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NOTES

Oxidative Modification of NK30424A and B Enhance Inhibitory Effect on Lipopolysaccharide-induced Tumour Necrosis Factor-α Promoter Activity

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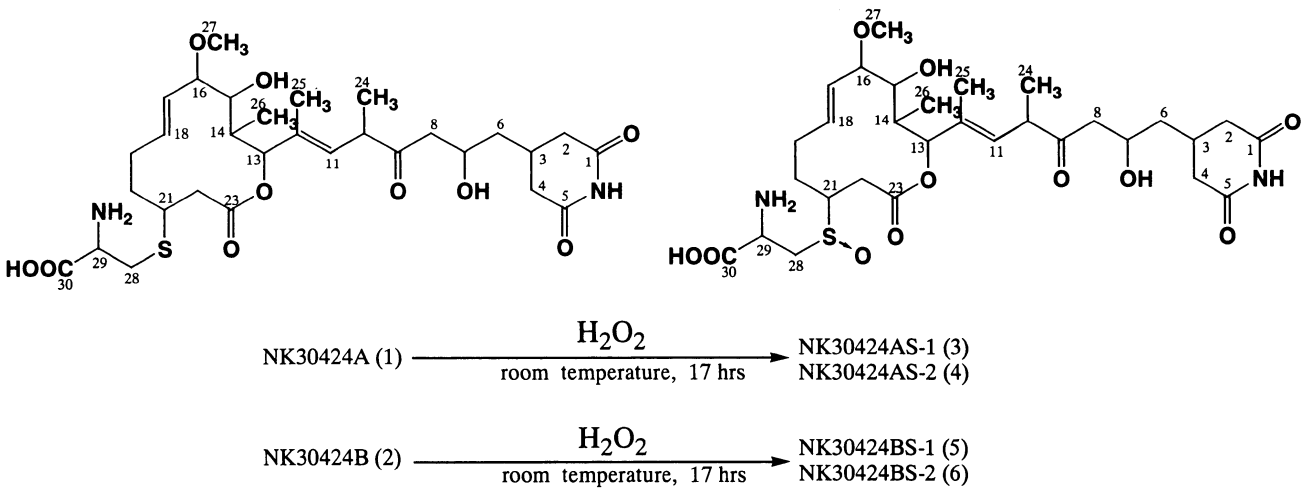
Novel potent inhibitors of lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α promoter activity were isolated from the culture broth of *Streptomyces* sp. NA30424. Previously, we discovered TNF-α inhibitors, NK30424A (compound **1**) and NK30424B (compound **2**) in the same culture broth[1]. Compounds **1** and **2** are stereoisomers, and their IC_{50} values against LPS-induced TNF-α promoter activity are 640 nM and 440 nM, respectively. In addition, we found four minor components in the broth with more strong activity. In this paper, the preparation and characteristics of the new components showing more potent inhibitory effects on TNF-α promoter activity are described.

These new components have oxidized structures of compound **1** or **2**. Their HPLC analysis using reverse phase column showed that these new components had higher polarity than compounds **1** and **2**. All of them have very close but clearly different retention time in this analysis. Mass spectra of all four components showed the same $(M+H)^+$ peak at *m/z* 643 giving them a molecular weight of 642 : 16 mass units larger than that of compound **1** or **2**. These data support that four components have one more oxygen atom in their structures than compound **1** or **2** having a glutarimide structure with an unsaturated 12-membered lactone containing a cysteine moiety (Fig. 1).

To confirm their structures, S-oxide products were chemically prepared with hydrogen peroxide, because the S-atom in the structure of compound **1** or **2** is a susceptible position for oxidation. The new sulfoxide products, named

Fig. 1. Structures of NK30424A (**1**), NK30424B (**2**) and their sulfoxides (**3**, **4**, **5** and **6**).



NK30424A (1) and B (2) are the stereoisomers, having $[\alpha]_D$ (c 0.5, 20℃) ; A: +10.8 (H_2O), B: +11.2 (H_2O). The isometric positions are under study.

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Table 1. ^{1}H NMR spectral data of NK30424 sulfoxides.

Position	^{1}H NMR (400MHz, D_2O) δ ppm [a]			
	NK30424AS-1(3)	NK30424AS-2(4)	NK30424BS-1(5)	NK30424BS-2(6)
2	2.41 (1H, m)	2.46 (1H, m)	2.42 (1H, m)	2.42 (1H, m)
2	2.78 (1H, m)	2.77 (1H, m)	2.76 (1H, m)	2.79 (1H, m)
3	2.41 (1H, m)	2.40 (1H, m)	2.42 (1H, m)	2.42 (1H, m)
4	2.41 (1H, m)	2.46 (1H, m)	2.42 (1H, m)	2.46 (1H, m)
4	2.78 (1H, m)	2.77 (1H, m)	2.76 (1H, m)	2.76 (1H, m)
6	1.50 (1H, m)	1.48 (1H, m)	1.49 (1H, m)	1.49 (1H, m)
6	1.58 (1H, m)	1.57 (1H, m)	1.58 (1H, m)	1.59 (1H, m)
7	4.17 (1H, m)	4.15 (1H, m)	4.17 (1H, m)	4.18 (1H, m)
8	2.69 (2H, d, 6.3)	2.68 (2H, d, 6.3)	2.70 (2H, m)	2.72 (2H, m)
10	3.62 (1H, m)	3.62 (1H, m)	3.60 (1H, m)	3.62 (1H, m)
11	5.24 (1H, d, 9.8)	5.23 (1H, d, 9.8)	5.24 (1H, d, 9.6)	5.27 (1H, d, 9.7)
13	4.91 (1H, d, 2.9)	4.88 (1H, d, 2.5)	5.07 (1H, bs)	5.08 (1H, d, 2.1)
14	2.07 (1H, m)	2.06 (1H, m)	2.06 (1H, m)	2.09 (1H, m)
15	3.75 (1H, dd, 9.4, 5.4)	3.74 (1H, dd, 9.4, 5.7)	3.67 (1H, dd, 9.2, 3.7)	3.67 (1H, dd, 9.3, 3.3)
16	3.60 (1H, m)	3.59 (1H, m)	3.60 (1H, m)	3.60 (1H, m)
17	5.58 (1H, dd, 16.2, 6.1)	5.59 (1H, dd, 16.1, 5.3)	5.58 (1H, dd, 16.0, 6.3)	5.55 (1H, dd, 16.0, 6.2)
18	5.82 (1H, m)	5.83 (1H, ddd, 16.1, 8.3, 3.8)	5.78 (1H, m)	5.77 (1H, m)
19	2.16 (1H, m)	2.08 (1H, m)		
19	2.66 (1H, m)	2.64 (1H, m)	2.35 (2H, m)	2.33 (2H, m)
20	1.84 (1H, m)	1.70 (1H, m)	1.68 (1H, m)	1.81 (1H, m)
20	2.19 (1H, m)	2.09 (1H, m)	2.10 (1H, m)	2.15 (1H, m)
21	3.46 (1H, m)	3.50 (1H, m)	3.54 (1H, m)	3.36 (1H, m)
22	2.60 (1H, dd, 16.0, 10.5)	2.64 (1H, m)	2.50 (2H, m)	2.49 (2H, m)
22	3.00 (1H, d, 16.0)	3.08 (1H, m)		
24	1.13 (3H, d, 6.8)	1.12 (3H, d, 6.8)	1.13 (3H, d, 6.7)	1.13 (3H, d, 6.8)
25	1.77 (3H, d, 0.6)	1.76 (3H, s)	1.78 (3H, s)	1.79 (3H, s)
26	0.97 (3H, d, 7.1)	0.97 (3H, d, 7.1)	1.00 (3H, d, 7.2)	0.98 (3H, d, 7.2)
27	3.30 (3H, s)	3.30 (3H, s)	3.32 (3H, s)	3.32 (3H, s)
28	3.38 (1H, m)	3.03 (1H, dd, 13.8, 7.7)		3.23 (1H, dd, 13.9, 7.8)
28	3.49 (1H, m)	3.34 (1H, dd, 13.8, 5.8)	3.32 (2H, m)	3.49 (1H, dd, 13.9, 6.0)
29	4.27 (1H, dd, 7.8, 3.6)	4.23 (1H, dd, 7.7, 5.8)	4.27 (1H, dd, 8.0, 2.9)	4.28 (1H, dd, 7.8, 6.0)

a : Sodium 3-(trimethylsilyl)propionic-2,2,3,3-d $_4$ acid as internal standard

Table 2. ^{13}C NMR spectral data of NK30424 sulfoxides.

Position	^{13}C NMR (100MHz, D_2O) δ ppm [a]			
	NK30424AS-1(3)	NK30424AS-2(4)	NK30424BS-1(5)	NK30424BS-2(6)
1	179.5(s) [b]	179.5(s) [b]	179.5(s) [b]	179.5(s) [b]
2	39.2(t) [c]	39.2(t) [c]	39.1(t) [c]	39.1(t) [c]
3	29.4(d)	29.4(d)	29.4(d)	29.4(d)
4	40.2(t) [c]	40.1(t) [c]	40.1(t) [c]	40.1(t) [c]
5	179.6(s) [b]	179.6(s) [b]	179.6(s) [b]	179.6(s) [b]
6	43.6(t)	43.6(t)	43.5(t)	43.6(t)
7	67.4(d)	67.4(d)	67.4(d)	67.4(d)
8	50.8(t)	50.5(t)	50.7(t)	50.8(t)
9	218.3(s)	218.3(s)	218.2(s)	218.2(s)
10	48.8(d)	48.8(d)	48.8(d)	48.8(d)
11	128.6(d)	128.5(d)	128.5(d)	129.1(d)
12	137.1(s)	137.2(s)	137.6(s)	137.3(s)
13	82.8(d)	82.7(d)	82.4(d)	83.2(d)
14	40.9(d)	40.9(d)	41.4(d)	41.1(d)
15	76.1(d)	76.3(d)	77.3(d)	76.7(d)
16	84.8(d)	84.7(d)	84.7(d)	84.7(d)
17	130.7(d)	130.6(d)	133.3(d)	133.2(d)
18	138.4(d)	138.4(d)	136.5(d)	136.4(d)
19	33.0(t)	33.4(t)	31.3(t)	30.9(t)
20	28.6(t)	28.1(t)	29.0(t)	30.3(t)
21	60.0(d)	60.1(d)	57.7(d)	58.2(d)
22	35.1(t)	33.9(t)	35.4(t)	34.6(t)
23	174.1(s)	174.3(s)	174.0(s)	174.1(s)
24	18.0(q)	18.0(q)	18.0(q)	18.0(q)
25	16.3(q)	16.3(q)	16.5(q)	16.4(q)
26	13.6(q)	13.6(q)	13.6(q)	13.6(q)
27	58.8(q)	58.8(q)	58.9(q)	59.0(q)
28	50.7(t)	48.9(t)	49.8(t)	51.7(t)
29	53.2(d)	53.9(d)	53.2(d)	53.9(d)
30	174.9(s)	175.2(s)	174.4(s)	174.4(s)

a : Sodium 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid as internal standard

b,c : Assignments may be interchanged

NK30424AS-1 (compound **3**) and AS-2 (compound **4**), were obtained as S–O diastereomers from compound **1**, and NK30424BS-1 (compound **5**) and BS-2 (compound **6**) from compound **2**. These structures were confirmed by FAB-MS, IR and 2D-NMR (^{1}H-^{1}H COSY, ^{13}C-^{1}H HMQC and ^{13}C-^{1}H HMBC) analysis. ^{1}H and ^{13}C NMR assignments for compounds **3**~**6** are summarized in Tables 1 and 2. IR spectra of these compounds showed 1014~1022 cm^{-1} absorption according to the S–O bond. Thus, the structures of mono-oxidation products from compounds **1** and **2** have been elucidated as their sulfoxides, although the stereochemistries of these compounds remain to be defined. In HPLC analysis, compounds **3**~**6** have identical peaks to those of the minor components in the NA30424 culture broth.

Not only HPLC analysis but also biological assay supported that the minor components in the culture broth might be S-oxide analogues. The IC_{50} values of compounds **3**~**6** for inhibition of LPS-induced TNF-α promoter activity in J774.1 murine macrophage-like cells were 8.9, 22, 6.5 and 3.5 nM, respectively. They are approximately 30~120 times more potent than compounds **1** (640 nM) and **2** (440 nM). These results indicate the sulfoxide groups of compounds **3**~**6** are necessary for their potent activities.

NK30424 compounds inhibited LPS-induced TNF-α promoter activity, and their target is suggested to be a molecule in a NF-κB signaling pathway. It is well known that NF-κB is the indispensable transcription factor in LPS-induced TNF-α production[2]. In addition, it is considered that NF-κB is a promising target molecule for anti-inflammatory agents, because of its ability to activate transcription of various genes involved in inflammation. Furthermore, increasing evidence supports its role in regulating cell growth, oncogenesis, and protection from cell death[3]. The determination of NK30424-compounds target molecule is now under way, and recent unpublished data suggest that these compounds can inhibit the NF-κB signaling pathway. In the comparison with compounds **1** and **2**, compounds **3**~**6** have more powerful activity. Hence, they would be better candidates for treatment of pathophysiologic diseases promoted by constitutive activation of the NF-κB signaling pathway.

Experimental

IR spectra were recorded on a JEOL JIR-6500 infrared spectrometer. FAB-MS spectra were recorded on a Micromass Auto Spec Q spectrometer. ^{1}H (400 MHz) and ^{13}C (125 MHz) NMR spectra were obtained on a Bruker model AVENCE-400 spectrometer.

NK30424AS-1 (3) and AS-2 (4)

NK30424AS-1 (compound **3**) and AS-2 (compound **4**) were prepared by compound **1** oxidization. Under ice-cooling, 30% hydrogen peroxide aqueous solution (0.02 ml) was added to a solution of compound **1** (9 mg, 14.4 μmol) in 10 mM sodium acetate buffer (pH 5, 1.5 ml), and the mixture was stirred at room temperature for 17 hours. The resulting solution contained compounds **3** and **4** was concentrated under reduced pressure, and was purified by a preparative HPLC (PEGASIL ODS; 10 mm×250 mm) using with the mobile phase of 17% CH_3CN in 10 mM ammonium phosphate buffer (pH 7) at flow rate of 2.5 ml/minute. Each compound **3** and **4** was eluted on retention time at 24.4 and 27.8 minutes, respectively. Eluates were evaporated to remove CH_3CN and each resultant aqueous layer was applied on a column of Diaion HP-20 resin (1 ml), respectively. The column was washed with water (2 ml) and 20% aq. MeOH (2 ml), and eluted with 80% aq. MeOH (4 ml). These eluates were concentrated and lyophilized to yield the colorless powder of compounds **3** (4.2 mg, 6.5 μmol, Y.=45.1%) and **4** (2.4 mg, 3.7 μmol, Y.=25.7%). Compound **3**: IR (KBr) cm^{-1} 3433, 2933, 1697, 1641, 1380, 1267, 1155, 1095, 1020; FAB-MS (pos.) *m/z* 643 $(M+H)^+$. Compound **4**: IR (KBr) cm^{-1} 3437, 2933, 1702, 1641, 1383, 1267, 1155, 1095, 1022; FAB-MS (pos.) *m/z* 643 $(M+H)^+$.

NK30424BS-1 (5) and BS-2 (6)

For the preparation of compounds **5** and **6**, the same procedure described above was carried out. From compound **2** (43 mg, 68.7 μmol), each lyophilized colorless powder of compounds **5** (15.0 mg, 23.4 μmol, Y.=34.1%) and **6** (12.9 mg, 20.1 μmol, Y.=29.3%) was obtained. During the procedure, Compound **5** had a peak at retention time 43.2 minutes by HPLC preparation (Capcell Pak ODS; 20 mm×250 mm) with the mobile phase of 16% CH_3CN in 10 mM ammonium phosphate buffer (pH 7) at flow rate of 8 ml/minute. In the same condition, compound **6** was eluted at 52.2 minutes. Characteristics of those compounds were as follows; Compound **5**: IR (KBr) cm^{-1} 3444, 2933, 1699, 1653, 1385, 1265, 1153, 1103, 1014; FAB-MS (pos.) *m/z* 643 $(M+H)^+$. Compound **6**: IR (KBr) cm^{-1} 3444, 2933, 1699, 1653, 1385, 1265, 1153, 1103, 1014; FAB-MS (pos.) *m/z* 643 $(M+H)^+$.

Assay for TNF-α Promoter Activity

Measurement of TNF-α promoter activity was performed as described previously[1]. Briefly, TNF-α

promoter activity was detected as β-galactosidase activity in a murine cell line J744.1 transfected with β-galactosidase reporter plasmid containing human TNF-α promoter DNA. β-Galactosidase activity was measured after 6 hours LPS (1 μg/ml) stimulation, using 4-methylumbellyferyl-D-β-garactopyranoside as a substrate. Cells incubated without LPS served as controls for the baseline activity of TNF-α promoter. Cultures incubated with LPS in the absence of test samples served as positive controls for activation of TNF-α promoter activity.

References

1) TAKAYASU, Y.; K. TSUCHIYA, T. AOYAMA & Y. SUKENAGA: NK30424A and B, novel inhibitors of lipopolysaccharide-induced tumour necrosis factor alpha production, produced by *Streptomyces* sp. NA30424. J. Antibiotics 54: 1111~1115, 2001
2) SHAKHOV, A. N.; M. A. COLLART, P. VASSALLI, S. A. NEDOSPASOV & C. V. JONGENEEL: κB-Type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumour necrosis factor-α gene in primary macrophages. J. Exp. Med. 171: 35~47, 1990
3) RAYET, B. & C. GÉLINAS: Aberrant *rel/nfkb* genes and activity in human cancer. Oncogene 18: 6938~6947, 1999