterol lowering. Wechter reported assays of a number of methyl ether variants of 3',5'-dimethyl-3,5-diiodothyronine in 1965 including the ethanol, methyl acetate, and acetic acid analogs, which had roughly the same cholesterol lowering activity as their parent phenolic compounds. [68] Blank reported a set of analogs in 1963 that were N,N-diethylaminoethyl esters of 3,3',5-triiodothyroalkanoic acids, which included both phenolic and methyl ethers in the 4' position that were assayed for their ability to reduce serum cholesterol. [138] The phenolic and methyl ether benzoic acid analogs were roughly equipotent, with the methyl ether possessing slightly more activity, while the acetic and propionic acid methyl ether analogs had roughly half the activity of the phenolic analogs. Utley reported in 1964 that in control, hypophysectomized, and thyroidectomized rats that SKF 13364-A, the N,N-diethylaminoethyl ester methyl ether acetate analog from Blank's 1963 study, produced significantly less cardiac hypertrophy and less change in liver oxygen consumption than L-T3 or SKF 12276, the 3'-isopropyl analog of L-T3. [152] These results were further evidence of the selective properties of methyl ether compounds, but the small number of compounds evaluated in the study limited the structure-activity relationship information that could be gleaned from the results. Blank returned with a more comprehensive report in 1964 that

investigated various ethers of 3,5-diiodothyroalkanoic acids with either 3'-iodo or 3'-isopropyl substitutions. [153] The study found that the methyl ethers of the acetic acid analogs with either 3'-iodo or 3'-isopropyl substitutions possessed the greatest separation between cholesterol lowering activity and elevation of oxygen consumption and cardiac hypertrophy, suggesting that they would have a significant safety margin in comparison to the other analogs. The study also investigated N,N-diethylaminoethyl ethers of the acetic and propionic acid analogs with 3'-iodo substitutions and found that they were universally inactive, which confirmed that larger ether substitutions were not tolerated, while larger esters were acceptable.

Zenker and Jorgensen synthesized[154] and assayed[155] a series of phenoxy-3,5-diiodo-DL-phenylalanine derivatives with 2'-alkyl substitutions, expanding on the standard analogs with substitutions only at the 3' or 5' positions. An unexpected result from this study was that the 2',3'-dimethyl analog had significant thyromimetic activity in rat-based antigoiter and oxygen consumption assays. This was contrary to the expectation that it would be an antagonist and was instead a full agonist that enhanced the effect of co-administered L-T4. This result was particularly surprising as it does not have a 4'-hydroxyl group and the 2',5'-dimethyl and 2',4'-dimethyl analogs were both partial antagonists. The 2',3'-dimethyl-4'-hydroxy and 2',5'-dimethyl-4'-hydroxy and 2'-isopropyl-4'-hydroxy

and 2'-isopropyl-3'-methyl-4'-hydroxy analogs were all agonists rather than antagonists, suggesting that 2'-alkyl substitutions were tolerated, albeit with ~1% of the potency of L-T4.

1.5 Thyromimetic Development by Smith Kline & French

The modern era of thyromimetics was ushered in by research done at Smith French & Kline during the 1980s as increased understanding of the mechanisms of thyroid hormone action pushed chemists to probe a broader chemical space around the basic thyronine core and new biochemical assays allowed more precise testing.

New assays were developed as researchers discovered enzymatic systems that were influenced by thyroid hormone. For instance, a single dose of T4 induced a ten-fold increase in glycerol-3-phosphate dehydrogenase (GPDH) activity either in culture or *in vivo*, which made it useful as a measurement of thyroid hormone analog potency. [156,157] These measurements could be carried out *in vitro*, leading to greater precision and accuracy than previous physiological assays. Despite these advances, the lack of a unified mechanism for thyroid hormone action led researchers down spurious paths as studies claimed that thyroid hormones could directly modulate enzymatic pathways. Bronk reported in 1959 that increases in oxygen consumption and phosphate incorporation

could be stimulated by thyroid hormones and thyroid hormone analogs in purified mitochondria, but the concentrations necessary to elicit these effects were orders of magnitude above the physiological concentrations of thyroid hormones. [158] Similarly, assays of *in vitro* cholesterol synthesis in the presence of thyroid hormones and thyroid hormone analogs found that they reduced cholesterol synthesis, but only if the concentrations were in the range of 10 mM. [159] These effects required concentrations far above the roughly 2 nM endogenous concentration of total T3, calling their physiological relevance into question.

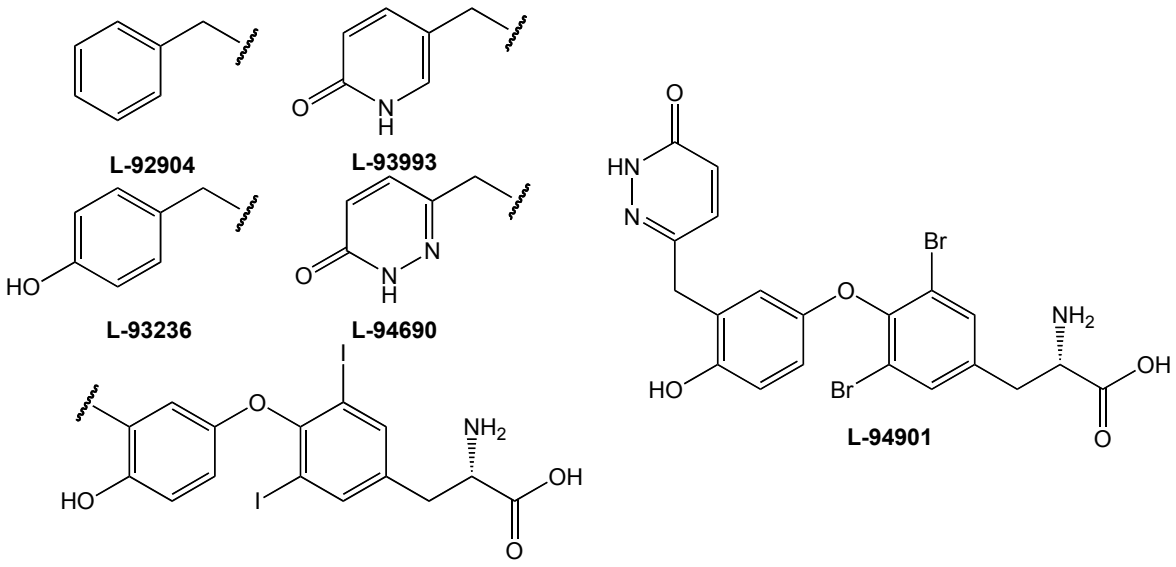

Figure 1.8

SKF analogs

Underwood reported a series of compounds in 1986 that was shaped around the question of how lipophilicity governed the activity of thyromimetics by exploring new modifications of the 3' position. [160] The first compound in the series, L-92904, was a simple 3'-benzyl substitution of 3,5-diiodo-L-thyronine. While a relatively weak compound in comparison to L-T3, it demonstrated selectivity in two *in vivo* assays - enhanced activation of GPDH in the liver compared to activation in the heart and superior binding to liver nuclei compared to heart nuclei. Increasing the polarity by replacing the 3'-benzyl with a 3'-(4-hydroxybenzyl) group to give L-93236 reduced GPDH activation in the heart but not the liver and improved *in vivo* binding to liver nuclei more than heart nuclei, significantly boosting selectivity. Continuing the trend, the 3'-pyridone derivative L93993 was completely inactive in the heart, with only slightly reduced activity in the liver, boosting the selectivity overall. Further increasing the polarity but slightly muddling the hypothesis, the pyridazinone derivative L94690 had roughly the same activity in heart and liver as L-93236, but the *in vivo* binding to liver nuclei was significantly enhanced. In a surprising turn, replacing the inner ring iodines with bromines while retaining the 3'-pyridazinone to give L-94901 boosted GPDH activation in the liver almost ten-fold and *in vivo* binding to liver nuclei almost two-fold while maintaining low activity and binding in the heart.

This last compound was selected for further *in vivo* testing in hypothyroid rats. L-94901 was equipotent with L-T3 in increasing GPDH activity in the liver and reducing serum cholesterol, but ten thousand times less potent than L-T3 at increasing GPDH activity in the heart and arterial rate, and at suppressing TSH. The mechanism by which L-94901 reduced LDL while increasing HDL was reported by Ness in 1998, who found it increases membrane-associated LDL receptor levels as well as stimulating the expression and activity of HMG-CoA and apo A-I. [161] A further refinement of the mechanism of L-94901 was reported by Ichikawa in 2000, who found that it preferentially displaced radiolabeled T3 from the nuclei of intact cultured hepatic cells compared to cultured pituitary cells, but the selective binding disappeared when the plasma and nuclear membranes were disrupted, suggesting that its selectivity was based primarily on differential uptake. [162] Three different methods were subsequently reported for synthesizing L-94901, [163-165] the last of which focused on iodonium salts for forming the critical diaryl ether bridge. Iodonium salts would go on to become the most commonly used method for synthesizing diaryl ether thyromimetics.

Leeson reported an expansion of this work in 1988, which both increased the chemical space and used quantitative structure-activity relationships (QSARs) to more precisely determine the optimal 3' substituent on a thyronine

core. [166] A major finding was that while the hydrophobicity of the 3' substituent was critical for selective binding affinity, the optimal size was significantly larger than either iodine or isopropyl groups. At the same time, there was a significant drop in binding affinity when hydrophobic substituents - such as phenethyl or n-heptyl groups - became too large, suggesting steric limits at the 3' position. Additionally, 3' substituents with less steric bulk or hydrophobicity than an iodo or isopropyl group at that position significantly reduced binding affinity. Lastly, it was found that ethers built from 3'-benzyl alcohols or amides built from 3'-benzylamines could also produce relatively high-affinity compounds as long as the substituent extended a hydrophobic appendage into the binding pocket. A further set of analogs and QSAR investigations were reported by Leeson in 1989 that varied the basic thyronine core at almost every position[167]. The compounds were tested in rat liver and heart using the same *in vitro* and *in vivo* assays of nuclear binding and induction of GPDH activity.

The first set of compounds were developed in an effort to further understand the properties of substituted 3'-benzyl derivatives of 3,5-diiodo-L-thyronine, producing two major findings. First, the activity of 3'-benzyl derivatives was very sensitive to substituent effects, with most changes eliminating activity in the liver. Out of twenty compounds, the only analogs with significant activity in the liver were the 4-hydroxy, 4-fluoro, pyridin-3-ol,

pyridone, pyridazinone, and pyridin-4-one derivatives and only the last four showed the desired lack of activity in the heart. Second, almost every compound in the series demonstrated significant binding to liver and heart nuclei, which suggested that correlations between binding and activity were not strong. The next set kept the 3'-pyridonemethyl group that had proven effective in the first series while varying the 3,5 positions, diaryl ether, and alanine side chain. There was no clear trend with substitutions at the 3,5 positions, with the shift from iodines to other halogens or methyl groups sometimes improving activity and sometimes decreasing activity in a context-dependent manner, with the unsatisfying conclusion that all permutations needed to be tried to find the optimal set of inner ring groups. This was also true with replacement of the diaryl ether with a diaryl thioether - there were no clear trends for why activity was increased or decreased depending on the other substituents of the analog. When the L-alanyl side chain was modified, the D-alanyl analog was equivalent to its L-alanyl parent, while the deaminated analogs were more varied, with the acetic and especially butanoic acid analogs displaying good activity and selectivity, while the propionic acid analog lost its selective properties. The ultimate conclusion of this study was that each substituent change influenced the others, so it was not strictly possible to mix and match chemical features without perturbing the overall activity. However, some of this was due to the limitations

of the assay methods used in the study. As the authors noted, increases in activity for analogs substituted with bromine, chlorine, or methyl groups on the inner ring in comparison to iodines might represent improved metabolic stability rather than improved target engagement, but without pharmacokinetic measurements it was not possible to know how each element contributed to the final measurements of activity.

1.6 Thyromimetic Development by Ciba-Geigy Pharmaceuticals

Ciba-Geigy Pharmaceuticals based their thyromimetic research on two major ideas, one chemical and one biological. The chemical insight was that a wider variety of groups substituting for either the alanyl or alkanoic acids of most earlier thyromimetics could be both active and easier to synthesize. The biological basis of their research was a series of papers purporting to demonstrate both high affinity, specific binding of thyromimetics to the cell membrane and rapid biological effects evoked by that specific binding. Ciba's series of thyromimetics were optimized for high nuclear affinity and low

membrane affinity, which was claimed to produce more selective physiological action.

This theory came from a report published by Pliam in 1977 claiming that purified rat liver plasma membranes contained two saturable binding sites: one high affinity, low capacity site and a second low affinity, high capacity site that were selective for L-T3 over D-T3 and L-T4. [168] This was a plausible mechanism for a number of rapid effects at the membrane stimulated by T3 including increased uptake of amino acids[169] and sugars. [170] These effects were also demonstrated to be specific for L-T3 - L-T4, rT3, and 3,5-T2 had orders of magnitude less activity. [171] In 1981 Segal reported that T3 stimulated increases in sugar uptake were mediated by cAMP and independent of protein synthesis, which bolstered the argument that the effects were not due to thyroid hormone action in the nucleus. [172]

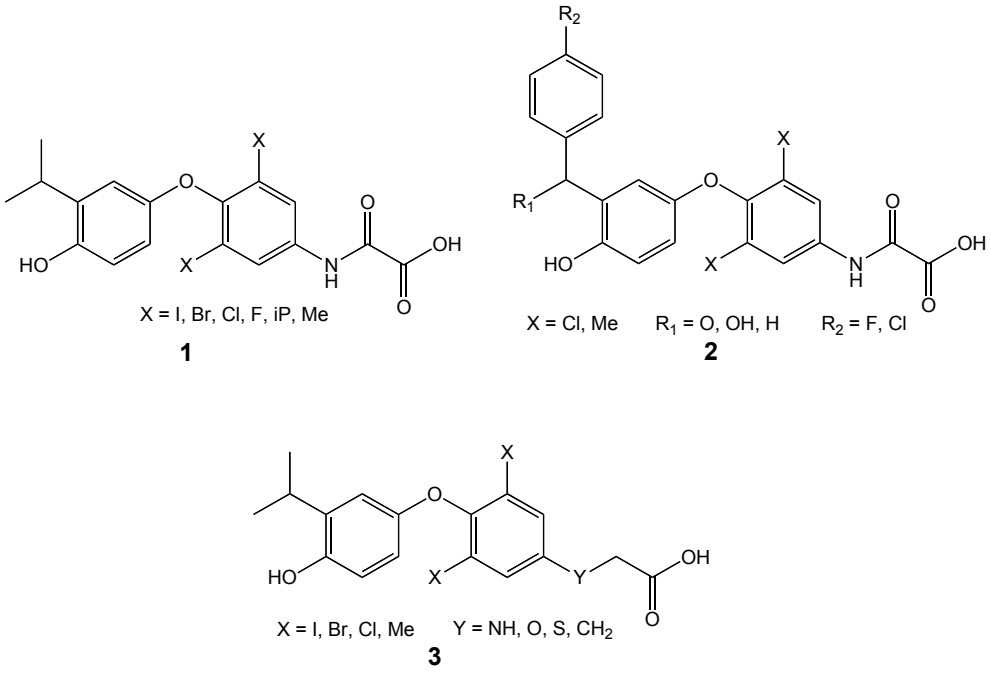

Figure 1.9

Ciba-Geigy Pharmaceuticals analogs.

The first round of Ciba-Geigy Pharmaceuticals thyromimetics were reported by Yokoyama in 1995, which encompassed three broad categories of compounds. [173] The first set (Fig 1.9 - **1**) was a series of oxalic acid derivatives coupled to the biaryl ether core through an amide linkage and with a 3'-isopropyl substitution. All of the compounds in this first series demonstrated selectivity for nuclear binding over membrane binding, with the 3,5-dimethyl analogs demonstrating the greatest selectivity. A surprising result was that ethyl and benzyl esters of these compounds retained almost full nuclear binding. The second series (Fig 1.9 – **2**) elaborated on the results of the first by retaining the oxalic amides and varying the 3' position with benzyl, benzyl carbinol, or bezyl

ketone substitutions with halogens in the 4-position of the benzyl ring. While some of the compounds in this series matched the nuclear binding affinity and selectivity of the first series, none were significant improvements. The third series (Fig 1.9 – **3**) was a group of amino, oxy, and thio acetic acids built on a standard biaryl ether core as well as two propionic acid derivatives directly coupled to the core, all returning to the original 3'-isopropyl substitution. The two best compounds out of this series were marginal improvements on the first two series - both 3,5-dibromo derivatives with either a propionic acid or oxyacetic acid appendage. Four of the best compounds from the first two series of compounds - the 3'-isopropyl 3,5-dichloro and 3,5-difluoro oxalic acid amides and 3,5-dimethyl ethyl oxalate amide derivative with a racemic 3'-(4-fluorobenzyl) carbinol substitution (CGS 26214) - were selected for *in vivo* testing. In hypercholesterolemic rats these compounds demonstrated significant cholesterol lowering at doses 4-10 times less than those necessary to see a comparable effect with L-T3 along with a lack of cardiotoxicity. Further *in vivo* testing of CGS 26214 was reported by Stephan in 1996, who confirmed its selective effects in hypercholesterolemic rats while providing a mechanism of action. [174] CGS 26214 showed more than one hundred-fold selectivity for binding to the nuclei of intact HepG2 cells compared to neonatal rat cardiac myocytes as well as upregulation of LDL receptor activity in HepG2 cells and in

rat liver cells after oral dosing for one week. In hypercholesterolemic rats CGS 26214 had a ten-fold greater impact on cholesterol than L-T3 at low doses. Doses sufficient to produce robust cholesterol lowering with CGS 26214 were less than those that evoked cardiac hypertrophy and were also less than those necessary to produce cholesterol lowering with L-T3, demonstrating the safety of the compound. CGS 26214 was renamed Axitirome when it entered Phase I clinical testing in 1997, but was withdrawn as a clinical candidate by Novartis in 1999. [175]

Similar results were reported by Taylor in 1997 for CGS 23425, the 3,5-dimethyl-3'-isopropyl oxalic acid amide derivative. [176] The report elaborated on the mechanism by showed that it upregulated the expression of apolipoprotein AI, the major protein constituent of HDL and that the stimulation was selectively mediated by the TR β-subtype (TRβ). A later report by Wada in 2000 showed that in rats CGS 23425 reduces the levels of ApoB-100, a protein constituent of chylomicrons, low density lipoprotein (LDL), and very low density lipoprotein (VLDL), which would all contribute to overall reductions in LDL. [177] A later report by Adamson in 2004 compared the array of genes stimulated by T3 and CGS 23425 and found that the results were largely consistent both with the analog's β-selective binding and with its reported lack of cardiotoxicity. [178] A related series of compounds were reported by Stanton in

2000, which focused on modifying the 3' position with phenyl and cyclohexyl groups. [179] Two compounds in the series, the 3'-cyclohexyl and 3'-phenyl analogs were found to produce significant decreases in serum cholesterol in rats fed high-fat diets. This did not correlate with their *in vitro* binding to rat hepatic nuclei, as other compounds in the series had better affinities, but did not produce the same cholesterol-lowering effects. Additionally, the cyclohexyl and phenyl analogs were tested for cardiac side effects and the cyclohexyl analog increased heart rate and weight while the phenyl analog produced decreases in heart weight and rate. Despite these positive initial results, no further reports of these compounds have been published.

While Ciba's thyromimetic program did not produce an approved drug, it had the important result of opening up the chemical space that later analogs would explore as well as directly influencing some of the most successful thyromimetics in the field.

1.7 Modern Thyromimetic Development

During the 1960s and 1970s investigations into the mechanisms of thyroid hormones began to focus on the nucleus as the primary site of action. Tata in 1966[180] and Nakagawa in 1967[181] reported that thyroid hormones stimulated the

incorporation of ^{14}C-labeled orotic acid and ^{32}P-labeled phosphate into RNA in both a dose- and time-dependent fashion. This led researchers to look in the cell nucleus for thyroid hormone binding sites. Oppenheimer, Koerner, Schwartz, and Surks published a series of papers between 1972 and 1975 demonstrating that unlabeled T3 could displace radiolabed T3 *in vivo* from the nuclear fractions of rat liver and kidney, [182] that thyroid hormone analogs could also displace radiolabeled T3 *in vivo* from the nuclear fractions of rat liver and heart roughly in proportion to their physiological activity, [183] and that the same process could be observed *in vitro* with T3[184] and thyroid hormone analogs[185] in isolated rat liver nuclei. Importantly, the relative binding constants of T3 and thyroid hormone analogs for nuclei, either *in vivo* or *in vitro*, were demonstrated to be on the same order of magnitude as the physiological concentration of T3, which suggested that these interactions were more relevant than those found for isolated enzyme systems. Additionally, these methods allowed researchers to assess the contribution of tissue uptake by measuring the difference between displacement from intact cells and partially solubilized cells[162] or isolated nuclei. [174] Work reported by Spindler in 1975 showed that the hormone binding sites were located on chromatin and demonstrated that this localization was not dependent on the presence of thyroid hormone. [186] Latham reported in 1976 that a soluble binding protein for thyroid hormone and thyroid hormone analogs had been

isolated from rat liver nuclei and was estimated to be 50.5 kDa. [187] This protein had the same binding constants for thyroid hormones that had been found for whole nuclei, suggesting that it was the primary site of thyroid hormone binding. Little more was accomplished in further characterizing the binding for nearly a decade due to the low abundance of thyroid hormone receptors, which are on the order of 10^3 to 10^4 per cell, and the difficulty in purifying low-abundance proteins to homogeneity. Competition binding to nuclei, either *in vitro* or *in vivo* remained the standard technique for measuring binding constants of thyroid hormone analogs for many years. While these new methods bypassed many of the constraints imposed by physiological assays of whole animals or isolated tissues and allowed for far more precise comparisons between analogs, it was still limited by the imperfect relationship between receptor or nuclear binding and physiological action.

The first large-scale purifications of thyroid hormone receptors from rat livers was reported by Ichikawa in 1987 and Aprilletti in 1988, which produced pure receptor in quantities sufficient to definitively demonstrate their mass, binding constants, [188] and that they bound to specific DNA sequences. [189] The binding constants obtained in these studies were comparable to those obtained with isolated nuclei, suggesting that they were not more precise than the former method. But given the low yield of the purifications, there was no obvious

upside to using purified receptors. The discovery that thyroid hormone receptors are expressed in multiple forms, similar to other nuclear receptors such as estrogen receptors, made purification a high priority. This required the tools of molecular biology as the TR subtypes α_1 and β_1 have roughly the same mass and T3 binds to them with equal affinity, which made biochemical separation impractical. The sequence of one form of the thyroid hormone receptor was first reported simultaneously by Weinberger[130] and Sap[190] in 1986, who determined that it was homologous with the retroviral onocogene *c-erbA*. Researchers quickly determined that other variants of the thyroid hormone receptor could be found endogenously and that they had the same binding constants for T3 when expressed *in vitro.* [191] The surprising result was that while T3 and T4 had roughly equal affinities for the α and β subtypes, other analogs such as triac had different binding affinities for each subtype. [192] Combined with the discovery that the subtypes had varying expression levels in each tissue, [193,194] this suggested that subtype selectivity could be a powerful route for creating analogs with selective effects. Reports in the early-1990s demonstrated that functional thyroid hormone receptors could be expressed heterologously in *E. coli* - the α-subtype ligand binding domain (LBD) [195] and the full-length β-receptor by itself[196] or as a fusion with GST. [197] This made it practical to perform binding assays on individual TR subtypes to determine selectivity.

The ability to express TRs in heterologous systems also made it possible to obtain them in the quantities necessary for growing single crystals. An x-ray crystal structure of the TRα LBD bound to L-T3 was reported by Wagner in 1995, which opened up the field to allow true rational design of thyromimetics[198]. The TRβ LBD bound to T3 and cocrystallized with the coactivator GRIP1 was reported by Darimont in 1998, which allowed researchers to make structural comparisons between the two TR LBDs and gave insight into how the LBD interacted with coactivators. [199,200] More recently crystal structures of a β-selective thyromimetic bound to the TRα and TRβ LBDs by Bleicher in 2008 gave significant insight into the conformational changes between the receptors that are believed to be the primary basis of the ligand's β-selectivity. [201]

In parallel with the mounting evidence that the physiologically relevant binding sites for T3 were located within the cell nucleus, reports were published demonstrating that thyroid hormone could stimulate the expression of specific protein products. Shambaugh III reported in 1969 that thyroid hormone stimulated the the activity of carbamoyl phosphate synthetase in tadpole and frog liver preparations, which was shown to be due to increases in protein expression rather than by changing the kinetic parameters of the enzyme. [202] Importantly they demonstrated that T4, T3, and 3,3',5-triiodothyropropionic acid all stimulated this increase, while 3,5-diiodothyronine and thyronine did

not, confirming that it was a specific effect correlated with the previously observed physiological activities of the various thyronines. Similar results were reported by Lin in 1978 for (Na+, K+)-ATPase in multiple rat tissues, which confirmed that thyroid hormone increased protein concentration without altering the binding or catalytic activity of the ion pump itself. [203] As the focus of thyroid hormone action shifted towards the nucleus, it became clear that TRs regulated specific sets of genes. A report by Evans in 1982 detailed how thyroid hormones regulate the expression of growth hormone genes in cultured rat pituitary tumor cells - addition of thyroid hormone increased growth hormone mRNA levels four-fold. [204] A report from Gurr in 1983 demonstrated that thyroid hormone could also negatively regulate expression of target genes such as TSH. [205] A critical element of that study was the observation that closely related genes could be regulated independently - TSHβ was reduced significantly while TSHα was only reduced in some samples. Reports by Larsen in 1986 and Wight and Glass in 1987 delineated some of the promoter sequences that are recognized and bound by the thyroid hormone receptor upstream of TR-responsive genes. [206-208] They also demonstrated that the sequence was modular and could be inserted upstream of a different gene, rendering it responsive to thyroid hormone activation. With these molecular tools in hand, Thompson reported in 1989 that it was possible to co-transfect mammalian cells

with plasmids coding for a thyroid hormone receptor and a synthetic construct containing a reporter gene driven by a thyroid hormone response element. [209] Because each receptor subtype could be overexpressed separately, this created a powerful tool for assessing compound selectivity and potency. The method was patented by Evans in 1991, which largely prevented the use of this tool in industry for many years, though it was taken up more readily by academics. [210]

These two critical tools - *in vitro* binding and cell-based transactivation assays - allowed researchers to precisely delineate the selectivity of new thyromimetics and ushered in the current era of thyroid hormone analog development.

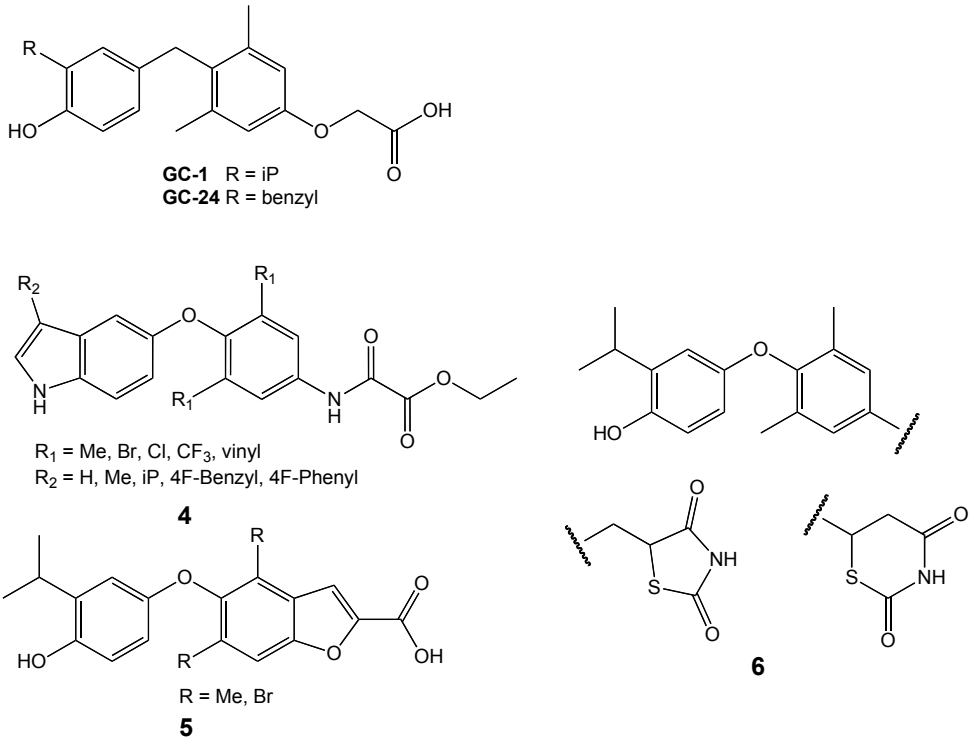

Figure 1.10

The GC series developed by the Scanlan group and fused ring thyromimetics developed by Bayer AG.

The first compound to fully exploit these methods was **GC-1** (Fig 1.10), reported by Chiellini in 1998. [211] Building on the work of Yokoyama, [173] it combined the 3,5-dimethyl-3'-isopropyl substitutions and oxyacetic acid group that had displayed such good nuclear binding while replacing the diaryl ether with a methylene bridge to produce a novel compound. Importantly, it demonstrated 10-fold selectivity for TRβ compared to TRα in both *in vitro* receptor binding and cell-based transactivation assays. GC-1 was an excellent clinical candidate for cholesterol lowering because the desired liver-mediated

cholesterol lowering effects were found to be regulated by TRβ while the cardiac side effects were regulated by TRα. The selectivity was demonstrated unequivocally *in vivo* with a *Xenopus laevis* metamorphosis assay. [212] GC-1 induced reabsorption of the gills and tail, which are mediated by TRβ, without stimulating the formation of hind limbs, which is mediated by TRα. When the compound was tested in hypothyroid mice it was as or more effective than L-T3 at producing decreases in serum cholesterol and triglycerides, while almost no increases in heart weight to body weight ratio or other hemodynamic parameters were observed. [213] While GC-1 was shown to suppress TSH at high doses, there was significant separation between the doses needed to produce TSH suppression and cholesterol lowering. In addition to its previously demonstrated receptor selectivity, it was found that GC-1 partitioned strongly to the liver, while concentrations in heart, muscle, and brain were low, which provided a secondary mechanism to explaining its selective physiological effects. These selective effects of GC-1 were also demonstrated in rats and cynomolgus monkeys. [214] The patent for GC-1 was licensed by QuatRx Pharmaceuticals in 2005, who gave it the trade name sobetirome and carried out a Phase I clinical study. The single and multiple dose studies demonstrated significant decreases in serum cholesterol without evoking hypo- or hyperthyroidism or other serious adverse effects. While development of GC-1 as a treatment for

hypercholesterolemia was suspended, new patents have been filed for its use in treating x-linked adrenoleukodystrophy[215] and demyelination diseases such as multiple sclerosis. [216]

Despite the fact that most chemical features of GC-1 had been chosen primarily for synthetic tractability rather than through rational design, [217] many of them turned out to be critical for its potency and selective effects. The diaryl ether and propionic acid analogs first reported by Yokoyama[173] were tested for binding affinity, which demonstrated that the methylene bridge improved binding at both receptor subtypes by four-fold while the oxyacetic acid group improved binding to TRβ by two-fold while having no effect on binding to TRα. [218] The methylene bridge similarly enhanced binding for an analog of DIMIT, improving binding to both receptors subtypes by four-fold, suggesting that this effect was not limited to the chemical context of GC-1. These results suggested that GC-1 was already significantly optimized, but one site for refinement was found in the synthesis of **GC-24** (Fig 1.10), an analog that replaced the 3'-isopropyl group of GC-1 with a 3'-benzyl group. [219] This modification significantly reduced the binding affinity of GC-24 for TRα while leaving the affinity for TRβ intact, increasing the selectivity of the compound. It was found that this was due to the hydrophobic pocket accessed by the 3' substituent being

able to rearrange to accommodate the benzyl substitution in TRβ but not in TRα, which was determined by mutational analysis of the receptors.

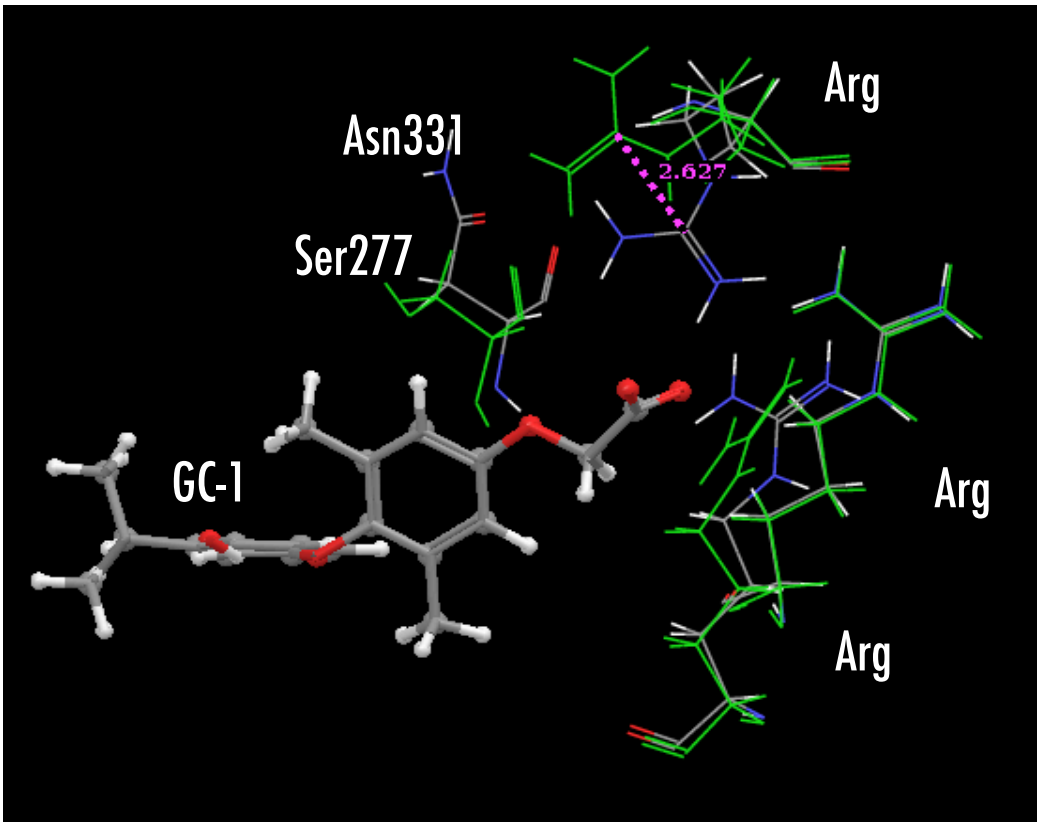

Figure 1.11
Critical residues in the ligand binding domains of TRα (colored by atom) and TRβ (green) implicated in the binding selectivity of GC-1.

Ser-277 in TRα and Asn-331 in TRβ are the only residues on the inner surface of the ligand binding pocket that vary between the two receptors and these play a critical role in arranging a series of Arg residues in the binding pocket (Fig 1.11). Changing Ser-277 to Asn in TRα and Asn-331 to Ser in TRβ will reverse the binding selectivity for GC-1, suggesting that the arrangement of Arg residues in that pocket is the primary determinant of its selectivity.

Conversely, GC-24 has low binding affinity for both mutants, which suggests that other structural features of the ligand binding domain are also critical for its selectivity and vary significantly between the two receptors.

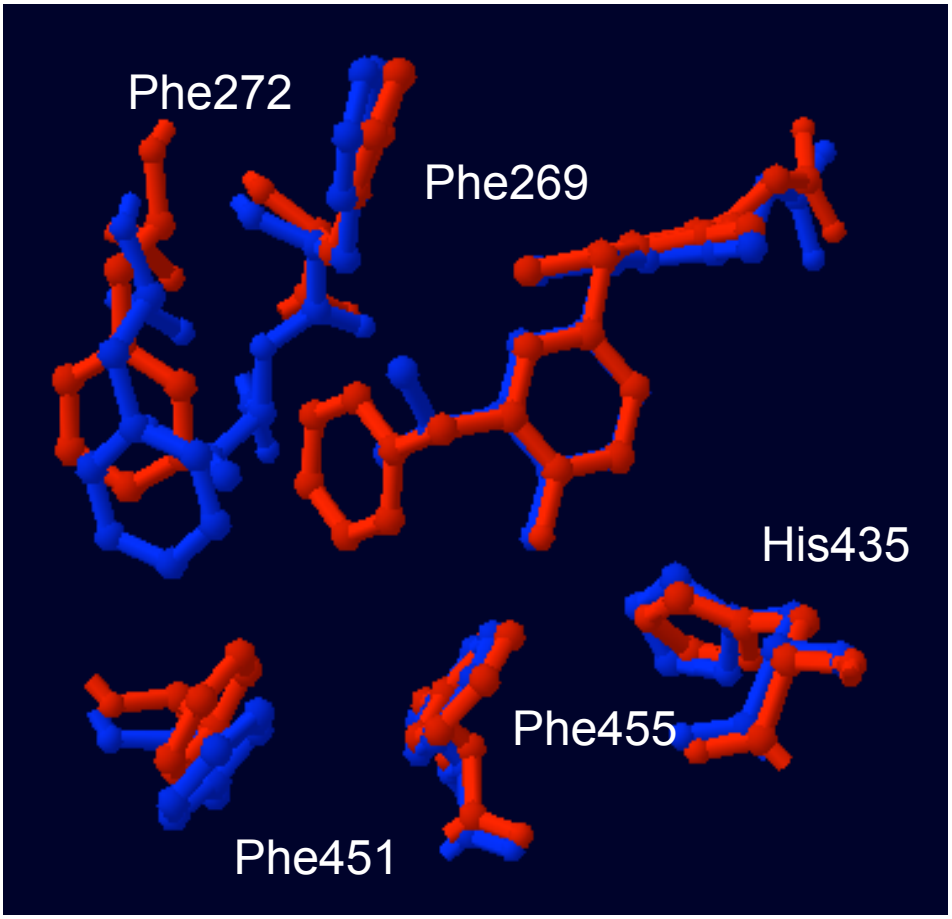

Figure 1.12
Phenylalanine residues in the TRβ ligand binding domain that influence the binding of GC-1 (blue) (PDB 3IMY) and GC-24 (red) (PDB 1Q4X).

Crystal structures of GC-24 and GC-1 bound to TRβ found two critical structural changes that provided a mechanism for the behavior of these ligands. First, the hydrophobic pocket rearranged a number of critical Phe residues to accommodate the larger benzyl substitution (Fig 1.12). Second, when compared

to previously reported TRα crystal structures it was found that helix 11 packed more tightly in the TRα LBD than in the TRβ LBD, giving TRα significantly less conformational flexibility to accommodate the extra steric bulk of the benzyl group on GC-24. [220]

In 2008 Hashimoto reported a small number of GC-1 derivatives that coupled fluorocein through two different linkers. [221] The first analog MP01 placed a 2-(4-hexyloxy)phenyl)acetic acid amide at the 5' position of GC-1 and was found to have no measurable binding affinity for either TR. The second analog JZ01 placed a 6-(4-ethynylphenyl)hexanoic acid group at the 5' position, which was conjugated to fluorescein through an amide. JZ01 displayed high binding affinity for TRβ at 21 nM and could be competitively displaced by both T3 and GC-1, confirming that it was binding to the same site. Fluorescence images taken *in vivo* found that the compound localized almost exclusively to the nucleus in cells transiently transfected with TRβ and the fluorescence could be blocked by the addition of T3. Despite these promising results, no further work with this compound has been reported.

This period saw many of the major pharmaceutical firms pursue thyromimetics programs, largely in an effort to develop LDL cholesterol lowering agents. This led to the exploration of novel chemotypes as research groups worked around the patented chemical space, vastly expanding the range

of known active motifs. While most investigated one or two novel chemical modifications, a few went much further.

Bayer pursued a strategy of replacing the inner and outer rings of the traditional thyromimetic core with fused ring groups to increase the rigidity of the resulting molecules and improve their selectivity. The first set of compounds (Fig 1.10 – **4**) were patented in 2001[222] and reported by Haning in 2005 - these replaced the outer ring phenol with a series of 3'-substituted indoles. [223] Activity was reported using the uncommon technique of measuring TRE-driven luciferase activity in HepG2 cells containing both TRα and TRβ, which provided a compound metric of transactivation potency for both subtypes rather than individually to give information on subtype selectivity. Individual EC_{50} values for each receptor were left unreported, but selectivity was assessed in a separate assay. Three compounds in the series exhibited moderate 10X TRβ-selectivity and EC_{50} values in the nanomolar range. As L-T3 was the only benchmark, comparisons with other TRβ-selective thyromimetics are difficult to draw given the unique assay methodologies, but the lack of subsequent reports suggest that this series was not developed further. The second series (Fig 1.10 – **5**, **6**) was patented in 2002[224] and reported by Haning in 2007, which shifted focus to the inner ring. [225] Benzofurans with carboxylic acid appendages were synthesized that mimicked oxyacetic and oxypropionic acid groups while

reducing their conformational flexibility. Some compounds in this series demonstrated nanomolar and sub-nanomolar potency, but none were selective *in vitro*. The lack of selectivity was confirmed *in vivo* with Naval Medical Research Institute mice - the most potent compound in the series produced both cholesterol lowering and increases in heart weight with a non-linear dose-response relationship, cementing its lack of suitability as a therapeutic agent.

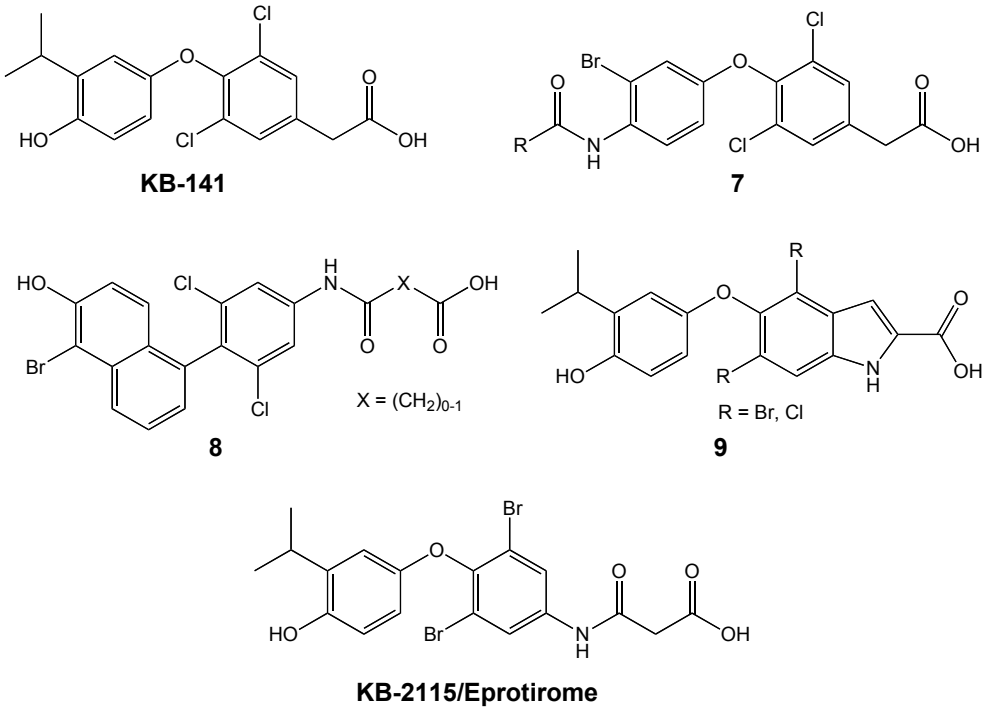

Figure 1.13

Karo Bio thyromimetics.

Karo Bio reported its first series of analogs in 2003[226] based on patents filed in 1999 and 2000[227,228] in partnership with Bristol-Myers Squibb. This series built on earlier iodine-based thyroalkanoic acid analogs by replacing the

iodines with 3'-isopropyl and either 3,5-dichloro or 3,5-dibromo groups to improve metabolic stability in comparison to iodine substitutions. TRβ selectivity peaked at 14-fold in the 3,5-dichloro series with the acetic acid analog, while the benzoic and propionic acid analogs had only modestly 3-fold selective binding. This trend was confirmed in a cell-based transactivation assay. In a different trend, binding affinity increased directly with chain length and the propionic acid analog had 10-100 fold greater affinity than the acetic acid analog. The *in vitro* selectivity translated *in vivo*, with the acetic acid analogs demonstrating ~10-fold selectivity for cholesterol lowing compared to cardiotoxicity in rats, but in both cases suppression of TSH occurred at doses below those necessary to elicit robust cholesterol lowering. The 3,5-dichloro acetic acid analog was designated as KB-141 (Fig 1.13) and Grover reported further evaluations that confirmed the results of Ye, while also demonstrating that the cholesterol lowering and lack of cardiotoxicity previously seen in rats carried into cynomolgus monkeys treated with KB141. [229] The claim was made that the tissue distribution of KB141 was comparable to that of T3, suggesting that its selective effects were due entirely to its TRβ selectivity. [229] An extension of the series was reported by Hangeland in 2004 that retained the 3,5-dichloro or 3,5-dibromo acetic acid core and varied the 3' position with substituted phenyl and heterocyclic groups. [230] The first set of phenyl substitutions

determined that *meta*-substituted phenyl groups at the 3' position had the greatest TRβ affinity and selectivity, with the trifluoromethyl and ethyl substitutions producing the best results. Notably none of the 3,5-dibromo analogs reported in the 2004 study were able to achieve the selectivity of the 3,5-dichloro analogs, but they are not directly comparable as the 3,5-dibromo analogs explored a different set of 3' substitutions. Another attempt to modulate the selectivity of KB-141 was reported by Garg in 2007 that coupled a series of amino acids through the acetic acid group and added 5'-substituents. [231] Both potency and selectivity were reduced across the board, with larger amino acids or 5'-substituents producing the most pronounced reductions. Two compounds in the series were tested *in vivo* and found to have greater than 10-fold preference for cholesterol lowering compared to elevation of heart rate, but TSH suppression occurred at doses too close to those necessary for cholesterol lowering, eliminating their potential usefulness as therapeutics. A final variation on the series (Fig 1.13 – **7**) was a set of compounds reported by Li in 2006 with the 3,5-dichloro acetic acid core, a 3'-bromo substitution, and replacements of the 4'-hydroxyl group with a series of straight and branched chain amides built on a 4'-amino group. [232] No compound in this series improved on the selectivity or potency of KB-141 or the 3'-*meta*-ethylphenyl analog previously reported by Hangeland. So while a fair amount of chemical space around this basic structure

had been explored, the relatively simple KB-141, based on the structure of triac, remained the best out of this series.

A new set of compounds (Fig 1.13 – **8**) reported by Karo Bio took the novel approach of replacing the standard biaryl core with a phenyl-naphthalene core, as reported by Hangeland in 2005. [233] The phenyl-naphthelene cores were made roughly isosteric with the standard biaryl ether cores while altering the conformations they could assume by placing the outer ring hydroxyl group at the 6' position. Out of this series, only one compound demonstrated improved selectivity in comparison to KB141 - it placed an isopropyl group at the 5' position on the naphthyl ring and used a malonic acid amide conjugated through an aniline on the phenyl ring to give 25-fold selective affinity for TRβ in a competition binding assay. Following this Karo Bio pursued a similar strategy to Bayer in synthesizing thyromimetics with fused ring heterocycles (Fig 1.13 – **9**). Collazo reported a series in 2006 where the inner ring was replaced by bicyclic nitrogen five- and six-membered rings with carboxylic acid appendages. [234] One compound in the series - an indole with a 2-substituted carboxylic acid - displayed moderate 14-fold selective binding affinity for TRβ, though this translated into only 2-fold selectivity in a cell-based transactivation assay. No additional information about either of these series has been published by Karo

Bio, suggesting that they were not considered sufficiently promising for additional development.

Karo Bio's best compound KB2115 (Eprotirome) (Fig 1.13), was patented in 2004 under Bristol-Myers Squibb. [235] The compound was built on a 3'-isopropyl-3,5-dibromo ether bridged core, with a malonic acid amide conjugated through an aniline group on the inner ring, bringing together chemical features that had been successful in other compounds produced during the earlier phase of their research program. An *in vitro* binding assay of KB2115 demonstrated 22-fold TRβ-selectivity, [236] but suggestions have also been made that preferential liver uptake played a role in its selective effects. [237] A Phase I fourteen-day, once-daily dosing clinical trial was reported in 2008, which showed that KB2115 could lower plasma total and LDL cholesterol with no observed drug-induced effects on cardiac parameters in overweight and hypercholesterolemic subjects. [238] Complicating the results, free T4 and, to a lesser degree, free T3 were suppressed in a dose-dependent fashion, but TSH remained comparable to placebo at all doses. This could have resulted from the induction of type 1 deiodinase in the liver, which is regulated by TRβ, increasing the conversion of T4 to T3 and T3 to 3,5-T2. [239] A Phase II clinical trial involving once-daily dosing over twelve weeks was reported in 2010 that investigated the effects of KB2115 in addition to simvastation or atorvastatin. [240] KB2115 plus a statin

produced significantly larger decreases in serum cholesterol and LDL compared to a statin alone without cardiotoxicity or biomarkers of bone turnover, but continued to evoke the same decreases in free T4. Bolstering their utility case, it was found that KB2115 also reduced triglycerides and Lp(a) lipoprotein, which were not observed with statin treatment alone. The positive results obtained in the first two trials led to a larger Phase III trial with heterozygous familial hypercholesterolemia patients currently receiving the standard of care. [241] The study was initiated in 2011 and scheduled to end in 2014, but was discontinued early due to adverse results from a large animal toxicology study in dogs over 12 months, which reported that KB2115 induced chondrodysplasia at high doses. Though it is unclear how the drug induced cartilage damage, further development of the drug was halted.

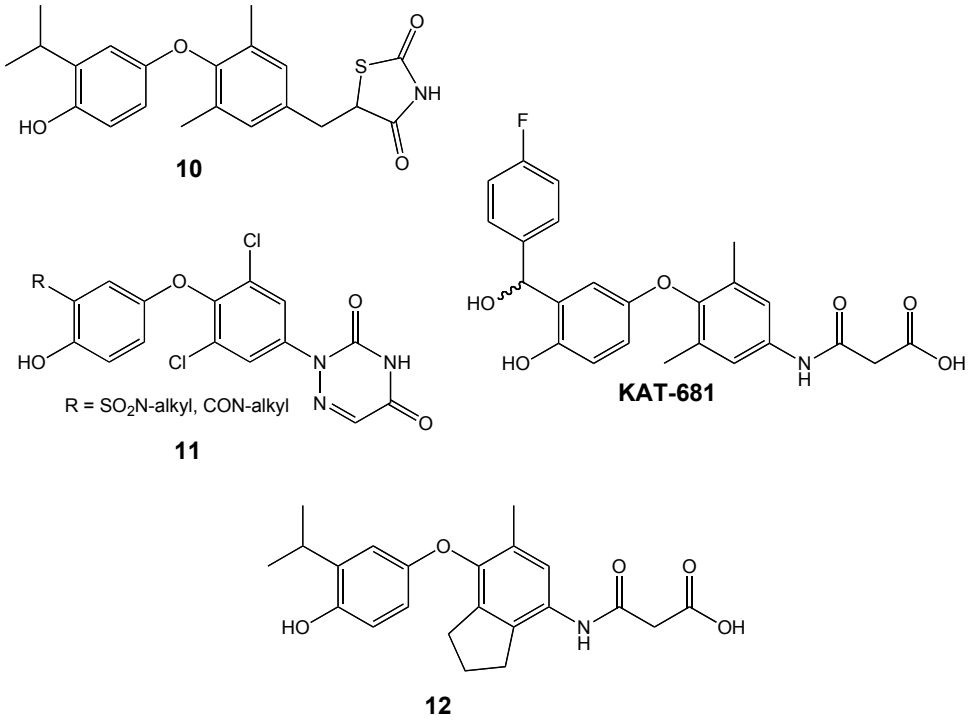

Figure 1.14

Thyromimetics developed by Iyaku Bushi Sekkei Kenkysho, Pfizer, and Kissei Pharmaceutical.

Iyaku Bunshi Sekkei Kenkyusho received a patent for a series of thiazolidinedione-based thyromimetics in 2001. [175] These were reported by Ebisawa in 1999 and based on the observation that thiazolidinedione moieties were carboxylic acid bioisosteres in other ligands. [242] The thiazolidinedione moiety was linked to standard thyromimetic scaffolds by either a methylene (Fig 1.14 – **10**) or an olefin. The methylene linked compounds were significantly more potent than the olefinic compounds in activating TRα-mediated TRE-driven luciferase expression, which was consistent with the activities of acrylic

and acetic acid analogs of T3. Despite these promising *in vitro* results, no *in vivo* data has been reported.

Pfizer's thyromimetic program began with a series of 6-azauracil derivatives that had originally been synthesized as anticoccidial agents. The structural similarities between the anticoccidal series and thyroid hormone analogs was noted and new analogs were synthesized that more closely mimicked a standard thyromimetic core. The first compound in the series with 3'-isopropyl-3,5-dimethyl substitutions already possessed good binding affinity for TRβ and ~8-fold TRβ/TRα selectivity. [243] While this was a promising lead compound, further refinements at the 3' position were investigated (Fig 1.14 – **11**) to improve TRβ selectivity. A range of moieties including sulfonamides and amides were chosen due to their synthetic tractability. Additionally, the 3,5 position was modified from the original dimethyl substitutions to include 3-chloro-5-methyl and 3,5-dichloro substitutions in an effort to boost potency. The best initial 3' substitution, a piperidinyl sulfonamide, showed further enhancement of TRβ binding affinity and TRβ/TRα selectivity as each methyl group in the 3,5 positions was replaced by a chlorine. While other 3' substitutions such as cyclobutylamino and spiro-tetrahydrofuranyl provided greater TRβ/TRα selectivity, it was always at the cost of reduced TRβ affinity. The 3'-amide derivatives were nearly all more selective than the suflonamides,

with the (±)-*exo*-2-norborynl derivative providing the greatest TRβ/TRα affinity at >100-fold. The (R)-nopinone derivative displayed the best combination of TRβ affinity and TRβ/TRα selectivity at 0.06 nM and 58.2 fold respectively. While originally envisioned as treatments for hypercholesterolemia, they found new utility when it was observed that the compounds could stimulate TRβ-mediated hair growth. Li reported in 2010 that two compounds from the original series as well as a 3'-(4-fluorobenzyl) analog were TRβ-selective and stimulated hair growth at minimum effective doses of <0.1% w/v in C3H/HeN mice and had a unique metabolic profile that reduced systemic exposure after topical application. [244] Despite these *in vitro* and *in vivo* results, no further reports from Pfizer have been published.

Kissei Pharmaceutical filed a patent in 2001 covering a range of malonic acid-based thyromimetics. [245] While claims were made for the traditional role of thyroid hormone analogs in treating hypercholesterolemia, they also staked out unique therapeutic territory. An association between hypothyroidism and hepatocelluar carcinoma has been observed[246], suggesting that increased activation of thyroid hormone receptors in the liver could ameliorate the condition. The lead compound **KAT-681** (Fig 1.14) modified a previously reported compound from Ciba Pharmaceuticals[173] by expanding the oxamic acid to a malonic acid. While the first report detailed the chiral resolution of KAT-

2003, an ethyl ester of KAT-681, and noted that the (+) enantiomer was more active than the (-) enantiomer, [247] subsequent reports of its biological activity used racemic KAT-681. The first *in vivo* data focused on the inhibition of hepatocarcinoma in rats induced with 2-acetylaminofluorene over two or five weeks, which demonstrated significant reductions in proliferation index and lesion size from treatment compared to vehicle. [248,249] Subsequent reports returned to KAT-681, now renamed T-0681, as a treatment for hypercholesterolemia. These demonstrated that T-0681 could dramatically reduce serum cholesterol and atherosclerosis in rabbits[250] and mice[251] without significantly increasing heart rate. No subsequent reports have been published, suggesting that KAT-681/T-0681 did not advance to clinical development for either indication. Kissei published data on a final series of thyromimetics (Fig 1.14 – **12**) with di- and tri-substitutions on the inner ring that utilized combinations of halogen, alkyl, and fused ring indane and tetralin groups. [236,252] While some compounds in the series demonstrated greater *in vitro* TRβ-selective binding affinity than the benchmark KB-2115, it was always at the cost of reduced potency. More important were the *in vivo* results with cholesterol-fed rats showing that the separation between the EC_{50} for significant reductions in cholesterol and the EC30 for suppression of T4 were not directly correlated with *in vitro* TRβ-selectivity - one compound in the series with superior TRβ-

selectivity and one with equal affinity for each receptor were roughly matched in terms of *in vivo* selectivity for cholesterol lowering compared to T4 suppression. The lack of subsequent reports suggests that this series did not advance into clinical development.

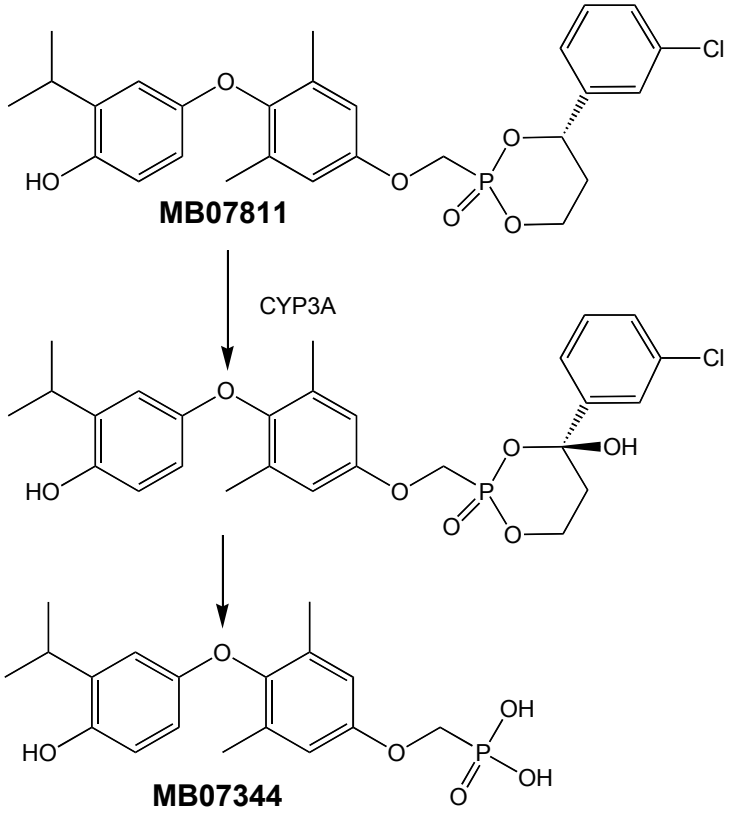

Figure 1.15

Metabolic activation of thyromimetic prodrug MB07811 developed by Metabasis Therapeutics.

Metabasis Therapeutics patented a series of thyromimetics in 2004 that took a novel approach to selectivity - prodrugs designed to be metabolized in the liver into an active ligand to reduce peripheral exposure and increase selectivity.[253] The compounds were a series of phosphonic acid derivatives coupled to a

standard thyromimetic core through a variety of alkyl, ether, thioether, and alkylamine bridges. Due to the high charge density of phosphonic acid groups, an array of phosphonate ester and phosphonamide derivatives of the series were synthesized to increase membrane permeability of the prodrug while releasing the active phosphonic acid either through simple hydrolysis or active metabolism in the liver. *in vitro* binding assays reported by Boyer in 2008 found that the active forms of the series had low binding affinity and selectivity in comparison to benchmarks such as L-T3, GC-1, KB-141, and KB-2115. [254] *in vivo* results were more promising - a number of compounds in the series reduced serum cholesterol by 20-48% in cholesterol-fed rats at doses of 0.2 mg/kg administered by ip injection. Two of the active compounds - phosphonic acid analogs of GC-1 and triac - were selected for further testing and demonstrated selective activation of liver hGPDH compared to heart hGPDH, which suggested that they would have cardiac-sparing properties. The phosphonic acid analog of GC-1 was derivatized into a series of prodrugs and oral dosing demonstrated significant efficacy for most of the series. The cyclic 1-(3-chlorophenyl)-1,3-propanyl prodrug was selected as the best of the series due to acceptable oral bioavailability (10%) and low serum esterase activity resulting in low extrahepatic concentrations of the active form due to specific CYP3A-mediated metabolism of the cyclic ester. This prodrug was designated **MB07811** and the

active form was designated **MB07344** (Fig 1.15). Full *in vivo* characterization was reported by Erion in 2007. [255] MB07811 administered once daily to rats at 50 mg/kg did not evoke significant increases in heart rate, hemodynamic parameters, or heart weight. Further validating its safety, expression of TR target genes in the heart, pituitary, and thyroid were not significantly altered by a single oral dose of MB07811 administered at up to 4 mg/kg. MB07811 entered into Phase 1a and 1b clinical trials in 2006 and reports in 2008 indicated that the drug was well-tolerated with healthy patients in both rising single-dose and rising multiple-dose trials with significant reductions in serum cholesterol at doses ranging from 0.25 to 5 mg/kg. However these positive results were accompanied by dose-dependent reductions in endogenous thyroid hormone levels, suggesting that the liver-selective prodrug strategy was not effective. A phase 2a trial in patients with hypercholesterolemic patients was withdrawn before initiation in 2009, suggesting that development on MB07811 had been terminated.

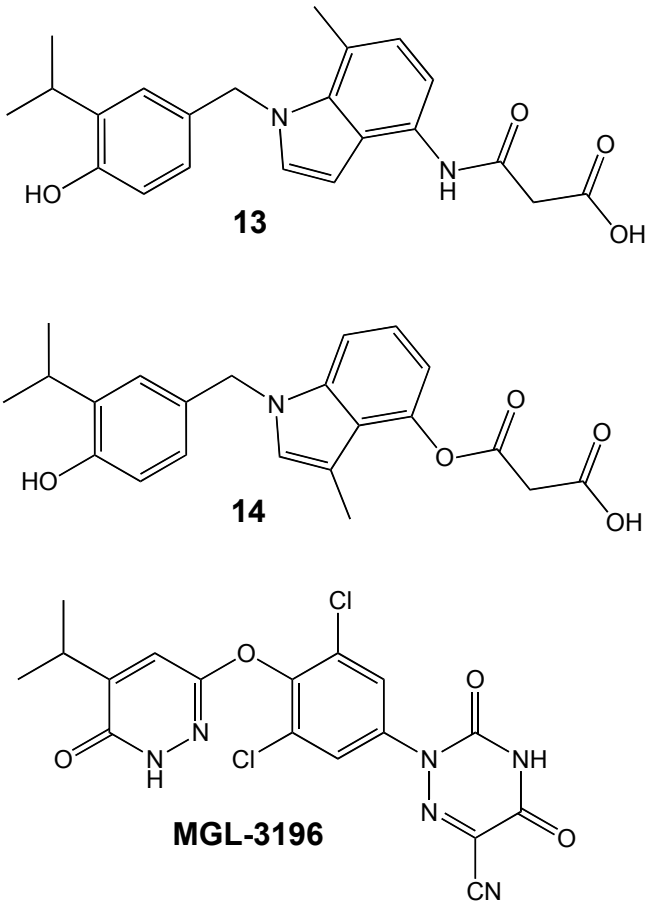

Figure 1.16

Thyromimetics developed by SKK, Eli Lily, and Madrigal Pharmaceuticals.

Sanwa Kagaku Kenkyusho (SKK) patented a series of indole-based thyromimetics (Fig 1.16 – **13**) in 2009[256] and a report on this series was published by Takahashi in 2013. [257] Unlike Bayer's indole-based series of thyroid hormone analogs, the SKK series placed the indole on the inner ring and conjugated it through the indole nitrogen to a series of outer ring groups via simple benzyl chloride alkylations. The carboxylic acid moiety was connected to the 3-position of the indole through a variety of straight chain alkyl or amide

linkages, which gave a maximum of ~20-fold TRβ-selectivity, but binding affinity was relatively poor, at best in the 100 nanomolar range compared to the 3 nM affinity of T3 in their assays. Two malonic amides similar to KB-2115 also displayed significant TRβ-selectivity in cell-based transactivation assays, which led to *in vivo* testing in cholesterol-fed rats. 6 or 30 mg/kg/day oral dosing for the respective compounds in cholesterol-fed rats over one week resulted in roughly 50% reductions in serum cholesterol and triglycerides, without statistically significant increases in heart rate or suppression of TSH. A follow-up report by the same authors in 2014 pointed towards liver-selective distribution as the main factor in the preference for cholesterol lowering compared to cardiotoxic effects or HPT perturbation. [258] Compared to the TRβ-selective thyromimetic GC-1, the SKK compounds demonstrated far higher liver:heart and liver: plasma ratios, though it is difficult to assess the validity of the data as only one animal was used per time point per compound. Despite these positive results, it was also shown that one SKK compound significantly reduced T4 after administration for two or four weeks, suggesting that HPT perturbation could be a long-term problem in a clinical setting.

Eli Lily reported a series of similar indole-based thyromimetics (Fig 1.16 – **14**) in 2015 that utilized an oxyacetic acid group instead of the alkyl and amide linkages of the SKK series. [259] Methyl or chlorine substitutions at the 2-, 3- and

7-positions of the indole had significant effects on TRβ binding affinity. The 3-methyl substitution providing the greatest selectivity at 20-fold, but this sacrificed 10-fold binding affinity compared to the more tightly binding 2-methyl-7-chloro compound that displayed more modest 10-fold TRβ-selectivity. Building on the selectivity of the 3-methyl compound, variations on the 3'-isopropyl group were tested, with an expected trend of bulkier substituents reducing the binding affinity for TRα and concomitantly improving TRβ-selectivity, which peaked at 68-fold for the 3'-benzyl substitution. For reasons not stated by the authors the less selective 3'-isopropyl compound was picked for oral administration in fructose-fed rats, a model of diet-induced metabolic syndrome. Maximum effects included HDL elevation by 55% at a 1 mg/kg dose and serum triglyceride reduction by 64% at a 10 mg/kg dose. No data has been presented on cardiac or HPT effects, so it is unclear whether the *in vitro* TRβ-selectivity translates into physiological selectivity.

Madrigal Pharmaceuticals, previously known as VIA Pharmaceuticals, first reported their clinical candidate thyromimetic **MGL-3196** (Fig 1.16) in an abstract in 2009. [260] The compound had initially been discovered by Roche, who licensed it to Madrigal for further development. Synthetic, *in vitro*, and pre-clinical *in vivo* data were reported by Kelly in 2014. [261] The series containing MGL-3196 was built around a pyridazinone outer ring connected to a collection

of inner rings with straight chain carboxylic acids, amides, and azauracil heterocycles related to those utilized by Pfizer. *in vitro* activity was determined in a cell-free FRET-based coactivator recruitment assay, which showed that the azauracil derivatives possessed the greatest combination of potency and selectivity. Expression of α-MHC mRNA in the heart was used to assay physiological selectivity, which found high variability in exposure and activity between compounds with only small structural differences. One compound was selected for *in vivo* testing in diet-induced obesity (DIO) mice and demonstrated significant reductions in serum cholesterol without reducing bone mineral density, increasing heart weight, or perturbing the HPT axis. Madrigal has carried out three separate Phase 1 clinical trials with MGL-3196 in 2011, 2012, and 2015. The results of the first two studies were reported by Taub in 2013 and indicated that MGL-3196 produced the desired outcomes - reductions in serum cholesterol and lipids without changes in heart rate or TSH. [262] There was a trend towards reduced T4 that was significant at the 200 mg dose, which the authors suggest was due to increased expression of DIO-1. Madrigal was granted a new patent for use of MGL-3196 to treat resistance to thyroid hormone in 2015. [263]

1.8 Mutant-Selective Thyromimetics

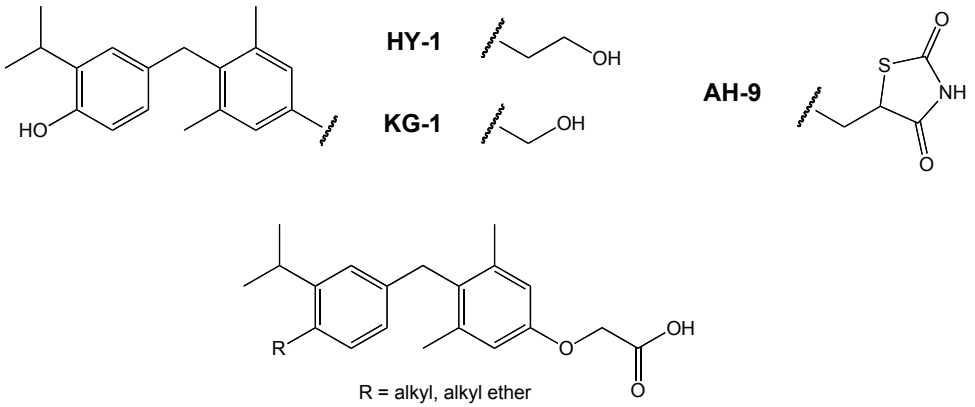

Figure 1.17

Mutant-selective thyromimetics.

Resistance to thyroid hormone is a syndrome encompassing a number of mutations in the thyroid hormone receptor ligand binding domain that reduce or block thyroid hormone binding. A number of compounds have been synthesized that selectively bind to these mutant receptors, in some cases creating orthogonal ligand/receptor pairs. Ye reported **HY-1** (Fig 1.17), the first mutant-selective thyroid hormone analog in 2001. [264] It was based on the structure of GC-1, but replaced its oxyacetic acid with an ethanol. This made it roughly 10-fold selective for TRβ mutant R320C compared to TRα or TRβ. Shi reported a second series of ligands in 2005 that expanded on HY-1 with hydroxyl groups linked to the thyromimetic core through a series of alkyl, alkyl ether, and alkyne linkers. [265] While HY-1 was only selective for R320C, the benzyl analog **KG-1**

(Fig 1.17) was 4-5 fold selective for TRβ mutants R316H, R320H, and R320C. Hashimoto reported a new series of mutant-selective ligands in 2005, which also modified the structure of GC-1 by replacing the carboxylic acid with bioisosteric tetrazole and thiazolidinedione groups. [266] The thiazolidinedione ligand **AH-9** (Fig 1.17) activated TRα, TRβ, and TRβ mutants R320H and R320C at roughly equal EC_{50} values in a cell-based transactivation assay, but the EC_{50} for the TRβ R316H mutant was 196 times greater. This made it a more potent compound for rescuing R320 mutations than HY-1 or KG-1, but it did not display the mutant selectivity of those earlier compounds. A final paper published by Hassan in 2006 focused on the opposite end of both the ligand binding domain and the thyromimetic structure[267]. His435 in TRβ typically interacts with the phenol of thyroid hormones and thyroid hormone analogs, so the H435A mutant significantly decreases the binding affinity of those compounds for the mutant receptor. Replacement of 4'-phenol in GC-1 with a series of alkyl ethers and alkyl groups (Fig 1.17 – **16**) produced the ethyl ether analog QH-2 that was a full agonist and had an EC_{50} of 6.4 nM for TRβ H435A in a cell-based transactivation assay. It proved to be orthogonal to TRα and TRβ with EC_{50} values nearly one-hundred times greater for the WT receptors.

1.9 Diiodothyronine-Based Analogs

While less extensively studied than analogs of T4 or T3, analogs of 3,5-diiodothyronine (T2) have been investigated since the first synthesis of thyroid hormone produced it as an intermediate. [8] The first reports on the activity of T2 were by Gaddum in 1927 and 1930, who noted that it was roughly 40-fold less potent than thyroxine by weight in a tadpole metamorphosis assay[14] and 15-fold less potent than thyroxine in stimulating oxygen consumption by rats. [17] Its use as a clinical substitute for thyroxine was investigated by Anderson and reported in 1933 as an acceptable replacement, relieving the symptoms of hypothyroidism without adverse effects. [268]

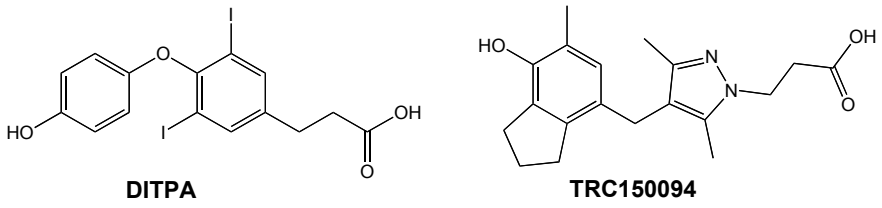

Figure 1.18

Diiodothyronine (T2) analogs.

The first reported T2 analog was diiodothyropropionic acid (DITPA) (Fig 1.18). The first synthesis and chemical characterization of DITPA was reported by Wawzonek in 1950 along with a series of thyrocinnamic acids. [65] This work was followed up with biological assays reported by Barker in 1951 that found DITPA to have 1-2% of the activity of DL-thyroxine in stimulating oxygen

consumption in rats[66] and a report by Stasilli in 1959 that found DITPA to have less than 3% of the activity of L-T4 in goiter-prevention and calorigenic assays. [269] With the exception of a brief report from Eades Jr. in 1963 that found DITPA effectively blocked the incorporation of radiolabeled acetate into cholesterol in rat liver homogenates, [270] the compound received almost no further study for several decades.

A review by Morkin in 1983 summarized the then-known effects of thyroid hormone on cardiac output, including mechanical, ionotropic, and biochemical changes. [271] The article concluded by suggesting that thyroid hormone analogs with the ability to selectively induce expression of high-activity type myosin isoenzyme without increasing myocardial oxygen demands could be clinical therapeutics for treating cardiac hypertrophy and congestive heart failure. A report by Pennock in 1992 was the first to suggest that DITPA was such an analog. [272] They found that DITPA induced α-MHC mRNA and increased cardiac output in thyroidectomized rats while producing smaller increases in heart rate or oxygen consumption than L-T4. This occurred despite the lack of selective binding of DITPA to either TR subtype. A follow-up study reported by Morkin in 1993 found that in a rat model of heart failure short-term administration of L-T4 increased cardiac output while longer-term administration produced increases in heart rate and regression of cardiac output.

[273] In contrast they found that DITPA produced similar increases in cardiac output with reduced side-effects when given in combination with the ACE inhibitor captopril. As rats are not an ideal model of myocardial infarction due to different compositions of MHC and other contractile proteins, Mahaffrey reported in 1995 that a similar experiment with rabbits produced comparable results to the studies in rats. [274] These positive preclinical results led to Phase I clinical trials in heart failure patients that found significant improvements in diastolic function without any change in systolic function or heart rate. [275] In addition, cholesterol and triglycerides were significantly reduced, but TSH and total T4 were suppressed while rT3 rose significantly, indicating that DITPA had noticeable effects on the HPT axis and liver. Phase II trials in similar patient populations were pursued by Morkin in partnership with Titan Pharmaceuticals. Studies were registered in 2002 and 2005, but both were terminated before completion. Goldman reported in 2009 that the study had been discontinued due to adverse effects and that little if any quantifiable improvement had been demonstrated in patients receiving the drug[276]. A potential explanation for these results were offered by Hassan Talukder in 2011. [277] The group found that administration of DITPA to mice in an ischemia-reperfusion model of mycardial infarction increased the occurrence of arrhythmias and mortality during ischemic events. This suggests that long-term treatment could be extremely

hazardous for patients already at increased risk for additional cardiac events. A Johns Hopkins sponsored clinical trial of DITPA as a treatment for hypercholesterolemia was registered in 2006, but it was also terminated before completion.

A new indication for DITPA was reported by Di Cosmo in 2009, who suggested that it could be a treatment for patients with monocarboxylate transporter 8 (MCT8) mutations and deficiencies. [278] In humans these mutations eliminate the transport of thyroid hormone across the blood brain barrier, which produces CNS hypothyroidism that leads to major developmental defects and peripheral hyperthyroidism. In a mouse MCT8 knockout strain DITPA was able to normalize TSH and serum thyroid hormone levels without eliciting thyrotoxic effects, suggesting that its entry into the brain was MCT8-independent. Similar results were reported by Ferrara in 2015 in correcting metabolic parameters in MCT8 knockout mice. [279] A separate study reported by Ferrara in 2014 found that DITPA administered to dams of fetal MCT8 knockout mice could pass through the placental barrier to suppress TSH and partially correct the expression of TH-responsive genes, further demonstrating its utility in MCT8 deficiency[280]. A small-scale compassionate use trial was carried out at multiple centers with children suffering from MCT8-deficiency, which found that administration of DITPA over 26-40 months corrected TSH,

T3, and T4 while reducing heart rate and elevating cholesterol, suggesting that peripheral hyperthyroidism had been corrected by normalization of the HPT axis. [281]

A patent granted in 2008 to Torrent Pharmaceuticals included the second major T2 analog to receive serious study. [282] The series was based around a pyrazole inner ring functionalized with either propionic acid or tetrazole substituents and linked to either 2',3'-dihydroinden-4-ol or indole outer rings via a methylene bridging group. Initial results were reported by Cioffi in 2010, which indicated that the best analog in the series, TRC150094 (Fig 1.18), had little to no agonist activity through either TR in cell-based transactivation assays but it normalized body weight by increasing oxygen consumption and reducing cholesterol and triglycerides in a rat diet-induced obesity model without impacting TSH, T4, T3, or cardiac parameters. [283] A report by Zambad in 2011 indicated that TRC150094 could also improve glucose tolerance and glycemic profile while ameliorating hypertension in a similar rat diet-induced obesity model. [284] A proteomic analysis reported by Silvestri in 2012 indicated that TRC150094 increased the expression of a number of metabolic and mitochondrial pathways in liver and skeletal muscle, providing a potential mechanism for the previously reported *in vivo* results. [285] These positive preclinical results led to a Phase I clinical trial to assess changes in insulin

sensitivity in patients with metabolic syndrome. [286] While no overt toxicity was observed, there was no improvement in insulin sensitivity or other metabolic parameters in the treated patients compared to the placebo group. To date no further studies of TRC150094 have been initiated.

1.10 Thyroid Hormone Antagonists

The first thyroid hormone antagonists were stumbled upon accidentally, as synthetic intermediates reported by Woolley in 1946 during an effort to produce benzyl ether analogs of N-acetyl diiodotyrosine. [287] The para-nitrobenzyl ether (Fig 1.19 – **15**) and para-nitrobenzylethyl ethers of N-acetyl diiodotyrosine were found to significantly antagonize the effects of DL-thyroxine in an amphibian metamorphosis assay, as well as in a rat-based methyl cyanide protection assay. However, these compounds were not potent, requiring two hundred- to two thousand-fold excess of inhibitor to block a given quantity of thyroxine and displayed significant toxicity when administered in the absence of exogenous thyroxine. Frieden and Winzler extended this work in 1949 by synthesizing and evaluating a number of benzyl ethers of diiodotyrosine and 4-hydroxy-3,5-diiodobenzoic acid (Fig 1.19 – **16**), which were demonstrated to have K_i values ten to one hundred times lower than Woolley's N-acetyl analogs.

[288] These antagonists were also shown to be competitive inhibitors of both thyroxine and a number of thyroxine analogs by the methods of Lineweaver and Burk. Unfortunately the benzoic acid analog, which was the most potent of the series, also demonstrated significant toxicity.

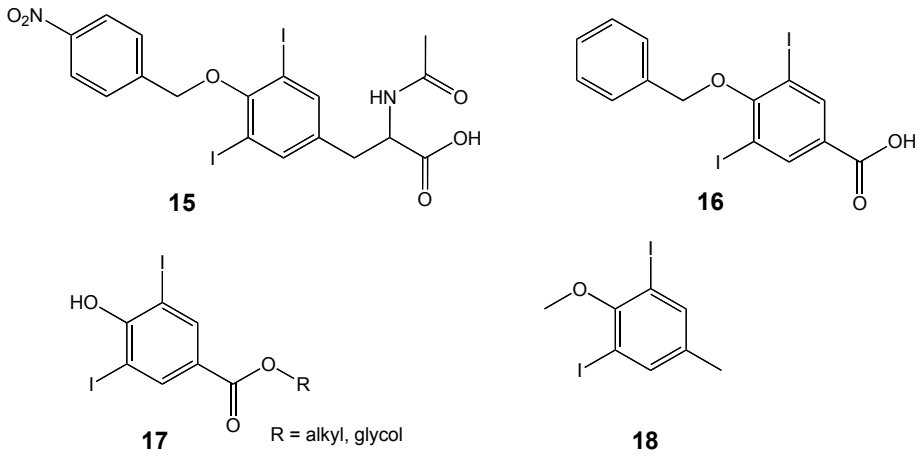

Figure 1.19

First generation thyroid hormone antagonists.

Cortell assayed a range of thyronine analogs in 1949 in a rat goiter prevention assay that had been previously synthesized by Neimann including fluorinated thyronines, 2',6'-diiodothyronine, and 3',5'-diiodo-4-(4'-hydroxyphenoxy)-3,5-diiodohippuric acid. [29] Out of the series, only 2',6'-diiodothyronine diplayed significant antagonist activity, but at roughly a 150-fold molar ratio of inhibitor to block a given quantity of administered thyroxine.

Sheahan, Wilkinson, Sprott and Maclagan released a series of papers in 1951-1953 that investigated the antagonist properties of 4-hydroxy-3,5-

diiodobenzene-based compounds. These were designed to mimic features of the inner ring of thyroxine while utilizing the increased potency of benzoic acid derivatives found by Frieden and Winzler. The first was a series of n-alkyl 4-hydroxy-3,5-diiodobenzoate esters (Fig 1.19 – **17**), which displayed significant antagonism at chain length between one to four carbons. [289] The n-butyl derivative was most potent and was able to reduce increased DL-thyroxine-mediated oxygen consumption by roughly 50% at a dose twelve and a half times that of the administered DL-thyroxine. Unlike the benzyl ethers of Woolley, Frieden, and Winzler, these n-alkyl 4-hydroxy-3,5-diiodobenzoates had significant therapeutic windows of thirty to four hundred depending on the route of administration. Building on the previous work with alkyl and benzyl ethers, the same researchers demonstrated that alkylating the 4-hydroxy position produced compounds that still displayed antagonism, but in all cases they were significantly less potent than the parent 4-hydroxy compounds. [290] In the same issue they also reported a similar series where the benzoate group was replaced by a benzaldehyde. [291] As with the benzoate ethers, these compounds were either of lower potency or displayed no activity in comparison to the original set of 4-hydroxy3,5-diiodobenzoates. The final set of analogs explored 4-hydroxy-3,5-diiodobenzoate esters of glycols. [292] Once again there were tight structural constraints and only the short chain bis-hydroxy esters displayed significant

inhibitory activity, while longer chain bis-hydroxy esters or glycerol esters were completely inactive.

Having explored antagonists constructed from analogs of the inner ring, Sheahan, Wilkinson, and Maclagan turned to analogs of the outer ring. [293] The analogs were a mix of 3,5- and 2,6-diiodophenols and anilines. The most potent analog from the series was 3,5-diiodomethyoxytoluene (Fig 1.19 – **18**), which was roughly as potent as n-butyl 4-hydroxy-3,5-diiodobenzoate in the same mouse oxygen consumption assay.

Pitman and Barker published several papers in 1959 exploring a number of antagonists based on the structure of 3,3',5'-triiodothyronine, now known as reverse T3 (rT3). It had previously been demonstrated that rT3 had almost no agonist activity, even at very high doses. [294] From this observation, both rT3 and rT3 analogs were assayed for their ability to antagonize T3-evoked increases in oxygen consumption. DL-rT3 was able to almost completely block increases in oxygen consumption, but it required molar ratios of one hundred to two hundred time the dose of L-thyroxine to produce full inhibition. [295] The propionic analogs of rT3 and 3,3'-T2 proved to be roughly as potent inhibitors of L-thyroxine action as DL-rT3, while the acetic acid analog produced only 50% inhibition at the same molar ratios. [296]

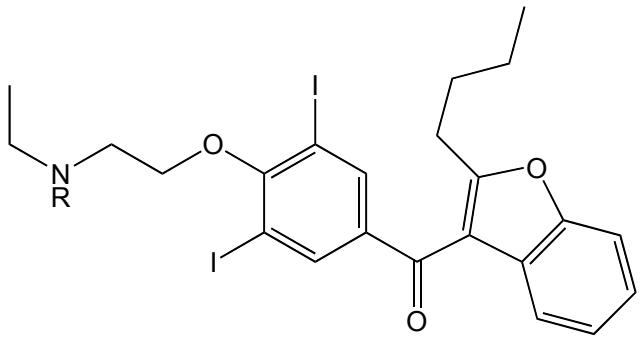

Figure 1.20

Amiodarone and desmethylamiodarone.

The field of thyroid hormone receptor antagonists remained fallow for nearly three decades until it was spurred back into life by Amiodarone (Fig 1.20), a drug originally used as an anti-arrhythmic. [297] While structurally unrelated to classic thyromimetics, amiodarone does have a bicyclic structure and 3,5-diiodo motif. Competition binding assays with radiolabeled T3 demonstrated micromolor affinity for liver and pituitary tumor cell nuclear extracts and roughly ten-fold less for brain nuclear extracts. The K_i in T3-induced transactivation and IC_{50} for uptake of radiolabeled T3 into intact cells was similarly in the high micromolar range. While the initial report found that amiodarone was a competitive antagonist of TR, it was reported by Bakker in 1994 that one of its metabolites, desethylamiodarone (Fig 1.20), was a non-competitive inhibitor of TRβ while amiodarone itself had no inhibitory effect. [298] Bakker attributed the antagonism of amiodarone that has been observed by

Norman to the use of Tween-80, a detergent that has been used to solubilize the compound. A concurrent report from Hudig in 1994 showed that amiodarone could block the effects of T3 *in vivo*, inducing hypercholesteremia in rats by downregulating the LDL receptor. [299] However, no effort was made to determine whether this was a direct effect of amiodarone or due to the desethyl metabolite reported by Bakker. Ultimately the low potency and non-selective pharmacodynamics of amiodarone limited its usefulness either as a tool for studying thyroid hormone activity *in vitro* or as a therapeutic.

Rationally designed thyroid hormone antagonists have developed out of the body of knowledge that accumulated during the 1980s[300] and 1990s[301] about the structural similarities between nuclear hormone receptors. Nuclear hormone receptors are modular polypeptides that contain both a DNA binding domain and a ligand binding domain that also serves as a platform for dimerization and coregulator binding. These domains also contain similar secondary structure motifs that translate into similar conformational shifts during ligand binding. These parallels allow structural features of ligands that antagonize one nuclear hormone receptor to be applied to ligands for other nuclear hormone receptors to transform them into antagonists.

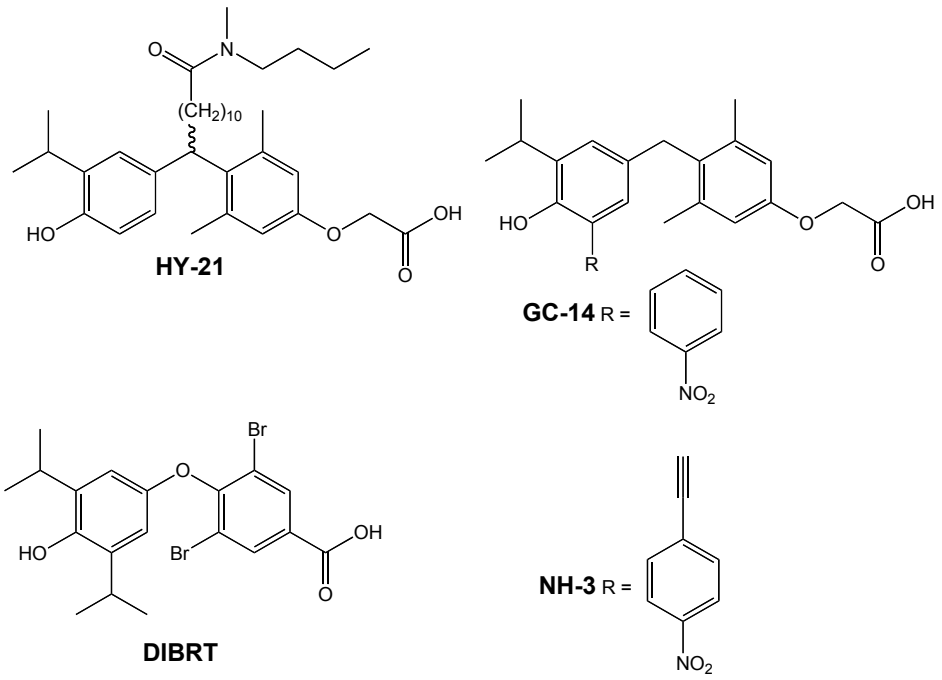

Figure 1.21

Thyroid hormone antagonists developed by the Scanlan group.

The first rationally designed TR antagonist was the HY series reported by Yoshihara in 2001, [302] which utilized structural features of the estrogen receptor antagonist ICI-164,384. [303] Large hydrophobic groups were appended to the bridging methylene of GC-1, which were believed to sterically inhibit formation of the activated TR conformation. These compounds had a number of limitations. Predictably, binding affinity was significantly reduced, with the most potent inhibitor HY-21 (Fig 1.21) demonstrating an IC_{50} of roughly 1 μM. Surprisingly, the loss of affinity across the series was not directly correlated with steric bulk. Second, the subtype selectivity found in the parent molecule did not

translate into most of the series, with only the least sterically bulky modification retaining modest selectivity.

The next series of antagonists reported by Chiellini in 2002 used a similar strategy of sterically bulky modifications to GC-1, but transferred the modifications from the bridging group to the 5' position to block translocation of helix 12 and limit coactivator recruitment, which is generally described as the extension hypothesis. [304] This continuation of the GC series demonstrated that steric bulk was not the only determining factor, as a number of positional isomers of nitrobenzene were appended at the 5' position, but only the *para*-nitro analog GC-14 (Fig 1.21) displayed significant, but not full, antagonist character, while the *meta*- and *ortho*-nitro analogs GC-19 and GC-20 were full agonists at TRβ.

A similar strategy was used in designing the compound DIBRT (Fig 1.21), which had isopropyl groups at both the 3' and 5' positions. [305] While coactivator binding assays demonstrated that DIBRT could block T3-induced GRIP1 recruitment to a significant degree, transactivation assays demonstrated complex agonist-antagonist behavior across the range of tested drug concentrations. From these two compound series, it seemed that steric bulk at the 5' position was insufficient to transform a full agonist into a full antagonist.

The most successful series of TR antagonists has been the NH series described in two reports by Nguyen from 2002[306] and 2005. [307] This series built on the SAR of GC-14 with a different but related set of bulky hydrophobic groups appended to the 5' position of GC-1. The extensions were made larger and more rigid by the insertion of an ethynyl group between the GC-1 inner ring and the aryl groups found in the GC antagonist series. Similar SAR exists in the NH series - electron withdrawing groups in the *para* position such as aryl nitro (NH-3), trifluoromethyl (NH-5), azide (NH-7), ketone (NH-9), or fluoride (NH-10) produced full antagonists with varying IC_{50} values. Electron donating groups in the *para* position produced partial agonists, while electron withdrawing groups in the *meta* or *ortho* positions produced weak antagonists or inactive compounds with appreciable binding affinity but no ability to induce transactivation on their own or block induction by 1 nM T3. The *in vitro* results were validated *in vivo* when NH-3 (Fig 1.21) was tested in rats and found to block the effects of T3 at moderate doses, but also exhibited weak agonist effects at high doses. [308] While the increased potency and antagonist activity of this series partially validates the extension hypothesis, the electronic and positional requirements on the aryl component of the extension suggest that it is not a simple steric effect. Unfortunately no crystal structures of TR complexed with NH-3 exist, so we are currently left to guess at the basis of this SAR.

In 2008 Hashimoto reported a new antagonist based on the NH series scaffold. [221] Instead of the *p*-nitro group on the benzene ring found in NH-3, JZ07 replaced it with a n-hexanoic acid amide of propargylamine. JZ07 was a full antagonist in a cell-based transactivation assay and retained the TRβ-selectivity of the parent compound with EC_{50} values comparable to or slightly better than NH-3.

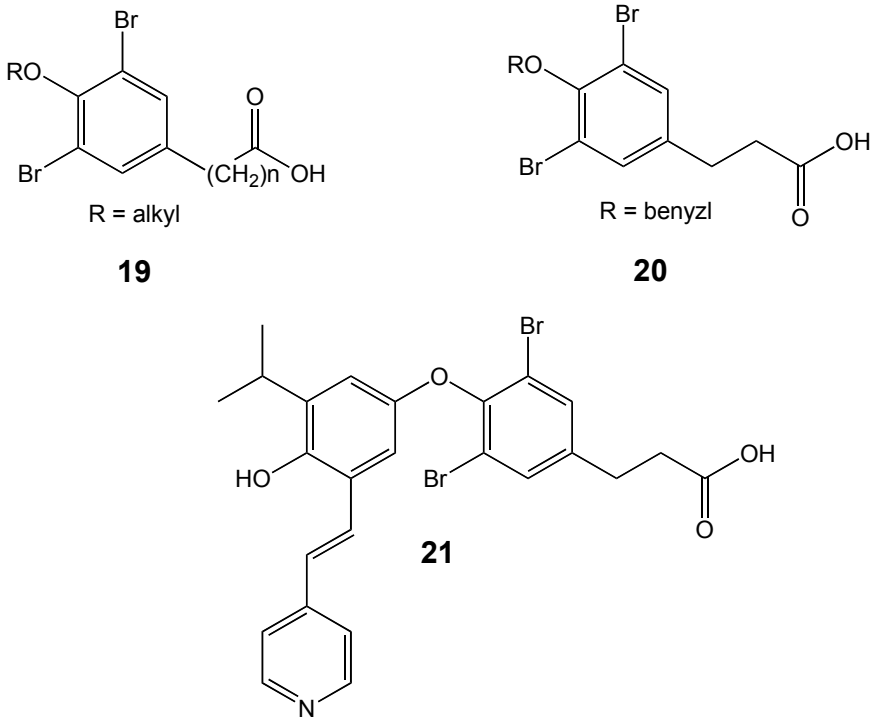

Figure 1.22

Direct and indirect antagonists synthesized by Karo Bio.

A number of attempts have been made by Karo Bio to produce direct and indirect antagonists. A set of 3,5-dibromo-4-alkoxyphenylalkanoic acid derivatives was described by Hedors in 2005 that returned to the ideas of

Woolley, Frieden, and Winzler by replacing the outer ring of classic thyroid hormone analogs with a series of alkyl ether groups (Fig 1.22 – **19**). [309] This set of antagonists was relatively poor, with K_d and IC_{50} values in the micromolar range. The results of Hedor's work with the 3,5-dibromo-4-alkoxyphenylpropanoic acid skeleton were built upon to produce a second generation of analogs that were reported by Malm in 2007. [310] Instead of a cyclohexyl appendage in place of the outer ring, benzyl ethers (Fig 1.22 – **20**) with either electron-donating or electron-withdrawing substituents were synthesized and tested. The 3-bromo and 3-ethylamine analogs displayed the best K_d and IC_{50} values of the series, which were in the nanomolar range. Consistent with the GC and NH series, the electron-withdrawing 3-bromo analog displayed partial antagonist activity while the electron-donating 3-ethylamine analog was a partial agonist. In 2006 Koehler reported a single compound that utilized a strategy similar to NH-3 by modifying the parent agonist[173] at the 5' position, this time with a pyridyl vinyl group (Fig 1.22 – **21**). [311] This compound had K_d and IC_{50} values in the same mid-nanomolar range similar to NH-3, but did not retain the TRβ-selectivity of its parent agonist.

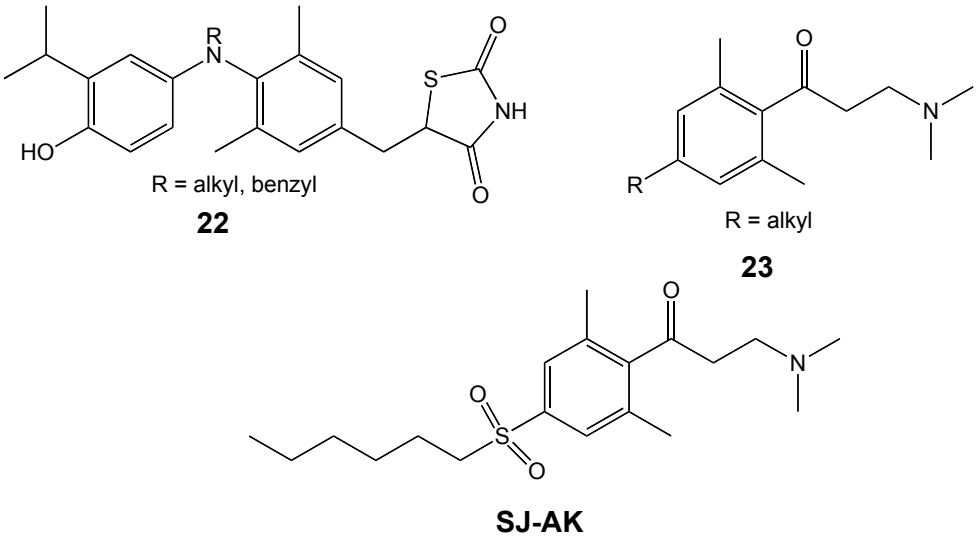

Figure 1.23

Diphenylamine direct antagonist and mechanism-based inhibitors of TR coactivator recruitment.

The last major class of direct antagonists were the diphenylamines (Fig 1.23 – **22**) reported by Komatsu in 2007. [312] These ligands utilized a standard thyromimetic core that used a diaryl amine bridging group and a thiazolidinedione polar group, which was reported to be an effective carboxylate mimetic by Ebisawa in 1999. [242] Relatively small alkyl appendages added to the diphenyl amine were sufficient to transform the initial agonist into a series of antagonists. However, 50% inhibition of T3-mediated transactivation for even the best compounds in the series occurred only at high nanomolar or micromolar doses and no compound showed full antagonism.

A series of inhibitors reported by Arnold in 2005 took the novel route of physically blocking the interaction between the receptor and coactivators, in

contrast to competitive antagonists that bound in the ligand binding pocket. [313] Because they bind to a protein-protein interface, they were structurally dissimilar from the thyroid hormone analogs that had been the basis of previous antagonists. A library was screened in a high-throughput assay of coactivator binding, [314] which found a group of β-aminoketones (Fig 1.23 – 23) that almost completely blocked recruitment at 30 μM. The best compound in the series had an IC_{50} of 2.1 μM and was found to form a reactive enone that formed a covalent bond between the inhibitor and the TR surface through a cysteine. Structural modifications of these initial hits were reported in 2007, with only modest improvements in potency but some improvements in subtype selectivity. [315] These compounds had limited use as all demonstrated cytotoxicity at concentrations comparable to their IC_{50} values. Sadana reported a refined antagonist, SJ-AK (Fig 1.23), in 2011, which improved on the previous series by adding a sulfonyl group to the ring to enhance the reactivity of the electrophile while limited the previously observed cytotoxicity. [316] While SJ-AK blocked only 50% of T3-mediated transactivation in a cell-based assay at the tested dose, microarray analysis showed that it blocked unique set of endogenous TR-regulated genes that partially overlapped with those blocked by NH-3. While this made SJ-AK a novel tool for exploring the molecular

mechanisms of TR-mediated transactivation, the low efficacy of the compound limit its utility.

Chapter 2 – Development of Novel TRα-Selective Thyromimetics

2.1 - Introduction

The thyromimetic literature is replete with TRβ-selective analogs[175,317] and it is likely that industry libraries contain even more that have not become public. However, there is a distinct lack of potent TRα-selective thyroid hormone analogs. This leaves researchers with an incomplete toolkit for understanding the molecular basis of thyroid hormone action.

A number of approaches have been used to understand how TR subtypes control gene expression in the context of a complex regulatory system. [318] TR knockouts in systems ranging from cell culture to mice have revealed distinct and overlapping functions for each subtype. [319] Double knockouts are not simply additive, but also reveal defects not found in either single knockout model, [320] which is strong evidence for functional redundancy with a large set of genes that are regulated in tandem plus smaller sets of genes that are independently regulated. But knockout studies suffer from limitations - deletion of a TR does not simply remove its ability to transactivate target genes, but also relieves the transrepression of another set of target genes by the absence of the unliganded TR. [301] Additionally, TRs operate within a complex network of other regulatory factors - deletion of one TR will free coactivators and corepressors to bind other nuclear receptors, perturbing transcriptional pathways and making it difficult to

assess direct effects from indirect effects. [321,322] A further complication of TR knockouts is that deiodinase activity is regulated by TRs. The three deiodinase subtypes remove iodines from either the inner ring, outer ring, or both, which will influence the balance of endogenous thyronines. Activation of TRα upregulates the expression of type 3 deiodinase, so deletion of TRα will decrease the production of rT3 from T4 as well as the disposal of T3. [322] Activation of TRβ upregulates the expression of type 1 deiodinase, so deletion of TRβ will result in significantly elevated levels of T4 and T3. [239] Either state will potentially confound knockout studies on the organismal level, as thyroid status is perturbed and further alters gene expression, unless performed on thyroidectomized animals.

Studies using transient overexpression of TR subtypes in tissue culture bolster the hypothesis that TR subtypes regulate overlapping sets of genes. The majority of genes that are up- or down-regulated when each subtype is overexpressed overlap, while a smaller number are uniquely regulated by each subtype. [323] But these conclusions are limited by the methodology. Overexpression seeks to swamp out activation of endogenous TRs, but it is difficult to do so without producing confounding effects. Expression levels will sometimes be so high that excess TRs are exported to the cytosol, with unknown effects. [324] And as with TR knockouts, overexpressing TRs will change the

availability of corepressors and coactivators, which may modulate gene transcription by TRs as well as other nuclear recepors in ways that that may confound interpretation of the results.

Natural TR mutations provide another route to understanding the genetic role of TRs. Many of these mutations modify the ligand binding domain of the TR in ways that reduce or eliminate T3 binding while leaving the DNA binding domain unperturbed. [325,326] These mutations often play a role in resistance to thyroid hormone, a clinical syndrome where patients present with HPT axis dysregulation due to the inability of T3 to bind to TRs in the hypothalamus. Circulating thyroid hormone levels are higher than normal, but without the suppression of TSH. In some cases the increased TH levels compensate for reduced ligand binding affinity, but complete elimination of binding can present more overt symptoms. These mutations tend to operate in a dominant-negative fashion, suppressing the effects of the WT allele in heterozygous animals as the mutated receptor is locked in the repressive unliganded conformation, blocking T3-mediated transactivation and recruiting corepressors to TREs. Symptoms vary widely, ranging from apparently euthyroid individuals with elevated thyroid hormone levels to tachycardia from excess stimulation of the functional TRα. Knock-in mouse models with these types of mutations in either TRα or TRβ recapitulate the clinical symptoms and analysis of gene expression profiles

in these mouse strains have confirmed that overlapping and distinct sets of genes are regulated by each subtype. [327]

Pharmacological methods have their own limitations. Currently they must operate via inference. Cells or organisms are treated with T3 or a TRβ-selective analog. It is inferred that genes activated or repressed by T3 but not by the TRβ-selective analog are regulated by TRα. There is significant power in this approach as it utilizes intact biological systems and avoids the dosage issues of the genetic approaches - it is also incomplete as there is no way to selectively activate TRα-controlled genes. As it has been noted, TRs operate within a complex network of coactivators and corepressors. This means that agonist and antagonist are not purely binary categories for thyroid hormone analogs. Initial screens that classify analogs as agonists or antagonists tend to use artificial *in vitro* assays that evaluate the induction or inhibition of a single gene, which may not adequately model the more complex behavior *in vivo*. For instance, the antagonist NH-3 not only blocks coactivator recruitment, but also blocks corepressor recruitment, [328] which means that its effects on gene transcription will be different than either a TR knockout or a knock-in mutant TR that blocks ligand binding. The wide range of structural motifs that have been used to construct thyroid hormone analogs can be expected to change the conformation of the ligand-binding domain in a non-binary fashion, which will have

differential effects on specific coactivator and corepressor recruitment, changing the suite of genes that are activated or repressed by a given analog. On an organismal level PK-ADME plays an important role. Evaluating drug concentrations in target tissues is critical for understanding transcriptional outcomes - low induction may be due to the drug not reaching a sufficient concentration in a target tissue, while non-selective effects may be evoked by bioaccumulation that saturates both TRs in a target tissue.

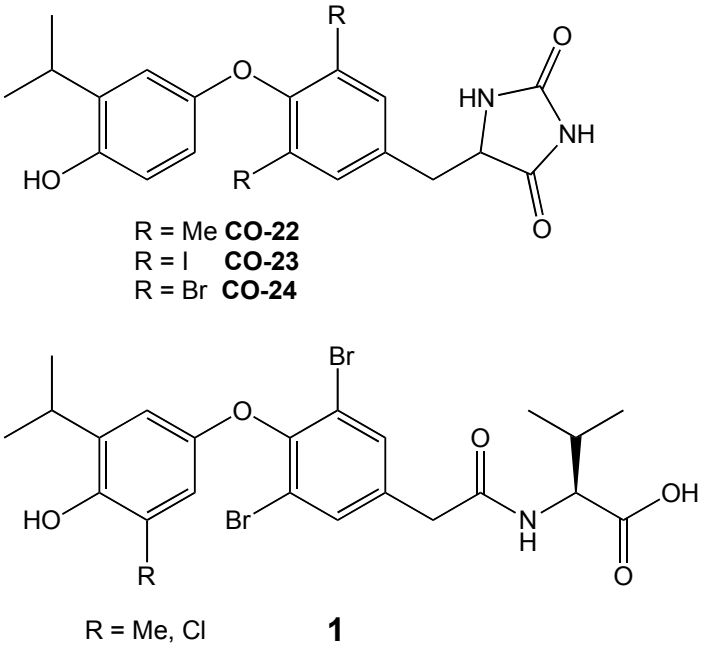

Figure 2.1

TRα-selective thyromimetics: the CO series from the Scanlan group and compounds produced by Karo Bio.

Attempts have been made to complete the pharmacological toolkit by creating TRα-selective thyromimetics. The first rationally designed TRα agonist was reported by Ocasio in 2006. [329] It was built on the hypothesis that the polar

tail is the primary structural determinant of selective TR activation. The first reported compound, CO-22 (Fig 2.1), used a standard iodine-free thyromimetic core coupled to an imidazolidinedione by a short linker. While CO-22 displayed no binding selectivity, it had a mid-micromolar EC_{50} for TRα-mediated transactivation of a reporter gene in U2OS cells, while showing only partial agonism for TRβ-mediated transactivation. Drawing on well-known SAR, replacement of the inner ring methyl groups of CO-22 with iodines gave the second-generation compound CO-23 (Fig 2.1) with significantly improved potency. The *in vitro* binding affinity of CO-23 was improved more than one hundred-fold compared to CO-22, though it remained unselective. The EC_{50} of TRα-mediated transactivation was improved by the same factor and had ten-fold selectivity over TRβ-mediated transactivation in U2OS cells and six-fold selectivity in HeLa cells. The *in vitro* selectivity was confirmed in a tadpole metamorphosis assay - TRα-mediated hind limb growth, but not TRβ-mediated tail reabsorption, were observed when tadpoles were treated with CO-23. The TRβ-selective compound GC-1 induced tail reabsorption but not hind limb growth, while T3 induced both hind limb growth and tail reabsorption. The combination of CO-23 and GC-1 completely recapitulated the effects of T3 administration, suggesting that they activated complementary TR subtypes.

Other features of the ligand scaffold were explored in a 2008 report from Ocasio, which focused on modifying the inner and outer ring hydrophobic groups to improve selectivity. [330] Replacement of the 3'-isopropyl group of CO-23 with smaller alkyl or halogen substituents improved selectivity, but at the cost of weaker *in vitro* binding and less potency in transactivation assays in U2OS cells. In contrast, replacement of the inner ring iodines with bromines to give CO-24 (Fig 2.1) reduced activity at both receptors and decreased selectivity in U2OS cells, but improved EC_{50} in HeLa cells. The selectivity of CO-24 was confirmed in a tadpole metamorphis assay, with even more pronounced hind limb growth than that induced by CO-23. It was hypothesized that the tadpole results were due largely to the improved metabolic stability of aryl bromides in comparison to aryl iodides. Broadly it was demonstrated that the SAR for this series was extremely tight, with even minor modifications drastically altering binding, potency, and selectivity.

In the studies of the CO series there was a consistent trend that the compounds displayed no binding selectivity, but cell-based transactivation and tadpole metamorphosis assays showed that they preferentially activated TRα compared to TRβ. While other thyromimetics had demonstrated selective physiological effects without selective binding, this had generally been attributed to selective tissue uptake. The selectivity of the CO series represented a novel

mode of selectivity that was hypothesized to be due to conformational selectivity - the compounds induced different changes in receptor conformation that led to selective gene activation with the same change in Gibbs free energy upon binding to each subtype. This hypothesis was challenged by a report from Grijota-Martínez in 2011 comparing activation of TRα and TRβ target genes in rats by GC-24 - a TRβ-selective analog - and CO-23. [331] GC-24 selectively activated TRβ-regulated genes in the liver, heart, and brain, confirming its *in vitro* selectivity. In comparison, CO-23 was found both to be a weaker agonist than *in vitro* results would have suggested and to be just as unselective as T3. While it was unclear what contribution tissue distribution made to these unselective effects, it was clear that if CO-23 retained any selectivity at the receptor level this was not enough to preclude it from activating TRβ target genes at high doses. This severely weakened any case to be made for using CO-23 to elicit selective TRα-mediated therapeutically beneficial effects in humans.

Two TRα-selective analogs were coincidentally synthesized by Karo Bio and reported by Garg in 2007 as part of a series of TRβ-selective thyromimetics. [231] The series was built around an iodine-free thyroacetic acid core conjugated to a range of amino acids (Fig 2.1 – 1). While the valine conjugates had roughly 10-fold TRβ-selective binding, the addition of either a 5'-chlorine or a 5'-methyl transformed them into roughly 3-fold TRα-selective compounds. Complicating

these results, the EC_{50} values in a cell-based transactivation assay for the TRα-selective compounds were roughly 10-fold more potent than the IC_{50} values from the competition-binding assay, suggesting that something may have gone wrong with their assays because it has been consistently reported in the thyromimetic literature that *in vitro* binding affinity IC_{50} values are significantly lower than cell-based transactivation EC_{50} values. Additionally the TRα-selective compounds were only partial agonists in comparison to the full agonism demonstrated by CO-23.

Ultimately the full range of tools available for investigating TR-mediated gene activation are all necessary and complementary as they perturb the system in different ways. Comparisons between these data sets that take their varying mechanisms into account will allow us to develop a fuller and more complete understanding the critical role of TRs in development and homeostasis.

The structure-activity relationship (SAR) information available within the broad thyromimetic field and the specific information gleaned from the work of Ocasio[329,330] in developing rationally designed TRα-selective analogs make it clear that a strategy to develop new analogs requires exploring a broad range of chemotypes to find novel compounds that selectively activate TRα. This necessitates a synthetic strategy that allows new compounds to be created rapidly and in high yield to produce diversity. Benzamides are an obvious choice

due to the well-established amide coupling chemistry and the wide array of commercially available amines.

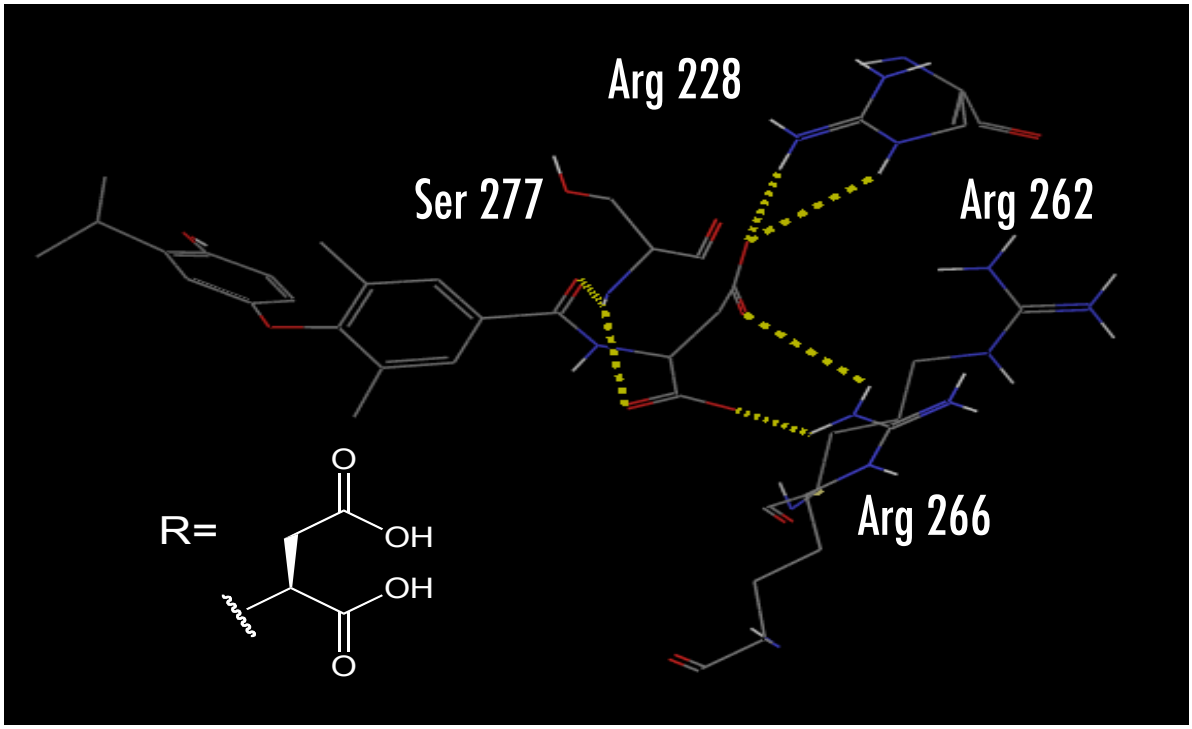

Figure 2.2

Docking of a putative TRα-selective ligand (JD-16) docked to TRα (PDB 3ILZ) showing bonding interactions with critical Arg residues on the inner surface of the ligand binding pocket.

Modeling studies using Schrödinger's Glide software with X-ray crystal structures of the TRα and TRβ ligand binding domains (LBD) reported by Bleicher[201] suggested that ligands with multiple acidic or hydrogen bond acceptor groups facing the arginine cluster in the ligand binding pocket would have greater affinity for TRα than TRβ by interacting favorably with Arg228 in TRα that has greater conformationally flexibility than the corresponding residue

Arg 282 in TRβ. This provided a testable hypothesis for establishing TRα-selectivity.

2.2 - Results

The first TRα-selective series in this report was built on the same basic thyromimetic scaffold used in the CO series. [329] Instead of an imidazolidinedione moiety, this series linked amino acids and amino alcohols groups through a benzoic acid on the inner ring to form a series of benzamides (Fig 2.3). Building the critical diaryl ether used the same copper(II) acetate-based Evans coupling[332] utilized in the CO series rather than the more popular hypervalent iodine or Ullman-type couplings[333] due to the ease of synthesizing aryl boronic acids and the broader range of suitable protecting groups compatible with this chemistry. Coupling the benzoic acid to the amines was accomplished through standard amide coupling methods that delivered the protected products in high yield and purity with only a simple workup. Standard deprotection methods produced the first series of final compounds as either free acids or lithium salts suitable for biological assays.

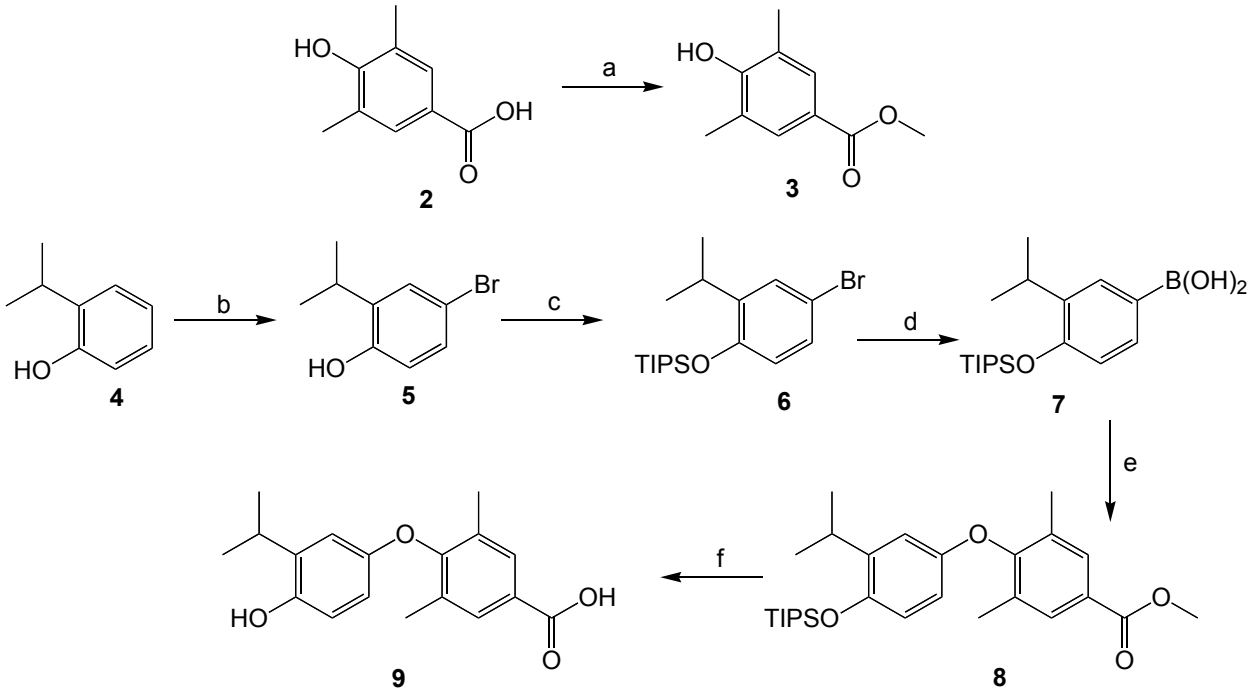

Figure 2.3

Synthesis of intermediate **9**. Reagents and conditions: (a) H_2SO_4, MeOH, 65° C; (b) Br_2, DCM, 0° C; (c) TIPS•Cl, imidazole, DCM, 0° C to RT; (d) (i) nBuLi, THF, -78° C (ii) $B(OiPr)_3$; (e) 3, $Cu(OAc)_2$, DIEA, pyridine, DCM, RT; (f) KOH, MeOH, THF, RT.

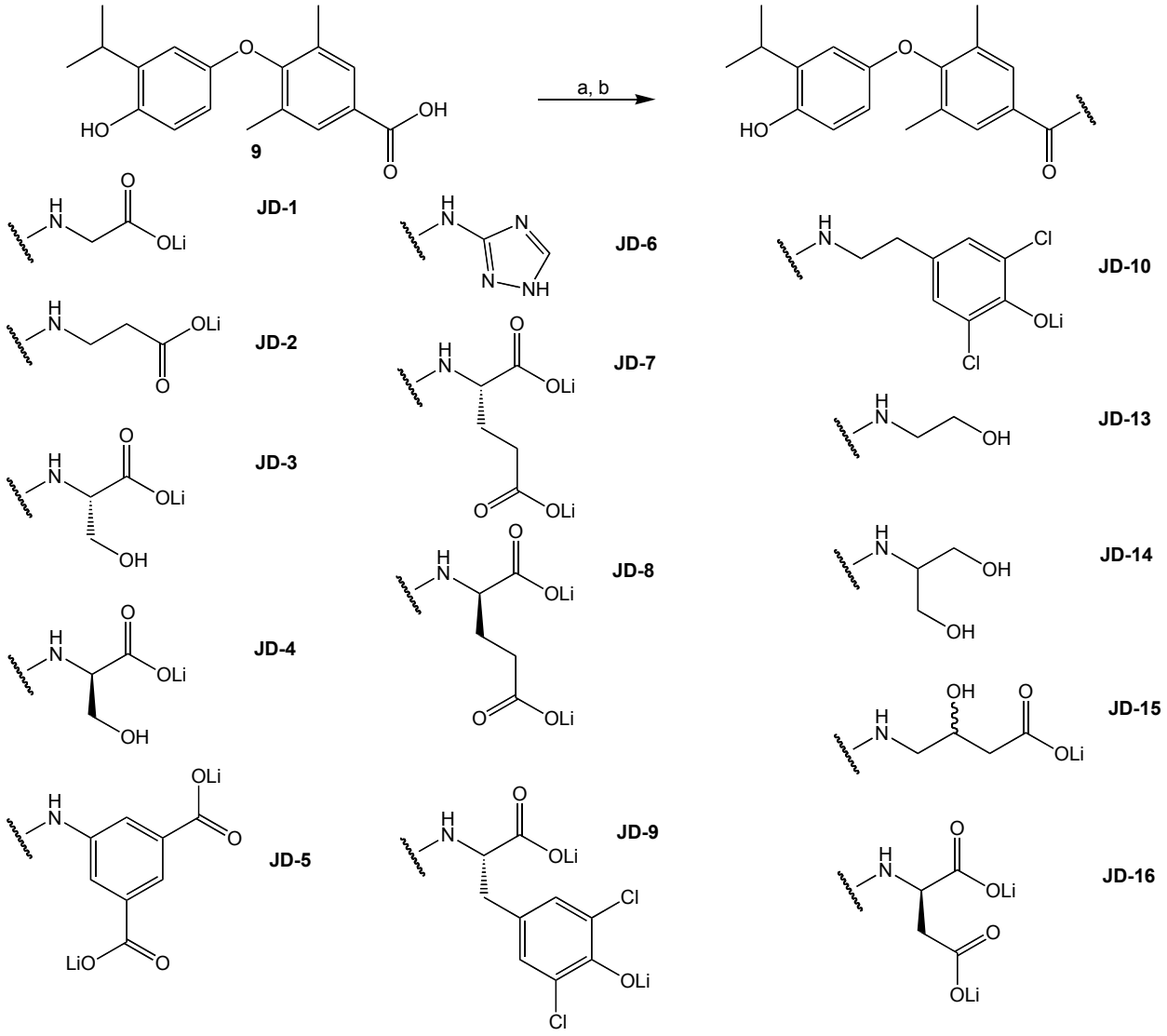

Figure 2.4

Synthesis of **JD-1** to **JD-16**. Reagents and conditions: (a) (i) EDC•Cl, HOBt, DIEA, DMF, RT (ii) amine, RT; (b) LiOH, MeOH, THF, RT.

Cell-based transactivation assays were used to evaluate thyroid hormone analogs for efficacy and selectivity. HEK293 cells were transiently transfected with plasmids for CMV-TRα or CMV-TRβ, a DR4 TRE-driven firefly luciferase, and an SV40 plasmid containing the *Renilla* luciferase gene. After incubating the transfected cells with the drugs to be tested the assays were

quantified using the Promega DualGlo luciferase kit. The first series of compounds encompassed a wide range of chemotypes (Fig 2.5) and were tested at 1 μM. These assays found that only a handful possessed the desired selectivity.

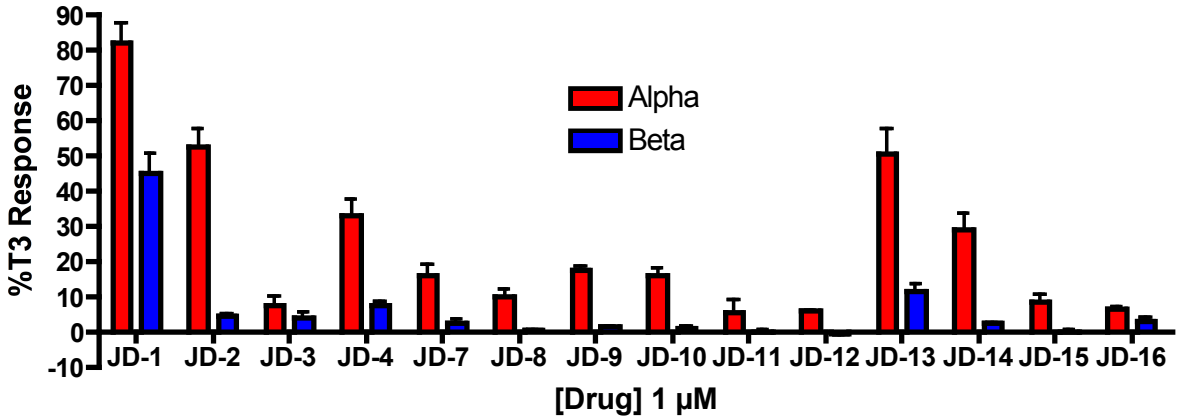

Figure 2.5

TRE-driven dual luciferase assay of $hTR\alpha_1$ and $hTR\beta_1$ transiently transfected in HEK293 cells with the series built on **9** (Fig 2.3 and 2.4) tested at 1 μM doses. Drug responses are normalized to maximal T3 response. Plot shows means of triplicates with error bars.

Out of that subset only **JD-1** at 1μM reached 100% of the maximal T3 response at TRα. Critical SAR information was found in this first series. Most important was that the simple glycine (**JD-1**) and β-alanine (**JD-2**) analogs showed a trend towards improved selectivity but decreasing efficacy with increasing chain length. Second were the differences between **JD-3** and **JD-4**, amides of the (S)- and (R)-enantiomers of serine. While the (S)-serine **JD-3** had low efficacy at both receptors and little selectivity, the (R)-serine **JD-4** had better efficacy and showed moderate selectivity. This suggested that the R-group

of the serine moiety was participating in a polar interaction and that only one enantiomer could favorably position the hydroxyl. Third, the ethanolamine **JD-13** and 2-aminoglycerol **JD-14** both showed reasonable efficacy and potency in the single dose experiment, which could be rationalized as being roughly isosteric with the glycine and serine amides.

Full dose-response assays (Table 2.1) showed that **JD-1** and **JD-2** were full selective agonists while **JD-13** and **JD-14**, were TRα-selective, but only partial agonists at the maximum concentration tested.

These initial results required modifications to the core that would improve the affinity for the TR ligand-binding domain without losing selectivity. The first path forward was found from SAR studies of the TRβ-selective thyromimetic GC-1, which suggested that replacing the diaryl ether with a diaryl methane would enhance binding affinity for both subtypes, boosting potency while retaining selectivity. [218]

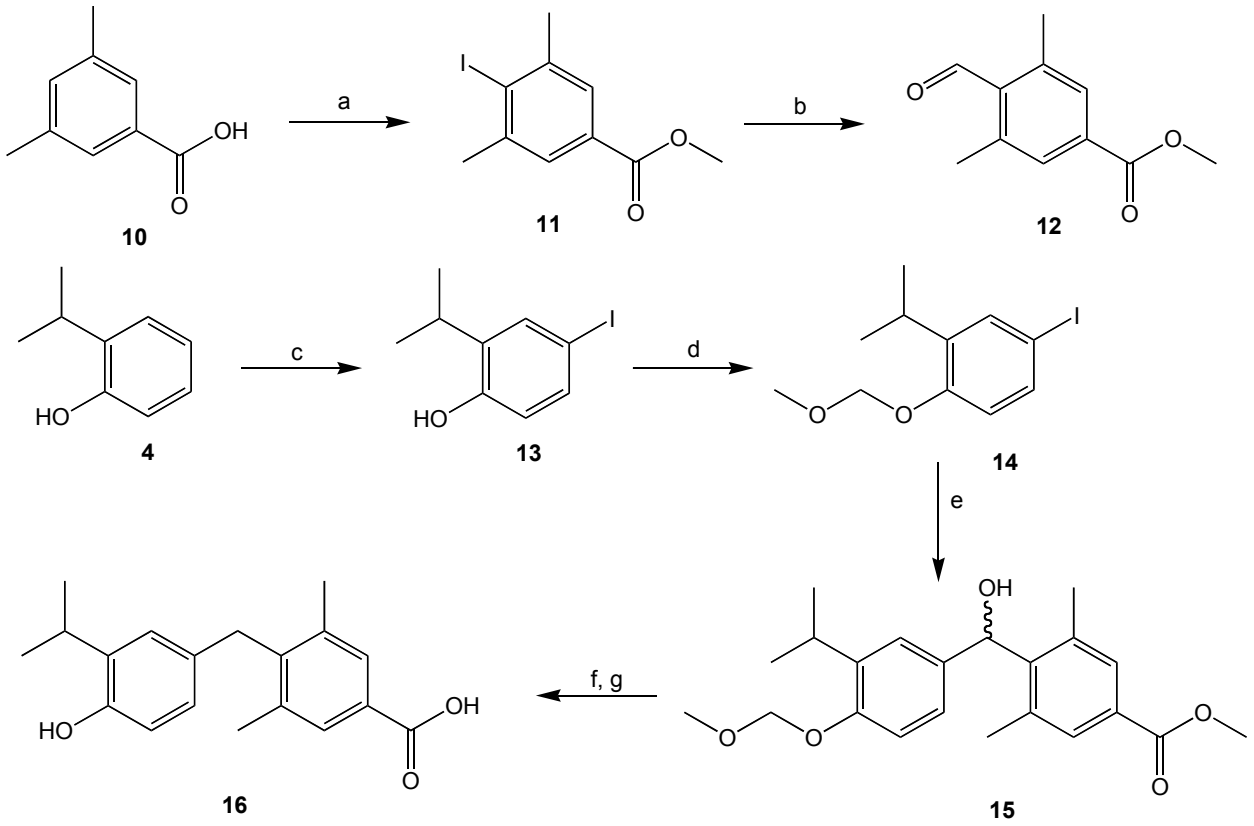

Figure 2.6

Synthesis of intermediate **16**. Reagents and conditions: (a) (i) I_2, H_2SO_4, 100° C (ii) H_2SO_4, MeOH, 65° C; (b) (i) iPrMgCl, THF, -20° C to RT (ii) DMF, RT; (c) NaI, NaOCl, NaOH, MeOH, H_2O, 4° C; (d) MOM•Cl, TBAI, NaOH, DCM, H_2O, RT; (e) (i) iPMgCl, THF, 0° C to RT (ii) **12**, -78° C; (f) TFA, Et_3SiH, DCM, 0° C to RT; (g) KOH, MeOH, THF, RT.

Unlike the initial diaryl ether series, it was not possible to purchase the inner ring as an advanced intermediate. 3,5-dimethylbenzoic acid was chosen as the starting material and selectively iodinated at the 4-position using *N*-iodosuccinimide and hot sulfuric acid as catalyst, activator, and solvent. The reaction proceeded selectively and in relatively high yield with minimal purification. After protection the benzoic acid as a simple ester the intermediate was formylated by halogen-magnesium exchange at the aryl iodide with

isopropyl magnesium chloride, then quenched with DMF to form the benzaldehyde. Importantly, this reaction was fully compatible with the methyl ester due to the reduced reactivity of the Grignard in comparison to aryl lithiums and the steric hinderance of the adjacent 3,5-dimethyl substitution. Carbon-carbon bond formation between the two aryl rings was carried out using the same procedure as that for GC-1 reported by Placzek. [334] The resulting carbinol was reduced using a trifluoroacetic acid/triethylsilane system in DCM at room temperature, then the ester was converted to the acid using standard base hydrolysis. A single final compound with a β-alanine amide, **JD-22**, was produced through the same amide coupling and deprotection strategy employed for the first series.

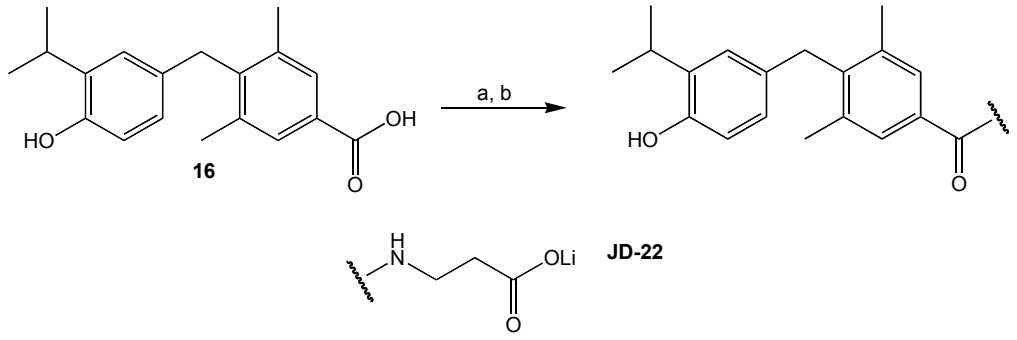

Figure 2.7

Synthesis of **JD-22**. Reagents and conditions: (a) (i) EDC•Cl, HOBt, DIEA, DMF, RT; (ii) β-alanine methyl ester•HCl; (b) LiOH, MeOH, THF, RT.

Contrary to expectations, **JD-22** demonstrated equivalent potency to **JD-2** in TRα-mediated transactivation and slightly lower potency in TRβ-mediated transactivation, improving selectivity but providing no increase in potency at

either receptor (Table 2.1). While this was a useful result, a second modification was necessary to boost the potency to useful levels.

Ocasio had documented similar challenges in developing TRα-selective thyromimetics. [330] The initial compound CO-22 (Fig 2.1) used inner ring 3,5-dimethyl groups, but it suffered from inadequate efficacy and potency. [329] Replacing the 3,5-dimethyl groups of CO-22 with 3,5-diiodo substitutions to give CO-23 provided the necessary potency boost, but introduced a potential metabolic liability due to the activity of deiodinases *in vivo*. A third analog, CO-24, used 3,5-dibromo substitutions, and was less active than CO-23, but still more potent than CO-22.

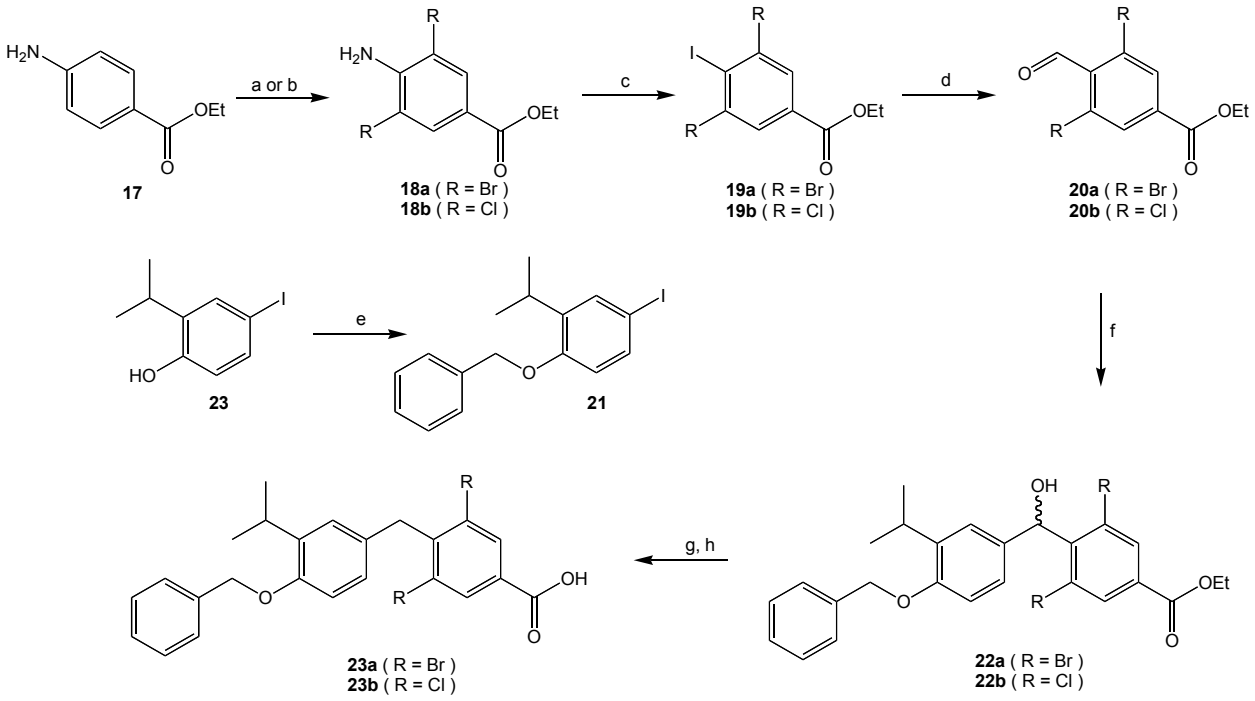

Figure 2.8

Synthesis of intermediates **23a** and **23b**. Reagents and conditions: (a) Br_2, DCM, RT; (b) $SOCl_2$, MeCN, 0° C; (c) (i) $NaNO_2$, HBF_4, AcOH, H_2O, RT (ii) KI, $CHCl_3$, H_2O, RT; (d) (i) iPrMgCl, THF, -40° C (ii) DMF, -40° C; (e) BnBr, TBAI, NaOH, DCM, H_2O, 55° C; (f) (i) **31**, iPrMgCl, THF, 0° C to RT (ii) **20a** or **20b**, -78° C; (g) TFA, Et_3SiH, DCM, 0° C to RT; (h) KOH, MeOH, THF, RT.

Introducing a 3,5-dibromo substitution into this series of thyromimetics required a new synthetic strategy. A report from Modrakowski in 2001 provided a route for producing the critical intermediate, ethyl 4-iodo-3,5-dibromobenzoate, from readily available ethyl 4-aminobenzoate, commonly known as benzocaine. [335] The original approach utilized a standard aqueous Sandmeyer reaction to convert ethyl 4-amino-3,5-dibromobenzoate into an aryl iodide, but was hindered by the low pKa of the protonated aniline limiting the solubility of the intermediate in aqueous acid. The issue was resolved by

adapting the method for diazonium formation reported by Filimonov in 2008, using acetic acid as the primary solvent and *p*-toluenesulfonic acid hydrate or aqueous tetrafluoroboric acid as the activating acid. [336] The resulting tosylate or tetrafluoroborate diazonium salts could be precipitated in a less polar solvent such as diethyl ether and collected by filtration, completely removing any organic soluble residual starting materials, side products, or solvents. Despite being stable enough to be purified in solid form, the diazonium salts were still active in standard Sandmeyer conditions and produced the appropriate aryl iodide in good yield and purity. This intermediate was transformed into a benzaldehyde using the same Grignard chemistry as before, followed by the same carbon-carbon bond formation, carbinol reduction, and deprotection to give the halogenated core. The glycine and β-alanine amides were synthesized using the same coupling methods and assayed.

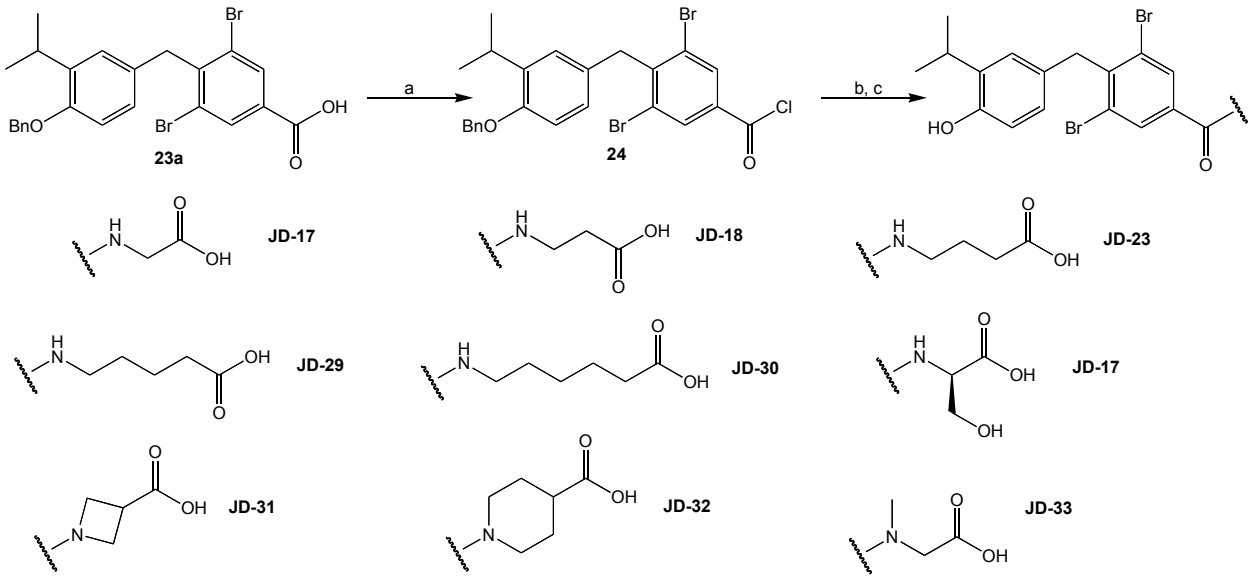

Figure 2.9

Synthesis of **JD-17** to **JD-33**. Reagents and conditions: (a) $(COCl)_2$, DCM, 0° C to RT; (b) Amine, DIEA, DCM, RT; (c) BCl_3, pentamethylbenzene, DCM, -78° C.

The combination of diaryl methane and 3,5-dibromo substitutions boosted the potency of **JD-17** and **JD-18** by roughly 10-fold in comparison to **JD-1** and **JD-2** while maintaining their TRα-selectivity (Table 2.1).

Additional analogs were synthesized to explore the structural basis of TRα-selectivity. Chain length was extended with γ-aminobutyric acid (**JD-23**), 5-aminovaleric acid (**JD-29**), and 6-aminohexanoic acid (**JD-30**) - these were found to have diminishing selectivity and activity in comparison to the glycine and β-alanine analogs. Because of the selectivity demonstrated by the D-serine amide **JD-4**, a D-serine amide was built on the dibromo core (**JD-19**) and found to have equal or better potency and selectivity than **JD-18**, suggesting

that the R-group makes an additional polar interaction in comparison to **JD-17**, boosting its selectivity.

These results set a limit on the maximum straight chain length, but analogs with cyclic amino acids displayed surprising results - the 3-carboxyazetidine (**JD-31**) and 4-carboxypiperidine (**JD-32**) analogs had nearly the same potency and selectivity as the glycine analog (**JD-17**). To test the role of the amide NH proton, the sarcosine analog (**JD-33**) was synthesized and found to have low potency and almost no selectivity, likely due to the *cis-trans* distribution of the amide.

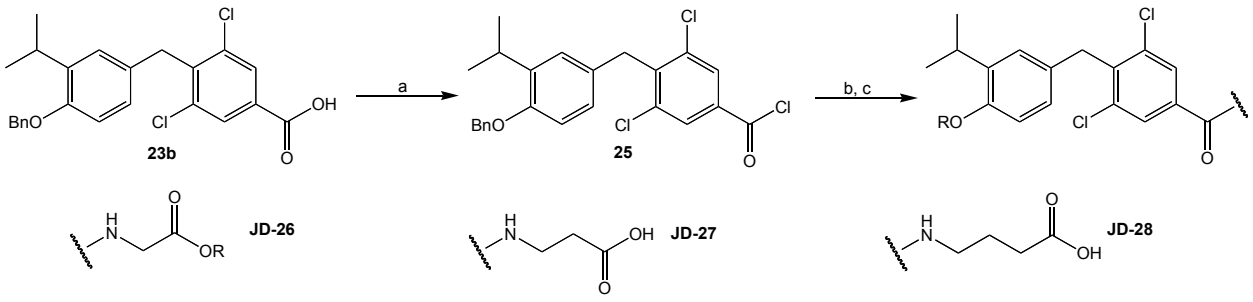

Figure 2.10

Synthesis of **JD-26** to **JD-28**. Reagents and conditions: (a) $(COCl)_2$, DCM, 0° C to RT; (b) amine, DIEA, DCM, RT; (c) BCl_3, pentamethylbenzene, DCM, -78° C.

A new core with inner ring chlorines instead of bromines was synthesized from the same ethyl 4-aminobenzoate starting material. Three amide analogs were built on this core - glycine (**JD-26**), β-alanine (**JD-27**), and γ-aminobutyric acid (**JD-28**) - and tested, demonstrating a significantly different pattern of transactivation activity. **JD-26** had roughly the same potency and

selectivity as **JD-17**, but **JD-27** and **JD-28** had significantly diminished potency and selectivity in comparison to **JD-18** and **JD-23**.

Compound	EC_{50} TRα	% Activation	EC_{50} TRβ	% Activation	Selectivity
JD-1	500 ±162	100	800 ±185	100	1.6
JD-2	1500 ±242	100	19000 ±7810	100	12.7
JD-13	1640 ±540	47	13200 ±4390	100	8.2
JD-14	5400 ±1930	44	N.D.	N.D.	N.D.
JD-22	2300 ±2990	100	51000	100	22.3
JD-17	48 ±72	100	327 ±75	100	6.5
JD-18	102 ±118	100	871 ±2000	100	8.5
JD-19	82 ±61	100	891 ±267	100	10.9
JD-23	226 ±39	100	272 ±62	100	1.2
JD-29	353 ±512	57	535 ±718	100	1.5
JD-30	241 ±381	51	155 ±388	100	0.6
JD-31	99 ±7	100	346 ±821	100	3.5
JD-32	129 ±88	100	443 ±526	100	3.4
JD-33	1070 ±755	100	3000 ±2860	100	2.7
JD-26	31 ±93	100	171 ±39	100	5.58
JD-27	>1000	N.D.	27300 ±62400	100	N.D.
JD-28	>1000	N.D.	37000 ±84600	100	N.D.

Table 2.1

Subtype selectivity measured by EC_{50} values from TRE-driven dual luciferase transactivation assays. EC_{50} values reported ±SEM in nM. Values from dose-response curves that did not plateau are reported as Not Determined (N.D.).

2.3 - Discussion

While TRβ-selective thyromimetics are extremely common within the literature, TRα-selective compounds are much more rare. While some of this is explained by TRβ being a far more important target for clinical therapeutics in conditions such as hypercholesterolemia, the combination of low hit rates during rational design efforts and the lack of TRα-selective ligands incidentally found

during screens and SAR studies suggest that it is inherently difficult to achieve tight binding to TRα with ligand structures that deviate from the natural hormone T3.

While there is no published crystal structure of TRs bound to a TRα-selective ligand to illuminate the factors governing selective binding and activation, crystal structures of the TRβ-selective thyromimetic GC-1 bound to both TR subtypes reported by Bleicher in 2008 provide some strong hints. [201] While most of the residues on the interior surface of the ligand binding pocket are not significantly changed between the two subtypes, there are critical differences in the arginine residues that interact with the carboxylic acid moiety of GC-1. The TRβ-ligand complex is found in a single productive conformation where Asn331 interacts with Arg282 to lock the arginine in a position where it can favorably interact with the ligand's carboxylic acid moiety. The TRα-ligand complex is found in multiple conformations, many of them unproductive where Arg228 has moved several angstroms away from the ligand's carboxylic acid and interacts instead with Ser277, which would significantly decrease the electrostatic interaction between the arginine and the ligand. Second, this suggests far greater conformational flexibility for Arg 228 in the TRα structure than Arg282 in the TRβ structure. While the TRβ structure is already in a productive conformation, allowing the ligand-receptor complex to gain the

maximal enthalpic benefit from the interaction, adopting the productive conformation in TRα will have an entropic cost from reducing the degrees of freedom for Arg 282 and an enthalpic cost by reducing its interaction with Ser277. These factors may limit the maximum favorable change in Gibb's free energy of a thyromimetic binding to TRα.

A second factor limiting the ability to develop TRα-selective ligands is the pocket that the 3' hydrophobic group of most thyromimetics fit into. Crystal structures with the TRβ-selective thyromimetic GC-24 demonstrated that the TRβ ligand-binding domain has greater conformational flexibility in this region and can accommodate much larger substitutions at this position, while the limited flexibility of the corresponding pocket in TRα reduces the binding affinity of ligands with large substituents at the 3' position. This creates a relatively simply method for producing TRβ-selective ligands by increasing the steric bulk at this position, increasing selectivity by decreasing the affinity for TRα. Conversely Ocasio found that smaller, more polarizable halogen substitutions at the 3' position marginally increased TRα-selectivity at a significant cost in potency. [330] This can be rationalized from X-ray crystal structures of 3,3',5-trioodothyronine (T3) bound to TRα and TRβ LBDs which show that the backbone carbonyl of G290 in TRα is 0.1 Å closer to the 3'-iodine than the backbone carbonyl of G344 in TRβ, suggesting a stronger halogen

bonding interaction with TRα than TRβ. [337] While a 3'-iodine may be one method for improving the TRα-selectivity, the loss of potency observed by Ocasio and the metabolic instability of that substitution make it unsuitable.

One of the most common themes in thyromimetic drug design is the outsized impact of small changes. Single atom changes can radically alter binding affinity and selectivity, indicating that much of the receptor binding pocket can only accommodate minor changes while still adopting a fully active conformation. This suggests that there may be a limit to the maximum potency that can be achieved for TRα-selective ligands, a suggestion that is supported by the continued absence of available TRα-selective ligands.

2.4 - Experimental

Chemistry

Chemistry General. ^{1}H NMR were taken on a Bruker 400. All ^{1}H NMR were calibrated to the NMR solvent reference peak (D_6-acetone, MeOD, D_2O, $CDCl_3$). Anhydrous tetrahydrofuran (THF) and dimethylformamide (DMF) were obtained from a Seca Solvent System. All other solvents used were purchased from Sigma-Aldrich or Fisher. Purity analysis of final compounds was determined to be >95% by HPLC. HPLC analysis was performed on a Varian ProStar HPLC with an Agilent Eclipse Plus C18 5 uM column (4.6 x 250 mm) with a gradient of 10% to 95% acetonitrile (0.1% TFA) over 15 minutes.

Methyl 4-hydroxy-3,5-dimethylbenzoate. 2 (4.98 g, 30 mmol) was dissolved in 125 mL of MeOH with 1 mL of H_2SO_4. The solution was refluxed at 80° C for 18 hr. Solvent was stripped under vacuum, then the crude was quenched with 25 mL of saturated $Na_2S_2O_3$. The aqueous layer was extracted 3X with EtOAc, then the organic fractions were combined, washed 2X with 20 mL of brine, dried with $MgSO_4$, filtered, and concentrated to give 5.05 g of **3**

(93% yield). 1H NMR (400 MHz, CDCl3) δ 7.72 (s, 2H); 5.06 (s, 1H); 3.90 (s, 3H); 2,78 (s, 6H).

4-bromo-2-isopropyl phenol. 4 (2.72 g, 20 mmol) was dissolved in 100 mL of DCM, then the solution was cooled to –20° C. Bromine (2.04 mL, 40 mmol) was added drop wise, waiting for color to disappear between additions. The reaction was quenched with 20 mL of saturated aqueous $Na_2S_2O_3$. The organic layer was washed 1X with 20 mL of water and 2X with 20 mL of brine, then dried with $MgSO_4$, filtered, and concentrated to give **5** in quantitative yield. 1H NMR (400 MHz, CDCl3) δ 7.29 (d, 1H); 7.18 (dd, 1H); 6.65 (d, 1H); 4.75 (s, 1H); 3.19 (m, 1H); 1.25 (d, 6H).

(4-bromo-2-isopropylphenoxy)triisopropylsilane. 5 (16.39 g, 76.2 mmol) and imidazole (15.56 g, 228.6 mmol) were dissolved in 125 mL of DCM. The solution was cooled to 0° C, then triisopropylsilyl chloride (27.7 mL, 129.54 mmol) was added. The reaction was stirred at RT for 3 hours. The reaction was diluted with 250 mL of Et_2O, washed 2X with 100 mL of 1 N aqueous HCl, 1X with 100 mL of saturated aqueous sodium bicarbonate, and 2X with 100 mL of brine, then dried with $MgSO_4$, filtered, and concentrated. The crude oil was purified by flash chromatography (silica gel, hexanes) to give **6** as a reddish oil. 1H NMR (400 MHz, CDCl3) δ 7.27 (d, 1H); 7.11 (dd, 1H); 6.63 (d, 1H); 3.31 (m, 1H); 1.27 (m, 3H); 1.20 (d, 6H); 1.12 (d, 18H).

4-(triisopropylsilyloxy)-3-isopropylphenylboronic acid. 6 (7.43 g, 20 mmol) was loaded into a flame-dried flask. 100 mL of dry THF were added, degassed, and flushed with argon, then the solution was cooled to –78° C. n-butyllithium (2.5M, 16 mL, 40 mmol) was added drop wise, then the lithium-halogen exchange was stirred for 1 hr at –78° C under argon. Triisopropylborate (9.2 mL, 40 mmol) was added, the reaction was stirred for 1 hr at –78°C, then quenched with 100 mL of 1N aqueous HCl. The aqueous layer was extracted 3X with 100 mL of EtOAc. The organic fractions were combined, washed 2X with 100 mL of brine, then dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was purified by flash chromatography (silica gel, DCM/methanol, 0-10%) to give 5.62 g of **7** as an off-white solid (90% yield).

Methyl 4-(4-(triisopropylsilyloxy)-3-isopropylphenoxy)-3,5-dimethylbenzoate. Vacuum dried **7** (0.36 g, 2 mmol), **3** (1.35 g, 4 mmol), and copper(II) acetate (0.36 g, 2 mmol) were added to a flame-dried flask with freshly activated 4 Å molecular sieves. 20 mL of dry DCM, pyridine (0.8 mL, 10 mmol), and DIEA (1.74 mL, 10 mmol) were added and degassed, then flushed with dry air. The reaction was stirred for 96 hours under dry air. The reaction was diluted with 60 mL of Et_2O, filtered through celite, washed 2X with 20 mL of 1N aqueous HCl and 2X with 20 mL of brine, then dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was purified by flash

chromatography (silica gel, hexanes/EtOAc, 0-20%) to give 439 mg of **8** as a clear oil (47% yield).

4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzoic acid. 8 (405 mg, 0.86 mmol) was dissolved in 20 mL of MeOH. Sodium fluoride (0.5 g, 8.6 mmol) and 10 M aqueous NaOH (0.86 mL, 8.6 mmol) were added, then the reaction was stirred for 24 hr at RT. The reaction was concentrated, redissolved in 10 mL of H2O, washed 2X with 10 mL of Et_2O, then acidified with 10 mL of 3 N aqueous HCl. The aqueous layer was acidified and the precipitate was collected by filtration and dried to give 232 mg of **9** as an off-white solid (82%). 1H NMR (400 MHz, MeOD) δ 7.73 (s, 2H); 6.56 (s, 2H); 6.21 (d, 1H); 3.19 (m, 1H); 2.09 (s, 6H); 1.09 (d, 6H).

Methyl 4-iodo-3,5-dimethylbenzoate. 10 (3 g, 20 mmol) was dissolved in 20 mL of H_2SO_4, then heated to 60° C. N-iodosuccinamide (5.4 g, 24 mmol) was added in three 1.8 g portions at 15 min intervals. The reaction was stirred for an additional 15 min, then poured over ice. The precipitated product was collected by filtration, washed with H2O followed by hexanes, then used without additional purification. 1H NMR (400 MHz, d4-methanol) δ 7.71 (s, 2H); 2.53 (s, 6H). The crude product was redissolved in 50 mL of MeOH. 0.5 mL of H_2SO_4 were added, then the solution was refluxed at 80° C for 18 hr. The reaction was concentrated, then redissolved in 50 mL of EtOAc. The organic

layer was washed 2X with 20 mL of saturated aqueous sodium bicarbonate and 2X with 20 mL of brine, then dried with $MgSO_4$, filtered, and concentrated to give 2.315 g of **11** as a tan solid (37% yield). 1H NMR (400 MHz, $CDCl_3$) δ 7.71 (s, 2H); 3.92 (s, 3H); 2.54 (s, 6H).

4-formyl-3,5-dimethylbenzoate. A flask was loaded with 0.5 g of 4Å molecular sieves then flame-dried under vacuum. After cooling under argon **11** (1.45 g, 5 mmol) was loaded, then the flask was sealed, evacuated, and flushed with argon. 20 mL of THF were added and degassed, then the solution was cooled to –15° C. iPMgCl in THF (2 M, 2.5 mL, 5 mmol) was added, then the reaction was stirred for 1 hr at RT. DMF (3.8 mL, 50 mmol) was added, then the reaction was stirred for 30 min at RT. The reaction was decanted into 20 mL of 1 N aqueous HCl, then the aqueous layer was extracted 3X with 20 mL of EtOAc. The organic fractions were combined, washed 2X with 20 mL of brine, then dried with $MgSO_4$, filtered, and concentrated. The crude was precipitated from cold hexanes to give 375 mg of **12** (39% yield). 1H NMR (400 MHz, $CDCl_3$) δ 10.67 (s, 1H); 7.77 (s, 2H); 3.96 (s, 3H); 2.66 (s, 6H).

4-iodo-2-isopropylphenol. 4 (6.8g, 50 mmol) and NaI (7.5 g, 50 mmol) were dissolved in 70 mL of MeOH. 10 M aqueous NaOH (5 mL, 50 mmol) was added, then the solution was cooled to 0° C. 6.25% w/v aqueous NaOCl (62.5 mL, 50 mmol) was added drop wise over 24 hr at 0° C. The reaction was

acidified to pH 7 with 12 N aqueous HCl, then quenched with 10 mL of saturated aqueous $Na_2S_2O_3$. The aqueous layer was extracted 3X with Et_2O. The organic fractions were combined, washed 2X with brine, then dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was purified by flash chromatography (silica gel, hexane/ethyl acetate, 1-20%) to give 11.35 g of **13** (87% yield) as a reddish oil. 1H NMR (400 MHz, $CDCl_3$) δ 7.47 (d, 1H); 7.36 (dd, 1H); 6.54 (d, 1H); 4.78 (s, 1H); 3.16 (m, 1H); 1.25 (d, 6H).

4-iodo-2-isopropyl-1-(methoxymethoxy)benzene. 13 (2.62 g, 10 mmol) and tetrabutylammonium iodide (369 mg, 1 mmol) were dissolved in 100 mL of DCM. 10 mL of 10 M aqueous NaOH were added, followed by 5 mL of 6 M chloromethyl methyl ether in MeOAc. The reaction was stirred for 30 min at RT, then diluted with 200 mL of Et_2O. The organic layer was washed 2X with 100 mL of H2O and 2X with 100 mL of brine, then dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was purified by flash chromatography (silica gel, hexane/ethyl acetate, 1-20%) to give 2.48 g of **14** as an oil. 1H NMR (400 MHz, $CDCl_3$) δ 7.47 (d, 1 H); 7.41 (dd, 1H); 6.83 (d, 1H); 5.12 (s, 2H); 3.47 (1, 3H); 3.27 (m, 1h); 1.20 (d, 6H).

Methyl 4-(hydroxy(3-isopropyl-4-(methoxymethoxy)phenyl)methyl)-3,5-dimethylbenzoate. A flask was loaded with 4 Å molecular sieves and flame-dried under vacuum. **14** (0.61 g, 2 mmol) was loaded and the flask was sealed,

evacuated, and flushed with argon. 8 mL of dry THF were added and degassed, then the solution was cooled to 0° C. Isopropylmagnesium chloride (2 M, 1.5 mL, 3 mmol) was added, then the reaction was stirred for 2 hours at RT. A second flask was loaded with 4 Å molecular sieves and and flame-dried under vacuum. **12** (0.2 g, 1 mmol) was loaded and the flask was sealed, evacuated, and flushed with argon. 4 mL of dry THF were added and degassed. The arylmagnesium solution was cooled to –78° C, then the benzaldehyde solution was added drop wise via cannula and the reaction was stirred for 1 hour at –78° C. The reaction was quenched with 10 mL of 1 N aqueous HCl. 10 mL of Et_2O were added. The organic layer was washed 1X with 10 mL of aqueous HCl and 2X with 10 mL of brine. The organic layer was dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was purified by flash chromatography (silica gel, hexanes/EtOAc 2-20%) to give 127 mg of **15** as a clear oil (34% yield). 1H NMR (400 MHz, $CDCl_3$) δ 7.72 (s, 2H); 7.22 (d, 1H); 6.97 (d, 1H); 6.88 (dd, 1H); 6.34 (d, 1H); 3.93 (s, 3H); 3.50 (s, 2H); 3.33 (m, 1H); 2.35 (s, 6H); 1.20 (t, 6H).

4-(4-hydroxy-3-isopropylbenzyl)-3,5-dimethylbenzoic acid. 15 (127 mg, 0.34 mmol) was dissolved in 1 mL of DCM. Triethylsilane (0.54 mL, 3.4 mmol) was added, then the solution was cooled to 0° C. Trifluoroacetic acid (0.26 mL, 3.4 mmol) was added, then the reaction was stirred for 1 hour at RT.

The reaction was concentrated, then dried under vacuum. The crude product was redissolved in 3 mL of THF, then 0.68 mL of 5 M potassium hydroxide in MeOH were added. The reaction was stirred for 1 hour at RT, then concentrated. The crude was redissolved in 10 mL of H2O, washed 2X with 5 mL of Et_2O, then acidified with 5 mL of 3 N aqueous HCl. The precipitate was collected by filtration, then dried under vacuum to give 73 mg of **16** (72% yield). 1H NMR (400 MHz, MeOD) δ 7.72 (s, 2H); 6.84 (s, 1H); 6.61 (d, 1H); 6.52 (d, 1H); 4.03 (s, 2H); 3.22 (m, 1H); 2.30 (s, 6H); 1.14 (d, 6H).

General procedure for EDC•HCl mediated amide coupling. Starting benzoic acid, EDC•HCl (31 mg, 0.2 mmol), HOBt (27 mg, 0.2 mmol), and DIEA (104 µl, 0.6 mmol) were dissolved in 1 mL of DMF. The reaction was stirred for 1 hr at RT, then amine (0.2 mmol) was added and the reaction was stirred for an additional 18 hr. The reaction was diluted with 10 mL of EtOAc, washed 2X with 5 mL of 1 N aqueous HCl, 1X with 5 mL of saturated aqueous sodium bicarbonate, and 2X with 5 mL of brine, then dried with $MgSO_4$, filtered, and concentrated.

General procedure for ester deprotection. Alkyl ester (0.1 mmol) was dissolved in 1 mL of MeOH. 1 mL of 1 M LiOH in MeOH was added, then the reaction was stirred for 2 hr at RT. The reaction was decanted into vigorously

stirring Et_2O at 0° C and the precipitate was collected by filtration then dried under vacuum.

Lithium 2-(4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamido)acetate. 18 mg of **JD-1**, 50% yield. 1H NMR (400 MHz, MeOD) δ 7.60 (s, 2H); 6.56 (m, 2H); 6.24 (dd, 1H); 3.96 (s, 2H); 3.18 (m, 1H); 2.10 (s, 6H); 1.08 (d, 6H).

Lithium 2-(4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamido)propanoate. 36 mg of **JD-2**, 95% yield. 1H NMR (400 MHz, MeOD) δ 7.53 (s, 2H); 6.55 (m, 2H); 6.23 (dd, 1H); 3.58 (t, 2H); 3.18 (m, 1H); 2.57 (t, 2H); 2.09 (s, 6H); 1.08 (d, 6H).

(*S*)-lithium 2-(4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamido)-3-hydroxypropanoate. 37 mg of **JD-3**, 97% yield. 1H NMR (400 MHz, MeOD) δ 7.61 (s, 2H); 6.55 (m, 2H); 6.23 (dd, 1H); 4.47 (s, 1H); 3.92 (s, 2H); 3.17 (m, 1H); 2.09 (s, 6H); 1.07 (d, 6H).

(*R*)-lithium 2-(4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamido)-3-hydroxypropanoate. 33 mg of **JD-4**, 84% yield. 1H NMR (400 MHz, MeOD) δ 7.70 (s, 2H); 6.63 (d, 2H); 6.31 (dd, 1H); 4.53 (m, 1H); 3.96 (d, 2H); 2.17 (s, 6H); 1.15 (d, 6H).

Lithium 5-(4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamido)benzene-1,3-dioate. 7 mg of **JD-5**, 15% yield. 1H NMR

(400 MHz, MeOD) δ 8.60 (d, 2H); 8.38 (t, 1H); 7.71 (s, 2H); 6.59 (m, 2H); 6.28 (dd, 1H); 3.19 (m, 1H); 2.15 (s, 6H); 1.10 (d, 6H).

3-(4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamido)-1,2,4-triazole. 13 mg of **JD-6** (35% yield). 1H NMR (400 MHz, $CDCl_3$) δ 7.95 (s, 2H); 7.55 (s, 1H); 7.01 (s, 2H); 6.78 (d, 1H); 6.59 (d, 1H); 6.26 (dd, 1H); 3.32 (m, 1H); 2.19 (s, 6H); 1.09 (d, 6H).

(*S*)-lithium 2-(4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamido)pentanedioate. 44 mg of **JD-7** (quant yield). 1H NMR (400 MHz, MeOD) δ 7.09 (s, 2H); 5.95 (d, 1H); 5.91 (d, 1H); 5.63 (dd, 1H); 3.87 (m, 1H); 2.92 (m, 1H); 1.81 (m, 2H); 1.71 (m, 2H); 1.61 (s, 6H); 0.52 (d, 6H).

(*R*)-lithium 2-(4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamido)pentanedioate. 44 mg of **JD-8** (quant yield). 1H NMR (400 MHz, MeOD) δ 7.41 (s, 2H); 6.27 (d, 1H); 6.22 (d, 1H); 5.95 (dd, 1H); 4.19 (m, 1H); 3.18 (m, 1H); 2.09 (m, 2H); 2.04 (m, 2H); 1.92 (s, 6H); 0.84 (d, 6H).

(*S*)-lithium 2-(4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamido)-3-(3,5-dichloro-4-hydroxyphenyl)propanoate. 7 mg of **JD-9** (13% yield). 1H NMR (400 MHz, MeOD) δ 7.36 (s, 2H); 6.87 (s, 2H);

6.40 (d, 1H); 6.35 (d, 1H); 6.07 (dd, 1H); 4.42 (m, 1H); 2.95 (m, 1H); 2.79 (m, 2H); 2.00 (s, 6H); 0.95 (d, 6H).

***N*-(3,5-dichloro-4-hydroxyphenethyl)-4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamide.** 21 mg of **JD-10** (42% yield). 1H NMR (400 MHz, $CDCl_3$) δ 7.43 (s, 2H); 7.15 (s, 2H); 6.72 (d, 1H); 6.59 (d, 1H); 6.28 (t, 1H); 6.20 (dd, 1H); 3.64 (q, 2H); 3.21 (m, 1H); 2.83 (t, 2H); 2.12 (s, 6H); 1.21 (d, 6H).

4-(4-hydroxy-3-isopropylphenoxy)-*N*-(2-hydroxyethyl)-3,5-dimethylbenzamide. 2.27 mg of **JD-13** (7% yield). 1H NMR (400 MHz, $CDCl_3$) δ 7.50 (s, 2H); 6.84 (t, 1H); 6.70 (d, 1H); 6.56 (d, 1H); 6.17 (dd, 1H); 6.02 (s, 1H); 3.82 (t, 2H); 3.62 (m, 2H); 3.20 (m, 1H); 2.09 (s, 6H); 1.18 (d, 6H).

4-(4-hydroxy-3-isopropylphenoxy)-*N*-(1,3-dihydroxypropan-2-yl)-3,5-dimethylbenzamide. 2.11 mg of **JD-14** (6% yield). 1H NMR (400 MHz, MeOD) δ 7.60 (s, 2H); 6.59 (d, 1H); 6.55 (d, 1H); 6.27 (dd, 1H); 4.13 (t, 1H); 3.71 (d, 4H); 3.21 (m, 1H); 2.12 (s, 6H); 1.09 (d, 6H).

Lithium 4-(4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamido)-3-hydroxybutanoate. 2.3 mg of **JD-15** (6% yield). 1H NMR (400 MHz, MeOD) δ 7.56 (s, 2H); 6.54 (m, 2H); 6.23 (dd, 1H); 4.19 (m, 1H); 3.42 (m, 2H); 3.16 (m, 1H); 2.51 (m, 2H); 2.09 (s, 6H); 1.07 (d, 6H).

(*S*)-lithium 2-(4-(4-hydroxy-3-isopropylphenoxy)-3,5-dimethylbenzamido)-3-(3,5-dichloro-4-hydroxyphenyl)butanoate. 43 mg of **JD-16** (quant yield). 1H NMR (400 MHz, MeOD) δ 7.41 (s, 2H); 6.27 (d, 1H); 6.22 (d, 1H); 5.95 (dd, 1H); 4.19 (m, 1H); 3.18 (m, 1H); 2.11 (m, 2H); 1.92 (s, 6H); 0.84 (d, 6H).

Lithium 3-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dimethylbenzamido)propanoate. 23 mg of **JD-22** (78% yield). 1H NMR (400 MHz, D_2O) δ 7.35 (s, 2H); 6.77 (s, 1H); 6.46 (m, 2H); 3.87 (s, 2H); 3.46 (t, 2H); 3.08 (m, 1H); 2.38 (t, 2H); 2.18 (s, 6H); 0.94 (d, 6H).

Ethyl 3,5-dibromo-4-formylbenzoate. 17 (16.5 g, 0.1 mol) was dissolved in 400 mL of DCM, then cooled to 0° C. Bromine (15.4 mL, 0.3 mol) was added, then the reaction was stirred for 30 min at RT. The resulting precipitate was collected by filtration and washed with DCM, then dried under vacuum to give **18a** in quantitative yield. 1H NMR (400 MHz, $CDCl_3$) δ 8.10 (s, 2H); 5.00 (s, 2H); 4.35 (q, 2H); 1.39 (t, 3H). **18a** (32.3 g, 0.1 mol) was dissolved in 200 mL of AcOH with aqueous tetrafluoroboric acid (5.7 M, 53 mL, 0.3 mol). Solid sodium nitrite (10.3 g, 0.15 mol) was added over 1 hour with significant gas evolution until the starting aniline had been consumed. The reaction was decanted into 1.6 L of Et_2O at 0° C to precipitate the diazonium tris-tetrafluoroborate salt, which was collected by filtration and washed with Et_2O,

then dried under vacuum to give a pale yellow solid (46.9 g, 78% yield). 1H NMR (400 MHz, D_2O) δ 8.61 (s, 2H); 4.35 (q, 2H); 1.28 (t, 3H). Potassium iodide (104.6 g, 0.63 mol) was dissolved in 630 mL of water, then 630 mL of DCM were added to form a biphasic mixture. Solid diazonium tetrafluoroborate was added, then the reaction was stirred for 18 hours at RT. The reaction was diluted with 1.2 L of Et_2O, washed 2X with 200 mL of saturated aqueous $Na_2S_2O_3$, and 2X with 200 mL of brine, then dried with $MgSO_4$, filtered, and concentrated. The crude solid was recrystallized from boiling EtOH to give 19.9 g of **19a** as red-orange needles (58% yield). 1H NMR (400 MHz, $CDCl_3$) δ 8.18 (s, 2H); 4.40 (q, 2H); 1.42 (t, 3H). A flask was loaded with 4 Å molecular sieves and flame-dried under vacuum. **19a** (2.17 g, 5 mmol) was loaded and the flask was sealed, evacuated, and flushed with argon. 25 mL of dry THF were added and degassed, then the solution was cooled to –40° C. Isopropylmagnesium chloride in THF (2 M, 5 mL, 10 mmol) was added, then the reaction was stirred for 1 hour at –40° C. Dry DMF (1.9 mL, 25 mmol) was added, then the reaction was stirred for 1 hour at –40° C. The reaction was decanted into 25 mL of 1 N aqueous HCl. The aqueous layer was extracted 2X with 25 mL of Et_2O, then the organic fractions were combined, washed 2X with 25 mL of brine, dried with $MgSO_4$, filtered, and concentrated. The crude was resuspended in hexanes, then cooled to –20° C for 18 hours to precipitate **20a**, which was collected by

filtration and dried to give 1.378 g (41% yield). 1H NMR (400 MHz, $CDCl_3$) δ 10.27 (s, 1H); 8.28 (s, 2H); 4.44 (q, 2H); 1.44 (t, 3H).

1-((4-iodo-2-isopropylphenoxy)methyl)benzene. 13 (9.17 g, 35 mmol) and TBAI (1.29 g, 3.5 mmol) were dissolved in 175 mL of DCM. 35 mL of 10 M aqueous NaOH was added, followed by benzyl bromide (4.16 mL, 35 mmol), then the reaction was refluxed at 50° C for 3 hours. The reaction was diluted with 350 mL of Et2O, washed 1X with 10 mL of 1 N aqueous HCl, 1X with 100 mL of saturated aqueous $Na_2S_2O_3$, and 2X with 100 mL of brine, then dried with $MgSO_4$, filtered, and concentrated. The crude oil was purified by flash chromatography (silica gel, hexanes/DCM 6-50%) to give 8.9 g of **21** as and orange solid (72% yield). 1H NMR (400 MHz, CDCl3) δ 7.45 (m, 7 H); 6.68 (d, 1H); 5.08 (s, 2H); 3.36 (m, 1H); 1.23 (d, 6H).

Ethyl 4-((4-(benzyloxy)-3-isopropylphenyl)(hydroxy)methyl)-3,5-dibromobenzoate. A flask was loaded with 4 Å molecular sieves and flame-dried under vacuum. **21** (2.82 g, 8 mmol) was loaded and the flask was sealed, evacuated, and flushed with argon. 32 mL of dry THF were added and degassed, then the solution was cooled to 0° C. Isopropylmagnesium chloride (2 M, 6 mL, 12 mmol) was added, then the reaction was stirred for 2 hours at RT. A second flask was loaded with 4 Å molecular sieves and and flame-dried under vacuum. **20a** (1.34 g, 4 mmol) was loaded and the flask was sealed, evacuated,

and flushed with argon. 16 mL of dry THF were added and degassed. The arylmagnesium solution was cooled to –78° C, then the benzaldehyde solution was added drop wise via cannula and the reaction was stirred for 1 hour at –78° C. The reaction was quenched with 50 mL of 1 N aqueous HCl. 100 mL of Et_2O were added. The organic layer was washed 1X with 50 mL of aqueous HCl and 2X with 50 mL of brine. The organic layer was dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was purified by flash chromatography (silica gel, hexanes/EtOAc 2-20%) to give 1 g of **22a** as a clear oil (44% yield). 1H NMR (400 MHz, $CDCl_3$) δ 8.26 (s, 2H); 7.40 (m, 5H); 7.26 (d, 1H); 6.92 (dd, 1H); 6.84 (d, 1H); 6.63 (d, 1H); 5.08 (s, 2H); 4.42 (q, 2H); 3.51 (d, 1H); 3.41 (m, 1H); 1.44 (t, 3H); 1.22 (t, 6H).

4-(4-(benzyloxy)-3-isopropylbenzyl)-3,5-dibromobenzoic acid. 22a (1 g, 1.8 mmol) was dissolved in 18 mL of DCM. Triethylsilane (0.57 mL, 3.6 mmol) was added, then the solution was cooled to 0° C. Trifluoroacetic acid (1.38 mL, 18 mmol) was added drop wise, then the reaction was stirred for 1.5 hours at 0° C. The reaction was concentrated under vacuum to give the product in quantitative yield. 1H NMR (400 MHz, $CDCl_3$) δ 8.23 (s, 2H); 7.4 (m, 5H); 7.15 (d, 1H); 6.89 (dd, 1H); 6.79 (d, 1H); 5.05 (s, 2H); 4.40 (m, 4H); 3.38 (m, 1H); 1.42 (t, 3H); 1.22 (d, 6H). The intermediate (1.8 mmol) was redissolved in 9 mL of THF then 3.6 mL of 10 M KOH in MeOH (18 mmol) were added. The

reaction was stirred for 1 hr at RT then concentrated. The crude was redissolved in a minimal amount of H2O then acidified with aqueous HCl and the precipitate was collected by filtration. The off-white solid was dried under vacuum to give 752 mg of **23a** (80% yield). 1H NMR (400 MHz, d_6-acetone) δ 8.23 (s, 2H); 7.50 (d, 2H); 7.42 (t, 2H); 7.33 (t, 1H); 7.19 (d, 1H); 6.92 (m, 2H); 5.11 (s, 2H); 4.44 (s, 2H); 3.35 (m, 1H); 1.19 (d, 6H).

Ethyl 3,5-chloro-4-formylbenzoate. 17 (16.5 g, 0.1 mol) was dissolved in 400 mL of MeCN, then cooled to 0° C. Sulfuryl chloride (16.2 mL, 0.2 mol) was added, then the reaction was stirred to RT over 18 hours. The resulting precipitate was collected by filtration. The filtrate was concentrated and additional product was recrystallized from EtOH/water at 0° C. The combined product was dried under vacuum to give 7.553 g of **18b** (32% yield). **18b** (7.02 g, 30 mmol) was dissolved in 60 mL of AcOH with aqueous tetrafluoroboric acid (5.7 M, 15.8 mL, 90 mmol). Solid sodium nitrite (3.1 g, 45 mmol) was added over 1 hour with significant gas evolution until the starting aniline had been consumed. The reaction was decanted into 480 mL of Et_2O at 0° C to precipitate the diazonium tetrafluoroborate salt, which was collected by filtration and washed with Et_2O, then dried under vacuum to give a pale yellow solid (13.96 g, 92% yield). 1H NMR (400 MHz, D_2O) δ 8.46 (s, 2H); 4.37 (q, 2H); 1.29 (t, 3H). Potassium iodide (49.8 g, 0.3 mol) was dissolved in 300 mL of

water, then 300 mL of chloroform were added to form a biphasic mixture. Solid diazonium tetrafluoroborate was added, then the reaction was stirred for 18 hours at RT. The reaction was diluted with 600 mL of Et_2O, washed 2X with 100 mL of saturated aqueous $Na_2S_2O_3$, and 2X with 100 mL of brine, then dried with $MgSO_4$, filtered, and concentrated. The crude solid was recrystallized from boiling EtOH to give **19b** as red-orange needles. 1H NMR (400 MHz, $CDCl_3$) δ 7.98 (s, 2H); 4.40 (q, 2H); 1.42 (t, 3H). A flask was loaded with 4 Å molecular sieves and flame-dried under vacuum. **19b** (5 mmol) was loaded and the flask was sealed, evacuated, and flushed with argon. 25 mL of dry THF were added and degassed, then the solution was cooled to –40° C. Isopropylmagnesium chloride in THF (2 M, 5 mL, 10 mmol) was added, then the reaction was stirred for 1 hour at –40° C. Dry DMF (1.9 mL, 25 mmol) was added, then the reaction was stirred for 1 hour at –40° C. The reaction was decanted into 25 mL of 1 N aqueous HCl. The aqueous layer was extracted 2X with 25 mL of Et_2O, then the organic fractions were combined, washed 2X with 25 mL of brine, dried with $MgSO_4$, filtered, and concentrated. The crude was resuspended in hexanes, then cooled to –20° C for 18 hours to precipitate the product. The off-white solid was collected and dried under vacuum to give 0.94 g of **20b** (76% yield) 1H NMR (400 MHz, $CDCl_3$) δ 10.51 (s, 1H); 8.04 (s, 2H); 4.44 (q, 2H); 1.44 (t, 3H).

Ethyl 4-((4-(benzyloxy)-3-isopropylphenyl)(hydroxy)methyl)-3,5-dichlorobenzoate. A flask was loaded with 4 Å molecular sieves and flame-dried under vacuum. **21** (1.76 g, 5 mmol) was loaded and the flask was sealed, evacuated, and flushed with argon. 25 mL of dry THF were added and degassed, then the solution was cooled to 0° C. Isopropylmagnesium chloride (2 M, 3.75 mL, 7.5 mmol) was added, then the reaction was stirred for 2 hours at RT. A second flask was loaded with 4 Å molecular sieves and and flame-dried under vacuum. **20b** (0.62 g, 2.5 mmol) was loaded and the flask was sealed, evacuated, and flushed with argon. 12.5 mL of dry THF were added and degassed. The arylmagnesium solution was cooled to –78° C, then the benzaldehyde solution was added drop wise via cannula and the reaction was stirred for 1 hour at –78° C. The reaction was quenched with 25 mL of 1 N aqueous HCl. The aqueous layer was extracted 3X with 5 mL of EtOAc. The organic fractions were combined and washed 2X with 55 mL of brine. The organic layer was dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was purified by flash chromatography (silica gel, hexanes/EtOAc 2-20%) to give 848 mg of **21b** as a clear oil (72% yield). 1H NMR (400 MHz, $CDCl_3$) δ 8.02 (s, 2H); 7.43 (m, 6H); 6.94 (d, 1H); 6.84 (d, 1H); 6.62 (d, 1H); 5.08 (s, 2H); 4.42 (q, 2H), 3.41 (m, 2H); 1.45 (t, 3H); 1.22 (t, 6H).

4-(4-(benzyloxy)-3-isopropylbenzyl)-3,5-dichlorobenzoic acid. 21b (0.85 g, 1.8 mmol) was dissolved in 18 mL of DCM. Triethylsilane (0.57 mL, 3.6 mmol) was added, then the solution was cooled to 0° C. Trifluoroacetic acid (1.38 mL, 18 mmol) was added drop wise, then the reaction was stirred for 1.5 hours at 0° C. The reaction was concentrated under vacuum to give 0.54 g of product (74% yield). 1H NMR (400 MHz, $CDCl_3$) δ 8.00 (s, 2H); 7.42 (m, 5H); 7.18 (d, 1H); 6.94 (dd, 1H); 6.80 (d, 1H); 5.05 (s, 2H); 4.40 (q, 2H); 4.32 (s, 2H); 3.37 (m, 1H); 1.42 (t, 3H); 1.22 (d, 6H). The intermediate (0.61 g, 1.33 mmol) was dissolved in 6.6 mL of THF, then 2.66 mL of 10 M KOH in MeOH (13.28 mmol) was added to the reaction, which was stirred for 1 hr. The reaction was concentrated, redissolved in 20 mL of H2O, then acidified with 3 N aqueous HCl. The aqueous fraction was extracted 3X with 10 mL of EtOAc. The organic fractions were combined, washed 2X with 5 mL of brine, then dried with $MgSO_4$, filtered, and concentrated. The white solid was dried under vacuum to give 0.54 g of **23b** (95% yield). 1H NMR (400 MHz, d_6-acetone) δ 8.01 (s, 2H), 7.51 (d, 2H); 7.41 (t, 2H); 7.39 (t, 1H); 7.21 (s, 1H); 6.94 (d, 2H); 5.11 (s, 2H); 4.35 (s, 2H); 3.36 (m, 1h); 1.19 (d, 6H).

General procedure for acid chloride formation. Starting benzoic acid (**23a** or **23b**) (0.1 mmol) was resuspended in 2 mL of DCM. 2 µL of DMF were added, then the mixture was cooled to 0° C. Oxalyl chloride (0.4 mmol) was

added, then the reaction was stirred for 1 hr at RT. The reaction was concentrated, redissolved in hexanes, filtered through glass wool, concentrated again, then dried under vacuum to remove residual oxalyl chloride.

4-(4-(benzyloxy)-3-isopropylbenzyl)-3,5-dibromobenzoyl chloride. 53 mg of **72** (99% yield). 1H NMR (400 MHz, $CDCl_3$) δ 8.30 (s, 2H); 7.39 (m, 5H); 7.16 (d, 1H); 6.89 (dd, 1H); 6.80 (d, 1H); 5.06 (s, 2H); 4.44 (s, 2H); 3.39 (m, 1H); 1.23 (d, 6H).

4-(4-(benzyloxy)-3-isopropylbenzyl)-3,5-dichlorobenzoyl chloride. 44 mg of **71** (98% yield). 1H NMR (400 MHz, $CDCl_3$) δ 8.10 (s, 2H); 7.41 (m, 5H); 7.19 (d, 1H); 6.94 (dd, 1H); 6.80 (d, 1H); 5.06 (s, 2H); 4.35 (s, 2H); 3.39 (m, 1H); 1.23 (d, 6H).

General procedure for acid chloride amide coupling. The acid chloride intermediate was redissolved in 2 mL of DCM with DIEA (0.1 mL, 0.6 mmol). Amine (0.2 mmol) was added, then the reaction was stirred for 18 hr at RT, diluted with 10 mL of Et_2O, washed 2X with 5 mL of 1 N aqueous HCl, 1X with 5 mL of saturated aqueous sodium bicarbonate, and 2X with 5 mL of brine, then dried with $MgSO_4$, filtered, and concentrated to give the amide product.

General procedure for benzyl deprotection. A flask was loaded with molecular sieves, then flame-dried under vacuum. After cooling under argon, starting benzyl ether (0.1 mmol) and pentamethylbenzene (0.6 mmol) were

loaded in 7 mL of dry DCM. The solution was degassed, then cooled to -78° C. 1 M BCl_3 in DCM (0.6 mL, 0.6 mmol) was added then the reaction was stirred for 30 min at -78° C under argon. 5 mL of 1 M aqueous sodium hydroxide were added to quench the reaction. The aqueous layer was washed 2X with 5 mL of EtOAc, acidified with 5 mL of 3 N aqueous HCl, then extracted 3X with 5 mL of EtOAc. The organic fractions were combined, washed 2X with 5 mL of brine, then dried with $MgSO_4$, filtered, and concentrated to give the product.

2-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dibromobenzamido) acetic acid. 28 mg of **JD-17** (58% yield). 1H NMR (400 MHz, d_6-acetone) δ 8.35 (t, 1H); 8.19 (s, 2H); 7.09 (d, 1H); 6.75 (m, 2H); 4.36 (s, 2H); 4.13 (d, 2H); 3.27 (m, 1H); 1.17 (d, 6H).

2-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dibromobenzamido) propionic acid. 28 mg of **JD-18** (56% yield). 1H NMR (400 MHz, $CDCl_3$) δ 7.95 (s, 2H); 7.10 (d, 1H); 6.80 (m, 2H); 6.63 (d, 1H); 4.37 (s, 2H); 3.74 (q, 2H); 3.18 (m, 1H); 2.75 (t, 2H); 1.25 (d, 6H).

2-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dibromobenzamido) butanoic acid. 10 mg of **JD-23** (19% yield). 1H NMR (400 MHz, $CDCl_3$) δ 7.96 (s, 2H); 7.11 (d, 1H); 6.80 (dd, 1H); 6.64 (d, 1H); 6.57 (t, 1H); 4.37 (s, 2H); 3. 54 (q, 2H); 3.18 (m, 1H); 2.50 (t, 2H); 2.00 (m, 2H); 1.23 (d, 6H).

2-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dibromobenzamido) pentanoic acid. 24 mg of **JD-29** (45% yield). 1H NMR (400 MHz, d_6-acetone) δ 8.15 (s, 2H); 8.02 (t, 1H); 7.09 (d, 1H); 6.75 (m, 2H); 4.35 (s, 2H); 3.43 (m, 2H); 3.27 (m, 1H); 2.35 (m, 2H); 1.67 (m, 4H); 1.18 (d, 6H).

2-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dibromobenzamido) hexanoic acid. 30 mg of **JD-30** (55% yield). 1H NMR (400 MHz, $CDCl_3$) δ 7.95 (s, 2H); 7.15 (d, 1H); 6.88 (dd, 1H); 6.79 (d, 1H); 6.27 (t, 1H); 4.40 (s, 2H); 3.49 (q, 2H); 3.38 (m, 1H); 2.38 (t, 2H); 1.69 (m, 4H); 1.45 (m, 2H); 1.22 (d, 6H).

(*R*)-2-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dibromobenzamido)-3-hydroxypropanoaic acid. 19 mg of **JD-19** (36% yield). 1H NMR (400 MHz, D_2O) δ 8.00 (s, 2H); 6.87 (s, 1H); 6.68 (m, 1H); 6.40 (dd, 1H); 4.35 (m, 1H); 4.20 (s, 2H); 3.84 (m, 2H); 3.11 (m, 1H); 0.94 (d, 6H).

1-(3,5-dibromo-4-(4-hydroxy-3-isopropylbenzyl)benzoyl)azetidine-3-carboxylic acid. 14 mg of **JD-31** (27% yield) 1H NMR (400 MHz, $CDCl_3$) δ 7.85 (s, 2H); 7.17 (d, 1H); 6.90 (dd, 1H); 6.80 (d, 1H); 4.43 (m, 4H); 3.55 (m, 1H); 3.39 (m, 1H); 1.22 (d, 6H).

1-(3,5-dibromo-4-(4-hydroxy-3-isopropylbenzyl)benzoyl)piperidine-4-carboxylic acid. 41 mg of **JD-32** (76% yield) 1H NMR (400 MHz, $CDCl_3$) δ

2-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dibromo-*N*-methylbenzamido)acetic acid. 19 mg of **JD-33** (38% yield). 1H NMR (400 MHz, $CDCl_3$) δ

2-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dichlorobenzamido)acetic acid. 34 mg of **JD-26** (86% yield). 1H NMR (400 MHz, d_6-acetone) δ 8.30 (t, 1H); 8.16 (s, 1H); 7.97 (s, 2H); 7.39 (m, 10H); 7.11 (d, 1H); 6.80 (dd, 1H); 6.72 (d, 1H); 4.28 (s, 2H); 4.16 (d, 2H); 3.38 (m, 1H); 1.19 (d, 6H).

2-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dichlorobenzamido) propionic acid. 17 mg of **JD-27** (41% yield). 1H NMR (400 MHz, d_6-acetone) δ 8.15 (s, 1H); 8.06 (s, 1H); 7.93 (s, 2H); 7.11 (d, 1H); 6.79 (dd, 1H); 6.71 (d, 1H); 4.26 (s, 2H); 3.44 (q, 2H); 3.26 (m, 1H); 2.67 (t, 2H); 1.17 (d, 6H).

2-(4-(4-hydroxy-3-isopropylbenzyl)-3,5-dichlorobenzamido) butanoic acid. 12 mg of **JD-28** (28% yield). 1H NMR (400 MHz, d_6-acetone) δ 8.15 (s, 1H); 8.04 (s, 1H); 7.94 (s, 2H); 7.12 (d, 1H); 6.79 (dd, 1H); 6.72 (d, 1H); 4.26 (s, 2H); 3.45 (m, 2H); 3.38 (m, 1H); 2.43 (t, 2H); 190 (t, 2H); 1.22 (d, 6H).

General procedure for amino acid benzyl ester protection. Starting amino acid (2 mmol) was dissolved in 6 mL of benzyl alcohol. Trimethylsilyl chloride (0.76 mL, 6 mmol) was added then the reaction was refluxed at 100° C for 18 hr. The reaction was decanted into 40 mL of vigorously stirring hexanes

at 0° C to precipitate the product, which was collected by filtration then dried under vacuum.

Glycine benzyl ester hydrochloride. 305 mg of product (76% yield). 1H NMR (400 MHz, D_2O) δ 7.34 (s, 5H); 5.18 (s, 2H); 3.83 (s, 2H).

β-alanine benzyl ester hydrochloride. 376 mg of product (87% yield). 1H NMR (400 MHz, D_2O) δ7.33 (s, 5H); 5.09 (s, 2H); 3.17 (t, 2H); 2.73 (t, 2H).

γ-aminobutyric acid benzyl ester hydrochloride. 429 mg of product (93% yield). 1H NMR (400 MHz, D_2O) δ 7.23 (s, 5H); 5.06 (s, 2H); 2.89 (t, 2H); 2.43 (t, 2H); 1.84 (m, 2H).

5-aminovaleric acid benzyl ester hydrochloride. 431 mg of product (88% yield). 1H NMR (400 MHz, D_2O) δ 7.32 (s, 5H); 5.04 (s, 2H); 2.85 (t, 2H); 2.36 (t, 2H); 1.55 (m, 4H).

Sarcosine benzyl ester hydrochloride. 185 mg of product (43% yield). 1H NMR (400 MHz, D_2O) δ 7.35 (s, 5H); 5.19 (s, 2H); 3.91 (s, 2H); 2.66 (s, 3H).

Azetidine-3-carboxylate benzyl ester hydrochloride. 21 mg of product (5% yield).

Piperidine-4-carboxylate benzyl ester hydrochloride. 461 mg of product (90% yield). 1H NMR (400 MHz, $CDCl_3$) δ 9.71 (s, 1H); 9.55 (s, 1H); 7.36 (m, 5H); 5.16 (s, 2H); 3.36 (m, 2H); 3.05 (m, 2H); 2.67 (m, 1H); 2.20 (m, 4H).

Materials and Methods

Transactivation Assay. Human epithelial kidney cells (HEK 293) were grown to 80% confluency in Dubelcco's modified Eagles 4.5 g/L glucose medium (high glucose DMEM) containing 10% fetal bovine serum, 50 units/mL penicillin and 50 µg/mL streptomycin. The cells were trypsinized with 0.25% trypsin then diluted to $5x10^5$ cells/mL with high glucose DMEM. Cells were added to Costar 3917 96-well plates at $5x10^4$ cells/well then incubated at 37° C for 24 hours.

1.5 µg of TR expression vector (full length TRα-CMV or TRβ-CMV), 1.5 µg of a reporter plasmid containing a DR4 thyroid hormone response element (TRE) direct repeat spaced by four nucleotides (AGGTCAcaggAGGTCA) cloned upstream of a minimal thymidine kinase promoter linked to a firefly luciferase coding sequence, and 0.75 µg of a pRL-SV40 constitutive *Renilla* luciferase reporter plasmid were diluted into 540 µl of OptiMEM. 27 µL of

Lipofectamine 2000 reagent was diluted into 540 µL of OptiMEM. The plasmid and lipofectamine dilutions were combined then incubated at RT for 10 min. The mixture was then diluted into 4.29 mL of OptiMEM. Plates were washed with 100 µL of phosphate buffered saline (PBS) at pH 7.2 without magnesium or calcium chloride per well. Transfection mixtures were added at 50 µL per well, then incubated at 37° C for 4 hours. Modified DME/F-12 Ham's medium without phenol red containing 15 mM HEPES and bicarbonate, 5 mM L-glutamine, charcoal-stripped FBS, 50 units/mL penicillin and 50 µg/mL streptomycin was added at 50 µL per well, then the plates were incubated at 37° C for 20 hours.

Drug stocks were made at 10 mM in DMSO or DME/F-12 Ham's, then serially diluted to 1X concentrations in DME/F-12 Ham's. Plates were washed with 100 µL of PBS (pH 7.2) per well. 100 µL of each drug stock was added to the wells in triplicate then the plates were incubated at 37° C for 24 hours.

Cells were assayed for luciferase activity using the Promega DualGlo kit. 50 µl of Luciferase Reagent were added per well, the plate was rocked for 15 min at RT then the plate was read of firefly luciferase activity. 50 µl of Stop & Glo Reagent were added per well then the plate was read for *Renilla* luciferase activity. Data normalized to *Renilla* internal control were analyzed with

GraphPad Prism v.4a using the sigmoid dose response model to generate EC_{50} values ±SEM.

Chapter 3 – Enhancing TRβ-Selective Thyromimetics

3.1 - Introduction

Thyroid hormone (TH) is a key signal for oligodendrocyte differentiation and myelin formation during development and also stimulates remyelination in adult models of multiple sclerosis (MS). [338] However, TH is not an acceptable long-term therapy due to there being no therapeutic window in which remyelination can be achieved while avoiding the cardiotoxicity and bone demineralization associated with chronic hyperthyroidism. Some thyroid hormone analogs can activate thyroid hormone-responsive genes while avoiding the associated downsides of TH by exploiting molecular and physiological features of thyroid hormone receptors. [339] These receptors are expressed in two major forms with heterogenous tissue distribution and overlapping but distinct sets of target genes. [340] TRα is enriched in the heart, brain, and bone while TRβ is enriched in the liver. [341] Developing selective thyromimetics has been challenging due to the high sequence homology of thyroid hormone receptor subtypes - only one amino acid residue on the internal surface of the ligand binding domain cavity varies between the α and β forms. GC-1 (Fig 3.1) was one of the first potent analogs that demonstrated significant TRβ-selectivity *in vitro*, [211,218] and *in vivo*. [213,214,342] While GC-1 was designed as a cardiac-sparing treatment for hypercholesterolemia by activating TRβ in the liver, recent studies

have demonstrated its clinical potential in human demyelination diseases ranging from multiple sclerosis[343] to X-linked adrenoleukodystrophy. [344]

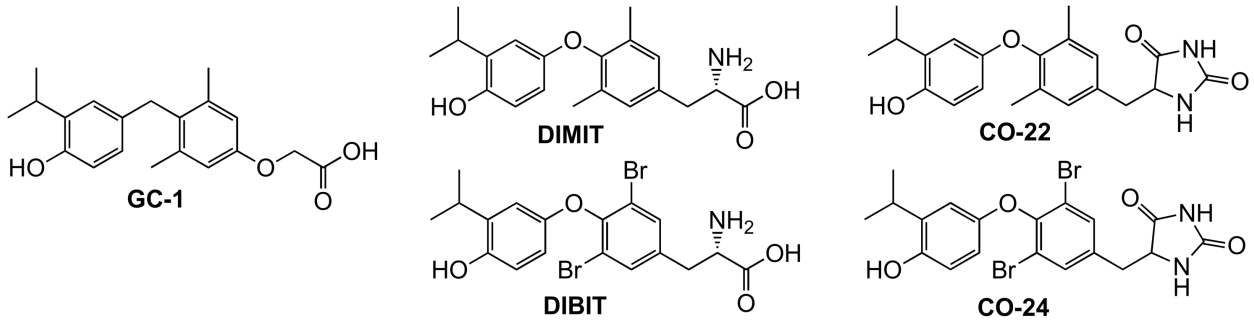

Figure 3.1

Chemical structures of TR agonists GC-1, DIMIT, DIBIT, CO-22, and CO-24

Despite these promising results, the utility of GC-1 in treating demyelination is potentially limited by low brain uptake[213] and reduced receptor activation compared to T3. While many of the structural features of GC-1 are critical for its binding affinity and receptor selectivity, [218] the 3,5-dimethyl constituents are not optimal. There is a large body of structure-activity relationship and quantitative structure-activity relationship data demonstrating that thyromimetics with inner ring methyl substitutions have significantly reduced activity in comparison to structurally similar analogs with inner ring halogen substitutions. The iodine-free analog 3'-isopropyl-3,5-dibromo-L-thyronine (DIBIT) (Fig 3.1) was 2- to 7-fold more potent than L-T4 in rat heart rate elevation and anti-goiter assays, [140] while the halogen-free analog 3'-

isopropyl-3,5-dimethyl-DL-thyronine (DIMIT) had little measurable activity in the same assays. [142] For the TRα-selective compounds CO22 and CO24, replacement of inner ring methyl groups with bromines improved binding affinity by 15-fold. [329,330] A QSSR study of thyroid hormone analogs suggested a mechanism for these findings - inner ring halogens can form a dipole-dipole interaction with a backbone carbonyl in the TR ligand binding domain, which influences binding affinity and selectivity. [345] These data suggest that GC-1 could be improved by synthesizing new analogs that replace the inner ring methyl groups with halogens.

3.2 - Results

Replacing the inner ring methyl groups of GC-1 with halogens required a new synthetic approach. Work by Dabrowski[346] provided a template for producing the necessary 4-hydroxy-2,6-dihalobenzaldehyde intermediates by selective deprotonation of the 4-position of silyl protected 3,5-dihalophenols with lithium amide reagents. The method was improved by replacing the methyl ether and trimethylsilyl ether protecting groups used by Dabrowski with the more sterically bulky triethylsilyl ether protecting group, which significantly improved the selectivity of the deprotonation. These intermediates were used in a slightly

altered version of the recently reported improved GC-1 synthesis. [334] The 4-hydroxy-2,6-dihalobenzaldehyde intermediates could not be alkylated with *tert*-butyl chloroacetate using the standard cesium carbonate/DMF conditions due to the halogen substitutions reducing the nucleophilicity of the phenol. However, the reaction went to completion and in good yield after converting the alkyl chloride into an alkyl iodide via an *in situ* Finklestein reaction. After forming the *tert*-butyl oxyacetate intermediate, the carbon-carbon bond formation proceeded in the same fashion as with GC-1 by forming an arylmagnesium with **7** that attacked the benzaldehyde to form a carbinol intermediate. The arylmagnesium nucleophile was critical to the success of the synthesis as it will not exchange with aryl chlorides or bromides at cryogenic temperatures and is compatible with the *tert*-butyl ester protecting group. [347] Reduction of the carbinol and deprotection of the *tert*-butyl ester and methoxymethyl ether protecting groups proceeded simultaneously with TFA and triethylsilane in dichloromethane. The dibromo analog JD-20 was synthesized in 27% overall yield and the dichloro analog JD-21 was synthesized in 17% yield, both in five steps.

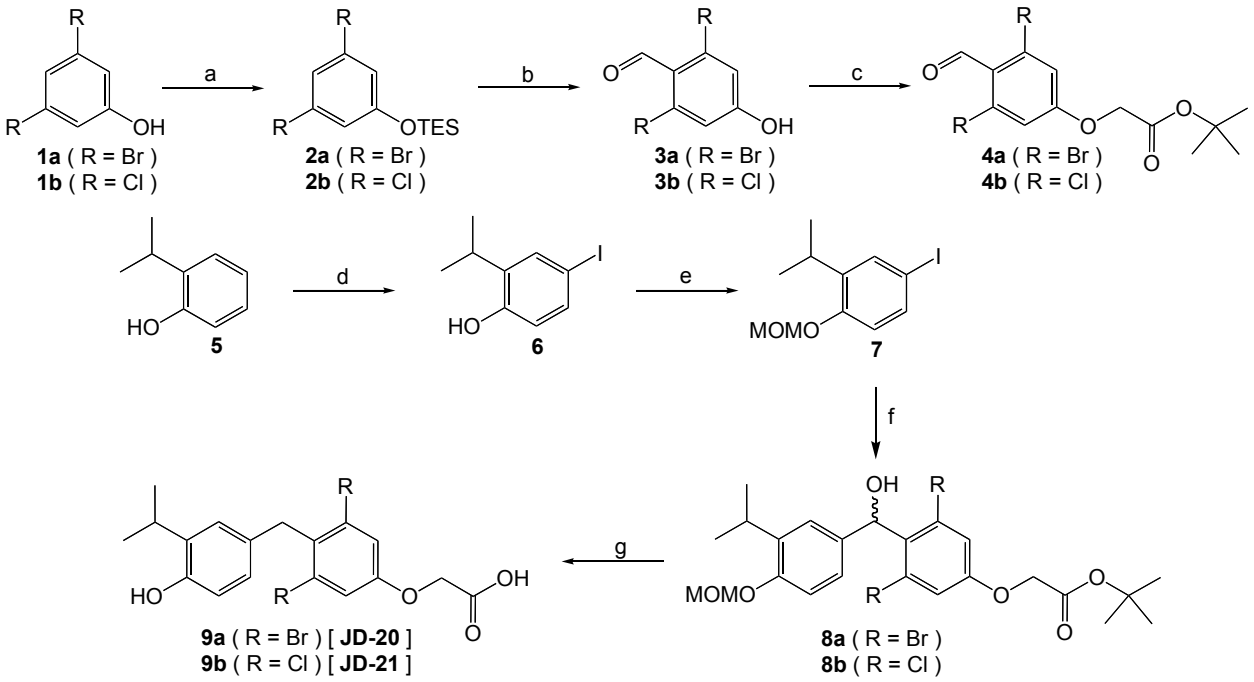

Figure 3.2

Synthesis of JD-20 **9a** and JD-21 **9b**. Reagents and Conditions: (a) triethylsilyl chloride, imidazole, DCM, 0°C, 95%; (b) (i) nBuLi, DIA/TMP, THF, -78° C (ii) DMF, 56-67%; (c) *tert*-butylchloroacetate, NaI, $CsCO$, acetone, 60-65° C, 84-88%; (d) NaI, NaOH, NaOCl, MeOH, HO, 87%; (e) MOMCl, TBAI, NaOH, DCM, HO, 81%; (f) (i) iPrMgCl, THF, 0° C to RT (ii) 4, -78° C, 54-79%; (g) TFA, triethylsilane, DCM, 0° C to RT, 58-69%.

A electroporation-based transfection system with U2OS or HeLa cells has previously been used for measuring the potency of thyroid hormone receptor-mediated transactivation. [211,330] Previous reports suggested that lipofectamine reagents sequestered lipophilic compounds such as GC-1, [302] but the electroporation method was limited by poor transfection efficiencies and limited dynamic range that delivered inconsistent results and frequently reported

significantly different EC_{50} values for T3 at each receptor subtype. The previous assay was modified and optimized using Lipofectamine 2000 and HEK293 cells. In comparison to the previous method, the new method has significantly higher transfection efficiencies and a greater dynamic range while being operationally simpler and delivered similar EC_{50} values for T3 at both subtypes.

Assays with the new transactivation assay showed that JD-20 and JD-21 have improved potency in comparison to their parent compound GC-1. Increases in potency at TRα were significant, with more modest improvements at TRβ, boosting the analogs to match the EC_{50} of T3 (Fig 3.3).

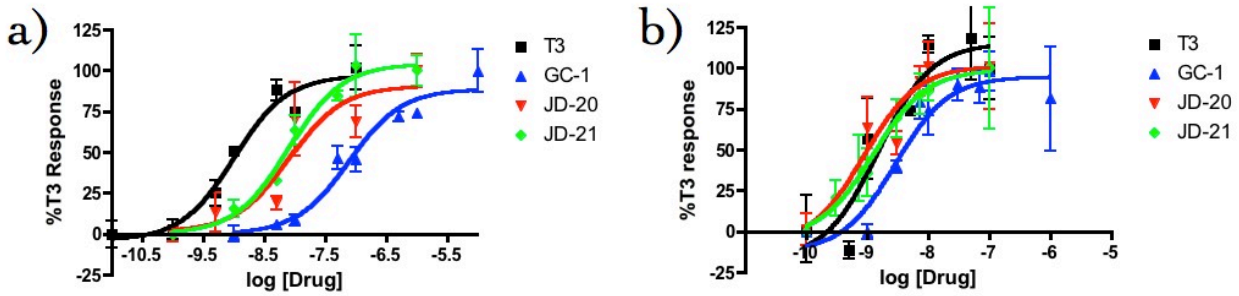

Figure 3.3

TRE-driven dual luciferase transactivation assays with calculated sigmoidal dose-response curves against a) hTRα and b) hTRβ in transiently transfected HEK293 cells. Plots show means of triplicates with error bars normalized to T3 response.

Compound	EC_{50} TRα (nM)	EC_{50} TRβ (nM)	TRα/TRβ
T3	1.01 ±0.4	1.49 ±1.57	0.678

GC-1	74.7 ±28.9	2.82 ±1.81	26.5
JD-20	7.96 ±6.95	0.88 ±1.12	9.04
JD-21	7.82 ±3.61	1.24 ±1.30	6.31

Table 3.1

Subtype selectivity measured by EC_{50} values from TRE-driven dual luciferase transactivation assays.

A distribution study was carried out in C57BL/6J mice to determine the concentrations in brain and serum after systemic (ip) administration. Mice were given single 9.14 µmol/kg doses of GC-1, JD-20, or JD-21. Tissue and blood were collected 1 hr post-injection and the concentration of the drugs was determined by LC-MS/MS analysis (Fig 3.4). JD-21 showed roughly comparable brain uptake compared to GC-1 while JD-20 was somewhat lower. Serum levels of JD-20 and JD-21 were both significantly lower than GC-1. These gave JD-21 a higher brain:serum ratio than GC-1 while JD-20 had a brain:serum ratio comparable to GC-1.

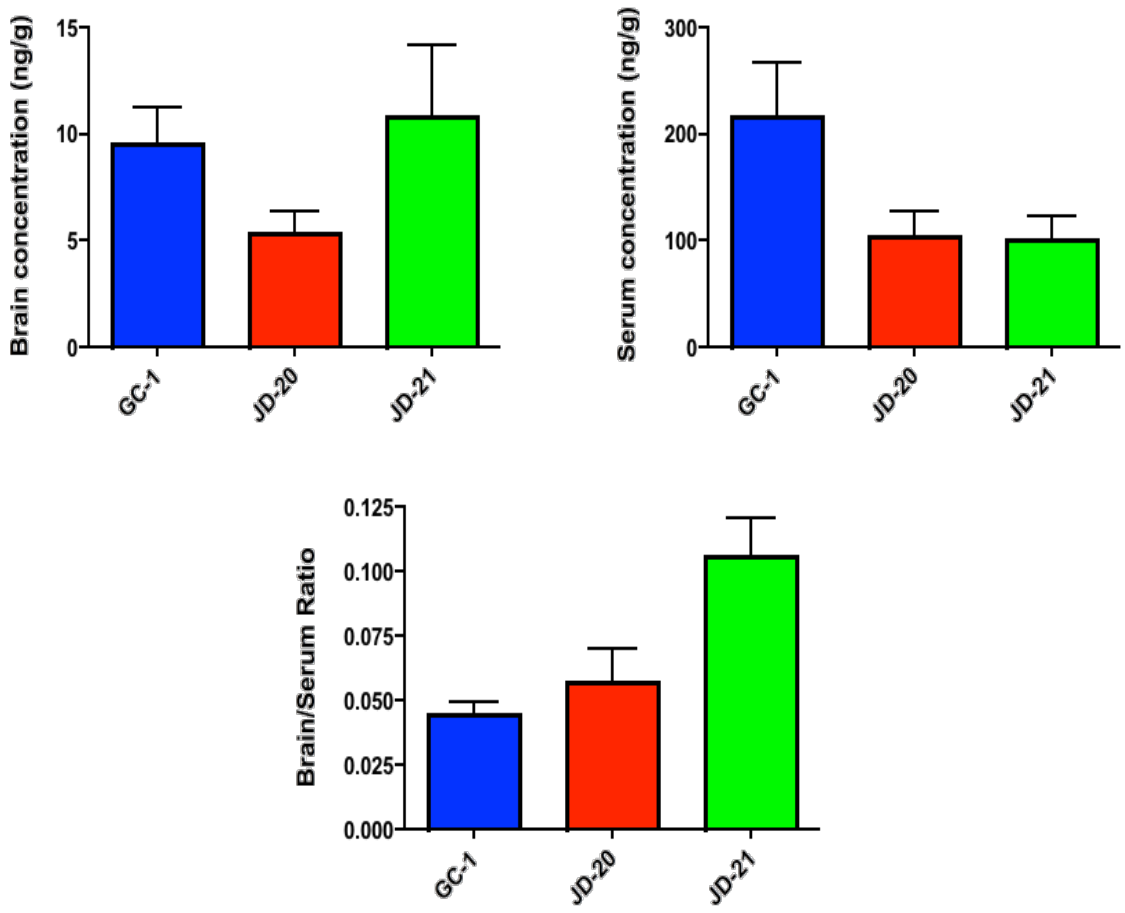

Figure 3.4

in vivo concentrations of GC-1, JD-20, and JD-21 in C57/B mouse tissues 1 hr after systemic administration (ip) of GC-1, JD-20, and JD-21 9.14 µmol/kg doses measured by LC-MS/MS in brain and serum.

Induction of *Hairless* (*Hr*), a TR target gene, mRNA expression in the brain was determined by qPCR and normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA (Fig 3.5). Vehicle (1:1 saline/DMSO) was used as a negative control and saturating doses of T3 (0.305 µmol/kg) and GC-1 (9.14 µmol/kg) were used as positive controls. JD-20 and JD-21 at 0.914

μmol/kg had comparable *Hr* induction to GC-1 at the same dose, suggesting roughly 10-fold greater potency than GC-1.

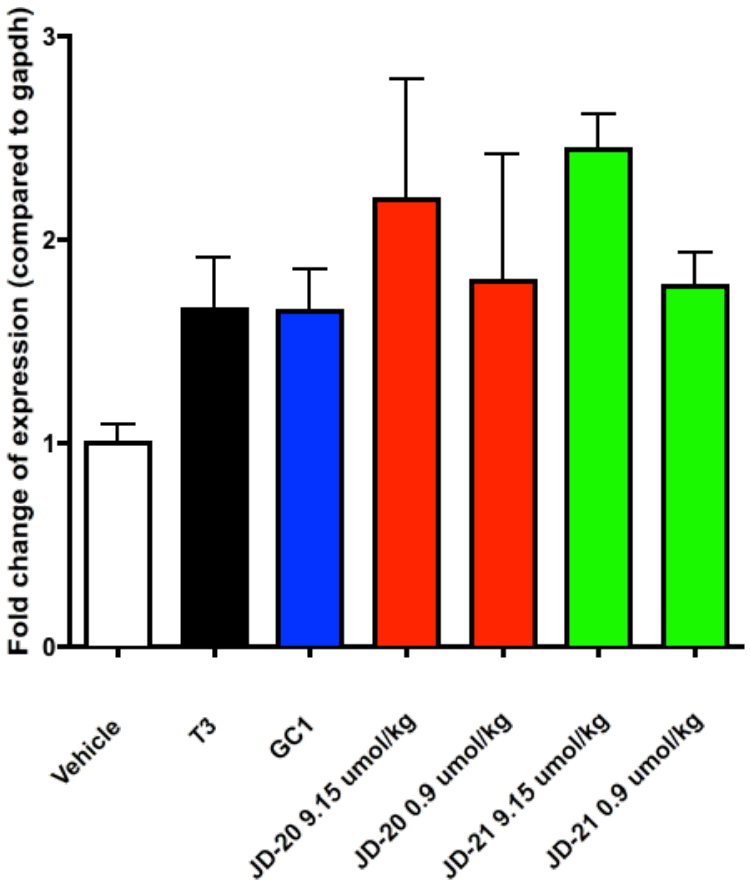

Figure 3.5

Expression of TR regulated gene *Hairless* (*Hr*) mRNA normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA measured by qPCR in C57/B mouse brain (3 mice/dose) 2 hr after systemic administration (ip) of saturating doses of T3 (0.305 μmol/kg) or GC-1 (9.14 μmol/kg) plus escalating doses of JD-20 and JD-21 (0.914 and 9.14 μmol/kg).

The EC_{50} values of *Hr* mRNA induction in the brain normalized to *GAPDH* mRNA expression by GC-1, JD-20, and JD-21 (Fig 3.6) were determined using the same experimental protocol. The EC_{50} for GC-1 was 8.20 ±12.65 µmol/kg, the EC_{50} for JD-20 was 1.49 ±1.08 µmol/kg, and the EC_{50} for JD-21 was 1.21 ±1.75 µmol/kg, making the halogenated analogs roughly 6-fold more potent than GC-1 at inducing *Hr* mRNA expression in the brain.

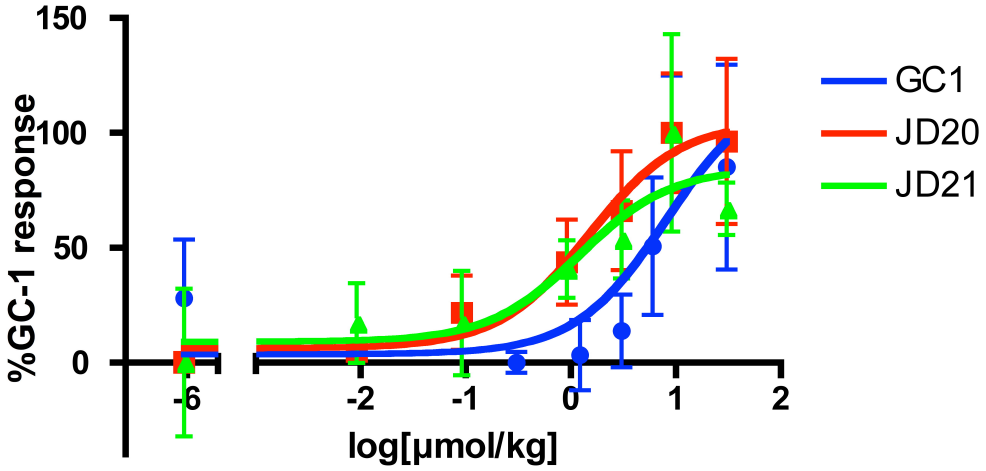

Figure 3.6

Expression of TR regulated gene *Hairless* (*Hr*) mRNA normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA measured by qPCR in C57/B mouse brain (3 mice/dose) 2 hr after systemic administration (ip) of GC-1, JD-20, and JD-21.

3.3 - Discussion

While GC-1 has become one of the standard TRβ-selective thyromimetics in the field, this study makes clear that a simple structural change - replacing the inner ring methyl groups with bromine or chlorine - produces significantly improved compounds that maintain critical selectivity and brain uptake properties of the parent, likely due to a halogen bonding interaction between the ligand and a backbone carbonyl in the TR ligand binding domain. The TRβ-selectivity and CNS penetration of GC-1 are preserved in JD-20 and JD-21, suggesting that they should be even more useful clinical candidates than GC-1 for treating the demyelination diseases such as X-linked adrenoleukodystrophy and multiple sclerosis.

The improved potency of JD-20 and JD-21 is consistent with numerous thyromimetic SAR studies that have found superior activity for analogs with halogens in the 3,5-position compared to similar analogs with methyl groups at those positions. What is surprising in this instance is that by most measures JD-20 and JD-21 appear to have very similar properties. In previous thyromimetic SAR studies changing halogens frequently produced dramatic changes in both potency and selectivity. [345] On this scaffold the only major difference is found in

brain uptake, where JD-20 has a reduced uptake in comparison to GC-1 and JD-21. This hints that these compounds may be transported across the blood-brain barrier by an active rather than a passive mechanism, where halogen bonding or sterics might change the relative rates at which the analogs pass through the putative transporter. Further experiments are needed to determine whether or what fraction of the uptake of these analogs into the brain occurs via a selective, saturable transport mechanism.

3.4 - Experimental

Material and Methods

Transactivation Assay. Human epithelial kidney cells (HEK 293) were grown to 80% confluency in Dubelcco's modified Eagles 4.5 g/L glucose medium (high glucose DMEM) containing 10% fetal bovine serum, 50 units/mL penicillin and 50 µg/mL streptomycin. The cells were trypsinized with 0.25% trypsin, then diluted to $5x10^5$ cells/mL with high glucose DMEM. Cells were added to Costar 3917 96-well plates at $5x10^4$ cells/well, then incubated at 37° C for 24 hours.

1.5 µg of TR expression vector (full length TRα-CMV or TRβ-CMV), 1.5 µg of a reporter plasmid containing a DR4 thyroid hormone response element (TRE) direct repeat spaced by four nucleotides (AGGTCAcaggAGGTCA) cloned upstream of a minimal thymidine kinase promoter linked to a firefly luciferase coding sequence, and 0.75 µg of a pRL-SV40 constitutive *Renilla* luciferase reporter plasmid were diluted into 540 µl of OptiMEM. 27 µL of Lipofectamine 2000 reagent was diluted into 540 µL of OptiMEM. The plasmid and lipofectamine dilutions were combined then incubated at RT for 10 min. The mixture was then diluted into 4.29 mL of OptiMEM. Plates were washed with 100 µL of phosphate buffered saline (PBS) at pH 7.2 without magnesium or calcium chloride per well. Transfection mixtures were added at 50 µL per well, then incubated at 37° C for 4 hours. Modified DME/F-12 Ham's medium without phenol red containing 15 mM HEPES and bicarbonate, 5 mM L-glutamine, charcoal-stripped FBS, 50 units/mL penicillin and 50 µg/mL streptomycin was added at 50 µL per well, then the plates were incubated at 37° C for 20 hours.

Drug stocks were made at 10 mM in DMSO, then serially diluted to 1X concentrations in DME/F-12 Ham's. Plates were washed with 100 µL of PBS (pH 7.2) per well. 100 µL of each drug stock was added to the wells in triplicate, then the plates were incubated at 37° C for 24 hours.

Cells were assayed for luciferase activity using the Promega DualGlo kit. 50 µl of Luciferase Reagent were added per well, the plate was rocked for 15 min at RT, then the plate was read of firefly luciferase activity. 50 µl of Stop & Glo Reagent were added per well, then the plate was read for *Renilla* luciferase activity. Data normalized to *Renilla* internal control were analyzed with GraphPad Prism v.4a using the sigmoid dose response model to generate EC_{50} values ±SEM.

Animal Studies. Experimental protocols were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Oregon Health & Science University Institutional Animal Care & Use Committee. Wild type male C57BL/6J mice, aged 8–10 weeks, were housed in a climate controlled room with a 12 hour light-dark cycle with ad libitum access to food and water.

Distribution Studies. Mice were injected once intraperitoneally (ip) with GC-1 at 9.14 µmol/kg, and analogs at 0.914, 9.14, and 30.5 µmol/kg. Euthanasia was performed on three mice per dose at 1 hr and the tissues and blood were harvested. Tissues were immediately frozen and blood was kept on ice for a minimum of 30 minutes and then spun down at 7,500 x G for 15 minutes. Serum (100uL) was collected and was stored with tissues at -80C until samples were processed.

Serum Processing. The serum samples were warmed to RT and 10 uL of 2.99 µM internal standard (D-GC-1) was added to them. Acetonitrile (500 uL) was added and the sample was vortexed for 20 seconds. The sample was then centrifuged at 10,000 x G for 15 minutes at 4 C. Next, 90% of the upper supernatant was transferred to a glass test tube and concentrated using a speedvac for 1.5 hr at 45° C. The dried sample was then dissolved in 400 µL of 50:50 ACN:H$_2$O and vortexed for 20 seconds. The resulting mixture was transferred to an eppendorf and centrifuged at 10,000 x G for 15 minutes. The supernatant was filtered with 0.22 µM centrifugal filters and submitted for LC-MS/MS analysis. The standard curve was made with 100 µL of serum from a 8-10 week old mouse not injected with T3, GC-1, or analogs. The processing was performed exactly the same except after filtering the sample was split amongst 6 vials. To 5 out of the 6 vials was added GC-1, JD-20, and JD-21 to make final concentrations of each compound in matrix of (0.1 pg/µL, 1 pg/µL, 10 pg/µL, 100 pg/µL, and 1000 pg/µL).

Brain Processing. The brain samples were warmed to RT and transferred to a homogenizer tube with 5 GoldSpec 1/8 chrome steel balls (Applied Industrial Technologies). The resulting tube was weighed and then 1 mL of H$_2$O was added, followed by 10 uL of 2.99 µM internal standard (D-Sobetirome). The tube was homogenized with a Bead Bug for 30 seconds and then transferred

to a falcon tube containing 3 mL of ACN. ACN (1 mL) was used to wash homogenizer tube and the solution was transferred back to the falcon tube. The sample was then processed using the same method for the serum processing except the sample was concentrated in a glass tube using a speed vac for 4 hr at 45C.

Gene activation. Mice were injected once intraperitoneally (ip) with vehicle (1:1 saline/DMSO), T3 at 0.305 μmol/kg, GC-1 at 9.14 μmol/kg, and analogs at 0.914, 9.14, and 30.5 μmol/kg. Euthanasia was performed on three mice per dose at 2 hr and the tissues were harvested. The brain tissues collected for qPCR analysis were processed according to a protocol for RNA extraction using Trizol reagent and the PureLink RNA mini kit, using Qiagen RNase-free DNase kit during the optional DNase treatment step. 1 μg of extracted RNA was used to synthesize cDNA via a reverse transcription (RT) reaction using the Qiagen QuantiTect Reverse Transcription kit. DNA contamination was controlled for by duplicating one sample without the addition of RT enzyme. Expression of the *Hairless* (*Hr*) gene was measured by QPCR using the QuantiTect SYBR green PCR kit from Qiagen. The primer sequences for hairless (Fwd: CCAAGTCTGGGCCAAGTTTG; Rev: TGTCCTTGGTCCGATTGGAA) were previously described by Barca-Mayo[322]. The template cDNA was diluted 2-fold to minimize the interference of

RT reagents in the qPCR reaction. Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) was the housekeeping gene used for normalizing between samples. Data analysis for single dose experiment was done using the comparative C, method to look at the relative differences in *Hr* gene expression. Data analysis for dose-response experiment was done using GraphPad Prism v.4a with the sigmoid dose response model to generate EC_{50} values ±SEM.

Chemistry

Chemistry General. ^{1}H NMR were taken on a Bruker 400. All ^{1}H NMR were calibrated to the NMR solvent reference peak (D_6-acetone, $CDCl_3$). Anhydrous tetrahydrofuran (THF) and dimethylformamide (DMF) were obtained from a Seca Solvent System. All other solvents used were purchased from Sigma-Aldrich or Fisher. Purity analysis of final compounds was determined to be >95% by HPLC. HPLC analysis was performed on a Varian ProStar HPLC with an Agilent Eclipse Plus C18 5 uM column (4.6 x 250 mm) with a gradient of 10% to 95% acetonitrile (0.1% TFA) over 15 minutes.

Synthesis

(3,5-dibromophenoxy)triethylsilane (2a). **1a** (5.04g, 20 mmol) and imidazole (4.09 g, 60 mmol) were dissolved in 80 mL of DCM. The solution was cooled to 0° C, then triethylsilyl chloride (5.03 mL, 30 mmol) was added, then the reaction was stirred at 0° C for 30 min. The reaction was diluted with 160 mL of Et_2O, washed 2X with 50 mL of H_2O and 2X with 50 mL of brine, then dried with $MgSO_4$, filtered, and concentrated to give the **2a** in quantitative yield, which was used without purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (t, 1H), 7.10 (d, 2H), 1.02 (t, 9H), 0.82 (q, 6H).

4-hydroxy-2,6-dibromobenzaldehyde (3a). A flask was loaded with molecular sieves, then flame-dried under vacuum. After cooling under argon, **2a** (5.49 g, 15 mmol) was loaded, then the flask was sealed, evacuated, and flushed with argon. 30 mL of dry THF were added and degassed, then the solution was cooled to -78° C. A second flask was loaded with molecular sieves, then flame-dried under vacuum. After cooling under argon, diisopropylamine (4.6 mL, 33 mmol) was added, followed by 60 mL of dry THF, then the solution was degassed and cooled to -78° C. 2.5 M n-butyllithium solution in hexanes (12 mL, 30 mmol) was added, then the solution was stirred for 1 hr at -78° C. The lithium diisopropylamide solution was transferred dropwise via cannula to the **2a** solution, then the deprotonation was stirred for 1 hr at -78° C. 5.8 mL of dry DMF (75 mmol) were added, then the reaction was stirred for 1 hr at -78° C.

The reaction was decanted into 50 mL of 1 N aqueous HCl. The aqueous layer was extracted 3X with 90 mL of Et_2O. The organic fractions were combined, washed 2X with 50 mL of brine, then dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was precipitated from hexanes at -78° C to give 2.8 g of **3a** (67% yield). ^{1}H NMR (400 MHz, d_6-acetone) δ 10.16 (s, 1H), 7.27 (s, 2H).

***tert*-butyl 2-(3,5-dibromo-4-formylphenoxy)acetate (4a).** **3a** (2.8 g, 10 mmol), sodium iodide (3 g, 20 mmol), and cesium carbonate (3.24 g, 10 mmol) were dissolved in 40 mL of acetone. 2.86 mL *tert*-butyl chloroacetate (20 mmol) were added, then the reaction was refluxed at 65° C for 2 hr. The reaction was diluted with 80 mL of Et_2O, washed 2X with 30 mL of water and 2X with 30 mL of brine, then dried with $MgSO_4$, filtered, and concentrated. The product was precipitated from hexanes and collected by filtration, then dried under vacuum to give 3.49 g of **4a** (88% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.23 (s, 1H), 7.19 (s, 2H), 4.59 (s, 2H), 1.52 (s, 9H).

(3,5-dichlorophenoxy)triethylsilane (2b). **1b** (6.54g, 40 mmol) and imidazole (8.18 g, 120 mmol) were dissolved in 160 mL of DCM. The solution was cooled to 0° C, then triethylsilyl chloride (10 mL, 60 mmol) was added, then the reaction was stirred at 0° C for 30 min. The reaction was diluted with 320 mL of Et_2O, washed 2X with 100 mL of H_2O and 2X with 75 mL of brine, then

dried with $MgSO_4$, filtered, and concentrated to give **2b**, which was used without purification and weighed 10.58 g after drying (95% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 (t, 1H), 6.76 (d, 2H), 1.02 (t, 9H), 0.77 (q, 6H).

4-hydroxy-2,6-dichlorobenzaldehyde (**3b**). A flask was loaded with molecular sieves, then flame-dried under vacuum. After cooling under argon, **2b** (3.6 g, 13 mmol) was loaded, then the flask was sealed, evacuated, and flushed with argon. 13 mL of dry THF were added and degassed, then the solution was cooled to -78° C. A second flask was loaded with molecular sieves, then flame-dried under vacuum. After cooling under argon, 2,2,6,6-tetramethylpiperdidine (1.84 g, 13 mmol) was added, followed by 13 mL of dry THF, then the solution was degassed and cooled to -78° C. 2.5 M n-butyllithium solution in hexanes (5.2 mL, 13 mmol) was added, then the solution was stirred for 20 min at 0° C. The lithium TMP solution was transferred dropwise via cannula to the **2b** solution, then the deprotonation was stirred for 30 min at -78° C. 5 mL of dry DMF (65 mmol) were added, then the reaction was stirred for 30 min at -78° C. The reaction was decanted into 15 mL of 1 N aqueous HCl. The aqueous layer was extracted 3X with 15 mL of EtOAc. The organic fractions were combined, washed 2X with 15 mL of brine, then dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was recrystallized from hexanes

at -20° C to give 1.39 g of **3b** (56% yield). ^{1}H NMR (400 MHz, d_6-acetone) δ 10.37 (s, 1H), 7.01 (s, 2H).

***tert*-butyl 2-(3,5-dichloro-4-formylphenoxy)acetate (4b).** **3b** (1.15 g, 6 mmol), sodium iodide (1.8 g, 12 mmol), and cesium carbonate (1.94 g, 6 mmol) were dissolved in 24 mL of acetone. 1.72 mL *tert*-butyl chloroacetate (12 mmol) were added, then the reaction was refluxed at 60° C for 24 hr. The reaction was diluted with 30 mL of Et_2O, washed 2X with 10 mL of water and 2X with 10 mL of brine, then dried with $MgSO_4$, filtered, and concentrated. The crude oil was redissolved in a minimal amount of Et_2O then added dropwise to 100 mL of vigorously stirring hexanes at -78°C. The precipitate was collected by filtration and dried under vacuum to give 1.545 g of **4b** (84% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.43 (s, 1H), 6.92 (s, 2H), 4.59 (s, 2H), 1.52 (s, 9H).

4-iodo-2-isopropylphenol (6). **5** (6.8g, 50 mmol), NaI (7.5 g, 50 mmol) were dissolved in 70 mL of MeOH. 10 M aqueous NaOH (5 mL, 50 mmol) was added, then the solution was cooled to 0° C. 6.25% w/v aqueous NaOCl (62.5 mL, 50 mmol) was added drop wise over 24 hr at 0° C. The reaction was acidified to pH 7 with 12 N aqueous HCl, then quenched with 10 mL of saturated aqueous $Na_2S_2O_3$. The aqueous layer was extracted 3X with Et_2O. The organic fractions were combined, washed 2X with brine, then dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was purified

by flash chromatography (silica gel, hexane/ethyl acetate, 1-20%) to give 11.35 g of **6** (87% yield) as a reddish oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (d, 1H), 7.36 (dd, 1H), 6.54 (d, 1H), 3.16 (m, 1H), 1.25 (d, 6H).

4-iodo-2-isopropyl-1-(methoxymethoxy)benzene (7). 6 (2.62 g, 10 mmol) and tetrabutylammonium iodide (369 mg, 1 mmol) were dissolved in 100 mL of DCM. 10 mL of 10 M aqueous NaOH were added, followed by 5 mL of 6 M chloromethyl methyl ether in MeOAc. The reaction was stirred for 30 min at RT, then diluted with 200 mL of Et_2O. The organic layer was washed 2X with 100 mL of H_2O and 2X with 100 mL of brine, then dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was purified by flash chromatography (silica gel, hexane/ethyl acetate, 1-20%) to give 2.48 g of **7** (81% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (d, 1H), 7.42 (dd, 1H), 6.83 (d, 1H), 5.18 (s, 2H), 3.47 (s, 3H), 3.27 (m, 1H), 1.20 (d, 6H).

***tert*-butyl 2-(3,5-dibromo-4-(hydroxy(3-isopropyl-4-(methoxymethoxy)phenyl)methyl)phenoxy)acetate (8a)**. A flask was loaded with 4 Å molecular sieves and flame-dried under vacuum. **7** (1.47 g, 4.8 mmol) was loaded and the flask was sealed, evacuated, and flushed with argon. 24 mL of dry THF were added and degassed, then the solution was cooled to 0° C. Isopropylmagnesium chloride (2 M THF, 5.5 mL, 7.2 mmol) was added, then the reaction was stirred for 2 hours at RT. A second flask was loaded with 4 Å

molecular sieves and and flame-dried under vacuum. **4a** (946 mg, 2.4 mmol) was loaded and the flask was sealed, evacuated, and flushed with argon. 12 mL of dry THF were added and degassed. The arylmagnesium solution was cooled to –78° C, then the **4a** solution was added drop wise via cannula and the reaction was stirred for 1 hour at –78° C. The reaction was quenched with 10 mL of 1 N aqueous HCl. The aqueous layer was extracted 3X with 10 mL of EtOAc. The organic fractions were combined and washed 2X with 10 mL of brine. The organic layer was dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was purified by flash chromatography (silica gel, hexanes/EtOAc 4-40%) to give 1.089 g of **8a** (79% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 (d, 1H), 7.17 (s, 2H), 7.00 (d, 1H), 6.90 (dd, 1H), 6.51 (d, 1H), 5.21 (s, 2H), 4.53 (s, 2H), 3.49 (s, 3H), 3.34 (m, 3H), 1.52 (s, 9H), 1.21 (t, 6H).

2-(3,5-dibromo-4-((3-isopropyl-4-hydroxyphenyl)methyl)phenoxy)acetic acid (**9a**). **8a** (1.089 g, 1.9 mmol) was dissolved in 19 mL of DCM with 1.21 mL of triethylsilane (7.58 mmol). The solution was cooled to 0°C, then 4.35 mL of trifluoroacetic acid (56.9 mmol) were added and the reaction was stirred for 30 min at 0° C, then 2 hr at RT. Solvent was removed under vacuum, then the product was precipitated by the addition of hexanes and collected by filtration. The solid was dried under vacuum to give 505 mg of **JD-20** (**9a**) (58% yield). ^{1}H NMR (400 MHz,

$CDCl_3$) δ 7.19 (s, 2H), 7.10 (d, 1H), 6.82 (dd, 1H), 6.64 (d, 1H), 4.68 (s, 2H), 4.28 (s, 2H), 3.18 (m, 1H), 1.24 (d, 6H).

***tert*-butyl 2-(3,5-dichloro-4-(hydroxy(3-isopropyl-4-(methoxymethoxy)phenyl)methyl)phenoxy)acetate (8b)**. A flask was loaded with 4 Å molecular sieves and flame-dried under vacuum. **7** (459 mg, 1.5 mmol) was loaded and the flask was sealed, evacuated, and flushed with argon. 6 mL of dry THF were added and degassed, then the solution was cooled to 0° C. Isopropylmagnesium chloride (2 M THF, 1.125 mL, 2.25 mmol) was added, then the reaction was stirred for 2 hours at RT. A second flask was loaded with 4 Å molecular sieves and and flame-dried under vacuum. **4b** (305 mg, 1 mmol) was loaded and the flask was sealed, evacuated, and flushed with argon. 4 mL of dry THF were added and degassed. The arylmagnesium solution was cooled to –78° C, then the **4b** solution was added drop wise via cannula and the reaction was stirred for 1 hour at –78° C. The reaction was quenched with 5 mL of 1 N aqueous HCl. The aqueous layer was extracted 3X with 5 mL of EtOAc. The organic fractions were combined and washed 2X with 5 mL of brine. The organic layer was dried with $MgSO_4$, filtered, and concentrated to give the crude product, which was purified by flash chromatography (silica gel, hexanes/EtOAc 2-20%) to give 260 mg of **8b** (54% yield). ^{1}H NMR (400 MHZ, $CDCl_3$) δ 7.26 (d, 1H), 6.99 (dd, 1H), 6.94 (d, 1H), 6.93 (s, 2H), 6.50 (d, 1H),

5.21 (s, 2H), 4.53 (s, 2H), 3.50 (s, 3H), 3.33 (m, 1H), 3.23 (d, 1H), 1.52 (s, 9H), 1.21 (t, 6H).

2-(3,5-dichloro-4-((3-isopropyl-4-hydroxyphenyl)methyl)phenoxy)acetic acid (9b). **8b** (260 mg, 0.54 mmol) was dissolved in 5.4 mL of DCM with 0.345 mL of triethylsilane (2.16 mmol). The solution was cooled to 0°C, then 1.24 mL of trifluoroacetic acid (16.2 mmol) were added and the reaction was stirred for 30 min at 0° C, then 2 hr at RT. Solvent was removed under vacuum, then the product was precipitated by the addition of hexanes and collected by filtration. The solid was dried under vacuum to give 137 mg of **JD-21 (9b)** (69% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.12 (d, 1H), 6.95 (s, 2H), 6.86 (dd, 1H), 6.64 (d, 1H), 4.68 (s, 2H), 4.18 (s, 2H), 3.17 (m, 1H), 1.24 (d, 6H).

Chapter 4 – Summary and Future Directions

4.1 Summary

The history of thyromimetic development has passed through a series of phases as basic biological discoveries opened up new techniques for assaying analogs and revealed new targets. Most recent efforts have focused on selectively activating TRβ with the goal of lowering serum cholesterol by activating TRβ in the liver, these efforts have been stymied by side-effects and increasingly high regulatory hurdles. [348] Efforts have been made to produce TRα-selective thyromimetics, [329,330] but these have failed to maintain their selectivity when used in mammalian model organisms. [331] These results suggest that new efforts in chemistry and biology are necessary to develop new thyromimetics and find new targets.

This dissertation set out to test a hypothesis that thyromimetics with multiple acidic groups or other hydrogen bond donor would favorably interact with the more flexible arginine groups within the TRα ligand-binding pocket producing TRα-selective ligands. To do this a scaffold was developed and derivativzed with a range of benzamides. While the initial series showed a handful of ligands with different chemotypes display favorable selectivity, none had sufficient efficacy. Several rounds of development were necessary to create a

series that retained the desired selectivity combined with acceptable potency in the one hundred nanomolar range.

From these compounds a number of structural insights were gained. The first was that one path to TRα-selectivity was through the length of the benzamide portion of the ligand. Selectivity improved from the glycine amide to the β-alanine amide, then fell off abruptly when the chain length was increased beyond three carbons. However, this could be partially overcome by using cyclic amino acids such as 3-carboxyazetidine or 4-carboxypiperidine amides instead of longer straight chain amino acids. This suggests that the cyclic amino acids have conformational selectivity that favorably situates their carboxylic acid groups in comparison to the more flexible straight chain amino acids. Selectivity could also be achieved with a D-serine amide, which is the best evidence supporting the original hypothesis.

The second major structural insight was that replacing the inner ring methyl groups of the first series with halogens dramatically improved potency. Bromines were most favored, boosting potency across the board, while chlorines were more mixed. The dichloro glycine analog matched the dibromo glycine analog, but any extension of the chain length reduced potency by at least an order of magnitude. This is difficult to rationalize, but suggests that the halogen bond[345] that is the putative basis of the improved potency displayed by ligands

with halogens on the inner rings instead of methyl groups plays an important role in positioning the ligand within the pocket and the smaller chlorine substitutions make larger ligands fit unfavorably in the rest of the pocket.

The structure-activity relationship information from the TRα-selective analogs project was used to improve one of the best TRβ-selective thyromimetics, GC-1.[211] GC-1 has inner ring methyl groups and it was hypothesized that making isosteric ligands with halogens in these positions might display improved potency compared to the parent. This proved to be synthetically tractable and the new ligands displayed the predicted improvements in potency in both cell-based transactivation assays and TR-regulated gene activation *in vivo*. Brain uptake studies with these ligands showed that their uptake was comparable to GC-1 suggesting that the improved potency in gene activation is primarily due to the improved potency at the receptor seen in the cell-based assays.

4.2 Future Directions

The most important question coming from the development of TRα-selective analogs is whether the potency and selectivity can be further enhanced. While there may be thermodynamic limits and metabolic constraints on what is

possible, there is still chemical space to explore. The potency and selectivity of the D-serine analogs suggests that other ligands with multiple acidic groups may provide stronger interactions with the arginine cluster, but these may require ester prodrug strategies to mask some or all of the acidic groups to so that they can pass through the plasma membrane without an active uptake mechanism.

An important question arising from the results of these studies is whether the improved potency shown by replacing the inner ring methyl groups of thyromimetics with halogens is generally applicable. An obvious candidate is the thyroid hormone antagonist NH-3 that is based on the structure of GC-1.[306] It has a large 5' extension that causes the ligand to bind with lower affinity to thyroid hormone receptors than GC-1. Replacement of the inner ring methyl groups with halogens could recover that lost binding energy and produce a more potent inhibitor of thyroid hormone action.

Most importantly the biology of the upgraded TRβ-selective thyromimetics needs to be further explored. While they display significantly improved potency in gene activation assays, further work is needed to fully understand their PK-ADME properties. There are hints of different distribution profiles, which may be due to active transport of the compounds by different tissues. Additionally while aryl bromides and chlorides are generally stable *in vivo*, it needs to be confirmed that their elimination and toxicity profiles are

similar to that of GC-1. Finally, there is a significant amount of work to be done to further understand their potential applications in demyelination diseases such as X-linked adrenoleukodystrophy and multiple schlerosis. GC-1 has already shown significant promise in lysolecithin and cuprazone models of these diseases and these preliminary results suggest that the halogen analogs will be even more effective.

Chapter 5 - References

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Appendix A: NMR Spectra

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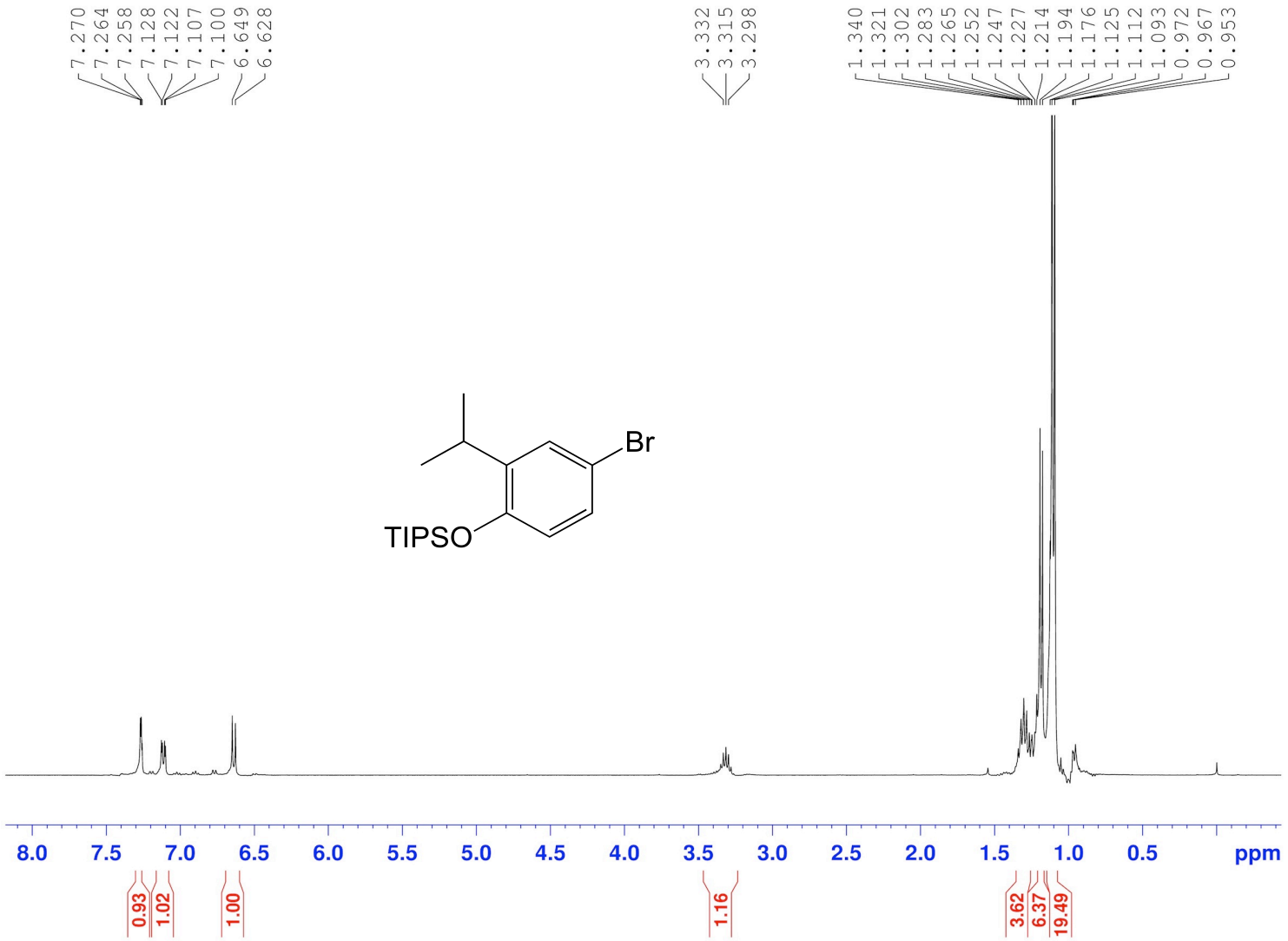
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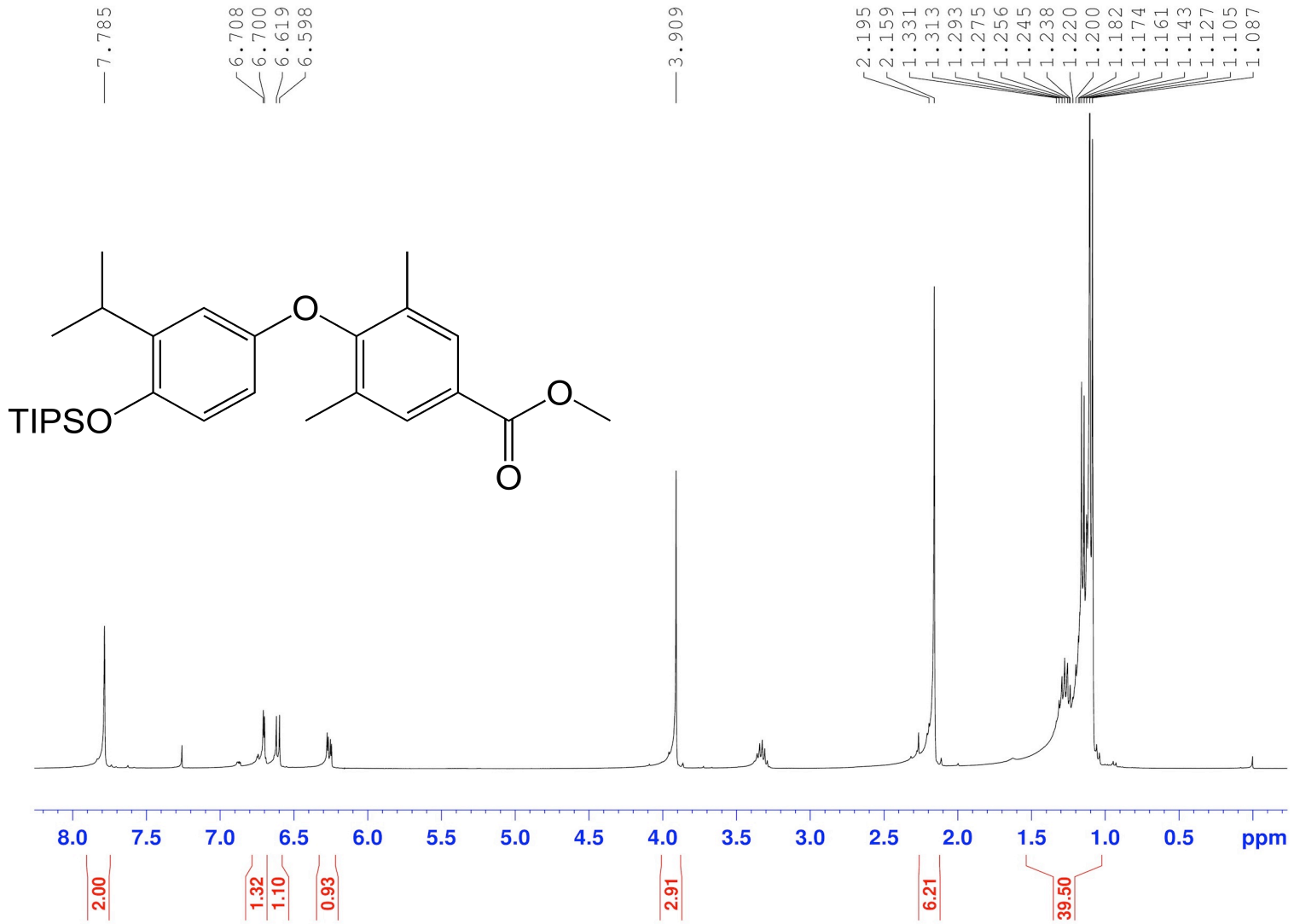
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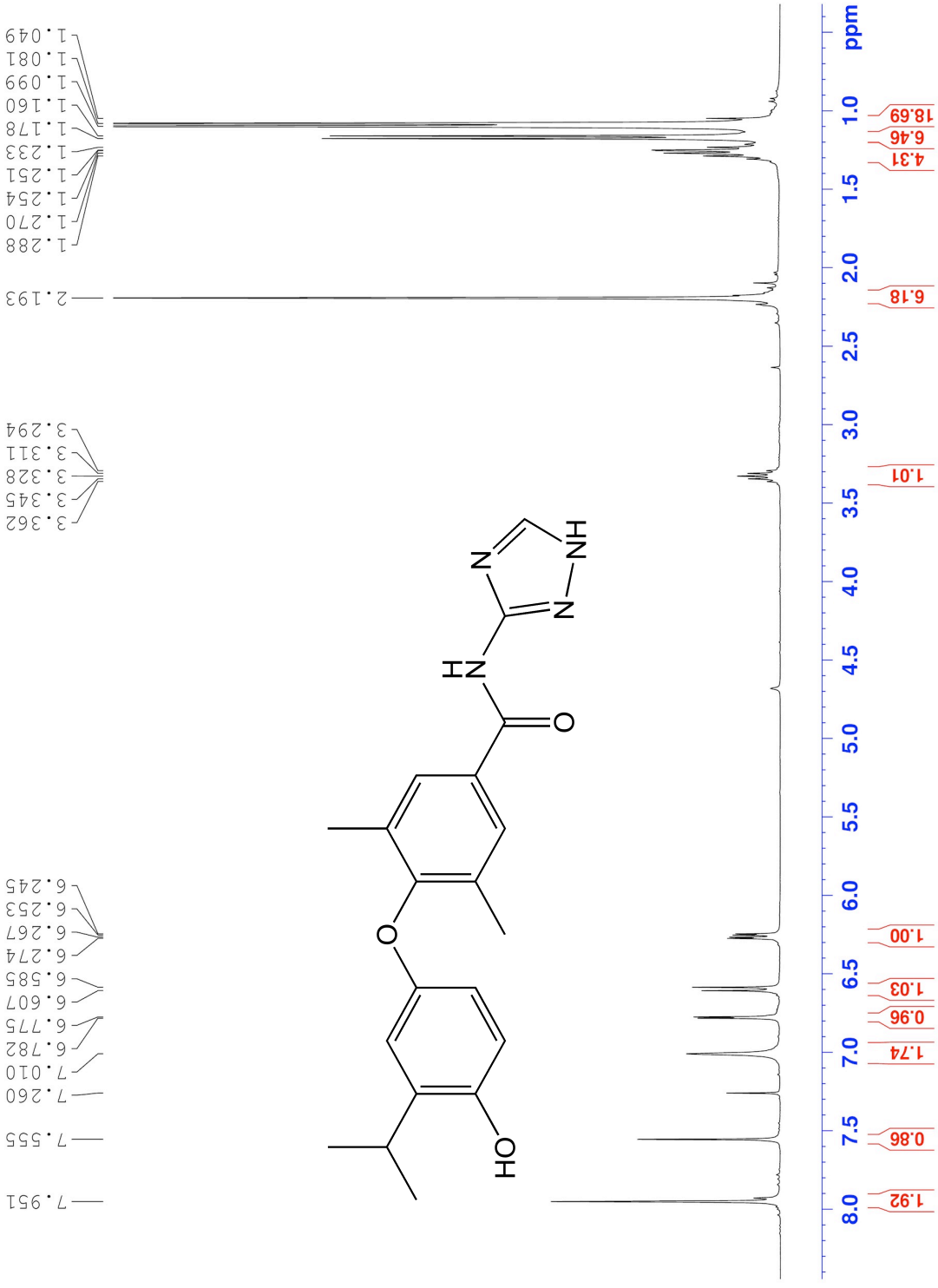
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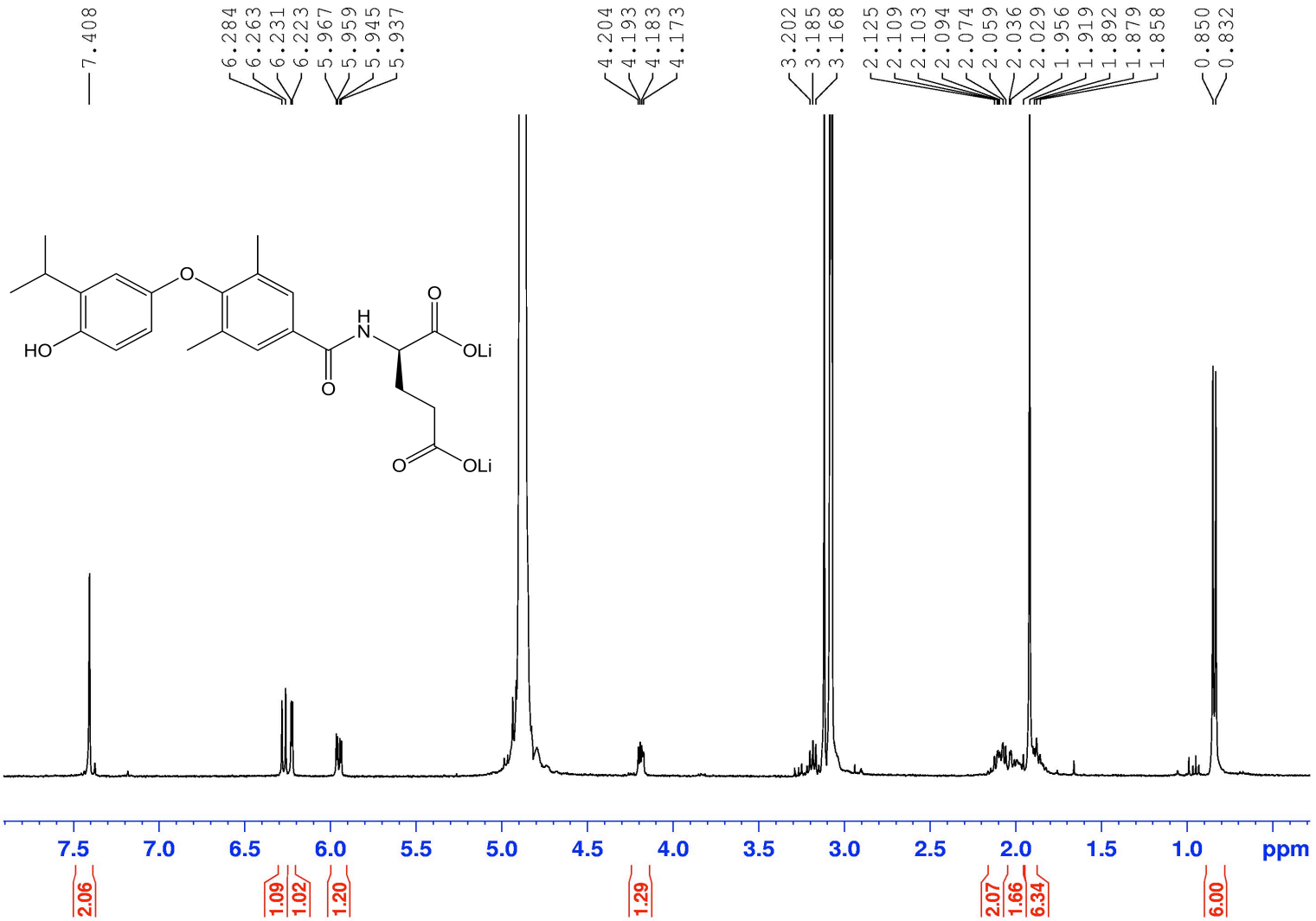
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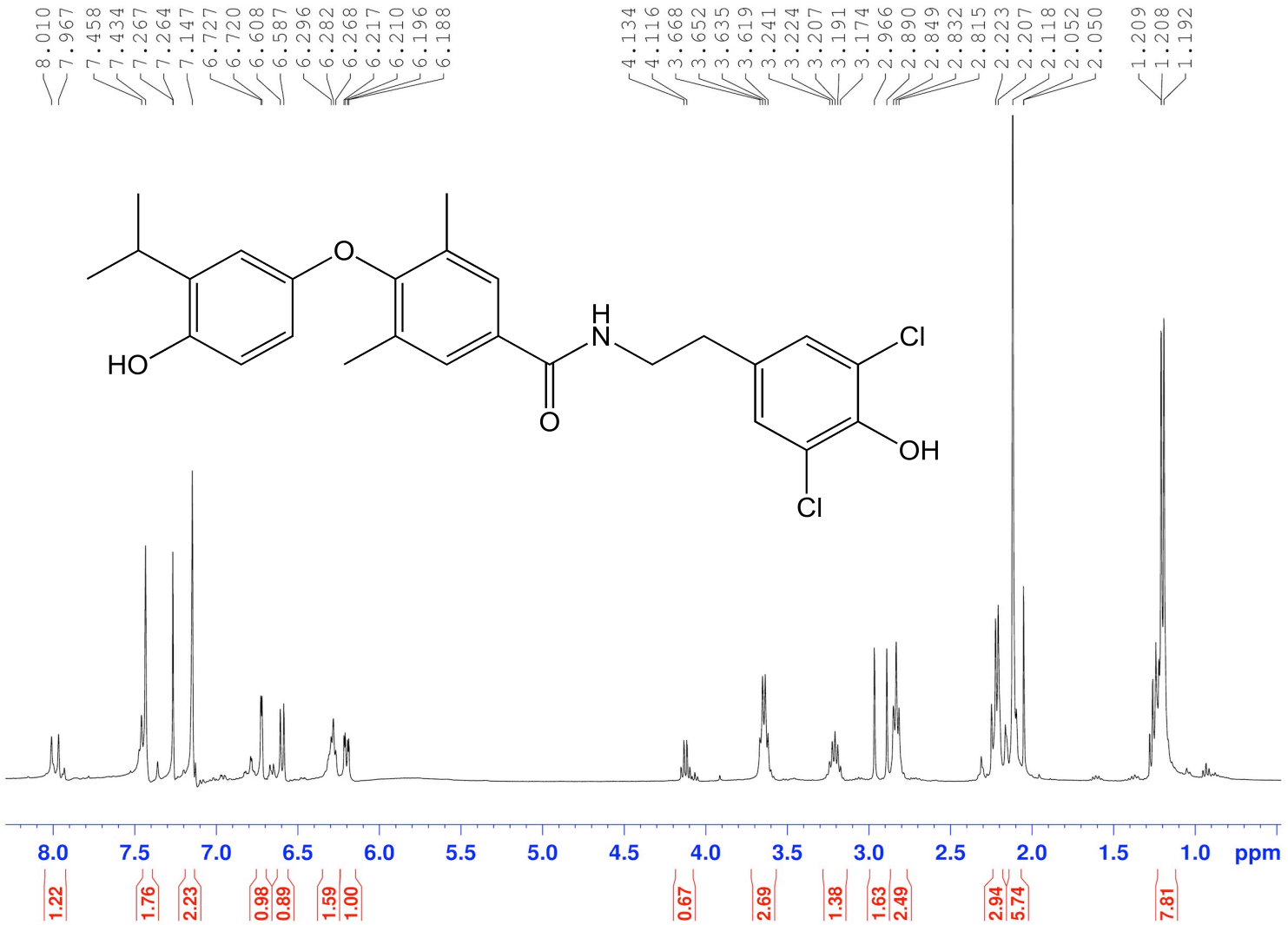
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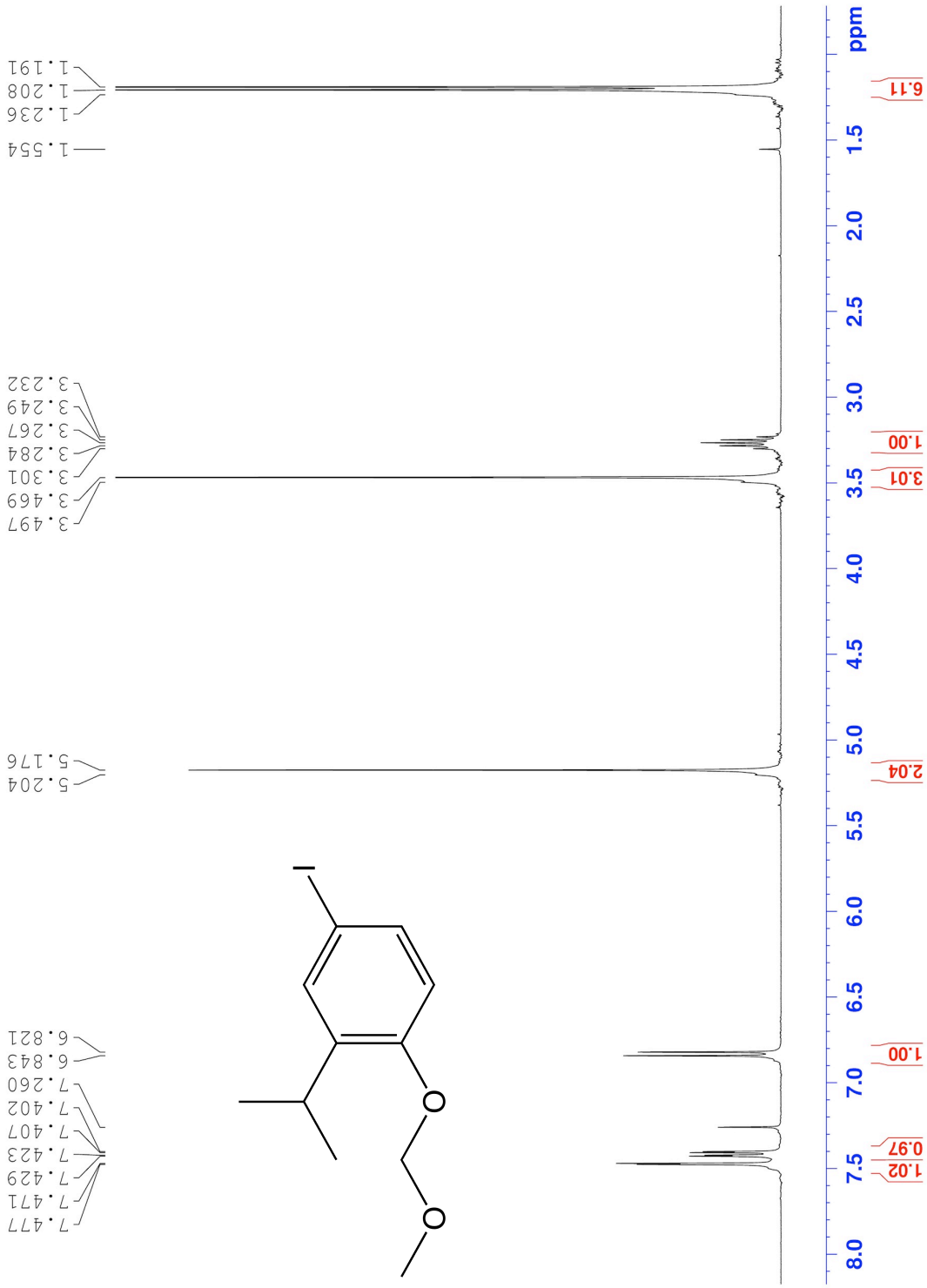
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O
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O
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OH
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N
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O
HO
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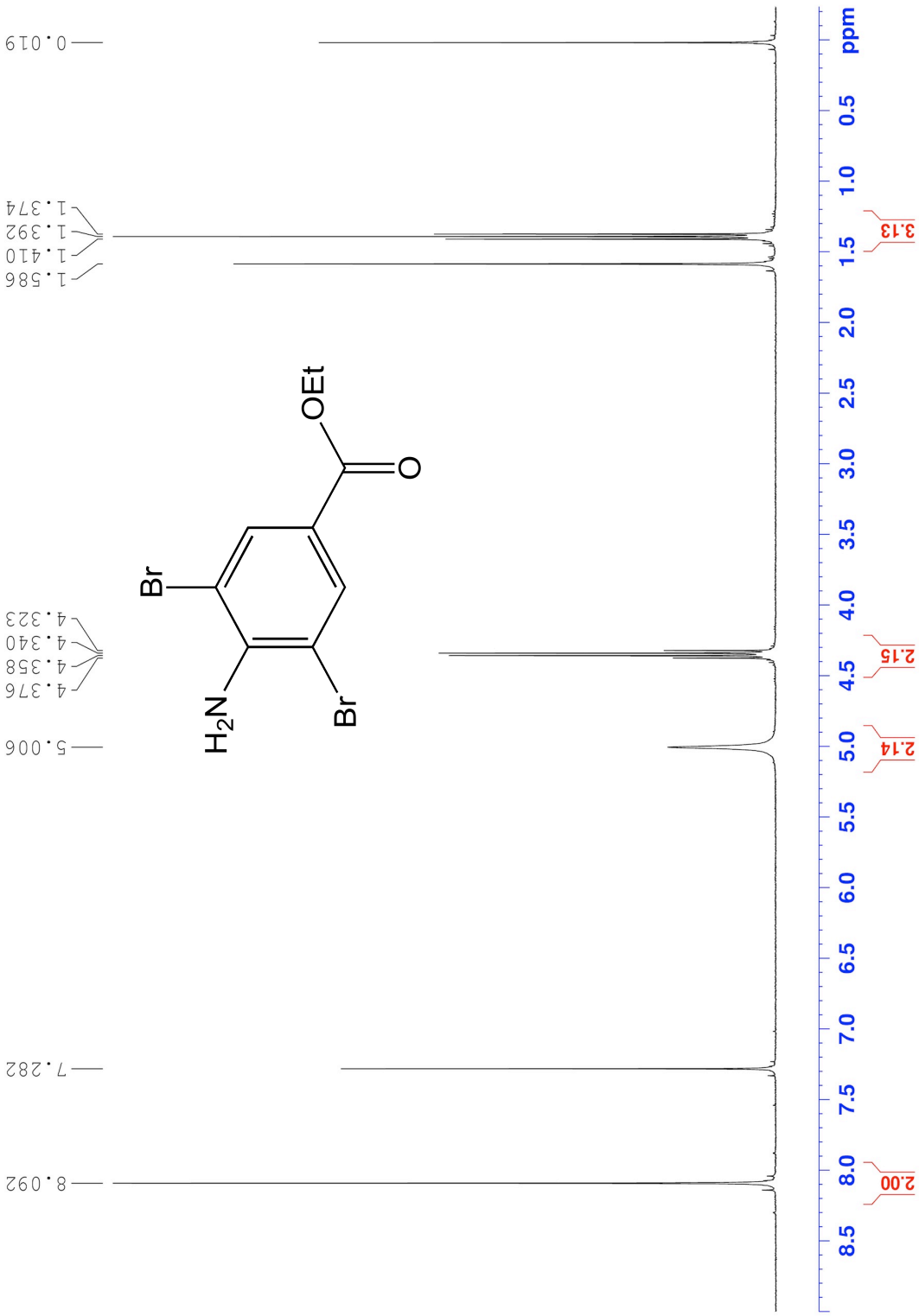
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Br
OEt
O
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6.5
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7.5
8.0
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2.14
2.00

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4.411
4.393
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I
Br
O
OEt

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6
7
8
9
10
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O
Br
Br
O

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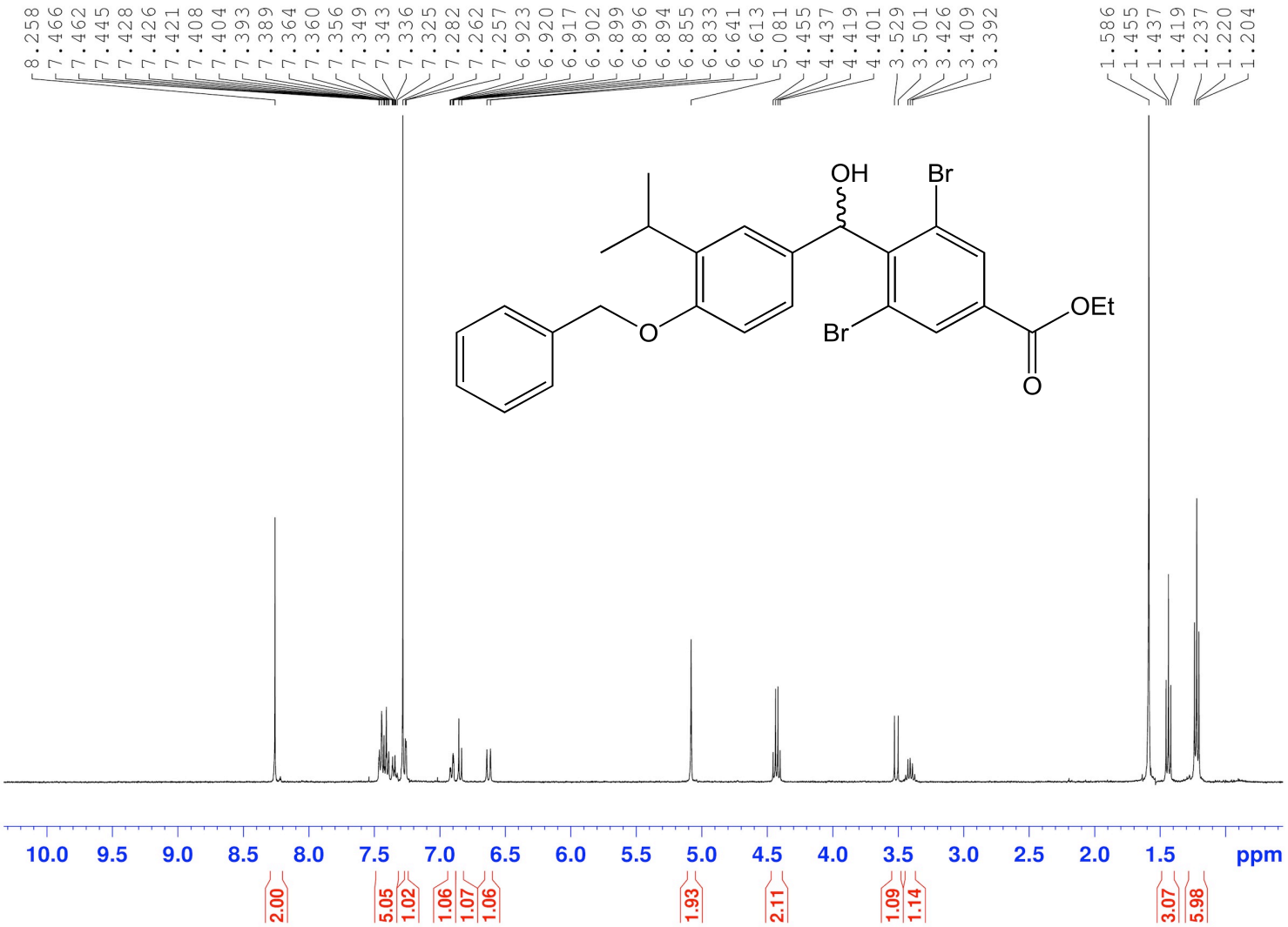
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7.257
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3.501
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3.409
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1.437
1.419
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OH
Br
Br
OEt
O
O
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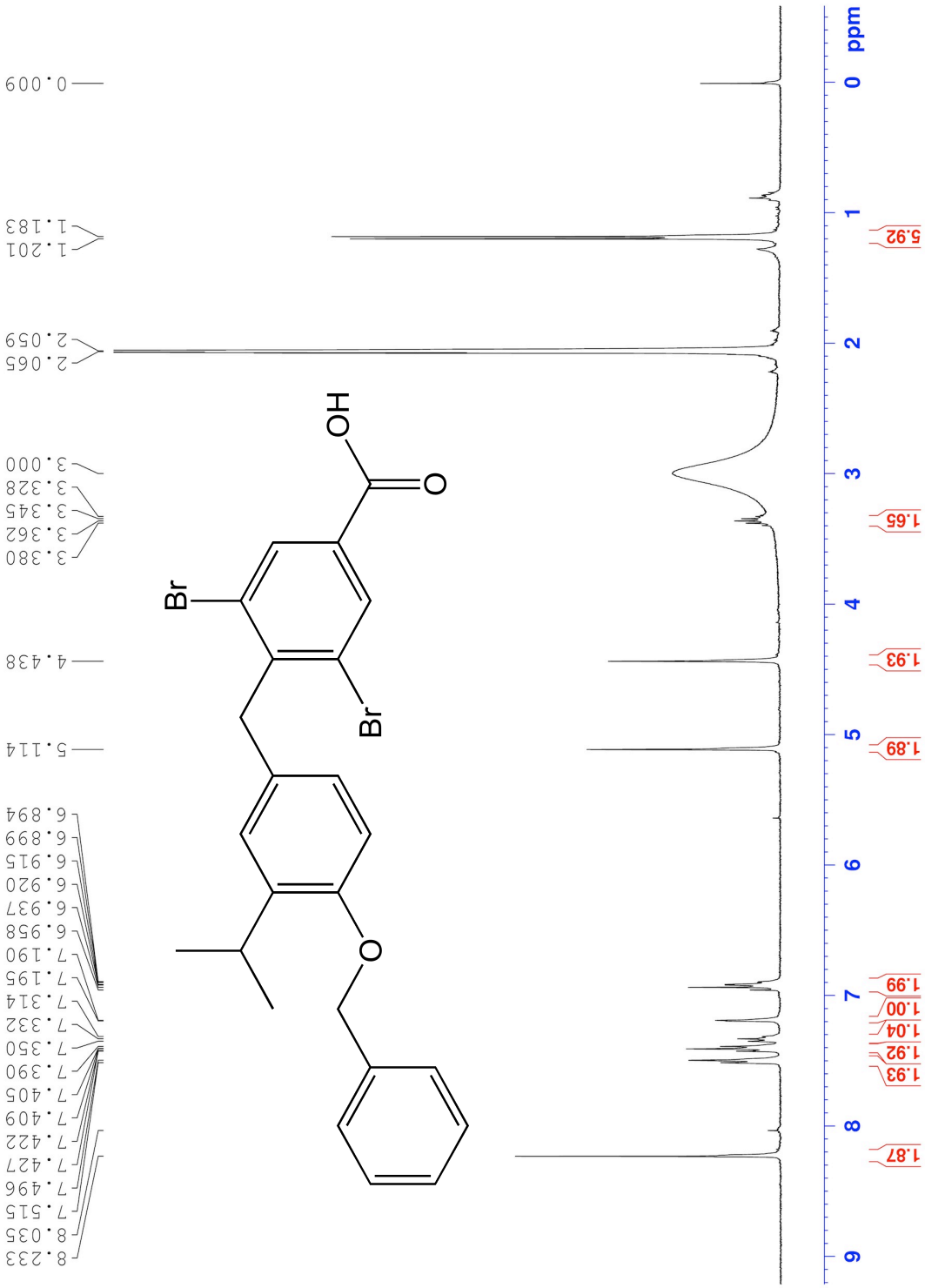
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O
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Br
O
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3
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6
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9
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Br
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O
OH
N
H
O
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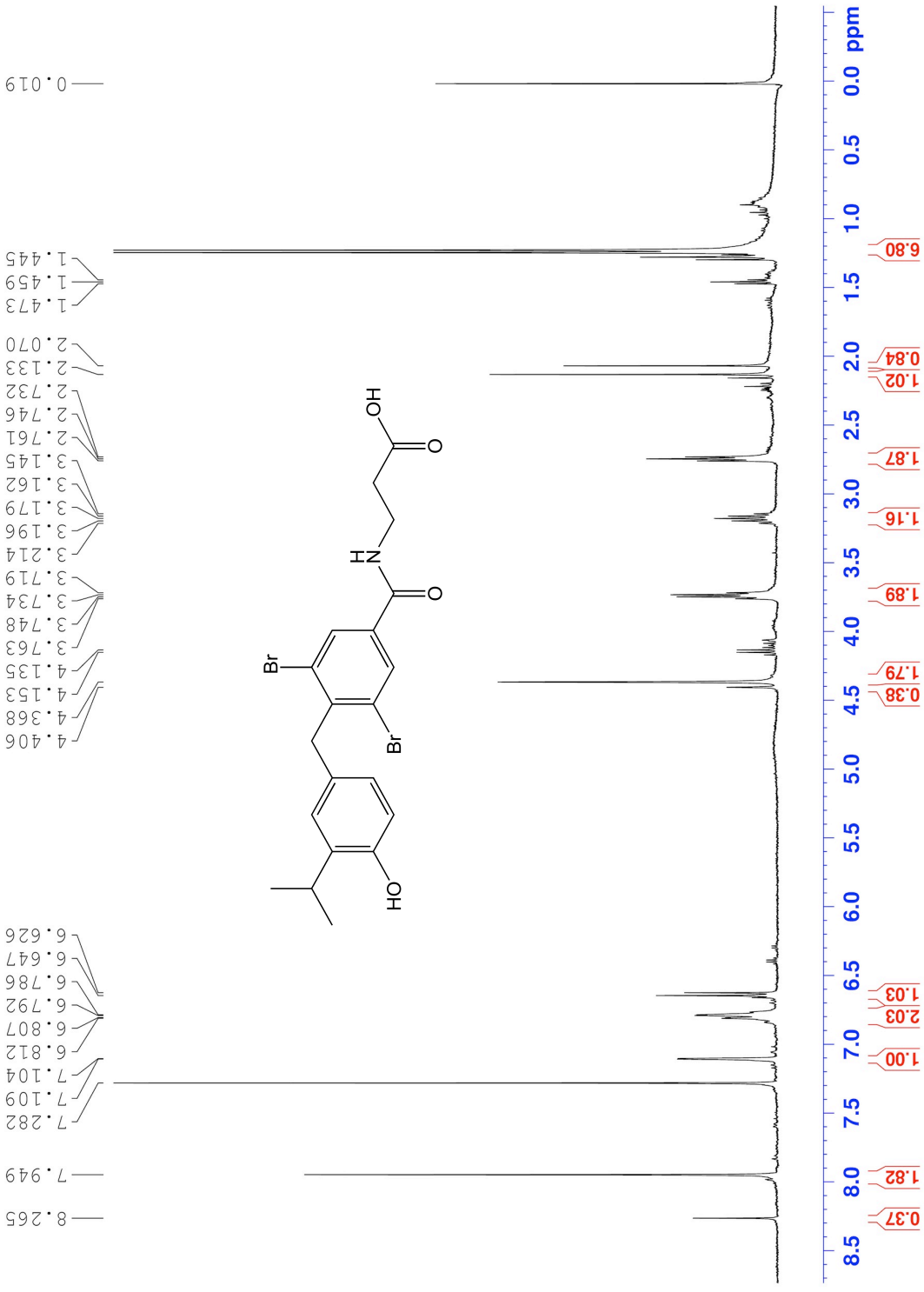
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O
OH
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H
N
OH
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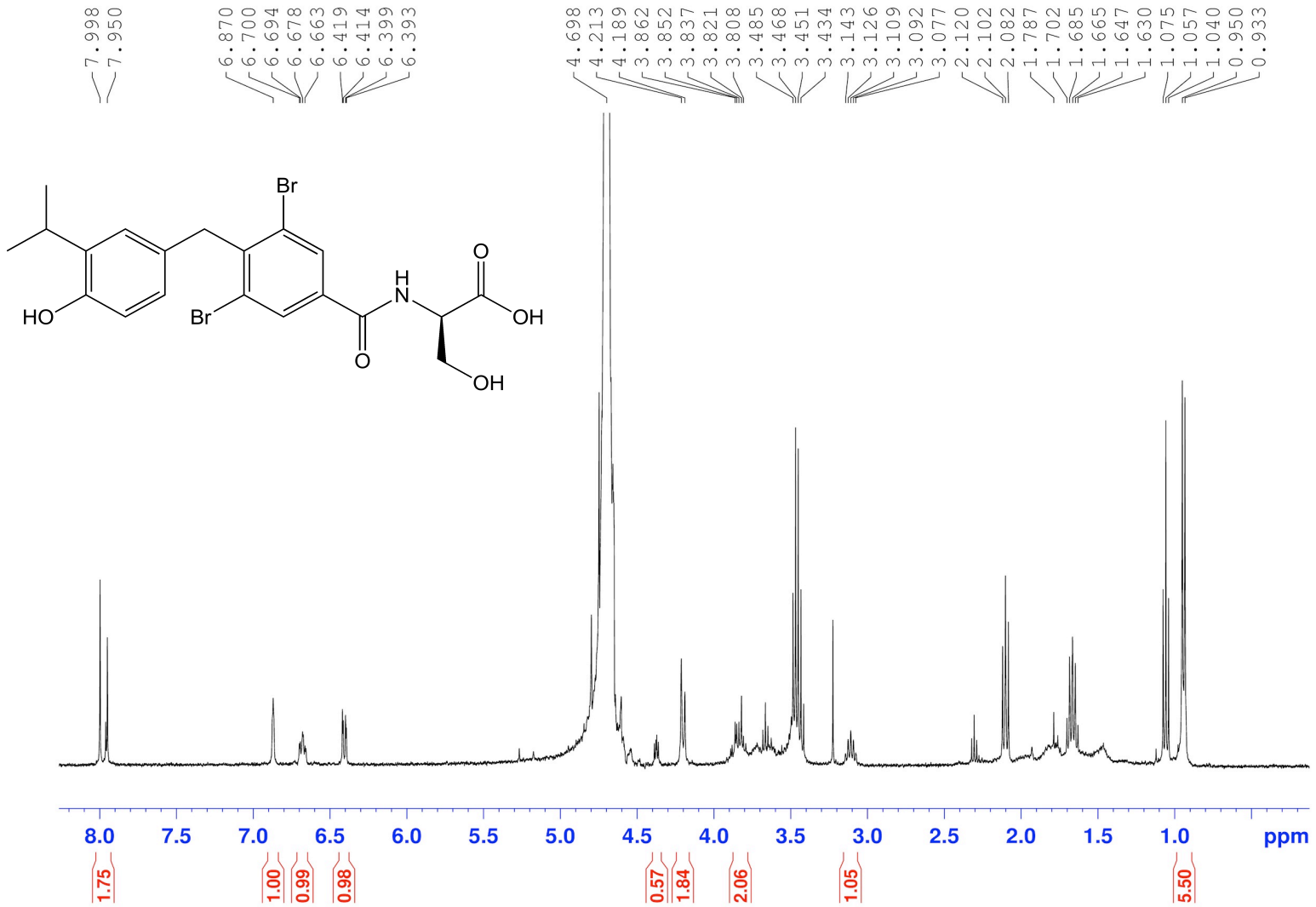
Br
Br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HO
Br
H
N
O
OH
O
OH
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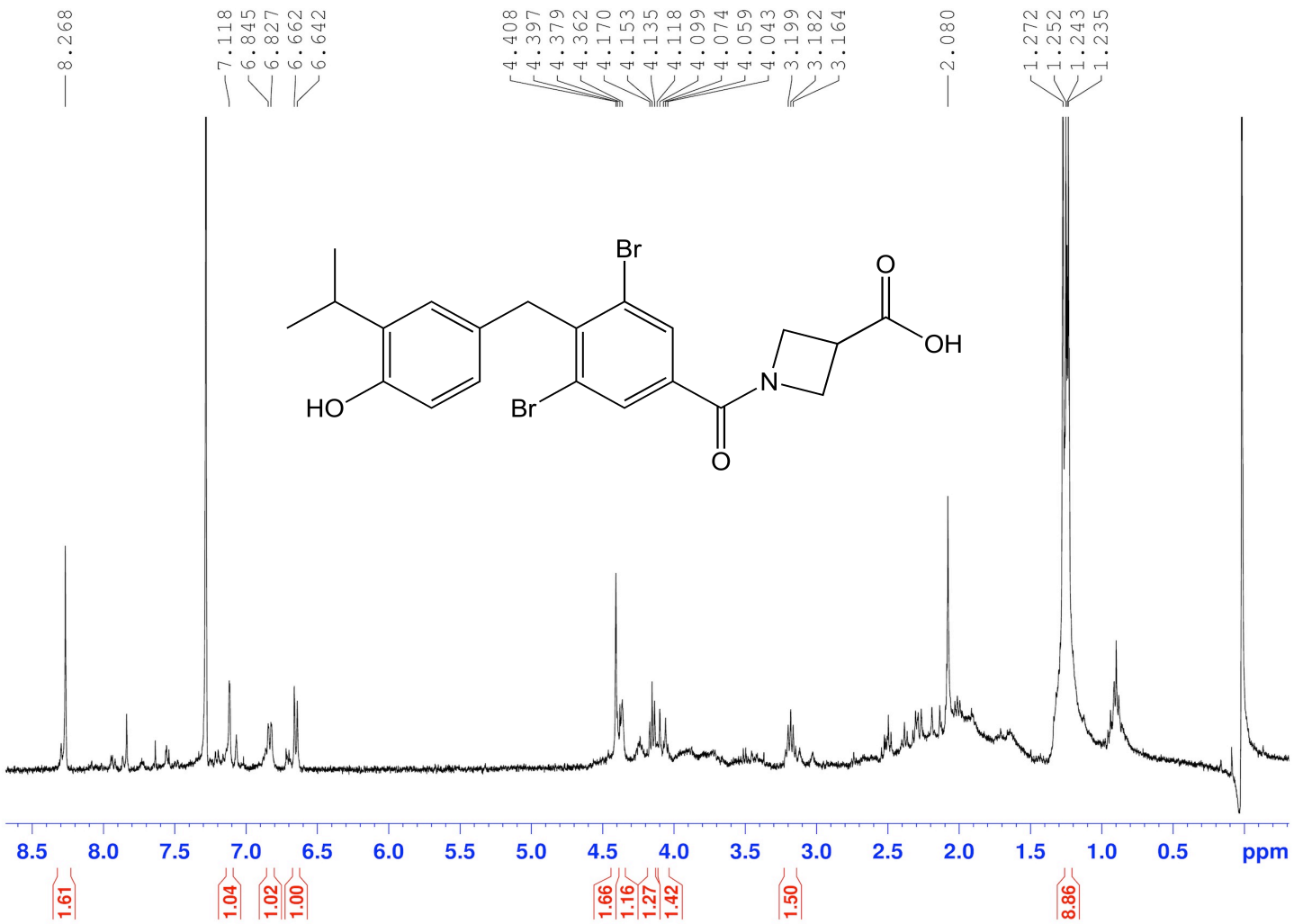
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HO
Br
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O
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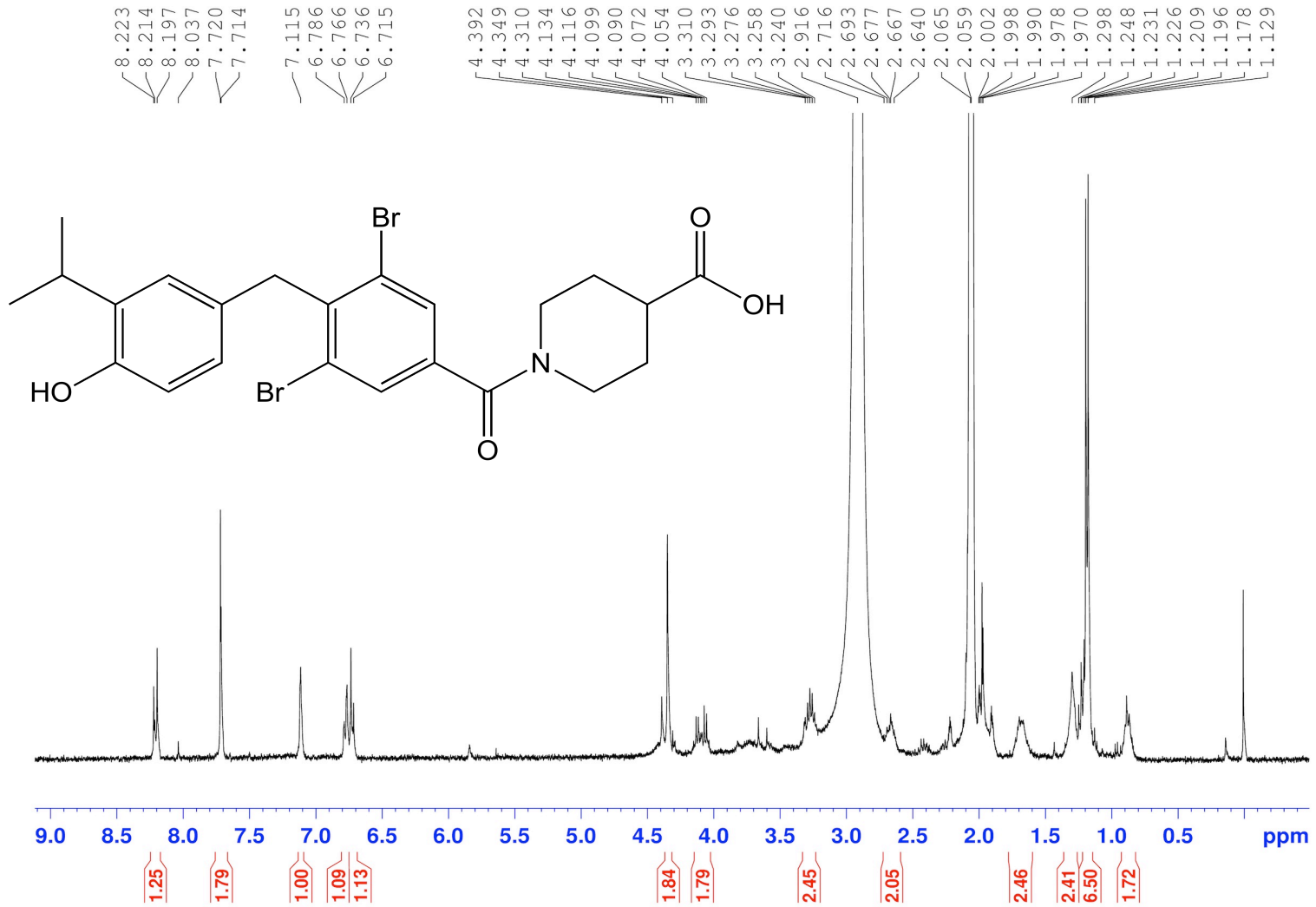
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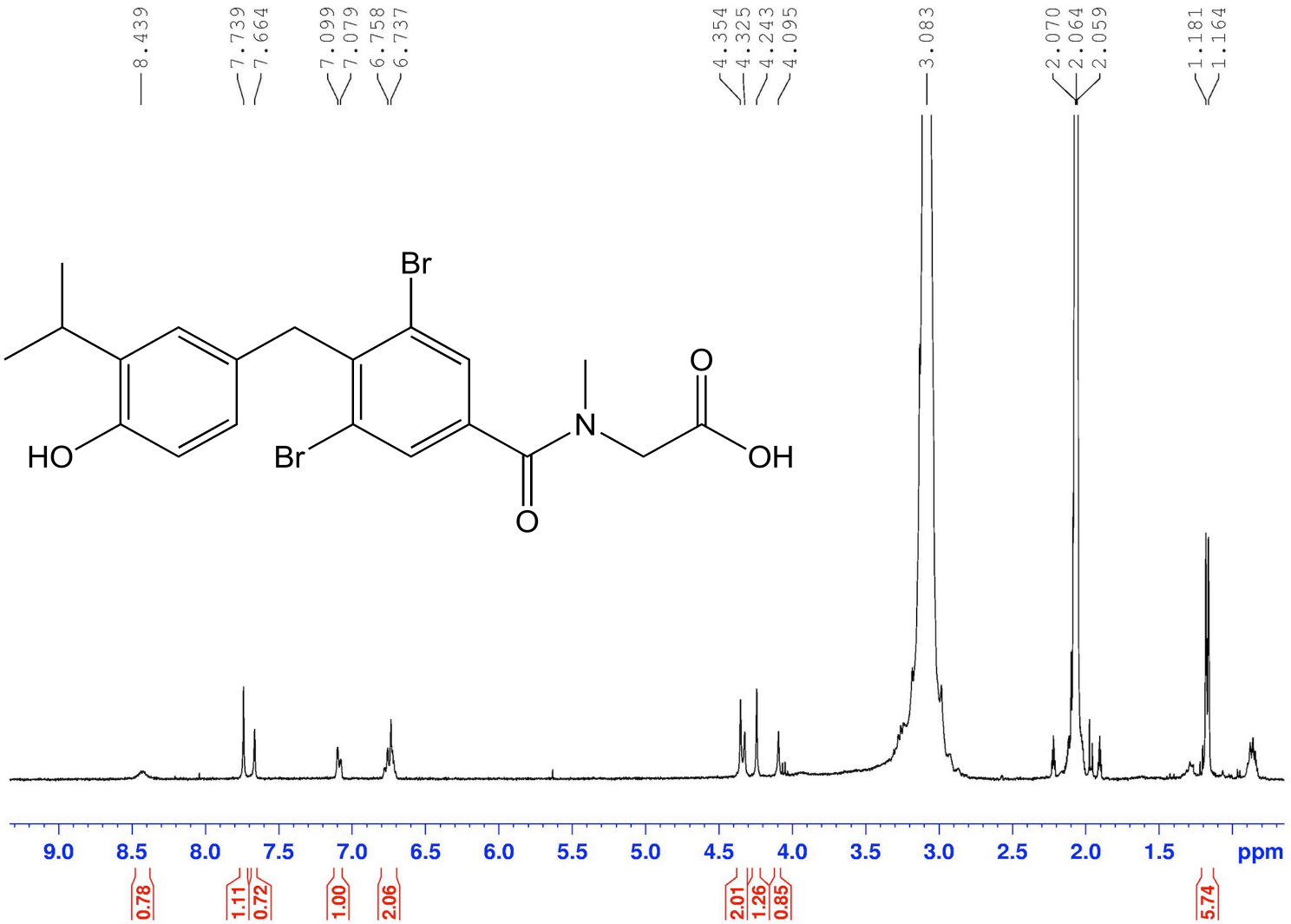
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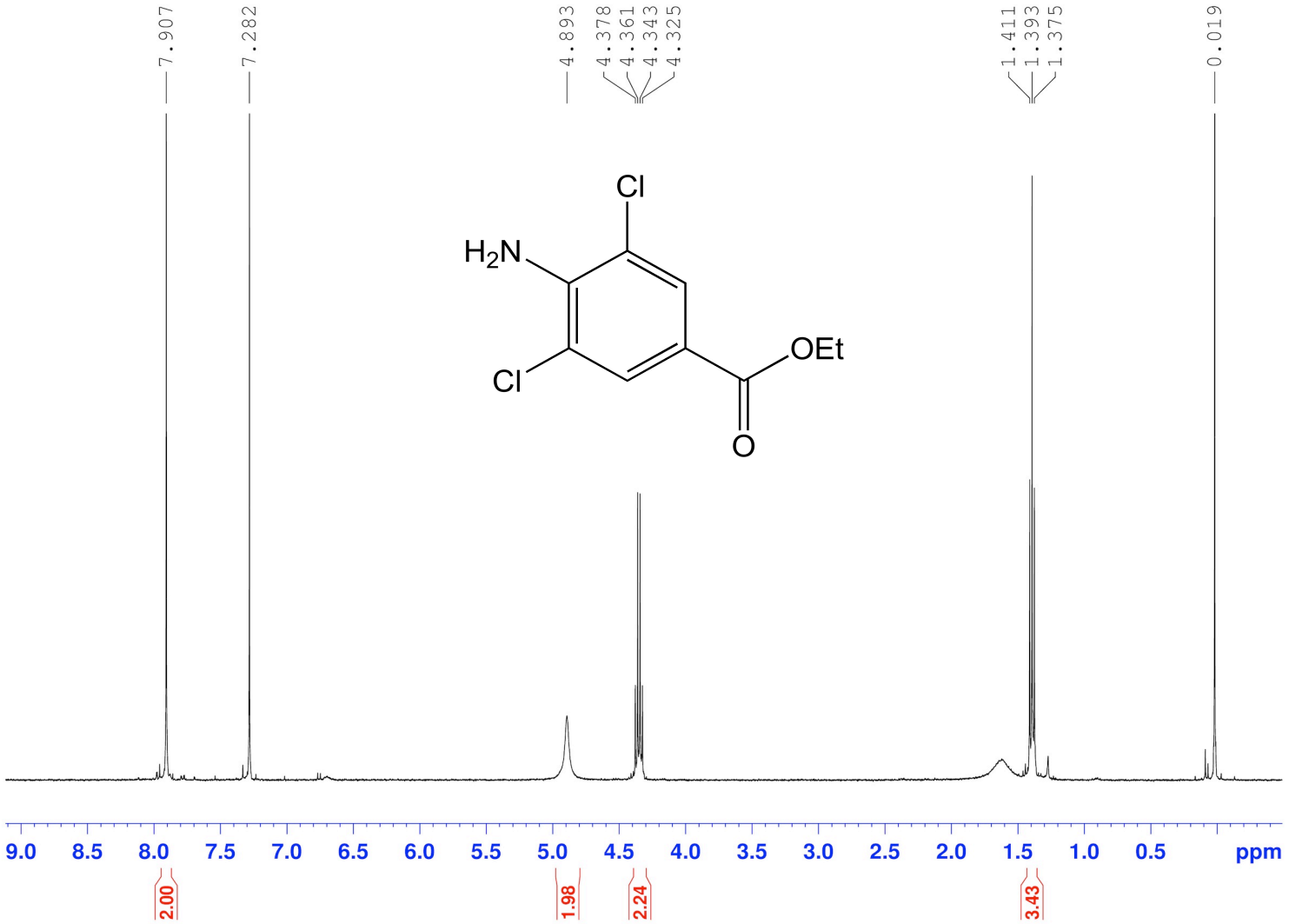
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Cl
OEt
O
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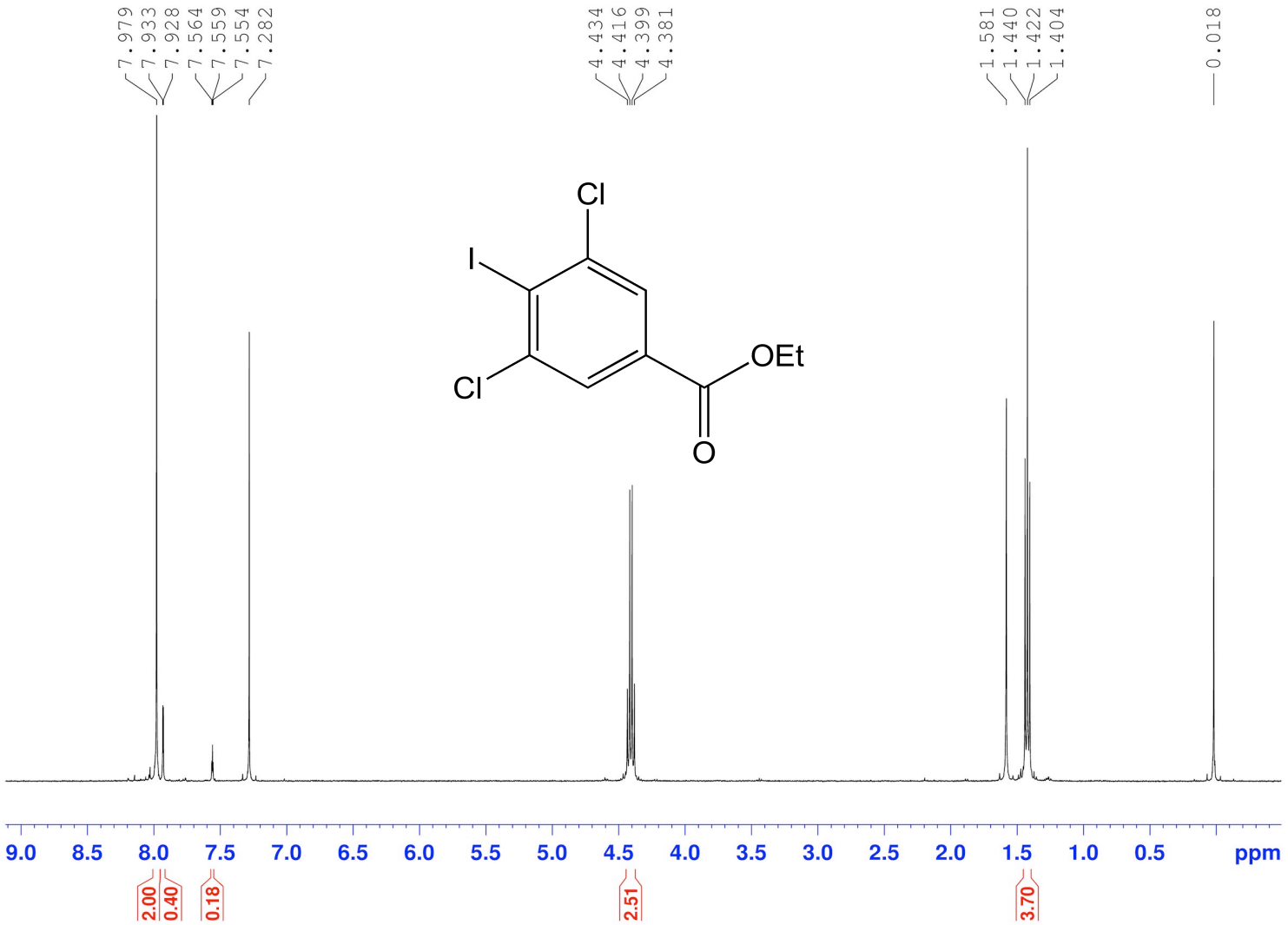
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I
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OEt
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6.5
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5.5
5.0
4.5
4.0
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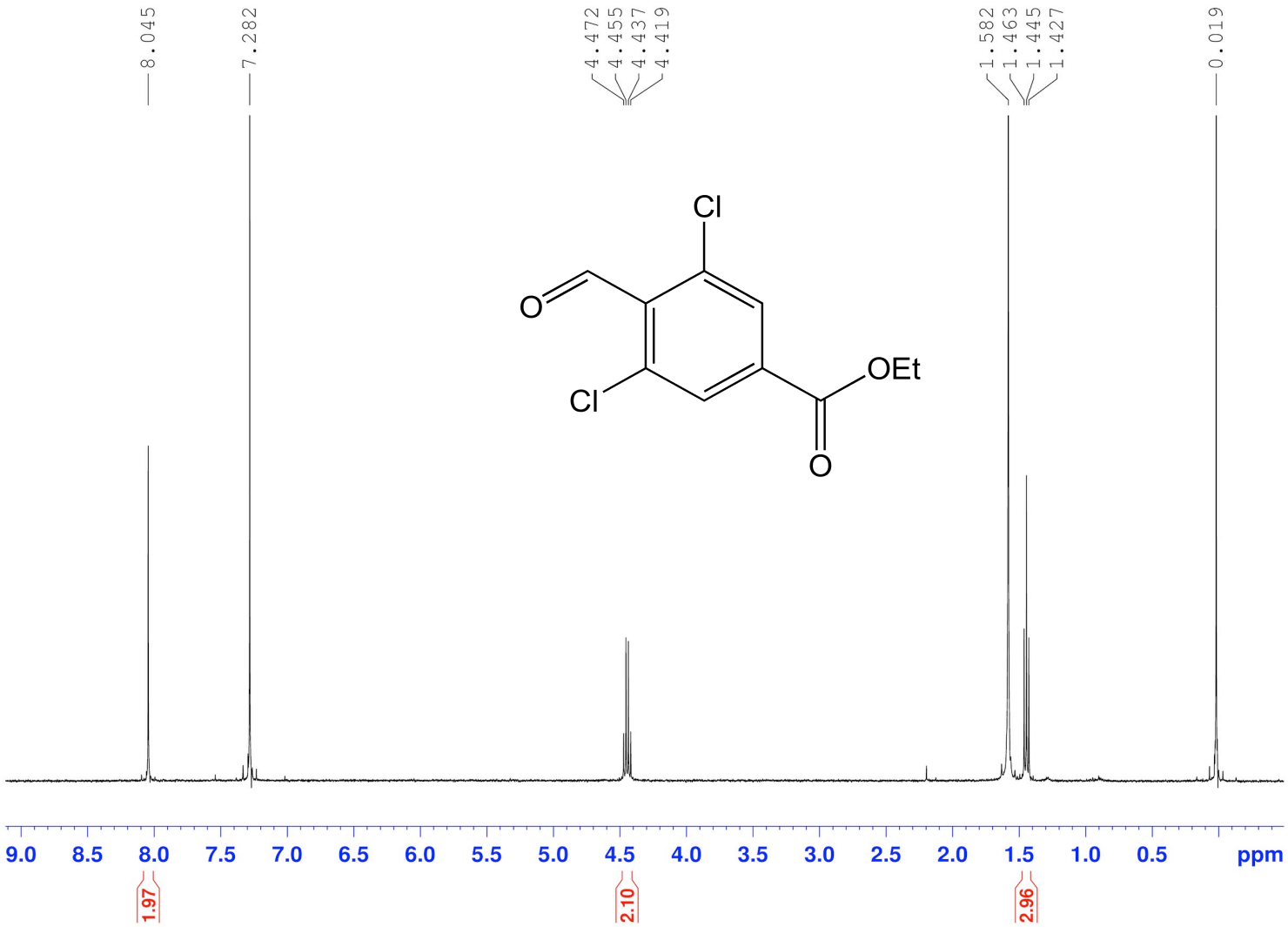
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O
Cl
OEt
O
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
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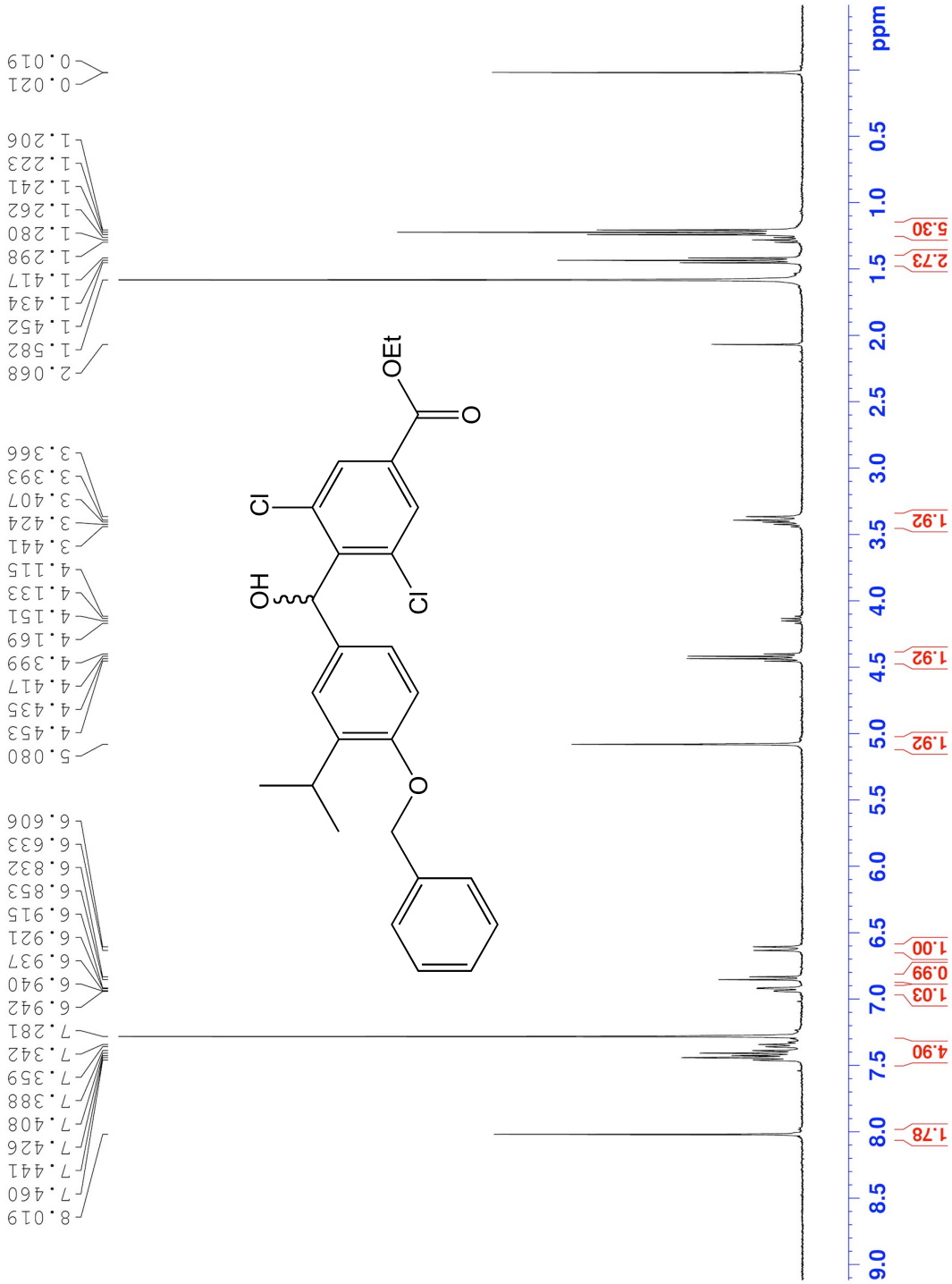
2.96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5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
1.78
4.90
1.03
0.99
1.00
1.92
1.92
1.92
2.73
5.30
OEt
O
Cl
OH
Cl
O
8.019
7.460
7.441
7.426
7.408
7.388
7.359
7.342
7.281
6.942
6.940
6.937
6.921
6.915
6.853
6.832
6.633
6.606
5.080
4.453
4.435
4.417
4.399
4.169
4.151
4.133
4.115
3.441
3.424
3.407
3.393
3.366
2.068
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1.280
1.262
1.241
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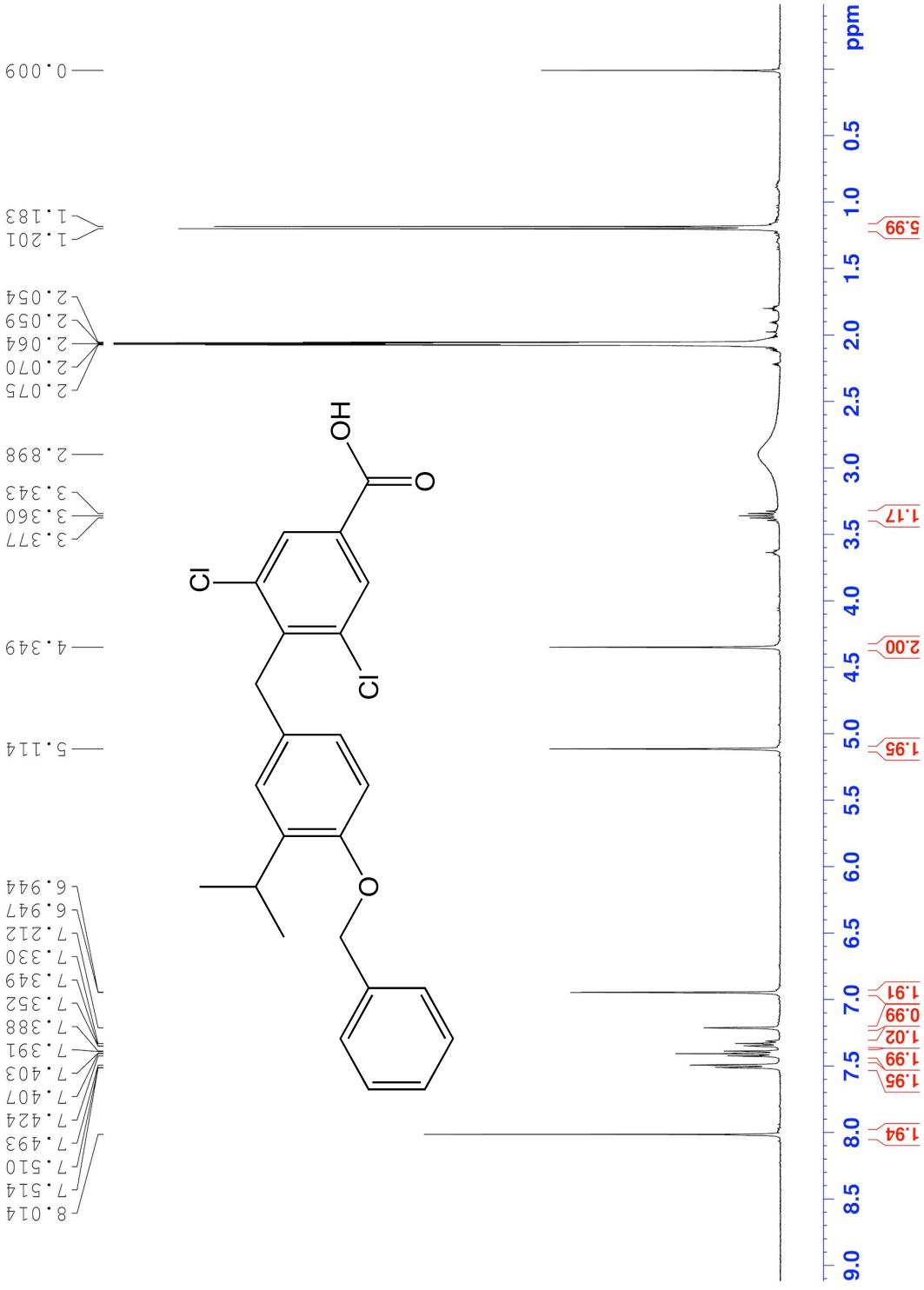
0.019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5.114
6.944
6.947
7.212
7.330
7.349
7.352
7.388
7.391
7.403
7.407
7.424
7.493
7.510
7.514
8.014
Cl
Cl
OH
O
O
ppm
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
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1.99
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0.99
1.91
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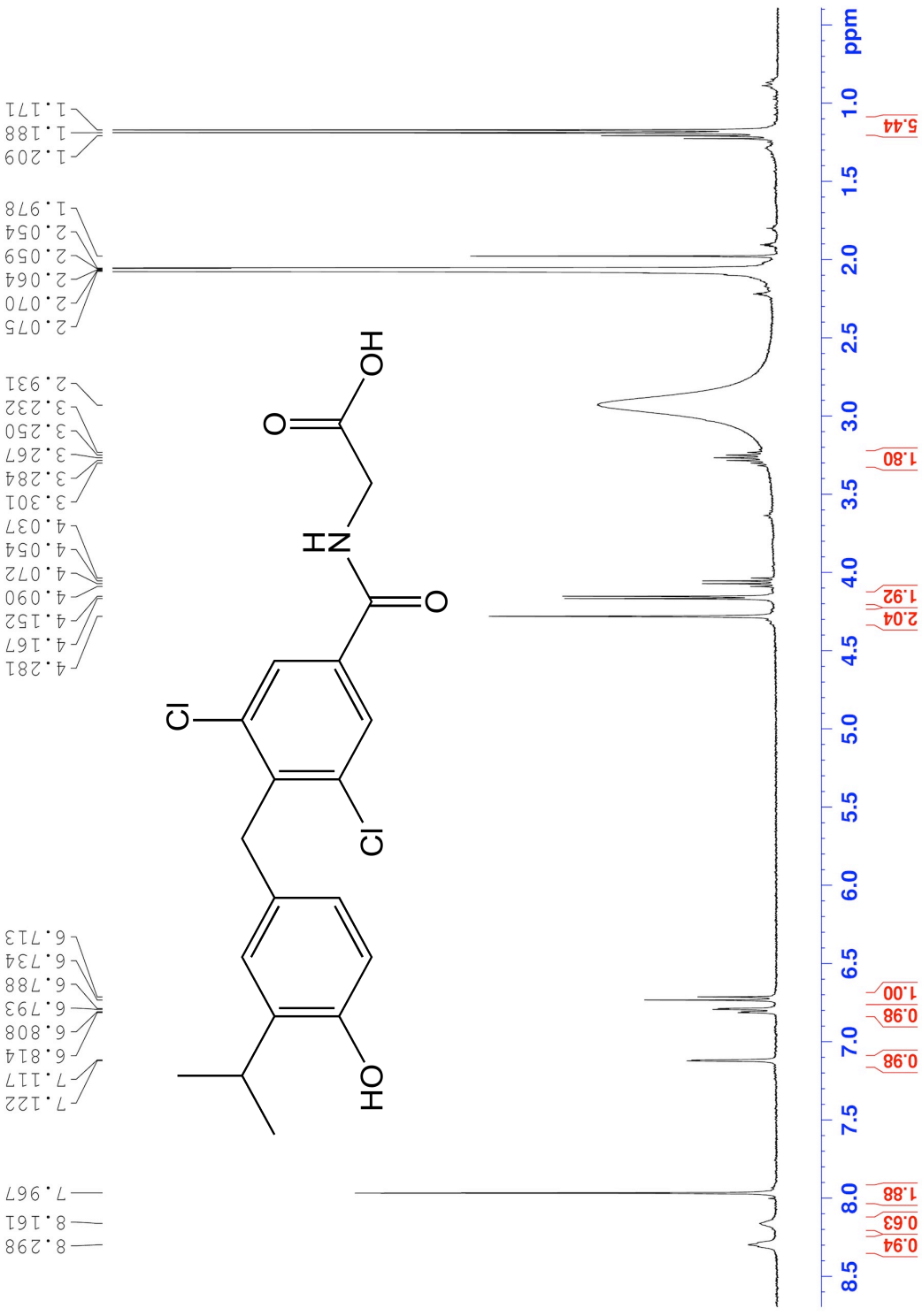
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6.734
6.713
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4.167
4.152
4.090
4.072
4.054
4.037
3.301
3.284
3.267
3.250
3.232
2.931
2.075
2.070
2.064
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2.054
1.978
1.209
1.188
1.171
ppm
8.5
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7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.94
0.63
1.88
0.98
0.98
1.00
2.04
1.92
1.80
5.44
HO
O
NH
O
Cl
Cl

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7.928
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7.111
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6.799
6.784
6.778
6.728
6.708
4.264
4.090
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4.054
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3.659
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3.636
3.628
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2.059
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0.89
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1.00
1.04
1.00
1.97
2.12
1.28
2.03
5.56
ppm
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6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
HO
Cl
Cl
NH
O
O
OH

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6.789
6.730
6.710
4.264
4.090
4.072
4.070
4.055
4.037
3.803
3.636
3.493
3.476
3.462
3.446
3.309
3.281
3.266
3.248
2.881
2.847
2.443
2.424
2.406
2.226
2.220
2.218
2.215
2.087
2.076
2.073
2.070
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2.065
2.062
2.059
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2.054
2.051
1.978
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1.224
1.209
1.207
1.191
1.189
1.172
1.169
OH
O
NH
O
Cl
Cl
ppm
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
1.00
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1.00
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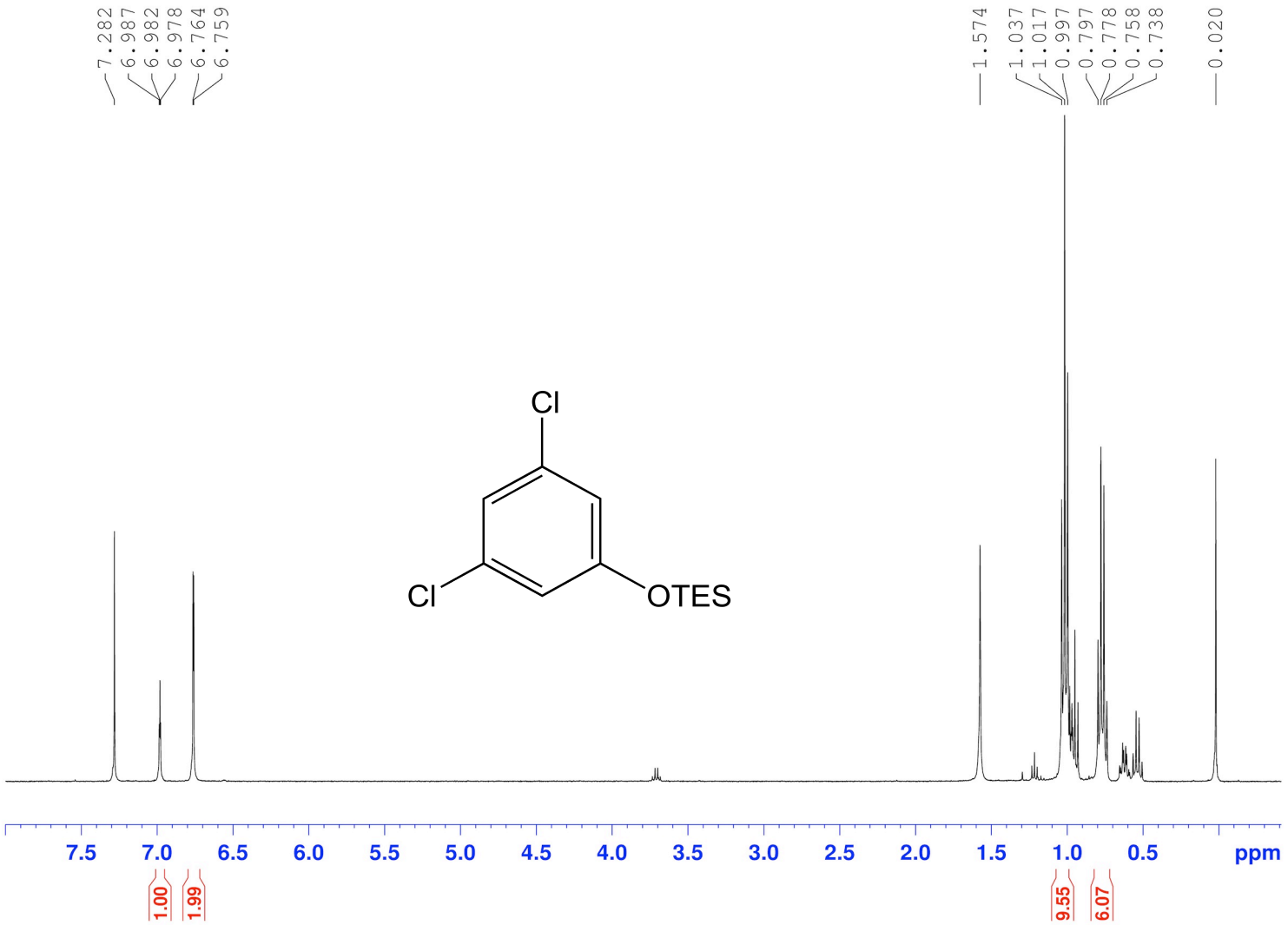
1.52
2.61
2.23
2.09
2.65
5.25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1.037
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0.997
0.797
0.778
0.758
0.738
0.020
Cl
Cl
OTES
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
1.00
1.99
9.55
6.07

10.368
7.011
2.867
2.064
0.010
2.00
1.05
0 ppm
1
2
3
4
5
6
7
8
9
10
OH
Cl
Cl
O

10.435
7.282
6.922
4.592
2.193
1.585
1.519
0.018
1.00
2.05
2.19
9.41
ppm
1
2
3
4
5
6
7
8
9
10
11
Cl
Cl
O
O
O
O

7.473
7.468
7.376
7.371
7.356
7.350
7.282
6.554
6.533
4.780
3.194
3.177
3.160
3.143
3.126
1.259
1.242
I
OH
ppm
1.03
0.09
1.03
1.00
1.10
1.05
6.01

7.282
7.262
7.257
7.003
6.982
6.938
6.936
6.932
6.930
6.920
6.517
6.490
5.207
4.533
3.500
3.372
3.354
3.337
3.320
3.303
3.251
3.224
2.068
1.585
1.520
1.232
1.215
1.198
0.019
OH
Cl
Cl
O
O
O
MOMO
ppm
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
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1.00
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1.97
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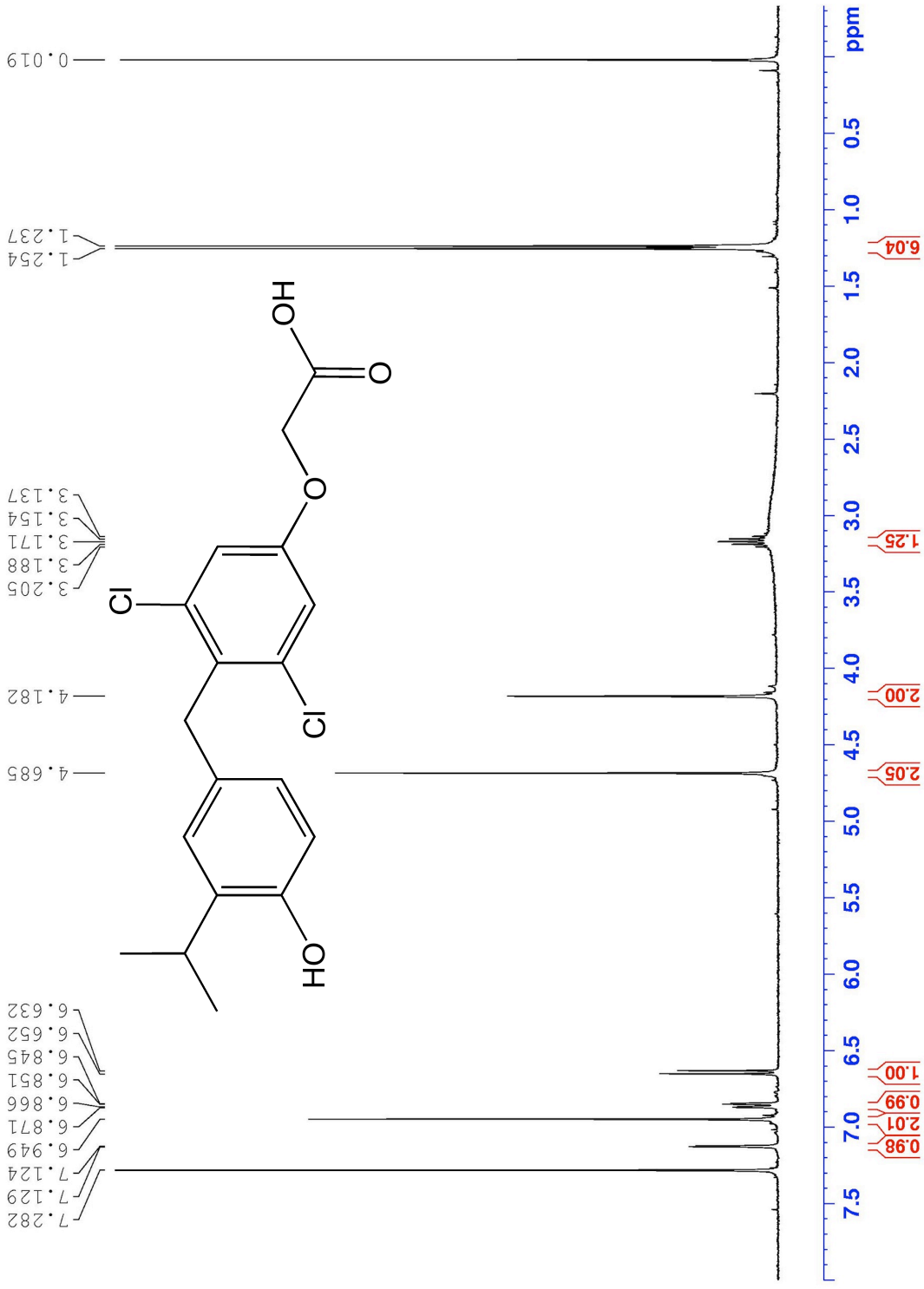
1.15
1.02
9.34
5.89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6.851
6.866
6.871
6.949
7.124
7.129
7.282
Cl
Cl
O
OH
O
HO
ppm
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
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7.5
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2.05
1.00
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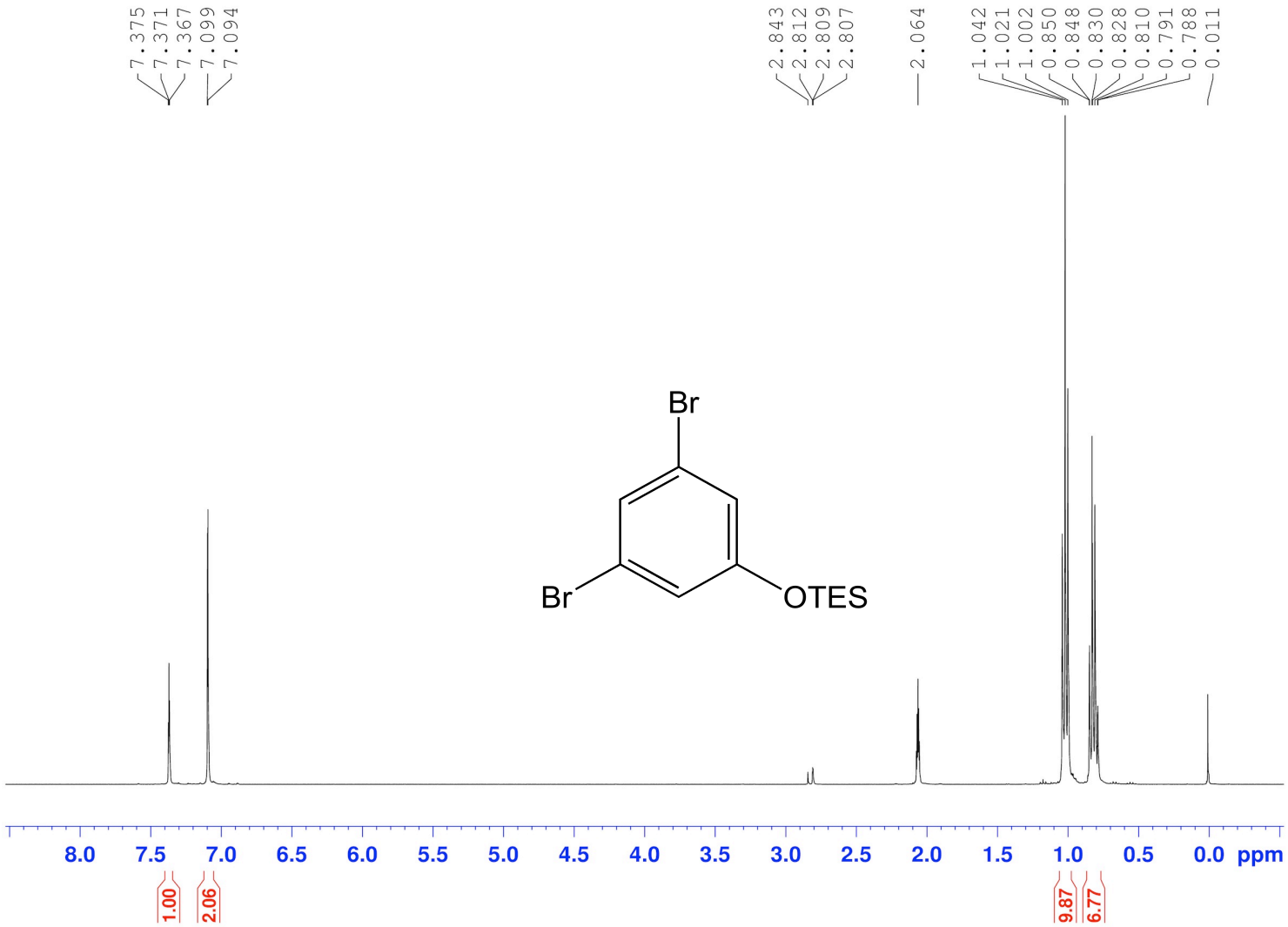
0.98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0.848
0.830
0.828
0.810
0.791
0.788
0.011
Br
Br
OTES
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm
1.00
2.06
9.87
6.77

10.161
7.266
2.966
2.064
0.009
1.00
1.93
0 ppm
1
2
3
4
5
6
7
8
9
10
11
OH
Br
Br
O

10.229
7.282
7.193
4.591
1.577
1.521
0.019
1.00
2.15
2.18
9.78
ppm
1
2
3
4
5
6
7
8
9
10
Br
Br
O
O
O
O

0.019
1.196
1.212
1.228
1.521
1.589
2.068
3.305
3.322
3.339
3.348
3.357
3.375
3.488
3.502
4.115
4.133
4.150
4.168
4.533
5.207
5.210
6.501
6.528
6.895
6.899
6.916
6.922
6.979
7.000
7.169
7.239
7.244
7.267
ppm
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1.5
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2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
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5.95
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2.07
2.86
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1.96
1.00
1.01
1.08
1.92
1.12
Br
OH
Br
O
O
O
MOMO

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3.161
3.178
3.196
3.213
3.495
4.257
4.279
4.506
4.685
6.633
6.653
6.813
6.833
7.109
7.152
7.187
7.282
Br
Br
HO
O
O
OH
ppm
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1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
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1.18
3.05
2.64
1.00
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1.03
2.06