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Semisynthesis and pharmacological activities of Tetrac analogs: Angiogenesis modulators

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ABSTRACT

Novel Tetrac analogs were synthesized and then tested. Anti-angiogenesis efficacy was carried out using the Chick Chorioallantoic Membrane (CAM) model and the mouse matrigel model for angiogenesis. Pharmacological activities showed Tetrac can accommodate numerous modifications and maintain anti-angiogenesis activity.

Angiogenesis is a vascular event, which partly induces the growth of new blood vessels into tumors by cell adhesion to the ECM (Extra Cellular Matrix) and allows for tumor progression. To improve treatment for brain cancer better, an orally active compound with optimal therapeutic index which would inhibit pathological angiogenesis with minimal impact on physiological processes is critically needed. The thyroid hormone antagonists (Tetrac and Triac) were found to perturb the angiogenesis process stimulated by VEGF (Vascular Endothelial Growth Factor) or FGF (Fibroblast Growth Factor) without influencing the preexisting blood vessels.[1] The site of action of the compounds was then found to be at or near the integrin $\alpha_V\beta_3$'s binding pocket.[2,3] As in the case of the achiral Tetrac **1**, the only possible structural isomer (Cs) is a low molecular weight compound with a core constituted by a phenol linked to a phenylacetic acid function ($pK_a = 4.3$) by an ether bond and substituted by four iodide atoms. A MM2 energy minimization revealed the most stable conformation was obtained when the two aromatic rings were in perpendicular planes and the iodides to be space filling and predominant in size (Fig. 1). This lipophilic core together with the hydrophilic head may confer to Tetrac an ease to the passive diffusion across the lipid membrane of cells by which the compound was observed to reach nuclear receptors.[4] During the course of the planed semisynthesis of Tetrac nanoparticles, which would allow keeping the neovascular activity without interfering with the vascular events, a suitable protecting group at the phenylacetic acid end of Tetrac was required as a first step in the synthesis of the nanoparticle precursors. The molecule was thus modulated in new more lipophilic ester analogs. Since the association to the nano scale sized particles was conceived to occur at the phenol's OH, it was alkylated after the formation of the car-

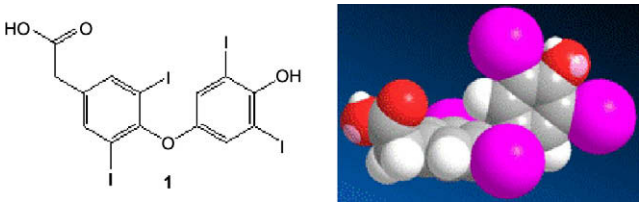

Figure 1. Structure and 3D representation (MM2 energy minimization, cambridge Soft Chem 3D Ultra program) of Tetrac **1**.

Abbreviations: ECM, extra cellular matrix; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; TEA, triethylamine; DCM, dichloromethane; TiPSCl, triisopropylsilyl chloride; Cs_2CO_3, cesium carbonate; MeOH, methanol; HPLC, high pressure liquid chromatography; EtOH, ethanol; ddH_2O, double distilled water; Tetrac-OTiPS, triisopropylsilyl-2-(4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl)acetate; EBH, epibromohydrin; DEBHT, oxiran-2-ylmethyl-2-(4-(3,5-diiodo-4-(oxiran-2-ylmethoxy)phenoxy)-3,5-diiodophenyl)acetate; EBH-Tetrac-OTiPS, triisopropylsilyl-2-(4-(3,5-diiodo-4-(oxiran-2-ylmethoxy)phenoxy)-3,5-diiodophenyl)-acetate; SM, starting material.

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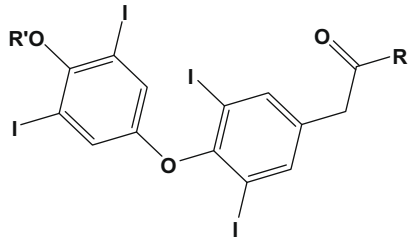
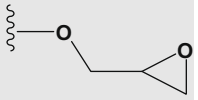
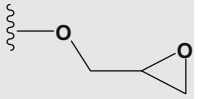
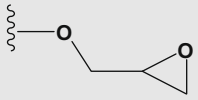
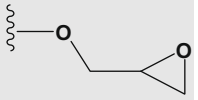
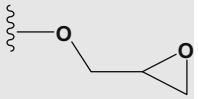
Table 1
Structure of Tetrac analogs **2–9**



2-9

Compounds	R	R′
2	OMe	H
3	O^{t}Bu	H
4		H
5	OTiPS	H
6		
7	OTiPS	
8		
9	H	

bon and silicon based esters (Table 1). The compounds were then tested for their inhibition of angiogenesis; perhaps the epoxide, when linked at the phenol's OH, could interact with Serine or Tyrosine which were reported at or near the binding site of the integrin receptor by Xiong et al.[5,6]

Table 2
Comparison between the reactivities of acetic acid and Tetrac

Reagents	Acetates	Tetrac esters
MeI	**10**, bp = 57 °C[a]	×
EtI	**11**, bp = 77 °C[a]	×
^{t}BuI	**12**, bp = 98 °C[a]	×
CBzCl	×[b]	×
TMSCl	**13**, bp = 121 °C[a]	×
TiPSCl	**14**, bp = 153 °C[a]	**5**, mp = 146 °C
$TiPSN_3$	×	×
4-CF_3Ph-$(EtO)_3$Si	×	×

[a] Values were compared to those reported in the MERCK Index IXth edition.
[b] × = no product was formed.

The first Tetrac analogs (Table 1) were prepared following literature methods. The methyl ester, analog **2**, was prepared following a described method[7,8] in 61% yield. Analogs **3** and **4** were new products of the same reaction and were prepared following another described method for the synthesis of *tert*-butyl esters.[9] Unexpectedly, the main product of the reaction was **4** (30% yield).[10] Compound **5** was new and synthesized by de-protonation of Tetrac with TEA or Cs_2CO_3 in anhydride DCM and then by addition of TiPSCl.[11]

Plus, as shown on Table 2, Tetrac was found to hardly react with most of the esterifying halogenated agents used to probe the formation of other esters.

This interesting feature was thought to be due to the deactivating effect of the 4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl. This was proven hereafter as the reactivity of acetic acid in comparison to Tetrac was studied. All the compounds were submitted to a general method[12] which consisted in adding a series of several carbon based and silicon based protecting groups to the starting material activated by Cs_2CO_3 towards the formation of the esters (**5**, **10**–**14**) (Fig. 2, Table 2). In the course of this study, the formation of **2** was observed in high yield (90%) when Tetrac was reacted with benzyl chloroformate in MeOH and at room temperature. Analog **6** was then obtained by refluxing epibromohydrin with **4** in anhydride dioxane (Table 1). Then numerous attempts to condensate epibromohydrin with compound **5** were accomplished by varying the concentration of the epoxide containing alkylating agent (i.e., 1.0 equiv, 10.0 equiv, 100.0 equiv) and led to use the HPLC technique to study the course of the reaction.

While optimizing the reaction conditions of the condensation of **5** (Tetrac-OTiPS (triisopropylsilyl-2-(4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl)acetate)) with epibromohydrin (EBH (epibromohydrin)), **8** (DEBHT (oxiran-2-ylmethyl-2-(4-(3,5-diio-

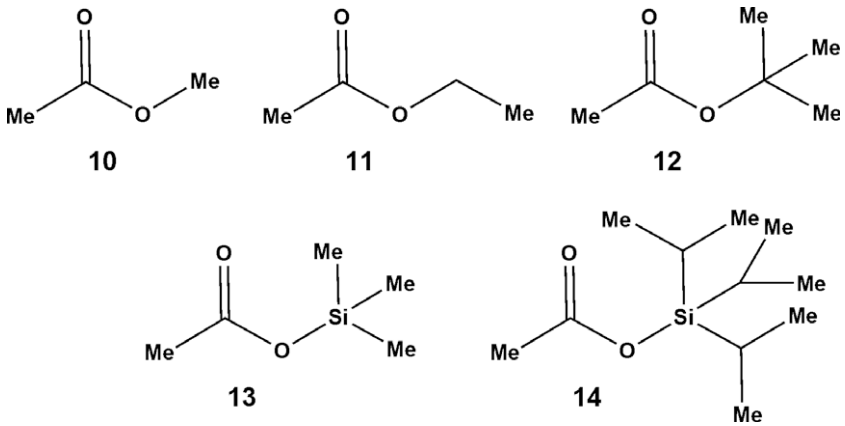


Figure 2. Structures of **10**–**14**.

do-4-(oxiran-2-ylmethoxy)phenoxy)-3,5-diiodophenyl)acetate)) was found to be the major side product. During the first attempt, compound **8** was formed equivalently as **7** (EBH-Tetrac-OTiPS (triisopropylsilyl-2-(4-(3,5-diiodo-4-(oxiran-2-ylmethoxy)phenoxy)-3,5-diiodophenyl)acetate)). In order to minimize the formation of **8**, five experiments were subsequently completed and were monitored by HPLC (Fig. 2). At first, several reaction conditions were used to minimize the formation of **8** by temperature/time control. The starting material was completely transformed at 30 min and no evolution in the **7**/**8** ratio was detected over 90 min (Fig. 3A). The second experiment analyzed the effect of using an excess of the base (Fig. 3B). Larger amount was found to have minor effect on the course of the reaction as the **7**/**8** ratio was just slightly higher at 30 min. In the third experiment, the reaction kinetic was monitored at 5-min intervals from 0 to 30 min (Fig. 3C). The starting material was nearly consumed at 20 min and the **7**/**8** ratio decreased from 0.94 to 0.29 from 5 to 30 min. Interestingly, when using 1.0 equiv of TEA, no de-protection of **5** was detected over a period of 30 min (Fig. 3D) as only one additional peak corresponding to the retention time of **7** was detected on the chromatogram by lowering the heating temperature to 55 °C using 1.0 equiv of base and a large excess of epibromohydrin at a reaction time of 150 min. Also, the reaction was found to be neater in those conditions at a reaction time of 30 min than longer. Thereafter, the reaction was performed on a larger scale (300 mg) with 1.0 equiv of base and monitored over a 150 min period (Fig. 3E).

From 30 min to 150 min or longer, the **7**/**8** ratio stayed the same (i.e., 0.73) together with the yield of transformation of the starting material which was quantified to 47%. Using the optimized conditions, the two steps synthesis developed to obtain compounds **5** and **7** were then condensed via a one pot semi-synthetic process to obtain **7** in an overall 40% yield.[13] Product **9** was then easily re-crystallized in EtOH.

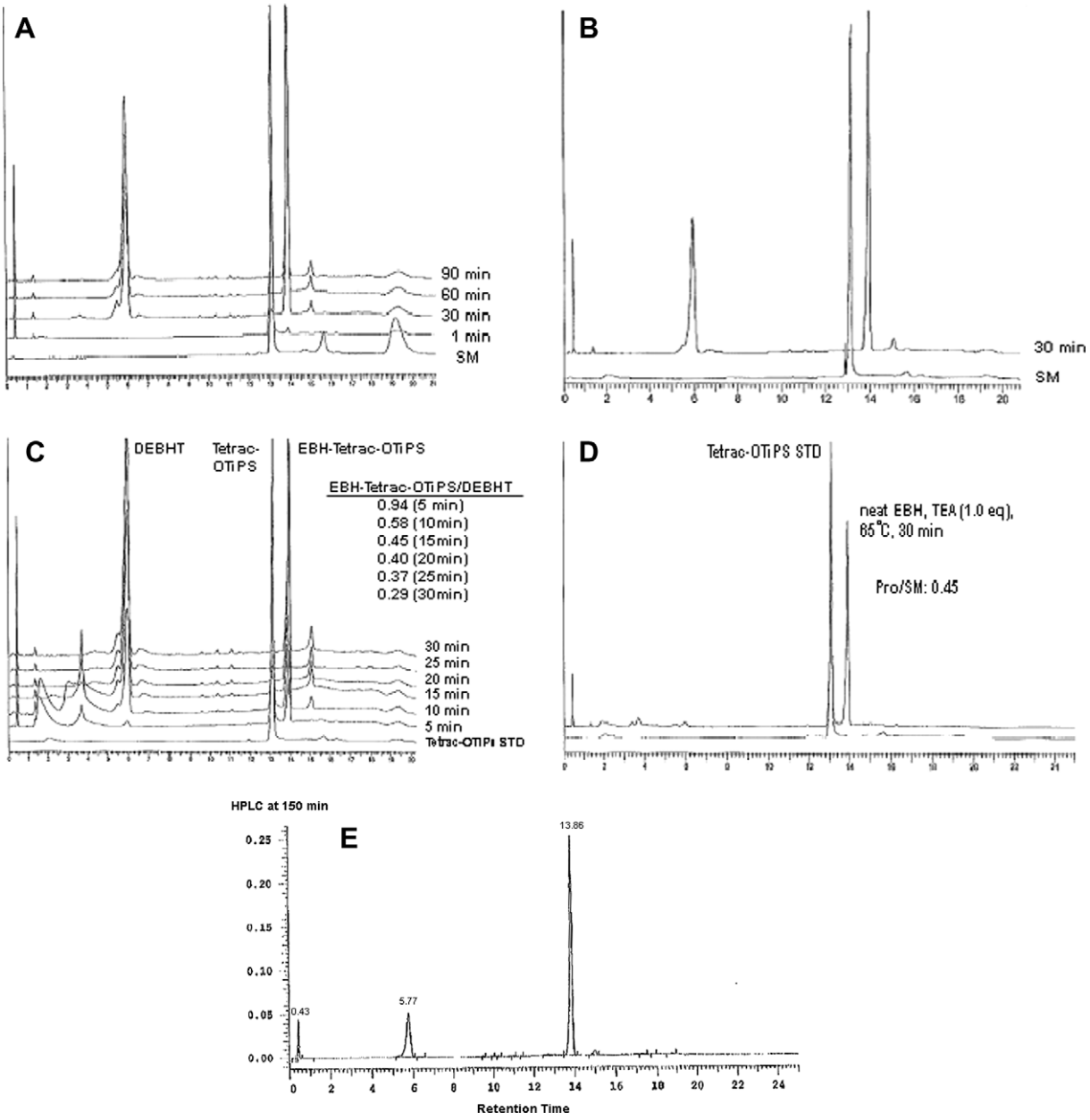


Figure 3. HPLC chromatograms showing the optimization steps for the synthesis of **7**.

To investigate the structure–activity relationships, each compound was tested for its ability to block angiogenesis in comparison to Tetrac. Inhibition of angiogenesis was measured by the chicken embryo ChorioAllantoic Membrane (CAM) model[14] (Fig. 4), a widely used in vivo study, which exposed the live chicken embryo membrane to various stimulating and inhibitory compounds and by the matrigel assay[15] which exposed test angiogenesis-inducing and inhibitory compounds to cold liquid matrigel, a mix of several proteins which, after subcutaneous injection, solidified and permitted the formation of new blood vessels. FGF and Tetrac were used as controls in both assays (Tables 3 and 4).

Compounds were considered to be strong inhibitors if they achieved more than 50% inhibition as compared to the control. In the CAM experiments, a majority of Tetrac analog compounds showed a strong inhibitory effect on new blood vessel formation within the range from 75% to 97% (Table 3), comparable to 85% of Tetrac. In parallel, synthesized analogs **3**, **4**, **5**, **6**, **7**, **8** and **9** were tested for their ability to inhibit hemoglobin formation from matrigel. Tetrac (10 μg) significantly inhibited FGF induced angiogenesis (90% inhibition). Tetrac analog compounds also blocked the FGF induced angiogenesis in a comparable fashion to Tetrac (Table 4). Inhibition with compounds **3**, **4**, **5**, **6**, **7**, **8** and **9** was observed to be strong. Any modulations on either or both sides of Tetrac did not affect greatly the antagonist effect of the parent compound. Though the inhibition of angiogenesis in the CAM assay by compounds **4**, **6** and **8** (Table 3) were not as strong as compounds **3**, **5**, **7** and **9**, their activities were better in the matrigel assay.

As the highly stable *tert*butyl-Tetrac ester **3**, which can only be deprotected in acidic conditions and the analog **7** which was protected at both ends showed the same activity at inhibiting FGF-induced angiogenesis, the phenol and carboxylic acid were proven here not to be mandatory for the activity of the molecule. Those results tend, in fact, to support the hypothesis of a specific binding site on the integrin designed to interact with the tetraiodophenoxyphenol core of the thyroid hormones.

In summary, the chemical reactivity of Tetrac was probed by esterification and phenol alkylation reactions. An HPLC assisted study allowed to optimize a convenient synthesis pathway to the formation of the nanoparticle. The biological activities showed any modulations did not suppress Tetrac's potency at inhibiting angiogenesis stimulated by FGF. The potent precursor **7** was considered for the conjugation to PLGA based nanoparticles.

Table 3
Anti-angiogenesis efficacy of Tetrac analogs in the CAM model

Treatment	Mean branch points[a]	Mean % inhibition
PBS	73 ± 6	—
FGF(1.25 μg/ml)	130 ± 11	—
FGF + **Tetrac**	81 ± 8	85 ± 14[c]
FGF + **3**	79 ± 7	88 ± 13[c]
FGF + **4**	121 ± 4	15 ± 8
FGF + **5**	87 ± 11	75 ± 19[c]
FGF + **6**	105 ± 7	44 ± 13[b]
FGF + **7**	75 ± 11	96 ± 21[c]
FGF + **8**	112 ± 10	31 ± 18
FGF + **9**	94 ± 10	62 ± 18[b]

[a] Data representing mean ± SEM, $n = 8$.
[b] $p < 0.05$.
[c] $p < 0.001$.

Table 4
Anti-angiogenesis efficacy of Tetrac analogs in the mouse-matrigel model

	Hemoglobin (mg/ml) ± SEM[a]	Mean inhibition of angiogenesis (%)[b]
Control	0.3 ± 0.1	—
FGF 100 ng	2.4 ± 0.4	—
FGF + **Tetrac**	0.5 ± 0.1[c]	90 ± 13
FGF + **3**	0.4 ± 0.1[c]	95 ± 14
FGF + **4**	0.2 ± 0.1[c]	104 ± 12
FGF + **5**	0.2 ± 0.1[c]	104 ± 14
FGF + **6**	0.7 ± 0.1[c]	80 ± 16
FGF + **7**	0.3 ± 0.1[c]	100 ± 9
FGF + **8**	0.3 ± 0.1[c]	100 ± 14
FGF + **9**	0.3 ± 0.1[c]	100 ± 12

[a] Hemoglobin data represent mean ± SEM, $n = 7–14$.
[b] Tetrac and analogs were tested at 10 μg/plug.
[c] $p < 0.001$.

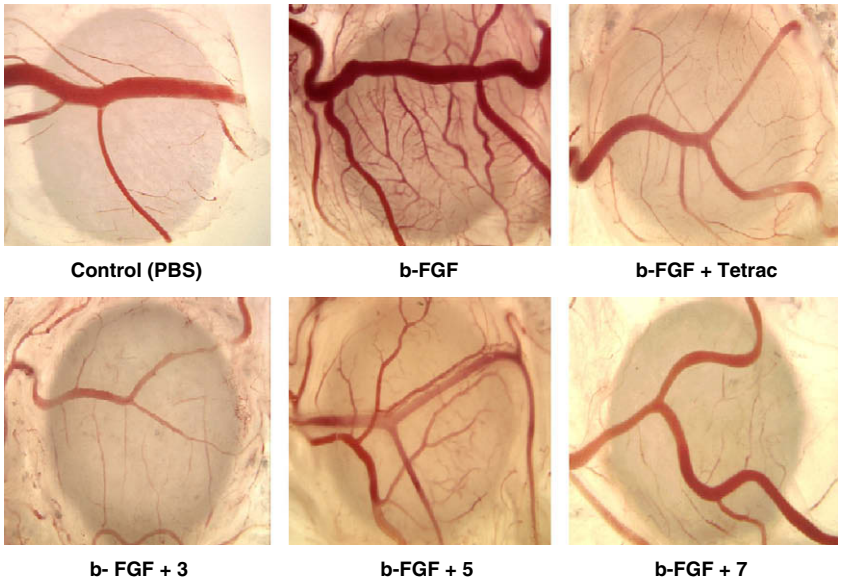


Figure 4. Representative illustrations of the anti-angiogenesis effects of Tetrac and Tetrac analogs **3**, **5** and **7**.

Acknowledgments

Thanks to Azopharma's contribution in regards to the synthesis and analytical characterization of the EBH-Tetrac-OTiPS compound, the Rensselaer Polytechnic Institute (NMR analyses), to the University at Albany Proteomics Facility, the Center for Functional Genomics for the MS analysis, to Dr. Huazhong HE, PRI for reviewing this article and to Dr. Jay Jayaraman, PRI for his help and constant support. We appreciate the partial funding of this work by the Charitable Leadership Foundation and the Medical Technology Acceleration Program and the Pharmaceutical Research Institute.

Supplementary data

Supplementary data for publication contain informations on the protocols and the analytical characterizations of all molecules. Plus were included the protocols for the angiogenesis assays, the NMR spectra of compound **2**, **7** and a 2D NMR analysis on the connectivity of compound **9**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.094.

References and notes

1. Mousa, S. A.; Bergh, J. J.; Dier, E.; Rebbaa, A.; O'Connor, L. J.; Yalcin, M.; Aljada, A.; Dyskin, E.; Davis, F. B.; Lin, H. Y.; Davis, P. J. *Angiogenesis* **2008**, *11*, 183.
2. Cody, V.; Davis, P. J.; Davis, F. B. *Steroids* **2007**, *72*, 165.
3. Bergh, J. J.; Lin, H. Y.; Lansing, L.; Mohamed, S. N.; Davis, F. B.; Mousa, S.; Davis, P. J. *Endocrinolgy* **2005**, *146*, 2864.
4. Hill, S. R., Jr.; Barker, S. B.; McNeil, J. H.; Tingley, J. O.; Hibbett, L. L. *J. Clin. Invest.* **1960**, *39*, 523.
5. Xiong, J.-P.; Stehle, T.; Diefenbach, B.; Zhang, R.; Dunker, R.; Scott, D. L.; Joachimiak, A.; Goodman, S. L.; Arnaout, M. A. *Science* **2001**, *294*, 339.
6. Xiong, J.-P.; Stehle, T.; Zhang, R.; Joachimiak, A.; Frech, M.; Goodman, S. L.; Arnaout, M. A. *Science* **2002**, *296*, 151.
7. Wilkinson, J. H. *Biochem. J.* **1956**, *63*, 601.
8. Wilkinson, J. H. Int. Patent WO/805761, 1958.
9. Neises, B.; Steglich, W. *Org. Synth.* **1990**, *7*, 93.
10. Obtained after activation of Tetrac with dicyclohexylcarbodiimide (DCC) in presence of 4-dimethylaminopyridine (DMAP).
11. *Synthesis of triisopropylsilyl-2-(4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl) acetate* (**5**): In anhydrous conditions, Tetrac (2.1 g, 2.9 mmol, 1.0 equiv) was suspended in THF. TEA (402 μL, 2.9 mmol, 1.0 equiv) was added. After stirring at room temperature for 5 min, TiPSCl (618 μL, 2.9 mmol, 1.0 equiv) was added drop by drop. After stirring for 20 min, the solvent was evaporated and the crude product was precipitated with diethylether, filtrated and then recrystallized to give triisopropylsilyl-2-(4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl)acetate (2.1 g, 2.3 mmol) as a powder.
Yield: 80%; white powder; recrystallization solvent: EtOH; TLC: 0.77 (DCM); mp = 146 °C; IR (ν cm^{-1}): 1701 (CO); UV–vis (DMSO): λ_{max} nm = 228; HPLC (μBondapak C18): rt = 1.5 min (CH_3CN/buffer pH 4.0 70:30); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.81 (s, 2H, ArH), 7.11 (s, 2H, ArH), 5.56 (br, 1H, OH), 3.61 (s, 2H, CH_2), 1.27–1.33 (m, 3H, 3 CH), 1.05–1.17 (m, 18H, 6 CH_3); MS (ESI-) *m/z*: 903.3 [$(M-H)^-$, 100]; Anal. Calcd for $C_{23}H_{28}I_4O_4Si$: C, 30.55; H, 3.12. Found: C, 30.47; H, 2.83.
12. *Synthesis of triisopropylsilylacetate* (**14**): In anhydrous conditions, acetic acid (0.5 mL, 8.7 mmol, 1.0 equiv) was suspended in anhydrous DCM. Cs_2CO_3 (2.84 g, 8.7 mmol, 1.0 equiv) was then added. After stirring at room temperature for 5 min, TiPSCl (1.86 mL, 8.7 mmol, 1.0 equiv) was added drop by drop. The product was filtered and then concentrated.
Yield: 99%; clear oil; TLC: 0.77 (DCM); bp = 153 °C; IR (ν cm^{-1}): 1723 (CO); ^{1}H NMR ($CDCl_3$) δ (ppm): 2.07 (s, 3H, CH_3), 1.32 (m, 3H, 3 CH), 1.08–1.10 (m, 18H, 6 CH_3).
13. *Triisopropylsilyl-2-(4-(3,5-diiodo-4-(oxiran-2-ylmethoxy)phenoxy)-3,5-diiodophenyl)acetate* (**7**).
In anhydrous conditions, Tetrac (3.0 g, 4.0 mmol, 1.0 equiv) was suspended in anhydrous THF. TEA (559 μL, 4.0 mmol, 1.0 equiv) was then added. After stirring at room temperature for 5 min, TiPSCl (858 μL, 4.0 mmol, 1.0 equiv) was added drop by drop. After stirring for 20 min, TEA (559 μL, 4.0 mmol, 1.0 equiv) was added and then epibromohydrin (3.3 mL, 40.0 mmol, 10.0 equiv) was added and the reaction medium was stirred at reflux (55 °C) and monitored by TLC. After 12 h, the reaction medium was cooled, filtered and the organic phase was evaporated. The crude product was then separated by column chromatography (DCM/cyclohexane 90:10) to give triisopropylsilyl-2-(4-(3,5-diiodo-4-(oxiran-2-ylmethoxy)phenoxy)-3,5-diiodophenyl)acetate (1.5 g, 1.6 mmol) as a solid.
Yield: 40%; white powder; recrystallization solvent: EtOAc; TLC: 0.24 (DCM); mp = 120 °C; IR (ν cm^{-1}): 1711 (CO); UV–vis (DMSO): λ_{max} nm = 256; HPLC (μBondapak C18): rt = 10.6 min (CH_3CN/H_2O 90:10); ^{1}H NMR (DMSO-d_6) δ (ppm): 7.80 (s, 2H, ArH), 7.16 (s, 2H, ArH), 4.10–4.15 (dd, J = 4.0 Hz, 1H, CH), 3.99–4.06 (dd, J = 5.5 Hz, 1H, CH), 3.61 (s, 2H, CH_2), 2.92–2.93 (t, J = 4.0 Hz, 1H, CH), 2.78–2.79 (dd, J_1 = 4.0 Hz, J_2 = 10.0 Hz, 1H, CH), 1.28–1.33 (m, 3H, 3 CH), 1.05 (s, 18H, 6 CH_3); MS (ESI+) *m/z*: 804.3 [$(M-C_9H_{21}Si+H)^+$, 87], 826.9 [$(M-C_9H_{21}Si+Na)^+$, 100]; MS (APCI+) *m/z*: 961.8 [$(M+H)^+$, 20]; Anal. Calcd for $C_{26}H_{32}I_4O_5Si$: C, 32.52; H, 3.36. Found: C, 32.70; H, 3.13.
14. *Chick ChorioAllantoic Membrane model*. Ten-day-old fertilized chicken eggs were incubated at 37 °C with 55% relative humidity. In the dark with the help of candling lamp and using a hypodermic needle a small hole was punctured in the shell covering the air sac. A second hole was punctured on the wider side of the egg above an avascular area of the embryonic membrane. An artificial air sac was created below the second hole by applying gentle vacuum to the first hole using a small rubber squeeze bulb. The vacuum caused the separation of ChorioAllantoic Membrane (CAM) from the shell. An approximately 1.0 cm^2 window was cut in the shell over the dropped CAM with the use of a mini drill. The underlying CAM was accessed through this small window. Filter disks were punched using a small puncher from filter paper #1 (Whatman International, United Kingdom). Filter disks were soaked in 3 mg/mL cortisone acetate solution (95% ethanol and water) and air-dried under sterile condition. For inducing angiogenesis, sterile filter disks were saturated with FGF (1 μg/mL) and control disks were saturated with PBS without calcium and magnesium.
Using sterile forceps one filter/CAM was placed from the window. The window was sealed with Highland brand transparent tape. After 1 h, 10 μL of inhibitor (1 μM) was added on the FGF-stimulated CAMs topically. Control filter disks received PBS without calcium and magnesium. After 48 h, CAM tissue directly beneath filter disk was harvested and placed in a 35-mm Petri dish. Ten eggs/treatments were used.
15. *Mouse matrigel model of angiogenesis*. Normal male mice (C57BL/6NCr) 6–8 weeks of age and weighing ~20 g were purchased from Taconic farm. Animals were housed 4 per cage, in controlled conditions of temperature (20–24 °C); humidity (60–70%) and 12 h light/dark cycle provided with food and water ad libitum. Experimental protocol was approved by VA IACUC. Mice were allowed to acclimate for 5 days prior to the start of treatments. Matrigel (BD Bioscience, San Jose CA) was thawed overnight at 4 °C and placed on ice. Aliquots of matrigel were placed into cold polypropylene tubes to prevent the matrigel from solidifying and the angiogenesis promoter added to matrigel with or without antagonist. Matrigel was subcutaneously injected as triple injection per animal at 100 μL/animal. At day 14 post plug implant all animals were killed in CO_2 chamber and matrigel plugs were collected. Plug hemoglobin content was analyzed from three implants/mice (n = 6 per group) to measure angiogenesis.
Hemoglobin determination of angiogenesis in Matrigel plugs. The Matrigel plugs dissected from the mice were carefully stripped of any remaining peritoneum. The plugs were placed into 0.5 mL tube of ddH_2O and homogenized for 5–10 min. The samples were spun at 4000 rpm on a centrifuge for 10 min and the supernatants collected for hemoglobin measurement. Fifty microliters of supernatant were mixed with 50 mL Drabkin's reagent and allowed to sit at room temperature for 15–30 min, and then 100 mL of this mixture was placed in a 96-well plate. Absorbance was read with a Microplate Manager ELISA reader at 540 nm. Hemoglobin (Hb) concentration was determined by comparison with a standard curve in mg/mL. Hemoglobin concentration is a reflection of the number of blood vessels in the plugs.