Discovery of Novel and Potent Small-Molecule Inhibitors of NO and Cytokine Production as Antisepsis Agents: Synthesis and Biological Activity of Alkyl 6-(N-Substituted sulfamoyl)cyclohex-1-ene-1-carboxylate

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To develop a new therapeutic agent for sepsis, screening of the Takeda chemical library was carried out using mouse macrophages stimulated with lipopolysaccharide (LPS) to identify a new class of small-molecule inhibitors of inflammatory mediator production. The lead compound **5a** was discovered, from which a series of novel cyclohexene derivatives **I** bearing a sulfamoyl and ester group were designed, synthesized and tested for their inhibitory activity against nitric oxide (NO) production. Derivatives **I** were synthesized by the coupling of sulfonyl chlorides and anilines with concomitant double bond migration in the presence of triethylamine, and phenyl ring substitution and modification of the ester and cyclohexene moieties were carried out. Among the compounds synthesized, ethyl (6*R*)-6-[*N*-(2-chloro-4-fluorophenyl)sulfamoyl]-cyclohex-1-ene-1-carboxylate [(*R*)-(+)-**5n**, TAK-242] was found to exhibit the most potent suppressive activity for the production of not only NO but also inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) induced by LPS-stimulated mouse macrophages with IC_{50} values of 1.8, 1.9 and 1.3 nM, respectively. It shows marked beneficial effects in vivo also. Intravenous administration of (*R*)-(+)-**5n** at doses of 0.1 mg/kg or more suppressed the production of NO and various cytokines [TNF-α, IL-6 and IL-1*β*] in the mouse endotoxin shock model. Furthermore, it protected mice from death dose-dependently and all mice survived at a dose of 3 mg/kg. The minimum effective dose to protect mice from lethality in this model was 0.3 mg/kg, which was consistent with those for inhibitory effects on the production of NO and cytokines. Compound (*R*)-(+)-**5n** is currently undergoing clinical trials for the treatment of sepsis.

Introduction

The understanding of the pathophysiology of systemic inflammatory response syndrome (SIRS) has increased markedly over the past few years.[1] Sepsis is generally defined as SIRS induced by bacterial infection, and severe sepsis is defined as sepsis associated with acute organ dysfunction or hypoperfusion abnormalities.[2] Septic shock is a subset of severe sepsis and is characterized by hypotension refractory to treatment. In the United States, more than 500 000 patients per year develop sepsis. In particular, sepsis and SIRS are increasingly the chief causes of death in intensive care units, with mortality rates between 30 and 70%.[1b,3] Despite more than two decades of extensive research, the heterogeneity of the syndrome has meant that the development of an anti-sepsis agent has proved remarkably difficult.[4] Recently, Drotrecogin-α (Xigris), a recombinant human activated protein C, was launched to the market.[5] It lowered the mortality rate in severe sepsis patients from 31−25%[5c] and is the only drug to be approved for the treatment of severe sepsis. However, the observed mortality difference between Drotrecogin-α and placebo was limited to patients with high risk of death. In addition, since Drotrecogin-α increases the risk of bleeding, it is contraindicated in patients with active internal bleeding and recent intracranial surgery. Therefore, there is still a major unmet medical need for more effective and safer new antisepsis agents.

It is known that sepsis is caused by some bacterial components such as lipopolysaccharide (LPS), lipoteichoic acid, and peptidoglycan, which prompt host immune cells to activate and stimulate the production of various kinds of mediators [cytokines, nitric oxide (NO), lipid metabolites, etc.].[6] Overwhelming inflammatory and immune responses are essential features of sepsis and play a central part in the pathogenesis of tissue damage, multiple organ failure, and death induced by sepsis.[5] A number of inflammatory modifiers that suppress single inflammatory mediator production, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), NO, or platelet activating factor, have already entered clinical trials for the treatment of sepsis.[7] Unfortunately, most of the candidates which have been shown to be effective in preclinical studies have not demonstrated significant efficacy for sepsis patients.[4,7] Clinical trials of some of them are still ongoing. Thus, we considered that a drug suppressing multiple inflammatory mediators is necessary for clinical effectiveness.

LPS is an important component of the outer membrane of Gram negative bacteria and has a pivotal role in inducing sepsis.[6,8] LPS triggers the activation of the immune system through the induction of cytokine

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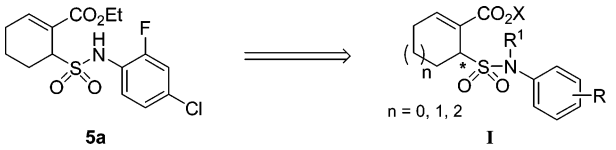


Figure 1. Structure of lead compound **5a** and cyclohexene derivatives **I**.

release, which leads to damaged blood vessels and a decrease in vascular resistance and subsequent collapse of organs and death. Therefore, one promising approach for treating sepsis is to target LPS itself. Various anti-LPS agents, such as anti-LPS antibodies, LPS-neutralizing proteins, polymyxin B, and LPS antagonists (for instance, lipid A analogue[9]) have been evaluated.

To discover new small molecule inhibitors of multiple inflammatory mediator production from mouse macrophages stimulated with LPS, screening of the Takeda chemical library was carried out. Thus, a novel cyclohexene derivative **5a** bearing a phenylsulfamoyl and ethyl ester group was identified, which showed suppressive effects on the production of NO, TNF-α, and interleukin-6 (IL-6) with 50% inhibitory concentration (IC_{50}) values of less than 1 μM in vitro. Furthermore, intraperitoneal administration of **5a** showed a potent protective effect at a dose of 10−30 mg/kg in mouse endotoxin shock model, which is a commonly used sepsis model.

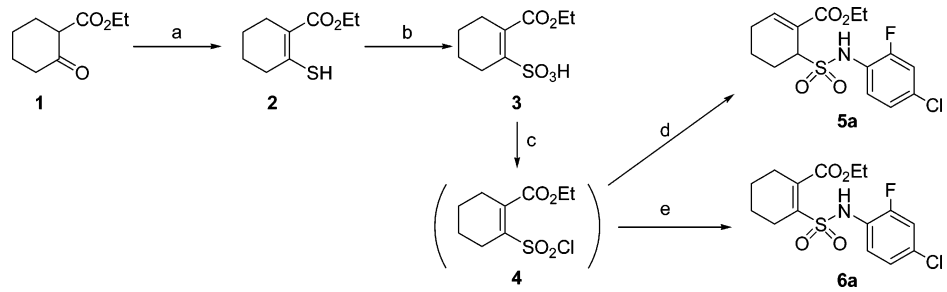
Chemical modification of lead compound **5a**, focusing on the benzene ring substituents, the ester moiety, and the cyclohexene ring (Figure 1) was carried out to improve the suppressive effects on the production of NO and various cytokines. In this paper, we describe the synthesis, structure−activity relationships, and biological properties of this novel series of cyclohexene derivatives.

Chemistry

The synthetic route to compound **5a** is shown in Scheme 1. The thioenolate **2**, prepared from commercially available ethyl 2-oxocyclohexanecarboxylate (**1**) according to the literature method,[10] was oxidized with sodium trioxoborane tetrahydrate in acetic acid (AcOH) to give the crude sulfonic acid **3**. Subsequent chlorination was carried out by reaction of **3** with thionyl chloride ($SOCl_2$), and the resulting sulfonyl chloride **4** was used without purification. Coupling of **4** with 4-chloro-2-fluoroaniline in the presence of triethylamine (Et_3N) gave exclusively the double bond isomerized product **5a**. The structure of **5a** was determined by the ^1H NMR chemical shifts of the cyclohexene, where the olefinic and methine protons of **5a** were observed at δ 7.12 (1H) and 4.32 (1H) and were assigned to the 2- and 6-position of the cyclohexene ring, respectively. However, when reaction of **4** with 4-chloro-2-fluoroaniline was carried out in the absence of Et_3N in *N*,*N*-dimethylformamide (DMF), only product **6a** (structure determined by ^1H NMR) was obtained in a low yield without double bond isomerization.

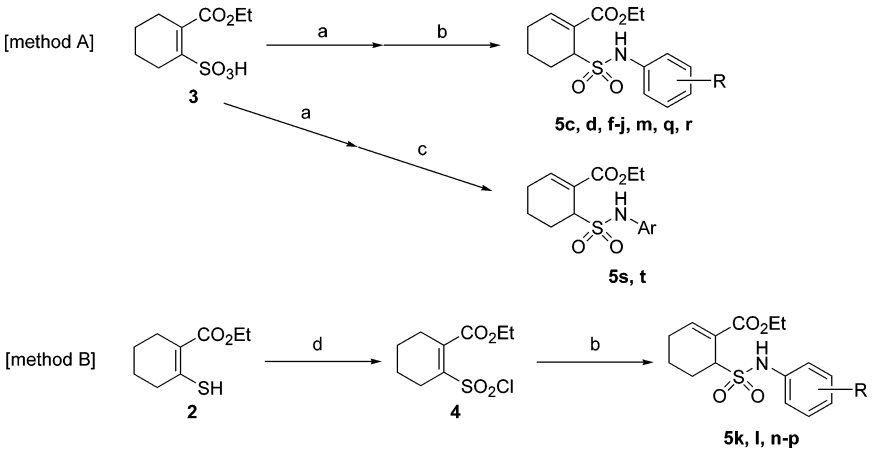
Chemical modification of the substituents on the phenyl ring was performed according to the synthetic route shown in Scheme 2. Substituted aniline derivatives **5c**,**d**,**f**−**j**,**m**,**q**,**r** were prepared from **3** under the same conditions as used in the synthesis of **5a** (method A). Thus, the sulfonic acid **3** was converted to sulfonyl

Scheme 1. Synthesis of Compound **5a**[a]



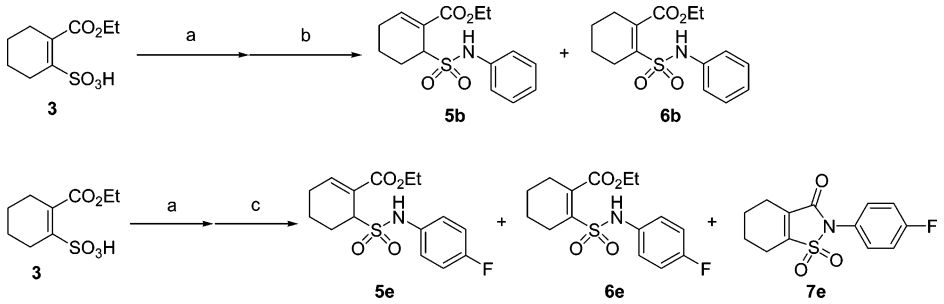
[a] Reagents: (a) H_2S/HCl, EtOH; (b) $NaBO_3 \cdot 4H_2O$, AcOH; (c) $SOCl_2$; (d) 4-chloro-2-fluoroaniline, Et_3N, AcOEt; (e) 4-chloro-2-fluoroaniline, DMF.

Scheme 2. Synthesis of the *N*-Arylsulfamoyl Derivatives[a]



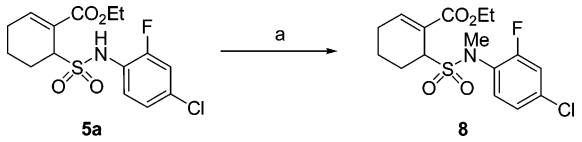
[a] Reagents: (a) $SOCl_2$; (b) substituted aniline, Et_3N, AcOEt; (c) 2-aminothiazole or 2-aminothiadiazole, Et_3N, AcOEt; (d) Cl_2, AcOH−H_2O.

Scheme 3. Synthesis of *N*-(Phenyl- and 4-Fluorophenyl)sulfamoyl Derivatives[a]



[a] Reagents: (a) $SOCl_2$; (b) aniline, Et_3N, AcOEt; (c) 4-fluoroaniline, Et_3N, AcOEt.

Scheme 4. N-Methylation of Compound **5a**[a]



[a] Reagents: (a) MeI, K_2CO_3.

chloride without purification and subsequent treatment with various anilines in the presence of Et_3N to give **5c**,**d**,**f**−**j**,**m**,**q**,**r**. With a similar method, thiazole and thiadiazole derivatives **5s**, **5t** were synthesized. Alternatively, chlorination of **2** with chlorine gas in AcOH-water followed by chromatography on silica gel gave purified sulfonyl chloride **4**,[11] which were reacted with anilines in the presence of Et_3N to give compounds **5k**, **l**, **n**−**p** (method B). In most cases, both methods A and B exclusively gave the double bond isomerized products **5**. However, when unsubstituted aniline was used, **5b** and nonisomerized product **6b** were obtained, as shown in Scheme 3. Additionally, the reaction of **4** with 4-fluoroaniline gave a mixture of **5e**, nonisomerized product **6e** and cyclized product **7e**, which was formed by intramolecular cyclization of **6e**. On the other hand, the reaction of **4** with 4-methoxyaniline gave predominantly the nonisomerized product (data not shown). From these results, it seems that the formation of the isomerized cyclohexene derivative depends on the nucleophilicity of the reactant aniline.

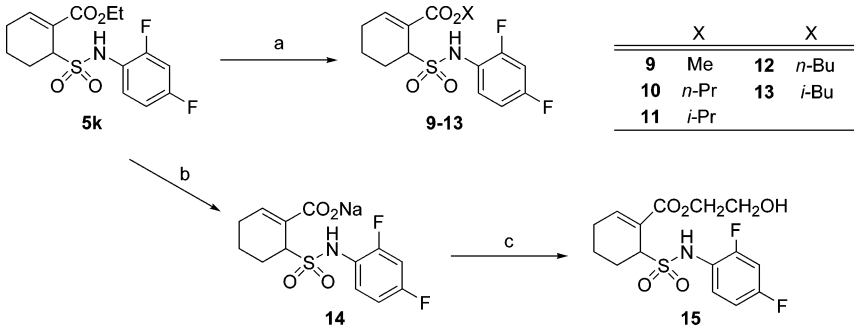
Methylation of the sulfonamide nitrogen with methyl iodide and potassium carbonate proceeded in near-quantitative yield to give the *N*-methylsulfonamide **8** (Scheme 4).

Conversion of the ethyl ester was carried out using the 2,4-difluorophenyl derivative **5k** shown in Scheme 5, since **5k** showed more potent activity than **5a**. The esters **9**−**13** were prepared by transesterification with the appropriate alcohol in the presence of a catalytic amount of concentrated sulfuric acid. Hydroxyethyl ester **15** was synthesized via the sodium salt of carboxylic acid **14**. Thus, **5k** was converted to sodium carboxylate **14** by alkaline hydrolysis with sodium hydroxide (NaOH) followed by purification using highly porous polymer resin (CHP-20) and subsequent alkylation of **14** with 2-bromoethanol gave **15**.

The synthesis of cyclopentene and cycloheptene derivatives, **19** and **23**, was accomplished as outlined in Scheme 6. Compound **17** obtained from the commercially available cyclopentene ester **16** using Lawesson's reagent was converted in three steps into the corresponding sulfonyl chloride **18** by the same synthetic route used for **5a**. After purification of **18** using silica gel chromatography, coupling reaction with 2-chloro-4-fluoroaniline gave **19**. The cycloheptene derivative **23** was also prepared from the cycloheptene ester **20**, derived from cycloheptanone according to Mague's method,[12] using the same conditions employed for the preparation of **19**. The ring-opened derivative **28** was synthesized from **25**, prepared from ethyl 3-oxohexanoate,[13] via the corresponding sulfonyl chloride **27** by the same procedure used for **5a**. Benzenesulfonyl chloride **29** was reacted with 2-chloro-4-fluoroaniline to give **30**, followed by treatment with sodium methoxide in ethanol to afford the benzene derivative **31** (Scheme 6).

Among the compounds prepared, compound **5n** showed the most potent suppressive effects on the production of NO, and therefore separation of the enantiomers of **5n** was carried out. Racemic mixture **5n** was optically resolved by preparative high-performance liquid chromatography (HPLC) using a chiral column [CHIRALPAK AD,[14] hexane/ethanol = 9:1] to give each enantiomers, (+)-**5n** and (−)-**5n**, in high enantiomeric purity (>99%ee). These enantiomers were submitted for X-ray crystallographic analysis and the absolute configuration

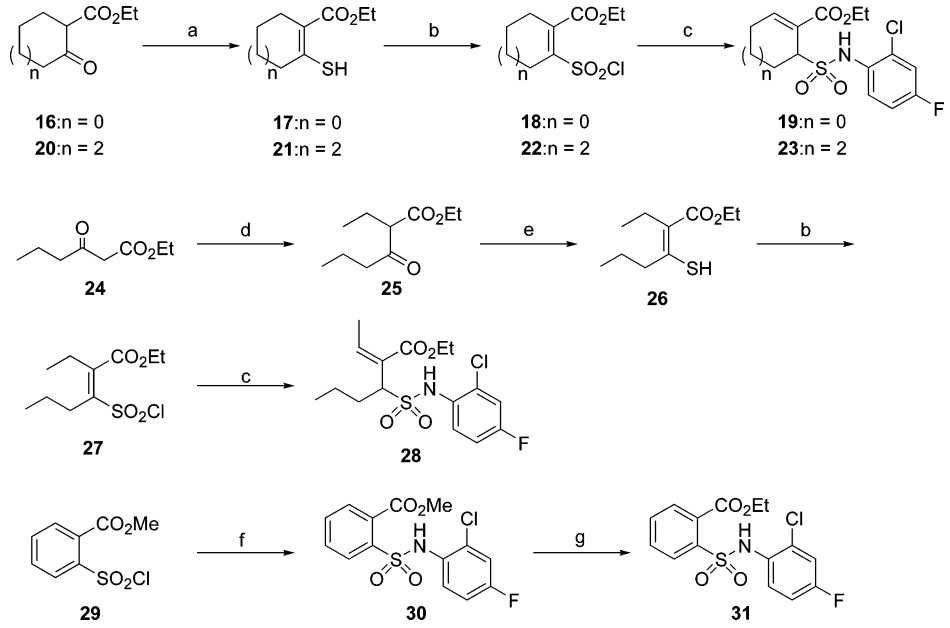
Scheme 5. Variation of the Ester Group Using 2,4-Difluorophenyl Derivative **5k**[a]



	X		X
9	Me	**12**	*n*-Bu
10	*n*-Pr	**13**	*i*-Bu
11	*i*-Pr		

[a] Reagents: (a) X-OH, c.H_2SO_4; (b) 1 N NaOH, MeCN; (c) $Br(CH_2)_2OH$, DMF.

Scheme 6. Modification of the Cyclohexene Ring[a]



[a] Reagents: (a) Lawesson's reagent; (b) $NaBO_3 \cdot 4H_2O$, AcOH, then $SOCl_2$; (c) 2-chloro-4-fluoroaniline, Et_3N, AcOEt; (d) EtI, NaOEt, EtOH; (e) H_2S, HCl, EtOH; (f) 2-chloro-4-fluoroaniline, pyridine, CH_2Cl_2; (g) NaOMe, EtOH.

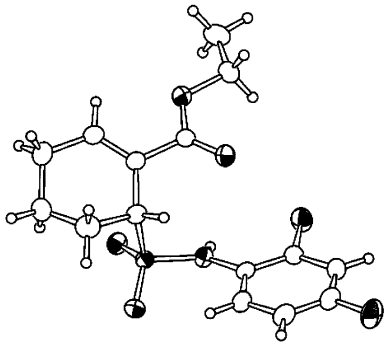


Figure 2. X-ray crystal structure of (*R*)-(+)-**5n**.

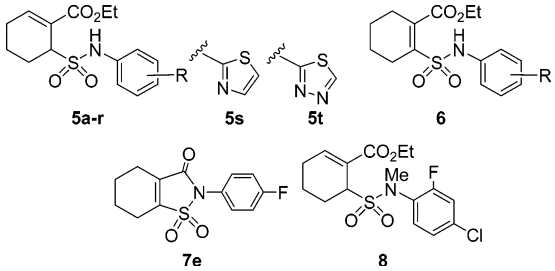
of (+)-**5n** and (−)-**5n** were confirmed to be (*R*)- and (*S*)-form, respectively. The X-ray crystal structure of (*R*)-(+)-**5n** is shown in Figure 2.

Results and Discussion

The compounds were evaluated for their suppressive activity against NO production using a murine macrophage cell line, RAW264.7, induced by LPS as the first in vitro screen. The results are summarized in Tables 1−4 as IC_{50} values.

We initially examined the effect of the substituent on the phenyl ring of **5a**. As shown in Table 1, lead compound **5a** with 4-chloro-2-fluorophenyl showed moderate potency (IC_{50} = 160 nM), but removal of the two halogen atoms on the phenyl ring resulted in a ca. 2-fold decrease in activity (**5b**, IC_{50} = 260 nM). Introduction of a fluorine atom at the 2-, 3- or 4-position of the phenyl ring resulted in high potency (**5c**−**e**) compared to unsubstituted compound **5b**, with 2-fluoro derivative **5c** (IC_{50} = 75 nM) exhibiting activity over 3-fold greater than that of **5b**. In the case of substitution with a chlorine atom, 2-chloro derivative **5f** (IC_{50} = 12 nM) showed improved activity compared to 3- or 4-chloro derivatives (**5g**, **h**) and was 22-fold more potent than that of **5b**. These results suggested that either a chlorine

Table 1. Inhibitory Activities of *N*-Arylsufamoyl Derivatives **5a**−**t**, **6a**,**b**,**e**, **7e**, and **8** on NO Production



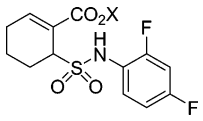
compd	R	IC_{50}[a] (nM)	compd	R	IC_{50}[a] (nM)
5a	2-F, 4-Cl	160 ± 65	**5n**	2-Cl, 4-F	3.2 ± 0.89
5b	H	260 ± 113	**5o**	2-Cl, 4-Me	41 ± 14
5c	2-F	75 ± 45	**5p**	2-Cl, 4-CN	1600 ± 172
5d	3-F	150 ± 14	**5q**	2-Et	130 ± 16
5e	4-F	110 ± 11	**5r**	2-CO_2Me	1100 ± 194
5f	2-Cl	12 ± 1.2	**5s**	−	>10000
5g	3-Cl	66 ± 19	**5t**	−	>10000
5h	4-Cl	400 ± 279	**6a**	2-F, 4-Cl	1700 ± 495
5i	2,3-F_2	140 ± 49	**6b**	H	>8200
5j	2,6-F_2	160 ± 4.9	**6e**	4-F	1400 ± 363
5k	2,4-F_2	16 ± 1.4	**7e**	−	4100 ± 1742
5l	2,4,5-F_3	30 ± 1.8	**8**	−	230 ± 45
5m	2,4-Cl_2	20 ± 2.0			

[a] The inhibitory activity is shown as an IC_{50} value, which is the concentration of test compound required to suppress the production of NO by 50% of control. Values are the mean ± SD of two or three experiments.

or fluorine atom at the 2-position on the phenyl ring significantly increased the inhibitory activity against NO production.

Next, the effect of di- or trihalogeno substitution on the phenyl ring was examined. Introduction of a fluorine atom at the 3- or 6-position of the 2-fluorophenyl ring resulted in lower activity, while the 2,4-difluoro derivative **5k** (IC_{50} = 16 nM) showed a 5-fold enhancement in potency compared to 2-fluoro derivative **5c**. However, 2,4,5-trifluorophenyl derivative **5l** was less potent than 2,4-difluorophenyl **5k**. Furthermore, 2-chloro-4-fluorophenyl derivative **5n** showed strong activity with IC_{50}

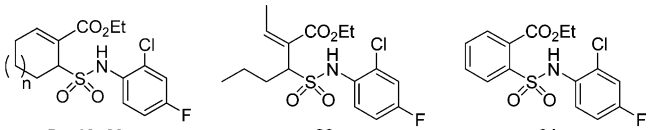
Table 2. Inhibitory Activities of Cyclohexene Derivatives **5k**, **9**−**13**, and **15** on NO Production



compd	X	IC_{50}[a] (nM)
5k	Et	16 ± 1.4
9	Me	57 ± 5.4
10	*n*-Pr	29 ± 7.2
11	*i*-Pr	110 ± 11
12	*n*-Bu	140 ± 5.3
13	*i*-Bu	49 ± 26
15	$(CH_2)_2OH$	330 ± 184

[a] The inhibitory activity is shown as an IC_{50} value, which is the concentration of test compound required to suppress the production of NO by 50% of control. Values are the mean ± SD of two or three experiments.

Table 3. Inhibitory Activities of Compounds **5n**, **19**, **23**, **28**, and **31** on NO Production



5n, 19, 23 **28** **31**

compd	*n*	IC_{50}[a] (nM)
5n	1	3.2 ± 0.89
19	0	110 ± 16
23	2	240 ± 44
28	–	2600 ± 124
31	–	>10000

[a] The inhibitory activity is shown as an IC_{50} value, which is the concentration of test compound required to suppress the production of NO by 50% of control. Values are the mean ± SD of two or three experiments.

value of less than 10 nM (IC_{50} = 3.2 nM), although 2,4-dichlorophenyl and 4-chloro-2-fluorophenyl derivatives (**5m** and **5a**) showed less activity than **5f** and **5c**, respectively. From these results, introduction of a fluorine atom at the 4-position of the phenyl ring resulted in a 4-fold enhancement in potency, whereas substitution with a chlorine atom at the 4-position decreased the inhibitory activity.

When an electron-withdrawing cyano group was introduced at the 4-position of the phenyl ring in the 2-chloro derivative **5f**, a remarkable decrease of activity was observed. In contrast, introduction of a small, slightly electron-donating methyl group in **5f** retained activity. In addition, substitution at the 2-position of the phenyl ring with ethyl (**5q**) and methoxycarbonyl (**5r**) groups showed less potency than for the 2-chlorophenyl derivative **5f**. Replacement of the phenyl group with heteroaryl groups such as thiazole (**5s**) and thiadiazole (**5t**) resulted in a loss of potency.

Comparison of the substituted phenyl derivatives indicated that the compounds having 2,4-dihalogeno or 2-halogeno substituents on the phenyl ring were found to have a strong activity and the order of their potency was 2-Cl-4-F > 2-Cl; 2,4-F_2; 2,4-Cl_2; 2-Cl-4-Me; 2-F >2-F-4-Cl. These results suggest that the potency of inhibitory activity depends significantly on the position and electronic properties of the phenyl ring substituents.

Introduction of a methyl group at the sulfamoyl moiety resulted in a slight decrease in potency. Furthermore, the IC_{50} values of the nonisomerized compounds **6a**, **6b**, and **6e**, and the ring closed compound **7e**, were 1700, 8200, 1400, and 4100 nM, respectively, which were over 10-fold less potent than the corresponding isomers **5a**, **5b**, and **5e**. It is suggested that the position of the double bond in the cyclohexene ring is crucial and plays an important role in its inhibitory effect.

Next, attention was focused on exchange of the ethyl ester group in 2,4-difluorophenyl derivative **5k** (Table 2). Replacement of the ethyl group with a methyl group resulted in a 4-fold reduction in activity. Substitution with isopropyl (**11**), butyl (**12**) and isobutyl (**13**) groups also showed over 3-fold less potency than **5k**, while the propyl ester **10** maintained activity. Additionally, the 2-hydroxyethyl ester derivative (**15**) showed 20-fold less potency than ethyl ester **5k**, suggesting that hydrophobic substituents are favorable for this moiety. Finally, we examined the effect of changing the size of the cyclohexene ring. As shown in Table 3, ring contraction of **5n** to the five-membered ring showed over 30-fold reduction in potency. Ring expansion to seven-membered ring also exhibited a 75-fold less potency than **5n**. Furthermore, ring-opening of cyclohexene (**28**) and replacement of cyclohexene ring with benzene ring (**31**) caused a marked decrease of potency.

From the results of the SAR study, the cyclohexene derivatives bearing the ester group at 1-position and substituted phenylsufamoyl group at 6-position had the inhibitory activity. It was suggested that the activity was influenced by the shape of molecule from the results of changing the size of cyclohexene ring, and that there might be at least two pockets in the binding site on target molecule (for the ester moiety and the phenyl moiety), since it seemed that each moiety was strictly recognized as mentioned above. Among all the compounds prepared, **5n**, having the combination of cyclohexene ring, ethyl ester group, and 2-chloro-4-fluorophenylsufamoyl group, exhibited the most potent suppressive activity for NO production, and the activities of the two enantiomers, (*R*)-(+)-**5n** and (*S*)-(−)-**5n**, were evaluated in order to investigate the stereochemical requirement for inhibition. As expected, a significant difference was observed between the enantiomers, and (*R*)-(+)-**5n** (IC_{50} = 1.8 nM) exhibited 350-fold more activity than (*S*)-(−)-**5n** (IC_{50} = 640 nM). In addition, the suppressive effects of **5n** and both enantiomers on the production of cytokines from LPS-stimulated RAW264.7 cells were examined. (*R*)-(+)-**5n** strongly suppressed the production of TNF-α and IL-6 with IC_{50} values of 1.9 and 1.3 nM, respectively (Table 4), which was 120-fold stronger than those of lead compound **5a**.

The in vivo efficacy of (*R*)-(+)-**5n** which was the highest inhibitor in the in vitro assay was examined using mouse endotoxin shock model. When administrated intraveniously 1 h before lethal LPS challenge, (*R*)-(+)-**5n** rescued mice in a dose-dependent manner (Figure 3). At a dose of 3 mg/kg, all mice survived and the minimum effective dose (ED_{50}) value of (*R*)-(+)-**5n** to protect mice from lethality was 0.3 mg/kg. In this model, TNF-α, IL-6, IL-1β, and NO levels in serum increased significantly after LPS challenge, and (*R*)-(+)-**5n** (0.1−3 mg/kg) suppressed the production of all cytokines and NO induced by LPS in a dose-dependent manner. At a

Table 4. In Vitro Inhibitory Activities of NO, TNF-α, and IL-6 Production of **5a**,**n**, (*R*)-(+)-**5n**, and (*S*)-(−)-**5n**

compd	IC_{50}[a] (nM)		
	NO	TNF-α	IL-6
5a	160 ± 65	230 ± 47	170 ± 20
5n	3.2 ± 0.89	3.4 ± 1.3	2.5 ± 0.24
(*R*)-(+)-**5n** (**TAK-242**)	1.8 ± 0.30	1.9 ± 0.54	1.3 ± 0.12
(*S*)-(−)-**5n**	640 ± 365	740 ± 242	480 ± 10

[a] The inhibitory activity is shown as an IC_{50} value, which is the concentration of test compound required to suppress the production of NO, TNF-α, and IL-6 by 50% of control. Values are the mean ± SD of three or four experiments.

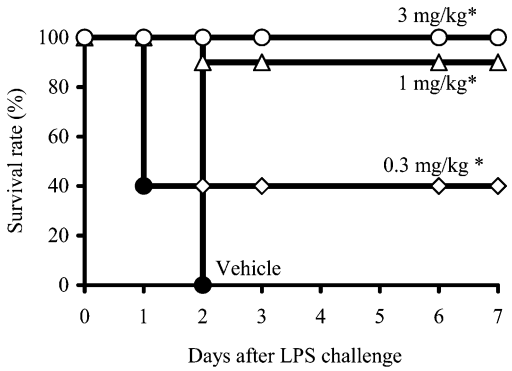


Figure 3. Effect of (*R*)-(+)-**5n** on LPS-induced lethality in mice. (*R*)-(+)-**5n** (0.3−3 mg/kg) was administered intravenously 1 h before LPS challenge (7 mg/kg, ip). *, $p \leq 0.05$ (Tarone's test with a simple closed step-down method) vs vehicle. Each group consisted of 10 mice.

dose of 0.1 mg/kg, (*R*)-(+)-**5n** significantly inhibited the production of all cytokines and NO (Figure 4).

Conclusion

In this study, screening for new small-molecule inhibitors of NO and inflammatory cytokines production led to discover of the lead compound **5a**. Cyclohexene derivatives **I** bearing a 6-(substituted phenyl)sulfamoyl and 1-(alkyl ester) group were designed and synthesized by coupling of sulfonyl chlorides with a variety of anilines in the presence of Et_3N with concomitant migration of the double bond. Chemical modification of the substituents on the phenyl ring, ethyl ester moiety, and cyclohexene ring was carried out and an identified novel series of cyclohexene derivatives exhibited strong suppressive effects on NO production from mouse macropharges stimulated with LPS. Among all the compounds prepared, (*R*)-(+)-**5n** exhibited the most potent inhibitory effect on the production of inflammatory cytokines such as TNF-α and IL-6 as well as NO. These IC_{50} values were approximately 100-fold stronger than those of lead compound **5a**. Furthermore, (*R*)-(+)-**5n** showed significant in vivo efficacies in mouse endotoxin shock model. The ED_{50} value to protect mice from lethality was 0.3 mg/kg, and at a dose of 3 mg/kg all mice survived. In addition, (*R*)-(+)-**5n** suppressed the increase in serum levels of inflammatory mediators such as TNF-α, IL-6, IL-1β, and NO.

Compound (*R*)-(+)-**5n** (TAK-242) was selected as a candidate for clinical investigation and is a promising new class of therapeutic agent for the treatment of sepsis. Although the molecular target of (*R*)-(+)-**5n** has not yet been identified, it is presumed that (*R*)-(+)-**5n** selectively inhibits the Toll-like receptor (TLR) 4-dependent signaling pathway.[15]

Experimental Section

Chemistry. All melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Gemini-200 (200 MHz) spectrometer and Varian Mercury-300 (300 MHz) in the solvent indicated. Chemical shifts are given in δ values (ppm) with

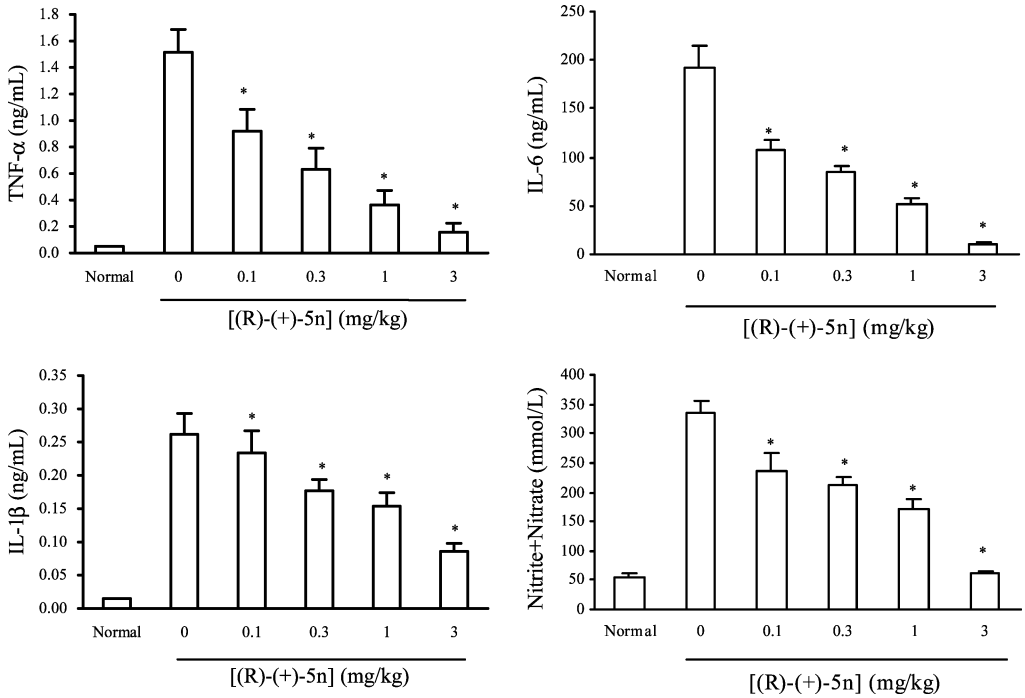


Figure 4. Effects of (*R*)-(+)-**5n** on LPS-induced increase in serum NO and proinflammatory cytokine levels in mice. (*R*)-(+)-**5n** (0.1−3 mg/kg) was administered intravenously 1 h before LPS challenge (7 mg/kg, ip). Sera for TNF-α, IL-6, IL-1β, and NO were collected at 1, 2, and 6 h after LPS challenge, respectively. Data are expressed as means ± SE of 8 mice. *, $p \leq 0.025$ (the one-tailed Shirley-Williams or Williams test) vs vehicle (0 mg/kg).

tetramethylsilane as an internal standard. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br = broad. Coupling constants (*J* values) are given in hertz (Hz). Infrared absorption spectra (IR) were recorded on a JASCO IR-810 spectrometer. The secondary ion mass spectra (SI-MS) were recorded with a Hitachi M-80A mass spectrometer. Optical rotations were determined with a JASCO DIP-181 or DIP-370 digital polarimeter. Elemental analyses were carried out by Takeda Analytical Laboratories Ltd. and were within ±0.4% of the theoretical values for the elements indicated unless otherwise noted.

Reactions were carried out at room temperature unless otherwise noted and followed by thin-layer chromatography (TLC) on silica gel 60 F_{254} precoated TLC plates (E. Merck) or by HPLC using an octadecyl silica (ODS) column (A-303, 4.6 mm i.d. × 250 mm, YMC Co., Ltd.). Standard workup procedures were as follows. The reaction mixture was partitioned between the indicated solvent and water. Organic extracts were combined and washed in the indicated order using the following aqueous solutions: water, 5% aqueous sodium carbonate solution (aqueous $NaHCO_3$), saturated sodium chloride (NaCl) solution (brine), 1 N aqueous sodium hydroxide solution (1 N NaOH) and 1 N hydrochloric acid (1 N HCl). Extracts were dried over anhydrous magnesium sulfate ($MgSO_4$), filtered, and evaporated in vacuo. Chromatographic separations were carried out on Silica gel 60 (0.063−0.200 mm, E. Merck) or ODS (CPO-273L, prepacked column, 22 mm × 300 mm, Kusano Kagaku Kikai Co.) using the indicated eluents. Yields were not maximized.

Ethyl 2-Mercaptocyclohex-1-ene-1-carboxylate (2).[10] Hydrogen sulfide (H_2S) gas was bubbled through a solution of ethyl 2-oxocyclohexanecarboxylate (**1**) (100 g, 538 mmol) in ethanol (1.0 L) at −50 °C for 2 h, and then hydrogen chloride (HCl) gas was bubbled into the reaction mixture at −20 °C for 2 h, followed by the bubbling of H_2S gas at −20 °C for 2 h. After having been allowed to stand for 14 h, the reaction mixture was poured into an ice−water (600 mL) and worked up [hexane (2.0 L); water (800 mL × 5)]. The residue was purified by distillation [bp 136−139 °C/15−16 mmHg (bp 134−135 °C/15−16 mmHg)[10]] to give **2** (79.5 g, 73%) as a colorless oil: IR ν_{max}(KBr), cm^{-1}: 2936, 1728, 1696, 1593, 1366, 1279, 1242, 1181, 1061. ^{1}H NMR ($CDCl_3$): δ 1.30 (3H, t, J = 7.0 Hz), 1.67 (3H, t, J = 3.0 Hz), 1.82−1.91 (1H, m), 2.24−2.35 (2H, m), 2.48 (2H, br s), 3.95 (1H, s), 4.22 (2H, q, J = 7.0 Hz).

2-(Ethoxycarbonyl)cyclohex-1-ene-1-sulfonic Acid (3). A solution of **2** (30.0 g, 161 mmol) in AcOH (50 mL) was added dropwise to a stirred solution of sodium trioxoboran tetrahydrate (74.3 g, 483 mmol) in AcOH (400 mL) for 2 h at 50−55 °C. The resulting mixture was stirred for 3 h at 50−55 °C and for 9 h at 80−85 °C, and which was then cooled and evaporated under reduced pressure to dryness. Acetonitrile (MeCN) (660 mL) was added to the residue, and the whole was stirred for 1 h followed by filtration to remove the insoluble substances. After the filtrate was evaporated under reduced pressure to dryness, the residue was dissolved in MeCN (500 mL) and then stirred for 2 h. The resulting precipitate was filtered off, and the filtrate was evaporated under reduced pressure to dryness. Diisopropyl ether (1.0 L) was added to the residue to afford **3** (55 g, containing ca. 50% inorganic substances) as a white powder: ^{1}H NMR (DMSO-d_6): δ 1.17 (3H, t, J = 7.0 Hz), 1.53 (4H, br), 2.08−2.09 (2H, m), 2.22−2.24 (2H, m), 3.99 (2H, q, J = 7.0 Hz).

Ethyl 6-[*N*-(4-Chloro-2-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5a). Method A. Compound **3** (7.8 g) obtained above was dissolved in thionyl chloride ($SOCl_2$) (21.0 mL) and heated under reflux for 14 h, and then the reaction mixture was evaporated under reduced pressure to dryness. The residue was subjected three times to the procedure involving an addition of hexane (30 mL) followed by evaporation under reduced pressure to dryness to yield ethyl 2-(chlorosulfonyl)cyclohex-1-ene-1-carboxylate (6.8 g). This (6.2 g) was combined with ethyl acetate (20 mL), and the resultant mixture was added to a solution of 4-chloro-2-fluoroaniline (3.64 g, 24.5 mmol), Et_3N (3.41 mL, 24.5 mmol), and ethyl acetate (54 mL) and then stirred for 18 h. The reaction mixture was diluted with ethyl acetate (50 mL), and the whole was then worked up [water (200 mL), brine (100 mL × 3)]. The residue was crystallized from diisopropyl ether (8.0 mL), and the precipitate was washed with diisopropyl ether (10 mL) and ethyl acetate (8.0 mL) to yield **5a** (1.60 g, 21.2% from **2**) as colorless crystals. The mother liquor and the washings were combined and subjected to silica gel column chromatography (hexane/ethyl acetate, 5:1 to 4:1) followed by crystallization from ethyl acetate/diisopropyl ether to give second crop of **5a** (1.41 g, 18.7% from **2**) as colorless crystals. mp 127−129 °C (diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.10 (3H, t, J = 7.2 Hz), 1.57−1.82 (2H, m), 1.98−2.44 (4H, m), 4.02 (2H, q, J = 7.2 Hz), 4.32 (1H, d, J = 4.4 Hz), 7.12 (1H, t, J = 3.4 Hz), 7.23−7.31 (1H, m), 7.45−7.54 (2H, m), 10.04 (1H, s). Anal. ($C_{15}H_{17}ClFNO_4S$) C, H, N.

The following compounds **5c**, **d**, **f**−**j**, **m**, **q**−**t** were prepared by a manner similar to that used for **5a**.

Ethyl 6-[*N*-(2-Fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5c): Yield 43%; mp 110−111 °C (diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.07 (3H, t, J = 7.2 Hz), 1.58−1.82 (2H, m), 2.05−2.46 (4H, m), 4.01 (2H, q, J = 7.2 Hz), 4.32 (1H, d, J = 4.6 Hz), 7.09−7.32 (4H, m), 7.44−7.54 (1H, m), 9.91 (1H, s). Anal. ($C_{15}H_{18}FNO_4S$) C, H, N.

Ethyl 6-[*N*-(3-Fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5d): Yield 29%; mp 113−114 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.16 (3H, t, J = 7.0 Hz), 1.60−1.80 (2H, m), 2.00−2.33 (4H, m), 3.98−4.15 (2H, m), 4.37 (1H, d, J = 4.8 Hz), 6.87 (1H, dt, J = 8.4, 7.0 Hz), 7.00−7.17 (3H, m), 7.34 (1H, dt, J = 8.4, 7.0 Hz), 10.33 (1H, brs). Anal. ($C_{15}H_{18}FNO_4S$) C, H, N.

Ethyl 6-[*N*-(2-Chlorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5f): Yield 19%; mp 210−211 °C (ethyl acetate/hexane); ^{1}H NMR (DMSO-d_6): δ 1.05 (3H, t, J = 7.0 Hz), 1.55−1.84 (2H, m), 1.99−2.58 (4H, m), 4.00 (2H, q, J = 7.0 Hz), 4.30 (1H, d, J = 5.2 Hz), 7.11 (1H, br), 7.19−7.39 (2H, m), 7.48−7.56 (2H, m), 9.66 (1H, s). Anal. ($C_{15}H_{18}ClNO_4S$) C, H, N.

Ethyl 6-[*N*-(3-Chlorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5g): Yield 10%; mp 113−114 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.16 (3H, t, J = 7.0 Hz), 1.54−1.81 (2H, m), 1.94−2.38 (4H, m), 4.00−4.15 (2H, m), 4.36 (1H, d, J = 4.4 Hz), 7.07 (1H, br), 7.11−7.37 (4H, m), 10.29 (1H, s). Anal. ($C_{15}H_{18}ClNO_4S$) C, H, N.

Ethyl 6-[*N*-(4-Chlorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5h): Yield 16%; mp 145−146 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.15 (3H, t, J = 7.0 Hz), 1.51−1.78 (2H, m), 1.95−2.20 (4H, m), 3.96−4.13 (2H, m), 4.32 (1H, d, J = 4.0 Hz), 7.13 (1H, t, J = 4.0 Hz), 7.20−7.24 (2H, m), 7.34−7.39 (2H, m), 10.17 (1H, s). Anal. ($C_{15}H_{18}ClNO_4S$) C, H, N.

Ethyl 6-[*N*-(2,3-Difluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5i): Yield 34%; mp 111−113 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.00 (3H, t, J = 7.0 Hz), 1.58−1.83 (2H, m), 1.98−2.43 (4H, m), 4.02 (2H, q, J = 7.0 Hz), 4.38 (1H, d, J = 4.4 Hz), 7.13−7.36 (4H, m), 10.22 (1H, s). Anal. ($C_{15}H_{17}F_2NO_4S$) C, H, N.

Ethyl 6-[*N*-(2,6-Difluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5j): Yield 15%; mp 117−118 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.00 (3H, t, J = 7.0 Hz), 1.59−1.88 (2H, m), 2.08−2.56 (4H, m), 3.97 (2H, q, J = 7.0 Hz), 4.39 (1H, d, J = 5.0 Hz), 7.07−7.15 (4H, m), 7.34−7.50 (1H, m), 9.70 (1H, brs). Anal. ($C_{15}H_{17}F_2NO_4S$) C, H, N.

Ethyl 6-[*N*-(2,4-Dichlorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5m): Yield 15%; mp 93−94 °C (ethyl acetate/hexane); ^{1}H NMR (DMSO-d_6): δ 1.07 (3H, t, J = 7.0 Hz), 1.54−1.82 (2H, m), 1.95−2.45 (4H, m), 4.01 (2H, q, J = 7.0 Hz), 4.32 (1H, d, J = 4.8 Hz), 7.12 (1H, br), 7.40−7.67 (3H, m), 9.81 (1H, s). Anal. ($C_{15}H_{17}Cl_2NO_4S$) C, H, N.

Ethyl 6-[*N*-(2-Ethylphenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5q): Yield 14%; mp 62−63 °C (ethyl acetate/hexane); ^{1}H NMR (DMSO-d_6): δ 1.06 (3H, t, J = 7.0 Hz), 1.16

(3H, t, J = 7.6 Hz), 1.52−1.86 (2H, m), 1.99−2.50 (4H, m), 2.72 (2H, q, J = 7.6 Hz), 4.01 (2H, q, J = 7.0 Hz), 4.39 (1H, d, J = 4.8 Hz), 7.10 (1H, br), 7.16−7.38 (4H, m), 9.18 (1H, s). Anal. ($C_{17}H_{23}NO_4S$) C, H, N.

Ethyl 6-[*N*-(2-Methoxycarbonylphenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5r): Yield 18%; mp 91−92 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.21 (3H, t, J = 7.0 Hz), 1.68−2.36 (6H, m), 3.90 (3H, s), 3.93−4.07 (2H, m), 4.50 (1H, d, J = 4.4 Hz), 7.15−7.23 (2H, m), 7.61−7.69 (2H, m), 8.00 (1H, d, J = 8.8 Hz), 10.39 (1H, s). Anal. ($C_{17}H_{21}NO_5S$) C, H, N.

Ethyl 6-[*N*-(1,3-Thiazol-2-yl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5s): Yield 0.9%; mp 156.5−157.5 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.15 (3H, t, J = 7.0 Hz), 1.44−1.78 (2H, m), 2.04−2.36 (4H, m), 4.04 (2H, m), 4.17 (1H, d, J = 4.0 Hz), 6.79 (1H, q, J = 4.6 Hz), 6.97 (1H, t, J = 4.0 Hz), 7.21 (1H, d, J = 4.6 Hz), 12.60 (1H, brs). Anal. ($C_{12}H_{16}N_2O_4S_2 \cdot 1/4H_2O$) C, H, N.

Ethyl 6-[*N*-(1,3,4-Thiadiazol-2-yl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5t): Yield 4.1%; amorphous powder; ^{1}H NMR (DMSO-d_6): δ 1.16 (3H, t, J = 7.0 Hz), 1.15−1.77 (2H, m), 2.00−2.40 (4H, m), 3.98−4.15 (2H, m), 4.20 (1H, d, J = 5.4 Hz), 6.55 (1H, s), 7.01 (1H, t, J = 5.4 Hz), 8.70 (1H, s).

Ethyl 6-(*N*-Phenylsulfamoyl)cyclohex-1-ene-1-carboxylate (5b) and Ethyl 2-(*N*-Phenylsulfamoyl)cyclohex-1-ene-1-carboxylate (6b). Compound **3** (670 mg) was treated with $SOCl_2$ (2.0 mL) and aniline (280 mg) according to a procedure similar to that described for the preparation of **5a**. The obtained residue was purified by silica gel column (hexane/ethyl acetate, 4:1) and ODS column chromatography (methanol/water, 7:3) followed by crystallization from methanol/water to afford **5b** (56 mg, 11% from **2**) and **6b** (37 mg, 7% from **2**) as colorless crystals, respectively.

5b: mp 110−111 °C (methanol/water); ^{1}H NMR (DMSO-d_6): δ 1.14 (3H, t, J = 7.2 Hz), 1.55−1.74 (2H, m), 1.98−2.42 (4H, m), 3.97−4.12 (2H, m), 4.32 (1H, d, J = 4.8 Hz), 7.02−7.35 (6H, m), 10.03 (1H, brs). Anal. ($C_{15}H_{19}NO_4S$) C, H, N.

6b: mp 112−120 °C (methanol/water); ^{1}H NMR (DMSO-d_6): δ 1.23 (3H, t, J = 7.0 Hz), 1.54 (4H, br), 2.25 (4H, br), 4.14 (2H, q, J = 7.0 Hz), 7.02−7.32 (5H, m), 10.13 (1H, brs). Anal. ($C_{15}H_{19}NO_4S$) C, H, N.

Ethyl 6-[*N*-(4-Fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5e), Ethyl 2-[*N*-(4-Fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (6e), and 2-(4-Fluorophenyl)-5,6,7,7a-tetrahydro-1,2-benzisothiazol-3(2*H*)-one 1,1-Dioxide (7e). Compound **3** (720 mg) was treated with $SOCl_2$ (2.1 mL) and 4-fluoroaniline (340 mg) according to a procedure similar to that described for the preparation of **5a**. The obtained residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1) to give **7e** (33 mg, 6% from **2**) as white powdery crystals. The second eluent was purified with ODS column chromatography (methanol/water, 7:3) to afford **5e** (36 mg, 12% from **2**) and **6e** (25 mg, 5% from **2**) as colorless crystals, respectively.

5e: mp 125−126 °C (methanol/water); ^{1}H NMR (DMSO-d_6): δ 1.14 (3H, t, J = 7.2 Hz), 1.55−1.77 (2H, m), 1.98−2.44 (4H, m), 3.97−4.13 (2H, m), 4.28 (1H, d, J = 4.2 Hz), 7.10−7.28 (5H, m), 10.03 (1H, brs). Anal. ($C_{15}H_{18}FNO_4S$) C, H, N.

6e: mp 113−118 °C (methanol/water); ^{1}H NMR (DMSO-d_6): δ 1.20 (3H, t, J = 7.2 Hz), 1.54 (4H, br), 2.25 (4H, br), 4.11 (2H, q, J = 7.2 Hz), 7.12 (2H, s), 7.16 (2H, s), 10.11 (1H, brs). Anal. ($C_{15}H_{18}FNO_4S$) C, H, N.

7e: mp 150−153 °C (diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.75−1.88 (4H, m), 2.42−2.64 (4H, m), 7.40−7.49 (4H, m). Anal. ($C_{13}H_{12}FNO_3S$) C, H, N.

Ethyl 2-[*N*-(4-Chloro-2-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (6a). Compound **3** (2.0 g) was dissolved in $SOCl_2$ (5.0 mL) and heated under reflux for 14 h, and then the reaction was evaporated under reduced pressure to dryness. The residue was subjected three times to the procedure involving an addition of hexane (10 mL) followed by evaporation under reduced pressure to dryness to yield ethyl 2-(chlorosulfonyl)cyclohex-1-ene-1-carboxylate. This was combined with ethyl acetate (30 mL), and the resultant mixture was added to a solution of 4-chloro-2-fluoroaniline (0.55 g, 3.7 mmol) in DMF (5.0 mL). After having been stirred for 18 h, the reaction mixture was diluted with ethyl acetate (100 mL), and the whole was then worked up (water, brine). The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1) followed by crystallization from ethyl acetate/diisopropyl ether to give **6a** (44 mg, 3.3% from **2**) as colorless crystals: mp 105−106 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.06 (3H, t, J = 7.2 Hz), 1.62 (4H, br), 2.25 (2H, br), 2.39 (2H, br), 3.95 (2H, q, J = 7.2 Hz), 7.23−7.37 (2H, m), 7.47−7.52 (1H, m), 10.11 (1H, s). Anal. ($C_{15}H_{17}ClFNO_4S$) C, H, N.

Ethyl 2-(Chlorosulfonyl)cyclohex-1-ene-1-carboxylate (4). Chlorine (Cl_2) gas was bubbled through a solution of **2** (1.40 g, 75.2 mmol) in AcOH (18 mL) and water (2.0 mL) below 15 °C for 20 min. After having been stirred for 30 min, nitrogen gas was bubbled for 10 min. The mixture was concentrated in vacuo, and the residue was worked up [ethyl acetate (20 mL × 2); water (20 mL), brine (40 mL)]. The residue was purified by silica gel chromatography (hexane/ethyl acetate, 9:1) to afford **4** (1.40 g, 74%) as pale yellow crystals: mp 32−33 °C (ethyl acetate); IR ν_{max}(KBr), cm^{-1}: 1738, 1375, 1279, 1254, 1173, 604, 599. ^{1}H NMR ($CDCl_3$): δ 1.34 (3H, t, J = 7.0 Hz), 1.70−1.90 (4H, m), 2.51−2.67 (4H, m), 4.30 (2H, q, J = 7.0 Hz).

Ethyl 6-[*N*-(2,4-Difluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5k). Method B. A solution of **4** (19.3 g, 76.4 mmol) in ethyl acetate (100 mL) was added dropwise for 40 min to an ice-cooled solution of 2,4-difluoroaniline (11.6 g, 90.3 mmol), Et_3N (21.1 mL, 151 mmol), and ethyl acetate (100 mL) under nitrogen atmosphere. After having been stirred for 14 h, the reaction mixture was diluted with ethyl acetate (250 mL), and the whole was then worked up (water, 0.5 N HCl, water, brine). The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1) followed by crystallization from ethyl acetate/diisopropyl ether to give **5k** (19.9 g, 75.4%) as colorless crystals: mp 123−124 °C; ^{1}H NMR (DMSO-d_6): δ 1.07 (3H, t, J = 7.2 Hz), 1.46−1.82 (2H, m), 1.97−2.50 (4H, m), 4.01 (2H q, J = 7.2 Hz), 4.28 (1H, d, J = 4.8 Hz), 7.04−7.15 (2H, m), 7.29−7.54 (2H, m) 9.86 (1H, brs). Anal. ($C_{15}H_{17}F_2NO_4S$) C, H, N.

The following compounds **5l**, **5n**, **5o**, and **5p** were prepared by a manner similar to that used for **5k**.

Ethyl 6-[*N*-(2,4,5-trifluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5l): Yield 52%; mp 143−144 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.13 (3H, t, J = 7.0 Hz), 1.55−1.85 (2H, m), 1.96−2.48 (4H, m), 4.05 (2H, q, J = 7.0 Hz), 4.35 (1H, d, J = 4.4 Hz), 7.14 (1H, br), 7.47−7.71 (2H, m), 10.17 (1H, s). Anal. ($C_{15}H_{16}F_3NO_4S$) C, H, N.

Ethyl 6-[*N*-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5n): Yield 78%; mp 102−103 °C (ethyl acetate/hexane); ^{1}H NMR (DMSO-d_6): δ 1.05 (3H, t, J = 7.0 Hz), 1.52−1.83 (2H, m), 1.98−2.46 (4H, m), 4.00 (2H, q, J = 7.0 Hz), 4.29 (1H, d, J = 4.8 Hz), 7.10 (1H, br), 7.20−7.30 (1H, m), 7.49−7.58 (2H, m), 9.80 (1H, s). Anal. ($C_{15}H_{17}ClFNO_4S$) C, H, N.

Ethyl 6-[*N*-(2-Chloro-4-methylphenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5o): Yield 46%; mp 76−77 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.06 (3H, t, J = 7.0 Hz), 1.51−1.83 (2H, m), 1.99−2.46 (4H, m), 2.29 (3H, s), 4.00 (2H, q, J = 7.0 Hz), 4.29 (1H, d, J = 5.4 Hz), 7.08 (1H, br), 7.12−7.16 (1H, m), 7.33−7.41 (2H, m), 9.53 (1H, s). Anal. ($C_{16}H_{20}ClNO_4S$) C, H, N.

Ethyl 6-[*N*-(2-Chloro-4-cyanophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (5p): Yield 28%; mp 95−96 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.12 (3H, t, J = 7.0 Hz), 1.56−1.84 (2H, m), 1.95−2.42 (4H, m), 4.03 (2H, q, J = 7.0 Hz), 4.46 (1H, d, J = 4.8 Hz), 7.20 (1H, br), 7.70−7.84 (2H, m), 8.07 (1H, br), 10.09 (1H, s). Anal. ($C_{16}H_{17}ClN_2O_4S$) C, H, N.

Ethyl 6-[*N*-(4-Chloro-2-fluorophenyl)-*N*-methylsulfamoyl]cyclohex-1-ene-1-carboxylate (8). A mixture of **5a** (250 mg, 0.69 mmol), methyl iodide (118 mg, 1.38 mmol), K_2CO_3 (191 mg, 1.38 mmol) and DMF (2.5 mL) was stirred for 1

h and worked up (ethyl acetate; water). The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1) to give **8** (50 mg, 96%) as a colorless oil: ^{1}H NMR (DMSO-d_6): δ 1.17 (3H, t, J = 7.2 Hz), 1.56−2.44 (6H, m), 3.19 (3H, s), 4.12 (2H, q, J = 7.2 Hz), 4.64 (1H, d, J = 4.4 Hz), 7.16 (1H, t, J = 3.6 Hz), 7.33−7.39 (1H, m), 7.54−7.62 (2H, m). Anal. ($C_{16}H_{19}ClFNO_4S$) C, H, N.

Methyl 6-[*N*-(2,4-Difluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (9). A mixture of **5k** (300 mg, 0.87 mmol), concentrated H_2SO_4 (0.4 mL), and methanol (6.0 mL) was stirred under reflux for 8 days. The reaction mixture was concentrated in vacuo, and the residue was then worked up (ethyl acetate; water). The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 5:1 to 2:1) followed by crystallization from diisopropyl ether to give **9** (95 mg, 33%) as colorless crystals: mp 117−118 °C; ^{1}H NMR (DMSO-d_6): δ 1.58−1.82 (2H, m), 1.98−2.42 (4H, m), 3.56 (3H, s), 4.30 (1H, d, J = 4.6 Hz), 7.05−7.15 (2H, m), 7.28−7.55 (2H, m), 9.85 (1H, brs). Anal. ($C_{14}H_{15}F_2NO_4S$) C, H, N.

Propyl 6-[*N*-(2,4-Difluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (10). Compound **10** was prepared in 19% yield from **5k** and 1-propanol according to a procedure similar to that described for the preparation of **9**, as colorless crystals: mp 110−111 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 0.79 (3H, t, J = 7.4 Hz), 1.38−1.82 (4H, m), 2.02−2.45 (4H, m), 3.91 (2H, t, J = 6.4 Hz), 4.27 (1H, d, J = 4.8 Hz), 7.05−7.12 (2H, m), 7.28−7.53 (2H, m), 9.86 (1H, s). Anal. ($C_{16}H_{19}F_2NO_4S$) C, H, N.

Isopropyl 6-[*N*-(2,4-Difluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (11). Compound **11** was prepared in 10% yield from **5k** and 2-propanol according to a procedure similar to that described for the preparation of **9**, as white powder: mp 134−135 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.04 (3H, d, J = 6.4 Hz), 1.09 (3H, d, J = 6.4 Hz), 1.58−1.82 (2H, m), 2.02−2.45 (4H, m), 4.25 (1H, d, J = 4.8 Hz), 4.83 (1H, septet, J = 6.4 Hz), 7.05−7.15 (2H, m), 7.30−7.54 (2H, m), 9.86 (1H, s). Anal. ($C_{16}H_{19}F_2NO_4S$) C, H, N.

Butyl 6-[*N*-(2,4-Difluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (12). Compound **12** was prepared in 27% yield from **5k** and 1-butanol according to a procedure similar to that described for the preparation of **9**, as white crystals: mp 85−86 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 0.83 (3H, t, J = 7.0 Hz), 1.18−1.82 (6H, m), 2.00−2.42 (4H, m), 3.95 (2H, br), 4.24 (1H, d, J = 4.4 Hz), 7.09 (2H, br), 7.30−7.49 (2H, m), 9.86 (1H, brs). Anal. ($C_{17}H_{21}F_2NO_4S$) C, H, N.

Isobutyl 6-[*N*-(2,4-Difluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (13). Compound **13** was prepared in 16% yield from **5k** and 2-butanol according to a procedure similar to that described for the preparation of **9**, as white crystals: mp 93−94 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 0.80 (6H, d, J = 6.8 Hz), 1.58−1.84 (2H, m), 2.00−2.47 (4H, m), 3.35−3.45 (1H, m), 3.75 (2H, d, J = 6.8 Hz), 4.27 (1H, d, J = 4.8 Hz), 7.03−7.13 (2H, m), 7.27−7.53 (2H, m), 9.85 (1H, s). Anal. ($C_{17}H_{21}F_2NO_4S$) C, H, N.

Sodium 6-[*N*-(2,4-Difluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (14). Compound **5k** (2.50 g, 7.22 mmol) was dissolved in MeCN (288 mL), and then 1 N NaOH (288 mL) was added to the solution. The mixture was stirred at 55 °C for 12 h and concentrated in vacuo. The residue was chromatographed on CHP-20P column (water to 50% aqueous methanol solution). The eluent was concentrated in vacuo, and the residue was dissolved in water (10 mL) and lyophilized to yield **14** (0.50 g, 15%) as a white powder: SI-MS *m/z*: 340 (M + H). ^{1}H NMR (DMSO-d_6): δ 1.50−1.65 (2H, m), 1.78−2.41 (4H, m), 4.13 (1H, d, J = 4.0 Hz), 6.88−6.98 (2H, m), 7.09−7.20 (1H, m), 7.42 (1H, dt, J = 9.0, 6.2 Hz). Anal. ($C_{13}H_{12}F_2NO_4SNa\cdot H_2O$) C, H, N.

2-Hydroxyethyl 6-[*N*-(2,4-Difluorophenyl)sulfamoyl]-cyclohex-1-ene-1-carboxylate (15). 2-Bromoethanol (81 mg, 0.65 mmol) was added to a solution of **14** (100 mg, 0.30 mmol) in DMF (2.0 mL) at 0 °C. The mixture was stirred for 72 h at room temperature and then worked up (ethyl acetate; water, aqueous $NaHCO_3$, and brine). The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 1:1) followed by crystallization from diisopropyl ether to give **15** (35 mg, 33%) as white powdery crystals: mp 110−111 °C (ethyl acetate/diisopropyl ether); ^{1}H NMR (DMSO-d_6): δ 1.58−1.81 (2H, m), 2.00−2.42 (4H, m), 3.51 (2H, br), 4.00 (2H, t, J = 5.0 Hz), 4.34 (1H, d, J = 4.4 Hz), 4.77 (1H, br), 7.02−7.20 (2H, m), 7.26−7.37 (1H, m), 7.44−7.56 (1H, m), 9.82 (1H, br). Anal. ($C_{15}H_{17}F_2NO_5S$) C, H, N.

Ethyl 2-(Chlorosulfonyl)cyclopent-1-ene-1-carboxylate (18). Compound **17**[10] (3.90 g, 22.6 mmol), derived from **16** using Lawesson's reagent, was treated according to a procedure similar to that described for the preparation of **3** to afford 2-(ethoxycarbonyl)cyclopent-1-ene-1-sulfonic acid (7.8 g) as white powder containing inorganic substances. This powder (1.0 g) was dissolved in $SOCl_2$ (3.0 mL) and then stirred at 80 to 90 °C for 15 h. The mixture was evaporated under reduced pressure to dryness and then worked up [ethyl acetate (50 mL); water (50 mL), brine (50 mL)]. The residue was purified by flash chromatography on silica gel column (hexane/ethyl acetate, 5:1) to yield **18** (154 mg, 22% from **17**) as a yellow oil: IR ν_{max}(KBr), cm^{-1}: 1738, 1377, 1300, 1265, 1177, 613, 579, 550. ^{1}H NMR ($CDCl_3$): δ 1.35 (3H, t, J = 7.0 Hz), 2.18 (2H, quintet, J = 8.0 Hz), 2.92−3.08 (4H, m), 4.33 (2H, q, J = 7.0 Hz).

Ethyl 2-(Chlorosufonyl)cyclohept-1-ene-1-carboxylate (22). Compound **22** was prepared in 34% yield from **21**, which was synthesized from **20**, according to a procedure similar to that described for the preparation of **18**, as a brown oil: IR ν_{max}(KBr), cm^{-1}: 2934, 1811, 1736, 1273, 1194, 810. ^{1}H NMR ($CDCl_3$): δ 1.34 (3H, t, J = 7.4 Hz), 1.60−2.00 (6H, m), 2.40−2.90 (4H, m), 4.29 (2H, q, J = 7.4 Hz).

Ethyl 5-[*N*-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclopent-1-ene-1-carboxylate (19). Compound **19** was prepared in 23% yield from **18** and 2-chloro-4-fluoroaniline according to a procedure similar to that described for the synthesis of **5k**, as pale yellow crystals: mp 116−117 °C (diisopropyl ether/hexane); ^{1}H NMR (DMSO-d_6): δ 1.15 (3H, t, J = 7.0 Hz), 2.22−2.74 (4H, m), 4.07 (2H, q, J = 7.0 Hz), 4.50 (1H, d, J = 8.0 Hz), 7.10 (1H, s), 7.18−7.28 (1H, m), 7.47−7.56 (2H, m), 9.70 (1H, s). Anal. ($C_{14}H_{15}ClFNO_4S$) C, H, N.

Ethyl 7-[*N*-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohept-1-ene-1-carboxylate (23). Compound **23** was prepared in 3.5% yield from **22** and 2-chloro-4-fluoroaniline according to a procedure similar to that described for the synthesis of **5k**, as pale yellow crystals: mp 71.5−72.5 °C (hexane); ^{1}H NMR (DMSO-d_6): δ 1.23 (3H, t, J = 7.0 Hz), 1.19−1.38 (1H, m), 1.67−1.81 (3H, m), 2.02−2.15 (1H, m), 2.15−2.76 (3H, m), 4.05 (2H, q, J = 7.0 Hz), 4.80 (1H, t, J = 4.6 Hz), 7.17−7.27 (1H, m), 7.44−7.59 (3H, m), 9.59 (1H, s). Anal. ($C_{16}H_{19}ClFNO_4S$) C, H, N.

Ethyl (2*Z*)-3-(Chlorosulfonyl)-2-ethylhex-2-enoate (27). Compound **25**[13] (44.2 g, 908 mmol) was treated with H_2S and HCl gases according to a procedure similar to that described for the preparation of **2** to afford **26** (35.1 g) containing byproduct. The resulting **26** (10.0 g) was treated with sodium trioxoboran tetrahydrate (22.8 g), followed by $SOCl_2$ (45.6 mL) to give **27** (1.76 g, 9.7% from **25**): ^{1}H NMR ($CDCl_3$): δ 0.93−1.44 (9H, m), 1.70−2.82 (6H, m), 4.15−4.37 (2H, m).

Ethyl (2*E*)-3-[(2-Chloro-4-fluorophenyl)sulfamoyl]-2-ethylidenhexanoate (28). Compound **28** was prepared in 12% yield from **27** and 2-chloro-4-fluoroaniline according to a procedure similar to that described for the synthesis of **5k**, as a brown solid: mp 47−48 °C (hexane); IR ν_{max}(KBr), cm^{-1}: 3260, 2965, 1719, 1495, 1333, 1229, 1159, 1049, 887. ^{1}H NMR (DMSO-d_6): δ 0.86 (3H, t, J = 7.2 Hz), 1.13 (3H, t, J = 7.0 Hz), 1.19−2.17 (4H, m), 2.02 (3H, d, J = 7.2 Hz), 4.08 (2H, q, J = 7.0 Hz), 4.28 (1H, dd, J = 11.6, 3.6 Hz), 6.51 (1H, q, J = 7.2 Hz), 7.19−7.28 (1H, m), 7.46−7.53 (2H, m), 9.59 (1H, s). Anal. ($C_{16}H_{21}ClFNO_4S$) C, H, N. The obtained compound **28** was determined to be the (*E*)-isomer by NOE experiment.

Methyl 2-[*N*-(2-Chloro-4-fluorophenyl)sulfamoyl]benzoate (30). To an ice-cooled solution of methyl 2-(chlorosulfonyl)benzoate (**29**) (1.06 g, 4.52 mmol) in CH_2Cl_2 (10 mL) were

added 2-chloro-4-fluoroaniline (0.27 mL, 2.26 mmol) and pyridine (0.77 mL, 9.94 mmol), and then the mixture was stirred for 3 days. After compound **29** (500 mg, 2.13 mmol) and pyridine (0.41 mL, 2.34 mmol) were added, the mixture was stirred for 12 h further and then worked up [ethyl acetate (50 mL); 1 N HCl (50 mL × 2), brine (50 mL)]. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 20:1 to 5:1) followed by crystallization from hexane to give **30** (127 mg, 17%) as white powdery crystals: mp 139.0−139.5 °C; IR ν_{max}(KBr), cm^{-1}: 1725, 1493, 1292, 1173. ^1H NMR ($CDCl_3$): δ 4.05 (3H, s), 6.94−7.04 (2H, m), 7.49−7.70 (3H, m), 7.80−7.90 (2H, m), 8.44 (1H, s). Anal. ($C_{14}H_{11}ClFNO_4S$) C, H, N.

Ethyl 2-[*N*-(2-Chloro-4-fluorophenyl)sulfamoyl]benzoate (31). To a solution of compound **30** (70 mg, 0.20 mmol) in ethanol (5.0 mL) was added 28% sodium methoxide in methanol (39 mg, 0.20 mmol), and then the mixture was stirred for 3 days under reflux. The reaction mixture was concentrated in vacuo and worked up [ethyl acetate (20 mL); 1 N HCl (30 mL), brine (30 mL)]. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 10:1 to 5:1) followed by crystallization from hexane to give **31** (46 mg, 64%) as white powdery crystals: mp 138−139 °C; IR ν_{max}(KBr), cm^{-1}: 1715, 1495, 1281, 1173. ^1H NMR ($CDCl_3$): δ 1.47 (3H, t, J = 7.2 Hz), 4.52 (2H, q, J = 7.2 Hz), 6.94−7.04 (2H, m), 7.48−7.90 (5H, m), 8.46 (1H, s). Anal. ($C_{15}H_{13}ClFNO_4S$) C, H, N.

Ethyl (6*R*)-6-[(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate [(*R*)-(+)-5n] and Ethyl (6*S*)-6-[(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate [(*S*)-(−)-5n]. The racemic mixture **5n** (2.01 g) was separated by preparative HPLC using CHIRALPAK AD[14] (10.0 mm i.d. × 250 mm) under detection at 254 nm. Elution with a mixture of hexane/ethanol (9:1) at a flow rate of 8.0 mL/min at room temperature gave two enantiomers, (*R*)-(+)-**5n** (959 mg) and (*S*)-(−)-**5n** (979 mg), as pale yellow oils. The obtained enantiomers were purified by silica gel column chromatography (hexane/ethyl acetate, 9:2) followed by recrystallization from diisopropyl ether/hexane to give (*R*)-(+)-**5n** (634 mg) and (*S*)-(−)-**5n** (681 mg) as colorless crystals, respectively.

(*R*)-(+)-5n (TAK-242): mp 54−55 °C; $[\alpha]_D^{20}$ +111.0° (*c* 1.0, methanol). IR ν_{max}(KBr), cm^{-1}: 3245, 2969, 1720, 1649, 1491, 1329, 1256, 1150, 1067, 889. ^1H NMR (DMSO-d_6): δ 1.05 (3H, t, J = 7.0 Hz), 1.56−1.83 (2H, m), 2.01−2.43 (4H, m), 4.00 (2H, q, J = 7.0 Hz), 4.30 (1H, d, J = 5.0 Hz), 7.10 (1H, br), 7.20−7.30 (1H, m), 7.50−7.58 (2H, m), 9.74 (1H, s). Anal. ($C_{15}H_{17}ClFNO_4S$) C, H, N.

(*S*)-(−)-5n: mp 54−55 °C; $[\alpha]_D^{20}$ −111.0° (*c* 1.0, methanol). IR ν_{max}(KBr), cm^{-1}: 2936, 1709, 1605, 1493, 1314, 1250, 1128, 1061. ^1H NMR (DMSO-d_6): δ 1.05 (3H, t, J = 7.0 Hz), 1.55−1.84 (2H, m), 1.96−2.43 (4H, m), 4.00 (2H, q, J = 7.0 Hz), 4.29 (1H, d, J = 5.0 Hz), 7.10 (1H, br), 7.20−7.30 (1H, m), 7.50−7.58 (2H, m), 9.73 (1H, s). Anal. ($C_{15}H_{17}ClFNO_4S$) C, H, N.

The enantiomeric excess of (*R*)-(+)-**5n** and (*S*)-(−)-**5n** were determined by HPLC to be >99%, respectively [column: CHIRALPAK AD[14] (4.6 mm i.d. × 250 mm), room temperature; eluent: hexane/ethanol (9:1); flow rate: 0.6 mL/min; detection: UV at 254 nm; t_R of (*R*)-(+)-**5n**, 20.0 min; t_R of (*S*)-(−)-**5n**, 16.6 min].

Biological Methods. Cells, Animals, and Materials. Murine macrophage cell line RAW 264.7 was purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI1640 (Nikken Bio Medical Laboratories, Kyoto, Japan) containing 10% heat-inactivated fetal bovine serum (FBS) and 10 μg/mL kanamycin at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Female BALB/c mice (6-week-old) weighing 15−21 g were obtained from Charles River Japan Inc. (Kanagawa, Japan), and allowed to acclimate for at least one week before use. LPS (*Escherichia coli* O111:B4, Sigma, St. Louis, MO) was dissolved in saline at a concentration of 10 mg/mL in advance, and kept at −80 °C until use. The LPS stock solution was diluted to appropriate concentrations with saline or culture medium before use.

Assay for Inhibitory Activity against NO Production. RAW264.7 cells were plated at 1×10^5 cells/well in 96-well culture plates (Nunc, Rochester, NY) and incubated overnight. After removing cell culture supernatants, cells were stimulated with 10 ng/mL LPS in the presence of various concentrations of test compounds for 20 h in stimulation medium (phenol red-free RPMI1640 containing 1% heat-inactivated FBS and 10 μg/mL kanamycin) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The test compounds were dissolved at 10 mmol/L in DMF, diluted with an RPMI-1640 medium to the appropriate concentrations, and added to the culture.

Production of NO was estimated by measuring the amount of nitrite, a stable metabolite of NO, according to a fluorometric method using 2,3-diaminonaphthalene (DAN, Dojindo Laboratories, Kumamoto, Japan). Briefly, 25 μL of 20 μg/mL DAN was added to 50 μL of the culture supernatant and incubated at room temperature for 10 min. After adding 25 μL of 0.5 N NaOH, a fluorescence at 460 nm (excitation wavelength: 355 nm) was measured.

Assay for Inhibitory Activity against Cytokines Production. Concentrations of TNF-α, and IL-6 in the culture supernatants were determined using specific enzyme-linked immunosorbent assay (ELISA) (Amersham Life Science, Buckinghamshire, UK) according to the manufacturer's instructions.

Endotoxin Shock Model. Mice were intraperitoneally injected with LPS at a lethal dose of 7 mg/kg. Survival of mice was recorded for 7 days following LPS challenge. Compound (*R*)-(+)-**5n** was dissolved in 10% (w/v) Glucuronylglucosyl-β-cyclodextrin sodium salt (Wako, Osaka, Japan) and administered intravenously 1 h before the LPS injection.

Evaluation of Cytokine and NO Production In Vivo. The blood was collected at the indicated times, and allowed to clot at room temperature. The serum was separated by centrifugation and stored at −80 °C until analysis. The serum levels of TNF-α, IL-6, and IL-1β were determined by specific ELISA (Amersham Life Science) according to the manufacturer's instructions.

The serum levels of nitrite and nitrate, which are stable end products of NO metabolism were measured by a fluorometric method.[16] Briefly, 10 μL aliquots of sample were mixed with 20 μL of β-NADPH (0.2 mmol/L, Roche, Mannheim, Germany) and 20 μL of *Aspergillus* nitrate reductase (0.25 U/mL, Roche) and incubated for 20 min at room temperature to convert nitrate to nitrite. To these samples was added 25 μL of DAN (25 μg/mL, Dojindo Laboratories) in HCl (2 mol/L) and incubated for 10 min at room temperature. NaOH (25 μL) (0.5 mol/L) was added, and the fluorescence (excitation: 355 nm, emission: 460 nm) was measured. The nitrite concentrations were calculated from a standard curve with sodium nitrite (Wako).

Statistical Analysis. For analysis of the production of cytokine and NO, one-tailed Williams or Shirley-Williams test was used. The survival rate of mice was evaluated by Tarone's test with a simple closed step-down method.

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Supporting Information Available: X-ray crystallographic data for (*R*)-(+)-**5n** and elemental analyses for the new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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