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Total synthesis of eryvarin H and its derivatives and their biological activity as ERRγ inverse agonist†

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Total synthesis of eryvarin H and a biological investigation of its analogues as a potential inverse agonist of ERRγ are described here. Among the 13 analogues prepared by the modular synthetic route, eryvarin H and compound 12 showed meaningful ERRγ inverse agonistic activities along with moderate selectivity over ERα and other nuclear receptors in the cell-based reporter gene assay.

Nuclear receptors (NRs) have been recognized as one of the most important targets for the development of novel therapeutic agents *via* regulating various disease-relevant cellular functions at the transcription level.[1] NRs are described as transcriptional factors mainly regulated by endogenous ligands in biological systems. Among them, a series of NRs are categorized as orphan nuclear receptors when they do not have any identified endogenous ligands, and some of the orphan nuclear receptors still possess constitutive transcriptional activities without their endogenous ligands.[2] Therefore, the identification of bioactive small molecules that can specifically control their activity can provide an important clue to the development of novel therapeutic agents as well as a research tool for deciphering complex events in biological systems.[3] Unlike the genetic approach, bioactive small molecules can perturb particular functions of wild-type gene products in a temporal and reversible manner.[4] Therefore, we aimed to identify new synthetic ligands for the transcriptional regulation of nuclear receptors, especially orphan nuclear receptors.

We have been interested in estrogen-related receptors (ERRs) that are orphan NRs and closely related to estrogen receptors (ERs) in their sequence homology. But their difference in the ligand binding domain (LBD) differentiates their characteristics from those of ERs.[5] ERRs have various functional roles associated with diseases including cancer and metabolic diseases as ER does.[6] Among the members of the ERR family, we paid particular attention to ERRγ due to its crucial role in various biological events, such as hepatic insulin signaling, gluconeogenesis, regulating oxidative metabolism, suppressing tumor growth of prostate cancer cells, and modulating cell proliferation and estrogen signaling in breast cancer.[6–8] ERRγ is a third subtype receptor and is highly expressed in various human adult tissues including brain, kidney, skeletal muscle, heart, and placenta.[9] Based on these observations, ERRγ became an emerging target for cancers and metabolic diseases, even though there are a limited number of ERRγ agonists and inverse agonists that control its downstream activity.[10–12] In addition, most of the reported ligands suffered non-specific interactions with various gene products and caused functional cross-talks with other nuclear receptors, especially with ERα, in the estrogenic signaling pathway.[10,11] Therefore, it is essential to discover novel bioactive small molecules that selectively regulate ERRγ to elucidate its functional roles in our body. As a continuation of our efforts[7,10] in this field, we pursued the identification of novel ERRγ inverse agonists to deliver new potential drug-like molecules to the biomedical community.

Under this mission statement, we searched for the new chemical entity from the collection of natural products using structure-based *in silico* analysis. With the X-ray co-crystal structure of ERRγ with GSK5182, a known ERRγ inverse agonist, we performed a docking simulation study of the natural product collection at the LBD of ERRγ, which allows the identification of potential ligands for ERRγ. After computer-based screening of 4000 structurally diverse natural products from the Korea Bioactive Natural Material Bank, we selected the natural product

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eryvarin H (**1**) as a potential inverse agonist of ERRγ. Eryvarin H was first isolated from the roots of *Erythrina* species, *Erythrina variegata*[13] or *Erythrina abyssinica* in 2003.[14] In the past, eryvarin H has been known to be an anti-microbial agent[14] or a cellular radical scavenger.[15] However, eryvarin H (**1**) used in previous reports was extracted from natural resources with a limited quantity. Therefore, herein, we accomplished the first total synthesis of eryvarin H (**1**) and its derivatives using a modular synthetic route. The resulting eryvarin H and its derivatives were evaluated for their function as selective ligands for ERRγ.

For the discovery of a novel ERRγ inverse agonist, we first explored the *in silico* docking study of structurally diverse natural products with ERRγ co-crystallized with GSK5182 (PDB ID: 2GPU).[12] GSK5182 is a confirmed ERRγ inverse agonist and binds at its ligand binding site. From the collection of natural products, we searched for potential ligands that can bind at the LBD of ERRγ in a similar way to GSK5182. Based on the binding scores in computational docking analysis, we selected a list of natural products. Among them, we selected eryvarin H (**1**) as a potential inverse agonist of ERRγ. As shown in Fig. 1a and b, eryvarin H (**1**) nicely occupies the empty space at the active site of ERRγ with dipole–dipole interaction as well as hydrophobic interactions, which was clearly visualized in the electrostatic potential clouds of eryvarin H at the LBD of ERRγ. The whole amino acid map within the range of 2 Å also reveals the specific interactions, such as hydrogen bonding of Asp273, Tyr326 and Asn346 with hydroxyl groups on eryvarin H (**1**) and dipole–dipole interaction of Cys269 with ether linkage on eryvarin H (**1**) (Fig. 1c). However, even though there is a series of potential specific interactions of eryvarin H with ERRγ, it is essential to have a structural modularity to ensure the specificity due to the high degree of structural similarity of ERRγ with ERα. In addition, the extraction from natural resources only provides the limited quantity of eryvarin H without an access to its analogues. Therefore, we pursued the total synthesis of eryvarin H (**1**) and the subsequent structural modification for structure–activity relationship study.

Eryvarin H (**1**) is composed of two aromatic rings, 2*H*-chromen-7-ol and electron-rich aryl ring, that can be connected *via* C–C bond formation using Pd-mediated cross-coupling (Fig. 1e). Based on this retrosynthetic analysis of eryvarin H (**1**), we prepared the two cross-coupling partners, vinyl bromide **2a** and arylboronic ester **3**. For the preparation of the vinyl bromide (**2a**) part, resorcinol was first transformed to 7-hydroxychroman-4-one (**4**) by the treatment of 3-chloropropionic acid *via* acid-catalyzed electrophilic aromatic acylation, followed by *O*-alkylation.[16] The mono-bromination at the α position to carbonyl in compound **4** led to the formation of compound **5**. The *tert*-butyldimethylsilyl (TBS) protection of phenol in compound **5**, carbonyl reduction using $NaBH_4$, and the subsequent acid-catalyzed dehydration allowed the formation of desired vinyl bromide (**2a**) in good yields (Scheme 1a). The synthesis of its cross-coupling counterpart, arylboronic ester (**3**), was started by the treatment of 2,5-dimethoxybenzaldehyde with Br_2 under mild acidic conditions to provide its brominated compound **5**.[17] This aryl bromination was quite regioselective at the C-4 position due to the presence of aldehyde moiety as a biasing element, which was interconverted to phenol moiety through Baeyer–Villiger

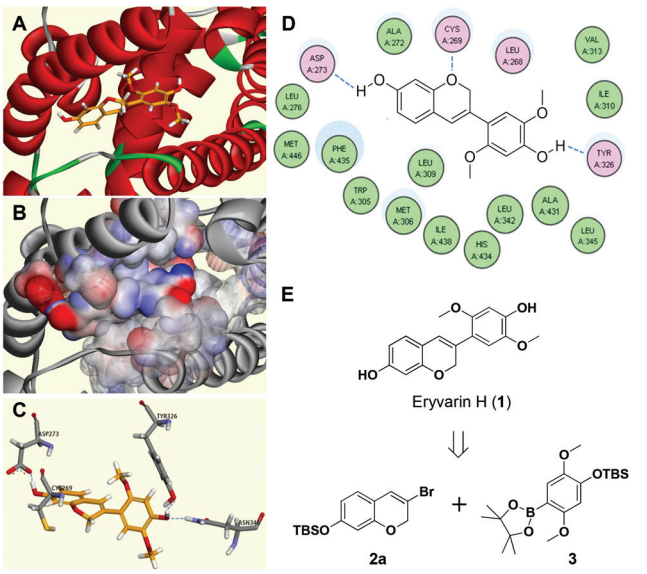
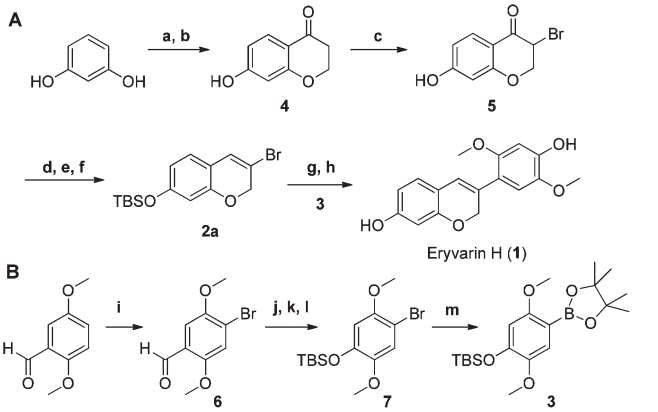


Fig. 1 Docking simulation of eryvarin H at the ligand binding pocket of ERRγ. (a) Potential binding mode of eryvarin H; (b) possible hydrophobic and dipole–dipole interactions of eryvarin H with residues at the ligand binding site of ERRγ shown in a space filling model. The red color represents partial positive charge, and the blue color represents partial negative charge. (c) Potential hydrogen bonding interactions of eryvarin H with polar amino acids (Asp273, Tyr326, and Asn346); (d) schematic diagram of amino acids within the 2 Å range from eryvarin H; (e) chemical structure of eryvarin H and its retrosynthetic analysis.



Scheme 1 Synthetic route for total synthesis of eryvarin H *via* C–C bond formation between two coupling partners (**2a** and **3**). Reagents and conditions: (A) Preparation of eryvarin H (**1**) through **2a**. (a) 3-Chloropropionic acid, TfOH, 80 °C, 2 h; (b) 2 N NaOH, 0 °C to r.t., 3 h, 62% (2-step yield); (c) $CuBr_2$, EtOAc–$CHCl_3$–MeOH, 70 °C, 4 h, 67%; (d) TBSCl, imidazole, DCM, r.t., 1 h; (e) $NaBH_4$, EtOH, r.t., 1 h; (f) TsOH–H_2O, toluene, 80 °C, 120 W μW, 20 min, 61% (3-step yield); (g) **3**, $Pd(PPh_3)_4$, Na_2CO_3, toluene–EtOH–H_2O, 80 °C, 2 h; (h) HF/pyridine, THF, r.t., 3 h, 73% (2-step yield). (B) Preparation of **3**. (i) Br_2, acetic acid, r.t., 12 h, 62%; (j) *m*-CPBA, DCM, r.t., 3 h; (k) NaOH, methanol, r.t., 3 h; (l) TBSCl, imidazole, DCM, r.t., 3 h, 95% (3-step yield); (m) pinacolborane, $Pd(OAc)_2$, DPEphos, TEA, 1,4-dioxane, 100 °C, 12 h, 63%.

oxidation of **6** and the direct hydrolysis of the resulting ester in a basic methanol solution. The resulting phenol was then protected by TBSCl to prepare **7** in excellent 3-step yields. Finally, various conditions were tested for the introduction of C–C cross-coupling moiety, but Pd-catalyzed boronic ester formation with pinacolborane, DPEphos, triethylamine (TEA) in 1,4-dioxane only yielded the desired product **3** in a reasonable yield (Scheme 1b). After the preparation of both key partners, we successfully finished up the first total synthesis of eryvarin H (**1**) *via* the formation of C–C covalent bonds between vinyl bromide **2a** and arylboronic ester **3** using Suzuki–Miyaura coupling reaction, followed by TBS deprotection with HF/pyridine with 8 linear steps in 18% overall yield (see ESI†).

After the completion of total synthesis of eryvarin H, we envisioned the preparation of its systematic analogues for the molecular level understanding of its binding event with ERγ. On the basis of our *in silico* docking simulation of eryvarin H at the LBD of ERγ, we hypothesized that the specific interaction of 2′,5′-dimethoxy-4′-hydroxyphenyl on eryvarin H at the deep binding pocket of ERγ is crucial for its functional modulation. We also revealed some flexible and empty regions at the ligand binding site in the docking structure of ERγ with eryvarin H, which might provide an opportunity to induce some additional binding interactions for enhanced potency and selectivity toward ERγ over ERα. Therefore, we prepared 12 derivatives (**8–19**) of eryvarin H using the modular synthetic procedure (see ESI†). As shown in Fig. 2, we focused on the diversification of aryl substituents to decipher the origin of specific hydrogen bondings at the ligand binding pocket. Dimethyl-substituted vinyl bromide **2b**, TIPS-protected 3-bromo-2,2-dimethyl-2*H*-chromen-7-ol, was synthesized using a reported procedure.[18]

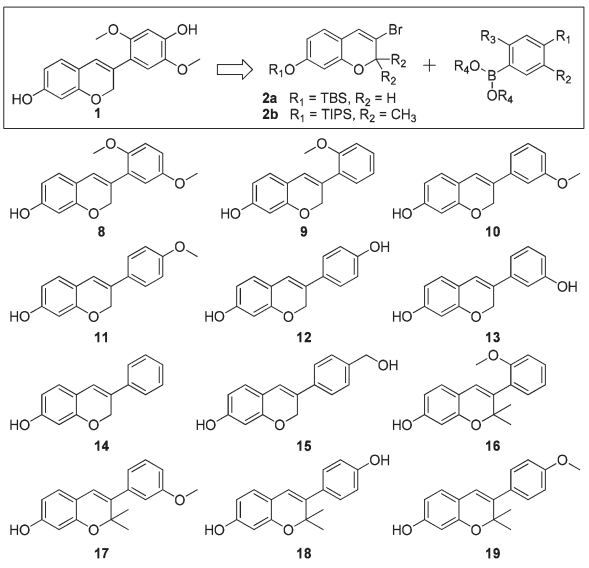


Fig. 2 Derivatives of eryvarin H containing isoflav-3-ene structural motif (**8–19**). These derivatives were systematically prepared by Pd-mediated Suzuki cross-coupling between aryl bromide structures (**2a** or **2b**) and various boronic esters/boronic acids.

After the preparation of eryvarin H (**1**) and its analogues (**8–19**), their biological activities on the transcriptional regulation toward ERγ were evaluated using the cell-based reporter gene assay to confirm their roles as inverse agonists. In this assay, we measure the chemoluminescence induced by the expression changes of luciferase upon treatment of small molecules after the transient transfection of DNA plasmid (Gal4-fused ERγ-LBD construct) to HEK-293 T human kidney cell line by calcium phosphate transfection protocol.[19] The resulting luminescence signal was normalized with β-gal expression to minimize the false positives caused by cellular cytotoxicity.[20] As shown in Fig. 2, we confirmed that eryvarin H (**1**) is an ERγ inverse agonist and showed a drastic reduction in the chemoluminescence signal of downstream luciferase. Interestingly, all 13 analogues including eryvarin H showed some levels of inverse agonistic activities toward ERγ, though their relative luminescence signals were varied on the basis of chemical structure (see ESI†). Among them, compounds **1**, **11**, and **12** showed more potent inverse agonism. Speaking of their structure–activity relationship, either a hydroxy or methoxy group at the C-4′ position is essential for their activity and the steric or hydrophobic elements are not necessary at the C-2 position in the case of analogues (**16–19**) containing 2*H*-chromenol with dimethyl substituents instead of two hydrogens. The additional bulky dimethyl substituents at the C-2 position might block the specific interactions with the ligand binding pocket of ERγ, which significantly reduced the inverse agonistic activity.

Furthermore, transcriptional activities of these 13 analogues towards other kinds of NRs (ERα, mCAR, HNF4, SF-1)[21] were measured by reporter gene assay systems to investigate the selective ERγ inverse agonistic activity over other NRs (Fig. 4). Owing to the high degree of structural similarity and DNA sequence homology of ERγ with ERα, the ligand-induced transcriptional activity of ERγ is compared with that of ERα. As shown in Fig. 3 and 4, eryvarin H and compound **12** showed the selective inverse agonistic activity toward ERγ

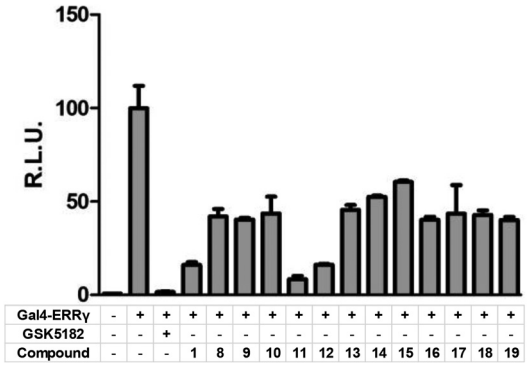


Fig. 3 Biological evaluation of eryvarin H (**1**) and its derivatives (**8–19**) as inverse agonists using the cell-based reporter gene assay in HEK-293 T cells after transfection of Gal4-ERγ, pFR-Luc, and β-gal plasmids. Compounds **1**, **11** and **12** showed good inverse agonistic activities among all tested compounds. All compounds including GSK5182 were treated at the final concentration of 10 μM. R.L.U. is relative luminescent unit.

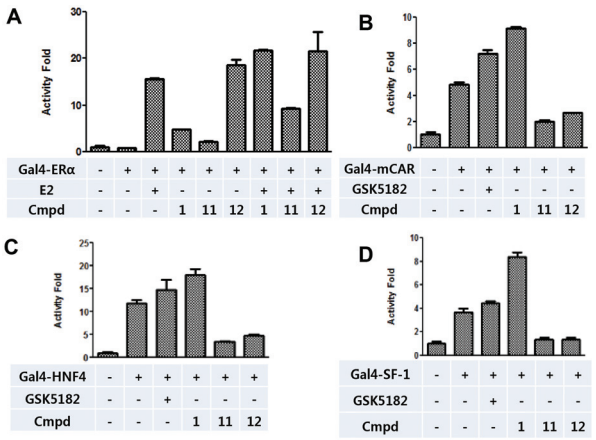


Fig. 4 Transcriptional activities of eryvarin H, **11**, and **12** towards other NRs: (a) ERα, (b) mCAR, (c) HNF4 and (d) SF-1. Normalized luciferase activities of each NR were tested in HEK-293. T cells upon treatment of three compounds to test their selective inverse agonism over that in ERRγ. E2 refers to estradiol.

over ERα. However, the efficacy and specificity of eryvarin H we observed in this study are not comparable to those of GSK5182 that we have used for the biochemical studies of ERRγ.

In conclusion, natural products have been serving as essential perturbagens in various biological processes due to their evolutional heritage and structural diversity. As a continuation of our effort at the functional modification of ERRγ, we aimed to develop a novel ERRγ inverse agonist *via in silico* docking analysis with a collection of natural products. The computer-aided ligand discovery allowed the identification of eryvarin H (**1**) as a potential inverse agonist of ERRγ. Along with a limited supply of eryvarin H *via* extraction from natural resources, the inability for a structure–activity relationship of this natural product against ERRγ led us to pursue the first total synthesis of eryvarin H and its structural analogues. In addition, we wanted to decipher the molecular basis of its interaction with residues at the ligand binding pocket of ERRγ, which has high structural similarity and sequence homology with ERα. Therefore, we prepared two key intermediates, vinyl bromides (**2a**) and arylboronic ester (**3**), and successfully completed the total synthesis of eryvarin H through Pd-mediated Suzuki–Miyaura cross-coupling of two intermediates in reasonable overall yields. With this modular synthetic route, we also prepared 12 derivatives (**8–19**) of eryvarin H simply by changing the substituents on arylboronic esters or introducing a dimethyl substituent at the C-2 position of TBS-protected 3-bromo-2*H*-chromen-7-ol. The resulting 13 analogues including eryvarin H were subjected to the cell-based reporter gene assay using DNA plasmid containing Gal4-fused ERRγ LBD to measure their biological activity as ERRγ inverse agonists. Among these derivatives, eryvarin H and compound **12** showed meaningful ERRγ inverse agonistic activities along with moderate selectivity over ERα and other NRs. However, the level of efficacy and specificity of eryvarin H toward ERRγ over ERα is not comparable to that of GSK5182 that we have used for the biochemical studies of ERRγ. Therefore, we unfortunately discontinued the follow-up *in vivo* biological study of eryvarin H and its analogues due to their limited potential as a selective ERRγ inverse agonist. Even though this study failed to produce a selective and potent inverse agonist of ERRγ, this study clearly exemplified the rational drug discovery procedure using *in silico* docking simulation with a collection of natural products, followed by total synthesis of a natural product and its analogues as a potential perturbagen of proteins-of-interest. We envision that this rational approach can be adapted to identify a new small molecule that modulates the function of new therapeutic targets and key signaling pathways.

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