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# Discovery of a potent, metabolically stabilized resorcylic lactone as an anti-inflammatory lead

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ABSTRACT

With bioactivity-guided phenotype screenings, a potent anti-inflammatory compound f152A1 has been isolated, characterized and identified as the known natural product LL-Z1640-2. Metabolic instability precluded its use for the study on animal disease models. Via total synthesis, a potent, metabolically stabilized analog ER-803064 has been created; addition of the (*S*)-Me group at C4 onto f152A1 has resulted in a dramatic improvement on its metabolic stability, while preserving the anti-inflammatory activities.


At Eisai, natural product inspired drug discovery has been one of the key research endeavors in several disease areas. With bioactivity-guided phenotype screenings, researchers at Eisai Tsukuba Laboratories detected a potent anti-inflammatory activity in the fraction #152 obtained from the fermentation broth of a fungus, *Curvularia verruculosa*, in the mid 1990s. They isolated and characterized the active metabolite, named as f152A1, thereby establishing that f152A1 is identical with a previously reported natural product LL-Z1640-2 **1**.[1] Several related natural products are known in literature, some of which are listed in Figure 1.[2,3] In 1978, the Lederle group reported that **1** exhibited modest inhibitory activity against growth and motility of ciliated protozoan *Tetrahymena Pyriformis*.[1] More recently, **1** and **2** were reported to exhibit potent inhibition of PDGFR signaling and IL-1β release from inflammatory cells.[4]

We became interested in f152A1 (**1**), because it exhibited potent inhibitory activity in cell signaling ($IC_{50}$ of 11 nM in a TNFα-PLAP reporter assay), but showed much weaker activity in a counter screen assay designed to assess non-specific cytotoxic effects ($IC_{50}$ of 2000–3000 nM in a β-actin-PLAP reporter assay). Despite its poor PK properties, f152A1 showed an in vivo activity indicating its inhibition of LPS-stimulated TNFα production in mice.

Our investigations suggested that its pharmacological effects could be attributable to inhibition of two kinases MEKK1 and MEK1 associated with inflammatory signaling.[5] These discoveries were consistent with similar findings reported by a Merck group on a related compound L-783,277 (**3**).[2b,6] In this Letter, we report a potent and serum stable analog ER-803064 (**15**) of f152A1 (**1**), along with some selected SAR studies.

In an early phase of study, we recognized that f152A1 (**1**) exhibits very potent inhibitory activities in vitro (Table 1). Unfortunately, f152A1 was rapidly inactivated in human or mouse microsomes and plasma (Fig. 2). Analysis of the metabolites in mouse plasma indicated that *cis*-to-*trans* isomerization of the enone is the major deactivating process (Supplementary Fig. S1A). The *trans*-isomer **7** was found to be approximately 50-fold less potent ($IC_{50}$ = 349 nM) than the parent *cis*-isomer (Fig. 4). We hypothesized that the *cis*-to-*trans* isomerization of the enone takes place via Michael addition/elimination and that glutathione (GSH) and glutathione transferase (GST) in biological fluid are responsible for the isomerization. Indeed, we found that the enone is unstable in the presence of GSH in PBS (Supplementary Fig. S1A) and the deactivation process is accelerated with the combined use of GST

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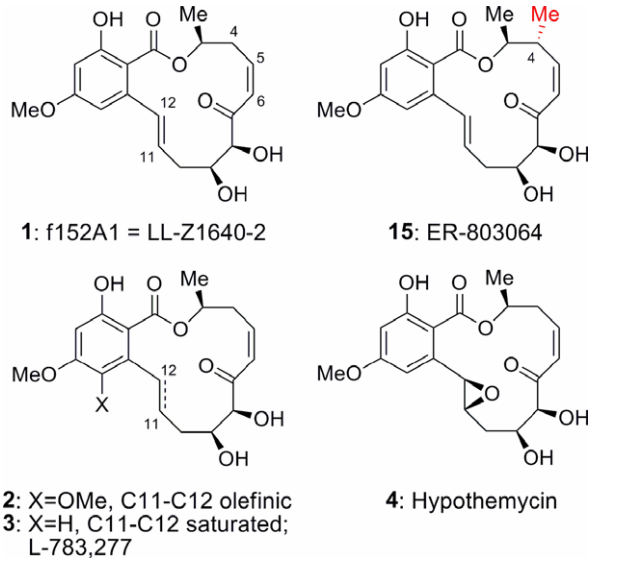



**Figure 1.** Structure of f152A1 (LL-Z1640-2), closely related natural products[2,3] and ER-803064.

**Table 1**
Mouse PK data of selected compounds

| PK parameter iv dosing | **1** (f152A1)[a] | **15** (ER-803064)[b] |
|---|---|---|
| CL (L/kg/h) | 42.6 | 5.51 |
| VDss (L/kg) | 3.3 | 1.69 |
| *t*/2 (h) | 0.05 | 3.44 |
| AUC/Dose (ng h/mL/kg) | 23.8 | 181.35 |

[a] Plasma level of f152A1 was only detected at two of the earliest time points after intravenous administration (3 mg/kg) to BALB/c mice. Oral administration of f152A1 resulted in no detectable plasma level.
[b] ER-803064 showed 9% oral BA, results not shown.

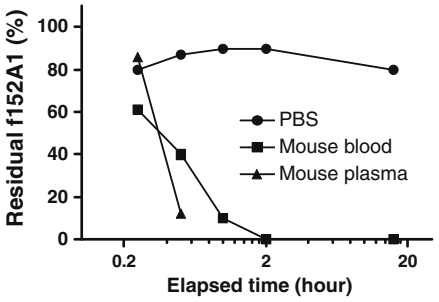



**Figure 2.** Stability of f152A1 in PBS, mouse blood and plasma. See Ref. 11 in Supplementary data notes.

and GSH (Supplementary Fig. S1B). Based upon these results, we concluded that an enzymatic process catalyzed by GST is the main cause for metabolic instability of f152A1 in vivo. Therefore, we first focused to find a lead compound that would maintain the inhibitory activities with an improved metabolic stability. To achieve this goal, we planned to take two different approaches: (1) to search for a metabolically stable pharmacophore equivalent to the *cis*-enone and (2) to prevent or suppress *cis*-to-*trans* isomerization of the enone in biological systems.

In order to carry out these approaches, we had to assure an access to properly modified analogs of **1** and decided to rely on total synthesis. At the start of this medicinal chemistry effort, there was no reported total synthesis of f152A1.[7,8] Through retro synthetic

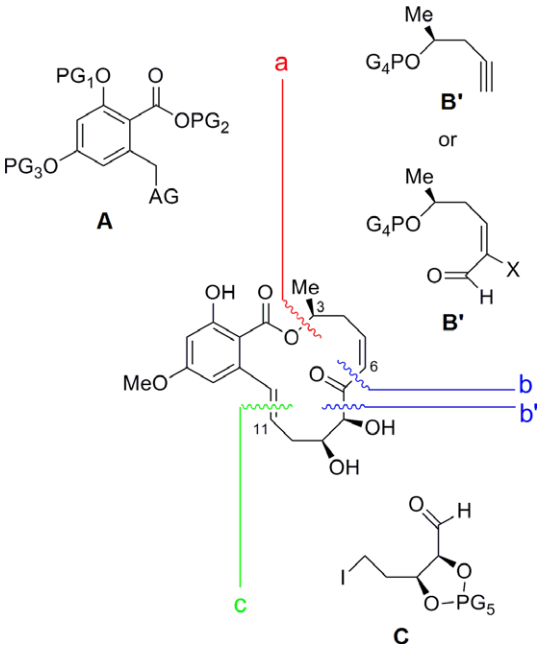



**Figure 3.** Key fragments and bond formations: Three key fragments **A**–**C** with various protecting groups were used. Assembly of these fragments involve in general: Disconnection a: macrolactonization at the final assembly stages using the standard Yamaguchi or Mukiyama protocols; Disconnection b: C3–C6 acetylene or its equivalent were coupled with C7–C11 aldehyde, to give the C5–C6 *cis*-olefin; Disconnection b': Alternative route for modification on C5 or C6-enone, C3–C7 fragment with *cis*-C5–C6 olefinic configuration could be coupled with an acetylene to provide the C3–C11 acyclic fragment for assembly; Disconnection c: a aromatic benzylic selenide could be coupled with alkyl iodide C3–C11 or C7–C11, to form C11–C12 *trans*-olefin. Analog synthesis used these synthetic routes or variation of Ref. 9.

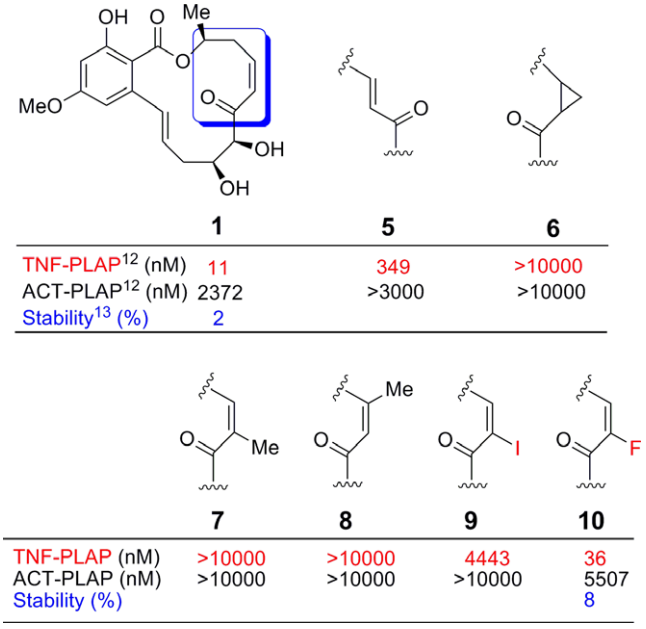
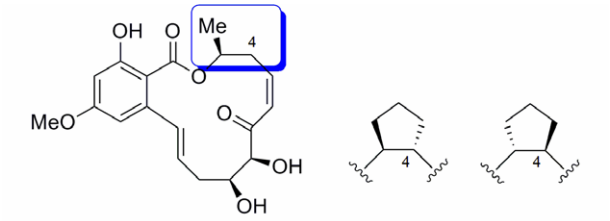
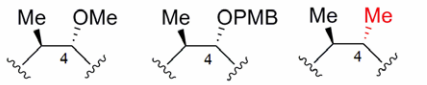



**Figure 4.** Selected examples for the enone modification. TNF/PLAP and Actin/PLAP were employed to assess potency and specificity. Their experimental protocols are given in note 12 in Supplemental data. Stability test (2 h at 37 °C) was conducted with use of mouse plasma, with the amount (%) of non-metabolized parent compound being indicated (see note 13).

analysis, we divided f152A1 into three segments, **A**, **B** and **C** (Fig. 3) and developed efficient and flexible syntheses. Three synthetic routes are outlined in Supplementary data (Schemes 1–4).



| | 1 | 11 | 12 |
|---|---|---|---|
| TNF-PLAP (nM) | 11 | 667 | 4000 |
| ACT-PLAP (nM) | 2372 | 3000 | >30000 |
| Stability (%) | 2 | 77 | 34 |



| | 13 | 14 | 15 (ER-803064) |
|---|---|---|---|
| TNF-PLAP (nM) | 239 | 888 | 136 |
| ACT-PLAP (nM) | 7597 | >10000 | >10000 |
| Stability (%) | 25 | | >80 |

**Figure 5.** Representative examples for the C3–C4 modification.

With the developed synthetic routes, we synthesized dozens analogs bearing the enone replacements (Fig. 4 and Supplementary Table S1). Overall the integrity of the enone is critical for biologic activity. Replacing the enone, with cyclopropyl, analogs **6,** or substitution on the enone with larger size substuents, α-methyl **7**, β-methyl **8** or α-iodide **9**, proved to be detrimental. However, the C6-fluoro enone analog, **10** retained good potency (TNF-PLAP, $IC_{50}$ = 36 nM), while maintaining low level actin-PLAP activity. **10** also afforded some stability improvements (~8% remaining vs ~2% of f152A1). In this fluoro-enone series, the potency of (8*R*,9*R*) diol-epimers **23** (Supplementary Table S1) were reduced, suggesting importance of these hydroxyl groups in binding and bioactivity.

In the second approach, we synthesized analogs with modified patterns of the C3–C4 substitution. Among them, C3–C4 fused

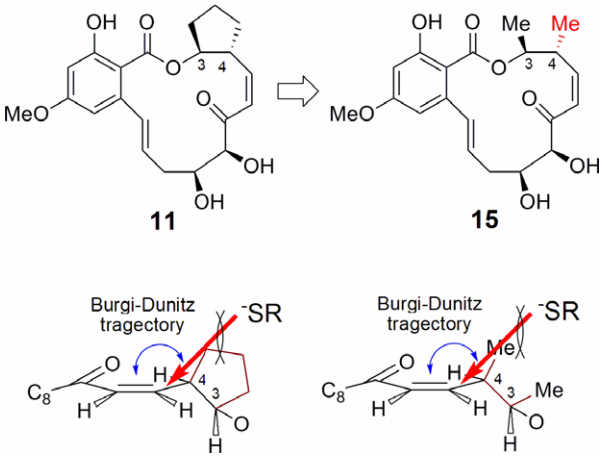



**Figure 6.** Trajectory model of **11** and proposed **15**.[10]

cyclopentane (3*S*,4*S*)-**11**, (3*R*,4*R*)-**12**, and C4(*S*)-OMe **13** showed considerable improvement in metabolic stability, with maintaining decent bio-activities (Fig. 5); for example, (3*S*,4*S*)-**11** showed a dramatic improvement in mouse plasma stability over f152A1 (**1**) (Fig. 5). Interestingly, the (3*S*,4*S*)-configuration was found to be essential to maintain a potency in bioactivity (additional data in Supplementary Table S2).

Using the AMBER program (version 3.0, revision A), molecular dynamic calculations were performed on f152A1, resulting in two low-energy conformers. Based on these conformers, we advanced a possible rationale to explain the observed, improved metabolic stability of **11** over **1**. As discussed earlier, the metabolic inactivation likely takes place via Michael addition/elimination. In both low-energy conformers, the *cis*-enone presents a clear convex face for a Michael donor to approach (see Supplementary Fig. S1B). Interestingly, the Bürgi-Dunitz trajectory for a nucleophile to approach from the convex face to the *cis*-enone is blocked by one of the methylene present in the cyclopentane ring (Fig. 6). This rationale immediately suggested the possibility that the metabolic stabilization should be observed for C4 (*S*)-methyl analog **15** (Fig. 6). Interestingly, a replacement of the cyclopentane ring in

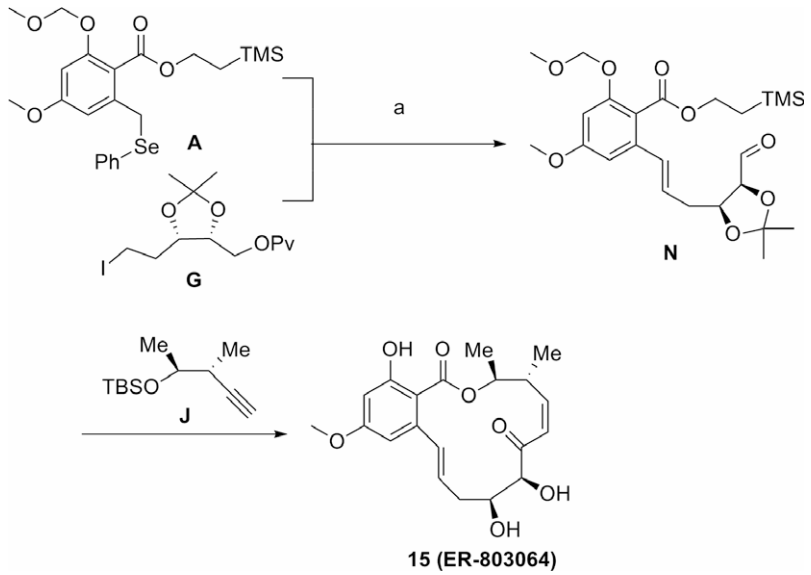



**Figure 7.** First synthesis of **15** (ER-803064). Reagents: (a) (1) LiHMDS, THF/HMPA; (2) *m*CPBA; (3) TEA; (4) base; (5) oxidation; (b) (1) *n*-BuLi, 78%; (2) lindlar, $H_2$; (3) BzCl, 94% (two steps); (4) TBAF, 68%; (5) $Cl_3PhCOCl$, DMAP, toluene, 62%; (6) base, quantitative; (7) oxidation, quantitative; (8) HF, 86%. For fragment A, G, J, see Supplementary Scheme 1.

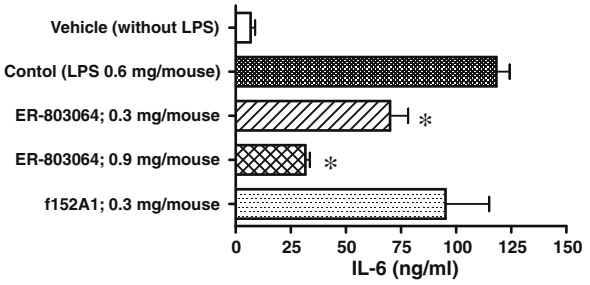



**Figure 8.** Effects of ER-803064 and f152A1 on LPS-induced IL-6 Production in Mice (for detailed description see Supplementary data note 14).

**11** with C4 (*S*)-Me group should result in a release in ring-strain of the macrolactone ring, thereby making **15** a mimic conformationally closer to the parent f152A1.

With these predictions, we synthesized analog **15** (Fig. 7). To our delight, **15** (ER-803064) indeed restored the potency ($IC_{50}$ = 136 nM), with the excellent metabolic stability (>80% in 2 h vs ~2% for f152A1). Further expanding SAR on C4 substitutions, it showed that C4 (*S*)-Me analog was the most potent among the series (Table S4). With all these properties, **15** (ER-803064) now serves us as the new lead for further optimization and also as the pharmacological benchmark.

In comparison to f152A1, mouse PK parameters of ER-803064 were then studied, thereby demonstrating clear improvements; in particular, clearance improved from 42.6 L h/kg to 5.51. The overall exposure increased approximately six-fold, normalized by dose (Table 1). Although ER-803064 lost in vitro potency by nearly 10-fold in suppression of LPS-stimulated cytokine production from macrophages, the short term LPS challenge in vivo model showed much stronger suppression of cytokine production (IL-6) in mice than f152A1 (**1**) at similar dosing (Fig. 8).[14]

In conclusion, our medicinal chemistry study has led to the discovery of ER-803064 (**15**), a serum- and plasma-stable resorcylic acid analog of ER-803064 (**1**). Addition of the (*S*)-Me group at C4 has resulted in a dramatic improvement in metabolic stability with retention of bioactivity. Based on these findings, we make efforts for development of an optimum drug candidate.

## Acknowledgments

Fermentation broth of a fungus, *Curvularia verruculosa*, was offered from Biosource Laboratories, Mercian Corporation. We thank Drs. Seiichi Kobayashi (Eisai, Japan) and Ieharu Hishinuma (Eisai Research Institute of Boston Inc.) for discussion and helpful advices.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.08.096.

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11. a) The experimental details for mouse stability is summarized as following: The test compound was either diluted in blood to the final concentration of 100 μM or 20 μM depending on the concentration of DMSO stock solution, or in pooled mouse plasma containing EDTA obtained from Cocalico Biologicals, Inc., or in PBS (1X) either made in the lab or purchased from Gibcol BRL. The spiked body fluids and PBS were incubated for different time period, usually 0, 15, 30, 60, 120, and 960 min at 37 °C with gentle shaking. 250 μL out of the spiking fluids was mixed with 100% MeOH at the end of each time point. HPLC analysis: First Waters Nova-Pak C18 column (80 × 100 mm) was applied. The HPLC running condition was as follows: Flow rate: 2 ml/min; Mobile phases: A: 250 mM $NH_4Ac$, pH 4.9, B: 100% AcCN; Gradients (B): 30% (0-18 min), 50% (19-24 min), 95% (25-30 min), and 30% (31 min and after); Detection: monitor at 235 nm with UV scanning between 220-400 nm; b) Sample (80 μM) was added to PBS containing GSH (4 mM) alone, or GSH (4 mM) and glutathione S-transferase (GST; 0.5 U/μL). The total reaction volume was 250 μL. The reaction mixture without GSH and GST was applied as control. The reaction was carried out for 40 min. The sample was prepared and analyzed as described in (a).
12. The experimental description of assays: **TNF-PLAP(**TNFα-PLAP reporter cells,THP-1-33 cells): A TNFα-PLAP plasmid (TNFα-promoter + 5'-UTR (1.4 kb) + PLAP + SV40 polyA + PGK-neo, Goto, M. et al. Mol. Pharmacol. 1996, 49, 860) was constructed with slight modification in which TNFα-3'-UTR (772 b.p.) was inserted between PLAP and SV40 polyA (TNFα-promoter + 5'-UTR (1.4 kb) + PLAP + TNFα-3'-UTR + SV40 polyA + PGKneo). Then the TNFα-PLAP reporter cells were established by stably transfecting the modified TNFα-PLAP plasmid into THP-1 cells. **Actin-PLAP (**β-actin-PLAP reporter cells,B164 cells)**:** In order to simultaneously evaluate non-specific effects of test compounds on transcription, β-actin-PLAP reporter cells were also established by stably transfecting β-actin-PLAP plasmid (β-actin-promoter (4.3 kb) + PLAP + SV40 polyA + PGKneo) into B164 cells. Cell-based assays with TNFα-PLAP and β-actin-PLAP reporter cells: cells were maintained in RPMI1640 containing 10% heat-inactivated endotoxin-free fetal bovine serum (FBS) and G418 (1 mg/mL). These cells were seeded at a density of $1.0 \times 10^4$ cells/well onto 96-well plate, then were cultured in the presence or absence of test compounds for 30 min, followed by stimulation with 100 ng/ml of lipopolysaccharide (LPS; *E.coli* 0127:B08 or 011:B4). Total volume of the reaction mixture was 200 μL. After the cultivation for 24-48 hrs, culture supernatant was harvested and alkaline phosphatase activity in the supernatant was measured.
13. Experimental description for mouse plasma stability: Each compound was dissolved in freshly prepared heparinized mouse blood at a concentration of 50 μg/ml or 50 μM, and incubated at 37 C for 0, 30 and 120 min. For HPLC analysis, the sample of each time point was directly extracted with MeOH containing 0.2 % perchloric acid immediately after the incubation. The HPLC column is YMC J'sphere ODS H80 75x4.6 mm. The solvents is 30% acetonitrile in 0.15% $KH_2PO4$ water solution buffered with phosphoric acid to pH=3.5. The pump speed is 1.1 mL/min. The amount of unchanged form was estimated from the peak area, and the remaining % at each time point of the incubation was calculated by comparing with the area at 0 min.
14. The test compounds were co-injected with LPS through tail vain in the formulation with 10 % cyclodextrin. At 3 hours after the injection, the peripheral blood was harvested and plasma was separated for the determination of IL-6 by ELISA. Each column represents mean with SEM (n = 6 - 8). *P<0.05; statistically significant as compared with the LPS-treated control group in Dunnett's multiple comparison post hoc analysis.