Determination of (*R*)-Xanthoanthrafil, a Phosphodiesterase-5 Inhibitor, in a Dietary Supplement Promoted for Sexual Enhancement

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We describe here the first case of the finding of xanthoanthrafil, a phosphodiesterase-5 inhibitor, in a dietary supplement. A methanol extract of the supplement product was first analyzed by TLC and HPLC. The results indicated that the extract contained an unknown compound. The molecular weight of the compound was 389 and the accurate mass showed its elemental composition to be $C_{19}H_{23}N_3O_6$. Combined with this data, NMR analysis revealed the planar structure of the unknown compound to be *N*-(3,4-dimethoxybenzyl)-2-(1-hydroxypropan-2-ylamino)-5-nitrobenzamide. The *R*-configuration of this compound had been synthesized as a phosphodiesterase-5 inhibitor, formerly reported as FR226807 by Fujisawa Pharmaceutical Co., Ltd. The absolute configuration of the isolated compound was estimated to have *R*-configuration by its optical rotation. Considering its general properties, this compound is renamed as (*R*)-xanthoanthrafil with the agreement of Astellas Pharma Inc. which is the successor of Fujisawa Pharmaceutical Co., Ltd. Quantitative analysis revealed that the content of (*R*)-xanthoanthrafil in the product was about 31 mg/capsule.

Key words xanthoanthrafil; TLC; HPLC; liquid chromatography-mass spectrometry; NMR; penile erectile dysfunction

Many kinds of dietary supplements are sold over the internet. Some of these products are unlawfully advertised as effective for weight loss, sexual enhancement, and diabetes. Over the past few years, it has been reported that undeclared active drug ingredients have been detected in some dietary supplements.[1—4)] These products are considered as unapproved pharmaceutical products and are regulated by the Pharmaceutical Affairs Law of Japan. Moreover, undeclared active drug ingredients in these products may have serious side effects. Fenfluramine, *N*-nitroso-fenfluramine, and sibutramine have been detected in dietary supplements for weight loss, which may affect the health of consumers. Therefore, examination of these so-called dietary supplements is an important step in preventing the serious consequences caused by undeclared active drug ingredients in these products.

Sildenafil, vardenafil, and tadalafil, known as active drug ingredients for the treatment of penile erectile dysfunction (ED), have also been detected in dietary supplements for sexual enhancement for men. Recently, some ingredients that have similar or modified structures to known active drug ingredients have been detected, especially in dietary supplements intended for sexual enhancement. Shin *et al.* showed that a new compound found in a functional food was structurally similar to sildenafil and named it homosildenafil.[5)] Similar kinds of analogues were detected in Korea,[6,7)] the Netherlands,[8)] Singapore,[9)] the U.S.A.,[10)] and Japan.[4)] These analogues were deduced to be phosphodiesterase-5 inhibitors because of their structural resemblance to well known active ingredients used for ED therapy.

Here we report the first case of the finding of a phosphodiesterase-5 inhibitor, which is not known as an active drug ingredient, in a supplement product that is illegally marketed on websites for enhancing the sexual performance of men. This compound was finally identified as FR226807, which was synthesized as a new drug for ED by Fujisawa Pharmaceutical Co., Ltd. (currently Astellas Pharma Inc., Tokyo, Japan), but reported as a phosphodiesterase-5 inhibitor by Hosogai *et al.*[11)] To the best of our knowledge, any of the illegal compounds found in sexual enhancing supplement products are known as active drug ingredients or their analogues; however, this compound is not an analogue of approved drug ingredients and has a new type of structure among illegal compounds for ED.

Experimental

Chemicals and Reagents The standard of FR226807 was kindly provided by Astellas Pharma Inc. HPLC-grade acetonitrile and all other chemicals (analytical grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Sample The examined product was composed of four pieces of white capsules, which contained 0.23 g of yellowish powder. The words "Power capsule" were marked on the package of the product, suggesting the effect it may have.

Preparation of Sample Solution The contents of a single capsule were extracted in 50 ml of methanol with a reciprocating shaker. Then, 40 ml of the extracted solution was evaporated to dryness and reconstituted with 2 ml methanol to prepare the TLC sample solution. A portion of the extracted solution was diluted 5-fold with methanol for HPLC analysis and 200-fold for liquid chromatography-mass spectrometry analysis and filtered using a 0.45-μm syringe filter.

TLC Analysis An HPTLC silica gel 60 F_{254} plate (thickness, 0.25 mm; Art. 5642, Merck, Tokyo, Japan) was used. One microliter of the TLC sample solution mentioned above was spotted. The plate was equilibrated with a mixture of ethyl acetate, methanol, and 28% ammonia solution (9 : 1 : 0.1) in a sealed chamber for 30 min. Then, the plates were developed to a distance of about 60 mm. After air-drying, the plates were examined under fluorescent light.

HPLC Analysis A reversed-phase HPLC system consisting of a Waters Alliance 2695 separation module equipped with a photodiode array (PDA) detector model 2996 (Nihon Waters K.K., Tokyo, Japan) was used. The sample solution was separated using a Unison US-C18 column (150×4.6 mm, 5 μm particle size; Imtakt Corporation, Kyoto, Japan) coupled to a TCI Opti-Guard™ Fit ODS guard column (15×1 mm, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The column was kept at 40 °C during the run. The mobile phase consisted of acetonitrile (eluent A) and 0.02 mol/l ammonium acetate buffer (pH 4.0 with acetic acid, eluent B). The gradient elution was started at 20% eluent A held for 2 min, linearly increased to 30% A in 5 min, to 50% A in 20 min. The flow rate was set at 1.0 ml/min. Injection volume was 2 μl. The wavelength of the PDA detector for screening was set from UV 210 to 600 nm, and monitoring of chromatographic peaks was per-

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formed at UV 254 nm. Data storage and processing were performed using Empower software (Nihon Waters K.K.).

Liquid Chromatography-Mass Spectrometry Analysis The sample solution and the yellowish spot extract detected by TLC analysis were analyzed with a liquid chromatography-hybrid triple quadrupole linear ion trap mass spectrometer (Qtrap® LC/MS/MS system; Applied Biosystems Japan Ltd., Tokyo, Japan) consisting of an Agilent 1100 series binary pump, an autosampler with sample cooler, and a column oven (Agilent Technologies Japan Ltd., Tokyo, Japan), equipped with the mass spectrometer. The solutions were separated using a Cadenza CD-C18 column (150×2.0 mm, 3 μm particle size; Imtakt Corporation) coupled to the guard column kept at 40 °C. The mobile phase consisted of 0.01 mol/l ammonium acetate buffer (pH 4.0 with acetic acid, eluent A) and acetonitrile (eluent B). Gradient elution was started at 30% eluent B held for 2 min, linearly increased to 60% B in 10 min, and then held for 13 min. The flow rate was set at 0.2 ml/min. Injection volume was 5 μl. Electrospray ionization (ESI) on both positive and negative modes was used for the analysis. The temperature of the turbo ion spray and ion spray voltages for positive and negative modes on ESI were set at 450 °C, 5000 V, and −4000 V, respectively. We used enhanced mass spectrum (EMS) scan as the scan mode. The declustering potential, collision energy, and the spread were 30 V, 30 V, and 0 V for the positive scan and −30 V, −30 V, and 15 V for the negative scan, respectively. Data storage and processing were performed with Analyst software (Applied Biosystems Japan Ltd.).

Measurement of Accurate Mass Accurate mass analysis was performed by direct inlet-electron ionization (DI-EI) using a magnetic sector-type mass spectrometer (GCmate II; JEOL Ltd., Tokyo, Japan). Perfluorokerosene was used as the mass marker. The TLC sample solution mentioned above was used. The temperature of the ion chamber was set at 100 °C and increased linearly to 250 °C. The measured mass was used for the calculation of the element components. The following restrictions were applied: mass error limit 3 milli atomic mass units, element options C, H, $N_{0—7}$, and $O_{0—8}$. In addition, the nitrogen rule was applied.

NMR Analysis ^{1}H- and ^{13}C-NMR analyses were performed. The TLC sample solution was purified by HPLC as follows: A Cadenza CD-C18 column (150×10 mm, 3 μm particle size; Imtakt Corporation) was used for separation by isocratic flow with a mixture of 0.01 mol/l ammonium acetate buffer (pH 4.0) and acetonitrile (50 : 50, v/v). The collected fraction was diluted with ultrapure water prior to application to an Oasis HLB cartridge (Nihon Waters K.K.) for desalting and purification. The methanol elute was dried with a gentle nitrogen stream and resuspended in $CDCl_3$. ^{1}H- and ^{13}C-NMR spectra were recorded on an ECA-800 spectrometer (JEOL Ltd.).

Determination of Optical Rotation The optical rotation of extracted xanthoanthrafil and FR226807 were measured using a P-1020 polarimeter (JASCO Corporation, Tokyo, Japan) with a glass cell 100 mm in length and with the D line of sodium as the light source. For the test solution, 14 mg of extracted xanthoanthrafil, which had been purified by recrystallization, was dissolved in 1.4 ml of chloroform, making a 1% solution. Then, 15 mg of FR226807 was dissolved in 1.5 ml chloroform to obtain the control solution for optical rotation.

Determination of Xanthoanthrafil The 30 mg capsule content was shaken for 30 min with 30 ml methanol three times. All extracted solutions were combined into a 100 ml volumetric flask and adjusted with methanol exactly as the test solution. Three independent replicates were prepared. One milligram of FR226807 standard was solved in methanol and diluted to prepare a 0.04 mg/ml standard solution. Both solutions were analyzed with a reversed-phase HPLC system. An isocratic flow of a buffer mixture of acetonitrile and 0.02 mol/l ammonium acetate (pH 4.0) (35 : 65, v/v) was used as the mobile phase. Injection volume was 10 μl. The wavelength of the PDA detector was set at UV 390 nm. Other conditions of HPLC analysis are described in the section HPLC analysis.

Results and Discussion

In this study, we reported for the first time, the identification of xanthoanthrafil from an illegal dietary supplement. We found that this compound has a novel structure, which is not usually observed in anti-ED drugs.

TLC and HPLC analyses, which were adopted as the screening methods, led to the identification of xanthoanthrafil. In fact, a characteristic yellowish spot was detected by TLC analysis from the sample solution. The retention factor (R_f) of the spot was 0.63. In addition, only one unknown peak was detected at 18.4 min from the sample solution by the HPLC analysis (Fig. 1a). The PDA-sliced UV spectrum of the peak exhibited a maximum at 390—392 nm and a minimum at 296—298 nm (Fig. 1b). The maximum absorption was satisfactory in accordance with the yellowish spot detected by TLC analysis. These characteristics were completely different from those of known phosphodiesterase-5 inhibitors, such as sildenafil, vardenafil, and tadalafil, which have been detected in some kinds of dietary supplement.[2,5—10,12] Therefore, it was possible that the ingredient was an unknown compound not found hitherto in dietary supplements.

An unknown compound in the sample solution was detected at 14.0 min in both positive and negative EMS scans by the Qtrap® LC/MS/MS analysis. In the positive EMS scan, major ion peaks at *m/z* 428 and 151 were detected in the spectrum of the peak (Fig. 2a). Further, a major ion peak at *m/z* 388 was detected in the negative EMS scan (Fig. 2b). These results suggested that the molecular-related ions of the

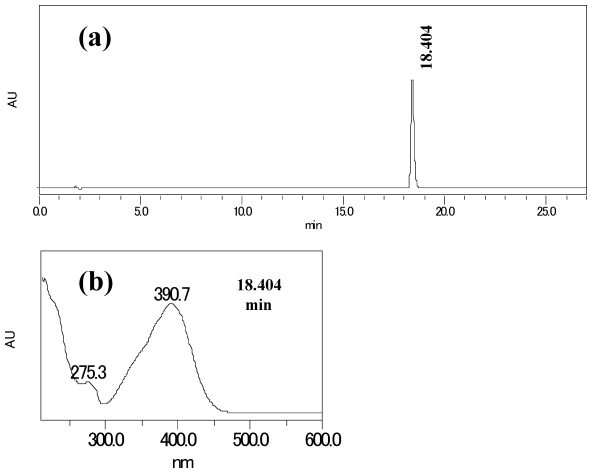

Fig. 1. HPLC Chromatogram of the Sample Solution and the UV Spectrum of the Detected Peak

Representative chromatogram of the sample solution monitored at UV 254 nm (a). The UV spectrum is obtained by PDA slicing from the detected peaks of the sample solution (b).

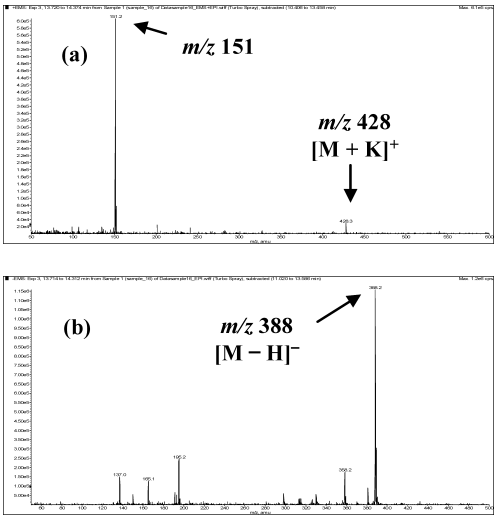

Fig. 2. Mass Spectra of the Detected Peak by Qtrap LC/MS/MS Analysis

Positive (a) and negative (b) EMS scans.

compound were $[M+K]^+$ at *m/z* 428 and $[M-H]^-$ at *m/z* 388, indicating that the molecular weight was 389. In addition, a methanol extract of the yellowish spot mentioned in the TLC analysis showed the same results, closely coincident with those of the sample solution.

The accurate mass of the $M^{+\cdot}$ ion was *m/z* 389.1594, giving estimated elemental compositions of $C_{20}H_{19}N_7O_2$, $C_{22}H_{21}N_4O_3$, $C_{17}H_{21}N_6O_5$, $C_{19}H_{23}N_3O_6$, and $C_{21}H_{25}O_7$. Only two of these elemental compositions, $C_{20}H_{19}N_7O_2$ and $C_{19}H_{23}N_3O_6$, complied with the limitation rules.

The planar structure of the unknown compound was finally identified by ^{1}H- and ^{13}C-NMR analyses including various 2D NMR techniques. The ^{1}H-NMR spectrum of the compound exhibited 20 non-exchangeable protons, including two methoxyl signals at δ 3.89 (3H, s) and δ 3.90 (3H, s), one secondary methyl signal at δ 1.31 (3H, d, J=6.9 Hz), and two sets of ABX-type aromatic proton signals at δ 6.77 (d, J=9.6 Hz), 8.15 (dd, J=2.3, 9.6 Hz), 8.31 (d, J=2.3 Hz), and δ 6.87 (d, J=7.8 Hz), 6.89 (d, J=2.3 Hz), 6.91 (dd, J=2.3, 7.8 Hz). The ^{1}H-NMR spectrum also exhibited two characteristic signals assignable to amine protons at δ 6.48 (br s) and 8.90 (br d, J=7.7 Hz). The ^{13}C-NMR spectrum of the compound showed 19 carbon signals, including two methoxyl carbons, one methyl, two methylenes with one oxygenated carbon (δ 66.2), seven methines, and one carbonyl function (δ 167.8). Interpretation of the ^{1}H–^{1}H COSY and HMQC spectra of the compound suggested the presence of four partial structures (Fig. 3A—D), in addition to the two methoxyl groups and one carbonyl group. The connectivity of these partial structures was deduced from the HMBC spectrum (Fig. 3). These data were in agreement with the planar structure of the unknown compound as *N*-(3,4-dimethoxybenzyl)-2-(1-hydroxypropan-2-ylamino)-5-nitrobenzamide, shown in Fig. 4. The assignments of the ^{1}H- and ^{13}C-NMR signals are summarized in Table 1. The elemental composition, $C_{19}H_{23}N_3O_6$, corresponded to the result of the accurate mass analysis. The *R*-configuration of the deduced structure is coincident with that of FR226807, which has already been reported as a selective phosphodiesterase-5 inhibitor.[11] The ^{1}H- and ^{13}C-NMR data of FR226807 were superimposable on it. To identify the absolute configuration, the optical rotation of the ingredient was measured, since FR226807 has one chiral carbon at the C8 position in the molecule. The control solution of FR226807 showed that the angular rotation was +0.0962° (CV, 0.3%), indicating $[\alpha]_D^{20}$ +9.62° for the weak specific rotation. The angular rotation of the solution of the isolated compound showed +0.0974° (CV 1.7%), indicating robust similarity to that of the control. Based on this result, we deduced that the absolute configuration of the isolated compound was the *R*-configuration, which is identical to that of FR226807. Considering its general properties, this compound is renamed as (*R*)-xanthoanthrafil with the agreement of Astellas Pharma Inc., because this compound is a yellowish anthranilic derivative. This is the first case in which xanthoanthrafil has been detected from a so-called dietary supplement.

Finally, quantitative analysis of (*R*)-xanthoanthrafil in the supplement product was determined by an HPLC method. The retention time of this compound was 10.8 min. The content of this compound in the capsule was 31 mg. Since the product packaging gives the dosage as two capsules, about 62 mg of (*R*)-xanthoanthrafil would be taken in a single dose.

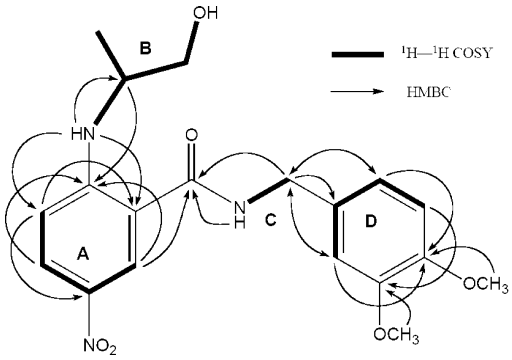

Fig. 3. ^{1}H–^{1}H and Major Long-Range ^{1}H–^{13}C Correlations of the Unknown Compound

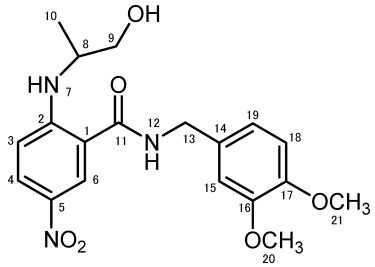

Fig. 4. Structure of Xanthoanthrafil

Table 1. ^{1}H- and ^{13}C-NMR Chemical Shifts of the Unknown Compound

Position	^{1}H[a)]	^{13}C[b)]
1		113.2
2		153.9
3	6.77 d (9.6)	111.2
4	8.15 dd (2.3, 9.6)	128.8
5		135.4
6	8.31 d (2.3)	124.7
7	8.90 br d (7.7)	
8	3.84 m	50.0
9 (a)	3.69 br dd (5.5, 10.6)	66.2
(b)	3.78 br d (10.6)	
10 (3H)	1.31 d (6.9)	17.1
11		167.8
12	6.48 br s	
13 (2H)	4.53 m	43.9
14		129.8
15	6.89 d (2.3)	111.3
16		149.3
17		148.8
18	6.87 d (7.8)	111.4
19	6.91 dd (2.3, 7.8)	120.4
20 (3H)	3.90 s	56.0
21 (3H)	3.89 s	56.0

a) *J* values (in Hz) in parentheses. *b*) Assigments were made from the HMQC spectrum.

Although xanthoanthrafil has not been classified as an active drug ingredient in Japan or abroad, the compound is now classified as a raw material that is exclusively used in pharmaceuticals in Japan, considering its activity with regard to a phosphodiesterase-5 inhibitor and its purpose to the synthesis. Therefore, whenever it is found in any dietary supplement, the supplement product is regarded as contravening the Pharmaceutical Affairs Law of Japan. Any illegal dietary supplement containing any drug substance or new illegal

compound potentially poses life-threatening health risks for consumers, because no study regarding its safety have been conducted yet. Therefore, we have to continuously monitor such compounds in dietary supplements. In addition, we must not only enlighten manufacturers about the safety of their products but also educate people who consume these products about the risk to their health.

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