Determination of a New Type of Phosphodiesterase-5 Inhibitor, Thioquinapiperifil, in a Dietary Supplement Promoted for Sexual Enhancement

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A new type of phosphodiesterase-5 (PDE-5) inhibitor, thioquinapiperifil (1), was found in dietary supplements. LC-MS analysis indicated that the supplements contain two major compounds. One was identified as thiodenafil (synonym: thiosildenafil) by direct comparison with the authentic compound. The other showed a molecular weight of 448, and accurate mass measurement showed its elemental composition to be $C_{24}H_{28}N_6O_1S_1$. Together, the mass and NMR spectrometric data revealed that the compound is an imidazoquinazoline derivative: 3-ethyl-1,3-dihydro-8-[[[2-[4-(hydroxymethyl)-1-piperidinyl]phenyl]methyl]amino]-2*H*-imidazo[4,5-*g*]quinazoline-2-thione. This compound had been synthesized as a PDE-5 inhibitor, formerly reported as KF31327 by Kyowa Hakko Kogyo Co., Ltd. Considering this compound's general properties, it has been renamed thioquinapiperifil with the agreement of Kyowa Hakko Kogyo Co., Ltd. The detection of imidazoquinazoline-type compounds in dietary supplements has not been reported. Quantitative analysis showed that the contents of 1 and thiodenafil in the products were about 13—15 mg/tablet (43—48 μg/mg) and about 0.4 mg/tablet (1 μg/mg), respectively.

Key words thioquinapiperifil; phosphodiesterase-5 inhibitor; LC-MS; NMR; erectile dysfunction

Recently, many kinds of dietary supplements have become available directly to the public *via* the internet. 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, vardenafil, and tadalafil, known as active drug ingredients for the treatment of penile erectile dysfunction (ED).[1—3] These pharmaceuticals selectively inhibit the phosphodiesterase-5 (PDE-5) enzyme, thus raising cyclic guanosine monophosphatase (cGMP) levels to cause a vasodilatory effect. Considering their risk, these products should be used only under the supervision of medical experts.[1]

Recently, various ingredients with structures similar to or modified from those of such compounds have been newly detected.[1,4—21] By 2007, over 10 different analogs of sildenafil, tadarafil, and vardenafil had been reported, and new analogs are still being found.[4—18,21] These analogs are deduced to be PDE-5 inhibitors because of their structural resemblance, and in fact they exhibit this inhibitory activity.[4]

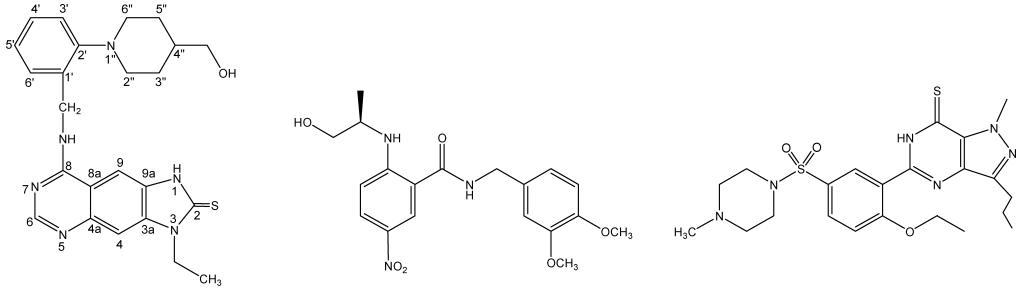
In 2007, (*R*)-xanthoanthrafil, an anthranilic acid derivative, was found in a dietary supplement advertising sexual enhancement for men (Fig. 1).[22,23] The compound's structure is not like those of the ingredients of any well-known drug. (*R*)-Xanthoanthrafil was first synthesized as a candidate compound for the treatment of ED by Fujisawa Pharmaceutical Co., Ltd. (currently Astellas Pharma Inc., Tokyo, Japan),[24] and was reported as a PDE-5 inhibitor, FR226807, after discontinuation of its development process to an approved drug.

In this paper, we report the identification and analysis of another new type of PDE-5 inhibitor, an imidazoquinazoline derivative, in dietary supplements. 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.[25—27]

Experimental

Chemicals and Reagents HPLC-grade acetonitrile and all other chemicals (analytical grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Centrifugal filter devices (Ultrafree-MC, 0.45 μm filter unit) were from Millipore (Bedford, MA, U.S.A.). Authentic thiodenafil (synonym: thiosildenafil) was synthesized in our laboratory[28,29] and identified as KJH-1002 by comparison with the reported data.[20]

Samples Three kinds of products (five products in all) were purchased at an porno shop in Japan or *via* the internet (from October to December 2007). These products were composed of 2 or 10 sand-colored tablets (13—15 mg of product per tablet).



compound **1** (*R*)-xanthoanthrafil thiodenafil

Fig. 1. Structures of Compound **1** and Related Compounds

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Preparation of Sample Solution A tablet (260 mg) was crushed into powder in a mortar with a pestle. Then, 20 mg of the powder were immersed with 2 ml of a solution of 1% formic acid/acetonitrile (20 : 80, v/v) and sonicated for 5 min. After centrifugation (3 min at 1500 rpm), 1 ml of solution was diluted with 1 ml of 5 mM ammonium formate (pH 3.5)/acetonitrile (75 : 25, v/v) and filtered through a centrifugal filter device.

Liquid Chromatography-Mass Spectrometry Analysis The sample solutions were qualitatively analyzed by using a liquid chromatography-electrospray ionization-mass spectrometer (LC-ESI-MS) consisting of an Agilent 1100 series HPLC system equipped with an 1100 series LC/MSD SL (Agilent Technologies, Palo Alto, CA, U.S.A.). The sample solutions were separated using an Inertsil ODS-3 column (2.1 i.d.×150 mm, 5 μm; GL Sciences Inc., Tokyo, Japan) at 40 °C. The following gradient system was used with a mobile phase A (5 mM ammonium formate buffer (pH 3.5)/acetonitrile (75 : 25, v/v) and a mobile phase B (acetonitrile) delivered at 0.3 ml/min; A : B 100 : 0 (0—3 min)–70 : 30 (13—20 min)–50 : 50 (30—50 min). The injection volume was 1 μl. For the detection system, a tandem setting of a photo diode array (PDA) and a mass detector (MSD) was adopted. The wavelength of the PDA detector for screening was set from UV 190 to 400 nm, and chromatographic peaks were monitored at UV 270 and 290 nm. Mass analysis by the ESI was used in a positive mode. Nitrogen gas was used for nebulization at a flow rate of 13 l/min at 350 °C. The nebulizer pressure was 60 psi, the vaporizer temperature was 350 °C, the capillary voltage was 3000 V, and the fragment voltage was 230 or 350 V. MS data were recorded in the full scan mode (*m/z* 50—600). The chromatographic peaks were detected and integrated by the Agilent Chemistation data analysis system (Agilent Technologies).

HPLC Analysis For the quantitative and qualitative analysis of the sample solutions, an HPLC system consisting of a Shimadzu 10A VP series equipped with a PDA detector model SPD-M10A (Shimadzu Co., Kyoto, Japan) was used. The sample solution was separated using an Inertsil ODS-3 column (4.6 i.d.×150 mm, 5 μm; GL Sciences Inc.) delivered at 1 ml/min and kept at 40 °C. The wavelength of the PDA detector for monitoring the chromatographic peaks was set at UV 350 nm. Data storage and processing were performed using CLASS-VP software (Shimadzu Co.). Other conditions of HPLC analysis are described in the LC-MS analysis section.

Standard Solutions To prepare standard solutions ranging from 0.01 to 1 mg/ml, 1 mg of thiodenafil standard was dissolved in methanol and diluted with mobile phase A. An isolated compound **1** was diluted with methanol to prepare standard solutions with the same concentration as that of thiodenafil. The HPLC analysis conditions are described in the HPLC analysis section.

Isolation of Compound 1 Several tablets were crashed into powder in a mortar with a pestle. Then, 800 mg of the powder were immersed with 20 ml of methanol and sonicated for 20 min. After the solution was centrifuged, the supernatant was evaporated to dryness and purified by HPLC as follows. An Inertsil ODS-3 column (20 i.d.×250 mm, 5 μm; GL Sciences Inc.) coupled to a guard column (7.6 i.d.×30 mm, 5 μm; GL Sciences Inc.) was used for separation by isocratic flow with a mixture of ultra-pure water and acetonitrile (60 : 40, v/v). The collected fraction was dried under vacuum to afford compound **1** as a yellowish amorphous solid.

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 (Thermo Fisher Scientific Inc.). MS data were recorded in the full scan mode (*m/z* 100—1000).

NMR Analysis DMSO-d_6 (99.96%) was 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-600 spectrometer (JEOL Ltd., Tokyo, Japan) equipped with an ATH5FG probe (JEOL Ltd.) and a Varian C13 cold probe (Varian, Inc., Palo Alto, CA, U.S.A.). Assignments were made *via* ^{1}H, ^{13}C-NMR, heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC), double quantum filtered correlation spectroscopy (DQF-COSY), and rotating frame nuclear Overhauser effect (ROE) spectra.

Results and Discussion

In this study, we reported **1** as a newly identified compound from an illegal dietary supplement. We found that this compound has a novel structure that is not usually observed in anti-ED drugs.

In the sample solution of each of the five products, two main peaks were detected by LC-ESI-MS analysis (Figs. 2A, B). One peak, detected at 15.8 min, exhibited a major ion peak at *m/z* 491 $[M+H]^+$ in the positive scan mode. A comparison with the authentic compound revealed this peak to represent thiodenafil (Fig. 1), which has been synthesized and reported as a PDE-5 inhibitor named KJH-1002.[19,20] The other unknown peak in the sample solution was detected at 9.6 min in positive scan mode (Figs. 2A, B). The PDA-sliced UV spectrum of the peak exhibited maxima at 211, 268, 363 nm and minima at 234 and 299 nm (Fig. 2C). These characteristics were completely different from those of known PDE-5 inhibitors, such as thiodenafil (UV λ_{max} nm: 227, 295, 353 and λ_{min} nm: 276, 315, Fig. 2C),[21] sildenafil, vardenafil, and tadalafil, which have been detected in some kinds of dietary supplements.[30] Therefore, we concluded that the ingredient was an unknown compound (**1**) not found hitherto in dietary supplements.

The accurate mass of the $[M+H]^+$ ion of **1** was *m/z* 449.21181 giving an estimated elemental composition of $C_{24}H_{29}N_6O_1S_1$ (*m/z* 449.21236, 0.55 mmu) as the most approximate result.

The ^{1}H-NMR spectrum[31] of **1** exhibited 28 non-exchangeable protons, including a methyl signal at δ 1.28 (3H, t, J=7.2 Hz), AA′BB′-type aromatic proton signals at δ 7.12 and 7.15 (each 1H, dd, J=7.6, 1.0 Hz), 6.96 and 7.19 (each 1H, td, J=7.6, 1.0 Hz), and three other aromatic protons at δ 7.64, 8.09, and 8.37 (each 1H, s). In addition, seven methylene proton signals at δ 1.33—4.84 (14H), and a characteristic signal assignable to amine proton at δ 13.24 (1H, s) were observed. The ^{13}C-NMR spectrum[31] of **1** showed 24 carbon signals, including one methyl, seven methylenes with one oxygenated carbon (δ 66.1), one methine, and one thiocarbonyl carbon (δ 172.0). The presence of three partial structures (a 1,2-substituted phenyl group, a 4-hydroxymethylpiperidine group, and a 3-ethyl-2*H*-imidazo[4,5-*g*]quinazoline-2-thione group) was suggested from its DQF-COSY, HMQC, and HMBC spectra. The connectivity of the 1′ position of the 1,2-substituted phenyl and the 3-ethyl-2*H*-imidazo[4,5-*g*]quinazoline-2-thione groups through the iminomethylene bridge was also deduced from the HMBC spectrum. In addition, the selected ROE correlations between the equatorial proton at 2″ position (δ 3.09) and the iminomethylene proton (δ 4.84) suggested the linkage between the 1″ position of the piperidine group and the 2′ position of the phenyl group. Therefore, the structure of **1** is finally elucidated as 3-ethyl-1,3-dihydro-8-[[[2-[4-(hydroxymethyl)-1-piperidinyl]phenyl]methyl]amino]-2*H*-imidazo[4,5-*g*]quinazoline-2-thione, as shown in Fig. 1.

The deduced structure is coincident with that of KF31327, which has already been reported as a selective PDE-5 inhibitor.[25—27] Comparison of the ^{1}H- and ^{13}C-NMR data of the unknown compound with those of KF31327 revealed that the isolated compound is KF31327.[25,31] Considering its general properties, this compound is renamed thioquinapiperifil (**1**) with the agreement of Kyowa Hakko Kogyo Co., Ltd.

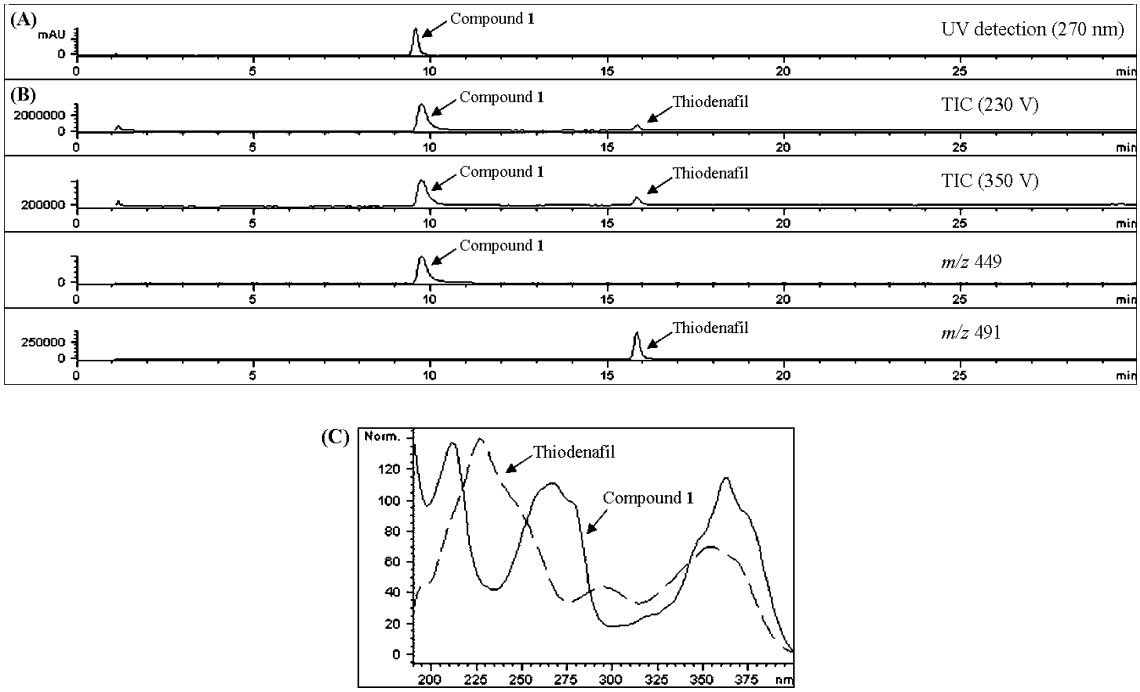

Fig. 2. (A) HPLC-UV (270 nm) and (B) -MS Chromatogram of the Sample Solution, and (C) UV Spectra of the Detected Peaks (Compound **1** and Thiodenafil) Obtained from the Analysis of LC-MS Coupled with a PDA

This is the first case in which **1** has been detected in a so-called dietary supplement.

Then, quantitative analysis of **1** in the supplement product was performed using HPLC. The content of this compound in the tablet was 13—15 mg. Since the product packaging gives the dosage as two tablets, about 26—30 mg of **1** would be taken in a single dose. Additionally, the same tablet contained 0.4 mg of thiodenafil.

This is the first report of a new type of PDE-5 inhibitor, imidazoquinazoline derivative (**1**), contained in some dietary supplements promoted for sexual enhancement. Until now, as far as we know, all new illegal compounds identified in dietary supplements promoted for sexual enhancement for men are analogs of approved drugs, such as sildenafil, tadarafil, and vardenafil, except for one case. That is, in 2007, Kumasaka *et al.* identified a new type of ingredient, (*R*)-xanthoanthrafil, which until then had been identified as a PDE-5 inhibitor in a paper but had never been sold as a drug ingredient.[23] Our identification of thioquinapiperifil is the second case in which a non-analog of approved drugs has been identified.

Kyowa Hakko Kogyo Co., Ltd.,[25—27] has reported some analogs of thioquinapiperifil and has described their synthesis with limited pharmacological data. This situation alerts us that thioquinapiparifil analogs may be found in dietary supplements in the near future. To avoid health problems caused by illegal dietary supplements containing any drug ingredients, which are classified as a raw material that is exclusively used in pharmaceuticals in Japan, or a new illegal compound, we have to continuously monitor such compounds in dietary supplements.

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