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Synthesis of indolyl-3-acetonitrile derivatives and their inhibitory effects on nitric oxide and PGE_2 productions in LPS-induced RAW 264.7 cells

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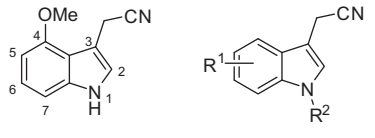
ABSTRACT

Arvelexin is one of major constituents of *Brassica rapa* that exerts anti-inflammatory activities. Several indolyl-3-acetonitrile derivatives were synthesized as arvelexin analogs and evaluated for their abilities to inhibit NO and PGE_2 productions in LPS-induced RAW 264.7 cells. Of the indolyl-3-acetonitriles synthesized, compound **2k**, which possesses a hydroxyl group at C-7 position of the indole ring and an *N*-methyl substituent, more potently inhibited NO and PGE_2 productions and was less cytotoxic than arvelexin on macrophage cells.

Inflammation is one of the protective immune responses to tissue injury caused by harmful stimuli, such as invading pathogens, damaged cells or irritants.[1] Paradoxically, persistent and excessive immune response can promote tissue damage, resulting in chronic inflammation. This chronic inflammation is a part of many human diseases, including arteriosclerosis,[2] inflammatory bowel disease,[3] arthritis,[4] cancer[5] and Alzheimer's disease.[6] In the inflammatory state, activated immune cells, such as macrophages secrete large amounts of proinflammatory cytokines, nitric oxide (NO), and prostaglandin E_2 (PGE_2). However, high levels of NO and PGE_2 in a chronic inflammation state can result in various pathological conditions.[7,8] Accordingly, control of the production of NO and PGE_2 in macrophages are current research topics for the development of new anti-inflammatory agents.

Arvelexin (2-(4-methoxy-1*H*-indol-3-yl)acetonitrile, **1**) is a crucifer phytoalexin, biosynthesized de novo as a defense metabolite in response to various forms of stress including salt, drought, UV irradiation, or pathogens.[9] Arvelexin has been isolated from the plants of Brassicaceae family including *Thlaspi arvense* (stinkweed),[10] seeds of *Thellungiella halophila* [11] and the roots of *Brassica campestris* ssp *rapa*.[12] Arvelexin was known to possess antifungal activity,[11] however, studies on other biological activities are scarce. Furthermore, structure–activity relationship studies were not investigated intensively by the synthesis of arvelexin analogs. Recently, we first reported that arvelexin is an anti-inflammatory constituent of *Brassica rapa* because it down-regulates inflammatory *i*-NOS, COX-2, TNF-α, IL-6, and IL-1β gene expression via NF-κB inactivation.[13]

In the present study, we prepared various indolyl-3-acetonitriles **2a**–**2n** as arvelexin analogs with the aim to study structure–activity relationships, and thereby provide new leads to compounds possessing enhanced inflammatory activity and reduced cytotoxicity. More specifically, we synthesized indolyl-3-acetonitriles possessing methoxy, benzyloxy, or hydroxyl substituent (R^1) at the benzene ring of indole structure and introduced methyl or several acyl substituents (R^2) at nitrogen. The anti-inflammatory activities of arvelexin analogs were evaluated by



1, arvelexin **2a-2n**, target compounds

Figure 1. Structures of arvelexin and target compounds.

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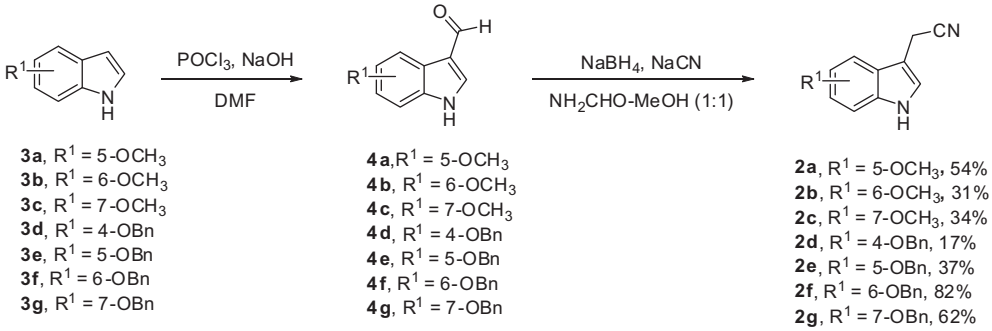
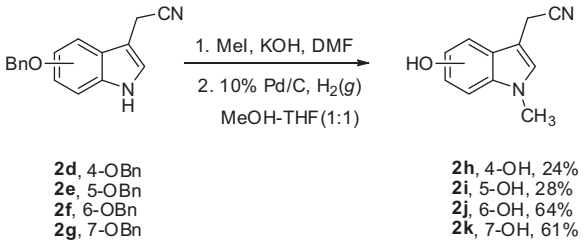


Scheme 1. Synthesis of arvelexin analogs **2a**–**2g**.



Scheme 2. Synthesis of arvelexin analogs **2h**–**2k**.

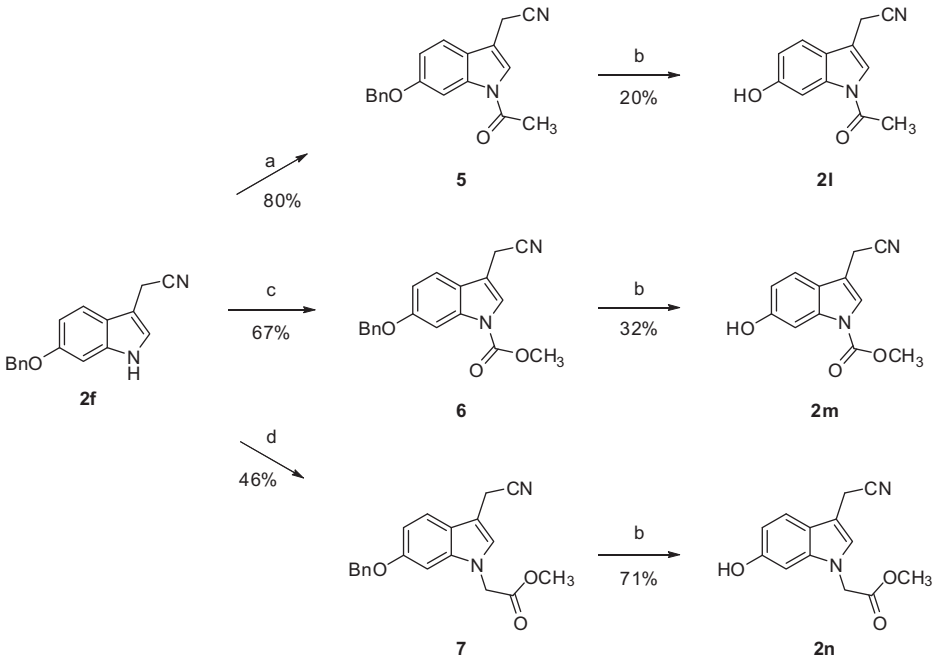
measuring the inhibitions of the productions of NO and PGE_2 in LPS-induced RAW 264.7 cells (Fig. 1).

Indolyl-3-acetonitrile derivatives **2a**–**2g**, which has a methoxy or benzyloxy substituent at the benzene ring, were synthesized as shown in Scheme 1 using a modification of previously reported procedure. Commercially available indole derivatives **3a**–**3g**, which already possess a methoxy or benzyloxy substituent at the benzene ring, were used as starting materials. Compounds **3a**–**3g** were converted into indole-3-carboxaldehydes **4a**–**4g** by a Vilsmeier–Haack reaction with phosphorus oxychloride and dimethyl formamide.[14] Treatment of these carboxaldehydes with NaCN in the presence of $NaBH_4$ in NH_2CHO–MeOH afforded indolyl-3-acetonitriles **2a**–**2g**.[15]

Indolyl-3-acetonitriles **2h**–**2k**, which has a hydroxy substituent at the benzene ring and a methyl substituent at nitrogen, were prepared from **2d** to **2g** as shown in Scheme 2. The methylation at nitrogen with methyl iodide and KOH in DMF followed by removal of benzyl group in **2d**–**2g** by the catalytic hydrogenation over 10% Pd–C in MeOH and THF produced **2h**–**2k**.[16]

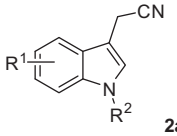
To examine further the influence of the substituents at nitrogen on the anti-inflammatory activity, *N*-acyl or ester containing indolyl-3-acetonitriles **2l**–**2n** were also synthesized (Scheme 3). Treatment of (2-(4-benzyloxy-1*H*-indol-3-yl)acetonitrile **2f**) with acetic anhydride, methyl chloroformate, or methyl bromoacetate, followed by debenzylation with 5% Pd–C afforded **2l**–**2n**. In case of using 10% Pd–C at the debenzylation step, the isolation yields of products were very low probably due to side reactions such as hydrogenation at the double bond of the indole ring.

Inflammation is a protective immune response against tissue damage induced by external stimulants in the body.[2] However, the inflammatory response can also result in considerable damage to the host, because microbial components like lipopolysaccharide



Scheme 3. Synthesis of arvelexin analogs **2i**–**2n**. Reagents and conditions: (a) Ac_2O, NEt_3, reflux; (b) 5% Pd/C, H_2, MeOH–THF; (c) methyl chloroformate, NaH, THF; (d) methyl bromoacetate, NaH, DMF.

Table 1
Effects of arvelexin and its analogs **2a**–**2n** on cytotoxicity and productions of NO and PGE_2 in LPS-induced RAW 267.4 cells



2a~2n

Entry	R^1	R^2	IC_{50}[a] (μM)		
			Cytotoxicity	NO production	PGE_2 production
2a	5-OCH_3	H	99.61 ± 5.01	>100	>100
2b	6-OCH_3	H	161.14 ± 5.72	>100	83.57 ± 10.33
2c	7-OCH_3	H	>400	>100	>100
2d	4-OBn	H	105.04 ± 16.97	45.61 ± 9.64	32.44 ± 10.8
2e	5-OBn	H	82.68 ± 5.79	19.45 ± 0.14	>25
2f	6-OBn	H	53.25 ± 8.10	20.44 ± 3.87	>25
2g	7-OBn	H	>400	48.74 ± 2.10	73.09 ± 16.4
2h	4-OH	CH_3	>400	57.08 ± 16.69	16.01 ± 6.55
2i	5-OH	CH_3	325.15 ± 5.50	>100	57.72 ± 7.76
2j	6-OH	CH_3	371.68 ± 10.96	80.10 ± 17.94	11.72 ± 3.56
2k	7-OH	CH_3	>400	39.76 ± 14.13	6.22 ± 2.75
2l	6-OH	$COCH_3$	>400	82.07 ± 2.79	41.27 ± 2.73
2m	6-OH	CO_2CH_3	339.08 ± 23.76	>100	15.18 ± 3.05
2n	6-OH	$CH_2CO_2CH_3$	>400	>100	>100
1, arvelexin			206.6 ± 2.32	83.60 ± 9.05	13.92 ± 8.52

[a] Data are presented as the means ± SDs of three independent experiments.

(LPS) stimulate macrophages to produce pro-inflammatory cytokines, *i*-NOS, and COX-2. In particular, the large amounts of NO and PGE_2 secreted by activated immune cells in the inflammatory state can induce various pathological conditions. Thus, inhibition of the production of inflammatory mediators is a potential strategy for the treatment of many acute and chronic inflammatory diseases. Recently, we found that arvelexin inhibited production of NO and PGE_2.[13] In the present study, we synthesized several indolyl-3-acetonitriles as arvelexin analogs to determine whether any compounds had better anti-inflammatory activity and less cytotoxicity than arvelexin. The synthesized indolyl-3-acetonitriles **2a**–**2n** were assessed in terms of their ability to inhibit the production of inflammatory mediators, NO and PGE_2, in LPS-induced RAW 264.7 cells.[17–19] Arvelexin (**1**) was used as a reference for comparisons. The cytotoxic effects of the indolyl-3-acetonitriles on RAW 264.7 cells were also evaluated using the MTT assay[20] to test whether the inhibitory effects on the productions of NO and PGE_2 were due to cytotoxic effects.

Indolyl-3-acetonitriles synthesized showed varying cytotoxicities and inhibitory activities on the LPS-induced productions of NO and PGE_2 (Table 1). When the 4-methoxy substituent of the indole ring in arvelexin (**1**) was moved to 5-, 6-, or 7-position to lead indolyl-3-acetonitriles **2a**–**2c**, inhibitory activities on LPS-induced NO and PGE_2 productions were decreased. On the other hand, compounds **2d**–**2g**, which possess a benzyloxy substituent instead of methoxy group, more potently inhibited NO production than arvelexin. However, except for compound **2g**, these inhibitory effects appeared to be due in part to their cytotoxic effects. When the hydroxy group and methyl group were introduced at indole structure (**2h**–**2k**), both NO and PGE_2 production were more inhibited with reduced cytotoxicity compared to arvelexin. Interestingly, compounds with C-7 substituent (**2c**, **2g**, **2k**) had no cytotoxic effect on RAW 264.7 cells at concentrations up to 400 μM, irrespective of the pattern of the substituents. Substitution of acyl or other ester groups at nitrogen of 6-hydroxy-indolyl-3-acetonitrile (**2l**–**2n**) did not enhance the inhibitory activities on NO and PGE_2 productions. Of the compounds, **2k** exhibited well-balanced and enhanced activity profiles with respect to the inhibitions of the productions of NO (IC_{50} = 39.76 μM vs 83.60 μM) and PGE_2 (IC_{50} = 6.22 μM vs 13.92 μM), and cytotoxicity (IC_{50} = >400 μM vs 206.6 μM) than arvelexin.

In conclusion, we synthesized indolyl-3-acetonitriles as arvelexin analogs in order to explore the relationship between structures of indolyl-3-acetonitriles and their anti-inflammatory activities. Synthesized compounds showed varying cytotoxicities and inhibitory activities on the LPS-induced productions of NO and PGE_2. Introduction of benzyloxy or hydroxyl group at 7-position of the indole ring enhanced NO productions and reduced cytotoxicity on RAW 264.7 cells. Compound **2k** exhibited the most potent and enhanced inhibitory activities on both NO and PGE_2 productions in LPS-induced RAW 264.7 cells with reduced cytotoxicity compared to arvelexin.

Acknowledgments

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16. *Physical and spectral data of selected compounds*. Compound **2j**: mp 160 °C (dec); IR (KBr, cm^{-1}) ν 3386, 2937, 2364, 1626, 1223; ^{1}H NMR (400 MHz, CD_3OD) δ 7.27 (1H, d, J = 8.5 Hz, H-4), 6.88 (s, 1H, H-2), 6.63 (1H, d, J = 2.0 Hz, H-7), 6.66 (1H, dd, J = 8.5, 2.0 Hz, H-5), 3.76 (3H, s, –NCH_3), 3.56 (2H, s, –CH_2CN); ^{13}C NMR (100 MHz, CD_3OD) δ 155.02, 139.86, 127.16, 121.87, 120.03, 119.78, 113.23, 111.25, 110.74, 104.48, 103.35, 95.97, 32.70; HR-DART-MS m/z 187.0860 (calcd for $C_{11}H_{11}N_2O$ $[M+H]^+$, 187.0871). Compound **2k**: mp 140 °C (dec); IR (KBr, cm^{-1}) ν 3379, 2938, 2367, 1581, 1274; ^{1}H NMR (400 MHz, CD_3OD) δ 6.89–6.91 (2H, m, H-2,4), 6.75 (1H, t, J = 7.9 Hz, H-5), 6.42 (1H, d, J = 7.9 Hz, H-6), 3.94 (3H, s, –NCH_3), 3.74 (2H, s, –CH_2CN); ^{13}C NMR (100 MHz, CD_3OD) δ 144.90, 129.30, 127.96, 126.59, 119.87, 118.57, 109.08, 106.57, 102.85, 34.86, 12.76; HR-DART-MS m/z 187.0860 (calcd for $C_{11}H_{11}N_2O$ $[M+H]^+$, 187.0871).
17. *Cell culture and sample treatment*: the RAW 264.7 macrophage cell line was obtained from the Korea Cell Line Bank (Seoul). Cells were grown at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) in a humidified 5% CO_2 atmosphere. Cells were incubated with various concentrations of tested samples and then stimulated with LPS 1 μg/mL for the indicated time.
18. *Nitrite determination:* RAW 264.7 cells were plated at 4×10^5 cells/well in 24 well-plates and then incubated with or without LPS (1 μg/mL) in the absence or presence of various concentrations (3.15, 6.25, 12.5, 25, 50 and 100 μM) of tested samples for 24 h. Nitrite levels in culture media were determined using the Griess reaction and presumed to reflect NO levels. Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine–HCl], and incubated at room temperature for 10 min. Absorbance was then measured at 540 nm using a microplate reader (Perkin Elmer Cetus, Foster City, CA, USA). Fresh culture media were used as blanks in all experiments. The amount of nitrite in the samples was measured with the sodium nitrite serial dilution standard curve and nitrite production was measured.
19. *PGE_2 assay*: RAW 264.7 cells were pretreated with tested samples for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. Levels of PGE_2 in the culture media was quantified using EIA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.
20. *MTT assay for cell viability*: RAW 264.7 cells were plated at a density of 10^5 cells/well in 96-well plates. To determine the appropriate concentration not toxic to cells, cytotoxicity studies were performed 24 h after treating cells with various concentrations of tested compounds. Cell viabilities were determined using colorimetric MTT assays, as described previously.[21]
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