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

Nahoko UCHIYAMA, Kazuhiro SAISHO, Ruri KIKURA-HANAJIRI, Yuji HAISHIMA, and Yukihiro GODA*

National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan.
Received April 7, 2008; accepted July 8, 2008; published online July 10, 2008

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]

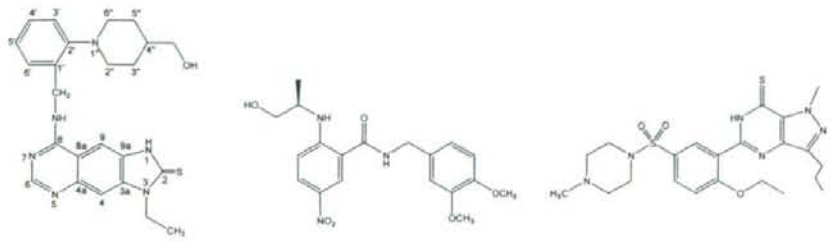
In 2007, (*R*)-xanthoanthrafil, an anthranilic acid derivative, was found in a dietary supplement advertising sexual enhancement for men (Fig. 1).[22,23] The compound's structure is not like those of the ingredients of any well-known drug. (*R*)-Xanthoanthrafil was first synthesized as a candidate compound 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

* To whom correspondence should be addressed. e-mail: goda@nihs.go.jp

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.

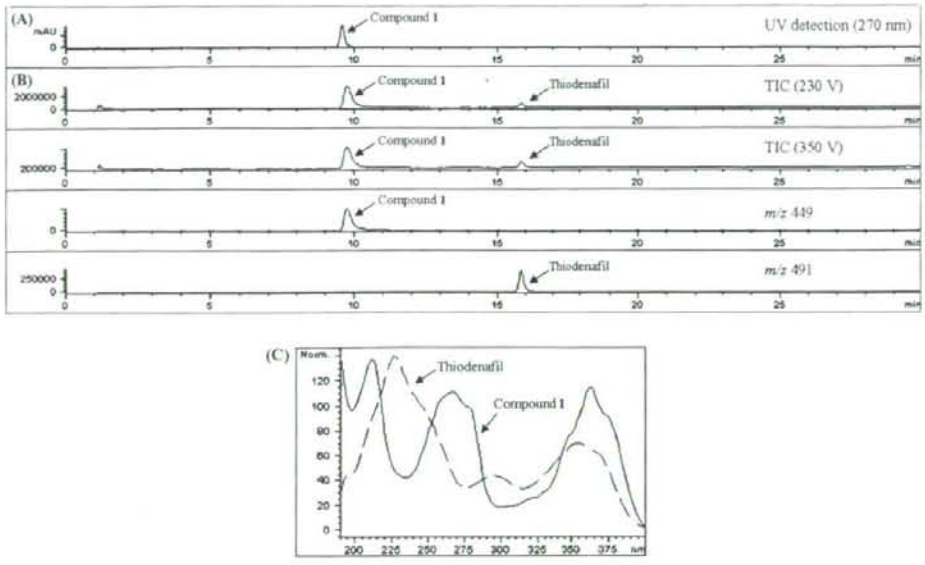


Fig. 2. (A) HPLC-UV (270 nm) and (B) -MS Chromatogram of the Sample Solution, and (C) UV Spectra of the Detected Peaks (Compound **1** and Thiodenafil) Obtained from the Analysis of LC-MS Coupled with a PDA

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.

Acknowledgments Part of this work was supported by a Health and Labor Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan.

References and Notes

1) Japan, Ministry of Health, Labour and Welfare, websites: listed in (http://www.mhlw.go.jp/kinkyu/diet/other/050623-1.html) [in Japanese].
2) Moriyasu T., Shigeoka S., Kishimoto K., Ishikawa F., Nakajima J., Kamimura H., Yasuda I., *Yakugaku Zasshi*, **121**, 765—769 (2001).
3) Zhu X., Xiao S., Chen B., Zhang F., Yao S., Wan Z., Yang D., Han H., *J. Chromatogr. A*, **1066**, 89—95 (2005).
4) Venhuis B. J., Barends D. M., Zwaagstra M. E., De Kaste D., "RIVM Report 370030001/2007," National Institute for Public Health and the Environment, the Netherlands, 2007.
5) Shin M. H., Hong M. K., Kim, W. S., Lee Y. J., Jeoung Y. C., *Food Addit. Contam.*, **20**, 793—796 (2003).
6) Shin C., Hong M., Kim D., Lim Y., *Magn. Reson. Chem.*, **42**, 1060—1062 (2004).
7) Park H. J., Jeong H. K., Chang M. I., Im M. H., Jeong J. Y., Choi D. M., Park K., Hong M. K., Youm J., Han S. B., *Food Addit. Contam.*, **24**, 122—129 (2007).
8) Blok-Tip L., Zomer B., Bakker F., Hartog K. D., Hamzink M., ten Hove J., Vredenbregt M., de Kaste D., *Food Addit. Contam.*, **21**, 737—748 (2004).
9) Hou P., Zou P., Low M. Y., Chan E., Koh H. L., *Food Addit. Contam.*, **23**, 870—875 (2006).
10) Zou P., Hou P., Low M.-Y., Koh H. L., *Food Addit. Contam.*, **23**, 446—451 (2006).
11) Zou P., Hou P., Oh S. S., Low M. Y., Koh H. L., *Rapid Commun. Mass Spectrom.*, **20**, 3488—3490 (2006).
12) Gratz S. R., Flurer, C. L., Wolnik K. A., *J. Pharm. Biomed. Anal.*, **36**, 525—533 (2004).
13) Gratz S. R., Gamble B. M., Flurer R. A., *Rapid Commun. Mass Spectrom.*, **20**, 2317—2327 (2006).
14) Lai K. C., Liu Y. C., Tseng M. C., Lin J. H., *J. Food Drug Anal.*, **14**, 19—23 (2006).
15) Reepmeyer J. C., Woodruff J. T., *J. Chromatogr. A*, **1125**, 67—75 (2006).
16) Reepmeyer J. C., Woodruff J. T., d'Avignon D. A., *J. Pharm. Biomed. Anal.*, **43**, 1615—1621 (2007).
17) Reepmeyer J. C., Woodruff J. T., *J. Pharm. Biomed. Anal.*, **44**, 887—893 (2007).
18) Lam Y. H., Poon W. T., Lai C. K., Chan A.Y. W., Mak T. W. L., *J. Pharm. Biomed. Anal.*, **46**, 804—807 (2008).
19) Cho E. Y., Chung S. H., Kim J. H., Kim D. K., Jin C., *J. Appl. Phar-*

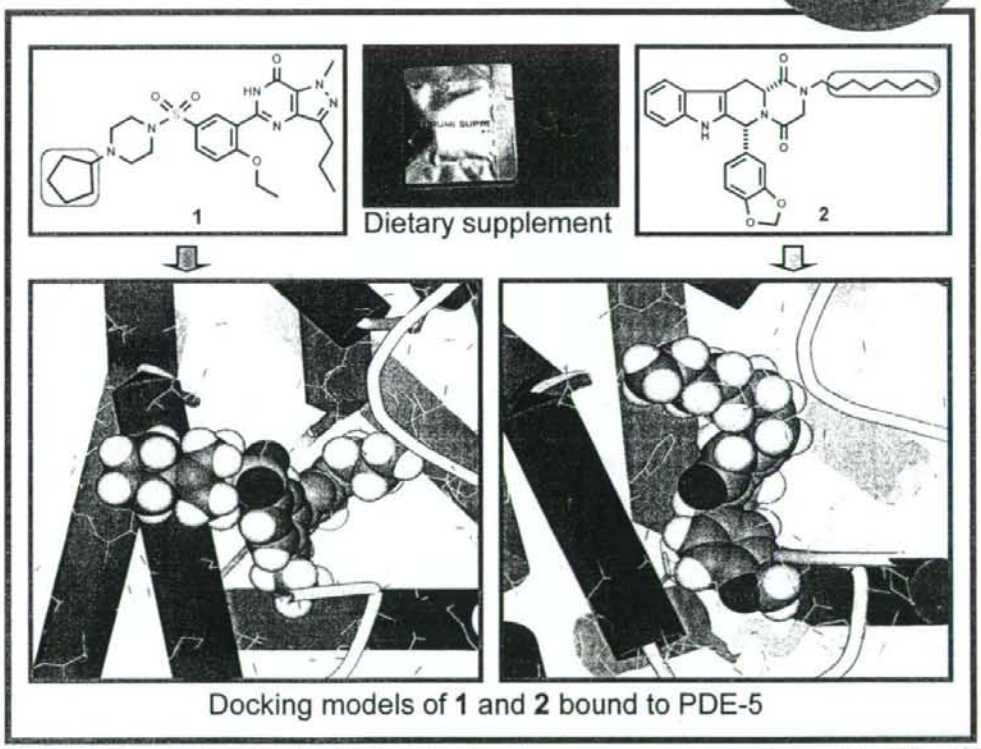
macol., **11**, 232—237 (2003).
20) Kim J. H., Kim Y., Choi K., Kim D. H., Nam G., Seo J. H., WO 2002102802 (2002) [*PCT Int. Appl.*, (2002)].
21) Zou P., Hou P., Oh S. S., Chong Y. M., Bloodworth B. C., Low M. Y., Koh H. L., *J. Pharm. Biomed. Anal.*, **47**, 279—284 (2008).
22) Kumasaka K., Doi K., Kojima T., Kawahara N., Goda Y., Proceedings of the 127th Annual Meeting of the Pharmaceutical Society of Japan, Toyama, Japan, 28—30 March, 2007.
23) Kumasaka K., Kawahara N., Doi K., Kojima T., Goda Y., *Chem. Pharm. Bull.*, **56**, 227—230 (2008).
24) Hosogai N., Hamada K., Tomita M., Nagashima A., Takahashi T., Sekizawa T., Mizutani T., Urano Y., Kuroda A., Sawada K., Ozaki T., Seki J., Goto T., *Eur. J. Pharm.*, **428**, 295—302 (2001).
25) Fujino K., Takami H., Atsumi T., Ogasa T., Mohri S., Kasai M., *Org. Process Res. Dev.*, **5**, 426—433 (2001).
26) Onoda Y., Nomoto Y., Ohno T., Yamada K., Ichimura M., WO9808848 (1998) [*PCT Int. Appl.*, (1998)].
27) Hirose R., Okumura H., Yoshimatsu A., Irie J., Onoda Y., Nomoto Y., Takai H., Ohno T., Ichimura M., *Eur. J. Pharmacol.*, **431**, 17—24 (2001).
28) Kamakura H., Hirama Y., Hayashi T., Kanetoshi A., Goda Y., Proceedings of the 128th Annual Meeting of the Pharmaceutical Society of Japan, Yokohama, Japan, 26—28 March, 2008.
29) Thomsen I., Clausen K., Scheibye S., Lawesson S. O., "Org. Synth.," Vol. 62, ed. by Danheiser R. L., Organic Syntheses Inc., New York, 1984, pp. 158—161.
30) Zou P., Oh S. S., Hou P., Low M. Y., Koh H. L., *J. Chromatogr. A*, **1104**, 113—122 (2006).
31) Detailed NMR data with assignments are available from the corresponding author upon request.

毎月1回1日発行　57巻2号平成21年2月1日発行　昭和35年8月15日　第3種郵便物認可　ISSN 0009-2363

CHEMICAL & PHARMACEUTICAL BULLETIN

VOL.57 No.2　CPBTAL 57 (2) 117-228 (2009)

FEBRUARY 2009



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

pp. 185—189

http://cpb.pharm.or.jp

PHARMACEUTICAL SOCIETY OF JAPAN

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

Takashi HASEGAWA,[a] Kazunaga TAKAHASHI,[a] Masaaki SAITO,[a] Toshiyasu ISHII,[a] Tomoko NAGATA,[a] Masaaki KURIHARA,[b] Yuji HAISHIMA,[b] Yukihiro GODA,*[,b] and Nobuo KAWAHARA[b]

[a] *Chiba Prefectural Institute of Public Health; 666–2 Nitona-cho, Chuo-ku, Chiba, Chiba 260–8715, Japan: and* [b] *National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan.*
Received October 29, 2008; accepted November 19, 2008; published online November 21, 2008

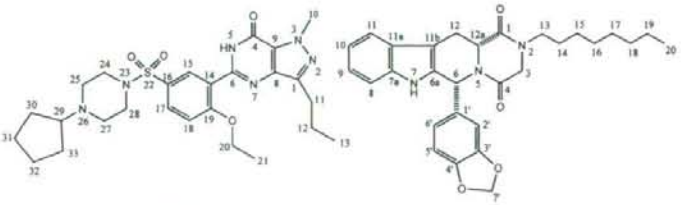
A new sildenafil analogue, cyclopentynafil (1) and a new tadalafil analogue, *N*-octylnortadalafil (2) were isolated from a dietary supplement illegally marketed for erectile dysfunction. The structures of the sildenafil and tadalafil analogues were elucidated by using HPLC-photodiode array (PDA), LC-MS, 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]



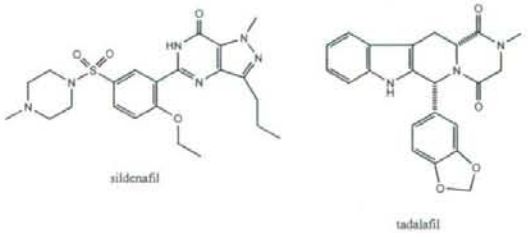


Fig. 1. Structures of Compounds **1**, **2** and Related Compounds

* To whom correspondence should be addressed. e-mail: goda@nihs.go.jp

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

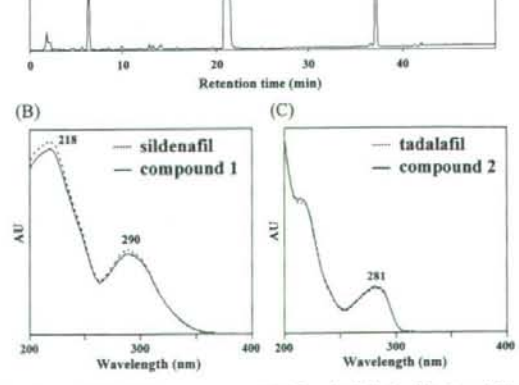


Fig. 2. (A) HPLC Chromatogram of the Sample Solution Monitored Max Plot (200—400 nm) and (B) the Overlaid UV Spectra of Sildenafil and Compound **1**, and (C) the Overlaid UV Spectra of Tadalafil and Compound **2**

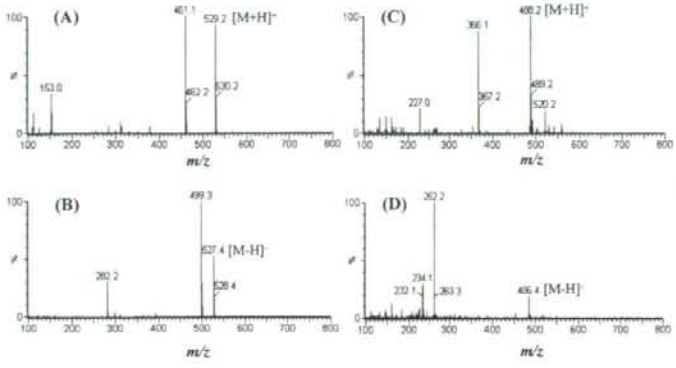


Fig. 3. Mass Spectra of Compounds **1** and **2** by LC-ESI-MS Analysis

(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.

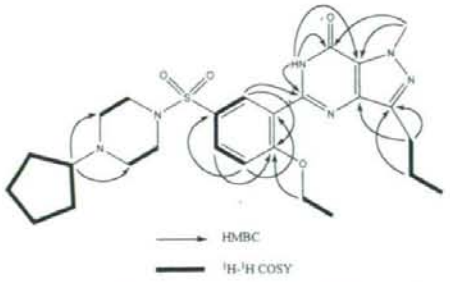


Fig. 4. ^{1}H–^{1}H and Major Long-Range ^{1}H–^{13}C Correlations of Compound **1**

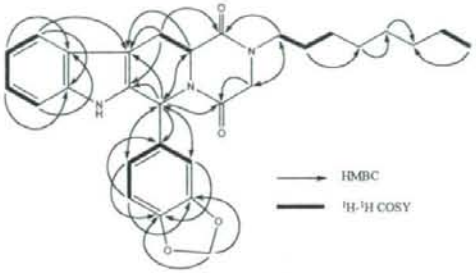


Fig. 5. ^{1}H–^{1}H and Major Long-Range ^{1}H–^{13}C Correlations of Compound **2**

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.

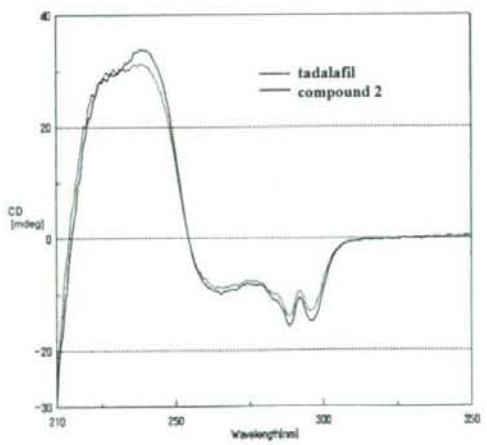


Fig. 6. Overlaid CD Spectra of Tadalafil and Compound **2**

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.

Acknowledgement This study was partly supported by a Health and Labour Science Research Grant from the Ministry of Health, Labour and Welfare of Japan.

References

1) Ministry of Health, Labour and Welfare of Japan, websites: ⟨http://www.mhlw.go.jp/kinkyu/diet/other/050623-1.html⟩
2) Moriyasu T., Shigeoka S., Kishimoto K., Ishikawa F., Nakajima J., Kamimura H., Yasuda I., *Yakugaku Zasshi*, **121**, 765—769 (2001).
3) Zhu X., Xiao S., Chen B., Zhang F., Yao S., Wan Z., Yang D., Han H., *J. Chromatogr. A*, **1066**, 89—95 (2005).
4) Hasegawa T., Saijo M., Ishii T., Nagata T., Haishima, Y., Kawahara N., Goda Y., *J. Food Hyg. Soc. Jpn.*, **49**, 311—315 (2008).
5) Jefferson D. R., Natarajan S., Ganesan A., *Synlett*, **2004**, 1428—1430 (2004).
6) Venhuis B. J., Barends D. M., Zwaagstra M. E., De Kaste D., "RIVM Report 370030001/2007," National Institute for Public Health and the Environment, the Netherlands, 2007.
7) Shin M. H., Hong M. K., Kim W. S., Lee Y. J., Jeoung Y. C., *Food Addit. Contam.*, **20**, 793—796 (2003).
8) Shin C., Hong M., Kim D., Lim Y., *Magn. Reson. Chem.*, **42**, 1060—1062 (2004).
9) Park H. J., Jeong H. K., Chang M. I., Im M. H., Jeong J. Y., Choi D. M., Park K., Hong M. K., Youm J., Han S. B., *Food Addit. Contam.*, **24**, 122—129 (2007).
10) Blok-Tip L., Zomer B., Bakker F., Hartog K. D., Hamzink M., ten Hove J., Vredenbregt M., de Kaste D., *Food Addit. Contam.*, **21**, 737—748 (2004).
11) Hou P., Zou P., Low M. Y., Chan E., Koh H. L., *Food Addit. Contam.*, **23**, 870—875 (2006).
12) Zou P., Hou P., Low M.-Y., Koh H. L., *Food Addit. Contam.*, **23**, 446—451 (2006).
13) Zou P., Hou P., Oh S. S., Low M. Y., Koh H. L., *Rapid Commun. Mass Spectrom.*, **20**, 3488—3490 (2006).
14) Gratz S. R., Flurer, C. L., Wolnik K. A., *J. Pharm. Biomed. Anal.*, **36**, 525—533 (2004).
15) Gratz S. R., Gamble B. M., Flurer R. A., *Rapid Commun. Mass Spectrom.*, **20**, 2317—2327 (2006).
16) Lai K. C., Liu Y. C., Tseng M. C., Lin J. H., *J. Food Drug Anal.*, **14**, 19—23 (2006).
17) Reepmeyer J. C., Woodruff J. T., *J. Chromatogr. A*, **1125**, 67—75 (2006).
18) Reepmeyer J. C., Woodruff J. T., d'Avignon D. A., *J. Pharm. Biomed. Anal.*, **43**, 1615—1621 (2007).
19) Reepmeyer J. C., Woodruff J. T., *J. Pharm. Biomed. Anal.*, **44**, 887—893 (2007).
20) Lam Y. H., Poon W. T., Lai C. K., Chan A.Y. W., Mak T. W. L., *J. Pharm. Biomed. Anal.*, **46**, 804—807 (2008).
21) Zou P., Hou P., Oh S. S., Chong Y. M., Bloodworth B. C., Low M. Y., Koh H. L., *J. Pharm. Biomed. Anal.*, **47**, 279—284 (2008).
22) Cho E. Y., Chung S. H., Kim J. H., Kim D. K., Jin C., *J. Appl. Pharmacol.*, **11**, 232—237 (2003).
23) Kim J. H., Kim Y., Choi K., Kim D. H., Nam G., Seo J. H., WO 2002102802 (2002).
24) Kumasaka K., Kawahara N., Doi K., Kojima T., Goda Y., *Chem. Pharm. Bull.*, **56**, 227—230 (2008).
25) Hosogai N., Hamada K., Tomita M., Nagashima A., Takahashi T., Sekizawa T., Mizutani T., Urano Y., Kuroda A., Sawada K., Ozaki T., Seki J., Goto T., *Eur. J. Pharm.*, **428**, 295—302 (2001).
26) Uchiyama N., Saisho K., Kikura-Hanajiri R., Haishima Y., Goda Y., *Chem. Pharm. Bull.*, **56**, 1331—1334 (2008).
27) Fujino K., Takami H., Atsumi T., Ogasa T., Mohri S., Kasai M., *Org. Process Res. Dev.*, **5**, 426—433 (2001).
28) Onoda Y., Nomoto Y., Ohno T., Yamada K., Ichimura M., WO9808848 (1998).
29) Hirose R., Okumura H., Yoshimatsu A., Irie J, Onoda Y., Nomoto Y., Takai H., Ohno T., Ichimura M., *Eur. J. Pharm.*, **431**, 17—24 (2001).
30) Toque F. H. A., Priviero F. B. M., Teixeira C. E., Perissutti E., Fiorino F., Severino B., Frecentese F., Lorenzetti R., Baracat J., Santagada V., Caliendo G., Antunes E., De Nucci G., *J. Med. Chem.*, **51**, 2807—2815 (2008).

—Reviews—

健康食品の安全性確保と基原の重要性

合田幸広

The Safety of Health Foods and Importance of Their Origin

Yukihiro GODA
National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

(Received February 18, 2008)

The safety guideline for voluntary inspections on the ingredients used for capsulated or pellet food, announced by the director of the department of food safety of the Ministry of Health, Labor and Welfare on February 1, 2008 states that "how to guarantee the origin" is the top priority to ensure safety. However, in the course of our continuous investigation of the origin of natural products, the ingredients of some health food products such as chondroitin sulfate, white kwao keur (*Pueraria candollei* var. *mirifica*) and black cohosh did not originate from the labeled material. The usage of the correct origin is the fist step for the quality assurance of "health food". Therefore, we believe that regulatory requirements for accurately indicating the origin of "health foods" and effective enforcement of these requirements are needed.

Key words——health food product; food safety; correct origin

平成17年2月1日に，厚生労働省医薬食品局食品安全部長通知（食安発第0201003号）として，「錠剤，カプセル状等食品の原材料の安全性に関する自主点検ガイドライン」が提出された．本通知は，食品の安全性は長い食経験を通じて担保されたものであるが，食品によっては，食経験のみによって安全性を担保できない場合もあり，また摂取形態が変わると過剰摂取の可能性があることを念頭に，錠剤，カプセル状等食品の原材料の安全性を点検するための自主点検フローとして示されたものである．この自主点検フローの上位ステップでは，基原材料の基原，使用部位及び原材料の製造方法等について保証する方法が明確であり，一定の品質(成分)が常に保証されていることが述べられている．一方，われわれの検討では，様々な健康食品で，表示と異なる基原のものが使用されていることが明らかとなってきた．

例えば，コンドロイチン硫酸では，入手した12の健康食品のうち9製品がサメ由来であることが予想される表示が行われていたが，成分である*N*-アセチルガラクトサミン誘導体を分析したところ，そのうちの2製品は，ほ乳類由来と考えられる*N*-アセチルガラクトサミン誘導体を大量に含み，表示と基原が異なっていることが確認された.[1] また，プエラリア（white kwao keur）含有を標榜する17製品について同植物に特徴的な二次代謝物質であるdeoxymiroestrol, miroestrol, isomiroestrol及びkwakhurin及びイソフラボン類の分析を行ったところ，8製品でこれら4成分が検出されたが残りの9製品からは，puerarinやdaidzein等のイソフラボン類は検出されたものの同植物に特徴的な4成分は検出されなかった．さらに，粉末及びカプセル状の13製品（特徴的成分が検出されたもの6製品）についてgenomic DNAを抽出し遺伝子解析を行ったところ，特徴成分が検出された製品では，基原植物である*Pueraria candollei* var. *mirifica*の配列が検出されたが，それ以外の製品では，7製品では，同植物の配列は全く検出されず，*Medicago sativa*（ムラサキウマゴヤシ＝アルファルファ），*Glycyrrhiza glabra*（スペインカンゾウ），*Ipomoea batatas*（サツマイモ），*Triticum aestirum*（パンコムギ），

国立医薬品食品衛生研究所（〒158-8501 東京都世田谷区上用賀1-18-1）
e-mail: goda@nihs.go.jp
本総説は，日本薬学会第127年会シンポジウムS9で発表したものを中心に記述したものである．

Pachyrhizus erosus（クズイモ），*Nelumbo ncifera*（ハス）等の配列が認められ，他の植物が原材料であることが明らかとなった.[2] さらにまた，ブラックコホッシュ（*Cimicifuga recemosa*）では，入手した6製品のうちLC-ELSD分析等により特徴的な二次代謝成分actein が確認されたものは3製品のみであり，賦形剤のみが検出されたものが2製品，残り1製品は基原の異なる植物が使用された可能性が高いことが判った.[3]

このように，いわゆる健康食品においては，正しい基原の原材料が用いられず，表示と基原が異なったものが商品として流通している場合が多いことが確認された．今回紹介した製品の場合，これが意図的なものなのか，原材料を入手した段階から，原材料の基原を確認しないまま，加工し製品として販売したものかは不明である．野菜や生薬の場合，五感や形態等で見分けることも消費者に知識があれば可能となる．一方，粉末や錠剤，カプセル等に加工された製品の場合，消費者のレベルでは原材料について判断する手掛かりがなくなり，その点をついて違法な販売を行っているものと考えられる．

医薬品である生薬の場合，品質確保の第一歩は，基原の正しい植物を使用することであり，基原は，適否の判断基準であることが日本薬局方の生薬総則で明示されている．生薬の場合，日本薬局方や局方外生薬規格において，学名で基原が規定されるとともに，公的な植物和名が同時に記載されるため，原料植物に対する混乱が起こり難い．また，古来より日本で生育してきた植物には，標準和名があり，和名でその植物をある程度規定できる場合が多い．他方，健康食品の場合，原材料について学名で規定するというルールがないだけでなく，外来植物や，外国産の植物が原材料になっている場合が多い．したがって，表示に用いられる植物名は，原材料の導入者が，その都度販売目的で正当な根拠なく付ける場合がみられる．また植物和名と生薬名も，混同し易く，健康食品の表示では，よく混乱が生じている．例えば，植物和名エゾウコギ（*Eleutherococcus senticosus*＝*Acanthopanax senticosus*）には，シゴカ（刺五加）という生薬名があり，局方で規定されているが，健康食品販売での通称名はシベリアニンジンと呼ばれる場合が多い．また，植物和名ウコン（*Curucuma longa*）は，生薬名もウコンであり，これらは局方で規定されているが，健康食品では，別植物である所謂ハルウコン（*C. aromatica*）と区別するためアキウコンやクスリウコンと呼称する場合が多い．一方，*C. aromatica* の植物和名はキョウオウ（姜黄）であるが，中国では，姜黄（Jianghuang）は，*C. longa* となる．このような名称の混乱も，表示と中身が一致しない原因の1つであるものと考えられる．

天然物の品質確保の第一歩は，基原の正しい原材料を使用することである．含量規格や不純物規格に合っていても，原材料の基原が間違ったものを使用していれば，品質が確保されたとはいえない．この考えは，対象が健康食品であったとしても，当然守られるべき原則であろう．食品分野においても，法で規制されている特定保健用食品の場合は，審査の際に基原や製造方法まで確認されるため，間違った基原のものが使用される可能性はほとんどない．他方，いわゆる健康食品では，基原の表示について法的な規制が行われていないため，この原則が守られていない．健康食品の品質と安全性確保のためには，まず，正しい基原と部位の原材料を常に使用することが重要であり，われわれは，この点に関して，なんらかのルール作りの必要性があると考える．

謝辞　本報告で述べた研究結果は，厚生労働省の食品等検査費及び厚生労働科学研究費補助金により行われたものであり，ここに深謝します．

REFERENCES

1) Sakai S., Otake R., Toida T., Goda Y., *Chem. Pharm. Bull.*, **55**, 299–303 (2007).
2) Goda Y., *Farumashia*, **42**, 905–907 (2006).
3) Ohnuma M., Kobayashi S., Hakamatsuka T., Goda Y., Abstracts of papers, the 13th Annual Meeting of Japanese Society of Food Chemistry, Tokyo, May 2007, p. 44.

合田幸広

東京大学大学院薬学系研究科博士課程昭和60年修了，昭和61年国立衛生試験所食品添加物部研究員，平成4年同食品添加物部主任研究官，平成8年同食品部第3室室長，平成13年国立医薬品食品衛生研究所生薬部長，現在の研究テーマ：生薬，漢方処方，食薬区分，法規制薬物等に関するレギュラトリーサイエンス

性機能改善薬との関連事例

合田 幸広　鎌倉 浩之
国立医薬品食品衛生研究所

いわゆる健康食品から検出される性機能改善薬とその類縁体について，薬事法上の取り扱い，検出時期，構造，検出される含量等について概説した。さらに，ホスホジエステラーゼ（PDE）阻害剤である点を念頭に，これらの化合物が健康食品に含まれることによって予想される有害事象について解説を行った。最も危険な有害事象は，PDE6の非選択性阻害であり，最悪の場合，使用者が失明する可能性がある。したがって，このような健康食品については，積極的な監視，取り締まりが必要と考えられる。

キーワード　性機能改善薬，ED治療薬，性機能改善薬構造類似体，ED治療薬構造類似体，専ら医薬品成分，PDE5阻害薬，健康被害，危険性

1 性機能改善薬とは

性機能改善薬（ED治療薬）として知られている薬物には，Viagra®（sildenafil: シルデナフィル），Cialis®（tadalafil: タダラフィル），Levitra®（vardenafil: バルデナフィル）がある．日本では，シルデナフィルはクエン酸シルデナフィル，バルデナフィルは塩酸バルデナフィル水和物の形で処方せん医薬品として認可されている。さらに昨年（2007年）タダラフィルも処方せん医薬品として認可され，3化合物とも薬事法上「専ら医薬品成分」として扱われる．

これらの医薬品の活性ターゲットは，ホスホジエステラーゼ（PDE）5であり，本酵素を阻害することで，陰茎の海綿体の平滑筋の弛緩を引き起こし，勃起不全を改善する。シルデナフィルと比較して，タダラフィルはより長い作用持続時間が特長である。また，バルデナフィルは10倍以上酵素阻害活性が強く，より少ない投与量で活性がある[1]。

2 日本国内での健康食品からの検出[2]

これらの成分のうち特にシルデナフィルは，2003年より，日本で何らかの形で強壮を標榜・暗示する健康食品から検出されるようになり，2004年になってさらにタダラフィルが，2005年になってバルデナフィルが検出されるようになった。また，2003年

1882-3971/08/￥500/論文/JCLS

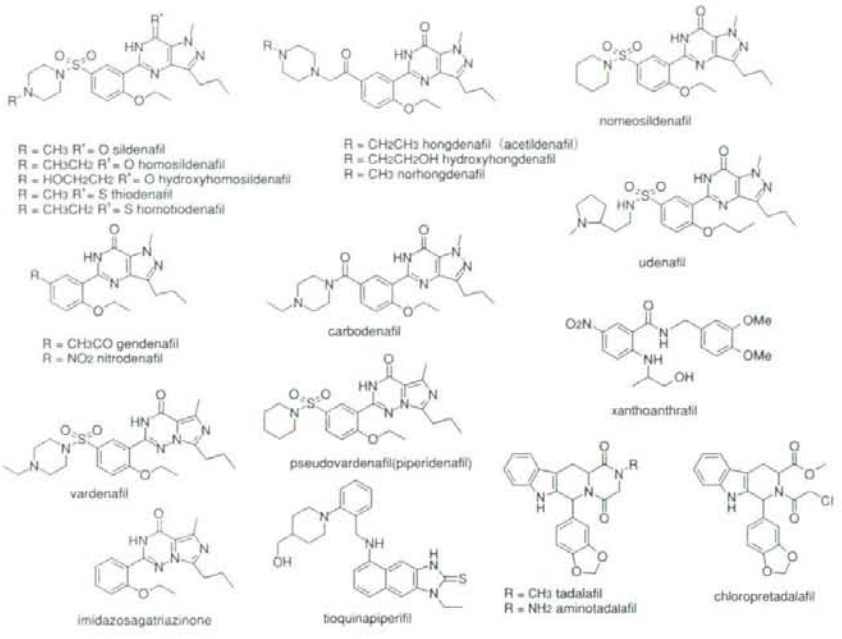


図1 ● 強壮を標榜・暗示する健康食品から検出・同定された化合物（合成品）

の段階で，すでにホモシルデナフィルといったこれらの医薬品成分の構造類似体が検出されている。さらに，2004年には新規化合物であったヒドロキシホモシルデナフィル，最初に韓国で発見されたホンデナフィル（アセチルデナフィル），2005年にはシルデナフィルの構造の部分構造を欠損させたゲンデナフィル，バルデナフィルの部分構造を欠損させたイミダゾサガトリアジノン，2006年にはタダラフィルの合成前駆体であるクロロプレタダラフィル，2007年には旧藤沢薬品が論文発表した化合物であるキサントアントラフィル（医薬品としては未承認），2008年には，協和発酵が論文発表した化合物であるチオキナピペリフィル（医薬品としては未承認）等，様々なタイプの化合物が検出されている。

これらの化合物はすべて，PDE5阻害活性を持つか，あるいはPDE5の活性部位との結合ミュレーションにより，同阻害活性を持つことが予測されており，すべて「専ら医薬品成分」に指定されている。これまで無承認無許可医薬品成分として，強壮を標榜・暗示する健康食品から検出・同定された化合物の構造を図1に示す。

3. 健康食品中の含量

いわゆる強壮/性機能改善を標榜・暗示する健康食品の場合，何らかの健康被害が出ても，羞恥心から被害者が公に届けることはほとんどないものと考えられている。

表1 ● ED治療薬および類似成分のヒト血小板由来PDE5阻害活性と健康食品中の含量

化合物	IC50値(nM)	カプセル・錠剤中での含量(mg)
aminotadalafil(アミノタダラフィル)	18.0	200
pseudovardenafil(プソイドバルデナフィル)	0.5	20
imidazosagatriazinone(イミダゾサガトリアジノン)	4.5	100
gendenafil(ゲンデナフィル)	15.0	200
sildenafil(シルデナフィル)	3.5-4.3*	25-100
vardenafil(バルデナフィル)	0.091-0.7*	2.5-20
tadalafil(タダラフィル)	0.9-1.8*	5-20
xanthoanthrafil(キサントアントラフィル)	1.1*	30

*文献値

したがって，日本で具体的な健康被害が報告されているものは，筆者の知る限りない。通常，これらの健康食品は，カプセルや錠剤のタイプで販売されている。したがって，1カプセル／錠あたり，賦形剤も含めておよそ250mg程度の化合物が入っていることになる。検出された化合物のヒト血小板由来のPDE5阻害活性とカプセル内の含量についてまとめたものを表1に示す。

このように，酵素活性が強いものの場合は数～30mg程度，酵素活性が弱いものでは100～200mg程度と，ある程度その活性を考慮した量がカプセル内に含まれているため，勃起不全（ED; penile erectile dysfunction）について改善が見られた場合には，購入者はそれなりの満足を得ている可能性もある。しかし，もし医薬品として認可された成分が入っていたとしても，医師や薬剤師のコントロール外で用いられているため，使用禁忌等が考慮されず，問題が生じている可能性が高い。

4. 医薬品としての禁忌

ED治療薬で最も重要な禁忌は，硝酸剤およびNO供与剤（ニトログリセリン，亜硝酸アミル，硝酸イソソルビド等）との併用で，その場合，降圧作用が増強し，過度に血圧が低下することになる[3]。実際，いわゆるラブホテルで，亜硝酸系の化合物を含む違法薬物と，強壮を標榜する健康食品を併用したため，過度の血圧降下が起こり，救急病院に搬送された事例がある。また，これらの性機能改善薬は，死亡例を含む心筋梗塞等の重篤な心血管系の有害事象が報告されている。

したがって，重度の肝機能障害のある者，低血圧の者，治療による管理がなされていない高血圧の者，脳梗塞・脳出血や心筋梗塞の既往歴が最近6カ月以内にある者には，使用が禁忌となっている[3]。さらに，網膜色素変性症の者は，PDEの遺伝障害を有する可能性があり禁忌となっている[3]。また，バルデナフィルの場合，特にQT延長（心電図上でQT時間の延長が起こる）が特有の副作用として報告されており，先天性のQT延長者に対しての投与や，延長作用のある薬剤（抗不整脈薬）との併用は禁忌である[3]。さらに，シルデナフィルと比較してより作用が強いため，各種P450の阻害活性を持つ薬物との併用，血管拡張作用を示すα遮断薬との併用も禁忌となっている[3]。

5. PDE5選択阻害の意味

PDEは，セカンドメッセンジャーである

環状ヌクレオチド（cAMPおよびcGMP）を加水分解する酵素で，その構造，酵素学的性質等から11種類のファミリーに分類される。さらにアイソザイムを含めるとPDEファミリーは21遺伝子から構成される[1]。それぞれのPDEは生体の様々な部位や組織で特異的に発現し，特定の蛋白質と相互作用することが報告されている。したがって，シルデナフィル，バルデナフィル，タダラフィルは，ある程度PDE5に選択阻害活性を持つことで医薬品化されたといえる。

しかし，網膜に分布するPDE6に対しても数分の1程度の阻害活性を示すシルデナフィルの場合には，副作用として色覚異常が報告されている[3]。

6. 構造類似体の危険性

医薬品として承認された化合物の構造類似体の場合，最も危険と考えられる副作用は，PDEの非特異的な酵素阻害による事象である。シルデナフィルのスルホニル基がアセチル基に置換されたホンデナフィルの場合，PDE6に対してもPDE5と同様に阻害することが報告されており[4]，使用者にシルデナフィルより強い色覚障害が生じる可能性が高く，悪くすれば失明することも考えられる。また，アミノタダラフィルの場合，メチルアミンの代わりに，ヒドラジンが使用されて化合物が合成された結果，反応性が高いため様々なPDEと反応し，不可逆的な酵素阻害を起こす可能性が高い。したがって予想外の作用が生じている可能性がある[4]。

さらに，プソイドバルデナフィル（ピペリドバルデナフィル/ピペリデナフィル）の場合，その末端構造からバルデナフィルと比較して代謝されにくいことが予想される。したがって血液中の濃度が減衰されにくく，さらにより脂溶性が高いことから，脳血液関門を通過しやすいものと推定され，神経系にもダメージを与える可能性が示唆されている[4]。

キサントアントラフィル[5]やチオキナピペリフィル[6]の場合，医薬品としての開発が何らかの障害があって中止された化合物であるものと推定される。公表されている論文からは，その原因は明らかではないが，PDEが生体の様々な部位で重要な役割を果たしていること，未承認のPDE合成阻害剤を含む健康食品を食することは非常にリスクの高い行為であると考えられる。

7. その他の危険性

ある程度安全性が確認された化合物で有害事象が起こる場合，その原因が不純物である場合が多い。L-トリプトファン含有健康食品の場合はその典型的な例と考えられるが，シルデナフィル等，承認された医薬品成分を含む健康食品でも，同様の危険性は十分に考えられる。事実，強壮を標榜・暗示する健康食品を分析すると，マイナー化合物として合成中間体が検出される場合がある。また，ヨヒンビン等を含む天然成分との混合物である場合もあり，成分同士の相互作用による危険性も十分に予想される。

さらに，1カプセル／錠ごとの含量が正確ではなく，ときに活性成分が全く含まれていないカプセル／錠がある場合もあり，使用者が正確に量のコントロールをできないことも危険度を高めている。また，製品名が同じであっても，含まれている化合物が異なることはよくある事例である。

おわりに

強壮を標榜・暗示する健康食品は，使用

者がその使用を隠す場合が多いため，健康被害の実態はほとんど明らかになっていない。しかし，その使用には上記に述べたような様々な危険性があることが予想されている。特に，PDE6阻害による失明の可能性は，非常に重篤な有害事象と考えられ，今後も，積極的な監視，取締りが必要であるものと考える。

プロフィール

合田 幸広（ごうだ ゆきひろ）
1980年東京大学薬学部卒業，1985年同大学大学院薬学系研究科博士課程修了（薬学博士），1986年国立衛生試験所食品添加物部研究員，1992年食品添加物部主任研究官，1996年食品部第三室長，2001年国立医薬品食品衛生研究所生薬部長，現在に至る。専門：生薬，漢方処方，食薬区分，法規制薬物，違法ドラッグ，無承認無許可医薬品に関するレギュラトリーサイエンス。

■文献

1) 小寺淳ほか: 環状ヌクレオチドホスホジエステラーゼ研究の最近の進展. 日薬理誌126: 121-127, 2006
2) 医薬品成分（シルデナフィル及び類似成分）が検出されたいわゆる健康食品について（厚生労働省）http://www.mhlw.go.jp/kinkyu/diet/other/050623-1.html
3) 日本医薬品集フォーラム: 日本医薬品集医療薬2008年版, じほう, 東京
4) Venhuis BJ et al: Recent development in counterfeits and imitations of Viagra, Cialis and Levitra. RIVM Report 370030001, 2007
5) Kumasaka K et al: Determination of (R)-xanthoanthrafil, a phosphodiesterase-5 inhibitor, in a dietary supplement promoted for sexual enhancement. Chem Pharm Bull 56: 227-230, 2008
6) Uchiyama N et al: Determination of a new type of phosphodiesterase-5 inhibitor, thioquinapiperifil, in a dietary supplement promoted for sexual enhancement. Chem Pharm Bull 56(9), in press