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Novel Sesquiterpenoids from the Fermentation of *Xylaria persicaria* Are Selective Ligands for the NPY Y5 Receptor

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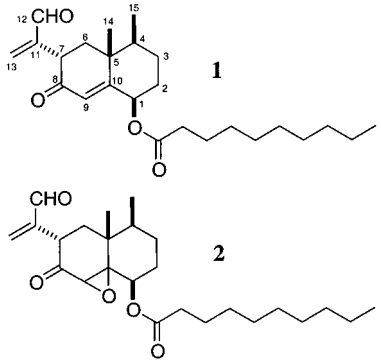
Abstract: Neuropeptide Y (NPY) is a polypeptide found in the peripheral and central nervous system and is involved in the regulation of feeding. Antagonists of NPY receptor activation could therefore have potential for development as antiobesity drugs. Fermentation of an isolate of *Xylaria persicaria* yielded two novel eremophilane sesquiterpenoids xylarenals A (**1**) and B (**2**). These compounds are selective for the NPY Y5 receptor but have only modest affinity. The isolation, structure elucidation, and biological activities of these compounds are described.

Neuropeptide Y (NPY) is a 36-amino acid polypeptide that is widely distributed in the peripheral and central nervous system where it functions as a neurotransmitter, neuromodulator, and neurohormone.[1] NPY has been associated with a variety of physiological functions, including energy metabolism, vasoconstriction, hormone release, gastric secretion, and circadian rhythms, as well as behaviors such as feeding, satiety, anxiety, memory, and learning.[1] NPY shares sequence homology and tertiary structure similarity with peptide YY (PYY) and pancreatic polypeptide (PP).[2] These three peptides bind, with varying affinities, to six distinct NPY receptors, five of which have been cloned (Y1, Y2, Y4, Y5, y6), and one has been characterized pharmacologically.[3] All six receptors are found in the brain, although Y4 is more abundant in peripheral tissues and y6 is missing from rat and truncated in primates.

Experiments to determine which NPY receptors mediate the various actions of NPY have used genetic models and a limited set of pharmacological tools. Genetically modified mice have been made, including those with Y1, Y2, and Y5 receptor knockouts[4] and those with inactivation of NPY, with NPY overexpression or with PP overexpression.[5] Several ligands have been developed to evaluate the role of NPY, including peptide derivative agonists with varying potencies at the NPY receptors and nonpeptide antagonists selective for Y1 or Y5.[6] The genetic and pharmacological studies suggest that the Y5 receptor is involved in mediating food intake and body weight.[4c,6a,7]

In this report we present the identification of two novel natural products that bind selectively to the Y5 receptor, although they have only modest affinity for the receptor.

Xylaria persicaria (Schwein.) Berk. & M.A. Curtis (Ascomycotina, Xylariaceae) occurs on fallen fruits of *Liquidambar styraciflua* L. in eastern North America.[8] The fungus was fermented on a solid vermiculite-based medium on a 2 L scale and then extracted with MEK. The crude MEK extract (500 mL) was fractionated by step gradient flash C_{18} chromatography (water/MeOH). The MeOH fraction was found to contain the activity against the Y5 receptor and HPLC showed the presence of two major peaks. This fraction was further purified by preparative C_{18} HPLC to give xylarenals A (**1**) and B (**2**).



Xylarenal A (**1**) was obtained as a pale yellow oil that showed a protonated molecular ion in the positive ion

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TABLE 1. ^{1}H and ^{13}C NMR Data for Xylarenal A (1)

atom	δ_C[a]	δ_H[a]		HMBC correlations (7 Hz)
1	74.07	5.41	1H, pseudo t, 3.0 Hz	C-2, C-3, C-5, C-9, C-10, C-1′
2	31.32	1.99	1H, m	C-1
		1.72	1H, m	C-1, C-3, C-4
3	25.68	1.75	1H, m	C-1, C-4
		1.45	1H, m	C-1, C-2, C-4, C-5, C-15
4	43.23	1.50	1H, m	
5	39.51			
6	42.96	1.98	2H, m	C-4, C-8, C-10, C-11
7	43.66	3.66	1H, dd, 13.2, 5.5 Hz	C-5, C-6, C-8, C-10, C-11, C-12, C-13
8	197.88			
9	128.66	5.93	1H, s	C-1, C-5, C-7
10	162.54			
11	148.91			
12	193.55	9.53	1H, s	C-11
13	136.11	6.32	1H, s	C-7
		6.21	1H, s	C-7, C-11, C-12
14	17.55	1.27	3H, s	C-4, C-5, C-6
15	15.21	0.93	3H, d, 6.7 Hz	C-3, C-4, C-5
1′	172.59			
2′	34.98	2.28	2H, pseudo q, 7.5 Hz	C-1, C-1′, C-3′, C-4′
3′	25.24	1.59	2H, m	C-1′, C-5′
4′[b]	29.80	1.29	2H, m	
5′[b]	29.64	1.29	2H, m	
6′[b]	29.61	1.29	2H, m	
7′[b]	29.48	1.29	2H, m	
8′	23.04	1.28	2H, m	
9′	32.25	1.27	2H, m	
10′	14.24	0.88	3H, t, 6.7 Hz	C-8′, C-9′

[a] CD_2Cl_2 was used as the solvent and the internal standard. [b] Assignments may be interchanged.

APCI-MS spectrum at *m*/*z* 403 $(M + H)^+$. A HRESI-MS measurement on this peak revealed that the compound had the molecular formula $C_{25}H_{38}O_4$. The ^{13}C and ^{1}H NMR data are shown in Table 1. The ^{1}H NMR spectrum showed signals for three methyl groups (a singlet at δ 1.27, a doublet at δ 0.93, and a triplet at δ 0.88), an oxygenated methine multiplet at δ 5.41, an olefinic singlet at δ 5.93, vinyl group singlets at δ 6.32 and 6.21, and an aldehyde singlet at δ 9.53. The ^{13}C NMR spectrum of **1** showed 25 signals comprised of 3 methyls, 11 methylenes, an olefinic methylene, 2 methines, an oxygenated methine, an olefinic methine, a quaternary carbon, 2 olefinic quaternary carbons, and an aldehyde, ketone, and ester carbonyl carbon. The hydrogen-bearing carbons were assigned using one-bond proton carbon correlations observed in a HSQC experiment. Inspection of data generated by the analysis of 2D NMR experiments such as GCOSY, TOCSY, and GHMBC allowed the assignment of three separate proton spin systems and isolated olefinic methylene (C-13) and methine (C-9) carbons (Figure 1).

The first proton spin system was found to be a methyl followed by a methine and then two adjacent methylene groups, followed by an oxygenated methine (C-1, C-2, C-3, C-4, C-15). The second consisted of a methylene adjacent to a methine (C-6−C-7). The third spin system was found to be a decanoyl group (C-1′−C-10′). More evidence for the presence of this spin system was provided by the observation of a peak at *m*/*z* 231 in the positive ion APCI mass spectrum, which corresponded to the loss of a decanoyl group.

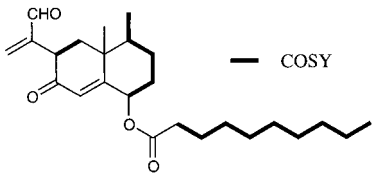

FIGURE 1. COSY correlations observed for xylarenal A (**1**).

The GHMBC experiment was used to both confirm the above proton spin-system assignments and establish the connectivity between the fragments described above. The methyl group at δ 1.27 showed correlations with C-4, C-5, and C-6 allowing the placement of a quaternary carbon (C-5) between the above fragments. This was confirmed by the observation of correlations between the methylene at δ 1.98 and C-4 and between the methyl group at δ 0.93 and C-5. A further correlation from the methyl group at δ 0.93 confirmed that it was directly attached to the C-4 methine. The vinylic methylene group was assigned at the β-position of an α,β-unsaturated aldehyde moiety by the observation of correlations between the vinyl protons at δ 6.32 and 6.21 with C-11 and C-12. This assignment was confirmed by the observation of a correlation between the aldehyde proton at δ 9.53 and C-11, and the chemical shift of the aldehyde carbonyl carbon was consistent with a conjugated carbonyl. Correlations observed between the vinylic protons at δ 6.32 and 6.21 and C-7, between the methylene at δ 1.98 and C-11, and between the methine at δ 3.66 and C-11, C-12, and C-13 allowed the assignment of C-7 as being directly attached to C-11 of the α,β-unsaturated aldehyde moiety. A further correlation observed between the signal at δ 3.66 and C-8 directly attached a keto-carbonyl group at the final valency position of C-7. The chemical shift of the keto-carbonyl group suggested that it was α,β-unsaturated, and the olefinic methine was consequently assigned at the α-position of an α,β-unsaturated ketone moiety, after the observation of correlations between the signal at δ 5.93 and C-5 and C-7. The assignment of this six-membered ring was confirmed by the observation of correlations between the methylene at δ 1.98 and C-8 and C-10. Correlations between the olefinic methine at δ 5.93 and C-1 and between the oxygenated methine at δ 5.41 and C-5, C-9, and C-10 confirmed the bicyclic sesquiterpenoid structure of **1**. The acylation of C-1 with the decanoyl group was assigned by observing three-bond proton−carbon correlations between H1 and C-1′ and between H2′ and C-1.

The relative stereochemistry of **1** was assigned by an analysis of the coupling constants with aid from a 1D TOCSY experiment and analysis of NOE data from a 2D NOESY experiment. The C-7 methine proton appeared as a doublet of doublets with coupling constants of 13.2 and 5.5 Hz arising from coupling with the axial and equatorial protons of the C-6 methylene, respectively. This allowed the assignment of the C-7 methine proton configuration as axial. This assignment was supported by the observation of a NOE between the C-7 methine and the C-14 methyl group. The ring junction methyl group at C-14 also showed strong NOEs with the α-methylene group of the fatty acyl chain (C-2′) and the C-3 methylene proton at δ 1.75 indicating that this proton and methyl and acyl groups had the axial configuration.

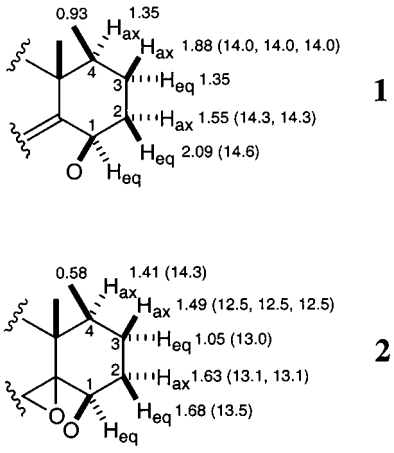

FIGURE 2. Coupling constant analysis of 1D TOCSY data for xylarenals A (**1**) and B (**2**) (only diagnostic vicinal axial−axial and geminal coupling constants are shown).

The C-1 methine proton appeared as a pseudo triplet with a coupling constant of 3.0 Hz arising from its scalar coupling with the axial and equatorial protons of the C-2 methylene. This ensured that the C-1 proton was in the equatorial configuration. Due to severe signal overlap, it was not possible to directly measure the coupling constants for the C-4 methine proton at δ 1.50 from the 1D ^{1}H NMR spectrum to determine the stereochemistry at C-4, so a 1D TOCSY experiment was employed. Selective excitation at δ 5.41 (C-1) followed by a TOCSY mixing period produced a clean subspectrum of the C-1, C-2, C-3, C-4, and C-15 spin system. These measurements were performed in benzene-d_6, wherein the subspectrum showed good dispersion allowing direct measurement of the relevant coupling constants. Figure 2 shows the proton chemical shifts in benzene-d_6 of this spin system along with the large-magnitude coupling constants for each signal. Assuming that these large coupling constants (>10 Hz) arise only from geminal and vicinal axial−axial couplings, it was possible to assign each proton in the spin system as either axial or equatorial. This analysis clearly showed that the C-4 methine proton had the axial configuration. This assignment was supported by the observation of a NOE between the C-4 methine proton and the axial proton of the C-2 methine at δ 1.72. The relative stereochemistry of xylarenal A was therefore assigned as that depicted in **1**.

Xylarenal B (**2**) was obtained as a pale yellow oil that showed a protonated molecular ion in the positive ion APCI-MS spectrum at *m*/*z* 419 $(M + H)^+$. A HRESI-MS measurement on this peak revealed that the compound had the molecular formula $C_{25}H_{38}O_5$. The ^{13}C and ^{1}H NMR data are shown in Table 2. The data were very similar to that of xylarenal A (**1**) except in the vicinity of C-8, C-9, and C-10. The olefinic ^{13}C NMR signals for C-9 and C-10 at δ 128.7 and 162.5, respectively, for **1** were replaced by saturated oxygenated signals at δ 62.7 and 67.1, respectively, for **2**. The chemical shift of the carbonyl carbon at C-8 was slightly more deshielded for **2** than for **1**. All other 1- and 2-D NMR data were similar to that of **1**. These data were interpreted such that the structure of **2** consisted simply of the replacement of the C-9−C-10 double bond of **1** with an epoxide. The relative stereochemistry of **2** was determined using an analysis similar to that for **1** (Figure 2) and was found to be identical in all positions except for the additional chiral center at C-9. This proton appeared as a singlet and showed a solitary NOE with the C-1 methine proton. The distance between this proton and the C-1 methine proton is similar in both of the possible configurations for C-9.

TABLE 2. ^{1}H and ^{13}C NMR Data for Xylarenal B (2)

atom	δ_C[a]	δ_H[a]		HMBC correlations (7 Hz)
1	74.18	4.39	1H, t, 2.8	C-2, C-3, C-5, C-9, C-10, C-1′
2	29.47	1.88	1H, m	C-1, C-3, C-4, C-10
		1.29	1H, m	
3	25.79	1.73	1H, m	C-5, C-15
		1.49	1H, m	C-5, C-15
4	39.71	1.72	1H, m	C-5, C-14
5	37.09			
6	37.22	1.79	1H, t, 12.7 Hz	C-4, C-5, C-7, C-10, C-11, C-14
		1.61	1H, m	C-5, C-7, C-8, C-10, C-15
7	43.59	3.32	1H, dd, 12.8, 6.3 Hz	C-6, C-8, C-11, C-12, C-13
8	203.79			
9	62.74	3.36	1H, s	C-1, C-7, C-8, C-10
10	67.11			
11	149.80			
12	192.69	9.45	1H, s	C-7
13	136.50	6.32	1H, s	C-7, C-11
		6.16	1H, s	C-7, C-11, C-12
14	15.76	1.19	3H, s	C-4, C-6, C-10
15	15.45	0.90	3H, d, 6.3 Hz	C-3, C-4, C-5
1′	172.67			
2′	34.84	2.31	2H, dt, 7.5, 3.2 Hz	C-1′, C-3′, C-4′
3′	25.22	1.61	2H, m	C-5′
4′[b]	29.79	1.29	2H, m	
5′[b]	29.63	1.29	2H, m	
6′[b]	29.61	1.29	2H, m	
7′[b]	29.50	1.29	2H, m	
8′	23.05	1.28	2H, m	
9′	32.24	1.27	2H, m	C-7′
10′	14.25	0.88	3H, t, 7.0 Hz	C-8′, C-9′

[a] CD_2Cl_2 used as solvent and internal standard. [b] Assignments may be interchanged.

To facilitate the assignment of the relative stereochemistry at C-9, an attempt was made to hydrolyze the acyl chain of **1** using base catalysis, but the reaction gave numerous products. Similar problems have been observed in attempts to hydrolyze the acyl chain of the structurally related integric acid.[9b] The relative stereochemistry at C-9 of **2** was therefore left unassigned.

The purified compounds were evaluated for their affinity against the five cloned mouse NPY receptors (Table 3). Xylarenal B (**2**) had a Y5 IC_{50} of 3.6 ± 0.3 μM, and xylarenal A (**1**) had a slightly higher Y5 IC_{50}, 6.8 ± 1.5 μM. Both compounds had such low affinities for the Y1, Y2, Y4, and y6 receptors that an IC_{50} could not be determined.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were performed at 25 °C on a spectrometer with a deuterium lock. Spectra were referenced to residual undeuter-

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TABLE 3. Affinity of Novel Xylarenals for Mouse NPY Receptors

compound	Y1	Y2	Y4	Y5[a]	y6
			(IC_{50}, μM)		
xylarenal A (**1**)	>20	>20	>20	6.8 ± 1.5	>20
xylarenal B (**2**)	>20	>20	>20	3.6 ± 0.3	>20

[a] Data, ± SEM, is the average of four determinations.

ated solvent peaks or solvent ^{13}C signals. Analytical HPLC was conducted using a HPLC system equipped with a diode-array detector and a 4.6 × 250 mm column, particle size 5 μm, pore size 120 Å. Preparative HPLC was conducted using a solvent delivery system equipped with a single-wavelength detector (set at 220 nm) using a 20 × 250 mm column, particle size 5 μm, pore size 120 Å, equipped with a 20 × 50 mm guard column, particle size 5 μm, pore size 120 Å.

Receptor Binding Assays. DNA encoding the ORFs of the mouse NPY receptors were isolated by PCR from genomic DNA (Y2, Y4, Y5) or from cDNA clones (Y1, y6) obtained by hybridization of Y1 probes to a mouse hypothalamic cDNA library.[10] The cDNAs encoding the receptors were cloned into expression vector pciNeo (Promega, Madison, WI) with an optimal Kozac sequence: GCCGCCACCATG. Clones were confirmed by DNA sequencing and electroporated into COS-7 cells.[11] COS-7 cells transiently expressing recombinant receptors were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin-G (100 IU/mL) and streptomycin (100 μg/mL). These cells were grown in a 95% air, 5% CO_2 humidified atmosphere at 37 °C. Membranes were prepared 72 h post-transfection as described previously.[11] For competition binding, membranes (1−10 μg of protein) were incubated with ^{125}I−PYY or ^{125}I−PP (for Y4; 90 pM) in the presence or absence of 1 μM unlabeled PYY (or PP) or serial dilutions of various fractions or compounds. Binding reactions were performed in 0.25 mL of 50 mM Tris, pH 7.4, containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 0.1% bovine serum albumin, 10 μM phosphoramidon, 4 μg/mL leupeptin, and 40 μg/mL bacitracin. After 2 h at room temperature, the binding reaction was terminated by filtering over GF/C filters, and the radioactivity bound to the filters was quantified with a gamma counter. IC_{50} values were determined using nonlinear regression performed by PRISM, version 3.0 (GraphPad).

Mycology. *X. persicaria* was collected on fallen *L. styraciflua* fruits in North Edison, Middlesex County, New Jersey, on September 14, 1994. Identification of the stromata was based on data of Rogers.[8] The strain MF6722 (= GB4350) was initiated from germinating conidia from stromata surfaces on malt−yeast extract agar. Frozen vegetative mycelia (−80 °C) are maintained in the Merck Microbial Resources Collection.

Fermentation. The culture was inoculated into seed flasks by aseptically transferring a 1 mL aliquot of the frozen stock culture into a 250 mL Erlenmeyer flask containing 50 mL of the following medium (in g/L): corn steep powder, 2.5; tomato paste, 40; oat flour, 10; glucose, 10; trace elements solution, 10 mL/L; pH to 6.8. The trace elements solution for the seed medium consisted of the following (in g/L): $FeSO_4 \cdot 7H_2O$, 1.0; $MnSO_4 \cdot H_2O$, 1.0; $CuCl_2 \cdot 2H_2O$, 0.025; $CaCl_2$, 0.1; H_3BO_3, 0.056; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.019; $ZnSO_4 \cdot 7H_2O$, 0.2. The culture was incubated at 25 °C, 220 rpm, for 4 days prior to inoculation of the production medium. The production phase was performed on a solid substrate medium, in two 2 L roller bottles containing approximately 675 mL of large-particle vermiculite (measured by volume), with 220 mL of a liquid nutrient solution poured over it. The nutrient solution was formulated with the following (in g/L): sucrose, 60; glucose, 80; glycerol, 60; tomato paste, 5; ardamine pH, 5; $(NH_4)_2SO_4$, 2; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 0.5; trace elements, 1 mL/L; pH to 7.0. The trace elements consisted of the following (in g/L): $FeCl_3 \cdot 6H_2O$, 5.8; $MnSO_4 \cdot H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.015; $Na_2MoO_4 \cdot 2H_2O$, 0.012; $ZnCl_2$, 0.02; $SnCl_2 \cdot 2H_2O$, 0.005; H_3BO_3, 0.01; KCl, 0.02. The solid and liquid portions of the production medium were combined at the time of inoculation. Each bottle was inoculated with 8−10 mL of the vegetative seed and shaken to coat the vermiculite with the seed growth and nutrient solution. Inoculated bottles were incubated on a rolling machine (4 rpm), at 22 °C for 17 days. Fungal growth in each bottle was extracted with approximately 250 mL of methyl ethyl ketone.

Isolation and Purification. The 500 mL of MEK extract was concentrated in vacuo to afford a crude extract. The extract was dissolved in water/MeOH (1:1, 10 mL) and fractionated by step gradient flash C_{18} chromatography (40 × 150 mm, water (480 mL), 50% water in MeOH (480 mL), MeOH (480 mL), 50% MeOH in CH_2Cl_2 (480 mL)). The four fractions collected were analyzed by HPLC and NPY-1 filter-binding assay. The MeOH fraction was found to contain the NPY-5 activity, and HPLC showed the presence of two major peaks. This fraction (557.4 mg) was further purified by preparative C_{18} HPLC (isocratic at 20% water in MeOH), to give xylarenal A (**1**) (5.9 mg, yield = 11.8 mg/L) and B (**2**) (3.6 mg, yield = 7.2 mg/L) as yellow oils.

Xylarenal A (1): $[\alpha]_D^{25}$ +30° (*c* 0.40, CH_2Cl_2); IR (film) ν_{max} 1723 (C=O), 1682 (C=O) cm^{-1}; ^1H and ^{13}C NMR data are shown in Table 1; APCIMS *m/z* 403 $[M + H]^+$; HRESI MS *m/z* 403.2841 (calcd for $C_{25}H_{39}O_4$, 403.2848).

Xylarenal B (2): $[\alpha]_D^{25}$ −19° (*c* 0.20, CH_2Cl_2); IR (film) ν_{max} 1725 (C=O), 1694 (C=O) cm^{-1}; ^1H and ^{13}C NMR data are shown in Table 2; APCIMS *m/z* 419 $[M + H]^+$; HRESI MS *m/z* 419.2777 (calcd for $C_{25}H_{39}O_5$, 419.2798).

Supporting Information Available: ^1H and ^{13}C NMR spectra for xylarenals A (**1**) and B (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

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