A. graveolens, are known to contain 3-*n*-butylphthalide derivatives with their characteristic fragrance [11], and these distinctive compounds were not observed in our isolation study of the Indian celery.

In India, various celery-related products are used as folk medicines and/or spices: ajmud (*A. graveolens*), ajwain (*Trachyspermum ammi*), Radhuni (*Carum roxburghianum*) and wild celery (*A. leptophyllum*). The contradicting constituents of celery seeds reported by the Indian [2–10] and the Japanese researchers [11] are possibly due to the indiscriminative handling of celery and celery-related seeds in Indian (and also Pakistani) crude drugs/spices markets.

In order to solve this anomaly, we investigated the botanical origin of the Indian celery by using the analysis of the internal transcribed spacer (ITS) sequence of nuclear rDNA. Furthermore, we collected the seed samples sold as 'celery seeds' from local markets in Pakistan and related countries and classified the source plants of the procured samples based on their ITS genotypes, morphological features and chemical compositions to rationalize their regional characteristics regarding the local names and the botanical source in Pakistan.

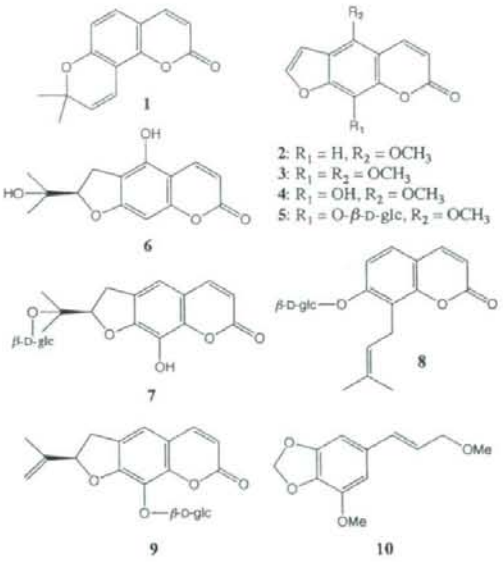

Fig. 1 Structures of the constituents isolated from Indian celery seed: seselin (**1**), bergapten (**2**), isopimpinellin (**3**), 8-hydroxy-5-methoxypsoralene (**4**), 5-methoxy-8-*O*-β-D-glucosyloxypsoralene (**5**), celereoin (**6**), isorutarin (**7**), vellein (**8**), apiumetin glucoside (**9**), anthriscinol methyl ether (**10**)

Materials and methods

Materials

The seeds of the authentic celery-related plants were generously supplied by each university or herbarium (Table 1). The seeds of *A. graveolens* used in this study were the same as those studied by the researchers at Showa Pharmaceutical University in their phytochemical studies [11], and they were originally imported from India by Asaoka Spice Co., Ltd. The Indian celery samples were purchased from local markets in Pakistan and related countries. The sample details are summarized in Table 2 together with their ITS genotypes. For the morphological observation and TLC analysis, Ce-27, Ce-30 and Ce-33 were used as the representatives of the major genotypes in the Pakistani markets.

ITS sequence analysis

Each seed sample (20 mg) was crushed in a mixer mill, MM-300 (Qiagen, Germany), under liquid N_2. Genomic DNA was extracted from the powdered samples by using a DNeasy Plant Mini Kit (Qiagen, Germany). The ITS region (small subunit rDNA-ITS1-5.8S rDNA-ITS2-large subunit rDNA) of nuclear rDNA was amplified by a polymerase chain reaction (PCR) by using the obtained genomic DNA as a template. PCR was catalyzed using KOD DNA polymerase (Toyobo, Japan) with the following program in a Takara Thermal Cycler MP (Takara Bio, Japan) or a DNA engine PTC-200 (MJ Research, USA; currently Bio-Rad, USA): 94°C, 4 min; 40 cycles of 98°C, 15 s, 55°C, 5 s and 74°C, 30 s; 74°C, 4 min. The primers were designed for the conserved sequence among plant rDNA genes. The sequence information is as follows: ITS-S1, 5′-CTT TAT CAT TTA GAG GAA GGA G-3′; ITS-AS1, 5′-TTT TCC

Table 1 Details of the authentic plants used in this study

Sample no.	Scientific name	Source	Vouchor no.
Ce-1	*Apium graveolens*	Prof. Junichi Kitajima, Showa Pharmaceutical University, Japan	None
Ce-62	*Cyclospermum leptophyllum* (Pers.) Sprague ex Britt. & Wilson	USDA, ARS Plant Genetic Resource Unit, Cornell University, USA	PI 325867 94CI1
Ce-63	*Seseli diffusum* (Roxb. ex Sm) Santapau & Wagh	Herbarium FARILLE, Jardin Botanique Alpin, La Jaysinia, Samoens, France	n 84-272

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Table 2 Details of the celery seed samples purchased in local markets

No.	Sample no.	Local name	Market place	Genotype*
1	Ce-2	Ajmod	Karachi	B
2	Ce-3	Ajmod	Karachi	B
3	Ce-4	Ajmod	Karachi	B
4	Ce-5	Ajmod	Karachi	B
5	Ce-6	Wal-ajowain	Sialkot	A
6	Ce-8	Ajmodh	Sargodha	A
7	Ce-9	Unknown	Peshawer	A
8	Ce-10	Ajmodh	Peshawer	A
9	Ce-11	Ajmodh	Peshawer	A
10	Ce-12	Ajmodh Karafas	Peshawer	A
11	Ce-13	Unknown	Mongora	A
12	Ce-14	Unknown	Hasan Abdal	C
13	Ce-15	Unknown	Dera Ismail Khan	A
14	Ce-16	Indian Ajowain	Lahore	B
15	Ce-17	Unknown	Taxila	A
16	Ce-18	Unknown	Peshawer	A
17	Ce-19	Unknown	Jalalabad, Afganistan	A
18	Ce-20	Celery	Karachi	D
19	Ce-23	Juwano	Kathmandu, Nepal	D
20	Ce-24	Jwano	Kathmandu, Nepal	D
21	Ce-25	Ajwain	Dibrugarh, India	D
22	Ce-26	Ajmodh Karafas	Peshawer	A
23	Ce-27	Ajmod	Sindh	B
24	Ce-28	Wal-ajowain	Bahawalpur	B
25	Ce-30	Ajmod, Karfas	Bahawalpur	A
26	Ce-31	Wal-ajowain	Bahawalpur	B
27	Ce-32	Wal-ajowain	Sialkot	D
28	Ce-33	Wal-ajowain	Sialkot	D
29	Ce-34	Ajmod	Sialkot	A
30	Ce-35	Wal-ajowain	Sialkot	B
31	Ce-36	Ajmud, Ajwain karfas	Murree	A
32	Ce-37	Wal-ajowain	Murree	B
33	Ce-38	Wal-ajowain	Rawalpindi	B
34	Ce-39	Wal-ajowain	Rawalpindi	B
35	Ce-40	Thick ajowain	Peshawer	D
36	Ce-41	Thin ajowain Desi ajowain	Peshawer	D
37	Ce-43	Indian ajowain Thick ajowain	Peshawer	D
38	Ce-44	Thin ajowain	Peshawer	D
39	Ce-45	AjowainKharasani	Peshawer	D
40	Ce-46	Wal-ajowain	Manshera	B
41	Ce-47	Wal-ajowain	Abbottabad	B
42	Ce-48	Indian ajowain Thick ajowain	Abbottabad	D
43	Ce-50	Wal-ajowain	Lahore	B
44	Ce-51	Ajowain wel	Lahore	B
45	Ce-52	Ajowain wel	Lahore	B
46	Ce-53	Wel-ajowain	Lahore	B
47	Ce-54	Wel-ajowain	Sukher	B
48	Ce-55	Wel-ajowain	Sukher	B
49	Ce-56	Wel-ajowain	Sukher	B
50	Ce-57	Wel-ajowain	Sukher	B
51	Ce-58	Wel-ajowain	Quetta	A
52	Ce-59	Ajowain	Quetta	B

*Sequence types A to D have high similarity or are identical to *Apium graveolens* (U30552, U30553), the authentic *Seseli diffusum* (AB243688; this study), *Conium maculatum* (U79609, U79612) and *Trachyspermum ammi* (U78380, U78440), respectively

TCC GCT TAT TGA TAT GC-3′. After the removal of excess primers and dNTPs from the reaction mixture by Montage-PCR (Millipore, USA), the amplicons with a length of about 700 base pairs (bp) were directly sequenced on an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, USA). A cycle sequencing reaction was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The DNA sequences were aligned on the basis of the Clustal W program [12].

Morphological observation

Twenty-kernel weight of each mericarp was measured in triplicate. The length, width and thickness of the mericarps were measured with a digital vernier caliper ($n = 20$). Microscopic observations of fruit and mericarp were performed using a digital microscope zoom lens, VH-Z25 (Keyence).

TLC analysis

Ten milligrams of each sample was extracted with 200 μL methanol at 60°C for 1 h. After centrifugation, the supernatant was charged on a TLC plate Silica gel 60 F_{254} (Merck) and developed with hexane/ethyl acetate (4:1).

Results and discussion

Botanical origin of Indian celery containing furocoumarins

We investigated the origin of the Indian celery, whose main principles were furocoumarin derivatives, using the

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ITS sequence analysis. The ITS region is often used for molecular identification of various plant species owing to its high copy numbers and the presence of both conserved and variable segments as well as its variation rate suitable for the phylogeny.

The ITS sequences of the authentic samples (*A. graveolens*, Ce-1; *Cyclospermum leptophyllum*, Ce-62; *Seseli diffusum*, Ce-63) and the Indian celery (Ce-2) used for the phytochemical study were determined. The ITS sequence of the authentic *A. graveolens* (Ce-1) was identical to the corresponding sequences of *A. graveolens* (Acc. nos. U30552 and U30553) in the International Nucleotide Sequence Database (DDBJ/EMBL/GenBank: INSD) except for only one nucleotide in the ITS2 region. The ITS sequences of *C. leptophyllum* (Ce-62) and *S. diffusum* (Ce-63) showed high sequence similarities (86–97%) with *Bunium* sp. (Ex. Acc. nos. DQ435226, DQ435220, DQ435265 and DQ435259) and *Psammogeton* sp. (Acc. nos. AF008630, AF164839, AF009109, AF164864) in the INSD, respectively. However, no identical sequences to them were found in the databases. The ITS sequences of Ce-62 and Ce-63 were registered in the INSD as accession numbers AB243689 and AB243688, respectively. The sequence similarities among Ce-1, Ce-62 and Ce-63 were 82.9% for Ce-1 versus Ce-62, 83.0% for Ce-1 versus Ce-63 and 83.9% for Ce-62 versus Ce-63.

The ITS sequence of the Indian celery (Ce-2) was completely identical to that of the authentic *S. diffusum* (Ce-63) in ITS1 and ITS2 regions. *S. diffusum* (Roxb. *ex* Sm.) Santapau & Wagh (Syn: *Ligusticum diffusum*) is native to India. Späth et al. [13]. also reported the isolation of the same coumarin derivatives (**1–3**) from the fruits of *S. indicum* (syn *S. diffusum* [14]). In addition to these data originating from the genetic and phytochemical approaches, the fruits of *S. diffusum* are known to be carminative and to be used in India to expel roundworms [15], and the plant has also been reported to be in the Rawalpindi dist., Pakistan [16]. These facts indicate that the Indian celery seeds used in our (and also the Indian authors') study are not derived from *A. graveolens* but from *S. diffusum*. In the future, the botanical identity of the Indian celery as *S. diffusum* might need to be carefully confirmed because only the one sample was used for the authentic *S. diffusum* in this study.

Market survey in the Pakistani and related countries' crude drugs/spice markets

Because it was found that the Indian celery differs from *A. graveolens*, we investigated different forms of celery seeds available in Pakistani and related countries' markets to clarify and establish the relationship between their local names, market places and the botanical sources. Fifty-two celery seed samples collected during the market survey were applied to the ITS sequence analysis and were classified into four genotypes designated for types A–D on the basis of their ITS sequences (Table 2, Fig. 2). The ITS sequence alignment of these four genotypes is shown in Fig. S1, Supplementary material. Type A sequence completely matched that of Ce-1, and hence the source plant corresponding to this sample type was identified as *A. graveolens*. This genotype was found in 16 samples, most of which were obtained as 'Ajmod (h)' or 'Karafas' in the northern region of Pakistan. The celery, *A. graveolens*, is expressed as 'Ajmoda' in Sanskrit, Hindi and Unani, whereas the names 'Bazr-ul-karafs' and 'Tukhm-e-karafs' are used in Arabian and Persian, respectively [17]. These vernacular names probably shifted into the above local names in Pakistan. Type B sequence was completely identical to the authentic *S. diffusum* (Ce-63), and found in 23 samples. Type C showed the identical sequence to that of *Conium maculatum* (Acc. nos. U79609 and U79612) in the INSD except for one nucleotide in the ITS2 region. Only one sample, Ce-14, was classified into this genotype. This sample was easily distinguished from the other types by its appearance. This sample is considered to be a rare adulterant of celery in Pakistani crude drug markets.

Type D sequence found in 12 samples showed a genotype that correlated with that of *Trachyspermum ammi* (Acc. nos. U78380 and U78440) in the INSD except for

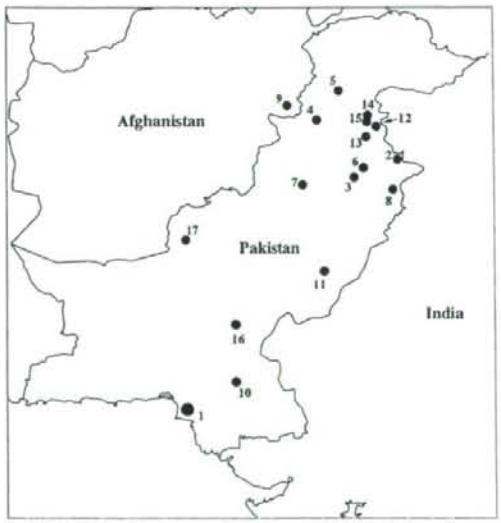

Fig. 2 Distribution map of the local markets surveyed in this study: *1* Karachi, *2* Sialkot, *3* Sargodha, *4* Peshawer, *5* Mongora, *6* Hasan Abdal and Taxila, *7* Dera Ismail Khan, *8* Lahole, *9* Jalalabad, *10* Sindh, *11* Bahawalpur, *12* Murree, *13* Rawalpindi, *14* Manshera, *15* Abbottabad, *16* Sukher, *17* Quetta

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two nucleotides in the ITS1 region. The fruits of *T. ammi* are used as 'Carvi oriental' or 'ajawain' for treating stomach troubles and whooping cough by household ladies in northern India [18]. This fact and our sequence data indicate that the type D sample is derived from *T. ammi*. Various celery-related products derived from *A. graveolens*, *T. ammi*, *Carum roxburghianum*, *A. leptophyllum* and *P. crispum* are used for folk medicines and/or spices in India. The last three species were not observed in our market survey targeted at 'celery seeds' in Pakistan. Therefore, our results suggest that these three species are not common in the country or are not recognized as 'celery seeds' by Pakistani people.

Unlike type A samples found only in the northern area, samples of types B and D were distributed in the markets of most regions surveyed. Most of the type B samples have been sold as 'wal ajowain' or 'wel ajowain' whose meaning was found to be a wild form of 'ajowain' (*T. ammi*) through interviews during market survey. On the other hand, type B samples have been obtained as 'ajmud' in the Karachi area, although the name is applied to *A. graveolens* in the northern area. Furthermore, the local name of type D samples (*T. ammi*) was also variable: (thick/thin/Indian) ajowain, wal ajowain and so on. These data mean that the relationship between the local name and the botanical source of celery seeds varied depending on the market place. It is thought that each type of celery seeds is selectively used on the basis of their pharmacological effects, traditional cultures, rituals, usages and commercial values in indigenous communities of Pakistan. Therefore, the confusion regarding their botanical source should be resolved for their appropriate use.

Microscopic observation and TLC analysis of celery-related seeds from Pakistani markets

For rapid identification of the source plants of Indian celery and its analogues, we investigated their morphological features and chemical composition. Microscopic images of the major genotypes (types A, B and D) found in the Pakistani markets and three authentic samples (Ce-1, Ce-62 and Ce-63) are shown in Fig. S2, Supplementary material. They were cremocarp characteristic of the Umbelliferae plant and their morphological features are as follows:

Type A (*A. graveolens*)—The fruit was a laterally compressed globose. The size of a mericarp was 1.60 ± 0.14 mm long, 0.75 ± 0.07 mm wide and 0.71 ± 0.08 mm thick. The twenty-kernel weight of the mericarps was 9.37 ± 0.85 mg. Each mericarp had five small prominent ribs.

Type B (*S. diffusum*)—The fruit was almost globose. Each mericarp was flat and ellipsoid in a commissural face and had a semicircular cross section. The size of a mericarp was 2.01 ± 0.18 mm long, 1.35 ± 0.16 mm wide and 0.85 ± 0.11 mm thick. The twenty-kernel weight of the mericarps was 13.4 ± 0.53 mg. Each mericarp had five large prominent ribs.

Type D (*T. ammi*)—The fruit was a laterally compressed ovoid. The commissural face of each mericarp was lanceolate and curved directed on the long side. The cross section of each mericarp was semicircular. The size of a mericarp was 2.35 ± 0.26 mm long, 0.95 ± 0.09 mm wide and 0.83 ± 0.08 mm thick. The twenty-kernel weight of mericarps was 19.6 ± 0.67 mg. Each mericarp had five prominent ribs.

Type A and type B were morphologically quite consistent with their corresponding authentic plants (Ce-1 and Ce-63, respectively). The sizes of the ribs significantly varied among the three genotypes in the order type B > type D > type A. A comparison of the rib size in section view can easily discriminate each genotype from the others.

In addition, the TLC analysis of the methanol extracts of the seeds is useful for the detection of the celery containing furocoumarins (Fig. S3, Supplementary material). The authentic *S. diffusum* (Ce-63) and type B samples (Ce-2 and Ce-27) have pale yellow and blue fluorescent spots characteristic of the furocoumarin derivatives, whereas the other samples (Ce-1 and Ce-30 for *A. graveolens* and Ce-33 for *T. ammi*) have no fluorescent spots.

The presence/absence of furocoumarin derivatives in feeding materials is an important factor from the viewpoint of the risk assessment for foods and drugs because the linear furocoumarin derivatives are well known to have phototoxicity and carcinogenic properties [19–21]. Our study indicated that the reported isolation of these compounds from *A. graveolens* seeds by the Indian researchers [2–10] was attributable to the misidentification of the material. Simultaneously, it was found that the unexpected intake of furocoumarin derivatives which pose a health risk to humans could occur by contamination of the common Indian celery with *S. diffusum*. The exact identification of the botanical source is thus important for the safety of the resulting foods and drugs.

Conclusions

In this study, we proved that the Indian celery seeds containing coumarin derivatives (**1**–**9**) are not derived from *A. graveolens* by using genetic and phytochemical approaches, even though Indian workers reported that *A. graveolens* seeds contain these compounds [2–10]. Furthermore, our results strongly suggested that the seeds were derived from *S. diffusum* based on their ITS sequences, characteristic components and distribution area. The

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market survey in Pakistan and related countries established that the celery seeds available in Pakistani markets are mainly composed of three species (*A. graveolens*, *T. ammi* and probably *S. diffusum*) and that the relationship between the local name and their botanical source varied depending on the market place. The ambiguous botanical origin of the celery seeds in the Indian and Pakistani local markets could therefore have caused the disputable phytochemical results reported by the Indian researchers.

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いわゆる健康食品及び違法ドラッグ製品に含まれた植物由来成分の検出事例について

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永田　知子、川原　信夫[1]、合田　幸広[1]

Plant Components in Dietary Supplements and Illegal Drug

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Tomoko NAGATA, Nobuo KAWAHARA[1] and Yukihiro GODA[1]

要　旨

不明ピークを有する健康食品及び違法ドラッグ3製品について更なる検討を行ったところ、植物由来成分であるルチン、クエルセチン、クエルシトリン、イソクエルシトリン、フォルモノネチン、バイオカニンA、ヒドロキシカビコールが検出された。

キーワード：違法ドラッグ、健康食品、HPLC/PDA、LC/MS
Keywords : illegal drugs, dietary supplement, HPLC/PDA, LC/MS

はじめに

近年、「いわゆる健康食品」のうち、効能効果を高めることを目的として医薬品成分として扱われる成分を入れた製品が出回るようになり、その製品によると思われる健康被害が多数発生している[1~3]。「いわゆる健康食品」に含まれる医薬品成分の中には、現在医薬品として使用されている成分の他にそれら既存成分の構造を一部変更した成分が含まれていることや[4~7]、既存成分の類似体ではないが医薬品成分と同様の作用を有すると考えられる成分が検出されることがあり[8,10]、「いわゆる健康食品」に関する試験検査を複雑化している。

違法ドラッグは「麻薬又は向精神薬には指定されておらず、麻薬又は向精神薬に類似の有害性を有することが疑われる物質（人為的に合成されたもの、天然物及びそれに由来するものを含む。）であって、専ら人に乱用させることを目的として製造、販売等がされるもの」とされ[11]、インターネットなどを通じて乱用が拡大している[12]。違法ドラッグには、既存違法ドラッグ成分の一部の構造を変えた「デザイナードラッグ」と呼ばれる類似薬物が含まれていることがあり、毎年のように新規薬物が検出され[13,14]、違法ドラッグの試験検査を「いわゆる健康食品」の試験検査と同様に煩雑化させる要因となっている。

千葉県では「違法ドラッグ対策事業及び無承認無許可医薬品取締事業」として健康食品及び違法ドラッグの試買調査を行っているが[15,16]、試験検査を実施している中で、多くの不明ピークが検出される。今回、不明ピークを有する3製品について更なる検討を行ったところ、新規化合物ではなかったが、先のピーク成分を同定することによって、今後の試験検査に有用な情報となったので報告する。

実験方法

1．試料

1）製品

違法ドラッグ対策事業及び無承認無許可医薬品取締事業として試買した製品のうち不明ピークを有した3製品。以下に剤形、表示内容量及び表示原材料を示す。

（1）製品1

剤形：カプセル、表示内容量：250mg×10、表示原材料：ブラックコホッシュエキス、レッドクローバーエキス、プエラリアエキス、パフィア4倍濃縮エキス、カツアバ4倍濃縮エキス、アリアムサテバ

（2）製品2

剤形：カプセル、表示内容量：350mg×2、表示原材料：セントジョーンズワートエキス末、ショ糖エステル

（3）製品3

剤形：錠剤、表示内容量：600mg×7、表示原材料：乳糖、キシマ、コーンスターチ、ペパーミントオイル、ビンロウジ、甘味料（アスパルテーム）、炭酸カルシウム、タルカム、アカシア、クスノキ、香料（*d*-ボルネオール、*l*-メントール）

2）植物類：セントジョーンズワートは市販の物を用いた。レッドクローバーは千葉県薬草園の標本を用いた。

2．試薬及び試液

1）標準品：ルチン、クエルセチン及びアスパルテームは和光純薬工業㈱製、イソクエルシトリン及びクエルシトリンはSIGMA社製、バイオカニンAはMP Biomedicals社製、フォルモノネチンはLKT Laboratories社製を用いた。

2）その他の試薬

1）国立医薬品食品衛生研究所
受理年月日　2008年9月30日

メタノールは残留農薬・PCB試験用、アセトニトリルはHPLC用及びLC/MS用、1-ヘキサンスルホン酸ナトリウムはイオンペアクロマトグラフ用（いずれも、和光純薬工業㈱製）を用いた。重メタノールはAcros organics社製を用いた。水は日本ミリポア社製MILLI-Q Labo超純水製造装置により精製した超純水を用いた。その他の試薬はすべて市販の試薬特級品を用いた。

3．標準溶液の調製

各標準品を1000μg/mLとなるようメタノールに溶解させ調製した（標準原液）。標準溶液はこれら標準原液をメタノールで100μg/mLに適宜希釈し調製した。

4．試験溶液の調製

試料は粉末とし、約0.10gを精密に量り取り、70%メタノール10mLを加え15分間超音波抽出後、0.45μmのメンブランフィルターでろ過し、試験溶液とした。

5．装置

1）フォトダイオードアレイ検出器付き高速液体クロマトグラフ（以下HPLC/PDAと略す）

日本分光㈱製PU-2089型ポンプ、同AS-2055型オートサンプラー、同CO-2065型カラムオーブン、同MD-2015型フォトダイオードアレイ検出器を用いた。

2）高速液体クロマトグラフ質量分析計（以下LC/MSと略す）

Waters社製2695型セパレーションモジュール、同ZQ 4000型質量分析計を用いた。

3）NMR装置

JEOL製ECA-800を用いた。

6．測定条件

1）HPLC/PDA測定条件

既報[15]の一斉分析法に従い行った。

カラム：TSK-GEL ODS-80 Ts(4.6mm i.d.×150mm、5μm)、カラム温度：40℃、移動相A液：アセトニトリル/水/リン酸混液（100：900：1、5mmol/Lヘキサンスルホン酸ナトリウム含有）、移動相B液：アセトニトリル/水/リン酸混液（900：100：1、5mmol/Lヘキサンスルホン酸ナトリウム含有）、グラジエント条件：0分(A:B=90:10)→25分(A:B=55:45)→44-49分(A:B=10:90)、流速：1.0mL/min、注入量：20μL、測定波長：200-400nm (Max Plot)

2）LC/MS測定条件

以下のイオン化法及びコーン電圧条件以外は既報[15]のグラジエント条件2に従い行った。

カラム：Atlantis dC18（2.1mm i.d.×150mm、3μm)、カラム温度：40℃、移動相A液：0.1%ギ酸溶液、B液：0.1%ギ酸含有アセトニトリル、グラジエント条件：0分(A:B=95:5)→15分(A:B=80:20)→30-35分(A:B=20:80)、流速：0.2mL/min、注入量：10μL、イオン化法：ESIポジティブ又はESIネガティブ、コーン電圧：ESIポジティブ　10、20、30、60及び90V、ESIネガティブ 10、20、40、50、90V、測定質量電荷比範囲：*m/z* 100-800

3）NMR測定条件

NMR測定溶媒には重メタノールを用い、^{1}H-及び^{13}C-NMR測定等1-D測定、^{1}H-^{1}H COSY、HMQC及びHMBC等の2-D測定を行った。

結果及び考察

1．製品1の分析

製品1をHPLC/PDAで分析した結果、保持時間20.2分（ピーク1）と25.8分（ピーク2）に比較的大きなピークが観察され、それぞれのピークをフォトダイオードアレイ検出器でモニターすると、ピーク1は242nmと299nmに極大吸収を、218nmと287nmに極小吸収を持つ吸収スペクトルを、ピーク2は257nmに極大吸収、227nmに極小吸収を持つ吸収スペクトルを示した（図1A)。この製品の原材料表示はブラックコホシュエキス，レッドクローバーエキス、プエラリアエキス等とされており、当研究室において標本として保有していたレッドクローバーを70%メタノールで超音波抽出しHPLC/PDAで分析を行った。その結果、レッドクローバーのクロマトグラムにおいて製品1と同様の保持時間及び吸収スペクトルを有するピークが確認された。レッドクローバーはマメ科の植物で、更年期症状を軽減するサプリメント等でよく用いられているハーブの一種であり[17]、主な成分はフォルモノネチン及びバイオカニンAである[18],[19]。そこで、フォルモノネチン及びバイオカニンA標準溶液をHPLC/PDAで分析した結果、フォルモノネチンは製品1のピーク1と、バイオカニンAは製品1のピーク2と保持時間及び吸収スペクトルが一致した（図2)。また、製品1をLC/MSのESIポジティブモードによりコーン電圧30Vで分析した結果、保持時間29.3分及び31.3分にピークが確認された。マススペクトルでは保持時間29.3分のピークは*m/z* 269に$[M+H]^+$イオンが観察され、これはフォルモノネチン標準溶液から得られたマススペクトルと同様であった（表1)。また、保持時間31.3分のピークは*m/z* 285に$[M+H]^+$イオンが観察され、これはバイオカニンA標準溶液から得られたマススペクトルと同様であった（表1)。以上のことから、製品1の2つの不明成分はフォルモノネチンとバイオカニンAであると判断した。

2．製品2の分析

製品2をHPLC/PDAで分析した結果、保持時間6.6分（ピーク1)、6.9分（ピーク2)、7.2分（ピーク3)、8.9分（ピーク4）及び13.5分（ピーク5）に大きなピークが観察され、各ピークをフォトダイオードアレイ検出器でモニターすると、ピーク1～4は254nmと350nm付近に極大吸収を、235nmと280nm付近に極小吸収を持つ吸収スペクトルを、ピーク5は251nmと368nmに極大吸収を、236nmと280nmに極小吸収を持つ吸収スペクトルを示した（図1B)。この製品の表示にはセントジョーンズワート加工食品と記載されていたので、市販のセントジョーンズワートをHPLC/PDAで分析したところ、製品1とほぼ同様のクロマトグラムを示し、各ピークの吸収スペ

クトルも同様であった。セントジョーンズワートの成分としてフラボノイドのルチン、イソクエルシトリン、クエルシトリン、クエルセチンが知られている[20)-22)]。そこで、ルチン、イソクエルシトリン、クエルシトリン、クエルセチン標準溶液をHPLC/PDAで分析したところ、ルチンは製品2のピーク1と、イソクエルシトリンはピーク3と、クエルシトリンはピーク4と、クエルセチンはピーク5とそれぞれ保持時間及び吸収スペクトルが一致した（図2）。また、製品1と同様にLC/MSのESIポジティブモードで製品2及びルチン、イソクエルシトリン、クエルシトリン、クエルセチン標準溶液を分析したところ、製品2から22.3分に $[M+H]^+$ イオンが m/z 611を示しフラグメントイオンが m/z 303及び465を示すピークが、22.8分に $[M+H]^+$ イオンが m/z 465を示しフラグメントイオンが m/z 303を示すピークが、24.0分に $[M+H]^+$ イオンが m/z 449を示しフラグメントイオンが m/z 303を示すピークが、26.5分に $[M+H]^+$ イオンが m/z 303を示すピークが確認され、各ピークはそれぞれ、ルチン、イソクエルシトリン、クエルシトリン、クエルセチン標準溶液から得られたLC/MS分析結果と保持時間及びマススペクトルが一致した（表1）。以上のことから製品1の不明ピーク成分の中の4つはルチン、イソクエルシトリン、クエルシトリン、クエルセチンであると判断した。製品2で検出されたピーク2については、吸収スペクトルがルチンやイソクエルシトリン、クエルシトリンとほぼ同様の吸収スペクトルを示したことから、セントジョーンズワートに含まれているとされる他のヒペロシド[21),22)]などのフラボノイドではないかと推測された。

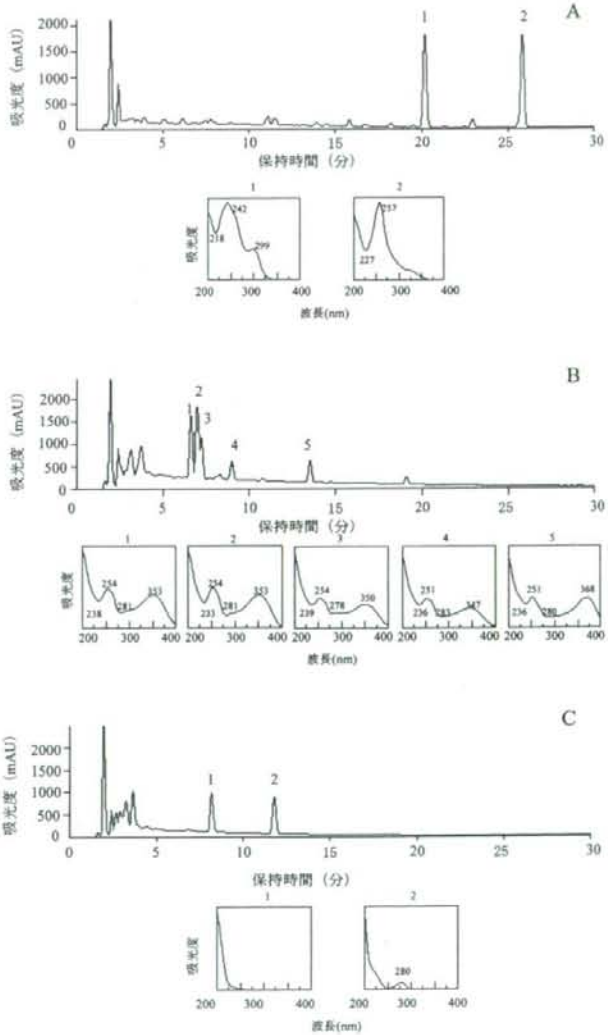

図1　各製品の最大吸光度によるHPLCクロマトグラム及び検出されたピークの吸収スペクトル
A：製品1、B：製品2、C：製品3

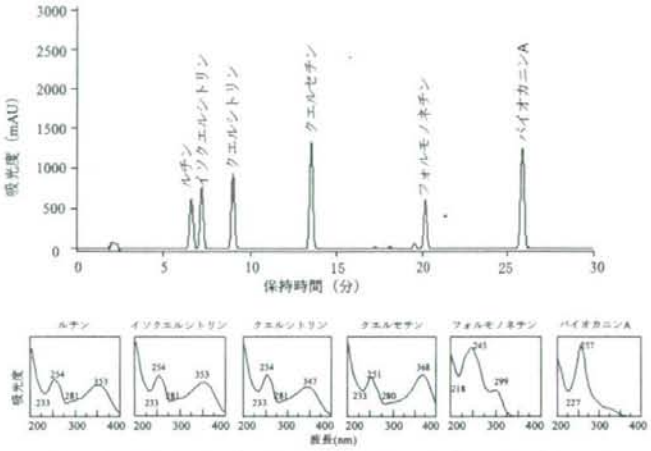

図2　各標準溶液の最大吸光度によるHPLCクロマトグラム及び吸収スペクトル

表1　LC/MSにおける各標準溶液の分析結果

成分	極性	コーン電圧(V)	保持時間（分）	$[M+H]^+$イオン (*m/z*)	フラグメントイオン (*m/z*)
フォルモノネチン	ポジティブ	30	29.3	269	
バイオカニンA	ポジティブ	30	31.3	285	
ルチン	ポジティブ	30	22.3	611	465, 303
イソクエルシトリン	ポジティブ	20	22.8	465	303
クエルシトリン	ポジティブ	20	24.0	449	303
クエルセチン	ポジティブ	60	26.5	303	

3．製品3の分析

製品3をHPLC/PDAで分析した結果、保持時間8.1分（ピーク1）と11.8分（ピーク2）に比較的大きなピークが観察された（図1C）。ピーク1の保持時間と吸収スペクトルはアスパルテーム標準溶液をHPLC/PDAで分析して得られた保持時間及び吸収スペクトルと一致した。また、LC/MSのESIポジティブモードによる分析では製品3から15.7分に$[M+H]^+$イオンが*m/z*295を示すピークがみられ、そのピークはアスパルテーム標準溶液から得られた保持時間及びマススペクトルと一致した。そのため、ピーク1はアスパルテームであると判断した。ピーク2をフォトダイオードアレイ検出器でモニターすると280nm付近に極大吸収を持つ吸収スペクトルを示した（図1C）。また、製品3をLC/MSのESIネガティブモードによりコーン電圧40Vで分析するとピーク2に該当すると思われるピークが保持時間25.7分にみられ、$[M-H]^-$イオンと思われるピークが*m/z*149に観察され、分子量150が推定された（図3）。本ピークはアスパルテームと同程度のピーク強度を有しており、医薬品成分あるいは何らかの薬理作用を有する物質である可能性が考えられた。そこで、この不明な化合物を単離精製し、NMR測定を行った。^{13}C-NMRデータにおける9本のシグナルより、LC/MSの結果と合わせて、本化合物は分子式$C_9H_{10}O_2$が推定された。本化合物の^{1}H-NMRにおいてδ6.65（1H, d, *J*=7.8Hz）、δ6.59（1H, d, *J*=1.8Hz）及びδ6.47（1H, dd, *J*=7.8, 1.8Hz）より1、2、4置換ベンゼン環の存在が示唆された。さらに、δ3.20（2H, d, *J*=6.4Hz）、δ4.97（1H, dd, *J*=10.1、0.9Hz）、δ5.01（1H, dd, *J*=17.0, 0.9Hz）、δ5.90（1H, ddt, *J*=17.0, 10.1, 6.4 Hz）よりベンゼン環に結合した2-プロペニル基（アリル基）に特徴的なシグナルパターンが観測された。一方、^{13}C-NMRにおいて観測された2つの芳香族炭素（δ146.2及びδ144.5）のシグナルより、本化合物は2つの水酸基を有するアリルベンゼン誘導体と推定された。さらにHMBCスペクトル解析の結果、本未知化合物の構造を最終的にヒドロキシカビコールと決定した（図4、5）。ヒドロキシカビコールはキンマに含まれる植物成分で[23),24)]、スーパーオキシドスカベンジャーとしての作用やシクロオキシゲナーゼ阻害作用が知られている[25),26)]。製品3の原材料名としてキンマが記載されており、検出

されたヒドロキシカビコールはそのキンマに由来しているのではないかと推察された。ビンロウの種子（ビンロウジ）を石灰にまぶしキンマ葉で包んだものをベテルチューイングといい、咀嚼嗜好品としてインドや東南アジアなどで供されているが[7),8)]、本製品の原材料名にキンマの他にビンロウジが記載されていることから、本製品はそのベテルチューイングを模したものではないかと思われる。

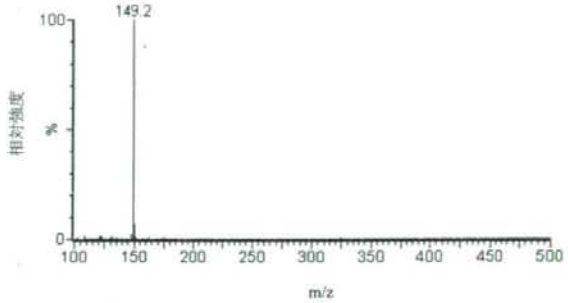

図3　製品3未知成分のマススペクトル（LC/MS ESI ネガティブモード　コーン電圧：40V）

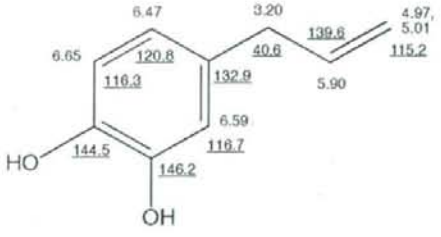

図4　製品3不明化合物における重メタノール中の ^{1}H 及び ^{13}C-NMR データ

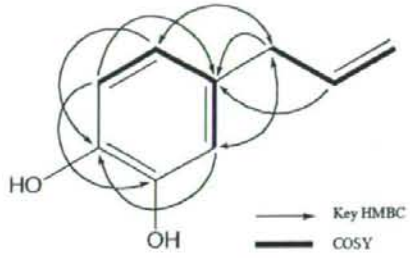

図5　製品3不明化合物における ^{1}H-^{1}H COSY 及び ^{1}H-^{13}C HMBC 測定結果

まとめ

いわゆる健康食品及び違法ドラッグ製品からフォルモノネチン、バイオカニンA、ルチン、クエルセチン、クエルシトリン、イソクエルシトリン及びヒドロキシカビコールが検出された。今回検出された成分は、全て製品原材料に表示されていた植物に含まれる成分であり、その植物由来であったと考えられる。これら検出された成分の保持時間及び吸収スペクトルをライブラリーに登録後、他の「いわゆる健康食品」及び違法ドラッグ製品を試験検査したところ、いくつかの製品からこれら植物由来成分が検出された。

いわゆる健康食品及び違法ドラッグ製品から新規医薬品成分が次々と検出される現状においては、試験検査を行っていく中で、検出されたピークが既存の医薬品成分に該当しない不明なピークであったとしても、そのピーク成分が新規医薬品成分に該当するかどうか調べるための検査を行わなければならないことがあり、更なる時間と労力がかかってしまう。今回検出された植物由来成分のような医薬品成分に該当しない成分もスペクトルライブラリーに登録しておけば、不明ピークを減らすことにつながり、有用であると思われる。

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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

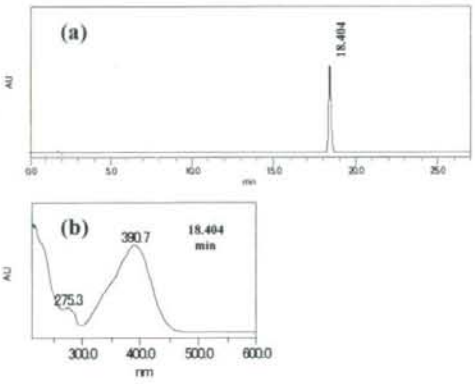

Fig. 1. HPLC Chromatogram of the Sample Solution and the UV Spectrum of the Detected Peak

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).

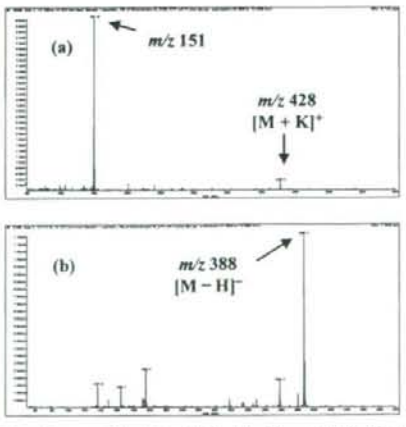

Fig. 2. Mass Spectra of the Detected Peak by Qtrap LC/MS/MS Analysis

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^+ 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.

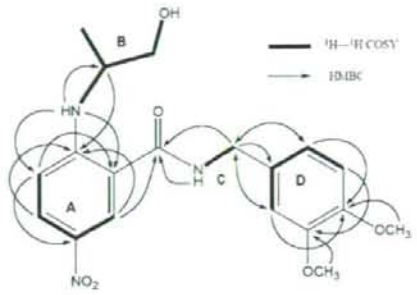

Fig. 3. ^{1}H–^{1}H and Major Long-Range ^{1}H–^{13}C Correlations of the Unknown Compound

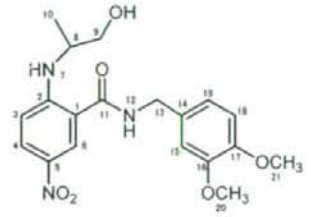

Fig. 4. Structure of Xanthoanthrafil

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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Note

Structural Elucidation of a Tadalafil Analogue Found in a Dietary Supplement

(Received November 30, 2007)

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A tadalafil analogue was detected in a dietary supplement marketed for tonic effect, along with hydroxyhomosildenafil and aminotadalafil. The tadalafil analogue was isolated by preparative thin layer chromatography (TLC) and its structure was elucidated using high-performance liquid chromatography (HPLC), liquid chromatography electrospray ionization-mass spectrometry (LC-ESI-MS), Fourier transform-ion cyclotron resonance-mass spectrometry (FT-ICR-MS) and nuclear magnetic resonance (NMR) spectroscopy. The compound was determined to be methyl-1-(1,3-benzodioxol-5-yl)-2-(chloroacetyl)-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole-3-carboxylate. This is the first report of detection of this compound in a dietary supplement.

Key words: tadalafil analogue; tonic effect; dietary supplement; HPLC; NMR

Introduction

Dietary supplements are widely used in Japan. There are many dietary supplements advertised for weight reduction, hyperglycemia and tonic effect, but some are adulterated with synthetic drugs[1)-4)], and associated health hazards have been reported in the past 5 years*1. It is potentially dangerous for consumers to take adulterated dietary supplements. Moreover, not only prescription drugs but also their analogues such as *N*-nitroso-fenfluramine[5)], homosildenafil[6)], acetildenafil[7)], hydroxyhomosildenafil[7)] and aminotadalafil[8)] have been detected. We have already established a HPLC screening method for synthetic drugs in dietary supplements, such as tonic medicines, anorexic agents, hypoglycemic agents, and so on*2. Inspections of 41 dietary supplements marketed for tonic effect were carried out in 2007. During the inspection, a tadalafil analogue was detected in a dietary supplement, along with hydroxyhomosildenafil and aminotadalafil. Here we report a newly found tadalafil analogue, which was isolated by preparative TLC and identified by means of HPLC, LC-ESI-MS, FT-ICR-MS and NMR.

*1 Pharmaceutical and Food Safety Bureau-Health, Labor and Welfare Ministry, Japan (2004) Information on health hazards and non-approved and non permitted pharmaceuticals. Available from: http://www.mhlw.go.jp/kinkyu/diet.html

*2 Saijo, M., Ishii, T., Hasegawa, T., Nagata, T. Medical components detected in dietary supplements with suggestive for tonic and weight reduction. Chibaken Eiseikenkyusho Nenpo (Annual Report of the Chiba Prefectural Institute of Public Health), (in press)

Materials and Methods

Sample

Capsules containing "khaki powder", marketed for tonic effect, were purchased through the internet.

Standard and reagents

Tadalafil was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Aminotadalafil and hydroxyhomosildenafil were obtained from the National Institute of Health Sciences (Tokyo, Japan). HPLC grade acetonitrile, methanol and all other reagents (analytical grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dimethylsulfoxide (DMSO-d_6) used for NMR analysis was purchased from Isotec, Inc. (Miamisburg, OH, U.S.A.).

Mixed standard solutions

A mixed standard solution of aminotadalafil, hydroxyhomosildenafil and tadalafil (30 μg/mL each) was prepared in methanol and used for HPLC and LC-ESI-MS.

Sample preparation for HPLC and LC-ESI-MS analysis

The sample powder (10 mg) was ultrasonically extracted in 10 mL methanol for 15 min. The extract was filtered through a 0.45 μm PTFE filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and the filtrate was applied to HPLC and LC-ESI-MS.

HPLC analysis

HPLC analysis was performed using a Waters LC system equipped with a model 616 pump, a model CHM

column oven, and a model 996 PDA detector (Waters Co., Milford, MA, U.S.A.). A TSKgel ODS-80Ts column (150×4.6 mm i.d., 5 μm, Tosoh Co., Tokyo, Japan) was used. The mobile phase was 0.1% (v/v) phosphoric acid aqueous solution (eluent A) and acetonitrile containing 0.1% (v/v) phosphoric acid (eluent B). The gradient elution began at 80% A and linearly decreased to 35% A in 35 min. The flow rate of the mobile phase was 1.0 mL/min and the injection volume was 20 μL. The column temperature was 40℃. The wavelength of PDA was 210 to 400 nm; and monitoring of chromatograms was performed at 280 nm.

LC-ESI-MS analysis

LC-ESI-MS analysis was performed in positive ionization mode using a Waters Alliance 2695 separation module and ZQ mass spectrometer (Waters Co.). An Atlantis dC18 column (150×2.1 mm i.d., 3 μm, Waters Co.) was used. The mobile phase was 0.1% formic acid aqueous solution (eluent A) and acetonitrile containing 0.1% formic acid (v/v) (eluent B). The gradient elution began at 80% A and linearly decreased to 20% A in 50 min. The flow rate of the mobile phase was 0.2 mL/min and the injection volume was 10 μL. The column temperature was 40℃. The instrument parameters were: source temperature, 120℃; desolvation temperature, 350℃; capillary voltage, 3 kV; cone voltage, 30 V; and desolvation gas flow, 500 L/hr. The mass range of the spectra was from *m/z* 100 to *m/z* 800.

Purification

The sample powder (240 mg) was ultrasonically extracted twice in 25 mL methanol for 15 min. The extract was filtered and the filtrate was concentrated to 2 mL under vacuum. Then, the residual solution was applied to three silica gel 60 F_{254} TLC plates (20×10 cm with 2.0 mm thickness, Merck, Darmstadt, Germany) in bands. The plates were developed with a chloroform/ethyl acetate mixture (2 : 1) to a distance of about 7 cm. After air-drying, the plates were examined under ultraviolet (UV) light (wavelength: 254 nm). The band of the unknown compound, *Rf* value 0.92, was taken and dissolved in 50 mL methanol. The methanol solution was filtered, the filtrate was evaporated to dryness, and the residue was reconstituted in 3 mL methanol. This solution was filtered and the filtrate was evaporated to dryness. The residue was dissolved in diethyl ether, the solution was filtered, and the filtrate was evaporated to dryness. The purity of the compound was determined by normalization of the peak areas detected by HPLC with UV detection at 210 nm and 280 nm.

NMR analysis

^1H and ^{13}C NMR data were acquired on a JNM ECA-800 (JEOL Ltd., Tokyo, Japan) operating at 800 MHz and 200 MHz, respectively. DMSO-d_6 was used as a solvent. Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane as an internal reference; coupling constants (J) are given in Hz. Conventional pulse sequences were used for correlated spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC) and rotating frame nuclear Overhauser effect spectroscopy (ROESY).

FT-ICR-MS analysis

Accurate mass of the unknown compound was measured with an LTQ OrbiTrap XL instrument (Thermo Fisher Scientific K.K., San Jose, CA, U.S.A.) with direct infusion in ESI positive ion mode, under conditions of solvent flow rate 5 mL/min, sheath gas flow rate 20 arb, auxiliary gas flow rate 10 arb, spray voltage 5 kV, capillary temperature 275℃, capillary voltage 4 V, and tube lens voltage 60 V. Tyrosin 1,3,6 standard was used as a mass calibrant of the FT mass analyzer (resolution =100,000), and tyrosin 3 standard was used as a lock mass ion (*m/z* 508.20783) during the measurement. Theoretical mass and delta values (mmu) were calculated by using the elemental composition tool of Xcalibur/Qual Browser software.

Results and Discussion

HPLC chromatograms of a mixed standard solution and the sample extract are shown in Fig. 1 and the corresponding UV spectra are shown in Fig. 2. Three major peaks were detected in the sample extract at 14.47 min (peak 1), 19.38 min (peak 2) and 33.22 min (peak 3). The compounds eluted at peak 1 and peak 2 were identified as hydroxyhomosildenafil and aminotadalafil, respectively, by comparing retention time, UV spectra (Fig. 2) and mass spectra (data not shown) with those of reference standards. Although the UV spectrum of peak 3 exhibited a similar spectrum to those of tadalafil and aminotadalafil, the retention time of peak 3 (unknown compound) was later than those of tadalafil and aminotadalafil. As shown in Fig. 3, in LC-ESI-MS

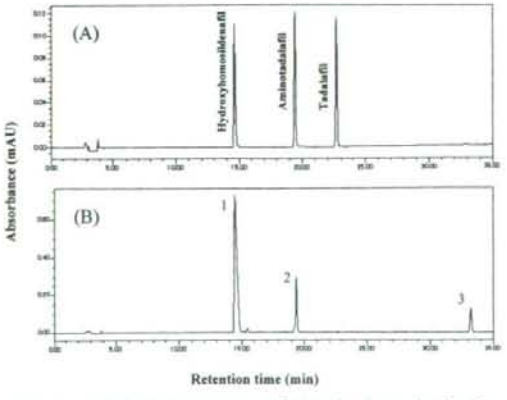

Fig. 1. HPLC chromatograms of (A) mixed standard solution and (B) sample extract

The concentration of mixed standard solution was 30 μg/mL each of aminotadalafil, hydoroxyhomosildenafil and tadalafil.

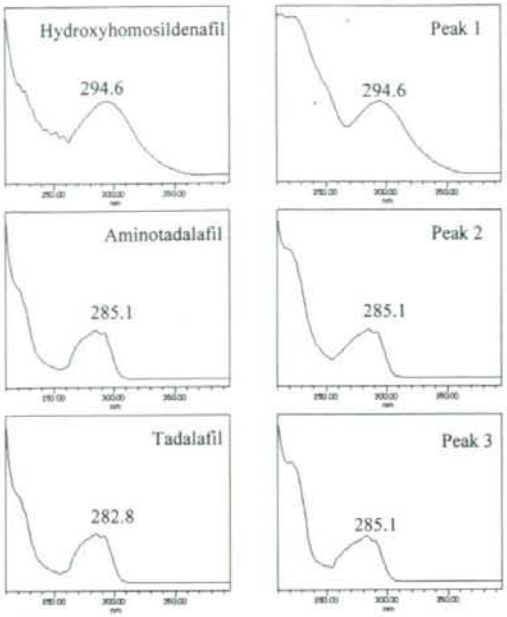

Fig. 2. UV Spectra of the peaks in the HPLC chromatograms of mixed standard solution and sample extract

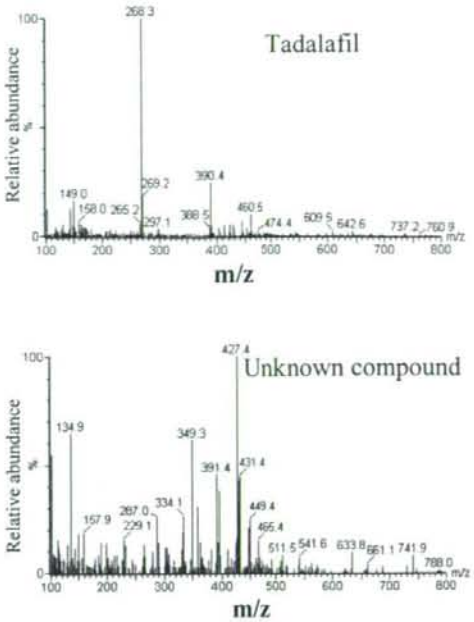

Fig. 3. LC-ESI-MS spectra of tadalafil and the unknown compound

Table 1. NMR data of tadalafil and the unknown compound[a]

Tadalafil			Unknown compound		
Position no.	^1H (δ_H)	^{13}C (δ_X)	Position no.	^1H (δ_H)	^{13}C (δ_X)
1		166.8	1	6.75 (1H, s)	51.3
3	3.94 (1H, d, J=16.9)	51.3	3	5.19 (1H, d, J=6.9)	52.3
	4.18 (1H, d, J=16.9)		4	3.07 (1H, dd, J=6.9, 14.7)	21.0
4		166.5		3.45 (1H, d, J=14.7)	
6	6.13 (1H, s)	55.4	4a		106.2
6a		136.1	4b		125.8
7	11.02 (1H, s)		5	7.54 (1H, d, J=7.8)	118.1
7a		136.9	6	7.02 (1H, dd, J=7.3, 7.8)	118.7
8	7.29 (1H, d, J=7.5)	111.2	7	7.09 (1H, dd, J=7.3, 8.3)	121.6
9	6.99 (1H, q, J=7.5, 6.0)	118.8	8	7.27 (1H, d, J=8.3)	111.2
10	7.06 (1H, q, J=7.1, 6.4)	121.1	8a		136.3
11	7.54 (1H, d, J=7.2)	118.0	9	10.87 (1H, s)	
11a		125.6	9a		129.9
11b		104.6	1′		133.5
12	2.97 (1H, dd, J=11.3, 4.5)	23.0	2′	6.63 (1H, br s)	109.1
	3.52 (1H, dd, J=11.7, 3.8)		3′		146.9
12a	4.40 (1H, dd, J=7.2, 4.1)	55.2	4′		146.6
13	2.93 (3H, s)	32.8	5′	6.80 (1H, d, J=8.2)	107.6
1′		133.8	6′	6.44 (1H, br d, J=8.2)	122.4
2′	6.86 (1H, s)	106.8	7′	5.97 (2H, d, J=16.6)	101.0
3′		146.9	$\underline{C}OCH_2Cl$		166.8
4′		145.9	$CO\underline{CH_2}Cl$	4.44 (1H, d, J=13.8)	43.2
5′, 6′	6.78 (2H, d, J=1.14)	107.9		4.83 (1H, d, J=13.8)	
		119.2	$\underline{C}OOCH_3$		170.4
7′	5.92 (2H, s)	100.8	$COO\underline{CH_3}$	3.02 (3H, s)	51.8

[a] All measurements were made in DMSO-d_6

analysis, the base peak of the unknown compound was observed at m/z 427 and this ion was inferred to be the $[M+H]^+$ ion.

In the purification using preparative TLC, three well-separated bands, the Rf values of which were 0.92 (unknown compound), 0.75 (aminotadalafil) and 0.32

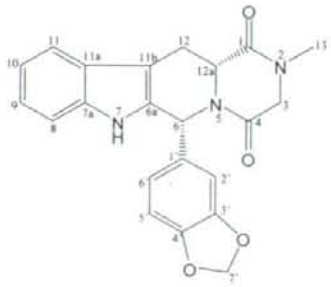

Fig. 4. Chemical structure of tadalafil

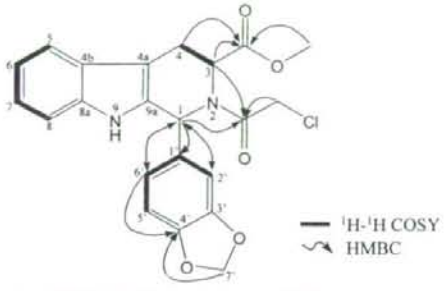

Fig. 5. ^{1}H-^{1}H COSY and selected HMBC correlations of the unknown compound

(hydroxyhomosildenafil), were obtained from the sample solution. The band of the unknown compound afforded approximately 1.7 mg yellow powder, the purity of which was determined to be more than 96%, based on HPLC with detection at both 210 and 280 nm.

The structure of the unknown compound was further elucidated using NMR analysis. NMR spectral data of tadalafil and the unknown compound are shown in Table 1. The signals of tadalafil were in agreement with published data[8)-10)]. The chemical structure of tadalafil is shown in Fig. 4. The ^{1}H NMR and ^{13}C NMR spectroscopic data of the unknown compound were similar to those of tadalafil except for the lack of a *N*-methyl group and the a methylene protons of the glycine moiety in the dioxopiperazine ring. The ^{1}H NMR signals at δ 4.44 (1H, d, J=13.8 Hz), 4.83 (1H, d, J=13.8 Hz) and 3.02 (3H, s) indicated the presence of a chloromethylene group and a carbomethoxy group. This suggests that the dioxopiperazine ring was cleaved into a carboxylic methylester group and chloromethylene group. The connectivity of these groups was deduced from the HMBC correlations (Fig. 5). The methylene protons (H_2-4), methine proton (H-3), and methyl proton (H_3-CH_3) correlated to an ester carbon (C=O). In addition, the methine proton (H-1), methine proton (H-3), and chloromethylene protons (CH_2Cl) showed correlations to a carbonyl carbon (C=O). The relative stereochemistry between H-1 and H-3 was determined by its ROESY correlation (Fig. 6). A ROESY correlation between H-1 and H-3 indicated that the two protons, H-1 and H-3, were oriented *cis* to each other. This is the same as the stereochemistry of

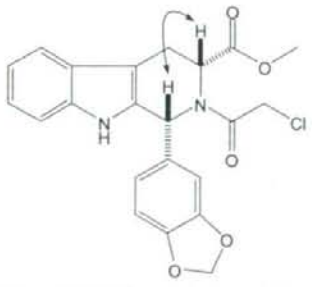

Fig. 6. Selected ROESY correlation of the unknown compound

tadalafil. The absolute configuration of the unknown compound remains to be determined.

The FT-ICR-MS measurement was carried out in order to confirm the molecular formula. Accurate FT-ICR-MS measurement on the unknown compound revealed the $[M+Na]^+$ ion at m/z 449.0874 (calc. 449.0880) (base peak) and $[M+H]^+$ ion at m/z 427.1055 (calc. 427.1061) (relative intensity: 30%), corresponding to the molecular formula of $C_{22}H_{19}ClN_2O_5$.

From these results, the unknown compound was determined to be methyl-1-(1,3-benzodioxol-5-yl)-2-(chloroacetyl)-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole-3-carboxylate. Literature search indicated that the planar structure of this compound corresponds to a tadalafil synthetic precursor[11)]. This compound was detected for the first time in a dietary supplement.

Tadalafil is a PDE-5 inhibitor and is used to treat penile erectile dysfunction. Pharmacological studies indicate that interaction between PED-5 inhibitors and nitrates may cause severe blood pressure reduction[12)]. Although there have been no systematic studies on the efficacy and toxicology of PDE-5 inhibitor analogues, one case of liver function impairment in Japan may have been due to the use of products containing hydroxyhomosildenafil[*3]. It is potentially dangerous for consumers to take dietary supplements adulterated with PED-5 inhibitors analogues. Therefore, inspection of synthetic drugs in dietary supplements is important and should be carried out in the future.

Acknowledgments

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