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# Characterization of thyroid hormone receptor α (TRα)-specific analogs with varying inner- and outer-ring substituents

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**Abstract**—Analogs of the TRα-specific thyromimetic **CO23** were synthesized and analyzed in vitro using competitive binding and transactivation assays. Like **CO23**, all analogs bind to both thyroid hormone receptor subtypes with about the same affinity; however, modification of **CO23** by derivatization of the 3′ position of the outer-ring or replacement of the inner-ring iodides with bromides attenuates binding. Despite lacking a preference in binding to TRα, all analogs display TRα-specificity in transactivation assays using U2OS and HeLa cells. At best, several agonists exhibit an approximately 6–12-fold preference in transactivation when tested with TRα in HeLa cells. One analog, CO24, showed in vivo TRα-specific action in a tadpole metamorphosis assay.


## 1. Introduction

Thyroid hormone is a classic endocrine signaling hormone that mediates a wide variety of regulatory events affecting growth, development, and metabolism.[1–3] In circulation, thyroid hormone exists as a pro-hormone, 3,5,3′,5′-tetraiodo-L-thyronine (**T**$_4$, Fig. 1), but is converted to its principal active form, 3,5,3′-triiodo-L-thyronine (**T**$_3$, Fig. 1), by deiodination of one outer-ring position by deiodinases.[4,5] Compound **T**$_3$ exerts its actions by translocating into the nucleus of target cells and binding to the ligand binding domain (LBD) of thyroid hormone receptors (TRs) which are members of the nuclear receptor superfamily of ligand responsive transcriptional regulators.[4] There are two genes for TR, TRα and TRβ, that give rise to an ensemble of four different isoforms by means of alternative splicing or differential promoter usage: TRα$_1$, TRα$_2$, TRβ$_1$, and TRβ$_2$.[4,6] Ligand binding to TR induces a conformational change in the LBD allowing it to induce or repress gene expression by recruitment of coactivator or corepressor proteins.[4] Selective thyromimetics are T3 analogs that, unlike T3, have tissue selective actions.[1,2] A current guiding hypothesis is that TR subtype selectivity may correlate with tissue selective actions and TRβ-selective compounds such as GC-1 (Fig. 1) are being developed as potential therapeutic agents for hyperlipidemia and obesity. Until recently, little success had been reported on the development of TRα-selective thyromimetics.

We recently reported on the synthesis and characterization of **CO23**, the first potent thyromimetic with TRα-specific effects in vitro and in vivo. This compound demonstrated 3- to 5-fold TRα-specificity in transactivation assays using U2OS and HeLa cells, respectively.[7] Despite not having an overwhelming preference for TRα activation, **CO23** has profound effects on precocious *Xenopus laevis* tadpole metamorphosis that correlates with the selective activation of TRα. In this study, we have prepared a focused panel of **CO23** analogs and evaluated them for TRα selectivity in vitro and in vivo.

*Keywords*: Thyroid hormone receptors; Thyroid hormone receptor α; Thyroid hormone receptor β; **CO23**; **CO23** analogs; GC-1; Cardiac specific; Liver specific; *Xenopus laevis*; Metamorphosis; Induced metamorphosis; In vivo.
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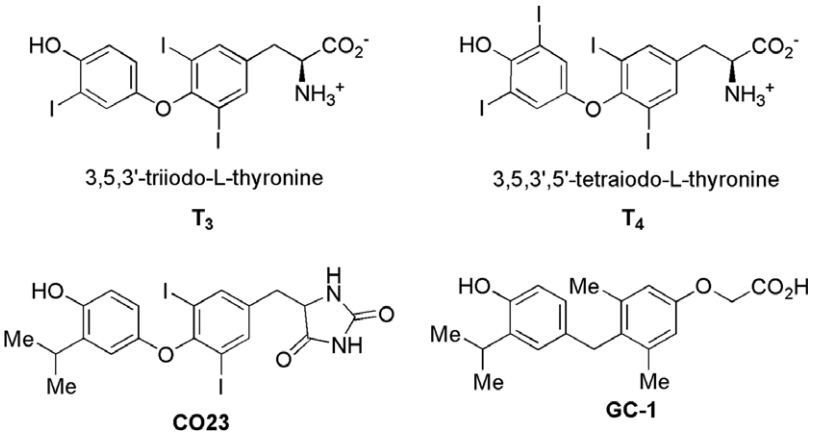



**Figure 1.** Structures of $T_3$, $T_4$, **CO23**, and **GC-1**.

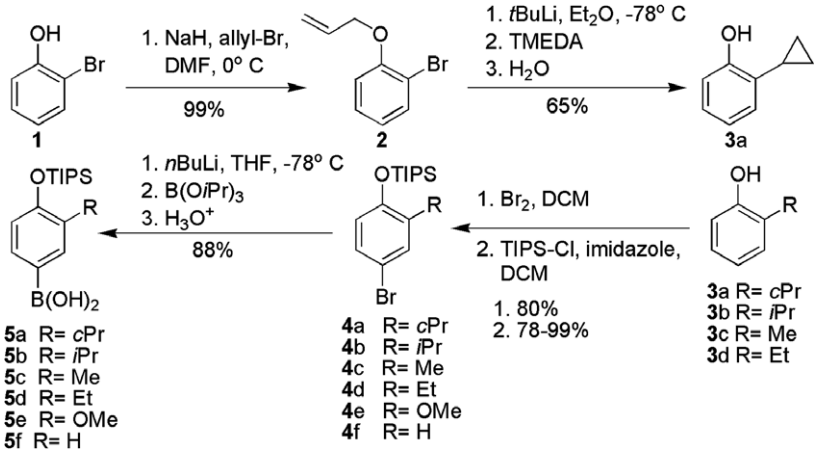
## 2. Chemistry

The hydantoin moiety attached to position one of the inner-ring by a methylene linker was deemed necessary for conferring TRα-specificity,[7] and hence to improve TRα-specificity, additional modifications to inner- and outer-ring substituents were examined. Substitution of the outer-ring was achieved by preparation or purchase of *para*-brominated phenols with varying groups in the *ortho*-position followed by TIPS protection (Scheme 1). One of the rare *para*-brominated phenols was generated by protection of 2-bromophenol (**1**) using allyl bromide which gives rise to allyl 2-bromophenyl ether (**2**). Lithiation of **2** causes it to undergo an intramolecular carbolithiation/1,3-elimination reaction that gives rise to 2-cyclopropylphenol (**3a**).[8] After generation of TIPS protected bromophenols (**4a**–**f**), they were all converted to boronic acids (**5a**–**f**) by treatment with *n*-butyllithium followed by addition of triisopropylborate and 3 N hydrochloric acid and used at a later stage for the generation of biaryl ethers (Scheme 2).[7]

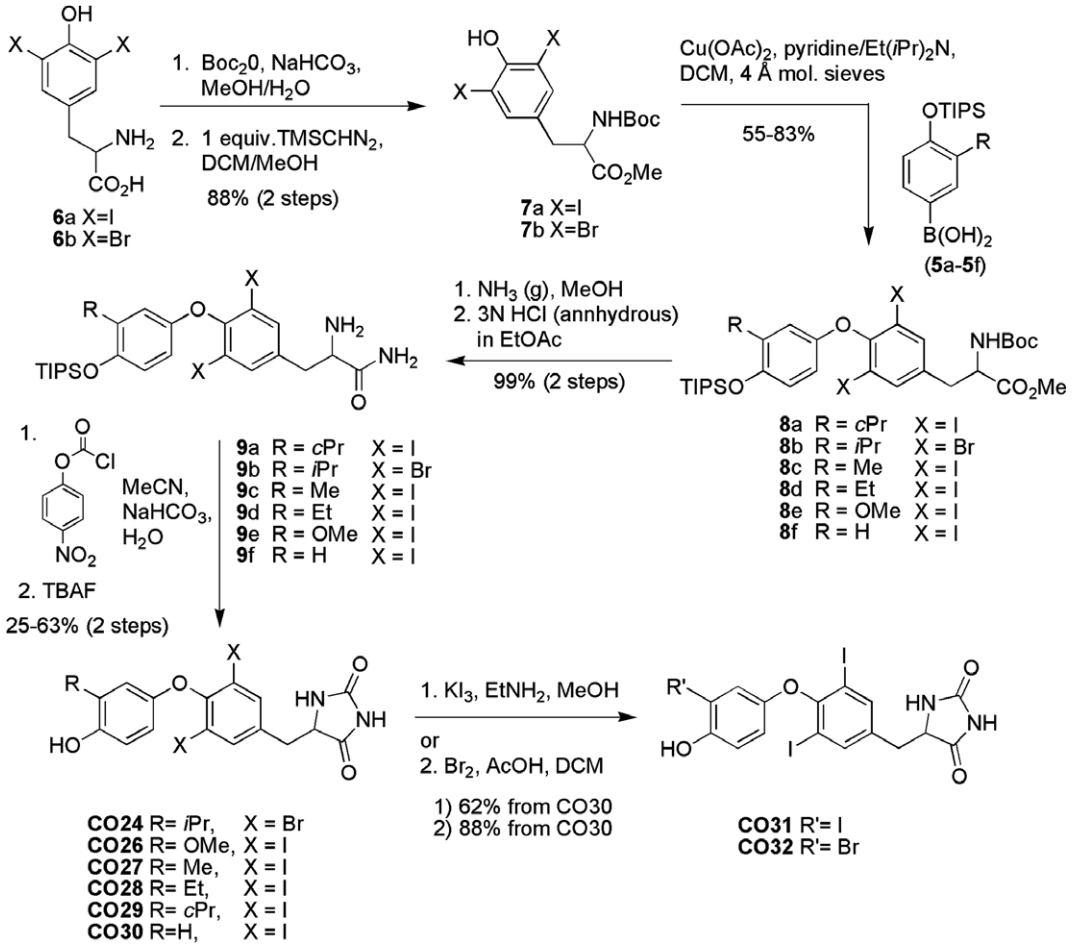
At this stage, another level of thyromimetic diversity is achieved by starting with either diiodo-L-tyrosine or dibromo-L-tyrosine (**6a**–**b**) and converting them to *N*-Boc-3,5-dihalo-L-tyrosine methyl esters (**7a**–**b**).[7] Compounds **7a**–**b** and boronic acids **5a**–**f** were coupled under Evan's conditions using cupric acetate as a catalyst leading to biaryl ethers (**8a**–**f**).[7] Amidation of **8a**–**f** in methanol saturated with ammonia gas and Boc-deprotection yields biaryl ethers with amino acid amide side chains (**9a**–**f**).[7] This side chain undergoes cyclization to form the imidazolidinedione after treatment with *para*-nitrophenylchloroformate, sodium bicarbonate, and water.[7] Deprotection of the TIPS groups with tetrabutylammonium fluoride leads to several **CO23** analogs (**CO24** and **CO26**–**CO30**). The iodination[9] or bromination[7] of the 3′ position of **CO30** leads to two further analogs, **CO31** and **CO32**.

## 3. Biological evaluation

The biological activity of the aforementioned **CO23** analogs was measured in vitro using $^{125}I$–$T_3$ competitive binding and transactivation assays. Replacement of inner-ring iodides with bromides causes a ~10-fold decrease in binding (**CO24** vs **CO23**). TR ligand activation in U2OS cells (Table 1) showed that **CO24** was not TRα-specific compared to the $T_3$ control. In U2OS cells it is important to compare the potencies




**Scheme 1.** Synthetic route used for the synthesis of TIPS-protected, 4-hydroxyphenyl boronic acid.




**Scheme 2.** Synthesis of **CO24** and **CO26–CO32**.

of test ligands to that of $T_3$ as thyroid hormone shows a difference in activation of TR$\alpha_1$ and TR$\beta_1$ using a synthetic thyroid hormone response element driven luciferase reporter construct. However, in HeLa cells, a cell line where thyroid hormone consistently shows equal activation of both TR subtypes, not only does **CO24** show fourfold TR$\alpha$-selectivity in potency, it is also TR$\alpha$-selective in terms of efficacy in that it causes transcriptional activity to plateau at a level that is twice as high as $T_3$ (Table 1 and Fig. 2a). In vivo,

**Table 1.** Binding affinity and potency of **CO23** analogs

| Compound | $K_d$ and $EC_{50}$ values (nM) | | | | | |
|---|---|---|---|---|---|---|
| | Binding affinity ($K_d$)[a] | | Transactivation in U2OS cells ($EC_{50}$)[b] | | Transactivation in HeLa cells ($EC_{50}$)[b] | |
| | Trα | TRβ | Trα | TRβ | TRα | TRβ |
| $T_3$[c] | 0.058 | 0.081 | 2.4 ± 0.4 | 11 ± 2 | 2.4 ± 0.5 | 2.4 ± 0.5 |
| **CO23**[c] | 1.2 ± 0.2 | 1.7 ± 0.3 | 34 ± 4 | 390 ± 3 | 11 ± 1 | 58 ± 1 |
| **CO24** | 17 ± 1 | 18.4 ± 1 | 128 ± 5 | 421 ± 3 | 8.4 ± 3 | 32 ± 3 |
| **CO26** | 82 ± 21 | 119 ± 30 | 870 ± 160 | 8000 ± 1800 | 42 ± 6 | 265 ± 10 |
| **CO27** | 42 ± 13 | 53 ± 17 | 146 ± 28 | 4000 ± 1000 | 27 ± 6 | 180 ± 30 |
| **CO28** | 15 ± 6 | 18 ± 2 | 87 ± 14 | 1400 ± 170 | 10 ± 1 | 27 ± 6 |
| **CO29** | 25 ± 7 | 49 ± 5 | 145 ± 10 | 2000 ± 290 | 39 ± 4 | 100 ± 14 |
| **CO30** | 1700 ± 10 | 2000 ± 360 | >20,000[d] | >20,000[d] | >10,000[d] | >10,000[d] |
| **CO31** | 24 ± 3 | 36 ± 1 | 105 ± 8 | 2000 ± 170 | 21 ± 3 | 216 ± 80 |
| **CO32** | 43 ± 6 | 68 ± 10 | 206 ± 41 | 5200 ± 670 | 44 ± 1 | 530 ± 170 |

[a] Determined by means of an $^{125}I$–$T_3$ competitive binding assay and data are reported as mean $K_d$ ± standard error of the mean, $n = 3$.

[b] Determined through use of a TRE-driven dual-luciferase reporter assay in U2OS or HeLa cells and the data are reported as mean $EC_{50}$ values ± standard error of the mean, $n = 3$.

[c] See Ref. 7.

[d] Dose–response curves generated using **CO30** in transactivation assays did not plateau, and hence the $EC_{50}$ value is an approximated value based on extrapolation.

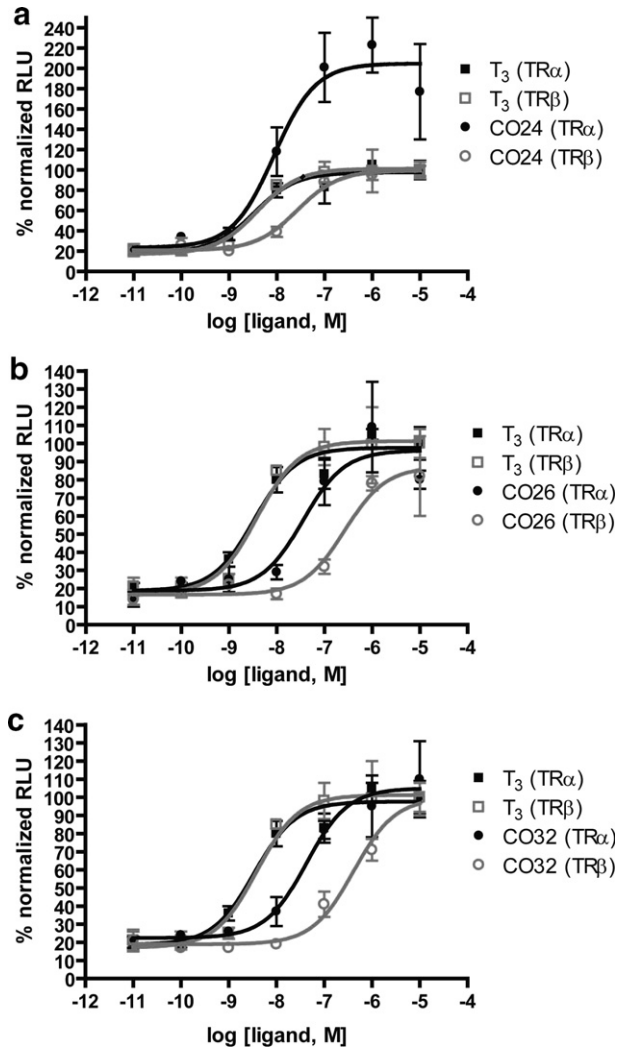



**Figure 2.** TRE-driven dual-luciferase reporter assays showing transactivation curves for $T_3$, (a) **CO24**, (b) **CO26**, and (c) **CO32** against $hTR\alpha_1$ and $hTR\beta_1$ in HeLa cells. Plots show mean of triplicates with SD.

*X. laevis* tadpoles precociously induced to undergo metamorphosis revealed gross morphological changes compared to untreated tadpoles, some of which are consistent with enhanced TRα activity. Tadpoles treated with 30 nM $T_3$ and 300 nM **CO24** both experienced resorption of tissue in the head and tail, experienced an overall decrease in size, and developed Meckel's cartilage (lower jaw); however, **CO24** treated tadpoles exhibited massive hind leg and fore leg development, a noticeably larger body size, and less resorption of tissue in the head compared to $T_3$ treated tadpoles (Fig. 3). Compared to **CO23** induced metamorphosis, **CO24** had similar effects on tadpole metamorphosis with some exceptions. For example, both **CO24** and **CO23** treated tadpoles developed more massive fore and hind limbs than $T_3$ treated tadpoles, but tadpoles treated with **CO24** developed the most massive limbs overall. Furthermore, **CO24** treated tadpoles experienced slightly greater resorption of larval tissue compared to **CO23** treated tadpoles, particularly in the head and tail, but this may be due to a slight decrease in TRα-specificity compared to **CO23**.[7]

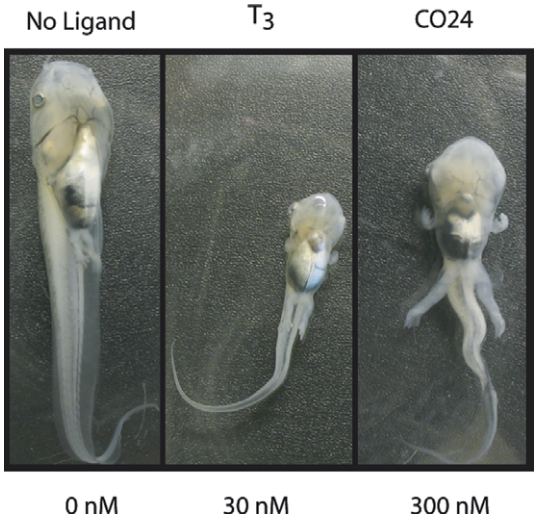



**Figure 3.** In vivo analysis of **CO24**. Induced metamorphosis of stage-53/54 tadpoles, $n = 3$, treated for 4-days with DMSO, 30 nM $T_3$, and 300 nM **CO24**.

**CO23** analogs with outer-ring substitutions all displayed a decrease in binding affinity and potency when tested in U2OS cells compared to **CO23** (Table 1). In U2OS cells, the potency of transactivation of the following substituents against TRα decreases from left to right: Ethyl > iodo > cyclopropyl > methyl > bromo > methoxy > hydrogen (Table 1). Unlike **CO23**, displaying only modest transcriptional activity in U2OS cells, they activate in the presence of TRβ very poorly. The best outer-ring analog had an $EC_{50}$ value of 1.4 μM compared to 390 nM for **CO23** when tested in U2OS cells in the presence of TRβ (Table 1).

Like **CO24**, in order to get a more direct measure of TRα-specificity, all analogs were assayed in HeLa cells. With the exception of **CO30**-only because its lack of activity in both U2OS and HeLa cells made it impossible to calculate and compare $EC_{50}$ values from their dose–response curves-all analogs demonstrated TRα-specificity with a few proving superior to **CO23**. Compounds **CO26**, **CO27**, **CO31**, and **CO32** demonstrated 6-, 6-, 10-, and 12-fold TRα-specificity, respectively, all of which are an improvement over **CO23**'s 5-fold TRα-specificity. Dose–response curves for **CO26** and **CO32** showing transactivation in the presence of TRα and TRβ clearly demonstrate TRα-specificity despite the compounds' inferior potencies compared to **CO23** (Fig. 2b and c). The rank order potency from left to right in HeLa cells is also impressive as some compounds are about equipotent to **CO23**-mediated TRα transactivation: Ethyl > iodo > methyl > cyclopropyl > methoxy > bromo > hydrogen.

## 4. Discussion

**CO23**, the first potent thyromimetic to demonstrate TRα-specificity in vitro and in vivo,[7] was derivatized at the 3, 5, and 3′ positions in order to generate thyromimetics with enhanced TRα-specificity. Small substitu-

ents with varying electronic properties were selected to be placed on the 3′ position as the structure activity data correlate with a decrease in potency with substituents larger than an isopropyl group.[5] In terms of the 3 and 5 positions, bromides were selected as previous studies show a dramatic decrease in potency with methyl groups on the inner-ring.[7] In HeLa cells, four of eight ligands showed greater TRα-specificity with **CO32** achieving greater than a twofold gain in specificity. Although all analogs bound poorly to both $TR\alpha_1$ and $TR\beta_1$ and with about equal binding affinity to both receptor subtypes, analogs **CO24** and **CO28** were about equipotent to **CO23** in their ability to cause TRα-mediated transcription. This phenomenon brings attention to a mode of subtype specificity which has gone unnoticed for some time. Analogs reported herein are not the first to demonstrate functional specificity. Considering that there is only one amino acid side chain difference in the TR LBD, Ser277 (TRα) to Asn331 (TRβ), the similarity in affinity is not surprising.[10] However, previous studies of related estrogen receptors demonstrate that subtle differences in the induced conformations of amino acid side chains may result in this selectivity.[11] In this case, the same ligand may cause minor differences in one receptor LBD that causes amino acid side chains to perturb the conformationally mobile helix-12, an important mediator of coactivator recruitment, gene regulation, and potentiation of interactions with the transcriptional machinery.[10]

In vivo, **CO24** led to changes that are consistent with enhanced TRα-activity compared to $\mathbf{T}_3$ treated controls, particularly as demonstrated by the massive hind and fore leg emergence in precociously induced *X. laevis* tadpoles. The inner-ring bromides probably confer resistance to dehalogenation by deiodinases, and therefore provide one explanation for CO24's potency and efficacy in vivo and in vitro. Although it is a leap to suggest from studies on amphibian metamorphosis that these analogs would have beneficial effects in mammals and possibly humans, other studies with TRα and cardiac-selective ligands suggest that TRα-specific thyromimetics may have therapeutic utility in the area of heart disease. Finally, TRα-specific thyromimetics, like their predecessor TRβ-selective counterparts, may be useful probes of TR biology.

## 5. Experimental

### 5.1. General

All chemicals used for organic synthesis were purchased from Aldrich, Sigma-Aldrich, Fluka, or Acros and were used without further purification. Anhydrous conditions were maintained under argon using standard schlenk line techniques and oven-dried glassware. Anhydrous THF, DCM, pyridine, and diisopropyl ethylamine were available in house and dispensable from a solvent purification system. Compounds were purified by either flash chromatography using silica gel (VWR Scientific) or through preparatory thin layer chromatography (prep TLC) using Analtech prep-TLC plates ($20 \times 20$ cm, 1000 μm). $^1$H NMR spectra were taken on the Varian Utility 400 MHz spectrometer in $CDCl_3$ or DMSO-$d_6$ solvents and chemical shifts were reported as $\delta$ (parts per million) downfield of the internal control trimethylsilane (TMS) for all solvents. High resolution mass spectrometry (HRMS) using electrospray ionization was performed by the National Bio-Organic, Biomedical Mass Spectrometry Resource at UCSF. All compounds were at least 95% pure as determined by HPLC analysis using a Waters 2695 instrument and an Xterra 3.5 μM reverse-phase $C_{18}$ $2.1 \times 50$-mm column. HPLC grade Acetonitrile and $H_2O$ were purchased from Fisher.

### 5.2. $^{125}$I–$T_3$ competitive binding assay

Full-length $hTR\alpha_1$ and $hTR\beta_1$ were expressed using a TNT T7 quick-coupled transcription translation system (Promega). Competition assays for binding of unlabeled $\mathbf{T}_3$ and **CO23** analogs were performed using 1 nM $^{125}$I–$T_3$ in a gel filtration binding assay as described.[12]

### 5.3. Transactivation assay

Human bone osteosarcoma epithelial (U2OS) cells or human uterine cervix cancer (HeLa) cells (Cell Culture Facility, UCSF) were grown to ~80% confluency in Dulbecco's modified Eagle's (DME)/H-21, 4.5 g/L glucose medium containing 10% newborn calf serum (NCS) or fetal bovine serum (FBS), respectively, (both heat-inactivated), 2 mM glutamine, 50 U ml$^{-1}$ penicillin, and 50 μg ml$^{-1}$ streptomycin. Cells ($\sim 1.5–2 \times 10^6$) were collected and resuspended in 0.5 ml of electroporation buffer (Dulbecco's phosphate-buffered saline containing 0.1% glucose and 10 mg/ml bioprene) with 1.5 μg of a TR expression vector (full-length $hTR\alpha_1$-CMV or $hTR\beta_1$-CMV), 0.5 μg of pRL-TK constitutive *Renilla* luciferase reporter plasmid (Promega), 5 μg of a reporter plasmid containing a synthetic TR response element (DR-4) containing two copies of a direct repeat spaced by four nucleotides (AGGTCAcaggAGGTCA) cloned immediately upstream of a minimal thymidine kinase promoter linked to a luciferase coding sequence.[13] Cells were electroporated using a Bio-Rad gene pulser at 350 V and 960 microfarads in 0.4-cm cuvettes, pooled in DME/F-12 Ham's 1:1 without phenol red (U2OS) or DME/H-21 (HeLa) supplemented as above except that NCS and FBS were hormone stripped using dextrose-coated charcoal, and plated in 96-well (U2OS) or 12-well (HeLa) plates to a final density of 20,000 cells/well and 100,000 cells per well, respectively. After a 2-h incubation period, compounds in 1% dimethylsulfoxide (DMSO) were added to the cell culture medium in triplicate. After an additional 16-h incubation period, cells were harvested and assayed for luciferase activity using the Promega dual-luciferase kit (Promega) and an Analyst AD (Molecular Devices). Data normalized to the *Renilla* internal control were analyzed with GraphPad Prism, v4, using the sigmoid-dose response model to generate $EC_{50}$ values; $EC_{50}$ values were obtained by fitting data to the following equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{((\log EC_{50} - X) * \text{HillSlope})})$.

### 5.4. Preparation of chemicals

Stocks of $\mathbf{T_3}$ and **CO23** analogs were prepared with DMSO at a concentration of 10 mM and stored at −20 °C until use. All other chemicals were purchased from Sigma unless otherwise indicated. About 0.1% aminobenzoic acid ethyl ester (Tricaine or MS222) was made fresh in sterile $ddH_2O$ and kept at 4 °C for no longer than 1 week.

### 5.5. General *Xenopus laevis* tadpole procedures

*Xenopus laevis* stage-53/54 tadpoles were purchased from NASCO, Inc. and staged according to Nieuwkoop and Faber.[14] Upon receipt, tadpoles were allowed to set overnight at room temperature (18–25 °C) in order to recover from shipping shock, after which half of the initial rearing water was replaced with 0.1× Marc's Modified Ringer's (MMR) buffer (10× solution consists of 100 mM NaCl (Fisher), 2 mM KCl (Fisher), 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, and 5 mM Hepes, pH 7.8). Tadpoles were ultimately maintained in fresh 0.1× MMR buffer, changed every 2-days. After completion of experiments, live tadpoles were euthanized by treatment with 0.01% Tricaine, exposure to an ice-bath, and either fixed in phosphate-buffered saline containing 3.5% formalin or decapitated in order to ensure death. Animals were photographed with a Canon PowerShot A510 and images were processed with Adobe Photoshop CS, v8, and Adobe Illustrator CS, v11. All tadpole experiments were conducted in accordance with Institutional Animal Care and Use Committee approval (animal protocol #: A7228-23070-01).

### 5.6. Induced metamorphosis experiments

Stage-53/54 tadpoles were added to Extra-Deep Petri dishes (Fisher) in triplicate containing 50 mL of 0.1× MMR buffer and vehicle or the appropriate concentration of ligand ($\mathbf{T_3}$ or **CO23** analog). The final DMSO concentration was 0.1%. Induced metamorphosis experiments were repeated at least threefold.

### 5.7. Chemistry

**5.7.1. 2-Cyclopropylphenol (3a).** To 2-bromophenol **1** (8 g, 46.2 mmol) in 100 ml of dimethylformamide at 0 °C was added NaH (2.6 g of a 60% suspension, 64.7 mmol). The reaction mixture was stirred for about 10 min after which allyl bromide was added dropwise. After 30 min, the reaction mixture was treated with water and extracted with diethyl ether. The organic portion was dried over $MgSO_4$, filtered, and concentrated in vacuo to give the crude product, which was purified by flash chromatography (silica gel, hexane/ethyl acetate, 5:95) to give allyl 2-bromophenyl ether **2** (9.7 g, 45.5 mmol, 99%) as a white solid. This material was carried on to the next reaction to make **3a**. A dry round-bottom flask was charged with **2** (11 g, 51.6 mmol) and 260 ml of anhydrous diethyl ether and stirred at −78 °C. To this solution was added drop-wise 1.7 M *tert*-BuLi in hexanes (60.7 ml, 103.3 mmol) after which stirring commenced for 30 min. To this solution was added *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (17 ml, 113.5 mmol) and stirring commenced for 45 min before warming to room temperature. The reaction mixture was allowed to stir overnight before addition of water. The aqueous phase was extracted with EtOAc. The combined organic layers were washed with water, brine, and 3 N HCl, dried over $MgSO_4$, concentrated in vacuo, and purified by flash chromatography (silica gel, hexane/ethyl acetate, 20:80) to give **3a** (5.1 g, 38.0 mmol, 74%) as a white solid. $^1$H NMR ($CDCl_3$) $\delta$ 7.36 (d, $J$ = 8 Hz, 1H), 7.06 (m, 2H), 6.85 (d, $J$ = 8 Hz), 5.45 (s, 1H), 1.81 (m, 1H), 0.95 (m, 2H), 0.64 (m, 2H).

### 5.8. General procedure for preparation of TIPS-protected, 4-hydroxyphenyl boronic acids substituted at the 3-position (5a–f)

2-Isopropylphenol 4 (10.0 g, 73.4 mmol) was added to a dry three-neck round-bottom flask fitted with an addition funnel and an exhaust line that runs into a base trap (6 M KOH). This solution was allowed to stir at 0 °C after which $Br_2$ (4.5 ml, 88.1 mmol) was added dropwise over a period of 15 min. Stirring commenced for an additional 3-h before addition of sat. $NaHCO_3$, water, and EtOAc. The aqueous phase was extracted with EtOAC and the combined organic layers were washed with brine and then dried over $MgSO_4$. After concentration of the organic phase in vacuo, the crude product was purified by flash chromatography (silica gel, hexane/ethyl acetate, 10:90) to give a slightly yellow clear oil (11.8 g, 55.1 mmol, 75%). This material (5 g, 23.3 mmol) was combined with TIPS-Cl (5.9 ml, 27.8 mmol) in a dry round-bottom flask containing 50 ml of anhydrous DCM and stirred at 0 °C. To this solution was added imidazole (3.9 g, 58 mmol) and stirring commenced for an additional 18-h. The next day, the reaction was quenched with water and the aqueous phase extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, concentrated in vacuo, and then purified with a short-path distillation column under high vacuum (0.5 mTorr). Pure fractions were collected at 130 °C to give **4b** as a clear white solid (6.9 g, 18.6 mmol, 80%). This material was carried on to make **5b**. A dry round-bottom flask was charged with **4b** (6.9 g, 18.6 mmol) and 100 ml of anhydrous THF and stirred at −78 °C. To this solution was added drop-wise 2.5 M *n*-BuLi in hexanes (9.7 ml, 24.2 mmol) after which stirring commenced for 30 min. To this solution was added triisopropyl borate (8.7 ml, 37.2 mmol) and stirring commenced for 45 min before warming to room temperature. After 1 h, the reaction was quenched with 3 N HCl and the aqueous phase extracted with EtOAc. The combined organic layers were washed with water and brine, dried over $MgSO_4$, concentrated in vacuo, and purified by flash chromatography (silica gel, hexane/ethylacetate, 10–40%) to give **5b** (5.5 g, 16.4 mmol, 88%) as a white solid.

### 5.9. General procedure for preparation of *N*-Boc-3,5-dihalo-L-tyrosine methyl esters (7a–b)

L-Diiodotyrosine (**6a**) (5 g, 11.5 mmol) was added to a round-bottom flask and dissolved in $MeOH/H_2O$ (2:1).

To this mixture was added $NaHCO_3$ (2.9 g, 34.5 mmol) followed by $Boc_2O$ (3.97 ml, 17.3 mmol). The reaction mixture was allowed to stir until completion as determined by TLC analysis (product should turn blue when tested with *p*-anisaldehyde). Upon completion, the mixture was acidified to pH 4.5 and extracted with EtOAc. The combined organic layers were washed with water and brine and then dried over $MgSO_4$. The crude material (5.9 g, 11.1 mmol, 96%) was then utilized in the next reaction. To this material (5.9 g, 11.1 mmol) in toluene/MeOH (9:1) was added $TMSCHN_2$ (0.5 M, 22 ml, 11.6 mmol) drop-wise over 30 min at room temperature using a syringe pump. The reaction mixture was allowed to stir until completion as determined by TLC analysis and then washed with 0.5 M HCl and water. The aqueous phase was extracted with EtOAc and the organic layers washed with brine, dried over $MgSO_4$, and concentrated in vacuo to give *N*-Boc-3,5-diiodo-L-tyrosine methyl ester (**7a**) (5.4 g, 9.9 mmol, 89%).

**5.9.1. *N*-Boc-3,5-diiodo-L-tyrosine methyl ester.** The preparation of **7a** was effected using the general procedure for the preparation of Boc-protected dihalo-L-tyrosine methyl esters to give 5.4 g (89%) of the titled compound as a white solid. $^1H$ NMR ($CDCl_3$) $\delta$ 7.44 (s, 1H), 5.01 (s, 1H), 4.49 (dd, $J = 4.0$ Hz, $J = 8.0$ Hz, 1H), 3.74 (s, 3H), 3.00 (dd, $J = 4.0$ Hz, $J = 14.0$ Hz, 1H), 2.91 (dd, $J = 8.0$ Hz, $J = 14.0$ Hz, 1H), 1.45 (s, 9H).

**5.9.2. *N*-Boc-3,5-dibromo-L-tyrosine methyl ester.** The preparation of **7b** was effected using the general procedure for the preparation of Boc-protected dihalo-L-tyrosine methyl esters to give 1.1 g (87%) of the titled compound as a white solid. $^1H$ NMR ($CDCl_3$) $\delta$ 7.22 (s, 1H), 5.02 (s, 1H), 4.50 (dd, $J = 4.0$ Hz, $J = 8.0$ Hz, 1H), 3.74 (s, 3H), 3.05 (dd, $J = 4.0$ Hz, $J = 14.0$ Hz, 1H), 2.93 (dd, $J = 8.0$ Hz, $J = 14.0$ Hz, 1H), 1.44 (s, 9H).

### 5.10. General procedure for the preparation of biaryl ethers (8a–f)

4 molecular sieves were flame-dried under high vacuum in a dry round-bottom flask. To this flask were added boronic acid (3 mmol) and copper acetate (dried to a verdigris color). These components were dissolved in 10 ml anhydrous DCM after which anhydrous pyridine (5 mmol) and diisopropyl ethylamine (5 mmol) were added. This mixture was then allowed to stir at room temperature for 5 min before addition of phenol (1 mmol) in three portions separated by 5 min each. At this point, the flask was fitted with a drying tube containing drierite and allowed to stir under ambient air overnight, ~16–24-h. After this time, the reaction mixture was concentrated in vacuo and purified by flash chromatography to give products **8a**–**f** (yield generally from 55% to 83%).

### 5.11. General procedure for the preparation of 5-(4-(4-hydroxyphenoxy)-3,5-dihalobenzyl)imidazolidine-2,4-diones (CO24 and CO26–CO30)

Biaryl ether **8b** (510 mg, 0.69 mmol) was dissolved in 20 ml MeOH and saturated with ammonia gas. After 16–18 h, the mixture was purged with argon, concentrated in vacuo, and dissolved in anhydrous 3 N HCl in EtOAc/Ether. After 3 h, the mixture was quenched with water, the pH was adjusted to 4.5, and the aqueous phase extracted with EtOAc. The organic layers were washed with water and brine, dried over $MgSO_4$, and the crude material **9b** (410 mg, 0.67 mmol, 99%) was carried on to make **CO24**. Compounds **9b** (410 mg, 0.67 mmol), 4-nitrophenyl chloroformate (160 mg, 0.78 mmol), and $NaHCO_3$ (218 mg, 2.6 mmol) were added to a dry round-bottom flask containing 10 ml anhydrous MeCN. The reaction mixture was allowed to stir overnight followed by addition of 6.5 ml of $H_2O$. The solution should quickly turn yellow due to generation of nitrophenol. After 6 h, the reaction mixture was roto-vapped in order to remove MeCN, acidified to pH 5, and then extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, and reconstituted in 10 ml THF. Deprotection of the TIPS group afforded **CO24** (204 mg, 0.41 mmol, 63%, 2-steps from **9b**) after purification by prep TLC (silica gel, hexane/ethyl acetate, 40:60).

**5.11.1. Preparation of CO24.** The preparation of **CO24** was effected using the general procedure for the preparation of 5-(4-(4-hydroxyphenoxy)-3,5-dihalobenzyl)imidazolidine-2,4-diones to give 204 mg (63%, 2-steps from **9b**) of the titled compound as a white solid. $^1H$ NMR (DMSO-$d_6$) $\delta$ 10.65 (s, 1H), 9.06 (s, 1H), 7.99 (s, 1H), 7.58 (s, 2H), 6.64 (d, $J = 8.0$ Hz, 1H), 6.63 (d, $J = 4.0$ Hz, 1H), 6.20 (dd, $J = 4.0$ Hz, $J = 8.0$ Hz, 1H), 4.39 (dd, $J = 4.0$ Hz, $J = 8.0$ Hz, 1H), 3.15 (heptet, $J = 8.0$ Hz, 1H), 2.99 (dd, $J = 4.0$ Hz, $J = 14.0$ Hz, 1H), 2.87 (dd, $J = 8.0$ Hz, $J = 14.0$ Hz, 1H), 1.10 (d, $J = 8.0$ Hz, 6H). HPLC (MeCN/water, 50–100%, 15 min): retention time 3.1 min HR-MS calcd for $C_{19}H_{18}Br_2N_2O_4$: 497.9613. Found: 497.9607.

**5.11.2. Preparation of CO26.** The preparation of **CO26** was effected using the general procedure for the preparation of 5-(4-(4-hydroxyphenoxy)-3,5-dihalobenzyl)imidazolidine-2,4-diones to give 80 mg (41%, 2-steps from **9e**) of the titled compound as a white solid. $^1H$ NMR (DMSO-$d_6$) $\delta$ 10.61 (s, 1H), 8.65 (s, 1H), 7.99 (s, 1H), 7.75 (s, 2H), 6.64 (d, $J = 8.0$ Hz, 1H), 6.50 (d, $J = 4.0$ Hz, 1H), 5.90 (dd, $J = 4.0$ Hz, $J = 8.0$ Hz, 1H), 4.37 (dd, $J = 4.0$ Hz, $J = 8.0$ Hz, 1H), 3.72 (s, 3H), 2.94 (dd, $J = 4.0$ Hz, $J = 14.0$ Hz, 1H), 2.81 (dd, $J = 8.0$ Hz, $J = 14.0$ Hz, 1H). HPLC (MeCN/water, 50–100%, 15 min): retention time 1.8 min HR-MS calcd for $C_{17}H_{14}I_2N_2O_5$: 579.8992. Found: 579.9004.

**5.11.3. Preparation of CO27.** The preparation of **CO27** was effected using the general procedure for the preparation of 5-(4-(4-hydroxyphenoxy)-3,5-dihalobenzyl)imidazolidine-2,4-diones to give 50 mg (25%, 2-steps from **9c**) of the titled compound as a white solid. $^1H$ NMR (DMSO-$d_6$) $\delta$ 10.65 (s, 1H), 9.09 (s, 1H), 7.97 (s, 1H), 7.77 (s, 2H), 6.66 (d, $J = 8.0$ Hz, 1H), 6.48 (d, $J = 4.0$ Hz, 1H), 6.26 (dd, $J = 4.0$ Hz, $J = 8.0$ Hz, 1H), 4.40 (dd, $J = 4.0$ Hz, $J = 8.0$ Hz, 1H), 2.94 (dd, $J = 4.0$ Hz, $J = 14.0$ Hz, 1H), 2.81 (dd, $J = 8.0$ Hz, $J = 14.0$ Hz, 1H), 2.07 (s, 3H). HPLC (MeCN/water,

50–100%, 15 min): retention time 1.9 min HR-MS calcd for $C_{17}H_{14}I_2N_2O_4$: 563.9043. Found: 563.9045.

**5.11.4. Preparation of CO28.** The preparation of **CO28** was effected using the general procedure for the preparation of 5-(4-(4-hydroxyphenoxy)-3,5-dihalobenzyl)imidazolidine-2,4-diones to give 91 mg (35%, 2-steps from **9d**) of the titled compound as a white solid. $^1$H NMR (DMSO-$d_6$) $\delta$ 10.60 (s, 1H), 8.94 (s, 1H), 7.98 (s, 1H), 7.75 (s, 2H), 6.64 (d, $J$ = 8.0 Hz, 1H), 6.53 (d, $J$ = 4.0 Hz, 1H), 6.24 (dd, $J$ = 4.0 Hz, $J$ = 8.0 Hz, 1H), 4.36 (dd, $J$ = 4.0 Hz, $J$ = 8.0 Hz, 1H), 3.16 (q, 2H), 2.95 (dd, $J$ = 4.0 Hz, $J$ = 14.0 Hz, 1H), 2.85 (dd, $J$ = 8.0 Hz, $J$ = 14.0 Hz, 1H), 1.11 (t, 3H). HPLC (MeCN/water, 50–100%, 15 min): retention time 2.1 min HR-MS calcd for $C_{18}H_{16}I_2N_2O_4$: 577.9199. Found: 577.9198.

**5.11.5. Preparation of CO29.** The preparation of **CO29** was effected using the general procedure for the preparation of 5-(4-(4-hydroxyphenoxy)-3,5-dihalobenzyl)imidazolidine-2,4-diones to give 1.0 g (67%, 2-steps from **9a**) of the titled compound as a white solid. $^1$H NMR (DMSO-$d_6$) $\delta$ 10.60 (s, 1H), 9.00 (s, 1H), 7.98 (s, 1H), 7.74 (s, 2H), 6.62 (d, $J$ = 8.0 Hz, 1H), 6.24 (d, $J$ = 4.0 Hz, 1H), 6.13 (dd, $J$ = 4.0 Hz, $J$ = 8.0 Hz, 1H), 4.37 (dd, $J$ = 4.0 Hz, $J$ = 8.0 Hz, 1H), 3.17 (m, 1H), 2.94 (dd, $J$ = 4.0 Hz, $J$ = 14.0 Hz, 1H), 2.85 (dd, $J$ = 8.0 Hz, $J$ = 14.0 Hz, 1H), 0.84 (m, 2H), 0.53 (m, 2H). HPLC (MeCN/water, 50–100%, 15 min): retention time 2.0 min HR-MS calcd for $C_{19}H_{16}I_2N_2O_4$: 589.9199. Found: 589.9209.

**5.11.6. Preparation of CO30.** The preparation of **CO30** was effected using the general procedure for the preparation of 5-(4-(4-hydroxyphenoxy)-3,5-dihalobenzyl)imidazolidine-2,4-diones to give 1.3 g (65%, 2-steps from **9f**) of the titled compound as a white solid. $^1$H NMR (DMSO-$d_6$) $\delta$ 10.61 (s, 1H), 9.09 (s, 1H), 7.98 (s, 1H), 7.76 (s, 2H), 6.67 (d, $J$ = 8.0 Hz, 2H), 6.51 (d, $J$ = 8.0 Hz, 2H), 4.36 (dd, $J$ = 4.0 Hz, $J$ = 8.0 Hz, 1H), 2.95 (dd, $J$ = 4.0 Hz, $J$ = 14.0 Hz, 1H), 2.84 (dd, $J$ = 8.0 Hz, $J$ = 14.0 Hz, 1H). HPLC (MeCN/water, 50–100%, 15 min): retention time 1.7 min HR-MS calcd for $C_{16}H_{12}I_2N_2O_4$: 549.8886. Found: 549.8900.

**5.11.7. Preparation of 5-(4-(4-hydroxy-3-iodophenoxy)-3,5-diiodobenzyl)imidazolidine-2,4-dione (CO31).** 5-(4-(4-Hydroxyphenoxy)-3,5-diiodobenzyl)imidazolidine-2,4-dione (**CO30**, 100 mg, 0.2 mmol) in 0.2 ml of MeOH was added to a round-bottom flask at −5 °C and dissolved in 5 ml of a 70% solution of aqueous ethylamine. To this mixture was added drop-wise Iodine ($I_2$) as a 1 N aqueous solution saturated with KI (0.24 ml, 0.24 mmol). After 6 h, the reaction mixture was acidified to pH 4.5, extracted with EtOAc, concentrated in vacuo, and purified by flash chromatography (silica gel, hexane/ethyl acetate, 40:60) to give **CO31** (85 mg, 0.13 mmol, 62%). $^1$H NMR (DMSO-$d_6$) $\delta$ 10.62 (s, 1H), 9.97 (s, 1H), 8.00 (s, 1H), 7.77 (s, 2H), 7.02 (d, $J$ = 4.0 Hz, 1 H), 6.81 (d, $J$ = 8.0 Hz, 1H), 6.58 (dd, $J$ = 4.0 Hz, $J$ = 8.0 Hz, 1H), 4.37 (dd, $J$ = 4.0 Hz, $J$ = 8.0 Hz, 1H), 2.95 (dd, $J$ = 4.0 Hz, $J$ = 14.0 Hz, 1H), 2.84 (dd, $J$ = 8.0 Hz, $J$ = 14.0 Hz, 1H). HPLC (MeCN/water, 50–100%, 15 min): retention time 2.1 min HR-MS calcd for $C_{16}H_{11}I_3N_2O_4$: 675.7853. Found: 675.7877.

**5.11.8. Preparation of 5-(4-(4-hydroxy-3-iodophenoxy)-3,5-diiodobenzyl)imidazolidine-2,4-dione (CO32).** 5-(4-(4-Hydroxyphenoxy)-3,5-diiodobenzyl)imidazolidine-2,4-dione (**CO30**, 100 mg, 0.2 mmol) was added to a round-bottom flask and dissolved in 2 ml of DCM and 0.25 ml of glacial acetic acid at 0 °C. To this mixture was added drop-wise bromine (12.3 μl, 0.24 mmol) in 1 ml of DCM. After 1 h, the reaction mixture was extracted with EtOAc, concentrated in vacuo, and purified by flash chromatography (silica gel, hexane/ethyl acetate, 40:60) to give **CO32** (109 mg, 0.18 mmol, 88%). $^1$H NMR (DMSO-$d_6$) $\delta$ 10.61 (s, 1H), 9.90 (s, 1H), 7.98 (s, 1H), 7.76 (s, 2H), 6.87 (d, $J$ = 8.0 Hz, 1H), 6.82 (d, $J$ = 4.0 Hz, 1H), 6.55 (dd, $J$ = 4.0 Hz, $J$ = 8.0 Hz, 1H), 4.36 (dd, $J$ = 4.0 Hz, $J$ = 8.0 Hz, 1H), 2.96 (dd, $J$ = 4.0 Hz, $J$ = 14.0 Hz, 1H), 2.79 (dd, $J$ = 8.0 Hz, $J$ = 14.0 Hz, 1H). HPLC (MeCN/water, 50–100%, 15 min): retention time 2.0 min HR-MS calcd for $C_{16}H_{11}BrI_2N_2O_4$: 627.7992. Found: 627.7981.

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