# Discovery and Synthesis of Novel Compounds for Asthma Treatment

(喘息治療を目的とした新規化合物の創製と合成)

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2003

# Preface

The thesis contains the author's study at International Research Laboratories, Ciba-Geigy Japan, Ltd. during 1992-1996 and at Research Center Kyoto, Bayer Yakuhin, Ltd. during 1999-2000. The aim of the author's work described in this thesis is to develop novel medications for the treatment of asthma.

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# CONTENTS

# General Introduction

Asthma is one of the most serious allergic diseases, which causes a chronic inflammation of the airways of the lung. Chronically inflamed airways are hyper-responsive. They become obstructed and airflow is limited by bronchoconstriction, mucus plugs, and increased inflammation when exposed to a variety of stimuli, which include environmental antigens, such as dust mite, fur, pollen, mold, tobacco smoke, air pollutant, exercise, strong emotional stress, or chemical irritants. Asthma causes recurring episodes of coughing, wheezing, chest tightness, and difficulties in breathing, and asthma attacks can be life threatening.[1]

Asthma is one of the most common chronic diseases worldwide, and is increasing in prevalence, particularly in children. In fact, the number of children with asthma has doubled in the last 10 years in Japan.[2] Asthma was quite rare at the beginning of the 20$^{th}$ century, and is still rare in developing countries. In the popular imagination, asthma is the result of air pollution. There is no doubt that air pollution can exacerbate existing asthma. However, pollution in most westernized countries has declined dramatically at the same time that the prevalence of asthma has been increasing.[3] Several surveys suggest that asthma is associated with a clean westernized

environment.[4] Therefore, it is concerned that the prevalence of asthma may increase even in developing countries with their urbanization.

Asthma is a chronic disorder requiring long-term management, and many patients have to take preventive medication every day. The Global Initiative of Asthma (GINA: Global strategy for asthma management and prevention, National Institutes of Health National Heart, Lung, and Blood Institute) classifies medications for asthma as controllers or relievers (rescue medication).[5] Relievers include short-acting bronchodilating medications, which act quickly to relieve bronchoconstriction and accompanying acute symptoms such as cough, chest tightness, and wheezing, but have no effect on the inflammation, which is underlying in asthma. Controllers are medications taken daily on a long-term basis that are useful in getting and keeping persistent asthma under control. Corticosteroids have been commonly used as the anti-inflammatory treatment of asthma. However, these drugs require inhalers to be used, and many people are concerned about side-effect of the steroids.

Currently available medications provide symptom control in mild to moderate asthma. Despite increases in daily doses, however, moderate to severe asthma is not well controlled. Adverse reactions are a concern when higher doses are used, particularly with inhaled corticosteroids.[6]

Consequently, there is a need for novel, orally active agents that are as effective as steroids, but which are better tolerated and show disease-modifying activity. In particular, such agents are especially needed for the treatment of moderate to severe asthma in adults as well as children.

This thesis deals with a development of both relievers and controllers as novel medications for the treatment of asthma. In Chapter 1 and 2, the discovery and the synthesis of endothelin antagonists are described. The endothelin antagonists are expected to work as relievers with a novel mechanism for the bronchoconstriction in the respiratory diseases, such as asthma. In Chapter 3, the discovery and the synthesis of novel and potent IKK-β (IκB kinase β) inhibitors are described. The IKK-β inhibitors are expected to suspend the signaling pathways from a variety of inflammatory stimuli by inhibiting activation of the transcription factor, Nuclear Factor kappa B (NF-κB), thereby work as controllers with strong anti-inflammatory effect for asthma.

## References


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[2] Statically health survey for school children, Ministry of Education, Culture, Sports, Science and Technology, Japan, December (**2002**).

[3] von Mutius, E.; Weiland, S.K.; Fritzsch, C.; Duhme, H.; Keil, U. *Lancet,* **1998,** *351,* 862-866.

[4] (a) von Mutius, E.; Fritzsch, C.; Weiland, S.K.; Roll, G.; Magnusson, H. *Brit. Med. J.* **1992,** *305,* 1395-1399. (b) von Mutius *et al. Am. J. Respir. Crit. Care Med.* **1994,** *149,* 358-364. (c) Bjorksten B. *et al. Eur. Respir. J.* **1998,** *12,* 432-437.

[5] Global Initiative for Asthma (GINA, **1993**) Global strategy for asthma management and prevention NHLBI/WHO workshop report; see also: Sly, R.M. *Ann. Allergy. Asthma. Immunol.* **1997,** *78,* 427-437.

[6] Barnes, P.J.; Pedersen, S.; Busse, W.W. *Am. J. Respir. Crit. Care. Med.* **1998,** *157,* S1-S53.

# Part 1. Novel and potent endothelin antagonists for asthma treatment

## Chapter 1. Discovery of IRL 2500: a novel and potent $ET_B$ selective endothelin antagonist[1]

### 1. Introduction

Endothelins are the most potent vasoconstrictors secreted from endothelial cells, and have been paid considerable attentions since their discovery in 1988.[2] The endothelins are a family of 21 amino acid peptides, of which there are three distinct isopeptides (ET-1, ET-2 and ET-3), as shown in Figure 1. The isoforms ET-2 and ET-3 differ from ET-1 by two and six amino acids, respectively, and share significant homology. Surprisingly, the structures of the endothelins are quite similar to that of a type of viper venom, sarafotoxin, which causes strong vasoconstriction through the endothelin receptors. **These peptides have been suggested to play an important role in the pathophysiology of a large number of diseases such as renal failure, vasospasm, hypertension and asthma.**[3]

In the airway, ET-1 is localized primary to the bronchiral smooth muscle with low expression in the epithelium.[4] The lung has the highest levels of ET-1, and ET-1 expression in the airways is regulated by inflammatory mediators. **Eosinophilic airway inflammation, as may be seen in severe asthma, is associated with increased ET-1 levels in the lung,[5] indicating that .the endothelins are noteworthy target for the treatment of asthma.**

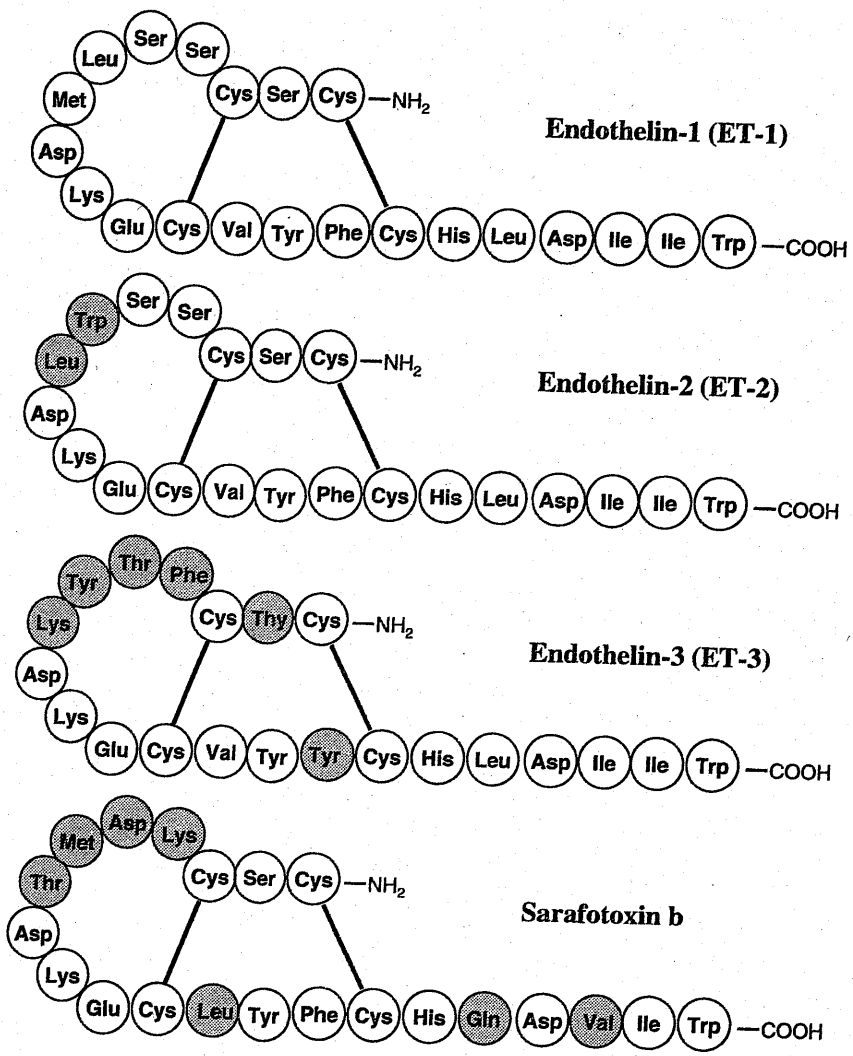
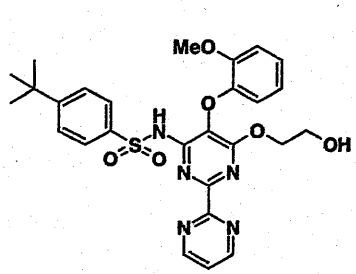


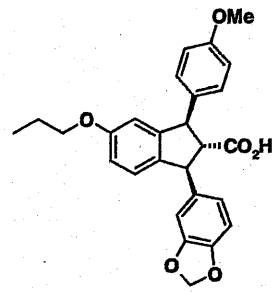

**Figure 1.** Endothelin family

The pharmacological actions of the endothelins are mediated by two distinct subtypes of endothelin receptors[6], $ET_A$ and $ET_B$, which are known as G-protein coupled receptors and a member of the seven transmembrane helix family of proteins. The $ET_A$ receptor is characterized by distinct selectivity for ET-1 and ET-2 over the isopeptide ET-3, and two biological actions mediated by the $ET_A$ receptor are vasoconstriction and vascular smooth muscle cell proliferation.[7] Hence, the antagonism of the $ET_A$ receptor is thought to be effective for a treatment of cardiovascular diseases, such as

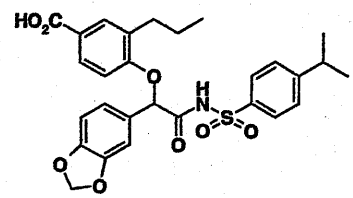
hypertension. In contrast, the $ET_B$ receptor shows equivalent affinity towards ET-1, ET-2 and ET-3, and mediates vasoconstriction and bronchoconstriction.[7] **Therefore, the antagonism of the $ET_B$ receptor relieves the bronchoconstriction in the respiratory diseases and is expected to be a novel mechanism for a treatment of asthma and COPD[8] (chronic obstructive pulmonary disease).**



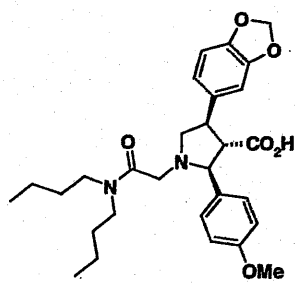
**Bosentan (Ro 47-0203)**
**(Hoffmann-La Roche)**
**Ki ($ET_A$) = 6.5 nM**
**Ki ($ET_B$) = 340 nM**



**SB-209670**
**(GlaxoSmithKline)**
**Ki ($ET_A$) = 0.43 nM**
**Ki ($ET_B$) = 15 nM**



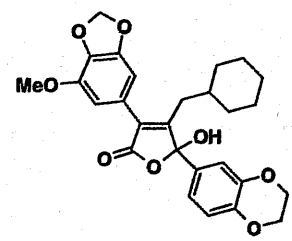
**L-754142**
**(Merck)**
**Ki ($ET_A$) = 0.06 nM**
**Ki ($ET_B$) = 2.2 nM**



**A-127722**
**(Abbott Laboratories)**
**Ki ($ET_A$) = 0.15 nM**
**Ki ($ET_B$) = 166 nM**

**Structure has not been disclosed.**

**A-158112**
**(Abbott Laboratories)**
**Ki ($ET_A$) = 0.8 nM**
**Ki ($ET_B$) = 0.56 nM**



**PD-160874**
**(Parke-Davis)**
**Ki ($ET_A$) = 4.9 nM**
**Ki ($ET_B$) = 9.7 nM**

**Figure 2. Known non-peptidic $ET_A$ selective and $ET_A$ / $ET_B$ dual antagonists**

The development of potent endothelin antagonists, selective as well as non-selective, is necessary to determine the pathophysiological role of the endothelins and their receptor subtypes. Various peptidic compounds have been reported[9] as

endothelin-receptor antagonists which are selective for $ET_A$ (BQ-123 and FR139317), $ET_B$ (BQ-788 and RES-701-1) or exhibit dual $ET_A$ / $ET_B$ antagonism (PD 142893, PD 145065 and TAK-044). A number of non-peptidic endothelin antagonists have also been reported (Figure 2).[9,10] These include the $ET_A$ selective antagonists (A-127722, L-754142, Ro 47 0203 (bosentan), and SB 209670) and the dual $ET_A$ / $ET_B$ antagonists (A-158112 and PD 160874). However, Neither a non-peptidic nor a peptidic $ET_B$ selective antagonist has been reported to date. In this chapter, a development of novel low molecular weight $ET_B$ selective endothelin antagonists and their pharmacological effects are described.

## 2. Discovery of lead compound (search for important binding areas of endothelin)

When our endothelin antagonist discovery program had been initiated in 1990, only limited information for finding endothelin antagonists was available. Although several peptidic endothelin antagonists and the amino acid sequences of the endothelins had been known, there were no reports on the non-peptidic endothelin antagonists and the 3-dimensional structure of endothelins. There were a few reports for the endothelins 3-dimensional structure estimated by NMR study. But, it's been recently suggested that the crystal structure[11] prepared from aqueous solution of ET-1 is very different from all of the NMR models of endothelins, indicating that the NMR models of endothelins are unreliable. It is well recognized in the pharmaceutical companies that the most common way for the lead finding process is a high-throughput screening of a compound library. Actually, the known non-peptidic endothelin antagonists

should have been discovered by a high-throughput screening followed by the lead optimization. However, back then, we had neither a compound library nor the system for a high-throughput screening. **Thus, in order to identify a new low molecular weight $ET_B$ selective ET antagonist, we mainly adopted two unique strategies as follows.**

**(1) Glycine substitution scan of the C-terminal fragment of ET-1**

As the C-terminal tryptophan has been already identified as an important residue for binding,[12] we concentrated our efforts on the hydrophobic C-terminus of endothelins. Fortunately, a different group in Ciba-Geigy has identified a dodecapeptide IRL 1543[13] as a potent and selective $ET_B$ receptor agonist [Ki ($ET_B$) = 0.077 nM]. In order to elucidate amino acid sequences of endothelins responsible for the strong receptor binding affinity, the shortest C-terminal fragment of ET-1 and IRL 1543 with very strong binding potency was submitted to a glycine substitution scan. After one amino acid was replaced by a glycine at a time, each inhibition of [$^{125}$I]ET-3 binding to the $ET_B$ receptor as well as [$^{125}$I]ET-a binding to the $ET_A$ receptor was determined. The results are summarized in Figure 3.

While in all of the glycine scan modifications of IRL-1543 the binding potency decreases, there are two areas, which are more sensitive towards an amino acid exchange than others. Replacing an amino acid in -Ile-Ile-Trp- with a glycine resulted in a remarkable decrease in activity. But, the glycine scan in –Ala-His-Leu-Asp- didn't affect the activity so much. For the *N*-terminal area, replacement of Phe with a glycine led to a decrease in activity, but the glycine scan in –Ala-Val- maintained the activity. **Consequently, the glycine scan study suggests that the C-terminal -Ile-**

**Ile-Trp- and the *N*-terminal -Phe- are the most important areas of ET-1 and IRL-1543 for the $ET_B$ receptor binding.**

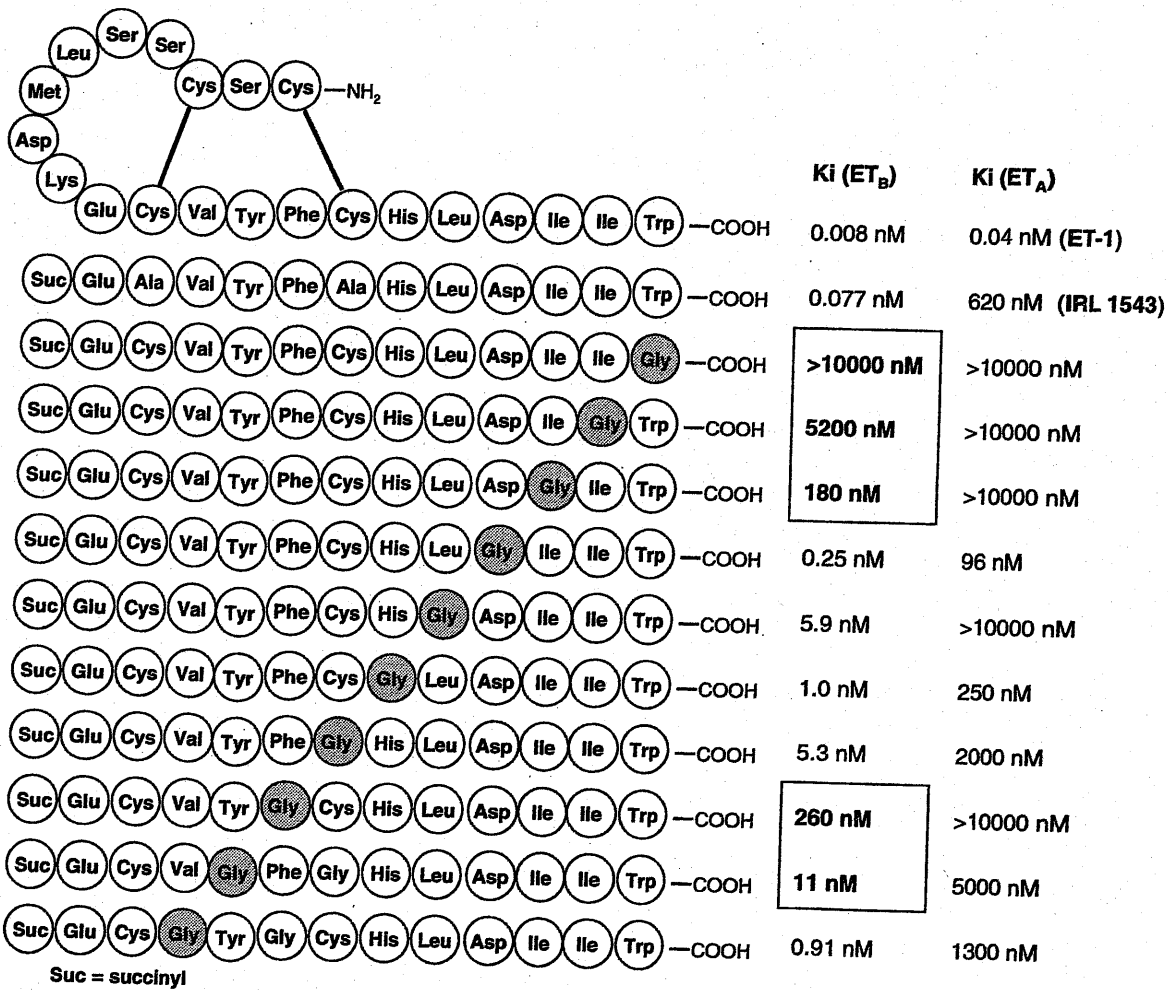



**Figure 3. Glycine substitution scan of the C-terminal ~~dodeca~~peptide fragment of ET-1.**

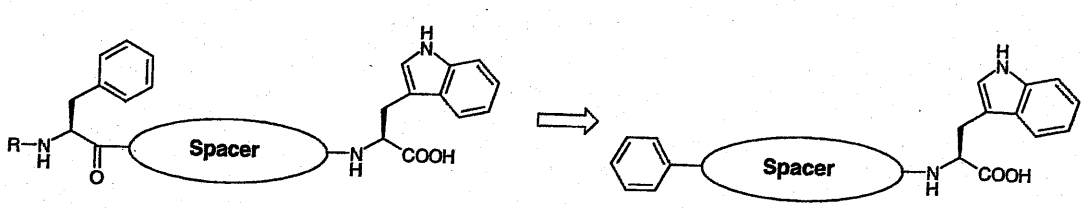
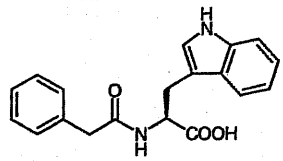
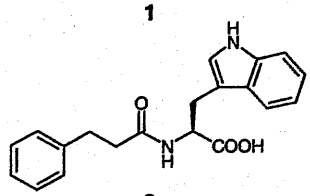
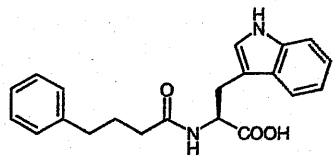
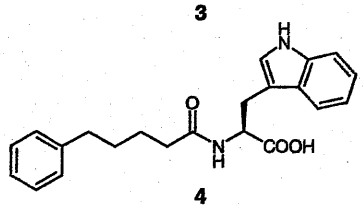
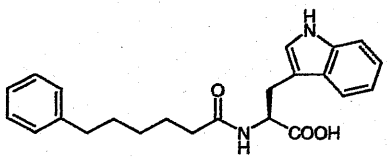
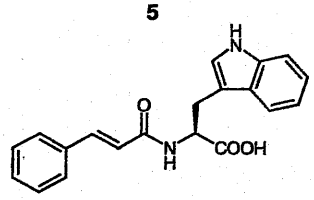
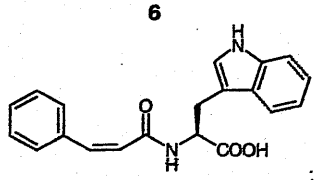
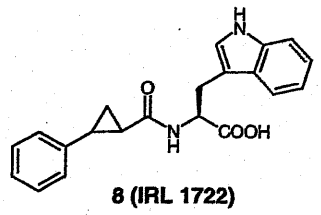



**Figure 4. First strategy to discover endothelin antagonists**

**Table 1.** First series of designed compounds with endothelin receptor binding activities

| | Ki ($ET_A$) | Ki ($ET_B$) |
|---|---|---|
|  **1** | Undetectable | Undetectable |
|  **2** | Undetectable | 91 µM |
|  **3** | Undetectable | 68 µM |
|  **4** | Undetectable | 104 µM |
|  **5** | Undetectable | 78 µM |
|  **6** | Undetectable | 44 µM |
|  **7** | Undetectable | Undetectable |
|  **8 (IRL 1722)** | 73 µM | 16 µM |

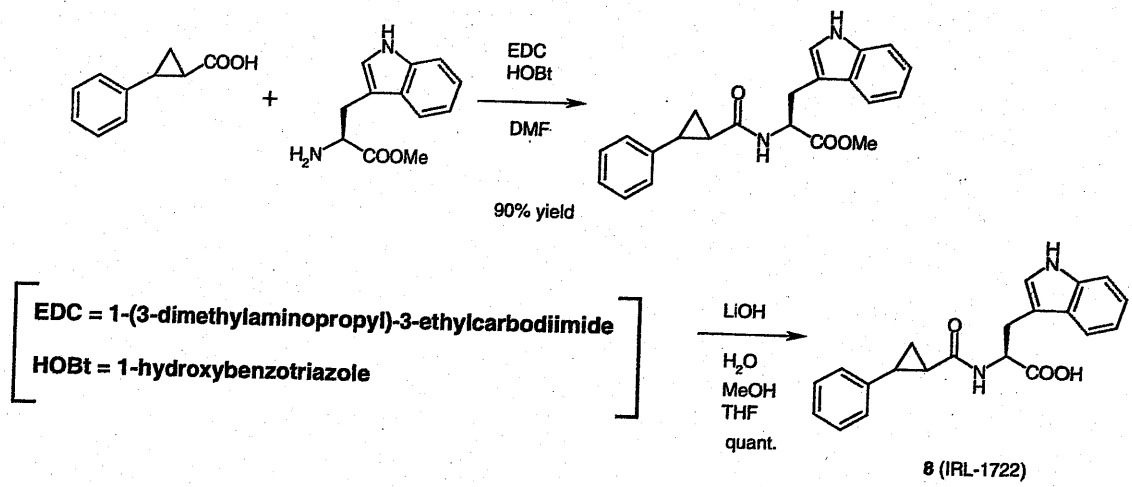



**Figure 5. Synthesis of the first lead compound, IRL 1722**

Based on this evidence a small series of simple analogs having an aromatic moiety attached through a spacer to the amino group of tryptophan was synthesized, as outlined in Figure 4. Following this quite simple concept, several compounds were synthesized and tested to examine the $ET_B$ receptor binding affinity, as shown in Table 1. The synthetic methods are quite straightforward, and just require the amide formation and the basic hydrolysis to make the analogs in Table 1, as exemplified in Figure 5.

As we expected, some of the compounds showed weak but detectable receptor binding activities. Compound **1** with one carbon chain between the tryptophan and the phenyl didn't show the activity. However, compound **2** with two-carbon chain was weakly active in the $ET_B$ receptor-binding assay. Introduction of longer carbon chain resulted in a decrease in activity, as exemplified by compounds **3**, **4** and **5**. The *trans*-cinnamamide analog **6** exhibited more potent activity than the corresponding simple carbon chain **2**. On the other hand, the *cis*-cinnamamide analog **7** was inactive, suggesting that the tertiary structure as well as the carbon chain length is important for the activity. **Out of this series of weak inhibitors *N-trans*-2-phenylcyclopropanoyl**

**tryptophan IRL 1722 [Ki($ET_B$) = 16000 nM] was identified as the first (albeit weak) lead structure.**

**(2) Screening of substance P antagonists towards the $ET_B$ receptor binding affinity.**

At this point when the first lead compound IRL-1722 had been discovered, an extremely helpful idea came from a very different direction. An extensive homology study[14] of the rhodopsin superfamily of seven-transmembrane receptors showed greatest homology of the endothelin receptors with bombesin receptors and tachykinin receptors, suggesting a close evolutionary relationship between these receptors.

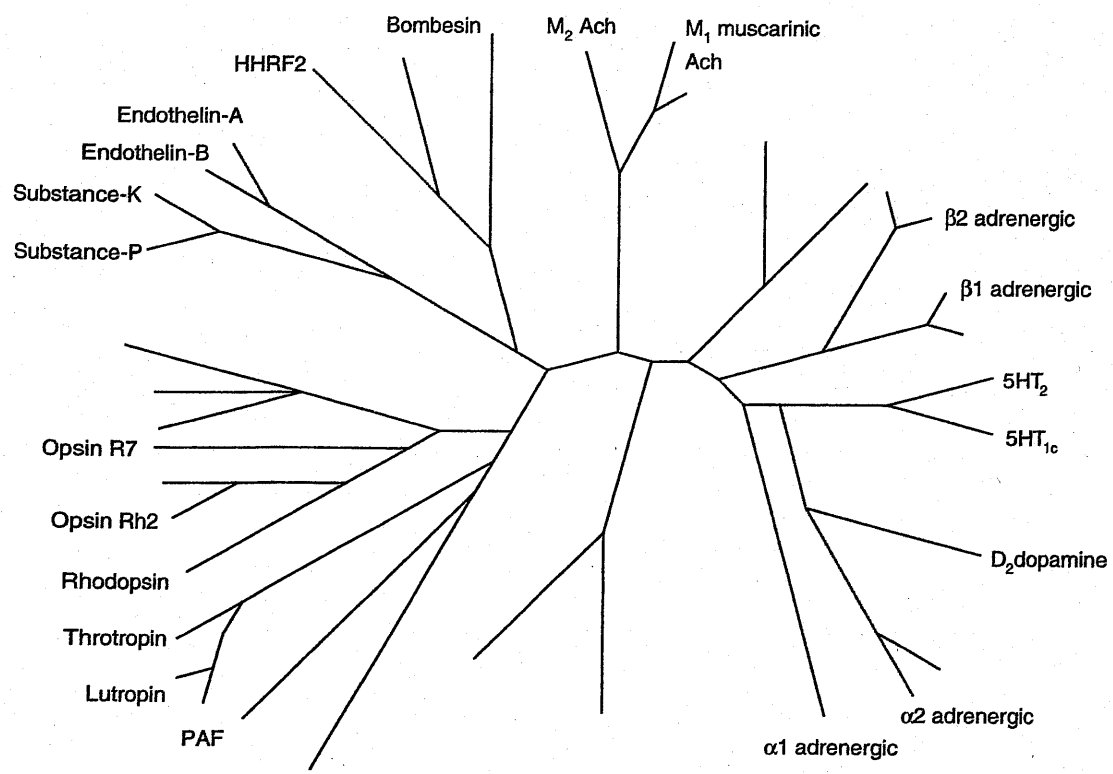



**Figure 6. Unrooted phylogenetic tree of peptide receptors of the rhodopsin superfamily[14]**

Both subtypes of the endothelin receptors, together with two subtypes of bombesin receptors ($BB_1$ and $BB_2$ receptors) and these subtypes of tachykinin receptors ($NK_1$ (substance P), $NK_2$ and $NK_3$ receptors), are part of a cluster distinct from other clusters in a phylogenetic tree of the superfamily (Figure 6).

In general, the tertiary structure of a protein is more conserved than the primary structure during evolution. It is also thought that this general rule may be applicable to the structure of the ligand-binding domain of the receptor. Fortunately, there was a research program for substance P antagonist in Ciba-Geigy, Switzerland. Thus, recognition of the close evolutionary relationship between endothelin receptors and tachykinin receptors prompted us to a limited random screening of Ciba-Geigy compounds identified from the substance P antagonist program. Out of randomly selected 140 compounds with substance P activity three compounds showed weak $ET_A$ and $ET_B$ receptor binding activity (Ki = 5 – 20 μM). **Among them, CGP 49941 (9) was found to show the highest affinity for the $ET_B$ receptor [Ki ($ET_B$) = 5000 nM].**

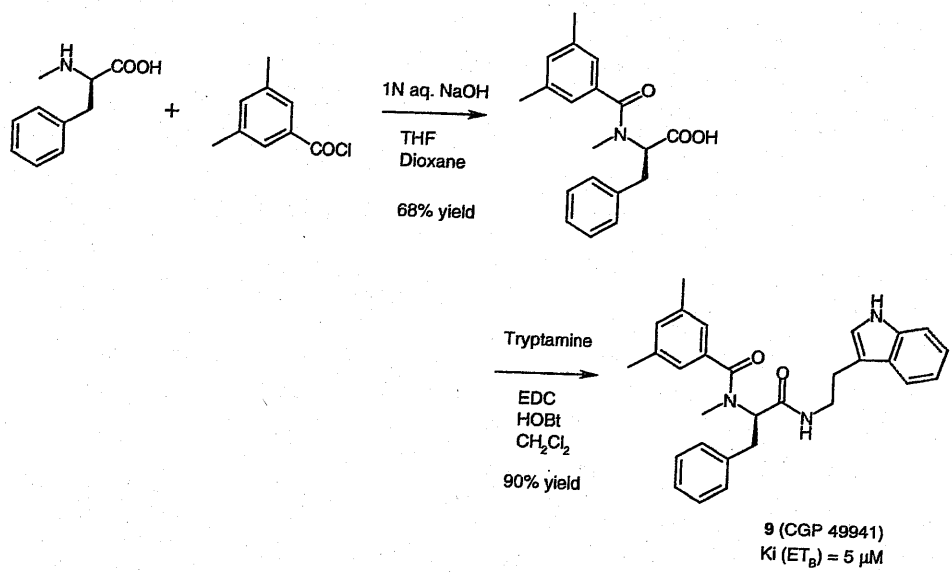



**Figure 7. Synthesis of CGP 49941 and the $ET_B$ receptor antagonistic activity**

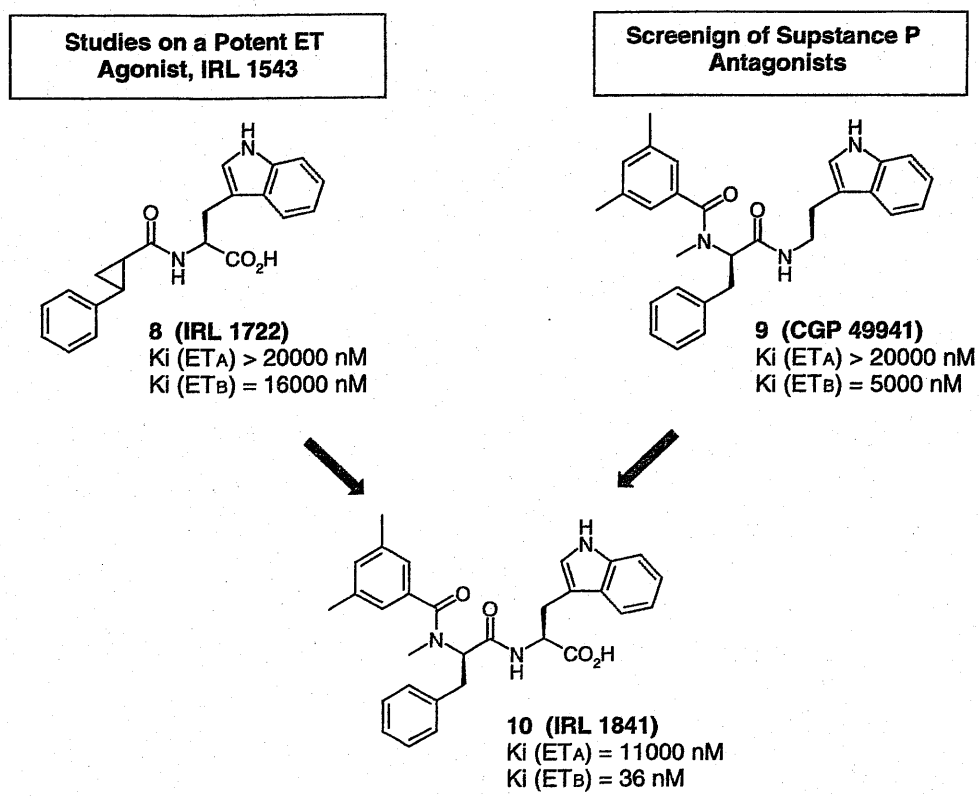



**Figure 8. Combination of the structural elements of IRL 1722 and CGP 49941**

Based on the two strategies above, we identified two weakly active $ET_B$ inhibitors, compound **8** (IRL 1722) and compound **9** (CGP 49941). The CGP 49941 identified from the substance P antagonists screening have not only promising binding properties but also very interesting structural similarities with the first lead compound, IRL 1722, identified from the study on the glycine substitution scan of the potent endothelin agonist, IRL 1543. The indole group (tryptophan vs. tryptamine) as well as the phenyl group (phenyl-cyclopropane vs. phenylalanine) appears to be in very similar steric arrangements. The major differences are the *N*-acyl group on the CGP 49941 and the carboxylic acid group on IRL 1722. **The combination of the structural elements of IRL 1722 and CGP 49941 resulted in IRL 1841 with a notable $ET_B$-selective binding affinity [Ki($ET_B$) = 36 nM; Ki($ET_A$) = 11000 nM] (Figure 8).**

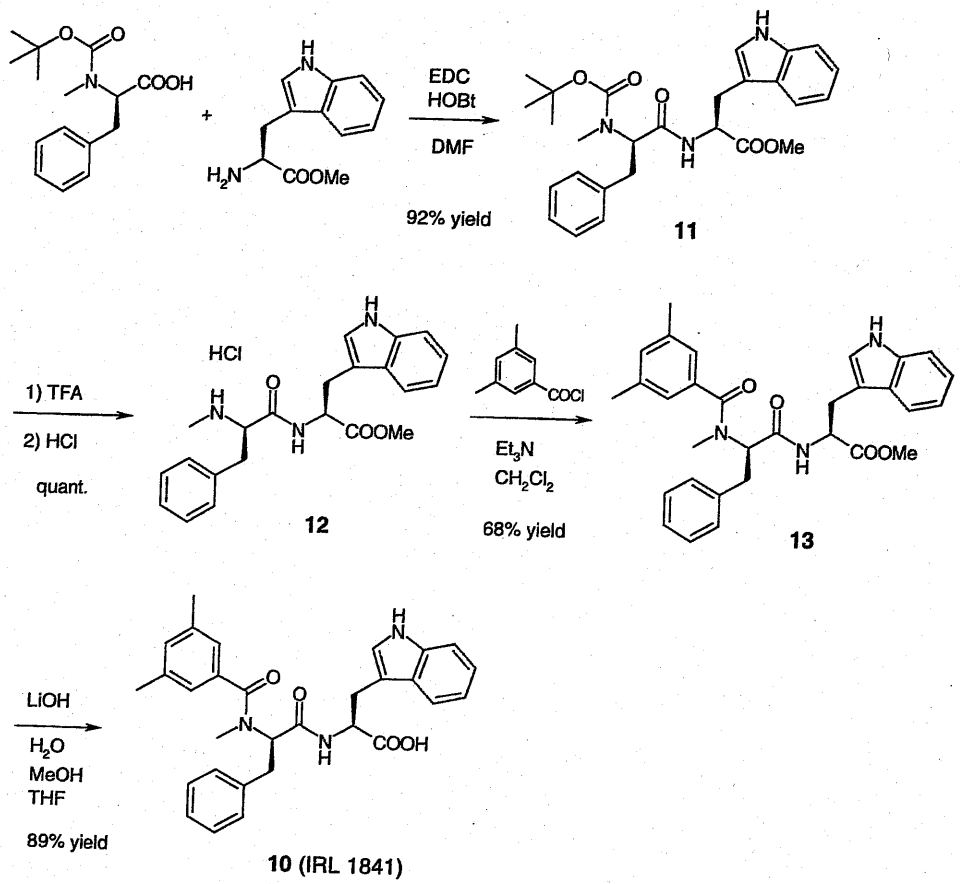



**Figure 9. Synthesis of $ET_B$ receptor selective antagonist, IRL 1841**

Since the starting material, *N*-(*tert*-butoxycarbonyl)-*N*-methyl-(*L*)-phenylalanine, was commercially available, synthesis of compound **10** (IRL 1841) was rather simple as shown in Figure 9. All reaction steps proceeds without racemization, which was determined by the HPLC analyses.

## 3. Discovery of a potent $ET_B$ selective antagonist, IRL 2500,[15] and the functional characterizations

Figure 10 illustrates a summary of the structure-$ET_B$ receptor binding activity relationships for IRL 1841. All of the initial modifications resulted in a drastic

decrease or loss of activity. For instance, compounds with different stereo configurations like SR, RR and SS were found to be almost inactive. Removal of the *N*-methyl moiety on the benzoylamide group resulted in a decrease of activity [Ki ($ET_B$) = 1 μM], as did replacement of the 3,5-dimethylphenyl with a simple phenyl [Ki ($ET_B$) = 1 μM]. These results indicate that the structure of IRL 1841 seems to be already quite optimized in respect to its binding to the $ET_B$ receptor. However, **a further intensive chemical optimization led to a discovery of the p-phenyl-phenyl alanine analog 14 (IRL 2500) with greatly high $ET_B$ receptor-affinity [Ki ($ET_B$) = 1 nM; Ki ($ET_A$) = 440 nM].**

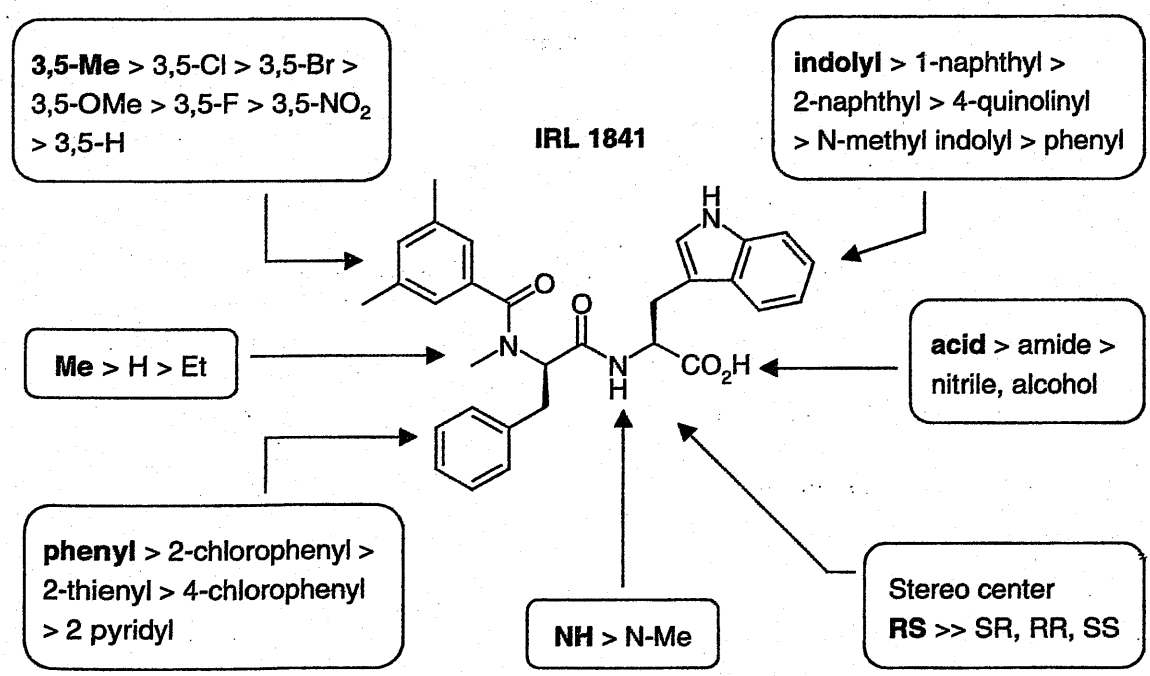



**Figure 10. Summary of the structure-activity relationships study for IRL 1841**

Thus, the $ET_B$ selective antagonists, IRL 1841 **10** and IRL 2500 **14** were discovered by a modification of the substance P antagonist, CGP 49941 **9**, but they

showed only weak affinity to substance P receptors (Figure 11). While CGP 49941 inhibited the $^3$H-substance P binding to bovine retina[16] with an $IC_{50}$ of 800 nM, IRL 1841 showed a weaker binding ($IC_{50}$ = 2 μM) and IRL 2500 was even weaker ($IC_{50}$ = 10 μM). It should be noted here that this modification resulted in an improvement of the $ET_A$ receptor affinity as well as the $ET_B$ receptor affinity, indicating the possibility to discover dual $ET_A$ / $ET_B$ antagonists and $ET_A$ selective antagonists by a modification of the structure of IRL 1841 and IRL 2500.

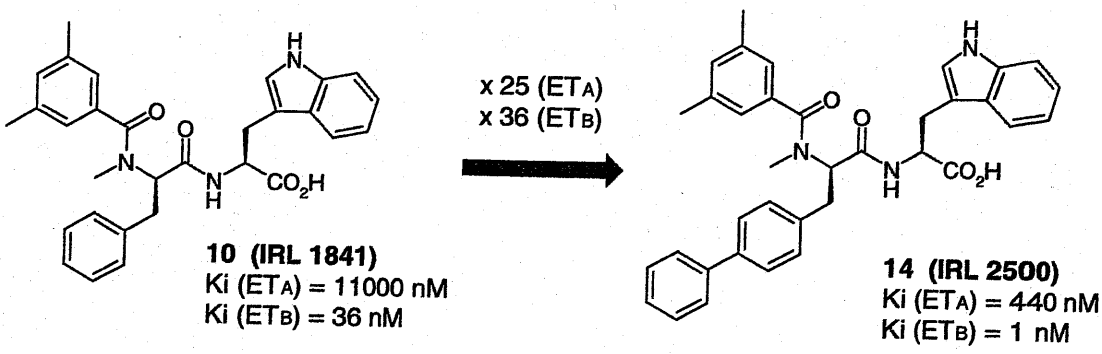



**Figure 11. Discovery of IRL 2500 by a modification of IRL 1841**

Synthesis of IRL 2500 is illustrated in Figure 12. At first, introduction of a methyl group on the benzoylamide moiety was implemented using methyl iodide and sodium hydride. However, high extent of the racemization (about 20%) occurred due to the strong basic conditions. In order to avoid the racemization, a unique *N*-methylation reaction was adopted.[17] The optically pure 4-phenyl-phenylananine ester was treated with freshly distilled cyclopentadiene and formaldehyde to give the Diels-Alder adduct, which was then subject to a *retro*-Diels-Alder reaction in the presence of triethylsilane and TFA to provide the optically pure *N*-methylamino acid ester analog. This method has no deprotonation step using strong base, thereby proceeds without racemization.

After the hydrolysis using lithium hydroxide without racemization, the carboxylic acid analog was coupled with (L)-tryptophan methyl ester using EDC [1-(3-dimethylaminopropyl)-ethylcarbodiimide][18] and HOBt (1-hydroxybenzotriazole)[19] to give the dipeptide ester analog with a slight racemization. It is well known for long in peptide chemistry that the activate benzoylamino acid esters can be easily racemized via the oxazolone formation in the amidation step,[20] as shown in Figure 13. However, the racemized dipeptide analog was purified by the recrystallization to provide the optically pure analog. Final hydrolysis step using lithium hydroxide gave IRL 2500 without racemization.

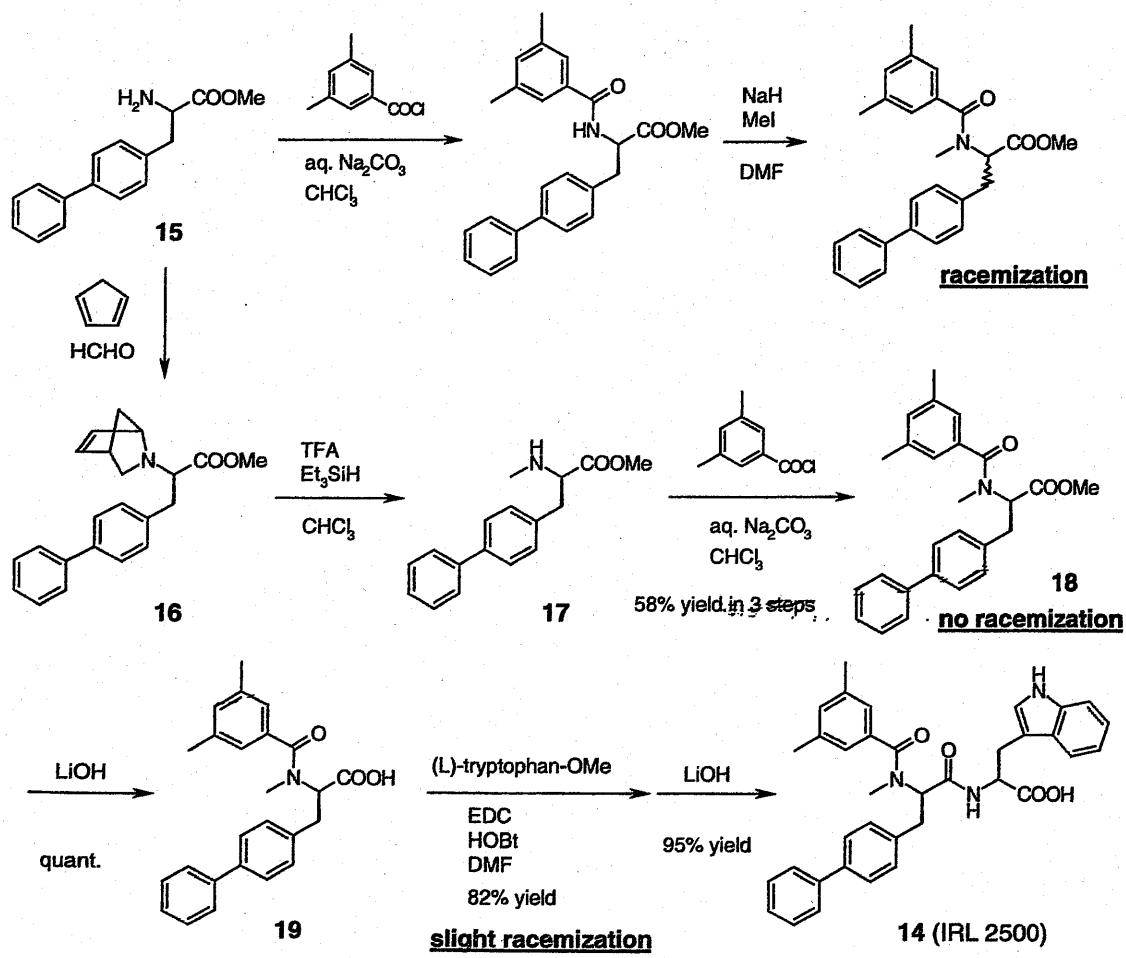



**Figure 12. Synthesis of IRL 2500**

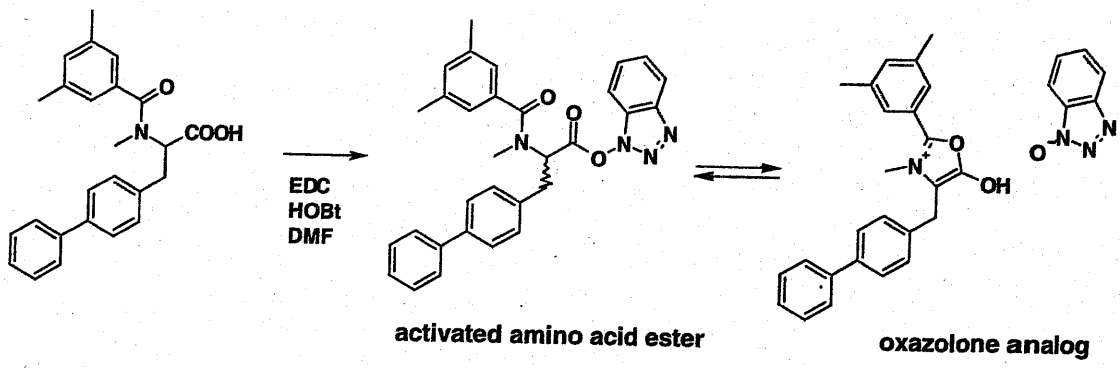



**Figure 13. Possible mechanism for the racemization in the amide formation step**

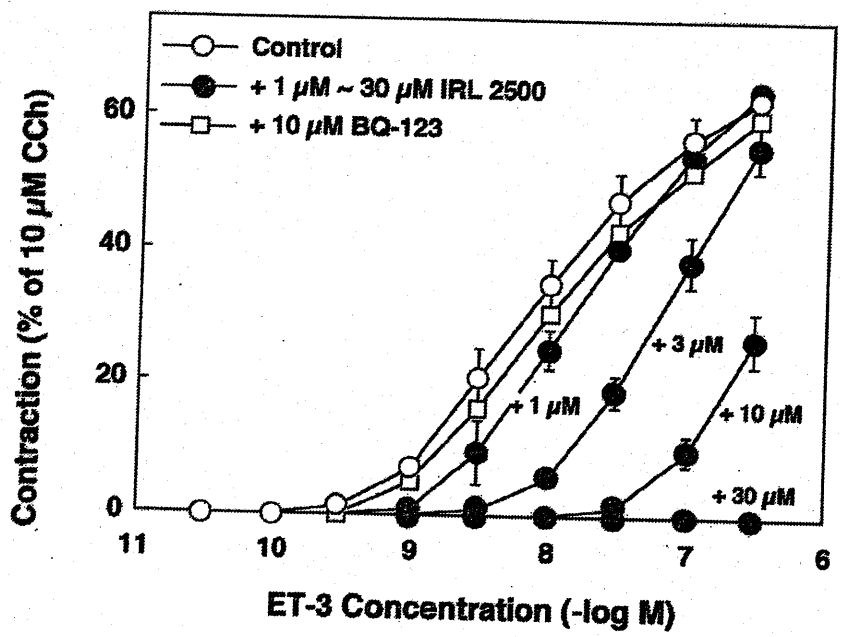
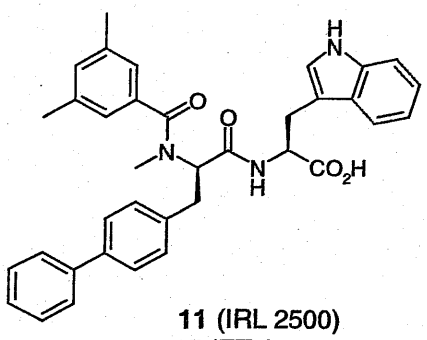



**Figure 14. Effect of IRL2500 on ET-3-induced contraction in epithelium-denuded guinea pig trachea**

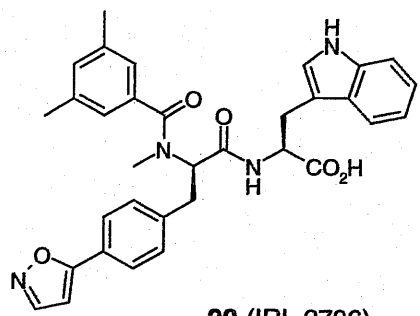
Functional characteristics of IRL 2500 were investigated in *ex vivo* assays using isolated guinea pig trachea and rat thoracic aorta denuded of epithelium and endothelium, respectively. The guinea pig trachea smooth muscle expresses both $ET_A$ and $ET_B$ receptors[21] and rat aortic smooth muscle possesses predominantly the $ET_A$

receptor.[22] IRL 2500 showed no agonistic activity in both tissues at least up to 30 μM. In the guinea pig tracheal tissue with both $ET_A$ and $ET_B$ receptors, IRL 2500 concentration-dependently antagonized the $ET_B$-mediated tracheal contraction induced by ET-3 which was hardly affected by an $ET_A$ specific antagonist, BQ-123,[23] of 10 μM (Figure 14). On the other hand, IRL 2500 had no effect on the ET-1 induced contraction of the rat aorta with the $ET_A$ receptor. **These results indicate that IRL 2500 behaves as an antagonist selective to the $ET_B$ receptor. Moreover, it should be noted that antagonism of the $ET_B$ receptor is a novel and effective target for the trachea smooth muscle relaxation for the asthma patients without affecting the cardiovascular system, such as hypotensive effect.**

## 4. Further optimization of IRL 2500 to develop more potent $ET_B$ selective antagonists



**11** (IRL 2500)
Ki ($ET_A$) = 440 nM
Ki ($ET_B$) = 1 nM



**20** (IRL 2796)
Ki ($ET_A$) = 45 nM
Ki ($ET_B$) = 0.21 nM

**Figure 15. Optimization of the biaryl moiety**

In the previous sections, a rational approach for the discovery of IRL 2500 by combination of sequence studies of an ET-1 analog and a homology study of the rhodopsin superfamily of seven transmembrane receptors. **As it was found from the**

**initial SAR study of IRL 1841 10 that a clue to improvement of the activity was a modification of the biphenyl moiety on IRL 2500 (14), further optimization efforts focused on replacement of the biphenyl moiety with heteroaromatic diaryl group.**[24] In this section, the development of IRL 2796 **20**, one of the most potent $ET_B$ selective antagonists known to date, is described.

## Chemistry

Synthesis of compound **20** is illustrated in Figure 16. The 5-Isoxazolyl ring was constructed from 4' methylacetophenone according to the reported procedure.[25] Bromination on the benzyl position of 4-(5-isoxazolyl)toluene (**23**) gave 4-(5-isoxazolyl)benzyl bromide (**24**), which was then coupled with diphenylmethylene-glycine ethyl ester in the presence of phase-transfer catalyst, tetrabutylammonium hydrogen sulfate, under basic two-phase conditions followed by deprotection of the *N*-diphenylmethylene group in aqueous hydrochloric acid solution to provide 4-(5-isoxazolyl)phenylalanine ester (**25**).[26] Amide formation of the unnatural amino acid ester with 3,5-dimethylbenzoic acid, *N*-methylation using methyl iodide and sodium hydride, and saponification using lithium hydroxide gave the left-hand fragment (**28**) in good yield. Coupling reaction of the racemic unnatural amino acid (**28**) with (*L*)-tryptophan methyl ester yielded a 7:3 mixture of two diastereomers, which could be easily separated by medium pressure liquid chromatography (MPLC, Kusano KHLC-201-43 type III). Saponification of the less polar isomer (major product) with *R,S*-configuration afforded the more potent isomer **20** [IRL 2796, Ki ($ET_B$) = 0.21 nM]. The more polar isomer (minor isomer) with *S,S*-configuration provided the less potent isomer **21** [IRL 2797, Ki ($ET_B$) = 16 nM].

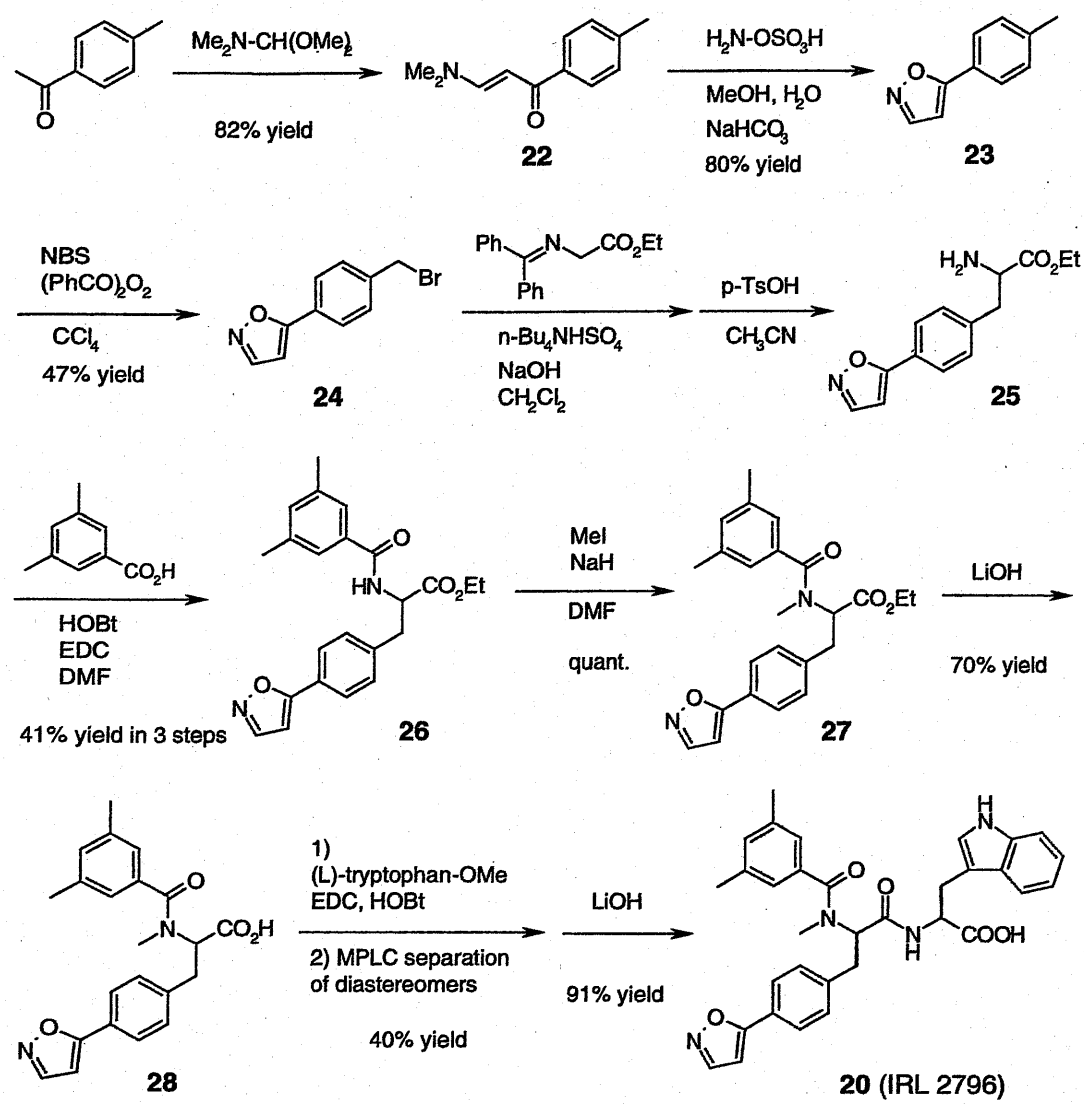



**Figure 16. Synthesis of compound 20 (IRL 2796)**

At this point, there was no clear evidence to prove the stereo configurations. Although it's indirect demonstration, the SAR studies around both IRL 1841 and IRL 2500 support the hypothesis that the *R,S*-isomers always exhibit better activity than that of *S,S*-isomers. Thus, the more potent isomer was determined as *R,S*-configuration. Latterly the absolute configuration was completely proved by the X-ray analysis of the sulfonamide analog of compound **20**. The detailed discussions are described in

Chapter 2.

The corresponding 4-(3-Thienyl)phenylalanine analog **29** (IRL 2659) was synthesized as shown in Figure 17. The 4-(3-thinenyl)toluene (**32**) was prepared by an aryl-aryl coupling reaction between 4-toluene magnesium bromide (**30**) and 3-boromothiophene (**31**) using a catalytic amount of [1,2-bis(diphenylphosphino)-ethane]nickel(II) chloride, and then brominated on the benzyl position by a radical bromination using benzoyl peroxide as the initiator. Introduction of the amino acid moiety was conducted by the procedure analogous to that for the 4-(5-isoxazolyl)phenylalanine analog **20** (IRL 2796).

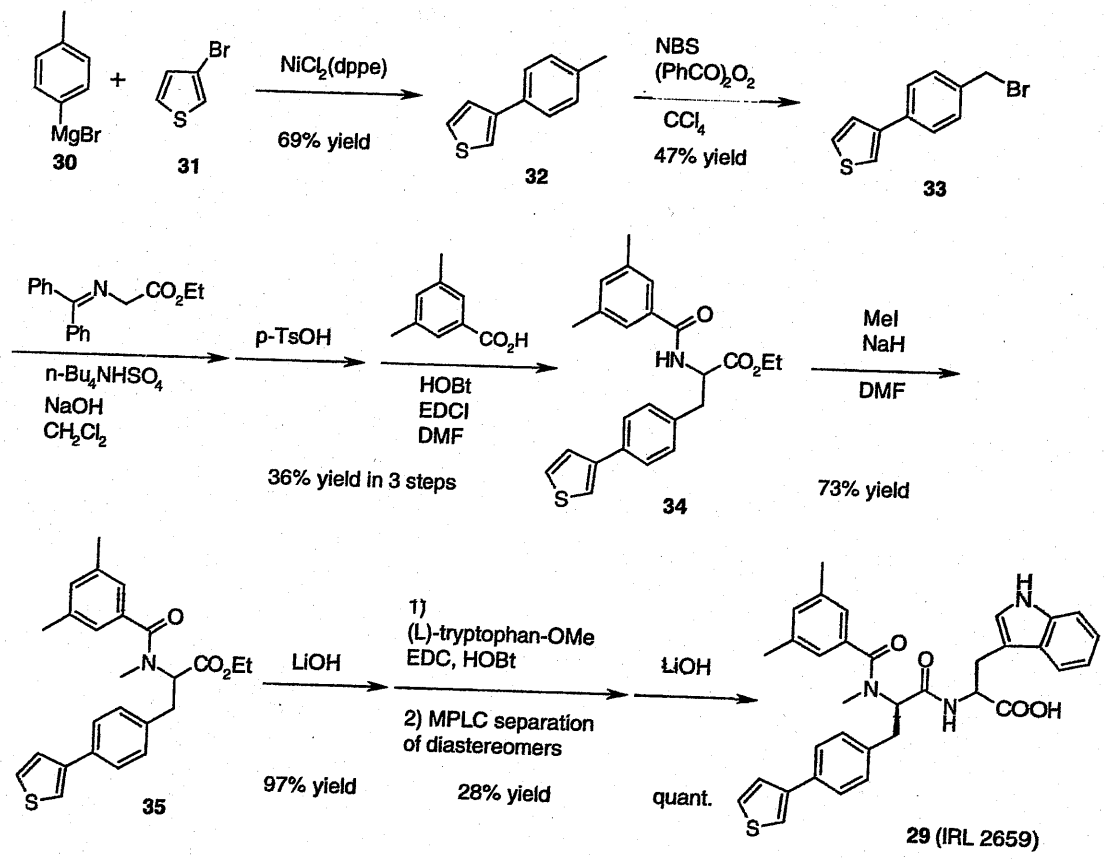
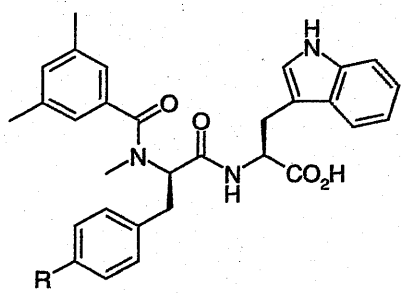


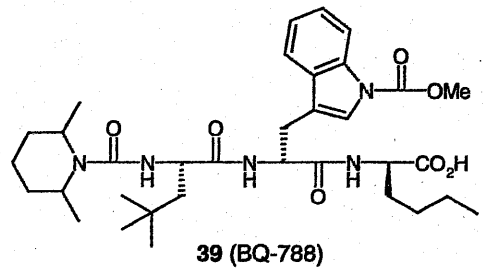

**Figure 17. Synthesis of compound 29 (IRL 2659)**

**Structure-activity relationships**

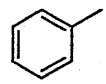
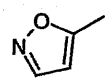
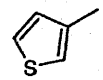
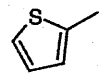
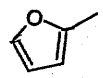
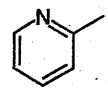
Table 2 summarizes structure-activity relationships (SAR) study for the 4-substututed phenylananyltryptophan derivatives.

**Table 2. SAR for the 4-substituted phenylalaninyltryptophan derivatives**





**39** (BQ-788)

| No | R | Ki ($ET_B$) (nM) | Ki ($ET_A$) (nM) | Ratio A/B | $pA_2$ [a] |
|---|---|---|---|---|---|
| 10 (IRL 1841) | H | 36 | 11000 | 305 | 6.3 |
| 14 (IRL 2500) |  | 1 | 440 | 440 | 6.7 |
| 20 (IRL 2796) |  | **0.21** | 45 | 214 | **7.6** |
| 29 (IRL 2659) |  | **0.23** | 54 | 235 | **7.0** |
| 36 |  | 0.6 | 87 | 145 | — |
| 37 |  | 0.76 | 250 | 329 | — |
| 38 [b] |  | 4.4 | 2300 | 523 | — |
| 39 (BQ-788) | — | 0.72 | 75 | 104 | — |

a) Antagonistic activities ($pA_2$) were determined for guinea pig tracheal contraction induced ET-A. b) racemic phenylalanin analog

An aromatic ring at the 4-position of the phenylalanine moiety shows a significant effect in improving the binding affinity for $ET_A$ receptor as well as $ET_B$ receptor, suggesting that the 4-position is an important portion to bind to a common hydrophobic pocket on endothelin receptors. Among several derivatives, replacement of this position with 5-isoxazolyl group (**20**) and 3-thienyl (**29**) most remarkably contributed to the increase in the binding affinities. The well-known peptidic $ET_B$ receptor selective antagonist, BQ-788,[27] was used as a reference compound to compare the activity with our antagonists. In our assays, BQ-788 exhibited quite potent activity [Ki ($ET_B$) = 0.72 nM, Ki ($ET_A$) = 75 nM]. However, the 5-isoxazolyl (**20**) and 3-thienyl (**29**) analogs displayed 3 times more potent binding affinity to $ET_B$ receptor and better selectivity to $ET_A$ receptor than those of BQ-788. The potent analogs (**20**, **29**) were found to antagonize the $ET_B$-mediated tracheal contraction induced by ET-3, and demonstrated more potent activity than IRL 1841 and IRL 2500 as expected (Table 2).

## 5. Conclusions

The small molecular-weight compounds, such as IRL 2500 (**14**), was rationally developed as potent and highly selective antagonists for the $ET_B$ receptor. The rational approach, based on the structure-activity correlation of ET-1 fragment and structural homology of the rhodopsin superfamily of receptors, successfully aided the quick discovery of early lead compounds. With an increase in evidence for the involvement of the $ET_B$ receptor in a multitude of endothelin actions, these novel $ET_B$ receptor antagonists will be useful tools for demonstrating the role of $ET_B$ receptor subtypes in physiological study. The potent analogs (**20**, **29**) can be categorized as the most potent $ET_B$ selective antagonists known to date. These analogs would provide a novel

mechanism for the trachea smooth muscle relaxation for the asthma patients.

## 6. Experiments

### General

$^1$H NMR spectra were recorded using Bruker DRX-400 (400MHz for $^1$H) spectrometer in $CDCl_3$ or DMSO-d6. Chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as an internal standard at zero ppm. Coupling constant (J) are given in hertz and the abbreviations s, d, t, q, and m refer to singlet, doublet, triplet, quartet and multiplet, respectively. The abbreviation "br" refer to "broad". Mass spectroscopy data were recorded on a FINNIGAN MAT 95. Melting points are uncorrected. TLC was performed on a precoated silica gel plate (Merck silica gel 60 F-254). Silica gel (WAKO-gel C-200 (75-150 $\mu$ m))was used for all column chromatography separations. All chemicals were reagent grade and were purchased from Sigma-Aldrich, Wako pure chemical industries, Ltd., Tokyo kasei kogyo co. Ltd.

### Synthesis of peptides

ET-1 analogs were synthesized by a solid phase procedure on an Applied Biosystems Model 431A automated peptide synthesizer applying Fmoc strategy. The following side-chain-protected Fmoc-amino acid derivatives were used for the synthesis: Lys(Boc), His(Tri), Asp(OtBu), Glu(OtBu) and Tyr(tBu). The peptides were constructed on 0.25 mmol Fmoc-Trp linked to p-benzoyloxybenzyl alcohol resin. The Fmoc group was removed by treatment with 20% piperidine in 1-methyl-2-pyrrolidone before each condensation reaction step. The peptide chain was elongated by *in situ*

prepared 1-hydroxybenzotriazole esters using four-fold excess of Fmoc amino acid derivatives. The fully protected peptide resin was subjected to a piperidine cycle to remove the terminal Fmoc group. The peptide-resin was treated with succinic anhydride to obtain $N^{\alpha}$-succinyl derivatives. The peptide resin was then treated with trifluoroacetic acid/ $H_2O$/ 1,2-ethanedithiol (76/4/20, v/v/v) to remove all the protecting groups and the resin. The desired peptides were purified by preparative reverse-phase HPLC using a Tosoh TSKgel ODS 120-T column (21.5 mm I.D. x 300 mm) or a Shiseido CAPCELL PAK C18 column (20 mm I.D. x 250 mm). The homogeneity of the peptides was confirmed by analytical reverse-phase HPLC and amino acid analysis.

**_N_-[(*trans*-2-phenylcyclopropyl)carbonyl]-(L)-tryptophane (8)**

To a cold (0 °C) mixture of *trans*-2-phenyl-1-cyclopropane carboxylic acid (0.30 g, 1.8 mmol), (L)-tryptophan methyl ester hydrochloride (0.50 g, 2.0 mmol), HOBt (0.6 g, 4.4 mmol) [HOBt = 1-hydroxybenzotriazole] and DMF (10 mL) was added EDC (0.3 ml, 2.5 mmol) [EDC = 1-(3-dimethylaminopropyl)-ethylcarbodiimide]. The mixture was allowed to warm to room temperature, and the stirring was continued for 3 hours. The reaction mixture was partitioned between ethyl acetate and water. The separated organic phase was washed with water and brine successively, dried over $Na_2SO_4$, filtered, and dried in vacuo. The residue was purified by column chromatography on silica gel (hexane/ ethyl acetate, 4:1) to give *N*-[(*trans*-2-phenylcyclopropyl)carbonyl]-(L)-tryptophane methyl ester (0.60 g, 90% yield).

A mixture of *N*-[(*trans*-2-phenylcyclopropyl)carbonyl]- (L)-tryptophane methyl ester (0.50 g, 1.4 mmol), THF (6 mL) and water (6 mL)was treated at 0 °C with lithium hydroxide monohydrate (65 mg, 1.5 mmol). After 1 hour, the mixture was allowed to

warm to room temperature, and the stirring was continued for 2 hours. The resulting mixture was diluted with water, and washed twice with ether. The separated aqueous phase was acidified to pH 2 with 1N hydrochloric acid, and then extracted twice with ethyl acetate. The combined organic extracts was washed with water and brine successively, dried over $MgSO_4$, filtered and concentrated in vacuo to give the title compound (0.49 g, quant.) as a white solid; m.p. 124-126°; $[\alpha]_D$ = -5 (c = 1.15, ethanol); HPLC (Chiralcel OD, hexane/ isopropanol/ TFA, 900: 100: 3) ee > 95%; $^1$H NMR (DMSO-*d6*, 400 MHz) δ 12.62 (1H, s), 10.84 (1H, s), 8.15 (1H, d, *J* = 7.9 Hz), 7.52 (1H, d, *J* = 7.9 Hz), 7.33 (1H, d, *J* = 7.9 Hz), 7.29 – 7.13 (6H, m), 7.06 (1H, t, *J* = 6.9 Hz), 7.00 (1H, t, *J* = 6.9 Hz), 4.46 – 4.43 (1H, m), 3.14 (1H, dd, *J* = 5.0, 14.5 Hz), 2.99 (1H, dd, *J* = 8.8, 14.8 Hz), 2.40 - 2.38 (1H, m), 1.82 - 1.79 (1H, m), 1.44 - 1.42 (1H, m), 1.35 - 1.32 (1H, m).

**_N_-[2-(1H-indol-3-yl)ethyl]-[_N_-(3,5-dimethylbenzoyl)-_N_-methyl]-(D)-phenylalaniamide (9)**

To a cold (0 °C) solution of *N*-methyl-(D)-phenylalanine (0.30 g, 1.7 mmol), 1,4-dioxane (8 mL) was added 1N sodium hydroxide (4 mL) followed by a solution of 3,5-dimethylbenzoyl chloride (0.35 g, 2.1 mmol) in THF (5 mL). After 30 min, the mixture was allowed to warm to room temperature, and the stirring was continued 1 hour. The mixture was diluted with water, and washed twice with ether. The separated aqueous phase was acidified to pH 2 with 1N hydrochloric acid, and extracted twice with ethyl acetate. The combined organic extracts were washed with water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to give *N*-(3,5-dimethylbenzoyl)-*N*-methyl-(D)-phenylalane (0.35 g, 68% yield).

To a cold (0 °C) mixture of *N*-(3,5-dimethylbenzoyl)-*N*-methyl-(D)-phenylalane

(0.30 g, 0.96 mmol), tryptamine (0.17 g, 1.06 mmol), HOBt (0.3 g, 2.2 mmol) and dichloromethane (7 mL) was added EDC (0.4 mL, 2.3 mmol). After 30 min, the mixture was allowed to warm to room temperature, and the stirring was continued overnight. The mixture was diluted with water, and extracted twice with ethyl acetate. The combined organic extracts were washed with water and brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/ ethyl acetate, 4:1) to give the title compound (0.39 g, 90% yield) as a white solid; m.p. 160-161 °C; $^1$H NMR ($CDCl_3$, 400 MHz) δ 8.29 (s), 8.11 (s), 7.59(d, *J* = 7.5 Hz), 7.33-7.90 (m), 6.81 (br), 6.69 (br), 6.52 (s), 5.95 (br), 5.86 (s), 5.35 (dd, *J* = 7, 9.5 Hz), 4.2 (br), 3.76 (m), 3.62 (dd, *J* = 7, 13 Hz), 3.52 (m), 3.35 (dd, *J* = 7, 15 Hz), 3.15 (dd, *J* = 10, 15 Hz), 3.05-2.8 (m), 2.72 (s), 2.22 (s), 1.97 (s).

**_N_-(3,5-Dimethylbenzoyl)-_N_-methyl-(D)-phenylalanyl-(L)-tryptophan (10)**

To a stirred solution of *N*-Boc-*N*-methyl-(D)-phenylalanine (2.1 g, 7.5 mmol) [Boc = tert-butyloxycarbonyl] in dry DMF (15 mL) were added (L)-tryptophan methyl ester hydrochloride (2.0 g, 7.8 mmol) and HOBt (1.2 g, 8.8 mmol). The mixture was cooled to 0 °C, and then EDC (1.7 mL, 9.2 mmol) was added dropwise. The reaction mixture was slowly warmed to room temperature and the stirring was continued for 2 hours. The homogeneous mixture was diluted with ethyl acetate (500 mL) and washed with three portions of water (200 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated in vacuo to give *N*-Boc-*N*-methyl-(D)-phenylalanyl- (L)-tryptophan methyl ester **(11)** (3.3 g, 92% yield)as a white foam. $[\alpha]_D = +42$ (c = 1.0, ethanol).

The above crude material **(11)** was dissolved in a mixture of TFA (6 mL) and

ethanedithiol (1.5 mL) and stirred under $N_2$ atmosphere at room temperature for 1 hour. A 4M solution of hydrogen chloride in 1,4-dioxane was added. The hydrochloride salt precipitated by addition of ether (400 mL) and hexane (200 mL) was collected by filtration, washed with ether and dried in vacuo to give *N*-methyl-(D)-phenylalanyl-(L)-tryptophan methyl ester hydrochloride (**12**) (2.6 g, quant.) as a white powder. $[\alpha]_D$ = -29 (c = 1.0, ethanol).

A solution of the above hydrochloride salt (**12**) (200 mg, 0.48 mmol) and 3,5-dimethylbenzoic acid (87 mg, 0.57 mmol) in DMF (1 mL) was treated at 0 °C with EDC (0.11 mL, 0.6 mmol). The reaction mixture was allowed to warm to room temperature and the stirring was continued for 2 hours. The homogeneous mixture was diluted with ethyl acetate (100 mL) and washed with three portions of water (70 mL). The separated organic layer was dried over $MgSO_4$, filtered and concentrated in vacuo. Chromatography on silica-gel (ethyl acetate: hexane, 1:1) afforded *N*-methyl-(D)-phenylalanyl-(L)-tryptophan methyl ester (**13**) (165 mg, 68% yield) as a white form, which was then hydrolyzed at 0 °C with lithium hydroxide monohydrate (20 mg, 0.47 mmol) in MeOH/water, 2:1 (9 mL). After 3 hours, the reaction mixture was diluted with water (200 mL) and washed with water (100 mL). The combined aqueous layers were acidified to pH = 2 with 1M hydrochloric acid and extracted twice with ethyl acetate. The combined organic extracts were dried over $MgSO_4$, filtered and concentrated in vacuo to give the title compound (**10**) (140 mg, 89% yield) as a white foam; m.p. 91-94 °C. FAB-MS *m/z* 498 $(M+H)^+$. $[\alpha]_D$ = -46 (c = 1.095, ethanol). HPLC (Chiralcel OD, hexane/ isopropanol/ TFA, 900:100:3) ee > 95%. $^1H$ NMR ($CDCl_3$, 400 MHz) δ 8.29 (s), 8.15 (s), 7.56 (d, *J* = 7.8 Hz), 7.47 (d, *J* = 7.8 Hz), 7.3 – 6.7 (m), 6.48 (s), 5.92 (s), 5.41 (dd, *J* = 6.8, 9.7 Hz), 4.84 (dd, *J* = 5.8, 13.2 Hz), 4.33

(dd, $J$ = 2, 7.2 Hz), 3.4 – 2.75 (m), 2.70 (s), 2.18 (s), 1.91 (s).

**$N$-(3,5-Dimethylbenzoyl)-$N$-methyl-(D)-(4-phenylphenyl)alanyl-(L)-tryptophan (14)**

A solution of thionylchloride (6.5 mL) in dry methanol (280 mL) at –20 °C was treated with (D)-(4-phenylphenyl)alanine (3.7 g, 13.3 mmol).[28] The reaction mixture was stirred at reflux overnight and then concentrated in vacuo. Recrystallization from methanol and ether gave (D)-(4-phenylphenyl)alanine methyl ester hydrochloride (**15**); $[\alpha]_D$ = +13 (c = 1.025, methanol).

A solution of the above material (**15**) (315 mg, 0.94 mmol) in dry THF (0.4 mL) was treated at room temperature with water (0.4 mL), formalin (0.15 mL, 1.88 mmol) and freshly distilled cyclopentadiene (0.3 mL, 3.63 mmol). The pale yellow solution was stirred at room temperature for 2 hours, then washed with hexane (100 mL), diluted with 4% aqueous $NaHCO_3$ solution (199 mL), and extracted with chloroform (200 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated in vacuo to give a crude bicyclic intermediate (**16**) (0.39 g). This material was dissolved in chloroform (4.7 mL) and treated with TFA (4.7 mL) and triethylsilane (0.45 mL). The reaction mixture was stirred at room temperature under $N_2$ atmosphere for 20 hours, and then concentrated in vacuo. The crude product was dissolved in ethyl acetate (200 mL) and washed with 1M hydrochloric acid (100 mL) followed by saturated aqueous $NaHCO_3$ solution (100 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated in vacuo to give $N$-methyl-(D)-(4-phenylphenyl)alanine methyl ester (**17**) as a white form.

A solution of the above material in chloroform (5 mL) was treated with 2M aqueous $Na_2CO_3$ solution (0.6 mL) and 3,5-dimethylbenzoyl chloride (0.3 mL, 1.4

mmol). The reaction mixture was stirred at room temperature for 2.5 hours, then diluted with ethyl acetate (200 mL), washed with 4% aqueous $NaHCO_3$ solution (100 mL), water (100 mL), 1M hydrochloric acid (100 mL) and water (100mL) successively. The organic layer was dried over $MgSO_4$, filtered and concentrated in vacuo. Chromatography on silica-gel (ethyl acetate/ hexane, 4:1) afforded *N*-(3,5-dimethylbenzoyl)-*N*-methyl-(D)-(4-phenylphenyl)alanine methyl ester **(18)** (280 mg, 58% yield); $[\alpha]_D$ = +48 (c = 0.685, methanol; ee > 98% (HPLC: Chiralcel OF).

This material (110 mg, 0.27 mmol) was treated at 0 °C with lithium hydroxide monohydrate (13 mg, 0.31 mmol) in methanol (0.8 mL), water (0.4 mL) and THF (0.4 mL). After being stirred for 2 hours, the reaction mixture was diluted with ether (200 mL) and washed three times with water. The combined aqueous layers were acidified to pH = 2 with 1M hydrochloric acid and extracted twice with ethyl acetate (200 mL). The organic extracts were dried over $MgSO_4$, filtered and concentrated in vacuo to give *N*-(3,5-dimethylbenzoyl)-*N*-methyl-(D)-(4-phenylphenyl)alanine **(19)** (106 mg, quant.) as a white foam; $[\alpha]_D$ = + 7.5 (c = 1.0, methanol).

A solution of the above material (103 mg, 0.27 mmol), (L)-tryptophan methyl ester hydrochloride (100 mg, 0.39 mmol) and HOBt (70 mg, 0.52 mmol) in dry DMF (3 mL) was treated at 0 °C with EDC (0.07 mL, 0.38 mmol). The mixture was allowed to warm to room temperature, and the stirring was continued overnight. The homogeneous mixture was diluted with ethyl acetate (100 mL) and washed three times with water. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. Chromatography on silica-gel (ethyl acetate/ hexane, 1:1) afforded *N*-(3,5-dimethylbenzoyl)-*N*-methyl-(D)-(4-phenylphenyl)alanyl-(L)-tryptophane methyl ester (130 mg, 82% yield) as a white foam; de > 98% (HPLC: Chiralcel OD).

This material (125 mg, 0.21 mmol) was hydrolyzed at 0 °C with lithium hydroxide monohydrate (10 mg, 0.23 mmol) in methanol (2 mL), THF (1 mL) and water (1 mL). After being stirred for 3 hours, the reaction mixture was diluted with ether (100 mL) and washed with three times with water. The combined aqueous layers were acidified to pH = 2 with 1M hydrochloric acid and extracted with twice with ethyl acetate (100 mL). The ethyl acetate extracts were dried over $MgSO_4$, filtered and concentrated in vacuo to give the title compound **(14)** (114 mg, 95% yield) as a white foam; FAB-MS *m/z* 574 $(M+H)^+$; $[\alpha]_D$ = + 2.5 (c = 1.0, ethanol); $^1H$ NMR ($CDCl_3$, 400 MHz) δ 8.32 (s), 8.22 (s), 7.6-6.8 (m), 6.93 (s), 6.8 (m), 6.51 (s), 5.97 (s), 5.46 (t, *J* = 8 Hz), 4.85 (q, *J* = 6 Hz), 4.36 (m), 3.4-2.8 (m), 2.73 (s), 2.15 (s), 1.85 (s).

***N*-(3,5-Dimethylbenzoyl)-*N*-methyl-(D)-3-[4-(5-isoxazolyl)-phenyl]alanyl-(L)-tryptophan (20)**

A mixture of 4-methylacetophenone (100 g, 0.75 mol) and *N,N*-dimethyl-formamide dimethyl acetal (200 mL, 1.51 mol) was heated at reflux for 20 hours. After cooled to room temperature, the reaction mixture was concentrated in vacuo to give crude 3-dimethylamino-1-p-tolylprop-2-ene-1-one **(22)** (99g, 82% yield) as a pale yellow oil.[25]

To a solution of crude 3-dimethylamino-1-p-tolylprop-2-ene-1-one (148 g, 0.92 mol) in dry methanol (1 L) at 0 °C was added a solution of hydroxylamine-*O*-sulfonic acid (93 g) in dry methanol (700 mL). The mixture was allowed to warm to room temperature and the stirring was continued for 20 min, then carefully poured into a solution of $NaHCO_3$ (150 g) in water (11 L). After standing at room temperature over night, the resultant precipitate was collected by filtration, and dried in vacuo to give 5-(4-methylphenyl)isoxazole **(23)** (117 g, 80% yield). In order to the pure product, the

crude material can be purified by column chromatography on silica-gel (hexane: ethyl acetate, 4:1).

To a solution of 5-(4-methylphenyl)isoxazole (17 g, 0.094 mol) and *N*-bromosuccinimide (19 g) in tetrachloromethane (500 mL) under $N_2$ atmosphere was added benzoyl peroxide (0.43 g), and the mixture was stirred at reflux overnight. After cooled to room temperature, the mixture was evaporated under reduced pressure, and the residue was purified by column chromatography on silica-gel (hexane/ ethyl acetate, 4:1) to give 5-(4-bromomethylphenyl)isoxazole **(24)** (10.5 g, 47% yield); $^1$H NMR ($CDCl_3$, 400 MHz) δ 8.32 (1H, d, $J$ = 1.8 Hz), 7.78 (2H, d, $J$ = 8.2 Hz), 7.51 (2H, d, $J$ = 8.2 Hz), 6.54 (1H, d, $J$ = 1.8 Hz), 4.52 (2H, s).

To a mixture of 5-(4-bromomethylphenyl)isoxazole (700 mg, 2.9 mmol), *N*-diphenylmethyleneglycine ethyl ester (890 mg), tetrabutylammonium hydrogen sulfate (1.0 g) and dichloromethane (20 mL) was added 2.5N aqueous sodium hydroxide solution (20 mL), and the mixture was vigorously stirred at room temperature overnight. The organic layer was separated and concentrated in vacuo. The residue was partitioned between ether and water. The separated organic phase was washed with water and brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo to give crude *N*-diphenylmethylene-3-[4-(5-isoxazolyl)phenyl]alanine ethyl ester, which was used for the next step without further purification.

Crude *N*-diphenylmethylene-3-[4-(5-isoxazolyl)phenyl]alanine ethyl ester (280 mg) was treated with p-toluenesulfonic acid monohydrate (100 mg) in a mixture of acetonitrile (35 mL) and water (3.5 mL) at room temperature for 3.5 hours. The reaction mixture was concentrated in vacuo, and the residue was partirioned between ether and 1N sodium hydroxide solution. The separated organic phase was washed

with brine, dried over $MgSO_4$, filtered and concentrated in vacuo to give crude 3-[4-(5-isoxazolyl)phenyl]alanine ethyl ester (**25**), which was used for the next step without further purification.

Crude 3-[4-(5-isoxazolyl)phenyl]alanine ethyl ester (660 mg) was dissolved in chloroform (6.6 mL), stirred vigorously with 2N aqueous sodium carbonate solution (1.4 mL), and after cooling to 10 °C, 3,5-dimethylbenzoylchloride (0.7 mL) was added. Stirring was continued at 10 °C for 1 hour, and at room temperature for 2 hours. The reaction mixture was partitioned between dichloromethane and water. The separated organic phase was washed with 10% aqueous citric acid and brine successively, dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by column chromatography on silica-gel (hexane/ ethyl acetate, 4:1) to give *N*-(3,5-dimethylbenzoyl)-3-[4-(5-isoxazolyl)phenyl]alanine ethyl ester (**26**) (470 mg, 41% yield in 3 steps) as a white amorphous; $^1$H NMR ($CDCl_3$, 400 MHz) δ 8.28 (1H, d, $J$ = 1.8 Hz), 7.72 (2H, d, $J$ = 8.2 Hz), 7.33 (2H, s), 7.26 (2H, d, $J$ = 8.2 Hz), 7.15 (1H, s), 6.65 (1H, d, $J$ = 7.3 Hz), 6.50 (1H, d, $J$ = 1.8 Hz), 5.09 (1H, m), 4.24 (2H, q, $J$ = 4.24 Hz), 3.32 (2H, m), 2.34 (6H, s), 1.29 (3H, t, $J$ = 7.1 Hz).

A solution of *N*-(3,5-dimethylbenzoyl)-3-[4-(5-isoxazolyl)phenyl]alanine ethyl ester (3.8 g, 9.7 mmol) and methyl iodide (1.8 mL, 29 mmol) in dry DMF (40 mL) was cooled in an ice bath, and sodium hydride (60% in oil, 390 mg) was added in portions. The mixture was allowed to warm to room temperature, and the stirring was continued for 5 hours, then poured into water, extracted with ethyl acetate. The separated organic phase was washed with water and brine successively, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/ ethyl acetate, 3:1) to give *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4- (5-

isoxazolyl)phenyl]alanine ethyl ester (**27**) (3.9 g, quant.); Rf = 0.45 (ethyl acetate/ hexane, 1:1); $^1$H NMR ($CDCl_3$, 400 MHz) δ 8.30 (1H, br d), 7.75 (2H, m), 7.41 (1H, br d), 7.12 (1H, br d), 6.97 (0.5 H, s), 6.93 (0.5 H, s), 6.70 (1H, s), 6.53 (1H, br d), 6.36 (1H, br d), 5.40 (0.5H, m), 4.58 (0.5H, m), 4.27 (2H, m), 3.54 (m, 0.5 H), 3.25 (1H, m), 3.05 (2H, m), 2.78 (1.5H, s), 2.23 (3H, s), 2.13 (3H, s), 1.27 (3H, m).

*N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4- (5-isoxazolyl)phenyl]alanine ethyl ester (37 mg, 0.091 mmol) was treated with lithium hydroxide monohydrate (5 mg, 0.1 mmol) in methanol (0.5 mL), THF (0.25 mL) and water (0.25 mL) at room temperature for 3 hours. The mixture was then partitioned between ether and water. The separated aqueous phase was acidified to pH 2 with 1N hydrochloric acid, and subsequently extracted with ethyl acetate. The separated organic phase was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to give *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)phenyl]alanine (**28**) (24 mg, 70% yield); Rf = 0.51 (chloroform/ methanol, 9:1 with 0.1% acetic acid); $^1$H NMR ($CDCl_3$, 400 MHz) δ 8.48 (s), 8.30 (s), 7.80 (m), 7.43 (m), 7.13 (m), 7.03 (s), 6.94 (s), 6.75 (s), 6.69 (s), 6.54 (s), 6.36 (s), 5.16 (m), 4.65 (m), 3.55 (m), 3.40 (m), 3.25 (m), 3.10 (m), 2.80 (s), 2.26 (s), 2.14 (s).

To a cold (0 °C) ~~mix~~ture of *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)phenyl]alanine (445 mg, 1.18 mmol), (L)-tryptophane methyl ester hydrochloride (400 mg), HOBt (330 mg) and DMF (24 mL) was added EDC (0.32 mL). After 1 hour, the mixture was allowed to warm to room temperature, and the stirring was continued overnight, then the mixture was partitioned between ethyl acetate and 10% aqueous citric acid solution. The separated organic phase was washed with 4% aqueous $NaHCO_3$ solution, water and brine successively, dried over $Na_2SO_4$, filtered and

concentrated in vacuo. Flash chromatography on silica gel (hexane/ ethyl acetate, 2:1) afforded the product as a mixture of diastereomers. The mixture was separated by medium pressure liquid chromatography (Kusano KHLC-201-43 type III) on silica gel using ether/ dichloromethane, 1:1 as the eluent to give *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)phenyl]-(D)-alanyl-(L)-tryptophane methyl ester (270 mg, 40% yield) as a colorless amorphous; $^1$H NMR ($CDCl_3$, 400 MHz) δ 8.60 (s), 8.30 (m), 7.70 (m), 7.55 (m), 7.10 (m), 6.90 (m), 6.50 (m), 5.85 (s), 5.43 (m), 4.90 (m), 4.33 (m), 3.75 (s), 3.70 (s), 3.35 (m), 3.15 (m), 2.85 (s), 2.57 (s), 2.35 (s), 2.20 (s), 1.93 (s), 1.85 (s). The other fraction, *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl) phenyl]-(L)-alanyl-(L)-tryptophane methyl ester, was used for the synthesis of *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)phenyl]-(D)-alanyl-(L)-tryptophane **21** by the similar manner described here.

*N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)phenyl]-(D)-alanyl-(L)-tryptophane methyl ester (50 mg, 0.086 mmol) was treated with lithium hydroxide monohydrate (3.8 mg) in methanol (1 mL), THF (0.5 mL) and water (0.5 mL) at 0 °C for 1 hour, and at room temperature for 2 hours. The mixture was then partitioned between ether and water. The separated aqueous phase was acidified to pH 2 with 1N hydrochloric acid, and subsequently extracted with ethyl acetate. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo to give *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)phenyl]-(D)-alanyl-(L)-tryptophane (**20**) (44 mg, 91% yield); $^1$H NMR ($CDCl_3$, 400 MHz) δ 8.29 (s), 8.12 (s), 7.70 (m), 7.55 (m), 7.28 (m), 7.17 (m), 7.07 (m), 6.98 (m), 6.86 (m), 6.61 (s), 6.53 (s), 6.49 (s), 5.97 (s), 5.43 (m), 4.87 (m), 4.40 (m), 3.90 (m), 3.33 (m), 2.08 (m), 2.77 (s), 2.18 (s), 1.89 (s).

**_N_-(3,5-Dimethylbenzoyl)-_N_-methyl-(D)-3-[4-(3-thienyl)-phenyl]alanyl-(L)-tryptophan (29)**

A solution of 4-bromotoluene (0.9 mL, 7.3 mmol) in THF (3 mL) was added to dried magnesium turnings (0.815 g, 33.5 atoms) under $N_2$ atmosphere. After the exothermic reaction initiated, a solution of 4-bromotoluene (3.16 mL, 25.7 mmol) in THF (3 mL) was added dropwise, and the stirring was continued for 10 min. The resultant Grignard reagent was dropped to a suspension of 3-bromothiophene (2.8 mL, 29.9 mmol) and [1,2-bis(diphenylphosphino)ethane]nickel (II) chloride (0.72 g, 1.4 mmol) in diethyl ether (50 mL). The reaction mixture was stirred at room temperature for 15 min, and at reflux for 18 hours. After cooled to room temperature, the reaction mixture was quenched with 1N hydrochloric acid, and extracted with ether. The separated organic phase was washed with saturate $NaHCO_3$ solution and brine successively, dried over $MgSO_4$, and concentrated in vacuo. The residue was recrystallized from ethanol to give 4-(3-thienyl)toluene **(32)** (3.57 g, 69% yield).

To a solution of 4-(3-thienyl)toluene (3.56 g, 20.5 mmol) in $CCl_4$ (100 mL) was added *N*-bromosuccinimide (3.64 g, 20.5 mmol) followed by benzoyl peroxide (80 mg, 3.3 mmol). The mixture was heated at reflux 24 hours, and then concentrated in vacuo. The crude residue was purified by recrystallization from ethanol to give 4-(3-thienyl)benzyl bromide **(33)** (2.45 g, 47% yield); Rf = 0.2 (hexane).

To a mixture of 4-(3-thienyl)benzyl bromide (1.94 g, 7.67 mmol), *N*-diphenylmethyleneglycine ethyl ester (2.28 g, 8.10 mmol), tetrabutylammonium hydrogen sulfate (2.65 g, 7.80 mmol) and dichloromethane (50 mL) was added 2.5N aqueous sodium hydroxide solution (60 mL), and the mixture was vigorously stirred at room temperature overnight. The organic layer was separated and concentrated in

vacuo. The residue was partitioned between ether and water. The separated organic phase was washed with water and brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo to give crude *N*-diphenylmethylene-3-[4-(3-thienyl)phenyl]alanine ethyl ester (1.80 g), which was used for the next step without further purification; Rf = 0.43 (hexane/ ethyl acetate, 4:1).

Crude *N*-diphenylmethylene-3-[4-(3-thienyl)phenyl]alanine ethyl ester (1.80 g) was treated with p-toluenesulfonic acid monohydrate (0.8 g, 4.2 mmol) in a mixture of acetonitrile (270 mL) and water (27 mL) at room temperature for 3.5 hours. The reaction mixture was concentrated in vacuo, and the residue was partitioned between ether and 1N sodium hydroxide solution. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered and concentrated in vacuo to give crude 3-[4-(3-thienyl)phenyl]alanine ethyl ester (1.84 g) including a small amount of benzophenone, which was used for the next step without further purification.

To a cold (0 °C) mixture of the crude 3-[4-(3-thienyl)phenyl]alanine ethyl ester (1.84 g), 3,5-dimethylbenzoic acid (0.81 g, 5.3 mmol), HOBt (0.70 g, 5.2 mmol) and DMF (30 mL) was added EDC (1.0 mL, 5.5 mmol). The mixture was allowed to warm to room temperature, and the stirring was continued overnight. The mixture was partitioned between ethyl acetate and 10% aqueous citric acid solution. The separated organic phase was washed with brine, saturated $NaHCO_3$ solution, water and brine successively, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude product was purified by column chromatography on silica-gel (hexane/ ethyl acetate, 4:1) to give *N*-(3,5-dimethylbenzoyl)-3-[4-(3-thienyl)phenyl]alanine ethyl ester **(34)** (1.12 g, 36% yield in 3 steps) as a white amorphous; Rf = 0.20 (hexane/ ethyl acetate, 4:1).

To a suspension of sodium hydride (60% in oil, 0.18 g, 4.5 mmol) in dry DMF (4

mL) was added a solution of *N*-(3,5-dimethylbenzoyl)-3-[4-(3-thienyl)phenyl]alanine ethyl ester (**34**) (1.05 g, 2.58 mmol) in dry DMF (4 mL). After the mixture was stirred at room temperature for 1 hour, methyl iodide (0.25 mL, 4.0 mmol) was added, and the stirring was continued for 4 hours. The mixture was poured into water, extracted with ethyl acetate. The separated organic phase was washed with water and brine successively, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/ ethyl acetate, 4:1) to give *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(3-thienyl)phenyl]alanine ethyl ester (**35**) (0.791 g, 73% yield); Rf = 0.33 (hexane/ ethyl acetate 2:1); FAB-MS *m/z* 422 $(M+H)^+$.

To a solution of the above product (0.782 g, 1.86 mmol) in methanol (4 mL), THF (3 mL) and water (2.5 mL) was added lithium hydroxide monohydrate (0.102 g, 2.43 mmol), and the stirring was continued at room temperature for 45 min. The mixture was diluted with water, and extracted twice with water. The separated aqueous phase was acidified to pH 2 with 1N hydrochloric acid, and then extracted with ethyl acetate. The separated ethyl acetate layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to give *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(3-thienyl) phenyl]alanine (0.712 g, 97% yield); $^1H$ NMR ($CDCl_3$, 400 MHz) δ 8.50 (1H, s), 7.80 (1H, m), 7.45 (2H, m), 7.13 (1H, m), 7.00 (1H, s), 6.85 (1H, s), 6.63 (1H, s), 6.54 (2H, m), 5.16 (1H, m), 4.65 (1H, m), 3.55 (1H, m), 3.40 (1H, m), 3.25 (1H, m), 3.10 (1H, m), 2.80 (2H, s), 2.26 (2H, s), 2.15 (3H, s).

To a cold (0 °C) mixture of *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(3-thienyl)phenyl]alanine (135 mg, 0.33 mmol), (L)-tryptophane methyl ester hydrochloride (110 mg, 0.43 mmol), HOBt (58 mg, 0.43 mmol) and DMF (3 mL) was added EDC (0.08 mL, 0.43 mmol). After 2 hour, the mixture was allowed to warm to

room temperature, and the stirring was continued overnight, then the mixture was partitioned between ethyl acetate and 10% aqueous citric acid solution. The separated organic phase was washed with 4% aqueous $NaHCO_3$ solution, water and brine successively, dried over $Na_2SO_4$, filtered and concentrated in vacuo. Flash chromatography on silica gel (hexane/ ethyl acetate, 1:1) afforded the product as a mixture of diastereomers. The mixture was separated by medium pressure liquid chromatography (Kusano KHLC-201-43 type III) on silica gel using ether/ dichloromethane, 1:1 as the eluent to give *N*-(3,5-dimethylbenzoyl)-*N*- methyl-3-[4-(3-thienyl)phenyl]-(D)-alanyl-(L)-tryptophane methyl ester (55 mg, 28% yield) as a colorless amorphous.

*N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(3-thienyl)phenyl]-(D)-alanyl-(L)-tryptophane methyl ester (50 mg, 0.092 mmol) was treated with lithium hydroxide monohydrate (6 mg, 0.14 mmol) in methanol (1 mL), THF (0.5 mL) and water (0.5 mL) at 0 °C for 1 hour, and at room temperature for 2 hours. The mixture was then partitioned between ether and water. The separated aqueous phase was acidified to pH 2 with 1N hydrochloric acid, and subsequently extracted with ethyl acetate. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo to give *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(3-thienyl) phenyl]-(D)-alanyl-(L)-tryptophane (**29**) (49 mg, quant.); FAB-MS *m/z* 580 $(M+H)^+$; $^1H$ NMR ($CDCl_3$, 400 MHz) δ 9.10 (s), 8.00 (m), 7.58 (m), 7.29 (m), 7.07 (m), 6.99 (m), 6.88 (m), 6.61 (s), 6.41 (m), 6.50 (s), 5.98 (s), 5.41 (m), 4.97 (m), 3.91 (m), 3.30 (m), 2.10 (m), 2.78 (s), 2.28 (s), 2.08 (s).

**Pharmacological Experiments**

**Endothelin (ET) receptor binding assay**

The binding affinity to the ET receptor of the compounds was determined according to the reported procedure.[13] ET-1 and ET-3 are purchased from Peptide Institute Inc. (Osaka, Japan). [$^{125}$I]ET-1 and [$^{125}$I]ET-3 (~74 TBq/mmol each) are purchased from Amersham International (Bucks, U.K.).

The plasma membrane of porcine lung (2 mg of protein) was incubated at 37 °C for 1 hour with 30 pM [$^{125}$I]ET-1 or 10 pM [$^{125}$I]ET-3 in the absence or presence of various amounts of nonlabeled ligands in a total volume of 1mL of 20 mM HEPES (pH 7.4), containing 145 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA 1 mg/mL bovine serum albumin, and 0.2 mg/mL bacitracin. After the incubation, unbound [$^{125}$I]ETs were separated by centrifugation at 20,000 xg for 20 min at 4 °C followed by aspiration of the supernatant. The radioactivity in the membrane pellet was measured in Wallac-1470 Wizard autogamma counter (Pharmacia). Nonspecific binding was defined as membrane-associated radioactivity in the presence of saturating concentrations of ETs (100 nM). Nonspecific binding was subtracted from the total binding and the difference was defined as specific binding. Total binding was always less than 15% of the total radioactivity added.

The binding to the $ET_A$ receptor was determined with [$^{125}$I]ET-1 in the presence of 1 nM nonlabeled ET-3 and the binding to the $ET_B$ receptor with [$^{125}$I]ET-3 alone. By Scatchard analysis, the $ET_A$ receptor showed an apparent dissociation constant (Kd) of 44 pM and maximum binding sites (Bmax) of 362 fmol/mg protein. From the inhibition curves for the binding of [$^{125}$I]ETs, the apparent binding affinity constant (Ki) of each analog was calculated as a parameter of the affinity for the $ET_A$ and $ET_B$ receptors.

**Functional assays (contractions of guinea pig trachea and rat thoracic aorta)**

Epithelium- and endothelium-denuded ring preparations (~2 mm width) from guinea pig trachea and rat thoracic aorta, respectively, were placed into a 4.0 ml organ bath containing the oxygenated (95% $O_2$ – 5% $CO_2$) Krebs-Henseleit solution (composition, mM: 113.0 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 5.5 glucose and 0.01 EDTA; pH 7.4; 37 °C) for isometric tension measurement at a resting tension of 1 g. Each preparation was first stimulated with 10 μM carbachol for the trachea or with 100 nM norepinephrine for the aorta to use as a reference standard for the responses to ET-3 for the trachea or ET-1 for the aorta. An endothelin antagonist was dissolved in dimethyl sulfoxide, the solution was diluted to 1/1000 with the Krebs-Henseleit solution, and then the whole volume of the Krebs-Henseleit solution in the organ bath was replaced with the solution containing the endothelin antagonist.

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## Chapter 2. Discovery and synthesis of novel and potent $ET_A$ and $ET_B$ dual endothelin antagonists [1]

### 1. Introduction

As it is described in the previous chapter, endothelins are a family of 21 amino acid peptides with a wide range of activities in the airways and pulmonary vascular system through two distinct endothelin receptors, $ET_A$ and $ET_B$. All three endothelins cause bronchoconstriction in intact airways, with ET-1 being the most potent. The vast majority of ET-1 binding sites on bronchial smooth muscle are $ET_B$ receptors, and bronchoconstriction in human bronchi is not inhibited by $ET_A$ antagonists but augmented by $ET_B$ receptor antagonists.[2] Therefore, it is expected that $ET_B$ selective antagonists relieve the symptom of asthma and COPD (chronic obstructive pulmonary disease) by inhibiting the bronchoconstriction. Actually, IRL 2500 and other selective $ET_B$ selective antagonists demonstrated to inhibit trachea smooth muscle contraction induced by ET-3.[3]

However, it is lately reported that inhibition of $ET_A$ or combined $ET_A$ and $ET_B$ receptors additionally leads to decreased airway inflammation in antigen-challenged animals, suggesting that the proinflammatry effects of ET-1 in the airway are mediated by $ET_A$ receptors.[4] It is well-known that there are two distinct antigen-induced responses, immediate and late asthmatic responses, in patients with asthma.[5] The both responses can be suppressed considerably by anti-ET antiserum, providing the direct evidence that endothelins are involved in the asthmatic responses, as shown in Figure 1. The $ET_B$ selective antagonist, BQ-788,[6] significantly inhibited only the immediate asthmatic responses, which can be suppressed by well-known bronchodilators, β2

agonists and histamine antagonists, such as diphenhydramine. On the other hand, the late asthmatic response, which can be controlled by inflammatory agents like steroids, was significantly blocked by the $ET_A$ selective antagonist, BQ-123.[7] **These results suggest that inhibition of both $ET_A$ and $ET_B$ receptors would suppress the late asthmatic response as well as the immediate asthmatic response. Thus, our next strategies focused on a development of $ET_A$ and $ET_B$ dual antagonists by an optimization of the $ET_B$ selective antagonists for treatment of asthma patients.**

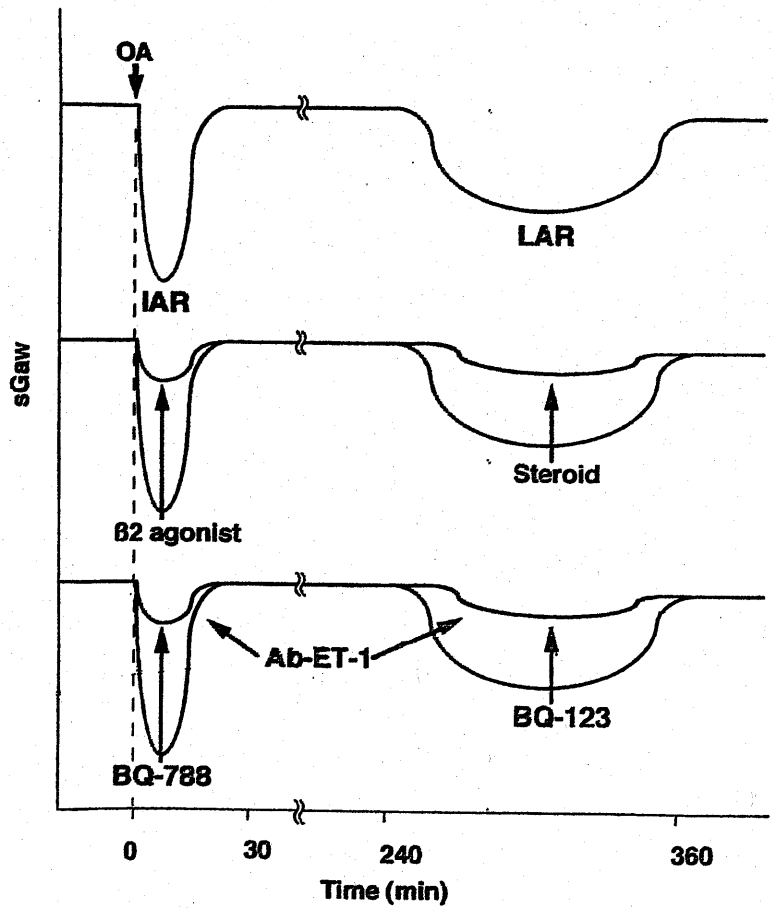



**Figure 1. Possible contributory role of endogenous endothelins on ovalbumin (antigen)-induced immediate and late asthmatic responses.**[4(a)] sGAW (Specific airways conductance), OA (Ovalbumin), IAR (Immediate asthmatic response), LAR (Late asthmatic response), BQ-788 ($ET_B$ selective antagonist), BQ-123 ($ET_A$ selective antagonist), Ab-ET-1 (anti-ET antiserum).

## 2. Discovery of a first $ET_A/ET_B$ dual antagonist, IRL 2674, from the $ET_B$ selective antagonist, IRL 2500.

As shown in the previous chapter, introduction of a phenyl ring at the 4-position of the phenylalanine on IRL 1841 (**10**) resulted in a potent $ET_B$ selective antagonist, IRL 2500 (**14**). This modification improved not only the $ET_B$ receptor affinity by 36-fold but also the $ET_A$ receptor affinity by 28-fold, suggesting that the $ET_A$ receptor affinity can be increased by the modification of the biphenyl ring on IRL 2500 in order to provide potent $ET_A/ET_B$ dual antagonists. In fact, replacement of the phenyl ring on IRL 2500 with a 3-thienyl ring led to an improvement of $ET_A$ receptor affinity by 8-fold along with quadrupling the $ET_B$ receptor affinity. However, further improvement of the $ET_A/ET_B$ ratio cannot be achieved by modification of the aromatic ring.

A great breakthrough was attained in the course of a systematic SAR (structure-activity relationships) study for modification of the carboxylic acid moiety. It was found that the $ET_A$ receptor binding affinity was dramatically improved by attaching a sulfonamide group to the C-terminus of tryptophan with retaining the $ET_B$ receptor binding affinity, resulting in the more balanced $ET_A/ET_B$ binding affinity. For instance, replacement of the carboxylic acid moiety on IRL 2500 with a phenyl sulfonamide group resulted in an increase of $ET_A$ receptor affinity by 20-fold without altering the $ET_B$ receptor affinity.

The aromatic ring on the (D)-phenylalanine moiety and the sulfonamide moiety on the C-terminus were found to be important to enhance the $ET_A$ receptor binding affinity. Thus, the structurally important elements for $ET_A$ receptor binding affinity were combined to provide the first balanced $ET_A$ and $ET_B$ dual antagonist, IRL 2674 (**41**), as illustrated in Figure 2. Then further optimization of the sulfonamide moiety

was implemented using the 5-isoxazole analog, IRL 2796 (**20**), with more potent activity than that of the corresponding 3-thienyl analog, IRL 2659 (**29**), (Table 1).

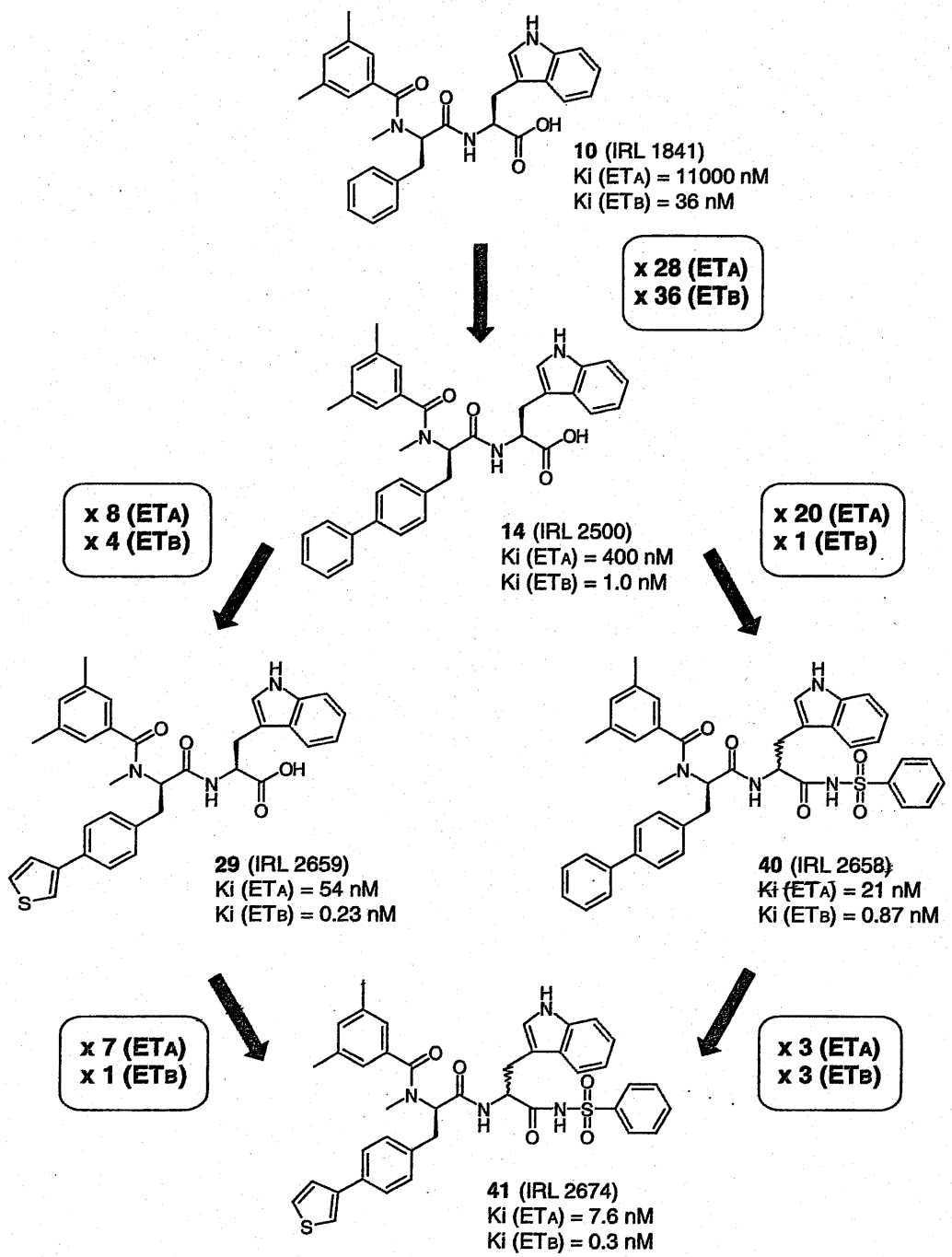
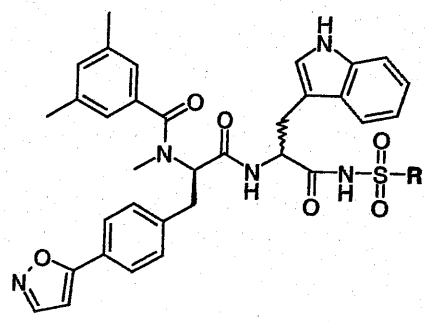



**Figure 2. Development of the first $ET_A/ET_B$ dual antagonist**

**Table 1. Optimization of the sulfonamide moiety for the 4-isoxazolylphenylalanyltryptophan derivatives**



| No | R | Ki ($ET_B$) (nM) | Ki ($ET_A$) (nM) | Ratio A/B |
|---|---|---|---|---|
| 42 | ethyl | 0.36 | 5.3 | 14 |
| 43 | vinyl | 0.14 | 1.9 | 14 |
| 44 | **n-propyl** | **0.24** | **2.2** | **9** |
| 45 | 1-propenyl | 0.39 | 3.9 | 10 |
| 46 | 2-propenyl | 0.23 | 0.89 | 4 |
| 47 | iso-propyl | 0.38 | 10 | 26 |
| 48 | **n-butyl** | **0.24** | **2.9** | **12** |
| 49 | ethoxyethyl | 0.23 | 6.0 | 26 |
| 50 | phenyl | 0.34 | 2.3 | 7 |
| 51 | benzyl | 0.50 | 5.4 | 11 |
| 52 | L-749,329 | 150 | 5.0 | 0.03 |
| 53 | SB 209670 | 1.2 | 0.21 | 0.18 |

All alkyl and aromatic sulfonamide derivatives exhibited greatly high affinity in both $ET_A$ and $ET_B$ receptors. It should be noted here that the aromatic analog **50** (IRL 2847) is easily metabolized in animal plasmas to cleave the sulfonamide bond [degradation rate: 57% (rat), 61% (mouse)]. On the other hand, the alkyl sulfonamide analogs, such as n-butyl analog **48**, are rather stable in the rat and human plasmas.

Base on the biological and chemical properties of all sulfonamide analogs synthesized, the n-propyl and n-butyl sulfonamide were selected for further optimization study. The vinyl (**43**) and propenyl (**45**, **46**) also exhibited potent activities, but these were excluded due to the chemically stability issues. The in-house results of competitors' compounds (**52**, **53**) indicate that our endothelin antagonists have rather balanced $ET_A$ and $ET_B$ antagonistic activity compared with the competitors' compounds.

### 3. First approach to synthesize the sulfonamaide derivatives, IRL 2674.

First syntheses of the sulfonamide analogs are shown in Figure 3. As illustrated in Figure 17 of Chapter 1, the enantiomeric pure carboxylic acid analogs, IRL 2659 (**29**) and IRL 2660, were synthesized by an MPLC separation of the corresponding racemic ester analogs followed by saponification of each enantiomeric pure esters using lithium hydroxide. The (R, S)-isomer, IRL 2659 (**29**), displayed much better activity than the (S, S)-isomer, IRL 2660. The relationships between the stereo configurations and the biological activities of IRL 2659 derivatives (**29**) are consistent with the results for SAR study of the phenylalanine analogs (IRL 1841) and the biphenylalanine analogs (IRL 2500). In all cases observed, the (R, S)-isomer was much superior to other isomers for both $ET_A$ and $ET_B$ receptor affinities.

The each carboxylic acid analogs were converted to *N*-hydroxysuccinimide analogs, which were then treated with a potassium salt of benzenesulfonamide to give acylated phenylsulfonamide analogs, IRL 2674 (**41**) and IRL 2971. The HPLC analysis of IRL 2674 (**41**) using a chiral column showed two peaks at 12.2 min and 16.8 min with 6:4 ratio (Chiralcel OD 4.6 x 250 mm, solvent iso-propanol/ hexane, 35: 65 with 0.1% TFA). IRL 2971 was also analyzed by HPLC under the same conditions,

and showed separated peaks at different retention times, 9.5 min and 33 min. These results indicate that the possible 4 isomers are clearly separated in the chiral column HPLC analysis, and the chiral center on the tryptophan moiety instead of phenylalanine moiety was racemized during the sulfonamide formation steps.

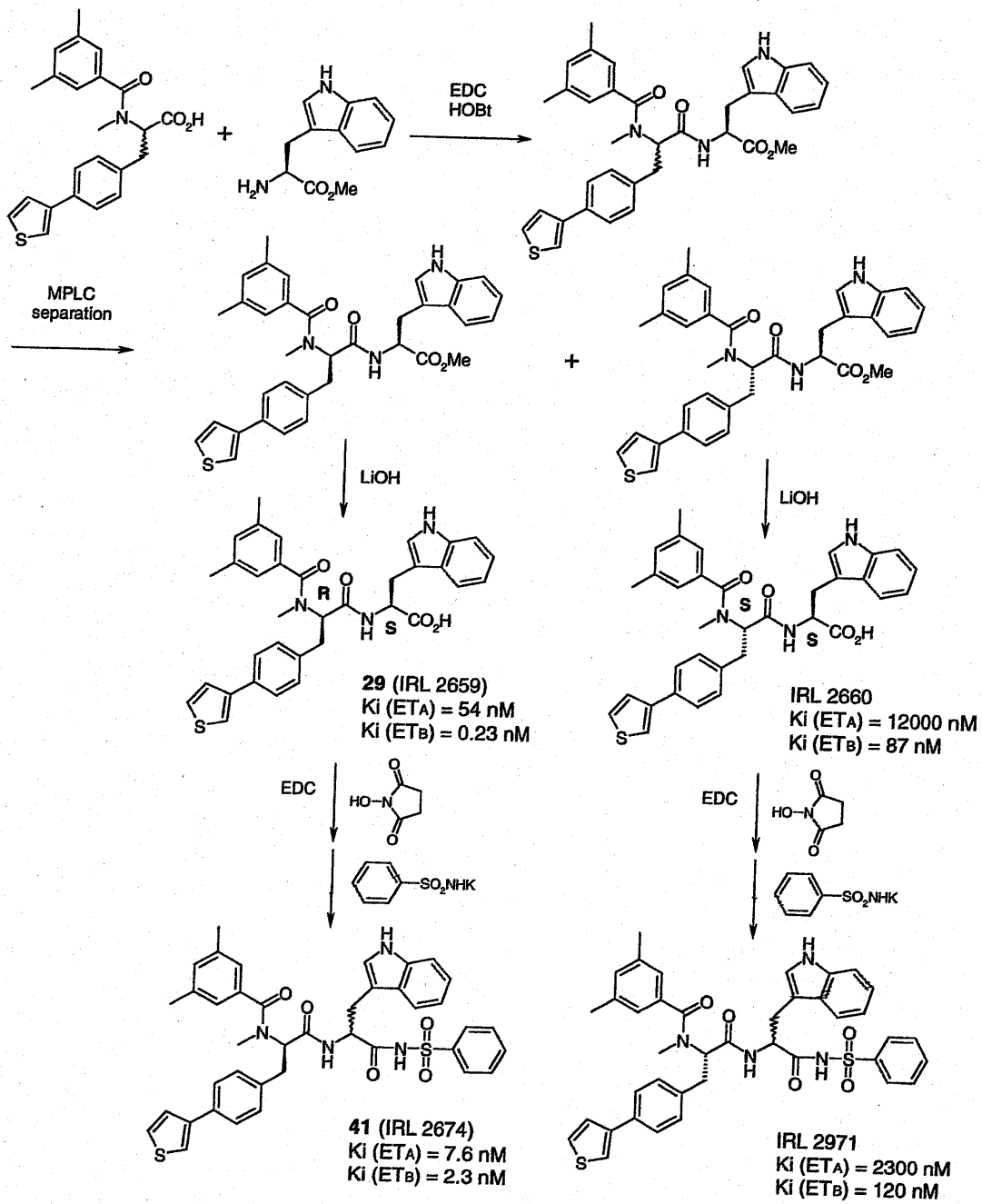



**Figure 3. Synthesis of the sulfonamide analog, IRL 2674**

The biological activities also support the evidence that the chiral center on the tryptophan moiety was racemized during the sulfonamide formation steps. If the phenylalanine moiety was racemized, IRL 2674 (**41**) and IRL 2971 should have similar binding affinity. However, IRL 2674 (**41**) is about 300 times more potent than IRL 2971 for the $ET_A$ receptor binding affinity, indicating that the racemization occur on the tryptophan moiety. A mechanism of the complete racemization only on the tryptophane moiety can be explained by the oxazolone formation as shown in Figure 4. As already discussed in Figure 13 of Chapter1, this behavior has been known for long in peptide chemistry, the razemization in peptide coupling of *N*-acyl-amino acids being ascribed to oxazolone (azlactone) intermediate.[8] Therefore, alternative approach for the acyl-sulfonamide formation reaction is necessary to prevent the racemization on the asymmetric center.

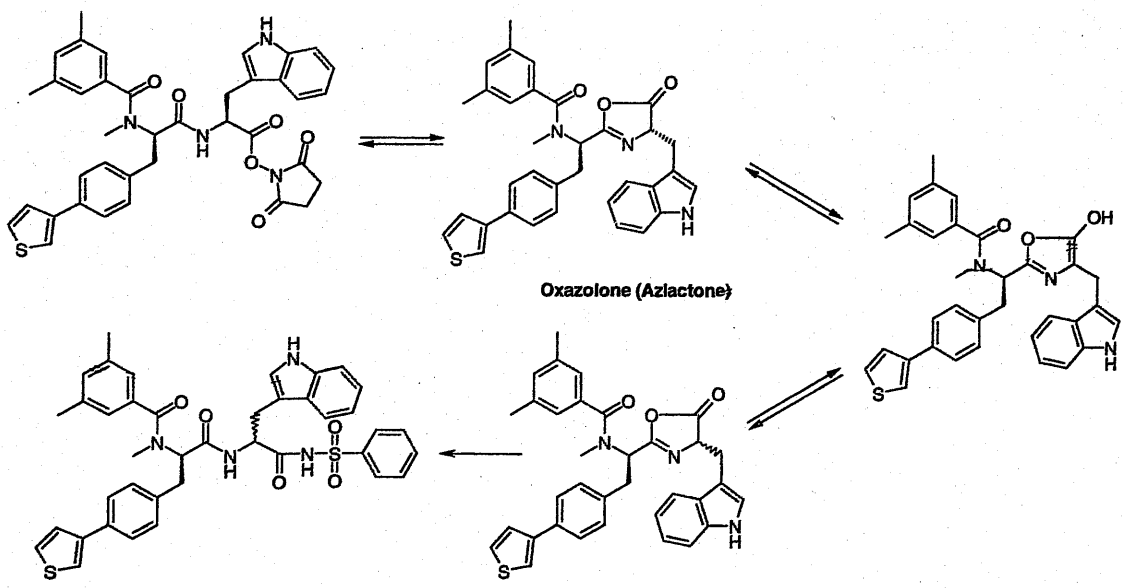



**Figure 4. Possible mechanism for the racemization in the sulfonamide formation.**

All attempts for synthesis of the optically pure enantiomers of IRL 2674 (**41**) were unsuccessful. Thus, the biological activities of all compounds listed in Table 1 were evaluated as a mixture of diastereomers. In order to confirm the endothelin receptor binding affinities of each enantiomers, IRL 2674 (**41**) was separated by preparative HPLC (Shimadzu Shim-pak ODS 50 x 250 mm, $CH_3CN/ H_2O$ = 68/32 with 0.1% TFA), as shown in Figure 5. One enantiomer, IRL 2974, exhibited higher affinity than the other one, especially for the $ET_A$ receptor. While the absolute configurations were not determined at this point, the biological results suggest that the more potent compound, IRL 2974, is (R,S)-isomer and the less potent compound, IRL 2973, is (R,R)-isomer.

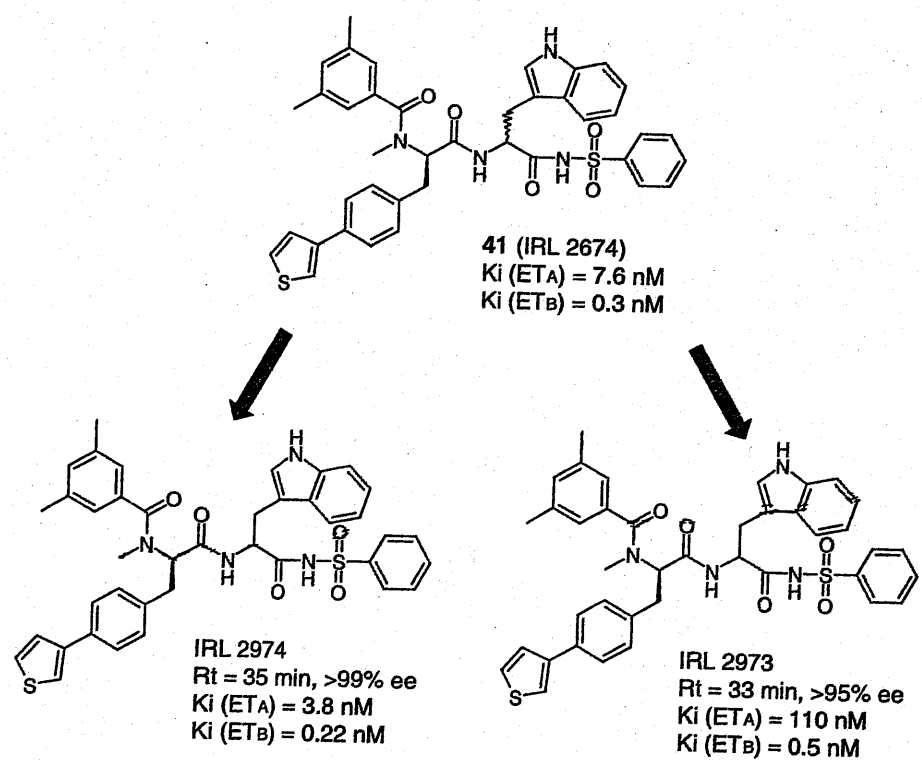



**Figure 5. Separation of each enantiomers of IRL 2674 by HPLC**

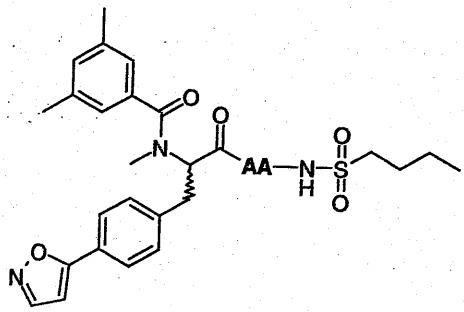
**4. Optimization of the right-hand side, tryptophan moiety.**

As described in Chapter 1, these endothelin antagonists were identified from the two approaches, studies on the potent endothelin agonist and the screenings of the substance P antagonists. The studies on the potent endothelin agonist, ET-1 and IRL 1543 suggested that both phenylalanine and tryptophan moieties are important for the affinity with endothelin receptors. Based on this hypothesis, the greatly potent $ET_B$ receptor selective antagonists and $ET_A$ /$ET_B$ dual antagonists have successfully been discovered. Therefore, our main efforts focused on the modification with keeping the phenylalanine-tryptophan core structure. However, it was found from the further extensive SAR study that the tryptophan moiety on the right-hand side could be replaced with various amino acids to maintain the endothelin binding affinities when the sulfonamide moiety was attached (Table 2). The SAR study on the optimization of the right-hand amino acid moiety was implemented for the 4-isoxazolylphenylalanine moieties as the left-hand side, all compounds listed Table 2 were synthesized as a mixture of diastereomers.

While the tryptophan derivative **54** was the most potent $ET_B$ selective antagonist ($ET_A/ET_B$ = 12) among all compounds synthesized, it is of great interest to find that the β-branched α-amino acids (**59-62**) have well balanced binding affinities between $ET_A$ and $ET_B$ receptors. It was also found that the β-branched α-amino acids (**59-62**) are much more stable to rat and mouse plasmas metabolism compared than the other analogs, such as the tryptophan analog **54**, suggesting that the pharmacokinetic profiles are improved by adding the β-branched alkyl moiety on the right-hand amino acid group. **Among them, the valine derivative, IRL 3461 (59), exhibited the highest binding affinity to both $ET_A$ and $ET_B$ receptors with good stability against plasmas**

of different animal species.

**Table 2. SAR for the right-hand amino acid moiety of the 4-isoxazolylphenylalanine derivatives**



| No | AA [a] (amino acid) | Ki ($ET_B$) (nM) | Ki ($ET_A$) (nM) | Ratio A/B |
|---|---|---|---|---|
| 54 | tryptophan | 0.24 | 2.9 | 12 |
| 55 | 2-naphthylalanine | 0.59 | 3.1 | 5 |
| 56 | ethylglycine | 0.7 | 5.5 | 8 |
| 57 | methionine | 0.49 | 5.2 | 11 |
| 58 | leucine | 1.3 | 8.0 | 6 |
| 59 | valine [b] | 1.2 | 1.8 | 1 |
| 60 | isoleucine | 3.5 | 6.9 | 2 |
| 61 | cyclohexylglycine | 3.1 | 8.6 | 3 |
| 62 | threonine | 3.2 | 9.1 | 3 |

a) (L)-isomer, b) IRL 3461

## 5. Development of a mild sulfonamide formation reaction to avoid the racemization.

Since the coupling reaction of the *N*-hydroxysuccinimide ester with potassium salt of sulfonamides always resulted in the racemization of the asymmetric center on the phenylalanine moiety as shown in Figure 3, it was necessary to develop a novel

sulfonamide formation reaction without racemization. In general, *N*-acyl-amino acid is more easily racemized than *N*-alkoxycarbonyl-amino acids, such as Boc (*tert*-butoxycarbonyl) and Cbz (benzyloxycarbonyl) protection. It is known that the racemization in peptide couplings of *N*-acyl-amino acids is ascribed to a formation of oxazolone (azalactone) intermediates,[8] as exemplified in Figure 4. Thus, our efforts focused on the formation of the acyl-sulfonamide bond using Boc-protected amino acids under a novel and mild reaction conditions to avoid the racemization.

Since the amino moiety of the sulfonamides has poor nucleophilic property, the nucleophilicity has to be enhanced for the mild the acyl-sulfonamide bond formation. It has long been known that a wide variety of covalent halides react with silylamines under mild conditions to cleave the silicon-nitrogen bond (eq 1). This reaction has been extensively used in organometallic synthesis. It has also reported the similar reaction can be used for a mild amide bond formation (eq 2).[9]

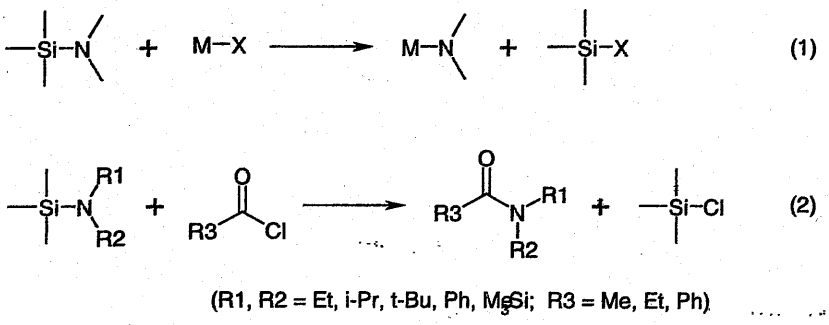



**Figure 6. Reaction of *N*-silylated amine with electrophiles**

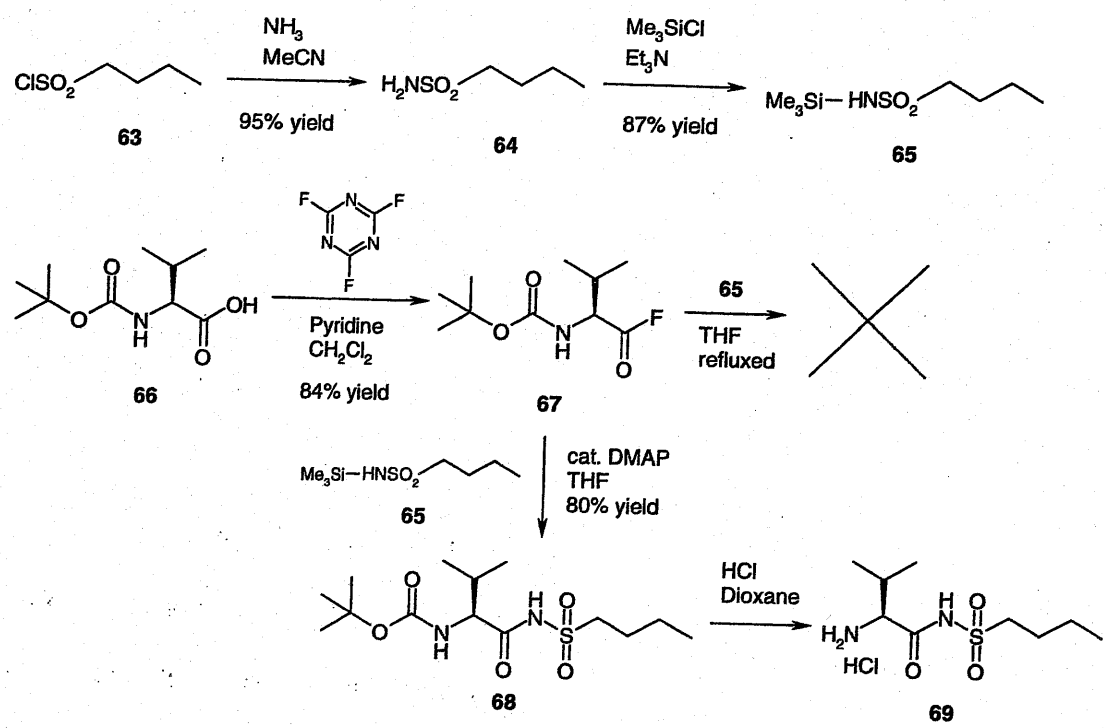



**Figure 7. Synthesis of the optically pure acyl-sulfonamide**

This methodology prompted me to apply it to the acyl-sulfonamide bond formation. The alkylsilyl-sulfonamide analog (**65**) was synthesized according to the reported procedure (Figure 7),[10] and these acy-sulfonamide analogs are turned our to be stable enough under the amide bond formation conditions. The amino acid analog (**66**) was converted into the acyl-fluoride analog (**67**) because Si-F bond (135 kcal/mol) has rather high strength compared with other silyl single bonds, such as Si-Cl (90 kcal/mol), Si-Br (70 kcal/mol) and Si-O (110 kcal/mol), [11] which should facilitate the bond forming reaction. The Boc-protected valine fluoride (**67**) was prepared using cyanuric fluoride without the racemization according to the reported procedure.[12] Although the reaction of the acyl fluoride (**67**) with the silyl-sulfonamide analog (**65**) just under refluxing conditions was unsuccessful, addition of a catalytic amount of 4-dimethylaminopyridine (DMAP) led to an acceleration of the reaction even at room

temperature to give the acyl-sulfonamide derivative (**68**) in good yield. Besides, racemization of the amino acid moiety was not observed in this reaction, indicating that the oxazolone formation in the acyl-sulfonamide bond formation owing to the mild reaction conditions and the Boc-protection group. **This novel procedure would be useful and feasible for a synthesis of the optically pure amino acid sulfonamide derivatives.**

## 6. Stereoselective synthesis of bifunctional endothelin antagonist, IRL 3630[13]

As shown in Table 2, IRL 3461 exhibited potent and balanced $ET_A$ and $ET_B$ receptor binding affinities [Ki ($ET_A$) = 1.8, Ki ($ET_B$) = 1.2]. The IRL 3461 was synthesized by a coupling reaction of the racemic 4-isoxazolylphenylalanine analog with *N*-butanesulfonyl *L*-valineamide using EDC [1-(3-dimethylaminopropyl)-ethylcarbodiimide][14] and HOBt (1-hydroxybenztriazole)[15] to give a 7:3 mixture of the two diastereomers, (D,L)-isomer **70** and (L,L)-isomer **71** (Figure 8).

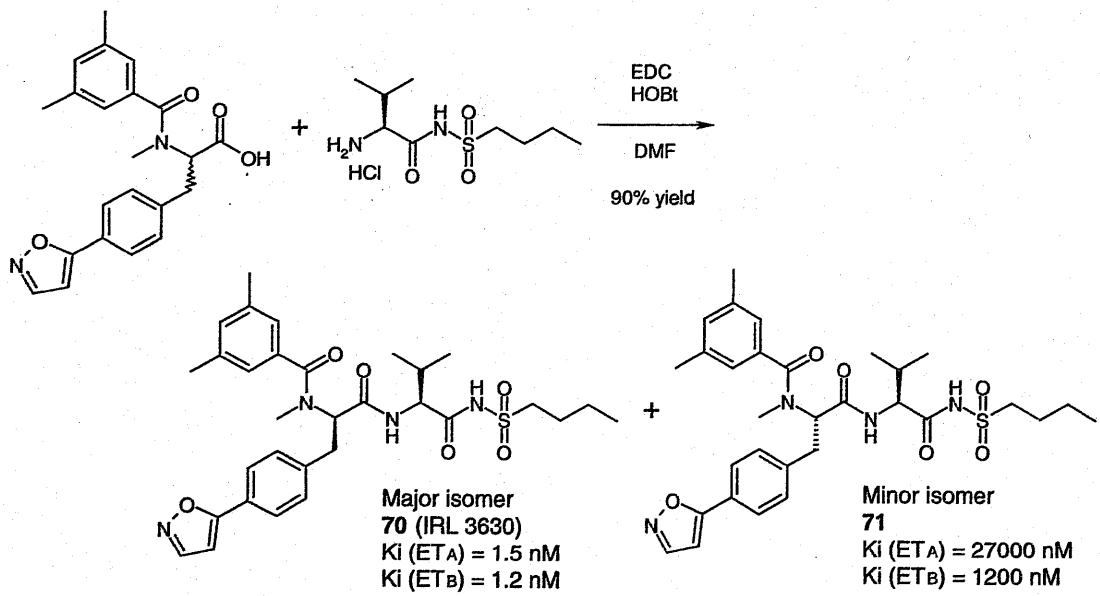



**Figure 8. Synthesis of a potent $ET_A/ET_B$ dual antagonist, IRL 3630**

The isomers were separated by HPLC (Shimadzu Shim-pak (H), hexane: isopropanol: TFA = 90: 10: 0.5, 1.0 ml/min) to give the major isomer, **70** (IRL 3630), at the retention time of 38.6 min and the minor isomer **71** at 43.8 min. The major isomer **70** exhibited greatly more potent biological activity than the minor isomer **71**, and the absolute configuration of the major isomer, IRL 3630, was determined as (D,L)-configuration by the X-ray analysis (Figure 9).

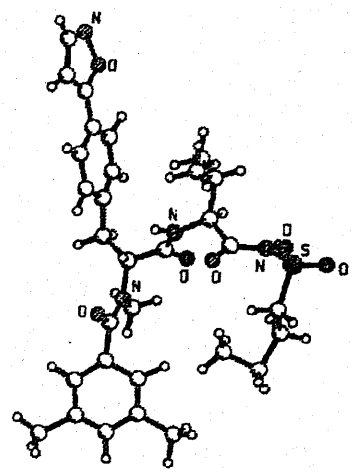


**Figure 9. X-ray structure of IRL 3630 (70)**

The unbalanced formation of the diastereomers can be explained by a kinetic resolution with *in-situ* recycling of the substance, so-called "dynamic kinetic resolution".[16] As already discussed in Figure 13 of Chapter1 and Figure 4 of this Chapter, when an activated *N*-acyl-amino acid couples with an amine nucleophile in peptide synthesis, racemization often occurs as a result of the competing intramolecular cyclization reaction to form the oxazolone (azlactone).[8] The oxazolone undergoes nucleophilic attack of amines just as does the activated ester, however, because of its chiral instability by the aromatization under these conditions, it racemizes almost completely before coupling.[17] The amide product provided from the amination of the racemic oxazolone is nevertheless not a 1:1 mixture because asymmetric induction occurs during attack by the chiral amine at the carbonyl carbon atoms of the oxazolone enantiomers.[18] The same phenomenon was observed in the amide bond formation between of the racemic 4-isoxazolylphenylalanine analog with *N*-butanesulfonyl *L*-valineamide to give a 7:3 mixture of the two diastereomers (Figure 10).

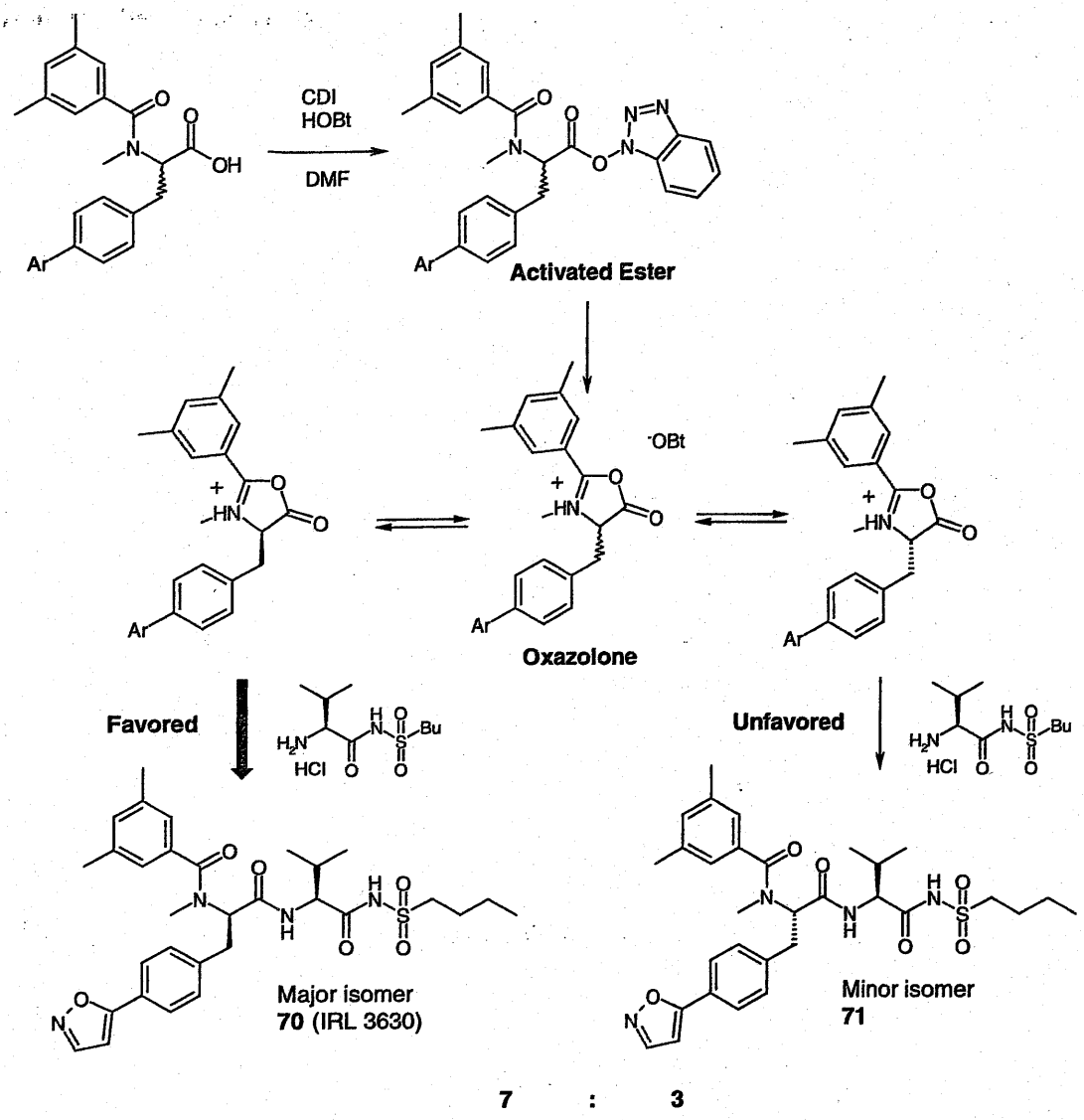



**Figure 10. Asymmetric induction in the amide formation**

Figure 11 illustrates the presumed mechanism to explain the favorable formation of the (D,L)-isomer. In the favored stereo configuration, the (L)-valine analog can readily approach to ~~the oxazoline~~ analog from the opposite side of the 4-isoxazolylphenyl moiety. On the other hand, in the unfavored one, when the (L)-valine analog approaches to the opposite side of the 4-isoxazolylphenyl moiety as well, there appears to be a steric collision between the (L)-valine analog with the 3,4-

dimethylbenzene ring. As a result, the (D,L)-isomer **70** (IRL 3630) was predominantly formed over the (L,L)-isomer **71**.

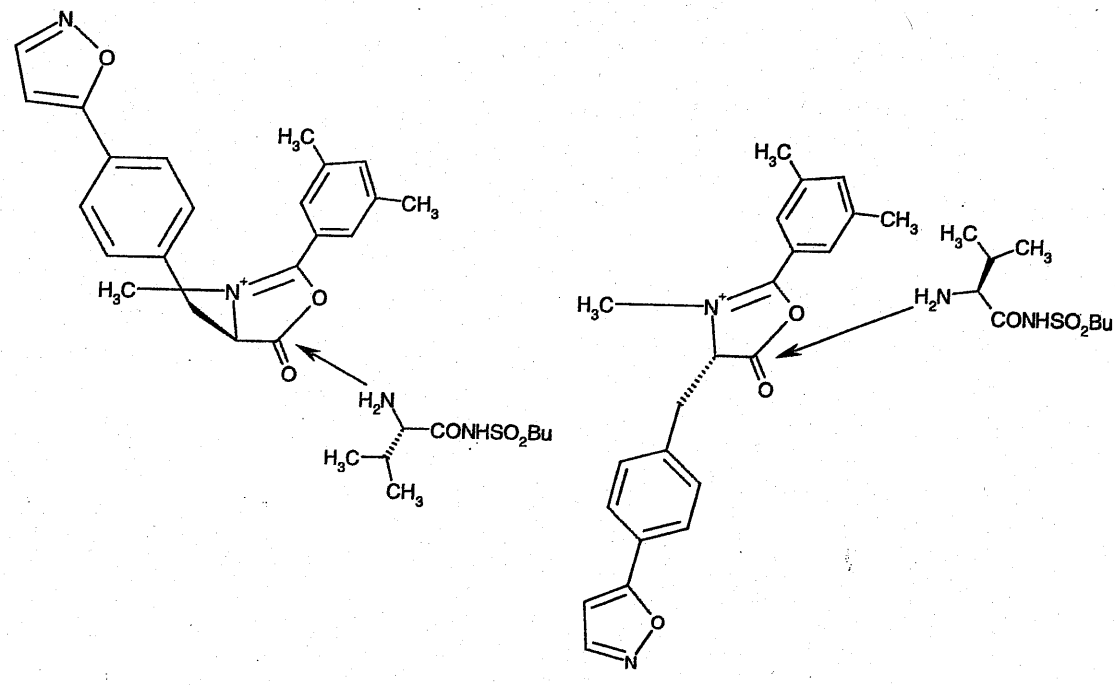
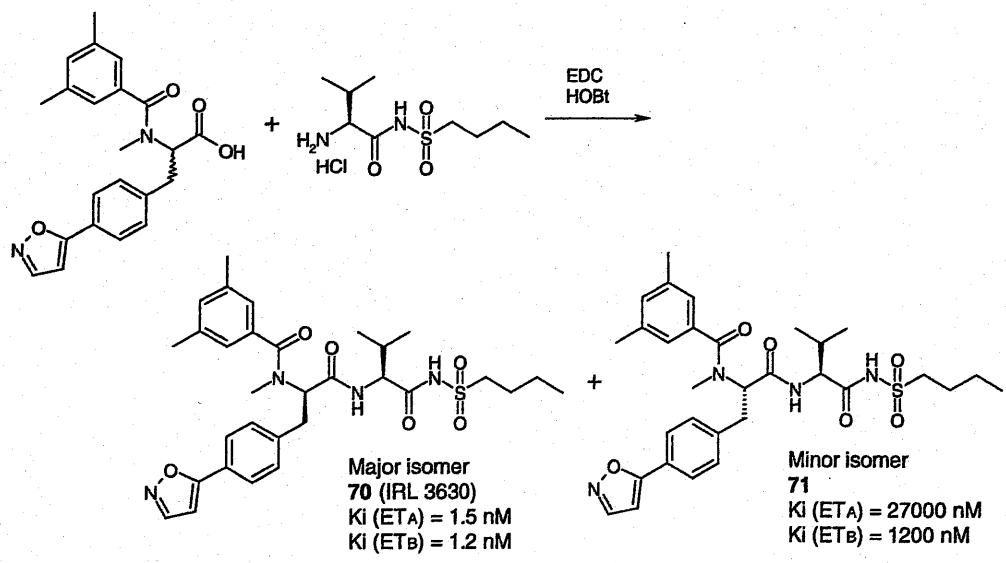



**Figure 11. Mechanism for the asymmetric induction via the oxazolone formation**

In the course of examining the reaction conditions to improve the diastereoselectivity, it was found that a two-phase solvent system ($CH_2Cl_2$ –$H_2O$) using EDC and HOBt as the coupling reagents remarkably accelerated the isomerization of the 4-isoxazolylphenylalanine chirality, thereby the (D,L)-isomer **70** (IRL 3630) and the (L,L)-isomer **71** were formed in a 12:1 ratio in 82% yield (Table 3). Moreover, a single crystallization of the crude product provided the pure (D,L)-isomer **70** (IRL 3630), which was the most potent isomer of all the stereo isomers.

**Table 3. Diastereoselectivity in the coupling reaction using EDC and HOBt**




| solvent | Ratio of diastereomers **70 : 71** | Yield % |
|---|---|---|
| DMF | 7 : 3 | 90 |
| $CH_2Cl_2 - H_2O$ | 12 : 1 | 82 |

The possible mechanism for the high asymmetric induction in the two-phase solvent system is depicted in Figure 12. At the very initial stage of this reaction, the water-soluble reagents, EDC, HOBt and the (L)-valine sulfonamide, should be mainly dissolved in the aqueous phase, and the hydrophobic 4-isoxazolylphenylalanine analog exists in the organic phase. In the next stage, 4-isoxazolylphenylalanine analog is allowed to react with EDC and HOBt on the boundary layer to form the oxazolone analog, which still has highly hydrophobic property and is dissolved in the organic phase. The oxazolone analog has rather less opportunities to react with the (L)-valine sulfonamide in the two-phase system compared with the reaction in the single-phase system like DMF. Thus, the slow reaction of the oxazolone analog results in an acceleration of the isomerization of the 4-isoxazolylphenylalanine chirality in the

organic phase, and the stereoselective coupling reaction to provide the (D,L)-isomer **70** (IRL 3630) predominantly. Benoiton *et al.*[19] and Miyazawa *et al.*[20] have separately studied the solvent effect on the asymmetric induction in the amide formation of the oxazolones from racemic *N*-benzoylamino acids with (L)-amino acid esters, and have similarly showed that synthesis of the (D,L)-isomers is more predominantly observed in a non polar solvent like $CH_2Cl_2$ rather than in a polar solvent like DMF, suggesting that $CH_2Cl_2$ is a suitable solvent to form the (D,L)-dipeptide isomer in this type of reactions. These results are totally consistent with our observations in the two-phase coupling reaction using $CH_2Cl_2$ and water.

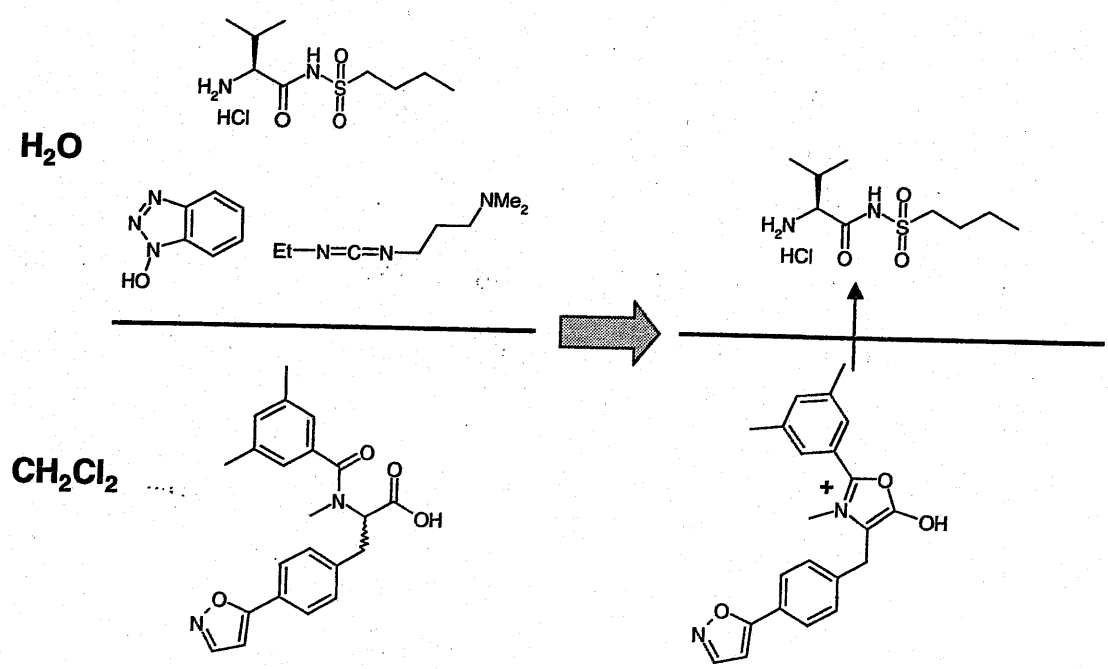



**Figure 12. Possible mechanism for the asymmetric induction in the two-phase solvent.**

## 7. Conclusions

The extensive SAR (structure-activity relationships) study around the $ET_B$

receptor selective antagonists successfully led to a discovery of the balanced $ET_A$ and $ET_B$ dual antagonists incorporating the acyl-sulfonamide moiety as the characteristic structure. While the initial synthetic approach to introduce the sulfonamide moiety into amino acids gave rise to a complete racemization on the asymmetric center, the acyl-sulfonamide formation reaction was improved to provide the single isomer in a highly diastereomeric excess. The new procedure was applied to a synthesis of a single isomer of the most potent $ET_A$ and $ET_B$ dual antagonist, IRL 3630, by a kinetic resolution with *in-situ* recycling of the substrate. With an increase in evidence for the involvement of both $ET_A$ and $ET_B$ receptors in the asthmatic responses, these novel $ET_A$ and $ET_B$ dual antagonists will be useful tools for demonstrating the role of both receptors in physiological studies for asthma and other inflammatory diseases.

## 8. Experiments

### General

$^1H$ NMR spectra were recorded using Bruker DRX-400 (400 MHz for $^1H$) spectrometer in $CDCl_3$ or DMSO-d6. Chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as an internal standard at zero ppm. Coupling constant (J) are given in hertz and the abbreviations s, d, t, q, and m refer to singlet, doublet, triplet, quartet and multiplet, respectively. The abbreviation "br" refer to "broad". Mass spectroscopy data were recorded on a FINNIGAN MAT 95. Melting points are uncorrected. TLC was performed on a precoated silica gel plate (Merck silica gel 60 F-254). Silica gel (WAKO-gel C-200 (75-150 $\mu$ m))was used for all column chromatography separations. All chemicals were reagent grade and were purchased

from Sigma-Aldrich, Wako pure chemical industries, Ltd., Tokyo kasei kogyo co. Ltd.

**_N_-Benzenesulfonyl-[_N_-(3,5-dimethylbenzoyl)-_N_-methyl-3-(biphenyl-4-yl)-(D)-alanyl]-(D,L)-tryptopanamide (40)**

A solution of the [_N_-(3,5-dimethylbenzoyl)-_N_-methyl-3-(biphenyl-4-yl)-(D)-alanyl]-(L)-tryptophan (**14**) (115 mg, 0.20 mmol) and _N_-hydroxysuccinimide (28 mg, 0.24 mmol) in dry DMF (2 mL) was treated with EDC [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride] (46 mg, 0.24 mmol) and stirred at room temperature overnight. The homogeneous mixture was diluted with ethyl acetate (100 mL) and washed three times with water (70 mL). The separated organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo to give the corresponding activated ester (130 mg, 97% yield) as a white foam. This material was dissolved in DMF (2 mL) and allowed to cool to 0 °C. To the sold mixture was added dropwise a solution of potassium benzenesulfonylamide (50 mg, 0.25 mmol) in DMSO (1 mL). After being stirred at 0 °C for 1 hour, the reaction mixture was diluted with ethyl acetate (100 mL) and washed three times with water (79 mL). The separated organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (ethyl acetate/ hexane, 1:1 with 0.1% TFA) to give the title compound (93 mg, 67% yield) as a white foam; FAB-MS *m/z* 711 $(M-H)^-$; NMR (DMSO-*d6*, 400 MHz) δ 7.90 (m), 7.58 (m), 7.46 (m), 7.34 (m), 7.20 (m), 7.07 (t, $J = 7$ Hz), 6.98 (m), 6.50 (m), 4.8 (s, br), 3.4-2.4 (m), 2.19 (s).

**_N_-Benzenesulfonyl-{_N_-(3,5-dimethylbenzoyl)-_N_-methyl-3-[4-(3-thienyl)phenyl]-(D)-alanyl}-(D,L)-tryptopanamide (41)**

Following the procedure for compound **40**, {_N_-(3,5-dimethylbenzoyl)-_N_-methyl-

(D)-3-[4-(3-thienyl)phenyl]-alanyl}-(L)-tryptophan (**29**) (61 mg, 0.11 mmol) was coupled with *N*-hydroxysuccinimide (61 mg, 0.14 mmol) to give the activated ester as a white foam. This material was dissolved in DMF (2 mL) and allowed to cool to 0 °C. To the sold mixture was added dropwise a solution of potassium benzenesulfonylamide (40 mg, 0.20 mmol) in DMSO (1 mL). After being stirred at 0 °C for 1 hour, the reaction mixture was diluted with ethyl acetate (100 mL) and washed three times with water (79 mL). The separated organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (ethyl acetate/ hexane, 1:1 with 0.1% TFA) to give the title compound (47 mg, 60% yield in 2 steps) as a white foam; FAB-MS *m/z* 717 $(M-H)^-$.

**_N_-Benzenesulfonyl-{_N_-(3,5-dimethylbenzoyl)-3-[4-(5-isoxazolyl)-phenyl-(D)-alanyl]-(D,L)-tryptophanamide (50)**

To a solution of a {*N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)-phenyl]-(D)-alanyl}-(L)-tryptophan **20** (340 mg, 0.602 mmol) in ethyl acetate (3.5 mL) under a nitrogen atmosphere were added triethylamine (0.096 mL, 0.69 mmol) and isopropyl chloroformate (0.077 mL, 0.65 mmol), and the stirring was continued at room temperature for 1 hour. The resulting mixture was filtered under a nitrogen atmosphere, and the filtrate was added dropwise to a solution of potassium benzenesulfonamide (153 mg, 0.78 mmol) in DMSO (7 mL). After being stirred at room temperature for 6 hours, the mixture was partitioned between ethyl acetate and water. The separated organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude product was purified by preparative thin layer chromatography on silica gel (ethyl acetate/ hexane, 2:1 with 0.1% acetic acid) afforded the title compound (245 mg, 58% yield); FAB-MS *m/z* 702 $(M-H)^-$.

***N*-Butanesulfonyl-{*N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)phenyl]-(D)-alanyl}-(L)-valineamide (70)**

***N*-Butanesulfonyl-valineamide hydrochloride (69)**

Ammonia gas was bubbled into a solution of 1-butanesulfonyl chloride **(63)** (2.0 g, 13 mmol) in acetonitrile (20 mL) at room temperature for 30 min. The mixture was filtered, and the filtrate was concentrated in vacuo. The residue was diluted with water (20 mL) and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo to give 1-butanesulfonylamide **(64)** (1.7 g, 95% yield) as colorless oil.

A solution of 1-butanesulfonamide obtained above **(64)**, triethylamine (1.84 mL, 13.2 mmol) and chlorotrimethylsilane (1.60 mL, 12.6 mmol) in toluene (30 mL) was heated at reflux under a nitrogen atmosphere for 4 hours. The mixture was filtered, and the filtrate was concentrated in vacuo to give *N*-butanesulfonyl-*N*-trimethylsilylamide **(65)** (2.46 g, 87% yield) as brown oil.

To a cold (-15 °C) solution of t-butoxycarbonylvaline **(66)** (1.0 g, 4.6 mmol) and pyridine (0.39 mL) in methylene chloride (11 mL) was added dropwise cyanuric fluoride (1.87 mL, 13.8 mmol), and the mixture was stirred at –15 °C for 1 hour. The reaction mixture was diluted with methylene chloride and crashed ice, and filtered on Celite. The filtrate was diluted with waterand extracted with methylene chloride. The organic layer was separated, dried over $MgSO_4$, filtered and concentrated in vacuo to give tert-butoxyvaline fluoride **(67)** (0.85 g, 84% yield), which was used for the next step without further purification..

To a solution of *N*-butanesulfonyl-*N*-trimethylsilylamide **(65)** (1.72 g, 8.28 mmol) and *tert*-butoxycarbonylvaline fluoride **(67)** (1.01 g, 4.6 mmol) in THF (25 mL) was

added 4-dimethylaminopyridine (220 mg, 1.84 mol), and the stirring was continued for 1 hour. The mixture was diluted with 10% aqueous citric acid solution, and extracted with ethyl acetate. The organic layer was washed with water and brine successively, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (ethyl acetate/ hexane, 3:1) to give *N*-butanesulfonyl-(t-butoxycarbonyl)valineamide **(68)** (1.11 g, 72% yield).

To a solution of *N*-butanesulfonyl-(t-butoxycarbonyl)valineamide **(68)** (0.70 g, 2.08 mmol) in 1,4-dioxane (25 mL) was added 4N HCl/1,4-dioxane (30 mL) at room temperature under a nitrogen atmosphere, and the mixture was stirred for 8 hours. The reaction mixture was concentrated in vacuo, and the solid residue was washed with ether and dried to give *N*-butanesulfonyl-valineamide hydrochloride **(69)** (0.58 g, quant.) as a white solid.

**Coupling reaction to synthesize compound (70)**

**Procedure 1 (DMF)**

To a cold (0 °C) solution of 1-hydroxybenzotriazole (2.57 g, 19.02 mmol), *N*-butanesulfonyl-(L)-valineamide hydrochloride **(69)** (2.94 g, 10.76 mmol) and *N*-(3,5-dimethylbenzoyl)-*N*-mehtyl-3[4-(5-isoxazolylphenyl)-(D,L)-alanine (4.10 g, 10.82 mmol), prepared for a synthesis of compound **20** in Chapter 1, in DMF (100 mL) under a nitrogen atmosphere was added dropwise 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (1.97 mL, 10.75 mmol) with stirring. After being stirred for 2 hours, the reaction mixture was slowly warmed to room temperature and stirring was continued overnight. The reaction mixture was diluted with water, and extracted twice with ethyl acetate. The separated organic layer was washed with water and brine successively, dried over $Na_2SO_4$, filtered, and concentrated in vacuo to give the crude product as a 7:3

mixture of the diastereomers. The crude material was purified by HPLC (column, Shinwakako ULTRON VX-SIL 5 μm silica gel 250 x 50 mm; hexane/ isopropanol/ TFA, 90:10:0.5; 40 ml/min, Rt = 59 min) to give *N*-Butanesulfonyl-{*N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)phenyl]-(D)-alanyl}-(L)-valineamide **70** (3.46 g, 5.80 mmol) as a colorless crystal.

**Procedure 2 (DMF)**

To a cold (0 °C) solution of 1-hydroxybenzotriazole (2.57 g, 19.02 mmol), *N*-butanesulfonyl-(L)-valineamide hydrochloride (**69**) (2.94 g, 10.76 mmol) and *N*-(3,5-dimethylbenzoyl)-*N*-mehtyl-3[4-(5-isoxazolylphenyl)-(D,L)-alanine (4.10 g, 10.82 mmol), prepared for a synthesis of compound **20** in Chapter 1, in $CH_2Cl_2$ (30 mL) and water (30 mL) was added dropwise 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (1.97 mL, 10.75 mmol) with stirring. After being stirred for 2 hours, the reaction mixture was slowly warmed to room temperature and stirring was continued overnight. The organic layer was separated and the aqueous layer was extracted with CH2Cl2. The combined organic layer was washed with water and brine successively, dried over $MgSO_4$, filtered, and concentrated in vacuo to give the crude product as a 12:1 mixture of the diastereomers. The crude product was purified by recrystallization from isopropanol to give *N*-Butanesulfonyl-{*N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)phenyl]-(D)-alanyl}-(L)-valineamide **70** (5.27 g, 82% yield) as a colorless crystal; Rf = 0.24 (chloroform/ methanol, 19:1); m.p. 194-196 °C; $[\alpha]_D$ = + 104.5 (c = 1.00, chloroform); chiral HPLC analysis Rt = 64.7 min (Daicel Chiralpak AD 4.6 x 250 mm, 1.0 ml/min, hexane/ EtOH/ TFA = 85: 15: 0.5).

**X-ray structure analysis of IRL 3630 (70)**

The relative stereochemistry was determined by a single crystal structure analysis.

A platelet shaped crystal measuring 0.32 x 0.38 x 0.01 $mm^3$ was investigated on a Enraf-Nonius CAD4 diffractometer with graphite monochromated CuKα radiation. The crystallographic data are summarized as follows: $C_{31}H_{40}N_4O_6S$, tetragonal, space group $P4_122$, a = 8.459 (1) Å, c = 89.535 (5) Å, V = 6406 (2) $Å^3$, Z = 8, Dcalc = 1.237 $g/cm^3$. A total of 4719 independent intensities were measured of which 1011 were classified as observed with $I > 3\sigma$ (1). The structure was solved by direct methods (Program SHELXS86). The structure was refined using full matrix least squares calculations with isotropic displacement parameters for S and anisotropic ones for all the other non-hydrogen atoms (Program CRYSTALS). The positions of the hydrogen atoms were calculated and not refined. The final R-factor for 157 variables was 0.086.

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# Part 2. Novel and potent IKK-β inhibitors for asthma treatment

## Chapter 3. Discovery and synthesis of novel and potent IKK-β inhibitors[1]

### 1. Introduction

Endothelin antagonists would be used as effective bronchodilating medications that open airways by relaxing the smooth muscles around the airways that tighten during an asthma attack. The bronchodilators suspend the symptoms of the asthma attack but they don't cure the airway inflammation, which is underlying in asthma. When patients need to use bronchodilators, such as β-agonist, several times in a single day, it should mean that the swelling in the airways is getting worse and another kind of medicine is needed for such chronic airway inflammation. Corticosteroids have been commonly used as the anti-inflammatory treatment of asthma. However, these drugs require inhalers to be used, and many people are concerned about side-effect of the steroids, such as osteoporosis.[2] Therefore, there is a clear significant need for novel disease modifying drugs to cure and prevent the airway inflammations for asthma.

IκB kinase β (IKK-β) is a 756 amino acid-containing serine-threonine protein kinase, and exists as the IKK-complex containing IKK-α, IKK-γ, NIK and various other known and unknown proteins in most cell types.[3] The signaling pathways from a variety of inflammatory stimuli converge on IKK-β, which is a central modulator for the activation of various inflammatory genes, as shown in Figure 1. IKK-β functions in this role by activating the transcription factor Nuclear Factor kappa B (NF-κB). Because IKK-β plays such a central role, inhibiting its activity would be an innovative

approach for a disease modifying therapy for asthma.

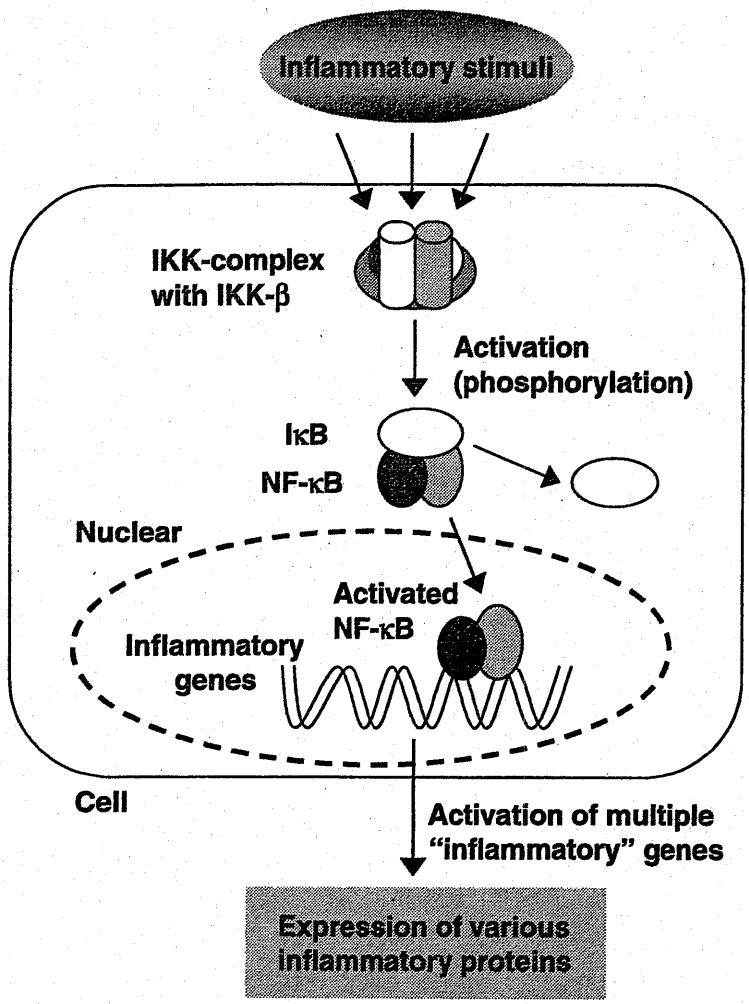



**Figure 1. Signal pathways leading to the transcription factor NF-κB**

NF-κB is an inducible transcription factor that is thought to be a pivotal target for drugs for chronic inflammatory diseases.[4] In fact, several experiments indicate that the activated NF-κB contributes to asthma pathophysiology and that inhibition of NF-κB activation will improve asthma symptoms. It has been reported that the activated NF-κB has been identified in the airways of asthma patients, but not in normal ones.[5] In

an animal model, NF-κB knock-out mice were incapable of mounting an eosinophilic airway inflammation as compared to wild-type mice.[6] **These experimental results suggest that inhibition of IKK-β and thereby inhibition of NF-κB activation can be a novel mechanism for anti-asthma drugs.**

## 2. Known IKK-β inhibitors

A number of reports on IKK-β inhibitors exist (Figure 1). The non-specific protein kinase inhibitors, quercetin and staurosporine,[7] are known to inhibit IKK-β kinase.[8] While the natural protein kinase inhibitor staurosporine and quecetin potently inhibit IKK-β, they also showed strong inhibitory activity towards other kinases. Well-known anti-inflammatory agents such as aspirin[9] and cyclopentenone prostaglandins[10] are also reported as IKK-β inhibitors. More recently, it has been reported that a novel IκB kinase (IKK) inhibitor, PS-1145, specifically blocks TNFα-induced NF-κB activation, resulting in a suppression of the release of various cytokines both *in vitro* and *in vivo*.[11]

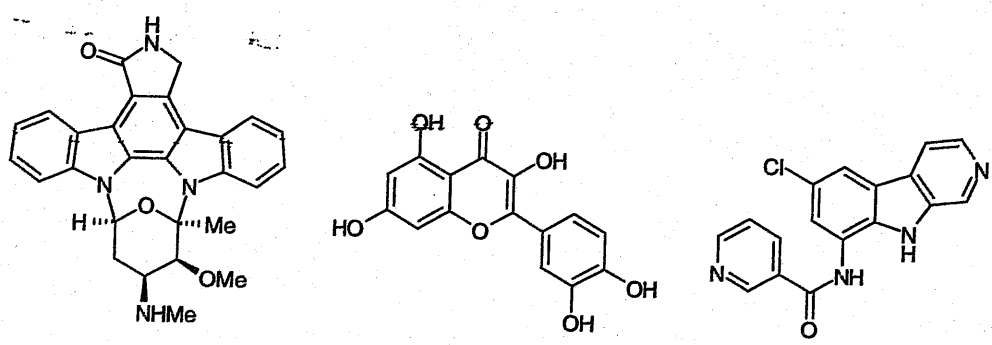
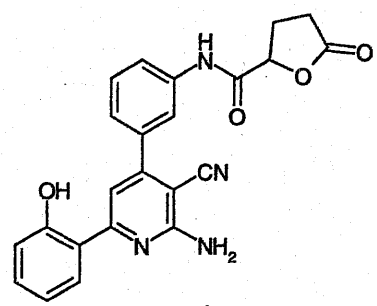



**Figure 2. Structure of known IKK-β inhibitors**

## 3. Lead Finding: Discovery of a new class of IKK-β inhibitors

From high-throughput screening of the Bayer compound library, the 2-amino-3-cyano-4-aryl-6-(2-hydroxy-phenyl)pyridine analogs **1** and **8** (shown in Scheme 1) were identified as novel potent IKK-β inhibitors (Table 1).

**Table 1. Inhibitory activities of the lead compound 1 in various kinase and cellular assays**




**1**

$IC_{50}$ = 1.5 μM (IKK-β)
$IC_{50}$ > 20 μM (IKK-α, Syk and MKK4)

| Cells/Cell Line | Stimulus | Read-Out | $IC_{50}$, μM[a] |
|---|---|---|---|
| A549 | TNFα | RANTES | 8 |
| Jurkat T-cell | anti-CD3/anti-CD28 | IL-2 | 15 |
| HEK293 | TNFα | NF-κB-Luciferase | 8 |
| Mouse B-cells | LPS/IL-4 | IgE | 0.35 |
| Human PBMCs | LPS | TNFα | 10 |

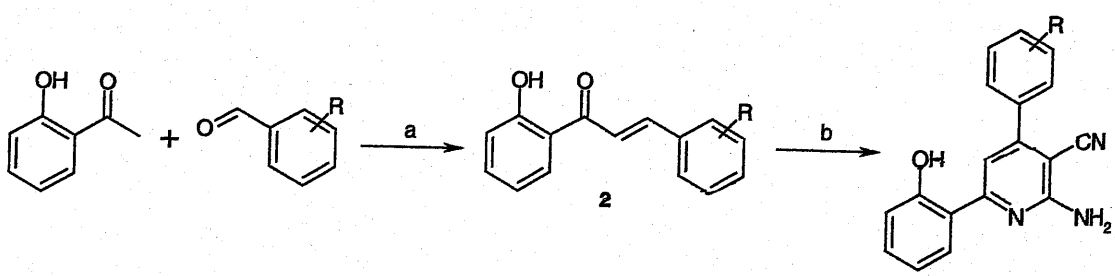
[a]Values are means of more than three experiments.

The lead compound **1** shows potent inhibitory activity against IKK-β ($IC_{50}$ = 1.5 μM) and excellent selectivity vs. other kinases such as IKK-α, Syk and MKK4 (mitogen-activated protein kinase kinase 4) ($IC_{50}$ values > 20 μM). Furthermore, this lead compound inhibits NF-κB-dependent expression of several reporter genes,

chemokines, cytokines and IgE production in various functional cellular assays (Table 1). These results suggest that compound **1** is a lead structure for specific inhibitors of the IKK-complex activated by various inflammatory stimuli and it can be therefore pharmacologically confirmed that IKK-β is a key component of the signal transduction pathway in those physiological responses.

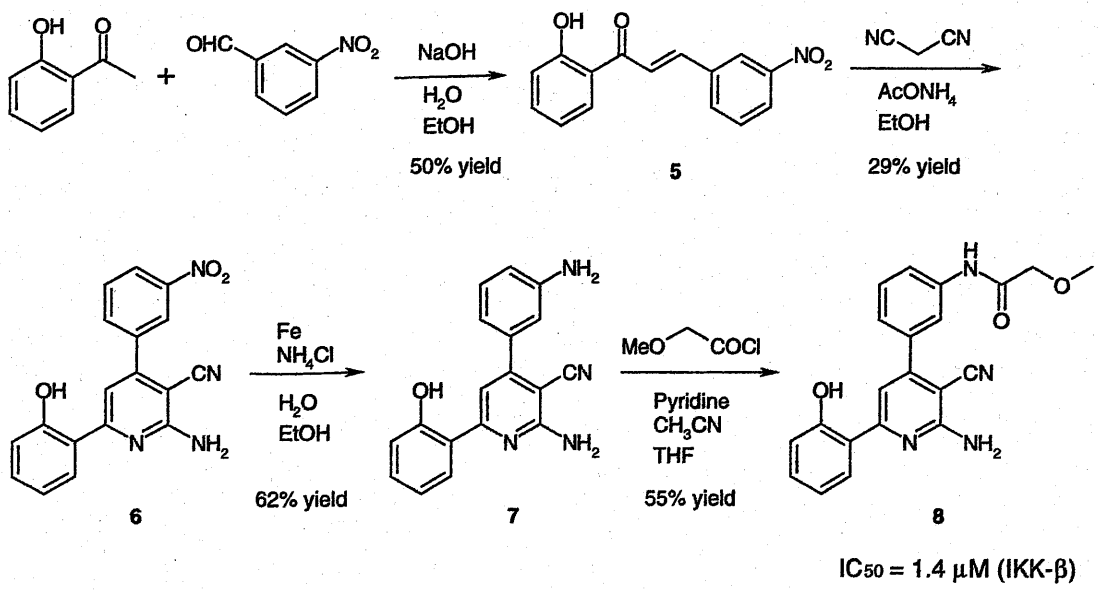
## 4. Synthesis of 2-amino-3-cyano-4,6-diarylpyridines as novel IKK-β inhibitors

Synthesis of the 2-amino-3-cyano-4,6-diarylpyridines has been reported in several publications.[12] Manna *et al.* actually synthesized the 2-amino-3-cyano-4-aryl-6-(2-hydroxyphenyl)pyridine analogs **3** and **4** by a reaction of substituted 1,3-diaryl-2-propen-1-ones **2** with malononitrile in the presence of ammonium acetate (Scheme 1).[13] Although the *o*-hydroxyacetophenone was utilized for the pyridine contraction reaction without protection of the phenolic hydroxyl group, protection became necessary for further modifications of the substituents on the aromatic group at the 4-position of the pyridine.




**3** (R = 4-$NO_2$)
**4** (R = 4-$NMe_2$)

**Scheme 1.** (a) 30% NaOH, EtOH, rt; (b) malononitrile, ammonium acetate, EtOH, reflux, 24 h, 51% (**3**, R = 4-$NO_2$), 11% (**4**, R = 4-$N(CH_3)_2$).[14]

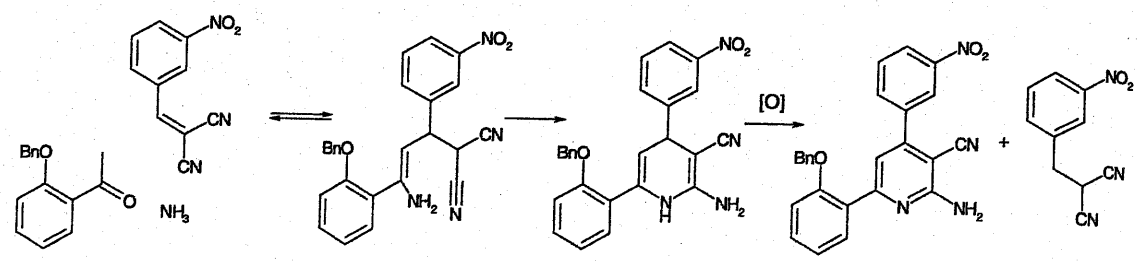



**Scheme 2. First synthesis of the 2-amino-3-cyano-4,6-diarylpyridine 8**

The high-throughput screening hit **8** was initially synthesized following the reported procedure.[13] Reaction of the 2'-hydroxyacetophenone with 3-nitro-benzaldehyde under basic conditions gave the chalcone derivative **5**, which underwent the pyridine ring formation by treatment with malononitrile and ammonium acetate. Reduction of the nitro moiety on compound **6** was carried out using iron powder in the presence of ammonium chloride as the proton source. The selective amide formation on the aniline group proceeded using acyl chloride and pyridine to give compound **8**.

When protected 2'-hydroxyacetophenones were used as the starting materials, the 2-amino-3-cyano-4,6-diarylpyridine core structures were simply constructed using a one-pot coupling reaction of four components, acetophenone, benzaldehyde, malononitrile and ammonium acetate, as exemplified in Scheme 4. Lately, the similar reaction was found in a literature precedent.[15] Various ether moieties, such as silyl, benzyl and methoxyethyl ethers, could be utilized as the protective group for the phenol of 2'-hydroxyacetophenones. Although the chemical yields for the four-components

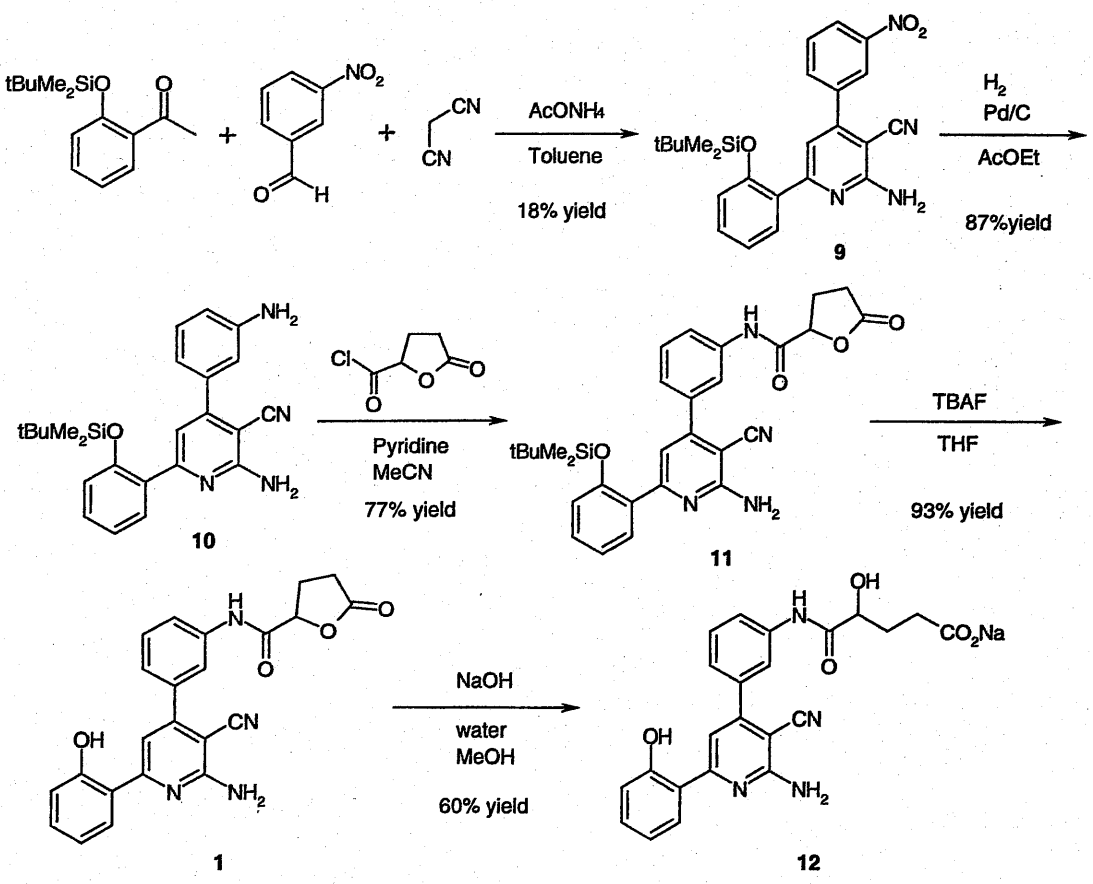
coupling reaction were not so sufficient, the straightforward method facilitates the analog synthesis for SAR study of the IKK-β inhibitors.




**Scheme 3. Reaction mechanism for the four-components coupling**

The possible reaction mechanism is illustrated in Scheme 3. The initial reaction step should be the Knoevenagel condensation between benzaldehyde and malononitrile because the Knoevenagel adduct could be actually prepared just by refluxing benzaldehyse and malononitrile in the presence of ammonium acetate.[16] Next, the Michael addition of acetophenone into the Knoevenagel adduct followed by the cyclization with ammonia probably take place to give the dihydropyridine analog. In fact, a small amount of the dihydropyridine analog was observed in the crude product. Although an oxidative step should be subsequently required to form the pyridine analog, a clear-cut oxidizing reagent is not included in the starting materials. In order to translate the oxidative step in the pyridine construction, a doubtful oxidizer, oxygen, was excluded by bubbling argon gas as much as possible. But, the pyridine construction proceeded very smoothly under such conditions, indicating that oxygen isn't taken part in the pyridine ring formation. After thorough investigations of the reaction, a saturated Knoevenagel adduct was detected in the crude product (Scheme 3).[17] In addition, the excess amount of benzaldehyde and malononitrile was necessary

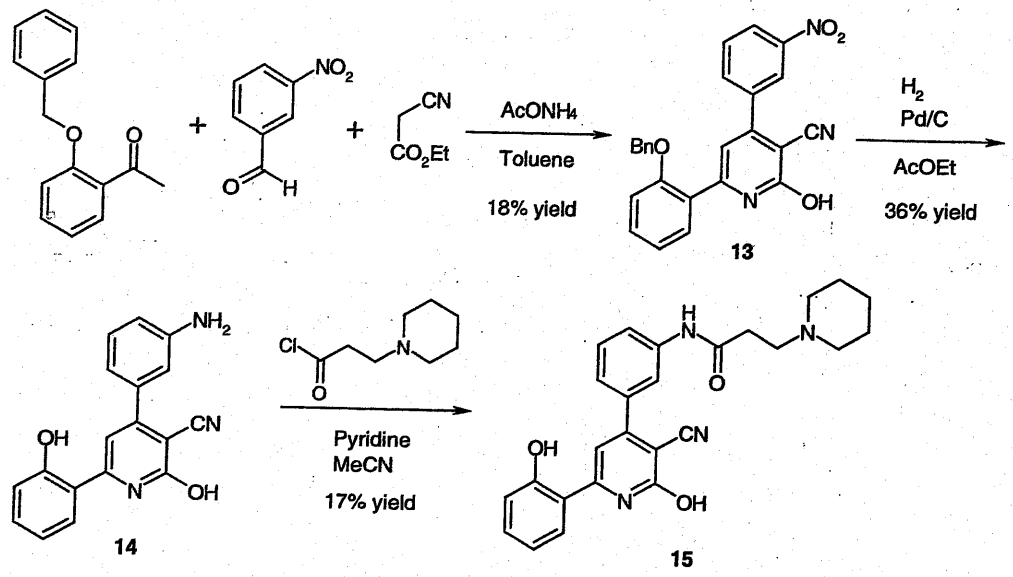
for the good chemical yields for the four-components coupling. These results suggest that the Knoevenagel adduct is not only the component for the pyridine ring but also works as an oxidizer to form the pyridine ring.




**Scheme 4. Synthesis of lead compound (1) and the water-soluble analog (12)**

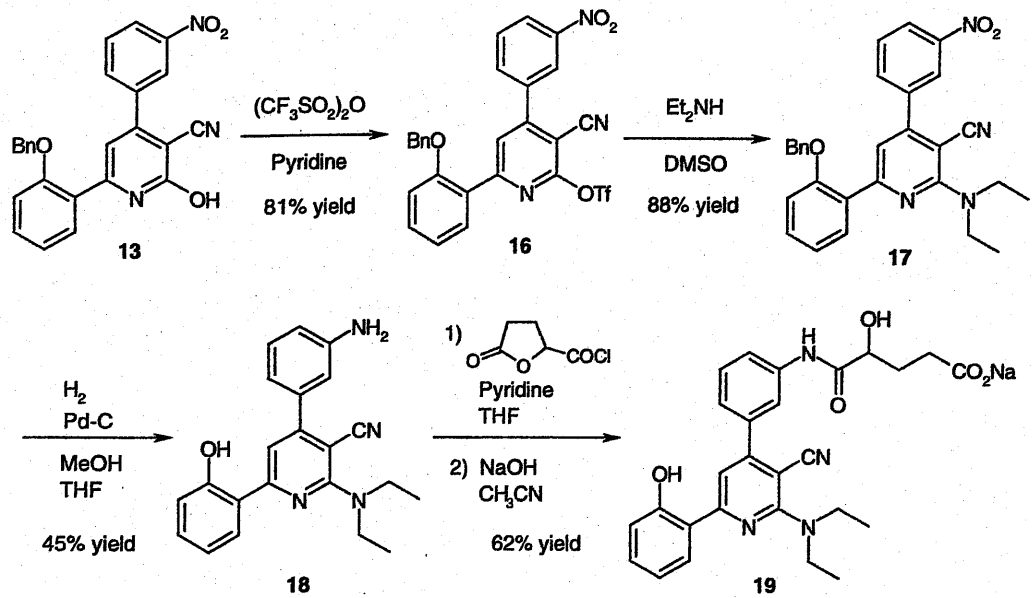
Synthesis of the first lead compound **1** is shown in Scheme 4. The lead compound **1** had limited solubility in organic solution as well as aqueous solution. In general, insoluble properties of the compound often cause problems for *in vitro* and *in vivo* assays. Hence, initial optimization efforts focused on the introduction of hydrophilic substituents to improve the aqueous solubility as well as the IKK-β inhibitory activity. In order to improve the aqueous solubility of compound **1**, it was

treated with aqueous sodium hydroxide solution to prepare its sodium salt. However, under the basic conditions, compound **1** was subject to ring opening of the lactone moiety to provide the sodium salt **12**. Moreover, the water-soluble derivative **12** was found to show better IKK-β inhibitory activity ($IC_{50}$ = 0.9 μM) than the parent compound **1** ($IC_{50}$ = 1.5 μM).

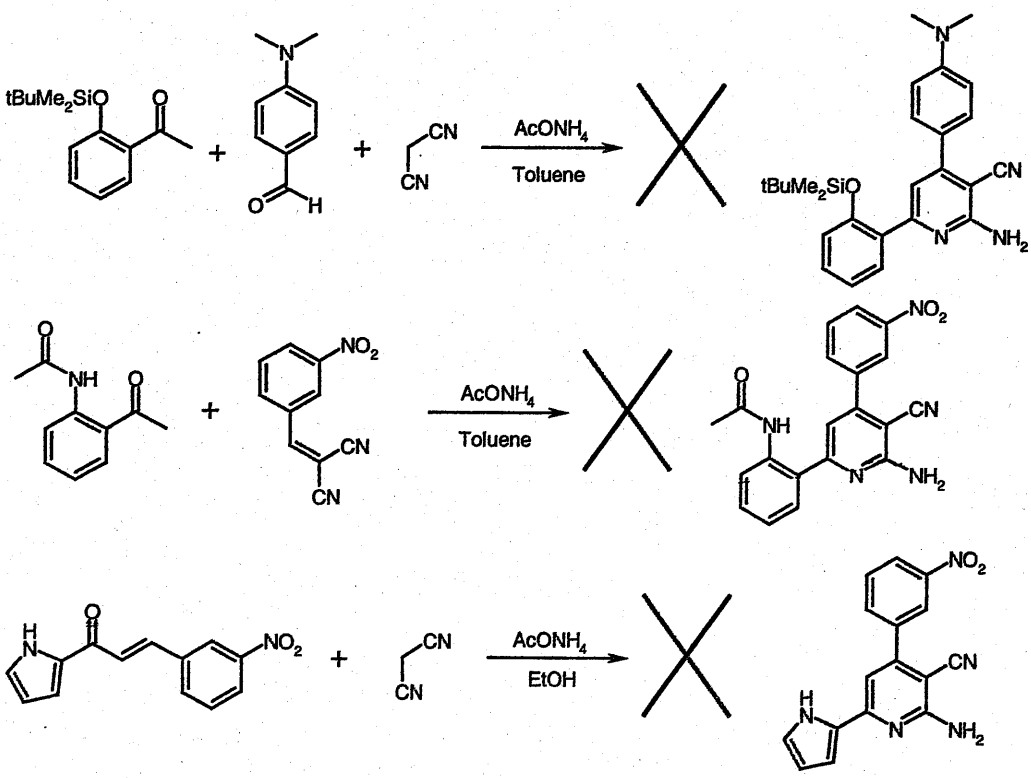



**Scheme 5. Synthesis of the pyridone analog (15)**

The corresponding pyridone analog **15** was prepared by the similar one-pot reaction using ethyl cyanoacetate instead of malononitrile (Scheme 5), and was subsequently utilized to synthesize the 2-diethylaminopyridine analog **19** (Scheme 6). The pyridone analog **15** was treated with triflic anhydride to provide the corresponding triflate **16**, which underwent a nucleophilic addition of diethylamine at 40 °C in DMSO to give the 2-diethylaminopyridine analog **17**. Hydrogenolysis using Pd-C catalysis followed by amide formation with acyl chloride and hydrolysis gave the desired product **19**.




**Scheme 6. Synthesis of 2-dialkylaminopyridine derivative (19)**

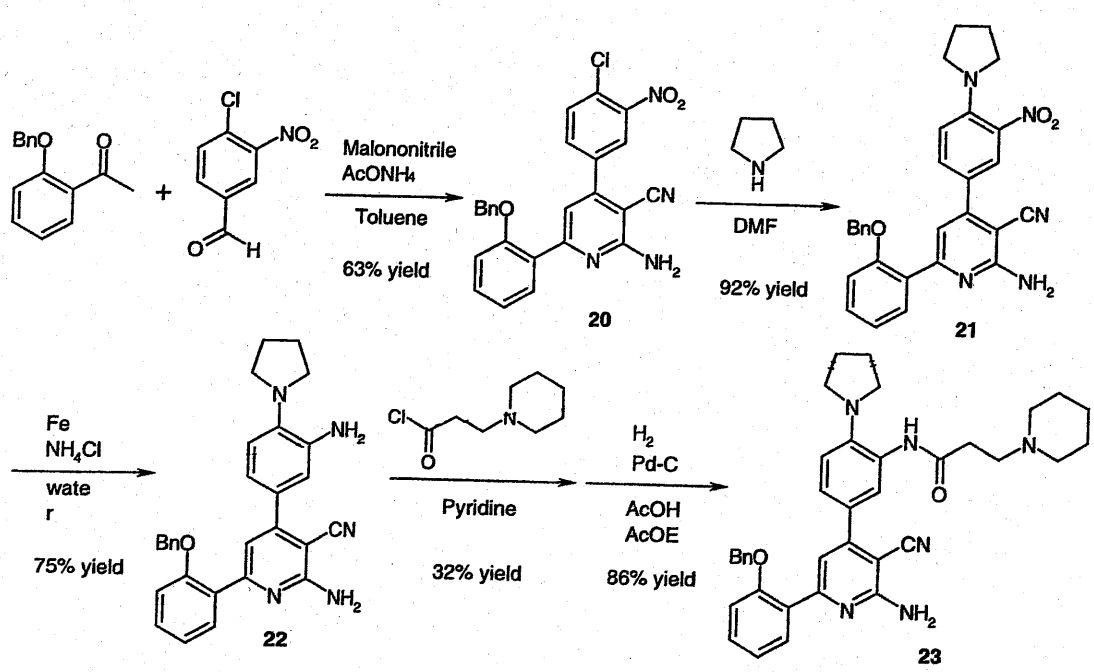



**Scheme 7. Unsuccessful examples for the four-components coupling**

In most cases, the four-component coupling reaction proceeds very smoothly to construct the pyridine core structure. However, there are some limitations for the

applicable substituent in this reaction, as shown in Scheme 7. The starting materials including basic alkylamine, amide group and heterocycles with an acidic proton didn't undergo the pyridine construction reaction. These observations suggest that this reaction requires neutral conditions, and the slight deviation to either acidity or basicity seems to prevent the pyridine construction.

In order to introduce an alkylamino group onto the lead structure, 4-chloro-3-nitrobenzaldehyde was used as the starting material (Scheme 8). The four-components coupling proceeded smoothly to give the pyridine analog **20** in relatively high chemical yield, suggesting that the benzylether is one of the most suitable protecting groups of the 2'-hydroxyacetophenones in this reaction. Since the nitro group increased the electrophilicity on the aromatic ring, introduction of pyrrole was simply achieved in DMF by heating at 60 °C for 2 hours to give the compound **21** in 92% yield.




**Scheme 8. Introduction of alkylamino group on the C-4 phenyl moiety**

## 5. SAR study for the 2-amino-3-cyano-4,6-diarylpyridines

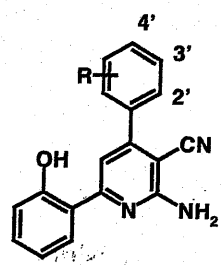
Recombinant human IKK-β was used to measure kinase activity *in vitro*. To test cellular efficacy of IKK-β inhibition, an ELISA assay measuring TNFα-induced RANTES production was employed.

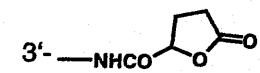
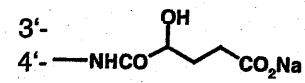
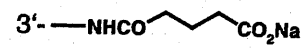
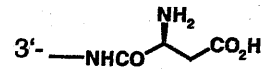
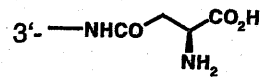
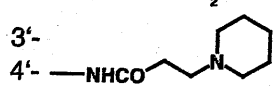
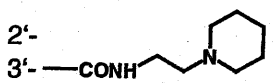
We initially synthesized *para*-nitro (**3**) and *para*-dimethylamine (**4**) analogs to assess their IKK-β inhibitory activity because these compounds have been reported to exhibit anti-inflammatory activity in animal models (paw edema tests using carrageenin).[13] However, these compounds showed no inhibitory activity in our *in vitro* assays (Table 2). It was found that the phenyl group at the 4-position of the pyridine could accommodate a variety of substituents. Since the lead compound **1** is sparingly soluble in an aqueous solution, our initial optimization efforts focused on the introduction of hydrophilic substituents onto this phenyl group with the aim to improve the solubility as well as the inhibitory activity. Various hydrophilic functionalities, such as alcohol, carboxylic acid and amine, are tolerated at this position, suggesting the hypothesis that this portion of the molecule may interact with the hydrophilic pocket of the enzyme.

The functionality on the phenyl group appears to affect cellular activity (TNFα-induced RANTES production inhibitory activity) significantly. Introduction of a basic ~~amino~~ moiety on the side chain of the phenyl group (**32** and **34**) improved cellular activity. However, the carboxylic acid analogs (**12, 24-26, 28-29**) exhibited either no or weak cellular activity, albeit with potent IKK-β inhibitory activity, suggesting poor cell membrane permeability of these derivatives. While displacement of substituents from the 3' to the 4'-position on the phenyl group did not significantly impact IKK-β

inhibitory activity, such modifications resulted in a complete loss of cellular activity (**12** vs. **28**, **32** vs. **33** and **35** vs. **26**).

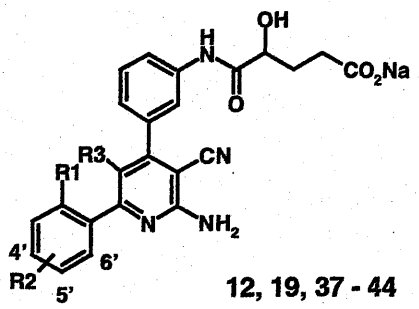
**Table 2. Modification of the substituents on the C-4 phenyl group**



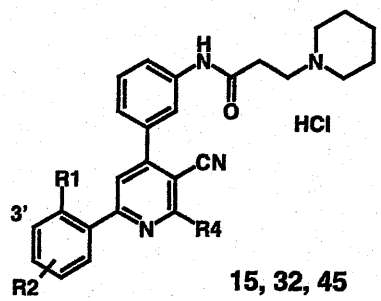

| Compound | R | $IC_{50}$ (μM) | |
|---|---|---|---|
| | | IKK-β[a] | RANTES[b] |
| 3 | 4'-NO2 | >20 | >50 |
| 4 | 4'-$N(CH_3)_2$ | >20 | >50 |
| 24 | 2'-$CO_2Na$ | 0.7 | >50 |
| 25 | 3'-$CO_2Na$ | 2.5 | >50 |
| 26 | 4'-$CO_2Na$ | 0.7 | >50 |
| 27 | 3'-$NH_2$ | 20 | >50 |
| 8 | 3'-  | 1.4 | >50 |
| 1 | 3'-  | 1.5 | 8 |
| 12 | 3'-  | 0.9 | 20 |
| 28 | 4'- | 0.5 | >50 |
| 29 | 3'-  | 1.4 | >50 |
| 30 | 3'-  | 0.6 | 15 |
| 31 | 3'-  | 0.6 | 35 |
| 32 | 3'- | 0.6 | 7 |
| 33 | 4'-  | 1.0 | >50 |
| 34 | 2'- | 0.5 | 6 |
| 35 | 3'-  | 1.8 | 15 |
| 36 | 4'- | 0.6 | >50 |

[a] Enzyme inhibition using recombinant human IKK-β. [b] Inhibition of TNFα-induced RANTES production in the human epithelial cell line A549.

**Table 3. Modification of the C-6 phenol group and the core ring**




12, 19, 37 - 44




15, 32, 45

| Compound | R1 | R2 | R3 | R4 | $IC_{50}$ (μM) IKK-β [a] |
|---|---|---|---|---|---|
| 12 | -OH | -H | -H | | 0.9 |
| 37 | -H | -H | -H | | >20 |
| 38 | -H | 4'-OH | -H | | >20 |
| 39 | $-OCH_3$ | -H | -H | | >20 |
| 40 | -OH | 5'-F | -H | | 1.7 |
| 41 | -OH | 5'-$OCH_3$ | -H | | 5.3 |
| 42 | -OH | 6'-$OCH_3$ | -H | | 2 |
| 43 | -OH | 6'-$CH_3$ | -H | | >20 |
| 44 | -OH | -H | $-CH_3$ | | >20 |
| 19 | -OH | -H | | $-NEt_2$ | >20 |
| 32 | -OH | -H | | $-NH_2$ | 0.6 |
| 45 | -H | 3'-OH | | $-NH_2$ | >20 |
| 15 | -OH | -H | | -OH | >20 |

[a] Enzyme inhibition using recombinant human IKK-β.

In contrast to the diversity of substitution tolerated on the phenyl group at the 4-position, modification of the phenol group at the 6-position appears to be rather restrictive (Table 3). Removal of the phenolic hydroxide (**37**) results in a complete loss of activity. Change of the substitution position (**38** and **45**) or replacement of the hydroxy group with a methoxy group (**39**) also yielded inactive compounds, suggesting that the 2'-phenol group is necessary for activity.

It was of interest that the 2'-phenol analog **44** incorporating a methyl group on the 5-position of the pyridine ring exhibited no inhibitory activity. The common

characteristic of the inactive compounds was found to be a lack of hydrogen-bonding interaction between the phenolic hydroxide and the pyridine nitrogen atom, which could typically be observed via the chemical shift of the phenolic hydroxide proton in the $^1H$ NMR spectrum (Figure 3). The hydrogen-bonding interaction of the 5-methyl analog **44** appears to be interrupted by the steric collision of the methyl group with the hydrogen atom at the 6'-position of the phenol group. Thus, the attainment of a geometry allowing for the internal hydrogen bond appears to play a significant role in achieving IKK-β inhibitory activity.

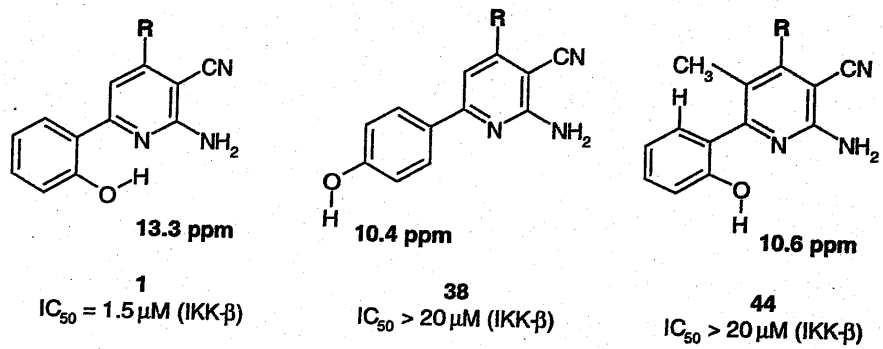
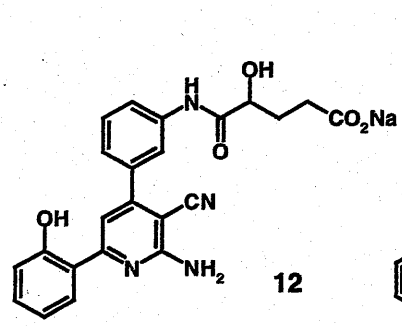



**Figure 3. Correlations between phenol proton chemical shift and activity.** The hydrogen-bonding interaction between the phenolic hydroxide and the pyridine nitrogen atom was estimated by the chemical shift of the phenolic hydroxide proton in the $^1H$ NMR spectrum, which was measured on a Brucker 300 MHz spectrometer using DMSO-*d6* as solvent and tetramethylsilane ($Me_4Si$) as an internal standard (0 ppm). When the structure is suitable for the hydrogen-bonding, the chemical shift of this proton is approximately 13 ppm. On the other hand, in the absence of intramolecular hydrogen bonding, the chemical shift shifts upfield, toward 10 ppm.

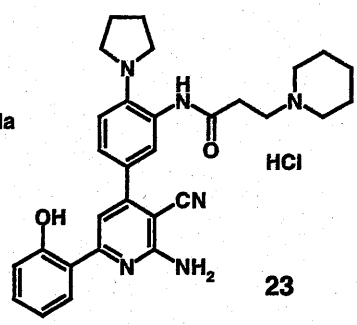
Addition of a substituent at the 5'-position on the 2'-phenol group usually resulted in a loss of cellular activity, despite maintaining IKK-β inhibitory activity (**40-41**). The introduction of a methyl group at the 6'-position gave an inactive compound **43**, in which the methyl group possibly interrupts the hydrogen-bonding interaction.

The 2-aminopyridine ring also appears to be important for inhibitory activity (Table 3). Replacement of the amino moiety by a hydroxy moiety (**15**) leads to a loss of activity, as does substitution of the amino moiety by a diethylamino group (**19**), suggesting that the 2-amino moiety on the pyridine ring is essential for the IKK-β inhibitory activity.

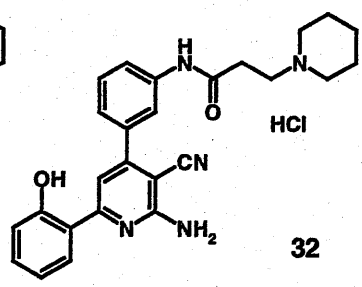
**Table 4. Modification of the C-4 phenyl group and In vivo evaluation of the potent IKK-β inhibitors**




**12**




**23**




**32**

| Compound | in vitro $IC_{50}$ (μM) | | in vivo $ED_{50}$ (mg/kg)[a] | |
|---|---|---|---|---|
| | IKK-β | RANTES | i.p.[b] | p.o.[c] |
| **1** | 1.5 | 8 | 10 | >30 |
| **12** | 0.9 | 20 | 1.3 | 19 |
| **23** | 1.0 | 0.8 | 0.8 | 10.1 |
| **32** | 0.6 | 7 | < 0.03 | 2 |

[a] *In vivo* TNFα induction by LPS in mice. [b] Intraperitoneal administration 30 min prior to i.p. injection of LPS [c] Oral administration 60 min prior to i.p. injection of LPS.

Introduction of an alkylamine group at the 4'-position on the phenyl ring was tolerated for IKK-β inhibition, and the pyrrolidine analog **23** was shown to be 10-fold more potent than the parent compound 1 in the cellular assay (Table 4).

In order to investigate the pharmacological profile of these potent IKK-β inhibitors, we tested their activity in an acute model of cytokine release (LPS-induced

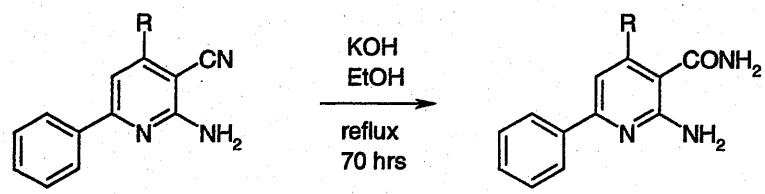
TNFα production in mice), which is considered to be one of the most mechanism-relevant models for the evaluation of IKK-β inhibition (Table 4). The initial lead compound **1** exhibits *in vivo* activity only when administered i.p. (intraperitoneally) but lacks oral efficacy due to low oral bioavailability. On the other hand, the more hydrophilic analogs, such as the carboxylic acid analog **12** and the basic amino analogs (**23, 32**), demonstrate potent oral activity as well as high potency when administered i.p.. The piperidyl analog **32** inhibits LPS-induced TNFα production with an $ED_{50}$ of 0.03 mg/kg and 2 mg/kg after intraperitoneal and oral administration, respectively. The compound **32** identified by the optimization efforts of this compound class maintains excellent selectivity vs. other kinases such as IKK-α ($IC_{50}$ = 20 μM), Syk and MKK4 ($IC_{50}$ > 20 $\mu$M).

In summary of this section, screening of the Bayer compound library resulted in the identification of a novel class of IKK-β inhibitors. Optimization of the lead compound **1** resulted in improvements in both *in vitro* and *in vivo* potency. As a result, compound **32** has been identified as a selective and potent inhibitor of the IKK-β which is orally bioavailable in mice, and which demonstrates significant *in vivo* activity in an acute model of cytokine release (LPS-induced TNFα).

## 6. Development of a new pyridine synthesis for modification of the nitrile moiety[18]

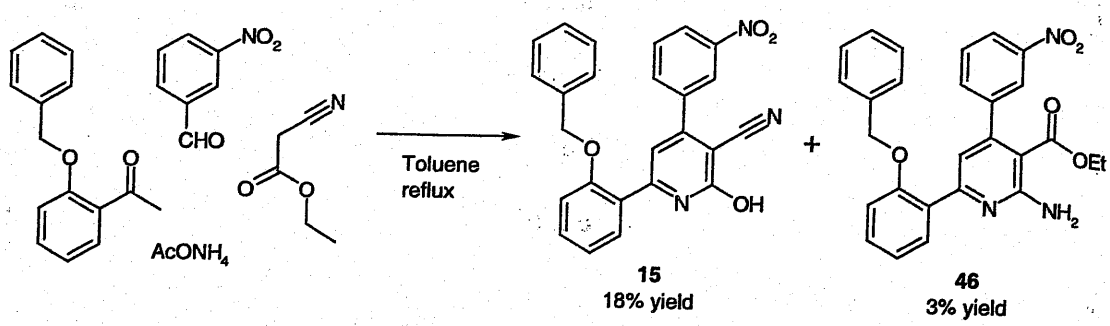
The initial SAR study of the first lead compound **1** indicated that various functional groups were tolerated on the 4-phenyl ring without losing the activity whereas the *ortho*-phenol and the 2-aminopyridine were demonstrated to be essential

moieties for activity. Hence, the emphasis of our initial synthetic efforts was focused on a modification of the substituents on the 4-phenyl ring. For the further extensive SAR study, our synthetic strategy was next shifted to a modification of the C-3 nitrile moiety. However, it turned out that the nitrile moiety at the 3-position on the pyridine ring was chemically too inert to convert it into other functional moieties, such as carboxylic acid. For instance, the 2-amino-3-cyanopyridine derivatives could be converted into the corresponding carboxamide analogs under strong basic conditions, as shown in Scheme 9. However, further hydrolysis to form the carboxylic acid analogs seems to need more harsh conditions (KOH, glycerol, 200 °C), as described in the precedent as well.[12] Thus, we tried to find new synthetic procedures for further chemical derivatization of the C-3 nitrile moiety.

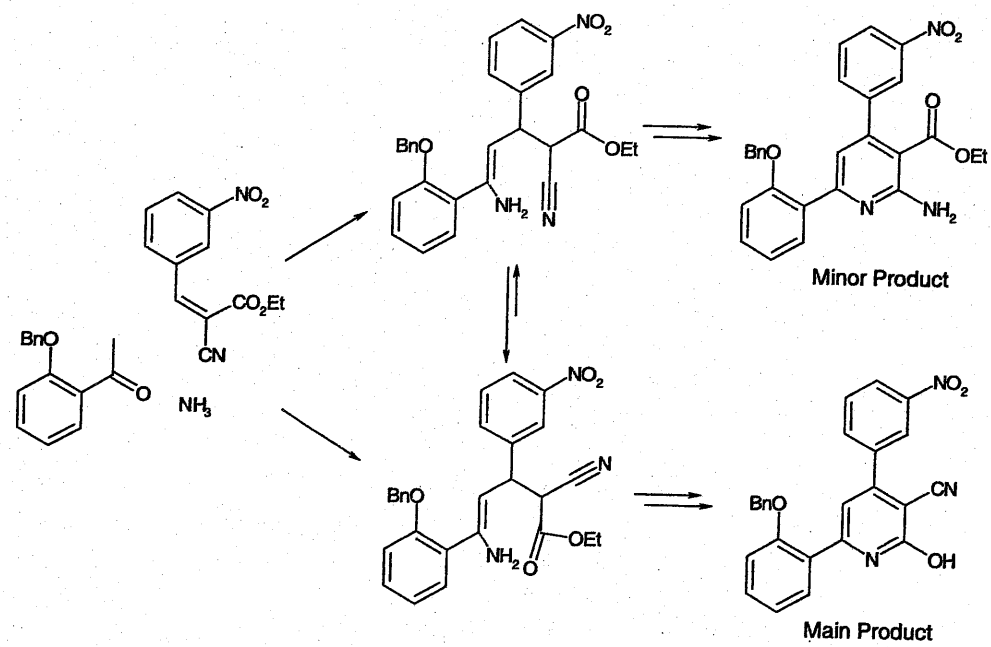



**Scheme 9. Hydrolysis of 2-amino-3-cyanopyridine derivative**

As shown in Scheme 5, the pyridone analog **15** was prepared by a four-components coupling reaction using acetophenone, benzaldehyde, ammonium acetate and ethyl cyanoacetate. In this reaction, formation of a small amount of 2-aminonicotinic acid ester **46** was found as the by-product (Scheme 10). This finding prompted me to modify the reaction to synthesize the 2-aminonicotinic acid ester derivatives more selectively.



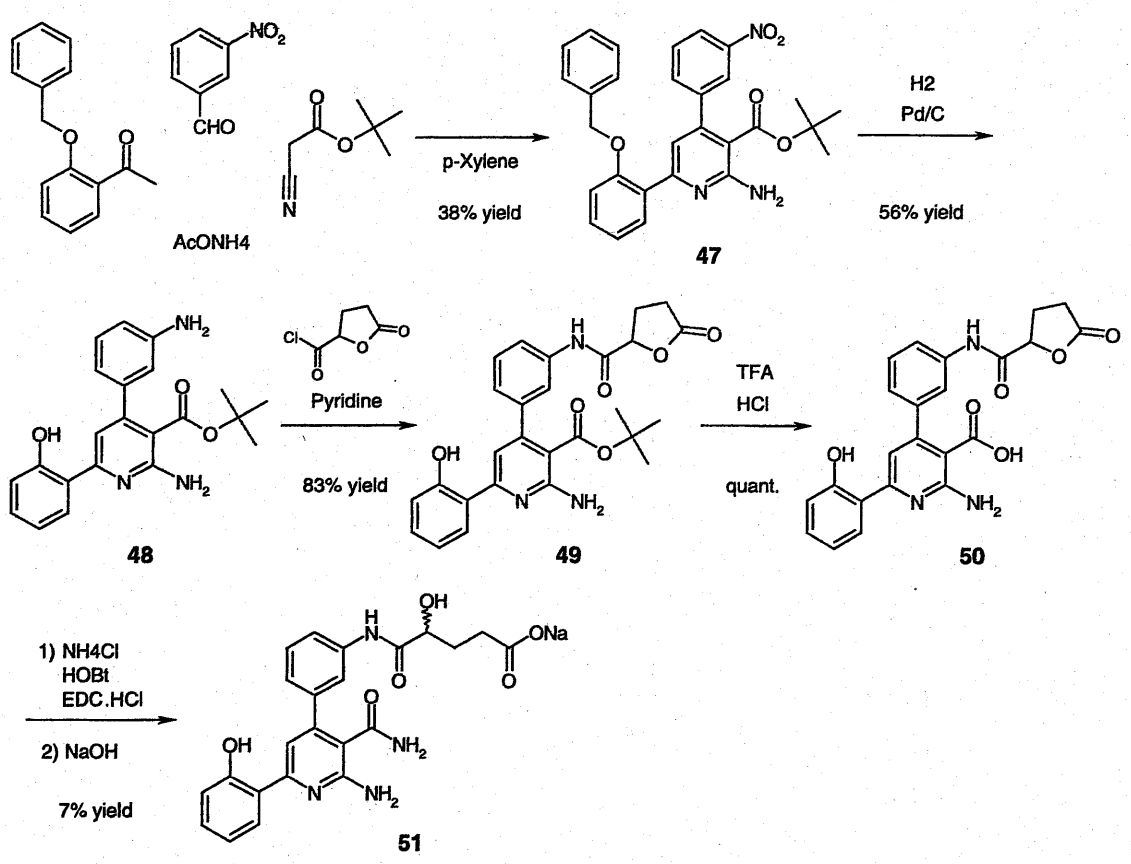

**Scheme 10. Formation of 2-aminonicotinic acid ester as a by-product**




**Scheme 11. Reaction mechanism for formation of 2-aminonicotinic acid ester**

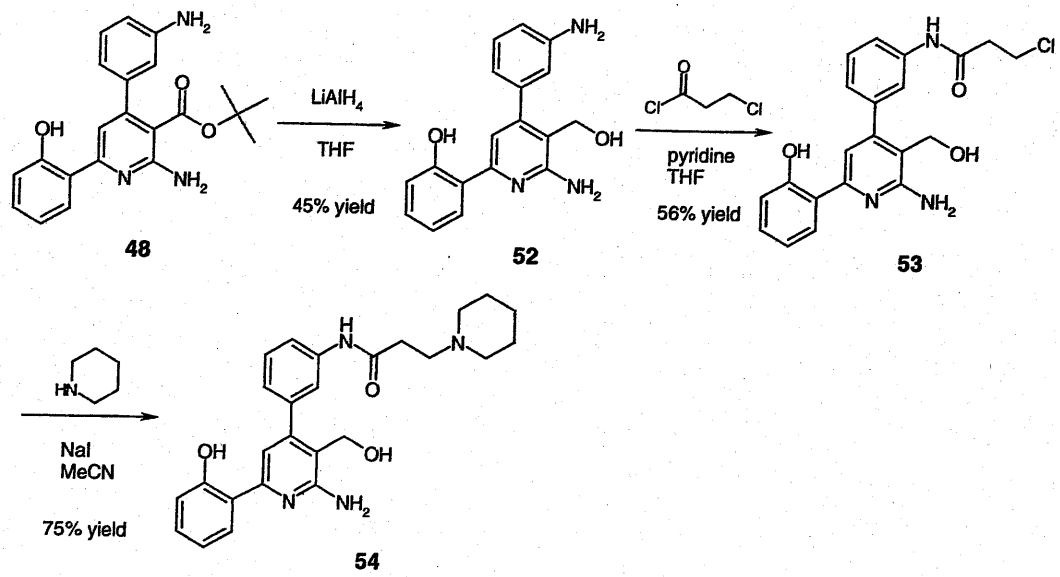
The proposed mechanism consists of the ~~Michael addition~~ of the acetophenone into the ~~Knovenagel~~ adduct with ammonia and the subsequent cyclization step, in which amino moiety attacks either the nitrile or the ester. In this case, the nucleophilic attack of the amine occurred mainly on the ester moiety to give the pyridone analog because the electrophilicity of the ester is superior to that of the nitrile moiety. Therefore, in order to reverse the reaction selectivity, the α-cyanoacetic acid ester with more bulky esters instead of ethyl cyanoacetate were attempted to use as the starting material for the

four-components coupling reaction. Consequently, it was found that *tert*-butyl cyanoacetate was subject to the selective cyclization to give the 2-aminonicotinic acid ester derivatives (Scheme 12). In this reaction, the corresponding pyridone analog was not detected in the crude product.




**Scheme 12. Synthesis of the carboxylic acid analog 50 and carboxamide analog 51**

Hydrogenolysis of the benzylether (**47**) followed by the selective amide formation using the acyl chloride and pyridine gave the *tert*-butyl ester derivative **49**, which was treated under acidic conditions for deprotection of the *tert*-butyl ester group to provide the carboxylic analog **50** (Scheme 12). Amide formation of the carboxylic analog **50** followed by the lactone ring opening under basic conditions provided the carboxamide **51**.




**Scheme 13. Synthesis of the hydroxymethyl analog 54**

The *tert*-butyl ester group (**48**) underwent reduction with lithium aluminum hydride or Red-Al® [a solution of sodium bis(2-methoxyethoxy)aluminum hydride in toluene] to yield the hydroxymethyl analog **52** (Scheme 13). The compound **52** was subject to selective amide formation on the aniline moiety with the acyl chloride to give compound **53**, which was then treated with piperidine in the presence of sodium iodide to provide the compound **54**.

## 7. Discovery of novel IKK-β inhibitors with amino acid residue[19]

It has been demonstrated from the initial SAR study of the first lead compound **1** that modification of the substituents on the C-4 phenyl ring are tolerated to some extent, as shown in Table 2. Hence, the emphasis of our initial synthetic efforts was next shifted to replacing the 4-pheny ring itself by other substituent, such as alkyl moieties. As the result, a novel analog **55** with a phenylalanine residue at the 4-position was

found to be 25 times more potent than the lead compound **1** for the IKK-β inhibitory activity (Figure 4).

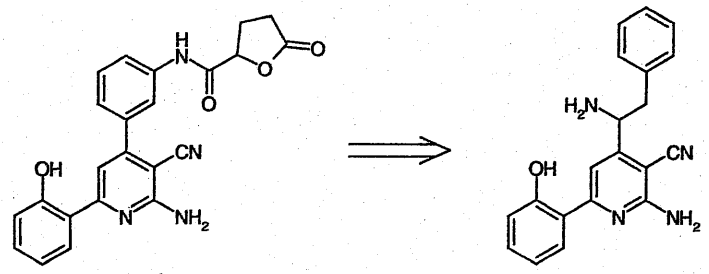
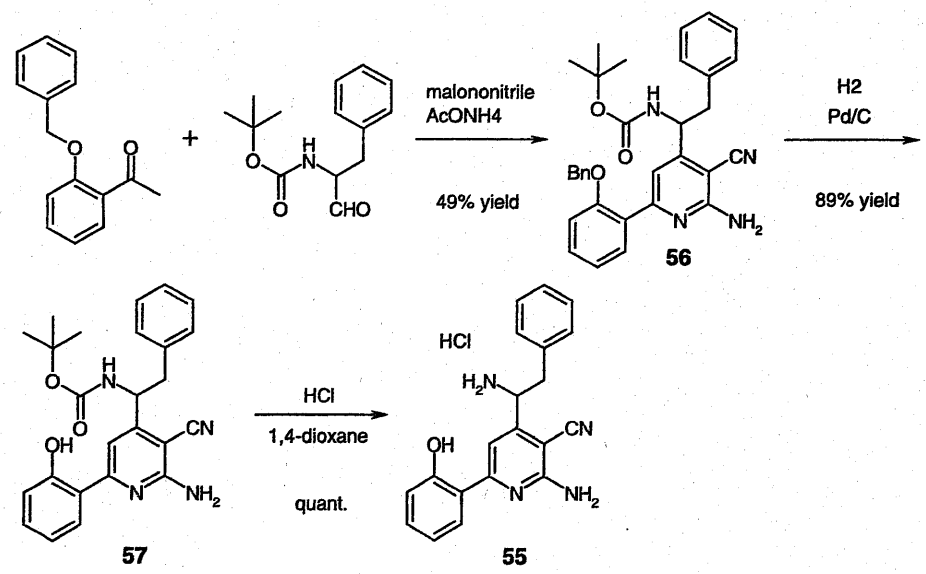



**Figure 4. Replacement of the C-4 phenyl ring with a phenylalanine residue**

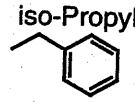
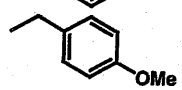
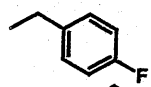
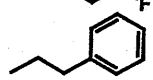
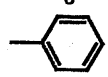


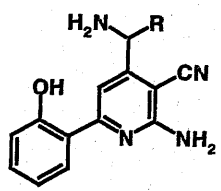

**Scheme 14. Synthesis of compound 55 with a phenylalanine residue**

The synthesis of compound **55** could be achieved with the four-components coupling reaction using the commercially available *N*-(*tert*-butoxycarbonyl)-phanylalaninal (Scheme 14). Although the optically pure phenylalaninal analog was used as the starting material, complete racemization on the asymmetric center occurred

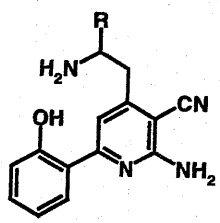
during the four-components coupling to form the racemic product **55**. After the pyridine core structure formation, the benzyl ether on the intermediate **56** was removed by hydrogenolysis to give compound **57**, which was then treated under acidic conditions for deprotection of the Boc group to yield compound **55** with greatly potent IKK-β inhibitory activity.

**Table 5. SAR study for the IKK-β inhibitors with amino acid residue**

| Compound | R | $IC_{50}$ (μM) | | |
|---|---|---|---|---|
| | | IKK-β[a] | RANTES[b] | TNFα[c] |
| 58 | $-CH_3$ | 0.15 | 4 | 3 |
| 59 | n-Butyl | 0.48 | 15 | n.d. |
| 60 | iso-Propyl | 0.24 | 5 | n.d. |
| 55 |  | 0.06 | 8 | 5 |
| 61 |  | 0.08 | 5 | 4 |
| 62 |  | 0.04 | 5 | 7 |
| 63 |  | 0.73 | 15 | n.d. |
| 64 | -H | 0.07 | 0.8 | 1.5 |
| 65 | $-CH_3$ | 0.47 | 3 | 2 |
| 66 |  | 0.11 | 2 | 2 |



55, 58 - 63



64 - 66

[a] Enzyme inhibition using recombinant human IKK-β. [b] Inhibition of TNFα-induced RANTES production in the human epithelial cell line A549. [c] Inhibition of LPS-induced TNFα production in human PBMCs. n.d.: not done

The structure-activity relationships study on the IKK-β inhibitors with an amino acid residue is illustrated in Table 5. Various aminomethyl derivatives (**55, 58 – 63**), demonstrated high activity. Among them, p-fluorobenzyl analog **62** was found to show the most potent IKK-β inhibitory activity ($IC_{50}$ = 40 nM). Aminoethyl derivatives (**64**

– **66**) also exhibited quite high *in vitro* activities analogous to those of the corresponding aminomethyl analogs. Especially, the simple aminoethylanalog **64** demonstrated not only potent IKK-β kinase inhibitory activity but also high cellular activity in the TNFα-induced RANTES production assay, suggesting that the compound **64** might have excellent cellular permeability as compared with other analogs.

## 8. Conclusion

From high-throughput screening of the Bayer compound library, the 2-amino-3-cyano-4-aryl-6-(2-hydroxyphenyl)pyridine analog **1** was identified as a novel and selective IKK-β inhibitor. The pyridine core structures were simply constructed using a one-pot coupling reaction of four components, acetophenone, benzaldehyde, malononitrile and ammonium acetate. Improvement of the pyridine synthesis provided 2-amino-4,6-diarylnicotinic acid derivatives, which have high potential for further derivations at the C-3 moiety on the core structure. Finally, optimization of the lead compound resulted in a marked improvement in IKK-β inhibitory activity, as exemplified by compound **55** with a phenylalanine residue ($IC_{50}$ = 0.06 μM).

## 9. Experiments

### General

Melting points are uncorrected. $^1H$ NMR spectra were recorded using either Bruker DRX-300 (300MHz for $^1H$) or 500 Bruker UltraShield™ (500MHz for $^1H$) spectrometer in $CDCl_3$ or DMSO-d6. Chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as an internal standard at zero ppm. Coupling constant (J) are given in hertz and the abbreviations s, d, t, q, and m refer to singlet,

doublet, triplet, quartet and multiplet, respectively. The abbreviation "br" refer to "broad". FAB (Fast-atom-bombardment) Mass spectroscopy data were recorded on a FINNIGAN MAT 95. Liquid chromatography – Mass spectroscopy (LCMS) data were recorded on a Micromass Platform LC with Shimadzu Phenomenex ODS column (4.6 x 30 mm) flushing a mixture of acetonitrile and water (9:1 to 1:9) at 1 ml/min of the low rate. The MASS specta were obtained using electrospray (ES) ionization techniques. TLC was performed on a precoated silica gel plate (Merck silica gel 60 F-254). Silica gel (WAKO-gel C-200 (75-150 $\mu$ m))was used for all column chromatography separations. All chemicals were reagent grade and were purchased from Sigma-Aldrich, Wako pure chemical industries, Ltd., Tokyo kasei kogyo co. Ltd.

**[Preparation of the starting materials]**

**2'-[*tert*-Butyl(dimethyl)silyl]oxyacetophenone**

To a stirred solution of 2'-hydroxyacetophenone (10.000 g, 73.447 mmol) in DMF (150 ml) was added imidazole (6.000 g, 88.137 mmol) and *tert*-butyl dimethylchlorosilane (12.177 g, 80.792 mmol). The mixture was stirred at room temperature for 60 hours. The reaction mixture was diluted with diethyl ether and washed with water and aqueous potassium hydrogen sulfate solution. The organic layer was dried over magnesium sulfate, filtered and concentrated. Purification was carried out by silica gel column (hexane/ethyl acetate =9/1-4/1) to give the desired product (19.83 g, 92% yield) as a colorless oil; LCMS (ES) *m/z* 251 $(M+H)^+$; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 0.26 (6H, s), 1.00 (9H, s), 2.60 (3H, s), 6.85 – 7.00 (2H, m), 7.31 – 7.61 (2H, m).

**1-[2-(Benzyloxy)phenyl]ethanone**

A mixture of 2'-hydroxyacetophenone (68.1 g, 0.500 mol), benzylbromide (94.1 g, 0.550 mol) and $K_2CO_3$ (103 g, 0.750 mol) in acetone (1.0 L) was heated at reflux, and the stirring was continued overnight. After cooled to room temperature, the mixture was concentrated under reduced pressure. The residue was diluted with water, and extracted with ethyl acetate. The separated organic phase was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by distillation under reduced pressure to give the title product (100 g, yield; 88% yield) as a colorless oil; LCMS (ES) *m/z* 227 $(M+H)^+$.

**[Synthesis of target compounds]**

***N*-{3-[2-amino-3-cyano-6-(2-hydroxyphenyl)-4-pyridinyl]phenyl}-2-methoxyacetamide (8)**

To a mixture of 2'-hydroxyacetophenone (7.00 g, 51.4 mmol), 3-nitro-benzaldehyde (7.77 g, 51.4 mmol) and ethanol (40 mL) was added dropwise 50% aqueous NaOH solution (31 mL), and stirring was continued at room temperature overnight. The resultant precipitate was collected by filtration and washed with H2O. The solid obtained was suspended in boiled ethanol. After cooled to room temperature, the precipitate was filtered, washed with ethanol, and dried in vacuo to give 1-(2-hydroxyphenyl)-3-(3-nitrophenyl)propenone (**5**) (6.97 g, 50% yield) as a brown solid; FAB-MS *m/z* 270 $(M+H)^+$.

A mixture of the product above (**5**) (3.00 g, 11.1 mmol), malononitrile (0.736 g, 11.1 mmol), ammonium acetate (6.87 g, 89.1 mmol) and ethanol (30 mL) was heated at reflux overnight. After cooled to room temperature, the resulting solid was collected by filtration and washed with ethanol. The solid obtained was suspended in boiled

ethanol. After cooled to room temperature, the precipitate was collected by filtration, washed with ethanol, and dried in vacuo to give 2-amino-6-(2-hydroxyphenyl)-4-(3-nitrophenyl)nicotinonitrile (**6**) (1.07 g, 29% yield) as a brown solid; LCMS (ES) *m/z* 333 $(M+H)^+$.

A mixture of the product above (**6**) (1.46 g, 4.39 mmol), Fe powder (2.00 g, 35.8 mmol), ammonium chloride (0.200 g, 3.74 mmol), water (12 mL) and ethanol (37 mL) was heated at reflux for 1 hour. The mixture was filtered to remove the Fe powder and washed with ethanol and ethyl acetate. The combined filtrates were concentrated in vacuo to give B as a yellow solid, which was used for the next step without further purification. 2-amino-4-(3-aminophenyl)-6-(2-hydroxyphenyl)nicotinonitrile (**7**) (0.817 g, 62% yield) as a yellow solid; LCMS (ES) *m/z* 303 $(M+H)^+$.

To a solution of the above product (**7**) (0.20 g, 0.66 mmol) and pyridine (0.054 mL, 0.66 mmol) in $CH_3CN$ (2 mL) and THF (4 mL) at 0 °C under argon atmosphere was added dropwise a solution of methoxyacetyl chloride (0.072 g, 0.66 mmol) in CH3CN (0.4 mL). After being stirred at 0 °C for 1 hour, the mixture was partitioned between ethyl acetate and water. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The crude product was suspended in ethyl acetate, collected by filtration, washed with ethyl acetate, and dried in vacuo. The yellow solid obtained was suspended in boiled $CH_3CN$. After cooled to room temperature, the precipitate was collected by filtration, washed with $CH_3CN$, and dried in vacuo to give the title compound (**8**) (0.14 g, 55% yield) as a pale yellow solid; m.p. >260 °C; LCMS (ES) *m/z* 375 $(M+H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 3.40 (1H, s), 4.40 (1H, s), 6.86 – 6.93 (2H, m), 7.31 – 7.53 (6H, m), 7.90 (1H, d, $J$ = 9.1 Hz), 7.96 (1H, s), 8.02 (1H, dd, $J$ = 1.3, 8.1 Hz), 9.93 (1H, s), 13.33 (1H, s).

In similar manners as described in the example above to synthesize compound (**8**), following compounds (**3 – 4**) shown in Table 2 were synthesized.

**2-Amino-6-(2-hydroxyphenyl)-4-(4-nitrophenyl)nicotinonitrile** (**3**); LCMS (ES) *m/z* 333 $(M+H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 6.80 – 6.93 (4H, m), 7.20 – 7.33 (4H, m), 7.63 (2H, d, *J* = 8.6 Hz), 8.12 (1H, d, *J* = 6.8 Hz), 13.33 (1H, br).

**2-Amino-4-(4-dimethylaminophenyl)-6-(2-hydroxyphenyl)nicotinonitrile** (**4**); LC-MS (ES) *m/z* 331 $(M+H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 3.01 (6H, s), 6.83 – 6.90 (4H, m), 7.28 – 7.37 (4H, m), 7.60 (2H, d, *J* = 9.0 Hz), 8.03 (1H, d, *J* = 6.8 Hz), 13.50 (1H, br).

***N*-{3-[2-amino-3-cyano-6-(2-hydroxyphenyl)-4-pyridinyl]phenyl}-5-oxotetrahydro-2-furancarboxamide (1)**

A mixture of 2'-[*tert*-butyl(dimethyl)silyl]oxyacetophenone (20.0 g, 79.9 mmol), 3-nitrobenzaldehyde (12.1 g, 79.9 mmol), malononitrile (5.3 g, 79.9 mmol), ammonium acetate (9.2 g, 119.8 mmol) and toluene (25 mL) was stirred under reflux for 3 hr. The reaction mixture was extracted with ethyl acetate and water. The separated organic phase was dried over $Na_2SO_4$, filtrated and concentrated under reduce pressure. The crude product was purified by column chromatography on silica gel (hexane/ ethyl acetate, 9 : 1 – 4 : 1) to give 2-amino-6-(2-{[*tert*-butyl(dimethyl)silyl]oxy}phenyl)- 4-(3-nitrophenyl)nicotinonitrile (**9**) (6.5 g, 18%) as a white solid.

A mixture of the product above (**9**) (3.2 g, 7.2 mmol), 10% Pd-C (0.25 g) and ethyl acetate (1 L) was stirred at room temperature for 12 hours under a hydrogen atmosphere (2.5 atm). The reaction mixture was filtered on Celite® and the filtration

was concentrated under reduce pressure to give 2-amino-4-(3-aminophenyl)-6-(2-{[*tert*-butyl(dimethyl)silyl]oxy}phenyl)nicotinonitrile (**10**) (2.6 g, 87%), which was used for the next step without further purification.

To a cold (0 °C) mixture of pyridine (0.20 mL, 2.424 mmol), the above product (**10**) (1.010 g, 2.424 mmol), acetonitrile (10 mL) and THF (2 mL) was added dropwise a solution of 5-oxotetrahydro-2-furancarbonyl chloride (0.360 g, 2.424 mmol) in acetonitrile (4 mL), and the stirring was continued for 2 hours. The mixture was allowed to warm to room temperature, the stirring was continued for 1 h, and then extracted with ethyl acetate and water. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduce pressure. The residue was purified by column chromatography on silica gel (hexane: Ethyl acetate, 7:3 - 5:5) to give *N*-{3-[2-amino-6-(2-{[tert-butyl(dimethyl)silyl]oxy}phenyl)-3-cyano-4-pyridinyl]phenyl}-5-oxotetrahydro-2-furancarboxamide (**11**) (0.982 g, 77%) as a white solid.

To a cold (0 °C) solution of the above product (**11**) (0.400 g, 0.757 mmol) in THF (5 mL) was added dropwise a solution n-tetrabutylammonium fluoride in THF (1M, 2 mL). After being stirred for 30 min, the mixture was quenched with water (5 mL). The resulting precipitate was collected by filtration, washed with water and ethanol, and dried under reduce pressure to give *N*-{3-[2-amino-3-cyano-6-(2-hydroxyphenyl)-4-pyridinyl]phenyl}-5-oxotetrahydro-2-furancarboxamide (**1**) (0.293 g, 93%) as a white solid; m.p. >260 °C; LCMS (ES) *m/z* 415 $(M+H)^+$; $^1$H-NMR (300 MHz, DMSO-*d6*) δ 2.23 - 2.38 (2H, m), 2.55 - 2.59 (2H, m), 5.08 - 5.12 (1H, m), 6.87 - 6.93 (2H, m), 7.32 - 7.47 (4H, m), 7.50 - 7.56 (2H, m), 7.82 (1H, d, J = 8.3 Hz), 7.91 (1H, s), 8.02 (1H, d, J = 8.0 Hz), 10.47 (1H, s), 13.33 (1H, s).

**Sodium 5-({3-[2-amino-3-cyano-6-(2-hydroxyphenyl)-4-pyridinyl]phenyl}amino)-4-hydroxy-5-oxopentanoate (12)**

To a suspension of *N*-{3-[2-amino-3-cyano-6-(2-hydroxyphenyl)-4-pyridinyl] phenyl}-5-oxotetrahydro-2-furancarboxamide **(1)** (0.250 g, 0.603 mmol) in methanol (10 mL) was added dropwise 2N NaOH solution (0.3 mL). After the mixture was stirred for 10 min, the resulting precipitate was filtered off. The transparent solution was concentrated under reduce pressure and the residue was crystallized from methanol and ethyl ether. The resulting solid was collected by filtration under an argon atmosphere, washed with ethyl ether and dried under reduce pressure to give the title compound **(12)** (0.165 g, 60 %) as a pale yellow solid; m.p. 199 (dec.); LCMS (ES) *m/z* 433 $(M - Na + 2H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ (300 MHz, DMSO-d6): 1.72 - 1.98 (2H, m), 2.18 - 2.25 (2H, m), 4.01 (1H, dd, J = 3.2, 7.5 Hz), 6.60 - 6.85 (2H, m), 7.09 - 7.30 (3H, m), 7.32 (1H, d, J = 7.9 Hz), 7.46 (1H, t, J = 7.9 Hz), 7.67 (1H, br), 7.93 (1H, d, J = 8.2 Hz), 8.01 - 8.05 (2H, m), 9.91 (1H, s), 10.38 (1H, br s).

In similar manners as described in the example above to synthesize compounds **(1)** and **(12)**, following compounds **(27 - 31)** shown in Table 2 and compounds **(37 - 44)** shown in Table 3 were synthesized.

**2-Amino-4-(3-aminophenyl)-6-(2-hydroxyphenyl)nicotinonitrile (27)**; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 5.31 (2H, s), 6.70 – 6.80 (3H, m), 6.85 – 6.93 (2H, m), 7.18 (1H, t, *J* = 7.9 Hz), 7.31 (1H, s), 7.32 – 7.25 (1H, m), 7.39 (2H, s), 8.00 (1H, d, *J* = 6.8 Hz), 13.38 (1H, s).

**Sodium 4-{4-[2-amino-3-cyano-6-(2-hydroxyphenyl)pyridin-4-yl]-phenyl-**

**carbamoyl}-4-hydroxybutyrate (28)**; LCMS (ES) *m/z* 433 (M - Na + 2H)$^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 1.79 – 1.97 (2H, m), 2.13 – 2.27 (2H, m), 4.03 (1H, dd, *J* = 3.0, 7.2 Hz), 6.85 – 6.92 (2H, m), 7.30 – 7.42 (4H, m), 7.65 (2H, d, *J* = 8.7 Hz), 7.93 (2H, d, *J* = 8.7 Hz), 8.06 (1H, d, *J* = 7.9 Hz), 9.99 (1H, s), 10.31 (1H, br s), 13.38 (1H, br s).

**Sodium 4-{3-[2-amino-3-cyano-6-(2-hydroxyphenyl)-pyridin-4-yl]-phenyl-carbamoyl}butyrate (29)** ; LCMS (ES) *m/z* 417 (M - Na + 2H)$^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 1.51-2.02 (4H, m), 4.10 (1H, dd, *J* = 3.2, 7.5 Hz), 6.90 (1H, dd, *J* = 5.0, 8.7 Hz), 7.26-7.63 (6H, m), 7.90-8.00 (3H, m), 9.98 (1H, s), 10.45 (1H, br s), 13.15 (1H, br s).

**3-Amino-*N*-{3-[2-amino-3-cyano-6-(2-hydroxyphenyl)pyridin-4-yl]phenyl}-succinamic acid (30);** LCMS (ES) *m/z* 418 (M + H)$^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 2.94 and 3.00 (2H, dd, *J* = 5.3, 17.3 Hz), 4.30 (1H, br), 6.87 – 6.94 (2H, m), 7.32 – 7.43 (3H, m), 7.56 (1H, t, *J* = 7.9 Hz), 7.80 (1H, d, *J* = 9.0 Hz), 7.89 (1H, s), 8.00 (1H, d, *J* = 6.8 Hz), 8.46 (3H, br), 10.96 (1H, s).

**2-Amino-*N*-{3-[2-amino-3-cyano-6-(2-hydroxyphenyl)-pyridin-4-yl]phenyl}-succinamic acid (31);** LCMS (ES) *m/z* 418 (M + H)$^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 3.02 and 3.07 (2H, dd, *J* = 5.3, 17.0 Hz), 4.27 (1H, q, *J* = 5.3 Hz), 6.86 – 6.94 (2H, m), 7.32 – 7.37 (3H, m), 7.51 (1H, t, *J* = 7.9 Hz), 7.76 (1H, d, *J* = 7.9 Hz), 7.88 (1H, s), 8.00 (1H, d, *J* = 6.8 Hz), 8.36 (3H, br), 10.61 (1H, s).

**Sodium 4-[3-(2-amino-3-cyano-6-phenylpyridin-4-yl)phenylcarbamoyl]-4-hydroxy-butyrate (37);** m.p. 180 °C (dec.); LCMS (ES) *m/z* 417 (M -Na + 2H)$^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 1.76-1.96 (2H, m), 2.16-2.21 (2H, m), 4.01 (1H, dd, *J* = 3.3, 7.6 Hz, 6.98 (2H, br s), 7.26 (1H, s), 7.34-7.52 (5H, m), 7.95 (1H, d, *J* = 8.2 Hz), 8.03-8.14 (3H, m), 9.89 (1H, s), 10.37 (1H, br s).

**Sodium 4-{3-[2-amino-3-cyano-6-(4-hydroxyphenyl)pyridin-4-yl]phenyl-carbamoyl}-4-hydroxy-butyrate (38);** LCMS (ES) *m/z* 433 (M -Na + 2H)$^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 1.75 – 1.96 (2H, m), 2.10 - 2.70 (2H, m), 3.99 (1H, dd, *J* = 3.0, 7.5 Hz), 6.38 – 6.70 (4H, m), 6.97 (1H, s), 7.28 (2H, d, *J* = 7.9 Hz), 7.30 – 7.45 (m, 2H), 7.79 (2H, d, *J* = 8.7 Hz), 7.90 (1H, d, *J* = 7.9 Hz), 7.96 (1H, s), 9.86 (1H, s), 10.40 (1H, s).

**Sodium; 4-{3-[2-amino-3-cyano-6-(2-methoxyphenyl)pyridin-4-yl]phenyl-carbamoyl}-4-hydroxy-butyrate (39);** LCMS (ES) *m/z* 447 (M -Na + 2H)$^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 1.74 – 1.92 (2H, m), 2.13 – 2.28 (2H, m), 3.83 (3H, s), 3.98 (1H, q, *J* = 5.0 Hz), 6.88 (2H, s), 6.91 – 7.17 (2H, m), 7.18 (1H, s), 7.30 (1H, d, *J* = 8.0 Hz), 7.40 – 7.48 (2H, m), 7.76 (1H, dd, *J* = 1.8, 7.7 Hz), 7.85 (1H, d, *J* = 9.2 Hz), 8.08 (1H, s), 9.92 (1H, s), 10.69 (1H, s).

**Sodium 4-{3-[2-amino-3-cyano-6-(5-fluoro-2-hydroxyphenyl)pyridin-4-yl]phenyl-carbamoyl}-4-hydroxybutyrate (40);** m.p. 245 °C (dec.); LCMS (ES) *m/z* 451 (M -Na + 2H)$^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 1.73-1.96 (4H, m), 4.01 (1H, dd, *J* = 3.2, 7.5 Hz), 6.90 (1H, dd, *J* = 5.0, 8.9 Hz), 7.27-7.52 (6H, m), 7.92-8.02 (3H, m), 9.89 (1H, s), 10.47 (1H, br s), 13.16 (1H, br s).

**Sodium 4-{3-[2-amino-3-cyano-6-(2-hydroxy-5-methoxyphenyl)pyridin-4-yl]-phenylcarbamoyl}-4-hydroxybutyrate (41);** LCMS (ES) *m/z* 463 (M -Na + 2H)$^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 1.74 – 1.90 (2H, m), 2.07 – 2.18 (2H, m), 3.75 (3H, s), 3.98 (1H, dd, *J* = 3.0, 7.2 Hz), 6.83 (1H, d, *J* = 8.7 Hz), 6.96 (1H, dd, *J* = 3.0, 9.0 Hz), 7.32 (1H, d, *J* = 7.9 Hz), 7.38 (1H, br s), 7.44 – 7.51 (3H, m), 7.95 (1H, d, *J* = 8.3 Hz), 8.01 (1H, s), 9.89 (1H, s), 10.73 (1H, br s).

**Sodium 4-{3-[2-amino-3-cyano-6-(2-hydroxy-6-methoxyphenyl)pyridin-4-yl]-**

**phenylcarbamoyl}-4-hydroxybutyrate (42);** LCMS (ES) *m/z* 463 $(M - Na + 2H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 1.70 – 1.94 (2H, m), 2.09 – 2.28 (2H, m), 3.80 (3H, s), 3.99 (1H, dd, *J* = 3.0, 7.5 Hz), 6.55 (2H, t, *J* = 8.3 Hz), 7.20 – 7.36 (5H, m), 7.46 (1H, t, *J* = 7.9 Hz), 7.82 (1H, d, *J* = 9.4 Hz), 8.11 (1H, s), 9.94 (1H, s), 10.52 (1H, br), 12.49 (1H, br).

**Sodium 4-{3-[2-amino-3-cyano-6-(2-hydroxy-6-methylphenyl)pyridin-4-yl]-phenylcarbamoyl}-4-hydroxybutyrate (43);** LCMS (ES) *m/z* 447 $(M - Na + 2H)^+$; $^1H$ NMR (500 MHz, DMSO-*d6*) δ 1.73 – 1.94 (2H, m), 2.10 – 2.19 (2H, m), 2.21 (3H, s), 3.99 (1H, br d, *J* = 4.9 Hz), 6.77 – 6.87 (1H, m), 7.24 (1H, d, *J* = 7.4 Hz), 7.33 – 7.37 (2H, m), 7.44 – 7.49 (3H, m), 7.89 (1H, d, *J* = 7.4 Hz), 7.96 (1H, d, *J* = 7.9 Hz), 8.03 (1H, s), 9.90 (1H, s), 10.78 (1H br s), 13.91 (1H, br).

**Sodium 4-{3-[2-amino-3-cyano-6-(2-hydroxyphenyl)-5-methyl-pyridin-4-yl]-phenylcarbamoyl}-4-hydroxybutyrate (44);** LCMS (ES) *m/z* 447 $(M - Na + 2H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 1.73 (3H, s), 1.75 – 1.94 (2H, m), 2.08 – 2.23 (2H, m), 3.98 (1H, dd, *J* = 3.0, 7.9 Hz), 6.63 (2H, s), 6.83 – 6.89 (2H, m), 7.00 (1H, d, *J* = 7.5 Hz), 7.14 – 7.22 (2H, m), 7.43 (1H, dd, *J* = 7.5, 8.3 Hz), 7.80 (2H, br), 9.69 (1H, br), 9.90 (1H, s), 10.57 (1H, s).

***N*-{3-[3-Cyano-2-hydroxy-6-(2-hydroxyphenyl)pyridin-4-yl]phenyl}-3-piperidin-1-yl-propionamide hydrochloride (15)**

A mixture of 2'-hydroxyacetophenone (3.00 g, 13.3 mmol), 3-nitrobenzaldehyde (2.00 g, 13.3 mmol), ethyl cyanoacetate (1.50 g, 13.3 mmol), ammonium acetate (3.07 g, 39.8 mmol) and p-xylene (10 mL) was stirred at 120 °C for 2 hours. After cooled to room temperature, the mixture was diluted with ethanol. The resultant precipitate was

collected by filtration, washed with ethanol, dried in vacuo to give 6-(2-benzyloxyphenyl)-2-hydroxy-4-(3-nitrophenyl)nicotinonitrile **(13)** (1.01 g, 18% yield) as a pale yellow solid; LCMS (ES) *m/z* 424 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 5.18 (2H, s), 6.70 (1H, br), 7.12 (1H, t, *J* = 7.0 Hz), 7.29 – 7.37 (4H, m), 7.45 – 7.62 (4H, m), 7.84 (1H, t, *J* = 8.0 Hz), 8.05 (1H, d, *J* = 8.0 Hz), 8.40 (1H, dd, *J* = 2.2, 8.3 Hz), 8.49 (1H, d, *J* = 1.9 Hz), 12.77 (1H, br s).

The filtrate was concentrated in vacuo, and the residue was extracted with ethyl acetate and water. The separated organic phase was washed with brine, dried over $Na_2SO_4$ and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane: ethyl acetate, 4:1) to give 2-amino-6-(2-benzyloxyphenyl)-4-(3-nitrophenyl)nicotinic acid ethyl ester **(46)** (0.30 g, 67% purity, 3% yield) as a pale yellow oil; LCMS (ES) *m/z* 470 $(M + H)^+$.

A mixture of compound **13** (0.200 g, 0.600 mmol), 10% Pd-C (0.05 g), and ethyl acetate (5 mL) was hydrogenated at 5 atm overnight. The reaction mixture was filtered on Celite®, washed with methanol, $CH_3CN$, THF and ethyl acetate successively. The combined filtrates were concentrated in vacuo. The residue was suspended in DMF, diluted with ethyl acetate, and then passed through column chromatography on silica gel with ethyl acetate eluent. The combined ethyl acetate fractions were concentrated in vacuo. The residue was suspended in ethanol, collected by filtration, washed with ethanol, and dried in vacuo to give 4-(3-aminophenyl)-6-(2-hydroxyphenyl)-2-hydroxynicotinonitrile **(14)** (0.065 g, 36% yield) as a yellow solid; m.p. >260 °C; LCMS (ES) *m/z* 304 $(M + H)^+$.

To a suspension of the above product **(14)** (0.040 g, 0.13 mmol) in $CH_3CN$ (2.0 mL) and THF (2.0 mL) including 3-piperidin-1-yl-propionyl chloride hydrochloride

(0.034 g, 0.16 mmol) at 0 °C was added triethylamine (0.022 mL, 0.16 mmol) followed by pyridine (0.021 mL, 0.26 mmol). After 2 hours, the mixture was allowed to warm to room temperature, and stirred at room temperature for 4 hours. The suspension was diluted with water and ethyl acetate, and then the insoluble material was collected by filtration, and washed with ethyl acetate. The solid obtained was suspended in methanol, filtered, and washed with methanol. The crude product was dissolved in a mixture of $CH_3CN$ and THF, treated with excess 4N HCl in 1,4-dioxane, and concentrated in vacuo. The resulting solid was suspended in ethyl acetate, filtered, washed with ethyl acetate, and dried in vacuo to give *N*-{3-[3-cyano-2-hydroxy-6-(2-hydroxyphenyl)pyridin-4-yl]phenyl}-3-piperidin-1-yl-propionamide hydrochloride (**15**) (0.011 g, 17% yield) as a pale gray solid; m.p. >260 °C; LCMS (ES) *m/z* 443 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 1.30 – 1.85 (6H, m), 2.80 – 3.06 (4H, m), 3.30 – 3.55 (4H, m), 6.58 (1H, br), 6.90 (1H, t, *J* = 7.8 Hz), 7.02 (1H, d, *J* = 8.2 Hz), 7.33 – 7.39 (2H, m), 7.48 – 7.54 (2H, m), 7.76 (1H, d, *J* = 8.4 Hz), 7.93 (1H, s), 9.75 (1H, br), 10.50 (1H, s), 12.48 (1H, br).

**Sodium 4-{3-[3-cyano-2-diethylamino-6-(2-hydroxyphenyl)pyridin-4-yl]-phenyl carbamoyl}-4-hydroxybutyrate (19)**

To a cold (0°C) solution of 6-(2-benzyloxyphenyl)-2-hydroxy-4-(3-nitrophenyl) nicotinonitrile (**13**) (0.500 g, 1.18 mmol) in pyridine (5 mL) under an argon atmosphere was added dropwise triflic anhydride (0.199 mL, 1.18 mmol) After being stirred for 4 hours, the reaction mixture was quenched with water, and extracted with ethyl acetate and 1N HCl solution. The separated organic phase was washed with water and brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The residue was purified by

column chromatography on silica gel (hexane: ethyl acetate, 4:1) to give trifluoromethanesulfonic acid 6-(2-benzyloxy-phenyl)-3-cyano-4-(3-nitro-phenyl) pyridin-2-yl ester (**16**) (0.531 g, 81% yield) as a pale yellow foam; LCMS (ES) *m/z* 556 $(M + H)^+$.

To a solution of the compound (**16**) (0.250 g, 0.450 mmol) in DMSO (2 mL) was added diethylamine (0.116 mL, 1.13 mmol), and the mixture was stirred at 40 °C for 1 hour. The resulting mixture was partitioned between ethyl acetate and water. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane: ethyl acetate, 4:1) to give 6-(2-benzyloxyphenyl)-2-diethylamino-4-(3-nitrophenyl)nicotinonitrile (**17**) (0.190 g, 88% yield) as a pale yellow solid; LCMS (ES) *m/z* 479 $(M + H)^+$.

A mixture of the compound (**17**) (0.180 g, 0.376 mmol), 10% Pd-C (0.050 g), methanol (5 mL) and THF (5 mL) was hydrogenated at 3 atm overnight. The mixture was filtered on Celite®, and washed with THF, ethyl acetate and methanol successively. The combined filtrates were concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane: ethyl acetate, 4:1 - 2:1) to give a yellow oil, which was then crystallized with methanol. The solid obtained was collected by filtration, washed with methanol, dried in vacuo to give 4-(3-aminophenyl)-2-diethylamino-6-(2-hydroxyphenyl)nicotinonitrile (**18**) (0.061 g, 45% yield) as a colorless solid; LCMS (ES) *m/z* 359 $(M + H)^+$.

To a cold (0°C) solution of the compound (**18**) (0.050 g, 0.139 mmol) in THF (1.5 mL) including pyridine (0.015 mL, 0.18 mmol) was added 5-oxotetrahydrofuran- 2-carbonyl chloride (0.027 g, 0.18 mmol). After 2 h, the mixture was allowed to warm

to room temperature, and stirring was continued for 2 hours. The mixture was partitioned between ethyl acetate and water. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane: ethyl acetate, 3:4) to give 5-oxotetrahydrofuran-2-carboxylic acid {3-[3-cyano-2-diethylamino-6-(2-hydroxy-phenyl)pyridin-4-yl] phenyl}amide (0.045 g, 69% yield) as a yellow oil.

The product above (0.040 g, 0.085 mmol) was dissolved in $CH_3CN$ (1 mL), and treated with 1N aqueous sodium hydroxide solution (0.5 mL). The mixture was purified by Prep-HPLC (Shimadzu Prep-ODS 20 mm x 25 cm, 10% $CH_3CN$/H2O -30 min- 90% $CH_3CN$/H2O, 10 ml/min, 254 nm). The combined fractions were concentrated in vacuo to give the title compound **(19)** (0.039 g, 90% yield) as a yellow solid; m.p. 217 – 220 °C; LCMS (ES) *m/z* 489 $(M - Na + 2H)^+$; $^1H$ NMR (500 MHz, DMSO-*d6*) δ 1.29 (6H, t, $J$ = 6.9 Hz), 1.69 – 1.93 (2H, m), 2.12 – 2.25 (2H, m), 3.77 (4H, q. $J$ = 6.9 Hz), 6.88 – 6.95 (2H, m), 7.30 – 7.38 (2H, m), 7.48 (1H, t, $J$ = 8.5 Hz), 7.54 (1H, s), 7.95 (1H, d, $J$ = 8.2 Hz), 8.00 (1H, s), 8.19 (1H, dd, $J$ = 2.1, 8.2 Hz), 9.88 (1H, s), 10.54 (1H, br), 13.07 (1H, br).

**_N_-[5-[2-amino-3-cyano-6-(2-hydroxyphenyl)-4-pyridinyl]-2-(1-pyrrolidinyl)phenyl]-3-(1-piperidinyl)propanamide hydrochloride (23)**

A mixture of 2-benzyloxyacetophenone (5.00 g, 22.10 mmol), 4'-chloro-3'-nitrobenzaldehyde (8.20 g, 44.19 mmol), malononitrile (2.92 g, 44.19 mmol) and ammonium acetate (8.52 g, 110.48 mmol) in toluene (15 mL) was stirred at 130 °C for 3 hours. After cooled to room temperature, the mixture was diluted with ethyl acetate and THF. The organic phase was washed twice with water, dried over $Na_2SO_4$, filtered

and concentrated under reduce pressure. The residue was suspended in ethanol. The precipitate was collected by filtration, washed with ethyl acetate and dried under reduce pressure to give 2-amino-6-(2-{[*tert*-butyl(dimethyl)silyl]oxy}phenyl)-4-(4-chloro-3-nitrophenyl)nicotinonitrile (**20**) (6.40 g, 63% yield) as a white solid; $^1$H NMR (500 MHz, DMSO-*d6*) δ 5.16 (2H, s), 7.06 (2H, br s), 7.11 (2H, dd, *J* = 7.5, 7.7 Hz), 7.26 - 7.32 (4H, m), 7.38 - 7.47 (3H, m), 7.69 (1H, dd, *J* = 2.1, 8.4 Hz), 7.85 (1H, dd, *J* = 1.7, 7.7 Hz), 7.90 (1H, d, *J* = 8.4 Hz), 8.19 (1H, d, *J* = 2.1 Hz).

To a cold (0°C) solution of the above product (**20**) (1.000 g, 2.189 mmol) in DMF (10 mL) was added pyrrolidine (0.778 g, 10.943 mmol). The mixture was stirred at room temperature for 2 hr and at 60°C for 2 hours. The reaction mixture was partitioned between ethyl acetate and water. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduce pressure. The residue was suspended in ethanol, and the precipitate was collected by filtration, washed with ethyl acetate and dried under reduce pressure to give 2-amino-6-(2-{[tert-butyl (dimethyl)silyl]oxy}phenyl)-4-[3-nitro-4-(1-pyrrolidinyl)phenyl]nicotinonitrile (**21**) (0.990 g, 92% yield) as a yellow solid.

To a suspension of the above product (**21**) (1.000 g, 2.034 mmol) and Fe powder (3.000 g) in ethanol (180 mL) were added water (10 mL) and then ammonium chloride (1.000 g). The mixture was stirred under reflux for 3 h. The mixture was filtered on Celite® Pad and the filtrate was concentrated under reduce pressure. The residue was suspended in ethanol, and the precipitate was collected by filtration, washed with ethyl acetate and dried under reduce pressure to give 2-amino-4-[3-amino-4-(1-pyrrolidinyl) phenyl]-6-(2-{[tert-butyl(dimethyl)silyl]oxy} phenyl)nicotinonitrile (**22**) (0.700 g, 75% yield) as a white solid.

To a stirred solution of the above product **(22)** (0.166 g, 0.360 mmol) in pyridine (3 mL) was added 3-(1-piperidinyl)propanoyl chloride hydrochloride (0.153 g, 0.719 mmol) at room temperature and the stirring was continued for 3 hours. The reaction was extracted with ethyl acetate and water. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduce pressure. The residue was suspended in ethyl acetate and ethanol, and then the precipitate was collected by filtration, washed with ethanol and dried under reduce pressure to give *N*-[5-[2-amino-6-(2-{[tert-butyl(dimethyl)silyl]oxy}phenyl)-3-cyano-4-pyridinyl]-2-(1-pyrrolidinyl) phenyl]-3-(1-piperidinyl)propanamide (0.070 g, 32% yield) as a white solid.

A mixture of the product above (0.140 g, 0.233 mmol) and 10% Pd-C (0.150 g), ethyl acetate and acetic acid (2.0 mL) was stirred at room temperature under a hydrogen atmosphere for 12 hours. The reaction mixture was filtered on Celite® and the filtrate was concentrated under reduce pressure. The residue was dissolved in THF (3 mL) and 4N HCl in 1,4-dioxane (0.5 mL) was added to the solution. The resulted yellow solid was collected by filtration, washed with THF and dried under reduce pressure to give *N*-[5-[2-amino-3-cyano-6-(2-hydroxyphenyl)-4-pyridinyl]-2-(1-pyrrolidinyl)-phenyl]-3-(1-piperidinyl)propanamide hydrochloride (0.110 g, 86% yield) **(23)**; m.p. 152 °C; LCMS (ES) *m/z* 511 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 1.35 - 1.40 (1H, m), 1.67 - 1.80 (6H, m), 1.92 (4H, s), 2.89 - 2.98 (4H, m), 3.29 - 3.57 (8H, m), 3.58 - 3.60 (1H, m), 6.87 - 6.95 (2H, m), 7.02 (1H, d, J = 7.9 Hz), 7.30 - 7.37 (3H, m), 7.53 - 7.56 (2H, m), 7.98 (2H, d, J = 7.2 Hz), 9.85 (1H, s), 10.45 (1H, br s).

**Sodium 3-[2-amino-3-cyano-6-(2-hydroxyphenyl)-4-pyridinyl]benzoate (25)**

A mixture of 2-{(4-methoxybenzyl)oxy}acetophenone (0.26 g, 1.00 mmol), methyl 3-formbenzoate (0.16 g, 1.00 mmol), malononitrile (0.07 g, 1.00 mmol), ammonium acetate (0.23 g, 3.00 mmol) and xylene (10 mL) was heated at 120 °C (bath temperature) overnight. The resulting mixture was concentrated under reduce pressure, and the residue was diluted with water and extracted with ethyl acetate. The separated organic phase was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduce pressure. The crude material was purified by column chromatography on silica gel (hexane: ethyl acetate, 70:30) to give 2-amino-6-{2-[(4-methoxybenzyl)oxy] phenyl}-4-(3-methoxycarbonylphenyl)nicotinonitrile (0.200 g, 43% yield).

A mixture of the product above (0.20 g, 0.43 mmol), aqueous 4N NaOH solution (1.0 mL) and THF (5.0 mL) was stirred at room temperature overnight. The reaction mixture was poured into water and extracted with ethyl ether twice. To the separated aqueous phase was added 1N $KHSO_4$ solution until the mixture became acidic. The resulting precipitate was collected by filtration and dried under reduce pressure to give 2-amino-6-{2-[(4-methoxybenzyl)oxy]phenyl}-4-(3-hydroxycarbonylphenyl)nicotinonitrile (0.16 g, 81% yield) as a gray powder.

A mixture of the product above (0.16 g, 0.36 mmol), trifluoroacetic acid (3.0 mL), anisole (0.50 mL), and water (0.50 mL) was stirred at room temperature overnight. The reaction mixture was diluted with toluene and concentrated under reduced pressure. The residue was dissolved in THF and then crystallized with hexane. The resulting precipitate was collected by filtration and washed with hexane, and then dried under reduce pressure to give 2-amino-6-(2-hydroxyphenyl)-4-(3-hydroxycarbonylphenyl) nicotinonitrile (0.12 g, 74% yield).

The product above (0.02 g, 0.045 mmol) was dissolved in 4N NaOH solution (2

mL), and the resulting solution was purified by HPLC (ODS reverse phase column, 10% $CH_3CN/H_2O$ - 90% $CH_3CN/H_2O$) to give the title compound **(25)** . (0.015 g, 95% yield) as a pale yellow powder; m.p. >260 °C; LCMS (ES) *m/z* 332 $(M - Na + 2H)^+$; $^1H$ NMR (500 MHz, DMSO-*d6*) δ 6.85 - 6.93 (2H, m), 7.31 - 7.56 (5H, m), 7.58 (1H, d, J = 3.0 Hz), 7.99 - 8.06 (2H, m), 8.10 (1H, s), 13.40 (1H, br s).

**N-{3-[2-amino-3-cyano-6-(2-hydroxyphenyl)-4-pyridinyl]phenyl}-3-(1-piperidinyl)propanamide hydrochloride (32)**

To a cold (0°C) solution of 3-(1-piperidinyl)propanoic acid (0.676 g, 3.601 mmol) in dichloromethane was added oxalyl chloride (0.471 mL, 5.401 mmol) and stirred at room temperature for 12 hr. The mixture was concentrated under reduce pressure. The residue was added to the solution of 2-amino-4-(3-aminophenyl)-6-(2- {[tert-butyl(dimethyl)silyl]oxy}phenyl)nicotinonitrile **(10)** (1.500 g, 3.601 mmol) in acetonitrile (3 mL), and the resulting mixture was stirred at 0°C for 1 hr. The reaction mixture was extracted with ethyl acetate and water. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduce pressure. Purification was carried out by silica gel column chromatography (chloroform/ methanol, 10 : 1) to give *N*-{3-[2-amino-6-(2-{[*tert*-butyl(dimethyl)silyl]oxy}phenyl)-3-cyano-4-pyridinyl]phenyl}-3-(1-piperidinyl)propanamide (0.860g, 45% yield) as a white solid.

To a stirred solution of the above product (0.150 g, 0.270 mmol) in 1,4-dioxane (5 mL) was added 4N HCl in dioxane (2 mL). The mixture was stirred at room temperature for 12 hr. The resulted precipitate was collected by filtration, washed with ethanol and dried under reduce pressure to give the title compound **(32)** (0.011g, 9%

yield) as a colorless foam; LCMS (ES) *m/z* 442 $(M + H)^+$; $^1H$ NMR (500 MHz, DMSO-*d6*) δ 1.38 - 1.50 (1H, m), 1.71 - 1.84 (5H, m), 2.76 - 3.04 (4H, m), 3.35 - 3.51 (4H, m), 6.90 - 6.98 (2H, m), 7.38 - 7.41 (3H, m), 7.54 (1H, t, J = 7.9 Hz), 7.83 (1H, d, J = 8.2 Hz), 7.95 (1H, s), 8.06 (1H, dd, J = 1.3, 8.2 Hz), 10.42 (1H, br s), 10.64 (1H, br s).

**3-[2-amino-3-cyano-6-(2-hydroxyphenyl)-4-pyridinyl]-*N*-[2-(1-piperidinyl)ethyl]-benzamide (35)**

To a cold (0 °C) mixture of 3-(2-amino-3-cyano-6-{2-[(4-methoxybenzyl)oxy] phenyl}-4-pyridinyl)benzoic acid [an intermediate for compound (**25**)] (1.35 g 3.00 mmol), *N*-(2-aminoethyl)piperidine (0.420 g 3.30 mmol), and 1-hydroxybenzotriazole (0.490 g 3.60 mmol) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.69 g, 3.60 mmol) with stirring. The mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was concentrated under reduce pressure. The residue was dissolved in ethyl acetate and partitioned between ethyl acetate and water. The separated organic phase was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduce pressure. The solid obtained was triturated with ethyl ether and dried under reduce pressure to give 2-(1-piperidinyl)ethyl 3-(2-amino-3-cyano-6-{2-[(4-methoxybenzyl)oxy]phenyl}-4-pyridinyl)benzoate (0.98 g, 58% yield).

A mixture of the above product (0.14 g, 0.25 mmol), trifluoroacetic acid (3.00 mL), anisole (0.50 mL) and water (0.50 mL) was stirred at room temperature overnight. The reaction mixture was diluted with toluene and concentrated under reduced pressure. The residue was purified by HPLC (ODS reverse phase column, 10% $CH_3CN/H_2O$ - 90% $CH_3CN/H_2O$) to give the title compound (**35**) (0.061 g, 44% yield) as a pale yellow

solid; m.p. 131 °C (dec.); LCMS (ES) *m/z* 442 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 1.36 - 1.86 (6H, m), 2.95 (2H, q, J = 10.5 Hz), 3.26 - 3.28 (2H, m), 3.56 (2H, d, J = 12.4 Hz), 3.66 (2H, J = 6.6 Hz), 6.88 - 6.95 (2H, m), 7.01 (1H, t, J = 7.91 Hz), 7.45 (1H, s), 7.53 (2H, s), 7.70 (1H, t, J = 7.9 Hz), 7.88 (1H, d, J = 7.54 Hz), 8.05 (2H, t, J = 7.0 Hz), 8.12 (1H, s), 8.84 (1H, t, J = 5.7 Hz), 9.02 (1H, br s), 13.31 (1H, s).

**Sodium salt of 2-amino-6-(2-hydroxyphenyl)-4-(3-{[(5-oxotetrahydro-2-furanyl) carbonyl]amino}phenyl)nicotinic acid (50)**

A mixture of 2'-benzyloxyacetophenone (3.00 g, 13.25 mmol), 3'-nitrobenzaldehyde (2.00 g, 13.25 mmol), tert-butyl cyanoacetate (1.87 g, 13.25 mmol), ammonium acetate (3.07 g, 39.77 mmol) and p-xylene (10 mL) was stirred at 120 °C for 2 hours. After cooled to room temperature, the reaction mixture was partitioned between ethyl acetate and water. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtrated and concentrated under reduce pressure. The crude product was purified by column chromatography on silica-gel (hexane: ethyl acetate, 5:1 - 2:1) to give *tert*-butyl 2-amino-6-[2-(benzyloxy)phenyl]-4-(3-nitrophenyl) nicotinate **(47)** (2.51 g, 38% yield) as a yellow oil; LCMS (ES) *m/z* 498 $(M + H)^+$.

A mixture of the compound **(47)** (2.50 g, 5.02 mmol), 10% Pd-C (0.20 g) and ethyl acetate (20 mL) was stirred at 2 atm under a hydrogen atmosphere for 5 h. The mixture was filtered on Celite® pad to remove Pd-C and washed with ethyl acetate. The filtrate was concentrated under reduce pressure, and the residue was purified by column chromatography on silica-gel (hexane: ethyl acetate, 4:1 - 3:2) to give *tert*-butyl 2-amino-4-(3-aminophenyl)-6-(2-hydroxyphenyl)nicotinate **(48)** as a pale yellow oil. (1.05 g, 56% yield); LCMS (ES) *m/z* 378 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ

1.18 (9H, s), 5.17 (2H, s), 6.47 – 6.52 (2H, m), 6.60 – 6.64 (1H, m), 6.82 – 6.90 (m, 2H), 7.03 (2H, s), 7.09 (1H, t, $J$ = 7.5 Hz), 7.13 (1H, s), 7.28 (1H, dd, $J$ = 1.5, 8.3 Hz), 7.93 (1H, dd, $J$ = 1.5, 7.9 Hz), 13.82 (1H, s).

To a cold (0 °C) solution of the compound (**48**) (0.626 g, 1.659 mmol) in THF (7 mL) including pyridine (0.148 mL, 1.824 mmol) was added a solution of 5-oxotetrahydro-2-furancarbonyl chloride (0.271 g, 1.824 mmol) in THF (3 mL). After 1 h, the mixture was allowed to warm to room temperature and the stirring was continued for 2 hours. The mixture was partitioned between ethyl acetate and water. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduce pressure. The residue was purified by column chromatography on silica-gel (hexane: ethyl acetate, 1:4 - 1:19) to give *tert*-butyl 2-amino-6-(2-hydroxyphenyl)-4-(3-{[(5-oxotetrahydro-2-furanyl)carbonyl]amino}-phenyl)nicotinate (**49**) (0.677 g, 83% yield) as a pale yellow foam; LCMS (ES) *m/z* 490 $(M + H)^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 1.12 (9H, s), 2.16 – 2.27 (m, 2H), 2.51 – 2.59 (2H, m), 5.03 – 5.08 (1H, m), 6.82 – 6.92 (2H, m), 7.11 (1H, d, $J$ = 7.8 Hz), 7.18 (1H, s), 7.19 (2H, s), 7.29 (1H, dd, $J$ = 1.5, 6.9 Hz), 7.44 (1H, t, $J$ = 7.8 Hz), 7.64 (1H, t, $J$ = 1.7 Hz), 7.72 (1H, d, $J$ = 8.0 Hz), 7.95 (1H, dd, $J$ = 1.4, 8.2 Hz), 10.34 (1H, s), 13.74 (1H, s).

A mixture of the compound (**49**) (0.025 g, 0.051 mmol) and trifluoroacetic acid (1 mL) was stirred at room temperature for 2 h. After the mixture was concentrated under reduce pressure, the residue was dissolved in acetonitrile (0.6 mL) and then treated with saturated $NaHCO_3$ solution (0.2 mL). The resulting mixture was purified by HPLC (ODS reverse phase column, 10% $CH_3CN/H_2O$ - 90% $CH_3CN/H_2O$) to give a sodium salt of 2-amino-6-(2-hydroxyphenyl)-4-(3-{[(5-oxotetrahydro-2-furanyl)

carbonyl]amino}phenyl)nicotinate (**50**) (0.009 g, 39% yield) as a pale yellow solid; LCMS (ES) *m/z* 434 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 2.19 – 2.56 (4H, m), 5.07 – 5.22 (1H, m), 6.77 – 6.84 (2H, m), 6.96 (2H, s), 7.00 (1H, s), 7.14 – 7.29 (3H, m), 7.60 (1H, s), 7.64 (1H, d, *J* = 8.2 Hz), 7.83 (1H, d, *J* = 7.0 Hz), 10.43 (1H, s), 14.60 (1H, s).

**Sodium 5-({3-[2-amino-3-(aminocarbonyl)-6-(2-hydroxyphenyl)-4-pyridinyl] phenyl}amino)-4-hydroxy-5-oxopentanoate (51)**

To a cold (0 °C) mixture of the compound (**50**) (0.070 g, 0.15 mmol), ammonium chloride (0.016 g, 0.30 mmol), 1-hydroxybenzotriazole hydrate (0.032 g, 0.24 mmol) and DMF (2 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.034 g, 0.18 mmol) followed by triethylamine (0.042 mL, 0.30 mmol). After 1 hour, the mixture was allowed to warm to room temperature and the stirring was continued overnight. The resulting mixture was diluted with water and extracted with ethyl acetate. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduce pressure. The residue was purified by HPLC (ODS reverse phase column, 50% $CH_3CN/H_2O$ - 90% $CH_3CN/H_2O$) to give a carboxamide analog of the starting material. The product was dissolved in acetonitrile (0.7 mL) and then treated with aqueous 1N NaOH solution (0.6 mL). The resulting mixture was purified by HPLC (ODS reverse phase column, 10% $CH_3CN/H_2O$ - 90% $CH_3CN/H_2O$) to give the title compound (**51**) (0.005 g, 7% yield) as a yellow solid; LCMS (ES) *m/z* 451 $(M - Na + 2H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 1.70 – 1.96 (2H, m), 2.13 – 2.24 (2H, m), 3.97 – 4.00 (1H, m), 6.46 (2H, br), 6.82 (2H, br), 7.16 (1H, d, *J* = 7.7 Hz), 7.19 – 7.37 (5H, m), 7.84 (1H, s), 7.87 (1H, d, *J* = 8.1 Hz), 7.95 (1H,

dd, $J$ = 1.3, 8.0 Hz), 9.75 (1H, s), 10.65 (1H, s), 13.91 (1H, s).

**$N$-{3-[2-amino-3-(hydroxymethyl)-6-(2-hydroxyphenyl)-4-pyridinyl]phenyl}-3-(1-piperidinyl)propanamide (54)**

To a cold (0 °C) suspension of lithium aluminum hydride (0.077 g, 1.62 mmol) in THF (3 mL) under an argon atmosphere was added dropwise a solution of *tert*-butyl 2-amino-4-(3-aminophenyl)-6-(2-hydroxyphenyl)nicotinate (**48**) (0.353 g, 0.935 mmol) in THF (2 mL) with stirring. After 30 min, the mixture was allowed to warm to room temperature, and the stirring was continued at room temperature for 2h and at 50 °C overnight. After cooled to room temperature, the reaction mixture was quenched with water and extracted with ethyl acetate. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduce pressure. The residue was purified by recrystallization from methanol to give 2-[6-amino-4-(3-aminophenyl)-5-(hydroxymethyl)-2-pyridinyl]phenol (**52**) (0.133 g, 45% yield) as a pale yellow solid; LCMS (ES) *m/z* 308 $(M + H)^+$; $^1$H NMR (500 MHz, DMSO-*d6*) δ 4.37 (2H, d, $J$ = 5.0 Hz), 5.05 (1H, t, $J$ = 5.0 Hz), 5.16 (2H, s), 6.42 (2H, s), 6.56 – 6.64 (3H, m), 6.77 – 6.86 (2H, m), 7.06 (1H, s), 7.09 (1H, d, $J$ = 7.2 Hz), 7.21 (1H, dd, $J$ = 1.4, 6.9 Hz), 7.85 (1H, dd, $J$ = 1.4, 8.0 Hz), 13.96 (1H, s).

To a cold (0 °C) solution of the above product (**52**) (0.100 g, 0.325 mmol) in THF (3 mL) including pyridine (0.028 mL, 0.34 mmol) was added 3-chloropropionyl chloride (0.043 g, 0.34 mmol). After being stirred for 30 min, the mixture was allowed to warm to room temperature and the stirring was continued for 2 hours. The resulting mixture was diluted with water and extracted with ethyl acetate. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduce

pressure. The residue was crystallized with ethyl ether. The resulting solid was collected by filtration, washed with ethyl ether and dried under reduce pressure to give *N*-{3-[2-amino-3-(hydroxymethyl)-6-(2-hydroxyphenyl)-4- pyridinyl]phenyl}-3-chloro-propanamide **(53)** (0.072 g, 56% yield) as a pale yellow solid; LCMS (ES) *m/z* 398 (M + H)$^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 2.85 (2H, t, *J* = 6.2 Hz), 3.89 (2H, t, *J* = 6.2 Hz), 4.34 (2H, d, *J* = 5.0 Hz), 5.08 (1H, t, *J* = 5.0 Hz), 6.49 (2H, s), 6.78 – 6.87 (2H, m), 7.10 (1H, s), 7.16 (1H, d, *J* = 7.8 Hz), 7.21 (1H, dd, *J* = 1.4, 8.3 Hz), 7.42 (1H, t, *J* = 7.8 Hz), 7.63 (1H, s), 7.70 (1H, d, *J* = 8.2 Hz), 7.87 (1H, dd, *J* = 1.3, 8.0 Hz), 10.17 (1H, s), 14.21 (1H, s).

To a solution of the above product **(53)** (0.065 g, 0.16 mmol) and sodium iodide (0.007 g, 0.05 mmol) in acetonitrile (5 mL) was added piperidine (0.16 mL, 1.63 mmol), and the mixture was stirred at 75 °C overnight. After cooled to room temperature, the resulting mixture was diluted with water and extracted with ethyl acetate. The separated organic phase was washed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduce pressure. The residue was purified by column chromatography on silica-gel (chloroform: methanol, 85:15) to give the title compound **(54)** (0.055 g, 75% yield) as a white foam; LCMS (ES) *m/z* 447 (M + H)$^+$; $^1$H NMR (300 MHz, DMSO-*d6*) δ 1.30 – 1.60 (6H, m), 2.29 – 2.75 (8H, m), 4.34 (2H, d, *J* = 5.0 Hz), 5.08 (1H, t, *J* = 5.0 Hz), 6.49 (2H, s), 6.78 – 6.87 (2H, m), 7.10 (1H, s), 7.14 (1H, d, *J* = 7.7 Hz), 7.19 – 7.26 (1H, m), 7.40 (1H, t, *J* = 7.8 Hz), 7.61 (1H, *J* = 8.1 Hz), 7.86 (1H, dd, *J* = 1.4, 8.1 Hz), 10.28 (1H, s), 14.22 (1H, s).

**2-Amino-4-(1-amino-2-phenyl-ethyl)-6-(2-hydroxy-phenyl)-nicotinonitrile hydrochloride (55)**

A mixture of 1-[2-(benzyloxy)phenyl]ethanone (2.00 g, 8.84 mmol), *N*-(*tert*-butyloxylcarbonyl)phenylalaninal (4.41 g, 17.68 mmol), malononitrile (1.17 g, 17.68 mmol), ammonium acetate (1.70 g, 22.10 mmol) and toluene (30 mL) was stirred at 120 °C for 3 hours in a sealed reaction vessel. After cooled to room temperature, the reaction mixture was diluted with ethyl acetate. The organic layer was washed with aqueous saturated $NaHCO_3$ solution and brine successively, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane: ethyl acetate, 4:1 to 2:1) to give {1-[2-Amino-6-(2-benzyloxy-phenyl)-3-cyanopyridin-4-yl]-2-phenylethyl}carbamic acid *tert*-butyl ester **(56)** (2.246 g, 49% yield) including some impurities as an amorphous solid; LCMS (ES) *m/z* 521 $(M + H)^+$.

A mixture of the compound **(56)** (1.10 g, 2.11 mmol), 10% Pd-C (0.30 g), ethyl acetate (30 mL) and THF (5 mL) was hydrogenated at 3 atm for 3 days. The mixture was diluted with THF, filtered on Celite®, and washed with THF. The combined filtrates were concentrated in vacuo. The residual solid was washed with ether, and dried in vacuo to give {1-[2-Amino-3-cyano-6-(2-hydroxyphenyl)pyridin-4-yl]-2-phenylethyl}carbamic acid *tert*-butyl ester **(57)** (0.810 g, 89% yield) as a white solid; LCMS (ES) *m/z* 431 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 1.28 (9H, s), 2.90 – 2.93 (2H, br m), 4.86 – 4.92 (1H, m), 6.90 – 6.99 (2H, m), 7.19 – 7.39 (6H, m), 7.37 (2H, s), 7.55 (1H, s), 7.68 (1H, d, $J$ = 8.6 Hz), 7.90 (1H, dd, $J$ = 1.3, 8.1 Hz), 13.37 (1H, s).

To a suspension of the compound **(57)** (0.410 g, 0.952 mmol) in 1,4-dioxane (7 mL) was added a 4N HCl solution in 1,4-dioxane (10 mL), and the stirring was continued 3 hours. The resultant precipitate was collected by filtration, washed with

1,4-dioxane, and dried in vacuo to give the title compound **(55)** as a pale yellow solid; m.p. 190 °C (dec.); LCMS (ES) *m/z* 331 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 3.18 (1H, dd, J = 9.4, 13.2 Hz), 3.21 (1H, dd, J = 5.7, 13.2 Hz), 4.54 (1H, br), 6.91 - 6.99 (2H, m), 7.10 - 7.13 (2H, m), 7.20 - 7.60 (7H, m), 8.06 (1H, s), 8.32 (1H, d, J = 7.9 Hz), 9.05 (3H, br).

In similar manners as described in the example above to synthesize compound **(57)**, following compounds **(58 –66)** shown in Table 5 were synthesized.

**2-Amino-4-(1-aminoethyl)-6-(2-hydroxyphenyl)nicotinonitrile (58)**; LCMS (ES) *m/z* 255 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 1.58 (3H, d, J = 6.9 Hz), 4.45 (1H, m), 6.91 - 6.96 (2H, m), 7.34 - 7.40 (1H, m), 7.90 (1H, s), 8.06 (1H, d, J = 8.0 Hz), 8.94 (1H, br s).

**2-Amino-4-(1-aminopentyl)-6-(2-hydroxyphenyl)nicotinonitrile (59)**; LCMS (ES) *m/z* 297 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 0.85 (3H, t, J = 6.7 Hz), 1.17 - 1.30 (4H, m), 1.88- 1.94 (1H, m), 1.97 - 2.12 (1H, m), 4.29 (1H, br s), 6.93 (2H, t, J = 6.5 Hz), 7.37 (1H, t, J = 7.6 Hz), 7.60 (1H, br s), 7.92 (1H, s), 8.07 (1H, dd, J = 7.8, 14.5 Hz), 8.99 (2H, s).

**2-Amino-4-(1-amino-2-methylpropyl)-6-(2-hydroxyphenyl)nicotinonitrile (60)**; LCMS (ES) *m/z* 283 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 0.64 (3H, d, J = 6.8 Hz), 1.08 (3H, d, J = 6.9 Hz), 4.96 (1H, s), 6.90 - 6.95 (2H, m), 7.36 (1H, t, J = 8.4 Hz), 7.61 (1H, s), 7.72 (2H, br s), 8.05 (1H, d, J = 7.0 Hz), 10.21 (1H, s), 13.65 (1H, s).

**2-Amino-4-[1-amino-2-(4-methoxyphenyl)-ethyl]-6-(2-hydroxyphenyl) nicotinonitrile (61)**; LCMS (ES) *m/z* 361 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 3.11 - 3.24 (1H, m), 3.27 - 3.45 (1H, m), 3.68 (3H, s), 5.24 (1H, br s), 6.79 (2H, d, J =

8.8 Hz), 6.88 - 6.99 (2H, m), 7.08 (2H, d, J = 8.8 Hz), 7.33 - 7.41 (3H, m), 7.63 (1H, s), 8.00 (1H, dd, J = 1.3, 8.2 Hz), 10.14 (1H, s).

**2-Amino-4-[1-amino-2-(4-fluorophenyl)-ethyl]-6-(2-hydroxyphenyl)nicotinonitrile (62)**; LCMS (ES) *m/z* 349 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 3.15 - 3.23 (1H, m), 3.47 - 3.53 (1H, m), 4.47 - 4.51 (1H, m), 6.91 - 6.98 (2H, m), 7.06 - 7.20 (4H, m), 7.35 - 7.48 (1H, m), 8.11 (1H, s), 8.13 - 8.17 (1H, m), 9.10 (2H, br s).

**2-Amino-4-(1-amino-3-phenylpropyl)-6-(2-hydroxyphenyl)nicotinonitrile (63)**; LCMS (ES) *m/z* 345 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 2.08 (1H, m), 5.03 (1H, dd, J = 4.1, 6.8 Hz), 6.92 (2H, m), 7.15 - 7.39 (6H, m), 7.70 (3H, d, J = 5.6 Hz), 8.06 (1H, dd, J = 1.3, 8.3 Hz), 8.87 (1H, br), 9.25 (1H, m), 10.34 (1H, m), 13.69 (1H, s).

**2-Amino-4-(2-aminoethyl)-6-(2-hydroxyphenyl)nicotinonitrile (64)**; LCMS (ES) *m/z* 255 $(M + H)^+$; $^1H$ NMR (500 MHz, DMSO-*d6*) δ 3.03 (2H, t, J = 7.5 Hz), 3.23 (2H, t, J = 7.7 Hz), 6.89 - 6.99 (2H, m), 7.35 (2H, t, J = 7.1 Hz), 7.41 (2H, br s), 7.88 - 7.98 (4H, m), 13.38 (1H, s).

**2-Amino-4-(2-aminopropyl)-6-(2-hydroxyphenyl)nicotinonitrile (65)**; LCMS (ES) *m/z* 269 $(M + H)^+$; $^1H$ NMR (300 MHz, DMSO-*d6*) δ 1.21 (3H, d, J = 6.4 Hz), 2.88 - 3.08 (2H, m), 3.73 (1H, br s), 6.89 (1H, s), 6.92 (1H, br s), 7.35 (2H, t, J = 6.8 Hz), 7.43 (1H, br s), 7,95 (3H, br s), 13.36 (1H, s).

**2-Amino-4-(2-amino-2-phenylethyl)-6-(2-hydroxyphenyl)nicotinonitrile (66)**; FAB-MS *m/z* 331 $(M + H)^+$; 3.26 - 3.34 (1H, m), 3.48 - 3.57 (1H, m), 4.02 - 4.80 (1H, m), 6.87 - 6.93 (2H, m), 7.30 - 7.43 (1H., m), 7.53 (2H, d, J = 6.4 Hz), 7.89 (1H, d, J = 8.8 Hz), 8.79 (2H, br s).

**[Pharmacological Experiments]**

**IKK-β kinase inhibitory assay**

(1) Preparation of IKK-β kinase protein.

A cDNA fragment encoding human IKK-β open reading frame was generated by PCR with the use of a pair of primers designed from the published sequence (Woronicz JD et al. (1997) Science 278, 866-869). A template was obtained from Quickclone cDNA (Clontech) using Elongase™ Amplification kit (Life Technologies). The DNA fragments generated by PCR were gel-purified and subcloned into pBluescript. The cDNA fragment cloned in pBluescript was inserted into pcDNA3.1/His C KpnI/NotI, and transferred into pVL1393 SmaI/XbaI (Pharmingen) to construct a baculovirus transfer vector. Then the vector, together with the linearized baculovirus (BaculoGold™, Pharmingen) was used to transfect Sf21 cells (Invitrogen, San Diego, CA). Generated recombinant baculovirus was cloned and amplified in Sf21 cells, grown in TNM-FH insect cell medium (Life Technologies, Inc.) supplemented with 10% FCS, 50 g/ml Gentamycin, 0.1% Pluronic F-68 (Life Technologies, Inc.) as suspension culture (200 ml in 1 L Erlenmeyer flask; 27°C; 130 rpm). Sf21 cells were infected with this amplified virus with a multiplicity of infection of 5 following standard protocols (Crossen R, Gruenwald S (1997) Baculovirus Expression Vector System Instruction Manual, Pharmingen Corporation) and harvested 48 hrs later. The cells were lysed to obtain the produced chimeric protein of IKK-β kinase fused by histidine (His-tagged IKK-β).

(2) The preparation of purified GST-IκBα fusion proteins

An expression vector containing the nucleotide sequence encoding fusion protein of GST with amino acid residues 1 to 54 of IκBα under the control of an IPTG-

inducible promoter was constructed. The expression vector was introduced in E. coli and the transformant was cultured and lysed to obtain a GST-IκBα fusion protein. Then the resulting GST-IκBα fusion protein was purified and biotinated for kinase assay.

(3) The measurement of IKK-β kinase activity

The 96-well format kinase assay of IKK-β were performed to test the inhibitory activity of the compounds of the present invention. First, 5 μl of a test compound was put in the presence of 2.5% dimethyl sulfoxide (DMSO) in each well in a U-bottomed 96-well plate (Falcon). For control wells of background (BG) and total phosphorylation (TP), 5 μl of 2.5% DMSO was put. Recombinant IKK-ß (final 0.6 μg/ml) and bio-GST-IκBα(1-54) (final 0.2 μM) were diluted in 25 μl of 2 x kinase buffer ß (40 mM Tris-HCl, pH 7.6, 40 mM $MgCl_2$, 40 mM ß-glycerophosphate, 40 mM p-nitrophenylphosphate, 2 mM EDTA, 40 mM creatine phosphate, 2 mM DTT, 2 mM $Na_3VO_4$, 0.2 mg/ml BSA and 0.8 mM phenylmethylsulfonyl fluoride) and transferred to the 96-well plate. Bio-GST-IκBα(1-54) in 25 μl of 2 x kinase buffer ß without IKK-ß was transferred to BG wells. Then 20 μl of 12.5 μM ATP, 62.5 μCi/ml [$\gamma$-$^{33}$P] ATP (Amersham Pharmacia Biotech) was added and the resulting mixture was incubated for 2 hours at room temperature. The kinase reactions were terminated by the addition of 150 μl of termination buffer (100 mM EDTA, 1 mg/ml BSA, 0.2 mg $NaN_3$). One handred and fifty μl of the sample were transferred to a streptavidin-coated, white MTP (Steffens Biotechniche Analysen GmbH #08114E14.FWD) to capture the biotinylated substrates. After 1 hr of incubation, non-bound radioactivity was eliminated by washing the wells five times with 300 μl of washing buffer including 0.9 % NaCl and

0.1% (w/v) Tween-20 with the use of a MW-96 plate washer (BioTec). The bound radioactivity was determined after the addition of 170 µl MicroScint-PS scintillation cocktail (Packard) using a TopCount scintillation counter.

**The measurement of RANTES production in response to TNF-α from A549 cells**

(1) Preparation of A549 cells

The A549 human lung epithelium cell line (ATCC #CCL-885) was maintained in Dulbecco's modified Eagle's medium (D-MEM, Nikken Biomedical Institute) supplemented with 10% FCS (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine (culture medium). Forty thousand ($4 \times 10^4$) cells (80 µl/well) were seeded in each well of 96 well flat-bottom tissue culture plate (Falcon #3072). The plate was allowed to stand for 2 hrs, thus the cells were adhered to the bottom of each well. To the each well was added 10 µl vehicle (1% DMSO), serial dilutions of test compounds in 1% DMSO, or 5 nM Dexamethasone in 1% DMSO as a reference. The mixture (90 µl/well) was incubated for 1 hr at 37°C. After 1 hr, 1 µg/ml TNF-α (10 µl) in culture medium was added to the mixture to obtain 100 µl of reaction mixture. The reaction mixture was cultured for 24 hrs to stimulate the cells with 100 ng/ml TNF-α. Cells with vehicle without TNF-α stimulation were also prepared.

(2) Measurement of RANTES production

Then the concentration of RANTES released from the cells in the supernatants of each well was determined using a quantitative sandwich enzyme immunoassay technique. First, 2µg/ml mouse anti-huRANTES mAb (R&D Systems, #mAb678) in PBS buffer (pH 7.4, 100µl) was put in each well of 96-well NUNC fluoro plate (Nalge

Nunc, New York USA) (Final 200ng/well) and the plate was allowed to stand for overnight at 4°C to be coated by the antibody. Each well of the plate was then washed with 350 µl wash buffer (0.05% Tween-20, 0.85% NaCl, and 25 mM Tris/HCl pH7.4) for three times. Blocking buffer containing 1% BSA (Sigma、99% pure, 100 g), 5% sucrose (Nacalai tesque, 99% pure, 500 g), and 0.02% azide (Nacalai tesque, 100%, 500 g) were added (200 µl) to each well and then the plate was allowed to stand for 4 hours to stabilize the coated antibody. Next, 50 µl supernatants of cell culture prepared in (1) above were put in each well of the 96-well NUNC fluoro plate with coated antibody. Recombinant Human RANTES (Pepro Tech, Inc. #300-06) was used as the standard for the determination of RANTES production (linear range between 1 and 10 ng/ml). Eu-labelled mouse anti-huRANES mAb (60 ng/ml: R&D Systems, #mAb278) in PBS supplemented by 1% BSA and 0.05% Tween 20 was added (50 µl) to each well. The reaction mixtures were incubated at room temperature for 4 hrs. After washing with wash buffer (0.05% Tween-20, 0.85% NaCl, and 25 mM Tris/HCl pH7.4, 350 µl/well) for 5 times with the use of a Sera Washer (Bio-Tech, #MW-96R), the enhancement solution (DELFIA, #1244-405, 100 µl/well) was added to each well. The plate was incubated for 10 minutes at room temperature with moderate shaking. Fluorescent intensity was measured using a DELFIA fluorimeter (Wallac). Excitation was performed at 340 nm and emission was measured at 615 nm.

**Mouse LPS-induced TNF-α production**

Eight weeks old BALB/c female mice were placed into two groups, a control group and a treated group. A solution containing 200 µg/mouse of LPS in 0.9%

physiological salt was administered by intraperitoneal (ip) injection into the control mice. Mice in the treated group were first injected ip with compounds of the present invention 30 minutes prior to the LPS injection. Under anesthesia with pentobarbital (80 mg/kg, i.p.), blood was collected from the posterior venous cavity of the treated and control mice at 90 min post-LPS injection into 96-well plate containing 2% EDTA solution. The plasma was separated by centrifugation at 1800 rpm for 10 minutes at 4°C and then diluted with four times volumes of phosphate buffer saline (pH 7.4) containing 1% bovine serum albumin. TNF-α concentration in the sample was determined using an ELISA kit (Pharmingen, San Diego, CA.)

The mean TNF-α level in 5 mice from each group was determined and the percent reduction in TNF-α levels was calculated. The treated mice showed significant decrease in the level of TNF-α as compared to the control mice. The result indicates that the compounds of the present invention can restrain LPS-induced cytokine activity.

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[17] 2-(3-Nitrobenzyl)malononitrile; m.p. 133.4-134.5 °C; $^1$H NMR (300 MHz, $CDCl_3$) δ 3.42 (2H, d, $J$ = 6.8 Hz), 4.02 (1H, t, $J$ = 6.8 Hz), 7.62 – 7.73 (2H, m), 8.22 – 8.30 (2H, m).

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# Conclusion

The aim of the research described in this thesis is to develop and synthesize novel compounds for the treatment of asthma, which is one of the most common chronic diseases worldwide and increasing in prevalence. In this thesis, developments of both relievers and controllers as novel medications for asthma are discussed. The results obtained through this work are summarized as follows.

In Chapter 1, discovery and synthesis of endothelin $ET_B$ selective antagonists are described. Combination of rational approaches, based on the structure-activity correlation of ET-1 fragment and structural homology of the rhodopsin superfamily of receptors, successfully led to the discovery of early lead compounds, which was further optimized the more potent and selective $ET_B$ antagonists with high inhibitory activity on ET-3-induced trachea contraction. With an increase in evidence for the involvement of the $ET_B$ receptor in a multitude of endothelin actions, these novel $ET_B$ receptor antagonists will be useful tools for demonstrating the role of $ET_B$ receptor subtypes in physiological study.

In Chapter 2, discovery and synthesis of novel and potent $ET_A$ and $ET_B$ dual endothelin antagonists are described. The $ET_A$ and $ET_B$ dual endothelin antagonists were identified from further optimization of the $ET_B$ selective antagonists described in Chapter 1. For a chiral synthesis of the promising analogs, new synthetic methodologies, the synthesis of acylsulfonamide derivatives without racemization and the stereoselective amide formation by a kinetic resolution with *in-situ* recycling of the substance, were developed. The potent $ET_A$ and $ET_B$ dual endothelin antagonists are expected to work as relievers with a novel mechanism for the bronchoconstriction in the

respiratory diseases, such as asthma.

In Chapter 3, discovery and synthesis of novel and potent IKK-β inhibitors are described. The lead compound, 2-amino-3-cyano-4-aryl-6-(2-hydroxyphenyl)pyridine analog, was identified from high-throughput screening of the Bayer compound library. The pyridine core structures were simply constructed using a one-pot coupling reaction of four components, acetophenone, benzaldehyde, malononitrile and ammonium acetate. Improvement of the pyridine synthesis provided 2-amino-4,6-diarylnicotinic acid derivatives, which have an ample possibility for further derivations at the C-3 moiety on the core structure. Optimization of the lead compound resulted in a marked improvement in IKK-β inhibitory activity ($IC_{50}$ < 100 nM). These selective and potent IKK-β inhibitors are expected to have a strong anti-inflammatory effect with the potential of improving the underlying causes of asthma and replace glucocorticoids in asthma treatment.

## List of Publications


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## List of Supplementary Publications

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