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## SYNTHESIS AND DNA CLEAVAGE ACTIVITY OF NOVEL SPIRO PYRAZOL-3-ONES CONTAINING ISOXAZOLINE MOIETY

Eiichi Masumoto, Emi Shirouzu, Nobuhiro Kashige, Fumi Okabe-Nakahara,  
Fumio Miake, Kenji Yamagata, and Hiroshi Maruoka\*

Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma,  
Jonan-ku, Fukuoka 814-0180, Japan. E-mail: maruoka@fukuoka-u.ac.jp

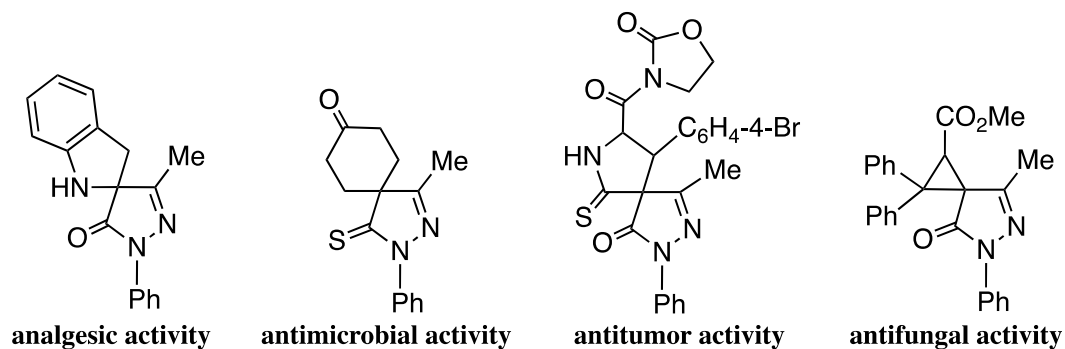
**Abstract** – An approach to the synthesis of novel spiro pyrazol-3-ones containing isoxazoline moiety is described. A plausible mechanism for the conversion is proposed. The spiro compounds were formed *via* potassium carbonate-assisted intramolecular cyclocondensation reaction of  $\beta$ -hydroxy ketoximes, which were prepared from pyrazol-3-ones containing  $\beta$ -hydroxy ketone moiety through an oximation. On the other hand, treatment of the  $\beta$ -hydroxy ketoximes with [hydroxy(tosyloxy)iodo]benzene (HTIB) caused an oxidative N–O coupling reaction to give the spiro pyrazol-3-ones containing isoxazoline *N*-oxide moiety. All the synthesized compounds were characterized by spectroscopic analysis and were tested for their DNA cleavage activity.

Spiro compounds, especially nitrogen-containing spiro heterocycles, served as an important structural unit in many bioactive natural products, pharmaceuticals, and agricultural chemicals.<sup>1</sup> Among them, spiro heterocycles containing pyrazole moiety also have biological activities, such as analgesic,<sup>2a</sup> antimicrobial,<sup>2b</sup> antitumor,<sup>2c</sup> and antifungal<sup>2d</sup> activities (Figure 1). Hence, their synthesis continues to attract attention and provides an interesting challenge.

On the other hand, many papers have accounted in the last decade for the synthesis and occurrence of isoxazoline derivatives in nature and medicinal chemistry.<sup>3</sup> Besides its natural occurrence, isoxazoline is also an important skeleton found in many synthetic bioactive compounds. Isoxazoline derivatives have wide-ranging collection of conventional biological activities, for example, antimicrobial,<sup>4</sup> anticancer,<sup>5</sup> anti-inflammatory,<sup>6</sup> and antiplatelet<sup>7</sup> activities. In this context, several synthetic methods used for the formation of isoxazole and its derivatives have recently been reported in the literature.<sup>8</sup>

Prompted by these observations, we focused our work on the synthesis of novel spiro pyrazol-3-ones containing isoxazoline moiety, which might have useful biological and therapeutic activities. In

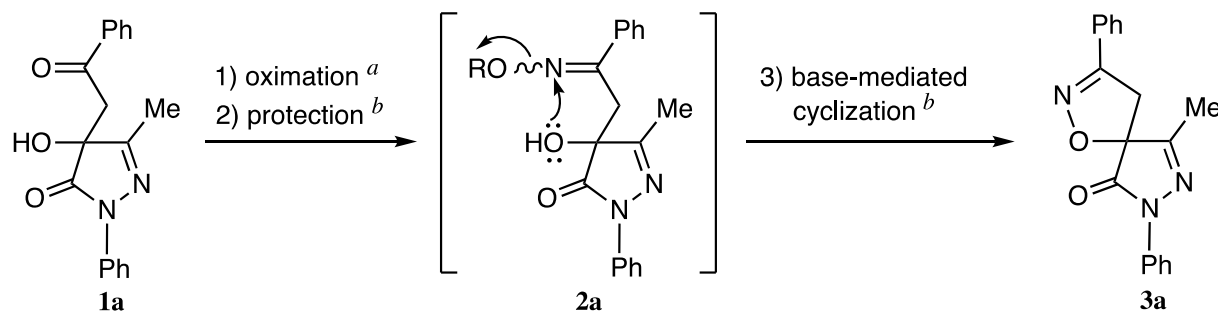
connection with the synthesis of spiro pyrazol-3-one derivatives,<sup>2d,9</sup> we report herein the results of our investigation. The key element of our approach was the optimization of potassium carbonate-assisted cyclocondensation and HTIB-mediated<sup>10</sup> oxidative N–O coupling of pyrazol-3-ones containing  $\beta$ -hydroxy ketoxime moiety to yield the spiro-isoxazoline-pyrazol-3-ones.



**Figure 1.** Selected bioactive spiro heterocycles containing pyrazole moiety

Initially, we examined the intramolecular cyclization reaction of pyrazol-3-ones containing  $\beta$ -hydroxy ketoxime moiety in the presence of a base (Table 1). The starting material, pyrazol-3-one **1a** containing  $\beta$ -hydroxy ketone moiety, was prepared according to our previous procedure.<sup>9a</sup> To simplify the operation, the key intermediate  $\beta$ -hydroxy ketoxime was used without isolation and purification.<sup>11</sup> The base-mediated cyclization reaction of the  $\beta$ -hydroxy ketoxime free without protection in the presence of a base such as pyridine failed. Therefore, we carried out several experiments on the spiro compound **3a**, testing different reaction conditions, for example, base, solvent, reaction temperature, and reaction time, but our attempts were unacceptable with respect to yield. Thus, treatment of *O*-acetylated, mesylated, or tosylated oxime **2a** with a base caused an intramolecular  $S_N2$ -type reaction<sup>12</sup> with oxygen nucleophile on oxime nitrogen to give **3a** in lower yields (entries 1–3 and 5–12). Under some conditions, **3a** could not be detected at all and instead **2a** was obtained (entries 1, 3, 7, and 11). Fortunately, best result was obtained when a mixture of  $\beta$ -hydroxy ketoxime, which was prepared from **1a** and hydroxylamine, and acetic anhydride (1.2 equiv.) in the presence of potassium carbonate (2.5 equiv.) in refluxing toluene was stirred for 3 h, the desired spiro compound **3a** was obtained in 3 steps 57% yield (entry 4).

With the optimized reaction conditions in hand, treatment of **1b,c** with hydroxylamine hydrochloride/sodium acetate and subsequent acetic anhydride/potassium carbonate combinations caused an intramolecular  $S_N2$ -type reaction of the key intermediate *O*-acetylated oximes **2b,c** via an elimination of acetyloxy group to give the corresponding spiro compounds **3b,c** in moderate yields (Scheme 1). In the case of the reaction of **1d** as the substrate, only a trace of the expected spiro compound **3d** could be observed and the reaction was not clean. Fortunately, we found the reaction condition using tosylated oxime **2d** under which **3d** could be isolated in 3 steps 40% yield (Scheme 1).

**Table 1.** Optimization of the reaction conditions for the synthesis of **3a**

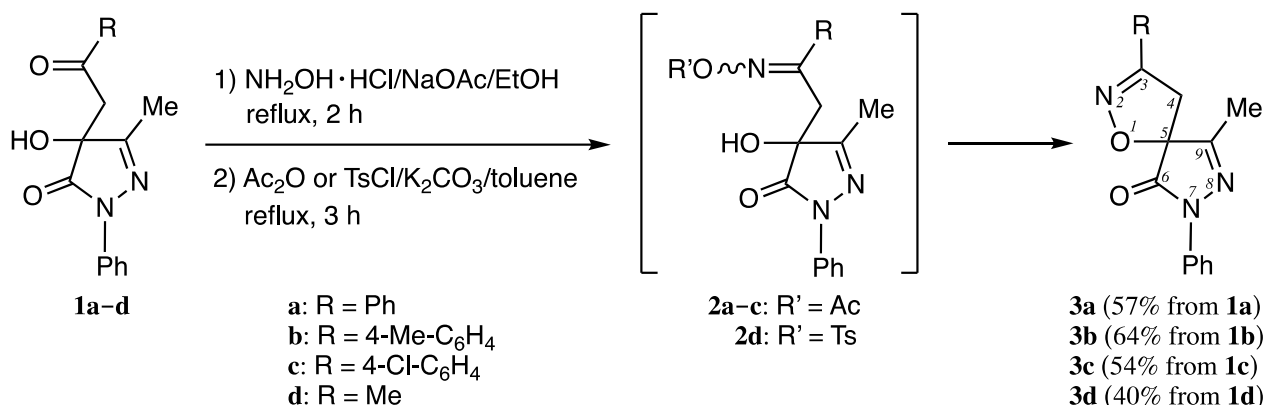
Entry	R	Base (Equiv.)	Solvent	Conditions	Yield (%) <sup>c</sup> of <b>2a</b>	Yield (%) <sup>d</sup> of <b>3a</b>
1	Ac	Et <sub>3</sub> N (2.5)	CHCl <sub>3</sub>	reflux, 5 h	54	trace
2	Ac	Et <sub>3</sub> N (2.5)	toluene	reflux, 3 h	0	34
3	Ac	DIPEA <sup>e</sup> (2.5)	AcOEt	rt, 12 h	90	0
4	Ac	K <sub>2</sub> CO <sub>3</sub> (2.5)	toluene	reflux, 3 h	0	57
5	Ms	Et <sub>3</sub> N (2.5)	CHCl <sub>3</sub>	reflux, 5 h	0	trace
6	Ms	Et <sub>3</sub> N (2.5)	toluene	reflux, 3 h	0	trace
7	Ms	DIPEA <sup>e</sup> (2.5)	AcOEt	rt, 12 h	56	trace
8	Ms	K <sub>2</sub> CO <sub>3</sub> (2.5)	toluene	reflux, 3 h	0	12
9	Ts	Et <sub>3</sub> N (2.5)	CHCl <sub>3</sub>	reflux, 5 h	0	37
10	Ts	Et <sub>3</sub> N (2.5)	toluene	reflux, 3 h	0	15
11	Ts	DIPEA <sup>e</sup> (2.5)	AcOEt	rt, 12 h	20	0
12	Ts	K <sub>2</sub> CO <sub>3</sub> (2.5)	toluene	reflux, 3 h	0	37

<sup>a</sup> NH<sub>2</sub>OH·HCl (2.0 equiv.), NaOAc (2.0 equiv.), EtOH, reflux, 2 h.

<sup>b</sup> Ac<sub>2</sub>O (1.2 equiv.), MsCl (1.2 equiv.), or TsCl (1.2 equiv.), base, solvent, conditions.

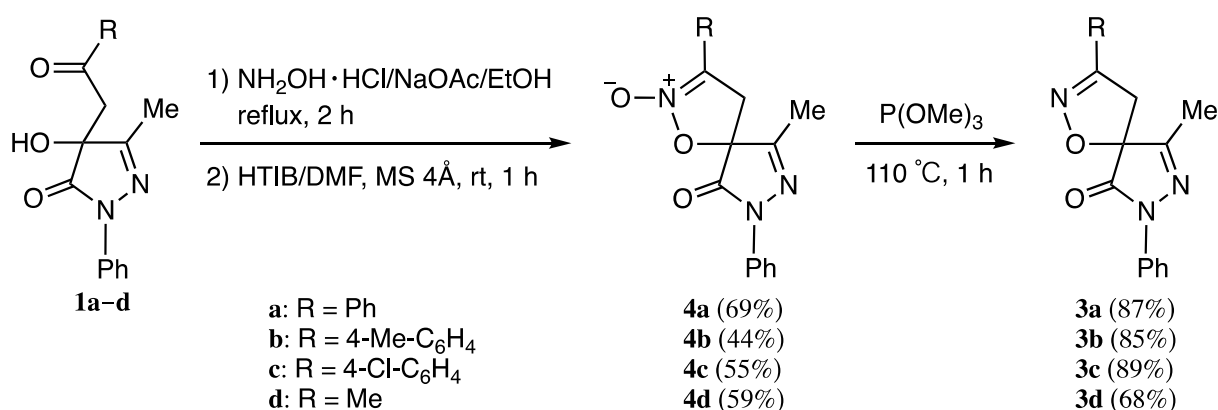
<sup>c</sup> Isolated yield. <sup>d</sup> Isolated yield. <sup>e</sup> *N,N*-Diisopropylethylamine.

These products **3a–d** gave satisfactory elemental analyses and spectroscopic data (IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, and MS) consistent with their assigned structures (see experimental section). For example, IR spectrum of **3a** displays a band at 1698 cm<sup>-1</sup> because of a carbonyl group. The <sup>1</sup>H NMR spectrum of **3a** in CDCl<sub>3</sub> exhibits a three-proton singlet at δ 2.16 assignable to the methyl protons and two one-proton doublets at δ 3.85 and 4.11 assignable to the methylene protons. The <sup>13</sup>C NMR spectrum of **3a** in CDCl<sub>3</sub> shows a signal at δ 13.0 because of the methyl carbon, a signal at δ 42.2 because of the methylene carbon, a signal at δ 87.0 because of the spiro carbon, and a signal at δ 170.2 because of the carbonyl carbon.



Scheme 1

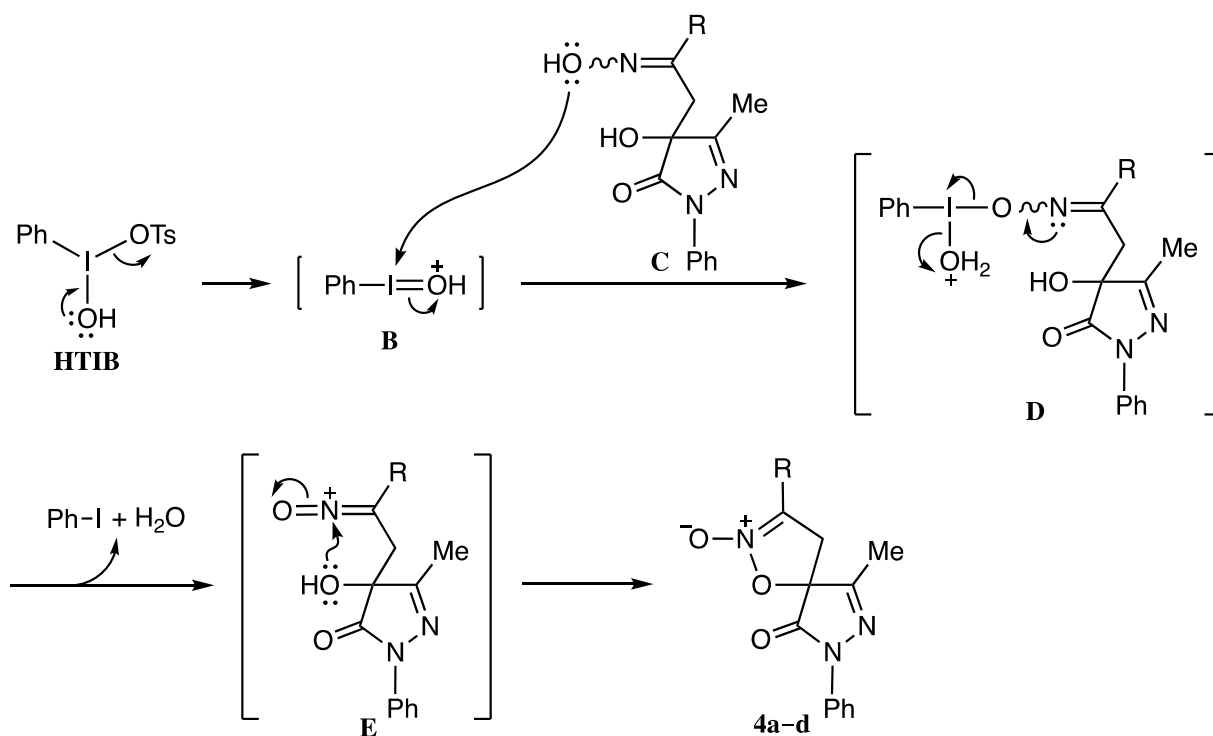
We next carried out another intramolecular cyclization reaction of  $\beta$ -hydroxy ketoximes with HTIB<sup>10</sup> as an oxidizing agent to obtain spiro pyrazol-3-ones containing isoxazoline *N*-oxide moiety. After some optimization, the results are presented in Scheme 2. Thus,  $\beta$ -hydroxy ketoximes, which were prepared from **1a-d** and hydroxylamine, were reacted with HTIB in *N,N*-dimethylformamide (DMF) at room temperature for 1 h to provide the corresponding isoxazoline *N*-oxides **4a-d** with 2 steps 44–69% isolated yields. These products **4a-d** were characterized by spectroscopic analyses (see experimental section). Although we tested the oxidative N–O coupling reaction under a variety of other conditions such as a phenyliodine(III) diacetate (PIDA)/DMF and phenyliodine(III) bis(trifluoroacetate) (PIFA)/DMF system, those attempts were not successful. Furthermore, solvent effects were observed with DMF giving the highest yield of the isoxazoline *N*-oxide **4a**, while other solvents such as MeOH, THF, MeCN,  $\text{CH}_2\text{Cl}_2$ , and AcOEt gave somewhat lower yields of **4a**. It makes us believe that the oxidative N–O coupling reaction can only be promoted by using HTIB/DMF system.



Scheme 2

A plausible mechanism for this cascade cyclization was proposed, as shown in Scheme 3. HTIB would undergo a release of tosylate anion to form hydroxyphenyliodonium ion **B** as an active species in the

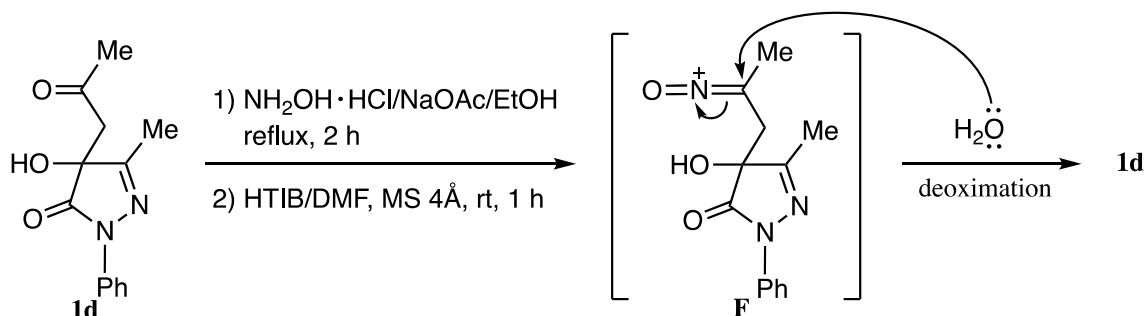
oxidative N–O coupling reaction. The reaction of **B** with **C** probably causes a nucleophilic addition of oxime hydroxyl group of **C** to **B** via a proton migration, giving the intermediate nucleophilic adduct **D**, which would undergo an elimination of iodobenzene and H<sub>2</sub>O to produce the key intermediate *N*-oxonitrenium ion **E**. The neighboring alcoholic oxygen of **E** would attack the nitrogen center and then the desired isoxazoline *N*-oxides **4a–d** would be formed through a deprotonation. It is noteworthy that HTIB has a diverse array of applications in synthetic chemistry.<sup>10</sup>



Scheme 3

In the case of the oxidative N–O coupling reaction of **1d** in the absence of 4Å molecular sieves (MS 4Å), the desired isoxazoline *N*-oxide **4d** was isolated in lower yield of 40% and the reaction was not clean. The reason for this change of behavior is not very clear at present. One explanation could rely on the fact that H<sub>2</sub>O would attack the carbon center of **F** and then the result would be the formation of the deoxygenation product **1d** (Scheme 4). Moreover, when there is not an excess amount of MS 4Å as a dehydrating agent in the reaction system, it seems that the decomposition of the β-hydroxy ketoxime into **1d** would proceed easily.

On the basis of all of the aforementioned results, we examined the deoxygenation of isoxazoline *N*-oxides **4a–d** to confirm the structures of spiro compounds **3a–d**. Subsequently, thermal treatment of **4a–d** with trimethyl phosphite at 110 °C for 1 h without solvent caused a deoxygenation to give the corresponding spiro compounds **3a–d** in 68–89% moderate to good yields (Scheme 2), which were identical with authentic samples prepared according to Scheme 1.



Scheme 4

Table 2. DNA cleavage by **3a–d** and **4a–d** in the absence and/or presence of  $\text{Cu}^{2+}$  <sup>a</sup>

Entry	Compound	DNA type	Relative amounts of DNA (%)	
			Without $\text{Cu}^{2+}$ <sup>b</sup>	With $\text{Cu}^{2+}$ <sup>c</sup>
1	Control <sup>d</sup>	ccc- oc-	100 0	100 0
2	<b>3a</b> <sup>e</sup>	ccc- oc-	100 0	100 0
3	<b>3b</b> <sup>e</sup>	ccc- oc-	100 0	100 0
4	<b>3c</b> <sup>e</sup>	ccc- oc-	100 0	87 13
5	<b>3d</b> <sup>e</sup>	ccc- oc-	100 0	0 100
6	<b>4a</b> <sup>e</sup>	ccc- oc-	100 0	73 27
7	<b>4b</b> <sup>e</sup>	ccc- oc-	100 0	83 17
8	<b>4c</b> <sup>e</sup>	ccc- oc-	100 0	86 14
9	<b>4d</b> <sup>e</sup>	ccc- oc-	79 21	77 23

<sup>a</sup> Incubation for 3 h. <sup>b</sup>  $\text{CuCl}_2$ : 0 mM. <sup>c</sup>  $\text{CuCl}_2$ : 1 mM. <sup>d</sup> Amount: 0 mM. <sup>e</sup> Amount: 10 mM.

As activity was accelerated upon addition of  $\text{Cu}^{2+}$ , the quantity of compounds and the incubation time were minimized until differences in activity could be observed.

Finally, we have tested *in vitro* DNA cleavage activity of the synthesized spiro compounds **3a–d** and **4a–d**. The values obtained for activity were based on the remaining amounts of covalently closed circular duplex DNA, namely ccc-DNA, of plasmid pBR322.<sup>13</sup> The data of DNA cleavage activity is summarized in Table 2. Indeed, in the absence of  $\text{Cu}^{2+}$ , the tested compounds other than **4d** showed no DNA cleavage activity. These activities of compounds **3c,d** and **4a–c**, however, were obviously accelerated by the addition of 1 mM  $\text{Cu}^{2+}$  (entries 4–8). Interestingly, it was found that compound **3d** showed high DNA cleavage activity *in vitro* with  $\text{Cu}^{2+}$  (entry 5).

In conclusion, we have prepared new functionalized spiro pyrazol-3-ones containing isoxazoline moiety. The key element of our approach was the potassium carbonate-assisted cyclocondensation and HTIB-mediated oxidative N–O coupling of pyrazol-3-ones containing  $\beta$ -hydroxy ketoxime moiety to yield the spiro-isoxazoline-pyrazol-3-ones. In this present work, we have found that the synthesized spiro compounds **3c** and **4a–d** showed moderate DNA cleavage activity *in vitro* with  $\text{Cu}^{2+}$ . In addition, **3d** exhibited high DNA cleavage activity. Spiro-isoxazoline-pyrazol-3-ones are important building blocks in organic synthesis and for the preparation of biologically active compounds with interest in medicinal chemistry. Further synthetic applications for functionalized spiro pyrazol-3-one derivatives containing isoxazoline moiety are in progress.

## EXPERIMENTAL

All melting points are uncorrected. The IR spectra were recorded on a Thermo Fisher Scientific Nicolet iS5 FT-IR spectrometer equipped with an iD7 diamond ATR accessory. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were measured with a JEOL JNM-ECZ600R/S1 spectrometer at 600.17 and 150.91 MHz, respectively. The  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts ( $\delta$ ) are reported in parts per million (ppm) relative to TMS as internal standard. Positive FAB MS spectra were obtained on a JEOL JMS-700T spectrometer. Elemental analyses were performed on YANACO MT-6 CHN analyzer. The starting compounds **1a–d** were prepared in this laboratory according to our previous procedure reported in the literature.<sup>9a</sup>

### General procedure for the preparation of spiro pyrazol-3-ones **3a–d** containing isoxazoline moiety from **1a–d** via oximation/acetylation or tosylation/potassium carbonate-assisted cyclocondensation.

A mixture of **1a–d** (1.0 mmol), hydroxylamine hydrochloride (0.138 g, 2.0 mmol), and sodium acetate (0.164 g, 2.0 mmol) in EtOH (10 mL) was refluxed for 2 h. After removal of the solvent *in vacuo*, cold  $\text{H}_2\text{O}$  was added to the residue with stirring and ice cooling. The resulting mixture was extracted with AcOEt (60 mL). The extract was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated *in vacuo* to give crude  $\beta$ -hydroxy ketoximes. A mixture of the crude  $\beta$ -hydroxy ketoximes, acetic anhydride (0.122 g, 1.2 mmol, in the case of the preparation of **3a–c**) or *p*-toluenesulfonyl chloride (0.228 g, 1.2 mmol, in the case of the preparation of **3d**), and potassium carbonate (0.345 g, 2.5 mmol) in toluene (5 mL) was refluxed for 3 h. After removal of the solvent *in vacuo*, a 5% HCl solution (20 mL) was added to the reaction mixture with stirring and ice cooling. The resulting mixture was extracted with  $\text{CHCl}_3$  (60 mL). The extract was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with  $\text{CHCl}_3$  as the eluent to afford **3a–d**.

**9-Methyl-3,7-diphenyl-1-oxa-2,7,8-triazaspiro[4.4]nona-2,8-dien-6-one (3a):** Colorless needles (0.174 g, 57%), mp 144–145 °C ( $\text{Et}_2\text{O}$ /petroleum ether); IR (ATR):  $\nu$  1698  $\text{cm}^{-1}$  (CO);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.16 (s, 3H, 9-Me), 3.85 (d,  $J = 17.9$  Hz, 1H, 4-H), 4.11 (d,  $J = 17.9$  Hz, 1H, 4-H), 7.20–7.23 (m, 1H, Ph-H),

7.42–7.52 (m, 5H, Ph-H), 7.70–7.72 (m, 2H, Ph-H), 7.77–7.79 (m, 2H, Ph-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  13.0 (9-Me), 42.2 (C-4), 87.0 (C-5), 118.8, 125.8, 127.8, 128.1, 129.56, 129.63, 131.6, 137.9 (Ph-C), 157.2 (C-3), 159.5 (C-9), 170.2 (C-6); MS:  $m/z$  306  $[\text{M}+\text{H}]^+$ . Anal. Calcd for  $\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_2$ : C, 70.81; H, 4.95; N, 13.76. Found: C, 70.85; H, 4.99; N, 13.77.

**9-Methyl-3-(4-methylphenyl)-7-phenyl-1-oxa-2,7,8-triazaspiro[4.4]nona-2,8-dien-6-one (3b):**

Colorless needles (0.204 g, 64%), mp 167–168 °C ( $\text{Et}_2\text{O}$ /petroleum ether); IR (ATR):  $\nu$  1698  $\text{cm}^{-1}$  (CO);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.15 (s, 3H, 9-Me), 3.81 (d,  $J = 17.9$  Hz, 1H, 4-H), 4.07 (d,  $J = 17.9$  Hz, 1H, 4-H), 7.20–7.22 (m, 1H, Ph-H), 7.28–7.32 (m, 2H, Ph-H), 7.42–7.46 (m, 2H, Ph-H), 7.56–7.62 (m, 2H, Ph-H), 7.77–7.79 (m, 2H, Ph-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  13.0 (9-Me), 21.6 (4-Me- $\text{C}_6\text{H}_4$ ), 42.3 (C-4), 86.8 (C-5), 118.7, 125.3, 125.8, 127.7, 129.6, 130.1, 137.9, 141.5 (Ph-C), 157.0 (C-3), 159.5 (C-9), 170.2 (C-6); MS:  $m/z$  320  $[\text{M}+\text{H}]^+$ . Anal. Calcd for  $\text{C}_{19}\text{H}_{17}\text{N}_3\text{O}_2$ : C, 71.46; H, 5.37; N, 13.16. Found: C, 71.61; H, 5.43; N, 13.16.

**3-(4-Chlorophenyl)-9-methyl-7-phenyl-1-oxa-2,7,8-triazaspiro[4.4]nona-2,8-dien-6-one (3c):**

Colorless needles (0.185 g, 54%), mp 162 °C ( $\text{Et}_2\text{O}$ /petroleum ether); IR (ATR):  $\nu$  1693  $\text{cm}^{-1}$  (CO);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.15 (s, 3H, 9-Me), 3.85 (d,  $J = 17.9$  Hz, 1H, 4-H), 4.10 (d,  $J = 17.9$  Hz, 1H, 4-H), 7.20–7.22 (m, 1H, Ph-H), 7.42–7.45 (m, 2H, Ph-H), 7.55–7.57 (m, 2H, Ph-H), 7.71–7.73 (m, 2H, Ph-H), 7.77–7.79 (m, 2H, Ph-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  13.0 (9-Me), 42.0 (C-4), 87.2 (C-5), 118.8, 125.9, 127.0, 129.57, 129.63, 129.7, 136.2, 137.8 (Ph-C), 156.5 (C-3), 159.3 (C-9), 170.0 (C-6); MS:  $m/z$  340  $[\text{M}+\text{H}]^+$ . Anal. Calcd for  $\text{C}_{18}\text{H}_{14}\text{ClN}_3\text{O}_2$ : C, 63.63; H, 4.15; N, 12.37. Found: C, 63.60; H, 4.13; N, 12.37.

**3,9-Dimethyl-7-phenyl-1-oxa-2,7,8-triazaspiro[4.4]nona-2,8-dien-6-one (3d):** Colorless needles (0.098 g, 40%), mp 74–75 °C ( $\text{Et}_2\text{O}$ /petroleum ether); IR (ATR):  $\nu$  1716  $\text{cm}^{-1}$  (CO);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.10 (s, 3H, 3-Me), 2.15 (s, 3H, 9-Me), 3.15–3.41 (m, 2H, 4-H), 7.17–7.20 (m, 1H, Ph-H), 7.37–7.41 (m, 2H, Ph-H), 7.83–7.86 (m, 2H, Ph-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  12.6 (3- and 9-Me), 45.9 (C-4), 85.8 (C-5), 118.7, 125.5, 129.0, 137.6 (Ph-C), 154.2 (C-3), 158.2 (C-9), 170.0 (C-6); MS:  $m/z$  244  $[\text{M}+\text{H}]^+$ . Anal. Calcd for  $\text{C}_{13}\text{H}_{13}\text{N}_3\text{O}_2$ : C, 64.19; H, 5.39; N, 17.27. Found: C, 64.23; H, 5.40; N, 17.28.

**General procedure for the preparation of spiro pyrazol-3-ones 4a–d containing isoxazoline N-oxide moiety from 1a–d via an oximation and HTIB-mediated oxidative N–O coupling.** A mixture of  $\beta$ -hydroxy ketoximes, which were prepared from **1a–d** according to the same procedure as described above, HTIB (0.431 g, 1.1 mmol), and MS 4Å (0.050 g) in DMF (5 mL) was stirred at rt for 1 h and then MS 4Å was removed by filtration and washed with DMF. The filtrate was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with  $\text{CHCl}_3$  as the eluent to give **4a–d**.

**9-Methyl-3,7-diphenyl-1-oxa-2,7,8-triazaspiro[4.4]nona-2,8-dien-6-one 2-oxide (4a):** Colorless needles (0.222 g, 69%), mp 175–176 °C (MeOH/ $\text{Et}_2\text{O}$ ); IR (ATR):  $\nu$  1692  $\text{cm}^{-1}$  (CO);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.29 (s, 3H, 9-Me), 3.65 (d,  $J = 16.5$  Hz, 1H, 4-H), 3.93 (d,  $J = 16.5$  Hz, 1H, 4-H), 7.21–7.26 (m, 1H,



Ph-H), 7.39–7.53 (m, 5H, Ph-H), 7.80–7.89 (m, 4H, Ph-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  12.9 (9-Me), 38.1 (C-4), 78.8 (C-5), 110.7 (C-3), 118.7, 125.3, 125.9, 126.3, 129.1, 129.2, 130.4, 137.4 (Ph-C), 157.3 (C-9), 168.1 (C-6); MS:  $m/z$  322  $[\text{M}+\text{H}]^+$ . Anal. Calcd for  $\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_3$ : C, 67.28; H, 4.71; N, 13.08. Found: C, 67.19; H, 4.79; N, 13.00.

**9-Methyl-3-(4-methylphenyl)-7-phenyl-1-oxa-2,7,8-triazaspiro[4.4]nona-2,8-dien-6-one 2-oxide (4b):**

Colorless needles (0.147 g, 44%), mp 168–169 °C (MeOH/Et<sub>2</sub>O); IR (ATR):  $\nu$  1699  $\text{cm}^{-1}$  (CO);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.28 (s, 3H, 9-Me), 2.39 (s, 3H, 4-Me-C<sub>6</sub>H<sub>4</sub>), 3.62 (d,  $J$  = 16.5 Hz, 1H, 4-H), 3.90 (d,  $J$  = 16.5 Hz, 1H, 4-H), 7.18–7.29 (m, 3H, Ph-H), 7.38–7.45 (m, 2H, Ph-H), 7.72–7.75 (m, 2H, Ph-H), 7.84–7.88 (m, 2H, Ph-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  12.9 (9-Me), 21.6 (4-Me-C<sub>6</sub>H<sub>4</sub>), 38.2 (C-4), 78.8 (C-5), 110.7 (C-3), 118.7, 122.4, 125.8, 126.2, 129.1, 129.9, 137.4, 141.0 (Ph-C), 157.4 (C-9), 168.2 (C-6); MS:  $m/z$  336  $[\text{M}+\text{H}]^+$ . Anal. Calcd for  $\text{C}_{19}\text{H}_{17}\text{N}_3\text{O}_3$ : C, 68.05; H, 5.11; N, 12.53. Found: C, 68.06; H, 5.16; N, 12.54.

**3-(4-Chlorophenyl)-9-methyl-7-phenyl-1-oxa-2,7,8-triazaspiro[4.4]nona-2,8-dien-6-one 2-oxide (4c):**

Colorless scales (0.195 g, 55%), mp 173–174 °C (MeOH/Et<sub>2</sub>O); IR (ATR):  $\nu$  1699  $\text{cm}^{-1}$  (CO);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.29 (s, 3H, 9-Me), 3.62 (d,  $J$  = 16.5 Hz, 1H, 4-H), 3.89 (d,  $J$  = 16.5 Hz, 1H, 4-H), 7.18–7.26 (m, 1H, Ph-H), 7.37–7.48 (m, 4H, Ph-H), 7.76–7.89 (m, 4H, Ph-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  12.9 (9-Me), 37.9 (C-4), 78.8 (C-5), 110.2 (C-3), 118.7, 123.9, 125.9, 127.4, 129.1, 129.5, 136.3, 137.3 (Ph-C), 157.0 (C-9), 168.0 (C-6); MS:  $m/z$  356  $[\text{M}+\text{H}]^+$ . Anal. Calcd for  $\text{C}_{18}\text{H}_{14}\text{ClN}_3\text{O}_3$ : C, 60.77; H, 3.97; N, 11.81. Found: C, 60.79; H, 3.89; N, 11.89.

**3,9-Dimethyl-7-phenyl-1-oxa-2,7,8-triazaspiro[4.4]nona-2,8-dien-6-one 2-oxide (4d):**

Colorless needles (0.152 g, 59%), mp 128–129 °C (MeOH/Et<sub>2</sub>O); IR (ATR):  $\nu$  1709  $\text{cm}^{-1}$  (CO);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.07 (s, 3H, 3-Me), 2.24 (s, 3H, 9-Me), 3.17–3.46 (m, 2H, 4-H), 7.19–7.22 (m, 1H, Ph-H), 7.38–7.41 (m, 2H, Ph-H), 7.82–7.84 (m, 2H, Ph-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  12.8 (3-Me), 12.5 (9-Me), 39.7 (C-4), 78.4 (C-5), 108.4 (C-3), 118.7, 125.8, 129.1, 137.4 (Ph-C), 157.2 (C-9), 168.4 (C-6); MS:  $m/z$  260  $[\text{M}+\text{H}]^+$ . Anal. Calcd for  $\text{C}_{13}\text{H}_{13}\text{N}_3\text{O}_3$ : C, 60.22; H, 5.05; N, 16.21. Found: C, 60.23; H, 5.09; N, 16.26.

**General procedure for the preparation of 3a–d from 4a–d and trimethyl phosphite.** A mixture of **4a–d** (1 mmol) and trimethyl phosphite (0.620 g, 5 mmol) was stirred at 110 °C for 1 h. To the reaction mixture, a 5% H<sub>2</sub>SO<sub>4</sub> solution (20 mL) was added with stirring and ice-cooling. The resulting mixture was extracted with CHCl<sub>3</sub> (60 mL). The extract was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with CHCl<sub>3</sub> as the eluent to provide **3a** (0.265 g, 87%), **3b** (0.270 g, 85%), **3c** (0.303 g, 89%), and **3d** (0.167 g, 68%), respectively.

**Reaction of plasmid pBR322 with compounds 3a–d and 4a–d.** The method of assaying the DNA cleavage activity, using a covalently closed circular duplex DNA (ccc-DNA) of plasmid pBR322 as a substrate, was described in our previous investigation.<sup>13</sup> The results are listed in Table 2. The reaction mixture (100  $\mu\text{L}$ ) containing 1  $\mu\text{g}$  of ccc-DNA of plasmid pBR322, 10 mM of **3a–d** and **4a–d**, and 50

mM Tris-HCl buffer (pH 7.4), was incubated at 37 °C. At interval, 20 µL of the reaction was mixed with 2 µL of 10 × Loading Buffer (TAKARA BIO INC. Shiga, Japan). The resulting mixture was directly by 1.0% agarose gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide (0.5 µg/mL) for 20 min. Under these conditions the order of anodal migration for the tree topological forms of the DNA was ccc-DNA, full-length linear duplex DNA (linear-DNA), and nicked open circular duplex DNA (oc-DNA). The ccc-DNA produced oc-DNA after single strand scission and linear-DNA after double-strand scission. They were all detected as clearly separated bands in agarose gels. The stained DNA bands were made visible using BioDoc-It™ Imaging Systems (UVP, Upland, CA) and then took the JPEG image file. For quantitative analysis of DNA on the gels, densitometric analyses of the images file were carried out using QuantiScan densitometry software (BIOSOFT, Cambridge, U.K.). The area under the ccc-DNA was multiplied by a factor of 1.42 to correct for its reduced binding of ethidium bromide as indicated by Lloyd and coworkers.<sup>13a</sup>

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