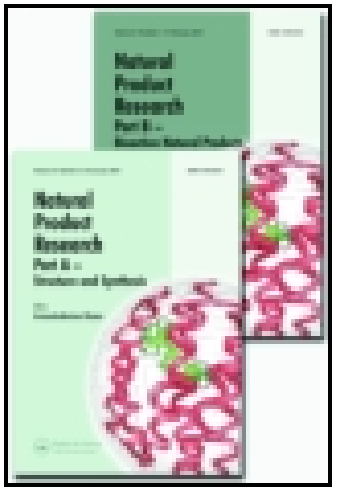

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Synthesis and biological evaluation of nigranoic acid esters as novel human neutrophil elastase inhibitors

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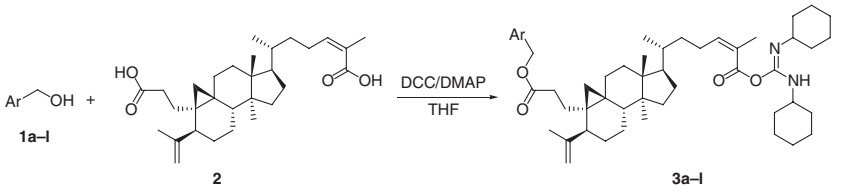

Human neutrophil elastase (HNE) has been implicated as a major contributor in the pathogenesis of diseases, such as lung disorders and other inflammatory diseases. A series of 12 new nigranoic acid esters were regioselectively synthesised in good yields and evaluated for HNE inhibitory activity. Nigranoic acid exhibited significant inhibitory activity against HNE with the IC_{50} value of 3.77 μM, and six esters displayed considerable inhibitory effects on HNE with IC_{50} values in the range of 2.61–8.95 μM. The nigranoic acid esters having phenyls substituted with bromine and trimethoxyls (**3h** and **3b**) showed stronger inhibitory activity on HNE than nigranoic acid.

Keywords: nigranoic acid esters; human neutrophil elastase; inhibition

1. Introduction

Human neutrophil elastase (HNE, E.C. 3.4.21.37) is a 30 kDa serine protease stored in the azurophilic granules of neutrophils. The intracellular HNE at high concentration (5 mM) breaks down foreign proteins (e.g. those from invading bacteria), whereas extracellular HNE, released by neutrophils and mostly bound to the neutrophil plasma membrane, assists neutrophil migration to inflammation sites by degrading various host proteins, such as extracellular matrix proteins (Siedle et al. 2007). Under normal physiological conditions, HNE is controlled by endogenous inhibitors, including α_1-trypsin (α_1-AT), secretory leucocyte proteinase inhibitor, α_2-macroglobulin and elafin (Rubin 1996; Fitch et al. 2006; Pham 2006; Williams et al. 2006). However, an imbalance between HNE and its endogenous inhibitors can stimulate inflammatory lung disorders through HNE's role in the inflammatory process, mucus overproduction and lung tissue damage (Wright et al. 2002; Caldwell et al. 2005; Bergin et al. 2008). HNE plays an important role in a variety of lung inflammatory diseases, including acute lung injury, acute

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respiratory distress syndrome, bronchiectasis, pulmonary emphysema and chronic obstructive pulmonary disease (Stevens et al. 2011). Sivelestat sodium hydrate (ONO-5046) is the only clinically registered synthesised selective HNE inhibitor. However, the use of ONO-5046 is limited by its poor pharmacokinetics and by the fact that it presents risks of organ toxicity (Ohbayashi 2002; Huang et al. 2008; Stevens et al. 2011).

Furthermore, nigranoic acid is an A ring-secocycloartene triterpenoid isolated from the leaves and stems of *Schisandra lancifolia* and has been reported to show a variety of biological and medicinal activities, including cytotoxic activity towards leukaemia and HeLa cells, and inhibition of expression of HIV reverse transcriptase and polymerase (Sun et al. 1996, 2011; Chen et al. 2001; Xiao et al. 2006; Xu et al. 2010). Recently, nigranoic acid was isolated from *Schisandra propinqua*, which inspired us to study the structure–activity relationship of nigranoic acid analogues by the structural modification. Six new hydroxyl derivatives of nigranoic acid were obtained by microbial transformation (Dong, Chen, Song, He, et al. 2007; Dong, Chen, Song, Zhu, et al. 2007; Yang et al. 2012), and only two-hydroxyl analogues exhibited weak anti-HIV activity (Yang et al. 2012). Following our work on the structural modification and biological activity of nigranoic acid analogues, we now report the synthesis and identification of 12 nigranoic acid aromatic ester derivatives by the reaction of nigranoic acid and benzyl alcohols in the presence of *N*,*N*′-dicyclohexylcarbodie (DCC)/4-dimethylaminopyridine (DMAP). In addition, the activities of these compounds were determined through HNE inhibitory activity bioassay, and some compounds exhibited considerable inhibitory effects on HNE with IC_{50} values in the range of 2.61–8.95 μM.

2. Results and discussion

2.1. *Chemistry*

Benzyl alcohols were prepared by stirring aromatic aldehydes with excess $NaBH_4$ in methanol for 2 h. Twelve nigranoic acid esters were synthesised in the presence of DCC/DMAP with the nigranoic acid and benzyl alcohols as starting materials. The synthetic route of the compounds is outlined in Figure 1.

According to the literature (Melzig et al. 2001), in the presence of 2.2 equiv DCC/DMAP, nigranoic acid aromatic ester derivatives have been synthesised by the reaction of nigranoic acid (1.0 equiv) and benzyl alcohol (2.2 equiv) in THF at room temperature. The reaction afforded good yield (82–98%) and obtained single regioisomer. With the help of ^1H, ^{13}C NMR and HMBC spectrum of **3a**–**l** (Figures S1–S12), proton H-31 showed a key HMBC correlation with the carboxyl carbon C-3, suggesting a linkage between C-31 and C-3 in the product, that is to say, the esterification regioselectively reacted at C-3 position, not C-27 position, maybe because of the steric hindrance and links of different carbon chains (saturated aliphatic carbon chain for C-3 and α,β-unsaturated carbon chain for C-27). The structures of the synthesised compounds were confirmed by ^1H, ^{13}C NMR spectra and HR-MS (Table 1).

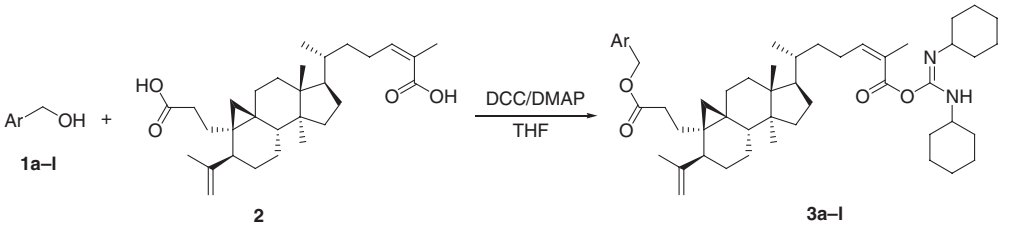
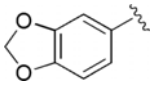
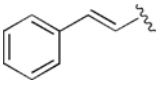
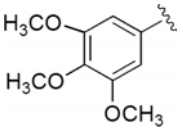
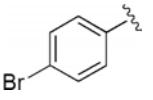
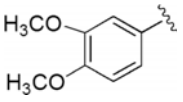
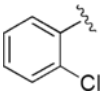
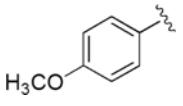
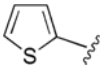
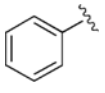
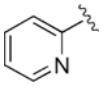
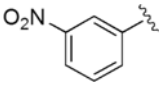
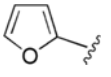


Figure 1. Synthesis of nigranoic acid esters **3a**–**3l**.

Table 1. Synthesis of nigranoic acid esters (**3a**–**3l**).

Entry	Ar	Product[a] (yield %)[b]	Entry	Ar	Product[a] (yield %)[b]
1		**3a** (85)	7		**3g** (94)
2		**3b** (89)	8		**3h** (88)
3		**3c** (94)	9		**3i** (82)
4		**3d** (87)	10		**3j** (91)
5		**3e** (85)	11		**3k** (98)
6		**3f** (89)	12		**3l** (84)

[a] All the products are confirmed by using NMR.
[b] Yield refers to pure products after column chromatography.

2.2. *HNE activity*

The inhibitory effects of the nigranoic acid and its esters were investigated, the concentration gradients of the tested compounds at 1, 3, 10, 30 and 100 μM. As positive control, ONO-5046 exhibited potent inhibitory activity, with an IC_{50} value of 87.05 nM (Feng et al. 2012). HNE activity was strongly and directly inhibited by nigranoic acid, with the IC_{50} value of 3.77 μM, whereas nigranoic acid esters having phenyls substituted with bromine and trimethoxyls (**3h** and **3b**) showed slightly more inhibitory potency than that of nigranoic acid, with IC_{50} values of 2.61 and 3.25 μM, respectively. Compound **3h** exhibited HNE inhibition with more than 83.21% at 10 μM ($p < 0.01$), and compound **3b** imposed a considerable inhibitory effect on HNE in a dose-dependent manner, with a minimal effective concentration of 1 μM (28.25% inhibition). The nigranoic acid esters bearing phenyls substituted with dimethoxyls and chlorine (**3c** and **3i**), and thiophene (**3j**) showed slightly less potency than that of nigranoic acid, with the IC_{50} values ranging from 3.89 to 4.83 μM. The nigranoic acid ester with cinnamenyl group (**3g**) showed the least inhibitory potency (IC_{50} value: 8.95 μM) among the tested compounds (Table 2). The other six analogues showed no activity.

Table 2. Anti-HNE activity of nigranoic acid esters (mean ± SD), $n = 3$.

Compound (μM)	1	3	10	30	100	IC_{50}
ONO-5046						0.087
Nigranoic acid	1.048 ± 0.201	0.806 ± 0.095*	0.365 ± 0.026*	0.071 ± 0.007*	0.060 ± 0.006*	3.77
3b	0.943 ± 0.056*	0.688 ± 0.176*	0.373 ± 0.102*	0.176 ± 0.027*	0.167 ± 0.011*	3.25
3c	0.589 ± 0.073*	0.443 ± 0.066*	0.124 ± 0.027*	0.130 ± 0.008*	0.147 ± 0.013*	3.89
3g	0.660 ± 0.016	0.529 ± 0.009*	0.148 ± 0.011*	0.288 ± 0.122*	0.165 ± 0.012*	8.95
3h	0.494 ± 0.051*	0.365 ± 0.002*	0.117 ± 0.006*	0.128 ± 0.006*	0.144 ± 0.016*	2.61
3i	0.558 ± 0.045*	0.410 ± 0.018*	0.106 ± 0.011*	0.126 ± 0.011*	0.153 ± 0.009*	4.01
3j	0.550 ± 0.033*	0.386 ± 0.030*	0.113 ± 0.010*	0.255 ± 0.090*	0.196 ± 0.021*	4.83

Note: The absorbance at 405 nm is expressed as mean ± standard deviation.
*$P < 0.01$ versus the control group.

3. Experimental

3.1. *General*

TLC analyses were performed on commercial glass plates bearing 0.25-mm layer of Merck Silica gel 60F254. Silica gel (200–300 mesh) was used for column chromatography. ^{1}H, ^{13}C NMR and HMBC spectra were recorded on a Bruker AVANCE III 500 MHz spectrometer (Bruker BioSpin Group, Rheinstetten, Germany) in $CDCl_3$ solution. ESI-MS and HR-MS were measured on a VG Auto Spec-3000 spectrometer (VG PRIMA, Birmingham, England). Unless otherwise noted, the starting materials were either commercially available from Aladdin in Shanghai, China or synthesised according to the references cited. Nigranoic acid (**2**) was isolated from the Chinese medicine *S. propinqua* and identified unambiguously by NMR spectroscopy and MS, and by comparison with authentic samples from the China Institute for Control of Pharmaceutical and Biological Products (Beijing, China). The purity was >99% based on high-performance liquid chromatography and thin-layer chromatography analyses.

3.2. *General procedure for the synthesis of nigranoic acid esters (3a–3l)*

The mixture of nigranoic acid (0.235 g, 0.5 mmol) and DCC (0.227 g, 1.1 mmol) was dissolved in dry THF (5 mL) and stirred under 0°C for 10 min, to which the solution of benzyl alcohol (1.1 mmol) and DMAP (0.134 g, 1.1 mmol) in dry THF (5 mL) was added and stirred for 30 min at the same temperature. The reaction mixture was warmed to room temperature and stirred overnight. The solvent was removed by vacuum and the residue was purified by column chromatography over silica gel (ethyl acetate/petroleum ether = 1:4) to afford the target product.

3.3. *HNE activity inhibitory bioassay*

HNE (EC 3.4.21.37) from human leucocytes was purchased from Innovative Research Company (Novi, Michigan). ONO-5046, HNE substrate (MeO–Suc–Ala–Ala–Pro–Val–pNA), soybean trypsin inhibitor and DMSO were obtained from Sigma (St. Louis, MO, USA) and used without further purification. All other reagents were of analytical grade.

The assay on the direct inhibition of HNE activity was performed as previously described (Shelkov et al. 2004), and carried out with ONO-5046 as positive control (Feng et al. 2012). Briefly, 200 μL substrate solution (1.4 mM MeO–Suc–Ala–Ala–Pro–Val–pNA in Tris-HCl buffer, 10 mM, pH 7.5) was mixed with different concentration gradients of the test compound solution; stock solutions of triterpenes were dissolved in DMSO, which was restricted to 0.5% and diluted with Tris-HCl buffer to produce the final sample concentrations. 0.01 U HNE was added to the solution, and then the resultant mixture was incubated for 1 h at 37°C in the dark. Subsequently, the reaction was quenched by adding 200 μL soybean trypsin inhibitor at a concentration of 0.2 mg/mL. The intensity of the colour developed by the digested substrate was then immediately measured at 405 nm using an automatic microplate reader (Bio-Tek, Synergy HT, San Diego, CA, USA), and the control background was subtracted. The group in which the HNE substrate was replaced with 10 mM Tris-HCl buffer solutions of pH 7.5 was used as the zero alignment of the test group. The inhibitory activity of the nigranoic acid aromatic ester derivatives was derived using the following equation:

$$\text{Inhibitory ratio} = \frac{A_{\text{control}} - (A_{\text{sample}} - A_{\text{zero}})}{A_{\text{control}}} \times 100\%,$$

where A_{control} is the absorbance of the group containing only human NE and the substrate, A_{sample}

is the absorbance of the group containing the test compounds, the substrate and NE, and A_{zero} is the absorbance of the group containing the test compounds, Tris-HCl buffer solution and HNE.

The absorbance data were expressed as mean ± standard deviation. The calculation was carried out in triplicate in three separate experiments. The data were statistically analysed by one-way ANOVA followed by Dunnett-*t* test using SPSS 16.0.

4. Conclusions

In conclusion, a series of 12 new nigranoic acid esters **3a**–**3l** were efficiently prepared by nigranoic acid with benzyl alcohols in excellent yields. Though HNE activity inhibitory bioassay *in vitro*, nigranoic acid exhibited significant inhibitory activity against HNE with the IC_{50} value of 3.77 μM, whereas nigranoic acid esters having phenyls substituted with bromine and trimethoxyls (**3h** and **3b**) showed slightly more inhibitory potency than that of nigranoic acid, with the IC_{50} values of 2.61 and 3.25 μM, respectively. The nigranoic acid esters bearing phenyls substituted with dimethoxyls and chlorine (**3c** and **3i**), and thiophene (**3j**) showed slightly less potency than that of nigranoic acid, with the IC_{50} values ranging from 3.89 to 4.83 μM. The nigranoic acid ester with cinnamenyl group (**3g**) showed the least inhibitory potency (IC_{50} value: 8.95 μM) among the tested compounds. The other six analogues showed no activity.

Supplementary material

Supplementary material relating to this article is available online, alongside Figures S1–S24 and spectral data.

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Note

1. Guoli Huang and Li Feng contributed equally to this work.

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