Isolation and Structural Elucidation of Cyclopentynafil and *N*-Octylnortadalafil Found in a Dietary Supplement

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A new sildenafil analogue, cyclopentynafil (1) and a new tadalafil analogue, *N*-octylnortadalafil (2) were isolated from a dietary supplement illegally marketed for erectile dysfunction. The structures of the sildenafil and tadalafil analogues were elucidated by using HPLC-photodiode array (PDA), LC-MS, high-resolution MS, NMR and circular dichroism (CD). These compounds were determined to be 5-[2-ethoxy-5-(4-cyclopentylpiperazin-1-ylsulfonyl)phenyl]-1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one and (6*R*,12a*R*)-2-octyl-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydropyrazino[1′,2′:1,6]pyrido[3,4-*b*]indole-1,4-dione, respectively. Recently, a large number of phosphodiesterase-5 (PDE-5) inhibitors, including their analogues, have been isolated from dietary supplements, while cyclopentynafil and *N*-octylnortadalafil are the first compounds reported to be new sildenafil and tadalafil analogues, respectively. Quantitative HPLC analysis showed that the contents of 1 and 2 in the product were about 130 mg/tablet (301 μg/mg) and about 27 mg/tablet (64.1 μg/mg), respectively.

Key words cyclopentynafil; *N*-octylnortadalafil; phosphodiesterase-5 inhibitor; LC-MS; NMR; erectile dysfunction

Recently, along with the rise in health consciousness, the consumption of dietary supplements has increased year by year. In Japan, some of these products are illegally advertised as effective for sexual enhancement. Consumers take these products without knowing that most are adulterated with synthetic compounds, such as sildenafil (Fig. 1), vardenafil and tadalafil (Fig. 1), all of which are known as active drug ingredients for the treatment of penile erectile dysfunction (ED).[1—3)]

In our previous paper, we identified a new tadalafil analogue, chloropretadalafil,[4)] which had been synthesized as a tadalafil precursor,[5)] from a dietary supplement along with hydroxyhomosildenafil and aminotadalafil.

Thus far, a large number of analogues of sildenafil, tadarafil and vardenafil have been reported,[6—23)] while a new type of phosphodiesterase-5 (PDE-5) inhibitor, (*R*)-xanthoanthrafil, an anthranilic acid derivative, has been found in a dietary supplement advertising sexual enhancement for men.[24)] (*R*)-Xanthoanthrafil was first synthesized as a candidate compound for the treatment of ED by Fujisawa Pharmaceutical Co., Ltd. (currently Astellas Pharma Inc., Tokyo, Japan),[25)] and was reported as a PDE-5 inhibitor, FR226807, after the manufacturer discontinued the process of developing the drug for approval. Furthermore, another new type of PDE-5 inhibitor, thioquinapiperifil, an imidazoquinazoline derivative, was also detected in a dietary supplement.[26)] This compound was first synthesized as KF31327 by Kyowa Hakko Kogyo Co., Ltd., and Hirose *et al.* reported that it was a more potent and selective PDE-5 inhibitor than sildenafil.[27—29)]

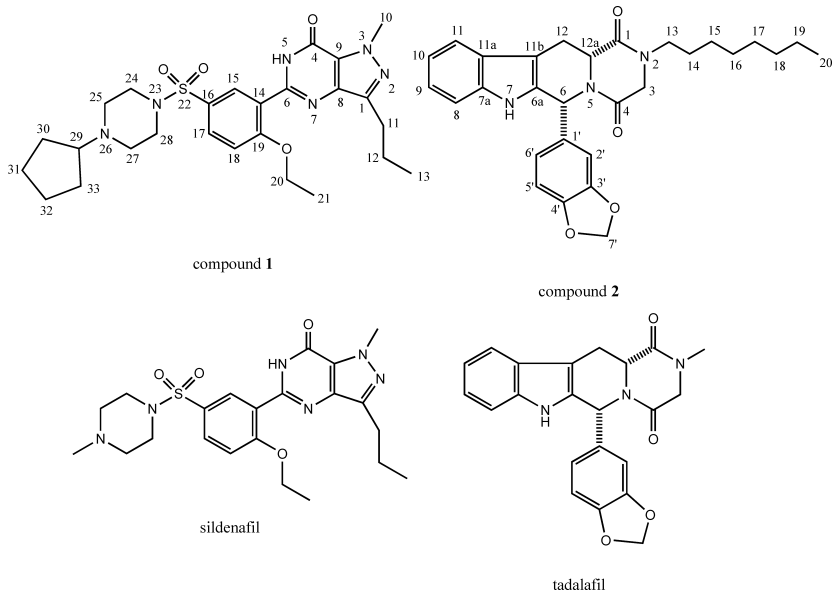

Fig. 1. Structures of Compounds **1**, **2** and Related Compounds

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In this paper, we report the analysis and structural elucidation of a new sildenafil analogue, cyclopentynafil and a new tadalafil analogue, *N*-octylnortadalafil, that were isolated from a dietary supplement illegally marketed for erectile dysfunction.

Experimental

Chemicals and Reagents HPLC-grade acetonitrile and all other reagents (analytical grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Sample The examined product was purchased as a processed food composed mainly of walnuts through the Internet and was composed of four pieces of ivory tablets (400 mg). The product properties were as follows: product name; Highperwalnup 2, producing company; Art Creation Co., Ltd., sales company; Ogawa Planning Co., Ltd., date of purchase; December 28, 2007.

Preparation of Sample Solution One tablet was finely powdered, and 100 mg of the powder was ultrasonically extracted in 10 ml of 70% methanol for 15 min. The extract was centrifuged at 1700×***g***. The supernatant was filtered through a 0.45 μm filter. The filtrate was used for HPLC, and a portion of it was diluted 10-fold with methanol for liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis.

HPLC Analysis HPLC analysis was performed using a JASCO PU-2089 apparatus equipped with a photodiode array (PDA) detector model MD-2015 (JASCO Corporation, Tokyo, Japan). The sample solution was separated by using a TSK-GEL ODS-80Ts column (150×4.6 mm i.d., 5 μm, Tosoh Co., Tokyo, Japan). The mobile phase was an acetonitrile/water/phosphoric acid (100 : 900 : 1) mixture solution containing 5 mmol/l sodium hexanesulfonate (eluent A) and an acetonitrile/water/phosphoric acid (900 : 100 : 1) mixture solution containing 5 mmol/l sodium hexanesulfonate (eluent B). The gradient elution was started at 90% eluent A, and was linearly decreased to 55% A in 25 min and to 10% A in 44—49 min. The flow rate of the mobile phase was set at 1.0 ml/min, and the injection volume was 20 μl. The column temperature was maintained at 40 °C. The PDA detection wavelength was set from ultraviolet (UV) 200 to 400 nm, and max-plot chromatographic monitoring was performed (200—400 nm).

LC-ESI-MS Analysis LC-ESI-MS analysis was performed using a Waters alliance 2695 separation module and ZQ mass spectrometer (Waters Corporation, Milford, MA, U.S.A.). The sample solution was separated by using an Atlantis dC18 column (150×2.1 mm i.d., 3 μm, Waters Corporation). The mobile phase was 0.1% formic acid aqueous solution (eluent A) and acetonitrile containing 0.1% formic acid (eluent B). The gradient elution began at 95% eluent A, and linearly decreased to 80% A in 15 min and to 20% A in 30—35 min. The flow rate of the mobile phase was set at 0.2 ml/min, and the injection volume was 10 μl. The column temperature was maintained at 40 °C. ESI on both positive and negative modes was used for the analysis. The instrument parameters were as follows: source temperature, 120 °C; desolation temperature, 350 °C; capillary voltage, 3 kV; cone voltage, 30, 60 V (ESI positive), −60 V (ESI negative); and desolation gas flow, 600 l/h. The mass range of the spectra was *m*/*z* 100 to *m*/*z* 800.

Isolation of Compounds 1 and 2 Sample powder (300 mg) was dissolved in 20 ml water, and the solution was extracted with 40 ml diethyl ether for 10 min, three times. All of the diethyl ether layers were combined, dehydrated with anhydrous sodium sulfate for 1 h, and filtrated by filter paper. The filtrate was evaporated to dryness then reconstituted with 3 ml methanol. The methanol solution was centrifuged, and the precipitate was dried *in vacuo* to afford compound **1** (18.8 mg). The supernatant was applied to silica gel $60F_{254}$ TLC plates (20×10 cm with 1.0 mm thickness, Merck, Darmstadt, Germany) in a band. The plates were developed using a saturated tank with a hexane/ethyl acetate/acetic acid mixture (50 : 50 : 1) to a distance of about 7 cm. After air-drying, the plates were examined using UV light (wavelength: 254 nm). A band with an *Rf* value of 0.39 was scraped and dissolved in 120 ml of methanol. The methanol solution was filtered, and the filtrate was evaporated to dryness. The residue was reconstituted in 10 ml diethyl ether. This solution was filtered, and the filtrate was evaporated to dryness to afford compound **2** (4.4 mg).

Measurement of Accurate Mass The accurate mass of the target compound was measured by the LTQ OrbiTrap XL instrument (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) with the direct-infusion ESI positive-ion mode under the following conditions: solvent flow rate 5 μl/min, sheath gas flow rate 20 arb, aux gas flow rate 10 arb, spray voltage 5 kV, capillary temperature 275 °C, capillary voltage 4 V, and tube lens 60 V. Tyrosine 1, 3, 6 standard was used as a mass calibrant of FT mass analyzer (resolution=100000), and tyrosine 3 standard was used as a lock mass ion (*m*/*z* 508.20783) during the measurement. Theoretical mass and delta value (mmu) were calculated by using the elemental composition tool of Xcalibur/Qual Browser software.

NMR Analysis $CDCl_3$ (99.96%) and CD_3OD (99.96%) were purchased from ISOTEC Inc., which is part of Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). The NMR spectra were obtained on an ECA-800 spectrometer (JEOL Ltd., Tokyo, Japan) equipped with HCNFG and CH5FG probes (JEOL Ltd.). The ^{1}H- and ^{13}C-NMR chemical shifts of compounds **1** and **2** were assigned by heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC), ^{1}H–^{1}H shift correlation spectroscopy (^{1}H–^{1}H COSY) and nuclear Overhauser effect (NOE) spectra.

Measurement of Circular Dichroism The circular dichroism (CD) spectra of compound **2** and tadalafil were measured by using a J-720 spectrophotometer (JASCO Corporation, Tokyo, Japan) with a quartz cell 10 mm in length. The concentration in methanol solution of compound **2** and tadalafil were 0.041 mmol/l and 0.044 mmol/l, respectively.

Docking Study of Compounds 1 and 2 with PDE-5 Docking models of compounds **1** and **2** bound to PDE-5 were constructed by conformational search simulation (Mixed MCMM/Low Mod). AMBER* was used as force field. Calculations were performed by *MacroModel* (ver. 8.1). 1UDT (PDB ID) and 1UDU, crystal structures of PDE-5 were used for docking models of compounds **1** and **2**, respectively.

Results and Discussion

In this study, we reported **1** and **2** as newly isolated compounds from an illegal dietary supplement. Figure 2A shows the HPLC chromatograms of an extract of the supplement. Two main peaks were detected in the extract, one at 20.9 min (compound **1**) and the other at 37.2 min (compound **2**). The PDA-sliced UV spectrum of **1** exhibited a quite similar profile (λ_{max} nm: 218, 290, Fig. 2B) to that of sildenafil; however, **1** eluted at a later retention time (20.9 min) than sildenafil (18.3 min) under the same chromatographic conditions. Meanwhile, the PDA-sliced UV spectrum of **2** showed a quite similar profile (λ_{max} nm: 281, Fig. 2C) to tadalafil, but **2** eluted at a later retention time (37.2 min) than tadalafil

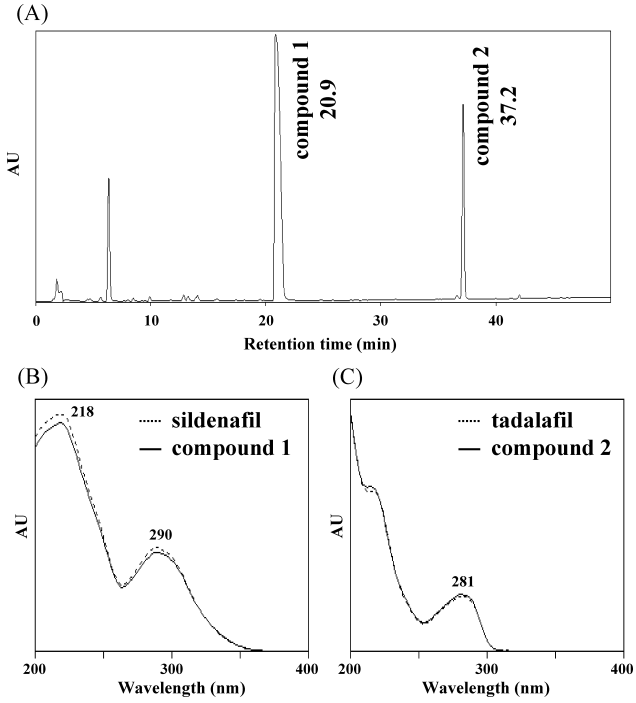

Fig. 2. (A) HPLC Chromatogram of the Sample Solution Monitored Max Plot (200—400 nm) and (B) the Overlaid UV Spectra of Sildenafil and Compound **1**, and (C) the Overlaid UV Spectra of Tadalafil and Compound **2**

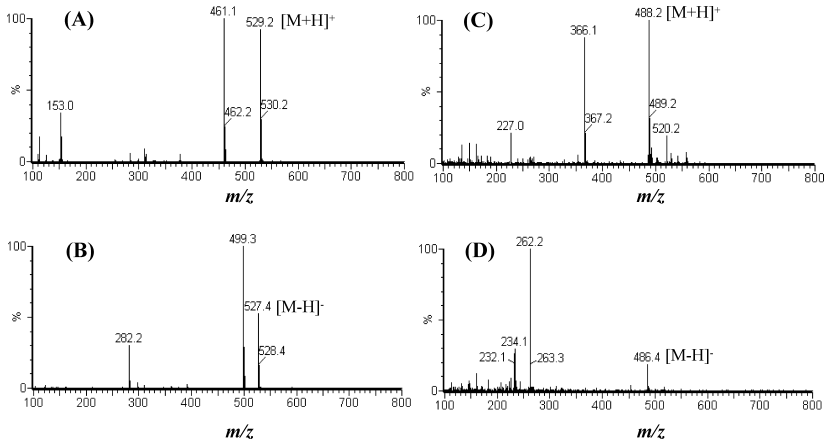

Fig. 3. Mass Spectra of Compounds **1** and **2** by LC-ESI-MS Analysis

(A) Compound **1** (positive, cone voltage: 60 V), (B) compound **1** (negative, cone voltage: −60 V), (C) compound **2** (positive, cone voltage: 30 V), (D) compound **2** (negative, cone voltage: −60 V).

(20.0 min). Furthermore, the peak of **1** exhibited major ion peaks at *m/z* 529 $[M+H]^+$ in the positive scan mode and at *m/z* 527 $[M-H]^-$ in the negative scan mode by LC-ESI-MS analysis (Figs. 3A, B). Also major ion peaks at *m/z* 488 $[M+H]^+$ and at *m/z* 486 $[M-H]^-$ were detected in the peak of **2** (Figs. 3C, D). These data strongly suggested that **1** is a sildenafil analogue and **2** a tadalafil analogue.

Compound **1** formed a colorless amorphous powder and was determined by accurate mass measurement to have the molecular formula $C_{26}H_{36}N_6O_4S$ with a quasimolecular ion at *m/z* 529.2590 (Calcd 529.2597) $[M+H]^+$. The ^{1}H-NMR spectrum of **1** exhibited 35 nonexchangeable protons, including a methyl signal at δ 4.28 (3H, s), an ethoxyl group of signals at δ 1.65 (3H, t, J=6.9 Hz), 4.37 (2H, q, J=6.9 Hz), a *n*-propanyl group of signals at δ 1.01 (3H, t, J=7.3 Hz), 1.86 (2H, sext, J=7.3 Hz), 2.92 (2H, t, J=7.3 Hz) and ABX-type aromatic proton signals at δ 7.14 (1H, d, J=8.7 Hz), 7.83 (1H, dd, J=2.3, 8.7 Hz), 8.83 (1H, d, J=2.3 Hz). The ^{13}C-NMR spectrum of **1** showed 3 methyls, 11 methylenes, including an oxygenated carbon (δ 66.1), 4 methines including 3 aromatic carbons (δ 113.0, 131.3, 131.8) and 7 aromatic quaternary carbons (δ 121.1, 124.5, 128.6, 138.4, 146.4, 147.0, 159.3), and a carbonyl group (δ 153.6). These signals are very similar to those of sildenafil (Table 1), except for the disappearance of an *N*-methyl group and the presence of a methine signal at δ 2.51 (1H, quint, J=7.6 Hz) and two sets of equivalent methylene signals at δ 1.53 (2H, m) and 1.63 (2H, m), and 1.28 (2H, m) and 1.83 (2H, m).

Interpretation of the ^{1}H–^{1}H COSY and HMQC spectra of **1** indicated the presence of a cyclopentyl group (Fig. 4). The connectivity of this group was deduced from the HMBC spectrum (Fig. 4). The methine proton at δ 2.51 (H-29) of the cyclopentyl group showed correlations to the methylene carbons at δ 51.2 (C-25, C-27) of sildenafil. These data determined the structure of **1** as 5-[2-ethoxy-5-(4-cyclopentylpiperazin-1-ylsulfonyl)phenyl]-1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one. The assignments of the ^{1}H- and ^{13}C-NMR signals of **1** are summarized in Table 1. Considering its properties, compound **1** is named as cyclopentynafil.

Compound **2** formed a colorless amorphous powder and was determined by accurate mass measurement to have the molecular formula $C_{29}H_{33}N_3O_4$ with a quasimolecular ion at *m/z* 510.2362 (Calcd 510.2363) $[M+Na]^+$. The ^{1}H-NMR spectrum of **2** exhibited 32 nonexchangeable protons, including a methyl signal at δ 0.89 (3H, t, J=7.3 Hz), a methylenedioxyl group signal at δ 5.85 (2H, d, J=6.9 Hz), ABX-type aromatic proton signals at δ 6.68 (1H, d, J =7.8 Hz), 6.78 (1H, d, J=1.9 Hz) and 6.79 (1H, dd, J=7.8, 1.9 Hz), and AB-type aromatic proton signals at δ 7.02 (1H, br t, J=7.8 Hz), 7.07 (1H, br t, J=8.2 Hz), 7.27 (1H, d, J= 8.2 Hz) and 7.52 (1H, d, J=7.8 Hz). The ^{13}C-NMR spectrum of **2** showed 1 methyl, 9 methylenes, including a methylenedioxy carbon (δ

Table 1. ^{1}H- and ^{13}C-NMR Chemical Shifts of Compound **1** and Sildenafil in $CDCl_3$

Position	**1** (^{1}H[a])	Sildenafil (^{1}H[a])	**1** (^{13}C[b])	Sildenafil (^{13}C[b])
1			147.0	146.4
4			153.6	153.6
5	10.80 s	10.82 s		
6			146.4	147.0
8			138.4	138.4
9			124.5	124.5
10 (3H)	4.28 s	4.28 s	38.2	38.2
11 (2H)	2.92 t (7.3)	2.93 t (7.2)	27.8	27.7
12 (2H)	1.86 sext (7.3)	1.86 sext (7.2)	22.3	22.2
13 (3H)	1.01 t (7.3)	1.02 t (7.2)	14.0	14.0
14			121.1	121.1
15	8.83 d (2.3)	8.82 d (2.4)	131.3	131.2
16			128.6	129.0
17	7.83 dd (2.3, 8.7)	7.84 dd (2.4, 8.6)	131.8	131.7
18	7.14 d (8.7)	7.15 d (8.6)	113.0	113.0
19			159.3	159.3
20 (2H)	4.37 q (6.9)	4.37 q (6.9)	66.1	66.1
21 (3H)	1.65 t (6.9)	1.65 t (6.9)	14.6	14.5
24 (2H)	3.10 br s	3.11 br s	46.1	45.9
25 (2H)	2.59 br s	2.50 br s	51.2	54.0
27 (2H)	2.59 br s	2.50 br s	51.2	54.0
28 (2H)	3.10 br s	3.11 br s	46.1	45.9
29	2.51 quint (7.6)	2.28 (3H) s	66.8	45.7
30 (2H)	1.28 m, 1.83 m		30.4	
31 (2H)	1.53 m, 1.63 m		24.0	
32 (2H)	1.53 m, 1.63 m		24.0	
33 (2H)	1.28 m, 1.83 m		30.4	

a) Recorded in 800 MHz and *J* values (in Hz) in parentheses. *b*) Recorded in 200 MHz.

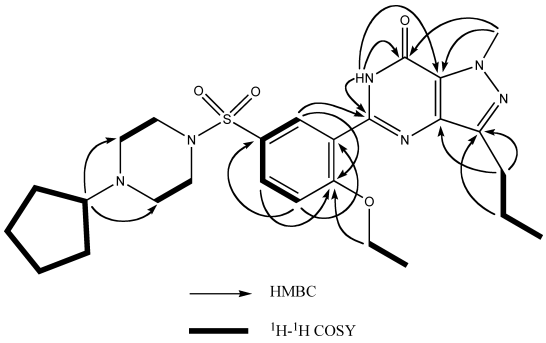

Fig. 4. ^{1}H–^{1}H and Major Long-Range ^{1}H–^{13}C Correlations of Compound **1**

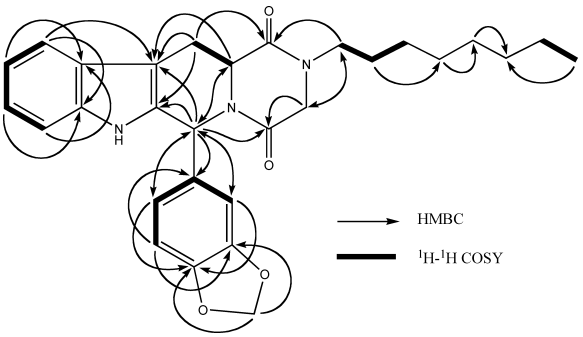

Fig. 5. ^{1}H–^{1}H and Major Long-Range ^{1}H–^{13}C Correlations of Compound **2**

102.4), 9 methines including 7 aromatic carbons (δ 108.2, 109.0, 111.2, 118.9, 120.3, 121.1, 122.8) and 7 aromatic quaternary carbons (δ 106.1, 127.4, 134.6, 137.6, 138.3, 148.3, 149.2), and 2 carbonyl groups (δ 169.1, 169.6). These signals are very similar to those of tadalafil (Table 2), except for the disappearance of an *N*-methyl group and the presence of 7 methylene signals, including an *N*-methylene group signal at δ 3.49 (2H, br t, J=7.4 Hz) and a methyl signal at δ 0.89 (3H, t, J=7.3 Hz).

Interpretation of the ^{1}H–^{1}H COSY and HMQC spectra of **2** indicated the presence of an *N*-octyl group (Fig. 5). The connectivity of an *N*-octyl group was deduced from the HMBC spectrum (Fig. 5). The methylene proton at δ 3.49 (H-13) of the *N*-octyl group showed correlations to the carbonyl carbon at δ 169.1 (C-1) and a methylene carbon at δ 51.2 (C-3) of tadalafil. Also, methylene protons at δ 3.94 and 4.24 (H_2-3) showed correlations to the methylene carbon at δ 47.2 (C-13). These data determined the planar structure of **2**, as shown in Fig. 5.

The relative configuration between two methine protons at C-6 and C-12a was established a *cis* configuration by the NOE experiment. Furthermore, the CD spectrum of **2** is superimposable with that of tadalafil (Fig. 6), and it is clear that the absolute stereochemistry of two methine protons at C-6 and C-12a are the same as that of tadalafil. These results enabled us to elucidate the structure of **2** as (6*R*,12a*R*)-6-(1,3-benzodioxol-5-yl)-2-octyl-2,3,6,7,12,12a-hexahydropyrazino[1′,2′ : 1,6]pyrdo[3,4-*b*]indol-1,4-dione. The assignments of the ^{1}H- and ^{13}C-NMR signals of **2** are summarized in Table 2. Considering its properties, compound **2** is designated as *N*-octylnortadalafil.

Table 2. ^{1}H- and ^{13}C-NMR Chemical Shifts of Compound **2** and Tadalafil in CD_3OD

Position	**2** (^{1}H^a)	Tadalafil (^{1}H^a)	**2** (^{13}C^b)	Tadalafil (^{13}C^b)
1			169.1	168.9
3	4.24 br d (17.4)	4.20 br d (17.0)	51.2	52.9
	3.94 d (17.4)	3.97 d (17.0)		
4			169.6	169.0
6	6.24 s	6.17 s	57.5	58.0
6a			134.6	134.7
7a			138.3	138.4
8	7.27 d (8.2)	7.25 d (8.3)	111.2	112.2
9	7.07 br t (8.2)	7.06 br t (8.3)	122.8	122.7
10	7.02 br t (7.8)	7.02 br t (7.8)	120.3	120.3
11	7.52 d (7.8)	7.51 d (7.8)	118.9	118.9
11a			127.4	127.4
11b			106.1	106.3
12	3.62 dd (15.6, 5.1)	3.66 dd (15.6, 4.6)	24.2	24.7
	3.12 br dd (15.6, 11.5)	3.11 br dd (15.6, 11.9)		
12a	4.44 br dd (11.5, 5.1)	4.40 br dd (11.9, 4.6)	57.4	57.6
13 (2H)	3.49 br t (7.4)	3.03 (3H) s	47.2	33.8
14 (2H)	1.59 m		27.9	
15 (2H)	1.30 m		27.8	
16 (2H)	1.26 m		30.4	
17 (2H)	1.32 m		30.4	
18 (2H)	1.28 m		33.0	
19 (2H)	1.31 m		23.7	
20 (3H)	0.89 t (7.3)		14.4	
1′			137.6	137.7
2′	6.78 d (1.9)	6.80 d (1.4)	108.2	108.3
3′			149.2	149.1
4′			148.3	148.2
5′	6.68 d (7.8)	6.68 d (8.2)	109.0	108.9
6′	6.79 dd (7.8, 1.9)	6.82 dd (8.2, 1.4)	121.1	121.3
7′ (2H)	5.85 d (6.9)	5.84 d (9.1)	102.4	102.4

a) Recorded in 800 MHz and *J* values (in Hz) in parentheses. *b*) Recorded in 200 MHz.

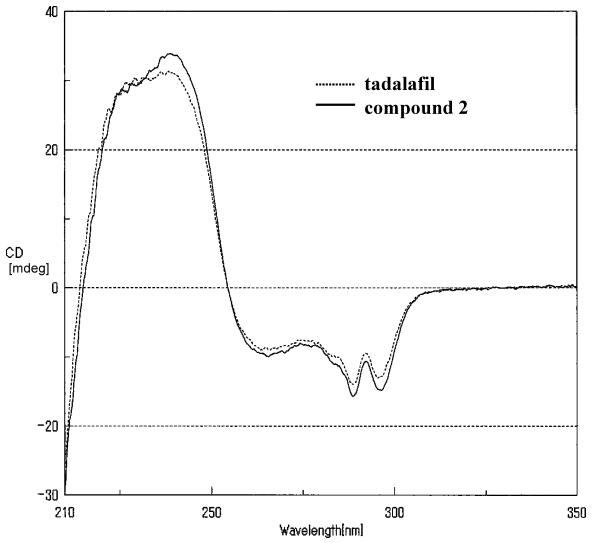

Fig. 6. Overlaid CD Spectra of Tadalafil and Compound **2**

Furthermore, quantitative analyses of **1** and **2** in the supplement product were determined by HPLC. The contents of **1** and **2** in the product were about 130 mg/tablet (301 μg/mg) and about 27 mg/tablet (64.1 μg/mg), respectively.

Finally, we calculated to make docking models of **1** and **2** bound to PDE-5. Compounds **1** and **2** were well fitted to the cavity of PDE-5 like sildenafil and tadarafil, respectively.

Therefore, both compounds are expected to have inhibitory activities against PDE-5.

In conclusion, a new sildenafil analogue, cyclopentynafil and a new tadalafil analogue, *N*-octylnortadalafil were isolated from a dietary supplement illegally marketed in Japan for erectile dysfunction. Their structures were elucidated by using HPLC-PDA, LC-MS, high-resolution MS, NMR and CD. Recently, Toque *et al.* synthesized a new cyclohexyl type of sildenafil analogue and its IC_{50} value as PDE-5 inhibitor was almost same as sildenafil,[30] whereas cyclopentynafil and *N*-octylnortadalafil are the first compounds reported to be new sildenafil and tadalafil analogues, respectively, and their inhibitory activities against PDE-5 are expected by docking study. Thus, tremendous risk is faced by patients who unknowingly look to dietary supplements, which are adulterated with such analogues for ED treatment.

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