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PF1270A, B and C, Novel Histamine H3 Receptor Ligands Produced by *Penicillium waksmanii* PF1270

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Abstract Three novel histamine H3 receptor (H3R) ligands, PF1270A (**1**), PF1270B (**2**) and PF1270C (**3**) were isolated from the culture broth of the fungal strain PF1270. The strain was identified as *Penicillium waksmanii* on the basis of morphological characteristics. These compounds were obtained from the culture broth by solvent extraction and chromatographic purification. Their structures were established by spectroscopic methods and X-ray crystallographic analysis. They possess pentacyclic spiroindolinone skeletons. **1**, **2** and **3** displayed high affinity for the rat H3R (*Ki*=0.058, 0.17 and 0.19 μM, respectively) and human H3R (*Ki*=0.047, 0.12 and 0.22 μM, respectively). Moreover, **1**, **2** and **3** acted as potent agonists with the EC_{50} values of 0.12, 0.15 and 0.20 μM, respectively.

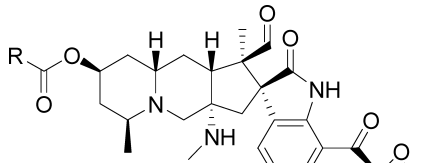
Keywords histamine H3 receptor ligand, *Penicillium waksmanii*, PF1270A, PF1270B, PF1270C

Introduction

Histamine is one of the biological amines broadly distributed in various mammalian tissues. Its pharmacological activities are transferred into cells via histamine receptors existing on the cell membrane. Histamine H1, H2, H3 and H4 receptors have been reported as isoforms of histamine receptors [1～4].

Histamine H3 receptors (H3Rs) that are distributed in the brain and peripheral tissues were identified pharmacologically in 1983 by Arrang *et al.* [3]. H3Rs regulate synthesis and release of histamine as autoreceptors [5, 6]. Further, they play roles as heteroreceptors that control the release of various neurotransmitters such as serotonin, noradrenalin and dopamine in the brain [7～9]. Therefore, H3R ligands are potential therapeutic agents for diabetes, obesity, and central nervous system (CNS) disorders such as attention-deficit hyperactivity disorder (ADHD), cognitive disorders, depression, epilepsy and sleep disorders [10～13].

In this paper, we describe the taxonomy and fermentation of the strain PF1270, the isolation, physico-chemical properties, structure elucidation and biological activities of the novel H3R ligands, PF1270A (**1**), PF1270B (**2**) and PF1270C (**3**) (Fig. 1).



1 R = $CH_2CH_2CH_3$
2 R = CH_2CH_3
3 R = CH_3

Fig. 1 Structures of PF1270A (**1**), B (**2**) and C (**3**).

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Materials and Methods

General

FAB-MS and HR-FAB-MS were measured with a JEOL JMS-FABmate mass spectrometer and a JEOL JMS-700 mass spectrometer, respectively. UV and IR spectra were recorded with a Shimadzu UV-260 spectrometer and a Shimadzu FTIR-8100 spectrometer, respectively. Optical rotations were obtained with a JASCO DIP-370 digital polarimeter using a 10 cm cell. Melting points were determined with a Yanaco MP-J3 micro melting point apparatus. NMR spectra were measured with a JEOL JNM-LA400 spectrometer. X-ray diffraction measurement was performed on a Rigaku AFC7R diffractometer with graphite monochromated Cu-Kα radiation and rotating anode generator.

Microorganism

The fungal strain PF1270 was isolated from a soil sample collected in Shimane Prefecture, Japan. The strain has been deposited at the International Patent Organism Depositary, the National Institute of Advanced Industrial Science and Technology, Japan under the accession number FERM BP-08610.

Taxonomy

Taxonomic studies of the strain PF1270 were performed according to the methods of Pitt [14]. For the evaluation of culture characteristics, Czapek's yeast extract agar (CYA, K_2HPO_4 0.1%, yeast extract 0.5%, sucrose 3.0%, $NaNO_3$ 0.3%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, agar 1.5%) and malt extract agar (MEA, malt extract 2.0%, peptone 0.1%, glucose 2.0%, agar 1.5%) were used. The color was identified in accordance with the Methuen Handbook of Colour [15]. Morphology was observed under an optical microscope.

Fermentation

A slant culture of the strain PF1270 was used to inoculate fifteen 100-ml Erlenmeyer flasks. Each contained 20 ml of a seed medium consisting of glucose 1.0%, soluble starch 2.0%, yeast extract 0.3%, polypeptone 0.5%, soybean meal 0.2%, wheat germ 0.6% and $CaCO_3$ 0.2% in deionized water adjusted to pH 7.0 with NaOH solution prior to sterilization. The flasks were incubated at 25°C for 72 hours on a rotary shaker at 220 rpm. Portions of 3.0 ml of this seed culture were transferred into one hundred 500-ml Erlenmeyer flasks, each of which contained soybean meal 2.5% and water-absorbed rice 100 g as solid production medium. The flasks were thoroughly stirred and then statically cultured at 25°C for 14 days. After incubation, 10 kg portion of the obtained culture was extracted with 20 liters of 67% aqueous acetone.

Cell Culture

CHO cells stably expressing recombinant human H3Rs prepared by Meiji Seika Kaisha, Ltd. were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 1.0 mg/ml G418 at 37°C in 5.0% CO_2.

Membrane Preparation of rat H3Rs

Forebrain from a Sprague-Dawley (SD) rat was homogenized at 4°C in 10 volumes of 0.32 M sucrose solution in a Teflon-glass homogenizer. The homogenate was centrifuged at 1,500×g for 10 minutes. The supernatant was further centrifuged at 40,000×g for 20 minutes at 4°C. The pellets were suspended in 10 volumes of buffer (50 mM Tris-HCl, pH 7.4, 5.0 mM EDTA) and centrifuged under the above-mentioned conditions. This step was repeated and the resulting membranes were stored at −80°C.

Cell Membrane Preparation of Human H3Rs

CHO cells stably expressing recombinant human H3Rs were harvested and centrifuged at 1,500×g for 5 minutes at 4°C. The pellets were suspended in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4 and homogenized by sonication (Sonifier, Branson), which was then centrifuged at 40,000×g for 20 minutes at 4°C. This step was repeated and the resulting cell membranes were stored at −80°C.

Ligand Binding Assay

Membranes expressing rat H3Rs (225 μg/ml) or recombinant human H3Rs (25 μg/ml) were incubated in 200 μl of Tris-assay buffer (50 mM Tris-HCl, pH 7.4, 5.0 mM EDTA) on the 96-well plate. 1.0 nM *N*-α-[methyl-^{3}H]-histamine dihydrochloride (3.0 TBq/mmol, PerkinElmer) and various concentrations of compounds were added to the buffer. Non-specific binding was determined with 10 μM thioperamide (Tocris). The samples were incubated for 60 minutes at room temperature. The reaction was stopped by rapid filtration through 96-well microplate, (UniFilter GF/B, PerkinElmer) soaked in polyethyleneimine (0.3%) using a cell harvester (FilterMate, PerkinElmer). Filters were washed five times with Tris-assay buffer and dried for 60 minutes at 50°C. Then, 30 μl of MicroScinti-20 (PerkinElmer) was dispensed to each well. The radioactivity was measured by a liquid scintillation counter (TopCount96, PerkinElmer). The *Ki* values were calculated from IC_{50} values according to the Cheng-Prusoff equation.

[^{35}S] GTPγS Binding Assay

Cell membranes expressing recombinant human H3Rs (10 μg/ml) were incubated in 250 μl of assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 3.0 mM $MgCl_2$, 0.2 M EGTA, 1.0 mM DTT) on the 96-well plate. 200 pM guanosine 5′[γ-^{35}S] thiotriphosphate, triethylamine salt (38 TBq/mmol, GE Healthcare), 10 μM GDP (Sigma-Aldrich) and various concentrations of compounds were added to the buffer with or without 10 nM (*R*)-(−)-α-methylhistamine (Tocris) as an antagonist format or as an agonist format. The samples were incubated for 60 minutes at room temperature. The reaction was stopped by rapid filtration through 96-well microplate, (UniFilter GF/B, PerkinElmer) using a cell harvester (FilterMate, PerkinElmer). Filters were washed eight times with wash buffer (50 mM Tris-HCl, pH 7.4, 3.0 mM $MgCl_2$) and dried for 60 minutes at 50°C. Then, 30 μl of MicroScinti-20 (PerkinElmer) was dispensed to each well. The radioactivity was measured by a liquid scintillation counter (TopCount96, PerkinElmer). Agonistic activity was calculated as follows: Agonistic activity (% effect)=(radioactivity with tested sample)/(radioactivity without tested sample)×100. EC_{50} values were calculated from a sigmoid curve of agonistic activities.

Results

Taxonomy

The cultural characteristics of the strain PF1270, grown at 25°C for 14 days, were as follows. Colonies on CYA reached 18～26 mm in diameter and produced abundant conidiogenesis. The colony surface was velvety, plane and grayish green. The reverse was dense yellow. Colonies on MEA reached 17～19 mm in diameter and produced abundant conidiogenesis. The colony surface was velvety, plane and grayish green. The reverse was dense yellow. The strain PF1270 did not grow on either medium at 37°C.

Morphological characteristics of the strain PF1270 were as follows. Conidiophores were borne from the substrate and aerial hyphae, and penicilli were monoverticillate or biverticillate. Phialides were ampulliform and 6～8×1.5～2 μm. Conidia were globose to subglobose, smooth, 2～3 μm and borne in long chains (Fig. 2). Based on the above characteristics, the strain PF1270 was identified as *Penicillium waksmanii*.

Isolation

The culture broth was extracted with 20 liters of 67% aqueous acetone. The filtrate of the extracts was concentrated *in vacuo* to remove acetone and adjusted to pH 7.0 with 1 N NaOH solution. The aqueous solution was adsorbed onto a column of DIAION HP-20 (Mitsubishi Chemical Corporation). The column was successively washed with 3.0 liters of water and 3.0 liters of 50% aqueous acetone, and then eluated with 3.0 liters of acetone. To this acetone eluate was added 3.0 liters of water and the mixture concentrated under reduced pressure. The resulting aqueous solution was adjusted to pH 9.0 with 1 N NaOH solution and extracted with an equal volume of ethyl acetate. Evaporation of the ethyl acetate layer gave 3.7 g of a crude extract which was subjected to a silica gel column chromatography (Wako Gel C-300, Wako Pure Chemical Industries) with stepwise elution using hexane - ethyl acetate (90 : 10, 50 : 50, 30 : 70 and neat ethyl acetate). The combined active fractions were concentrated *in vacuo*. The residue was applied onto a column of silica gel and eluted stepwise with hexane - ethyl acetate (90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50, 40 : 60 and 30 : 70). The active fractions were collected and concentrated *in vacuo*. The residue was subjected to a silica gel column chromatography and eluted with chloroform - methanol (50 : 1). The active fractions were combined and concentrated *in vacuo* to give a small volume of methanol-soluble crude extract containing **1** and precipitates containing **1**, **2** and **3**. The methanol-soluble crude extract was purified by six preparative TLCs (Silica gel 60 F_{254}, 0.5 mm, Merck) with hexane - ethyl acetate (1 : 10) to give 72.3 mg of **1** as a yellowish white powder. The precipitates containing **1**, **2** and **3** were purified by preparative HPLC (Inertsil ODS-2, 20 mm i.d.×250 mm, GL Science, acetonitrile/0.005% phosphoric acid=22 : 78) to give 70.2 mg of **1**, the residue containing **2** and the residue containing **3**. The residue containing **2** was further purified by a preparative TLC (Silica gel 60 F_{254}, 0.5 mm, Merck) with hexane - ethyl acetate (1 : 5) to give 17.0 mg of **2** as a yellowish white powder. The residue containing **3**, upon a preparative TLC (Silica gel 60 F_{254}, 0.5 mm, Merck) with hexane - ethyl acetate (1 : 5), gave 6.0 mg of **3** as a

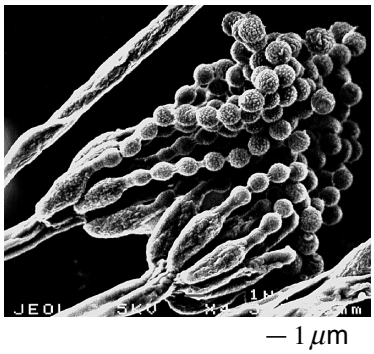

Fig. 2 Scanning electron micrograph of strain PF1270.

Table 1 Physico-chemical properties of PF1270A (**1**), PF1270B (**2**) and PF1270C (**3**)

	1	**2**	**3**
Appearance	Yellowish white powder	Yellowish white powder	Yellowish white powder
Molecular formula	$C_{32}H_{43}N_3O_6$	$C_{31}H_{41}N_3O_6$	$C_{30}H_{39}N_3O_6$
FAB-MS (*m/z*)	566 $(M+H)^+$	552 $(M+H)^+$	538 $(M+H)^+$
HR-FAB-MS (*m/z*)			
Found	566.3224 $(M+H)^+$	552.3077 $(M+H)^+$	538.2917 $(M+H)^+$
Calcd.	566.3230 (for $C_{32}H_{44}N_3O_6$)	552.3073 (for $C_{31}H_{42}N_3O_6$)	538.2917 (for $C_{30}H_{40}N_3O_6$)
$[\alpha]_D^{25}$ (*c* 1.0, CH_3CN)	+75.0°	+82.8°	+79.6°
UV $\lambda_{max}^{CH_3CN}$ nm (ε)	202 (25200), 228 (18800), 246 (24600), 334 (9030)	201 (23800), 228 (17200), 246 (22400), 334 (8110)	199 (20700), 228 (13300), 247 (17700), 334 (6380)
IR ν_{max} (KBr) cm^{-1}	3384, 2934, 1721, 1674, 1601, 1445, 1381, 1254, 1184, 1090, 1069, 764	3411, 2942, 1728, 1673, 1603, 1450, 1381, 1255, 1188, 1092, 1069, 762	3382, 2964, 1732, 1676, 1605, 1453, 1381, 1250, 1190, 1103, 1069, 758
Melting point	173~175°C	176~178°C	187~189°C
Solubility			
soluble	$CHCl_3$, MeOH, Acetone, CH_3CN, EtOAc, DMSO	$CHCl_3$, MeOH, Acetone, CH_3CN, EtOAc, DMSO	$CHCl_3$, MeOH, Acetone, CH_3CN, EtOAc, DMSO
insoluble	Hexane, H_2O	Hexane, H_2O	Hexane, H_2O

yellowish white powder.

1 was recrystallized from acetone - H_2O for X-ray crystallographic analysis.

Physico-chemical Properties

The physico-chemical properties of **1**, **2** and **3** are summarized in Table 1. These data suggest that they are structurally related. The ^{1}H- and ^{13}C-NMR spectral data of **1**, **2** and **3** observed in $CDCl_3$ are summarized in Table 2.

Structure Elucidation

The molecular formula of **1** was established as $C_{32}H_{43}N_3O_6$ by HR-FAB-MS. The ^{1}H-NMR spectrum data of **1** showed one aldehyde proton signal at H-29 (δ 9.64) and three arotamic proton signals at H-4 (δ 7.94), H-5 (δ 7.16) and H-6 (δ 7.76). The ^{13}C-NMR and HSQC spectra showed 32 carbon atoms (six methyl, seven methylene, nine methine and ten quaternary carbons). The ^{1}H-^{1}H COSY and HMBC correlations of **1** are summarized in Fig. 3. In the ^{1}H-^{1}H COSY spectrum, sequential proton networks were observed from H-27 to H-18, from H-2′ to H-4′ and from H-4 to H-6. In the HMBC spectrum, cross peaks were observed from NH-1 to C-3, C-3a and C-7a, from H-4 to C-6 and C-7a, from H-5 to C-3a and C-7 and from H-6 to C-4 and C-7a, respectively. These correlations indicated the connectivity of an indolinone ring (from NH-1 to C-7a). Cross peaks were also observed from H-8 to C-2, C-3, C-3a, C-9, C-10, C-18 and C-19, from H-10 to C-9, C-16 and C-18, respectively. These correlations revealed the connectivity between dodecahydrocyclopenta[*b*]quinolizine (N-11, C-3 and from C-8 to C-19) and the indolinone ring through the spiro carbon (C-3). Cross peaks were observed from H-26 to C-9, from H-28 to C-3, C-18, C-19 and C-29 and from H-29 to C-19. These correlations and the carbon chemical shift of C-26 suggested the connectivity from C-26 to C-9 through NH-25 and from C-28 and C-29 to C-19, respectively. Cross peaks were observed from H-6 to C-20, from H-21 to C-20 and C-22, from H-23 to C-21, C-22 and C-24 and from H-24 to C-21, C-22 and C-23. These correlations indicated the connectivity between the indolinone ring and the side chain (from C-20 to C-24). The structure of **1** was determined as shown in Fig. 3 based on all instrumental analyses data. Moreover, using the data of X-ray crystallographic analysis of **1** (Fig. 4), the relative configuration of **1** was established as shown in Fig. 1. The crystal data of **1** are as follows: Compound formula; $C_{32}H_{43}N_3O_6$. Molecular weight; 565.7. Crystal system; orthorhombic. Space group; $P2_12_12_1$. Lattice parameters; a=14.088 Å, b=25.809 Å, c=8.051 Å, α=90°, β=90°, γ=90°, Z=4, V=2927 Å^3, d=1.28 g/cm^3. Final R and weighted R values were 0.039 and 0.059, respectively.

The molecular formula of **2** was established as $C_{31}H_{41}N_3O_6$ by HR-FAB-MS. The ^{1}H- and ^{13}C-NMR spectra of **2** were similar to those of **1** except for the absence of one methylene group at the acyl side chain. The structure of **2** was elucidated as shown in Fig. 1.

The molecular formula of **3** was established as $C_{30}H_{39}N_3O_6$ by HR-FAB-MS. The ^{1}H- and ^{13}C-NMR

Table 2 ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) data of **1**, **2** and **3** in $CDCl_3$

Position	**1**		**2**		**3**	
	δ_C [a]	δ_H [b] (m [c], *J* in Hz)	δ_C [a]	δ_H [b] (m [c], *J* in Hz)	δ_C [a]	δ_H [b] (m [c], *J* in Hz)
1		9.64 (br s)		9.65 (br s)		9.64 (br s)
2	182.1 s		182.1 s		182.1 s	
3	58.7 s		58.7 s		58.7 s	
3a	134.5 s		134.5 s		134.5 s	
4	134.2 d	7.94 (d, 7.6)	134.2 d	7.94 (d, 7.8)	134.3 d	7.94 (d, 7.6)
5	121.8 d	7.16 (t, 7.6)	121.8 d	7.16 (t, 7.8)	121.8 d	7.16 (t, 7.6)
6	127.4 d	7.76 (d, 7.6)	127.4 d	7.76 (d, 7.8)	127.4 d	7.76 (d, 7.6)
7	117.2 s		117.2 s		117.2 s	
7a	143.0 s		143.0 s		143.0 s	
8	44.8 t	1.98 (d, 13.4)	44.8 t	1.97 (d, 13.4)	44.7 t	1.96 (d, 13.4)
		2.13 (d, 13.4)		2.13 (d, 13.4)		2.13 (d, 13.4)
9	62.7 s		62.7 s		62.7 s	
10	56.7 t	2.34 (d, 11.2)	56.7 t	2.34 (d, 11.2)	56.7 t	2.34 (d, 11.0)
		3.01 (d, 11.2)		3.00 (d, 11.2)		3.00 (d, 11.0)
12	53.9 d	3.07 (br t, 6.3)	53.9 d	3.06 (br t, 6.4)	53.9 d	3.06 (br t, 6.1)
13	35.5 t	1.85 (m)	35.4 t	1.85 (m)	35.3 t	1.88 (m)
		2.00 (m)		1.99 (m)		1.98 (m)
14	68.0 d	5.10 (br t, 2.9)	68.2 d	5.08 (br t, 2.9)	68.4 d	5.07 (br t, 2.9)
15	37.8 t	1.50 (br t, 12.9)	37.8 t	1.50 (br t, 13.0)	37.8 t	1.50 (br t, 12.7)
		1.85 (m)		1.85 (m)		1.88 (m)
16	47.0 d	2.79 (br t, 9.8)	47.0 d	2.81 (br t, 9.3)	47.0 d	2.82 (br t, 9.5)
17	30.5 t	1.20 (m)	30.5 t	1.21 (m)	30.5 t	1.21 (m)
		1.67 (m)		1.66 (m)		1.67 (m)
18	50.6 d	2.49 (dd, 2.9, 13.7)	50.5 d	2.49 (dd, 3.4, 13.7)	50.5 d	2.49 (dd, 2.9, 13.4)
19	61.5 s		61.5 s		61.5 s	
20	194.7 s		194.7 s		194.7 s	
21	64.2 d	4.05 (s)	64.2 d	4.05 (s)	64.2 d	4.05 (s)
22	61.5 s		61.5 s		61.5 s	
23	18.6 q	1.26 (s)	18.6 q	1.25 (s)	18.6 q	1.26 (s)
24	24.3 q	1.61 (s)	24.3 q	1.60 (s)	24.3 q	1.61 (s)
26	29.2 q	2.25 (s)	29.2 q	2.25 (s)	29.2 q	2.25 (s)
27	13.2 q	1.14 (d, 6.6)	13.2 q	1.13 (d, 6.8)	13.1 q	1.14 (d, 6.8)
28	17.9 q	1.14 (s)	17.9 q	1.15 (s)	17.9 q	1.14 (s)
29	201.9 d	9.35 (s)	201.9 d	9.33 (s)	201.9 d	9.34 (s)
1′	173.1 s		173.9 s		170.6 s	
2′	36.8 t	2.30 (dt, 3.1, 7.3)	28.1 t	2.34 (dq, 2.7, 7.6)	21.5 q	2.07 (s)
3′	18.4 t	1.67 (tq, 7.3, 7.3)	9.1 q	1.15 (t, 7.6)		
4′	13.7q	0.98 (t, 7.3)				

[a] $CDCl_3$ was used as the internal standard (δ_C 77.0 ppm).

[b] $CHCl_3$ was used as the internal standard (δ_H 7.26 ppm).

[c] Multiplicity.

spectra of **3** were similar to those of **1** except for the absence of two methylene groups at the acyl side chain. The structure of **3** was elucidated as shown in Fig. 1.

Biological Activities

1, **2** and **3** displayed high affinity for both rat and human H3Rs (Table 3). The *Ki* values of rat H3R were 0.058, 0.17 and 0.19 μM, respectively and the *Ki* values of human H3R were 0.047, 0.12 and 0.22 μM, respectively. Moreover, these compounds acted as potent agonists with the EC_{50} values of 0.12, 0.15 and 0.20 μM, respectively. **1**, **2** and **3** did not affect antagonist format (data not shown).

1 did not exhibit antimicrobial activities at 20 μg/paper disk.

Discussion

In the course of our screening for H3R ligands from microorganism, we found three novel compounds, **1**, **2** and **3** from the culture broth of *Penicillium waksmanii* PF1270. These compounds possess pentacyclic spiroindolinone skeletons. Several similar structure compounds such as citrinadin A and citrinadin B [16,17] have been isolated from fungi but their stereochemistry at the spiro carbon (C-3) is opposite to that of PF1270s.

Most of the H3R ligands such as (*R*)-α-methylhistamine as an agonist and thioperamide as an antagonist have an imidazole ring that is present in the structure of histamine [18]. However, **1**, **2** and **3** are novel H3R agonists without an imidazole ring in their molecules. These compounds displayed high affinity for rat H3R and human H3R. Moreover, **1**, **2** and **3** acted as potent agonists with the EC_{50} values of 0.12, 0.15 and 0.20 μM, respectively. The highest affinity of **1** toward the H3R may be due to relatively long acyl chain length.

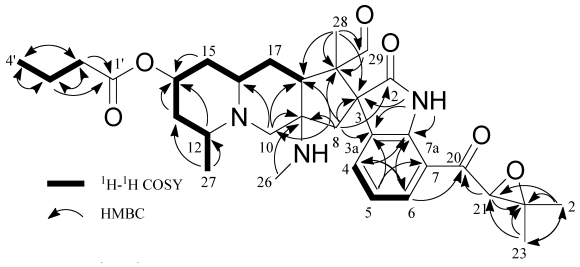

Fig. 3 ^{1}H-^{1}H COSY and HMBC correlations of **1**.

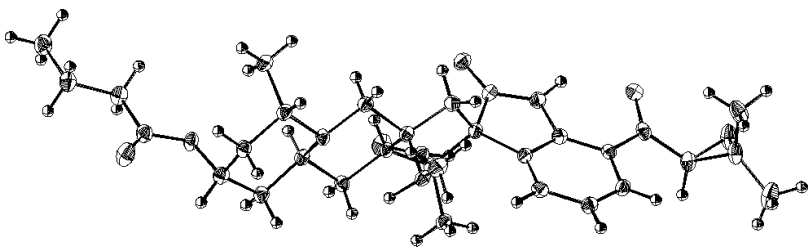

Fig. 4 ORTEP drawing of **1**.

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Table 3 Binding affinity (*Ki*) and potency (EC_{50}) of **1**, **2** and **3** at histamine H3 receptors

Compound	Rat H3R *Ki* (μM)	Human H3R *Ki* (μM)	Human H3R EC_{50} (μM)
1	0.058	0.047	0.12
2	0.17	0.12	0.15
3	0.19	0.22	0.20

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