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Molecular Editing of Kinase-Targeting Resorcylic Acid Lactones (RAL): Fluoroenone RAL

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Kinases have become one of the most intensely pursued target class for therapeutic intervention, particularly for applications in oncology.[1] Most small-molecule inhibitors are heterocycles reminiscent of the adenosine scaffold, which target the nucleotide binding site of the kinase. Resorcylic acid lactones (RAL) bearing a suitably positioned *cis*-enone, such as hypothemycin,[2,3] LL-Z1640-2,[4,5] L-783277,[6] radicicol A[7,8] (Figure 1), have been shown to irreversibly inhibit select kinases and represent a unique pharmacophore that has been shown to be effective in vivo.[9] Through a bioinformatic analysis of the kinome, Santi and co-workers identified 46 kinases that could potentially be targeted by this family of compounds.[2] Our efforts to expand the diversity of this natural pharmacophore has led to the identification of several tolerated modifications, such as substitution of the benzylic carbon by an oxygen which offers simpler synthetic access.[7,10,11]

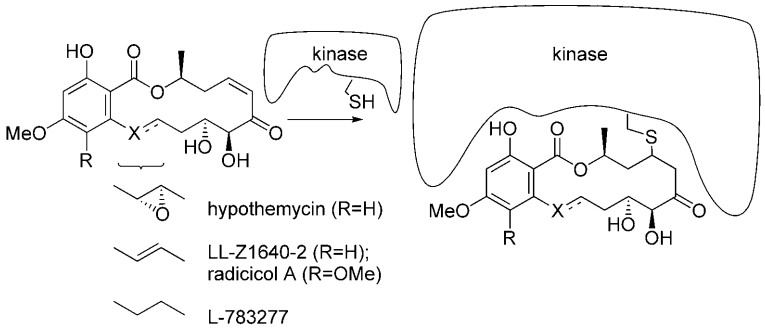

Figure 1. Irreversible inhibition of kinases bearing a suitably positioned cysteine residue in the nucleotide binding site (most prominently: VEGFRs, MEKs, FLT3, GAK, MKNKs, PDGFRs, TAK1, KIT) by *cis*-enone resorcylic acid lactones.

Based on the fact that these compounds react with the cysteine residue in the kinase nucleotide binding site through a Michael addition, modulation of the electronic properties of the enone may further enhance the inhibitory properties of this pharmacophore. Previously, we have shown that substitution at the β-position of the enone with a methyl group abolishes activity.[11] These results were concurrently confirmed by researchers from Eisai.[12]

To further pursue modulation of the Michael acceptor, we reasoned that a fluorine substituent at the α-position of the enone may accentuate the Michael acceptor properties while constituting a fairly neutral steric permutation. This "molecular editing"[13] may provide further benefits from a synthetic perspective, as well as favorably impede the propensity of these *cis*-enones to isomerize to the thermodynamically more favorable and biologically less active *trans*-enone. As shown in Figure 2, the synthesis could capitalize on our previously established strategy,[7,10,14] namely, disconnection of the ester and benzylic position. However, the difference of reactivity between a fluoroalkene and an alkene could be harnessed to introduce the diol moiety by dihydroxylation chemistry.

The synthesis of fluoroenones **1** and **2** began with the straightforward conversion of ester **5** into fluoroenal **7** via aldehyde **6** relying on a Horner–Wadsworth–Emmons reaction with triethyl 2-fluoro-2-phosphonoacetate to install the desired *E*-fluoroenoal (Scheme 1). Reaction of aldehyde **7** with alkyl lithium **8** afforded the desired alcohol as a diastereomeric mixture (diastereoisomeric ratio (dr), 1:1), which was reduced to the *cis*-alkene using Lindlar's catalyst (H_2, $Pd/CaCO_3$) to afford compound **9**. As the hydroxy group will ultimately be oxidized to the ketone, the lack of selectivity is inconsequential.

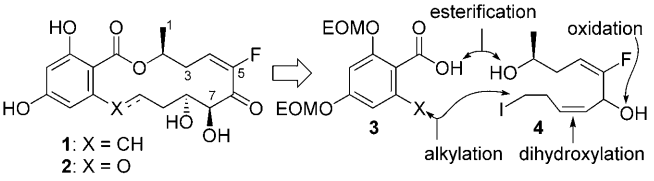

Figure 2. Key disconnections for the synthesis of 5-fluoro-*cis*-enone resorcylides.

Benzoyl protection of alcohol **9** thus afforded **10** wherein the terminal OPMB group was converted to an iodide **11** (DDQ; I_2, PPh_3, imidazole). Attempts to alkylate a phenol using **11** led to rapid elimination of the iodide to give a conjugated triene (product not shown). To circumvent this elimination, which was thought to be facilitated by the presence of the alkene, compound **10** was dihydroxylated (dr, 2.5:1 in favor of the desired isomer **12**) and converted to acetonide **13**. Conversion of the terminal protected PMB ether into an iodide afforded the key intermediate **14**.

Fluoroenones **1** and **2** were synthesized from key intermediate **14** via the route shown in Scheme 2. Following previously established chemistry, fragment **14** was coupled to the aromatic moiety **15** bearing a benzylic selenide by alkylation followed by oxidation/elimination of the selenide to afford **17**. Con-

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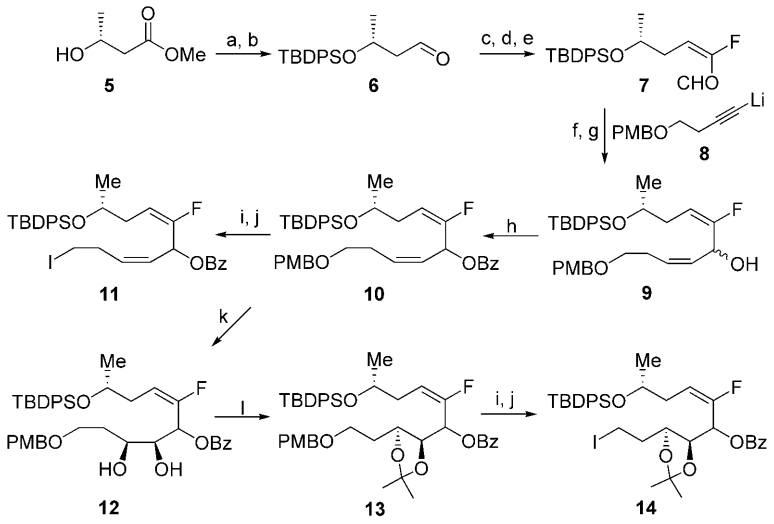
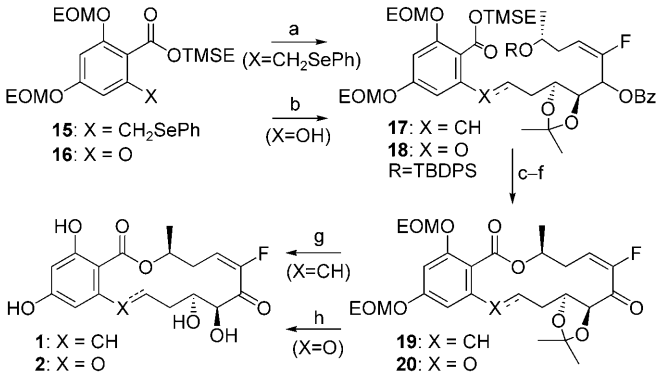


Scheme 1. Synthesis of key intermediate **14** from ester **5**. *Reagents and conditions*: a) TBDPSCl (1.1 equiv), imidazole (2.0 equiv), CH_2Cl_2, 23 °C, 13 h, 90%; b) Dibal-H (1.1 equiv), PhMe, −78 °C, 1 h, 85%; c) 1. $(EtO)_2POCHFCO_2Et$, *n*BuLi, THF, 0 °C, 1 h; 2. **6** (1.0 equiv), 0→23 °C, 12 h, 83%; d) Dibal-H (2.5 equiv), CH_2Cl_2, 0→23 °C, 85%; e) $(COCl)_2$ (1.5 equiv), DMSO (2.45 equiv), Et_3N (4.0 equiv), CH_2Cl_2, 1 h, 98%; f) 1. $PMBO(CH_2)_2CCH$ (2.1 equiv), *n*BuLi (2.0 equiv), THF, −78 °C, 1 h; 2. **7** (1.0 equiv), −78 °C, 1 h, 62%; g) H_2, Pd/$CaCO_3$, MeOH, 23 °C, 25 min, 97%; h) BzCl (1.5 equiv), pyridine (2.5 equiv), CH_2Cl_2, 0→23 °C, 14 h, 91%; i) DDQ (1.2 equiv), CH_2Cl_2/H_2O, 23 °C, 3 h, 92%; j) PPh_3 (1.8 equiv), imidazole (3.0 equiv), I_2, CH_2Cl_2, 23 °C, 1.5 h, 90%; k) OSO_4 (1%), NMO·H_2O (2.0 equiv), THF, 23 °C, 15 h, 78%; l) 2-methoxy propene (1.5 equiv), PPTS (0.1 equiv), CH_2Cl_2, 23 °C, 1 h, 93%. Abbreviations: Bz, benzoyl; Dibal-H, diisobutylaluminium hydride; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; NMO, *N*-methylmorpholine *N*-oxide; PPTS, pyridinium *p*-tolenesulfonate; TBDPS, *tert*-butyldiphenylsilyl.



Scheme 2. Synthesis of fluoroenones **1** and **2** from key intermediate **14**. *Reagents and conditions*: a) 1. LDA (2.0 equiv), THF/HMPA, −78 °C, 10 min; 2. **14** (1.0 equiv), THF, −78 °C, 20 min, 80%; 3. H_2O_2 (2.0 equiv), THF, 23 °C, 3 h, 92%; b) K_2CO_3 (2.0 equiv), **14** (1.0 equiv), DMF, 80 °C, 12 h, 99%; c) TBAF (10 equiv), THF, 23 °C, 48–56 h; d) PPh_3 (2.0 equiv), DIAD (2.0 equiv), PhMe, 23 °C, 2-4 h, 70–74% (two steps); e) 1% NaOH in MeOH, 50 °C, 2–2.5 h, 69–76%; f) DMP (1.5 equiv), CH_2Cl_2, 23 °C, 14 h, 80–88%; g) 40% aq HF in CH_3CN (1:10), 23 °C, 6.5 h, 65%; h) PS-SO_3H (10 equiv), MeOH, 23 °C, 9 h, 70%. Abbreviations: DIAD, diisopropyl azodicarboxylate; LDA, lithiumdiisopropylamide; HMPA, hexamethylphophoramide; DMF, *N*,*N*-dimethylformamide; DMP, Dess–Martin periodinane; TBAF, tetrabutylammoniumfluoride; PS, polystyrene supported.

versely, alkylation of phenol **16** with fragment **14** yielded the ether analogue **18** in excellent yield. Global deprotection of the silyl groups (TMSE and TBDPS) followed by Mitsunobu macrocyclization, selective benzoate deprotection and oxidation of the resulting alcohol afforded products **19** and **20** from **17** and **18**, respectively, in good overall yield. Global deprotection of **19** and **20** using aqueous hydrofluoric acid and resin-bound sulfonic acid, respectively, afforded the desired fluoroenone resorcylic acids **1** (from **19**) and **2** (from **20**). We and others had previously noted the sensitivity of the *cis*-enone system of LL-Z1640-2 and related analogues to acid conditions that could lead to isomerization.[10,15,16] As anticipated based on the higher thermodynamic stability of *E*-fluoroalkene, analogues **1** and **2** proved to be more resistant to epimerization, and no trace of isomerization was observed even with prolonged reaction times. Interestingly, the different diastereoisomers originating from the non-selective dihydroxylation proved to have significant difference in the kinetics of deprotection, which offered a convenient means to separate them.

To gage the indiscriminate reactivity of these enone resorcylic acids with thiols, fluoroenone **2** and the parent analogue lacking the fluoride were treated with one equivalent of DTT (2 mM) as a prototypical thiol nucleophile in phosphate-buffered saline (pH 7.4) containing 1% DMSO. Interestingly, a clear difference in reactivity was observed with the fluoroenone **2** reacting slower. With fluoroenones **1** and **2** in hand, we tested their efficacy against VEGF-R2, which was frequently found to be the most inhibited kinase in our profile of *cis*-enone resorcylic acid library,[7,11] and KIT, which represents a less inhibited kinase. As shown in Table 1, fluoroenones **1** and **2** are less active than the natural

Table 1. Inhibition of VEGF-R2 and KIT (biochemical assay).

Compound	VEGF-R2 [nM]	KIT [nM]
LL-Z1640-2	2.63	57.8
fluoroenone **1**	6.8	221
fluoroenone **2**	60.5	1600

Assays were performed in duplicate

product LL-Z1640-2, however, they maintain an inhibition level that is interesting: 6.8 nM and 60.5 nM for **1** and **2**, respectively, against VEGF-R2 compared with 2.63 nM for LL-Z1640-2, and their selectivity for VEGF-R2 relative to KIT remains unaffected. The products originating from the minor diastereoisomers of the dihydroxylation were significantly less active.

The cellular activity of fluoroenones **1** and **2** against VEGF-R2 was tested in HUE cells, a spontaneously immortalized HUVEC clone known to overexpress VEGF-R2. The results paral-

leled those of the enzyme inhibition assays. As shown in Figure 3, the natural product LL-Z1640-2 is a very potent inhibitor with an IC_{50} value of 6.5 nM. Direct comparison with fluoroenone **2** revealed an IC_{50} value of 17 nM, while the analogue containing the synthetically more accessible ether macrocycle remained very potent with an IC_{50} value of 70 nM.

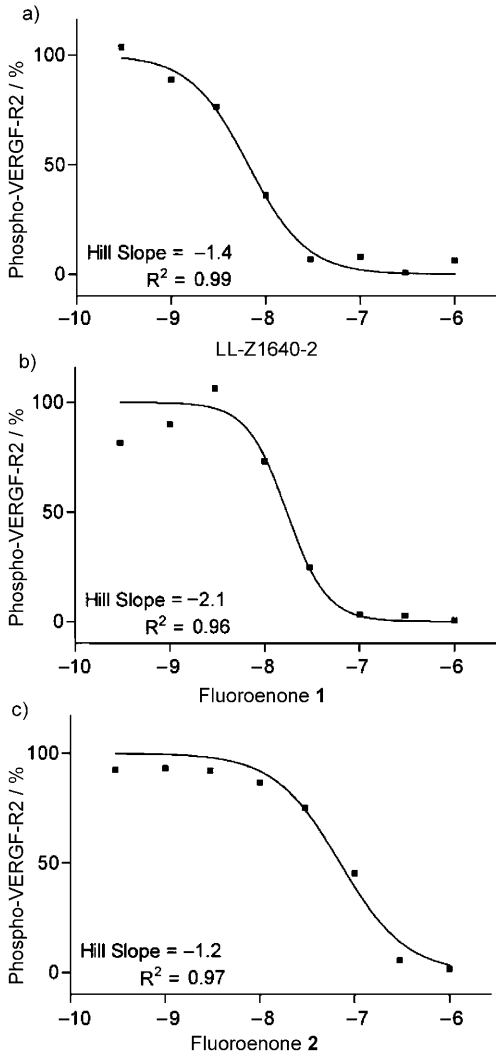


Figure 3. Cellular inhibition of VEGFR2 by a) LL-Z1640-2 (IC_{50} = 6.5 nM), b) fluoroenone **1** (IC_{50} = 17 nM) and c) fluoroenone **2** (IC_{50} = 70 nM). Assays were performed in triplicate.

Thus the fluoroenone modification is well tolerated but does not enhance the cellular efficacy of VEGF-R2 inhibition. During the course of our investigation, a similar modification was reported by researchers from Esai,[12] however, their medicinal chemistry efforts focused on the inhibition of inflammation-related pathways (MEKs and their down-stream regulatory effect on cytokines).[12,17] A selected analogue (E6201) indeed shows a >60- and >200-fold selectivity[17] for MEK1 relatively to VGF-R2 and PDGFRβ, which is a significantly different pattern relatively to the parent natural product (LL-Z1640-2) for which VEGF-R2 and PDGFRβ are most inhibited.[11] It was further shown that the addition of a methyl substituent at the γ-position relative to the enone (C3, numbering shown in Figure 2) enhanced the metabolic stability with a tolerable loss of activity (tenfold).[12] It should be noted, however, that several *cis*-enone have already been shown to be effective in vivo. As the inhibition is irreversible, the relative rate of metabolic instability and irreversible inhibition coupled to target turn over should be considered.

Experimental Section

Detailed procedures for the synthesis of compounds **1** and **2** are described in the Supporting Information.

Fluoroenone **1**: R_f = 0.19 (CH_2Cl_2/MeOH, 9:1); ^{1}H NMR (CD_3OD, 400 MHz): δ = 6.99 (d, J = 15.3 Hz, 1H), 6.40 (d, J = 2.4 Hz, 1H), 6.28 (d, J = 2.4 Hz, 1H), 6.09 (ddd, J = 15.1, 8.9, 6.4 Hz, 1H), 5.95 (ddd, J = 21.9, 11.6, 2.7 Hz, 1H), 5.28–5.20 (m, 1H), 4.90 (br s, 1H), 4.09–4.04 (m, 1H), 3.50–3.39 (m, 1H), 2.62–2.53 (m, 1H), 2.31–2.17 (m, 2H), 1.50 ppm (d, J = 6.2 Hz, 3H), n.b., 4OH signals not visible; ^{13}C NMR (CD_3OD, 100 MHz, 25 °C): δ = 196.4 (d, $^2J_{C,F}$ = 36.8 Hz, C=O), 172.8 (C=O), 166.9 (C), 164.1 (C), 155.2 (2×d, $^1J_{C,F}$ = 254.6 Hz, C), 145.0 (C), 133.9 (CH), 132.3 (CH), 123.9 (d, $^2J_{C,F}$ = 18.4 Hz, CH), 109.4 (CH), 103.5 (C), 102.9 (CH), 81.7 (d, $^3J_{C,F}$ = 3.8 Hz, CH), 74.8 (CH), 74.2 (CH), 36.6 (CH_2), 33.4 (d, $^3J_{C,F}$ = 6.1 Hz, CH_2), 20.9 ppm (CH_3); HRMS (MALDI-TOF): *m/z* $[M+Na]^+$ calcd for $C_{18}H_{19}FO_7Na$: 389.1013; found: 389.1024.

Fluoroenone **2**: R_f = 0.18 (CH_2Cl_2/MeOH, 9:1); ^{1}H NMR (CD_3OD, 400 MHz): δ = 6.02 (d, J = 1.9 Hz, 1H), 5.98 (d, J = 1.9 Hz, 1H), 5.88 (ddd, J = 21.8, 11.8, 2.7 Hz, 1H), 5.44–5.29 (m, 1H), 5.03 (d, J = 4.3 Hz, 1H), 4.24–4.18 (m, 2H), 3.97–3.88 (m, 1H), 3.11–3.00 (m, 1H), 2.55–2.44 (m, 1H), 1.94–1.86 (m, 1H), 1.74–1.61 (m, 1H), 1.38 ppm (d, J = 5.9 Hz, 3H), n.b., 4OH signals not visible; ^{13}C NMR (CD_3OD, 100 MHz, 25 °C): δ = 196.8 (d, $^2J_{c,F}$ = 35.9 Hz, C=O), 172.6 (C=O), 167.1 (C), 165.4 (C), 163.6 (C), 155.6 (2xd, $^1J_{C,F}$ = 255.3 Hz, C), 119.5 (d, $^2J_{C,F}$ = 17.6 Hz, CH), 96.6 (CH), 96.4 (C), 92.8 (CH), 80.8 (d, $^3J_{C,F}$ = 2.9 Hz, CH), 73.1 (CH), 69.5 (CH), 65.8 (CH_2), 33.1 (d, $^3J_{C,F}$ = 5.9 Hz, CH_2), 31.3 (CH_2), 20.8 ppm (CH_3); HRMS (MALDI-TOF): *m/z* $[M+Na]^+$ calcd for $C_{17}H_{19}FO_8Na$: 393.0962, found: 393.0942.

Enzymatic inhibition of VEGF-R2 and KIT: A radiometric protein kinase assay (^{33}PanQinase® Activity Assay) was used for measuring the kinase activity of VEGF-R2 and KIT kinases (Proqinase, Freiburg, Germany). All kinase assays were performed in 96-well FlashPlates™ from Perkin–Elmer (Boston, USA) using 50 μL of assay buffer (60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 μM Na_3VO_4, 1.2 mM DTT, 50 μg mL^{-1} PEG2000, 1 μM [γ-^{33}P]ATP), 20 ng of kinase and a generic substrate (polyGluTyr) with 1% DMSO. The test compound concentration ranged from 10 μM to 0.1 nM (semilog dilution). The assays were performed by premixing the ATP solution with the test compound and addition of this solution to the kinase/substrate solution. After 60 min at 30 °C, the reaction was stopped with 50 μL of 2% H_3PO_4, plates were aspirated and treated with 200 μL of 0.9% NaCl (2×) and the level of ^{33}P incorporation was determined with a microplate scintillation counter (MicroBeta, Wallac). Assays were run in duplicate.

Cellular VEGF-R2 inhibition assay: HUE cells, a spontaneously immortalized HUVEC clone known to overexpress VEGF-R2, were plated in ECGM (PromoCell, Germany), supplemented with 10% fetal calf serum (FCS), with 25.000 cells per well in 48-well cell culture dishes. After 6 h, the medium was exchanged against ECBM (PromoCell, Germany) without FCS, and the cells were starved

overnight. Prediluted test samples in DMSO (from 0.1 mM to 30 nM in half logarithmic steps) were added to the cell culture medium (1:100) resulting in a final DMSO concentration of 1%. After incubation for 90 min at 37 °C, cells were stimulated for 7 min at room temperature with 100 ng mL^{-1} hVEGF-A165. As a positive control, one row in each plate was treated with the known kinase inhibitor staurosporine. The mean value of those wells was used as the background, which was substracted from all other data points. One row was treated with DMSO alone and represents the maximal gain in activation with cells that received 1% DMSO and had been stimulated with hVEGF-A165. The mean of those wells was set to 100%. Quantification of VEGF-R2 phosphorylation was assessed in 96-well plates via sandwich ELISA using a respective VEGF-R2-specific capture antibody and an antiphosphotyrosine detection antibody. Raw data were converted into percent receptor autophosphorylation relative to stimulated controls, which were set to 100%. IC_{50} value determination was done with GraphPad Prism 5.01 software with constrain of bottom to 0 and top to 100 using a nonlinear regression curve fit with variable hill slope. The equation is a four-parameter logistic equation. All compounds were tested in triplicate.

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Keywords: fluorine · irreversible inhibitors · kinases · resorcylic acid lactones · VEGF

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